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Proceedings of the ESF Workshop

Protein Arrays – Bridging the Gap Between Physics and Biomedicine

Jena, Germany, 1 – 3 April 2004

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Protein Arrays – Bridging the Gap Between Physics and Biomedicine

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PROGRAMME

All talks will be 20 min + 10 min discussion

Thursday 1 April 2004

from 14:00 — *Registration*

19:30 — Get together reception

Dinner

Friday 2 April 2004

09:00 – 09:15 — **Hui Wang**, Strasbourg, France
Representative of the Standing Committee for the European Medical Research Councils
Presentation of the European Science Foundation (ESF)

Session 1 - Bioconjugates, Biomodifications

Chair: Bo Liedberg

09:15 – 09:45 — **Christof Niemeyer**, Dortmund, Germany
Self-Assembled Bioconjugates for Biochip Technologies

09:45 – 10:15 — **Anthony Cass**, London, UK
The role of protein engineering in designing content

30 min coffee break

Session 2 - Detection techniques for protein arrays: optical spectroscopy, microscopy

Chair: Matthias Vaupel

10:45 – 11:15 — **H. A. O. Hill**, Oxford, UK
Proteins at electrode surfaces: examination by Scanning Probe Microscopy

11:15 – 11:45 — **Tero Soukko**, Turku, Finland
Time-resolved fluorescent labels in protein recognition

11:45 – 12:05 — **Peter Schellenberg,** Jena, Germany
Analysing protein microarrays by utilizing intrinsic time resolved UV fluorescence

12:10 — Lunch

Session 3 – Detection techniques for protein arrays: ellipsometry, mass spectroscopy

Chair : H. A. O. Hill

14:00 – 14:30 **Bo Liedberg**, Sweden
Microarray production on polymeric hydrogels using microcontact printing

14:30 – 15:00 **Matthias Vaupel**, Germany
Quality control and kinetics recording on micro arrays with imaging ellipsometry

15:00 – 15:30 **Ferdinand von Eggeling**, Jena, Germany
Biomarker discovery with ProteinChip technology

30 min coffee break

Session 4 – Substrates, coatings, chemistry

Chair: Wilhelm Ansorge

16:00 – 16:30 **Holger Eickhoff**, Berlin, Germany
Protein Chip data bridging data sets from 2D Gels and DNA –microarrays: Applications in neurology and immunology research

16:30 – 17:00 **Gérard Siest**, Nancy, France
Will the proteinchips in clinical chemistry be useful in preventive medicine

17:00 – 17:30 **Finn Edler von Eyben**, Odense, Denmark
Gene expression in abdominal fat

17:30 – 17:50 **Hans Martin Striebel** , Jena, Germany
Supports and probe immobilization for protein microarray analysis by time resolved UV fluorescence

Dinner/Buffet Restaurant SCALA, Intershop-Tower
City tour from top of the building

Saturday 3 April 2004

Session 5 - Protein array assembly: supports, immobilization techniques

Chair: Jörg Hoheisel

09:00 – 09:30 **Eginhard Schick**, Switzerland
Cell Lysate Arrays- Protein Profiling in Cellular Systems

09:30 – 10:00 **Derek Murphy**, Ireland
Generation and some applications of protein arrays

10:00 – 10:30 **Stefan Pabst**, Tübingen, Germany
Protein Microarray Technology

30 min coffee break

Session 6 - Protein array applications: screening, medical diagnosis, antibody arrays, translation profiling

Chair: Gérard Siest

11:00 – 11:30 **Wilhelm Ansorge**, Heidelberg, Germany
Microarray techniques in biomedical research

11:30 – 12:00 **Jörg Hoheisel**, Heidelberg, Germany
Combining proteomics and genomics for cancer analysis

12:00 – 12:30 **Gregory Michaud**, New Haven, CT, USA
Applications of functional proteome scale microarrays

12:30 – 13:00 **Ian Humphery-Smith**, The Netherlands
Proteins arrays and a new paradigm in lead optimization

13:00 Lunch

Session 7 – Micro probe arrays, special applications

Chair: Ian Humphery-Smith

14:30 – 15:00 **Mingyue He**, Cambridge, UK
Cell-free protein arrays from DNA

15:00 – 15:30 **Karl Otto Greulich**, Jena, Germany
Ubiquitous cancer genes – candidates for protein chips?

15:30 **Discussion:** Future prospects

16:30 **End of workshop**

Dinner

Sunday 4 April 2004

Breakfast

Departure

The Role of Protein Engineering in Designing Content

Tony Cass
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S. Kensington Campus, London SW7 2AZ, UK

Introduction: Although proteins have many valuable properties as analytical reagents they also suffer for several serious drawbacks. This lack of 'fitness for purpose' when seeking to use them in the bioanalytical arena is not surprising as these molecules have evolved to fulfil a specific purpose ***in a biological context*** and expecting them to perform similarly well in the artificial environment of a microarray is a demanding requirement.
We have sought to overcome the inherent limitations of natural proteins whilst retaining many of their admirable properties by introducing mutations or additional elements of sequence using the tools of protein design and engineering.

There are a variety of targets for engineering proteins for microarray production including:

- Attachment chemistry for functional immobilisation that is generic and so can work with many different proteins
- Signal generation to transduce the binding of analyte to the protein into a measureable physicochemical change
- Binding site affinity, matching the affinity of the binding site to the expected concentration of analyte yields maximum sensitivity.
- Binding site specificity determines the range of analytes **and sample matrices** that can be studied.
- Improved stability results in long-term storage without special precautions

A protein engineering approach allows us to treat the molecule as a series of modules that can (ideally) be independently manipulated and certainly in the case of immobilisation, signal transduction and binding site properties this can be straightforwardly achieved.

Immobilisation: Traditionally immobilisation of proteins has involved 'random' chemical cross-linking or strong adsorption methods with high surface area supports. This has often resulted in significant loss of activity (upto 90%) however where the proteins were cheap and efficiency not important this was acceptable. Protein microarrays have neither the luxury of cheap content nor the signal to noise characteristics that can tolerate large amounts of non-functional protein. An approach that has proved quite robust and generic has been to use a C- or N-terminal peptide fusion that interacts strongly and specifically with the surface to allow functional and oriented immobilisation:

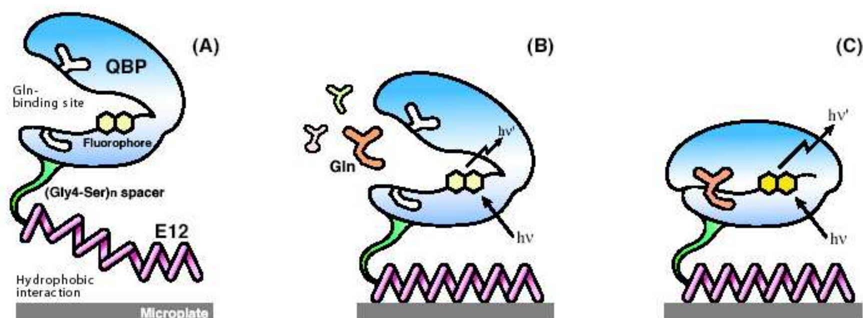


Figure 1. Fusion 'tag' based immobilisation

The E12 tag shown in Figure 1 is a hydrophobic sequence derived from elastin that adsorbs strongly to hydrophobic surfaces.

Signal Transduction: The direct readout of a protein ligand interaction offers the most convenient way of transducing analyte binding to signal generation and whilst various label free methods such as surface plasmon resonance imaging have been employed we have chosen to use fluorescence. Engineering in a fluorescent reporter group that is sensitive to its environment means that ligand binding is converted in to a change in fluorescence intensity that can be readily measured with a conventional microarray scanner:

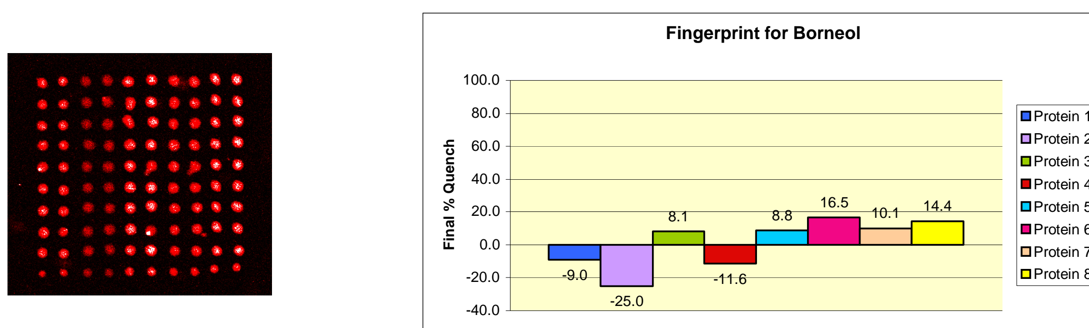
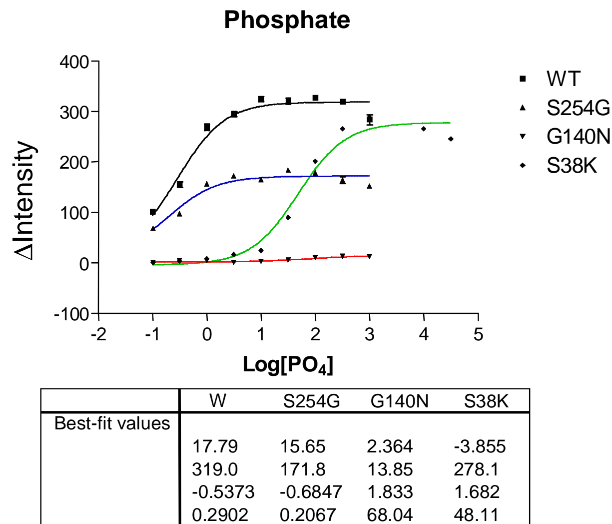


Figure 2: Left a binding protein microarray showing fluorescence from protein spots labelled with an environment sensitive fluorophore. Right the pattern of intensity changes seen with a terpene.

Binding Site Modification: The Kd of a binding protein indicates the concentration of ligand at which it is maximally sensitive. To span a range of concentrations and also to accommodate changes in specificity the residues in the binding site can be altered by mutagensis:



	W	S254G	G140N	S38K
Best-fit values				
	17.79	15.65	2.364	-3.855
	319.0	171.8	13.85	278.1
	-0.5373	-0.6847	1.833	1.682
	0.2902	0.2067	68.04	48.11

Figure 3: Mutations that change the phosphate affinity for the phosphate binding protein (pbp)

Point mutations in either the binding site or elsewhere in the protein can shift Kd values and hence analytical range by upto 3 orders of magnitude such that at low concentrations high affinity spots show intensity changes and at high concentrations where these are saturated the low affinity spots then respond.

Conclusions: It is now generally recognised that the provision of suitable content is a limiting factor in producing protein microarrays. Rationale protein design can go a long way towards providing both diverse content and also in modifying proteins to better suit them to their role in this powerful emerging analytical tool.

Protein microarray data bridging data sets from 2D gels and DNA microarrays

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When proteins misbehave they can influence the health of individuals in many ways, from the amyloid proteins that aggregate in the brains of Alzheimer's or Huntingtons Chorea patients to the proteins that cause unpredictable cancer-cell growth. Understanding disease and disease progress more effectively means getting a better idea on how proteins work and interact—or fail. The most emerging tools in analysing protein-protein interactions are micro-arrays, small chips containing thousands of protein samples that can be utilized to analyze protein populations of cells quickly. Protein Chips can be essential tools to get a deeper insight how diseases develop and progress, how drugs work and how to find new drugs. Therefore protein microarray research and development is one of the central parts in drug discovery and drug development processes in industry and academia.

When a protein microarray is exposed to biochemicals or solutions of other proteins, some of those molecules will stick and some will wash off; the ones that stick can be identified by various markers, such as fluorescent tags or just by their molecular mass or other fingerprints. Molecules that adhere strongly to specific proteins are valuable leads in the search for new medications, because binding characteristics are essential for effective pharmaceuticals and diagnostic purposes.

