

EXPERIMENTAL APPROACHES USED TO QUANTIFY PHYSICAL PARAMETERS ON CELLULAR AND SUBCELLULAR LEVEL

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From a mechanical point of view, plant and hyphal cells are more complex than their animal counterparts, since the variety of structural components determining cellular architecture is bigger. In addition to cytoskeletal elements and plasma membrane, the cell wall and the turgor pressure equip plant and hyphal cells with an exoskeleton and a hydroskeleton, respectively. To quantify the physical properties of plant and hyphal cells, a plethora of experimental methods has been developed. This review provides an overview over experimental approaches that have been used to measure turgor pressure and to determine the mechanical properties of the plant cell wall on subcellular level. It is completed by a glimpse into the arsenal of techniques that has been used to characterize the physical properties of the cytoskeletal elements. These have mostly been used on animal cells but might hopefully find their way into plant cell research. Finally, assays and tests to measure the generation of forces by cells and subcellular structures are discussed.

Key words: cytomechanics, cell biomechanics, cell wall, cytoskeleton, hypha, plant cell, tip growth, turgor

The mechanical behavior of organisms and organs is determined by the physical properties of their building blocks, the cells and their components. Cells act as mechanical structures that are able to resist external physical forces such as gravity and to exert forces by way of growth processes. On the other hand, cells are also influenced by external physical forces. These play a fundamental role in the regulation of cell functions, including gene induction, protein synthesis, cell growth, and death. It has been known for a long time that tissues differentiate, grow and remodel in response to changes in mechanical forces. To appreciate the mechanical aspect of cellular architecture and to understand how cells are able to perceive and to react to applied mechanical forces, we need to investigate and quantify their physical properties.

The principle concept of quantitative mechano-physical studies consists in the application of calibrated loads or deformations on an object and the subsequent quantification of its response. Assessing physical properties on the cellular and subcellular level implies a technical challenge, since experimental devices in the appropriate dimension have to be developed.

Depending on the structure under investigation and the precise question to be answered, different approaches have been realized to deform cells or cellular structures and to measure their reactions. Deformations can either be local or global, they can address either an individual cellular component or the cell as a physical entity. This review summarizes some of the experimental techniques and methodological approaches that have been developed to measure the physical properties of cells and cellular components. The focus will be on approaches realized on plant and hyphal cells, but because of its technical advancement the occasional glimpse into progress achieved

in mammalian cells is also provided.

All eukaryotic cells share at least two structural building elements that compose cellular architecture and determine cellular morphology: the cytoskeleton and the plasma membrane. The cytoskeletal proteins function as rod shaped building blocks that are able to resist both compression and tensile stresses. Furthermore, these polymers are dynamic and hence able to actively exert forces through their assembly and disassembly as well as by way of motor proteins. The plasma membrane surrounds the cell and from a mechanical point of view can be modeled as an envelope characterized by its pliability and elasticity.

In plants, fungi and water molds two additional features contribute to the mechanics of cellular structure - the cell wall and the turgor pressure. The cell wall surrounds the cell and depending on its biochemical composition it forms a more or less rigid shell. The presence of this shell allows the establishment of considerable pressure in the protoplast, mainly by way of vacuole swelling. From an architectural point of view plant and fungal cells are, therefore, much more complex than animal cells and all of the subcellular structural building blocks and their interactions have to be considered when attempting to quantitatively assess the mechanical parameters governing cellular functioning. In the following, experimental approaches to assess the physical properties and the mechanical behavior of the three most important structural elements in plant and hyphal cells are discussed: the cytoskeleton, the turgor pressure and the cell wall. The final chapter reviews devices that are used to measure dynamic cellular activities and the generation of forces on cellular and subcellular level.

1 BUCKLING AND BENDING - THE CYTOSKELETON IS AN ELASTIC SCAFFOLD

The elements of the cytoskeleton have multiple mechanical functions. Among others they form the rails along which intracellular transport takes place, they govern cell division and provide the force to contract or to move the leading edge of many animal cells forward (Mitchison and Cramer, 1996). Cytoskeletal elements are also involved in the mechanotransduction of mechanical stresses (Wang et al., 1993). In animal cells the cytoskeleton has an important architectural function, since it determines the shape of the cell. This is particularly obvious in cells that are able to undergo repeated deformations such as erythrocytes on their way through minute capillaries. Maintenance of shape does not seem to be an important cytoskeletal function in plant cells given that plant protoplasts generally round up after having been isolated from the surrounding cell wall. Detailed studies on this topic are lacking, however. Some of the better known mechanical functions of the plant cell cytoskeleton include the interaction with statoliths during graviperception and the formation of the mitotic spindle during cell division, functions that are associated with providing internal structure.

The question how the cytoskeletal elements interact to provide a cell with structure that is able to resist deforming forces has mostly been measured using mammalian cells because of the absence of a surrounding cell wall. This permits the deformation of cytoskeletal elements from the outside, through the membrane. Another reason for the relative abundance of biomechanical data on mammalian cells is the fact that they allow quantitative studies on the change in the physical properties of cells and biomolecules that occur with the progression of certain human diseases such as malaria, sickle cell anemia and cancer. This research therefore enjoys strong support in the medical field (Wang and Thampany, 2006). For an excellent review of the fundamental approaches to cytoskeletal mechanics in terms of flexible polymers and networks refer to Boal (2002).

One of the methods used to probe the mechanical properties of a cell and in particular of the cytoskeleton is *micropipet aspiration* which consists in measuring the suction pressure necessary to partially or wholly suck a single cell into a micropipette (Figure 1A). The diameter of the pipette may range from less than 1 μm to 10 μm and video microscopy is used to record the shape change of the cell (Hochmuth, 2000). Besides the underlying cytoskeleton, this technique also measures the membrane elasticity. This approach to completely deform the cell is particularly relevant in those mammalian cells for which the ability to repeatedly undergo shape changes is pivotal for cellular functioning such as erythrocytes. Other techniques to globally change cellular shape in order to gain insight into cellular mechanics are based on manipulations of cells using *microneedles* (Dennerll et al., 1989) or *optical tweezers* (Hénon et al., 1999). The latter technique uses a laser to trap, control and manipulate

minute objects or particles - hence its other name *optical trap*. The principle of this technique is the light pressure (or gradient force) that moves a particle with higher refractive index than that of the surrounding medium to the focal point of the laser beam and exerts a net force to keep it there (Figure 1B). Among other applications optical tweezers have been used to probe the elasticity of single DNA molecules (Bustamante et al., 2003), protein-protein interaction forces (Litvinov et al., 2002) and the mechanical properties of cytoskeletal elements *in vitro* (Kuo et al., 1995; Kurachi et al., 1995; Tsuda et al., 1996). A list of other applications can be found in Lang and Block (2003).

