Mechanical Load Induces a 100-Fold Increase in the Rate of Collagen Proteolysis by MMP-1
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Supplemental Information

Materials:

The collagen model peptide gene was synthesized by DNA 2.0 (Menlo Park, CA). Biotin maleimide, bovine serum albumin (BSA) and 4-aminophenylmercuric acetate (APMA) were purchased from Sigma Aldrich (St. Louis, MO). Mouse Anti-*Myc,* streptavidin coated magnetic beads (Dynal MyOne T1 and Dynal M-280), DNA from λ-phage, T4 DNA ligase (5 U/μL) were purchased from Invitrogen (Carlsbad, CA). Coverslips were purchased from VWR International (West Chester, PA). MMP-1 cDNA (*Homo sapiens*) was obtained from Harvard Plasmid Database (Harvard, MA) and cloned into the pJexpress 414 vector by DNA Express (Ontario, Canada).

Collagen model peptide expression and purification:

The collagen model peptide consists of a *N*-terminal 6xHis-tag for purification, followed by a 5x *myc* tag, $(GPP)_{10}$ to enforce triple helix formation, the collagen α1 residues 772-786 (GPQGIAGQRGVVGL), which form the MMP-1 recognition site, the foldon sequence (GSGYIPEAPRDGQAYVRKDGEWVLLSTFL), and a *C*-terminal KKCK to facilitate labeling with biotin-maleimide. Foldon derives from the T4-phage protein fibritin, and stabilizes collagen model trimers when fused at either the *N*- or *C*-terminus(*1-2*). The plasmid was transformed into chemically competent BL21(DE3) *E. coli* cells. The cells were grown to an OD = 0.6 at 37 °C, and then induced by adding IPTG for five hours at 25 °C. The cells were harvested by centrifuging at 4000xG for 30 minutes at 4 °C. The cell pellet was resuspended in PBS and centrifuged at 4000xG for 30 minutes at 4 °C. The cells were resuspended in 50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole buffer (pH 8.0). 5 μM each of lysozyme, DNAse I, pepstatin A, leupeptin, and PMSF were added to the suspension and gently mixed at 4 °C for 45 minutes. The mixture was sonicated on ice for 5 cycles (30 seconds each), and then centrifuged at 6000 x G for 30 minutes at 4 °C.

Nickel-NTA agarose (Qiagen) was added (2 mL per 5 mg of protein) to the supernatant and gently mixed at 4 °C for 90 minutes. The protein was purified using His-Tag affinity chromatography. Briefly, the supernatant with the nickel agarose beads was added to a 10 mL column, and the flow through was collected. The resin was allowed to settle (not allowed to dry) and washed twice with 5 mL of solution containing 50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole buffer (pH 8.0). The protein was eluted with eight 500 µL aliquots of 50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole buffer (pH 8.0). All the samples were run on a denaturing poly acrylamide gel to determine which eluent had the highest concentration of protein. The eluent fractions with the highest concentration of the desired protein were dialyzed against a buffer of 5 mM sodium phosphate (pH 7.4), 150 mM sodium chloride and 50% (v/v) glycerol overnight. The concentration of the protein was determined using the Bradford assay, and the protein was stored long term at -20°C.

MMP-1 expression and purification:

Full-length MMP-1 (minus the signal peptide) was cloned into a pET 28b+ vector for expression. The protein was expressed in chemically competent BL21(DE3) *E. coli* cells. The cells were grown to an OD = 0.6 at 37 °C, and protein expression induced by adding IPTG for five hours at 25 °C. The cells were pelleted by centrifuging at 4000 x G for 30 minutes at 4 °C. The pellet was collected and resuspended in PBS and centrifuged at 4000 x G for 30 minutes at 4 °C. The cells were frozen overnight at -20 °C (the freeze thaw step aids in cell lysis). The pellet was resuspended in 50 mM Tris HCl (pH 8.0), 100 mM sodium chloride, 200 mM EDTA, 1 mM DTT and 25% (w/v) glucose for 30 minutes. 1 mg of lysozyme was added, followed by 30-minute incubation at 4 °C. Next, an equal volume of lysis buffer (50 mM Tris HCl (pH 8.0), 100 mM NaCl, 200 mM EDTA, 1 mM DTT, 2% triton-X 100) was added, followed by a further 15-minute incubation at 4 °C. The mixture was sonicated on ice for 5 cycles of 30 seconds each. The sonicated suspension was centrifuged at 6000xG for 30 minutes at 4°C to harvest the inclusion bodies. The pellet was collected and suspended in 6 M urea and 20 mM Tris HCl (pH 8.0) for 2 hours at

