DOI 10.1002/pmic.200600762

Proteomics 2007, *7*, 1786–1799

RESEARCH ARTICLE

Antibody microarray-based profiling of complex specimens: systematic evaluation of labeling strategies

Wlad Kusnezow[1], Virryan Banzon[1], Christoph Schröder[1], René Schaal[1], Jörg D. Hoheisel[1], Sven Rüffer[1], Petra Luft[2], Albert Duschl[2] and Yana V. Syagailo[1]

[1] Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany
[2] Institute of Chemistry and Biochemistry, University of Salzburg, Salzburg, Austria

Antibody microarrays have often had limited success in detection of low abundant proteins in complex specimens. Signal amplification systems improve this situation, but still are quite laborious and expensive. However, the issue of sensitivity is more likely a matter of kinetically appropriate microarray design as demonstrated previously. Hence, we re-examined in this study the suitability of simple and inexpensive detection approaches for highly sensitive antibody microarray analysis. N-hydroxysuccinimidyl ester (NHS)- and Universal Linkage System (ULS)-based fluorescein and biotin labels used as tags for subsequent detection with anti-fluorescein and extravidin, respectively, as well as fluorescent dyes were applied for analysis of blood plasma. Parameters modifying strongly the performance of microarray detection such as labeling conditions, incubation time, concentrations of anti-fluorescein and extravidin and extent of protein labeling were analyzed and optimized in this study. Indirect detection strategies whether based on NHS- or ULS-chemistries strongly outperformed direct fluorescent labeling and enabled detection of low abundant cytokines with many dozen-fold signal-to-noise ratios. Finally, particularly sensitive detection chemistry was applied to monitoring cytokine production of stimulated peripheral T cells. Microarray data were in accord with quantitative cytokine levels measured by ELISA and Luminex, demonstrating comparable reliability and femtomolar range sensitivity of the established microarray approach.

Received: July 13, 2006
Revised: January 24, 2007
Accepted: March 2, 2007

Keywords:
Antibody microarray / Detection strategies / Microspot kinetics / Protein profiling / Sensitivity

Correspondence: Dr. Wlad Kusnezow, Division of Functional Genome Analysis, DKFZ, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany
E-mail: w.kusnezow@googlemail.com
Fax: +49-6221-424 687

Abbreviations: **ALB**, human serum albumin; **Bio**, biotin; **GPTS**, (3-glycidoxypropyl)trimethoxy-silane; **Flu**, fluorescein; **IFNG**, interferon-gamma; **IL**, interleukin; **KLH**, keyhole limpet hemocyanin; **LR**, labeling ratio; **NHS**, N-hydroxysuccinimidyl ester; **PBMC**, peripheral blood mononuclear cells; **PSA**, prostate specific antigen; **rSI**, relative signal intensity; **rS/N ratio**, relative signal-to-noise ratio; **SI**, absolute signal intensity; **S/N ratio**, absolute signal-to-noise ratio; **TG**, thyroglobulin; **TF**, transferrin; **ULS**, Universal Linkage System

1 Introduction

In the last few years, antibody microarrays are continuously increasing in importance as prospective tools for the functional analysis of cellular activity and regulation as well as for diagnosis of disease [1, 2]. A wide range of immobilization [3, 4] and detection [5–7] technologies is now available for a designer of microspot assays. Nevertheless, microarray-based immunoassays, whenever applied to analysis of complex specimens, have had only limited success and had to overcome again and again their main hurdle, the failure to detect low abundant proteins. Hence, the improvement of sensitivity is going to be the most important challenge of this technology nowadays [7, 8].

The current generation of successful and sensitive antibody microarray applications for protein profiling uses mostly sandwich assay designs and strong signal generation systems by rolling circle amplification (RCA) or resonance light scattering particles (RLS) [6, 9–11]. While such systems were demonstrated to be useful for microarrays with tens and even up to a few hundreds of antibody spots, they are still difficult to apply for assays with a significantly higher content, like a 750-feature microarray published by us last year [8]. One problem is the need to find an equivalent number of matched specific and often rare secondary antibodies. In addition, the cost of such analysis may be hundreds of dollars per microarray due to the expense for the large number of secondary antibodies required and for the signal generating systems (see, *e.g.* RLS-kit; www.invitrogen.com). In summary, these approaches are quite laborious and expensive, limiting the fast processing of large numbers of samples and thus restricting this technology to a few specialized laboratories worldwide.

Investigating antibody microarrays from a physicochemical point of view, we demonstrated in our previous studies [8, 12–14] that the main physicochemical limitation of this technology is a strong dependence of microspot reaction on mass transport (or diffusion). This results in long incubation times, which may reach hundreds of hours for achieving the thermodynamic equilibrium [13, 14]. The sensitivity problem is therefore the issue of a kinetically appropriate design of microarray experiments, which may improve the performance of an assay by many orders of magnitude [14]. Therefore, current efforts focused on development of strong signal generating systems may not still be decisive in the context of the sensitivity and reproducibility of this technology [8, 13, 14]. On the contrary, the simpler and cheaper a detection approach, the more suitable it is for high-throughput analysis. In an assay optimized with respect to maximization of the reaction velocity, sensitivities in the low fM–high aM range could be demonstrated in our recent studies [13, 14], and in fact by using only simple detection strategies (*e.g.* direct protein labeling with Cy3-NHS).

However, only a few simple detection approaches like labeling with fluorescent dyes or haptens found application in antibody microarray technology. Classical Cy3/Cy5 labeling, often applied for profiling purposes [15, 16], is an approach originating from the related DNA microarray technology. Streptavidin-biotin (Bio) detection is also frequently used in profiling experiments [17–19]. Evaluations of the label-based detection strategies in some studies [18, 20] were still quite artificial as only signal intensities (SI) obtained from merely a few antigen-antibody interactions were analyzed. Therefore, there is a gap in our knowledge caused primarily by the assumption that such detection strategies yield insufficient sensitivity for protein profiling purposes.

To find an optimal detection strategy and chemistry, different Bio and fluorescein (Flu) labels and fluorescent dyes functionalized with classical N-hydroxysuccinimidyl ester (NHS)- or novel Universal Linkage System (ULS™) [21]-reactive groups (Fig. 1) were investigated in this study. NHS groups react covalently with primary amines of proteins (on lysine residues and at the amino terminus). ULS technology is based on the stable, coordinative binding properties of a platinum complex to methionines, cysteines and histidines. ULS technology was evaluated for labeling of nucleic acids in fluorescent *in situ* hybridization (FISH) and various microarray technologies [22] as well as for total serum labeling in microtiter well plate-based ELISA [23]. Their suitability for antibody microarrays is, however, more ambiguous and is also investigated here. Upon optimization and analysis of different detection chemistries, the capability of the most suitable and sensitive one was demonstrated on an example of a real expression profiling and expression profiles obtained from microarray were matched against quantitative data gained with classical ELISA and Luminex-system.

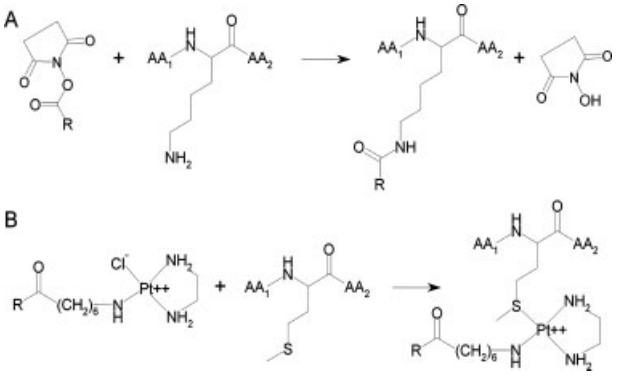

Figure 1. General reaction scheme of NHS (A) and ULS (B) labeling reagents reacting with primary amine and SH-group-containing amino acids, respectively. Note that ULS also coordinates with histidine residues in proteins. R- reporter functional groups, *e.g.* fluorophores or haptens. AA- other amino acids.

2 Materials and methods

2.1 Materials

All chemicals were purchased from Fluka (Taufkirchen, Germany), Sigma-Aldrich (Munich, Germany) or SDS (Peypin, France), unless stated otherwise. Untreated slides were purchased from Menzel-Gläser (Braunschweig, Germany). Milk powder and (3-glycidoxypropyl)trimethoxy-silane (GPTS) were obtained from Sigma-Aldrich. For the homemade antibody microarray, antibodies against human serum antigens were purchased from the following suppliers: anti-interleukin-1β1 (anti-IL1B), anti-IL6, anti-IL8, anti-IL10, anti-TGFB and anti-prostate-specific antigen (anti-PSA) were obtained from Acris Antibodies (Hiddenhausen, Germany); anti-IL2, anti-IL4, and anti-thyroglobulin (anti-TG) from HyTest (Turku, Finland); anti-interferon-γ (anti-IFNG), anti-keyhole limpet hemocyanin (KLH), anti-transferrin (TF),

