



---

# Quantification of absolute labeling efficiency at the single-protein level

---

In the format provided by the authors and unedited

## Supplementary Information

Supplementary Table 1 | Labeling efficiency – Tag-proteins

Target	Binder	Clone	Terminus	Total area ( $\mu\text{m}^2$ )	Target density ( $\mu\text{m}^{-2}$ )	Labeling efficiency (%)	Target-Reference offset (nm)	Cells (n)	Repeats (n)
CD86 - mEGFP	Nanobody	1H1	C	$354.0 \pm 57.4$	$48.8 \pm 33.7$	$47.3 \pm 2.7$	$10.3 \pm 0.7$	6	3
CD86 - mEGFP	Nanobody	1B2	C	$547.5 \pm 77.4$	$22.3 \pm 8.3$	$43.8 \pm 2.0$	$10.3 \pm 0.5$	5	2
CD86 - ALFA-tag	Nanobody	1G5	C	$525.4 \pm 130.5$	$29.6 \pm 17.0$	$42.0 \pm 5.1$	$11.8 \pm 0.3$	6	3
CD86 - mEGFP	Nanobody	1H1 1B2	C	$331.8 \pm 0.0$	$28.9 \pm 15.9$	$61.8 \pm 5.4$	$13.5 \pm 1.7$	5	2
CD86 – ALFA-tag-mEGFP	Nanobody	1H1 (GFP) 1B2 (GFP) 1G5 (Alfa)	C	$337.4 \pm 68.0$	$19.5 \pm 8.4$	$76.2 \pm 8.4$	$15.0 \pm 0.1$	6	2
CD86 - mCherry	Nanobody	2B12	C	$360.3 \pm 73.0$	$47.1 \pm 33.7$	$19.7 \pm 2.5$	$11.3 \pm 0.5$	7	3
CD86 - TagRFP	Nanobody	1H7	C	$404.8 \pm 88.2$	$26.0 \pm 15.2$	$41.4 \pm 3.4$	$11.8 \pm 0.4$	5	2
CD86 - mEOS2	Nanobody	1E8	C	$391.6 \pm 115.3$	$13.3 \pm 8.0$	$5.8 \pm 1.3$	$11.8 \pm 0.4$	5	2
CD86 - mNeonGreen	Nanobody	1E2	C	$388.7 \pm 105.0$	$40.3 \pm 30.4$	$35.7 \pm 4.1$	$11.9 \pm 0.3$	7	3
CD86 - SPOT-tag	Nanobody	bivBC2	C	$245.6 \pm 56.7$	$15.0 \pm 5.8$	$10.6 \pm 1.8$	$19.0 \pm 1.2$	5	2
CD86 - mEGFP	Nanobody	1H1	N	$287.6 \pm 74.5$	$57.3 \pm 33.3$	$45.2 \pm 4.8$	$10.0 \pm 0.0$	6	3
CD70 - mEGFP	Nanobody	1H1	N	$259.9 \pm 39.1$	$9.3 \pm 4.5$	$44.3 \pm 5.1$	$11.7 \pm 0.4$	6	3
CD70 - mEGFP	Nanobody	1H1	C	$331.8 \pm 0.0$	$26.9 \pm 17.3$	$45.6 \pm 6.5$	$10.7 \pm 0.4$	7	3
Nup96-mEGFP	Nanobody	1H1	C	---	---	$44.7 \pm 0.5$	---	2	2

Supplementary Table 2 | Labeling efficiency – Antibodies

Target	Antibody conjugation	Total area ( $\mu\text{m}^2$ )	Target density ( $\mu\text{m}^{-2}$ )	Labeling efficiency (%)	Target-Reference offset (nm)	Cells (n)	Repeats (n)
PD-L1	TransGlutaminase	$412.9 \pm 102.9$	$62.0 \pm 37.2$	$49.8 \pm 14.2$	$9.4 \pm 0.5$	9	3
PD-L1	GlyCLICK	$534.9 \pm 98.3$	$31.2 \pm 5.8$	$41.3 \pm 4.2$	$9.7 \pm 0.4$	7	3
CD80	TransGlutaminase	$327.1 \pm 9.3$	$15.4 \pm 6.7$	$40.5 \pm 6.6$	$8.7 \pm 1.0$	7	3
CD80	GlyCLICK	$316.6 \pm 78.1$	$32.2 \pm 30.0$	$5.9 \pm 3.7$	$9.6 \pm 0.3$	10	3
CD86	TransGlutaminase	$322.4 \pm 58.0$	$18.2 \pm 3.6$	$39.5 \pm 8.3$	$8.0 \pm 1.4$	7	3
CD86	GlyCLICK	$298.7 \pm 71.3$	$25.7 \pm 8.3$	$34.2 \pm 12.7$	$7.8 \pm 0.4$	5	3

