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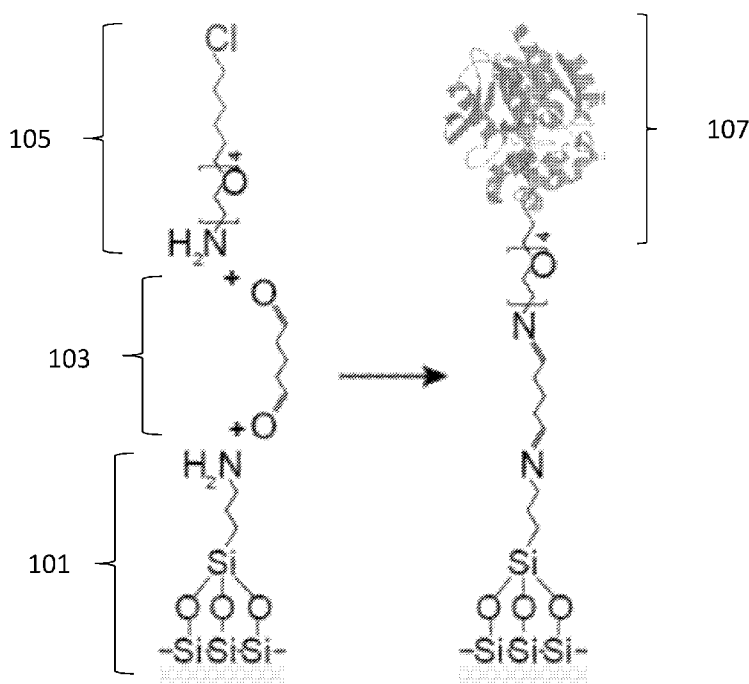


Fig. 1A

(57) Abstract: The present disclosure relates to new systems and methods for applying a force to a molecule in a controlled manner for studying molecule dynamics. An exemplary system can include a magnetic component attached to a voice coil; a substrate positioned opposite from the magnetic component for attachment to a first end of a molecule; a camera for obtaining images; and a positioning device for adjusting a focus of the camera, wherein the voice coil is configured to apply a predetermined amount of force to the molecule by adjusting a position of the magnetic component relative to a second end of the molecule.



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MAGNETIC TWEEZERS, CALIBRATION METHODS AND USES THEREOF**CROSS-REFERENCE TO RELATED APPLICATIONS**

[001] This application claims benefit under 35 U.S.C. 119(e) to U.S. Provisional Patent Application No. 62/110,901, filed on February 2, 2015, the entirety of which is explicitly incorporated by reference herein.

[002] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The patent and scientific literature referred to herein establishes knowledge that is available to those skilled in the art. The issued patents, applications, and other publications that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. In the case of inconsistencies, the present disclosure will prevail.

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT**

[003] This invention was made with government support under Grant Nos. HL66030 and HL061228 awarded by the National Institutes of Health (NIH) and under Grant No. NSF1252857 awarded by the National Science Foundation (NSF). The government has certain rights in the invention.

BACKGROUND

[004] Force-clamp spectroscopy allows for measurement of protein dynamics in response to well defined force protocols. This technique, implemented with the atomic force microscope (hereafter, AFM), has been used to measure the rates of protein unfolding, of chemical reactions such as thiol-disulfide exchange, disulfide isomerization, and protein folding. Force-clamp AFM makes use of an active feedback with a time constant that can approach 1ms. A known limitation of this approach is the mechanical drift of the AFM instrument. Drift limits the duration of a single molecule experiment to a few minutes and decreases accuracy at low forces.

[005] Magnetic tweezers have tremendous potential for protein force-spectroscopy and are useful in a wide variety of contexts. Magnetic tweezers allow low force measurements over extended periods of time. One particular goal in developing improved systems is to provide magnetic tweezers that can aid in accurately measuring the force being applied to short molecules (including, for example, but not limited to, single proteins) undergoing dynamic changes in length as they fold and unfold. Recent advances in covalent tethering techniques applied to force spectroscopy now allow for the study of covalently anchored proteins without fear of detachment. None of the prior tools are especially well optimized for accurately measuring the dynamics of protein(s) over a wide range of forces over extended periods of time (e.g., several hours, several days, several weeks and longer time periods).

SUMMARY

[006] In certain aspects, the present disclosure relates to systems and methods for studying the structure, dynamics, and chemistry of molecules such as proteins, DNA, and RNA with high accuracy and low cost. In certain embodiments, the present disclosure relates to systems and methods for measuring the force being applied to molecules under study. In certain aspects, a molecule under study (e.g., engineered modular protein) is used to calibrate the force being applied to the molecule under study and derive a magnet law based on the distance between a magnetic component and a measuring paramagnetic bead for accurate measurement of molecule dynamics. The magnet law, as discussed herein, relates to the relationship between force applied to a paramagnetic bead attached to a molecule under study and a separation distance between the paramagnetic bead and the magnetic tweezer magnets. In certain embodiments, a magnet law, derived using the disclosed magnetic tweezers, may be

used to determine a precise amount of force needed to cause a desired change in length of any molecule under study (e.g., proteins, DNA, RNA). In certain aspects, a magnet law discussed herein can be used to accurately measure molecule dynamics over a wide range of forces with little dispersion from bead to bead and for durations of several hours, days, or weeks.

[007] In certain embodiments, a system for applying a controlled amount of force to a molecule includes a voice coil actuator attached to a magnetic component of magnetic tweezers, a molecule attached at one end to a paramagnetic bead and at the other end to a substrate, a camera, a positioning device (e.g., nanopositioning device), and a reference bead.

[008] In certain aspects, the present disclosure relates to magnetic tweezers comprising a voice coil mechanism. In certain aspects, the present disclosure relates to magnetic tweezers having greater speed and accuracy of the magnet positioner (which is used to apply the force), due to the implementation of a voice coil mechanism that positions the magnets. In certain aspects, the voice coil mechanism has a feedback system and positions the magnets with high resolution (e.g., 200 micron resolution). In certain aspects, the present disclosure relates to magnetic tweezers with low noise and high acquisition rate (~ 1000 Hz) due to camera and computation improvements. In certain aspects, the present disclosure relates to magnetic tweezers useful for applying picoNewton (pN) range forces to single molecules with high force resolution and for extended periods of time without mechanical drift. In certain aspects, the present disclosure relates to magnetic tweezers useful for changing the force with high-speed. In certain aspects, the present disclosure relates to magnetic tweezers capable of measuring DNA, RNA, proteins and other molecules. In certain aspects, the present disclosure relates to magnetic tweezers useful for high throughput screening, and total internal reflection microscopy.

[009] One aspect of the present disclosure relates to a system for applying force (e.g., picoNewton range force, e.g., up to 65 pN) to a molecule (e.g., protein, DNA, RNA). The system may include a magnetic component; a voice coil attached to the magnetic component; a substrate positioned opposite from the magnetic component for attachment to a first end of the molecule; a camera for obtaining images; and a positioning device for adjusting a focus of the camera, wherein the voice coil is configured to apply a predetermined amount of force to the molecule by adjusting a position of the magnetic component relative to a second end of the molecule.

[010] In some embodiments, the system may comprise the molecule, wherein the first end of the molecule is attached to the substrate and the second end of the molecule is attached to a paramagnetic bead, the paramagnetic bead being positioned between the magnetic component and the substrate.

[011] In some embodiments, the system may comprise a reference bead disposed on the substrate at an initial position proximate (e.g., close to and in the same plane as) the first end of the molecule. In some embodiments, the reference bead is a non-magnetic bead.

[012] In some embodiments, the positioning device may continuously adjust the focus of the camera to maintain the reference bead at the initial position (e.g., such that the reference bead does not move from its initial position).

[013] In some embodiments, the camera obtains a first set of images of the paramagnetic bead and the reference bead prior to application of force at different focus positions and a live image of the paramagnetic bead and the reference bead during application of force.

[014] In some embodiments, the system determines the force by correlating a radial profile of a live image during application of force with radial profiles of the first set of images.

[015] In some embodiments, the system comprises a position adjustment stage for controlling a position of the paramagnetic bead.

[016] In some embodiments, the system comprises a microscope. In some embodiments, the system comprises a total internal reflection module for the microscope. In some embodiments, the system comprises a fluorescence camera. In some embodiments, the system comprises a laser/diode.

[017] In some embodiments, the substrate is or comprises glass.

[018] In some embodiments, the molecule is selected from the list consisting of protein, DNA, and RNA. In some embodiments, the first end of the molecule is attached to the substrate via HaloTag or AviTag method. In some embodiments, the first end of the molecule is attached to the substrate via any suitable attachment methods.

[019] In some embodiments, the predetermined amount of force is in picoNewton range (e.g., up to and including 65pN).

[020] In some embodiments, the voice coil applies the predetermined amount of force for periods of time of at least one hour without visible drift. In some embodiments, the voice coil applies the predetermined amount of force for periods of time of at least one day without visible drift. In some embodiments, the voice coil applies the predetermined amount of force for periods of time of at least several days (e.g., more than 1 day, more than 2 days, more than 3 days, more than 4 days, more than 7 days) without visible drift. In some embodiments the voice coil is controlled by a computer. In some embodiments, the voice coil is controlled by a command voltage sent from a computer through a DAQ card.

[021] Another aspect of the present disclosure relates to a method for controlling an amount of force applied to a molecule, comprising obtaining, with a camera a first set of images, the camera being positioned proximate a substrate for attachment to a first end of the molecule; applying a force to the molecule by adjusting, with a voice coil attached to a magnetic component, a position of the magnetic component relative to a second end of the molecule causing the paramagnetic bead to translate from a first position to a second position relative to a central axis of the molecule, wherein the magnetic component is disposed opposite the substrate; obtaining, with the camera, a live image of the second end of the molecule; correlating the live image with the first set of images to determine the second position; and measuring the force applied to the molecule as a function of the position of the second end of the molecule.

[022] In some embodiments, the method comprises attaching the first end of the molecule to the substrate; and attaching the second end of the molecule to a paramagnetic bead. In some embodiments, the method comprises attaching a reference bead to the substrate at an initial location proximate the first end of the molecule.

[023] In some embodiments, the first set of images comprises images of the paramagnetic bead and the reference bead. In some embodiments, the first set of images is obtained at various focal distances prior to application of the force. In some embodiments, the live image is an image of the paramagnetic bead and the reference bead. In some embodiments, the live image is obtained during application of the force.

[024] In some embodiments, the method comprises continuously adjusting, by a positioning device, a focus of the camera to maintain the reference bead at the initial position to correct drift.

[025] A further of the present disclosure relates to a system for applying a force (e.g., picoNewton range force) to a molecule (e.g., protein, DNA, RNA). The system may include a magnetic component attached to a voice coil; a substrate positioned opposite from the magnetic component, wherein a first end of the molecule is attached to the substrate; a reference bead disposed on the substrate at an initial position proximate the first end of the molecule; a paramagnetic bead attached to a second end of the molecule, the paramagnetic bead being positioned between the magnetic component and the substrate, wherein the voice coil applies a predetermined amount of force to the molecule by adjusting a position of the magnetic component relative to the paramagnetic bead; a camera for obtaining images of the paramagnetic bead and the reference bead; and a positioning device for maintaining the reference bead at the initial position.

[026] In some embodiments, the voice coil is controlled by a computer. In some embodiments, the positioning device is controlled by a computer.

[027] Another aspect of the present disclosure relates to a method for controlling an amount of force applied to a molecule, comprising: attaching a reference bead to a substrate at an initial location; attaching a first end of the molecule to the substrate proximate the reference bead; attaching a second end of the molecule to a paramagnetic bead; obtaining, with a camera positioned proximate the surface, a first set of images of the magnetic probe and the reference bead; applying a force to the molecule by adjusting, with a voice coil, a position of a magnetic component relative to the paramagnetic bead causing the paramagnetic bead to translate from a first position to a second position relative to a central axis of the molecule, wherein the magnetic component is disposed opposite the surface; obtaining, with the camera, a live image of the magnetic bead and the reference bead; correlating the live image with the first set of images to determine the second position of the paramagnetic bead; and measuring the force applied to the molecule as a function of the position of the paramagnetic bead.

BRIEF DESCRIPTION OF THE FIGURES

[028] Figures 1A-1D show Magnetic Tweezers and HaloTag attachment measuring of the folding dynamics of polypeptides under force, according to some aspects of the present disclosure.

[029] Figures 2A-2C show length scaling of protein under force for determination of magnet law, according to some aspects of the present disclosure.

[030] Figure 2D depicts histograms of step-size measurements of different magnet positions, according to embodiments of the present disclosure.

[031] Figure 2E depicts results of testing of the magnet law by varying different parameters: variation of the position of the DNA B-S overstretching transition (left), variation of the persistence length (middle), and variation of the contour length increment (right), according to embodiments of the present disclosure.

[032] Figures 2F-2G depict length scaling of several proteins under force, according to embodiments of the present disclosure.

[033] Figures 3A-3D depict fiduciary marker for the magnet law, using the B-S overstretching transition, according to some embodiments of the present disclosure.

[034] Figures 4A-4D illustrates stability of the voice-coil tweezers over hours-long recordings of HaloTag anchored proteins, according to some embodiments of the present disclosure.

[035] Figures 5A-5C is a two-week long recording of a single HaloTag anchored protein L construct, according to some embodiments of the present disclosure.

[036] Figures 6A-6E depicts a chamber design for long-term single molecule measurements, according to some embodiments of the present disclosure.

[037] Figures 7A-7E depicts measuring the z-position of beads using image processing, according to embodiments of the present disclosure.

[038] Figure 7A depicts a 128x128 pixel region of interest (ROI) of a 2.8 μm bead at focal position 1000 nm (top) and 1600 nm (bottom), according to embodiments of the present disclosure.

[039] Figure 7B is a FFT of the images in Figure 8A. The red line marks a radial vector. The black dots mark the pixels that contribute to three different radii; 48, 94, 140 (pixels x4), according to embodiments of the present disclosure.

[040] Figure 7C depict radial profiles resulting from the FFT's shown in Figure 7B and computed using the FKA algorithm. The black dots identify radial vector positions 48, 94, 140 (see Figure 7B), according to embodiments of the present disclosure.

[041] Figure 7D depicts a stack of radial vectors obtained by moving the objective focal plane (z position) through the bead shown in (Figure 7A) over 2 μm , in steps of 20 nm, according to embodiments of the present disclosure.

[042] Figure 7E depicts correlation profiles between measured radial vectors (inset) and the stack at two different z positions. The correlations are fit with a Gaussian distribution (solid lines), where the mean reports the location of the bead in the z-axis, at nm resolution, according to embodiments of the present disclosure.

[043] Figure 8 depicts a comparison between the magnet law obtained with calibration methods according to embodiments of the present disclosure and magnet laws obtained with fluctuation method of long DNA tethers.

DETAILED DESCRIPTION

[044] In some embodiments, the present disclosure relates to systems and methods using magnetic tweezers for accurate measurement of protein dynamics under force. Folding and unfolding transitions of molecules (e.g., proteins, e.g., short recombinant proteins) are dependent on applied force. Some embodiments discussed herein relate to determining a relationship between force applied to a paramagnetic bead and separation distance between a paramagnetic bead and a magnetic component of the magnetic tweezers. Some embodiments discussed herein relate to using the determined relationship between the force and the separation distance to precisely calculate the amount of force applied to any molecule (e.g., protein, DNA, RNA) under study.

[045] Some embodiments discussed herein demonstrate a novel approach to the use of magnetic tweezers instrumentation to study molecule (e.g., protein) dynamics under force for extended periods of time (e.g., several hours, several days, several weeks). In order to study molecule dynamics for extended periods of time, it is important to be able to accurately measure the amount of force that is being applied to said molecules. Some embodiments relate to determining a relationship between the separation distance between a magnetic component of magnetic tweezers and a paramagnetic bead attached to a molecule under study (e.g., protein, DNA, RNA) and an amount of force applied to said molecule using a magnetic tweezer setup as discussed herein. This relationship may be used to study dynamics of any molecule under study using a magnetic tweezer setup as discussed herein.

[046] In some embodiments, molecules (e.g., proteins, DNA, RNA) are attached to a substrate (e.g., glass surface on the lower side of a fluid chamber) using standard chemistry methods (e.g., HaloTag, AviTag attachment) and paramagnetic beads are flushed inside the fluid cell. The beads react with the opposite end of the molecule (e.g., through biotin-streptavidin interaction). Non-magnetic reference beads are linked to the surface during the surface functionalization procedure. Force is applied to single molecules by adjusting the separation between the paramagnetic beads and a pair of permanent magnets. The position of the permanent magnets is adjusted using a voice coil actuator, which may be controlled by a command voltage sent from a computer through a DAQ card. In some embodiments, the voice coil actuator can position the permanent magnets with about 200 micron resolution. In some embodiments, this precise positioning, together with the fact that the magnetic field gradient (which gives the magnetic force) decays on mm range, allows for calculation of the force with minimum error.

[047] In some embodiments, two regions of interest (of, e.g., about 128x128 pixels) encompassing the reference and magnetic beads are selected and image stack libraries for each of these two beads are obtained by changing the focus position in steps of 20 nm. In some embodiments, the z position of each bead may be measured by correlating the radial profile of the Fourier transform of the current image from the camera live stream with the profiles from the saved image-stack libraries, obtained at different focal distances, which will be discussed in further detail below. In some embodiments, the correlation between the actual images of the beads can be used, instead of their Fourier transforms, to obtain the z- position of the bead; such embodiments may involve finding centers of the beads. The change in

length of the tethered molecule as a function of force is reported from the difference between the absolute positions of the magnetic and reference beads. In turn, this allows for precise calculation of the force applied to the molecule. During the experiment, a positioning device (e.g., piezo nanofocusing device) may be continuously adjusting the focus in steps such that the reference bead is maintained at the same initial position. This approach corrects any drift and allows for hour-long, days-long, or even weeks-long recordings.

[048] In some embodiments, an engineered modular protein can be used to calculate the force being applied to a molecule (e.g., short protein). Or, high force-sensitivity of protein L can be used as a molecular template to generate a magnet-law (relationship) based solely on the distance between the magnet and the measuring paramagnetic bead. The dynamics of protein L can be accurately measured over a wide range of forces, with little dispersion from bead to bead and for durations of several hours, several days, and several weeks using the systems and methods discussed herein. In some embodiments, the use of a voice-coil actuator for fast and high resolution magnet positioning, as described herein, ensures the reproducibility of the magnet law over weeks-long single molecule experiments. This magnet law may be used to accurately study the dynamics of various molecules.

[049] Magnetic tweezers are an analytical tool that can be used for studying the structure, dynamics and chemistry of single molecules, such as proteins and DNA/RNA. Magnetic tweezers may be specifically suited to apply low forces without visible drift for extensive periods of time of several hours (or longer time periods). In some embodiments, magnetic tweezers enable increased speed and accuracy of the magnet positioner (which is used to apply the force), due at least in part to the implementation of a voice coil mechanism. In some embodiments, the use of the magnetic tweezers results in low noise and high acquisition rate because of a new image processing algorithm.

[050] Single molecule studies of proteins under force have been so far limited by the range of applied force and the time span that a single protein could be exposed to a mechanical perturbation. Typically single molecules can be probed with force spectroscopy either at high forces using atomic force microscopy (AFM) or at low forces using optical/magnetic tweezers, but never simultaneously over an extended range of forces. Earlier attempts to bridge this gap by using electro-magnetic tweezers were marred by difficulties in determining precisely the applied force and a very low number of observations caused by the frequent

detachment of the molecules from their anchors. Although it was clear that magnetic tweezers held great promise, these technical challenges made it difficult to obtain reliable data that could be used as a benchmark to examine single proteins under force. Only recently has covalent attachment been established as a reliable technique to anchor and pull single molecules in force spectroscopy measurements. This covalent attachment approach now allows tethering of single proteins without the limitations in measuring time and force range of nonspecific anchors.

[051] Unlike other single molecule techniques, such as AFM, where the force is continuously measured, in the disclosed magnetic tweezers the force is estimated based on previous calibrations obtained at different magnet positions. A commonly used method to calibrate the force in magnetic tweezers is based on analyzing the thermal fluctuations of the tethered magnetic bead. In this case, the estimated force F is directly proportional to the absolute length of the tether z , and inversely proportional to δ_x^2 , the square of the variance of the fluctuations along a coordinate perpendicular to the direction of stretching: $F = zk_B T / \delta_x^2$. Calibrating the force from bead fluctuations using long DNA linkers of several microns and high-speed camera acquisition rates can provide an accurate measure of the magnetic force over an extended range.

