Toward high-throughput biomechanical phenotyping of single molecules

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Two high-throughput single-molecule force spectroscopy platforms expand the reach of this technology for biomechanical molecular phenotyping.

Single-molecule force spectroscopy (SMFS) is a powerful tool for mechanical manipulation of single biomolecules such as DNA, RNA, sugars, proteins and lipids. Typically, a molecule of interest attached to a surface at one end is pulled from the other end using a highly precise force-transducing device while the molecular response is recorded. Through this manipulation it is possible to determine structural, mechanical and adhesive properties of biomolecules, as well as to observe their folding and misfolding pathways or approximate their thermodynamic and kinetic parameters. It is even possible to determine and mechanically control the functional state of single proteins or to characterize how this state interacts with drugs or other functional biomolecules. This has opened an exciting avenue toward manipulating and understanding biomolecular systems *in vitro* and *in vivo*. Nevertheless, the widespread use of single-molecule methods has been hampered by their low experimental throughput. Two recent papers published in *Nature Methods* give general guidelines for the design, optimization and validation of high-throughput SMFS. If combined, these techniques could open the door to a new bioanalytical dimension through highly parallelized and accurate measurements of the biomechanical phenotypes of thousands of proteins[1,2].

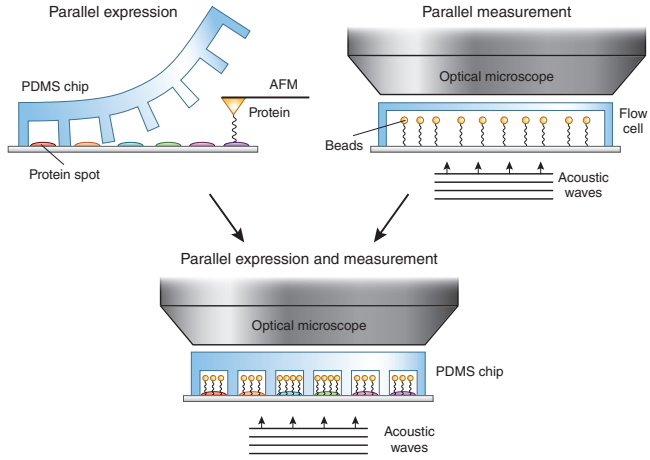

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Figure 1 | We envision that combining the high-throughput techniques of Otten *et al.*[1] (top left) and Sitters *et al.*[2] (top right) may provide new ways to characterize the mechanical properties of proteins expressed *in situ* in microfluidic chips (bottom).

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Improvements over the last 20 years have brought forth a powerful SMFS toolbox, including atomic force microscopy (AFM), optical tweezers, magnetic tweezers, microneedle manipulation, biomembrane force probe and flow-induced stretching. These SMFS techniques, which differ in the force-measuring device used (i.e., AFM tip, bead, needle or membrane) and how force is measured (i.e., AFM cantilever, optical or magnetic trap, or membrane deformation), have their own intrinsic advantages and disadvantages[3,4]. Nevertheless, the power of these techniques is highlighted by the wide diversity of measurements they can perform. Currently, the SMFS capacity spans six orders of magnitude in length (subångström to micrometer range) and four orders of magnitude in force (subpiconewton to nanonewton range), allowing measurements ranging from the strength of a single bond to the analysis of the force-extension or force-time relationship of individual molecules.

An important advantage of single-molecule techniques is that they do not suffer from problems associated with population averaging inherent to ensemble measurements. So far, however, this strength has also been a weakness because, in spite of their high sensitivity, single-molecule methods can be time consuming and experimentally challenging. Nonspecific interactions of the sample with the force-measuring device often cause SMFS to show low yields of interpretable single-molecule manipulations (between 0.1% and 10%). A way to improve this yield is to engineer a specific handle that allows tethering of the extremities of a targeted biomolecule to the force-measuring device and to the support. To drastically improve measurement statistics, this tethering should ideally be reversible so that after characterization of one biomolecule, the next one can be picked up and characterized. Finally, the need to prepare the biomolecule, force-measuring device and support separately increases the experimental workload and gives rise to experimental uncertainties.