**Supplementary Table 3 | Labeling efficiency – EGFR binders**

Target	Binder	Clone	Total area ( $\mu\text{m}^2$ )	Target density ( $\mu\text{m}^2$ )	Labeling efficiency (%)	Target-Reference offset (nm)	Cells (n)	Repeats (n)
EGFR	Affibody	AB95116	260.7 $\pm$ 26.3	30.9 $\pm$ 7.3	17.0 $\pm$ 6.8	15.1 $\pm$ 0.5	7	3
EGFR	Nanobody	1H1 1B2	296.5 $\pm$ 91.7	38.6 $\pm$ 12.8	62.5 $\pm$ 4.3	5.1 $\pm$ 0.2	6	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	199.12 + kLC 1A23	301.7 $\pm$ 50.0	38.2 $\pm$ 10.6	39.5 $\pm$ 5.8	14.6 $\pm$ 0.4	11	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	D38B1 + IgG 10E10	354.0 $\pm$ 104.2	24.0 $\pm$ 8.5	29.8 $\pm$ 6.1	12.8 $\pm$ 1.1	12	3

**Supplementary Table 4 | EGFR oligomerization**

Target	Binder	Clone	Condition	Total area ( $\mu\text{m}^2$ )	Target density ( $\mu\text{m}^2$ )	Labeling efficiency (%)	Target-Reference offset (nm)	Monomers (%) – LE-corrected	Dimers (%) – LE-corrected	Monomers (%)	Dimers (%)	Cells (n)	Repeats (n)
EGFR (mEGFP)	Nanobody	1H1 1B2	Non-stimulated	276.5 $\pm$ 89.9	58.6 $\pm$ 20.8	62.2 $\pm$ 4.8	5.2 $\pm$ 0.3	90.5 $\pm$ 4.1	9.5 $\pm$ 4.1	93.7 $\pm$ 2.6	6.3 $\pm$ 2.6	6	3
EGFR (mEGFP)	Nanobody	1H1 1B2	EGF-stimulated	264.6 $\pm$ 76.2	129 $\pm$ 82	59.0 $\pm$ 3.6	5.1 $\pm$ 0.2	49.3 $\pm$ 8.0	50.7 $\pm$ 8.0	67.9 $\pm$ 6.7	32.1 $\pm$ 6.7	9	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	199.12 + kLC 1A23	Non-stimulated	276.5 $\pm$ 89.9	38.8 $\pm$ 13.7	43.5 $\pm$ 7.9	14.2 $\pm$ 0.6	93.7 $\pm$ 3.4	6.3 $\pm$ 3.4	98.7 $\pm$ 1.2	1.3 $\pm$ 1.2	6	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	199.12 + kLC 1A23	EGF-stimulated	264.6 $\pm$ 76.2	87.6 $\pm$ 46.0	49.3 $\pm$ 4.0	12.0 $\pm$ 1.3	51.9 $\pm$ 8.5	48.1 $\pm$ 8.5	78.7 $\pm$ 3.4	21.3 $\pm$ 3.4	9	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	D38B1 + IgG 10E10	Non-stimulated	276.5 $\pm$ 89.9	28.6 $\pm$ 12.6	36.3 $\pm$ 8.1	14.2 $\pm$ 0.6	94.5 $\pm$ 2.7	5.5 $\pm$ 2.7	100.0 $\pm$ 0.0	0.0 $\pm$ 0.0	6	3
EGFR	Antibody + 2 <sup>nd</sup> nanobody	D38B1 + IgG 10E10	EGF-stimulated	264.6 $\pm$ 76.2	38.5 $\pm$ 21.6	35.4 $\pm$ 8.6	13.6 $\pm$ 0.8	49.1 $\pm$ 7.4	50.9 $\pm$ 7.4	86.6 $\pm$ 4.0	13.4 $\pm$ 4.0	9	3

**Supplementary Table 5 | Docking sequences**

Docking strand name	Sequence
5xR1	TCCTCCTCCTCCTCCTCCT
7xR3	CTCTCTCTCTCTCTCTC
7xR4	ACACACACACACACACA
5xR6	CAACAACAACAACAAC