[052] In contrast to DNA, proteins are much shorter tethers, which show force dependent folding fluctuations. The highest source of error when analyzing the motion of a bead tethered to a short protein linker is typically given by the estimation of the absolute tether length, z . This value of the absolute measured length is prone to two sources of error. A first source of error comes from the physics of proteins under force. Protein domains unfold and refold, continuously changing their contour length. These folding transitions dominate the motion of the magnetic bead and introduce a significant error in estimating the thermal fluctuation of the magnetic bead. A second source of error comes from how length is measured with magnetic tweezers. As discussed in further detail below, in some embodiments, two image stack libraries are obtained for the reference and magnetic beads respectively, that reflect their absolute positions and the length is given by the difference between the positions of these two beads. At zero length, obtained during the acquisition of the two stack libraries, the protein is already extending due to the Brownian motion of the magnetic bead. While magnetic tweezers can accurately report relative changes in the end-to-end length of a tethered molecule, the absolute length is not directly measured. A single stack

library obtained with the same magnetic bead type, glued to the surface, could in principle be used to obtain the absolute value of the tether length.

[053] Some embodiments discussed herein relate to new approaches to measure the force at a given magnet sample separation. In some implementations, this method is based on the properties of a homopolyprotein composed of protein L domains. Proteins are unique as they display step-changes in the measured length due to folding transitions. These changes in length are highly sensitive to the applied force, making them ideal nano-scale rulers for the magnetic force. The force dependency of protein folding transitions is combined with the known force of the DNA overstretching transition to obtain a general magnet law, as will be discussed in further detail below, describing the change in force with magnet-sample separation. In the sampled force range, a single exponential relationship accurately describes the variation in force with magnet position. Using a single magnet law for a given combination of paramagnetic beads and magnet geometry is justified by the measured small force variance of only 4% of the location of the B-S DNA overstretching transition. Other types of magnetic beads with higher dispersion or the use of less accurate positioning devices for the magnets might require a separate calibration on a bead-to-bead basis. In such a case, the folding step sizes of the protein domains at different magnet positions can provide a direct measurement of the pulling force. To obtain this calibration, the persistence and contour length increment of the studied protein is useful. While the persistence length is generally protein independent, the contour length increment can be easily measured with AFM or estimated from structural analysis, if the crystal structure of the protein is known.

[054] Magnetic tweezers can apply force in the pN range to proteins and other molecules that are tethered between a glass surface and a magnetic bead. In some embodiments, this instrument is based on a voice coil system capable of positioning the magnets with submicron resolution at speeds of about -0.7 m/s over a about 1 cm range. In some embodiments, the magnetic tweezers setup includes a cooling system for the camera to prevent fan-related vibrations. In some embodiments, the magnetic tweezers can apply force to a single molecule for several hours without the drift limitations of other single molecule techniques. In some embodiments, the components of the magnetic tweezers can be available “off-the-shelf” allowing for the cost of the setup to remain low and affordable.

[055] The low drift and high force resolution of magnetic tweezers combined with HaloTag anchored proteins allows studying protein dynamics at low forces. A limiting factor in the use of magnetic tweezers to study proteins at low force is given by the difficulty in estimating precisely the applied force. The standard approach is to measure the Brownian fluctuations of the tethered paramagnetic bead, from which a force can be calculated. This approach can be used for measuring the force being applied to micrometer-long DNA tethers. However, its direct use is not suitable in the study of protein folding/unfolding reactions.

[056] Under mechanical force, proteins show folding transitions as step-changes in the measured end-to-end length. These folding transitions dominate the motion of the paramagnetic bead at low forces, severely limiting the use of spectral analysis to precisely obtain the pulling force (Figure 1A-D). Furthermore, owing to their short length (e.g., about 20-300 nm), the measuring paramagnetic bead is forced to operate near a surface with its associated effects on viscosity and anisotropy.

[057] Figure 1A shows HaloTag surface chemistry, according to aspects of the present disclosure. An amine-terminated silanized surface (101) is cross-linked to an amine-terminated chloroalkane ligand (105) using glutaraldehyde (103). A fusion protein consisting of a HaloTag followed by eight repeats of protein L and terminated by an AviTag (HaloTag-(protein L)₈-AviTag) (107) was studied. The HaloTag was reacted with the chloroalkane ligand on the glass surface (102), forming a covalent anchor (104) for the construct.

[058] Figure 1B shows the reaction between the AviTag at the other end of the construct being reacted with a streptavidin-coated paramagnetic bead (gold) (106), according to aspects of the present disclosure. The pulling force can be adjusted by positioning a pair of permanent magnets (110) at a fixed distance (Magnet Position) above the glass surface (102). The length of the protein under force is measured with respect to a fiduciary non-magnetic bead (108) affixed to the glass surface (grey) (102). The insert in Figure 1B shows a HaloTag protein between a glass surface (e.g., 102) and another protein domain.

[059] In some embodiments, molecules (e.g., proteins, DNA, RNA) can be attached to a substrate (e.g., glass surface 102) via any standard chemistry methods. In some embodiments, the standard chemistry methods are selected from, for example, AviTag, HaloTag, His-Tag, SpyTag, SNAP Tag, thiol chemistry, and antibodies. In some embodiments, the protein is any protein that would denature within the range of applied forces.

[060] In some embodiments, the paramagnetic bead (106) is DYNABEADS (R) M-270 Streptavidin having a diameter of or about 2.8 microns, product size of 2 mL, concentration of 10 mg/ml, binding property of about 650-1350 pmoles/mg beads, isoelectric point of about pH 4.5, size distribution CV< about 3%, and an iron content (ferrites) of about 14%. In some embodiments, the paramagnetic beads have hydrophilic and carboxylic acid surface functionality. In some embodiments, the paramagnetic beads are uniform and have a monolayer of recombinant streptavidin covalently coupled to the surface.

[061] In some embodiments, the paramagnetic bead (106) is DYNABEADS (R) M-280 Streptavidin having a diameter of or about 2.8 microns, product size of about 2 mL, concentration of 10 mg/ml, binding property of 650-900 pmoles/mg beads, isoelectric point of about pH 5.0, size distribution CV< about 3%, and an iron content (ferrites) of about 14%. In some embodiments, the paramagnetic beads have hydrophobic and tosylactivated surface functionality. In some embodiments, the paramagnetic beads are uniform and have a monolayer of recombinant streptavidin covalently coupled to the surface and further blocked with BSA.

[062] In some embodiments, the paramagnetic bead (106) is DYNABEADS(R) M-450 Tosylactivated having a diameter of or about 4.5 microns and a product size of 5 mL. In some embodiments, the paramagnetic beads have hydrophobic and tosylactivated surface functionality. In some embodiments, the paramagnetic beads contain surface tosyl groups. In some embodiments, the paramagnetic beads covalently bind primary amino and sulfhydryl groups in antibodies to position them in the optimal orientation for cell-surface protein binding.

[063] In some embodiments, the paramagnetic beads have a diameter between about 1 micron and 5 microns. In some embodiments, the paramagnetic beads are coated with antibodies. In some embodiments, the paramagnetic beads have a monolayer of recombinant streptavidin covalently coupled to the surface, which may optionally be further blocked with BSA. In some embodiments, the paramagnetic beads have a binding property of about 650-900 pmoles/mg beads, about 650-1350 pmoles/mg beads, or above about 1300 pmoles/mg beads. In some embodiments, the paramagnetic beads have surface functionality selected from the list consisting of: hydrophilic, carboxylic acid, tosylactivated, hydrophobic, and any

suitable combinations thereof. In some embodiments, the paramagnetic beads have amine, carboxylic acid or epoxy groups, which can be used to obtain new attachment chemistries.

[064] In some embodiments, the paramagnetic bead is selected from MAGNALINK™ from Solulin and PROMAG™ from Bangs Laboratories.

[065] In some embodiments, the reference bead (108) is in the same size range as the paramagnetic bead (106) (e.g., between about 1 micron and about 5 microns). In some embodiments, the reference beads may include, but are not limited to, POLYBEAD® Amino Microspheres 3.00µm (17145-5) from Polysciences, Amino-polystyrene Particles 2.5-2.9 µm (AP-25-10) from Spherotech, IDC™ Latex Particles - Aliphatic Amine Latex, 2% w/v 3 µm, (A37368), from Life Technologies.

[066] In some embodiments, the substrate (102) is or comprises glass. In some embodiments, the substrate (e.g., glass) has a thickness between about 0.1-0.2 microns). In some embodiments, Micro Cover Glasses, 22 x 40 mm x 0.13 - 0.16 mm thick (260144) Ted Pella may be used.

[067] Figure 1C shows schematics of the voice-coil tweezers setup (100), according to aspects of the present disclosure. The applied force on the paramagnetic bead is precisely controlled by attaching a pair of permanent magnets (110) to a voice-coil actuator (112) with a 10 mm range and a maximal velocity of or about 0.7 m/s. An embedded encoder (114) reports on the magnet position with 150 nm resolution. The position of the permanent magnets is adjusted using the voice coil actuator (112), which is controlled by a command voltage sent from the computer (126) through the DAQ card (128). This arrangement allows for the application of fast, arbitrary and repeatable force pulses to the protein. The remainder is a standard magnetic tweezers setup including a piezo nanofocusing device (120), a light source (122), an inverted microscope and a high speed CCD camera (124). The piezo nanofocusing device (120) can continuously adjust focus with nm or sub-nm resolution.

[068] In some embodiments, the magnetic tweezers include the following components: (1) voice coil (e.g. LFA2010/SCA814, Equipment Solutions); (2) camera (e.g., Zyla from Andor, Pike 032B/C from Allied Vision Technologies); (3) piezo nanofocusing device (e.g., Fast PIFOC, Physik Instrumente); (4) microscope (e.g., Olympus IX71, Olympus Corporation); (5) data acquisition (“DAQ”) card (e.g., USB-6289, National Instruments); (6) optical

components (e.g., Olympus, Zeiss); and (7) a computer. In some embodiments, the magnetic tweezers include only the seven components listed above. In some embodiments, the magnetic tweezers also include one or more (or any combination of) the following additional components: (8) position adjustment stage; (9) vibration isolation table; (10) magnetic and non-magnetic beads; and (11) control software (written, for example, in Igor Pro, Wavemetrics). In some embodiments, the magnetic tweezers also include one or more (or any combination of) the following additional components: (12) Total Internal Reflection module for the microscope; (13) fluorescence camera; (14) laser; (15) custom-made microscope.

[069] Figure 1D is a graph showing results of a long-term experiment using the voice-coil tweezers in accordance with some embodiments discussed herein. The top trace of Figure 1D shows the protein length, and the bottom trace of Figure 1D reports the magnet position. The protein is first fully unfolded at MP=1.4 mm (high force), refolded at MP = 4.3 mm (low force), and finally allowed to equilibrate over 3 hours at MP = 3.3 mm. The folding/unfolding dynamics of the protein precludes the use of fluctuation analysis to measure the applied force.

[070] In some embodiments, the folding dynamics of protein L are used to establish a novel approach for calibrating the pulling force applied by magnetic tweezers to short recombinant proteins. Protein L binds immunoglobulins through L chain interaction. Protein L consists of 719 amino acid residues. Protein L binds a wide range of antibody classes, including representatives of all antibody classes. The size of the folding/unfolding steps is highly sensitive to force and can be described using standard polymer elasticity models, such as the worm-like chain (WLC). This characteristic makes protein L an ideal molecular template for reporting the force as a function of magnet position. In some embodiments, the force dependent step sizes of the folding/unfolding transitions can be used to derive a magnet law that precisely calculates the force applied to a protein (or another molecule), solely based on the distance between magnets and paramagnetic bead. In certain embodiments, the force sensitivity of protein L is combined with the DNA overstretching force standard to determine the magnet law. In certain embodiments, this calibration method is validated by analyzing the unfolding rate of protein L at high forces and examining the size of the folding steps at low forces.

[071] The use of a magnet law demands exacting and stable positioning of the magnets, in order to trust that the applied force is not drifting over time and can be reproduced from one experiment to the next. Furthermore, it is also essential that the magnets can be positioned very fast and with very high accuracy. In some embodiments, a voice-coil actuator is used for fast positioning of permanent magnets over 1 cm travel with 150 nm resolution. As discussed in further detail below, in some embodiments, voice-coil magnetic tweezers demonstrate remarkable stability over hours-long recordings of protein L dynamics at low forces. In addition, in further experiments, measuring time of these experiments was expanded to up to two weeks and the dynamics under force of the same protein were measured with minimal drift.

[072] As magnetic tweezers becomes the technique of choice to study the dynamics of proteins at low forces, the use of protein constructs as nano-scale rulers provides an accurate and highly reproducible approach to measure the force when using short protein tethers.

[073] In some embodiments, the systems and methods discussed herein may be useful in studying various diseases. In particular, the systems and methods discussed herein may be useful in studying diseases that involve misfolding of proteins (e.g., Huntington's disease) as the systems and methods discussed herein allow for precise application of force to proteins under study. In some embodiments, the systems and methods discussed herein may be useful in drug discovery applications. For example, the systems and methods discussed herein may be useful for developing drugs that allow for treatment of diseases at the protein level. The systems and methods discussed herein may be useful, for example, in developing antibiotics, for example by preventing formation of certain bonds and preventing bacteria from attaching to certain hosts and spreading disease.

[074] The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[075] The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

EXAMPLES

[076] Example 1: A nano-scale ruler for calibrating magnetic tweezers.

[077] The experimental approach described herein is based on magnetic tweezers, a technique capable of applying mechanical force to single molecules using paramagnetic beads (Figure 1A-D). This approach is complemented by the use of HaloTag based covalent attachment chemistry, which permits exposing single proteins to high mechanical perturbations for extended periods of time without detachment. In magnetic tweezers, the applied force is often calculated using a calibration curve that measures force as a function of the distance between the magnet and the paramagnetic bead tethered to the molecule under study. The applied force directly depends on the size and magnetic properties of the beads and the geometry and strength of the magnets. It was recently proposed that a single magnet law would suffice to accurately estimate the force on micrometer long DNA molecules. Folding step sizes of protein L were used as a nano-scale ruler for the change in force with magnet position.

[078] When exposed to a constant force, protein L domains undergo unfolding transitions as step-size increases in the measured end-to-end length (Figure 2A). Figure 2A shows unfolding traces of the eight-domain protein L construct (top) measured at three different magnet positions (bottom). Protein L unfolds within several seconds in steps of 15.3 nm at magnet position 1.3 mm (red) and within two minutes in steps of 12.3 nm at magnet position 2.5 mm (yellow). At magnet position 3.3 mm protein L reaches a steady state where unfolding and refolding steps of 8.7 nm are observed over an extended period of time (blue, Figure 2A). The force scaling of the unfolding step sizes is also apparent in the length of the fully unfolded protein (eight domains in all cases; Figure 2A). Both the unfolding rate and the step sizes scale with the applied force. Folding steps also scale with the pulling force and, for a given force, have the same size as the unfolding steps (Figure 2D).

[079] Figure 2B recapitulates these data showing how the step sizes vary with magnet position. The solid line in Figure 2B is a fit of Equation 4 to the data, and the shaded area shows the 95% confidence contour.

[080] Figure 2C shows magnet law derived from Figure 2B, solid line, Equation 5. The dots in Figure 2C correspond to the force calculated from the WLC model for the step sizes

measured at different magnet positions. The diamond in Figure 2C marks the force and magnet position for the B-S transition (transition from B-DNA to highly overstretched S-DNA having about 1.7-fold longer form at 65pN).

[081] Figure 2D depicts histograms of step-size measurements of different magnet positions, according to embodiments of the present disclosure. Length histograms were obtained from the data corresponding to each step and the absolute position was measured using Gaussian fits. The size of each step was measured as the difference between the centers of the two peaks. The standard deviation of each step was 1-2 nm. The populations of the folding step sizes are normally distributed around their average.

[082] Studies on the unfolding of titin (also known as connectin, a protein greater than about 1 micron in length) molecules under force showed that the stepwise increases in contour length of an unfolding protein, as can be described by simple polymer physics models. Both the Worm Like Chain (WLC) and Freely Jointed Chain (FJC) models produce equivalent results and they are used indistinctively through the force spectroscopy literature. Similarly, the data shown in Figure 2B can be accurately fit by the WLC model using the protein L parameters of persistence length $p = 0.58$ nm and contour length of $\Delta L_c = 18.6$ nm, assuming an exponential magnet law given by:

$$F(MP) = a \cdot e^{b(MP)} \quad \text{Eq. 1}$$

[083] When magnet law is combined with the WLC model, it gives:

$$MP(x) = \frac{1}{b} \ln \left\{ \frac{k_B T}{p} \left[\frac{1}{4} \left(1 - \frac{x}{\Delta L_c} \right)^{-2} - \frac{1}{4} + \frac{x}{\Delta L_c} \right] / a \right\} \quad \text{Eq. 2}$$

[084] where MP is the magnet position, x is the observed step size, and a and b are fitting parameters. Fitting the data of Figure 2B using Equation 2 gives values of $a = 177 \pm 17$ pN and $b = -1.07 \pm 0.05$ nm⁻¹.

[085] The B-S overstretching transition of DNA molecules is used as a force standard to evaluate this approach in the context of short tethers. This transition takes place at exactly 65 pN, independent of the force loading rate. By including this measurement, the magnet law becomes:

$$F(MP) = F_{B-S} \cdot e^{b(MP_{B-S} - MP)} \quad \text{Eq. 3}$$

[086] where F_{B-S} is the force at which the B-S transition is observed in DNA (65 pN), and MP_{B-S} the magnet position where the B-S transition is observed in the present magnetic tweezers instrument. MP_{B-S} was measured with the experimental approach shown in Figure 3. Toward this end, polyprotein containing a HaloTag followed by eight domains of protein L and a terminal cysteine anchored to a 605 bp DNA linker were engineered (Figure 3A). When force was applied to this construct ($MP = 1.4$ mm; Figure 3A), ~14 nm unfolding steps were observed. As the magnet position was decreased linearly (ramp-increasing force from $MP=1.4$ to $MP=0.9$ mm), the overstretching transition was observed as a sharp extension of 127 ± 28 nm (Figures 3A, B), at a magnet position of $MP_{B-S} = 0.99 \pm 0.05$ mm ($n = 34$; Figure 3D). A similar construct composed of eight repeats of the more mechanically stable I27 protein and the same DNA segment first showed the overstretching transition at the same 0.99 mm magnet position, followed by eight ~25 nm steps, characteristic of the unfolding of I27 (Figure 3C). In Figure 3C, the I27 domains unfolded at forces higher than the B-S transition. Figure 3D is a histogram of magnet position values where the B-S transition was measured in both I27 and protein L constructs. The line in Figure 3D was a Gaussian fit with $MP = 0.99 \pm 0.05$ mm ($n=34$ different beads).

[087] Equation 3 is combined with the WLC model, giving:

$$MP(x) = MP_{B-S} - \frac{1}{b} \ln \left\{ \frac{k_B T}{p} \left[\frac{1}{4} \left(1 - \frac{x}{\Delta L_c} \right)^{-2} - \frac{1}{4} + \frac{x}{\Delta L_c} \right] / F_{B-S} \right\} \quad \text{Eq 4}$$

[088] Equation 4 provides a closed-form for $MP(x)$, with b as the only fitting parameter. Equation 4 was then fit to the data of Figure 2B using the Levenberg-Marquardt least orthogonal distance method with a confidence level of 95% (solid line; Figure 2B). From the fit, the following parameters were obtained, $b = 0.90 \pm 0.03$ mm⁻¹, for $F_{B-S} = 65$ pN. The fit was weighted with the standard deviation of the measured extension steps (Figure 2B) and a standard deviation for the magnet position of 0.05 mm, as measured from the DNA-protein experiments (Figure 3D). The shading in Figure 2B marks the upper and lower confidence contours of the fit.

[089] As such, the magnet law as defined by Equation 3 is:

$$F(MP) = 65 \cdot e^{0.9(0.99-MP)} \quad \text{Eq. 5}$$

[090] The accuracy of this magnet law provided by Equation 5 was verified in Figure 2C. The predicted force for each step size calculated using the WLC was plotted as a function of the MP where the steps were measured (red dots; Figure 2C). The position of the overstretching B-S transition in DNA (open square; Figure 2C) was marked in Figure 2C as open squares. The data shown in Figure 2C was compared with the magnet law given by Equation 5 (solid line with shading at 95% confidence; Figure 2C). In Figure 2E, the parameter sensitivity of the magnet law was explored. Deviations of 5 pN for F_{B-S} , of 0.1 nm for p , and of 0.3 nm for ΔL_c were imposed. The impact of these parameter variations on the magnet law was within the 95% confidence level.

[091] Figure 2E-left shows the effect of the variation of the position of the DNA B-S overstretching transition with $F_{B-S} = 65 \pm 5$ pN.

[092] Figure 2E-middle shows the effect of the variation of the persistence length $p = 0.58 \pm 0.1$ nm.

