Protocol: optimised electrophysiological analysis of intact guard cells from *Arabidopsis*

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**Abstract**

Genetic resources available for *Arabidopsis thaliana* make this species particularly attractive as a model for molecular genetic studies of guard cell homeostasis, transport and signalling, but this facility is not matched by accessible tools for quantitative analysis of transport in the intact cell. We have developed a reliable set of procedures for voltage clamp analysis of guard cells from *Arabidopsis* leaves. These procedures greatly simplify electrophysiological recordings, extending the duration of measurements and scope for analysis of the predominant K⁺ and anion channels of intact stomatal guard cells to that achieved previously in work with *Vicia* and tobacco guard cells.

**Keywords:** Microelectrode, K⁺ channel (voltage-gated), Cl⁻ channel, Voltage-gated, Membrane conductance, Mutant analysis, Arabidopsis

**Introduction**

Stomata are pores, commonly found in the epidermis of leaves, and are surrounded by a pair of specialised cells known as guard cells. Guard cells regulate the size of the stomatal pore to balance the exchange CO₂ for photosynthesis with the need to conserve water [1]. The acquisition of stomata and the leaf cuticle are considered to be key elements in the evolution of advanced terrestrial plants [2] allowing plants to inhabit different and often fluctuating environments while controlling water content. Stomatal pores typically occupy less than 5% of the leaf surface, but they provide for over 90% of the CO₂ entering the leaf and over 70% of water loss from the plant as a whole [3]. Guard cells respond to a number of well-defined signals – including hormones, light and atmospheric CO₂ concentration – integrating these signals to regulate stomatal aperture [4,5].

In the past few decades, the combination of physiological and molecular biological methods in the model plant *Arabidopsis thaliana* has greatly advanced our understanding of stomata [1,4-7]. Among these, voltage clamp methods have proven powerful in connecting the molecular and physiological frameworks in an understanding of stomatal function. The voltage clamp itself lies at the core of a toolchest of techniques and provides the essential utility to bring the driving force of membrane voltage under experimental control. By so doing, it enables the dissection, identification and monitoring of ionic currents carried by individual ion transporters – ATP-dependent pumps, ion-coupled carriers and ion channels – across biological membranes [8]. Classic voltage clamp methods rely on impalements with two microelectrodes (or a single microelectrode with two separate barrels) that are used to measure membrane voltage and to pass current for voltage clamping, respectively [8,9]. Because a defined spatial geometry is essential for quantifying current spread under clamp conditions [8-10], these methods have proven highly successful for work primarily on a small number of single-celled species as well as cell types that are easily isolated from their surrounding tissues [11-17].

Since its wider introduction in the 1980’s [18,19], the patch clamp variant of the voltage clamp has been widely used in studies of plant ion channels [8,20]. The patch clamp offers a number of advantages for work on plant cells, the most important being the facility for electrical recordings from single cells isolated from almost any surrounding tissue, thereby avoiding the difficulties of electrical coupling via plasmodesmata between cells in situ [21]. It also presents some difficulties. For patch clamp recordings from plant cells it is essential to remove the cell wall, commonly by enzymatic digestion,
and to stabilise the protoplast against osmotic swelling in the absence of turgor. Both manipulations affect the underlying homeostatic properties of the cells and must influence their physiological behaviour [22,23]. Additionally, obtaining electrically and mechanically robust seals between the patch electrode and protoplast, and retaining stable measurements without significant “rundown” of currents over long periods of time are often challenging [20,24].

By contrast with many plant cell types [but see Chen et al. [15]], guard cells at maturity do not retain electrical connections with their neighbours [11,25]. They are easily separated by mechanical peeling of leaves [1] and recovered intact with their cell wall within the monolayer of epidermal cells. These features greatly simplify their handling for voltage clamp recordings and analysis, avoiding the need to isolate protoplasts and the technical challenges of the patch clamp. Despite the obvious advantages, only a very few studies [26-28] have made use of micro-electrode impalements and classic voltage clamp methods with intact Arabidopsis guard cells. A major difficulty in this case has been to obtain reliable measurements over 20–30 min or more, time periods long enough for physiological and pharmacological studies with single cells. Thus, many researchers have relied on statistical approaches in patch recordings from populations of guard cell protoplasts, often without an internal reference for comparisons; simply put, impalement methods have not offered significant benefits in overcoming the problem of ‘rundown’ in channel activities common to patch clamp recording [20,24].

