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Super-long single-molecule tracking reveals dynamic-anchorage-induced integrin function

Taka A. Tsunoyama¹ , Yusuke Watanabe², Junri Goto², Kazuma Naito², Rinshi S. Kasai³, Kenichi G. N. Suzuki^{2,4}, Takahiro K. Fujiwara² and Akihiro Kusumi^{1,2,3*}

¹Membrane Cooperativity Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Onna, Japan. ²Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto, Japan. ³Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan.

⁴Present address: Center for Highly Advanced Integration of Nano and Life Sciences (G-CHAIN), Gifu University, Gifu, Japan. *e-mail: akihiro.kusumi@oist.jp

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Note: Supplementary Videos are available on the Nature Chemical Biology website.

Supplementary Tables

Supplementary Table 1 | Summary of photobleaching lifetimes, fluorescence intensities, single-molecule localization precisions, the numbers of inspected fluorescent spots, and the degrees of freedom for fitting, for all fluorophores observed under all of the conditions employed in this study. Grey shading: control. Orange shading: optimal conditions. See **Fig. 1d** and **Supplementary Fig. 7** for graphical summaries.

Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴) ^c	v ^d
Halo-R110	Outside	Live	21	-	5.2 ± 0.07	1.0 ± 0.02	1.0 ± 0.02	32.5	14.8	897
				TX	8.4 ± 0.06	1.6 ± 0.03	1.1 ± 0.01	-	20.1	897
				TQ20	4.0 ± 0.02	0.8 ± 0.02	1.1 ± 0.01	-	6.0	897
				TQ40	1.1 ± 0.01	0.2 ± 0.01	1.0 ± 0.01	-	2.9	897
			2	-	5.1 ± 0.08	1.0 ± 0.02	0.9 ± 0.01	-	6.2	897
				TX	7.4 ± 0.06	1.4 ± 0.02	1.2 ± 0.01	-	17.5	1497
				TQ20	3.9 ± 0.03	0.8 ± 0.01	1.0 ± 0.02	-	6.1	1497
				TQ40	1.9 ± 0.01	0.4 ± 0.01	0.9 ± 0.01	-	3.1	897
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	5.2 ± 0.06	1.0 ± 0.02	0.9 ± 0.01	-	10.3	897
				TQ40	6.5 ± 0.05	1.3 ± 0.02	0.9 ± 0.01	-	9.9	897
Halo-R110	Inside	Live	21	-	4.8 ± 0.03	1.0 ± 0.01	1.0 ± 0.04	32.5	11.2	897
				TX	7.5 ± 0.05	1.5 ± 0.01	0.9 ± 0.01	-	9.0	897
				TQ20	3.7 ± 0.02	0.8 ± 0.02	1.0 ± 0.01	-	9.4	897
				TQ40	1.7 ± 0.01	0.4 ± 0.01	0.9 ± 0.02	-	4.1	897
			2	-	4.9 ± 0.01	1.0 ± 0.01	0.9 ± 0.02	-	10.0	897
				TX	6.9 ± 0.12	1.4 ± 0.03	0.9 ± 0.02	-	15.4	1497
				TQ20	1.9 ± 0.02	0.4 ± 0.01	1.1 ± 0.01	-	3.1	1497
				TQ40	1.6 ± 0.05	0.3 ± 0.01	1.1 ± 0.01	-	2.6	897
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	5.8 ± 0.10	1.2 ± 0.02	1.0 ± 0.01	-	4.7	897
				TQ40	5.7 ± 0.06	1.2 ± 0.01	1.1 ± 0.02	-	5.8	897

To be continued

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Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴)	v ^d
Halo-AL488	Outside	Live	21	-	2.4 ± 0.01	1.0 ± 0.02	1.0 ± 0.01	26.7	6.7	897
				TX	4.2 ± 0.04	1.8 ± 0.02	1.1 ± 0.02	-	8.7	897
				TQ20	N. A. ^e		N. A. ^e	-		
				TQ40	N. A. ^e		N. A. ^e	-		
			2	-	2.3 ± 0.08	0.9 ± 0.03	0.9 ± 0.01	-	1.6	897
				TX	4.9 ± 0.09	2.1 ± 0.04	1.2 ± 0.01	-	9.8	897
				TQ20	N. A. ^e		N. A. ^e	-		
				TQ40	N. A. ^e		N. A. ^e	-		
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	N. A. ^e		N. A. ^e	-		
				TQ40	N. A. ^e		N. A. ^e	-		
ACP-AT488	Outside	Live	21	-	2.8 ± 0.02	1.0 ± 0.01	1.0 ± 0.01	27.6	4.7	1497
				TX	2.9 ± 0.02	1.0 ± 0.01	1.1 ± 0.01	-	9.9	1497
				TQ20	4.5 ± 0.06	1.6 ± 0.03	1.2 ± 0.01	-	6.5	1497
				TQ40	2.4 ± 0.05	0.9 ± 0.02	1.0 ± 0.01	-	4.6	1497
			2	-	2.0 ± 0.03	0.7 ± 0.01	1.0 ± 0.01	-	2.8	1497
				TX	2.9 ± 0.03	1.0 ± 0.01	0.9 ± 0.01	-	5.4	1497
				TQ20	12.4 ± 0.35	4.4 ± 0.13	0.9 ± 0.01	-	19.1	1497
				TQ40	4.8 ± 0.05	1.7 ± 0.02	0.9 ± 0.01	-	3.8	1497
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	5.7 ± 0.14	2.0 ± 0.05	1.0 ± 0.01	-	11.7	1497
				TQ40	6.3 ± 0.06	2.2 ± 0.03	1.1 ± 0.01	-	11.5	1497
Halo-TMR	Outside	Live	21	-	5.1 ± 0.03	1.0 ± 0.01	1.0 ± 0.02	24.7	7.5	1497
				TX	12.7 ± 0.04	2.5 ± 0.02	1.0 ± 0.01	-	19.5	1497
				TQ20	7.1 ± 0.10	1.4 ± 0.02	1.0 ± 0.01	-	13.3	1497
				TQ40	5.0 ± 0.14	1.0 ± 0.03	0.9 ± 0.01	-	6.5	1497
			10	-	3.7 ± 0.03	0.7 ± 0.01	0.9 ± 0.01	-	7.5	1497
				TX	13.4 ± 0.09	2.6 ± 0.02	1.0 ± 0.01	-	19.2	1497
				TQ20	9.1 ± 0.15	1.8 ± 0.03	1.0 ± 0.01	-	15.4	1497
				TQ40	7.6 ± 0.01	1.5 ± 0.01	0.9 ± 0.01	-	7.7	1497
			5	-	4.4 ± 0.11	0.9 ± 0.02	0.8 ± 0.01	-	9.3	1497
				TX	18.2 ± 0.11	3.6 ± 0.03	0.9 ± 0.01	-	34.4	1497
				TQ20	9.6 ± 0.35	1.9 ± 0.07	1.0 ± 0.01	-	23.8	1497
				TQ40	7.8 ± 0.21	1.5 ± 0.04	0.9 ± 0.01	-	16.8	1497

To be continued

Continued (3/6)

Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴)	v ^d
Halo-TMR	Outside	Live	2	-	3.8 ± 0.02	0.5 ± 0.01	0.7 ± 0.02	-	3.8	1497
				TX	31.0 ± 0.47	6.1 ± 0.10	0.9 ± 0.01	25.8	40.3	1497
				TQ20	17.4 ± 0.27	3.4 ± 0.06	1.0 ± 0.01	-	21.1	1497
				TQ40	11.6 ± 0.21	2.3 ± 0.04	1.0 ± 0.01	-	20.4	1497
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	9.5 ± 0.21	1.9 ± 0.04	0.8 ± 0.01	-	20.0	1497
				TQ40	17.3 ± 0.10	3.4 ± 0.03	0.8 ± 0.01	-	34.4	1497
Halo-TMR	Outside	Fixed	21	-	4.6 ± 0.11	1.0 ± 0.03	1.0 ± 0.01	24.7	7.7	1497
				TX	12.2 ± 0.27	2.6 ± 0.05	1.0 ± 0.01		24.1	1497
			2	TX	29.1 ± 0.50	6.3 ± 0.19	0.9 ± 0.01	25.8	53.5	1497
			0	TX	N. A. ^e		N. A. ^e			
Halo-TMR	Inside	Live	21	-	5.3 ± 0.02	1.0 ± 0.01	1.0 ± 0.01	24.7	9.4	1497
				TX	6.5 ± 0.09	1.4 ± 0.02	1.0 ± 0.01	-	10.2	1497
				TQ20	7.3 ± 0.03	1.6 ± 0.01	1.0 ± 0.01	-	8.7	1497
				TQ40	3.4 ± 0.07	0.7 ± 0.01	1.0 ± 0.01	-	5.2	1497
			10	-	4.0 ± 0.04	0.9 ± 0.01	0.9 ± 0.01	-	8.9	1497
				TX	13.6 ± 0.18	2.9 ± 0.04	0.9 ± 0.02	-	23.5	1497
				TQ20	13.7 ± 0.35	3.0 ± 0.07	0.9 ± 0.01	-	25.2	1497
				TQ40	7.2 ± 0.04	1.6 ± 0.01	0.8 ± 0.01	-	15.0	1497
			5	-	4.5 ± 0.05	1.0 ± 0.01	0.9 ± 0.01	-	8.2	1497
				TX	12.0 ± 0.13	2.6 ± 0.03	0.9 ± 0.01	-	14.4	1497
				TQ20	13.7 ± 0.16	3.0 ± 0.03	1.0 ± 0.01	-	23.4	1497
				TQ40	10.6 ± 0.11	2.3 ± 0.01	1.1 ± 0.02	-	19.3	1497
			2	-	3.6 ± 0.04	0.8 ± 0.02	0.9 ± 0.01	-	6.9	1497
				TX	32.4 ± 0.37	7.0 ± 0.08	0.9 ± 0.02	25.8	55.6	1497
				TQ20	26.1 ± 0.65	5.7 ± 0.12	0.9 ± 0.01	-	51.2	1497
				TQ40	16.1 ± 0.34	3.5 ± 0.07	0.9 ± 0.01	-	25.2	1497
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	13.5 ± 0.27	2.9 ± 0.01	0.9 ± 0.02	-	36.0	1497
				TQ40	18.3 ± 0.29	4.0 ± 0.01	0.9 ± 0.01	-	33.7	1497
ACP-TMR	Outside	Live	21	-	1.8 ± 0.04	1.0 ± 0.05	1.0 ± 0.02	-	5.0	497
			2	TX	4.1 ± 0.05	2.3 ± 0.06	1.0 ± 0.01	-	10.4	497

To be continued

Continued (4/6)

Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴)	v ^d
ACP-DY547	Outside	Live	21	-	1.5 ± 0.01	1.0 ± 0.04	1.0 ± 0.01	30.0	4.4	597
				TX	1.1 ± 0.01	0.7 ± 0.04	1.1 ± 0.01	-	1.7	597
				TQ20	1.3 ± 0.04	0.9 ± 0.05	1.2 ± 0.01	-	2.1	597
				TQ40	1.5 ± 0.03	1.0 ± 0.04	1.2 ± 0.01	-	3.9	597
			2	-	1.8 ± 0.04	1.2 ± 0.04	1.1 ± 0.01	-	5.2	597
				TX	3.2 ± 0.01	2.1 ± 0.06	1.3 ± 0.01	-	3.2	597
				TQ20	1.8 ± 0.01	1.2 ± 0.05	1.1 ± 0.01	-	3.4	597
				TQ40	2.2 ± 0.01	1.5 ± 0.05	1.1 ± 0.01	-	2.4	597
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	N. A. ^e		N. A. ^e	-		
				TQ40	N. A. ^e		N. A. ^e	-		
ACP-AT594	Outside	Live	21	-	9.2 ± 0.01	1.0 ± 0.01	1.0 ± 0.02	26.1	14.0	1497
				TX	7.7 ± 0.17	0.8 ± 0.02	0.8 ± 0.01	-	13.4	1497
				TQ20	12.8 ± 0.16	1.4 ± 0.02	1.2 ± 0.01	-	32.5	1497
				TQ40	6.7 ± 0.25	0.7 ± 0.03	1.2 ± 0.02	-	12.5	1497
			10	-	8.4 ± 0.17	0.9 ± 0.02	1.1 ± 0.01	-	7.1	1497
				TX	8.8 ± 0.23	1.0 ± 0.03	1.0 ± 0.01	-	14.0	1497
				TQ20	8.8 ± 0.38	1.0 ± 0.04	1.1 ± 0.02	-	27.7	1497
				TQ40	19.0 ± 0.31	2.1 ± 0.03	1.0 ± 0.01	-	35.5	1497
			5	-	7.7 ± 0.09	0.8 ± 0.01	1.0 ± 0.01	-	18.6	1497
				TX	7.9 ± 0.05	0.9 ± 0.01	0.9 ± 0.02	-	9.4	1497
				TQ20	13.7 ± 0.08	1.5 ± 0.02	1.0 ± 0.01	-	31.2	1497
				TQ40	15.8 ± 0.17	1.7 ± 0.01	1.1 ± 0.01	-	44.0	1497
			2	-	6.7 ± 0.06	0.7 ± 0.01	0.9 ± 0.02	-	11.9	1497
				TX	6.1 ± 0.11	0.7 ± 0.01	0.8 ± 0.01	-	18.2	1497
				TQ20	14.9 ± 0.39	1.6 ± 0.04	1.2 ± 0.02	-	17.6	1497
				TQ40	25.3 ± 0.25	2.8 ± 0.03	1.2 ± 0.02	-	53.8	1497
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	18.6 ± 0.20	2.0 ± 0.02	1.0 ± 0.01	-		
				TQ40	20.8 ± 0.30	2.3 ± 0.03	1.0 ± 0.01	-		
Halo-AT594	Outside	Live	21	-	8.6 ± 0.21	1.0 ± 0.03	1.0 ± 0.01	-	37	1497
			2	TQ40	24.8 ± 0.46	2.9 ± 0.09	1.2 ± 0.02	-	64	1497

To be continued

Continued (5/6)

Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴)	v ^d
ACP-DY647	Outside	Live	21	-	1.9 ± 0.02	1.0 ± 0.02	1.0 ± 0.01	30.0	4.5	597
				TX	0.7 ± 0.03	0.4 ± 0.02	1.1 ± 0.01	-	1.1	597
				TQ20	1.1 ± 0.01	0.6 ± 0.01	1.1 ± 0.02	-	1.9	597
				TQ40	1.2 ± 0.03	0.6 ± 0.02	1.1 ± 0.01	-	1.3	597
			2	-	1.8 ± 0.03	1.0 ± 0.02	1.0 ± 0.02	-	2.8	597
				TX	2.2 ± 0.03	1.2 ± 0.02	1.1 ± 0.02	-	5.6	597
				TQ20	7.0 ± 0.17	3.8 ± 0.11	1.1 ± 0.02	-	14.2	597
				TQ40	6.6 ± 0.23	3.6 ± 0.13	1.2 ± 0.02	-	10.3	597
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	31.7 ± 0.46	17.1 ± 0.34	0.9 ± 0.01	-		1497
				TQ40	33.6 ± 0.40	18.1 ± 0.32	0.7 ± 0.02	-	64.9	1497
									52.9	
ACP-AT647N	Outside	Live	21	-	9.5 ± 0.17	1.0 ± 0.03	1.0 ± 0.02	30.1	22.1	1497
				TX	6.7 ± 0.05	0.7 ± 0.01	1.0 ± 0.01	-	19.6	1497
				TQ20	8.8 ± 0.21	0.9 ± 0.03	1.0 ± 0.01	-	20.3	1497
				TQ40	8.9 ± 0.07	0.9 ± 0.02	1.1 ± 0.02	-	11.0	1497
			2	-	10.8 ± 0.16	1.1 ± 0.03	1.0 ± 0.01	-	26.3	1497
				TX	5.8 ± 0.04	0.6 ± 0.01	0.8 ± 0.01	-	15.1	1497
				TQ20	51.5 ± 1.37	5.4 ± 0.17	1.3 ± 0.01	-	73.1	2997
				TQ40	27.3 ± 0.90	2.9 ± 0.11	1.2 ± 0.01	-	41.7	2997
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	35.1 ± 0.89	3.7 ± 0.11	1.3 ± 0.04	-	76.8	2997
				TQ40	13.8 ± 0.28	1.5 ± 0.04	1.1 ± 0.01	-	31.9	2997
ACP-ST647	Outside	Live	21	-	16.2 ± 0.06	1.0 ± 0.01	1.0 ± 0.02	23.0	25.2	5997
				TX	26.0 ± 0.03	1.6 ± 0.01	1.0 ± 0.01	-	34.4	5997
				TQ20	39.6 ± 0.69	2.4 ± 0.04	1.1 ± 0.03	-	29.5	5997
				TQ40	41.7 ± 0.62	2.6 ± 0.04	1.0 ± 0.01	-	52.3	5997
			2	-	23.4 ± 0.09	1.4 ± 0.01	1.1 ± 0.02	-	56.1	5997
				TX	61.1 ± 1.01	3.8 ± 0.06	1.0 ± 0.02	-	63.1	5997
				TQ20	105.8 ± 0.78	6.5 ± 0.05	1.4 ± 0.01	21.5	269.8	5997
				TQ40	72.1 ± 0.70	4.5 ± 0.05	1.2 ± 0.01	-	139.4	5997
			0	-	N. A. ^e		N. A. ^e	-		
				TX	N. A. ^e		N. A. ^e	-		
				TQ20	33.5 ± 0.12	2.1 ± 0.01	1.3 ± 0.01	-	74.6	5997
				TQ40	38.5 ± 0.18	2.4 ± 0.01	1.1 ± 0.01	-	56.8	5997

To be continued

Continued (6/6)

Tag protein-Dye	Outside/ Inside the cell	Live/ Fixed cells	O ₂ (%)	ROXS	Photobleaching lifetime (s) ^a	Relative prolongation (fold) ^a	Relative intensity (fold) ^b	Precision (nm)	N (x10 ⁴)	v ^d
Halo-ST647	Outside	Live	21	-	17.8 ± 0.09	1.0 ± 0.01	1.0 ± 0.01	-	25.4	5997
			2	TQ20	107.1 ± 0.59	6.0 ± 0.05	1.3 ± 0.02	-	178.3	5997
ACP-ST647	Outside	Fixed	21	-	15.5 ± 0.11	1.0 ± 0.01	1.0 ± 0.01	23.0	33.6	5997
			21	TQ20	42.1 ± 0.66	2.7 ± 0.05	1.1 ± 0.01	-	106.8	5997
			2	TQ20	99.0 ± 1.57	6.4 ± 0.11	1.4 ± 0.01	21.5	120.8	5997
			0	TQ20	47.2 ± 1.30	3.0 ± 0.09	1.3 ± 0.02	-	91.7	5997
Halo-TMR-TX	Outside	Live	21	-	4.0 ± 0.08	1.0 ± 0.01	0.9 ± 0.01	26.5	4.9	997
			10	-	4.4 ± 0.11	1.1 ± 0.02	0.9 ± 0.01	-	10.1	997
			5	-	3.5 ± 0.05	0.9 ± 0.01	0.9 ± 0.01	-	5.6	997
			2	-	3.4 ± 0.05	0.8 ± 0.01	0.9 ± 0.01	27.3	3.9	997
			0	-	6.5 ± 0.10	1.6 ± 0.02	0.8 ± 0.01	28.4	17.3	997
ACP-AT594-TX	Outside	Live	21	-	13.1 ± 0.07	1.0 ± 0.01	0.9 ± 0.01	27.4	28.1	1497
			10	-	13.8 ± 0.21	1.0 ± 0.02	0.8 ± 0.01	-	19.1	1497
			5	-	18.8 ± 0.18	1.4 ± 0.02	0.8 ± 0.01	-	42.1	1497
			2	-	39.3 ± 0.70	3.0 ± 0.06	0.7 ± 0.01	28.7	25.4	1497
			0	-	30.4 ± 0.57	2.3 ± 0.04	0.9 ± 0.01	-	50.1	1497
Halo-Cy3-TX	Outside	Live	21	-	1.7 ± 0.02	1.0 ± 0.02	1.0 ± 0.01	22.2	3.1	997
			10	-	2.6 ± 0.06	1.5 ± 0.04	1.0 ± 0.01	-	3.4	997
			5	-	2.7 ± 0.05	1.6 ± 0.04	1.1 ± 0.01	-	6.9	997
			2	-	4.1 ± 0.08	2.4 ± 0.06	0.9 ± 0.01	23.8	11.4	997
			0	-	3.0 ± 0.02	1.7 ± 0.03	0.9 ± 0.01	-	3.1	997
Halo-Cy5-TX	Outside	Live	21	-	0.7 ± 0.01	1.0 ± 0.01	1.0 ± 0.02	25.1	0.8	5997
			10	-	2.5 ± 0.07	3.6 ± 0.11	1.0 ± 0.01	-	3.8	5997
			5	-	17.2 ± 0.52	25.4 ± 0.78	0.9 ± 0.01	-	28	5997
			2	-	40.3 ± 0.44	59.5 ± 0.69	0.9 ± 0.01	26.3	101.6	5997
			0	-	42.8 ± 0.36	63.2 ± 0.59	0.9 ± 0.01	26.7	68.5	5997

^aThe photobleaching lifetime (exponential decay time constant [= mean] ± the standard error) obtained by counting the number of fluorescent spots in each frame followed by the fitting with a single exponential function (see **Online Methods**).

^bThe fluorescence intensities (the mode values $\pm$ the standard errors) obtained by the log-normal curve fitting (see **Supplementary Fig. 6b** and its caption for the detailed method).

^cThe total numbers of fluorescent spots detected in all video frames in six independent movies.

^dThe degree of freedom for curve fitting ([the number of bins] – [the number of free parameters for fitting]).

^eNot analyzed due to extensive blinking ("ON" times mostly less than 66 ms; see **Online Methods**).

Supplementary Table 2 | Summary of TALL lifetimes ($\tau_1=1/k_{M,1}$, $\tau_2=1/k_{M,2}$, and $\tau_3=1/k_{M,3}$ with a $1/k_B$ of 83.5 s), time fractions against the total TALL period, and total numbers of cells and TALL events examined. The cover glasses used for culturing/observing cells were coated with fibronectin except for the specimens shown as “+ VN” (vitronectin coating).

a. Inside FA

	TALL lifetime (s) (time fraction [%]) ^a			# of observed cells	# of observed TALLs	# of shorter TALLs	# of longer TALLs	χ^2 ^b
	τ_1	τ_2	τ_3					
Itg $\beta 1$ (WT)	0.51 ± 0.013 (6)	4.3 ± 0.46 (12)	43 ± 5.1 (82)	22	1155	785	370	294
Itg $\beta 1$ (WT) + Mn^{2+}	0.79 ± 0.021 (8)	5.2 ± 0.82 (16)	40 ± 6.4 (76)	21	1250	918	332	294
Itg $\beta 1$ (D130A)	0.65 ± 0.0060 (27)	5.7 ± 0.15 (73)	– (0)	25	1192	1025	169	296
Itg $\beta 1$ (Y783A/Y795A)	0.48 ± 0.080 (33)	4.7 ± 0.21 (67)	– (0)	30	587	521	66	296
Itg $\beta 1$ (D130A/Y783A/Y795A)	0.24 ± 0.012 (81)	1.3 ± 0.073 (19)	– (0)	25	452	430	22	296
Itg $\beta 1$ (WT) + 2 μ M Y27632	0.40 ± 0.019 (8)	2.3 ± 0.16 (15)	19 ± 2.0 (77)	28	1145	865	280	294
Itg $\beta 1$ (WT) + 10 μ M Y27632	0.52 ± 0.0070 (25)	4.3 ± 0.10 (75)	– (0)	26	1437	1245	192	296
Itg $\beta 1$ (WT) + VN	0.38 ± 0.015 (19)	6.0 ± 0.33 (81)	– (0)	33	606	488	118	296
Itg $\beta 3$ (WT)	0.66 ± 0.016 (2)	9.4 ± 1.5 (13)	79 ± 10 (85)	33	1376	909	467	294
Itg $\beta 3$ (WT) + Mn^{2+}	0.61 ± 0.022 (2)	6.2 ± 1.7 (4)	87 ± 10 (94)	47	921	529	309	294
Itg $\beta 3$ (N305T)	0.66 ± 0.16 (6)	8.4 ± 1.1 (11)	99 ± 11 (88)	39	1093	724	369	294
Itg $\beta 3$ (WT) + 2 μ M Y27632	0.67 ± 0.015 (6)	7.7 ± 1.5 (16)	67 ± 12 (78)	45	1052	831	221	294
Itg $\beta 3$ (WT) + 10 μ M Y27632	0.46 ± 0.010 (17)	6.7 ± 0.26 (83)	– (0)	39	998	880	118	296
Itg $\beta 3$ (WT) + VN	0.68 ± 0.0098 (8)	6.3 ± 1.1 (11)	50 ± 6.7 (81)	41	1415	1154	261	294

b. Outside FA

	TALL lifetime (s) (time fraction [%]) ^a			# of observed cells	# of observed TALLs	# of shorter TALLs	# of longer TALLs	χ^2 ^b
	τ_1	τ_2	τ_3					
Itg $\beta 1$ (WT)	0.35 ± 0.0051 (26)	2.0 ± 0.095 (16)	20 ± 2.3 (58)	22	4595	N.D. ^c	N.D.	294
Itg $\beta 1$ (WT) + Mn^{2+}	0.36 ± 0.0066 (23)	1.6 ± 0.060 (17)	15 ± 1.0 (60)	21	3986	N.D.	N.D.	294
Itg $\beta 1$ (D130A)	0.38 ± 0.0041 (45)	2.4 ± 0.039 (55)	– (0)	25	4115	N.D.	N.D.	296
Itg $\beta 1$ (Y783A/Y795A)	0.33 ± 0.0026 (51)	2.6 ± 0.033 (49)	– (0)	30	2831	N.D.	N.D.	296
Itg $\beta 1$ (D130A/Y783A/Y795A)	0.40 ± 0.0030 (61)	3.0 ± 0.087 (39)	– (0)	25	1815	N.D.	N.D.	296
Itg $\beta 1$ (WT) + 2 μ M Y27632	0.36 ± 0.0066 (26)	1.3 ± 0.040 (23)	8.0 ± 0.39 (51)	28	5451	N.D.	N.D.	294
Itg $\beta 1$ (WT) + 10 μ M Y27632	0.48 ± 0.0035 (46)	2.8 ± 0.050 (54)	– (0)	26	5267	N.D.	N.D.	296
Itg $\beta 1$ (WT) + VN	0.47 ± 0.0080 (34)	4.3 ± 0.18 (49)	– (0)	33	2260	N.D.	N.D.	296
Itg $\beta 3$ (WT)	0.76 ± 0.015 (10)	3.4 ± 0.59 (8)	21 ± 1.9 (82)	33	2656	N.D.	N.D.	294
Itg $\beta 3$ (WT) + Mn^{2+}	0.43 ± 0.010 (6)	2.6 ± 0.11 (9)	61 ± 4.2 (85)	47	2471	N.D.	N.D.	294
Itg $\beta 3$ (N305T)	0.35 ± 0.0074 (9)	2.1 ± 0.10 (7)	31 ± 2.0 (84)	39	2548	N.D.	N.D.	294
Itg $\beta 3$ (WT) + 2 μ M Y27632	0.47 ± 0.0060 (18)	2.3 ± 0.13 (13)	20 ± 1.6 (69)	45	3505	N.D.	N.D.	294
Itg $\beta 3$ (WT) + 10 μ M Y27632	0.35 ± 0.0053 (23)	1.8 ± 0.061 (19)	14 ± 0.91 (58)	39	4072	N.D.	N.D.	294
Itg $\beta 3$ (WT) + VN	0.55 ± 0.0049 (23)	3.6 ± 0.34 (17)	21 ± 3.7 (66)	41	2194	N.D.	N.D.	294

^aExponential decay time constant ($= \text{mean} \pm \text{the standard error}$).

^bThe degree of freedom for curve fitting ([the number of bins] – [the number of free parameters for fitting]).

^cN.D. : Not determined.

Supplementary Table 3 | Summary of Akaike's and Bayesian information criteria (AIC and BIC, respectively) for the fitting of TALL lifetimes (**Supplementary Table 2**), using a sum of 1, 2, 3, and 4 exponential functions (single, double, triple, and quadruple expo.). For n , see **Supplementary Table 2**.

a. Inside FA

	Information criteria (AIC, BIC)							
	single expo.		double expo.		triple expo.		quadruple expo.	
Itg β 1 (WT)	128	139	-374	-356	-448	-415	-415	-448
Itg β 1 (WT) + Mn^{2+}	165	176	-421	-402	-534	-501	-511	-485
Itg β 1 (D130A)	-54	-43	-1018	-989	-1005	-972	-999	-973
Itg β 1 (Y783A/Y795A)	-49	-38	-671	-633	-644	-618	-644	-617
Itg β 1 (D130AY783A/Y795A)	-523	-511	-1020	-998	-943	-971	-379	-346
Itg β 1 (WT) + 2 μ M Y27632	208	220	-411	-392	-611	-585	-607	-574
Itg β 1 (WT) + 10 μ M Y27632	50	61	-846	-828	-924	-898	-919	-886
Itg β 1 (WT) + VN	-307	-318	-185	-167	-194	-162	-181	-155
Itg β 3 (WT)	453	464	-211	-192	-265	-239	-261	-228
Itg β 3 (WT) + Mn^{2+}	344	355	-90	-71	-121	-95	-152	-119
Itg β 3 (N305T)	395	407	-217	-199	-291	-265	-303	-271
Itg β 3 (WT) + 2 μ M Y27632	126	137	-335	-317	-356	-330	-411	-378
Itg β 3 (WT) + 10 μ M Y27632	222	233	-621	-599	-607	-581	-603	-570
Itg β 3 (WT) + VN	-27	-16	-606	-588	-650	-624	-650	-617

b. Outside FA

	Information criteria (AIC, BIC)							
	single expo.		double expo.		triple expo.		quadruple expo.	
Itg β 1 (WT)	-246	-236	-1004	-985	-1228	-1202	-1223	-1190
Itg β 1 (WT) + Mn^{2+}	-196	-185	-964	-945	-1377	-1344	-1314	-1288
Itg β 1 (D130A)	-187	-176	-1321	-1299	-1295	-1262	-1268	-1242
Itg β 1 (Y783A/Y795A)	-173	-161	-1445	-1421	-1436	-1409	-1431	-1398
Itg β 1 (D130AY783A/Y795A)	-472	-460	-1335	-1316	-1312	-1286	-1291	-1258
Itg β 1 (WT) + 2 μ M Y27632	-193	-182	-1122	-1103	-1610	-1584	-1675	-1642
Itg β 1 (WT) + 10 μ M Y27632	-292	-281	-1362	-1344	-1543	-1510	-1533	-1507
Itg β 1 (WT) + VN	-72	-60	-675	-656	-886	-860	-882	-849
Itg β 3 (WT)	66	77	-758	-740	-837	-812	-833	-800
Itg β 3 (WT) + Mn^{2+}	19	31	-553	-535	-864	-838	-859	-826
Itg β 3 (N305T)	24	35	-605	-586	-1025	-991	-1007	-974
Itg β 3 (WT) + 2 μ M Y27632	-187	-176	-972	-954	-1350	-1317	-1229	-1203
Itg β 3 (WT) + 10 μ M Y27632	-136	-125	-987	-968	-1382	-1349	-1363	-1337
Itg β 3 (WT) + VN	-305	-294	-1094	-1076	-1192	-1166	-1187	-1154

Supplementary Table 4 | Summary of % time fractions (against the sum of the observation periods for all of the molecules, T_{total} ; Mean $\pm$ SEM. For n , see **Supplementary Table 2**). The cover glasses used for culturing/observing cells were coated with fibronectin except for the specimens shown as “+ VN” (vitronectin coating).

a. Inside FA

	$f_{\text{ExpAATI}} (\%)^a$	$f_{\text{AATI}} (\%)^b$	$f_{\text{MLI}} (\%)^c$	True TALL (%) ^d
Itg β 1 (WT)	9.6 $\pm$ 1.7	8.6 $\pm$ 2.8	0.99 $\pm$ 3.3	78 $\pm$ 4.9
Itg β 1 (WT) + Mn ²⁺	10.8 $\pm$ 1.7	8.3 $\pm$ 3.9	2.6 $\pm$ 4.3	89 $\pm$ 2.6
Itg β 1 (D130A)	1.5 $\pm$ 1.9	0.14 $\pm$ 0.022	1.4 $\pm$ 1.9	39 $\pm$ 5.9
Itg β 1 (Y783A/Y795A)	0.53 $\pm$ 2.0	0.077 $\pm$ 0.017	0.45 $\pm$ 2.0	32 $\pm$ 6.3
Itg β 1 (D130AY783A/Y795A)	1.5 $\pm$ 1.5	0.0017 $\pm$ 0.00060	1.5 $\pm$ 1.4	13 $\pm$ 4.6
Itg β 1 (WT) + 2 μ M Y27632	0.72 $\pm$ 1.6	2.3 $\pm$ 0.43	-1.6 $\pm$ 1.6	76 $\pm$ 4.9
Itg β 1 (WT) + 10 μ M Y27632	2.5 $\pm$ 1.2	0.12 $\pm$ 0.0098	2.4 $\pm$ 1.2	54 $\pm$ 3.8
Itg β 1 (WT) + VN	0.74 $\pm$ 3.3	0.25 $\pm$ 0.037	0.50 $\pm$ 3.3	56 $\pm$ 5.6
Itg β 3 (WT)	20 $\pm$ 4.0	18 $\pm$ 7.6	2.0 $\pm$ 8.6	83 $\pm$ 9.3
Itg β 3 (WT) + Mn ²⁺	26 $\pm$ 1.7	25 $\pm$ 9.3	1.1 $\pm$ 9.4	95 $\pm$ 10
Itg β 3 (N305T)	26 $\pm$ 2.3	27 $\pm$ 11	-1.2 $\pm$ 12	95 $\pm$ 13
Itg β 3 (WT) + 2 μ M Y27632	12 $\pm$ 4.3	13 $\pm$ 9.5	-0.72 $\pm$ 10.4	75 $\pm$ 11
Itg β 3 (WT) + 10 μ M Y27632	1.1 $\pm$ 1.5	0.32 $\pm$ 0.036	0.74 $\pm$ 1.5	58 $\pm$ 4.9
Itg β 3 (WT) + VN	15 $\pm$ 1.8	11 $\pm$ 4.4	3.4 $\pm$ 4.7	88 $\pm$ 6.6

b. Outside FA

	$f_{\text{ExpAATI}} (\%)^a$	$f_{\text{AATI}} (\%)^b$	$f_{\text{MLI}} (\%)^c$	True TALL (%) ^d
Itg β 1 (WT)	3.2 $\pm$ 1.4	1.4 $\pm$ 0.34	1.8 $\pm$ 1.4	51 $\pm$ 4.4
Itg β 1 (WT) + Mn ²⁺	2.0 $\pm$ 1.1	1.2 $\pm$ 0.16	0.87 $\pm$ 1.1	64 $\pm$ 3.3
Itg β 1 (D130A)	0.86 $\pm$ 1.3	0.013 $\pm$ 0.0025	0.85 $\pm$ 1.3	21 $\pm$ 4.0
Itg β 1 (Y783A/Y795A)	0.36 $\pm$ 1.5	0.018 $\pm$ 0.0034	0.34 $\pm$ 1.5	25 $\pm$ 4.7
Itg β 1 (D130AY783A/Y795A)	1.2 $\pm$ 1.0	0.0091 $\pm$ 0.0025	1.2 $\pm$ 1.0	13 $\pm$ 3.3
Itg β 1 (WT) + 2 μ M Y27632	0.99 $\pm$ 0.94	0.27 $\pm$ 0.024	0.72 $\pm$ 0.90	51 $\pm$ 3.0
Itg β 1 (WT) + 10 μ M Y27632	0.81 $\pm$ 1.3	0.033 $\pm$ 0.0034	0.77 $\pm$ 1.3	42 $\pm$ 4.1
Itg β 1 (WT) + VN	3.0 $\pm$ 0.94	0.061 $\pm$ 0.0071	3.0 $\pm$ 1.3	30 $\pm$ 2.4
Itg β 3 (WT)	4.0 $\pm$ 1.3	1.5 $\pm$ 0.27	2.5 $\pm$.3	40 $\pm$ 4.1
Itg β 3 (WT) + Mn ²⁺	15 $\pm$ 0.98	13 $\pm$ 2.6	1.9 $\pm$ 2.8	76 $\pm$ 3.4
Itg β 3 (N305T)	7.2 $\pm$ 0.49	4.1 $\pm$ 0.49	3.1 $\pm$ 0.70	56 $\pm$ 3.1
Itg β 3 (WT) + 2 μ M Y27632	1.5 $\pm$ 1.1	1.2 $\pm$ 0.20	0.34 $\pm$ 1.1	40 $\pm$ 3.5
Itg β 3 (WT) + 10 μ M Y27632	2.2 $\pm$ 1.4	0.53 $\pm$ 0.096	1.6 $\pm$ 1.4	34 $\pm$ 4.5
Itg β 3 (WT) + VN	3.0 $\pm$ 1.3	0.061 $\pm$ 0.0071	3.0 $\pm$ 1.3	38 $\pm$ 3.2

^aTime fractions of molecules (in %) that were experimentally and directly determined to be immobile at time 0 and photobleached before starting motion (% Time fraction of experimentally-observed apparent all-time immobile periods). See **Eq. 39** in

Supplementary Note 1.

^bTime fractions (in %) in which the observed molecules are expected to exhibit the AATI behavior (f_{AATI}) in the absence of “alternative molecules” undergoing immobilization for periods much longer than τ_{rec} (**Eq. 44** in **Supplementary Note 1**), i.e., the time fraction calculated using the parameters determined by $G_{\text{TALL}}(\delta)$ (**Eq. 29** in **Supplementary Note 1**).

^c $f_{\text{ExpATI}} - f_{\text{AATI}}$, representing the time fraction (in %) of “alternative molecules” exhibiting Much Longer Immobilization (molecules that are immobilized for periods much longer than τ_{rec}). See **Eq. 39** in **Supplementary Note 1**. Note that this time fraction is much less than the immobile time fraction previously determined by FRAP⁵⁴⁻⁵⁶, which was 55~67% in the time scale between 20-200 s. Since the longest τ_3 TALLs found here were 43 s (~15% time fraction) for integrin $\beta 1$ and 79 s (~30% time fraction) for integrin $\beta 3$ (**Supplementary Figs. 23 and 24**), they might have been counted as immobile fractions in FRAP observations. In addition, since three types of immobilization durations existed, the FRAP evaluation of immobile components might have been quite difficult.

