Supporting Information

Going Vertical to Improve the Accuracy of Atomic Force Microscopy Based Single-Molecule Force Spectroscopy

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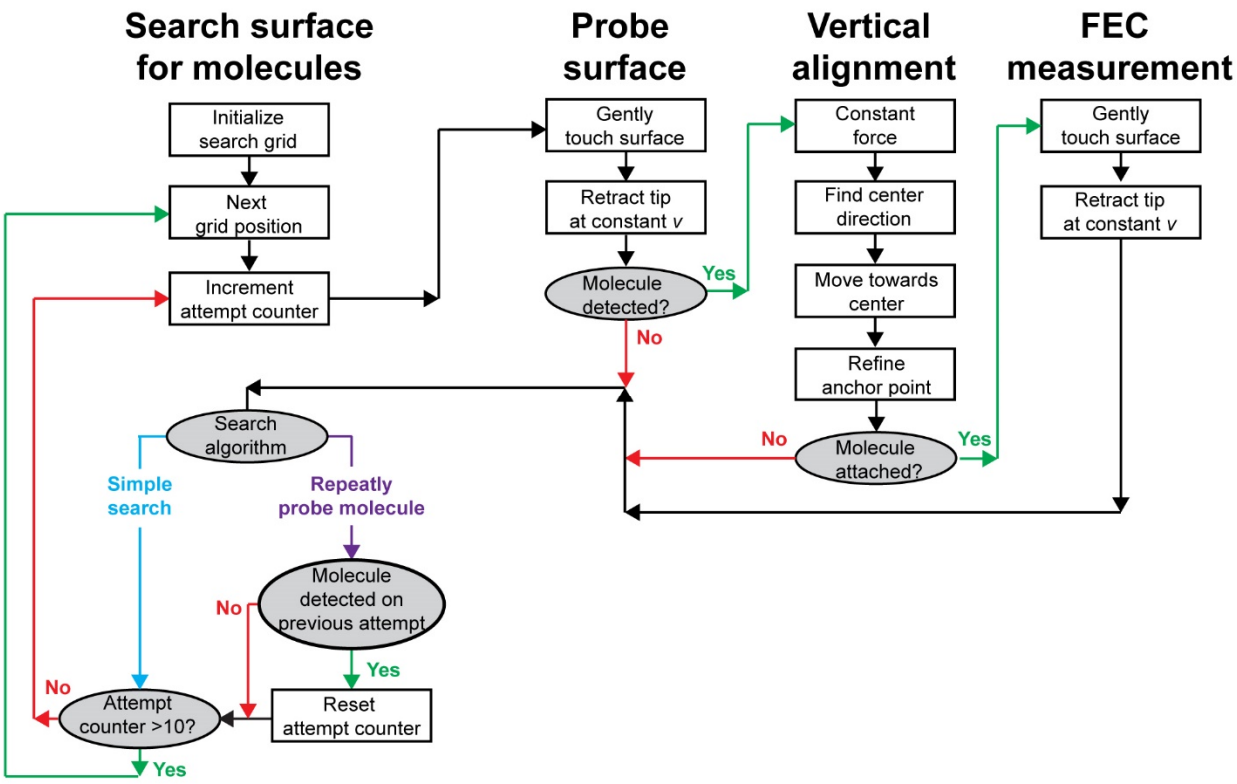

Figure S1. Process diagram for automated data acquisition using the vertical alignment algorithm for DNA molecules. For the polyprotein- and PEG-based assays, the steps "Find center direction" and "Move towards center" were omitted due to the smaller initial r_{offset}.