4°C to dissolve the cell debris. The suspension was centrifuged at 6000xG for 30 minutes at 4 °C and the inclusion bodies collected as the pellet. The inclusion bodies were dissolved overnight in 6 M Gdn-HCl, 20 mM Tris-HCl (pH 7.0), 10 mM DTT and 20 mM cystamine. The suspension was centrifuged at 6000xG for 30 minutes at 4°C and the supernatant collected. The protein concentration was measured using a Bradford Assay. The solubilized, denatured protein solution was diluted to a concentration of 0.12 mg mL^{-1} in 6 M Gdn-HCl, 20 mM Tris-HCl (pH 7.0), 10 mM DTT and 0.05% Brij-35 and gently mixing at 4°C for two hours. The solution was then dialyzed overnight against 20 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, 0.1 mM zinc chloride, 150 mM NaCl, 5 mM β-mercaptoethanol, 1 mM 2-hydroxyethyldisulfide and 0.05% Brij-35. Next day the protein was dialyzed against 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, 50 μM zinc chloride, and 0.02% sodium azide. The purity of the protein was checked using SDS-PAGE, and since the protein was sufficiently pure (Fig. S1A) no further purification steps were used. The protein was flash frozen and stored at -80°C.

Prior to use, MMP-1 was activated by 3.5 mM APMA: A 35 mM stock of APMA was made by adding APMA to 0.1 NaOH and rigorously vortexing. 1 volume of APMA was added to 10 volumes of MMP and gently mixed using a pipettor. The activation was carried out at 37 °C for three hours. The activation results in a cleavage of the pro-peptide and this was confirmed by a downshift in the gel (Fig. S1A)

Collagen bulk proteolysis:

1 μM activated MMP-1 was added to 80 μM collagen model protein and incubated at room temperature. Aliquots were taken at 10, 20, 30, 40, 50 and 60 minutes. The reaction was quenched by adding 10 mM EDTA and briefly boiling the samples. The samples were analyzed using SDS-PAGE, and the results confirm collagen proteolysis by MMP-1. (Fig. S1B).

MALDI-Mass Spec:

MALDI mass spectrometry (MALDI-MS) confirms that the monomeric collagen model protein has a mass of 14.4 kD identical to the predicted mass. Native PAGE confirms that the collagen oligomerize, with negligible amounts of dimer, monomer, or higher order oligomers observed. The proteolysis products of collagen model trimer were analyzed by MALDI-MS to determine the proteolysis site(s). 70 μM collagen was proteolyzed by 1 μM MMP-1 at room temperature for 45 minutes. 10 mM EDTA was added to quench the reaction. The sample was flash frozen for preservation. MALDI-MS was done at the Stanford University Protein and Nucleic Acid Facility on a Perseptive Voyager MALDI-TOF mass spectrometer. The mass spec shows a prominent peak at 9750 Da, corresponding to proteolysis at GPQG---IAGQRGVVGL (--- denoting the cleavage site), as predicted based on previous studies (*3*).

Magnetic tweezers setup:

The magnetic tweezer was built on a Zeiss Axiovert 100TM microscope (Fig. S2). Two rare earth magnets separated by a 1 mm circular aperture were mounted on an L-bracket, which is attached to a vertical motorized translator MTS50-Z8, (Thorlabs, Newton, NJ). The magnetic field, and hence applied force, was controlled by moving the magnets using the stepping stage.

