3. Materials

3.1 Chemicals

ABTS	Sigma-Aldrich Chemie, Munich, Germany
Ammonium persulfate	Bio-Rad Laboratories, Hercules, CA, USA
Ampicillin	Sigma-Aldrich Chemie, Munich, Germany
Betaine	Sigma-Aldrich Chemie, Munich, Germany
Bind-silane	Amersham Biosciences, Uppsala, Sweden
Bromphenol blue	Sigma-Aldrich Chemie, Munich, Germany
Chloramphenicol	Sigma-Aldrich Chemie, Munich, Germany
Coomassie brilliant blue R 250	Sigma-Aldrich Chemie, Munich, Germany
Coomassie Plus-200 protein assay reagent	Pierce Biotechnology, Rockford, IL, USA
Cy3-monoreactive dye	Amersham Biosciences, Uppsala, Sweden
Cy5-monoreactive dye	Amersham Biosciences, Uppsala, Sweden
DDAO phosphate	Sigma-Aldrich Chemie, Munich, Germany
DTT	Serva, Heidelberg, Germany
EDTA	Sigma-Aldrich Chemie, Munich, Germany
Glycerol	Sigma-Aldrich Chemie, Munich, Germany
Glycine	Sigma-Aldrich Chemie, Munich, Germany
Imidazole	Sigma-Aldrich Chemie, Munich, Germany
IPTG	Sigma-Aldrich Chemie, Munich, Germany
Kanamycin	Sigma-Aldrich Chemie, Munich, Germany
Ni-NTA-agarose	Qiagen, Hilden, Germany
Non-fat dry milk	Bio-Rad Laboratories, Hercules, CA, USA
PEG 3350	Sigma-Aldrich Chemie, Munich, Germany
PETG	Sigma-Aldrich Chemie, Munich, Germany
Poly-L-lysine solution	Sigma-Aldrich Chemie, Munich, Germany
Protease inhibitor cocktail	Sigma-Aldrich Chemie, Munich, Germany
Repel-silane ES	Amersham Biosciences, Uppsala, Sweden
Rotiphorese Gel 30 (29% acrylamide, 0.8% bisacrylamide)	Carl Roth, Karlsruhe, Germany
SDS	Sigma-Aldrich Chemie, Munich, Germany
Sodium azide	Sigma-Aldrich Chemie, Munich, Germany

TEMED	Life Technologies, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich Chemie, Munich, Germany
Tween 20	Sigma-Aldrich Chemie, Munich, Germany
Tryptone	Difco Laboratories, Detroit, MI, USA
Yeast extract	Difco Laboratories, Detroit, MI, USA
ZPPD	Merck Biosciences, Darmstadt, Germany

Anorganic salts, acids and bases and alcohols were *pro analysi* quality from Sigma-Aldrich Chemie, Munich or Merck, Darmstadt, Germany.

3.2 Media, Buffers and Solutions

ABTS-Buffer

25 mM sodium citrate
25 mM citric acid
0.015% (v/v) H_2O_2
adjust to pH 4.0

AP-Buffer

1 mM $MgCl_2$
100 mM Tris-HCl
adjust to pH 9.5

Bind-Silane-Solution

0.1% (v/v) acetic acid (glacial) abs.
0.3% (v/v) Bind-Silane
99.6% (v/v) ethanol abs.

Cleaning solution for poly-L-lysine coating:

0.25 M NaOH
50% (v/v) ethanol abs.

Destaining solution:

15% (v/v) methanol
10% (v/v) acetic acid

Elution Buffer for Ni-NTA-Agarose

50 mM $NaH_2 PO_4$
300 mM NaCl
250 mM imidazole
adjust to pH 8.0

5x Laemmli Buffer

0.125 M Tris-Base
1.25 M Glycine
0.05% (w/v) SDS

4x Loading Buffer for denaturing SDS-PAGE

0.2 M Tris-HCl pH 6.8
0.8% (w/v) SDS
0.4% (w/v) bromophenol blue
40% (v/v) glycerol

Lysis Buffer

50 mM $NaH_2 PO_4$
300 mM NaCl
10 mM imidazole
adjust to pH 8.0

1x PBS pH 7.5

0.137 M NaCl
0.0027 M KCl
0.012 M Na_2HPO_4
1.76 mM KH_2PO_4
adjust to pH 7.5
autoclave

PBS-T

99.9% (v/v) PBS

0.1% (v/v) Tween-20

8% Polyacrylamide Gel Solution

26% (v/v) Rotiphorese Gel 30

0.05% (w/v) ammonium persulfate

0.2% (v/v) TEMED

12.5% Polyacrylamide Separation Gel Solution

42% (v/v) Rotiphorese Gel 30

25% (v/v) 1.5 M Tris, pH 8.8

0.001% (w/v) SDS

0.45% (w/v) ammonium persulfate

0.5% (v/v) TEMED

Poly-L-lysine coating solution:

10% (v/v) poly-L-lysine solution

10% (v/v) 1x PBS

Sodium Citrate Buffer

20 mM sodium citrate

adjust to pH 3.5

autoclave

Sodium Carbonate Buffer

1 M sodium carbonate

adjust to pH 9.6

autoclave

4.0% Stacking Gel Solution

13.4% (v/v) Rotiphorese Gel 30

25% (v/v) 0.5 M Tris, pH 6.8

0.025% (w/v) SDS

0.05% (w/v) ammonium persulfate

0.1% (v/v) TEMED

Staining solution

2.5 g/l Coomassie brilliant blue R 250

45% (v/v) Ethanol abs.

