Use of the pressure probe in studies of stomatal function

Peter J. Franks¹
School of Tropical Biology, James Cook University, PO Box 6811, Cairns, QLD 4870, Australia

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Abstract

Over the past few decades the pressure probe has been used extensively in studies of the hydro-mechanical and osmotic properties of plant cells. However, although pressure probe techniques have been employed successfully in the study of stomatal function, there is no detailed account of this special application of the pressure probe technique. This paper describes the construction and use of the pressure probe in studies relating to stomatal function, and reviews the current state of knowledge of stomatal function in relation to guard cell and leaf hydromechanical properties.

Key words: Guard cell turgor pressure, pressure probe, stomata, stomatal mechanics, transpiration.

Introduction

Stomatal guard cells, with their ability to sense and integrate many internal and external environmental signals directly, are amongst the most physiologically complex cells in higher plants (Cowan, 1977; Raschke, 1979; Farquhar et al., 1980; Willmer and Fricker, 1996; MacRobbie, 1998; Assmann and Shimazaki, 1999; Schroeder et al., 2001; Zeiger et al., 2002). Although there have been significant advances in understanding stomatal function over the past few decades, there is still much that is not understood. Stomata are self-powered turgor-operated valves and, although operating as a coordinated population within the leaf epidermis, each precisely controlled stomatal pore is the result of a continuously shifting balance between forces generated within the guard cells and those in neighbouring epidermal cells. Until only recently, data on the basic relationship between guard cell pressure and stomatal aperture were unavailable, largely because of technical difficulties. This considerably limited the development of mechanistic models of stomatal function. However, development of the pressure probe has enabled exploration of the biophysical principles of stomatal function at the cellular level. The purpose of this article is to describe in detail how the pressure probe is used to study stomata, and to discuss some of the main findings.

The cell pressure probe was first used in the study of hydromechanical properties of plant cells by Hüsken et al. (1978). It has since been used in the study of water and solute relations of a variety of plant tissues, including leaf epidermis (Zimmermann et al., 1980; Tomos et al., 1981; Tyerman and Steudle, 1982; Shackel, 1987), mesophyll (Steudle et al., 1980; Nonami and Schulze, 1989), xylem (Balling, 1990; Wei et al., 1999) and roots (Steudle and Frencsch, 1989; Azaizeh, 1991; Zimmermann et al., 1992). There are several excellent technical and review papers on the general use of the modern cell pressure probe. Steudle (1993) provides a detailed description of theory and application of the cell pressure probe to studies of water and solute relations in various plant tissues. Boyer (1995) also presents a detailed chapter on cell pressure probe design and application. More recently, Tomos and Leigh (1999) provided a comprehensive review of the pressure probe literature. However, use of the pressure probe in studies of stomatal function represents a special case that to date has not been well described.

Although some of the most useful data on the biomechanics of stomatal movement have come from direct measurement and manipulation of guard cell pressure, application of the cell pressure probe to studies of stomatal function is not limited to the probing of guard cells alone. Although guard cells can sense and respond independently to several environmental stimuli, their functioning in the intact leaf is intricately linked via hydraulic, mechanical and chemical interactions to the whole plant. Therefore, use of the pressure probe in studies of stomatal function can involve measurements not only on stomata, but on tissues located throughout the plant.

¹E-mail: peter.franks@jcu.edu.au
Pressure probe design and application

As with most physiological measurement techniques involving precision instruments, experiments using the pressure probe are best carried out in the air-conditioned and dust-free environment of a standard laboratory. However, this need not limit its use to specialized research laboratories. Most field stations are equipped with such facilities, and thus there is no real impediment to using the pressure probe in remote locations. Furthermore, the pressure probe itself (excluding microscope and data acquisition hardware) meets several additional criteria that make it worthy of consideration for use in field laboratories: it is light, compact, robust, and cheap.

