Multiplexed Single-Molecule Force Proteolysis Measurements Using Magnetic Tweezers

Keywords: Single-molecule spectroscopy, magnetic tweezers, force proteolysis, collagen, MMP-1

Short Abstract

In this article we discuss the use of magnetic tweezers to study the effect of force on proteolysis at the single molecule level in a highly parallelizable manner.

Abstract

Generation and detection of mechanical forces is a ubiquitous aspect of cell physiology, with direct relevance to cancer metastasis[1], atherogenesis[2] and wound healing[3]. In each of these examples, cells both exert force on their surroundings and simultaneously enzymatically remodel the surrounding extracellular matrix (ECM). The direct effect of force on the enzymatic remodeling of the ECM proteins has thus become an area of considerable interest due to its likely biological and medical importance[4-7].

Single molecule techniques such as optical trapping[8], atomic force microscopy[9], and magnetic tweezers[10,11] allow researchers to probe the function of enzymes at a molecular level by exerting forces on individual proteins. Of all techniques listed above, magnetic tweezers (MT) are notable for their low cost and high throughput. MT exert forces in the range of ~1-100 pN and can provide millisecond temporal resolution[12], qualities that are well matched to the study of enzyme mechanism at the single-molecule level. Here we report a highly parallelizable MT assay to study the effect of force on the proteolysis of single protein molecules. We present the specific example of the proteolysis of trimeric collagen peptide by matrix metalloproteinase 1 (MMP-1); however, this assay can be easily adapted to study other pairs of substrates and proteases.

Protocol

1. Flow Cell Preparation

1.1) Coverslips (#1.5, 22×22 and 22×40 mm, VWR) are cleaned using sonication.

1) Add the coverslips to a small glass container capable of holding coverslips and fitting in the sonicator (see step 2).

2) Fill the container with isopropanol and sonicate in a bath sonicator for 20 minutes.

3) Discard the isopropanol and rinse the coverslips with copious quantities of deionized water produced by a Barnsted MilliQ apparatus or similar device. Fill the container with water and sonicate for 20 minutes.

4) After sonication, dry the coverslips in a stream of filtered, dust-free air. Use only coverslips that appear clean (i.e. no smudges).

5) Gently flame the coverslips for a few seconds to clean them of any remaining dust and moisture: pass the coverslips through a gas flame produced with a bunsen burner, taking care not to warp the coverslip due to excess heat. This step removes residual surface contaminants.

1.2) Using double-sided scotch tape, attach the two coverslips together. Cut the tape ~3 cm in length, and 0.3 cm in width. Attach the tape on 22x40 coverslip leaving a 1.6 cm channel in the middle (the width of the channel can be adjusted according to need). Use a pipette tip to gently press down on the coverslip to ensure that the tape has properly adhered.

2. Magnetic tweezers setup and calibration

2.1) Magnetic tweezers were setup using permanent rare-earth magnets as described previously[10,12] An aluminum L-bracket was constructed with a 1 mm diameter pinhole and mounted on a vertical z-translator (Thorlabs) to modulate the height of the tweezers. Two permanent rare-earth magnets were attached to the bracket on either side of the pinhole to create the magnetic trap (figure 1).

2.2) DNA preparation: DNA from lambda phage functionalized by oligonucleotides labeled at its 5′ and 3′ ends with biotin and digoxigenin (IDT DNA, San Diego, CA) was used to calibrate the magnetic tweezers.

1. Mix 50 μL of 4 nM λ-DNA with 2 μL of 40 nM biotin conjugated oligonucleotide and heat at 60 °C for 10 minutes. Then slowly cool at room temperature over 1 hour.

2. Add DNA ligase according to manufacturer's specifications and ligate at room temperature for 3 hours.

3. Add 2 μL of 400 nM digoxigenin conjugated oligonucleotide and incubate at room temperature for 45 minutes.

4. Add 1.5 μL of 10 mM ATP and continue ligation of the digoxigenin conjugated oligonucleotide to the λ-DNA for 3 hours.

5. Remove the excess oligonucleotides and the DNA ligase using 100 kDa spin filters (MicroconUltracell YM-100). A total of 1000-fold buffer exchange in TE buffer is adequate to

remove the excess oligos. Note: It is important not to use spin speeds >500×G because that will lead to the shearing of the DNA.

6. Run the DNA on a 0.7% agarose gel to ensure that the DNA is not denatured or sheared.

7. Measure concentration of DNA. The use of Nanodrop UV-Vis spectrometer (Thermo Scientific) is recommended because the volume may be very small.

