Supplementary Tables

Supplementary Table 1 | SOMAmer reagents

SOMAmer reagent	Target	Design/Modifications	Base Count	MW (kDa)	K_d (nM)
Cyanine-3-P1-SL1070	GFP	Cy3-5'-ATACATCTA-TT-SOMAmer-3'-inverted-dT	50	21.0	0.2
Cyanine-3-P1-SL1069	EGFR (ERBB1)	Cy3-5'-ATACATCTA-TT-SOMAmer-3'-inverted-dT	50	21.6	1.6
P1-SL1069	EGFR (ERBB1)	5'-ATACATCTA-TT-SOMAmer-3'-inverted-dT	50	21.1	1.4
Fl-SL1071-P5	Catalase	Fluorescein-5'-SOMAmer-TT-TCAATGTAT-3'	50	20.5	0.1
Fl-SL1072-P3	LIMP2	Fluorescein-5'-SOMAmer-TT-TCTTCATTA-3'	50	20.8	40
P1- SL1073	HSP60	5'-ATACATCTA-TT-SOMAmer-3'-inverted-dT	74	28.4	3
Fl- SL1074-P6	HSP90a/b	Fluorescein-5'-SOMAmer-TT-TTAGGTAAA-3'	50	21.0	2
Fl-SL1075-P3	ErbB2	Fluorescein-5'-SOMAmer-TT-TCTTCATTA-3'	50	17.4	10

Supplementary Table 2 | Tested SOMAmer reagents for PFA and methanol fixation protocol

Target	SOMAmer ID	PFA fixation	Methanol fixation
EGFR	P1-SL1069	+	+
EGFR	Cyanine-3-P1-SL1069	+	+
ErbB2	Fl-SL1075-P3	+	+
ErbB2	FI-SL1081-P3	-	-
ErbB2	SL1082-P3	+	+
HSP90a/b	Fl_SL1074-P6	+	+
LIMP2	Fl-SL1072-P3	+	-
HSP60	P1-SL1073	Low signal	+
HSP60	Cyanine-3-P1-SL1076	Low signal	+
Catalase	Fl-SL1071-P5	+	-
Calreticulin	Fl-SL1077-P6	-	-
LaminB	Fl-SL1078-P3	Low signal	Not tested
GTP-binding nuclear protein Ran	Fl-SL1079-P5	-	-
DnaJ homolog TIM14	Fl-SL1080-P3	-	-
GFP	Cyanine-3-P1-SL1070	+	Not tested

Supplementary Notes

Supplementary Note 1: Use of polyanionic competitors during staining

The addition of competitors during the SOMAmer staining reaction can help to reduce non-specific binding and thus improve the signal-to-noise ratio. Although SOMAmers typically have very high, antibody-like affinities to their targets, cells contain many proteins with a modest nonspecific affinity towards the SOMAmers, such as highly abundant plasma proteins that can bind DNA. Another source of non-specific SOMAmer binding is electrostatic attraction of the polyanionic aptamers to positively charged regions of some proteins, such as histones in the nucleus. Here, we used two different competitors simultaneously: The first is low molecular weight dextran sulfate (6.5–10 kDa), typically used during SOMAmer selection as kinetic challenger, which has been shown to reduce nonspecific binding in cell staining[1]. High concentrations of dextran sulfate drastically decreased off-target binding of the SOMAmers in the nucleus (**Supplementary Figure 11**). The second competitor we used is a DNA-based polyanionic competitor named Z-Block (SomaLogic). Z-Block contains the modified nucleotides (Bn-dU) as some of the SOMAmers (30-mer, [5'-AC[Bn-dU]$_2$]$_7$AC-3']) and strongly reduces intracellular off-target interactions of the SOMAmers. An increase in the Z-Block concentration led to a highly improved signal-to-noise level (**Supplementary Figure 12**). The optimum concentration of competitors was evaluated for every SOMAmer construct individually. We found that a molar excess of 1,000 times for Z-Block and 10,000 times for dextran sulfate are a good starting point to reduce unspecific binding to a minimum and to obtain the maximum signal for specific staining at the same time.

