UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 474

Validation of antibodies for protein profiling

A study using immunohistochemistry on tissue microarrays

LINDA PAAVILAINEN

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2009

ISSN 1651-6206
ISBN 978-91-554-7588-8
urn:nbn:se:uu:diva-107471

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöldsv 20, Uppsala, Friday, September 25, 2009 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract
Paavilainen, L. 2009. Validation of antibodies for protein profiling. A study using immunohistochemistry on tissue microarrays. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 474. 54 pp. Uppsala. ISBN 978-91-554-7588-8.

The field of proteomics has rapidly expanded due to the completion of the human genome sequence. This thesis validates affinity-purified monospecific antibodies of polyclonal origin, for protein profiling in a broad spectrum of normal tissues and cells. Validation of antibodies is crucial for development of reliable binders for target proteins and this thesis evaluates the generation and application of large sets of msAbs in different settings. MsAbs were generated towards recombinant Protein Epitope Signature Tag (PrEST) antigens using a stringent affinity-purification strategy, presented in the first study. The specificity of msAbs was studied using reverse phase protein arrays and immunohistochemistry (IHC), and results presented over 90% success rate in the protein array analysis. In IHC, 81% of the msAbs displayed apparent specific staining in normal tissues. MsAbs were also compared with commercial analogs (cAbs) using IHC and Western blot. Results presented similar outcome between msAbs and cAbs in both applications, although interpretation suggested more extensive IHC staining patterns with msAbs than with monoclonal analogs. For antibody validation, an approach called paired antibodies was presented and involved the generation of two msAbs towards non-overlapping epitopes on the same protein. Similarities in protein detection between paired antibodies were studied using three different antibody-based methods. Similar results were observed in several applications, indicating that this strategy can be a useful tool for studying known and unknown proteins. Given the reliability of msAbs, they were also applied in a study investigating the impact of tissue fixatives on protein detection. The study showed that different fixation mechanisms appeared to affect protein recognition by indicating that aldehyde-based fixation, e.g. induced by neutral buffered formalin, was preferred for tissues used in IHC and non-aldehyde based fixation was applicable for tissues used in protein extraction analysis and Western blotting.

Conclusively, validation results suggest that msAbs are reliable affinity binders that can be used as valuable tools for proteome-wide protein profiling in tissues and cells.

Keywords: antibody validation, monospecific antibodies, protein profiling, immunohistochemistry, tissue microarrays, western blot, fixatives

Linda Paavilainen, Department of Genetics and Pathology, Rudbeck laboratory, Uppsala University, SE-75185 Uppsala, Sweden

ISSN 1651-6206
ISBN 978-91-554-7588-8
urn:nbn:se:uu:diva-107471 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-107471)

Ju mer man tänker, desto mer inser
man att det inte finns något enkelt svar.

Nalle Puh – A.A Milne

List of Papers

This thesis is based on the following scientific papers, which are referred to in the text by their roman numerals.

I Nilsson P, **Paavilainen L**[*], Larsson K[*], Ödling J[*], Sundberg M, Andersson A-C, Kampf C, Persson A, Al-Khalili Szigyarto C, Ottosson J, Björling E, Hober S, Wernérus H, Wester K, Pontén F and Uhlén M. Towards a human proteome atlas: High-throughput generation of mono-specific antibodies for tissue profiling. *Proteomics 2005, 5:4327-4337.*

II **Paavilainen L**, Wernérus H, Nilsson P, Uhlén M, Hober S, Wester K and Pontén F. Evaluation of monospecific antibodies: A comparison study with commercial analogs using immunohistochemistry on tissue microarrays. *Appl Immunohistochem Mol Morphol 2008, 16(5):493-502.*

III **Paavilainen L**, Edvinsson Å, Asplund A, Hober S, Kampf C, Pontén F and Wester K. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. *Submitted.*

IV **Paavilainen L**, Sivertsson Å, Oskarsson L, Persson J, Lundberg E, Gry M, Asplund A, Fagerberg L, Wester K, Hober S, Uhlén M and Pontén F. Paired antibodies for multi-application validation of antibody specificity. *Manuscript.*

[*] Authors contributed equally.

Contents

Abbreviations

2-DE	Two-dimensional gel electrophoresis
ABP	Albumin binding protein
AR	Antigen retrieval
cAb	Commercial antibody
DAB	3,3′-diaminobenzidine
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FFPE	Formalin-fixed paraffin embedded
HIAR	Heat-induced antigen retrieval
HPA	Human Protein Atlas
HRP	Horseradish peroxidase
Ig	Immunoglobulin
Ip	Isoelectric point
IHC	Immunohistochemistry
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ ionization
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
msAb	Monospecific antibody
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PrEST	Protein epitope signature tag
PTM	Post-translational modification
RCA	Rolling circle amplification
scFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMA	Tissue microarray
ZBF	Zinc-based fixative

Introduction

The human being consists of a machinery of millions of cells, constantly working together in a complex system for maintaining life. The cells are programmed according to the genetic code, thus organizing and supervising molecular mechanisms and reactions throughout the body. The genetic code comprises millions of nucleotides, enfolded into a double helix spiral called deoxyribonucleic acid or most commonly known as DNA[1]. The DNA encodes for proteins, which in turn perform designated functions in the cells. Today, the human genome consists of 22,258 known protein-coding genes (Ensembl version 55.37)[2]. The first draft of the human genome sequence was reported in 2001 [3,4] and three years later the human genome sequence was considered as completed [5]. The effort of publishing the human genome sequence has made it possible for scientists all over the world to access genomic information through web-based databases such as, UniProt and Ensembl [2,6]. These databanks contain comprehensive information regarding genes and their protein products, and are highly valuable assets for the research community.

Revealing the human genome sequence has resulted in enhanced interest in the structure and function of proteins, and their potential role in cancer and other diseases. An attempt to generate a protein database containing binders for all human protein encoding genes started in 2003 with The Human Protein Atlas (HPA) project [7]. So far the protein database has resulted in over 7,000,000 images of protein expression in normal and cancer tissues and cells. This large-scale effort is further described in the affinity reagent chapter (page 18).

This thesis is based on the work of four research papers and the following chapters will give an introduction to the protein field and some of the most common techniques used for investigating our extraordinary proteins.

Proteins

Proteins are present in all living cells, performing functions that are controlled by advanced mechanisms. Far from everything is completely understood and research is essential to fully comprehend the complexity of cellular mechanisms and protein interactions. The central dogma in molecular biology describes the flow of genetic information in a cell, involving replication of our genetic code, transcription of DNA into messenger RNA (mRNA) and translation into protein (Figure 1)[8]. In order to become functional, most proteins undergo post-translational modifications (PTMs) that are performed by enzymes in different cellular compartments, such as the endoplasmic reticulum (ER) and Golgi apparatus. PTMs include for instance, glycosylations, phosphorylations and proteolytic cleavage [9,10].

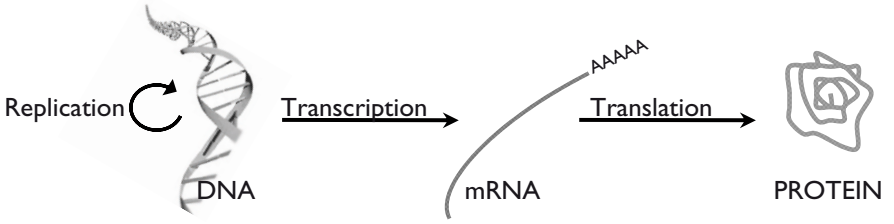

Figure 1. The central dogma of molecular biology. The dogma involves replication of DNA, transcription into messenger RNA (mRNA) and translation into proteins.

Proteins perform several functions in the living cell, e.g., catalyzing biochemical reactions, transport nutrients or providing structure of cells by involvement in the extracellular matrix. Proteins are also involved in blood clotting, muscle contractions and in the immune response system.

Proteins are polymers consisting of a chain of amino acids (polypeptide). The amino acids represent building blocks that exist in twenty different variants. These variants can be assembled into various combinations, forming the primary structure of the protein. The linear structure of the chain folds into α-helical structures and β-sheets, characterizing the secondary structure. Finally, the protein folds into a three-dimensional shape, the tertiary structure, to become functionally active. Some proteins consist of multiple inter-

acting polypeptide chains and can thereby form a quaternary structure. Factors such as amino acid composition and structure determine the physical and chemical properties of the protein, thus deciding its designated function and localization [9,11].

The amount of a certain protein differs in various cell types or even in different cellular compartments within the cell. Some proteins are present in low levels while others are continuously highly expressed. Circumstances such as cell division, activation of the defense system or disease can also affect protein fluctuations. Variations in protein expression levels are therefore of interest in biomarker discovery, where proteins can be used as therapeutic tools for instance in cancer treatments [12-14].

Proteomics

To describe all proteins expressed by a genome, the term "proteome" is used [15]. As previously mentioned, the human proteome consists of genes coding for around 22,000 non-redundant proteins, given that every gene locus encodes for one single protein. Since proteins exist in different isoforms and variants due to events such as alternative splicing and PTMs, the total number of proteins within the human proteome differs. Alternative splicing is estimated to generate 50,000 to 100,000 protein variants, thus resulting in different quantities depending on protein classification [16].

Within the proteomics field, platforms for protein characterization can be divided into two main technological strategies: separation-based and affinity-based techniques. Separation-based techniques involve separation of proteins on a matrix, generally using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and two-dimensional electrophoresis (2DE). The gel is subjected to a field current, enabling separation based on protein mass and isoelectric point (Ip) [17]. Visualization of proteins is accomplished by staining with Coomassie blue or the more sensitive silver staining [18]. Inability to detect proteins of low levels and lack of protein quantification in multiple samples simultaneously, has led to the development of the difference gel electrophoresis (DIGE) technique [19]. This approach enables protein detection in several samples on the same gel and visualization is performed using fluorescent dyes [20].

Protein identification, when using separation-based techniques, is frequently performed in the combination with mass spectrometry (MS) and bioinformatic tools. MS measures mass-to-charge ratio (m/z) of charged ions by ionization of molecular samples. The two main techniques for ionization involve electrospray ionization (ESI) and matrix-assisted desorption/ionization (MALDI) [21,22]. ESI provides direct ionization of proteins and peptides in solution and the particles are then selectively released to a detector for registration of the number of ions at each m/z value. In the MALDI

technique, samples are mixed with an organic compound (matrix) prior to ionization and the technique is often used in combination with the time-of-flight (TOF) mass analyzer, resulting in peptide mass mapping (fingerprinting)-[23,24].

The technology can be used for different types of protein investigations, such as defining protein interactions, analysis of PTMs or studying the proteome content in tissue samples [10,25]. The affinity or probe-based detection is the other approach for characterization of proteins and will be described in the following chapters.

Affinity reagents for protein detection

Protein detection using affinity reagents provide a large resource within clinical pathology and life science. Affinity or probe-based protein detection techniques have in common that a probe or binder needs to be present. Affinity reagents can e.g. be antibodies, antibody fragments or oligonucleotides. This chapter will describe different types of antigens and affinity reagents with special focus on antibodies.

Antigens

An antigen is defined as a foreign substance that induces an immune response, i.e. generation of antibodies, when administered into host species. The small site on the antigen, where the antibody binds to its complementary position, is called the epitope. In order for the antibody to recognize its corresponding antigen, it is essential that the epitope is available for binding and not altered due to environmental changes, such as denaturation. Denaturation can occur through protein fixation methods, pH changes or heating, and can affect the ability of the antibody to bind to the protein [26].

Antigens are often proteins or polysaccharides but can also include nucleic acids or lipids. Smaller peptides or other substances (called haptens) can also function as antigens but often need to be coupled to a larger carrier protein to induce an immune response.

