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Supplemental Information

High-Affinity Bent β_2-Integrin Molecules in Arresting Neutrophils Face Each Other through Binding to ICAMs In *cis*

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Supplemental Figures:

Figure S1

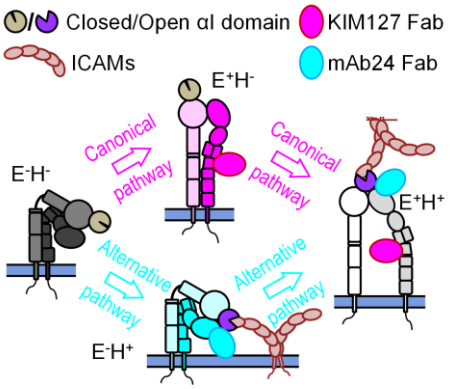


Figure S1. Two activation pathways and four conformations of β_2 integrins, Related to Figure 7

KIM127 (magenta) can specifically detect integrin extension (E^+) and mAb24 (cyan) can specifically detect headpiece-opening (H^+). Switchblade pathway: E^-H^- ($KIM127^-mAb24^-$) → E^+H^-($KIM127^+mAb24^-$) → E^+H^+ ($KIM127^+mAb24^+$); Alternative pathway (Fan et al., 2016): E^-H^- ($KIM127^-mAb24^-$) → E^-H^+ ($mAb24^+KIM127^-$) → E^+H^+ ($KIM127^+mAb24^+$)[8].

Figure S2

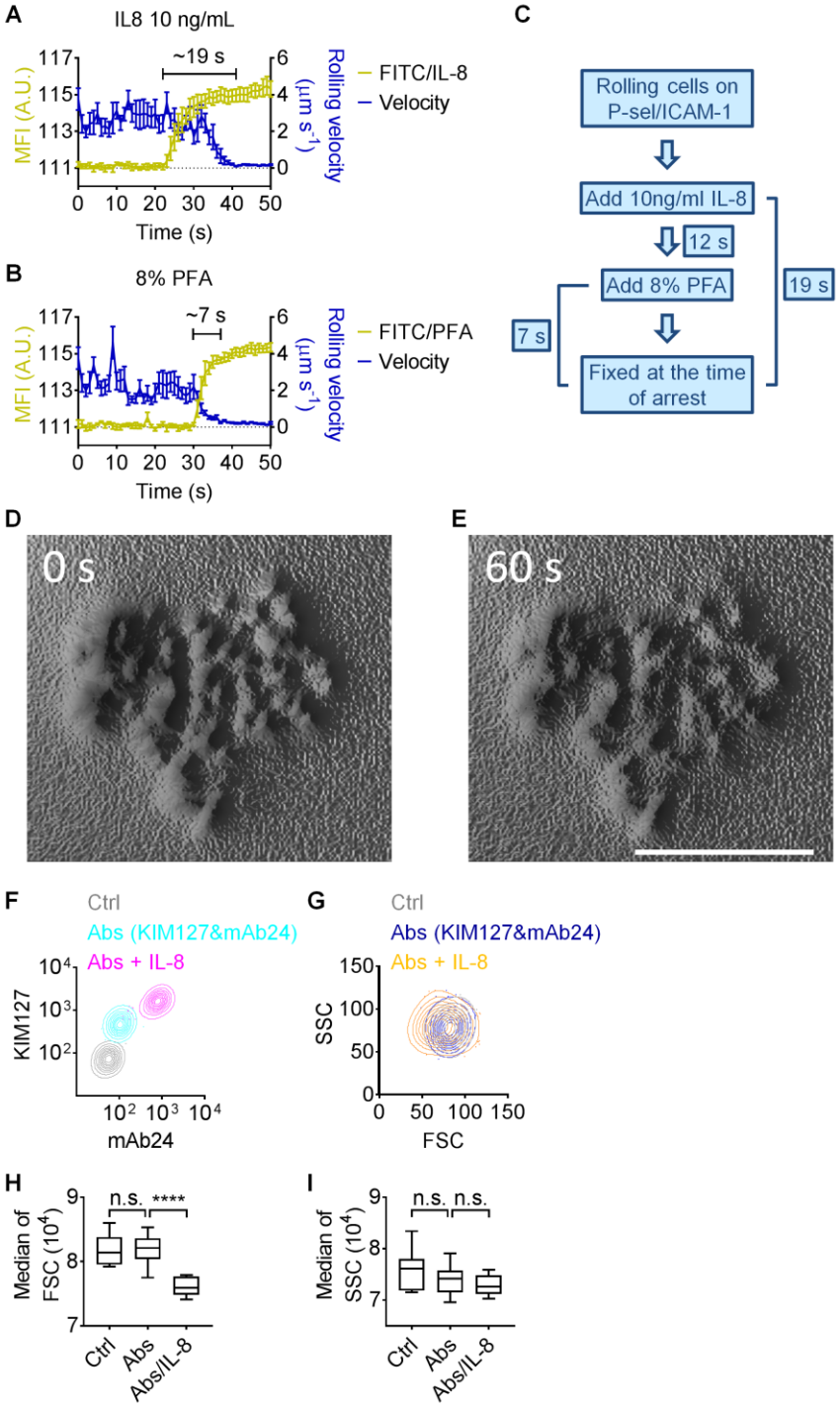

A
IL8 10 ng/mL
~19 s
MFI (A.U.)
Rolling velocity (μm s-1)
FITC/IL-8
Velocity
Time (s)
B
8% PFA
~7 s
FITC/PFA
Velocity
C
Rolling cells on P-sel/ICAM-1
Add 10ng/ml IL-8
12 s
Add 8% PFA
7 s
19 s
Fixed at the time of arrest
D
0 s
E
60 s
F
Ctrl
Abs (KIM127&mAb24)
Abs + IL-8
KIM127
mAb24
G
Ctrl
Abs (KIM127&mAb24)
Abs + IL-8
SSC
FSC
H
Median of FSC (10^4)
n.s.
****
Ctrl
Abs
Abs/IL-8
I
Median of SSC (10^4)
n.s.
n.s.
Ctrl
Abs
Abs/IL-8

Figure S2. Fixing neutrophils at the time of arrest, Related to Figure 1

(A-B) Primary human neutrophils were allowed to roll on coverslips coated with P-selectin (2 μg/ml) and ICAM-1 (10 μg/ml) at a wall shear stress of 6 dyn/cm^2. Then IL-8 (10 ng/ml, A) or PFA (8%, B) was perfused and the rolling velocity was monitored (blue curves) to identify the arrest or fixation, respectively. FITC was perfused together to monitor the arrival of IL-8 or PFA (yellow curves). IL-8 takes ~19 seconds to arrest neutrophils. PFA takes ~7s to fix neutrophils. Mean ±SD, n=10 (C) To fix neutrophils at the time of arrest, PFA was added 12s after IL-8. (D-E) Top-view 3D topography of neutrophil footprint before (D) and sixty seconds after (E) fixation in 8% PFA. The cell membrane was identified by the labeling of GPI-anchored protein CD16 (AF488 conjugated antibody, clone 3G8) on neutrophils. Microvilli were identified as hills in the topography. (F-I) IL-8 stimulation but not antibody incubation affects the phenotype of neutrophils. Changes of KIM127/mAb24 MFI (F) and FSC/SSC (G) before (grey) and after adding antibodies (cyan in F, blue in G) and IL-8 (magenta in F, orange in G) were shown as contour plots. Medians of FSC (H) and SSC (I) of neutrophils before and after adding antibodies and IL-8 from five independent experiments were shown. n.s. $P>0.05$, ****$p<0.0001$ by one-way ANOVA test.

