Fluorescent T7 display phages obtained by translational frameshift

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ABSTRACT

Lytic phages form a powerful platform for the display of large cDNA libraries and offer the possibility to screen for interactions with almost any substrate. To visualize these interactions directly by fluorescence microscopy, we constructed fluorescent T7 phages by exploiting the flexibility of phages to incorporate modified versions of its capsid protein. By applying translational frameshift sequences, helper plasmids were constructed that expressed a fixed ratio of both wild-type capsid protein (gp10) and capsid protein fused to enhanced yellow fluorescent protein (EYFP). The frameshift sequences were inserted between the 3′ end of the capsid gene and the sequence encoding EYFP. Fluorescent fusion proteins are only formed when the ribosome makes a −1 shift in reading frame during translation. Using standard fluorescence microscopy, we could sensitively monitor the enrichment of specific binders in a cDNA library displayed on fluorescent T7 phages. The perspectives of fluorescent display phages in the fast emerging field of single molecule detection and sorting technologies are discussed.

INTRODUCTION

A major challenge in the post-genome era is to unravel protein–protein interactions which are involved in transmission of information within cells, the so-called interactome. Identification of these interacting molecules would highly support the understanding of how cells function and also provide leads to the development of new drugs. To map the interactome a range of high-throughput screening technologies is employed, including yeast two-hybrid, mass spectroscopy of co-immunoprecipitated protein complexes, protein arrays and phage display, all with their particular strengths and weaknesses (1–4). For phage display these strengths are the large libraries that can be created, the level of control over the binding conditions, the ease of identifying interactors by PCR (the phenotype and genotype are coupled) and the possibility to screen for interactions with almost any kind of substrate, from small chemical compounds to post-translationally modified proteins or complete cells (5–7). Since its introduction 20 years ago, phage display has played a crucial role in selecting interacting molecules from large libraries. The filamentous phage M13 predominated for a long time in the phage display field and is still a key player for the selection of recombinant antibody fragments (8–11). However, for the assembly of the M13 phage particles all viral proteins need to be transported through the bacterial inner membrane. Sequence and folding characteristics of cytosolic proteins are often incompatible with this translocation process, which imposes serious constraints on the display of cDNA libraries on M13. To circumvent this drawback of M13, lytic phages like T4, T7 and lambda have been adapted for display applications (12–14). Lytic phage display does not rely on the *Escherichia coli* secretory mechanism, because the phages are assembled in the cytoplasm and released by lysis of the bacterium.

The commercially available T7 phage display system (T7Select; Novagen) is at the moment the most widely used lytic phage display system and has successfully been employed to reveal interactions between proteins and between proteins and chemical compounds (6,15–18). The T7 phage allows the fusion of large protein fragments, up to 1000 amino acids in a low copy number, to its capsid protein. Further advantages are the availability of an efficient packaging system and the fact that due to its fast amplification, multiple selection rounds can be performed per day.

Here we report a method to construct fluorescent T7 display phages. Fluorescent phage particles enable a direct visualisation of the interaction between displayed proteins and their binding partners, which has several advantages. The enrichment of specific binders in a display library can be followed directly by standard fluorescence microscopy during the affinity selection procedure, obviating the need for more laborious and time consuming procedures like ELISA

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and plaque lift assays. Directly monitoring the enrichment allows making a more considered decision on when to stop the selection procedure, thereby avoiding unnecessary amplification steps. Furthermore, future developments in advanced microscopic techniques may enable the detection of single fluorescent phages in extremely small volumes, which opens the door to sensitive biolibrary sorting platforms (19,20).

To obtain fluorescent phage particles we incorporated the enhanced yellow fluorescent protein (EYFP) in the phage capsid by fusing it to the T7 capsid protein (gp10). The T7 phage will allow only a limited proportion of its capsid proteins to be fused to other proteins while maintaining its infectivity. For this reason, we constructed helper plasmids that express both the wild-type capsid protein and the fluorescent protein from the same gene fusion by introducing a regulated translational frameshift site between the two fusion partners. The frameshift sequence was placed in such a way that when the bacterial ribosome follows the normal reading frame it will encounter a stop codon after the capsid protein gene, but once it shifts to the −1 frame it will read through into the EYFP gene. Regulated translational frameshifts are a relatively common phenomenon in nature and have been found in viruses, prokaryotes and eukaryotes (21–27). They are based on secondary structures in mRNA that combined with specific RNA sequences will cause the ribosomes to leave the original reading frame so that codons are redefined or translational bypasses occur. As far as we know this is the first report of using a translational frameshift for a biotechnological purpose.

MATERIALS AND METHODS

Helper plasmid construction

The frameshift helper plasmids were based on the helper plasmid present in the BLT5403 *E.coli* strain (Novagen), which carries the T7 gene 10 under the T7 φ*10* promoter. A fragment of gene 10 was amplified using the primers 10A-F (ACTATAGGGAGACCACAACGG) and 10A-EcoRI-mut-R (CTTCAAGAATTCTTA**C**TCCACTTT**G**AA**A**ACCACTGC-ACCAGCAGC) to restore the natural frameshift sequence by the introduction of three mutations (underlined). The PCR fragment was introduced in p5403 by exchanging the sequence between the KpnI and EcoRI restriction sites. The EYFP gene (Clontech) was amplified to introduce an EcoRI site on both ends, using the primers YFP-EcoRI-F (GTAAGAATTCTTATGGTGAGCAAGGGCGAG) and YFP-EcoRI-R (GTGAATTCTTAGCTCATGACTGACTTG-TAGAG). The ligation of the resulting fragment into the modified p5403 resulted in p10-EYFP, wherein the *EYFP* gene is present in the −1 reading frame relative to gene 10.

To create pX1-EYFP and pX2-EYFP, first an EcoRI and a HindIII site were introduced in p5403 upstream of the stop codon of gene 10 by introducing in between the BsgI and EcoRI site a DNA fragment formed by annealing the oligonucleotides EH-F (GAATTCCGGTTCTTAAAAGCTTTAAC) and EH-R (AATTGTTAAAGCTTTTAAGAACCGGAAT-TCTC). The new EcoRI and HindIII sites were used to introduce the *EYFP* gene amplified with YFP-EcoRI-F (GTAAGAATTCCATGGTGAGCAAGGGCGAG) and

YFP-HindIII-R (GCAAGCTTTTAGCTCATGACTGACT-TGTAGAG). This resulted in p5403-EH-EYFP. The *DnaX* frameshift cassettes were constructed from complementary oligonucleotides. The *DnaX* cassette with stem–loop (X1) with the following oligo pairs: XU1 (GCAGGGAGCA-ACCAAAGCAAAAAAG)/XL1 (ACTCTTTTTTGCTTTG-GTTGCTCCCTGCTC), XU2 (AGTCAACCGGCAGCCGC-TACCCGC)/XL2 (CGCGCGGGTAGCGGCTGCCGGTTG) and XU3 (GCGCGGCCGGTG**TGA**ATTCCATGCCCA)/XL3 (AGCTTGGGCATGGAAT**TCA**CACCGGCCG). The underlined nucleotides represent the introduced stop codon responsible for terminating the translation when no frameshift occurs. The ligation fragment was purified from gel and ligated into the BsgI and HindIII digested p5403-EH-EYFP, creating pX1-EYFP. The pX2-EYFP helper plasmid was constructed the same way, but with a cassette based on the following complementary primers: XSU1 (GCAGGGAG-CAACCAAAGCAAAAAAGAGGTGAATTCCATGCCCA)/XSL1 (AGCTTGGGCATGGAATTCACCTCTTTTTTGCT-TTGGTTGCTCCCTGCTC). All constructs were sequenced to check if sequences were correct.

