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Polarized focal adhesion kinase activity within a focal adhesion during cell migration

In the format provided by the authors and unedited

Supplemental Information

1. Supplementary Figures 1-24

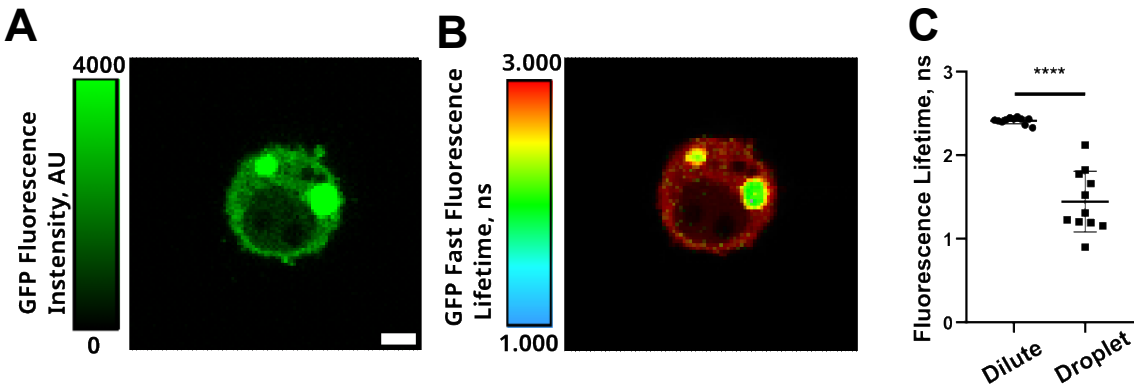
2. Supplementary Tables 1-2

3. Supplementary Movies 1-15.

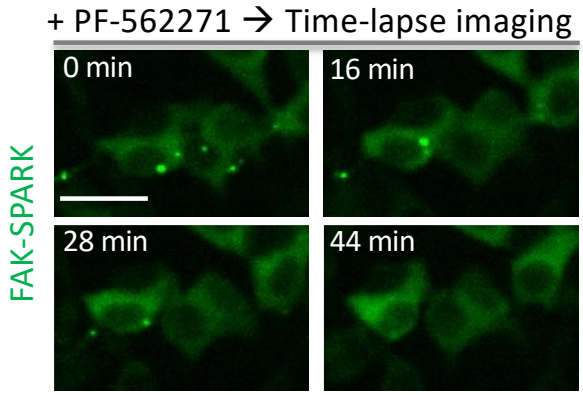
4. Supplementary References

5. Uncropped data of blots in Supplementary Figure 9A

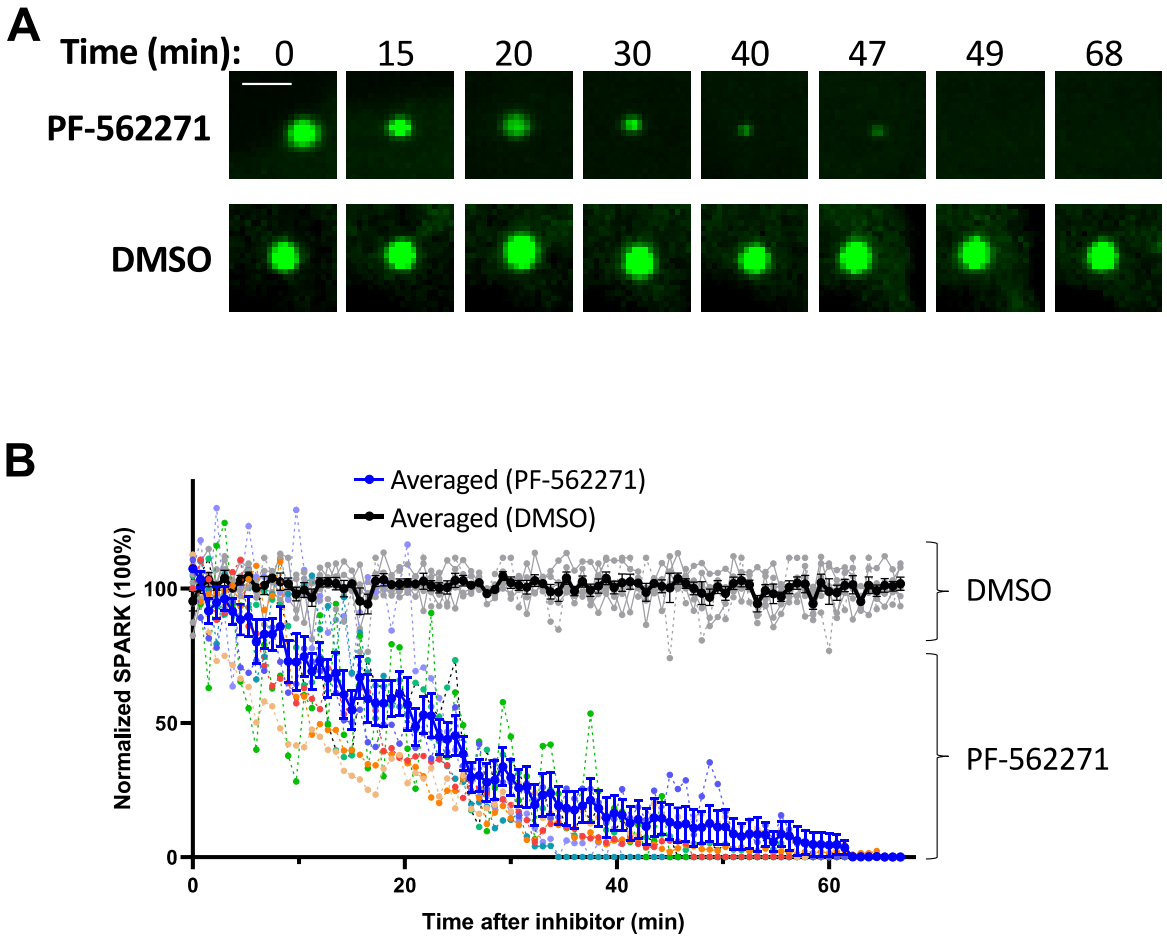
Supplementary Figures



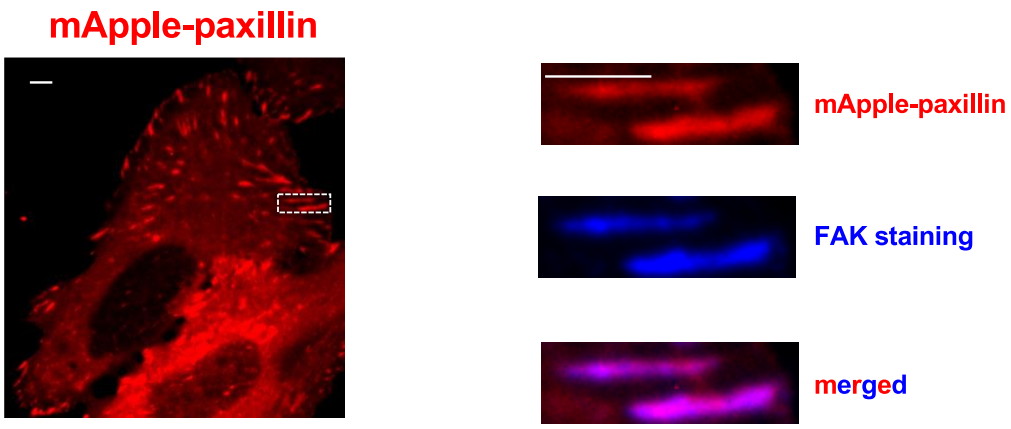
Supplementary Figure 1. Fluorescence lifetime of GFP in diffuse and condensed states. A, Fluorescence intensity images of GFP in cells expressing FAK-SPARK. This observation is reproducible in 11 cells tested. **B**, Fluorescence lifetime images of GFP in cells expressing FAK-SPARK using fast fluorescence lifetime imaging microscopy (FLIM). **C**, Quantification of GFP fluorescence lifetime in diffuse state and droplets. Average lifetime is 1.445 ns for GFP in FAK-SPARK droplets and 2.415 ns for GFP in diffuse state. Data represent mean ± SD (n = 11 cells) $p=2.71x10^{-8}$. Scale bar, 5 µm. ****, p value < 0.001



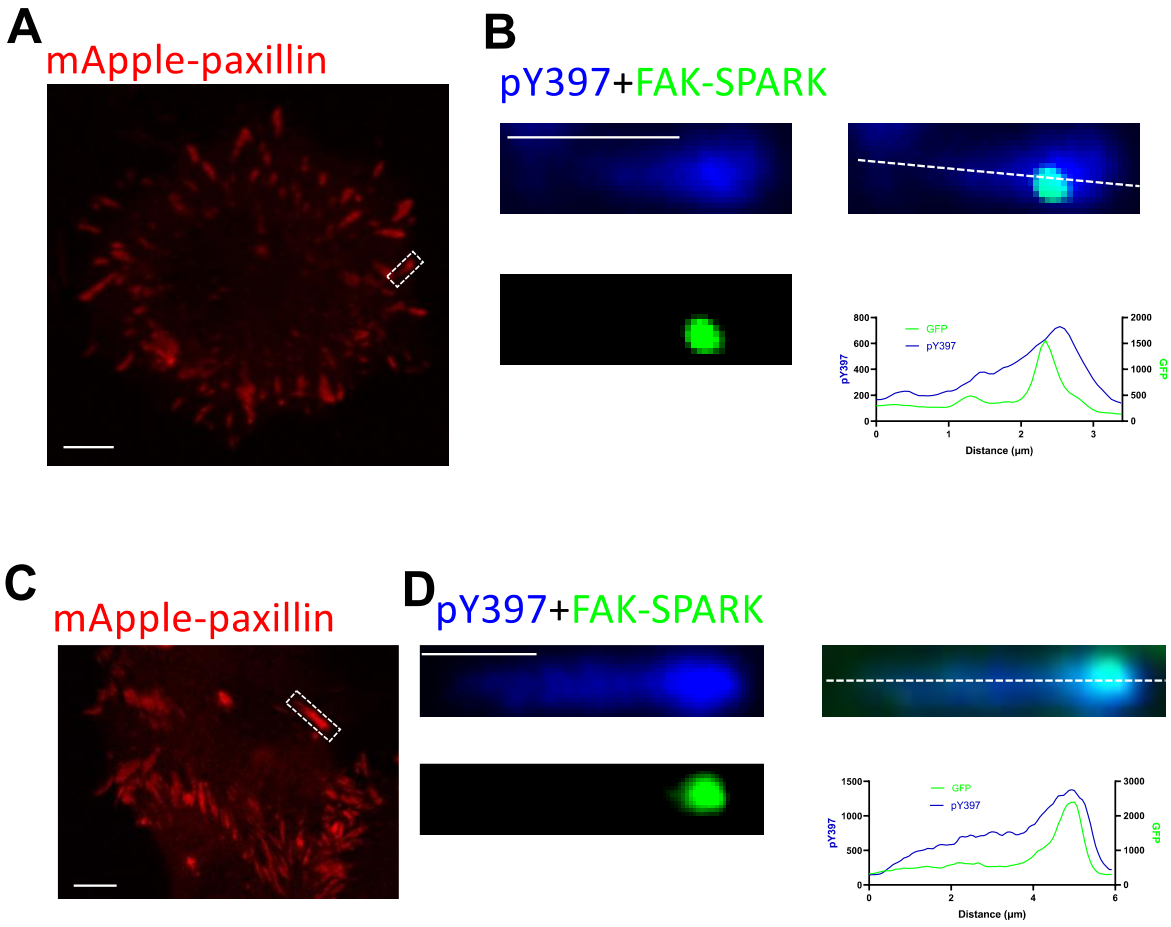
Supplementary Figure 2. FAK-SPARK is reversible. HEK293 cells expressing FAK-SPARK was incubated with FAK inhibitor PF-562271, followed by time-lapse imaging. This experiment was repeated for three times independently with similar results. Scale bar, 10 µm.



