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Presented by

Mohamed Saiel Saeed Alhamdani

(M.Sc. in Clinical Biochemistry)

Born in Nineveh, Iraq

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Antibody microarray-based cellular oncoproteomics

Referees:

PD Dr. Stefan Wiemann

Prof. Dr. Harald Hermann-Lerdon

CONTENTS

SUMMARY

Since its recent introduction, antibody microarray technology has emerged as a powerful, robust, and sensitive tool for proteomic analyses. It enables a broad spectrum of applications, with a particular focus in biomedical sciences and clinical research. However, to date, antibody array platform protocols had been designed predominantly for the analyses of serum or plasma samples. The analysis of cell lysates and tissue homogenates posed therefore a significant challenge with respect to quality and reliability. In the work presented here, several technical issues that are crucial for the performance of such studies were established and applied to the analysis of cancer cells and tissues.

The process of extracting comprehensive proteome representations is a critical step in any proteomic investigation. While many such techniques have been described and are even commercially available, the existing processes for protein extraction from tissues were entirely inadequate for antibody array studies and thus addressed in detail. As a result, a single-step extraction procedure was developed for the isolation of proteins from mammalian tissues under native conditions in an effective and reproducible manner. Compared to existing processes, a substantially higher protein recovery was achieved, particularly of membrane as well as compartmental proteins. Also, an overall much better preservation of protein functionality was achieved. The resulting protein extracts exhibit a high compatibility with antibody microarray studies.

Moreover, assay protocols were established, refined and optimized so that robust analyses of protein extracts from mammalian tissues and cells became possible. The optimized analytical factors of the array assay were (i) the buffer composition for blocking the array surface against unspecific protein binding, (ii) blocking duration, (iii) protein handling and processing, (iv) labeling parameters like type of dye, molar ratio of label versus protein, and dye removal, as well as (v) incubation parameters such as buffer composition, duration, temperature, and sample agitation.

With the new, optimized setup, the cellular proteomes of 24 pancreatic cancer cell lines and two controls were investigated using an antibody microarray that targets 741 cancer-related proteins. The protocol is being used further for the analysis of hundreds of pancreatic cancer tissue samples in an ongoing application of the array in oncoproteomics.

ZUSAMMENFASSUNG

Seit ihrer kürzlichen Einführung haben sich Antikörper-Microarrays schnell als leistungsstarke, robuste und empfindliche Methode zur Untersuchung von Veränderungen des Proteoms etabliert. Sie erlauben ein breites Spektrum an Anwendungen, mit Schwerpunkt im Bereich biomedizinischer Wissenschaft und klinischer Forschung. Allerdings waren Antikörper-Microarrays bisher hauptsächlich für die Analyse von Serum- und Urinproben ausgelegt. Zell-Lysate und Gewebe-Extrakte stellten eine große Herausforderung hinsichtlich Qualität und Verlässlichkeit dar. In der hier präsentierten Arbeit wurden eine Reihe technischer Aspekte zur Anwendungsreife gebracht, die für eine Analyse von Zell-Extrakten essenziell sind, und für Studien an Tumorzellen und Krebsgeweben genutzt.

Die Extraktion einer möglichst vollständigen Repräsentation eines zellulären Proteoms ist ein kritischer Schritt für jede Art der Proteinanalyse. Zwar gab es bereits Methoden und entsprechende Reagenzien sind kommerziell erhältlich, aber die vorhandenen Verfahren für ein Proteinextraktion aus Zellen und Geweben waren völlig unzureichend für Studien mit Antikörper-Microarrays und wurden deshalb detailliert bearbeitet. Als Ergebnis wurde ein effektives Ein-Schritt-Extraktionsverfahren von hoher Reproduzierbarkeit entwickelt, das es erlaubt, Proteine unter nativen Bedingungen aus Zellen und Geweben zu isolieren. Im Vergleich zu bekannten Methoden sind die Ausbeuten signifikant besser, speziell auch für membran-assoziierte Proteine und Moleküle aus Zellkompartimenten. Gleichzeitig wird die Funktionalität der Proteine wesentlich besser erhalten. Die mit dem Protokoll gewonnenen Proteinextrakte zeigen eine hohe Kompatibilität mit Studien auf Antikörper-Microarrays.

Zusätzlich wurden Parameter der Array-Analyse untersucht und optimiert, so dass eine robuste Analyse von komplexen zellulären Proteinextrakten möglich wurde. Unter anderem wurden Faktoren wie (i) die Pufferzusammensetzung zur Blockierung der Oberflächen gegen unspezifische Bindung, (ii) die Dauer der Blockierung, (iii) Proteinhandhabung und Prozessierung, (iv) Parameter des Markierungsprozesses und des Entfernens überschüssigen Farbstoffs, als auch (v) Inkubationsparameter wie etwa Pufferzusammensetzung, Dauer, Temperatur und Probenmischung analysiert und optimiert.

Mit den entwickelten Verfahren wurden die Proteome von 24 Pankreaskrebs Zelllinien und zwei Kontroll-Zelllinien mittels eines Microarrays untersucht, der 810 Antikörper gegen 741 Proteine trug, die mit Krebserkrankungen assoziiert sind. Weiterhin wurden in einer Studie zum Verständnis der molekularen Hintergründe von Pankreaskrebs mehr als vierhundert Gewebeproben von Tumorpatienten und aus gesundem Gewebe analysiert.

INTRODUCTION

The availability of high-throughput technologies such as DNA-microarrays (Hoheisel, 2006) and next-generation sequencing (Schadt et al., 2010) along with user-friendly bioinformatic tools (Mychaleckyj, 2007) has paved the way for researchers towards non-reductionist approaches in investigating biological phenomena, laying the foundations for a new kind of biological studies that may be hypothesis-free initially or entirely based on data produced elsewhere. Parallel to the massive developments at the genetic level, there has been a growing interest in proteomics, the comprehensive analysis of proteins and protein networks (Wilkins et al., 2006). Since the term proteomics was first coined by Marc Wilkins and colleagues (Wilkins et al., 1996), the field has evolved enormously, especially from a biomedical perspective. The intrinsic advantage of proteomics over genomics comes from the fact that the majority of pharmacological interventions as well as diagnostic tools are directed at proteins rather than genes (Martin and Nelson, 2001).

However, owing to the complexity and immense diversity of proteins as compared to genes, analogous high-throughput tools like genome-wide expression profiling by DNA-microarrays and next generation sequencing to explore the proteome are still missing and therefore in constant demand, although protein arrays and mass spectrometry strongly move in this direction. Classical gel- and mass spectrometry-based methods represent currently the most widely used analytical instruments in proteomics and have evolved into indispensable tools for proteomic research (Han et al., 2008, Wittmann-Liebold et al., 2006). However, these approaches still suffer from limitations in terms of resolution, sensitivity, quantification and cost (Koomen et al., 2005, Diamandis and van der Merwe, 2005, Bunai and Yamane, 2005, Beranova-Giorgianni, 2003). Therefore, there is the need for more sensitive and robust techniques that can cope with the vast proteome complexity. On the other hand, affinity-based methods, such as antibody microarrays, seem to be a good and complementary alternative that can deal with the sensitivity, specificity and technical difficulties that are inherent to other methodologies and contribute substantially to global proteomic profiling.

(For more details refer to own publications I, II and III)

Technical aspects of antibody microarrays

Antibody microarray is an affinity based proteomic approach that uses miniaturized analytical systems generated by spatially arraying small volumes (nanoliter scale or less) of individual antibodies at discrete positions on a solid support (Fig. 1) (Lv and Liu, 2007, Kusnezow et al., 2006a, Angenendt, 2005, Pavlickova et al., 2004, Haab, 2003, Kusnezow and Hoheisel, 2003, Glokler and Angenendt, 2003, Kusnezow and Hoheisel, 2002). The number of antibodies used in an assay has varied from a few to several hundred, and was rarely larger than one thousand. This miniaturized and multiplexed immunoassay was first discussed by Ekins in the late 1980s (Ekins and Chu, 1991, Ekins, 1989). The generated arrays are incubation with small quantities of complex protein sample, and depending on the detection method and/or platform setup (direct or sandwich antibody array) the amount of bound antigen is determined for each spot. The arrays are scanned to acquire the images containing signal intensities, which can then be converted to numerical values using special software to reflecting protein expression within the sample. Assay sensitivities in the picomolar to femtomolar range have been reported (Kusnezow et al., 2006b). The technical

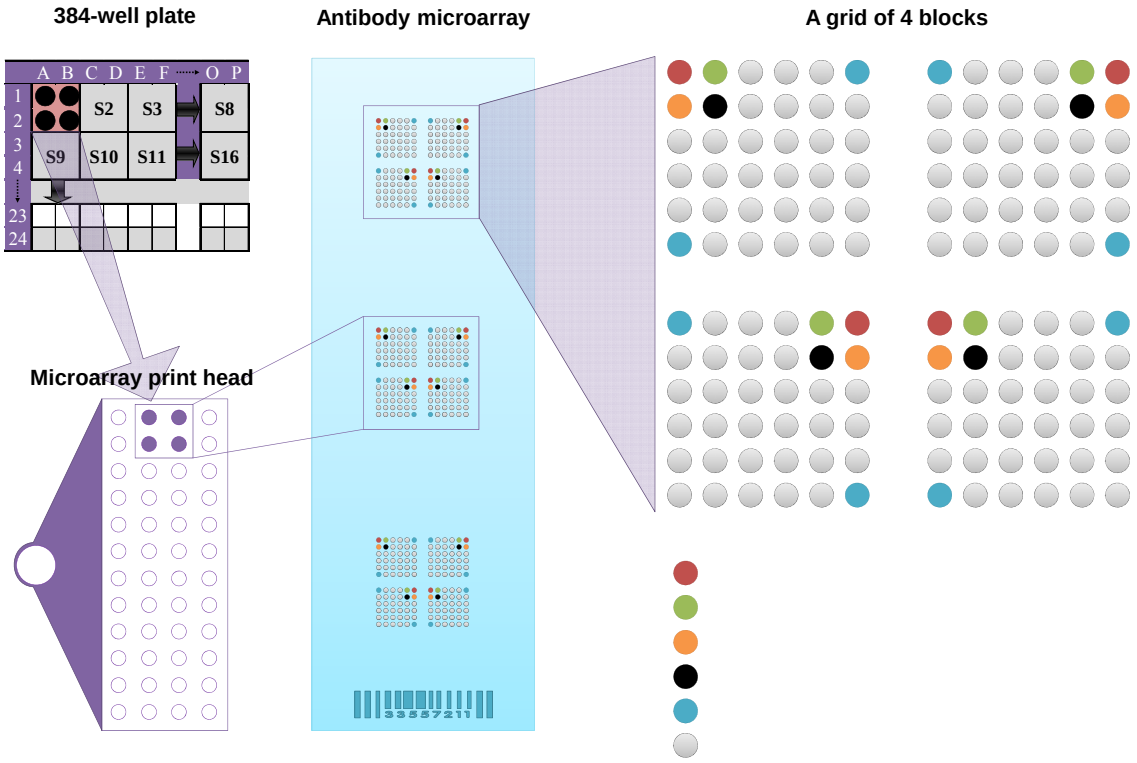

Figure 1. An example on the orientation of antibodies in 384-well plate and the position 4 pins in the spotter pin head tool. The pattern of target and control antibodies can be generated depending on the number of pins, distribution of the antibodies in the 384-well plate, the spot pattern setup in the spotter software, and the number of replicates.

factors that determine the performance of antibody microarrays are the array surface, the antibody quality, sample processing, incubation conditions, signal detection methods and data analysis approach (Fig. 2).

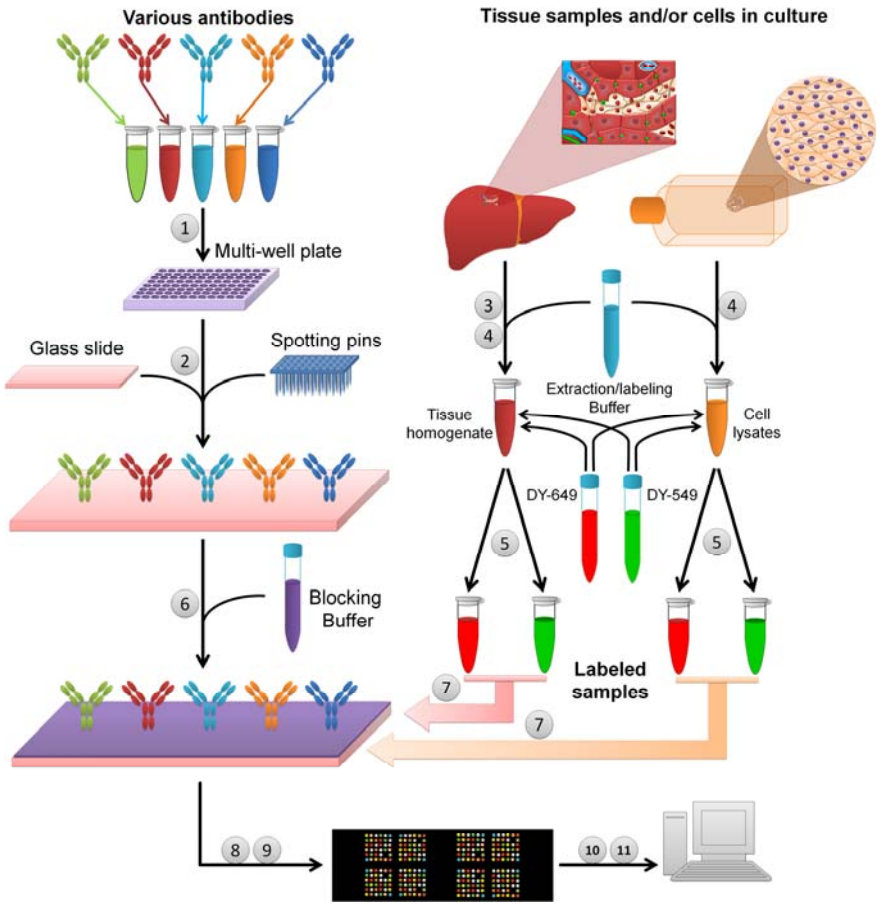

Figure 2. A schematic diagram showing the protocol work flow. 1) Pipetting various antibodies to the multi-well plate, 2) Spotting antibodies on slides, 3) tissue samples homogenization, 4) incubation of tissue or cell culture with the extraction/labeling buffer, 5) protein extract labeling, 6) spotted slides washing and blocking, 7) incubation with labeled protein samples, 8) Washing and drying, 9) scanning, 10) image analysis and 11) data analysis.

Attaching antibodies to a solid support can generally be done by chemical (covalent) or physical (affinity or adsorption) methods and each has its advantages and disadvantages. The governing factors here are the nature of protein(s), the mode of attachment, the compatibility with available hardware (e.g. contact or non-contact printing), the array density, and the sample size or reagent consumption. It should be considered that due to the labile nature of proteins, attachment to a solid support is not without the risk of compromising protein functionality, and consequently, performance. Microscopic glass slides are the most

frequently used solid support for the fabrication antibody arrays. The surface is usually modified chemically in order to enhance the attachment. Example of surfaces for affinity or adsorption are nitrocellulose (Huang et al., 2001, Knight et al., 2004), agarose (Afanassiev et al., 2000) or hydrogel (Rubina et al., 2003, Zhou et al., 2004) which hold the advantage of preserving protein function and storage longevity (Kusnezow and Hoheisel, 2003). However, these surfaces are less robust than covalent attachment (Soellner et al., 2003) as with epoxy- (Letarte et al., 2005, Seong, 2002, Angenendt et al., 2003, Kusnezow et al., 2003) and aldehyde-coated surfaces (MacBeath and Schreiber, 2000, Hahn et al., 2007), which have shown a better performance in this regard (Olle et al., 2005, Angenendt et al., 2002). However, covalent attachment may occur at random, which has the disadvantage of potentially hindering the epitope-specific part of the antibody.

One of the most challenging issues currently is having access to well-validated affinity reagents for profiling the human proteome (Taussig et al., 2007, Uhlen, 2008). Antibody-based arrays have been fabricated using monoclonal (Barber et al., 2009, Ehrlich et al., 2008, Chaga, 2008, Christopherson et al., 2006, Belov et al., 2005, Woolfson et al., 2005, Yeretssian et al., 2005, Hudelist et al., 2004) and polyclonal (Rivas et al., 2008, Han et al., 2006, Schröder et al., 2010a) antibodies, as well as recombinant antibody fragments such as $F(ab')_2$ and scFv (Song et al., 2007, Ingvarsson et al., 2007). Additionally, other types of affinity reagents have been proposed as capture molecules (Plückthun, 2009), such as affibodies (Tolmachev et al., 2007, Nygren, 2008), small molecule scaffold binders (Xiao et al., 2009, Prakesch et al., 2008), aptamers (nucleic acid scaffolds) (Stoevesandt and Taussig, 2007, Wilson et al., 2001, Tuerk and Gold, 1990), peptides (Nygren and Skerra, 2004), proteins like lipocalins (Beste et al., 1999) ankyrin repeat proteins (Stumpp and Amstutz, 2007, Binz et al., 2004), fibronectin (Xu et al., 2002), Zn-finger (Bianchi et al., 1995) and other small chemical entities (Peczuh and Hamilton, 2000, Schuffenhauer et al., 2005, Roque et al., 2004). Nevertheless, antibodies and their fragments are still the predominant affinity reagents in the production of antibody microarrays. Methods and initiatives are now in progress to create a global resource of well-characterized affinity reagents for an analysis of the human proteome (Alhamdani and Hoheisel, 2011). The main objectives of these efforts are the generation of highly specific antibodies using high-throughput technologies, such as recombinant-antibody phage display (Winter et al., 1994), ribosome display (He and Taussig, 2005), RNA display libraries (Lipovsek and Plückthun, 2004), bacterial surface display

(Jostock and Dübel, 2005), and yeast surface display (Levy et al., 2007), validation of the established antibodies, and developing novel non-antibody affinity reagents.

The ability to isolate an organism's protein in a reproducible and representative manner has a tremendous impact on the reliability of the description of the biological events. The complexity and the enormous dynamic range of molecule concentrations in a proteome as well as the susceptibility of proteins to even small changes in their environment provide a formidable challenge. However, once a protein sample is adequately brought into solution, whether in a denatured or a native form, it can be subjected to antibody array analysis. However, protein preparation under native conditions could be advantageous, since functionality may be preserved and the detection of protein isoforms may be possible (Alhamdani et al., 2010b). Protein isoforms are important to be identified for pharmacological reasons, for example. High sample complexity could cause unspecific binding and complicate uniform labelling in other proteomic approaches. For antibody microarrays, however, it has been shown that depletion of highly abundant proteins had no significant impact on the quality of the assay (Schröder et al., 2010a) but, on the contrary, introduced a considerable bias in proteome representation. Since plasma is easy to collect in a nearly non-invasive process, the application of antibody microarrays for studying this type of specimens has created much interest (Schröder et al., 2010a, Bergsma et al., 2010, Rimini et al., 2009, Lal et al., 2009, Li et al., 2009, Zeng et al., 2009, Lukesova et al., 2008, Hon et al., 2008, Sun et al., 2008, Loch et al., 2007). Conditioned media of cultured cells received similarly wide attention (Chou et al., 2008, Huang et al., 2001, Gruber et al., 2009, Grassel et al., 2009, Chen et al., 2009, Cai et al., 2009, Inai et al., 2008, Lee et al., 2008, Ohshima et al., 2008, Seeber et al., 2008, Perera et al., 2008, Neuhoff et al., 2007, Sze et al., 2007, Ebihara et al., 2007, Lu et al., 2007). However, also other types of specimens were investigated, such as urine (Schröder et al., 2010a, Hu et al., 2009, Liu et al., 2006), prostatic fluid (Fujita et al., 2008), cerebrospinal fluid (Dhungana et al., 2009, Tsai et al., 2008), tears (Leonardi et al., 2009), saliva (Lal et al., 2009), exhaled breath (Barta et al., 2010), cultured cells (Wong et al., 2009, Lin et al., 2003, Sreekumar et al., 2001) and tissue biopsies (Zander et al., 2009, Hao et al., 2008, Moschos et al., 2007, Hudelist et al., 2004, Anderson et al., 2003). Owing to the differences in proteome composition and complexity, sample processing should be optimized empirically. The tissue proteome for example is more complex and gives rise to higher background noise as compared to plasma (Haab, 2003) (Fig. 3).

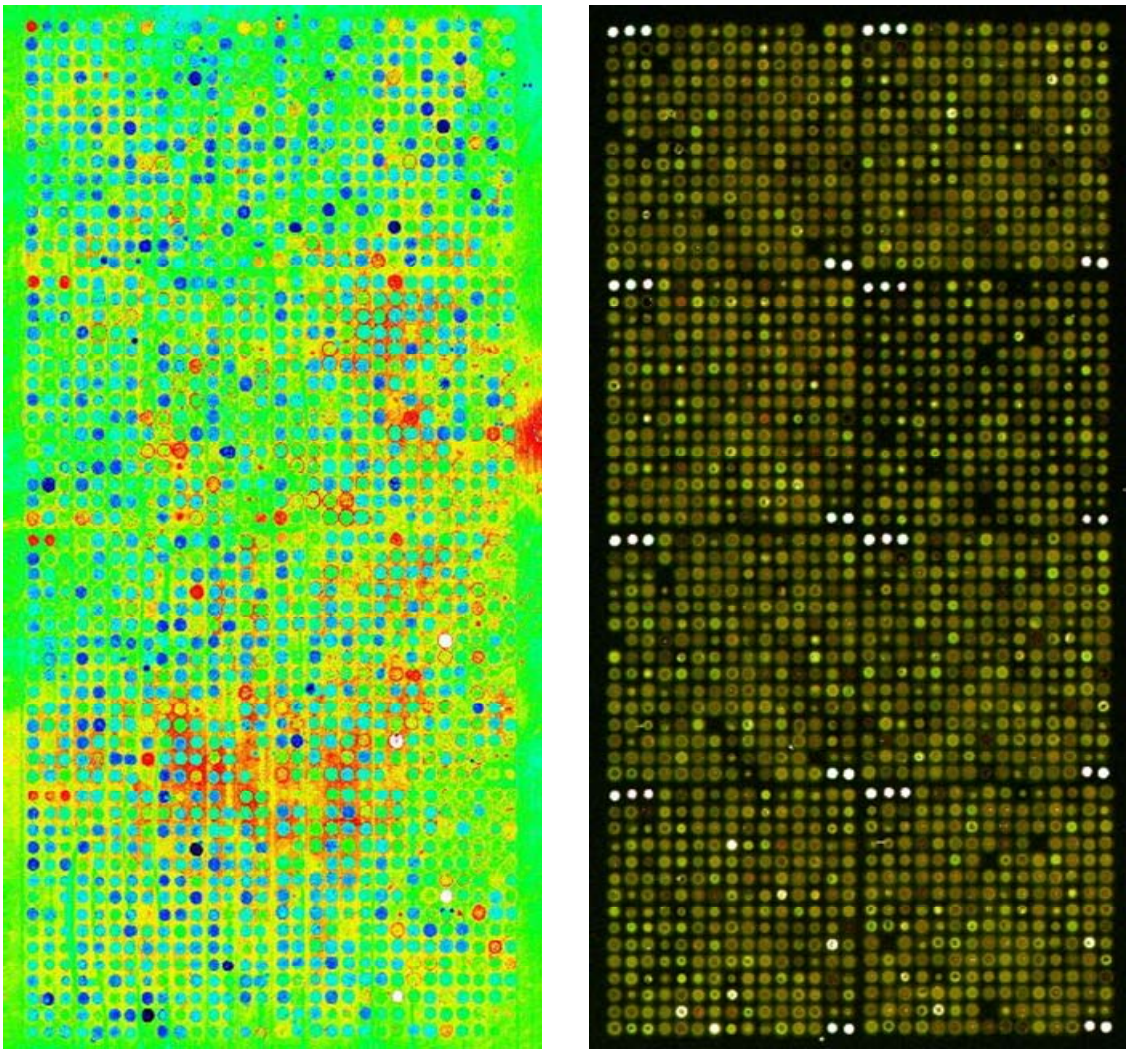

Figure 3. The difference in quality of antibody microarray between plasma (right array) and cell lysate samples (left array) analyzed under similar conditions.

When protein sample is prepared, the subsequent analysis can either be done in a label-based or label-free procedure. Protein labelling is predominantly used for the assays due to simplicity, robustness and sensitivity (Kusnezow et al., 2007, Wingren et al., 2007, Kusnezow et al., 2006b, Zhou et al., 2004, Kusnezow et al., 2003), and limit of detection can be attained in the range of picomolar to femtomolar without even the necessity of signal amplification (Wingren et al., 2007, Kusnezow et al., 2006b). Labelling of proteins can be achieved with fluorescent dyes (such as Cy3 or Cy5 dyes) or with a hapten such as biotin or biotin derivatives. Recently, dual-color labelling approach was introduced to antibody microarray analysis, resulting in a substantially improvement of the microarray performance in terms of reproducibility and resolution capability (Schröder et al., 2010a). However, labelling of protein should be done with precaution since the introduction of too many label

molecules may affect the epitopes required for the formation of the antibody-antigen complex. Label-free techniques, on the other hand, are currently gaining more recognition as a good alternative to overcome the unfavourable effect of labelling methods. As compared to labelling, these new methods currently still exhibit lower sensitivity, the need for expensive materials or pieces of equipment, and restrictions to multiplexing (Ray et al., 2010).

Antibody array applications

With their rapid development during the past ten years, antibody microarrays have seen a rise in applications, especially for clinically relevant analyses and investigations of the pathophysiology of human diseases. A broad range of human diseases has been investigated, predominantly in the field of oncology (Mustafa et al., 2011, Alhamdani et al., 2009, Alhamdani and Hoheisel, 2011). The platform have shown a promising potential in cancer diagnostics, biomarker discovery, therapy monitoring and the identification of new drug target leads (Alhamdani et al., 2009, Chatterjee et al., 2009, Loch et al., 2007, Sanchez-Carbayo, 2006, Borrebaeck, 2006, Haab, 2005). Microarrays of both commercial resource and homemade origin have been applied in this quest. Generally, studies using home-made arrays were more focused on global proteomics profiling, while those in which commercial arrays were used in conjunction with other methodologies. Of note is that homemade arrays demonstrated better functionality, performance and flexibility than those from commercial sources, which is probably the result of the ease of access to the operator of the technology and its components, which has benefits for troubleshooting and tracking errors.

Pancreatic cancer

Pancreatic cancer is one of the most lethal malignancies. Most patients die within a year of diagnosis and only about 3% survive five years or longer (Warshaw and Fernandez-del Castillo, 1992). The poor prognosis can be attributed to late presentation, aggressive local invasion, early formation of metastases, and poor response to chemotherapy (1999, Rosty and Goggins, 2002). Additionally, diagnosis by conventional means is very difficult due to the anatomic location of the pancreas (Misek et al., 2007). Patients with pancreatic tumours often present themselves with vague complaints of gastrointestinal complications at late stages of the disease. Surgery on such late-stage patients is frequently impossible or of only limited

effect (Yokoyama et al., 2009). The establishment of procedures for an earlier diagnosis of pancreatic cancer poses a challenge (Hingorani et al., 2003) since the appropriate molecular information is lacking. It is nearly impossible to obtain clinical samples from patients with premalignant or early stage malignant disease that may provide this data.

Although ductal cells make up only about 10% of all pancreatic cells and 4% of the pancreatic volume, more than 90% of human pancreatic cancers are morphologically and biologically consistent with a classification as cells that belong to the ductal cell lineage of the exocrine pancreas (Hingorani et al., 2003). The processes that lead to malignant alterations in the pancreas and the reasons why particularly ductal cells are affected are poorly understood. Current knowledge of the biological properties of pancreatic ductal adenocarcinoma (PDAC) is in part derived from *in vitro* studies of pancreatic tumour cell lines. Although they are an artificial system, cultured cells provide an important model for studying physiologic, pathophysiologic, and differentiation processes in a controlled manner. A substantial number of PDAC cell lines of different characteristics have been established and provide a good material source for investigating various molecular aspects of the devastating disease.

Aim of study

The first obstacle in processing cellular proteome is the availability of suitable protein extraction formula that allows representative and reproducible cellular protein isolation (Alhamdani and Hoheisel, 2011, Alhamdani et al., 2009, Alhamdani et al., 2010c). Extraction and solubilization of proteins are critical factors for the experimental outcome (Leimgruber, 2005) in every proteomic study. The complexity of the proteome and its vast dynamic range as well as the susceptibility of proteins to minimal changes in the milieu and their relative abundance, all rendering the processing of a sample for proteomic analysis a challenging task. Proteins were mostly isolated by procedures adapted from immunoblotting or enzyme-linked immunosorbent assay (ELISA), or extraction buffers from commercial sources were used. However, buffers for immunoblotting or ELISA are aiming at the extraction of certain proteins or a group of proteins rather than a full representation of the cellular proteome. Commercial buffers, on the other hand, are frequently unsatisfactory due to a rather limited extraction capacity and/or difficulties in identifying the source of error in downstream analysis owing to the unknown nature of their formulation.

The second obstacle is the incompatibility of the platform to cellular proteome analysis (Alhamdani et al., 2010a). This explains the low quality and high background usually observed with the analysis of cellular in comparison with plasma proteome (Haab, 2003) (Fig. 3). Since its introduction, antibody microarray has been applied predominantly in the analysis of protein samples acquired from body fluids or conditioned cell culture media (see above). To a lesser extent, cellular proteomes from tissue homogenate and cell extract have shown minimal application.

The work presented in this thesis reports a single step extraction buffer and protocol for the isolation of cellular proteome in a rather reproducible a representative manner along with a high compatibility with antibody microarray analysis and better preservation of protein functionality for other proteomics application. The work also presents a thorough evaluation and concomitant optimization of the analysis parameters of antibody microarray for proteomic analysis of mammalian protein extracts. Critical issues such as blocking the microarray surface, sample manipulation before and after labeling, the actual protein labeling as well as the incubation conditions all were looked at and the effects of the various aspects on each other were evaluated. Antibody microarrays that are more amenable to studying protein extracts from tissue homogenates or cultured cells will add to a broader spectrum of applications and deepen the confidence in the quality of the data that can be obtained, particularly toward a use of the technique in biomedical studies.

The optimized platform generated from the above was applied to investigate the cellular proteome of 24 pancreatic cancer cell lines and two additional normal lines as controls (Tab. 1). The identification of cellular variations may be utilised for the provision of molecular evidence for disease characteristics. To this end, an antibody microarray made of 810 antibodies that permits the analysis of the expression levels of 741 distinct proteins (Schröder et al., 2010b) was used. Antibodies were selected on the basis of a thorough analysis of transcriptional profiling data and other available information to be highly associated with the occurrence of pancreatic adenocarcinoma, colon and breast cancer (Bauer et al., 2009, Buchholz et al., 2005, Notterman et al., 2001).

Table 1. Charactaristic of normal and pancreatic cancer cell lines.

#	Cell line	Source of cells	Cell type	Differentiation according to histol. grade of tumor
1	**HPDE**	Normal pancreas	Ductal	-
2	**HUVEC**	Umbilical vein	Endothelial	-
3	**BxPC-3**	Primary tumor	Ductal	moderate-well, G2-G3
4	**FAMPAC**	Primary tumor	Ductal	poor
5	**IMIM-PC1**	Primary tumor	Ductal	moderate
6	**IMIM-PC2**	Primary tumor	Ductal	well
7	**MDA-Panc28**	Primary tumor	Ductal and Acinar	G4
8	**MIA PaCa-2**	Primary tumor	Ductal	-
9	**PANC-1**	Primary tumor	Ductal	G4
10	**SK-PC-1**	Primary tumor	Ductal	well
11	**SU.86.86**	Primary tumor	Ductal	G2-G3
17	**Capan-1**	Liver metastasis	Ductal	
18	**Capan-2**	-	Ductal	G1
19	**CFPAC-1**	Liver metastasis	Ductal	G1
23	**Suit-2**	Liver metastasis	Ductal	G2
20	**Suit-007**	Liver metastasis	Ductal	moderate
21	**Suit-020**	Liver metastasis	Ductal	moderate
22	**Suit-028**	Liver metastasis	Ductal	moderate
24	**Colo357**	Lymph node metastasis	Ductal	-
25	**T3M4**	Lymph node metastasis	Ductal	-
12	**A818-1**	Ascites	Ductal	well, G1
13	**A818-4**	Ascites	Ductal	well, G1
14	**A818-7**	Ascites	Ductal	well, G1
15	**AsPC-1**	Ascites	Ductal	moderate-well, G2
16	**HPAF-II**	Ascites	Ductal	G1-G2
26	**BON-1**	pancreas	-	-

MATERIALS AND METHODS

For a full disclosure of this section refer to own publications IV, V and VI

RESULTS AND DISCUSSION

Cellular proteome extraction buffer

The protein isolation buffer was formulated to provide a high qualitative and quantitative extraction capacity, in particular for membrane and compartmentalized proteins of the cell, all in a single step. In addition, the chemical composition of each component allows for the extraction with minimal perturbation of protein functionality. Consideration was taken into account to avoid substances like reductants or quenchers that interfere with protein labelling by fluorescent dyes. Moreover, the effect of cellular biomolecules such as nucleic acids, which may negatively affect the extracted protein quality and quantity, was taken into consideration in the extraction formula.

The buffer composition was made basically from one of Good's buffers (HEPES). This group of buffers are characterized by their high compatibility with biological analysis, good solubility in water, minor salt effects and minimal interference with biological functions (Good et al., 1966). The effect of nucleic acids was controlled by the inclusion of Benzonase enzyme to the mix which destroys chromosomal DNA. The proteases and phosphatases activities were controlled by the addition of inhibitors cocktail, hence preserving proteins from modification. Glycerol was also included to allow for the storage of samples for long time at -20°C without losing of protein functionality due to repeated freezing/thawing cycles. Most critical to a successful isolation, however, are the detergents used in the process. They were selected from groups of non-ionic, ionic and zwitterionic non-denaturing detergents. In addition to studying the effect of individual detergents, two detergent mixtures were formulated to assess the combined effect of these on cellular protein recovery. The detergent mixes were composed of up to four detergents that are of entirely different chemical structure and represent a broad spectrum of detergents generally used for protein extraction from biological specimens. The chemical classes to which the chosen detergents belong are polymeric phenylethylene glycols, long-chain alkyl amidosulfobetaines, cholic acid derivatives and long-chain alkylglycosides. A mixture of chemical structures should be superior to overcome the steric obstacle posed by the lipids engulfing protein geometry. Additionally, different classes of detergents have demonstrated their preference for isolating proteins from particular cellular compartments (Borner et al., 2009, Ramsby and Makowski, 1999). A combination may add more power to isolating proteins from the various cellular organelles. After preliminary tests (data not shown), we ended up with two mixtures that exhibited good results (Alhamdani et al., 2010b).

The buffer was evaluated with cells in culture (BxPC-3 and SU-8686) and various hard (heart and lung) and soft (liver and pancreas) organs of laboratory animals, as well as with human pancreatic tissues. As compared with various standard extraction buffers in common use as well as commercial protein extraction kits from different sources, the extraction buffer from the present study has expressed higher protein extraction efficiency in terms of quality and quantity. Protein quantification by BCA method and immunoblotting showed a superiority of the current extraction buffer over commercial ones. Similar findings were observed with protein function as judged by the assessment of the activity of cytosolic (aldehyde dehydrogenase) membrane bound (gamma-glutamyltransferase) and membrane/cytosolic (Glutathione S-transferase) enzymes. The positive effect of the extraction buffer was nicely mirrored on the antibody microarray quality and outcome and demonstrated high compatibility with the platform as compared with the other buffers. Finally, individual compartmentalized proteins including γ-GT (plasma membrane), flotillin (plasma membrane), caveolin (plasma membrane), α-actinin (cytoskeleton), TGN-46 (Golgi apparatus), calreticulin (endoplasmatic reticulum), catalase (peroxisome), cathepsin D (lysosome), lamin A (nucleus) and cytochrome C (mitochondria) were tested to assess the capacity of the buffer to recover proteins from various cellular organelles. Again, higher signals were found on both immunoblot bands and array spots for the extraction buffer as compared with control. The quality and compatibility of the current buffer with antibody microarray were evaluated using different quality measures such as signal-to-noise ratio (SNR), signal intensity and spot morphology. Results have shown favourable effect of the extraction buffer over the commercial control.

(For more details refer to own publication IV)

Optimization of the analysis of cellular proteome with antibody microarray

The presence of proteins as a predominant bio-molecule in the serum and other body fluids has made it easier to generate sufficient quality array upon analysis. However, this is not the case when dealing with cellular protein extracts. The presence of nucleic acids, lipids, and metabolites has its drastic impact on array performance and data quality. Although, improvements have been made by adapting protein preparation (Alhamdani et al., 2010b), still, there are additional intrinsic differences in complexity and dynamic mass of proteins. To date, no optimization for cellular proteomes had been performed. In order to make the

methodology amenable to the analysis of protein extracts from mammalian tissues, steps involving blocking (blocking buffer, time, temperature, etc.) , incubation (protein quantity, incubation medium, temperature, time, mode of mixing, and delipidation), labeling (type of fluorescent dye, labeling ration, and dye removal) and washing conditions were all studied.

The effects of buffer type, incubation time, temperature, and sample agitation method were also evaluated. In this regard, earlier studies have provided only limited information concerning to blocking condition in cellular proteome analysis with antibody microarray. In most of those studies, undisclosed recipes were used or there was no mentioning of this essential step. Moreover, none provided quality control measures of the results. For example, Knezevic *et al*. (Knezevic et al., 2001) used 1% BSA for incubation of tissue lysates for 8-12 h at 4°C. In another study (Lin et al., 2003), 5% BSA was used for incubation of tissue and cell lysates. Others provided no information. Ten commercial and home-made buffers were compared in this study. Quality was assessed in terms of spot signal uniformity and SNR. We found BSA associated with lower quality, while superior results were obtained with 10% milk-PBST80. Also, a time-dependent decrease in local and global background intensity was observed. However, blocking for more than 3 h resulted in a tenfold increase of the percentage of spots flagged as absent. The incubations at 4°C overnight or at room temperature for 1 h produced similar quality. This conforms with the proposition that for antibody-antigen complexes, which fit to a 1:1 Langmuir association model, the dissociation rate constant is more temperature-dependent than the association rate constant (Johnstone et al., 1990). Besides temperature, sample agitation is also critical for array performance (Kusnezow et al., 2006c). We compared mechanical agitation (Quadriperm) and surface acoustic wave stimulation (Slidebooster). The former produced a higher SNR.

The extraction under native conditions resulted in a better SNR than extraction with protein denaturation. Also, the removal of lipids from the sample was tested. Lipids like phosphatidyl-ethanolamine may undergo labeling and bind to the hydrophobic slide surface producing background signal. Sample delipidation was tested with Cleanascite reagent and a substantial improvement of the array quality was observed. Lipid removal was only necessary for tissues homogenates, however, and mainly in those with higher lipid content.

Protein labeling has been intensively investigated previously. Recently, it has been shown that direct labeling using a two-color approach can substantially improve microarray performance in terms of reproducibility and discriminative power (Schröder et al., 2010b). Here, we extend this issue by analyzing dye-pairs and assessing the dye/protein (D/P) molar

ratio suitable for optimal cellular proteome labeling. Five fluorescent dye-pairs were tested (Cye, Dyomics-7, Dyomics-9, ATTO and Oyster dye). Dye bias was less pronounced with increasing polarity of the dyes. Cy3 and Cy5 were second to DY-549 and DY-649 in water solubility but performed slightly better with tissue homogenates. Maximal labeling efficiency was achieved at a D/P molar ratio of 14-22. Gel electrophoresis, on the other hand, Gel electrophoresis showed a continuous increase in the fluorescence intensities of protein bands even at high D/P ratios. Increasing the D/P molar ratio has been suggested as beneficial for the array sensitivity (Kopf et al., 2005). We found, however, that ratios higher than 22 reduced the sensitivity, presumably as a result of masking of the antigenic sites by excessive dye molecules. We also evaluated the impact of dye removal after labeling. Usually, dialysis or gel filtration is applied. We found that the removal step is superfluous since un-reacted NHS-ester moieties of the dye undergo spontaneous hydrolysis in the aqueous extraction medium. The ionic strength and detergent of the washing buffer minimizes the chances of non-covalent interaction of the dye with protein molecules, hence the influence of access un-reacted dye is negligible. Besides cutting expenses, avoiding dye removal has other advantages, too, such as shortening the time required for the assay, minimizing technical complexity, and – most importantly – avoiding a loss of small proteins or peptides which may occur during dialysis or gel filtration.

