nature chemical biology

REVIEW ARTICLE

PUBLISHED ONLINE: 17 OCTOBER 2012 | DOI: 10.1038/NCHEMBIO.1082

Force probing of individual molecules inside the living cell is now a reality

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Biological systems can be quantitatively explored using single-molecule manipulation techniques such as optical or magnetic tweezers or atomic force microscopy. Though a plethora of discoveries have been accomplished using single-molecule manipulation techniques *in vitro*, such investigations constantly face the criticism that conditions are too far from being physiologically relevant. Technical achievements now allow scientists to take the next step: to use single-molecule manipulation techniques quantitatively *in vivo*. Considerable progress has been accomplished in this realm; for example, the interaction between a protein and the membrane of a living cell has been probed, the mechanical properties of individual proteins central for cellular adhesion have been measured and even the action of molecular motors in living cells has been quantified. Here, we review the progress of *in vivo* single-molecule manipulation with a focus on the special challenges posed by *in vivo* conditions and how these can be overcome.

The ability to manipulate individual macromolecules while measuring forces has the potential to reveal fundamental chemical, biological and physical properties often hidden in ensemble experiments. If a macromolecule exerts a force, its action can be probed by single-molecule force spectroscopy techniques capable of manipulating individual macromolecules and operating in force-distance ranges relevant at the cellular level. *In vitro* single-molecule force spectroscopy has revealed fundamental properties and mechanisms of several of the cellular workhorses. Indeed, the molecular motor kinesin has been found to move in a stepwise hand-over-hand fashion, and its stalling force, the conversion between biochemical and mechanical energy and details regarding its force-generating mechanisms have been discovered[1–3]. Several of the myosin family motors, polymerase, topoisomerase and the ribosome have also been studied in great detail *in vitro*[4–8]. Of interest to *in vivo* studies are also measurements of the mechanical strength of DNA[9–11] and of nonmotor proteins[12,13].

In vitro, it is possible to isolate the influence of one relevant parameter, and the results are relatively 'clean'. However, the results obtained *in vitro* may not faithfully reflect properties of the same molecule *in vivo*. For instance, the diffusion of membrane proteins *in vivo* has been shown to be rather different from that *in vitro* in artificial membranes, as the protein motility depends on cellular metabolism[14]. Also, molecular motors seem to have somewhat different characteristics *in vivo* than *in vitro*, dyneins have shorter runs inside a living cell[15] than in a test tube[16], and ribosomes seem to translate over ten times slower along mRNAs *in vitro*[6] than they do in ensemble *in vivo* measurements[17]. Differences between *in vitro* and *in vivo* performances of individual molecules are probably caused by different experimental environments. By investigating individual molecules in their natural environment inside a living cell, it may be possible to understand the impact of the cell's physiological state and metabolism on molecular task and performance. Also, the coupling between biochemical and mechanical energy that allows the cell to convert the energy stored in, for example, ATP to a mechanical motion may be more easily elucidated inside a living cell, which has all of the components necessary for the conversion.

Several techniques capable of manipulating individual molecules have emerged during the last decades. The first atomic force microscope (AFM) was invented in the 1980s[18]. Built on the scanning tunneling microscope, the AFM has the advantage that it does not require a conductive sample and can be readily used under ambient pressure and even in aqueous environments; hence, it has the potential for *in vivo* investigations. In the 1990s, it was shown that individual microorganisms could be confined in space by a single tightly focused laser beam, that the microorganisms appeared to stay alive and that forces exerted by molecular motors acting inside the cell could even be measured[19,20]; this technique, which later found widespread use within the physical, chemical and biological disciplines, is denoted 'optical tweezers'. In parallel with using light to confine dielectric particles, magnetic fields have also been used to manipulate micron-sized magnetic particles attached to molecules of interest. Compared to optical tweezers, magnetic tweezers have the additional advantage that they make it relatively easy to rotate the particles[21,22], and as biological material essentially does not react to magnetic fields, magnetic tweezers are only minimally invasive when used for *in vivo* studies[23]. Other single-molecule techniques that have certain advantages were also developed, such as the bio-force probe, which uses the deformation of a soft object (for example, a vesicle or a blood cell) as a measure of the applied force[24], or micropipettes or needles applied in different forms. Several reviews have focused on the application of single-molecule manipulation techniques *in vitro* explaining the technical details, advantages and disadvantages of each technique[25–28].

