Video Article

Demonstrating the Uses of the Novel Gravitational Force Spectrometer to Stretch and Measure Fibrous Proteins

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Abstract

The study of macromolecular structure has become critical to the elucidation of molecular mechanisms and function. There are several limited, but important bioinstruments capable of testing the force dependence of structural features in proteins. Scale has been a limiting parameter on how accurately researchers can peer into the nanomechanical world of molecules, such as nucleic acids, enzymes, and motor proteins that perform life-sustaining work. Atomic force microscopy (AFM) is well tuned to determine native structures of fibrous proteins with a distance resolution on par with electron microscopy. However, in AFM force studies, the forces are typically much higher than a single molecule might experience^{1,2}. Optical traps (OT) are very good at determining the relative distance between the trapped beads and they can impart very small forces³. However, they do not yield accurate absolute lengths of the molecules under study. Molecular simulations provide supportive information to such experiments, but are limited in the ability to handle the same large molecular sizes, long time frames, and convince some researchers in the absence of other supporting evidence^{2,4}.

The gravitational force spectrometer (GFS) fills a critical niche in the arsenal of an investigator by providing a unique combination of abilities. This instrument is capable of generating forces typically with 98% or better accuracy from the femtonewton range to the nanonewton range. The distance measurements currently are capable of resolving the absolute molecular length down to five nanometers, and relative bead pair separation distances with a precision similar to an optical trap. Also, the GFS can determine stretching or uncoiling where the force is near equilibrium, or provide a graded force to juxtapose against any measured structural changes. It is even possible to determine how many amino acid residues are involved in uncoiling events under physiological force loads². Unlike in other methods where there is extensive force calibration that must precede any assay, the GFS requires no such force calibration⁵. By complementing the strengths of other methods, the GFS will bridge gaps in understanding the nanomechanics of vital proteins and other macromolecules.

Protocol

Introduction to the Novel GFS Configuration

The GFS consists of several essential components: A regular light microscope, an equatorial mount, a camera, and a computer [Figure 1]. The sealed flow-cell chamber which holds the sample is also indispensable according to the GFS design. The Light microscope is mounted onto the equatorial mount so the scope can be rotated into different orientations in space. This ability allows the static vector of gravity to be exploited so that the samples can be dynamically oriented in relation to the vector so the force of gravity can impart piconewton-range force loads to the samples. The camera replaces the light microscope's ocular lens so it can record changes in the orientation of the sample. This raw data is digitized and manipulated by the computer to interpret the data into actual force and distance measurements. The sealed flow-chamber is designed to allow all degrees of freedom in space without sample loss. In the chamber resides the sample molecule which is tethered near one terminus to an "anchored" bead that is glued to the surface of the chamber. The opposite terminus is tethered to a "mobile" bead that is free from the surface of the chamber. It is this mobile bead, free in the assay buffer that can be acted upon by gravitational force thereby stretching the tethered molecule at such low force loads [Figure 2]. The sample simply looks like a pair of microspheres under the microscope, although it does take some experience to discern good usable pairs coupled by their attachment to a molecule from pairs where both beads are sitting on the surface of the flow-chamber. One modification to the system is the addition of a floating platform which holds the GFS and is suspended by springs. In this configuration, once the sample has been rotated into a position where gravitational force can act on the sample, the platform and all its components can be dropped against the spring constant. Near free-fall, the force acting on the mobile bead is close to zero and at the springs' maximum extension, the gravitational force is multiplied by as much as two times. In this way, a graded force/distance response can be graphed to measure the behavior of a single molecule at different force loads.

