Supplementary Material

Biased activation of the receptor tyrosine kinase HER2

Claudia Catapano[1], Johanna V Rahm[1], Marjan Omer[2.3], Laura Teodori[2.3], Jørgen Kjems[2.3], Marina S Dietz[1] & Mike Heilemann[1,*]

[1]Institute of Physical and Theoretical Chemistry, Goethe-University Frankfurt, Max-von-Laue-Str. 7, 60438 Frankfurt, Germany

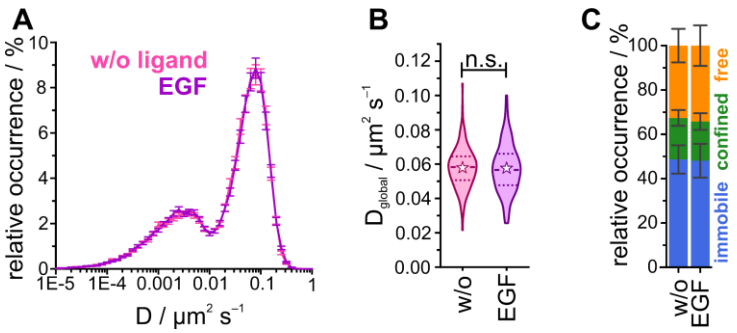
[2]Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark

[3]Center for Cellular Signal Patterns (CellPAT), Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark

*Correspondence: heilemann@chemie.uni-frankfurt.de

Supplementary Note 1

We compared our finding with receptor expression levels in HeLa cells. RNA expression levels were compared from the Human Protein Atlas v21.1 (https://www.proteinatlas.org/, **Figure S8A**) (Uhlén et al. 2015). RNA-seq data indicated the highest expression level for HER2 in HeLa cells, closely followed by that of EGFR. Comparably, RNA expression levels for HER3 and HER4 are relatively low, although that of HER4 is found at a slightly higher value. The protein expression levels of the ErbB family in HeLa cells reports the highest expression level for EGFR, with slightly lower expression levels for HER2 and HER4, while data for HER3 is not available (https://www.proteomicsdb.org/, **Figure S8B**) (Schmidt et al. 2018). Other studies reported no detectable HER3 receptors on the plasma membrane of HeLa cells using fluorescence activated cell sorting (FACS) (Chen B, Mao R, Wang H, She J. 2010) or confocal microscopy (Belleudi et al. 2012).



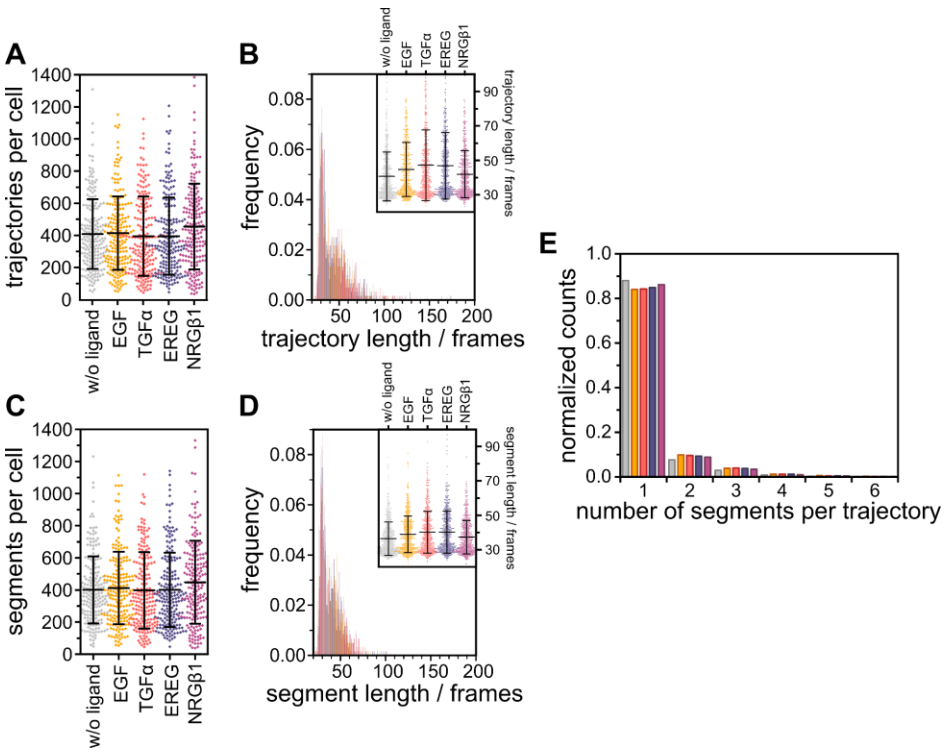
Supplementary Figure S1. Diffusion dynamics of mEGFP-TMD labeled with an anti-GFP nanobody in untreated and EGF-treated HeLa cells