Unfortunately the generation of protein chips requires much more efforts than DNA microchips. While DNA is DNA and a variety of different DNA molecules behave stable in a hybridisation experiment, proteins are much more difficult to produce and to handle. Proteins are uniquely folded strings of subunits of amino acids, and a lot of what a specific protein does depend on the precise three-dimensional pattern that the string folds into. Outside of a narrow range of environmental conditions, proteins will "denature"—the amino-acid chain will lose its three-dimensional structure and a lot of its specificity and activity. One big challenge in Protein Microarray technology is to produce native and functional proteins and store them in a native and special environment for every single spot on an array. Although applications like antibody profiling are possible on denatured arrays, the major aim is to develop native protein arrays. These arrays can be application or pathway specific or representing a full proteome.

Ubiquitous Cancer Genes: Candidates for Protein - Chips ?

Brigitte Altenberg [1], Christine Gemünd [1], Karl Otto Greulich [2]

[1] European Molecular Biology Laboratory, Meyerhofstr. 1, D 69126 Heidelberg, [2] Institute of Molecular Biotechnology, Beutenbergstr.11, D 07745 Jena

Introduction

In contrast to DNA chips carrying up to more than 10 000 different sequences, "multi - purpose" protein chips may be more difficult to generate, since the chemistry for binding proteins to chip surfaces is more difficult than DNA binding techniques. Chips for routine use in medical diagnostics ideally should carry only a limited number of selected molecules relevant for the special diagnosis. Otherwise, for small labs, interpretation of the results would be too difficult. Thus, small specialized protein chips will play a larger role than small chips in DNA technology. This requires efficient selection of the corresponding proteins. For cancer chips, proteins relevant in as many as possible cancers, would be helpful, since they can be used at least to some extent in a "multi - purpose" manner. With this motivation, we have searched data bases such as CancerGene, Tumor Gene and dbEST for genes which are overexpressed in the 52 different known cancer classes. 119 000 ESTs were checked, representing essentially all 30 - 40 000 human genes.

Materials and Methods

The database dbEST, part of the Cancer Gene Anatomy Project (CGAP) made available by the NIH, provides access to the majority of published gene expression data and unifies data generated using different experimental approaches. If in literature, for a given gene, expression data are available for a given cancer and its corresponding normal tissue, the ratio of expression levels and a confidence level judging the statistical quality are given in a dbEST subprogram called "Virtual Northern". It turned out that expression ratios > 1.5 and confidence values $p < 0.05$ are statistically meaningful to state overexpression of a gene in a given cancer.

Results

Table 1 gives a list of human genes (rows in table 1) among the 30 000 - 40 000 human genes, which are overexpressed in a large number of cancer classes (columns in table 1). We classify the corresponding genes as "ubiquitous(ly overexpressed) cancer genes" when they are overexpressed in more than 15 cancers. Surprisingly, only 31 genes (approx. 0.1 % of the human genes) are ubiquitous. No gene is overexpressed in all cancers. Using such ubiquitous genes one needs only a limited number of corresponding proteins to characterize "cancer". In addition, the overexpression of a gene in so many phenotypically different cancers suggests that their overexpression may be a cause, not just a consequence of cancer.

Table 1: Genes of the human genome which have been found to be overexpressed in more than 15 cancer types. Each row represents a gene, each column on the right a given cancer. If a gene has been found to be overexpressed this is marked by an "+" in the corresponding table - position. The column most right gives the number of cancers in which overexpression has been found. The row at the bottom indicates, how many of the 31 genes are expressed in the cancer given in the corresponding column.

Gene	bone	bone marrow	brain	cartilage	cervix	colon	endocrine	eye	gastrointestinal tract	genitourinary	head and neck	kidney	liver	lung	lymph node	lymphoreticular	mammary gland	muscle	nervous	ovary	pancreas	pituitary gland	placenta	prostate	skin	stomach	synovium	testis	thymus	uterus	white blood cells	sum up OVER
GAPD (glyceraldehyde-3-phosphate dehydrogenase)			+	+	+	+		+		+	+	+	+	+	+	+	+		+	+	+		+	+	+	+		+		+		22
(Homo sapiens mRNA expressed only in placental villi, clone SMAP83.)	+			+	+	+	+	+		+		+	+	+	+	+	+	+			+		+	+	+	+		+		+		21
EEF1A1 (eukaryotic translation elongation factor 1 alpha 1)	+	+			+	+	+	+		+		+	+	+	+	+	+	+					+	+	+		+	+		+	+	21
K-ALPHA-1 (tubulin, alpha, ubiquitous)			+	+	+	+		+				+	+	+	+		+	+		+	+		+	+	+	+		+		+	+	20
PKM2 (pyruvate kinase, muscle)			+	+		+		+			+	+	+	+	+		+	+	+	+	+		+	+	+	+		+		+		20
RPL4 (ribosomal protein L4)	+		+		+	+		+				+	+	+	+	+	+	+	+		+		+	+	+	+		+		+		20
ACTG1 (actin, gamma 1)			+	+	+			+	+			+	+	+		+	+	+		+	+		+	+	+			+		+	+	19
CCT7 (chaperonin containing TCP1, subunit 7 (eta))			+			+		+				+	+	+	+	+	+	+	+	+	+		+	+	+	+		+		+		19
HSPCB (heat shock 90kDa protein 1, beta)		+	+			+		+				+	+	+	+	+	+	+		+	+		+	+	+	+		+		+		19
EIF4A1 (eukaryotic translation initiation factor 4A, isoform 1)	+		+			+		+				+	+	+	+	+	+	+		+	+		+	+	+	+		+		+		19
OK/SW-cl.56 (beta 5-tubulin)				+		+		+				+	+	+	+	+	+	+	+	+	+		+	+	+	+		+		+		19
RPL7A (ribosomal protein L7a)	+		+	+		+		+				+	+	+		+	+	+	+	+			+	+	+			+		+		18
LAMR1 (laminin receptor 1 (ribosomal protein SA, 67kDa))	+		+			+		+		+		+	+	+	+	+	+	+	+				+	+	+			+		+		18
CCT3 (chaperonin containing TCP1, subunit 3 (gamma))			+			+		+				+	+	+	+	+		+	+	+	+		+	+	+	+		+		+		18
RPS2 (ribosomal protein S2)	+		+		+	+		+				+	+	+	+		+	+		+	+		+	+	+			+		+		18
HMGA1 (high mobility group AT-hook 1)	+		+			+		+					+	+	+	+		+	+	+	+		+	+	+	+		+		+		18
ACTB (actin, beta)			+	+			+				+	+	+		+	+	+	+		+	+		+	+	+	+		+		+		18
ENO1 (enolase 1, (alpha))			+		+	+		+				+	+	+	+			+	+	+	+		+	+	+	+		+		+		18
ALDOA (aldolase A, fructose-bisphosphate)			+					+			+	+	+	+	+		+		+	+	+		+	+	+	+		+		+	+	18
RPS3 (ribosomal protein S3)	+		+			+		+				+	+	+	+		+	+	+			+	+	+	+			+		+		17
ATP5B (ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide)			+					+				+	+	+	+	+	+	+	+		+		+	+	+	+		+		+		17
RPL3 (ribosomal protein L3)	+	+	+					+				+	+	+	+	+	+	+	+				+	+	+			+		+		17
EEF1G (eukaryotic translation elongation factor 1 gamma)		+	+		+	+		+				+	+	+	+	+	+	+					+	+	+			+		+		17
NPM1 (nucleophosmin (nucleolar phosphoprotein B23, numatrin))		+	+			+		+		+		+	+		+		+	+	+				+	+	+			+		+		16
GNB2L1 (guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1)	+		+		+	+		+				+	+	+	+		+	+					+	+	+			+		+		16
FTH1 (ferritin, heavy polypeptide 1)			+		+						+	+	+	+	+		+	+		+			+	+	+			+		+	+	16
(Homo sapiens transcribed sequence with strong similarity to protein ref)			+			+		+				+	+	+	+	+	+	+		+			+	+	+			+		+		16
NONO (non-POU domain containing, octamer-binding)			+			+		+				+	+	+	+	+	+	+	+				+	+	+	+				+		16
LDHB (lactate dehydrogenase B)	+	+	+			+		+					+	+	+		+	+			+		+	+	+			+		+		16
RPLP0 (ribosomal protein, large, P0)	+		+			+		+				+	+	+	+		+	+					+	+	+			+	+	+		16
EIF3S8 (eukaryotic translation initiation factor 3, subunit 8, 110kDa)			+			+		+			+	+	+	+	+		+	+		+	+		+	+	+					+		16
	13	6	28	8	11	25	3	29	1	5	6	29	31	29	29	19	28	29	16	18	19	1	31	31	31	16	1	29	1	31	5	

Discussion

One may ask if the classification "ubiquitous" is justified when a gene is found to be overexpressed in 15 / 52, i.e. in less than 1 / 3 of all known cancers. We think that this indeed is justified for two correlated reasons. First, for a number of less prevalent or less frequently investigated cancers, so far data on overexpression are simply not yet available. With increasing data sets in literature, these cancer classes may also contribute to the list of overexpressed genes and consequently the threshold for classifying a gene as ubiquitous may be increased. Second, most of the diseases listed in table 1 are among the more common cancers. A rough estimate based on a list of all human cancer cases worldwide indicates, that the cancers in table 1 represent 70 - 80 % of all human cancer cases. Thus we have covered, in terms of patients and years lost from cancer, the majority of all cases.

With a chip carrying antibodies against the products of the genes listed in table 1, one will probably be able to distinguish between almost all cancers listed in the columns of table 1, since for each cancer another pattern should "light up". In liver cancer and in the sex specific cancers of the placenta, uterus and prostate, all 31 genes are overexpressed, while particularly for cancers of the gastrointestinal tract, pituitary gland synovium and thymus overexpression is reported only sporadically. Probably, a chip carrying all 31 proteins represented in table 1 will not be optimal, since differential expression and translation of genes overexpressed only 1.5 or 2 fold may be too difficult to detect. But even a chip containing only a subset of proteins in table 1 may be sufficiently informative.

When the chip does not carry the antibodies against the informative proteins, but the proteins themselves, binding partners of the latter can be fished from cell extracts of the corresponding tissues. These can be characterized for example by mass spectrometry and thus binding partners of these critical proteins and mechanistic information may be derived for the different classes of cancer.

In conclusion, table 1 can provide the basis for small, efficient and problem oriented protein or antibody chips containing a few tens of proteins. Certainly, thorough optimization is required, but finally a set of proteins suitable for a number of specific diagnostic tasks may be envisioned.

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Cell-free protein arrays from PCR DNA

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We have developed a cell-free 'protein *in situ* array' method (PISA) for single-step generation of protein arrays from PCR DNA through combination of *in vitro* expression and *in situ* protein immobilisation (1). In this method, individual tagged proteins are synthesised in a cell free system on a tag-capturing surfaces, such that the proteins are immobilised on a surface as they are produced. Alternatively, individuals proteins are synthesised on a ligand-coated surface, molecules binding to the ligand are captured on the surface.

PISA technology is particular useful for arraying proteins and domains which cannot be functionally produced by heterologous expression *in vivo* or for which the cloned DNA is not available. We have successfully used it to generate array elements from different proteins including single-chain antibodies, ligand-binding domains and enzymes.

We also combine PISA with our cell-free ribosome display technology (2) for studying protein-protein interactions. This approach is designed to use PISA-generated arrays to screen a ribosome display library containing **p**rotein-**r**ibosome-**m**RNA (PRM) complexes. After interactions, proteins in PISA format are known from their locations and the interacting PRM complexes will be identified through analysis of RT-PCR product from the attached mRNA. The combination of PISA and ribosome display method can provide a powerful tool for 'library against library' screening of potential interacting partners.

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Proteins at electrode surfaces: examination by Scanning Probe Microscopy

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The major problem in attempting to understand *any* electrochemical phenomenon is the nature of the electrode surface at which the reaction occurs. It is pointless trying to rationalise reactions that occur at, say, a glassy carbon electrode when the nature of that electrode surface is not understood. This seemed particularly relevant to our studies on bioelectrochemistry where the electroactive species were usually proteins and it was well known that the latter had strict requirements concerning the electrode surface, whether modified or not, for the very reaction to occur. Thus we embarked on the study of the Scanning Probe Microscopy of electrode surfaces and proteins adsorbed thereon. The advantage of this method is that one obtained detailed information, often at the atomic level, of the electrode surface and that could well be in aqueous solution: the disadvantage of the method is that one had to use reasonably 'flat' surfaces thus excluding, say, edge plane graphite electrodes which had proved valuable in bioelectrochemical investigations.