Despite high interest in performing single-molecule manipulation and quantitative force measurements inside the complex cytoplasm of a living cell, results are still relatively sparse. The delay in obtaining *in vivo* results at the single-molecule level is probably caused by the technical challenges related to operating on the single-molecule level under *in vivo* conditions[29]. Through new developments in optical and magnetic trapping as well as atomic force microscopy (reviewed in **Box 1** and **Table 1**), these techniques are now meeting most of the *in vivo* challenges and are being used for *in vivo* single-molecule manipulation. Here, we present the route to obtaining successful force probings of individual molecules *in vivo* as well as recent results in this realm.

Getting a handle on the biological system

To manipulate individual molecules inside a living organism, the molecule of interest needs to be attached to a handle through which force can be transduced. The handle should be bio-orthogonal and allow for manipulation or visualization of the molecule of interest. It is crucial that the handle is indeed specifically attached to the system of interest. It is equally important to take precautions to eliminate

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Box 1 | *In vivo* single-molecule techniques.

Optical tweezers are formed by tightly focusing a laser beam. It is the only nanotool capable of manipulating naturally occurring cytoplasmic organelles without penetrating the cellular membrane. Any object with an inducible dipole will be drawn along the gradient of the intensity profile. Precise force-distance measurements are typically done using one or two traps, although a large number of traps can also work in parallel[78,79]. The spatial resolution of an optical trap can be brought down to 0.2 nm, and the temporal resolution can be easily brought down to μs. An optical trap exerts a harmonic potential on the trapped object with a typical stiffness of ~0.01–1 pN nm^{-1}, depending on laser power and alignment. Naturally occurring endogenous lipid granules[58,57,80,81] can be used as handles for an optical trap. Individual gold nanorods[32] or spheres[82–84] as slender as 8 nm in diameter can also be optically manipulated, their tiny size making them attractive probes for intracellular force investigations. **Figure 1** shows optical trapping of a force-transducing handle attached to a kinesin molecule stepping along microtubules inside a living cell. Heating[33,84,89,90] and creation of radicals can lead to physiological damage of optically trapped living organisms[85–87]. Anyhow, microorganisms trapped with an infrared laser remain physiologically competent if held at reasonable laser powers with exposure times below 10 min[88]. Proper force calibration inside a living organism poses a challenge, but methods exist that take into account the viscoelastic nature of the cytoplasm[91–95].

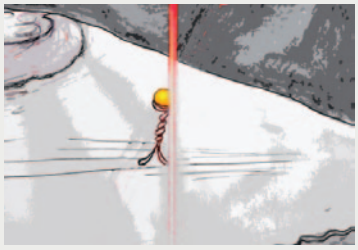


Figure 1 | A kinesin motor carrying a force-transducing handle and monitored by optical tweezers inside a living cell. Using the optical trap (red line), the step size and the force production by an individual kinesin motor can be quantified.

Magnetic tweezers are able to reach inside living organisms in an essentially noninvasive manner as biological material is not very susceptible to magnetism. However, as there are no permanent nor inducible magnets present inside a living cell, most commonly, a superparamagnetic particle is inserted into the organism (**Fig. 2a**), specifically attached to the molecule of interest and then used as a force-transducing handle (**Fig. 2b**). The typical diameter of such a magnetic particle is between 0.5 μm and several microns, and it feels a force proportional to the gradient of the field intensity. The position of the magnetic particles in the field is monitored by a camera, and image analysis provides a spatial resolution of 5–10 nm in three dimensions[21,22,96]. The image-based calibration procedures function equally well inside a living cell. Forces are typically in the pN-to-nN range, enough to rupture most covalent bonds. One notable difference between magnetic tweezers and optical tweezers is that magnetic tweezers can be set up such that the force on the magnetic bead is nearly constant (varying only ~0.01 pN over distances as large as 10 μm). As the entire sample is subject to the magnetic field, magnetic tweezers have the advantage that several molecules can be manipulated simultaneously, but this could potentially be a drawback as a particular magnetic bead cannot be selected. By rotating the magnets, a controlled torque can be transferred to the magnetic bead, causing it and the attached molecule to rotate[97] (**Fig. 2b**). Also, optically trapped objects can be rotated in a controlled fashion, but this is technically more cumbersome[98].