10% (v/v) acetic acid (glacial)

TBS

0.15 M NaCl

10 mM Tris HCl

adjust pH to 7.5

autoclave

TBS-T

99.9% (v/v) TBS

0.1% (v/v) Tween-20

TBS-TT

99.8% (v/v) TBS

0.1% (v/v) Tween-20

0.1% (v/v) Triton X-100

Wash Buffer for Ni-NTA-Agarose

50 mM $NaH_2 PO_4$

300 mM NaCl

20 mM imidazole

adjust to pH 8.0

2YT Medium

16 g/l tryptone

10 g/l yeast extract

5 g/l NaCl

adjust pH to 7.0

autoclave

3.3 Other Materials

Airpore tape sheets	Qiagen, Hilden, Germany
Aldehyde slides	Telechem International, Sunnyvale, CA, USA
Amine slides	Telechem International, Sunnyvale, CA, USA
Aminosilane slides	Schott Nexterion, Mainz, Germany
BenchMark pre-stained protein ladder	Invitrogen, Karlsruhe, Germany
Dendrimer slides	
Epoxy slides	Telechem International, Sunnyvale, CA, USA
1.5 ml-Eppendorf tubes	Eppendorf, Hamburg, Germany
2 ml-Eppendorf tubes	Eppendorf, Hamburg, Germany
Falcon 96-well MicroTest III	BD Labware, Oxnard, CA, USA
FAST slides	Schleicher & Schuell Biosciences, Keene, NH, USA
Glass microscope slides	Menzel Gläser, Braunschweig, Germany
Glass slide holder and incubation chamber	Neolab, Heidelberg, Germany
Hydrogel Slides	PerkinElmer, Wellesley, MA, USA
Mauly letter clips	Jakob Maul, Bad König, Germany
MaxiSorb slides	Nalge Nunc International, Rochester, NY, USA
MaxiSorb slides (black)	Nalge Nunc International, Rochester, NY, USA
Metal stripe (d=0.03 mm)	Perschmann GmbH, Braunschweig, Germany
Millex® Filter Units (pore size: 0.2 µm)	Millipore, Billerica, MA, USA
Multiscreen-DV plate	Millipore, Billerica, MA, USA
Parafilm	American National Can, Menasha, WI, USA
Piece of leather	Rewe Handelsgruppe, Cologne, Germany
Plastic boxes for microscope slides	Neolab, Heidelberg, Germany
Plastic slide racks and incubation chamber	Carl Roth; Karlsruhe, Germany

Polysine^{TM} Slides	Menzel Gläser
1 ml Polypropylene columns	Qiagen, Hilden, Germany
15 ml Polypropylene tubes	Greiner Bio-One, Frickenhausen, Germany
Polystyrene slides	Nalge Nunc International, Rochester, NY, USA
Reflective slides	Amersham Biosciences, Uppsala, Sweden
Silanated slides	Telechem International, Sunnyvale, CA, USA
50 ml Sterile plastic tubes	Greiner Bio-One, Frickenhausen, Germany
Ultrafree centrifugal filters	Millipore, Billerica, MA, USA
96 v-well microtitre plate for cell cultures	Nalge Nunc International, Rochester, NY, USA
384 v-well polystyrene plates	Genetix Ltd, Hampshire, UK
96-well Cliniplate	Thermo Labsystems, Helsinki, Finland
96-well glass microplate	Micronit Microfluidics, Enschede, Netherlands

PEG-epoxy slides were a donation of Jens Sobek, Functional Genomics Centre, Zurich, Zurich, Switzerland. Two pIVEX2.4 vectors containing genes coding wt-GFP and β-galactosidase were obtained by courtesy of Dr. Erhardt Fernholz, Roche Diagnostics GmbH (Penzberg, Germany).

3.4 Proteins, Antibodies and Enzymes

Alkaline phosphatase	Sigma-Aldrich Chemie, Munich, Germany
Alkaline phosphatase-conjugated goat anti-Mouse IgG (Fc specific)	Sigma-Aldrich Chemie, Munich, Germany
β-Galactosidase	Sigma-Aldrich Chemie, Munich, Germany
BSA	Sigma-Aldrich Chemie, Munich, Germany
Cathepsin D	Merck Biosciences, Darmstadt, Germany
Fibrinogen	Sigma-Aldrich Chemie, Munich, Germany
Goat Cy5-anti-mouse IgG (H+L)	Jackson ImmunoResearch Laboratories, West Grove, PA, USA

Goat Cy3-anti-mouse IgG (H+L)	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Goat Cy3-anti-rabbit-IgG (H+L)	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Goat Cy5-anti-rabbit-IgG (H+L)	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Helper phage M13K07	Amersham Biosciences, Uppsala, Sweden
Horseradish peroxidase	Sigma-Aldrich Chemie, Munich, Germany
Horseradish peroxidase conjugated protein L	Affitech, Oslo, Norway
HSA	Sigma-Aldrich Chemie, Munich, Germany
Lysozyme	Roche Applied Science, Mannheim, Germany
Monoclonal mouse anti-fibrinogen antibody (Clone: 2C2-G7)	BD Biosciences, San Jose, Ca, USA
Monoclonal mouse anti-HSA antibody (Clone: 1C8)	DPC Biermann, Bad Naunheim, Germany
Monoclonal mouse anti-tubulin-alpha Ab-2 antibody (Clone DM1A)	Biocarta Europe, Hamburg, Germany
Polyclonal rabbit anti-fibrinogen antibody	Calbiochem-Novabiochem, San Diego, CA, USA
Polyclonal rabbit anti-HSA antibody	DPC Biermann, Bad Naunheim, Germany
Thrombin	Sigma-Aldrich Chemie, Munich, Germany

All other proteins were purified and expressed from clones of the human fetal brain library (85) in previous projects (117).