[093] Figure 2E-right shows the effect of the variation of the contour length increment $\Delta L_c = 18.6 \pm 0.3$ nm. The points were calculated from the measured extension as a function of force (Figure 3B). The black line represents the fit using the parameters $F_{B-S} = 65$ pN, $p = 0.58$ nm and $\Delta L_c = 18.6$ nm. The blue and green lines represent the magnet law evaluated at the upper and lower bounds of the fit parameter set as one standard deviation from the mean. The standard deviations in each of the three fit parameters were taken from previous studies.

[094] In addition to protein L, unfolding and folding step sizes for ubiquitin and I27 polyproteins were measured (Figure 2F-G). The increase in contour length upon unfolding (ΔL_c) is protein specific: $\Delta L_c = 18.6$ nm for protein L, $\Delta L_c = 24.5$ nm for ubiquitin, and $\Delta L_c = 28.4$ nm for I27. The force dependency of the steps sizes for these proteins was reproduced by using Equation 4 with the corresponding values of ΔL_c (Figure 2F-G).

[095] Figure 2F shows the average step size of unfolding and folding transitions as a function of magnet position/force for four proteins: I27 from human titin, ubiquitin, and protein L. The solid lines represent the WLC law assuming a single exponential variation of force with magnet position and a contour length of 28.4 nm for I27, and of 24.5 nm for Ubiquitin. The step sizes at low force were obtained from folding transitions, while the high force points were obtained from unfolding transitions. Protein L showed folding transitions

over the entire force range with smaller standard deviation (1.1 nm) than I27 (3.9 nm) and ubiquitin (1.8 nm), making it an ideal nano-scale ruler for calibrating the magnetic force.

[096] Figure 2G shows the normalized folding transitions to their contour length that collapse on a single master curve.

[097] The magnet law defined by equation 5 above is valid as long as the instrument remains stable during the measurements, implying that the relationship between magnet position and force is time independent. Due to the exponential dependency of force with magnet position, it can be expected that errors may become more significant at high forces. As a proxy for errors in the high force regime, the unfolding rates of protein L over extended periods of time were measured (Figure 4A, C). A single protein L construct (anchored HaloTag-(protein L)₈-AviTag protein obtained by alternating the force from 54 pN to 4.3 pN (MP = 1.2 mm – 4 mm)) was continuously exposed to unfolding-refolding cycles for >8 hours (Figure 4A). The inset on the right of Figure 4A magnifies a single unfolding trace from the 8h long recording (left; red box). These cycles consisted of unfolding the protein with 30-second pulses to 54 pN (MP = 1.2 mm), after which the protein was allowed to refold at around 4 pN (MP = 4 mm). A moving average of 20 unfolding traces was fit with a single exponential to measure the unfolding rate over the 8 h long experiment.

[098] Figure 4B shows similar recording of folding/unfolding cycles as in Figure 4A but pulsing at a lower force of 8 pN (MP = 3.3 mm).

[099] Figure 4C shows the measured unfolding rates (mean rate of $0.6 \pm 0.06 \text{ s}^{-1}$ at 54 pN) over the full length of the trace, obtained from a moving box average of 20 consecutive traces taken from the trace in Figure 4A.

[0100] Figure 4D shows the average step size per pulse from Figure 4B, $9.0 \pm 0.4 \text{ nm}$. Given that the unfolding rate is exponentially dependent on the force, its stability is a sensitive measure of the force variations at the high end of the force law. The observed rate variations, if they arise solely from instrumental error, were calculated to correspond to changes in force of $\pm 3 \text{ pN}$.

[0101] Another measure of the stability of the present magnetic tweezers instrument is the distribution of unfolding and refolding step sizes, which were used as the basis for determining the magnet law (Figure 2B). Following the shape of the WLC model of polymer

elasticity, the step sizes are relatively invariant at the high end of the force law. However, at forces below 20 pN, the step sizes change rapidly with force, hence providing a good measure of stability in this range.

[0102] Figure 4B shows several 25 min long unfolding pulses at 8 pN (MP=3.3 mm) showing numerous unfolding and refolding steps as the protein equilibrates. From these data the average step size in each pulse (Figure 4D) was measured, showing that these measurements were particularly stable. The mean value of the step-sizes at 8 pN was 9.00 ± 0.04 nm (Figure 4D), which is very close to the value predicted by the WLC model at this force (8.9 nm).

[0103] The experiment shown in Figure 5A-C further extends the observations shown in Figure 4, to a single protein L construct experiment lasting 14 days. In this case, a single protein L construct is probed (measured daily, for several pulses) by unfolding at 45 pN for 45 s (MP=1.4 mm; Figure 5A).

[0104] Figure 5A shows daily unfolding pulses to 45 pN (MP = 1.4 mm) tracking the unfolding kinetics of the same single HaloTag-(protein L)₈-AviTag molecule over 14 days. The molecule was allowed to rest throughout the day in the folded state (2-4 pN; MP 4.6-4 mm) with the microscope lights turned off, and was tested daily in the evening (~5 pm; pulses).

[0105] Figure 5B shows superimposed traces from Figure 5A, showing the full unfolding of the protein L construct at day 1 (red), day 6 (blue), day 9 (green), day 11 (magenta), and day 14 (gold). The unfolding rate stayed very similar throughout.

[0106] Figure 5C shows average step sizes measured from the unfolding staircases. The step sizes remained constant throughout the two weeks.

[0107] For such long experiments, the chances of biotin-streptavidin detachment from the paramagnetic bead were minimized by probing the molecules only once or twice each day in order to minimize their exposure to 45 pN. In between force pulses, the molecule was held at 4 pN (MP=4.0 mm) to allow for domain refolding. Importantly, rates of unfolding were comparable on each day (Figure 5B). Moreover, the unfolding step sizes measured each day were within error of the predicted 15.2 nm, calculated from the WLC model for MP=1.4 mm

(Eq. 2), and betrayed no directional drift (Figure 5C). Thus, the magnetic tweezers setup and magnet law are suitable for reliable long-term force measurements.

[0108] To resolve individually addressable HaloTag-protein L molecules, the magnetic tweezers microscope stage was modified with a xy-positioning stage with 100 nm resolution. Further, HaloTag-Ligand-functionalized fluid chambers having a $50\ \mu\text{m}^2$ grid array were developed. Using this combination ensured that it was possible to reliably return to individual beads on successive days. The molecule shown in Figure 5A-C was the longest lasting before detachment (14 days), from an initial group of 17 molecules that were tracked simultaneously and that lasted more than one day (7-1 day; 3-2 days; 1-3 days; 1-4 days; 1-5 days; 1-6 days; 1-9 days; 1-10 days; 1-14 days). It is likely that the loss of anchoring occurred at the non-covalent streptavidin-biotin interface with the paramagnetic bead rather than the HaloTag-chloroalkane interface with the glass surface, which is covalent. Replacing the biotin-streptavidin interface with an orthogonal covalent Tag will solve this problem allowing, in principle, for protein L constructs to be probed indefinitely.

[0109] Another potential source of error in the use of the instant magnet law results from bead-to-bead variations. However, the instant measurements indicate that for a given magnet position, M-270 paramagnetic beads generate a reproducible force. This is demonstrated in Figure 3D, where the overstretching B-S transition (65 pN) occurs over a very narrow distribution of magnet positions. In some embodiments, it can be important to be able to position the magnets in a reproducible manner so that any variation results solely from the beads themselves. The voice-coil design with the submicron encoder solves this problem by giving a reliable positioning of the magnets between different experiments. Hence, it can be concluded that there is little variation in force amongst M-270 beads.

[0110] Figure 8 compares the data and magnet law discussed herein, with magnet laws obtained by others using thermal fluctuations of micrometer long DNA tethers and the same paramagnetic beads (M-270; DYNABEADS(R)). There is remarkably good agreement between these different methods at forces below 65 pN. While the change in force with magnet position has been shown to follow a single exponential, several studies have proposed that a double exponential magnet law might be needed at higher forces.

[0111] Figure 8 depicts a comparison between the magnet law obtained according to some embodiments discussed herein and magnet laws obtained with fluctuation method of long

DNA tethers. The points were obtained using the measured extension at different magnet positions (circles) and the B-S DNA overstretching transition (diamond). The black line represents the magnet law in accordance with certain embodiments discussed herein. The red line represents the magnet law obtained in reference Yu, Z.; Dulin, D.; Cnossen, J.; Köber, M.; van Oene, M. M.; Ordu, O.; Berghuis, B. A.; Hensgens, T.; Lipfert, J.; Dekker, N. H., A Force Calibration Standard for Magnetic Tweezers. *Rev. Sci. Instrum.* 2014, 85 (12) for the same M-270 paramagnetic beads, and using a gap between magnets of 0.3 mm: $F = -0.019 + 60.4 \cdot \exp(-(MP - 0.4)/0.563) + 56.9 \cdot \exp(-(MP - 0.4)/1.46)$. The blue line represents the magnet law obtained in reference Chen, H.; Yuan, G. H.; Winardhi, R. S.; Yao, M. X.; Popa, I.; Fernandez, J. M.; Yan, J., Dynamics of Equilibrium Folding and Unfolding Transitions of Titin Immunoglobulin Domain under Constant Forces. *JACS* 2015, 137 (10), 3540-3546 for the same M-270 paramagnetic beads and the same type of magnets: $F = C \cdot (\exp(-MP/0.36) + 0.48 \cdot \exp(-MP/1.12))$, where $C = 290$ pN. The figure shows good agreement between these different calibration curves at forces below ~65 pN.

[0112] Magnetic tweezers, combined with HaloTag anchoring techniques have opened up the possibility to study protein dynamics with remarkable stability and accuracy under force clamp conditions. The methods demonstrated herein provide a robust approach to estimating the force applied to a protein in the low force regime below ~ 60 pN and for very extended periods of time (e.g., several hours, several days, several weeks). These techniques complement force-clamp AFM spectroscopy that excels in speed and resolution at higher forces, but remains limited by mechanical drift in the low force regime. To the inventors' knowledge, the mechanical study of a single protein over a period of 14 days is the longest yet reported. Such long-term recordings open up the possibility of beginning to study protein mechanics on the human timescale, where protein folding and misfolding occur over a lifetime, as, for example, with ocular cataracts or chronic traumatic brain injury. This long-term recording was enabled by the HaloTag covalent chemistry used for surface conjugation. Engineering of a second covalent anchor to replace the non-covalent biotin-Streptavidin attachment to the magnetic bead would further improve the stability of the tethers, and could offer the potential for month-long or year-long single molecule studies where rare events such as those that take place in-vivo, become detectable.

[0113] The force calibration method demonstrated herein, based on nanoscale protein fingerprints, provides a robust approach to measuring the force in magnetic tweezers, when

using protein tethers. HaloTag covalent tethering of proteins combined with Streptavidin-AviTag attachment allows for extremely long recordings and any force drift can be estimated from both the unfolding rates variation at high forces and the step sizes at high magnet positions.

[0114] Example 2: Protein expression, purification and modification

[0115] Polyprotein constructs were engineered using a combination of *Bam*HI, *Bgl*II, and *Kpn*I restriction sites, as described previously. The protein constructs had eight repeats of protein L (B1 domain from *Peptostreptococcus magnus*), I27, or ubiquitin flanked by an N-terminal HaloTag enzyme (Promega) and a C-terminal AviTag. Proteins cross-linked with DNA had eight protein L and I27^{C47/63A} engineered between a HaloTag at the N-terminus and a cysteine residue at the C-terminus. For purification purposes, a His6-tag was also present before the AviTag or the cysteine residue. The multidomain proteins were expressed and purified as follows. BLR or ERL cells were grown at 37° C until OD 600 nm ~0.6–0.8. The protein overexpression was induced with 1 mM IPTG overnight at 25° C. Cells were re-suspended in sodium phosphate buffer pH 7.0, 300 mM NaCl, 10% glycerol and 1 mM DTT, and disrupted by French press. The proteins were purified from the lysate following a two-step procedure: with a Ni-NTA histidine affinity purification column (GE healthcare), followed by size exclusion chromatography using a Superdex-200 HR column (GE healthcare) in 10 mM HEPES buffer pH 7.2, 150 mM NaCl, 10 % glycerol and 1 mM EDTA. The purified multidomain proteins containing the AviTag sequence were pooled and concentrated to 100-150 µM before biotinylation. Biotinylation was performed in 50 mM Bicine buffer pH 8.3, 10 mM magnesium acetate, 10 mM ATP, 100 µM biotin and 2.5 µg biotin ligase BirA enzyme (Avidity), at 30° C, for 4 hours. The biotinylation of the multidomain protein was confirmed through western blotting, using conjugated streptavidin horseradish peroxidase (GE healthcare).

[0116] For the DNA-protein constructs, a DNA segment from λ-phage DNA (Thermo Scientific) was ligated with amine and biotin primers (IDT) using a standard PCR mix (New England Biolabs), which resulted in a 605 bp DNA linker. The amine end of DNA was reacted with sulfo-SMCC (Thermo Scientific) for 1 h in a solution of borax buffer pH 8.5. The sulfo-SMCC was removed by cleaning the DNA using a PCR clean-up kit (NucleoSpin)

and eluting with water. The cross-linking reaction was confirmed using SDS polyacrylamide gels, stained with ethidium bromide.

[0117] Example 3: Fluid chamber preparation

[0118] The single molecule magnetic tweezers measurements were done in fluid chambers made of two glass coverslips (Ted Pella) separated by strips of parafilm. The bottom surfaces were successively cleaned by sonication for 20 min in 1% Hellmanex (Hellma), acetone and ethanol (both from Sigma-Aldrich). After drying with air, the bottom surfaces were exposed to air plasma for 20 min, and silanized by immersion for 20 min in a solution of (3-aminopropyl)-triethoxysilane (Sigma-Aldrich) 0.1 % v/v, in ethanol. After washing unreacted silane with ethanol, the surfaces were cured at 100° C for over an hour. The top glass surfaces were cleaned by sonication in 1% Hellmanex for 20 min and washed with ethanol. To eliminate reflection of incident light from the magnets, the top glass was coated with either a non-reflective black tape or a layer of 60 nm of Ni/Cr and 40 nm of gold (Good Fellow, using an Edwards Auto 306 Vacuum Coating System). For long-term magnetic tweezers experiments, glass coverslips with an imprinted 50 μm^2 grid array (Ibidi) were used to allow for repeated addressing of individual beads over multiple days (Figure 6A-E). These coverslips were cleaned similarly, with the exception of air plasma and silane curing at 100°C was replaced with a 24 h vacuum step.

[0119] Figure 6A is a schematic of Ibidi μ Dish fluid chamber positioned beneath a set of permanent magnets. Figure 6B depicts magnification of the 25 x 25 grid array at the center of the fluid chamber. Each cell is 50 μm^2 . Figure 6C shows that cells in the grid array are addressable by alphanumeric coordinates. Figure 6D is a screenshot of the grid showing the reference (R, blue square) and paramagnetic (M, green square) beads. Figure 6E shows images of the paramagnetic bead (green square) probed over a period of 14 days (Figure 5A-C). The bead was readily addressable each day in grid position G17 at x-y coordinates of 12.99 mm, 9.60 mm.

[0120] After assembly, the chambers were incubated for 1 hour with a solution of v/v glutaraldehyde 1% (Sigma Aldrich) diluted in PBS buffer, pH 7.2. The fluid chamber was then filled with 0.025% w/v amine-terminated nonmagnetic polystyrene beads with diameter of 2.89 μm (Spherotech), diluted in PBS. After 10 min, the beads that did not adsorb were washed with 100 μL PBS buffer. The chambers were then reacted for > 4 hours with a

solution of 10 $\mu\text{g/mL}$ HaloTag amine (O4) ligand (Promega), diluted in the same PBS buffer. Finally, the fluid chambers were blocked for 12 hours with TRIS buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl_2 and 1% w/v sulfhydryl blocked-BSA (Lee Biosolutions).

[0121] Example 4: Magnetic tweezers setup

[0122] The setups described herein can be built on top of an inverted microscope (e.g., Olympus IX-71/Zeiss Axiovert S100 or another suitable microscope) using a 63x oil-immersion objective (Zeiss/Olympus), mounted on a nanofocusing piezo device/actuator (P-725; Physik Instrumente) and a 1.6x optivar lens. The fluid chamber was illuminated using a collimated cold white LED (Thor Labs). Images were acquired using a CCD Pike F-032b camera (Allied Vision Technologies) operating at 280 Hz or a Zyla 5.5 sCMOS camera (Andor), operating at 1030 Hz. Paramagnetic DYNABEADS(R) M-270 beads with a diameter of 2.8 μm (Invitrogen) were exposed to force using a pair of permanent neodymium grade N52 magnets (D33, K&J Magnetics), approaching the fluid cell from the top (Figure 1A-D). The position of the magnets was controlled with a linear voice coil (LFA-2010; Equipment Solutions), which is capable of moving 10 mm with ~ 0.7 m/s speed and 150 nm position resolution. The data acquisition and control of the voice coil and piezo nanofocusing device were done using a multifunction DAQ card (NI USB-6341, National Instruments). For long-term recordings, a *xy*-stage moving with ~ 100 nm resolution (M-686, Physik Instrumente) was incorporated in order to address identical bead coordinates over separate days. The image processing and control of the instrument were performed in real-time using custom-written software in Igor Pro (WaveMetrics).

[0123] Example 5: Single-molecule measurements

[0124] The protein was freshly diluted in the same TRIS blocking buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl_2 and 1% w/v sulfhydryl blocked-BSA) to ~ 0.01 μM and left to adsorb on the surface for 10 min. After washing with TRIS buffer, streptavidin coated magnetic beads (DYNABEADS(R) M-270, Invitrogen) were left to react with the protein for ~ 1 min, before approaching the magnets to a low force position (4 mm). The protein experiments were performed in TRIS blocking buffer, while the protein- DNA experiments were performed in the same TRIS buffer lacking MgCl_2 . Two regions of interest of 128×128 pixels encompassing the reference and magnetic beads were selected and image stack libraries for each of these two beads were obtained by changing the focus position in steps of

20 nm. The z position of each bead was measured by correlating the radial profile of the FFT of the current image with the profiles from the saved image libraries, obtained at different focal distances. The z-positions of the two beads, reporting on the change in tether length with force, were computed in real-time, close to the camera acquisition rate (~280 Hz for Pike AVT/~1000 Hz for Andor) using computers with i7 Intel processors, overclocked to 4.6 GHz. Throughout the recordings, the objective was kept in focus by maintaining the reference bead at a constant position. In long-term recordings (> 1 h) a drift in the z position of the magnetic beads becomes apparent (5-10 nm/h).

[0125] The step sizes describing the unfolding and refolding of individual protein domains were measured from individual traces (Figure 2E). For the low force conditions, a boxcar filter (typically 50 points) was applied beforehand. Length histograms were obtained from the data corresponding to each step and the absolute position was measured using a Gaussian fit. The size of each step was then obtained from the position of two consecutive histograms (Figure 2E). The unfolding rates were calculated from the average traces assuming a single exponential unfolding kinetics. The error of the unfolding force was estimated as S.E.M. using bootstrapping.

[0126] Example 7: Magnetic Tweezer Embodiment

[0127] In certain embodiments, the magnetic tweezers described herein can apply force in the pN range to proteins and other molecules that are tethered between a glass surface and a magnetic bead.

[0128] One embodiment of the magnetic tweezers described herein is based on a voice-coil system capable of positioning the magnets with submicron resolution at speeds of ~0.7 m/s over a 1 cm range. A custom-made cooling system was designed for the camera to prevent fan-related vibrations. This instrument can apply force to a single molecule for several hours without the drift limitations of other single molecule techniques.

[0129] Example 8: Image Processing

[0130] Image processing was done using custom-written software in Igor Pro (Wavemetrics), which is described in further detail below. In brief, the z-position displacement of a bead was determined in a three step process: (1) Fourier transform (FFT) of the bead was acquired; (2) the radial profile was computed from the FFT with a pixel-

addressing algorithm (FKA algorithm); (3) radial profiles were correlated to a z-stack library of the bead acquired prior to the experiment (Figure 7A-E). The z-position displacement was calculated for the paramagnetic bead tethered to protein, and for a local fixed reference bead used to correct for instrumental vibration and focal drift. With this procedure, frames rates of ~1 kHz are achievable.

[0131] Magnetic tweezers depend on image processing to determine the z-position of the two beads: a paramagnetic bead to which the protein is tethered, and a non-magnetic fixed reference bead which is used to subtract out instrumental vibration and focal drift. Several approaches based on highly parallelized computing using the computer's graphic processing units have been developed. The instant approach uses solely the computer's central processing unit (CPU) to calculate in real-time the position of the beads. To achieve satisfactory results, the CPU was overclocked (i7 Intel CPU, overclocked to 4.6 GHz) and the image processing algorithms were optimized.