Figure S3

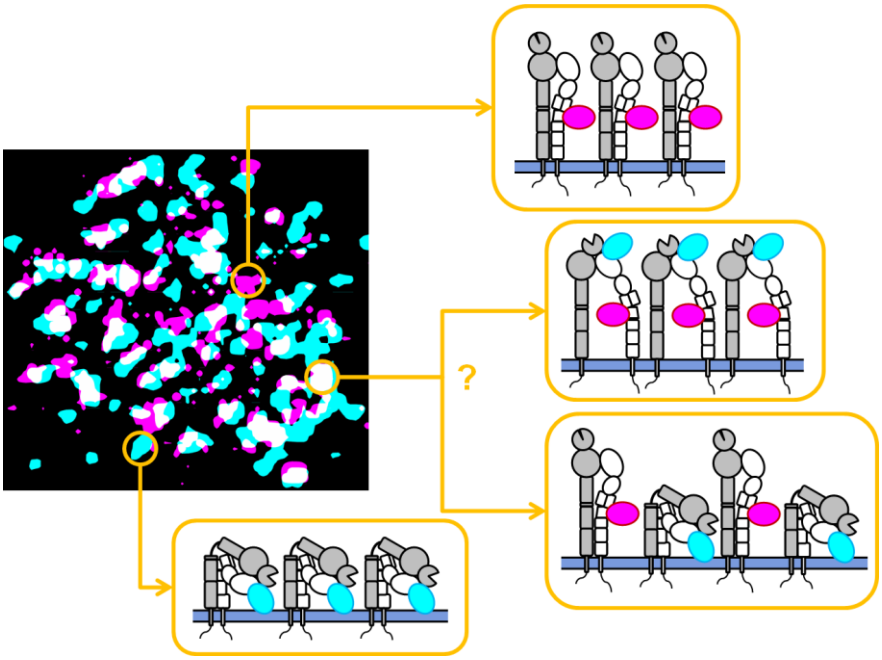


Figure S3. TIRF has insufficient resolution to identify true E^+H^+ β_2 integrin clusters, Related to Figure 1

In the binary cluster image of β_2 integrin activation on a typical TIRF footprint of an arrested human neutrophil, a magenta cluster contains mostly $KIM127^+mAb24^-$ (E^+H^-, top right), a cyan cluster contains mostly $KIM127^-mAb24^+$ (E^-H^+, bottom left), but when KIM127 and mAb24 are colocalized in a white cluster, this cluster may contain E^+H^+ β_2 integrins (middle right) or a mixture of E^+H^- and E^-H^+ β_2 integrins (bottom right).

Figure S4

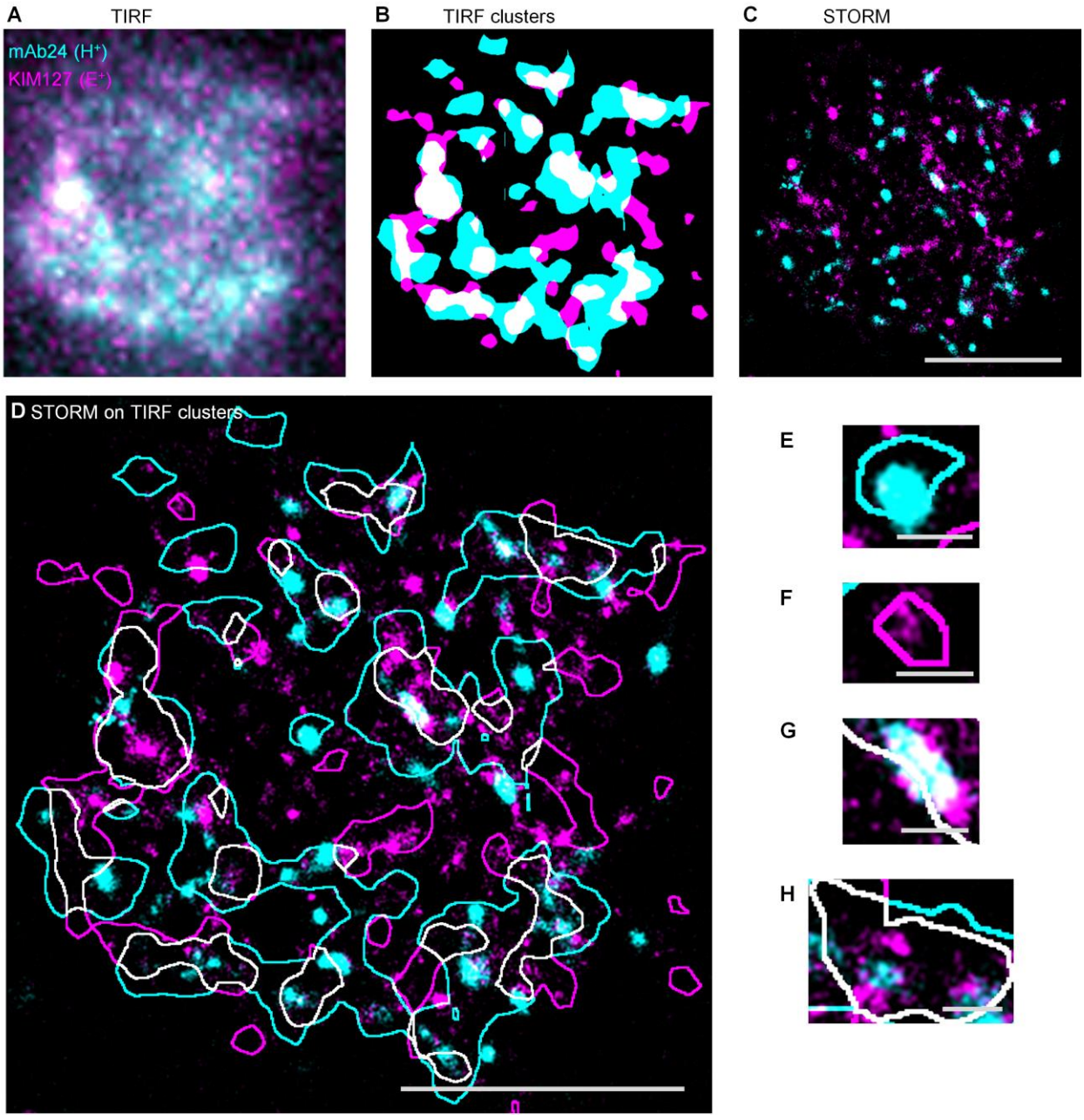


Figure S4. Super-resolution STORM imaging of β_2 integrin activation on arrested human monocytes, Related to Figure 1

