Making it small:

Protein Microarrays for Tumour Analysis

Dr. Markus Templin

Head Assay Development

NMI Natural and Medical Sciences Institute

at the University of Tübingen

Erlenbach, September 13th 2007
Protein Profiling @ NMI

- Systems biology approach to analyze cell signalling
- Proteomic and clinical research

- Miniturized and parallelized immunoassays
- Automation, throughput, flexibility
- Minimal amount of samples, e.g. fine needle biopsies
„Making It Small“ …
… Driven by Limiting Sample Material

- solid tissue (parts)
- body fluids
- needle biopsies
- tissue sections
- cell cultures

Material:
- > 100 mg
- ~ 20 mg
- < 1 mg

Microarray based Protein Profiling
Microarrays

Solid Phase Assay System

Specific Capture Molecules

*Picograms to nanograms of DNA/Peptide/Protein per Spot!*

Highly Parallel

Highly Miniaturised

*Low Sample Consumption*

**SENSITIVE!**
Arrays: Planar vs Bead Technology

Spatial address for identification
Confocal scanner for readout

Colour coded bead populations
FACS for readout

Luminex 100
BD FACSarray
Beads vs. Planar Arrays

Planar Array

• up to 20,000 analytes
• up to 100 samples a day
• image processing necessary
• good reproducibility
• array production, quality control and automation complex

Bead Array

• max. 100 analytes
• thousands of samples a day
• no image processing
• good reproducibility
• simple production and quality control
• automation available
Analysis of Breast Cancer Biopsies

- > 100 samples, comprehensive clinical data
- Tissue weight: 6-30 mg (ø 19.9 ± 6.3 mg)
- Tissue Solubilisation: Bead-based Antibody Array (Luminex)

multiplex analysis
>100 BREAST BIOPSIES
High-quality:
3D-US, fast processing,
~ 100% central hits

ASSAY DEVELOPMENT
Multiplex on
Luminex platform

CLINICAL DATA
accurate, comprehensive

ANALYSIS
of complex data sets

VALIDATION

54 PARAMETERS / BIOPSY

INFORMATIVE SET OF ANALYTES

PROGNOSIS

PREDICTION OF THERAPEUTIC RESPONSE

Cooperation: U. Sauer, H. Deissler, EOL, University of Ulm
Assay Development: Her-2 – clinical IHC vs. Luminex

- IHC 0
  - n=61
  - p = 4.268E-5*

- IHC 1+
  - n=28
  - p = 0.669*

- IHC 2+
  - n=8
  - p = 1.92E-3*

- IHC 3+
  - n=16
  - p = 0.182*

- Normal tissue
  - n=10
  - 6 individual

- Luminex
  - 0.5 AU

- Luminex
  - 35.3 AU

* t-Test
# Focused Protein Profiling

## Breast Tumour Tissue

### Multiplex 1
- HER-2: 11.9 ng/ml
- ER: 14.1 ng/ml
- PR: 1.10 ng/ml
- TIMP-1: 30.1 ng/ml
- PAI-1: -
- IGF-1R: 1.43 ng/ml

### Multiplex 2
- EGFR: 1.65 ng/ml
- VEGFR-1: 29.4 ng/ml
- VEGFR-2: -
- VEGFR-3: -
- TIMP-2: 13.1 ng/ml
- uPA: 1.42 ng/ml
- β-catenin: 117 ng/ml

### Multiplex 3
- MMP-1: 22.1 ng/ml
- MMP-2: -
- MMP-3: 32.6 pg/ml
- MMP-7: 3.85 ng/ml
- MMP-8: 3.90 ng/ml
- MMP-9: 7.74 ng/ml
- MMP-10: 0.40 ng/ml
- MMP-13: 1.45 ng/ml

### Multiplex 4
- Fas ligand: -
- ANP-2: 0.41 ng/ml
- IGFBP-3: 7.54 ng/ml

### Multiplex 5
- TNFR-1: 0.33 ng/ml
- TNFR-2: 0.40 ng/ml
- VCAM-1: 4.52 ng/ml
- ICAM-1: 90.2 ng/ml
- gp130: 5.56 ng/ml
- Fas: 0.39 ng/ml
- IL-2R: -
- MIF: 19.4 ng/ml

### Multiplex 6 (commercial)
- EGF: -
- VEGF: 1.32 ng/ml
- FGF-2: 1.59 ng/ml
- PDGF-AB/BB: 0.26 ng/ml
- PDGF-AA: 0.16 ng/ml
- Flt-3 ligand: -