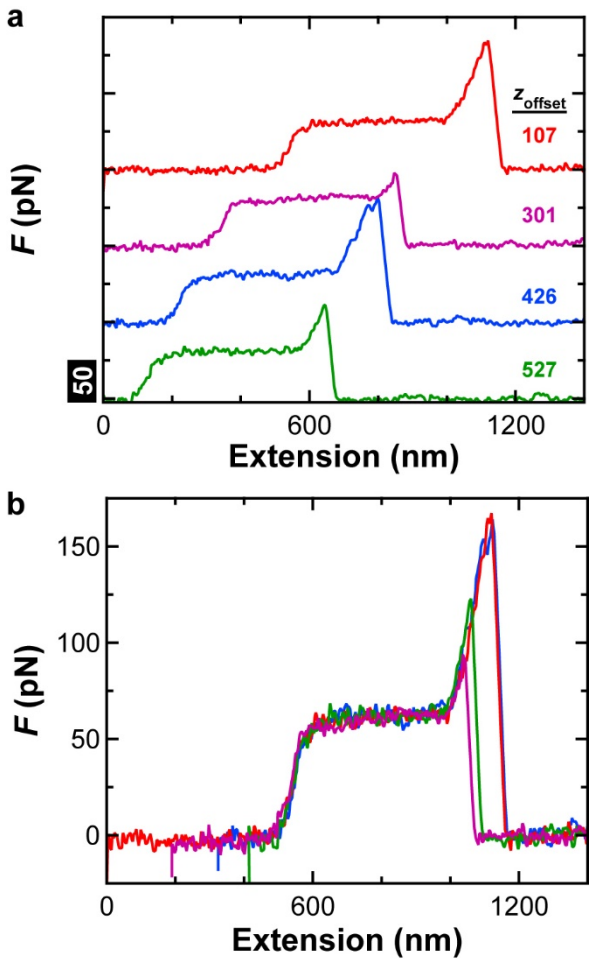

Figure S2. Vertically aligned force-extension curves exhibit the canonical values for DNA overstretching. (a) Examples of force-extension curves for 650-nm DNA with varying z_{offset} (107, 301, 426, and 527 nm). Force-extension curves are vertically offset for clarity. (b) Laterally shifted force-extension curves illustrate that overstretching transition does not vary with z_{offset}. As expected, the extension plateau occurs at the canonical of ~65 pN for DNA overstretching and plateau width was ~70% of the contour length (450 nm) for 650 nm regardless of z_{offset}. Note, small downward spikes below 0 pN are associated with contact with the surface.

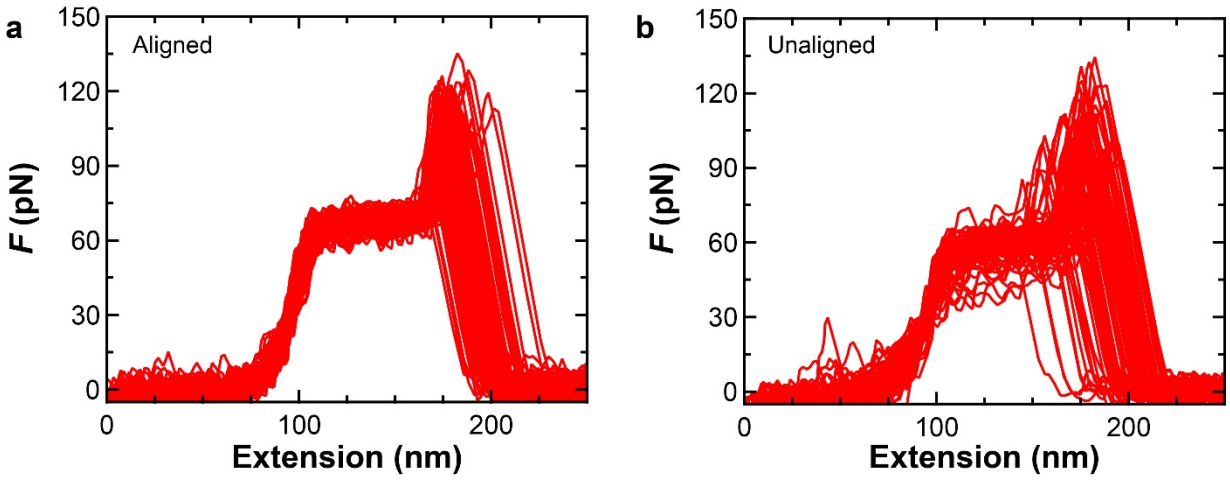

Figure S3. Improved precision and accuracy in a DNA-based assay. (a) An overlay of 93 force-extension curves of distinct 100-nm DNA molecules stretched at 4 μm/s that were laterally aligned. (b) An overlay of 84 force-extension force (FEC) without such lateral alignment. In both cases, hydrodynamic drag on the cantilever was accommodated for by setting the force after rupture to $F = 0$ pN. To avoid a distracting visually artifact due to lateral alignment at 30 pN, a small random lateral offset was added to the FEC curves by sampling from a Gaussian distribution with a width of 1.5 nm, equivalent to the random noise on the FECs This process does not alter the vertical spread in the overlay of the traces, the key scientific point of this comparison. Finally, we note that the larger lateral spread in the length of the overstretching transition arises from using DNA molecules containing multiple biotin labels which leads to a variation in the effective contour length depending on which biotins are attached to the tip and the orientation of the DNA backbone relative at the attachment point to the tip relative to the stretching axis, since it is not free to rotate when attached by multiple bonds.

