Video Article

A Simple Method for Imaging *Arabidopsis* Leaves Using Perfluorodecalin as an Infiltrative Imaging Medium

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Abstract

The problem of acquiring high-resolution images deep into biological samples is widely acknowledged[1]. In air-filled tissue such as the spongy mesophyll of plant leaves or vertebrate lungs further difficulties arise from multiple transitions in refractive index between cellular components, between cells and airspaces and between the biological tissue and the rest of the optical system. Moreover, refractive index mismatches lead to attenuation of fluorophore excitation and signal emission in fluorescence microscopy. We describe here the application of the perfluorocarbon, perfluorodecalin (PFD), as an infiltrative imaging medium which optically improves laser scanning confocal microscopy (LSCM) sample imaging at depth, without resorting to damaging increases in laser power and has minimal physiological impact[2]. We describe the protocol for use of PFD with *Arabidopsis thaliana* leaf tissue, which is optically complex as a result of its structure (**Figure 1**). PFD has a number of attributes that make it suitable for this use[3]. The refractive index of PFD (1.313) is comparable with that of water (1.333) and is closer to that of cytosol (approx. 1.4) than air (1.000). In addition, PFD is readily available, non-fluorescent and is non-toxic. The low surface tension of PFD (19 dynes cm^{-1}) is lower than that of water (72 dynes cm^{-1}) and also below the limit (25 - 30 dyne cm^{-1}) for stomatal penetration[4], which allows it to flood the spongy mesophyll airspaces without the application of a potentially destructive vacuum or surfactant. Finally and crucially, PFD has a great capacity for dissolving CO_2 and O_2, which allows gas exchange to be maintained in the flooded tissue, thus minimizing the physiological impact on the sample. These properties have been used in various applications which include partial liquid breathing and lung inflation[5,6], surgery[7], artificial blood[8], oxygenation of growth media[9], and studies of ice crystal formation in plants[10]. Currently, it is common to mount tissue in water or aqueous buffer for live confocal imaging. We consider that the use of PFD as a mounting medium represents an improvement on existing practice and allows the simple preparation of live whole leaf samples for imaging.

Video Link

The video component of this article can be found at http://www.jove.com/video/3394/

Protocol

The protocol given below describes a simple method for using PFD as an infiltrative mounting medium in *Arabidopsis thaliana* leaves, but we anticipate that this method may be used in a variety of applications where imaging air-rich tissues is desired.

1. Mounting leaf samples in PFD

1. Prepare a microscope slide with a gas-permeable gasket of polydimethylsiloxane (PDMS, Carolina Observation Gel). PDMS is a viscoelastic polymer and can be molded to provide a chamber tailored to experimental requirements.
2. Equilibrate PFD with air. This may be achieved by bubbling with air or by shaking a small volume of PFD in an air-filled bottle.
3. Decant PFD into an open Petri dish and float a whole seedling or excised leaf on PFD for 5 minutes. The tissue should become translucent, reminiscent of vitrified tissue (**Figure 2 (a)**). Leaves may appear darker or lighter than before exposure to PFD, depending on lighting conditions and the age of tissue used.
4. Fill the PDMS chamber with air-equilibrated PFD and carefully transfer the tissue samples from the incubation Petri dish to the PDMS chamber. Seal the slide with a coverslip and image according to experimental requirements.
5. Note: PFD also performs well in an open chamber constructed on a coverslip or in a perfusion chamber and is compatible with silicon grease. PFD is not compatible with Teflon components as it dissolves them.

2. Representative Results:

Microscopic inspection of PFD-incubated leaf samples shows that the majority of airspaces are flooded. **Figure 2 (b)** shows airspaces flooded with recombinant GFP suspended in PFD. It is apparent that the leaf is flooded upon incubation with PFD, but that occasional pockets of air may remain. Water does not flood the leaf under these conditions. LSCM of samples incubated and mounted in air, water or PFD shows that PFD gives an advantage over water and air in the depth of imaging possible (**Figure 3**). The example given in **Figure 3** shows air, water and PFD

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mounted *Arabidopsis* leaves expressing cytosolically localized Venus, a variant of YFP[11] that is expressed constitutively under the 35S promoter. We can see an approximate 2-fold increase in depth of imaging when comparing PFD and water. It should be noted, however, that the precise improvement seen when using PFD varies with numerical aperture of the lens and the type of tissue used. We have seen cytoplasmic streaming and root hair elongation in PFD treated seedlings, each indicative of healthy plants. In addition we have shown[2] that *Fv/Fm*, a measure of the photosynthetic functioning of plants[12] remains within tolerable limits over timescales used in imaging.