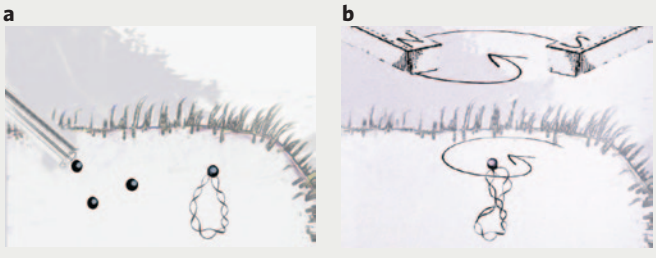


Figure 2 | Magnetic beads are microinjected, attached to DNA plasmids and manipulated by magnetic tweezers. (**a**) A microneedle penetrates the bacterial outer membrane and delivers magnetic particles into the cytoplasm. The magnetic particles specifically attach to plasmid DNA. (**b**) Magnetic tweezers twist the magnetic particles, thus inducing a controlled number of supercoils in plasmid DNA inside the living bacteria.

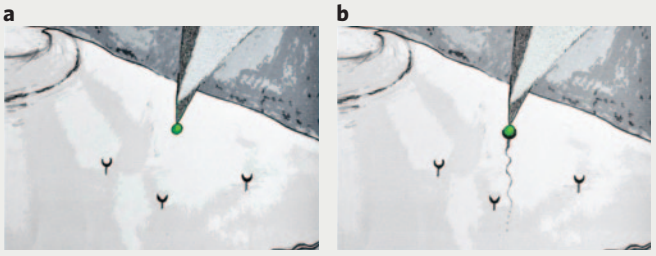


Figure 3 | An AFM maps out a cellular surface, attaches specifically to a protein and performs force spectroscopy on the protein. (**a**) Mapping out the surface, locating receptors matching the antibody on the tip. (**b**) The tip is firmly attached to a receptor matching the antibody, the tip is contracted, and the receptor is pulled out of the membrane surface.

An AMF senses its environment through mechanical interaction with a microscopic tip attached to a cantilever. The AFM is particularly well suited for *in vivo* measurements of molecules on the surfaces of cells because it works in solutions and at physiological temperatures[99]. Measuring inside the cytoplasm of a living cell would require that the cantilever penetrates the cell wall, a relatively invasive event potentially affecting the physiological state of the cell. In contrast to the magnetic and optical tweezers platforms, high-quality AFMs are commercially available and are probably the single-molecule technique that has gained the most widespread use. A piezo-electric motor positions the cantilever precisely, and the tip can be functionalized for specific attachment. The typical AFM cantilever has a spring constant somewhat higher than that of an optical trap, thus allowing for application and measurements of higher forces, and the spatial resolution can be brought down to 1 nm. If operated in the scanning mode (**Fig. 3a**), the AFM can map out a two-dimensional cellular surface with nanometer resolution. It can also be used as a one-dimensional force measuring tool—a 'molecular force probe' (**Fig. 3b**). The Bell-Evans model can be used for data analysis[35], predicting that the rupture force of a particular bond increases linearly with the logarithm of the loading rate. This is true for most chemical bonds, but not for the so-called 'catch bonds' optimized for binding at a particular loading rate[100].

or minimize unspecific bindings. If the force-transducing handle is outside the living organism, for example, during the study of membrane protein motility or of the proteins involved in cellular adhesion, unspecific bindings can be efficiently suppressed by adding BSA, α-casein or other proteins to the sample, as is routinely done in *in vitro* single-molecule experiments. However, if the handle is inside the cytoplasm, nonspecific bindings are essentially unavoidable, and clever control experiments such as calibrating without the specific binding to the molecule of interest have to be done.

Typical handles. Polystyrene, metallic and magnetic beads are commercially available in many sizes with a variety of coatings. Also, user-friendly kits to conjugate most antibodies to the handle exist. AFM tips are commercially available with a variety of specific coatings. For AFM experiments, it is often advantageous to have a linker between the cantilever and the molecule of interest as the linker reduces the risk of nonspecific binding between cantilever and substrate and also assists in avoiding a direct coupling between fluctuations of the cantilever and the molecule of interest[30]. One can use a polymeric linker, which has a series of binding sites to the molecule of interest[30]; thus, a force with multiple peaks is obtained during each retraction curve, and the distance between peaks assists in determining whether the probed binding was specific or not.

A promising force-transducing handle for *in vivo* single-molecule optical manipulation is a colloidal quantum dot. Quantum dots are easy to localize inside the cytoplasm, and they can be individually optically manipulated[31]. Also, their characteristic blinking can be used to quantify them at a particular location. Also of interest for *in vivo* labeling and force-transduction is a gold nanorod, which is also extremely luminescent, does not bleach and can be optically manipulated and aligned[32]. If resonant with the confining laser light, however, gold nanorods can heat considerably, the exact temperature being dependent on laser power and orientation[33].