Protein production

BL21(DE3) cells transformed with either pX1-EYFP, pX2-EYFP or p10-EYFP were grown at 37°C in 8 ml liquid Luria–Bertani (LB) with 100 µg/ml ampicillin (LB-amp). The protein production was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cultures reached an optical density (OD_{600}) of 0.5. After induction the temperature was lowered to 28°C. After 3 h the cells were pelleted and the produced protein was analysed on 10% SDS–PAGE. For western blot biotinylated anti-T7 (T7-Tag antibody, Novagen) was used diluted 1:10 000 in phosphate-buffered saline (PBS) containing 0.1% BSA and 0.1% Tween-20 (PBSBT), and the antibody was detected with 1:2000 streptavidin-alkalic phosphatase (AP) conjugate (Sigma). EYFP was detected with 1:200 anti-GFP (Clontech) and 1:5000 anti-Rabbit-AP (Jackson), both diluted in PBSBT.

T7 Select cDNA library construction and bio-panning procedures

For the construction of the cDNA display library, mRNA was extracted from total RNA of PVY[c] infected *Nicotiana benthamiana* leaf material with poly(dT) beads (Genoprep). cDNA was synthesized with the OrientExpress cDNA synthesis kit (Novagen) with random hexanucleotide primers. The cDNA fragments between 100 and 1500 bp length were ligated in the T7Select10-3 vector arms (Novagen) via a directional cloning strategy. After packaging the library in T7 phages the number of individual clones (5×10^5 p.f.u.) was determined by titering and the average insert size (∼500 bp) was determined by PCR amplification of 30 individual phage plaques. Three rounds of bio-panning against the 5H6 monoclonal antibody were performed. The selection procedure consisted of incubating 2×10^8 phages with 3 µg 5H6 antibody coated in microtiter plate wells for 3 h at room temperature. Non-binding phages were removed by washing 5× with PBST. Eluted phages were amplified after each round in 2 ml BLT5403 culture. Enrichment in phages displaying binding protein fragments was monitored by

plaque lifts on nitrocellulose filter (500–1500 plaques per selection round); the plaques of binding phage were detected with alkaline phosphatase-conjugated bait (5H6). The inserts from 25 binding phage clones from the third selection round were sequenced.

T7 amplification and purification

The effect of the frameshift helper plasmids on the efficiency of the phage amplification was tested by growing *E.coli* BL21(DE3) containing one of the helper plasmid constructs, in 8 ml of LB-amp at 37°C. At an OD_{600} of 0.5, the temperature was lowered to 28°C, and the cells were induced with 1 mM IPTG and simultaneously infected with 2×10^8 p.f.u. Phage titers were determined by mixing serial dilutions of the produced phages to an overnight culture of cells containing the original helper plasmid (BLT5403). The bacteria and phages were mixed with warm top agar (48°C) and plated on LB agar plates and incubated at 37°C.

Phages needed for purification were amplified in a 300 ml culture of *E.coli* containing the required helper plasmid by infecting it with 4×10^{10} p.f.u. of T7. After lysis of the bacteria, 50 µl Dnase I (2 mg/ml) was added and the phages were incubated for a further 20 min. NaCl was added to a final concentration of 500 mM and the phages were separated from the cell debris by centrifugation for 10 min at 8000 r.p.m. in a Sorval SLA-3000 rotor at 4°C. 10% (w/v) PEG 6000 was added to the supernatant. The lysate-PEG mixture was stored overnight at 4°C. The next day the phages were pelleted (8000 r.p.m. at 4°C in a Sorval SLA-3000 rotor). The pellet was dissolved in 5 ml of 1 M NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA. The resuspended phages were loaded on a CsCl gradient (20–62% w/v) and purified in a Beckman SW41 rotor centrifuged at 35 000 r.p.m. for 1 h at 20°C. The phages were collected and dialysed against PBS and stored at 4°C until further use.

Phage labelling with Alexa Fluor 488

Of 1 M sodium carbonate 10 µl was added to 100 µl of the purified phages (3×10^{12} particles/ml), mixed with one vial Alexa Fluor 488 tetrafluorophenyl (TFP) ester from the Alexa Fluor 488 protein labelling kit (Molecular Probes, Product #A10235) and incubated for 1 h at room temperature. The phages were separated from the unincorporated Alexa dye by size exclusion spin column chromatography. The labelled phages were stored at 4°C in PBS buffer.

Fluorescent correlation spectroscopy

For fluorescence correlation spectroscopy (FCS) a Zeiss-EVOTEC ConfoCor® system was used in combination with an argon ion laser supplying a 488 nm wavelength. The laser power was reduced with a 2.0 density filter and focused in the sample by a water immersion objective (Zeiss C-Apochromat 40×, N.A 1.2). The phage samples were measured in a glass bottom 96 well plate. Data traces were analysed in FCS Data processor 1.4.1 (SSTC) and analysed using a triplet-state model (28).

Antibodies biotinylation and coating of microspheres

Prior to the biotinylation reaction 40 µl of 0.5 M sodium carbonate of pH 9.5 was added to 400 µl of anti-T7, 5H6 (Y-5) or anti-myc (9E10) (2 mg/ml in PBS). To this mixture 4 µl *N*-hydroxysuccinimido-D-biotin (3 mg/ml) (Sigma) dissolved in DMSO was added and then incubated for 4 h at room temperature, while gently mixing. The free biotin was removed by dialysis against PBS at room temperature. Sodium azide was added and the biotinylated proteins were stored at 4°C till further use. The streptavidin-coated microspheres (1% solids) from Bangs Laboratories with a diameter of 0.95 µm had a capacity to bind 0.38 µg biotinylated FITC per ml beads. Microspheres were vortexed and 10 µl of the suspension was mixed with 180 µl PBS containing 0.1% BSA and 0.1% Tween-20 (PBSBT). To the diluted microspheres 10 µl of the biotinylated antibody were added, and incubated for 45 min at room temperature. During incubation the mixture was briefly vortexed every 10 min. The microspheres were pelleted by centrifugation (15 min, 1500 r.c.f.). The supernatant was removed and the pellet was resuspended in 200 µl PBSBT. To remove most unbound antibody the microspheres were pelleted and resuspended in fresh PBSBT three times in total.

Fluorescent imaging of the phage interacting with antibody-coated microspheres

The microspheres coated with antibodies (25 µl) were incubated with 100 µl fluorescent phages (5×10^{12} phages/ml) for 3 h at room temperature prior to observing the interaction in glass-bottomed 96 well plates (Whatman). Images were made on a Biorad Radiance 2100 MP-VIS system coupled to a Nikon TE300 inverted microscope. The sample was excited by an Argon laser at 488 nm. Fluorescence was collected using a Nikon Plan Apochromat 60× water immersed objective (N.A. 1.2) and filtered by a BP505-560 emission filter (Omega).

RESULTS

Vector construction and frameshift induction in *E.coli* cells

Three helper plasmids were constructed to obtain genetically fluorescent T7 phage. The function of helper plasmids is to provide sufficient wild-type capsid protein in addition to the library-displaying capsid protein that originates from the T7 genome. Different translational frameshift cassettes were placed at the 3′ end of the sequence encoding the wild-type capsid protein (gp10) in the helper plasmid p5403. The expression of gp10 in these constructs is regulated by the T7 φ10 promoter. The first constructed vector, named pX1, contained three recoding elements from the *E.coli dnaX* gene; the frameshift inducing sequence enclosed by a Shine-Dalgarno motif at one side and a stem–loop sequence at the other. The latter two elements influence the frequency of the frameshift event, which in wild-type *dnaX* occurs in 50% of the translations. A second helper plasmid, named pX2, was constructed by including the *dnaX* frameshift sequence and the Shine-Dalgarno sequence only, which was predicted to have a lower frameshift frequency (29). A third helper plasmid, named p10, was constructed by re-introducing a short frameshift sequence found in the wild-type gene 10 of T7 at the end of the capsid protein

gene in p5403. In the wild-type T7 this sequence is responsible for a −1 frameshift that causes the formation of the minor capsid protein 10B instead of the major capsid protein10A in ~10% of the translations (30–32). To complete the construction of the plasmids the gene coding for the EYFP, Clontech was placed in the −1 reading frame directly downstream of the frameshift cassettes, thereby creating the vectors pX1-EYFP, pX2-EYFP and p10-EYFP (Figure 1). In all three vectors stop codons were introduced right after the frameshift cassettes in the zero reading frame, so that if no frameshift takes place no extensive amino acid sequence will be added to the capsid protein. However, when the ribosome shifts from the initial reading frame into the −1 frame, the translation continues and a capsid protein-EYFP fusion (gp10-EYFP) is formed.

