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Methods

Perfluorodecalin enhances *in vivo* confocal microscopy resolution of *Arabidopsis thaliana* mesophyll

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Key words: Arabidopsis, confocal microscopy, fluorescence, mesophyll, perfluorocarbon, perfluorodecalin.

Summary

- Air spaces in the leaf mesophyll generate deleterious optical effects that compromise confocal microscopy.
- Leaves were mounted in the nontoxic, nonfluorescent perfluorocarbon, perfluorodecalin (PFD), and optical enhancement and physiological effect were assessed using confocal microscopy and chlorophyll fluorescence.
- Mounting leaves of *Arabidopsis thaliana* in PFD significantly improved the optical qualities of the leaf, thereby enabling high-resolution laser scanning confocal imaging over twofold deeper into the mesophyll, compared with using water. Incubation in PFD had less physiological impact on the mounted specimen than water.
- We conclude that the application of PFD as a mounting medium substantially increases confocal image resolution of living mesophyll and vascular bundle cells, with minimal physiological impact.

Introduction

Laser scanning confocal microscopy (LSCM) is a widely used cell fluorescence imaging technique that enables clear optical sectioning of the mounted specimen. Several factors, including the wavelength and intensity of the excitation laser, the magnification and numerical aperture of the objective lens, the signal intensity from the mounted specimen and the position of the pinhole that filters out-of-focus information, define the criteria for confocal image acquisition (Cheng, 2006). These criteria are demanding when imaging through several cell layers or when the tissue is differentially refractive, as is the case for the spongy mesophyll of plant leaves. The spongy mesophyll is located adjacent to the abaxial (lower) epidermis, contains numerous air spaces and may be several cells thick. These characteristics result in high amounts of reflection, refraction and diffraction within the mesophyll that, together, produce spherical aberrations that impair confocal image quality (Inoue, 2006). In addition, there is a progressive attenuation of excitation laser intensity through the mesophyll and a concomitant decrease in fluorescence emission intensity that reduces detectable signal. Simply increasing the power of the excitation laser to image deeper into leaves or cotyledons often results in cellular damage or rapid bleaching of the chromophores. Increasing the sensitivity or gain of the emission detector reduces the signal-to-noise ratio, which also compromises image quality. Optical resolution can be enhanced by improved lenses or by sample fixation and subsequent immersion or embedment procedures (Haseloff *et al.*, 1997). When *in vivo* imaging is required, it can be enhanced by infiltrating the mesophyll air spaces with a medium of similar refractive index to that of the cytosol. To this end, plant tissues are generally mounted in water or a dilute aqueous solution. However, under atmospheric pressure, water does not readily infiltrate plant leaves because of the presence of the hydrophobic cuticle and the surface tension at the interface of the stomatal pores (Schönherr & Bukovac, 1972).

Perfluorocarbons are colourless, nontoxic liquids with among the lowest known surface tensions (Sargent & Seffl, 1970). In addition, perfluorocarbons have a high capacity for dissolving O_2 and CO_2. Biological applications include the production of artificial blood substitutes

(Lowe, 2003), respiratory medicine in premature babies (Davies, 1999), eye and pancreatic surgery (Crafoord *et al.*, 1995; Brandhorst *et al.*, 2005) and drug delivery (Krafft, 2001). In plants, perfluorocarbons have been almost exclusively used to increase solubility of gases in media for tissue culture (Wardrop *et al.*, 1997a) and micropropagation (Wardrop *et al.*, 1997b).

We have found that the nonvolatile perfluorocarbon, perfluorodecalin (PFD), rapidly and uniformly infiltrated the leaves of the model angiosperm *Arabidopsis thaliana*, without the application of a vacuum and with minimal physiological impact. Mounting leaves in PFD significantly improved the resolution of confocal images of the mesophyll and enabled imaging over twofold deeper into the tissue compared with using water as a mounting medium.

Materials and Methods

Plant culture

Arabidopsis thaliana (L.) Heynh (Col-0 ecotype) and transformants that stably and constitutively express a cytoplasmically localized 'Venus' yellow fluorescent protein (SEYFP-F46L; Nagai *et al.*, 2002) were used in this study. Seeds were surface-sterilized for 3 min with 70% ethanol and then for 5 min with 10% sodium hypochlorite. Seeds were washed five times in water and suspended in 0.1% agar. Seeds were stratified at 4°C, in the dark, for 48 h before being germinated and grown at 20°C, in a 16 : 8 h light : dark photoperiod on 0.5 × MS agar medium (2.15 g l^{-1} MS salts with Gamborg's vitamins (Melford Laboratories Ltd., Ipswich, UK); 0.8% w/v phytagel, pH 5.7)

Fluorescence spectrophotometry

Three-dimensional fluorescence spectra of water, immersion medium (Zeiss Immersol W, Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK) and PFD (F2 Chemicals, F2 Chemicals Ltd., Lea Town, Lancashire, UK) were acquired using an Aminco-Bowman series 2 spectrophotofluorometer (Thermo Electron Scientific Instruments Corp., Madison, Wisconsin, USA). Excitation wavelengths used were every 10 nm, from 200 to 700 nm, and the fluorescence emission was recorded every 10 nm, from 200 to 700 nm. To ensure clarity, Rayleigh/Tyndall scatter was removed following the scan and spectra were plotted using Origin software (OriginLab Corp., Northampton, Massachusetts, USA).