Magnetic Trap Force Calibration:

DNA from lambda phage was used for calibrating the magnetic tweezer. The sticky ends of the λ-DNA were functionalized by oligo-nucleotides conjugated with biotin and digoxigenin (IDT DNA, San Diego, CA). 50 μL of 4 nM λ-DNA was mixed with 2 μL of 40 nM biotin conjugated oligo and heated at 60 °C for 10 minutes and slowly cooled at room temperature for 1 hour. DNA ligase was added according to manufacturer's specifications, and ligation was carried out at room temperature for 3 hours. 2 μL of 400 nM digoxigenin conjugated oligonucleotide was added and incubated at room temperature for 45 mins. Finally, 1.5 μL of 10 mM ATP was added and ligation was continued for 3 hours. The excess oligos were removed using a 100 kDa spin filters (Microcon Ultracell YM-100).

For DNA attachment to the flow cell, the anti-digoxigenin antibody (sheep IgG) (1 μg mL^{-1}; Roche Bioscience, Manheim, Germany) was added to the flow cell and incubated for 15 minutes. The surface was passivated by washing the flow cell with 2 mg mL^{-1} BSA, 0.1% Tween-20 in PBS followed by incubation at room temperature for 45 minutes. This step was repeated once. ~50 pM of fuctionalized λ-DNA was added, and allowed to attach to the coverslip for 15 minutes. The excess DNA was washed with BSA buffer. Streptavidin-coated superparamagnetic beads (1 μm beads = 2 μg mL^{-1}; 2.8 μm beads = 20 μg mL^{-1}) were then added and allowed to attach to the immobilized DNA for 15 minutes.

Fields of view that contained several single beads (Fig S2) were picked. Videos were captured at 79.96 Hz for 500 frames. The procedure was repeated by varying the height of magnets (controlled by a motorized *z*-translator) and force was calculated at various magnet distances from the flow cell surface (Fig S3). The force was calibrated by measuring the Brownian fluctuations of the beads (*4*) and confirmed using power spectrum analysis (*5*). We used a custom-written bead tracking program in MATLAB to measure the force due to Brownian motion. Bead centers were tracked by first selecting the center of the bead to extract a 40x40 pixel image centered at the selection and fitting a 2D Gaussian mask to the resulting 40x40 pixel image. The location of each bead was tracked over the entire 500 frame video resulting in 500 x-y positions for the bead. Force was calculated as:

$$F = \frac{k_B T * l}{\sigma^2} \qquad \text{Eqn. 1}$$

Here k_BT is the energy due to Brownian fluctuations (4.2 pN nm), l is the length of the λ-DNA (16.3 μm) and σ^2 is the variance in x or y due to the bead position fluctuation.

The force generated by the magnetic trap was confirmed using power spectrum analysis. The x and y positions of the bead were tracked using the method described above. The autocorrelation of the beads were calculated using MATLAB's autocorr() function. The power spectrum was then obtained by taking

the magnitude of the Fourier transform of the autocorrelation using MATLAB's abs() and fft() functions. The resulting power spectrum was plotted against a frequency and fit to a Lorentzian plus constant:

$$PS(f) = \frac{a}{f^2 + f_o^2} + c \qquad \text{Eqn. 2}$$

Where f_o represents the rolloff frequency as described in ref. *5*. (NOTE: typical Lorentzian does not include the constant "*c*". However, the discreteness of the data set causes the autocorrelation function to not actually decay to exactly zero and so the constant is added to account for that fact.) Once the rolloff frequency is determined, the force is determined to be $12\pi^2\mu a f_o L$ where μ is the fluid viscosity (assumed to be 1cP as water) and a is the bead radius (0.5 μm or 1.4 μm). Comparisons of the force calculated by the two methods agree to within 20%.