[0132] The main steps of the disclosed program are: acquisition of a frame by a camera and its transfer to the computer, obtaining the region of interest (ROI) around the beads of interest (of 128x128 px²), computing Fourier transforms (FFT) from the two ROIs, calculating the radial profile and the correlation with the stack library and fitting the correlation profile to determine the position of each bead (Figure 7A-E).

[0133] The two slowest steps in the instant approach are the computation of the FFT and its radial profile. To improve the calculation of the radial profile, a pixel-addressing algorithm (FKA algorithm) was utilized. Figure 7A is a simplified illustration of the pixel-addressing algorithm in a generated 128x128 pixel image. Its corresponding FFT is a matrix of 65x128 pixels (Figure 7B). The radial profile address function r_0 of the pixel coordinates is:

$$r_0 = p \cdot \left\| \sqrt{(W_x - x_c)^2 + (W_y - y_c)^2} \right\|, \quad \text{Eq. 6}$$

where p is a digital zoom factor ($p = 4$ in Figure 7B), W_x and W_y are matrices having the same number of elements as the FFT matrix, with their rows and columns respectively increasing in increments of one, and x_c and y_c are the expected centers of the FFT. Using the address matrix the value of each point in the radial profile can be computed by simply averaging the pixels within a given address number.

[0134] In some embodiments, to reduce the computation time during image processing, a specified pixel-addressing algorithm (FKA algorithm) was written to construct the radial profile from an FFT. Given that each pixel always contributes to a unique radial position, all the values in the radial vector can be calculated by averaging the intensities (I0) of the contributing pixels. There are exactly 8320 pixels in a 128 x 65 FFT (Figure 7B). Below, the radial vector (x4) contributions to positions 48, 94 and 140 (black pixels in Figure 7B and the corresponding circles in Figure 7C) were computed:

[0135] Profile[48]=(I0[3380]+I0[3381]+I0[3450]+I0[3583]+I0[3649]+I0[3846]+I0[4107]+I0[4172]+I0[4237]+I0[4496]+I0[4689]+I0[4753]+I0[4880]+I0[4940]+I0[4941])/15

[0136] Profile[94]=(I0[2670]+I0[2738]+I0[2939]+I0[3005]+I0[3203]+I0[3269]+I0[3662]+I0[3858]+I0[4508]+I0[4702]+I0[5089]+I0[5153]+I0[5345]+I0[5409]+I0[5598]+I0[5660])/16

[0137] Profile[140]=(I0[1885]+I0[1886]+I0[1887]+I0[1958]+I0[2027]+I0[2094]+I0[2161]+I0[2228]+I0[2361]+I0[2823]+I0[3020]+I0[3151]+I0[3282]+I0[3413]+I0[3674]+I0[4065]+I0[4130]+I0[4195]+I0[4260]+I0[4325]+I0[4714]+I0[4973]+I0[5102]+I0[5231]+I0[5360]+I0[5553]+I0[6001]+I0[6128]+I0[6191]+I0[6254]+I0[6317]+I0[6378]+I0[6435]+I0[6436]+I0[6437])/35

[0138] The change in the end-to-end length of the tethered protein was determined from the diffraction pattern around the bead, which is highly sensitive to the position of the bead in the Z-plane. Regions of interest (ROI) of 128x128 px² were selected around the addressed beads and their FFT was computed (Figure 7B). The FFT centers the bead in the frequency space and positions the pixels in place, independent of the thermal fluctuations of the beads.

Furthermore, working in the Fourier space reduces correlation errors coming from camera noise and from changes in illumination during the experiment. Preceding the FFT, a Kaiser window was applied to reduce artifacts from the boundaries of the image. The sub-pixel alignment of the beads in the Fourier space between different frames allowed for employment of the pixel-addressing algorithm (FKA algorithm) to compute the radial profile of the FFT image. For the instant experiments, the radial profile was obtained using a digital zoom factor of $p = 4$, which further increases the resolution. For beads with diameter of $\sim 3 \mu\text{m}$ measured at magnifications of 100x it was found that the range between 40 to 140 points in the profile gives the best results (corresponding to a disc between pixels 10 and 35 around the center of the bead's FFT). This range ignores the low frequencies, which are dominated by the bead

and are insensitive to focal changes, and the high frequencies, which are altered by the camera sampling and acquisition noise.

[0139] To determine the position of the paramagnetic and reference beads along the field of view, their radial profile was correlated against a previously obtained stack library (Figure 7D). Prior to the measurement, a paramagnetic and reference bead were chosen. For each paramagnetic and reference bead, a stack library at a magnet position was saved where the force was high enough to limit the changes in height due to the thermal motion of the tethered bead, but low enough to prevent the unfolding of protein domains (generally, the magnets were positioned at a separation of 4 mm for this step). To record the stack library, the piezo was moved in equal steps of 20 nm, and in each step the radial profile of the beads of interest was saved (Figure 7D). During the measurement, the Pearson correlation was computed between the radial profile of the bead obtained during live stream and the pre-recorded stack library (Figure 7E). Around the maximum, the correlation had a “bell-curve” shape. By simply fitting a Gaussian function the position of the beads can be found with nanometer resolution.

[0140] Computer Systems

[0141] The methods and computations described herein can be performed using a variety of hardware and/or software based system. When used in connection with the methods described herein, such hardware and/or software based systems can comprise a engine. In certain embodiments, an engine can be a system that employs parallel computation. Parallel computation can use a variable number of interconnected computation nodes. Parallel computation can also use a general-purpose computer for each node where each node is interconnected using one or more data links or networks.

[0142] The methods of the present disclosure can be implemented in digital electronic circuitry, or in computer hardware, firmware, software, or in combinations thereof. Computer assistance allows manipulations of measurement data and permit automation. Furthermore, computer assistance makes possible the simultaneous comparison of multiple measurements. The instructions and systems can be implemented in a computer program product tangibly embodied in a machine-readable storage device for execution by a programmable processor; and method actions can be performed by a programmable processor executing the instructions to perform molecular measurements by operating on input data and generating output.

[0143] The steps of the modeling methods can include both steps implemented by commercially available software packages, and steps implemented by instructions provided by a scripting language (e.g., Perl, Python), or a compiled language (e.g., C, Fortran). Also, the steps can be integrated using instructions provided with a computer language, such as those mentioned above.

[0144] The methods and systems of the present disclosure can be implemented advantageously in one or more computer programs that are executable on a programmable system including at least one programmable processor coupled to receive data and instructions from, and to transmit data and instructions to, a data storage system, at least one input device, and at least one output device. Suitable processors include, by way of example, both general and special purpose microprocessors. Generally, a processor will receive instructions and data from a read-only memory and/or a random access memory. Generally, a computer can include one or more mass storage devices for storing data files; such devices include magnetic disks, such as internal hard disks and removable disks; magneto-optical disks; and optical disks. Storage devices suitable for tangibly embodying computer program instructions and data include all forms of non-volatile memory, including, by way of example, semiconductor memory devices, such as EPROM, EEPROM, and flash memory devices; magnetic disks such as, internal hard disks and removable disks; magneto-optical disks; and CD-ROM disks. Any of the foregoing can be supplemented by, or incorporated in, ASICs (application-specific integrated circuits). For better performance, computers can have overclocked CPUs or can use GPU as processing unit(s).

[0145] By way of example a computer system suitable for use with the methods described herein can comprise a programmable processing system suitable for implementing or performing the apparatus or methods of the present disclosure. The system can include a processor, a random access memory (RAM), a program memory (for example, a writable read-only memory (ROM) such as a flash ROM), a hard drive controller, and an input/output (I/O) controller coupled by a processor (CPU) bus. The system can be preprogrammed, in ROM, for example, or it can be programmed (and reprogrammed) by loading a program from another source (for example, from a floppy disk, a CD-ROM, or another computer).

[0146] The hard drive controller can be coupled to a hard disk suitable for storing executable computer programs, including programs embodying the present disclosure, and data including

storage. The I/O controller can be coupled by means of an I/O bus to an I/O interface, that can include one or more of the following: a monitor, a mouse, a keyboard or other input device. The I/O interface receives and transmits data in analog or digital form over communication links such as a serial link, local area network, wireless link, and parallel link.

[0147] The following examples illustrate the present disclosure, and are set forth to aid in the understanding of the present disclosure, and should not be construed to limit in any way the scope of the present disclosure as defined in the claims which follow thereafter.

[0148] It is to be understood that the disclosed subject matter is not limited in its application to the details of construction and to the arrangements of the components set forth in the following description or illustrated in the drawings. The disclosed subject matter is capable of other embodiments and of being practiced and carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein are for the purpose of description and should not be regarded as limiting.

[0149] As such, those skilled in the art will appreciate that the conception, upon which this disclosure is based, may readily be utilized as a basis for the designing of other structures, methods, and systems for carrying out the several purposes of the disclosed subject matter. It is important, therefore, that the claims be regarded as including such equivalent constructions insofar as they do not depart from the spirit and scope of the disclosed subject matter.

[0150] Although the disclosed subject matter has been described and illustrated in the foregoing exemplary embodiments, it is understood that the present disclosure has been made only by way of example, and that numerous changes in the details of implementation of the disclosed subject matter may be made without departing from the spirit and scope of the disclosed subject matter, which is limited only by the claims which follow.

What is claimed is:

1. A system for applying a force to a molecule, comprising:
 - a magnetic component;
 - a voice coil attached to the magnetic component;
 - a substrate positioned opposite from the magnetic component for attachment to a first end of the molecule ;
 - a camera for obtaining images; and
 - a positioning device for adjusting a focus of the camera,wherein the voice coil is configured to apply a predetermined amount of force to the molecule by adjusting a position of the magnetic component relative to a second end of the molecule.
2. The system of claim 1, comprising the molecule, wherein the first end of the molecule is attached to the substrate and the second end of the molecule is attached to a paramagnetic bead, the paramagnetic bead being positioned between the magnetic component and the substrate.
3. The system of claim 2, comprising a reference bead disposed on the substrate at an initial position proximate the first end of the molecule.
4. The system of claim 3, wherein the reference bead is a non-magnetic bead.
5. The system of claim 3, wherein the positioning device continuously adjusts the focus of the camera to maintain the reference bead at the initial position.
6. The system of any one of claims 3-5, wherein the camera obtains a first set of images of the paramagnetic bead and the reference bead prior to application of force at different focus positions and a live image of the paramagnetic bead and the reference bead during application of force.
7. The system of claim 6, wherein the system determines the force by correlating a radial profile of a live image during application of force with radial profiles of the first set of images.
8. The system of any one of claims 3-5, comprising a position adjustment stage for controlling a position of the paramagnetic bead.
9. The system of any one of claims 1-5, comprising a microscope.

10. The system of claim 9, comprising a total internal reflection module for the microscope.
11. The system of any one of claims 1-5, comprising a fluorescence camera.
12. The system of any one of claims 1-5, comprising a laser/diode.
13. The system of any one of claims 1-5, wherein the substrate is or comprises glass.
14. The system of any one of claims 2-5, wherein the molecule is selected from the list consisting of protein, DNA, and RNA.
15. The system of any one of claims 2-5, wherein the first end of the molecule is attached to the substrate via HaloTag or AviTag method.
16. The system of any one of claim 1-5, wherein the predetermined amount of force is in picoNewton range.
17. The system of any one of claims 1-5, wherein the voice coil applies the predetermined amount of force for periods of time of at least one hour without visible drift.
18. The system of any one of claims 1-5, wherein the voice coil applies the predetermined amount of force for periods of time of at least one day without visible drift.
19. A method for controlling an amount of force applied to a molecule, comprising:
 - obtaining, with a camera a first set of images, the camera being positioned proximate a substrate for attachment to a first end of the molecule;
 - applying a force to the molecule by adjusting, with a voice coil attached to a magnetic component, a position of the magnetic component relative to a second end of the molecule causing the second end of the molecule to translate from a first position to a second position relative to a central axis of the molecule, wherein the magnetic component is disposed opposite the substrate;
 - obtaining, with the camera, a live image of the second end of the molecule;
 - correlating the live image with the first set of images to determine the second position; and
 - measuring the force applied to the molecule as a function of the position of the second end of the molecule.
20. The method of claim 19, comprising attaching the first end of the molecule to the substrate; and attaching the second end of the molecule to a paramagnetic bead.

21. The method of claim 20, comprising attaching a reference bead to the substrate at an initial location proximate the first end of the molecule
22. The method of claim 21, wherein the first set of images comprises images of the paramagnetic bead and the reference bead.
23. The method of claim 21, wherein the first set of images is obtained at various focal distances prior to application of the force.
24. The method of any one of claims 22-23, wherein the live image is an image of the paramagnetic bead and the reference bead.
25. The method of claim 24, wherein the live image is obtained during application of the force.
26. The method of any one of claims 19-23, comprising, continuously adjusting, by a positioning device, a focus of the camera to maintain the reference bead at the initial position to correct drift.

