

# CONFOCAL FLUORESCENCE MICROSCOPY OF MUNG BEAN LEAVES

Zhiwei Chen<sup>1,2,\*</sup>, Dongwu Liu<sup>1,2</sup>

<sup>1</sup> *Analysis and Testing Center, Shandong University of Technology, Zibo, Shandong Province, P. R. China 255049*

<sup>2</sup> *School of Life Sciences, Shandong University of Technology, Zibo, Shandong Province, P. R. China 255049*

\* *Corresponding author, Address: Analysis and Testing Center, Shandong University of Technology, Zibo 255049, Shandong Province, P. R. China, Tel: +86-0533-2786781, Fax: +86-0533-2786781, Email: chen@sdut.edu.cn*

**Abstract:** Recently, confocal microscope has become a routine technique and indispensable tool for cell biological studies and molecular investigations. The light emitted from the point out-of-focus is blocked by the pinhole and can not reach the detector, which is one of the critical features of the confocal microscope. In present studies, the probes acridine orange (AO) and rhodamine-123 were used to research stoma and mitochondria of mung bean leaves, respectively. The results indicated that the stomatal guard cells and mitochondria were clearly seen in epidermic tissue of mung bean leaves. Taken together, it is a good method to research plant cells with confocal microscope and fluorescence probes.

**Keywords:** confocal microscope, mung bean leaves, chloroplast, mitochondria, guard cell

## 1. INTRODUCTION

Confocal microscope is one of the important advances in the development history of optical microscopes. At present, confocal microscope has become a routine technique and indispensable tool for cell biological studies and molecular investigations (Lichtman, 1994). Confocal microscope works by exciting fluorescence with a highly focused beam of laser light. The light emitted from the point out-of-focus is blocked by the pinhole and can not

reach the detector, which is one of the critical features of the confocal microscope. Thus only an image of the fluorescence from the focal plane is observed. In addition, with the laser scanning over the sample from point to point, a single two-dimensional image of the optical section is obtained (Lichtman, 1994). Moreover, a series of optical sections at different focal planes can be acquired, and a three-dimensional image of the sample reconstructed (Webb, 1999). Since sectioning is performed using optics, living cells can also be analyzed, which is another important feature of the confocal microscope (Blancaflor and Gilroy, 2000).

With confocal microscope, the researchers could obtain the images of the cells much more clearly than conventional microscope. There have been numerous scientific papers employing confocal microscope in plant biology since it was first used in plant science research (Hepler and Gunning, 1998; Taira et al., 2004; Meckel et al., 2007). Confocal microscope has been applied to determine heterogeneity of plant mitochondrial responses (Armstrong et al., 2006), chloroplast structure and arrangement of chlorophyll-protein complexes (Garstka et al., 2007), oxidative stress in plant cells (Li et al., 2007), microtubule arrays and arabidopsis stomatal development (Lucas et al., 2006), and mitochondrial localization (Yang et al., 2006). However, previous reports mainly concern the cultured plant cells, and there are few reports about analyzing the whole plant leaves. Since confocal microscope is an appropriate method for qualitative analysis of plant cells, the aims of the present studies are to analyze the mitochondria and stoma in mung bean leaves with confocal microscope.

## **2. MATERIALS AND METHODS**

### **2.1 Plant material and growth conditions**

Seeds of mung bean were germinated on moist filter paper at room temperature. Seedlings were then transferred to 4-inch pots containing clay soil and were watered three times per week. Plants were grown in extraventricular chambers. Mature leaves from 25-day-old plants were used for analyses.

### **2.2 Mitochondria visualization**

Leaf disks were incubated in the presence of 10  $\mu$ M Rhodamine-123 (Molecular Probes) at room temperature for 1 h. At the end of the incubation period, the mung bean leaves were rinsed briefly with deionized water to remove excess dye from the surface of the leaves, wiped to remove excess

water. Samples treated with water alone served as controls.

### **2.3 Stoma visualization**

Leaf disks were incubated in the presence of 0.01% acridine orange (AO) (Molecular Probes) at room temperature for 10 min. Excess dye was eliminated by washing the disks three times in PBS buffer.

