6th ESF Workshop on Affinity Proteomics

Alpbach, Austria, March 11-13 2013

Summary

The ESF/AFFINOMICS 6th Affinity Proteomics workshop, held from March 11-13th 2013 in Alpbach (Austria), was attended by 104 academic and industrial participants from Europe and the USA. As in previous years, the focus of the event was the current state of affinity methods for proteome analysis. It covered a wide area, including the Human Protein Atlas and human proteome projects, biomarker discovery, the application of binders in cell signalling research, and complementarity of affinity methods to more conventional mass spectrometry proteomics. New technologies were represented, from recombinant binder production methods to array-based analyses and microfluidics systems. In addition to 16 principal invited speakers, there were 18 short talks selected from submitted abstracts and 20 poster presentations. The programme comprised two and a half days of talks, with time set aside for outdoor activities. The meeting was co-funded by the ESF Programme Frontiers of Functional Genomics and the EU Framework 7 collaborative project AFFINOMICS.

Description of the scientific content of and discussion at the event

The aim of the meeting programme was to address recent developments in characterisation of proteins on a genome-wide scale using specific binding reagents, such as antibodies, protein scaffolds, aptamers and peptides. In order to achieve this, there is an acknowledged need to establish comprehensive resources of well-characterised affinity binding reagents together with technologies for their application. The production of such resources also has important potential clinical applications in diagnostics and therapeutics, as well as providing a major 'post-genome' challenge with significance for both basic and medical research. A central aspect is the systematic generation of binders by modern high throughput technologies, particularly recombinant methods. However, the technology of binder production is only one aspect; such projects also need to begin with a clear definition and prioritisation of target molecules. In the case of the EU AFFINOMICS project, the focus is on cell signal transduction molecules, both for their intrinsic importance in all cellular activity and response, but also because of their frequent involvement in cancer as a result of mutation. A related area of medical importance where binders are actively employed is in the definition of disease-specific biomarkers, particularly at the plasma level; it is likely that combinations of altered concentrations of several proteins will provide diagnostic signatures which can have predictive value. Such developments and applications of binders will directly relate to personalised medicine and the individualisation of patient treatment, the new paradigm of healthcare. These topics were covered in different presentations through the meeting.

In the opening session (Monday 14th March), **Garry Nolan** (Stanford) presented a keynote lecture on 'A definable "structure" for the immune system and cancers at the single cell level.' Using a next-generation single-cell 'mass cytometry' platform, Nolan's group has quantified surface and cytokine or drug responsive indices of kinase target with 45 or more parameter analysis (e.g. 45 antibodies, viability, nucleic acid content and relative cell size). They have recently extended this parameterisation to mRNA with the capability to measure down to 5 molecules per cell in combination with any other set of previously created markers. **Ulf Landegren** (Uppsala) then presented 'High-performance protein measurement in single cells and plasma samples'. Sensitivity and specificity of protein detection are key limiting factors for basic research, in the search for new biomarkers and for advanced diagnostics. The proximity ligation or proximity extension assays developed by his group provide improved performance over earlier assay formats, expand the scope for parallel measurements from small sample aliquots and can help visualise interacting proteins reflecting activity steps of cells and tissues. Professor Landegren described results using recombinant affinity reagents for localised detection of protein complexes in cells and also illustrated the possibility of measuring sets of protein molecules present in individual cells, revealing cellular heterogeneity at the protein level. These technologies have been adopted for commercialisation by OLINK AB, as presented by **Mats Gullberg**, who described their system called 'Proseek' for protein biomarker research. The Proseek Oncology I 96x96 includes a panel of 96 immunoassays, consisting of 92 potential human protein biomarkers as well as 4 controls, to be analysed in 96 samples simultaneously. The technology is based on the Proximity Extension Assay, which combines antibody-based protein detection with a quantitative real-time PCR read-out. Proseek Oncology is the first product in a series of several up-coming panels from the company. **Mathias Uhlén** (Stockholm) described progress in the human protein atlas project, perhaps the most complete example to date of an affinity approach to defining the human proteome. The aim is to generate a first draft on a whole-proteome level of protein localisations in human cells, tissues and organs, including various disease-related tissues. The current version 10.0 of the Human Protein Atlas (HPA) (www.proteinatlas.org) contains more than 17,000 validated antibodies targeting 14,000 genes corresponding to 70% of the protein-encoded genes in humans. The atlas contains more than 13 million high-resolution images generated by immunohistochemistry and confocal microscopy. The antibodies have been generated to regions of low homology and the long-term objective is to generate paired antibodies towards the protein targets with separate and non-overlapping epitopes to provide protein evidence for all human protein-coding genes. Pilot projects have also been initiated to generate recombinant affinity reagents, a pilot version of a Rodent Brain Protein Atlas and to study human biology. In addition, Professor Uhlén's group have developed a targeted MS-proteomics strategy based on the recombinant protein fragments (PrESTs) generated within the framework of the Protein Atlas project. They have used the HPA to study global protein expression patterns in human cells, tissues and organs, as well as a discovery tool to find potential biomarkers for cancer diagnostics and to develop new potential antibodies for therapeutics. This was