(For more details refer to own publications V and VI)

Application of antibody microarray in the analysis of pancreatic cancer cell lines

Cell lines provide a good model to investigate parts of the molecular basis of cancer and could consequently permit an identification of both biomarkers and new therapeutic avenues. Here, we evaluated the cellular proteome of 24 pancreatic cancer cell lines using an antibody microarray targeting 741 proteins (Schröder et al., 2010b). About half of the differentially expressed proteins between cancer and normal cell lines had already been documented to have diagnostic application in pancreatic cancer such in diagnosis, prognosis, and effect of therapy. Seventy-three other regulated proteins were found and never been reported previously. Interestingly, all extracellular proteins with a regulatory effect on PDAC had a lower expression level in the cancer cell lines. However, in a separate study, we found concomitantly a strongly increased abundance in the secretome of serum-free medium of PDAC cells. Of those, IL6 was highly secreted by 16 of the pancreatic cancer cell lines

(unpublished data).In agreement with this finding, a higher diagnostic and prognostic value of serum IL-6 than that of CRP, CEA and CA 19-9 was documented in pancreatic cancer previously (Mroczko et al., 2010).

A closer look at the molecular feature of metastatic pancreatic cancer may provide valuable answers why PDAC cells harbour and thrive in certain organs more than others and may provide valuable decision regarding surgical intervention. Additionally, regulated proteins between primary and metastatic tumors may serve as new drug targets candidates. Comparing pancreatic cell lines on the basis of whether originated from metastases or from primary tumour revealed 187 regulated proteins, most of which are cell surface proteins that promote invasion and metastasis, including cell adhesion molecules such as ICAM1, VCAM1, Junctional Adhesion Molecule B (JAM2), Occludin and Selectin, molecules mediating homotypic interaction during invasion and metastasis such as E-Cadherin, Afadin (MLLT4), molecules mediating the interaction with the extracellular matrix such as MMP1, MMP2 and TIMP1, antigenic glycoproteins like CEACAM5, MADCAM1 and LAMP2, receptors like FAS, IL1A, IL1RN, IL2R and IL2RG and recognition proteins for immune cells like CD44, GCA. Matrix metaloproteinases (MMPs) are known proteolytic enzymes used by tumour cells for detachment and invasion. In pancreatic cancer, the imbalance between MMPs and their tissue inhibitors has been reported (Bramhall et al., 1997). Our finding showed an increased expression of MMP-7 and MMP-12 in cell originated from ascites, MMP1 in cell from liver metastasis, MMP-11 and MMP-14 in both primary and liver groups, and MMP-2 in all groups. The inhibition of MMP-2 by RO28–2653 or MMI-166 showed an effective reduction of liver metastasis in an animal model of pancreatic cancer (Kilian et al., 2006), which is an example of drug targeted metastatic tumors.

The degree of cancer cell differentiation corresponds strongly with the disease progression and its aggressiveness and therefore indicates processes that may allow for accurate prognosis and therapeutic success. The majority of PDAC tumours are well- to moderately differentiated, while poorly differentiated tumours are less common (Kloppel et al., 2000). In PDAC, the hallmarks of cell differentiation are tubular structures, papillae, cycts and secreted mucins (Adsay et al., 2005). The last are a group of glycoproteins that are mainly produced by ductal and granular epithelial cells. Some of these, such as MUC4, were suggested as a potential tumour marker for the diagnosis of pancreatic cancer (Andrianifahanana et al., 2001). Among all secreted mucins, MUC2 is considered to be the molecule most correlated to inflammation and cancer (Velcich et al., 2002). In our analysis, a

significant decrease in MUC2 expression was observed across all differentiation stages, with the lowest level being found in poorly differentiated cell lines, which in agreement with previous reports (Terada et al., 1996, Andrianifahanana et al., 2001). Apart from the mucins, 61 proteins involved in the regulation of differentiation were similarly regulated in cancer cells irrespective of the actual differentiation stage. The majority are extracellular and nuclear proteins and, to a lesser extent, proteins bound to the plasma membrane. Most of those function as transcription regulators, cytokines, growth factors and trans-membrane receptors. Some are well-established key players in the events leading to the development of PDAC, such as p53 (Casey et al., 1993), NFκB (Wang et al., 1999), ERBB2 (Safran et al., 2001), p38 MAPK (Giehl et al., 2000), CDK4 (Al-Aynati et al., 2004) and SMAD4 (Tascilar et al., 2001), which all were found to be overexpressed in the cancer cells. However, besides the proteins common to all cancer cell lines, also proteins were found that were regulated only at a particular stage of differentiation. It is intriguing that the number of specifically regulated proteins increased with the decrease of cell differentiation, being highest in the poorly differentiated cell lines.

(For more details refer to own publications VII)

CONCLUSIONS

An extraction buffer was established with high compatibility and applicability to antibody microarray-based but also other proteomic analysis. It was superior to the best systems currently available. The buffer is formulated of a detergents cocktail that includes at least one polymeric phenylethylene glycol, a long-chain amidosulfobetaine, cholate and a long-chain alkylglycoside in combination with a protease and phosphatase inhibitor cocktail, an anti-freeze agent and benzonaze, all buffered in a biocompatible manner. Application of the buffer is for reproducible extraction of proteins from cell lysates and tissue homogenates in a simple one-step manner, with good protein functionality preservation and representation of cellular organelle proteins. Although designed for antibody microarray experimentation, the buffer showed its applicability for protein extraction for a broad range of other proteomic applications such as in enzymology and immunoblotting.

Furthermore, a combination of measures, modifications and adaptations was introduced to the protocol of cellular protein analysis by antibody microarrays, which led to a substantial improvement in data quality of studies of complex protein samples from tissues and cell cultures. The protocol at stages of sample preprocessing, labeling parameters, blocking and incubation conditions were all brought to optimal setup that made antibody microarray amenable for processing cellular proteome with a procedure that is rather to perform and yields reproducible and quantifiable data.

Both the optimized approaches above were employed at the proteomic profiling of 24 pancreatic cancer and two normal cell lines aided by complex antibody microarrays generated from a panel of 810 different antibodies, each spotted in duplicate. In this analysis, 73 distinct disease marker proteins between normal and cancer cell lines were identified that had not been described before. Additionally, categorizing cancer cells in accordance to their original location (primary tumour, liver metastases, or ascites) was made possible. A comparison of the cells' degree of differentiation (well, moderately, or poorly differentiated) resulted in unique marker sets of high relevance. Last, 187 proteins were differentially expressed in primary versus metastatic cancer cells, of which the majority is functionally related to cellular movement.

In addition to the cell lines, the protocol is currently used for the analysis of several hundred pancreatic tissues from both normal and pancreatic cancer subjects. Preliminary data obtained from the analysis of 412 samples showed a promising list of regulated proteins that may help in the prediction and diagnosis of this devastating disease.

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LIST OF OWN PUBLICATIONS

- ***Publication I (Pages 32-38)***
  - **ALHAMDANI, M. S.**, SCHRÖDER, C. & HOHEISEL, J. D. (2009) Oncoproteomic profiling with antibody microarrays. Genome Med, 1, 68. (AMS contribution 75%)
- ***Publication II (Pages 39-63)***
  - **ALHAMDANI, M. S.** & HOHEISEL, J. D. (2011) Antibody microarrays in proteome profiling. IN RAPLEY, R. & HARBRON, S. (Eds.) Molecular Analysis and Genome Discovery. 2nd ed., Wiley. (AMS contribution 80%)
- ***Publication III (Pages 64-70)***
  - MUSTAFA, S. A., HOHEISEL, J. D. & **ALHAMDANI, M. S.** (2011) Secretome profiling with antibody microarrays. Mol Biosyst, 7, 1795-801. (AMS contribution 65%)
- ***Publication IV (Pages 71-79)***
  - **ALHAMDANI, M. S.**, SCHRÖDER, C., WERNER, J., GIESE, N., BAUER, A. & HOHEISEL, J. (2010) Single-step procedure for the isolation of proteins at near-native conditions from mammalian tissue for proteomic analysis on antibody microarrays. J Proteome Res, 9, 963-971. (AMS contribution 75%, Thesis work 40%)
- ***Publication V (Pages 80-103)***
  - **ALHAMDANI, M. S.**, SCHRODER, C. & HOHEISEL, J. D. (2010) Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays. Proteomics, 10, 3203-7. (AMS contribution 75%, Thesis work 40%)
- ***Publication VI (Pages 104-122)***
  - SCHRÖDER, C., **ALHAMDANI, M. S. S.**, FELLENBERG, K., BAUER, A., JACOB, A. & HOHEISEL, J. D. (2011) Robust protein profiling with complex antibody microarrays in a dual-colour mode. Methods Mol Biol, 203-221. (AMS contribution 30%)
- ***Publication VII (Pages 123-153)***
  - **ALHAMDANI, M. S.**, YOUNS, M., BAUER, A., SCHRÖDER, C. & HOHEISEL, J. D. (2011) Immunoassay-based protein profiling of 24 pancreatic cancer cell lines reveals characteristics of the disease, the degree of cell differentiation, the source of tumor cells, and their metastatic potential. . Mol Cell Proteomics, (submitted). (AMS contribution 75%, Thesis work 20%)

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PD Dr. Stefan Wiemann

APPENDICES: OWN PUBLICATIONS

Genome Medicine

Review

Oncoproteomic profiling with antibody microarrays

Mohamed SS Alhamdani, Christoph Schröder and Jörg D Hoheisel

Address: Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany.

Correspondence: Mohamed Saiel Saeed Alhamdani. Email: m.alhamdani@dkfz-heidelberg.de

Abstract

The incidence of cancer and its associated mortality are increasing globally, indicating an urgent need to develop even more effective and sensitive sets of biomarkers that could help in early diagnosis and consequent intervention. Given that many cellular processes are carried out by proteins, cancer research has recently shifted toward an exploration of the full proteome for such discovery. Among the advanced methodologies that are being developed for analyzing the proteome, antibody microarrays have become a prominent tool for gathering the information required for a better understanding of disease biology, early detection, discrimination of tumors and monitoring of disease progression. Here, we review the technical aspects and challenges in the development and use of antibody microarray assays and examine recently reported applications in oncoproteomics.

Introduction

Over the past two decades, there have been tremendous advances in the understanding of the molecular processes by which normal cells transform into cancer and of the importance of signaling pathways in cancer initiation and progression. This progress has paved the way for the development of numerous therapeutic leads. In addition, the enormous leap in biotechnology and bioinformatics raises hopes for substantial progress in cancer diagnosis and treatment. Despite the increased knowledge and improved technical capabilities, however, global mortality from cancer is projected to continue rising, mainly because of the aging of the population, with an estimated 9 million people dying from cancer in 2015 and 11.4 million in 2030 [1]. A major obstacle to the reversion of this trend is the fact that cancer is frequently detectable only at late stages. Current cancer diagnosis also still relies on the testing of classical cancer markers, such as cancer antigen (CA)-125, CA19-9, CA72-4 and carcinoembryonic antigen (CEA), in combination with histopathological examination of tissue biopsies. Furthermore, there is a growing need for individual monitoring of the response to therapy and disease progression, as the effect of a particular treatment is not uniform among affected subjects with the same diagnosis. In consequence, approaches are urgently required that enhance the power of detection and diagnosis of cancer at early stages.

Prompted by the sequencing of the human genome, high-throughput technologies have evolved, shifting attention towards a non-reductionist approach to investigating biological phenomena. The explosion of interest in exploring the genome and proteome for biomarkers has already provided a better understanding of the molecular basis of cancer. Among the high-throughput technologies, DNA analysis by microarrays [2] and, more recently, second-generation sequencing [3] have become prominent approaches. However, the similarity in genetic alteration shared among various cancers limits the possibility of linking the genetic portrait to a particular disease feature [4]. The genomic sequence does not specify which proteins interact, how interactions occur or where in a cell a protein localizes under various conditions. Transcript abundance levels do not necessarily correlate with protein abundance [5], and frequently one cannot tell from the sequence whether a gene is translated into protein or rather functions as RNA.

Recent developments in genetic analysis have been paralleled by a surge in interest in the comprehensive study of proteins and protein networks. From a biomedical perspective, the field of proteomics has great potential because most pharmacological interventions and diagnostic tests are directed at proteins rather than genes. The inherent advantage of proteomics over genomics is that the identified protein itself is the biological end-product [6]. There are several sophisticated technologies that enable proteome-wide analysis of multiple proteins in a variety of specimens. Among these, two-dimensional gel electrophoresis and mass spectrometry have been widely used and have evolved into indispensable tools for proteomic research [7,8]. Optimization processes have been significantly improved with regard to their performance at handling small sample sizes and analyzing complex protein

CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; MALDI-TOF, matrix assisted laser desorption ionization time of flight; MAP, mitogen activated protein; scFv, single-chain variable fragment.

mixtures [9]. However, they still suffer from limitations in terms of resolution, sensitivity and reproducibility, high cost and the great amount of time and labor required. Affinity protein-array technology seems to be a promising tool to overcome some of these limitations.

Technical aspects of antibody microarrays

Antibody microarrays are miniaturized analytical systems generated by spatially arraying small amounts (volumes at a picoliter scale or less) of individual capture molecules, mostly antibodies, onto a solid support (Figure 1) [10-14]. So far, the number of antibodies has varied from a few to several hundred. Upon incubation with a protein sample, bound antigens are detected by fluorescence detection or surface plasmon resonance, for example. The acquired signal intensity images are converted to numerical values reflecting the protein profiles within the samples. Assay sensitivities in the picomole to femtomole range have been reported [15,16]. Although antibody microarrays were introduced after DNA microarrays, the feasibility of miniaturized and multiplexed immunoassays was first reported and discussed by Ekins in the late 1980s [17,18]. The technical factors that determine the set-up of a high-performing antibody microarray are the array surface, the antibodies, sample processing, incubation and signal generation and data analysis.

Array surface

The choice of surface is critical for array performance because, unlike DNA, proteins are very divergent and inhomogeneous in structure and properties and prone to loss of function by denaturation and/or modification [19]. The most frequently used solid supports for antibody microarrays are microscopic glass, plastic or silicon slides that are coated with a variety of substrates [20-22]. Examples of chemical substrates are nitrocellulose, aldehydes, amino-polyethylene glycol, Ni-nitrilotriacetic acid, streptavidin, epoxysilane and polyacrylamide-gel coatings. The choice of a specific substrate depends on several factors, such as the complexity and nature of the analyzed sample (whether it consists of individual proteins, proteins from plasma or other body liquids, or samples from cultured cells or tissues), the mode of antibody coupling, biocompatibility and array density. In addition to flat slides, arrays using nanovials and attovials [23] have been used in an attempt to enhance sensitivity and multiplexing.

Antibodies

There are several types of affinity reagents that can act as capture molecules, such as monoclonal and polyclonal antibodies, recombinant antibody fragments (scFab, scFv, and so on), binders with different scaffolds (such as affibodies or anchorins), nucleic acid scaffolds (aptamers), peptides and small chemical entities [24]. Each molecule class has its advantages and disadvantages. Nevertheless, currently antibodies and antibody fragments continue to be the most

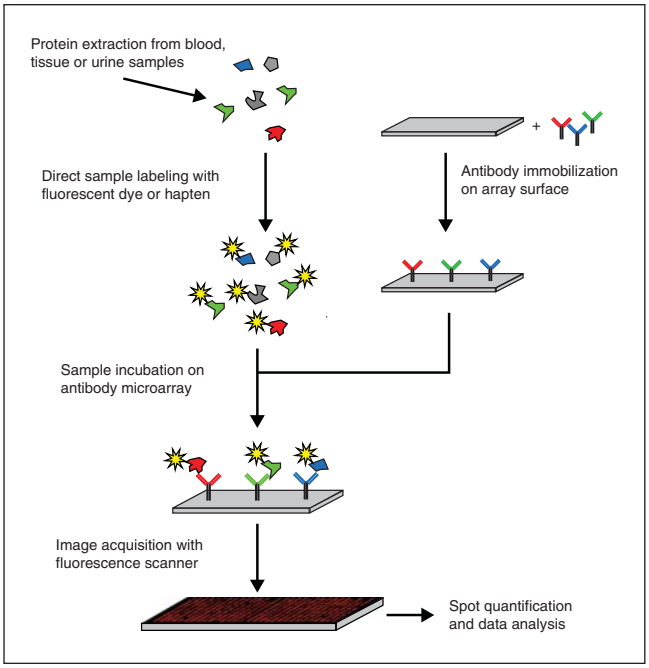

Figure 1

Schematic diagram of the basic processes of analyzing protein extracts on antibody microarrays. Although many details such as the binder type, the protein labeling, the surface structure of the solid support or the detection procedure may change considerably, the principal components and steps of the assay remain the same.

attractive affinity probes. Mono-specific polyclonal antibodies [25] are attractive because of the cooperative effect obtained from the generation of a mixture of antibodies to several epitopes of the target protein. This allows more antibodies to bind to each target, concomitantly improving affinity, and makes the binding assay less dependent on a single epitope. This is particularly important for multi-platform applications, in which the protein target may be denatured in different ways by factors such as detergent, alcohol, formalin or mechanical stress.

So far, the vast majority of microarrays have been generated using monoclonal and polyclonal antibodies from commercial sources. Concerns are rising, however, over how many of these commercial antibodies meet the expected performance and specificity requirements [26]. In addition, there is an imbalance in representation. Hundreds of antibodies exist against particular targets - for instance, more than 900 antibodies for p53 - whereas none are available for many others. Recombinant-antibody phage-display libraries have been suggested as a way to reduce the limitations associated with monoclonal and polyclonal antibodies in terms of specificity, functionality, stability and availability [10,14]. Furthermore, programs have been initiated for the creation of a global resource of well characterized affinity reagents for an analysis of the

human proteome, most prominently the Swedish Human Proteome Atlas project [27] or, transnationally, the European ProteomeBinders consortium [26].

Sample processing

In any proteomic study, sample preparation is a critical factor. Owing to the complexity of the proteome, the enormous dynamic range in concentration and the susceptibility of proteins to minimal changes in the milieu and the relative abundance in a mixture, processing a sample for proteomic analysis is a challenging task. The majority of recent antibody microarray applications studied serum samples. However, other types of specimens were also targeted, such as extracts of cell surface proteins [28], cultured cells [29] or tissue biopsies [30]. Although many reports have focused on the optimization of protocols for protein extraction from mammalian cells for gel separation and mass spectrometry [31], proteins for antibody microarray assays are mostly isolated by procedures long used for immunoblotting or enzyme-linked immunosorbent assays (ELISAs). Introducing an advanced protein extraction protocol that is more representative of a whole cellular proteome would be advantageous for microarray-based global protein analysis. In all proteomic approaches, sample complexity can give rise to non-specific binding and complicate uniform labeling. Strategies have been developed to remove high-abundance proteins [32] or to fractionate the proteins [33] in order to reduce complexity.

Sample labeling and signal read-out

Subsequent to isolation, samples are further processed by labeling either directly with fluorescent dyes or indirectly with biotin or biotin derivatives. Biotin is recognized by labeled streptavidin. Testing of labeling tags showed a superior sensitivity and signal-to-background ratio when samples were labeled with biotin [34,35]. However, although sample-labeling approaches allow high-sensitivity detection in the picomole to femtomole range, there are some concerns regarding the introduction of too many label molecules, which might affect the antibody-antigen binding capacity. There are several label-free detection techniques that bypass labeling complications. The oldest one is the sandwich approach known from ELISA, in which two antibodies are used for selective binding to a specific protein. An arrayed antibody serves as capture reagent. Upon protein binding, the bound molecule is detected by the second antibody, which carries the label directly or is identified by a third, labeled antibody. However, since the process necessitates a working pair of antibodies for each individual analyte, technological issues prevent this approach for multiplex arrays that consist of several hundreds to thousands of antibodies.

Emerging methods use matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [36], surface plasmon resonance [37], nanowires [38], micro-cantilevers [39], quartz crystal microbalances [40] or light scattering [41] for read-out. However, with fluorescence-based array detection approaching single-molecule sensitivity [42], the alternative methods still need to prove their practical feasibility and competitiveness. Incubation conditions have been found to be critical for analysis, especially in view of the huge dynamic range of protein concentrations. Mass transport and kinetics are crucial for reproducible and sensitive studies [16]. Appropriate mixing, for example, is of critical importance to such ends [16].

Data analysis

Data analysis and interpretation are usually carried out using approaches adopted directly from DNA microarray studies. Data normalization can be tackled with a variety of methods, such as an internally normalized ratio algorithm following dual-color labeling [43], spike-in protein control(s) of known concentration, and relative normalization to a particular analyte assayed independently by other methods (such as ELISA) [44]. In addition to measurements at equilibrium, new technology enables the analysis of association and particularly dissociation [45], adding extra quality to the analysis.

Antibody microarrays in oncoproteomics

Although still very much under development, the antibody microarray technique has already shown wide application potential for clinical cancer research and diagnostics [45]. Table 1 lists some recent applications of antibody microarrays in oncoproteomics. The antibody platforms had either been fabricated in-house or obtained from commercial sources. The number of binders varied from a few tens, as in the analysis of cytokine networks [46] or functional pathways [47], to hundreds, as in studies focused on a more global protein expression analysis [29,30,48]. Several sources of samples have been used, including culture cell extracts [29,49-51], dissected tissue biopsies [25,51-53], exhaled breath [46] and body fluids [30,54-60]. Nevertheless, the most studied specimens were sera taken from both cancer patients and healthy controls [30,54-56,58-60]. The rationale is that serum reflects the body's whole cellular metabolic harvest, and leakage of proteins from a particular organ or group of cells to the circulation provides some reflection of biochemical alterations during disease. In addition, in more technical terms, protein complexity is relatively low in serum and protein extraction is easy to perform.

Hudelist *et al.* [52] used antibody microarrays for profiling expressed proteins in normal and malignant breast tissues. They found increased expression levels of several proteins in malignant breast tissues, such as casein kinase Ie, p53, annexin XI, the cell-cycle protein CDC25C, the general transcription initiation factor eIF-4E and mitogen-activated protein (MAP) kinase 7, using commercial arrays of 378

Table 1

Application of antibody microarrays in cancer research

Cancer type	Sample source	Assay platform	Number of antibodies	References
Angiogenesis	Cell line	ProteoChip	60	[47]
Bladder	Human sera	Nitrocellulose FAST slides	254	[53]
Breast	Cell line, human sera and tissue	Hypromatrix, RayBio, Proteome Profiler, MaxiSorp slide, nitrocellulose membrane, Panorama cell signaling, BD antibody microarray 380	400, 174, 42, 129, 312, 224, 378	[29,49-52, 54,61,62, 70-73]
Colon	Cell line	Poly-L-lysine or superaldehyde coated glass slides	146	[63]
Colorectal	Human tissue, cell line	Lab Vision, Panorama cell signaling	720, 224	[30,64,74]
Gastric	Human sera	Lab Vision	720	[30]
Intestinal	Mouse sera	Nitrocellulose-coated slides	40	[75]
Leukemia	Human sera	DotScan	82	[76]
Liver	Human tissue, cell line	Hypromatrix	400	[48]
Lung	Human sera and exhaled breath condensate, cell line	Cytokine antibody array VI+VII, Panorama cell signaling, Nitrocellulose-coated slides	120, 224, 84	[46,55,77,78]
Melanoma	Cell line	RayBio	174	[79]
Ovarian	Human sera	Hydrogel-coated glass slides	320	[57,60]
Pancreatic	Human sera	Nitrocellulose-coated slides, MaxiSorp slide	129, 48, 90	[44,56,59,68,69]
Prostate	Cell line, human sera and prostate fluid	Phosphorylation antibody array, RayBio, hydrogels or poly-L-lysine coated slides	71, 174, 184, 86	[65-67,77,80]
Renal	Human sera	RayBio	20	[58]

antibodies. In another report [61], 224 antibodies revealed proteins that are related to doxorubicin therapy resistance in breast cancer cell lines. A decrease in the expression of MAP kinase-activated monophosphotyrosine, cyclin D2, cytokeratin 18, cyclin B1 and heterogeneous nuclear ribonucleoprotein m3-m4 was found to be associated with doxorubicin resistance. Other recent investigations helped identify a marker involved in invasion (interleukin (IL)-8) [62]. Studying the serum proteome from metastatic breast cancer patients and healthy controls with recombinant single-chain variable fragment (scFv) microarrays [54], breast cancer was identified with a specificity and sensitivity of 85% on the basis of 129 serum analytes.

In bladder cancer, an array of 254 antibodies showed 93.7% sensitivity to discriminate between serum samples of 58 healthy subjects versus 37 bladder cancer patients [53]. The impact of radiation treatment was evaluated in LoVo colon carcinoma cells [63]. An array of 146 antibodies showed increased expression of apoptosis regulators paralleled by downregulation of CEA, pointing to a possible application for monitoring response to radiation therapy in colon cancer. In colorectal cancer, the marker IPO-38 [30], cytokeratin 13, calcineurin, the serine/threonine kinase CHK1, clathrin light chain, MAP kinase 3, phospho-protein tyrosine kinase 2 (also called focal adhesion kinase, phosphorylated at Ser-910) and the p53 regulator MDM2 [64] were found as possible biomarkers. They were further validated with standard protocols such as ELISA, immunoblotting, immunohistochemistry and MALDI-TOF/TOF mass spectroscopy. However, the number of patients evaluated in these colorectal cancer studies was low. The application of antibody microarrays to prostate cancer also identified several potential marker proteins [65,66]. Analysis of cytokines from prostate fluid of patients with minimal and maximal cancer volume revealed a possibility for early detection of the disease [67].

Several publications have recently reported the use of antibody microarrays in assessing markers of lung cancer, which is the leading cancer-related cause of death. Kullmann *et al.* [46] tested cytokine profiles with a 120-antibody array in breath condensates of 50 smoking lung cancer patients and 25 smokers without clinical or radiological sign of a pulmonary tumor and were able to differentiate the two groups by nine cytokines, including eotaxin, fibroblast growth factors, IL-10 and macrophage inflammatory protein (MIP)-3. However, the results were not stratified according to stages and histological subtypes owing to the use of pooled samples. Gao *et al.* [55] constructed an array of 48 antibodies against distinctive serum proteins. They analyzed 24 newly diagnosed subjects with lung cancer, 24 healthy controls and 32 subjects with chronic obstructive pulmonary disease. C-reactive protein,

serum amyloid A, mucin 1 and α1-antitrypsin were among the proteins that showed higher abundances in the lung cancer samples than in the control samples.

Pancreatic cancer has received much attention, being one of the most deadly forms of cancer with basically no current treatment available. Initial observations of serum profiles came from Haab and colleagues [59], revealing individual and combined protein markers associated with pancreatic cancer and variations in specific glycans on multiple proteins. In another study from the same group [68], antibody microarrays were used to analyze post-translation modification of serum protein in pancreatic cancer patients. By profiling both protein and glycan variations [69], they found cancer-associated glycan alteration on the proteins MUC1 and CEA [68]. The Borrebaeck group [56] used an array of recombinant scFv antibodies in an attempt to classify sera derived from pancreatic adenocarcinoma patients versus samples from healthy subjects. They reported a protein signature based on 19 non-redundant analytes discriminating between cancer patients and healthy subjects.

Conclusions and future perspectives

The antibody microarray is a technology that still requires maturation. Although some technical factors have been dealt with, others remain to be optimized. In particular, appropriate binders need to be produced and validated. However, from the initial and mostly still rather preliminary studies, one can already conclude that important information can be gathered in an efficient and probably even quantitative process. The technology has the advantage of targeting the actual effector molecules of many biochemical processes, thus providing information that is of immediate clinical relevance. Sensitivity issues should be overcome by new detection modes, which could enable sensitivity up to the level of counting individual molecules. The method's practical usefulness will be particularly enhanced once the analysis of samples obtained by non-invasive means provides the required clinical information.

As is the case for other profiling procedures, indirect biomarkers - molecules that indicate a cellular state without necessarily being the cause for it - provide only limited diagnostic and prognostic accuracy if studied individually. Indeed, the use of multiple biomarkers rather than a single one improves diagnostic accuracy, enhances the predictive power for patient outcome and may enable adequate monitoring of the response to treatment. Because of the decisive role of proteins in cellular activities, antibody or other binder microarrays have the potential to quickly become a routine diagnostic tool, eventually even in relatively simple formats with few binder molecules.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed equally to this work.

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10

Antibody Microarrays in Proteome Profiling

Mohamed Saiel Saeed Alhamdani and Jörg D. Hoheisel

Introduction

Following the completion of sequencing of the first human genome, high-throughput technologies such as DNA-microarrays (Hoheisel 2006) and – more recently – second-generation sequencing (Ansorge 2009) have become prominent and formidable analytical tools in biology. Adding to this instrumentation, the availability of powerful and user-friendly bioinformatics tools (Mychaleckyj 2007) has paved the way for researchers to deal with the massive amount of data generated by these technologies. In consequence, non-reductionist approaches have become possible and opened a new epoch in investigating biological phenomena, laying the foundations for a new biology. In extension to this tremendous development at the molecular-genetic level, there has been a surge in interest in the comprehensive analysis of proteins and protein networks (Wilkins *et al.* 2006). The field of proteomics, a term coined by Marc Wilkins and colleagues (Wilkins *et al.* 1996), evolved out of necessity, especially from a biomedical perspective. The intrinsic advantage of proteomics over genomics is that to a large extent the proteome is the biological end-product of the genome (Martin and Nelson 2001), although more and more information becomes available about the direct involvement of nucleic acids in regulation, structure building and functional activities. However, genomic sequences do not offer any apparent information on protein interaction, localization and post-translation modification. Also, transcript abundance levels do not necessarily predict the corresponding protein level (Schmidt *et al.* 2007), nor do

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they provide information about its regulatory status, which is usually governed by endogenous and exogenous factors. Most pharmacological interventions are directed at proteins rather than genes.

With this in mind, it is obvious that tools for an analysis of the proteome are of crucial importance. While for example genome-wide expression profiling by DNA-microarrays and sequencing have become routine, analogous tools are not yet available for proteins, although protein arrays and mass spectrometry strongly move in this direction. In addition to the technical shortcomings, proteins are immensely more diverse in structure and biophysical properties than nucleic acids. Post-translation modifications add even another level of complexity. In simple terms, an organism has only one genome but, at the same time, several proteomes.

So far, two-dimensional gel electrophoresis and mass spectrometry represent the most widely used analytical tools in proteomics and have evolved into indispensable tools for proteomic research (X. Han, Aslanian and Yates 2008; Wittmann-Liebold, Graack and Pohl 2006). Since the mid-1990s, the performance of these technologies in handling small sample sizes and analysing complex protein mixtures (Aebersold and Mann 2003) have been improved significantly. With the continuing need for more sensitive and robust techniques that can cope with very high complexity, however, these classical approaches still have limitations in resolution, sensitivity and cost (Bunai and Yamane 2005; Diamandis and van der Merwe 2005; Koomen *et al.* 2005; Beranova-Giorgianni 2003). Assays with affinity-reagents are a good and complementary alternative, since they are technically based on a different principle and additionally already well established in clinical diagnostics. Therefore, protein-array technology seems to be well suited to overcome some of the limitations inherent to other methodologies and to contribute substantially to the tool set used in global proteomic profiling.

Technical aspects

Antibody microarrays are miniaturized analytical systems generated by spatially arraying small amounts (volumes at a nanolitre scale or less) of individual capture molecules – mostly antibodies – at discrete positions on a solid support (Figure 10.1) (Lv and Liu 2007; Kusnezow *et al.* 2006a; Angenendt 2005; Pavlickova, Schneider and Hug 2004; Glokler and Angenendt 2003; Haab 2003; Kusnezow and Hoheisel 2003; Kusnezow and Hoheisel 2002). The feasibility of such miniaturized and multiplexed immunoassays was first discussed by Ekins in the late 1980s (Ekins and Chu 1991; Ekins 1989). To date, the number of antibodies used in an assay has varied from a few to several hundred, and has rarely been larger than one thousand. Upon incubation with a complex protein sample (in micro-liter quantities), the amount of bound antigen is determined for each spot. The acquired images contain signal intensities, which are converted to numerical values reflecting the protein profile within the biological sample. Assay sensitivities in the picomolar to femtomolar range have

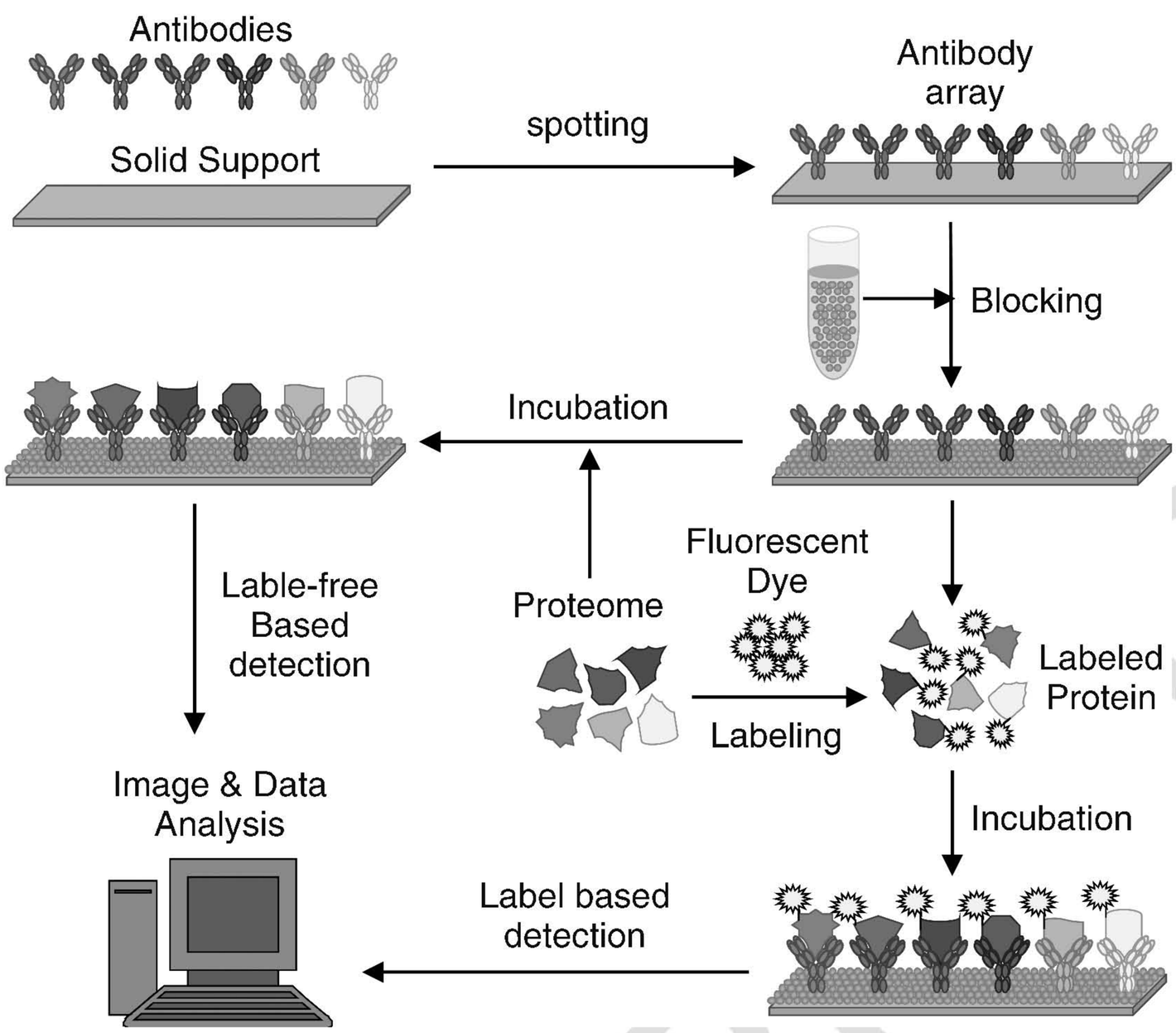

Figure 10.1 A schematic representation of the steps of an antibody array analysis

been reported (Kusnezow *et al*. 2006a). The technical factors that determine the performance of antibody microarrays are the array surface, the antibody quality, sample processing, incubation conditions, signal detection and data analysis.

The solid support

Unlike DNA, proteins have very divergent inhomogeneous structures and biophysical and chemical properties and are prone to perturbation by denaturation and/or modification (Doerr 2005). Hence, attaching proteins to a solid support is not without the risk of compromising functionality and, consequently, performance. There have been continuous efforts to generate support media that have a minimal detrimental effect on the arrayed proteins. The selection of the appropriate solid support is usually governed by several factors such as the nature of protein(s), the mode of attachment, the compatibility with available hardware (e.g. contact or non-contact printing), the array density, and the sample size or reagent consumption. Generally, microscopic glass slides are the most frequently used solid support for antibody microarray fabrication. The surface is usually coated with substrates that facilitate the attachment. A physical or chemical interaction between substrate and antibodies takes place by means of affinity, adsorption or covalent binding.

Choosing the appropriate solid support amongst the plethora of commercially available support media could be difficult. Care should be taken to consider factors such

as surface hydrophilicity, orientation of immobilization and the chemistry of attachment, since all affect antibody–antigen interaction. Proteins can bind strongly to hydrophobic surfaces, which may contribute to increasing signal intensity and detection limit. The tendency of proteins to denature, however, increases on hydrophobic surfaces (Sorribas, Padeste and Tiefenauer 2002). Polystyrene-based plastic surfaces, such as those used in enzyme-linked immunosorbent assay (ELISA) plates, represent an efficient and simple way to immobilize antibodies, yet they show a significant degree of inactivation due to denaturation and steric obstruction (Butler *et al.* 1993, 1992).

Surfaces coated with a highly hydrophilic substrate such as nitrocellulose (Knight *et al.* 2004; Huang *et al.* 2001), agarose (Afanassiev, Hanemann and Wolfl 2000) or hydrogel (Zhou *et al.* 2004; Rubina *et al.* 2003) could be advantageous for preserving protein function and storage longevity (Kusnezow and Hoheisel 2003). Hydrophilic surfaces allow protein immobilization by adsorption, which is less robust than covalent attachment (Soellner *et al.* 2003). Epoxy-coated surfaces (Letarte *et al.* 2005; Angenendt *et al.* 2003; Kusnezow *et al.* 2003; Seong 2002) and aldehyde (Hahn *et al.* 2007; MacBeath and Schreiber 2000) have shown a better performance in this regard (Olle *et al.* 2005; Angenendt *et al.* 2002). Covalent attachment makes use of side-chain reactive groups such the amino-group of lysine or the thiol-group of cysteine, which are present in virtually all proteins. However, covalent reaction may occur at random, with the disadvantage that the protein may bind somewhere near to the active site. Attaching proteins in a uniform rather than random manner can provide a substantial advantage in assay sensitivity (Peluso *et al.* 2003). For antibodies, methods have been implemented that benefit from their unique structure and allow the establishment of an orientation that leaves the antigenic sites well exposed for capturing the target proteins. These methods depend on the affinity of certain molecules such as proteins A, G, A/G and L (Yuan, He and Lee 2009; Bonroy *et al.* 2006; Danczyk *et al.* 2003; Podlaski and Stern 2000) and synthetic peptides (Jung *et al.* 2008) to a certain antibody segment, usually the F_c part (constant region) (Ghose, Hubbard and Cramer 2007; Arora, Hammes and Oas 2006; H. Yang, Gurgel and Carbonell 2005; Guss *et al.* 1986). Other methods target the carbohydrate moiety (Nisnevitch *et al.* 2000) or the hinge sulfohydryl-group (Peluso *et al.* 2003) as site of attachment.

Affinity reagents

The quality and robustness of microarrays rely strongly on the affinity molecules used in their fabrication. Currently, access to well-validated affinity reagents is one of the challenging issues in profiling the human proteome (Uhlen 2008; Taussig *et al.* 2007). Antibody-based arrays have been fabricated using monoclonal antibodies (Barber *et al.* 2009; Chaga 2008; Ehrlich *et al.* 2008; Christopherson *et al.* 2006; Belov *et al.* 2005; Woolfson *et al.* 2005; Yeretssian *et al.* 2005; Hudelist *et al.* 2004)

and polyclonal (Schröder *et al.* 2010; Rivas *et al.* 2008; M.K. Han *et al.* 2006), as well as recombinant antibody fragments such as $F(ab')_2$ and scFv (Ingvarsson *et al.* 2007; Song *et al.* 2007). Still, there are several other types of affinity reagents that can act as capture molecules (Plückthun 2009), such as affibodies (Nygren 2008; Tolmachev *et al.* 2007), small molecule scaffold binders (Xiao *et al.* 2009; Prakesch *et al.* 2008), aptamers (nucleic acid scaffolds) (Stoevesandt and Taussig 2007; Wilson, Keefe and Szostak 2001; Tuerk and Gold 1990), peptides (Nygren and Skerra 2004), proteins such as lipocalins (Beste *et al.* 1999), ankyrin repeat proteins (Stumpp and Amstutz 2007; Binz *et al.* 2004), fibronectin (Xu *et al.* 2002), Zn-finger (Bianchi *et al.* 1995) and other small chemical entities (Schuffenhauer *et al.* 2005; Roque, Taipa and Lowe 2004; Peczuh and Hamilton, 2000). Each molecule class has its advantages and disadvantages. Nevertheless, currently antibodies and antibody fragments are still the most attractive affinity probes. In the analysis, it seems to be advantageous to use at least two binders, recognizing two non-overlapping epitopes of the same target (Uhlen and Hober 2009; Uhlen 2008; Stoevesandt and Taussig 2007). Mono-specific polyclonal antibodies are therefore highly attractive probes because of the cooperative effect of a mixture of antibodies that bind to several epitopes of a particular target protein (Nilsson *et al.* 2005).

