Microbiology (2013), **159**, 107–111 DOI 10.1099/mic.0.062711-0

DNA unmasked in the red rain cells of Kerala

Rajkumar Gangappa and Stuart I. Hogg

Department of Science and Sport, University of Glamorgan, Pontypridd CF37 4AT, UK

Correspondence
Stuart Hogg
sihogg@glam.ac.uk

Received 3 August 2012
Revised 1 November 2012
Accepted 20 November 2012

Extraordinary claims have been made for the biological properties of the red rain cells of Kerala, including a suggestion that they lack DNA. We have investigated the fluorescence properties of red rain cells, and the solubility of the red pigment in a variety of solvents. Extraction of the pigment with DMSO allowed successful demonstration of DNA using DAPI staining. Cellular impermeability to staining reagents due to the red pigment is the likely explanation for the failure of previous efforts to demonstrate DNA in red rain cells.

INTRODUCTION

For a total of three months in the summer of 2001, coloured rain fell intermittently over Kerala, Southern India. The rain was characterized by red pigmented particles that were initially characterized as *Trentepohlia* algae, fungal spores, desert sand or even red blood cells, but were shown to have structural features consistent with bacterial spores (Louis & Kumar, 2006). The inability of those authors to detect DNA by conventional staining methods prompted them to suggest that the red rain cells might have an extraterrestrial origin. This claim has stimulated much discussion in the popular press and online forums, but no investigations confirming or refuting their findings have subsequently been published in peer-reviewed journals. In the absence of further evidence, no satisfactory explanation has been proposed for the failure to detect DNA. Unpublished results reported by DiGregorio (2007) suggested that the red pigment of these cells was insoluble in many organic solvents. This has led us to propose that the negative outcome of the staining experiments of Louis & Kumar (2006) could be due to an impermeability of the peripheral cellular envelope to chemical reagents. In this study, we investigated the nature of the pigmentation of red rain cells and its possible role in the exclusion of stains such as DAPI from the cell's interior.

METHODS

Red rain cell samples. Samples of red rain were obtained from Professor M. Burchell, School of Physical Sciences, University of Kent, UK.

Bright-field, differential interference contrast (DIC) and polarizing light microscopy. Washed red rain cells were resuspended in DMSO, acetone, butanol, chloroform, ethanol, ethyl acetate/methanol (1 : 1) and concentrated HCl or H_2SO_4, and incubated for 20 min at room temperature. Treated and untreated cells were then viewed at $\times 1000$ magnification by using bright-field, DIC and polarizing optics.

Analysis of fluorescent properties. Red rain samples were analysed for fluorescence emission properties in red, green and blue regions at excitation wavelengths of 405, 458, 488 and 543 nm by using confocal scanning laser microscopy (CSLM) (Leica TCS SP2 AOBS).

DMSO-treated cells were also viewed by using phase-contrast fluorescence microscopy (Axiovert 10, Zeiss) under filters with excitation and emission wavelengths as shown in Table 1.

Staining tests. Red rain cells were centrifuged at 13 000 ***g*** for 10 min, and the precipitate was washed twice in double-distilled water (ddH_2O). The washed pellet was suspended in 300 µl ddH_2O or 99 % DMSO and incubated at room temperature for 15–20 min. Cells were further washed and resuspended in ddH_2O. Samples (20 µl) were heat-fixed and stained according to the procedures below.

Gram stain. Heat-fixed samples were stained with ammonium oxalate crystal violet for 30 s, washed off with ddH_2O and flooded with Lugol's iodine for a further 30 s and washed again with ddH_2O. Decolourization with 95 % ethanol was followed by counterstaining with neutral red for 30 s. Counterstained cells were washed with ddH_2O and air-dried.

Acid-fast stain. Heat-fixed cells were flooded with Much stain (phenol/methyl violet), washed with ddH_2O and treated with Lugol's iodine for 5 min. Samples were decolourized with 95 % ethanol for 1 min, washed and counterstained with neutral red for 1 min.

Spore-specific staining (Schaeffer and Fulton method). Heat-fixed samples were flooded with 5 % aqueous malachite green and heated until steaming for 5 min. Slides were then washed with ddH_2O and counterstained with 1 % neutral red for 30 s.