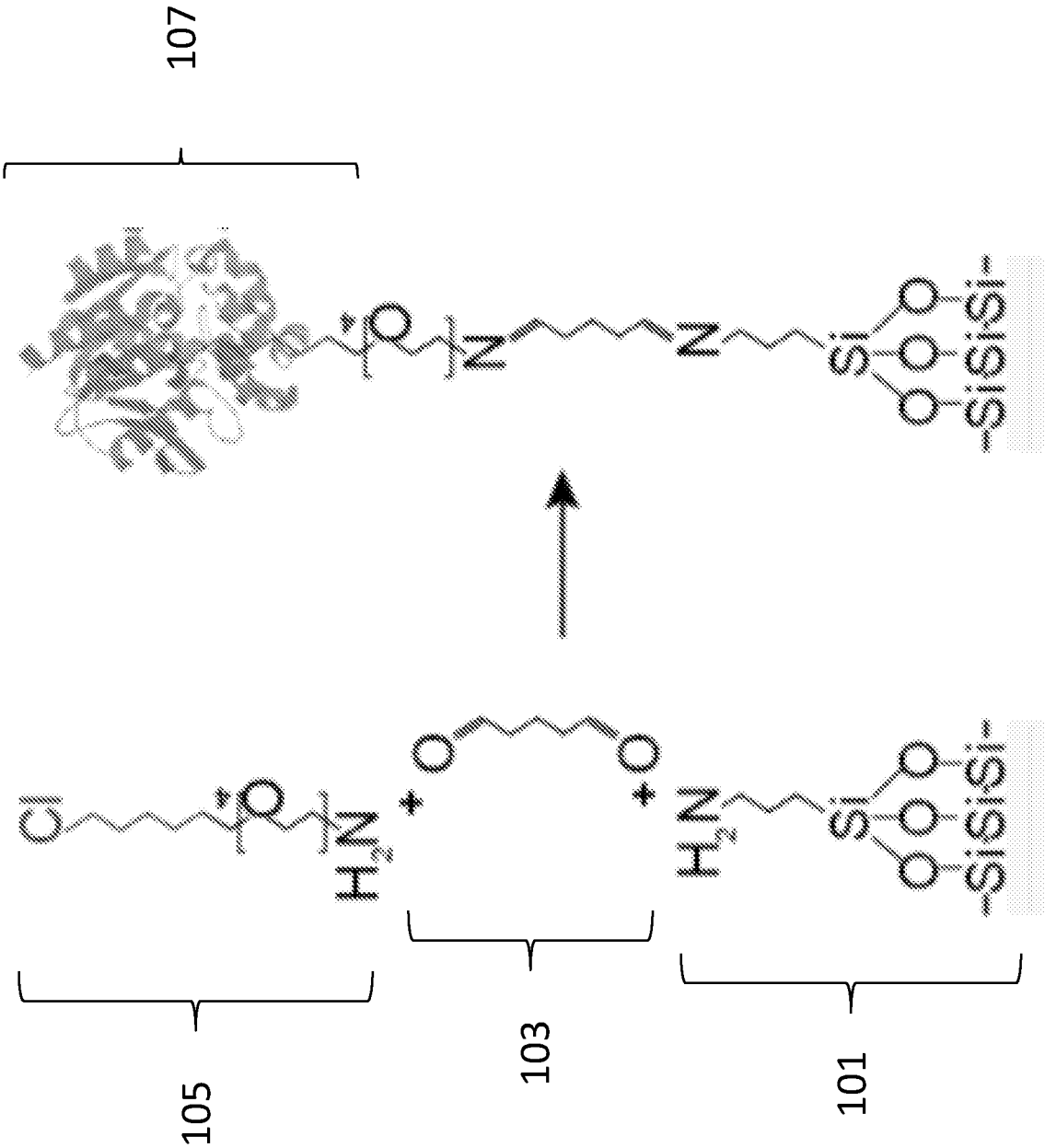


Fig. 1A

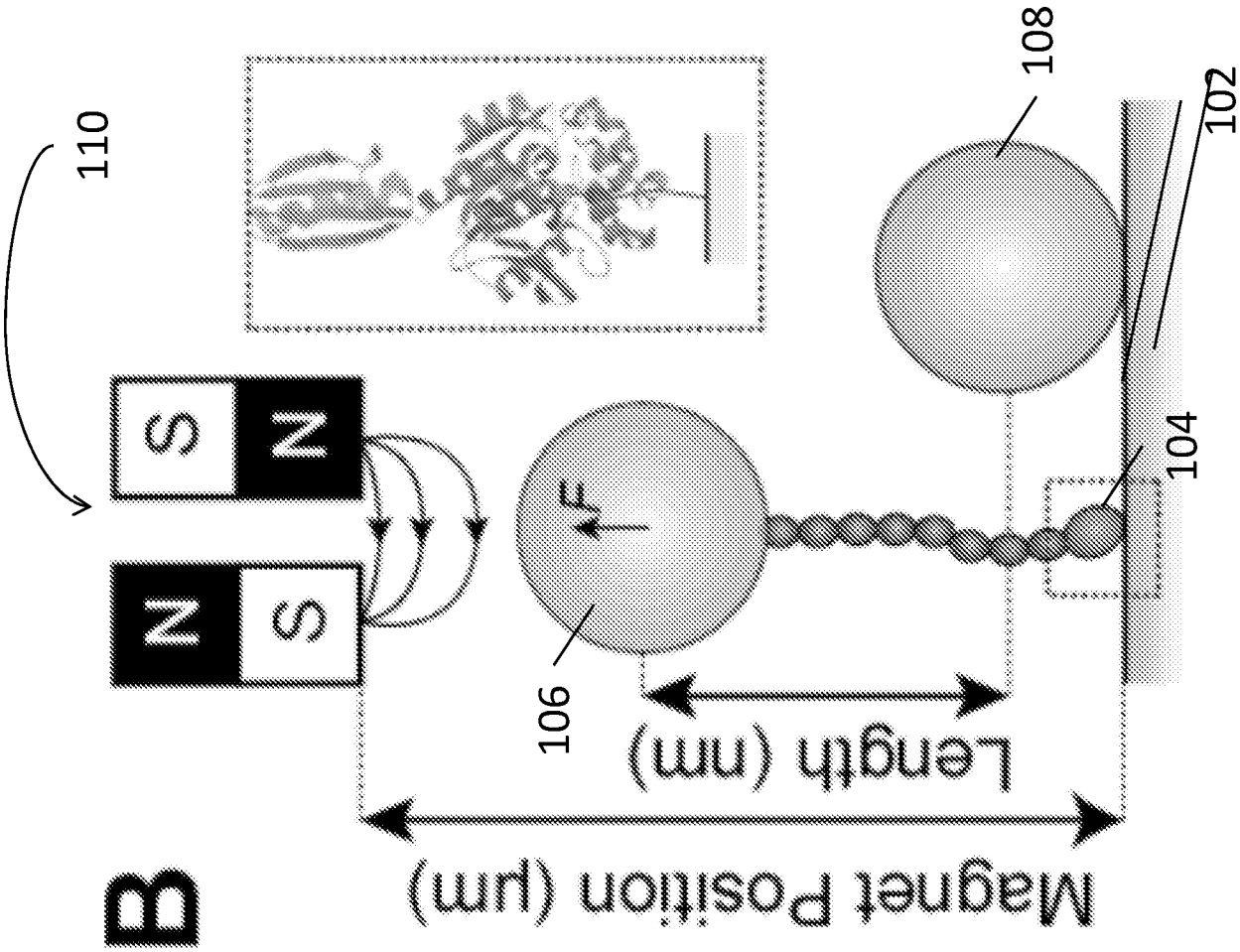


Fig. 1B

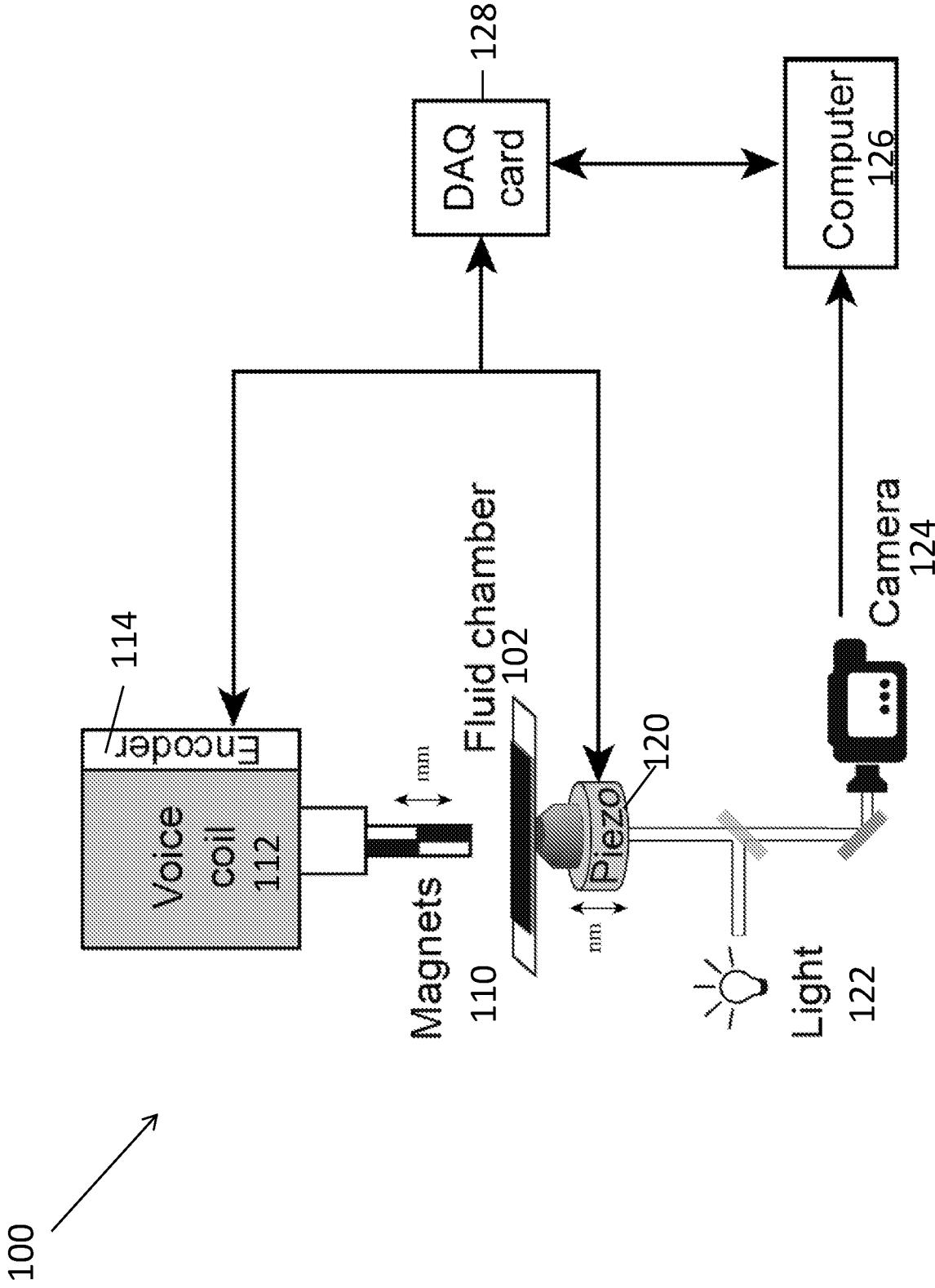


Fig. 1C

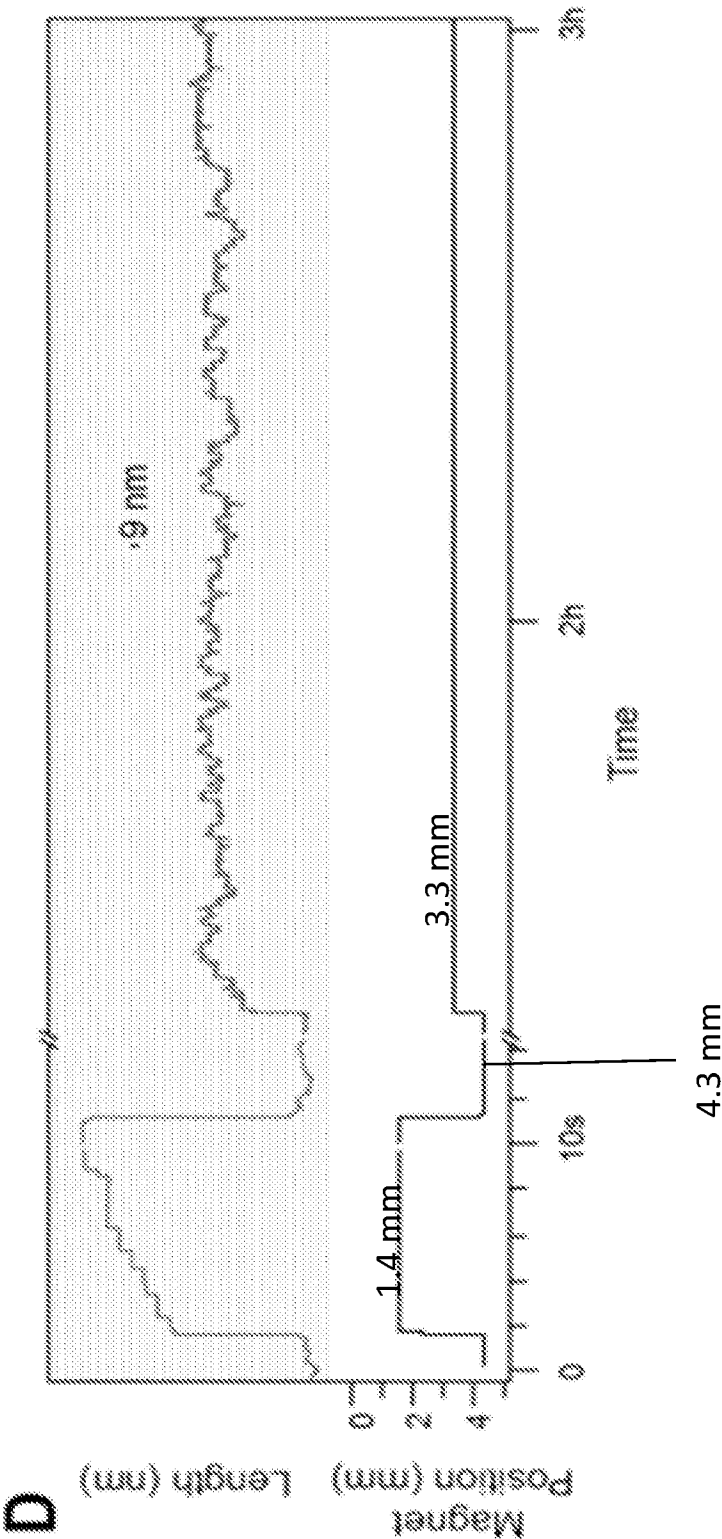


Fig. 1D

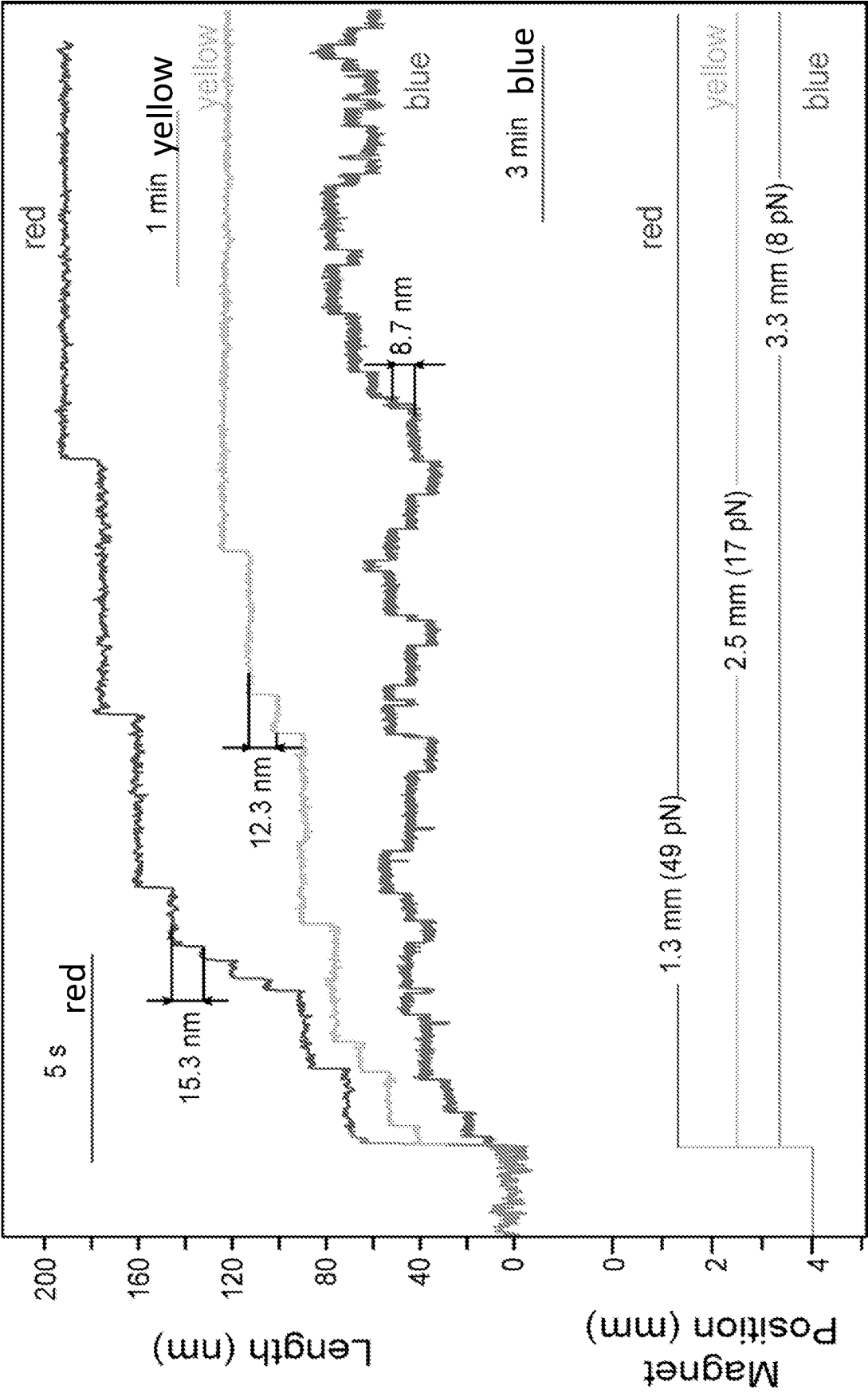