To date, most antibody arrays have been produced from monoclonal and polyclonal antibodies obtained from commercial sources. Commercial antibodies, however, are generally manufactured to serve particular applications such as ELISA, Western blot, immunoprecipitation (IP), immunohistochemistry (IHC), or immunocytochemistry (ICC). Some antibody manufacturers are now starting to include information about the suitability for microarray applications in their data sheets. However, as there are about 22 000 protein-encoding genes in the human genome (Birney *et al.* 2007; Clamp *et al.* 2007), there is a considerable imbalance in antibody coverage. It is possible to find hundreds of antibodies against particular targets – for instance, there are more than 900 antibodies against p53 – while none are available for many others. To fill this gap, several initiatives exist for creating a global resource of well-characterized affinity reagents for an analysis of the human proteome (Table 10.1). Among the main tasks of these efforts are the generation of highly specific antibodies using high-throughput technologies, such as recombinant-antibody phage display

Table 10.1 Initiatives for a global production of human protein affinity reagents

Initiative	Website	Reference
Human Proteome Atlas	http://www.proteinatlas.org/	(Berglund *et al.* 2008; Uhlen *et al.* 2005)
ProteomeBinders	http://www.proteomebinders.org/	(Taussig *et al.* 2007)
Clinical Proteomic Technologies Initiative	http://proteomics.cancer.gov/	
Antibody Factory	http://www.antibody-factory.de/	(Konthur, Hust and Dübel 2005)

(Winter *et al.* 1994), ribosome display (He and Taussig 2005), RNA display libraries (Lipovsek and Plückthun 2004), bacterial surface display (Jostock and Dübel 2005), and yeast surface display (Levy *et al.* 2007), validation of the established antibodies, and developing novel non-antibody affinity reagents.

Protein samples

Sample processing procedures that isolate an organism's proteome in a representative and reproducible manner are another crucial factor. The complexity and the enormous dynamic range of molecule concentrations in a proteome as well as the susceptibility of proteins to even small changes in their environment provide a formidable challenge. However, once a protein sample is adequately brought into solution, whether in a denatured or native form, it can be subjected to antibody array analysis. However, protein preparation under native conditions could be advantageous, since functionality may be preserved and the detection of protein isoforms may be possible (Alhamdani *et al.* 2010), as these are important to be identified for pharmacological reasons, for example. High sample complexity could cause unspecific binding and complicate uniform labelling in other proteomic approaches. For antibody microarrays, however, it has been shown that depletion of highly abundant proteins had no significant impact on the quality of the assay (Schröder *et al.* 2010) but, in contrast, introduced a considerable bias in proteome representation.

Since plasma is easy to collect in a nearly non-invasive process, the application of antibody microarrays for studying this type of specimen has created much interest (Bergsma *et al.* 2010; Schröder *et al.* 2010; Lal *et al.* 2009; C. Li *et al.* 2009; Rimini *et al.* 2009; Zeng *et al.* 2010; Hon *et al.* 2008; Lukesova *et al.* 2008; Sun *et al.* 2008; Loch *et al.* 2007). Conditioned media of cultured cells has received similarly wide attention (Cai *et al.* 2009; L.L. Chen *et al.* 2009; Grassel *et al.* 2009; Gruber *et al.* 2009; Chou *et al.* 2008; Inai *et al.* 2008; Y.C. Lee *et al.* 2008; Ohshima *et al.* 2008; Perera *et al.* 2008; Seeber *et al.* 2008; Ebihara *et al.* 2007; Lu *et al.* 2007; Neuhoff *et al.* 2007; Sze *et al.* 2007; Huang *et al.* 2001). However, other types of specimens have also been investigated, such as urine (Schröder *et al.* 2010; Hu *et al.* 2009; Liu *et al.* 2006), prostatic fluid (Fujita *et al.* 2008), cerebrospinal fluid (Dhungana, Sharrack and Woodroofe 2009; Tsai *et al.* 2008), tears (Leonardi *et al.* 2009), saliva (Lal *et al.* 2009), exhaled breath (Barta *et al.* 2010), cultured cells (Wong *et al.* 2009; Lin *et al.* 2003; Sreekumar *et al.* 2001) and tissue biopsies (Zander *et al.* 2009; Y. Hao *et al.* 2008; Moschos *et al.* 2007; Hudelist *et al.* 2004; Anderson *et al.* 2003). Owing to the differences in proteome composition and complexity, sample processing should be optimized empirically. The tissue proteome for example is more complex and gives rise to higher background noise as compared to plasma (Haab, 2003). Procedures have been established to overcome this obstacle (Alhamdani *et al.* 2010).

Labelling and detection strategies

Once a protein sample is isolated, the subsequent analysis may involve either label-based or label-free processing. Currently, labelling is predominantly used for the assays because of its simplicity, robustness and sensitivity (Kusnezow *et al.* 2003, 2006a, 2007; Wingren *et al.* 2007; Zhou *et al.* 2004). Sensitivities in the range of picomolar to femtomolar can be achieved, which is comparable to ELISA but without the necessity of signal amplification (Wingren *et al.* 2007; Kusnezow *et al.* 2006a). Proteins can be labelled either directly with fluorescent dyes (such as Cy3 or Cy5 dyes) or indirectly with a hapten such as biotin or biotin derivatives, followed by incubation with a fluorescently tagged molecule (such as streptavidin in the case of biotin) that has a high affinity to the hapten. There is a strong preference for indirect protein labelling with one colour, as this approach has enhanced sensitivity compared to direct labelling (Kusnezow *et al.* 2007; Wingren *et al.* 2007). Recently, direct labelling using a two-colour approach has been reported, substantially improving microarray performance for reproducibility and discriminative power (Schröder *et al.* 2010).

Despite the favourable virtues of sample-labelling approaches, there are concerns that the introduction of too many label molecules might affect the epitopes required for the formation of the antibody–antigen complex. Hence, label-free techniques are currently gaining more recognition. The simplest example of a label-free method is the sandwich assay (Ekins 1998). This approach is similar to ELISA, in which a specific protein is targeted by two antibodies. The first antibody is arrayed on a solid support and serves as a capture reagent. A second antibody is required to bind to the same target molecule. Usually it carries a label directly or is in turn identified by a third labelled antibody. However, the need for two highly specific antibodies and particularly the lack of scalability for a complex array, are severely limiting factors for such an approach (Templin *et al.* 2004). No more than about 30 antibody pairs can be used at the same time. Other more promising methods are emerging and may replace labelling once their practical feasibility and competitiveness have been demonstrated. Compared to labelling, these new methods currently still exhibit lower sensitivity, the need for expensive materials or equipment and restrictions to multiplexing. Table 10.2 lists some of these technologies, which were reviewed in more detail by Ray, Mehta and Srivastava (2010).

Bioinformatics

Since the principle is nearly identical, antibody array data analysis and interpretation can be carried out using the means developed for DNA-microarray studies. A major difference is data normalization, since reliable house-keeping proteins for data

Table 10.2 Label-free detection technologies

Technology	Sensitivity/ resolution	Array complexity	Reference
Surface plasmon resonance (SPR)	10 ng/ml	High	(Nedelkov, Tubbs and Nelson 2006; Usui-Aoki *et al.* 2005)
SPR-imaging (SPRi)	nM-zM	Very high	(Ladd *et al.* 2009; Lausted, Hu and Hood 2008; Suraniti *et al.* 2007; H.J. Lee, Nedelkov and Corn 2006; Kyo, Usui-Aoki and Koga 2005)
Oblique-incidence reflectivity difference-based (OI-RD)		Very high	(Fei *et al.* 2008; Zhu *et al.* 2007)
Scanning Kelvin nanoprobe (SKN)	<50 nm	High	(Sinensky and Belcher 2007; Thompson *et al.* 2005; Cheran *et al.* 2004)
Atomic force microscope (AFM)	Picolitre volume	High	(Kim *et al.* 2009; Huff *et al.* 2004)
Nanowires and nanotubes	nM-fM	Very high	(Drouvalakis *et al.* 2008; L. Yang *et al.* 2008; Okuno *et al.* 2007; Zheng *et al.* 2005; Cui *et al.* 2001)
Biological compact disk (BioCD)	30–70 pg/ml	High	(Morais *et al.* 2008; X. Wang, Zhao and Nolte 2008)
Ellipsometry	1 ng/ml	High	(Valsesia *et al.* 2006; Z.H. Wang *et al.* 2006; G. Jin *et al.* 2004; Z.H. Wang and Jin 2003)
Microcantilevers	0.2 ng/ml	High	(Backmann *et al.* 2005)

normalization cannot be picked easily and array complexity is frequently insufficient to use the majority of (unchanged) signals for normalization (Royce *et al.* 2006). To overcome the problem, other approaches have been used, such as an internally normalized ratio algorithm after dual-colour labelling (Andersson *et al.* 2005), spike-in protein control(s) of known concentration (Hamelinck *et al.* 2005) or relative normalization to particular probes, which are also assayed by another method (e.g. ELISA) (Hamelinck *et al.* 2005). With normalized data at hand, subsequent analyses such as the search for differentially expressed proteins, clustering or validation by Western blotting or, reversely, qRT-PCR, are performed as for the results from DNA-microarray.

Antibody array applications

With their rapid development during the past 10 years, antibody microarrays have seen a rise in applications, especially for clinically relevant analyses and

Table 10.3 Application of antibody arrays in human studies

Disease	Reference
Allergy	(Leonardi *et al.* 2009; Zander *et al.* 2009)
Bone Diseases	(Jarvinen *et al.* 2008)
Brain Diseases	(Tsai *et al.* 2008; Hergenroeder *et al.* 2010)
Cardiovascular Diseases	(Dhungana, Sharrack and Woodroofe 2009; Lal *et al.* 2009, 2004)
Chronic Fatigue Syndrome	(Cho *et al.* 2009)
Cystic Fibrosis	(Srivastava *et al.* 2006)
Dermatitis	(Harper *et al.* 2010; Hon *et al.* 2008)
HIV	(Wu *et al.* 2007, 2008)
Bowl Disease	(Kader *et al.* 2005)
Kidney Diseases	(Hu *et al.* 2009; Kaukinen *et al.* 2008; Liu *et al.* 2006)
Muscle Diseases	(Anderson *et al.* 2003)
Pulmonary Diseases	(Barreiro *et al.* 2008)
Transplantation	(Hu *et al.* 2009; Lal *et al.* 2004)
Cancer	
Bladder	(Sanchez-Carbayo *et al.* 2006)
Breast	(Carlsson *et al.* 2008; Smith *et al.* 2006; Hudelist *et al.* 2004)
Colorectal	(Toh *et al.* 2009; Madoz-Gurpide *et al.* 2007; Spisak *et al.* 2010)
Gastric	(Ellmark *et al.* 2006)
Leukemia	(Scupoli *et al.* 2008; Belov *et al.* 2003, 2006)
Liver	(Lausted, Hu and Hood 2008; Sun *et al.* 2008; Nonomura *et al.* 2007)
Lung	(Barta *et al.* 2010; M.K. Han *et al.* 2009; Kullmann *et al.* 2008; Gao *et al.* 2005)
Lymphoma	(Belov *et al.* 2006)
Melanoma	(Moschos *et al.* 2007)
Multiple Myeloma	(Kline *et al.* 2007)
Pancreas	(Schröder *et al.* 2010; C. Li *et al.* 2009; Ingvarsson *et al.* 2008; S. Chen *et al.* 2007; Hamelinck *et al.* 2005)
Prostate	(Fujita *et al.* 2008; Iizumi *et al.* 2008; Shafer *et al.* 2007; Orchekowski *et al.* 2005)
Renal	(Lukesova *et al.* 2008)

investigations of the pathophysiology of human diseases. A broad range of human diseases has been investigated, predominantly in the field of oncology (Table 10.3). However, more basic biologically oriented investigations have also been performed (Table 10.4). Reviewing all applications in detail is beyond the scope of this chapter. In summary, however, the technique has demonstrated its potential and versatility in multiplexed proteomics profiling. Antibody arrays are promising tools for cancer diagnostics, biomarker discovery, therapy monitoring and the identification of new drug target leads (Alhamdani, Schröder and Hoheisel 2009; Chatterjee, Wojciechowski and Tainsky 2009; Loch *et al.* 2007; Borrebaeck, 2006; Sanchez-Carbayo 2006; Haab 2005).

Table 10.4 Biological fields of application

Angiogenesis	(Xi *et al.* 2009; Karaca *et al.* 2008; Ahn *et al.* 2006; Azizan *et al.* 2006)
Apoptosis	(Ray *et al.* 2010)
Biomarker discovery	(Schröder *et al.* 2010; Barber *et al.* 2009; M.K. Han *et al.* 2009; C. Li *et al.* 2009; Carlsson *et al.* 2008; Hao *et al.* 2008; Ingvarsson *et al.* 2008; Rivas *et al.* 2008; Sun *et al.* 2008; Loch *et al.* 2007; Shafer *et al.* 2007; Song *et al.* 2007; Wu *et al.* 2007; Christopherson *et al.* 2006; Sanchez-Carbayo *et al.* 2006; Spisak *et al.*)
Cell cycle analysis	(Uemura *et al.* 2009; Jamesdaniel *et al.* 2008; Tapias *et al.* 2008; X. Li *et al.* 2007);
Cell signaling	(Qi *et al.* 2009; Uemura *et al.* 2009; Wong *et al.* 2009; Zhong *et al.* 2009; Dewing *et al.* 2008; Iiizumi *et al.* 2008; Korf *et al.* 2008; Pelech *et al.* 2008; Skalnikova *et al.* 2008; Madoz-Gurpide *et al.* 2007)
Cytokine profiling	(L.L. Chen *et al.* 2009; Rads *et al.* 2009; Fenton *et al.* 2009; Gasparrini *et al.* 2009; Haddad and Belosevic 2009; Q. Hao, Wang and Tang 2009; Jiang *et al.* 2009; Takeda *et al.* 2009; Bandyopadhyay, Romero and Chattopadhyay 2008; Chang *et al.* 2008; Chou *et al.* 2008; Inai *et al.* 2008; K.B. Jin *et al.* 2008; Karaca *et al.* 2008; S. Li *et al.* 2008; Lu *et al.* 2008; Ohshima *et al.* 2008; Oscarsson *et al.* 2008; Huang 2007; Neuhoff *et al.* 2007; Watanabe *et al.* 2005; Foster *et al.* 2010; Yin *et al.* 2010)
Glycoproteomics	(C. Li *et al.* 2009; Zeng *et al.* 2010; Bereczki *et al.* 2007; S. Chen *et al.* 2007)
Monitoring of drug treatment and therapy	(Y.A. Lee, Cho and Yokozawa 2010; Cao *et al.* 2009; M.H. Chen *et al.* 2009; Cho *et al.* 2009; M.H. Lee *et al.* 2009; Qi *et al.* 2009; Zander *et al.* 2009; Jarvinen *et al.* 2008; K.B. Jin *et al.* 2008)
Phosphoproteomics	(Chung and Chin 2009; Fischer *et al.* 2009; Nystrom *et al.* 2009; Qi *et al.* 2009; Wong *et al.* 2009; Pelech *et al.* 2008; Rajala 2008; Gembitsky *et al.* 2004; Y.A. Lee, Cho and Yokozawa 2010)
Stem cell research	(Skalnikova *et al.* 2008; Tomchuck *et al.* 2008; Sze *et al.* 2007; Ko, Kato and Iwata 2005)

The microarray platforms used in these studies were either home-made or commercial. Currently, there are several manufacturers who provide arrays with a fixed antibody number (e.g. RayBiotech, www.raybiotech.com; Full Moon Biosystems, www.fullmoonbiosystems.com; Sigma, www.sigmaaldrich.com; R&D Systems, www.rndsystems.com) or offer customized arrays (e.g. Lampire Biological Laboratories, www.lampire.com; Kinexus, www.kinexus.ca). Generally, in studies involving home-made arrays, the focus was more on global proteomics profiling, while those with commercial arrays used them as a secondary tool among other methodologies. Interestingly, many home-made arrays have shown better functionality, performance and flexibility than those from commercial sources. Their superiority may result from the transparency of the system and its components to the operator, which has benefits for troubleshooting and tracking the sources of

error. Also, the propriety nature and the price of commercial kits and arrays may restrict the performance of replicate experiments.

Summary

Antibody arrays are powerful, robust and sensitive proteomic tools that enable a broad spectrum of applications, to date particularly in biomedical sciences and clinical research. They provide expression and/or conformational data on a currently still limited set of proteins consuming only small amounts of both antibody and protein. Many technical issues have been addressed successfully, improving the performance of the best protocols to and beyond that of ELISA assays, the current standard format of immuno-based protein analysis. Further improvement can be expected from the increase in the number and quality of the affinity reagents. Access to more antibodies or equivalent binder molecules of enhanced specificity and affinity will add substantially to the power of the technology. Technically, there is no major obstacle to scale-up microarray analyses to several 10 000 probe molecules The production of solid support media that preserve protein functionality and allow for a well-oriented attachment could improve resolution and detection limit and strongly affect the ability to discriminate between protein isoforms. The assay format could also benefit from ongoing developments in detection methodologies. Surface Plasmon Resonance imaging methods, for example, are currently showing sensitivities comparable to those of fluorescence-based imaging and may become an alternative in future. However, fluorescence is likely to develop to single-molecule sensitivity, thus enabling real quantitative counting of bound protein molecules rather than the relative measurement of signal intensity.

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REVIEW

Secretome profiling with antibody microarrays

Shakhawan Abdulrahman Mustafa,[ab] Jörg D. Hoheisel[a] and Mohamed Saiel Saeed Alhamdani*[a]

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Following the advances in human genome sequencing, attention has shifted in part toward the elucidation of the encoded biological functions. Since proteins are the driving forces behind very many biological activities, large-scale examinations of their expression variations, their functional roles and regulation have moved to the central stage. A significant fraction of the human proteome consists of secreted proteins. Exploring this set of molecules offers unique opportunities for understanding molecular interactions between cells and fosters biomarker discovery that could advance the detection and monitoring of diseases. Antibody microarrays are among the relatively new proteomic methodologies that may advance the field significantly because of their relative simplicity, robust performance and high sensitivity down to single-molecule detection. In addition, several aspects such as variations in amount, structure and activity can be assayed at a time. Antibody microarrays are therefore likely to improve the analytical capabilities in proteomics and consequently permit the production of even more informative and reliable data. This review looks at recent applications of this novel platform technology in secretome analysis and reflects on the future.

In the past few decades, the field of molecular biology has witnessed yet another leap forward, which is associated with the deciphering of the human genome. Genomics established itself as a field of molecular biology concerned with the elucidation and analysis of the information contained in the cellular nucleic acids. Genomic techniques have been developed that permit whole genome sequencing of individuals,[1] the unravelling of epigenetic modifications and gene regulation processes[2] as well as the identification of transcriptional variations,[3] for example. Despite the remarkable progress in our understanding of the complex biological processes

[a] *Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany. E-mail: m.alhamdani@dkfz-heidelberg.de; Fax: +49-6221-42-4687; Tel: +49-6221-42-2718*
[b] *Center of Molecular Biotechnology, University of Bonn, 53115 Bonn, Germany*

Shakhawan Abdulrahman Mustafa

Shakhawan Abdulrahman Mustafa earned his BSc in biology from Sulaimani University (2004), member of Kurdistan establishment for scientific research in Sulaimani, Kurdistan region, Iraq. He is currently finishing his master studies in molecular biotechnology at the University of Bonn and the Division of Functional Genome Analysis at the Deutsches Krebsforschungszentrum (DKFZ; German Cancer Research Center) in Heidelberg, Germany. His scientific interests are cancer proteomics, with focus on pancreatic cancer secretome and the role of microenvironment in disease progression and resistance to therapy.

Jörg D. Hoheisel

Jörg D. Hoheisel is Head of Division and Chairman of the Scientific Council at the Deutsches Krebsforschungszentrum (DKFZ; German Cancer Research Center) in Heidelberg, Germany. Prior to joining DKFZ in 1993, he worked for five years at the Imperial Cancer Research Fund in London, UK, the initial two years being funded by a post-doctoral EMBO fellowship. Previously, he had been trained as a molecular biologist at the University of Constance, Germany, where he also did his diploma and PhD graduation.

involved in disease pathogenesis at the level of nucleic acids, our insights into many other molecular levels remain incomplete and blurred. Already the surprisingly small number of (protein-encoding) genes found by genome sequencing indicated and emphasised the fact that regulation and activity occur at many molecular levels.[4] Genomic data are generally inadequate to predict dynamic protein properties, for example. Consequently, the scope of analysis was expanded beyond the merely genomic and transcriptomic approaches to address also events at the proteome level.[5–7]

The term proteome was coined in 1996 by Marc Wilkins and colleagues and is defined as the analysis of the complete set of proteins expressed in a cell or tissue.[8] Proteins are the molecules that execute many biological functions in a cell, and many regulatory processes take place at the protein level. The proportion and importance of protein modification is reflected by the fact that 5% to 10% of mammalian genes encode for proteins that modify other proteins. Proteins are involved in basically all vital biological processes in cells. Consequently, 98% of all therapeutic targets are proteins currently. Their obvious central role in understanding cellular activity at a molecular level promoted proteomics already early on as a second pillar of comprehensive molecular analyses.[9–11]

From proteomics to secretomics

As the proteome as a whole and the very many individual proteins continuously undergo dynamic changes, proteomics faces the challenge of detecting and analysing all these variations. For lack of processes for a really comprehensive investigation, studies are usually aiming at one major type of objective currently, such as functional[12] or structural aspects,[13] which deals with particular protein modifications, for example phosphoproteomics[14] or glycoproteomics,[15] or concentrates on a physiologically or biologically defined sub-proteome. One such sub-population is formed by the proteins that are secreted from cells into the extracellular medium. The term secretome refers to this class of proteins as released under defined conditions at a given time.[16] It is estimated that about 10% of all genes in the human genome encode for proteins of this class;[17] the *Secreted Protein Database* (http://spd.cbi.pku.edu.cn/) lists more than 18 000 entries of secreted proteins along with their sequences.[18] Secreted proteins are considered to be the main group of molecules for intracellular communication. They participate in most physiological processes, such as cell signalling, differentiation, invasion, metastasis, cell adhesion and binding, angiogenesis, and apoptosis. Common proteins in any secretome include cytokines, chemokines, hormones, immunoglobulins, neuroproteins, lipoproteins, growth factors and extracellular matrix degrading proteinases.[19–21] However, most secreted proteins are expressed during specific growth stages, by particular cell types or during specific cellular responses. Therefore, they could represent a reliable source of biomarkers in body fluids.[22,23]

Current techniques used in secretome analysis

Currently, mass spectrometry (MS), either coupled to other preparative and analytical methods or on its own, is the main method in proteomic research, even achieving in few cases a data quality and reproducibility that is sufficient for use in a clinical setting. The classical approach is a combination of two-dimensional gel electrophoresis and MS. More recently, a modified version called differential in-gel electrophoresis (DIGE) has improved performance at the gel-based part.[24–26] Overall, however, analysis is moving away from gel-based systems,[27] with chromatography and MS taking over. Multidimensional protein identification technology (MudPIT) and isotope-coded affinity tags (ICATs) are two more recently developed methods in this field. There are also numerous MS-techniques and adaptations; matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS), surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS), and electrospray ionisation mass spectrometry (ESI–MS) are the most prominent forms.

Antibody microarrays

Antibody microarray analyses represent a methodology that is complementary to the MS techniques, adding quite a few features toward an overall comprehensive analysis.[23,28–33] As depicted in Fig. 1, the procedure is equivalent to the chip-based transcriptional profiling analyses. Antibodies (or other appropriate binder molecules) are arrayed on a solid support. The relevant protein mixture of interest is isolated, labelled with a fluorescence dye and applied to the array. As with transcript analyses, two samples labelled with two dyes can be applied at a time. Signal intensities obtained at the various binder molecules provide the basic information.

Antibody microarray analyses have the big advantage that different kinds of data can be obtained in a single assay. Aspects that can be studied include variations in the abundance of proteins,[23] the occurrence of structural differences in the form of protein isoforms[34] or protein modifications[35] and the definition of biochemical activities and regulative

Mohamed Saiel Saeed Alhamdani

Mohamed Saiel Saeed Alhamdani earned his BSc in chemistry from the College of Science, Al-Mustansiriya University and his MSc in clinical biochemistry from the College of Medicine, Al-Mustansiriya University in Baghdad, Iraq. He worked as a Junior Research Scientist and Lecturer at the College of Pharmacy, Al-Mustansiriya University. He is currently finishing his PhD in molecular biology at the Faculty of Biosciences, University of Heidelberg and the Division of Functional Genome Analysis at the Deutsches Krebsforschungszentrum (DKFZ; German Cancer Research Center) in Heidelberg, Germany.

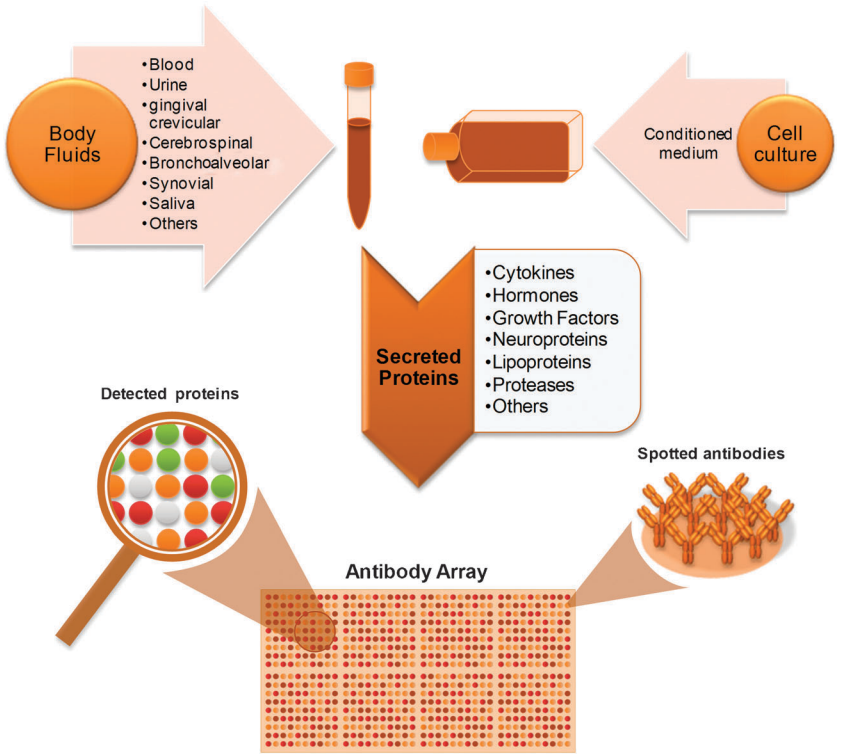

Fig. 1 Scheme of the protein profiling process with antibody microarrays. Proteins are isolated from the respective samples, mostly labelled with a fluorophor and applied to the array. If two different samples are applied that are labelled with different dyes, not only signal intensity but also the colour at the spots provides information about variations in protein abundance.

processes by virtue of detecting interaction partners, for example. Technically, assay processes have been established which permit analyses with a degree of robustness and reproducibility that meets the requirements of clinical applications.[23] Although in complex analyses a sandwich assay format is made impossible by the number of antibodies displayed on the array platform, specificities better than those achieved with ELISA assays are possible. Mass transport and kinetics were identified as factors that limited severely the speed and the sensitivity of analysis. To overcome this, appropriate processes were established that on standard detection devices permit sensitivities in the attomolar range without signal amplification[36,37] and allow to reach binding equilibrium and thus reproducible and quantifiable measurements within reasonable time periods. More recently, using appropriate hardware, sensitivities down to single-molecule detection were achieved.[38] Also, various auxiliary facets such as appropriate protocols for protein extraction have been established,[39] although this is a relatively minor aspect with regard to secretome analysis.

The main obstacle for thorough proteome studies is the availability of antibodies or other binders.[40] There are many different types of affinity reagents available today. However, currently still missing is a comprehensive set of binders of appropriate specificity and affinity that covers all human proteins. Efforts are ongoing, however, toward the provision of a global resource of well-characterised affinity reagents for the mapping of the human proteome (*e.g.*, *ProteomeBinders*, www.proteomebinders.org; *Affinomics*, www.affinomics.org; the *Clinical Proteomic Technologies Initiative*, proteomics.cancer.gov; and the *Antibody Factory*, www.antibody-factory.de). The *Human Proteome Atlas* activity (www.proteinatlas.org) is currently the most advanced initiative.[41] While availability of binders for all kinds of analysis purposes will take a long time still to be established, considering that there are probably more than a million of protein isoforms and modifications, a set of molecules for the detection of a "basic human proteome" of some 22 000 proteins (assuming one protein per human gene) will be available soon.

Applications of antibody microarrays in secretome analysis

There are numerous reports about antibody microarray analyses, ranging from small concept studies to actual proteome expression profiling efforts with several hundred antibodies, using a large variety of platforms of home-made or commercial design. Apart from many technically oriented reports, the objectives were manifold, including the identification of disease associated biomarkers,[42] cell phenotyping,[43] bacterial serotyping,[44] oncoproteomic analyses,[7] investigation of drug abuse,[45] or the definition of signatures of hereditary diseases.[46] The secretomes from many sources were studied, such as the protein content of plasma or serum, urine, cerebrospinal fluid, tears, saliva and of conditioned media of cultured cells. Investigations of human plasma samples represent the largest group of reports. They have been reviewed before,[47,48] however, and are not discussed herein. Instead, this review focuses on the profiling of cellular secretomes and body fluids other than serum or plasma.

Table 1 Applications of antibody microarrays in cellular secretome analyses

Cells (stimulus)	Antibody targets	Biomarkers	Ref.
Stem cells			
Rat bone marrow-derived mesenchymal progenitor cells	Cytokines	TIMP-1, MCP-1, VEGF-164, CINC-2	49
Human embryonic stem cell-derived mesenchymal stem cells HuES9.E1	Cytokines	IGFBP2, TIMP1 and TIMP2	50
Adipose tissue-derived stem cells	Cytokines	CXCL5	51
Epithelial cells			
Normal ovarian surface cells	Cytokines	LIF, IL-10 and IL-4	52
Human bronchial epithelial cell line BEAS-2B	Cytokines	MCP-1, IL-8, RANTES, ENA-78, GROα, VEGF, CXCL16, MMP-9	53
BEAS-2B (carbon nanotubes)	Cytokines	IL-6, IL-8 and MIF	54
Human prostate cells (IFN-γ, IL-1β and IL-2)	Cytokines	IL-6, IL-8, GROα, ENA-78, CXCL-16 and MCP-1	55
Retinal pigment cells	Angiogenesis	IL-6, IL-8, MCP-1, TIMP-1, TIMP-2, VEGF	56
Lens epithelial cells	Cytokines	TGF-β2, IL-4 and VEGF	57
Thymic epithelial cells	Cytokines	IL-6, IL-8, GRO, GRO-α and MCP-1	58
Other non-cancer cells			
Prostatic stromal cells	Cytokines	MCP-1	55
Trabecular meshwork cells	Cytokines	TGF-β2, IL-4 and VEGF	57
Periodontal ligament fibroblasts	Cytokines	IL-1, -6, -8, -10, MCP-1, -2, -3, GDNF, VEGF and IGFBP-2	58
Human bone marrow mononuclear cells (HBMC)	Cytokines	IL-6, IL-8, GROα, ENA-78 and CXCL-16	59
Human umbilical cord blood derived mononuclear cells (EGF, FGF, all-trans retinoic acid)	Cytokines	IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-16, Ang, VEGF, BDNF, GDNF, NT-3, NT-4, PDGF-B, EGF, HGF, MCP-1, MCP-4, MIP-1β, MIP-3α, SDF-1, Etx-2, PARC, MIG and GRO	60
Human stromal cell line HS-5 (drug treatment with Ara-C, Dau, Dox and Vin)	Cytokines	CKβ, IL-12, IL-13, IGFBP-2, MCP-1, MCP-3, MCP-4, MDC, MIP-1β and MIP-1σ	61
Astrocytes and primary cortical neurons (Huntingtin protein)	Cytokines	CCL5 and RANTES	62
Monocyte derived macrophages U937, human gingival and pulp fibroblasts (triethylene glycol dimethacrylate)	Cytokines	MCP-1	63
Ganglion neuron SC-DRG (cryo-shock)	Cytokines	IL-1α, MIP-4, MIP-5, leptin, IL-15, ICAM-1, TNFRI and TNFRII	64
Human dermal fibroblasts, human dermal microvascular endothelial cells, WM1158 melanoma cell line	Cytokines	IL-8, MCP-1 and TIMP-2	65
Human umbilical vein endothelial cells	Cytokines	VEGF, IL-8 and amphiregulin	66
Annulus cells (prostaglandin E1)	Cytokines	IL-6 and EGFs	67
Human myeloma ILKM-3	Cytokines	IL-1α, IL-6, IL-8, RANTES, TNFα-receptor 1, VEGF and CTLA	68
Macrophage (bacterial material)	Cytokines	TNF-α, IL-6, IL-17, MCP-1, RANTES and IFN-γ	69
Carcinoma associated fibroblast	Cytokines	VEGF and IL-1α	70
Major pelvic ganglia, penile smooth muscle cells	Cytokines	CXCL5	51
Preantral mouse follicles (hMG and rFSH)	Cytokines	TECK, sTNFRI and SDF-1α	71
Dental pulp and odontoblasts (TGF-β1)	Cytokines	IL-1α, -1β, -2, -6, -7 and -12	72
Neurons (glucose/oxygen/serum deprivation)	Cytokines	TGF-β1, GDNF, NT-3 and leptin	73
Endothelial cells (Angiotensin-II)	Other selection	IP-10	74
Cancer cells			
Breast cancer cell lines MCF-7, MDA-MB231	Cytokines, angiogenesis	VEGF, IL-8, amphiregulin. MCP-1 and IL-6	66, 75
MCF-7 (leptin)	Cytokines	FGF9, TNFβ, MCSF, IGFBP-3 and TGF-b3	76
Non-small cell lung cancer cell lines A549, H1299	Cytokines	MCP-1, IL-8, RANTES, ENA-78, GROα, VEGF, C–X–C motif, CXCL16 and MMP-9	53
Myeloid leukaemia cell line K562	Cytokines	CKβ, IL-12, IL-13, IGFBP-2, MCP-1, MCP-3, MCP-4, MDC, MIP-1β and MIP-1σ	61
Epithelial ovarian carcinoma	Cytokines	LIF, IL-10 and IL-4	52
Prostate cancer cell lines PC-3, LNCaP, C4-2B	Cytokines	IL-1α, MIP-4, MIP-5, leptin, IL-15, ICAM-1, TNFRI, TNFRII, MCP-1, IL-6, IL-8, GROα, ENA-78 and CXCL-16	59, 64
Prostate cancer cell line (quercetin and kaempferol)	Cytokines	GM-CSF	77
Primary renal cell carcinoma A-498 cells (IL-4, TNF-α)	Cytokines	IL-4, TNF-α, IL-8, TIMP-1, GRO, IL-6, MCP-1, M-CSF, GDNF, HGF and RANTES	78
Thymic carcinoma cell line ThyL-6	Cytokines	IL-1α, IL-6, IL-8, RANTES, soluble TNFα-receptor 1, VEGF and CTLA	68
Oral squamous cell carcinoma cell lines Ca9.22, YD-38 (CCL7)	Cytokines	VEGF and IL-1α	70

Cellular secretomics

Most investigations on conditioned media from cultured cells used a sandwich-assay format for detection, which has the advantage of being able to deal with low protein concentrations, since no labelling is required, but has the disadvantage that the degree of multiplexing is very limited. Therefore, only few molecules can be studied at a time, rendering the whole approach of a highly parallel assay format less useful. In most studies, arrays from commercial sources with antibodies against cytokines were applied; only few studies aimed at other target molecules (Table 1). This restriction to a particular, although important class of proteins is strongly limiting the amount of information that could be gathered from the studies. In addition, the analyses done to date are individually motivated experiments; no concerted activity has yet been organised.

The cells used in the studies were of diverse origin (Table 1). Next to stem cells and stem cell-like cells, also a large body of other cell types has been utilised. Quite a few of them were epithelial cells. In addition, conditioned media from different cancer cell lines were subjected to an analysis on antibody microarrays as part of a molecular assessment of several types of cancer such as lung, breast, ovarian, renal, prostate, thymic and oral cancer as well as leukemia. Some of the cellular systems looked at were not cell lines but had been obtained directly from animals and human tissues.

Co-culturing cells with conditioned media is an interesting scheme for analysing the effects of secreted proteins on microenvironment and cell-to-cell communication. Furthermore, a wide range of secretomics expression analyses was performed after various types of stimulus or induction (Table 1). This ranged from cryotherapy effects and glucose/oxygen/serum deprivation to inductions with factors such as hormones, growth factors, cytokines, chemicals, proteins or bacterial extracts. In addition, secretome profiling was used to explore biological events like cell differentiation, prospective therapy, angiogenesis, neurotrophic action, infantile aphakic glaucoma, prostatic enlargement, inflammation, bone resorption and cytotoxicity.

Body fluid secretomics

As it is obvious that cellular secrets will be transferred to the body fluids, secretome profiling was also performed on various body fluids (Table 2). Unlike the platforms applied to analysing cellular secretomes, the array content used in these studies varied a lot in terms of the number of printed antibodies and the kind of proteins targeted by the antibodies. There have been only relatively few publications about such analyses to date, which is surprising given the fact that body fluids can be obtained easily and may provide in part an even non-invasive means of diagnostics. Maybe the relatively trivial fact that a high protein concentration is needed for a successful labelling has hampered progress. Since water itself interferes with the standard ester reactions that are frequently used for attaching directly a fluorescence label to the proteins,

Table 2 Applications of antibody microarrays in body fluid analyses

Body fluid	Disease/condition	Antibody targets	Biomarkers	Ref.
Urine	Pancreatic cancer	Other selection	TSN8, TBB5, TRI22, AKA12, TEP1, MLP3B and RBM3	23
	Systemic juvenile idiopathic arthritis	Other selection	TIMP1, IL-18, P-Selectin, MMP9 and L-Selectin.	79
	Prostate cancer	Cytokines	IL-18BP	80
	Chronic kidney disease	Other selection	MIG, IP-10, MIP-1delta, osteoprotegerin	81
		Cytokines	LIX, MCP-1, beta-NGF and TIMP-1	82
	Chronic kidney disease	Cytokines	MCP-1, RANTES, TIMP-1, TNF-alpha, VEGF, E-selectin, Fas, IL-2, MMP-2, TGFβ	83
Tears	Contact lenses	Other selection	Cystatin, secretoglobin, lysozyme and S100 A8	84
	Vernal kerato conjunctivitis	Stationary phase proteins	IL-4, IL-5, IL-8, IL-10, bFGF, HB-EGF, VEGF, HGF, MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10	85
	Allergy	Stationary phase proteins	IL-8	86
	Giant papillary conjunctivitis	Other selection	Eotaxin, eotaxin-2, IL-4, IL-6, IL-6sR, IL-7, IL-11, MCP-1, MIP-1delta, MIG, TIMP-2 and M-CSF	87
	Allergy	Membrane-bound proteins	EGF, MCP-1, VEGF, IL-8, TIMP-1, -2, ANG, IP-10, GRO, ENA-78 and MIP-3α	88
Cerebrospinal fluid	Idiopathic intracranial hypertension	Cytokines	CCL2	89
	Spinal cord injury	Cytokines	IL-6, -8, MCP-1, NAP-2, ICAM-1, soluble Fas, TIMP-1 and MMP-2, -9	90
Synovial fluid	Rheumatoid arthritis and osteoarthritis	Chemokines	MDC, CTACK, ENA78, SDF1α, TECK, IP10, XCL1, MCP1, Eotaxin2, NAP2	91
	Arthritis	Other selection	Citrullinated fibrinogen	92
Bronchoalveolar lavage fluid	Hyperoxia	Cytokines	LIX, sTNF-R1, MIP-1γ, IL-6, MCP-1	93
Wound fluid	Chronic venous leg ulcers	Cytokines	IL-1α, β, MIP-1delta, IL-8, MIP-1α, Lcn-2, TLR-2 and TLR-4	94
Prostatic fluid	Prostate disease	Cytokines	HGF and IL18Bpa	95
Gingival crevicular fluid	Chronic periodontitis	Cytokines	TIMP-2, TNF-beta, GRO, IP-10, Ang, VEGF,IGFBP-3, OPG, EGF, GDNF, PARC,OSM, FGF-4, IL-16, LIGHT and PlGF	96
Saliva	Chronic obstructive pulmonary disease	Cytokines	IL-8, TIMP-1, EGF, MCP-1 and IP-10.	97

achieving a high protein concentration prior to labelling is crucial for success.