We have studied a number of redox-active proteins and enzymes, particularly at Au (111) surfaces. We have been concerned, where possible, to ensure that the biological material is still electroactive because there was the concern that, when adsorbed on a clean electrode surface, the protein would be, not that one had a detailed understanding of the meaning of denaturation in this context. We should, using metallothionein that this $zinc_7$-protein was adsorbed (see Figure 1) on to a gold surface and there appeared to be only a slight change in the dimensions of the protein when adsorbed. Such minor changes could be sufficient, of course, to lead to a loss of, for example, enzyme activity and thus, when examining the enzyme, cytochrome P450, we considered it essential to examine genetic hybrids of this enzyme having a cysteine introduced on the surface and hence able to bind to gold. Images of this protein adsorbed on gold were obtained and even single molecules were observed (figure 2). The same type of information on the copper-containing protein, azurin, was derived and, as expected, the electrochemical properties were not significantly affected. More recently, we have studied the other proteins in the electron transfer 'chain' of the P450 system and we have even found it possible to observe complexes formed between P450 and its partner protein, putidaredoxin.

The importance of this work stemmed from the involvement of the group in the genesis of a glucose sensor that employed a ferrocene as mediator in the electrochemical reaction involving glucose oxidase. We managed to model the system at a *slightly* modified basal plane graphite electrode and observed the Scanning Tunnelling Microscopy of individual molecules of glucose oxidase *under conditions where the electroenzymatic reaction was occurring*. It should be possible, in

the future, to obtain similar information for any electroenzymatic reaction: whether it will be possible to observe electron transfer of a *single* molecule of an enzyme remains to be seen.

Acknowledgements: The work referred to above depended of the efforts of my colleagues, Drs. J. J. Davis, D. Djuricic and L-L. Wong and, of course, the funding agencies, the EU, EPSRC, BBSRC and Abbott Laboratories.

Figure 1

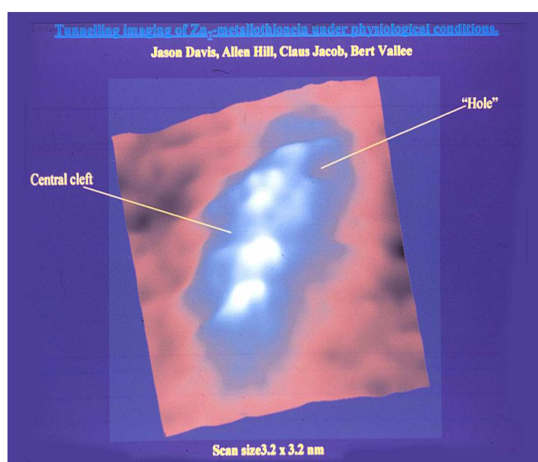
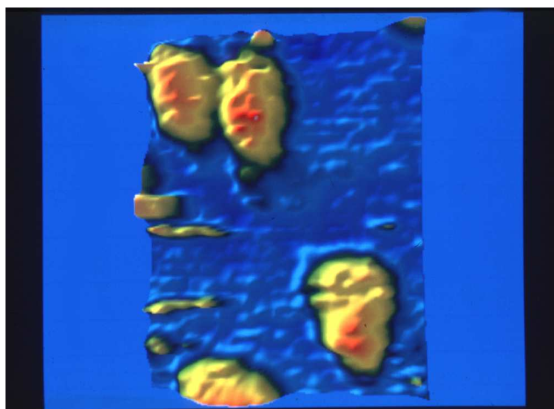


Figure 2 The STM of a cytochrome P450 in buffer



Proteins arrays and a new paradigm in lead optimization

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In the laboratory when examining reaction kinetics, the options provided for biomolecule binding are often very restricted. As a consequence, it is tempting to forget that intracellularly these biomolecules might undertake a variety of other functions and with varying degrees of specificity. For example, results from specific screening of monoclonal antibodies clearly demonstrate a continuum of above-background binders to a large diversity of potential interaction partners, particularly, if the latter are presented at similar concentrations and degrees of molecular accessibility on protein arrays. Intracellularly, the situation is further complicated as work is undertaken by populations of molecules which must attain physiological thresholds as the end-point(s) of one or more reaction pathways so as to commit the cell to a particular process or task, e.g. division or death.

Biomolecules do work by interacting either alone or as a complex via what are termed “interaction patches”. In order to better quantify the potential binding-site diversity presence in human cells, the mean interaction energy of 37 antibody-antigen binding events was accurately determined from known crystal structures and used to determine the interaction patch radius. The highly non-globular nature of antibodies means that such estimates for more general protein-protein interactions will be at the lower end of the intermolecular spectrum. Nonetheless, this information could then be exploited in conjunction with a minimalist model based upon perfect spheres as representative of populations of globular proteins. The latter was then corrected by accurate surface area calculations obtained from some 680 known SCOP domain crystal structures. Subsequent calculations were based on the binding-site diversity present in 34,881 non-denatured Human proteins, gene size; and reliable estimates of the number of protein isoforms engendered in human cells per Open Reading Frame. Very rapidly, these predictions can be extrapolated to as many as 600 trillion potential intra-cellular interactions in human beings. In the face of such complexity, biology must set-about reducing dimensionality.

Spatial and temporal dimensionality reduction is achieved by on- and off-rate affinities, reaction times, localised target and ligand concentrations, the size and number of interaction partners, the extent of reaction reversibility required for the biological task at hand, oxygen partial pressures, intra-cellular compartmentalisation and tissue differentiation. As a result of such processes, biomolecular interactions must be brought into and out of play in an ordered manner, e.g. inactive precursors, lateral

sequestration, etc. Some of the physiological consequences able to be derived form these predictions included the minimal information threshold required for MHC-peptides and the obligatory nature of polyclonality for both cellular and humoral immunity. The latter are likely to be governed by similar mathematical constraints as bioinformatic tools employed in proteomics, namely combinatory sieving.

This manner of modelling molecular biology is of paramount importance to improving target selectivity of both traditional small-molecule drugs and therapeutic biomolecules, both of which are faced with the same never-ending plethora of potential binding-sites within the human body and their associated potential deleterious side-effects. If the genomic revolution is to impact positively improved medicines and healthcare delivery, this knowledge base must now be better applied to the reduction of Adverse Drug Effects that currently account for hundreds of thousands of deaths, many millions of hospitalizations and many more patient ill-effects globally per annum.

Microarray production on polymeric hydrogels using microcontact printing

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Abstract

Microcontact printing (μCP) [1] has become an attractive and flexible technique for microarray and biochip production. We describe herein a series of experiments where μCP has been used to introduce molecular patterns on planar gold substrates as well as on supported polymeric hydrogels. Most emphasis is focussed on so called temporary hydrophobic barriers printed on carboxymethylated dextran (CMD) hydrogels using a cationic surfactant molecule tetraoctadecylammonium bromide (TOAB). The fabrication process is characterized using microscopic wetting, atomic force microscopy (AFM), imaging null ellipsometry, scanning electron microscopy (SEM) and infrared spectroscopy (IR). AFM showed that the printed barriers consisted of aggregated TOAB molecules. The distribution of the aggregates varied with size of the printed barriers most likely because of an edge transfer lithography (ETL) mechanism. The microarray spots were activated with a series of protein ligands and characterized with respect to loading capacity and homogeneity using fluorescence microscopy and surface plasmon microscopy (SPM). The introduced barriers were removed after ligand activation using either a sodium chloride salt solution pH 11 or tert-butyl alcohol to reduce non-specific binding. Finally, the potential of using the well established CMD hydrogel chemistry as a platform for protein microarrays was exploited using surface plasmon microscopy.

Microarray production

The printing process is performed by inking the elastomeric PDMS stamp with a 3 mM TOAB ($CH_3(CH_2)_{17})_4N^+Br^-$) in chloroform [2]. The PDMS stamp is carefully dried and then brought in contact with the dextran hydrogel [3], Figure 1a, for 15-25 minutes. The fastest and simplest way to illustrate

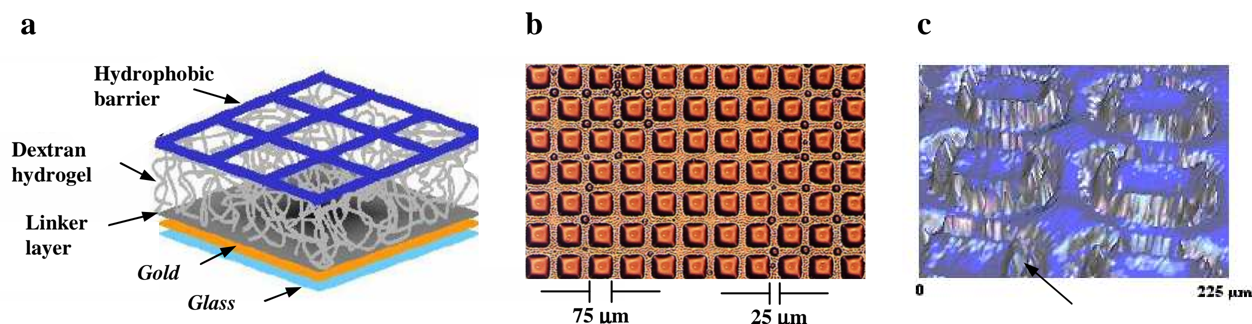

Figure 1. a) Hydrophobic barriers of TOAB on a dextran hydrogel. b) Microwetting image of a 75x75 μm^2 array. c) Null ellipsometry image showing the ETL effect of TOAB aggregates on the dextran hydrogel. The printed barriers are 75 μm wide and the TOAB aggregates are preferentially accumulated along the rim of the spots.

the outcome of the printing process is to mount the sample on a cooled stage and study the condensation pattern of water on the sample surface using an optical microscope. A typical microwetting image is seen in Figure 1b, where the spots 75x75 μm^2 are separated by 25 µm wide TOAB barriers. AFM (data not shown) reveals that the printed barriers consist of aggregates of TOAB molecules forming disk like structures of varying size, typically 100-600 nm in diameter and 20-100 nm in height. The transfer of TOAB aggregates from the PDMS stamp to the dextran surface is fairly homogeneous as long as the barriers are less than 50 µm wide. However, the transfer becomes inhomogeneous when the size of the printed barriers exceeds 50 µm. This is most likely due to so-called edge transfer lithography (ETL) a phenomenon related to the dewetting process occurring on the stamp surface during solvent evaporation [4]. The recesses of the stamp (especially the walls) are selectively filled with ink molecules, and when the stamp is brought into contact with the surface, the ink molecules are transferred primarily at the edges of the recesses, thereby resulting in an inhomogeneous barrier layer, as in Figure 1c. Despite ETL, we found that the inhomogeneous pattern of TOAB aggregates are sufficient to hold large quantities of activation and ligand solutions, most likely because of the sharp and well-defined edge structures around the spots, Figure 1c.

Ligand immobilization and characterization

The distribution of piezodispensed protein ligands (Protein A, transferrin, anti-myoglobin, BSA) within the individual spots is investigated by fluorescence and surface plasmon microcopy [5]. Coffee ring stains are often seen for dispensed protein ligands, a well-known phenomenon associated with microarray production. They appear because of the rapid evaporation rate of small droplets and a simultaneous outward flux of non-volatile ink molecules. Several strategies are tested in this work to suppress such evaporation either by adding glycerol (1%, 5%, 10% and 20%), increasing the RH up to 65%, or by increasing the RH up to 65% at the same time as the temperature is lowered to ~16 °C (the dew point). The latter approach turned out to give the most homogeneous immobilization of protein ligands, as revealed by fluorescence and surface plasmon microscopy, and this procedure was used to test and evaluate several model systems.