We have revisited the problems of voltage clamp recording from intact Arabidopsis guard cells and offer here a few simple procedures that enable classic, two-electrode voltage clamp recordings. Included with this protocol are summaries of results demonstrating its utility in characterising the major ion channel currents and their stability over time periods of one hour or more. The impalement approach greatly simplifies experimental access to these currents and enables physiological studies to be carried out on a cell-by-cell basis.

**Materials**

**Plant materials**

- *Arabidopsis thaliana*. For purposes of demonstration, we included with wild-type (Col0) the nitrate reductase-null mutant *nia1-1/nia2-5* (*nia1nia2*) [29], the ABA-receptor quadruple mutant *pyr1/pyl1/pyl2/pyl4* (QC3) [30], the vesicle-trafficking mutant *spy121 (=syr1/pen1)* and its complementation with *SYP121* [31,32], the dehydroascorbate reductase mutant *dhar1-3* [33], and the K⁺ channel mutant *kc1-2* [31].

**Reagents**

- KCl, Ca(OH)₂, NaOH, HCl, CsCl, tetraethylammonium chloride (TEA-Cl), potassium acetate (K⁺-Ac), and 2-(N-morpholino)ethanesulfonic acid (MES) analytical grade.
- Opening Buffer (OB) for pretreating the stomatal guard cells, comprising 50 mM KCl and 10 mM MES, titrated to its pH 6.1 with NaOH, without added Ca²⁺.
- Recording Buffer 1 (RB1) for voltage clamp measurements of K⁺ channel currents, comprising 10 mM KCl and 5 mM MES, titrated to pH 6.1 with Ca(OH)₂ ([Ca²⁺] = 1 mM).
- Recording Buffer 2 (RB2) for voltage clamp measurements of the Cl⁻/anion channel currents, comprising 15 mM TEA-Cl, 15 mM CsCl and 5 mM MES, titrated to pH 6.1 with Ca(OH)₂ ([Ca²⁺] = 1 mM).

**Equipment**

- Environment-controlled growth room
- Refrigerator for stratifying seeds at 4°C
- Narashige PD5 multi-purpose microelectrode puller or equivalent, modified for multibarrelled microelectrodes [9].
- High-impedance (>10¹¹ Ω), multi-channel voltage clamp amplifiers and probes [8,9]
- Desktop computer and data acquisition system [8,9]
- Light microscope with a total magnification at least 400× or higher
- 12-volt battery for DC power to supply microscope
- Huxley-type micromanipulator with carrier (see below) incorporating light-weight micropositioner (e.g. Narishige C2-type micromanipulator)
- Faraday cage
- Anti-vibration table
- Gravity-feed system for switching between experimental solutions [9]
- Optically clear and pressure-sensitive silicone adhesive [8,9,12]
- Fine-tipped forceps, dressing forceps and razor blades
- Glass capillaries for double-barrelled microelectrodes [9]
- Two-ml polypolyethylene pipettes, silicon rubber and 0.5-mm diameter Ag wire for halfcells (see [9] and below)

**Protocol**

**Key steps for growing Arabidopsis plants and selecting guard cells for voltage clamp**

Growth history has an appreciable impact on stable voltage clamp recordings in Arabidopsis guard cells.
1. Pretreat compost with Intercept 70WG (Scotts, Ipswich, UK), a systemic insecticide.

2. Sow seeds onto the nutrient-rich Levington F2 + S 3 compost (Coulders, Glasgow, UK) in 60 mm pots covered with polyester mesh (Remnant Kings, Glasgow, UK Figure 1A) to avoid soil contact of the abaxial leaf surface and soil-borne stress factors.

3. Stratify seeds at 4°C, once sown, for 48 hours and leave the seed to germinate under a plastic lid (>95% RH) for one week.

4. Cultivate plants in a controlled environment growth room under long day conditions with $100 \mu \text{mol m}^{-2} \text{s}^{-1}$ light and a light/dark cycle of 16 h/8 h, 22/18°C, and 55/70% RH. Evenly and regularly water plants from below.

5. Transfer pots after one week to propagators. We use propagators with NITEX mesh fabric (mesh opening 200 μm diameter; Sefar, Heiden, Switzerland) over the sides of the covers to permit free air exchange while keeping out insects.