(A) Primary human monocytes rolling on P-selectin and ICAM-1 at a wall shear stress of 6 dyn/cm^2 were exposed to CCL2 and Fab fragments of the β_2 integrin extension reporter KIM127 (E^+, magenta) and the high-affinity reporter mAb24 (H^+, cyan), immediately fixed and imaged by

TIRF (entire TIRF footprint, raw image shown). (B) Binary image of A using smart segmentation as in (Fan et al., 2016) (C) STORM buffer was introduced and blinking events were recorded for 10,000 frames per channel over 10 minutes, corrected for stochastic motion and drift as in STAR Methods to obtain a raw super-resolution STORM image of the footprint of arrested human monocytes. (D-H) STORM image overlaid with the outlines of binary TIRF clusters (from B). Zoomed-in examples of $E^{-}H^{+}$ (cyan, E) and $E^{+}H^{-}$ TIRF clusters (magenta, F). Some white $E^{+}H^{+}$ TIRF clusters showed true colocalization of KIM127 and mAb24 in STORM (G**,** white). H shows examples of clusters that appeared colocalized in TIRF but were truly composed of $E^{-}H^{+}$ and $E^{+}H^{-}$ areas as revealed by STORM. Scale bars are 3μm for A-D, and 300nm for E-H.

Figure S5

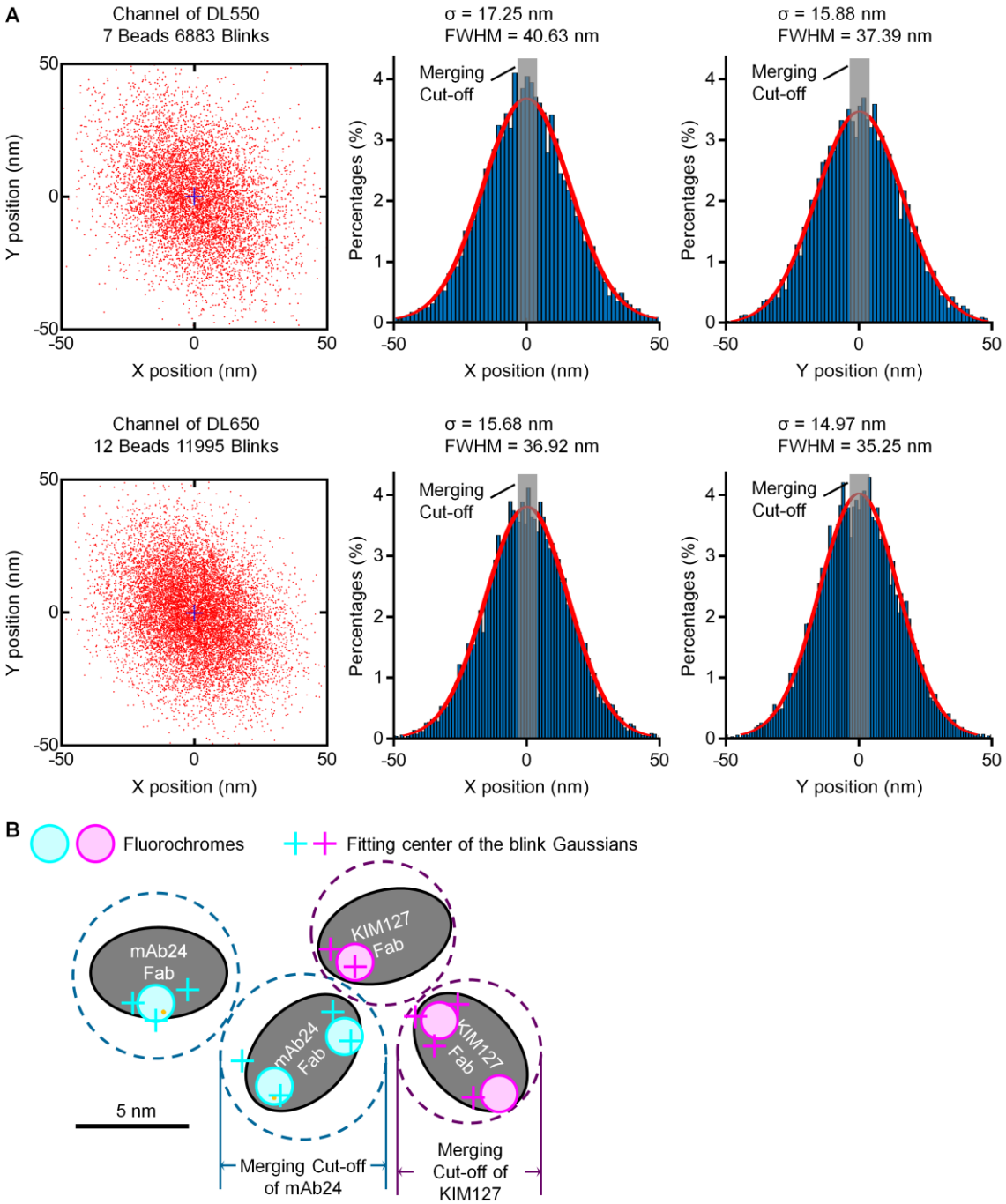

A
Channel of DL550
7 Beads 6883 Blinks
σ = 17.25 nm
FWHM = 40.63 nm
σ = 15.88 nm
FWHM = 37.39 nm
Merging
Cut-off
Y position (nm)
X position (nm)
Percentages (%)
Channel of DL650
12 Beads 11995 Blinks
σ = 15.68 nm
FWHM = 36.92 nm
σ = 14.97 nm
FWHM = 35.25 nm
B
Fluorochromes
Fitting center of the blink Gaussians
mAb24
Fab
KIM127
Fab
5 nm
Merging Cut-off
of mAb24
Merging
Cut-off of
KIM127

Figure S5. STORM resolution, Related to Figure 1 and 3

(A) Position as determined by molecular modeling (merging cut-off, grey) overlaid on the Gaussian of localization accuracy. The resolution of STORM imaging is defined as σ of the Gaussian. The localization data (left column), X localization accuracy (middle column), and Y localization accuracy (right column) of the two channels (upper row: DL550 for mAb24; lower row: DL650 for KIM127). (B) Schematic showing the merging of multiple blinks (crosses) of individual fluorochromes (circles) and multiple fluorochromes (circles) on the same Fab (grey) as shown in Fig. 3H-I (random simulation). All multiple blinks, whether from one fluorochrome or multiple fluorochromes in the same Fab, are merged to represent one Fab.

