From genes to protein mechanics on a chip

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Single-molecule force spectroscopy enables mechanical testing of individual proteins, but low experimental throughput limits the ability to screen constructs in parallel. We describe a microfluidic platform for on-chip expression, covalent surface attachment and measurement of single-molecule protein mechanical properties. A dockerin tag on each protein molecule allowed us to perform thousands of pulling cycles using a single cohesin-modified cantilever. The ability to synthesize and mechanically probe protein libraries enables high-throughput mechanical phenotyping.

Mechanical forces play a pivotal role in biological systems by performing tasks such as guiding cell adhesion1, inducing gene expression patterns2 and directing stem cell differentiation3. At the molecular level, mechanosensitive proteins act as sensors and transducers, communicating the presence and direction of applied forces to downstream signaling cascades. Conformational changes in response to mechanical forces4 and energetic barriers along unfolding pathways can be probed by single-molecule force spectroscopy (SMFS) techniques4. Such techniques, including optical tweezers, magnetic tweezers and atomic force microscopy (AFM), have been used to interrogate high-affinity receptor-ligand binding5, measure unfolding and refolding dynamics of individual protein domains6–8, observe base-pair stepping of RNA polymerases9 and identify DNA stretching and twisting moduli10.

Despite these successes, SMFS experiments have been limited by low throughput. Experimental data sets typically contain a majority of unusable force-distance traces owing to the measurement of multiple molecular interactions in parallel or a lack of specific interactions. Typical yields of interpretable single-molecule interaction traces in SMFS experiments vary between 1% and 25%. The incapacity of SMFS to quickly screen libraries of molecular variants has hindered progress toward understanding sequence-structure-function relationships at the single-molecule level. In particular, the need to prepare each protein sample and cantilever separately increases experimental workload and gives rise to calibration uncertainties. Therefore, methods to interrogate the mechanical behavior of different proteins in a parallel and streamlined format with the same cantilever would offer distinct advantages. Such a screening approach could characterize single-molecule properties such as unfolding forces, interdomain mechanical signatures and mechanically activated catch-bond behavior1. Screening of these properties could find applications in biotechnology and human health studies in which mechanical dysregulation or misfolding is suspected to play a role in pathology11.

Here we developed a platform for parallel characterization of individual protein mechanics in a single experiment (Fig. 1). Microspotted gene arrays were used to synthesize fusion proteins in situ using cell-free gene expression. Proteins were covalently immobilized inside multilayer microfluidic circuits. A single cantilever was then positioned above the protein array and used to probe the mechanical response of each individual protein via a common C-terminal dockerin (Doc) fusion tag. Genes of interest were chosen such that each gene product exhibited an identifiable unfolding pattern when loaded from the N to the C terminus. Each target protein was expressed with an N-terminal 11-amino-acid ybbR tag, which was used to covalently and site-specifically link the protein to the surface via Sfp synthase–catalyzed reaction with coenzyme A (CoA)12. At the C terminus the proteins contained a 75-amino-acid celluloseosomal Doc from Clostridium thermocellulum13 as a specific handle targeted by the cohesin (Coh)-modified cantilever.

The gene microarray was aligned and reversibly bonded to a microfluidic chip known as MITOMI (mechanically induced trapping of molecular interactions). The chip has been used in the past for screening transcription factors14,15 and mapping interaction networks16. More recently, our group employed MITOMI chips for molecular force assays17. In this work, MITOMI chips featured 640 dumbbell-shaped unit cells in a flow layer and 2,004 micromechanical valves in a control layer. Each unit cell was equipped with pneumatic 'neck,' 'sandwich' and 'button' valves (Fig. 1a) according to design principles of soft lithography18. Each neck valve protected the microspotted DNA in the back chamber from exposure to other reagents during surface patterning in the front chamber. The sandwich valves prevented chamber-to-chamber cross contamination, ensuring that only a single protein variant was present in each sample spot. For surface chemistry in the front chamber, the button valves were actuated to shield the sample spots, allowing n-dodecyl β-D-maltoside passivation in the surrounding area. Releasing the button valves allowed subsequent functionalization with CoA-poly(ethylene glycol) (CoA-PEG) in the sample area under the buttons serving as the protein immobilization site. We expressed the genes by