### Multiplex 7 (commercial)
- IL-6: -
- IL-7: -
- IL-8: 0.12 ng/ml
- MCP-1: 80.8 pg/ml
- RANTES: 1.36 ng/ml

### Multiplex 8 (commercial)
- IL-1α: 0.21 ng/ml
- IL-1β: -
- IL-10: -
- IL-13: -
- IP-10: 6.60 ng/ml
- MIP-1α: -
- eotaxin: -
- GM-CSF: -
- INF-γ: -
- TNFα: -
- IL-12 (p40): -
**Lymph node involvement**

- Tumour: n=101
- Sentinel lymph node: n=39 affected
- Non-sentinel lymph node: n=24 affected

*Identification of differentially expressed proteins*
Prediction of nodal involvement

- Analysis of primary tumour for predictive patterns
- Nodal involvement can be predicted for analysis of the primary tumour

Screening with 54 assays + standard parameters (SP)

- Measuring protein concentrations

36 informative assays + SP

- Univariate analyses

15 assays + 2 SP with p < 0.2

- Multivariate logistic regression

5 assays + 1 SP

ROC AUC = 0.83

15 assays + 2 SP

ROC AUC = 0.87
Assay Formats – A Comparison

Forward Phase Protein Microarray (μELISA)

* captures many analytes in one sample

Reverse Phase Protein Microarray (Lysate Array)

* measures one analyte in many samples
Reverse Phase Arrays (RPA)

- array of samples
- minimal sample and reagent requirement
  e.g. only μg’s of protein for hundreds of assays
- crude extracts (complete cell content)
- conditions comparable to Westerns
- quantitative and dynamic read-out
- one quality controlled antibody per analyte and assay (rapid & robust results)
- related sample sets on the very same array

up to 320 sample points / array

8 data points / sample
(4 dilutions, duplicate spots)
40 samples
From Cells to Results

< 1 mg
samples

cell cultures

lysates
arraying chips

sections

assay
readout analysis

biopsies

> 100 mg tissue

solid tissues

ab#1
ab#2
ab#3

etc.
Lysate Array Platform

Array Spotting:
- 1 droplet per spot
- 3 jets simultaneously
- ~0.4 nl spotting volume
- <4% spotting CV

Array Read-Out:
Planar Waveguide Technology

GeSiM NP 2
Some Numbers: Analysis - Sensitivity

Analysis of sample signal (8 spots):

- Lysates spots at 100 … 400 μg/ml total crude protein
- ~1 cell equivalent (100 pg) per spot
- ~10^9 proteins per spot
- Down to ~10^-6 analyte fraction detectable (as determined in a spiking exp. with rec. phospho-p44/42 MAPK)
- Detection sensitivity in a spot: ~1000 analyte copies (zeptomole)

Robust signal generation by averaging over spot signals at 4 dilutions in duplicates
Performance:
Cellular signaling in treated cell cultures

- Dynamic cell signaling events
- Quantification of \( \geq 20\% \) changes of protein abundance and activation state (e.g. phosphorylation) levels!
- Good correlation to Western results
- Good reproducibility

<table>
<thead>
<tr>
<th>CV spot-to-spot signal</th>
<th>3-5 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV sample signals (8 spots) (intra-array)</td>
<td>6-13 %</td>
</tr>
<tr>
<td>CV sample signals (8 spots) (array-to-array)</td>
<td>7-16 %</td>
</tr>
</tbody>
</table>

Jurkat cells treated with CD3 / CD28 to induce MAP kinase signaling
Quantitative Tumour Proteomics
(collaboration with Prof. S. Stevanovic, Univ. Tübingen)

Material

• Comparison study:
  40 pairs of tumor and healthy tissue

• Solid fresh tissues, shock-frozen immediately after surgery

• Different carcinoma:
  Renal cell (RCC), Prostate, Colon, Rectum

RPA protein expression studies

• Verification of marker candidates as protein tumor markers
  e.g. ß-catenin, CEA, p53, EGFR, Carbo-anhydrase 9, Muc1, cMet, Her2, etc.