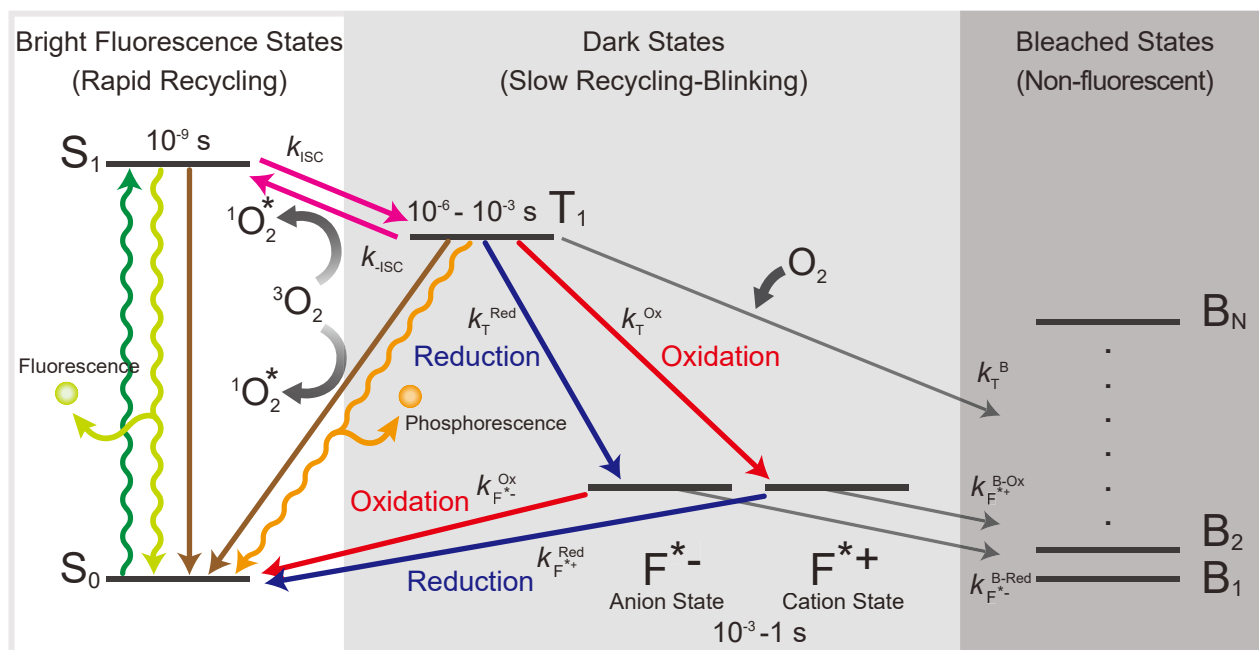
^dThe time fraction of “True TALL” periods (against τ_{total}), calculated as $100 - \% \text{Mobile} - f_{\text{MLII}}$.

Supplementary Table 5 | Summary of *P* values (two-sided Mann-Whitney U test) for the cell area (*n* = 20 cells) shown in **Supplementary Fig. 14**.

Integrin expression	ROXS conditions	Time after plating (min)			
		15	30	60	90
None	Control	-	-	-	-
	2%O ₂	0.95	0.56	0.12	0.97
	2%O ₂ +TX	0.93	0.30	0.22	0.13
	2%O ₂ +TQ20	0.70	0.66	0.29	0.36
	2%O ₂ +TQ40	0.78	0.24	0.60	0.84
	0%O ₂	1.6 x 10 ⁻⁴	6.7 x 10 ⁻⁴	6.0 x 10 ⁻⁴	0.028
Itgβ1	Control	-	-	-	-
	2%O ₂	0.14	0.19	0.31	0.54
	2%O ₂ +TX	0.93	0.71	0.12	0.70
	2%O ₂ +TQ20	0.73	0.97	0.086	0.56
	2%O ₂ +TQ40	0.056	0.64	0.82	0.86
	0%O ₂	3.3 x 10 ⁻⁴	1.0 x 10 ⁻⁴	1.0 x 10 ⁻⁶	4.1 x 10 ⁻⁶
ACP-Itgβ1	Control	0.76	0.41	0.053	0.068
	2%O ₂	0.90	0.51	0.22	0.12
	2%O ₂ +TX	0.064	0.44	0.95	0.81
	2%O ₂ +TQ20	0.37	0.25	0.84	0.80
	2%O ₂ +TQ40	0.80	0.68	0.46	0.98
ACP-Itgβ1(D130A)	Control	N.D. ^a	3.3 x 10 ⁻⁷	3.9 x 10 ⁻⁸	1.0 x 10 ⁻⁴
ACP-Itgβ1(Y783A/Y795A)	Control	N.D. ^a	5.2 x 10 ⁻⁷	3.9 x 10 ⁻⁸	6.6 x 10 ⁻⁷
ACP-Itgβ1(D130A/Y783A/Y795A)	Control	N.D. ^a	1.1 x 10 ⁻⁸	6.8 x 10 ⁻¹⁰	6.9 x 10 ⁻⁸

^aN.D. : Not determined because no data were obtained.

Supplementary Figures



Supplementary Figure 1 | The effects of molecular oxygen and ROXS on the probabilities of transitions among various energy states of fluorescent molecules, schematically shown based on a Jablonski diagram.

Vibration-induced sublevels are omitted for clarity. The triplet state T_1 is considered to be most relevant to photobleaching. Fluorophores in the T_1 state often react with other compounds such as molecular oxygen to form non-fluorescent compounds, due to its higher energy and longer lifetime. This is the major cause of photobleaching.

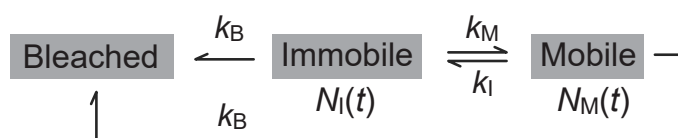
During the continuous excitation of a fluorophore, it undergoes the excitation-deexcitation cycle, moving back and forth between the ground state S_0 and the excited singlet state S_1 , and thus a single fluorescent molecule becomes observable because it emits many fluorescent photons during the integration time of the detector.

However, a fluorescent molecule in the excited state S_1 sometimes (with certain probabilities) transitions to the T_1 state and then to the ion states, F^{*-} and $F^{*+57-59}$. Since the lifetimes of these states are longer than that of S_1 , often by a factor of a million or more, once a fluorescent molecule enters these states, it cannot be excited for long periods of time (and thus cannot emit any photons) until it relaxes to the ground state S_0 . Therefore, these states are called dark states. When the fluorescent molecule finally returns back to the

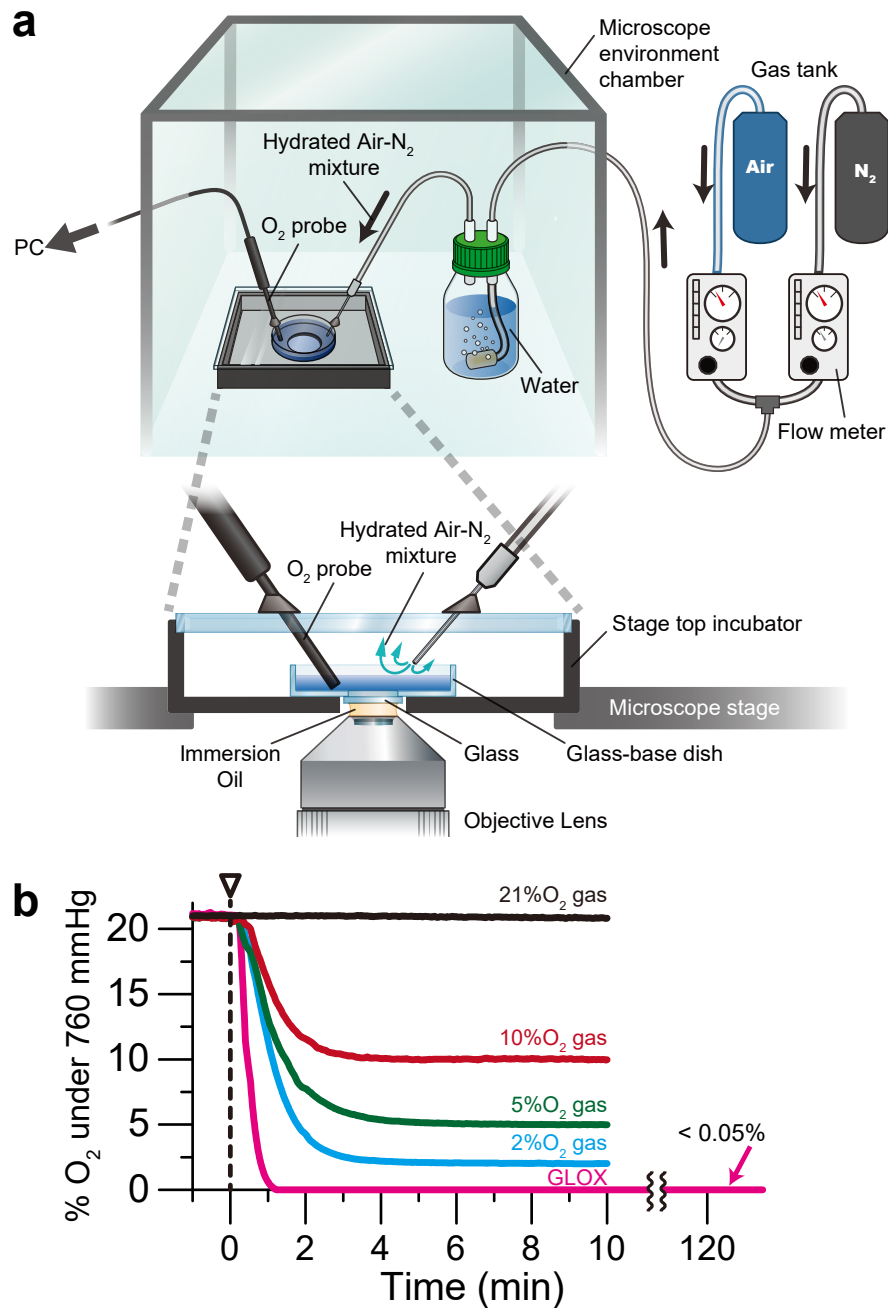
ground state S_0 , it can be excited again and resume the excitation-emission cycle. Namely, when a fluorescent dye molecule cycles between bright and dark periods, it blinks, and this phenomenon is called photoblinking⁶⁰⁻⁶².

In ROXS, both reducing and oxidizing reagents are included in the specimen, and the molecules in the T_1 state react with either the reducing or oxidizing reagent to transition to the ion states (F^{*-} or F^{*+} , respectively) and then are further oxidized or reduced, respectively, by the added reagent to return to the ground state S_0 . In the ROXS experiments, there was generally no dissolved molecular oxygen present in the specimen⁹.

In its ground state, molecular oxygen is in the triplet spin state. Therefore, it undergoes spin exchange reactions with the dye molecules in the T_1 state, bringing the dye molecules back to the ground state S_0 directly or indirectly by way of the excited singlet state S_1 , but the reactive singlet molecular oxygen produced by the exchange reaction might in turn react with the dye molecule, inducing photobleaching of the dye molecule. Thus, the presence of molecular oxygen in the specimen could induce photobleaching of the dye molecule, but at the same time could reduce photobleaching by reducing the lifetime of T_1 . Furthermore, molecular oxygen could participate in the oxidizing reactions of the dye (working as an oxidizing reagent in a ROX system). Therefore, if a good compromise of the dual functions of molecular oxygen (inducing and reducing photobleaching) by varying its concentrations (used together with the separately added ROXS), it would be desirable. Furthermore, for the research using living cells, because the cell, for its proper function, requires environmental molecular oxygen concentrations in the range between 1 and 13%¹⁸⁻²⁰ (for cells in culture, 2% is better than 21%¹²), molecular oxygen should be present in the medium for fluorescence observations. Therefore, we set out to find the dyes and the observation conditions (ROXS + proper oxygen concentrations) that can allow us to observe fluorescence molecules in living cells as long as possible.



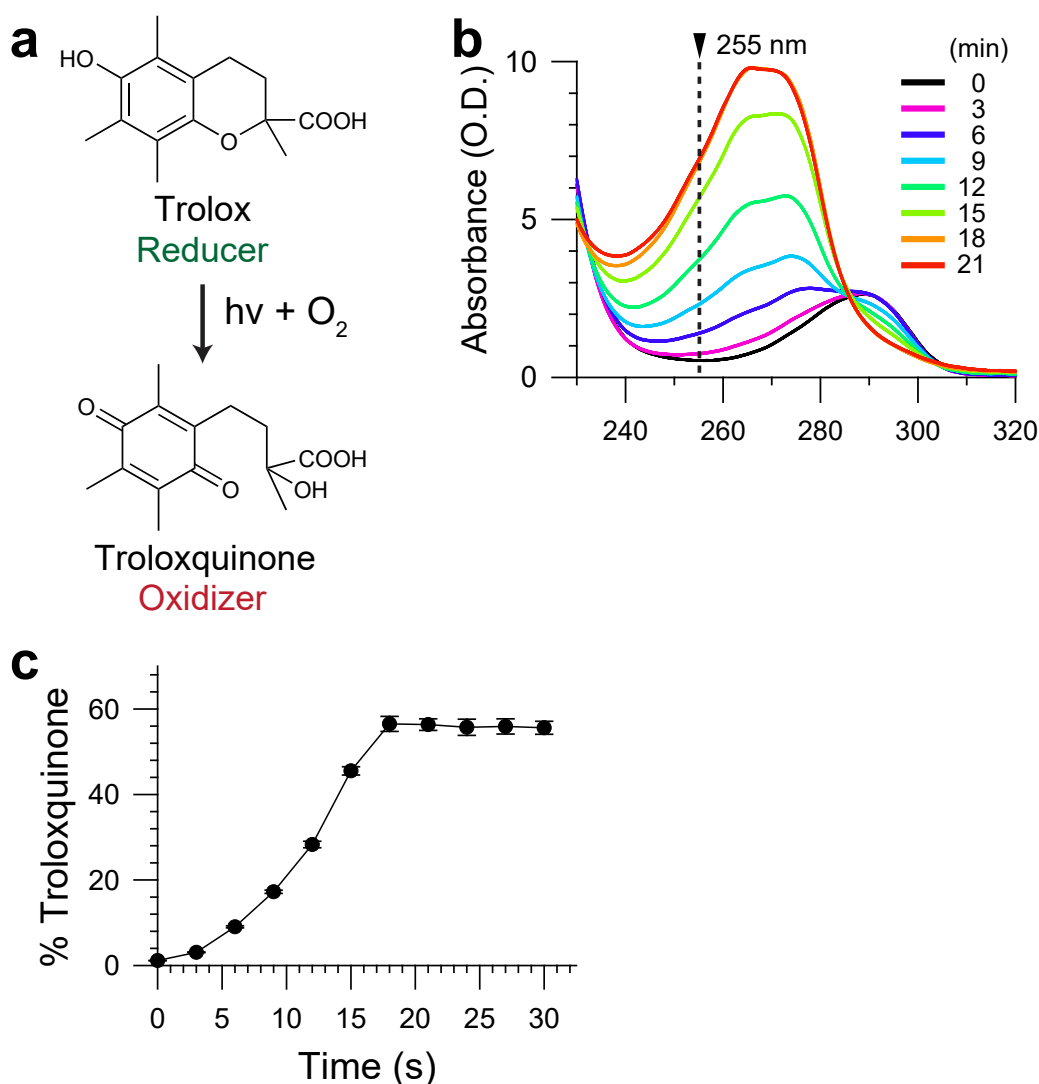
Supplementary Figure 2 | Schematic diagram showing the equilibrium of mobile (non-bleached), immobile (TALL; non-bleached), and bleached (mobile and TALL) states, with the kinetic rate constants for the transitions between these states. The final objective is to develop a theory to evaluate k_M (and k_I) from the results obtained by single fluorescent-molecule imaging.



Supplementary Figure 3 | The set up to control the dissolved oxygen concentrations in the cell culture medium in the glass-base dish (the cells were cultured on the cover-glass bottom of this dish) placed on the microscope stage in the gas-environment chamber (**a**), and the time course of the decrease of the dissolved O₂ concentration in the cell culture medium when the injected gas mixture was changed from normal air to an air-nitrogen mixture (**b**). 0% O₂ (below the sensor's detection limit of 0.05% O₂) was achieved enzymatically, using the GLOX buffer.

(a) Schematic figure showing the set up to control the gas-environment and the temperature of cells cultured in the glass-base dish during the microscope observations. In a home-built microscope environment chamber made of thermo- and electric-field-insulating plastic, which covers the entire microscope except for the excitation arm and the detection arms, and contains three heating circulators, an additional smaller chamber was placed on the microscope stage, for further stabilization of the specimen temperature and for varying the partial pressure of molecular oxygen in the gas surrounding the specimen. The bottom plane of the chamber has a hole in which a glass-base dish can be placed snugly, so air escapes from there only slowly. On the lid of this chamber, two small holes allow access for the tubing for injecting the humidified gas mixture and for placing the micro-electrode for monitoring the dissolved oxygen concentrations. The N₂ gas and air (79% N₂ + 21% O₂) were supplied from each tank and mixed with a multi gas mixer with flow meters. The gas mixture was humidified by passage through a bottle filled with ultrapure water, and then continuously supplied onto the surface of the solution covering the cells attached to the glass-base dish, at a flow-rate of 200 ml/min.

(b) Time courses of the decrease of dissolved O₂ concentration in the cell culture medium when the injected gas mixture was changed from normal air to an air-nitrogen mixture at time 0. To reach 0% O₂; *i.e.*, below the sensor's detection limit of 0.05% O₂, an enzymatic method was employed using the GLOX buffer. For the details of the method, see **Online Methods**.



Supplementary Figure 4 | Troloxquinone preparation from trolox by UV irradiation.

(a) Troloxquinone was generated from trolox by UV ($\lambda_{\text{max}} \sim 302$ nm)-induced oxidation.

(b) Time-dependent changes of the absorption spectrum of the trolox solution ($\lambda_{\text{max}} \sim 290$ nm) induced by UV irradiation, showing the formation of troloxquinone ($\lambda_{\text{max}} \sim 270$ nm), with an isosbestic point of ~ 285 nm. The optical density (O.D.) refers to that of the UV-irradiated solution before dilution for the absorption measurement (among 10 independent experiments.). Absorption at 255 nm was used for determining the concentrations of trolox and troloxquinone¹¹. No difference was found between the spectra obtained at 18 and 21 min. The total amount of TX and TQ was 1 mM. Representative spectra among 10 totally independent experiments.

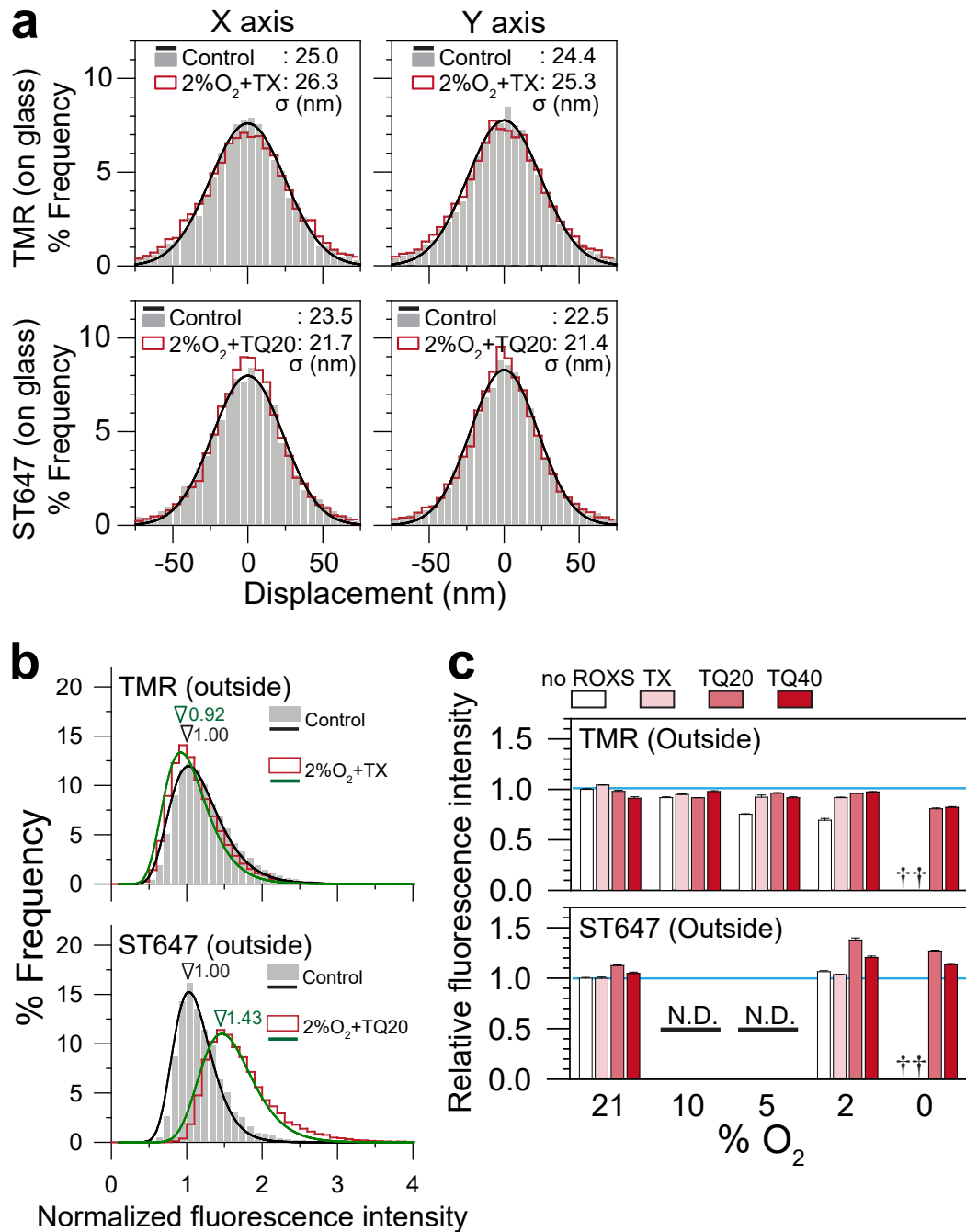
(c) The time-dependent increase of the troloxquinone fraction (vs. trolox + troloxquinone, mean $\pm$ SEM values, $n = 10$ independent experiments) after UV irradiation, reaching

quasi-photoequilibrium at ~60% troloxquinone (*vs.* 40% trolox) after 20 min UV irradiation under the conditions employed here. For details, see **Online Methods**.

influx of fluorescently-labeled CD47 molecules into the illumination area from the outside by diffusion is negligible and the laser illumination was rather uniform.

As shown in the figure, in the case of T24 cells, since almost the entire basal PM was illuminated at the intensity close to the center of the spot (>75%), the major problem for evaluating photobleaching by counting the spot numbers was caused by the influx of fluorescently-labeled CD47 molecules, diffusing into the counting area from the apical PM. Since the effective macroscopic diffusion coefficient of CD47 was $0.25 \pm 0.14 \mu\text{m}^2/\text{s}$ (mean $\pm$ SD), CD47 can diffuse within areas with radii of ~ 10 and $20 \mu\text{m}$ during 50 and 200 s, respectively (using mean diffusion coefficient + SD $\times 2$, $\sim 95.5\%$ of CD47 molecules). Therefore, the area used for counting fluorescent CD47 spots was limited to a small square region of $17.5 \times 17.5 \mu\text{m}$ (half the diagonal length = $12.4 \mu\text{m}$, which is smaller than $20 \mu\text{m}$ [the cell radius of $30 \mu\text{m}$ minus $10 \mu\text{m}$ of the CD47 diffusion radius during 50 s]) for 50-s observations or $10.0 \times 10.0 \mu\text{m}$ (half the diagonal length = $7.1 \mu\text{m}$, which is smaller than $10 \mu\text{m}$ [the cell radius of $30 \mu\text{m}$ minus $20 \mu\text{m}$ of the CD47 diffusion radius during 200 s]) for 200-s observations in the center of the laser-illuminated area.

With regard to the uniformity of laser illumination, in these square areas for counting the number of fluorescent spots, the laser intensity was likely reduced from the laser center by only 4.8% or less. Therefore, the illumination is considered to be rather uniform in the area used for counting the number of fluorescent spots.



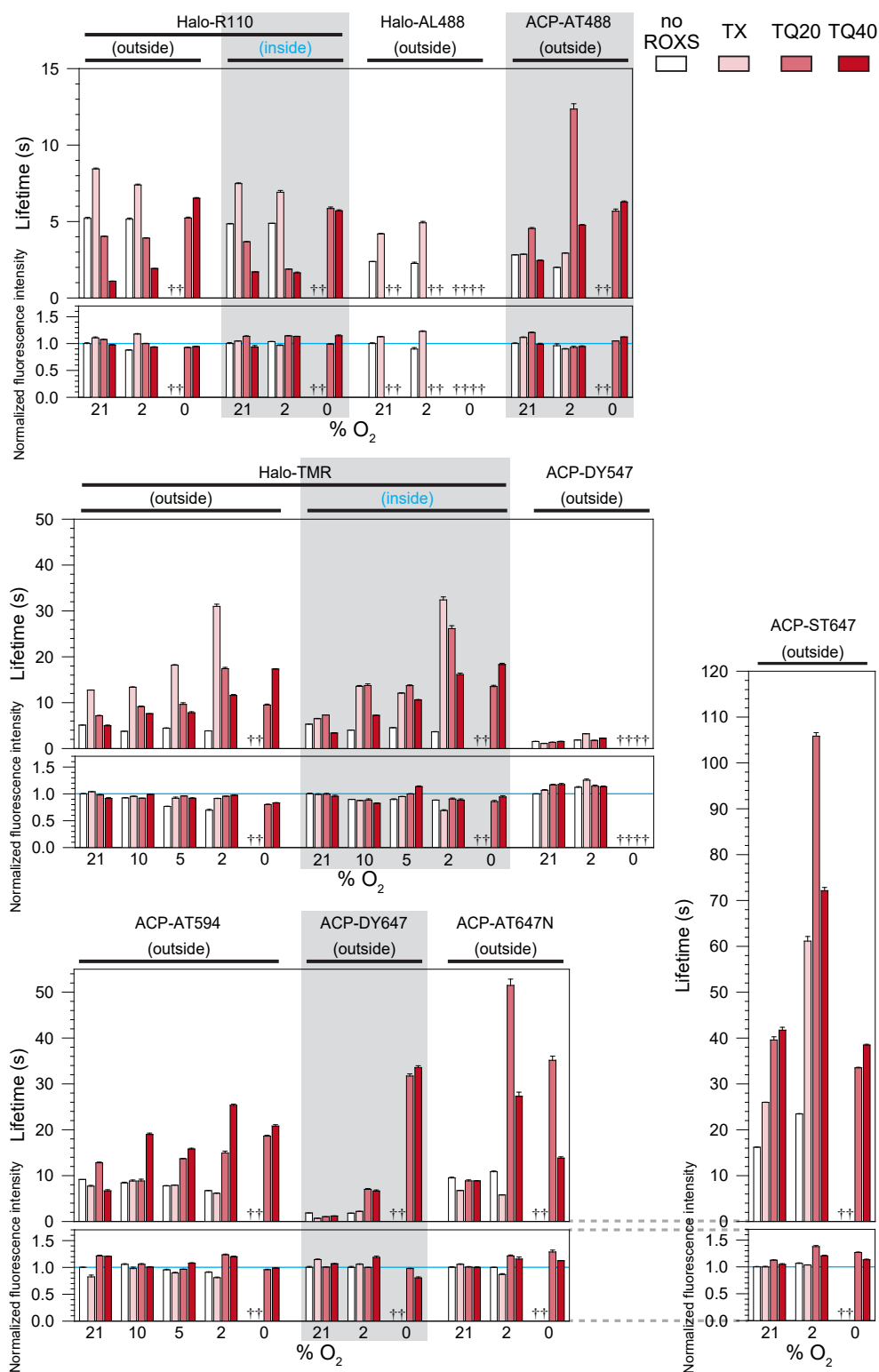
Supplementary Figure 6 | Single-molecule localization precisions and fluorescence signal intensities of single TMR and ST647 molecules (located on the outer cell surface) were either unchanged or only slightly affected when the observation conditions were changed from the control conditions to the optimized low oxygen + ROXS conditions.

(a) The locations (x and y coordinates) of single TMR (**top**) and ST647 (**bottom**) molecules attached to the glass-base dish in T-HBSS, determined for 128 and 151 molecules (TMR and ST647, respectively; after 30 coordinate determinations for each molecule, their mean value

was subtracted from each of the 30 determined coordinates). The standard deviations determined by best-fit Gaussian functions (black curves for the control specimens; the curves for the 2%O₂+ROXS specimens were close to the black curves and are not shown here) provided the single-molecule localization precision.

(b) The distributions of the fluorescence signal intensities (after background subtraction for individual spots) of TMR bound to Halo-CD47 (**top**) and ST647 bound to ACP-CD47 (**bottom**), located on the outer surface of living cells. Distributions could be fitted by log-normal functions^{63,64}, providing the mode values (triangles; the mode is the signal intensity value at which the log-normal probability distribution function has its maximal value). The intensities were normalized by the mode value determined under control conditions.

(c) The mode values and standard errors of fluorescence intensities obtained by the log-normal fitting of TMR (**top**) and ST647 (**bottom**) located outside the cell, under various %O₂ + ROXS conditions. [†]Unable to determine due to extensive blinking, providing trajectories mostly as short as 1-2 frames (33 and 66 ms), which could not be distinguished from the noise signal. N.D., not done. For *n*, see **Supplementary Table 1**.



Supplementary Figure 7 | Summary of photobleaching lifetimes (obtained by counting the number of fluorescent spots in each video frame in live cells; exponential decay time constant [= mean] $\pm$ the standard error) and fluorescence intensities of organic fluorophores (the mode values $\pm$ the standard errors obtained by log-normal fitting)

examined in this study, under various dissolved oxygen concentrations and ROXS conditions. Also see **Supplementary Table 1**. For η , see **Supplementary Table 1**.

(Top row) Blue-excitable dyes: Rhodamine 110 (R110), Alexa488 (AL488), and ATTO488 (AT488).

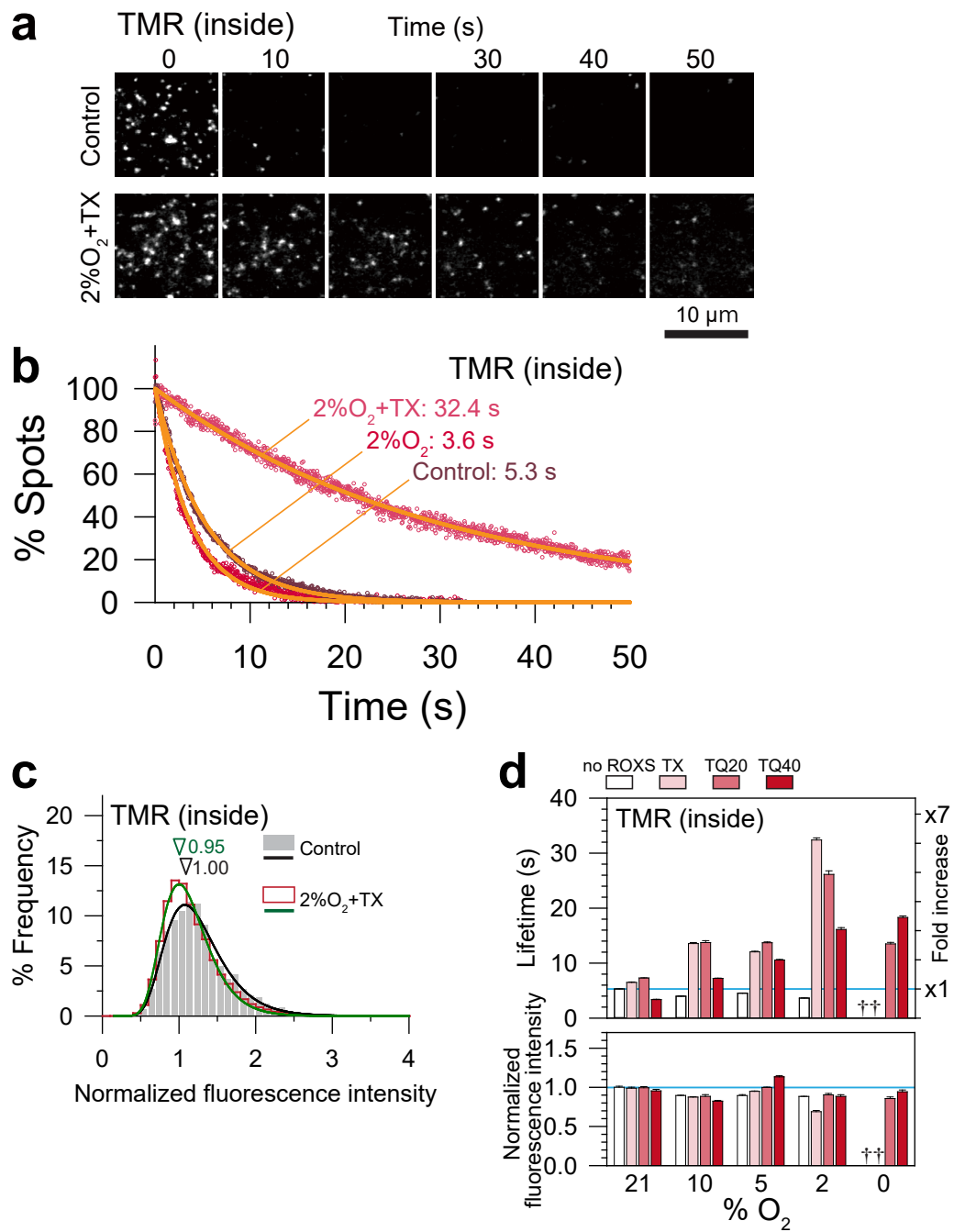
(Middle row) Green-excitable dyes: TMR and DY547.

(Bottom row) Orange-red-excitable dyes: AT594, DY647, ATTO647, and ST647.

The top box in each row shows photobleaching lifetimes, whereas the bottom box in each row shows fluorescence intensities. For the dependence on tag proteins (Halo and ACP), see

Supplementary Fig. 9. [†]Unable to determine due to extensive blinking, providing trajectories mostly as short as 1-2 frames (33 and 66 ms), which could not be distinguished from the noise signal.

To examine fluorophores located outside the cell, R110-, AL488-, TMR-, TMR-TX-, Cy3-TX-, and Cy5-TX-Halo ligands were conjugated to Halo-CD47, and AT488-, DY547-, AT594-, AT594-TX-, DY647-, AT647N-, and ST647-CoA were linked to ACP-CD47. To examine fluorophores located in the cytoplasm, R110 and TMR were attached to CD47-Halo.



Supplementary Figure 8 | TMR (Halo ligand conjugated with TMR) is a membrane permeable dye useful to label the Halo-tag protein inside the cell (CD47-Halo). Its photobleaching *inside* the living cells (in the cytoplasm) can be suppressed by low O₂ concentrations + ROXS, as effectively as that of TMR located outside the cell.

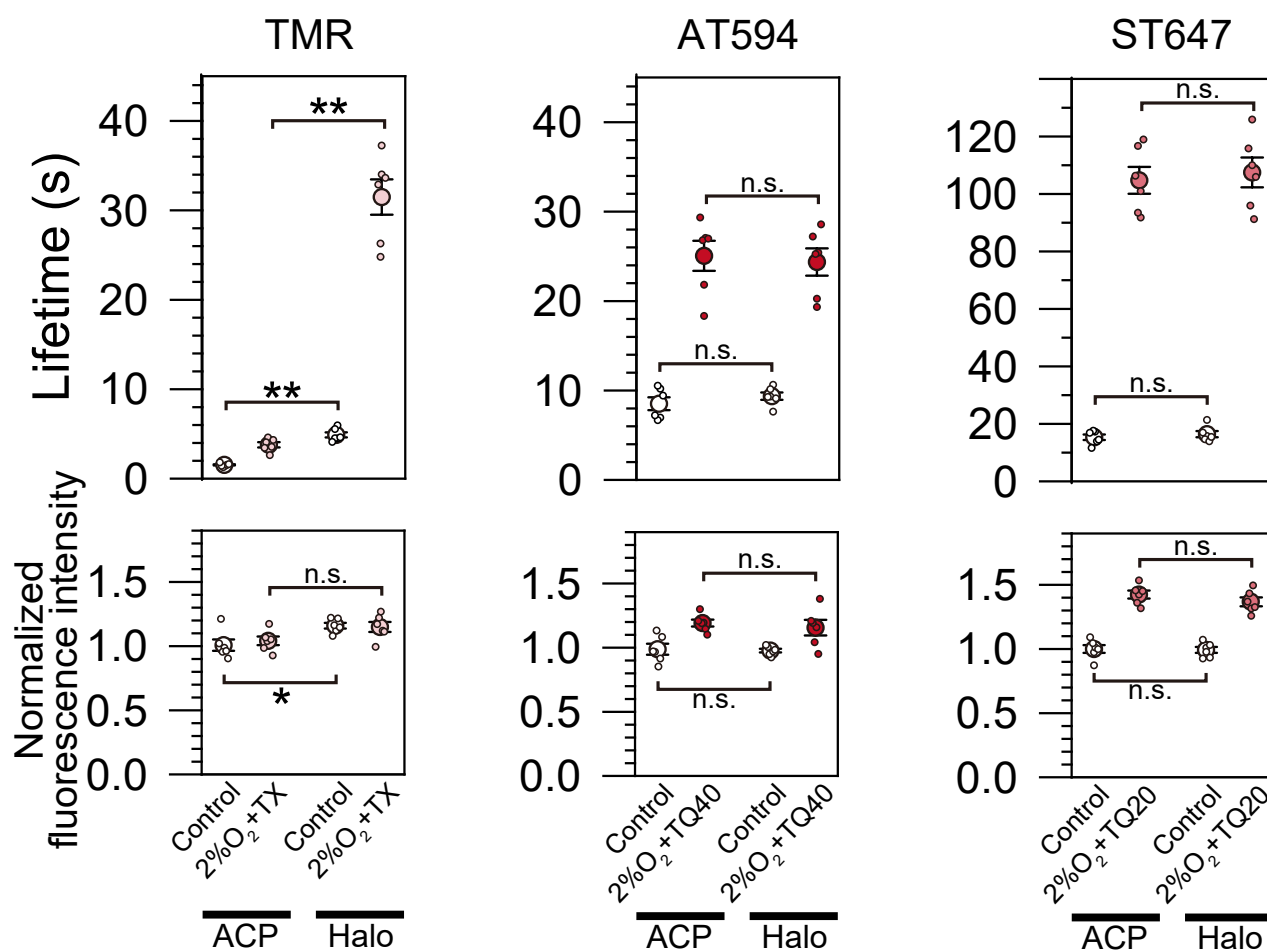
(a) Representative (among six independent experiments) time series of TIRF images of TMR bound to CD47-Halo (TMR located inside the cell) in a single cell.

(b) The time-dependent decreases of the numbers of fluorescent-spots of TMR inside the cell counted in each frame, observed in a single cell. Orange curves represent the best-fit single exponential functions.

(c) The distributions of the fluorescence signal intensities (after background subtraction for individual spots) of TMR bound to CD47-Halo located in the cytoplasm of a single living cell. Distributions could be fitted by log-normal functions, providing the mode values (triangles). The intensities were normalized by the mode value determined under control conditions.

(d) The photobleaching lifetimes (single-molecule tracking lifetime; exponential decay time constant [= mean] $\pm$ the standard error) (**top**) and the fluorescence intensities (the mode values $\pm$ the standard errors obtained by log-normal fitting) (**bottom**) of TMR bound to CD47-Halo, under various %O₂ + ROXS conditions. Photobleaching lifetimes inside the living cells were prolonged to about the same extent as those located outside the cells. [†]Unable to determine due to extensive blinking, providing trajectories mostly as short as 1-2 frames (33 and 66 ms), which could not be distinguished from the noise signal. For *n*, see

Supplementary Table 1.