**Supplementary Table 6 | Acquisition parameters for binder specificity experiments**

Cell	Target	Binder	Clone	Imager-Sequence	Exposure (ms)	Frames (n)	Power (at objective, mW)	Imager concentration (pM)	Cluster radius xy	Cluster N <sub>min</sub>
CHO-K1	mEGFP	Nanobody	1H1	R3	75	30 000	25	500	10	10
CHO-K1	mEGFP	Nanobody	1B2	R3	75	30 000	25	500	10	10
CHO-K1	ALFA-tag	Nanobody	1G5	R3	75	30 000	25	500	10	10
CHO-K1	mEGFP	Nanobody	1H1 1B2	R3	75	30 000	25	500	10	10
CHO-K1	mEGFP ALFA-tag	Nanobody	1H1 1B2 1G5	R3	75	30 000	25	500	10	10
CHO-K1	mCherry	Nanobody	2B12	R3	75	30 000	25	500	10	10
CHO-K1	TagRFP	Nanobody	1H7	R3	75	30 000	25	500	10	10
CHO-K1	mEOS2	Nanobody	1E8	R3	75	30 000	25	500	10	10
CHO-K1	mNeonGreen	Nanobody	1E2	R3	75	30 000	25	500	10	10
CHO-K1	SPOT-tag	Nanobody	bivBC2	R3	75	30 000	25	500	10	10
CHO-K1	mEGFP	Nanobody	1H1	R3	75	30 000	25	500	10	10
CHO-K1	PD-L1	Antibody	10F.9G2	R4	75	30 000	25	500	10	10
CHO-K1	CD80	Antibody	16-10A1	R3	75	30 000	25	500	10	10
CHO-K1	CD86	Antibody	GL-1	R1	75	30 000	25	500	10	10
CHO-K1	EGFR	Affibody	AB95116	R3	75	30 000	25	500	10	10
CHO-K1	mEGFP	Nanobody	1H1 1B2	R3	75	30 000	25	500	10	10
CHO-K1	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	199.12 + kLC 1A23	R3	75	30 000	25	500	10	10
CHO-K1	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	D38B1 + IgG 10E10	R3	75	30 000	25	500	10	10

**Supplementary Table 7 | Acquisition parameters for labeling efficiency experiments**

Cell	Construct	Target	Binder	Clone	Imager-Sequence	Reference	Binder	Clone	Imager-Sequence	Exposure (ms)	Frames (n)	Power (at objective, mW)	Imager conc. (pM)	Cluster radius xy	Cluster N <sub>min</sub>
BSC1	ALFA-CD86-mEGFP	mEGFP	Nanobody	1H1	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	mEGFP-CD86-ALFA	mEGFP	Nanobody	1H1	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
U2OS	Nup96-mEGFP	mEGFP	Nanobody	1H1	R3	---	---	---	---	150	30 000	18	500	10	10
BSC1	ALFA-CD86-mEGFP	mEGFP	Nanobody	1B2	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10

BSC1	mEGFP- CD86- ALFA-tag	ALFA-tag	Nanobody	1G5	R3	mEGFP	Nanobody	1H1	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	mEGFP	Nanobody	1H1 1B2	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	mEGFP ALFA-tag	Nanobody	1H1 1B2 1G5	R3	CD86	Antibody	GL-1	R1	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	mCherry	Nanobody	2B12	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	TagRFP	Nanobody	1H7	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	mEOS2	Nanobody	1E8	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	mNeonG reen	Nanobody	1E2	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD86- mEGFP	SPOT-tag	Nanobody	bivBC2	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	ALFA- CD70- mEGFP	mEGFP	Nanobody	1H1	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
BSC1	mEGFP- CD70- ALFA	mEGFP	Nanobody	1H1	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
CHO- K1	PD-L1- ALFA- mEGFP	PD-L1	Antibody	10F.9 G2	R4	ALFA-tag	Nanobody	1G5	R3	75	30 000	25	500	10	10
CHO- K1	CD80- ALFA- mEGFP	CD80	Antibody	16- 10A1	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
CHO- K1	CD86- ALFA- mEGFP	CD86	Antibody	GL-1	R1	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
CHO- K1	EGFR- ALFA- mEGFP	EGFR	Affibody	AB951 16	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
CHO- K1	EGFR- ALFA- mEGFP	mEGFP	Nanobody	1H1 1B2	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
CHO- K1	EGFR- ALFA- mEGFP	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	199.12 + kLC 1A23	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10