Table 1. List of tested labeling substances and their suppliers

Name	MW	Supplier	max./opt. LR[a)]
Biotin group			
Detection: Cy3-labeled extravidin; Abs/Em[b)]: 548/562; MEC[c)]: 150 000			
Bio-LC-NHS	454.5	Pierce (Rockford, IL, USA)	2–4/0.5
Bio-ULS	808.3	Kreatech Biotechnology (Amsterdam, The Netherlands)	>8/0.5
Bio-PEG_4-NHS	588.7	Quanta BioDesign Ltd. (Powel, OH, USA)	1/1
Bio-PEG_{68}-NHS	3400	Nektar Therapeutics (San Carlos, CA, USA)	2/1
Fluorescein group			
Detection: Dy647-labeled anti-fluorescein; Abs/Em: 653/672; MEC: 250 000			
Flu-NHS	473.4	Molecular Probes™-Invitrogen (Karlsruhe, Germany)	4/<4
Flu-ULS	764.13	Kreatech Biotechnology	>4/>4
Flu-PEG_{68}-NHS	3400	Nektar Therapeutics	-/-
Direct labeling group			
Abs/Em: Cy3 - 548/562; Dy547 - 557/574; Dy647 653/672			
MEC: Cy3 and Dy547 -150 000; Dy647- 250 000			
Cy3-NHS	765.9	Amersham Biosciences (Uppsala, Sweden)	4/2
Dy547-NHS	735.8	Dyomics (Jena, Germany)	1/2
Dy547-ULS	1089.6	Kreatech Biotechnology	>4/0.5
Dy547-PEG-ULS	~3000	Kreatech Biotechnology	>4/0.5
Dy647-ULS	1115.7	Kreatech Biotechnology	>2/0.5

a) LR of interest were defined from two points of view: the maximal LR (max.) is the lowest LR enabling to achieve the highest overall SI on array; the optimal LR (opt.) is defined for the highest overall rS/N ratio obtained in our test system. The maximal LR represents the highest appropriate label concentration, which may be useful, *e.g.* for systems with lower sample complexity. The optimal LR were used in the comparison experiment in Fig. 7.

b) Abs/Em-absorption and emission maxima.

c) MEC-molar extinction coefficient in $(Mcm)^{-1}$.

anti-serum albumin (ALB) as well as antigens such as recombinant IFNG, KLH and TG from Sigma-Aldrich. Different labeling substances were purchased from providers as indicated in Table 1.

2.2 Production of antibody arrays

Homemade epoxysilanized slides were manufactured according to the following protocol. Untreated slides were treated with 3 M NaOH for 5 min, incubated in freshly prepared Piranha solution (3:1 ratio of sulfuric acids and hydrogen peroxide, respectively) for 2 h, rinsed four times in water and derivatized in a 100% GPTS solution at room temperature for 3 h. After silanization, GPTS-treated slides were washed thoroughly with dichloroethane and dried with gaseous N_2. The 1x PBS buffer supplemented with 0.5% trehalose was used as spotting buffer [24]. The antibodies were spotted using an SDDC-2 Micro-Arrayer from Engineering Services (Toronto, Canada). After spotting, the slides were incubated at 4°C overnight and subsequently blocked for 3 h at room temperature in PBST (1x PBS and 0.05% Tween20) supplemented with 4% of milk powder.

2.3 Sample preparation and processing

Blood plasma obtained from healthy donors was used in the optimization experiments in this study (Figs. 2–7). Blood plasma was prepared using BD Vacutainer spray-coated K_2 EDTA tubes (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Protein concentration in blood plasma was measured using the NanoOrange protein quantification kit (Molecular Probes™-Invitrogen, Karlsruhe, Germany) as indicated by the manufacturer. Plasma was supplemented with KLH, recombinant IFNG and TG to a final concentration of 5 nM each in the incubation solution. Together with ALB, these represent the set of high abundant proteins. The remaining antibodies printed on microarrays such as anti-IL4, anti-IL2, anti-IL1B and anti-PSA bind low abundant proteins, which are present in low-pM and middle-fM concentrations in blood [25].

For validation of the established antibody microarray system, peripheral blood mononuclear cells (PBMC) were prepared from buffy coats of healthy donors by density gradient centrifugation using Ficoll-Paque Plus (Amersham, Arlington Heights, IL). After washing two times with RPMI 1640 medium cells were incubated for 1 h in plastic tissue-culture dishes at 37°C in a humidified atmosphere

containing 5% CO_2. Nonadherent lymphocytes were collected and cultured without or with 100 ng/mL anti-CD3 and anti-CD28 (both generous gifts of Novartis, Vienna, Austria), with or without patulin (Sigma-Aldrich) for 48 h in RPMI 1640 + 10% FCS (heat inactivated), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The collected culture supernatants from non-stimulated, CD3/CD28- and CD3/CD28/patulin-stimulated lymphocytes were analyzed by microarray containing antibodies against following cytokines: IL1B, IL2, IL4, IL6, IL8, IL10, IFNG and TGFB1. The levels of IFNG, IL10, IL4, and IL8 were also measured by classical commercially available ELISA (BD, Belgium). To quantify levels of very low concentrated cytokines such as IL1B, IL6 and TGFB, the Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria) based on a Luminex 100 LabMap System (Luminex, Austin, TX, USA) was applied.

2.4 Sample labeling

Sample labeling using NHS-containing substances was performed according to a common protocol: diluted serum containing 3–5 mg protein/mL was labeled in 0.1 M Sodium Bicarbonate solution for 1 h at 4°C. Non-reacted dye was blocked from further reaction by addition of hydroxylamine to a final concentration of 1 M followed by 10 min incubation at room temperature. Samples were labeled with ULS-containing substances overnight in a hybridization oven at 37°C with continuous stirring in ULS-protein labeling buffer according to the manufacturer's instructions (Kreatech Biotechnology BV, Amsterdam, The Netherlands). The reaction was stopped by addition of Kreastop solution. Several concentrations of substances were used for labeling, which are subsequently indicated as "labeling ratio" or LR and were calculated as follows: *e.g.* LR of 4 means that four mols of the respective label was used to label one virtual mol of total proteins, the molecular weight of which is assumed for simplicity to be 10 kDa. In other words, to label 1 mg of total plasma proteins (corresponding to 0.1 μmol of virtual protein), a fourfold higher mol quantity of label substance (0.4 μm) was applied. LR from 0.5 to 8 were usually analyzed for every substance, aiming at labeling a substantial portion of the low-molecular weight proteins in plasma (*e.g.* cytokines between 10 and 30 kDa) even at the lowest LR used.

Non-reacted fluorescent dyes were separated from the labeled proteins using PD-10 columns (Sephadex™ G-25, Amersham Biosciences). Bio-containing reagents were separated, depending on the obtained volume, using Microcon™ YM-10 centrifugal units (maximal volume 500 μL) or Amicon Ultra 10K device (maximal volume 4 mL) from Millipore (Schwalbach, Germany). Both centrifugal filter devices were also used to adjust the concentration of labeled serum to 4 mg/mL if needed.

2.5 Incubation

Incubation of the microarrays with labeled blood plasma was performed in Flexiperms (Sigma-Aldrich), microtiter plate well-like incubation chambers, attached to the surface of the slides with double adhesive tape. The incubation solution (300 μL) was incubated at room temperature using a SlideBooster (Advalytix, Brunnthal, Germany) about 7 h, if not stated otherwise. After incubation of the microarrays with Bio- or Flu-containing substances, the slides were washed four times (5 min each)with PBST and incubated for 1 h with 10 nM extravidin (Sigma-Aldrich) labeled previously with Cy3 or 40 nM anti-Flu-DY-647 (Kreatech Biotechnology BV) also in the SlideBooster. Finally, the slides were washed five times (5 min each) with PBST. Slides incubated with plasma, which was labeled with dyes from the group of direct labels (Table 1), were washed six times (5 min each) with PBST. All slides were centrifuged 10 min at 1500 rpm to dry.

The antibody microarray system used here has the same design parameters as described previously in [13, 14], so that many quantitative reaction characteristics, such as diffusion coefficients, mass-transport constants, binding-site density, reaction durations, *etc.* can be obtained for our assay from [13, 14].

2.6 Scanning and data analysis

Fluorescence signals were recorded using a Scan Array5000 unit (Packard Biosciences, Billerica, MA, USA) and analyzed with the GenePix software package (Axon Instruments, Union City, CA, USA). The results were stored and managed in an appropriate Microsoft Access database. The complete dataset analyzed consists of more than 8000 records, including signal intensities and separately measured background signals, both obtained from more than 300 individual arrays scanned at various scanner adjustments. All data points in this work represent an average of three-to-four individual measurements. Signal-to-noise (S/N) ratio was defined as signal intensity (SI) in proportion to mean background measured on arrays between spots by a special grid. Only spots with absolute signal-to-noise (S/N) ratio of more than 2 were included in the analysis. Arrays with less than seven such spots (of nine altogether) were excluded from the analysis and graphical presentation.

When trying to compare slides incubated with differently treated samples, we found it unpractical and mathematically incorrect to present the averages from SI or S/N ratios obtained for very different antigen-antibody pairs on a chip, the way it is usually done. The contribution of particular SI or S/N ratios to the average values differs by many orders of magnitude and does not allow estimating the actual impact of the parameter of interest on the overall microarray performance. To avoid this and to bring absolute values obtained from different antibody-spots on one scale, all data presented

in Figures below were converted into relative, normalized form.