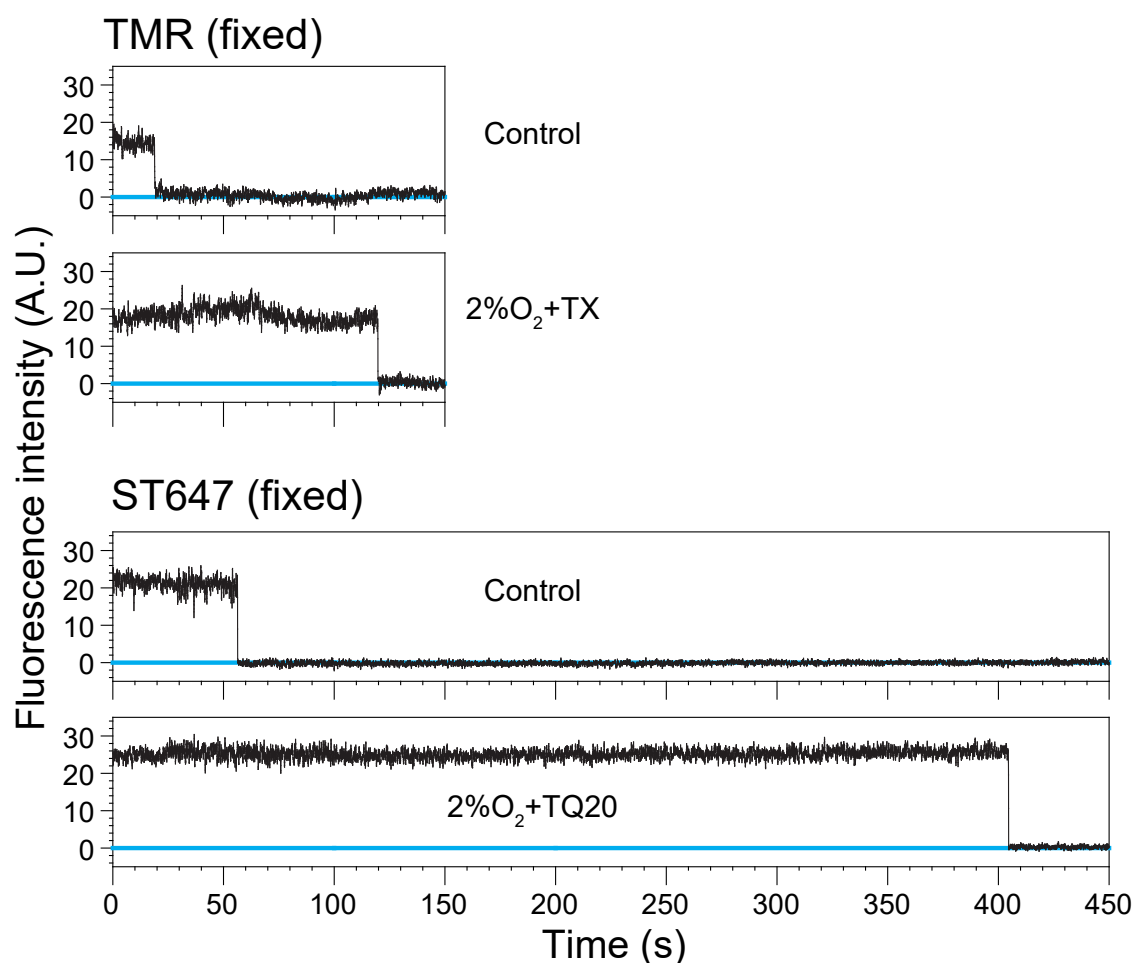
Supplementary Figure 9 | Comparison between Halo and ACP tags, in terms of the photostabilization effect by 2%O₂+ROXS on TMR, AT594, and ST647 bound to these protein tags (linked to the N-terminus of CD47, located outside the cell).

Six independent experiments were performed for each dye under a given ROXS condition. For each experiment, the photobleaching lifetime (exponential decay time constant, **top**) and the mode of the signal intensity of single spots (obtained by the log-normal fitting; see **Supplementary Fig. 6b, bottom**) were obtained. These values were averaged to obtain the mean $\pm$ SEM of the six independent experiments. ROXS conditions were those optimized for the Halo (for TMR) or ACP (for AT594 and ST647)-tag protein and its fluorescent ligands (**Fig. 1d**).

The photobleaching lifetime of TMR bound to the Halo-tag protein was significantly longer than that bound to the ACP-tag protein under both the control and 2%O₂+ROXS conditions. Meanwhile, no statistically significant differences were detectable between Halo- and ACP-tag proteins for the dyes of AT594 and ST647. Two-sided Welch's T test: [*P* for ACP

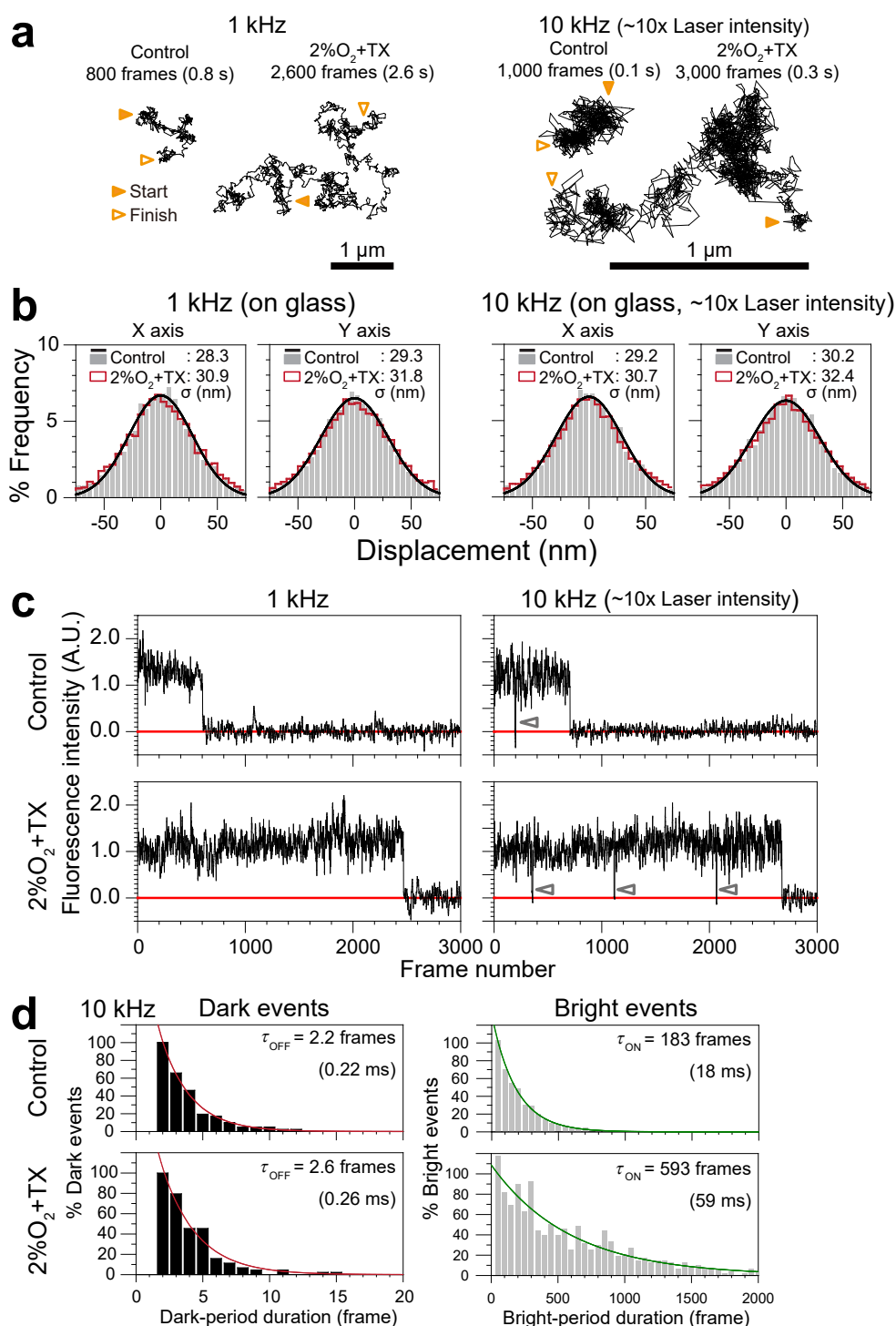
vs. Halo (control), P for ACP vs. Halo (optimized conditions)] = [4.5×10^{-5} , 2.6×10^{-5}] for TMR, [0.33, 0.76] for AT594, and [0.49, 0.70] for ST647).

For the fluorescent intensities, significant difference was found only between ACP-TMR and Halo-TMR under the control conditions (two-sided Welch's T test: [P for ACP vs Halo (control), P for ACP vs Halo (optimized conditions)] = [0.017, 0.065] for TMR, [0.81, 0.61] for AT594, and [0.84, 0.25] for ST647). For number of fluorescent spots, n , see **Supplementary Table 1**. **: $P < 0.01$, *: $P < 0.05$, n.s.: $P > 0.05$



Supplementary Figure 10 | TMR (bound to Halo-CD47) and ST647 (bound to ACP-CD47) hardly exhibited blinking at a time resolution of 33 ms under either the control or 2%O₂+ optimized ROXS conditions. To avoid other difficulties of tracking moving molecules, the cells were chemically fixed⁶⁵.

Typical (for n , see **Table 1**) time traces of fluorescence intensities of single molecules of TMR (**two top boxes**) and ST647 (**two bottom boxes**). Trajectories with durations of $\sim 4\times$ lifetimes (**Table 1**) were selected arbitrarily. Note that blinking (temporary transition to the dark state) is undetectable even in these long traces.



Supplementary Figure 11 | Ultrafast single fluorescent-molecule tracking at a 0.1-ms resolution revealed TMR blinking, with alternating dark-and-bright states with lifetimes of 0.22 and 18 ms (0.26 and 59 ms), respectively, under the control (optimized; 2%O₂+TX) conditions. TMR bound to Halo-CD47 in the live-cell PM (outside the cell) (**a**, **c**, **d**) or TMR attached to the coverslip surface (**b**) was observed.

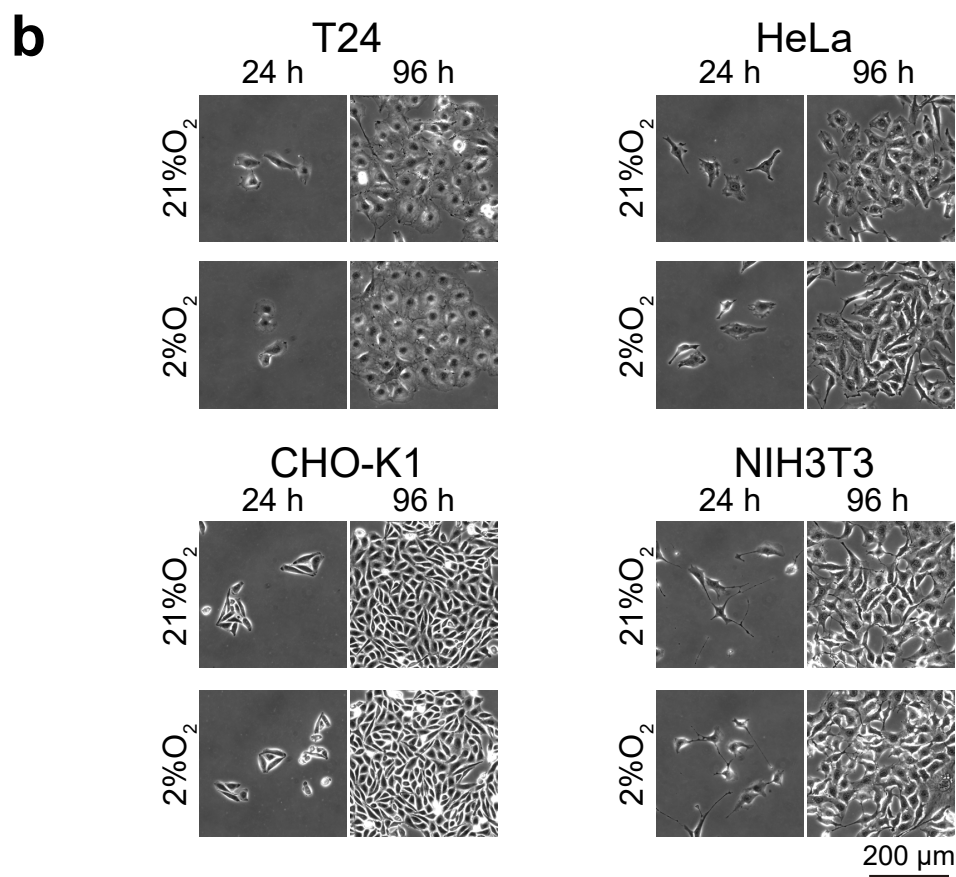
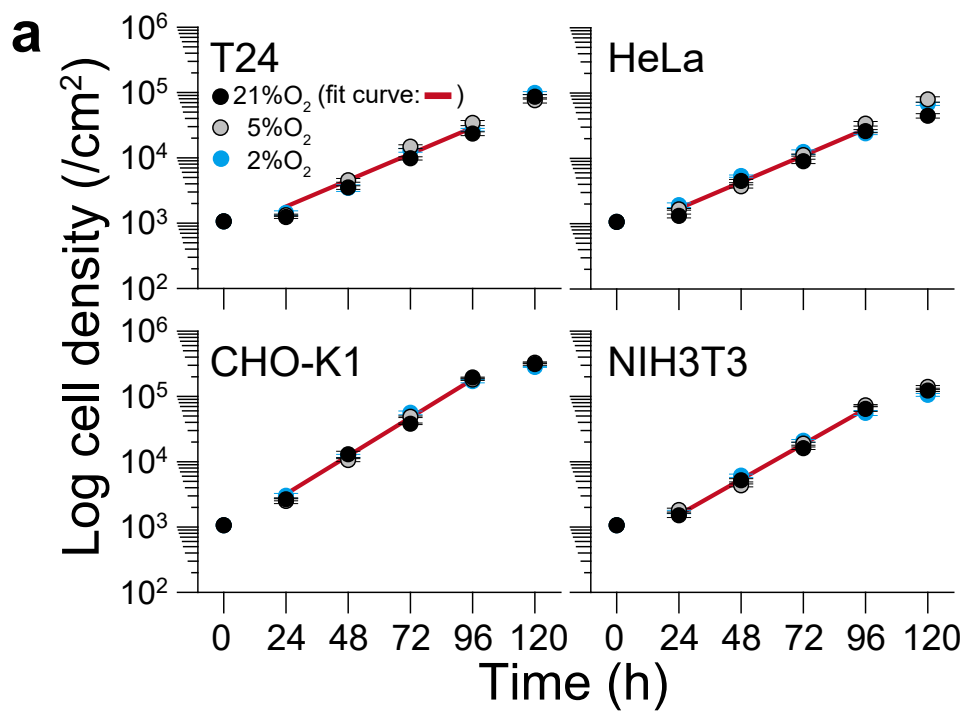
(a) Typical trajectories (among 331, 585, 401 and 583 trajectories from left to right) of TMR bound to Halo-CD47 in the live-cell PM (outside the cell) at observation frame rates of 1 **(left)** and 10 kHz **(right)** (integration times of 1 and 0.1 ms, respectively) under the control and optimized (2%O₂+TX) conditions. A few % of TMR molecules could be tracked without photoblinking and photobleaching for periods of ~1,000 and ~3,000 frames under the control and optimized conditions, respectively, under single-molecule tracking conditions at both 1 and 10 kHz (in terms of the number of frames, the observable length remained the same for both 1 and 10 kHz when the laser intensity was increased by a factor of ~10 at 10 kHz). This way, the number of emitted photons from a single molecule per frame would remain about the same. No non-linear effects were observed at the laser powers employed here. The integrated laser intensity during a single frame was adjusted to become about the same for the observations at both frame rates. Note that the trajectory lengths in terms of the actual time are different by a factor of ~10 for the two frame rates, and therefore, the trajectories are shown in different scales. Here, the integrated laser intensities during a single frame are ~60% of those employed at video rate. The actual laser intensities employed are described in the Online Methods section "Single fluorescent-molecule imaging".

(b) Single-molecule localization precisions (on the x and y axes, representing the horizontal and vertical axes of the camera) for TMR attached to the glass observed at frame rates of 1 and 10 kHz, determined for 140 and 131 molecules (1 and 10 kHz, respectively; after 30 coordinate determinations for each molecule, their mean value was subtracted from each of the 30 determined coordinates. Grey closed bars (green best-fit Gaussian curves) and red open bars are for the control and optimized conditions. The curves for the 2%O₂+ROXS specimens were close to the black curves and are not shown here. The precision is given as the standard deviation of the best-fit Gaussian functions. By varying the excitation laser intensity by a factor of ~10, the single-molecule localization precisions at 1 and 10 kHz were largely equalized.

(c) Typical (among the same number of trajectories shown in **a**) time traces of fluorescence intensities of single molecules of TMR bound to Halo-CD47 in the live-cell PM under the control and optimized conditions (**top and bottom boxes, respectively**), observed at frame rates of 1 and 10 kHz (**left and right boxes, respectively**). At an observation frame rate of 10 kHz, blinking (temporary transition to the state of no signal intensity) of

TMR was detected (triangles). The x-axis is given as the frame number. The actual time is 1 ms/frame for 1 kHz and 0.1 ms/frame for 10 kHz.

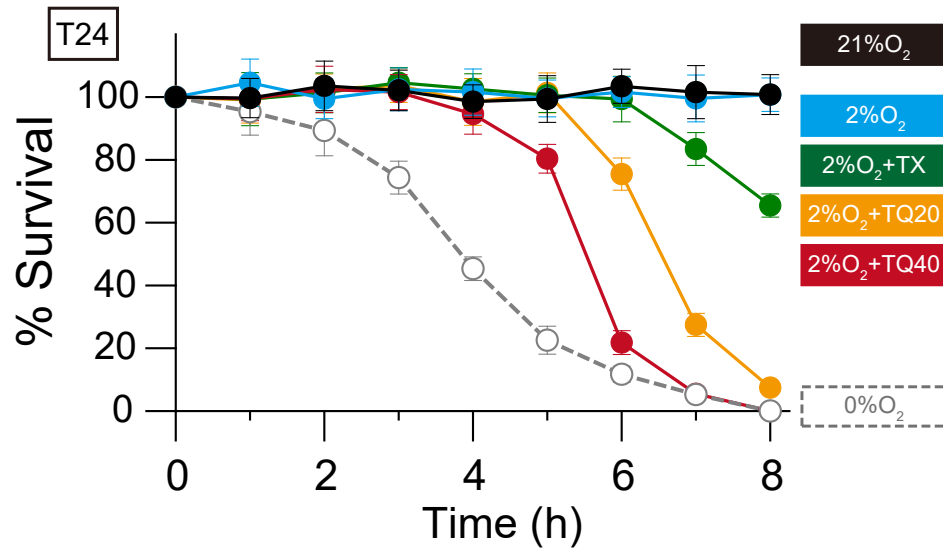
(d) Distributions of the dark (**left**) and bright (**right**) periods detected at 10 kHz. Red and green curves are best-fit single exponential functions, providing the lifetimes of the dark and bright states of 0.22 ± 0.0050 and 18 ± 0.33 ms, respectively, under the control conditions, and 0.26 ± 0.011 and 59 ± 3.5 ms, respectively, under the optimized (2%O₂+TX) conditions (exponential decay time constant [= mean] $\pm$ its standard error; n for dark and bright events = 115 and 585 for control, and 140 and 583 for 2%O₂+TX conditions). These results suggest that blinking was rarely detectable at lower frame rates, such as 1 kHz and 30 Hz, due to averaging over 1 and 33 ms (much longer than the dark periods).



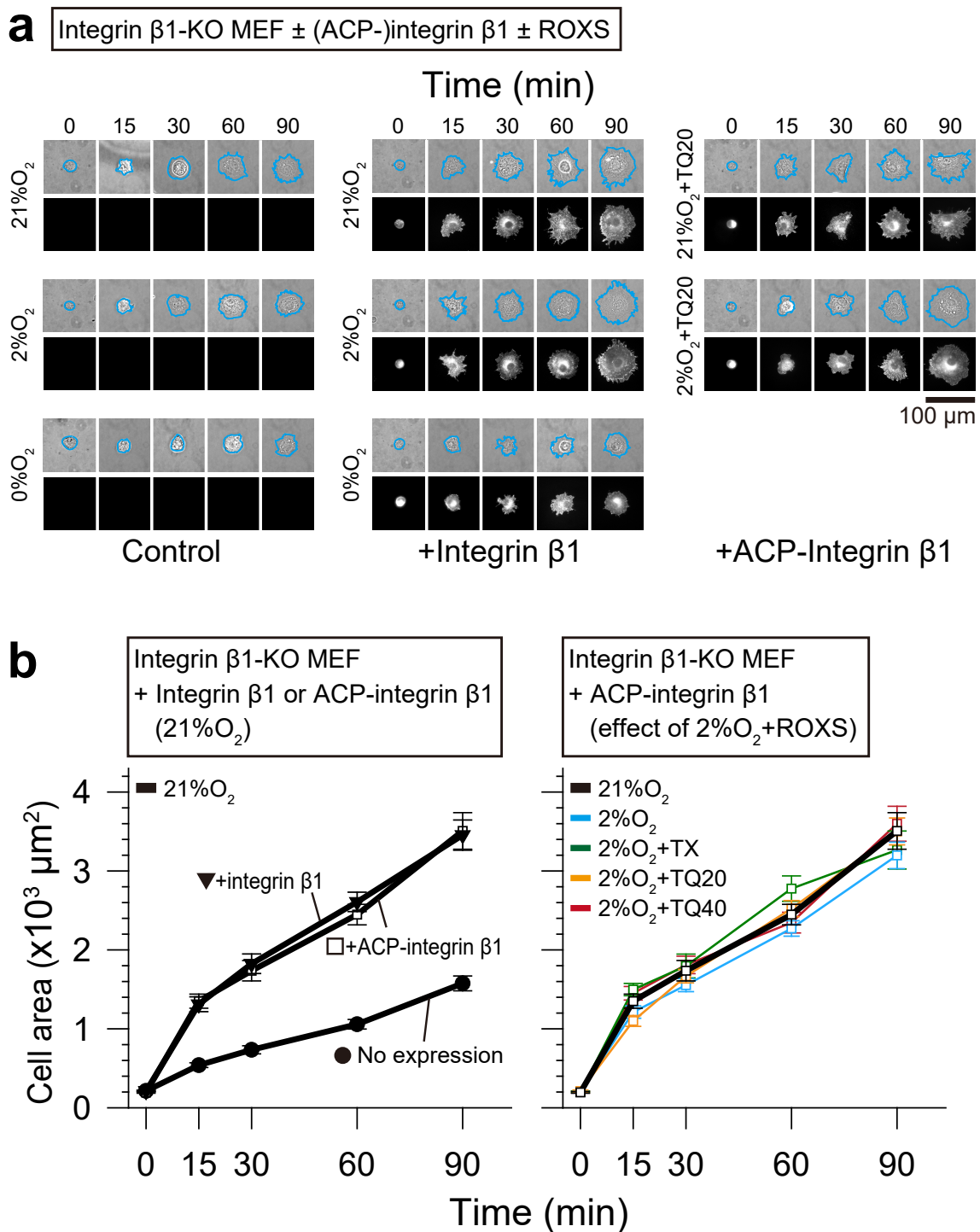
Supplementary Figure 12 | Cells grew at about the same rate under 2, 5, and 21% oxygen atmospheres.

(a) The number densities of T24, HeLa, CHO-K1, and NIH3T3 cells cultured with complete growth medium under 5% CO₂ in the presence of 2, 5, and 21% oxygen atmospheres, plotted as a function of time after inoculation (mean $\pm$ SEM, $n = 6$). The doubling time was obtained by fitting the cell density between 24 and 96 h with a single exponential growth curve, and shown in **Fig. 3a** (note that the y-axis is in the log scale). The data at ~ 126 h were not used for the evaluation of the doubling time, because the cell confluencies were high at this time point, which might have already induced contact inhibition of growth. Note that the results obtained under the conditions of 2, 5, and 21%O₂ were similar to each other for each cell line.

(b) Typical (among 6 fields for each picture) phase-contrast images of cells cultured under 21 or 2%O₂ conditions for 24 or 96 h after plating.



Supplementary Figure 13 | Percentages of surviving T24 cells, under various low-oxygen + ROXS conditions, determined by the trypan-blue exclusion assay (mean $\pm$ SEM, $n = 6$ independent experiments).



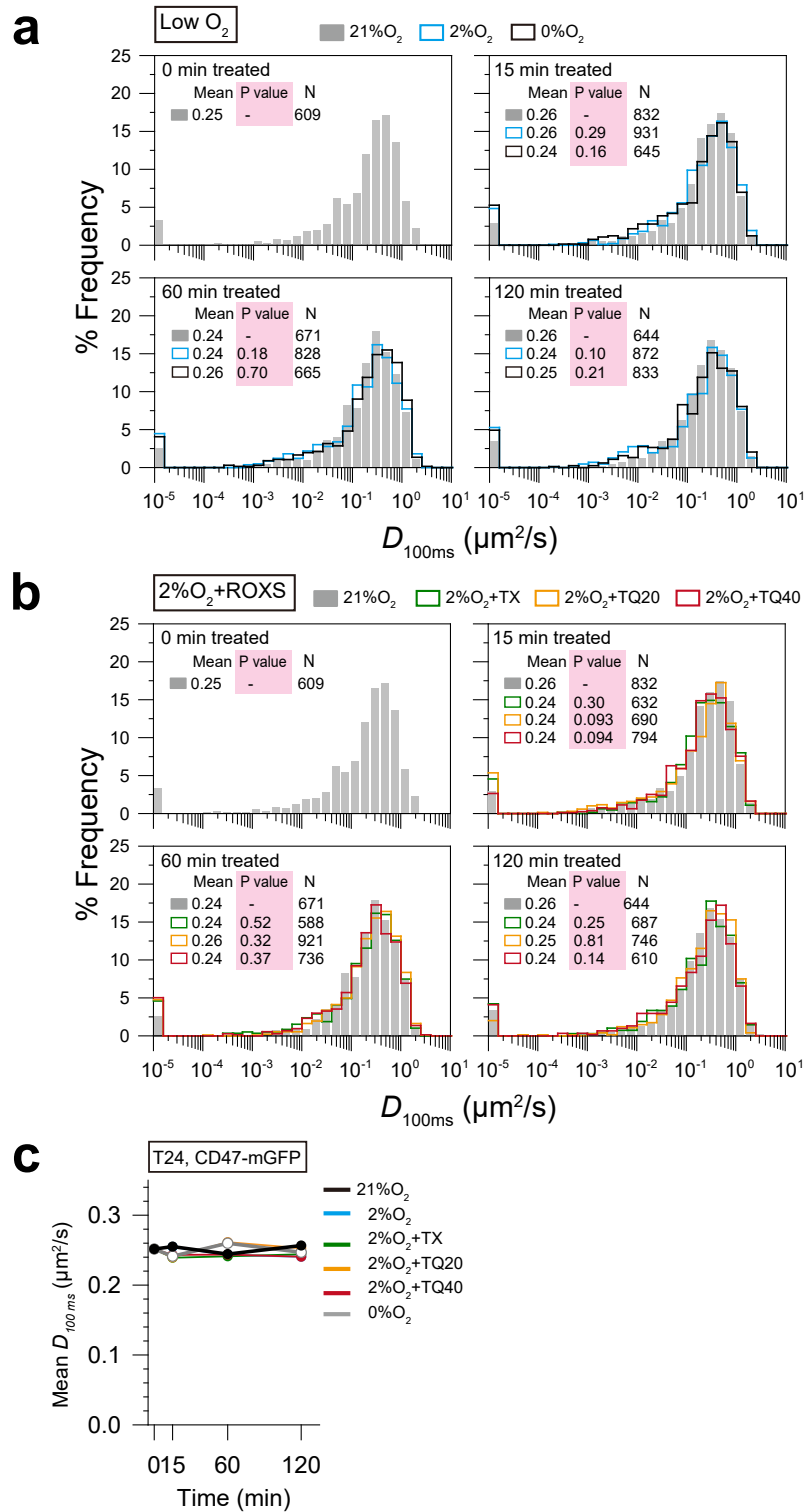
Supplementary Figure 14 | Spreading of integrin $\beta 1$ -KO MEF cells before and after transfection of the integrin $\beta 1$ or ACP-integrin $\beta 1$ cDNA was inspected under 0, 2, and 21%O₂ conditions as well as those with 2%O₂+ROXS (TX, TQ20, and TQ40). Differences in cell spreading were undetectable between the 21%O₂ and 2%O₂+ROXS (TX, TQ20, and TQ40) conditions for at least 90 min.

(a) Representative (among 20 cells inspected closely) phase-contrast (**top**) and anti-integrin $\beta 1$ immunofluorescence (epi-fluorescence, **bottom**) images of integrin $\beta 1$ -KO MEFs with or without transfection-expression of integrin $\beta 1$ (no tag) or ACP-integrin $\beta 1$, at 0, 15, 30, 60, and 90 min after plating on FN-coated glass-base dishes. The anti-integrin $\beta 1$ monoclonal antibody used here (K20) can bind to both active and inactive integrin $\beta 1$. The cells with similar integrin $\beta 1$ expression levels were selected for comparing cells cultured under different oxygen concentrations. Cyan lines in the phase-contrast images indicate cell peripheries, and by measuring the area within the cyan line, the cell area attached to the glass was evaluated (see **b**). Non-transfected integrin $\beta 1$ -KO MEF cells spread more slowly than transfected cells. Cells under 2% oxygen appeared to spread as well as those under 21% oxygen, whereas cells under 0% oxygen spread much more slowly.

(b) The time course of cell spreading after plating, as measured by the cell area attached to the glass observed by phase-contrast microscopy (mean cell area $\pm$ SEM, $n = 20$).

(left) Expression of integrin $\beta 1$ or ACP-integrin $\beta 1$ at similar levels enhanced cell spreading after plating to similar extents. This result suggests that ACP attached to the N-terminus of integrin $\beta 1$ does not strongly inhibit integrin $\beta 1$ function. As plotted in **Fig. 3d** (based on the data shown in **a**), under 0%O₂, cell spreading was greatly suppressed in cells with and without integrin $\beta 1$ expression. No significant difference was found between integrin $\beta 1$ and ACP-integrin $\beta 1$ up to 90 min (two-sided Mann-Whitney U test; for the actual P values, see **Supplementary Table 5**).

(right) The time course of spreading of integrin $\beta 1$ -KO MEFs expressing ACP-integrin $\beta 1$ was not affected for at least 90 min under the conditions of 2%O₂+ROXS (TX, TQ20, and TQ40). The statistical significance was evaluated by the two-sided Mann-Whitney U test (for the actual P values, see **Supplementary Table 5**).

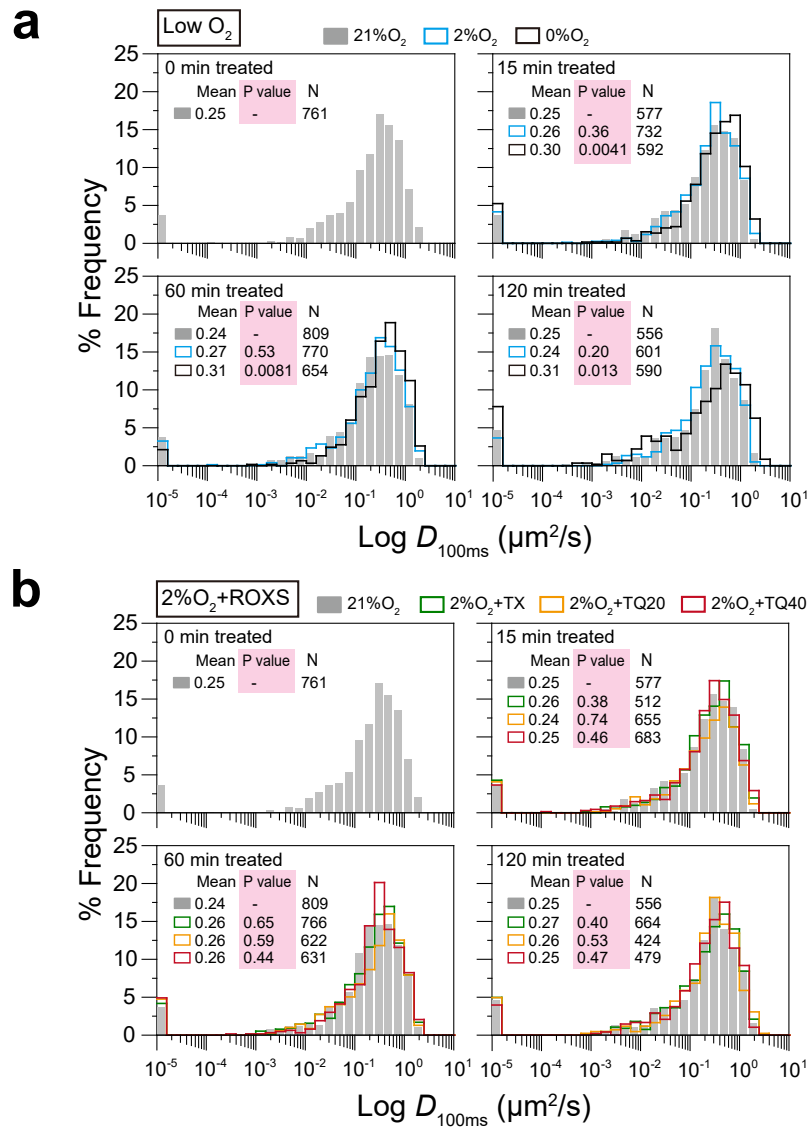


Supplementary Figure 15 | The diffusion of CD47-mGFP in the PM of T24 cells did not significantly change under various O₂ and ROXS conditions. The mGFP probe was employed here, because the photo-properties of GFP were hardly affected by the O₂ concentration and the ROXS conditions.

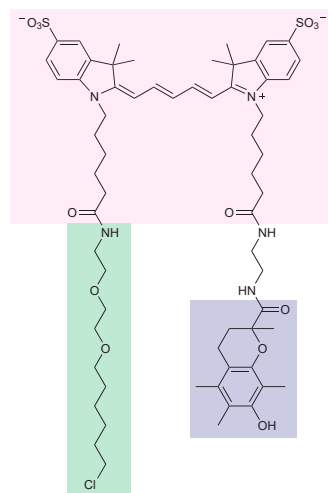
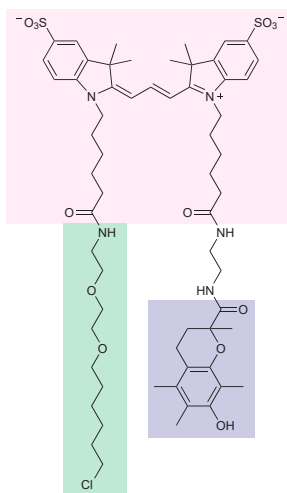
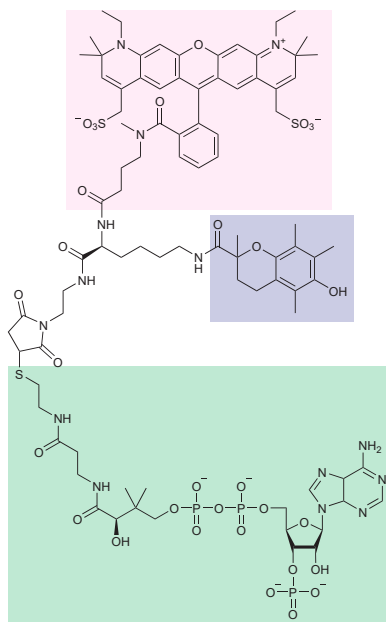
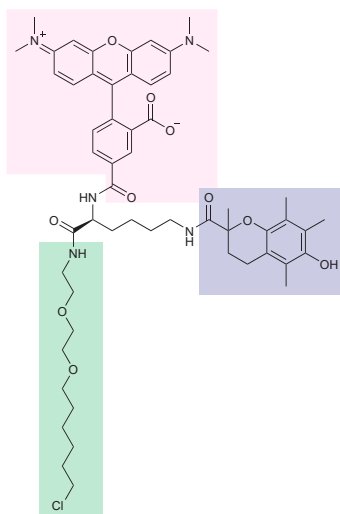
(a) The distributions of the diffusion coefficients (in the time scale of 100 ms, $D_{100\text{ms}}$) of CD47-mGFP when T24 cells were incubated in 21, 2, and 0% oxygen for various time periods up to 120 min. The mean value, the P value (compared with the distribution under control conditions [21%O₂], using the two-sided Mann–Whitney U-test), and the N value (total number of trajectories inspected here) are shown in each box.

(b) The distributions of the diffusion coefficients of CD47-mGFP when T24 cells were incubated under various ROXS conditions (TX, TQ20, and TQ40; all in the presence of 2% O₂) for various time periods up to 120 min. The results were compared with the $D_{100\text{ms}}$ distributions under 21%O₂ conditions after the given treatment periods. The mean value, the P value (compared with the distribution under control conditions [21%O₂], using the two-sided Mann–Whitney U-test), and the N value (total number of trajectories inspected here) are shown in each box.

(c) Mean diffusion coefficients of CD47-mGFP in T24 cells plotted as a function of time under various conditions. As shown in **(a)** and **(b)**, under all conditions examined here, the diffusion coefficient of CD47-mGFP was not statistically significantly affected, at least for 120 min, in T24-cell PM. This result suggests that the T24-cell PM (with its associated actin meshwork) is less sensitive to 0%O₂ as compared with the HeLa-cell PM.