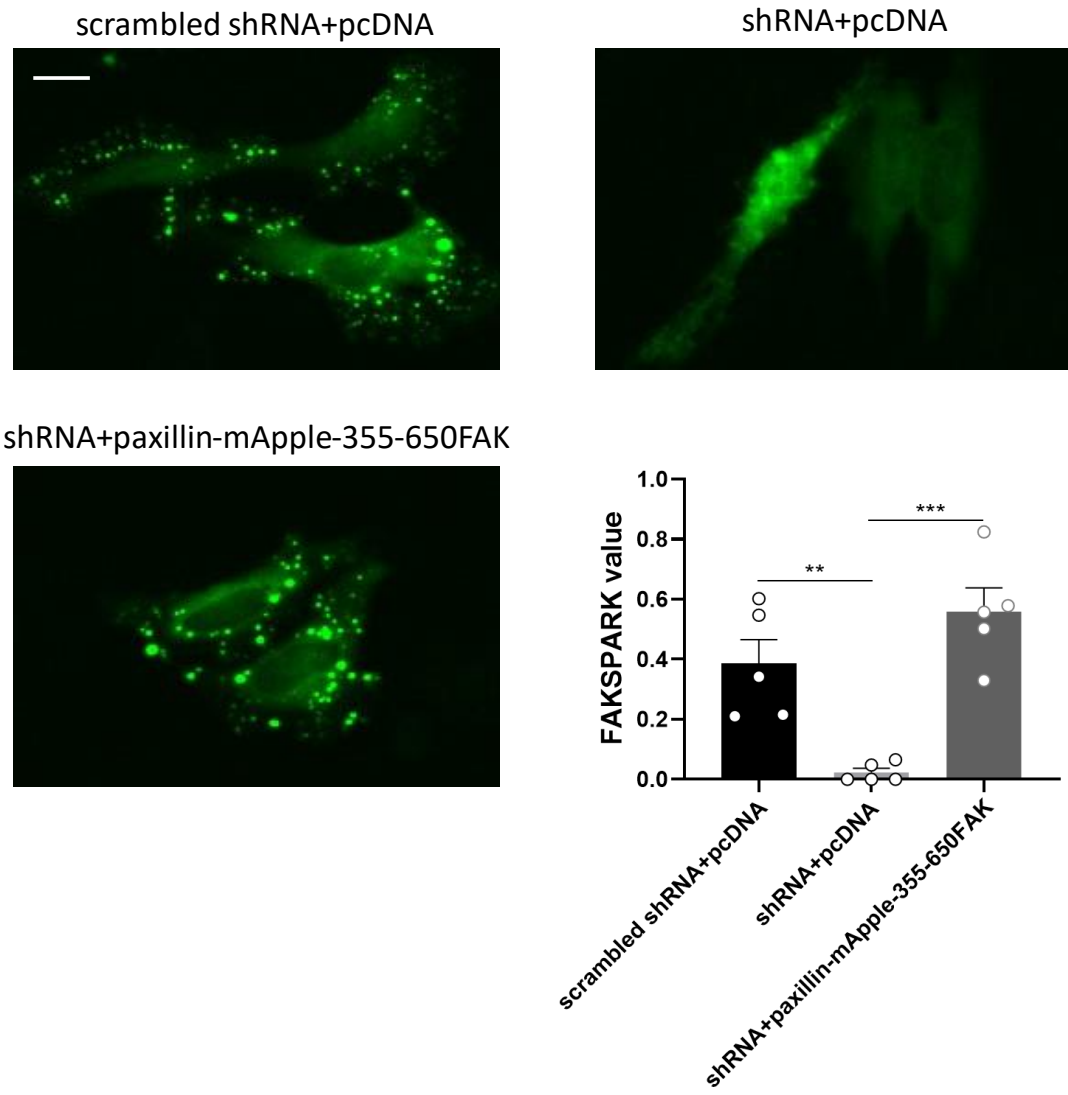
Supplementary Figure 3. Disassembly of single FAK-SPARK droplets upon PF-562271 treatment. HEK293 cells expressing FAK-SPARK were treated with PF-562271 and imaged under 60X to follow the fate of single droplets. **A**, Representative images. **B**, Quantification of the FAK-SPARK droplets over time. SPARK signal is normalized to 1 at time 0. Data represent mean ± SEM (n = 8 and 6 for PF-562271 and DMSO, respectively). Scale bar, 2 µm.



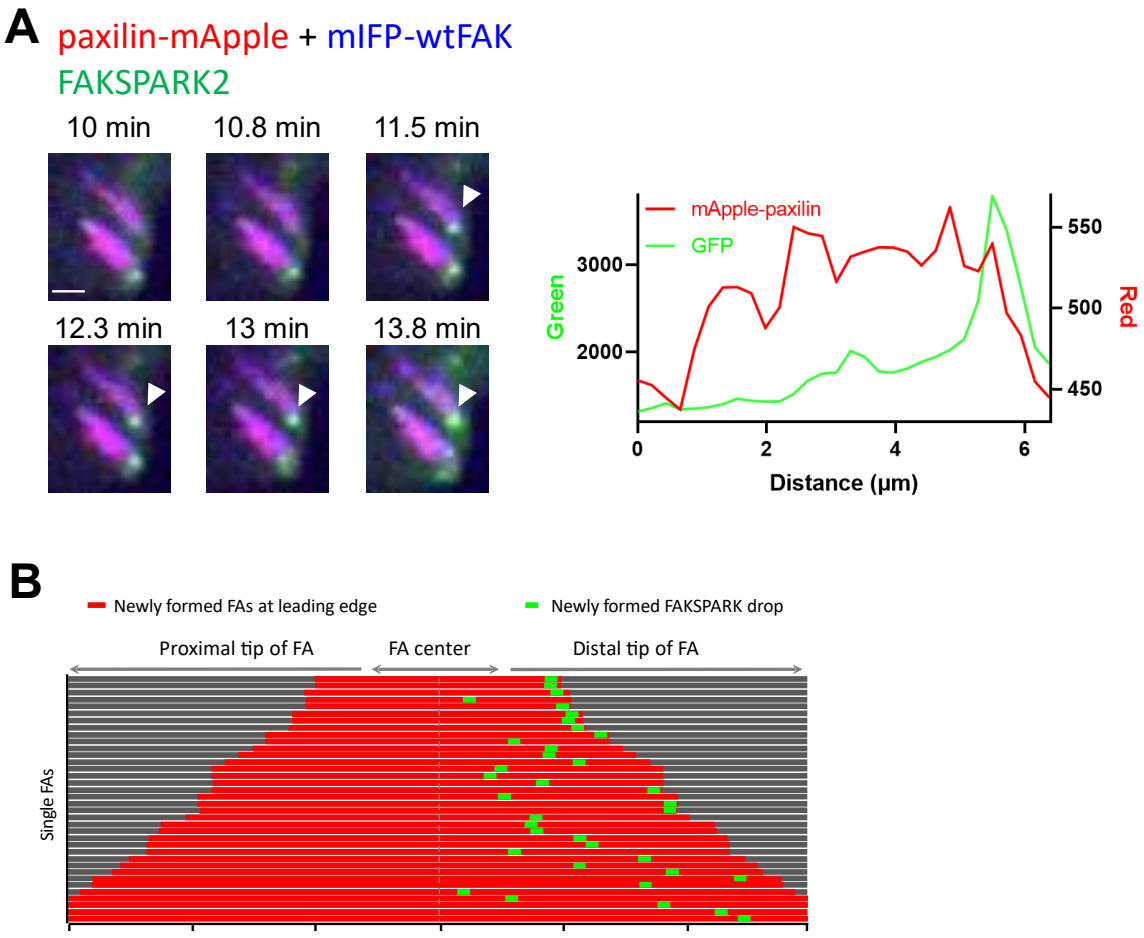
Supplementary Figure 4. Distribution of FAK in focal adhesions. HeLa cells expressing mApple-paxillin were stained with the antibody against FAK. Left: fluorescence images showing focal adhesions. Right: zoom-in of the focal adhesions in the boxed area shown in the left panel. Immunofluorescence of FAK was shown in blue. Similar observation was made independently for three times. Scale bar, 5 μm.



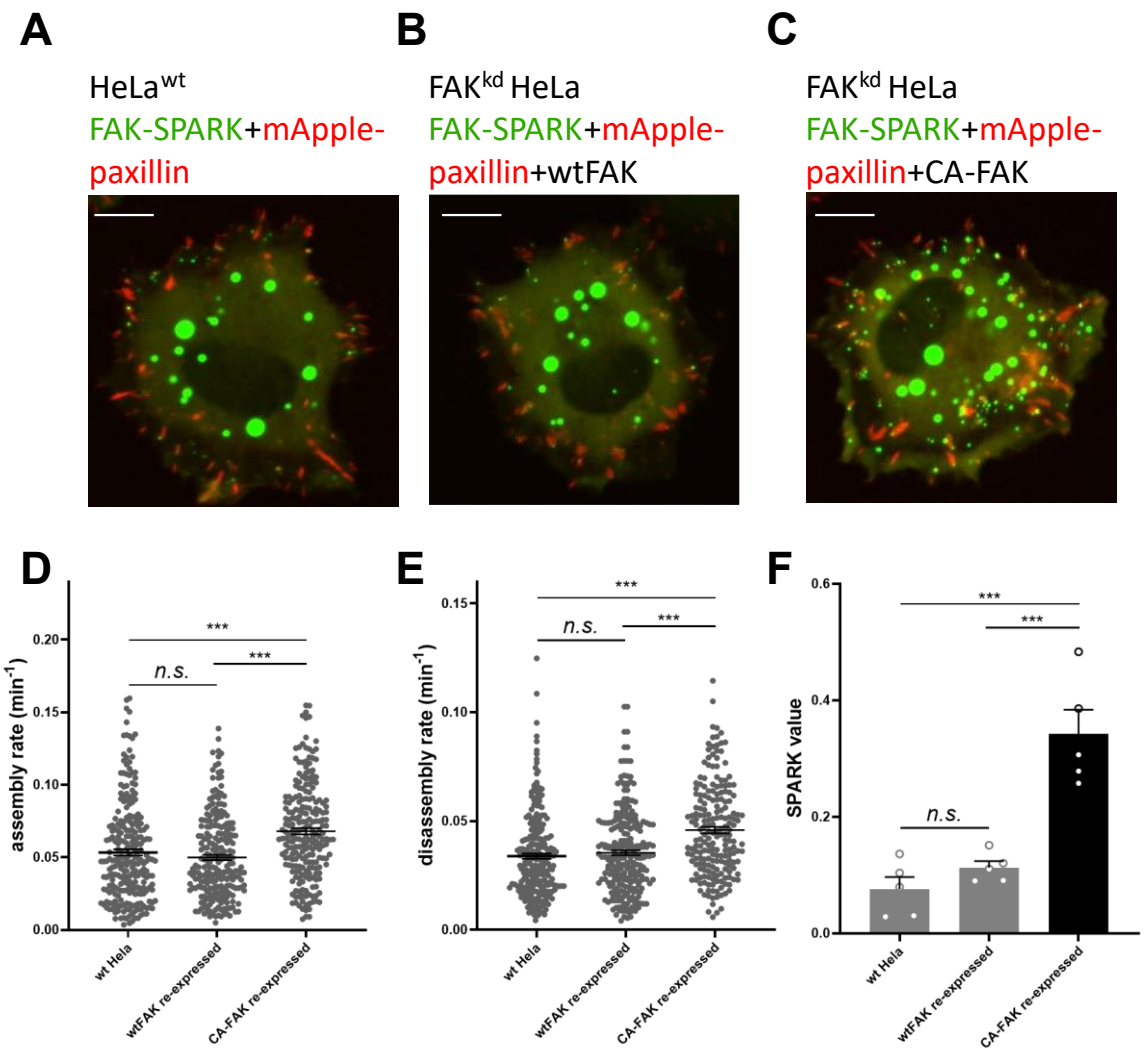
Supplementary Figure 5. Polarized FAK-SPARK signal is consistent with the gradient pattern of immunofluorescence against pY397 of FAK in HeLa cells during cell spreading (A & B) or migration (C & D). **A** & **C**, Representative images showing mApple-paxillin-labeled focal adhesions in spreading **A** and migrating **C** cells. **B** & **D**, zoom-in images of the boxed area (in A and C) showing antibody staining against pY397 of FAK (blue) and FAK-SPARK signal (green). Lower right panels in **B** & **D** shows fluorescence intensity of pY397 and FAK-SPARK over distance along the FA. Similar observation was made independently for three times. Scale bar, 5 μm (**A** & **C**), 2 μm (**B** & **D**).