Figure S6

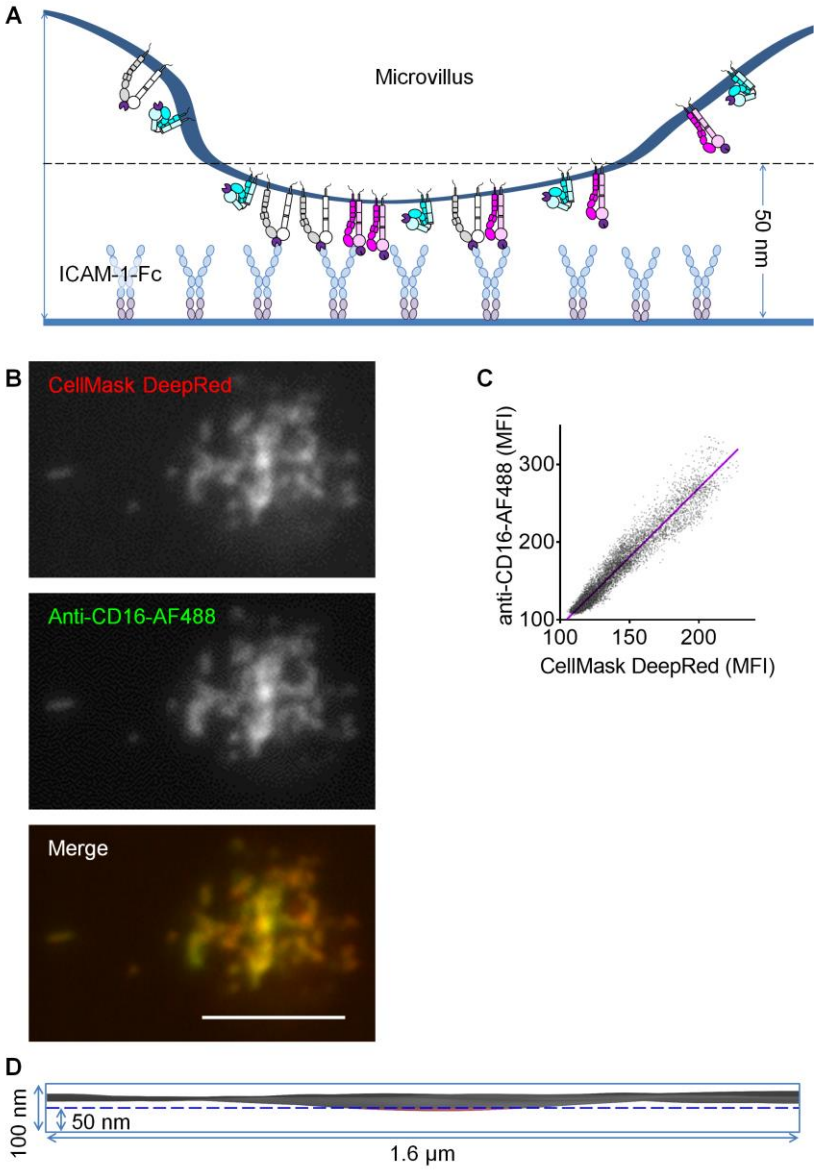
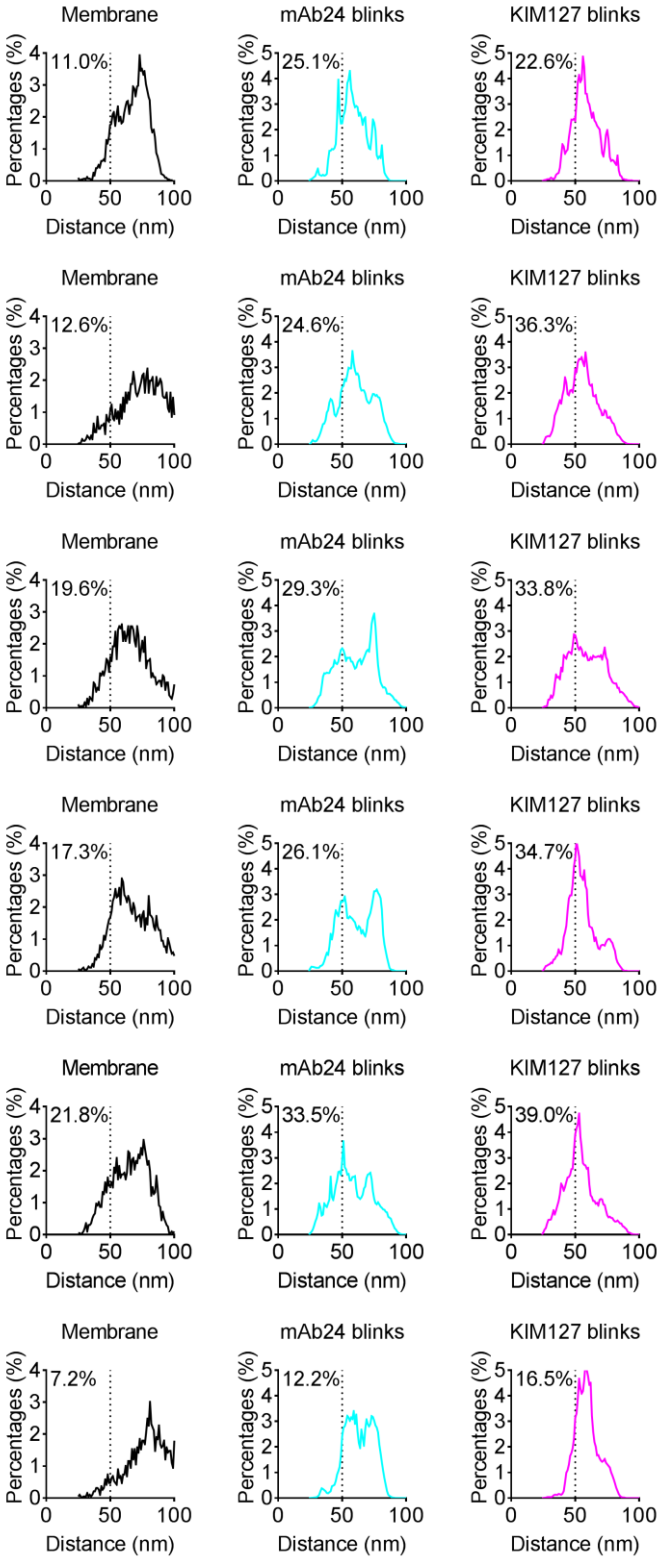