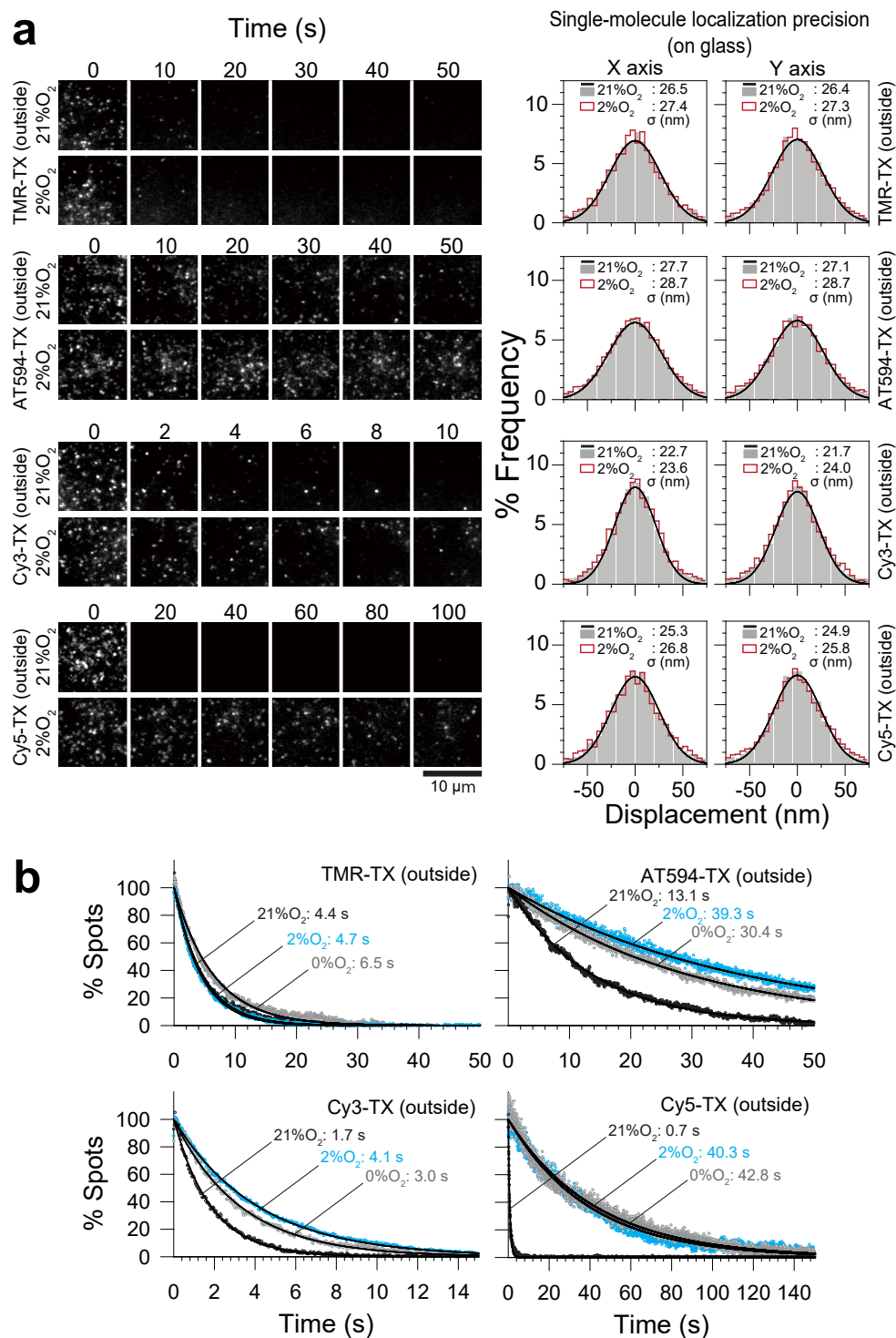


Supplementary Figure 16 | The diffusion of CD47-mGFP in the PM of HeLa cells was *enhanced* even 15 min after the cells were placed under 0% oxygen, at variance with the diffusion of CD47-mGFP in T24 cells. The figure arrangements, keys, and the numbers related to statistics are the same as those of **Supplementary Fig. 15a and b**.



Trolox-conjugated fluorophores for avoiding photobleaching were independently developed by two groups^{1,2,4,6}. However, TX-conjugated TMR (TMR-TX-Halo ligand, **a**) and AT594 (AT594-TX-CoA, **b**) have never been synthesized or examined. In addition, in the present study, we employed the Halo ligand and CoA (for conjugation to ACP), rather than the SNAP ligand, because the Halo ligand and the CoA compound worked better for single-molecule

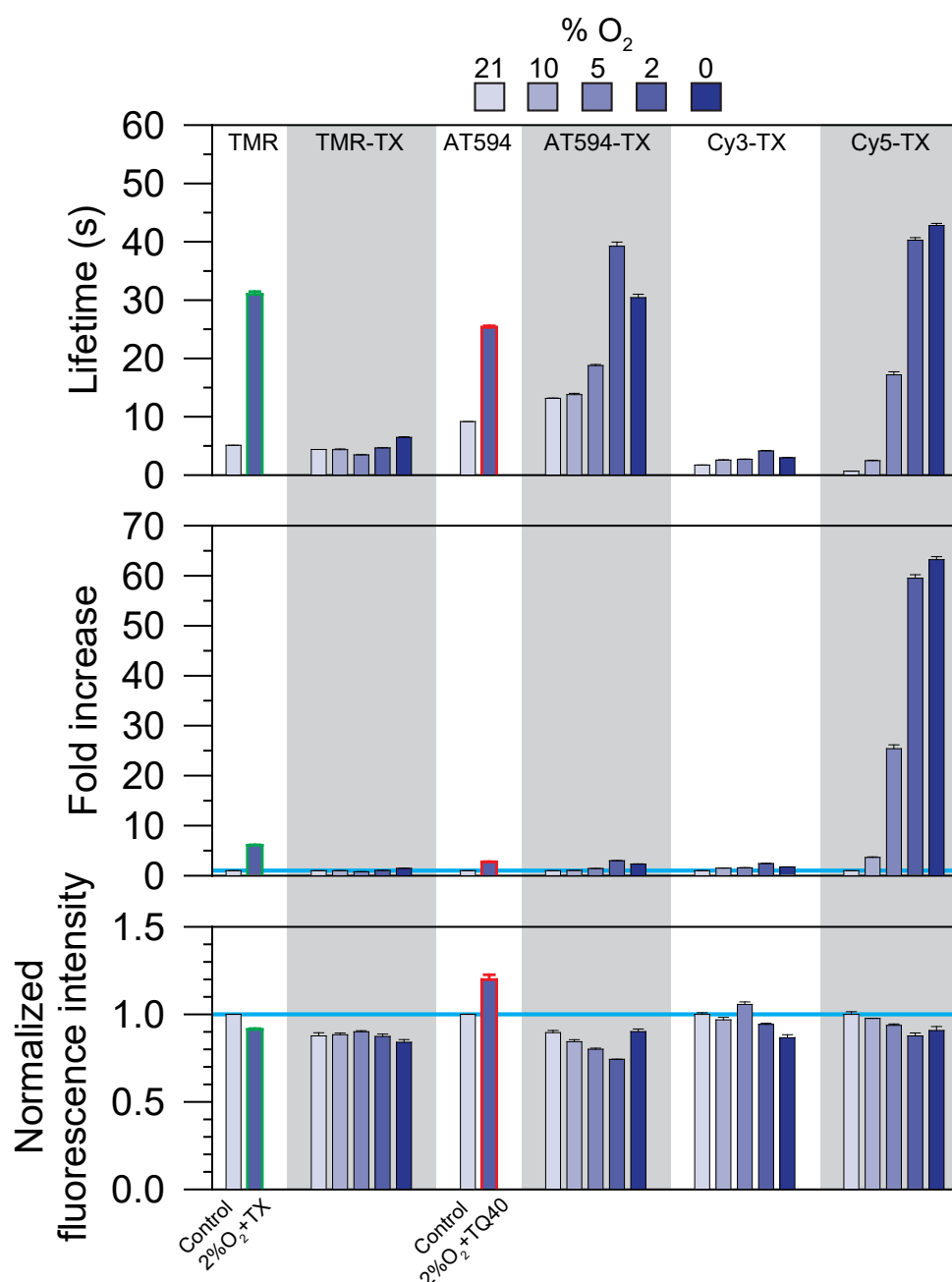
tracking of CD47, due to lower non-specific binding to the PM (Cy3-TX-Halo ligand, **c**, and Cy5-TX-Halo ligand, **d**). The fluorescent dye moiety (magenta shading) was conjugated to trolox (purple shading) and either the halo ligand or the ACP ligand (coenzyme A) (green shading).



Supplementary Figure 18 | Photobleaching time courses of trolox-conjugated fluorescent dyes (TMR-TX-, Cy3-TX-, and Cy5-TX-Halo-ligands and AT594-TX-CoA) under single fluorescent-molecule imaging conditions at localization precisions in the range of 21.7~28.7 nm, under 21 and 2%O₂ conditions (no ROXS here because TX is already covalently linked to the dye moiety), observed in T24 cells.

(a) Representative (among six independent experiments) time series of TIRF images (**left**) of TMR-TX, AT594-TX, Cy3-TX, and Cy5-TX bound to Halo-CD47 (probes located outside the cell) and single-molecule localization precisions of those dyes attached to the glass, determined for 137, 151, 143 and 135 molecules of TMR-TX, AT594-TX, Cy3-TX, and Cy5-TX, respectively (see **Supplementary Fig. 6a** for details) (**right**). The results were compared between 21 and 2%O₂. Grey closed bars (black best-fit Gaussian curves) are for 21%O₂ and red open bars are for 2%O₂ conditions. The best-fit Gaussian curves for the 2%O₂ conditions are not shown here, as they almost entirely overlapped with the black best-fit Gaussian curves. The precision is given as the standard deviation of the best-fit Gaussian functions.

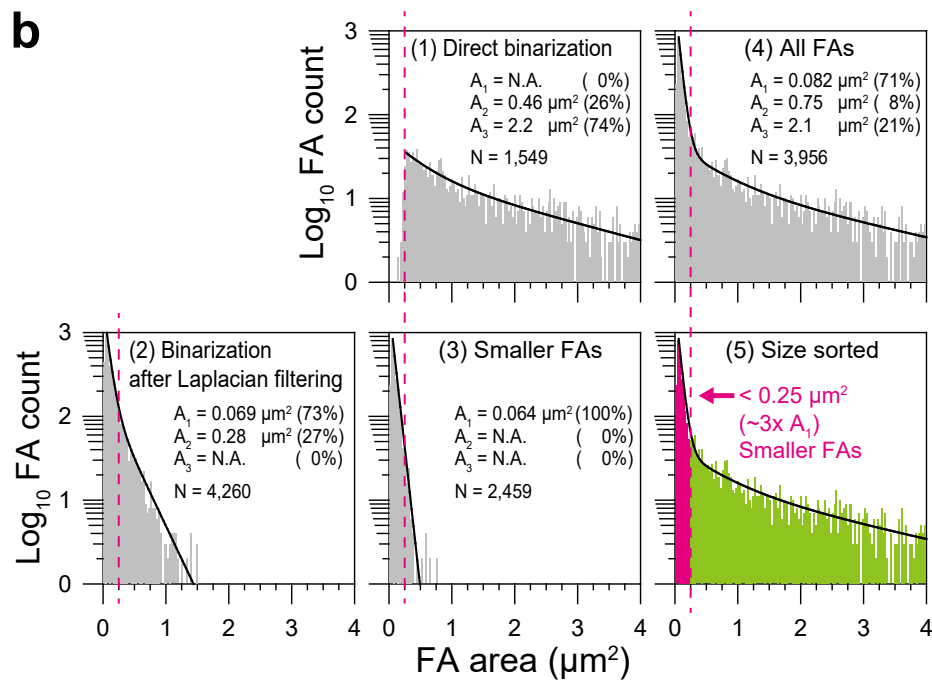
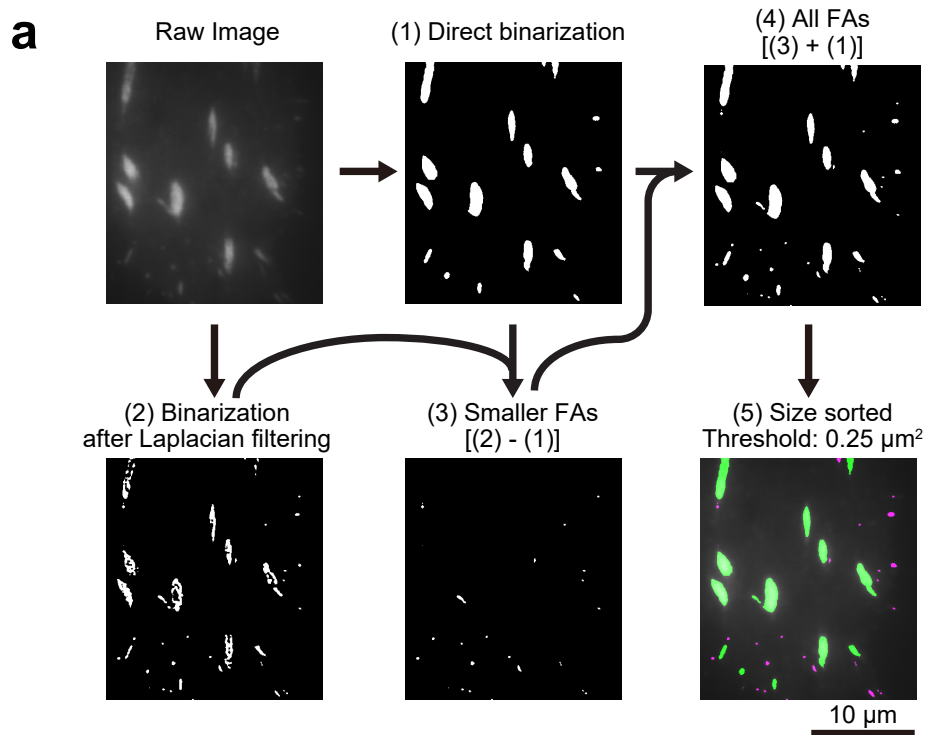
(b) The time-dependent decreases of the numbers of fluorescent spots found in each frame. Black curves represent the best-fit single exponential functions. Note that the x-axes for Cy3-TX and Cy5-TX are different from those for TMR-TX and Atto594-TX, due to the much shorter and much longer photobleaching lifetimes, respectively. Among the four trolox-conjugated dye molecules, Cy5-TX exhibited the best performance. Under the 2%O₂ conditions, the Cy5-TX photobleaching lifetime was extended to 39 s with a localization precision of ~25 nm.



Supplementary Figure 19 | Photobleaching lifetimes (number-counting lifetimes) of trolox-conjugated fluorescent dyes, TMR-, AT594-, Cy3-, and Cy5-TX bound to Halo-CD47 (+ TMR and AT594 without TX conjugation under the control and optimized 2%O₂ + ROXs conditions, for comparison) in the T24-cell-PM under 21, 10, 5, 2, and 0%O₂ atmospheres and single fluorescent-molecule imaging conditions, with localization precisions in the range of 21.7~28.7 nm. The photobleaching lifetime (exponential decay time constant [= mean] ± the standard error), its fold-increase as compared with that under control conditions (21% O₂) (**top and middle, respectively**), and the fluorescence

intensity (mode $\pm$ the standard error obtained from the log-normal fitting; **bottom**) are shown. For n , see **Supplementary Table 1**.

The AT594- and Cy5-TX photobleaching lifetimes could be extended to 35~47 s, respectively, under 2 and 0%O₂ conditions. Nevertheless, it should be noted that ST647 under optimized conditions (2%O₂+TQ20) exhibited lifetimes about two-fold longer than the best TX-conjugated dyes employed here. TMR under the optimal 2%O₂ + TX conditions exhibited a longer photobleaching time than TMR-TX, whereas AT594-TX performed better than AT594 under the optimal 2%O₂ + TQ40 conditions.

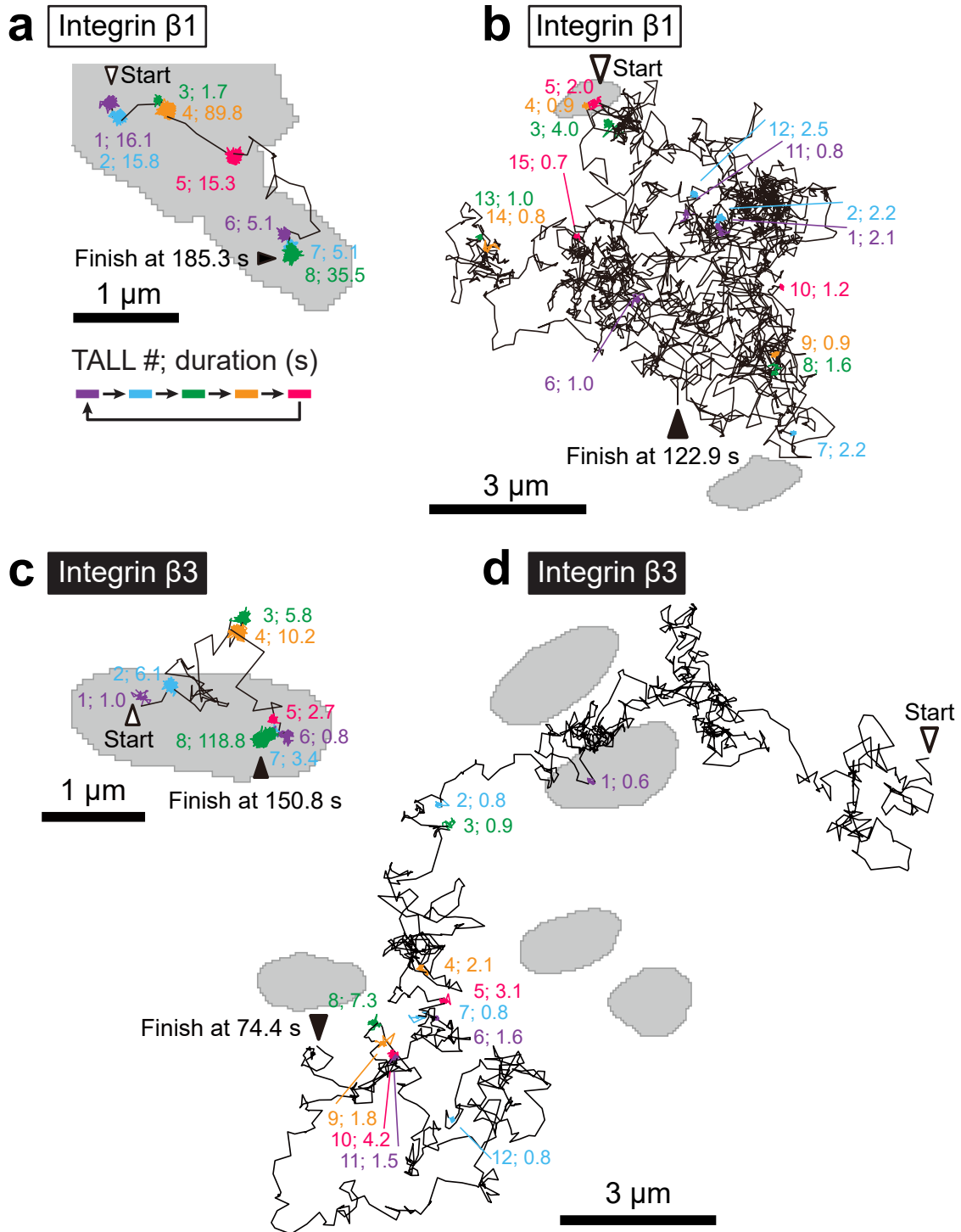


Supplementary Figure 20 | The method for defining the FA region using a binarization method developed for identifying both larger and smaller FAs (larger and smaller than $0.25 \mu\text{m}^2$, respectively).

(a) The process of binarization for defining larger and smaller FA regions, employed in the present research. It is shown by using a typical raw image. (Top, left) A raw image of

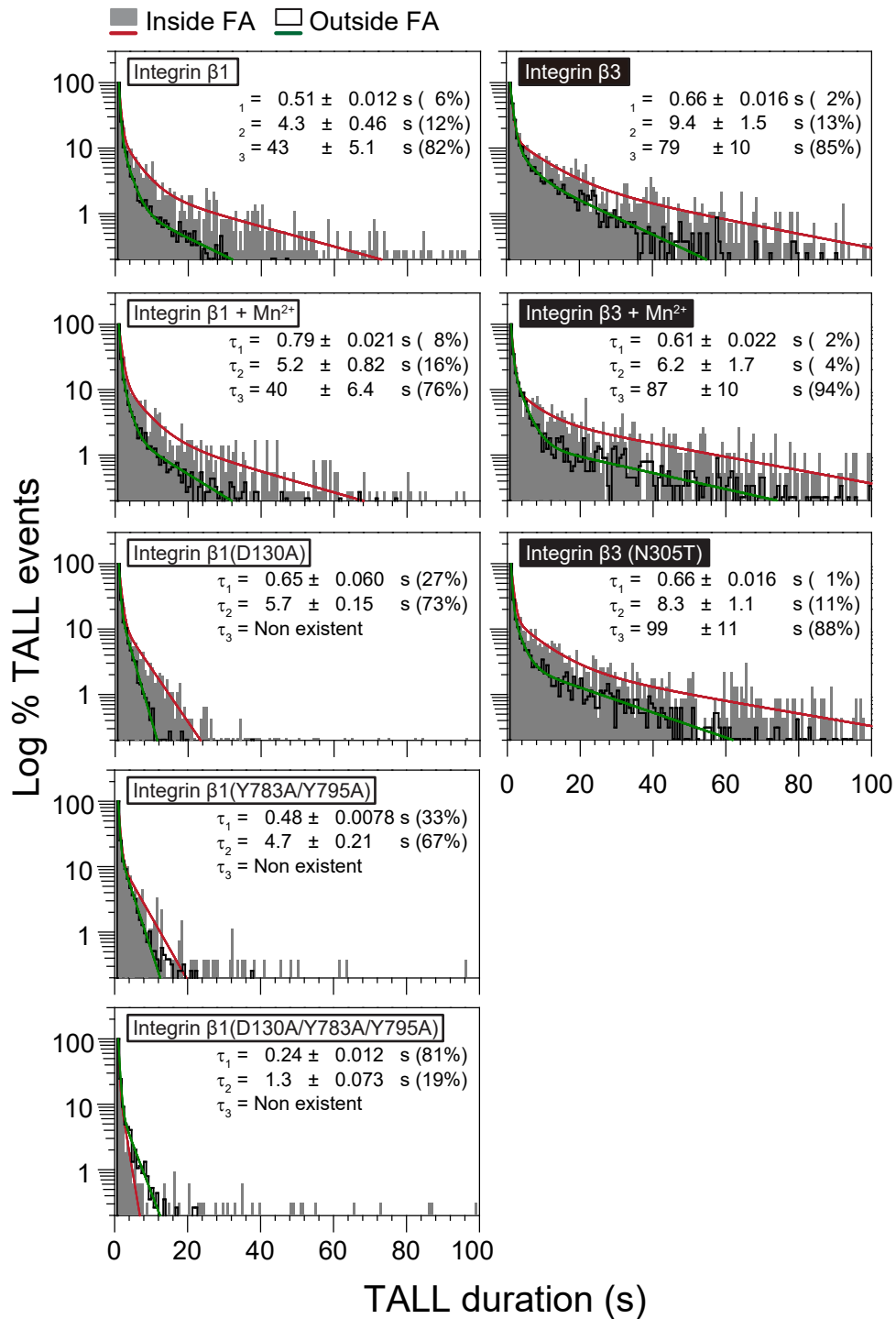
mGFP-paxillin. (**Top, middle**) The binarized image from the raw image (1: "Direct binarization"; see **Online methods** for details). However, the binarized images tended to lose smaller FAs. To circumvent this problem, we first applied a Laplacian filter (5x5 matrix with a value of 24 at the center pixel and -1 in other pixels; total =0) and then a 5x5 pixel median filter to the raw image, and then binarized the image (2: "Binarization after Laplacian filtering", **bottom left**). This procedure enhanced the images of smaller FAs (quantitative results shown in **b**). However, it caused the fragmentation of larger FAs, which we assumed to be artifactual, considering the spatial resolution of normal TIRF microscopy (240 nm). Therefore, to identify only the smaller FAs, in image 2, we removed the FAs that colocalized with the FAs in image 1, to obtain image 3 ("Smaller FAs", **bottom middle**). Finally, to show all of the FAs, we combined (superimposed) image 1 and image 3 (4: "All FAs"). The FAs in image 4 are shown in two colors; those larger and smaller than $0.25 \mu\text{m}^2$ are shown in green and magenta, respectively. The basis for this is clarified in **b**.

(**b**) The distributions of the FA area sizes. These distributions could operationally be fitted by an exponential function or the sum of two or three exponential functions. The best number of exponentials for each distribution was determined by both Akaike's and Baysean information criteria. The exponential decay constants (in terms of the area size) are shown in each box (A_1 , A_2 , and A_3), together with the number of FAs examined (N). The number fractions of each exponential component are shown in parentheses. The boxes are arranged in the same manner as in **a**. The size distribution for image 3 ("Smaller FAs", bottom, middle) could be fitted by a single exponential function with an A_1 of $0.064 \mu\text{m}^2$, which is very similar to the A_1 of image 2 ($0.069 \mu\text{m}^2$). This result supports the idea that the image processing method developed here works well for identifying FAs. Note that the distributions for image 1 ("Direct binarization") and image 3 ("Binarization after Laplacian filtering") lack the smallest (A_1) and largest (A_3) components, respectively. The distribution for image 4 could be fitted by the sum of three exponential functions, with the A_1 value close to that for images 2 and 3, the A_2 value somewhat close to those for images 1 and 2, and the A_3 value close to that for image 1, quantitatively supporting the image processing conducted in **a**. The threshold value between the smaller and larger FAs (magenta and green, respectively, in distribution 5) employed was $0.25 \mu\text{m}^2$, which was $\sim 3 \times A_1$ determined in the distribution for image 4. Smaller FAs might include those often called nascent adhesions or focal complexes^{22,23}.



Supplementary Figure 21 | Typical trajectories (additional examples, among 1729 and 2714 trajectories for integrins $\beta 1$ and $\beta 3$, respectively) of ST647 bound to ACP-integrin $\beta 1$ and ACP-integrin $\beta 3$ using super-long single-molecule tracking under the conditions of 2%O₂+TQ20 in HeLa cells, showing that integrins move in and out of the FA zones, frequently exhibiting temporary immobilization (TALL) events both inside and outside the FA zone.

FA zones (grey areas) are shown as the binarized images of mGFP-paxillin. Trajectories shown in **(a and c)** and **(b and d)** represent those located mostly inside and outside the FA, respectively. The colored parts of the trajectories indicate TALL events, occurring in the order of purple, cyan, green, orange, magenta, and then back to purple. The sequence of TALL occurrences is also shown by the integer, and the number that follows the semicolon indicates the TALL duration in seconds.



Supplementary Figure 22 | The distributions of the TALL durations of integrin $\beta 1$ (WT, D130A exoplasmic mutant [no RGD binding], the Y783A/Y795A cytoplasmic mutant [no linkage to actin filaments], and the combination of the two [D130A/ Y783A/Y795A], as well as WT with the addition of Mn^{2+}) and integrin $\beta 3$ (WT with the addition of Mn^{2+} , and the extracellular hybrid domain N305T constitutively active mutant).

(**Solid gray bars and red curves**) TALLs that occurred inside the FA.

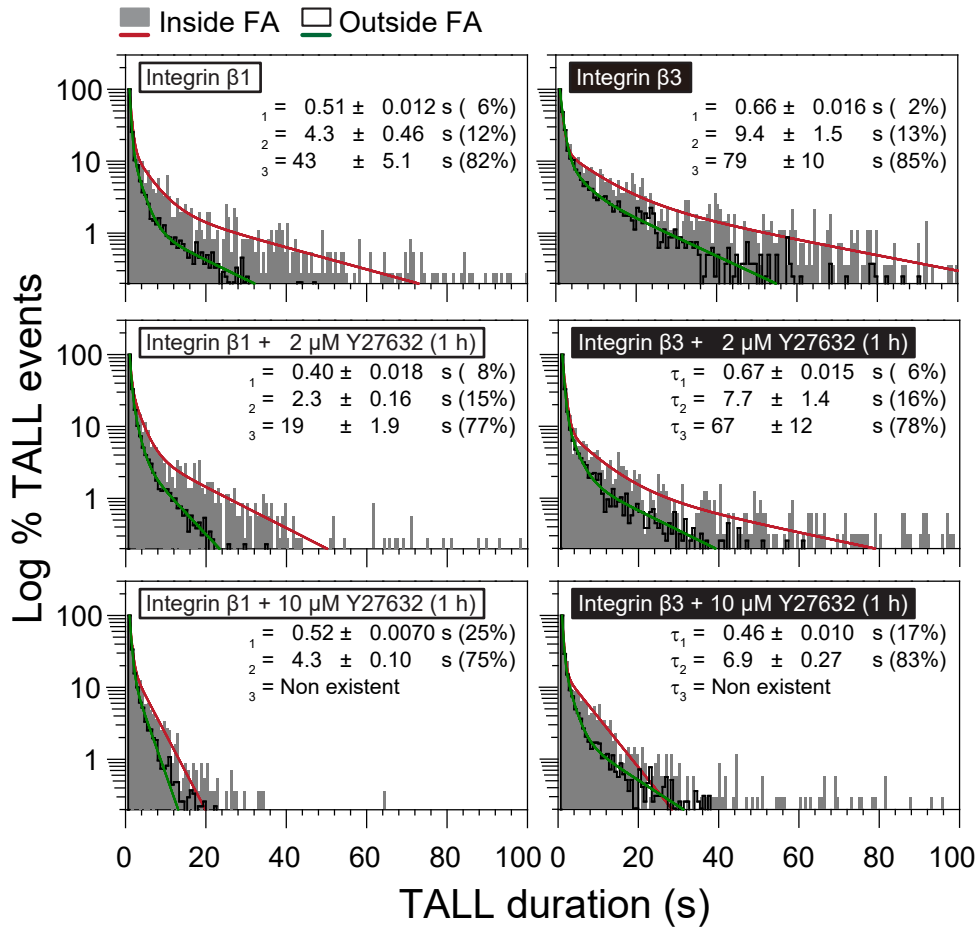
(**Open black bars and green curves**) TALLs that occurred outside the FA.

The WT-integrin $\beta 1$ and $\beta 3$ histograms indicate that virtually all TALL durations last for less than 100 s, and could operationally be fitted by the sum of three exponential functions, providing three lifetimes for the TALLs that occurred in the FA areas, of (1) < 1 s ($< 10\%$ of the entire TALL time), (2) several seconds (< 10 s; $\sim 15\%$ of the entire TALL time), and (3) ~ 40 s for integrin $\beta 1$ and ~ 80 s for $\beta 3$ ($\sim 80\%$ of the entire TALL time). To avoid over-fitting, the number of exponential functions for the fitting was optimized with Bayesian and Akaike's information criteria (**Supplementary Table 3**). These data were obtained in the HeLa-cell-PM.

The time constant of each exponential component for the TALLs that occurred inside the FA (mean) with the SEM (estimated as the fitting error of the 68.3% confidence limit) is shown in each box (after correction for the photobleaching lifetime). %Time fraction against the total TALL duration is shown in the parenthesis (for the data of outside the FA, the total number of TALL events and the total number of cells, see **Supplementary Table 2**). The reduction of the bin size from 20 frames to 2 frames only slightly increased the τ_3 lifetime, but hardly affected τ_1 and τ_2 as well as the time fractions of individual components.

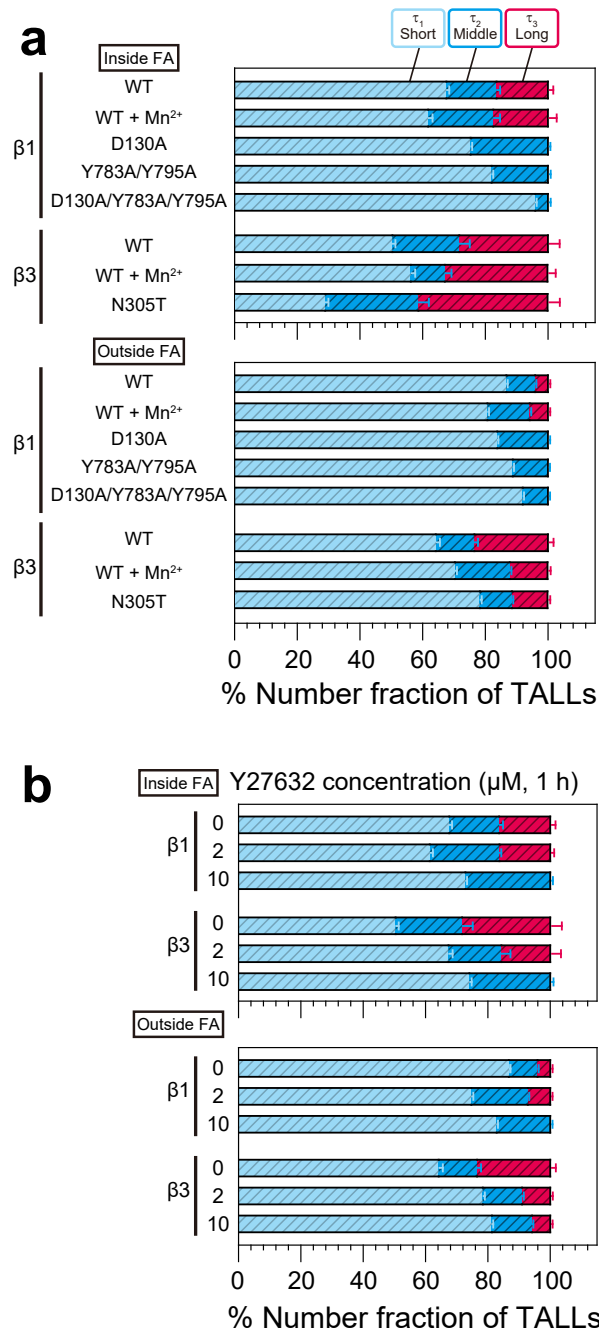
The addition of Mn^{2+} , an integrin activator, only slightly affected the TALL behaviors of integrin $\beta 1$, but its effect on the TALL behaviors of integrin $\beta 3$ was much greater.

The integrin $\beta 1$ D130A exoplasmic mutant (no RGD binding) and the Y783A/Y795A cytoplasmic mutant (no linkage to actin filaments due to the lack of binding to talin, tensin, filamin, or kindling) exhibited no τ_3 -component. The double mutant combining these two mutations (D130A/Y783A/Y795A) exhibited no τ_3 -component and only a small fraction of the τ_2 -component. These results suggest that the longest τ_3 -component was mainly induced by integrin $\beta 1$'s binding to both the extracellular matrix and actin filaments (via cytoplasmic FA components). The integrin $\beta 3$ (N305T), the N305T hybrid domain swing-out mutant of integrin $\beta 3$, exhibited a slightly elongated τ_3 , (and an increased time fraction for the τ_3 component, as shown in **Fig. 4b; Supplementary Fig. 24; Supplementary Table 2**), suggesting that the hybrid domain swing-out conformational change is important for inducing the longest TALL events of integrin $\beta 3$.



Supplementary Figure 23 | The distributions of the TALL durations of integrin $\beta 1$ and $\beta 3$ before and after the addition of the ROCK inhibitor Y27632.

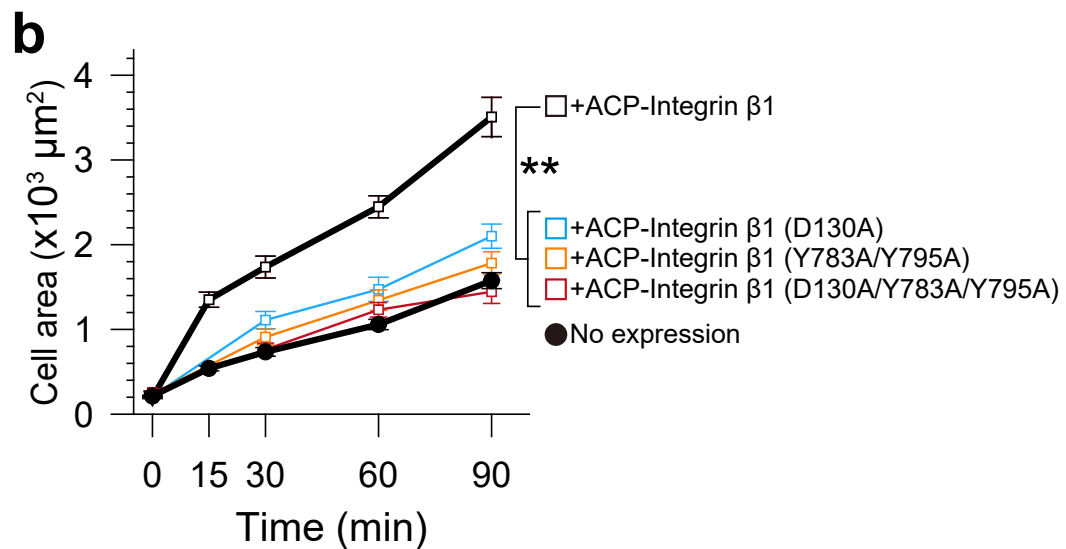
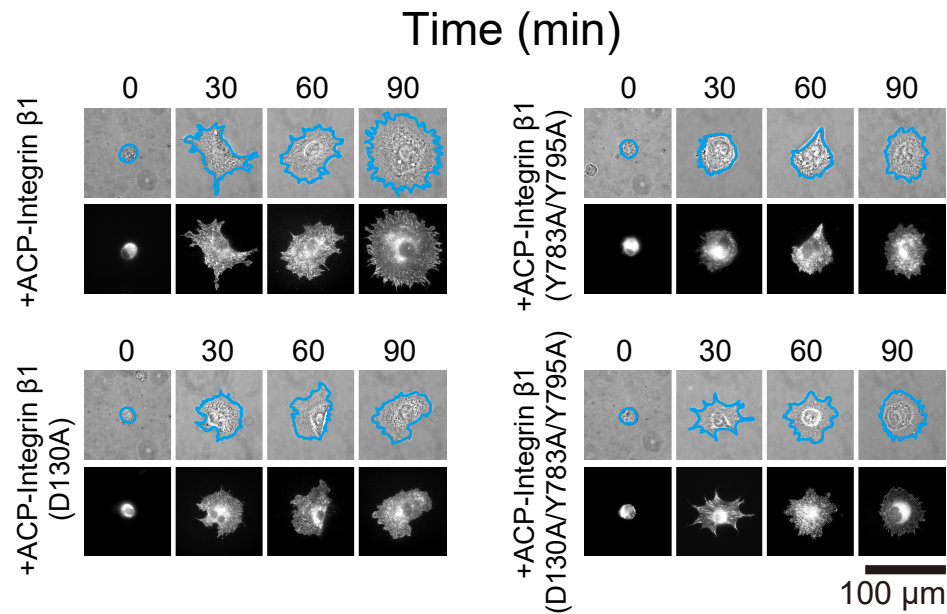
The figure arrangements and keys are the same as those of **Supplementary Fig. 22**. The treatment of cells with the ROCK inhibitor Y27632 greatly reduced the τ_3 component of integrin $\beta 3$ in a concentration-dependent manner, just as it did to the τ_3 component of integrin $\beta 1$ (**Fig. 4c**), suggesting the necessity of the myosin II-induced traction force by way of the actin filament for inducing integrin-based long-term (τ_3 periods) links between the extracellular matrix and the cytoplasmic actin filaments.