There is no commercially available pressure probe suitable for general stomatal studies. However, the instrument is simple to construct if one has access to machining facilities. The pressure probe operates on the principle of regulated volume displacement. Once the probe is inserted into a cell, a closed, elastic-walled, fluid-filled system is formed, where the oil within the pressure probe interfaces with the cellular contents. The pressure is regulated by sliding a steel piston into or out of the oil-filled reservoir of the pressure probe.

Main components

A schematic diagram of a pressure probe suitable for studies of stomatal function is shown in Fig. 1. A list of equipment suppliers is provided in the appendix. In principle, this instrument is similar to pressure probes described elsewhere (Steudle, 1993), except for two main features. First, the piston and transducer housing are separated from the glass capillary holder by a length of flexible HPLC (PEEK) tubing. This adaptation, first described by Murphy and Smith (1994), greatly facilitates high precision orientation of the glass capillary, which is important because much of the success in taking measurements directly on guard cells depends on where and how the capillary is inserted into the guard cell. Second, the stomatal pressure probe needs to be capable of operation at relatively high pressures. Guard cells can require up to 5 MPa to achieve full opening (Franks et al., 1998). The two seals most likely to leak are the o-rings around the sliding steel piston and around the glass capillary. However, provided these fittings are machined to fine tolerances and shaped appropriately, a high-pressure seal can be maintained.

The transducer and piston housing (Fig. 1a, A–F) forms the bulk of the instrument and is machined from a block of Plexiglass. The simplest design is to bore a “T” and then at each opening tap threads to hold the transducer, piston guide and PEEK tube connector. The micrometer screw moves the piston in and out to regulate pressure. It may be operated by an electric motor (Hüsken et al., 1978) or, if fitted with a wheel of about 7 cm diameter, can be operated easily by hand.

A suitable pressure transducer is the piezoresistive strain gauge type (e.g. XTL-190-1000-A, rated at 1000 psi, Kulite Semiconductor Products, Inc., Leonia, USA). Apart from being small and rugged, these sensors offer high signal output, excellent repeatability and infinite resolution (limited only by that of the recording instrument). They are also temperature compensated and tolerant of considerable overpressure. The recommended silicone oil (Wacker AS 4, Wacker-Chemi, Berlin) is a phenylmethyl polysiloxane with low kinematic viscosity (4 mm² s⁻¹) and low compressibility.

To observe the probe tip during insertion into cells and to monitor the oil–sap meniscus, a high-powered microscope is necessary. If working with epidermal peels an inverted microscope allows unhindered probe access. When working with intact plant material a standard
compound microscope with epi-illumination is required. In this case, a long working distance objective is required to enable probing of cells while viewing (40× magnification with a working distance of 7 mm is sufficient). A microscope with a fixed stage is best.

Due to the highly delicate nature of plant cells, accidental mechanical shocks and vibration due to nearby machinery can be a problem. These disturbances are not always present, but at some locations can be severe enough to impede work with the pressure probe or any other type of microprobe. In many cases the vibrations and mechanical shocks can be dampened sufficiently with a ‘steady bench’. Depending on the severity of the vibration problem, these benches can vary in type from a simple slab of rock (about 60×60×5 cm), to a slab of rock or other high-density material mounted on air cushions that can be tuned for optimal dampening.

There are several different types of micromanipulator suitable for pressure probe work, and the choice can be a matter of personal preference. However, highly recommended is the hydraulic type in which the controls are separated from the moving probe holder by a length of fine hydraulic tubing. This ensures that random hand movements do not hinder probe manipulations.

When working with stomata it is often desirable to capture images for later analysis of stomatal apertures. For this purpose a standard CCD camera and image capture software can be used. Similarly, although in many instances a chart recorder or voltmeter may be sufficient for transducer output recordings, time-series measurements and analyses involving pressure and volume manipulations will require a data acquisition card and computer software.