To be able use optical tweezers to apply large deformations on entire cells silica beads are attached at diametrically opposite points of the cell. One bead is trapped in the laser beam whereas the other one is attached to a glass slide and moved thus stretching the cell (Figure 1B). This approach was successfully applied on red blood cells to investigate the change in mechanical properties due to malaria infection (Mills et al., 2004). The variety of experimental approaches illustrates the versatility of the optical tweezer method. It has the advantage of not necessitating any physical contact with the sample, but the drawbacks are the possible photo damage effect on biological samples and the relatively low maximum force achievable which is a few hundred pN.

While the optical trap is therefore unlikely to be able to cope with the forces necessary to deform walled plant cells in their entirety, it most certainly has potential for the investigation of the plant cell cytoskeleton. In the early days of the optical trap, it was employed to investigate the viscoelastic properties of the plant cell cytoplasm which is determined by the cytoskeletal configuration (Ashkin and Dziedzic, 1989) as well as that of Hechtian strands (Buer et al., 2000). The effect of cytoskeletal structure and the movement of organelles such as statoliths has been investigated in *Chara* rhizoids. The laser-guided displacement of statoliths allowed conclusions on the forces acting on individual organelles by axially oriented actin filaments (Leitz et al., 1995).

Other experimental approaches to measure the physical properties of the cytoskeleton *in vivo* are applied from the outside of the cell but act more locally and thus provide information with subcellular resolution. Among them is *atomic force microscopy (AFM)*, a technique that can act as both a powerful imaging tool and a force sensor with piconewton force resolution. AFM is based on a very sharp tiny tip mounted at the end of a flexible cantilever (Figure 1C). The interaction force between the tip and the sample surface causes the deflection of the cantilever which is signalled by a laser beam reflected off the back of the cantilever and recorded by a photodiode. The sensitivity of the application is high enough to be able to measure interactions between individual biomolecules (Willemsen et al. 2000). As far as intact cells are concerned, the spatial distribution of stiffness of cellular surfaces can be mapped using the Hertz contact theory, which provides analytical solutions to the stress distribution at the interface between and within two elastic bodies.

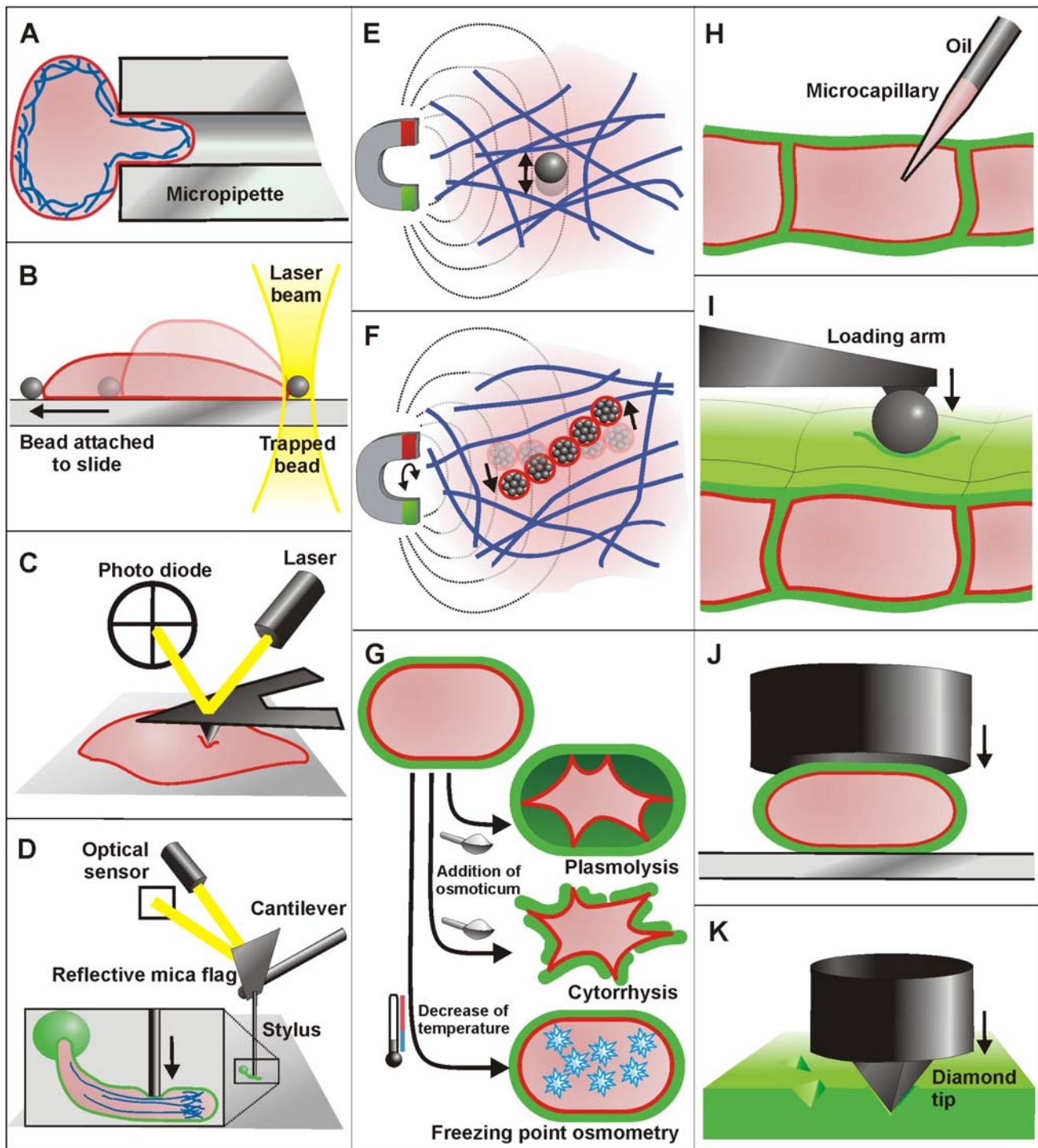


Figure 1

Simplified schematic drawings of cytomechanical approaches. Objects are not drawn to scale. The plasma membrane is depicted in red, cytoskeletal elements in blue, and cell walls in green.