(A) Distribution of diffusion coefficients from uPAINT experiments for resting (pink, D_{global} = 0.058 ± 0.004 µm^2 s^{-1}) and EGF-treated (lilac, D_{global} = 0.058 ± 0.004 µm^2 s^{-1}) living HeLa cells (N = 140) at 22 °C.

(B) Global diffusion coefficients displayed as violin plots with dotted lines marking the quartiles, dashed lines the median, and stars representing mean values (p = 0.696).

(C) Relative occurrences of immobile, confined, and freely diffusing particles.

Error bars are defined by SEMs; p > 0.05 no significant difference (n.s.).



Supplementary Figure S2. Analysis of segments and trajectories per cell

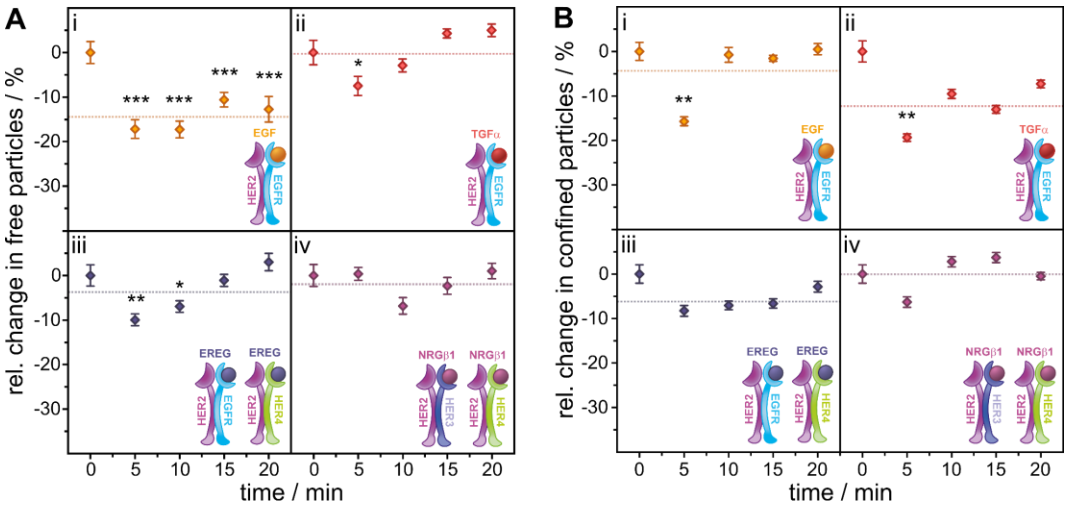
(A) Diamonds indicate the mean number of trajectories per cell with the interval representing the overall mean value ± its standard deviation. Overall mean values per condition are 411 ± 217 for resting HER2, 417 ± 230 for EGF, 398 ± 247 for TGFα, 398 ± 241 for EREG, and 458 ± 266 for NRGβ1 stimulated HER2.

(B) Mean trajectory length per cell plotted as histogram and overlaid for all four conditions. The inlay displays the distribution of trajectory lengths per cell. Diamonds indicate the mean trajectory length per cell with the interval representing the overall mean value ± its standard deviation. Overall mean values per condition are 41 ± 8 for resting HER2, 45 ± 9 for EGF, 47 ± 13 for TGFα, 47 ± 11 for EREG, and 42 ± 8 for NRGβ1 stimulated cells.

(C) Diamonds indicate the mean number of analyzed segments per cell with the interval representing the overall mean value ± its standard deviation. Overall mean values per condition are 400 ± 209 for resting HER2, 412 ± 225 for EGF, 398 ± 238 for TGFα, 401 ± 231 for EREG, and 448 ± 259 for NRGβ1 stimulated cells.

