Eur Biophys J (2002) 31: 179–184
DOI 10.1007/s00249-001-0204-0

ARTICLE

**Lutz Jermutus · Reto Kolly · Zeno Földes-Papp
Jozef Hanes · Rudolf Rigler · Andreas Plückthun**

Ligand binding of a ribosome-displayed protein detected in solution at the single molecule level by fluorescence correlation spectroscopy

Received: 11 September 2001 / Revised: 5 December 2001 / Accepted: 6 December 2001 / Published online: 30 January 2002

Abstract Interaction of a single-chain antibody fragment (scFv) with its cognate antigen while still attached to the ribosome was studied by fluorescence correlation spectroscopy (FCS). In experiments with purified scFv, FCS was capable of resolving the difference in diffusion time between free and antibody-bound labelled antigen. Ribosome-displayed antibody fragments generated by in vitro translation, in which neither the protein nor the mRNA leaves the ribosome owing to the absence of a stop codon and stabilizing buffer conditions, could be shown to specifically bind the antigen. The antibody-antigen interaction was specific, as shown by inhibition or displacement with unlabelled antigen and by control experiments with a non-cognate antibody fragment.

Keywords Fluorescence correlation spectroscopy · Single molecule analysis · Ribosome display · Single-chain antibody fragment · scFv

Introduction

Recent advances in fluorescence and micro-mechanical technology have decreased the detection limit down to the level of single molecules. Fluorescence correlation spectroscopy (FCS), based on the detection of single molecules in a confocal volume element (Eigen and Rigler 1994; Nie et al. 1994; Rigler 1995), provides a highly sensitive way to study biomolecular interactions (Rigler and Elson 2001). In FCS, a mixture of soluble fluorescent molecules with different molecular weights can be analyzed by a single measurement. Up to three species with different diffusion times can be resolved and their amounts quantitated using a single fluorophore. Therefore, the amount of ligand being in the free state (fast diffusion) and the receptor-bound state (slow diffusion of the complex) can be quantitated and measured as a function of concentration and, thereby, affinity and interaction kinetics can be determined. Moreover, measurements can be performed in small volumes (down to 1 μL) within a short experimental time (1–60 s). During this time period the fluorescence intensity of molecules in a small volume element (0.2 fL) is recorded and correlated in time. By using such a small measurement volume, background fluorescence can be minimized since contaminating fluorescence molecules will only rarely enter the measurement volume. The fluorescence fluctuations in the measurement volume are due to the variations in concentration of the fluorescent dye by Brownian diffusion of single molecules. From the autocorrelation function the average number of molecules and their respective diffusion coefficients can be calculated.

Since the detection method is so sensitive, it would be attractive to study the protein directly after in vitro translation. Moreover, upon in vitro translation of mRNA, a ternary mRNA-ribosome-polypeptide complex can be formed by omitting a stop codon and by using stabilizing buffer conditions, such that the nascent protein remains attached to the ribosome translating the encoding mRNA. Formation of this complex, in which the genetic information and the protein encoded by that gene are linked, allows evolution and selection of binding proteins (Amstutz et al. 2001).

Several previous reports on FCS described the interaction of ligands with their receptor embedded in the cell

L. Jermutus[1] · R. Kolly · J. Hanes[3] · A. Plückthun (✉)
Biochemisches Institut, Universität Zürich,
Winterthurerstrasse 190, 8057 Zürich, Switzerland
E-mail: pluecthun@biocfebs.unizh.ch
Fax: +41-1-6355712

Z. Földes-Papp[2] · R. Rigler (✉)
Department of Medical Biophysics (MBB),
Karolinska Institutet, 17177 Stockholm, Sweden
E-mail: rudolf.rigler@mbb.ki.se
Fax: +46-8-326505

Present addresses:
[1]Cambridge Antibody Technology,
The Science Park, Melbourn, Cambridgeshire SG8 6JJ, UK

[2]Clinical Immunology and Jean Dausset
Laboratory, Graz University M.S. and Hospital,
Auenbruggerplatz 8, 8036 Graz, LKH, Austria

[3]Evotec Neuroscience GmbH,
Schnackenburgallee 114, 22525 Hamburg, Germany

membrane (Hasler et al. 1999; Rigler et al. 1999; Schuler et al. 1999; Van Craenenbroeck and Engelborghs 1999; Wohland et al. 1999) or in solution (Boldicke et al. 2000; Hink et al. 2000). In this study we report for the first time the detection by FCS of the specific interaction of both purified and in vitro translated, ribosome-bound antibody fragments with their labelled cognate antigen.