PFD infiltration and whole-leaf imaging

Images of whole leaves from 14-d-old plants were acquired with a Leica DCF300FX digital camera coupled to a Leica MZ16F dissecting microscope (Planapo ×1.0 lens; 2.5 zoom), using Leica FireCam Software (Leica Microsystems (UK) Ltd., Milton-Keynes, Buckinghamshire, UK). Exposure was fixed at 100 ms. Leaves were placed on a dimpled microscopy slide and positioned over a '+' symbol that was printed on acetate film and illuminated from below. Leaves were first imaged in air then immersed in water and imaged a second time. The leaves were then blotted dry, immersed in 200 µl PFD and imaged again. Identical results were obtained for leaves that were immersed in PFD without a previous immersion in water.

LSCM imaging of apoplastic green fluorescent protein

Recombinant green fluorescent protein (GFP) was dissolved in PFD to a final concentration of 0.1 µg μl^{-1}. Leaves were excised from *A. thaliana* seedlings, incubated in the GFP/PFD solution for 5 min and mounted in PFD on a microscope slide. Leaves were imaged using a Zeiss Axiovert 510 Meta LSCM equipped with a ×10/0.3 Plan-Neofluar lens. Light paths and wavelengths were controlled by HFT 405/488/543/633 nm and NFT 635 dichroic mirrors. GFP fluorescence was excited at 488 nm using a 30 mW argon laser set to 2% transmission intensity. Chlorophyll fluorescence was excited at 633 nm using a 30 mW argon laser set to 50% transmission intensity. Pixel dwell time was 4.20 µs. GFP fluorescence was recorded at 505–507 nm. Chlorophyll fluorescence was recorded at 647–711 nm. The pinhole was set at 72 µm (1.75 Airy units (AU)) for GFP fluorescence and at 92 µm (1.03 AU) for chlorophyll fluorescence. Images were integrated and processed using Zeiss 510 software.

LSCM imaging of cytoplasmically localized Venus

Confocal imaging was performed using a Zeiss Axiovert 510 Meta LSCM equipped with a ×63/1.2 water-corrected oil immersion C-Apochromat lens unless otherwise stated. The immersion medium was Zeiss Immersol W. Light paths and wavelengths were controlled by a 458/514 nm dichroic mirror. The pinhole was set at 142 µm (1.95 AU). Images were integrated and processed using Zeiss 510 software. Images of Venus and chlorophyll fluorescence in intact *Arabidopsis* leaves were collected with excitation at 514 nm with a 30 mW argon laser, 6.1 A, 19.8% transmission intensity and a pixel dwell time of 4.20 µs, unless otherwise stated. Emission was recorded at 518–604 nm for Venus and at 657–679 nm for chlorophyll. Raw data were used to reconstitute *z*-stacks and orthogonal sections and all data were processed identically between samples. *z*-stacks measured as follows: *x*, 152.80 µm; *y*, 31.74 µm; and *z*, 95.00 µm. Orthogonal projections were created from *z*-stacks containing 95 images acquired at intervals of 1 µm. Excised leaves were maintained in air or floated on water or PFD for 5 min and mounted in the same medium for

imaging. Projections in the *z*-plane were integrated with a first angle of 180°. Figures were assembled in Adobe Photoshop (Adobe Systems Inc., San Jose, California, USA). Where images were acquired using the ×40/1.30 Oil DIC lens, Venus fluorescence was excited at 514 nm (6.1 A, 10.1% transmission) and emission was imaged at 530–600 nm. Chloroplast fluorescence was excited at 633 nm (2.0% transmission) and emission was collected using a LP 650 nm filter. For clarity of reproduction, the gamma (γ) values for images presented in Fig. 3(a) were adjusted such that for images (i)–(iii), γ = 1; for images (iv)–(vi), γ = 1.4; and for images (vii)–(ix), γ = 1.9.