Three types of antigens are used in the field of antibody-based proteomics: small peptides, full-length proteins and recombinant proteins. A peptide comprises a small part of a protein, usually consisting of 15 amino acids, thus making the limited size small enough for linear conformation [27,28]. On the other hand, full-length proteins can adopt the conformation of the native protein, but to ensure proper folding they should be expressed in a system where correct folding is possible e.g. in mammalian cells [29]. Antibody-based proteomics with full-length protein antigens may not be the optimal choice due to the slow production procedure and laborious handling of these antigens. The third antigen type, recombinant proteins, is generated by fusion of one part of the protein to a tag. The recombinant protein can after host cell immunization, be utilized for affinity-purification of the antibodies. The length of the recombinant protein is not restricted and can thus be longer than peptide antigens. Monoclonal and polyclonal antibodies have success-

fully been generated using recombinant antigens [30,31]. One example is Protein Epitope Signature Tags (PrESTs), which are designed using gene sequence information and bioinformatic software tools [2]. Each PrEST consists of 50-150 amino acid residues and is selected avoiding homology regions, transmembrane regions and hair-pin loops. Antibody cross-reactivity is prevented by carefully designing PrESTs with low sequence identity to other proteins. The recombinant PrEST protein consists of the PrEST sequence together with histidine and albumin binding protein (ABP) [32]. The histidine tag is used for PrEST purification prior to immunization, to ensure correct antigen. Albumin binding protein is used for enhanced immune response and together with the histidine-tag later used for affinity-purification of the polyclonal antibodies.

Antibodies

For many decades, antibodies have played an essential role in the history of protein detection. Antibodies naturally exist as part of the specific immune system and their main function is to detect foreign substances and target them for elimination. Foreign substances can for instance be pathogens such as bacteria or viruses invading the human body.

Antibodies are glycoproteins belonging to the immunoglobulin (Ig) family and are produced by white blood cells, B-lymphocytes, either as membrane bound or secreted. The secreted antibodies recognize antigens that trigger the immune response, while antibodies bound to the membrane of B-lymphocytes have receptor functions for antigens [33,34]. The Ig-family comprises five antibody classes (isotypes) but the most frequently used are IgG and IgM. The antibody molecule is composed of two identical light polypeptide chains (L) and two identical heavy polypeptide chains (H), linked together by disulphide bonds (Figure 2). The structure also contains a hinge region, providing flexibility to the antibody molecule. The polypeptide chains are further divided into variable (V) and constant (C) regions, where the variable regions of the heavy (V_H) and light (V_L) chains form the antigen-binding site. The N-terminal part of the antigen-binding site consists of six hypervariable domains, providing wide molecular recognition. The range of recognition is determined by the diversity of antibody gene combinations [35,36].

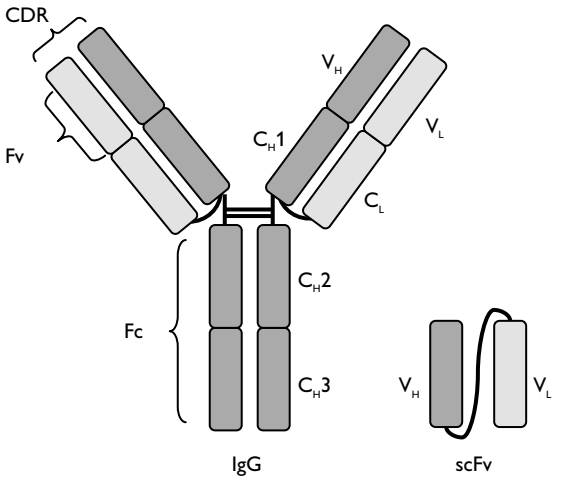

Figure 2. Schematic illustration of an antibody molecule (IgG) and scFv fragment. The IgG molecule consists of two heavy chains (dark grey) and two light chains (light grey), while the scFv only contains the variable regions of chains.

Different types of antibodies are available, e.g. monoclonal (mAb), polyclonal (pAb) and monospecific (msAb), with various properties and production strategies. Depending on target molecule for antibody binding and protein detection method used, different types of antibodies are applicable.

Monoclonal antibodies

Monoclonal antibodies (mAbs) are widely used in clinical diagnostics and research due to their ability of binding molecular targets with high specificity [37]. The antibodies are generated by immunization of antigen into host species, commonly mice. This was first described in 1975 by Köhler and Milstein, who successfully fused mouse B-lymphocytes with myeloma cells, in order to form immortal hybridoma cells [38]. The advantage of using hybridomas is the rapid and continuous generation of identical antibodies. MAbs recognize only one single epitope on the antigen and are thus advantageous to use in e.g. therapeutic applications. However, mAbs can be vulnerable to chemical treatment on target cells, that can cause loss of epitopes [33]. For large-scale efforts or multi-application strategies, mAbs may be less suitable [39].

Polyclonal antibodies

Polyclonal antisera are pools of antibodies recognizing multiple epitopes on the antigen [40]. Polyclonal antibodies (pAb) can be raised in different host species, but rabbits are frequently used. Production of pAbs in rabbits is advantageous primarily due to their easy maintenance and high yield of serum. The antigen is injected intradermally or subcutaneously and in order to maintain or increase antibody levels, booster injections are administered repeatedly. Polyclonal antiserum is collected and can be used in an unpurified or purified form. Blood, from which clotting proteins and red blood cells have been removed, is referred to as antiserum [34]. Purification of antiserum can be performed using affinity chromatography. Because pAbs recognize several epitopes on the antigens, they are more tolerant towards epitope modifications due to e.g. chemical treatment. This feature is advantageous for protein detection in multiple applications [39,41]. However, lack of reproducibility and the relatively low yield of affinity-purified pAbs from each immunization, make them less favorable in some settings.

Monospecific antibodies

Monospecific antibodies (msAbs) are multi-epitope binders of polyclonal origin that are generated using unique PrESTs as antigens (described in the Antigen section). The recombinantly generated PrEST protein is immunized into host species (preferably rabbits) and polyclonal antiserum is collected and affinity purified [42]. The polyclonal antibodies are called “monospecific” because the collected serum is purified in two steps. Firstly, using a depletion column where antibodies towards histidine and ABP are removed, and secondly, with affinity chromatography using the recombinant PrEST protein as ligand, thus generating specific msAbs recognizing various epitopes on the antigen with less unspecific binding [43]. Due to the relatively convenient generation, the msAbs are suitable for large-scale production and can successfully be used in several high-throughput applications [16,44]. The major disadvantage with all antibodies of polyclonal origin is the lack of a renewable source. Previously designed antigens can be re-immunized into host species, but the polyclonal sera will not contain identical antibodies possessing the same properties as previously generated antibody batch.

Large-scale generation of monospecific antibodies

The Swedish Human Atlas project is a large-scale effort aiming to map the human proteome using msAbs [7,45]. The project has over the years produced over 8000 monospecific antibodies towards known and unknown proteins of the human proteome [46]. Validation of the monospecific antibodies is important and several techniques are utilized for quality assurance. The msAbs are used for screening proteins in normal and cancer tissues and images of pro-

tein expression are displayed in a web-based atlas (www.proteinatlas.org), available for the scientific society to exploit [47,48].

Other affinity binders

Other affinity binders, with versatile molecular binding properties, are available. These binders are generated by *in vitro* methodologies with the advantage of being reproducible, and thereby circumvent the need for immunization in host species [49,50].

Antibody fragments can be utilized as affinity reagents for protein detection assays. The single chain variable fragment (scFv) is a well-known antibody fragment obtained by protease digestion. The variable regions of the heavy and light chains, V_H and V_L , are separated from the antibody molecule and linked together artificially by a peptide linker, thus generating the scFv (Figure 2) [51]. The small size of scFvs, with maintained antigenicity, can sometimes be an advantage compared to larger antibody molecules. Another advantage is the generation of fragments via recombinant selection and expression by e.g. phage display technology [52].

Recombinant antibodies or peptide ligands that bind targets of interest, is another example of affinity binders that can be selected using the phage display technology. The technology is based on libraries of phages presenting different proteins, including antibodies or peptides, on their surface [53]. The protein of interest is fused together with a coat protein of a bacteriophage, and the complex is later inserted into the phage genome, resulting in expression of the target protein on the surface of the phage. The phage library is incubated with the target (e.g. on a plate or bead) and phages that bind specifically are eluted and amplified. This process is repeated until a desired pool of phages is collected and characterized [54-56]. Examples of affinity binders that can be selected by the phage display technology are Affibodies. They are small proteins of 58 amino acids, based on the IgG antibody domain of protein A (a surface protein of *staphylococcus aureus*). Affibody libraries consist of a multitude of ligand variants with identical backbone but variable surface-binding properties. Affibodies are mainly used in biotechnology research but also in biotherapeutics [57].

Aptamers are affinity reagents that are composed of single stranded DNA or RNA molecules, selected from *in vitro* generated libraries. The aptamers form a complex three-dimensional shape with high affinity for the target molecule [58]. SELEX (Systemic Evolution of Ligands by EXponential Enrichment) is an *in vitro* procedure for identifying aptamers and the process involves generation of large populations of nucleic acid molecules. The population is incubated with the target of interest (e.g. a peptide, protein, or small molecule) and molecules with highest affinity for the target are selected. These target bound molecules are separated, purified and thereafter

amplified using polymerase chain reaction (PCR) [59]. The molecules are used for generating a new population library for further selection cycles. This procedure is repeated several times until individual molecules, i.e. aptamers, with high affinity for the target are selected [60,61].

Affinity-based detection

Among the affinity-based detection techniques, various protein array formats are included. The techniques are based platforms for the affinity reagent to bind the protein of interest and include enzyme-linked immunosorbent assay (ELISA), Western blotting, immunohistochemistry (IHC) and immunofluorescence.

The ELISA assay is based on measuring the amount of single proteins in solutions and has extensively been used for many years [41]. However, in the past few years, the interest of measuring various proteins simultaneously in a solution has increased. This has lead to the development of antibody or protein arrays in three different formats: direct antibody arrays, sandwich arrays and reverse phase protein arrays [62-64]. In the direct antibody array, antibodies are coated to the platform and already labeled proteins are allowed to bind. In the sandwich array, a dual-antibody format is used. This begins with one antibody already fixed onto the platform when proteins are added. The detection is completed when a second antibody, tagged with an enzyme for visualization, is added and binds to the antibody-protein complex. In the reverse phase protein array, the proteins are coated onto the platform and detection is visualized by the binding of labeled antibodies [65].

Western blot is a frequently used antibody-based detection technique, in which proteins are separated according to their weight, using gel electrophoresis (Figure 3)[66]. The methodology can be used for identifying target molecules in complex protein mixtures. SDS-PAGE gels are often employed for protein separation and transfer of proteins onto membranes, usually polyvinylidene difluoride (PVDF) or nitrocellulose, enabling recognition by antibodies towards the protein target of interest. The antibody-antigen complex can be visualized using e.g. enzyme-based detection. During preparation of the samples used in Western blot, the proteins are denatured and epitopes are linearized, thereby limiting the types of antibodies suitable for this application [67].

IHC is widely used as protein detection method and is considered golden standard in clinical pathology. Tissues and cell samples are frequently used and evaluation is performed using light microscopy. This method will be further presented in the next chapter. Immunofluorescence is also commonly used, generally for cells or cell lines. In this protein detection method antibodies towards the target proteins are coupled to fluorophores and together

with organelle-specific markers visualization is established (Figure 3). Confocal microscopy is used for evaluation of protein detection.