Conjugation. The most commonly used conjugations between a single molecule of interest and a handle are receptor-ligand or antibody-antigen couplets, and these can be highly specific and stronger than the force applied through the single-molecule technique. However, in reality, most chemical bonds, including those to the handles, have strengths that are comparable to the forces exerted, for example, in a typical AFM experiment. Also, the force required to rupture a particular band is strongly dependent on the force loading rate[34–36]. One conjugation that has been quite successful for attachment of handles for single-molecule manipulation, also *in vivo*[37], is the biotin-streptavidin bond, whose strength is nearly equal to that of a covalent bond. Another commonly used specific conjugation system relies on digoxigenin-antidigoxigenin binding, though this is not as strong as the biotin-streptavidin bond. Histidines or reactive cysteines within proteins provide another means through which specific attachments can be made. A new alternative to this with special appeal to single-molecule experiments is the so-called 'affinity clamp', which is based on the peptide–PDZ domain interactions[38] and seems to be robust, specific and able to withstand substantial forces.

One-to-one correspondence. To investigate the action of a single molecule, it is crucial that there is a one-to-one correspondence between the handle and the molecule of interest. When viewed in a normal microscope, the image one sees of a particle is its point-spread function (PSF). The PSF has a linear dimension of minimum 250 nm, the optical resolution of a normal microscope. If the handle is larger than its PSF, a multiplicity of handles is directly visible in the microscope. However, if the nanoparticles used as handles are smaller than their PSF, it is not possible to determine through normal microscopy whether one or more particles are within their PSF. Hence, a signal originating from several nanoparticles each attached to at least one molecule within one PSF might be falsely interpreted as stemming from a single particle or a single molecule. It is equally important to ensure that only one active molecule is attached to each handle. This is not trivial, especially in the crowded cytoplasm, though the likelihood of attaching only a single molecule to the handle can be increased by lowering the concentration of the chemicals for bioconjugation; for example, if the biotin-streptavidin conjugation is used, only beads sparsely coated with streptavidin should be used or only a very minor fraction of the molecules of interest should be biotinylated.

Internalizing the handle. One challenge for *in vivo* single-molecule manipulation is to get the handle inside the cytoplasm. Unlike the AFM, both magnetic and optical tweezers can operate inside a living cell without mechanically penetrating the membrane. Optical tweezers can use endogenously occurring dense particles with optical contrast, such as lipid granules, as handles. However, for all magnetic tweezers and most optical tweezers applications *in vivo*, it is necessary to insert a force-transducing handle into the organism. Several eukaryotic cells readily take up microscopic and nanoscopic particles through endocytosis, which is one way to internalize particles[39,40]. However, the efficiency of endocytosis depends on particle size, and endocytosed particles will be actively transported, typically along microtubules to an endosomal storing site, rendering them unavailable for manipulation at any cytoplasmic location of interest.

Micropipetting is an alternative to internalizing a handle. Cells with relatively soft outer membranes can be penetrated by a glass micropipette injecting the desired handle at most intracellular

Table 1 | How single-molecule techniques comply to *in vivo* conditions

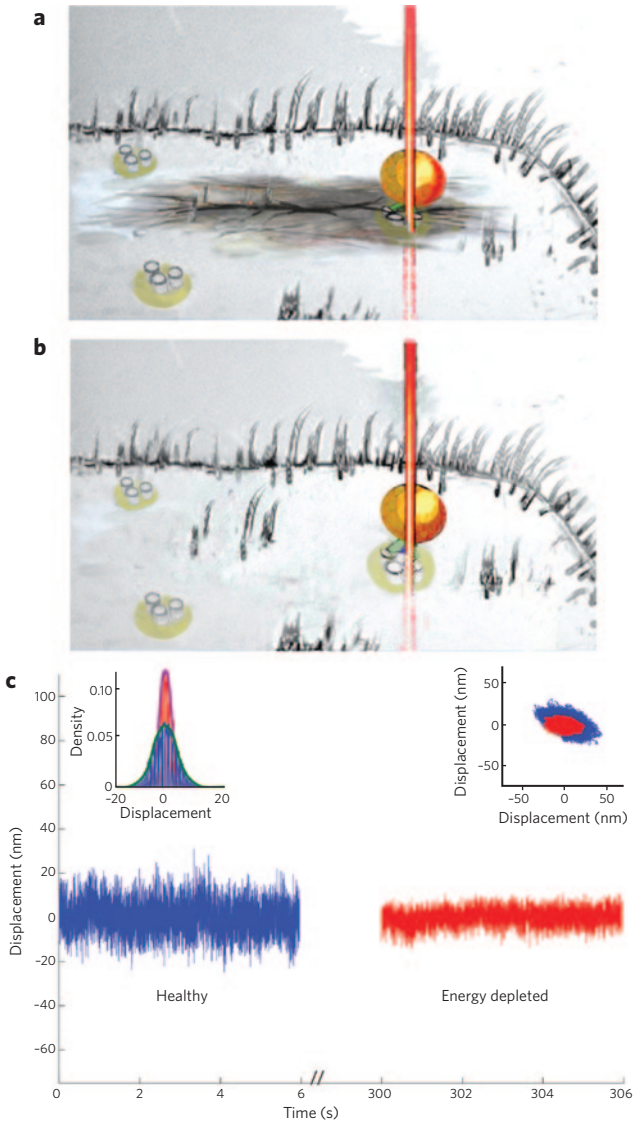
In vivo condition or technique	Optical tweezers	Magnetic tweezers	Atomic force microscopy
Operate on whole cell or organism	Yes	Yes	Yes
Physiological conditions: aqueous environment, pH ~7, body temperatures	Yes	Yes	Yes
Noninvasive method	Possible photodamage and heating	Yes	Can be used on cell surfaces only; attachment of tip might be invasive
Method works inside a cell	Yes	Yes	No, only on cellular surfaces
Handle inside cell	Can use endogenously occurring particles	Must be injected or endocytosed	Not possible without penetrating the membrane
One-to-one correspondence between molecule and handle	With care	With care	With care
Elimination of unspecific bindings	With care	With care	With care
Reliable *in vivo* calibration	Yes[94,95]	Yes[22]	Can only be calibrated outside the cell