A. Micropipette aspiration. A microcapillary is placed on the surface of the cell and a suction pressure is applied to force the cell into the opening.

B. Global cellular deformation with optical tweezers. Two beads are attached at opposite ends of the cell. One bead is trapped in the laser beam, the other is attached to the microscope slide and can thus be moved away to stretch the cell.

C. Atomic force microscopy. A microscopic tip attached to a flexible cantilever locally deforms the surface of the cell. The bending of the cantilever monitored by reflected laser light provides information on the applied force.

D. Micro-indentation. A calibrated horizontal glass beam serves as cantilever to which a glass stylus is attached that performs local deformations on a cell (in this case a pollen tube). A reflective micaflag allows the monitoring of the vertical position of the stylus.

Figure 1 continued

E. Magnetic bead rheometry. Ferromagnetic beads move upon exposure to an external magnetic field. The movements are monitored optically and provide information on the density and configuration of the cytoskeleton.

F. Magnetic rotational microrheology. Magnetic nanoparticles accumulate in endosomes which form chains in the cytoplasm. These move upon exposure to an external magnetic field that changes direction.

G. Indirect methods to measure turgor pressure. In incipient plasmolysis and cytorrhysis the internal osmotic value is determined by balancing it with the osmotic value of the surrounding medium. In freezing point osmometry the temperature that causes ice crystals to appear provides information on the osmotic value of the cytoplasm.

H. Pressure probe. Turgor pressure is monitored by inserting an oil-filled microcapillary into the cell. It measures the pressure necessary to stabilize the meniscus between the cytoplasm and the oil by way of an electronic pressure sensor.

I. Ball tonometer. A glass ball attached to a cantilever applies a spherical load. The contact surface between ball and cell surface is monitored optically and provides information on the pressure inside the cell.

J. Compression of single cells. A cell is compressed between two plates and the physical properties of the cell wall is calculated from the cell's reaction with the help of a theoretical model.

K. Nano-indentation. A minute diamond tip is used to locally deform a material with a calibrated force. From the depth of the indentation the physical properties of the material can be determined.

In mammalian cells, cytoskeletal fibers underlying the cell membrane can thus be "seen" and their rigidity quantified through the relatively pliable plasma membrane (Simon et al., 2004).

This approach to measure cytoskeletal mechanical properties from the outside is of rather limited use for plant cells because of the surrounding cell wall which is likely to partially or completely mask the stiffening effect of the cytoskeletal elements. It might be possible in situations where the surrounding wall is very thin or absent such as in isolated plant cell protoplasts. A technique similar to AFM, **micro-indentation**, has been applied to thin-walled pollen tubes (Geitmann et al., 2004; Geitmann and Parre, 2004; Bolduc et al., 2006). It consists of a glass stylus connected to a horizontal beam with a known bending constant which gauges an object's resistance to deformation (Figure 1D). Being in the order of few micrometers the deformation amplitude is bigger than that common for the AFM. Importantly, due to the length of the micro-indenter stylus, the horizontal beam is completely out of focus and allows simultaneous microscopic observation of the deformed cellular region (Figure 2). It is thus possible to monitor the deformation proper as well as cellular dynamic processes in the inverted microscope on which the indenter is mounted. This is of particular interest in rapidly growing cells such as pollen tubes or fungal hyphae, since the position of the deforming stylus relative to the growing apex of the cell changes constantly.

Micro-indentation has been successfully used to characterize the cytoskeleton based stiffness of animal cells such as leukocytes (Zahalak et al., 1990). In pollen tubes the cellular stiffness measured by micro-indentation coincided well with the non-uniform distribution of actin filaments in these cells. However, this parameter has yet to be discriminated from the effect of the non-uniform distribution of the mechanical properties of the surrounding cell wall (Geitmann and Parre, 2004). This example illustrates that in plant cells quantifying the cytoskeletal mechanics by way of loads applied from the outside requires complex analysis of the data, even in thin-walled cells.

Approaches that act within the cell might therefore be more promising for the quantification of cytoskeletal mechanics in plant cells. In addition to optical tweezer applications, several other methods are of potential interest for plant cells: In **magnetic bead rheometry** ferromagnetic beads with sizes of few micrometers are taken up by living cells through phagocytosis and tracked optically while exposed to a magnetic field (Figure 1E). The movements provide information on the viscoelastic moduli of the cytoplasm (Bausch et al., 1999). Since plant cells generally are not able of phagocytosis, insertion of such a bead could probably be achieved with particle gun bombardment or microinjection.

A similar principle is realized in **magnetic rotational microrheology** which is based on the analysis of the rotational dynamics of chains of magnetic endosomes in response to a rotation of the external magnetic field (Figure 1F) (Wilhelm, 2003). These endogenous magnetic probes were obtained inside living cells by labeling intracellular compartments with magnetic nanoparticles, following endocytosis and their subsequent concentration in endocytotic compartments. Endosome movements provided information on the viscosity governing the long time flow of the medium surrounding the endosome chains and the relaxation time reflecting the proportion of solidlike versus liquidlike behavior - properties that reflect the configuration of the cytoskeleton.

Instead of the forces applied by a magnetic field, the natural Brownian motion of particles embedded in the cytoskeletal network is monitored in **laser tracking microrheology**. Using a focused, low-power laser beam, a probe particle is tracked by monitoring its forward-scattered light with a quadrant photodiode detector which allows the calculation of the viscoelastic shear moduli of the cytoplasm (Yamada et al., 2000).