1. Microsphere Preparation

1. Submerge about 10 mg of glass or silica beads in 0.04% 3-Aminopropyltriethoxysilane (cut with acetone) for two minutes.
2. Rinse with two changes of double distilled water and centrifuge to pellet at 2000 X G for 5 minutes. Discard supernatant.
3. Add 5 mL of coupling buffer (0.01 M pyridine cut with double distilled water and adjust pH to 6.0), and shake this mixture vigorously. Centrifuge as described above. Repeat this step three times.
4. To wet cake of beads, add 2 mL of 5% glutaraldehyde solution (cut glutaraldehyde with coupling buffer). Shake vigorously.
5. Under a hood, rotate bead/glutaraldehyde mixture for 3 hours at room temperature.
6. Centrifuge as above and aspirate the supernatant.
7. Wash the beads in 5 mL of coupling buffer by shaking them vigorously and centrifuge and aspirate the supernatant. Repeat this three more times.
8. Add about 15 µL of the desired antibody to beads and shake vigorously. The beads should be rotated for 16-24 hours.

9. Under the hood, add 5 mL of 1 M glycine quenching solution (cut glycine with double distilled water and adjust pH to 7.0). Shake this mixture vigorously and rotate for 30 minutes.
10. Centrifuge and aspirate the supernatant.
11. Add 5 mL of wash buffer (0.01 M Tris, pH 7.0; 0.1% sodium azide; 0.1% BSA; 0.15 M NaCl; and 0.001 M EDTA). Shake this vigorously, centrifuge and aspirate the supernatant. Repeat this step three additional times.
12. Change buffer to low-salt buffer (0.1 M KCl; 0.02 M imidazole; 5 mM MgCl₂; adjust to pH 7.0). Repeat three times.

2. Sample Attachment to Microspheres

1. Take a small amount of prepared beads (about 2 µL from each cake of beads however, if there is a large discrepancy in diameter between batches, it is advantageous to use around an 8:1 ratio of large to small beads) and add them to a microcentrifuge tube with assay buffer. Reduce the concentration of your protein to around 5 µM, using the assay buffer. Prepare at least a total volume of 400 µL including the buffer, the protein, and the beads.
2. Rotate this mixture at around 1 RPM for 3 hours (if the protein is agitated too much, it will aggregate and become useless).

3. Slide Chamber Preparation

1. Coat a thick microscope slide with 0.01% nitrocellulose (in amyl acetate). Let this slide dry for about 10 minutes.
2. With a sharp glass cutter, cut cover slide so as to make a chamber. This requires four strips of glass.
3. Use the factory edge of the glass and rake it across a smear of vacuum grease on both sides of the edge.
4. Press grease coated strips onto dried nitrocellulose covered slide to create a box on the surface of the slide.
5. Pipette about 2 µL of bead/protein mixture by tapping pipetter onto surface of glass inside the box.
6. Add about 20-400 µL of low salt buffer depending on how large the chamber is.
7. Press a cover slip on top of the box which is already coated with vacuum grease to finish the sealed and buffered chamber.
8. Let slide sit on a level place so the beads have ample time to anchor themselves to the nitrocellulose.

4. GFS Data Acquisition

1. Mount slide onto GFS stage
2. Monitor what the GFS camera is recording and search for legitimate "bead pairs," in which the small microsphere is attached near the equator of the large microsphere.
3. When a potential bead pair is found, go through the depth of focus to determine if the "mobile" bead is not resting on the surface of the slide.
4. Once a suitable pair is identified, note angle mobile bead is away from $d_{\max}$ ($d_{\max}$ = the maximum distance between the centroids of the coupled microspheres). Move the scope into position to acquire video.
5. The angle of travel of the GFS should be sufficient to record $d_{\min}$, $d_{\max}$, and $d_{\min}$ which might call for 25-90 degrees depending on the length of the molecule.
6. RECORD as the pair travels from $d_{\min}$ to $d_{\max}$ and back to $d_{\min}$.
7. It is a good idea to also shoot a movie of the background so it can be subtracted later for analysis.
8. If performing a GFS drop, move the scope back to $d_{\max}$ and record the drop at at least 60 frames per second. The critical part is the first oscillation, but long record times can also be used for dynamic study.

