Supporting Information

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SI Text

Methods

Scaffold and Dual Random Loop Library Creation. To create the scaffold libraries, the inserted loop regions were kept constant while the remaining GFPM scaffold was mutated (see Fig. S3 for schematic and Table S1 for primers used). Starting with pCT-ESO-GFPM-H3L3, 3 PCRs were carried out to amplify regions I, II, and III as indicated in Fig. S3. Each PCR consisted of plasmid template (250 ng), 10 μM of dNTP mix (Invitrogen), 10× buffer ($-MgCl_2$), 50 mM $MgCl_2$, 1 μL Platinum Taq (Invitrogen), and sterile water to bring the reaction volume to 50 μL. The reaction was carried out using a MJ Research PTC-200 thermocycler. The 3 PCR products were gel purified using a gel extraction kit (Qiagen) and 1 μL was used for a mutagenic PCR. Mutagenesis was carried out by error-prone PCR using the nucleotide analogues 2′-deoxy-*p*-nucleoside-5′-triphosphate and 8-oxo-2′-deoxyguanosine-5′-triphosphate (TriLink Biotech) as described previously (1). The mutated fragments were subsequently amplified by PCR and 10 μL of each were used in a 100-μL primer-less PCR to assemble the entire scaffold ORF. After gel purification and pellet paint precipitation, the amplified scaffold library was then created in yeast by homologous recombination with scaffold PCR product and pCT-ESO acceptor vector as described earlier (2). For the second round of directed evolution, the library was created by first shuffling first round clones 20–1-3, 20–1-6, and 20–1-8 followed by the above mutagenesis procedure to incorporate additional mutation. The third and fourth rounds involved only error-prone PCR as described above in the absence of shuffling. The fluorescence of evolved GFP scaffolds measured by flow cytometry is contributed to by the GFP fluorescence from proteins inside the cell as well as from those being displayed on the surface of yeast. Thus, to verify that we were evolving the scaffold to be more fluorescent on the surface of yeast and/or expressed at higher levels on the surface and not just evolving an increase in intracellular retained fluorescent protein, the surface protein was stripped to determine the external fluorescence contribution for each clone. After measuring the overall GFP fluorescence, the cells were treated with 0.5 M DTT (Sigma Aldrich) for 60 min to selectively remove surface-displayed (external) GFP and the GFP fluorescence measured again using a flow cytometer to quantify the fluorescence contribution derived from surface-displayed GFP.

To create the dual random loop libraries, the scaffold including the added restriction sites was left unaltered while the 9 amino acids inserted at Asp-102/Asp-103 and the 8 amino acids inserted at Glu-172/Asp-173 were simultaneously randomized (see Fig. S3 for schematic and Table S1 for primers used). Briefly, starting with a selected scaffold, primers encoding for NNK sequences (3) were used to create regions I and III having randomized DNA sequences in place of the CDR sequences. The regions of I and III homologous to the central region II were extended, and assembly PCR was used to create a single assembled fragment which was further amplified. Homologous recombination was used to create the resultant dual loop-inserted libraries. For the creation of the 37–2-7 dual loop library where the randomized loops contained only tyrosine and serine residues (4), the above procedure was followed with primers instead containing TMY repeat sequence.

Library Screening and Selection. For isolation of streptavidin PE binders, the GFAb library was incubated with streptavidin PE at a concentration of 9.4 nM. To isolate phycoerythrin binders, we treated the GFAb library with biotinylated PE (Invitrogen) at a concentration of 1 μM. Recombinant monomeric human tyrosine kinase receptor B (TrkB) (R&D Systems) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from human erythrocytes (Sigma Aldrich) were biotinylated using Sulfo-NHS-LC-biotin (Pierce). The GFAb library pool was incubated with biotinylated TrkB at a concentration of 250 nM. The same procedure was followed to isolate GFP-based binders against biotinylated GAPDH which was used at a concentration of 500 nM. Secondary fluorophores were alternated between streptavidin PE, anti-biotin (Neomarkers) followed by anti-mouse PE, and anti-biotin followed by anti-mouse Alexa 647 (Invitrogen) to lessen the likelihood of recovering binders against the secondary reagents. The potential for such secondary binders is present in all naïve libraries, and as indicated by our pilot streptavidin PE and biotinylated PE selections these also exist in the GFAb library. Any such instances of secondary reagent binding are indicated in Table 2. The biotinylation of GAPDH and TrkB did not effect GFAb binding as binding could be detected with the unmodified antigen using anti-GAPDH (Chemicon and Millipore) and anti-TrkB (R&D Systems) antibodies for detection on the surface of yeast. Recombinant human TrkB/Fc chimera (R&D Systems), recombinant human TrkA/Fc (R&D Systems), and recombinant human TrkC/Fc (R&D Systems) were used to test T3 specificity.