followed by an account by **Jochen Schwenk** (Stockholm) of 'Affinity proteomic profiling in plasma biobanks' in which antibody suspension bead arrays have been developed within the HPA and non-fractionated, biotinylated and heat-treated samples are profiled with 384 antibodies at a time. There followed a presentation by **Edouard Nice** (Monash, Australia) on 'Design, operation and application of an automated high throughput monoclonal antibody facility to support global proteomics initiatives'. Monash University has established a state of the art, high throughput, robotic platform, based on a model originally established at EMBL, to produce custom made, high quality, high-affinity mouse or rat monoclonal antibodies against protein or peptide targets with the capacity to provide thousands of novel antibodies per year to a global clientèle. Using Antigen-MicroArray (AMA) technology for primary screening, multiple antigens can be screened simultaneously for specific binding and, by differential staining, IgG-secreting clones can be specifically selected. The session concluded with a talk by **Victoria Newman** (London) on 'Antibodypedia, an open-access resource for antibody validation and commenting'. Antibodypedia, an online resource cataloguing antibodies, validation data and commentary, and run in collaboration with the Nature Publishing Group, attempts to create transparency for antibody users. Reagents are annotated with experimental data, both positive and negative, from the research community and suppliers alike; links to scientific literature citing their use are included as well as user-submitted comments on performance. Since Antibodypedia's re-launch in December 2011, its listings have grown to well over 500,000 antibodies, along with 250,000 data images. 89% of proteins encoded by human ORFs are detectable by at least one Antibodypedia antibody.

In the following session (Tuesday 12th March), **Hanno Langen** (Roche, Basel) introduced the subject of biomarker discovery and validation, which he described as a 'stony path'. Although only a few novel diagnostic markers have been introduced into the market in recent years, proteomics technologies are now offering unique chances to identify new candidate markers. He pointed out that before a marker can be introduced into the market, three successive developmental phases have to be completed: the discovery phase, in which a variety of proteomics technologies are applied to identify marker candidates; the prototype developmental phase, in which immunological assays are established and validated in defined sample collectives; and finally the product development phase, with assay formats suitable for automated platforms. The discovery aspect was illustrated by **Carl Borrebaeck** (Lund), by means of antibody microarray, a high-throughput technology for protein expression profiling, which is now generating data for improved diagnosis and patient stratification, as demonstrated in several clinical studies. His group uses human recombinant scFv antibody fragments, microarray adapted by molecular design as probes and displaying an outstanding on-chip functionality and stability. The applicability of the platform for differential high-content screening of clinical samples has been exploited in a set of key applications within oncoproteomics (breast cancer and pancreatic cancer), autoimmunity (systemic lupus erythematosus) and inflammatory diseases (pancreatitis).

Paul Ko Ferrigno (Leeds) then described 'A high-content Affimer microarray platform for serum biomarker detection in inflammatory disease', in which he identified peptide 'Affimers' specific for purified C-Reactive Protein that also recognise CRP in patient samples. The data indicate that it may be possible to identify patients with SLE or Bechet's disease amongst the more prevalent cases of rheumatoid arthritis or vasculitis.