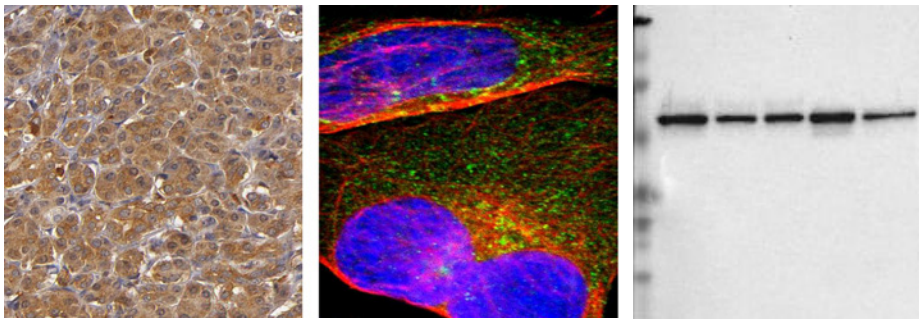

Figure 3. Examples of affinity-based protein detection techniques. In this figure the same antibody has been used for IHC staining in pancreas tissue (brown color represents protein detection), immunofluorescence in cell lines (green color = protein detection, blue color = cell nucleus, red color = cytoskeleton) and Western blot.

A rather recent technology based on affinity reagents containing oligonucleotide extensions, is proximity ligation [68]. Two or more reagents (or probes) need to bind the target protein, and upon binding, the DNA strands can be ligated together in a reaction dependent on the proximity of the binders [69]. In order to get an enhanced signal for protein detection, the strands are amplified using PCR. The PCR products, i.e., ligation products, reflect the amount of target molecules (proteins) detected in the sample. For visualization, fluorescence is used. In order to estimate the protein amount, the fluorescent light is compared to standard protein dilution series. This technique enables detection of very low amounts of proteins and the sensitivity of the detection is generally dependent on the affinity of the binders. The probes used in proximity ligation are preferably of high affinity, e.g., aptamers. One drawback is the limited availability of different aptamers and therefore mAbs or pAbs are often modified into suitable probes by addition of oligonucleotide sequences [70].

Immunohistochemistry

Immunohistochemistry (IHC) is a powerful technique widely used in clinical histopathological diagnosis and research for detection of proteins in tissues and cells. The technology has developed during the recent years, improving specificity and sensitivity. Today, the IHC application can be used in a high-throughput fashion for studying proteins in various settings [71]. Standardized procedures for the IHC process would be desirable but is unfortunately an issue today [72]. Inter- and intra laboratory differences are major concerns and standardized procedures are ongoing to strengthen reliability of IHC outcomes [73]. The reliability of the technique is not only dependent on antibody quality, but also other important factors such as, tissue fixation and processing, antigen retrieval and sensitivity of the protein detection system. This chapter will give insight into the IHC process and also discuss validation of the immunostaining results.

Tissue fixation and processing

In order to avoid enzymatic degradation of proteins and preserve cellular components, tissues used in IHC require fixation. Fixatives stabilize cells and tissues, and also prevent autolysis by inactivating lysosomal enzymes and inhibiting growth of bacteria and molds. A commonly used fixative is neutral-buffered formalin (10%), containing 4% formaldehyde, owing to its low cost, simple preparation and good preservation of morphology [74-76]. The fixation process should start immediately after surgical removal of tissue (maximum 30 min delay) and requires 24 to 48 hours [73]. The fixation process is followed by dehydration (in graded alcohols and xylene) and embedding [71,77]. Paraffin is frequently used for embedding and denatures proteins to some extent, thus requiring deparaffinization and most often antigen retrieval prior to IHC stainings [73].

An alternative to paraffin embedded formalin fixed (FFPE) tissues is the use of frozen tissues. The tissues are snap frozen and do not require fixation, although fixation is sometimes used for improving morphology [76,78].

Fixation mechanisms

Pathology archives consist of extensive amounts of formalin-fixed paraffin embedded (FFPE) tissue samples from patients. Formalin provides fixation of proteins by a cross-linking mechanism. The cross-linking involves the formation of hydroxymethylene bridges between proteins or between proteins and nucleic acids [79,80]. Several attempts have been made to elucidate the mechanism for formalin fixation and it is thought to affect the side-chain groups on proteins by breaking hydrogen bonds and influence electrostatic interactions, thus altering the three-dimensional structure of the protein. The conformational changes induced by formalin affect antigen-binding sites, thus generally requiring antigen retrieval (unmasking of epitopes) (AR) for successful antibody binding [80,81]. Morgan et al also proposed that calcium ions play a role in the fixing mechanism [82]. Formalin is an aldehyde-based fixative and due to its drawbacks, such as modification of epitopes and the toxicity of formaldehyde, other fixatives have over the years been presented. Glyo-fixx is a dialdehyde-based fixative that induces only partial cross-linking based on its chemical composition, including alcohol and glyoxal. The dialdehyde resembles the formaldehyde molecule but owns other properties [83]. The active chemical agent, glyoxal, has in several studies provided well-preserved morphology and high-quality IHC staining results [84,85]. Advantages with glyo-fixx are that AR is not always required and that it is less toxic. Another aldehyde-based fixative presenting high-quality immunostaining results is Zink formalin [86-88]. The use of zinc ions in the fixative solution is supposed to stabilize macromolecules towards changes caused by formaldehyde, preserving the three-dimensional structure of proteins. It has been suggested that one zinc ion forms a complex with four ligands e.g., amino acids such as cysteine and histidine, which in turn creates a stabilizing effect [89].

Non-aldehyde based fixatives also exist and include e.g., alcohol, acetone or salt-based solutions. Acetone and alcohol-based solutions fix tissues and cells by coagulation and is often used for cytological preparations. This process is irreversible and thus does not necessitate AR [90]. Alcohol-based fixatives are usually combined with heavy metals and/or different buffers, and are known for preserving RNA and DNA in tissue and cell samples [91-94]. On the other hand, tissues fixed with these fixatives often lack the well-preserved morphology, otherwise provided by aldehyde-based fixatives. An example of a non-aldehyde based fixative that has been investigated is the zinc-based fixative (ZBF) introduced by Beckstead in 1994. The fixative consists of zinc salts in a Tris-Ca acetate buffer and the gentle fixation mechanism of ZBF has shown to be suitable for certain proteins [95,96-98].

Antigen retrieval

In 1947-1948 Fraenkel-Conrat et al originally observed that the cross-linking mechanism of formalin could be reversed by high temperature or strong alkaline hydrolysis [99-101]. The earliest methods were based on proteolytic digestion by enzymes such as trypsin, pepsin or proteinase K [102]. The digestion was thought to break methylene bridges caused by formalin fixation. In 1991 Shi et al further presented the concept of performing microwave heating as antigen retrieval prior to IHC [103]. The mechanism for heating, in order to break methylene bridges and display the antigen for antibody recognition, is not fully known. Several studies have proposed models for the AR mechanism, concluding that heat appears to be an important factor [104 81,105,106]. Despite the unclear mechanism of AR, the method is widely used in pathology laboratories as standard procedure for FFPE tissues [73,107,108]. The technique is termed Heat Induced Antigen Retrieval (HIAR) and can be implemented using e.g., a decloaking chamber, microwave or autoclave [109-111].

Other factors that appear to play a role in the efficacy of AR prior to IHC are the chemical composition and the pH of the retrieval solution [112,113]. Distilled water can be used as AR solution, but antigens in general require buffers with certain pH and ionic strength to achieve strong and specific stainings in IHC [114]. Frequently used AR solutions are e.g., citrate, Tris-EDTA or urea [82,115]. Studies have also shown that the use of metal ions in the AR solution can improve IHC outcome [116,117].

The concern with HIAR is that only some cross-links, in the form of Schiff bases, are reversible. Multi-epitope binding pAbs can therefore be advantageous to use, rather than mAbs, only recognizing single epitopes [118,119]. However, mAbs are widely used in clinical diagnostics on FFPE tissues, indicating applicability in this setting. Sompuram et al investigated the effect of HIAR on binding of mAbs to protein epitopes [120]. The study suggested that well validated mAbs selected for clinical diagnostics recognize linear epitopes, thus making denaturation of epitopes due to heat irrelevant. The authors suggest that heat does not provide enough energy to break peptide bonds of the linear determinants and would therefore explain the fact that clinically relevant mAbs recognize target proteins in FFPE tissues [121].

Protein detection systems

Detection systems have become more sensitive over recent years and automation has improved reproducibility [71]. Different detection systems can be utilized in the IHC procedure to provide strong and specific stainings [122]. The protein detection generally starts with binding of a primary antibody towards the molecular target of interest. An enzyme-labeled secondary reagent directed against the primary antibody is then added (Figure 4). Examples of

secondary reagents, which in previous studies have shown strong signal in FPPE tissues, are enzyme-linked dextran polymers (illustrated to the right in Figure 4). They contain labeled secondary antibodies that can recognize primary antibodies raised in several species [123]. This is advantageous for high-throughput IHC when using e.g. both mAbs and pAbs originated from mice and rabbits, respectively.

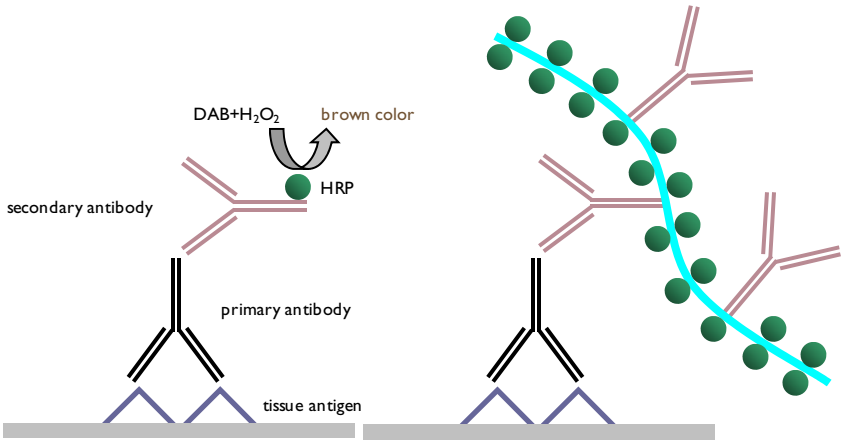

Figure 4. Schematic figure of the immunohistochemical staining reaction using two different secondary reagents. The figure to the left represents the IHC staining procedure with a primary antibody together with an enzyme-labeled secondary antibody. The figure to the right is a simplified illustration of the IHC reaction when an enzyme-linked dextran polymer is used as secondary reagent.

The IHC technique utilizes enzyme-substrate reactions generating color visualization of the antigen-antibody complex. The secondary reagents are usually labeled with enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) [34]. The HRP enzyme forms a complex with a substrate, e.g. hydrogen peroxide (H_2O_2), in the presence of an electron donor. The electron donor "drives" the reaction and thus terminates it when absent. The donors become colored upon oxidation and are therefore called chromogens. There are several chromogens available, for instance 3,3´diaminobenzidine (DAB) that generates a brown color reaction product insoluble in organic solvents, and aminoethylcarbazole (AEC), resulting in a red color reaction product that is soluble in alcohol [37]. The color reaction provides the final outcome of IHC to be evaluated using light microscopy.

Amplification of protein detection

Proteins present in low levels in tissue and cells can benefit from enhancement of the detection signal by e.g. tyramide amplification [124]. The technique can provide an increase of the detection signal up to a thousand fold, when deposition of highly reactive biotinylated tyramide signals bind protein molecules where peroxidase is present [125,126]. Another approach for signal enhancement is the rolling circle amplification (RCA). The technique was initially intended for detection of nucleic acids, but can be applied to IHC [127]. In RCA, the enhancement of signal is achieved by DNA replication. Nucleic acid targets are recognized by an immuno-conjugate, and hybridization of a circular nucleotide acid probe to a RCA primer enables DNA replication. An enzyme labeled complex visualizes the signal enhancement and the technique has been reported to generate a 10^5 increase in signal [128].