Measurement of soluble protein fluorescence properties. Purified GFPM and 37–2-7 were diluted to prepare 5 samples with OD_{488} 0.1 and lower. The emission spectrum for each dilution was measured (488 nm excitation) and the area under the curve computed. A plot of area under the curve for various dilutions was plotted for the samples. Sodium fluorescein (Sigma Aldrich) was diluted in 0.1 M NaOH and used as a standard with a quantum yield of 0.92. A similar trace of area under the curve versus dilutions used was prepared to compute the quantum efficiencies of GFPM and clone 37–2-7. All measurements were performed on a Jovan Yvon Horiba FluoroMax-3. To measure the extinction coefficient at 488 nm, the protein sample concentration was measured using the BCA kit (Pierce). The optical density was measured and divided by the concentration of the protein sample to obtain the extinction coefficient. For all other scaffolds and GFAbs, single dilution extinction coefficient and quantum yield measurements were performed using the same procedure as above except relative protein concentration measurements were made by western blotting.

Measurement of Soluble GFAb-binding Properties. HEK 293 T/17 cells (human embryonic kidney cell line) were cultured using DMEM media (Invitrogen) in 24-well plates. The cells were washed twice using PBS supplemented with BSA at 10 mg/mL. They were fixed for 5 min using a 1:1 mixture of methanol and acetone. The cells were washed twice with PBS-BSA and incubated for 90 min with 150 μL GAPDH binder G6 or an equal concentration of the scaffold 20–5-8 as a negative control. The cells were subsequently washed twice and fixed using 4% paraformaldehyde for 4 min. The cells were imaged using an Olympus IX70 fluorescence microscope with an excitation wavelength of 445 ± 20 nm and an emission wavelength of 509 ± 24 nm. As a positive control, HEK cells were labeled with anti-GAPDH monoclonal antibody (1:100) (Chemicon and Millipore) for 1 h followed by anti-mouse Alexa 488 (Molecular Probes, Invitro-

gen) for 30 min. DAPI was used as a nuclear stain. For the streptavidin-PE binder, 1.3 GFAb, which binds to streptavidin alone, 5 μL streptavidin-coated polystyrene beads (Spherotech) with a mean particle diameter of 3.2 μm were washed with PBS-BSA (1 g/L). The beads were incubated with PBS-BSA for 20 min followed by incubation with 500 nM of 1.3 GFAb. An equal concentration of scaffold 20–5-8 was used as a control, and 400 nM streptavidin-PE was used in some samples as a competitive inhibitor. After 1 h, the beads were washed with PBS-BSA and viewed on an Olympus IX70 fluorescence microscope and analyzed by flow cytometry.

Measurement of soluble T3 GFAb affinity was performed using antigen-loaded beads combined with flow cytometry. Briefly, streptavidin-coated polystyrene beads were washed in PBS-BSA (1 g/L). The beads were coated with biotinylated monomeric TrkB (approx 0.45 mg/mL) for 1 h at room temperature on a rocker. The beads were blocked with of PBS-BSA (10 g/L) for 1 h at room temperature. The TrkB-loaded beads were then incubated with serial dilutions of GFAb T3 always kept in molar excess by using increasing volumes (5) for 1 h with rocking. The beads were washed in PBS-BSA (1 g/L) and GFP fluorescence quantified by flow cytometry. The equilibrium dissociation constant was then determined by fitting the binding curve to a 2 parameter equilibrium binding model as described previously (5). Soluble T3 GFAb affinity was also estimated by competition assay. Briefly, soluble T3 GFAb was incubated with soluble TrkB at known concentrations. Yeast displaying T3 GFAb were then used to measure the unbound concentration of TrkB in the solution phase binding mixture by FACS. Using equilibrium binding models representing both the solution phase interaction and the yeast surface interaction, the solution phase affinities were measured to be approximately 10 nM.