Supplementary Figure 24 | The *number* fractions (in contrast to the *time* fractions shown in **Fig. 4b, c**) of TALL occurrences (rather than TALL periods), of the short, middle, and long TALL events (τ_1 , τ_2 , and τ_3 , components, respectively), determined from the fitting of three exponential functions (**Supplementary Figs. 22 and 23**), showing that shorter TALLs occur very frequently, whereas the time fraction of the entire TALL time is dominated by the τ_3 -component (see **Fig. 4b, c**). Mean $\pm$ SEM, $n = 21$ -45 cells (for n for each condition, see **Supplementary Table 2**). Based on these observations, we propose that single integrin molecules are continually brought to their working sites (*i.e.*, their binding sites on

actin filaments and the ECM) one after another and work as a component for both the force sensor and clutch (partially consistent with previous proposals^{38,66-70}); during the short-periods of integrin binding which occurs very often, the integrin-containing molecular complexes work as a sensor for the myosin II-induced tension (as shown later) and when the integrin-containing molecular complexes sense the tension, they start working as a clutch by letting integrin undergo long-term binding to both the ECM and actin filaments for periods on the order of several 10s of seconds. The reason why many integrin molecules must be brought to the binding sites one after another is probably because keeping a single or a few molecules of unbound integrin in small areas proximal to the binding sites would be difficult.

a Integrin $\beta 1$ -KO MEF + ACP-integrin $\beta 1$ (wt and mutants) under 21%O₂

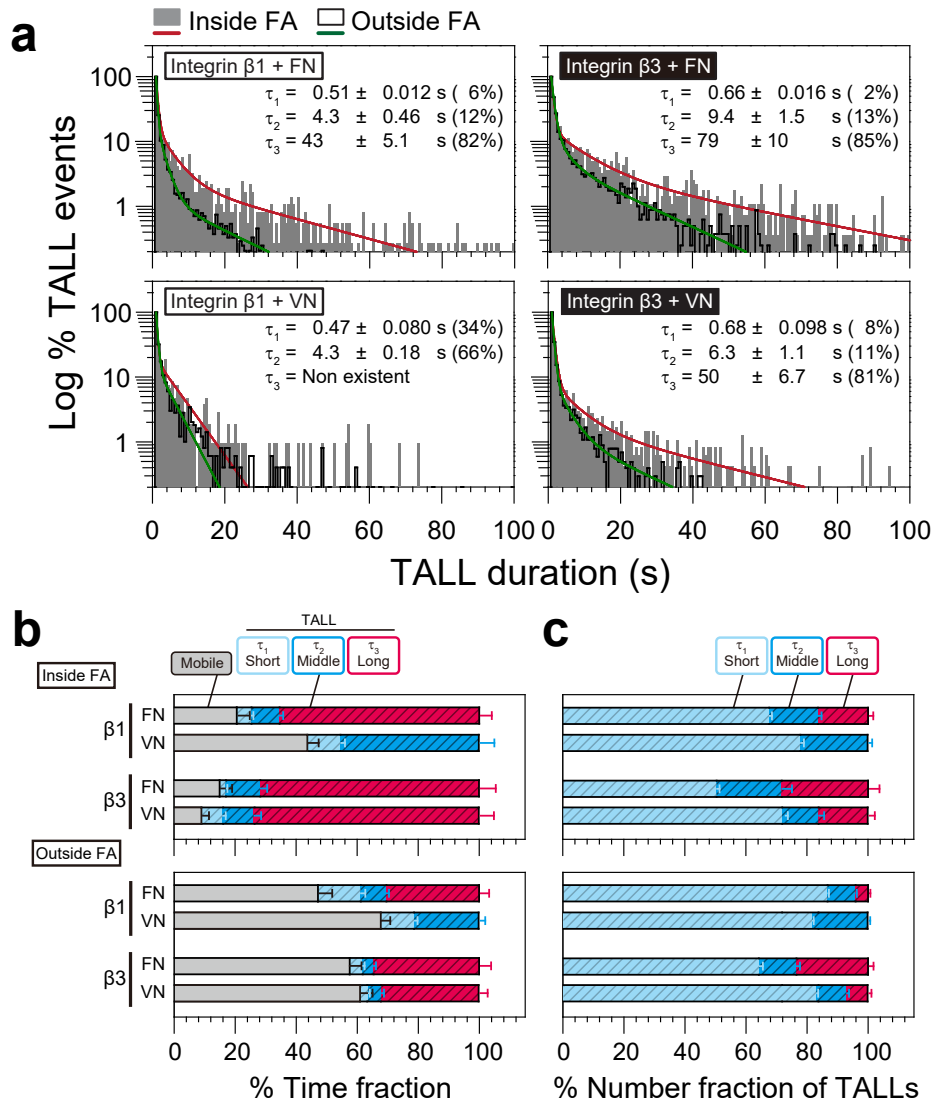


Supplementary Figure 25 | Time courses of the spreading of integrin $\beta 1$ -KO MEF cells after transfection-expression of ACP-integrin $\beta 1$ and its mutants that fail to bind to the extracellular RGD domain (D130A) and the cytoplasmic FA components, talin, tensin, filamin, and kindling (and thus no binding to actin filaments) (Y783A/Y795A), or that binding to neither of the two (D130A/Y783A/Y795A), showing that none of these mutants aided cell spreading.

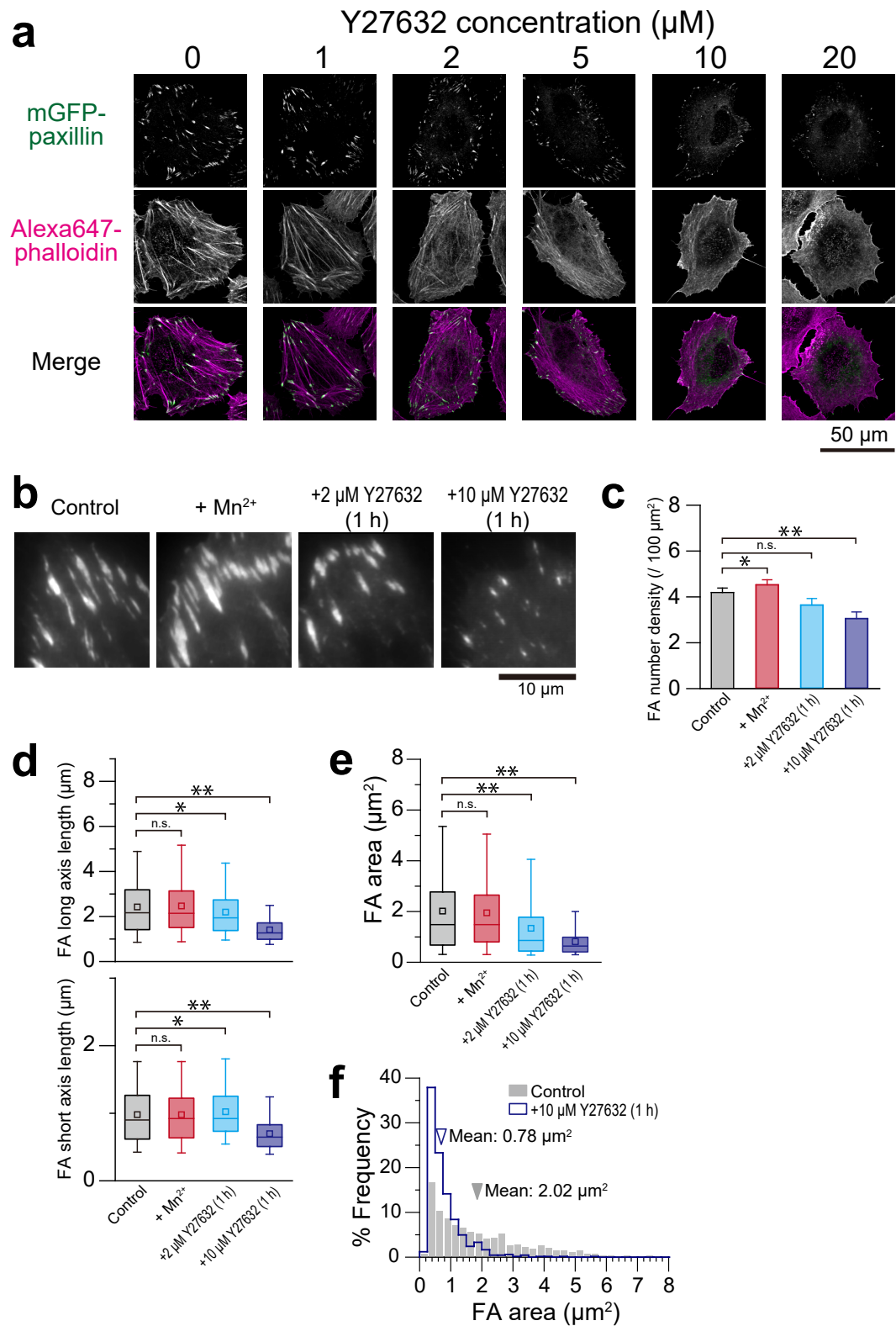
(a) Representative (among 20 examined cells) phase-contrast (**top**) and anti-integrin $\beta 1$ immunofluorescence (epi-fluorescence, **bottom**) images of integrin $\beta 1$ -KO MEFs with or

without transfection-expression of ACP-integrin $\beta 1$, at 0, 15, 30, 60 and 90 min after plating on FN-coated glass-base dishes. The anti-integrin $\beta 1$ monoclonal antibody used here (K20) can bind to both active and inactive integrin $\beta 1$. The cells with similar ACP-integrin $\beta 1$ (or its mutant) expression levels were selected for comparing the effects of expressing these molecules. Cyan lines in the phase-contrast images indicate cell peripheries, and by measuring the area within the cyan line, the cell area attached to the glass was evaluated (see **b**). Non-transfected integrin $\beta 1$ -KO MEF cells spread more slowly than transfected cells (see **Supplementary Fig. 14**).

(b) Time courses of cell spreading after plating, as measured by the cell area attached to the glass observed by phase-contrast microscopy (mean cell area $\pm$ SEM, $n = 20$ cells). Expression of integrin $\beta 1$ or ACP-integrin $\beta 1$ at similar levels enhanced cell spreading after plating, to similar extents (**Supplementary Fig. 14**). Meanwhile, the mutant ACP-integrin $\beta 1$ molecules failed to aid cell spreading (like the integrin $\beta 1$ mutants without ACP). $**P < 0.01$ (two-sided Mann–Whitney U-test; for the actual P values, see **Supplementary Table 5**). Integrin $\beta 1$ -KO MEF cells (control) without additional expression of integrin $\beta 1$ -related molecules can still spread (although slowly) probably due to the presence of other integrins, such as integrin $\beta 3^{43}$.

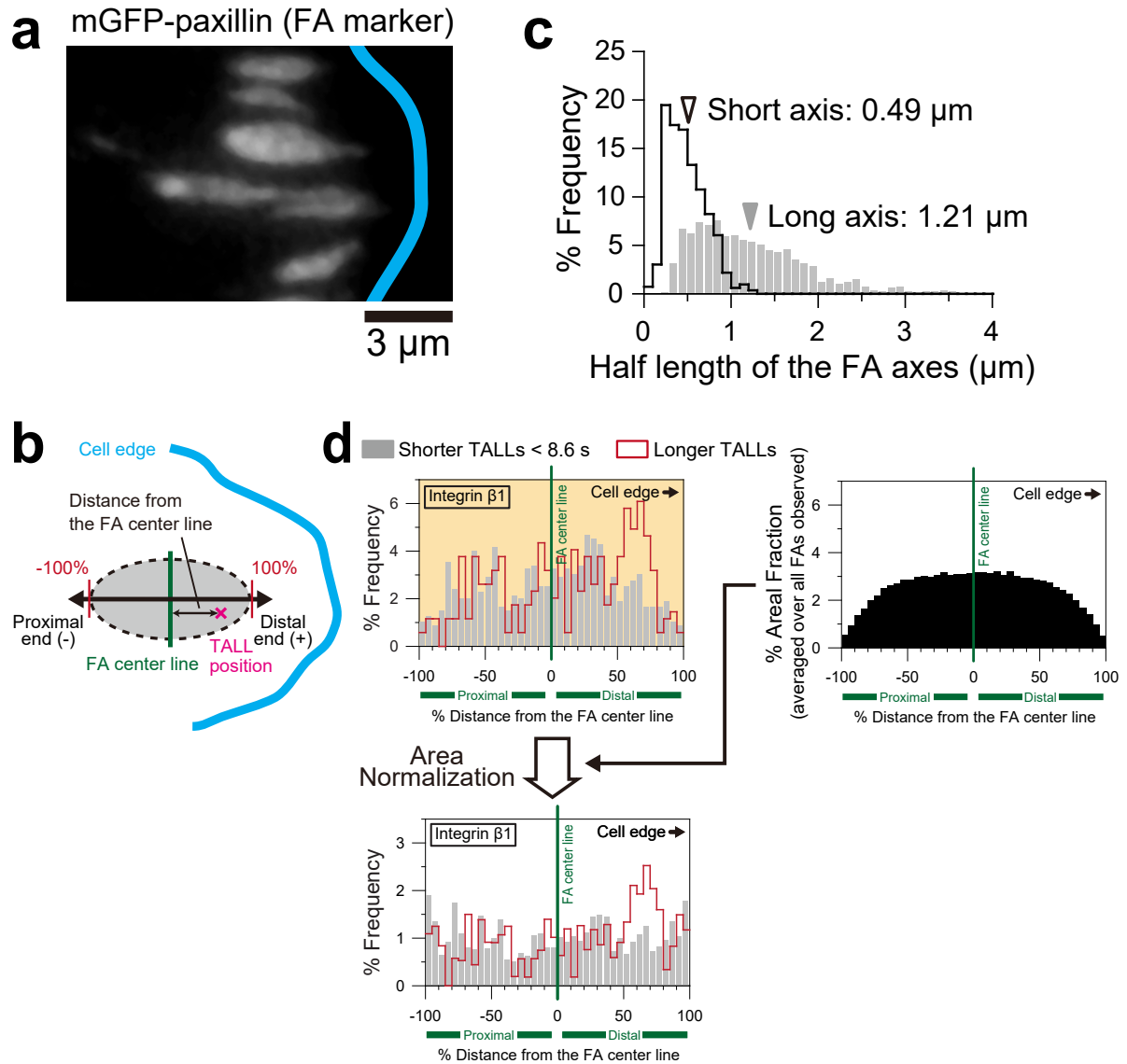


Supplementary Figure 26 | Differences between fibronectin and vitronectin coating for the TALL behaviors of integrins $\beta 1$ and $\beta 3$, in terms of **(a)** the distributions of the TALL durations, **(b)** the time fractions and **(c)** the *number* fractions of mobile periods and the three TALL periods (mean $\pm$ SEM, $n = 21$ -45 cells (for n for each experiment, see **Supplementary Table 2**). For detailed data, see **Supplementary Note 1** and **Supplementary Tables 2-4**.



Supplementary Figure 27 | Treatments with Y27632, the ROCK inhibitor, leading to the blockage of myosin II function. Y27632 reduced the number density and the size of larger FAs ($> 0.25 \mu\text{m}^2$) and modified the actin organization.

- (a)** Representative (among 15 images) confocal images of HeLa cells expressing mGFP-paxillin (as an FA marker) incubated in T-HBSS containing 0, 1, 2, 5, and 10 μM Y27632 for 1 h, followed by 4%-paraformaldehyde fixation, permeabilization, and staining with Alexa647-phalloidin (labeling actin stress fibers).
- (b)** Representative (among 30 images) TIRF images of mGFP-paxillin in HeLa cells incubated with 1 mM Mn^{2+} , or 2 or 10 μM Y27632 for 1 h.
- (c)** The number density of larger FAs ($> 0.25 \mu\text{m}^2$) evaluated from the TIRF images of mGFP-paxillin (Mean $\pm$ SEM, $n = 30$ cells). Incubation with Mn^{2+} slightly increased the FA density, whereas incubation with Y27632 slightly decreased it. The statistical tests were performed using the two-sided Welch's T test: [P for control vs. $+\text{Mn}^{2+}$, 2 μM Y27632, and 10 μM Y27632, respectively] = 0.048, 0.34, and 1.3×10^{-4} . *: $P < 0.05$, **: $P < 0.01$, n.s.: $P > 0.05$ (the same for **d** and **e**).
- (d)** Box plots of the FA long-axis (top) and short-axis (bottom) of the FAs larger than $0.25 \mu\text{m}^2$ (Mean $\pm$ SEM: $n = 828, 926, 659$, and 665 FAs for control, $+\text{Mn}^{2+}$, 2 μM Y27632, and 10 μM Y27632, respectively). Boxes extend from the 25th to 75th percentiles, with a line at the median and an internal box at the mean. Whiskers extend from the 5th to 95th percentiles. The incubation with 10 μM Y27632 for 1 h significantly decreased both the long and short axes of the FA. The statistical tests were performed using the two-sided Mann–Whitney U test: P for control vs. [$+\text{Mn}^{2+}$, 2 μM Y27632, 10 μM Y27632] = [0.75, 0.018, 4.8×10^{-10}] for the long-axis, and [0.96, 0.011, 9.0×10^{-15}] for the short-axis.
- (e)** Box plots of the areas of larger FAs ($> 0.25 \mu\text{m}^2$) after 1-h incubations with 1 mM Mn^{2+} , or 2 or 10 μM Y27632 for 1 h (the keys and the numbers of inspected FAs are the same as those in **(d)**). The statistical tests were performed using the two-sided Mann–Whitney U test: P for control vs. [$+\text{Mn}^{2+}$, 2 μM Y27632, 10 μM Y27632] = [0.96, 1.4×10^{-12} , 3.6×10^{-18}].
- (f)** Distributions of individual FA areas (FAs with areas $> 0.25 \mu\text{m}^2$) with (grey bars) and without (open blue bars) an incubation with 10 μM Y27632 for 1 h.



Supplementary Figure 28 | The method to describe the spatial distributions of the TALL locations along the FA long axis (relative to the half-axis length).

(a) A representative TIRF image of FA marked by mGFP-paxillin. The cyan curve represents the cell edge.

(b) Schematic drawing of the FA zone (the grey area surrounded by the black dashed curve) modeled as an ellipse with the short and long axes, shown together with the cell periphery (cyan curve). In the following analysis of TALL locations in FAs, each FA region was modeled as an ellipse. For this purpose, the binarized images of FAs (**Supplementary Fig. 20**) were imported into Matlab. Ellipses were fit to each isolated FA region using the "BWLABEL and REGIONPROPS" functions of Matlab, and the major and minor axes were determined^{71,72}.

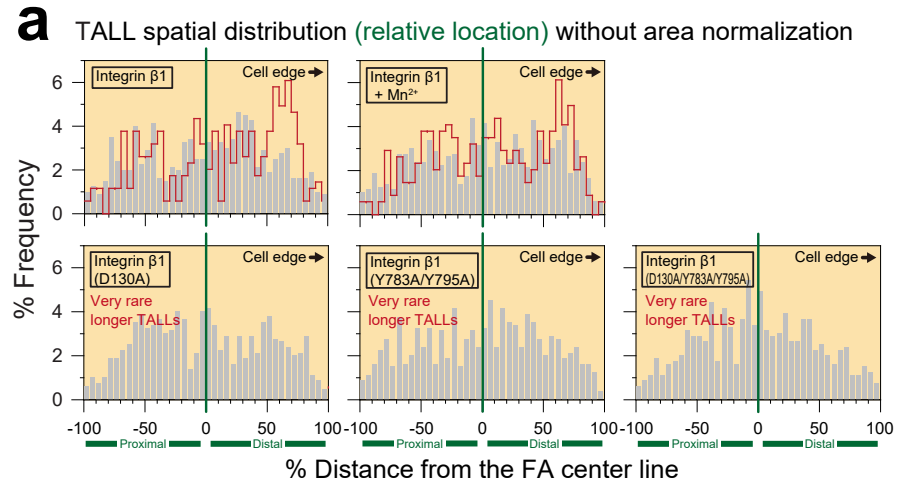
The position where an integrin molecule undergoes TALL is shown by a magenta cross, and its distance to the short axis (along the long axis) is shown by the smaller black arrow. The positive (negative) sign before the distance indicates that the TALL location is on the distal (proximal) side in the FA.

(c) Distributions of the half-lengths of FAs' long and short axes. Triangles indicate mean values. $n = 821$.

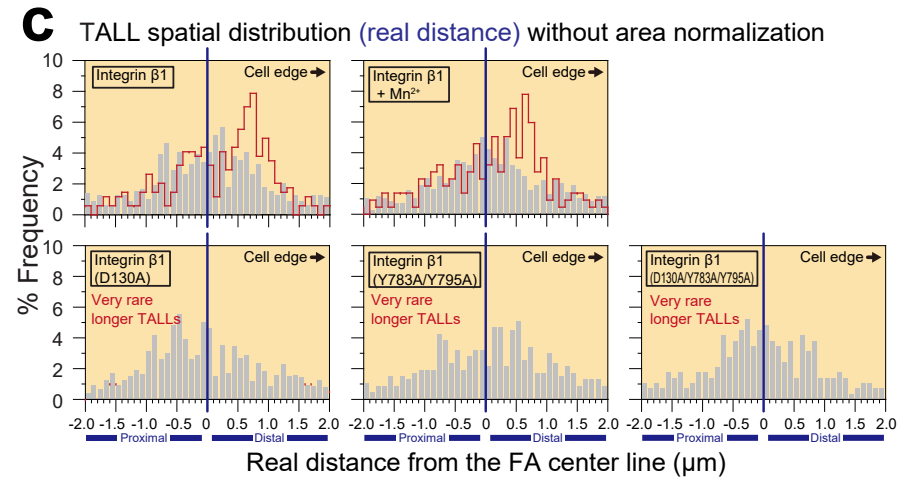
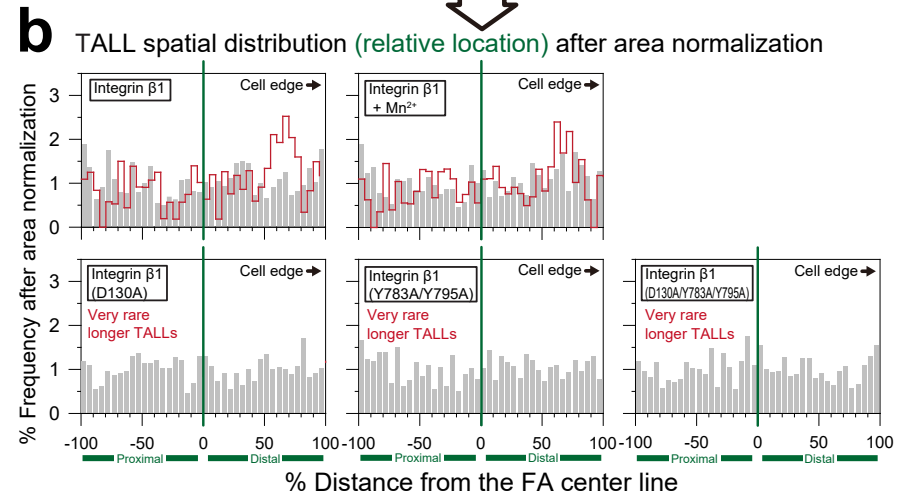
(d) (top) The spatial distribution of TALL frequency plotted as a function of the % distance along the long axis (relative to the half-length of the long axis, from the FA center line). Another useful display of the spatial TALL distribution employed here was the frequency per unit area in the FA (**bottom**), which was obtained by normalizing the original distribution (**top**) with the area size distributions (*i.e.*, against the spatially random TALL occurrences in the FA region modeled as in **b; right**).

In the present report, we show the histograms both before normalization (proportional to the actual TALL frequency at a given distance from the center line) and after normalization (proportional to the TALL frequency in a unit area at a given distance).

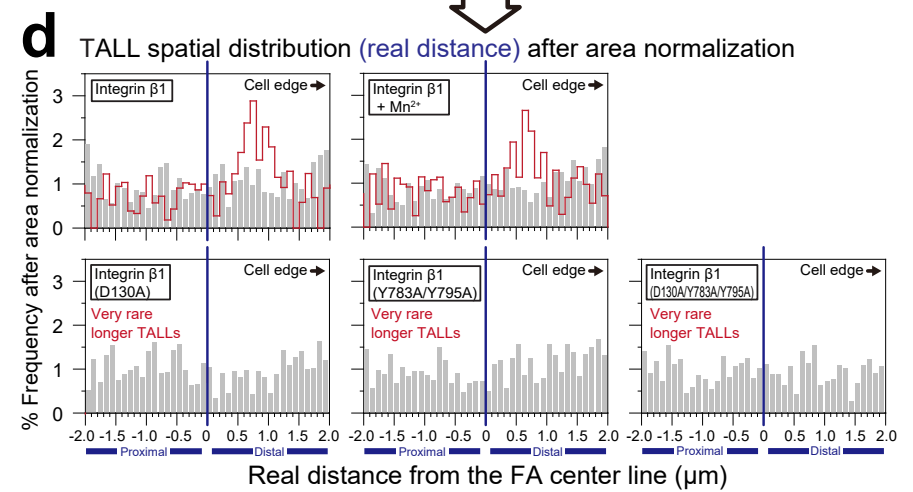
■ Shorter TALLs < 8.6 s (Integrin $\beta 1$) or 18.8 s (Integrin $\beta 3$) □ Longer TALLs



Area normalization



Area normalization

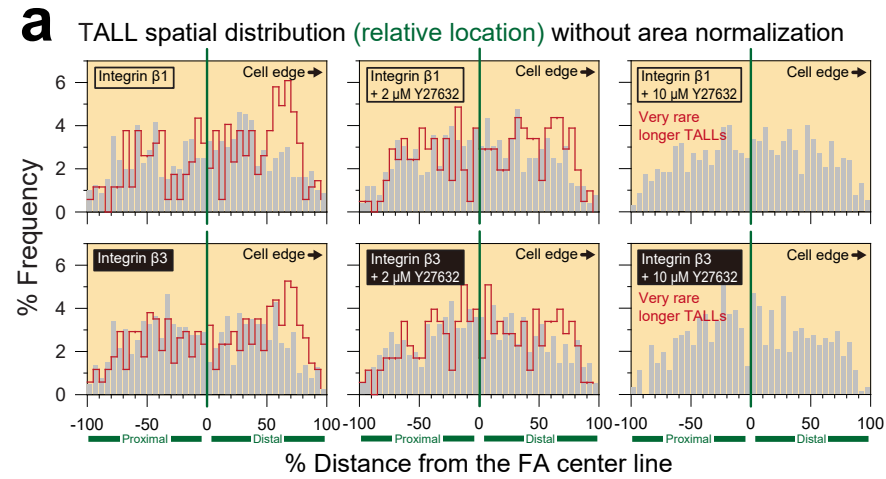


Supplementary Figure 29 | Longer TALLs of integrin $\beta 1$ most frequently occurred two-thirds of the distance away from the FA center toward the distal end, whereas three integrin $\beta 1$ mutants (no RGD binding and/or no linkage to actin filaments) rarely exhibited longer TALLs.

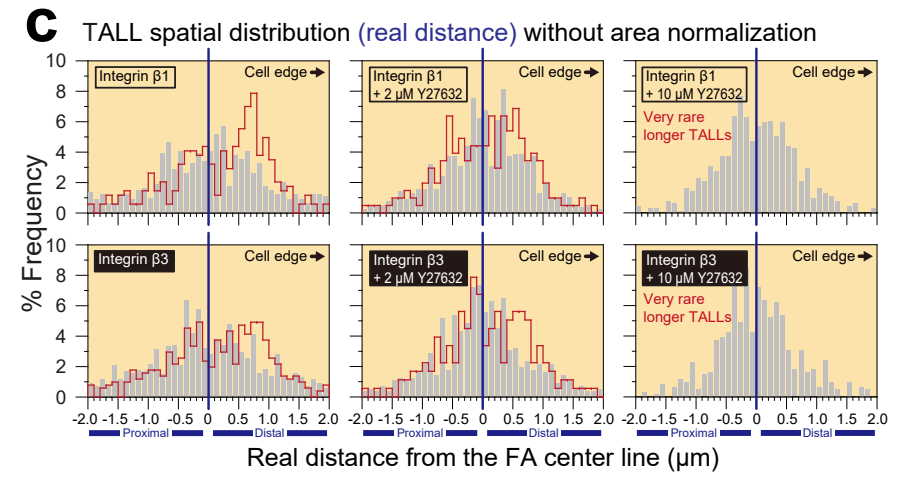
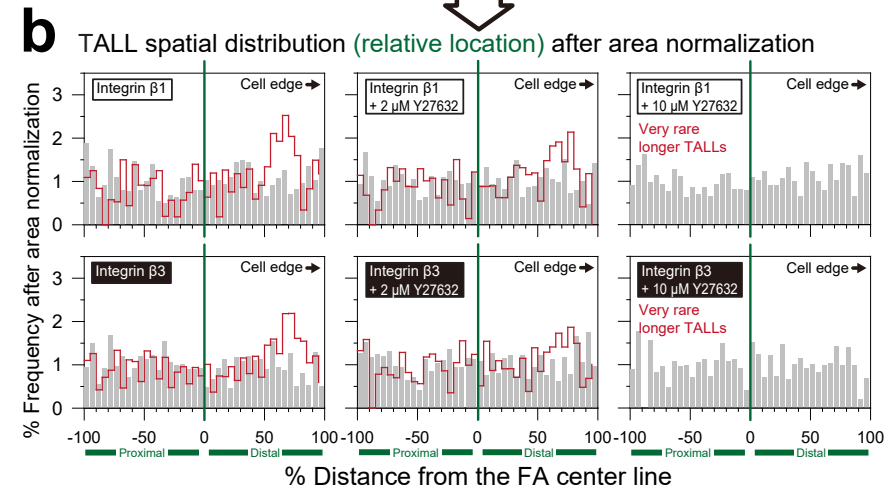
Frequency of TALLs of integrins in the FA, plotted as a function of the location along the FA long axis. The distributions without area normalization are shown in **a** and **c**, whereas **b** and **d** show the distributions after area normalization (the frequency per unit area). The x-axes in **a** and **b** indicate the distance relative to the half-length of the long axis, whereas those in **c** and **d** indicate the actual distances (with positive and negative signs) from the short axes. For the total number of TALLs examined for each molecule, see **Supplementary Table 2**.

The spatial distributions of longer TALLs of WT integrin $\beta 1$ (also after the addition of Mn^{2+}) showed a clear peak around two-thirds of the distance away from the FA center toward the distal end (or $\sim 0.7 \mu m$ away from the FA center toward the distal end). Note that these peaks were found even in the distributions without area normalization. For integrin $\beta 1$'s D130A, Y783A/Y795A, and D130A/Y783A/Y795A mutants, data of longer TALLs could not be plotted, because they hardly exhibited longer TALLs.

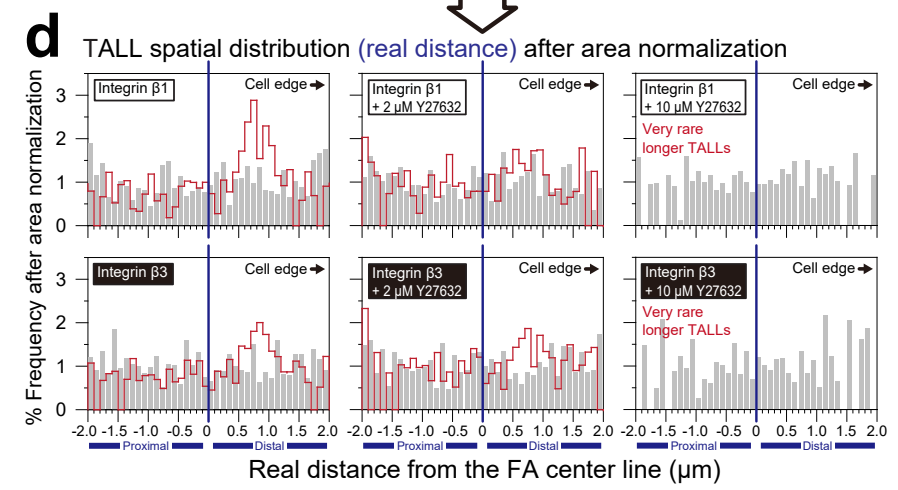
■ Shorter TALLs < 8.6 s (Integrin $\beta 1$) or 18.8 s (Integrin $\beta 3$) □ Longer TALLs



Area normalization

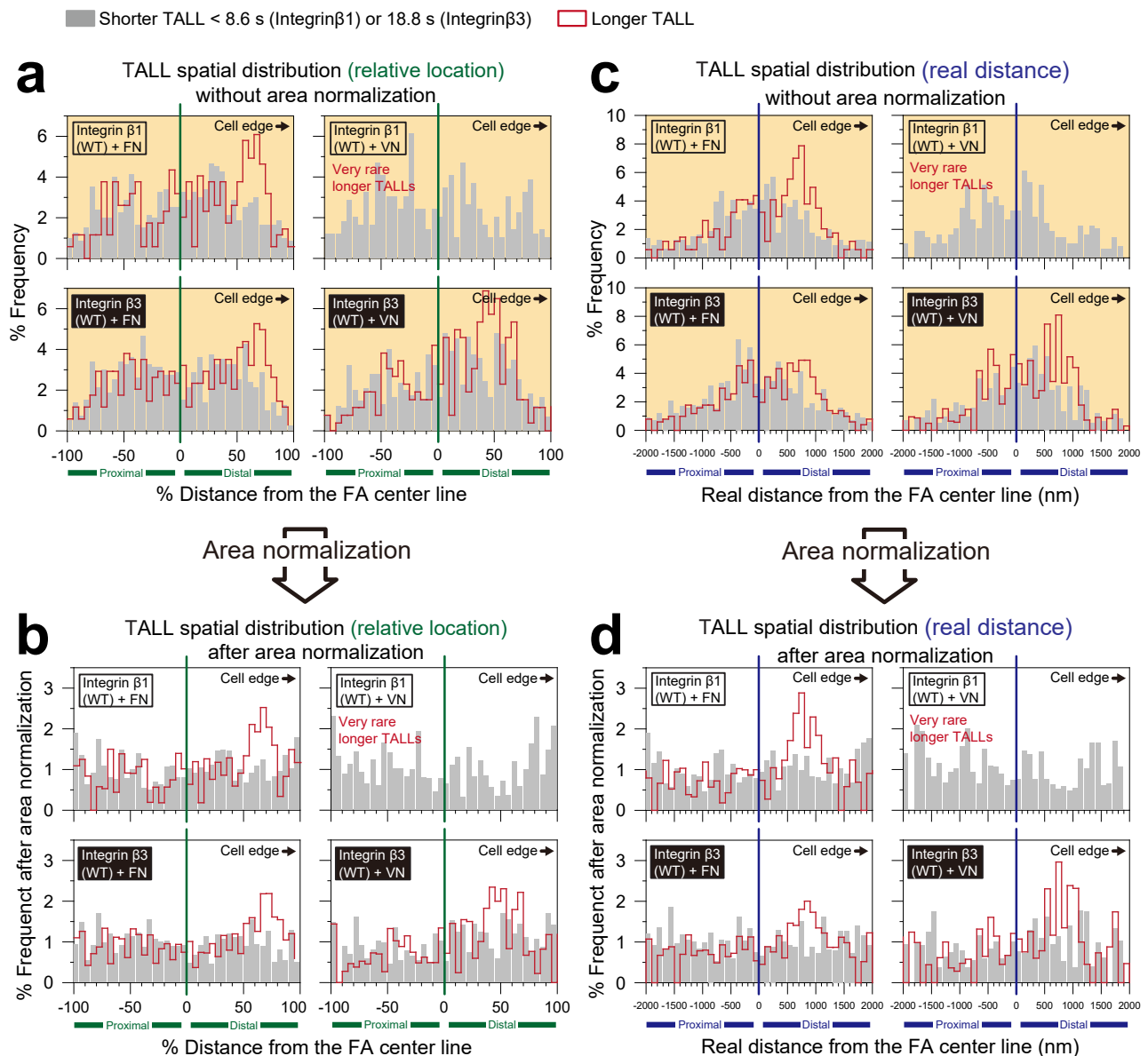


Area normalization

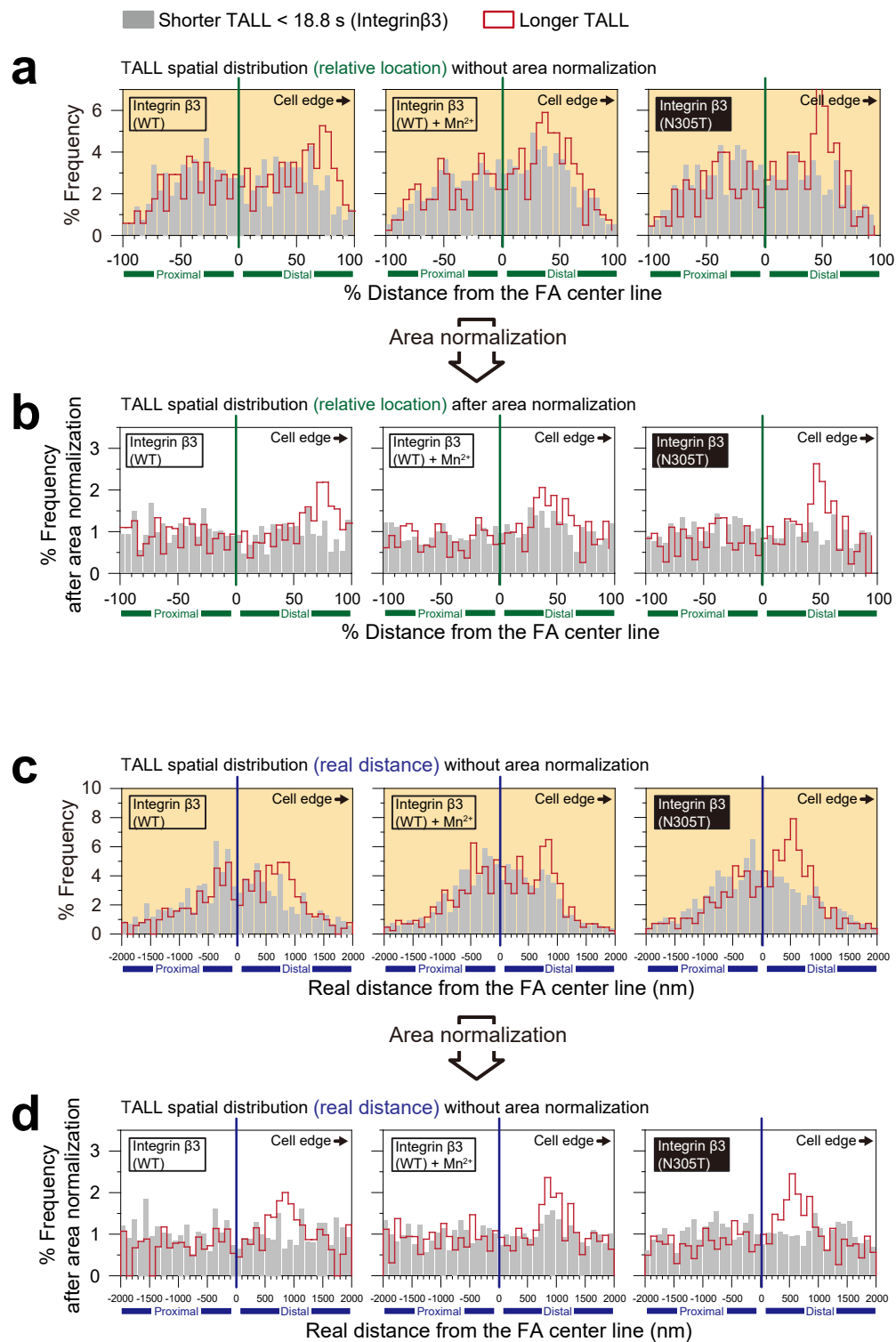


Supplementary Figure 30 | The effect of the Y27632 addition on the spatial distributions of TALLs in the FA for integrin $\beta 1$ and $\beta 3$.