Single molecule collagen proteolysis by MMP-1:

The flow chamber was a coverslip sandwich made of 22x22 mm and 22x40 mm, no. 1.5 coverslips. It was found that the particular brand of coverslips (VWR International) was important for the surface attachments to work properly. Unless mentioned otherwise, all protein solutions were in diluted in 5 mM sodium phosphate (pH 7.4), 150 mM sodium chloride buffer. Anti-*myc* (15 μg mL^{-1}) was added to the flow cell and incubated at room temperature for 20 minutes. 5 mg mL^{-1} BSA was added to the flow cell and incubated for 1 hour to passivate the surface and prevent non-specific sticking of proteins and beads. The flow cell was washed with BSA buffer and incubated for 15 minutes. 150 pM collagen was added and allowed to attach to the antibody via the *myc* tag for 45 minutes. Excess collagen molecules were washed out and streptavidin coated super paramagnetic beads (1 μm beads = 2 μg $mL^{-1}$and 2.8 μm beads = 20 μg mL^{-1}) were then added followed by a 45 minute incubation.

The concentration of antibody was used such that there are only sparse attachments dependent on the presence of both antibody and biotinylated (vs. non-biotinylated) collagen were observed (Table S1). The

cleavage kinetics we observe are single-exponential under all conditions, strongly supporting the conclusion that only one collagen trimer tethers each magnetic bead to the surface is the great majority of cases.

The collagen trimer consists of three peptides, each of which has a 5x *myc* tag and a biotin for attachment. There is thus the possibility of anywhere between 1-3 attachments at either end. However, the single-exponential kinetics we observe strongly argue for a mechanically and chemically homogeneous population of surface-immobilized trimers. The trimers are held together by an *N*-terminal $(GPP)_{10}$ repeat and *C*-terminal foldon domains, both of which strongly enforce trimerization. Given the simple kinetics we observe, plus the known robustness of similar collagen model proteins, the most likely explanation is that the $(GPP)_{10}$ and foldon domains distribute applied force throughout the trimer irrespective of the number of attachments.

Activated MMP-1 (diluted in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, 50 μM zinc chloride) was added to the flow cells and the cells were introduced to the magnetic trap. As soon as the cells were introduced, the data point was recorded as time = 0. Multiple fields of views were sampled at each time point, and the total beads per field of view were averaged. At each time point, the average number of beads were normalized to the average beads at time = 0. This gives a normalized number of remaining beads (or number of collagen molecules uncleaved) at a given time point. This ratio was plotted as a function of time and fit to

$$f(t) = ae^{-bt} + c \qquad \text{Eqn. 3}$$

where *f(t)* is the ratio of the beads attached (or collagen molecules unproteolyzed), *t* is time, *a*+*c* is the number of beads that are attached at t = 0, *b* is the decay rate constant, and *c* is the ratio of beads that are attached non-specifically. The same experiment was done at varying force and MMP-1 concentrations to elucidate the effect of force on collagen proteolysis by MMP-1.

Controls: To confirm specific attachment of collagen to antibody, and beads to collagen, the following controls were done. All the experiments were done as above with the following modifications. Each control experiment was done four different times on different days.

Table S1. Number of beads observed during control experiments

BSA only + Beads	0-2/80,000 μm^2
Anti-*myc* + BSA + Beads	0-3/80,000 μm^2
Anti-*myc* + BSA + Collagen + Beads	0-3/80,000 μm^2
BSA + Collagen + Beads	0-2/80,000 μm^2
BSA + Biotin-Collagen + Beads	0-4/80,000 μm^2
Anti-*myc* + BSA + Biotin-Collagen + Beads	30-40 or 80-200/80,000 μm^2*

*~30-40 beads for 2.8 μm beads and 80-200 beads for 1 μm beads

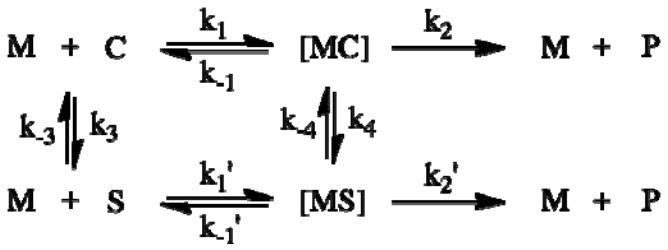
Model Development:

Collagen proteolysis in the absence of force can be modeled using Michaelis-Menten-like kinetics:

Where M is MMP, C is collagen, MC is the uncut collagen-MMP complex, and P is the cleaved collagen product. Since the enzyme concentration (μM) is much greater than the substrate concentration (pM), the free enzyme concentration can be approximated as a constant. Furthermore, k_2 is likely much slower than k_{-1} which allows the rapid equilibrium approximation(*6*). Consequently, the rate of product formation is approximated as:

$$\frac{dP}{dt} = \frac{k_2[M]}{K_D + [M]}(C_0 - P) \qquad \text{Eqn. 4}$$

Where C_0 is the total number of collagen trimers observed at time zero and K_D is the MMP dissociation constant: k_{-1}/k_1. Although multiple mechanistic models likely fit the force-dependent data, a general framework is (*6*):



Here S is the stretched collagen conformation, and k_3, k_{-3}, k_4, and k_{-4} are the rates of the presumably rapid, force-dependent equilibration between the stretched and unstretched states. Although other models are possible, the discrete helical and stretched conformations assumed here are consistent with the generally cooperative nature of collagen unfolding (*7*).

$$K_3 = \frac{k_3}{k_{-3}} = K_3^0 e^{\frac{FD_3}{k_B T}} \qquad \text{Eqn. 5}$$

Where K_3^0 is the equilibrium constant in the absence of load. D_3 is the sum of the distances to the transition state from the C and S ground-state conformations: $D_3 = |\delta_3| + |\delta_{-3}|$. Note that in this particular case D_3 also corresponds simply to the difference in length between the C and S collagen conformations.

A more general product formation rate can be achieved by invoking the assumptions of rapid equilibrium and constant free enzyme concentration, as well as the mass balance on the collagen substrate:

Mass Balance

$$C_0 = C + S + MC + MS + P = C + K_3 C + K_1 M * C + K_2 K'_1 M * C + P \quad \text{Eqn. 6}$$

Product Formation Rate

$$\frac{dP}{dt} = k_2[MC] + k'_2[MS] = \frac{(k_2 K_1 + k_2 ' K'_1 K_3)M}{1 + K_2 + (K_1 + K'_1 K_3)M}(C_0 - P) \quad \text{Eqn. 7}$$

In this analysis the equilibrium constants, K_1, K_2, K_3, K_4, are all association constants. Rearrangement of the decay rate constant derived from the product formation rate yields expressions for the parameters used in the empirical hyperbolic fit to the data (Eqn. 1, Main Text):

$$k_{cat} = \frac{k_2 K_1 + k'_2 K'_1 K_3}{K_1 + K'_1 K_3} = \frac{k_2 K_1 + k'_2 K_1 K_4}{K_1 + K_1 K_4} \quad \text{Eqn. 8}$$

$$K_D = \frac{1 + K_3}{K_1 + K'_1 K_3} = \frac{1 + K_3}{K_1 + K_1 K_4} = \frac{1 + K_3}{K_1(1 + K_4)} \quad \text{Eqn. 9}$$

$$\frac{k_{cat}}{K_D} = \frac{k_2 K_1 + k'_2 K'_1 K_3}{1 + K_3} = \frac{K_1 k_2 + k'_2 K_1 K_4}{1 + K_3} \quad \text{Eqn. 10}$$

It should be noted that due to the rapid equilibrium assumption, $K'_1 K_3 = K_1 K_4$. We note that k_{cat} is algebraically equivalent to the weighted average of k_2 and k_2' at a given force. Similarly, the equation for K_D can be obtained by finding the enzyme concentration at which the fraction of bound substrates to unbound substrates is unity:

$$k_{cat} = \frac{k_2[MC] + k'_2[MS]}{[MC] + [MS]} = \frac{k_2 K_1 + k'_2 K_1 K_4}{K_1 + K_1 K_4} \quad \text{Eqn. 11}$$

$$\frac{Bound}{Unbound} = 1 = \frac{[MC] + [MS]}{[C] + [S]} = \frac{K_1 + K'_1 K_3}{1 + K_3}[M] \quad \text{Eqn. 12}$$