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incubating an in vitro transcription and translation cell extract at 37 °C with the spotted DNA in the back chamber. The synthesized proteins then diffused to the front chamber, where they were covalently linked to the surface via an Sfp-catalyzed reaction of surface-bound CoA with solution-phase N-terminal ybbR peptide tags (Fig. 1b). Partial pressurization of the button valve19 was used for tagging an outer concentric portion of the sample area with a fluorescently (TagRFP) tagged Coh that specifically bound to the C-terminal Doc tag of each target protein, thereby confirming successful protein synthesis and surface immobilization (Supplementary Fig. 1). Finally, the microfluidic device was removed from the glass slide to provide access to the protein array from above. Using this approach, we generated microarrays of site-specifically and covalently immobilized proteins for subsequent SMFS experiments, starting from a conventional gene array. An inverted three-channel total-internal-reflection fluorescence/atomic force microscope (TIRF-AFM)20 was used to position the cantilever in the center of the fluorescent rings in the protein array and perform SMFS measurements (Fig. 1c). The Coh-modified cantilever was used to probe the surface for expressed target proteins containing the C-terminal Doc tag. Upon surface contact of the cantilever, formation of a Coh-Doc complex allowed measurement of target-protein unfolding in a well-controlled pulling geometry (N to C terminus). We retracted the probe at constant velocity and recorded force-extension traces that characterized the unfolding fingerprint of the target protein. This approach-retract process could be repeated many times at each array address to characterize each expression construct. Several unique features of the C-terminal Doc tag make it particularly suitable as a protein handle for SMFS. Its small size of 8 kDa does not notably add to the molecular weight of the gene products, which is advantageous for cell-free expression. Additionally, Doc exhibits a specific and high-affinity interaction with Coh domains from the C. thermocellum scaffold protein CipA. Coh was used both for fluorescence detection of the gene products, which is advantageous for cell-free expression. Additionally, Doc exhibits a specific and high-affinity interaction with Coh domains from the C. thermocellum scaffold protein CipA. Coh was used both for fluorescence detection of the gene products, which is advantageous for cell-free expression.
that the gene of interest was completely expressed through to the C terminus (Fig. 2). Furthermore, this double rupture peak indicated that the interaction with the Coh-modified cantilever was specific and that the pulling geometry was strictly controlled such that force was applied to the molecule of interest from the N to the C terminus.

To validate and demonstrate our approach, we expressed genes of interest comprising well-known fingerprint domains in the SMFS literature. We produced multimeric polyproteins including tetrameric human type-III fibronectin (FBN) and dimeric chicken brain α-spectrin (SPN). We also synthesized monomers of endo-1,4-xylanase T6 from *Geobacillus stearothermophilus* (XYL) superfolder GFP (GFP) and twitchin kinase. In all cases, the target proteins were fused to N-terminal ybbR and C-terminal Doc tags (Supplementary Figs. 2–6). Unfolding data for FBN, SPN, XYL and GFP were obtained using a single cantilever to probe a single microarray (Figs. 2 and 3). Twitchin kinase was found not to express in sufficient yield to provide reliable unfolding statistics.