5. GFS Data Analysis

1. Transform raw video into digitally "thresholded" image and run macro in imageJ to determine the centroid position of each bead in every frame of video. This is also for the drop video.
2. Dump the X,Y and area data from ImageJ into Excel and plot the points.
3. If a proper bead pair was acquired by video, a noticeable hump in the graph is indicative of the beads being at their closest ($d_{\min}$) and at the apex of the graph is the position of $d_{\max}$.
4. Using this data, the radius of each bead should be determined precisely in ImageJ and all this information is put into the nested equation:

$$d = [(g \sin \alpha)^2 + (g \cos \alpha + d_{\max} - g)^2]^{0.5}$$

$$g = [d_{\min}^2 + r_b^2 - (r_a + r_b)^2]^{0.5} = r_b \sin \beta$$

$$c = d_{\max} - r_a - g$$
 (d = distance between centroids; g = force of gravity; α = the angle in degrees parallel to the objective lens; r_b = radius of mobile bead; r_a = radius of anchored bead; β = angle of attachment off the axis of the equator of the anchored bead.)
5. Using the fitted radius of the mobile bead which also yields its volume, and given the density of the bead, the force the mobile bead imparts on the molecule can be calculated in piconewtons after the buoyancy of the solvent is subtracted. This method measures the force on the tethered molecule in piconewtons and calculates the absolute molecule length between antibody attachments in nanometers. $F = V(d-b)a$ (F = force, V = volume, d = density of the glass microsphere, a = acceleration due to gravity, b = the density of the displaced water).

6. Representative Results:

If the bead prep is done correctly, there will be minimum bead aggregation although there may still be an occasional bead clump. When viewed through the scope, there should be a reasonable distribution of beads whether paired or not in the chamber.

It is important to minimize vibrations as much as possible; to do this either an air table, special shock absorbing feet for a tripod that holds an EQ mount, or on the system that utilizes springs can be used for vibration isolation.

Another useful tip concerning the sealed flow chamber is to let it stand for about five minutes on a level table after it has been fully constructed. This allows any unattached larger beads to float down through the buffer and rest in the layer of nitrocellulose. If the slide were instead mounted directly upon completion, the investigator would continually have to deal with beads literally flying through the field of view; and if this happens during video acquisition it can corrupt the experiment. If this is done properly, bead flight is significantly minimized and cleaner video results.

When a potential bead pair is identified by the GFS operator, it is useful to put it through a preliminary rotation to monitor the behavior of the pair. Rarely, the large bead is not firmly affixed to the slide. If this happens, there is no use in utilizing the pair because it is critical that the larger anchored bead stay in a fixed position through the duration of the acquisition. If the pair is stable and does not exhibit "anchored bead roll," then it is suitable for experimentation.

A plot of bead separation distances versus change in angle relative to gravity can be used to evaluate the acquired data. A good representative result would show little separation on a plateau and as the mobile bead frees itself from the anchored bead because of the influence of gravity, the graph will show more separation. This continues until a nice peak is reached which is called $d_{\max}$ and the curve starts downward again returning to the baseline $d_{\min}$ [Figure 1]. In ideal conditions, this signature is symmetrical. A non-representative result would show a graph with incoherent patterns showing no distinct $d_{\min}$ - $d_{\max}$ - $d_{\min}$ pattern; or it would show separations that are orders of magnitude too high for a single molecule indicating maybe there was a piece of dust between the beads, or maybe that the mobile bead was simply not attached at all. The process of finding the pair, shooting it, processing it, and analyzing it has many stop gaps where improper bead pairs are culled out. So, if you get to the end of the entire process and you have a molecule length that is consistent with previous results, one can be very confident that the original bead pair is representative and can be included in the results. On a good day, about half of the bead pairs shot can be taken through to become a legitimate data point. For instance, the length of the coiled coil of myosin between MF20 and MF30 antibodies, is known based on EM data and AFM, data to approach 100 nm ^{2,7,8,9}. If the result is several times this length, the sample has aggregated. The results presented here are from standard GFS rotation experiments and demonstrate a distance of 96 nm $\pm$ 5 nm, [Figure 2] which agrees closely with the measurements of the MF30 (which binds at the N-terminus of myosin subfragment-2) and MF20 (which binds in the light meromyosin) antibody separation distance on myosin deduced from the literature values [Figure 3].