All the above stained cell samples were viewed and analysed with the bright-field mode of a Leica TCS SP2 AOBS spectral confocal microscope.

DAPI staining. Both the cell suspensions prepared as above were heat-fixed at 50 °C for 15 min and covered with 50 µl DAPI solution (3 µg ml^{-1}). Preparations were incubated for 10 min at room temperature in the dark, then rinsed twice in PBS. Excess liquid was drained off from the slide and samples were allowed to dry in the dark. Cells were then viewed in the transmitted light mode and

Abbreviations: CFP, cyan fluorescent protein; CSLM, confocal scanning laser microscopy; ddH_2O, double-distilled water; DIC, differential interference contrast; ET-DsRed, red fluorescent protein; YFP, yellow fluorescent protein.

Table 1. Excitation and emission wavelengths of filters used in fluorescence microscopy

Filter	Excitation (nm)	Emission (nm)
GFP	470/40	525/50
ET-DsRed	545/25	605/70
YFP	450/490	520
CFP	395/440	470

excited at 405 nm for DNA-DAPI specific fluorescence emission (461 nm) using a Leica TCS SP2 AOBS spectral confocal microscope.

RESULTS AND DISCUSSION

Morphology

Red rain cells were confirmed by bright-field microscopy to have the appearance of red, non-motile, unicellular micro-organisms (Fig. 1a). The red pigment fills the entire cell, masking any internal morphology; however, the red colour of the outer envelope is more intense than the central core, suggesting a maximum concentration of pigment within this region (Fig. 1b, c). The cell highlighted in Fig. 1(a) may represent the remains of a cell after the loss of its internal contents. Subsequent analysis by DIC optics revealed an orange-yellow birefringence in the external thick layer of the cells (Fig. 1b), which may be attributed to the presence of crystalline substructures in the cellular periphery. The birefringence appears to be arranged in four characteristic packets inside the external thick layer surrounding the central core (Fig. 1b), separated by a space of nearly 0.5 μm in size. Each packet measures 1.25 μm wide and some 4–5 μm in length, occupying a large portion of the cell volume. Additional observations made by polarizing microscopy for the determination of outer layer birefringence were consistent with the DIC images. The birefringent features observed under polarizing light optics suggest the presence of anisotropic materials in the outer coating of these cells. Fig. 1(c) shows an orange-yellow polarization colour, corresponding on the Michel Levy chart to the value for dolomite [calcium magnesium carbonate, $CaMg(CO_3)_2$]. Further analysis of red rain cell morphology by electron microscopy will be discussed elsewhere.

Solubility of red pigment

Red rain cells were treated in various organic solvents in an effort to ascertain the solubility of the red pigmentation. With the exception of DMSO, none of the solvents employed had any noticeable effect on the red cells, which retained their red coloration and refractivity to light. Cells treated with DMSO, in contrast, lost all red pigmentation and became transparent (Fig. 1d).

Under DIC and polarizing optics (Fig. 1e, f), the DMSO-treated red rain cells are viewed as highly resolved phase-contrast images. The absence of any birefringence or anisotropic properties from the outer envelope of DMSO-treated cells when compared with untreated red rain cells is attributed to the complete removal of the red pigmentation.

Untreated red rain cells are refractive to high-energy radiation

The effect of extraction with DMSO can also be seen by comparing the transmitted light images of untreated (Fig. 2a–d) and DMSO-treated (Fig. 2e–h) cells.

At excitation wavelengths of 405, 458 and 488 nm, untreated red rain cells displayed no fluorescence in the red, green or blue region, whilst at 543 nm they showed fluorescence in the red region only (Fig. 2d). Results with DMSO-treated red rain cells contrasted strongly with these. With excitation at 405 and 458 nm, fluorescence emission was seen in the red and green regions, and none in the blue region (Fig. 2e–f). Excitation of treated cells at 488 and 543 nm, respectively, resulted in strong fluorescence in both red and green regions, and in the red region only (Fig. 2g, h). The fluorescence emission appeared to be limited to the central core of the cells. Subsequent analysis by fluorescence microscopy revealed a similar fluorescence emission pattern.