Almost all of the methods investigating cytoskeletal mechanics that are described in this chapter have been applied only on animal cells so far. Most of them are suitable for use in walled plant and hyphal cells, however, and might therefore be employed in future research.

2 UNDER PRESSURE - ERECTILE FORCES BASED ON TURGOR

The concept of a stable structure being generated by internal pressure is realized in certain animal organs, but in the plant and fungal kingdoms it is a fundamental

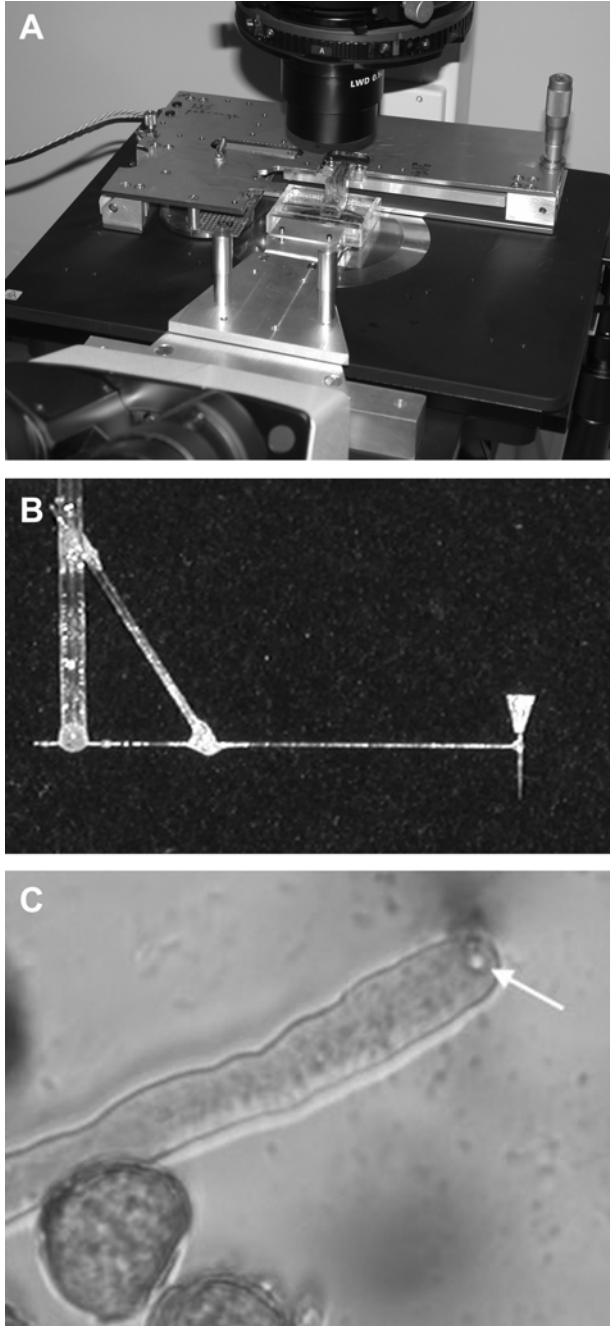


Figure 2. Microindenter.

A. Setup of the micro-indenter device on the stage of an inverted microscope.

B. Cantilever and attached stylus with reflective mica-flag.

C. Microscope image of the micro-indenter stylus touching a pollen tube (arrow). The dark blur above the white contact patch is the out of focus portion of the stylus.

architectural principle of all organisms. By putting cell walls under tension stress, the turgor functions like a hydroskeleton thus contributing significantly to the mechanical properties of plant organs, especially those that are herbaceous. The precise control of the pressure does not only serve to allow for structural stability, but it is also employed for growth and for movement. The technical principle behind most growth processes in plant cells is *irreversible* increase in cell volume driven by water uptake. This process is driven by a difference in the water potential between the protoplast and the apoplast that in turn is caused by stress relaxation in the cell wall temporarily reducing the turgor (Schopfer, this volume). On the other hand, many plant movements are based on the ability of certain strategically located cells to rapidly increase and decrease their volume through water uptake or loss in *reversible* fashion, regulated by the osmotic pressure of the protoplast. The opening and closing of stomatal guard cells, the leaf and branch movements caused by bulliform cells and pulvini are only few examples where this mechanical principle has been realized in order to allow movements on individual cellular or on organ level.

Turgor and water movements therefore play an important role for various physiological functions and their quantification has been an important challenge. Turgor measurements have been attempted with various indirect methods that are based on the quantification of the difference between the osmotic pressure of the protoplast and that of the surrounding medium, since in fully turgid cells this value is approximately equal to the turgor pressure in the cell. *Psychrometric techniques* are based on the fact that the vapor pressure of water is lowered as its water potential is reduced. Psychrometers measure the water vapor pressure of a solution or plant sample, on the basis of the principle that evaporation of water from a surface cools the surface (Boyer, 1995). Many variations of the techniques exist, such as *isopiestic psychrometry* in which the vapor pressure of a tissue is compared to that of a droplet of a defined solution (Nonami et al., 1987).

Other approaches to indirectly assess turgor pressure include *incipient plasmolysis*, in which the osmotic potential can be found by balancing a tissue with a defined external solution so that it is neither turgid nor flaccid (Figure 1G). *Incipient cytorrhysis* or *cell collapse* use the same principle by exposing tissues or cells to a range of external solutions with different concentrations of osmotica and determining at what osmotic value the cells collapse (Figure 1G). This technique was used to determine the turgor pressure in the penetration organs of fungal hyphae, the appressoria, which can reach up to 8 MPa (Howard and Valent, 1996). To appreciate this number it might help to realize that an inflated car tire typically has a pressure of 0.2 MPa and household water pressure amounts to around 0.3 MPa. Various osmometric methods (*freezing point osmometry, melting point osmometry, vapor pressure deficit osmometry*) use the principle that changes in the solute content alter the physical characteristics of a solution such as freezing and melting points (Kaminskyj et al., 1992) (Figure 1G).