A
Microvillus
50 nm
ICAM-1-Fc
B
CellMask DeepRed
Anti-CD16-AF488
Merge
C
anti-CD16-AF488 (MFI)
300
200
100
100
150
200
CellMask DeepRed (MFI)
D
100 nm
50 nm
1.6 µm

Figure S6. The area within 50 nm of the substrate contains the E^+H^+ integrin molecules that can bind ICAM-1 in trans, Related to Figure 2

(A) Schematics showing E^-H^+ (cyan), E^+H^- (magenta), and E^+H^+ (white) integrins on a microvillus of the neutrophil. Only integrins within 50 nm of the substrate can bind the ligand (human recombinant ICAM-1-Fc) in trans and support neutrophil arrest. The area within 50 nm of the substrate is at the tip of microvilli and nearly parallel to the substrate. (B) Typical TIRF images of a neutrophil cell footprint labeled with both the cell membrane dye CellMask DeepRed (Red) and AF488-conjugated CD16 antibody (Green). The scale bar is 5 μm. (C) The pixel-to-pixel correlation of the MFI of CellMask DeepRed and AF488-conjugated CD16 antibody. (D) 3D topography of a microvillus with X, Y, and Z at the same scale. Data from a TIRF image of anti-CD16-AF488. The area within 50 nm of the substrate is highlighted in red.

93 Figure S7



94
95

Figure S7. The proportion of cell footprint and integrin activation within 50 nm of the coverslip, Related to Figure 2.

Histograms showing distributions of distances between the neutrophil membrane and the coverslip (left panels), mAb24 blinks and the coverslip (middle panels), and KIM127 blinks and the coverslip (right panels) from six cells.

Figure S8

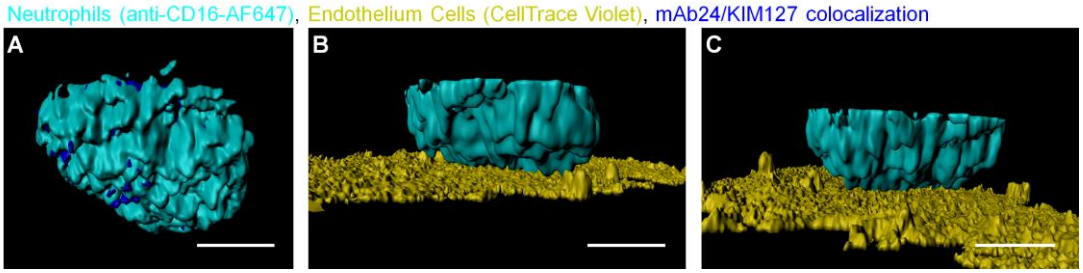


Figure S8. The 3D reconstruction of a neutrophil adhered on endothelial cell monolayers (HUVECs) acquired by the AiryScan confocal imaging, Related to Figure 2.

(A) Bottom view of a neutrophil adhered on endothelial cell monolayers under a shear stress of 2 dyn cm^{-2}. The arrest is triggered by the perfusion of 10 ng ml^{-1} IL-8. The neutrophil surface was labeled with AF647-conjugated anti-CD16 (cyan). Clusters of $E^{+}H^{+}$ (blue) β_2 integrins were observed. Clusters of $E^{-}H^{+}$ and $E^{+}H^{-}$ were not shown here. (B-C) The side view of the neutrophil-HUVEC interaction. HUVECs were labeled with CellTrace Violet (yellow). A few protrusions of HUVECs were observed. Only one or two of them are under the neutrophil and fill the "valleys" of neutrophil microvilli. Most area of the endothelial surface has no protrusion.

Figure S9

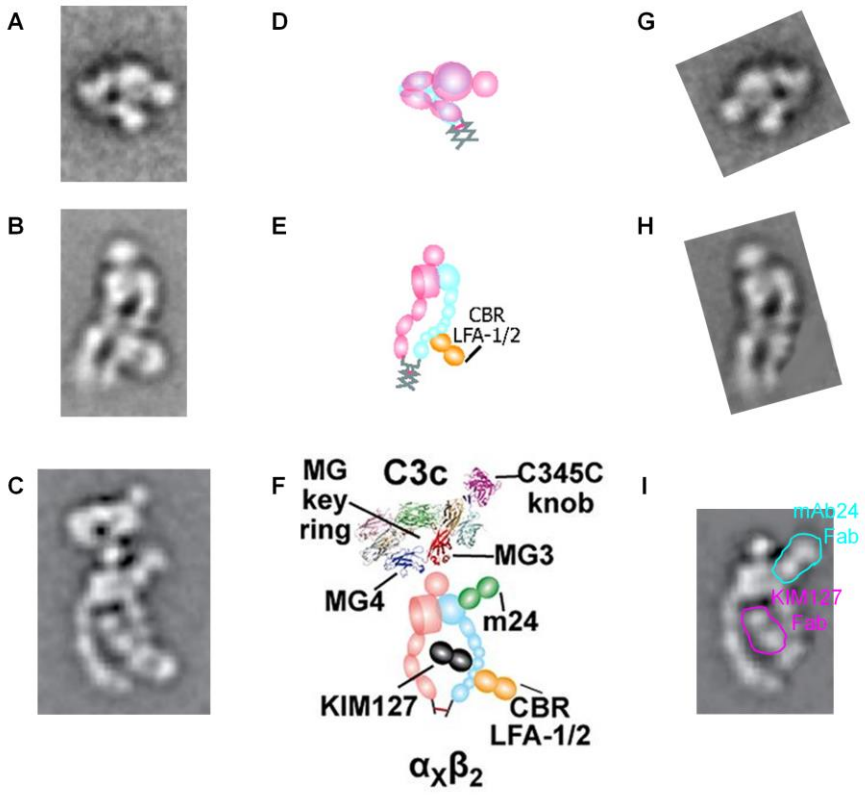

A
D
G
B
E
CBR
LFA-1/2
H
C
F
MG
key
ring
C3c
C345C
knob
MG3
MG4
m24
KIM127
CBR
LFA-1/2
αXβ2
I
mAb24
Fab
KIM127
Fab

Figure S9. The modification of EM images in Fig 2, Related to Figure 3

(A-B) EM images of bent (E^-, A) and extended (E^+, B) $\alpha_X\beta_2$ were adopted directly from Fig. 2A (right panel) and B (left panel) in Chen et al., 2010, respectively. The E^+ $\alpha_X\beta_2$ was bound with the Fab of monoclonal antibody CBR LFA-1/2. (C) The EM image of extended-open (E^+H^+) $\alpha_X\beta_2$ was adopted directly from Fig. 2B (panel 1) in Chen et al., 2012. The E^+H^+ $\alpha_X\beta_2$ was bound with the ligand C3c, Fabs of monoclonal antibodies mAb24 (m24), KIM127, and CBR LFA-1/2. (D-F) Schematics of the EM images were shown. D and E were adopted directly from Fig. 2A and B in Chen et al., 2010, respectively. F was adopted directly from Fig. 2C in Chen et al., 2012. (G-I) EM images of E^-, $E^{+,}$ and E^+H^+ β_2 integrins showed in Figure 3B. G was rotated and flipped horizontally from A. H was rotated from B, and the Fab of CBR LFA-1/2 was photoshopped out. In I, The ligand C3c and the Fab of CBR LFA-1/2 were photoshopped out from C. The Fabs of mAb24 and KIM127 were circled by cyan and magenta, and labeled, respectively.