IHC validation

A complex issue regarding immunohistochemical techniques is standardization [129,130]. As previously mentioned, variations in tissue sample preparation and fixation times lead to discrepancies in protein detection results [72,131,132]. In order to use IHC as validation tool, it is critical that the outcome is reproducible and reliable. A factor that should be taken into consideration when evaluating antibodies towards known proteins using IHC, is literature research. Information about splice variants of genes, protein functions and expression in tissues are valuable when analyzing IHC results [6].

The use of automated instruments performing IHC is an approach that increases reproducibility and provides a high-throughput system [71]. The automated instruments provide hundreds of tissue sections to be immunostained simultaneously under equal conditions. To further improve quality assurance, the use of tissue microarrays (TMAs) is an efficient method for including control samples into the same block later used in IHC [133,134]. TMAs contain a multitude of tissues in the same block and can be treated equally as a single FFPE tissue block. The TMAs can be used for optimizing antibody titrations or effectively screen several tissues simultaneously with antibodies. The concept of using TMAs in an automated IHC setting can therefore be a valuable tool for investigating antibodies in a large-scale setting. The TMA technology will be further discussed in the next chapter.

Interpretation of IHC outcome is also a crucial factor affecting reliability. Manual interpretation using light microscopy is highly subjective and standardization is therefore desirable. Although efforts have been made to develop scoring guidelines or systems for determination of protein expression, subjectivity is still an issue [135-137]. Today, digital image analysis can be utilized and thus provides an interesting and important approach for analyzing

immunohistochemical stainings [138-140]. Automated systems for evaluation of staining patterns are available and used for protein detection in tissues and cells [141,142]. In a high-throughput IHC setting, automated image analysis of TMAs is a useful strategy for analyzing large amounts of samples. For tissues, however, there are issues that limit this application. Due to the heterogeneous composition of tissues, i.e. containing cell types and structures of various sizes, the definition of immunoreactivity is challenging. For cells, high-throughput image analysis is available, for instance through the TMAx software tool. The software is part of an automated image analysis system that identifies fraction of positive cells and also determines staining intensity levels (weak, moderate or strong staining)[143].

Tissue microarray technology

Traditional methods of molecular pathology are often time consuming and large tissue resources are needed. The tissue microarray technology provides a high-throughput method that is time efficient and where the use of tissue resources are reduced [144]. The tissue microarray (TMA) technology developed by Kononen et al, originated from the "sausage block" where a multitude of different tissues were be embedded into a single paraffin block [134,145]. The further developed TMA technology provided structure by placing tissue cores into a paraffin block in an organized grid-like fashion, thus effectively utilizing the space on one block (Figure 5) [146,147]. The TMA is constructed using donor blocks, usually containing FFPE tissues, although frozen tissues, *in vitro* cultured cell lines and proteins have previously also been introduced [148-151]. Cylindrical cores, from 0.6-2 mm in diameter, are punched out from a donor block and transferred into a recipient paraffin block [152]. This procedure is performed for every tissue core and is today also available in an automated setting.

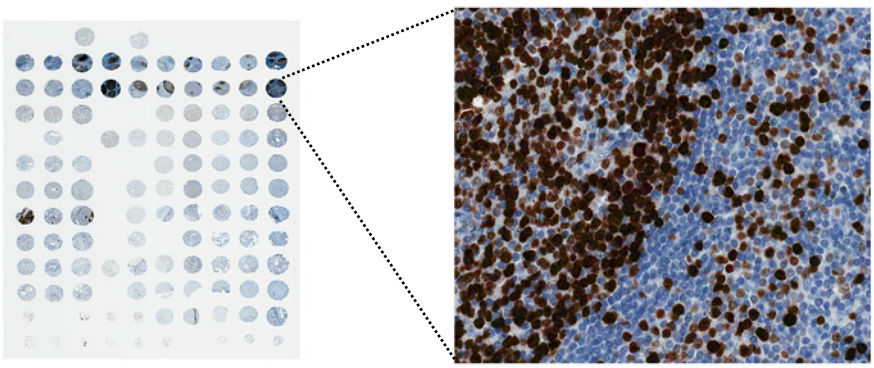

Figure 5. IHC stained tissue microarray section with a magnified image of immunoreactivity (brown color) in tonsil tissue.

The core depth is important for the amount of sections that can be obtained from an array. Usually a depth of 3 mm is used thus resulting in over 200 TMA sections (4 µm thick) available for IHC [153,154]. Tissue heterogeneity has

been a common concern when using small cores (0.6 mm). This problem can however be reduced by selection of the area of interest by experienced pathologists and representation of tissue cores in at least duplicates [155-157]. Histological and molecular detection techniques that can be used with regular sections, can also be used with TMAs. Immunohistochemical detection of protein expression in tissue specimens is a common application [144,158,159].

TMAs are utilized for different purposes and can be designed and constructed according to study interest. An efficient approach for screening of potential diagnostic biomarkers is a TMA block composed of biopsies from multiple tumor types [160]. TMAs can also be designed for studying variations in tumor progression or prognostic features from patients with clinical follow-up data [161,162,163].

Present investigation

Aims of the present investigation:

- Evaluate affinity-purified monospecific antibodies for protein profiling in normal tissues
- Investigate the impact of different fixation mechanisms on protein detection in tissue and cells
- Evaluate the strategy of using paired antibodies towards the same protein, as a tool for antibody validation of unknown proteins

Results and discussion

This thesis is based on validation and application of monospecific antibodies in a high-throughput setting. This chapter presents results and discussion of each paper and in the end, conclusions and future perspectives.

Paper I

Towards a human proteome atlas: High-throughput generation of monospecific antibodies for tissue profiling.

The generation of specific binders for proteome profiling is crucial and in paper I, a strategy for producing antibodies using stringent purifications methods in a high-throughput setting is presented. Protein-specific antibodies of polyclonal origin, called monospecific antibodies (msAbs), were generated and investigated regarding specificity in a novel reverse phase protein assay. Antibodies were generated by immunization of host species (rabbits) with a recombinant PrEST antigen, consisting of a dual tag (histidine and albumin-binding protein). Polyclonal sera were collected and purified in two steps, firstly by depletion of tag-specific binders and secondly, affinity purified using the recombinant PrEST protein as ligand, thus generating msAbs. To evaluate antibody specificity towards the target protein, protein microarray analysis was performed. 464 msAbs were analyzed on arrays containing 72 different recombinant PrEST proteins in duplicates. Results indicated that 96% (446 out of 464) of the msAbs were specific for their target antigen.

190 msAbs were further evaluated regarding protein target specificity in human tissues using immunohistochemistry (IHC). IHC was performed on tissue microarrays (TMAs) containing 48 normal tissues in triplicates, and evaluation of IHC stainings was performed by judging intensity and fraction of positive cells in each tissue type. Results demonstrated that 81% (154 out of 190 msAbs) recognized corresponding target antigens by presenting specific staining patterns in normal tissues. To further confirm true antibody-binding in tissues, adsorption assays were performed for 25 msAbs. The msAbs were incubated with corresponding PrEST protein ligand prior to immunohistochemical staining and results revealed that all msAbs presented a negative staining pattern, i.e. disappearance of positive cells in the tissues.

Finally, four msAbs, designed towards well-characterized proteins, were compared with monoclonal commercial analogs regarding protein expression patterns in tissues. The proteins were selected to represent different subcellular compartments such as, cytoplasmic (leukocyte common antigen), nuclear (estrogen receptor and proliferation related protein Ki-67) and membranous (HER-2 receptor) localization. MsAbs and corresponding commercial analogs presented similar immunohistochemical staining results, indicating reliable protein recognition with msAbs in this setting. Minor discrepancies were however noted, for example, the msAb towards leukocyte common antigen (LCA) showed stronger staining intensity than the monoclonal commercial analog. This staining intensity differences could perhaps be explained by tissue variations (due to different patient biopsies), and/or loss of epitopes due to denaturating procedures during tissue processing prior to IHC. It could be further speculated that msAbs, due to their polyclonal properties, recognize several epitopes compared to monoclonal antibodies (mAbs), thereby perhaps explaining some differences in expression patterns.

Paper II

Evaluation of monospecific antibodies: A comparison study with commercial analogs using immunohistochemistry on tissue microarrays.

In paper II, the strategy of using msAbs for profiling the human proteome was further investigated by comparing expression patterns with a larger set of well-established commercial analogs, widely used in pathology departments for histopathological diagnosis and for research. Paper II is therefore based on a more substantial comparison of msAbs and commercial antibodies (cAbs), including both monoclonal and polyclonal analogs, using IHC and Western blot.

In total, 48 msAbs and cAbs were selected and compared regarding protein distribution in tissue microarrays containing 40 normal tissues from 144 individuals, using IHC. The antibodies were also compared regarding protein detection in cell lines (RT-4, U251 and A431) and tissue lysates (from liver and tonsil), using Western blot. For the cAbs, both monoclonal and polyclonal analogs were selected in order to elucidate speculations made in Paper I, regarding that staining differences could be explained by single or multiepitope recognition.

Results from the antibody comparison demonstrated that 92% (44 out of 48) of the msAbs and cAbs presented similar protein expression patterns in IHC stainings of normal tissues. A general interpretation was that msAbs presented a more extensive protein distribution in immunohistochemical stainings than monoclonal antibodies (mAbs), but with less background staining when compared to polyclonal analogs. The IHC stainings on 40

tissue types were judged and scored according to staining intensity and fraction of positive cells, following certain criteria (see material and methods section in the article). 34 out of 48 (71%) antibody pairs presented similar staining patterns in ≥ 30 normal tissue types, whereas 44 out of 48 (92%) antibodies showed similar pattern in ≥ 20 tissue types. Four antibody pairs presented inconsistent staining patterns and were considered as dissimilar. The use of TMAs containing same tissue type but from different patients could perhaps affect the staining results.

In the Western blot application, 21 of the msAbs and 22 of the cAbs detected exclusively proteins of expected size. 7 of the msAbs and 15 of the cAbs did not detect proteins and were considered as negative, whereas 14 msAbs and 7 cAbs detected proteins of incorrect size. Antibodies not belonging to these categories were regarded as uncertain. The relatively high number of negative results for the cAbs may be justified by the fact that not all cAbs were recommended for this application by the distributor. Other negative Western blot results could be due to the protein not being present in the samples. For antibodies regarded as uncertain, i.e. by detecting proteins of incorrect size, different post-translational modifications (PTMs) could be one explanation for the results. PTMs can alter protein structure and thereby possibly result in bands of other sizes.

Generally, different factors such as PTMs or tissue fixation can influence comparison results but also the epitope-binding properties of the different antibody types. The advantage of using polyclonal antibodies (pAbs) is the multi-epitope binding of antigens, making them less vulnerable to epitope loss as compared to mAbs. In this study, we found that msAbs and pAbs presented more similar staining outcome than monoclonal cAbs. MsAb are of polyclonal origin but further purified than polyclonal cAbs, and could thus be an alternative for exploring known and unknown proteins of the human proteome. Contradictive results in Western blot and IHC (similar in one application but dissimilar in the other) may be explained by alternative protein denaturation procedures in the two applications.

Paper III

The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells.

The involvement of tissue fixatives in protein detection is an important factor for understanding antibody-binding properties. Today, 10% neutral buffered formalin (NBF), consisting of 4% formaldehyde, is commonly used for preservation of tissues in pathology departments and research facilities. Although NBF is widely used, its cross-linking mechanism for fixation may not always be suitable. NBF fixed samples most often require antigen re-

trieval, e.g., through heat (HIAR), for antibody-based protein detection and one concern is that all epitopes may not be retrieved by HIAR and thereby perhaps not available for antibodies to bind. Formaldehyde is also considered as carcinogenic and therefore an alternative fixative would be desirable.