### **2.4 Laser-scanning confocal microscopy**

For microscopy, 3×5 mm leaf cuttings were put under an inverted microscope (Leica DM IRB, Germany). A laser-scanning confocal microscope (Leica TCS SP2, Germany) with an air-cooled, argon-ion laser as the excitation source at 488 nm was used to view the sites of stoma, and 514 nm was used to view the sites of mitochondria. Moreover, He-Ne laser as the excitation source at 633 nm was used to view the sites of chloroplast. Images were obtained with a HCX PLAN Apo 20.0×0.7 PH2 objective lens. Mitochondria and stoma were respectively detected in the yellow and green channel. The channel settings of pinhole, detector gain, and amplification offset and gain were adjusted to provide an optimal balance of fluorescent intensity of the targeted cells and background. Data were collected by a computer attached to the instrument, stored on the hard drive, processed with a Leica TCS Image Browser, and transferred to Adobe Photoshop CS2 for preparation of figures.

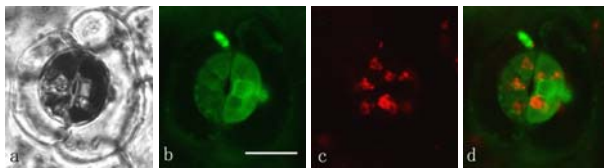
## **3. RESULTS AND DISCUSSION**

Exchange of water and CO<sub>2</sub> between leaves and the ambient air is one of the important plant processes. In this plant process, heat is dissipated and a primary substrate for photosynthesis is taken up. Stoma plays a key role in the control of water efflux and CO<sub>2</sub> influx between the inside leaf and the ambient air. In the epidermis of plant leaves, stoma is surrounded by two guard cells. Recently, guard cells have become a highly developed model system for characterizing early signal transduction mechanism in plants (Garcia-Mata and Lamattina, 2007; Garcia-Mata et al., 2007; MacRobbie and Kurup, 2007). It has been found that acridine orange (AO) is the most popular fluorochrome for studies on whole blood, reticulocyte counting, and identification of nucleic acids (Anderson, 1957; Armstrong and Niven, 1957; Armstrong, 1956). In present studies, stomatal guard cells stained with AO were imaged with confocal microscope, and guard cells and chloroplasts in guard cells were clearly seen (Figs 1B, C, D). The results indicate that AO is

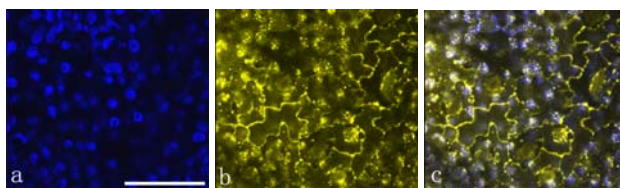
easily detectable using the confocal microscope, and AO is an appropriate probe for living guard cells studies.

Rhodamine 123, which is a potentiometric sensor and nontoxic dye, can accumulate in mitochondria for the mitochondrial membrane potential. Moreover, rhodamine-123 is easily detectable using an epifluorescent or confocal microscope, which highlights heterogeneity of mitochondrial shape, size, position, and dynamic within living plant cells. Mitochondrial morphology and dynamics have been studied extensively in eukaryotic cells. The results of Logan and Leaver indicate that the shape of mitochondria is varied depending on the cell type (Logan and Leaver, 2000). As early as 1914, the mitochondrial shape was studied with simple bright field microscope (Lewis and Lewis, 1914). Since then there have been numerous studies investigating mitochondrial polymorphism and physiology. Ultrastructural mitochondrial shapes and sizes within fixed cells are usually studied using the electron microscope. However, it is not possible to dismiss the possibility that any heterogeneity observed resulted from preparation or fixation artifacts (Logan and Leaver, 2000). Here we used rhodamine 123 as fluorescence probe, and mitochondria was clearly seen in epidermic tissue of mung bean leaves (Fig. 2B, C).

Taken together, the developments in technology and reagents that brought fluorescence-based methods to microscopy have become prevalent in plant cell biology. Our results indicate that it is a good method and simple way to research mung bean leaves with confocal microscopy and fluorescence probes AO and rhodamine-123.



*Fig. 1:* Laser-scanning confocal microscope images of stomatal guard cells of mung bean leaves. Stomatal guard cells were labeled with AO. (A) transmission channel, showing phase contrast; (B) green channel, showing DNA and RNA fluorescence; (C) Red channel, showing chlorophyll fluorescence in chloroplasts of epidermic cells; (D) B and C combined. Scale bars, 12.58  $\mu\text{m}$ .



