Supplementary Discussion

Typically in SMFS experiments, rupture force – loading rate plots are used to characterize k_{off} and Δx, the unbinding (or unfolding) probability per time unit and the distance to the transition state along the reaction coordinate, respectively, providing direct information about the energy landscape governing protein folding[1]. SMFS experiments are also complemented by all-atom simulations of such systems *in silico*. Recently, it was shown that high speed SMFS experiments could be performed at speeds achievable in molecular dynamics simulations[2], overcoming a long standing discrepancy between experiment and simulation.

In analyzing single-molecule unfolding curves (i.e., **Fig. 2**), we note that the spotted DNA at the measured array addresses correctly corresponded to the domain of interest encoded by the corresponding spotted DNA at that position. For example, the fibronectin tetramer was measured at array position (237), the spectrin dimer at position (239), the xylanase monomer at position (196), and the sfGFP monomer at position (238), corresponding to the correct genes deposited into the expression chambers at those array positions (**Fig. 2**). Typically 10–15 immobilization chambers per microarray were measured. Typically several thousand force curves were acquired giving rise to dozens of interpretable single-molecule interaction curves.

Upper force limit

Here we extend the discussion regarding the upper force limit for the SMFS-MITOMI system. In all force-distance data traces, the last rupture events represent unbinding of the Coh-Doc complex, not unfolding of a domain. This rupture force of the Coh-Doc complex represents an upper limit in force for the entire construct, since the Doc is used as a handle sequence grabbed by the Coh-modified cantilver. The system we described can therefore interrogate domains with mechanical rupture forces that lie below that of Coh-Doc (~125 pN at 10 nN/s). If proteins with larger unfolding forces should be investigated, other Coh-Doc domains that show even higher complex rupture forces can be used. The Coh-Doc pair from *R. flavefaciens*, for example (PDB 4IU3) exhibits rupture forces over 600 pN at these loading rates (unpublished data). This could alternatively be used as a handle sequence to interrogate mechanically more stable domains of interest.

Computerized image analysis can be used to automate cantilever positioning above the fluorescent rings and subsequent acquisition of unfolding traces at each array address in combination with online force curve analysis to further increase throughput. Additionally, well-characterized reference proteins on the same chip may serve as calibration standards further minimizing uncertainty in absolute force values.

It is possible to operate the MITOMI device in a simplified way without the need for microspotting template DNA and chip alignment. This manual option should encourage the interested community to apply the suggested method to their single molecule force spectroscopy experiments. MITOMI enables the experimenter to prepare up to 16 different constructs in one column with 40 repeats each by flow-loading the DNA. Since

the valves are pressure sensitive it is also possible to operate these manually. This way it is possible to make use of the parallelized method without having the automation tools.Supplementary Materials & Methods

DNA Sequences

Supplementary Table 1. Overview of primers

	Name	Sequence
1	FW-w/o C-Tags MCS	TAACTCGAGTAAGATCCGGCTGC
2	REV-N-Tags MCS	GCTAGCACTAGTCCATGGGTG
3	FW-DocI GA	AAAGTGGTACCTGGTACTCC
4	REV-XylDocI-GA	CGGATCTTACTCGAGTTAGTTCTTGTACGGCAATGTATC
5	FW 10FNIII GA	CGCACCGGCTCTGGCTCTGGCTCTGTTAGTGATGTTCCGCGTG
6	REV 10 FNIII GA	GGAGTACCAGGTACCACTTTGGTGCG
7	REV 10FNIII (auf GS Li) GA	ACTAACAGAGCCAGAGCCAGAGCCGGTGCGATAATTGATTGAAATC
8	FW sfGFP (auf MCS) GA	CACCCATGGACTAGTGCTAGCAGCAAAGGTGAAGAACTGTTTAC
9	REV sfGFP (auf DocI) GA	GGAGTACCAGGTACCACTTTCTTATACAGCTCATCCATACCATG

Supplementary Table 2. Overview of DNA plasmids available at Addgene database

Addgene ID	Construct
58708	pET28a-ybbR-HIS-sfGFP-DocI
58709	pET28a-ybbR-HIS-CBM-CohI
58710	pET28a-StrepII-TagRFP-CohI
58711	pET28a-ybbR-HIS-Xyl-DocI
58712	pET28a-ybbR-HIS-10FNIII(x4)-DocI
58713	pET28a-ybbR-HIS-Spec(x2)-DocI

Multiple cloning site for the protein of interest:

N terminal region

T7 promoter | *lac operator* | *RBS* | *ATG* | *ybbr Tag* | *HRV 3C protease site* | *HIS Tag (x6)*