1. Zaccolo M, Williams DM, Brown DM, Gherardi E (1996) An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. *J Mol Biol* 255:589–603.
2. Swers JS, Kellogg BA, Wittrup KD (2004) Shuffled antibody libraries created by in vivo homologous recombination and yeast surface display. *Nucleic Acids Res* 32:15.
3. Wolkowicz R, Nolan GP (2003) Retroviral technology - Applications for expressed peptide libraries. *Front Biosci* 8:D603–D619.
4. Fellouse FA, Li B, Compaan DM, Peden AA, Hymowitz SG, Sidhu SS (2005) Molecular recognition by a binary code. *J Mol Biol* 348:1153–1162.
5. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD (2006) Isolating and engineering human antibodies using yeast surface display. *Nat Protoc* 1:755–768.

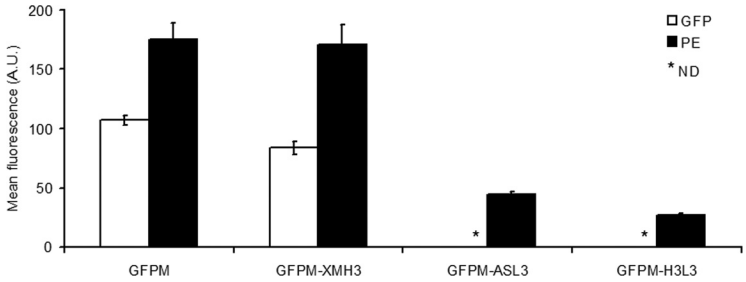
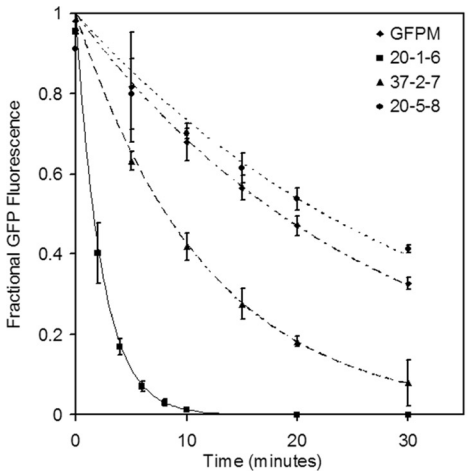


Fig. S1. Properties of dual surrogate loop-inserted GFPM. Quantitative expression (PE) and fluorescence (GFP) flow cytometric data are presented as the mean ± SD of triplicate independent yeast transformants. ND denotes the absence of a detectable signal.



Protein	Denaturation Rate Constant (x 10^3 min^{-1})	Half-life Surface (min)	Half-life Soluble (min)
GFPM	37 ± 1	19	12
20-1-6	410 ± 10	2	-
37-2-7	81 ± 2	9	7
70C-3	33 ± 1	21	17
20-4-8	38 ± 2	18	-
20-5-8	26 ± 2	26	-

Fig. S2. Thermal denaturation characteristics of the evolved scaffolds. Scaffolds were denatured on the yeast surface for the indicated times at 70 °C and remaining fluorescence quantified by flow cytometry. First order exponential denaturation rate constants were determined and the exponential fits along with experimental data are shown in the plot. Rate constants are listed in the accompanying table along with the fluorescence half-life for each mutant (half-life surface). The data represent triplicate samples throughout the entire timecourse. A similar procedure was performed with several of the purified, soluble scaffold proteins with fluorescence half-life monitored by 70 °C denaturation in solution using a thermocyler equipped with fluorescence detection capability (half-life soluble).