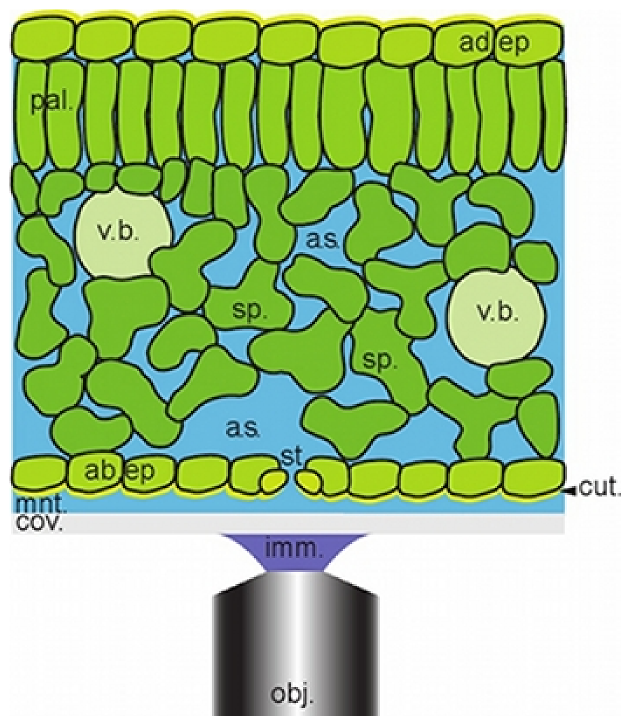

Figure 1. Plant leaf complexity and optical implications
Diagrammatical representation showing the anatomical features of the *A. thaliana* leaf in relation to the optical set-up. Abbreviations used are obj. = objective lens, imm. = immersion fluid, cov. = coverslip, mnt. = mountant, cut. = cuticle, ad. ep. = adaxial epidermis, st. = stomatal pore, sp. = spongy mesophyll, a.s. = air space, pal. = palisade mesophyll, v.b. = vascular bundle, ad. ep. = adaxial epidermis. Cell walls are indicated by black lines.

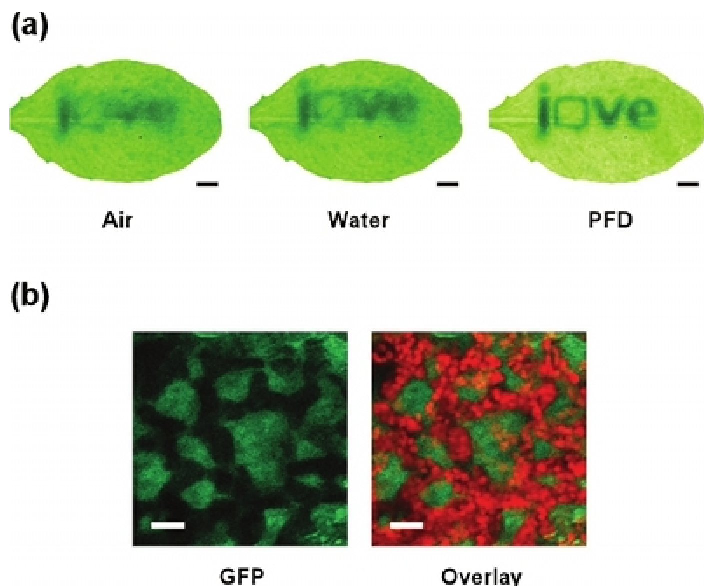

Figure 2. PFD readily penetrates *Arabidopsis* leaves
(a) Leaves incubated in air, water or PFD for 5 minutes and imaged using a Leica DCF3000FX digital camera with a Leica MZ16F microscope (Leica Microsystems (UK) Ltd., Milton-Keynes, UK). The JoVE logo was printed on acetate film and illuminated from below with a light box. Exposure time was 89 ms and all images were collected and processed identically. Scale bars represent 2 mm.