Red rain cells suspended in DMSO showed fluorescence when observed through all excitation filters, suggesting the presence of heterogeneous compounds within the core region (Fig. 3). Those analysed under the red fluorescent protein (ET-DsRed) filter displayed additional fluorescence in the external envelope. As highlighted by the arrow in Fig. 3, the translucent matrix masking the cells is brightly fluorescent under all excitation filters. This translucent matrix may be the internal content of the red rain cells, which has possibly leaked out due to the weakening of outer cell layers caused by DMSO. The fluorescence at a wide range of emission wavelengths suggests the presence of an ensemble of heterogeneous compounds.

Though the molecular properties and role of red pigmentation and inner content of these cells remain to be determined, the results presented here clearly demonstrate that the greater proportion of pigments that give these cells their colour is located in their outer thick envelope. The resistance of untreated cells to high-energy radiation and organic solvents clearly suggests a role for the red pigments in the protection of the central core, facilitating resistance to hostile conditions. Therefore, we propose that the removal of these red compounds from the external layers could enhance their permeability to chemical reagents, allowing DAPI and other stains to gain access to the targeted areas of the interior of these cells.

Extraction of red pigment with DMSO allows permeability to staining reagents

Attempts were made to stain the red rain cells by a variety of standard methods. Heat-fixed cells stained by using

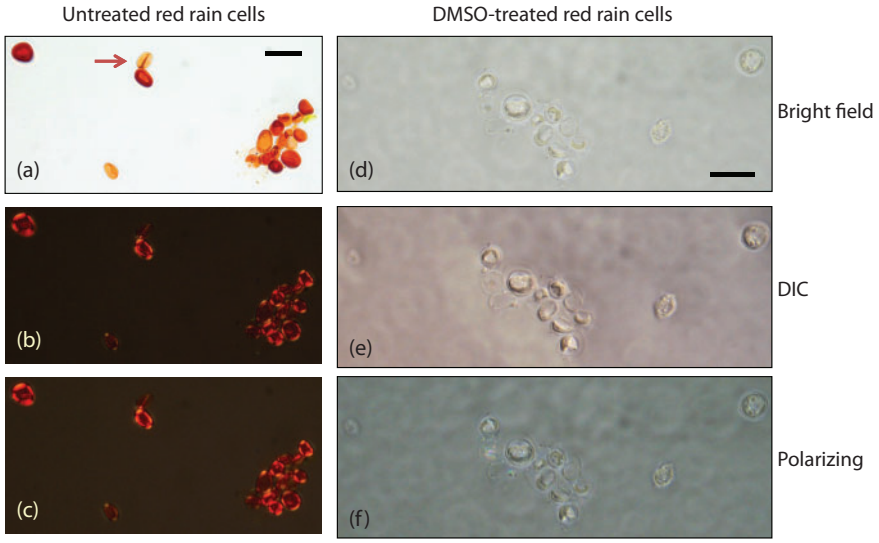

Fig. 1. Analysis of untreated and DMSO-treated red rain cells by bright-field, DIC and polarizing microscopy. The arrow in (a) points to an empty external outer sheath of a red rain cell. Bars, 10 μm.

Gram, acid-fast and spore-specific staining appeared red, with an intensity similar to that of unstained controls (Fig. 4a–c). In some cases there appeared to be an accumulation of staining reagents on the periphery of the cells, suggesting that they were unable to penetrate further. However, DMSO-treated red rain cells, when subjected to similar staining tests, showed intense staining in their outer envelope and throughout the cytoplasm (Fig. 4d–f). The general morphology of these cells remained similar to that of untreated cells. The cells showed a positive reaction for both the Gram and acid-fast stains (Fig. 4d, e); however, spore-specific staining was ambiguous, with some cells appearing blue, while others stained red (Fig. 4f). Spore staining was used in view of the morphological similarities

