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Measuring the osmotic water permeability coefficient (Pf) of spherical cells: isolated plant protoplasts as an example --Manuscript Draft--

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Abstract:	Studying AQP regulation mechanisms is crucial for the understanding of water relations at both the cellular and the whole plant levels. Presented here is a simple and very efficient method for the determination of the osmotic water permeability coefficient (Pf) in plant protoplasts, applicable in principle also to other spherical cells such as frog oocytes. The first step of the assay is the isolation of protoplasts from the plant tissue of interest by enzymatic digestion into a chamber with an appropriate isotonic solution. The second step consists of an osmotic challenge assay: protoplasts immobilized on the bottom of the chamber are submitted to a constant perfusion starting with an isotonic solution and followed by a hypotonic solution. The cell swelling is video-recorded. In the third step, the images are processed offline to yield volume changes, and the time course of the volume changes is correlated with the time course of the change in osmolarity of the chamber perfusion medium, using a curve-fitting procedure written in Matlab (the 'Pffit'), to yield Pf.
Author Comments:	Material for free download: (1) The 'PffitInstaller_web.exe' with the 'Pffit.exe' program, bundled with example data files and the two ImageJ plugins, 'Image Explorer' and 'Protoplast Analyzer'; this is available on http://departments.agri.huji.ac.il/plantscience/staff-eng/moran-pffit.html (2) the 'Pffit User Guide' as a Supplemental File on JoVE's website.

	Ammendment to Author Contribution: Xavier Draye, contributed the 'Image Explorer' and 'Protoplast Analyzer' plugins for ImageJ and their descriptions for data analysis.
Additional Information:	
Question	Response
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האוניברסיטה העברית בירושלים
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THE ROBERT H. SMITH INSTITUTE OF PLANT SCIENCES AND GENETICS IN AGRICULTURE



31 March 2014

JoVE Editor

JoVE51652R2

Dear Michelle,

Enclosed please find our revised manuscript entitled: **"Measuring the osmotic water permeability coefficient (Pf) of spherical cells: isolated plant protoplasts as an example"** by Shatil-Cohen et al..

Our revision indeed took us a while, in particular, after we realized we had to revise the P_fFit program – as described in our reply to the reviewers' comments.

The list of changes in the MS appears within "our replies..." document.

Thanks for your patience.

We hope this revision is satisfactory.

Would it be possible to complete the arrangements of the MS for publication (including filming) before the 31st of May – the date of my departure to Australia for 5 months?

Sincerely,

Nava Moran

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-----1st Cover Letter -----

22. Sept. 2013

Dear JoVE Editor,

Enclosed please find our manuscript entitled: **"Measuring the osmotic water permeability coefficient (P_f) of isolated plant protoplasts and frog oocytes"** by Shatil-Cohen et al.

• ***Why this work should be published in JoVE's unique multimedia format***

We believe our method is relatively easy and efficient, more so than other published methods. Once assimilated by a user, it will enhance both data accumulation and analysis, and, hopefully, important results, as well.

Because visual demonstration does not rely on preformed (often mistaken) concepts as does verbal explanation, a video format will help familiarize the 'initiate' with our method much faster than by print.

• ***Author contributions***

Arava Shatil-Cohen designed, performed and analyzed the experiments, wrote the MS.

Hadas Sibony participated in the experiments.

Xavier Draye, contributed the "Protoplast Analyzer" plugin for ImageJ, and its textual description for data analysis.

François Chaumont, a co-developer, with MM and NM of the computerized approach to the determination of P_f based on data from non-instantaneous hypotonic-challenge to protoplasts (Moshelion et al., 2004), participated in MS discussion and writing.

Nava Moran, a co-developer, with MM and FC of the computerized approach to the determination of P_f based on data from non-instantaneous hypotonic-challenge to protoplasts (Moshelion et al., 2004), participated in MS discussion and writing. She is the Corresponding Author for this MS.

Menachem Moshelion, a co-developer, with NM and FC, of the computerized approach to the determination of P_f based on data from non-instantaneous hypotonic-challenge to protoplasts (Moshelion et al., 2004), participated in the design of experiments, MS discussion and writing. He is the project leader.

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- **The JoVE editor** we have been corresponding with is Zhao Chen.

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(we do not list opposed reviewers)

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6. **Steve Tyerman**, University of Adelaide, steve.tyerman@adelaide.edu.au

We hope this MS is found suitable for publication in JoVE.

Sincerely,



Nava Moran

TITLE:

Measuring the osmotic water permeability coefficient (P_f) of spherical cells: isolated plant protoplasts as an example

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KEYWORDS:

Osmotic water permeability coefficient, aquaporins, protoplasts, curve fitting, non-instantaneous osmolarity change, volume change time course.

SHORT ABSTRACT:

Measuring the osmotic water permeability coefficient (P_f) of cells can help understand the regulatory mechanisms of aquaporins (AQPs). P_f determination in spherical plant cell protoplasts presented here involves protoplasts isolation and numerical analysis of their initial rate of volume change as a result of an osmotic challenge during constant bath perfusion.

LONG ABSTRACT:

Studying AQP regulation mechanisms is crucial for the understanding of water relations at both the cellular and the whole plant levels. Presented here is a simple and very efficient method for the determination of the osmotic water permeability coefficient (P_f) in plant protoplasts, applicable in principle also to other spherical cells such as frog oocytes. The first step of the assay is the isolation of protoplasts from the plant tissue of interest by enzymatic digestion into a chamber with an appropriate isotonic solution. The second step consists of an osmotic challenge assay: protoplasts immobilized on the bottom of the chamber are submitted to a constant perfusion starting with an isotonic solution and followed by a hypotonic solution. The cell swelling is video-recorded. In the third step, the images are processed offline to yield volume changes, and the time course of the volume changes is correlated with the time course of the change in osmolarity of the chamber perfusion medium, using a curve-fitting procedure written in Matlab (the 'Pfit'), to yield P_f .

INTRODUCTION:

Water uptake and flow across cellular membranes is a fundamental requirement for plant existence at both the cellular and the whole-plant levels. At the cellular level, aquaporins (AQPs) play a key role in the regulation of the osmotic water permeability coefficient (P_f) of the cell membrane¹⁻³.

To date, several methods have been employed in measuring the endogenous P_f of protoplast from different plant organs (i.e. roots, mesophyll, endodermis, etc., reviewed by Chaumont et al. ⁴). One of the approaches to measure P_f is to expose the protoplasts to an osmotic challenge and to monitor the initial rate of its volume change (i.e., the slope of the early linear phase of the volume change). Two different methods were previously described based on this approach, both based on an instantaneous exchange of solutions. The first one consists of immobilizing the protoplast with a suction micropipette and switching the solution flow⁵ and the second one of transferring the protoplast from one solution to another using a micropipette⁶. These suction-micropipette and transferring-micropipette methods, which allow image acquisition at the very start of the fast solution exchange (to capture the early linear phase of volume change), likely involve a physical stress to protoplasts and require specialized equipment and expert micromanipulation.

The method described here minimizes the disturbance to the cells, involves no micromanipulation and permits derivation of P_f when the bath perfusion is *not* instantaneous.

After the enzymatic digestion, the protoplasts, submerged in an isotonic solution, are immobilized on the coverslip-glass bottom of a plexiglass (aka Lucite or perspex) chamber by charge interaction. Then, during a constant bath perfusion, the isotonic solution is flushed away by a hypotonic solution generating a hypo-osmotic challenge to the protoplasts. The swelling of the protoplast is video-recorded and then, by combining the information about the time course of the bath perfusion and the time course of the cell swelling, the P_f is determined by image processing and curve-fitting procedures.

The advantages of this method are that the experiment is very efficient, i.e. it is possible to monitor a few cells simultaneously in a single assay, and that it does not require special equipment or particular micromanipulation skills. Several applications for this method are possible. For example, determination of the native P_f of a variety of cells from different tissues and plants, such as mesophyll and bundle sheath cells from Arabidopsis leaf⁷, maize leaf mesophyll or root cortex cells⁸⁻¹⁰ or suspension cultured cells^{11,12}. In addition, it is possible to determine P_f of spherical animal cells such as oocyte cells¹¹. Another example involves examination of AQP activity by transient expression of their gene in the protoplasts (or any other genes which may affect them; e.g., genes of kinases) and determination of their contribution to P_f ; for example, expression of tomato AQP SITIP2;2 in Arabidopsis mesophyll protoplasts by PEG transformation and determination the SITIP2;2-related P_f ¹³. Finally, examination of the effect on P_f of different molecules/substances (drugs, hormones, etc.) added to the solutions can also be examined, for example of the AQP blocker HgCl₂⁷.

The following protocol describes the isolation of protoplasts of Arabidopsis mesophyll cells and determination of their P_f .

PROTOCOL:

1) Preparation of Solutions

1.1) Prepare isotonic (600 mOsm) and hypotonic (500 mOsm) solutions containing 10 mM KCl, 1 mM CaCl₂, and 8 M 2-(N-morpholine)-ethanesulphonic acid (MES), pH 5.7 and adjust osmolarity with the appropriate amounts of D-sorbitol: 540 mM for the isotonic and 440 mM for the hypotonic solution. Verify the osmolarity of the solution (within 3 % of the target value) using an osmometer.

1.2) Prepare a dry stock of 'enzymatic mix' containing the following enzymes: 0.55 g cellulase, 0.1 g pectolyase, 0.33 g polyvinylpyrrolidone K 30, 0.33 g BSA (see Table No. 1 below), mix the dry powder by vortex, make 5.7 mg aliquots and store at -20°.

2) Isolation of Arabidopsis Mesophyll Protoplasts

2.1) Prepare a Petri dish (10 cm) with about 6 drops (approx. 30 µL each) of isotonic solution.