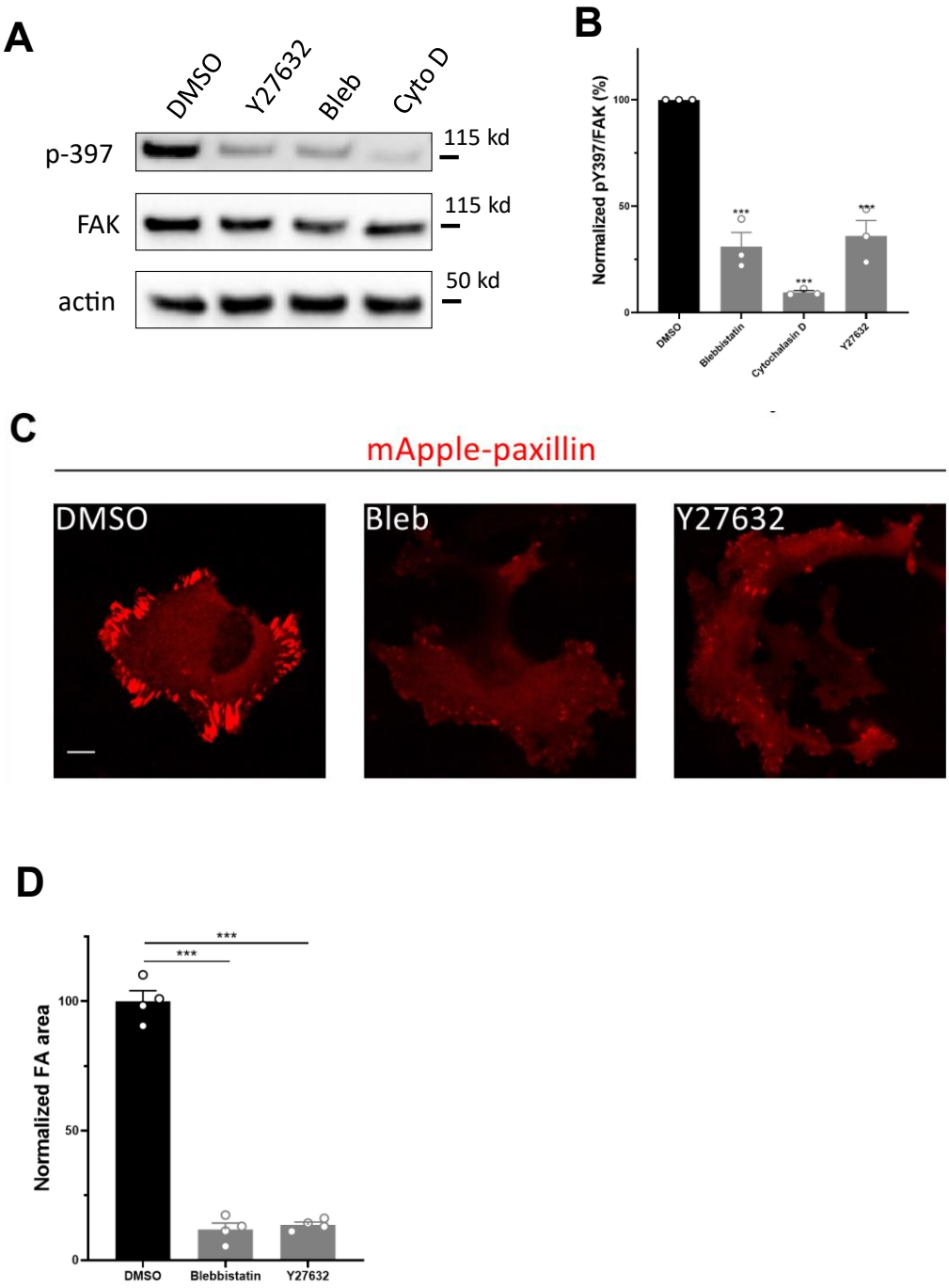
Supplementary Figure 6. FAK knockdown by shRNA eliminated FAK-SPARK signal. FAK was knocked down in HeLa cells. Co-expression of the constitutively active FAK (355-690 aa) recover the FAK activity. Data are mean ± SEM (n = 3 biological replicates). two-sided nonpaired t-test, p=0.0025 for scrambled shRNA and shRNA, p=0.00016 for shRNA+paxillin-mApple-355-690FAK. **: p value < 0.01. ***: p value < 0.001. Scale bar, 20 µm



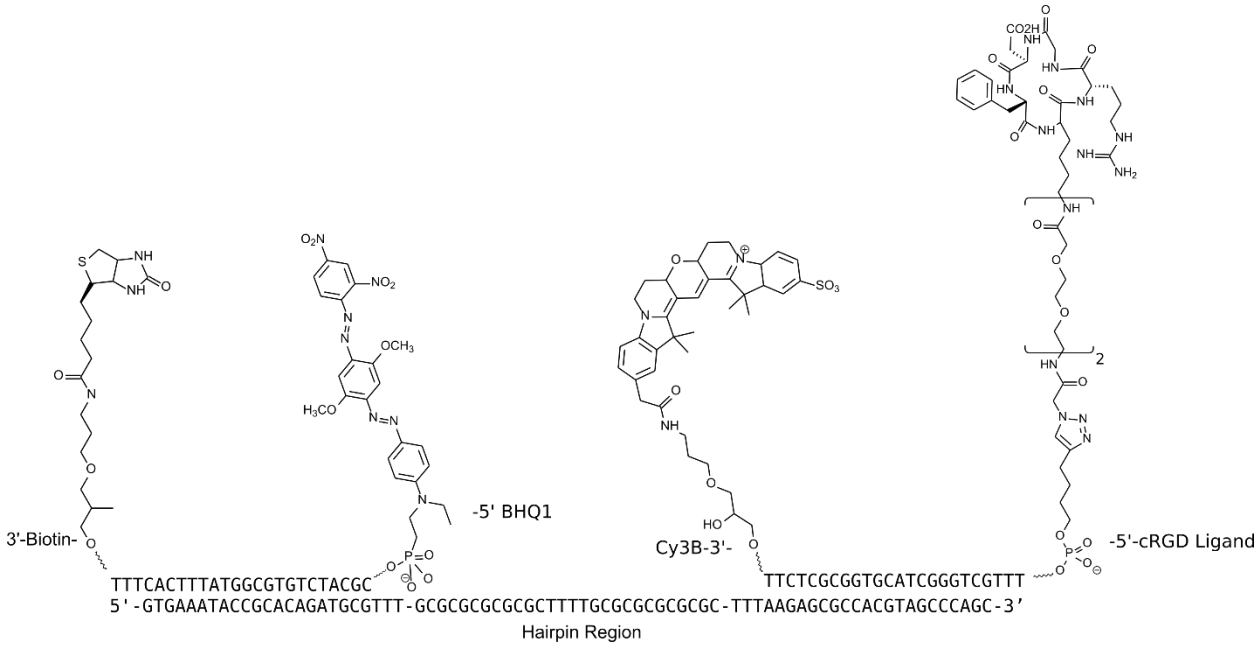
Supplementary Figure 7. Polarized FAK activity within single focal adhesions in FAK-KO HeLa cells that re-express FAK (wild type). A, Left: fluorescence images showing FAK activity at the distal site of the focal adhesion (FA). Wild type (WT) FAK were re-expressed in the Hela cells with FAK knockdown (FAK-KD). The cells co-expressed FAK-SPARK. Right: Fluorescence intensity over distance along the single FA. Similar observation was made independently for three times. **B**, Distribution of newly formed FAK-SPARK droplets in single FAs in the FAK-KD HeLa cells that re-express FAK. Scale bar, 2 μm.



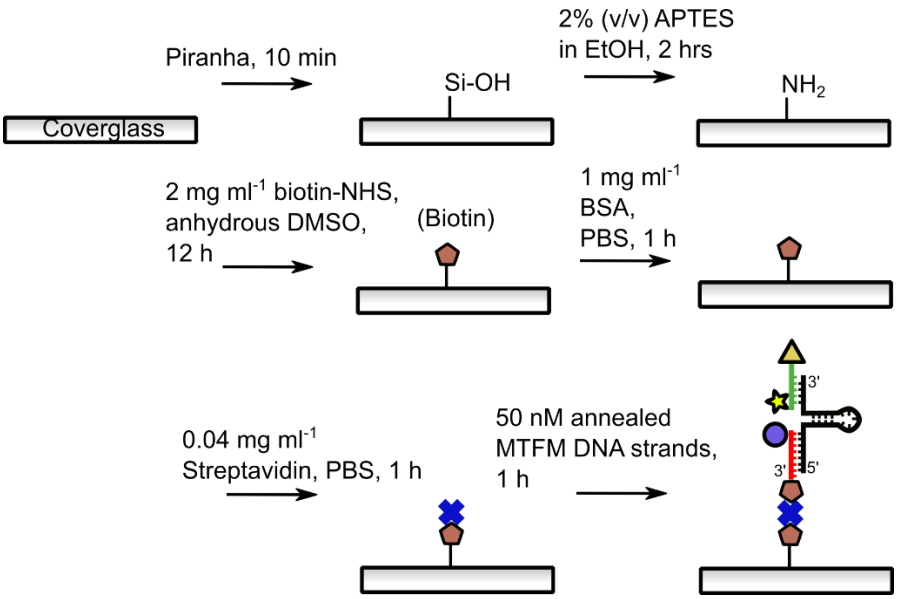
Supplementary Figure 8. Dynamics of focal adhesion assembly and disassembly rate and FAK-SPARK signaling during cell spreading in HeLa cells expressing wild type FAK (wtFAK) or constitutively active FAK (CA-FAK). A, **B** & **C**, Representative images. **D**, **E** & **F**, Quantification of FA assembly rate (**D**, n=248, 234 and 225 FAs for wtHela, wtFAK reexpressed and CA-FAK re-reexpressed, respectively), disassembly rate (**E**, n=245, 265 and 201 FAs for wtHela, wtFAK reexpressed and CA-FAK re-reexpressed, respectively) and FAK-SPARK value (**F**, n=5 biological replicates). two-sided nonpaired t-test for **D**, **E** & **F**. For **D**, p= 0.23 between wtHela and wtFAK reexpressed, $p=2.32x10^{-6}$ between wtHela and CA-FAK re-reexpressed, $p=4.20x10^{-10}$ between wtFAK reexpressed and CA-FAK re-reexpressed. For **E**, p= 0.56 between wtHela and wtFAK reexpressed, $p=4.05x10^{-9}$ between wtHela and CA-FAK re-reexpressed, $p=1.24x10^{-8}$ between wtFAK reexpressed and CA-FAK re-reexpressed. For **F**, p= 0.16 between wtHela and wtFAK reexpressed, p=0.00043 between wtHela and CA-FAK re-reexpressed, p=0.00068 between wtFAK reexpressed and CA-FAK re-reexpressed. Data represent mean ± SEM. *n.s.,* not significant. ***, $p<0.001$. Scale bar, 10 µm.



Supplementary Figure 9. Phosphorylation of Y397 of FAK is reduced upon drug treatment. A & **B** (n=3 biological replicates), Western blot analysis of pY397 after the HeLa cells were treated with Y27632, Blebbistatin or Cytochalasin D. **C** & **D**, Large reduction of focal adhesion size after Y27632 and Blebbistatin treatment. **C**, Representative images. **D**, Quantification of the FA area change (n=4 biological replicates). two-sided nonpaired t-test for comparing DMSO with other bars in both **B** and **D**. Exact p values, for B (from left to right): 0.00049, 4.72×10^{-8} and 0.00089; for D (from left to right): 1.64×10^{-6} and 8.77×10^{-7}. Data represent mean ± SEM. ***, $p<0.001$. Scale bar, 10 µm.