Fig. 2A

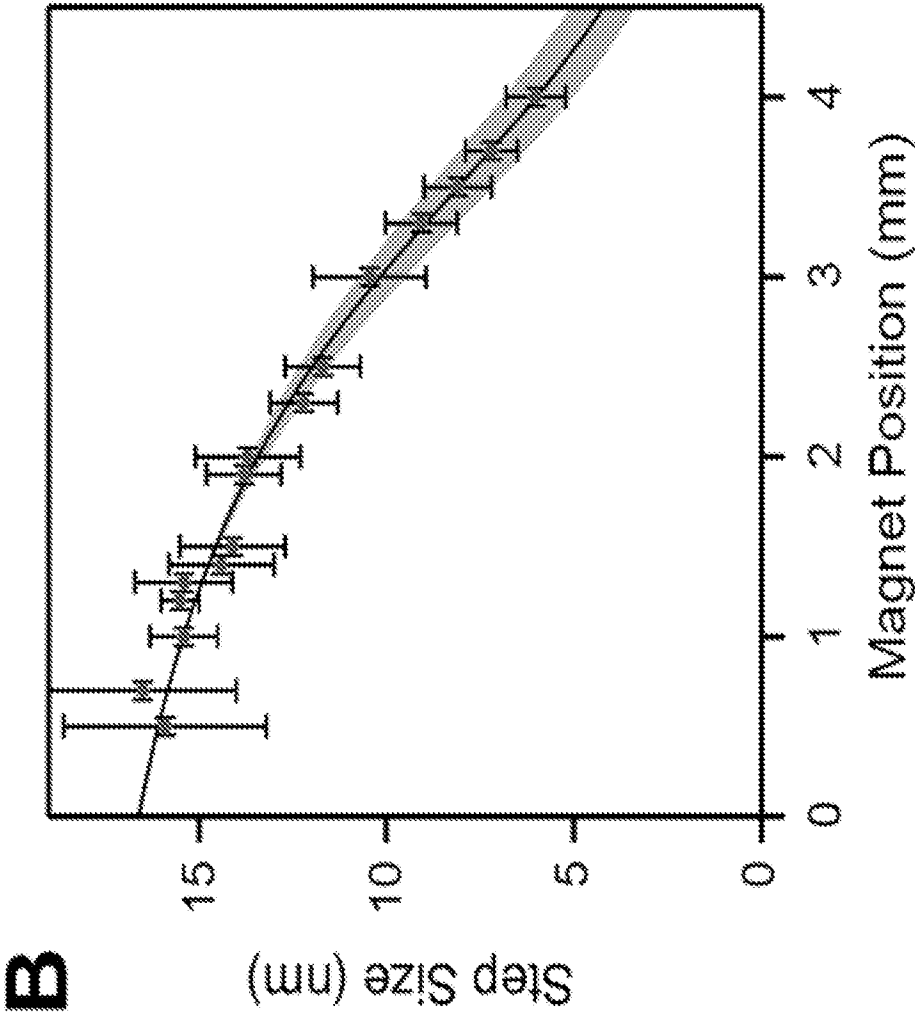


Fig. 2B

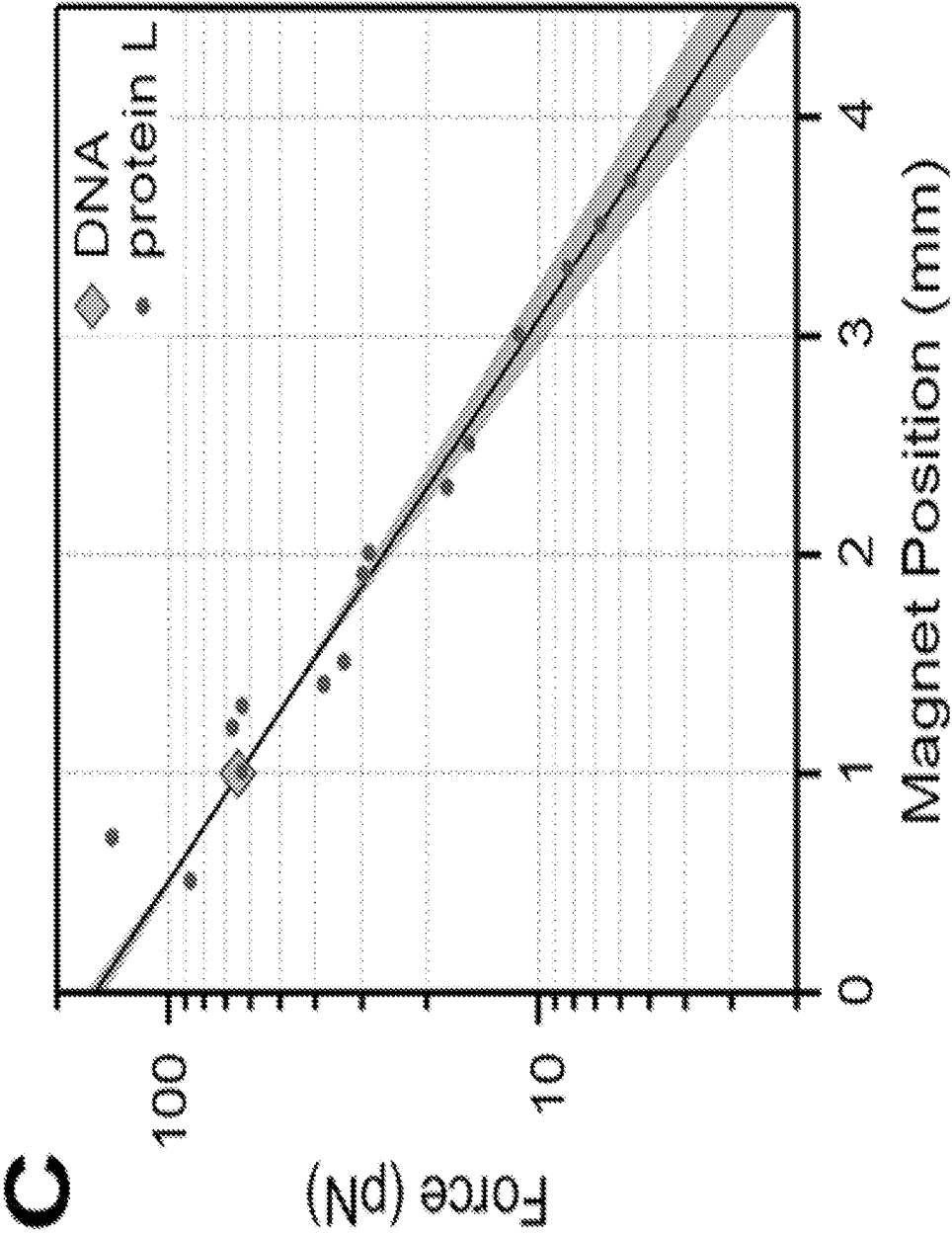


Fig. 2C

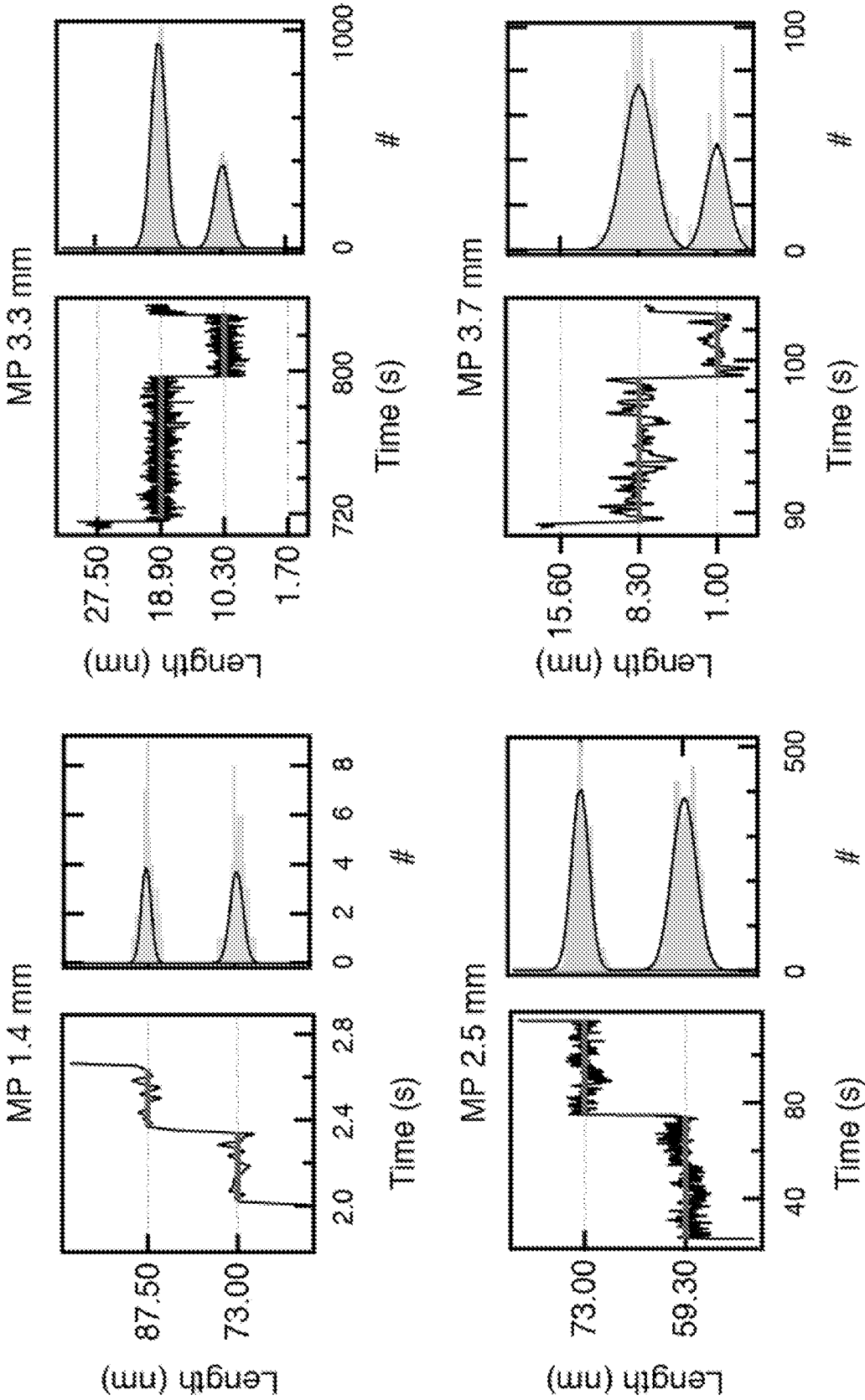


Fig. 2D

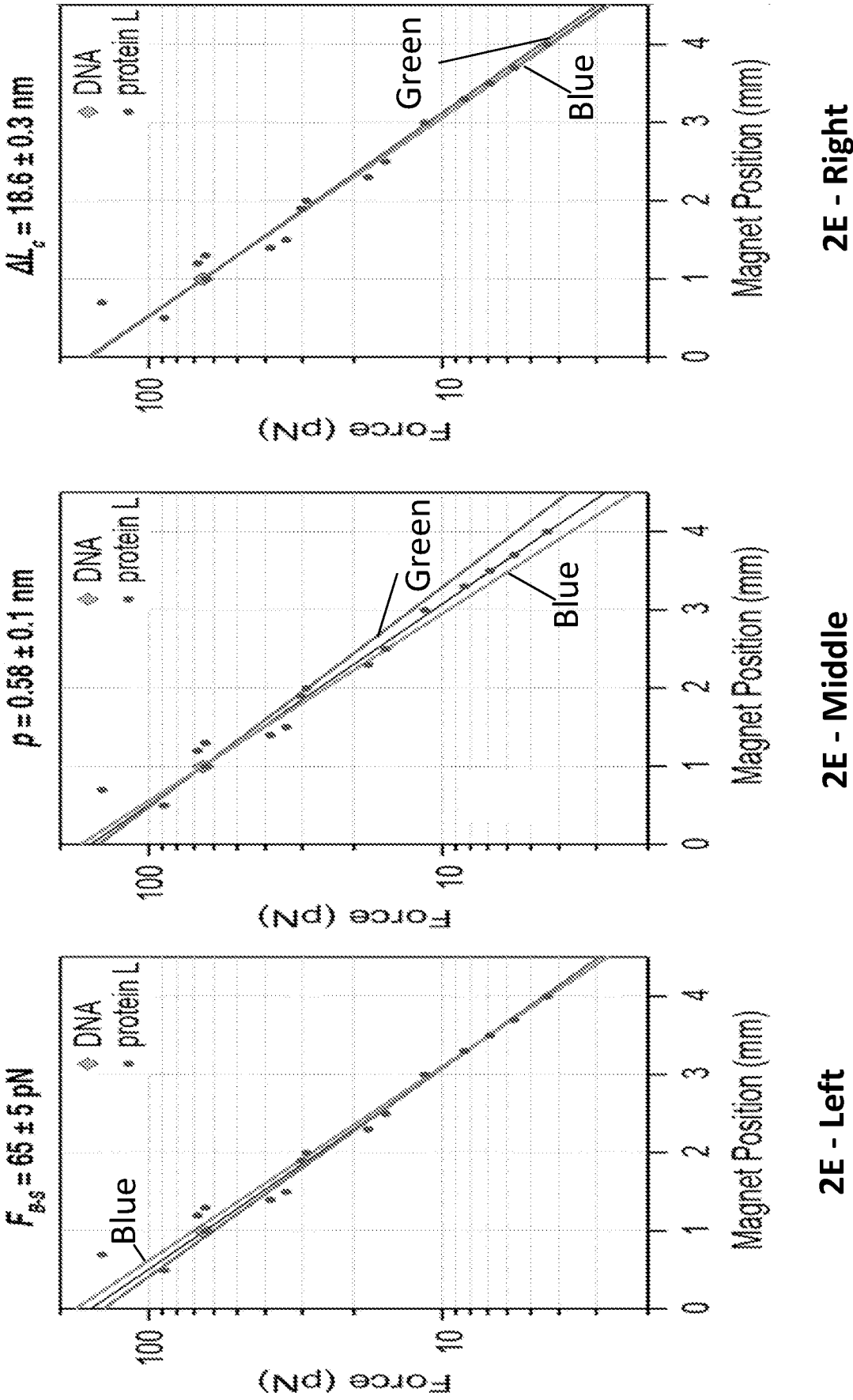
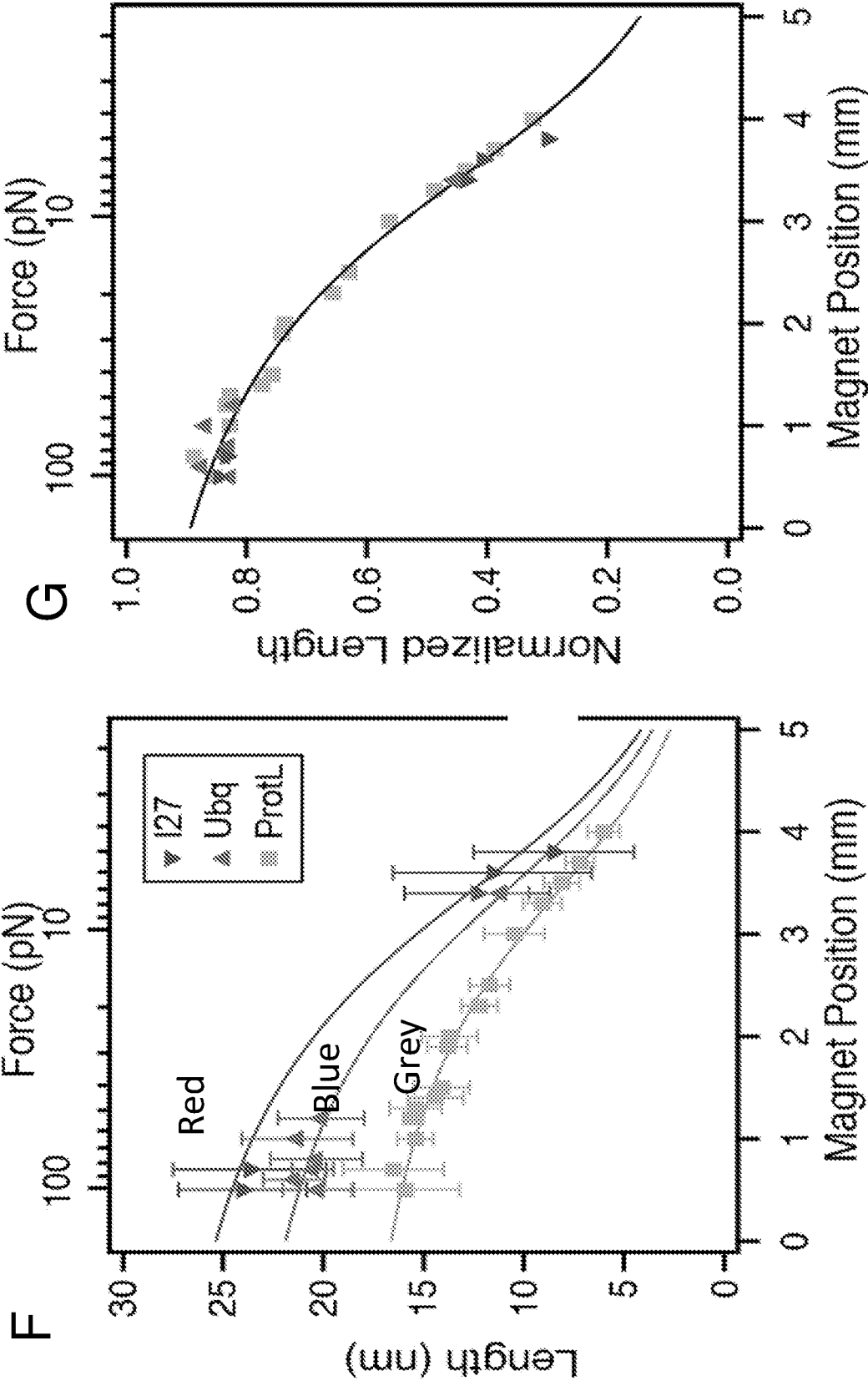