The expression of these constructs was tested in IPTG induced *E.coli* BL21(DE3) cells and the bacterial samples were subjected to SDS–PAGE. Coomassie brilliant blue staining of the proteins showed in all cases that after induction there was a high expression of wild-type coat protein without the EYFP fusion. Figure 2A shows that there are slight differences in molecular weight between the free coat proteins as they are expressed from the three vectors. These differences are caused by additional amino acids encoded in the frameshift cassettes of pX1-EYFP and pX2-EYFP (20 and 9 amino acids, respectively). The high concentrations of gp10-EYFP expressed from the vectors pX1-EYFP and pX2-EYFP are clearly visible in the Coomassie brilliant blue stained SDS–PAGE gel as bands with an approximate molecular weight of 70 kDa. The band intensity was used to estimate the ratio between gp10-EYFP and free capsid protein. These were ~1:1 for pX1-EYFP and 1:4 for pX2-EYFP. The western blot using antibodies that either recognize T7 gp10 or EYFP showed in both cases strong signals,
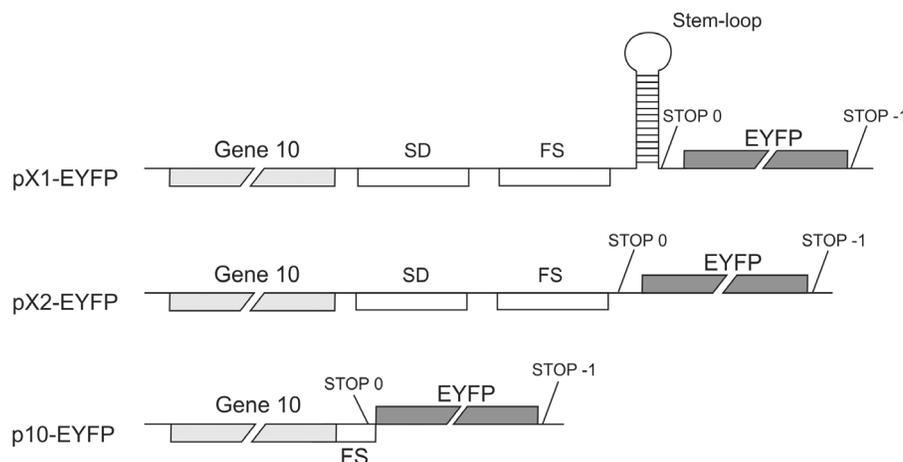
Figure 1. Schematic representation of the three translational frameshift constructs. The frameshift cassettes placed in the helper vector p5403 are located between gene 10 coding for the capsid protein (gp10) and the EYFP gene. The latter is placed in the −1 coding frame in relation to gene 10. Without recoding, the protein translation is terminated after the frameshift site (FS) indicated by STOP 0. In the −1 frame the stop codon is located beyond the EYFP gene; STOP −1. When the ribosome shifts to the −1 reading frame it produces the gp10-EYFP fusion. The constructs pX1-EYFP and pX2-EYFP contain frameshift elements originating from the *E.coli dnaX* gene; a Shine-Dalgarno (SD) sequence, the frameshift site (FS) and a stem–loop sequence. The pX2-EYFP vector is similar to pX1-EYFP, but lacks the stem–loop sequence. The frameshift site in p10-EYFP contains the short GGTTTTC frameshift inducing sequence present in T7 gene 10.
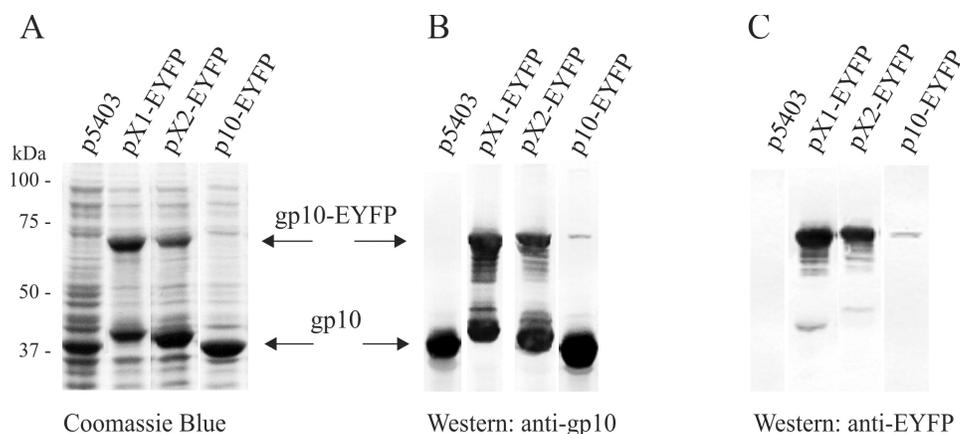
Figure 2. Different ratios of free capsid protein (gp10) and capsid protein fused to EYFP (gp10-EYFP) are produced from the three frameshift helper plasmids. Total cell samples of *E.coli* expressing the original helper plasmid (p5403), pX1-EYFP, pX2-EYFP and p10-EYFP were subjected to SDS–PAGE and analysed by Coomassie blue staining (**A**) or western blotting with anti-gp10 antibodies (**B**) and anti-EYFP antibodies (**C**). The positions on the gel of free gp10 and gp10-EYFP are indicated.

Table 1. Results of the FCS analysis of the phage variants and fluorescent molecules

Sample	τ_1 (μs)	D_1 (m^2 s^{-1})	%F1	τ_2 (ms)	D_2 (m^2 s^{-1})	%F2
Rhodamine green	23 (21–25)	2.8×10^{-10}	100	—	—	—
YFP (Citrine)	34 (32–35)	1.9×10^{-10}	100	—	—	—
T7-Alexa488	—	—	—	1.33 (1.27–1.35)	4.8×10^{-12}	100
T7-Pcp-EYFP	25 (23–26)	2.5×10^{-10}	77	1.7 (1.7–1.8)	3.8×10^{-12}	23
T7-Pcp	—	—	—	—	—	—

Diffusion time (τ) with SD derived from 10 measurements. Translational diffusion coefficient calculated as in Hink *et al.* (61). F1 and F2 denote the relative fractions of each component.

confirming that the bands at 70 kDa are indeed gp10-EYFP fusions, indicating that the fusion protein was abundantly expressed from pX1-EYFP and pX2-EYFP. The level of expression of gp10-EYFP produced from p10-EYFP was so low that the fusion protein could not be detected on the Coomassie brilliant blue stained gel. A western blot of this construct specifically showed a thin band at 70 kDa indicating the expression of the gp10-EYFP fusion protein (Figure 2B and C). It was estimated that a frameshift had taken place in <1% of the translations from the p10-EYFP vector.