3.5 Kits

Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit	Molecular Probes, Eugene, OR, USA
RTS 100 Cell-free Transcription and Translation Kit	Roche Applied Science, Mannheim, Germany

3.6 Strains

Escherichia coli HB2151	Amersham Biosciences, Uppsala, Sweden

Escherichia coli TG-1	Amersham Biosciences, Uppsala, Sweden
Escherichia coli BL-21	Stratagene, La Jolla, CA, USA
Escherichia coli SCS1	Stratagene, La Jolla, CA, USA

3.7 Laboratory Equipment

Centrifuges:

J6-HC	Beckman Coulter, Fullerton, CA, USA
Avanti-J25	Beckman Coulter, Fullerton, CA, USA
4K15	Sigma Laborzentrifugen, Osterode, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany

Freezer:

Forma (-80°C)	ThermoQuest Analytische Systeme, Egelsbach, Germany
freezer (+4°C to –20°C)	Robert Bosch, Gerlingen-Schillerhöhe, Germany

Gel electrophoresis apparatus:

Hoefer SE 200	Amersham Biosciences, Uppsala, Sweden
power supply	Bio-Rad Laboratories, Hercules, CA, USA

Incubator:

Series 6000	Heraeus Instruments, Hanau, Germany

Microarray Scanner:

ScanArray 4000	PerkinElmer, Wellesley, MA, USA

Nanodispensing device:

single channel piezodispensing head and control unit from sciFLEXARRAYER	Scienion, Berlin, Germany

Orbital shaker:

Rocky	Fröbel Labortechnik, Wasserburg, Germany

pH-meter:	
MP220	Mettler Toledo, Giessen, Germany
Pipettes:	
Pipetman P2	Abimed Analysen Technik, Langenfeld, Germany
Pipetman P20	Abimed Analysen Technik, Langenfeld, Germany
Pipetman P200	Abimed Analysen Technik, Langenfeld, Germany
Pipetman P1000	Abimed Analysen Technik, Langenfeld, Germany
SDS-PAGE scanner :	
ScanJet 5370C	Hewlett-Packard, Palo Alto, CA, USA
Spectral Photometer:	
SpektraMax 250	Molecular Devices, Sunnyvale, CA, USA
Spotting Robot:	
QArray Spotting Robot	Genetix Ltd, Hampshire, UK
Solid steel pins (100, 150 and 300 microns)	Genetix Ltd, Hampshire, UK
Thermocycler:	
PTC100	MJ Research., Watertown, USA
Ultrasonic homogeniser:	
Sonifier 250	Branson Ultrasonic, Danbury, CT, USA
Vortexer:	
MS1 Minishaker	IKA-Werke, Staufen, Germany

3.8 Software

Control Software of Spectral Photometer:	
SoftMax-Pro	Molecular Devices, Sunnyvale, CA, USA
Control Software of Microarray Spotter:	
Emily (Q soft 2000) – MicroArraying	Genetix Ltd, Hampshire, UK
Control Software of Microarray Scanner:	
ScanArray version 3.1	PerkinElmer, Wellesley, MA, USA
Quantification Software for Microarrays:	
GenePix Pro version 3.0, 4.1 and 5.0	Axon Instruments Inc., Union City, CA, USA

4. Methods

4.1 Derivatisation and Coating of Slides

4.1.1 Derivatisation of Glass Slides with Poly-L-Lysine

For the derivatisation of slides, glass slides are first cleaned and abraded with a mixture of NaOH and ethanol. Onto this surface polymers of L-lysine are added, which attach to the surface electrostatically.

The slides were placed in slide racks and the slide racks were placed in slide chambers. Cleaning solution for glass slides was poured into the chambers, the chambers were covered with lids and placed on an orbital shaker for 2 h. Then the racks were quickly transferred into fresh chambers filled with ddH_2O. The slides were rinsed vigorously by plunging racks up and down. This washing step was repeated six times with fresh ddH_2O each time and pH of the ddH_2O was checked after the final washing to ensure the total removal of NaOH. Then, the slides were transferred into poly-L-lysine coating solution and placed on an orbital shaker for 45 min. The slides were rinsed in ddH_2O, spun dry by centrifugation at 1000*g for 1 min and placed in sets of 5 into plastic boxes, which were sealed with parafilm. The slides were stored at 4°C and used within six weeks.

4.1.2 Coating of Glass Slides with an 8% Polyacrylamide Gel

The 8% (v/v) polyacrylamide gel is cast between two slides one of which is treated to be repellent and one to be adhesive. The thickness of the gel is determined by spacers, which keep the slides apart and produce a 30 µm-gap between both slides into which polyacrylamide solution is cast.

