Electronic Supplementary Material (ESI) for RSC Advances.

Recombinant protein linker production for noninvasive determination of single-cell yeast age in heterogeneous yeast populations

Marco Eigenfeld, [a] Roland Kerpes, *[a] Thomas Becker [a]

[a] Technical University of Munich, Chair of Brewing and Beverage Technology, Research Group Beverage and Cereal Biotechnology, Weihenstephaner Steig 20, 85354 Freising, Germany

E-mail [a]: roland.kerpes@tum.de

Supporting Information

1. Materials and methods

1.1 Cloning experiment

Using PTXB3 as a template, ChBD was amplified via polymerase chain reaction (PCR) according to the setup given in Table S1 and PCR program in Tables S2-4 with the following primers:

ChBD_for: 5'-ATC CGG CCG TAC GAC AAA TCC TGG TGT ATC -3' (forward primer)

ChBD_rev: 5'-AGC CTG CAG TCA TTG AAG CTG CCA CAA G-3' (reverse primer)

Annealing temperatures were calculated according to the NEB Tm calculator, and the estimated amplicon length was 156 bp. sfGFP was amplified in two steps, with an estimated length of 714 bp. The following primers were used for the first amplification:

sfGFP1_for: 5'-GGC GGT GGG TCC ATG AGT AAA GGA GAA GAA C-3' (forward primer)

sfGFP1_rev: 5'-ACC ACC GCC ACC TTT GTA GAG CTC ATC CAT GC-3' (reverse primer)

The amplification products generated were used in a second PCR to construct a glycine-serine (GS)-spacer with an estimated product length of 738 bp, with the following primers:

sfGFP2_for: 5'-ATA ACT AGT GGG TCC GGT GGC GGT GGG TCC ATG AG-3' (forward primer)

sfGFP2_rev: 5'-TTA ACG GCC GCC ACC GCT ACC ACC GCC ACC TTT G3' (reverse primer)

Table S1: Reaction setup for PCR and colony PCR reactions

Component	**25 µL PCR reaction**	**25 µL colony PCR reaction**
5 × Q5 reaction buffer	5 µL	5 µL
10 mM dNTPs	2.5 µL	2.5 µL
forward primer	1.25 µL	1.25 µL
reverse primer	1.25 µL	1.25 µL
Q5 DNA polymerase	0.25 µL	0.25 µL
Template	0.5 ng	14.75 µL ddH2O + picked colony
Nuclease-free water	to 25 µL	-

PCR, polymerase chain reaction.

* adopted from the protocol by New England Biolabs

Table S2: The PCR parameters for amplifying the chitin-binding-domain (ChBD).

PCR step	temperature [°C]	time [m:s]	goto step	loop
1	98	0:30		
2	98	0:10		
3	63	0:10		
4	72	0:10	2	9
5	98	0:10		
6	72	0:15	5	22
7	72	2:00		
8	16	pause		

Table S3: The PCR program for amplifying the superfolder GFP (sfGFP) with the sfGFP primers.

PCR step	temperature [°C]	time [m:s]	goto step	loop
1	98	0:30		
2	98	0:10		
3	56	0:20		
4	72	0:10	2	9
5	98	0:10		
6	72	0:25	5	22
7	72	2:00		
8	16	pause		

Table S4: The PCR parameters for amplifying the superfolder GFP with the sfGFP2 primers to include the GS-linker.

PCR step	temperature [°C]	time [m:s]	goto step	loop
1	98	0:30		
2	98	0:10		
3	72	0:30	2	9
4	98	0:10		
5	72	0:25	4	22
6	72	2:00		
7	16	pause		

Next, the ligation mixture was transformed into *E. coli* Top10 using the heat-shock method according to NEB instructions (NEB catalog #C2527). Therefore 100 µL chemicompetent *E. coli* cells were mixed with 1 ng/µL and transformed according to the protocol. 50 µL of transformed bacteria per plate were grown on a selective medium with 50 µg/mL of kanamycin as a selecting agent. Colony PCR was performed using grown clones according to the reaction setup given in Table 1, with sfGFP1_for and ChBD_rev to confirm the inserted fragment (fragment size = 921 bp).

Finally, plasmids were isolated using the Monarch® Plasmid Miniprep kit (NEB catalog #T1010L), and the correct sequence and size of the resulting *His6-SUMO-sfGFP-ChBD* construct in pET28b(+) was confirmed using Sanger sequencing (GATC Biotech, Konstanz, Germany) with the T7 and pET-RP standard primer and subsequently transformed into chemocompetent *E. coli* BL21 (DE3) cells by heat shock, as described earlier. Five clones were selected and screened for the correct plasmid size by colony PCR.

1.2 His6-SUMO-GFP-ChBD synthesis and purification

Buffer compositions

Buffer	Component	Molarity [mM]
Wasch- und Lysepuffer (pH 7,3)	NaCl	300
	MOPS	50
	EDTA	2
Binding buffer IMAC (pH 7,3)	NaCl	300
	MOPS	50
	Imidazol	20
Elutionspuffer IMAC (pH 7,3)	NaCl	100
	MOPS	50
	Imidazol	500
Bindepuffer AEC (pH 5,5)	Bis-Tris	20
Elutionspuffer AEC (pH 5,5)	Bis-Tris	20
	NaCl	1000

Chromatography setup

Immobilized metal-ion affinity chromatography (IMAC):

After equilibration of the column with twice the column volume (CV) of binding buffer, 20 mL of the supernatant was loaded on the column, followed by washing with 5 CVs of binding buffer. Purification was performed using step-wise gradient elution with 10%, 50%, and 75% elution buffer.