The gp10-EYFP: gp10 ratio affects the formation of infectious phage particles

Expression from each of the three frameshift constructs, pX1-EYFP, pX2-EYFP and p10-EYFP, results in the translation of both a wild-type capsid protein and a capsid protein-EYFP fusion protein in fixed ratios at three different levels. To test the functionality of the constructed helper plasmids, recombinant phages displaying a 95 amino acid fragment of the Potato Virus Y coat protein (Pcp) were amplified in bacteria containing either the helper plasmids pX1-EYFP, pX2-EYFP or p10-EYFP. It was expected that functional helper plasmids would lead to the formation of infectious phage particles with wild-type capsid protein, capsid protein fused to EYFP and capsid protein displaying Pcp. The bacteria containing the vector p10-EYFP or the original helper plasmid p5403 were fully lysed, 3 h after infection of the cultures, whereas the bacterial cultures containing pX1-EYFP or pX2-EYFP were still turbid. Absence of cell lysis implies the delay or inhibition of the phage amplification. To assess the efficiency of phage production for each of the helper plasmids the phage titers were determined. In the bacterial cultures containing pX1-EYFP and pX2-EYFP no infectious phage particles were detected. The titer of the phages produced from p10-EYFP was of the same order as the titer produced using the original p5403 helper plasmid (i.e. 2×10^{10} p.f.u. per ml). As from the three helper plasmids only p10-EYFP resulted in infectious phages, all further experiments were performed with this construct.

Fluorescence correlation spectroscopy measurements demonstrate the incorporation of gp10-EYFP in T7 capsids

FCS was applied to determine if the gp10-EYFP fusion protein was indeed incorporated in the phage particles produced from the p10-EYFP helper plasmid. FCS is a spectroscopic technique that provides information about the diffusion rate

and the average number of fluorescently labelled molecules in a solution. Fluorescent molecules diffusing through a confocal detection volume are detected as intensity fluctuations. By correlating these fluctuations in time the behaviour of multiple particles with different diffusion times can be resolved in a single measurement (33–35). Assembled phage particles have a diameter of ~60 nm, which is more than 10 times larger than free gp10-EYFP molecules, and are thus expected to have significantly longer diffusion times. Phages produced using p10-EYFP (T7-Pcp-EYFP) and p5403 (T7-Pcp) were purified and concentrated using ultracentrifugation on a CsCl gradient to eliminate most of the non-assembled gp10 molecules and bacterial debris. Similarly purified and Alexa488-labeled T7 phages served as comparison. FCS analysis of the Alexa488 labelled phage led to a diffusion time (τ) of 1.33 (1.27–1.35) ms (Table 1). The autocorrelation data of free EYFP could be fitted to a diffusion time of 34 μs (32–35). T7-Pcp phages do not contain any EYFP and excitation at a 514 nm wavelength resulted only in a low background fluorescence that could not be autocorrelated. Under the same conditions the T7-Pcp-EYFP phages, produced with the p10-EYFP helper plasmid, fluoresced with a maximal emission at 527 nm, typical for EYFP. Surprisingly, it was not possible to fit the autocorrelation data for T7-Pcp-EYFP for a single diffusion time. The autocorrelation curve showed that the sample contained two components. A two-component fit resulted in a relatively long diffusion time of 1.7 ms, comparable with the alexa488-labeled T7, and a short diffusion time of ~25 μs that was close to the value found for free EYFP and Rhodamine Green (Table 1). The exact diffusion time of the fast component could not be determined from these data. To exclude the possibility that this fast component was due to a photophysical phenomenon the measurements were repeated in a buffer with a higher viscosity. In the more viscous solution the diffusion times for both the fast and slow component were extended (data not shown), meaning that it must be a fluorescent particle, most likely non-incorporated gp10-EYFP. A photophysical artefact would have been independent of the viscosity (36).

The amplification temperature influences the formation of EYFP-labelled phages

T7 phage cDNA libraries are normally amplified in bacterial cultures incubated at 37°C. However, this is not the optimal temperature for the formation of the fluorophore in EYFP (37,38). A subset of gp10-EYFP formed at 37°C will not become fluorescent. To investigate the influence of the

incubation temperature during amplification of the library on the phage production and on the proportion of phage particles that show fluorescence, T7-Pcp-EYFP phages were amplified at 20, 28 and 37°C. Bacteria containing the p10-EYFP helper plasmid were first grown at 37°C till the OD_{600} reached 0.5 before the temperature was changed to the required amplification temperature, the protein production was induced and the cells were infected with the T7-Pcp phages. After complete lysis of the cells, the infectious phage concentrations were determined (Figure 3A). At 37°C ~10 times more infectious phages are formed than at 20°C. Amplification at 28°C showed an intermediate efficiency. FCS measurements could be used to determine the amount of fluorescent phages. Thereto the phages were first precipitated, purified on a CsCl gradient by ultracentrifugation and resuspended in 1.5 ml of PBS. The total phage concentration after purification was determined by measuring the optical density of the phage solution at 260 nm (Figure 3B). Although the amplification temperature clearly has an influence on the infectivity of the phages (Figure 3A), the total amount of phages, as determined by the optical density, did not differ significantly between the three growth temperatures (Figure 3B). Analysis of FCS measurements on the purified phages gave the average number of fluorescent phage particles present in the confocal detection volume (Figure 3C). The size of the detection volume was determined by analysis of compound with known diffusion coefficients, Rhodamine Green and EYFP. Knowing the size of the detection volume enabled us to calculate the concentration of fluorescent phages in the samples. The average concentration of fluorescent phages appeared to be the lowest at 37°C (Figure 3C). Comparison of the concentration of fluorescent phages (Figure 3C) with the total phage concentration (Figure 3B) indicates that the relative amounts of fluorescent phages are ~16% when amplified at 20°C, 14% at 28°C and 6% at 37°C. Based on these differences, although not statistically significant, and the infectivity

of the phages (Figure 3A) we considered 28°C the optimal amplification temperature for fluorescent phage production and consequently used this temperature in further experiments.