Two batches of glass slides were washed in technical ethanol for 10 min using glass racks and incubation chambers on an orbital shaker and dried in an incubator for 2 h at 55°C. The first batch of slides was treated by dispensing 200 µl bind-silane solution on one side of each slide, followed by a 30 minutes incubation under an extraction hood. The other batch of cleaned slides was incubated with repel-silane for 30 minutes under an extraction hood. Metal strips with a defined thickness of 30 µm were cut into pieces and used as spacers. Both sets of slides were cleaned with technical ethanol and wiped dry using a piece of cleaning leather. Bind-silane treated slides were placed bind-silane side up and metal spacers were put at both ends. Repel-silane treated slides were placed on the bind-silane slide leaving a protruding end of

about 1 mm and mauly letter clips were clipped on at both ends (Figure 9). An 8% (v/v) polyacrylamide gel solution was cast into the spacing between the two slides. After polymerisation, the mauly clips were removed and the slides were disassembled. The gel-coated slides were stored at 4°C until further use.

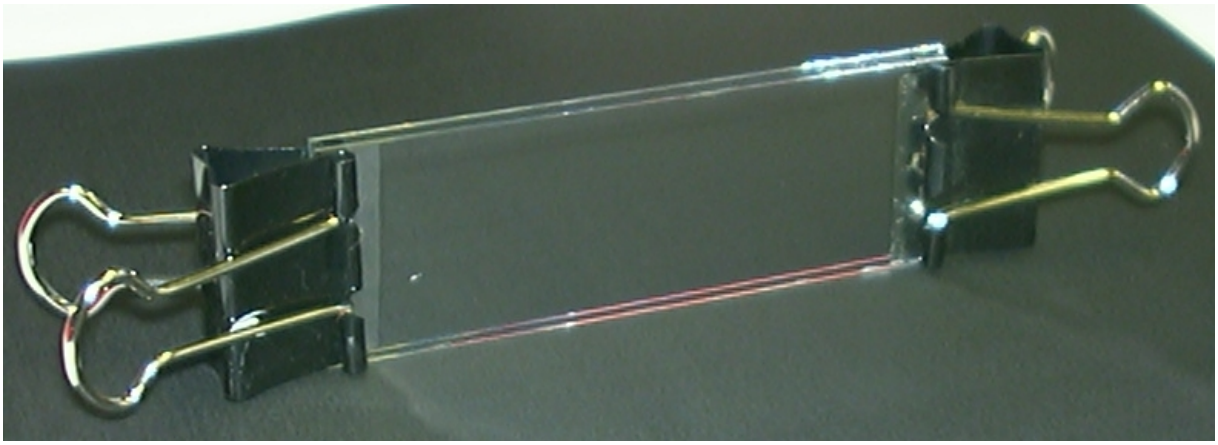

Figure 9: Photograph of assembled slides with a 30-µm gap by metal spacers.

4.1.3 Coating of Epoxy-Slides with BSA

For optimisation of enzymatic assays on chips, an epoxy-activated slide was coated with BSA to prevent direct enzyme surface contact.

All BSA-coated epoxy-slides were freshly prepared directly before use. Epoxy-coated slides were incubated with 2% (w/v) BSA dissolved in PBS for 30 minutes at 4°C. After incubation, the slides were rinsed with TBS and spun dry by centrifugation at 1000*g.

4.2 Fluorescent Labelling of Proteins and Antibodies

Several fluorophores are available for labelling of proteins, such as fluorescein, cyanine and its derivatives, Texas Red and several rhodamines. Fluorescein is a commonly used fluorophore with a high quantum yield. Nevertheless, it suffers severely from photobleaching, is pH dependent and requires anti-fading reagents such as n-propyl gallate. The development of a new generation of cyanine dyes resulted in fluorophores, which are brighter, more photostable, and give less background than most other fluorophores. Moreover, the development of long-wavelength fluorophores such as Cy5, with excitation wavelengths in the red spectra offer a significant advantage, since they benefit from decreased background fluorescence of biological specimens in these spectra (118).

The protein solution was diluted with 1x PBS to a final concentration of 1 mg/ml. 50 µl of sodium carbonate buffer (pH 9.6) was added to a total of 1 ml and mixed by pipetting up and down. Then protein solution was transferred to one vial of Cy3- or Cy5-monoreactive dye and the dye was resuspended by pipetting up and down. After incubation of the vial in the dark at room temperature for 30 minutes with mixing every 10 minutes, the labelled protein/dye mixture was transferred to a centrifugal filter with appropriate molecular weight cut-off and centrifuged for 10 minutes at 14000 rpm. Then the filtrate was discarded and the retentate was resuspended in 500 µl 1x PBS. This resuspension and centrifugation step was repeated until the filtrate was colourless. The labelled protein solution was then transferred into a 1.5 ml-eppendorf tube and resuspended to a final concentration of 1 mg/ml with 1x PBS/0.1% (w/v) NaN_3/20% (v/v) glycerol. Finally the protein was stored at –20°C.