Ribosome-displayed antibody fragments are detected, characterized and quantified at subnanomolar concentrations. In particular, it is shown that the ribosome-displayed antibody fragments specifically bind the antigen in solution. These experiments prove that cell-free translation provides sufficient material to study protein-protein interactions by FCS, provided that one partner can be fluorescently labelled.

Materials and methods

Peptide synthesis

The GCN4 (7P14P) peptide (NH_2-RMKQLEPKVEELL-PKNYHLENEVARLKKLVGER-CO_2H) was synthesized and N-terminally labelled with rhodamine green by Research Genetics (Huntsville, Ala., USA). Peptide synthesis was carried out with FMOC solid phase synthesis on chlorotrityl resins (Novabiochem, La Jolla, Calif., USA) on Advanced Chemtech 396-5000 multiple peptide synthesizers (ACT, Louisville, Ky., USA). Protected amino acids (Sygena-Genzyme, Cambridge, Mass., USA) were double coupled at 8-fold excess for 1 h. Resins were washed with DMF (EM Science, Gibbstown, NJ, USA) and MeOH (Fisher, Pittsburgh, Pa., USA) and coupled to rhodamine green carboxylic acid. The succinimidyl ester of the peptide was cleaved from the resin (Molecular Probes, Eugene, Ore., USA) in reagent R (TFA, EDT, thioanisole, anisole). The TFA mixture containing the peptide in solution was precipitated in ether and washed extensively. Preparative HPLC purification was accomplished by a gradient of 0–80% acetonitrile (EM Science, Gibbstown, NJ, USA) in 0.1% TFA. Lyophilization of the various fractions yielded the synthetic peptides as a TFA salt, as verified by MALDI-TOF using a Voyager mass spectrometer (PerSeptive, Foster City, Calif., USA). All other chemicals used in this study were of highest purity available from both Sigma and Fluka.

Protein purification and ELISA

The single-chain Fv antibody fragment (scFv) c11 was purified from *Escherichia coli* as described previously (Hanes et al. 1998). Purified protein at 10 nM concentration was assayed for functionality by ELISA according to published protocols (Hanes et al. 1998). For inhibition, the protein was equilibrated for 2 h with 0, 1 and 100 nM of labelled or unlabelled GCN4-7P14P peptide and then allowed to bind to immobilized antigen on a microtiter plate.

Fluorescence correlation spectroscopy

The FCS instrument used is a prototype built by Carl Zeiss. It is based on the ConfoCor device of Zeiss/Evotec and has been described previously (Rigler et al. 1998). Samples of 20 µL total volume were placed on a chambered coverglass (sterile borosilicate, Nunc) and measured at ~18 °C. The detection branch of the optical system (emission-measuring system) was focused in the object plane. Fluorescence emissions were collected by the objective (numerical aperture 1.2) and passed through a dichroic mirror. The transmitted light was focused by a lens on the pinhole. Since the excitation and emission optical pathways overlapped, out-of-focus emissions and scattered light were efficiently blocked by the pinhole. A pinhole size of 30 nm was used throughout the study. The emissions were focused by a lens and measured by an actively quenched avalanche photodiode (photon counting mode, SPCM 131-AQ, EG&G). The electronic output signals were autocorrelated by a two-channel ALV5000 correlator on a PC board. Data analysis was performed using in-house developed software based on the Marquardt non-linear least-squares parameterization for calculating the normalized mean square deviation between data and model (Marquardt 1963). Each sample was measured at least twice and each experiment was carried out at least three times.

In FCS the fluctuations δI of the emitted intensity around its mean value $<I>$ are measured and correlated. The autocorrelation function $G(\tau)$ is defined as:

$$G(\tau) = \langle I(t) + I(t+\tau)\rangle = \langle I\rangle^2 + \langle \delta I(t)\delta I(t+\tau)\rangle \quad (1)$$

where the brackets indicate the time average.