Seth Blackshaw (Johns Hopkins, Baltimore, USA) presented 'The NIH Protein Capture Initiative: generation of monospecific monoclonal antibodies against human transcription factors'. This programme is a parallel effort to AFFINOMICS in which the targets for monoclonal antibodies are human transcription factors. Antibodies that bind selectively to a single protein on the Human Proteome Microarray of >16,000 human proteins are then tested for usefulness in immunoprecipitation and, if successful, in ChIP-Seq using ENCODE cell lines. In contrast to these 'classical' spotted protein microarrays, **Oda Stoevesandt** (Cambridge) described a method for the rapid and economical 'printing' of protein microarrays directly from a DNA array template using cell-free protein synthesis (termed 'DNA array to protein array', DAPA). Since the experimental effort involved is comparable to the assembly of a Western blot, DAPA is an enabling technology, making customised protein microarrays affordable for laboratories with no access to routine microarray spotting. In a similar methodology, **Jörg Hoheisel** (Heidelberg) introduced the concept of 'Personalised proteomics by means of individualised protein microarrays', which takes advantage of sequence information from individuals for a directed characterisation of disease-specific protein isoforms (mutations, polymorphisms and splice variations), utilising a newly developed technique. First, a tissue's RNA/cDNA is copied onto the microarray by an on-chip PCR amplification, using gene-specific primer pairs that are attached to the chip surface. The arrayed DNA copies then act as templates for an *in situ* cell-free expression, yielding a protein microarray that presents the protein content of a particular tissue of an individual person.

In a session which brought the meeting up to date on alternatives to conventional antibodies, **Stefan Dübel** (Braunschweig) set the scene with 'Beyond natural antibodies: the power of *in vitro* antibody generation'. The Braunschweig pipeline using antibody phage display has yielded more than 1000 monoclonal human antibodies so far, most of them against human proteins. Thorough validation in many different assays indicated that the standardised recombinant antibody format has benefits over animal derived antibodies, for example when used on large protein microarrays. The theme was continued by **Tony Kossiakoff** (Chicago) who introduced the term Synthetic Antigen Binders (sABs) to describe a class of customised antibody-based reagents generated using novel phage display libraries and selection strategies. Their attributes provide for the ability to generate sABs that are engineered to: 1) target specific regions on the surface of the protein, 2) recognise specific conformational or oligomeric states, 3) induce conformational changes, and 4) capture and stabilise multi-protein complexes. As an illustration, conformation-specific sABs can

selectively capture either the open (apo-) or closed (ligand bound) conformational states of maltose binding protein to dramatically influence the equilibrium of ligand binding. In a demonstration of the combination of recombinant methods, **Andrew Bradbury** (Los Alamos) described 'Combining phage and yeast display to select antibodies' in which preliminary selections are made with phage display and subsequent analysis and additional sorting using yeast display. A novel method of engineering the Fc region of the classical IgG was presented by **Florian Rüker (Vienna)** in which structural loops in the CH3 domains are modified in order to create specific antigen binding sites, the resulting molecule being termed an 'Fcab'. They are not only attractive as stand-alone proteins, but they can also be used as modules in complete antibodies, replacing the wild-type Fc and thus allowing preparation of bispecific antibodies. Fcabs against a number of clinical targets have been selected and characterised.

Continuing the technology theme, **Margaret Kiss** (AxioMx, Branford CT) described 'An emulsion-based approach for the selection of recombinant affinity reagents'. A large library of antibody-displaying phage binds to beads in individual compartments within a water-in-oil emulsion and they have demonstrated the ability of micro-emulsion technology to identify novel scFvs against a phosphopeptide in a single round of selection. By minimising the selection rounds required for phage display and using a FACS machine as a 'colony picker' equivalent, this approach has the potential to dramatically increase the throughput of *in vitro* screening methods. This was followed by the transgenic mouse technology called the Crescendo Mouse **(Joyce Young,** Cambridge, UK**),** a transgenic mouse that lacks endogenous immunoglobulin and instead produces B cells expressing chimaeric heavy chain only antibodies (HcAb) in first-in-class 'triple knock-out' animals which lack expression from endogenous immunoglobulin heavy, kappa and lambda chain loci. Following immunisation with various antigens, robust antigen-specific HcAb responses were mounted which were readily detected by serum ELISA. The antigen-reactive VH sequences were efficiently mined using *in vitro* display techniques and diverse antigen-specific VH were isolated.