Conclusions and perspectives

The non-reductionist approach in assessing biological phenomena has opened a new era in the detection, management and monitoring of diseases. Deciphering the human genetic code triggered a chain reaction toward addressing at a similar level other molecule classes, in particular proteins and the proteome as a whole. The intimate involvement of proteins in basically all biological processes and their importance in regulating activities in cells and tissues made them already the prime target for drug administration. Also, there is a continuous increase of knowledge and subsequent utilisation with respect to protein-based molecular diagnostics. Secreted proteins represent a significant fraction of the proteome, are relatively easily accessible and are likely to be a promising source of biomarkers due to the numerous variations already observed between the protein profiles under normal and diseased conditions.

The technology, although new and still hampered by the relatively small number of available binder molecules, has demonstrated its potential for an increasing number of applications in secretome profiling. Particularly the ability to scale the degree of multiplicity not only with the number of analytes that should be studied but also with biologically relevant aspects, such as protein structure, amount and interaction, is a factor that is crucial for eventually successful application. At the same time, immunoassays are processes that are well known and established in many fields, including the most demanding pharmacological and clinical settings.

While there are enormous differences in the performance parameters of the reported systems, depending on the protocols and materials used, systems have been described that surpass standard assays such as ELISA by far in throughput, sensitivity, selectivity and cost and are clearly of a quality sufficient for applications. In these cases, the actual sensitivity, specificity and selectivity depend on the binder molecules only. The major challenge left for really comprehensive analyses is therefore getting more and better antibodies or other binders in sufficient numbers.

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Single-Step Procedure for the Isolation of Proteins at Near-Native Conditions from Mammalian Tissue for Proteomic Analysis on Antibody Microarrays

Mohamed Saiel Saeed Alhamdani,*,† Christoph Schröder,† Jens Werner,‡ Nathalia Giese,‡ Andrea Bauer,† and Jörg D. Hoheisel†

Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany, and Department of General Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany

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The process of extracting comprehensive proteome representations is a crucial step for many proteomic studies. While antibody microarrays are an evolving and promising methodology in proteomics, the issue of protein extraction from tissues for this kind of analysis has never been addressed. Here, we describe a single-step extraction buffer for the isolation of proteins from mammalian tissues under native conditions in an effective and reproducible manner. Protein was extracted from cell lines BxPC-3 and SU.86.86, rat organs (pancreas, liver, heart and lung) and human pancreatic cancer tissues using several buffer systems that contained individual nonionic or zwitterionic detergents in comparison to commercial extraction buffers. Also, detergent combinations were used that included at least one polymeric phenylethylene glycol, a long-chain amidosulfobetaine, cholate and a zwitterionic detergent. Extracts were analyzed for protein quantity and quality. The detergent cocktails exhibited superior extraction capacity. Additionally, they demonstrated a substantially higher recovery of membrane and compartmental proteins as well as much better preservation of protein functionality. Also, they did not interfere with subsequent analysis steps such as labeling. In Western blot and antibody microarray assays, they outperformed the other buffer systems, indicating that they should also be useful for other types of proteomic studies.

Keywords: Antibody microarray • protein extraction • detergents • compartmentalized protein

Introduction

Among the advanced methodologies that are being developed for analyzing the proteome, antibody microarrays emerge as a promising tool for gathering information at a global level that is required for the detection of disease-relevant variations, monitoring of disease progression and a better understanding of disease biology.[1,2] Antibody microarrays are an intrinsically robust and semiquantitative system that performs parallel measurements on sets of known proteins at a high-throughput.[3,4] By direct labeling of protein samples or label-free detection, the analysis is scalable to nearly any complexity. As in all kinds of proteomic studies, extraction and solubilization of proteins is a critical factor for the experimental outcome.[5] Owing to the complexity of the proteome, the enormous dynamic range in concentration, compartmentalization of proteins within cells and tissues as well as the susceptibility of proteins to minimal changes in the milieu and their relative abundance, an appropriate processing of a sample for proteomic analysis is a challenging task. The majority of recent antibody microarray studies analyzed serum samples. However, other types of specimens were investigated as well, such as extracts of cell surface proteins,[6] cultured cells[7] or tissue biopsies.[8] Proteins were mostly isolated by procedures adapted from immunoblotting or enzyme-linked immunosorbent assay (ELISA), or extraction buffers from commercial sources were used. However, buffers for immunoblotting or ELISA are aiming at the extraction of certain proteins or a group of proteins rather than a full representation of the cellular proteome. Commercial buffers, on the other hand, are frequently unsatisfactory due to a rather limited extraction capacity and/or difficulties in identifying the source of error in downstream analysis owing to the unknown nature of their formulation.

We are involved in studies of global expression variations by means of antibody microarray analysis. To this end, we established the processes for production, incubation and target labeling.[9−11] However, protein preparation remained a critical and complicated factor, especially for the analysis of tissues. Optimized procedures for isolating and solubilizing proteins for gel-based studies[12] or mass spectrometry[13] were found inapplicable to antibody microarrays. On the basis of recent experiences made for the isolation of protein fractions,[14] we

* Corresponding author: Mohamed Saiel Saeed Alhamdani, Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany. Tel.: [49] (6221) 42-4684. Fax: [49] (6221) 42-4687. E-mail: m.alhamdani@dkfz.de.
† Deutsches Krebsforschungszentrum (DKFZ).
‡ University of Heidelberg.

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looked into processes for isolating proteins in a comprehensive and simultaneously gentle manner, which deliver proteins in a near-native state. In addition, we aimed at a simple and highly reproducible process. Detergent-based protein extraction is one of the easiest, highly efficient and least harsh methods for isolating proteins from biological samples.[15] However, no zwitterionic or nonionic detergent and no detergent mixture was reported to solubilize all proteins.[5] Detergent selection for solubilization conditions has been an empirical and experimental process for a given set of samples.[16] Using a combination of detergents, which adds to the mixture the best properties of each, is likely to be more effective than using detergents individually.[17] Here, we describe a protein extraction formula for a one-step isolation of proteins from cell culture or mammalian tissues that is highly reproducible and effective as well as compatible with analysis on antibody microarrays. The buffer extracts proteins under near-native conditions and achieves an enrichment of membrane-associated and compartmentalized proteins.

Materials and Methods

Materials. All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise stated and were of highest purity or protein grade. Thirty-eight polyclonal and monoclonal antibodies were purchased from different commercial sources and used for printing the test array. Ten of these antibodies were also used in the Western blot experiments.

Buffer Formulation. The basic buffer composition was formulated after several tests. It consists of 20 mM HEPES buffer, pH 7.9, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF, 1 U/μL of Benzonase, 1× Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Bonn, Germany) and detergent(s). When detergents were added individually, their final concentration was 1% (w/v) for Nonidet P-40 substitute (NP-40S), Triton X100 (TrX100) and cholic acid sodium salt (Cholate) or 0.5% (w/v) for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Serva, Heidelberg, Germany), 3-(4-heptyl)phenyl-3-hydroxypropyl)dimethyl-ammoniopropanesulfonate (C7BzO), amidosulfobetaine-14 (ASB-14), *n*-dodecyl-β-D-maltoside (12-Malt) (GenaXXon Bioscience GmbH, Ulm, Germany) and *n*-octyl-β-D-glucoside (8-Glu). Also, two detergent cocktails were used: Mix1 was composed of the basic buffer and 0.5% TrX100, 0.5% NP-40S, 0.25% 12-Malt, 0.25% ASB-14 and 0.25% CHAPS; Mix2 contained as detergents 1% NP-40S, 0.5% cholate, 0.25% 8-Glu and 0.25% ASB-14. In addition, several commercial buffers were tested for their protein extraction efficiency. Because of their superior performance within the group of commercial buffers, the Q-Proteome Mammalian Protein Prep Kit (Q-Prot; Qiagen, Hilden, Germany) and the Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific) were selected for a more detailed comparison.

Protein Extraction from Cell Culture. The pancreatic cancer cell lines BxPC-3 and SU.86.86 were used in this experiment. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics at 37 °C. At 90% confluence, cells were collected by trypsination, washed three times with ice-cold PBS, lysed with the respective extraction buffer (10-times the volume of the packed cells), kept on ice for 30 min with occasional mixing, and then centrifuged at 20 000*g* for 20 min at 4 °C. The supernatant was aspirated with a fine needle in order not to disturb the upper layer or the pellet. Protein concentration in the supernatant was determined with the bicinchoninic acid (BCA) Protein Assay Reagent kit of Thermo Scientific (Bonn, Germany).

Protein Extraction from Tissues. Liver, heart, lung and pancreatic tissues were dissected under sterile conditions from adult male Wistar rats and stored at −80 °C until further processing. The animals were kept in accordance with the institutional and national guidelines. Twelve human pancreatic cancer tissue samples were selected randomly from a set of about 400 samples provided by the Surgery Department of the University of Heidelberg. Written informed consent was obtained from all patients and the local ethics committee of the University of Heidelberg approved the work.

Tissue samples were either immediately homogenized in 5-fold by weight the amount of extraction buffer with the aid of an Ultra Turrax homogenizer (IKA Werke GmbH, Staufen, Germany), a Dounce or a Potter-Elvehjem homogenizer, respectively. Alternatively, tissues were snap-frozen in liquid nitrogen followed by pulverization with a porcelain mortar and pestle in presence of liquid nitrogen and immediately resuspended in 5-fold by weight the amount of extraction buffer. Homogenates were incubated on ice for 30 min with occasional mixing and processed the same way as the cell culture samples.

Enzymatic Activity Assays. All enzymatic analyses were performed with unlabeled protein. Labeling of the protein samples with fluorescent dyes for incubation on the antibody microarrays may well have an effect on protein functions.

1. Gamma-glutamyltranspeptidase (γ-GT) Activity Assay. The activity of membrane bound enzyme γ-GT was assessed in protein extracts according to the procedure of Shaw et al.[18] In brief, the reaction mixture contained 4.4 mM γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 11 mM $MgCl_2$ and 100 mM Tris-HCl buffer (pH 7.9) in a final volume of 100 μL. The reaction was started by the addition of 20 μL of sample extract to the reaction mixture in a 96-well plate. The change in absorbance was monitored at 405 nm and 25 °C for 5 min in a Tecan Infinite-200 plate reader (Crailsheim, Germany). The activity was calculated from the extinction coefficient of *p*-nitroanilide (9900 M^{-1} cm^{-1}) and expressed as international units per milligram of protein (IU/mg protein).

2. Glutathione *S*-transferase (GST) Activity Assay. The total activity of GST (cytoplasmic and membrane bound) was determined in extracted protein samples as described previously,[19] with the modification of using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture was composed of 1.25 mM reduced glutathione, 1 mM CDNB, 1% ethanol and 100 mM potassium phosphate buffer, pH 6.5. For each sample, 5 μL was transferred into a well of a 96-well plate and the reaction was initiated by adding 215 μL of the reaction mixture. The increase in absorbance was followed at 340 nm and 25 °C for 10 min using the Tecan Infinite-200 plate reader. The activity was calculated from the extinction coefficient of 9600 M^{-1} cm^{-1} for CDNB.

3. Lactate Dehydrogenase (LDH) Activity Assay. LDH was assayed according to Babson and Babson.[20] Briefly, a color reagent was prepared by dissolving 40 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT), 100 mg of β-nicotinamide adenine dinucleotide oxidized (NAD) and 10 mg of phenazine methosulfate (PMS) in 20 mL of water. A sample volume of 20 μL was transferred into a well of a 96-well plate followed by the addition of 200 μL of substrate solution containing 50 mM lactate in 100 mM Tris-HCl buffer, pH 8.2. The temperature was adjusted to 30 °C. Then, 40 μL of the color reagent was added and the increase in absorbance

was monitored at 503 nm for 2 min using the Tecan Infinite-200 plate reader. LDH specific activity was calculated from the extinction coefficient of INT (19 300 M^{-1} cm^{-1}).

SDS-PAGE and Western Immunoblot Analysis. Samples of protein extracts were diluted in 2× Laemmli buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue), boiled in a heating block for 5 min and then loaded onto a 10% polyacrylamide gel for electrophoretic separation. Subsequently, the gels were either stained with EZBlue following the manufacturer's instructions or the proteins were transferred to a PROTRAN nitrocellulose membrane (Whatman, Dassel, Germany). The membranes were blocked with phosphate-buffered saline supplemented with 0.1% Tween-20 (PBST) and 5% nonfat dry milk (Bio-Rad, Munich, Germany). Following blocking, the membranes were incubated with primary antibodies at the adequate dilution in PBST supplemented with 5% milk overnight at 4 °C. Incubation with peroxidase-labeled secondary rat or mouse antibodies that bind to all primary antibodies was conducted at room temperature for 1 h, followed by extensive washing with PBST. Bands were visualized after addition of the Amersham ECL advanced Western blotting detection kit (GE Healthcare, Buckinghamshire, U.K.) with the aid of a Fujifilm LAS-3000 Documentation System (Fujifilm, Tokyo, Japan).

Antibody Microarray Printing. For assaying quantitatively the performance parameters of complex protein extracts on antibody microarrays, 38 mouse and rabbit antibodies from different sources were spotted six times on epoxy-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, U.K.) and SMP3B pins (Telechem, Sunnyvale, CA) at a humidity of 40−45%. The printing buffer was composed of 0.1 M borate buffer, pH 9.0, containing 0.006% Igepal CA-630, 0.05% sodium azide, 1% Trehalose and 1 mg/mL of the respective antibody. After printing, the slides were allowed to equilibrate at a humidity of 40−45% overnight and then stored in dry and dark conditions at 4 °C.

Protein Labeling with Fluorescent Dyes. The NHS-esters of the dyes DY-649 and DY-549 (Dyomics, Jena, Germany) were used for labeling extracted proteins. Samples from human pancreatic cancer tissue or cell culture were adjusted to a protein concentration of 4 or 2 mg/mL, respectively. The labeling reaction occurred in the dark in 0.1 M carbonate buffer, pH 8.5, with a dye/protein ratio of 30:1 mol at 4 °C for 2 h. An amount of 400 μg of protein was mixed with 200 nmol dye in a final volume of 200 μL of buffer. An average molecular weight of 60 000 Da was assumed for cellular proteins to calculate the above dye/protein ratio. Unreacted dye was removed using Zeba Spin Columns (Thermo Scientific) with buffer exchange to PBS according to the manufacturer's recommendation. After labeling, the measured dye/protein ratio was within a range of 0.75−1.25 in all experiments. Labeled samples were stored at −20 °C until use.

Incubation, Scanning, and Image Processing of the Microarrays. All subsequent procedures were performed in the dark. Before incubation with labeled proteins, slides were washed 4 times (5 min each) with PBST. Following washing, the slides were blocked with 5% nonfat dry milk in PBST for 3 h at room temperature. Blocked slides were incubated with 75 μg each of DY-549- and DY-649-labeled protein diluted in 5 mL of PBST, 5% milk at 4 °C overnight. The slides were then washed 4 times (5 min each) with PBST, rinsed with deionized water and dried in a ventilated oven at 37 °C. Scanning of the slides was performed using a ScanArray-4000XL (Perkin-Elmer, Waltham, MA) at constant laser power and PMT. The images were analyzed with the software GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). The detection limit was similar to that reported previously.[10,11]

Data Analysis. Results are presented as means (±SD) unless otherwise specified. Microarray data were analyzed without normalization using the software Acuity 4.0 (Molecular Devices, Sunnyvale, CA). Since each sample was labeled with both DY-549 and DY-649, the sum of medians was applied for comparing target signal intensities among tested buffers. The sum of medians is the sum of the medians of the pixel intensities at each wavelength, with the median background pixel intensity at each wavelength subtracted (GenePix Pro 6.0 analysis software tutorial, http://www.moleculardevices.com). The median of feature signal and background intensity was used to determine quality control measures like signal-to-noise ratio (SNR) and signal intensity thresholds. The SNR in both color channels was calculated as the mean (from three replicate arrays) of median signal intensity (background subtracted) divided by the standard deviation of the median local background intensities. Cutoff values of (mean − 1 × SD) for SNR and median signal intensity were used to threshold features in compliance with the quality measures. Statistical significance was determined with SSPS 16.0 for Windows (SPSS, Inc., Chicago, IL) using one-way analysis of variance (ANOVA) with post hoc LSD test for multiple analyses between groups. Results with p-values less than 0.05 were considered to be significant.

Results and Discussion

Like other proteomic methodologies, antibody microarrays make use of samples from different sources, ranging from material that requires minimal to no protein extraction, such as body fluids, to those that entail protein isolation step(s), such as cells in culture or tissue samples. As opposed to mass spectrometry analyses, the performance of antibody microarrays is strongly influenced by factors like protein structure (e.g., native or denatured), which is affected by the isolation procedure. In consequence, the protein extraction process is more critical than for methods which work with denatured molecules. However, in turn, this sensitivity permits studies on protein isoforms, for example, which are of particular interest for many pharmaceutical purposes. Therefore, we were interested in a process that preserves proteins in a near-native state.

Another major obstacle for establishing an appropriate protein extraction procedure was the requirement for compatibility of the resulting extracts with subsequent analytical procedures. Interfering substances that may affect protein quality or essential experimental processes, such as protein labeling, which is strongly influenced by the presence of nucleic acids, reductants or quenchers, for example, could influence the outcome of studies significantly. Also, complicated protocols have an intrinsically higher chance of introducing experimental bias. Thus, we aimed at a protocol, which consisted of a single incubation subsequent to the physical disruption of the cellular material.

Our criteria for formulating the basic composition of the protein extraction buffer were based on several factors: extraction should occur in a single step under mild, nondenaturing conditions; there should be no compounds that contain primary amines or thiols, which inhibit the fluorescence labeling; the effect of interfering biomolecules, such as nucleic acids should be minimized; and most importantly, solubiliza-

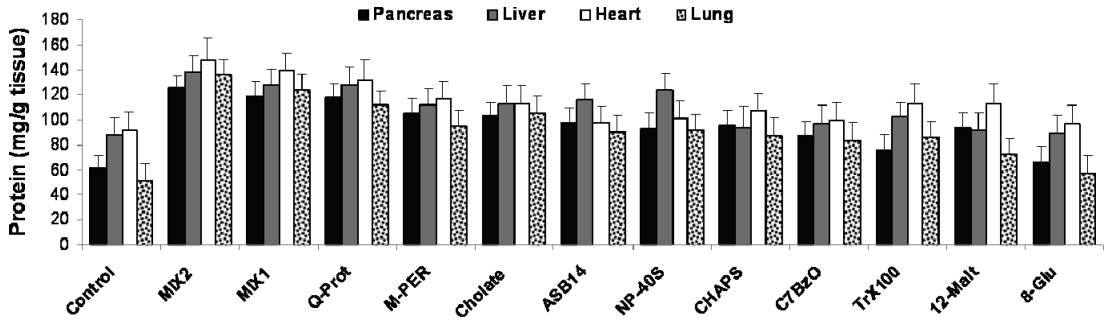

Figure 1. Protein yield with different detergents. The protein concentrations of extracts from pancreas, liver, heart and lung tissues are shown, which were obtained with the indicated buffer compositions. The average of three independent measurements is shown.

tion of compartmentalized proteins should occur in an effective and reproducible manner. As a consequence, substances commonly used for protein extraction, such as Tris-base, dithiothreitol and sodium dodecylsulfate (SDS), were excluded. As an alternative to Tris-base, we tested several other buffering substances like 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), 4-(*N*-morpholino)butanesulfonic acid (MOBS), 1,4-piperazinediethanesulfonic acid (PIPES) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES). This group of buffers, also known as Good's buffers,[21] are characterized by their high compatibility with biological analysis, good solubility in water, minor salt effects and minimal interference with biological functions. We found HEPES buffer to be the most suitable milieu for protein extraction. To block interference from nucleic acids, we added the enzyme Benzonase to the buffer, which destroys these macromolecules. To avoid protein degradation by the cellular protein-digesting machinery, a commercially available cocktail of protease and phosphatase inhibitors was added, and the temperature was kept at 0 °C or less throughout analysis. The presence of 20% glycerol prevented freezing of the solution.

Most critical to a successful isolation, however, were the detergents used in the process. They were selected from groups of nonionic, ionic and zwitterionic nondenaturing detergents. In addition to studying the effect of individual detergents, we formulated two detergent mixtures to assess the combined effect of these detergents on cellular protein recovery. The detergent mixes were composed of up to four detergents that are of entirely different chemical structure and represent a broad spectrum of detergents generally used for protein extraction from biological specimens. The chemical classes to which the chosen detergents belong are polymeric phenylethylene glycols, long-chain alkyl amidosulfobetaines, cholic acid derivatives and long-chain alkylglycosides. A mixture of chemical structures should be superior to overcome the steric obstacle posed by the lipids engulfing protein geometry. Additionally, different classes of detergents have demonstrated their preference for isolating proteins from particular cellular compartments.[14,22] A combination may add more power to isolating proteins from the various cellular organelles. After preliminary tests (data not shown), we ended up with two mixtures that exhibited good results.

The first detergent mixture (Mix1) has a composition somewhat similar to that routinely used for two-dimensional gel electrophoresis[12] with chaotropes like urea, thiourea and ampholytes being excluded. The presence of these substances is necessary in electrophoresis in order to prevent the precipitation of protein during the gel separation. They are not required for antibody array assays, however. On the contrary, since chaotropes are denaturant to proteins, their absence improves protein functionality. The second detergent mixture (Mix2) is composed of detergents that are mimicking in their chemical structure those of radioimmunoprecipitation assay (RIPA) buffer. In this mixture, Nonidet P-40 substitute, sodium cholate and ASB-40 were the substitutes for NP-40, sodium deoxycholate and SDS in RIPA buffer, respectively. However, both mixtures contained long-chain alkyl-glycosides, since they have been reported to have extraction capacity to integral membrane proteins.[5,12]

For a comparative evaluation of the overall procedure, we also analyzed several commercial buffer systems. In an initial examination, several of them exhibited a limited extraction capacity or an apparent bias for particular protein types or compartmental protein fractions (data not shown). From all buffers tested, the Q-Proteome Mammalian Protein Prep (Q-Prot) kit of Qiagen and Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific) were chosen as controls, since they showed the best results and are also meant to extract proteins at nondenaturing conditions.

Protein Levels Extracted from Rat Tissues and Cell Cultures. Our first experiments were aimed at evaluating the capacity of various detergents, either individually or in the two mixtures, to extract cellular proteins from solid (heart and lung) and soft (liver and pancreas) rat tissues. Tissue homogenization was performed using four mechanical approaches: homogenization with Ultra Turrax, Dounce and Potter-Elvehjem homogenizers or pulverization with mortar and pestle after freezing the tissue in liquid nitrogen. Generally, we found that pulverization of samples worked best for hard tissues like heart and lung tissue, while homogenization using a Potter-Elvehjem homogenizer gave the most satisfactory results for liver and pancreatic tissue (data not shown). However, since the differences were minor, we used the pulverization method for all tissue samples in order to minimize sources of variation and for the purpose of uniformity.

The efficiency of protein extraction was evaluated by measuring the amount of protein resulting from each preparation per gram of tissue (Figure 1) and comparing to a buffer devoid of detergent (control) and the commercial extraction systems. While there were some apparent differences between the four tested tissues, the overall tendency was the same across the experiment. Mix2, Mix1 and Q-Prot performed best, sodium cholate was the most effective individual detergent on all types of tissues studied, the polymeric and zwitterionic detergents showed moderate extraction efficiency, and 8-Glu delivered the least extraction potential.

Next, we studied the quantity and functionality of proteins extracted from human BxPC-3 and SU.86.86 cell lines. For both,

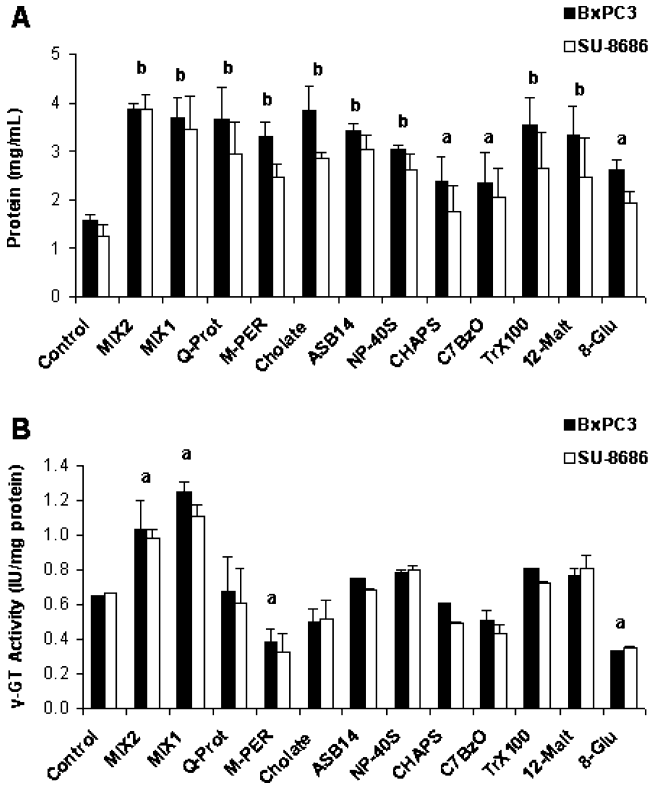

Figure 2. Protein extraction on BxPC-3 and SU.86.86 cell line lysates. The overall protein yield (A) and the specific γ-GT activity (B) are shown, which were obtained with three buffers. The average value from three independent measurements is shown. The significance compared to control buffer is $p < 0.01$ (a) and $p < 0.001$ (b), respectively.

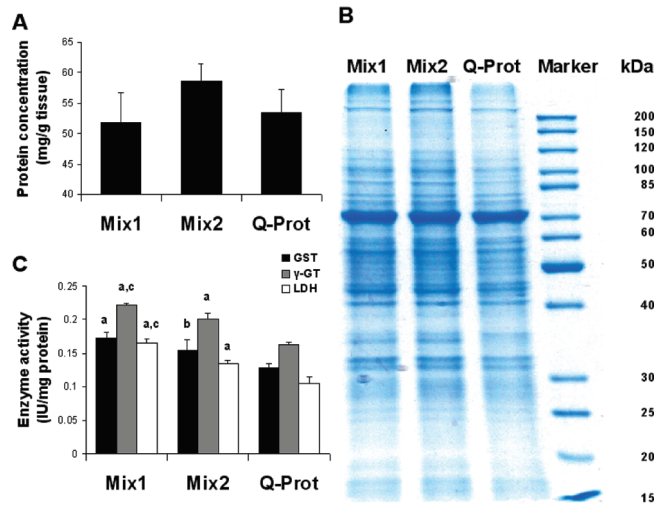

Figure 3. Quantitative and functional analysis of protein extracted with Mix1, Mix2 and Q-Prot, respectively, from a pool of 12 human pancreatic cancer tissues. The results are mean values (±SD) of three experiments. (A) The protein yield is shown. (B) The three different protein extracts were run on a 10% acrylamide gel and stained with Coomassie blue. (C) Measurements of the specific enzymatic activities of GST, γ-GT and LDH. Significances are (a) $p < 0.005$ vs Q-Prot, (b) $p < 0.05$ vs Q-Prot and (c) $p < 0.05$ vs Mix2.

the highest protein yield was obtained upon extraction with Mix1 and Mix2, followed by sodium cholate, ASB14 and the commercial Q-Prot buffer (Figure 2A). While the differences were not big between the best performing buffers, they were consistent throughout and differences were substantial between the best and worst performing buffers. The two detergent mixtures succeeded in preserving the enzymatic activity of γ-glutamyltranspeptidase (γ-GT) at the highest level (Figure 2B). Since a lower concentration of each individual detergent is used in the mixtures compared to buffers with just one detergent, the detergents may exert a less negative effect on protein integrity, while nevertheless in their sum guarantee effective extraction. Alternatively, the high activity could be explained by an enrichment of the membrane protein fraction upon extraction with the detergent cocktails; γ-GT is a membrane-associated enzyme.

Detergent Effects on Protein Extracted from Human Pancreatic Tissue. On the basis of the results with rat tissues and human cell lines, we performed studies with the three best buffer systems on 12 human pancreatic cancer tissues, which were randomly selected from a larger set of samples. Mix2 resulted in a significantly higher protein yield compared to Q-Prot and Mix1 (Figure 3A). Analysis of the enzymatic activity showed a different pattern, however. Protein extracted with Mix1 exhibited significantly higher enzymatic activities for γ-GT (membrane bound), GST (membrane bound and cytoplasmic) and LDH (cytoplasmic) as compared to the Q-Prot kit (Figure 3C). The lower enzymatic activity obtained by Mix2 extraction is probably a result of inhibitory effects of 8-Glu, which is present in this mixture. It has been shown that detergents with short (C_7−C_{10}) hydrocarbon chain like octylglucoside are more inactivating than the corresponding detergents with an intermediary (C_{12}−C_{14}) hydrocarbon chain like β-dodecylmaltoside.[23,24] Also in the cell culture experiments, the least γ-GT activity was found in protein extracted with 8-Glu (Figure 2B). We cannot comment on the reason for the lower enzymatic activity in extracts obtained with Q-Prot, since the composition of the buffer is not publicly known.

Effect of Extraction Buffers on Proteins from Cellular Organelles. To determine the efficiency of the extraction methods in recovering proteins from cellular organelles, we isolated proteins from human pancreatic cancer tissues followed by a qualitative and quantitative assessment of proteins by Western blot analysis and antibody microarrays. We looked at the following marker proteins: γ-GT [plasma membrane], flotillin [plasma membrane], caveolin [plasma membrane], β-actinin [cytoskeleton], TGN-46 [Golgi apparatus], calreticulin [endoplasmatic reticulum], catalase [peroxisome], cathepsin D [lysosome], lamin A [nucleus] and cytochrome C [mitochondria] (Figure 4). Overall, the results obtained by immunoblotting and microarray analysis, using the same antibodies, were in good agreement. With regard to protein yield, both Mix1 and Mix2 showed a much better efficiency in extracting membrane-associated proteins compared to Q-Prot.

Plasma membrane is one of the richest sources of cellular proteins that are important with respect to disease and therapy. Around 30% of the human proteins are embedded in the membrane.[25,26] Membrane proteins are involved in many important cellular events like cell signaling and signal transduction, transport, and cellular communication, besides others. It is well-documented that proteins of plasma membranes pose a complicating factor during proteomic analysis owing to the high hydrophobicity.[27] We investigated the capacity of the detergent cocktails for isolating highly hydrophobic proteins and found superior performance, revealed by both Western blot

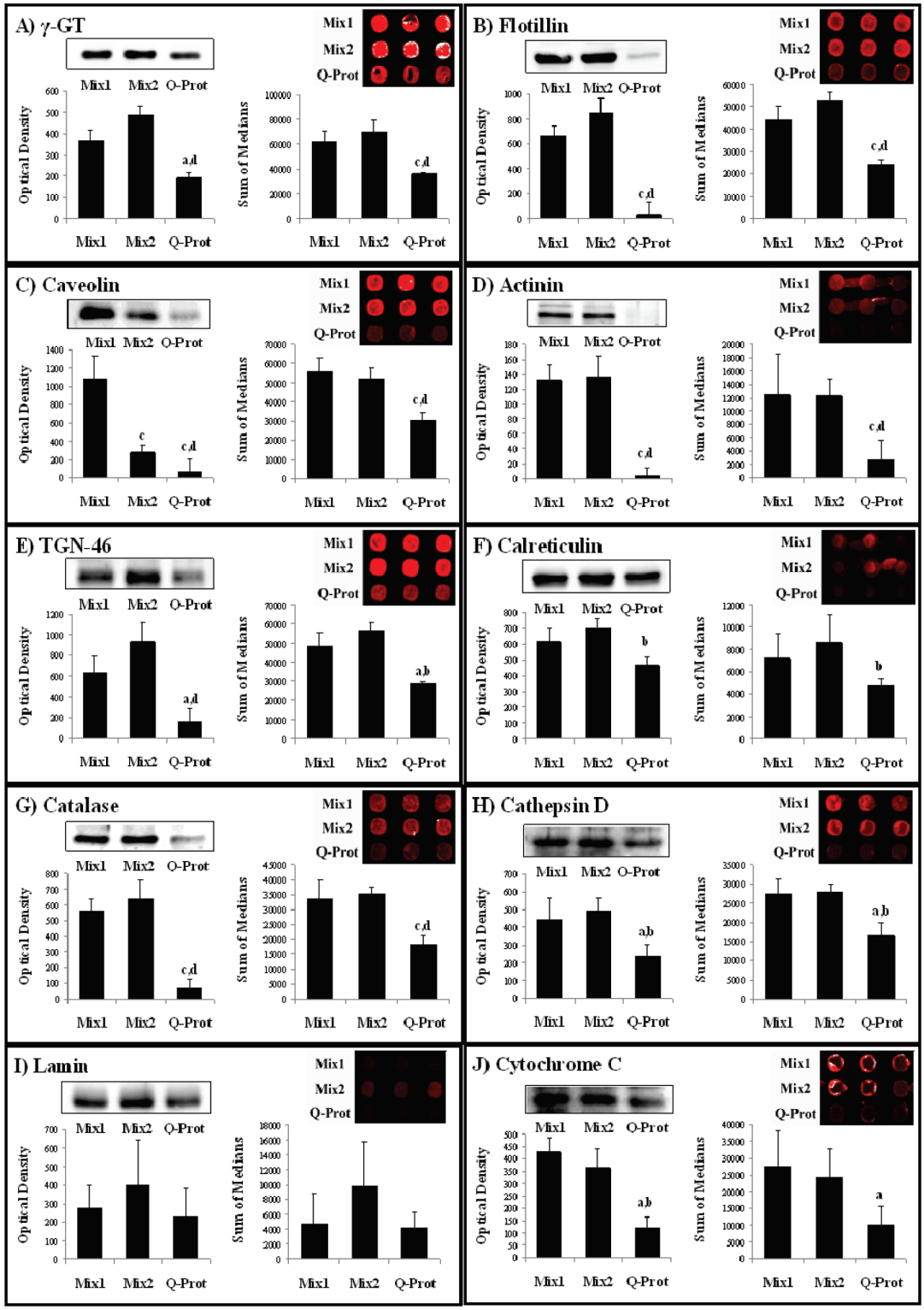

Figure 4. Western blot and antibody microarray results of cellular organelle marker proteins. In each panel, typical experimental results are shown. In addition, bar charts present the mean values of three separate experiments each; this equals 18 data points from microarray measurements. (A) γ-GT [plasma membrane], (B) flotillin [plasma membrane], (C) caveolin [plasma membrane], (D) β-actinin [cytoskeleton], (E) TGN-46 [Golgi apparatus], (F) calreticulin [endoplasmatic reticulum], (G) catalase [peroxisome], (H) cathepsin D [lysosome], (I) lamin A [nucleus] and (J) cytochrome C [mitochondria]. *P*-values: <0.05 vs Mix1 (a), <0.05 vs Mix2 (b), <0.005 vs Mix1 (c), and <0.005 vs Mix2 (d).

and antibody microarray analyses. Zwitterionic detergents and alkyl-glycoside have been suggested as better solubilizers of membrane proteins than polyethylene glycols like Triton X100 and NP-40.[12] We found no significant difference in the extraction efficiency between these detergent groups. However, in combination, they resulted in a much better yield of protein quantity and functional quality. The latter may be attributed to the lower concentration of each detergent individually.

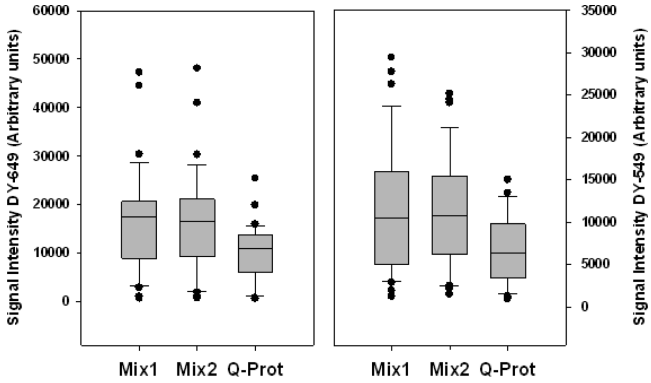

Figure 5. Box plot of the signal intensities obtained with samples prepared with Mix1, Mix2 and Q-Prot, respectively, and labeled with both red (DY-649) and green (DY-549) fluorescence dye. The values represent the means of three independent measurements.

Furthermore, cytoskeletal proteins are categorized among the hardest to solubilize. It has been shown that these proteins resist Triton X100 disruption.[28] He et al. recently reported a solubilization of cytoskeletal proteins using ASB-14.[29] Indeed, in our experiments, we were able to see apparent signals on the arrays and bands in the Western blots with both mixtures, each of which contained ASB-14.

Of the plasma membrane proteins, only caveolin showed a different result insofar as it was the only protein which could not be extracted similarly well with Mix2 as with Mix1. However, both buffers exhibited good solubilization of the cytoskeletal protein β-actinin and the other proteins from cellular organelles. Mix2 was almost always slightly better than Mix1 in protein extraction, although not significantly. Lamin A (nuclear envelope; Figure 4I) exhibited the smallest difference between the three buffer systems. However, microarray data for other nuclear proteins, which were not particularly confirmed by Western blot analysis, showed a higher recovery for all of these with Mix1 and Mix2 as compared to Q-Prot (Supporting Information).

Quality Control on Antibody Microarrays. We applied stringent quality control criteria to check the performance of the extracts on antibody microarrays. Quality control measures like signal intensity, signal-to-noise ratio (SNR) and dye effect were used to check the compatibility of tested buffers with the antibody microarrays. Overall, the detection limit of the antibody microarrays was similar to that reported previously.[10,11] For the signal-to-noise ratio, the threshold values (mean value $-$ 1 $\times$ SD) were 2.0 and 3.2 for DY-649 and DY-549, respectively. Similarly, only signal intensities (background subtracted) exceeding a cutoff (mean $-$ 1 $\times$ SD) value of 4243 and 3083 arbitrary units for the red and green detection channels, respectively, were considered adequate. Pancreatic cancer samples were labeled with the dyes DY-649 (red) and DY-549 (green), respectively. Two differently labeled protein preparations were then mixed and incubated on microarrays made of 38 antibodies, each spotted in six replicates. From the results, we calculated the percentage of microarray features that exhibited a value above the respective threshold. Uniform conditions were applied throughout the analysis with both fluorescent dyes. Protein extracts isolated with Mix1 and Mix2 produced the best results on the arrays followed by Q-Prot in terms of signal intensity (Figure 5) and overall SNR (Table 1). Q-Prot extracted fewer proteins that produced signal intensities which exceeded the threshold limit for both channels (Table 1). This result is important when detecting low-abundance proteins. Such proteins may go undetected not as a result of limited sensitivity of the array assay, but rather because of the inefficiency of the extraction buffer. In addition, the two dyes used in the analysis behaved very similarly in the labeling of samples obtained with both Mix1 and Mix2, while clear differences were observed with Q-Prot. As documented by this result, the unknown composition of commercial buffers could pose a serious limitation in condition optimization and detecting potential sources of error. All amines in Mix1 and Mix2 are tertiary amines, for example, and should therefore not affect the dye NHS-esters, which only react with primary amines.

Since each sample was labeled with both dyes, a similar pattern of signal intensities for target proteins is expected at both channels. This was the case with both Mix1 and Mix2. The results demonstrated a high correlation between the red and green channel detection for all 38 probes (Figure 6). Q-Prot, on the other hand, exhibited more variations, although identical conditions had been used in the experiments. This difference complicates two-color measurements considerably. As already mentioned above, we cannot comment on possible reasons for this effect for the lack of information about the composition of the commercial product. However, the very good correlation observed for protein samples extracted with Mix1 and Mix2 indicated that they have no significant effect on the labeling process.

Conclusions

Two buffer cocktails were established that demonstrated their compatibility with and applicability to proteomic analysis based on antibody microarrays and were superior to the best systems available to date. We currently use the cocktails for routine analyses of cell culture and tissue samples on complex antibody microarrays, made from a panel of 810 different

Table 1. Percent of Antibody Probes (Total Number is 38, Each Spotted in Six Replicates) That Showed Acceptable Quality in Terms of Signal Intensities and Signal-to-Noise Ratio[a]

	signal-to-noise ratio		signal intensity (minus background)	
	DY-649	DY-549	DY-649	DY-549
Mix1	93.86% ± 4.02%	90.35% ± 3.04%	85.96% ± 4.02%	89.47% ± 0.00%
Mix2	95.61% ± 1.52%	92.11% ± 2.63%	83.33% ± 1.52%	85.96% ± 1.52%
Q-Prot	94.74% ± 2.63%	82.46% ± 6.62%	79.82% ± 1.52%	77.19% ± 4.02%
	Threshold definition: mean value − 1 × SD		Threshold definition: mean value − 1 × SD	
	2.0	3.2	4243 units	3083 units

[a] The definition and actual values of the thresholds are given. The units for signal intensity are arbitrary values.