2.2) Peel the abaxial (lower) Arabidopsis leaf epidermis, cut the peeled *leaf* into squares of about 4x4 mm, then place the squares on the isotonic solution drops with the exposed abaxial side down, touching the solution.

2.3) Dissolve 5.7 mg of the enzyme mix in 165 μ L isotonic solution (3.3 % w/w) in a 1.5 mL tube, mix gently (by finger taps on the tube), and place several similar drops of the enzymatic solution in the same Petri dish.

2.4) Transfer the leaf pieces onto the *enzymatic* solution drops, close the dish sealing the lid with one round of parafilm and incubate for 20 min, floating the dish in a water bath set to 28 °C.

2.5) Add several more drops of the *isotonic* solution to the dish (2 drops per each enzyme sol. drop). Transfer each leaf piece to a new isotonic solution drop, then, sequentially, to a second drop (to wash the enzymatic solution away). Lift the piece by its edge using forceps, shake it in the second drop (like a tea bag) to release the protoplasts. Collect the drops with the protoplasts (using a clipped-off 100 μ L pipette tip) into a 1.5 mL tube.

3) The Hypotonic-Challenge Assay: Arabidopsis Mesophyll Cell Swelling

3.1) Prepare the perfusion system (**Figure 1A**) by filling one column with the isotonic solution and another column with the hypotonic solution. Open the valve, let some solution flow (first the hypotonic, then the isotonic) to fill the tubing all the way down to the inlet manifold (**Figure 1B**). Ensure there are no trapped air bubbles, and then close the valve.

3.2) Seal a coverslip, using silicone grease (Table 1), onto the bottom of the chamber within the plexiglass slide (**Figure 1B**; see also the schematics of the chamber in **Figure 1C**). To make the chamber bottom (the upward facing exposed surface of the coverslip within the grease ring) “sticky” for protoplasts, coat it with positive-charge-bearing protamine sulphate (1 % in water; Table 1) or poly-L-Lysine (0.1 % in water; Table 1). Spread this ‘glue’ over the coverslip using a pipette tip, wait for 1-2 min, rinse 3-4 times with the isotonic solution and shake away the remaining solution.

3.3) Fill the chamber up with the isotonic solution. Then, add a drop of protoplasts-containing solution to the chamber, using a clipped-off pipette tip and wait 3-4 min for the protoplasts to settle. Cover the chamber with a transparent cover (**Figures 1D, 1E**) touching the solution surface (avoid trapping air bubbles beneath).

3.4) Place the slide (gently!) on an inverted microscope table, connect it to the perfusion system and the pump (guarding against air bubbles in the tubing!) and turn on the isotonic solution flow for constant perfusion at 1 mL/min (faster rates can be used, up to 4 mL/min).

3.5) For recording volume changes, an inverted microscope is used, with a 20X objective and with a CCD video camera connected to a PC computer. Use the ‘CMU 1394 Camera Driver’ plugin of the ImageJ software (see the Table of Specific Materials for the download addresses of these two software pieces) to record a 60 s video movie of selected immobile protoplasts (presumably, those stuck to the bottom) at a rate of 1 image /sec (1 Hz). Start the recording with

a 15 s wash of the isotonic solution (this constitutes the baseline), switch to the hypotonic solution for 45 s (to complete a total 60 s from the start of perfusion). Save the movie in TIF format. **Note:** Choose a view field with as many cells as possible, fulfilling the following criteria: spherical in shape and with a well-focused cell contour at their largest perimeter (**Figure 2A**).

4) Analysis of the Cell Volume Change Using ImageJ

Note: To analyze the series of images of a swelling cell, use the ‘Image Explorer’ and ‘Protoplast Analyzer’ plugins in the ImageJ software (written by Xavier Draye)¹⁴. Starting with the chosen protoplasts at their first time point, the ‘Protoplast Analyzer’ plugin will detect automatically the protoplasts edges (contours) and calculate the time course of their areas during the experiment (the plugins are available with the Pfit analysis program, below).

4.1) Start ImageJ. To open the movie, click ‘File’ on the ImageJ panel, then, consecutively on the dropdown menus as they unfold: ‘Import’ then ‘Image Explorer’. Highlight the chosen movie, then right-click on it, then left-click on ‘Protoplast Analyzer’. Browse through the movie (using a slider at the protoplast image bottom) to identify protoplasts that remain largely immobile during the experiment – these will be analyzed. Back on the first image, using the mouse, draw circles (picked from the ImageJ drawing tools) around the selected protoplasts (**Figure 2B**), then click ‘OK’ in the table of ‘Detection parameters’ that appeared.

4.2) To launch the protoplast detection algorithm, click ‘Local’ on the protoplast image top panel, then ‘Process’ in the dropdown menu. Examine the green circles around the selected protoplasts (**Figure 2C**) throughout the movie. Save the ‘Result’ in an Excel file. Quit ImageJ. **Note:** In case a red dot appears (to indicate a bad contour fit – usually due to a poor image contrast), re-run with different parameters.

4.3) To determine the pixel-to- μm conversion factor for obtaining the real value of P_f , snap an image of a micrometer ruler via the same 20X microscope objective. Drag a line (picked from the ImageJ drawing tools) along the ruler image and read the pixel number equivalent to the ruler length at the bottom of the ImageJ main panel. Convert the arbitrary pixel area values in the Excel file into μm^2 . Save the areas time course as a text file (two columns of numbers only). **Note:** This will be an input to the volume-fitting ‘Pfit’ program.

5) Modeling the Rate of Osmolarity Change in the Experimental Chamber Using ImageJ and the Matlab Program Pfit

5.1) Add 2 mg xylene cyanol (Table 1, below) to 100 mL of the isotonic solution (to produce the ‘Indicator Dye’).

5.2) Prepare the perfusion system (as in 3.1) with the Indicator Dye and the non-dyed hypotonic solution.

5.3) Seal a cover slip using silicon grease to the bottom of the plexiglass chamber, then gently fill the chamber with the Indicator Dye, cover it with a cover slip (as with the protoplasts before) and place it on the microscope stage.

5.4) Connect the chamber to the perfusion system and the pump, and turn on the Indicator Dye flow for a constant perfusion at 1 mL/min.

5.5) Record a 60 s movie at the rate of 1 Hz. Start the recording with 15 s of Indicator Dye, switch to the hypotonic solution for 45 s. Stop filming. Flush with the Indicator Dye (at least for 30 s), then start a new movie. Repeat about 5-6 times and save all the movies

5.6) Use the ImageJ software to analyze the video images of the Indicator Dye transmittance to obtain an averaged time course of the changing transmittance.

5.6.1) Start ImageJ, click 'File', then, 'Open', and browse for the movie. For each movie, draw a 10 pixel-wide vertical rectangle anywhere on the 1st image of the movie. Click 'Image' on the ImageJ main panel, then click 'Crop' in the dropdown menu.

5.6.2) To align the 60 frames (of the 60 s movie) in one row, click again 'Image', then click consecutively in the dropdown menus as they unfold: 'Stacks' and 'Make Montage' (columns 60, rows 1). Draw a 1 pixel-high horizontal rectangle across the whole row of images and click 'Analyze' in the ImageJ main panel, then click 'plot profile' in the dropdown menu. **Note:** A 'Plot of Montage' window will appear (not shown), and a list of transmittance data can be opened from its menu. Each image of the movie is represented in this list by 10 transmittance values originating in its 10-pixel-wide rectangle and consequently the "time base" (the image sequential number) is 10-times longer.

5.6.3) Copy the lists of the transmittance data (one list per movie) to an Excel file. Average the transmittance time courses obtained from the several movies of the Indicator Dye flushes. Generate a real time base by multiplying the image sequential number by 0.1. **Save the averaged time course (two columns) to a text file.** **Note:** Before averaging, if desired, plot the individual time courses, to reject any irregularities. Ensure that the movie includes at least 5 final seconds of steady state transmittance of the Indicator Dye.

5.7) Start the Matlab fitting program P_fFit (the 'Indicator Fit' panel, **Figure 3**) to compute the various parameters of the osmolarity time course. **Note:** based on the known initial and final concentrations of the solution in the bath, the time course of the changing osmotic concentration of the solution is calculated from the concentration time course (calculated, in turn, from the Indicator Dye transmittance), assuming it follows the same dynamics as the dye concentration. P_fFit is a program available for use free of charge. The 'P_fFit_Installer_web.exe' can be downloaded from: <http://departments.agri.huji.ac.il/plantscience/staff-eng/moran-pffit.html> bundled with three example files. In addition, the 'P_fFit User Guide' with detailed explanations and definitions is accessible via Jove as a Supplemental file, which helps to familiarize the user with the P_fFit program.

5.8) In the 'Indicator Fit' panel, import the data of the mean time course of the Indicator Dye transmittance ('Indicator data file', **Figure 3A**) and insert manually the current experiment parameters and the initial guesses of the parameters 'width' and 't_{half}' describing the time course of the Indicator Dye concentration (**Figure 3B**). Click 'Run' to view the plots of the time

courses of the Indicator Dye concentration (real data and fit, **Figure 4A**), and of the modeled (calculated) bath osmolarity (**Figure 4B**). **Note:** a good fit to the data is essential (a recommendation: start with the values shown in **Figure 3**).

6) Determining the P_f using the Matlab Fitting Program PfFit

Note: In addition to the basic assumptions with regard to the behavior of a protoplast as a true and perfect osmometer¹¹, the determination of P_f rests on the presumption that P_f may change with time, that this dynamics of P_f underlies the time course of the cell volume change and that three parameters suffice to describe it: P_{fi} (the initial value of P_f), $Slope_{Pf}$ (the rate of the linear change of P_f) and Delay (the period from the start of the bath osmolarity change till the start of the cell volume change). Different models can be tested, including different combinations of these parameters and their values, including null values¹¹. PfFit searches for the best combination of these parameters to yield – by calculation – the most faithful reproduction of the experimental time course of the cell volume change¹¹, calculated, in turn, from the imported series of cell-contour areas (see also the Supplemental ‘PfFit User Guide’).