(D) Mean segment length per cell plotted as histogram and overlaid for all four conditions. The inlay displays the distribution of segment lengths per cell. Diamonds indicate the mean segment length per cell with the interval representing the overall mean value ± its standard deviation. Overall mean values per condition are 37 ± 6 for resting HER2, 39 ± 6 for EGF, 40 ± 7 for TGFα, 40 ± 7 for EREG, and 37 ± 6 for NRGβ1 stimulated cells.

(E) Mean number of segments per trajectory plotted as histogram with 85% of trajectories containing only one segment.



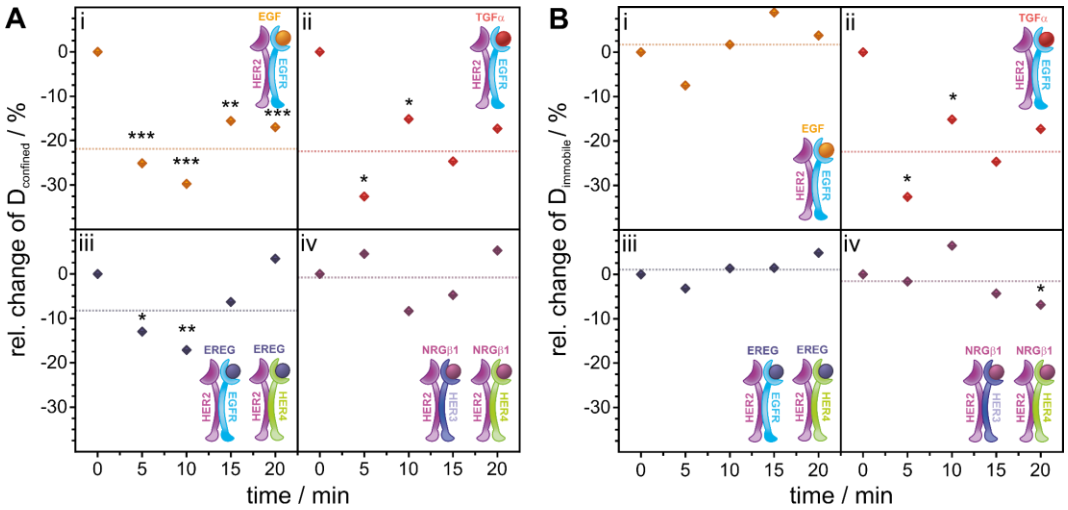
Supplementary Figure S3. Relative change in the temporal response of freely and confined moving HER2 in living HeLa cells upon ligand stimulation compared to the resting condition

Related to Figure 3A (N = 200).

(A) Relative change of molecules classified as freely diffusing plotted against the duration of ligand stimulation.

(B) Relative change of confined receptors plotted against the duration of ligand stimulation.

Relative changes were calculated from mean values of 40 cells per interval. Receptor models indicate the expected ligand-orchestrated interactions between HER2 and other receptors of the family. Dotted lines represent mean values of the relative change over the time of ligand stimulation. Error bars in dot plots represent the standard error of the difference (SED); for bar plots the standard error of the mean (SEM) is depicted. Significance was tested for stimulated cells vs. resting cells of the same samples before calculating the relative change with $p > 0.05$ no significant difference (no label), $p < 0.05$ significant difference (*), $p < 0.01$ very significant difference (**), $p < 0.001$ highly significant difference (***) between means.