4.3 Spotting, Postprocessing and Incubation

For robotic spotting a QArray robot controlled by the program Emily (Q soft 2000) – MicroArraying was used. The robot is capable of loading up to five 384-well source plates and can spot up to 84 slides simultaneously. For spotting up to 24 solid steel pins can be used with tip diameters of 100, 150 or 300-micron. Between the spotting of different samples, a wash routine was applied, which washed the head twice with ddH_2O and once with 80% (v/v) technical ethanol.

The proteins or antibodies for immobilisation were diluted in PBS and spotted in different patterns ranging from 4x4 to 11x11 spots per pin. After spotting, the slides were incubated overnight at 4°C. The next morning, the slides were rinsed with TBS and blocked in blocking solution (5% (w/v) non-fat dry milk dissolved in TBS-T) for 1 hour. After blocking, the slides were rinsed with TBS-T and incubated with analyte dissolved in blocking solution for 60 minutes at 4°C. In cases, in which the analyte was applied by another round of spotting using the multiple spotting technique, no incubation time is necessary, unless enzymatic activity is used for signal generation. In addition, different spotting buffers were used with the multiple spotting technique. For antibody-antigen interactions, the last spotting step was performed in PBS containing 8.7% (v/v) glycerol and 0.4 mg/ml BSA. For the scFv-screening on the chip, no additional substances were added to the expression cultures, while for enzymatic reactions 8.7% (v/v) glycerol was added to the appropriate reaction buffer (Chapter 4.12).

After incubation of all analytes, the slides were rinsed with TBS and washed twice in TBS-T for 15 minutes each. In the screening assay for scFv's TBS-TT was used for washing instead of TBS-T, while for enzymatic reactions no washing was performed. After washing of the slides in the immunosorbent assays, the slides were spun dry in a centrifuge.

4.4 Scanning and Analysis

Scanning was performed using the ScanArray® 4000 with a resolution of 5 or 10 µm. The scanner is equipped with filters and three lasers for the production of light with a wavelength appropriate for the excitation of dyes with similar properties than Cy3, Texas Red and Cy5. The excitation and emission wavelengths for all dyes scanned with this scanner used are listed in Table 1.

Dye	Excitation Maximum	Emission Maximum	Laser
Cy3	550 nm	570 nm	Green HeNe (543.5 nm)
Texas Red	595 nm	615 nm	Yellow HeNe (594 nm)
Cy5	649 nm	670 nm	Red HeNe (632.8 nm)

Table 1: Properties of the dyes used (119)

Scanning of nanowell arrays was performed using a high sensitivity laser scanner (a prototype developed by Perkin Elmer). A single laser beam at 488 nm scans the glass microplate in the field of 20x20 mm² containing 64 (8x8) wells. The resulting fluorescent signal at 535 nm is sent to a photomultiplier with photon counting. Quantification of fluorescent signals was performed with the software GenePix Pro using background subtracted median signal intensities of each spot.

4.5 Expression of Proteins

All expression cultures were obtained from the human fetal brain library (85) and sub-cloned libraries. Expression is controlled by a *lac* operon, which is diminished by glucose and induced by lactose or its non-cleavable analogue IPTG.

2YT medium containing 2% (w/v) glucose, 100 µg/ml ampicillin and 15 µg/ml kanamycin was inoculated with SCS1 expression cultures harbouring the pQE30-NST vector with the genetic information for cyclophilin A and poly-ubiquitin and the helper plasmid pSE111 with the *argU* gene, which codes for a rare arginine *t*RNA. BL-21 expression cultures containing

the pQE32-NST-BT-attB vector with the genetic information for PDLIM1 and CALM2 were inoculated in 2YT medium containing 2% (w/v) glucose and 100 µg/ml ampicillin. The cultures were incubated overnight at 37°C on a shaker. 500 ml of 2YT medium with appropriate antibiotics was inoculated with 4 ml of overnight culture and incubation was performed for 3 hours at 37°C. Protein expression was induced by addition of a final concentration of 1 mM IPTG and the cultures were overnight at 30°C on a shaker.

4.6 Purification of Proteins under Native Conditions and Buffer Exchange

Within native protein purification the cells are lysed using lysozyme and ultrasonic treatment. The recombinant proteins are purified by their His-tag, which binds onto Ni-NTA affinity resin. While lysis is performed in a buffer that contains only low concentrations of imidazole (10 mM), washing is done with a higher concentration of imidazole (20 mM) to increase stringency. Elution is performed using high levels of imidazole (250 mM) through competitive replacement of the His-tag by imidazole, which shows structural homologies to histidine. Washing and elution is done with buffers that provide a physiological environment in which proteins do not denature.

The cultures were centrifuged for 15 minutes at 4000 rpm in the J6-HC centrifuge. The supernatant was discarded and the pellets were stored overnight at –80°C. The next morning each pellet was weighed and resuspended in 5 ml lysis buffer, containing 1 mg/ml lysozyme, per gram wet weight. The suspension was incubated on ice for 15 minutes. The lysate was sonicated three times on ice for 30 seconds each with breaks for cooling until suspension was fluid. The lysate was transferred into 2 ml-eppendorf tubes and centrifuged for 30 minutes at 20,000*g. The supernatant was filled into a fresh plastic tube and 1 ml of 50% Ni-NTA slurry was added to 4 ml cleared lysate. The suspension was incubated for 2 hours at 4°C on a shaker and transferred to polypropylene columns. The column was washed with three bed volumes of wash buffer and protein was eluted with three times with half a bed volume elution buffer. All fractions were collected and checked by denaturing SDS-PAGE electrophoresis.