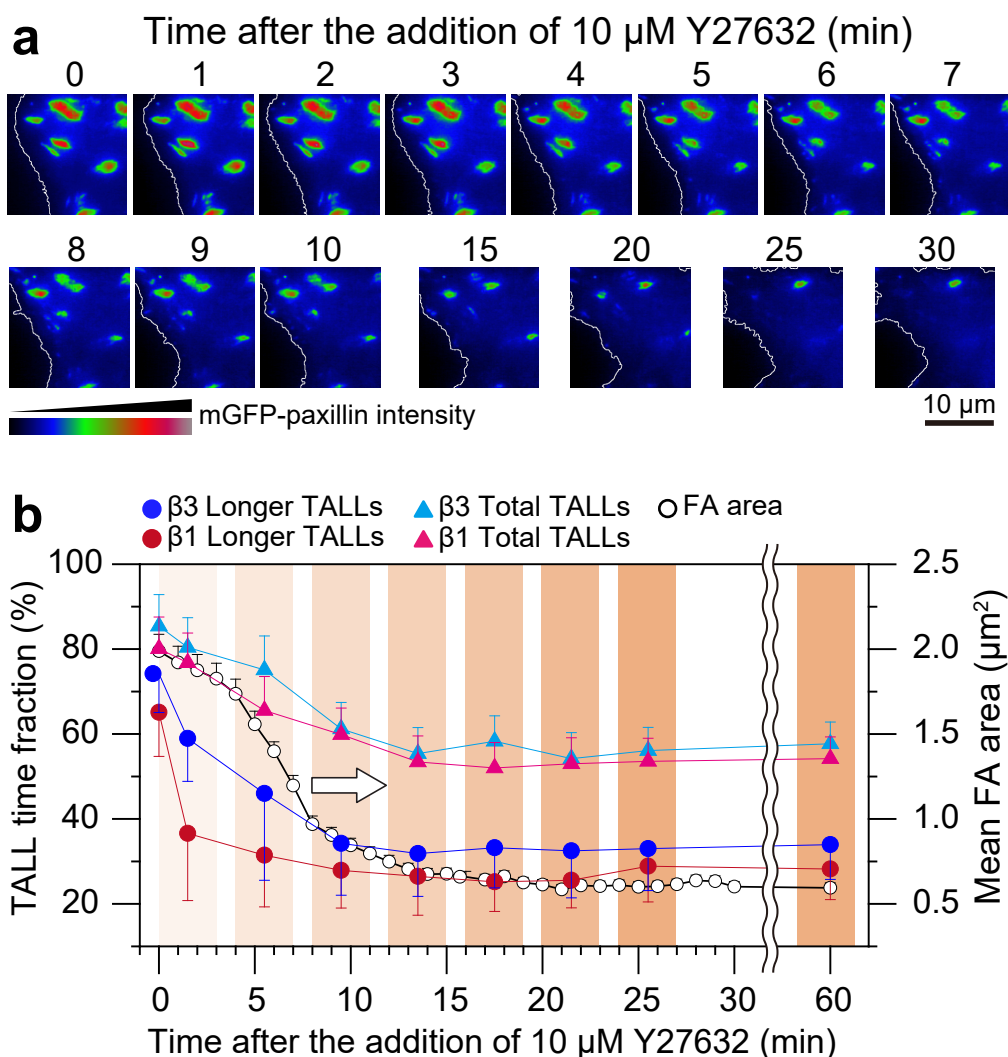
Frequencies of TALLs of integrins in the FA are plotted as a function of the location along the FA long axis. For four displays **a - d**, see the descriptions in the caption to **Supplementary Fig. 29**. For the total number of TALLs examined for each molecule, see **Supplementary Table 2**. The spatial distributions of longer TALLs of integrin $\beta 3$ showed peaks similar to those of integrin $\beta 1$. The addition of 10 μ M Y27632 almost entirely eliminated the longer TALLs.



Supplementary Figure 31 | Differences between fibronectin and vitronectin coating for the TALL behaviors of integrins β 1 and β 3, in terms of the spatial distribution of integrin's longer and shorter TALLs in the FA. For four displays **a - d**, see the descriptions in the caption to **Supplementary Fig. 29**. For the total number of TALLs examined for each molecule, see **Supplementary Table 2**.



Supplementary Figure 32 | The effect of the Mn^{2+} addition and the constitutively active N305T mutation on the spatial distributions of longer and shorter TALLs in the FA for integrin β 3. For four displays **a** - **d**, see the descriptions in the caption to **Supplementary Fig. 29**. For the total number of TALLs examined for each molecule, see **Supplementary Table 2**.

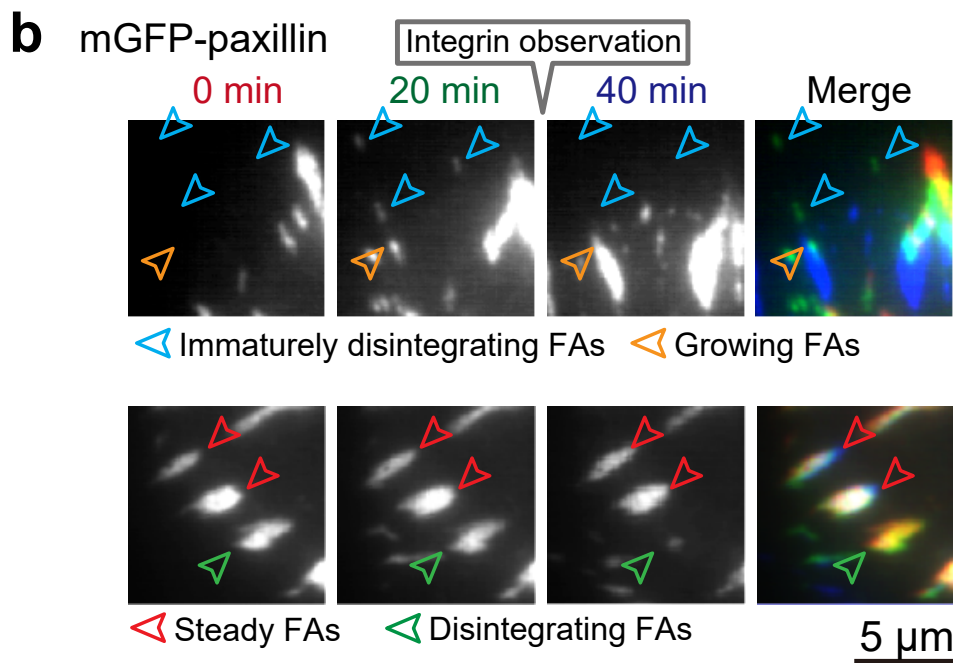
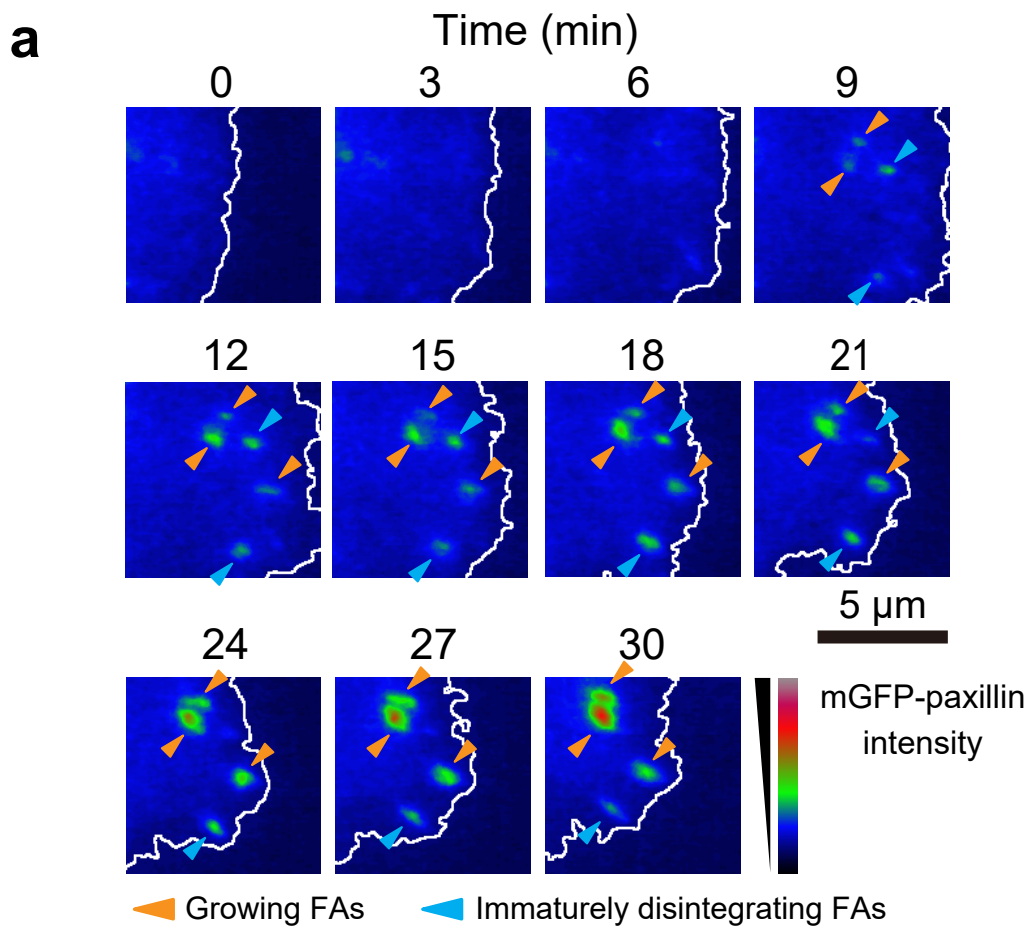


Supplementary Figure 33 | The cell treatment with 10 μM Y27632 induced a prompt and large reduction in the time fraction of integrin's longer TALLs (against the entire trajectory length), followed by a decrease of the mean FA area (visualized with mGFP-paxillin). These results suggest that integrins' longer TALLs might be important for maintaining the FA architecture.

(a) Representative time series of TIRF images of FAs visualized with mGFP-paxillin after the addition of 10 μM Y27632 (representative among 32 independent experiments). White curves represent the cell edges. See also **Supplementary Video 6**.

(b) The time courses of the time fractions of integrins' longer TALLs and total TALLs (against the entire trajectory length; see the y-axis on the left), as well as the mean FA area after the addition of 10 μM Y27632 (see the y-axis on the right). FA images were obtained every 1 min. Individual FA areas were first averaged within one cell, and then further

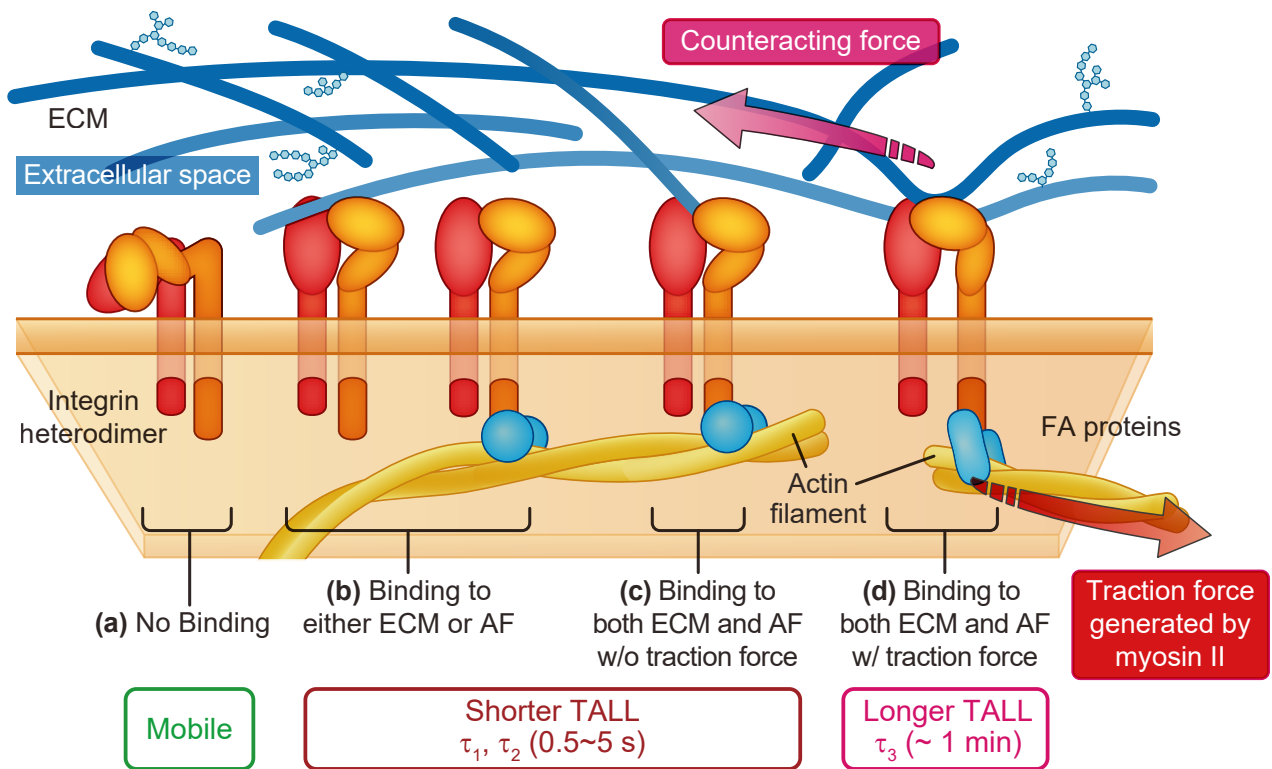
averaged over the results obtained from all of the observed cells (mean $\pm$ SEM, number of cells = 32). Integrin's trajectories were obtained during 3 min, starting from time 0, 4, 8, 12, 16, 20, 24 and 59.5 min after Y27632 addition, and the TALL time fractions were evaluated (mean $\pm$ SEM, n = 10 cells). For clarity, error bars (SEMs) are shown in only one direction.



Supplementary Figure 34 | FA turnover and dynamics.

(a) Representative (among 10 independent experiments) time-dependent changes of FAs on the basal PM visualized with mGFP-paxillin, showing that FAs form, disassemble, and slowly migrate continually in a matter of 30 min. White curves represent the cell edges. The original video is shown in **Supplementary Video 7**.

(b) Representative (among 10 independent experiments) time-lapse images of mGFP-paxillin-marked FAs. FAs were classified into larger or smaller ones, based on sizes larger or smaller than $0.25\ \mu\text{m}^2$, respectively (**Supplementary Fig. 20**). The four phases from the formation to the disintegration of FAs were defined as follows, based on the observations of FAs for 40 min: (1) growing FA, the FA that grew from a smaller FA to a larger FA (orange); (2) steady FA, the FA that was a larger FA at time 0, and remained as a larger FA (red); (3) disintegrating FA, FA that was a larger FA at time 0, and became a smaller FA or disappeared later (green); and (4) immaturely disintegrating FA, the FA that was a smaller FA at time 0, and disappeared later (blue). FAs that were smaller FAs at time 0 and remained as smaller FAs for 40 min scarcely existed, and therefore these FAs were ignored in this definition of the four phases of FA formation and disintegration.



Supplementary Figure 35 | Schematic figure showing how integrin heterodimers ($\alpha + \beta$ subunit; red and orange, respectively) link the ECM and the intracellular actin filaments.

(a) Integrins undergo diffusion in the PM, both outside and inside the FA, when they do not bind to the ECM or actin filaments (AF). **(b)** When integrins bind to either the ECM or AF, shorter TALLs of integrins (with lifetimes on the order of 0.5 - 5 s) take place. **(c)** Even when integrins bind to both the ECM and AF, the TALL lifetime do not change appreciably (the experiments employing a ROCK inhibitor Y27632). **(d)** When integrins bind to both the ECM and AF and traction force is exerted on the bound integrins, longer TALLs (with a lifetime of ~1 min) are induced. Namely, when each integrin dimer enters the FA region and binds to both the ECM and AF, and if it senses the force generated by myosin II, it mechanically couples the ECM and AF for ~1 min, and then departs from the binding site. These results indicate that the longer TALLs found in this study are the key molecular events for cell binding to the ECM.

We found that longer TALLs occur most often two-thirds of the distance away from the FA center toward the distal end. Larger traction forces would involve more frequent recruitment of integrin dimers; they would arrive one after another, each linking the ECM

and AF for periods on the order of 1 min. Since many integrin molecules would participate in crosslinking the ECM and AF, overall, the long-term linking of the ECM and AF could be achieved. This way, regulating the cell's binding to the ECM would be regulated more readily, rather than depending on long-term binding of much smaller numbers of integrin molecules. The spatial variations within the FA, in terms of the biochemical activation levels and the dynamics of vinculin and paxillin, as well as vinculin's z-axis distribution (spread toward the cytoplasm), have been reported⁷³⁻⁷⁵.

In this figure, note the three major conformations of an integrin molecule (dimers). **(a)** When integrin is not bound by ECM or AF and non-activated, the integrin dimer takes the "bent" conformation. **(b, c)** The ECM binding^{76,77}, cytoplasmic FA-protein binding (leading to the binding to AF⁷⁸), or Mn^{2+} addition^{76,79} induces conformational changes to the "stand (extended)" conformation. **(d)** When traction force is exerted on integrin, integrin further undergoes conformational changes in the hybrid head domain, from "closed" to "open" conformational changes, due to the swing out of the β subunit^{33-35,44}. Namely, two major conformational changes are required to fully activate integrins; from the inactive "bent-closed" conformation to the semi-active "stand-closed" conformation, and then to the fully active "stand-open" conformation^{33-35,44,80-82}.

pOStet15T3-SP(CD47)-SG-CD47

Kozack Signal peptide from CD47 ACP SG linker CD47 without signal peptide

gg cgc gcc ggt acc M ... A V D T G M ... A S G G G G S T S C T Q ... *
 ATG ... GCT gtc gac acc ggt ATG ... GCG AGC GGA GGA GGA GGA AGC act agt tgt aca CAG ... TAG ctg cag
 Ascl KpnI Sall AgeI SpeI BsrGI PstI

pOStet15T3-SP(IL6)-Halo7-SGX3-CD47

Kozack Signal peptide from interleukin6 Halo7 SGX3 linker CD47 without signal peptide

gg cgc gcc acc ATG ... P V D M ... G S G G G G S G G G G S G G G G T S C T Q ... *
 ATG ... CCA acc ggt ATG ... GGC TCT GGT GGC GGA GGG AGT GGC GGA GGT GGG TCA GGA GGT GGA GGC act agt tgt aca CAG ... TAG ctg cag
 Ascl AgeI SpeI BsrGI PstI

pOStet15T3-CD47-SGX3-Halo7, pEGFPN1-integrin β 1-SGX3-Halo7

Kozack CD47 or Integrin β 1 SGX3 linker Halo7

ggt acc M ... X E F C T T S S G G G G G S G G G G G S G G G G M ... *
 ATG ... XXX gaa ttc tgt aca act agt TCT GGT GGC GGA GGG AGT GGC GGA GGT GGG TCA GGA GGT GGA GGC ATG ... TAA GCG GCC GC
 KpnI EcoRI BsrGI SpeI NotI

pOStet15T3-CD47-SG-mEGFP, pEGFPN1-integrin β 1-SG-mEGFP

Kozack CD47 or Integrin β 1 SG linker mEGFP

ggt acc M ... X E F C T T S S G G G G G S M ... *
 ATG ... XXX gaa ttc tgt aca act agt AGC GGA GGA GGA GGA AGC ATG ... TAA GCG GCC GC
 KpnI EcoRI BsrGI SpeI NotI

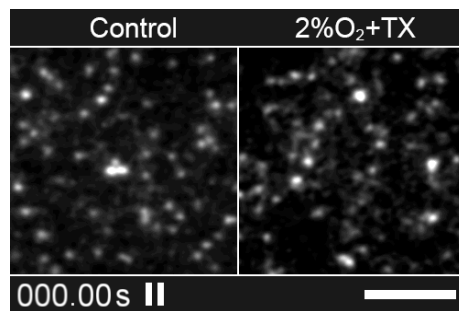
pEGFPN1-SP(CD8)-ACP-SG-integrins

Kozack Signal peptide from CD8 ACP SG linker Integrins without signal peptide

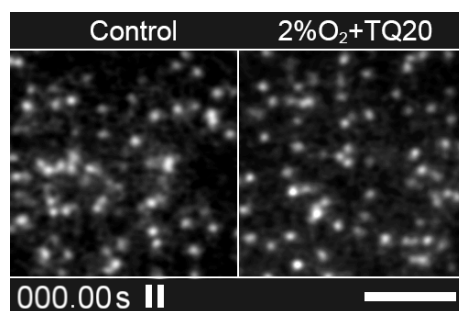
gg tac cgg cgc gcc agc ATG ... A T G M ... A S G G G G S T S R S X ... *
 ATG ... GCC acc ggt ATG ... GCG TCT GGT GGC GGA GGG AGT act agt aga tct XXX ... TAG gat atc gcg gcc gc
 KpnI Ascl AgeI SpeI BglII EcoRV NotI

Supplementary Figure 36 | Schematic structures of the parts of the cDNA constructs used in this study, showing the multiple cloning sites, inserted tag proteins, linkers, and the target proteins. See **Online Methods**.

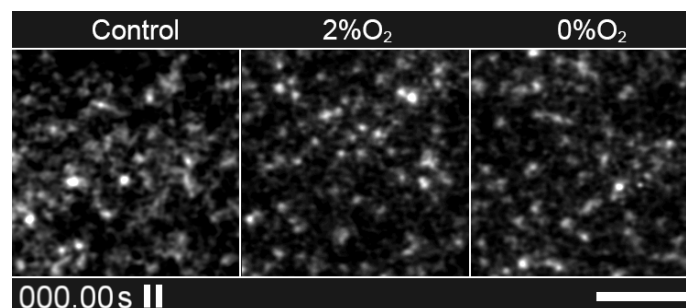
Supplementary Video Legends



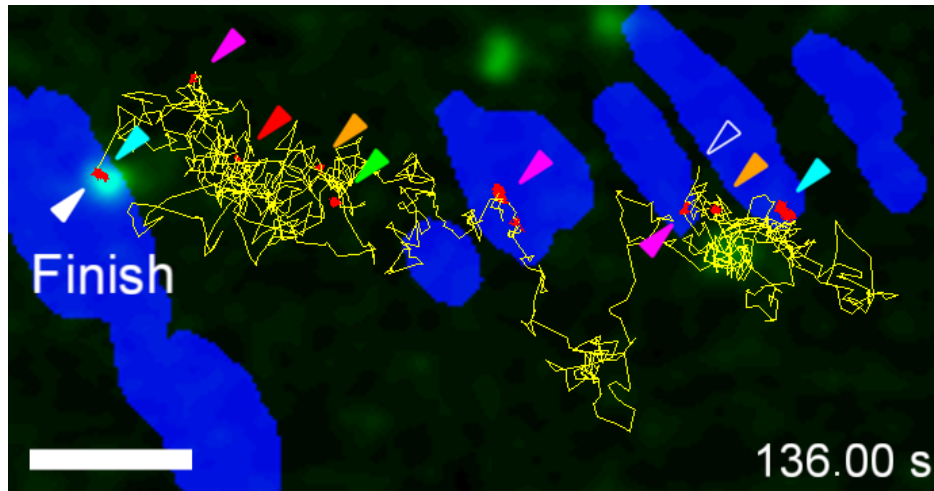
Supplementary Video 1 | Photobleaching of single TMR molecules attached to Halo-CD47 on the live-cell surface: control *vs.* 2%O₂+TX. Recorded at video rate (30 Hz) and replayed at 4x real time (the raw data for **Fig. 1a top**).



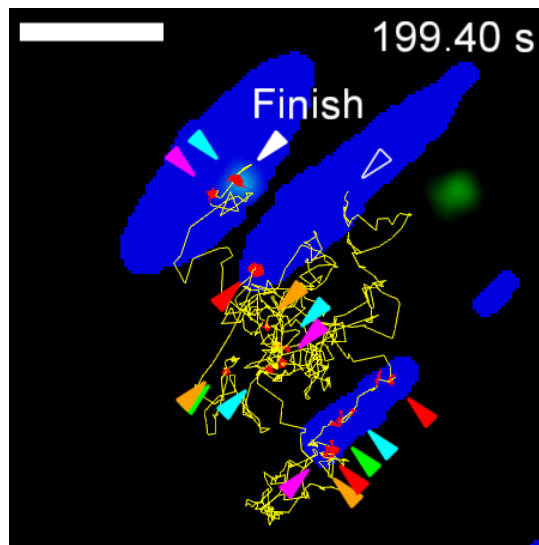
Supplementary Video 2 | Photobleaching of single ST647 molecules attached to ACP-CD47 on the live-cell surface: control *vs.* 2%O₂+TQ20. Recorded at video rate (30 Hz) and replayed at 4x real time (the raw data for **Fig. 1a bottom**).



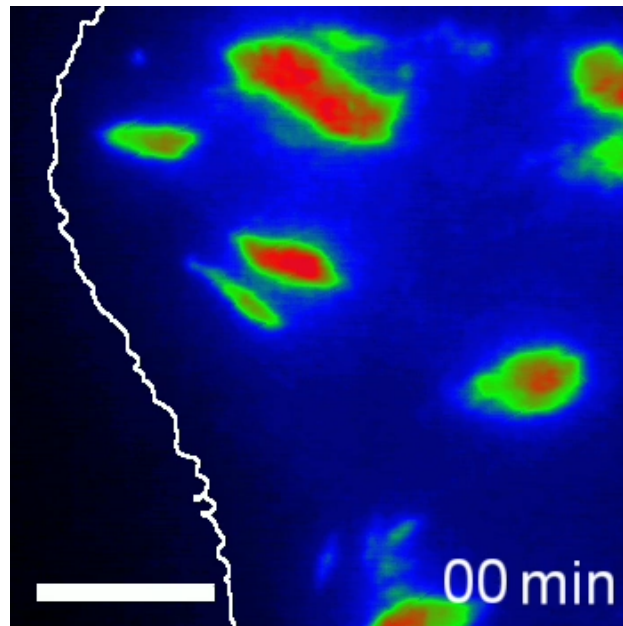
Supplementary Video 3 | Photobleaching of single Cy5-TX molecules attached to Halo-CD47 on the live-cell surface under control, 2%O₂, and 0%O₂ conditions. Recorded at video rate (30 Hz) and replayed at 4x real time (the raw data for **Supplementary Fig. 18a bottom**).



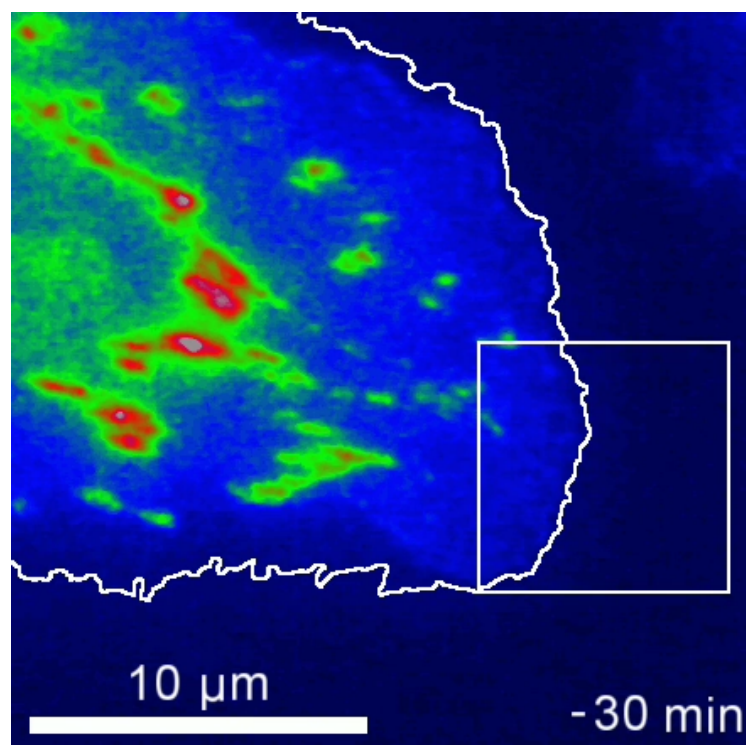
Supplementary Video 4 | Single molecules of ST647-labeled ACP-integrin $\beta 1$ (green spots) moved in and out of several FAs (blue binarized images of mGFP-paxillin), exhibiting alternating periods of TALL and thermal diffusion, both inside and outside the FAs (the raw data for **Fig. 4a left**), under 2%O₂+TQ20. A trajectory is overlaid for one of the ACP-integrin $\beta 1$ molecules.



Supplementary Video 5 | Single molecules of ST647-labeled ACP-integrin $\beta 3$, exhibiting intermittent TALLs similar to those of ACP-integrin $\beta 1$ (the raw data for **Fig. 4a right**).



Supplementary Video 6 | Time-course of FA diminution (visualized by mGFP-paxillin with color coding) after the addition of 10 μ M ROCK inhibitor Y26732 (see **Supplementary Fig. 32a** for the color scale).



Supplementary Video 7 | Time-lapse observation of FA formation and its termination near the cell periphery. FAs were labeled by mGFP-paxillin, and its signal intensity is color-coded (see **Supplementary Fig. 33a** for the color scale).

Supplementary References

(Continued from Main Text and Methods)

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Super-long single-molecule tracking reveals dynamic-anchorage-induced integrin function

Taka A. Tsunoyama, Yusuke Watanabe, Junri Goto, Kazuma Naito, Kenichi G.N. Suzuki,
Takahiro K. Fujiwara and Akihiro Kusumi

— Supplementary Note 1 —

**Theory for obtaining the correct distributions for the durations
of single-molecule events and the kinetic rate constants
in the presence of photobleaching and
under the conditions of limited recording durations**

In experiments using single fluorescent-molecule imaging and tracking, we often encounter interesting single molecule events, such as temporary arrest of lateral diffusion (TALL). To clarify and simplify the idea, in this theory part, we deal with the case where all molecules repeatedly undergo temporary simple-Brownian diffusion and TALL in the PM of living cells.

When we encounter TALL events, we often hope to evaluate the TALL lifetime or the distributions of the TALL durations by single-molecule tracking. Since we can see the behaviors of each single molecule, this evaluation might appear to be simple. However, the TALL-duration distribution measurements are often hampered by two problems: the photobleaching lifetime of the probe dye molecules and the limited durations of the image sequence recordings (from the initiation of the observation until the end of the recording of the image of the same view field, T_{rec}). In this **Supplementary Note 1** section, we develop a theory for obtaining the correct duration distributions of TALL events, by correcting the direct experimental results for photobleaching lifetimes and T_{rec} durations.

Consider many single fluorescent molecules in the PM undergoing repeated TALL events; *i.e.*, the molecules of interest are either mobile (undergoing simple-Brownian diffusion) or immobile (undergoing TALL), and they go back and forth between the mobile and immobile states. A molecule in the mobile (immobile) state can either transit to the immobile (mobile) state or become photobleached, as shown in the scheme in

Supplementary Fig. 2. To simplify the theory, we assume that the rate of every transition between any two states is described by a single kinetic rate constant (single exponential lifetime). This was shown to be correct for the photobleaching rates (k_B), for all of the fluorescent organic dye molecules examined in this study. Simply put, the aim of this theory is to obtain the rate constant for the transition from the immobile state to the mobile state, k_M , using single-molecule tracking. For this scheme to be adequate, we assume that the immobilizing domain is very small and all molecules in the immobilizing domain can face the PM bulk region within the image integration time (camera frame time). Under these conditions, we can also assume that a single rate constant for the transition from the mobile state to the immobile state (k_I) can be defined, which depends on the average time until the

molecules encounter the immobilizing small domains (and thus depends on the diffusion coefficient during the mobile period).

If no photobleaching of the fluorescent probes occurred and if the observation periods were much longer than the TALL durations, then the experimentally obtained TALL duration distribution would require no corrections. However, under the normal single-molecule observation conditions employed for live cells, photobleaching commonly occurs on the orders of 1-10 s, and the total duration of single-molecule observations on a single view field, T_{rec} , is quite limited, and normally less than a few 100 s.

First, we will explain how photobleaching affects the experimentally-observed distribution of individual TALL durations. The photobleaching of an ensemble of molecules quite often follows an exponential time course, as shown for all of the molecules employed in this study. Therefore, the photobleaching of each single molecule occurs stochastically, following the exponential time course. This means that in single-molecule tracking, the time window provided by each molecule will have a single exponential distribution, and thus when molecular events are observed by single-molecule tracking, it means that the events are observed through various time windows with single-exponential lifetime distributions. Therefore, to obtain the correct duration distribution of TALL events, this skewed lifetime distribution viewed through the looking glass of single molecules must be corrected.

Second, we will explain how the limited recording (observation) period, T_{rec} , influences the experimentally-observed distribution of individual TALL durations (**Supplementary Fig. 37**). This is because a large fraction of observed molecules might already be undergoing TALL at time 0, and those that start undergoing TALL during the observation period might still be undergoing TALL at the end of the observation period. Namely, the observed TALL events would include those with truncations at the beginning and ending portions of the TALL events, and this would prevent us from directly evaluating the TALL periods from the single-molecule tracking data.

In the following, we consider how these two effects can be taken into account for correctly obtaining the TALL duration distributions and the rate constants for the transition

from the immobile state to the mobile state, k_M . We also show that this evaluation is possible in the presence of an additional long-term immobilized component, with a lifetime much longer than T_{rec} .

Experimentally, we evaluate the TALL durations in the following way. First, we choose the field of view of the cell, using minimum irradiation to avoid photobleaching. Then, we start (time 0) recording a single-molecule movie, which often includes ~ 100 fluorescent spots. The observation lasts for a period called T_{rec} , which we simply call the “observation duration”. In this definition, T_{rec} represents the time at which a single observation (a recording of consecutive images) ends.

Second, these recordings are repeated many times (M times), and each time, we select a different view field (in the same cell culture dish or in a different cell culture dish), but we assume that the recording (observation) duration T_{rec} is the same for all of the recording sets. The observed duration for each molecule could be much shorter due to photobleaching, but T_{rec} simply represents the period in which a series of consecutive images (a movie) on a single view field is recorded. Therefore, the total time spent to finish a series of experiments will be $M \times T_{\text{rec}}$.

Third, we track all of the observed fluorescent spots and obtain their trajectories (neglecting fluorescent spots observed for periods shorter than 21 frames, this value is represented as T_{min} [in the case of video-rate observations, this time is 0.70 s]) and extract all of the TALL events found in all of the trajectories. This minimal length of the trajectory (21 frames) is required to define a TALL event. Here, we define another parameter, T_{total} , as “the sum of the time lengths of all of the trajectories (in time)” in an entire set of experiments. Note that $M \times T_{\text{rec}}$ should not be confused with T_{total} , as the former simply represents the total time of observations spent for a set of experiments, whereas the latter represents the length of all single-molecule trajectories obtained in an entire set of experiments (in the unit of seconds).

Fourth, we measure the duration of every TALL event and obtain the experimental histogram of TALL durations, without correction for photobleaching and the limited T_{rec} effect.

In this theory, we describe a method for correcting this raw distribution skewed by the limited photobleaching lifetime and T_{rec} , and for obtaining k_M , following the scheme shown in **Supplementary Fig. 2**.

(1) The equilibrium between mobile molecules and molecules undergoing TALL

In the scheme shown in **Supplementary Fig. 2**, a molecule in the mobile (immobile) state can either transit to the immobile (mobile) state or become photobleached. The time-dependent changes of the numbers of molecules in the immobile and mobile states, $N_I(t)$ and $N_M(t)$, respectively, are described by using the rate constants for the transition to the mobile and immobile states and photobleaching, k_M ($= 1/\tau$, where τ is the TALL lifetime), k_I , and k_B ($= 1/\tau_B$, where τ_B is the photobleaching lifetime), respectively, as

$$\frac{dN_I(t)}{dt} = -(k_M + k_B)N_I(t) + k_I N_M(t) \quad (1)$$

$$\frac{dN_M(t)}{dt} = -(k_I + k_B)N_M(t) + k_M N_I(t) . \quad (2)$$

Note that, in the present report, the numbers of molecules are not natural numbers, but the real numbers. In each recording, the numbers of molecules were counted, but after repeating the experiments M times, the average number will become the real number.

In the present report, time 0 ($t = 0$) is set at the time when the observation was initiated. We describe the numbers of fluorescent molecules in the immobile and mobile states at time 0 as N_{I0} and N_{M0} , respectively, and the total number of fluorescent molecules as N_0 i.e.,

$$N_I(0) = N_{I0} \quad (3)$$

$$N_M(0) = N_{M0} \quad (4)$$

$$N_0 = N_{I0} + N_{M0} . \quad (5)$$

Eqs. 1 and 2 are solved and $N_I(t)$ and $N_M(t)$ are written as

$$N_I(t) = \frac{k_I N_{M0} + k_I N_{I0}}{k_I + k_M} \exp(-k_B t) - \frac{k_I N_{M0} - k_M N_{I0}}{k_I + k_M} \exp[-(k_I + k_M + k_B) \cdot t] \quad (6)$$

$$N_M(t) = \frac{k_M N_{M0} + k_M N_{I0}}{k_I + k_M} \exp(-k_B t) + \frac{k_I N_{M0} - k_M N_{I0}}{k_I + k_M} \exp[-(k_I + k_M + k_B) \cdot t] . \quad (7)$$

At $t = 0$, the numbers of molecules in the mobile and immobile states are considered to be in equilibrium, *i.e.*,

$$k_M N_{I0} = k_I N_{M0} . \quad (8)$$

Combining Eqs. 5 and 8, N_{I0} and N_{M0} can be expressed as

$$N_{I0} = N_0 k_I / (k_I + k_M) \quad (9)$$

$$N_{M0} = N_0 k_M / (k_I + k_M) . \quad (10)$$

Using this relationship, Eqs. 6 and 7 can be written as

$$N_I(t) = \frac{k_I N_0}{k_I + k_M} \exp(-k_B t) \quad (11)$$

$$N_M(t) = \frac{k_M N_0}{k_I + k_M} \exp(-k_B t) . \quad (12)$$

Therefore, the following important relationship is obtained:

$$k_M N_I(t) = k_I N_M(t) . \quad (13)$$

Namely, if the numbers of molecules in the mobile and immobile states are in equilibrium at $t = 0$, and if the photobleaching rates are the same for both mobile and immobile molecules (which will generally be true), then the numbers of molecules that are still fluorescent (non-photobleached) at (any) time t in the mobile and immobile states are also in equilibrium.

(2) New definitions of the end of a TALL event and the TALL lifetime

In this report, the end of a TALL event is defined as either when a molecule in the immobile state transits to the mobile state (when it resumes diffusion) or when it is photobleached. Note that in this definition, the end of TALL is determined by either the molecule's restarting of motion or photobleaching, whichever occurred first, and that therefore this definition is different from our previous definition. In our previous reports, the end of a TALL event was solely defined by the restarting of the molecule's motion, and when photobleaching occurred before diffusion resumed, we did not measure the immobile duration. The effect of not including such TALL events was corrected, by using the photobleaching lifetime. However, as shown later, this correction method is only correct when the photobleaching lifetime and the TALL lifetime are much shorter than the recording (observation) duration (T_{rec}). In the present research, because we succeeded in substantially prolonging the photobleaching lifetime, the recording duration T_{rec} might not be much longer than the photobleaching lifetime. Therefore, to obtain the correct distribution of TALL durations and thus to evaluate the rate constant for the transition from the immobile state to the mobile state, k_M , we had to explicitly include both the effects of the photobleaching kinetics (with the kinetic constant k_B) and the limited recording duration (T_{rec}) in the theory. For this purpose, experimentally, we should measure the durations of all TALL events, regardless of whether they ended by photobleaching or by the resumption of thermal diffusion. The final conclusion is that the previous treatment is correct when the photobleaching lifetime is substantially shorter than the recording duration ($1/k_B < T_{\text{rec}}/2.4$), and that even when $1/k_B \sim T_{\text{rec}}$, if the TALL lifetime is substantially ($\sim 3\times$) shorter than the photobleaching lifetime $1/k_B$, then the influence of the limited recording time (T_{rec}) is limited.