Array evaluation

Non-specific binding (NSB) of proteins onto surfaces is an undesirable phenomenon that is hard to control and account for in label-free biosensing. NSB on hydrophobic surfaces (e.g. TOAB barriers) can severely influence the results from a microarray experiment. It may occur during the ligand immobilization step, where protein ligands interact and accumulate non-specifically with the TOAB barriers, as well as during the ligand-analyte binding step. It may therefore be wise to develop strategies to remove the hydrophobic barriers after ligand immobilization. We have tested a few alternative regeneration solutions; e.g. sodium chloride (NaCl) at pH 11, and *tert*-butyl alcohol (TBA). Both of them were able to remove the barriers without severely influencing the activity of the immobilized ligands. TBA turned out to be the most efficient one, and Figure 2 illustrates the effect

TBA has on the read-out accuracy of an image obtained by SPM. The NSB of the protein ligand, antimyoglobin, onto the barriers undoubtedly disturb the evaluation of the immobilization level of antimyoglobin, Figure 2a. However, exposing the array to a TBA regeneration solution for 10 minutes results in a microarray with very homogeneous spots (note also the flat baseline in b). The RMS roughness of the spot is 5 nm as revealed by AFM. Thus, TBA definitely improves the read out accuracy of the microarrays and we are currently evaluating a many ligand-analyte using the above approach in combination with SPM.

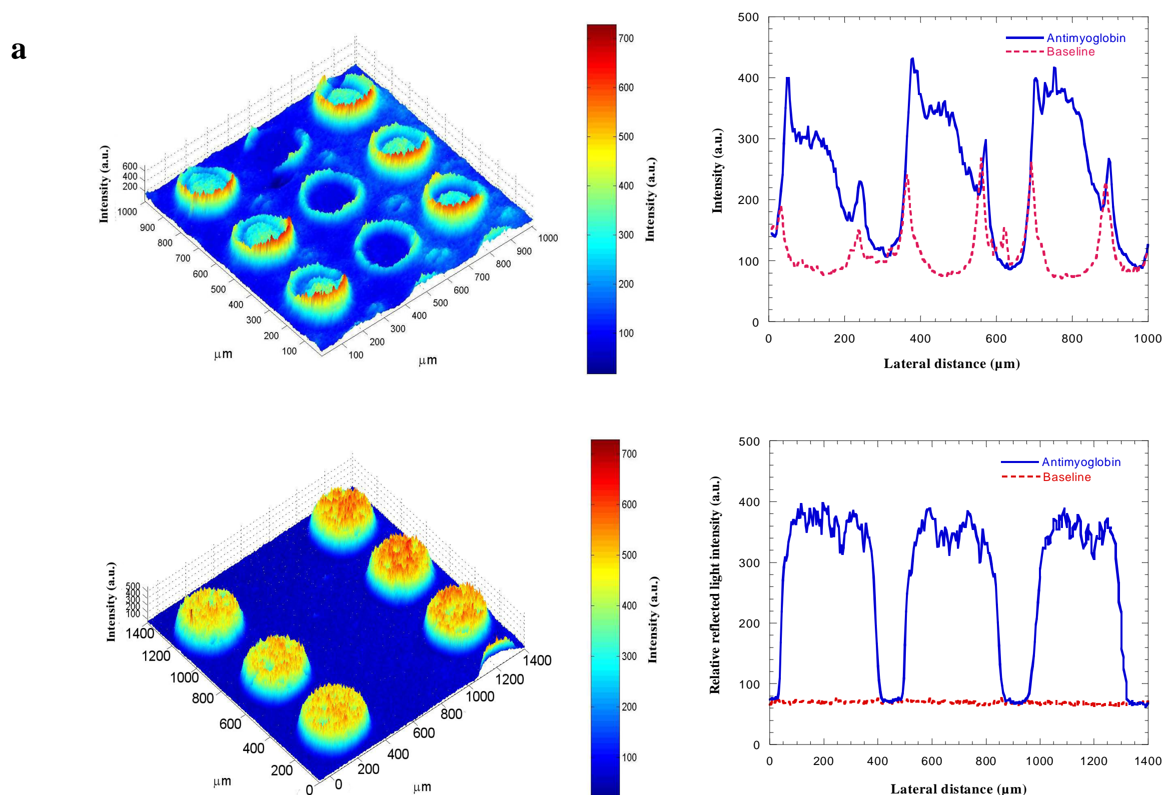

Figure 2. SPM images revealing the efficiency of the TBA regeneration solution. a) Antimyoglobin with a concentration of 0.5 mg/ml is dispensed into 200x200 μm^2 array elements defined by TOAB printed barriers. Line profile with (blue) and without (red) ligand immobilization. b) An identical sample as in a) treated with TBA solution for 10 minutes. Line profile with (blue) and without (red) ligand immobilization.

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Applications of functional proteome scale microarrays

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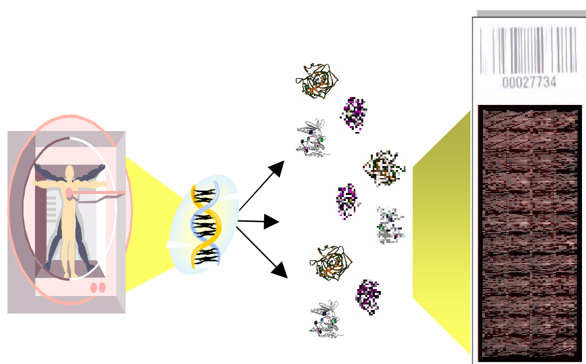
About the Company

PROTOMETRIX is pioneering the use of whole- or partial proteomes to improve the success rates of drug discovery. By rapidly converting gene sequences into arrays of functional proteins, Protometrix's technology can be applied to rapidly reveal new disease pathways, define previously unknown specificity and selectivity of potential drugs and quickly identify potential adverse effects associated with cross-reactivity.

Technology

Based upon the pioneering protein microarray research of Professor Michael Snyder of Yale University (***Science*** **293** (5573) 2101-05, 2001) Protometrix has developed an integrated, industrialized platform to rapidly clone, express, purify and array proteins from any organism. Through integrated robotics, informatics and artificial intelligence Protometrix can deliver the highest quality protein microarray products in the most cost-efficient manner.



Protometrix Technology rapidly takes genes from any organism and converts them into information-rich protein microarrays that can be used for a wide variety of interaction and biochemical studies.

Protometrix – Uniquely Harnessing the Proteome

Unprecedented Protein and Microarray Production

- Fully-automated gene-to-protein "engine" with highest quality
- Creating the largest collection of proteins, including human
- Highest success rate reported for protein functionality.

Unlimited Utility

- Easy-to-use, "universal" format
- Application protocols for:
  - Small molecules profiling
  - Protein-protein interactions
  - Biochemical (enzymatic) assays
  - Phospholipid studies
  - Pathway mapping.

Unmatched Performance

- Reveals new, important biology
- Enables rapidly, cost-effective and quantitative assessment of potential drugs.

Products, Services and Collaborations for Enhanced Discovery

- Products: sub-proteome arrays for purchase
- Custom Services: Production and/or profiling of protein arrays
- Research Collaborations:
  - Target, substrate or pathway identification
  - Sample profiling and biomarker discovery

Compound profiling and SAR

Bioconjugation Chemistry: Essential Means for Microarray Technologies

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Current developments in microarray technologies, concerning miniaturized, high throughput analyses in genome- and proteom research, biomedical diagnostics, drug screening and other applications, essentially depend on efficient chemical means for the conjugation of bioactive molecular components [1]. For instance, immobilization methods are required which allow for the functionalization of microstructured surfaces with nucleic acids, proteins, as well as small molecule analytes. This type of bioconjugation can be achieved using chemically activated substrates, prepared, for instance, by amino silylation of glass or metal oxides, and subsequent transformation with homo- and heterobifuctional crosslinking reagents. We have recently employed PAMAM dendrimers, containing a large number of primary amino groups in their outer sphere, as an intermedate layer between the bioactive component and the solid substrate [2]. This approach not only leads to an increase in signal intensity, and thus sensitivity in analytical assays, but also yields highly homogeneous biochips with an outstanding physico-chemical stability, thus enabling efficient preparation of protein and small-molecule biochips [3].

A second field of our research concerns semisynthetic bioconjugates of proteins and nucleic acids, which can be used as reagents in immunological diagnostics and microarray technologies [4,5]. As an example, a large number of different proteins can be simultaneously immobilized on a DNA microarray, using DNA-directed immobilization. This process, which extensively utilizes both covalent bioconjugation as well as non-covalent supramolecular selfassembly, allows one to prepare highly active protein arrays, useful for immunological diagnostics and proteome research [6-11]. The DNA-directed organization of proteins also enables the fabrication of nanostructured bioconjugates by self-assembly of semisynthetic DNA-protein conjugates and gold nanoparticles [12-14]. For instance, antibody-containing biofunctionalized hybrid nanoparticles are extraordinary stable and reveal the undisturbed bioactivity of the IgG molecules. Based on the gold particle-promoted silver development, such IgG-Au conjugates can be employed as signal-generating complexes in protein microarray analyses, enabling the site-specific detection of sub-femtomol amounts of antigens.

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Protein Microarray Technology

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Following the completion of the human genome sequencing project the combination of DNA microarrays with sophisticated bioinformatics allows scientists to take a global view into biological systems. In today's proteome era, the time is ready for protein microarrays for screening entire genomes for proteins that interact with particular factors, catalyse particular reactions, act as substrates for protein-modifying enzymes and/or as targets of autoimmune responses.

Besides planar microarrays, bead-based flow cytometry approaches have been developed that are very well suited for the multiplex detection of target molecules, these are especially useful when only a small number of parameters have to be determined simultaneously (see Figure 1). Bead-based assay systems employ colour-coded microspheres as the solid support for the capture molecules. A flow cytometer identifies each individual type of bead and quantifies the amount of captured target on each individual bead. Sensitivity, reliability and accuracy are similar to those observed with standard microtiter ELISA procedures while only a fraction of sample is required.

We have established different bead-based assays e.g. to analyse members of the epidermal growth factor receptor (EGFR)-family that play an important role in diagnosis and treatment of cancer. Using phosphospecific detection antibodies it is possible to quantify the amount of phophorylated receptors. Other molecules of interest involved in cancer progression are matrix metalloproteinases (MMPs). The MMPs are capable of degrading extracellular matrix components and they play a key role in cell migration of normal and malignant cells (e.g. involved in rheumatoid arthritis and metastasis of cancer cells). A miniaturised multiplexed sandwich immunoassay was developed, which allows the quantification of different MMPs from a few microliters of sample. The current assays exhibit limits of detection (LODs) in the range of 2-100 pg/ml for the different MMPs.

However, the basic requirement for multiplexed sandwich immunoassays is the availability of capture and detection antibodies exhibiting minimal crossreactivity. High numbers of binders can be enriched by classical ways (immunisation and hybridoma cell lines) and recombinant library technologies (phage display, ribosomal display and mRNA display). Currently, not the selection process, but the characterisation of the obtained binding molecules represents the bottleneck in the production process. To determine the properties of identified binders, array based methods have been developed. While the use of microarrays for analysing crossreactivity and for epitope mapping is evident, affinity measurements employing these miniaturised assay systems have not been described yet. The determination of the parameters that specify the antibody-antigen interaction (i.e. association and dissociation constants) is generally performed with kinetic measurements. While established methods e.g. surface plasmon resonance, give reliable results with a reasonable consumption of sample, their speed is a limiting factor for screening purposes. We developed an array based system for estimating the kinetic properties of a set of given recombinant antibodies. An apparent affinity value for the binders can be obtained and the binders can be ranked using a relatively simple experimental set-up. These values correlate with the corresponding K_D-values determined by surface plasmon resonance measurements. In addition, to allow a linear epitope mapping, arrays of overlapping peptides were analysed. When utilising N- and C-terminal shortened peptides on the array, the putative epitope can be further narrowed down. These first approaches look very promising in terms of a high-throughput characterisation of binders.