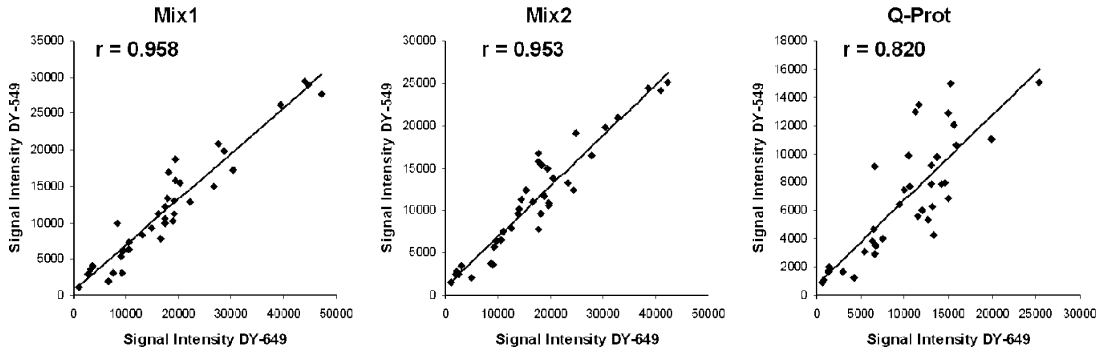

Figure 6. Correlation and Pearson correlation coefficient (*r*) between DY-649 and DY-549 labeled proteins extracted with Mix1, Mix2 and Q-Prot ($n = 38$).

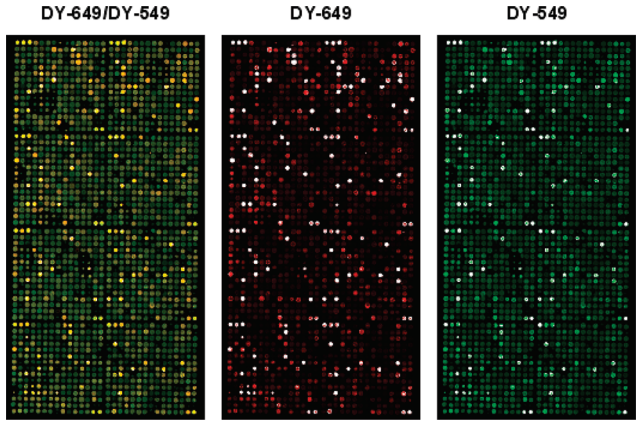

Figure 7. Result from a routine two-color expression measurement on a complex antibody microarray. Protein was extracted from BxPC-3 cell line with Mix2, labeled with DY-649 or DY-549, and incubated as described in Material and Methods on a complex microarray made of 810 different antibodies. This antibody panel targets proteins, whose genes had been identified to be differentially transcribed on the RNA-level in cancer versus normal tissue. Both the images of the individual detection channels and the combined image are shown.

antibodies, each spotted in duplicate. The studies aim at the elucidation of biological variations of cancer-relevant proteins. As part of these large-scale studies, also the identical samples are labeled with the two dyes and analyzed in competitive incubations on one microarray for the purpose of a continuous quality assessment (e.g., Figure 7), again demonstrating their assay compatibility. Both buffers are composed of detergent combinations that include at least one polymeric phenylethylene glycol, a long-chain amidosulfobetaine, cholate and a long-chain alkylglycoside. Their use leads to a reproducible and simple extraction of protein from tissues. While Mix2 has a higher overall yield judging from immunoblot experiments, Mix1 is slightly superior with regard to functional aspects. One should keep in mind, however, that functional aspects could well be affected by the addition of fluorophore labels. We aim at analyzing not only expression differences with regard to protein levels but also conformational variations, such as protein isoforms. Consequently, isolation of proteins in a structurally relevant form is critical. Nevertheless, factors such as chemical derivatization could influence the results considerably. Overall, the protein extracts resulting from incubations with Mix1 and Mix2 exhibited a good representation of membrane-associated proteins and worked with high efficiency. The latter is especially important for analyzing small clinical samples. While established for antibody array applications, the high agreement of the array data and the Western blot analyses indicates that the buffers could also be useful for other types of proteomic studies.

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Supporting Information Available: Figure 1S, antibody microarray results of the other 28 proteins for which no Western blot experiments were conducted. The values are arbitrary units (AU) of the mean of the sum of medians for signal intensities from three arrays. This material is available free of charge via the Internet at http://pubs.acs.org.

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TECHNICAL BRIEF

Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays

Mohamed Saiel Saeed Alhamdani, Christoph Schröder and Jörg D. Hoheisel

Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany

Antibody microarrays are a developing tool for global proteomic profiling. A protocol was established that permits robust analyses of protein extracts from mammalian tissues and cells rather than body fluids. The factors optimized were buffer composition for surface blocking, blocking duration, protein handling and processing, labeling parameters like type of dye, molar ratio of label *versus* protein, and dye removal, as well as incubation parameters such as duration, temperature, buffer, and sample agitation.

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In the last few years, antibody microarrays have had a significant impact on proteomic research [1, 2]. The format owes its success to the capacity to analyze proteomes globally in high throughput. However, in contrast to the growing need for studies of clinically relevant tissues, the platform still finds its major biomedical applications in the analysis of conditioned cell culture media, serum, and plasma samples as well as other body fluids like urine, cerebrospinal fluid, saliva, and tears (Supporting Information Table 1S). In our experience and that of others [3], the analysis of cellular proteins using current standard protocols, which were optimized for plasma or serum samples, failed to produce results of adequate quality. To date, there were only relatively few reports about analyses of cellular proteomes from tissue homogenates and cell lysates (Supporting Information Table 2S). Most of these used commercial antibody arrays, which display variable performances [2]. Furthermore, many protocols are time-consuming and involve the use of up to ten different buffers. This study reports on a thorough evaluation and concomitant optimization of the parameters for proteomic analysis of tissue protein extracts on antibody microarrays.

For analyses, the following standard protocol was established; more experimental detail is provided in the Supporting Information. Proteins were extracted from four pancreatic cancer cell lines and 18 human pancreatic cancer tissues as recently described [4]. Extracted protein was labeled with fluorescence dye DY-549 or DY-649 at a dye to protein (D/P) molar ratio of 18, with the assumption that 60 kDa is the average molecular weight of a protein. The protein concentration was adjusted to 2 mg/mL. Labeling occurred in the dark in 0.1 M carbonate buffer, pH 8.5, at 4°C for 2 h. Unreacted dye was quenched with 10% glycine for 30 min at 4°C in the dark. Labeled samples were stored at −20°C until use. Antibodies were spotted on epoxysilane-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP3B pins (Telechem, Sunnyvale, USA) at a humidity of 40–45%. The printing buffer was composed of 0.1 M carbonate buffer (pH 8.5) containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran, 5 mM magnesium chloride, 137 mM sodium chloride, and 1 mg/mL of the respective antibody. After printing, the slides equilibrated at a humidity of 40–45% overnight and were stored in dry and dark conditions at 4°C until use.

Printed slides were washed once for 5 min followed by another wash for 15 min with PBS (137 mM sodium

Correspondence: Dr. Mohamed Saiel Saeed Alhamdani, Functional Genome Analysis, Deutsches Krebsforschungszentrum Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany
E-mail: m.alhamdani@dkfz.de
Fax: +49-6221-42-4687

Abbreviations: D/P, dye to protein; **NHS-ester,** *N*-hydroxysuccinimide ester; **PBST80,** PBS plus Tween-80; **SNR,** signal-to-noise ratio

chloride, 2.7 mM potassium chloride, 10.0 mM disodium hydrogen phosphate, 1.76 mM sodium dihydrogen phosphate, pH 7.4) containing Tween-80 at a final concentration of 0.05% (PBST80). The slides were blocked with 5 mL of 10% non-fat dry milk (Biorad, Munich, Germany) in PBST80 for 3 h at room temperature using Quadriperm chambers (Greiner Bio-One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated in Quadriperm chambers with 50 µg labeled sample in 5 mL of 10% milk in PBST80 overnight in the dark at 4°C. The slides were then washed four times for 5 min in large volumes of PBST80, rinsed several times with deionized water, and dried in a ventilated oven at 22°C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer, Waltham, USA) at constant laser power and PMT. The images were analyzed with the software GenePix Pro 6.0

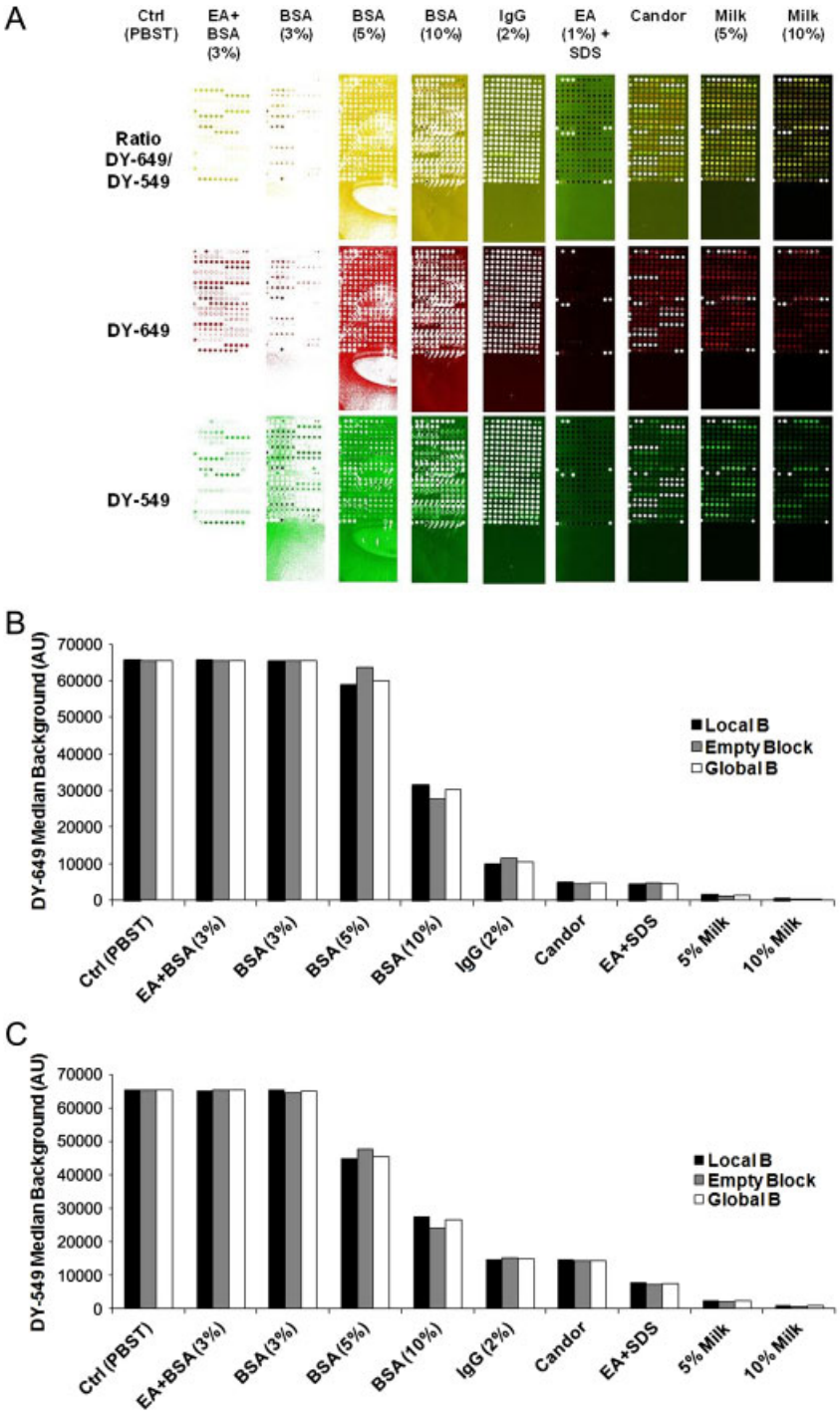

Figure 1. The effect of blocking buffers on background signal. In (A), the signals obtained with red and green dyes and the superimposed images are presented. All slides had been blocked for 3 h at room temperature followed by incubation with BxPC-3 lysate in Quadriperm chambers at 4°C overnight. The median background signal intensities from three slides for each buffer are shown for red (B) and green (C) fluorophors. Intensities were obtained at identical laser power and PMT for all slides.

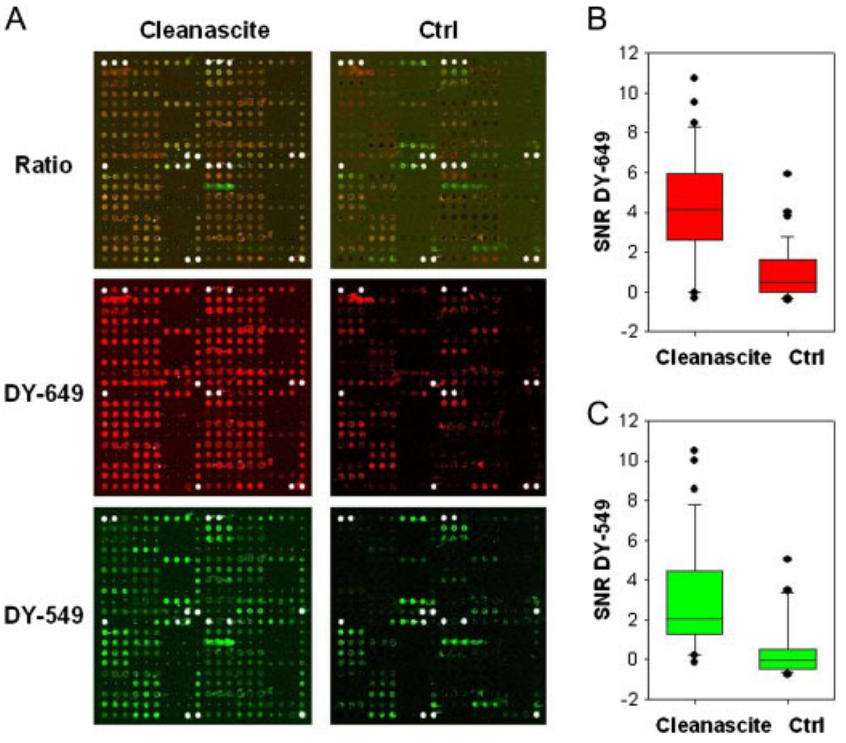

Figure 2. Lipid removal. Seven pancreatic cancer tissue samples with high lipid content were split into half. One half of each was treated with Cleanascite prior to labeling. The other half was not treated with Cleanascite. Treated and untreated samples were pooled separately, labeled, and analyzed in triplicate as described in the Supporting Information. In (A), the slide incubation results from the red and green channel scans and the superimposed image are shown. In (B) and (C) the respective SNRs of the feature intensities are presented.

(Molecular Devices, Sunnyvale, USA) and the software Acuity 4.0 (Molecular Devices).

The above protocol produced identical results for protein extracts from liquid samples as well as cells and tissues. While larger structures in serum or other body fluids are predominantly proteins, cells are generally more complex in their biomolecule content. The presence of nucleic acids, lipids, and metabolites drastically affects data quality. Improvements have been made by adapting protein preparation [4]. Still, there are additionally intrinsic differences in complexity and dynamic mass. To date, no optimization for cellular proteomes had been performed. In order to make the methodology amenable to the analysis of protein extracts from mammalian tissues, several steps were studied.

For blocking, ten commercial and home-made buffers were compared (see Supporting Information data for details). Earlier studies with tissues provided only limited information with regard to blocking (Supporting Information Table 2S). In most of them, undisclosed recipes were used or there was no mentioning of this essential step. In none of them, quality control measures were provided. We found that 10% milk in PBST80 produced best results (Fig. 1). Also, a time-dependent decrease in local and global background intensity was observed (Supporting Information Fig. 1S). However, blocking for more than 3 h increased tenfold the percentage of spots flagged as absent by the analysis software. Since the majority of antibodies used in our study were developed in rabbits, we also applied 2% IgG globulins from rabbit in PBS as blocking buffer. Although the background was significantly higher than with 10% milk, the slides nevertheless exhibited a slightly better signal-to-noise ratio (SNR). However, the use of IgG globulins is not feasible for economical reasons.

The incubation conditions have immediate consequences on quality and sensitivity and have been a focus of our work [5]. Here, the effects of buffer type, incubation time, temperature, and sample agitation method were evaluated. Quality was assessed in terms of spot signal uniformity and SNR. Knezevic *et al.* [6] used 1% BSA for incubation of tissue lysates for 8–12 h at 4°C. In another study [7], 5% BSA was used for incubation of tissue and cell lysates. Others provided no information. We found BSA associated with lower quality, while superior results were obtained with 10% milk-PBST80 (Supporting Information Fig. 2S).

Proteins are usually kept at low temperature to preserve their integrity. Microarray quality was significantly lower in overnight incubations at room temperature compared with 4°C (data not shown). However, incubations at 4°C overnight or at room temperature for 1 h produced similar quality. This conforms with the proposition that for antibody–antigen complexes, which fit to a 1:1 Langmuir association model, the dissociation rate constant is more temperature-dependent than the association rate constant [8]. Besides temperature, sample agitation is also critical for array performance [5]. We compared mechanical agitation (Quadriperm) and surface acoustic wave stimulation (Slidebooster; see Supporting Information). The former produced a higher SNR (Supporting Information Fig. 3S). As little as 10 µg of protein generated signals of sufficient quality (Supporting Information Fig. 4S).

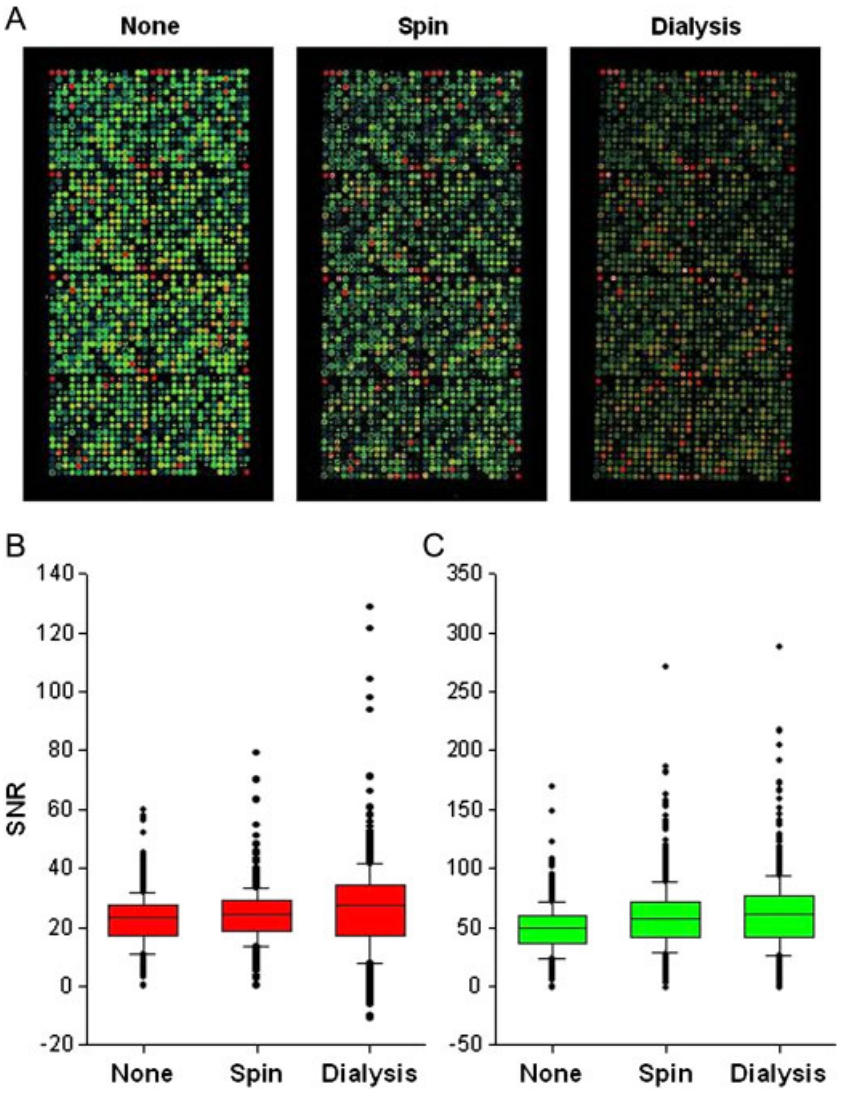

Figure 3. Removal of unincorporated dye molecules. Image scans of large antibody microarrays with some 1800 features produced from 810 cancer-associated antibodies are shown. Suit-007 and Suit-028 protein extracts were labeled with Cy3 and Cy5, respectively. Prior to incubation, the dye had not been removed (None) or removal occurred by spin column or dialysis (A). The quality of the microarrays is presented in terms of SNR (mean from triplicate experiments) in the red (B) and green (C) channels.

Protein processing also contributed to assay quality and reproducibility. Extraction under native conditions resulted in a better SNR than extraction with protein denaturation (Supporting Information Fig. 5S). Also, the removal of lipids from the sample was tested. Lipids like phosphatidyl-ethanolamine may undergo labeling and bind to the hydrophobic slide surface producing background signal. Sreekumar *et al.* [9] reported previously the use of Triton-X114 and ExtriGel beads to remove lipids from LoVo cells. However, no experimental details were provided. We tested sample delipidation with Cleanascite reagent and observed a substantial improvement of the array quality results (Fig. 2). Lipid removal was only necessary for tissues homogenates, however, and mainly in those with higher lipid content.

Protein labeling was investigated intensively before. Also, it has been shown that direct labeling using a two-color approach can substantially improve microarray performance in terms of reproducibility and discriminative power [10]. Here, we extend this issue by analyzing additional dye-pairs and assessing the D/P molar ratio. Five fluorescent dye-pairs were tested (Supporting Information). Dye bias was less pronounced with increasing polarity of the dyes (Supporting Information Fig. 6S). Cy3 and Cy5 were second to DY-549 and DY-649 in water solubility but performed slightly better with tissue homogenates. Maximal labeling efficiency was achieved at a molar ratio of 14–22 D/P (Supporting Information Fig. 7). Gel electrophoresis, on the other hand, showed a continuous increase in the fluorescence intensities of protein bands even at high D/P ratios (Supporting Information Fig. 8S). In a study with cell lysates, Kopf *et al.* [11] suggested that increasing the D/P molar ratio is beneficial for sensitivity. We found, however, that higher ratios induced a negative effect, presumably due to masking of the antigenic sites by excessive amounts of the dye.

We also evaluated the impact of dye removal after labeling. Usually, dialysis or gel filtration is applied. Fluorescent dyes or haptens like biotin in the form of NHS-esters are the

most commonly used conjugates, taking advantage of the abundance of lysine in proteins. It was shown that upon the attachment of unlabeled biotin, followed by an addition of fluorescence-labeled strepavidin, the removal of remaining biotin is unnecessary [12]. However, there is a substantial difference between a biotin-strepavidin system and direct protein labeling with NHS-ester fluorescence dyes. Fluorphores may interact non-covalently and unspecifically with hydrophobic proteins or the hydrophobic array surfaces and thus deteriorate image quality. Unlike most fluorescent dyes, biotin is highly soluble in water and removed during washing. We found that the removal step is superfluous (Fig. 3). Un-reacted NHS-ester moieties undergo spontaneous hydrolysis in the aqueous extraction medium, even if not quenched by glycine. Our experiment also precludes an effect of hydrogen-bonding, van-der-Waals, or other weak interaction forces. The ionic strength of PBS along with the amphiphilicity of Tween eliminates traces of the inactive dye during the washing steps (Supporting Information Fig. 9S). Besides cutting expenses, avoiding dye removal has other advantages, too, such as shortening the time required for the assay, minimizing technical complexity, and – most importantly – avoiding a loss of small proteins or peptides, which may occur during dialysis or gel filtration.

In conclusion, a combination of measures, modifications and adaptations was introduced to the process of protein analysis by antibody microarrays, which led to a substantial improvement in data quality of studies of complex protein samples from tissues and cell cultures. The entire procedure makes use of only one buffer (PBST80) throughout. The intra- and inter-array coefficient of variance for replicate spot intensities was less than 10 and 20%, respectively. We employ the protocol in ongoing studies on samples from pancreas and bladder cancer tissues and performed experiments on cell lines of different origin. In all experiments, the results obtained are in agreement with the data shown here (Supporting Information Fig. 10S), confirming the benefit of the refinements in a large number of samples.

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The authors have declared no conflict of interest.

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PROTEOMICS

Supporting Information for Proteomics

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Mohamed Saiel Saeed Alhamdani, Christoph Schröder and Jörg D. Hoheisel

Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays

Supplementary information

Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays

Mohamed Saiel Saeed Alhamdani, Christoph Schröder and Jörg D. Hoheisel

Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ),
Im Neuenheimer Feld 580, 69120 Heidelberg, Germany. Email: m-alhamdani@dkfz.de

Antibody microarrays still find their major biomedical applications in the analysis of conditioned cell culture media, serum and plasma samples as well as other body fluids like urine, cerebrospinal fluid, saliva, and tears (Tab. 1S).

Tab. 1S.
Biomedical applications in the analysis of serum and plasma or other fluidic samples.

Protein source	Reference
Serum and plasma samples	10, 13-26
Urine	10, 26, 27
Cerebrospinal fluid	28, 29
Saliva	30
Tears	31
Cell media	32-42

To date, there were only relatively few reports about antibody microarray analyses of cellular proteomes from tissue homogenates and cell lysates (Tab. 2S).

Tab. 2S.
Biomedical applications in the analysis of tissue homogenates and cell lysates.

Source	Reference	Array source	Blocking
Tissue homogenates	43	commercial	no information
	44	commercial	no information
	45	commercial	no information
	46	commercial	no information
	47	commercial	no information
	11	commercial	no information
	6	home-made	1% BSA
Cell lysates	48	home-made	5% BSA
	9	home-made	1% BSA

In this study, issues were looked at that are critical to the performance of accurate and sensitive measurements on cellular proteomes and had not been addressed before and the effects of the various aspects on each other were evaluated. The experimental parameters concerned are distributed across the entire analysis process at the levels of blocking the microarray surface, sample manipulation before and after labeling, the actual protein labeling as well as the incubation conditions.

Materials and Methods

Materials

All chemicals used in this study were purchased from Sigma-Aldrich unless stated otherwise and were of highest purity or protein grade. The microarrays used in testing the protocol were produced with thirty-eight polyclonal and monoclonal antibodies purchased from different commercial sources [Tab. 3S]. Antibodies supplied as ascites fluid, antisera or with stabilizer proteins were purified using the Nab Protein G Spin Kit (Thermo Scientific, Rockford, USA). The concentration of all antibodies was adjusted to 2 mg/ml by filtration with Microcon 100 kDa (Millipore, Schwalbach, Germany) prior to aliquoting and storage at -80°C. In addition, a much more complex, home-made microarray was used. It consists of 810 antibodies that target mostly cancer-related proteins and has been described in detail earlier [10].

Tab. 3S.
Sources of the commercial antibodies used in this study.

Target	Target full name	Company	City	Country
ACTB	Actin, beta	Sigma	Munich	Germany
ACTN1	Actinin, alpha	Sigma	Munich	Germany
AGR2	Anterior gradient protein 2 homolog	Biomol	Hamburg	Germany
APC	Adenomatous polyposis coli protein	Epitomics	Hamburg	Germany
BAX	Apoptosis regulator BAX	Eurogentec	Köln	Germany
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	Sigma	Munich	Germany
CAC1G	Voltage-dependent T-type calcium channel subunit alpha-1G	Sigma	Munich	Germany
CAD13	Cadherin-13	Biomol	Hamburg	Germany
CALR	Calreticulin	Biozol	Eching	Germany
CATD	Catalase	Calbiochem	Darmstadt	Germany
CATD	Cathepsin D heavy chain	Dianova	Hamburg	Germany
CAV2	Caveolin-2	Sigma	Munich	Germany
CD2A1	Cyclin-dependent kinase inhibitor 2A, isoforms 1/2/3	Eurogentec	Köln	Germany
CDN1C	Cyclin-dependent kinase inhibitor 1C	Sigma	Munich	Germany
CRP	C-reactive protein	ACRIS	Darmstadt	Germany
CYC	Cytochrome C	Dianova	Hamburg	Germany
DAPK1	Death-associated protein kinase 1	Abnova	Heidelberg	Germany

DKK1	Dickkopf-related protein 1	Biomol	Hamburg	Germany
ERBB3	Receptor tyrosine-protein kinase erbB-3	Acris	Darmstadt	Germany
FASTK	Fas-activated serine/threonine kinase	Santa Cruz Biotechnology	Heidelberg	Germany
FLOT1	Flotillin-1	Sigma	Munich	Germany
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Sigma	Munich	Germany
GSTP1	Glutathione S-transferase P	Immundiagnostik	Bensheim	Germany
IFNG	Interferon gamma	BD Pharmingen	Heidelberg	Germany
LAP-2	Lamina-associated polypeptide 2, isoform alpha	Sigma	Munich	Germany
LMNA	Lamin A	Biozol	Eching	Germany
MGMT	Methylated-DNA--protein-cysteine methyltransferase	Sigma	Munich	Germany
MUC6	Mucin-6	Biozol	Eching	Germany
PAI1	Plasminogen activator inhibitor 1	Eurogentec	Köln	Germany
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	Sigma	Munich	Germany
RUNX3	Runt-related transcription factor 3	R&D Systems	Wiesbaden	Germany
SFRP1	Secreted frizzled-related protein 1	Biomol	Hamburg	Germany
SFRP2	Secreted frizzled-related protein 2	Santa Cruz Biotechnology	Heidelberg	Germany
SOCS1	Suppressor of cytokine signaling 1	Millipore	Schwalbach	Germany
TGN-46	Trans-Golgi network integral membrane protein 2	Sigma	Munich	Germany
TIA1	Nucleolysin TIA-1 isoform p40	Santa Cruz Biotechnology	Heidelberg	Germany
TNAP3	Tumor necrosis factor, alpha-induced protein 3	Acris	Herford	Germany
γ-GT	Gamma-glutamyltransferase	Dianova	Hamburg	Germany

Antibody microarray printing

Antibodies of the small array were spotted in rows of five replicate spots each at two locations on epoxysilane-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP3B pins (Telechem, Sunnyvale, USA) at a humidity of 40% to 45%. The printing buffer was composed of 0.1 M carbonate buffer (pH 8.5) containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran, 5 mM magnesium chloride, and 1 mg/ml of the respective antibody. Cy3 or Cy5 labeled streptavidin molecules were spotted as dye controls. After printing, the slides were allowed to equilibrate at a humidity of 40% to 45% overnight and then stored in dry and dark conditions at 4°C until use. Details about the production of the large array are published elsewhere [10].

Protein extraction from cultured cells

Proteins were extracted from pancreatic cancer cell lines Suit-007, Suit-028, BxPC-3 and SU-8686 using a preparation protocol that was recently described in detail [4]. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C. At 90% confluence, cells were washed three times with ice-cold phosphate buffered saline (PBS), layered with a minimal volume of extraction buffer (Hepes-Mix: 20 mM Hepes buffer (pH 7.9), 1 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF, 1% NP-40 substitute, 0.5% sodium cholate, 0.25% n-dodecyl-β-D-maltoside (GenaXXon Bioscience, Ulm, Germany), 0.25% amidosulfobetaine-14, 1 U/µl of Benzonase (Merck Biosciences, Schwalbach, Germany) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Bonn, Germany), kept on ice for 30 min with occasional mixing, and then centrifuged at 20,000 g and 4°C for 20 min. The supernatant was aspirated with a fine needle and the protein concentration was determined with the bicinchoninic acid (BCA) Protein Assay Reagent kit of Thermo Scientific.

Protein extraction from tissues

Eighteen human pancreatic cancer tissue samples were kindly provided by the Surgery Department of the University of Heidelberg. Written informed consent was obtained from all patients and the local ethics committee of the University of Heidelberg approved the work. Tissue samples were directly snap-frozen in liquid nitrogen and stored at -80°C. Pulverization took place with a porcelain mortar and pestle in the presence of liquid nitrogen. The frozen powder was immediately resuspended in 10 µl extraction buffer per 1 mg of sample. The homogenates were incubated on ice for 30 min with occasional mixing and subsequently processed the same way as the cell culture samples. A pool of the processed samples was used in the analysis.

The standard protocol of antibody microarray analysis

The following protocol was used throughout. Whenever a particular condition was evaluated (see below), all other parameters were kept as described in this section.

Protein labeling

Extracted protein was labeled at a dye/protein (D/P) molar ratio of 18, with the assumption that 60 kDa is the average molecular weight of a protein [49]. Samples from human pancreatic cancer tissues or cell lysates were adjusted to a protein concentration of 2 mg/ml. The labeling reaction occurred in the dark in 0.1 M carbonate buffer, pH 8.5 at 4°C for 2 h. Unreacted dye was quenched with 10% glycine for 30 min at 4°C in the dark. Labeled samples were stored at -20°C until use.

Microarray slide blocking and incubation

Printed slides were washed once for 5 min followed by another wash for 15 min with PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10.0 mM disodium hydrogen phosphate, 1.76 mM sodium dihydrogen phosphate, pH 7.4) containing Tween-80 at a final concentration of 0.05% (PBST80). After washing, slides were blocked with 5 ml of 10% non-fat dry milk (Biorad, Munich, Germany) prepared in PBST80 for 3 h at room temperature using Quadriperm chambers (Greiner Bio-One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated with 50 µg of DY-549- or DY-649-labeled sample in 5 ml of 10% milk in PBST80 overnight in the dark at 4°C using Quadriperm chambers. The slides were then removed from the chambers, washed 4 times for 5 min each in large

volumes of PBST80, rinsed several times with deionized water, and dried at room temperature in a ventilated oven at 22°C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer, Waltham, USA) at constant laser power and PMT. The images were analyzed with the software GenePix Pro 6.0 (Molecular Devices, Sunnyvale, USA).

Tested parameters

Microarray blocking conditions

To study the effect of blocking time and the type of blocking buffer on array quality, the following buffers were tested: (i) PBS (control), (ii) the blocking solution from Candor Bioscience (Weissenberg, Germany), (iii) 1% ethanolamine (EA) plus 0.1% sodium dodecyl sulfate (SDS), (iv) 3% bovine serum albumin (BSA), (v) 5% BSA, (vi) 10% BSA, (vii) 1% EA plus 3% BSA, (viii) 5% milk-PBST80, (ix) 10% milk-PBST80, and (x) 2% rabbit serum IgG globulins (IgG). All blocking buffers except those containing EA were prepared in PBS. Slides were blocked with 7.5% milk-PBST80 for various time-intervals of up to 4 h at room temperature with continuous mixing using the Quadriperm slide chambers on an orbital shaker.

Microarray incubation conditions

All incubations were performed in the dark. Incubation variables like (i) type of buffer, (ii) temperature, (iii) time, (iv) mode of agitation and (v) probe concentration were investigated. The incubation buffers 2%BSA, 5% milk, and 10% milk, all prepared in PBST80, were used and compared with a buffer composed of PBST80 only. The effect of incubation time and temperature was done with a sample incubated either for 1 h at room temperature or overnight at 4°C. Agitation effects were evaluated with a sample incubated either in Quadriperm chambers on an orbital shaker or in the Advalytix Slidebooster (Olympus Life Science Research, Munich, Germany). Quadriperm chambers allow for liquid mixing by means of rotation on an orbital shaker. The Slidebooster achieves mixing by generating a micro-agitation environment in a stationary fluid by means of surface acoustic waves [50]. Labeled protein samples at quantities of 4, 8, 16, 32, 64 and 128 µg were diluted in 600 µl incubation buffer for the Slidebooster or 10, 20, 40, 80, 160, and 320 µg in 5,000 µl for the Quadriperm chamber, respectively, and used to define an optimal probe concentration for incubation.

Protein processing

Although proteins were extracted under near-native conditions [4], extraction under denatured conditions was tested as well, using modified radioimmunoprecipitation assay (RIPA) buffer. The modified RIPA buffer was composed of 0.1% SDS, 1.0% sodium deoxycholate, 1.0% NP-40 substitute and 0.1 M carbonate buffer (pH 8.5) instead of Tris buffer. Tris contains free amino groups that would compete with proteins for reacting with the NHS-ester dye and thus affect labeling. Additionally, a third buffer that is similar in composition to Hepes-Mix, but with 0.1 M carbonate, pH 8.5, replacing Hepes buffer was generated to study the effect of combining the extraction and labeling steps into a single buffer system. This alteration precludes the need for a buffer exchange step prior to labeling and, consequently, the loss of protein that may occur during this process.

The effect of lipid removal was examined with protein extracts using Cleanascite reagent (Biotech Support Group, New Jersey, USA) in a volume ratio of 1:4 (sample to

reagent) according to the manufacturer's instructions. Total lipid concentration was estimated using the sulfo-phospho-vanillin method as described elsewhere [51].

Protein labeling with fluorescent dyes

The type of labeling tag and labeling ratio were inspected. The NHS-esters of the dye pairs DY-649 and DY-549, DY-647 and DY-547 (Dyomics, Jena, Germany), Cy3 and Cy5 (GE Healthcare, UK), Oyster-650 and Oyster-550 (Denovo Biolabels, Münster, Germany) and ATTO-647 and ATTO-550 (Sigma-Aldrich) were used for direct labeling of protein extracts. Protein samples were labeled at incremental dye/protein ratios of 2 starting from 2 up to 28. Labeled samples were stored at -20°C until use.

Dye removal

The samples were incubated on the antibody microarrays in presence or absence of non-incorporated and inactivated dye molecules. Dye removal was achieved by using either Zeba Spin columns or dialysis with 3.5 kDa cut-off membranes (both Thermo Scientific) with a buffer change to PBS according to the manufacturer's recommendations.

Data analysis

Microarray data were analyzed without normalization using the software Acuity 4.0 (Molecular Devices, Sunnyvale, USA). The median of feature signal and background intensity was used to determine quality control measures like signal-to-noise ratio (SNR) and signal intensity thresholds. The SNR in both color channels was calculated as the mean (from three replicate arrays) of median signal intensity (background subtracted) divided by the standard deviation of the median local background intensities [52]. All comparisons and presented images of arrays were taken from scans using identical laser power and PMT.

Results

Blocking conditions

To determine the effect of various blocking buffers on the blocking and surface chemistry of epoxysilane slides, they were blocked with (i) Candor, (ii) 1% EA plus 0.1% SDS, (iii) 3% BSA, (iv) 1% EA plus 3% BSA, (v) 5% BSA, (vi) 10% BSA, (vii) 5% and (viii) 10% milk-PBST80, (ix) 2% IgG and (x) PBS alone as control, followed by incubation with DY-649 and DY-549 labeled BxPC-3 protein extract (Fig. 1; see main text). An empty surface area of similar dimensions to that containing the antibodies was analyzed to obtain the signal generated from the interaction of the sample with the blocked surface so as to exclude the effect of shedding signals from the spots. The signal across the entire surface area of the control slide reached saturation, clearly indicating how essential blocking is for this kind of analysis. Milk was the most effective blocking agent.

During blocking, a time-dependent decrease in local and global background intensity was observed using 10% milk-PBST80 (Fig. 1S), when slides were analyzed at hour intervals for up to 4 h. However, when blocking took longer than 3 h, the background for the red dye increased again. Also, longer blocking time resulted in an increase from 0.95% after 1 h to 9.74% after 4 h of spots that were flagged by the analysis software as not clearly distinguishable from background and thus called absent.

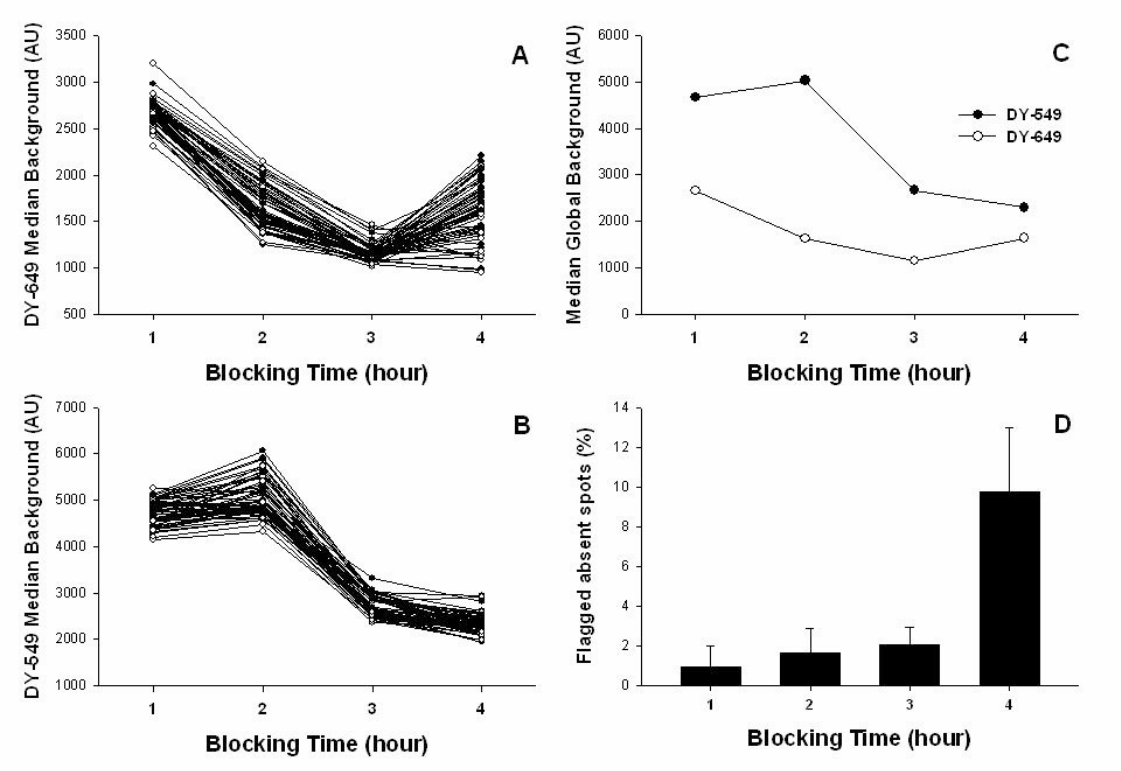

Fig. 1S. The effect of blocking time. Slides were blocked with 7.5% milk at room temperature for up to 4 h followed by incubation with BxPC-3 lysate in Quadriperm chambers at 4°C overnight. Variation over time is shown for the median local background intensity at the red (A) and green (B) channels, of the median global background intensity (C), and of the percentage of spots flagged absent in the analysis (D). The values are representing the mean of quadruplicate measurements for each time point.