Fig. 2E



Figs. 2F-2G

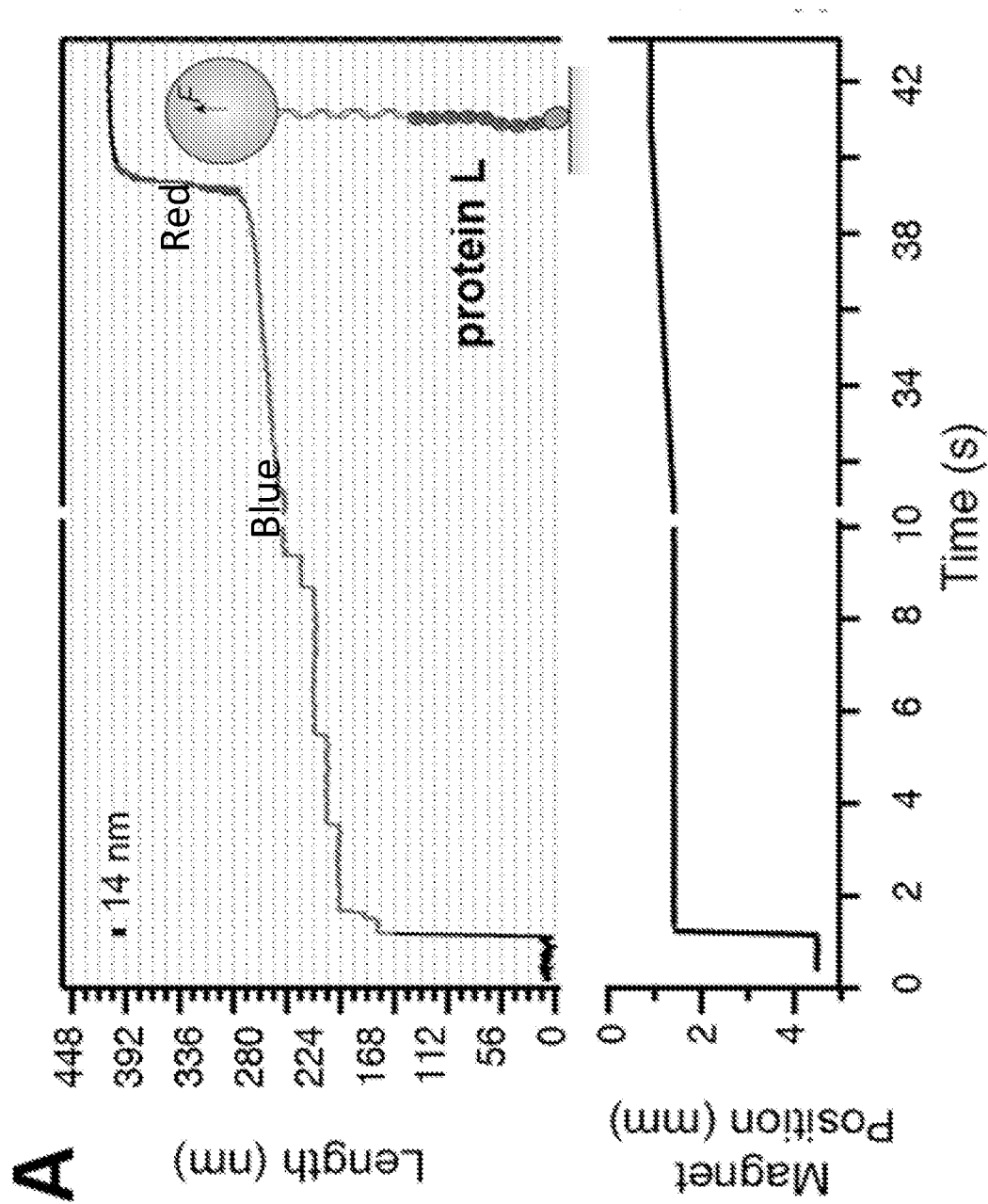


Fig. 3A

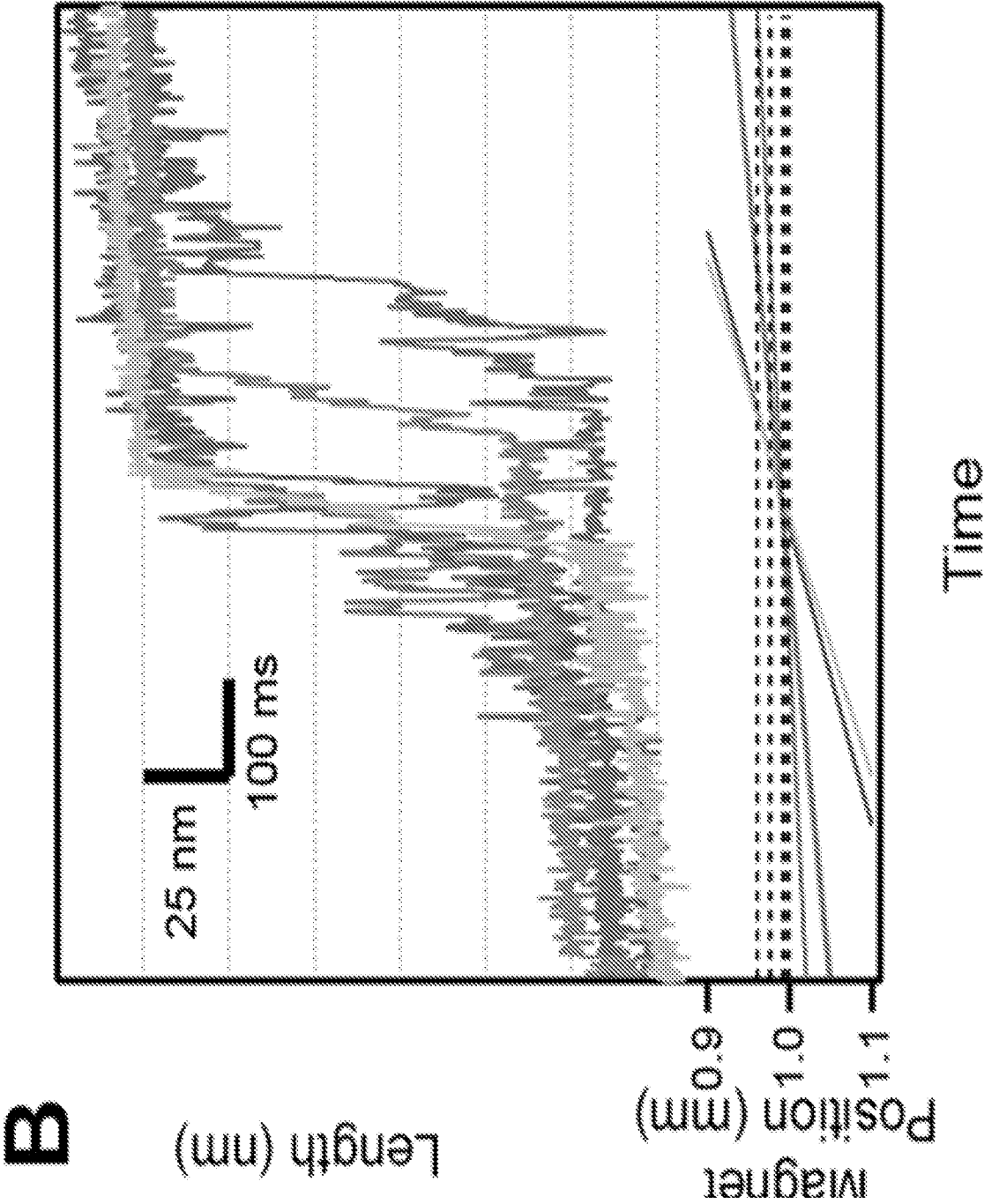


Fig. 3B

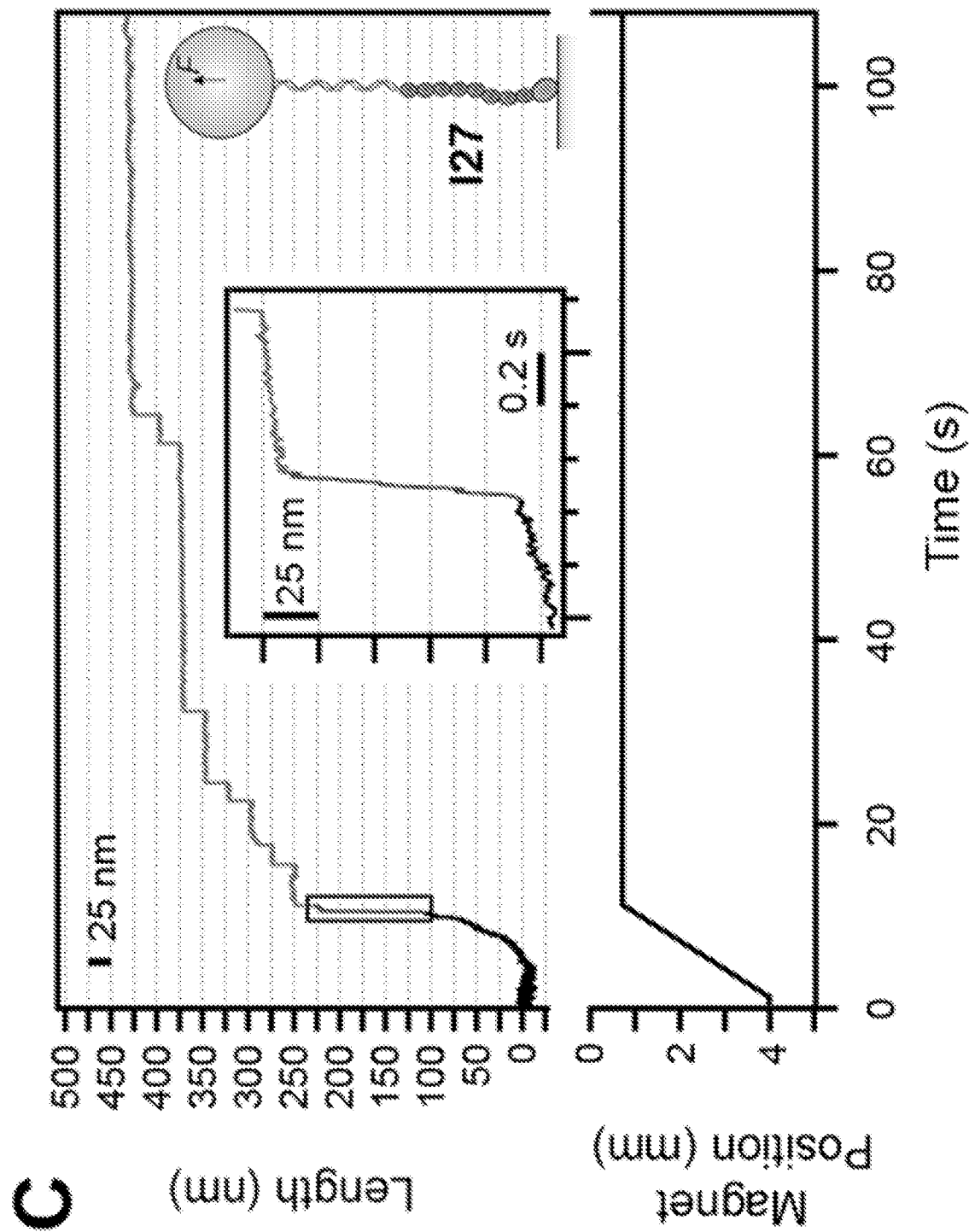


Fig. 3C

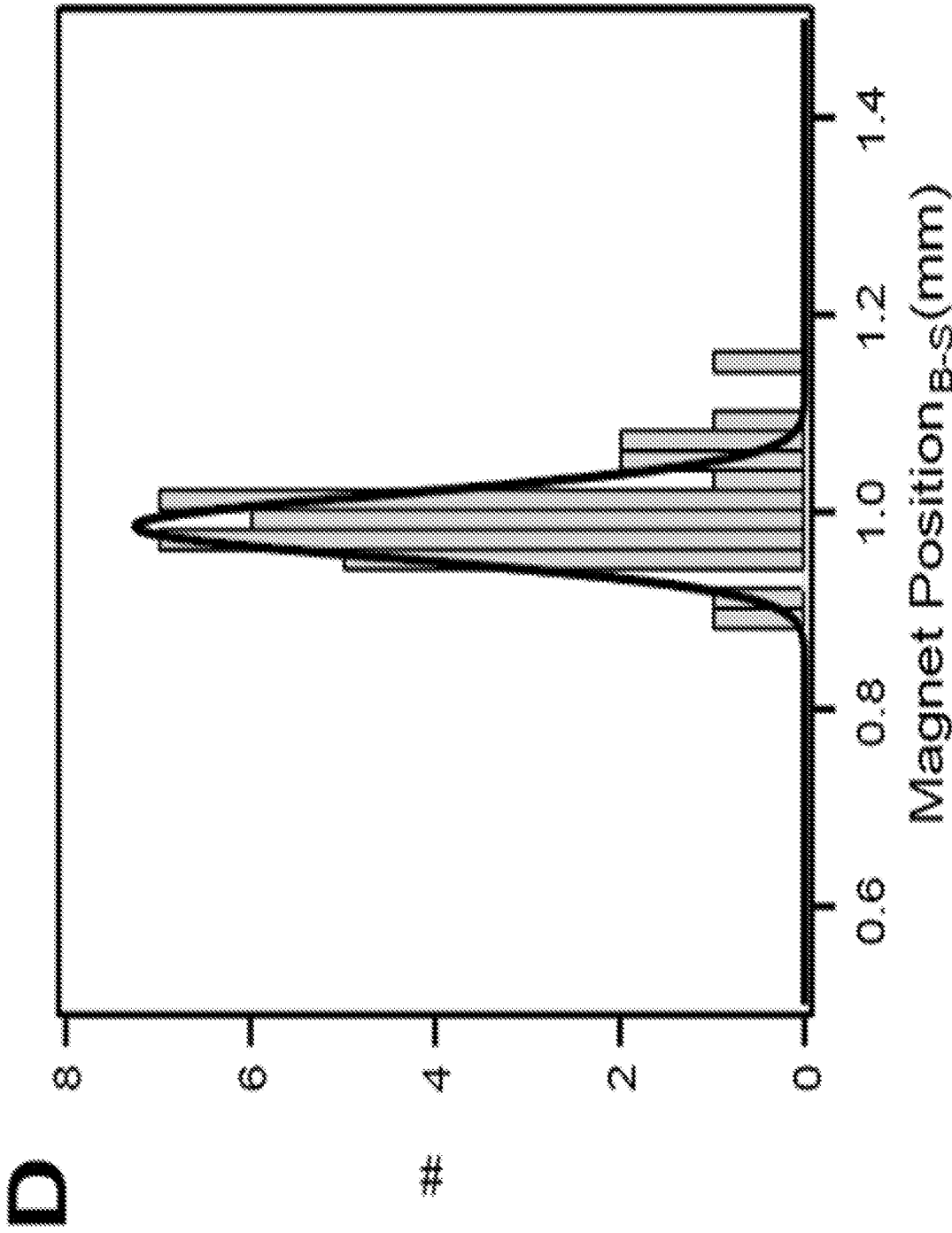
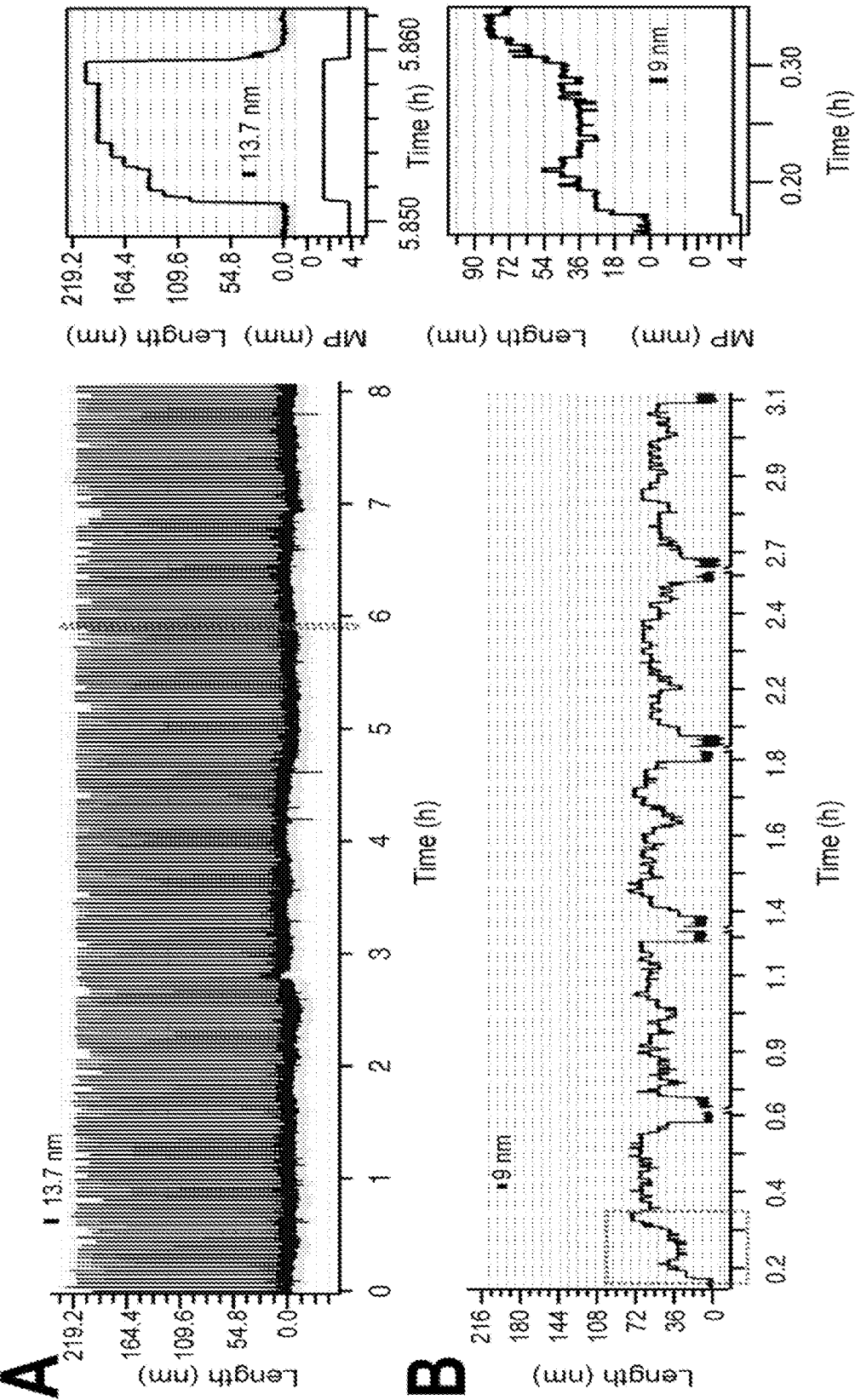
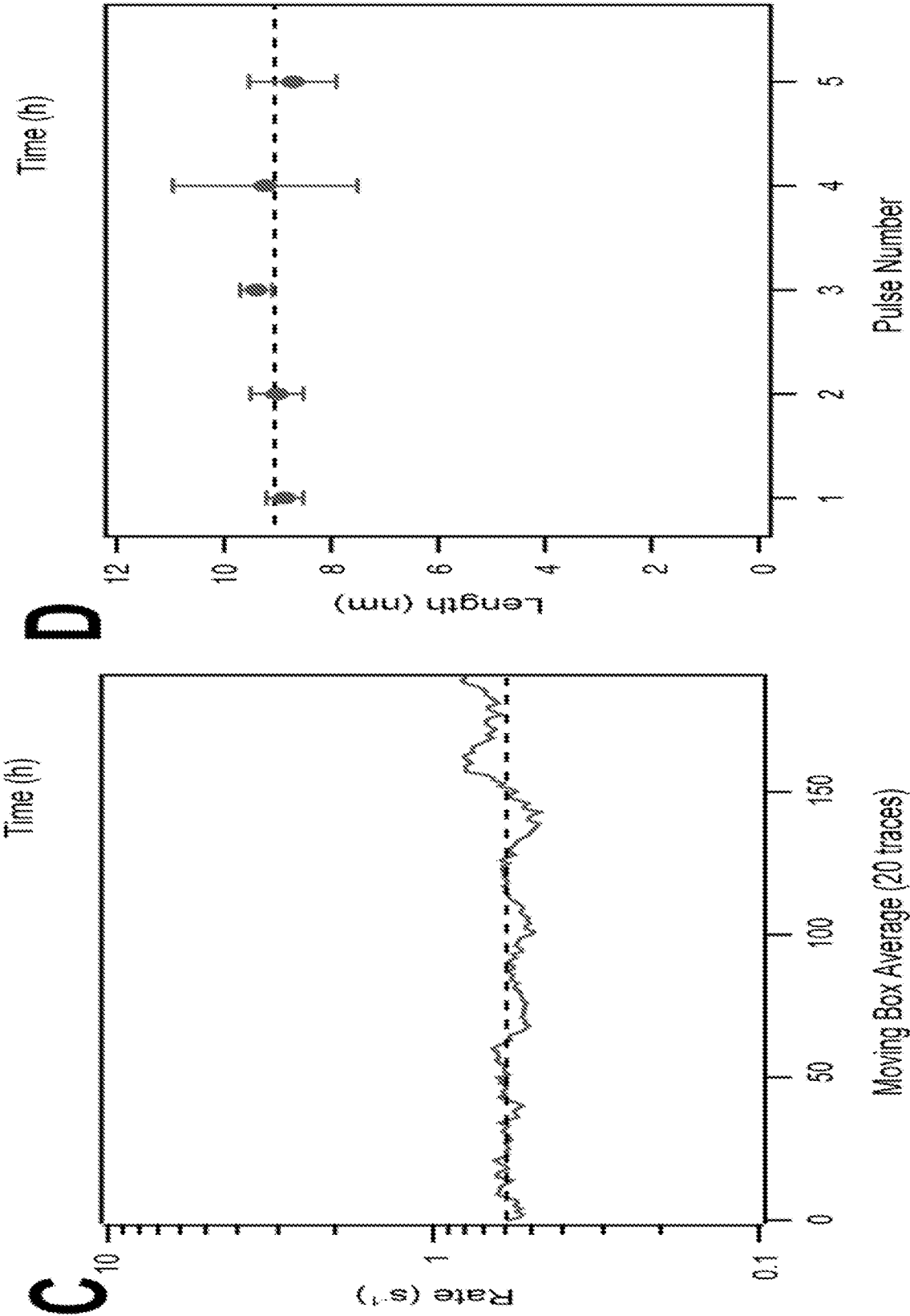