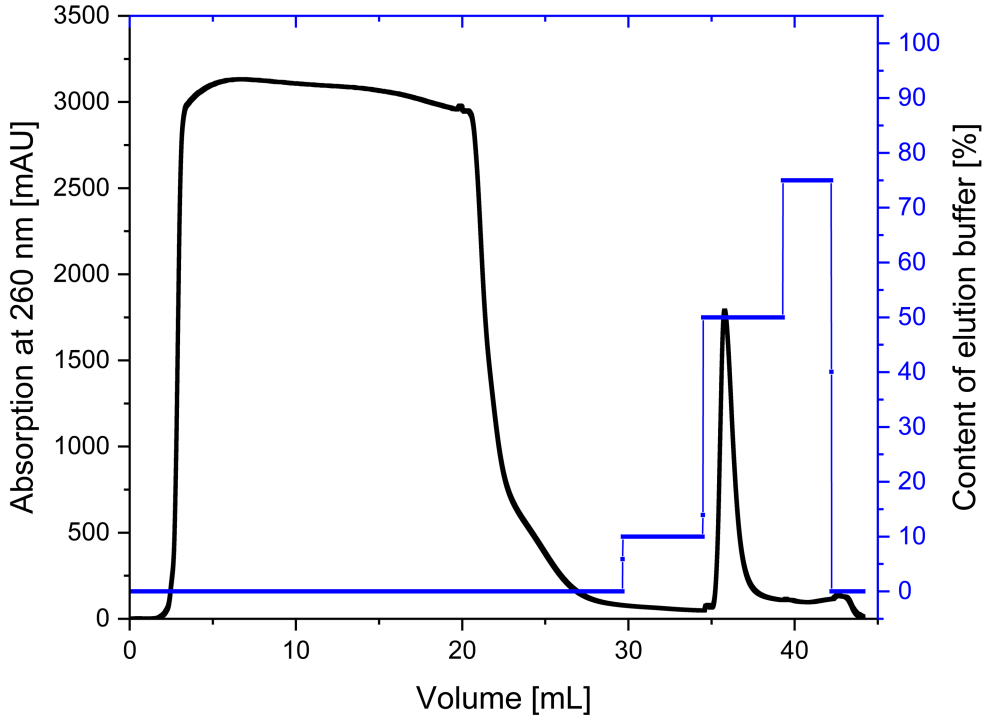

Figure S1: First purification of His6-SUMO-sfGFP-ChBD using IMAC.

Anion exchange chromatography (AEC):

Anion exchange column is equilibrated with binding buffer, and gradually eluted with 15 CVs ranging from 0% to 100% elution buffer according to Malho et al [90].

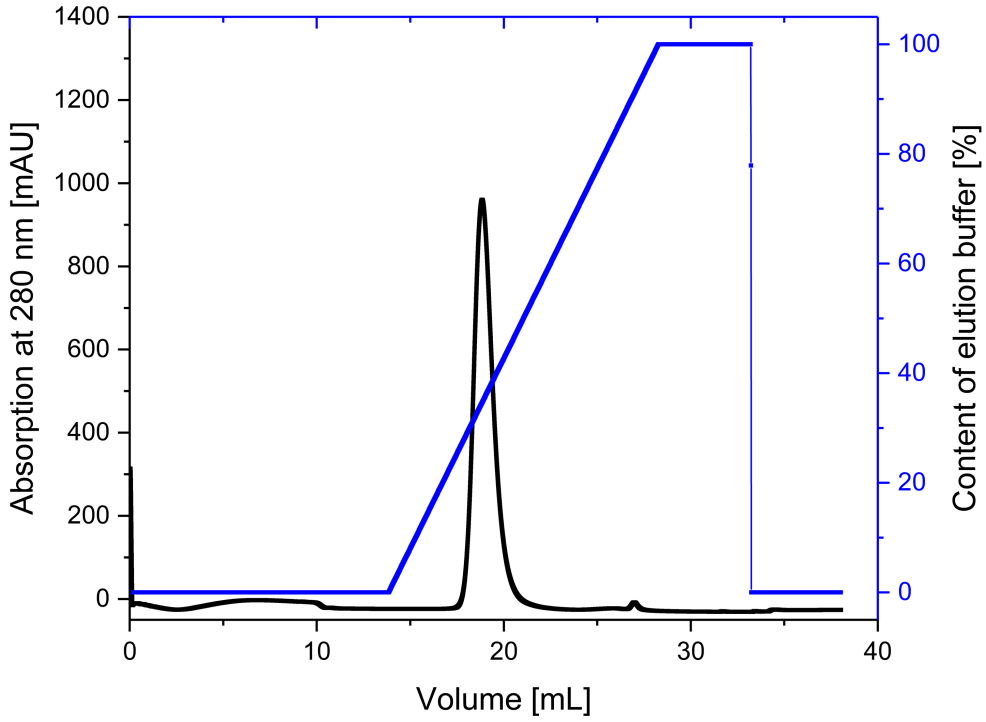

Figure S2: Second purification of His6-SUMO-sfGFP-ChBD using anion-exchange chromatography.