After denaturing SDS-PAGE electrophoresis, all elutions that contained recombinant protein were collected and filtered using Ultrafree centrifugal filters with an appropriate cut-off to retain the protein. The proteins were washed several times with PBS to replace the elution

buffer. Finally the proteins were reconstituted using PBS containing 17.4% (v/v) glycerol and frozen at –20°C until further use.

4.7 Bradford Assay

The principle of this detection method is based on the shift of the absorption maximum of Coomassie blue G-250, which changes upon binding to a protein from 465 nm to 595 nm. The absorption at 595 nm, which increases linearly with protein concentration, is measured and the protein concentration is calculated using a dilution row of BSA ranging from 0.01 mg/ml to 0.1 mg/ml diluted in the same buffer as the samples.

10 µl each BSA dilution step and the sample were transferred into the Falcon® 96-well MicroTest III and 100 µl of Coomassie® Plus-200 protein assay reagent were added. The plate was incubated for 15 minutes at room temperature and the absorption at 595 nm was measured after shaking the plate for 5 seconds in the SpectraMax 250. A standard curve was prepared from the BSA dilutions by the program Softmax Pro. The program determined the functional relation between absorption and protein content and calculated the protein concentrations of the samples.

4.8 Denaturing SDS-PAGE Electrophoresis

Sodium dodecyl sulfate (SDS) is an anionic detergent, which denatures proteins by assembling around the polypeptide backbone, causing a final mass ratio of SDS to amino acid of 1.4:1. Through assembly, SDS is covering the native charge of the protein and is charging the polypeptide in proportion to its length by its own negative charge. To gain a linear polypeptide it is often necessary to reduce disulfide bridges in proteins by DTT or β-mercaptoethanol. The proteins are then loaded onto a polyacrylamide gel and separated by denaturing SDS-PAGE according to their molecular weight. After electrophoresis the proteins are visualised by coomassie stain.

First the glass plates of the electrophoresis apparatus were cleaned with ethanol. Then the glass-plate sandwich of the electrophoresis apparatus was assembled according to manufacturer’s instructions using two clean glass plates and two spacers. The sandwich was locked to the casting stand and 12.5% (v/v) polyacrylamide separation gel solution was

prepared. Using a Pasteur pipette, separating gel solution was applied along an edge of one of the spacers until 2/3rd of the total height was covered and 1 ml of isopropanol was cast on the polyacrylamide solution to allow polymerisation. After polymerisation, 4% (v/v) stacking gel solution was prepared and poured. The comb was inserted and the gel was incubated for 30 to 60 min at room temperature for polymerisation. After polymerisation the electrophoresis apparatus was assembled.

The samples were prepared adding 4 µl 4x Loading buffer and 2 µl 1 M DTT to 10µl of protein sample. Protein samples were heated for 10 minutes at 100°C in a thermocycler. 1× Laemmli buffer was applied to the electrophoresis apparatus and the protein samples were transferred into the pockets of the gel. Then the gel was run for 20 minutes at 80V and for 45 minutes at 180V. After deconstruction of the chamber, the stacking gel was removed from the separation gel and the gel was placed in staining solution for 20 to 30 minutes. Afterwards the gel was washed shortly with ddH_20 and placed in destaining solution overnight. The next morning it was scanned using HP ScanJet 5370C.

4.9 Expression of scFv's

TG-1 cells from previous selection were used, which carry the genetic information of the scFv on a pIT2 vector under the control of a *lac* operon, which is blocked by glucose and induced by lactose or its non-cleavable analogue IPTG.

Expression media was prepared containing 2YT medium with 100 µg/ml ampicillin, 4.35% (v/v) glycerol, 0.1% (w/v) glucose and 1 mM IPTG, which allows inoculation and expression in a single step without manual interaction. Inoculation of the medium was performed with overnight cultures of TG-1 or HB2151 cells from previous phage display selections. Expression was performed either in a 10 ml batch using sterile 15 ml-polypropylene tubes or in 80 µl batches using 384-well polypropylene plates. The plate was sealed with an airpore tape sheet and the cultures were incubated overnight at 37°C in a shaker.

4.10 Transfection of Phagemids to HB2151

Expression of scFv's in TG-1 cells (an supE44-*amber* suppressor strain) will cause expression of all clones including those in which the scFv's contain internal TAG stop codons. Since the TAG stop codon between the scFv and the gIII gene is also suppressed, this leads to co-

expression of the scFv-pIII fusion, which tends to lower the overall levels of scFv expression, even in clones where there are no TAG stop codons in the scFv itself. To circumvent this problem, the selected phage can be used to infect HB2151 (a non-suppressor strain), which is then induced to give soluble expression of antibody fragments. ScFv genes that do not contain TAG stop codons will now yield higher levels of soluble scFv than in TG-1, but those that contain TAG stop codons will not produce any soluble scFv's (74, 120).