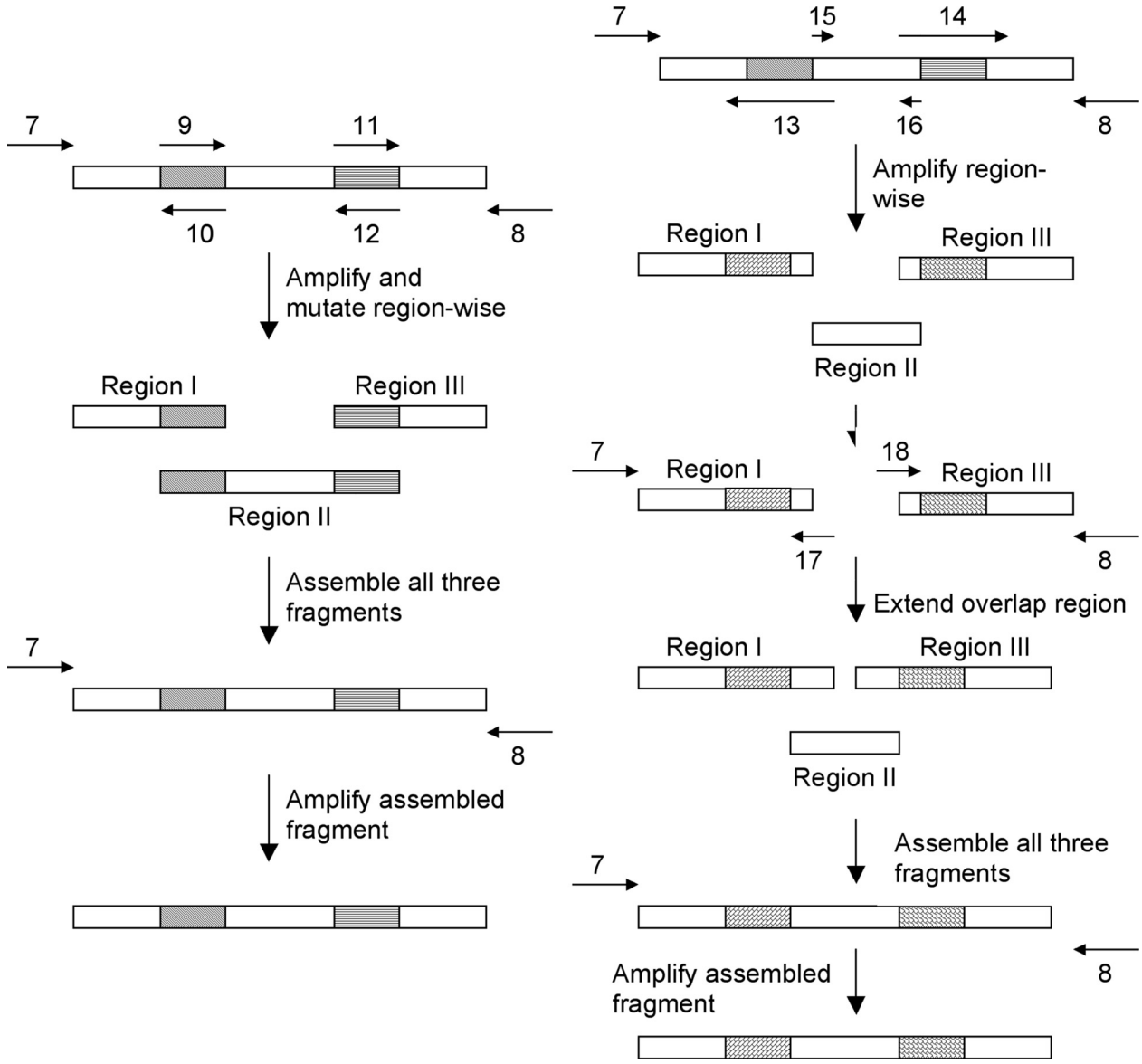
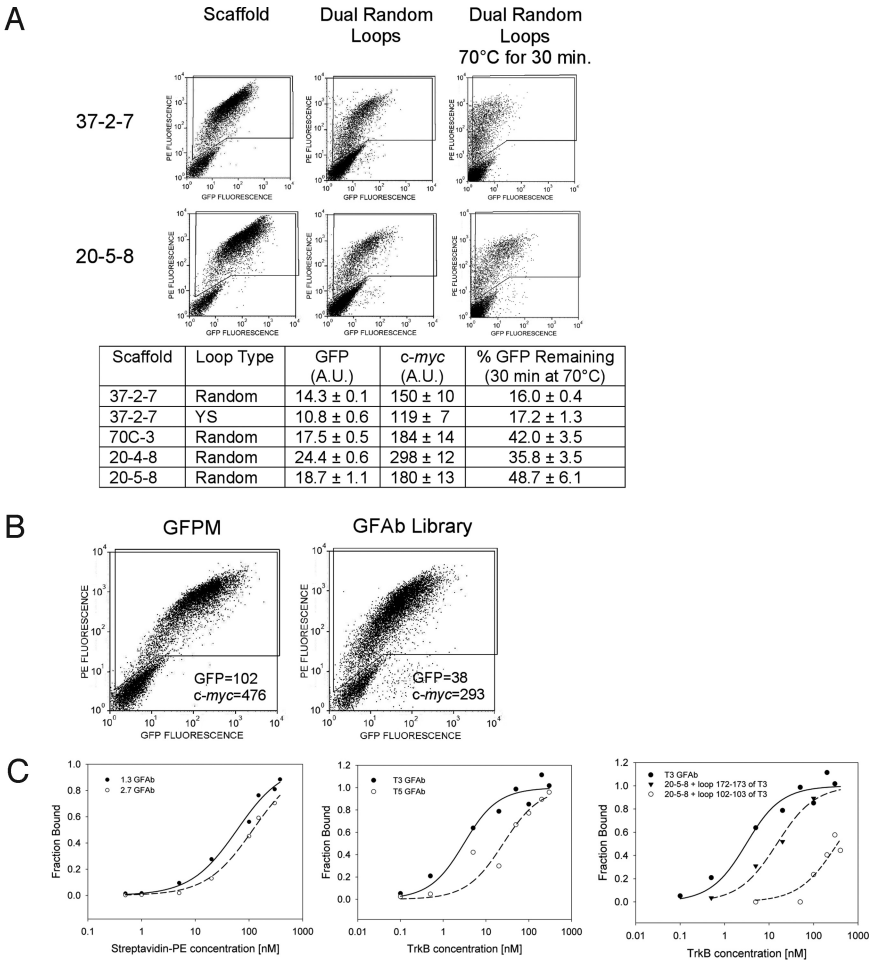