(3) The distribution of TALL durations (probability density function)

Using the new definition of the end of a TALL event, the probability density function of TALL duration [$P(\delta)$] is described using k_M and k_B (δ represents the TALL duration; $P(\delta)$ represents the probability density function without considering T_{rec} and T_{min}).

$$P(\delta) \propto \exp[-(k_B + k_M) \cdot \delta] . \quad (14)$$

The proportionality coefficient for this function is determined by normalization (because it is a probability density function)

$$P(\delta) = (k_B + k_M) \exp[-(k_B + k_M) \cdot \delta] . \quad (15)$$

Since the observation duration in which single-molecule imaging-tracking is conducted is limited (T_{rec} is not infinitely long), we consider the number of molecules that start TALL events (become immobile) at time t , $F_S(t)$. $F_S(t)$ is described by k_I and $N_M(t)$ as

$$F_S(t) = k_I N_M(t) . \quad (16)$$

Using Eq. 12, Eq. 16 is expressed as

$$F_S(t) = \frac{k_I k_M N_0}{k_I + k_M} \exp(-k_B t) . \quad (17)$$

The TALL events that occur during the observation duration between $t = 0$ and T_{rec} are classified into three cases, because TALLs might (a) be totally included within the observation period, but they also might (b) start after time 0 (at $t > 0$) and continue after the observation period, and (c) start at $t \leq 0$ before the observation period (see **Supplementary Fig. 37**). For simplicity, we neglect the TALLs that started before the observation and did not end during the observation period. If this frequently occurs, then the observation duration should be extended, perhaps by using time-lapse observations.

(Case a) For TALLs that were totally included within the observation period ($0 < t \leq T_{\text{rec}}$); the probability density function $G_a(\delta)$

Here we consider the duration (δ) distribution of TALLs (probability density function of δ) that started after $t = 0$ and ended before $t = T_{\text{rec}}$ (defined as $G_a(\delta)$; see “Case a” in **Supplementary Fig. 37**). When T_{rec} is very long, the number of TALL events that last for a duration δ can be expressed as

$$G(\delta) = \int_0^\infty F_S(t) \cdot dt \cdot P(\delta) . \quad (18)$$

Note that G is expressed as the number of TALLs/time, providing the distribution as a function of time δ (the height of each bar [the y-axis] in the histogram provides the number of occurrences per the bin size (in time), and so the dimensions of Eq. 18 are correct. This will apply to all of the histograms shown here). In the analysis of the experimental results, we usually obtain a histogram of the duration (δ), which we generally define as $H(\delta)$. $H(\delta)$ is simply a function obtained by multiplying the histogram's bin size Δt with $G(\delta)$.

Here we consider the case where T_{rec} is limited, and the molecules are mobile at $t = T_{\text{rec}}$. In this case, the upper end of the integration must be limited to $T_{\text{rec}} - \delta$; *i.e.*,

$$G_a(\delta) = \int_0^{T_{\text{rec}} - \delta} F_S(t) \cdot dt \cdot P(\delta) . \quad (19)$$

Using Eqs. 15 and 17, followed by integration, G_a is expressed as

$$G_a(\delta) = \frac{k_I k_M (k_B + k_M) N_0}{k_B (k_I + k_M)} \exp[-(k_B + k_M) \cdot \delta] - \frac{k_I k_M (k_B + k_M) N_0}{k_B (k_I + k_M)} \exp(-k_B T_{\text{rec}}) \exp(-k_M \delta) . \quad (20)$$

(Case b) For TALLs that started after time 0 (at $t > 0$) and continued after the observation period; the probability density function $G_b(\delta)$

Here, we consider the TALLs that initiated after $t = T_{\text{rec}} - \delta$ and did not end by $t = T_{\text{rec}}$ (*i.e.*, the TALL duration must be longer than δ ; see "Case b" in **Supplementary Fig. 37**). The duration (δ) distribution of the TALLs for this case (probability density function of δ), defined as $G_b(\delta)$, can be calculated as

$$G_b(\delta) = F_S(T_{\text{rec}} - \delta) \cdot \int_{\delta}^{\infty} P(\tau) \cdot d\tau . \quad (21)$$

Using Eqs. 15 and 17, followed by integration, $G_b(\delta)$ is expressed as

$$G_b(\delta) = \frac{k_I k_M N_0}{k_I + k_M} \exp(-k_B T_{\text{rec}}) \exp(-k_M \delta) . \quad (22)$$

(Case c) For TALLs that started at $t \leq 0$; the probability density functions G_c and $G_{\text{AATI}}(\delta)$

See “Case c” in **Supplementary Fig. 37**. First, we derive the duration (δ) distribution of TALLs (probability density function $G_c(\delta)$) in the case where a fluorescent molecule is immobile at $t = 0$ and becomes mobile at time δ (one of the cases of Case c). For this purpose, we consider the number of fluorescent molecules that are immobile at $t = 0$ and are still immobile at time t , and call it $N_I^0(t)$. The time-dependent changes of $N_I^0(t)$ are expressed as

$$\frac{dN_I^0(t)}{dt} = -(k_B + k_M)N_I^0(t) . \quad (23)$$

With the initial value N_{I0} (Eq. 3), the solution of Eq. 23 can be expressed as

$$N_I^0(t) = N_{I0} \exp[-(k_B + k_M) \cdot t] . \quad (24)$$

$$N_I^0(t) = \frac{k_I N_0}{k_I + k_M} \exp[-(k_B + k_M) \cdot t] . \quad (25)$$

$G_c(\delta)$ can be written as

$$G_c(\delta) = k_M N_I^0(\delta) . \quad (26)$$

Using Eq. 25, $G_c(\delta)$ can be expressed as

$$G_c(\delta) = \frac{k_M k_I N_0}{k_I + k_M} \exp[-(k_B + k_M) \cdot \delta] . \quad (27)$$

Second, we derive the duration (δ) distribution of TALLs (probability density function) in the case where a fluorescent molecule is immobile at $t = 0$ and becomes photobleached at time δ ($G_{\text{AATI}}(\delta)$; the other case of Case c; AATI represents Apparent All-Time Immobile). However, since some molecules might be totally immobilized during periods much longer than T_{rec} , which would represent alternative cases outside the scope of the TALL events considered here (see “Alternative case” in **Supplementary Fig. 37**), we will consider this in a later subsection “**(4) Estimating the time fraction of**

apparently-all-time-immobile molecules and dealing with molecules immobile for periods much longer than T_{rec} ".

(The full equation) The function to describe the TALL duration distribution; $G_{\text{TALL}}(\delta)$

Taking the results for cases a, b, and c together, the duration (δ) distribution of all observed TALL events $G_{\text{TALL}}(\delta)$ is given by

$$G_{\text{TALL}}(\delta) = G_a(\delta) + G_b(\delta) + G_c(\delta) \quad (28)$$

$$G_{\text{TALL}}(\delta) = \frac{k_I k_M (2k_B + k_M) N_0}{k_B (k_I + k_M)} \exp[-(k_B + k_M) \cdot \delta] - \frac{k_I k_M^2 N_0}{k_B (k_I + k_M)} \exp(-k_B T_{\text{rec}}) \exp(-k_M \delta) . \quad (29)$$

Namely, the distribution is given by the sum of two exponential functions, with rate constants of k_M and $k_B + k_M$. As stated (right after Eq. 18), in the analysis of the experimental results, we generally obtain a histogram of the durations (δ), which we define as $H_{\text{TALL}}(\delta)$. $H_{\text{TALL}}(\delta)$ can be obtained by multiplying the histogram's bin size Δt with $G_{\text{TALL}}(\delta)$ (because $G_{\text{TALL}}(\delta)$ is a distribution function with the dimensions of count/time).

$$H_{\text{TALL}}(\delta) = \frac{k_I k_M (2k_B + k_M) N_0 \cdot \Delta t}{k_B (k_I + k_M)} \exp[-(k_B + k_M) \cdot \delta] - \frac{k_I k_M^2 N_0 \cdot \Delta t}{k_B (k_I + k_M)} \exp(-k_B T_{\text{rec}}) \exp(-k_M \delta) . \quad (30)$$

Since k_B can be determined independently, and T_{rec} is an experimental parameter that the operator can determine, k_M can be determined by fitting the experimentally-obtained $H_{\text{TALL}}(\delta)$ by the sum of two exponential functions. The determination precision depends on the ratio of k_B vs. k_M , which will be further discussed in a later subsection **"(6) An approximate expression of the TALL duration distribution function under the experimental conditions employed here; $G_{\text{fit}}(\delta)$ "**

These results indicate that k_M can be determined by observing either one of the cases

of a, b, or c. For example, one could only observe molecules that are immobile at $t = 0$, and by simply measuring the times at which they become mobile, one could evaluate k_M using Eq. 27 (the bin size Δt should be multiplied for its histogram, as stated before for any probability density functions). This approach would be convenient if one could repeat many such experiments easily. Meanwhile, if repeating many experiments is difficult or too time consuming (for example, when repeatedly preparing specimens is difficult), one could observe all of the cases a, b, and c for the same viewfield (this way, the number of molecules observed for a single recording can be increased), and obtain k_M using Eq. 30.

When T_{rec} is infinitely long, Eq. 29 becomes

$$G_{\text{TALL}}(\delta, T_{\text{rec}} \rightarrow \infty) = \frac{k_I k_M (2k_B + k_M) N_0}{k_B (k_I + k_M)} \exp[-(k_B + k_M) \cdot \delta] , \quad (31)$$

which is consistent with the equation previously used to correct for photobleaching⁸³,

$$k_M = k_{\text{Observed}} - k_B , \quad (32)$$

or

$$1/\tau_M = 1/\tau_{\text{Observed}} - 1/\tau_B , \quad (33)$$

where k_{Observed} is a value determined by single-exponential fitting using Eq. 31, and τ_x is the inverse of k_x .

In the unlikely case of $k_B = 0$ (when no photobleaching occurs), Eqs. 15 and 17 become

$$P(\delta) = k_M \exp[-k_M \cdot \delta] . \quad (34)$$

$$F_S(t) = \frac{k_I k_M N_0}{k_I + k_M} . \quad (35)$$

respectively. Applying these equations to evaluate Eqs. 19 and 21, and directly using $k_B = 0$ in Eq. 27, the following equations are obtained

$$G_a(\delta) = (T_{\text{rec}} - \delta) \frac{k_I k_M N_0}{(k_I + k_M)} \exp[-k_M \delta] , \quad (36)$$

$$G_b(\delta) = \frac{k_M k_I N_0}{k_I + k_M} \exp[-k_M \delta], \quad (37)$$

$$G_c(\delta) = \frac{k_M k_I N_0}{k_I + k_M} \exp[-k_M \delta]. \quad (38)$$

respectively.

(4) Estimating the time fraction of apparently-all-time-immobile molecules and dealing with molecules immobile for periods much longer than T_{rec}

In the scheme shown in **Supplementary Fig. 37**, consider the case in which there are molecules that are immobile and coexist for periods much longer than T_{rec} , and therefore cannot be considered to undergo TALL ($k_M \sim 0$), which we call an “Alternative case”, as shown in **Supplementary Fig. 37**. When such molecules coexist in the viewfield of the microscope, it is difficult to differentiate these “alternative” molecules from those that undergo intermittent TALL events and are immobile at $t = 0$ (Case c), and then become photobleached before restarting their motion (see the bottom case of Case c in the scheme shown in **Supplementary Fig. 37**), which we call “apparently-all-time-immobile (AATI) molecules”.

Previously, such AATI molecules were simply called “all-time-immobile molecules” (for example, see **Refs. 53 and 83**). However, they are often a subfraction of the molecules that exhibit alternating mobile and immobile periods, and were incidentally and temporarily immobilized at time 0, and not molecules that are immobile for periods much longer than T_{rec} .

Here, we developed a method to evaluate the time fraction in which the molecules are expected to exhibit the AATI behavior (f_{AATI}) from the parameters determined by $G_{\text{TALL}}(\delta)$ (or $G_a(\delta)$, $G_b(\delta)$, or $G_c(\delta)$) (namely, assuming the absence of alternative molecules undergoing immobilization for periods much longer than T_{rec}). Once f_{AATI} is determined, we can experimentally determine the fraction of alternative molecules (which are immobile for

periods much longer than T_{rec} ; f_{MLI} , MLI represents “Much Longer Immobilization”), using the equation,

$$f_{\text{MLI}} = f_{\text{ExpATI}} - f_{\text{AATI}} \quad , \quad (39)$$

where f_{ExpATI} represents the fraction of molecules that were experimentally and directly determined to be immobile at time 0 and photobleached before starting motion.

The number of TALL events that end at time δ (duration δ , because the molecule is already immobile at time 0) due to photobleaching (the second case of Case c, G_{AATI}) can be expressed as

$$G_{\text{AATI}}(\delta) = k_{\text{B}} N_{\text{I}}^0(\delta) \quad . \quad (40)$$

By using the expression for $N_{\text{I}}^0(\delta)$ given in Eq. 25, Eq. 40 can be rewritten as

$$G_{\text{AATI}}(\delta) = \frac{k_{\text{B}} k_{\text{I}} N_0}{k_{\text{I}} + k_{\text{M}}} \exp[-(k_{\text{B}} + k_{\text{M}}) \cdot \delta] \quad . \quad (41)$$

The histogram for the duration (δ) of AATI molecules, $H_{\text{AATI}}(\delta)$, can be obtained by multiplying the histogram’s bin size Δt with $G_{\text{AATI}}(\delta)$, as we did to obtain Eq. 30. Namely,

$$H_{\text{AATI}}(\delta) = \frac{k_{\text{B}} k_{\text{I}} N_0 \cdot \Delta t}{k_{\text{I}} + k_{\text{M}}} \exp[-(k_{\text{B}} + k_{\text{M}}) \cdot \delta] \quad . \quad (42)$$

The time fraction f_{AATI} can be obtained by the following equation,

$$f_{\text{AATI}} = 1/T_{\text{total}} \cdot \int_{T_{\text{min}}}^{T_{\text{rec}}} \delta \cdot H_{\text{AATI}}(\delta) \cdot d\delta \quad . \quad (43)$$

After integration, the following equation is obtained:

$$f_{\text{AATI}} = \frac{k_{\text{B}} k_{\text{I}} N_0 \cdot \Delta t}{T_{\text{total}} (k_{\text{I}} + k_{\text{M}})} \left[-\frac{1}{(k_{\text{B}} + k_{\text{M}})^2} \exp[-(k_{\text{B}} + k_{\text{M}}) \cdot \delta] \right]_{T_{\text{min}}}^{T_{\text{rec}}} - \frac{1}{k_{\text{B}} + k_{\text{M}}} \cdot \delta \cdot \exp[-(k_{\text{B}} + k_{\text{M}}) \cdot \delta] \Big|_{T_{\text{min}}}^{T_{\text{rec}}} \quad . \quad (44)$$

As such, f_{AATI} can be obtained from experimentally determined parameters, and then by using Eq. 30, f_{MLI} can be determined. In other words, if f_{ExpATI} is close to f_{AATI} , then it indicates that the molecules undergoing immobilization for periods that are much longer than T_{rec} will not exist, as discussed in the subsection “Each TALL event of integrins” in

the main text.

(5) Testing the function $G_{TALL}(\delta)$ using Monte Carlo simulations of particles undergoing repeated intermittent TALL events

In the experiments conducted in the present research, under the conditions optimized for the lowest photobleaching rate (ST647, 2%O₂+TQ20), k_B was $\sim 1/83.5 \text{ s}^{-1}$, and our standard observation duration of integrin molecules (T_{rec}) was 200 s. We performed Monte Carlo simulations with transitions to mobile, immobile, or photobleaching states with probabilities given by their rate constants ($T_{rec} = 200 \text{ s}$ or 70 s : $k_B = 1/83.5 \text{ s}^{-1}$ or $1/4.3 \text{ s}^{-1}$; $k_M = 1/0.5, 1/5, 1/50, 1/70, 1/167$ or $1/500 \text{ s}^{-1}$; and $k_I = 1/1$, respectively; since k_I does not affect the exponential lifetimes, it is assumed to be 1), and obtained the TALL duration distributions. The simulation was conducted until TALL occurred 10^8 times (**Supplementary Figs. 38 and 39, left-top**).

These distributions were fitted by using $G_{TALL}(\delta)$ (Eq. 29, red solid line). The fits were excellent over the full ranges of δ (up to $200 \text{ s} = T_{rec}$) and the % frequency (y axis, in $4 \times \log_{10}$ cycles), suggesting that the $G_{TALL}(\delta)$ obtained here is correct. Next, we simulated the case where T_{rec} was changed from 200 s to 70 s, which was close to $1/k_B$ (with the same $k_B = 1/83.5 \text{ s}^{-1}$; using the same six k_M values) (**Supplementary Fig. 39, left-bottom**). The fits using $G_{TALL}(\delta)$ were excellent. As the next step, we simulated the case where the photobleaching rate was $\sim 20\times$ larger ($k_B = 1/4.3 \text{ s}^{-1}$) and T_{rec} was either 70 s or 200 s. Even under these conditions, the fits using $G_{TALL}(\delta)$ were excellent (**Supplementary Fig. 39, right boxes**), further supporting the proposal that $G_{TALL}(\delta)$ is the correct distribution function. Taken together, these results indicate that $G_{TALL}(\delta)$ in Eq. 29 represents the correct function for the TALL duration distribution.

(6) An approximate expression of the TALL duration distribution function (approximate probability density function of δ) under the experimental conditions employed here; $G_{fit}(\delta)$

Under actual experimental conditions, the attainable signal-to-noise ratios and the number of experiments that can be performed might be quite limited. Therefore, precisely fitting the TALL duration distribution histogram using $G_{\text{TALL}}(\delta)$ (or $H_{\text{TALL}}(\delta)$), as given in Eq. 29, might often be impractical, and thus one might want to fit the histogram using only the first term of $G_{\text{TALL}}(\delta)$, $G_{\text{fit}}(\delta)$. Namely,

$$G_{\text{fit}}(\delta) = \frac{k_I k_M (2k_B + k_M) N_0}{k_B (k_I + k_M)} \exp[-(k_B + k_M) \cdot \delta] , \quad (45)$$

which is the same as Eq. 30, meaning that it would be more practical to employ a long T_{rec} so that the second term in Eq. 30 could be neglected. In this subsection, we examine the cases where the corrections for T_{rec} could be neglected (*i.e.*, where $G_{\text{fit}}(\delta)$ could safely replace $G_{\text{TALL}}(\delta)$). For this purpose, we employed the somewhat standard experimental conditions of $T_{\text{rec}} = 200$ s and $k_B = 1/83.5$ s⁻¹, as described in the previous subsection (5).

As the first test, we examined the goodness of the fit when $G_{\text{fit}}(\delta)$ was used to replace $G_{\text{TALL}}(\delta)$ to fit the histograms shown in **Supplementary Figs. 38 and 39**. The best-fit curves obtained for $G_{\text{TALL}}(\delta)$ and $G_{\text{fit}}(\delta)$ are shown in these figures.

Under our standard conditions of $T_{\text{rec}} = 200$ s and $k_B = 1/83.5$ s⁻¹ (**Supplementary Figs. 38 and 39, top left**), the fit appeared to be quite good, particularly in the δ interval between 0 and ~ 83.5 s for all of the k_M values employed here, consistent with the concept in which the durations of the events shorter than the photobleaching time could be measured with good precision. The largest deviation appeared to occur for $k_M \sim 1/70$; *i.e.*, when the k_B and k_M values were close to each other (**Supplementary Fig. 39, bottom left**), showing that under these conditions, $G_{\text{TALL}}(\delta)$, rather than $G_{\text{fit}}(\delta)$, should be employed.

To quantify the goodness of fit for various k_M values (which are the parameters we hope to obtain in these experiments), the deviation (χ^2) was plotted against $1/k_M$ for various values of T_{rec} (200 s, which was our standard value, and 70 s) and k_B (1/83.5 s⁻¹, which was our standard value, and 1/4.3 s⁻¹) (**Supplementary Fig. 40**). Under our standard observation conditions of $k_B = 1/83.5$ s⁻¹ and $T_{\text{rec}} = 200$ s, the difference between the results using these two fit functions became the largest when $k_M \sim 1/70$ s⁻¹; *i.e.*, when k_M became

close to k_B . Compare this result with the $G_{TALL}(\delta)$ functions given in **Supplementary Fig. 38**. For $k_M \ll 1/70 \text{ s}^{-1}$, the decay lifetime in the second term was quite different from that in the first term, but the pre-exponential coefficient was small. For $k_M \gg 1/70 \text{ s}^{-1}$, the pre-exponential coefficient of the second term was large, but the decay lifetime in the second term became quite similar to that of the first term, and the pre-exponential coefficient was small. When $k_M \sim 1/70 \text{ s}^{-1}$ (*i.e.*, when $k_M \sim k_B$), such compensations were inoperative.

For the cases where the photobleaching rate was $\sim 20\times$ larger ($k_B = 1/4.3 \text{ s}^{-1}$) and T_{rec} was either 70 s or 200 s, the fits using $G_{fit}(\delta)$ were excellent for virtually all of the k_M values employed here (**Supplementary Figs. 39, right boxes and 40**). Taken together, these results indicate that when T_{rec} is greater than $1/k_B$ by a factor of 2.4 (200/83.5) or more, $G_{fit}(\delta)$, rather than $G_{TALL}(\delta)$, could be used.

However, note that we have discussed only the systematic differences between $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ under the conditions where the durations of 10^8 TALLs were determined. In practical experimental situations, where generally the durations of only a few hundred TALLs could be determined, the experimental errors in evaluating k_M values are expected to be much greater, as compared with the value evaluated after observing 10^8 TALLs (which must be close to the correct k_M value). Particularly, correctly determining the k_M value smaller than the k_B value (TALL durations longer than the photobleaching lifetime), using the approximate function $G_{fit}(\delta)$, could be extremely difficult. That is, in these situations, one might wonder how close the k_M value estimated by an approximate function $G_{fit}(\delta)$ would come to those obtained using the correct function $G_{TALL}(\delta)$. In unfavorable conditions for determining the k_M value, the error will become much larger, even when the correct function $G_{TALL}(\delta)$ is used for the fitting. However, if one employs $G_{fit}(\delta)$, rather than $G_{TALL}(\delta)$, will the deviation from the correct k_M value be greater than that when $G_{TALL}(\delta)$ is used?

To address this question, and also as the second test for replacing $G_{TALL}(\delta)$ with $G_{fit}(\delta)$, we examined the difference in the estimation of k_M , between the cases where $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ were used as fitting functions, under the conditions where the Monte Carlo

simulations were conducted until 500 TALLs were observed (as compared to 10^8 TALLs for previous figures) for each parameter set of k_M , k_B , and T_{rec} , to simulate the experimental conditions. Then, the fitting using $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ to evaluate k_M was performed. This process was repeated 1,000 times, to obtain the mean $\pm$ SEM of k_M s using these two functions.

The $1/k_M$ values obtained by the correct function $G_{TALL}(\delta)$ were plotted against those determined by using the approximate function $G_{fit}(\delta)$ (**Supplementary Figs. 41-44**). Interestingly and quite impressively, for virtually all combinations of the k_M , k_B , and T_{rec} values, both the mean and SEM values obtained by fitting with $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ were similar to each other. Even when the obtained mean values were quite different from the correct $1/k_M$ value and/or when the SEMs were very large, their values obtained by using $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ were quite similar to each other (except for the very unusual case shown in the bottom right box in **Supplementary Fig. 42**). These results support the proposal that $G_{fit}(\delta)$ can replace $G_{TALL}(\delta)$ well, virtually under all of the conditions examined here. Therefore, $G_{fit}(\delta)$ was employed to evaluate k_M for integrins in the present research. Furthermore, these results support the intuitive expectation that for measuring k_M correctly, the relationship of $1/k_M < 1/k_B < T_{rec}$ must be satisfied. When this condition is satisfied, the $1/k_M$ values determined by $G_{TALL}(\delta)$ and $G_{fit}(\delta)$ coincided within a 5% difference (**Supplementary Figs. 41- 44**).

(7) Practical ways of applying the theory to obtain k_M and the time fraction in which molecules exhibit the AATI behavior, f_{AATI}

In the ideal cases discussed within the previous subsections, N_0 is known. However, in experiments, due to the presence of f_{MLI} (alternative case), N_0 is unknown. Meanwhile, k_B can be determined independently, using the same set of trajectories. Under these conditions, k_M , the parameter we hope to determine most, can be determined in the following manner.

If we define C as

$$C = \frac{k_I k_M N_0}{k_B (k_I + k_M)} , \quad (46)$$

then Eq. 30 and Eq. 42 can be rewritten as

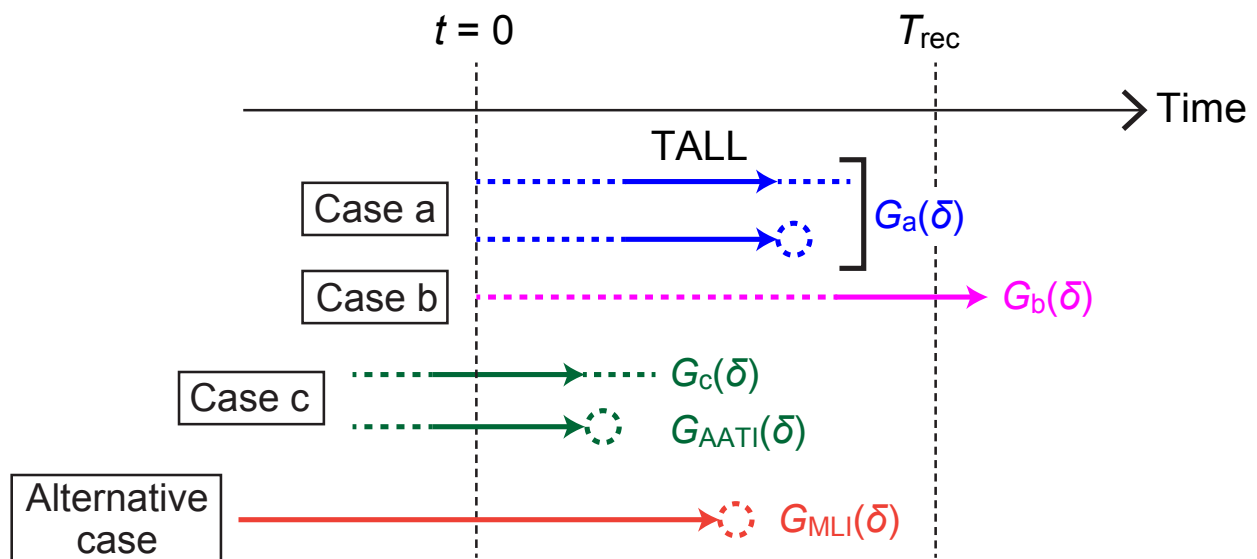
$$H_{\text{TALL}}(\delta) = C\Delta t (2k_B + k_M) \exp[-(k_B + k_M) \cdot \delta] - C\Delta t \cdot k_M \exp(-k_B T_{\text{rec}}) \exp(-k_M \delta) . \quad (47)$$

$$H_{\text{AATI}}(\delta) = C\Delta t \frac{k_B^2}{k_M} \exp[-(k_B + k_M) \cdot \delta] . \quad (48)$$

By fitting the histogram (distribution) using Eq. 47 or Eq. 48 (using C and k_M as free parameters), C and k_M can be determined. The time fraction in which molecules exhibit the AATI behavior, f_{AATI} (Eq. 44), can now be calculated using all of the determined values as

$$f_{\text{AATI}} = \frac{C\Delta t \cdot k_B^2}{T_{\text{total}} k_M} \cdot \left[-\frac{1}{(k_B + k_M)^2} \exp[-(k_B + k_M) \cdot \delta] \right]_{T_{\min}}^{T_{\text{rec}}} - \left[-\frac{1}{(k_B + k_M)} \cdot \delta \cdot \exp[-(k_B + k_M) \cdot \delta] \right]_{T_{\min}}^{T_{\text{rec}}} . \quad (49)$$

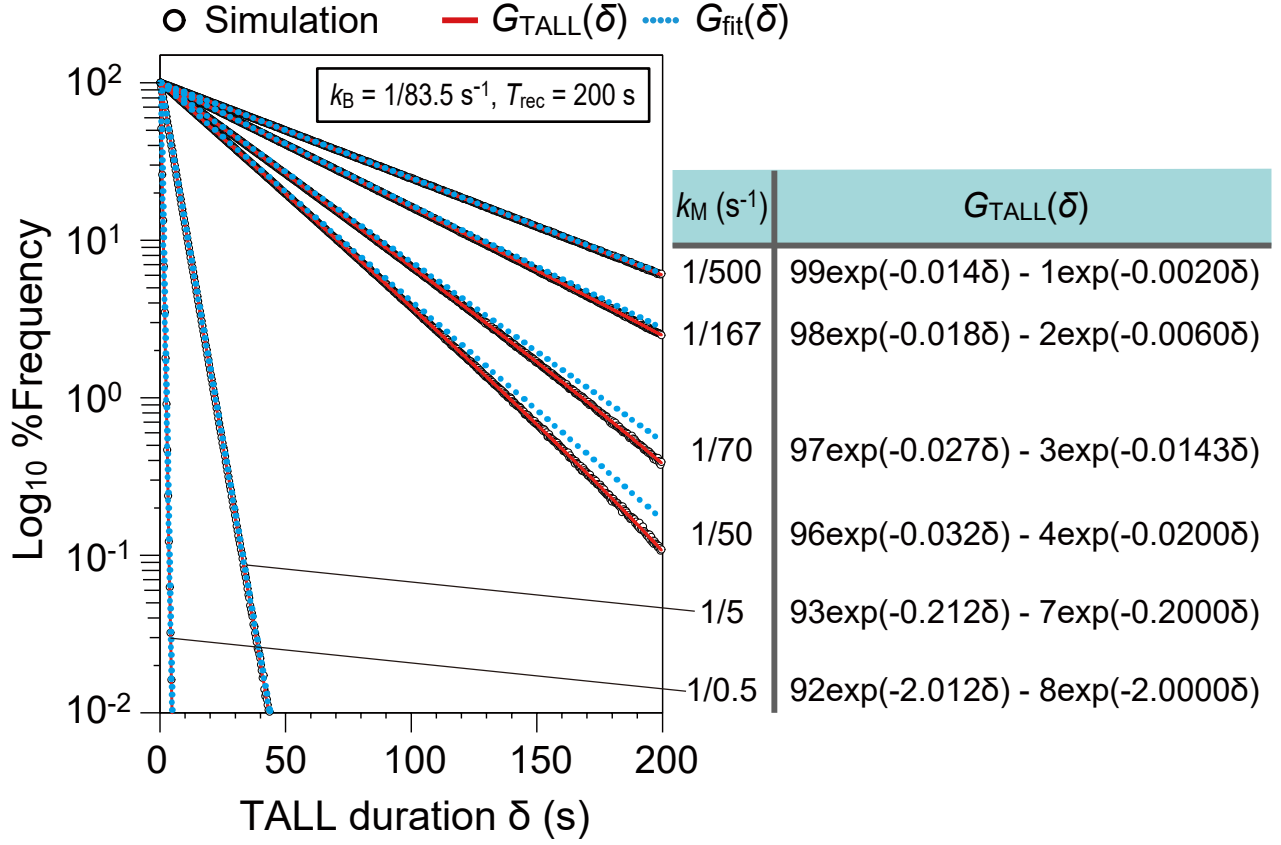
Supplementary Note 1 Figures



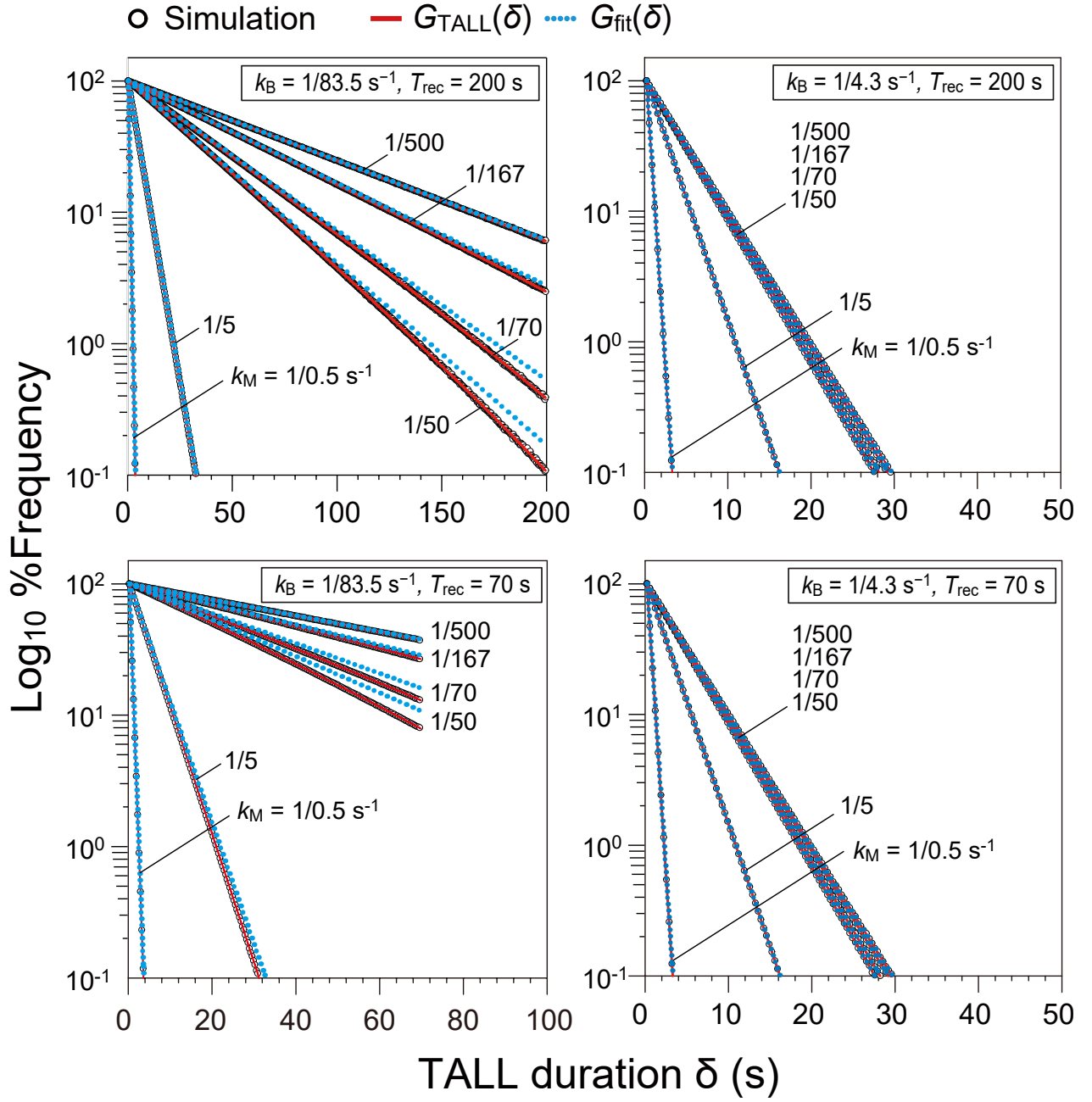
Supplementary Figure 37 | Timing diagram of TALL events (arrows) relative to the recording (observation) period, starting from $t = 0$ and ending at $t = T_{\text{rec}}$. Arrows indicate the TALL periods. TALLs can end by either restarting diffusion or photobleaching (dashed circles).

TALLs are classified into three categories, in terms of the temporal relationship with the beginning and the end of each recording of a single viewfield ($t = 0$ and T_{rec} , respectively). TALLs might be totally included within the recording period ($0 < t \leq T_{\text{rec}}$, Case a; $G_a(\delta)$), might start after time 0 (at $t > 0$) and continue after the recording period (Case b; $G_b(\delta)$), and might start at $t \leq 0$ (Case c; $G_c(\delta)$). The TALLs of Case c that ended by photobleaching have often been called “all-time immobile” events, as the molecules showing this behavior are immobilized throughout the recording period, because they are immobile at $t = 0$ and photobleached before restarting diffusion.

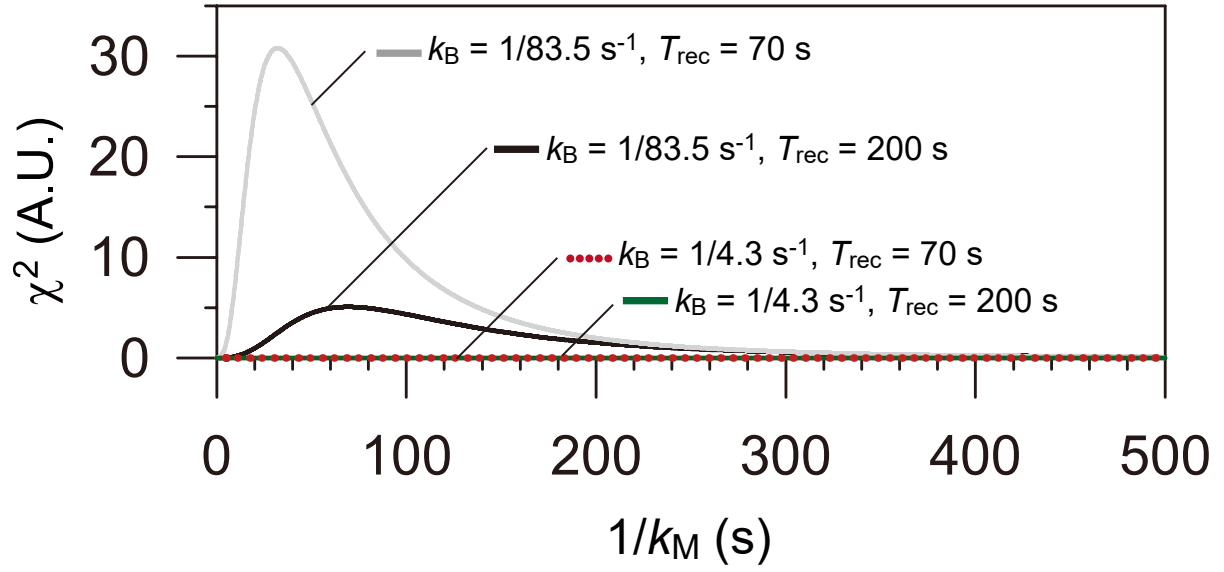
There might be some molecules in the viewfield that exhibit “much longer immobilization (MLI)”, as compared with T_{rec} (and thus with the TALL periods exhibited by the large majority of observed molecules), called “alternative molecules” (Alternative case). These molecules will be considered separately.



Supplementary Figure 38 | The distributions of TALL durations when $k_B = 1/83.5$ and $T_{rec} = 200$ s, obtained by Monte Carlo simulations of Brownian particles undergoing repeated TALL events and the best fit functions for the distributions using $G_{TALL}(\delta)$ (red curves) and $G_{fit}(\delta)$ (blue curves). The actual functions of $G_{TALL}(\delta)$ are provided for each k_M value, and their first terms represent $G_{fit}(\delta)$. The k_M values employed here were 1/0.5, 1/5, 1/50, 1/70, 1/167 and 1/500 s^{-1} .