6.1) Switch to the ‘Volume Fit’ panel (**Figure 5**). Choose for import the areas data file (the text file with the time course of the ‘areas’ of the analyzed protoplasts, **Figure 5A**). Choose ‘Last Indicator Fitting’ as the parameter source (**Figure 5B**; see the ‘PfFit User Guide’ for alternatives). **Note:** These parameters (**Figure 5D**) are then used to regenerate the osmoticum change in the bath for the volumes fitting procedure.

6.2) In the ‘Volume Fit’ panel (**Figure 5C**), initialize (fill in the initial guesses for) the P_f parameters: P_f , $Slope_{Pf}$ and Delay (a recommendation: start with 1, 1, and 30, respectively), Chose the model ‘Class’ (a recommendation: start with II and mark ‘checks’ for all three parameters to be fitted). Click ‘Run’, then eyeball the interim figure (**Figure 5E**) and adjust the Delay parameter and the length of the record, if needed.

6.3) Examine the results graph (**Figure 6**) to evaluate the fit quality and record the fit error. Change the initializing parameters a few-fold each, and re-‘Run’. **Note:** Don’t be discouraged when the program gets stuck – just restart the program!

6.4) Repeat this procedure several times, starting with different combinations of initialization parameters, *aiming for the lowest value of the fit error*.

6.5) Copy the list of the fit results directly from the screen, or find them in the PfFit-generated ‘_FIT_Vol_Results.txt’ file.

REPRESENTATIVE RESULTS:

In order to determine the P_f and compare the activity of different AQPs, mesophyll protoplasts from Arabidopsis leaf are used. These protoplasts were found to have low basal (background) P_f levels⁷ and can serve as a functional-expression system to enable reproducible P_f measurements. Protoplasts from a mature leaf from a 6 week-old Arabidopsis plant were isolated and three gene constructs with AQP genes from Arabidopsis (*AtPIP2;1*) and maize (*ZmPIP1;2* and *ZmPIP2;4*)

were transiently (and separately) expressed using the PEG transformation method¹⁵. Assuming that the event of transformation is simultaneous for a large number of plasmids applied to the cell irrespective of their nature and based on the results which showed a 100% success rate for synchronized transient expression of two plasmids in one cell reported previously for other plant systems^{15,16}, they were co-transformed with a vector encoding the enhanced green fluorescent protein (eGFP) in order to label the transformed protoplasts (**Figure 7**).

For the P_f assays, protoplasts were set in the experimental chamber (**Figure 1B**) and the GFP labeled protoplasts were monitored by video while they were flushed initially with the isotonic solution (600 mOsm), then with the hypotonic solution (500 mOsm), using the perfusion system (**Figure 1A**).

The time courses of the cell volume changes (**Figure 8A**) were obtained for each cell in two stages: first, the ‘Image Explorer’ and ‘Protoplast Analyzer’ plugins were used to generate the time course of changes in the cell contour area (**Figure 2**), then, the Matlab fitting program P_f Fit (**Figure 5**) was used to import these areas and convert them to cell volumes. The P_f values (**Figure 8C**) were derived for each cell using the P_f Fit program (**Figure 5**), based on the time course of the cell volumes and, additionally, on the imported averaged time course of the transmittance changes of the Indicator Dye (**Figure 3**), converted to the time course of the Indicator Dye concentration change (**Figure 4A**) and then – to the time course of the bath osmolarity change (**Figures 4B, 6A and 8B**). It is worth noting, that ΔC , the difference in osmotic concentrations in the cell (C_{in}) and in the bath (C_{out}), i.e., the driving force for the water influx, was due almost only to the change of C_{out} (**Figure 6A**). In this experiment, P_f increased during the assay (**Figure 6B**).

The P_f values of the protoplasts transformed with each of the three AQPs were significantly higher than the P_f of the control cell transformed with GFP alone (**Figure 8C**).

FIGURE LEGENDS

Figure 1: The volume-assay system. (A) The experimental setup: The perfusion system contains solution reservoirs (infusion columns, ‘Cols’), tubing (T), valves (V) and a peristaltic pump (P) connected to the plexiglass slide set on the microscope table. HS= hypotonic solution, IS isotonic solution, Cm camera. (B) An enlarged view of the plexiglass slide with the experimental chamber (Chr) and the tubing attached via an inlet (In) manifold connector. The solution is sucked from the chamber via an outlet (Out) to the pump. (C) A schematic drawing of the plexiglass slide (counterclock-wise: top view, long-side view and short-side view): a= glass cover slip, the central chamber bottom; b= clear adhesive tape (Table 1), serving as a bottom for the inlet and outlet solution grooves leading to and from the central chamber; when the Scotch tape is replaced (only occasionally), a hole is cut in it under the chamber; c= a plexiglass block glued to the slide; d= an outlet connector hole. Numbers are mm (but the drawing is not to scale). (D) An enlarged view of the center portion of the slide with the transparent cover (also plexiglass) partially covering the central chamber (arrows). (E) Schematic drawing (top and side views) of the transparent cover. The size of the transparent cover handle (green plastic in D) is arbitrary. Other details are as in C.

Figure 2: Analysis of swelling protoplasts images using the ‘Protoplast Analyzer’ plugin. (A) **a**, the first image of the movie with protoplasts, **b**, as in a, but yellow circles indicate the selection made after reviewing the movie, before the contours are auto-detected, **c**, from the first till the last image the green circles tightly follow the contours of the “well-behaved” protoplasts undergoing analysis. (B) ‘Time’-course plots (with units of image number on the abscissa) of the calculated areas within the protoplast contours (‘Area’, in square pixels), for each tracked (and numbered) protoplast. (C) The parameters input panel of the ‘Protoplast analyzer’ plugin. Four ‘detection parameters’ can be adjusted to fine-tune the protoplast detection algorithm. The ‘*number of border pixels*’ parameter sets the minimum thickness of the protoplast contour (default value: 5). The ‘*relative weight*’ parameter influences the grey-level threshold difference between the inner protoplast area and the outer border (default: 2). The ‘*maximum circumference ratio*’ defines a threshold for excluding protoplasts whenever their shape deviates from a circle. This parameter is the ratio of the protoplast circumference to the circumference of a perfect circle having the same area as the protoplast (default: 1.05). The ‘*maximum area increase*’ (% increase per time step) parameter excludes protoplasts with contour area increases above the parameter value (default value: 5%). Finally, the plugin also handles small protoplast movements but will stop tracking protoplasts that move rapidly or that disappear from the image area. The movie can be re-run as many times as necessary, and a single protoplast can be re-analyzed separately.

Figure 3: The ‘Indicator Fit’ panel of the P_fFit program. This part translates the indicator transmittance time course into bath osmolarity time course. (A) Browse for the saved data file containing the time course of transmittance changes of the Indicator Dye. (B) Either use the previously saved list of variables and parameters, or insert manually the 5 variable values of the current experiment: ‘true_C_init’ and ‘true_C_end’ (the osmolarities of the initial bath solution and the P_f-assay solution perfused via the bath), ‘t_start_wash’ (the duration of baseline sampling at the initial Indicator Dye level), ‘threshold_%’ (% of baseline value, at which the program detects automatically the departure from baseline transmittance; 1-5% are usually the most effective), ‘N_steady_st_pts’ (the number of samples – with 10 samples representing every Indicator Dye image taken – to be averaged at the end steady state level of the Indicator Dye, crucial for the conversion of the Indicator Dye concentration to the osmoticum concentration) and initial guesses for two of the four parameters of the Indicator Dye transmittance sigmoidal time course, ‘width’ and t_{half} (roughly related to the duration of the transition part of the sigmoid, and to its midpoint, respectively; t_{half} may be negative!). Two best fit parameters, in addition to ‘width’ and t_{half} are obtained without the need for initial guesses: lag (‘flush_lag’), the time between the valve opening to the arrival of the solution in the bath, and ‘C_init’, without a physical meaning, but necessary for the description of the osmolarity time course (see the Supplemental P_fFit User Guide).

Figure 4: The Indicator Dye concentration in the bath and the osmolarity of the medium. (A) The time course of the Indicator Dye concentration, calculated directly from data (dots) and from the best-fit parameters (line) as it is washed away by a non-dyed solution. (B) The calculated time course of the osmolarity change of the bath solution, assuming it follows the same dynamics as the change of the Indicator Dye concentration.

Figure 5: The ‘Volume Fit’ panel of the P_fFit program. (A) Browse for the area time-course data file of the analyzed protoplast. (B) Choose the ‘Last Indicator Fitting’ option to import the experiment parameters from the last run through the ‘Indicator Fit’ (see the Supplemental P_fFit User Guide for alternatives). (C) ‘Model Type’ / ‘Class’: Class I contains the simplest model 1, Class II – models 2-5, class III – models 6-8. The models differ with respect to which parameters are being fixed and which are being adjusted (i.e., freely variable) during the fitting procedure (tick the box to allow it to vary), and whether or not ‘Slope_{Pf}’ and/or ‘Delay’ are null. The models 1-6 are discussed at length by Moshelion *et al.*¹¹. ‘Combinations’ lists the parameter choices dictated by the choice of ‘Model Type’/‘Class’. Among models with a similar fit result – choose the simplest! Initialize the ‘P_f’, ‘Slope_{Pf}’ (‘Slope_Pf’) and ‘Delay’ parameters as shown (more details about ‘Delay’ in E below). (D) The variables and parameters describing the time course of the changing bath osmoticum are input either manually, or as described in B. (E) An interim plot, invoked by hitting ‘RUN’, of a time-course of volume change (calculated from the cell contour areas) to aid in the choice of the initial value for the ‘Delay’ parameter. Estimate, by eyeballing, the total length of the baseline from the 1st point till the start of cell volume change (the ‘inclusive delay: the sum of ‘t-start-wash’ + ‘lag’/‘flush-lag’ + the “physiological” ‘delay’). Insert this value as an input parameter for the ‘delay’ in the ‘VolumeFit’ panel and ‘Run’ again (see also the Supplemental P_fFit User Guide).

