

# Ball Tonometry: A Rapid, Nondestructive Method for Measuring Cell Turgor Pressure in Thin-Walled Plant Cells

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## ABSTRACT

In this article we describe a new method for the determination of turgor pressures in living plant cells. Based on the treatment of growing plant cells as thin-walled pressure vessels, we find that pressures can be accurately determined by observing and measuring the area of the contact patch formed when a spherical glass probe is lowered onto the cell surface with a known force. Within the limits we have described, we can show that the load (determined by precalibration of the device) divided by the projected area of the contact patch (determined by video microscopy) provides a direct, rapid, and accurate measure of the internal turgor pressure of the cell. We demonstrate, by parallel measurements with the pressure probe, that our method yields pressure data that are consistent with those from the pressure probe. Also, by incubating target tissues in stepped concentrations of mannitol to incrementally reduce the turgor pressure, we show that the pres-

ures measured by tonometry accurately reflect the predicted changes from the osmotic potential of the bathing medium. The advantages of this new method over the pressure probe are considerable, however, in that we can move rapidly from cell to cell, taking measurements every 20 s. In addition, the nondestructive nature of the method means that we can return to the same cell repeatedly for periodic pressure measurements. The limitations of the method lie in the fact that it is suitable only for superficial cells that are directly accessible to the probe and to cells that are relatively thin walled and not heavily decorated with surface features. It is also not suitable for measuring pressures in flaccid cells.

**Key words:** Turgor pressure; Plant growth; Tonometry; Pressure probe; Osmotic pressure; *Allium cepa* L.; *Phaseolus vulgaris* L.

## INTRODUCTION

The key role of turgor pressure in all aspects of plant growth and development has been recognized since

the time of Pfeffer (Pfeffer 1900, p. 134). Measurement of pressures in individual plant cells has remained problematic, however. For almost a century the only available method was the plasmolytic method, whereby plant cells were incubated in a graded series of osmotica. The cell turgor pressure is assumed to equal the osmotic potential of the solu-

tion that produced incipient plasmolysis. The method is based on the inference that the osmotic pressure of the incubating medium at incipient plasmolysis equals the cytosolic osmotic pressure and, hence, approaches the turgor pressure of the cell, assuming that the cell is bathed in pure water *in vivo*.

It was Paul Green who successfully developed a more direct measure of plant cell pressures and described in a 1967 article a method that became the precursor of the modern pressure probe (Green 1968; Green and Stanton 1967). In this ingenious experiment Green inserted a water-filled microcapillary, drawn to a fine open point at one end and sealed at the other, into the end of a *Nitella* internodal cell. The pressure within the cell compressed a bubble trapped within the capillary and allowed him to calculate the original pressure within the cell.

This simple experiment has given rise to a host of related methods, all of which can be roughly categorized as pressure probe methods. These methods attempt to improve on the original *Nitella* experiment in a number of ways. First, by replacing the trapped bubble with a semiconductor pressure transducer and reducing the size of the microcapillary to decrease the dead volume of the system; the sensitivity of the procedure can be increased significantly. Second, by providing a means for restoring the cell to its original volume after inevitably bleeding some of the cell sap into the micropipette during cell penetration; a truer picture of the original pressure in the target cell is possible. The most recent innovations in the use of the pressure probe involve adaptation for the measurement of high negative pressures in the transpiration stream (Wei and others 1999a, 1999b).

The pressure probe has one major flaw, however, in that it necessarily destroys the target cell, making it impossible to make repeated measurements on a single cell. It is also time consuming and technically demanding, making it almost impossible to take more than 5 or 10 good measurements in a day. Nor is it without its own set of assumptions, first among which is that the probed cell does not undergo significant electrochemical changes resulting from the breaching of its cell membrane. Second, one assumes that the flow of water and solutes and the forces driving those flows through the narrow pipette tip are not so large that the cell is unable to return to a stable thermodynamic equilibrium.

What we describe here is a method that when fully developed may obviate many of these difficulties, making it possible to take repeated measurements rapidly and without significant damage to the target cell. This method depends on the assumption that most actively growing plant cells can be treated

as thin-walled pressure vessels, in which case any rigid surface applied to the cell will produce a contact patch that is directly proportional to the applied load and inversely proportional to the turgor pressure in the cell. Thus, under ideal conditions the force applied to the cell surface through the probe, when divided by the projected area of the contact patch, will constitute a direct measurement of the internal pressure of the cell.