Absolute SI or S/N ratio (S_{ijk}) obtained for an *i*-antigen-antibody pair at *j*-parameter of interest on *k* replicate array were first normalized against the corresponding mean values ($\frac{1}{N}\sum_{n=1}^{N} S_{ijk}$) calculated from the total number of *i*-signals (*N*) within a group of tested parameters (time, labels, LR) for a particular antigen-antibody pair. For example, all absolute SI, which were obtained on IFNG-spots at different incubation times or for different LR, were divided by the mean value determined from these absolute SI. Subsequently, the obtained values for *K*-number of array replicates within *j*-parameter of interest were averaged out. More precisely, the calculation was done according to the following equation:

$$\overline{S}_{ij} = \frac{1}{K}\sum_{k=1}^{K}\left(\frac{NS_{ijk}}{\sum_{n=1}^{N} S_{ijk}}\right) \quad (1)$$

where $\overline{S}_{ij}$, which is subsequently indicated in the text as rSI or rS/N ratio, is the mean of relative values for *i*-antibody at *j*-parameter calculated from *K*-number of array replicates. For example, if the substance X was used for plasma labeling at LR 1, 2, 4 and 8 (j_1, j_2, j_4 and j_8) and every plasma sample was incubated on three single spotted arrays (k_1, k_2 and k_3) containing nine antibodies, then $K = 3$ for every *j*-parameter and $N = 12$ for every *i*-antibody. To determine the impact of parameter of interest on the overall array performance, $\overline{S}_{ij}$ values were averaged out over all antibodies or

$$\overline{S}_{j} = \sum_{i=1}^{I} \overline{S}_{ij}/I, \quad (2)$$

where $\overline{S_j}$ is the mean of relative values at *j*-parameter and *I* is the total number of different antibodies ($I = 9$ in example above). Subsequently, $\overline{S_j}$ is indicated as overall rSI or overall rS/N ratio.

To allow comparison between different antibodies $\overline{S_{ij}}$ and to obtain consequently proper $\overline{S_j}$ values, $\overline{S}_{ijk}$ for an antibody used in the analysis have to be present at all tested parameters. Therefore, in case that SI were missing for a parameter, the whole row of the respective antigen-antibody pair or alternatively this parameter were excluded from calculation. As will be demonstrated below such a presentation approach provides proper analytical values as well as an insight into the process influenced by the analyzed parameter. In our opinion, it can also be used for analysis of a variety of multiple other factors influencing the performance of a complex microarray assay (see graphical demonstration of this normalization method in Supporting Information).

3 Results

3.1 Experimental design

The assay used here, which was kinetically investigated and designed with respect to maximization of reaction rates on spots, results in strong and reproducible development of SI as demonstrated for IFNG, TG and KLH antigen-antibody pairs in our previous studies [13, 14]. Assessment of performance in a profiling experiment especially in the case of complex samples is a more difficult case, since it is performed with a multitude of interactions having unknown regime, velocity and duration of reaction as well as an unknown labeling extent of particular antigens. To avoid some potential fortuitousness in results and experimental interpretations, three experimental points were stipulated prior to analyzing detection approaches. (i) Two sets of antigen-antibody pairs for low (IL2, IL4, IL1B and PSA) and high (ALB, TG, TF, KLH and IFNG) abundant proteins were chosen for microarray construction to encompass two limits in bulk of interactions occurring while analyzing in a complex sample very long lasting and quickly saturated reactions, respectively. (ii) Kinetic behavior of these antigen-antibody interactions was analyzed for different detection approaches (see Section 3.3). (iii) The obtained data were analyzed and presented in relative terms enabling us to quantify and to compare the impact of parameters of interest on the whole array and without relation to a particular antigen-antibody pair (see also Supporting Information).

3.2 Optimization of labeling conditions

To minimize the denaturating effect of hydrophobic labels on proteins in blood plasma, various additives for the labeling buffer were tested in the first step. Addition of non-ionic detergents such as Triton X-100 was found to improve by manifold S/N ratios obtained on a chip as demonstrated for NHS-PEG_4-Bio and FLU-NHS in Fig. 2. This effect was also observed for different other substances having NHS- or ULS-reactive groups (data not shown). The optimal concentration of Triton X-100 in the labeling mixture was found to be 1–2%, a concentration range often applied for extraction of membrane proteins using this reagent [26]. This improvement is primarily attributed to lower and more even background signal on arrays (twice for NHS-PEG_4-Bio and threefold for FLU-NHS). In addition, SI slightly increased and even nearly doubled in the groups of high and low abundant proteins, respectively. Addition of other detergents such as Tween20 influenced the obtained S/N ratios only slightly (data not shown). In addition, continuous stirring of labeling mixture on a shaker at about 100 rpm resulted in a moderate improvement of the S/N ratios obtained on an array by about 10–20% (data not shown).

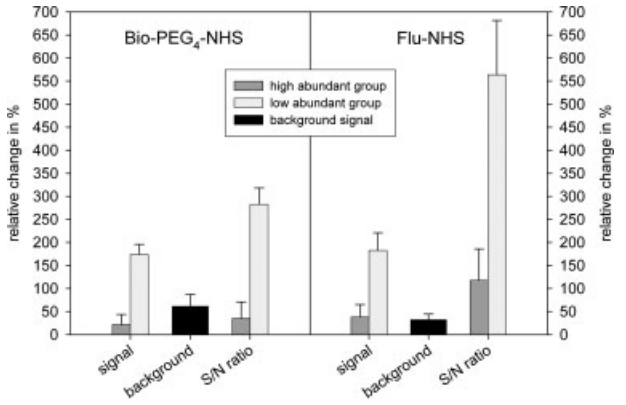

Figure 2. Relative change (in %) of SI, background signal and S/N ratios obtained on arrays upon addition of 2% Triton X-100 in the labeling mixture as compared to the data obtained without the detergent addition. SI and S/N ratios are separately presented for low and high abundant proteins. Plasma was labeled with NHS-PEG_4-Bio (left panel) and Flu-NHS (right panel).

3.3 Kinetic analysis of test system

To find optimal incubation conditions in terms of sensitivity and reproducibility and to analyze different direct and indirect detection strategies from a kinetic point of view, blood plasma samples were labeled with two Bio-substances, two fluorescent dyes, and FLU-NHS at LR of 1. Arrays were analyzed at different time points from 1 until 17 h of incubation.

The development of SI in the two groups of high and low abundant proteins was found to be extremely different. As demonstrated for the case of labeling with FLU-NHS (Fig. 3A), relative signal intensities (rSI) in the low abundant group increased linearly with the incubation time, while for high concentration proteins, saturation was reached within 1–4 h of incubation. The rSI increased in this case at 17 h compared to 1 h to various levels, ranging from insignificant (ALB) to about fivefold (IFNG). The same pattern was observed for all tested substances. Comparing 17-h *versus* 1-h incubation, the overall rSI rose about sixfold for Cy3-NHS and Flu-NHS and more than tenfold for DY547-NHS (Fig. 3B). In case of both Bio-containing substances, the overall rSI increased, however, only by about threefold. Another important parameter in our test assay was the change in S/N ratio with the time of incubation (Figs. 3C and D). The observed general trend was quite similar for all tested substances: The overall relative S/N (rS/N) ratios reached their optimum after 2–5 h of incubation (Fig. 3D), decreasing after that by about two- to- fourfold at later time points. However, the low abundant proteins attained their optimum in S/N ratios at significantly later time points (after about 5 h of incubation) (Fig. 3C).

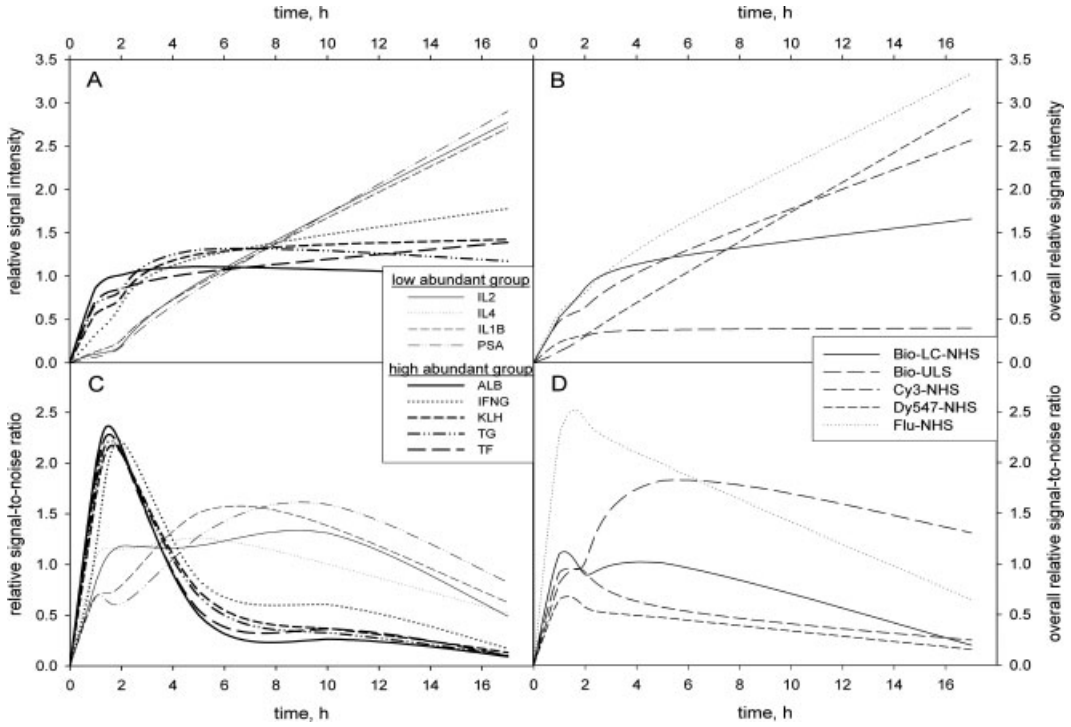

Figure 3. Normalized time-dependent development of rSI (A) and rS/N ratios (C) for nine antigen-antibody pairs (see panel on the graphs) as detected with FLU-NHS/anti-Flu system. Data are calculated according to Eq. (1), where *N* is the number of all replicates at all time points for a particular antigen antibody pair. Overall rSI (B) and rS/N ratios (D) for five different detection strategies (see panel on the graphs). Equation (2) is used for data calculation. *N* is the number of all replicates at all time points and for all tested substances for a particular antigen-antibody pair.