F_v/F_m quantification

Arabidopsis thaliana seedlings were germinated and grown on 0.5 × MS agar medium (2.15 g l^{-1} MS salts with Gamborg's vitamins; 0.8% w/v phytagel, pH 5.7) in glass Magenta Jars, at 20°C and in a 16 : 8 h light : dark photoperiod. Chlorophyll autofluorescence was quantified using a chlorophyll fluorescence imager system (Technologica Ltd., Colchester, Essex, UK) delivering an 800 ms pulse of actinic light (6349 μmol m^{-2} s^{-1} photon flux), controlled by FluorImager software (Technologica Ltd., Colchester, Essex, UK). FluorImager software was also used to calculate F_v/F_m values. Where appropriate, the aerial parts of the plants were fully submerged in 50 ml of water or PFD. Plants were incubated in the darkbox of the fluorimager for 1 h before imaging.

Effect of PFD on seed germination

Seeds were sterilized, sown on 0.5 × MS agar medium (2.15 g l^{-1} MS salts with Gamborg's vitamins; 0.8% w/v phytagel, pH 5.7) in glass Magenta jars, stratified and grown as detailed earlier. Fifty millilitres of water or PFD was added to the seeds after stratification, and seedlings were grown for 1 wk before being scored.

Results

PFD spectrophotometry

We used fluorescence spectrometry to record excitation and emission spectra of PFD and evaluate its suitability as a mounting medium. PFD was excited every 10 nm, from 200 to 700 nm, and the fluorescence emission recorded every 10 nm, from 200 to 700 nm. The fluorescence emission of PFD did not exceed 0.06 when excited from 200 to 700 nm (Fig. 1), which is similar to results obtained for water and the microscopy immersion medium used in this study (Zeiss Immersol W). The refractive index of PFD is 1.313 and very close to those of water (1.333) in which living specimens are usually mounted and Zeiss Immersol

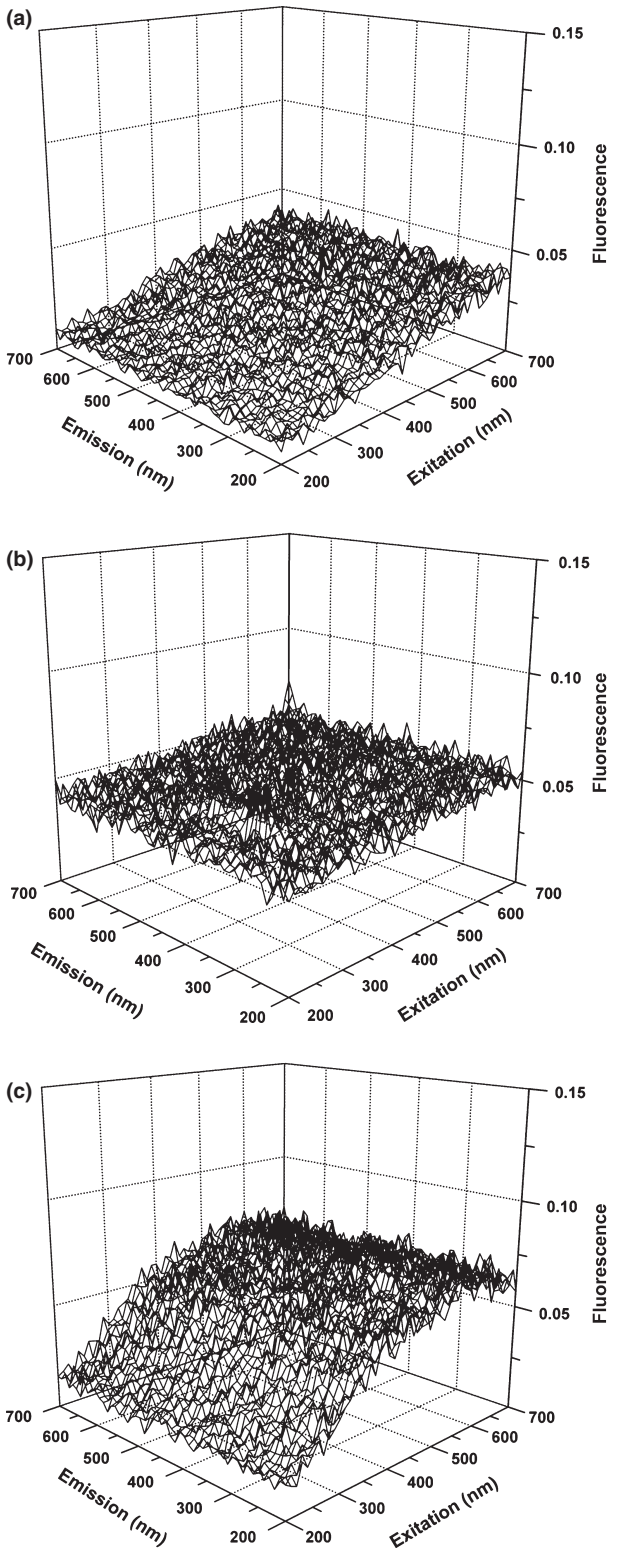

Fig. 1 Fluorescence emission spectra. Fluorescence excitation and emission of water (a), Zeiss Immersol W immersion medium (b), and perfluorodecalin (c). Excitation wavelengths used were every 10 nm, from 200 to 700 nm, and the fluorescence emission was recorded every 10 nm, from 200 to 700 nm. For clarity, Rayleigh-Tyndall scattering was removed before plotting.