6. In preparation for experiments excise either the 5th or 6th true leaf of three-week-old plants; these leaves display an elliptical shape and are more serrated compared to the older leaves. NOTE: There is a correlation between stomatal responsiveness and stomatal age, the most responsive stomata often occur on leaves with higher stomatal densities, many stomatal primordia and smaller epidermal cells (Figure 1B and C). Successful impalements yield similar currents under voltage clamp when recorded from guard cells of plants grown under long- and short-day conditions. Nonetheless, we favour plants grown under long days, as growth under short days gives lower stomatal densities (Figure 1D).

7. Pretreat the glass of the measuring chamber, coating it with Dow-Corning silicon prosthetic adhesive (Factor II, Tucson, USA; see [9]). NOTE: Silicon adhesive is pressure-sensitive and optically clear. Once dried, it remains useable for many weeks, even under water. However, the solvent used in the adhesive must evaporate before use or it will kill the cells.

8. Excise the epidermis of the leaf by wrapping the leaf over a finger, adaxial side down, cut into the mesophyll near the base of the mid-vein with

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Figure 1 Growth and selection of Arabidopsis guard cells on epidermal peels. (A) Rosette of a plant after 19-d growth at the stage from which epidermal peels were taken for impalements. Plants were grown in individual flower pots, covered with a polyester mesh. True leaves are numbered in order of their appearance. Scale bar, 1 cm. (B, C) Epidermal peels taken from plants grown under long- and short-day periods, respectively. Note the higher density of stomata and the smaller size of the epidermal cells in (B). Scale bars, 30 μm. Arrows in (B) indicate examples of guard cell pairs favoured for impalement (D) Stomatal densities of plants grown under long-day (LD) and short-day (SD) (n = 46). The significance level is indicated with asterisks (P < 0.01).
forceps, and lift the abaxial epidermis away from the mid vein towards the leaf margin. Gently replace peel against the mesophyll, keeping a gentle tension to avoid folds, then cut at the end of the peel near the leaf margin using a fresh (sharp) razor blade. **NOTE:** It is often easier to peel away the epidermis some minutes after excision when the leaf is less turgid, and to work from the petiole to the apex of the leaf. Ideally, epidermal peels should be free from wrinkles, folds, dirt and, once mounted, air bubbles. Successful impalements are best obtained from open stomata with young guard cells (arrows, Figure 1B), as judged by the thickness of the stomatal lip and squat shape of the guard cells.

9. Press the abaxial side of the leaf with the excised epidermal peel gently onto the prosthetic adhesive.

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**Figure 2** Mechanical improvements for *Arabidopsis* guard cell impalement. (A) Double-barrelled microelectrodes pulled with settings for *Vicia* (above) and for *Arabidopsis* (below), in the latter case showing a 1–1.5° taper to the final 10 μm of the tip. The extreme tips of both microelectrodes are below the resolution of the light microscope. Scale bars, 10 μm. (B) A custom-built brace with a fixed clamp (fc) for one amplifier headstage and a second, adjustable clamp (ac) provided by a Narashige C2 micromanipulator. The entire brace is fixed to the lateral, rack-and-pinion coarse movement of a Huxley-type micromanipulator visible behind. Scale bar, 1 cm. (C) Halfcells of the Ag|AgCl-KCl type constructed (left) using 0.5 mm diameter Ag wire soldered to a 2-mm diameter socket threaded in a PTFE sleeve and fitted with silicon and glass tubing, and (right) using 0.5 mm diameter Ag wire soldered to a 2-mm diameter socket and press-fit with a silicon plug behind the tip segment of a 2-ml graduated polypropylene pipette tip. Scale bar, 1 cm. When backfilled with KCl electrolyte, the halfcells weigh 5.5 g (left) and 0.6 g (right). For general details of halfcell construction, see [9].
coating of the measuring chamber glass. Remove the remaining leaf tissue and cover the epidermal peel immediately with OB to prevent it drying.

**Key steps for pulling microelectrodes**

The volume of an *Arabidopsis* guard cell is typically 10–15% that of *Vicia* and tobacco guard cells. Thus, microelectrodes with input resistances near 100 MΩ when filled with 200 mM K+-Ac, such as have been used in the past [13,34], are not suitable and generally give a low rate of success and a high leak conductance with little evidence of selective transport activity.