Representative graph showing relative distances between anchored and mobile bead centroids

Figure 1. GFS configuration. Major parts of GFS are labeled.

Figure 2. Schematic of GFS principle. Left side shows centroid of mobile bead at a minimum distance from centroid of anchored bead. As GFS is rotated, mobile bead aligns with vector of gravity which is also running parallel with the axis of the tethered molecule. In this position, the distance between the centroids of the mobile and anchored beads is at a maximum. Top right shows a representative slice from a GFS movie. Bottom right is an image of the GFS undergoing rotation.

Figure 3. Representative result showing $d_{\min}$ on the left of the graph; $d_{\max}$ at a relative separation of 17.78 microns; and a return to $d_{\min}$ around the baseline of about 17.75 microns of relative separation between the beads.

Figure 4. Representative result of GFS rotation experiment showing distance between MF20 and MF30 antibodies average 96 nm. This represents the approximate length of S2.

Figure 5. Myosin II dimer used to show possible attachments for use with the GFS including antibody and/or actin attachments. Different attachment possibilities allows for different GFS strategies to measure different regions, or to apply force perpendicular or parallel to the rod domain of the myosin dimer.

Discussion

When converting a movie to a digitally thresholded representation, it is crucial for the thresholded image to maintain the same area in each frame of video. Because the beads in a bead pair move independently of one another, any drift in the thresholded areas can also cause the relative distances between the centroids of the beads to drift and introduce significant error. Controlling the threshold area reduced the error five-fold in the distance measurements from 26 nm down to 5 nm. It is also crucial to obtain an accurate radius measurement for both beads as the ratio between the beads is important to the final calculation that yields both distance and force values.

The system can be modified in many ways. The most recent system imparts graded force to the tethered molecule by dropping the entire GFS against a spring constant. Once the spring constant is determined, a third-person video can be obtained at the same time the microscope video is taken and the two are synched together. Then each frame of video from the microscope camera can be given a force value according to the

position of the GFS as it is dropped in the third-person, synched movie. Each frame of video has a corresponding force value that the mobile bead is imparting during the fall and subsequent rebound of the spring system. Each frame of video is also analyzed to obtain the absolute molecule length. In this way, each discrete force can be correlated to each discrete distance measurement, thus a graded force/distance curve is available to see how one molecule behaves under increasing loads. This technique also serves to multiply the force a microsphere can impart up to two times gravity. Similarly, the volume of the bead is what ultimately determines the force profile as the system is designed to be dropped from the same distance every time. In contrast, the rotation experiment always only gives one force value. Additional accessories are also possible such as perfusion cells, fluorescence, increased automation, or even optical traps. Since one significant feature of the base system is its low cost and robustness, the system could be used for single molecule assay demonstrations in educational laboratories.

The applications for this system have already proven useful for determining the structural nature of the coiled coil of myosin as described in the representative results. In addition, it also confirmed the lengths of 12 bp DNA compared to AFM data and shown consistent results concerning the rupture force of the double stranded nucleotide¹⁰. Because of the flexibility provided by using site specific antibody attachments or other ligands or even toxins, the GFS is poised to generate much scientific structural data on the nanoscale of structure and the picoscale of forces. Molecules can be measured, stretched, and teased apart depending on the assay. Molecules already under force in the system can be subjected to reagents to determine possible signal transduction or enzymatic activity. The possibilities for single molecule studies seem endless at this point in time.

Disclosures

No conflicts of interest declared.

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