

Five challenges to bringing single-molecule force spectroscopy into living cells

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In recent years, single-molecule force spectroscopy techniques have been used to study how inter- and intramolecular interactions control the assembly and functional state of biomolecular machinery *in vitro*. Here we discuss the problems and challenges that need to be addressed to bring these technologies into living cells and to learn how cellular machinery is controlled *in vivo*.

The living cell is a highly specialized factory that has evolved ways to precisely control inter- and intramolecular interactions that regulate its molecular machinery. But how cells establish such biomolecular interactions remains mysterious. Single-molecule force spectroscopy (SMFS) devices have opened up a wide range of opportunities to quantify and manipulate the interactions of individual biomolecules. Nowadays, SMFS is increasingly used to explore the folding, conformational entropy, mobility, assembly and functionalities of molecular machinery of the cell *in vitro*. These SMFS studies have an inherent flaw: they use purified biomolecules that are removed from the cellular context. However, deciphering how cells control biomolecular interactions to drive their machinery in the highly complex and dynamic environment of their interior requires transferring SMFS into a living cell. Only with such a paradigm change will the combination of *in vitro* and *in vivo* SMFS measurements provide key insights into cellular processes. Here we scrutinize the

problems and challenges that need to be addressed to quantify interactions of single molecules *in vivo*.

Cellular complexity defines analytical approaches

Biomolecular interactions can generate tiny forces in the piconewton range that persist over time spans from milliseconds to many minutes or more. In the last two decades, tremendous progress has been made in developing SMFS methods (Fig. 1a) that can be used to quantify such forces that contribute to cellular and molecular systems^{1–6}. SMFS has provided insights into how biomolecular interactions guide protein folding, stability and functional state, macromolecular assembly, ligand and inhibitor binding, molecular transport, signal transduction, mechanosensing and cell adhesion, motility, sorting and differentiation. But nearly all current SMFS assays probe interactions of isolated biomolecules *in vitro* (Fig. 1b). Because biomolecules are highly controlled by their cellular environment, their interactions with the environment are different in the living cell than *in vitro*^{7–9}. Interactions that determine the assembly of cellular structures are hierarchical and the interaction cascades of biomolecular signaling pathways are structured in space, time and strength. Further increasing this complexity, the activity of many biomolecules is not only regulated by the cell but is also integrated into feedback mechanisms that control the cell.

Understanding which interactions the cell establishes to rule its own biological

processes and what feedback these processes induce requires characterizing these mechanisms in the living cell (Fig. 2). Accordingly, we have to develop methods that allow single biomolecules to be force-probed *in vivo*. This will entail bringing together nanoscale science, biophysics, engineering, and molecular and cell biology. Here we attempt to define five big needs that will allow SMFS to reach this goal. Some of these challenges overlap with those for applying SMFS *in vitro*. Therefore, we pay particular attention to the differences in addressing the challenges of *in vitro* to *in vivo* systems.

Define and overcome the limitations of current tools

There is a clear need to define the limitations of our research tools for force-probing single molecules in the cellular context. Historically, SMFS has been developed mainly to manipulate and probe isolated biomolecular systems such as receptor-ligand pairs, DNA, membranes and proteins^{4,10–12}. The SMFS methods used were based either on atomic force microscopy (AFM)¹³ or on optical or magnetic tweezers⁴. Pressurized microcapsules³ and microneedles¹ join this repertoire. These methods use mechanical probes such as cantilever styluses, beads, vesicles or microneedles to measure biomolecular forces. The large breadth in force resolution of these force probes (from $\sim 10^{-4}$ to 10^3 nanonewtons) has enabled measuring forces established by a few hydrogen bonds up to those required to separate two adhering cells.