The challenge presented by this approach lies in miniaturizing the apparatus such that single cells can be effectively targeted while retaining the ability to clearly visualize and measure the area of the contact patch. We have overcome this problem by constructing the probe as a submillimeter-diameter glass ball cemented to a coverglass fragment with optical adhesive of matching refractive index. The spherical surface of the probe is then applied to the target cell by means of a suitable loading assembly, and the contact patch is observed under the compound microscope through the flat upper surface of the cover glass.

The specimen is illuminated by epi-illumination with a spinning-disc confocal microscope, and the image of the contact patch is recorded and its area measured by video image analysis.

## MATERIALS AND METHODS

### Tonometer

The tonometric method requires, first of all, the ability to accurately position and quantify the load applied to the target cell, and second, the ability to visualize and capture the image of the contact patch. The probe that we have developed for this experiment consists of a small glass ball (Edmund Scientific Co., Barrington, NJ, and Mo-Sci Corporation, Rolla, MO) varying from 0.05 mm–0.5 mm in diameter, depending on the nature of the target tissue. The ball is cemented to a triangular fragment of microscope coverglass with a drop of UV-cured optical adhesive of matching refractive index (Edmund Scientific Co.). The ball assembly is then attached to the end of a slender rocker-arm that has as its fulcrum a jeweled needle bearing (Small Parts Inc., Miami, FL). The tail of the arm is extended by a fine plastic hair that serves as a goniometer pointer with a small mirror installed behind the arm so that its angle can be measured accurately. The total weight of the loading arms used in these experiments ranged between 1.1 g and 1.25 g. The needle-bearing and ball-tonometer assembly is supported by a bracket extending from a simple mechanical micromanipulator so that the ball indenter can be accurately

positioned under the microscope objective. The rocker arm assembly is trimmed such that when the upper surface of the coverglass is transverse to the gravity vector and the microscope axis, the attached ball applies a known axial load to the cell whose pressure is being measured. By adding some removable weights to the tail of the loading arm, the load applied by the ball to the cell can be adjusted to range between 20 mg and 75 mg. These loads were calibrated with an electronic balance (Mettler PM460, Highstown, NJ).

The specimen is positioned on the stage of a standard research microscope and observed through a low-power (10×–20×) objective. The ball is lowered onto the surface of the target cell and the microscope is focused through the coverglass onto the lower surface of the ball, where it contacts the cell surface.

Visualization of the contact patch is accomplished by means of white light epi-illumination through a spinning-disc confocal attachment (Technical Instruments Corp., San Francisco, CA) (Petran and Hadravsky 1989). This provides for rejection of out-of-focus light from below the plane of focus and increases the contrast between the contact patch and the surround.

Image capture is by means of a Pulnix black and white CCD camera (Pulnix America Inc., Sunnyvale, CA) attached to the trinocular head of the microscope, which transmits the image to an Optimas image analysis station (Media Cybernetics L. P., Bothell, WA), where standard computer morphometric methods can be used to circumscribe and derive the area of the contact patch.

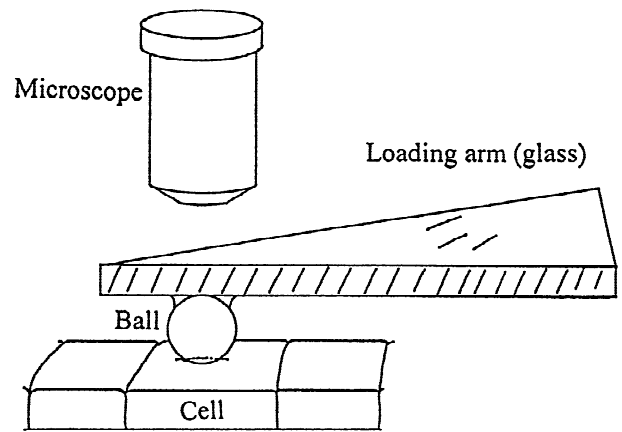
## Plant Material

Single adaxial epidermal cells from peels of fresh, Spanish onion leaf bases (*Allium cepa* L.) were chosen for tonometric measurement. Epidermal peels were mounted flat on large microscope slides in a drop of water or other osmoticum. Tonometry was carried out at five distinct load levels for each cell measured. In addition, separate peels were assessed after being mounted in a medium of known osmolarity to test the method under reduced turgor conditions.