2YT medium containing 100 µg/ml ampicillin and 1% (w/v) glucose was prepared and inoculated with TG-1 cells carrying monoclonal phagemids. The cells were incubated overnight at 37°C and replicated into 100 µl of fresh 2YT medium containing 100 µg/ml ampicillin. After shaking for 3 hours at 37°C, $2x10^{11}$ helper phage M13K07 diluted in 100 µl 2YT medium containing 100 µg/ml ampicillin were added to each well and incubated for 1 hour without shaking at 37°C. 50 µl of 2YT medium containing 100 µg/ml ampicillin and 60 µg/ml kanamycin were added and the cultures were incubated overnight at 30°C on a shaker. The next morning, the cells were pelleted by centrifugation and the supernatant was filtered using MultiScreen-DV plate carrying PVDF filters with a pore size of 0.65 µm. The filtrate containing the phage particles was stored at 4°C until further use.

A overnight culture of HB2151 was prepared in 2YT media and was incubated overnight at 37°C on a shaker. The next morning, the culture was diluted 1:1000 in 2YT and incubated for 2 hours at 37°C. Minute amounts of previously prepared filtrate containing phage particles were added using a replicator and the culture was incubated for 1 hour at 37°C without shaking. A equal volume of 2YT medium containing 200 µg/ml ampicilin, 17.4% (v/v) glycerol and 2% (w/v) glucose was added and the cultures was incubated for 6 hours at 37°C on a shaker. The cultures was frozen to –80°C until further use.

4.11 ELISA of scFv's

ELISA of the scFv's is performed by immobilisation of the antigen and subsequent incubation of the whole TG-1 cell culture. The *pelB* leader sequence in front of the genetic information for the scFv's causes a secretion of scFv's into periplasm and medium. Detection is performed by fluorescently labelled Protein L, which binds specifically to the kappa light chain of scFv's.

100 µl of a 10 µg/ml protein solution diluted in PBS was added in each well of a 96-well Cliniplate. After incubation for 60 minutes at room temperature, the plate was washed three times with 1x PBS-T. 400 µl of blocking solution (5% (w/v) non-fat dry milk dissolved in PBS-T) were transferred in each well for blocking and the plate was incubated at room temperature for 60 minutes. After rinsing the wells three times with 1x PBS-T, a dilution row of the TG-1 cultures was prepared in blocking solution containing 4.35% (v/v) glycerol. 100 µl of diluted cultures were added and incubated for 60 minutes at room temperature. The wells were rinsed three times with PBS-T and 100 µl 0.02% (v/v) of horseradish peroxidase conjugated Protein L dissolved in blocking solution were transferred to each well. After incubation for 60 minutes at room temperature, the wells were rinsed three times with PBS-T and 100 µl 0.5 mg/ml ABTS dissolved in a ABTS-buffer were added to each well. Scanning of the absorption at 405 nm was performed using the SpectraMax 250 at different time intervals.

The ELISA for the 384-well plate was done as described previously, with the exception that 60 µl of culture were used instead of 100 µl.

4.12 Enzymatic Assays on Microarrays

Enzymatic reactions on microarrays were performed in several spotting steps. In a first spotting step, the fluorogenic substrate was applied onto polystyrene slides, followed by an optional second spotting step, in which an inhibitor was spotted onto the same positions. In a final spotting step, enzyme solution was spotted onto the same spots as the substrate and the inhibitor. To prevent the spots from drying out, glycerol was added to the enzyme solutions.

4.12.1 Horseradish Peroxidase

All components of this assay with exception of the sodium azide and the polystyrene cell culture slides were part of the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit.

10 mM stock solutions of the fluorogenic substrate 10-acetyl-3,7-dihydroxyphenoxazine were prepared as described by the manufacturer. The stock solution was diluted down to 100 µM using 1x reaction buffer and spotted onto polystyrene cell culture slides. HRP and H_2O_2 stock solutions were diluted as described by the manufacturer and two aliquots of enzyme solution containing 0.2 U/ml horseradish HRP in 20 µM H_2O_2 and 8.7% (v/v) glycerol diluted in 1x reaction buffer were generated, one with and one lacking 1% (w/v) sodium azide. Both aliquots were spotted and the slides were scanned directly after spotting and after incubation for 2 hours in a dark and humidified chamber.

The determination of the detection limits was performed as in the assay of inhibition described above with the following exceptions: The fluorogenic substrate was dissolved to a concentration of 10 µM in ddH_2O. Dilution steps of HRP ranging from 16755 to 0 molecules per spot were prepared in 20 µM H_2O_2 and 8.7% (v/v) glycerol diluted in 1x reaction buffer and spotted in sets of 64 replicates per concentration step. Scanning was performed with the same scanner settings directly after spotting and after a 2-hour incubation in a dark humidified chamber at 37°C. The mean signal intensity of each concentration was calculated and the detection limit (mean + two standard deviations) was determined.

4.12.2 Alkaline Phosphatase

For the alkaline phosphatase (AP) assay, the fluorogenic substrate 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAO phosphate) was diluted to a concentration of 5 µM using ddH_2O and spotted as described previously. In a second spotting step different concentrations of EDTA ranging from 10 mM to 1 µM dissolved in ddH_2O as well as ddH_2O solely were spotted onto the immobilised DDAO phosphate. AP solution was prepared at a concentration of 981 mU/ml in AP-buffer containing 8.7% (v/v) glycerol and spotted in a third spotting step. Scanning and analysis was performed directly after spotting.