1. Pull microelectrodes to give tip resistances of 300–500 MΩ when filled with 200 mM K+-Ac.
2. For double-barrelled microelectrodes with the higher input resistances (and correspondingly lower electrolyte leakage rates), pull double-barrelled microelectrodes, after twisting 360° [9], using settings to give a pull time around 25 s. **NOTE:** We use settings similar to those used for *Vicia* and tobacco guard cells [34], but with the coil heat elevated to give pull times roughly 25% less than used for *Vicia* guard cells. The resulting microelectrodes have 1.8–2.0 cm-long shanks and tips that tapered with a 1–1.5° angle (Figure 2A).
3. Store microelectrodes in a glass desiccator and coat microelectrodes with paraffin before impalement for reducing capacitance [8,9,12].

**Key steps for impaling Arabidopsis guard cells**

**Before starting**

Electrical recordings using double-barrelled microelectrodes are carried out largely as described previously [12,35] with some modifications. For K⁺ currents, microelectrode barrels are filled with 200 mM K⁺-Acetate, pH 7.5, to minimise interference from the anion current and recordings are carried out in continuously-flowing RB1; for measurements of anion current, both electrode barrels are filled with 200 mM CsCl and the cells bathed in flowing RB2. Currents recorded under voltage clamp are normalised to the surface area of the impaled guard cells and, for K⁺ channel analysis, are corrected for background (instantaneous) currents as described previously [12,35] using Henry’s EP suite software (Y-Science, University of Glasgow, UK). **NOTE:** The typical length and radius of *Arabidopsis* guard cells are 20 and 5 μm, respectively. For the data summarised in the Tables, these parameters were 22 ± 0.6 μm² and 4 ± 0.1 μm, respectively. Assuming a spheroid geometry, the mean guard cell surface area and volume were 468 ± 12 μm² and 783 ± 21 μm³, respectively.

An essential prerequisite is the use of a stable microelectrode mount that can accommodate two amplifier headstages and halfcells with a minimum of mechanical relaxation over time. We have adapted a Huxley-type micromanipulator with a custom-machined aluminium brace that supports positioning clamps (Narashige, C2-type).

**Table 1 Effect of pretreatment with opening buffer (OB) on stomatal aperture in all Arabidopsis lines Col-0, *nia1nia2*, QC3, QC3, kcl-1, syp121, syp121ox, and dhar1-3**

| Aperture (μm) | Average of 8 lines | Col-0 | nia1nia2 | QC3 |
|--------------|-------------------|-------|----------|-----|
| Iἐm experiments | 2.95±0.04 (150)  | 3.22±0.09* (99)  | 3.05±0.07 (79)  | 3.55±0.15** (39)  | 2.41±0.11 (12)  | 2.87±0.09* (58)  | 3.12±0.09 (46)  | 3.63±0.15** (15) |
| Iἐvisc experiments | 2.89±0.06 (84)  | 3.48±0.09** (74)  | 2.89±0.06 (28)  | 3.45±0.13** (30)  | 2.47±0.11 (23)  | 3.00±0.12** (25)  | 3.17±0.09 (33)  | 3.83±0.16** (19) |

Data are means ± SE of (n) experiments. * P < 0.05; **P < 0.01 as compared with control.

**Table 2 Effect of pre-treatment with opening buffer (OB) on seal lasting time of guard cells from all Arabidopsis lines Col-0, *nia1nia2*, QC3, QC3, kcl-1, syp121, syp121ox, and dhar1-3**

| Time (min) | Average of 8 lines | Col-0 | nia1nia2 | QC3 |
|------------|-------------------|-------|----------|-----|
| I grad μA cm⁻² experiments | 21.2±4.0 (179)  | 34.4±3.8** (49)  | 19.2±1.5 (72)  | 36.8±3.1** (14)  | 18.6±2.6 (7)  | 33.7±2.7** (34)  | 28.8±3.6 (45)  | 40.0±6.8* (12)  |
| I grad μA cm⁻² experiments | 19.1±3.6 (45)  | 35.0±4.6** (29)  | 19.1±3.2 (12)  | 28.3±4.9* (9)  | 17.0±3.5 (15)  | 37.5±5.2** (12)  | 22.7±3.2 (13)  | 52.0±14.2** (5)  |

All the experiments lasted less than 10 min are discarded. Data are means ± SE of (n) experiments. * P < 0.05; **P < 0.01 as compared with control.
to stabilise paired amplifier headstages (Figure 2). Additionally, connections between the headstages and microelectrode barrels are made using Ag-AgCl|KCl halfcells similar to those described previously [9], but constructed around the light-weight polypropylene tubing from the tips of disposable 2-ml pipettes, which is essential to provide mechanical stability for long-term recordings (Figure 2).