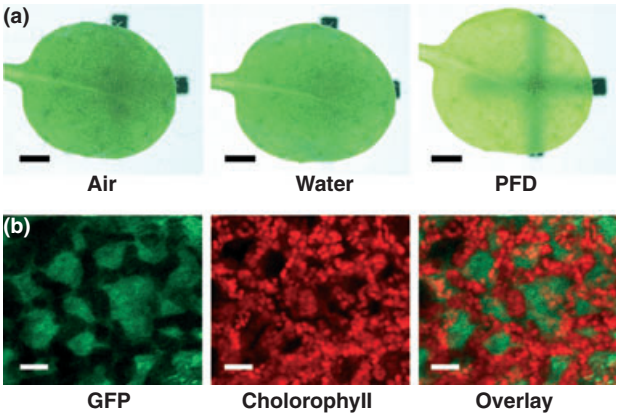

Fig. 2 Infiltration of intact *Arabidopsis thaliana* leaves by perfluorodecalin (PFD). (a) Bright-field images showing light transmission through an *A. thaliana* leaf in air, water or PFD. The '+' symbol is located under the leaf and only discernible when mesophyll air spaces are infiltrated with PFD, rendering the leaf translucent. Bars, 250 μm. (b) Laser scanning confocal microscopy (LSCM) images showing the fluorescence of recombinant green fluorescent protein (GFP) in the mesophyll air spaces of *A. thaliana*. GFP (0.1 μg μl^{-1}) dissolved in PFD was infiltrated into leaves. GFP fluorescence is shown in green and chlorophyll autofluorescence is shown in red. Bars, 25 μm.

W (1.334), and also to the value of 1.36 used by Sheahan (1996) for *Arabidopsis* leaf. The optical properties of PFD are therefore compatible with light microscopy and LSCM. While perfluorocarbons with other refractive indices are available, these tend to have a lower refractive index than water and PFD. A notable exception is perfluorohexyloctane, which has a refractive index of 1.34 (Hoerauf *et al.*, 2001). However, perfluorohexyloctane has some toxic effects (Mertens *et al.*, 2002), which precluded it from being used in this investigation.

PFD readily infiltrates the mesophyll

Perfluorodecalin has a low surface tension (Sargent & Seffl, 1970) that allows it to easily penetrate leaf stomatal pores and fill the intercellular air spaces of the mesophyll. Leaves kept in air or immersed in water are relatively opaque, whereas leaves bathed in PFD become translucent almost immediately (Fig. 2a). This increase in light transmission through the leaf when the mesophyll air spaces are infiltrated with PFD is attributable to reduced amounts of diffraction and refraction within the tissue that make it more optically homogeneous. To determine more accurately the extent of PFD infiltration into the mesophyll air spaces, a mature leaf was immersed in a 0.1 μg μl^{-1} solution of recombinant GFP in PFD. After *c.* 5 min incubation, GFP was distributed exclusively and relatively homogeneously within the spongy mesophyll air space (Fig. 2b), indicating that PFD had effectively infiltrated the mesophyll.