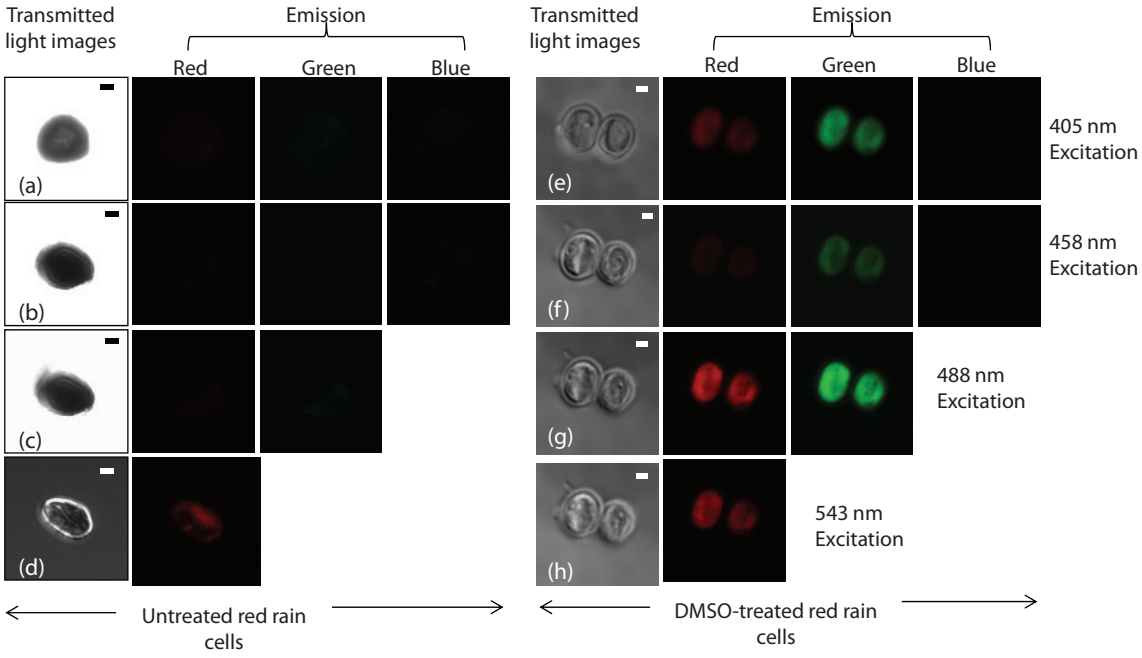

Fig. 2. Fluorescence emission of untreated red rain cells (a–d) and DMSO-treated red rain cells (e–h) in the red, green and blue regions, and their corresponding excitation wavelengths. Greyscale figures are transmitted light images. Bars, 1 μm.

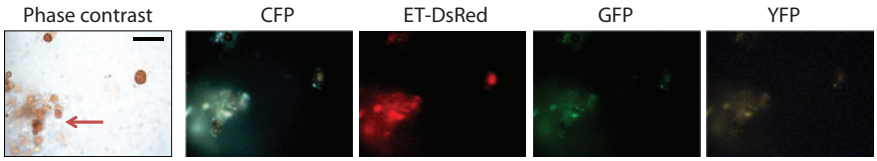

Fig. 3. Phase-contrast micrographs of red rain cells suspended in DMSO and their corresponding fluorescence emission under excitation filters [cyan fluorescent protein (CFP), ET-DsRed, GFP and yellow fluorescent protein (YFP)]. The arrow points to cells surrounded by invisible matrix, which appears to be their leaked inner contents fluorescing under all excitation filters. Bar, 10 μm.

of red rain cells to bacterial spores; however, there is insufficient evidence to confirm this identity. The DAPI staining method adopted in the present study was based on one that has been successfully applied to bacterial endospores (Schichnes *et al.*, 2006), but was found to give negative results with untreated red rain cells in the present study, a fact that may be attributable to the impermeability of their intensely pigmented outer layer.

When untreated red rain cells were subjected to DAPI staining and observed by transmitted light, they appeared completely opaque (Fig. 5a, b). DMSO-treated cells stained with DAPI, however, showed a transparent outer envelope and opaque central core (Fig. 5c, d), suggestive of DAPI penetration into the core. When exposed to 405 nm radiation, DAPI-stained untreated cells showed no fluorescence (Fig. 5a, b), whereas the central core of those treated with DMSO showed fluorescence emission in the blue region at 461 nm (Fig. 5c, d). This is consistent with DAPI–DNA complex specific fluorescence (Banerjee & Pal, 2008; Laflamme *et al.*, 2004; Tanious *et al.*, 1992; Setlow *et al.*, 1991; Hamada & Fujita, 1983), indicating the presence of DNA in the core of red rain cells. Certain nanoparticles fluorescing in blue are also apparent near the periphery of these cells (Fig. 5d). These could represent material that has leaked from the central core as an artefact of cell preparation.