1. Carry out impalement by first positioning the microelectrode to rest over one guard cell and present the tip across the stomatal pore before advancing along the axis of the microelectrode to impale the second guard cell. **NOTE:** The initial movement of the microelectrode towards the guard cell requires very gentle manipulation. A ‘snapping’ of the tip through the cell wall and into the guard cell should occur together with an increase in input resistance to approximately 1 GΩ and decrease (more negative) in membrane potential (see Additional file 1: Table S1).

2. Wait 2–3 min for a seal to stabilize after impalement. **NOTE:** As with Vicia guard cells [34], successful impalements show an increase in input resistance and membrane voltage over 2–3 minutes. Impalements carried out in RB1 buffer, but with 0.1 mM KCl, will give much larger changes in voltage as the microelectrode seals into the cell. For purposes of the comparisons below, we allowed recordings to stabilise under free-running conditions for 10 minutes before collecting voltage clamp data.

3. Switch to the RB1 or RB2 for K+ and anion currents measurements, respectively, using a gravity-fed system.

**Comments**

**Buffer pretreatment and recording stability**

Impalements are easier to achieve, and can be held for longer time when epidermal peels are pretreated with OB similar to that used by Allen et al. [36]. For comparison, the data in Tables 1, 2, 3, 4 and Additional file 1: Table S1 summarise measurements from the guard cells of 407 stomata, including measurements of stomatal aperture, free-running membrane voltage, inward- and outward-rectifying K+ currents, I_{Kin} and I_{Kout}, respectively, and in separate experiments of anion current, I_{Anion}. The data sets include measurements with and without OB pretreatment and show that stomata across all the lines tested were significantly more open (P < 0.05) following OB treatment: mean apertures following OB treatments were 3.22 ± 0.09 and 3.48 ± 0.09 μm compared to 2.95 ± 0.04 and 2.89 ± 0.06 μm without OB pretreatment (see Table 1). Most important, the comparison shows that OB pretreatment greatly extends the time over which impalements can be held. Stable current recordings were extended by 62% and 83% for K+ and anion current studies, respectively.

### Table 3 Analysis of maximal conductance (g_{max}), gating charge (δ), half maximal voltage (V_{1/2}) of I_{Kin} and I_{Kout} for the Arabidopsis lines Col-0, nia1nia2, QC3, QL3, kc1-3, syp121, syp121ox, and dhar1-3 in both opening and non-condition

| Channel | Parameters | Average of 8 lines | Control | Pretreatment | Control | Pretreatment | Control | Pretreatment | Control | Pretreatment |
|---------|------------|-------------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|
| I_{Kin} | g_{max}(mS cm^{-2}) | 2.3±0.1(103) | 2.0±0.2(67) | 2.2±0.2(26) | 2.6±0.2(15) | 0.3±0.1(10) | 0.9±0.1** (36) | 3.4±0.3(21) | 3.2±0.2(13) |
|         | δ          | 2.2±0.1(103) | 1.9±0.1* (67) | 2.4±0.1(26) | 2.0±0.1* (15) | 1.1±0.2(10) | 1.9±0.1** (36) | 2.1±0.2(21) | 1.9±0.2(13) |
| I_{Kout} | g_{max}(mS cm^{-2}) | 3.0±0.1(103) | 3.5±0.1* (67) | 2.6±0.2(26) | 3.8±0.6* (15) | 2.7±0.3(10) | 3.6±0.2* (36) | 3.9±0.3(21) | 3.5±0.3(13) |
|         | δ          | 1.6±0.1(103) | 1.6±0.1 (67) | 1.8±0.1(26) | 1.6±0.1 (15) | 1.5±0.1 (10) | 1.5±0.1 (36) | 1.5±0.2 (21) | 1.5±0.2 (13) |

Data are means ± SE of (n) experiments.

* P < 0.05; **P < 0.01 as compared with control.