CHO-K1	EGFR-ALFA-mEGFP	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	D38B1 + IgG 10E10	R3	ALFA-tag	Nanobody	1G5	R4	75	30 000	25	500	10	10
--------	-----------------	------	-------------------------------------	-------------------	----	----------	----------	-----	----	----	--------	----	-----	----	----

**Supplementary Table 8 | Acquisition parameters for EGFR experiments**

Cell	Construct	Target	Binder	Clone	Imager-Sequence	Reference	Binder	Clone	Imager-Sequence	Exposure (ms)	Frames TARGET / REFERENCE(n)	Power (at objective, mW)	Imager concentration -TARGET / REFERENCE (pM)	Cluster radius xy	Cluster N <sub>min</sub>
CHO-K1	EGFR-ALFA-mEGFP	mEGFP	Nanobody	1H1 1B2	R3	ALFA-tag	Nanobody	1G5	R4	75	50 000 / 30 000	25	250 / 500	10	10
CHO-K1	EGFR-ALFA-mEGFP	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	199.12 + kLC 1A23	R1	ALFA-tag	Nanobody	1G5	R4	75	50 000 / 30 000	25	250 / 500	10	10
CHO-K1	EGFR-ALFA-mEGFP	EGFR	Antibody + 2 <sup>nd</sup> Nanobody	D38B1 + IgG 10E10	R6	ALFA-tag	Nanobody	1G5	R4	75	50 000 / 30 000	25	250 / 500	10	10

**Supplementary Table 9 | Imager sequences**

Imager name	Sequence	3'-mod
R1	AGGAGGA	Cy3b
R3	AGAGAGA	Cy3b
R4	GTGTGT	Cy3b
R6	TGTTGTT	Cy3b

**Supplementary Table 10 | Binder specificity**

Target	Binder	Clone	Total area ( $\mu\text{m}^2$ )	Binder density ( $\mu\text{m}^2$ )	Cells (n)	Repeats (n)
mEGFP	Nanobody	1H1	331.8 $\pm$ 0.0	0.19 $\pm$ 0.05	5	2
mEGFP	Nanobody	1B2	331.8 $\pm$ 0.0	0.23 $\pm$ 0.05	5	2
ALFA-tag	Nanobody	1G5	404.8 $\pm$ 31.9	0.06 $\pm$ 0.05	5	2
mCherry	Nanobody	2B12	331.8 $\pm$ 0.0	0.61 $\pm$ 0.56	5	2
TagRFP	Nanobody	1H7	331.8 $\pm$ 0.0	0.41 $\pm$ 0.15	5	2
mEOS2	Nanobody	1E8	331.8 $\pm$ 0.0	0.35 $\pm$ 0.09	5	2
mNeonGreen	Nanobody	1E2	331.8 $\pm$ 0.0	0.30 $\pm$ 0.09	5	2
SPOT-tag	Nanobody	bivBC2	331.8 $\pm$ 0.0	0.46 $\pm$ 0.12	5	2
PD-L1	Antibody (TransGlutaminase)	10F.9G2	331.8 $\pm$ 0.0	0.31 $\pm$ 0.02	5	2
PD-L1	Antibody (GlyCLICK)	10F.9G2	331.8 $\pm$ 0.0	0.28 $\pm$ 0.10	5	2
CD80	Antibody (TransGlutaminase)	16-10A1	338.5 $\pm$ 13.0	0.21 $\pm$ 0.05	5	2
CD80	Antibody (GlyCLICK)	16-10A1	371.7 $\pm$ 31.9	0.39 $\pm$ 0.09	5	2
CD86	Antibody (TransGlutaminase)	GL-1	351.7 $\pm$ 26.0	0.18 $\pm$ 0.03	5	2
CD86	Antibody (GlyCLICK)	GL-1	345.1 $\pm$ 26.0	0.37 $\pm$ 0.05	5	2
EGFR	Affibody	AB95116	348.4 $\pm$ 32.5	0.62 $\pm$ 0.21	8	2
EGFR	Antibody + 2 <sup>nd</sup> Nanobody	199.12 + kLC 1A23	331.8 $\pm$ 0.0	0.37 $\pm$ 0.08	5	2
EGFR	Antibody + 2 <sup>nd</sup> Nanobody	D38B1 + IgG 10E10	331.8 $\pm$ 0.0	0.38 $\pm$ 0.11	5	2