Figure S10

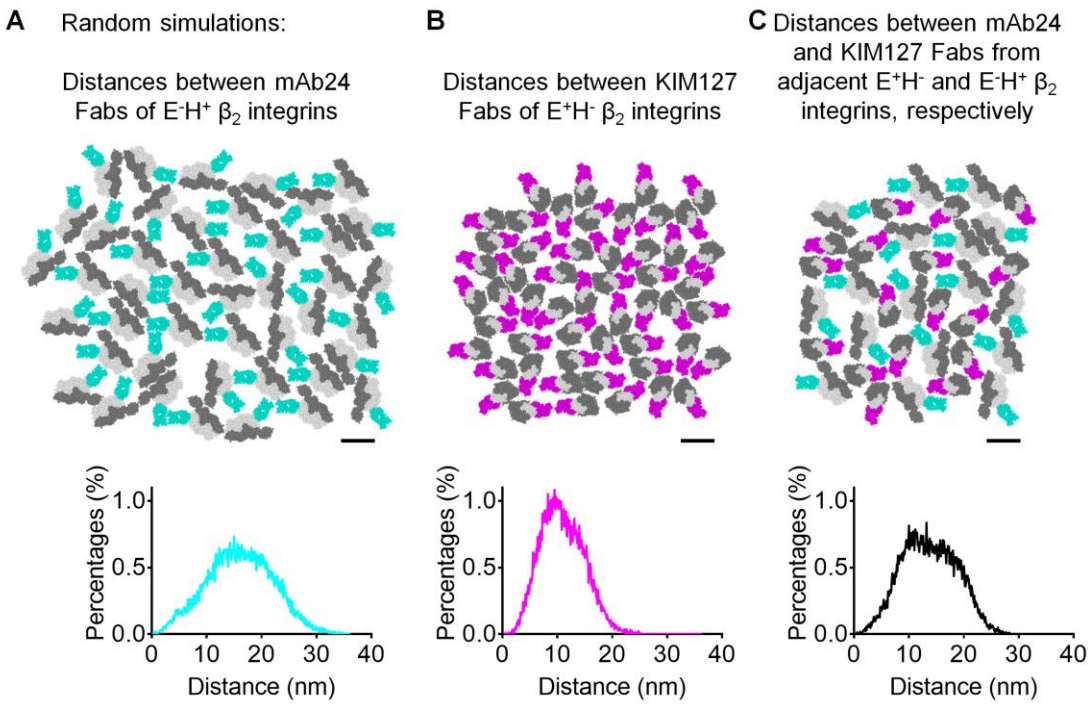
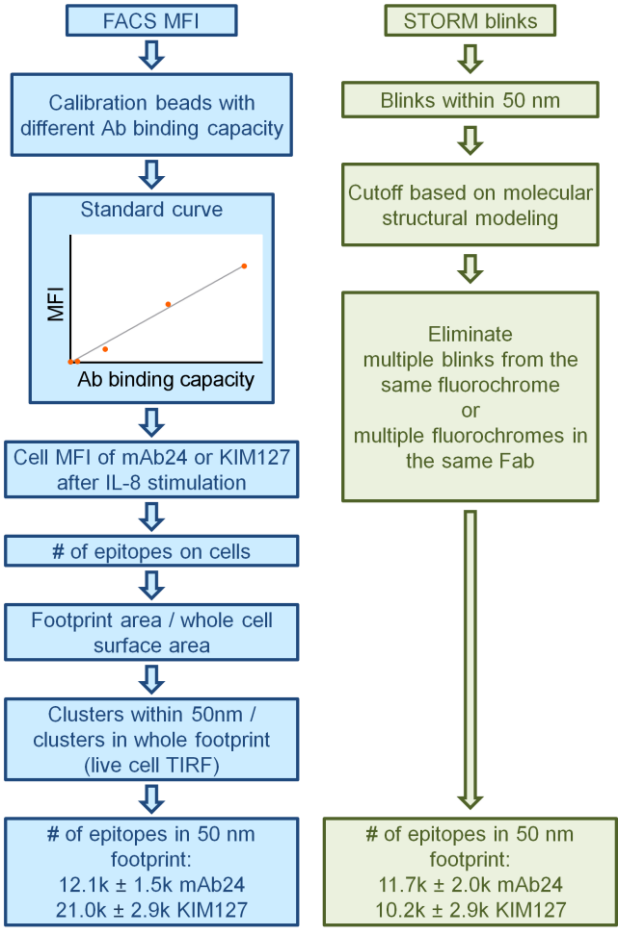


Figure S10. Random simulation of activated β_2 integrins in top view, Related to Figure 3 and 4

Space-filling top views of E^-H^+ (A), E^+H^- (B) β_2 integrins, and the mixture of E^-H^+ and E^+H^- (C) β_2 integrins with random adjacent directions and orientations. The histograms below show the distribution of distances between two blinks from two mAb24 Fabs in A (cyan curve), two KIM127 Fabs in B (magenta curve), one mAb24 Fab and one KIM127 Fab in C (black curve), respectively. Each histogram contains data from 1,000 space-filling random simulations of 64 conditions (8 directions × 8 orientations). Scale bars are all 10nm.

144 Figure S11



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146

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Figure S11. Comparison of the number of activated β_2 integrin molecules in footprints of arrested neutrophils based on flow cytometry (MFI, left) or STORM (right), Related to Figure 3

In the flow cytometry assay, calibration beads with five different antibody binding capacities were used. After incubating with the same antibody used for staining the cells, five different MFI were obtained. The standard curve correlates MFI with the number of epitopes (exactly one KIM127 or mAb24 epitope per integrin). MFI of mAb24 and KIM127 were determined on human neutrophils after IL-8 stimulation, yielding the number of mAb24 and KIM127 epitopes on the whole cell surface. This is multiplied with the ratio of the footprint area to the whole cell surface area as estimated by the cell diameter in epifluorescence imaging. This yields the number of $mAb24^+$ or $KIM127^+$ β_2 integrin molecules in the footprint. From the TIRF images of live neutrophils, at the time of arrest, we estimate the percentage of integrins within 50 nm to the substrate. This yields the number of $mAb24^+$ or $KIM127^+$ β_2 integrin molecules within 50 nm to the substrate. In STORM imaging, after filtering out blinks 50 nm away from the substrate, merging by the cutoff obtained from molecular modeling, the number of mAb24 and KIM127 epitopes is counted in the footprint. Both methods yield comparable numbers of mAb24 and KIM127 epitopes.