For the detection limit assay, the fluorogenic substrate DDAO phosphate was spotted at a concentration of 5 µM. In a second spotting step different amounts of AP ranging from 9433 to 0 molecules per spot were prepared in AP-buffer containing 8.7% (v/v) glycerol and spotted in sets of 64 spots per concentration step onto the immobilised DDAO phosphate. Scanning was performed with the same scanner settings directly after spotting and after a 2-hour incubation in a dark humidified chamber at 37°C. The mean signal intensity of each concentration was calculated and the detection limit (mean + two standard deviations) was determined.

4.12.3 β-Galactosidase

The assay of inhibition was performed by preparation of a stock solution of the fluorogenic substrate resorufin β-D-galactopyranoside with a concentration of 20 mg/ml using DMSO. Working dilutions of 0.2 mg/ml were prepared using ddH_2O and spotted as described above. Dilutions of 2-Phenylethyl β-D-thiogalactoside (PETG) were prepared ranging from 3.3 mM to 0.33 µM in ddH_2O and spotted onto the substrate. In a third spotting step, a β-galactosidase dilution of 1 U/ml was prepared in PBS containing 8.7% (v/v) glycerol and spotted. Scanning and analysis was performed directly after spotting.

The determination of the detection limits was carried out as in the assay of inhibition with the following exceptions: β-galactosidase dilutions ranging from 42.57 to 0 amol per spot were prepared in PBS containing 8.7% (v/v) glycerol and spotted in sets of 64 spots per concentration step onto the fluorogenic substrate. Scanning was performed with the same scanner settings directly after spotting and after a 2-hour incubation in a dark humidified chamber at 37°C. The mean signal intensity of each concentration was calculated and the detection limit (mean + two standard deviations) was determined.

4.12.4 Cathepsin D

For the determination of the detection limit, a 1 mg/ml stock solution of BODIPY TR casein was prepared by addition of 200 µl sodium citrate buffer (pH 3.5). Stock solution was dissolved down to 20 µg/ml BODIPY TR casein using 20mM sodium citrate (pH 3.5) containing 8.7% (v/v) glycerol as buffer and spotted as described previously. Dilution steps ranging from 4013 to 0 amol per spot were prepared using 20 mM sodium citrate (pH 3.5) containing 8.7% (v/v) glycerol and spotted as described previously. Scanning was performed directly and after a 1-hour incubation at 37°C with the same scanning settings.

4.12.5 Enzymatic Signal Amplification

For the assay a dilution row of monoclonal anti-HSA was prepared in PBS and spotted in sets of 10 spots per dilution on epoxy-coated slides, with dilution steps of 0 – 10.000.000 molecules per spot. After spotting, the slides were incubated overnight at 4°C and blocked in 3% (w/v) fat-free milk powder/TBST for 60 minutes. After rinsing with TBS, one slide was subjected to incubation with 1:1000 (v/v) alkaline phosphatase-conjugated anti-mouse antibodies dissolved in blocking solution for 2 hours, while another slide was incubated in parallel 1:1000 (v/v) Cy5-labelled anti-mouse antibodies dissolved in blocking solution. After

rinsing of both slides with TBS, the slides were washed twice in TBS-T for 15 minutes each. The slides were rinsed and spun dry by centrifugation. The slide, which was incubated with the Cy5-labelled antibodies, was then scanned, while the other slide was subjected to a second spotting round with 91.1% (v/v) AP-buffer/8.9% (v/v) glycerol containing 5 µM DDAO phosphate. After spotting, the slide was scanned immediately and after 2, 3 and overnight incubation in a humidified chamber at 37°C in the dark.

4.13 Liquid Handling for Nanowell Arrays

For the precision dispensing in nanolitre volume range, a single channel piezodispensing head with control unit from sciFLEXARRAYER was used. The head consists of a glass capillary with an integrated piezoceramic. The piezo nozzle has an inner diameter of 50 µm provides a droplet volume of 360 pl size. The volume range of 100-1000 nl was achieved through multiple droplet deposition at 100 Hz and 100 V.

All assays were performed in a 96-well glass microplate with a well volume of 1.5 µl. The well-to-well distance on the plate was 2.25 mm, which is identical to the distance used in standard 1536 plastic microplates.

4.14 Cell-free Transcription and Translation of Proteins

In contrast to cell-based systems, which involve the production of a protein from an expression construct within a living cell, cell-free protein expression removes the need for cell culture. The protein is expressed *in vitro* using cellular extracts that contain all the machinery and biochemical constituents required for transcription and translation. The cellular extract may be obtained either from prokaryotic or eukaryotic cells. Cell-free systems offer a multitude of advantages in comparison to cell-based systems, such as the expression of toxic proteins and enhanced yields of proteins that were only poorly expressible or insoluble in some cell-based systems.

For cell-free transcription and translation, the RTS 100 mix was used, which is derived from a modified lysate of *E. coli*. 50 µl-batches were prepared, as recommended by the manufacturer, with 12 µl *E. coli* lysate, 10 µl reaction mix, 12 µl amino acids without methionine, 1 µl methionine, 5 µl reconstitution buffer as well as 0.5 µg of circular DNA template having a T7-promotor, which were dissolved in 10 µl ddH_2O. The mix was

incubated overnight in a 30°C incubator without shaking. For the prediluted mix, the mix was prepared as described and diluted in 1x PBS prior to expression.