We transformed force-extension data (Fig. 2) into contour length space using the worm-like chain model and compared the measured contour length increments with the amino acid sequence lengths of each protein and literature values. The observed contour lengths and rupture forces were consistent with our expectations. FBN showed a fourfold-repeated sequence of rupture peaks at contour length increments of 32 nm (ΔL_{FBN}; Fig. 2a) frequently interrupted by an intermediate peak at 10–12 nm, both features characteristic of FBN. SPN showed two regular sawtooth-like peaks with contour lengths of 33 nm (ΔL_{SPN}; Fig. 2b). XYL exhibited a decreasing multipeaked unfolding fingerprint with a contour length increment of 92 nm (ΔL_{XYL}; Fig. 2c), occasionally showing additional increments corresponding to unfolding of remaining XYL subdomains, a result consistent with the prior study and accounting for N-terminal immobilization of XYL. GFP unfolding showed a contour length increment of 74 nm (ΔL_{GFP}; Fig. 2d). As each protein in the array contained the same C-terminal Doc tag, the final two rupture peaks in all force traces represented rupture of the Coh-Doc complex regardless of the protein of interest.

In our system, surface densities of expressed proteins were comparable to those obtained in conventional SMFS experiments. Uninterpretable and nonspecific interactions were excluded from the analysis (Supplementary Fig. 7). By collecting multiple unfolding traces, we assembled contour length diagrams for each protein of interest (Fig. 3a) and confirmed the predicted contour length increments on the basis of the encoded amino acid sequences in each DNA spot. Coh-Doc rupture events for all protein constructs in the array clustered to the same population in the force-loading rate plot, independently of the preceding rupture peaks from the protein of interest (Fig. 3b). The Coh-Doc ruptures agreed with previously reported values at similar loading rates. The unfolding events of the proteins of interest produced distinct populations in the force-loading rate plots (Fig. 3c). The unfolding events depended on the internal structure and the unfolding pathway of the fingerprint domain when stretched between its N and C termini. SPN, for example, an elongated 3-helix bundle, was previously reported to exhibit a broader energy well (Δx = 1.7 nm; ref. 23) and showed a flatter distribution of unfolding forces than that of the more compact globular FBN domain with a shorter, steeper potential (Δx = 0.4 nm; ref. 22).

In summary, our flexible approach efficiently streamlines protein expression, purification and SMFS into a single integrated platform (Supplementary Discussion). The approach should be compatible with other in vitro expression systems including extracts derived from insects, rabbit reticulocytes and human cell lines, and it is capable of introducing post-translational modifications and non-natural amino acids, allowing, for example, the screening of site-directed mutants. Our method allows for synthesis of cytotoxic proteins or proteins with a tendency to form inclusion bodies during bulk expression. In addition to providing greatly improved throughput, our system has the advantage of measuring multiple constructs with one cantilever, thereby eliminating errors introduced when performing multiple calibrations on different samples with uncertainties of ~10% (ref. 28). Detecting subtle differences in mechanical stability with this high-throughput approach could therefore be used to perform mechanical phenotyping experiments on similarly stable families of mutant proteins. This workflow opens the door to large-scale screening studies of protein nanomechanical properties.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Addgene: pET28a-ybbR-HIS-sfGFP-DocI, 58708; pET28a-ybbR-HIS-CBM-CohI, 58709; pET28a-StrepII-TagRFP-CohI, 58710; pET28a-ybbR-HIS-Xyl-DocI, 58711;
pET28a-ybbR-HIS-10FNIII(x4)-DocI, 58712; pET28a-ybbR-HIS-Spec(x2)-DocI, 58713.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.O., M.A.N. and H.E.G. designed the research; M.O., W.O., M.A.J. and T.V. performed experiments; d.A.P. helped with immobilization strategies; M.O., W.O., M.A.J., M.A.N. and H.E.G. cowrote the manuscript.

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The authors declare no competing financial interests.