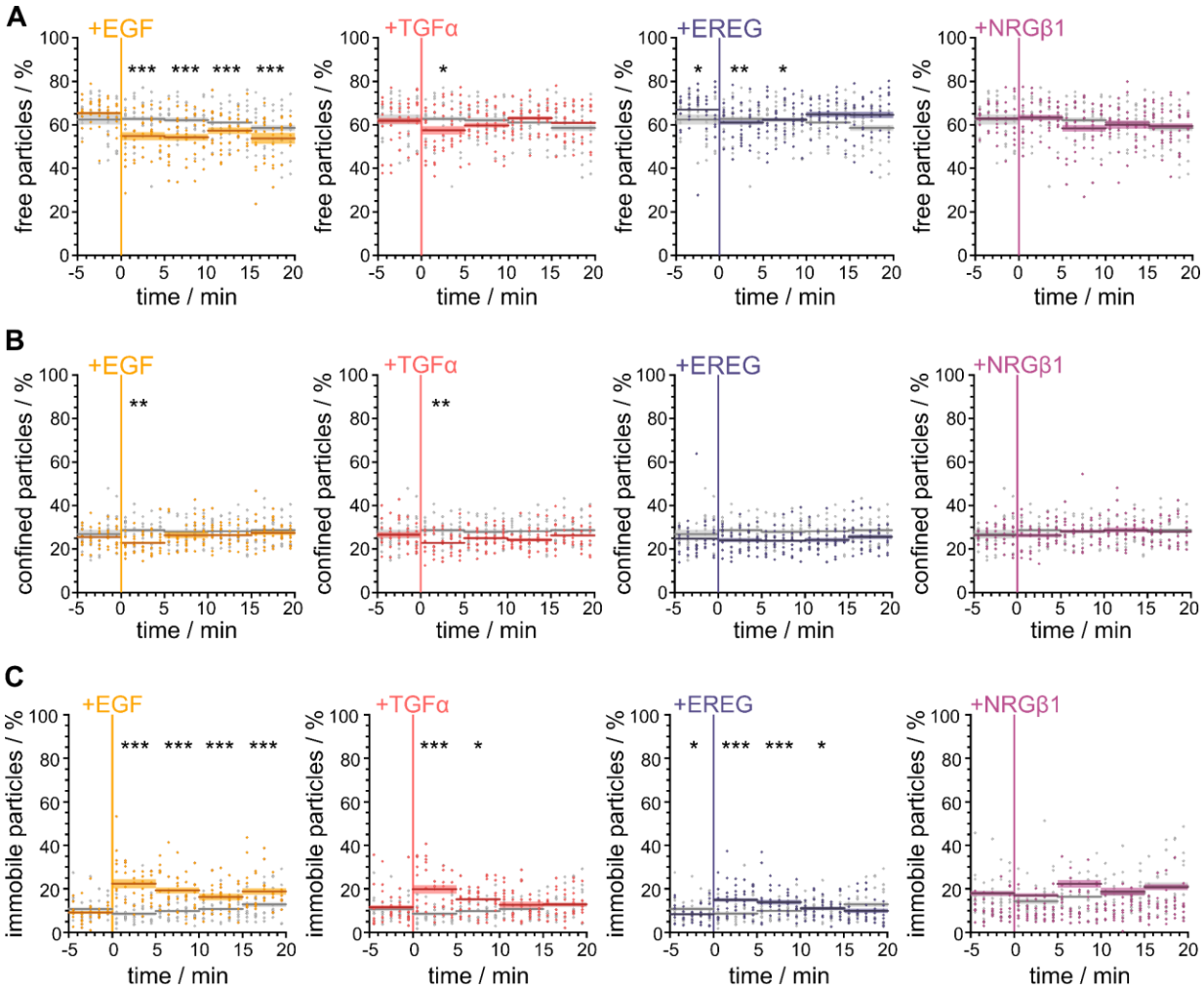
Supplementary Figure S4. Relative change in the temporal response of diffusion coefficients of confined and immobile HER2 in living HeLa cells upon ligand stimulation compared to the resting condition

Related to Figure 3B (N = 200).

(A) Relative change of the diffusion coefficient of confined moving particles plotted against the duration of ligand stimulation.

(B) Relative change of the diffusion coefficient of immobile particles plotted against the duration of ligand stimulation.