In Paper III, the impact of seven fixatives on protein recognition in tissues and cells was evaluated using IHC, protein extraction analysis and Western blot. The fixatives were chosen to represent different fixation mechanisms, divided into aldehyde-based (NBF, Glyo-fixx and Zn-formalin) and non-aldehyde based fixatives (FineFIX, HOPE, NEO-FIX and ZBF). Eight normal tissue types and three cancer cell lines were fixed with each fixative, and the samples were assembled into a fixative TMA. The TMA was utilized in IHC with 72 antibodies and protein expression was manually determined according to intensity and fraction and compared to corresponding NBF fixed tissue (considered as baseline).

Results indicated that tissues fixed with aldehyde-based fixatives showed superior morphology and also presented most similar staining intensity, compared to NBF fixed tissues. For tissues fixed with non-aldehyde based fixatives, the results were generally weaker IHC stainings, and poorer but acceptable morphological appearance. For cultured cells, NEO-FIX was considered as the superior fixative, providing cells with excellent morphology. In the immunohistochemical stainings, antibody dilutions were previously adapted for FFPE tissues and could therefore also partly explain weaker stainings for non-aldehyde fixed tissues. Approximately 15% of the antibodies presented discrepancies in staining patterns between NBF and alternative fixatives. Some alternatively fixed tissues displayed protein expression in concordance with literature findings, indicating that NBF not always is preferable. This could however not be related to certain types of proteins or particular sub-cellular compartments.

Protein extraction analysis was also performed in order to investigate protein yield from differently fixed tissues. Liver and tonsil lysates fixed in NBF, Glyo-fixx, FineFIX, NEO-FIX and HOPE, were investigated. The results displayed that HOPE fixed tissues presented the overall highest protein yield of 203-243 ng/mm^3, when including both tissue types. NBF fixed tonsil presented different protein yields in the two tissue samples, with the highest yield in tonsil tissue, 283 ng/mm^3, but among the lowest yield (84 ng/mm^3) of proteins from liver tissue. The lysates were further used in Western blot with 13 antibodies in order to investigate quality of the extracted proteins and also to study protein detection in differently fixed tissues with another antibody-based application. The results from the Western blot analysis indicated that tissues fixed in non-aldehyde based fixatives (HOPE, ZBF and NEO-FIX) detected most proteins and with highest quality. Only two proteins, keratin 17 and keratin 1, were detected in all fixed tissue lysates. It can be speculated that a fixative with a more gentle fixation mechanism, easier and independent of tissue type, can allow high amounts of proteins to

be extracted with good quality for Western blot analysis. In summary, the aldehyde-based fixatives were superior in morphology and IHC analysis but non-aldehyde based fixatives were preferable when performing protein extraction and Western blot analysis.

Paper IV

Paired antibodies for multi-application validation of antibody specificity.

The strategy of using msAbs for protein profiling has been investigated in previous papers. One challenge in the profiling of the human proteome is the investigation of unknown proteins, where no literature or information regarding protein expression is available. Paper IV presents an approach for generating two msAbs (called paired antibodies) towards non-overlapping epitopes on the same protein. These antibodies can thereby validate each other and thus be advantageous when studying e.g. unknown proteins.

2280 msAbs towards 1140 proteins were studied by antibody-based techniques in order to evaluate similarity in protein detection among the paired antibodies. The antibody pairs were divided into three cohorts based on previous validation data retrieved from the Human Protein Atlas project. 2280 antibodies belonged to cohort 1 and were selected based on supportive specificity results in protein arrays. In cohort 2, 702 antibodies were selected based on more extensive validation results, and these antibodies were also published in the Human Protein Atlas database (www.proteinatlas.org). Finally, cohort 3 consisted of 486 antibodies (from cohort 2) that were analyzed with three different protein detection methods (IHC, Western blot and immunofluorescence), while the previous cohorts only were analyzed with two methods (IHC and Western blot).

Antibody pairs were validated in several applications, IHC on tissues and cells, Western blot and immunofluorescence (IF) on three cancer cell lines. The results suggested that 28% of the antibody pairs in cohort 1 presented similar or partly similar results in IHC and Western blot applications. 8% antibody pairs were similar in both applications. For the further validated antibodies in cohort 2, 50% of the antibody pairs presented similar or partly similar results in the two applications, whereas 24% and 21% of the pairs were similar in IHC or Western blot respectively. In cohort 3, 42% of the paired antibodies were similar or partly similar in three applications, including IF, whereas 17% of the pairs were judged as similar. In total 55% of the antibody pairs presented similar results in at least two out of three applications.

The paired antibodies were also compared regarding other factors that might explain the similarity results, such as antibody concentration differences, number of splice variants etc. A statistically significant difference was

noted between antibody pairs presenting similar results in both IHC and Western blot. 53% of the antibodies in this category had a concentration above 0.17 mg/ml.

Correlation analysis was also performed to compare similarity results in tissues and cell lines. Correlation data was obtained for 331 of the antibody pairs belonging to cohort 2 (where this data was available) and the similarity results with IHC and Western blot were compared to automated image analysis data (TMAx) on cell lines. The correlation analysis verified the similarity results by presenting higher correlation among the antibody pairs considered as similar in both IHC and Western blot, and lower correlation for pairs demonstrating differences in one or both applications.

In summary, this study presented an extensive antibody pair strategy for validating protein detection in several applications. The approach of using two antibodies towards the same protein can be a useful tool for investigating known and unknown proteins of the human proteome.

Conclusions and future perspectives

This thesis is based on the use of monospecific antibodies for proteome wide profiling. The papers give insight into the performance of msAbs in different applications, comparisons and validation strategies. Paper I discusses the high-throughput setting of antibody production and confirms the strategy of generating specific polyclonal protein binders in this fashion. Paper II presents an extension of the previous paper, and focuses on a comparison of monospecific antibodies with well-established commercial analogs. The study brings additional understanding to the expression patterns of msAbs compared to polyclonal and monoclonal cAbs. Paper IV presents an attempt to approach the issue of characterizing unknown proteins by using paired antibodies. The paired antibodies are evaluated with different antibody-based techniques, generating similar results in several applications. Paper III, focuses on fixation mechanisms and their impact on protein detection when using antibody-based methods.

Altogether paper I, II and IV concludes that msAbs, from polyclonal origin, are beneficial in multiple application efforts and that these antibodies successfully can be used for protein profiling in human tissues and cells. Paper III indicates that fixation mechanisms can affect protein recognition, thus suggesting that various fixatives are applicable for different protein detection applications. The study concludes that aldehyde-based fixatives are preferred in applications involving morphology and immunohistochemical stainings and that non-aldehyde based fixatives may be suitable for protein extraction and western blot. A universal fixative still remains to be found, but this study gives some perspectives into fixation mechanisms and applications using a large set of antibodies.

For future studies, the first draft of the human proteome is estimated to be achieved in 2015 within the HPA project. The future for msAbs lies not only in the completion of the Human Protein Atlas, but they will hopefully be further utilized by researchers for investigation of protein functions, interactions and molecular biomarkers for disease. Already, potential biomarkers for various cancers have been identified [164,165]. MsAbs can further be used on differently designed TMAs, representing other diseases than cancer and perhaps also include larger quantities of other tissues and cell types. The use of TMAs with clinical follow-up data of patients with the same disease can give more insight into protein expression and future treatment strategies. Protein profiling in tissues and cells together with additional proteomics-based applications will perhaps enable personalized patient therapies in the future.

Since the proteome can be differently defined, containing millions of protein variants due to PTMs and alternative splicing, the designing of specific and reliable affinity binders will become more challenging. Validation of antibodies is crucial and requires further efforts to enable profiling of protein variants that might have large sequence similarities with each other. Antibodies will have an important role in future studies and perhaps the attempt of generating several antibodies towards non-overlapping epitopes on the same protein will become an implemented strategy for antibody validation.

MsAbs can also be utilized for protein profiling in different species. Although msAbs are raised towards human proteins, studies have shown successful protein detection in for instance rodent tissue [166]. This effort has resulted in a rodent protein atlas, using msAbs for protein profiling in brain tissue from rat [167]. Another ongoing study aiming at protein profiling using msAbs, is the comparison of protein expression in human and primate tissues. The study has so far presented promising results (unpublished) and will hopefully give insight into protein expression evolution.

The future for IHC will hopefully allow automated imaging of IHC stainings in tissues for high-throughput analysis of the immense amounts of data that constantly are generated in large-scale efforts. Also collaborations across fields are beneficial, such as proteomics using MS based technologies with histopathological research. Lately, strategies of using MS for revealing all proteins within one tissue section have been investigated, but the techniques still need further development in order to be utilized on the extensive pathology archival material consisting of FFPE tissues [168-170]. By aiming at studying proteins and proteomes from different perspectives, I believe the future will bring very interesting advancements to look forward to.

Populärvetenskaplig sammanfattning

Människan består av ungefär 22 000 gener som utgör vår genetiska kod, även kallad DNA. Den genetiska koden upptäcktes av forskare i HUGO projektet under 2003. I och med att den genetiska koden väl var tillgänglig så har arbetet kring vad dessa gener gör fortsatt. Generna fungerar som en programmeringskod för proteiner, som alla har viktiga funktioner i vår kropp. Ett forskningsprojekt med målet att karaktärisera alla människans proteiner är ”The Human Protein Atlas project” (HPA). Genom att till en början lokalisera i vilka vävnader proteinerna finns, kan man vidare studera deras funktion och struktur. I projektet studeras proteiner i normal- och cancervävnad, i hopp om att hitta olikheter i exempelvis proteiners lokalisering, vilket i sin tur kan leda till en diagnostisk markör för en viss cancerform. HPA-projektet har som mål att i ett första steg identifiera var i våra organ proteinerna finns och sedan publicera bilder av lokaliseringen i en webbaserad proteinatlas (www.proteinatlas.org).

Syftet med denna avhandling är att lokalisera människans proteiner i olika vävnader och celler med hjälp av målsökande molekyler som kallas för antikroppar. Dessa antikroppar är tillverkade i HPA-projektet och känner igen specifika proteiner och kan binda till dessa. Genom att använda metoder som exempelvis immunhistokemi, kan proteinerna upptäckas i vävnad med hjälp av en brun färgmarkör. Det första delarbetet i denna avhandling beskriver en strategi för att tillverka och utvärdera antikroppar med hjälp av en proteinplattform (protein array) och immunhistokemi. Plattformen innehåller hundratals proteiner och på denna kan en antikropp tillsättas som då binder in till proteinet som den känner igen. På detta sätt kan man säkerställa att antikroppen verkligen binder till ämnat protein och att den inte korsreagerar med andra proteiner. När man arbetar med proteiner så är det mycket viktigt att antikropparna som används är specifika, att de endast binder till avsett protein för att få ett tillförlitligt resultat. När denna specificitet har säkerställts utvärderas antikroppen på vävnader från patologiska arkiv innehållande biopsier från patienter. För att underlätta hanteringen av många vävnader och även reducera användandet av värdefullt patientmaterial, kan man tillverka ”vävnadsarrayer” (TMA). Detta innebär att istället för att testa en antikropp på en vävnad (från en klots), kan man montera ihop hundratals små delar av olika vävnader i en och samma klots. I dag finns det automatiserade instrument som tar små cylinderformade stansar (1 mm i diameter) från varje vävnadsklots och monterar dem samman i en enda TMA-klots. Klotsarna skivas

sedan i tunna skivor (4 μm) som kan läggas på glas som kan användas i mikroskop. Genom exempelvis immunhistokemi kan sedan antikroppen utvärderas. I mikroskop är det möjligt att genom den bruna färgmarkören lokalisera proteiner i olika vävnader. I det första delarbetet utvärderades antikropparnas specificitet d.v.s. hur väl de kände igen och band till respektive protein. Arbetet visade att de effektiva sätt (proteinplattform och immunhistokemi på TMA), som beskrivs ovan, att tillverka och utvärdera antikroppar fungerar för att generera pålitliga målsökande molekyler mot människans proteiner.

