

## Video Article

## Assessing Stomatal Response to Live Bacterial Cells using Whole Leaf Imaging

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## Abstract

Stomata are natural openings in the plant epidermis responsible for gas exchange between plant interior and environment. They are formed by a pair of guard cells, which are able to close the stomatal pore in response to a number of external factors including light intensity, carbon dioxide concentration, and relative humidity (RH). The stomatal pore is also the main route for pathogen entry into leaves, a crucial step for disease development. Recent studies have unveiled that closure of the pore is effective in minimizing bacterial disease development in *Arabidopsis* plants; an integral part of plant innate immunity. Previously, we have used epidermal peels to assess stomatal response to live bacteria (Melotto *et al.* 2006); however maintaining favorable environmental conditions for both plant epidermal peels and bacterial cells has been challenging. Leaf epidermis can be kept alive and healthy with MES buffer (10 mM KCl, 25 mM MES-KOH, pH 6.15) for electrophysiological experiments of guard cells. However, this buffer is not appropriate for obtaining bacterial suspension. On the other hand, bacterial cells can be kept alive in water which is not proper to maintain epidermal peels for long period of times. When an epidermal peel floats on water, the cells in the peel that are exposed to air dry within 4 hours limiting the timing to conduct the experiment. An ideal method for assessing the effect of a particular stimulus on guard cells should present minimal interference to stomatal physiology and to the natural environment of the plant as much as possible. We, therefore, developed a new method to assess stomatal response to live bacteria in which leaf wounding and manipulation is greatly minimized aiming to provide an easily reproducible and reliable stomatal assay. The protocol is based on staining of intact leaf with propidium iodide (PI), incubation of staining leaf with bacterial suspension, and observation of leaves under laser scanning confocal microscope. Finally, this method allows for the observation of the same live leaf sample over extended periods of time using conditions that closely mimic the natural conditions under which plants are attacked by pathogens.

## Protocol

## 1. Growing Plants and Preparing Bacteria

1. To begin this procedure sow *Arabidopsis* seeds on a 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite.
2. Grow the plants in a growth chamber (22°C, 12 hours of 100 µmol/mm<sup>2</sup>/sec daily light and 65±5% humidity) and water as needed. The plants are ready to use in 4-6 weeks when they have young fully expanded leaves prior to bolting.
3. Two days before starting the experiment prepare the bacterium culture. Streak *Pseudomonas syringae* from glycerol stock on modified LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl pH 7.0, and 1.5% agar) and incubate for 24 hours at 28°C. Use fresh culture to prepare the inoculum and always start culture plates from glycerol stocks as bacteria becomes less virulent after sub-culturing.
4. Use bacteria that grew on the plate to start a 10 mL liquid culture of *P. syringae* in a 50 mL Erlenmeyer flask. Incubate the culture overnight at 28°C with vigorous shaking until it reaches optical density or OD at 600 nm between 0.8 and 1.
5. Measure the OD at 600 and harvest the cells by centrifugation at 1360 xg for 10 minutes. Resuspend the cells in distilled water so that the final O.D is 0.2. This O.D corresponds to 10<sup>8</sup> colony forming units (CFU)/mL. With the bacteria ready, proceed to prepare the leaves and perform the assay.

## 2. Leaf Staining and Incubation with Bacteria

1. In order to stain the leaves first prepare 20 µM propidium iodide or PI solution in water. 10 mL of solution is sufficient to stain 3 small leaves at a time.
2. Retrieve the plants from the growth chamber and with forceps detach 3 young, fully-expanded leaves. Immerse the whole leaves in the PI solution for 5 minutes. Then remove the leaves and rinse them briefly with distilled water.
3. Next place a leaf with the lower surface facing down on a microscope slide. Cut the leaf with a sharp razor blade into four pieces excluding the mid vein so that the leaf lays flat on the slide.
4. To incubate the bacteria with the leaves, add 300 µL of bacterial suspension under the leaf pieces on the slide. Make sure that the lower surface of the leaf is in contact with the inoculum.
5. Incubate the samples under the same environmental conditions that were used to grow the plants. At the time to observe leaves under the microscope, transfer the leaf pieces to a new slide with lower surface facing up. Note that cover slip is not used when mounting the slides. The same leaf sample can be imaged multiple times during incubation and a time course may be set up according to the research objectives.