### Table 4 Effect of pre-treatment with opening buffer (OB) on the ‘rundown’ of I_{Kin} and I_{Kout} in Arabidopsis guard cells of Col-0, nia1nia2 and QC3

| Channel | Current (μA cm⁻²) | Col-0 | nia1nia2 | QC3 |
|---------|-----------------|-------|---------|-----|
|         | 10 min | 30 min | 10 min | 30 min | 10 min | 30 min |
| I_{Kin} | Control | -484.1 ±185.6 | -6.43 ±3.3** | -118.7±30.2 | -1.2±10.3** | -413.8±142.1 | -8.1±0.3** |
|         | Pretreatment | -586.3 ±40.7 | -431.9±75.2 | -223.0±49.5 | -247.1±54.8 | -602.1±112.4 | -543.2±44.3 |
| I_{Kout} | Control | 598.2 ±89.0 | 145.29±36.0* | 527.9±90.1 | 71.2±10.4** | 591.2±111.1 | 168.4±119.5** |
|         | Pretreatment | 901.0 ±271.5 | 921.6±236.4 | 698.8±147.2 | 669.9±174.9 | 741.3±198.8 | 732.3±127.0 |

Data are means ± SE from the analysis of >5 experiments extending over 60 min or more in each case.

* P < 0.01 as compared with control.
– to periods often in excess of one hour – compared with experiments in which guard cells were impaled immediately after peeling and mounting (see Table 2). The capacity to extend electrical recordings over this time scale ensures that experimental challenges such as exposures to hormones and different environmental parameters (for example CO$_2$, light, Ca$^{2+}$ and other ion concentrations) can be carried out on a cell-by-cell basis in Arabidopsis guard cells much as was pioneered in guard cells of Vicia and tobacco [12,13,35,37,38]. In effect, work over these timescales enables the use of each cell as its own control. The following summaries are provided in conjunction with the tabulated data.

**K$^+$ channel currents**

Out of 275 independent experiments with measurements of the K$^+$ currents 88% showed $I_{K_{in}}$ activity and 100% yielded $I_{K_{out}}$ activity as judged by the current activation kinetics, voltage dependencies and block by Cs$^+$ and TEA$^+$ (not shown, see Roelfsema and Prins...
Guard cells pretreated with OB showed appreciably greater stability in both $I_{K,in}$ and $I_{K,out}$ over extended time periods compared with guard cells impaled without pretreatment (Figures 3, 4 and Tables 3 and 4). Mean $I_{K,in}$ and $I_{K,out}$ amplitudes of all of the lines tested at 30 min, for example, decayed to less than 2% and 22%, respectively, of the initial amplitudes recorded 10 min after impalements in guard cells without OB pretreatment (see also [26]). By contrast, the $K^+$ currents showed less than a 5% change in amplitude over the same time period when guard cells were first pretreated in OB.

For quantitative comparisons, the steady-state kinetic characteristics for the $K^+$ currents were fitted either individually or jointly to a Boltzmann function of the form

$$I = \frac{g_{max}(V-E_K)}{(1 + \exp(-\delta(V_{1/2}-V)/F/RT))}$$

where $E_K$ is the equilibrium voltage for $K^+$ across the membrane, $g_{max}$ is the maximum ensemble conductance for the channels, $\delta$ is the voltage sensitivity coefficient or gating charge and $V_{1/2}$ is the voltage at

![Figure 4](http://www.plantmethods.com/content/8/1/15)

**Figure 4** Decay in $I_{K,in}$ and $I_{K,out}$ from guard cells of wild-type *Arabidopsis* plants without (no pretreatment) and with opening buffer pretreatment (pretreatment). Voltage clamp scans were carried out at intervals following impalements. Raw current traces are shown in for scans at 10, 20, and 30 min time points from two guard cells for $I_{K,out}$ (A) and $I_{K,in}$ (C). Scale: vertical, 500 $\mu$A cm$^{-2}$; horizontal, 2 s. Clamp scans were from a holding voltage of $-100$ mV with tail steps to $-100$ mV. Test voltage steps were to voltages between $-80$ and $+50$ mV for $I_{K,out}$ and to voltages between $-100$ and $-240$ mV for $I_{K,in}$. Data in (B) summarise the two current amplitude means ± SE (filled circles, no pretreatment; open circles, pretreatment) from 12 independent experiments with $I_{K,out}$ determined at $+40$ mV and $I_{K,in}$ determined at $-220$ mV. Note that currents recorded from guard cells in control experiments without OB pretreatment generally decayed with halftimes of 15–20 min.
which the ensemble conductance equals \( g_{\text{max}}/2 \). Both approaches yielded parameter values that are statistically indistinguishable (Table 3) and are similar to those obtained previously for Arabidopsis as well as Vicia and tobacco guard cells [13,24,26-28,37,40].