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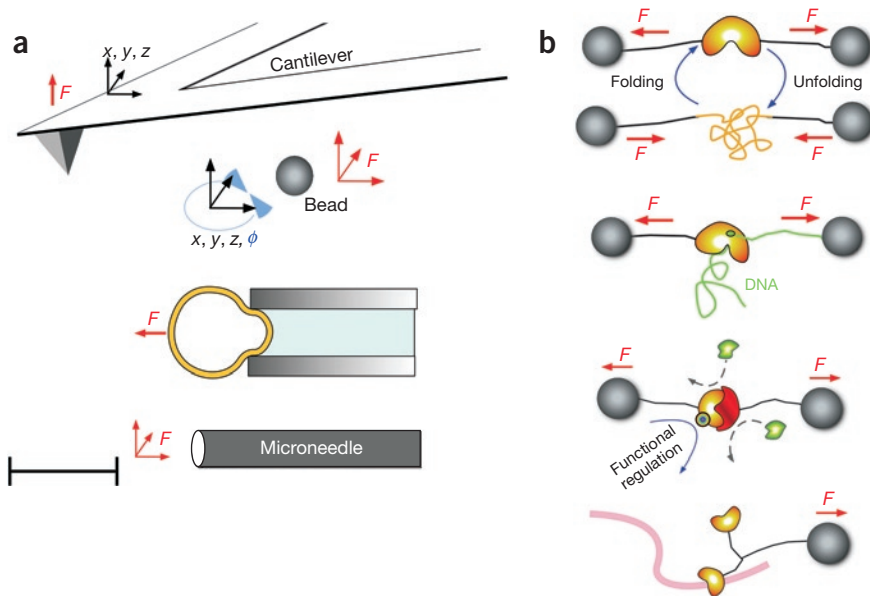


Figure 1 | SMFS of the cell's molecular machinery. (a) SMFS methods rely on different force probes to quantify interactions: AFM uses ~10–200-micrometer-long cantilevers^{5,13}, optical and magnetic tweezers use beads⁴, pressurized microcapsules use single cells or vesicles and microneedles^{1,3}. *F*, force. Scale bar, 20 μm . (b) Examples of using force probes (gray spheres) to quantify biomolecular interactions of single biomolecules *in vitro* (top to bottom): protein unfolding and folding, DNA-binding proteins, ligand-receptor bonds and cytoskeletal motor proteins.

Existing SMFS methods can be readily applied to measure forces at the cell surface and at times beneath the cell surface^{3,14}. SMFS at the cell surface reveals how a cell controls the assembly and functional state of cell surface receptors and provides insight into cellular adhesion. Approaching the cell from the outside provides limited insight into intracellular processes, however. So measuring intracellular interactions requires bringing the force-sensing probe inside the cell. This is the major hurdle, especially for SMFS methods, such as AFM, microneedle and pressurized microcapsule, where the force probe is mechanically connected to the instrument. Continuous technological developments have reduced the size and increased the sensitivity of the force probes, but existing cantilevers, beads, glass needles or pipettes can rarely be used inside living cells. Radical new concepts are needed.

Recent developments of optical tweezers attempted to overcome this limitation. By introducing small beads with diameters of about 200–400 nanometers into the cell, intracellular structures have been probed and contoured¹⁵, but the optical tweezers and force probe can easily interfere with cell function. Beads must become smaller to avoid this interference. This may be accomplished by engineering their optical

properties so that smaller beads can be more efficiently trapped than any cellular objects. An elegant way to circumvent such problems is to optically trap endogenous lipid droplets as actively transported cargo¹⁶. This approach allowed observation of single motor proteins transporting lipid droplets at piconewton force and sub-millisecond time resolution inside living cells. However, the intensity of the laser beams used to trap beads can be quite high (~0.1–1 watts) such that the cell is heated locally by a few degrees Celsius (~2–10 °C). Furthermore, laser-induced photodamage should not be neglected, and magnetic tweezers may therefore have advantages over optical tweezers *in vivo*⁴. But as cellular vesicles can not be trapped by magnetic tweezers, magnetic beads would need to be introduced into the cell to probe intracellular interactions.

A fundamentally different approach to force sensing is the use of molecular force probes. Molecular force probes are constructs that change state depending on the force applied to them. Several varieties based on fluorescence have been introduced. Fluorescent protein variants are routinely expressed as a fusion to label a protein of interest¹⁷. When a sufficient force is applied to it, the green fluorescent protein (GFP) changes its fluorescence

spectrum until it unfolds and fluorescence disappears. This effect was recently used to engineer a green fluorescent protein to detect strain in *Dictyostelium* Ax2 wild-type cells¹⁸. Similarly, fluorescence resonance energy transfer (FRET) pairs genetically engineered into vinculin were used to quantify mechanical forces in fibroblasts and endothelial cells¹⁹.

Other molecular force probes consist of a strained ssDNA loop flanked by two fluorescent dyes²⁰. Molecules, such as complementary ssDNA strands or proteins, interacting with this DNA loop can exert forces that change the distance between the dye molecules. As a result, the FRET efficiency decreases with increasing force stretching the DNA loop. Engineering the loop allows adjustment of its force sensitivity, and functionalization of the DNA would enable the sensing of specific biomolecular interactions of interest.

In the future, one can envision spectroscopically stable fluorophores that gradually change their emission spectra according to the force applied. Such fluorophores would then label biomolecules optically and quantify their forces.