2.3) Prepare flow cells as described in section 1.

2.4) For the attachment of the DNA to the flow cell, add anti-digoxigenin antibody (sheep IgG; 1 μg mL^{-1}; Roche Bioscience, Manheim, Germany) and allow it to adhere to the glass surface for 15 minutes.

2.5) Passivate the surface of the flow cell to prevent non-specific sticking of DNA by adding 2 mg mL^{-1} BSA, 0.1% Tween-20 in 1x PBS. Incubate at room temperature for 45 minutes.

2.6) Repeat step 2.5.

2.7) Add 50 pM of fuctionalized λ-DNA and allow to attach to the coverslip for 15 minutes.

2.8) Wash away excess DNA with 1x PBS.

2.9) Add streptavidin-coated superparamagnetic beads (1 μm beads = 2 μg mL^{-1}; 2.8 μm beads = 20 μg mL^{-1}) and allow them to attach to the immobilized DNA for 15 minutes.

2.10) Wash away excess beads with 1x PBS.

2.11) Magnetic Tweezers Calibration

1. Move the permanent magnet to a position that is far away from the sample surface (>15 mm).
2. Place the prepared flow cell onto the microscope.
3. Move the permanent magnet into position above the flow cell.
4. Choose the focal plane of λ-DNA functionalized beads by focusing on the beads away from both glass surfaces. The correct focal plane is one in which the desired beads have a bright center.
5. Take a video of bead motion at 80 Hz or greater for 500 frames.
6. Move the magnet closer to the sample surface. For distances beyond 5 mm, move in 1 mm increments. For distances between 1 and 5 mm, move in 0.5 mm increments. (For distances less than 1 mm, move in 0.25 mm increments).
7. Repeat steps 2.11.5 and 2.11.6 at each new magnet position until the magnet reaches the sample surface.
8. For each data set, track the center of the beads using a 2D Gaussian fit.
9. Calculate the force via Brownian fluctuations using:

$$F = \frac{k_B T * L}{\langle x^2 \rangle}$$

where k_BT is the Boltzmann thermal energy, L is the length of λ-DNA, and $\langle x^2 \rangle$ is the variance in centroid position.

10. Confirm the accuracy of the forces by checking the force calculation via a power spectrum Lorentzian fit in order to find the rolloff frequency. Applied force is related to the rolloff frequency by the relation:

$$F = 12\pi^2 \mu a f_o L$$

Where f_0 is the rolloff frequency, a is the bead radius, and μ is the fluid viscosity[12].

3. Collagen peptide attachment to flow cells

3.1) The collagen model peptide consists of a *N*-terminal 6x His-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[13,14].

3.2) The collagen peptide and MMP-1 proteins were expressed and purified as described previously[4,15].

3.3) Add anti-*myc* (15 μg mL^{-1}) to the flow cell and incubate at room temperature for 20 minutes to allow the anti-*myc* to attach to the surface of the flow cell.

3.4) Passivate the flow cell to prevent non-specific attachment of proteins using 5 mg mL^{-1} BSA in the same way as described in step 2.5

3.5) Add 150 pM collagen and allow it to attach to the antibody via the *myc* tag for 45 minutes.

3.6) Wash away excess collagen using PBS buffer.

3.7) Add streptavidin coated superparamagnetic beads (1 μm beads = 2 μg $mL^{-1}$and 2.8 μm beads = 20 μg mL^{-1}) and allow them to attach to the collagen molecules for 45 minutes. The 1 μm beads should be diluted to the desired concentration in PBS. The 3 μm beads should be separated from the solution using strong magnets, then resolubilized in PBS. This process should be repeated 3 times. We noticed that this step is essential to get the 3 μm beads to bind.

4. Force proteolysis assay

4.1) Once the flow cell is assembled with collagen and magnetic beads, image the flow cell in the magnetic trap under low, ~1 pN force to check if the beads are attached properly.

4.2) Add activated enzyme to the flow cell. In this case MMP-1 was pre-activated by adding 3.5 mM APMA (4-aminophenylmercuric acetate) and incubated at 37 °C for 3 hours. The activation was verified using SDS-PAGE.

4.3) As soon as the flow cell is re-introduced into the magnetic tweezers apparatus, record a video spanning a few fields of view, typically yielding several hundred attached beads. This will be t = 0.

4.4) Repeat the same process of recording several fields of view (while returning to the same field of view) at regular time points, till all the beads detach or no further proteolysis is discernable.