As the time-dependent increase of SI in the Bio/extravidin system was significantly lower in comparison to the detection by Flu/anti-Flu, we tried to analyze this further by testing different concentrations of both detecting molecules, anti-Flu (Fig. 4A) and extravidin (Fig. 4B). An increase in anti-Flu concentration from about 10 to 150 nM resulted in about 1.6-fold higher overall rSI, whereas the overall rS/N ratio, which had an optimum at about 40 nM, changed only slightly over the tested range. The overall rSI rose linearly nearly 70-fold if the concentration of extravidin was increased from 1 to 100 nM. Nevertheless, the background signal also strongly increased over this concentration range, resulting in an optimal rS/N ratio at an extravidin concentration of 10 nM.

3.4 Optimization of labeling substances

Next, all substances were analyzed separately to find the optimal concentrations for labeling of blood plasma. LR were usually from 0.5 to 8. However, high LR could not often be used for labels with high molecular weight (containing PEG or ULS) due to low solubility of these substances leading to a high labeling volume as well as a relatively large amount of them required for labeling (several mg *per* 1 mg of blood plasma). Therefore, such labeling conditions for these substances seem to be unpractical and expensive when applied for analysis of complex samples. To ensure comparability and reproducibility of data, blood plasma obtained from the same healthy donor was used in experiments on Figs. 5–7. For simplicity and clarity of presentation, the analyzed detection strategies were subdivided into three groups: Bio/extravidin, Flu/anti-Flu detection systems, and direct labels (Table 1).

3.4.1 Bio/extravidin detection system

All substances in this group could be successfully analyzed. Increase in the LR from 0.5 (Bio-LC-NHS) or 1 (Bio-ULS) to 8 resulted in a fourfold and tenfold increase in SI, respectively (Figs. 5A and D). The variability in the increase of the rSI was moderate between different proteins for both substances (mean about 20%). The lowest LR resulted in the highest rS/N ratios due to strong development of background signals with increasing concentrations of both labels. The PEG-linker containing labels showed different characteristics. With increasing LR, the SI failed to increase (Bio-PEG_4-NHS, Fig. 5B) or even decreased (Bio-PEG_{68}-NHS, Fig. 5C). The S/N behavior of the pegylated labels was also different: the background signal was not affected (Bio-PEG_4-NHS, Fig. 5B) or even substantially decreased in the case of Bio-PEG_{68}-NHS (Fig. 5B). Both PEG-labels could not be analyzed at LR 8 because absolute S/N ratios became too low under these conditions.

3.4.2 Flu/anti-Flu detection system

Labeling of blood proteins using Flu-PEG-NHS resulted in very low S/N ratios at all concentrations (data not shown). In contrast, the other labels, Flu-NHS and Flu-ULS, performed in general very well in terms of SI as well as S/N ratios. However, no general SI or S/N ratio trends for the whole set of analyzed antigens could be observed (Figs. 5E and F). Interestingly, we found that opposite trends could be easily obtained for high and low abundant proteins. *E.g.* with increasing LR of Flu-NHS (from 0.5 to 8) or Flu-ULS (from 1 to 4), SI for cytokine and PSA spots decreased by up to a factor of two to three, while signals obtained for the group of high abundant proteins increased by approximately the same factor. In general, higher labeling density did not decrease the overall S/N ratios or even improved this parameter in the case of Flu-ULS (about threefold) (Fig. 5F).

3.4.3 Direct labeling with fluorescent dyes

In general, S/N ratios obtained for this group of substances were moderate. S/N ratios for ULS-labels tended to decrease slightly with increasing LR (Figs. 6B, C and D). In contrast to

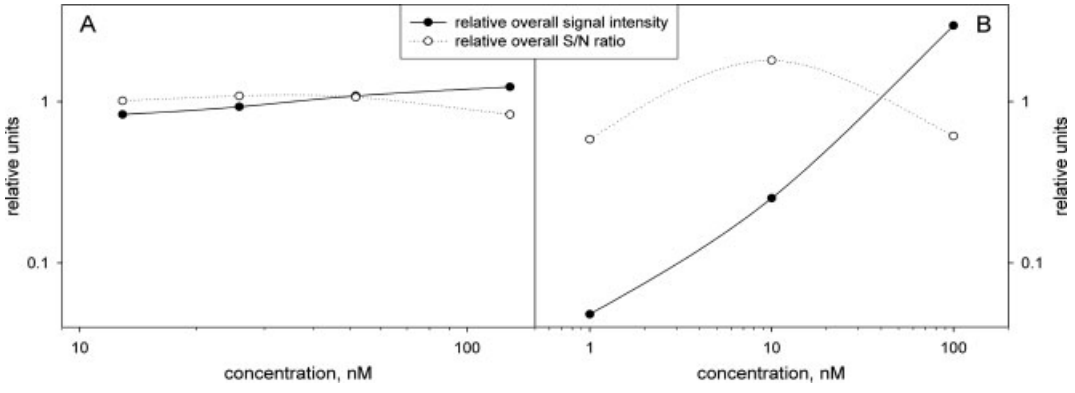

Figure 4. Overall rSI (–●–) and rS/N ratio (···○···) obtained at different concentrations of anti-Flu antibody (A) and extravidin (B). At an anti-Flu concentration of 1 nM, many spots could not be detected and, therefore, these data are not shown in panel (A). Blood plasma proteins were labeled with Flu-NHS (A) and Bio-LC-NHS (B). Equation (2) is used for data calculation. *N* is the number of all replicates at all time points for a particular antigen-antibody pair.

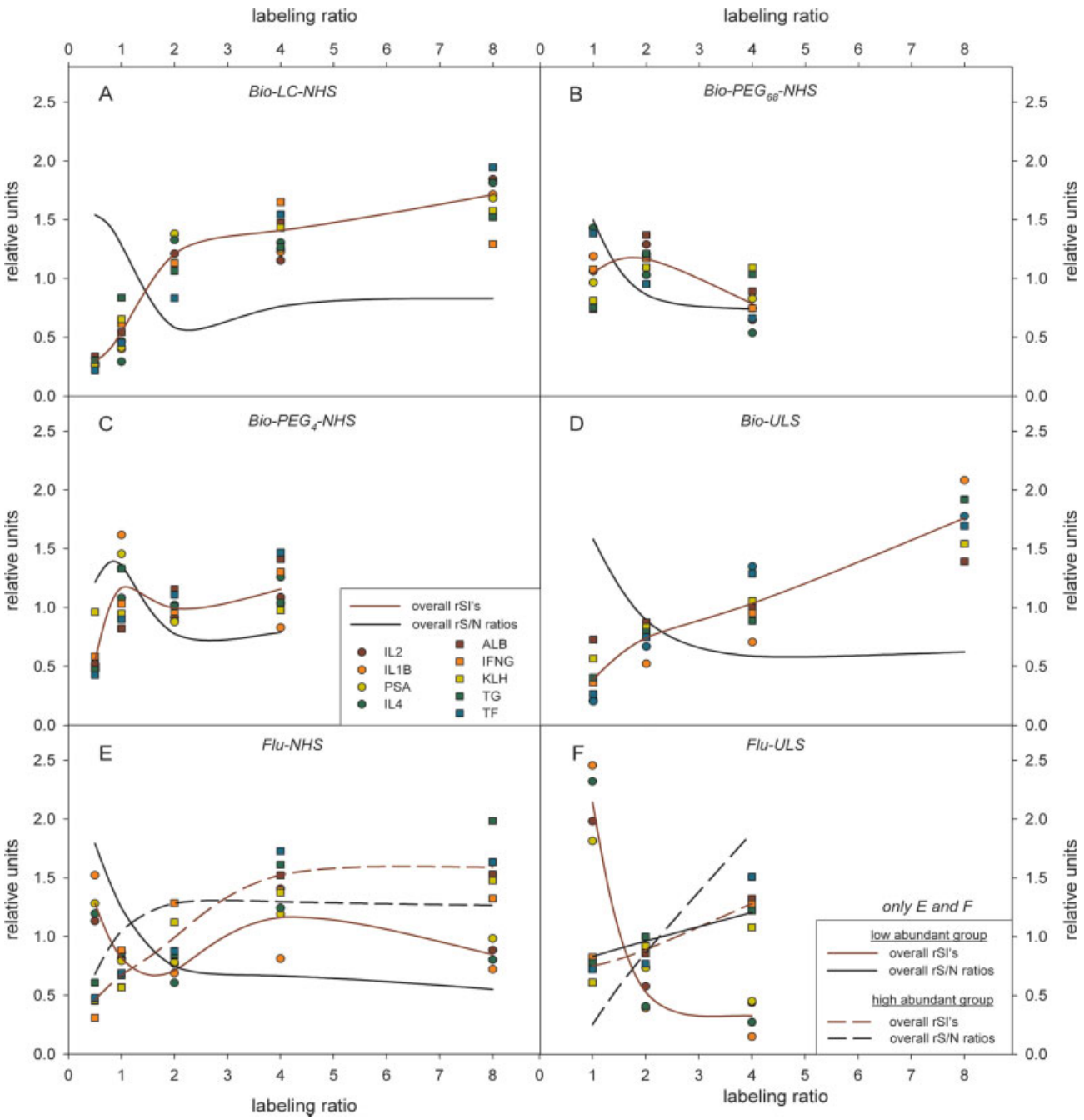

Figure 5. rSI and rS/N ratios obtained at different LR for different indirect detection strategies. Labeling substances used are: (A) BIO-LC-NHS, (B) BIO-PEG_{68}-NHS, (C) BIO-PEG_4-NHS, (D) BIO-ULS, (E) FLU-NHS and (F) FLU-ULS. Overall rSI and overall rS/N ratios are shown with brown and black lines, respectively. rSI for the particular proteins is depicted as ○ for low abundant and □ for high abundant proteins (see panel C). In the case of Flu-containing substances (E and F), overall rSI and rS/N ratios are additionally separated for these two groups: (—) low abundant and (—) high abundant proteins (see panel F). For data calculation, Eq. (1) for data points and Eq. (2) for lines are used. *N* is the number of all replicates at all LR for a particular antigen-antibody pair.

