

Measurement of Endogenous H₂O₂ and NO and Cell Viability by Confocal Laser Scanning Microscopy

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[Abstract] Recently, there is compelling evidence that hydrogen peroxide (H₂O₂) and nitric oxide (NO) function as signaling molecules in plants, mediating a range of responses including stomatal movement. Thus, the choice of sensitive methods for detection of endogenous H₂O₂ and NO in guard cells are very important for understanding the role of H₂O₂ and NO in guard cell signaling. In addition, besides stomatal closure caused by interfering guard cell signaling, it can also be caused by widespread, nonspecific damage to guard cells. To determine whether stomatal movement is caused by damage to guard cells, sensitive methods for detection of guard cell viability are often required.

The oxidatively sensitive fluorophore 2',7'-dichlorofluorescein (H₂DCF) is commonly employed to measure changes in intracellular H₂O₂ level directly. The non-polar diacetate ester (H₂DCFDA) of H₂DCF enters the cell and is hydrolysed into the more polar, non-fluorescent compound H₂DCF, which, therefore, is trapped. Subsequent oxidation of H₂DCF by H₂O₂, catalysed by peroxidases, yields the highly fluorescent DCF. Similarly, the cell-permeable, NO-sensitive fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) is widely used for the direct detection of NO presence in both animal and plant cells. The non-polar DAF-2DA enters the cell and is hydrolyzed by cytosolic esterase into the more polar, non-fluorescent compound DAF-2, which in the presence of NO is converted to the highly fluorescent triazole derivative DAF-2T. The fluorescent indicator dyes fluorescein diacetate (FAD) and propidium iodide (PI) are widely used for detection of cell viability. FAD passes through cell membranes and is hydrolyzed by intracellular esterase to produce a polar compound that passes slowly through a living cell membrane but fast through a damaged or dead cell membrane, and thus accumulates inside the viable cells and exhibits green fluorescence when excited by blue light. In contrast, PI passes through damaged or dead cell membranes and intercalates with DNA and RNA to form a bright red fluorescent complex seen in the nuclei of dying or dead cells but not living cells. Based on the above analysis, the fluorescent indicator dyes H₂DCFDA, DAF-2DA, FAD and PI load readily into guard cells, and their optical properties make them amenable to analysis by confocal laser scanning microscopy.

This protocol describes how to combine confocal laser scanning microscopy with fluorescent indicator dyes H₂DCFDA, DAF-2DA, FAD and PI respectively for measurement of H₂O₂ and NO and viability of guard cell in leaves of *Arabidopsis* (*Arabidopsis thaliana*).

Materials and Reagents

1. Leaves of *Arabidopsis* (*Arabidopsis thaliana*)
2. Ethanesulfonic acid (MES)
3. 2',7'-dichlorofluorescein diacetate (H₂DCFDA) (Sigma-Aldrich, catalog number: D6883)
4. 4, 5-diaminofluorescein diacetate (DAF-2DA) (Sigma-Aldrich, catalog number: D2813)
5. Propidium iodide (PI) (Sigma-Aldrich, catalog number: P4170)
6. Fluorescein diacetate (FDA) (Sigma-Aldrich, catalog number: F7378)
7. DMSO (Sigma-Aldrich, catalog number: D8418)
8. 10 mM MES/KCl buffer pH 6.15 (see Recipes)
9. Tris/KCl buffer pH 7.2 (see Recipes)
10. 10 mM H₂DCFDA (see Recipes)
11. 10 mM DAF-2DA (stock solution) (see Recipes)
12. 1 mg/ml PI (stock solution) (see Recipes)
13. 5 mg/ml FDA (stock solution) (see Recipes)

Equipment

1. TCS-SP2 Confocal Laser Scanning Microscopy (Leica Lasertechnik GmbH, Heidelberg)
2. 25 °C incubator
3. Glass slide and cover glass
4. Eyebrow brush (Yellow wolf hair, length of hair: 0.8 cm, width of hair: 0.8 cm)
5. Tweezers
6. 6-cm diameter Petri plate

Software

1. Leica confocal software (Leica Lasertechnik GmbH, Heidelberg)
2. Photoshop software

Procedure

1. Sampling.

Arabidopsis seedlings were grown in plant growth chambers under a 16-h light/8-h dark cycle, a photon flux density of 0.1 mmol/m²/s, and a day/night temperature cycle of 18 °C/22 °C for 4-6 weeks. The youngest, fully expanded and flat leaves were harvested for immediate use.

2. Opening the stomata.

For stomatal closing experiments, to ensure stomata at fully opened stage before starting of treatments, the freshly harvested flat leaves were first floated with their abaxial surfaces facing up on MES/KCl buffer (15 ml) in 6-cm diameter Petri plates for 2-3 h at 22 °C under light condition (0.1 mmol/m²/s) to open the stomata, and then for subsequent treatments.

3. Treating samples

Once the stomata were fully open (checked by microscope), the leaves were then floated on MES/KCl buffer alone or containing various compounds or inhibitors for required time at 22 °C under the same white light condition mentioned above or the desired conditions. Control treatments involved addition of buffer or appropriate solvents used with inhibitors.