On the second full day of the workshop, **Andreas Plückthun** (Zürich) explored whether binding scaffolds can exert strong biological effects themselves without any additional payload. A very versatile scaffold from his group is the Designed Ankyrin Repeat Protein (DARPin), which, because of favourable biophysical properties, can be engineered in many formats. Using DARPins generated against members of the EGFR family, and a combination of X-ray crystallography, signalling studies, and *in vivo* experiments, an investigation of the relationship between biological effect, target epitope and orientation was provided. **Larry Gold** (SomaLogic, Boulder, USA) then described 'SOMAmers: Amazing structures and many applications'. His company has developed nucleic acid SOMAmers as exquisite protein binders. Today they have roughly 2,000 SOMAmers directed at human proteins. Three SOMAmer-target co-crystals have been studied at high resolution so that one finally can see the reasons that SOMAmers are not "merely" aptamers. Low Kd's and slow off-rates provide

high SOMAmer specificity, leading to many applications. Continuing the theme of 'alternative' (to Ig) binder types, **John Löfblom** (Stockholm) reported on the 'Generation and characterisation of HER3-specific Affibody molecules', in particular intended for molecular imaging and biotherapeutic applications, while **Antonia Richter** (Munich) described 'Selection and characterisation of high-affinity Anticalins against VEGFR-3 for *in vivo* imaging of glioblastoma'. Both the Affibody and Anticalin platforms hold much promise for cancer imaging and to monitor therapies. The use of an exciting microfluidic system for directed evolution and affinity binder generation was presented by **H. Tom Soh (Santa Barbara),** providing theoretical and experimental evidence for extremely fast generation of affinity reagents.

Returning to the application of arrays, **Peter Nilsson** (Stockholm) presented results on 'Proteomic profiling of autoimmunity repertoires utilising antigen and high-density peptide microarrays'. This included different formats of antigen array, with either batches of 384 different antigens printed in 21 identical sub-arrays on each slide, 11,500 antigens printed in duplicate or high-density peptide microarrays with 2.1 million peptides and whole proteome coverage. **David Juncker** (Montreal) introduced the 'Antibody colocalisation microarray (ACM): A cross-reactivity "free" and scalable antibody microarray format' in which the number of parallel sandwich assays which can be performed on an array is increased when both capture and detection antibodies are physically colocalized by spotting to the same two-dimensional coordinate. ACMs with up to 50 targets were illustrated, along with a binding curve for each protein; proteins in the serum of breast cancer patients and healthy controls were quantified, and six candidate biomarkers identified. In contrast, bead based (rather than slide-based) antibody and protein arrays were presented by **Fridtjof Lund-Johansen** (Oslo) for high throughput validation of antibodies. Over the past years, he has developed a platform where samples are fractionated and all the sample fractions analysed with replicate antibody arrays. He presented a new and improved method for subcellular fractionation that yields highly pure fractions with proteins from four subcellular compartments, as well as data from testing several hundred commercially available antibodies on a bead-based array with 350 recombinant human proteins.