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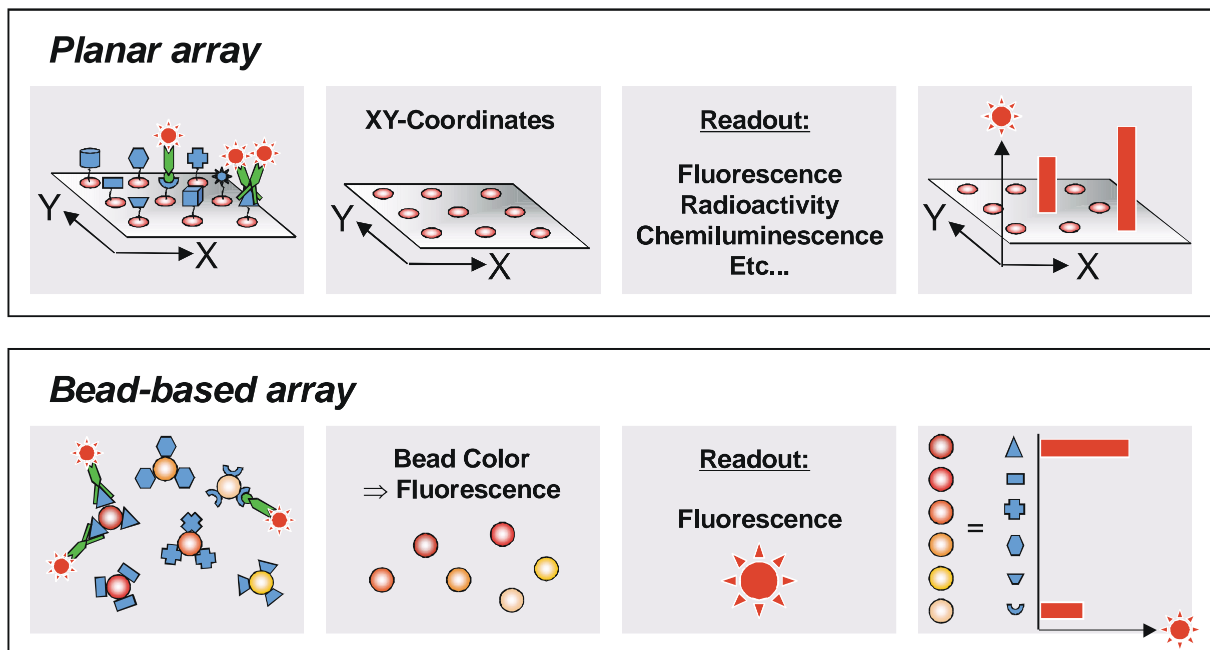

Figure 1: Types of protein microarrays. Planar microarrays and bead-based arrays represent two different microarray technologies that can be employed for multiplexed ligand-binding assays. Planar microarrays can be generated with hundreds and thousands of different capture spots. Multiplexing in bead-based arrays, however, is limited to the number of distinguishable beads. The separation of the beads is performed via colour-coding (e.g. Luminex-technology). The detection on planar arrays is performed using different detection principles, like chemiluminescence, radioactivity, mass spectrometry or fluorescence. The main readout for bound analytes in bead-based microarray assays to date is fluorescence.

Analysing protein microarrays by utilizing intrinsic time resolved UV fluorescence

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Label –free detection in protein microarray technology presents a challenge, which has yet been met only by a few methods, such as imaging ellipsometry, surface plasmon resonance, mass spectrometry and nanomechanical techniques. These methods may be favourably complemented by a new analytical approach based on lifetime measurements of the intrinsic fluorescence of proteins.
Generally, detection of biomolecules by fluorescence analysis belongs to the most sensitive methods available. For this reason, analysing protein- protein interactions on protein microarrays and their evaluation by using fluorescent labels has become a widely used method. However, to avoid chemical alteration of the biomolecules involved, one has to take advantage of the direct UV excitation of intrinsic aromatic amino acids, foremost tryptophan and tyrosine. Since the lifetime of excited electronic states of molecules is sensitive to environmental parameters, the protein-protein interaction will lead to changes in the fluorescence decay pattern. This may be explained, for example, by a structural change of the Tryptophan and Tyrosin environment, or by quenching due to Förster resonance energy transfer. It is difficult to determine precisely which mechanism is dominant for each individual pair of proteins, nevertheless the changes that we observe upon binding are sufficiently strong, sometimes as large as 30 % of the total fluorescence decay time.
In the experiment, the coupling of a specific biomolecule to an immobilized binding partner can be detected by comparing the fluorescence decay times of the immobilized partner prior and after incubation. This opens the possibility to detect proteins and their natural interactions without perturbation due to labelling.

The experimental set-up is depicted in Fig. 1. It consists of a self mode- locked frequency –tripled titanium –sapphire laser as excitation source for the aromatic amino acids around 280nm. The pulse picker reduces the repetition rate of the laser to ensure a complete decay of the fluorescence excited by the preceeding pulses. The fluorescence decay time traces are collected by means of time-correlated single photon counting. Alternatively, a streak camera can be used within the same set-up.

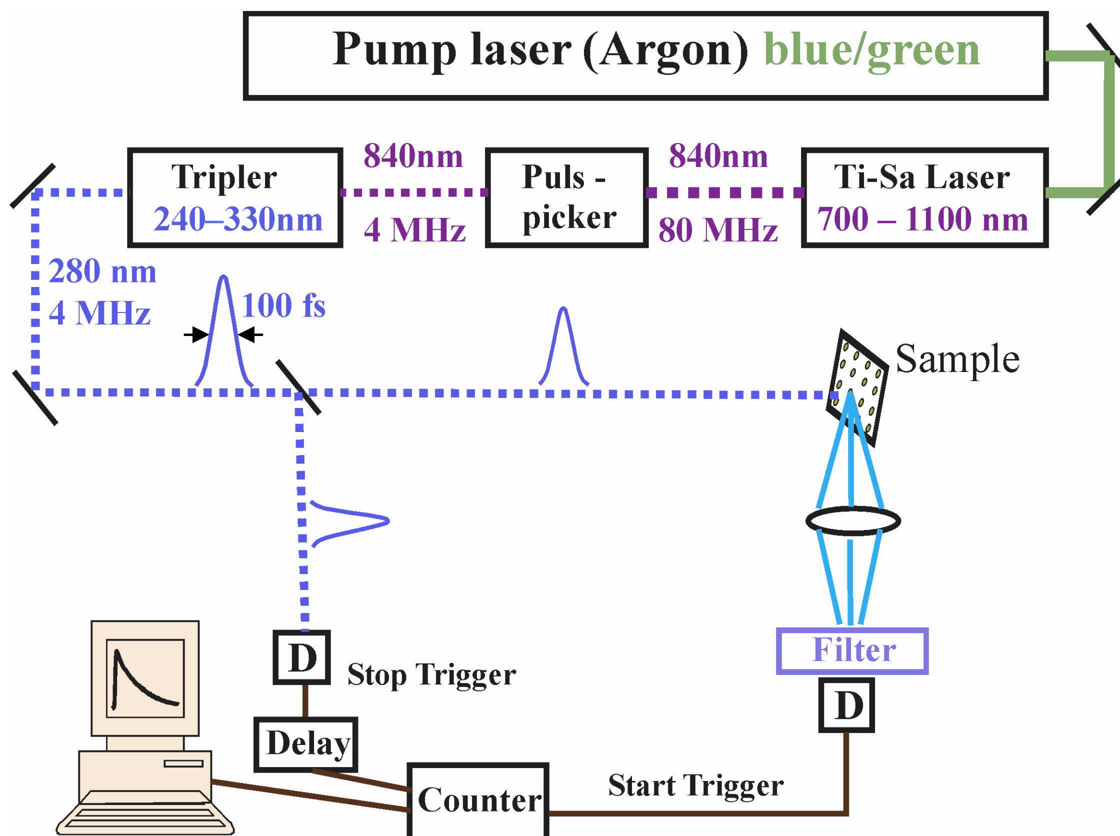

Figure 1: Experimental set-up

Due to the excitation in the UV range of the spectrum, special attention has to be given to sample preparation, for example for the prevention of background radiation, and to choose for proper coupling chemistry. These issues are addressed in a separate talk at this meeting.

We were able to establish the feasibility of label –free fluorescence lifetime analysis of protein microarrays for multiple binding partners including antibody / antigen pairs, specific protein pairs and protein / oligonicleotid-aptamer systems. For illustration, the change of the fluorescence lifetime as a result of binding between tubulin and kinesin is presented in Fig. 2:

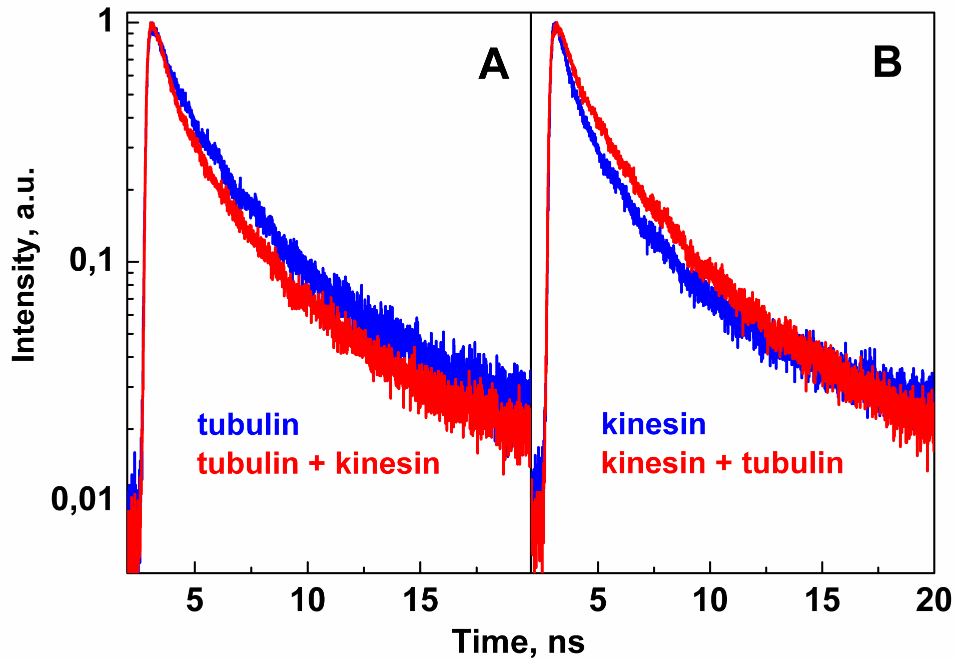

Figure 2: Fluorescence decay pattern, tubulin / kinesin pair

In this case, tubulin shows a longer fluorescence decay compared to kinesin. Accordingly, the fluorescence lifetime increases upon binding of tubulin to immobilized microtubule, and it decreases upon binding of microtubule to immobilized kinesin. In this example, the fluorescence of the bound system is determined by the difference in decay times of the individual proteins involved, and is not primarily a result of energy transfer. Incubation with proteins that are not supposed to bind to the immobilized microtubules or kinesin do not alter the fluorescence decay time, establishing the specificity of the method.

This work was supported by the BMBF (German Research Ministry), grant No. 13N8028, “SCREEN”.

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Cell Lysate Arrays – Protein Profiling in Cellular Systems

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Mapping of signaling pathways while studying biological systems is one of the key areas of interest in genomic and proteomic research. In genomic research, the parallel analysis of differential expression levels has driven DNA microarrays to become a routine method to find and validate relevant markers for specific biological effects. Novel technologies for hypothesis-driven studies in proteomic research, such as protein microarrays, now allow to quantify subtle changes of protein expression or protein activation (e.g. phosphorylation) in cellular systems, thus complementing the existing conventional analytical techniques (e.g. Western blotting) which are facing the limitations of throughput and economy-of-scale. In addition, the high sensitivity of microarrays will become of utmost importance when the amounts of sample material are limiting.

Zeptosens has developed the ZeptoMARK™ CeLyA Protein Profiling System, comprising the ZeptoREADER™ microarray analyzer, ZeptoMARK™ cell lysate arrays and assays, and ZeptoVIEW™ Pro microarray analysis software.

The ZeptoREADER™ is a highest sensitivity microarray reader, using a planar waveguide (PWG) based fluorescence imaging system for DNA and protein microarray applications. The major advantage of the PWG detection principle consists in the selective excitation of surface-bound fluorescent analytes in the evanescent field on the chip surface, whereas fluorescent molecules in solution do not contribute to the fluorescence signal. The resulting low background combined with the high excitation light field strength at the chip surface results in an increase of sensitivity by a factor 50-100 compared to today's state of the art microarray readout systems. The ZeptoREADER™ provides the ability to quantify e.g. biomarkers at low concentrations on micro-spots without signal amplification and allows the precise detection of small differences in expression and activation levels from limited amounts of e.g. differently treated samples.