1.3 Tricine SDS-PAGE

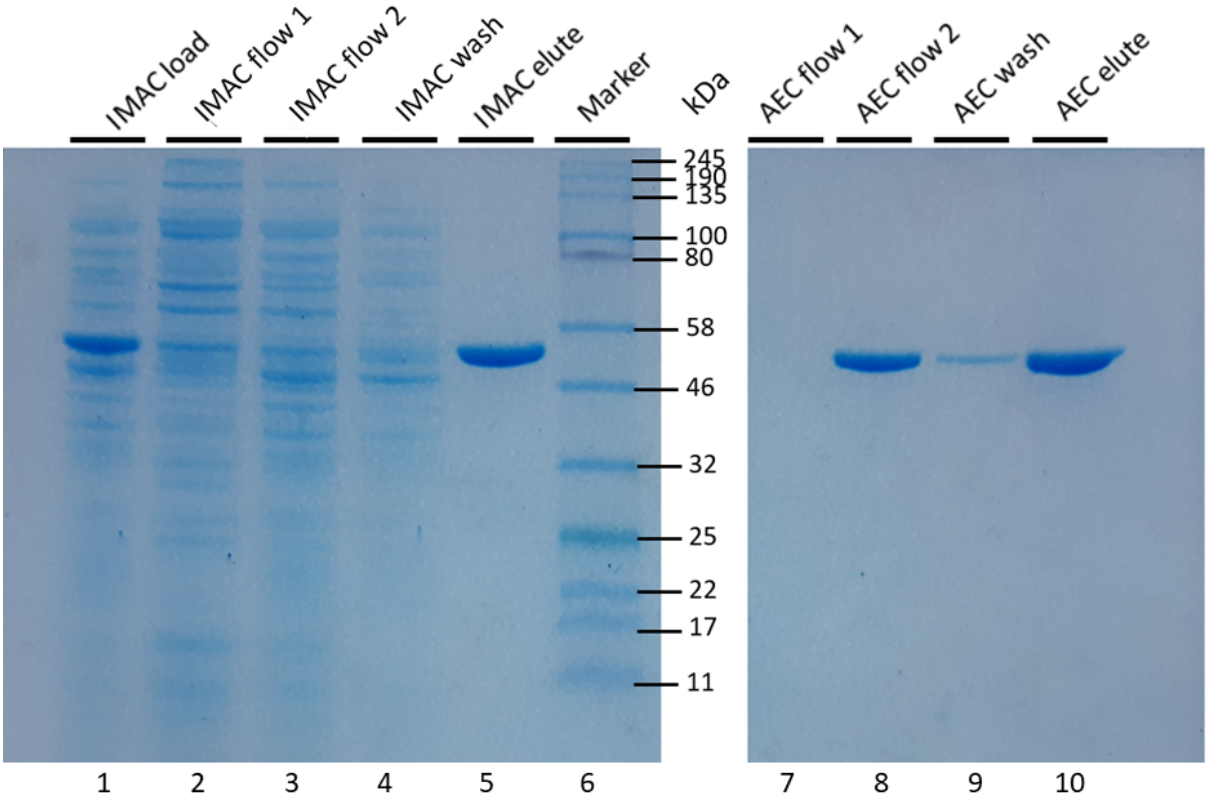

Figure S3: Tricine SDS-PAGE of FPLC fractions. (Left to right) Lane 1: supernatant of the cell lysate; lanes 2 and 3: protein flow fractions; lane 4: wash fraction; lane 5: target protein in elution fraction; lane 6: pre-stained protein standard marker (11–245 kD

1.4 Monodispersity check

We evaluated the size distribution of the purified protein sample by asymmetrical flow field-flow fractionation (AF4) coupled with multiangle light scattering (MALS), refractive index detection (dRI), and ultraviolet (UV) detection to prevent false-positive results due to immobilized dimers or trimers. Samples were filtered using a 0.2 µm pore sieve and diluted to a concentration of 0.25 g/L immediately before measurement. Protein sample elution was performed using 50 mM sodium nitrate buffer with 0.025 % sodium azide at a crossflow of 4 mL per min.

The polydispersity index (Đ) describes the width of the molecular weight (MW) distribution and is calculated by weight and number average MW (Mw/Mn). Molecularly uniform solutions have Đ = 1.[91]

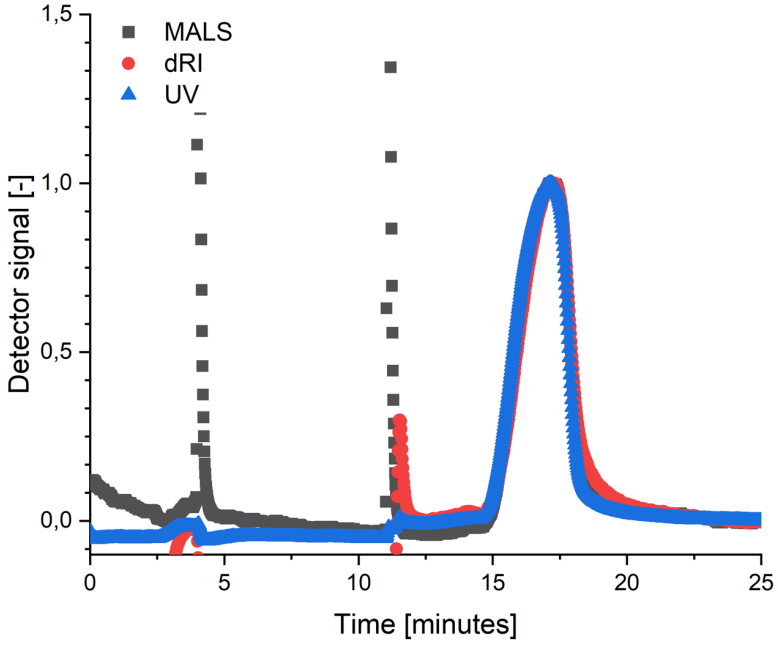

Figure S4: Asymmetric field flow fractionation chromatogram indicating monodispersity and purity of His6-SUMO-sfGFP-ChBD. 10 kDa regenerated cellulose membrane; 50 mM NaNO3 + 0.025% w/v NaN3; 350 µm spacer; crossflow: 4 mL/min; injection time: 1 min, 0.2 mL/min velocity.