Supplementary Figure 10. 19 pN MTFM probe diagram displaying molecular structures of modified DNA bases.



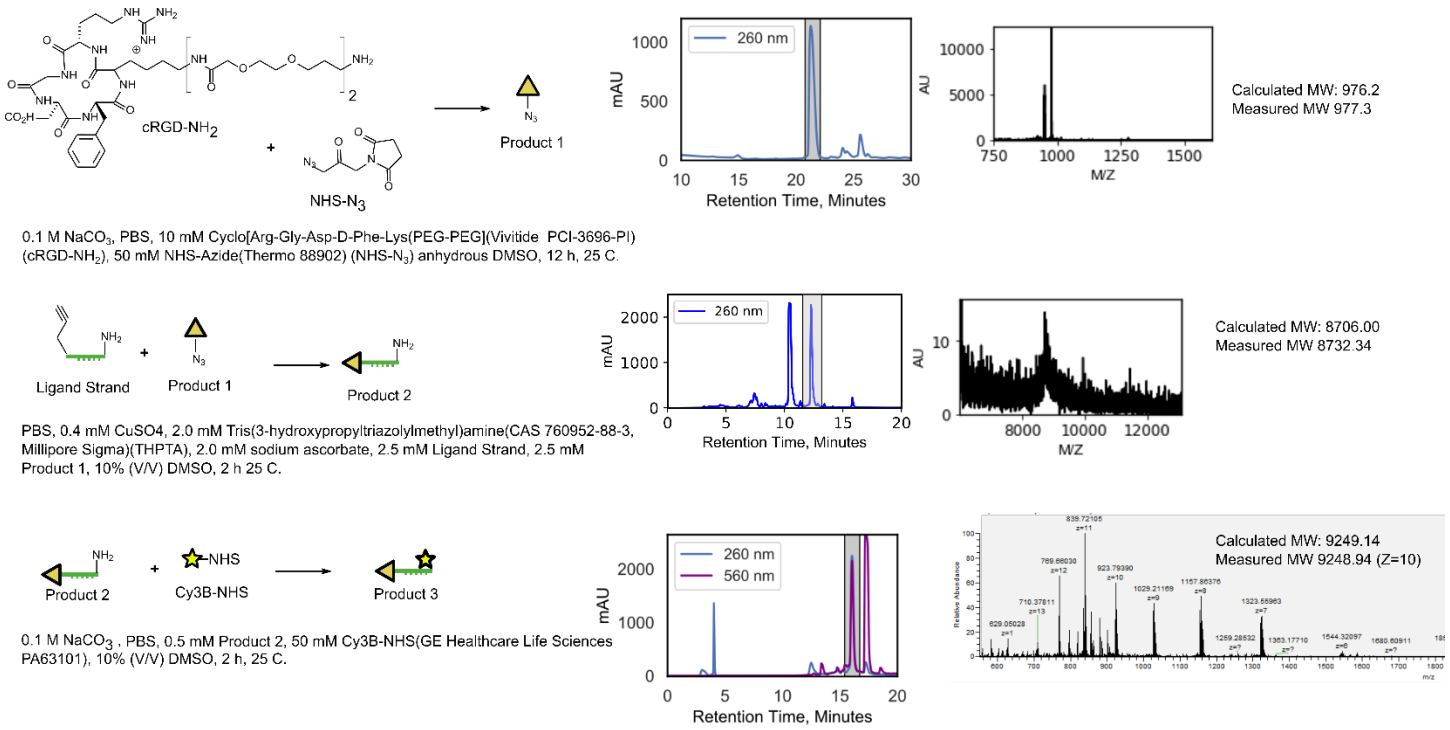
Supplementary Figure 11. Diagram of MTFM probe surface preparation.

$$\Delta G(F,x) = \Delta G_{fold} + \Delta G_{stretch} + F \cdot x$$

$$\Delta G_{stretch} = \frac{k_B T}{Lp} \frac{L_0}{4(1-\frac{x}{L_0})} [3\left(\frac{x}{L_0}\right)^2 - 2\left(\frac{x}{L_0}\right)^3]$$

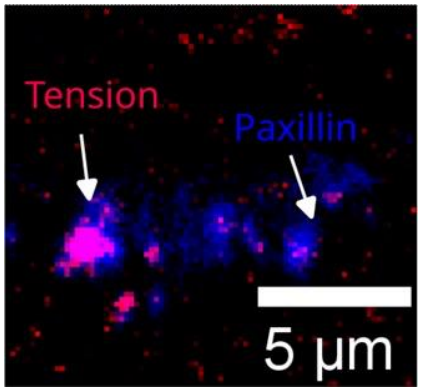
$$F_{1/2} = \frac{\Delta G_{fold} + \Delta G_{stretch}}{\Delta x}$$

Supplementary Figure 12. Calculation of $F_{1/2}$ of DNA hairpin. The $F_{1/2}$ threshold at which the DNA hairpins have a 50% probability of unfolding and generating MTFM fluorescence signal is based on assumptions and measurements made by Woodside *et al* [1]. Here, ΔG_{fold} is the free energy of hairpin unfolding without force application, F is the force applied on the hairpin, x is the extension of the hairpin, k_B is the Boltzmann constant $\Delta G_{stretch}$ is the free energy of extending the hairpin ssDNA from $F = 0$ to $F = F_{1/2}$ calculated from the worm-like chain model where Lp and L_0 are the persistence and contour length of the ssDNA hairpin respectively.

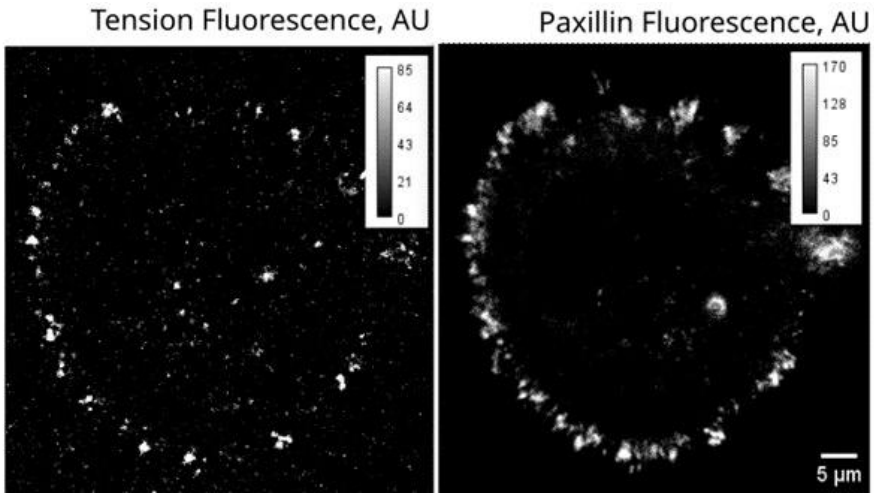


Supplementary Figure 13. Synthetic diagram of MTFM ligand modified oligonucleotide strand.