**Glass microcapillaries**

Borosilicate glass capillaries, typically 1.2 mm OD, 0.69 mm ID, are pulled into a fine point with a pipette puller. A programmable pipette puller (e.g. Model P-87, Flaming/Brown Micropipette Puller, Sutter Instrument Co., Novato, USA) allows tips with almost identical shape to be pulled repeatedly with ease. A suitable working tip diameter is typically around 1–2 μm. Often the probe tips are narrower than this when initially pulled and can be easily enlarged by scraping gently against the rough edge of a glass slide (using a micromanipulator and microscope). A probe tip that is either blocked or too narrow to allow the rapid movement of fluid in and out could lead to erroneously high pressures being recorded. However, such a constriction will be immediately apparent to the user. Before any measurements are made, the user should routinely check for unrestricted flow through the probe tip by ensuring that quick adjustments of pressure are accompanied by equally rapid movement and re-equilibration of the oil/sap meniscus.

**Filling the probe system**

After construction, the entire pressure probe reservoir must be filled with clean silicone oil, being careful to exclude bubbles. With the piston removed, the chambers in the plexiglass block and the PEEK tubing are filled. The piston is then inserted and tightened into place. When not holding a class capillary, the capillary holder attached to the end of the PEEK tubing should be capped to keep the contents clean and to prevent oil from evaporating or leaking. After a glass microcapillary is pulled (several may be pulled and kept in a container for later use), it is loaded with silicon oil using a fine syringe that can slide inside the capillary, filling it from the bottom up. When full, the capillary is screwed into the capillary holder, ensuring that no air bubbles are trapped when the oil in the capillary and holder are mated together. When tightening the capillary into place, it is important to monitor pressure and wind out the piston to avoid overpressure.

**Calibration**

Although transducers often come with a calibration certificate, a calibration check (transducer output voltage versus pressure) should be made. For this purpose a regulated high pressure air line and test gauge can be fitted via an adaptor to the capillary holder. The user should check for linearity and absence of hysteresis, and ensure that the performance conforms to the manufacturer’s specifications.

**Making measurements**

Although ideally suited to obtaining data on the relationship between guard cell turgor and stomatal aperture (Franks et al., 1995, 1998), the stomatal pressure probe described here is suitable for a variety of water relations studies on other types of cells. As mentioned earlier, stomatal function involves the co-ordination of many different types of cells, so measurements which aim to explore stomatal function are not limited to stomatal guard cells. What is limiting, however, is the size of the cells suitable for pressure probe measurements. Due to the dependence of several factors, including the wall properties of the cell, the optical and mechanical precision of the system being used, and the skill of the operator, there is no exact minimum size, but many researchers would agree that cells smaller than about 20 μm are extremely difficult to work with. Many plants have cells (including stomatal guard cells) that are this size or smaller, so this must be considered when planning experiments.

The basic procedure for measuring cell turgor is shown in Fig. 2. Upon insertion of the probe into a cell, the cell sap is forced back a short distance into the glass capillary. The pressure must then be increased via movement of the steel piston so that the sap–oil meniscus is brought back to the surface of the cell. The pressure measured in the system...
at this point is equal to the turgor pressure of the cell prior to probe insertion. For stomata, the technique of Franks et al. (1995) involves the manipulation of pressure in guard cells to obtain aperture versus pressure relationships. In this method, the probe is inserted into closed (deflated) guard cell pairs at their common ends, and the guard cells are pressurized (inflated) by extruding oil into them. In this case, the oil-sap meniscus is in the guard cells and its position is either advancing away from the probe tip, fixed, or retreating back toward the probe tip, depending on whether the operator is increasing pressure, holding it constant, or decreasing pressure. Detailed instructions on the measurement of cell wall elasticity, cell membrane hydraulic conductivity, osmotic pressure, and reflection coefficients are given in Steudle (1993), Boyer (1995) and Franks et al. (2001).