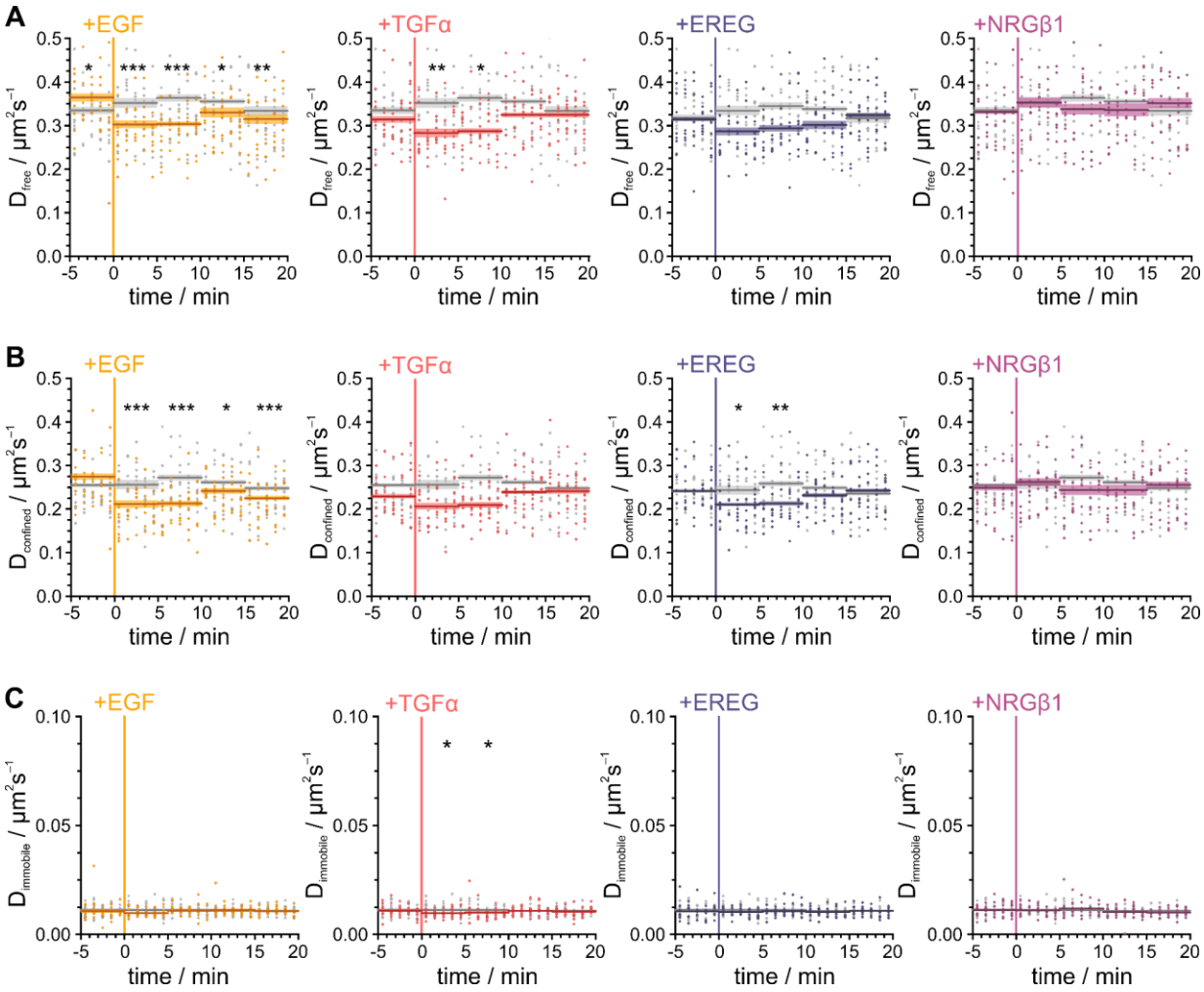
Relative changes were calculated from mean values of 40 cells per interval. Receptor models indicate the expected ligand-orchestrated interactions between HER2 and other receptors of the family. Dotted lines represent mean values of the relative change over the time of ligand stimulation. Error bars in dot plots represent the standard error of the difference (SED); for bar plots the standard error of the mean (SEM) is depicted. Significance was tested for stimulated cells vs. resting cells of the same samples before calculating the relative change with $p > 0.05$ no significant difference (no label), $p < 0.05$ significant difference (*), $p < 0.01$ very significant difference (**), $p < 0.001$ highly significant difference (***) between means.



Supplementary Figure S5. Temporal change of the relative occurrence of diffusion modes

Related to Figure 3A and S3.