Figure S12

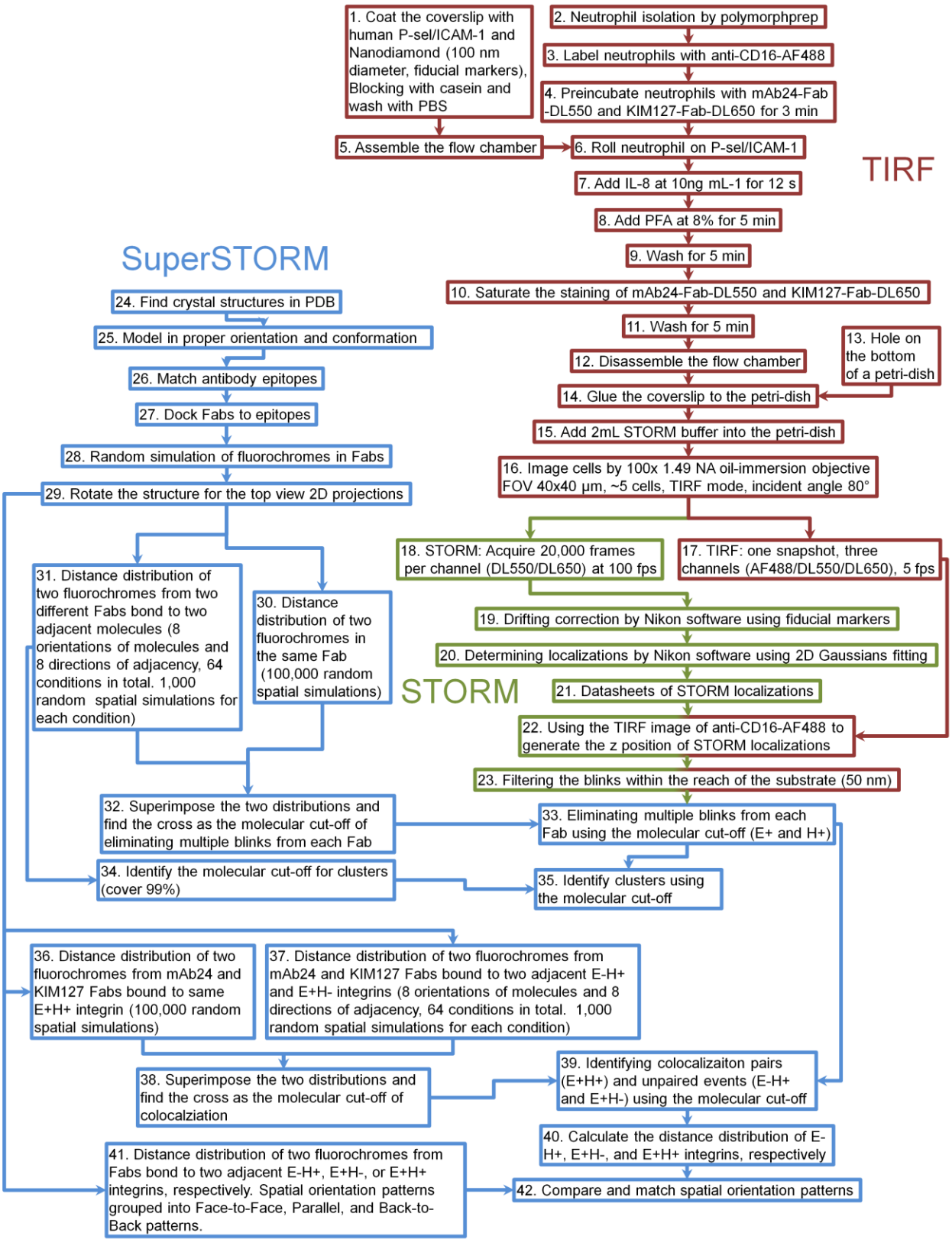
1. Coat the coverslip with human P-sel/ICAM-1 and Nanodiamond (100 nm diameter, fiducial markers), Blocking with casein and wash with PBS
2. Neutrophil isolation by polymorphprep
3. Label neutrophils with anti-CD16-AF488
4. Preincubate neutrophils with mAb24-Fab -DL550 and KIM127-Fab-DL650 for 3 min
5. Assemble the flow chamber
6. Roll neutrophil on P-sel/ICAM-1
TIRF
7. Add IL-8 at 10ng mL-1 for 12 s
8. Add PFA at 8% for 5 min
9. Wash for 5 min
10. Saturate the staining of mAb24-Fab-DL550 and KIM127-Fab-DL650
11. Wash for 5 min
12. Disassemble the flow chamber
13. Hole on the bottom of a petri-dish
14. Glue the coverslip to the petri-dish
15. Add 2mL STORM buffer into the petri-dish
16. Image cells by 100x 1.49 NA oil-immersion objective FOV 40x40 µm, ~5 cells, TIRF mode, incident angle 80°
17. TIRF: one snapshot, three channels (AF488/DL550/DL650), 5 fps
18. STORM: Acquire 20,000 frames per channel (DL550/DL650) at 100 fps
19. Drifting correction by Nikon software using fiducial markers
20. Determining localizations by Nikon software using 2D Gaussians fitting
STORM
21. Datasheets of STORM localizations
22. Using the TIRF image of anti-CD16-AF488 to generate the z position of STORM localizations
23. Filtering the blinks within the reach of the substrate (50 nm)
SuperSTORM
24. Find crystal structures in PDB
25. Model in proper orientation and conformation
26. Match antibody epitopes
27. Dock Fabs to epitopes
28. Random simulation of fluorochromes in Fabs
29. Rotate the structure for the top view 2D projections
30. Distance distribution of two fluorochromes in the same Fab (100,000 random spatial simulations)
31. Distance distribution of two fluorochromes from two different Fabs bond to two adjacent molecules (8 orientations of molecules and 8 directions of adjacency, 64 conditions in total. 1,000 random spatial simulations for each condition)
32. Superimpose the two distributions and find the cross as the molecular cut-off of eliminating multiple blinks from each Fab
33. Eliminating multiple blinks from each Fab using the molecular cut-off (E+ and H+)
34. Identify the molecular cut-off for clusters (cover 99%)
35. Identify clusters using the molecular cut-off
36. Distance distribution of two fluorochromes from mAb24 and KIM127 Fabs bound to same E+H+ integrin (100,000 random spatial simulations)
37. Distance distribution of two fluorochromes from mAb24 and KIM127 Fabs bound to two adjacent E-H+ and E+H- integrins (8 orientations of molecules and 8 directions of adjacency, 64 conditions in total. 1,000 random spatial simulations for each condition)
38. Superimpose the two distributions and find the cross as the molecular cut-off of colocalziation
39. Identifying colocalizaiton pairs (E+H+) and unpaired events (E-H+ and E+H-) using the molecular cut-off
40. Calculate the distance distribution of E-H+, E+H-, and E+H+ integrins, respectively
41. Distance distribution of two fluorochromes from Fabs bond to two adjacent E-H+, E+H-, or E+H+ integrins, respectively. Spatial orientation patterns grouped into Face-to-Face, Parallel, and Back-to-Back patterns.
42. Compare and match spatial orientation patterns

Figure S12. The workflow of experimental and image processing procedures, Related to STAR Methods.