EYFP-labelled phages enable the visualisation of protein–protein interactions

To visualize protein–protein interactions using fluorescent display phages we tested the physical interaction between the Pcp and a monoclonal antibody recognising this fragment [5H6; Y-5 in Ref. (39)]. To this purpose Pcp was displayed on fluorescent phages including gp10-EYFP (hereafter referred to as T7-Pcp-EYFP) and microspheres were coated 5H6. The 5H6-coated microspheres were expected to capture the T7-Pcp-EYFP phages, which could only be detected by fluorescence microscopy when the phage displays both the Pcp and EYFP molecules. Microspheres coated with an antibody to a *M*yc-tag (9E10) were used as a negative control; the phages do not contain the myc-tag and should not be recognized. Microspheres coated with an anti-T7 antibody that should bind all T7 phage particles were used as positive binding control. After incubation, the spheres were washed several times to remove unbound antibody. Antibody-coated microspheres that were not incubated with phages did not show a fluorescent signal and could not be detected by fluorescence microscopy under 514 nm excitation. The anti-T7 antibody-coated microspheres incubated with the T7-Pcp-EYFP phages were detected as spots on a dark background, indicating an interaction between the antibody and the fluorescent phage (Figure 4A). EYFP-fluorescence could also be detected on the 5H6 antibody-coated spheres after incubation with T7-Pcp-EYFP phages in a pattern similar to what was found with the anti-T7 antibody-coated spheres (Figure 4B). No fluorescence could be detected on the anti-myc antibody-coated spheres incubated with the phages, demonstrating that the fluorescence detected on the anti-T7
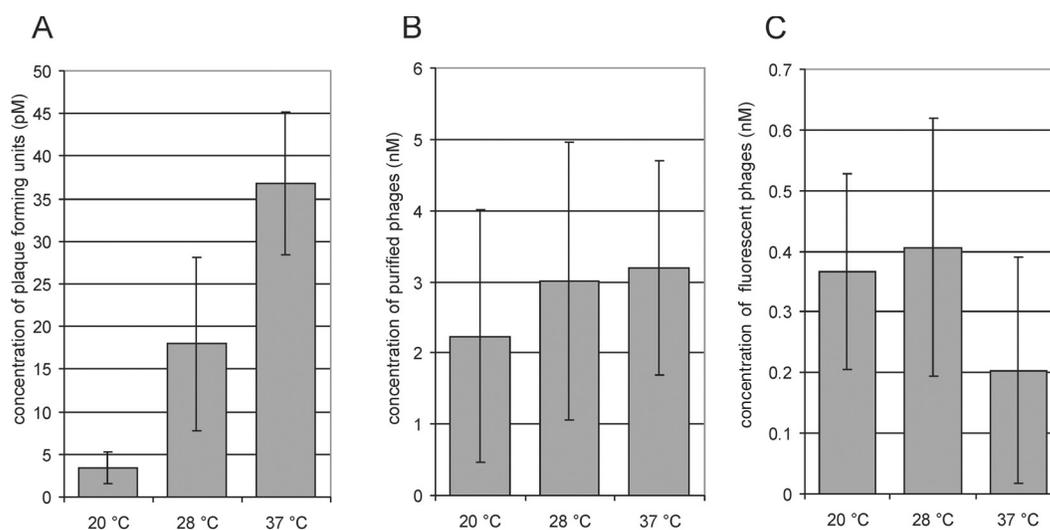
Figure 3. Influence of the temperature on the amplification efficiency and the formation of fluorescently labelled T7-Pcp-EYFP phages. Phages were amplified at 20, 28 and 37°C in 300 ml bacteria containing the p10-EYFP vector. The concentration of infectious phages was determined by titering the phages (**A**), concentration in pM, based on six samples. After CsCl purification the total phage concentration was determined by measuring the absorbance at 260 nm (**B**), based on four samples. The concentration of fluorescent phage particles was determined by FCS analysis in four (20 and 37°C) or five (28°C) independent samples (**C**).
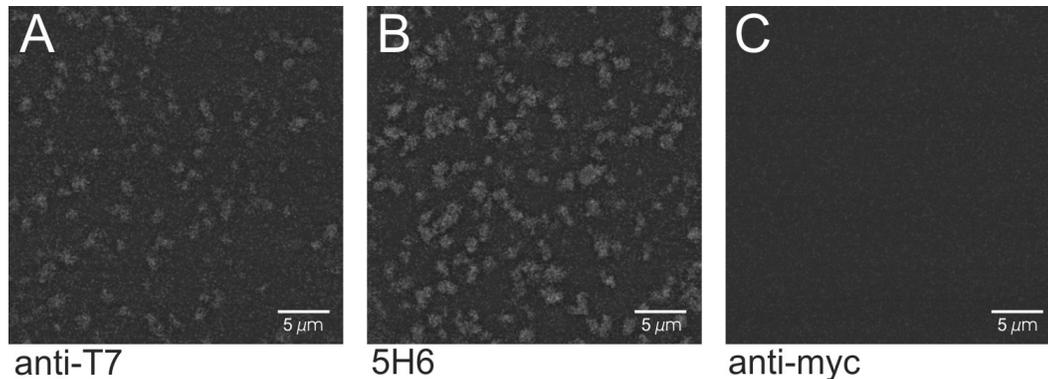
Figure 4. Visualization of the interaction between T7-Pcp-EYFP phages and antibody-coated microspheres (0.95 μm). The microspheres coated with the anti-T7 antibodies (**A**) or 5H6 antibodies against the Pcp (**B**) become fluorescent after incubation with the T7-Pcp-EYFP phage. Incubation of T7-Pcp-EYFP phages with anti-myc antibody-coated microspheres did not result in detectable EYFP-fluorescence on the beads (**C**).

and 5H6 antibody-coated spheres was due to specific binding of the phages (Figure 4C). From these results we conclude that the T7-Pcp-EYFP phage particles are labelled with EYFP and display the Pcp. Even though only a 7% of the phages contains a fluorescent EYFP molecule, as shown by FCS analysis, this is sufficient to detect the interaction between the phage and the bait-coated microspheres.

## Monitoring the enrichment of T7 phage display libraries by fluorescence microscopy

The next step was to examine the display of a diverse cDNA library on fluorescent phages. As a model system it was decided to use different well characterized selection stages from a cDNA display library bio-panning. The cDNA display library was constructed from PVY-infected *N.benthamiana* plants and inserted in the T7Select10-3 genome vector (Novagen). The library was selected against the 5H6 monoclonal antibody. Three selection rounds had resulted in subpopulations of this cDNA display library at different degrees of enrichment for phages displaying 5H6-binding protein fragments. The extent of enrichment was tested via plaque lift assays. Less than 0.5% of the phages in the original library displayed a 5H6-binding fragment, whereas the proportion increased to 5% after the first selection round, 31% after the second and 59% after the third selection round. PCR Amplification and sequencing of the cDNA inserts from interacting phages from the third selection round showed that the enrichment of the library was specific. In total 10 cDNA fragments of different lengths were found, all encoding fragments of the Pcp.

The original unselected library and the results from the first and third selection round were amplified in bacteria containing the p10-EYFP helper plasmid. The resulting phages were purified by ultracentrifugation on a CsCl gradient and incubated with a mixture of 5H6 and anti-myc coated microspheres (at a ratio of 1:9). The microspheres coated with 5H6 were labelled with a red fluorescent dye to distinguish them from the anti-myc coated microspheres. By fluorescence microscopy using two excitation wavelengths (514 and 633 nm) the specific interaction of the fluorescent T7 phages with the 5H6-coated microspheres was monitored. Excitation at 514 nm of the beads that were incubated with the phages

displaying the original library revealed faint dots that corresponded with the location of some of the 5H6-coated spheres as seen at 633 nm excitation (Figure 5A–D). However, there was also some EYFP-fluorescence visible on the anti-myc coated spheres. This indicates that in the unselected library the proportion of phages displaying a 5H6-binding peptide (∼0.5%) is still too low to elevate the signal above background level in this assay. The library that had been subjected to one round of selection shows a brighter EYFP signal on the 5H6-coated beads; the spheres that exhibit EYFP-fluorescence correspond here to the ones with the red fluorescence (Figure 5E–H). Incubation of phages from the third selection round, with 59% of the phages displaying a 5H6-binding peptide, resulted in the accumulation of a high amount of fluorescent phages specifically on the 5H6-coated microspheres (Figure 5I–L). The relative brightness of the beads was determined with Kodak 1D image analysis software. After one round of selection the beads were 2.5 times brighter than before selection. After three rounds the fluorescence intensity of the beads was 6.4 times higher than after incubation with the unselected library. The fluorescence screening was repeated with other ratios of 5H6- and anti-myc coated microspheres (9:1, 1:1, 1:50). A similar increase in fluorescence on the beads, even after one round of selection, could be seen (data not shown). This demonstrates that it is possible to apply the frameshift helper plasmid for the amplification and fluorescent labelling of a cDNA display library. Such a fluorescently labelled library can be used to directly monitor the enrichment in specific binders during an affinity selection procedure.