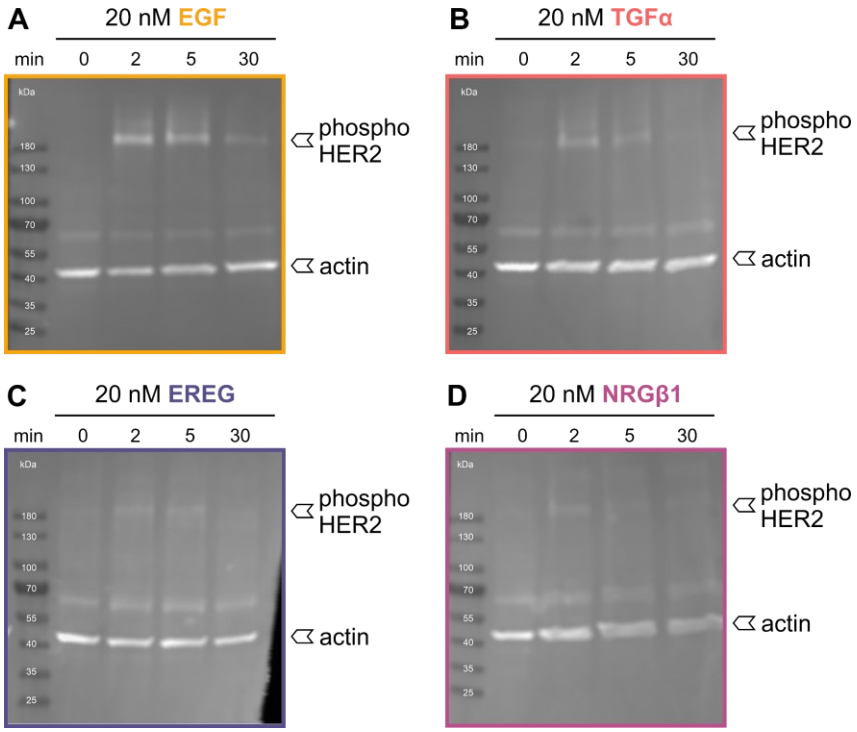
(A) The percentages of freely and (B) confined moving as well as (C) immobile HER2 after ligand stimulation in comparison with the resting condition in living HeLa cells (N = 200) are plotted against the time. Diamonds represent mean values per cell, lines indicate mean values over 5 min with confidence bands representing the SEM.



Supplementary Figure S6. Temporal change of the diffusion coefficients

Related to Figure 3B and S4.

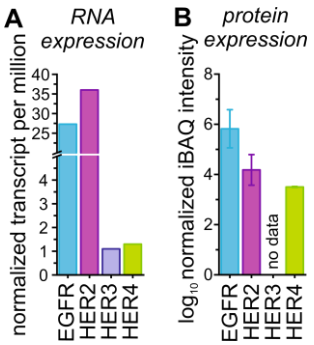
Diffusion coefficients of (A) freely and (B) confined moving as well as (C) immobile HER2 after ligand stimulation plotted in comparison with the resting condition in living HeLa cells (N = 200). Diamonds represent mean values per cell, lines indicate mean values over 5 min with confidence bands representing the SEM.



Supplementary Figure S7. Western blot analysis of ligand induced phosphorylation of HER2 in HeLa cells

Related to Figure 3.

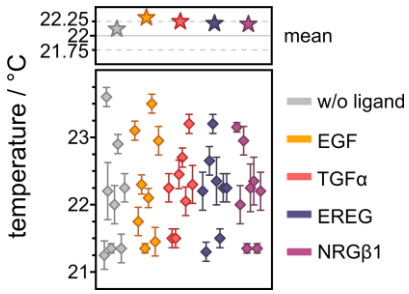
An antibody against the phosphorylated tyrosine residues Y1221/1222 of HER2 was applied next to an anti-actin antibody labeling actin as housekeeping gene. Page ruler served as a size marker on all blots. Tyrosine 1221/1222 was chosen as the target as this site serves as a junction to the Ras-Raf-MAPK pathway.



Supplementary Figure S8. RNA and protein expression levels of ErbB receptors

(A) RNA expression levels in normalized transcripts per million are taken from https://www.proteinatlas.org/.

(B) protein expression levels obtained by applying the intensity based absolute quantification (iBAQ) algorithm were taken from https://www.proteomicsdb.org/.



Supplementary Figure S9. Mean temperature per sample

Temperatures are color-coded with the respective ligand used for stimulation during that specific measurement. Diamonds represent mean temperatures per coverslip with the respective SEM. Stars represent overall mean values per condition.

Supplementary Table S1. Diffusion coefficients of mEGFP-TMD in unstimulated and EGF stimulated cells
Mean diffusion coefficients for 140 cells are listed next to mean values according to diffusion types with their respective SEMs.

