Fluorescent Dye Based Measurement of Vacuolar pH and K^+

Elias Bassil[1], Melanie Krebs[2], Stephen Halperin[3*], Karin Schumacher[3] and Eduardo Blumwald[3*]

[1]Department of Plant Sciences, University of California, Davis, USA; [2]Center for Organismal Studies, Ruprecht-Karls-Universitat, Heidelberg, Germany; [3]Horticulture Department, Pennsylvania State University, University Park, USA
*For correspondence: eblumwald@ucdavis.edu

[Abstract] Availability of ion specific fluorescent dyes has enabled the possibility to perform *in vivo* ion specific measurements using live cell imaging in many cellular compartments (Krebs *et al.*, 2010; Bassil *et al*., 2011; Halperin and Lynch, 2003; Swanson *et al.*, 2011; O'Connor and Silver, 2007). The importance of ion and pH homeostasis of intracellular compartments, including the vacuole, to cell growth is critical and well established (Krebs *et al.,* 2010; Bassil *et al.,* 2011).
* Dedicated to Stephen Halperin who tragically and unexpectedly passed away.

Materials and Reagents

A. Fluorescent dyes:
1. 2', 7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein acetoxymethyl (BCECF, AM) (Invitrogen, catalog number: B-1170; TEFLabs, catalog number: 0062)
2. Potassium-binding benzofuran isophthalate acetoxymethyl ester (PBFI, AM) (Life Technologies, Invitrogen™, catalog number: P-1267MP; TefLabs, catalog number: 0021)

B. Other materials:
1. Pluronic F127 (Life Technologies, Invitrogen™, catalog number: P-3000MP; Teflabs, catalog number: 2510)
2. Gramicidin (Sigma-Aldrich, catalog number: G5002)
3. 1/2 MS plates containing 0.8% sucrose, 0.8% agar pH 5.7 for seedling germination and growth
4. 1/10 strength MS medium (without sucrose) for dye loading
5. Sterile *Arabidopsis* seeds of choice
   Note: This method is optimized for Arabidopsis.
6. Small culture dishes (35 mm x 10 mm), small glass beaker (vol. 10 ml or smaller) or other small vessel for seedling incubation. An Eppendorf tube will work but caution should be

taken when adding or removing seedlings from these tubes causes damage. We prefer a vessel with a larger opening for easier access to seedlings.

Note: For PBFI loading, a sterile culture vessel is recommended because of the long incubation time.

7. Micropore tape (3 M, catalog number: 1530-1)
8. Dye loading medium (see Recipes)
9. *In situ* calibration buffer for BCECF (see Recipes)
10. *In situ* calibration buffer for PBFI (see Recipes)

Equipment

1. Confocal or epifluorescence microscope with appropriate filters (see below for spectral characteristics of the dyes)

Software

1. Open source software ImageJ (http://rsbweb.nih.gov/ij/index.html)

Procedure

A. Seedling growth

1. Vernalize sterile seeds in sterile water for 3 days at 4 °C. Seeds were sterilized according to the following protocol. In several Eppendorf tubes, aliquot 100 seeds approximately and place, without closing the lid, into a dessication jar. In the same desiccation jar, add 30 ml bleach to a small beaker and very carefully add 1 ml concentrated HCl to the bleach, while working in fume hood. Use the dessicator lid to shield from possible splashes. Close the lid immediately and make sure it is airtight. Leave for 3 h. Open in a clean bench and be cautious of inhaling any fumes as these are dangerous. Leave the room for 15 min. Close the tubes while in the clean bench. Seeds are now sterilized and can be vernalized by adding sterile water.
2. In a clean bench sow sterile seeds. Sterile pipette tips with a cut end large enough to allow imbibed seeds to pass are useful.

   Note: Using Micropore tape instead of parafilm to seal plates helps to reduce condensation in plates.
3. Place plates with seeds, vertically in a growth chamber 22 °C 16 h light. Growing seedling in vertically plates is intended to minimize damage to seedlings when moving them off the plated and into the dye-loading vessel.

4. Grow seedlings until cotyledons are fully expanded but before true leaves have emerged (approximately 4 days) and the seedlings are about 1 cm in length.
5. Seedlings are now ready for dye loading.