**Discussion**

**Guard cell wall properties**

Stomatal pores form because guard cells behave in a unique manner when internally pressurized. When the turgor pressure within a mature plant cell increases, the walls will, in most cases, stretch almost equally in all directions (isotropic expansion). With guard cells, however, stretching is not directionally uniform. Instead, pressurization results in greater stretching in the longitudinal direction than in the tangential. This unequal (anisotropic) cell wall stretching, which is dominated by the longitudinal expansion of the dorsal wall, is what bends the guard cells away from each other and creates the stomatal pore. All guard cells, regardless of shape, operate on the basis of this pattern of anisotropic wall expansion. It is thought that a combination of differential wall thickenings (thinner dorsal walls, thicker ventral walls), wall chemical composition and radial arrangement of microfibrils in the walls contributes to the unique mode of expansion of guard cells (Haberlandt, 1884; Aylor et al., 1973; Majewska-Sawka et al., 2002). However, the structure of guard cell walls is not well understood, and little is known about the physical changes that take place within the walls during stomatal opening and closing. The pressure probe is becoming an important tool for exploring these properties.

**Stomatal aperture versus pressure relationship**

Although it has long been known that stomata open and close by increasing and decreasing their turgor pressure (von Möhl, 1856; Heath, 1938; Meidner, 1982), there are only a limited number of studies in which the relationship between guard cell pressure and stomatal aperture has been measured. Using the apparatus and techniques described here, data have been obtained for several species (Franks et al., 1995, 1998). Typical characteristics for *Tradescantia virginiana* are shown in Fig. 3. While these data are far from comprehensive, the emerging pattern is that under the influence of the turgor pressure from neighbouring epidermal cells, the relationship between stomatal pore width \( a \) and guard cell turgor pressure \( P_g \) is sigmoidal. As epidermal turgor pressure \( P_e \) reduces to zero, this relationship tends toward a simple saturating curve. For an isolated guard cell, the change in volume \( V \) with a small change in \( P_g \) \( (dV/dP_g) \) always decreases with increasing \( P_g \) (Franks et al., 2001; Fig. 4). This explains the curve for \( P_e=0 \) in Fig. 3, since stomatal aperture is
linearly related to guard cell volume in isolated guard cells (Raschke, 1979; Fig. 5). However, the sigmoidal relationship between stomatal pore width and $P_g$ in the presence of epidermal turgor is due, in part, to the pressure-dependent influence of epidermal cell turgor on stomatal aperture. At low $P_g$, the closing force exerted on stomata by turgid epidermal cells is at its greatest, but diminishes with increasing guard cell turgor. Thus the mode of response of stomatal conductance to any environmental signal will depend significantly upon the magnitude of $P_g$ at the time the signal is received, such that responses to perturbations at low $P_g$ may differ in form from those occurring at high $P_g$. Evidence for this can be seen in stomatal responses to CO$_2$ (Wong et al., 1978) and leaf-to-air vapour pressure difference (Nonami et al., 1990) under high versus low light.

**Guard cell osmotic pressure**

The driving force behind active stomatal opening and closure is the regulation of guard cell osmotic pressure. As with most other living cells, guard cells conform to the general water potential model. Guard cell water potential, $\Psi_g$, will passively equilibrate with that of its surroundings through osmotic uptake or release of water. At any point in time guard cell water potential is defined by the difference between its turgor (or hydrostatic) pressure, $P_g$, and osmotic pressure, $\Pi_g$:

$$\Psi_g = P_g - \Pi_g$$  \hspace{1cm} (1)

By actively adjusting osmotic pressure, guard cell turgor increases or decreases so as to balance equation 1. When $\Psi_g$ is close to zero, such as in well-watered plants at high humidity, $P_g$ will approach $\Pi_g$ in magnitude. Using rapid plasmolytic techniques, Raschke (1979) measured around 4.0 MPa osmotic pressure in *Vicia faba* guard cells, and MacRobbie (1980) measured a similar value in *Commelina communis* guard cells. Using the same technique, Meidner and Bannister (1979) measured around 3.0 MPa osmotic pressure in *Vicia faba* guard cells, and 4.0 MPa in *Commelina communis*. Using freezing-point depression techniques, Bearce and Kohl (1970) report similar values for *Chrysanthemum* and *Pelargonium* guard cells. Therefore, osmotic pressures of around 3–4 MPa appear to be common in open stomata, suggesting that guard cell turgor pressures of this magnitude must also be common. This is not to say that they are always required. However, using a cell pressure probe technique, Franks et al. (1998) found that indeed, guard cell turgor pressures of about 4.0 MPa were required to achieve maximum stomatal opening in *Vicia faba*, *Tradescantia virginiana*, *Nephrolepis exaltata* and *Ginkgo biloba*.