Sample	D_{global} / $\mu m^2 s^{-1}$	$D_{immobile}$ / $\mu m^2 s^{-1}$	$D_{confined}$ / $\mu m^2 s^{-1}$	D_{free} / $\mu m^2 s^{-1}$
w/o ligand	0.058 ± 0.004	0.0039 ± 0.0006	0.062 ± 0.007	0.092 ± 0.005
EGF	0.058 ± 0.004	0.0038 ± 0.0005	0.064 ± 0.008	0.093 ± 0.005

Supplementary Table S2. Mann-Whitney-U test for comparison of global, immobile, confined, and freely diffusing mEGFP-TMD molecules of unstimulated cells with EGF stimulated cells
z-scores are derived from the *U*-statistic, *p*-values and levels of significance (*LOS*) are listed. Significance level α = 0.05, p ≥ 0.05 no significant difference (n.s.) p < 0.05 significant difference (*), p < 0.01 very significant difference (**), p < 0.001 highly significant difference (***).

Sample 1	Sample 2	global			immobile			confined			free		
		z	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*
w/o ligand	**EGF**	0.39	0.70	n.s.	1.05	0.29	n.s.	–1.99	0.046	*	–0.34	0.74	n.s.

Supplementary Table S3. Percentage of mEGFP-TMD molecules assigned to the three diffusion types immobile, confined, and free in unstimulated and EGF stimulated cells
Mean values for 140 cells are listed with their respective SEMs for each diffusion type.

Sample	immobile / %	confined / %	free / %
w/o ligand	33 ± 7	19 ± 4	49 ± 6
EGF	34 ± 9	18 ± 4	48 ± 8

Supplementary Table S4. Mann-Whitney-U test for comparison of the percentage of immobile, confined, and freely diffusing TMD-mEGFP molecules in EGF cells with ligand-stimulated cells

z-scores are derived from the *U*-statistic, *p*-values, and levels of significance (*LOS*) are listed. Significance level α = 0.05, p ≥ 0.05 no significant difference (n.s.) p < 0.05 significant difference (*), p < 0.01 very significant difference (**), p < 0.001 highly significant difference (***).

Sample 1	Sample 2	immobile			confined			free		
		z	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*
w/o ligand	**EGF**	–1.69	0.09	n.s.	2.24	0.02	*	0.78	0.43	n.s.

Supplementary Table S5. Binding of EGF family ligands to HER receptors summarized from published data

Interactions with monomeric receptors and heterodimers are listed. The relative strength of ligand binding/activation to receptors is listed in the right column while the relative strength in activation is listed in the lowest row.

Receptor / Ligand	EGF	TGFα	EREG	NRGβ1	Relative Binding Affinity
EGFR	x[a,b]	x[a,b]	x[a,b]		EGF > TGFα > EREG[a]
HER3				x[a,b]	
HER4			x[b]	x[a,b]	
HER2:EGFR	x[a]	x[a]	x[a,d]		EGF > TGFα > EREG[a]
HER2:HER3			x[a,c,d]	x[a]	NRGβ1 > EREG[a]
HER2:HER4	x[a]	x[a]	x[a,d]	x[a]	NRGβ1 > EGF > EREG, TGFα[a]
Relative Activation Strength	1:2 > 1:4[a]	1:2 >> 2:4[d]	1:2 > 2:3, 2:4[d]	HER4 > HER3[e,f] 1:3 > 1:4[a]	

[a] Jones 1999 (10.1016/s0014-5793(99)00283-5)
[b] Wilson 2008 (10.1016/j.pharmthera.2008.11.008)
[c] Barber 2019 (10.1093/jnci/djz231)

[d] Shelly 1998 (10.1074/jbc.273.17.10496)
[e] Pinkas-Kramarski 1998 (10.1128/MCB.18.10.6090)
[f] Tzahar 1994 (10.1016/S0021-9258(17)31521-1)

Supplementary Table S6. Diffusion coefficients of HER2 in unstimulated and ligand stimulated cells

Related to Figure 1CF, 2CF.

Mean diffusion coefficients for 128 cells are listed next to mean values according to diffusion types with their respective SEMs.