Incubation buffer conditions

Array quality was assessed in terms of spot signal uniformity and SNR at both red and green channels. To determine the effect of the incubation buffer, slides were first blocked with 10% milk in PBST80 followed by incubation with DY-649 and DY-549 labeled SU-8686 protein extract in PBST80 alone, 3% BSA-PBST80, 5% milk-PBST80 or 10% milk-PBST80, respectively. Fig. 2S shows images of parts of microarrays that were incubated at identical conditions but for the incubation buffers. The degree of homogeneity of pixel intensities in the spots can be represented in numerical values as standard deviation (SD) of signal intensity for red and green dyes (Fig. 2S B and C). Both 5% and 10% milk in PBST80 resulted in a better array quality and spot uniformity than PBST80 alone and 3% BSA, with slightly favorable results for 10% milk-PBST80. Incubation in 10% milk-PBST80 also produced the highest SNR (not shown).

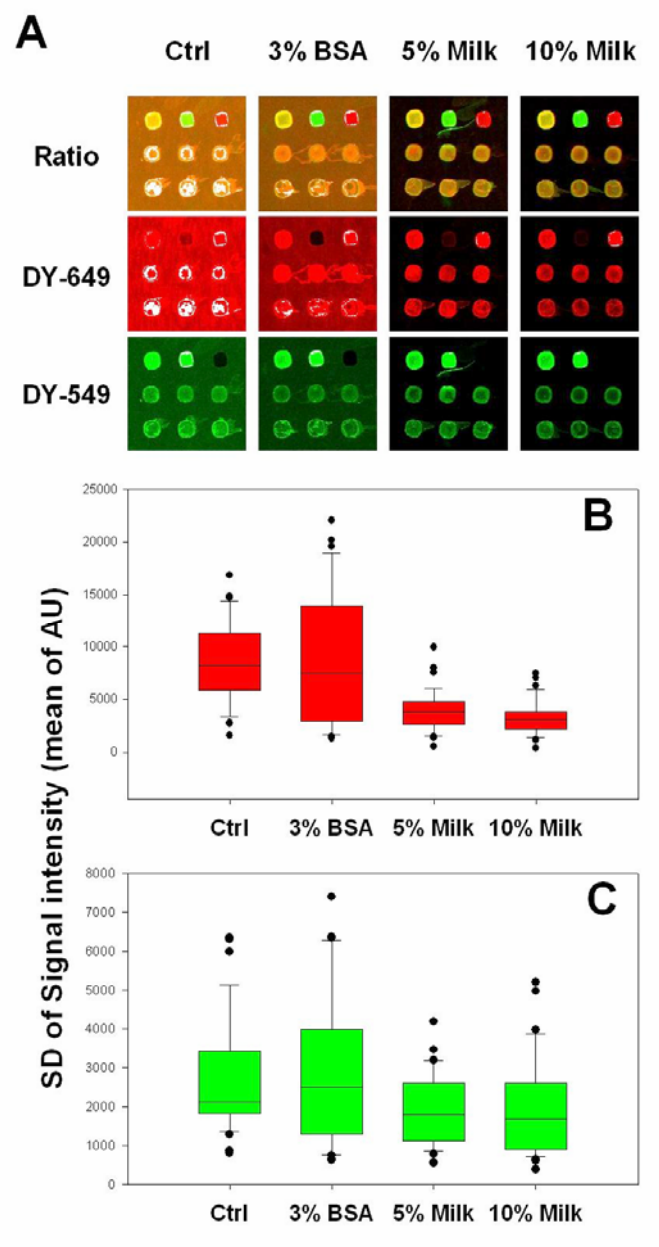

Fig. 2S. The effect of incubation buffers on array quality. Slides were incubated with SU-8686 lysate in Quadriperm chambers at 4°C overnight. The images obtained from scanning the red and green channels and the superimposed images (A) are shown. Also, the mean of SD of the features' signal intensities from triplicate experiments are given for red (B) and green (C) labeling with the Dyomics dyes.

Sample agitation

We used the Quadriperm and Slidebooster systems to compare their performance and determine the optimal protein concentrations for incubation on the microarrays. Both systems reduce mass transport and kinetic limitations my mixing the sample during incubation. We have shown previously that a detection limit in the femtomolar range can be achieved using this technology, much superior to the results with static systems [53]. Overall, agitation with the Quadriperm system produced a higher SNR as compared to the Slidebooster (Supplementary Fig. 3S). However, a much larger incubation volume is needed for the Quadriperm (5,000 µl) as compared to the Slidebooster (600 µl) system. Quantities of 4, 8, 16, 32, 64 and 128 µg labeled protein were incubated in the Slidebooster system and 10, 20, 40, 80, 160, and 320 µg in the Quadriperm system, respectively. The resulting images were analyzed at identical laser power and PMT gain (Fig. 4S).

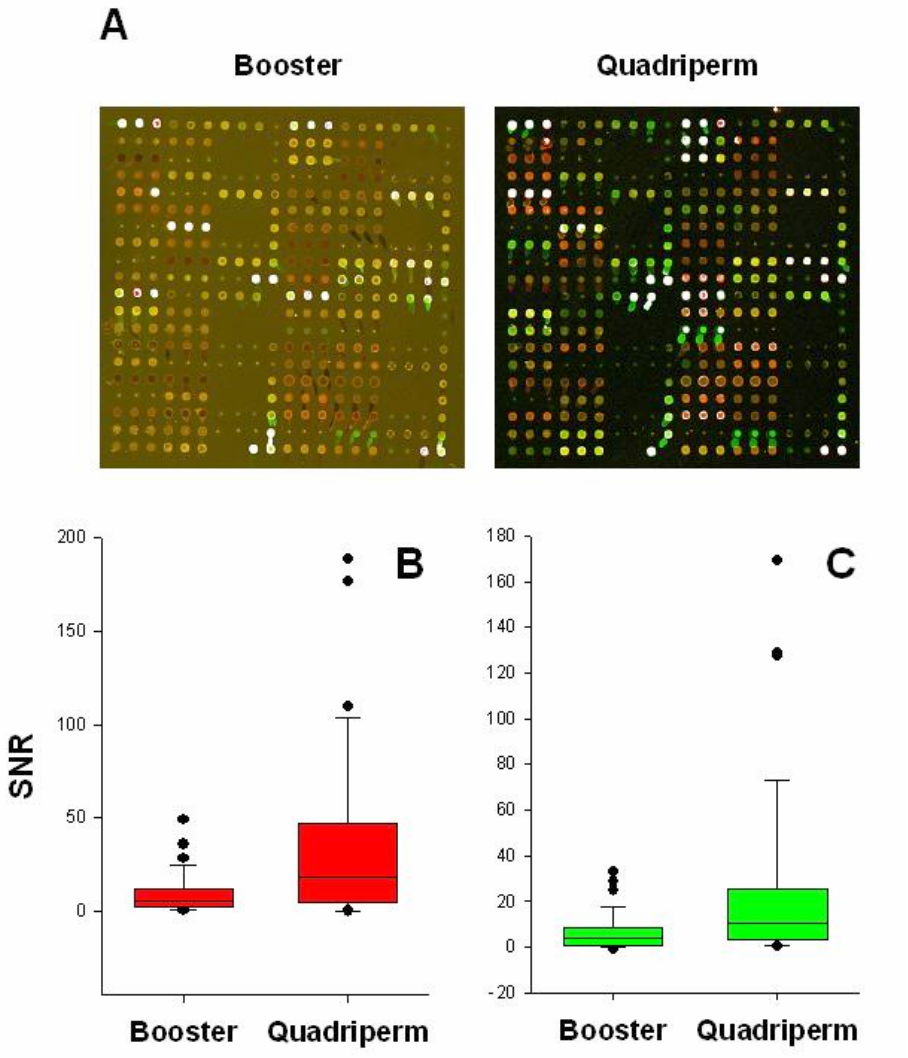

Fig. 3S. The effect of sample agitation methods during incubation. SU-8686 lysates were incubated using the Slidebooster or Quadriperm system at 4°C overnight as described in the text. The superimposed images (A) and the respective SNRs of feature intensities obtained in the red (B) and green (C) scanning channels are shown.

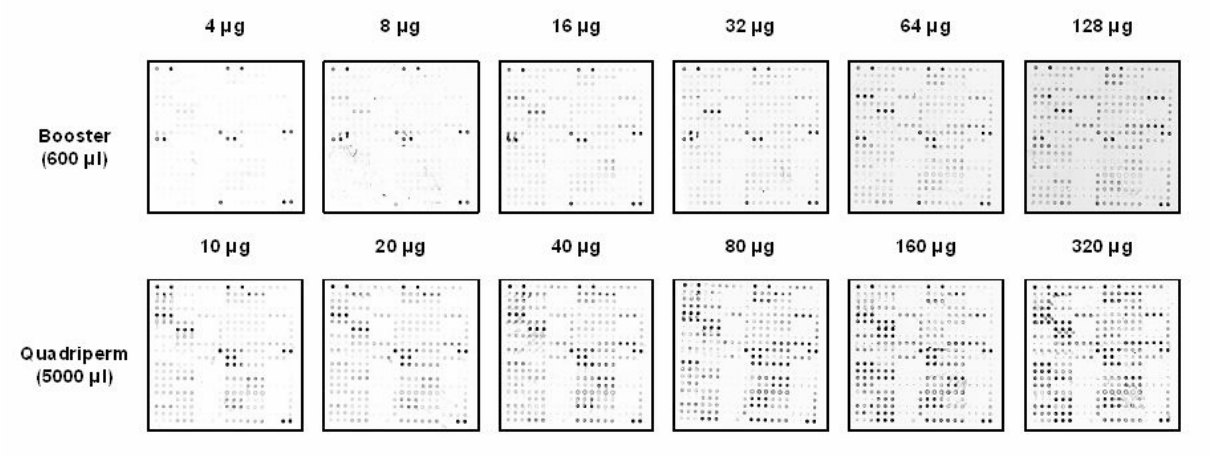

Fig. 4S. Protein concentration effects. Images are shown of arrays incubated with increasing quantities of DY-649 labeled protein extract from SU-8686 cells using either the Slidebooster or Quadriperm systems, which require 600 µl or 5000 µl of incubation volume, respectively.

Protein processing

Protein samples were manipulated during and after extraction in an attempt to find optimal conditions for achieving highest quality and reproducibility of the assay. For that purpose, proteins were extracted under native conditions as described [4] or denatured (RIPA buffer; see Methods part) conditions from human pancreatic tissues and cultured cells. Additionally, and for further simplification of the protocol, the buffer mix for the isolation of native proteins was modified by replacing Hepes with carbonate buffer, so as to avoid a buffer change between extraction and labeling. Extraction under native conditions resulted in a better SNR than extraction with protein denaturation (Fig. 5S). Furthermore, replacing Hepes with carbonate and thus using the same buffer during labeling had no significant effect on signal quality, although SNR was slightly better using Hepes.

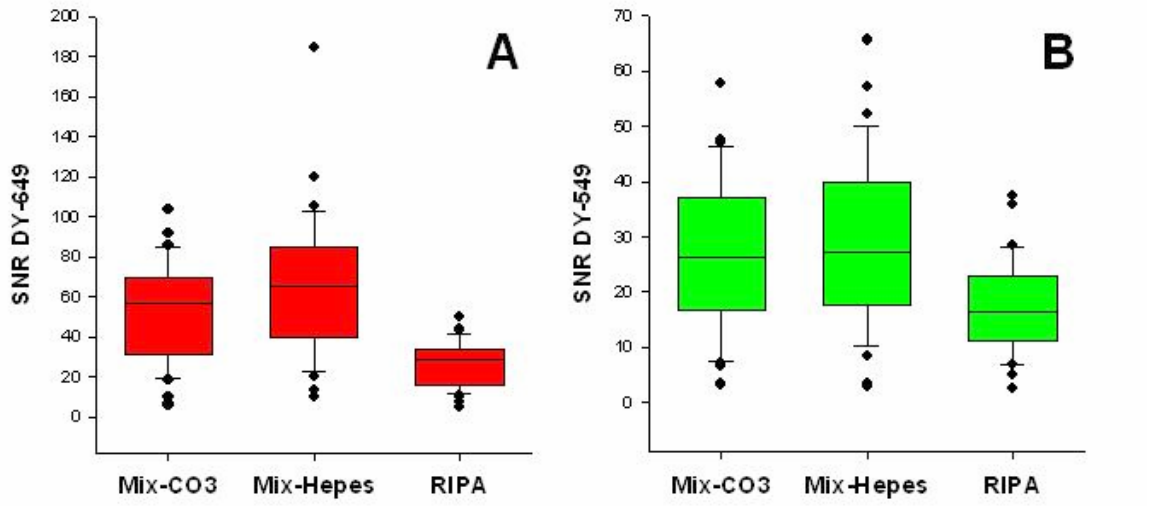

Fig. 5S. The effect of extraction. Protein was extracted from BxPC-3 or SU-8686 cells and pancreatic cancer tissue homogenates (pool of 11 samples) using the three buffer systems followed by labeling and analysis on arrays as described in Materials and Methods. The mean of SNRs of feature intensities from 6 experiments (two for each protein source) at both red (A) and green (B) scans are shown.

Protein labeling

Protein labeling was investigated intensively before [10, 54-58]. Here, five dye-pairs were tested to choose the pair that exhibits the least bias for labeling differences. The same protein sample extracted from SU-8686 cells was labeled at an 18 mole ratio of D/P using Cy3 and Cy5 (CyDye), ATTO-550 and ATTO-647 (Atto), Oyster-550 and Oyster-650 (Oyster), DY-547 and DY-647 (DY-7), as well as DY-549 and DY-649 (DY-9). The scatterplot of the signal intensities (background subtracted) at the green and red channel and the Pearson correlation coefficient (r) for each dye is shown in Fig. 6S.

We found that dye bias is less pronounced with increasing polarity of the dye-pair. The Dyomics dyes DY-549 and DY-649 contain three sulfonate groups each and are the most soluble pair among the tested molecules. Overall best correlation was found for DY-9 and CyDye followed by Oyster, DY-7, and then the Atto dye pair. However, when protein from tissues rather than cell lysate was used, CyDye slightly outperformed DY-9 (not shown).

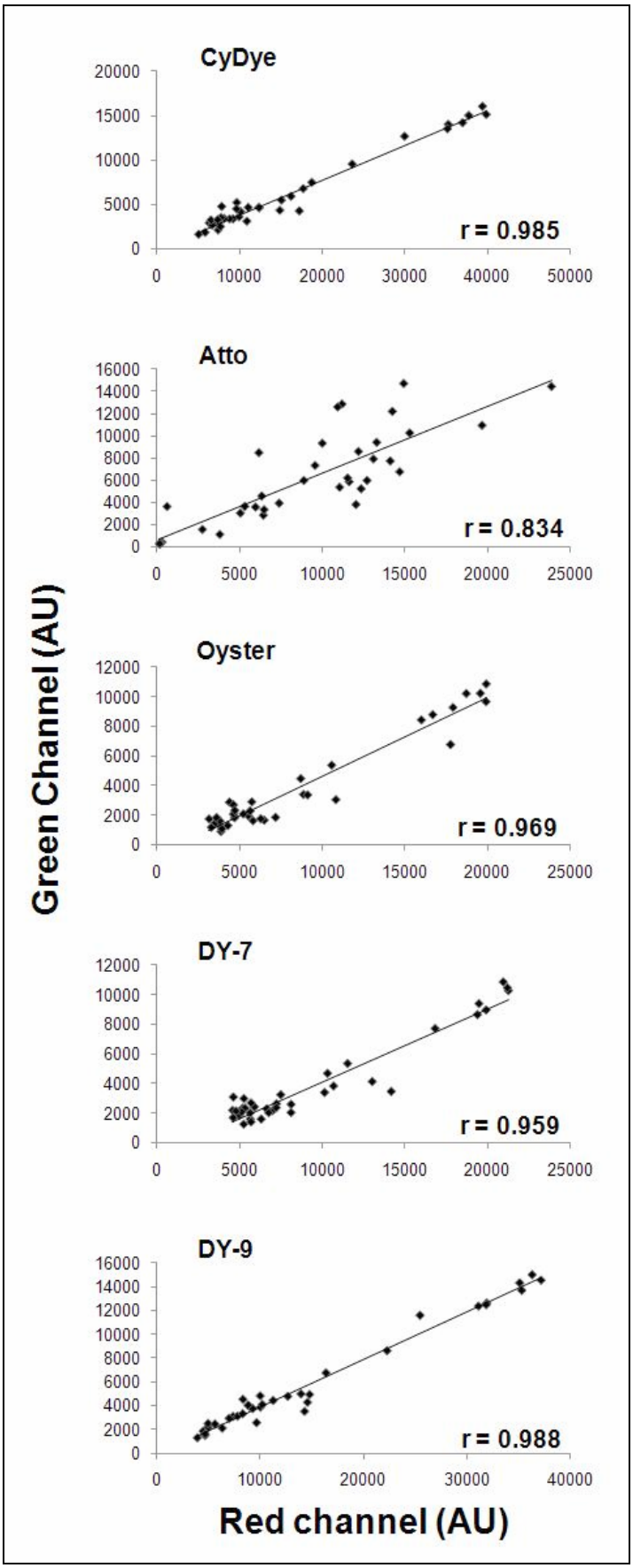

Fig. 6S. Labeling correlation of various dye pairs. SU-8686 protein extract was labeled with the five dye pairs and analyzed in duplicates as described in Materials and Methods. The scatterplots show the Pearson correlation coefficient and the relationship between each pair of the five tested dye pairs on an array of 38 antibodies

The best labeling ratio was determined after serially labeling protein samples at D/P molar ratios of 1, 2, 4, 6 and up to 28 with the DY-9 dye pair, assuming that the average molecular weight of a protein is 60 kDa [49]. Signal intensities at the individual spots of the antibody array increased gradually to a D/P of 14, followed by a steady signal at ratios of up to 22, and slightly declined at higher values (Fig. 7S). Spectrophotometric measurements of the degree of labeling achieved at a D/P of 14-22 revealed an actual ratio of 0.75-1.23. Gel electrophoresis, on the other hand, showed a continuous increase in the fluorescence intensities of protein bands even at high D/P ratios (Fig. 8S).

Interestingly, one of the antibodies used on the microarray targets the protein cyclin-dependent kinase inhibitor 2A (CD2A1), which lacks any lysine residue. In the assay, the signal at this antibody showed a constant intensity at both the red and green channel beyond a D/P labeling ratio of 14 (Fig. 7S). Due to the lack of lysines, CD2A1 can only be labeled at the terminal amino group. It therefore provided an excellent internal standard, showing the change in labeling efficiency and its effect on the recognition of capture antibodies to the labeled targets.

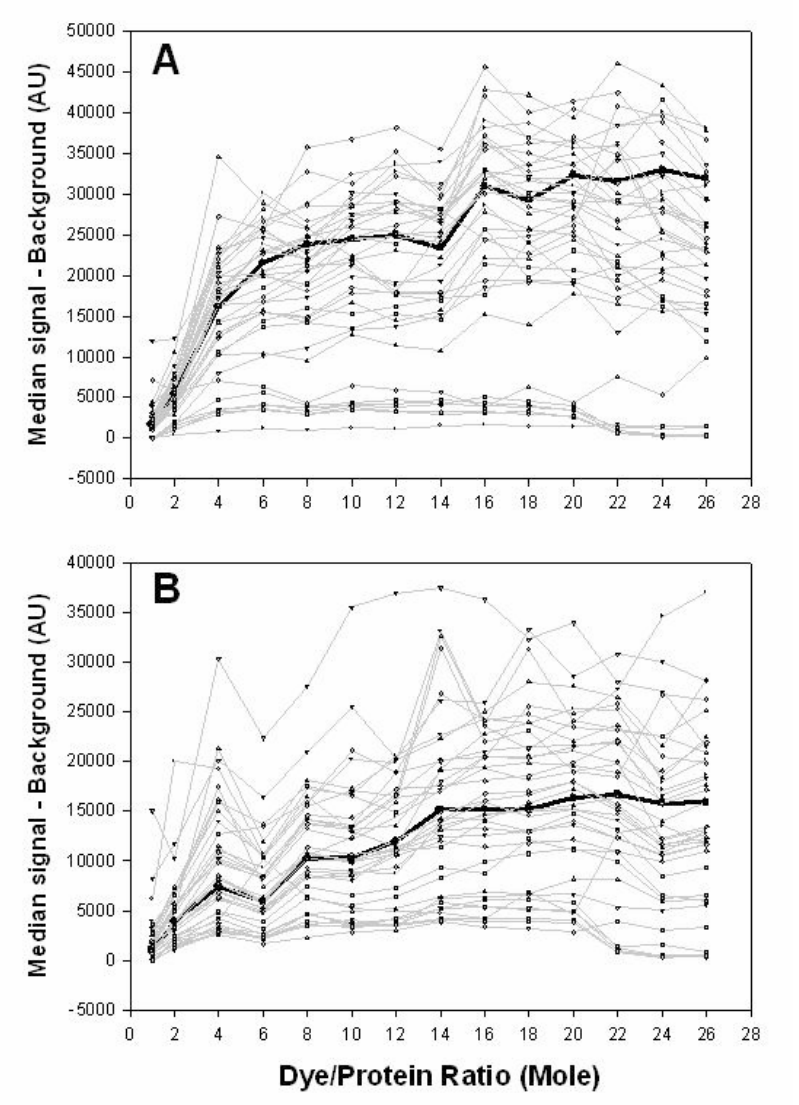

Fig. 7S. The effect of increasing Dye/Protein molar ratios on the median signal intensities of 38 antibodies is presented for red (A) and green (B) dyes. The bold lines represent the results for protein cyclin-dependent kinase inhibitor 2A (CD2A1), which contains no lysine residues for labeling. Each data point represents the mean signal intensity of duplicate experiments using BxPC-3 protein extract.

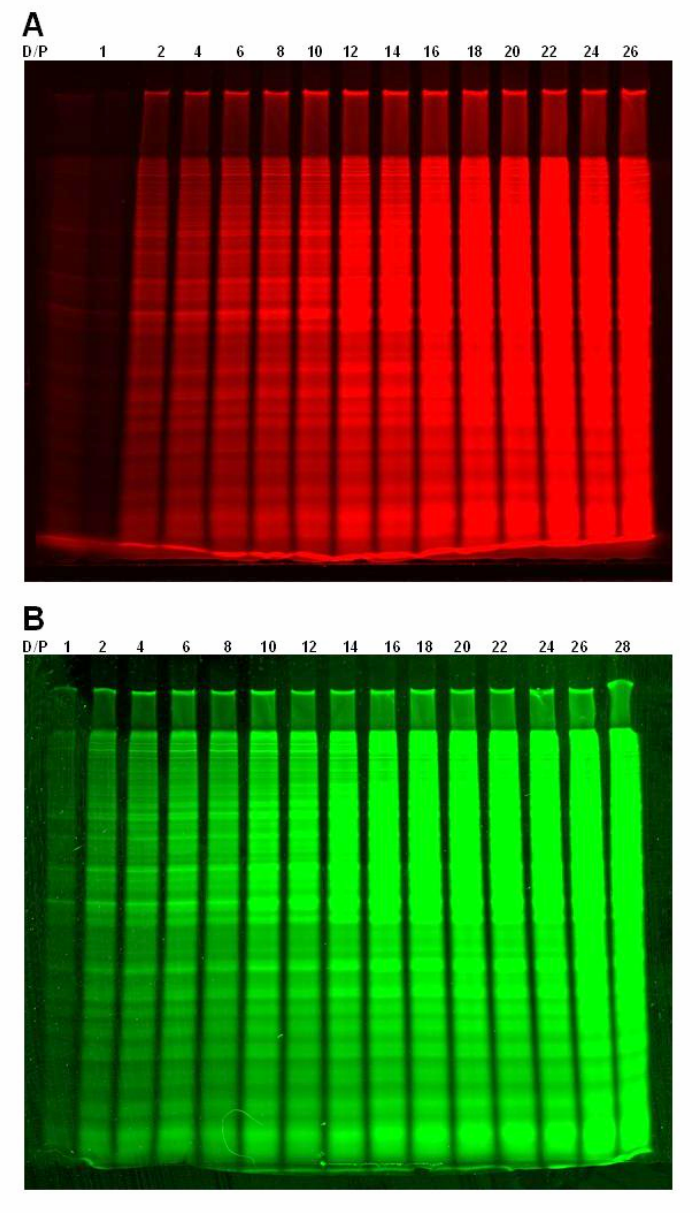

Fig. 8S. SDS-PAGE gel electrophoresis of protein samples labeled with DY-649 (A) and DY-549 (B). BxPC-3 samples were labeled at the indicated Dye/ Protein ratio (D/P), followed by gel separation and scanning at the red and green channels.

Dye removal after labeling

We also evaluated the impact of dye removal methods on SNR. After direct protein labeling, unreacted NHS-ester dye was first quenched with 10% glycine followed by either (i) dialysis against PBS for three days, (ii) buffer change to PBS using spin columns, or (iii) no removal at all. Surprisingly, we found no apparent effect for unreacted dye on the quality of the array results (Fig. 3; see main text). To investigate whether or not there is a possible interaction between the spots and the quenched dye remnants, we conducted a reaction of the NHS-ester dye and glycine alone in the absence of protein but at the conditions identical to that used for protein labeling. The inactivated dye was then incubated on arrays. No signals could be detected at the spot positions on the array when the dye alone was used (Fig. 9S).

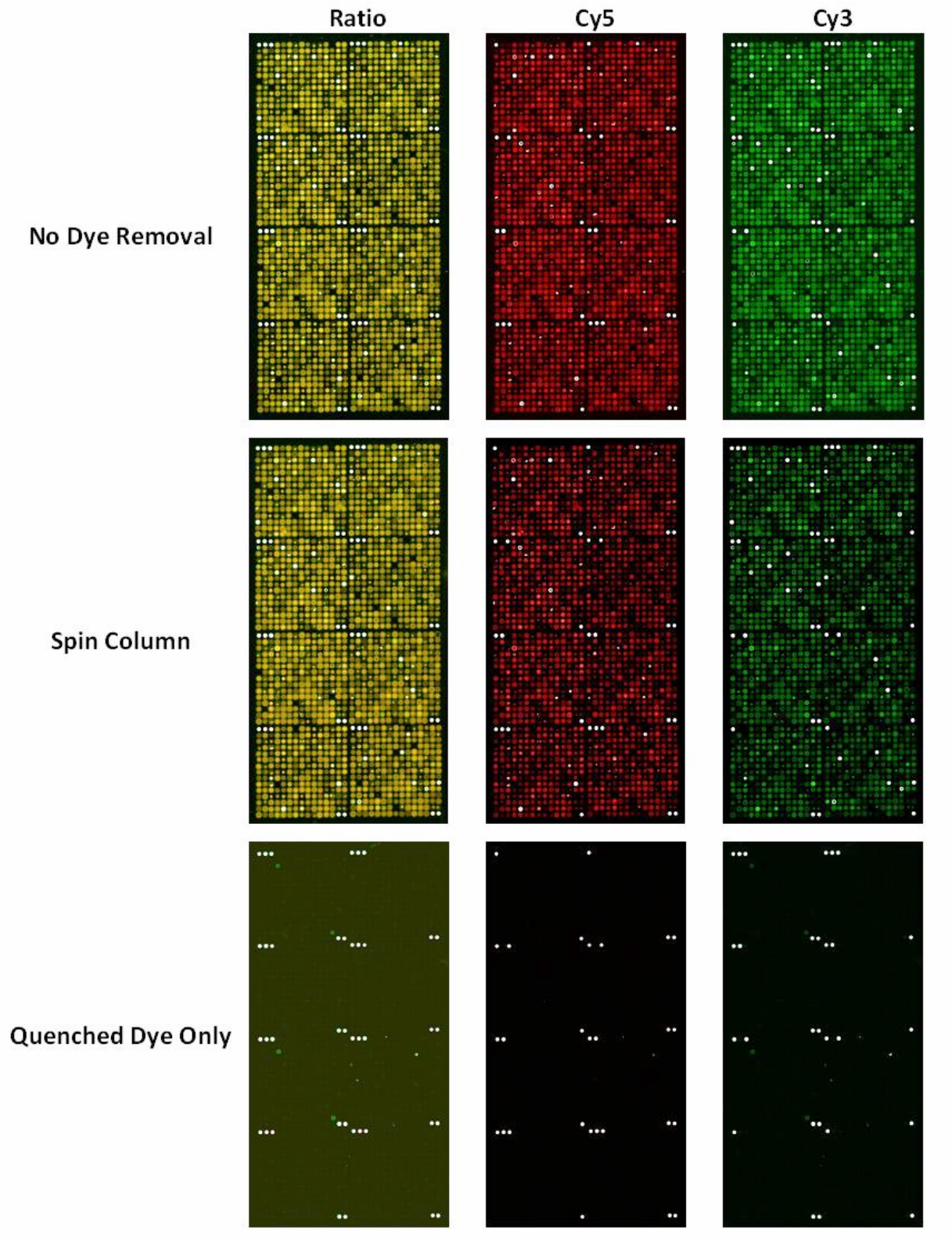

Fig. 9S. The effect of dye removal on data quality. Images are shown of large microarrays with some 1800 features made from 810 antibodies. Prior to incubation, unreacted dye was not removed (top panels) or separated from the labeled protein by a spin column (middle). The bottom row shows the results obtained in an experiment without protein; quenched dye alone did not generate any signal on the antibody arrays. Suit-007 (labeled with DY-549) and Suit-028 (labeled with DY-649) protein extracts were used.

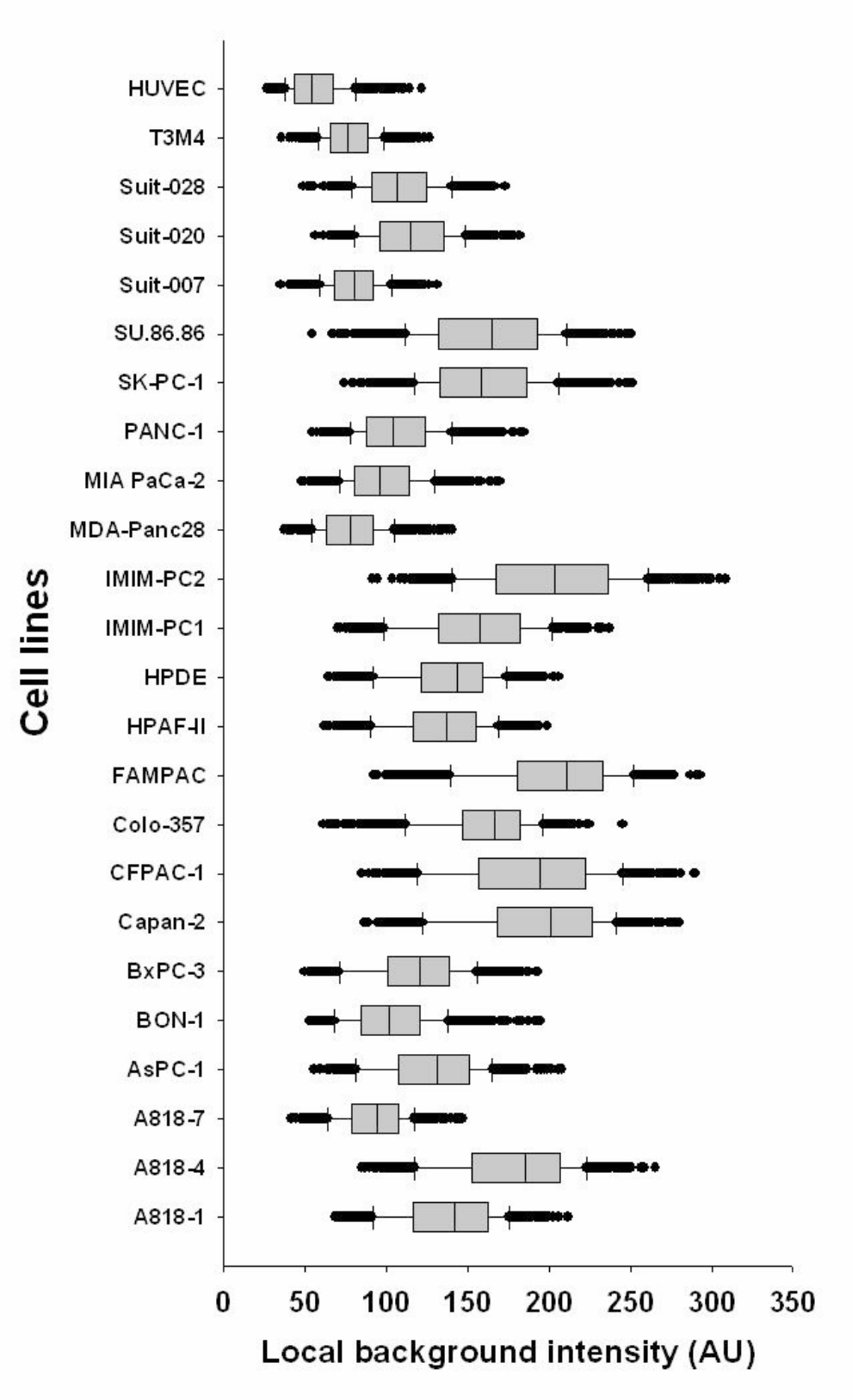

Fig. 10S. Variation among different samples. The background intensities for 24 different cell lines are shown. Cell lines were analyzed in duplicate with the large antibody array. For all cell lines, background intensities were reproducibly in a range of 20 to 300 arbitrary units, while signals were in a range of 10,000 to 40,000 units.

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Chapter 14

Robust Protein Profiling with Complex Antibody Microarrays in a Dual-Colour Mode

Christoph Schröder, Mohamed S.S. Alhamdani, Kurt Fellenberg, Andrea Bauer, Anette Jacob, and Jörg D. Hoheisel

Abstract

Antibody microarrays are a multiplexing technique for the analyses of hundreds of different analytes in parallel from small sample volumes of few microlitres only. With sensitivities in the picomolar to femtomolar range, they are gaining importance in proteomic analyses. These sensitivities can be obtained for complex protein samples without any pre-fractionation or signal amplification. Also, no expensive or elaborate protein depletion steps are needed. As with custom DNA-microarrays, the implementation of a dual-colour assay adds to assay robustness and reproducibility and was therefore a focus of our technical implementation. In order to perform antibody microarray experiments for large sets of samples and analytes in a robust manner, it was essential to optimise the experimental layout, the protein extraction, labelling and incubation as well as data processing steps. Here, we present our current protocol, which is used for the simultaneous analysis of the abundance of more than 800 proteins in plasma, urine, and tissue samples.

Key words: Antibody microarray, Proteomic profiling, Dual-colour analysis, direct sample labelling, Plasma profiling, Multiplexed immunoassay

1. Introduction

Antibody microarrays represent a relatively new technology in proteomics, which facilitates the analyses of hundreds of analytes in a parallel manner. Only small sample volumes are required (1–3). In the last decade, they have gained importance due to their advantageous combination of multiplexing capacity and very high sensitivities of low femtomolar range, even without signal amplification (4–6). Therefore, even for complex protein samples no expensive and elaborate protein pre-fractionation or protein depletion steps are needed (7). As with DNA microarrays, assay robustness and reproducibility could be improved dramatically by the

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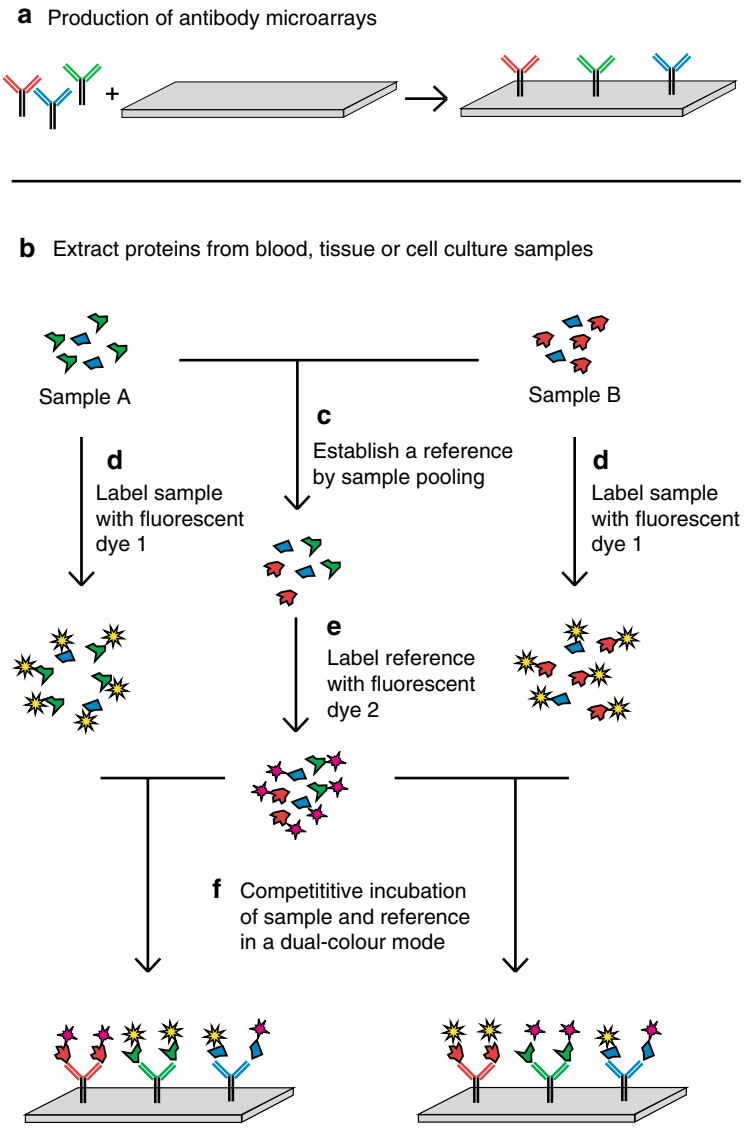

Fig. 1. Schematic representation of an antibody microarray experiment in a dual-colour mode and with a reference-based design.

implementation of a dual-colour assay (7, 8), which is therefore a standard element of our current protocol.

Antibody microarray experiments comprise five major steps: array production (Subheading 3.1), protein extraction (Subheading 3.2), sample labelling (Subheading 3.3), incubation (Subheading 3.4), and finally image acquisition and data analysis (Subheading 3.5). For array production (Fig. 1a), a set of different antibodies is immobilised at distinct locations on a planar surface. Protein samples are extracted from different sources such as plasma, serum, urine, tissue, or cell culture (Fig. 1b). Subsequently, there are different experimental design options for dual-colour assays. For a direct comparison, two

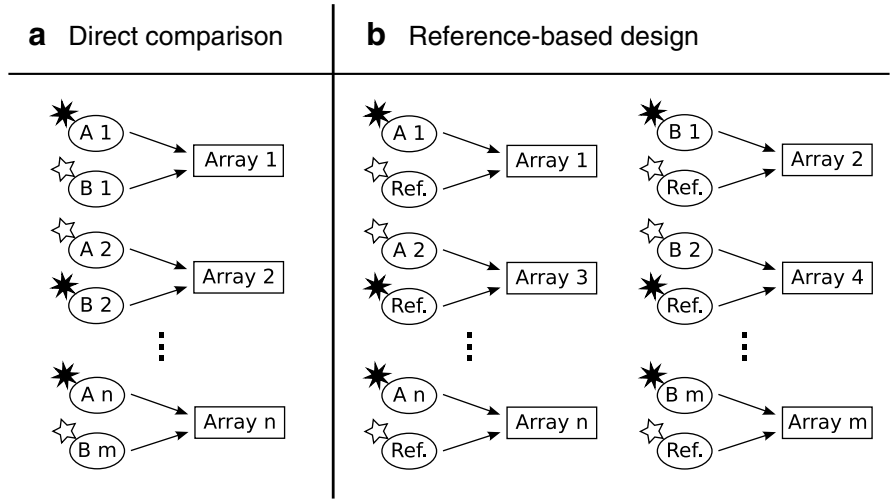

Fig. 2. Experimental layouts for microarray experiments. A scheme is shown for two different biological factors (**a**, normal; **b**, cancer) with biological replicates (1, 2, n, m). Samples can either be compared directly on the same array (**a**) or indirectly via a common reference (**b**).

samples or sample types are labelled with different fluorescent dyes and incubated competitively on the same array (Fig. 2a). Another option is a reference-based design (Fig. 2b). Herein, each sample is labelled with the same fluorescent dye (Fig. 1d) and incubated competitively with a common reference (Fig. 1f), which is labelled with a second dye (Fig. 1e). Such a reference sample can be established by pooling all samples (Fig. 1c) or a certain subset of samples represented in the study. The reference should be available in sufficient quantity for repeated incubation with all individual samples and encompass all sample types analysed in the study. A direct comparison is favourable for smaller studies in which a small number of parameters is analysed. The reference-based design facilitates the supplemental analyses of the impact of additional parameters such as gender, age, and the presence of a certain medication or disease without changes in the experimental design.