DISCUSSION

Translational frameshifts are a common phenomenon in nature. It is one of the mechanisms that enable cells to encode different variants of a protein in a single gene. Often these variants have specific functions in the cell. The frameshift sequence from the *E.coli dnaX* gene that is used in this study illustrates this quite well. Both the non-frameshifted product (τ) and the shorter, frameshifted variant (γ) are formed in a 1:1 ratio and serve as subunits of DNA polymerase III where they have distinct functions (29,40).
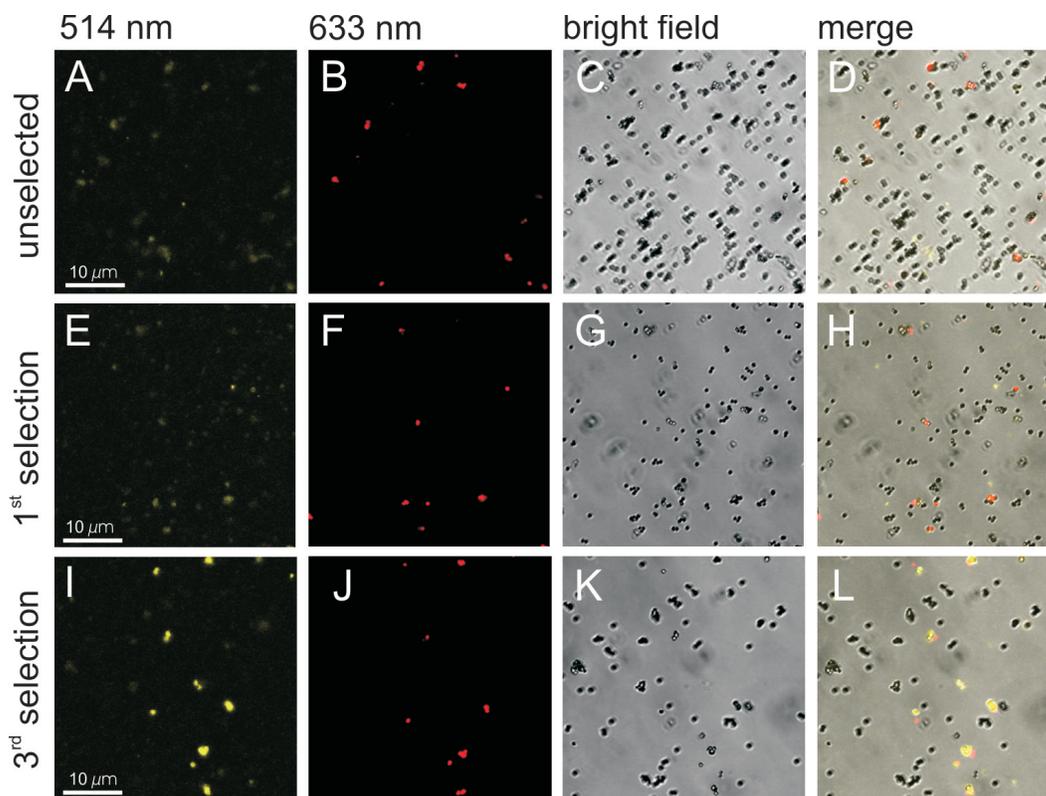
Figure 5. Microscopic images of antibody-coated microspheres incubated with EYFP-labelled T7 phages from different affinity selection rounds. Red fluorescent microspheres (0.95 μm) were coated with the 5H6 antibody against the Pcp and non-fluorescent microspheres with an anti-myc antibody. The microspheres were mixed in a 1:9 ratio. EYFP-labelled T7 phages displaying the unselected library (**A–D**), the library after one round of affinity selection against 5H6 (**E–H**) or the library after three rounds of selection (**I–L**) were incubated with the microspheres. EYFP-fluorescence was detected at 514 nm excitation (A, E and I), the red fluorescent beads at 633 nm (B, F and J). The bright field image (C, G and K) shows all beads. In (D, H and L) the superposition of the three channels is shown.

The result of a translational frameshift, or ribosome recoding, is the production of two proteins in a fixed ratio and with a particular nested sequence relationship. A feature that has interesting biotechnological implications as we show in this study. We employed ribosome recoding to produce fluorescence labelled T7 phages that are fully compatible with the commercially available T7 display system (T7Select; Novagen).

Using a recoding sequence to express two gene products has a number of advantages. In this case the expression of both the wild-type capsid protein and the capsid protein fused to EYFP were needed. If no translational recoding sequence is used, for each product a complete open reading frame should be present, either on one plasmid or on two different plasmids. In both options the presence of two homologous DNA sequences in the cell will adversely influence the plasmids stability (41). Furthermore, to get the desired ratio of the two products, the promoters, regulatory elements and copy numbers of the plasmids will have to be adjusted. With our system a certain ratio can be achieved by employing the right frameshift sequence, and many have been described already, with a wide range of frameshifting frequencies (29,42).

The fact that T7 phages can be used as a display system is based on their ability to incorporate capsid proteins with fusions into their capsids. However, fusions larger than about 50 amino acids cannot be displayed on every copy of the capsid protein. The T7 system used in this study (T7Select10-3) displays peptides from the cDNA library on 10–15 out of the 415 capsid proteins (43). It was not possible to predict how many of the 27 kDa EYFPs can be displayed in combination with the library. To find the right ratio we constructed three frameshift constructs with a range of shift efficiencies. The highest ratios of EYFP fused capsid protein, ∼50 and 25% (which would correspond to 200 and 100 fusions per phage head), did not sustain the correct formation of infectious phages. The frameshift sequence in the p10-EYFP helper plasmid directed the ribosome into the −1 frame to form the EYFP fusion in <1% of the translations. With this helper plasmid the capsid assembly was not hampered and amplification of a cDNA phage display library in *E.coli* was indistinguishable from the original helper plasmid. The presence of both the library derived proteins and the EYFP on the capsid surface was shown with the specific binding of these fluorescent phages to microspheres coated with specific antibodies, against which antigens were present in the library. It is likely that the capsid allows the inclusion of higher numbers of fluorescent fusions than tested in this study. Different frameshift sequences with recoding frequencies between 25 and 1% should be tested to find a ratio where most of the particles are fluorescent without interfering with the phage formation (44,45). Still, although the frameshift

frequency was not high enough to label every single phage particle, the fluorescent signal was sufficient for the detection of the phages and their interaction with the bait. We did not test our frameshift helper plasmid in combination with the other available T7Select vectors. The T7Select415 was designed for display of peptides smaller than 50 amino acids on every capsid protein and does not need a helper plasmid for amplification. If it would be combined with our helper plasmid a considerable fraction (50%) of unfused capsid protein derived from the helper plasmid would also be incorporated, thus lowering the copy number of the peptide fusions from the library. T7Select1-1 and 1-2 are designed for the display of large (maximum 1000 amino acids) and difficult to express proteins in a low copy number, which is comparable with the EYFP-fusion copy number resulting from the use of the frameshift helper plasmid. The fact that the library fragment would be incorporated in only 10% of the phages and the fluorescent fusion in ~14% means that an even smaller fraction of the phages will display both the library fragment and the EYFP-fusion. Thus, a larger number of phages would be necessary for screening.

The fluorescent phage particles could be characterized by means of microspectroscopic measurements. FCS was applied to confirm the incorporation of gp10-EYFP fusion proteins into the phage capsids and to determine what percentage of the phages carried EYFP. FCS is a very sensitive technique and is based on the measurement of fluorescence fluctuations that are caused by fluorescent particles moving in and out of the confocal detection volume. Autocorrelation of these fluctuations gives information about the translational diffusion coefficient of the fluorescent particles and their relative numbers (35). The diffusion of particles with fluorescence characteristics of EYFP and a diffusion coefficient corresponding to the 60 nm phages was measured. Comparing the total concentration of phages produced at 28°C with the number of fluorescent phages measured in the FCS experiments revealed that 14% of the phages is fluorescent under these conditions.

The large difference in size and thus diffusion coefficient between phages and most molecules that will normally be used as bait can be used to detect interactions between the two. Measuring the interaction between phage displayed proteins and their interactors by FCS has been attempted by Lagerkvist *et al.* (46). The interaction between an M13 phage displayed Fab fragment and its cognate antigen could be detected, but in their experiments the phages were labelled by fluorescent antibodies, causing aggregation and a significant loss of sensitivity in the detection of interactions.

Our data demonstrate that the enrichment of a library during affinity selection can be sensitively monitored with fluorescence microspectroscopy. When our library was enriched to 60% 5H6 binders by three rounds of bio-panning, the bright fluorescence of phages binding the 5H6-coated microspheres could easily be detected among the spheres coated with the anti-myc control antibody. Even after one round of panning, with ~5% of the phages displaying a 5H6-binding protein fragment, the difference was detectable between the fluorescence on the 5H6-coated beads and the beads with the control antibody. The specificity of the binding is evident by the lack of fluorescence on the beads coated with the control antibody. The clear signal of the fluorescent phage on the beads might be utilised in fluorescence based sorting systems like fluorescence activated bead sorting (FABS) or in microfluidic sorting systems. One could envisage even an setup wherein a fluorescent phage display library is screened against a library of bait molecules coated on beads or spotted on a chip surface (20,47).