Engineering optical molecular sensors to measure interactions inside a cell must fulfill prerequisites: sensors should be brought into the living cell without distorting the cellular integrity, be stable inside the cell and be insensitive to unwanted cellular interactions that alter their properties. In summary, developing noninvasive approaches to quantify biomolecular interactions of cellular processes will be a demanding task.

Establish standards and improve data quality

Establishing SMFS for sensing single-molecule interactions in living cells requires that the results obtained can be compared with each other. Experimental setups and standards need to be defined such that single molecules can be reproducibly force-probed. Adapting to functional requirements, the cell reassembles and changes the functional state of its molecular machinery. Hence, the cell's functional state must be known. This is achieved using molecular, cell-biological and genetic tools—that is, small interfering RNA, drugs and reporter constructs—that report and/or control the cell's functional state.

It is essential to define cellular standards for which a set of biological interactions can be reproducibly quantified before a novel

methodology is applied. Such cellular standards should be commercially available, and easy to culture and control. Suitable candidates such as HeLa cells are well characterized at the molecular and cellular level, and can be genetically modified and functionally controlled. Protocols to fluorescently label almost every biomolecule have already been established. Other well-characterized and controllable standards of bacterial, vertebrate and human origin must be found.

Cellular standards, however, are not sufficient for experimental reproducibility because of nonspecific interactions. Protocols have been developed to reliably functionalize force probes for the specific attachment of biomolecules, chemical compounds and even prokaryotic and eukaryotic cells^{2,5,14}. Nevertheless, the vast majority of interactions detected by SMFS are unspecific and of ambiguous origin. A major reason for such ambiguity is unspecific interactions with the force probe that superimpose with the specific biological interaction. In *in vitro* experiments, the idealized environmental conditions keep unspecific interactions to a minimum. When sensing interactions *in vivo*, however, the force probe may be contaminated after recording only a few force spectra^{2,14}.

To detect specific biomolecular interactions in the cellular environment we must design force probes that suppress unspecific interactions. Lipid vesicles in cells appear to show a limited number of unspecific interactions. As motor protein-mediated transport of lipid vesicles in living cells could be measured with single-molecule resolution¹⁶, it may be helpful to mask force probes with lipids. Such camouflaged force probes might be combined with polymeric linkers that have easily identifiable strain characteristics and to which biomolecules of interest are connected. The specific force spectrum of the biomolecule then combines with that of the linker and can be deconvoluted²¹.

It will not always be possible to avoid probe contamination, so procedures to identify force probe contamination during the course of an experiment will be crucial. These will save time and improve data quality. Minimizing contact area and experiment time also helps reduce contamination of the force probe. Therefore, the combination of SMFS and light microscopy should be further developed to optically guide force measurements on targeted areas of single live cells at a resolution of ~0.2–0.5