1.5 Adsorption isotherms

Binding experiments were conducted by incubating different amounts of His6-SUMO-GFP-ChBD with $2.1 \cdot 10^6$ *S. pastorianus var. carlsbergensis* yeast cells, 1200 Chitin resin particles

(positive control), or $8.5 \cdot 10^7$ *E. coli* DH5 α cells (negative control) for 30 min in darkness at 25°C under vigorous shaking. Then, the supernatant was separated from the pellet and analyzed with a Cytation5 multi-detection reader (BioTek, Germany) at the excitation of 488/9 nm and emission of 520/9 nm.

The binding properties of the yeast cells coupled to FITC-labeled WGA were also compared. 50 µL of yeast cell suspension at the OD_{600} of $7.5 \cdot 10^6$ cells/mL were incubated with 200 µL of WGA-FITC suspension for 15 min in darkness at room temperature and centrifuged for 1000 × g for 3 min. Then, the supernatant was analyzed with a Cytation5 multi-detection reader as described above.

Using the calibration curve in Figure S5, measured fluorescence units were calculated into the supernatant's corresponding protein concentrations and protein mass. Differences between initial protein mass and protein in the supernatant were divided by the number of cells/particles. The number of GFP molecules was calculated by use of the molar mass and Avogadro constant. Due to the curve, the Langmuir model was used for curve fitting.

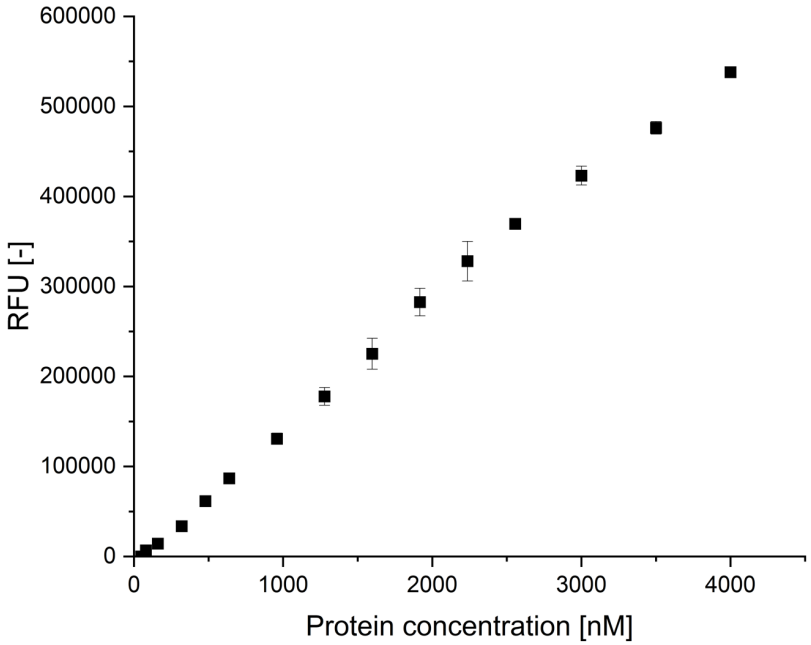

Figure S5: Calibration curve of known concentrations of His6-SUMO-sfGFP-ChBD and their corresponding relative fluorescence units (RFU). For every sample a new calibration curve was made.

1.6 Test of normally-distribution

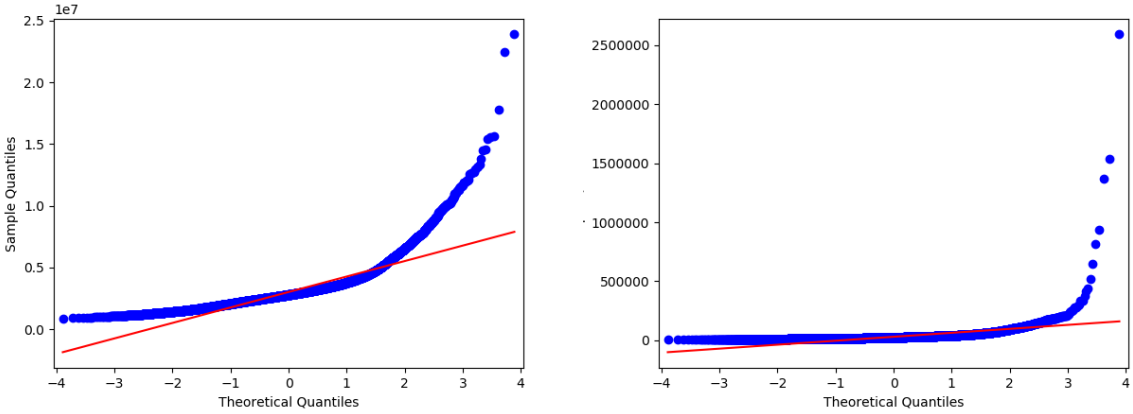

Figure S6: Q-Q plot indicating a non-normal distribution of FSC-A (left) and 525/40 - A data points (right). Red line indicates the normality line, which represents the ideal normal distribution.

1.7 Flow cytometry

Stained bud scar fluorescence intensities were measured performed using a Cytoflex S flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with an argon ion laser with 15 mW laser power and an excitation wavelength of 488 nm. Bud scar fluorescence was detected on an FL1 channel (525 nm) with at least 20,000 cells in each analysis (sample flow = max. 100 events/s; gain = 500). Each yeast suspension was analyzed independently in triplicate.