Comparisons of the intrinsic gating characteristics for the different Arabidopsis lines and the overall means showed that OB pretreatment had no substantive effect on either \( \delta \) or \( V_{1/2} \) (see also Figure 3). Values for \( g_{\text{max}} \) for \( I_{\text{K, out}} \) showed a significant increase in both the wild-type and nia1nia2 mutant lines, whereas \( g_{\text{max}} \) was largely unaffected in the QC3 mutant line (Table 3). These activities were reflected also in differences in the free-running membrane voltages (see Additional file 1: Table S1). We note, too, a close similarity in the gating parameters \( \delta \) and \( V_{1/2} \) between all of the lines, with the exception of the sypi21 and nia1nia2 mutants for which the genetic deletions are expected to affect channel gating or \( K^+ \) nutrition and balance [31]. Overall, these results confirm that the underlying gating properties for the two classes of \( K^+ \) channels were unaffected, at least during the first hour after impalements.

**Anion current**

To date, only Forestier et al. [28] reported \( I_{\text{anion}} \) in intact Arabidopsis stomatal guard cells, although components of \( I_{\text{anion}} \) have been identified with the SLAC1 and ALMT12 gene products [41-44]. We recorded \( I_{\text{anion}} \) in over 95% of cases from 158 guard cells in RB2 with current similar to past measurements from Arabidopsis, Vicia and tobacco [28,35,40]. The mean membrane voltage of \(-9.9 \pm 1.6 \text{ mV}\) in RB2 was also comparable to those recorded in these previous studies. We found no appreciable difference between guard cells with or without OB pretreatment (Additional file 1: Table S1) but, again, pretreatment prolonged the timeframe for \( I_{\text{anion}} \) recordings and experiments frequently extended over periods of one hour (Table 2 and Figure 5). Thus OB pretreatment improved the stability of \( I_{\text{anion}} \) recordings much as it did for those of \( I_{\text{K, in}} \) and \( I_{\text{K, out}} \).

**Summary**

Three key factors are essential for successful, two-electrode, voltage clamp recordings with Arabidopsis guard cells. First, the preparation and handling of the plants is important, incorporating a pretreatment regime with a stomatal opening buffer prior to the start of experiments; second, microelectrode design must meet the demands for impalements of very small cells, notably in the use of fine tips with input resistances roughly 5-fold higher than typically used for Vicia and tobacco guard cells; finally, a modified clamp and brace to carry the amplifier headstages and construction of light-weight, but rigid halfcells are essential prerequisites to provide stability without mechanical relaxation for long-term recordings. Overall, this combination of factors is sufficient to achieve measurements comparable to those with the much larger guard cells of Vicia and tobacco. These methods should now greatly speed the analysis of many mutants of Arabidopsis by simplifying electrophysiological studies of the guard cells.

**Additional file**

**Additional file 1: Table S1.** Effect of pre-treatment with opening buffer (OB) on guard cell membrane potential (\( E_m \)) in all Arabidopsis lines Col-0, nia1nia2, QC3, QL3, kc1-3, sypi21, sypi21ox, and dhart-1.3. Data are means ±SE of (n) experiments.
Authors’ contributions
ZHC carried out the electrophysiological studies and analysed the data together with XQX; CE carried out aperture measurements and image analysis; AH and MRB developed the software utilities, mechanical and electrical hardware for the voltage clamp recordings; ZHC, CE and MRB wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Anpamo Ruiz-Prado for growth room support. This work was funded by grants BB/F001673/1, BB/F001630/1 and BB/T02446/1 to MRB from the UK Biotechnology and Biological Sciences Research Council. CE was supported initially by a Glasgow University Open PhD Scholarship and by a 2011 UWS Research Travel Fellowship. Seed of the ptil1/pyr1/pyl4 mutants were gifts from Prof. S. Neill in University of the West England and Prof. S. Cutler of University of California in Riverside, respectively.

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Received: 13 August 2011 Accepted: 10 April 2012 Published: 6 May 2012

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doi:10.1186/1746-4811-8-15
Cite this article as: Chen et al.: Protocol: optimised electrophysiological analysis of intact guard cells from Arabidopsis. Plant Methods 2012 8:15.

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