B. Dye loading

1. Prepare 10 µM BCECF or 20 µM PBFI in Dye loading medium and add 0.02% Pluronic F-127. Mix gently but thoroughly. Typically 0.5 ml Dye Loading medium is sufficient to incubate approximately 10 seedlings, depending on the culture vessel used. Small 10 ml beakers or a 12 well culture plate work well for this.
   Note: Given the sensitivity of acetoxylmethyl ester (AM) dyes to hydrolysis, it is critical to use a fresh dye stock. We store our dye stock in aliquots at -20 °C in dark, sealed containers with silica dessicant beads.
2. Using forceps gently pick up seedlings under the cotyledons and place in dye loading medium. Sterile conditions are not necessary at this point. Care should be taken not to damage seedlings especially for loading with PBFI dye. We observed that poor handling leads to the bursting of many root hairs which coats the root surface with cytoplasmic matter that is strongly stained by PBFI. Staining outside roots reduces dye loading and creates a strong extracellular signal that interferes with the imaging of dye loaded into root cortical cells.
3. Incubate seedlings in dye loading medium in the dark at room temperature on a shaker at very low speed (enough to move the solution but not the seedlings).
4. Incubate seedlings loading with BCECF for 30 min to 1 h and 18 h to 20 h for PBFI.
   Note: Sterile conditions are necessary given the long incubation time and the presence of sucrose in the loading medium.
5. Carefully wash seedling with Dye loading medium to remove excess dye (5 min x 2). Care should be taken to prevent seedling damage and the bursting of root hairs (again it is critical in the in the case of PBFI loading).
6. Seedlings are now ready to image.
   Note: For PBFI, we found it difficult to measure root cortical cells near the root tip because root hairs nearest the tip are more prone to bursting, leading to the problem of strong fluorescence staining outside the roots described above in B-2. Reliable measurements using PBFI were made in mature zone root cortical cells and cells of the hypocotyl. For BCECF, imaging of all cell types was possible because this dye loads readily.

C. Imaging and image analysis

1. BCECF

a. BCECF is a dual-ratiometric dye that has been widely used to measure intracellular pH in various biological systems (Swanson *et al.*, 2011; O'Connor and Silver, 2007). Ratiometric measurements have several advantages over single emission or excitation dyes in that they are less affected by differences in amounts of dye loading or the volume of compartments where the dye is accumulating. In *Arabidopsis* root cells, BCECF specifically accumulates in the large central vacuole, making it an ideal tool for vacuolar pH measurements.

b. Seedlings can be imaged using a confocal or epifluorescent microscope. BCECF is sequentially excited using 458 and 488 nm. Fluorescence emission is detected for each of the two excitation wavelengths between 530 and 550 nm. Carefully adjust the imaging settings to account for that fact that the fluorescence intensity of BCECF will increase with rising pH and that it is best to detect fluorescence within a similar dynamic range. Avoid oversaturation since this will, underestimate fluorescent intensities and create artifacts in your pH measurements. Take into account that some light sources used for fluorescence microscopy such as argon gas lasers or mercury arc lamps require to be on for some time before they emit a stable non-fluctuating excitation light. A 20x objective is sufficient to collect images of many cortical cells. Root and hypocotyl cells stain more readily and are easier to image than shoot tissue.

   Note: Different cell types and tissues can have different vacuolar pH values (Bassil et al., 2011).

c. Image analysis can be done with the open source software ImageJ.

d. A background correction for each image is necessary before proceeding with the calculation of fluorescence intensity values. Images are corrected for background fluorescence using the subtract background function of ImageJ (found in the 'Process" pull down menu). Instructions for this type of background correction can be found here:
   (http://imagejdocu.tudor.lu/doku.php?id=gui:process:subtract_background&s[]=rolling&s[]=ball)

   It may be necessary to try different radius settings to obtain reasonable values which must be assessed empirically from the quality of the calibration curve (see below). A general rule is to obtain a calibration curve with a ratio range (*i.e.* the slope of the calibration curve) that would be large enough to allow small changes in pH to be determined (a 3 fold increase in the ratio over the pH range of 5.2-7.6 recommended here should be sufficient). The utility of the calibration curve depends greatly on the quality of the images collected and must therefore be worked out empirically.

Background correction can greatly influence the ratio values which must be kept in mind when using different background correction modification parameters.

e. From each background corrected image, an integrated pixel density value is obtained from the 'Measure' function of ImageJ (under the Analyze menu). Depending on the settings it may be necessary to first set measurements values to include 'integrated density' which can be done with the 'Set Measurements' command, also in the 'Analyze' menu. For each image (*i.e.* 488 nm and 458 nm) a single 'Integrated Density' measurement will be obtained. The measurements can be copied from the ImageJ output and pasted into Excel for further calculation. For the calculation, the Integrated Density value for the 488 nm excitation image is divided by the Integrated Density of the Ex458 nm image. This is repeated for the different pairs of images to obtain an average ratio and standard deviation to determine statistical significance between treatment samples.

f. Typically a ratio value that is the average of 10-20 images is collected from approximately 20 seedlings and would include 6-10 cortical cells for each image.