K_3 and K_4 are likely force sensitive. The effect of load on k_{cat}/K_D is thus:

$$\frac{k_{cat}}{K_D} = \frac{k_2 K_1 + k'_2 K_1 K_4^0 e^{\frac{FD_4}{k_B T}}}{1 + K_3^0 e^{\frac{FD_3}{k_B T}}} \qquad \text{Eqn. 13}$$

We define K_3^0 and K_4^0 as the inherent equilibrium constants in the absence of force, and D_3 and D_4 as the collagen extensions accompanying K_3 and K_4. We observe that k_{cat}/K_D is well fit by a single-exponential under the conditions of our experiment. If $K_3^0 << 1$ (on the order of 10^{-4}) and $D_3 < 2$ nm the denominator of Eqn. 13 remains effectively constant at forces below 13 pN, resulting in the simple exponential force dependence we observe.

Error Analysis:

The error on the number of beads counted at each time point is well-described as Poisson noise (verified using Pearsons Chi Squared test; all the data sets had a chi-squared value of less than 0.05). The error at each time point for n beads counted is thus $n^{1/2}$. The error in the fraction of beads attached was calculated by propagating the error. The data were fit with a non-linear least squares fitting using the Matlab curve fitting toolbox. The curve fitting toolbox in Matlab calculates 95% confidence intervals using the inverse of Student's t cumulative distribution function and the vector of diagonal elements from the estimated covariance matrix of the coefficient estimates. The 95% confidence interval was converted to a standard deviation as follows: s.d. = (|Value – 95% confidence limit|)/2.

The apparent bimolecular rate constant k_{cat}/K_D was calculated by fitting the proteolysis rates at 10.1, 8.6, 6.2, and 5.2 pN vs. MMP-1 concentrations by fitting the data to SI Eqn. 1. The bootstrap method(*8*) was used to calculate the error in the measurement. 1000 replicons were used to calculate the standard error by sampling from the normally-distributed error for each calculated rate. For forces of 13 pN and 11.5 pN the cleavage was very fast, making it impractical to measure cleavage rates at saturating MMP concentrations. Thus, the slope of the linear region was used to determine the k_{cat}/K_D values. For 1 pN, the reaction rate was very slow; thus, the linear regime was again utilized.

k_{cat}/K_D versus force was fit to an exponential with a constant prefactor. Fit error was determined by generating 1000 replications, generated by sampling normally-distributed error distributions for individual k_{cat}/K_D values (error in the determination of individual k_{cat}/K_D values was observed to be quasi-normally distributed).

Collagen Full Sequence

MHHHHHHEQKLISEEDLEQKLISEEDLEQKLISEEDLEQKLISEEDLEQKLISEEDLGPPGPPGPPGPPGPPGPP
GPPGPPGPPGPPGPPGPQGIAGQRGVVGLGSGYIPEAPRDGQAYVRKDGEWVLLSTFLKKCK

Cut sequence –(determined from MALDI)

MHHHHHHEQKLISEEDLEQKLISEEDLEQKLISEEDLEQKLISEEDLEQKLISEEDLGPPGPPGPPGPPGPPGPP
GPPGPPGPPGPPGPPGPQG

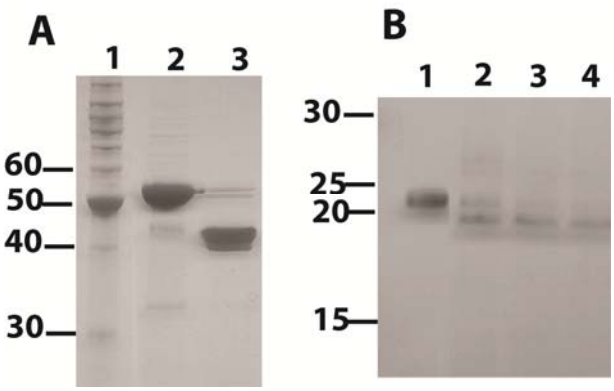


Figure S1. (**A**) MMP-1 Activation. Lane 1: Ladder, Lane 2: Inactive MMP-1, Lane 3: Activated MMP-1. (**B**) Collagen Bulk Proteolysis. Lane 1: Collagen monomer, Lane 2-4: Proteolysis at 20-minute intervals (50 µM collagen model trimer with 4 µM MMP – 1).