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ONLINE METHODS

Chip fabrication. Ready-to-use wafers for flow and control layers of the 640-chamber MITOMI design were obtained from Stanford Microfluidics Foundry (design name DTPAd)\textsuperscript{14}. The flow wafer features 15-µm-high features, rounded by photoresist reflow, whereas the control wafer features a rectangular cross-section. Microfluidic chips were cast in poly(dimethylsiloxane) (PDMS) from these wafers. For the control layer, Sylgard 184 (Dow Corning) base and curing agent were mixed at a ratio of 5:1 by weight, poured onto the wafer, degassed and partially cured for 20 min at 80 °C. For the flow-layer wafer, a 20:1 base–to–curing agent mixture of Sylgard 184 was spin-coated for 75 s at 1,600 r.p.m. and partially cured for 30 min at 80 °C. The control layer chips were cut out, inlet holes were punched and the chips were aligned onto the spin–coated PDMS on the flow-layer wafer. After the two-layer chips were baked for 90 min at 80 °C, they were cut and removed from the wafer, and inlet/outlet holes were punched. Microfluidic chips were stored for up to 6 weeks.

Cloning. For the construction of the fusion proteins, Gibson assembly\textsuperscript{29} was used. A ratio of 0.07 pmol vector to 0.3 pmol of insert was used for the fusion reaction. The primer sequences are provided in Supplementary Table 1. A pET28a plasmid was linearized with primers 1 and 2. The dockerin type I–encoding gene was isolated from the xylanase-dockerin type I construct\textsuperscript{27} with primers 3 and 4. Codon-optimized sequences were purchased from GeneArt/Invitrogen. The genes of interest were designed in such a way that they already contained sequences overlapping those of their neighboring partners (pET28a and dockerin type I). In the case of the spectrin, two domains were linked with a flexible glycine-serine (×6) linker. For fibronectin, four type III domains were fused separated by glycine-serine (×6) linkers. The expression vector in all cases was a pET28a plasmid with a modified multiple cloning site (sequence attached: plasmids are provided in Supplementary Table 1). A pET28a plasmid was linearized with primers 1 and 2. The dockerin type I–encoding gene was isolated from the xylanase-dockerin type I construct\textsuperscript{27} with primers 3 and 4. Codon-optimized sequences were purchased from GeneArt/Invitrogen. The genes of interest were designed in such a way that they already contained sequences overlapping those of their neighboring partners (pET28a and dockerin type I). In the case of the spectrin, two domains were linked with a flexible glycine-serine (×6) linker. For fibronectin, four type III domains were fused separated by glycine-serine (×6) linkers. The expression vector in all cases was a pET28a plasmid with a modified multiple cloning site (sequence attached: plasmids are available at Addgene, Supplementary Table 2). After construction, clones were verified via sequencing and amplified in NEB 5-alpha Escherichia coli cells. Following plasmid preparation, samples were concentrated up to 500 ng/µl before microspotting.

DNA microspotting. A 24 × 60-mm #1 thickness coverslip (Thermo Scientific) was silanized with 3-aminopropyltrimethyloxysilane (ABCR) following literature protocols\textsuperscript{30}. The DNA solution containing 1% (w/v) nuclease-free bovine serum albumin (Carl Roth) in nuclease-free water was microspotted under humid atmosphere onto the silanized coverslip using the GIX Microplotter II (Sonoplot) and a glass capillary with a 30-µm tip diameter (World Precision Instruments) according to the manufacturer’s instructions in a rectangular 40 × 16 pattern with 320-µm column pitch and 678-µm row pitch. Alignment of the DNA array and the microfluidic chip was done manually using a stereomicroscope. Bonding between the glass cover slip and microfluidic device was achieved by thermal bonding for 5 h at 80 °C on a hot plate.

Protein synthesis on-chip. The microfluidic device was operated at a pressure of 4 p.s.i. in the flow layer and 15 p.s.i. in the control layer. Operation started with the button and neck valves actuated for surface passivation. The flow layer was passivated by flushing through standard buffer (25 mM Tris, 75 mM NaCl, 1 mM CaCl₂, pH 7.2) for 5 min and 2% n-dodecyl β-D-maltoside (Thermo Scientific) in nuclease-free H₂O for 30 min (ref. 31). Next the button valve was opened, and borate buffer (50 mM sodium borate, pH 8.5) was flushed through for 30 min to deprotonate aminosilane groups on the glass surface.