4.5) Kinetic data analysis

1. Count the beads in the various fields of view at different time points and average them. Normalize the number of beads by dividing the average number of beads at each time point by the number of beads at t = 0. This ensures that we can compare the rates of proteolysis from different experiments, because the absolute number of beads will differ from experiment to experiment.
2. Plot the ratio as a function of time. In this case, we fitted it to an exponential decay plus a constant

$$f(t) = ae^{-bt} + c$$

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

Representative Results

The above protocol describes a novel use of magnetic tweezers (figure 1) for studying the effect of force on proteolysis. We calibrated the tweezers for 1 µm and 3 µm beads using both the magnitude of the observed Brownian fluctuations and roll-off frequencies (figure 2). In the force proteolysis experiments, the setup is similar except that the DNA is replaced with collagen (figures 3, 4). The normalized number of beads remaining can be plotted as function of time to get the proteolysis rates (figure 5), and this process can be repeated for various concentrations of enzyme at various forces.

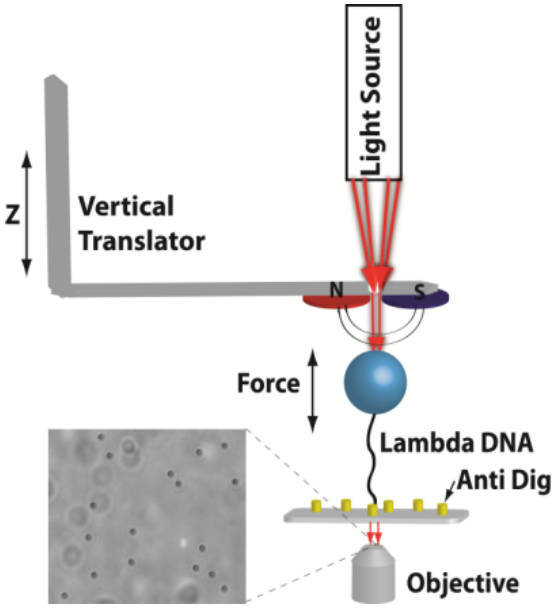

Figure 1. Schematic of the magnetic trap (not to scale). Two permanent rare earth magnets create a magnetic field that pulls on the superparamagnetic bead. Translating the magnet up and down adjusts the applied force. The beads are imaged using a conventional bright-field microscope with the light passing through a pin-hole between the two magnets. **Inset**: Image taken with a 40x air objective. The sharp spots correspond to the beads attached to the surface, and the out of the focus points are the detached beads.

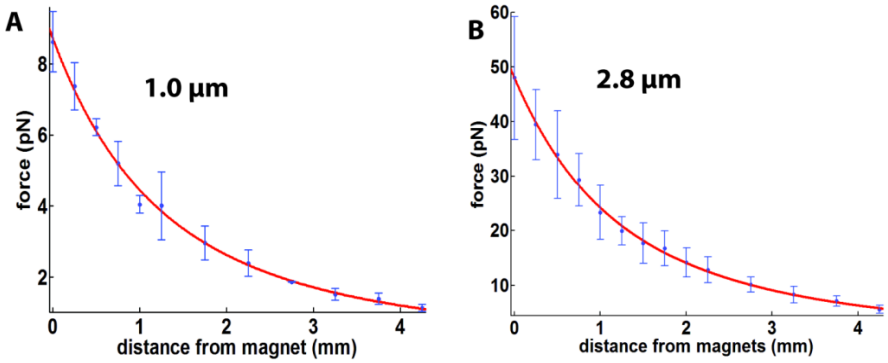

Figure 2. Plots of the calibrated force as a function of magnet distance from the sample surface for 1 µm (left) and 2.8 µm (right) beads. Data were fit to the empirical function $force = \frac{a}{x^2 + bx + c}$, where x is the distance from the magnet. a = 42.5, b = 3.54, and c = 4.89 for the 1 µm beads and a = 218, b = 3.49 and c = 4.55 for the 3 µm beads (these values are specific to our specific instrument and experimental geometry, and each instrument should be calibrated individually).