Figure 6: The results of fitting. (A) “Behind the scenes”: the calculated ultimate time courses of the osmoticum concentrations in the two compartments: the bath (C_{out}, green line) and the cell (C_{in}, blue line; C_{in} is calculated based on the protoplast volume change and an assumption that the plasma membrane is permeable only to water – the “perfect and true osmometer”¹¹), and the time course of the difference between them (delC, red line), which is the driving force for water flow, ‘Eo-tLag’ marks the end of the ‘flush-lag’ and the start of the hypotonic challenge (here only at about 21 s). Red box: the error of the fit value (fit-ERR, see definition in B below). (B) The ultimate result of fitting the volume time course; Green box: ‘INPUT VARIABLES’ are the values entered via the P_fFit/‘VolumeFit’ panel (defined in **Figure 5A** legend). Black box: ‘exptl-Vol’ and ‘fitted-Vol’ are the experimental data and the volume calculated using the best-fit parameters, respectively, ‘Eo-tLag’ is the same as in A, ‘Eo-Delay’ marks the the start of volume change. ‘Area up 3%’ marks the volume at which the surface area increased by 3%, the presumed limit to the cell membrane ability to stretch without rupturing. ‘Pf (scaled)’ is the time course of the fitting-based calculated P_f, spanning the values indicated below the red box as ‘Span of P_f’. Red box: ‘FITTED PARAMETERS’ are the values of the best-fit parameters: ‘P_f_i’ (the initial P_f), ‘delay’ (the period between the onset of the hypotonic challenge and the start of the volume change (which, according to the model 5 used in this example, is also the start in a change in the P_f value), and ‘slope-P_f’ (the constant rate of change in the P_f value. ‘fit_ERR’ shown in A – the minimization target of the Matlab fitting procedure – is the “root-mean-square” deviation (i.e., a square root of an averaged squared deviation) of a green dot from the black line), presented as % of the baseline volume. It is by this value that the relative success of repeated fitting with different parameter initialization values is judged. A NOTE OF CAUTION: As the best-fit parameter values could be the result of a local minimum found in the error minimization procedure – to verify that a global minimum has been found, several runs are required with different initialization values for these three parameters (and the lowest fit_ERR should be sought during these attempts. Blue box: DELTAs are the changes that occurred by the end of the fitted volume change period: ‘avg VOLm%’ is the relative extent of the calculated

protoplast volume change and 'avg Area%' is the relative change of the protoplast surface area. The initial size of the cell is given by 'radius', derived from the mean value of the protoplast basal contour area.

Figure 7: Epi-fluorescence microscopy view of mesophyll protoplasts from Arabidopsis leaf after PEG transformation with GFP, (A) under transmitted white light and (B) at 488 nm excitation and 520 nm emission. Scale bar: 100 μ m.

Figure 8: Volume change and the extracted osmotic water permeability, P_f . (A) Time course (60 s) of protoplast swelling upon exposure to hypotonic challenge (mean \pm SE). (B) The calculated osmoticum concentration in the bath during the hypotonic challenge. Note that while the hypotonic solution flow was switched on at 15 s, it reached the bath only after a lag, here of 5.9 s. (C) P_f (mean \pm SE). Asterisks indicate significant differences from control ($p \leq 0.05$). Data from at least three independent experiments for each treatment with a total of n protoplasts (control: n=52, AtPIP2;1: n=13, ZmPIP1;2: n=28, ZmPIP2;4: n=34).

DISCUSSION

Described here is a simple and very efficient procedure for measuring the P_f of isolated plant protoplasts, applicable in principle also to other spherical cells, e.g., frog oocytes¹¹. This method is based on measuring the P_f in response to an osmotic challenge to the cell. In contrast to the other methods based on this approach, however, the change of solutions, i.e., of the osmolarity, is not instantaneous, but gradual, during a constant bath perfusion, starting with the isotonic solution, in which the baseline cell volume is established. In addition, this method does not involve a suction pipette and therefore minimizes the disturbance to the protoplasts.

The approach presented here enables measurements from a variety of protoplasts, from different plants or tissues. Yet, because of the calculations involved, only spherical cells can be analyzed. Also, the enzymatic isolation of the protoplasts and the osmolarity of the solutions need to be adjusted to the assayed cells (for example, the enzymatic isolation of tomato mesophyll protoplasts takes about an hour, considerably longer than in the case of Arabidopsis protoplasts). The isolation of Arabidopsis mesophyll protoplasts according to the presented protocol is simple, rapid and efficient, yielding a high number of protoplasts. Notably, this, combined with their low basal P_f levels and their high transformation efficiency (**Figure 8**), makes them an attractive system for the functional expression of AQPs, to enable quantitative comparisons of P_f induced by different AQP isoforms. When expressing AQPs in these protoplasts with a marker gene (such as GFP), one can easily screen the protoplasts in the experimental chamber for fluorescing cells to analyze.

It is worthwhile to check whether this system is a viable alternative to oocytes for assaying AQPs even from animal sources (that functional animal proteins can be expressed in plant cells has been already demonstrated¹⁷).

Using the P_f Fit program, two more parameters, beside the P_f , are obtained for the description of the protoplast responses to hypotonic challenges: delay, the time between the onset of volume

change and the start of bath perfusion, and Slope_{P_f}, the rate of change in P_f during the osmotic challenge (described in detail in ¹¹).

For each experimental data set the volume fitting procedure needs to be performed several times, supplying different starting (initialization) values for these parameters, eventually choosing the fit with the lowest error. This error minimization process could be portrayed as seeking the deepest valley (a “global minimum”) in a landscape of valleys with different depths, among many hills, and attempting not be caught in a rather shallow valley (a “local minimum”).

Two types of P_f are obtained, P_f at the very beginning of the hypo-osmotic swelling response (‘P_f initial’) and P_f calculated at the end of 15 s of swelling, counting from the end of the delay (‘P_f final’). The difference between the two is discussed fully by Moshelion *et al.*¹¹, with regard to the 6 models analyzed.

There are two critical steps in the protocol: first, a good fit to the time course of the Indicator Dye concentration, second, a good fit to the time course of the volume of the swelling cell.

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DISCLOSURES:

We have nothing to disclose.

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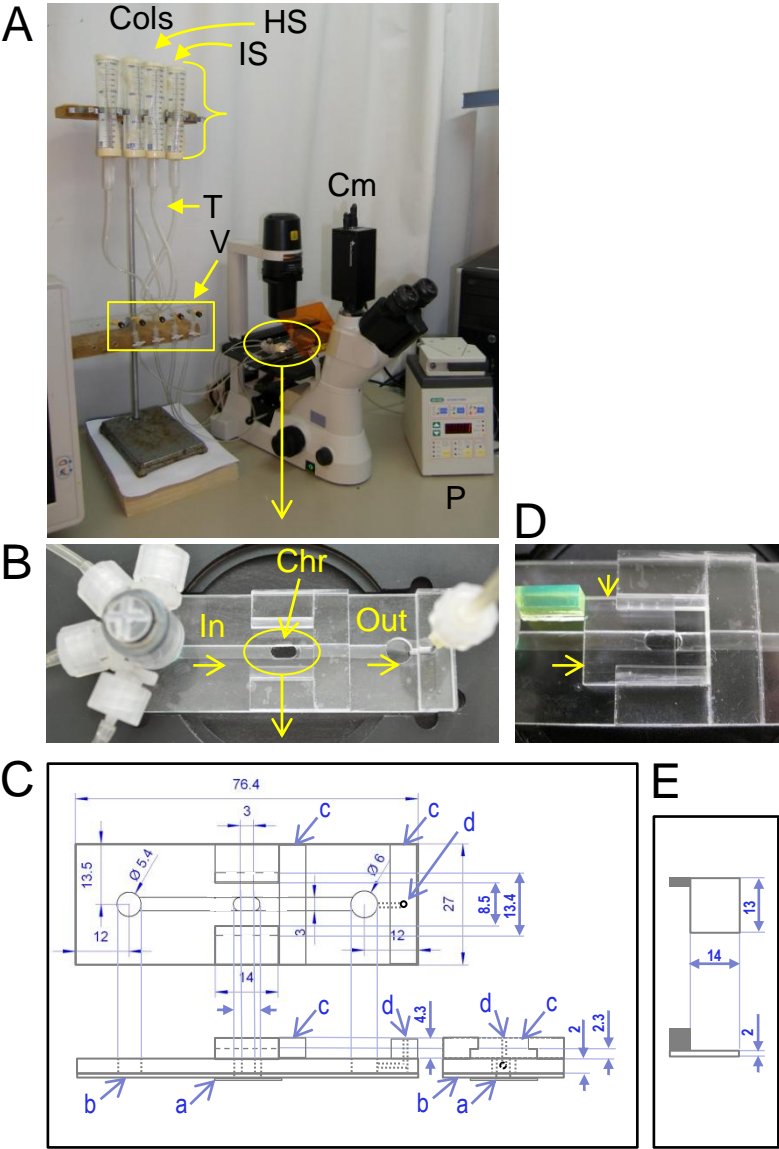