For maleimide/coenzyme A functionalization, a solution of 5 mM NHS-PEG-maleimide (MW = 513 Da, Thermo Scientific) in borate buffer was flushed through for 45 min. The device was then rinsed with nuclease-free H₂O for 5 min, followed by 30 min of 20 mM coenzyme A (Merck) in coupling buffer (50 mM sodium phosphate, pH 7.2, 50 mM NaCl, 10 mM EDTA). The button valve was then actuated to protect the functionalized surface area followed by 5 min of rinsing with standard buffer.

S30 T7 HY (Promega) in vitro transcription and translation mix, supplemented with 1 µL T7 polymerase (Promega) and 0.5 µL RNase inhibitor (Invitrogen), was then flushed into the chip, filling the DNA chambers (neck valve open).

The neck valve was then closed, and the channels were filled with 4'-phosphopantetheinyl transferase (Sfp synthase) in Sfp buffer (50 mM HEPES, 10 mM MgCl₂). The chip was then incubated at 37 °C on a hot plate. After 1 h of incubation, the neck and the button valves were opened to allow Sfp synthase–catalyzed linkage of expressed protein to the coenzyme A–functionalized area below the button. At the same time the sandwich valves were actuated to avoid chamber-to-chamber cross-contamination. After another 1.5 h of incubation, the neck and button valves were closed, the sandwich valves were opened and the chip was rinsed with standard buffer for 20 min.

To verify successful protein expression and immobilization on the functionalized surface area, a fluorescent detection construct (TagRFP–cohesin type I (2 µg/ml in standard buffer) was flushed through the device for 10 min with the button valve actuated. The sandwich valves were then actuated, and the button valve partially released by decreasing the pressure to 11 p.s.i. After 20 min of incubation at room temperature, the sandwich valves were opened, and the chip flushed with standard buffer for 20 min. Fluorescence images of all chambers were recorded on an inverted microscope with a 10× objective (Carl Zeiss), featuring an electron-multiplying charge-coupled device (EMCCD) camera (Andor). Prior to force spectroscopy experiments, the chip was stored in buffer at 4 °C.

Directly before measurement, the PDMS chip was peeled off from the glass substrate under buffer, revealing the microarray while avoiding drying of the functionalized surface. The array surface was then rinsed several times with buffer. We did not encounter any problems with cross-contamination between chambers.

Cantilever functionalization. A silicon-nitride cantilever bearing a silicon tip with a tip radius of ~8 nm (Biolever mini, Olympus) was silanized with ABCR as described previously\textsuperscript{30}. Protein functionalization was performed in a similar way as reported previously\textsuperscript{27,31}. Briefly, a 50 µM solution of CBM A2C–cohesin from C. thermocellum in standard buffer was incubated with 1:2 (v/v) TCEP beads (Tris (2-carboxyethyl) phosphine disulfide reducing gel, Thermo Scientific), previously washed with standard buffer, for 2.5 h. The cantilever was submerged in borate buffer for 45 min to deprotonate primary amine groups on the silanized surface and then incubated with 20 mM NHS-PEG-maleimide (MW = 5 kDa, Rapp Polymere) in borate buffer for 60 min.
The cantilever was rinsed sequentially in three beakers of deionized H₂O. TCEP beads were separated from the protein solution by centrifugation at 1,000g for 1 min. Next the cantilever was incubated for 60 min with reduced protein solution, which was diluted to a concentration of 1 mg/mL with standard buffer. Finally the cantilever was rinsed sequentially in three beakers of standard buffer and stored submerged in standard buffer in humid atmosphere at 4 ºC for up to 24 h before use.

**Force spectroscopy.** A custom-built TIRF (total internal reflection fluorescence)-AFM (atomic force microscope) hybrid was used to conduct the force spectroscopy measurements. The TIRF microscope was used to image fluorophores in up to three different color channels simultaneously using an iChrome MLE-S four-color laser (Toptica Photonics), an Optosplit III triple emission image splitter (Cairn Research) and a Xion3 EMCCD camera (Andor). A long-range stick-slip xy piezo nanopositioning system (ANC350, Attocube Systems) allowed access to the whole microchip array as well as fine spatial sampling of different surface molecules on the nanometer scale within each protein spot. Cantilever actuation in the z direction was performed by a LISA piezo-actuator (Physik Instrumente) driven by an MFP3D AFM controller (Asylum Research).