Other plant materials included bean hypocotyl (*Phaseolus vulgaris* L.). The bean materials were used predominantly for underwater trials and to test the reliability of the method with smaller cell sizes and smaller probe diameters.

## Verification

All tonometry measurements made on the onion adaxial epidermal tissue were verified by pressure



**Figure 1.** The relationship of tonometer probe assembly to the microscope axis and target tissue, generating a cup-shaped contact patch. A turgid cell is compressed by a ball-shaped indenter, resulting in a spherical, cup-shaped contact.

probe determination of cell turgor pressure. The pressure probe consisted of a water and silicone oil-filled glass micropipette ground to a tip diameter of approximately 4 µm. The probe chamber, transducer assembly, and associated electronics were built in the Laboratory of Dr. Ulrich Zimmermann at the University of Würzburg in Würzburg Germany and modified at the University of Vermont.

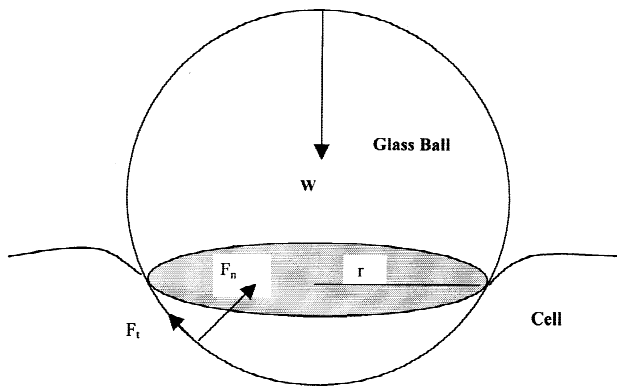
## Theoretical Background

A turgid cell can support a certain load: the heavier the load, the larger the contact area between the cell and the load. Figure 1 shows a turgid cell compressed by a ball-shaped indenter, resulting in a spherical, cup-shaped contact surface. Figure 2 illustrates the forces acting on the ball indenter. All these forces satisfy the equilibrium condition, written in vector form thus:

$$F_n + F_t + W = 0 \quad (1)$$

where  $F_n$  is the normal component of the supporting force,  $F_t$  is the tangential traction force from the cell, and  $W$  is the downward force including the weight of the ball and the downward force applied to the ball from the top.

The turgor pressure of the cell and the rigidity of the cell wall both contribute to  $F_n$ . If the cell wall is thin and flexible, which is the case for a growing cell, the turgor pressure of the cell will be the dominant factor in  $F_n$ . Tangentially acting forces, such as might arise because of friction between the cell wall and the ball or traction force caused by the existence of a viscous substance on the cell or a water menis-



**Figure 2.** 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 patch, and  $W$  is the load acting on the ball.

cus (if present) are represented by  $F_t$ . Obviously, as long as these forces act symmetrically about the vertical axis, the horizontal components will cancel.

The vertical scalar equation of Eq. (1) is:

$$\sum(F_n \cdot \cos\theta + F_t \cdot \sin\theta) = W \quad (2)$$

where  $\theta$  is the acute angle between  $F_n$  and vertical. Under condition of  $F_t \rightarrow 0$ , which we can achieve by eliminating the water meniscus and the viscous substance, we then have

$$\sum F_n \cdot \cos\theta = W \quad (3)$$

Because for a cell with a thin flexible wall, it is mainly the turgor pressure that generates  $F_n$ , the force in the vertical direction, then  $F_n = P(2\pi R \cdot dy)$ , where  $P$  is the turgor pressure,  $R$  is the radius of the ball, and  $dy$  is the incremental slice through the spherical contact zone. Notice that  $\cos\theta = y/R$ , the integration then becomes