The thermal motion (diffusion) through a Gaussian confocal volume element $G'(\tau)$ is given as (Rigler et al. 1993):

$$G'(\tau) = 1 + \frac{1}{N}\left(\frac{1}{1+\frac{\tau}{\tau_D}}\right)\left(\frac{1}{1+\left(\frac{\omega}{z}\right)^2\frac{\tau}{\tau_D}}\right)^{1/2} \quad (2)$$

with:

$$\tau_D = \omega^2/4D \quad (3)$$

where N is the mean number of fluorescent molecules in the volume element, τ_D the characteristic diffusion time, D the translational diffusion coefficient of the fluorescing species, and ω and z are the half axes of the cylindrical volume element. The parameters ω/z were determined from measurements using only the fluorescent dye rhodamine green. Equation (2) or its expansion to two or three components (molecular species) was fitted to the data of the FCS experiments.

In vitro translation for ribosome display

Ternary ribosomal complexes (mRNA-ribosome-scFv) were prepared as described, except that heparin was omitted (Hanes et al. 2000), and are depicted in Fig. 1. After translation, the diluted translation mix was layered on top of 2.5 mL of 20% sucrose in washing buffer (50 mM Tris-OAc, pH 7.5, 50 mM MgAc, 150 mM NaCl) and centrifuged through the sucrose cushion for 1 h at 100,000×*g*. Prior to FCS analysis, the pellet was air-dried on ice for several minutes and dissolved in 100 µL washing buffer by gentle agitation.

Results and discussion

Using FCS we detected the specific interaction of an scFv fragment with its cognate antigen on the single molecule level either with free scFv or while the scFv was still bound to the ribosome. For analyzing the free scFv, it was expressed in the periplasm of *E. coli* and subsequently purified. For analyzing the ribosome-associated protein, it was produced in a cell-free system based on an *E. coli* S30 extract with a construct and under conditions previously described (Hanes and Plückthun 1997; Hanes et al. 1998, 1999, 2000). We used the scFv c11 recognizing the 7P14P variant of the yeast GCN4 transcription factor with a dissociation constant (K_D) of 40 pM (Hanes et al. 1998). The peptide antigen was

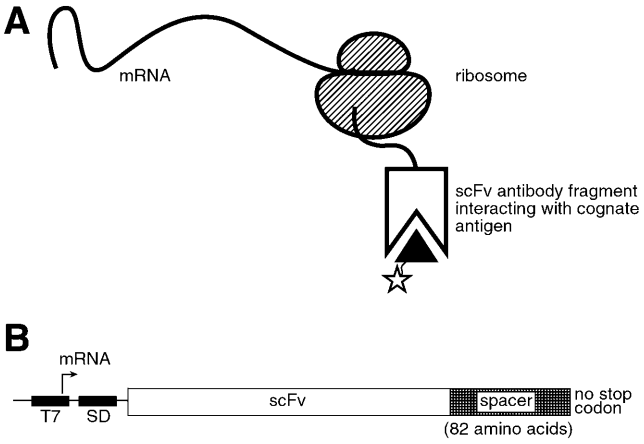

Fig. 1 **A** Schematic representation of the ribosomal complexes used in this report. ScFv antibody fragments were displayed on the ribosome for FCS using a rhodamine green-labelled antigen. **B** DNA construct used for generation of in vitro translated, ribosome-bound scFv antibody fragments. For details see Hanes et al. (2000)

N-terminally labelled with the fluorophore rhodamine green. The labelled 33-mer peptide was tested for functionality in an inhibition ELISA and shown to inhibit the binding of the purified scFv to immobilized antigen in the same concentration range as the unlabelled peptide (data not shown). This demonstrated that the binding of the scFv c11 to the antigen was not influenced by the coupling of the fluorophore.

FCS with purified antibody

Detection of antibody-antigen complex

The scFv c11 was expressed in the periplasm of *E. coli* and purified to homogeneity as described before (Hanes et al. 1998) (data not shown). For FCS measurements, purified scFv at 1 μM concentration was incubated with 1 nM labelled antigen. After 60 min incubation to reach equilibrium, fluorescence intensity fluctuations were recorded and autocorrelated. We could observe a molecular species with a diffusion time (τ_{Diff}) of about 260 μs, whereas the labelled peptide alone gave diffusion times of about 200 μs (Table 1). While the diffusion time of the antibody-antigen complex is as expected and corresponds to a globular molecule in the 30 kDa range, τ_{Diff} of the free peptide (molecular weight of 3 kDa) appears unusually high. This might be explained by the peptide structure. While the wild-type GCN4 sequence folds into a helical homodimeric coiled-coil structure, the introduction of two prolines at positions 7 and 14 in the 7P14P variant of the leucine zipper GCN4 results in the formation of a random coil, as observed by circular dichroism (Berger et al. 1999). Such an unstructured peptide would not be expected to be as compact as a globular protein of the same molecular weight and will diffuse more slowly through the volume element (Pan et al. 1997; Penkett et al. 1998). Furthermore, we found that the preparation of the labelled peptide contained approximately 25% free rhodamine green dye after extended storage. The diffusion time of the free dye was therefore included in all further data fittings.