The following force spectroscopy protocol was performed repeatedly in each functionalized protein target area. The cantilever approach velocity was 3,000 nm/s, dwell time at the surface was 10 ms and retract velocity was 800 nm/s. Data were recorded with 6,250-Hz sampling rate. The cantilever typically had a spring constant in the range of 100 pN/nm and a resonance frequency of 25 kHz in water. Accurate calibration of the system was performed by the nondestructive thermal method using corrections to account for discrepancies from the original theory.

**Data and statistical analysis.** The raw data were converted from photodiode voltages into force values in newtons, and the following standard corrections were applied. The zero force value for the unloaded cantilever in each curve was determined by finding the force value closest to zero in a small neighborhood of the first non-negative force value in the force-extension trace. The position of the surface was determined by finding the force value closest to 0 in a small neighborhood of the first non-negative force value in the force-extension trace. The z piezo position was corrected for the true tip-sample separation due to deflection of the lever as a function of the force for a Hookean spring.

A pattern-recognition software based on a package described previously and adapted in-house chose the curves showing worm-like chain force responses of the stretched protein constructs. Example curves showing multiple, unspecific or no interactions are shown in Supplementary Figure 7, together with a single xylanase trace for comparison. The expected protein backbone contour length increments for each construct were detected in contour length space: the real part of the following numerically solved inverse worm-like chain (WLC) formula was used to transform force-extension data into force–contour length space for every measured force curve:

\[
L(x) = \frac{x}{6u} \left( 3 + 4u + \frac{9 - 3u + 4u^2}{g(u)} + g(u) \right)
\]

where

\[
g(u) = \left[ 27 - \frac{27}{2} u + 36u^2 - 8u^3 + \frac{3\sqrt{3}}{2} \sqrt{-u^2 \left( 4u^3 - 3 - 108 \right)} \right]^{\frac{1}{3}}
\]

and

\[
u = \frac{F L_p}{kT}
\]

with \(L\) the contour length, \(x\) the extension, \(F\) the force, \(L_p\) the persistence length, \(k\) Boltzmann's constant and \(T\) the temperature. Transformed data points were combined in a Gaussian kernel density estimate with a bandwidth of 1 nm and plotted with a resolution of 1 nm. In these resulting energy-barrier position diagrams, the contour length increments could easily be determined. The transformation was performed with the following parameters: persistence length \(L_p = 0.4\) nm, thermal energy \(kT = 4.1\) pN nm. Force and distance thresholds were applied at 10 pN and 5 nm, respectively. The measurement data sets in each protein spot on the chip typically showed a yield of 0.5–5% specific interactions.

The force peaks corresponding to protein domain unfolding events, as well as those corresponding to final ruptures, were line fitted in force-time space to measure the loading rate of each individual event.

WLC fits for demonstrative purposes in Figure 2 were done by using the following formula:

\[
F(x) = \frac{kT}{L_p} \left( \frac{1}{4(1 - x / L)^2} + \frac{x}{L} - \frac{1}{4} \right)
\]

with \(F\) the force, \(k\) the Boltzmann's constant, \(T\) the temperature, \(L_p\) the persistence length, \(x\) the extension and \(L\) the contour length.

Discrepancies between contour length increments in fitted single-molecule traces and aligned contour length diagrams are artifacts caused by the fixed persistence length in the contour length transformation, whereas the WLC fits to single force traces treat both contour length and persistence length of each stretch as free parameters. An overview of the yield of interpretable curves of all constructs is available in Supplementary Table 3.

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Corrigendum: From genes to protein mechanics on a chip

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