In the final session, mass spectrometry was linked with binder reagents, first by **Bernd Wollscheid** (Zürich) who presented 'Ligand-based receptor capture (LRC) on living cells'. His group have developed cell surface capturing (CSC) technology for the unbiased identification and quantification of cell surface N-glycoproteomes by MS, sets of SRM assays for selected N-glycopeptides of clinical interest and the Cell Surface Protein Atlas (CSPA). This has demonstrated the powerful applicability of chemical reagents in the tagging of cell surface glycoproteins at carbohydrate groups and the subsequent purification of the corresponding peptides for MS analysis. They have now synthesised trifunctional cross-linkers for the ligand-based tagging of glycoprotein receptors on living cells and the purification of receptor-derived peptides for MS analysis. This ligand-based receptor capturing (LRC)

approach allows for the highly specific and sensitive detection of ligand interactions with their corresponding receptors under near-physiological conditions. **Walter Kolch** (Dublin) introduced protein-protein interactions in the regulation of the pro-apoptotic MST2/Hippo pathway. Using MS-based interaction proteomics to map kinase signalling networks that regulate cell proliferation and apoptosis revealed that the Raf-1 kinase can suppress apoptosis by inhibiting the MST2/Hippo kinase. By means of interaction proteomics, the downstream effectors and upstream regulators of the MST2 pathway were subsequently mapped. **Marius Ueffing** (Tübingen) considered 'Molecular dissection and multi-scale integration of functional protein networks in health and disease states: analysing the molecular basis of human vision'. By combining and integrating several layers of information gathering from proteome-centric biochemistry, *in silico* network analysis as well as structural biology, his group have developed an analytical approach to determine functional constraints in protein networks that drive biological functions and - when hit by mutation - cause disease. The biological system chosen for study is a highly compartmentalised organellar structure in mammalian photoreceptors called photoreceptor outer segments, which contain para-crystalline arrays of the light receptive GPCR rhodopsin, which are generated and compartmentalised via motor driven ciliary transport.

In two final short technical presentations, **Agata Zieba** (Uppsala) reported on the application of proximity ligation assays for the Human Protein Atlas antibody validation pipeline, and **Magnus Malmqvist** (Uppsala), a BIAcore pioneer, discussed 'Bridging the gap between molecular and cellular responses with real-time analysis of complex protein interactions'. His point was to evaluate complex interactions based on real-time analysis of cell binding following the complex interaction of EGF binding to EGFR family proteins and demonstrated a series of different binding patterns dependent on conditions and cell lines. **Mike Taussig** brought the workshop to a close with a resume of its main outcomes and thanks to his associates, Cheryl Smythe and Oda Stoevesandt, for their extensive work in the preparation and organisation of the very successful meeting.

Assessment of the results and impact of the event on the future direction of the field

This workshop brought together many leading European and US academic and industrial players in affinity proteomics, encouraging personal interactions and networking. Participant feedback praised the organisation and confirmed that this series of workshops is indeed now one of the best meetings in the field. The meeting highlighted recent progress by linking novel developments in proteome analysis using binding reagents, particularly in the key area of signal transduction, with the growing interest in personalised medicine, for which biomarker discovery and detection are key components. These topics will be of major importance for many years to come. The meeting also contributed significantly to promoting the linkage between EU projects such as AFFINOMICS with parallel activities in the USA; it was attended by representatives from the NIH and NCI responsible for organisation of the Protein Capture Reagents and Clinical Proteomics for Cancer initiatives

respectively, from which several groups funded by the US programmes were invited presenters. It is hoped that a number of papers by speakers at the meeting will be published in a special issue of the journal New Biotechnology accompanied by an extended meeting report.

Programme

Monday 11th March

16.00 **Mike Taussig (Cambridge)**
Welcome and Introduction
16.10 **Garry Nolan (Stanford)**
A Definable "Structure" for the Immune System and Cancers at the Single Cell Level
16.35 **Ulf Landegren (Uppsala)**
High-performance protein measurement in single cells and plasma samples
17.00 **Mats Gullberg (OLINK, Uppsala)**
Proseek for protein biomarker research
17.15 **Mathias Uhlen (Stockholm)**
Generating antibodies to the human proteome
17.40 **Jochen Schwenk (Stockholm)**
Affinity proteomic profiling in plasma biobanks
17.55 **Ed Nice (Monash)**
Design, Operation and Application of an Automated High Throughput Monoclonal Antibody Facility to Support Global Proteomics Initiatives
18.10 **Victoria Newman (London)**
Antibodypedia, an open-access resource for antibody validation and commenting.
18.25 **Discussion**
19.00 **Drinks and Dinner at the Alpbacherhof**