Note: As the epidermal strips is easier peeled from the abaxial surface than the paraxial surface of Arabidopsis leaves, we only peeled epidermal strips from abaxial surface of leaves for subsequent measurement. Thus, for treatments of UV-B radiation as well as other lights, to ensure the abaxial surface of leaves receiving same dose of UV-B radiation as well as other lights, the leaves were floated with their abaxial surfaces facing up and perpendicular to the light on MES/KCl buffer in all treatments including opening stomata.

4. Peeling epidermal strips.

After the above treatments, the leaf was taken out from MES/KCl buffer and a piece of filter paper was used to absorb the MES/KCl buffer on the surface of leaf. The leaf were flatly placed on a glass slide with its abaxial surfaces facing up, a tweezers was used to clamp a part of abaxial epidermis and mesophyll cells near the tip of leaf and the epidermal strips were quickly peeled along with the direction of the main leaf veins. Then, the peeled epidermal strips were immediately immersed in the corresponding treated buffer and pushed on the bottom of Petri plates by a forceps, the remained mesophyll cells were gently removed from epidermal strips by an eyebrow brush (Figure 1), and the tip of epidermal strip clamped by tweezers with more mesophyll cells was cut away, then epidermal strips were quickly used for loading of the fluorescent indicator dyes.

5. Loading fluorescent indicator dyes.

The peeled epidermal strips were immediately placed into Petri plates containing Tris-KCl buffer in the presence of H₂DCFDA at a final concentration of 50 μM for 10 min, DAF-2DA at a final concentration of 10 mM for 30 min, FAD at a final concentration of 10 μg·ml⁻¹ for 10 min, or PI at a final concentration of 5 μg·ml⁻¹ for 10 min respectively, in the dark at 25 °C to exclude the possibility of that the fluorescent probes were oxidized or hydrolyzed by UV-B or PAR radiation. Then, the epidermal strips were washed with fresh

Tris–KCl buffer without the fluorescent indicator dyes at least three times in dark to remove the excess dyes.

6. Examination of H₂O₂, NO and viability of guard cells by confocal laser scanning microscopy.

After loading of the fluorescent indicator dyes, the slides were made and an examination of the peels was immediately performed by TCS-SP2 confocal laser scanning microscopy with the following settings: excitation 488 nm and emission 530 nm for H₂DCFDA, DAF-2DA and FAD or excitation 536 nm and emission 617 nm for PI; normal scanning speed, frame 512 x 512. For example, by using these fluorescent indicator dyes and the confocal laser scanning microscope, Figure 1 clearly showed that guard cells of wild-type *Arabidopsis* under light alone had low levels of H₂O₂ (Figure 1A) and NO (Figure 1D), and high viability of guard cells (Figure 1G). However, 3 h of 0.5 W/m² UV-B radiation significantly induced production of H₂O₂ (Figure 1B) and NO (Figure 1E), and did not affect cell viability (Figure 1H) in wild-type guard cells, but did not induce H₂O₂ production in guard cells of *AtrbohD/F* double mutant (Figure 1C) or NO production in guard cells of *Nia1-2/Nia2-5* double mutant (Figure 1F). Furthermore, when wild-type leaves were exposed to 0.8 W/m² UV-B for 3 h, the guard cells were significantly damaged and clearly marked by the fluorescent dye PI (Figure 1I).

7. Analysis

Images acquired from the confocal microscope were analyzed with Leica confocal software to measure the average fluorescent pixel intensities in the guard cells following various treatments (such as in Figure 1J; the detailed procedure of analysis seen the following note) and processed with Photoshop software. In each experiment, three epidermal strips were at least measured, each of which originated from a different plant. Each experiment was repeated three times. The selected confocal image represented the same results from approximately nine time measurements. Data of fluorescence pixel intensities are statistically analyzed by one-way ANOVA and displayed as means ± SE (n = 60).

Note: Procedure for analysis of fluorescent intensity: On the “LAS AF” screen of Leica confocal software, click the “quality” button, select “Histogram” analysis method and circle guard cell to be analyzed, then “Statistics” shows the average fluorescent intensity of the circled guard cell, select “Export as” to save the “Statistics” displayed data in a text format.