A direct method to quantify the turgor pressure in the vascular system of shoots or parts thereof has been realized in form of the *pressure bomb*. By applying an external pressure until water extrudes from the organ it indicates the pressure necessary to balance the xylem pressure (Turner, 1988).

While providing accurate numbers, none of the above mentioned methods allowed the direct measurement of turgor pressure on the single cell level in real time, however. This was only possible with the development of the *pressure probe*. The first pressure probe was a water- and air-filled glass micro-capillary devised to make direct measurements of turgor in the giant-celled alga *Nitella* (Green, 1968). Using Boyle's Law, cellular pressure was estimated based upon the compression of an air bubble. The technique was then improved by the group of Zimmermann and Steudle who replaced the air bubble with an electronic pressure sensor attached to an oil-filled capillary (Figure 1H). Their model also included a piston that allowed turgor in the cell to be varied artificially, thus allowing for the assessment of parameters such as the half time for water exchange, hydraulic conductivity and cell wall volumetric elastic modulus. These parameters were first determined for a number of giant-celled alga such as *Nitella*, *Chara* and *Acetabularia*, in which values for turgor typically ranged from 0.1 to 0.6 MPa (Zimmermann and Steudle, 1978; Tomos, 1988). Further improvements of the device finally allowed the measurement of much smaller cells and thus higher plant cells such as stomatal guard cells could be studied (Hüsken et al., 1978; Franks, 2003). Values in well-watered higher plants were found to range from below 0.1 MPa to over 1 MPa. Plant cell walls therefore have to provide considerable mechanical resistance against these pressures.

While having provided important information, the pressure probe method necessitates the insertion of a micro-pipette into the cell and hence it is invasive and prone to artefacts. A non-invasive method of assessing turgor on single cell level was developed by Lintilhac and Wei, although its applicability is limited to surface cells with rather thin cell walls. They calibrated the cell's ability to bear a spherical load and related the load-induced deformation of its surface with the pressure inside the cell - the same principle as the medical device generally used to measure intraocular pressure (Figure 1I). Pressure is quantified by calibrating the size of the contact area between the deforming glass sphere and the underlying cell and can thus be assessed optically (Figure 3). This *tonometer* approach has been successfully applied on plant epidermal cells in which, if the cells were turgid, this non-invasive method produced results similar to those obtained with the pressure probe (Lintilhac et al., 2000; Wei et al., 2001).

3 PLASTIC OR VISCOELASTIC - THE CELL WALL FORMS A DEFORMABLE SHELL

The cell wall represents the most prominent structural

feature that distinguishes plant and hyphal cells from animal cells. By surrounding themselves with a more or less rigid exoskeleton, plant cells generate tissues that are structurally very different from their animal counterparts. From a mechanical point of view, in plant tissues the cell wall is the dominant load-bearing feature that determines structure. Two mechanical strategies can be identified: *a*) The ability of the cell wall to resist tensile stress allows for the establishment of the turgor based hydroskeleton and thus the generation of erectile forces that are able to withstand applied stresses. This principle is predominantly realized in cells with primary cell walls. *b*) Secondary cell

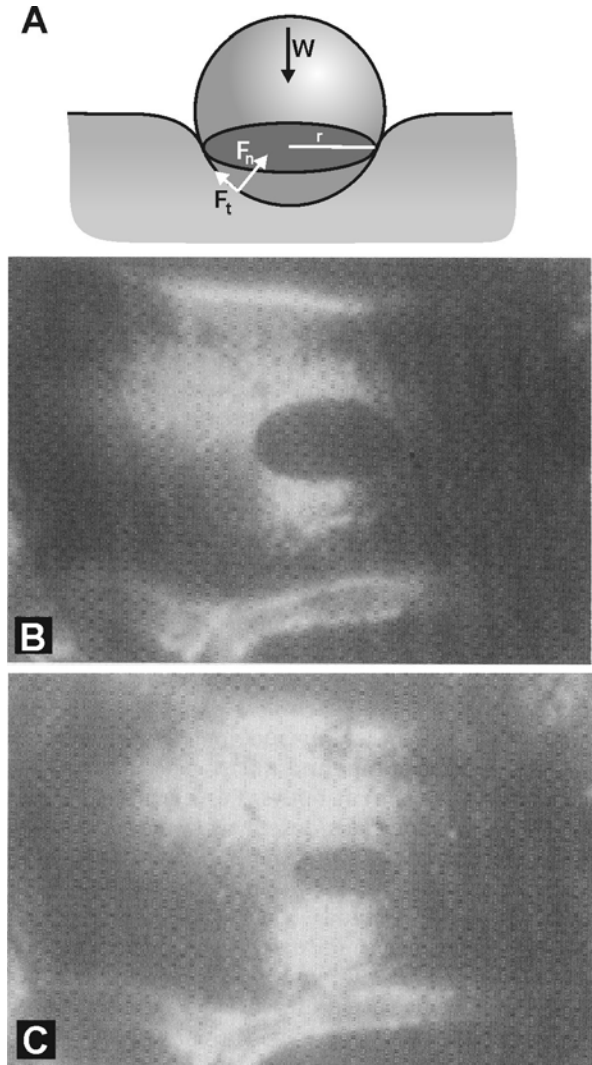


Figure 3. Ball tonometry.

A. The forces acting on the ball indenter. F_n is the normal component of the supporting force, F_t is the tangential traction force caused by friction with the cell surface, r is the radius of the projected contact path, and W is the load acting on the ball.

B, C. Image capture of the contact patch, showing the load-dependent change in contact area between a 300 μm ball probe and an onion cell. B shows the area of contact at 47 mg, C shows the area at 22 mg.

Figure A is redrawn after and Figures B and C taken from Lintilhac et al. (2000) with permission from the authors and the publisher.

walls typical for sclerenchymatic tissue are equipped to not only resist tensile but also compressive stresses. In this case the living protoplast and thus the turgor is dispensable for the cell's mechanical functioning and fully differentiated cells are often devoid of any cell component other than the cell wall.