Figure 1
Shatil-Cohen et al., 2014

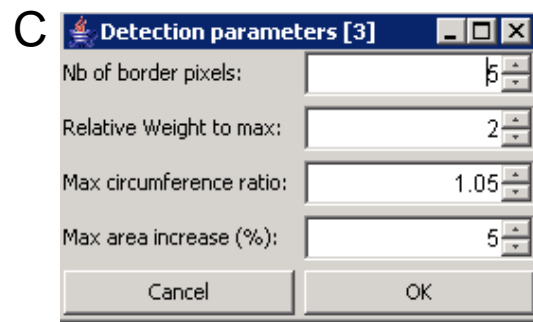
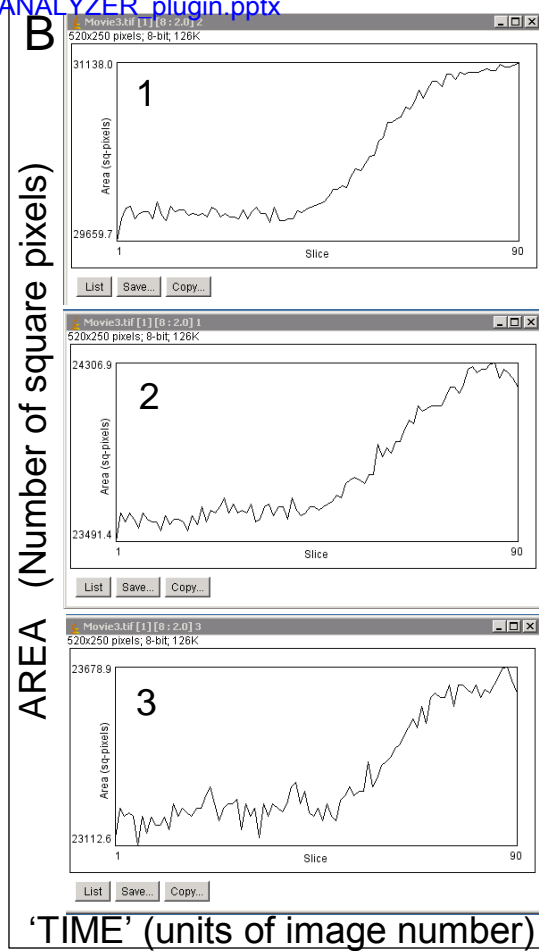
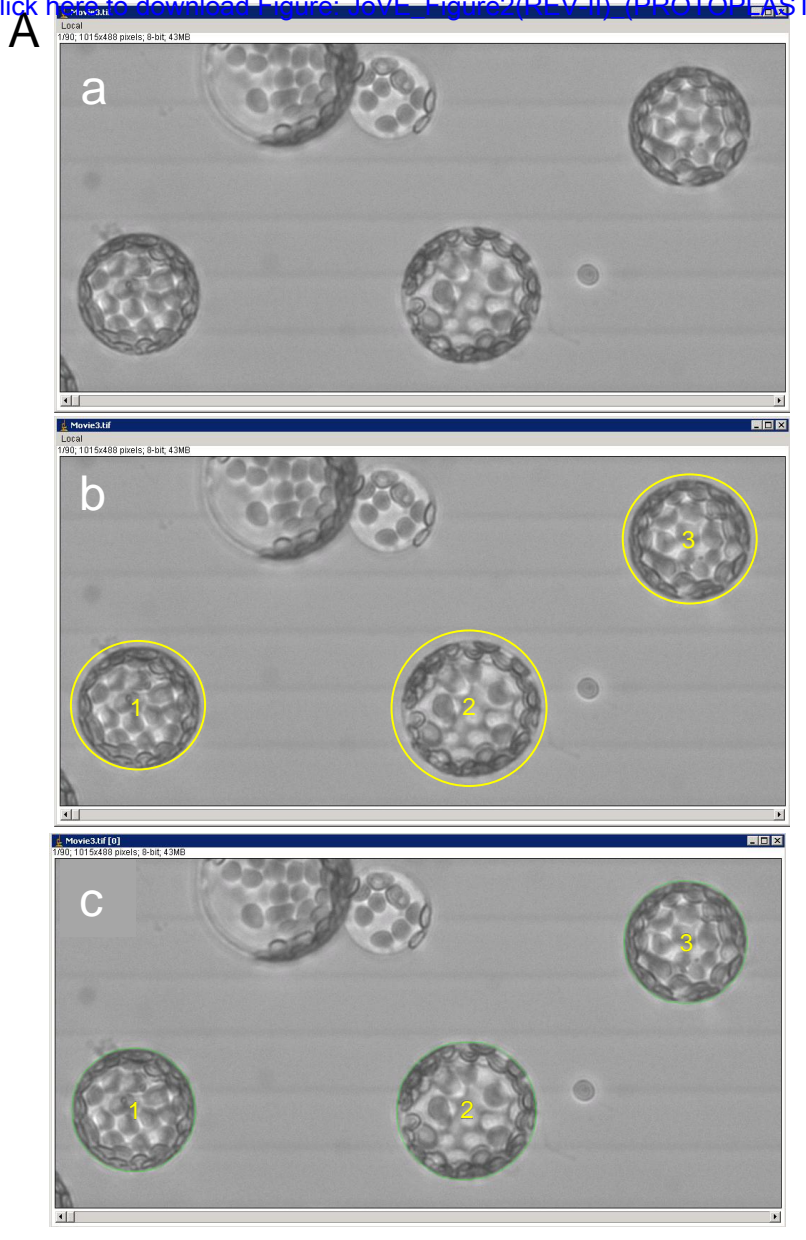


Figure 2
Shatil-Cohen et al., 2014

PfFit

Untitled 1

IndicatorFit ModelMake VolumeFit

A Indicator data file C:\PfFit\work\FlushOut_1av.txt

B Parameters Source Choose a File

true_C_init 0.6 Osm

true_C_end 0.5 Osm

t_start_wash 15 sec

Threshold_% 7 %

N_steady_st_pts 50

Guesses

width 7 sec

t_half -22 sec

Figure 3
Shatil-Cohen et al., 2014

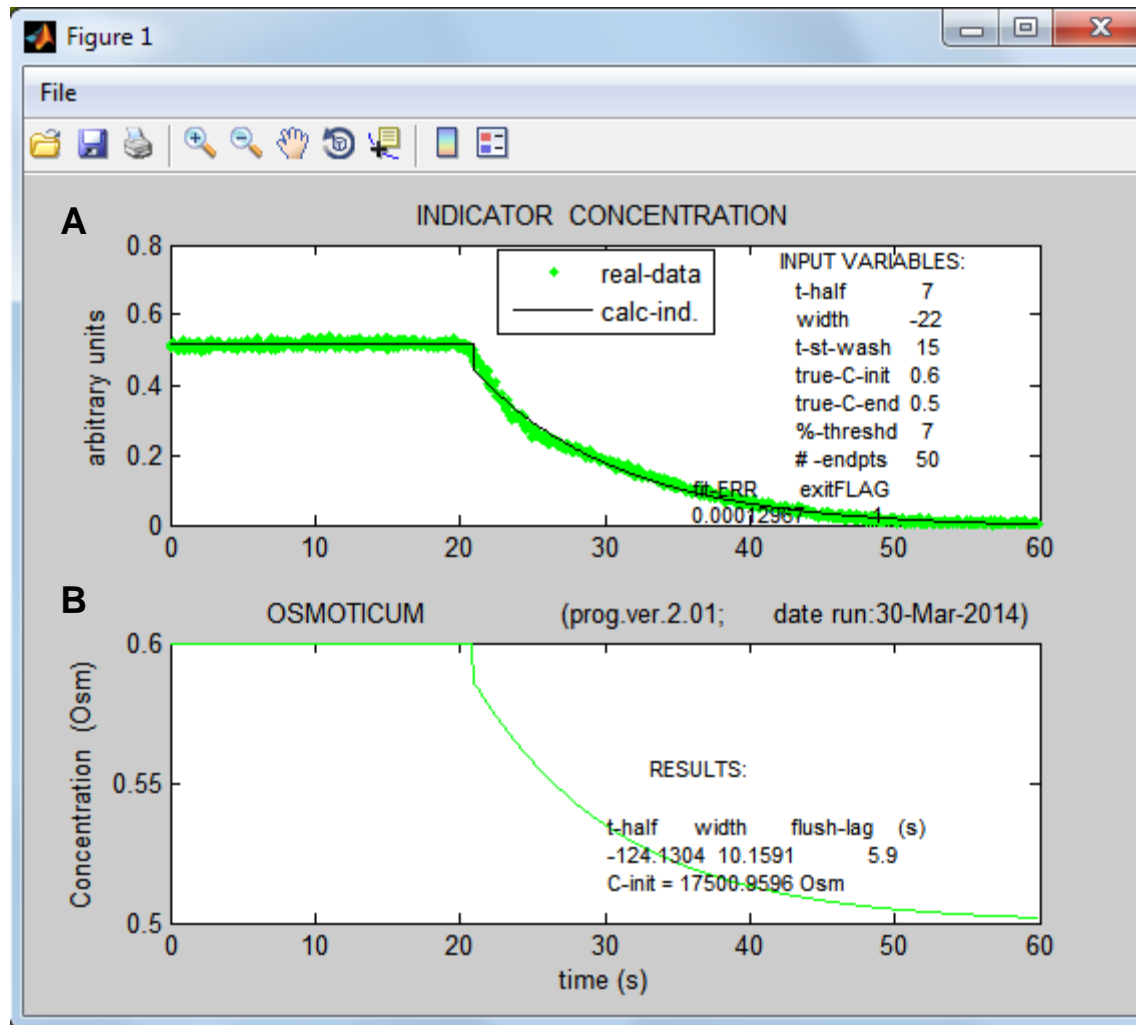


Figure 4
Shatil-Cohen et al., 2014

PfFit

Untitled 1

IndicatorFit ModelMake **VolumeFit**

A Areas data file C:\PfFitwork\AreasTimeCourse_micro

B Parameters Source Last Indicator Fitting

C

Model Type	Class II	Fit ?
Pf	1	um/se <input checked="" type="checkbox"/>
slope_Pf	1	um/sec^2 <input checked="" type="checkbox"/>
delay	30	sec <input checked="" type="checkbox"/>