Supplementary Note 2: Live-cell staining and DNA-PAINT single-particle-tracking

We tested if it is possible to use SOMAmers to label the extracellular EGFR in living A431 cells. We observed efficient membrane staining after 20 min of incubation at 4°C with the SOMAmers diluted in L-15 cell medium supplemented with 10 µM Z-Block (**Supplementary Figure 13b**). We show successful EGFR SOMAmer labeling with downstream fixation (4% PFA, 30 min), permeabilization and immunostaining of intracellular EGFR using primary and secondary antibodies. Co-localization of SOMAmer and antibody signal shows the specificity of the EGFR SOMAmer. We tested potential non-specific binding of the SOMAmers on living cells by using the GFP-SOMAmer as a negative staining control in A431 cells (**Supplementary Figure 13a**) and observed a low amount of non-specific binding, which could be efficiently reduced by the addition of 10 µM Z-Block.

Supplementary Methods

Secondary antibody conjugation to DNA

Anti-mouse secondary antibody was conjugated to a DNA-PAINT docking site (Thiol-5'-TTATACATCTA-3') using maleimide-PEG2-succinimidyl ester[2, 3].

Supplementary Figure 1

SK-BR-3 cells were fixed in pre-warmed (to 37°C) 4% paraformaldehyde in PBS for 15 min at room temperature, then washed twice with PBS and permeabilized with 0.25% Triton-X-100 in PBS for 15 min. The folded SOMAmer reagents against EGFR, ErbB2 and HSP90 were diluted to 100 nM in SOMAmer staining buffer (1×PBS, 5 mM $MgCl_2$, 1% BSA, 0.05% Tween-20, 0.1 mg/ml sheared salmon sperm DNA, 10 µM Z-Block, 1 mM dextran sulfate) and incubated with the cells overnight at 4°C. After SOMAmer incubation, the cells were washed three times with PBS + 5 mM $MgCl_2$. Post-fixation was performed using 4% paraformaldehyde in PBS + 5 mM $MgCl_2$ for 10 min at room temperature, then washed three times with PBS + 5 mM $MgCl_2$.

Imaging conditions

Imaging round	Imager strand	Imager concentration	Frames	Integration time	Laser power density
1. ErbB2	TAATGAAGA-Cy3b	0.75 nM	23,177	250 ms	0.83 kW/cm^2
2. EGFR	Cy3b-TAGATGTAT	3 nM	23,512	250 ms	0.83 kW/cm^2
3. HSP90	CTTTACCTAA-Cy3b	0.5 nM	30,000	250 ms	0.83 kW/cm^2

A cylindrical lens was used for 3D astigmatism imaging.

Supplementary Figure 2

SK-BR-3 cells were fixed with -20°C 100% methanol for 5 min at room temperature, then, washed three times with PBS. The folded SOMAmer reagents were diluted to 100 nM in SOMAmer staining buffer (1× PBS, 5 mM $MgCl_2$, 1% BSA, 0.05% Tween-20, 0.1 mg/ml sheared salmon sperm DNA, 10 µM Z-Block, 1 mM dextran sulfate) and incubated with the cells overnight at 4°C. After SOMAmer incubation, the cells were washed three times with PBS + 5 mM $MgCl_2$. Post-fixation was performed using 4% paraformaldehyde in PBS + 5 mM $MgCl_2$ for 10 min at room temperature followed by washing three times with PBS + 5 mM $MgCl_2$.

Imaging conditions

Imaging round	Imager strand	Imager concentration	Frames	Integration time	Laser power density
1. LIMP2	TAATGAAGA-Cy3b	1.5 nM	20,000	250 ms	0.41 kW/cm^2
2. HSP60	Cy3b-TAGATGTAT	0.3 nM	40,000	250 ms	0.62 kW/cm^2

A cylindrical lens was used for 3D astigmatism imaging.

