**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 Pf 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 Pf 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 SlopePf 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 osmometer[11](#_ENREF_11" \o "Moshelion, 2004 #2432), the determination of Pf is based on the presumption that Pf may change with time, that this dynamics of Pf underlies the time course of the cell volume change and that three parameters suffice to describe it: Pfi (the initial value of Pf), SlopePf (the rate of the linear change of Pf) 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 values[11](#_ENREF_11). 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 change[11](#_ENREF_11), calculated, in turn, from the imported series of cell-contour areas (see also the Supplemental ‘PfFit 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 (Pf) 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 (Pf) 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 Pf of isolated plant protoplasts, applicable in principle also to other spherical cells, e.g., frog oocytes[11](#_ENREF_11). **”**  
*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 could´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 PfFit (**Figure 5**) was used to import these areas and convert them to cell volumes. The Pf values **(Figure 8C)** were derived for each cell using the PfFit 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 osmoticum 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](#_ENREF_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 then 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/](http://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 PfFit 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 PfFit 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 PfFit User Guide). We therefore re-ran PfFit and produced new figures 3-6.*

***(2) The PfFit 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 PfFit User Guide).*

*-- Pf and PfFit have been changed to Pf and PfFit.*

*-- “ ” 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 PfFit 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 PfFit 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/*

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|  | 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](http://www.editorialmanager.com/jove) 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](http://www.jove.com)  
1 Alewife Center, Suite 200, Cambridge, MA 02140  
tel: 617 - 674 – 1888

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

Your JoVE Submission 51652

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| |  | | --- | | Michelle Kinahan michelle.kinahan@jove.com | | Jan 31  https://mail.google.com/mail/u/1/images/cleardot.gif |  | https://mail.google.com/mail/u/1/images/cleardot.gif  https://mail.google.com/mail/u/1/images/cleardot.gif |
| |  | | --- | | 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