PFD increases *z*-axis resolution in intact leaves

Leaves from *A. thaliana* plants constitutively expressing cytoplasmically localized Venus, a variant of enhanced yellow fluorescent protein (SEYFP-F46L; Nagai *et al.*, 2002), were imaged using a Zeiss Axiovert 510 Meta LSCM equipped with a ×63/1.2 water-corrected oil immersion C-Apochromat lens (Fig. 3a,b). Microscope settings were optimized for imaging at a depth of 25 μm in PFD, which resulted in saturated signal from the epidermis (Fig. 3a (iv,vii)). Optical sections were obtained every 25 μm (Fig. 3a – for clarity of reproduction, the γ-values for images presented were adjusted such that for images (i)–(iii), γ = 1; for images (iv)–(vi), γ = 1.4; and for images (vii)–(ix), γ = 1.9). Orthogonal projections (*x*/*z*-plane corresponding to a point on the *y*-axis) were calculated from a 95 μm *z*-stack (Fig. 3b). Leaves that were imaged without a mounting medium (i.e. in air) typically showed a high amount of reflection from the surface of the epidermis that impaired image quality and resulted in poor *z*-axis resolution (Fig. 3a(i,iv,vii); Fig. 3b(i)). Mounting leaves in water decreased the reflections from the surface of the leaf and enabled accurate imaging of the epidermis and of the mesophyll to a *z*-plane located *c.* 25 μm from the surface (Fig. 3-a(ii,v,viii); Fig. 3b(ii)). However, simply mounting leaves in PFD more than doubled the *z*-axis resolution of the ×63 objective compared with water, allowing clear images to be acquired from 25 μm and acceptable images to be acquired from 50 μm into the mesophyll (Fig. 3a(iii,vi,ix); Fig. 3-b(iii)). Although this evaluation of image quality is subjective, we considered it justified as tolerance of image quality is often user-defined and is largely dependent on the type of data required. While the absolute depth of acceptable imaging and increase in imaging depth compared with water varied with the age of the tissue, the exact method of slide preparation and the magnification of the objective lens, PFD consistently outperformed water as a mounting medium in every experiment we performed with leaf mesophyll. Most importantly, this improvement in *z*-axis resolution with PFD was achieved without increasing the power of the excitation laser, thereby reducing the potential for fluorophore bleaching and minimizing cell damage. Fig. 3(c) shows that infiltrating the leaf with PFD allows high-resolution imaging of the spongy mesophyll and cells surrounding the leaf vasculature. In addition to imaging the leaf, we also used PFD to mount roots, hypocotyls, anthers, carpels and female gametophytes for LCSM imaging. There was no stark improvement in using PFD rather than water for imaging any of these tissues, which may be attributed to the lack of intercellular air spaces within them (Supporting Information, Fig. S1). When seedlings were immersed in PFD, we observed root hair elongation and cytoplasmic streaming in all cell types observed, which are indicative of healthy tissues.