Det andra delarbetet består av en jämförelsestudie mellan de egentillverkade antikropparna (kallade monospecifika) och väletablerade kommersiella antikroppar som bl.a. används inom den patologiska diagnostiken. Totalt jämfördes 48 monospecifika antikroppar med kommersiella antikroppar (som kände igen 48 olika proteiner) genom två olika metoder för proteindetektion i normal vävnad. Studien visade att 92% av de egentillverkade monospecifika antikropparna hade liknande proteinlokalisering som de väletablerade kommersiella antikropparna. Studien visade att monospecifika antikroppar kan användas för vidare forskning av proteiner i vävnad.

Det tredje delarbetet fokuserar på hur man kan använda monospecifika antikroppar för att studera skillnader i proteinlokalisering i vävnader som behandlats på olika sätt. När vävnadsprover tas, exempelvis biopsier från patienter eller från operation, skickas de till patologilaboratoriet för diagnostisering. Det är mycket viktigt att vävnadsprovet snarast fixeras, t.ex. genom att lägga biten i en speciell fixeringslösning i 24 timmar. Fixeringen är nödvändig för att proteiner och celler ska bibehålla sin struktur och inte brytas ner av enzymer. I denna studie ville vi undersöka hur olika fixeringslösningar påverkar proteiners lokalisering i vävnad. I dag används generellt formalin som fixeringslösning inom patologisk diagnostik, men en universell fixeringslösning som passar för alla proteiner, vävnader och metoder finns ännu inte. I denna studie utvärderades sju olika fixeringslösningar baserade på olika mekanismer för att fixera proteiner och celler. Resultatet av undersökningen visade att fixeringslösningarna påverkade proteiners lokalisering till en viss del. Olika fixeringslösningar är lämpliga beroende på vilken metod man använder för att upptäcka proteiner.

I det fjärde delarbetet presenterades en strategi för att tillverka två monospecifika antikroppar (kallade par-antikroppar) som känner igen olika delar av samma protein. I denna studie undersöktes hur väl 1140 par-antikroppar detekterade proteiner med tre olika metoder. Studien visade att par-antikropparna till största del uppvisade likadan proteindetektion i en eller flera av metoderna. Förhoppningsvis kan strategin att tillverka par-antikroppar kunna öka tillförlitligheten av okända proteiners lokalisering i vävnader.

Alla delarbeten som ingår i denna avhandling pekar på att monospecifika antikroppar kan användas för att upptäcka proteiner i vävnader och celler. Vidare kan dessa antikroppar bidra till en tillförlitlig kartläggning av alla

människans proteiner, som i sin tur kan leda till framtida proteinmarkörer för olika sjukdomar.

Acknowledgements

This work was performed at the department of Genetics and Pathology at Uppsala University. The projects were supported by grants from the Knut and Alice Wallenberg foundation.

Jag skulle först och främst vilja tacka alla som på något sätt hjälpt mig att göra detta avhandlingsarbete möjligt! Det är några personer som jag speciellt skulle vilja rikta ett stort TACK till:

Min handledare Fredrik Pontén, för att du alltid har din dörr öppen och för din optimistiska attityd! Tack för all hjälp under årens gång och för din stora kunskap kring patologi och en massa annat! Du har en fantastisk förmåga att knyta ihop allting till något riktigt bra i slutändan.

Min andra handledare Kenneth Wester, för att du med din fantastiskt breda kunskap inom immunhistokemi guidat mig i rätt riktning och alltid haft tid för frågor och diskussioner. Det är skönt att veta att man aldrig är på jobbet tidigast av alla, för det är ju du ☺

Min bihandledare på KTH, Sophia Hober, för du alltid ställer upp när det behövs, speciellt under intensiva perioder under det senaste året. Sedan är det alltid trevligt med ett kvinnligt perspektiv på saker och ting också!

Caroline Kampf och Anna Asplund, för att ni finns tillgängliga för alla möjliga frågor. Kul också att ni engagerar er i att fixa med konferenser och andra roliga saker. Anna, speciellt tack för att du ställde upp (höggravid och allt ☺) och hjälpte till med fixpeket.

Hela HPR-gänget i Stockholm, för att jag fått komma och jobba hos er och för att ni alltid tar emot mig med ett varmt välkomnande. Speciellt tack till Emma Lundberg och Marie Skogs, för att ni ställde upp när jag var hos er och jobbade med IF. Vilka tjusiga bilder det blev tillslut!

Tack till alla medförfattare, för samarbete och bra synpunkter på manuskripten. Speciellt tack till Åsa Sivertsson, för att du med en positiv attityd och bra idéer varit en fantastisk hjälp och stöd under det senaste året. Tänk att det ”mycket komplexa” peket äntligen börjar bli något!

Jag vill även tacka Åsa Edvinsson som verkligen ställt upp och hjälpt till med fixpeket. Det är riktigt roligt att jobba tillsammans med en så positiv och engagerad medförfattare!

I would also like to thank Naim Matasci and Svante Pääbo from the Max Planck Institute in Leipzig, for fruitful collaboration that has given me a whole new perspective on protein profiling involving primates.

Till alla trevliga personer på Avdelningen för Pedagogisk utveckling (PU), för att jag har fått möjligheten att jobba hos er. Ni får nog se mer av mig i höst ☺

Till alla i HPR-Uppsala + alla ambitiösa studenter som varit hos oss genom åren! Detta trevliga gäng gör att man blir glad av att komma till jobbet, även de dagar som känns mindre roliga.

Till administratörerna på Rudbeck, för hjälp med diverse stressande moment inför disputationsanmälan, mammaledighet och annat. Tack även till dataexperterna Per-Ivan och Viktor, som kan rädda viktiga filer när datorer får för sig att krascha.

Alla roliga personer i skrivrummet: Carin, Marcus, Demet och Shyam för roliga diskussioner och trevligt umgänge. Jag är glad över att sitta i samma rum som er!

Goaste fd ”skrivrumsbrudarna”: Annika, Stina, Sara, Helena och Nurtena. Jag har alltid sett fram emot att komma till skrivrummet tack vare er! Tack för härliga vin- och sångkvällar, barnprat och mammaledighet tillsammans! Jag har verkligen fått vänner för livet.

Tack till Stina, som i rollen som ”dissertation planner” har koll på allt. Jag är imponerad av din multi-tasking-kapacitet och hoppas att den smittat av sig på mig!

Sara, min HPR-doktorandkollega under flera år, för allt kul vi haft på b.la. konferensresor och annat. Jag uppskattar alla bra tips och råd jag fått av dig!

Linda och Jonas, för att ni är sådana toppenvänner! Tack för mysiga middagar, musiktävlingar och barn/hund passning ☺ både på Öland och här i stan. Ni ställer upp i vått och torrt, speciellt nu när avhandlingsarbetet upptagit nästan alla lediga stunder. Ser fram emot fler roliga upptåg med er lilla familj.

Edith and Gerard, for our long and devoted friendship. We have done many fun things together, in Austria, Germany and Sweden. I hope to see you here every midsummer!

Johanna, min barndomsvän från Åkersberga sedan 22 år tillbaka. Tack för att du hejat på mig under våren och sommaren! Du har alltid stöttat mig i allt jag hittat på, om det så varit i Åkersberga, Stockholm eller Kalmar, och dessutom hittat mig en toppensambo!

Familjen Öland (som jag kallar er): Birgitta, Stefan, Sofia, Kristoffer, Chatrin, Niklas, Casper, Oliver och Filippa. För att vi får komma ner till er och "glömma" jobb och annat en stund. Med er har man aldrig tråkigt!

Till mina största supporters, min familj: Mamma, Pappa och Mia, för att ni alltid ställer upp och är intresserade av vad jag gör (jag vet att det inte är lätt att hänga med). Mamma och Pappa, för att ni alltid har stöttat, hejat och hjälpt mig oavsett vad det gällt. Mia, för att du är världens bästa syster ☺ Dessutom har du en grym argumentationstalang som jag hoppas kunna anamma åtminstone en bråkdel av. Tack även till Thomas, för att du är en så "morsom" norrman som tagit med oss till stugan i fjällen och lärt stadsråttor åka skidor ☺

Sist men inte minst riktar jag ett stort Tack till min egen lilla familj: Christer, Stella och Bellman, för att ni finns där och tar hand om mig! Utan er hade detta aldrig gått och jag är så tacksam över att ha en sambo som ställer upp i alla lägen! Bättre familj kan man inte önska sig, Ni är bäst!

References

1. Watson, J.D. & Crick, F.H. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737-738 (1953).
2. Hubbard, T.J.*, et al.* Ensembl 2009. *Nucleic Acids Res* **37**, D690-697 (2009).
3. Lander, E.S.*, et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).
4. Venter, J.C.*, et al.* The sequence of the human genome. *Science* **291**, 1304-1351 (2001).
5. Consortium, I.H.G.S. Finishing the euchromatic sequence of the human genome. *Nature* **431**, 931-945 (2004).
6. Consortium, T.U. The Universal Protein Resource (UniProt) 2009. *Nucleic Acids Res* **37**, D169-174 (2009).
7. Uhlen, M.*, et al.* A Human Protein Atlas for Normal and Cancer Tissues Based on Antibody Proteomics. *Mol Cell Proteomics* **4**, 1920-1932 (2005).
8. Crick, F.H. On protein synthesis. *Symp Soc Exp Biol* **12**, 138-163 (1958).
9. Brown, T.A. *Genomes*, (BIOS Scientific Publishers Ltd, Oxford, 1999).
10. Mann, M. & Jensen, O.N. Proteomic analysis of post-translational modifications. *Nat Biotechnol* **21**, 255-261 (2003).
11. Stryer, L. *Biochemistry*, (W.H. Freeman and Company, New York, 1995).
12. Gridelli, C. Targeted therapies and non-small-cell lung cancer: new developments. *Curr Opin Oncol* **19**, 75-77 (2007).
13. Stasi, R. Gemtuzumab ozogamicin: an anti-CD33 immunoconjugate for the treatment of acute myeloid leukaemia. *Expert Opin Biol Ther* **8**, 527-540 (2008).
14. Leader, B., Baca, Q.J. & Golan, D.E. Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov* **7**, 21-39 (2008).
15. Wilkins, M.R.*, et al.* From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (N Y)* **14**, 61-65 (1996).
16. Uhlen, M. & Ponten, F. Antibody-based proteomics for human tissue profiling. *Mol Cell Proteomics* **4**, 384-393 (2005).
17. O'Farrell, P.H. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007-4021 (1975).