Mechanical tests of cell walls have been carried out on various levels of organization: tissue, cell and molecule. Most studies used whole plant tissues, which are easy to handle, but the underlying cellular structure precludes direct interpretation in terms of cell wall material properties. On the other end of the scale, individual components of the plant cell wall have been purified or synthesized to measure their respective biochemical and biomechanical properties *in vitro*. Especially those components that in isolated form are of commercial interest such as cellulose and pectin, have been well characterized concerning their mechanical properties using *tensile* or *compression tests* or *rheometric assays* (Jarvis, 1984; Endress and al., 1996; Chanliaud and Gidley, 1999). Depending on the character of the material and the physical property to be assessed, the type of the test differs. In tensile and compression tests, typically applied for the crystalline component cellulose, blocks or strips of the purified compound are clamped and subjected to a deforming stress that stretches or compresses the object. The reaction of the specimen block is quantified and allows conclusions on its physical properties such as the Young's modulus of elasticity (Cosgrove, 1989; Cosgrove, 1993; Kutschera, 1996).

Visco-elastic materials like pectin gels are commonly tested by rheometers that measure the viscosity of the substance. This is done by plunging an object into the substance or by subjecting the material to an oscillating shear stress. The latter is also known as *small deformation oscillatory rheology* where a layer of the test substance is placed between two plates, one of which applies sinusoidal strains to the material. The movement transferred to the opposite plate is then registered and the behavior characterized (Endress and al., 1996; Whitney et al., 1999). Key parameters obtained by this method include the in-phase (elastic or storage) component of shear modulus and the dynamic viscosity.

To physically test cell walls in their natural state, large deformation tensile testing is generally performed by measuring the deformation of isolated cell walls or entire tissue blocks in response to forces applied externally, such as by *extensometers* or *instron-type stress-strain analysers* (Cosgrove, 1989; Cosgrove, 1993; Kutschera, 1996; Schopfer, this volume). These are based on the same principle as the tensile tests for *in vitro* cell wall components. Although such measurements can give answers concerning the mechanical properties, the extensions measured cannot always directly be related to the growth of living cells in a quantitative way, because the applied stress in most cases is unidirectional, whereas the *in vivo* stress in the cell wall due to turgor pressure is twodirectional.

While approaches to study cell wall components *in vitro* or entire cell walls in form of tissue blocks have provided

important information, quantitative physical data obtained on the level of individual living cells are scarce. There are several reasons for this lack of information. Most plant cells are present in the form of aggregates forming a tissue. Isolation of a single cell from such a tissue almost inevitably involves a disturbance of the cell wall structure, even though some authors have succeeded in minimizing this effect by peeling off individual cells instead of using chemical maceration techniques (Keckes et al., 2003). Secondly, while it is relatively easy to clamp a piece of tissue for a creep or bending experiment, the same manipulation on cellular level is not trivial, simply because of the size of the specimen. The development of micro- and nanomanipulation methods has therefore been an important step to obtain information at this scale. Some cells are big enough, however, to allow for manual excision of strips of cell wall that can be clamped into an extensometer. An example is *Chara* for which a detailed multi-azimuthal description of the anisotropic elastic modulus of the walls has been produced this way (Wei et al., 2006).

Another strategy to apply stress on cell walls of individual cells is their *compression* using a micromanipulation probe. This technique has allowed the estimation of the Young's modulus of their cell walls (Wang et al., 2004). The authors placed tomato cells grown in suspension culture into a device that compressed the entire cell between two plates (Figure 1J) and using a theoretical model for the force-deformation data they were able to conclude on the physical properties of the cell wall of these living cells. A similar principle has been realized by using a displacement transducer to obtain the volumetric elastic modulus of the cell walls of the huge *Chara* internode cells (Steudle et al., 1982).

Another method that has been used to measure the elastic modulus of the cell wall in living cells is the turgor *pressure probe*. Under conditions of changing turgor, the cell volume will change by an amount determined by the volumetric elastic modulus. The elastic modulus is therefore measured by determining the relative change in volume (measured by microscopy) in response to a change in turgor applied by the pressure probe (Tomos and Leigh, 1999).

A different concept lies behind the approach to use *nanoindentation* and *modulation force microscopy* (an application of the atomic force microscope) for the quantitative assessment of the physical properties of the plant and fungal cell walls. As mentioned earlier, the principle of such a technique is the application of a cycle of unloading and loading in form of a minute local deformation and the monitoring of the response of the deformed material both regarding its elasticity and plasticity (Figures 1C, 1K). Only very few applications have been published on living cells so far. Among these is an AFM study of the elastic properties of the cell wall of fungal hyphae (Zhao et al., 2005). AFM has mostly been applied on dead plant cells such as wood fibres. Given that wood fibres do not contain a living protoplast at maturity, the authors were able to cut open the cells horizontally and scan the section by applying local deforming forces in the

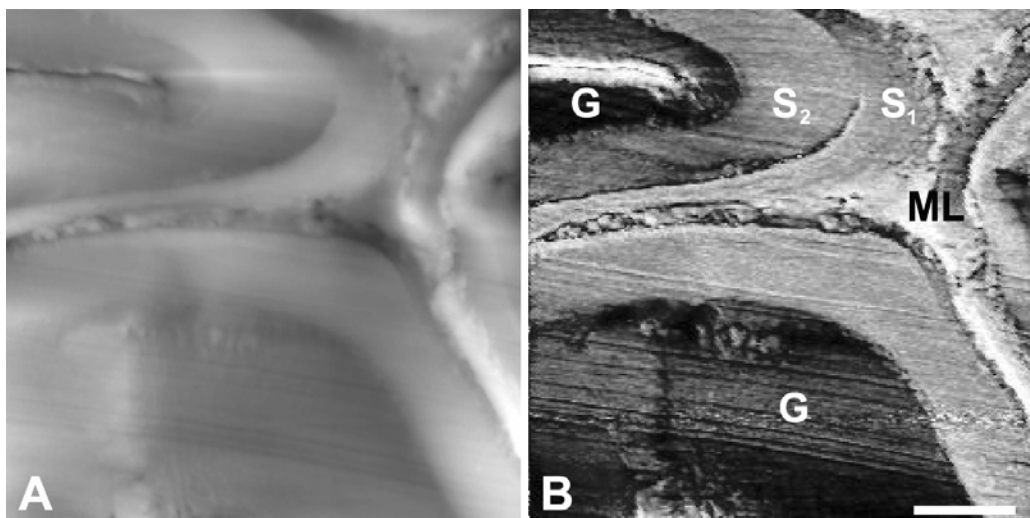


Figure 4. Cell wall layers of oak tension wood. A transverse section of wood was submitted to a scan in an atomic force microscope used in force modulation mode.