locations. Another alternative is to use a powerful and tightly focused laser to burn holes in the cell wall (photoporation) through which submicron particles or proteins can enter[41].

Manipulation inside the living organism

The most relevant environment for single-molecule manipulation is inside the living organism. In 1969, a pioneering study was published[42] where a microneedle was used to penetrate into the nucleus of living grasshopper spermatocytes. In the nucleus, the researchers were able to mechanically apply tension to individual chromosomes and study the influence of chromosome tension and orientation on anaphase segregation. Since then, several investigations overcoming all, or most of, the special *in vivo* challenges have been performed, thus shedding light on how individual molecules act in response to force or exert forces in their natural environment.

Membrane proteins. The first measurements on how individual proteins interact with the outer membrane of living animal cells were obtained by conjugating the membrane protein to a micron-sized bead and dragging this bead-protein complex through the cell wall by optical traps[43–45]. In this experiment, a relatively large force was applied to drag the protein a long distance through the membrane. As the membrane is compartmentalized by the presence of cytoskeletal structures and possibly lipid rafts, this type of measurement is rather invasive as the membrane structures are broken when the protein is forced to move a long distance (**Fig. 4a**).



To probe the local environment, a laser trap was used to confine the protein in its local, natural environment while probing its interaction with the membrane[46] (**Fig. 4b**). The protein is being manipulated in the situations depicted in both **Figure 4a** and **Figure 4b**: in the first by a large force dragging it through the membrane and in the latter with a weaker force confining the protein to a certain location. These results, along with results from video tracking of membrane proteins[47], gave rise to the celebrated fence-tether models, where the proteins can be envisioned as diffusing inside a localized environment, a confined membrane space termed a compartment, and occasionally jumping to a different compartment.

Animal cell walls are considerably different from prokaryotic membranes. Although most animal cell walls consist of a lipid bilayer with embedded proteins, the prokaryotic cell wall is composed of several layers. For instance, the cell wall of *Escherichia coli* consists of an outer lipopolysaccharide coat (the outer membrane), a central peptidoglycan layer and a lipid bilayer toward the cytoplasm. Hence, it is not unexpected that outer membrane proteins move rather differently in prokaryotic and eukaryotic membranes. Using the laser as a localization tool, the λ-receptor, a porin in the outer membranes of living *E. coli* (**Fig. 4b**), was shown to be connected more tightly to the membrane than typical eukaryotic membrane proteins and to have a higher diffusion constant[37,48]. Interestingly, the diffusion of the λ-receptor is dependent on the bacterial metabolism of the host cell: if the cell is depleted of energy, the proteins move significantly less[14,49]. **Figure 4c** shows a time series of the positions visited by a particular λ-receptor both before and after poisoning the cell with azide and arsenate to effectively stop electron transport and ATP synthesis. Hence, protein motility is not purely thermal and is an example of a biological system where the *in vivo* motility is fundamentally different from that *in vitro*. A couple of technical caveats are connected to determining the correct trajectory of a diffusion molecule. For instance, if the data acquisition rate is too slow, the diffusion constant will be underestimated[50].