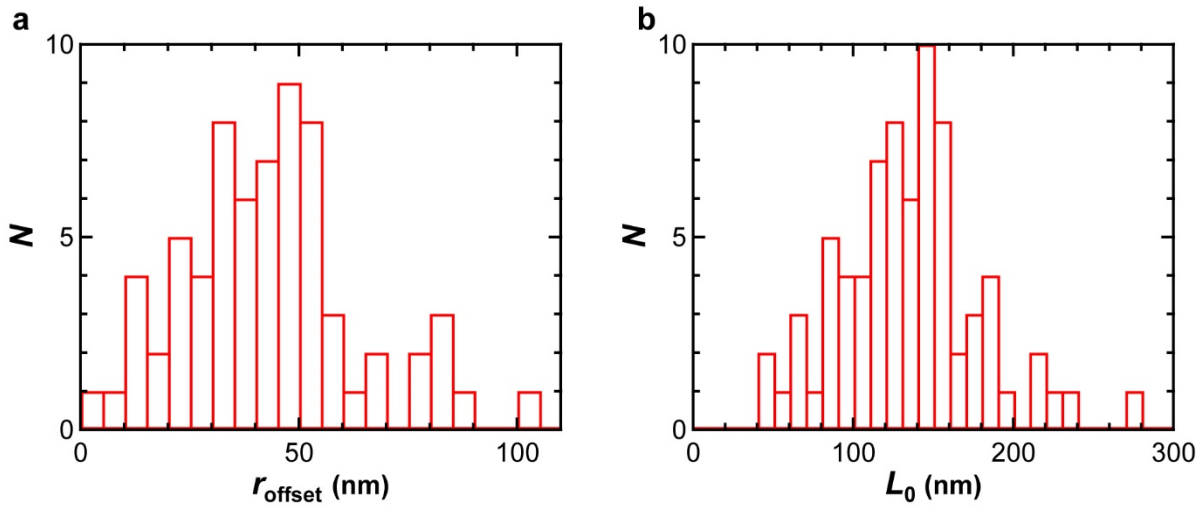

Figure S4. Geometric errors when vertically aligning a protein-ligand assay using $PEG_{MW=10k}$. (a) Histogram of the lateral offset (r_{offset}) for 68 PEG molecules that were geometrically aligned at 15 pN. Average lateral offset was 43 ± 20 nm (mean ± std. dev.). (b) Histogram of the associated measured contour lengths (L_0) yielded an average L_0 of 134 nm ± 44 nm (mean ± std. dev.) based on analysis of the aligned force-extension curves. While this average L_0 agreed with the expected value of ~130 nm based on chemical structure of full assay (*e.g.*, both PEG linkers, the size of the streptavidin, the length of the tether to the biotin), L_0 ranged from 43–270 nm, consistent with previous reports of polydisperity in PEG-based assays.[1] We note that a strict comparison of r_{offset}/L_0 between the polyprotein assay (L_0 = 92 nm) reported in Figure 6 and the longer PEG-based assay is simplistic because the polyprotein is initially folded. L_0 for the folded polyprotein is ~33 nm and thus similar to the $r_{offset}/L_0 \approx 1/3$ demonstrated here for a PEG-based assay. Unexpectedly, this ratio is larger than the for the DNA-based assay based on a much stiffer polymer and future work is needed to deduce if the origin of this result primarily arises from the polydisperity in the PEG, or other factors, such as density of PEG-linkers on the surface.

Molecule	Circle radius (nm)	Step distance (nm)	Step sample time (ms)	Cross distance (nm)	Sample rate (kHz)
650-nm DNA	75	150	250	150	1
100-nm DNA	20	20	250	50	1
NuG2 Polyprotein	-	-	-	50	1
PEG_{10k}	-	-	-	60	1

Table S1. Summary of value used by the geometric correction algorithm for the 650-nm DNA, 100-nm DNA, the NuG2 polyprotein and PEG_{10k}.

Primer	Sequence
4xBiotin-M13MP18-3520R	5'Biotin-TEG-TCA ATA A-iBiodT-CGG CTG TCT-iBiodT-TCC TTA TCA-iBiodT-TC
DBCO-M13MP18-1607F	5'DBCO-TEG-AGT TGT TCC TTT CTA TTC TCA CTC CGC
DBCO-M13MP18-3222F	5'DBCO-TEG-TTA GGC TCT GGA AAG ACG CTC GTT AG

Table S2. Primers used for 100- and 650-nm DNA constructs.

References

(1) Ott, W.; Jobst, M. A.; Bauer, M. S.; Durner, E.; Milles, L. F.; Nash, M. A.; Gaub, H. E., Elastin-Like Polypeptide Linkers for Single-Molecule Force Spectroscopy. *ACS Nano* **2017,** *11*, 6346-6354.