18. Sasse, J. & Gallagher, S.R. Staining proteins in gels. *Curr Protoc Immunol* **Chapter 8**, Unit 8 9 (2004).
19. Unlu, M., Morgan, M.E. & Minden, J.S. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* **18**, 2071-2077 (1997).
20. Minden, J.S., Dowd, S.R., Meyer, H.E. & Stuhler, K. Difference gel electrophoresis. *Electrophoresis* **30 Suppl 1**, S156-161 (2009).
21. Karas, M. & Hillenkamp, F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* **60**, 2299-2301 (1988).
22. Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F. & Whitehouse, C.M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**, 64-71 (1989).
23. Guerrera, I.C. & Kleiner, O. Application of mass spectrometry in proteomics. *Biosci Rep* **25**, 71-93 (2005).
24. Han, X., Aslanian, A. & Yates, J.R., 3rd. Mass spectrometry for proteomics. *Curr Opin Chem Biol* **12**, 483-490 (2008).
25. Lemaire, R.*, et al.* Direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections. *J Proteome Res* **6**, 1295-1305 (2007).
26. Shi, S.R., Cote, C., Kalra, K.L., Taylor, C.R. & Tandon, A.K. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. *J Histochem Cytochem* **40**, 787-792 (1992).
27. Shinnick, T.M., Sutcliffe, J.G., Green, N. & Lerner, R.A. Synthetic peptide immunogens as vaccines. *Annu Rev Microbiol* **37**, 425-446 (1983).
28. Hancock, D.C. & O'Reilly, N.J. Synthetic peptides as antigens for antibody production. *Methods Mol Biol* **295**, 13-26 (2005).
29. Geisse, S., Gram, H., Kleuser, B. & Kocher, H.P. Eukaryotic expression systems: a comparison. *Protein Expr Purif* **8**, 271-282 (1996).
30. Terpe, K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* **60**, 523-533 (2003).
31. Agaton, C.*, et al.* Selective enrichment of monospecific polyclonal antibodies for antibody-based proteomics efforts. *J Chromatogr A* **1043**, 33-40 (2004).
32. Lindskog, M., Rockberg, J., Uhlen, M. & Sterky, F. Selection of protein epitopes for antibody production. *Biotechniques* **38**, 723-727 (2005).
33. Polak, J.M., Van Noorden, S. *Immunocytochemistry*, (John Wright & Sons Ltd, Bristol, 1986).
34. Cuello, A.C. *Immunohistochemistry*, (John Wiley & Sons, Oxford, 1983).
35. Lipman, N.S., Jackson, L.R., Trudel, L.J. & Weis-Garcia, F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J* **46**, 258-268 (2005).

36. Kuby, J. *Immunology*, (W.H. Freeman and Company, 1997).
37. Wilson, K.W., J. *Principles and techniques of practical biochemistry*, (Cambridge University Press, Cambridge, 1994).
38. Kohler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497 (1975).
39. Uhlen, M. Mapping the human proteome using antibodies. *Mol Cell Proteomics* **6**, 1455-1456 (2007).
40. Coons, A.H., Creech, H.J. & Jones, R.N. Demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J Immunol* **45**, 159-170 (1942).
41. Engvall, E. & Perlmann, P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871-874 (1971).
42. Olive, C., Toth, I. & Jackson, D. Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies. *Mini Rev Med Chem* **1**, 429-438 (2001).
43. Agaton, C*., et al.* Affinity proteomics for systematic protein profiling of chromosome 21 gene products in human tissues. *Mol Cell Proteomics* **2**, 405-414 (2003).
44. Barbe, L*., et al.* Toward a confocal subcellular atlas of the human proteome. *Mol Cell Proteomics* **7**, 499-508 (2008).
45. Uhlen, M., Graslund, S. & Sundstrom, M. A pilot project to generate affinity reagents to human proteins. *Nat Methods* **5**, 854-855 (2008).
46. Berglund, L*., et al.* A whole-genome bioinformatics approach to selection of antigens for systematic antibody generation. *Proteomics* **8**, 2832-2839 (2008).
47. Ponten, F., Jirstrom, K. & Uhlen, M. The Human Protein Atlas--a tool for pathology. *J Pathol* **216**, 387-393 (2008).
48. Bjorling, E*., et al.* A web-based tool for in silico biomarker discovery based on tissue-specific protein profiles in normal and cancer tissues. *Mol Cell Proteomics* **7**, 825-844 (2008).
49. Better, M., Chang, C.P., Robinson, R.R. & Horwitz, A.H. Escherichia coli secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043 (1988).
50. Nord, K., Nilsson, J., Nilsson, B., Uhlen, M. & Nygren, P.A. A combinatorial library of an alpha-helical bacterial receptor domain. *Protein Eng* **8**, 601-608 (1995).
51. Bird, R.E*., et al.* Single-chain antigen-binding proteins. *Science* **242**, 423-426 (1988).
52. Skerra, A. & Pluckthun, A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science* **240**, 1038-1041 (1988).
53. Azzazy, H.M. & Highsmith, W.E., Jr. Phage display technology: clinical applications and recent innovations. *Clin Biochem* **35**, 425-445 (2002).
54. Smith, G.P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317 (1985).

55. Marks, J.D. & Bradbury, A. Selection of human antibodies from phage display libraries. *Methods Mol Biol* **248**, 161-176 (2004).
56. Bradbury, A.R.*, et al.* Antibodies in proteomics. *Methods Mol Biol* **248**, 519-546 (2004).
57. Nord, K.*, et al.* Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. *Nat Biotechnol* **15**, 772-777 (1997).
58. Borghouts, C., Kunz, C. & Groner, B. Peptide aptamers: recent developments for cancer therapy. *Expert Opin Biol Ther* **5**, 783-797 (2005).
59. Gold, L., Singer, B., He, Y.Y. & Brody, E. SELEX and the evolution of genomes. *Curr Opin Genet Dev* **7**, 848-851 (1997).
60. Brody, E.N.*, et al.* The use of aptamers in large arrays for molecular diagnostics. *Mol Diagn* **4**, 381-388 (1999).
61. Tuerk, C. & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505-510 (1990).
62. Schweitzer, B. & Kingsmore, S.F. Measuring proteins on microarrays. *Curr Opin Biotechnol* **13**, 14-19 (2002).
63. Haab, B.B., Dunham, M.J. & Brown, P.O. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol* **2**, RESEARCH0004 (2001).
64. Haab, B.B. Advances in protein microarray technology for protein expression and interaction profiling. *Curr Opin Drug Discov Devel* **4**, 116-123 (2001).
65. MacBeath, G. Protein microarrays and proteomics. *Nat Genet* **32 Suppl**, 526-532 (2002).
66. Burnette, W.N. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112**, 195-203 (1981).
67. Kurien, B.T. & Scofield, R.H. Western blotting. *Methods* **38**, 283-293 (2006).
68. Landegren, U.*, et al.* Molecular tools for a molecular medicine: analyzing genes, transcripts and proteins using padlock and proximity probes. *J Mol Recognit* **17**, 194-197 (2004).
69. Schallmeiner, E.*, et al.* Sensitive protein detection via triple-binder proximity ligation assays. *Nat Methods* **4**, 135-137 (2007).
70. Gullberg, M.*, et al.* Cytokine detection by antibody-based proximity ligation. *Proc Natl Acad Sci U S A* **101**, 8420-8424 (2004).
71. Warford, A., Howat, W. & McCafferty, J. Expression profiling by high-throughput immunohistochemistry. *J Immunol Methods* **290**, 81-92 (2004).
72. Shi, S.R., Liu, C., Perez, J. & Taylor, C.R. Protein-embedding technique: a potential approach to standardization of immunohistochem-

istry for formalin-fixed, paraffin-embedded tissue sections. *J Histochem Cytochem* **53**, 1167-1170 (2005).
73. Werner, M., Chott, A., Fabiano, A. & Battifora, H. Effect of formalin tissue fixation and processing on immunohistochemistry. *Am J Surg Pathol* **24**, 1016-1019 (2000).
74. Leong, A.S. & Gilham, P.N. The effects of progressive formaldehyde fixation on the preservation of tissue antigens. *Pathology* **21**, 266-268 (1989).
75. Puchtler, H. & Meloan, S.N. On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry* **82**, 201-204 (1985).
76. Taylor, C.R.*, et al.* Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. *Hum Pathol* **25**, 263-270 (1994).
77. Shi, S.R., Cote, R.J. & Taylor, C.R. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. *Appl Immunohistochem Mol Morphol* **9**, 107-116 (2001).
78. Pinkus, G.S. Diagnostic immunocytochemistry of paraffin-embedded tissues. *Hum Pathol* **13**, 411-415 (1982).
79. Mason, J.T. & O'Leary, T.J. Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J Histochem Cytochem* **39**, 225-229 (1991).
80. Helander, K.G. Kinetic studies of formaldehyde binding in tissue. *Biotech Histochem* **69**, 177-179 (1994).
81. Sompuram, S.R., Vani, K., Messana, E. & Bogen, S.A. A molecular mechanism of formalin fixation and antigen retrieval. *Am J Clin Pathol* **121**, 190-199 (2004).
82. Morgan, J.M., Navabi, H., Schmid, K.W. & Jasani, B. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. *J Pathol* **174**, 301-307 (1994).
83. Dapson, R.W. Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. *Biotech Histochem* **82**, 161-166 (2007).
84. Umlas, J. & Tulecke, M. The effects of glyoxal fixation on the histological evaluation of breast specimens. *Hum Pathol* **35**, 1058-1062 (2004).
85. Titford, M.E. & Horenstein, M.G. Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology. *Arch Pathol Lab Med* **129**, 502-506 (2005).
86. Herman, G., Chlipala, E., Bochenski, G., Sabin, I. & Elfont, E. Zinc formalin for automated tissue processing. *J Histotechnol* **11**, 85-89 (1988).
87. Tome, Y., Hirohashi, S., Noguchi, M. & Shimosato, Y. Preservation of cluster 1 small cell lung cancer antigen in zinc-formalin fixative

and its application to immunohistological diagnosis. *Histopathology* **16**, 469-474 (1990).
88. Kiernan, J.A. A system for quantitative evaluation of fixatives for light microscopy using paraffin sections of kidney and brain. *Biotech Histochem* **84**, 1-10 (2009).
89. Regan, L. & Clarke, N.D. A tetrahedral zinc(II)-binding site introduced into a designed protein. *Biochemistry* **29**, 10878-10883 (1990).
90. Brandtzaeg, P. Tissue preparation methods for immunocytochemistry. in *Techniques in Imunocytochemistry*, Vol. 1 (eds. Bullok, G. & Petruz, P.) 1-75 (Academic Press, New York, 1983).
91. Gillespie, J.W.*, et al.* Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am J Pathol* **160**, 449-457 (2002).
92. Cox, M.L.*, et al.* Assessment of fixatives, fixation, and tissue processing on morphology and RNA integrity. *Exp Mol Pathol* **80**, 183-191 (2006).
93. Delfour, C.*, et al.* RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. *J Mol Diagn* **8**, 157-169 (2006).
94. Witchell, J., Varshney, D., Gajjar, T., Wangoo, A. & Goyal, M. RNA isolation and quantitative PCR from HOPE- and formalin-fixed bovine lymph node tissues. *Pathol Res Pract* **204**, 105-111 (2008).
95. Beckstead, J.H. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J Histochem Cytochem* **42**, 1127-1134 (1994).
96. Wang, B.L., Springer, G.F. & Kaufman, M.W. Concurrent immunohistochemical staining of tumor-infiltrating lymphocytes and carcinoma-associated T (Thomsen-Friedenreich)/Tn antigens in human breast carcinoma. *J Histochem Cytochem* **44**, 187-191 (1996).
97. Wester, K.*, et al.* Zinc-based fixative improves preservation of genomic DNA and proteins in histoprocessing of human tissues. *Lab Invest* **83**, 889-899 (2003).
98. Hicks, D.J.*, et al.* Evaluation of zinc salt based fixatives for preserving antigenic determinants for immunohistochemical demonstration of murine immune system cell markers. *Biotech Histochem* **81**, 23-30 (2006).
99. Fraenkel-Conrat, H., Brandon, B. & Olcott, H.S. The reaction of formaldehyde with proteins. IV. Participation of indole groups. Gramicidin. *J Biol Chem* **168**, 99-118 (1947).
100. Fraenkel-Conrat, H. & Olcott, H.S. Reaction of formaldehyde with proteins; cross-linking of amino groups with phenol, imidazole, or indole groups. *J Biol Chem* **174**, 827-843 (1948).
101. Fraenkel-Conrat, H. & Olcott, H.S. The reaction of formaldehyde with proteins; cross-linking between amino and primary amide or guanidyl groups. *J Am Chem Soc* **70**, 2673-2684 (1948).

