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Arabidopsis thaliana Embryo Sac Mitochondrial Membrane Potential Stain

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[Abstract] The aim of this experiment is to study mitochondrial functional status in *Arabidopsis* embryo sacs using the membrane potential indicator JC-1. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules. The resulting equilibrium of ions leads in turn to the decoupling of the respiratory chain and the release of cytochrome c into the cytosol, a distinctive feature of the early stages of programmed cell death.

JC-1 is a lipophilic dye that can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases. In healthy cells with high mitochondrial potential, JC-1 spontaneously forms complexes with intense red fluorescence. On the other hand, in mitochondria with low mitochondrial potential, JC-1 remains in the monomeric form, which exhibits only green fluorescence (Martin *et al.*, 2013; Hauser *et al.*, 2006).

This protocol could be used in isolated mitochondria, and in a variety of cell types and different tissues of plants and other organism.

Materials and Reagents

1. Flowers at different developmental stages from an *Arabidopsis* inflorescence
2. JC-1 Dye (Life Technologies, Molecular Probes®, catalog number: T3168)
3. DMSO 99.9% (Sigma-Aldrich, catalog number: D8418)
4. Stock solution (10 $mg.ml^{-1}$ of JC-1 in DMSO)
5. Working solution (10 $ug.ml^{-1}$ of JC-1 in buffer A)
6. Buffer A (20 mM HEPES buffer, pH 7.2) (Sigma-Aldrich, catalog number: H3375) (see Recipes)

Equipment

1. Confocal microscope (Nikon Eclipse C1 Plus Confocal microscope, using EZ-C1 3.80 imaging software and Ti-Control)
2. Dissecting microscope (Nikon Corporation, model: SMZ800)
3. Coverslip (18 x 18 mm)
4. Microscopic slide (26 x 76 mm)
5. 1 ml insulin syringe with the 0.3 x 13 mm needle (BD)
6. Needle point tweezers

Software

1. NIH Image J software 1.47 for Windows (http://rsb.info.nih.gov/ij/)

Procedure

1. Pistils isolation (Figure 1, A-E).
   Using a pair of tweezers, take flowers, at different developmental stages from an *Arabidopsis* inflorescence (Alvarez-Buylla *et al.*, 2010). On a microscopic slide and under a dissecting microscope, remove sepals, petals, anthers and stigma using the needles of two 1 ml insulin syringes (0.3 x 13 mm) and make longitudinal superficial cuts on pistils at each side of the septum (Figure 1).
2. Pistils stain (Figure 1, F-G).
   a. Submerge the pistils in 100 µl of a solution containing 10 $\mu g.ml^{-1}$ of JC-1 in buffer A.
   b. Incubate for 30 min at room temperature without shaking. Protect from light, as the dye is photosensitive.
   c. Gently wash the pistils three times with buffer A.
3. Sample preparation for microscopy (Figure 1, H-L).
   a. On a microscopic slide under a dissecting microscope, use the needles of two 1 ml insulin syringes (0.3 x 13 mm) to dissect the pistils exposing the ovules
   b. Add a drop of buffer A and cover the sample with a coverslip.
   c. Immediately observe under a confocal microscope. The intensities of green (excitation/emission wavelength = 485/538 nm) and red (excitation/emission wavelength = 485/590 nm) are analyzed.