Table S1. Colocalization assays of anti-CD16-AF488 and CellMask DeepRed on neutrophils, Related to Figure 2

Cell #	Linear Correlation R^2	Person's R value (-0.12 to 1)	Li's ICQ value (-0.5 to0.5)	Kendall's Tau-b rank correlation value (-1 to 1)	Manders' M1 (0 to1)	Manders' M2 (0 to1)	Costes P-Value
1	0.9445	0.97	0.448	0.7822	1	1	1
2	0.9534	0.98	0.465	0.7472	1	1	1
3	0.9297	0.96	0.452	0.7243	1	1	1
4	0.9281	0.96	0.469	0.7408	1	1	1
5	0.9488	0.97	0.469	0.7944	1	1	1
6	0.9282	0.96	0.451	0.8093	1	1	1
Mean	0.9388	0.97	0.459	0.7664	1	1	1
SD	0.0105	0.01	0.009	0.0307	0	0	0

Table S2. Confidence intervals for the molecular cut-off in random simulations, Related to Figure 3

Cut-off for:	Value (nm)	False Negative Rate	False Positive Rate	Relative Figure
Merging H^+ blinks	7.3	0.93%	7.78%	Fig. 2l
Merging E^+ blinks	6.1	3.19%	11.47%	Fig. 2m
Colocalization	8.2	6.24%	14.36%	Fig. 3a

Table S3. Number of mAb24 (H^+)and KIM127 (E^+) events in each microllus tip within 50 nm of the coverslip, Related to Figure 2-4

Number	H^+	E^+	E^+H^+	Number	H^+	E^+	E^+H^+
Cell 1							
1	557	324	180	21	4	3	0
2	50	36	5	22	1393	1360	571
3	159	41	32	23	183	94	17
4	91	24	5	24	66	42	15
5	1	3	0	25	1	0	0
6	127	161	21	26	95	68	9
7	3	5	0	27	40	31	6
8	35	108	16	28	31	7	3
9	2	7	0	29	480	179	146
10	28	25	10	30	34	30	13
11	631	786	319	31	2	77	0
12	968	733	419	32	51	7	4
13	2558	2399	1323	33	37	16	6
14	2	0	0	34	12	18	9
15	716	669	263	35	0	0	0
16	1	2	0	36	28	52	2

17	0	2	0	37	27	63	10
18	29	8	2	38	211	341	179
19	10	7	1	39	3	5	0
20	1	3	0	40	3030	2493	1039
Cell 2							
1	4	18	1	20	1	10	0
2	136	363	44	21	0	7	0
3	75	205	25	22	41	144	22
4	90	143	40	23	35	80	20
5	16	41	11	24	0	1	0
6	6	41	4	25	1295	3100	632
7	0	1	0	26	50	150	45
8	98	318	81	27	43	125	12
9	5	33	4	28	161	660	75
10	3	39	2	29	12	58	5
11	5	31	4	30	6	52	2
12	19	55	10	31	796	1645	415
13	51	102	16	32	414	637	320
14	27	99	17	33	77	62	29
15	580	948	270	34	903	3028	496

16	2	15	1	35	1	4	0
17	18	32	10	36	0	5	0
18	1251	2578	736	37	3	27	0
19	2	8	0	38	7951	27724	6882
Cell 3							
1	0	1	0	12	4	36	4
2	0	1	0	13	87	334	45
3	0	2	0	14	17	126	5
4	1	4	0	15	5	16	2
5	12	116	7	16	0	2	0
6	18	113	7	17	821	4439	393
7	5	4	0	18	22	200	11
8	1	138	1	19	12	53	6
9	1	23	0	20	0	2	0
10	37	37	12	21	46	223	26
11	273	1104	94	22	6444	29941	2966
n	100	100	100	Average	336.80	897.33	184.35
SD	1093.87	4064.21	762.06	SEM	109.39	406.42	76.21

Table S4. Comparison of TIRF, STORM, and SuperSTORM in this study, Related to STAR Methods

	TIRF	STORM	SuperSTORM
Principle	Total internal reflective, evanescent wave illumination	TIRF based, STORM buffer, stochastic blinks, 2D Gaussian fitting	molecular modeling and spatial simulation to refine STORM data
Detector	ANDOR IXON3 Ultra DU897 EMCCD camera	ANDOR IXON3 Ultra DU897 EMCCD camera	ANDOR IXON3 Ultra DU897 EMCCD camera
Excitation	488 nm 561 nm 647 nm	561 nm 647 nm	561 nm 647 nm
Emission	503-550 nm 576-621 nm 658-800 nm	576-621 nm 658-800 nm	576-621 nm 658-800 nm
Resolution	~300 nm	~15 nm	~6 nm depending on molecular size

Supplemental Movies

Supplemental Movie 1. Structure of E^-H^+ β_2 integrin with mAb24 Fab bound, Related to Figure 3

E^-H^+ β_2 integrin is from the published crystal structure (PDB: 4NEH). α-chain in grey and β-chain in white. mAb24 Fab (cyan) is docked to its binding site.

Supplemental Movie 2. Structure of E^+H^- β_2 integrin with KIM127 Fab bound, Related to Figure 3

E^+H^- β_2 integrin is modeled by unfolding the headpiece of E^-H^- (PDB: 3K6S) to the extension. α-chain in grey and β-chain in white. KIM127 Fab (magenta) is docked to its binding site.

Supplemental Movie 3. Structure of E^+H^+ β_2 integrin with mAb24 and KIM127 Fabs bound, Related to Figure 3

For the E^+H^+ β_2 integrin modeling, the hybrid domain swing out of E^-H^+ β_2 integrin (PDB: 4NEH) is superposed with PDB: 3FCU, then the headpiece is unfolded. α-chain in grey and β-chain in white. mAb24 (cyan) and KIM127 (magenta) Fabs are docked to their binding sites. EM image of E^+H^+ β_2 integrin with mAb24 and KIM127 Fabs bound (Figure 3B, bottom) is used as a reference.