D

lag	5.9	sec
width	10.159	sec
t_half	-124.13	sec
C_init	17501	Osm
true_C_init	0.6	Osm
true_C_end	0.5	Osm
t_start_wash	15	sec

Figure 5
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Shatil-Cohen et al., 2014

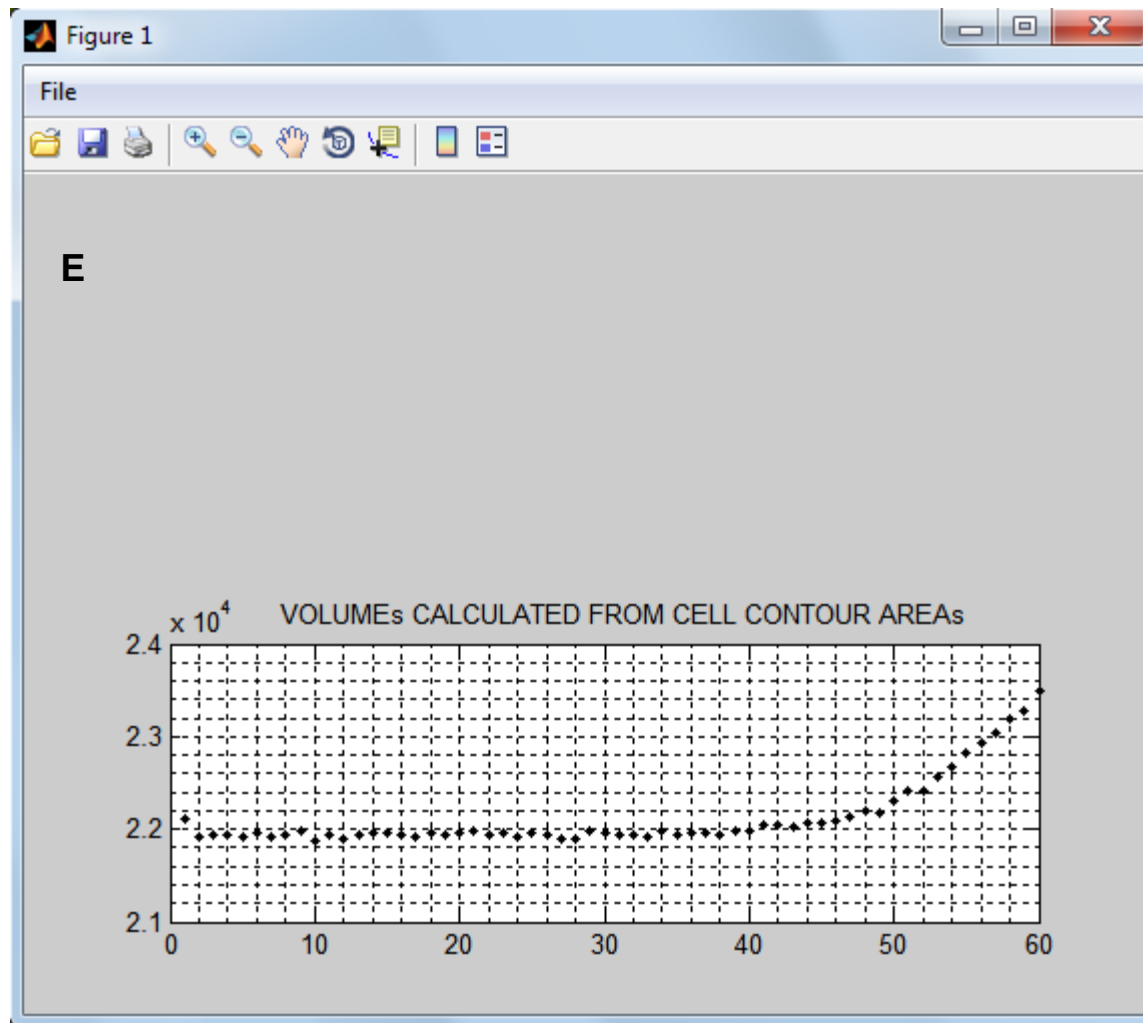


Figure 5
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Shatil-Cohen et al., 2014