A. Topographic image

B. Elasticity image made at 690 kHz providing qualitative information on stiffness: The darker zone corresponds to the stiffer cell wall layers, the G layer being the most rigid and the middle lamella (ML) the least. The lateral resolution of the images in the order of 20 to 50 nm is determined by the radius of the contact area between tip and sample. Bar = 2 μ m.

Images are taken from Clair et al. (2003) with permission from the authors and the publisher.

direction parallel to the cell surface. This method allowed them to assess the elastic properties of the individual cell wall layers present in the fibre cell wall (Wimmer and Lucas, 1997; Wimmer et al., 1997; Clair et al., 2003) (Figure 4). The mechanical properties of wood fibre cell walls were also assessed using tensile forces in combination with x-ray analysis of the microfibril angle (Keckes et al., 2003).

Micro-indentation has shown to be rather useful for the comparative studies analysing various treatments affecting cell wall properties in pollen tubes (Parre and Geitmann, 2005a; 2005b). While the deformations performed by this device are of a bigger amplitude and on a larger surface than those caused by an AFM tip, they are still small enough to provide spatial resolution on these cylindrical cells (Figure 1D). The experiments revealed that the non-uniform distribution of various cell wall components and their configurations are in part responsible for the characteristic gradient of physical properties along the longitudinal axis of pollen tubes.

4 GROWTH AND INVASION - GENERATION OF CELLULAR FORCES

While studying the cellular architecture means understanding a relatively static structure, cells are by no means motionless. They are able to move, change their shapes, form extensions and divide. In all of these activities, cellular forces are involved in generating the

necessary changes in cellular structure. In mammalian cells contractile movements such as cell crawling have been measured using *ruffling tests* that are based on the ruffling of a substratum caused by the traction forces exerted by attached cells (Oliver et al., 1999). The forces generated by the cells can be calibrated by measuring the distortions caused by microneedles with known bending constants. These experiments have demonstrated the central role of the cytoskeleton in generating the forces necessary for cellular movements in animal cells (Condeelis, 1993; Oliver et al., 1994).

Plant cells rarely contract or move in amoeboid manner, but they do share with animal cells their ability to grow, change shape and form cellular protrusions. In animal cells, the formation of cellular protrusions is powered by the cytoskeleton, either by way of motor proteins (myosin, kinesin) (Ishijima et al., 1991) or by the polymerization of cytoskeletal elements proper (Theriot, 2000). It was therefore important to quantify the propulsion and tensile forces that can actually be generated by the assembly (or disassembly) of individual cytoskeletal elements (Cooper, 1991). Such studies have been performed *in vitro* on microtubules which were assembled from purified monomers and made to interact with flexible or rigid barriers (Figure 5A). The subsequent *buckling* of the rod-shaped microtubule polymers was then analyzed to determine the force on each microtubule end (Dogterom and Yurke, 1997). These studies have helped to understand the mechanical function of pushing and pulling forces exerted by microtubules in the positioning of chromosomes

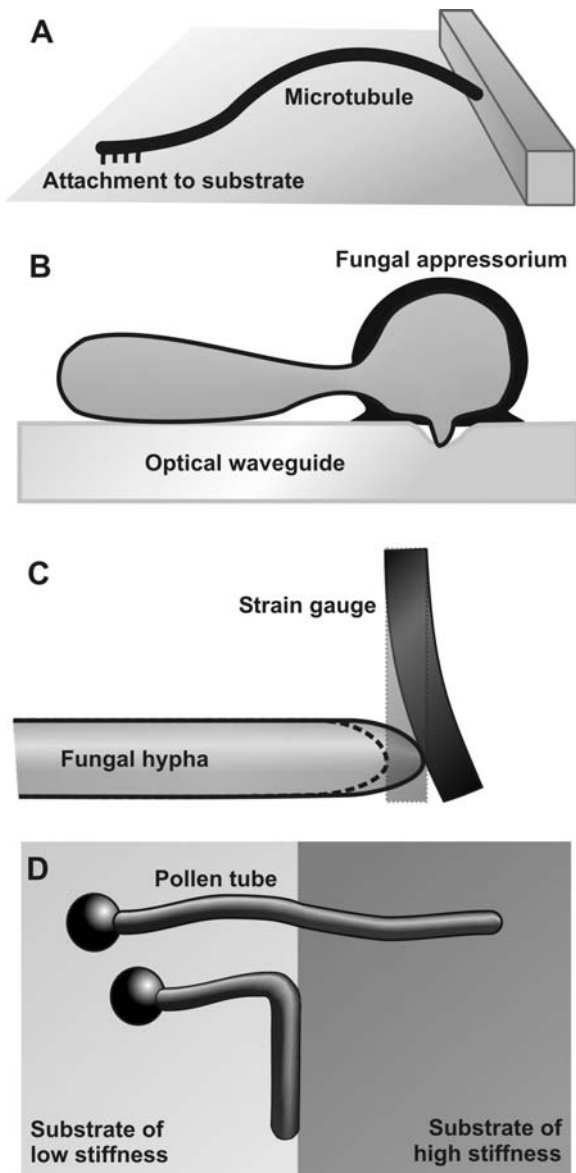


Figure 5. Simplified schematic drawings of cytomechanical approaches. Objects are not drawn to scale.

A. Microtubule buckling assay. Short microtubules attached to a substrate serve as nucleation centers for tubulin polymerization. An elongating microtubule buckles upon encountering a mechanical obstacle. The buckling pattern provides information on the force generated by the elongating polymer.