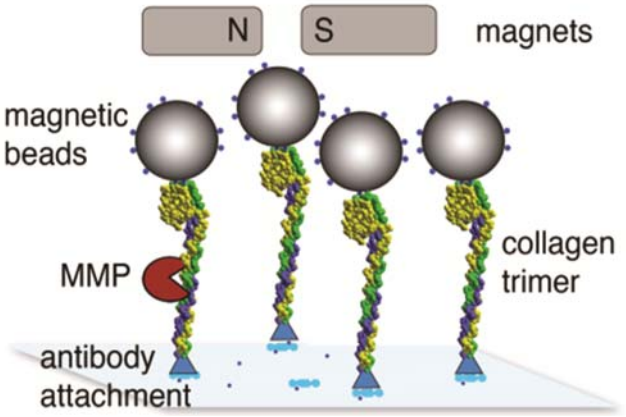

Figure 3. Force proteolysis assay setup (*Not to scale*). The collagen model trimer is attached to the surface of the coverslip via *myc*/anti-*myc* conjugation. The streptavidin-coated superparamagnetic beads are attached to the collagen via a biotin-streptavidin linkage. Activated MMP-1 cuts the collagen over time, causing the beads to detach from the surface and move away from the focal plane.

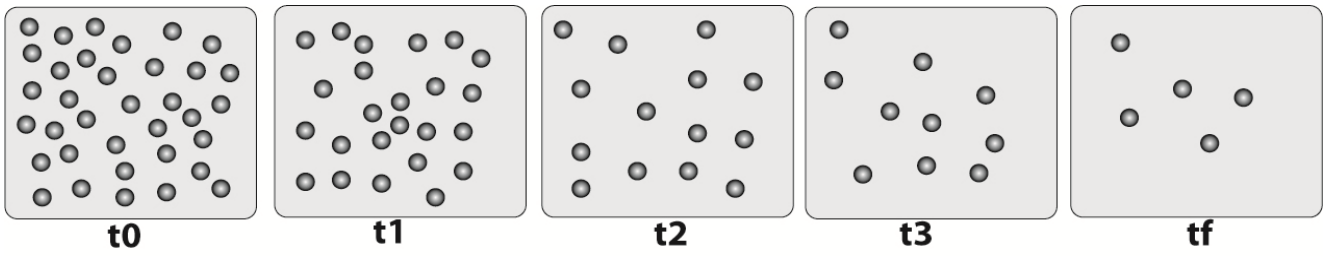

Figure 4. Schematic cartoon of proteolysis as a function of time. The cartoon shows a sample field of view over time as proteolysis occurs. Initially all the beads are attached to the collagen and are in the

focal plane. Over time, MMP-1 cuts the collagen and the beads detach and move away from the focal plane under the influence of the magnetic field.

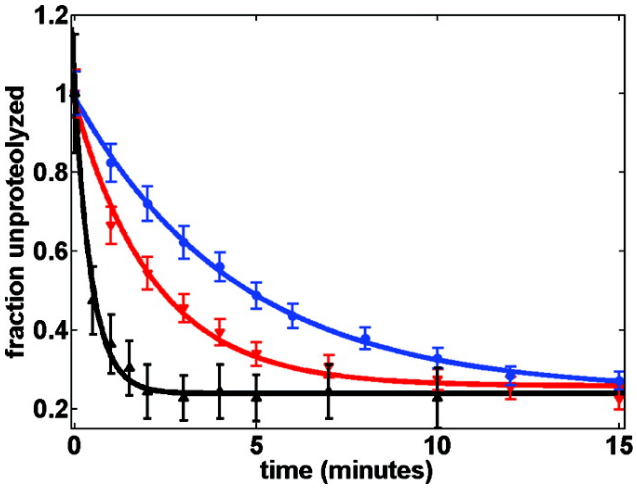

Figure 5. Proteolysis rates depend on applied force. Shown are data collected at 1.0 pN (3 μM MMP-1; *blue*), 6.2 pN (3 μM MMP-1; *red*) and 13 pN (0.2 μM MMP-1; *black*). The rates of proteolysis are: 0.22 ± 0.02 min^{-1} (1 pN), 0.46 ± 0.09 min^{-1} (6.2 pN), and 2.08 ± 0.18 min^{-1} (13 pN). Error bars correspond to the Poisson noise reflecting the number of observations at each time point.

Discussion

This protocol describes a new use for a classical single molecule technique. Magnetic tweezers allow medium to high-throughput single molecule assays in a cost-efficient manner. However, like all experimental techniques there are challenges and potential pitfalls.

Limitations of magnetic tweezers

Compared to an optical trap the spatial and temporal resolution of a MT apparatus is low. Moreover, the forces generated by the simple MT described here are 30 pN or less, significantly less than forces routinely accessed in AFM experiments. This limitation can be a virtue: MT are very well suited for applying sub-pN forces.