this, Dy547-NHS demonstrated saturation of SI as well as an optimum for S/N ratios at LR of 2 (Fig. 6A). Exceptionally strong SI increase over the tested concentration range (10–20-fold) was observed in this group for DY547-ULS and DY547-PEG-ULS, while the signals with NHS-labeled proteins increased only by a factor of 3–4. The differences in the relative increase of SI between different proteins were relatively low in this group of substances (usually less than 10%).

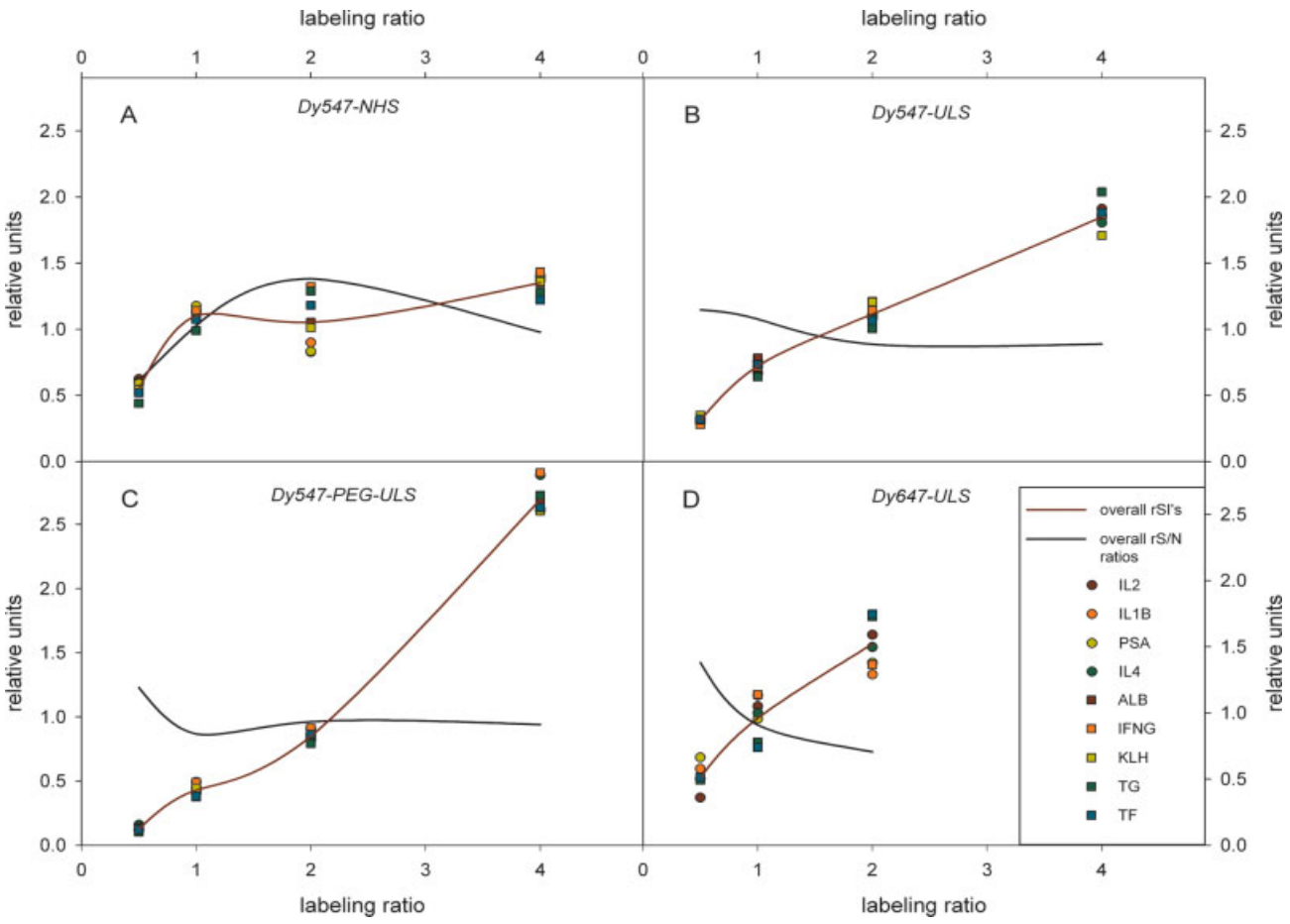

Figure 6. rSI and rS/N ratios obtained at different LR for different direct detection strategies. Labeling substances used are: (A) Dy547-NHS, (B) Dy547-ULS, (C) Dy547-PEG-ULS and (D) Dy647-ULS. Overall rSI and overall rS/N ratios are shown with brown and black lines, respectively. rSI for the particular proteins is depicted as ○ for low abundant and □ for high abundant proteins (see panel D). The data were calculated in the same way as in Figs. 5A–D.

3.5 Comparison of different detection approaches and labeling substances

The substances chosen for the next experiment were applied for labeling at their optimal LR in terms of S/N ratios (Table 1; Figs. 5 and 6). All labels with long PEG-linkers were excluded from the analysis because of much too low absolute S/N ratios obtained for these substances in previous experiments. Cy3-NHS, LR of which was also optimized (data not shown), was used in this experiment as reference substance at LR of 2.

By far the highest overall rSI on our arrays were obtained by labeling with DY647-ULS followed by Flu-NHS, Bio-LC-NHS and Bio-PEG_4-NHS (Fig. 7A). Some general differences between SI produced by NHS- and ULS-substances were observed in our experiments. Dependence of overall rSI on LR for all NHS-labels demonstrated saturation at LR of 1–2 or slightly more (Figs. 5 and 6). In contrast, overall rSI obtained for ULS-labels increased nearly proportionally with increasing LR (Flu-ULS seems to be an exception from this rule) and still remained many times lower compared to NHS-counterparts at similar LR (cf. Flu-NHS with Flu-ULS both applied at LR 4, and Bio-LC-NHS with Bio-ULS both applied at LR 0.5 in Fig. 7A). The latter effect is mainly attributed to the fact that especially the high abundant group produced many times lower SI with ULS as compared to NHS, while SI in the low abundant group remained relatively high (Figs. 7C and D).

In terms of overall S/N ratios, indirect detection strategies outperformed significantly the group of fluorescent labels (Figs. 7B, C and D) and demonstrated a nearly undetectable background signal (Fig. 7C). When using these detection approaches, S/N ratios of a few dozens and of a couple of hundreds could be obtained for low and high abundant groups, respectively (Fig. 7D). Interestingly, despite relatively low overall rSI, ULS-labels still demonstrated very high reliability in detection of low abundant antigens (Fig. 7D). The S/N ratios obtained from this group of pro-