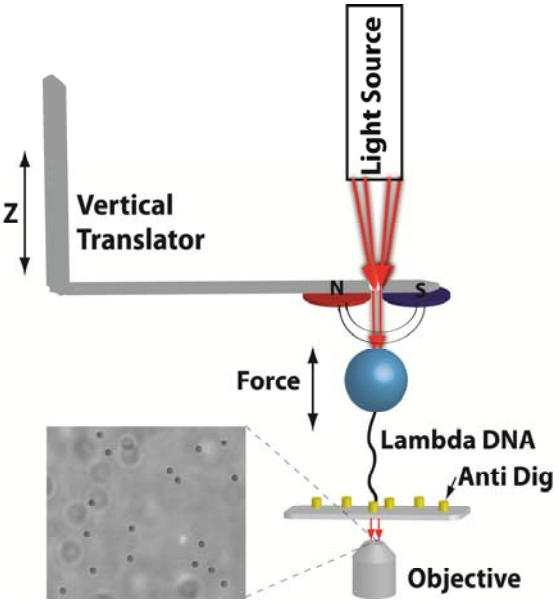


Figure S2. Magnetic tweezers setup. Inset: A sample field of view of beads.

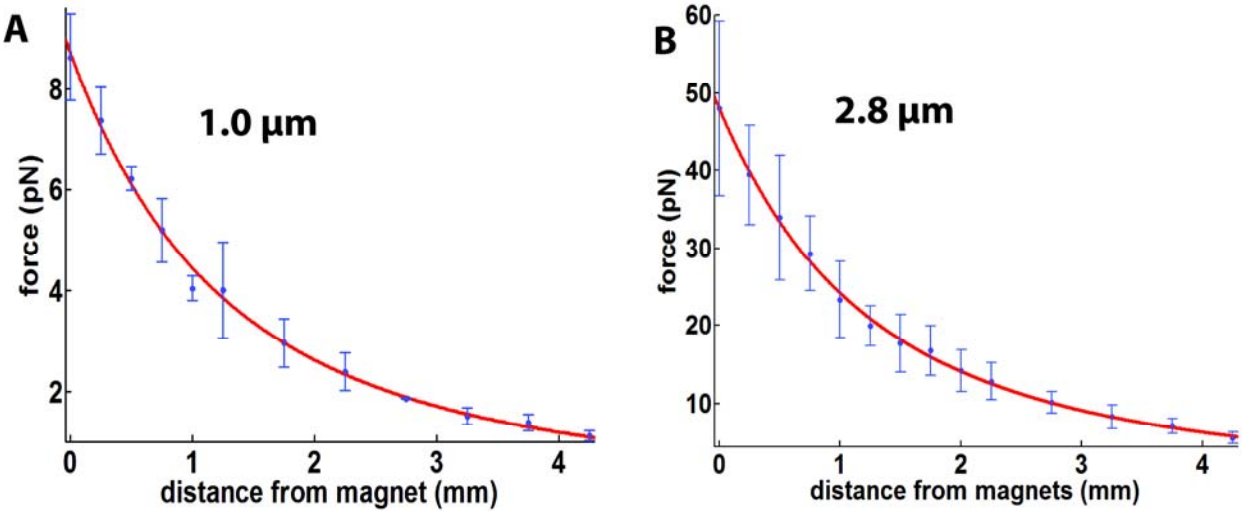


Figure S3. Magnetic trap force calibration curve. **(A)** 1 µm beads **(B)** 2.8 µm beads. The data was fitted to $force = \frac{a}{x^2 + bx + c}$, where x is the distance from the magnet.

References

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