Although the idea of enhancing the permeability of the outer envelope to various chemical solvents and staining reagents is well established for spores of bacteria (Denyer & Maillard, 2002; McDonnell & Russell, 1999; Russell, 1990), fungi (Kelly & Gay, 1969) and algae (Gunnison & Alexander, 1975), the use of DMSO in this investigation to increase the permeability of red rain cells to staining reagents represents a novel procedure for demonstrating the presence of DNA in these cells and facilitating their characterization by other staining methods.

We report results showing the location of a red pigment in the external layers of red rain cells and its apparent role in the impermeability to various staining reagents, including DAPI. We also show that extraction of this pigment from

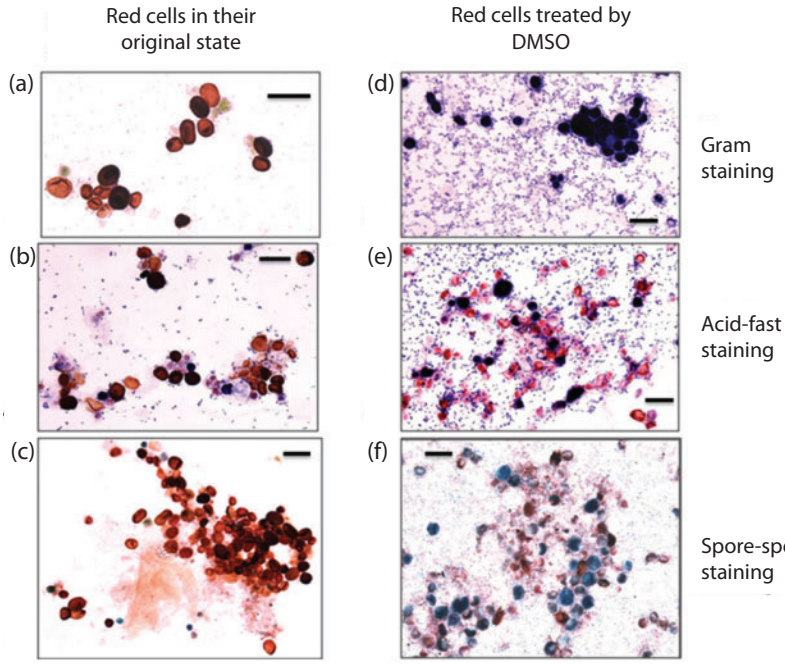

Fig. 4. Analysis of untreated red rain cells (a–c) and DMSO-treated red rain cells (d–f) by Gram, acid-fast and spore staining. Bars, 10 μm.