The interaction is specific

Two types of experiments demonstrated that the observed species with 260 μs diffusion times was a specific antibody-antigen complex. When the purified antibody was preincubated with an excess of unlabelled antigen (1 mM) to ensure complete saturation of binding sites and then incubated with the labelled peptide, or when complexes between the antibody and the labelled antigen were displaced by the same excess of unlabelled peptide, only a molecular species with a τ_{Diff} of about 200 μs, the free labelled peptide, could be detected (Table 1). These findings clearly showed that the complex with a τ_{Diff} of 260 μs is indeed the result of the specific association of the antibody fragment c11 with its cognate antigen, the GCN4 peptide derivative 7P14P.

FCS with ribosome-displayed antibody from in vitro translation

Antigen binding of the displayed antibody is specific

mRNA without a stop codon was generated from a PCR construct as illustrated in Fig. 1. The mRNA was purified and added to an S30 *E. coli* translation system. After a short incubation at 37 °C, ternary complexes of mRNA, ribosome and the folded polypeptide encoded by the mRNA (Fig. 1) were stabilized by rapid chilling and raising the Mg^{2+} concentration to 50 mM (Hanes

Table 1 Results from FCS studying purified scFv c11 with rhodamine green-labelled antigen

Experiment[a]	τ_{Diff} (μs)	*n*
Rhodamine green-labelled 7P14P GCN4 variant (antigen) at 1 nM	200 ± 15	7
Purified scFv c11 (1 μM) with 1 nM labelled antigen	260 ± 7	5
Purified scFv c11 (1 μM) with 1 nM labelled antigen after prior inhibition or displacement with 1 mM free, unlabelled antigen	200 ± 8	5

[a] Data were fitted by a two-state model as detailed in Materials and methods. Fitting the data with the three-state model resulted in significantly higher errors. In all experiments the second component was free rhodamine dye with a diffusion time of about 56 μs. τ_{Diff}: diffusion time; *n*: number of independent experiments

et al. 2000). Since the in vitro translation mix showed high autofluorescence primarily coming from the *E. coli* extract (data not shown), a purification step was necessary to reduce the background noise. For this purpose, the stopped translation mix was centrifuged through a sucrose cushion, and the pelleted ribosomal complexes were resuspended in washing buffer. This centrifugation reduced autofluorescence about 10-fold, which was sufficient to allow FCS experiments. The purified ribosomal complexes displaying scFv c11 were incubated with rhodamine green labelled antigen. FCS detected the presence of a species with a slow diffusion time of about 1.2 ms which made up between 10 and 15% of the population of fluorescent molecules (Table 2). Both inhibition and displacement experiments, carried out as described above, demonstrated the specificity of this complex, which disappeared after incubation with an excess of unlabelled antigen both before and after the incubation with labelled peptide (Fig. 2, Table 2).

The fraction of specifically labelled ribosomal complexes can be increased by removing free labelled antigen

To further increase the percentage of labelled ribosomal complexes, we first incubated the stopped translation mix with the rhodamine green-derivatized peptide antigen and then purified the ternary ribosome display complexes through a sucrose cushion. Hereby, we wanted to eliminate free labelled antigen which should not be able to enter the cushion. As expected, the percentage of the component with a diffusion time of 1.2 ms increased to 50% of the fluorescent molecule population.

The interaction is specific

In a further control experiment, we translated the anti-hag scFv (Hanes and Plückthun 1997) in the identical DNA construct and under identical conditions as the c11 scFv. This antibody fragment recognizes an unrelated peptide antigen, the hemagglutinin peptide of influenza virus (NH_2-KNSYPYDVPDYASLRS-CO_2H), and does not cross-react with the GCN4-7P14P peptide in inhibition ELISA (data not shown). Ternary ribosomal complexes of anti-hag scFv were incubated with labelled GCN4 variant peptide and purified over a sucrose cushion. FCS measurements did not reveal any component with a diffusion time in the ms range. Rather, we observed a molecular entity with a diffusion time of about 275 μs (Table 2). The same species had appeared in the preparation of the ribosomal complexes displaying c11, both after inhibition and displacement of these complexes with unlabelled peptide (Table 2). We therefore concluded that this species was most likely due to non-specific interactions of the dye molecule with other proteins of the *E. coli* extract used for in vitro translation.