Cell adhesion. One of the advantages of the AFM is that it can be used both to image a surface with a resolution high enough to see individual molecules and additionally in a mode where the cantilever moves in a direction orthogonal to the surface while simultaneously measuring corresponding values of force and distance at a high temporal rate, thus being able to detect very sudden transitions such as protein unfolding. These two scenarios are sketched in **Box 1**. Thus, in principle, an AFM can determine the location of particular proteins on the cellular surface and then force probe the proteins' adhesive or folding properties. As cellular adhesion typically involves forces higher than those easily achievable by optical traps or magnetic traps, AFM is the preferred single-molecule method for *in vivo* studies of cell adhesion.

Figure 4 | Probing membrane protein motility. (**a**) Sketch of how a gold nanoparticle attached to a membrane protein of interest is dragged through the membrane of a living cell using optical tweezers (red vertical line). The protein is dragged a certain distance through the outer membrane, a relatively invasive event for the cell. (**b**) The optical trap (red vertical line) restricts the motility of the bead-protein complex, thus probing protein motility of the protein in a more local environment. (**c**) Motility of the λ-receptor in the bacterial outer membrane as a function of time measured by the method sketched in **b**. Left, protein position versus time in a healthy cell. Right, position versus time of exactly the same protein but after poisoning the cell by azide and arsenate (energy depleted), thus shutting down cellular metabolism. The insets show the corresponding displacement histograms (left) and positions visited (right). Measurements taken before and after energy depletion are shown in blue and red, respectively. The image in **c** is reproduced from ref. 14 with permission (license number 2963650461819).

Indeed, experiments using AFMs have uncovered adhesion strengths of individual proteins involved in adhesion of living cells[51–53]. One challenge related to using AFM for *in vivo* measurements of adhesion and de-adhesion is that the forces measured also depend on the duration of contact between the cantilever and the sample and on the number and specificity of the adhering molecules. In an AFM experiment, a bone cell (or a small collection of bone cells) was brought into contact with different surfaces. Upon retraction of the cantilever, molecular de-adhesion forces were measured. A typical retraction force-distance curve is shown in **Figure 5** (ref. 52). Also, de-adhesion forces of *Dictyostelium discoideum* cells, which probably attached to the surface via csA glycoproteins, were also measured. The de-adhesion force of an individual csA molecule (or possibly of a csA dimer representing the functional unit) was found to be 23 pN, which is relatively small in comparison to most antibody-antigen interactions (which frequently exceed 50 pN)[52]. However, it is reasonable that adhesion forces are not too large because a cell that is part of a larger structure is often still motile.

The ability of an AFM to map out the distribution of individual proteins in the outer membrane of living cells was used in the study of clustering of membrane proteins of *Saccharomyces cerevisiae*[54]. Integrity sensor Wsc1 proteins were not uniformly distributed in the outer membrane but rather formed clusters with a linear dimension of 200 nm. Also, this study revealed that the signaling in *S. cerevisiae* is coupled to the localized distribution of sensors and receptors in membrane pockets.

Another promising technique for measuring forces related to adhesion dynamics is to use a protein involved in the adhesion process encoding a tension sensor module, which consists of two fluorophores separated by an elastic linker sequence. When force extends the linker, fluorescence resonance energy transfer (FRET) efficiency decreases. This method has been proven valid and capable of measuring forces with pN sensitivity in an assay where the mechanical tension across vinculin was measured and was shown to regulate focal adhesion dynamics[55].

Molecular motors. Given the success of applying single-molecule manipulation techniques to unravel the fundamental properties of molecular motors *in vitro*, the ultimate goal is to investigate molecular motors in their native environment. The first type of single-motor–mediated transport inside living cells to be investigated was the transport of mitochondria along microtubules inside the giant amoeba *Reticulomyxa*[20], a form of transport that is likely to be mediated by kinesin or dyneins. A stall force of an individual motor was found to be ~3 pN. Later, transport of vesicles along microtubules by dynein was investigated inside *Drosophila* embryos[15,56], and it was found that an individual dynein moving toward the minus end of microtubules could be stalled by a force of 1.1 pN. This was measured by optical tweezers using an endogenously occurring lipid granule transported by kinesin as a handle. More recent *in vivo* manipulation measurements show dynein stall forces of 7–8 pN and reveal the individual steps of dyneins inside a living cell[57]. Also, it was found that single lipid granules were often moved by multiple motors of the same type and that the runs were much shorter than expected from *in vitro* assays[16]. Hence, there are differences between *in vivo* and *in vitro* reports on the action of the dynein motor, and the present conclusion is that there exist important collective effects not captured by *in vitro* single-molecule manipulation