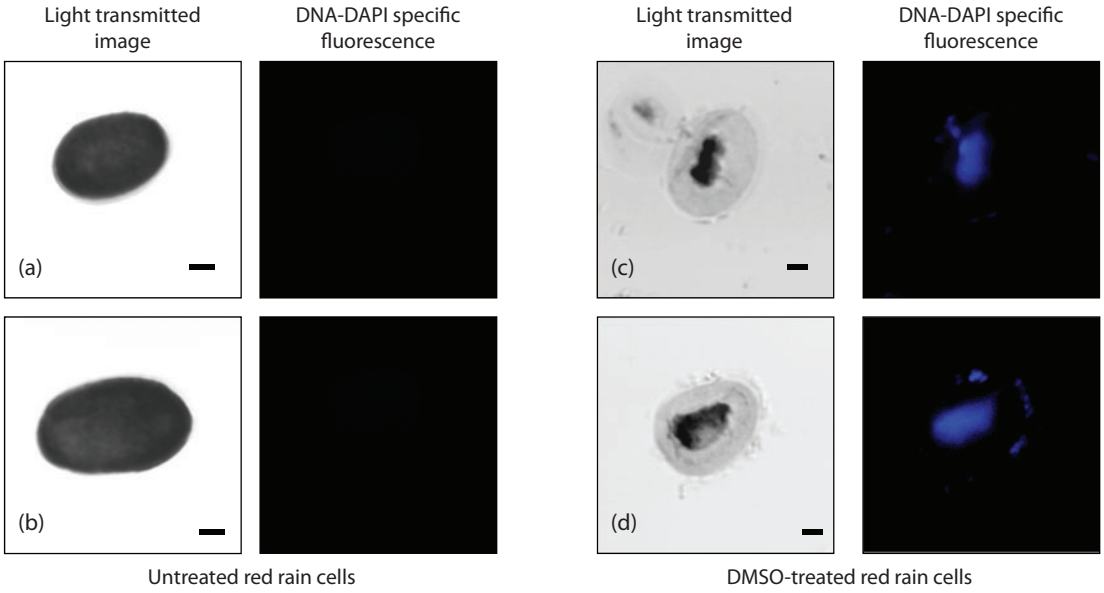

Fig. 5. DAPI staining of untreated red rain cells (a, b) and DMSO-treated red rain cells (c, d). Images of light transmitted and the corresponding DAPI-DNA specific fluorescence emission upon 405 nm irradiation are shown. Bars, 1 μm.

the outer layer with DMSO enhances the permeability of red rain cells to chemical stains, allowing positive DAPI staining, indicating the presence of DNA in the central core. Results based on polarizing microscopy indicate the crystalline nature of the red pigment in the peripheral layers. It is also evident that the removal of red pigment allows these cells to fluoresce at a wide range of excitation wavelengths, whilst untreated cells are completely refractive. Although the nature of the red compounds is still uncertain, it is clear from this investigation that these compounds firmly intercalate deep into the thick outer layers of these cells, which may facilitate resistance to hostile environments. The results presented here do not make the origin of the red rain cells of Kerala any clearer, but do clearly indicate the presence of DNA, and offer an explanation for the negative staining results reported by Louis & Kumar (2006).

ACKNOWLEDGEMENTS

We are grateful to Professor M. Burchell (School of Physical Sciences, University of Kent, UK) for providing red rain cell samples for this investigation, and to Professor A. K. Campbell (School of Pharmacy, Cardiff University, UK) for his assistance in fluorescence microscopy work. R. G. was in receipt of a scholarship from the Social Welfare Department of Bangalore, Karnataka, India.

REFERENCES

Banerjee, D. & Pal, S. K. (2008). Dynamics in the DNA recognition by DAPI: exploration of the various binding modes. *J Phys Chem B* **112**, 1016–1021.

Denyer, S. P. & Maillard, J.-Y. (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *J Appl Microbiol* **92** (Suppl. S1), 35S–45S.

DiGregorio, B. E. (2007). What made the rain red in India? Isotopic analysis points to a terrestrial origin for the unusual organic particles that colored the rain like blood. *Anal Chem* **79**, 3238.

Gunnison, D. & Alexander, M. (1975). Basis for the resistance of several algae to microbial decomposition. *Appl Microbiol* **29**, 729–738.

Hamada, S. & Fujita, S. (1983). DAPI staining improved for quantitative cytofluorometry. *Histochemistry* **79**, 219–226.

Kelly, M. S. & Gay, J. L. (1969). The basis of differential staining of ascospores and vegetative cells of yeasts. *Arch Mikrobiol* **66**, 1–13.

Laflamme, C., Lavigne, S., Ho, J. & Duchaine, C. (2004). Assessment of bacterial endospore viability with fluorescent dyes. *J Appl Microbiol* **96**, 684–692.

Louis, G. & Kumar, A. S. (2006). The red rain phenomenon of Kerala and its possible extraterrestrial origin. *Astrophys Space Sci* **302**, 175–187.

McDonnell, G. & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* **12**, 147–179.

Russell, A. D. (1990). Bacterial spores and chemical sporicidal agents. *Clin Microbiol Rev* **3**, 99–119.

Schichnes, D., Nemson, J. A. & Ruzin, S. E. (2006). Fluorescent staining method for bacterial endospores. *Microscope* **54**, 91–93.

Setlow, B., Magill, N., Febbroriello, P., Nakhimovsky, L., Koppel, D. E. & Setlow, P. (1991). Condensation of the forespore nucleoid early in sporulation of *Bacillus* species. *J Bacteriol* **173**, 6270–6278.

Tanious, F. A., Veal, J. M., Buczak, H., Ratmeyer, L. S. & Wilson, W. D. (1992). DAPI (4′,6-diamidino-2-phenylindole) binds differently to DNA and RNA: minor-groove binding at AT sites and intercalation at AU sites. *Biochemistry* **31**, 3103–3112.

Edited by: C.-C. Zhang