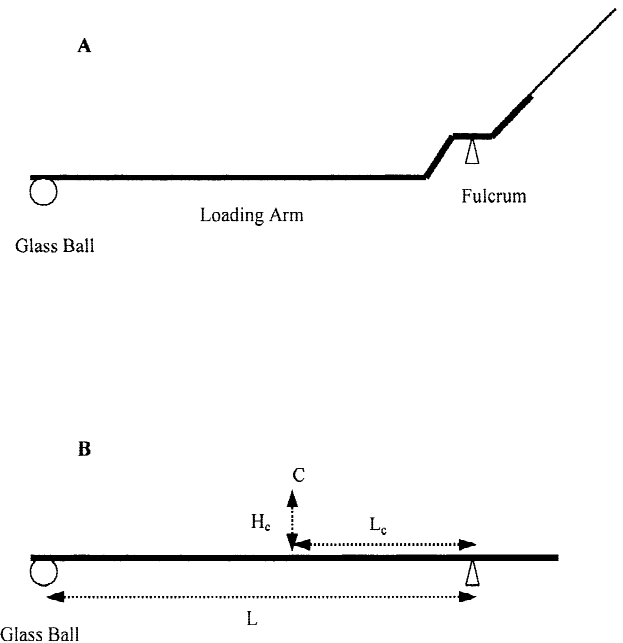
$$\int_a^R 2\pi P y \cdot dy = W$$

where  $a = \sqrt{R^2 - r^2}$  and  $r$  is the radius of the projected contact area (for a cell with a flat upper surface this area will be a circle). The preceding integration yields  $P\pi r^2 = W$ , or

$$P = W/(\pi r^2) = W/S \quad (4)$$

This equation is the basis of the ball-tonometer method. It states that knowing the load  $W$  on the ball, and the projected contact area  $S$  between the ball and the cell, we then can deduce the cell turgor pressure according to the Eq. (4).

It is clear then that for the method of ball tonometry to provide credible information we must assure



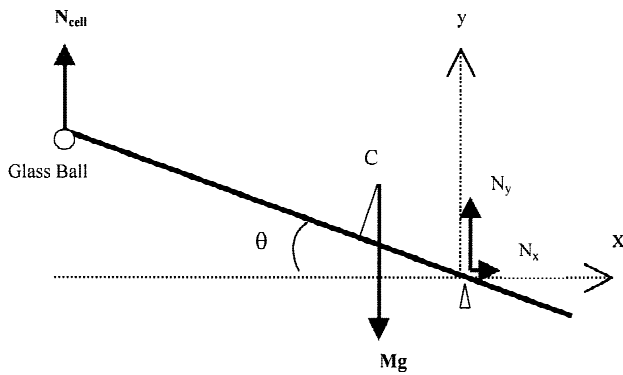
**Figure 3.** The structure of the loading arm. (A) shows the ball probe, loading arm, goniometer whisker, and the location of the needle-bearing fulcrum. (B) shows a simplified diagram of the loading arm assembly.  $C$  represents the center of mass of the assembly.  $L_c$  and  $H_c$  are the  $x$  and  $y$  coordinates of the center of mass that must be placed appropriately. See text.

ourselves that the following conditions are met. First, we must establish that the stiffness of the cell wall itself does not significantly support the ball and, second, we must establish that upwardly acting viscous forces and downwardly acting surface tension forces do not significantly affect the vertical balance of the probe.

Considering that the cell turgor pressure of many species is relatively high (for example, 0.2–0.7 MPa), most growing cells with primary walls still capable of expansion growth should meet the first criterion. Evidence that wall stiffness contributes to the support of the tonometer ball and arm would be found in a nonlinear relationship between the measured area and the applied load. The second criterion can be met by careful attention to methodological detail, for instance by taking care that both the probe and target cell surfaces are as clean as possible.