TAATACGACTCACTATAGG|GGAATTGTGAGCGGATAACAATTCC|CCTGTAGAAATAATTTTGT
TTAACTTTAAG|AAGGA|GATATACAT|ATG|GGTACC|GACTCTCTGGAATTCATCGCTTCTAA
ACTGGCT|CTGGAAGTTCTGTTCCAGGGTCCG|CTGCAG|CACCACCACCACCACCAC|CCATGG
ACTAGTGCTAGC

C terminal region

Dockerin Type I | T7 terminator

AAAGTGGTACCTGGTACTCCTTCTACTAAATTATACGGCGACGTCAATGATGACGGAAAAGTTAA
CTCAACTGACGCTGTAGCATTGAAGAGATATGTTTTGAGATCAGGTATAAGCATCAACACTGACA
ATGCCGATTTGAATGAAGACGGCAGAGTTAATTCAACTGACTTAGGAATTTTGAAGAGATATATT
CTCAAAGAAATAGATACATTGCCGTACAAGAAC|TAA|CTCGAGTAAGATCCGGCTGCTAACAAA
GCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAA|CTAGCATAACCCCTTGGGG
CCTCTAAACGGGTCTTGAGGGGTTTTTT

10 FibronectinIII (4x):

Glycin-Serin Linker (x6)

GTTAGTGATGTTCCGCGTGATCTGGAAGTTGTTGCAGCAACCCCGACCAGCCTGCTGATTAGCTG
GGATGCACCGGCAGTTACCGTTCGTTATTATCGTATTACCTATGGTGAAACCGGTGGTAATAGTC
CGGTTCAAGAATTTACCGTTCCGGGTAGCAAAAGCACCGCAACCATTAGCGGTCTGAAACCGGGT
GTTGATTACACCATTACCGTTTATGCCGTTACCGGTCGTGGTGATTCACCGGCAAGCAGCAAACC
GATTAGCATTAACTATCGTACCGGTAGCGGTAGTGGTAGCGTTTCAGATGTGCCTCGCGACCTGG
AAGTGGTGGCTGCCACACCGACCTCACTGCTGATCTCATGGGATGCCCCTGCCGTGACCGTGCGC
TATTATCGCATCACATATGGCGAGACAGGTGGCAATTCACCTGTGCAAGAATTCACAGTTCCTGG
TTCAAAAAGTACCGCCACAATTTCTGGCCTGAAACCTGGCGTGGATTACACAATCACAGTGTATG
CAGTGACAGGTCGCGGTGATAGTCCGGCAAGTTCAAAACCGATTTCAATCAATTATCGCACCGGC
TCTGGCTCTGGCTCTGTTAGTGATGTTCCGCGTGATCTGGAAGTTGTTGCAGCAACCCCGACCAG
CCTGCTGATTAGCTGGGATGCACCGGCAGTTACCGTTCGTTATTATCGTATTACCTATGGTGAAA
CCGGTGGTAATAGTCCGGTTCAAGAATTTACCGTTCCGGGTAGCAAAAGCACCGCAACCATTAGC
GGTCTGAAACCGGGTGTTGATTACACCATTACCGTTTATGCCGTTACCGGTCGTGGTGATTCACC
GGCAAGCAGCAAACCGATTAGCATTAACTATCGTACCGGTAGCGGTAGTGGTAGCGTTTCAGATG
TGCCTCGCGACCTGGAAGTGGTGGCTGCCACACCGACCTCACTGCTGATCTCATGGGATGCCCCT
GCCGTGACCGTGCGCTATTATCGCATCACATATGGCGAGACAGGTGGCAATTCACCTGTGCAAGA
ATTCACAGTTCCTGGTTCAAAAAGTACCGCCACAATTTCTGGCCTGAAACCTGGCGTGGATTACA
CAATCACAGTGTATGCAGTGACAGGTCGCGGTGATAGTCCGGCAAGTTCAAAACCGATTTCAATC
AAttaTCGCACC

sfGFP:

AGCAAAGGTGAAGAACTGTTTACCGGTGTTGTTCCGATTCTGGTTGAACTGGATGGTGATGTTAA
TGGCCACAAATTTTCAGTTCGTGGTGAAGGCGAAGGTGATGCAACCATTGGTAAACTGACCCTGA
AATTTATCTGTACCACCGGCAAACTGCCGGTTCCGTGGCCGACCCTGGTTACCACCCTGACCTAT
GGTGTTCAGTGTTTTAGCCGTTATCCGGATCATATGAAACGCCACGATTTTTTCAAAAGCGCAAT
GCCGGAAGGTTATGTTCAAGAACGTACCATCTCCTTTAAAGACGACGGTAAATACAAAACCCGTG
CCGTTGTTAAATTTGAAGGTGATACCCTGGTGAATCGCATTGAACTGAAAGGCACCGATTTTAAA
GAGGATGGTAATATCCTGGGCCACAAACTGGAATATAATTTCAATAGCCACAACGTGTATATCAC
CGCAGACAAACAGAAAAATGGCATCAAAGCCAATTTTACCGTGCGCCATAATGTTGAAGATGGTA
GCGTGCAGCTGGCAGATCATTATCAGCAGAATACCCCGATTGGTGATGGTCCGGTTCTGCTGCCG
GATAATCATTATCTGAGCACCCAGACCGTTCTGAGCAAAGATCCGAATGAAAAACGTGATCATAT
GGTGCTGCATGAGTATGTTAATGCAGCAGGTATTACCCATGGTATGGATGAGCTGTATAAG

alpha-Spectrin repeat 16 (chicken brain) (x2):