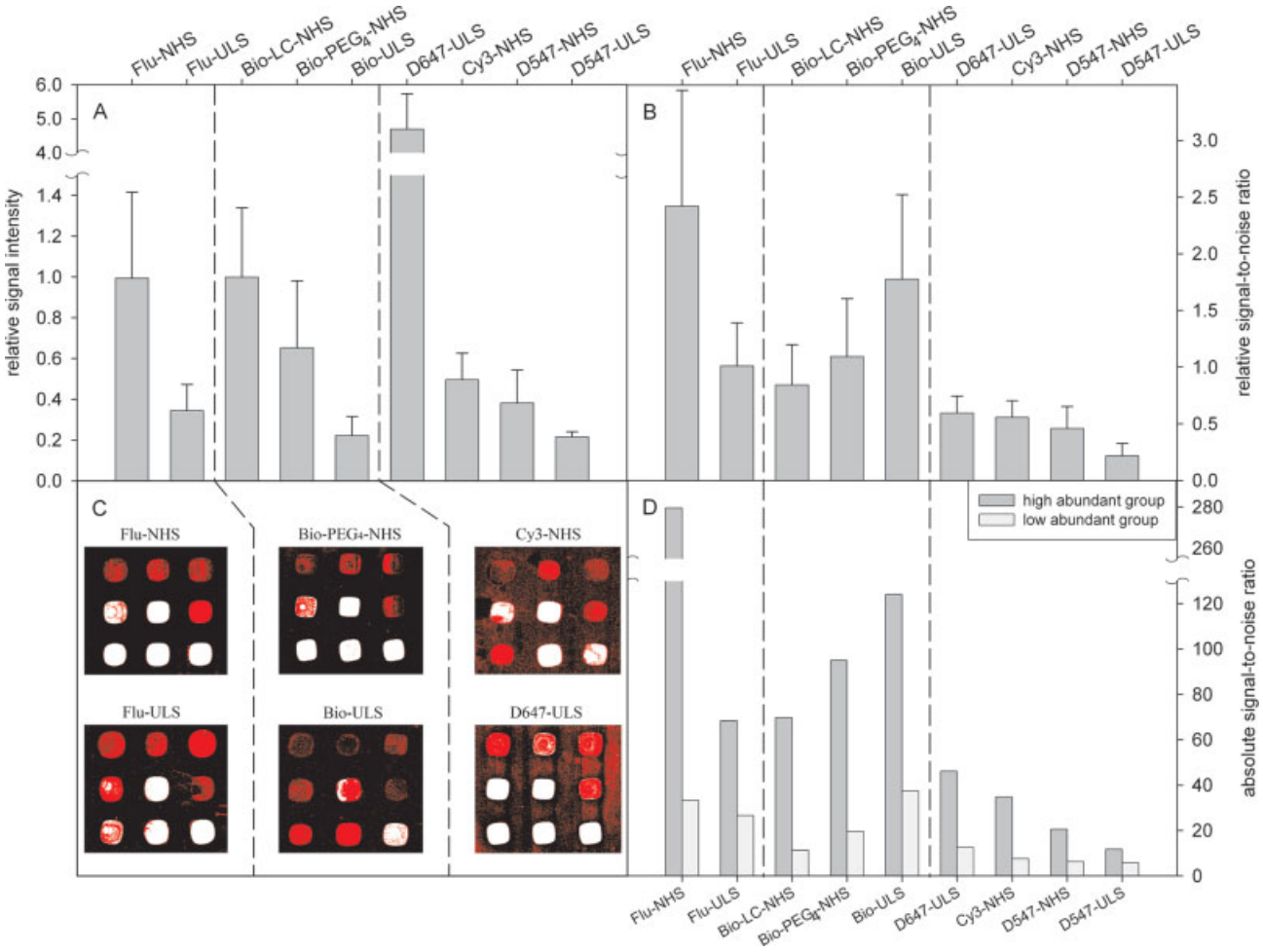

Figure 7. Comparison of overall rSI (A) and overall rS/N ratios (B) for different labeling substances. SD bars in panels (A) and (B) indicate the variations between rSI or rS/N ratios obtained for particular proteins ($\overline{S}_{ij}$ in Eq. (1)). Equation (2) was used for data calculation in panels (A) and (B). *N* is the number of all replicates at all labeling strategies used in an experiment for a particular antigen-antibody pair. (C) Pseudo-color images of arrays incubated with plasma labeled with six different substances (see corresponding bars in Fig. 7A). Antibodies were spotted as follows (from left to right): IL1B, IL4, IL2 in the first row, IFNG, TG, PSA in the second and KLH, TF, ALB in the third row. (D) Grey bars in panel (D) show mean over absolute S/N ratios for the group of high abundant proteins, white bars demonstrate the same parameter as calculated only for four low abundant proteins on the array.

teins, which usually are in the low pM–fM range in plasma, can be considered as an indication for attainable limits of detection. From this point of view, Flu-NHS, Bio-ULS, Flu-ULS, and Bio-PEG_4-NHS were assigned as the best suitable and most sensitive approaches for labeling and analysis of the complex specimens.

3.6 Assessment of reliability and sensitivity of the established system

In our previous publications, low fM sensitivities, dynamic range over five order of magnitude as well as good reproducibility could be attained in our antibody microarray system using direct Cy3 labeling [13] and Bio-PEG_4-NHS/extravidin detection strategy [14]. In particular, Bio-PEG_4-NHS-based detection was demonstrated to represent a significant improvement in comparison to the classical labeling with Cy-dyes [14]. To evaluate reliability and capability of the established antibody microarrays in real experimental conditions, a selected detection strategy, Flu-NHS/anti-Flu, was applied to analyze cytokine production by PBMC that were unstimulated, stimulated with anti-CD3 and anti-CD28, or with both antibodies plus patulin. Anti-CD3 and anti-CD28 are routinely used for activation of T cells for up-regulation of secretion of many cytokines. Patulin produced by common *Penicillium* and *Aspergillus* mould species can cause immunosuppressive effects [27], but it may also enhance immune reactions to environmental allergens. It was shown that patulin modulates strongly the level of several T cell-derived cytokines and skews the Th1/Th2 balance towards Th2 cells, which favors the development of allergies [28, 29]. The same samples were used for analysis of cytokine profile by means

of ELISA, Luminex-system and antibody microarrays (eight repetitions), which were expanded by additional anti-cytokines antibodies against IL6, IL8, IL10 and TGFB (Fig. 8).

The expression profiles obtained matched well between antibody microarrays and the other methods. The relative changes in cytokine levels as obtained from ratios P1/P2, P2/P3 or P1/P3 varied usually within 15%, while, in the case of IL1B, this variation was stronger at about 30–40%. Unfortunately, a row of the analyzed cytokine concentrations close or below the LOD of ELISA or Luminex-system and therefore could not be reliably measured by these methods. Moreover, ELISA completely failed to detect IL4 in any sample. In contrast to this, all cytokines in all samples could be successfully detected by antibody microarrays. In accordance with our previous reports, sensitivities in the middle-low fM range can be concluded on the basis of the quantitative data for the established microarray approach. The lowest cytokine concentrations as measured by antibody microarrays correspond to the following values: in the range of 10 pM for IL8 and IL10, about 1 pM for IFNG, 250 fM for IL8, 100–500 fM for TGFB (multimeric forms) and about 90 fM for IL1B, which was detected with lowest S/N ratio in this row of about three- to- fourfold.

4 Discussion

Physicochemical considerations have to be the starting point for design and development of practically every facet of antibody microarray technology. Due to the prolonged and complex microspot kinetics, SI as well as S/N ratio as measured at any time point is only a snapshot of a complex dynamic process, depending on affinity parameters, analyte concentration, mass-transport characteristics, reaction regime and adsorptive forces in the system. This situation requires a preceding experimental specification of a model microspot assay. Otherwise, it may lead to fortuitous interpretation of the experimental results [8].

A detection approach reflects the current state of the overall signal development on a complex chip. Our results indicate that SI and S/N ratios strongly changed in a non-linear manner and in dependence on applied detection strategy at short incubation times (Fig. 3). In general, microspot reaction predominantly develops at the beginning in proportionality to $\sqrt{t}$ and changes its regimes to $\sim t$ modus at longer incubation times [8, 12, 14]. In addition, spots detecting high abundant proteins reached saturation on our arrays within the early hours of incubation. Therefore, the contribution of particular interactions to the overall SI strongly changes at initial time points (Fig. 3A). Labels modifying these initial non-linear processes lead to a situation where it is difficult to establish a sequence of "good-bad" substances without relation to the time parameter (Fig. 3B). In contrast to this, an acceptable consistency in the row best-worst-case scenario could be observed at much longer incubation time. Therefore, incubation of about 5–10 h seems to be a good compromise, enabling to obtain good reproducibility as well as to detect all proteins with high S/N ratios.

Similarly to antigen-antibody binding on the spot, the interaction of detector molecules (*e.g.* extravidin or anti-Flu antibody in this study) with bound antigens can be limited in the same manner by mass transport. Using our theory of mass transport-dependent microspot reaction described in [8, 12–14] a simple relation can be derived for the concentration of detector molecules (L_d) required to detect a certain fractional occupancy of antibodies by analyte molecules (ϕ):

$$L_d = \frac{\pi \rho R \varphi}{4Dt} \quad (3)$$

where D is diffusion coefficient of detector molecules in cm^2/s (or effective diffusion coefficient in the case of stirring), t is the incubation time during the detection step in s, ρ is the density of binding sites on a spot in mol/cm^2 and R is the spot radius in cm. It has to be emphasized that Eq. (3) is only valid if the following conditions are true: (i) the reaction occurs in mass-transport limit or $k_+ \gg 4D/(\pi\rho\varphi R)$, where k_+ is the association rate constant of the detector molecules in $cm^3/(mol \times s)$; (ii) the dissociation of the bound analyte molecules is insignificant; (iii) L_d do not decrease because of the nonspecific adsorption on surface; (iv) $L_d \gg K_d$, where K_d is the binding affinity constant of the detector molecule in M (*e.g.* extravidin 10^{-15} M). The latter point ensures the fundamental possibility to detect all bound antigens according to the law of mass action. Taking now parameters from our previous publication as $D = 10^{-7} cm^2/s$, $\rho = 11^{-11}$ mol/cm^2 [14] and assuming R = 0.01cm, φ = *1* and t = 3600 s (1 h), one needs $L_d \approx$ 220 nM to be able to detect all bound analyte molecules on a saturated spot. In general, using any realistic parameters, at least some dozens or even many hundreds nM concentration of the detector molecules would be needed according to Eq. (3).