Supplementary Figures 6 and 7

Prior to fixation, A431 (Supp. Figure 6) or A549 (Supp. Figure 7) cells were serum depleted overnight and treated with 80 µM endocytosis inhibitor Dynasore (Sigma Aldrich, SML0340) for ~30 min. Cells were fixed with ice-cold 4% paraformaldehyde in PBS for 30 minutes at room temperature, then washed two times with PBS and permeabilized with 0.25% Triton-X-100 for 15 min.

SOMAmer staining: The folded SOMAmer against EGFR was diluted to 100 nM in SOMAmer staining buffer (1×PBS, 5 mM $MgCl_2$, 1% BSA, 0.05% Tween-20, 0.1 mg/ml salmon sperm DNA, 10 µM Z-Block, 1 mM dextran sulfate) and incubated with the cells overnight at 4°C. After SOMAmer incubation, the cells were washed three times with PBS + 5 mM $MgCl_2$. Post-fixation was performed using 1% paraformaldehyde and 0.1% glutaraldehyde in PBS + 5 mM $MgCl_2$ for 10 min at room temperature followed by washing three times with PBS + 5 mM $MgCl_2$.

EGFR immunostaining: Cells were blocked with 3% BSA in PBS for 60 min at room temperature. Primary antibody against the extracellular region of EGFR (Thermo Fisher, MA5-13319) was diluted (1:50) in 3% BSA in PBS and incubated with the cells overnight at 4°C. Cells were rinsed with PBS and washed three times with an incubation time of 5 min. Secondary anti-mouse antibody conjugated to a P1 docking site was diluted in 3% BSA in PBS and incubated with the cells for 1 h at room temperature. Cells were rinsed with PBS and washed three times with an incubation time of 5 min. Post-fixation was performed using 1% paraformaldehyde and 0.1% Glutaraldehyde in PBS + 5 mM $MgCl_2$ for 10 min at room temperature followed by washing three times with PBS + 5 mM $MgCl_2$.

Imaging conditions for Supplementary Figure 6:

	Imager strand	Imager concentration	Frames	Integration time	Laser power density
SOMAmer	Cy3b-TAGATGTAT	1.2 nM	50,000	150 ms	1.9 kW/cm^2
Antibody	CTAGATGTAT-Cy3b	0.25 nM	50,000	150 ms	1.9 kW/cm^2

For analysis, ~20,000 – 30,000 single EGFR proteins were selected and analyzed using Picasso's pick tool with a radius of 65 nm (adjusted to the experimental localization precision in order to fully select single receptors).

Imaging conditions for Supplementary Figure 7:

	Imager strand	Imager concentration	Frames	Integration time	Laser power density
SOMAmer	AGATGTAT-Cy3b	15 nM	40,000	100 ms	0.41 kW/cm^2
Antibody	AGATGTAT-Cy3b	2.5 nM	40,000	100 ms	0.41 kW/cm^2

For analysis, ~3,000 single EGFR proteins were selected and analyzed using Picasso's pick tool with a radius of ~90 nm (adjusted to the experimental localization precision in order to fully select single receptors).

Supplementary Figure 8

Imaging conditions:

Imaging Round	Imager strand	Imager concentration	Frames	Integration time	Laser power density
1. Catalase	CATACATTGA-Cy3b	1 nM	30,000	250 ms	0.83 kW/cm^2
2. PMP70	CTAGATGTAT-Cy3b	0.3 nM	30,000	200 ms	1.2 kW/cm^2

A cylindrical lens was used for 3D astigmatism imaging.

Supplementary References

1. Gupta, S. et al. *Appl Immunohisto M M* **19**, 273-278 (2011).
2. Agasti, S.S. et al. *Chem Sci* **8**, 3080-3091 (2017).
3. Schnitzbauer, J., Strauss, M.T., Schlichthaerle, T., Schueder, F. & Jungmann, R. *Nat Protoc* **12**, 1198-1228 (2017).