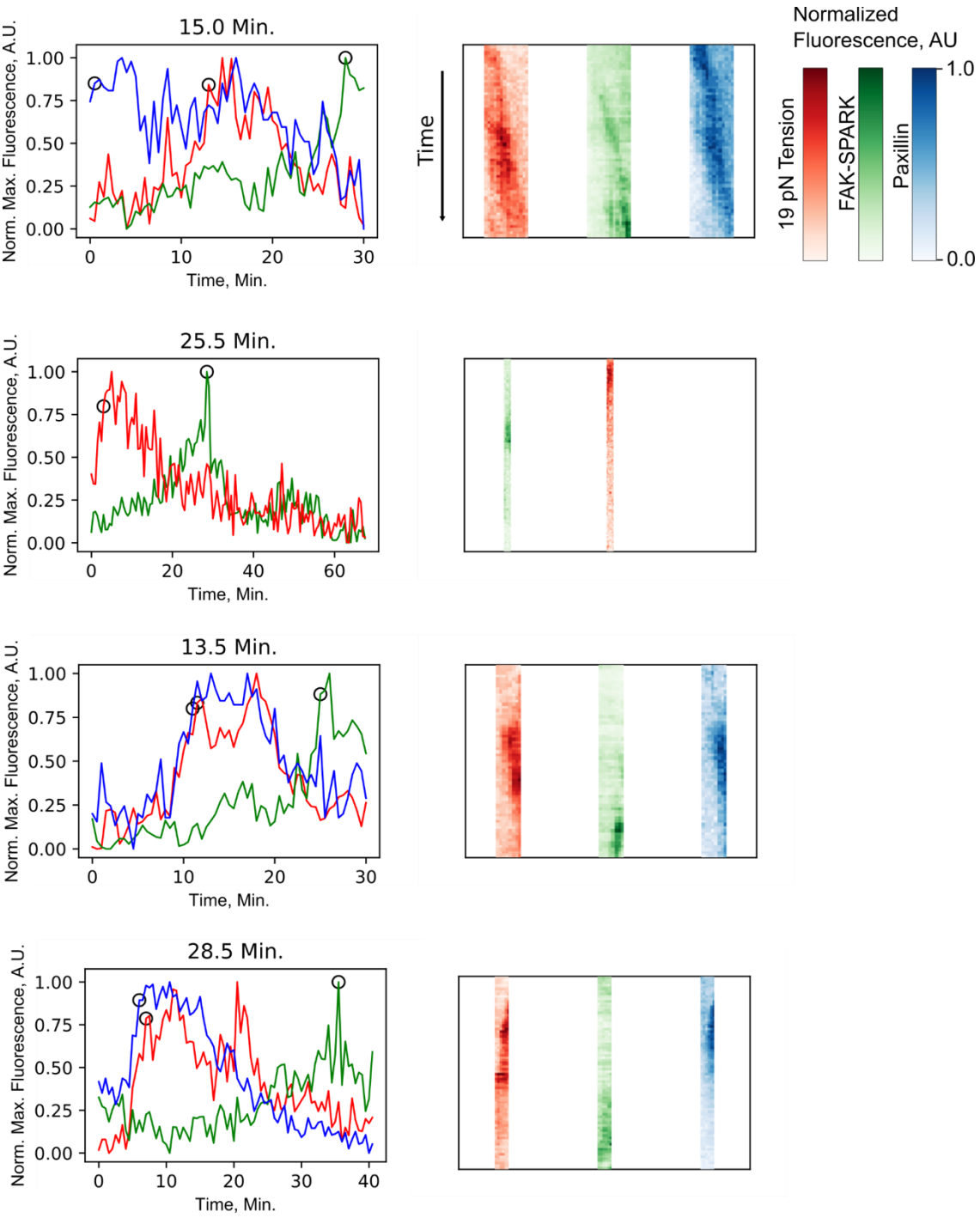
A



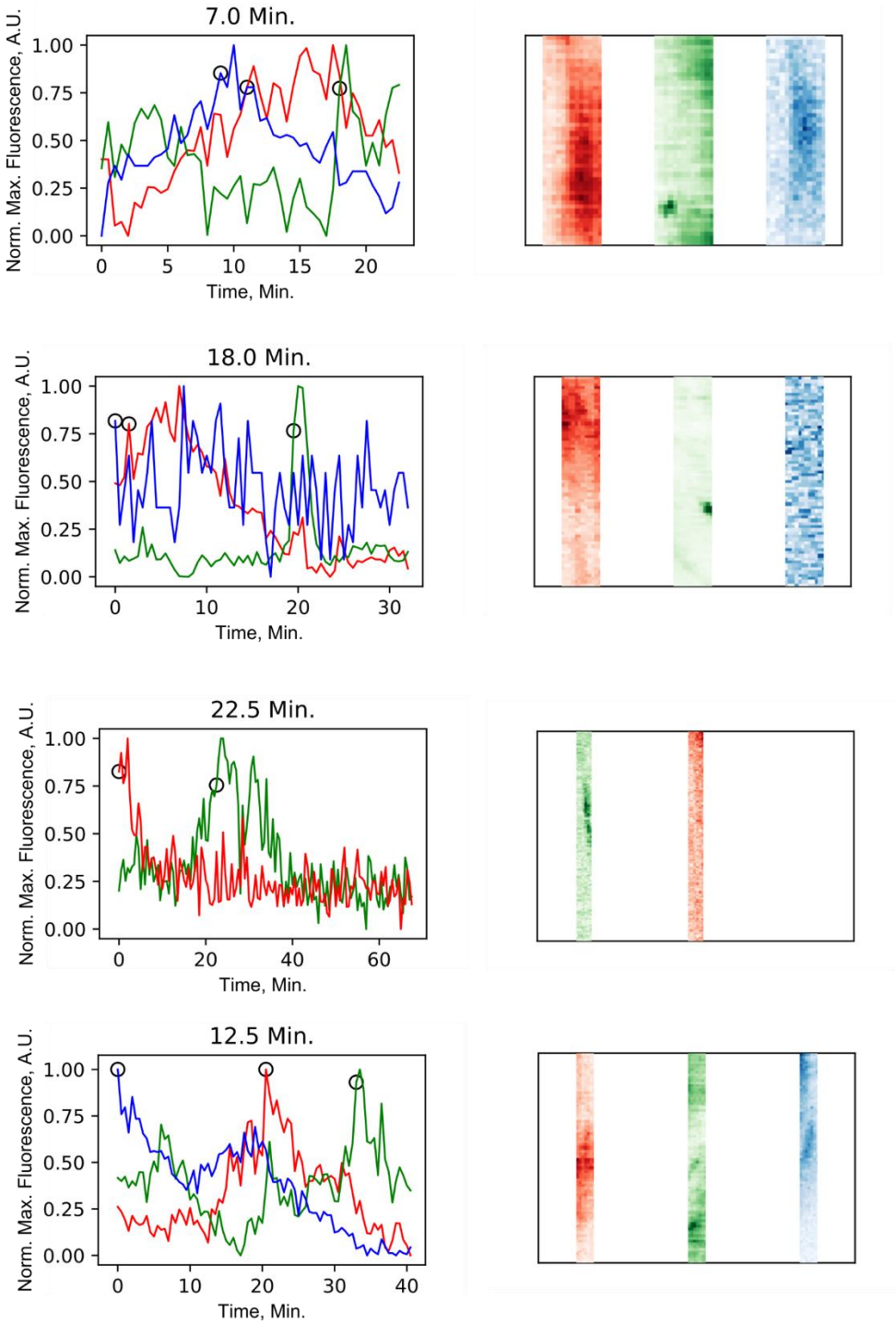
B



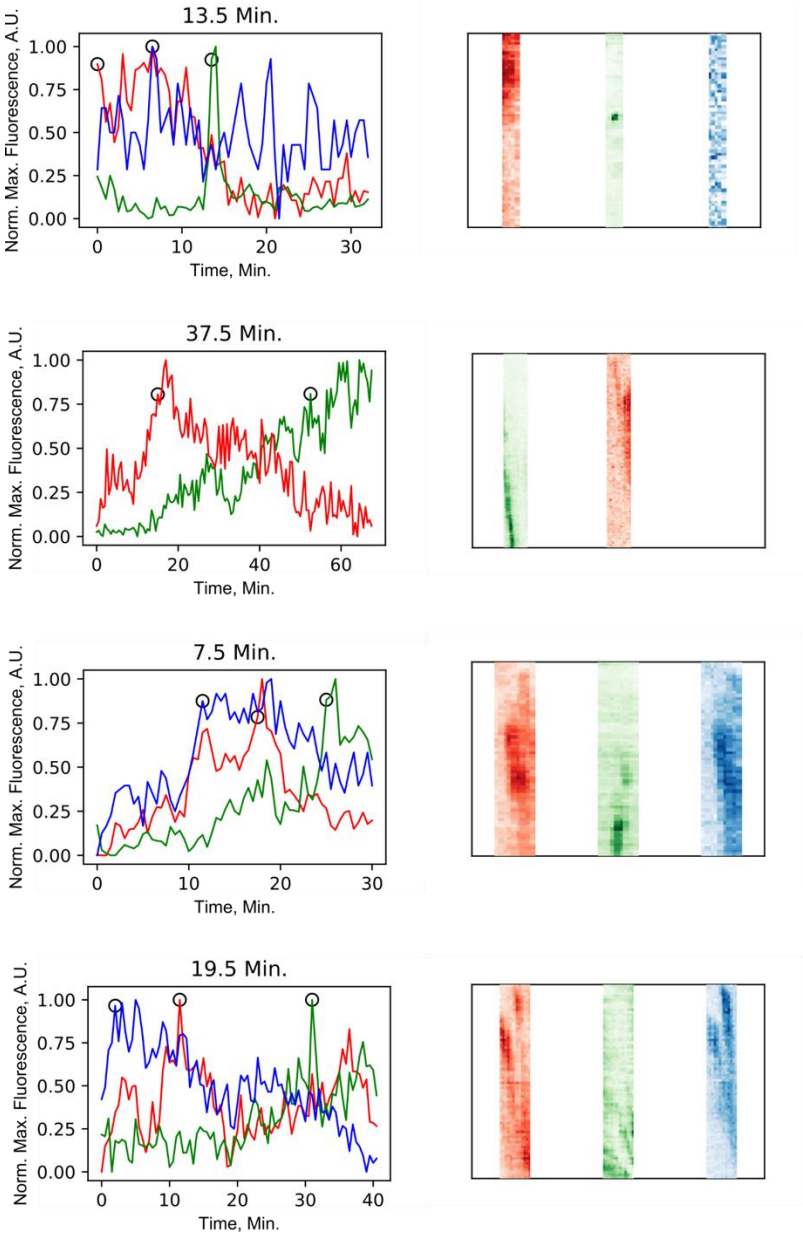
Supplementary Figure 14. Integrin tension is colocalized with paxillin and does not result from spectral bleed through. A, 19 pN hairpin tension and IFP2-paxillin fluorescence micrograph overlay demonstrating instances of paxillin recruitment (blue) in the presence (left arrow) and absence (right arrow), demonstrating that tension signal is not due to spectral bleed through from the paxillin fluorescence channel. **B**, Fluorescence micrographs of 19 pN hairpin tension signal(left) and IFP2-paxillin(right) of one cell, demonstrating the colocalization of the fluorescence signals. Similar observation in **A** and **B** was made independently for three times.



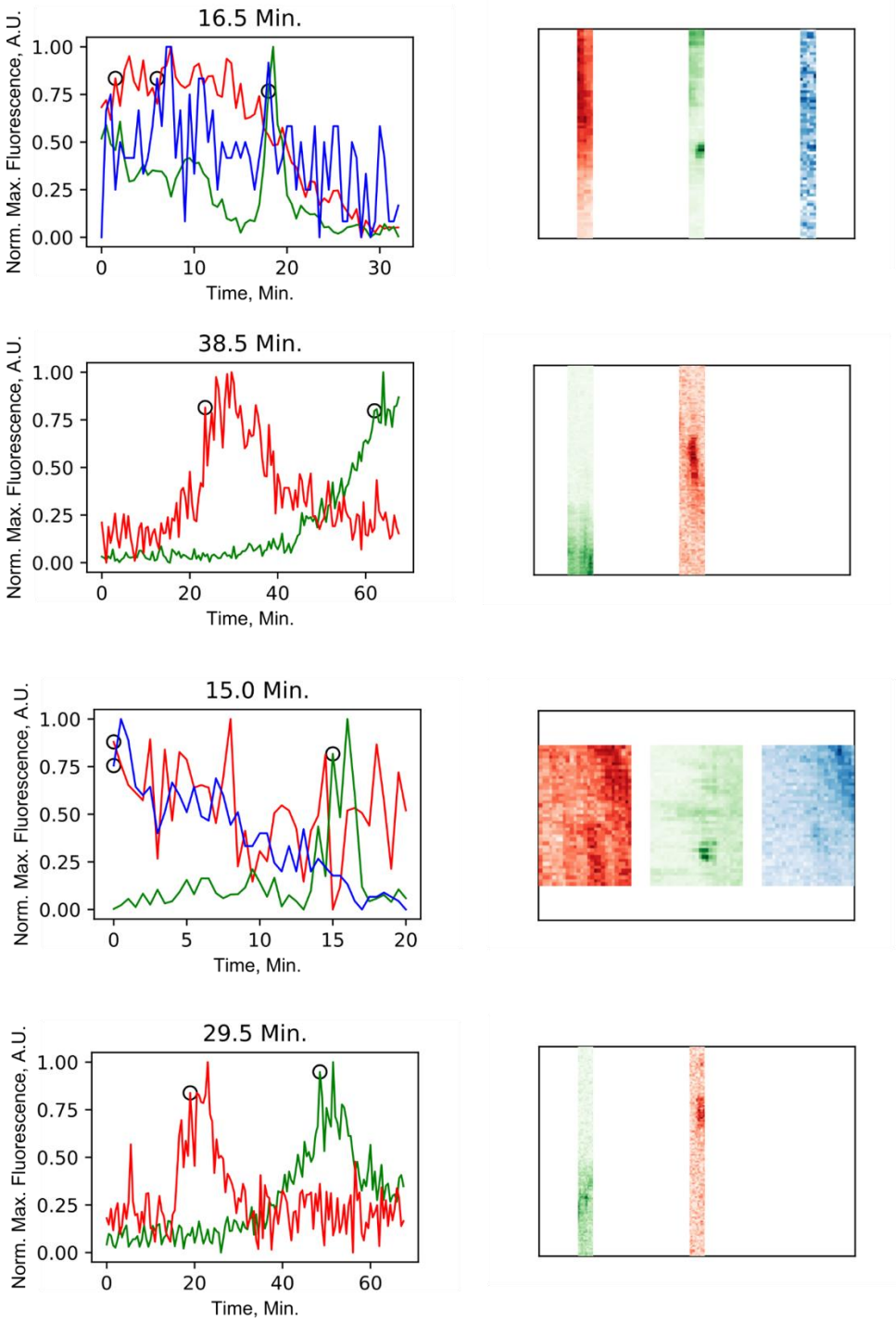
Supplementary Figure 15. (Cases 1-4) Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs.