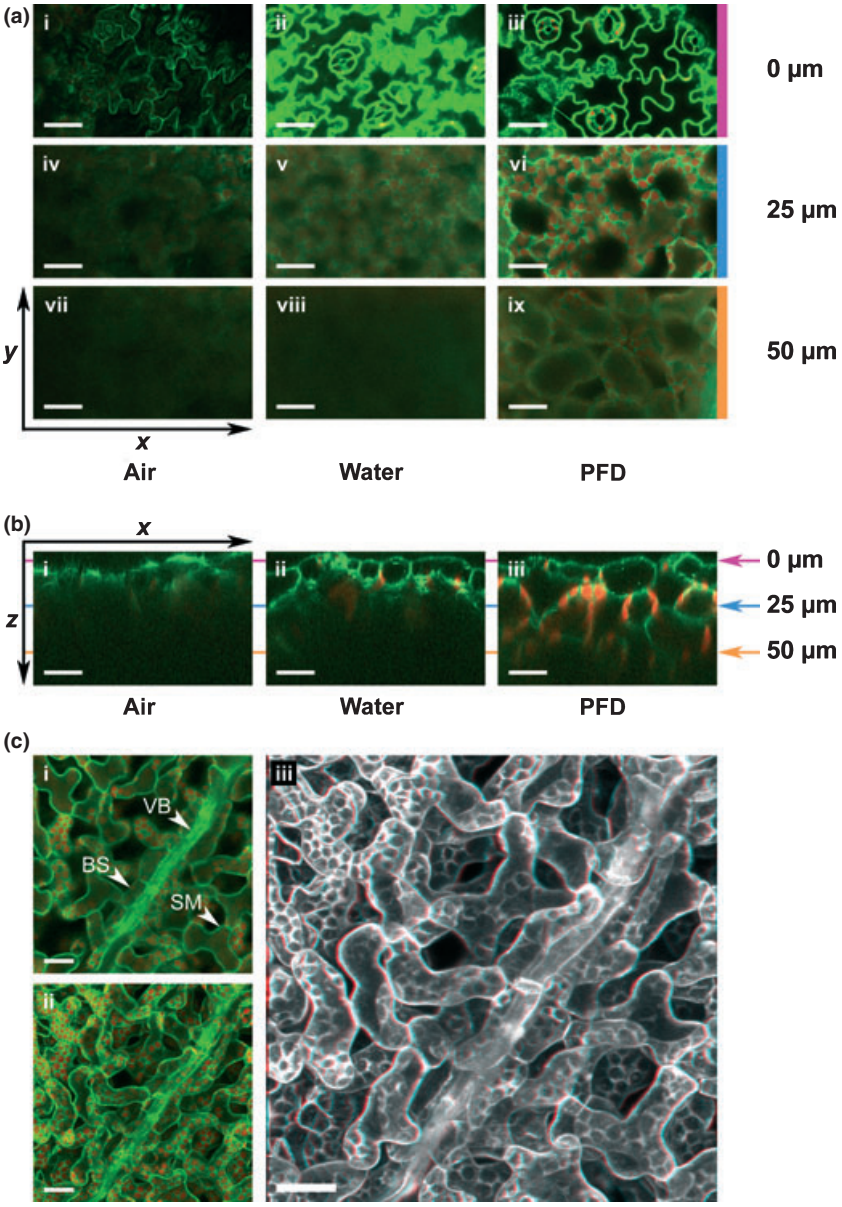

Fig. 3 *In vivo* imaging of intact *Arabidopsis thaliana* leaves is enhanced by mounting in perfluorodecalin (PFD). Laser scanning confocal microscopy (LSCM) images showing cytoplasmically localized Venus fluorescence (green) and chlorophyll autofluorescence (red) in intact *A. thaliana* leaves. Bars, 25 µm. Leaves were mounted in air, water or PFD, and imaged using a ×63 lens unless otherwise stated. (a) *A. thaliana* leaves imaged in air (i, iv, vii), water (ii, v, viii) or PFD (iii, vi, ix). Images are single confocal sections taken at the indicated distances (0, 25 and 50 µm) from the mid-epidermis. For clarity of reproduction, the γ-values for images presented were adjusted such that for images (i)–(iii), $\gamma = 1$; for images (iv)–(vi), $\gamma = 1.4$; for images (vii)–(ix), $\gamma = 1.9$. (b) Orthogonal projections calculated from a *z*-stack of 95 confocal images with 1 µm step resolution. Each image represents a single *x*/*z*-plane. Pink, blue and orange lines on the orthogonal projections correspond to depths of 0, 25 and 50 µm relative to the mid-epidermis. All images were processed identically. (c) Confocal images taken with a ×40 lens of the mesophyll and the vascular bundle in *A. thaliana* leaves mounted in PFD. (i) A single confocal section acquired 40 µm from the mid-epidermis. The white arrowheads indicate different cell types and structures within the leaf, including the spongy mesophyll (SM), bundle sheath cells (BS) and vascular bundle (VB). (ii) Confocal projection with 1 µm step resolution representing an optical slice 34–76 µm beneath the epidermis taken from a confocal *z*-stack and used to make a stereogram with difference angle of 4°(iii), illustrating effective imaging in the deep mesophyll.

PFD has minimal impact on leaf physiology

The air spaces of the mesophyll are essential for gaseous exchange and we were concerned that completely infiltrating the mesophyll with PFD might, while improving image resolution, have a deleterious effect on the physiology of the plant and negate any experimental advantage over using fixed samples. It was therefore essential to evaluate the impact of PFD infiltration on the physiology of the leaf.

Several parameters pertaining to the function of photosystem II (PSII) may be measured using diagnostic light treatments and by exploiting the fact that energy from light incident on the leaf and entering PSII may be dissipated as heat, re-emitted at a higher wavelength as chlorophyll fluorescence or used in PSII photochemistry. The maximum quantum efficiency of PSII photochemistry in dark-adapted leaves is widely used as an indicator of plant biotic and abiotic stresses (Baker, 2008). The maximum quantum efficiency of PSII is expressed as F_v/F_m (the efficiency of reduction of Q_A, the primary quinone electron acceptor of PSII), where F_v is the variable fluorescence of PSII (demonstrative of PSII ability to reduce Q_A) and F_m is the maximal fluorescence of PSII (measured in dark-adapted tissue, where Q_A is maximally reduced, and so energy that might otherwise be used in PSII photochemistry is emitted as chlorophyll fluorescence). As mesophyll is the primary photosynthetic tissue in higher plants and also the tissue that we were interested in imaging, we considered it reason-