Characterization and quantification of the ribosome-displayed scFv antibody

The diffusion time of the ribosomal complex of 1.2 ms corresponds to a molecular weight of about 2–3 MDa. This is in good agreement with the calculated size of the ternary ribosomal complex, which is mainly due to the molecular weight of the *E. coli* ribosome of 2.6 MDa.

Furthermore, we could calculate from these experiments the mean molar amount of functional ribosomal complexes displaying active scFv c11, recognized by its ability to bind labelled antigen. In a 100 μL in vitro translation we found this to be 0.1 pmol. This calculation was based on the average of 10 independent FCS measurements where data analysis determined the total number of fluorescent molecules and the relative fraction of each fluorescent component. Based on these values we could calculate the absolute amount of molecules with a diffusion time of 1.2 ms, which we had previously identified as the signal of the ternary ribosomal complexes. Owing to the high affinity of the displayed antibody and the excess of labelled antigen used, we assumed this number to be virtually identical to the total number of active ternary ribosomal complexes. This value is close to the 0.04 pmol complexes from a 110 μL reaction observed in our first description of the ribosome display method (Hanes and Plückthun 1997). The 2.5-fold difference can be explained by both improvements of the protocol (Hanes et al. 1999) and the high affinity of scFv c11 (Hanes et al. 1998).

Table 2 Results from FCS studying in vitro translated, ribosome-displayed scFv c11 with rhodamine green-labelled antigen

Experiment[a]	τ_{Diff1} (μs)	τ_{Diff2} (μs)	*n*
Ribosome-displayed scFv c11 with 1 nM labelled 7P14P GCN4 peptide (antigen)	1250 ± 140	275 ± 5	10
Ribosome-displayed scFv c11 with 1 nM labelled antigen after prior inhibition or displacement with 1 mM free, unlabelled antigen	280 ± 6	n.d.	4
Ribosome-displayed scFv hag with 1 nM labelled non-specific antigen	275 ± 5	n.d.	10

[a] Data were fitted by a three-state model as detailed in Materials and methods. In all experiments the third component was free rhodamine dye with a diffusion time of about 56 μs. τ_{Diff}: diffusion time; *n*: number of independent experiments; n.d.: not detectable (no further fluorescent species could be detected using a three-state model to fit the data)