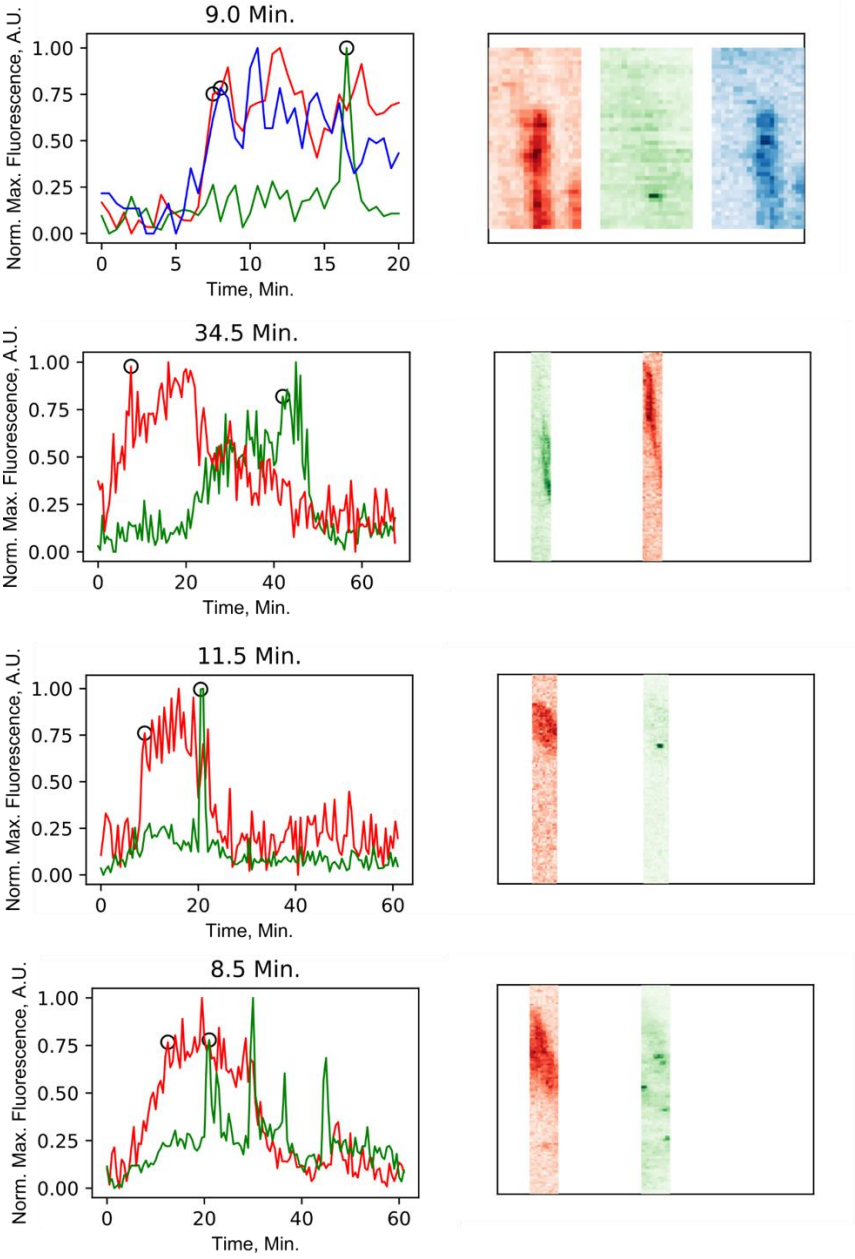
Supplementary Figure 16. **(Cases 5-8)** Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs. Please refer the scales to Supplementary Figure 15.



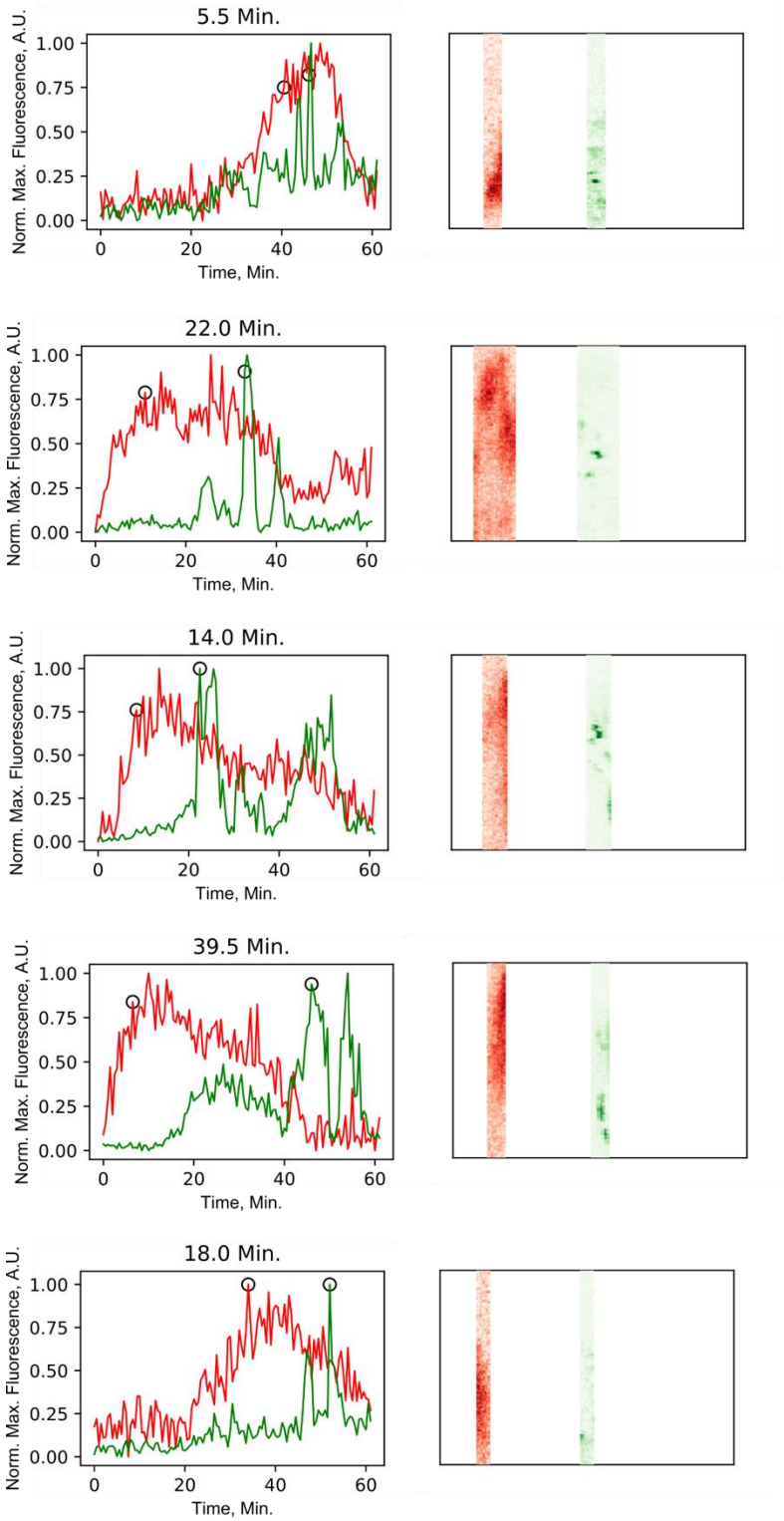
Supplementary Figure 17. (Cases 9-12) Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs. Please refer the scales to Supplementary Figure 15.



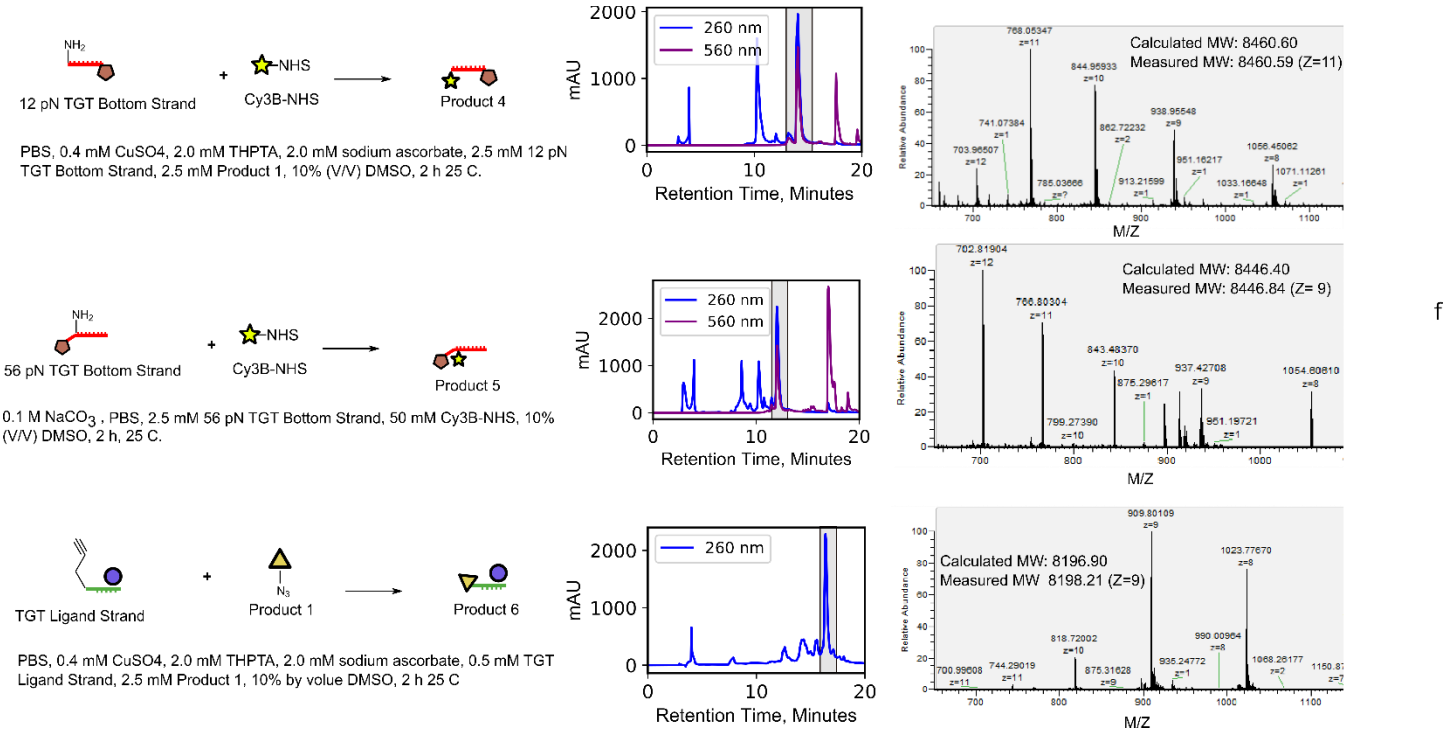
Supplementary Figure 18. (Cases 13-16) Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs. Please refer the scales to Supplementary Figure 15.



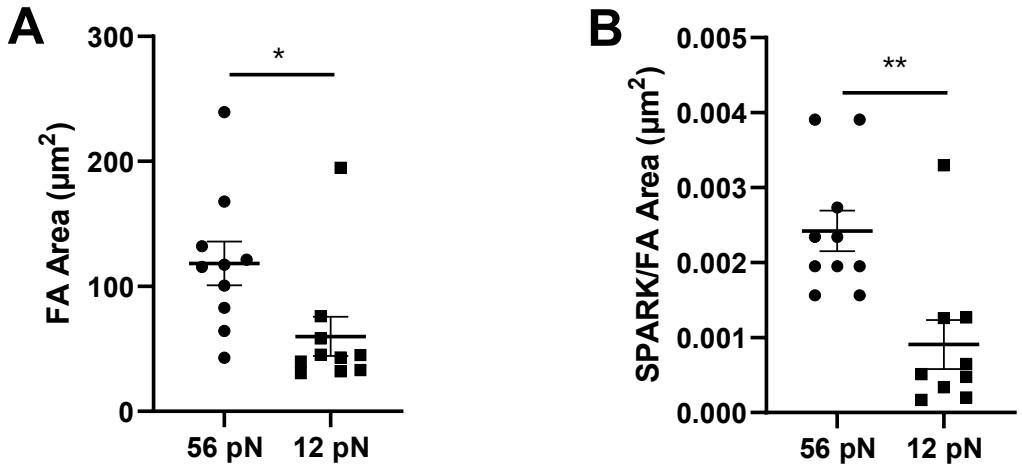
Supplementary Figure 19. (Cases 17-20) Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs. Please refer the scales to Supplementary Figure 15.