In principle there are alternative means to create fluorescent phages. Phages can for example be labelled with fluorescent chemical dyes (48–50). With a wide range of dyes available offering high quantum yields, very bright phage particles can be produced. In case of M13 phage the chemical dye labelling had no negative effect on the infectivity (51). With T7 probably being more vulnerable we experienced a 10-fold decrease of the infectivity after the chemical labelling (data not shown). Chemical labelling involves also extra handling steps and is needed each time the phages have been propagated in bacteria.

T7 is not the first phage that has been labelled with a fluorescent protein. Labelling of the lytic phage T4 with GFP has been reported. However, in these studies the phages were not used in a fluorescent phage display setup, but as model to study the T4 phage head structure or as detection system for *E.coli* (52–54). To display proteins on T4 phages fusions with either HOC (Highly antigenic Outer Capsid protein) or SOC (Small Outer Capsid protein) have been made (14,55–57). Both these proteins are not essential for particle formation and bind to the capsid with high copy number and in principle one could be used for labelling and one for display. However, T4 has the disadvantage of lacking an efficient *in vitro* packaging method, which makes it more difficult to create large display libraries. The proteins used for display in T4 are not incorporated in the plasmid, but bind to it with high affinity. This means the link between phenotype (the displayed protein) and genotype is not as strong as with T7 where the proteins used as display platform form an inextricable part of the capsid.

Combined with the standard bio-panning selection fluorescent display phages can give information about the enrichment of binders in a display library, which enables fine tuning of the selection procedure and obviates the need for more laborious ELISA or plaque lift assays. Furthermore, with the fast pace of developments in single molecule detection technologies and sorting systems, these fluorescent phages open the way to high-throughput platforms for the direct selection of binding molecules (58–60).

REFERENCES

1. Li,M. (2000) Applications of display technology in protein analysis. *Nat. Biotechnol.*, **18**, 1251–1256.
2. Tong,A.H.Y., Drees,B., Nardelli,G., Bader,G.D., Brannetti,B., Castagnoli,L., Evangelista,M., Ferracuti,S., Nelson,B., Paoluzi,S. *et al.* (2002) A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. *Science*, **295**, 321–324.

3. Ito,T., Tashiro,K., Muta,S., Ozawa,R., Chiba,T., Nishizawa,M., Yamamoto,K., Kuhara,S. and Sakaki,Y. (2000) Toward a protein–protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc. Natl Acad. Sci. USA*, **97**, 1143–1147.

4. Gavin,A.C., Bosche,M., Krause,R., Grandi,P., Marzioch,M., Bauer,A., Schultz,J., Rick,J.M., Michon,A.M., Cruciat,C.M. *et al.* (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*, **415**, 141–147.

5. Zozulya,S., Lioubin,M., Hill,R.J., Abram,C. and Gishizky,M.L. (1999) Mapping signal transduction pathways by phage display. *Nat. Biotechnol.*, **17**, 1193–1198.

6. Sche,P.P., McKenzie,K.M., White,J.D. and Austin,D.J. (1999) Display cloning: functional identification of natural product receptors using cDNA-phage display. *Chem. Biol.*, **6**, 707–716.

7. Geuijen,C.A., Bijl,N., Smit,R.C., Cox,F., Throsby,M., Visser,T.J., Jongeneelen,M.A., Bakker,A.B., Kruisbeek,A.M., Goudsmit,J. *et al.* (2005) A proteomic approach to tumour target identification using phage display, affinity purification and mass spectrometry. *Eur. J. Cancer*, **41**, 178–187.

8. Smith,G.P. (1985) Filamentous fusion phage—novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315–1317.

9. Devlin,J.J., Panganiban,L.C. and Devlin,P.E. (1990) Random peptide libraries—a source of specific protein-binding molecules. *Science*, **249**, 404–406.

10. Scott,J.K. and Smith,G.P. (1990) Searching for peptide ligands with an epitope library. *Science*, **249**, 386–390.

11. Griffiths,A.D., Williams,S.C., Hartley,O., Tomlinson,I.M., Waterhouse,P., Crosby,W.L., Kontermann,R.E., Jones,P.T., Low,N.M., Allison,T.J. *et al.* (1994) Isolation of high-affinity human-antibodies directly from large synthetic repertoires. *EMBO J.*, **13**, 3245–3260.

12. Yamamoto,M., Kominato,Y. and Yamamoto,F. (1999) Phage display cDNA cloning of protein with carbohydrate affinity. *Biochem. Biophys. Res. Commun.*, **255**, 194–199.

13. Sternberg,N. and Hoess,R.H. (1995) Display of peptides and proteins on the surface of bacteriophage-lambda. *Proc. Natl Acad. Sci. USA*, **92**, 1609–1613.

14. Ren,Z.J., Lewis,G.K., Wingfield,P.T., Locke,E.G., Steven,A.C. and Black,L.W. (1996) Phage display of intact domains at high copy number: a system based on SOC, the small outer capsid protein of bacteriophage T4. *Protein Sci.*, **5**, 1833–1843.

15. Sheu,T.J., Schwarz,E.M., Martinez,D.A., O'Keefe,R.J., Rosier,R.N., Zuscik,M.J. and Puzas,J.E. (2003) A phage display technique identifies a novel regulator of cell differentiation. *J. Biol. Chem.*, **278**, 438–443.

16. Danner,S. and Belasco,J.G. (2001) T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. *Proc. Natl Acad. Sci. USA*, **98**, 12954–12959.

17. Horibe,T., Gomi,M., Iguchi,D., Ito,H., Kitamura,Y., Masuoka,T., Tsujimoto,I., Kimura,T. and Kikuchi,M. (2004) Different contributions of the three CXXC motifs of human protein-disulfide isomerase-related protein to isomerase activity and oxidative refolding. *J. Biol. Chem.*, **279**, 4604–4611.

18. Krajcikova,D. and Hartley,R.W. (2004) A new member of the bacterial ribonuclease inhibitor family from *Saccharopolyspora erythraea*. *FEBS Lett.*, **557**, 164–168.

19. Bohmer,M. and Enderlein,J. (2003) Fluorescence spectroscopy of single molecules under ambient conditions: methodology and technology. *Chemphyschem*, **4**, 793–808.

20. Visser,A., Kunst,B.H., Keller,H. and Schots,A. (2004) Towards sorting of biolibraries using single-molecule fluorescence detection techniques. *Curr. Pharm. Biotechnol.*, **5**, 173–179.

21. Levin,M.E., Hendrix,R.W. and Casjens,S.R. (1993) A programmed translational frameshift is required for the synthesis of a bacteriophage-lambda tail assembly protein. *J. Mol. Biol.*, **234**, 124–139.

22. Brault,V. and Miller,W.A. (1992) Translational frameshifting mediated by a viral sequence in plant-cells. *Proc. Natl Acad. Sci. USA*, **89**, 2262–2266.

23. Mardon,G. and Varmus,H.E. (1983) Frameshift and intragenic suppressor mutations in a rous-sarcoma provirus suggest src encodes 2 proteins. *Cell*, **32**, 871–879.

24. Licznar,P., Mejlhede,N., Prere,M.F., Wills,N., Gesteland,R.F., Atkins,J.F. and Fayet,O. (2003) Programmed translational-1

25. Zheng,J.H. and McIntosh,M.A. (1995) Characterization of Is1221 from Mycoplasma-hyorhinis—expression of its putative transposase in *Escherichia coli* incorporates a ribosomal frameshift mechanism. *Mol. Microbiol.*, **16**, 669–685.

26. Matsufuji,S., Matsufuji,T., Miyazaki,Y., Murakami,Y., Atkins,J.F., Gesteland,R.F. and Hayashi,S. (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell*, **80**, 51–60.