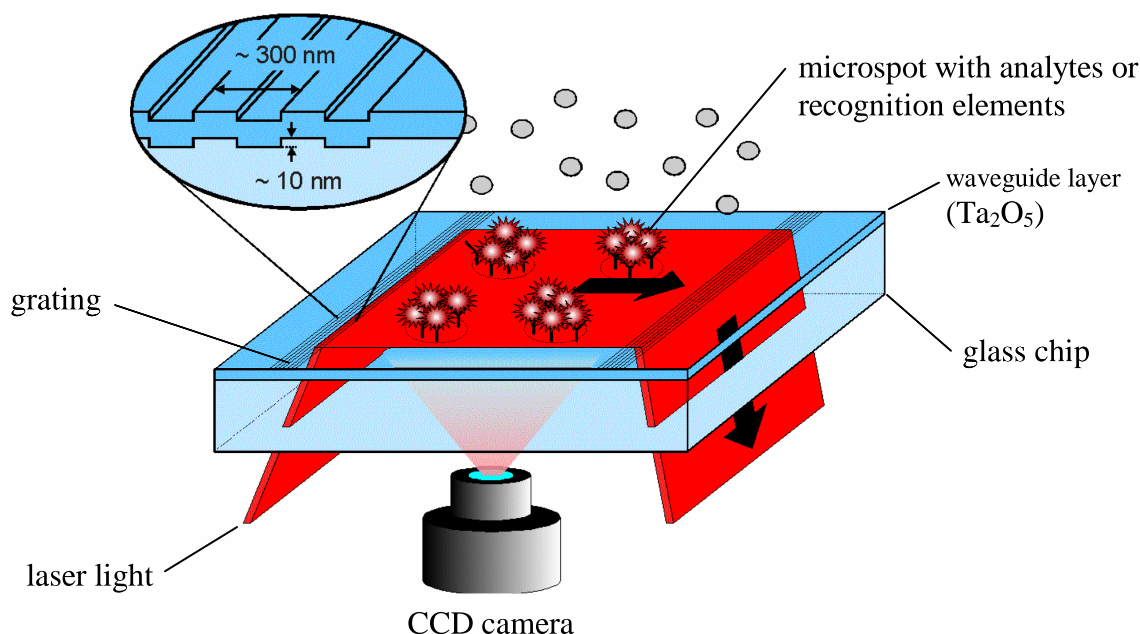

Figure 1: Schematic illustration of the planar waveguide detection principle. A laser beam of linear shape is coupled into the waveguide by means of a diffractive grating. The combination of a high field strength at the chip surface and the selective excitation of surface-bound fluorescent analytes leads to the high sensitivity of the system.

Cell lysate arrays (so-called reverse phase arrays) represent a new technology in the field of proteomics, which allows the investigation of protein analyte sets out of crude proteomic samples with low amounts of starting material. Interesting approaches include the comparative analysis of signaling pathway marker protein expression and/or activation (e.g. phosphorylation) in normal versus diseased tissue (e.g. for cancer) to identify abnormally expressed proteins or pathological alterations in signaling pathways, biomarker discovery or profiling of drug candidates in early drug discovery. In contrast to sandwich-type protein microarrays with immobilized capture molecules (e.g. antibodies), directed to the analytes of interest, in cell lysate arrays the whole proteome of a high number of cell or tissue samples is immobilized on the chip surface. These samples are then screened for the presence of defined target proteins or their activated states.

ZeptoMARK™ CeLyA is a complete microarray solution tailored to multi-parallel protein profiling from small amounts of cell or tissue samples, featuring high sensitivity (600 protein molecules/spot detectable), high throughput (up to 264 sample spots/array, up to 360 arrays/run) and highly economic use of reagents. The reverse phase array format involves the spotting of crude cell extracts onto the chip surface and the use of highly specific antibodies to detect the target proteins or protein

modifications of interest. Cell lysate arrays are preferentially applied when larger numbers of samples than numbers of protein targets are to be analyzed.

Examples of ZeptoMARK™ CeLyA applications in drug profiling and pathway mapping of different cell lines will be presented. The high array-to-array and chip-to-chip signal reproducibility allows the detection of 10-20% changes in protein expression and activation levels. Assay results of the lysate arrays show a good analytical correlation with conventional Western blotting data. Compared with Western blotting, a benefit factor of more than 100 in saving time, labor and reagent volumes can be achieved.

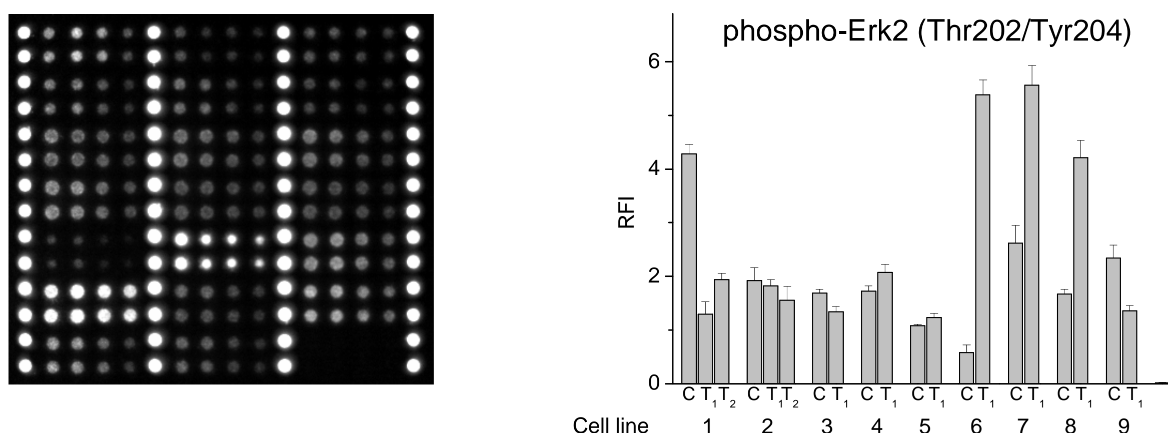

Figure 2: Example of a typical array image and signal bar plot for the profiling of the relative phospho-Erk2 concentrations in 9 different cell lines upon treatments (C = Control, T_1 = Treatment 1, T_2 = Treatment 2). Bars indicate normalized mean fluorescence signals with corresponding standard deviations, obtained from 8 array spots per lysate sample at different dilutions. RFI values correspond to the relative phospho-Erk2 concentrations in the different samples.

Time-resolved fluorescent labels in protein detection

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Addition of temporal resolution to spectrally and spatially resolved fluorometric measurement of biological samples improves both the sensitivity and quality of data, and enables also collection of more information in analysis. By temporal resolution the desired signal can be efficiently distinguished from the autofluorescence and other background noise [1]. Temporal resolution can also be exploited in a multilabel assay where each signal can be defined both by spectral and by temporal parameters [2].

The use of microsecond time-resolution and pulsed excitation in fluorometric measurement of bioanalytical assays dates back to late 1970's and the development of dissociation enhanced lanthanide fluoroimmunoassay technology [3, 4]. It was recognized that the time-resolved fluorometric measurement and the use of fluorescent rare-earth chelates with millisecond lifetime would allow enhanced sensitivity by development of both instrumentation and chemical methods. The lanthanide ions (e.g. samarium, europium, terbium dysprosium, erbium, ytterbium and neodymium) itself are only very weakly fluorescent as their molar absorptivity is very low, but the situation changes completely when these ions are chelated to appropriate organic ligands. Although the emission originates from the lanthanide ion, the organic ligand plays an important role. The long-lifetime fluorescence of lanthanide chelates is though to develop through separate excitation, energy transfer and emission phases [5]:

1. The organic ligand absorbs energy and is excited.

2. Excitation energy is transferred in the ligand from the singlet-excited to the tripled excited state (intersystem crossing).
3. Energy is transferred from the triplet state of the ligand to an appropriate resonance level of the lanthanide ion through intramolecular energy transfer.
4. The lanthanide ion releases energy as ion fluorescence.

Each of the lanthanide ions and their chelates is characterized by an emission spectrum typical of the metal ion. The lifetime and intensity of the emission are dependent on the chemical environment and thus on the properties of the ligand.

In pulsed-light time-resolved fluorometer the lanthanide chelates are excited with a xenon flash lamp with flash time typically 1 to 10 μs [6] or with nitrogen laser with sub-microsecond pulse width [7]. Emission is measured with a photomultiplier tube by photon counting using narrow band-pass emission filter. Photon counting is initiated by electronic gating for short period of time after a certain

delay from excitation. The flash frequency, the delay time and the counting time can be varied, and normally the total measurement time is 1 s comprising 1000 individual cycles with 400 μs delay and 400 μs microsecond counting time for europium chelates.

Development of fluorescent lanthanide chelate suitable for bioassays had several requirements: strong kinetic complexation with high thermodynamic stability and kinetic inertness; hydrophilicity; high molar absorptivity, high quantum yield; reactive group for coupling to biomolecules; and high photochemical stability. It was difficult to find suitable chelate, and initially the requirements were solved by using two different chelates. In Delfia technique, which was introduced in early 1980's [8, 9], a non-luminescent lanthanide chelate is used during an actual bioaffinity assay and the fluorescence is generated by addition of enhancement solution, which dissociates the lanthanide ion to be chelated with another ligand to form a highly fluorescent complex. In this technology, however, the spatial information is lost, i.e. it does not allow site-specific measurement. Thereafter, the development of intrinsically fluorescent stable lanthanide chelates has continued, and major advances have been achieved [10]. The new 9-dentate europium chelates (Fig. 1a) are thermodynamically very stable and they have high molar absorptivity and quantum yield [11].

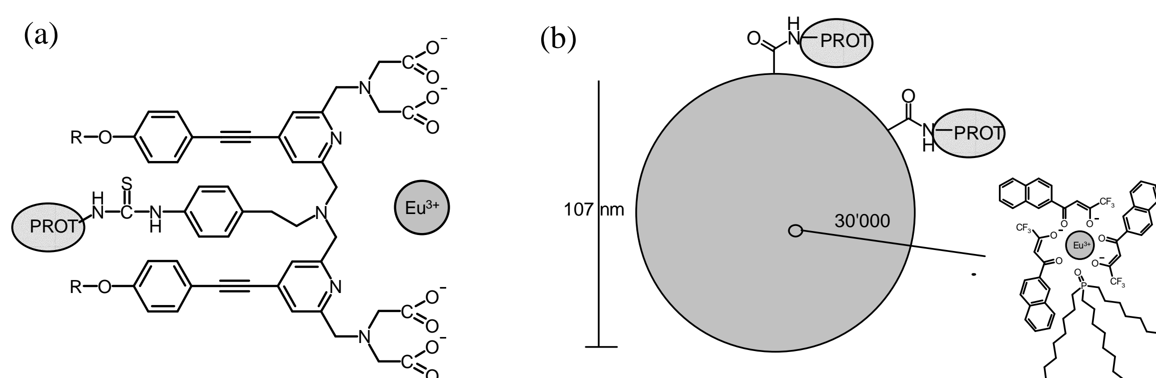

Figure 1. (*a*) Structure of intrinsically fluorescent 9-dentate europium-chelate coupled to protein using isothiocyanate reactive group [11]. (*b*) Nanoparticle dyed with tens of thousands of europium-chelates and coated covalently with multiple proteins [12].

The availability of an intrinsically fluorescent, inert, and stable europium chelate made it feasible to design new immunoassay methods based on time-resolved fluorometry for detection. One-step all-in-one dry reagent immunoassay [13, 14] is carried out in a microtitration well format and the concept is extremely simple to apply because only addition of sample and an assay buffer is needed. In the advanced version of the concept based on a moisture insensitive chelate the assay response can be measured directly after wash step from the well surface.

An alternative approach has also been studied to produce an extremely fluorescent lanthanide chelate label. A highly fluorescent but non-stable europium chelate complex, the same employed in the Delfia enhancement solution, has been impregnated to polystyrene nanoparticles and the obtained particles have been coated with proteins to produce a highly fluorescent bioconjugates (Fig. 1b). An individual nanoparticle can be dyed with tens of thousands of fluorescent lanthanide chelates, the dyed nanoparticles are monodisperse and no chelate leaks outside. The fluorescence of these dyed nanoparticles is so high, that even individual nanoparticles can be observed using a time-resolved fluorescence microscope on a solid surface and less than a zeptomole of these particles can be detected in a microtitration well using a rapid read-out.