Fig. 3D



Figs. 4A-4B



Figs. 4C-4D

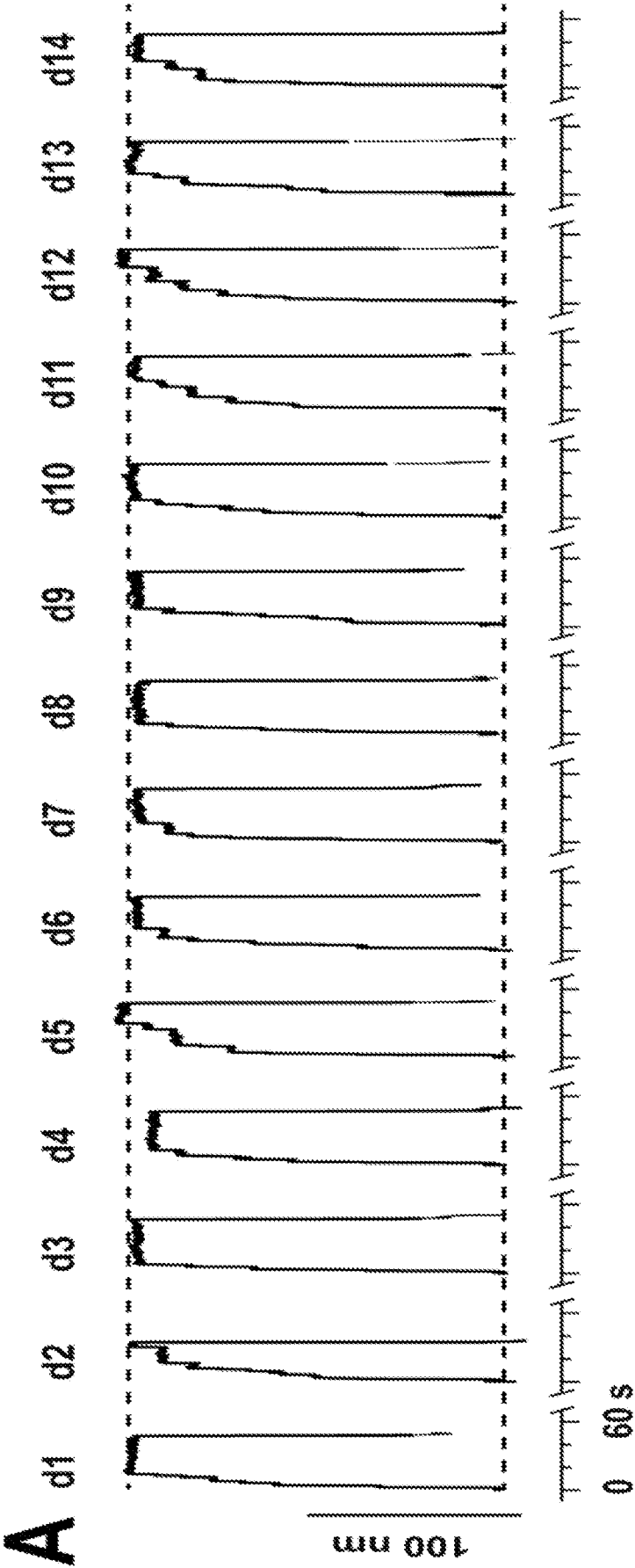


Fig. 5A

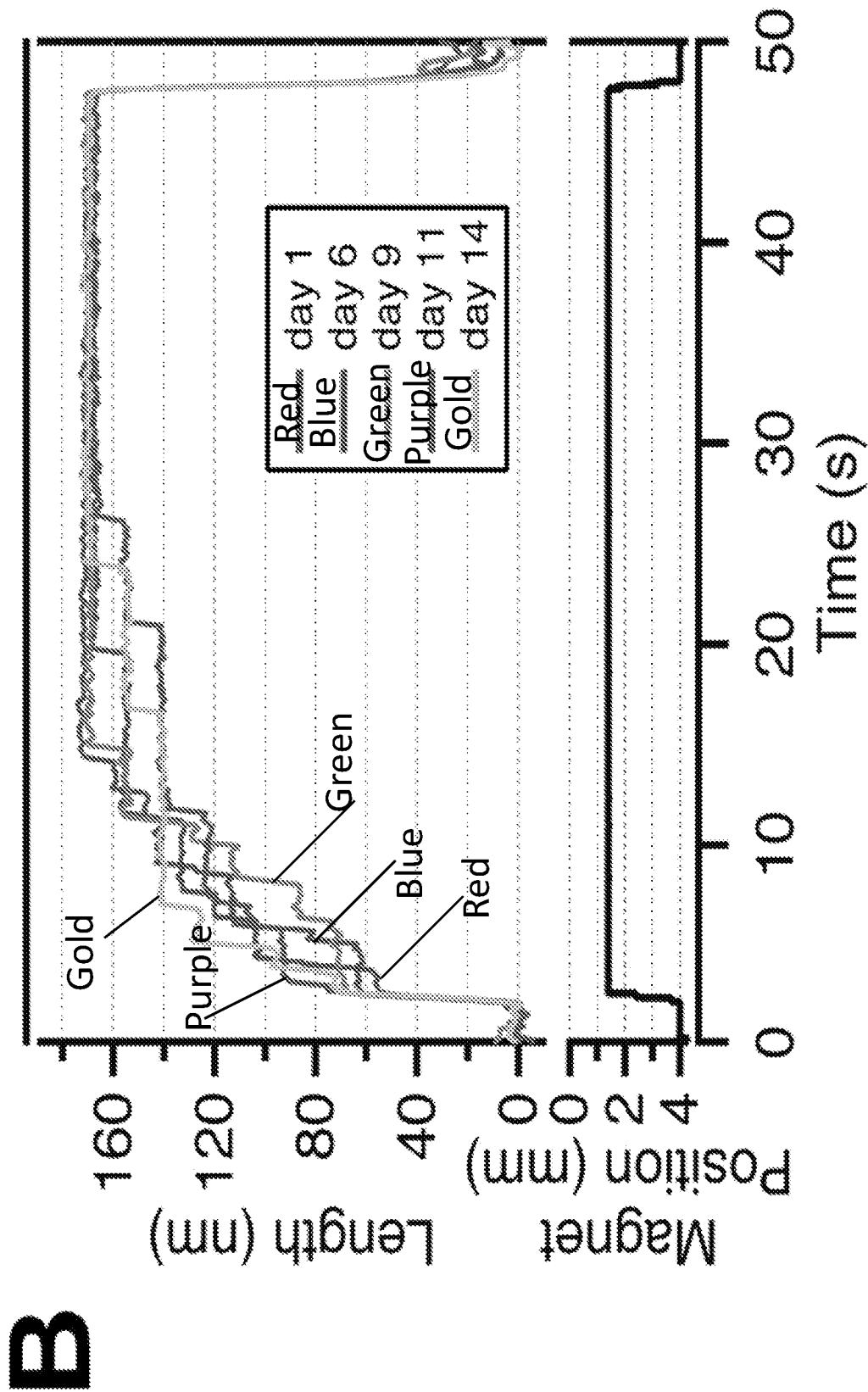


Fig. 5B

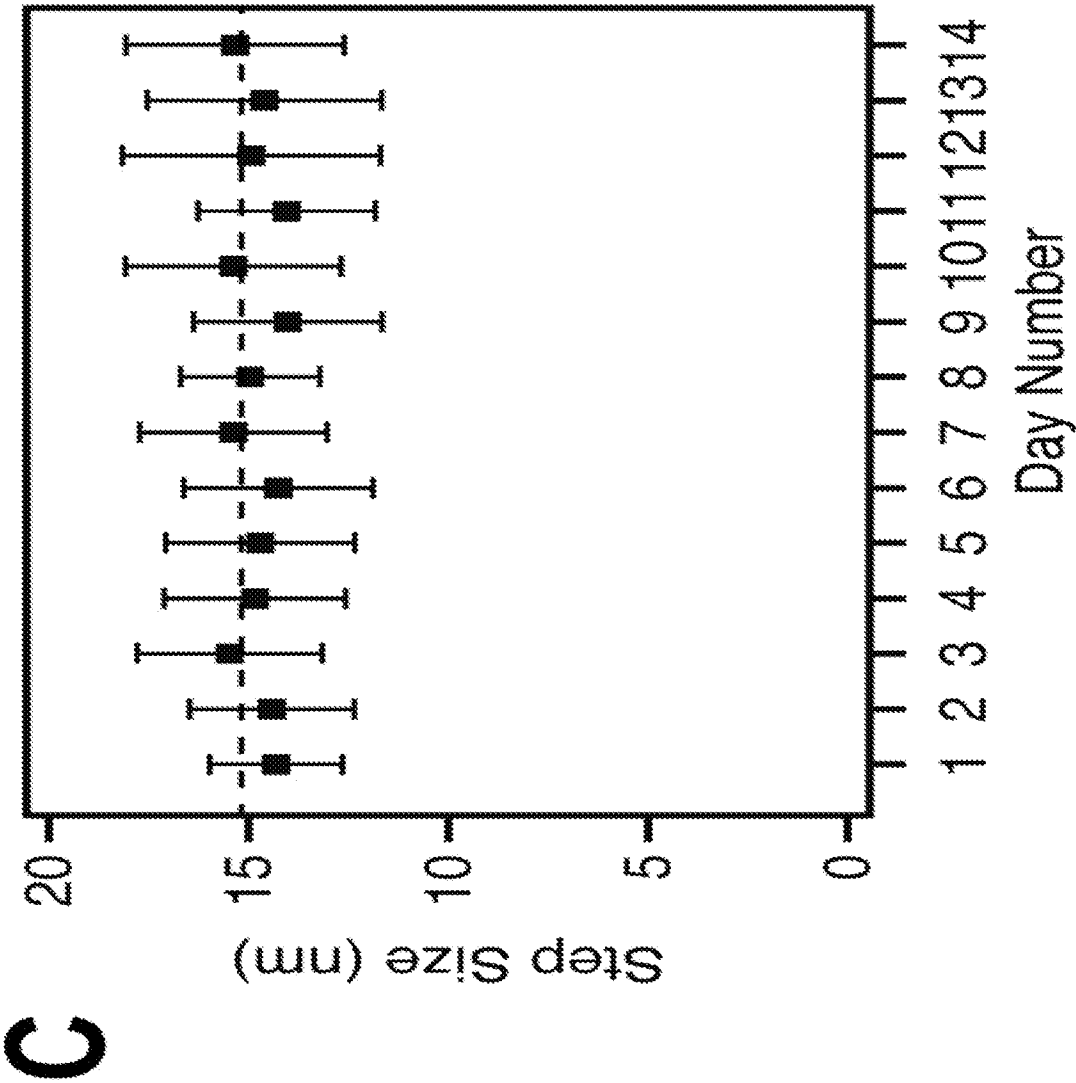
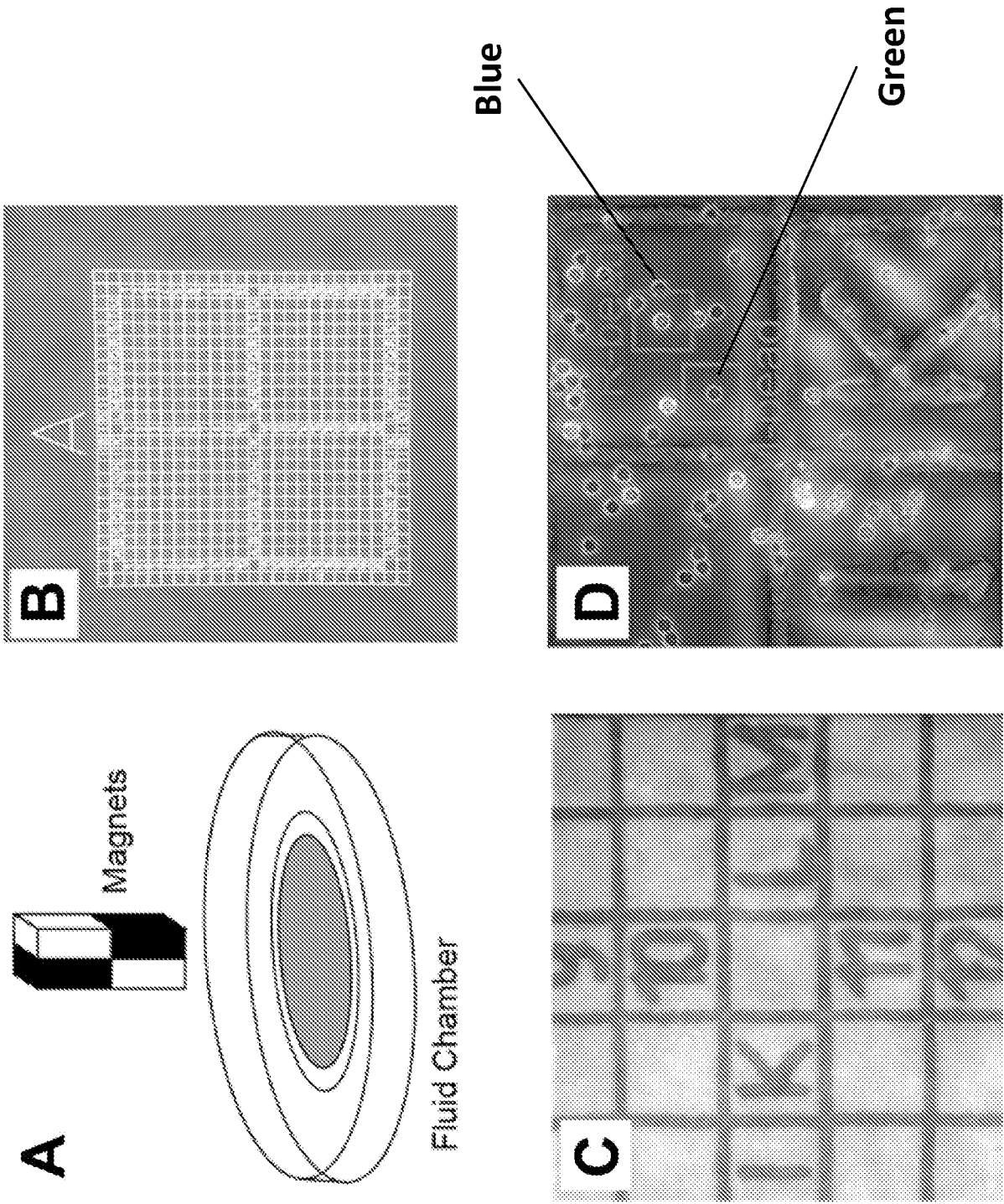


Fig. 5C



Figs. 6A-6D

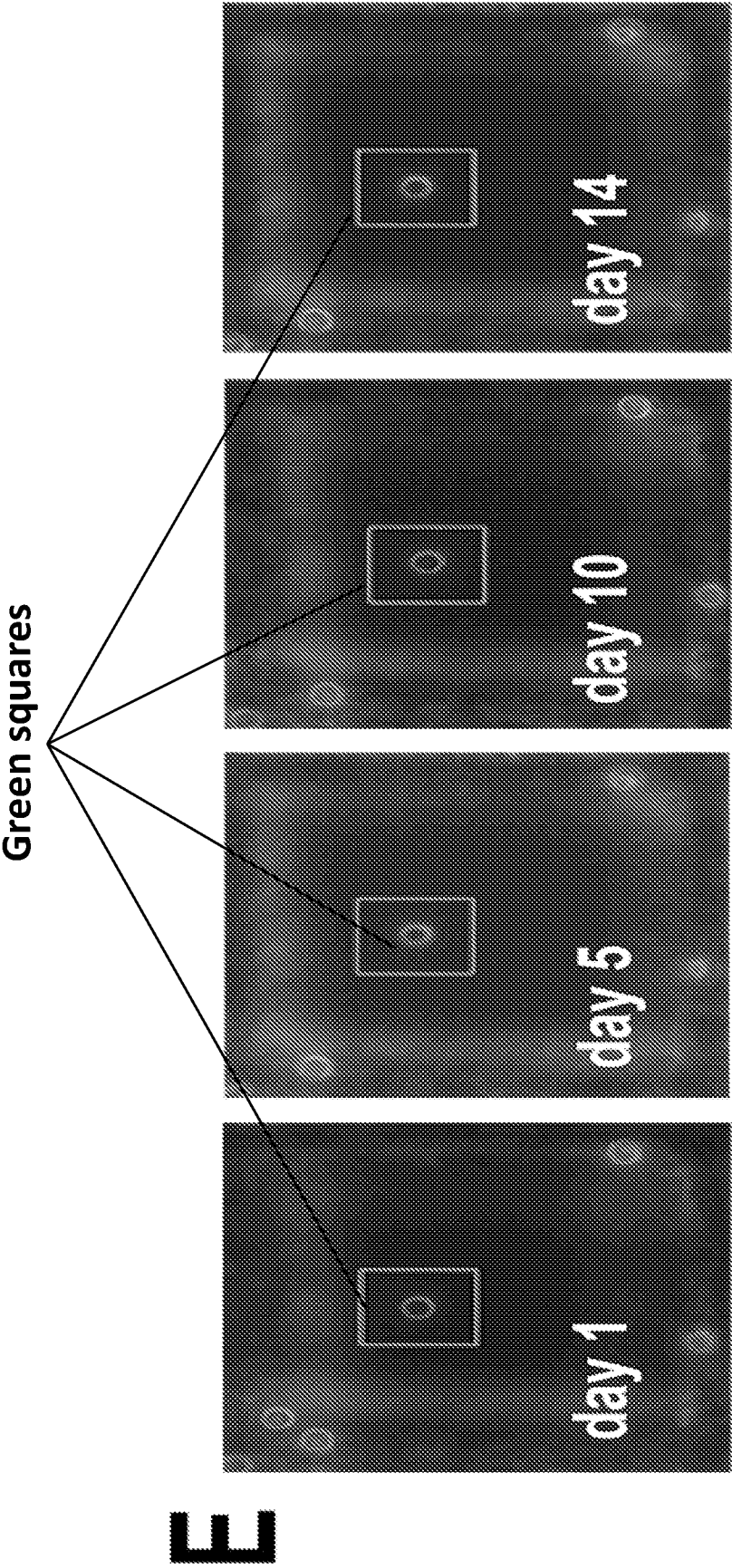
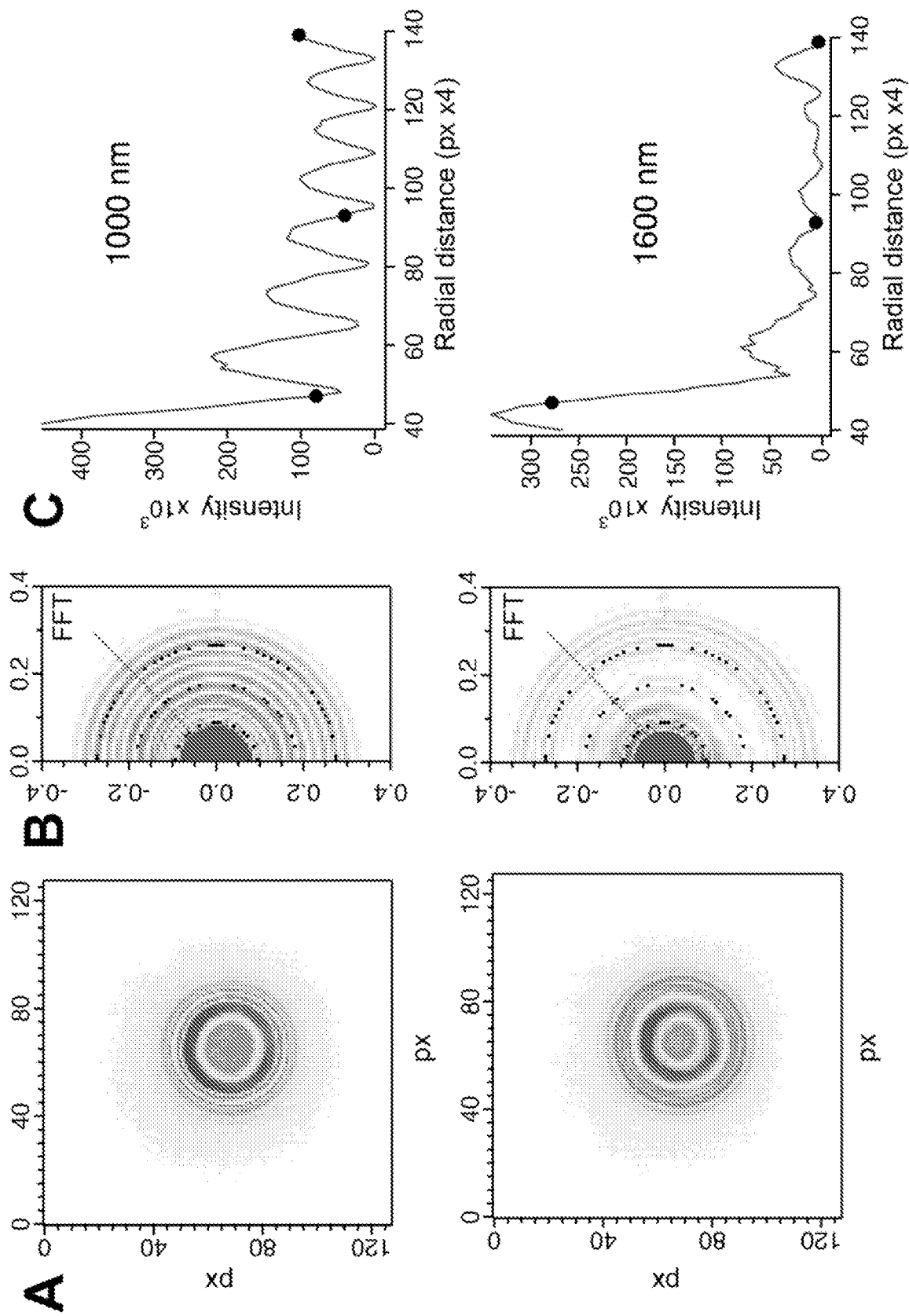
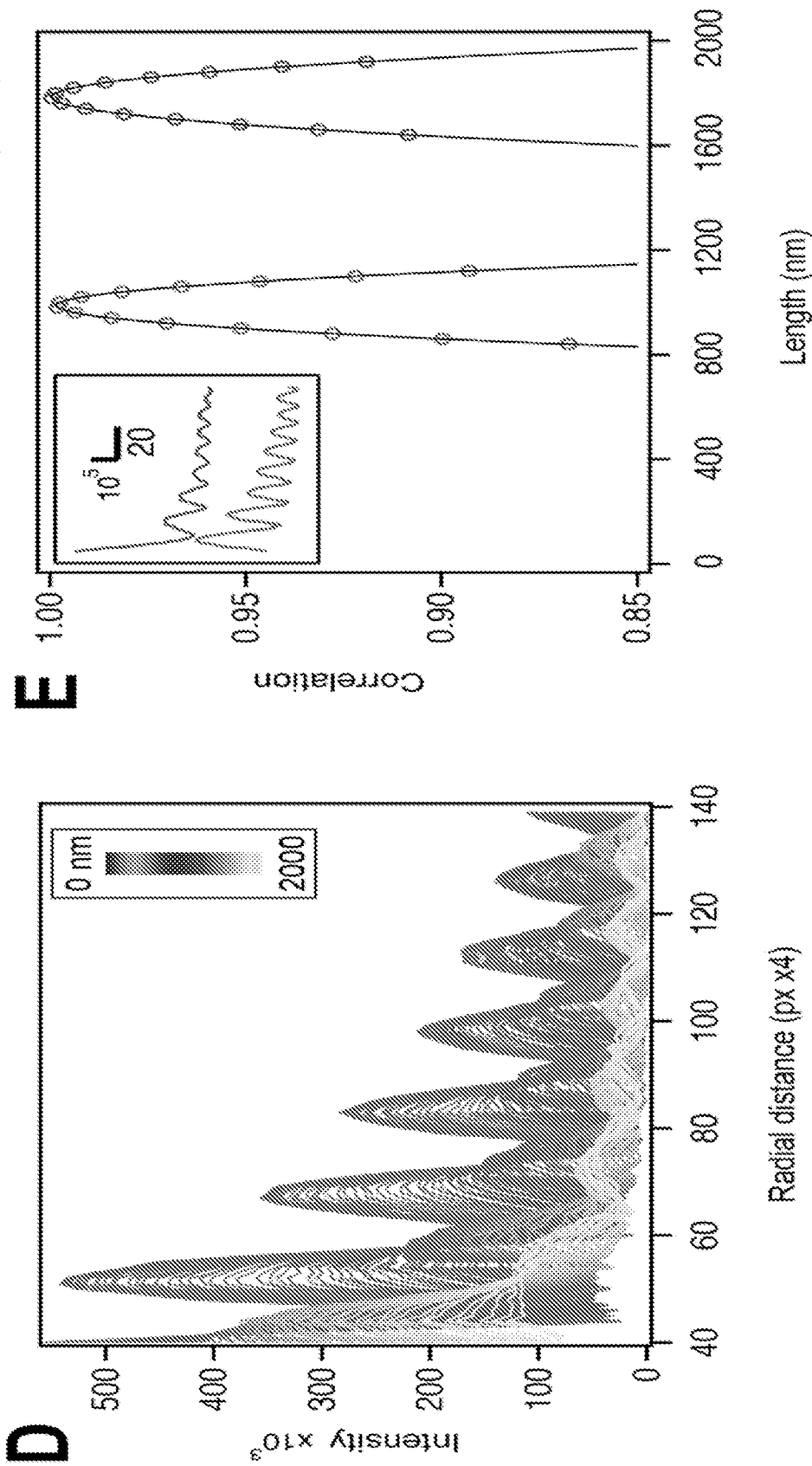


Fig. 6E



Figs. 7A-7C



Figs. 7D-7E

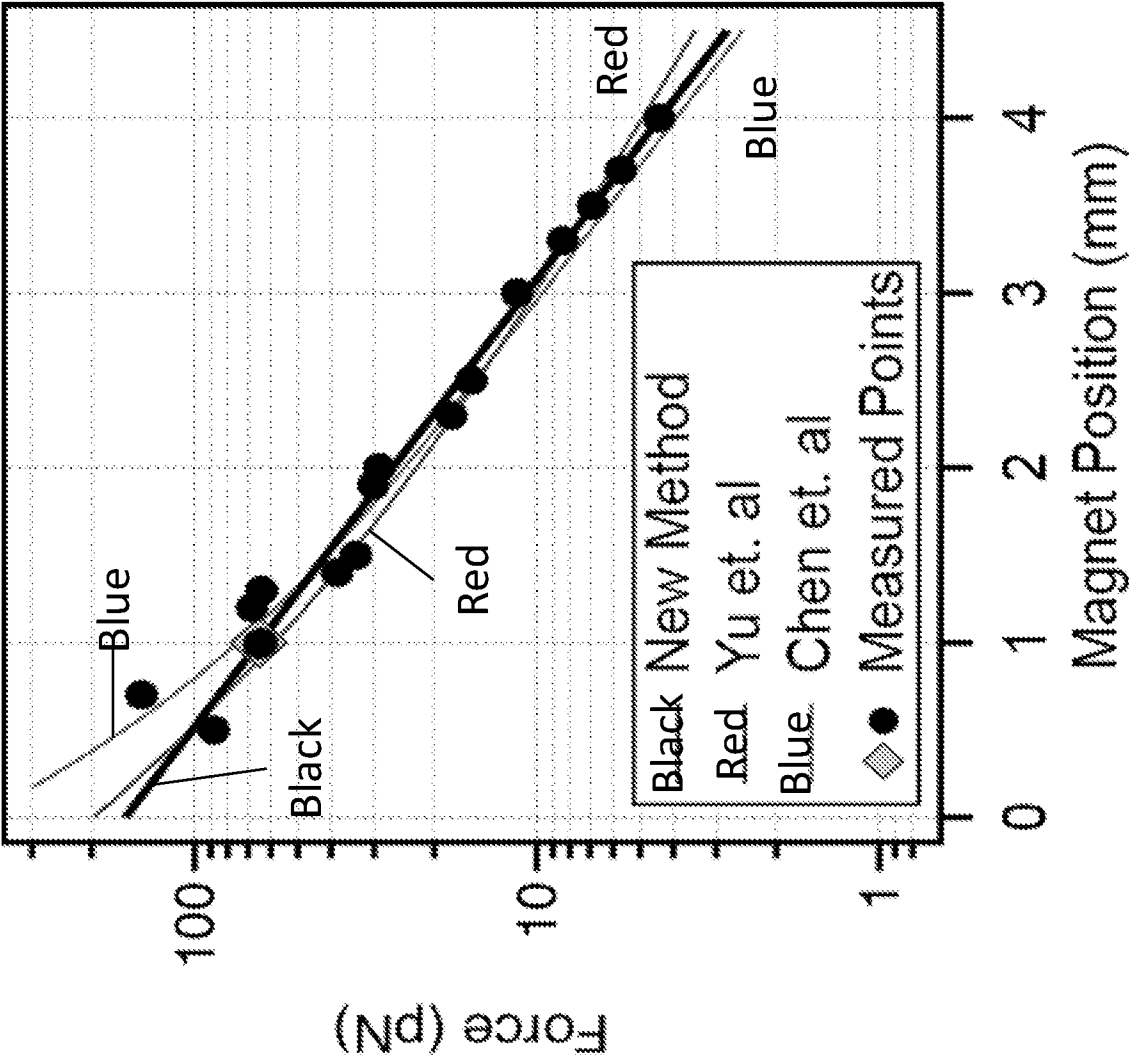


Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/16209

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01L 5/00; G01N 21/84; G02B 21/32 (2016.01)

CPC - G01Q 60/24, 60/38, 60/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): G01L 5/00; G01N 21/84; G02B 21/32 (2016.01)

CPC: G01Q 60/24, 60/38, 60/42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data) Google/Google Scholar, Ebsco, IEEE;

KEYWORDS: applying, forcing, molecule, protein, DNA, RNA, pragmatic bead, voice coil, voice wire, speech coil, speech wire, talk coil, talk wire, communication coil, communication wire, magnetic part, magnetic piece, magnetic tweezers, substrate, base, medium, camera

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 202229869 U (UNIV SHANGHAI JIAOTONG) May 23, 2012; See machine translation; claims 1 & 5, paragraphs [0018], [0024]-[0025], figure 1	1 & 19
A	LIU, R et al. "Mechanical Characterization of Protein L in the Low-Force Regime by Electromagnetic Tweezers/Evanescence Nanometry". Biophysics Journal, May 2009; [retrieved 2016-03-25]; Retrieved from the Internet <URL: http://ac.els-cdn.com/S0006349509005608/1-s2.0-S0006349509005608-main.pdf?_tid=3cae7de0-fb4c-11e5-992b-00000aa b0f6b&acdnat=1459874235_ed879bc780902bf35f5fa26b937506f1 >; figures 1b & 1c, page 3811, paragraph 1, page 3812, paragraphs 2-3 & 6, page 3813, paragraph 1	1 & 19
A	JP 3270365 B2 (Fujitsu LTD) April 02, 2002; See machine translation; figure 5, page 4, paragraph 1	1 & 19

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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