Tuesday 12th March

08.30 **Hanno Langen (Roche, Basel)**
The stony path: From Biomarker Discovery to Clinical validation
08.55 **Carl Borrebaeck (Lund)**
Next Generation Arrays
09.20 **Christer Wingren (Lund)**
Unlocking biomarker discovery - design of antibody-based micro- and nano-arrays for high-throughput disease proteomics
09.35 **Paul Ko Ferrigno (Leeds)**
A high-content Affimer microarray platform for serum biomarker detection in inflammatory disease
09.50 **Seth Blackshaw (Baltimore)**
The NIH Protein Capture Initiative: generation of monospecific monoclonal antibodies against human transcription factors.
10.05 **Oda Stoevesandt (Babraham)**
DNA Array to Protein Array
10.20 **Jörg Hoheisel (Heidelberg)**
Personalised proteomics by means of individualised protein microarrays
10.45 **Discussion**

11.00 **Coffee / Lunch**
11.30 **Skibreak**

16.00 **Tea**
16.30 **Stefan Dübel (Braunschweig)**
Beyond natural antibodies: the power of *in vitro* antibody generation
16.55 **Tony Kossiakoff (Chicago)**
Modifying biological function using conformational trapping by synthetic antibodies
17.20 **Andrew Bradbury (Los Alamos)**
Combining phage and yeast display to select antibodies
17.45 **Florian Rüker (Vienna)**
Fcabs – antigen binding Fc regions
18.00 **Margaret Kiss (AxioMx, Branford, CT)**
An emulsion-based approach for the selection of recombinant affinity reagents.
18.15 **Joyce Young (Crescendo, Cambridge)**
The Crescendo Mouse: An *in vivo* Route to Fully Human VH Therapeutics
18.30 **Break for boots**
18.45 **Depart to dinner at Rosmoos (uphill walking)**

Wednesday 13th March

08.30 **Andreas Plückthun (Zurich)**
Engineering strong biological effects into receptor-binding proteins
08.55 **Larry Gold (Boulder, CO)**
SOMAmers: Amazing structures and many applications
09.20 **John Löfblom (Stockholm)**
Generation and characterization of HER3-specific Affibody molecules
09.35 **Antonia Richter (Munich)**
Selection and characterization of high-affinity Anticalins against VEGFR-3 for *in vivo* imaging of glioblastoma
09.50 **Tom Soh (Santa Barbara, CA)**:
Directed Evolution in Microfluidic Systems
10.15 **Ralf Bischoff (Heidelberg)**
Next generation peptide microarrays
10.30 **Peter Nilsson (Stockholm)**
Proteomic profiling of autoimmunity repertoires utilising antigen and hih-denisty peptide microarrays
10.45 **Discussion**
11.00 **Coffee / Lunch**
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16.00 **Tea**
16.30 **David Juncker (Montreal)**
Antibody colocalization microarray: A Cross-reactivity “free” and scalable antibody microarray format
16.55 **Fridtjof Lund-Johansen (Oslo)**
Using antibody arrays and protein arrays for high throughput validation of antibodies
17.10 **Bernd Wollscheid (Zürich)**
Caught in the act: Ligand-based Receptor Capture (LRC) on living cells

17.35 **Walter Kolch (Dublin)**

Regulation of the pro-apoptotic MST2/Hippo pathway by protein-protein interactions

18.00 **Marius Ueffing (Tübingen)**

Molecular dissection and multi-scale integration of functional protein networks in health and disease states: analysing the molecular basis of human vision

18.25 **Agata Zieba (Uppsala)**

Application of PLA for the HPA antibody validation pipeline

18.40 **Magnus Malmqvist (Ridgeview Diagnostics, Uppsala)**

Bridging the gap between molecular and cellular responses with real-time analysis of complex protein interactions

18.55 **Mike Taussig (Cambridge)**

Winding up

19.30 **Dinner and Party**