Also, the related molecular motor kinesin, which moves cargo along microtubules, was investigated *in vivo* inside a *Drosophila* embryo where optical tweezers used the transported lipid granules as force-transducing handles[58]. The stalling force of an individual kinesin *in vivo* was found to be ~2.4 pN, considerably different from the 5–7 pN observed *in vitro*. In contrast to *in vitro* results, an increased number of motors resulted in neither longer travel distances nor higher velocities *in vivo*[58]; hence, several kinesins could easily be engaged simultaneously to carry a single cargo without affecting transport properties. Another approach to measuring force-velocity relations for vesicle transport in living neuron-committed teratocarcinoma cells was to video track the lipid granules and then use knowledge of the Stokes drag to calculate the force acting on each individual granule[59]. This resulted in force-velocity relations in agreement with *in vitro* single-molecule studies. Using optical trapping of lipid granules carried by kinesin inside living cultured mammalian cells, the regulation of kinesin activity *in vivo* was studied; it was found that the *in vivo* force production of kinesin depends on the amount of CK2 but not on CK2 kinase activity[60].

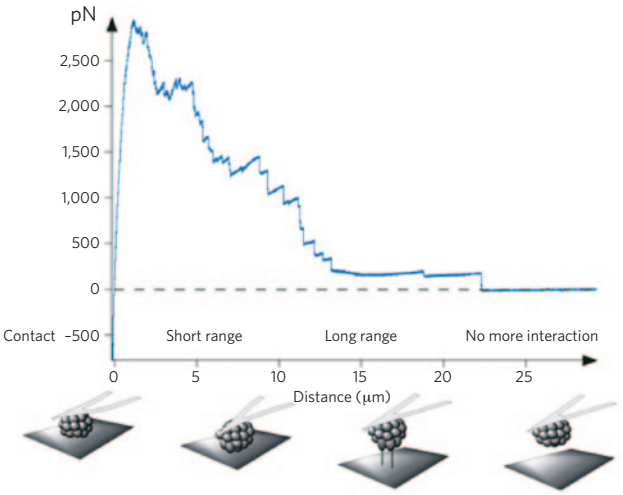


Figure 5 | AFM investigation of strength of individual proteins involved in cell adhesion. As illustrated, the AFM cantilever initially pushes a bone cell (or a collection of bone cells) toward a surface. The cantilever is then retracted, and individual adhesion bonds rupture. The graph shows the corresponding force-distance relation, where individual force steps indicate molecular de-adhesion events. The last three plateaus with steps probably originate from tether formation. Reproduced from ref. 52 (S. Karger AG, Basel) with permission.

The forces exerted by myosin molecules *in vivo* have been investigated by clever incorporation of a fluorescent strain sensor. This sensor was able to detect the interaction between the molecular motor myosin II and its substrate F-actin inside *Dictyostelium* cells, the assay being able to directly detect whether or not a force was acting on the lever arm of myosin II[61]. Using a fluorescence assay where individual myosin V molecules were marked by a quantum dot and internalized in a living cell, myosin V was shown to have relatively similar activity *in vivo* and *in vitro*, albeit with slightly increased velocity and processivity *in vivo*[62].

Magnetic tweezers have been used to study trafficking of endosomes along microtubules mediated by individual molecular motors. This was done in an assay where PC3 human prostatic adenocarcinoma cells were surrounded by magnetic microscopic particles that were readily endocytosed by the cells. With the magnetic trap, the motion of the endocytosed magnetic particles could be monitored as they were transported by molecular motors along microtubules, thus probing the local visco-elastic properties of the cell[63].

Future directions

Many of the challenges connected to applying single-molecule techniques *in vivo* can actually be met, thus opening the door for uncovering the action of individual molecules in their native environment. It is now realistic to quantitatively measure, for example, the forces applied by individual molecular motors as they operate inside the cell and to have an orchestra of different single-molecule manipulation and visualization techniques operating simultaneously on a cell (**Fig. 6**). During cell division, the forces exerted and