Flow cytometry of yeast cells with and without an immobilized protein linker was performed by fluorescence detection in a flow cytometric device (Cytoflex S flow cytometer). The resulting data were analyzed by focusing on forward scatter height (FSC-H), side scatter height (SSC-H), and 525 nm (525-40-A) fluorescence detectors. The flow cytometric data were evaluated using the software R.[57]

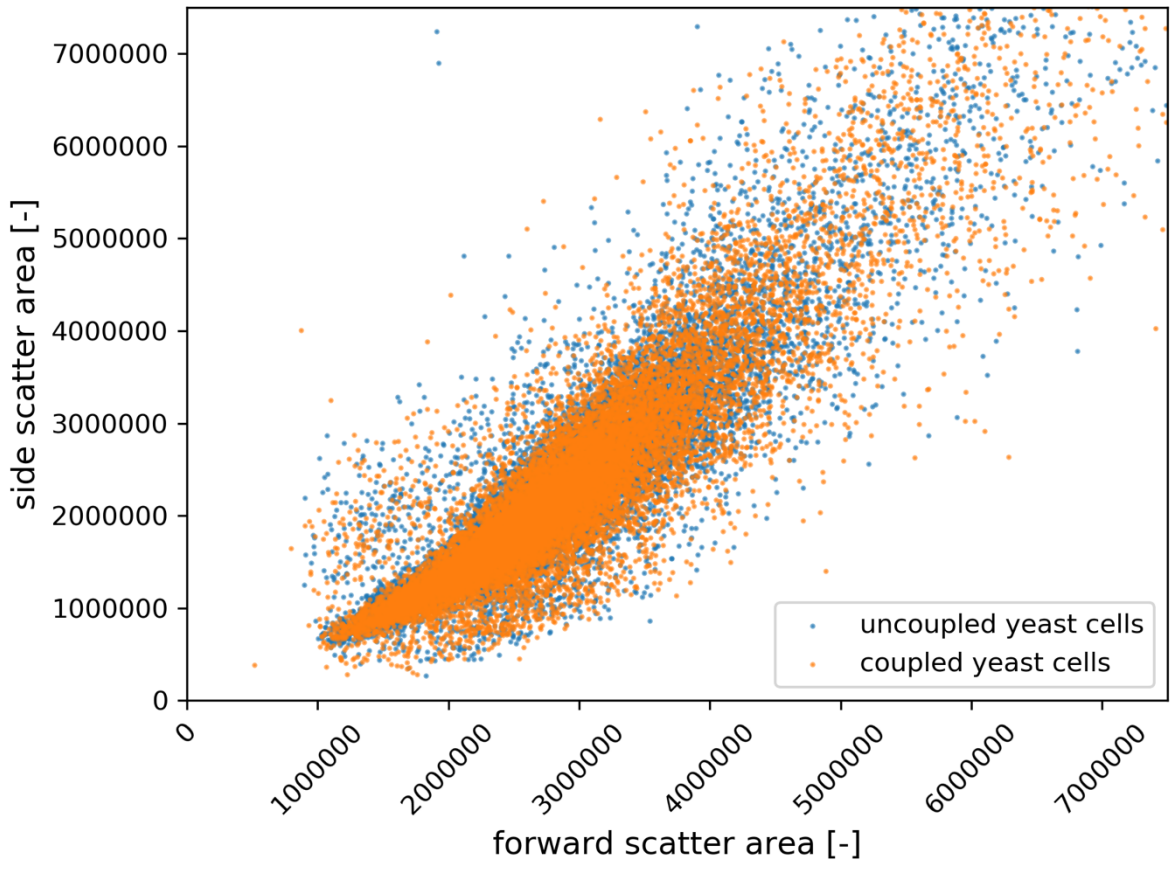

Figure S7: Graphical comparison of the front scatter signal, explaining the particle size, and the side scatter signal, an indicator of particle granularity, of yeast cells with and without immobilized protein linker.