### The Loading Arm

Figure 3A shows the structure of a loading arm. To ensure maximum accuracy, it is important to properly locate the center of mass, which can be estimated using the formulae:  $X_c = \sum m_i x_i / M$  and  $Y_c = \sum m_i y_i / M$ . The actual center of mass can be verified



**Figure 4.** Free-body diagram of the loading arm.  $Mg$  is the total weight of the loading arm,  $N_{cell}$  is the reaction-force of the cell on the ball indenter,  $C$  is the center of mass of the loading arm,  $\theta$  is the angle of the loading beam to the horizontal, which should be as close to horizontal as possible to minimize the effect of load variation with the angle of the arm, and  $N_x$  and  $N_y$  are the  $x$  and  $y$  components of the supporting force acting on the loading arm by the fulcrum.

by hanging the arm from both ends in turn, dropping a plumbline from each, and locating the point at which they intersect. Most of our loading arms had the following configuration. The distance between the ball and the fulcrum was approximately equal to 45 mm,  $X_c$  and  $Y_c$  (corresponding to the coordinates  $L_c$  and  $H_c$  shown in Figure 3B) were approximately equal to 3 mm and 4 mm, respectively.

Figure 3B shows a simplified diagram in which the loading arm has been represented by a lever. Its center of mass is indicated by  $C$ . For simplicity, the symbols  $X_c$  and  $Y_c$  have been replaced by  $L_c$  and  $H_c$ , respectively. During the turgor pressure measurement, the loading arm is in *static equilibrium*, satisfying the equations:

$$\sum \mathbf{F} = 0 \quad \text{and} \quad \sum \mathbf{M} = 0 \quad (5)$$

where  $\sum \mathbf{F}$  is the vector sum of all forces acting, and  $\sum \mathbf{M}$  is the vector moment of all forces relative to any point.

### The Static Equilibrium of the Loading Arm

Figure 4 is the free-body diagram of the loading arm. Suppose the angle between the loading arm and horizontal is  $\theta$ . A positive  $\theta$  means that the arm inclines upward (as shown in the figure), whereas a negative  $\theta$  implies that the arm declines downward. Because we are only interested in the  $N_{cell}$ , the supporting force from the cell to the ball, it is convenient to choose the fulcrum as the moment center. Thus, we need to consider the balance between two

moments only, the moment of  $N_{cell}$  and the moment of the arm weight,

$$N_{cell} \cdot L \cdot \cos\theta = Mg \cdot X_c \quad (6)$$

where  $L$  is the distance from the ball to the fulcrum,  $Mg$  is the total weight of the loading arm,  $X_c$  is the current coordinate of the center of mass,  $X_c = (L_c \cdot \cos\theta - H_c \cdot \sin\theta)$ .

Equation (6) gives

$$N_{cell} = Mg(L_c - H_c \cdot \tan\theta)/L \quad (7)$$

When  $\theta = 0$ ,  $N_{cell} = MgL_c/L$ , which is obviously the case when the arm is at horizontal. The sensitivity of  $N_{cell}$  to the  $\theta$  can be examined by taking the derivative of Eq. (7),

$$dN_{cell}/d\theta = -MgH_c/(L \cdot \cos^2\theta) \quad (8)$$

This result shows the following.

1. The point of least sensitivity of  $N_{cell}$  to the  $\theta$  occurs at  $\theta = 0$ , which is to say that to minimize the effect of variation of the load with the angle of the arm, we should perform our pressure measurement while the arm is as close to the horizontal as possible.
2. Accordingly, to minimize the  $dN_{cell}/d\theta$ , we should have a short  $H_c$  and long  $L$ , which is in turn a question of locating the center of mass properly, as previously noted.

## RESULTS

Figure 5A–C show successive images of projected contact areas obtained at three different loads on a single onion epidermal cell using a  $D = 300\text{-}\mu\text{m}$  glass ball. The loads applied in each case were: 47 mg (A), 32 mg (B), and 22 mg (C). These loads resulted in projected areas of  $801\ \mu\text{m}^2$ ,  $504\ \mu\text{m}^2$ , and  $339\ \mu\text{m}^2$ , respectively. For this one cell then, the turgor pressure was calculated as 0.59 MPa, 0.63 MPa, and 0.65 MPa, respectively. These results represent just one of many similar results that we obtained by this method.