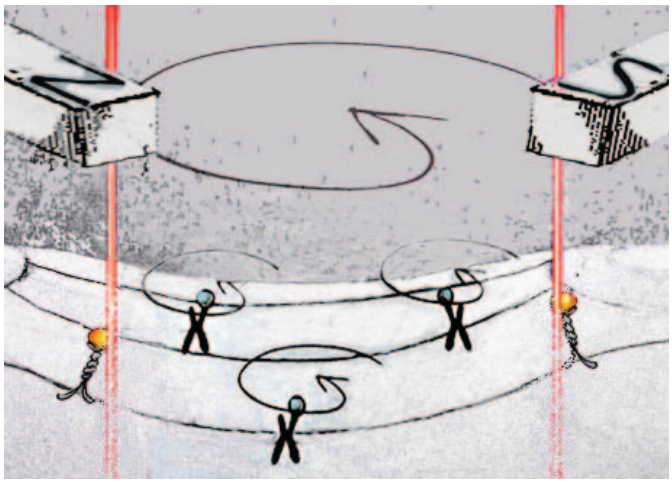


Figure 6 | *In vivo* quantitative measurements of forces involved in separating daughter nuclei during cell division measured by an orchestra of single-molecule manipulation methods. Optical tweezers measure forces exerted by individual kinesin and dynein molecules, pushing the two daughter nuclei apart. Simultaneously, the winding nature, the physical properties and the localization and orientation of chromatin are investigated by magnetic tweezers.

distances traveled by kinesins and dyneins could be studied by optical trapping while simultaneously monitoring the rotation of chromatin or by actively winding it, using magnetic tweezers operating on magnetic nanoparticles specifically attached to chromatin. These types of measurements would fill a large gap in our knowledge regarding the magnitude of forces involved in cell division in a living cell. Recently, the micromechanical properties of extracted single mitotic human chromosomes[64] and of the metaphase spindle in a *Xenopus laevis* egg extract[65] were investigated using microneedles, and the next exciting step will be to perform similar investigations inside a living cell on the single-molecule level.

The cellular transcription machinery could be followed by fluorescent staining or optical trapping while using magnetic traps to unwind and open chromatin, thus providing access to the genes to be transcribed. Also, a lipid granule could be used as a handle in an optical trapping assay with the goal of measuring forces related to polymerizing of microtubules[66] or actin[67] *in vivo*. In a magnetic tweezers assay, the magnetic handles need to be internalized into the cell, but once they are specifically attached to the molecules of interest, several motors can be quantitatively investigated simultaneously *in vivo*. Also, the rotational control of the magnetic tweezers will allow for *in vivo* studies of, for example, the rotation of the bacterial flagella of a living cell, DNA supercoiling, the action of topoisomerase or the rotary F1-F0-ATPase engine inside a living cell.

By attaching a single-stranded DNA tether to an AFM cantilever[68], specific bindings can be established between the tip and a variety of proteins and organelles of the living cell. Cells undergoing this type of attachment have proven superior in terms of cell viability after attachment in comparison to other commonly used specific attachment procedures; hence, this method has huge potential for *in vivo* AFM assays. Also, the prospects of using the same tip first to map out the surface of a living cell and then to choose a particularly interesting protein and subsequently perform force spectroscopy of that particular protein are promising.

The ability to visualize the localization and dynamics of individual proteins by fluorescent labeling is extremely powerful, in particular for investigating, for example, molecular motors or protein diffusion, as the intensity of the fluorescent marker can be used to ensure the 1:1 correspondence between handle and molecule. Therefore, combinations of fluorescent visualization, FRET and manipulation techniques have huge prospects for investigating how intramolecular and intracellular motion is related to force generation. Fluorescence has been combined with optical trapping, AFM and magnetic tweezers[69–72], but it remains to be proven that the powerful super-resolution microscopy techniques[73–75] can be combined with single-molecule manipulation tools. Such a combination would provide superior means for combined visualization and manipulation of individual molecules inside the living cell. One of the latest landmarks *in vitro* is the study of how individual molecular motors interact[76,77]. It would be extremely interesting to uncover collective phenomena among several molecular motors inside a living cell to investigate, for instance, how two polymerases transcribing against each other react upon the encounter; the presence of collective phenomena is probably one of the largest differences between classical *in vitro* studies and the reality inside a living cell. Hopefully, the present review will raise the awareness of scientists expert in central biological problems regarding the possibilities of manipulating individual molecules inside a living cell, thus resulting in a deeper understanding of the task of each individual molecule in the complex game of life.

Received 9 November 2011; accepted 22 August 2012; published online 17 October 2012

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Acknowledgments

The author acknowledges useful comments from E.J.G. Peterman, N.H. Dekker, C. Selhuber-Unkel and M.A. Sørensen and assistance with the illustrations from artist M. Høst. The author acknowledges financial support from the University of Copenhagen Excellence Program and from the Lundbeck Foundation.

Competing financial interests

The author declares no competing financial interests.

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