Consequently, the complete conversion of bound analyte molecules into SI can be supposed only for non-saturated spots using realistic optimal concentrations of detector molecules. Assuming the same parameter as above, φ-value would be in the range of $\varphi \leq 0.1$ in our experiments (few dozens nM), while the typical concentration of secondary antibodies in the literature of about 0.6 nM (0.1 μg/mL) [6, 10, 30] would enable to detect properly only spots with about $\varphi \leq 0.001$. In addition, a significant or even proportional to L_d change of SI could be expected as found in the experiment shown in Fig. 4. This effect may be additionally strengthen by a strong energy of nonspecific adsorption of the detector molecules as known, *e.g.* for streptavidin and its derivatives [31] (Fig. 4B). In accordance with our data, strong dependence of the generated SI on streptavidin concentration was also observed for the case of RP microarray [32]. It has to be emphasized that the maximal detectable φ-value may dramatically decrease, if other prerequisites for successful binding of detector molecules listed above are not fulfilled. Of note, and as a consequence, multi-step detection approaches as applied for

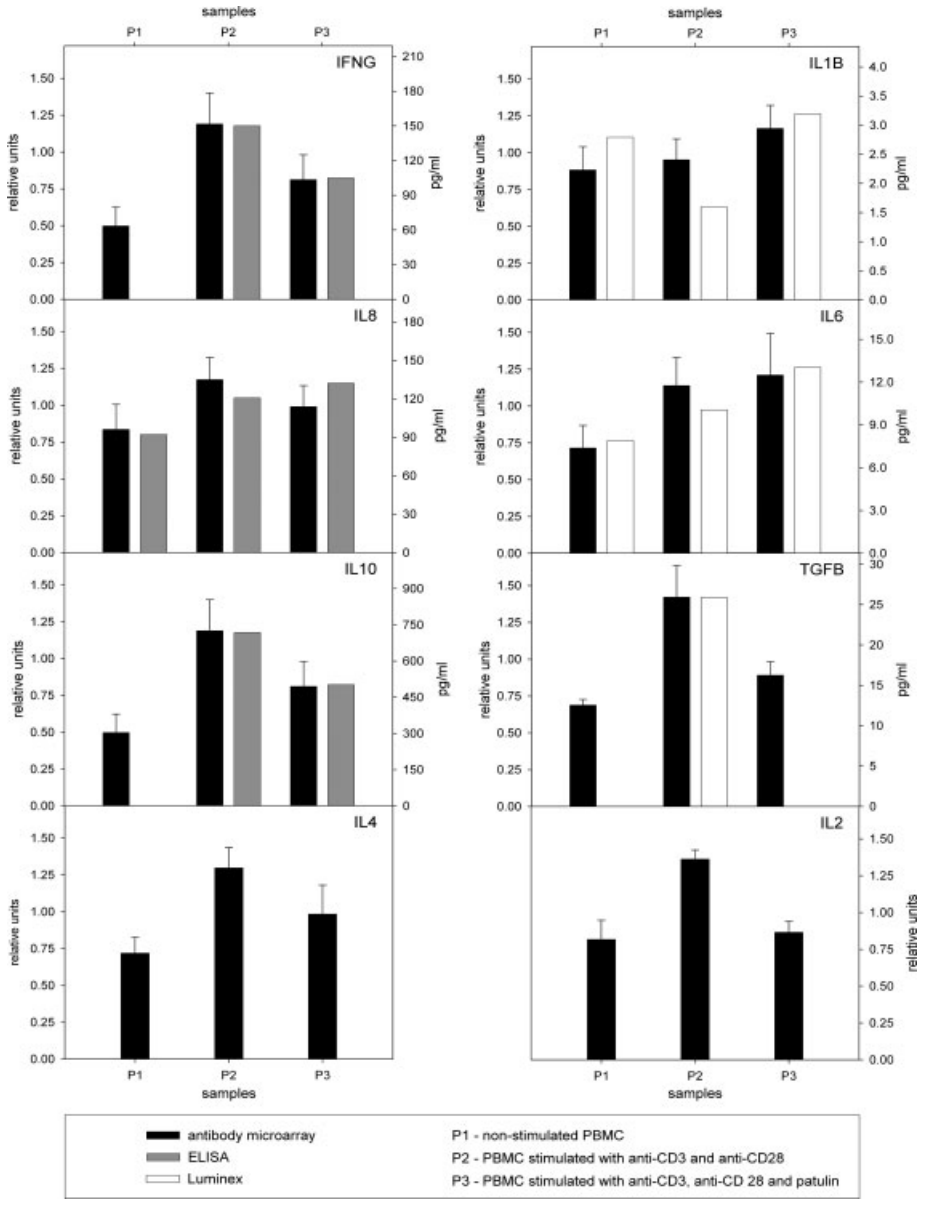

Figure 8. Comparison of expression profiles of different cytokines produced by unstimulated (P1), anti-CD3/anti-CD28 (P2) und anti-CD3/anti-CD28/patulin (P3) stimulated peripheral T cells as measured with antibody microarrays, ELISA and Luminex (see legend). The data obtained by different methods were first calculated using Eq. (1) with *K = 8, N = 24* for three parameters P1, P2 and P3 (i_1, i_2 and i_3) and compared to each other graphically. Microarray data are presented as relative units (left y-axis) and ELISA and Luminex data as pg/mL (right y-axis).

microarrays do not automatically result in stronger SI and concentration of detector molecules is a compromise between maximal S/N ratios on a chip and maximal detectable φ. Moreover, as shown in Fig. 7, the analyzed two-step detection approaches seem in general not to produce much stronger SI as compared to direct fluorescent labeling.

An explanation for better performance of two-step detections may be the fact that small amounts of free, non-reacted labels are still present after gel filtration. While fluorescent dyes sticking to the array surface directly give rise to high background fluorescence, haptens may not be easily accessible by the detector molecules due to steric hindrance. Because of the utmost importance of this issue, a new separation approach based on affinity towards label has been developed for highly efficient ULS removal (Kreatech's ULS-Trap columns, manuscript in preparation). Additionally, the detection complexes (primary antibody-antigen-detection molecules) may be stabilized by di- (antibody) or oligovalent (extravidin) binding of the detector molecules to the bound antigens. Especially during long washing steps, when dissociation of specifically bound analytes can be a strong factor, this may play a decisive role.

Detection approaches also represent an additional physicochemical parameter for an assay. While the diffusivity of proteins in solution can be only slightly influenced by low molecular weight substances, hydrophobic labels can obviously destabilize proteins leading to a partial denaturation and aggregation of proteins and can increase the non-

specific adsorption energies of proteins on surface. Experimental evidence of this is the increased background signal. As a result, these processes may significantly reduce the concentration of reactive analyte molecules as well as reaction rates on spots (for details of the reaction mechanisms see [8, 14]). These factors may contribute to the manifold differences of SI obtained for labels with comparable reactivity and molecular extinction coefficients (Fig. 7, *cf.* Dy647-ULS and Dy547-ULS).

Preservation of protein stability is, therefore, a crucial precondition and is mostly the result of empirical optimization. Independent of label used, non-ionic detergents such as Triton X-100 in the labeling buffer seem to affect more equal labeling conditions due to increased homogenization and solubility of proteins. This may prevent local over-labeling effects leading to aggregation of denatured proteins and increased background signal (Fig. 2). Depending on protein nature, Triton X-100 binds to the protein surface *via* hydrophobic and polar interactions enclosing the protein in a micelle [33]. Disrupting protein-protein/lipid interactions [26], this detergent still preserves and even improves the activity and stability of proteins [34, 35]. Interestingly, the addition of Triton X-100 especially improved the detectivity of low abundant proteins (Fig. 2), which are known to be associated with high abundant components or even lipid fraction in blood [25, 36, 37]. Most probably, this detergent acts as a sort of extracting agent for low abundant plasma proteome.

In addition, PEG-chemistry as applied for labeling is expected to have a protective effect on proteins. PEG binds strongly water molecules (two to three H_2O per each ethylene glycol subunit) [38] and creates a sort of hydrophilic coat around protein molecules. However, known properties of pegylated proteins such as shielding epitopes for recognition by an antibody [39] and strongly decreased mobility of PEG-modified proteins [40] seem to be responsible for malfunctioning of all long-PEG containing substances in this study. Quite the reverse, Bio-PEG_4-NHS performed well in comparison to the long-PEG labels as well as Bio-LC-NHS, indicating that a hydrophilic linker of optimal length may still have positive effects [41]. In confirmation of this, Geho *et al.* [32] could significantly improve the S/N ratios on an RP microarray by application of a pegylated form of streptavidin in a multi-step detection approach.

Although comparable performance for NHS- and ULS-derivatized substances is attainable, one should still pay attention to some fundamental differences between these two labels. Maximal SI are attainable with NHS-labels at an LR of about 1–2 and it is unpractical to use much higher label concentrations in view of any performance aspect. Applying ULS-labels, better performance may be still achieved even with higher LR. Obviously, the difference in the observed labeling efficiency between ULS- and NHS-substances may be based on the difference in target amino acids: thio-groups are rarer in proteins than primary amines, and a considerable part of them resides in the core of large proteins and may be, therefore, hardly accessible to the ULS-labels.

In general, the detection by hapten labeling using one of the four best performing substances (Flu-ULS, Bio-ULS, Flu-NHS and Bio-PEG_4-NHS) can be recommended for analysis of complex biological samples, whereas fluorescent dyes are reasonable to use only for samples with low complexity or for instance for interaction studies. While the presented approach is ready for use, optimal values obtained in this study for incubation time, LR, or concentration of detector molecules can be even corrected up with improvements in incubation buffer composition, surface chemistry or with decreasing samples complexity. Of note, the blood plasma used here for optimization is the most complex human proteome [25].