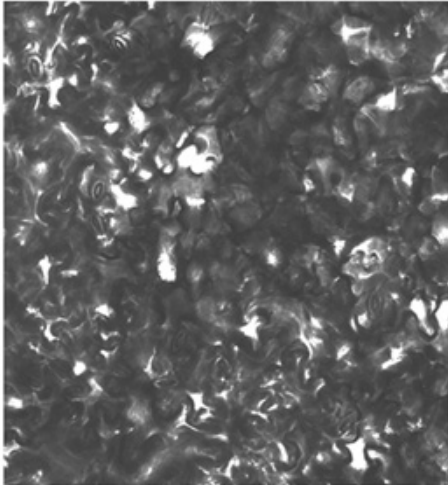
## 3. Microscopy, Measurement and Data Analysis

1. Here a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss Inc.) is used to examine the leaves. Observe the lower surface of the leaves to detect the fluorescence of PI (excitation 453 nm, emission 543 and 620 nm).
2. Using the same leaf samples capture images of stomata over time. Save the images for measuring the width of the stomatal aperture.
3. Measure stomatal aperture width of at least 60 stomata for each treatment at each time point using LSM image browser.

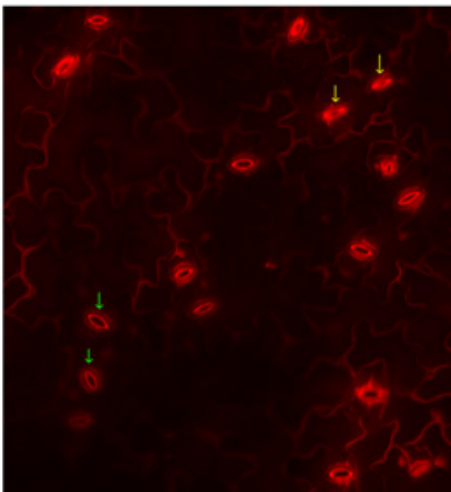
4. Calculate the average and standard error for the stomatal aperture width. Statistical significance of the results can be calculated using 2-tailed, paired wise Student's t-test.

#### 4. Representative Images of Stomatal Response to Incubation with Bacteria

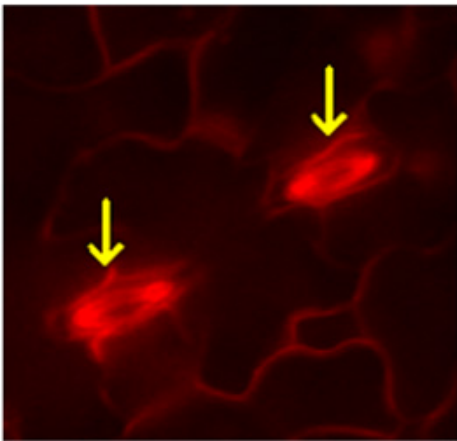
1. Here is a typical microscopic image (using a 20x objective) of an *Arabidopsis* leaf surface under laser scanning confocal microscope in bright field view (Figure 1).
2. Propidium iodide stains the cell wall of viable cells increasing the visibility of guard cells in addition to allowing for the identification of undamaged cells for data collection (Figure 2). A range of stomatal pore openings can be seen in these micrographs, from completely closed stomata to wide open stomata.
3. Completely closed stomata are identified by the shape of the guard cells (Figure 3). The aperture width is considered to be 0  $\mu\text{m}$ .
4. For open stomata (Figure 4) the aperture width shown is measured by using a straight line drawn across the widest area of the stomatal pore.
5. These are typical experimental results of this stomatal assay. *Arabidopsis* leaves were incubated in the dark with three different strains of *Pseudomonas syringae*. Only the bacterium Pst DC3000 was able to open dark-closed stomata, as measured by the aperture width of randomly selected 60 stomata per bacterial strain (Figure 5).