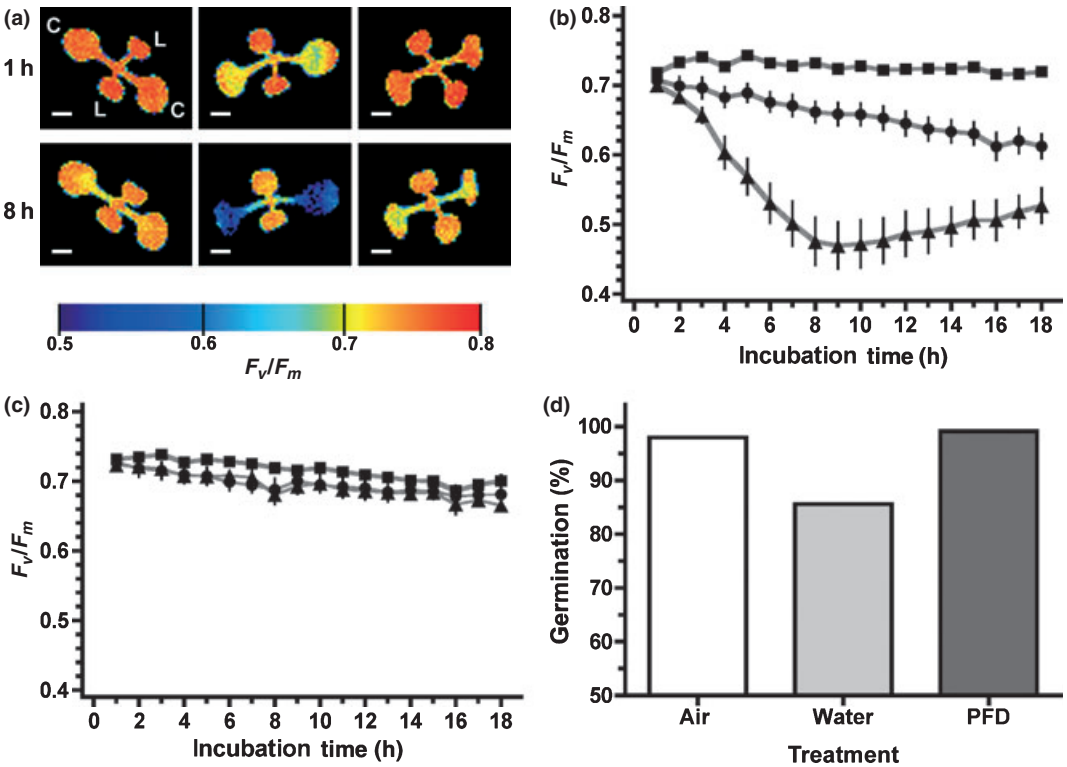

Fig. 4 Quantum efficiency of photosystem II and germination rate of *Arabidopsis thaliana* seedlings in air, water or perfluorodecalin (PFD). (a) False colour images of F_v/F_m in 10-d-old *A. thaliana* seedlings in air or immersed in water or in PFD. Images were taken every hour for 18 h and those presented are for 1 h or 8 h after transfer to the medium. C, cotyledons; L, leaves. Bars, 1 mm. F_v/F_m values range from 0.5 to 0.8, as indicated on the colour scale. (b) F_v/F_m of cotyledons of 10-d-old *A. thaliana* seedlings grown in air (squares) or immersed in water (triangles) or PFD (circles). Points represent the mean of at least six replicates with standard errors shown. (c) F_v/F_m of leaves from 10-d-old *A. thaliana* seedlings grown in air (squares) or immersed in water (triangles) or PFD (circles). Points represent the mean of at least six replicates with standard errors shown. (d) Germination rate (%) of *A. thaliana* seeds in air (white) or immersed in water (light grey) or PFD (dark grey). Seeds were sterilized, stratified and grown as detailed in the Materials and Methods section. After stratification, the seeds were submerged in 50 ml for either PFD or water. Seedlings were grown for 1 wk before being scored.

able to evaluate the physiological impact of PFD infiltration on plants by measuring F_v/F_m. *A. thaliana* seedlings were kept in air or immersed in water or in PFD and exposed to darkness for 1 h to inactivate the Calvin cycle and thereby allow an accurate measurement of F_v/F_m. Plants were then exposed to a 800 ms pulse of actinic light (6349 μmol m^{-2} s^{-1} photon flux), the fluorescence of the photosystem II reaction centre imaged (Fig. 4a) and F_v/F_m quantified for cotyledons (Fig. 4b) and leaves (Fig. 4c), at hourly intervals, for 18 h. Compared with control plants kept in air and for which F_v/F_m remained above 0.7, the F_v/F_m value for cotyledons of plants immersed in water reduced linearly from an average of 0.70 (± 0.01) at the start of the experiment to an average of 0.47 (± 0.03) at 8 h and 0.53 (± 0.02) at 18 h (Fig. 4b). This stark reduction in F_v/F_m was symptomatic of severe physiological stress, which is most probably the result of limited gaseous exchange within the mesophyll (MacDonald, 1975). Cotyledons immersed in PFD also showed a decrease in F_v/F_m, but to a much lesser extent than those in water; at 8 h, the mean F_v/F_m for cotyledons in PFD was 0.66 (± 0.01), from a starting value average of 0.71 (± 0.02), and at 18 h it was 0.61 (± 0.02). Conversely, the leaves of these plants showed no difference in F_v/F_m in air, water or PFD (Fig. 4c) as verified by an ANOVA (P = 0.5951).

As an additional test for plant health, *A. thaliana* seeds were immersed in water or in PFD and germination monitored after 7 d. Seeds were scored as germinated only if they possessed expanded, green cotyledons. Seeds submerged in PFD germinated as well as seeds kept in air and better than seeds immersed in water, indicating that PFD had no adverse effect on the critical stages of early development in *A. thaliana* (Fig. 4d). Seedlings that were germinated in PFD, however, were unable to expand true leaves. This observation confirms earlier experiments in which radish seedlings that were germinated on filter paper and submerged in a perfluorocarbon fluid showed growth inhibition after 3 d (Sukumaran *et al.*, 1972). Sukumaran *et al.*'s (1972) investigation also demonstrated that immersion for up to 2 d in perfluorocarbon had no deleterious effects on the aerial parts of several different plants, including *Pisum* (pea), *Malus* (apple), *Lilium* (lily) and *Allium* (onion). In

addition, we have observed cytoplasmic streaming in all cell types, including extending root hairs, when plants were mounted in PFD.