Today's best-performing protein profiling strategies by antibody microarrays are mostly based on sandwich detection systems aiming to amplify strongly the SI [6, 9–11]. However, these approaches are more expensive and complex, may result in a decrease in the number of detectable antigen molecules as well as increase loss of bound antigen molecules because of longer detection, and washing steps. The fM sensitivities in a protein profiling experiment are attainable even with simple detection approaches as demonstrated here as well as in our previous studies [13, 14]. Moreover, since the microarray data matched well with profiles obtained by alternative immunometric methods (Fig. 8), even small differences of 1.5 in antigen concentrations can be reliably detected by the established microarray approach. Furthermore, the observed expression profiles are also in good accordance with literature. Under comparable experimental conditions, stimulation of PBMC with anti-CD3/anti-CD28 is known to increase strongly the production of IFNG, IL4 [42, 43], IL2 and IL10 [42, 44]; an additional stimulation with patulin results in about 1.5–2-fold decrease of levels of IFNG and IL4 [28].

The suitability of such detection approaches entails a row of crucial benefits including simplicity, low costs and high speed of analysis. A larger spreading of this technology, which is today mostly limited to only a few laboratories in the world, is facilitated by these advantages.

In conclusion, this study provides a comprehensive investigation of various detection strategies. A series of Bio and Flu labels used as haptens with subsequent extravidin and anti-Flu detection, respectively, as well as fluorescent dyes were applied for analysis of complex specimens on antibody microarray. Reproducible analysis of different detection approaches could be done only at relatively long incubation times because of non-linear development of SI at initial time points. In case of Bio/extravidin and Flu/anti-Flu detection strategies, interaction of detector molecules did not guarantee the complete conversion of bound antigen molecules into SI. Probably due to some steric reasons, the two-step detection still performed better as compared to labeling with fluorescent dyes. Labels also influence the reaction rates on spots as well as nonspecific adsorption of proteins on surface. To prevent proteins from destabilization by labeling, addition of Triton X-100 in labeling buffer was found to improve strongly

the obtained S/N ratios especially in case of low abundant proteins. NHS- and ULS-derivatized labels targeting dissimilar amino acids significantly differed in labeling efficiency of individual proteins, while they still revealed comparable performance in detection of low abundant proteins. Flu-ULS, Bio-ULS, Flu-NHS and Bio-PEG_4-NHS were identified as most suitable labels enabling to attain many dozen-folds S/N ratios for low abundant blood cytokines. Finally, analyzing cytokine production by peripheral T cells, microarray expression profiles were found to match well with quantitative data obtained by ELISA and Luminex as well as with data obtained from literature. It indicates high reliability and fM sensitivities attained in the established antibody microarray approach.

We would like to thank Dr. Paul van der Hoeven, Dr. Frank J. M. Opdam and other co-workers from Kreatech Biotechnology BV for helpful discussions and critical reading of the manuscript. The work was funded by grants of the German Federal Ministry of Education and Research (BMBF) as part of the programs Proteomics, DHGP and NGFN as well as the MolTools project of the European Commission.

5 References

[1] Kusnezow, W., Hoheisel, J. D., *Biotechniques* 2002, Suppl, 14–23.

[2] Haab, B. B., *Mol. Cell. Proteomics* 2005, *4*, 377–383.

[3] Kusnezow, W., Hoheisel, J. D., *J. Mol. Recognit.* 2003, *16*, 165–176.

[4] Kusnezow, W., Pulli, T., Witt, O.,Hoheisel, J. D., in: Schena, M., (Ed.), *Protein microarrays*, Jones and Bartlett Publishers, Sudbury 2004, pp. 247–284.

[5] Schweitzer, B., Kingsmore, S. F., *Curr. Opin. Biotechnol.* 2002, *13*, 14–19.

[6] Saviranta, P., Okon, R., Brinker, A., Warashina, M. *et al., Clin. Chem.* 2004, *50*, 1907–1920.

[7] Espina, V., Woodhouse, E. C., Wulfkuhle, J., Asmussen, H. D. *et al., J. Immunol. Methods* 2004, *290*, 121–133.

[8] Kusnezow, W., Syagailo, Y. V., Goychuk, I., Hoheisel, J. D., Wild, D. G. *Expert Rev. Mol. Diagn.* 2006, *6*, 111–124.

[9] Rai, A. J., Gelfand, C. A., Haywood, B. C., Warunek, D. J. *et al., Proteomics* 2005, *5*, 3262–3277.

[10] Haab, B. B., Geierstanger, B. H., Michailidis, G., Vitzthum, F. *et al., Proteomics* 2005, *5*, 3278–3291.

[11] Schweitzer, B., Roberts, S., Grimwade, B., Shao, W. *et al., Nat. Biotechnol.* 2002, *20*, 359–365.

[12] Klenin, K. V., Kusnezow, W., Langowski, J., *J. Chem. Phys.* 2005, *122*, 214715.

[13] Kusnezow, W., Syagailo, Y. V., Ruffer, S., Klenin, K. *et al., Proteomics* 2006, *6*, 794–803.

[14] Kusnezow, W., Syagailo, Y. V., Rueffer, S., Baudenstiel, N. *et al., Mol. Cell. Proteomics* 2006, *5*, 1681–1696.

[15] Hamelinck, D., Zhou, H., Li, L., Verweij, C. *et al., Mol. Cell. Proteomics* 2005, *4*, 773–784.

[16] Sreekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R. *et al., Cancer Res.* 2001, *61*, 7585–7593.

[17] Knezevic, V., Leethanakul, C., Bichsel, V. E., Worth, J. M. *et al., Proteomics* 2001, *1*, 1271–1278.

[18] Li, Y., Reichert, W. M., *Langmuir* 2002, Published on Web, page est: 9.2.

[19] Madoz-Gurpide, J., Wang, H., Misek, D. E., Brichory, F., Hanash, S. M. *Proteomics* 2001, *1*, 1279–1287.

[20] Wiese, R. *Luminescence* 2003, *18*, 25–30.

[21] Heetebrij, R. J., Talman, E. G., v Velzen, M. A., van Gijlswijk, R. P. *et al., Chembiochem.* 2003, *4*, 573–583.

[22] van Gijlswijk, R. P., Talman, E. G., Janssen, P. J., Snoeijers, S. S. *et al., Expert Rev. Mol. Diagn.* 2001, *1*, 81–91.

[23] van Gijlswijk, R. P., Talman, E. G., Peekel, I., Bloem, J. *et al., Clin. Chem.* 2002, *48*, 1352–1359.

[24] Kusnezow, W., Jacob, A., Walijew, A., Diehl, F., Hoheisel, J. D., *Proteomics* 2003, *3*, 254–264.

[25] Anderson, N. L., Anderson, N. G., *Mol. Cell. Proteomics* 2002, *1*, 845–867.

[26] London, E., Brown, D. A., *Biochim. Biophys. Acta* 2000, *1508*, 182–195.

[27] Fung, F., Clark, R. F., *J. Toxicol. Clin. Toxicol.* 2004, *42*, 217–234.

[28] Wichmann, G., Herbarth, O., Lehmann, I., *Environ. Toxicol.* 2002, *17*, 211–218.

[29] Marin, M. L., Murtha, J., Dong, W., Pestka, J. J., *J. Toxicol. Environ. Health* 1996, *48*, 379–396.

[30] Zhou, H., Bouwman, K., Schotanus, M., Verweij, C. *et al., Genome Biol.* 2004, *5*, R28.

[31] Schetters, H., *Biomo. Eng.* 1999, *16*, 73–78.

[32] Geho, D., Lahar, N., Gurnani, P., Huebschman, M. *et al., Bioconjug. Chem.* 2005, *16*, 559–566.

[33] Singh, S. K., Kishore, N., *J. Phys. Chem. B Condens. Matter Mater. Surf. Interfaces Biophys.* 2006, *110*, 9728–9737.

[34] Choi, N. S., Hahm, J. H., Maeng, P. J., Kim, S. H., *J. Biochem. Mol. Biol.* 2005, *38*, 177–181.

[35] Stanton, J. B., Goldberg, A. F., Hoppe, G., Marmorstein, L. Y., Marmorstein, A. D., *Biochim. Biophys. Acta* 2006, *1758*, 241–247.

[36] Tirumalai, R. S., Chan, K. C., Prieto, D. A., Issaq, H. J. *et al., Mol. Cell. Proteomics* 2003, *2*, 1096–1103.

[37] Cho, S. Y., Lee, E. Y., Lee, J. S., Kim, H. Y. *et al., Proteomics* 2005, *5*, 3386–3396.

[38] Harris, J. M., Chess, R. B., *Nat. Rev. Drug Discov.* 2003, *2*, 214–221.

[39] Bhadra, D., Bhadra, S., Jain, P., Jain, N. K., *Pharmazie* 2002, *57*, 5–29.

[40] Kozlowski, A., Harris, J. M., *J. Control Release* 2001, *72*, 217–224.

[41] Caliceti, P., Veronese, F. M., *Adv. Drug Deliv. Rev.* 2003, *55*, 1261–1277.

[42] Raziuddin, S., Bahabri, S., Al-Dalaan, A., Siraj, A. K., Al-Sedairy, S., *Clin. Immunol. Immunopathol.* 1998, *86*, 192–198.

[43] Verhoef, C. M., Van Roon, J. A., Vianen, M. E., Glaudemans, C. A. *et al., Scand. J. Immunol.* 1999, *50*, 427–432.

[44] Schwarz, M., Majdic, O., Knapp, W., Holter, W., *Immunology* 1995, *86*, 364–371.