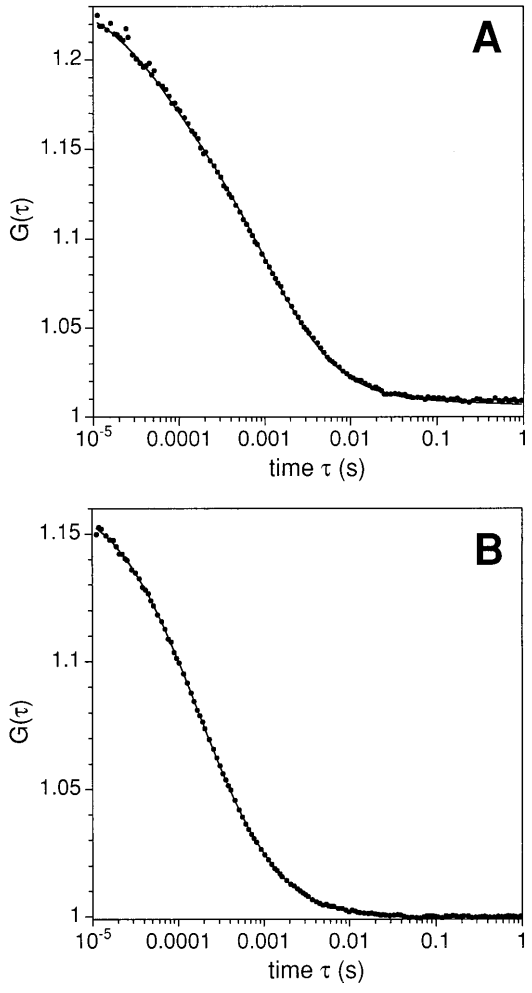

Fig. 2A, B FCS measurements with ribosome-displayed scFv antibody fragment c11. The correlation of fluorescence intensity fluctuations over time is plotted. The concentration of the labelled peptide antigen was 1 nM in all experiments. **A** Complex between ribosome-bound scFv c11 antibody fragment with its cognate rhodamine green-labelled antigen by using a sucrose cushion purification. Data were evaluated with a three-component model where the diffusion time of the first component was fixed to τ_{Diff} of the free rhodamine green dye (56 μs). The diffusion time of the second component in this experiment was 280 μs (error of 0.9%), τ_{Diff} of the third component was 1.21 ms (error of 7.7%) and the number of particles was 4.34 per volume element (*black dots*: observed data points; *line*: calculated correlation). **B** Results of both inhibition and displacement of the ribosome-bound antibody-antigen complex. Data could only be evaluated (with reasonable fitting errors) with a two-component model. The diffusion time of the first component was fixed to τ_{Diff} of the free rhodamine green dye (56 μs). The diffusion time of the second component in this experiment was 271 μs (error of 2.0%) and the number of particles 5.91 per volume element. (*black dots*: observed data points; *line*: calculated correlation)

From the measurement of the diffusion time of the purified scFv c11, a translational diffusion constant (D_{trans}) of 6.18×10^{-11} m^2 s^{-1} was calculated, which corresponds to a particle with an apparent hydrodynamic radius of 4.1 nm. This correlates well with a previously published report (Hink et al. 2000) describing D_{trans} for a scFv-GFP fusion of 6.05×10^{-11} m^2 s^{-1}. For the ribosome-displayed scFv, a value for D_{trans} of 1.27×10^{-11} m^2 s^{-1} was obtained, yielding a Stokes radius of 17.3 nm. Noller and colleagues recently reported (Cate et al. 1999) that the 70S ribosome has maximum dimensions of 21 nm.

In conclusion, this study demonstrated that ribosomal complexes displaying ligand-binding proteins can be purified sufficiently for FCS analysis. Moreover, the protein amount produced by cell-free expression is sufficient to characterize the binding properties of the synthesized polypeptide by FCS, even when stoichiometrically bound to the ribosome. Importantly, these results independently prove that a protein can be tethered to the ribosome, bound via its peptidyl-tRNA, and still functionally interact with its ligand, the principle of ribosome display (Hanes and Plückthun 1997). Recently, FCS has been used to characterize the binding of a phage-displayed antibody fragment (Lagerkvist et al. 2001). Here, we show the utility of FCS as an alternative method to study antibody-antigen interaction in solution even if one analyte is at low concentration, such as upon its production by cell-free translation. Currently, however, FCS works optimally at sub-nanomolar concentrations of fluorescent dyes, which limits the biological systems that can be efficiently and quantitatively studied by this method to interactions with sub-nanomolar dissociation constants.

The striking feature of this methodology is the short experimental time needed to quantitate the amount of functional ribosomal complexes, which would usually require performing a selection on a cognate ligand and to subsequently quantify the selected mRNA by real-time PCR analysis or Northern blotting (Hanes and Plückthun 1997). We therefore envision the presented technique to be useful in high-throughput applications of ribosome display, such as for quality control in automated selections or to quantitate the influence of various experimental conditions in optimization strategies. Moreover, these results set the stage for fluorescence sorting of ribosomal complexes according to the protein displayed. Both specific binding and low autofluorescence of the sucrose-cushion-purified ribosomal complexes could be demonstrated in the experiments described, thereby allowing the detection of a fluorescent ligand interacting with the displayed protein. Such flow sorting experiments could be a powerful step in automated evolution experiments. In further steps, we will need to demonstrate the possibility to sort ribosomes and to render such a process compatible with the demands of ribosome display regarding temperature and buffer composition.

Acknowledgements L.J. was supported by a predoctoral Kekulé fellowship from the Fonds der Chemischen Industrie (Frankfurt, Germany). This work was supported in part by the Human Frontier Science Program, grant RGP 0253/2001-M100.