The physiological impact of PFD on plants was therefore minimal when applied for a period of hours rather than days. This lack of deleterious effects may be explained by the exceptional O_2- and CO_2-carrying capacities of PFD, which readily permit gas exchange between tissues immersed in PFD and the medium.

Discussion

Confocal microscopy is an essential tool in understanding plant cell biology. However, the resolving power of the confocal microscope is reduced when imaging through several cell layers or when the tissue has an architecture that makes it differentially refractive, as is the case for the mesophyll of plant leaves or cotyledons. We have identified a novel application of the nontoxic perfluorocarbon, PFD, in improving confocal image resolution in *Arabidopsis* mesophyll. PFD is nonfluorescent and, because of its low surface tension, readily infiltrated plant leaves through the stomata. By mounting specimens of leaf in PFD, we considerably attenuated the problem of deleterious optical effects generated in the mesophyll and, in so doing, significantly increased the depth of confocal image acquisition of the ×63 objective lens. This increase in depth penetration was achieved without increasing the power of the excitation laser. While the experiments described here concentrate on LSCM imaging, the use of PFD in multiphoton microscopy may increase further the depth penetration of that technique (Blancaflor & Gilroy, 2000; Feijó & Moreno, 2004; Inoue, 2006).

Leaves of plants immersed in water or in PFD showed similar F_v/F_m values as plants maintained in air over an 18 h period. By contrast, the same physiological measure taken from cotyledons showed that immersion in PFD progressively impaired photosynthesis, although to a much lesser extent than did immersion in water. F_v/F_m values for cotyledons in PFD remained above 0.6, whereas F_v/F_m values for cotyledons in water fell below that value after *c.* 4 h. Germination and the early development of *A. thaliana* seedlings were also unaffected by immersion in PFD, although seedlings were unable to expand true leaves. It therefore appears that PFD has a minimal impact on plant physiology, especially when applied over the period of hours that is normal for microscopic imaging.

High-resolution, *in vivo* imaging has been instrumental in furthering our understanding of the physiology of various plant cell types such as stomatal guard cells, root hairs, pollen tubes, callus cells and algal zygotes (Gilroy & Jones, 1992; Berger & Brownlee, 1993; Allen *et al.*, 1999; Cutler & Ehrhardt, 2000; Monshausen *et al.*, 2007; Bove *et al.*, 2008; Poulter *et al.*, 2008). Owing to the technical difficulties of imaging deep inside tissues, especially highly autofluorescent tissues such as the leaf (Berg & Beachy, 2008), *in vivo* imaging has played a much lesser role in understanding the biology of parenchyma, compared with the more accessible cell types at the surface of the plant. Parenchymal cells perform critical functions, including: photosynthesis in the mesophyll; storage of starch, oil or protein in tubers, the seed endosperm or in cotyledons; nectar secretion; secondary meristematic development; and mechanical support. Moreover, parenchymal cells in leaves, stems or roots are prime targets for fungal and bacterial infection, which alone have been estimated to reduce crop yields by 15% (Oerke & Dehne, 2004). PFD significantly improves imaging of spongy mesophyll, allowing more precise imaging in this critical tissue. Mounting in PFD rather than in water did not improve the optical properties of roots, carpels, anthers or female gametophytes, which may be attributable to the lack of intercellular air spaces in these tissues, but PFD may confer a physiological advantage over water in these tissues, as it does in leaves. Numerous fluorescent markers are now available for imaging plant cell components, structures, proteins and metabolites (Fricker & Meyer, 2001; Deuschle *et al.*, 2005; Dixit *et al.*, 2006). Using PFD to enable high-resolution LSCM imaging deeper into spongy mesophyll than hitherto achievable will provide new insight into the development, differentiation and physiology of critical plant cell types within organs, and help refine our understanding of the process of pathogen invasion and survival within these tissues.

Conclusion

Perfluorodecalin has significant advantages as a mounting medium for *in vivo* LSCM of air-filled plant tissues, most notably the increase in *z*-axis resolution without a concomitant increase in excitation intensity that may damage cells. PFD is nonfluorescent, readily applied and has minimal physiological impact on the mounted specimen. We anticipate that the use of PFD as a mounting medium will increase the resolving potential of LSCM in tissues that are rich in air spaces and thus facilitate new discoveries.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 *In vivo* imaging of intact *Arabidopsis thaliana* hypocotyls and ovules, mounted in water or in perfluorodecalin (PFD).

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