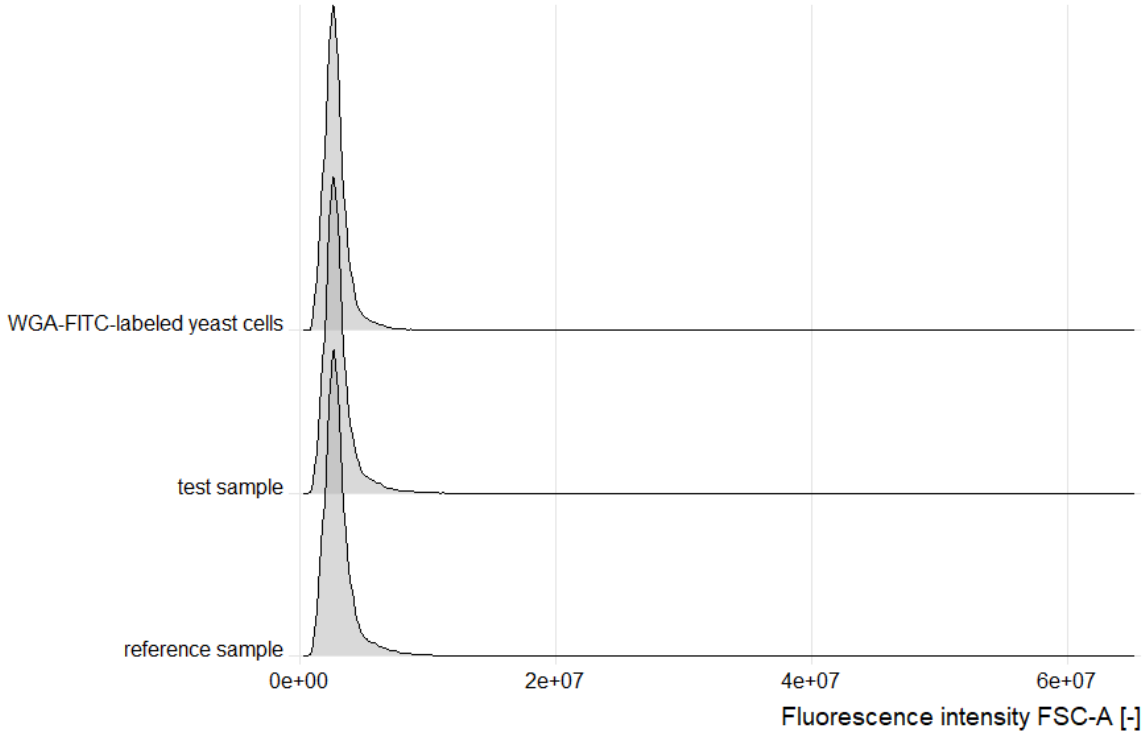

Figure S8: Histogram comparing yeast cells (reference sample), yeast cells coupled with His6-SUMO-sfGFP-ChBD (test sample) and yeast cells coupled with WGA-FITC at front scatter signal; N = 60,000 for each sample.

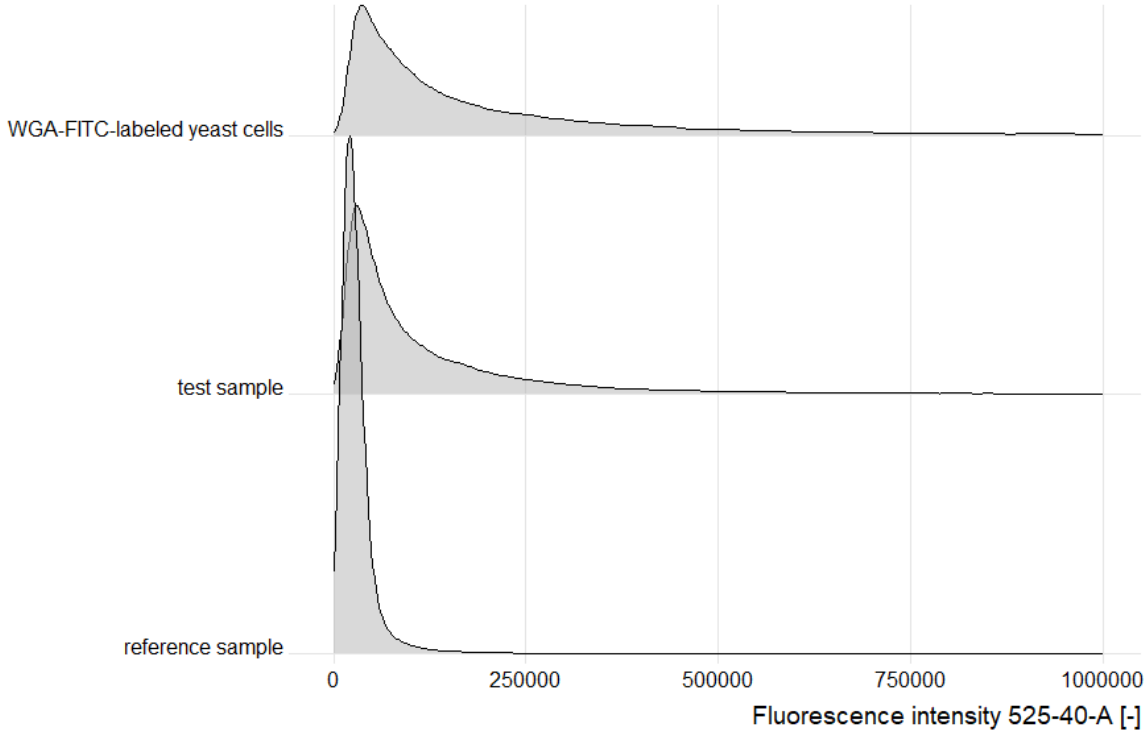

Figure S9: Histogram comparing yeast cells (reference sample), yeast cells coupled with His6-SUMO-sfGFP-ChBD (test sample) and yeast cells coupled with WGA-FITC at 525 nm fluorescence signal; N = 60,000 for each sample.