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(b) PFD carrying purified recombinant green fluorescent protein (GFP) delimits airspaces *in vivo*. GFP is false coloured green, and red is used to show chlorophyll auto-fluorescence, which delimits the mesophyll cells. (**Figure 2(b)** reproduced with permission and full technical details available in Littlejohn *et al.* 2010[2]). Scale bars represent 25 m.

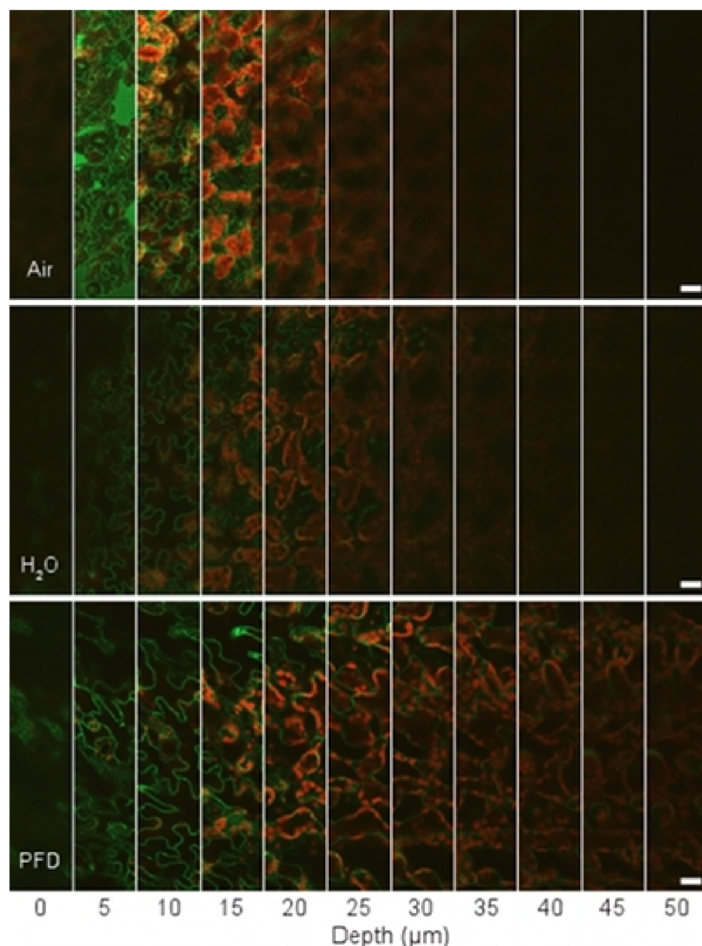

Figure 3. PFD improves confocal imaging in leaves
LSCM images showing cytoplasmically localised Venus fluorescence (green) and chlorophyll autofluorescence (red) in intact A. thaliana leaves imaged in air, water or PFD. Excitation wavelength was 514 nm and fluorescence emission was recorded at 518 nm 604 nm for Venus and at 657 nm 679 nm for chloroplasts. Each panel is a single confocal section taken from a Z-stack of 11 confocal images acquired at 5 µm intervals. These were extracted from a full Z-stack of 59 images acquired at 1 µm intervals, which have been used to produce supplementary movies. Depth measurements are given relative to the epidermal surface. Representative images, which were processed identically, are shown. Experimental details such as microscope settings are identical to those found in Littlejohn *et al*., 2010[2]. Scalebars represent 20 µm.

Discussion

This is a simple and easy to use technique to improve microscopy of air-filled or optically complex tissues. We have shown that the technique has some strong advantages and we hope that it will be used to elucidate biological questions pertaining to air-rich tissues. For example it would be a natural choice for studies of pathogen attack in plant mesophyll or in lungs. We also are aware of the limitations of the technique. We are working on better refractive index matching, use with other modes of microscopy, and perfluorocarbon mountant replenishment during long timescale experiments. We also recognise, though, that one of the main advantages of PFCs, namely being biologically inert has a flipside. PFCs do not readily dissolve biological molecules which also implies that they cannot be easily used to deliver compounds of interest such as hormones, drugs, and other small molecules or ions.

Disclosures

No conflicts of interest declared.

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