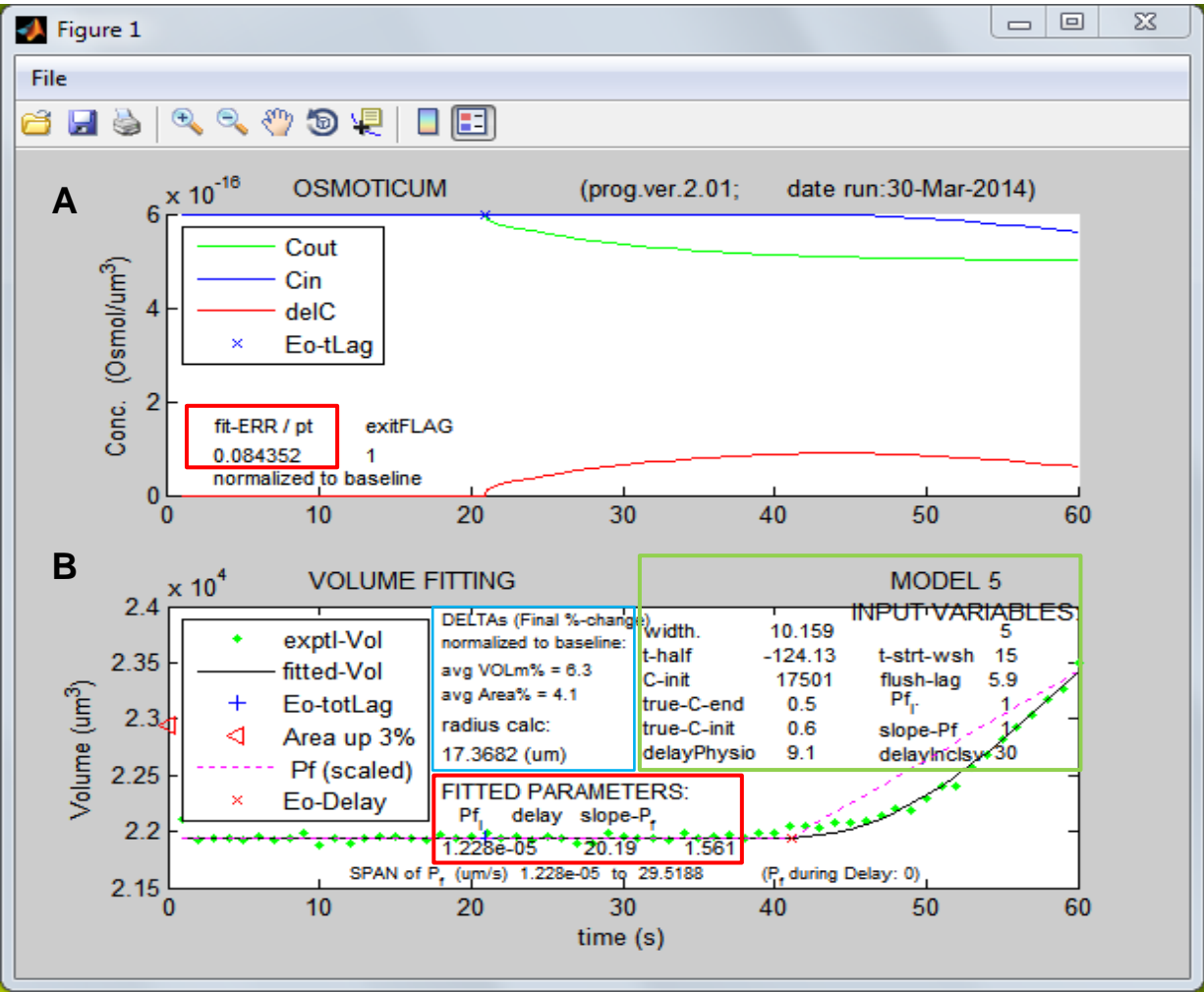


Figure 6

Shatil-Cohen et al., 2014

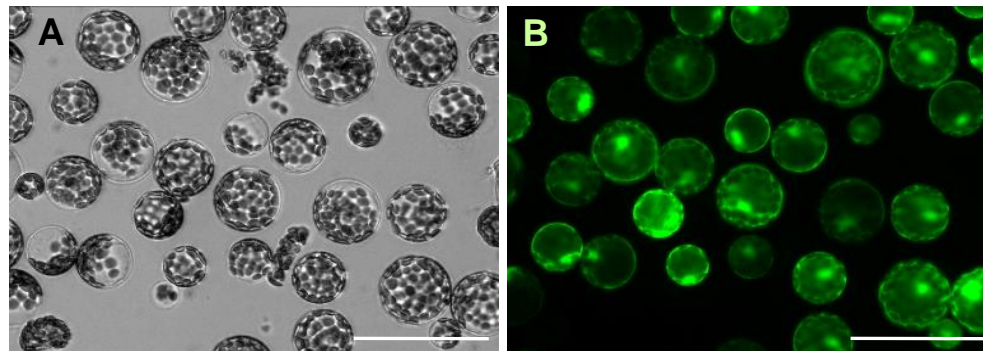


Figure 7
Shatil-Cohen et al., 2014

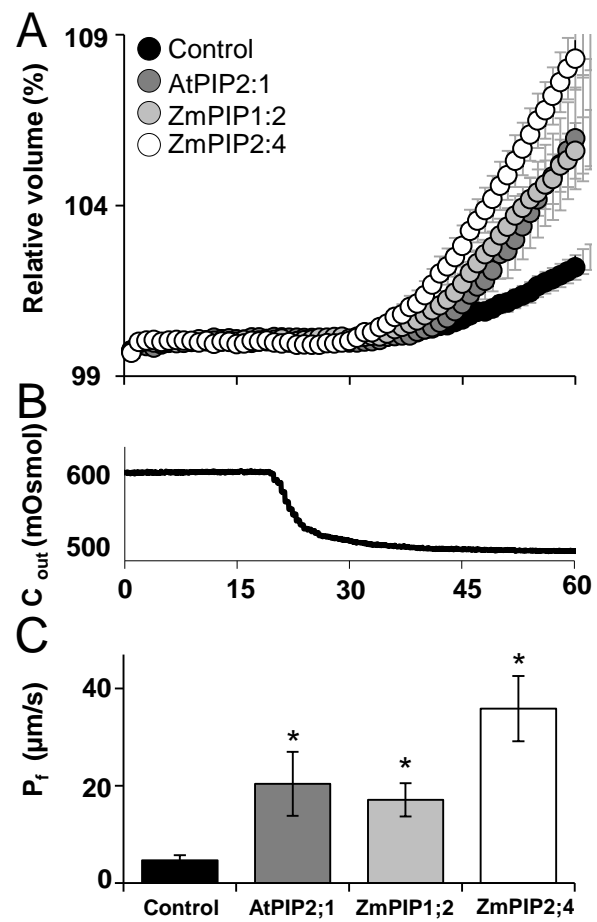


Figure 8
Shatil-Cohen et al., 2014

Table 1: Specific reagents and equipment

Name of the reagent	Company	Catalogue number
KCl	Chem-Impex International	01247-1
CaCl ₂	Merck	11718006
2-(N-morpholine)-ethanesulphonic acid (MES)	Sigma	15152002
D-Sorbitol	Sigma	18032003
Cellulase	Worthington, Lakewood, NJ, USA	LS002603
Pectolyase	Karlan, Phonix, AZ, USA	8006
Polyvinyl-pyrrolidone K 30 (PVP)	Sigma	81420
Bovine Serum Albumin (BSA)	Sigma	A9418-5G
Protamine sulphate	Sigma	P4380
Poly-L-Lysine	Sigma	P8920
Xylene cyanol	Sigma	X4126
Silicone vacuum grease heavy	Merck	107921
Inverted microscope	Nikon	Eclipse TS100/TS100F
Peristaltic pump	BIO-RAD	EP-1 Econo Pump
Grayscale digital camera	Scion Corporation	CFW-1308M
CMU 1394 Camera Driver' plugin for ImageJ	Carnegie Mellon	
ImageJ	NIH	
Econo Gradient Pump Fittings Kit	BIO-RAD	731-9006
Connectors, manifold	DirectMed	
Burette infusion sets (columns)	Welford	IF-BR-001
Tubing	TYGON	R-3603
Plexiglass slide etc.	Perspectiv	
3M packaging Scotch tape 1", clear	Viking Industrial, UK	VKMONO25

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http://www.chemimpex.com
http://www.merck.com
http://www.sigmaaldrich.com
http://www.sigmaaldrich.com
http://www.worthingtonbiochem.com
http://www.karlan.com
http://www.sigmaaldrich.com
http://www.sigmaaldrich.com
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http://www.sigmaaldrich.com
http://www.sigmaaldrich.com
https://merck-chemicals.co.id/chemicals/silicone-high-vacuum-grease-heavy/MDA_CHEM-1
http://www.nikoninstruments.com
http://www.bio-rad.com
http://www.scioncorp.com
http://www.cs.cmu.edu/~iwan/1394/download.html
http://rsb.info.nih.gov/ij/
http://www.bio-rad.com
http://directmed.com/main/Plastic-Medical-Tubing-Connectors.html?ACTION=S
http://www.welfordmedical.com/content.php?id=61
http://www.usplastic.com
http://www.perspectiv.co.il/index-en.html
http://www.vikingtapes.co.uk/c-428-vkmono-mono-filament-tape.aspx#.UuvqOfdy_8

Comment
Any source, anal. grade
Any source, anal. grade
Any source, anal. grade
Any source, anal. grade

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The Hebrew University of Jerusalem

Article Title:

Measuring the osmotic water permeability coefficient (Pf)
of isolated plant protoplasts and frog oocytes

Signature:



Date:

21 Sept. 2013

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MS # (internal use):

REPLIES TO Reviewers' comments _ JoVE51652R1

Dear Editor,

We thank the reviewers for their comments which led us to more detailed explanations.

We believe that this has made the MS more helpful to the potential users.

For our replies, we chose a different font style to distinguish them from the reviewers' comments.

At the end, we list additional changes not mentioned in the direct replies.

Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes. → *The figures and the MS have been downloaded and became the basis for changes.*

2) Please disregard the comment below if all of your figures are original. → *All are original.*
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version. → *Done.*

4) Prior to peer review, the highlighted portion of your protocol is close to or slightly over our 2.75 page highlighting limit. If, in response to peer review, additional details are added to the protocol, please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) to identify which portions of the procedure are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader. → *≤2.75 pages are yellow as of now (complete sentences).*

5) Please ensure that all images that you do not wish to be published are removed from the manuscript after you address these comments. → *All current images are to be included.*

Reviewer #1:

Manuscript Summary:

The manuscript describes a method of determining the osmotic water permeability coefficient P_f of

plant protoplasts from measurements of the time course of their swelling upon an osmotic challenge. A straightforward procedure for preparing fresh *Arabidopsis* mesophyll protoplasts is also presented and the perfusion system for the hypotonic challenge is described in detail. The analysis of the protoplast volume changes performed with the specially designed plugin "Protoplast analyzer" for the software platform ImageJ and the Matlab curve-fitting program used to determine the value of P_f are presented and illustrated with representative examples.

Major Concerns:

(1) --The explanations to Fig. 5 and Fig. 6 are fully comprehensive only for those familiar with the theoretical model put forward in the paper of Moshelion et al 2004 (reference 11), where the significance of the parameters Slope P_f and delay is explained. --This should be more clearly stated in the manuscript, in order to improve the intelligibility of the calculation procedure. → **A paragraph with a brief overview has been added in section 6 in the protocol (current rows 294-295):** "In addition to the basic assumptions with regard to the behavior of a protoplast as a true and perfect osmometer¹¹, the determination of P_f is based on the presumption that P_f may change with time, that this dynamics of P_f underlies the time course of the cell volume change and that three parameters suffice to describe it: P_{fi} (the initial value of P_f), Slope P_f (the rate of the linear change of P_f) and Delay (the period from the start of the bath osmolarity change till the start of the cell volume change). Different models can be tested, including different combinations of these parameters and their values, including null values¹¹. P_f Fit searches for the best combination of these parameters to yield – by calculation – the most faithful reproduction of the experimental time course of the cell volume change¹¹, calculated, in turn, from the imported series of cell-contour areas (see also the Supplemental 'P_fFit User Guide')."

Additionally, the legends to figures 3,5 and 6 have been expanded to include additional explanations about the fitting models and parameters (see also at the bottom, under "Additional changes/Figures").

(2) --Frog oocytes should be removed from the title they are not adequately addressed in the manuscript to warrant placement in the title. → **The mentions of oocytes have been altered to suit the reviewers' recommendations (the changes are highlighted here); The title has been changed to read: "Measuring the osmotic water permeability coefficient (P_f) of spherical cells: isolated plant protoplasts as an example"; Additionally, frog oocytes mentions are modified as follows: in the Long Abstract (updated also in the "Abstract" section of the on-line submission): "Presented here is a simple and very efficient method for the determination of the osmotic water permeability coefficient (P_f) in plant protoplasts, applicable in principle also to other spherical cells such as frog oocytes.", and in the Discussion: (rows 501-503) the sentence with a frog oocytes mention has been changed to read: "Described here is a simple and very efficient procedure for measuring the P_f of isolated plant protoplasts, applicable in principle also to other spherical cells, e.g., frog oocytes¹¹. "**

Minor Concerns:

(3) --I recommend the use of "immobilized" instead of "glued" when it refers to protoplasts firmly attached to the bottom of the measuring chamber. → **DONE (current rows 67, 99).**

(4) --On rows 115 and 116 the text should probably be: "...added to the solutions can also be examined, for example of..." → **DONE (current rows 117-118).**

(5) --In the Protocol, 1.2) enzyme amounts are given in "gr" which is wrong; SI mass unit is the gram (g).

→ **DONE**

(6) --Figure 6 is quite confusing, there are too much information confined to a small space. In Fig.6 I couldn't identify the "a." and "b." mentioned in the Figure legend. → *The Figure 6 legend has been rewritten extensively: we removed "a" & "b", added colored boxes in the figure to organize the details and added explanations and definitions of the abbreviations in the legend.*

(7) --Row 375 in the middle: there are two commas too many. → *Hopefully, all the typos are now gone.*

(8) --In the legend to Fig.8 there is a word missing on row 389 (*now, 493*) after "...bath during." → *Added: "the hypotonic challenge" after "during".*

(9) --On the row 390 (*now, 494*) there is a superfluous comma after "here". → **DONE**

(10) -- Row 432 (*now, 538*): areobtained should be separated. → **DONE**

(11) -- I do not understand why the authors consider the conversion from pixel numbers to lengths in mm to be a critical step. It is just a logical and easily done calibration step. → *We agree, we have changed this sentence to read (currently row 543-545, additions are highlighted here): "There are two critical steps in the protocol: first, a good fit to the time course of the Indicator Dye density, second, a good fit to the time course of the volume of the swelling cell."*

Additional Comments to Authors:

N/A

Reviewer #2:

The manuscript explains a simplified method for measuring osmotic water permeability of plant protoplasts. The explanation of the material and method is widely explained and the results are consistent with those that should be obtained with other methods. Therefore, it will merit publication in Journal of Visualized Experiments. However, there is a point that should be corrected before final acceptance.