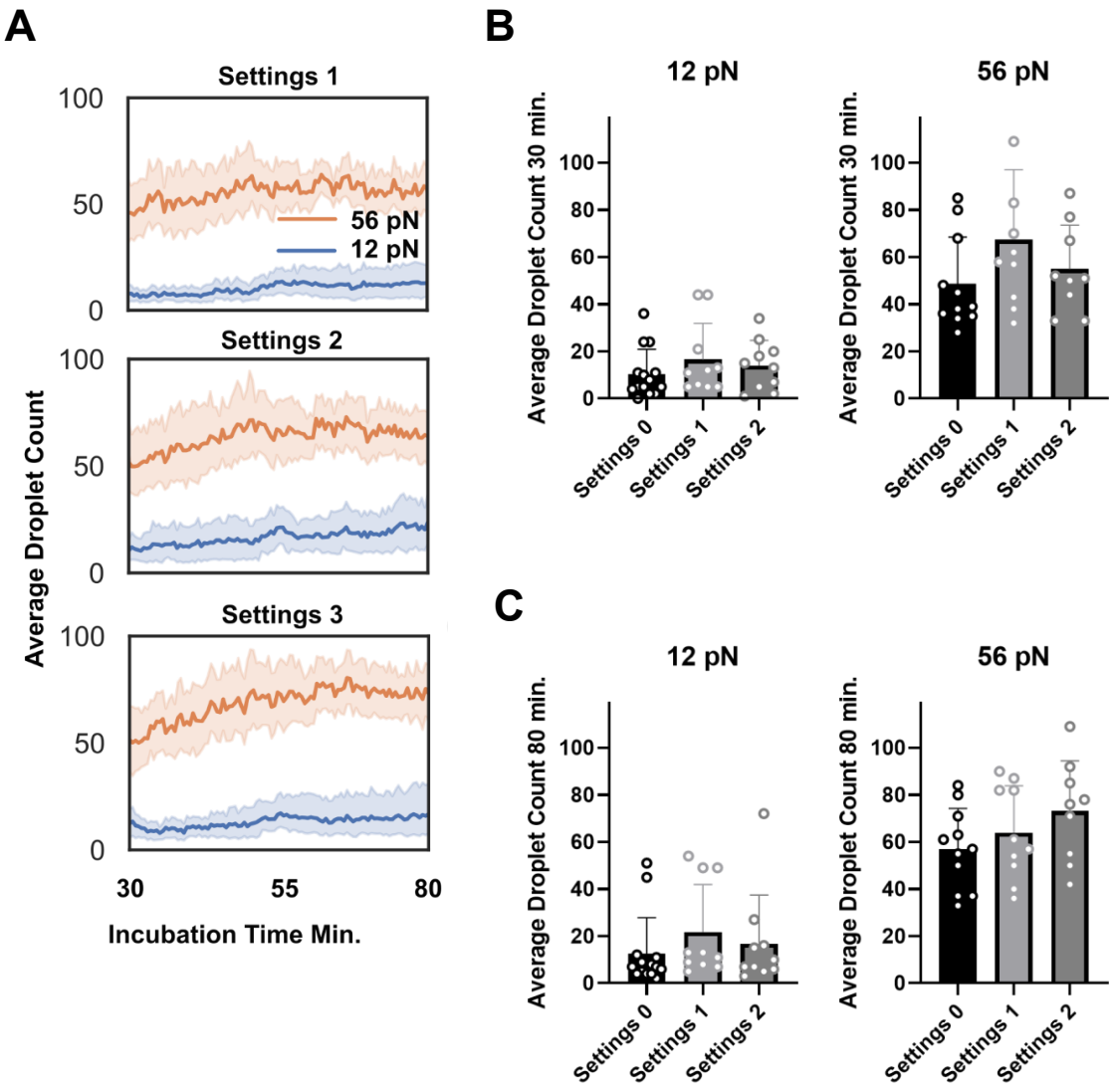
Supplementary Figure 20. (Cases 21-25) Kymographs and time delay graphs of individual focal adhesion used for time delay analysis of FAK-SPARK droplets(green), 19 pN tension(red), and IFP-2.0 paxillin(blue). Each plotted circle denotes the point at which the plot first reaches 75% of the maximum intensity. The time delay(min.) is displayed at the top of the graphs. Please refer the scales to Supplementary Figure 15.



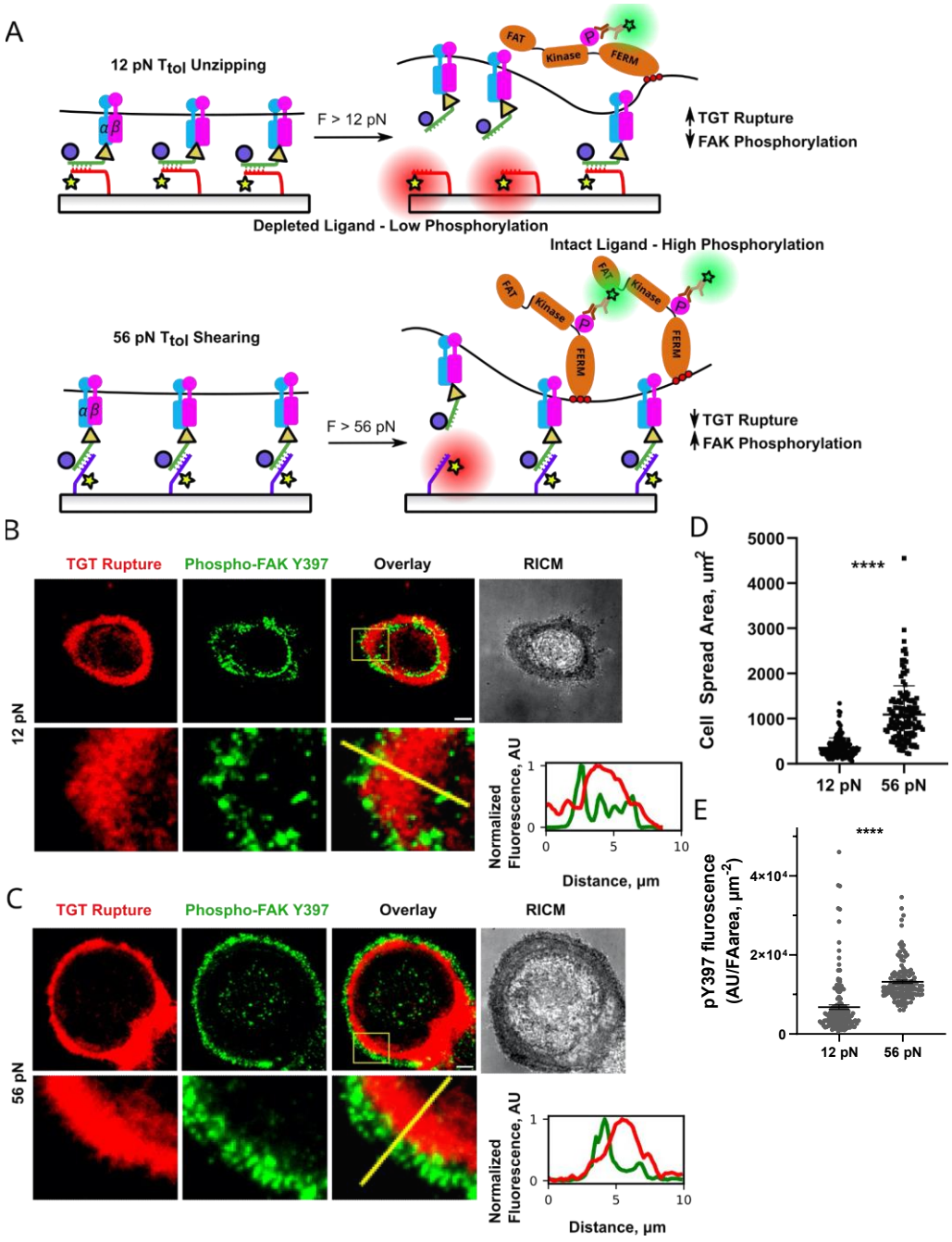
Supplementary Figure 21. Synthetic diagram of TGT modified oligonucleotide strands.



Supplementary Figure 22. FA area (A) and normalized FAK-SPARK value by FA area (B) in response to 56 pN and 12 pN tension using DNA-based tension gauge tether (TGT). A, Area of focal adhesion in HeLa cells reseeded on 12 pN or 56 pN TGT (n = 10 cells for both 56 and 12 pN). **B**, FAK-SPARK value normalized by area of focal adhesions (n = 9 and 10 cells for 56 and 12 pN, respectively). two-sided nonpaired t-test. p=0.02 for **A** and p=0.002 for **B**. Data represent mean ± SEM *, $p<0.05$, **, $p<0.01$



Supplementary Figure 23. A Average number of FAK-SPARK droplets under cells for each time point for cells incubated for 30 minutes and then undergoing a 50 minute spreading period on 12 and 56 pN TGTs (Supplementary Movies 14 & 15) as measured through different image segmentation algorithm settings, shaded region indicates standard deviation. Settings 1: an 8 µm2 maximum area threshold for identified particles without applying the watershed image segmentation operation to the binary mask image resulting from intensity thresholding Settings 2: an 8 µm2 maximum area threshold for identified particles and applying the watershed function, and Settings 3: a 24 µm2 maximum particle area for identified particles and applying the watershed function. **B** Average droplet count for cells incubated on 12 and 56 pN TGTs after 30 minutes of incubation for each identification algorithm setting. **C** Average droplet count after 80 minutes of incubation for each identification algorithm setting. Data collected for 10 cells from 3 bio-replicates for each 12 and 56 pN TGT group. One way-ANOVA, p=0.47 and 0.20 for 12pN and 56pN in **B**; p=0.48 and 0.20 for 12pN and 56pN in **C**. Data are mean $\pm$ SD.



Supplementary Figure 24. FAK phosphorylation scales with integrin ligand Ttol. **A** Diagram of the relationship between integrin ligand Ttol, TGT rupture mediated ligand depletion, and phosphorylation of FAK Y397. **B** Representative fluorescence micrographs and fluorescent intensity line-scans of Hela cells seeded on 12 and **C** 56 pN TGT surfaces and stained for phospho-FAK Y397 (green, TGT fluorescence red). **D** Cell spread area measured from RICM on 12 and 56 pN TGTs, Data are mean ± SD, $p=3.44x10^{-32}$, two-sided nonpaired t-test. **E** Phospho-FAK Y397 fluorescence intensity per cell on 12 and 56 pN TGTs. Each data point represents a single cell Data are mean ± SEM. $P=2.69x10^{-15}$, two-sided nonpaired t-test. N = 151 cells 12 pN, N = 135 cells 56 pN, 4 bio-replicates. Scale bar 5 µm. Note that cells display more frequent TGT rupture and lower cell spread area on 12 pN TGTs. This is consistent with previous reports and demonstrates the frequency of F> 12 pN compared to F>56 pNs by integrin receptors 2. When HeLa cells were plated on TGT surfaces for three hours and subsequently stained for phospho-FAK Y397, cells adhering to mechanically strong 56 pN TGTs displayed a higher level of FAK phosphorylation than the mechanically weaker 12 pN TGTs as quantified by fluorescence microscopy. This corroborates observations of the FAK-SPARK sensor activity on TGTs. Stronger integrin-ligand tension promotes FAK phosphorylation in the ligand presenting glass interface.