• Screening for new tumor markers: interacting candidates selected from known signaling pathways

• Currently 60 target proteins under investigation

Background

Samples characterized by

• Transcriptome analysis (Affymetrix GeneChip™)

• MS of MHC-presented peptide signatures

• Several marker candidates
"Differential Display" on MHC I

- MHC I: presents antigen peptides from cytosolic proteins
- HLA-Epitopes can be tumor associated
- Identification of differentially expressed tumor antigens

Heath et al. 2001
Nature Reviews Immunology
1. **Marker Identification**: differentially presented MHC-I peptides (Tübingen University)

2. **Antibody selection + characterisation**: Western Blot

2. **Array Generation and Quality Control**: On-Chip Protein stain and normalisation

4. **Assay**: Comparative Analysis Validation by independent approaches
# Tumour-associated Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipophilin</td>
<td>Adipocyten Differenzierung</td>
</tr>
<tr>
<td>Met</td>
<td>Proto-Oncogen; Tyrosin Kinase</td>
</tr>
<tr>
<td>Carboanhydrase 9</td>
<td>Säure-Base-Haushalt; CA9 einziges Tumor-assoziiertes Isoenzym</td>
</tr>
<tr>
<td>Regulator of G-protein signalling 5</td>
<td>Signal-Transduktion; GTPase Aktivierung</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Extrazellulär-Matrix (Abbau)</td>
</tr>
<tr>
<td>Muc1</td>
<td>Epitheliales Mucin; Glycoprotein; Drüngeweb</td>
</tr>
<tr>
<td>Nicotinamid-N-methyltransferase</td>
<td>oxidativer Stoffwechsel</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 3</td>
<td>Zellproliferation, Apoptose</td>
</tr>
<tr>
<td>Apolipoprotein L1</td>
<td>Cholesterin-Stoffwechsel</td>
</tr>
<tr>
<td>Survivin</td>
<td>Apoptose-Inhibitor</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>ER-lokalisiert; Stoffwechsel von Prokarzinogenen</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>CDK Kinase Regulator; Zellzyklus-Steuerung; (Tumorigenesis)</td>
</tr>
<tr>
<td>Antigen preferentially expressed in melanoma / PRAME</td>
<td>Cytotoxik T-Lymphocyte Antigen</td>
</tr>
<tr>
<td>Cytokeratin 18</td>
<td>Intermediärfilament; Zellstrukturmolekül</td>
</tr>
<tr>
<td>Carcinoembryonic antigen / CEA</td>
<td>Antigen mit Einfluß auf Apoptose und Metastasierung (CCA)</td>
</tr>
</tbody>
</table>

+ 45 additional proteins

antigens identified as differentially presented MHC-I peptides
Antibody Characterisation / Validation

Human renal tissue

Cell lines:
A: A431
B: HeLa
C: Jurkat

MHC-1 subunit

Human renal tissue

Cytokeratin 18

β-Catenin

Cytokeratin 18

β-Catenin

Accepted

Not accepted!
Lysate Arrays
Layout & Assay

- Comparative analysis:
  40 / 80 / 160 samples per array
- Pairs of samples (Tumour vs Normal)
- Normalisation by „On-Chip“ Protein stain
- 100+ validated, marker-specific antibodies used

320 data points / array

8 data points / sample
(4 dilutions, duplicate spots)
40 samples
Carboanhydrase 9 Expression

CA9: Carboxyjanhydrase 9, identified as tumor-associated HLA signature
Reverse Arrays for Tumour Analysis

- Up-regulation of different proteins in renal cancers:
  - mRNA data confirmed
  - up-regulation more drastic on the protein level
- Several known biomarkers confirmed
- Several biomarker candidates, identified from HLA peptide signatures, confirmed as cytosolic proteins (e.g. CA9, β2 microglobuline, .....)
- Phosphorylation!
Summary

• Robust and flexible screening platforms: Bead-based Systems - Reverse Phase Systems
  Sensitivity, reproducibility, throughput

• Little sample material
  Dozens of analytes from only μg’s of sample protein

• Large flexibility in applying different/new assays
  Western Blot antibodies for Reverse Arrays
  Adaptation of ELISA to Bead-System

• Clinically relevant results
Acknowledgements

Biochemistry
Thomas Joos
Silke Wittemann*
Nicole Schneiderhan-Marra
Oliver Pötz
Michael Hartmann
Cornelia Kazmaier
Hsin Yun Hsu
Katrin Lukert

University Ulm
Georg Sauer
Helmut Deissler

University Tübingen
Stefan Stevanovic

Insel Spital Bern
Prof. Markus Borner

Protein Profiling
Michael Pawlak
Berthold Gierke

Luminex
Jim Jacobsen, Luminex, Austin, TX, (USA)
Paul Ladestein, Luminex Europe, NL

Assay Development
Stefan Kramer
Thomas Knorpp
Anette Döttinger
Stefan Pabst
Sibylle Höppe
Sarah Mühl

Markus Ehrat, Zeptosens – A Division of Bayer, Witterswil, CH
Eginhard Schick, Zeptosens – A Division of Bayer, Witterswil, CH

Thomas Herget, Merck KGaA, Darmstadt (D)
Dominic Eisinger, Multiplex Biosciences, Lake Placid, NY (USA)