--In the title and in the abstract, author are pointing that the experiment are validated for frog oocytes. The experiments were only carried out with plant protoplasts. → *See our reply No. 2 to Reviewer 1 above.*

Minor Concerns:

I have only a few minor remarks and requests:

--1) Some material is not clearly indicated (and some features of Table 1 are disorted), in particular the important part, the plexiglass slide from Perspectiv cannot be clearly identified (I tried a search, but failed with this rudimentary information). → *The Excel table appears to have been fixed by the editors. The website address has been corrected to direct the user immediately to the section in English. Indeed, the slide does not appear among the various items listed on the site, but the manufacturer is very responsive and we included now the following comment in the Table: "Our slide was custom-made, it does not appear on the web site but a copy can be made to order as 'a copy of the slide already made for M. Moshelion'."*

-- In addition, a more detailed plan (side/ top views) could be added to allow manufacturing if a workshop is available at a university or research institute. → *As suggested, we have expanded the*

details of the schematics of the slide (Figure 1) to aid local manufacture.

--2) Page 7/ 312: Figure 8B should be 8C, Figure 4 should be 8B? → *We corrected and reworded this sentence to read as follows (now page 8/351-359): "The time courses of the cell volume changes (Figure 8A) were obtained for each cell in two stages: first, the 'Image Explorer' and 'Protoplast Analyzer' plugins were used to generate the time course of changes in the cell contour area (Figure 2), then, the Matlab fitting program P_fFit (Figure 5) was used to import these areas and convert them to cell volumes. The P_f values (Figure 8C) were derived for each cell using the P_fFit program (Figure 5), based on the time course of the cell volumes and, additionally, on the imported averaged time course of the transmittance changes of the Indicator Dye (Figure 3), converted to the time course of the Indicator Dye concentration change (Figure 4A) and then – to the time course of the bath osmolarity change (Figures 4B, 6A and 8B). "*

--3) Cin: although shown in figure 6, it is not clearly described how Cin was determined, what was assayed? → *It is now defined in Fig. 6A legend (the additions are highlighted here): "....the osmotic concentrations in the two compartments: the bath (Cout, green line) and the cell (Cin, blue line; Cin is calculated based on the protoplast volume change and an assumption that the plasma membrane is permeable only to water – the "perfect and true osmometer" ¹¹),"*

--4) Discussion: page 9/402 (now p. 12/ 506-508): I also agree that the perfusion system is nice and maybe more reproducible in handling (and less dependent on the operator), yet the eventual movement is a relative matter: sucking the protoplast is replaced by "only" moving the bath solution, in bath cases the protoplast surface experiences the forces of the moving liquid. → *Immobilizing the protoplast by a suction pipette by itself involves an active deformation of one part of the membrane vs. the other, on top of the relative fluid movement. In addition, we deem the shearing forces exerted on the protoplast by the relatively gentle flow of the bath solution during the assay described here much less disturbing than the sudden rush of solution during an 'instantaneous' bath exchange.*

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

This paper describes the instrumentation used by the group in their 2004 Plant Physiology paper (ref 11). The osmotic swelling assay is nicely explained and the technique described in the paper will likely be useful to a broad readership within the field of membrane transport - and osmotic transport in particular.

Major Concerns:

I have one major issue: the actual design of the chamber is not very well presented –

--The picture in Fig 1b is not consistent with the drawing in Fig. 1c. The authors should provide a full 3D detailed drawing of the chamber also showing how it is assembled with coverslips. → *We provide now an additional photo of the transparent chamber cover and additional schematic views of the slide and the cover, with indication of sizes, and the Figure 1 legend has been expanded accordingly.*

--Also, frog oocytes should not be in the title as they are not experimented on/with in the manuscript.

→ *We complied, see our reply No. 2 to Reviewer 1, above.*

www.perspectiv.co.il/ → *see our reply No. 1 to Reviewer 2, above.*

Minor Concerns:

It would facilitate the reading (and use) of the paper if the authors briefly reviewed the theory behind the experimental design - i.e. based on the S-1 Appendix to the original 2004 paper. → *We complied, see our reply No. 1 to Reviewer 1, above.*

ADDITIONAL CHANGES

(1) To prepare the P_fFit program for more convenient distribution (as the previous version was generated 10 years ago), we updated it to conform to the current version of Matlab (summarized in the P_fFit User Guide). We also changed slightly the appearance of the plots to improve their visibility and the correspondence of the concentration time courses between the “Indicator Dye” and the “osmoticum” (detailed in the P_fFit User Guide). We therefore re-ran P_fFit and produced new figures 3-6.

(2) The P_fFit User Guide is now included as a Supplemental File.

(3) In the MS, in general:

-- We chose to replace “Indicator Dye “density” with the more correct “Indicator Dye concentration”, and, in a few instances, where we refer directly to the recorded values -- with “Indicator Dye transmittance”; this can be seen under ‘track changes’ of the MS WORD (the conversion between the two is detailed in the Supplemental P_fFit User Guide).

-- P_f and P_fFit have been changed to P_f and P_fFit.

-- “ ” has been changed to ‘ ’ whenever the enclosed word is a reserved name of a variable, figure part, software, etc..

-- Various typos have been corrected, a few expressions have been reworded for clarity, all are visible under ‘track changes’.

(4) Protocol

-- Section 2.5: changed from 3 drops to 2 drops.

-- Section 3.3: renamed the chamber cover: “transparent cover” instead of “cover slip”.

-- Section 4: added the mention of the ImageJ plugin ‘Image Explorer’ to the protocol (it is mentioned later in section 4.1). Additionally, changed the download location of the two ImageJ plugins to: “ (the plugins are available with the P_fFit analysis program, below)”.

-- Section 4.3: removed the highlight.

-- Section 4.4: removed as unnecessary.

-- Section 5.3: removed the highlight.

-- Section 5.5: inserted filming directions.

-- Section 5.6.1: inserted ImageJ usage directions.

-- Section 5.6.2: added a detail to explain a result.

- Section 5.6.3: added directions for Indicator Dye data manipulation.
- Section 5.7: edited to direct the experimenter to the current version of the P_fFit Installer.
- Sections 6.2 and 6.3 are the result of splitting the former section 6.2.
- Section 6.4 is the former 6.3, edited.
- Section 6.5 is the former 6.4.

(5) Figures

- Fig. 1 and legend: added to part C: side-view schemata (long-side view and short-side view) of the chamber; added part D: the photo of the “transparent cover” and part E: schemata of the “transparent cover”; expanded legend to include the description of the added parts, and in particular, the Scotch tape glued semi-permanently to the slide bottom.
- Fig. 2: added “Number of” to the name of the ordinate, to precede “square pixels”. Legend unchanged.
- Fig. 3 and legend: the figure has been redrawn (otherwise unchanged); expanded the legend with definitions of the various variables and parameters.
- Fig. 4 and legend: the figure has been redrawn (otherwise unchanged); slightly edited the legend for clarity.
- Fig. 5 and legend: the figure has been redrawn and edited; marked “D” on the figure, added part E to the figure (an interim figure); extensively expanded the legend with an overview of models classification, definitions of the various variables and parameters, and the new part E.
- Fig. 6 and legend: the figure has been redrawn and edited; in part A: removed “/pt (um3)” following “fit-ERR”; in part B: added blue and green boxes for clarity, renamed “delay-prop” to “delayPhysio” (meaning: “Physiological delay”); extensively expanded the legend with definitions of the figure details.

(6) Table 1

- Added “for ImageJ” to follow “CMU 1394 Camera Driver’ plugin”
- Added details about a Scotch tape glued to the chamber slide bottom:
 ‘3M packaging Scotch tape 1”, clear’; ‘Viking Industrial, UK’; ‘VKMONO25’;
 ‘http://www.vikingtapes.co.uk/c-428-vkmono-mono-filament-tape.aspx#.UuvqOftdy_8’
- Added details for Silicone grease from Merck:
 107921 Silicone high vacuum grease heavy
https://merck-chemicals.co.id/chemicals/silicone-high-vacuum-grease-heavy/MDA_CHEM-107921/p_LMib.s1Oxr4AAAEvXHg49in.?SecurePage=true&SEO_ErrorPageOccurred=true&attachments=CoA/

Dec 02, 2013

To: "Nava Moran" nava.moran@mail.huji.ac.il
 From: "Justin Cherny" Justin.cherny@jove.com
 Subject: Your JoVE Submission JoVE 51652 R1

Dear Prof. Moran,

Your manuscript JoVE51652R1 'Measuring the osmotic water permeability coefficient (Pf) of isolated plant protoplasts and frog oocytes' has been peer-reviewed and the following comments need to be addressed. **Please keep JoVE's formatting requirements** and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

*Please use the **"track-changes"** function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please **also** upload **a list of changes**, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.*

To submit a revision, go to the [JoVE submission site](#) and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.

Sincerely,

Justin Cherny, Ph.D.

Science Editor

[JoVE](#)

1 Alewife Center, Suite 200, Cambridge, MA 02140

tel: 617 - 674 – 1888

Your revision is due by **Dec 20, 2013**.

Your JoVE Submission 51652

Michelle Kinahan michelle.kinahan@jove.com Jan 31

to me, Menachem, Arava

Dear Nava

Please take as much time as you need to revise your manuscript and resubmit it through our online submission system at your earliest convenience.

Best

Michelle

Supplemental Code File (if applicable)

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