Glycin-Serine Linker (x6)

CGTGCTAAACTGAACGAATCTCACCGTCTGCACCAGTTCTTCCGTGACATGGACGACGAAGAATC
TTGGATCAAAGAAAAAAAACTGCTGGTTTCTTCTGAAGACTACGGTCGTGACCTGACCGGTGTTC
AGAACCTGCGTAAAAAACACAAACGTCTGGAAGCTGAACTGGCTGCTCACGAACCGGCTATCCAG
GGTGTTCTGGACACCGGTAAAAAACTGTCTGACGACAACACCATCGGTAAAGAAGAAATCCAGCA
GCGTCTGGCTCAGTTCGTTGACCACTGGAAAGAACTGAAACAGCTGGCTGCTGCTCGTGGTCAGC
GTCTGGAAGAATCTCTGGAATACGGTAGCGGTAGCGGTTCACGTGCTAAACTGAACGAATCTCAC
CGTCTGCACCAGTTCTTCCGTGACATGGACGACGAAGAATCTTGGATCAAAGAAAAAAAACTGCT
GGTTTCTTCTGAAGACTACGGTCGTGACCTGACCGGTGTTCAGAACCTGCGTAAAAAACACAAAC
GTCTGGAAGCTGAACTGGCTGCTCACGAACCGGCTATCCAGGGTGTTCTGGACACCGGTAAAAAA
CTGTCTGACGACAACACCATCGGTAAAGAAGAAATCCAGCAGCGTCTGGCTCAGTTCGTTGACCA
CTGGAAAGAACTGAAACAGCTGGCTGCTGCTCGTGGTCAGCGTCTGGAAGAATCTCTGGAATAt

Xylanase:

AAGAATGCAGATTCCTATGCGAAAAAACCTCACATCAGCGCATTGAATGCCCCACAATTGGATCA
ACGCTACAAAAACGAGTTCACGATTGGTGCGGCAGTAGAACCTTATCAACTACAAAATGAAAAAG
ACGTACAAATGCTAAAGCGCCACTTCAACAGCATTGTTGCCGAGAACGTAATGAAACCGATCAGC
ATTCAACCTGAGGAAGGAAAATTCAATTTTGAACAAGCGGATCGAATTGTGAAGTTCGCTAAGGC
AAATGGCATGGATATTCGCTTCCATACACTCGTTTGGCACAGCCAAGTACCTCAATGGTTCTTTC
TTGACAAGGAAGGTAAGCCAATGGTTAATGAATGCGATCCAGTGAAACGTGAACAAAATAAACAA
CTGCTGTTAAAACGACTTGAAACTCATATTAAAACGATCGTCGAGCGGTACAAAGATGACATTAA
GTACTGGGACGTTGTAAATGAGGTTGTGGGGGACGACGGAAAACTGCGCAACTCTCCATGGTATC
AAATCGCCGGCATCGATTATATTAAAGTGGCATTCCAAGCAGCTAGAAAATATGGCGGAGACAAC
ATTAAGCTTTACATGAATGATTACAATACAGAAGTCGAACCGAAGCGAACCGCTCTTTACAATTT
AGTCAAACAACTGAAAGAAGAGGGTGTTCCGATCGACGGCATCGGCCATCAATCCCACATCCAAA
TCGGCTGGCCTTCTGAAGCAGAAATCGAGAAAACGATTAACATGTTCGCCGCTCTCGGTTTAGAC
AACCAAATCACTGAGCTTGATGTGAGCATGTACGGTTGGCCGCCGCGCGCTTACCCGACGTATGA
CGCCATTCCAAAACAAAAGTTTTTGGATCAGGCAGCGCGCTATGATCGTTTGTTCAAACTGTATG
AAAAGTTGAGCGATAAAATTAGCAACGTCACCTTCTGGGGCATCGCCGACAATCATACGTGGCTC
GACAGCCGTGCGGATGTGTACTATGACGCCAACGGGAATGTTGTGGTTGACCCGAACGCTCCGTA
CGCAAAAGTGGAAAAAGGGAAAGGAAAAGATGCGCCGTTCGTTTTTGGACCGGATTACAAAGTCA
AACCCGCATATTGGGCTATTATCGACCAC