Figure 6 shows a more comprehensive summary of results in which measurements obtained by ball tonometry are compared directly with measurements made on the same tissue sample with the pressure probe method. Each point consists of a single pressure probe reading plotted against the mean of five or more measurements obtained by ball tonometry, the reason being that tonometric measurements can be obtained much more rapidly. Clearly, there is excellent agreement between the two methods, indicating that over a 0.5-MPa (5 bar) range of cell turgors a measurement by one method

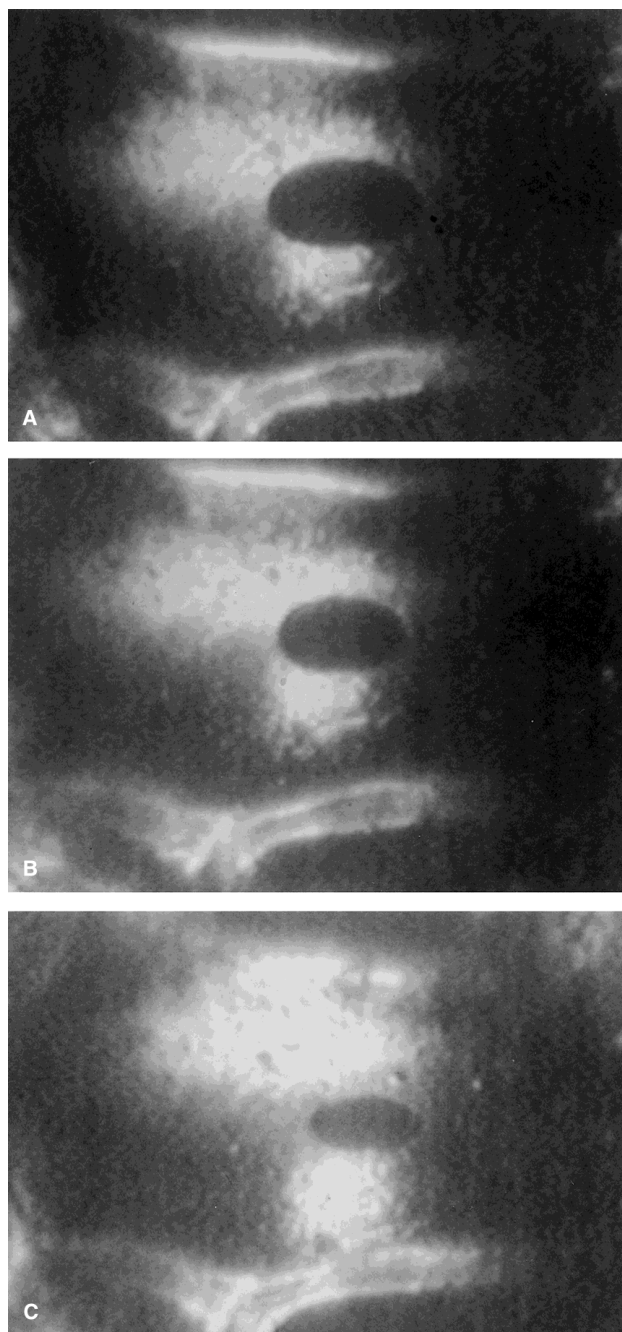


Figure 5. Image capture of contact patch, showing the load-dependent change in contact area between a 300-µm ball probe and an onion cell. (A) shows the area of contact at 47 mg. (B) shows the area at 32 mg. (C) shows the area at 22 mg.

would predict with reasonable accuracy a measurement by the other method. The slight offset of the curve from the origin can be explained either by a supporting effect caused by wall stiffness, leakage around the pressure probe, or, more likely, a slight strain-relief-related volume change in the cell