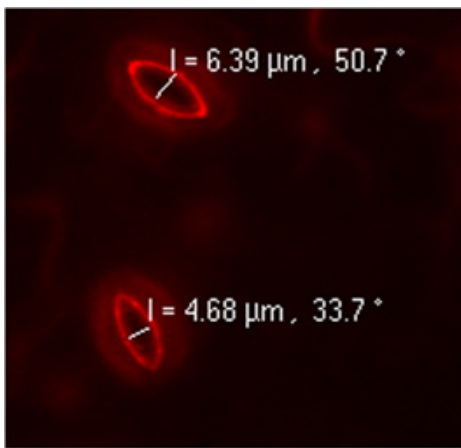
**Figure 1. Micrograph of the surface of an *Arabidopsis* leaf.** One field of view of the leaf surface under laser scanning confocal microscope (LSCM) using the 20x objective. Note that stomatal aperture is not as evident when compared to the fluorescent view aided by propidium iodide staining (Figure 2).



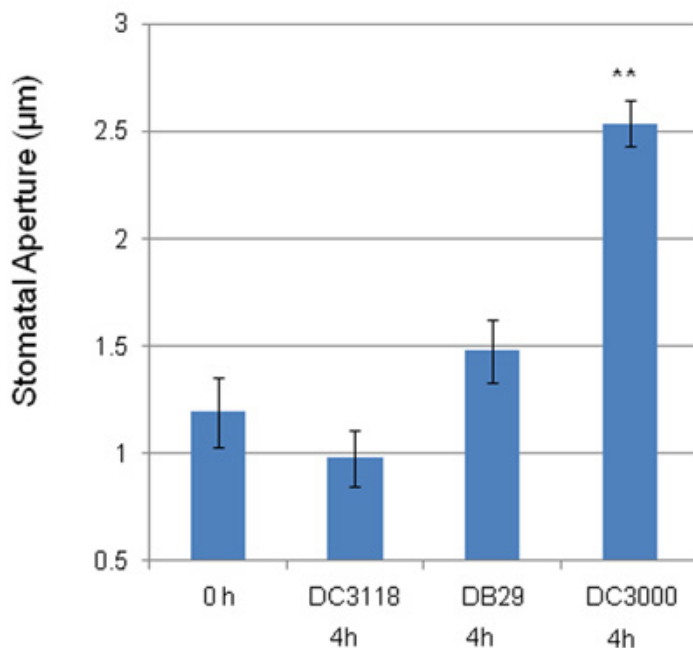
**Figure 2. Micrograph of the surface of a propidium iodide-stained *Arabidopsis* leaf.** One field of view of the leaf surface under laser scanning confocal microscope (LSCM) using the 20x objective. Note a range of stomatal pore opening. Yellow arrows are pointed to completely close stomata and green arrows are pointed to wide open stomata.



**Figure 3. Completely close stomata identified by the shape of and the opening between the guard cells.** The aperture width is considered to be 0  $\mu\text{m}$ .



**Figure 4. Open stomata showing the aperture width in  $\mu\text{m}$ .** Measurements were taken by using the LSCM browser based on a straight line drawn across the widest area of the stomatal pore.



**Figure 5. Stomatal aperture of *Arabidopsis* leaves incubated with three strains of *Pseudomonas syringae* pv. *tomato*: DC3118, DB29, and DC3000.** Plants were kept in the dark during the experimentation time. Results are shown as the mean ( $n=50-70$ )  $\pm$  standard error. Statistical significance was detected with two-tailed Student's t-test. The experiment was repeated three times with similar results.

## Discussion

We have presented a straight forward procedure to measure stomatal aperture in intact leaf tissue allowing for an easy assessment of stomatal response to different treatments.

Although we have presented results using the model system *Arabidopsis/Pseudomonas syringae*, the intact leaf stomatal assay can be potentially performed with any plant-bacterium combination. The protocol can easily be modified to fit growth requirements of other plants and bacterial pathogens. The overall principle and procedure remain the same. In addition, this method may be beneficial to researchers who wish to study functional output of guard cells not only to live microbes, but also to other stimuli and chemical agents under conditions that maintain the leaf's natural environment.

Whole leaf can be difficult to image due to its thickness and uneven topography. This problem can be alleviated by removing the mid vein of the leaf so it lays flat on the slide and using confocal microscopy. However, other types of fluorescence microscope can be used to obtain high resolution images of the leaf surface.

## Disclosures

No conflicts of interest declared.

## Acknowledgements

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