Detection construct RFP-Cohesin:

TagRFP-Cohesin:

T7 promoter | *lac operator* | *RBS* | *ATG* | *StrepII Tag* | *TagRFP* | *Linker* | *Cohesin* | T7 terminator

TAATACGACTCACTATAGG|GGAATTGTGAGCGGATAACAATTCC|CCTGTAGAAATAATTTTGT
TTAACTTTAAG|AAGGA|GATATACAT|ATG|GGTACC|TGGTCTCACCCGCAGTTCGAAAAA|G
TTTCTAAAGGTGAAGAACTGATCAAAGAAAACATGCACATGAAACTGTACATGGAAGGTACTGTT
AACAACCACCACTTCAAATGCACCTCTGAAGGTGAAGGTAAACCGTACGAAGGTACTCAGACCAT
GCGTATCAAAGTTGTTGAAGGTGGTCCGCTGCCGTTCGCTTTCGACATCCTGGCTACCTCTTTCA
TGTACGGTTCTCGTACCTTCATCAACCACACCCAGGGTATCCCGGACTTCTTCAAACAGTCTTTC
CCGGAAGGTTTCACCTGGGAACGTGTTACCACCTACGAAGACGGTGGTGTTCTGACCGCTACCCA
GGACACCTCTCTGCAAGACGGTTGCCTGATCTACAACGTTAAAATCCGTGGTGTTAACTTCCCGT
CTAACGGTCCGGTTATGCAGAAAAAAACCCTGGGTTGGGAAGCTAACACCGAAATGCTGTACCCG
GCTGACGGTGGTCTGGAAGGTCGTTCTGACATGGCTCTGAAACTGGTTGGTGGTGGTCACCTGAT
CTGCAACTTCAAAACCACCTACCGTTCTAAAAAACCGGCTAAAAACCTGAAAATGCCGGGTGTTT
ACTACGTTGACCACCGTCTGGAACGTATCAAAGAAGCTGACAAAGAAACCTACGTTGAACAGCAC
GAAGTTGCTGTTGCTCGTTACTGCGACCTGCCGTCTAAACTGGGTCACAAACTGAAC|GGCAGTG
TAGTACCATCAACACAGCCTGTAACAACACCACCTGCAACAACAAAACCACCTGCAACAACAATA
CCGCCGTCAGATGATCCGAATGCA|GGATCCGACGGTGTGGTAGTAGAAATTGGCAAAGTTACGG
GATCTGTTGGAACTACAGTTGAAATACCTGTATATTTCAGAGGAGTTCCATCCAAAGGAATAGCA
AACTGCGACTTTGTGTTCAGATATGATCCGAATGTATTGGAAATTATAGGGATAGATCCCGGAGA
CATAATAGTTGACCCGAATCCTACCAAGAGCTTTGATACTGCAATATATCCTGACAGAAAGATAA
TAGTATTCCTGTTTGCGGAAGACAGCGGAACAGGAGCGTATGCAATAACTAAAGACGGAGTATTT
GCAAAAATAAGAGCAACTGTAAAATCAAGTGCTCCGGGCTATATTACTTTCGACGAAGTAGGTGG
ATTTGCAGATAATGACCTGGTAGAACAGAAGGTATCATTTATAGACGGTGGTGTTAACGTTGGCA
ATGCAACA|TAA|CTCGAGTAAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTG
CTGCCACCGCTGAGCAATAA|CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTT
TTT

Molecular weights of synthesized fusion proteins

ybbR-(Fibronectin)$_4$-Dockerin Type I: 53 kDa
ybbR-(Spectrin)$_2$-Dockerin Type I: 40 kDa
ybbR-Xylanase-Dockerin Type I: 56 kDa
ybbR-sfGFP-Dockerin Type I: 39 kDa
ybbR-Twitchin-Dockerin Type I: 52 kDa

Supplementary Table 3. Yield of interpretable curves

Construct	**Interpretable Curves**
GFP	25 out of 15258 = 0.16 %
Fibronectin	27 out of 26653 = 0.1 %
Xylanase	91 out of 5553 = 1.64 %
Spectrin	50 out of 10344 = 0.48%

References

1. Merkel, R., Nassoy, P., Leung, A., Ritchie, K. & Evans, E. Energy landscapes of receptor–ligand bonds explored with dynamic force spectroscopy. *Nature* **397,** 50–53 (1999).
2. Rico, F., Gonzalez, L., Casuso, I., Puig-Vidal, M. & Scheuring, S. High-Speed Force Spectroscopy Unfolds Titin at the Velocity of Molecular Dynamics Simulations. *Science* **342,** 741–743 (2013).