Fig. S3. Schematic of strategy to create library of GFP scaffolds (left) and random loop libraries (right). Primer numbering matches description in Table S1.



Scaffold	Loop Type	GFP (A.U.)	c-*myc* (A.U.)	% GFP Remaining (30 min at 70°C)
37-2-7	Random	14.3 ± 0.1	150 ± 10	16.0 ± 0.4
37-2-7	YS	10.8 ± 0.6	119 ± 7	17.2 ± 1.3
70C-3	Random	17.5 ± 0.5	184 ± 14	42.0 ± 3.5
20-4-8	Random	24.4 ± 0.6	298 ± 12	35.8 ± 3.5
20-5-8	Random	18.7 ± 1.1	180 ± 13	48.7 ± 6.1

Fig. S4. Properties of random loop libraries and binding characteristics of isolated GFAb clones. (*A*) Dot plots indicating the full-length expression (PE) and fluorescence (GFP) of 2 scaffolds, 37–2-7 and 20–5-8, the dual random loop libraries created using these scaffolds and the resistance to thermal denaturation of the resultant libraries. Quantification of the gated regions in the dot plots was performed for all scaffolds and the data compiled in the accompanying table. All data are the result of duplicate independent library and denaturation samples. For comparison, trends in scaffold properties can be found in Fig. 1*C* and Fig. S2. (*B*) Comparison of the fitness of the GFAb library with that of non loop-inserted GFPM. Fluorescence (GFP) and expression (c-*myc*) statistics can be found in the *Inset*. (*C*) Yeast cell surface affinity titrations for selected GFAbs binding to streptavidin-PE (*Left*) or TrkB (*Center)*. Yeast cell surface affinity titrations for single loop knock-ins of TrkB-binding T3 GFAb indicating contribution of both inserted loops to the binding affinity of the dual loop T3 GFAb (*Right*). Shown are single sample titrations and equilibrium binding fits, while K_D with associated error from triplicate titration samples can be found in Table 2.