102. Battifora, H. & Kopinski, M. The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation. *J Histochem Cytochem* **34**, 1095-1100 (1986).
103. Shi, S.R., Key, M.E. & Kalra, K.L. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* **39**, 741-748 (1991).
104. Igarashi, H.*, et al.* Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *Apmis* **102**, 295-307 (1994).
105. Imam, S.A., Young, L., Chaiwun, B. & Taylor, C.R. Comparison of two microwave based antigen-retrieval solutions in unmasking epitopes in formalin-fixed tissue for immunostaining. *Anticancer Res* **15**, 1153-1158 (1995).
106. Bogen, S., Vani, K. & Sompuram, S. Molecular mechanisms of antigen retrieval: antigen retrieval reverses steric interference caused by formalin-induced cross-links. *Biotech Histochem*, 1-9 (2009).
107. Miller, R.T., Swanson, P.E. & Wick, M.R. Fixation and epitope retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. *Appl Immunohistochem Mol Morphol* **8**, 228-235 (2000).
108. Taylor, C.R. & Shi, S.R. Antigen retrieval: call for a return to first principles. *Appl Immunohistochem Mol Morphol* **8**, 173-174 (2000).
109. Norton, A.J., Jordan, S. & Yeomans, P. Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. *J Pathol* **173**, 371-379 (1994).
110. Powell, C. Pressure cooking retrieves "lost" antigens in tissue sections. Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. *Hum Exp Toxicol* **14**, 465-466 (1995).
111. Taylor, C.R.*, et al.* Comparative study of antigen retrieval heating methods: microwave, microwave and pressure cooker, autoclave, and steamer. *Biotech Histochem* **71**, 263-270 (1996).
112. Evers, P. & Uylings, H.B. Effects of microwave pretreatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. *J Neurosci Methods* **55**, 163-172 (1994).
113. Shi, S.R., Imam, S.A., Young, L., Cote, R.J. & Taylor, C.R. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J Histochem Cytochem* **43**, 193-201 (1995).
114. Emoto, K., Yamashita, S. & Okada, Y. Mechanisms of heat-induced antigen retrieval: does pH or ionic strength of the solution play a role for refolding antigens? *J Histochem Cytochem* **53**, 1311-1321 (2005).
115. Kawai, K., Serizawa, A., Hamana, T. & Tsutsumi, Y. Heat-induced antigen retrieval of proliferating cell nuclear antigen and p53 protein

in formalin-fixed, paraffin-embedded sections. *Pathol Int* **44**, 759-764 (1994).
116. Cattoretti, G.*, et al.* Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol* **171**, 83-98 (1993).
117. Morgan, J.M., Navabi, H. & Jasani, B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. *J Pathol* **182**, 233-237 (1997).
118. Bussolati, G. & Leonardo, E. Technical pitfalls potentially affecting diagnoses in immunohistochemistry. *J Clin Pathol* **61**, 1184-1192 (2008).
119. Shi, S.R., Cote, R.J. & Taylor, C.R. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem* **45**, 327-343 (1997).
120. Sompuram, S.R., Vani, K., Hafer, L.J. & Bogen, S.A. Antibodies immunoreactive with formalin-fixed tissue antigens recognize linear protein epitopes. *Am J Clin Pathol* **125**, 82-90 (2006).
121. Sompuram, S.R., Vani, K. & Bogen, S.A. A molecular model of antigen retrieval using a peptide array. *Am J Clin Pathol* **125**, 91-98 (2006).
122. Nadji, M. & Ganjei, P. Special report. Immunocytochemistry in diagnostic cytology: a 12-year perspective. *Am J Clin Pathol* **94**, 470-475 (1990).
123. Chilosi, M.*, et al.* A rapid immunostaining method for frozen sections. *Biotech Histochem* **69**, 235-239 (1994).
124. Gross, A.J. & Sizer, I.W. The oxidation of tyramine, tyrosine, and related compounds by peroxidase. *J Biol Chem* **234**, 1611-1614 (1959).
125. Adams, J.C. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem* **40**, 1457-1463 (1992).
126. Werner, M., Von Wasielewski, R. & Komminoth, P. Antigen retrieval, signal amplification and intensification in immunohistochemistry. *Histochem Cell Biol* **105**, 253-260 (1996).
127. Gusev, Y.*, et al.* Rolling circle amplification: a new approach to increase sensitivity for immunohistochemistry and flow cytometry. *Am J Pathol* **159**, 63-69 (2001).
128. Wiltshire, S.*, et al.* Detection of multiple allergen-specific IgEs on microarrays by immunoassay with rolling circle amplification. *Clin Chem* **46**, 1990-1993 (2000).
129. Reynolds, G.J. External quality assurance and assessment in immunocytochemistry. *Histopathology* **15**, 627-633 (1989).
130. Rudiger, T., Hofler, H. & Muller-Hermelink, H.K. [Immunohistochemistry Circle Trial of the University Institute of Pathology in Germany]. *Pathologe* **18**, 262-268 (1997).
131. Leong, A.S. Pitfalls in diagnostic immunohistology. *Adv Anat Pathol* **11**, 86-93 (2004).

132. Atkins, D.*, et al.* Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. *J Histochem Cytochem* **52**, 893-901 (2004).
133. Packeisen, J., Buerger, H., Krech, R. & Boecker, W. Tissue microarrays: a new approach for quality control in immunohistochemistry. *J Clin Pathol* **55**, 613-615 (2002).
134. Kononen, J.*, et al.* Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* **4**, 844-847 (1998).
135. Callagy, G.*, et al.* Molecular classification of breast carcinomas using tissue microarrays. *Diagn Mol Pathol* **12**, 27-34 (2003).
136. Walker, R.A. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment I. *Histopathology* **49**, 406-410 (2006).
137. Taylor, C.R. & Levenson, R.M. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment II. *Histopathology* **49**, 411-424 (2006).
138. McClelland, R.A.*, et al.* Automated quantitation of immunocytochemically localized estrogen receptors in human breast cancer. *Cancer Res* **50**, 3545-3550 (1990).
139. Ruifrok, A.C. & Johnston, D.A. Quantification of histochemical staining by color deconvolution. *Anal Quant Cytol Histol* **23**, 291-299 (2001).
140. Ruifrok, A.C., Katz, R.L. & Johnston, D.A. Comparison of quantification of histochemical staining by hue-saturation-intensity (HSI) transformation and color-deconvolution. *Appl Immunohistochem Mol Morphol* **11**, 85-91 (2003).
141. Bauer, K.D.*, et al.* Reliable and sensitive analysis of occult bone marrow metastases using automated cellular imaging. *Clin Cancer Res* **6**, 3552-3559 (2000).
142. Wang, S.*, et al.* Assessment of HER-2/neu status in breast cancer. Automated Cellular Imaging System (ACIS)-assisted quantitation of immunohistochemical assay achieves high accuracy in comparison with fluorescence in situ hybridization assay as the standard. *Am J Clin Pathol* **116**, 495-503 (2001).
143. Stromberg, S.*, et al.* A high-throughput strategy for protein profiling in cell microarrays using automated image analysis. *Proteomics* **7**, 2142-2150 (2007).
144. Moch, H., Kononen, T., Kallioniemi, O.P. & Sauter, G. Tissue microarrays: what will they bring to molecular and anatomic pathology? *Adv Anat Pathol* **8**, 14-20 (2001).
145. Battifora, H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab Invest* **55**, 244-248 (1986).
146. Kallioniemi, O.P., Wagner, U., Kononen, J. & Sauter, G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum Mol Genet* **10**, 657-662 (2001).

147. Hewitt, S.M. Design, construction, and use of tissue microarrays. *Methods Mol Biol* **264**, 61-72 (2004).
148. Moskaluk, C.A. & Stoler, M.H. Agarose mold embedding of cultured cells for tissue microarrays. *Diagn Mol Pathol* **11**, 234-238 (2002).
149. Fejzo, M.S. & Slamon, D.J. Frozen tumor tissue microarray technology for analysis of tumor RNA, DNA, and proteins. *Am J Pathol* **159**, 1645-1650 (2001).
150. Miyaji, T., Hewitt, S.M., Liotta, L.A. & Star, R.A. Frozen protein arrays: a new method for arraying and detecting recombinant and native tissue proteins. *Proteomics* **2**, 1489-1493 (2002).
151. Hewitt, S.M. & Star, R.A. Frozen protein arrays. *Methods Mol Biol* **441**, 105-112 (2008).
152. Bubendorf, L., Nocito, A., Moch, H. & Sauter, G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. *J Pathol* **195**, 72-79 (2001).
153. Rimm, D.L.*, et al.* Tissue microarray: a new technology for amplification of tissue resources. *Cancer J* **7**, 24-31 (2001).
154. Kampf, C.*, et al.* Antibody-based tissue profiling as a tool for clinical proteomics. *Clinical Proteomics* **1**, 285-299 (2004).
155. Camp, R.L., Charette, L.A. & Rimm, D.L. Validation of tissue microarray technology in breast carcinoma. *Lab Invest* **80**, 1943-1949 (2000).
156. Hoos, A. & Cordon-Cardo, C. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. *Lab Invest* **81**, 1331-1338 (2001).
157. Simon, R., Mirlacher, M. & Sauter, G. Tissue microarrays in cancer diagnosis. *Expert Rev Mol Diagn* **3**, 421-430 (2003).
158. Moch, H.*, et al.* High-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarray screening in renal cell carcinoma. *Am J Pathol* **154**, 981-986 (1999).
159. Mousses, S.*, et al.* Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. *Cancer Res* **62**, 1256-1260 (2002).
160. Uhlen, M. A new era for proteomics research? *Genome Biol* **9**, 325 (2008).
161. Bubendorf, L.*, et al.* Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. *Cancer Res* **59**, 803-806 (1999).
162. Nocito, A.*, et al.* Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. *J Pathol* **194**, 349-357 (2001).
163. Richter, J.*, et al.* High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* **157**, 787-794 (2000).

164. Ek, S.*, et al.* From gene expression analysis to tissue microarrays: a rational approach to identify therapeutic and diagnostic targets in lymphoid malignancies. *Mol Cell Proteomics* **5**, 1072-1081 (2006).
165. Bengtsson, S.*, et al.* Large-scale proteomics analysis of human ovarian cancer for biomarkers. *J Proteome Res* **6**, 1440-1450 (2007).
166. Mulder, J.*, et al.* Systematically generated antibodies against human gene products: high throughput screening on sections from the rat nervous system. *Neuroscience* **146**, 1689-1703 (2007).
167. Mulder, J.*, et al.* Tissue profiling of the mammalian central nervous system using human antibody-based proteomics. *Mol Cell Proteomics* **8**, 1612-1622 (2009).
168. Herring, K.D., Oppenheimer, S.R. & Caprioli, R.M. Direct tissue analysis by matrix-assisted laser desorption ionization mass spectrometry: application to kidney biology. *Semin Nephrol* **27**, 597-608 (2007).
169. Chaurand, P., Norris, J.L., Cornett, D.S., Mobley, J.A. & Caprioli, R.M. New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. *J Proteome Res* **5**, 2889-2900 (2006).
170. Groseclose, M.R., Massion, P.P., Chaurand, P. & Caprioli, R.M. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. *Proteomics* **8**, 3715-3724 (2008).

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 474

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-107471

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2009