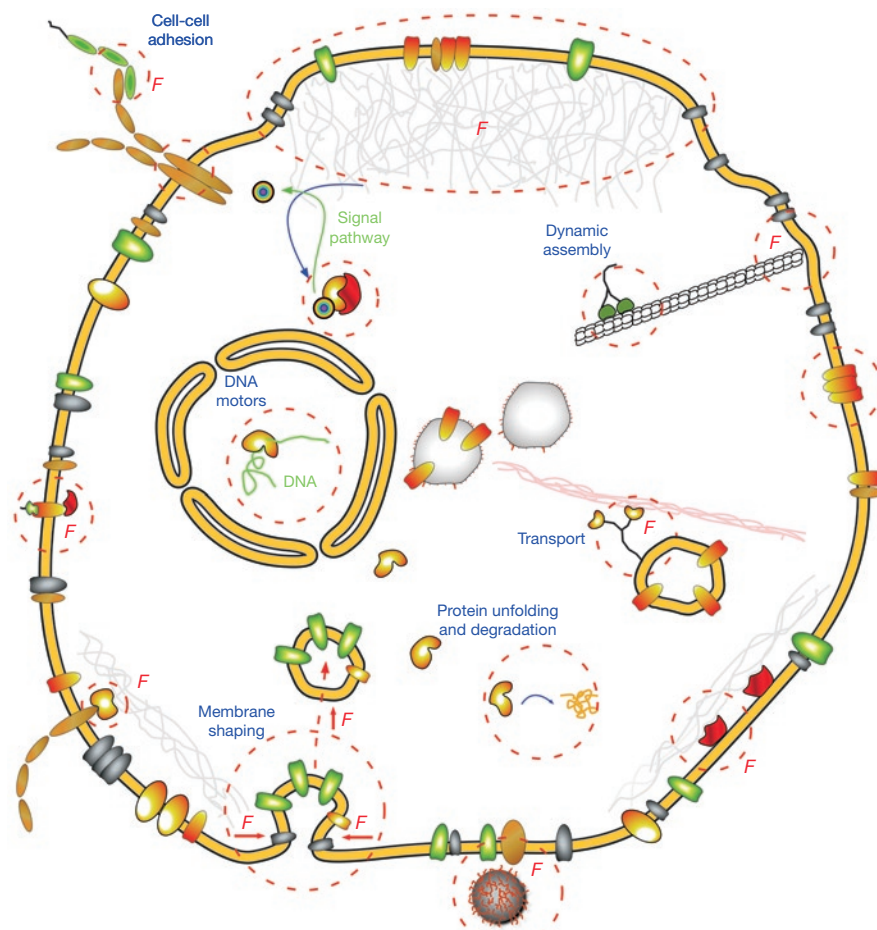


Figure 2 | Force-probing cellular interactions *in vivo*. SMFS offers exciting opportunities to sense interactions that drive the molecular machinery of the cell, including the dynamic assembly of supramolecular complexes, transport phenomena, protein folding, unfolding and degradation, membrane-protein insertion and folding, membrane shaping and reorganization, DNA-binding proteins, cell adhesion and signaling, signaling pathways or interactions of the cytoskeleton with membrane proteins.

micrometers. Force probes should be engineered that can be ‘autocleaved’ during the experiment. Photoactive surface coatings, whose chemical properties can be reversibly switched, may be suitable for accomplishing this.

Besides separating specific from unspecific interactions, the quality of SMFS data acquisition must be improved to increase the comparability of data between experiments and laboratories. Currently, the methods used to calibrate SMFS tools differ substantially in their accuracy⁴. A reason for such discrepancies is that the calibration methods for the different force sensors, such as cantilever, piezoelectric actuator, optical and magnetic field, bead size and molecule, differ. Often, multiple calibration procedures exist for each SMFS method and device. Unfortunately, different calibration procedures used for the same SMFS method

can reveal substantially different results, at times exceeding 10%. Suitable calibration methods should yield consistent results, be directly applicable *in vivo*, be simple to use and be consistently accurate within much less than 10%.

Develop ways to treat and interpret complex cellular data

Currently, theoretical models describe how biomolecular bonds rupture under certain experimental conditions^{3,6}—that is, if an externally applied force lowers the energy barrier that separates one state from another state (for example, a bound or folded state from an unbound or unfolded state). Inside the cell, membranes, polypeptides, polymers or nucleic acids are exposed to a variety of specific and unspecific interactions that are sensed by SMFS. This variety depends on the cell’s functional state and on the location

within the cell^{7–9}. Potentially, these cellular interactions alter the reaction pathway of a biomolecule from that examined *in vitro*. Moreover, interaction networks and reaction pathways continuously change in a cell, so even if a specific biomolecular interaction is sensed at a particular location in the cell, this interaction may be temporally modulated.

Consequently, the SMFS data will be more complex *in vivo* than *in vitro*, creating new challenges for data analysis. Classification of the sensed events may help understand how biomolecular interactions are established at given times and cellular locations. Ideally the complexity of the data will provide insight into the cellular environment in which an interaction was probed. As the experimental conditions used to probe dynamic biomolecular interactions *in vivo* differ from those gained using *in vitro* SMFS, which uses idealized conditions, these differences must be considered in theoretical models. To interpret such increasingly complex SMFS data, existing models must be extended or new ones must be developed.

It has been shown that a biological interaction exposed to a noncontinuous force changes strength and kinetic properties. Therefore, we also have to determine to what extent cellular dynamics directly acting on the force probe and thermal motions of the force probe change the properties of the biomolecular interaction being measured.

Improve data statistics and analysis

Analyzing the interactions and kinetics of single molecules in living cells means acquiring statistically relevant datasets with hundreds of measurements. As an analytical method, SMFS depends on application of force to quantify the transitions of biomolecules. Thus, force must be reproducibly applied as ascending or descending force ramps, force clamps or force oscillations. Only if the force applied shows exactly the same 'histories' can we test and compare well-defined populations of biomolecules. With appropriate amounts of data, increasing force sensitivity and temporal resolution, biomolecular kinetics may be assayed at several time points during a particular force experiment. Here we describe how these steps may be addressed for SMFS in living cells.