Unlike an optical trap or AFM, conventional MT, such as the apparatus here do not allow the manipulation of individual particles. Optical traps are usually used to apply force parallel to the coverslip, while MT are best-suited for vertical pulling experiments. Electromagnetic MT address these limitations, although at the cost of increased complexity.

Tweezer Calibration

It should be worth noting that both the Brownian fluctuation and power spectrum analysis for force calibration have their limitations. The Brownian fluctuation method, we found, is particularly sensitive to camera blur. As the frame rate increased (exposure time decreased) the calculated force decreased. In

practice, we increased frame rate until the forces calculated from Brownian fluctuations agreed with the forces calculated using the power spectrum to within 10%. Conversely, the power spectrum analysis is more robust against camera blur, but slightly more difficult to implement. We recommend performing both calculations to check for the robustness of the results.

We note that variations in bead size and magnetic particle content result in variations in applied force of ~10%. This effect was not important in our recent experiment because we average over hundreds of beads in each measurement. However, experiments that extract unique information from each tethered bead could potentially require a more accurate calibration method.

Controls to ensure single point attachments

Achieving specific, oriented, single-point attachments is often a major practical challenge in single molecule studies. The following controls confirmed specific attachment of collagen to antibody, and beads to collagen:

1. Added only beads to a flow cell passivated with BSA
2. Added anti-*myc*, passivated with BSA and then added beads
3. Added anti-*myc*, passivated with BSA, added non-biotinylated collagen and then added beads
4. Passivated with BSA, added non-biotinylated collagen and then added beads
5. Passivated with BSA, added biotinylated collagen and then added beads
6. Added anti-*myc*, passivated with BSA, added biotinylated collagen and then added beads.

In cases 1-5, where some component of the attachment series was deliberately omitted, anywhere from 0 to 4 beads were seen attached to the flow cell per field of view (80,000 μm^2). Only in case 6, where all the attachment moieties are present, we saw ~30-60 2.8 μm beads and 80-200 1 μm beads attached to the surface. The proteolysis kinetics observed are adequately fit by a single exponential plus a constant term, which strongly suggests that there is only one rate limiting step, and hence only a single tether. The constant term likely reflects beads that are non-specifically attached to the coverslip.

We report the concentrations of antibody and protein that worked for our experiment. A wide range of concentrations for each component should be examined when developing a new assay. BSA worked well as a surface passivation agent in our experiment. However, this method may not work in all circumstances. Other protocols for surface passivation have also been used with success. Examples include polyethylene glycol functionalized glass slides[16], supported lipid bilayers[17], or casein as a blocking agent.

Potential applications

We anticipate that similar experimental approaches may be broadly applicable in studying force dependent proteolysis[4,18], unfolding[19] and binding interactions[20]. We and others[21] have noted that the radius of diffraction rings for out of focus beads provides a sensitive means for tracking the position of the bead relative to the coverslip (usually denoted as the *z* axis).[21] Although generally not used in this

way, we believe that MT assays have the capacity to provide nanometer-precision measurements of protein structural dynamics. This additional information may be particularly valuable in the context of force-dependent proteolysis, binding or protein unfolding measurements.

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Table of specific reagents and equipment:

Micro Cover Glass #1.5 (22x22)	VWR	48366-067
Micro Cover Glass #1.5 (22x40)	VWR	48393-048
Lambda DNA	Invitrogen	25250-010
T4 DNA Ligase	Invitrogen	15224-041
Microcon Ultracel YM-100	Millipore	42413
Anti-Digoxigenin	Roche Diagnostics	11-333-089-001
Tween 20	Sigma	P9416-100ML
Anti-*myc* Antibody	Invitrogen	46-0603
Albumin Bovine Serum	Sigma	B4287-5G
Dynabeads M-280 Streptavidin	Invitrogen	658.01D
Dynabeads MyOne T1 Streptavidin	Invitrogen	658.01D
p-Aminophenylmercuric Acetate	Calbiochem	164610
Biotin-Maleimide	Sigma Aldrich	B1267
Biotin labeled oligonucleotide	IDT DNA	Custom synthesis
Digoxigenin labeled oligonucleotide	IDT DNA	Custom synthesis
Collagen peptide gene	DNA 2.0	Custom synthesis
MMP-1 cDNA	Harvard Plasmid Database	
z-translator	Thorlabs	MTS50
Servo controller for translator	Thorlabs	TDC001
Rare earth magnets	Grainger	2VAF2

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