Supplementary Table 1. Key resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Calcium Phosphate Transfection Kit	ThermoFisher Scientific	K278001
Lipofectamine-2000	ThermoFisher Scientific	11668030
Fibronectin human plasma	Sigma-Aldrich	F2006
Cytochalasin D	Sigma-Aldrich	250255
Y27632	Sigma-Aldrich	Y0503
(−)-Blebbistatin	Sigma-Aldrich	B0560
biliverdin HCl	Cayman Chemical	CAS 856699-18-8
compound 1652 (dual inhibitor of α5β1 and αvβ1)	Bill Degrado Lab, UCSF	Cite paper PMID: 33007395
PF562271	Target Molecule	T21768
P1D6, anti–integrin α5	Shepard Dean Lab, UCSF	Abcam, ab78614
Anti-FAK(phospho Y397) antibody EP2160Y	Abcam	ab81298
goat anti-rabbit IgG Alexa Fluor-488	Abcam	ab150077
rapamycin	Calbiochem	553210
CO2 Independent Medium	Gibco	18045088
Experimental Models: Cell Lines		
293T/17	ATCC	CRL-11268
Hela	Gift from Dengke Ma lab, UCSF	N/A
MDA-MB-231	UCSF Cell Culture Core Facility	N/A
U2OS	Gift from Dengke Ma lab, UCSF	N/A
Kelly	Gift from William A. Weiss lab, UCSF	N/A
SHEP	Gift from William A. Weiss lab, UCSF	N/A
SKNAS	Gift from William A. Weiss lab, UCSF	N/A
293FT cell	Gift from Young-wook Jun lab	N/A
MEF	Gift from Dean Sheppard Lab, UCSF	N/A
Recombinant DNA		
Plasmid: pcDNA3-H2B-mIFP-T2A-mCherry	This paper	N/A
Plasmid: pcDNA3-wtFAK-SPARK2	This paper	N/A
Plasmid: pcDNA3-mutFAK-SPARK2	This paper	N/A
Plasmid: pcDNA3-FAK-SPARK-NLS		
Plasmid: pLenti-wtFAK-SPARK	This paper	N/A
Plasmid: mApple-Paxillin-22	Nikon Imaging Centre, UCSF	Box 21, Position 6
Plasmid: pcDNA3-AuroraA-mKO3-CA-FAK	This paper	N/A
Plasmid: pcDNA3-HOTag1-NLS-mKO3_CA-FAK	This paper	N/A
Plasmid: pcDNA3-HOTag1-NLS-mKO3_FRB	This paper	N/A

Plasmid: pcDNA3-FKBP-IFP2-CA-FAK	This paper	N/A
Plasmid: pcDNA3-IFP-wtFAK	This paper	N/A
Plasmid: pcDNA3-IFP-Y180A/M183A-FAK	This paper	N/A
Plasmid: pcDNA3-IFP-FAK$^{KAKTLRK->AAATLAA}$	This paper	N/A
Plasmid: pcDNA3-FRNK-FAK (355-690)	This paper	N/A
Plasmid: pcDNA3-paxilin-mapple-CA-FAK (355-690)	This paper	N/A
Plasmid: pcDNA3-paxilin-IFP2	This paper	N/A
Plasmid: pcDNA3-CA-FAK (355-690)	This paper	N/A
p-Tol2-UAS-FAK-SPARK	This paper	N/A
p-Tol2-UAS-mutFAK-SPARK	This paper	N/A
Sigma Mission shRNA against human FAK	From Valerie Weaver Lab, UCSF	N/A
Software and Algorithms		
NIS-Element	Nikon	N/A
Fiji ImageJ	Opensource	https://fiji.sc/
Python	Opensource	Deposit by Dale

Supplementary Table 2 Oligonucleotide sequences as ordered from Integrated DNA Technologies with modified bp codes enclosed in forward slashes.

Name	**Sequence**
19 pN Hairpin	GTGAAATACCGCACAGATGCGTTTGCGCGCGCGCGCTTTTG CGCGCGCGCGCTTTAAGAGCGCCACGTAGCCCAGC
Ligand Strand	/5Hexynyl/TTTGCTGGGCTACGTGGCGCTCTT/3AmMO/
Anchor Strand	/BHQ1/CGCATCTGTGCGGTATTTCACTTT/Biotin/
TGT Ligand Strand	/5Hexynyl/GTG AAA TAG CGC ACA GAT GCG /3-BHQ2
12 pN TGT Bottom Strand	/5AmMC6/CGC ATC TGT GCG GTA TTT CAC TTT/3 Bio/
56 pN TGT Bottom Strand	/5Biosg/TT T/iUniAmM/C GCA TCT GTG CGG TAT TTC AC

Supplementary Movies 1-15.

Supplementary Movie 1. FAK-SPARK achieves spatiotemporal resolution in visualizing FAK activity in living cells. HOTag1-NLS-mKO3-Frb (blue), FAK-SPARK-NLS (green), FKBP-IFP2-CA-FAK (red). NLS: nuclear localization signal. Images were taken every 30 seconds per frame.

Supplementary Movie 2. FAK activation at the leading of a spreading cell. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 3. FAK activity in a living cell. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 4. A region of interest showing FAK activity in a living cell. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 5. FAK activation during assembly of a single focal adhesion in cells. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 6. FAK activation before disassembly of focal adhesions in cells. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 7. Disassembly of a single focal adhesion following FAK activation. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 8. FAK activation during sliding of single focal adhesions. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 9. FAK activation during turnover of focal adhesions in cells. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 10. A region of interest showing FAK activation during turnover of focal adhesions in cells. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 11. Polarized FAK activity at the distal tip of newly formed single focal adhesions in a migrating cell. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 12. No distal polarization of FAK activity when CA-FAK is fused to paxillin and targeted to focal adhesions in cells with endogenous FAK knocked down by shRNA against FAK. FAK-SPARK (green), mApple-paxillin (red). Images were taken every 30 seconds per frame.

Supplementary Movie 13. 19 pN hairpin Integrin tension, IFP2-Paxillin, and TIRF imaged FAK-SPARK droplets generated at regions experiencing 19 pN tension and enriched in paxillin.

Supplementary Movie 14. TGT rupture(red), IFP2-Paxillin (blue) and FAK-SPARK droplets(green), generated over time on 12 pN TGTs.

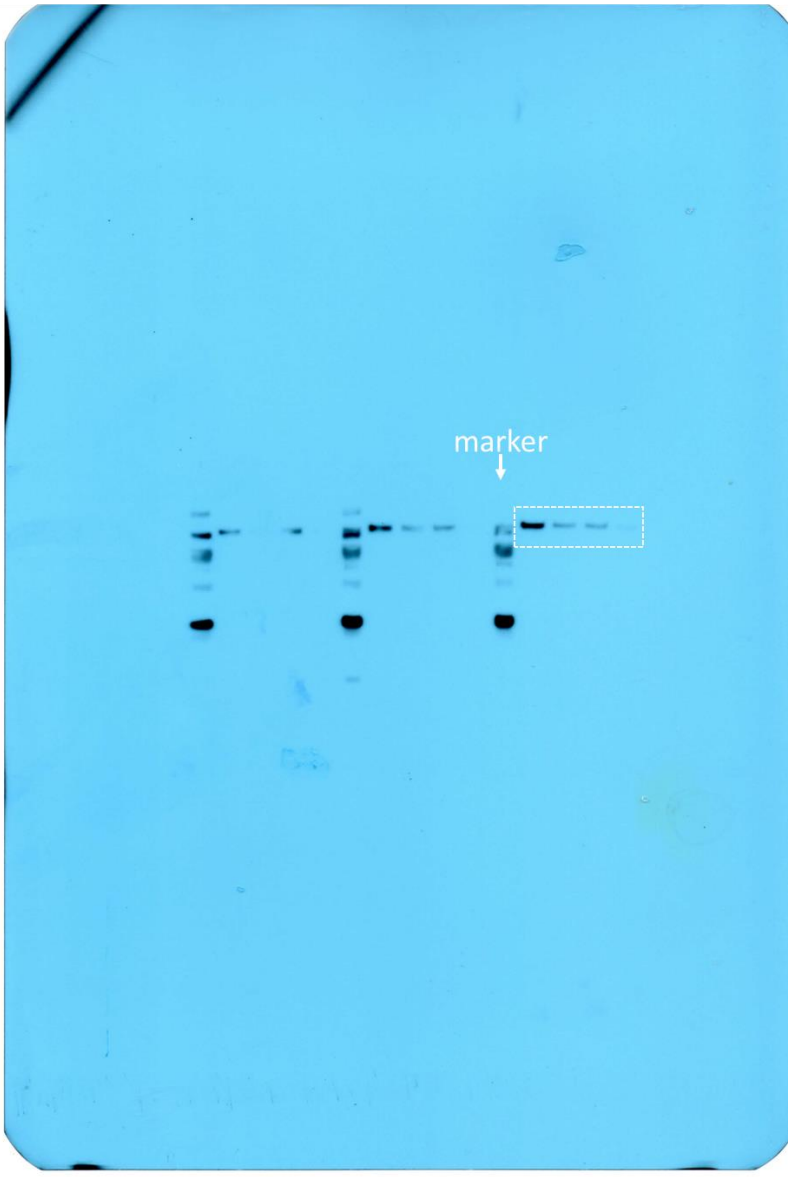
Supplementary Movie 15. TGT rupture(red), IFP2-Paxillin (blue) and FAK-SPARK droplets(green), generated over time on 56 pN TGTs.

References

1. Woodside, M. T. *et al.* Nanomechanical measurements of the sequence-dependent folding landscapes of single nucleic acid hairpins. *Proc Natl Acad Sci USA* **103,** 6190–6195 (2006).
2. Zhang, Y., Ge, C., Zhu, C. & Salaita, K. DNA-based digital tension probes reveal integrin forces during early cell adhesion. *Nature Communications* **5,** 5167–10 (2014).

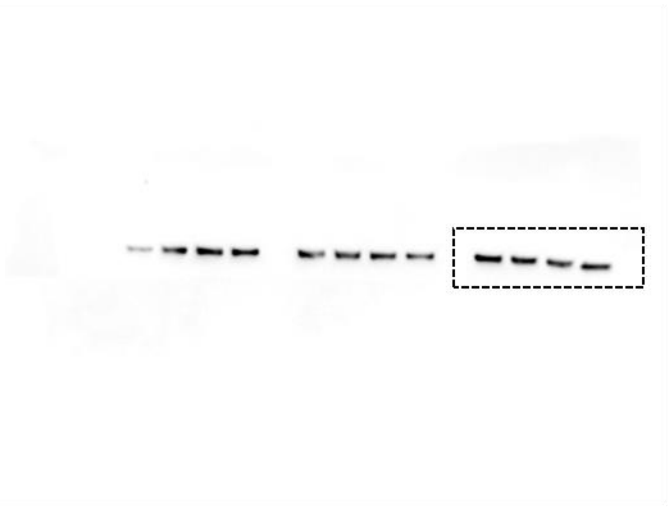
Uncropped data of blots in Supplementary Figure 9A, dashed line outlines cropped region

P-397 developed with film

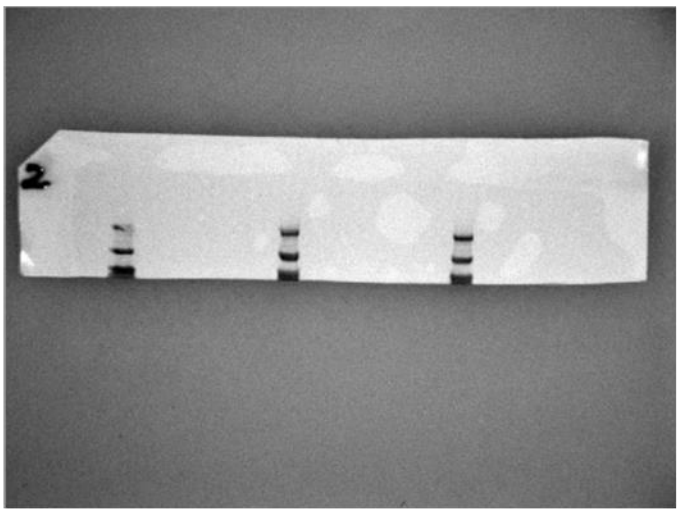


FAK and actin visualized with BIO-RAD Imager

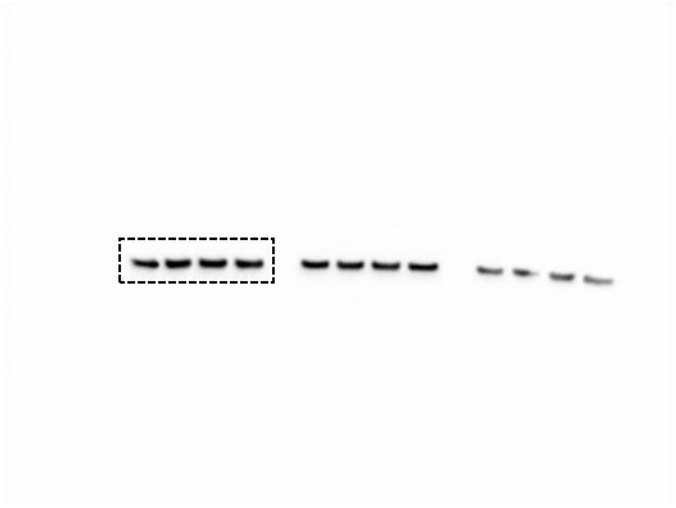
FAK blot signal



Brightfiled image for ladder



actin blot signal



Brightfiled image for ladder