Sample	D_{global} / $\mu m^2 s^{-1}$	$D_{immobile}$ / $\mu m^2 s^{-1}$	$D_{confined}$ / $\mu m^2 s^{-1}$	D_{free} / $\mu m^2 s^{-1}$
w/o ligand	0.274 ± 0.011	0.0110 ± 0.0019	0.246 ± 0.019	0.330 ± 0.014
EGF	0.222 ± 0.010	0.0106 ± 0.0013	0.211 ± 0.017	0.297 ± 0.013
TGFα	0.243 ± 0.010	0.0102 ± 0.0015	0.224 ± 0.018	0.305 ± 0.012
EREG	0.248 ± 0.010	0.0105 ± 0.0017	0.224 ± 0.017	0.301 ± 0.012
NRGβ1	0.267 ± 0.010	0.0108 ± 0.0017	0.239 ± 0.012	0.327 ± 0.013

Supplementary Table S7. Mann-Whitney-U test for comparison of global, immobile, confined, and free diffusion coefficients of HER2 in unstimulated cells with ligand stimulated cells

Related to Figure 1CF, 2CF.

z-scores derived from the *U*-statistic, *p*-values, and levels of significance (*LOS*) are listed. Significance level α = 0.05, p ≥ 0.05 no significant difference (n.s.) p < 0.05 significant difference (*), p < 0.01 very significant difference (**), p < 0.001 highly significant difference (***).

Sample 1	Sample 2	global			immobile			confined			free		
		z	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*
w/o ligand	**EGF**	7.1	$10 \cdot 10^{-13}$	***	2.1	0.04	*	6.0	$2 \cdot 10^{-9}$	***	4.8	$2 \cdot 10^{-6}$	***
	TGFα	4.4	$9 \cdot 10^{-6}$	***	3.5	$4 \cdot 10^{-4}$	***	3.8	$1 \cdot 10^{-4}$	***	3.6	$3 \cdot 10^{-4}$	***
	EREG	4.0	$7 \cdot 10^{-5}$	***	2.4	0.02	*	4.1	$5 \cdot 10^{-5}$	***	4.4	$1 \cdot 10^{-5}$	***

NRGβ1	0.7	0.5	n.s.	1.3	0.2	n.s.	1.0	0.3	n.s.	0.2	0.8	n.s.

Supplementary Table S8. Corrected percentage of HER2 molecules assigned to the three diffusion types immobile, confined, and free in unstimulated and ligand stimulated cells

Related to Figure 1E, 2E.

Mean values for 128 cells are listed with their respective SEMs for each diffusion type.

Sample	immobile / %	confined / %	free / %
w/o ligand	10.5 ± 0.4	28.1 ± 0.4	61.4 ± 0.6
EGF	20.7 ± 0.7	27.0 ± 0.5	52.2 ± 0.8
TGFα	14.2 ± 0.6	24.9 ± 0.4	60.9 ± 0.7
EREG	14.8 ± 0.5	26.5 ± 0.5	58.7 ± 0.7
NRGβ1	11.8 ± 0.5	28.3 ± 0.5	59.9 ± 0.8

Supplementary Table S9. Mann-Whitney-U test for comparison of the corrected percentage of immobile, confined, and freely diffusing HER2 in unstimulated cells with ligand stimulated cells

Related to Figure 1E, 2E.

z-scores are derived from the *U*-statistic, *p*-values, and levels of significance (*LOS*) are listed. Significance level α = 0.05, p ≥ 0.05 no significant difference (n.s.) p < 0.05 significant difference (*), p < 0.01 very significant difference (**), p < 0.001 highly significant difference (***).

Sample 1	Sample 2	immobile			confined			free		
		z	*p*	*LOS*	*z*	*p*	*LOS*	*z*	*p*	*LOS*
w/o ligand	**EGF**	–11.9	$1 \cdot 10^{-32}$	***	1.9	0.06	n.s.	8.6	$7 \cdot 10^{-19}$	***
	TGFα	–4.7	$3 \cdot 10^{-6}$	***	5.1	$4 \cdot 10^{-7}$	***	0.6	0.5	n.s.
	EREG	–7.1	$1 \cdot 10^{-12}$	***	2.6	0.01	**	3.2	$1 \cdot 10^{-3}$	**

NRGβ1	–2.1	0.03	*	–0.3	0.8	n.s.	1.5	0.1	n.s.