Despite significant progress toward understanding the process by which guard cell osmotic pressure is generated, much remains unknown about how it is regulated, or the relative quantities of osmotica that it comprises. By utilizing the pressure probe as a microsampling device, and applying micro-analytical techniques (Tomos and Sharrock, 2001) the nature and regulation of guard cell osmotic contents may be explored.

**Mechanical advantage of epidermal cells**

The term ‘mechanical advantage’, denoted here as $m$, refers to the extent to which forces in the epidermal cells counteract the movement of guard cells. An expression for the mechanical advantage was formalized by Cook et al. (1976) as follows:
which defines \( m \) as the ratio of sensitivities of \( a \) to \( P_e \) and \( P_g \). Due to this mechanical advantage, an equal increase in turgor pressure in both guard and epidermal cells will result in a reduction in stomatal aperture, and vice versa. For example, starting at the point \( P_e=0, P_g=1 \) MPa and \( a=10 \) \( \mu \)m in Fig. 3, if \( P_e \) and \( P_g \) both increase by 0.9 MPa, the net change in aperture will be a decrease from 10 \( \mu \)m to 1.3 \( \mu \)m. What is also evident from Fig. 3 is that this mechanical advantage is not constant, but varies widely with \( P_g \) and \( P_e \). It should be noted also that, although a given aperture can be achieved with a lower guard cell turgor, if epidermal turgor is reduced, the widest stomatal apertures still require high guard cell turgor in combination with low epidermal turgor.

This knowledge of \( m \) suggests that, if hydraulic coupling is close between guard and epidermal cells, any increase in epidermal water potential will, in the first instance, promote stomatal closure, and any decrease in epidermal water potential will promote opening. This seemingly counterproductive characteristic alludes to the special regulatory properties of stomata. In practice, the net result of such perturbations is usually the opposite. The almost universal observation is that in response to conditions that tend to reduce bulk leaf water potential, such as increasing transpiration rate (see reviews by Monteith, 1995; Buckley and Mott, 2002b) or decreased xylem hydraulic conductance (Hubbard et al., 2001; Cochard et al., 2002), stomata exhibit in the steady-state a net reduction in aperture.

**Stomatal control of transpiration rate**

Due to the pronounced diurnal and seasonal variations in transpiration potential (leaf-to-air vapour pressure difference, VPD), one of the key regulatory roles played by stomata is that of limiting transpirational water loss or, more precisely, minimizing transpiration-induced water deficit. The underlying mechanism of the so-called ‘humidity response’, where stomatal conductance decreases with increasing VPD, remains unknown (Jones, 1998; Meinzer, 2002), but the pressure probe is a tool that will allow such observations to make some important inroads into this subject (see below). Different types of plants are known to vary in the sensitivity of stomatal conductance to VPD (Tardieu and Simonneau, 1998; Franks and Farquhar, 1999). Those that are most sensitive are capable of maintaining a more constant leaf water potential, since they minimize any increase in transpiration rate with increasing VPD. Since stomatal conductance appears to be positively correlated with whole plant, leaf-specific, hydraulic conductance across species (Meinzer et al., 1995; Saliendra et al., 1995), differences in ability to minimize transpiration-induced reductions in bulk leaf water potential cannot be easily attributed to differences in xylem hydraulic conductance. Indeed, there is, as yet, little evidence to suggest a mechanistic link between xylem hydraulic conductance and stomatal sensitivity to VPD.