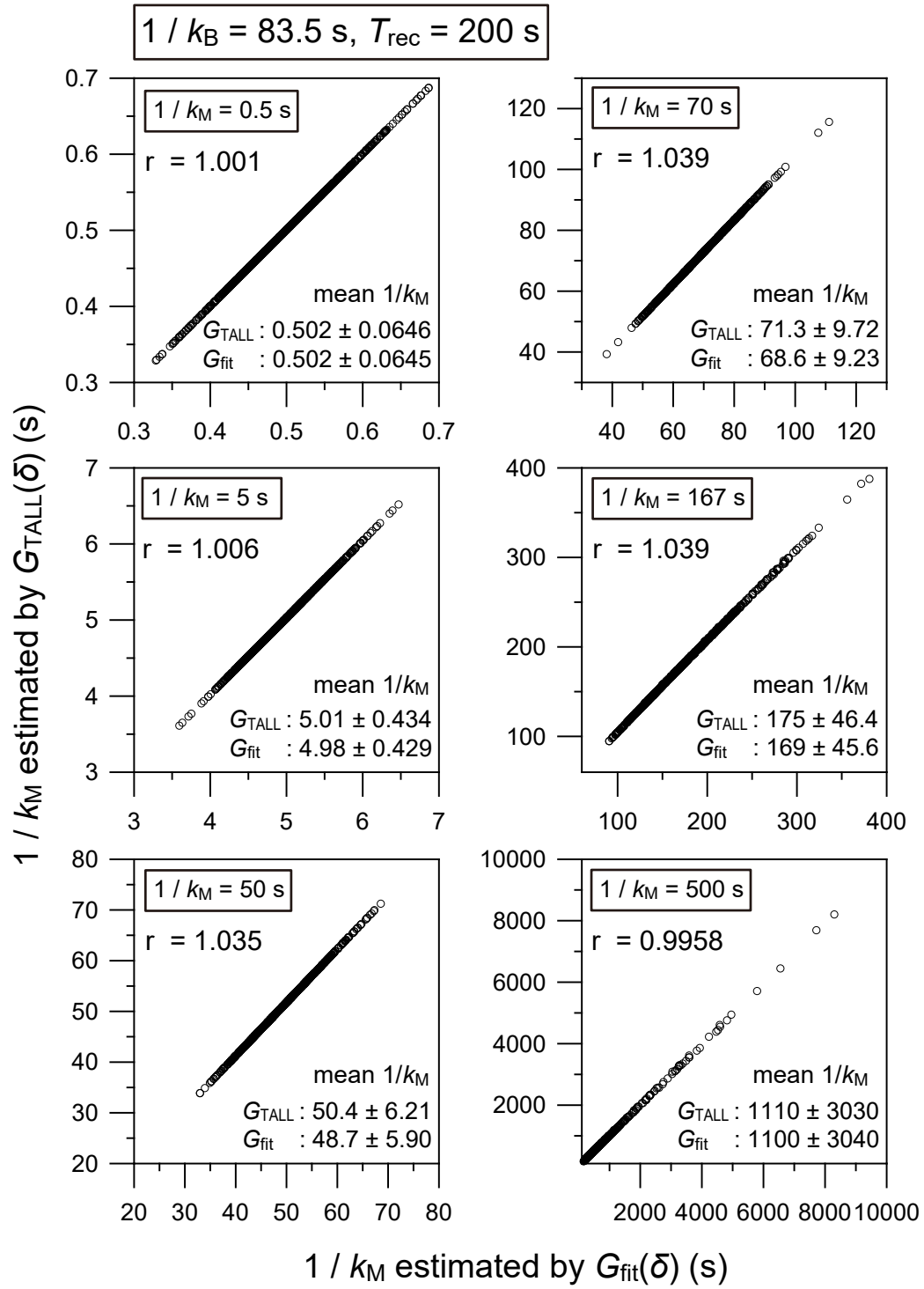


Supplementary Figure 39 | The distributions of TALL durations under four conditions of k_B ($1/83.5$ and $1/4.3 \text{ s}^{-1}$) $\times$ T_{rec} (200 and 70 s), obtained by Monte Carlo simulations of Brownian particles undergoing repeated TALL events and the best fit functions for the distributions using $G_{\text{TALL}}(\delta)$ (red curves) and $G_{\text{fit}}(\delta)$ (blue curves). The k_M values employed here were $1/0.5, 1/5, 1/50, 1/70, 1/167$ and $1/500 \text{ s}^{-1}$. The box at the top left is the same as that in **Supplementary Fig. 38**.



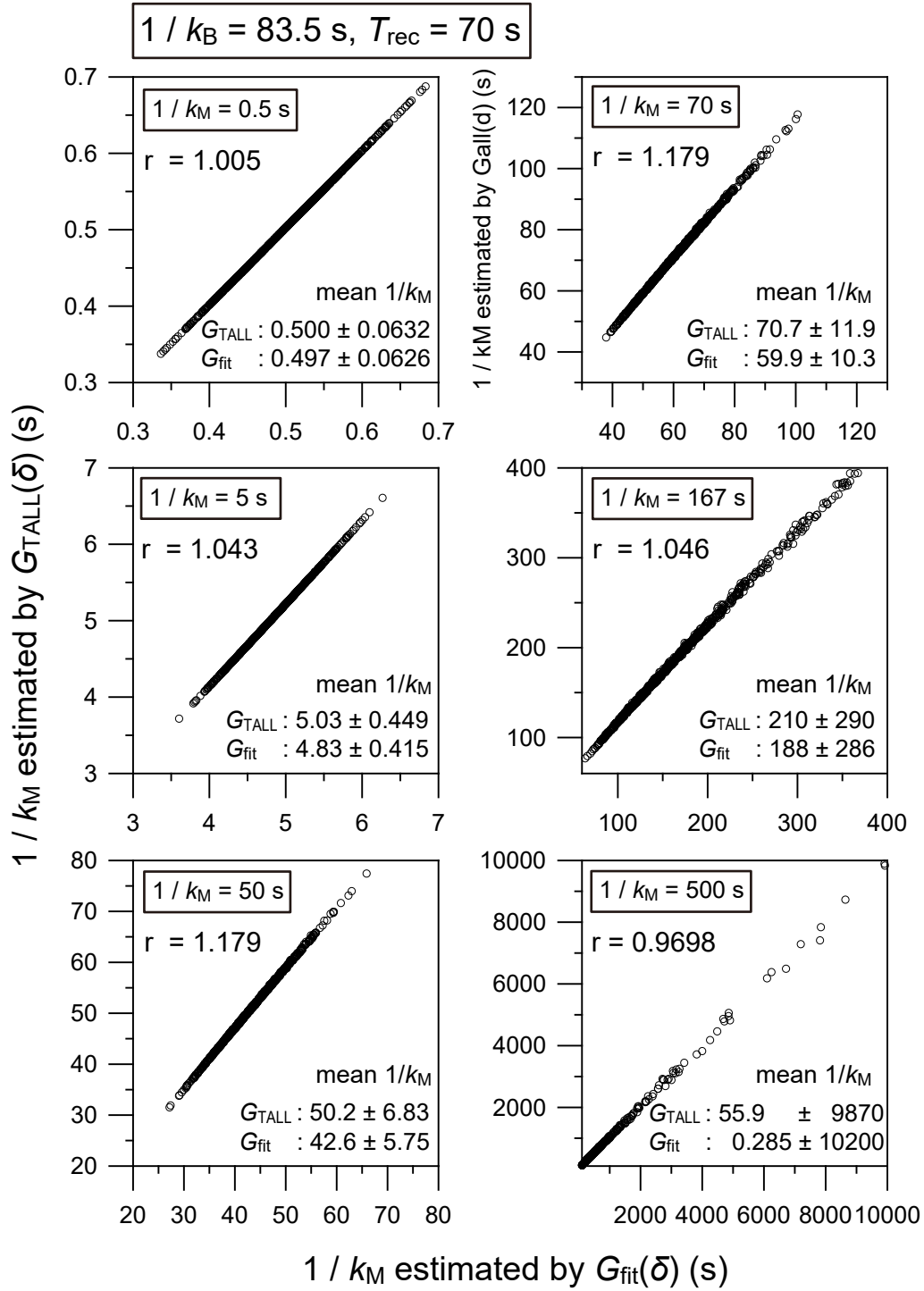
Supplementary Figure 40 | The difference between $G_{\text{TALL}}(\delta)$ and $G_{\text{fit}}(\delta)$ plotted against $1/k_M$, determined for the four conditions described in **Supplementary Fig. 39**. The difference was parameterized using χ^2 , defined as

$$\chi^2(1/k_M) = \int_0^{t_{\max}} \frac{[G_{\text{all}}(\delta) - G_{\text{fit}}(\delta)]^2}{G_{\text{fit}}(\delta)} \cdot d\delta$$

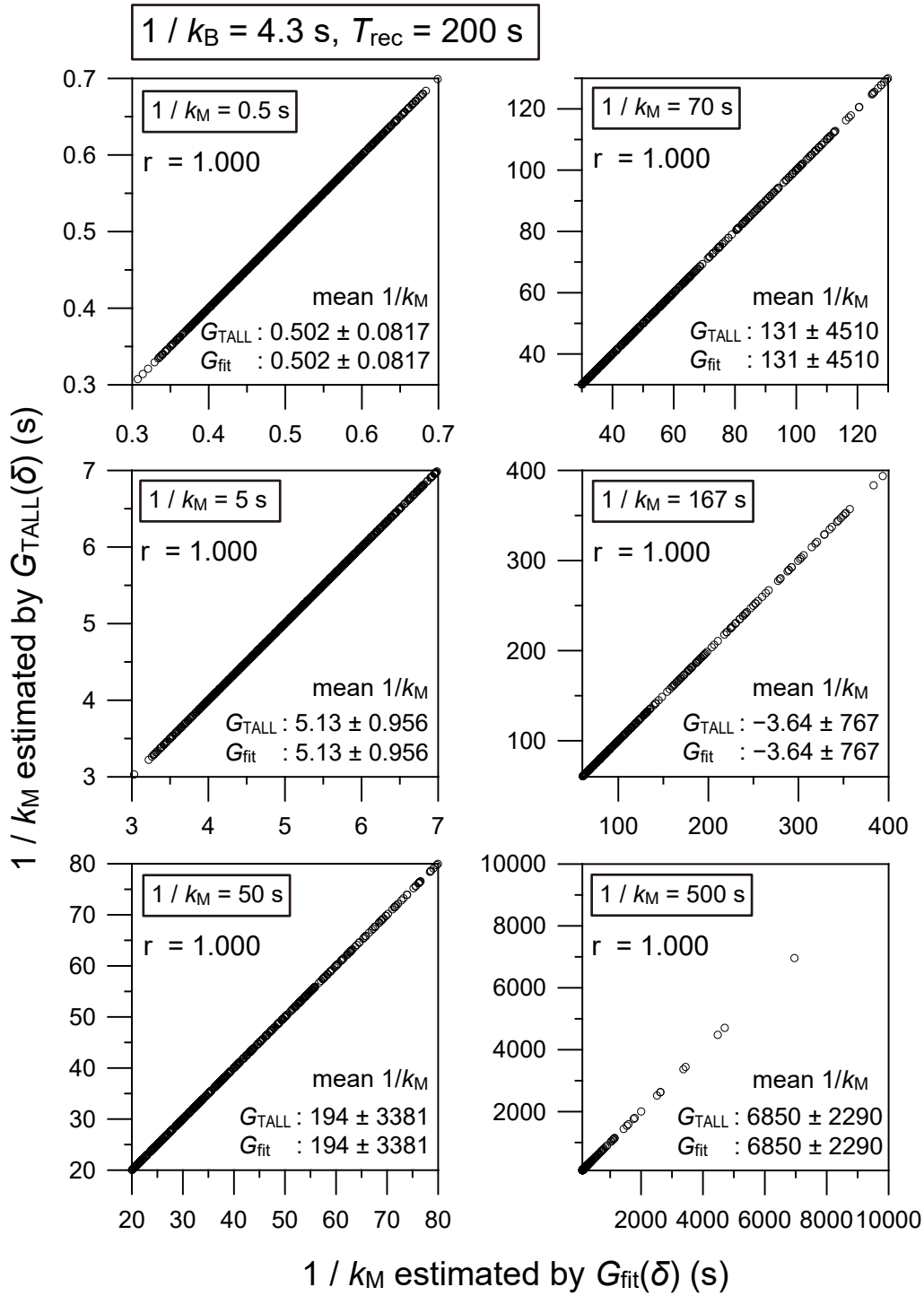


Supplementary Figure 41 | The correlation in the estimation of k_M between the cases where $G_{\text{TALL}}(\delta)$ and $G_{\text{fit}}(\delta)$ were used as fitting functions, for the histograms of TALL durations obtained by Monte Carlo simulations. The purpose of this examination is to determine whether $G_{\text{fit}}(\delta)$ can be used to replace $G_{\text{TALL}}(\delta)$ under practical experimental

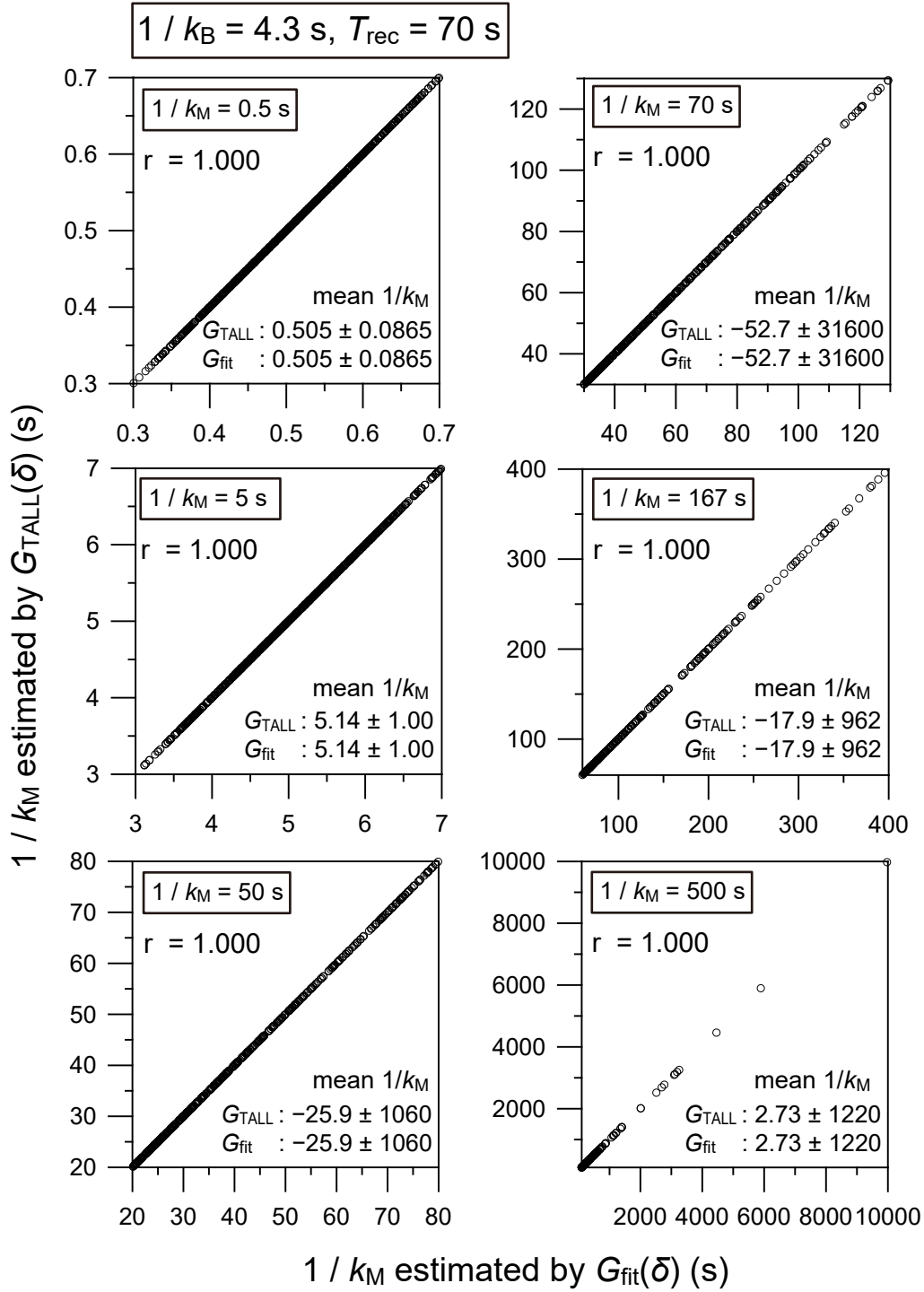
conditions where only a limited number of TALL events can be examined. For this purpose, in this simulation, we generated only 500 TALL events (as compared to 10^8 events for the results shown in **Supplementary Figs. 38 and 39**) for each parameter set, and to obtain the distribution, this was repeated 1,000 times. See the text for further details. The k_M values obtained by the correct function $G_{\text{TALL}}(\delta)$ were plotted against the k_M values obtained by using the approximate function $G_{\text{fit}}(\delta)$ (therefore, each plot in a box shown above contains 1,000 spots). The parameters employed here were $k_B = 1/83.5$ and $T_{\text{rec}} = 200$ s, with six k_M values ($1/0.5$, $1/5$, $1/50$, $1/70$, $1/167$ and $1/500$ s⁻¹).



Supplementary Figure 42 | The same as **Supplementary Fig. 40**, but with a different pair of k_B and T_{rec} ($k_B = 1/83.5 \text{ s}^{-1}$ and $T_{\text{rec}} = 70 \text{ s}$).



Supplementary Figure 43 | The same as **Supplementary Figs. 40 and 41**, but with a different pair of k_B and T_{rec} ($k_B = 1/4.3 \text{ s}^{-1}$ and $T_{\text{rec}} = 200 \text{ s}$).



Supplementary Figure 44 | The same as **Supplementary Figs. 40-42**, but with a different pair of k_B and T_{rec} ($k_B = 1/4.3 \text{ s}^{-1}$ and $T_{\text{rec}} = 70 \text{ s}$).

Supplementary Note 1 References

(Continued from Main Text, Methods and Supplementary Information)

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Super-long single-molecule tracking reveals dynamic-anchorage-induced integrin function

Taka A. Tsunoyama, Yusuke Watanabe, Junri Goto, Kazuma Naito, Kenichi G.N.

Suzuki, Takahiro K. Fujiwara and Akihiro Kusumi

— Supplementary Note 2 — Synthetic procedures

TMR-, AT594-, AT647N- and ST647-CoA were synthesized by conjugating the maleimide derivatives of these dye compounds (Life Technologies, ATTO-TECH GmbH and SETA Biomedicals) with coenzymeA-SH (CoA-SH, New England Biolabs). Briefly, the maleimide derivatives of these fluorescent molecules were dissolved in *N,N*-dimethylformamide (DMF), followed by the addition of CoA-SH in a tris(hydroxymethyl)aminomethane (Tris)-buffered aqueous solution (pH 7.5), and then the reaction mixture was stirred at RT for 2 h. The Dye-CoA conjugates were purified by preparative reverse-phase high performance liquid chromatography (HPLC; LC-2010A HT/SPD-M20A, Shimadzu, with a Gemini-NX-5u 18C column for AT594- and AT647N-CoA and a Zorbax Eclipse XDB-C18 column for ST657- and TMR-CoA).

The NH₂-Halo ligand was synthesized from 2-(2-aminoethoxy)ethanol and 6-chloro-1-iodohexane, as described in **Ref. 65**. The AT594- and ST647-Halo ligands were synthesized by conjugating the NHS derivatives of these dye compounds (ATTO-TECH GmbH and SETA Biomedicals, respectively) with the NH₂-Halo ligand. These starting compounds were dissolved in dichloromethane (DCM), followed by the addition of triethylamine, and then the reaction mixture was stirred at RT overnight. The Dye-Halo ligand conjugates were purified by preparative reverse-phase HPLC, with a Phenomenex Gemini-NX-5u 18C column for the AT594-Halo ligand and a Phenomenex Kinetex C18 column for the ST647-Halo ligand.

For the syntheses of the Cy3- and Cy5-TX-Halo ligands (**Supplementary Figs. 17c, d and 45**), the method of Altman *et al.* was employed, with slight modifications^{2,84}. Trolox was obtained from Sigma-Aldrich, and Cy3-bis-NHS and Cy5-bis-NHS were obtained from GE Healthcare. Briefly, compound **1** (trolox) was dissolved in DCM, followed by the addition of triethylamine (2.0 eq), ethylenediamine (10 eq), and BOP (1.5 eq), and then the reaction mixture was stirred at RT for 0.5 h. Compound **2** was obtained by silica gel column chromatography. To Compound **3** (Cy3-bis-NHS: n = 1, Cy5-bis-NHS: n = 2) dissolved in DCM, triethylamine (3.0 eq) was added, and to this mixture, compound **2** (0.8 eq) was slowly added, followed by an incubation with stirring at RT for 0.5 h to generate compound

4. Without purification, compound **5** (NH₂-Halo ligand, synthesized as described in a previous paragraph) was added, and the reaction mixture was further stirred at RT for 1 h to generate compound **6**, which was then purified by preparative reverse-phase HPLC with an Agilent Zorbax SB-C18 column (for Cy3-TX-Halo ligand) and a Phenomenex Kinetex5 μ C18 column (for Cy5-TX-Halo ligand).

For the synthesis of AT594-TX-CoA (**Supplementary Figs. 17b and 46a**), first, compound **7** (trolox-NHS) was synthesized from compound **1** (trolox), following a previously published procedure⁸⁵. Without purification, 9-fluorenylmethyloxycarbonyl (Fmoc)-Lys-OH (1.2 eq), DMF (2.2 eq), and *N,N*-diisopropylethylamine (DIPEA, 2.2 eq) were added, and then the reaction mixture was stirred at RT for 4 h. The resulting solution was diluted with ethylacetate, and then filtered. After concentration, compound **8** was purified by silica gel column chromatography (97% yield). Compound **8** (100 mg) and 33.4 mg *N*-(2-aminoethyl)maleimide hydrochloride (1.1 eq) were dissolved in prechilled dioxane (0°C), followed by the addition of 32 μ l DIPEA (1.1 eq) at 0°C and then 38 mg of *N,N'*-dicyclohexylcarbodiimide (DCC, 1.1 eq) at 0°C. The reaction mixture was stirred at RT overnight to generate compound **9**, which was then purified by silica gel column chromatography (final 59.3 mg, 99% yield). To compound **9** (59.3 mg) dissolved in DMF, 63 mg CoA-SH (1.0 eq) in 50 mM Tris (pH 7.5) was added, and the solution was stirred at RT for 4 h, and then the pH of the solution was adjusted to pH 8.0 by adding 1N NaOH. The resulting solution was further stirred at RT for 1 h, to generate compound **10**. Compound **10** was purified by preparative reverse-phase HPLC with an Agilent Zorbax SB-C18 column (80.6 mg, 66% yield). The Fmoc group of compound **10** was removed by dissolving 11.3 mg compound **10** in a water-DMF mixture (1:1 v/v), followed by the addition of 10 μ l piperidine (15 eq) and an incubation at RT for 10 min. The solvent was then evaporated, and compound **11** was purified by preparative reverse-phase HPLC with an Agilent Zorbax SB-C18 column (9.5 mg, 99% yield). To 3.3 mg compound **11** dissolved in DMF, 3.6 mg AT594-NHS (1.0 eq) dissolved in DMF and 0.68 μ l DIPEA (1.5 eq) were added, and then the reaction mixture was stirred at RT for 4 h, to generate compound **12**. Compound **12** was

purified by preparative reverse-phase HPLC with an Agilent Zorbax SB-C18 column (0.6 mg, 11% yield).

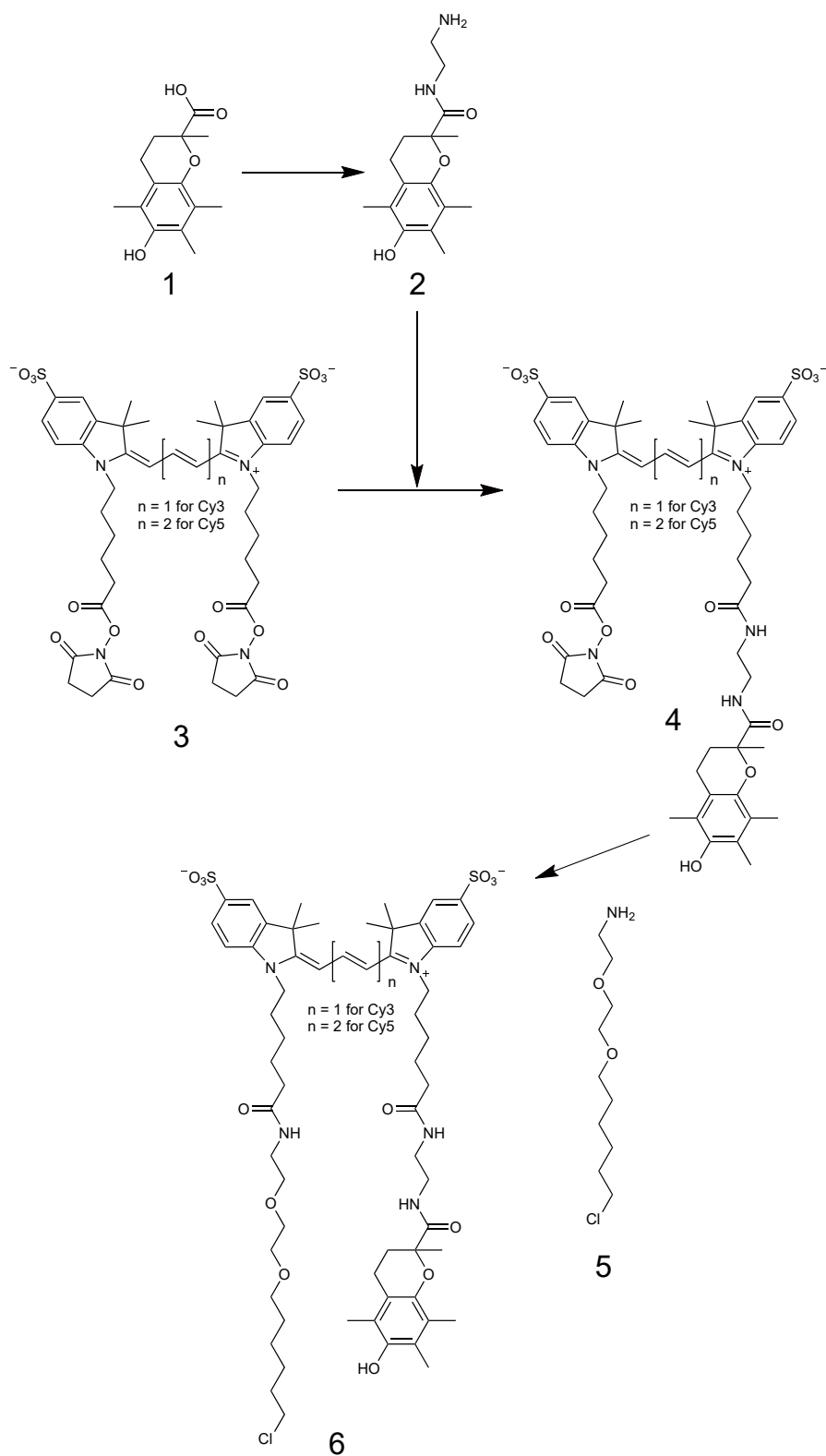
For the synthesis of the TMR-TX-Halo ligand (**Supplementary Figs. 17a and 46b**), 22.3 mg compound **5** and 29.3 mg compound **8** (1.0 eq) were dissolved in dioxane, and then 11 mg DCC (1.1 eq) and 9.4 μ l DIPEA (1.1 eq) were added. The mixture was stirred at RT for 4 h to form compound **13**. Compound **13** was purified by silica gel column chromatography (10.7 mg, 27% yield). The Fmoc group of compound **13** was removed by dissolving 10.7 mg compound **13** in DMF, followed by the addition of 10 μ l piperidine (1.2 eq) and an incubation with stirring at RT for 1 h. Compound **14** was purified by preparative reverse-phase HPLC with a Phenomenex Gemini NX column (2.6 mg, 33% yield), and then 2 mg TMR-NHS (1.2 eq) and 1.24 μ l DIPEA (2.2 eq) were added. The reaction mixture was stirred at RT overnight, and then the generated compound **15** was purified by preparative reverse-phase HPLC with a Phenomenex Gemini NX column (2.9 mg, 90% yield).

The purities of the final compounds were 95.67, 99.54, 99.61, and 90.62 for TMR-, AT594-, AT647N- and ST647-CoAs; 99.77 and 99.56 for AT594- and ST647-Halo ligands; 99.65 and 99.98 for Cy3- and Cy5-TX-Halo ligands; and 99.05 and 99.53% for AT594-TX-CoA and TMR-TX-Halo ligands, respectively, as determined by liquid chromatography-mass spectrometry (LC-MS; LCMS-2010A EV, Shimadzu, **Supplementary Fig. 47**).

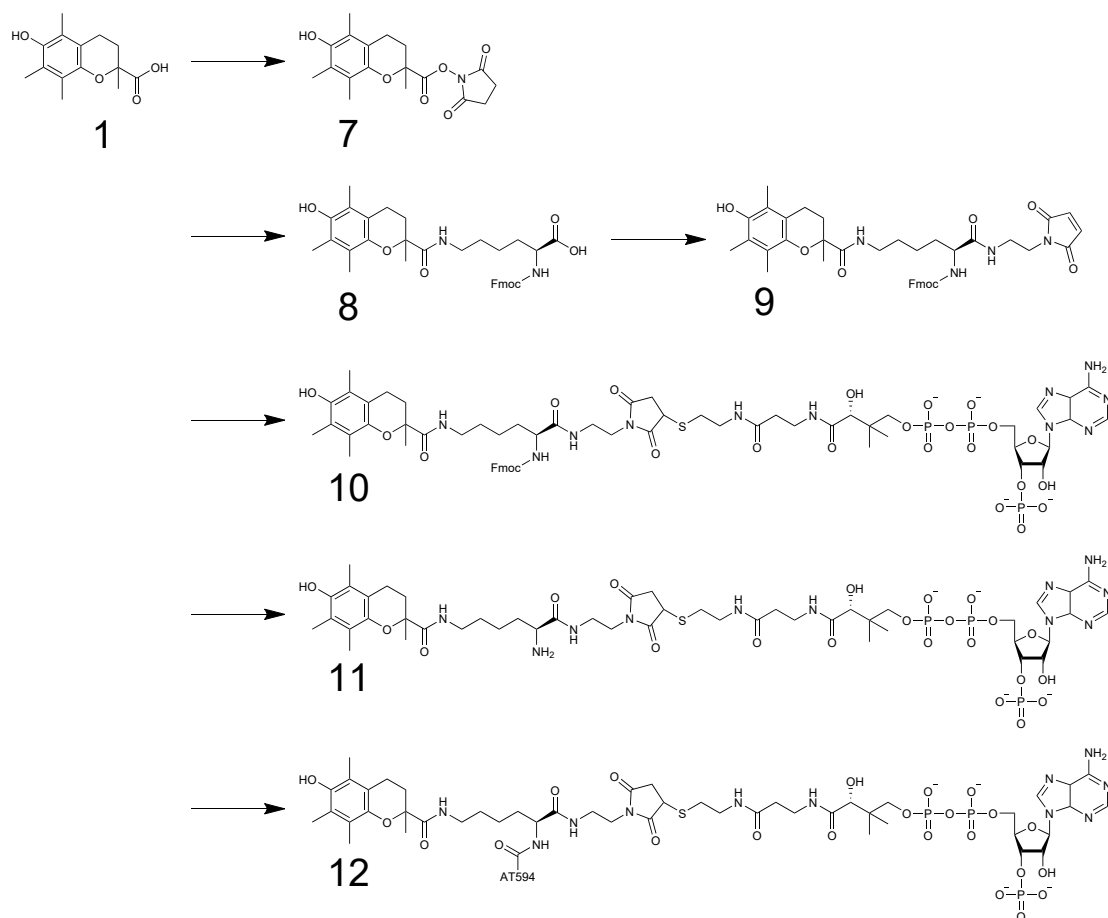
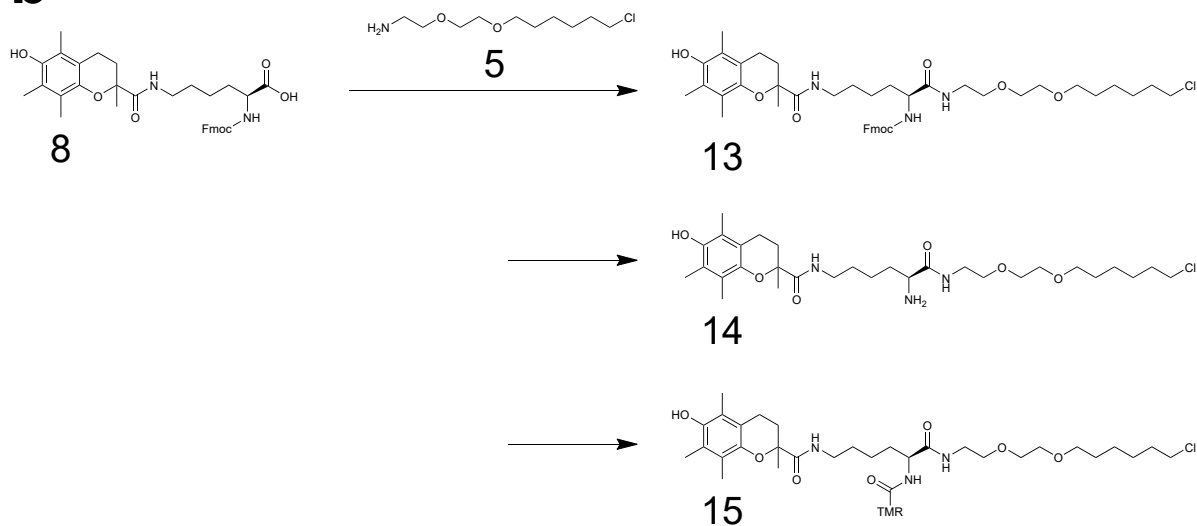
ACP-tagged CD47 and integrin wild-type and mutants expressed on the cell surface (ACP tag in the extracellular N-terminus) were covalently labeled with fluorescent dye compounds by incubating the cells with 50 nM dye-conjugated CoA and 2 μ M ACP synthase (New England Biolabs), in complete growth medium (containing 10 mM MgCl₂ sufficient for activating the synthase) at 37°C for 15 min. The Halo7-tagged proteins expressed in the PM (both inside and outside the cell) were covalently conjugated by fluorescent organic molecules, by incubating the cells with 5 nM Halo ligands linked to TMR or AL488 in complete growth medium at 37°C for 15 min, or 5 nM R110-Halo ligand in complete growth medium at 37°C overnight. Under these conditions, 10~20% of the expressed proteins

were fluorescently labeled.

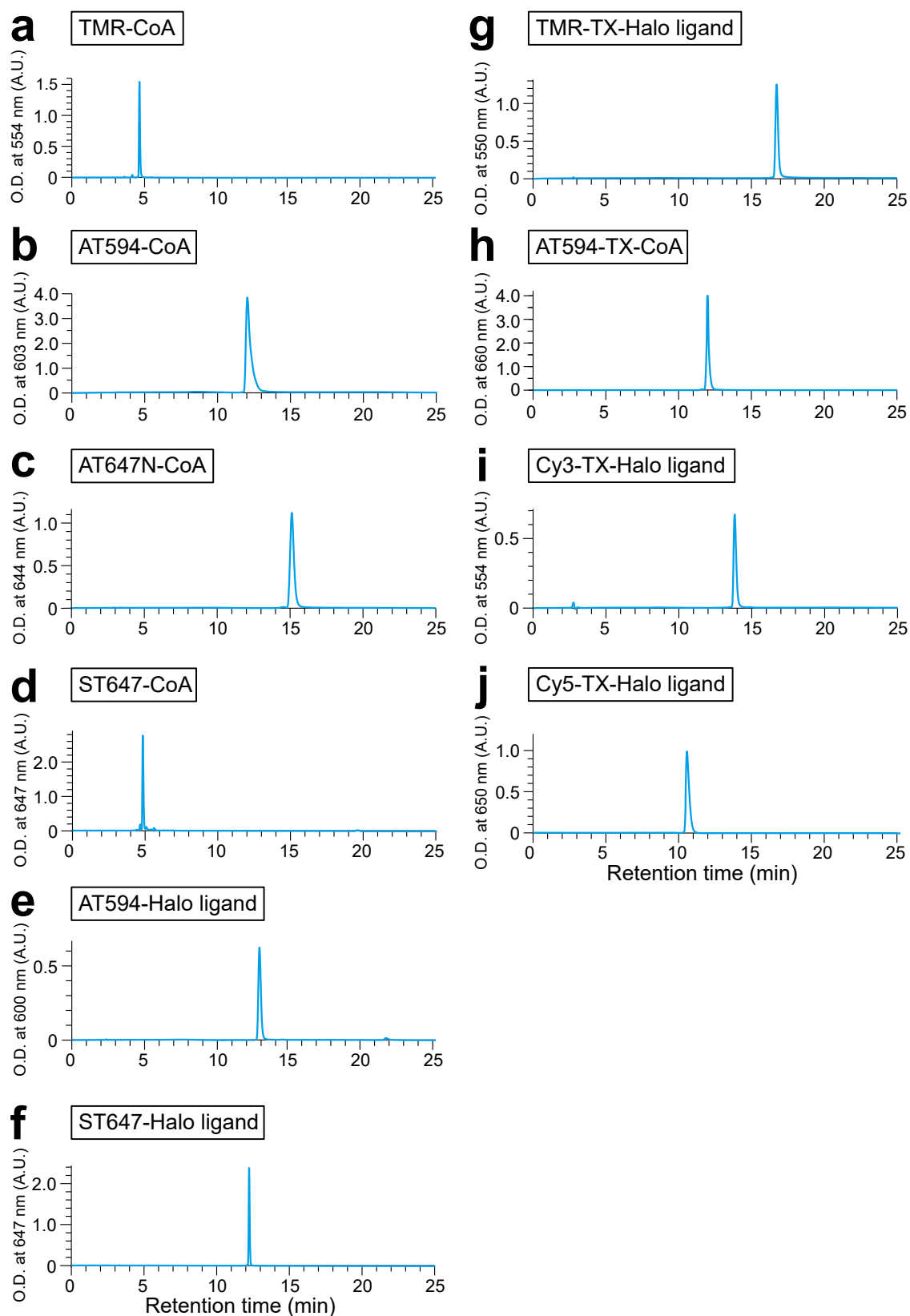
Supplementary Note 2 Figures



Supplementary Figure 45 | The scheme for synthesizing the Cy3- and Cy5-TX-Halo ligands.

a**b**

Supplementary Figure 46 | The schemes for synthesizing the AT594-TX-CoA (**a**) and TMR-TX-Halo (**b**) ligands.



Supplementary Figure 47 | LC-MS elution profiles of the final products synthesized in this study.

Supplementary Note 2 References

(Continued from Main Text, Methods, Supplementary Information, and Supplementary Note 1)

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85. Koufaki, M. *et al.* Synthesis of chroman analogues of lipoic acid and evaluation of their activity against reperfusion arrhythmias. *Bioorg. Med. Chem.* **12**, 4835–4841 (2004).