27. Ivanov,I.P., Matsufuji,S., Murakami,Y., Gesteland,R.F. and Atkins,J.F. (2000) Conservation of polyamine regulation by translational frameshifting from yeast to mammals. *EMBO J.*, **19**, 1907–1917.

28. Skakun,V.V., Hink,M.A., Digris,A.V., Engel,R., Novikov,E.G., Apanasovich,V.V. and Visser,A. (2005) Global analysis of fluorescence fluctuation data (vol 34, pg 323, 2005). *Eur. Biophys. J. Biophys. Lett.*, **34**, 972–972.

29. Larsen,B., Gesteland,R.F. and Atkins,J.F. (1997) Structural probing and mutagenic analysis of the stem–loop required for *Escherichia coli* dnaX ribosomal frameshifting: programmed efficiency of 50%. *J. Mol. Biol.*, **271**, 47–60.

30. Condron,B.G., Atkins,J.F. and Gesteland,R.F. (1991) Frameshifting in Gene-10 of Bacteriophage-T7. *J. Bacteriol.*, **173**, 6998–7003.

31. Sipley,J., Stassi,D., Dunn,J. and Goldman,E. (1991) Analysis of bacteriophage T7 gene 10A and frameshifted 10B proteins. *Gene Expr.*, **1**, 127–136.

32. Condron,B.G., Gesteland,R.F. and Atkins,J.F. (1991) An analysis of sequences stimulating frameshifting in the decoding of gene-10 of bacteriophage-T7. *Nucleic Acids Res.*, **19**, 5607–5612.

33. Dittrich,P., Malvezzi-Campeggi,F., Jahnz,M. and Schwille,P. (2001) Accessing molecular dynamics in cells by fluorescence correlation spectroscopy. *Biol. Chem.*, **382**, 491–494.

34. Maiti,S., Haupts,U. and Webb,W.W. (1997) Fluorescence correlation spectroscopy: diagnostics for sparse molecules. *Proc. Natl Acad. Sci. USA*, **94**, 11753–11757.

35. Visser,A. and Hink,M.A. (1999) New perspectives of fluorescence correlation spectroscopy. *J. Fluoresc.*, **9**, 81–87.

36. Enderlein,J., Gregor,I., Patra,D. and Fitter,J. (2004) Art and artefacts of fluorescence correlation spectroscopy. *Curr. Pharm. Biotechnol.*, **5**, 155–161.

37. Kimata,Y., Iwaki,M., Lim,C.R. and Kohno,K. (1997) A novel mutation which enhances the fluorescence of green fluorescent protein at high temperatures. *Biochem. Biophys. Res. Commun.*, **232**, 69–73.

38. Tsien,R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.*, **67**, 509–544.

39. Boonekamp,P.M., Pomp,H., Gussenhoven,G.C. and Schots,A. (1991) The use of immunochemical techniques and monoclonal-antibodies to study the viral coat protein-structure of potato virus-A, potato virus-Y and beet necrotic yellow vein virus. *Acta Botanica Neerlandica*, **40**, 41–52.

40. McHenry,C.S. (2003) Chromosomal replicases as asymmetric dimers: studies of subunit arrangement and functional consequences. *Mol. Microbiol.*, **49**, 1157–1165.

41. Baneyx,F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.*, **10**, 411–421.

42. Baranov,P.V., Gesteland,R.F. and Atkins,J.F. (2002) Recoding: translational bifurcations in gene expression. *Gene*, **286**, 187–201.

43. Rosenberg,A., Griffin,G., Studier,F.W., McCormick,M., Berg,J. and Mierendorf,R. (1996) T7Select phage display system: a powerful new protein display system based on bacteriophage T7. *inNovations* **6**, 1–6.

44. Gurvich,O.L., Baranov,P.V., Zhou,J., Hammer,A.W., Gesteland,R.F. and Atkins,J.F. (2003) Sequences that direct significant levels of frameshifting are frequent in coding regions of *Escherichia coli*. *EMBO J.*, **22**, 5941–5950.

45. Baranov,P.V., Gurvich,O.L., Hammer,A.W., Gesteland,R.F. and Atkins,J.F. (2003) Recode 2003. *Nucleic Acids Res.*, **31**, 87–89.

46. Lagerkvist,A.C., Foldes-Papp,Z., Persson,M.A.A. and 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.

47. Fu,A.Y., Spence,C., Scherer,A., Arnold,F.H. and Quake,S.R. (1999) A microfabricated fluorescence-activated cell sorter. *Nat. Biotechnol.*, **17**, 1109–1111.

frameshifting on hexanucleotide motifs and the wobble properties of tRNAs. *EMBO J.*, **22**, 4770–4778.

48. Gitis,V., Adin,A., Nasser,A., Gun,J. and Lev,O. (2002) Fluorescent dye labeled bacteriophages—a new tracer for the investigation of viral transport in porous media: 1. Introduction and characterization. *Water Res.*, **36**, 4227–4234.

49. Gitis,V., Adin,A., Nasser,A., Gun,J. and Lev,O. (2002) Fluorescent dye labeled bacteriophages—a new tracer for the investigation of viral transport in porous media: 2. Studies of deep-bed filtration. *Water Res.*, **36**, 4235–4242.

50. Oda,M., Morita,M., Unno,H. and Tanji,Y. (2004) Rapid detection of *Escherichia coli* O157:H7 by using green fluorescent protein-labeled PP01 bacteriophage. *Appl. Environ. Microbiol.*, **70**, 527–534.

51. Jaye,D.L., Geigerman,C.M., Fuller,R.E., Akyildiz,A. and Parkos,C.A. (2004) Direct fluorochrome labeling of phage display library clones for studying binding specificities: applications in flow cytometry and fluorescence microscopy. *J. Immunol. Methods*, **295**, 119–127.

52. Tanji,Y., Furukawa,C., Na,S.H., Hijikata,T., Miyanaga,K. and Unno,H. (2004) *Escherichia coli* detection by GFP-labeled lysozyme-inactivated T4 bacteriophage. *J. Biotechnol.*, **114**, 11–20.

53. Mullaney,J.M. and Black,L.W. (1998) Activity of foreign proteins targeted within the bacteriophage T4 head and prohead: implications for packaged DNA structure. *J. Mol. Biol.*, **283**, 913–929.

54. Mullaney,J.M., Thompson,R.B., Gryczynski,Z. and Black,L.W. (2000) Green fluorescent protein as a probe of rotational mobility within bacteriophage T4. *J. Virol. Methods*, **88**, 35–40.

55. Ren,Z.J. and Black,L.W. (1998) Phage T4 SOC and HOC display of biologically active, full-length proteins on the viral capsid. *Gene*, **215**, 439–444.

56. Jiang,J., AbuShilbayeh,L. and Rao,V.B. (1997) Display of a PorA peptide from Neisseria meningitidis on the bacteriophage T4 capsid surface. *Infect. Immun.*, **65**, 4770–4777.

57. Malys,N., Chang,D.Y., Baumann,R.G., Xie,D.M. and Black,L.W. (2002) A bipartite bacteriophage T4 SOC and HOC randomized peptide display library: detection and analysis of phage T4 terminase (gp17) and late sigma factor (gp55) interaction. *J. Mol. Biol.*, **319**, 289–304.

58. Auer,M., Moore,K.J., Meyer-Almes,F.J., Guenther,R., Pope,A.J. and Stoeckli,K.A. (1998) Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS. *Drug Discov. Today*, **3**, 457–465.

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

60. Ma,Y., Shortreed,M.R. and Yeung,E.S. (2000) High-throughput single-molecule spectroscopy in free solution. *Anal. Chem.*, **72**, 4640–4645.

61. Hink,M.A., Griep,R.A., Borst,J.W., van Hoek,A., Eppink,M.H., Schots,A. and Visser,A.J. (2000) Structural dynamics of green fluorescent protein alone and fused with a single chain Fv protein. *J. Biol. Chem.*, **275**, 17556–17560.