Inside the cell, force probes are exposed to an increased complexity of unspecific and specific interactions. Although, as discussed above, there may be ways to reduce unspecific

interactions, this issue will complicate the SMFS data recorded *in vivo*. Because SMFS experiments are generally used to measure the strength or lifetime of a biomolecular interaction far away from equilibrium, a few experimental measures are not sufficient to make statistically meaningful conclusions. The certainty P of detecting a minimum of κ interaction events that occur in one SMFS measurement with a probability p depends on the number of measurements (n) conducted. P decreases if the number of interaction events k to be detected in one experiment increases. The relation of these parameters is given by binominal statistics:

$$P(k \geq \kappa) = 1 - \sum_{i=0}^{\kappa-1} \binom{n}{i} p^i (1-p)^{n-i}$$

For example, at least 290 SMFS measurements must be conducted to detect, with a certainty of 95%, a biomolecular interaction that occurs with a probability of 10%. The number of measurements increases dramatically if several interactions of low probability are to be detected. This is the case *in vivo*, where the number of interaction events is dramatically higher than *in vitro*.

Understanding how cells control their molecular machinery requires studying how this machinery adapts to a cell's functional state. This necessitates being able to repeatedly force-probe the molecular machinery at different cellular locations and under different conditions. To reach this goal, SMFS techniques must be established for the high-throughput and automated quantification of biomolecular interactions in living cells. Fully automated SMFS robots that allow the characterization of isolated biomolecules have already been designed and combined with automated data analysis²². These methods now need to be adapted for use in living cells. Advanced signal processing tools appear to be useful for aligning, categorizing and averaging of single-molecule force spectra. Grouping force spectra into subpopulations unravels different hierarchical pathways. With such techniques, the use of SMFS should thus enable detection of subpopulations of cellular machinery systems that coexist in different functional states in the cell. Force spectra of subpopulations identified by multivariate statistical analysis can then be averaged allowing small but recurrent signals to be identified. These analyses will require parallelization of experiments so thousands of cellular interactions can be probed at the same time²³.

Microfluidic circuits may be useful to harbor arrays of single cells cultured under controlled conditions²⁴ for simultaneous probing with arrays of force probes^{13,23}.

Sense multiple parameters simultaneously

Currently, SMFS is used to sense one specific interaction at the time, but as we have discussed, biological processes involve complex networks of interactions. Recent AFM styluses resemble nanotechnological toolboxes that simultaneously measure multiple parameters^{5,13}. Transferring similar approaches to SMFS could provide multifunctional force probes that concurrently sense electrostatic potentials, ion currents, pH values, electrolytes, temperature, viscosity or the occurrence and concentration gradients of specific molecular compounds in the cell.

Even if all these parameters can be measured in a single location, a key question in cell biology is to understand how the structural heterogeneity of cells correlates with cellular interactions^{7–9,25}. Unfortunately, most SMFS methods lack sufficiently high spatial resolution (≤ 100 nm) to locate the interaction probed. Among these, AFM is the only one capable of imaging the surfaces of living cells at a resolution approaching ~ 50 nm^{2,14}. Using AFM in both imaging and SMFS modes allows visualization of the cell surface and localization and measurement of specific biomolecular interactions simultaneously. This capability has allowed investigation of the dynamic reassembly and functional adaptation of cell-surface receptors in response to the cellular environment^{2,14}, but new approaches will be needed to image and map biomolecular interactions inside the cell where AFM cannot be used.

Light microscopy, in contrast, has proven remarkably useful for studying the interior of living cells. New chemical and biological fluorescence labeling techniques have catalyzed new applications and insights in cell biology¹⁸. Theoretical and instrumental developments broke the resolution limit of conventional light microscopy. Using single-molecule FRET, individual macromolecules can be localized and their interactions and conformations can be qualitatively described^{19,25}. Such fluorescence tools provide unprecedented possibilities for tracking the real-time position, distances, distribution and dynamics of molecules in complex biological samples, at high spatial (few nanometers) and temporal (few milli-

seconds) resolution. Combining SMFS with light microscopy to simultaneously localize, quantify and manipulate biomolecular interactions *in vivo* appears to have the greatest promise for probing the complex landscape of interactions and forces in living cells.

Summary

We highlighted the challenges that, in our view, should be addressed to bring SMFS into the cell. Although SMFS is a well-established method to quantify biomolecular interactions *in vitro*, substantial hurdles remain to be tackled to quantify these interactions *in vivo*. We are confident that if addressed earnestly and competently, these hurdles can be overcome. At the end of this avenue, the possibility of using SMFS *in vitro* and *in vivo* will shine light into how cells establish and control interactions to guide their molecular machinery.

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The authors declare no competing financial interests.

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