The binding characteristics of nanoparticles coated with proteins, e.g. nanoparticle–antibody bioconjugates are substantially affected by the number of antibodies conjugated onto a single nanoparticle [15]. In reaction with solid-phase bound protein molecules, the second-order association rate and monovalent binding affinity constants of the bioconjugates have been shown to improve with the density of active binding sites on the surface, and eventually to exceed the intrinsic values of a conventionally labeled antibody. The immunoassay technology based on a nanoparticle label enables clinical studies at analyte concentrations not detectable with current immunoassays [16], and can challenge nucleic acid amplification-based technologies in the detection of pathogens such as viruses or bacteria, where the limit of detection is of vital importance.

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Supports and probe immobilization for protein microarray analysis by time resolved UV fluorescence

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UV-fluorescence life time measurement offers a new way for detection of proteins and protein-protein interactions. This technique allows label free detection and is solely based on the fluorescence of tryptophan and tyrosine, two amino acids occurring in 99% of all proteins.

Application of UV-fluorescence based measurements to protein microarrays requires consideration of a couple of basic rules with regard to protein immobilization and ligand binding. These practical requirements can be summarized in three categories:

a. substrates and substrate coating
b. immobilization chemistry
c. blocking and incubation techniques

Three classes of protein-protein interaction pairs were investigated for detection by UV-fluorescence lifetime technique: antibodies and their antigens, proteases and protease substrates, and singular protein pairs like the tubulin/kinesin system.

The use of UV radiation as an analytic tool excludes all kinds of aromatic or conjugated compounds in the detection system except those of the proteins themselves. Therefore, practically all plastic supports need to be replaced by UV transmittent materials. Suited best as support material is fused silica quartz (Litosil), or gold coated glass supports, but even some glass species may be used without yielding too much background. Common modification chemistries of quartz and glass surfaces include amination with aminosilane, or epoxydation with GOPS (3-glycido-oxypropyltrimethoxysilane). Generally, proteins should be kept in some distance to substrate surfaces in order to retain their native structures and to avoid quenching during measurement due to energy transfer between proteins and the support. This may be achieved best by either coating supports by a layer of biopolymers (i. e. oxidized agarose or other hydrogels), or by interposing homo- or heterobifunctional spacers between support and protein.

There exists a range of practically advantageous chemistries for native protein immobilization. These include EDC (N-[3-Dimethylaminopropyl]-N´-ethylcarbodiimide) mediated coupling of carboxyl functions to amino functions, NHS (N-hydroxysuccinimide) ester based crosslinking of aminofunctions, or epoxy based immobilization via ketene intermediates.

Protein immobilization by one of the described chemistries will leave unreacted areas of binding functions between protein spots, which have to be deactivated before incubation of the microarrays

with other proteins. As protein containing blocking solutions like casein or BSA (bovine serum albumine) may not be used in this detection method, alternative agents need to be considered. These include amines like Tris or ethanolamine for NHS-functionalized surfaces, or detergents like Tween 20 or CHAPS for general blocking purposes. Final incubation steps may be performed best in low ion strength phosphate buffers.

Determination of binding events by UV-fluorescence lifetime measurement requires two readings at each spot, one before and one after incubation with the ligand protein. At each reading, a fluorescence lifetime curve is taken (Fig. 1). Comparison of decay slopes of the normalized curves will show cases, where differences prior and after incubation occur. A final interpretation will assign presence or absence of a binding event to each spot. This will generate a pattern of binding/nonbinding representations on each protein microarray with regard to the particular protein that has to be analysed (Fig. 2).

Future applications will be focussed on more complex protein mixtures and cell extracts by this promising protein analysis method.

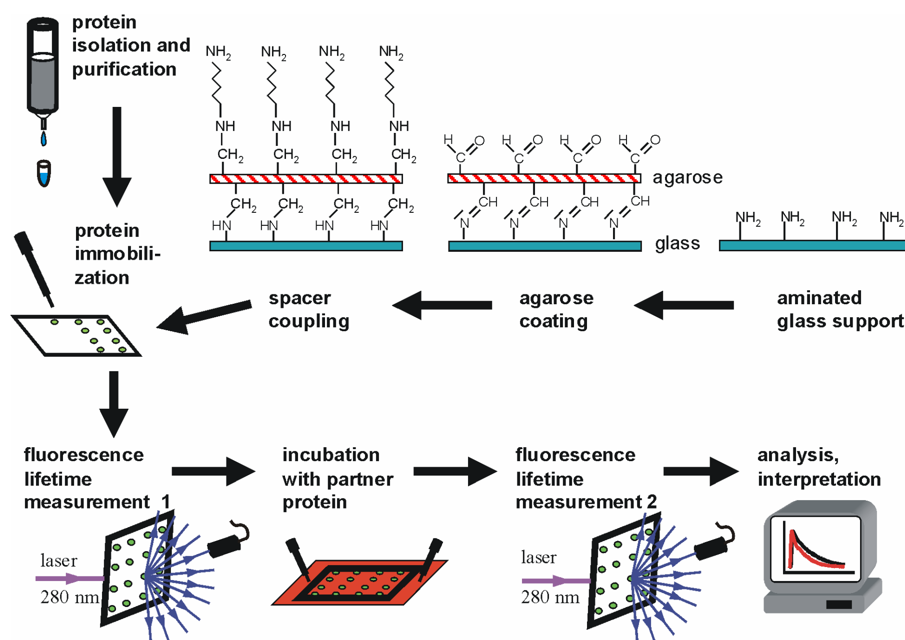

Figure 1: Principle of analysis with UV-fluorescence lifetime measurement

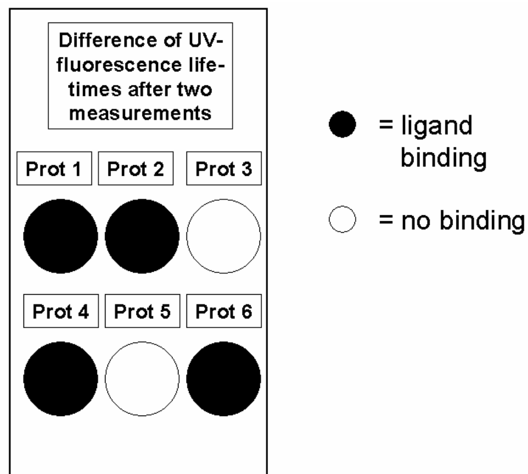

Figure 2: Final interpretation scheme of protein-protein interaction analysis

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Imaging Ellipsometry –
Marker-free Observation of Binding Kinetics and Quality Control

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In an imaging ellipsometer a polarized beam is reflected from the surface under investigation and passes an analyzer and an optical lens which makes an image of the illuminated surface on a CCD camera [1]. Each pixel of the camera is an indivudual detector of the ellipsometer. While rotating polarizer at fixed analyzer angle, Delta is simultaneously obtained for all pixels. In this way a thickness maps, e.g. Fig.1 is recorded within 30 seconds.

With imaging ellipsometry we prove binding reactions and we characterize quality parameters (shape, mass/thickness distribution, cleanness, homogeneity) of substrate and layers on biochips. Nanofilm's ArrayInspector needs 2 minutes for a scan with 8µm resolution of 10,000 DNA-spots on an ordinary glass slide. We worked with biochips consisting of glass or gold substrates coated with an activation layer on which the first binding partner, e.g. DNA or a Protein, is printed. In our experiments reaction kinetics (increasing thickness) of a second partner which is binding on the first (Fig.2) is recorded. Alternatively mass/thickness difference before and after binding is recorded (Fig.1). Test of binding can be done at each binding partner simultaneously. This enables principally high throughput screening (HTS) of up to 1000 reactions at once e.g. for pharmaceutical research.

Reactions are usually indicated by fluorescence from colored labels on the binding partners. By contrast imaging ellipsometry has some advantages, i.e. it is label free, cheap, and fast. We prove the reliability and comparability of those methodes while we obtained the same amount of binding mass of oligonucleotides with ellipsometry and fluorescence intensity measurements on the same samples.
In particular proteins can change their chemical functionality when they become labeled. Moreover very often protein concentrations are lower than e.g. DNA concentrations and therefore more difficult to detect. To solve those problems we use imaging ellipsometry on surface plasmon resonance (SPR) chips. We define ellipsometric sensitivities of mass/thickness and of refractive index noise of the solution of the binding partner. We compare the sensitivities of ellipsometry in air and in liquids on solid substrates, and on SPR-chips. We demonstrate that in SPR Delta has theoretically unlimited sensitivity where Psi and the in classical SPR measured reflection coefficient of p-polarization have constant sensitivity. In binding of avidin on biotin (Fig.1, Fig.2) 0.3 pm/mm^2 mass sensitivity (resolution) has been obtained where noise limited sensitivity has been 3 pm/mm^2 limited due to $6*10^{-6}$ refractive index fluctuations [3].
An electro-optically tunable multilayer substrate in shape of a micro resonator is under development to optimize sensitivity of SPR-chips. SPR and optical resonance couple in this chip. SPR is tuned while the optical resonator is tuned. Further advantages of this chip are: 2D-arrays of independently tunable

sensor fields on chip, angle of incidence is variable and suitable for imaging ellipsometry, and no rotating polarizers needed.

Tunable SPR-chips have high sensitivity but small dynamic range. By contrast high dynamic range at moderate sensitivity is obtained with ordinary ellipsometry on multilayer glass slides with linear response. Those slides are applied for simultaneous measurement of reaction channels with concentrations varying over several orders of magnitude. Reduction of noise by background substraction is demonstrated.

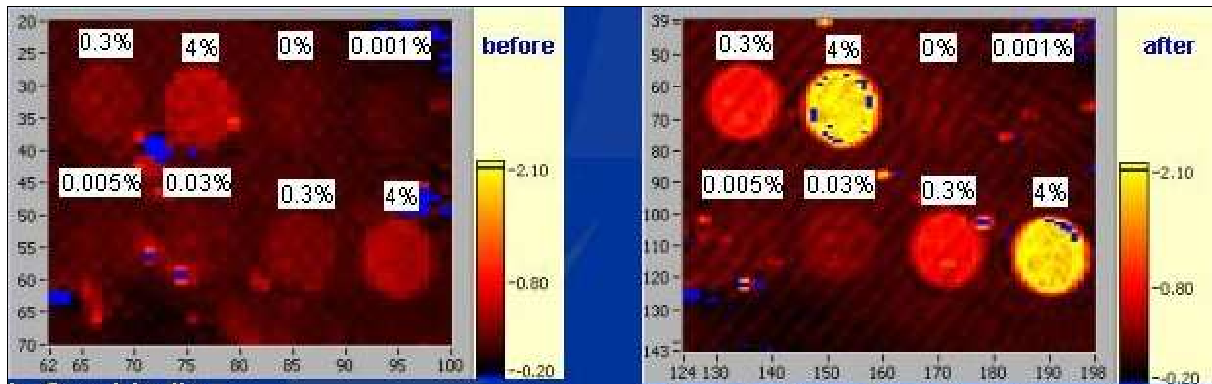

Fig. 1: Thickness maps [nm] of Microarray with different Protein concentrations on Gold SPR chip, field of view 5mm x 3mm, recorded before and after binding reaction with an imaging ellipsometer from Nanofilm.