1.8 Microscopic visualization

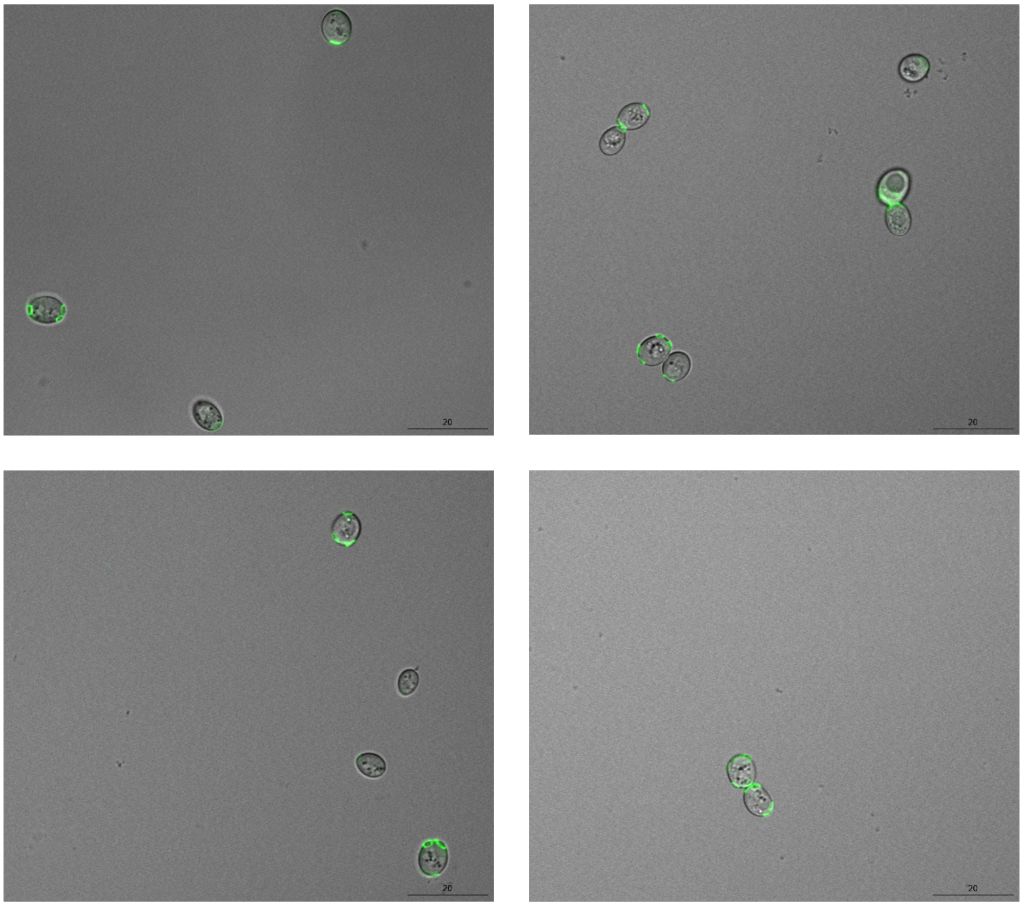

Figure S10: Overlay of the CLSM and transmission images of yeast cells successfully coupled with the protein linkers from different cultures; error bar indicates 20 µm.

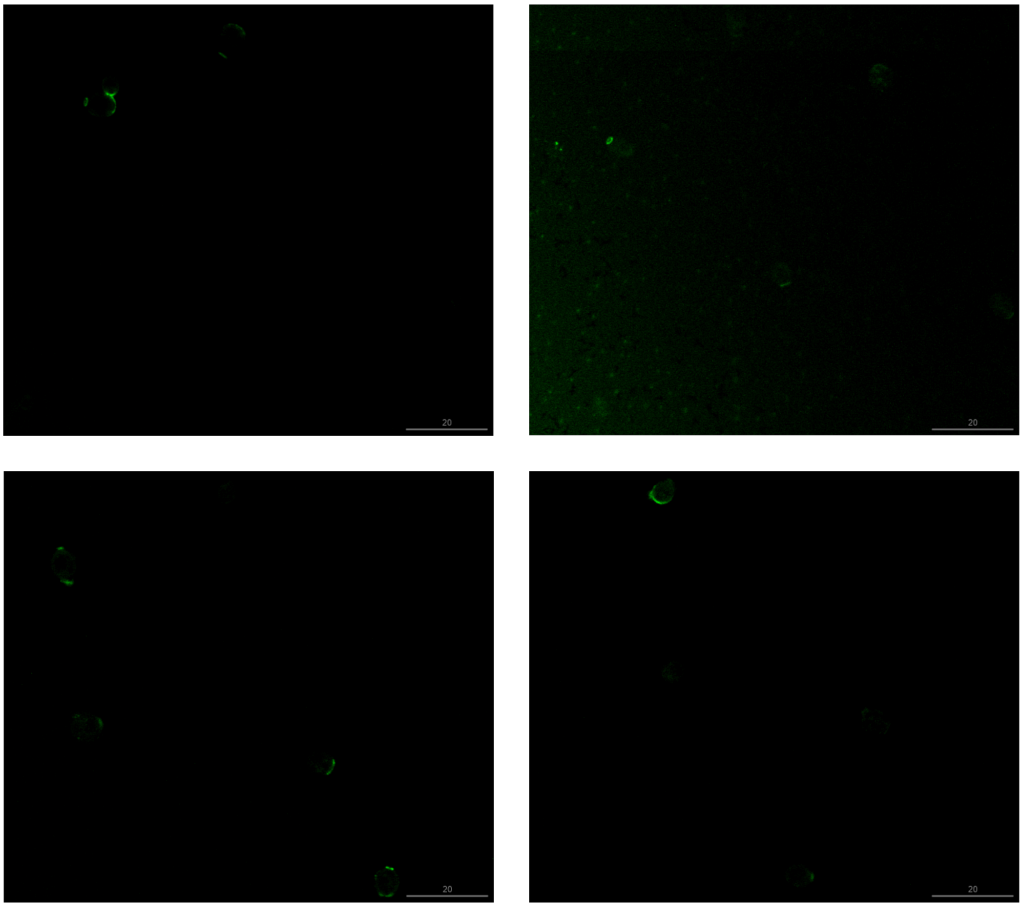

Figure S11: CLSM image of yeast cells successfully coupled with WGA-FITC protein linker. WGA, wheat germ agglutinin; FITC, fluoresceinisothiocyanate from different cultures; error bar indicates 20 µm.