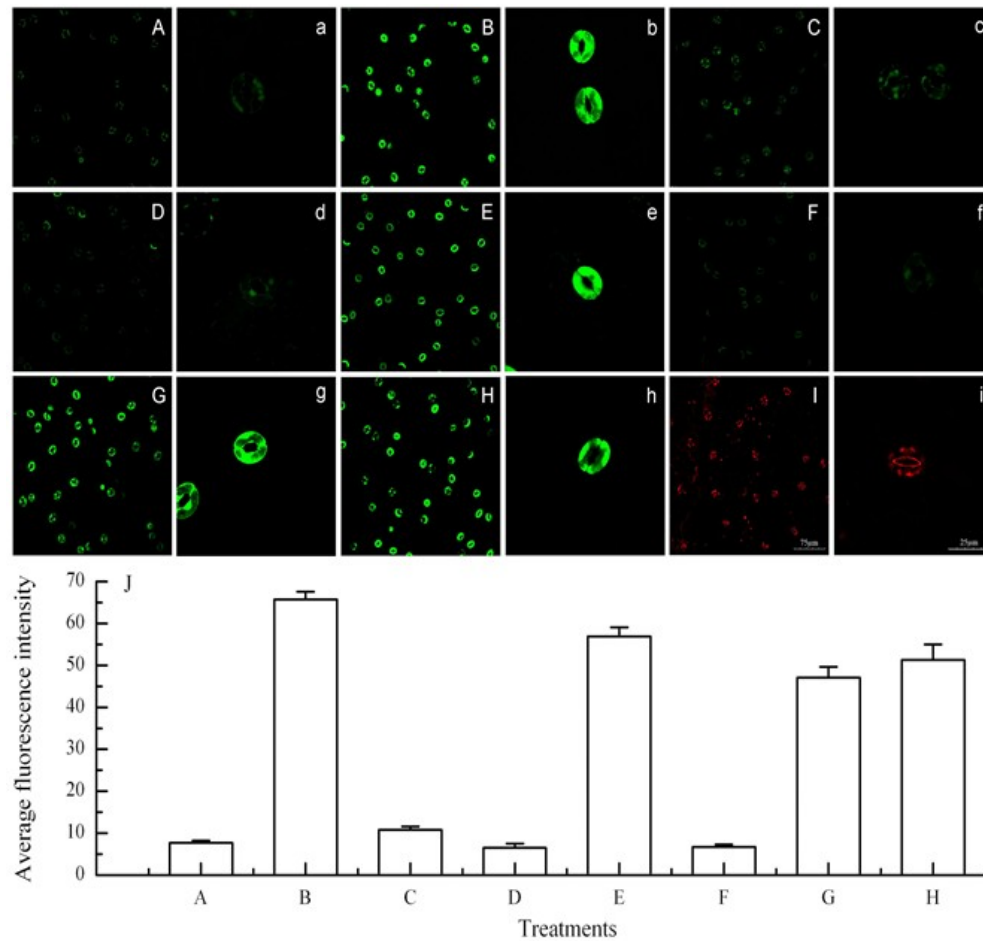


Figure 1. Effects of UV-B radiation on the production of H₂O₂ and NO and viability of *Arabidopsis* guard cells. A-C. Images of guard cells loaded with the fluorescent indicator dye H₂DCFDA. D-F. Images of guard cells loaded with the fluorescent indicator dye DAF-2DA. G and H. Images of guard cells loaded with the fluorescent indicator dye FAD. A, D and G. Wild-type guard cells exposed to light alone for 3 h. B, E and H. Wild-type guard cells exposed to light with 0.5 W m⁻² for 3 h. C and F. Double mutants *AtrbohD/F* and *Nia1-2/Nia2-5* guard cells respectively exposed to light with 0.5 W m⁻² for 3 h. I. Image of wild-type guard cells exposed to 0.8 W m⁻² UV-B for 3 h and loaded with the fluorescent indicator dye PI. J. The figure shows the average fluorescent intensities (means ± SE) of guard cells in images from A to H. The guard cells shown in images a-i are representative of guard cells shown in images A-I, respectively. Scale bars in image I (75 μm) and i (25 μm) are for images A-I and a-i, respectively.

Recipes

1. 10 mM MES/KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM CaCl₂, pH 6.15, 500 ml)

- 1.066 g MES
5.549 mg CaCl_2
1.86375 g KCl
Mix these chemicals with 400 ml dH_2O
Adjust pH to 6.15 with KOH
Add dH_2O to 500 ml
Stored at room temperature
2. Tris/KCl buffer (10 mM Tris and 50 mM KCl, pH 7.2, 500 ml)
0.6055 g Tris
1.86 g KCl
Mix these chemicals with 400 ml dH_2O
Adjust pH to 7.2 with HCl
Add dH_2O to 500 ml
Stored at room temperature
3. 10 mM H_2DCFDA (1 ml, stock solution)
Mix 4.8729 mg of H_2DCFDA with 1 ml DMSO
Stored at -20°C
This stock solution is diluted by Tris/KCl pH 7.2 buffer to get a working concentration of 50 μM
4. 10 mM DAF-2DA (stock solution)
Mix 1 mg of DAF-2DA with 224 μl DMSO to form 10 mM stock solution
Stored at -20°C
This stock solution is diluted by Tris/KCl pH 7.2 buffer to get a working concentration of 10 μM
5. 1 mg/ml PI (stock solution)
Mix 1 mg of PI with 1 ml dH_2O to make a stock solution
Stored at 4°C in a dark bottle
This stock solution is diluted by Tris/KCl pH 7.2 buffer to get a working concentration of 5 $\mu\text{g/ml}$
6. 5 mg/ml FDA (stock solution)
Mix 5 mg of FDA with 1 ml acetone to make a stock solution
Stored at 4°C in a dark bottle
This stock solution is diluted by Tris/KCl pH 7.2 buffer to get a working concentration of 10 $\mu\text{g/ml}$

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References

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