The pressure probe has contributed much toward unravelling the mechanistic basis of the humidity response. Figure 6 is a block diagram of the essential mechanistic elements of the stomatal response to humidity, adapted from the negative feedback model presented in Franks and Farquhar (1999). The two branches of the feedback loop represent the combined actions of epidermal and guard cells, whereby a common increase or decrease in turgor pressure leads to opposing effects on stomatal conductance. The net change in stomatal conductance (\( \Delta g \)) is the result of these stomatal and epidermal effects. Details of the model are explained in Franks and Farquhar (1999), but its operation may be summarized as follows: immediately after a change in VPD (before stomata have had time to respond) there is a change in transpiration rate (\( \Delta E \)). This sets in train a series of interrelated responses in the stomatal apparatus, as determined by specific hydraulic, osmotic and mechanical properties. The resulting change in stomatal conductance, \( \Delta g \), is the sum of the change due to guard cell effects alone (\( \Delta g \)) and the change due to epidermal cell effects alone (\( \Delta g \)). This gives a change in transpiration rate (\( \Delta E \)) that is determined entirely by this conductance change. The actual change in transpiration rate as a result of the VPD change, \( \Delta E \), is the sum of (\( \Delta E \)) and (\( \Delta E \)). With this mechanism, an increase in VPD will give a positive change in (\( \Delta E \)) and a negative change in

\[
m = -\left( \frac{\partial a}{\partial P_e} \right)_{P_g}
\]
Such that \( \Delta E \) will be smaller than it would be without the feedback mechanism.

By manipulating the components of the model in Fig. 6, observed responses to humidity can be simulated. However, such an exercise in itself is of little value without valid empirical data for each of the model parameters and, unfortunately, there is to date only limited information on these. The pressure probe studies by Shackel and Brinkmann (1985), Nonami and Schulze (1989) and Nonami et al. (1990) have provided important information on the hydraulic properties of the leaf epidermis. Their experiments with Tradescantia virginiana showed that the humidity response in T. virginiana is accompanied by a significant drawdown in water potential between xylem and subsidiary cells (about 0.1 MPa per kPa VPD under the experimental conditions; Fig. 7), while no comparable drawdown was observed in the xylem. The relatively constant xylem water potential, in this case, may be attributed to a relatively high xylem hydraulic conductance, since the relatively moderate stomatal response still allowed a considerable increase in transpiration rate. However, the results do indicate that a partial hydraulic decoupling of the epidermis from the xylem may form the basis of the, as yet unknown, sensor for the stomatal humidity response.

With the above hydraulic information, and the hydro-mechanical information obtained for T. virginiana by Franks et al. (1998), some of the blocks in the model in Fig. 6 can be parameterized. This in turn allows the confirmation of one further important requirement. Given the now known hydraulic and mechanical constraints of T. virginiana leaf tissue and stomatal guard cells, the stomatal response to humidity, in this plant at least, must include either or both of the following: (1) in addition to the drawdown in water potential between xylem and subsidiary cells, a further and substantial drawdown in water potential between subsidiary cells and sites of evaporation on guard cells, (2) an active change in guard cell osmotic pressure that is mediated by the ratio and magnitude of these two effects. There is no direct information on whether changes in stomatal conductance with VPD are accompanied by changes in guard cell osmotic pressure. However, if guard cell osmotic pressure were to be maintained constant, the hydraulic conductivity between xylem and guard cell evaporative sites would have to be around ten times lower than that between xylem and subsidiary cells just to overcome the effects of the mechanical advantage and induce stomatal closure rather than opening in response to increasing VPD (Fig. 8). The absence of data on subsidiary cell to guard cell hydraulic conductivity and VPD-induced changes in guard cell osmotic pressure leave only speculation on this topic. However, given that in most cases the stomatal closure in response to increasing VPD is much more dramatic than in Fig. 8 (dotted line), and in some cases beyond the theoretical limits of a fixed-gain negative feedback system, it is unlikely that guard cell osmotic pressure remains unchanged during the response. Furthermore, to explain some of the more extreme and less reversible stomatal humidity responses, there have been suggestions of diurnally regulated shifts in stomatal sensitivity characteristics (Franks et al., 1997; Mencuccini et al., 2000), altered
leaf hydraulic conductivity (Salleo et al., 2000; Buckley and Mott, 2002a, b), and ABA-mediated effects on stomatal osmotic pressure (Tardieu, 1993; Zhang and Outlaw, 2001; Wilkinson and Davies, 2002). Outlaw and De Vlieghere-He (2001) have also shown that under increased transpiration rate, the accumulation of apoplastic sucrose in the evaporative sites of guard cell walls of *Vicia faba* may lower the water potential of guard cell walls sufficiently to cause stomatal closure. Further advances and application of pressure probe techniques and related micro-techniques (Outlaw and Zhang, 2001; Zwieniecki et al., 2002) are essential for a more complete understanding of the complex cellular mechanisms underlying the multi-sensory behaviour of stomata.