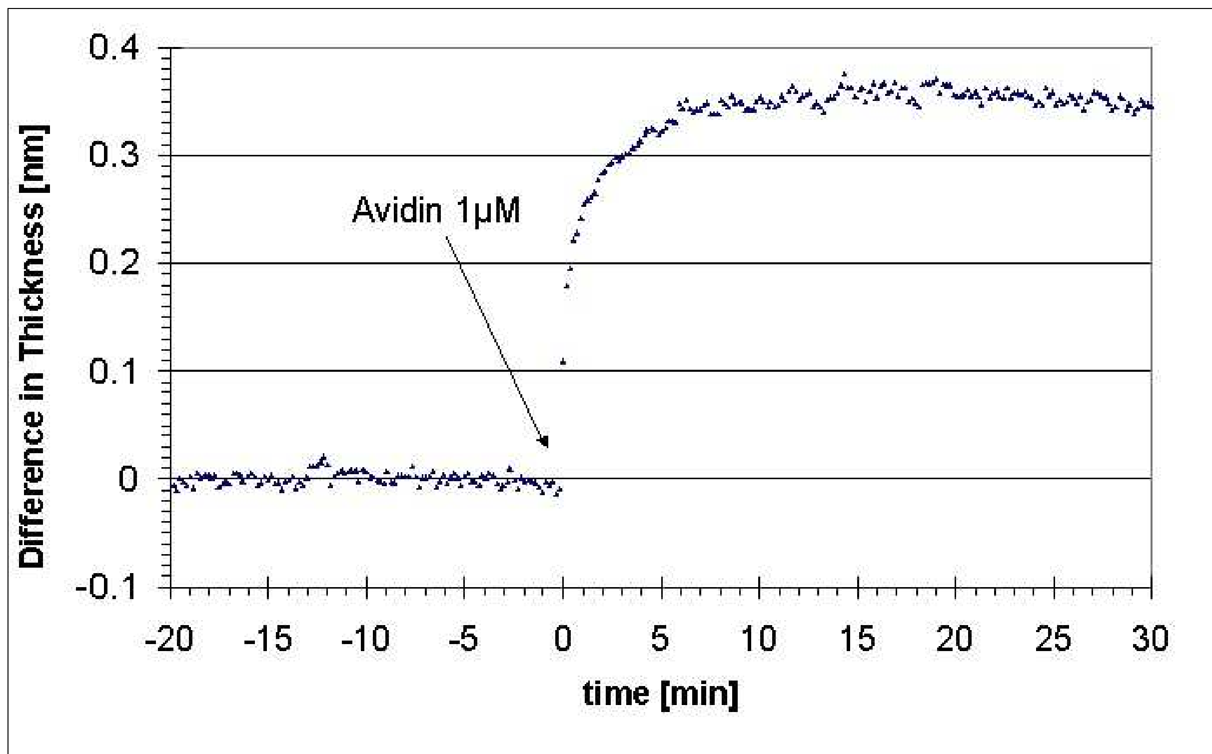

Fig.2: Binding of avidin to immobilized biotin

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Biomarker discovery with ProteinChip technology

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Head and neck cancer is a frequent malignancy with a complex, and up to now not clear etiology. Therefore, despite of improvements in diagnosis and therapy, the survival rate with head and neck squamous-cell carcinomas (HNSCC) is poor. For a better understanding of the molecular mechanisms behind the process of tumorigenesis and tumor progression we have analyzed changes of protein expression between microdissected normal and tumor tissue by ProteinChip® technology.

For this cryostat sections from head and neck tumors (n = 57) and adjacent mucosa (n = 44) were laser-microdissected (Fig. 1) and analyzed on ProteinChip arrays (Fig. 2). The derived mass spectrometry profiles (n = 47/41) exhibited numerous statistical differences. One peak significantly higher expressed in the tumor (p = 0.000029) was isolated by two-dimensional gel electrophoresis and identified as annexin V by in-gel proteolytic digestion, peptide mapping and Tandem MS/MS analysis. The relevance of this single marker protein was further evaluated by immunohistochemistry.

In this study we could show that biomarker in head and neck cancer can be found, identified and assessed by combination of ProteinChip technology, 2DE, and immunohistochemistry. Such studies, however, make only sense if a relatively pure microdissected tumor tissue is used. Only therewith minute changes of protein expression between normal and tumor tissue can be detected and it will become possible to educe tumor associated protein pattern which might be used as a marker for tumorigenesis and progression.

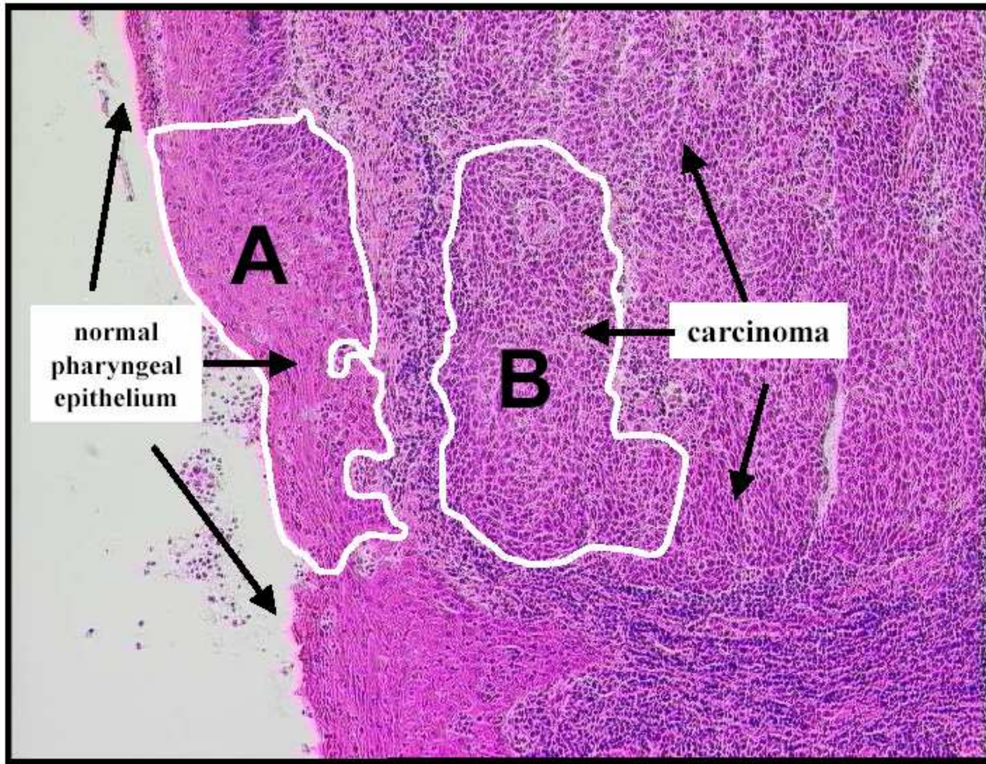

Figure 1: (LMPC; Palm System) Histological cancer section; the white line shows the area to be excised by Laser microdissection and pressure catapulting (LMPC; Palm)

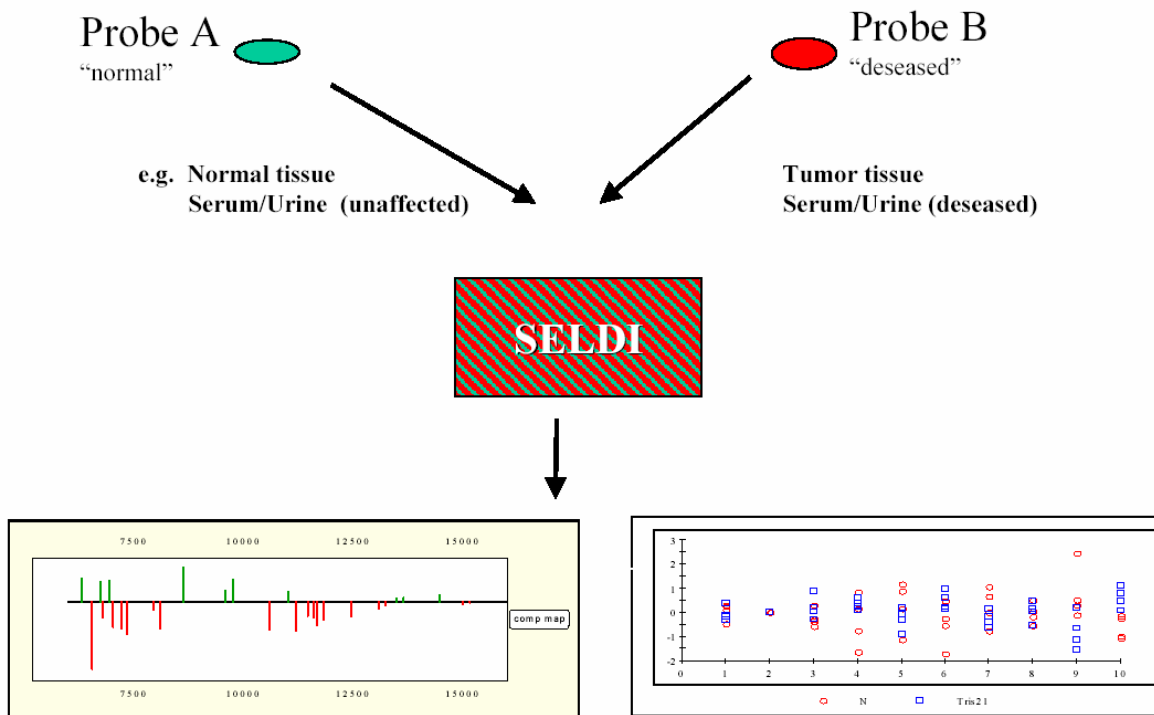

Figure 2: Searching for differentially expressed proteins by comparing microdissected normal und tumor tissue with ProteinChip technology (SELDI)

Gene Expression in Abdominal Fat: A Role for Protein Chips?

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Abstract

Obesity is a disease defined as a high body mass index (BMI 30 kg/m^2)(1). Other measurements of obesity refer to the regional distribution of excess fat in the body. Abdominal obesity can be measured as the waist circumference or the ratio between the waist and hip circumferences (Waist:Hip Ratio, WHR). Obesity causes hypertension, type 2 diabetes mellitus, and cardiovascular disease, and increases all-cause mortality. In foreseen future, obesity might supplant smoking as the leading cause of preventable deaths (2).

The health risk from abdominal obesity may mainly be due to intra-abdominal fat. This region of fat can be measured quantitatively e.g., by analyzing a slice of the abdomen from a CT scanning. A Danish study evaluated the relation between four different measurements of obesity and eight metabolic risk factors that are included in the definition of the metabolic syndrome (3,4). The study measured BMI, WHR, total fat percentage based on a dual energy x-ray absorptiometry (DEXA) scanning, and intra-abdominal fat (4). Intra-abdominal fat was the single obesity variable associated with the metabolic risk factors. In multiple regression analyses, intra-abdominal fat was significantly associated with six metabolic risk factors, whereas BMI, WHR, and fat percentage were not significant. The six metabolic risk factors were systolic and diastolic blood pressure, fasting concentrations of serum triglyceride, serum high-density lipoprotein (HDL) cholesterol, and capillary whole blood glucose, and plasma plasminogen activator inhibitor type 1 activity (PAI-1). In Intra-abdominal fat may explain 10% to 25% of the variation in the metabolic risk factors. The differences in abdominal fat, systolic blood pressure and serum HDL cholesterol have biological importance. Combined the three risk factors increase the risk of cardiovascular disease, and they are included as decision factors in the European recommendations for treatment of hypertension.

Intra-abdominal fat may induce such biological effects because it has a more extensive secretion of a series of adipocytokines or cytokines, like tumor necrosis factor alpha, and interleukin 6, into the blood system than subcutaneous fat (5). Linear relations between intra-abdominal fat and metabolic risk factors indicate that such biological mediators can cause the associations. The two fat

measurements may differ concerning their prediction of morbidity and all-cause mortality because intra-abdominal fat and overall fat differ in expression of a series of biological analytes.

New study

In our new study, we include six lean men (BMI<25) and six men with extreme obesity (BMI 40), and examine gene expression on mRNA level using microarrays, and on protein level using 2-dimensional proteomics. We will characterize differentially expressed genes by use of RT PCR and mass spectrometry. These genes may be among the 100+ candidate genes linked to the development of obesity, abdominal obesity, and type 2 diabetes mellitus, or other biologically relevant genes. We will use existing software for simultaneously analysing the expressions of many genes. In addition, use of bioinformatics may help to classify the biological function of genes that are differentially expressed.

Today, 2-dimensional proteomics are the gold standard for a wide search for divergent gene expressions on protein level. A question is whether protein chips prove a useful alternative for large-scale protein analyses in biomedicine. Multidisciplinary collaboration may explore whether protein chip is a relevant tool for exploratory studies. The investigations regarding the pathogenesis for obesity-related diseases point to new applications for protein chips.

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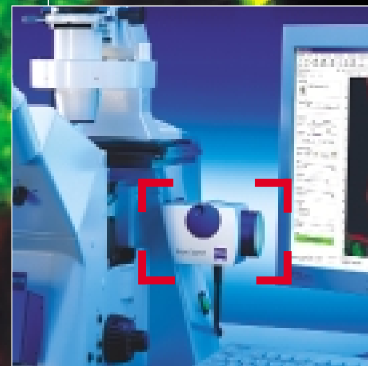
Confused

Convinced



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