2. PBFI

a. PBFI is also a ratiometric dye with dual excitation (360 nm & 380 nm) and emission above 500 nm. In general a similar approach is taken to collect and correct images of PBFI loaded root cells, except that imaging settings are Excitation 360 nm and collection of emission above 500 nm, and excitation 380 nm and collection of emission also above 500 nm.

b. Image processing is performed identically as that described for BCECF above C-1 c-f.

D. *In situ* calibration

1. BCECF

a. To obtain the calibration curve, dye loaded seedlings are incubated in each of the pH calibration buffers (see Recipes 2) for no longer than 15-20 min. Seedlings are carefully placed onto microscope slide with approximately 100 µl of dye and imaged immediately as described above.

b. The ratios for each pH incubation can now be plotted against pH to obtain the calibration curve. A sigmoidal regression (Boltzmann function) can be fitted to describe the calibration curve and to calculate subsequent pH values from the equation describing the curve.

2. PBFI

In situ calibration of PBFI loaded seedlings is performed by incubating dye loaded seedlings in PBFI *in situ* calibration buffers containing different K^+ concentrations.

Recipes

1. Dye loading medium
   1/10 strength MS
   5 mM MES (pH 5.7)
   0.5% sucrose
   Note: pH can be adjusted with KOH for BCECF loading but this will interfere with PBFI dye loading. For the later pH can be adjusted with BTP.
2. *In situ* calibration buffer for BCECF
   50 mM ammonium acetate
   50 mM Mes-BTP (pH 5.2-6.4) or 50 mM-HEPES-BTP (pH 6.8-7.6)
   Typically 6 or 7 buffers are adequate to cover the range pH 5.2 to 7.6
3. *In situ* calibration buffer for PBFI
   Dye loading medium
   2 µM Gramicidin
   Different solutions containing a range of KCl 0-100 mM should be prepared and typically 7 solutions (0, 10, 20, 40, 60, 80 and 100 mM KCl), are sufficient. It is important to note that *in situ* calibration of K^+ cannot be done at '0 mM K^+' because the tissue already contains some K^+. In this case measurements will fall below the lower limit of the calibration curve and it will be necessary to perform an *in vitro* calibration curve as well.

Acknowledgments

This protocol was modified in part from Krebs *et al.* (2010) and Halperin *et al.* (2003). This work was supported by grants from the National Science Foundation (MCB-0343279; IOS-0820112) and the Will W. Lester Endowment, University of California. It is dedicated to Stephen Halperin who tragically and unexpectedly passed away.

References

1. Bassil, E., Tajima, H., Liang, Y. C., Ohto, M. A., Ushijima, K., Nakano, R., Esumi, T., Coku, A., Belmonte, M. and Blumwald, E. (2011). The *Arabidopsis* Na^+/H^+ antiporters NHX1 and NHX2 control vacuolar pH and K^+ homeostasis to regulate growth, flower development, and reproduction. *Plant Cell* 23(9): 3482-3497.
2. Halperin, S. J. and Lynch, J. P. (2003). Effects of salinity on cytosolic Na^+ and K^+ in root hairs of *Arabidopsis thaliana*: *in vivo* measurements using the fluorescent dyes SBFI and PBFI. *J Exp Bot* 54(390): 2035-2043.

3. Krebs, M., Beyhl, D., Gorlich, E., Al-Rasheid, K. A., Marten, I., Stierhof, Y. D., Hedrich, R. and Schumacher, K. (2010). *Arabidopsis* V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proc Natl Acad Sci U S A* 107(7): 3251-3256.
4. O'Connor, N. and Silver, R. B. (2007). Ratio imaging: practical considerations for measuring intracellular Ca2+ and pH in living cells. *Methods Cell Biol* 81: 415-433.
5. Swanson, S. J., Choi, W. G., Chanoca, A. and Gilroy, S. (2011). *In vivo* imaging of Ca^{2+}, pH, and reactive oxygen species using fluorescent probes in plants. *Annu Rev Plant Biol* 62: 273-297.