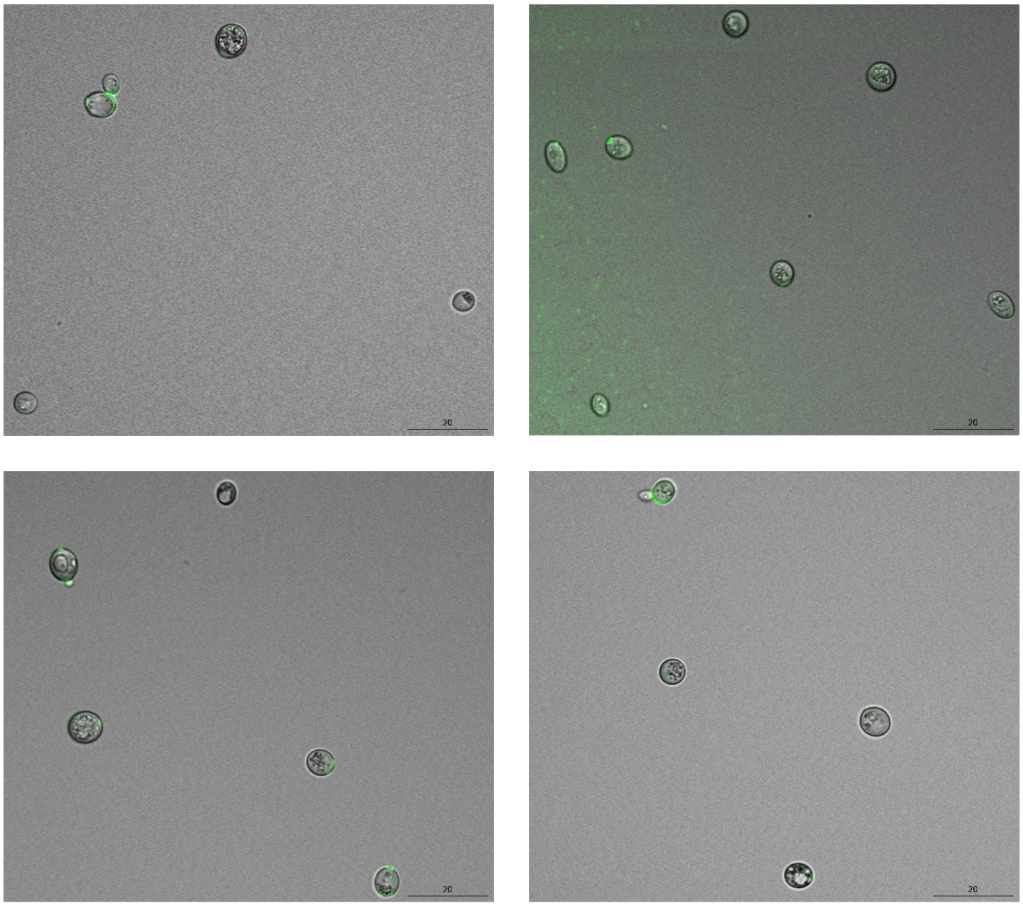

Figure 12: Overlay of CLSM and transmission pictures of yeast cells with successfully coupled WGA-FITC from different cultures; error bar indicates 20 µm.

1.9 Chitin determination

The chitin content of yeast cells was determined using a colorimetric assay according to the combination of two methods.[61, 62] In summary, a dried yeast sample of at least 40 mg or 0.5–12.5 mg of chitin powder (for calibration) was incubated with 75 µL of 72% (w/w) sulfuric acid on a thermal shaker. After 3 h, 1 mL of 0.3 mg/mL galactose was added to incubate for 4 hours at 100°C. The solution was then cooled down and adjusted to 4.5 mL by adding double-distilled water. Sulfate ions were removed by neutralizing the cell wall suspension with a saturated barium hydroxide solution. The solution was adjusted to 12 mL using double-distilled water and centrifuged for 5 min at 3800 × g. The supernatant was transferred to a new reaction tube and stored overnight for the total complexation of the sulfate ions. Afterward, the tubes were centrifuged again. Next, 100 µL of the supernatant was added to 900 µL of the detection solution (50 mmol/L Na_2SiO_3, 600 mmol/L Na_2MoO_4, 1.5 mol/L CH_3COOH, and 30% (v/v) dimethylsulfoxid solution), incubated for 30 min at 70°C for complexation, and cooled down for absorption measurement at 750 nm.

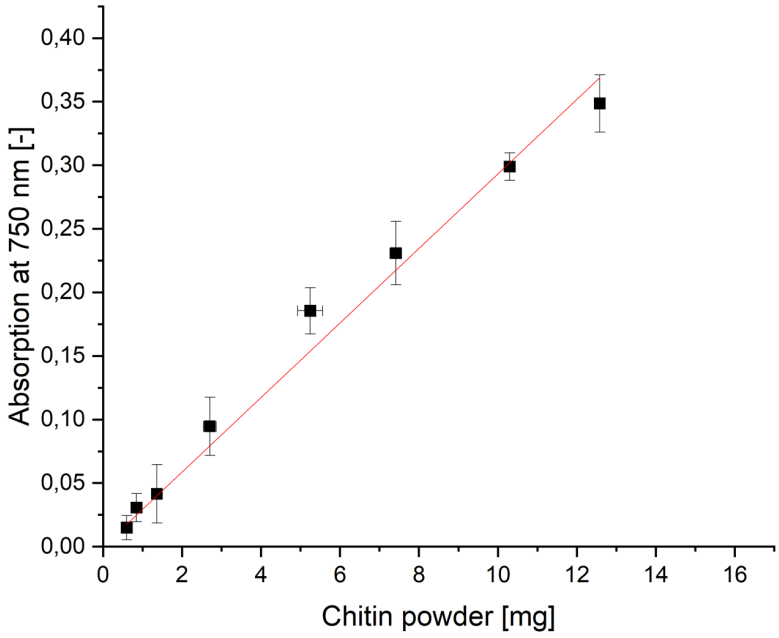

Figure S13: Calibration curve of chitin detection by acid hydrolysis and the indirect detection of chitin by the reduction of molybdenum by glucosamine forming molybdosilicate anions; R2 of 0.85. N = 3.

References:

90. J.-M. Malho, H. Heinonen, I. Kontro, N. E. Mushi, R. Serimaa, H.-P. Hentze, M. B. Linder and G. R. Szilvay, *Chemical Communications*, 2014, **50**, 7348-7351.
91. M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani and M. R. Mozafari, *Pharmaceutics*, 2018, **10**, 57.