B. Waveguide microscopy. A fungal appressorium attaches to a waveguide and deforms it by forming a penetration peg. The deformation of the waveguide is monitored optically and the invasive force exerted by the penetration peg is calculated with the help of the known mechanical properties of the waveguide.

C. Strain gauge. A growing fungal hyphae deforms the flexible beam of a strain gauge which allows the quantification of the growth force.

D. Pollen tubes formed in a substrate with low stiffness encounter a mechanical obstacle in form of a substrate with higher stiffness. The percentage of pollen tubes that cross this interface reflects the pollen tube's capacity to exert invasive forces.

and in the force generation at the cell cortex (Dogterom et al., 2005).

In plant and hyphal cell morphogenesis the cytoskeleton has a function in the spatial control of cell expansion (Smith and Oppenheimer, 2005). Whether or not the cytoskeletal elements contribute more directly by providing propulsion forces is very poorly understood even though speculations for possible mechanisms were put forward (Money, 1997). The mechanics of plant cell growth is presumed to be based mainly on an increase in the extensibility of the cell wall that reduces turgor thus causing water uptake leading to an increase in cell volume (Cosgrove, 1993; Kutschera, 2000; Schopfer, this volume). The monitoring of the osmotic and the turgor pressures and the characterization of local cell wall extensibility are therefore important approaches for the study of cellular dynamics and morphogenesis in plants.

In many cases the propulsion force driving cellular expansion does not only have to act against cellular limitation such as the plasma membrane and/or the plant cell wall, but cellular growth has to occur against mechanical resistance generated by the surrounding substrate. In no cell type is this more obvious than in tip growing cells, whose biological purpose it is to invade a relatively solid substrate: the soil in the case of root hairs, the transmitting tissue in the case of pollen tubes and any nutrient providing substrate in the case of hyphae. While the cells often use chemical means such as secreted enzymes or the induction of cell death (in the case of the transmitting tissue) to soften the growth substrate, unless this results in complete liquefaction, they are likely to meet substantial mechanical resistance which has to be overcome. Nowhere is this performance more impressive than in hyphal appressoria. These are specialized structures differentiated from the end of fungal germ tubes, which are able to penetrate mechanical obstacles such as epidermal plant tissue covered by thick cuticles or mammalian skin tissue (Ravishankar et al., 2001; MacDonald et al., 2002).

Attempts to measure the penetration force exerted by fungal appressoria have been made using various techniques (Bastmeyer et al., 2002). In very early studies Miyoshi (Miyoshi, 1895) let the appressoria penetration pegs *perforate gold membranes* (the material was chosen because it is inert and thus not prone to enzymatic digestion) and compared this with the forces necessary for a hyphal shaped glass needle to breach the membrane. More recently (Bechinger et al., 1999) used an *optical waveguide* for the same purpose (Figure 5B). The principle of this technique relies on total internal reflection that is realized in structures such as glass fibers and allows light propagation. The intensity of reflected light depends on the precise thickness of the waveguide and deformations caused by the penetration peg pushing against it can thus be detected and quantified. Provided that the elastic constants of the waveguide are known, the force exerted by the penetration peg can be calculated. Unfortunately, this technique necessitates tight adherence of an appressorium to the waveguide, and will thus be of limited use for other types of tip growing cells.

A different method was therefore used to quantify the invasive forces produced by vegetative hyphae, i.e. hyphae that do not form penetration pegs. The group of Money placed a miniature *strain gauge* in front of a growing hyphal apex and recorded the force exerted by the advancing cell (Money, 2001). When the hypha contacts the beam, the electrical output from the strain gauge immediately changes in proportion to the applied force and the pressure exerted by the cell is derived from dividing the measured force by the contact area with the beam (Figure 5C).

An indirect method to measure penetration force is the *agar penetration assay* that has been applied to both fungal hyphae (Bastmeyer et al., 2002) and pollen tubes (Gossot and Geitmann, submitted). The capacity of tip growing cells to grow within or to invade media stiffened with various concentrations of agarose allows conclusions on the role of various factors such as cell wall rigidity, turgor or cytoskeleton for the exertion of an invasive force (Figures 5D, 6). The use of a penetrometer that measures the mechanical resistance of the agarose allows a quantification of the penetration force.

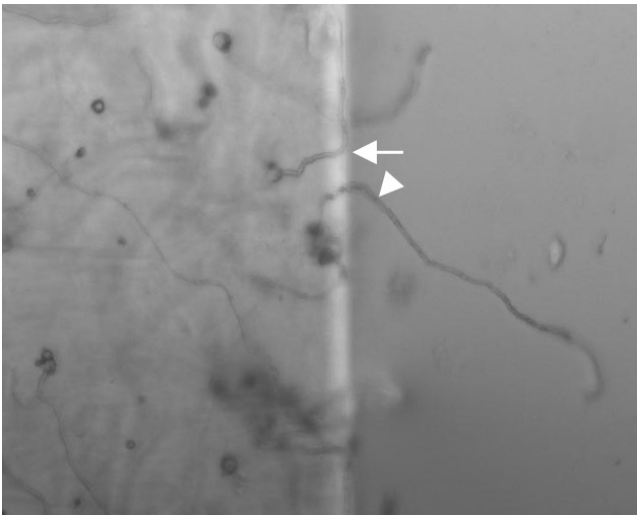


Figure 6

In vitro penetration assay for pollen tube invasive growth. Brightfield image of the experimental setup showing two portions of agarose stiffened growth medium. Pollen are placed in the medium with low stiffness (left). Pollen tubes that encounter the interface to the stiffer medium (right) are counted and categorized either as deviating (arrow) or penetrating (arrowhead).

5 SUMMARY

The quantification of the physical properties of cells and subcellular structures represents a challenge for practical experimentation because of the size of the specimens. A significant variety of approaches has been realized, but more sophisticated methods are certain to be developed,

especially considering that in the recent years micro- and nanotechniques have become increasingly available. Hopefully, plant cell biomechanical research will take advantage of the developments made in the animal field and also pursue new experimental strategies. This will lead to a better understanding of the mechanical aspects of cell physiology such as cellular growth, mechano-transduction and perception of mechanical and physical signals.

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