*Fig. 2:* Intracellular localization of mitochondria. Laser-scanning confocal microscope images of epidermic tissue of mung bean leaves. Epidermic cells were labeled with Rhodamine-123. (A) Blue channel, showing chlorophyll fluorescence in chloroplasts of epidermic cells; (B) yellow channel, showing mitochondria fluorescence; (C) A and B combined. Scale bars, 75  $\mu\text{m}$ .

#### 4. CONCLUSION

Taken together, the developments in technology and reagents that brought fluorescence-based methods to microscopy have become prevalent in plant cell biology. Our results indicate that it is a good method and simple way to research the mitochondria and stoma with confocal microscope and fluorescence probes AO and rhodamine-123.

#### ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation for Outstanding Young Scholars of Shandong Province, China (Grant No. 2007BS06021).

#### REFERENCES

- A. F. Armstrong, D. C. Logan, A. K. Tobin, et al. Heterogeneity of plant mitochondrial responses underpinning respiratory acclimation to the cold in *Arabidopsis thaliana* leaves, *Plant Cell Environ*, 2006, 29: 940-949
- B. Li, D. Xing, L. Zhang. Involvement of NADPH oxidase in sulfur dioxide-induced oxidative stress in plant cells, *Photochem Photobiol Sci*, 2007, 6: 628-634
- C. Garcia-Mata, L. Lamattina. Abscisic acid (ABA) inhibits light-induced stomatal opening through calcium- and nitric oxide-mediated signaling pathways, *Nitric Oxide*, 2007, 17: 143-151
- D. C. Logan, C. J. Leaver. Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells, *Journal of Experimental Botany*, 2000, 51: 865-871
- E. A. MacRobbie, S. Kurup. Signalling mechanisms in the regulation of vacuolar ion release in guard cells, *New Phytol*, 2007, 175: 630-640
- E. B. Blancaflor, S. Gilroy. Plant cell biology in the new millennium: new tools and new insights, *Am J Bot*, 2000, 87: 1547-1560
- E. S. Anderson. Visual observation of deoxyribonucleic acid changes in bacteria during growth of bacteriophage, *Nature*, 1957, 180: 1336-1338
- J. A. Armstrong, J. S. F. Niven. Fluorescence microscopy in the study of nucleic acids, *Nature*, 1957, 180: 1335-1336
- J. A. Armstrong. Histochemical differentiation of nucleic acids by means of induced fluorescence, *Exp Cell Res*, 1956, 11: 640-643
- J. R. Lucas, J. A. Nadeau, F. D. Sack. Microtubule arrays and *Arabidopsis* stomatal development, *J Exp Bot*, 2006, 57: 71-79
- J. W. Lichtman. Confocal microscopy, *Scientific American*, 1994, 271: 40-53
- M. A. Dong, J. L. Bufford, Y. Oono, et al. Heat suppresses activation of an auxin-responsive promoter in cultured guard cell protoplasts of tree tobacco, *Plant Physiol*, 2007, 145: 367-377

- M. Garstka, J. H. Venema, I. Rumak, et al. Contrasting effect of dark-chilling on chloroplast structure and arrangement of chlorophyll-protein complexes in pea and tomato: plants with a different susceptibility to non-freezing temperature, *Planta*, 2007, 226: 1165-1181
- M. R. Lewis, W. H. Lewis. Mitochondria (and other cytoplasmic structures) in tissue cultures, *American Journal of Anatomy*, 1914, 17: 339-401
- M. Taira, U. Valtersson, B. Burkhardt, et al. Arabidopsis thaliana GLN2-encoded glutamine synthetase is dual targeted to leaf mitochondria and chloroplasts, *Plant Cell*, 2004, 16: 2048-2058
- P. K. Hepler, B. E. S. Gunning. Confocal fluorescence microscopy of living cells, *Protoplasma*, 1998, 201: 121-157
- R. H. Webb. Theoretical basis of confocal microscopy, *Methods in Enzymology*, 1999, 307: 3-20
- T. Meckel, L. Gall, S. Semrau, et al. Guard cells elongate: relationship of volume and surface area during stomatal movement, *Biophys J*, 2007, 92: 1072-1080
- X. D. Yang, C. J. Dong, J. Y. Liu. A plant mitochondrial phospholipid hydroperoxide glutathione peroxidase: its precise localization and higher enzymatic activity, *Plant Mol Biol*, 2006, 62: 951-962