After incubation, slides are scanned and resulting fluorescence images transformed into signal intensities at the two colour channels using a software for spot recognition. The ratios of the two colour channels are used for the identification of differences. As with DNA-microarrays, most technical variation effects are eliminated by considering the ratios, leading to reproducible data (7). Besides sensitivity, a good reproducibility and consequently assay robustness are an essential prerequisite for proteomic profiling studies recording expression differences. In order to achieve high performance, we optimised the experimental layout, array production (4, 5, 9), protein extraction (10), labelling (4, 5) and sample incubation conditions (6) as well as data processing steps. Here, we present our current protocols which we use for a robust analysis of

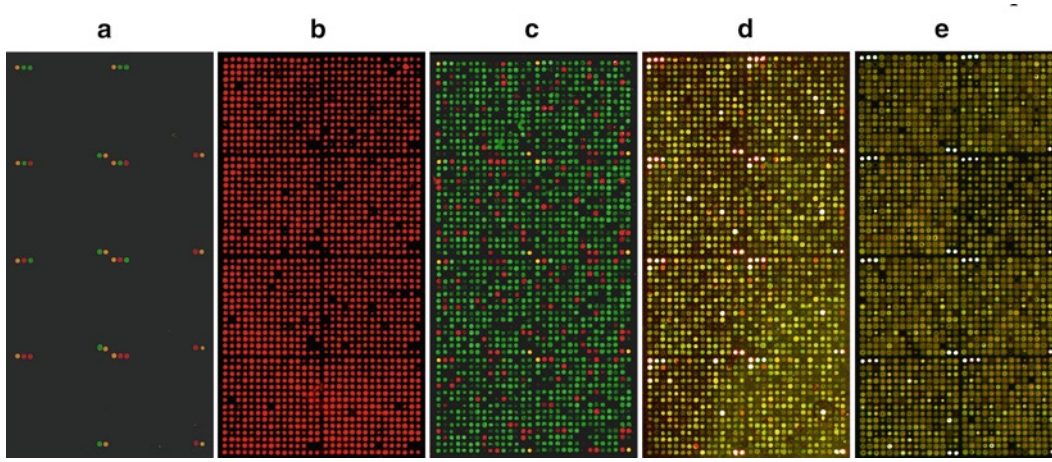

Fig. 3. Quality control of an antibody microarray consisting of some 1,800 features. Antibodies have been spotted in duplicates. (**a**) Two-colour positional controls facilitate easy tracking of the grid and verification of spot segmentation. (**b**) Sypro Ruby staining acts as a quality control measure of spotting. Negative controls do not show any immobilised protein. Panel (**c**) shows an antibody microarray incubated with 5 nM each of secondary fluorescently labelled antibodies against rabbit IgG (*green*) and mouse IgG (*red*). The majority of antibodies on the microarray were produced in rabbits. Panels (**d**) and (**e**) present antibody microarrays that were incubated with two human plasma (**d**) or cell culture samples (**e**) following the protocols presented here.

the abundance of more than 800 proteins in plasma, urine, and tissue samples (7, 10). Applying these protocols, we could demonstrate a high quality of the array production (Fig. 3). In a profiling study on urine samples, for example, pancreatic cancer patients and healthy controls could be differentiated on the basis of the protein patterns obtained (Fig. 4).

2. Materials

The following stock solutions and buffers are used in more than one of the methods. Stock solutions were prepared in ultrapure water, unless stated otherwise in the text.

1. 20% Triton X-100 stock solution (w/v); filter for sterilisation. *Attention: Triton X-100 is irritant and dangerous for the environment.*
2. 20% Tween-20 stock solution (w/v); filter for sterilisation.
3. 1 M Sodium bicarbonate buffer (pH 9.0); autoclave for sterilisation; store in aliquots at –20°C until use.
4. 5% Sodium azide stock solution (w/v).
5. 100× Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Bonn, Germany).
6. 10× PBS stock solution.

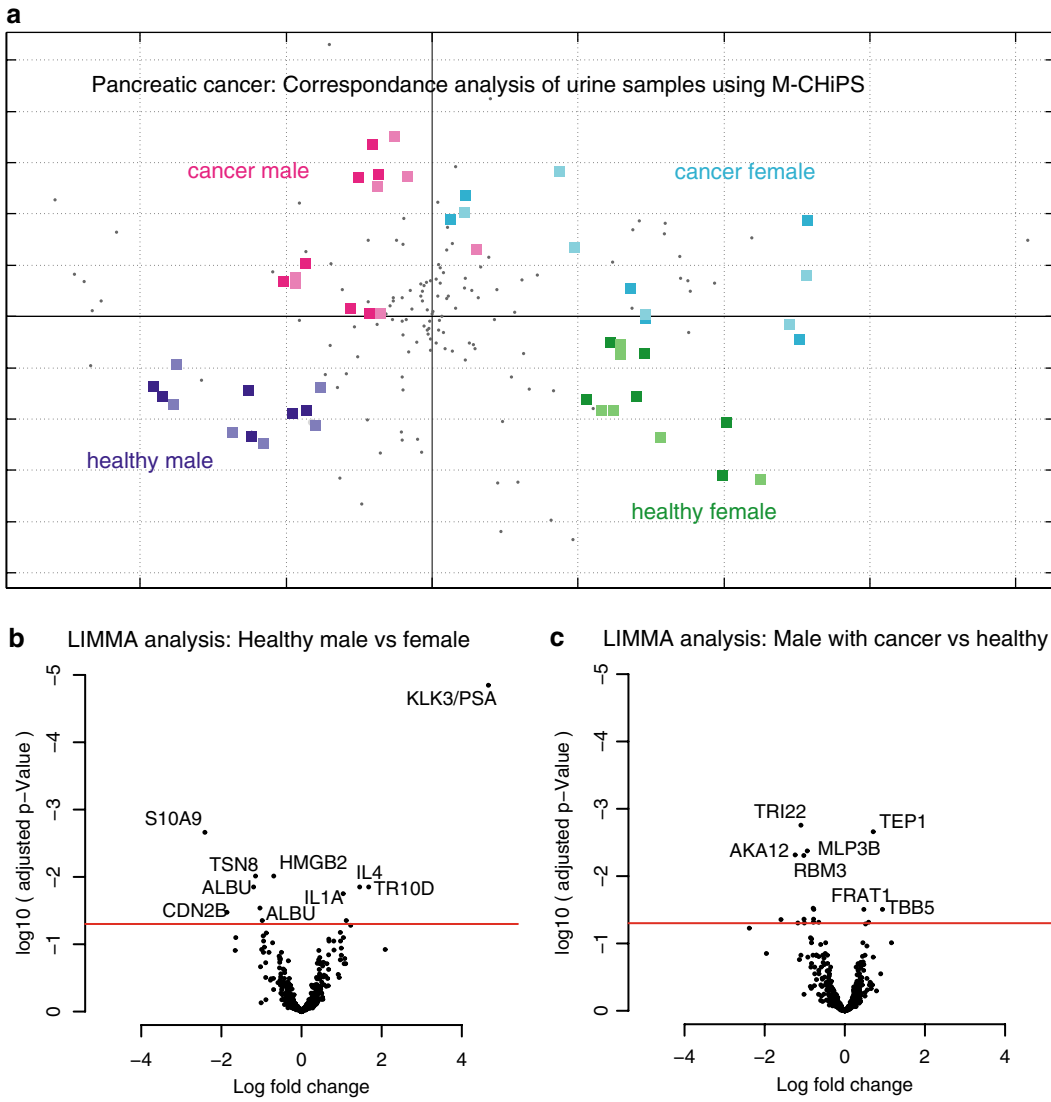

Fig. 4. The protocols presented here were applied for a profiling of urine samples from patients with pancreatic adenocarcinoma and healthy controls. (**a**) Correspondence analysis with M-CHiPS (15–17) resulted in a biplot of differentially abundant proteins and the samples. Samples are depicted as squares that are coloured according to disease-state and gender; black spots represent differentially expressed proteins. Samples located in the same direction from the centroid of the plot exhibit a similar expression pattern. The smaller the distance between two samples the higher is the concordance of their expression profiles. Proteins were found, which are particularly associated with the different sample groups. This association is indicated in the correspondence analysis plot by localization in the same direction off the centroid as the respective sample type. (**b**–**c**) Volcano plots summarise the results of LIMMA analyses (12). Log-fold changes and adjusted *p*-values are shown for gender-specific comparisons of the healthy (**b**) as well as disease-specific comparisons of the male subgroup (**c**). The *red line* marks a significance level of $p = 0.05$. This research was originally published in Molecular and Cellular Proteomics (7), © the American Society for Biochemistry and Molecular Biology.

7. Washing buffer A: 0.01% sodium azide (w/v), 0.05% Tween-20 (w/v), 0.05% Triton X-100 (w/v) in 1× PBS.
8. Washing buffer B: 0.5× PBS.

2.1. Antibody Microarray Production

1. Microarraying robot: several commercial models are available. Protocols have been established using the contact printers MicroGrid 2 (Genomic Solutions, Ann Arbor, USA) and SDDC-2 (ESI, Toronto, Canada) as well as the contact-free piezo spotter NP-2 (GeSiM, Großerkmannsdorf, Germany).
2. Centrifuge suitable for 384-well plates.
3. Epoxy-coated slides (Nexterion E, Schott, Jena, Germany).
4. Poly or monoclonal antibodies, affinity-purified in PBS with a concentration of 2 mg/mL (see Note 1 and Subheading 3.1.1).
5. Sypro Ruby protein blot stain (no. S4942, Sigma-Aldrich Corp., St. Louis, USA).
6. 10% Dextran (no. 31394, Sigma-Aldrich Corp., St. Louis, USA) stock solution (w/v) in H_2O; store at 4°C until use.
7. 10% Trehalose stock solution (w/v).
8. 1% Igepal CA-630 (no. I3021, Sigma-Aldrich Corp., St. Louis, USA) stock solution (v/v) in H_2O.
9. 100 mM Sodium borate buffer (pH 9.0).
10. 2× Spotting buffer: mix 2 mL 100 mM sodium borate buffer, pH 9.0, 20 µL 5% sodium azide, 0.5 mL 10% dextran stock solution, 10 µL 1% Igepal stock solution and adjust the volume to 10 mL with H_2O; filter for sterilisation. Prepare aliquots and store at –20°C until use.
11. Washing buffer C: 10% (v/v) methanol, 70% (v/v) acetic acid in H_2O.

2.2. Protein Extraction

1. Cooled centrifuge.
2. Small size porcelain mortar and pestle.
3. Cell scrapers.
4. 2–5 mL syringes with 25-gauge needles.
5. Liquid nitrogen.
6. Dulbecco's phosphate-buffered saline (DPBS).
7. 250 U/µL of benzonase.
8. 60 mM Magnesium chloride ($MgCl_2 \cdot 6H_2O$) stock solution.
9. 100 mM EDTA (pH 8.5) stock solution.
10. 200 mM Phenylmethanesulfonyl fluoride (PMSF) stock solution in iso-propanol; store in small aliquots at –20°C for up to 6 months.
11. 10% Nonidet P-40 substitute (NP-40S) in H_2O (w/v).
12. 10% Cholic acid sodium salt; filter for sterilisation (w/v).

13. 5% Amidosulfobetaine-14 (ASB-14) (w/v); filter for sterilisation and store up to 6 months at 4°C. ASB-14 may precipitate upon cooling, but can be solubilised again by bringing to room temperature while vortexing.
14. 2.5% *n*-dodecyl-β-d-maltoside (12-Malt) (w/v) (GenaXXon Bioscience GmbH, Ulm, Germany); filter for sterilisation and store up to 6 months at 4°C.
15. Glycerol (BioUltra grade).
16. Working solution of the extraction/labelling buffer: prepare from stock solutions by mixing 2.0 mL glycerol, 500 µL EDTA, 1.0 mL of each of carbonate buffer, NP-40S, cholate, 12-Malt, and ASB-14, 167 µL magnesium chloride, 50 µL PMSF, 4.0 µL benzonase, and 100 µL of proteases and phosphatases inhibitors cocktail. Complete the volume to 10 mL with H_2O, and keep on ice until use. The working solution is stable for 1 week at 4°C. However, PMSF loses its activity 30 min after dilution in the working buffer, and hence, always needs to be added directly before cell lysis.

2.3. Labelling of Protein Samples

1. *N*-Hydroxysuccinimide (NHS)-ester of fluorescent dyes (e.g., Dy549-NHS, DY649-NHS, Dyomics, Jena, Germany).
2. Pierce Zeba Spin Desalting columns (Thermo Fisher Scientific, Waltham, USA).
3. Hydroxylamine (Sigma-Aldrich Corp., St. Louis, USA).
   Attention: *hydroxylamine is harmful and dangerous for the environment.*

2.4. Sample Incubation

1. Advalytix Slidebooster (Olympus Life Science Research, Munich, Germany).
2. Homemade Plexiglas incubation chambers, which have a slightly larger inner dimension than the spotting area and can be reversibly attached to the slides by double-sided adhesive tape. As an alternative, LifterSlips or Gene Frames (Thermo Fisher Scientific, Waltham, USA) can be used (see Note 2).
3. Slide racks and containers (no. 2285.1, Carl Roth GmbH, Karlsruhe, Germany).
4. Blocking buffer: 5% non-fat dry milk (Biorad, Munich, Germany), 0.01% sodium azide, 0.05% (w/v) Tween-20 in 1× PBS. Mix well for at least 30 min on a magnetic stirrer in order to allow the milk powder to dissolve completely. Store at 4°C and use within a few days.

2.5. Scanning and Data Analysis

1. Microarray scanner: several commercial models are available. The protocols described here have been established using the ScanArray 4000XL (Perkin Elmer, Waltham, USA).
2. Software for image segmentation and spot recognition of the signals obtained on the microarrays: e.g., GenePix Pro

(Molecular Devices, Sunnyvale, USA), Mapix (Innopsys, Carbonne, France), or TIGR SpotFinder (11).

3. Software for data analysis: several open-source packages as well as commercial programmes are available (see Note 3).We used the LIMMA-package (12) of R-Bioconductor and M-CHiPS.

3. Methods

According to the experimental process, the protocols below are divided into five sections: antibody microarray production (Subheading 3.1), protein extraction (Subheading 3.2), sample labelling (Subheading 3.3), incubation of the samples on the microarrays (Subheading 3.4), and finally image acquisition and data analysis (Subheading 3.5).

3.1. Production of Antibody Microarrays

3.1.1. Pre-processing and Storage of Antibodies

Perform all subsequent steps at 4°C or on ice.

1. Purify antibodies that are delivered in a crude formulation like ascites fluid, whole antiserum, or in the presence of stabilisers such as bovine serum albumin (BSA) or gelatine. Use Protein A or G columns (e.g., Pierce Nab Protein G Spin Kit, Thermo Fisher Scientific, Waltham, USA) depending on the exact host used for antibody production and antibody isotype. Follow the instructions of the respective user manual.
2. For antibodies formulated in another buffer system or with the addition of glycerol, exchange buffer to PBS by dialysis (Pierce Slide-A-Lyzer, Thermo Fisher Scientific, Waltham, USA).
3. If necessary, adjust antibody concentration to 2 mg/mL (see Note 1) by filtration (Microcon YM-100, Millipore, Schwalbach, Germany) or dialysis (Pierce Slide-A-Lyzer and Pierce Slide-A-Lyzer concentrating solution; Thermo Fisher Scientific, Waltham, USA).
4. Prepare 5 µL aliquots (see Note 4) of antibody solution to avoid additional freeze–thaw cycles and store antibody aliquots at –20°C until use.

3.1.2. Preparation of Antibody Spotting Microtiter Plates

Prepare the spotting microtiter plate(s) directly prior to microarray spotting. Handle all tubes and plates on ice.

1. Thaw antibodies on ice.
2. Mix 5 µL antibody with 5 µL 2× spotting buffer (see Note 4) in order to have a final spotting concentration of 1 mg/mL.
3. Transfer the mix to the appropriate wells of the spotting microtiter plate. Pipette carefully in order to prevent any air bubbles.

4. Include positional controls: add 0.25 mg fluorescently labelled protein, 0.75 mg BSA, and 1× spotting buffer in a volume of 10 µL to wells of the spotting microtiter plate(s). Positional controls (Fig. 3a) facilitate an easy identification of slide orientation and grid recognition as well as spot segmentation during image processing.
5. Include negative controls: add 5 µL 2× spotting buffer and 5 µL PBS to some wells of the spotting microtiter plate(s).
6. Mix liquid in the microtiter plate wells thoroughly using a plate vortexer.
7. Centrifuge plate(s) at 1,000 × *g* for 2 min and keep at 4°C covered with a lid until array spotting.

3.1.3. Spotting Process

Prior to the final array production, a test-spotting run should be performed in order to assess the performance of the existing infrastructure. The test system should mimic the final production run with regard to the number of antibodies, complexity of the arrays as well as duration of spotting. BSA or immunoglobulins can be used for the test spotting (see Note 5). The quality of array production can be assessed as described in Subheading 3.1.4.

1. Program the robot and fill the washing buffer reservoirs and the air humidifier. If the robot has a cooling system, set temperature to 10°C.
2. For pin spotters, clean the pin tool and the pins thoroughly (see Note 6). Follow the manual of the pin manufacturer.
3. Start a pre-spotting by delivering at least 1,000 spots of 1× spotting buffer containing 1.0 mg/mL BSA. Make sure that all pins or piezo needles are performing well.
4. Place the slides in the robot using powder-free nitrile gloves. Pay attention not to touch the slides on the surface.
5. Place the spotting microtiter plates in the robot. If the robot has no cooling device, allow the plates to adapt to room temperature beforehand.
6. Allow the relative humidity in the robot to reach a value of about 50%.
7. Start the spotting process.
8. After spotting is finished, keep slides for two more hours within the robot at 50% humidity.
9. Leave the slides overnight at 4°C in the dark.
10. Perform quality control analysis (Subheading 3.1.4) for a part of the slides picked randomly from different positions within the microarraying robot.
11. Store slides dry at 4°C (e.g., in an exsiccator).

3.1.4. Quality Control

For quality control, immobilised antibodies can be stained after microarray production by a fluorescent dye such as Sypro Ruby (Fig. 3b). It is essential to perform such staining prior to blocking the slide surface.

1. Wash slides 4× 5 min on a shaker in washing buffer A.
2. Cover slides for 1 h with the ready-to-use Sypro Ruby staining solution.
3. Wash slides 4× 5 min in washing buffer C.
4. Wash slides 2× 5 min with H_2O.
5. Dry each slide individually by pointing a sharp stream of air to each spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
6. Scan the slides with a microarray fluorescence scanner recording the emission at 610 nm using excitation at 280 or 450 nm.

As an additional control, antibody microarrays can be incubated with 10 nM fluorescently labelled secondary antibodies (Fig. 3c) in blocking buffer for 2 h to control protein immobilisation and functionality. See Notes 7–10 for how to solve the most common problems faced in array production.

3.2. Protein Extraction

Sample handling has a major effect on the protein quality and composition. Therefore, treat all specimens within an experiment series in a uniform manner. Make sure that this happens also prior to their arrival in the microarray laboratory, e.g., during sampling. Typical factors that can affect quality are the kind of columns used for plasma/serum preparation, the period that a blood or tissue sample remains at room temperature prior to freezing, the time of adding protease inhibitors, and the number of freeze–thaw cycles. In general, all protein samples should be thawed and handled on ice in order to minimise degradation by proteases. Aliquot samples as soon as possible and avoid repeated freeze–thaw cycles.

While plasma or serum is prepared using standard procedures, the extraction of proteins from tissue or cell culture should be performed according to the protocols given below in order to facilitate an effective extraction, which is compatible with the subsequent label reaction and analyses on antibody microarrays (10).

Attention: *Human samples can potentially be infectious and should therefore be handled as biohazard.*

3.2.1. Tissue Samples

1. Prepare from stock solutions a working solution of the extraction/labelling buffer.
2. Mince tissues with a scalpel or a scissor to small pieces of 2–3 mm^3.

3. Immerse the mortar and pestle in liquid nitrogen for a minute until bubbling ceases. *Attention*: *Liquid nitrogen is extremely hazardous and may cause severe burns. Care should be taken to wear protective gear during handling* (see Note 11).
4. Snap-freeze the minced tissues in liquid nitrogen and transfer them immediately to the mortar. Layer the tissue with few millilitres of liquid nitrogen and pulverise with the pestle until the tissue has become a fine powder.
5. Transfer the powder into a pre-weighed microfuge tube and add 10 µL of the extraction/labelling buffer for each 1 mg of tissue (e.g., 500 µL buffer to 50 mg of tissue).
6. Vortex vigorously to disperse the sample in the buffer.
7. Keep it on ice for 20 min with occasional vortexing.
8. Pipette the sample up and down ten times with a small syringe and 25-gauge needle.
9. Centrifuge at 20,000 × *g* at 4°C for 20 min.
10. Aspirate the supernatant with a fine needle.
11. Label samples and store at –20°C.

3.2.2. Cell Culture

1. All buffers must be cooled and procedures must be carried out on ice (see Note 12).
2. Prepare from stock solutions a working solution of the extraction/labelling buffer.
3. Completely remove culture medium from the vessel (see Note 13).
4. Wash cells three times with ice-cold DPBS.
5. Remove completely the wash buffer and leave the vessel for 1 min in an upright position to drain minute amounts of remaining buffer.
6. Add the minimal volume of the extraction/labelling buffer, which is sufficient to cover the entire surface of the culture vessel (see Note 14).
7. Keep flask in flat position on ice or in a refrigerator for 20 min. Inspection of cells under the microscope may give a good indication for the lysis efficiency.
8. Collect cells with cell scraper and transfer them to a 2-mL Eppendorf tube.
9. Pipette the sample up and down ten times with a small syringe and 25-gauge needle. This operation ensures shredding of chromosomal DNA and facilitates the effect of benzonase.
10. Centrifuge at 20,000 × *g* at 4°C for 20 min.
11. Aspirate the supernatant with a fine needle.
12. Label samples and store them at –20°C.

3.3. Labelling of Protein Samples

3.3.1. Label Reaction

Fluorescent labels are covalently attached to the amino groups of the proteins using NHS-ester chemistry. For competitive two-colour assays with a common reference, all samples are labelled by a NHS-fluorescent dye (e.g., Dy-649). In addition, a reference sample is labelled with a second NHS-fluorescent dye (e.g., Dy-549). In order to have sufficient volume for all incubations in the study, the reference sample is usually labelled in multiple reactions and then mixed. For each label reaction

1. Thaw protein samples on ice.
2. Measure the protein concentration by BCA assay; the protein concentration should be at least 5 mg/mL for blood samples and not less than 1.0 mg/mL for proteins extracted from tissue or cell culture (see Note 15).
3. Label blood samples in a final concentration of 4 mg/mL with 400 µM NHS-ester of a fluorescent dye in 1% Triton X-100 and 100 mM sodium bicarbonate in a final volume of 250 µL (see Notes 16 and 17). For labelling of tissue and cell culture samples, use a protein concentration of 1 mg/mL and a dye concentration of 200 µM. After protein extraction according to Subheadings 3.2.1 or 3.2.2, proteins can be labelled directly without the addition of carbonate or detergent, since these are already present in the extraction/labelling buffer.
4. Incubate reaction tubes on a shaker (200 rpm) at 4°C, protected from light.
5. To stop the reaction, add to each reaction tube hydroxylamine to a final concentration of 1 M and incubate for 30 min at 4°C.
6. Use Zeba Desalt columns (Pierce) in order to remove unreacted dye and exchange buffer to PBS according to the protocol provided by the manufacturer.
7. Add 1× protease and phosphatase inhibitors cocktail.
8. Prepare aliquots and store them protected from light at –20°C.

3.4. Sample Incubation

1. Wash slides three times in washing buffer A by quickly moving the rack up and down.
2. Incubate slides in blocking buffer (see Note 18) for at least 3 h at room temperature with shaking at 150 rpm.
3. Wash slides 4× 5 min in washing buffer A.
4. Wash slides 2× 5 min in washing buffer B.
5. Dry each slide individually by aiming a sharp stream of air to each spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
6. Attach home-made Plexiglas incubation chambers to the slide using a double-adhesive tape (see Note 2).

7. Place the slides on a slidebooster instrument (see Note 19).
8. Prepare incubation buffer by adding Tween-20 to a final concentration of 1% (w/v) to the blocking buffer.
9. Dilute labelled blood samples and the common reference 1:20 each with incubation buffer (e.g., mix 30 µL sample A, 30 µL reference, and 540 µL incubation buffer). Instead of 1:20 use a ratio of 1:100 for tissue and cell culture samples. Transfer the incubation mix into the incubation chambers.
10. Cover incubation chambers, start mixing, and incubate overnight. Keep incubation time consistent for all samples.
11. Remove the incubation mix and wash each array 4× 5 min with washing buffer A under mixing conditions. Add the washing buffer quickly in order to keep the slide surface wet.
12. Stop the slidebooster and place slides in a container filled with washing buffer A.
13. Detach incubation chambers. Take care to remove all remaining residues of adhesives and keep the array surface wet during removal.
14. Immediately place arrays in a slide rack in a container filled with washing buffer A and wash for 3× 5 min.
15. Wash 2× 5 min in washing buffer B.
16. Dry each slide individually by aiming a sharp air stream at the spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
17. Store slides protected from light until scanning.

3.5. Scanning and Data Analysis

3.5.1. Scanning the Microarrays

Detect signal intensities in a microarray scanner. Beforehand, adjust the scanner settings of the photomultiplier tube (PMT) and the laser power (LP) in order to obtain visible signals for most spots, with only a small number of saturated spots for abundant proteins (see Note 20). For two-colour incubations, adjust scanner settings additionally in a way that the signal intensity distributions for both dyes match each other. Keep scanner settings fixed for all arrays within an experimental series.

3.5.2. Spot Recognition

Convert recorded image files into signal intensities by a software for semi-automatic spot recognition as well as signal quantification such as GenePix Pro, Mapix, or the freeware TIGR Spotfinder. Adjust the size of the spots and if possible flag the spots according to their quality.

3.5.3. Analysis of Differential Expression

There are several freeware and commercial analysis platforms available (see Note 3). For detailed information how to use M-CHiPS for correspondence analysis (Fig. 4a) refer to the online manuals

(http://www.mchips.org). Here, we describe the procedure for the identification of differential proteins using the LIMMA package within R-Bioconductor (Fig. 4b, c). More detailed information can be found in the user's guide available with the package (http://www.bioconductor.org/packages/release/bioc/html/limma.html).

1. Build up a "Targets.txt" file. This file contains the filenames of the results files after spot segmentation. In two additional columns, a definition of the sample types used for incubation in the two colour channels is provided. For an analysis of the factors cancer/healthy and male/female the following sample types would be defined in the two columns: "cancer_male", "cancer_female", "healthy_male", "healthy_female", or "reference".
2. Load the limma library.

```
library(limma)
```

3. Import the Targets.txt file.

```
targets <- readTargets()
```

4. Import the mean of the signal intensities and the median of the background intensities.

```
RG<-read.maimages(targets$FileName, source="genepix",
    columns=list(R="F649 Mean",G="F549 Mean",Rb="B649
    Median",Gb="B549 Median"))
```

5. Subtract the local background using the normexp method.

```
RGb <-backgroundCorrect(RG,method="normexp",
    offset=50)
```

6. Log-transform the data and normalise the two colour channels using loess normalisation (see Note 21) in one go.

```
MA<-normalizeWithinArrays(RGb,method="loess",
    iterations=10)
```

7. Assess the quality of your incubations and the efficiency of normalisation by inspecting signal intensity distributions and MA-plots prior (a) and after normalisation (b). For the MA-plots inspect each array by incrementing `i` from 1 to the number of arrays by `i=1; i=2` …

   (a) `plotMA(RGb(,i))` and `plotDensities(RGb)`

   (b) `plotMA(MA(,i))` and `plotDensities(MA)`

8. Build up a design matrix defining the experimental layout for the linear models as well as a contrasts matrix defining the type of comparisons to be performed.

```
design <- modelMatrix(targets, ref="Reference")
contrasts.matrix <- makeContrasts
(cancer=(cancer_male + cancer_female)-(healthy_
    male + healthy_female),
```

```
gender=(cancer_male + healthy_male)-(cancer_female +
    healthy_female),
levels=design)
```

9. Apply the linear models and search for differential expression.

```
fit <- lmFit(MA,design)
fit2 <- contrasts.fit(fit, contrasts.matrix)
fit2 <- eBayes(fit2)
```

List proteins with differential abundance for the disease (a) and gender-specific (b) comparison.

```
(a) topTable(fit2, coef=1, adjust="BH")
(b) topTable(fit2, coef=2, adjust="BH")
```

4. Notes

1. *Antibodies*: All antibodies that are working with high specificity and sensitivity in Western and ELISA assays can be used for antibody microarray experiments. Western blotting is the current method of choice to assess the specificity of antibodies. Antibodies should be purified and formulated in PBS without addition of stabilisers such as BSA, gelatine, or glycerol, which are negatively affecting the spotting process. It is beneficial to immobilise the antibodies in a comparably high concentration of 1 mg/mL after addition of the spotting buffer. However, it is possible to immobilise antibodies at lower concentrations, if sensitivity is of lower importance than antibody consumption.
2. *Incubation chamber*: Also LifterSlips or Gene Frames (Thermo Fisher Scientific, Waltham, USA) can be used to keep the incubation volume. However, higher sensitivity will be obtained with the increased volumes that were made possible by an adapted, homemade incubation chamber.
3. *Software for data analysis*: Many very versatile tools for the normalisation, filtering, and statistical testing are available within the Bioconductor package (13) for R. Most important functions are also integrated in the online analysis platform Expression Profiler (14). Also the freeware TIGR MultiExperiment Viewer (11) allows an application of many different analysis algorithms to the data. We used the LIMMA package (12) in R-Bioconductor and M-CHiPS (15–17). M-CHiPS is well suited especially for correspondence analysis and for correlating the samples to all given biological factors. In addition, there is a variety of commercial tools available for data analysis.

4. *Spotting volume*: The volume used in the spotting plates is dependent on the spotting robot and the number of replicates and slides. For one spot, usually between 0.5 and 10 nL are used depending on pin size (contact printing) or number of drops (non-contact printing). The volume in each sample uptake is 0.25–1.25 µL for pin spotters. Usually, the minimum volume, which can be handled by a microarraying robot is around 5 µL. A volume of 15 µL should be highly sufficient for most spotting projects.
5. *Fluorescently labelled protein in test runs*: Fluorescently labelled proteins should not be used for optimisation of the spotting process. Their increased hydrophobicity that is due to the dye hides problems that could occur with antibodies. In addition, artefacts are introduced, which would never occur with unlabelled proteins. Therefore, it is recommended to use unlabelled BSA for optimising the spotting parameters in a test run and to visualise immobilised proteins by additional staining with Sypro Ruby (Subheading 3.1.4).
6. *Pin handling*: Always use powder-free nitrile gloves for handling pins and pin tool. Clean pin tool and pins exactly as recommended by the manufacturer. In a last step, dip them in 100% ethanol and dry them completely using a stream of air. Do not use pressurised air canisters, which might contain organic propellants.
7. *Spots are missing*: If spots are missing at random, in most cases spotting pins got stuck in the pin tool. Clean and dry pins and pin tool thoroughly. If the problem is persistent, decrease the humidity in the spotter to a value of 35–45%. If spotting stops after a certain number of replicates or samples, perform an intensive pin cleaning protocol using special reagents according to the protocol of the pin manufacturer (e.g., http://arrayit.com/Products/MicroarrayI/PPCK80/ppck80.html). If problems persist, increase the pin washing time between sample uptakes, reload pins more often or increase the concentration of detergent in the spotting buffer. If spots can be observed optically after array production, e.g., making them visible by "breathing" at the surface, but no or few proteins can be detected after staining, make sure that the epoxy groups of the array surface are still active. Avoid TRIS or betaine additions to the spotting solution.
8. *Carryover*: If carryover in negative controls is observed, increase washing time between sample uptakes and make sure that pins are completely dried after washing. Exchange washing buffer more often, add detergents (e.g., 0.1% Tween-20) or use additional sonication.

9. *Inhomogeneous spot morphology*: To avoid doughnut-shaped spots, increase humidity during spotting process or add compounds to the spotting solution which are reducing the evaporation speed or are increasing surface tension. To avoid blurry spots, increase the detergent concentration of the spotting buffer.
10. *Inconsistent spot morphology*: Make sure that buffer formulation and concentration of the antibodies is consistent. If there is a systematic pattern, test the surface coating prior to spotting by breathing carefully at the surface. The absorption of vapour on the surface should be completely homogenous. Additionally, surface inhomogeneities (which sometimes pass quality control by the manufacturers) can be detected by scanning a slide at full laser power and PMT.
11. *Alternative to tissue homogenisation*: Alternative to the mortar and pestle method, minced tissue samples can be homogenised using Potter Elvehjem homogeniser. In this mode, minced tissues are first transferred into the homogeniser, topped with 10 µL lysis buffer for each 1 mg of tissue, and are then homogenised with 20–30 strokes.
12. *Preventing protein degradation*: Cell lysis is usually accompanied by the release of proteases and phosphatases, which may compromise protein structure and integrity. In addition to the favourable effect of including protease and phosphatase inhibitors to the extraction buffer, lowering the temperature contributes further to keeping the remaining activities to the minimum.
13. *Traces of medium*: Traces of medium may contain serum proteins that may introduce false-positive results on the array.
14. *Volume of extraction buffer*: We generally add 175, 350, and 1,000 µL of extraction buffer for 25T, 75T, and 175T culture flasks, respectively, followed by tilting the flask from side to side to spread the solution on the full surface. Depending on the type of cells, the protein concentration obtained this way is in the range of 1.75–3.75 mg/mL, which is sufficient for a successful labelling.
15. *Concentrating protein samples*: If the concentration of samples is too low but samples are available in sufficient quantity, they can be concentrated either by vacuum concentration or by filtration using Microcons (Millipore, Billerica, USA).
16. *Down-scaling of labelling reaction*: It is possible to perform label reactions with less starting material of your sample, although sensitivity may suffer. Reduce the overall reaction volume but keep the concentrations of protein and label reagent. Eventually, use smaller columns for the removal of unreacted dye.

17. *Dye handling*: Dissolve the label reagent in H_2O and use immediately in order to prevent hydrolysis of the NHS-esters. If the label reagent is delivered in larger quantities, it can be dissolved in DMSO or DMF and stored in aliquots under dry conditions at –20°C.
18. *Blocking and incubation buffer*: If increased background is observed, use "the blocking solution" (Candor Biosciences GmbH, Weißensberg, Germany) for blocking and incubation steps.
19. *Slidebooster*: If no slidebooster instrument is available, incubations can be performed in Quadriperm chambers (Greiner-Bio One, Germany) (18). However, larger incubation buffer volumes of 3–5 mL may be needed to cover the whole surface of a slide.
20. *Combining information derived from two scanner settings*: If it is not possible to obtain a representative majority of spots from one scanner setting, it is possible to perform multiple scans at different intensities and combine them using Masliner prior to data analysis (19).
21. *Array normalisation*: See the limma guide for additional normalisation methods or for the possibility to weight spots in the normalisation according to their quality flags introduced during spot recognition. For arrays with a small number of features and a high degree of differentially expressed features a normalisation based on selected non-differentially proteins can be beneficial (20).

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Immunoassay-based protein profiling of 24 pancreatic cancer cell lines reveals disease characteristics, the degree of cell differentiation, the source of tumour cells, and their metastatic potential

Mohamed Saiel Saeed Alhamdani[1], Mahmoud Youns[2], Andrea Bauer[1], Christoph Schröder[1] and Jörg D. Hoheisel[1]

[1] Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

[2] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of Helwan, Ain Helwan, Cairo, Egypt

SUMMARY

Pancreatic ductal adenocarcinoma is one of the most deadly forms of cancers, with a mortality that is almost identical to incidence. The inability to predict, detect or diagnose the disease early and its resistance to all current treatment modalities but surgery are the prime challenges to changing the devastating prognosis. Also, relatively little is known about pancreatic carcinogenesis. In order better to understand relevant aspects of pathophysiology, differentiation, and transformation, we analyzed the cellular proteomes of 24 pancreatic cancer cell lines and two controls using an antibody microarray that targets 741 cancer-related proteins. In this analysis, 73 distinct disease marker proteins were identified that had not been described before. Additionally, categorizing cancer cells in accordance to their original location (primary tumour, liver metastases, or ascites) was made possible. A comparison of the cells' degree of differentiation (well, moderately, or poorly differentiated) resulted in unique marker sets of high relevance. Last, 187 proteins were differentially expressed in primary versus metastatic cancer cells, of which the majority is functionally related to cellular movement.

INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies. Most patients die within a year of diagnosis and only about 3% survive five years or longer (1). The poor prognosis can be attributed to late presentation, aggressive local invasion, early formation of metastases, and poor response to chemotherapy (2, 3). Additionally, diagnosis by conventional means is very difficult due to the anatomic location of the pancreas (4). Patients with pancreatic tumours often present themselves with vague complaints of gastrointestinal complications at late stages of the disease. Surgery on such late-stage patients is frequently impossible or of only limited effect (5). The establishment of procedures for an earlier diagnosis of pancreatic cancer poses a challenge [6] since the appropriate molecular information is lacking. It is nearly impossible to obtain clinical samples from patients with premalignant or early stage malignant disease that may provide this data.

Although ductal cells make up only about 10% of all pancreatic cells and 4% of the pancreatic volume, more than 90% of human pancreatic cancers are morphologically and biologically consistent with a classification as cells that belong to the ductal cell lineage of the exocrine pancreas (6). The processes that lead to malignant alterations in the pancreas and the reasons why particularly ductal cells are affected are poorly understood. Current knowledge of the biological properties of pancreatic ductal adenocarcinoma (PDAC) is in part derived from *in vitro* studies of pancreatic tumour cell lines. Although they are an artificial system, cultured cells provide an important model for studying physiologic, pathophysiologic, and differentiation processes in a controlled manner. A substantial number of PDAC cell lines of different characteristics have been established and provide a good material source for investigating various molecular aspects of the devastating disease.

Despite the remarkable progress of our understanding of the complex biological processes involved in disease pathogenesis at the level of nucleic acids, our insight into the

molecular background of tumorigenesis remains partial. Many regulatory processes actually take place at the protein level. The proportion and importance of protein modification, for example, is reflected by the fact that 5% to 10% of mammalian genes encode for proteins that modify other proteins. Proteins are involved in basically all vital biological processes and execute many cellular functions. Consequently, 98% of all current therapeutic agents target proteins. Because of the obvious importance of variations that occur at the protein level, proteomic analyses have been performed on samples from pancreatic cancer patients for the identification of relevant biomarkers or possible therapeutic targets. Gel-based processes and mass spectrometry [reviewed in (7-9) as well as antibody microarrays were applied. The majority of analyses, however, investigated sera from pancreatic cancer patients (10-15). While potentially important for early diagnosis, sera analyses have limited relevance only with respect to understanding the particular tumoral features of pancreatic cancer.

In this study, the focus was on the identification of cellular variations that could be utilised for the provision of molecular evidence for disease characteristics. To this end, we took advantage of an antibody microarray made of 810 antibodies that permits the analysis of the expression levels of 741 distinct proteins [13]. They had been selected on the basis of a thorough analysis of transcriptional profiling data and other available information to be highly associated with the occurrence of pancreatic adenocarcinoma, colon and breast cancer (16-18). With this resource, the cellular proteomes of 24 pancreatic cancer cell lines and two control cells were studied. The molecular differences found in the analysis allow conclusions on the degree of cell differentiation, serve as indicator for defining the actual source of the tumour cells, and permit an assessment of their metastatic potential.