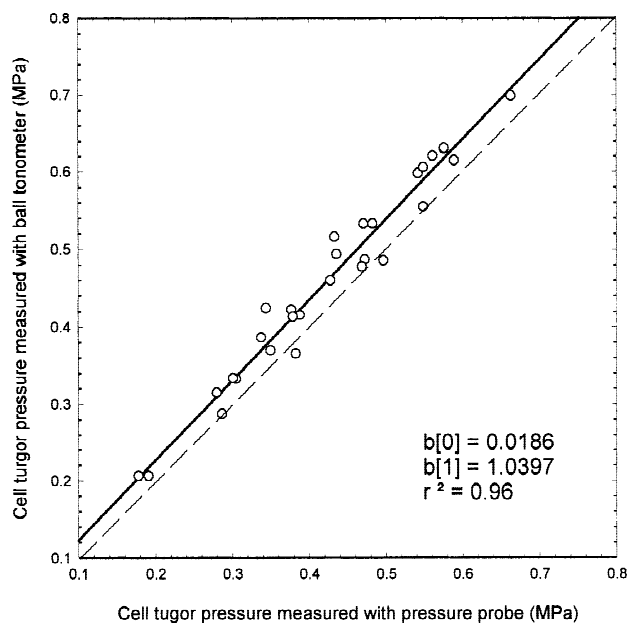


Figure 6. Direct comparison of tonometric and pressure probe measurements on turgid onion cells. Each point consists of one pressure probe measurement (*x*-axis) plotted against the mean of five tonometric measurements (*y*-axis). A perfect correspondence between the two methods would be represented by the dashed line passing through the origin. The offset reflects the slightly higher turgors measured by tonometry.

caused by the severing by the pressure probe tip of load-bearing tensile microfibrils in the wall.

Figure 7 illustrates how tonometrically derived pressure measurements vary in two parameters. Along the *y*-axis the five separate curves represent five separate tonometric runs carried out at five different osmotic concentrations, from distilled H<sub>2</sub>O ( $\pi = 0.0$  MPa) to  $\pi = 0.1$  MPa,  $\pi = 0.2$  MPa,  $\pi = 0.3$  MPa, and  $\pi = 0.4$  MPa, respectively. Along the *x*-axis we see the change in measured pressure at five different load levels. Each curve, therefore, follows the course of multiple measurements on a single cell, and each plotted point represents the mean of five separate tonometric measurements on the same cell. If measurement conforms to theory, the area of the contact patch will vary arithmetically with the applied load. The calculated pressure will consequently be load insensitive, resulting in five separate, horizontal curves, each representing a 0.1 MPa (1 bar) step down in equilibrium turgor pressure.

These results show unequivocally that real changes in cell turgor pressure produced by changing the osmotic environment of the cell are accurately reflected in tonometrically derived turgor pressure measurements. They also show that mea-

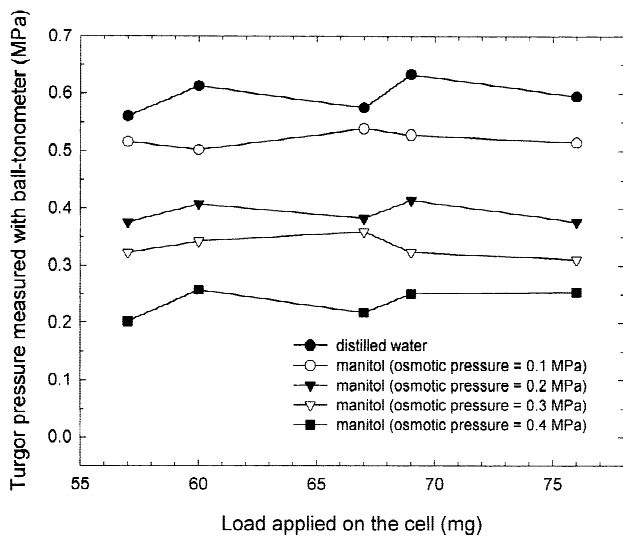


Figure 7. The invariance of measured turgor with load. The y-axis shows five separate tonometric runs on five separate cells, each bathed in media of successively higher osmotic pressure. The *upper curve* shows a cell at full turgor, bathed in distilled H<sub>2</sub>O. The *lower curves* were taken at incremental 0.1-MPa (1 bar) decreases in cell turgor. Each point along the x-axis is the mean of five measurements at the same load. Successive points are derived from measurements at an arbitrarily increased load. See Text.

surement by ball tonometry conforms to theory in that measured pressure does not vary significantly with the load acting on the tonometer arm.

In a separate and preliminary series of experiments, we attempted to overcome potential difficulties associated with measuring tissues and cells exposed to room air. The first of these difficulties is the lowering of cell turgor as a result of the gradual drying out of the tissue. The second is the error introduced into contact area measurements because of any meniscus that might be created around the contact zone by a surface film of water on the exposed cells.