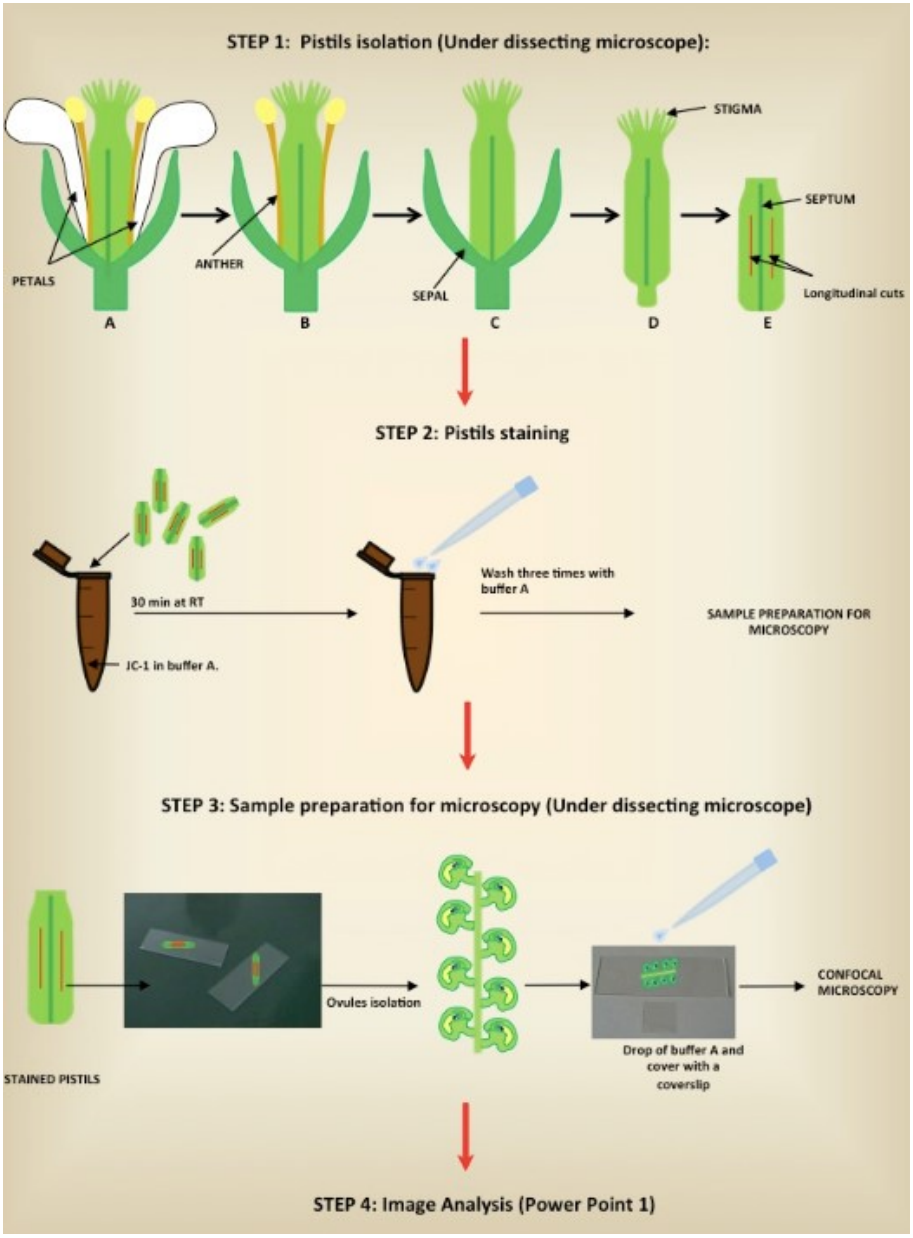

Figure 1. Schematic illustration showing the steps required for embryo sac mitochondrial staining

4. Image analysis (Power Point 1). The ratio of red to green fluorescence of JC-1 images is calculated using NIH Image J software.
   a. The outline of each embryo sac is delimited using the freehand tool to create a region of interest (ROI) and saved using the ROI manager tool (Analyze>tools>ROI manager>Add).

b. In the “Analyze” menu, select “set measurements” and click on area and “mean gray value”.
c. Using the image Menu, select “color” and then “split channels”.
d. Close the image in the blue channel. On the green channel, go to the analyze>tools>ROI manager and select the ROI added before. The ROI will appear on the image. Still in the ROI manager go to more>multi Measure. The result will appear in a new window.
e. Repeat this step with the image in the red channel.
f. Copy the results and paste them in an excel worksheet.
g. Calculate the red to green fluorescence ratio for each ROI.

Recipes

1. Buffer A
   20 mM HEPES buffer (pH 7.2)
   For 1 L of 1 M HEPES buffer:
   Dissolve 238.3 g HEPES (free acid) in 500 mL of ddH2O
   Stir while adjusting the pH 7.2 with 0.5 N NaOH
   Bring up the volume to 1 L with ddH_2O to prepare 1 L of 20 mM HEPES buffer (Buffer A)
   Add 20 ml of 1 M HEPES buffer in 980 ml of ddH_2O

Acknowledgments

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