EXPERIMENTAL PROCEDURES

Chemicals and antibodies – All chemicals used in this study were purchased from Sigma-Aldrich, unless stated otherwise, and were of highest purity or protein grade. In the analysis, a set of 810 antibodies was used as reported earlier (16-18). Their respective target protein and origin are listed in Supplementary Tab. S1. The majority had been produced on the basis of transcriptional studies on different cancer entities [e.g. 14-16], from which targets exhibiting differential expression were selected. For 668 of these targets, affinity-purified, peptide-specific, polyclonal antibodies from rabbit were provided by Eurogentec (Seraing, Belgium) on a complementary basis. They were characterized by means such as immunohistochemical analyses. An additional 142 antibodies were purchased from different commercial providers or obtained from collaborating partners. Antibodies supplied as ascites fluid, antisera or with stabilizer proteins were purified using the Nab Protein G Spin Kit (Thermo Scientific, Rockford, USA). The concentration of all antibodies was adjusted to 2 mg/ml by filtration with Microcon 100 kDa (Millipore, Schwalbach, Germany). The binders were aliquoted and stored at -80°C.

Cell culture – In total, 26 cell lines were used in this study (Tab. 1). All of them were tested to be negative for mycoplasma contamination. Usually, the cells were grown in IMDM medium (Invitrogen, Darmstadt, Germany) supplemented with 10% foetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C and 5% CO_2. Human umbilical endothelial cells (HUVEC) were cultured in complete endothelial culture medium (PromoCell, Heidelberg, Germany). The immortalized pancreatic cell line HPDE (19) was grown in keratinocyte serum free medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen).

Protein extraction – The cellular proteins were extracted using the optimized extraction process described in detail before (20). In brief, at 90% confluence, cells were

washed three times with ice cold phosphate buffered saline (PBS) and layered with the extraction buffer (Hepes-Mix: 20 mM Hepes buffer, pH 7.9, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40 substitute, 0.5% sodium cholate, 0.25% n-dodecyl-β-D-maltoside (GenaXXon Bioscience, Ulm, Germany), 0.25% amidosulfobetaine-14, 1 U/µl of Benzonase (Merck Biosciences, Schwalbach, Germany) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Bonn, Germany). After an incubation on ice for 30 min with occasional mixing, the cells were scraped off and pipetted several times through a fine needle syringe, followed by centrifugation at 20,000 g at 4°C for 20 min. The supernatant was aspirated with a fine needle in order not to disturb the upper layer or the pellet. Protein concentration in the supernatant was determined with the Bicinchoninic Acid Protein Assay Reagent kit (Thermo Scientific).

Protein labelling – Extracted protein was labelled as described in detail earlier (10, 21) using the fluorescence dyes DY-549 or DY-649 (Dyomics, Jena, Germany) at a molar dye-to-protein ratio of 7.5, with the assumption that 60 kDa is the average molecular weight of all proteins. The protein concentration was adjusted to 2 mg/ml. Labelling occurred in the dark in 0.1 M carbonate buffer, pH 8.5, at 4°C for 2 h. Unreacted dye was quenched with 10% glycine for 30 min at 4°C in the dark. Labelled samples were stored at -20°C until analysis.

Antibody microarray printing – A protocol was used, which has been described in very detail earlier (21). In short, the antibodies were spotted on epoxysilane-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP6B pins (Telechem, Sunnyvale, USA) at a humidity of 40 to 45%. The printing buffer was composed of 0.1 M carbonate buffer, pH 8.5, containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran sulphate, 5 mM magnesium chloride, 137 mM sodium chloride, and 1 mg/ml of the respective antibody. On each slide, the antibodies were

printed in quadruplicates. After printing, the slides equilibrated at a humidity of 40 to 45% overnight and were stored in dry and dark conditions at 4°C until use.

Sample incubation – Incubation of the microarrays with labelled samples was performed according to a protocol established recently (21). Briefly, printed slides were washed once for 5 min followed by another wash for 15 min with PBS containing Tween-20 at a final concentration of 0.05% (PBST20). The slides were blocked with 5 ml of 10% non-fat dry milk (Biorad, Munich, Germany) in PBST20 for 3 h at room temperature using Quadriperm chambers (Greiner Bio-One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated in Quadriperm chambers with 50 µg of DY-649 labelled sample and 50 µg of a DY-549 labelled pool of all samples in 5 ml incubation buffer containing 10% milk in PBST20 in the dark at 4°C overnight. The slides were then washed four times for 5 min in large volumes of PBST20, rinsed with deionised water, and dried in a ventilated oven at 22°C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer, Waltham, USA) at constant laser power and PMT. The images were analyzed with the software GenePix Pro 6.0 (Molecular Devices, Sunnyvale, USA).

Data analysis **–** GPR files of the scanned images were analyzed with the Chipster software (v1.4.6, CSC, Finland). Data were normalized using *normexp* with background correction offset [0, 50] as reported previously (22). The entire data set is accessible as supplemental information (Supplemental Tab. S2). Two-group comparisons, such as HUVEC versus all ductal cancer cells, primary versus metastatic cells and liver versus lymph node metastases, were performed using the EmpiricalBayes test with Bonferroni-Hochberg multiple testing correction and a cut-off at a p-value of 0.05 (23). Multiple-group comparisons, such as for cell origin and degree of differentiation, were performed using LIMMA with a p-value adjustment according to Bonferroni-Hochberg multiple testing correction (23). Cluster analysis was conducted using Pearson correlations and dendrograms

were constructed using the average linkage method (http://chipster.csc.fi). Biomarker and functional investigations were performed with the Ingenuity Systems Pathway Analysis tools (Ingenuity Systems, Redwood City, CA). The p-values were calculated using right-tailed Fisher's exact test (www.ingenuity.com). Protein annotation was conducted with the open-source STRAP software (24).

RESULTS

Cell line selection criteria **–** For the study, we collected several ductal adenocarcinoma cell lines so as to represent different cellular characteristics repeatedly (Tab. 1). Also, by using cells that exhibit cellular characteristics in variable combinations and have their origin from different sources, the chances were higher to identify common aspects. Using only a few cells could result in findings that are only specific to the particular tumour of the individual patient, from whom the respective cell line was isolated. As controls, two cell lines were utilized (Tab. 1). The Human Umbilical Endothelial Cell (HUVEC) has frequently been used as a control in comparative studies of pancreatic cancer (25, 26). More recently, the Human Pancreatic Duct Epithelial (HPDE) cell line was introduced, which exhibits a near normal phenotype and genotype except for the loss of the p53 functional pathway (27). This pancreatic duct epithelial cell had been immortalized after transfection with LXSN16-E6E7 retroviral expression vector for the E6 and E7 genes of human papilloma virus 16 (19). Of the cancer cell lines, nine had their origin from primary tumour, seven from liver metastasis, two from lymph node metastasis and five from ascites (Tab. 1). Since they all originated from exocrine tumours, we also studied BON-1 cells for comparison, which had been established from a pancreatic endocrine carcinoid tumour (28).

Cell line comparison **–** First, the protein levels of the various cell lines were compared to each other. In this initial analysis, it became apparent that overall the HPDE cells had at the protein level much more in common with the cancer cell lines than HUVEC, presumably due to the absence of cellular senescence. As a consequence of this resemblance of HPDE to PDAC cells, all comparisons were done with the HUVEC proteome acting as the basic control to which all expression measurements were related. For an overall comparison, hierarchical clustering was performed on the basis of the observed variations in protein abundance (Fig. 1). The clustering reflects to some extent the origin of the cell lines from primary tumour, metastases or ascites, although substantial differences were observed within the groups. Cell lines that are a sub-population of a parental cell line, like the Suit panel and the A818 cells, cluster intimately. Others, less directly related cells show similar results, such as the poorly differentiated primary cells Mia Paca-2 and PANC-1. In contrast, the two lymph node cell lines T3M4 and Colo-357 appear to be comparatively different. The cells IMIM-PC2 and SU.86.86, which both have their origin off primary tumours, exhibit a relatively high degree of similarity but are rather distant from the other cells from primary tumours. The FAMPAC cell line was established from a patient with a family history of pancreatic cancer (29). However, on the basis of the 741 proteins studied, no particular expression differences to the other pancreatic ductal cells was recorded. AsPC-1 seems to be exceptionally different from the other ascites cells and exhibited closer resemblance with cell lines from liver metastases or primary tumours. Finally, BON-1 is the cell line that is furthest apart from all others, which is not surprising, since it is an endocrine carcinoid tumour. The comparison with the exocrine tumour cells revealed a surprisingly large number of 489 proteins that were expressed at significantly different levels (Supplemental Tab. S3). The superiority of selecting HUVEC as control rather than HPDE was supported by a cluster

analysis based on a comparison of all cells to HPDE (not shown). In this, HUVEC was as distant from the PDAC cell lines as BON-1, as opposed to the result with HPDE in Fig. 1.

Variations in the protein expression of PDAC cells – The protein levels in the PDAC cell lines (all cell lines but BON-1 and HPDE) were compared to the proteome of HUVEC. There were 132 significantly regulated proteins (49 up- and 83 down-regulated). Only variations were considered that could be observed in at least 20 out of the 23 PDAC cell lines. Ingenuity biomarker analysis showed that 59 (44.7%) of them had already been reported as biomarkers at either the transcript or the protein level for diagnosis, prognosis, disease progression, treatment efficacy, response to therapy, and drug safety (Supplemental Tab. S4). The relatively high percentage is not surprising, since many antibodies used in the analysis had been selected on the basis of transcriptional variations observed in various tumour forms including pancreatic cancer. Therefore, some are bound to bind to known biomarkers. It is actually more surprising that about half of the differentially regulated molecules – 73 proteins (Tab. 2) – had not been defined before as markers for pancreatic cancer. An analysis of the functional aspects of these 73 PDAC-specific proteins revealed strong connections to cell death, cellular development, movement, growth and differentiation (Supplemental Tab. S5).

Comparison of cell lines from primary tumours and metastases – Distant metastases and multi-organ involvement are very common in advanced pancreatic cancer and among the main factors responsible for its dismal prognosis. Over 85% of patients with pancreatic cancer are presented with disease metastasis at the time of diagnosis. Surgery is contraindicative at this level and radio- and chemotherapy are the only available options yet disconcerting owing to tumour resistance. Better insight to the prime molecules involved in the interaction between the cancer cell and affected organ as well as the surrounding environment could allow for effectively targeting pancreatic cancer metastases. Cell lines

originating from primary tumours (BxPC-3, Capan-2, FAMPAC, IMIM-PC1, IMIM-PC2, MDA-Panc28, MIA-PaCa-2, PANC-1, SK-PC-1 and SU.86.86) and metastatic tumour cells (Capan-1, CFPAC-1, Colo357, Suit-007, Suit-020, Suit-028, Suit-2 and T3M4) were compared in order to find metastasis-specific changes. In total, 187 proteins were found to be differentially expressed (106 down, 81 up) between the two groups (Supplemental Tab. S6). An analysis of the functional annotations associated with the proteins revealed that 39% of them are involved in regulating cellular movement, migration, invasion and chemotaxis. Also their GO terms imply that the majority (84%) are incorporated in the regulation of such cellular processes or the function as binding molecules, interacting selectively and non-covalently) (Supplemental Fig. S1).

Assignment of the cell source – The PDAC cells in our analysis fall mainly into three groups that reflect the source from which they had been isolated: primary tumours (BxPC-3, Capan-2, IMIM-PC1, IMIM-PC2, MDA-Panc28, MIA-PaCa-2 and PANC-1), ascites fluid (A818-1, A818-4, A818-7, AsPC-1 and HPAF-II), and liver metastasis (Capan-1, CFPAC-1, Suit-007, Suit-020, Suit-028 and Suit-2). For each group, distinctively regulated proteins were found: 10 proteins were specific for primary tumours, 50 for ascites and 102 for liver metastases. (Fig. 2; Supplemental Tab. S7). There was an overlap of these proteins with the molecules that were found to be metastasis-specific. After their removal, 8, 47 and 63 unique proteins were left that act as indicators of the cellular source rather than its metastatic physiology.

Comparison of PDAC cells based on their degree of differentiation – In this analysis, only cell lines were included, for which a well-defined and particularly consistent degree of differentiation has been reported in the literature. For some of the studied cell lines, controversial results exist and they were not considered for this reason. Other cell lines have been described in the literature just once. Thus, no independent confirmation about their

degree of differentiation exists. We tried to corroborate the published information for these cell lines but were not successful in all cases. FAMPAC and MDA-Panc28 produced results that are in contrast to literature data. They had been described as poorly differentiated cells, based on the secretion of ductal proteins like MUC1 and cytokeratin-7 (29, 30). In our cultures, however, we could not confirm this (data not shown). Because of this disagreement, they were not included in the analysis.

Comparing the protein expression of PDAC cells with different degrees of differentiation (well differentiated: Capan-1, Capan-2, CFPAC-1 and SK-PC-1; moderately differentiated: A818-1, A818-4, A818-7, AsPC-1, T3M4 and BxPC-3; and poorly differentiated: MIA PaCa-2 and PANC-1), unique sets of proteins were identified that are associated with the differentiation degree (Fig. 3). Inversely to the decrease in the degree of cellular differentiation from well via moderate to poor, an increase in the number of regulated proteins from 5 via 34 to 107 proteins was observed (Supplemental Tab. S8, Fig3). Of all the 741 protein targets represented on the chip in terms of antibodies, 156 were involved in the regulation of cellular degree of differentiation. We found 61 proteins of the 156 to be significantly regulated among the three groups (Data not shown).

Acinar versus ductal cell type – All exocrine cell lines exhibit a ductal phenotype except of MDA-Panc-28, which displays both ductal and acinar features (30). A comparison revealed 87 proteins that differentiated the purely ductal tumour cells from MDA-Panc-28 (Supplemental Tab. S9). In particular the levels of the cell surface proteins mucin-2, tetraspanin-6 and C-C chemokine receptor type 7 were differentially expressed, suggesting that these molecules could be used for an immunohistochemical detection of cells from an acinar lineage.

DISCUSSION

Cell lines provide a model to investigate parts of the molecular basis of cancer and could consequently permit an identification of both biomarkers and new therapeutic avenues. For example, markers were detected that are associated with pancreatic cancer metastasis using the SW1990 cell line (31). In another study, the association between cell-surface markers and cancer tumorgenicity had been assessed (32). Here, we evaluated the cellular proteome of 24 pancreatic cancer cell lines using an antibody microarray with binders against 741 target proteins (10). About half of the proteins that were identified as differentially expressed in a comparison of cancer and normal cell lines had already been described for pancreatic cancer, for example acting as markers for the treatment efficacies with drugs like erlotinib (ERBB3 and VEGFA), bevacizumab (VEGFA), celecoxib (PTGS2), pioglitazone hydrochloride (INS), and tegafur, gimeracil, oxonic acid and gemcitabine (IL8, TP53 and VEGFA) or for predicting the success of radiotherapy (AREG and VEGFA) (ClinicalTrials.gov by Ingenuity). Also, IL10, TNF and VEGFA had been used as prognostic markers. Except for TP53 and AREG, the above mentioned markers were down-regulated in all tested cell lines, which is in agreement with previous reports which showed lower expression of these in the pancreatic cell lines PANC-1 (33, 34), MIA PaCa-2 (35) and MDA Panc-28 (34) as well as in the pancreatic duct of human adults (36, 37). Interestingly, all extracellular proteins with a regulatory effect on PDAC had a lower expression level in the cancer cell lines. However, in a separate study, we found concomitantly a strongly increased abundance in the secretome of PDAC cells. Especially IL6 was strongly secreted by 16 of the pancreatic cancer cell lines (unpublished data). Fittingly, in patients, a higher diagnostic and prognostic value of serum IL-6 was documented than that of CRP, CEA and CA 19-9 (38).

Proteins IL6, IL10, TNF, TP53 and VEGFA, along with other, up-regulated proteins like BRCA1, RPS19, RRM2 and SMAD4, have been reported to affect the development of

PDAC. It was shown, for example, that RRM2 contributes significantly to the invasiveness of PDAC (39), potentially through the up-regulation of MMP9. Silencing of the *RRM2* gene was associated with a decrease in cancer cells viability (40). Targeting RRM2 with gemcitabine is among the common therapeutic approaches for treating pancreatic cancer (41). Higher expression of this enzyme was documented in the pancreatic cell lines MIA PaCa-2 (39), Suit-2 (40) and Panc-1 (42). Fittingly, RRM2 was one of the most up-regulated proteins in the present study, exhibiting an increase in all cancer cell lines. Similarly, SMAD4 was shown to be expressed at an elevated level in several cancer cell lines (43-46), which is in agreement with our findings. Conversely, BRCA1, which plays a central role in DNA repair through promoting the cellular response to DNA damage (47) and functions as a tumour suppressor protein (48), was significantly less abundant in cancer as compared to normal cells. Mutations of the *BRCA1* gene have been reported for breast and ovarian (49) as well as pancreatic cancer (50).

Comparing cell lines that originated from pancreatic cancer metastases with cells from primary tumour, we found alteration in the expression of several cell surface proteins that promote invasion and metastasis, including cell adhesion molecules such as ICAM1, VCAM1, Junctional Adhesion Molecule B (JAM2), Occludin and Selectin, molecules mediating homotypic interaction during invasion and metastasis such as E-Cadherin, Afadin (MLLT4), molecules mediating the interaction with the extracellular matrix such as MMP1, MMP2 and TIMP1, antigenic glycoproteins like CEACAM5, MADCAM1 and LAMP2, receptors like FAS, IL1A, IL1RN, IL2R and IL2RG and recognition proteins for immune cells like CD44, GCA.

A closer look at the molecular feature of metastatic pancreatic cancer may provide valuable answers why PDAC cells harbour and thrive in certain organs more than others. At autopsy, the liver is the most frequent site of distant metastases, followed by the peritoneum,

pleura and lung, bones, and adrenal glands (51). Unlike the case with other cancers, such as colorectal cancer (52), surgical intervention of advanced pancreatic cancer is not an option for most cases (53), and chemotherapy may be the only treatment at this level, although again of little overall benefit. Additionally, pancreatic cancer commonly develops resistance to currently available therapeutic agents and novel drugs. The analysis of primary tumours, ascites and liver metastasis yielded proteins that may be potential targets for therapy. Among the proteins that were highly regulated only in cells from liver metastasis is serine/threonine-protein kinase B-raf, a cytoplasmic enzyme that is targeted with the compounds sorafenib and PLX4032 (54, 55). Sorafenibin in combination with gemcitabine has shown cytotoxicity against MIA PaCa-2, Capan-1 and Panc-1 cell lines (56). Unfortunately, however, similar findings could not be reproduced in phase II clinical trials (57, 58). Drugs like XR9576, OC 144-093, and valspodar have been used as inhibitors of the protein ATP-binding cassette (ABCB1), which involves in drug-resistance of certain tumours (59-61). ABCB1 is a P-glycoprotein that is not expressed by many cell types (59). Overexpression of ABCB1 was observed in our study only in cells that originated from liver metastasis. Transforming growth factor beta 2 (TGF-β2) was also among the proteins overexpressed in cells from liver metastases. This growth factor is known to affect negatively the immune response to cancer cells and promote cancer progression through proliferation, metastasis, and angiogenesis (62). Modulating of TGF-β2 level with AP-12009 has shown promising results in clinical trial for treatment of malignant gliomas (63).

Matrix metaloproteinases (MMPs) are known proteolytic enzymes used by tumour cells for detachment and invasion. In pancreatic cancer, the imbalance between MMPs and their tissue inhibitors has been reported (64). Our finding showed an increased expression of MMP-7 and MMP-12 in cell originated from ascites, MMP1 in cell from liver metastasis, MMP-11 and MMP-14 in both primary and liver groups, and MMP-2 in all groups. Inhibition

of MMP-2 by RO28–2653 or MMI-166 showed an effective reduction of liver metastasis in an animal model of pancreatic cancer (65).

The degree of cancer cell differentiation corresponds strongly with the disease progression and its aggressiveness and therefore indicates processes that may allow for accurate prognosis and therapeutic success. The majority of PDAC tumours are well- to moderately differentiated, while poorly differentiated tumours are less common (66). In PDAC, the hallmarks of cell differentiation are tubular structures, papillae, cycts and secreted mucins (67). The last are a group of glycoproteins that are mainly produced by ductal and granular epithelial cells. Some of them, such as MUC4, were suggested as a potential tumour marker for the diagnosis of pancreatic cancer (68). Among all secreted mucins, MUC2 is considered to be the molecule most correlated to inflammation and cancer (69). In our analysis, a significant decrease in MUC2 expression was observed across all differentiation stages, with the lowest level being found in poorly differentiated cell lines, which in agreement with previous records (68, 70). MUC1 was significantly higher only in the poorly differentiated cell lines. Since our control cell line, HUVEC, has the capacity to express MUC1 (71), it could be that the level of secreted MUC1 is comparable to that of the well and moderately differentiated cancer cells and thus the reason for the significant difference to poorly differentiated cells. Apart from the mucins, also 61 other proteins involved in the regulation of differentiation were similarly regulated in cancer cells irrespective of the actual differentiation stage. The majority are extracellular and nuclear proteins and, to a lesser extent, proteins bound to the plasma membrane. Most function as transcription regulators, cytokines, growth factors and trans-membrane receptors. Some are well-established key players in the events leading to the development of PDAC, such as p53 (72), NFκB (73), ERBB2 (74), p38 MAPK (75), CDK4 (76) and SMAD4 (77), which all were found to be overexpressed in the cancer cells. However, besides the proteins common to all cancer cell

lines, also proteins were found that were regulated only at a particular stage of differentiation. It is intriguing that the number of specifically regulated proteins increased with the decrease of cell differentiation, being highest in the poorly differentiated cell lines.

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Figure legend:

Figure 1. Hierachical clustering of the cell lines. Based on the protein expression, a cluster analysis was performed. At the bottom, the origin of each cell line is indicated. The colour-code used is identical to the one of Tab. 1.

Figure 2. Number of tissue-type specifically expressed proteins. A Venn-Diagram (A) shows the number of proteins that were regulated in cell lines originating from primary tumour, ascites or liver. In panels B, C and D, GO annotations of the regulated proteins are shown for biological processes, cellular components and molecular functions, respectively.

Figure 3. Comparison of cells based on their degree of differentiation. In the Venn-diagram, the number of regulated proteins are shown that exhibited variation in well, moderately or poorly differentiated cell lines.

Supplementary Figure 1. GO annotation analysis of regulated proteins between primary and metastatic cell lines. A) biological process, B) cellular component and C) molecular function.

Table 1.

#	Cell line	Source of cells	Cell type	Age of patient (years)	Gender	Disease	Class	Differentiation according to histol. grade of tumour	Differentiation *in vitro* / mouse xenograft	Tumorigenic
1	HPDE		Ductal			Normal, immortalized	Exocrine	-	-	No
2	HUVEC	Umbilical vein	Endothelial			Human endothelial normal cell	Endothelial	-	-	-
3	BxPC-3	Primary tumor	Ductal	61	female	adenocarcinoma	Exocrine	moderate-well, G2-G3	moderate / -	Yes
4	FAMPAC	Primary tumor	Ductal	43	female	adenocarcinoma; cystic fibrosis	Exocrine	poor	poor / poor	Yes
5	IMIM-PC1	Primary tumor	Ductal			adenocarcinoma	Exocrine	moderate	-	-
6	IMIM-PC2	Primary tumor	Ductal			adenocarcinoma	Exocrine	well	-	-
7	MDA-Panc28	Primary tumor	Ductal and Acinar	69	female	adenocarcinoma	Exocrine	G4	poor / poor	Yes
8	MIA PaCa-2	Primary tumor	Ductal	65	male	carcinoma	Exocrine		poor-mod. / poor-mod.	Yes
9	PANC-1	Primary tumor	Ductal	56	male	epithelioid carcinoma	Exocrine	G4	poor / poor	-
10	SK-PC-1	Primary tumor	Ductal			adenocarcinoma	Exocrine	well	-	-
11	SU.86.86	Primary tumor	Ductal	57	female	ductal carcinoma	Exocrine	G2-G3	moderate / -	Yes
17	Capan-1	Liver metastasis	Ductal	40	male	adenocarcinoma	Exocrine		well / well	Yes
18	Capan-2		Ductal	56	male	adenocarcinoma	Exocrine	G1	well / well	Yes
19	CFPAC-1	Liver metastasis	Ductal	26	male	adenocarcinoma; cystic fibrosis	Exocrine	G1	well / well	Yes
23	Suit-2	Liver metastasis	Ductal	73	male	adenocarcinoma	Exocrine	G2	mod.-well / mod.-well	Yes
20	Suit-007	Liver metastasis	Ductal	73	male	adenocarcinoma	Exocrine	moderate	-	-
21	Suit-020	Liver metastasis	Ductal	73	male	adenocarcinoma	Exocrine	moderate	-	-
22	Suit-028	Liver metastasis	Ductal	73	male	adenocarcinoma	Exocrine	moderate	-	-
24	Colo357	Lymph node metastasis	Ductal			adenocarcinoma	Exocrine		well / well	Yes
25	T3M4	Lymph node metastasis	Ductal	56	male		Exocrine		moderate / moderate	Yes
12	A818-1	Ascites	Ductal	75	female	adenocarcinoma	Exocrine	well, G1	well / -	-
13	A818-4	Ascites	Ductal	76	female	adenocarcinoma	Exocrine	well, G1	well / -	-
14	A818-7	Ascites	Ductal	77	female	adenocarcinoma	Exocrine	well, G1	well / -	-
15	AsPC-1	Ascites	Ductal	62	female	adenocarcinoma	Exocrine	moderate-well, G2	poor-mod. / -	-
16	HPAF-II	Ascites	Ductal	44	male	adenocarcinoma	Exocrine	G1-G2	well / mod.-well	Yes
26	BON-1			28	male	carcinoid tumor	Endocrine		-	Yes

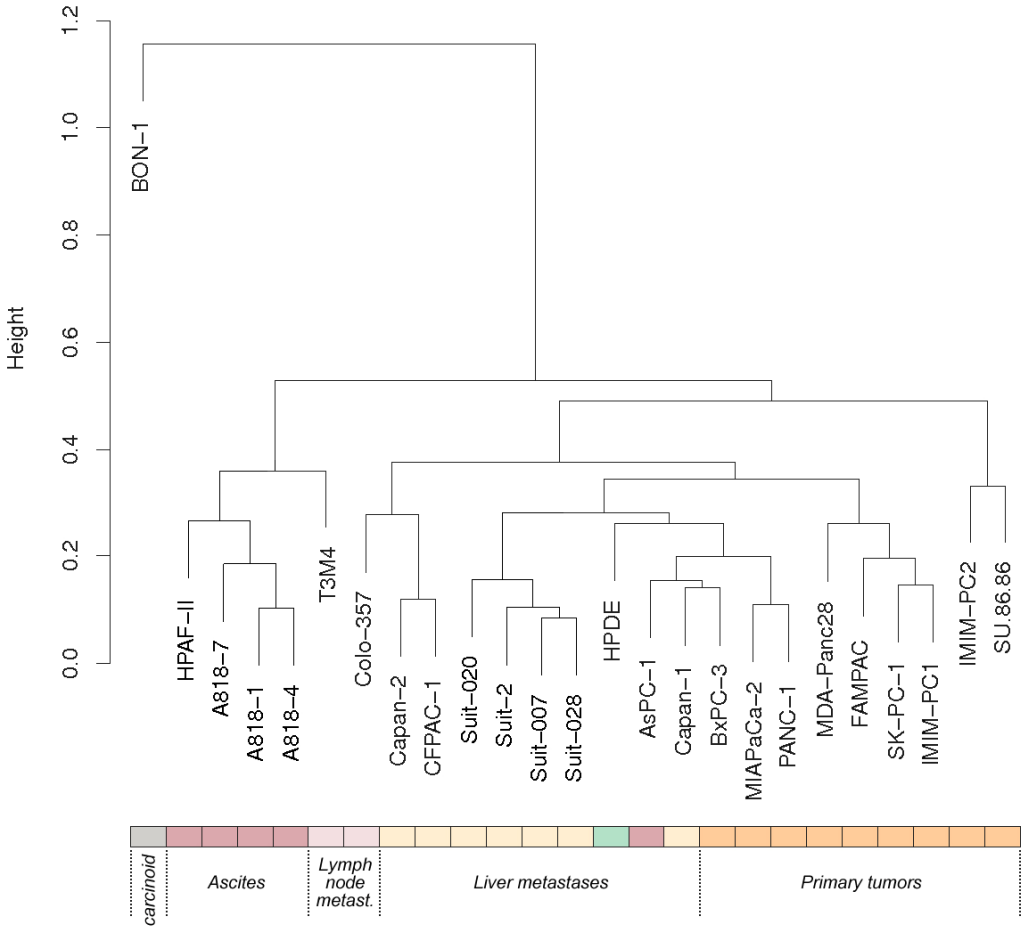
Table 2. Potential new markers regulated between normal and cancer cells with yet unspecified biomarker application.

ID	Symbol	Entrez Gene Name	Location	Family	p-value	Log-FC
Q16586	SGCA	sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)	Plasma Membrane	other	4.30E-05	-0.529
Q9BU70	C9orf156	chromosome 9 open reading frame 156	unknown	other	4.30E-05	0.395
P48637	GSS	glutathione synthetase	Cytoplasm	enzyme	7.20E-05	-0.447
O60844	ZG16	zymogen granule protein 16 homolog (rat)	Extracellular Space	other	3.72E-04	0.296
O43557	TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	Extracellular Space	cytokine	3.80E-04	-0.453
P51671	CCL11	chemokine (C-C motif) ligand 11	Extracellular Space	cytokine	4.64E-04	-0.522
P06681	C2	complement component 2	Extracellular Space	peptidase	4.97E-04	0.526
Q96CN7	ISOC1	isochorismatase domain containing 1	Cytoplasm	enzyme	5.89E-04	-0.434
Q13318	SPI1	spleen focus forming virus (SFFV) proviral integration oncogene spi1	Nucleus	transcription regulator	1.10E-03	-0.215
Q14934	NFATC4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nucleus	transcription regulator	1.10E-03	-0.66
Q9Y6I7	WSB1	WD repeat and SOCS box containing 1	unknown	other	1.40E-03	0.306
Q15011	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Cytoplasm	other	1.83E-03	-0.471
P48307	TFPI2	tissue factor pathway inhibitor 2	Extracellular Space	other	2.21E-03	-0.473
P20839	IMPDH1	IMP (inosine 5'-monophosphate) dehydrogenase 1	Cytoplasm	enzyme	2.51E-03	0.451
P29459	IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	Extracellular Space	cytokine	3.10E-03	-0.195
Q9UDY2	TJP2	tight junction protein 2 (zona occludens 2)	Plasma Membrane	kinase	4.18E-03	-0.338
P41250	GARS	glycyl-tRNA synthetase	Cytoplasm	enzyme	5.26E-03	-0.306
P63261	ACTG1	actin, gamma 1	Cytoplasm	other	5.64E-03	0.476
P19474	TRIM21	tripartite motif containing 21	Nucleus	enzyme	7.42E-03	-0.282
Q16864	ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F	Cytoplasm	transporter	7.65E-03	0.366
Q99832	CCT7	chaperonin containing TCP1, subunit 7 (eta)	Cytoplasm	other	7.65E-03	0.564
O43865	AHCYL1	adenosylhomocysteinase-like 1	Cytoplasm	enzyme	7.69E-03	0.497
Q02878	RPL6	ribosomal protein L6	Cytoplasm	other	8.02E-03	0.541
P35749	MYH11	myosin, heavy chain 11, smooth muscle	Cytoplasm	other	8.81E-03	0.567
Q14455	GNAS	GNAS complex locus	Plasma Membrane	enzyme	9.27E-03	0.274
P62841	RPS15	ribosomal protein S15	Cytoplasm	other	9.90E-03	0.468
Q9H5K9	C4orf41	chromosome 4 open reading frame 41	unknown	other	1.00E-02	0.362
Q92506	HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	Cytoplasm	enzyme	1.02E-02	-0.328

P25325	MPST	mercaptopyruvate sulfurtransferase	Cytoplasm	enzyme	1.03E-02	0.524
Q92985	IRF7	interferon regulatory factor 7	Nucleus	transcription regulator	1.07E-02	0.519
Q15393	SF3B3	splicing factor 3b, subunit 3, 130kDa	Nucleus	other	1.08E-02	0.301
P62993	GRB2	growth factor receptor-bound protein 2	Cytoplasm	other	1.11E-02	-0.259
P09758	TACSTD2	tumor-associated calcium signal transducer 2	Plasma Membrane	other	1.12E-02	0.488
P18510	IL1RN	interleukin 1 receptor antagonist	Extracellular Space	cytokine	1.15E-02	-0.327
P27797	CALR	calreticulin	Cytoplasm	transcription regulator	1.15E-02	-0.374
P09629	HOXB7	homeobox B7	Nucleus	transcription regulator	1.19E-02	0.397
P98066	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	Extracellular Space	other	1.41E-02	-0.329
P99999	CYCS	cytochrome c, somatic	Cytoplasm	enzyme	1.41E-02	0.411
Q15582	TGFBI	transforming growth factor, beta-induced, 68kDa	Extracellular Space	other	1.60E-02	0.198
O75947	ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d	Cytoplasm	enzyme	1.79E-02	-0.197
P51959	CCNG1	cyclin G1	Nucleus	other	1.90E-02	-0.433
Q53G59	KLHL12	kelch-like 12 (Drosophila)	unknown	other	1.98E-02	0.249
P09086	POU2F2	POU class 2 homeobox 2	Nucleus	transcription regulator	2.20E-02	-0.162
Q07826	NA	X-linked retinopathy protein	NA	NA	2.22E-02	-0.125
Q9H8T0	AKTIP	AKT interacting protein	Cytoplasm	other	2.22E-02	-0.204
P19387	POLR2C	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa	Nucleus	enzyme	2.40E-02	0.348
P62906	RPL10A	ribosomal protein L10a	unknown	other	2.47E-02	0.228
P02743	APCS	amyloid P component, serum	Extracellular Space	other	2.48E-02	0.257
P21333	FLNA	filamin A, alpha	Cytoplasm	other	2.74E-02	-0.205
Q99805	TM9SF2	transmembrane 9 superfamily member 2	Plasma Membrane	transporter	2.74E-02	-0.348
P21757	MSR1	macrophage scavenger receptor 1	Plasma Membrane	transmembrane receptor	2.81E-02	-0.24
Q16342	PDCD2	programmed cell death 2	Nucleus	other	2.82E-02	-0.196
P39019	RPS19	ribosomal protein S19	Cytoplasm	other	2.97E-02	0.322
P50991	CCT4	chaperonin containing TCP1, subunit 4 (delta)	Cytoplasm	other	2.99E-02	0.247
P22736	NR4A1	nuclear receptor subfamily 4, group A, member 1	Nucleus	ligand-dependent nuclear receptor	3.20E-02	-0.315
P32119	PRDX2	peroxiredoxin 2	Cytoplasm	enzyme	3.22E-02	0.45

P18124	RPL7	ribosomal protein L7	Cytoplasm	transcription regulator	3.26E-02	0.207
O43474	KLF4	Kruppel-like factor 4 (gut)	Nucleus	transcription regulator	3.41E-02	0.201
P24534	EEF1B2	eukaryotic translation elongation factor 1 beta 2	Cytoplasm	translation regulator	3.85E-02	0.402
P21453	S1PR1	sphingosine-1-phosphate receptor 1	Plasma Membrane	G-protein coupled receptor	3.97E-02	-0.305
P05204	HMGN2	high mobility group nucleosomal binding domain 2	Nucleus	other	4.10E-02	-0.203
P35442	THBS2	thrombospondin 2	Extracellular Space	other	4.39E-02	0.278
P20700	LMNB1	lamin B1	Nucleus	other	4.43E-02	-0.249
P07195	LDHB	lactate dehydrogenase B	Cytoplasm	enzyme	4.54E-02	0.147
P31483	TIA1	TIA1 cytotoxic granule-associated RNA binding protein	Nucleus	other	4.54E-02	-0.468
Q8IX18	DHX40	DEAH (Asp-Glu-Ala-His) box polypeptide 40	unknown	enzyme	4.54E-02	-0.231
O95865	DDAH2	dimethylarginine dimethylaminohydrolase 2	unknown	enzyme	4.73E-02	0.417
Q02505	MUC3A	mucin 3A, cell surface associated	Extracellular Space	other	4.80E-02	0.331
Q99439	CNN2	calponin 2	Cytoplasm	other	4.80E-02	-0.749
Q8TB96	ITFG1	integrin alpha FG-GAP repeat containing 1	Plasma Membrane	other	4.86E-02	-0.189
Q9UKR8	TSPAN16	tetraspanin 16	unknown	other	4.92E-02	0.329
Q9Y5Y6	ST14	suppression of tumorigenicity 14 (colon carcinoma)	Plasma Membrane	peptidase	4.93E-02	-0.224
P29320	EPHA3	EPH receptor A3	Plasma Membrane	kinase	4.95E-02	-0.296

Fig1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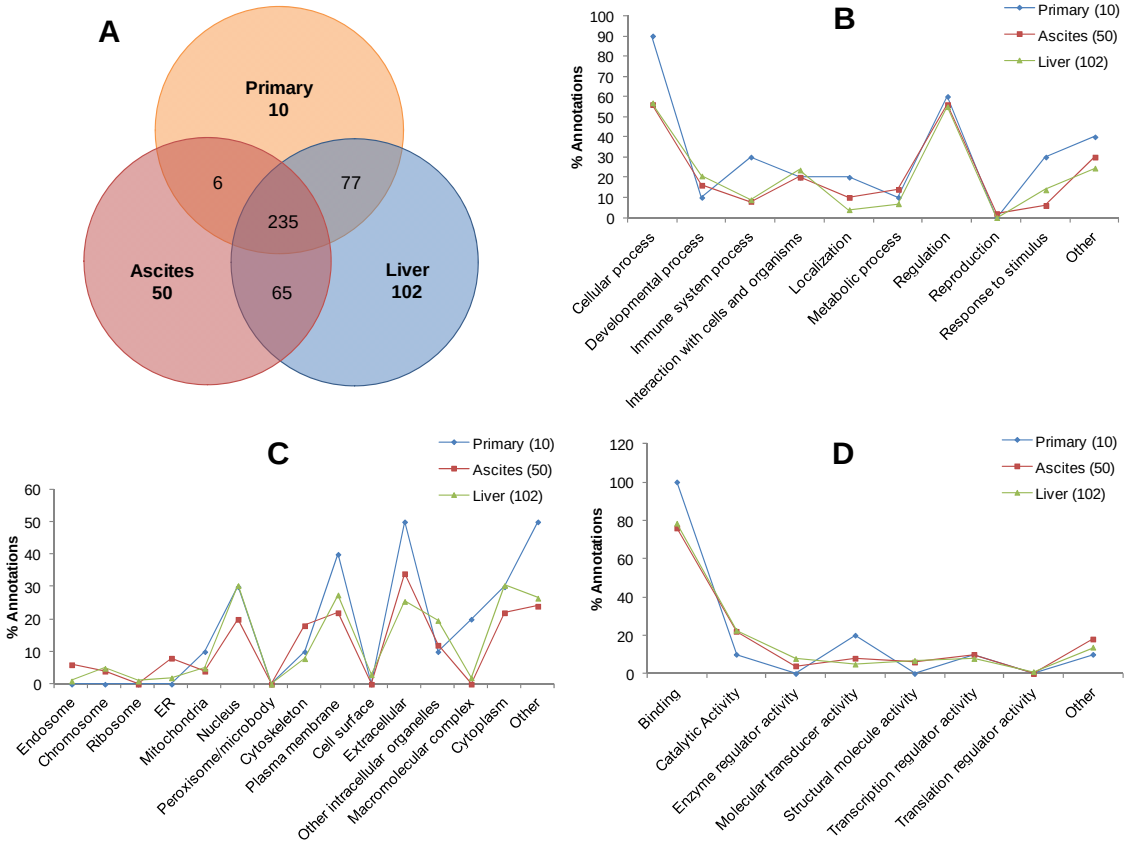
Ascites
Lymph node metast.
Liver metastases
Primary tumors

Fig 2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6
77
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Ascites
50
65
Liver
102
B
Primary (10)
Ascites (50)
Liver (102)
% Annotations
Cellular process
Developmental process
Immune system process
Interaction with cells and organisms
Localization
Metabolic process
Regulation
Reproduction
Response to stimulus
Other
C
Primary (10)
Ascites (50)
Liver (102)
% Annotations
Endosome
Chromosome
Ribosome
ER
Mitochondria
Nucleus
Peroxisome/microbody
Cytoskeleton
Plasma membrane
Cell surface
Extracellular
Other intracellular organelles
Macromolecular complex
Cytoplasm
Other
D
Primary (10)
Ascites (50)
Liver (102)
% Annotations
Binding
Catalytic Activity
Enzyme regulator activity
Molecular transducer activity
Structural molecule activity
Transcription regulator activity
Translation regulator activity
Other

Fig 3