To alleviate such throughput limitations in SMFS studies, Otten *et al.*[1] adopted a microfluidic platform, based on a mechanically induced trapping of molecular interactions

(MITOMI)[5,6] device, that creates up to 640 protein spots on a coverslip and readies them for AFM measurements (**Fig. 1**). MITOMI allows high-throughput *in situ* expression of proteins and their oriented covalent attachment using cell-free protein expression from microspotted gene arrays. The use of thousands of on-chip PDMS membrane valves allows for highly parallelized protein expression and preparation of various chemistries needed for mechanical phenotyping. Two different tags at each end of the protein are used to covalently anchor the protein to the coverslip and to noncovalently bind to a functionalized AFM cantilever. After successful expression has been confirmed by fluorescence of a fluorescent fusion protein, the microfluidic device is removed, resulting in a well-defined protein microarray. Finally, the protein microarray is probed by AFM, allowing for the mechanical phenotyping of different proteins, all carrying the same tag, using a single functionalized cantilever. The trick is that the interaction between the functionalized AFM cantilever and tagged protein is chosen to be reversible so that after a single-molecule experiment, the next protein carrying the same tag can be picked up and characterized by SMFS. Importantly, only fully expressed proteins carrying both the covalent anchor and the tag are picked up with this approach. The advantage of using a single cantilever is that it allows for direct comparison of the measured properties of different proteins, thereby removing uncertainties related to force-probe calibration. However, using a single cantilever is time consuming because the single-molecule experiments have to be done sequentially. Thus, the time needed to obtain a statistically relevant amount of data remains a limitation for this on-chip expression method.

An option to circumvent this issue could be to move toward using massively parallel force measurement assays. Among SMFS techniques, AFM[7], optical[8] and magnetic tweezers[9], and DNA-based force balances[10] show promise for such high-throughput screening applications. A new tool, acoustic tweezers[2], is a promising complement to the SMFS toolbox. Developed by Sitters *et al.*, this method relies on the use of acoustic force, in the range of subpiconewton values to hundreds of piconewtons, to simultaneously manipulate thousands of biomolecules attached to beads, with submillisecond response times. Acoustic waves generated from a voltage-driven liquid-coupled piezo plate are used to push or pull the beads at will, and when combined with simple phase microscopy, this allows one to record the positions, fluctuations and tension of thousands of bead-attached molecules in parallel (**Fig. 1**). This bead-based method allows cost-effective and massively parallel applications such as those needed to conduct statistically relevant numbers of single-molecule experiments[8,9]. Importantly, such experiments can be performed in a lab-on-a-chip device, as demonstrated by the authors using a simple flow cell[2]. Such setups, especially with the use of advanced microfluidic devices[11], could enable large-scale characterization of single proteins or other biomolecules carrying specific handles and when responding to changes in their environment (e.g., changes to pH, buffer or temperature, or the addition of drugs, chemical compounds or other molecules).

These two papers in *Nature Methods* constitute early demonstrations of highly parallelized preparation[1] and force measurement[2] of single biomolecules. We believe it is just a matter of time until such techniques are combined in truly integrated and highly capable systems (**Fig. 1**), which will revolutionize the mechanical phenotyping of single molecules. In such a scenario we envision that proteins expressed *in situ* and printed in microfluidic devices could be mechanically characterized on-site through reversible attachment of their tags to SMFS force transducers. This will allow massive amounts of mechanical data to be collected and enable, for example, the screening of point mutations leading to protein destabilization and misfolding, as occurs in neurodegenerative diseases, or the screening of factors that stabilize proteins for biotechnological or medical use. Such approaches will require automated data recording and analysis of single-molecule experiments and will provide detailed insights on behavior at scales ranging from the single molecule to the molecular ensemble. So far the microfluidic device is limited to characterizing water-soluble proteins, but membrane proteins, given their importance as major drug targets, may be a challenge that is soon taken on. Furthermore, these methods rely on the use of tags fused to the terminal ends of the protein. Thus, it must be carefully shown for each protein whether adding a tag to the N or C terminus influences functional or structural properties.

Excitingly, however, the mechanical phenotyping of single molecules in microfluidic devices can be extended by the microfluidic toolbox so that mechanical characterization meets the functional. Ultimately, this will bring together the mechanical, structural and functional properties of proteins and protein complexes.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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