Table S1. Oligonucleotides employed for library construction

	Oligonucleotide name	DNA sequence 5′→3′
1	A206Kupper	CTTATCCACTCAATCTAAGTTATCCAAAGATCCAAAC
2	A206K lower	GTTTGGATCTTTGGATAACTTAGATTGAGTGGATAAG
3	AflII-SpeI insertion upper	GAACTATTTTTTTCAAAGATCTTAAGACTAGTGACGGTAACTAC
4	AflIII-SpeI insertion lower	CTGGTCTTGTAGTTACCGTCACTAGTCTTAAGATCTTTGAAAAA
5	XhoI-MluI insertion upper	CAAAATTAGACACAACATTGAATCTAGAACGCGTGATGGTTCT
6	XhoI-MluI insertion lower	GCTAATTGAACAGAACCATCACGCGTTCTAGATTCAATGTTGT
7	ESO forward	GTGGAGGAGGCTCTGGTGGAGGCGGTAGCGGAGGCGGAGGGTCGGCTAGC
8	ESO reverse	GAACAAAAGCTTATTTCTGAAGAGGACTTGTAATAGCTGAGATCTGATA
9	Upper CDRL3	TTAAGCAACATTTTTGGAGTACTCCTCGGACGA
10	Lower CDRL3	CTAGTCGTCCGAGGAGTACTCCAAAAATGTTGC
11	Upper CDRH3	CTAGAGAGAGAGATTATAGGCTTGACTACA
12	Lower CDRH3	CGCGTGTAGTCAAGCCTATAATCTCTCTCT
13	Random loop 102–103	CTTGTAGTTACCGTCACTAGT $(MNN)_9$ CTTAAGATCTTTGAAAAAAAT
14	Random loop 172–173	AGACACAACATTGAATCTAGA $(NNK)_8$ ACGCGTGATGGTTCTGTTCAA
15	102–103 overhang	ACTAGTGACGGTAACTACAAG
16	172–173 overhang	TCTAGATTCAATGTTGTGTCT
17	Light extension	AAACTTGACTTCAGCTCTGGTCTTGTAGTTACCGTCACTAGT
18	Heavy extension	AATGGTATCAAAGCTAACTTCAAAATTAGACACAACATTGAATCTAGA

Table S2. Plasmids created in this study

Plasmid name	Amino acid insertion between Asp102-Asp103	Amino acid insertion between Glu172-Asp173
GFPM-AS	LKTS	–
GFPM-XM	–	SRTR
GFPM-ASXM	LKTS	SRTR
GFPM-ASL3	LKQHFWSTPRTTS	–
GFPM-XMH3	–	SRERDYRLDYTR
GFP-H3L3	LKQHFWSTPRTTS	SRERDYRLDYTR

Table S3. Spectral properties of selected scaffolds and GFAbs as soluble proteins

GFP variant	Quantum yield, Φ	Extinction coefficient, $M^{-1}cm^{-1}$	GFP/mol, % of GFPM*	GFP/mol, % of scaffold†
GFPM	0.60	37,000	100	
37–2-7	0.39	21,000	37	
20–5-8	0.73	20,000	66	
G6	0.72	25,000	82	120
T5	0.65	21,000	62	170
T3	0.20	32,000	28	43
1.3	0.83	7,300	27	41
2.7	0.23	36,000	39	60

*GFP per molecule (brightness) expressed as quantum yield x extinction coefficient and normalized to non-loop inserted GFPM.
†GFP per molecule (brightness) normalized to the parent scaffold to yield values found in Table 2.