**Conclusions and future challenges**

The pressure probe is used widely in studies of plant cell water relations and cell biomechanics. However, its potential for use in studies specific to stomatal function has yet to be fully realized. There are many interacting biochemical and biomechanical processes governing stomatal response to environmental signals, and many of these can be studied at the cellular level using the pressure probe. One of the primary applications of the pressure probe, in this context, is measuring the relationship between stomatal guard cell turgor, epidermal cell turgor, and stomatal aperture. This information is crucial for the development of mechanistic models of stomatal movement, so that patterns of leaf and canopy gas exchange might be better predicted.

The above discussion has reviewed the contribution that pressure probe techniques have made to current understanding of stomatal function. However, there is still much that remains unknown about the mechanism of stomatal movement and gas exchange regulation. Some key areas of future research focus are listed below.

1. More species: Data on stomatal biomechanics have been gathered for only a handful of species so far, and most of these are herbaceous plants. The link between stomatal mechanics, stomatal morphology and leaf gas exchange has barely been explored, but more information is needed on how these properties interrelate across plant life forms and environments.

2. Guard cell and leaf hydromechanics: In the intact leaf, stomatal aperture is the result of an intricate balance of forces and fluxes in and around the stomatal apparatus. While there is now some idea of the mechanical forces involved, and the dominant ionic and molecular fluxes, little is known about water fluxes and hydraulic resistances in the vicinity of the guard cells. Without this information, several current hypotheses about the stomatal mechanism will remain untested.

3. Guard cell osmotic pressure: Despite a good understanding of many of the components of guard cell osmotic regulation, much remains unknown about the control of guard cell osmotic pressure. Development of novel single-cell sampling and analysis techniques, using the pressure probe simultaneously to measure pressure and extract samples, will help advance current understanding in this area.

**Acknowledgements**

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**Appendix**

**Table A1. List of equipment and suppliers**

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer/supplier</th>
<th>Type/part no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope</td>
<td>Carl Zeiss</td>
<td>Inverted (for epidermal peels) Axiovert 35M</td>
</tr>
<tr>
<td>Microscope</td>
<td>Carl Zeiss</td>
<td>Compound, with 40× long working distance lens, for whole leaves</td>
</tr>
<tr>
<td>CCD camera</td>
<td>Diagnostic Instruments Inc., Burlingame, USA</td>
<td>Hydraulic</td>
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<tr>
<td>Image capture software</td>
<td>Diagnostic Instruments Inc., Burlingame, USA</td>
<td>1000 psi rated</td>
</tr>
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<td>Steady bench</td>
<td>Terra Universal Inc., Anaheim, CA, USA</td>
<td>Steel piston: Small Parts Inc. Miami Lakes, FL, USA</td>
</tr>
<tr>
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<td>Terra Universal Inc., Anaheim, CA, USA</td>
<td>1/32 inch diameter straight stainless steel wire</td>
</tr>
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<td>Pressure transducer</td>
<td>Kulite Semiconductor Leonia, NJ, USA</td>
<td>1/32 inch ID, 3/32 inch OD, buna-n type</td>
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<td>Pipette puller: Flaming Brown, Novato, CA, USA</td>
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<td>Glass capillaries: Small Parts Inc. Miami Lakes, FL, USA</td>
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