This experiment was carried out with the tissue and tonometer arm completely submerged in water; the observations and measurements were carried out as before except with water immersion optics.

The target tissue in this case was bean hypocotyl. Cells from each tissue were measured at decreasing loads of 56 mg, 43 mg, 31 mg, and 20 mg. For this experiment a total of 31 measurements was made, not including those that were thrown out for reasons of methodological uncertainty, in particular for cases in which the ball probe was suspected of touching two adjacent cells. For these submerged measurements we recorded a mean turgor of 0.92 MPa, with a standard deviation of 0.21 MPa. This

result represents our attempt to push the method to its limits.

It appears that the method can be successfully applied to submerged tissues, thereby extending the usefulness of the method to more delicate tissues and eliminating one potential source of artefactual error caused by the inflation of the contact area by surface films. However, several confounding factors need to be overcome, the first being the difficulty in achieving accurate load control, where buoyancy comes into play, and the second being a somewhat reduced contrast between the contact patch and the surround, making it more difficult to reliably trace out contact areas. It should be noted, too, that we have as yet been unable to verify our underwater results with parallel pressure probe measurements.

## DISCUSSION

Turgor pressure may be the most critical and yet the most elusive variable in plant growth. The destructive nature of existing measures of turgor pressure has meant that to some extent its role in routine studies of plant growth has been neglected. The method of turgor pressure measurement that we describe here has the potential to become truly routine. It is completely noninvasive and nondestructive. It is rapid, reproducible, and relatively undemanding in terms of technical expertise. It is also relatively easy to set up and in its simplest form requires only a compound microscope and suitable contrast enhancement optics. Furthermore, we believe that the methods described here will support considerable modification and improvement, potentially leading to greater accuracy in measurement than any other method.

The constraints on the system can be summarized as follows. First, the theoretical basis of the method requires that measured pressure does not vary with applied load, which we have shown to be true in a variety of subject tissues, but only if the loads are held within the range of 20–70 mg. Too heavy a load exceeds the elastic limits of the cell wall, whereas too light a load reduces the size of the contact patch to the point where variations in the measured area become unacceptable. It is also clear that the method works less well for cells that are approaching plasmolysis or that have thick or highly decorated walls. Furthermore, we have shown that in its present configuration, the loading arm should be as nearly perpendicular to the gravity vector as possible and that when this constraint is met we have been able to reduce the load error to less than 1 mg. Perhaps the major constraint of the system lies in the

optics, however. The ability to obtain reliable area measurements under working conditions appears to be largely a function of the ability to create sufficient contrast between the contact patch and the surround. The use of spinning-disc confocal microscopy appears to relieve this constraint to a large extent by eliminating scattered light from out-of-focus regions of the specimen. The spinning-disc method also provides a "real-time" white light image of the subject field. Nevertheless, anomalous refraction effects and inhomogeneities in the illumination of the contact patch continue to make automatic area measurements difficult, particularly when working under water with small cells, small probe diameters, and light loads, which is the current focus of work in our laboratory. We have, under ideal conditions, obtained good contact patches under water with ball diameters as small as 53  $\mu\text{m}$ .

Submerged probe measurements that use the present loading arm arrangement are difficult because of errors introduced during the calibration process. These errors arise from a combination of buoyancy effects and evaporation from the bath during calibration.

Future developments that we hope will obviate many of these problems include a strain-gauge instrumented loading arm capable of generating continuous real-time load information. This would free the operator from the orientation constraints imposed by gravitational loading, as well as sidestepping buoyancy effects and water menisci produced by surface films. We look forward to being able to track the behavior of single cells over extended time periods or to mapping cell-to-cell turgor differences over an entire organ surface, thereby relating

changes in cell turgor to tissue and organ level developmental changes.

In the spirit of Paul Green's continuing search for simple ways to explain complex phenomena on the basis of simple experiments with homemade tools, we offer this relatively simple method as a way to follow one of the central cellular parameters underlying all plant growth, namely, pressure-driven cell expansion. We hope that with further development this tool may even prove to be suitable for the study of one of Paul's favorite organisms: *Nitella*.

#### ACKNOWLEDGMENTS

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