References

Amstutz P, Forrer P, Zahnd C, Plückthun A (2001) *In vitro* display technologies: novel developments and applications. Curr Opin Biotechnol 12:400–405

Berger C, Weber-Bornhauser S, Eggenberger J, Hanes J, Plückthun A, Bosshard HR (1999) Antigen recognition by conformational selection. FEBS Lett 450:149–153

Boldicke T, Struck F, Schaper F, Tegge W, Sobek H, Villbrandt B, Lankenau P, Bocher M (2000) A new peptide-affinity tag for the detection and affinity purification of recombinant proteins with a monoclonal antibody. J Immunol Methods 240:165–183

Cate JH, Yusupov MM, Yusupova GZ, Earnest TN, Noller HF (1999) X-ray crystal structures of 70S ribosome functional complexes. Science 285:2095–2104

Eigen M, Rigler R (1994) Sorting single molecules: application to diagnostics and evolutionary biotechnology. Proc Natl Acad Sci USA 91:5740–5747

Hanes J, Plückthun A (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA 94:4937–4942

Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A (1998) Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. Proc Natl Acad Sci USA 95:14130–14135

Hanes J, Jermutus L, Schaffitzel C, Plückthun A (1999) Comparison of *Escherichia coli* and rabbit reticulocyte ribosome display systems. FEBS Lett 450:105–110

Hanes J, Jermutus L, Plückthun A (2000) Selecting and evolving functional proteins *in vitro* by ribosome display. Methods Enzymol 328:404–430

Hasler K, Panke O, Junge W (1999) On the stator of rotary ATP synthase: the binding strength of subunit δ to $(\alpha\beta)_3$ as determined by fluorescence correlation spectroscopy. Biochemistry 38:13759–13765

Hink MA, Griep RA, Borst JW, Hoek A van, Eppink MH, Schots A, Visser AJ (2000) Structural dynamics of green fluorescent protein alone and fused with a single chain Fv protein. J Biol Chem 275:17556–17560

Lagerkvist AC, Földes-Papp Z, Persson MA, Rigler R (2001) Fluorescence correlation spectroscopy as a method for assessment of interactions between phage displaying antibodies and soluble antigen. Protein Sci 10:1522–1528

Marquardt DW (1963) An algorithm for least-squares estimation of nonlinear parameters. J Soc Ind Appl Math 11:431–441

Nie S, Chiu DT, Zare RN (1994) Probing individual molecules with confocal fluorescence microscopy. Science 266:1018–1021

Pan H, Barany G, Woodward C (1997) Reduced BPTI is collapsed. A pulsed field gradient NMR study of unfolded and partially folded bovine pancreatic trypsin inhibitor. Protein Sci 6:1985–1992

Penkett CJ, Redfield C, Jones JA, Dodd I, Hubbard J, Smith RA, Smith LJ, Dobson CM (1998) Structural and dynamical characterization of a biologically active unfolded fibronectin-binding protein from *Staphylococcus aureus*. Biochemistry 37:17054–17067

Rigler R (1995) Fluorescence correlations, single molecule detection and large number screening. Applications in biotechnology. J Biotechnol 41:177–186

Rigler R, Elson ES (2001) Fluorescence correlation spectroscopy: theory and applications. (Springer series in chemical physics, vol 65) Springer, Berlin Heidelberg New York

Rigler R, Mets Ü, Widengren J, Kask P (1993) Fluorescence correlation spectroscopy with high count rate and low background: analysis of translational diffusion. Eur Biophys J 22:169–175

Rigler R, Földes-Papp Z, Meyer-Almes FJ, Sammet C, Volcker M, Schnetz A (1998) Fluorescence cross-correlation: a new concept for polymerase chain reaction. J Biotechnol 63:97–109

Rigler R, Pramanik A, Jonasson P, Kratz G, Jansson OT, Nygren P, Stahl S, Ekberg K, Johansson B, Uhlen S, Uhlen M, Jornvall H, Wahren J (1999) Specific binding of proinsulin C-peptide to human cell membranes. Proc Natl Acad Sci USA 96:13318–13323

Schuler J, Frank J, Trier U, Schafer-Korting M, Saenger W (1999) Interaction kinetics of tetramethylrhodamine transferrin with human transferrin receptor studied by fluorescence correlation spectroscopy. Biochemistry 38:8402–8408

Van Craenenbroeck E, Engelborghs Y (1999) Quantitative characterization of the binding of fluorescently labeled colchicine to tubulin *in vitro* using fluorescence correlation spectroscopy. Biochemistry 38:5082–5088

Wohland T, Friedrich K, Hovius R, Vogel H (1999) Study of ligand-receptor interactions by fluorescence correlation spectroscopy with different fluorophores: evidence that the homopentameric 5-hydroxytryptamine type 3As receptor binds only one ligand. Biochemistry 38:8671–8681