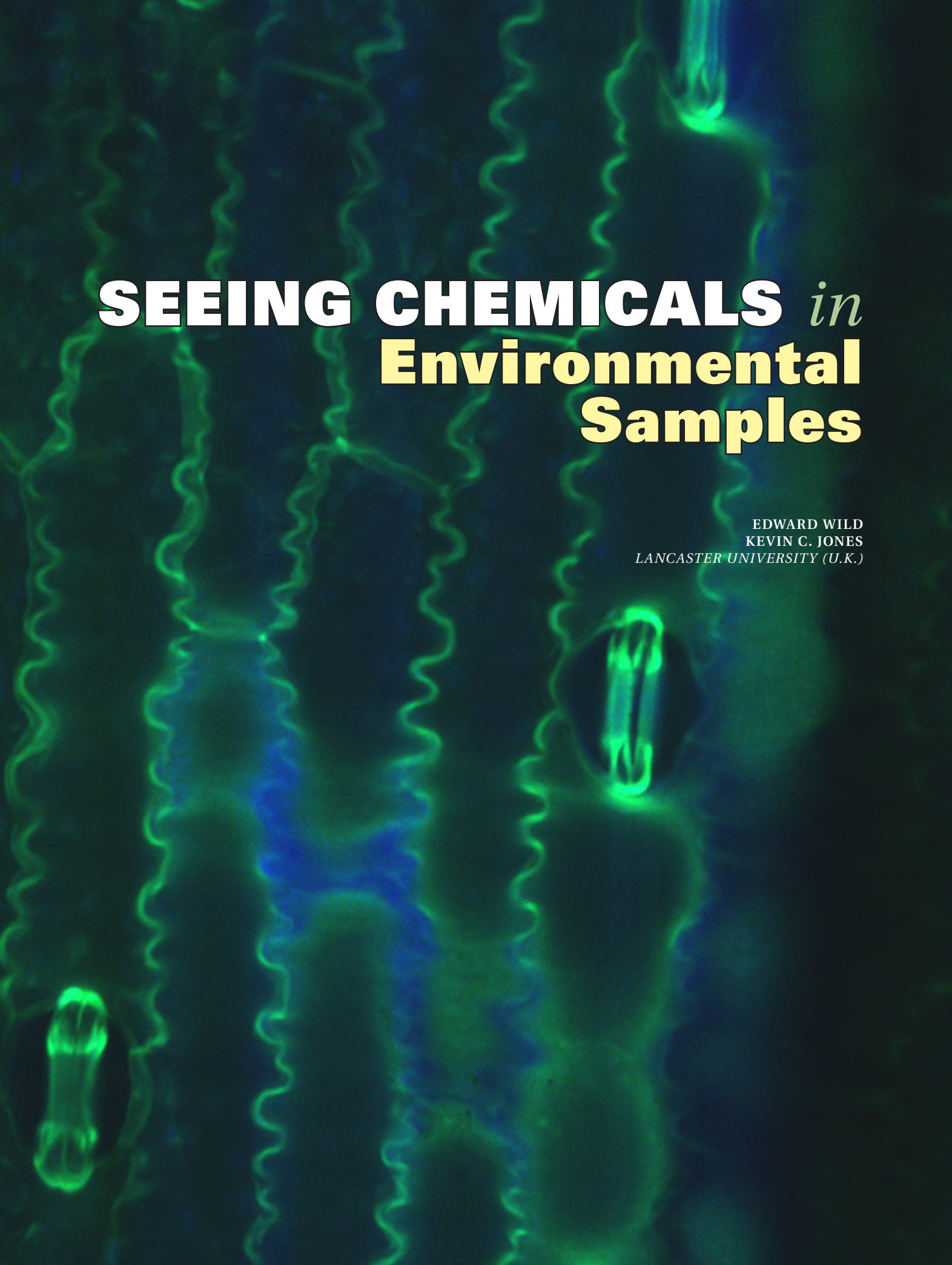
Seeing chemicals in environmental samples

ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · OCTOBER 2007

Impact Factor: 5.33 · DOI: 10.1021/es0726005 · Source: PubMed



SEEING CHEMICALS *in* Environmental Samples

EDWARD WILD
KEVIN C. JONES
LANCASTER UNIVERSITY (U.K.)

New techniques developed to visualize and track chemicals in plants can lead to improved process-based understanding and management of agrochemicals and to applications in other environmental fields.

Modern environmental chemistry relies on the destructive chemical extraction of analytes from samples to determine compound concentrations. However, environmental chemists and toxicologists are frequently interested in *where* the analyte resides within the sample. Are chemicals on surfaces, in matrices, or associated with particular phases or subcellular compartments? This knowledge determines a chemical's environmental significance by controlling its form, bioavailability, mobility, toxicity, and persistence. Unfortunately, sample extractions—at best—only allow inferences to be made about these issues (*1*). It has long been a goal for scientists to "see" chemicals in situ, in real time, in and on environmental surfaces and in matrices such as living plants and other organisms. This would improve mechanistic and process-based understanding and help scientists optimize the assessment and management of environmentally relevant chemicals. Now, for the first time, exciting developments in microscopy and imaging are helping to make in situ visualization of chemicals possible. We describe here how the new technique—two-photon excitation microscopy coupled with autofluorescence (TPEM-AF)—works, and how we initially developed and used it to directly observe the uptake, transport, storage, and degradation of PAHs in the cells and subcellular structures of intact living plants. We then go on to highlight some exciting new applications and speculate about possible future ones.

TPEM—what is it, and how does it work?

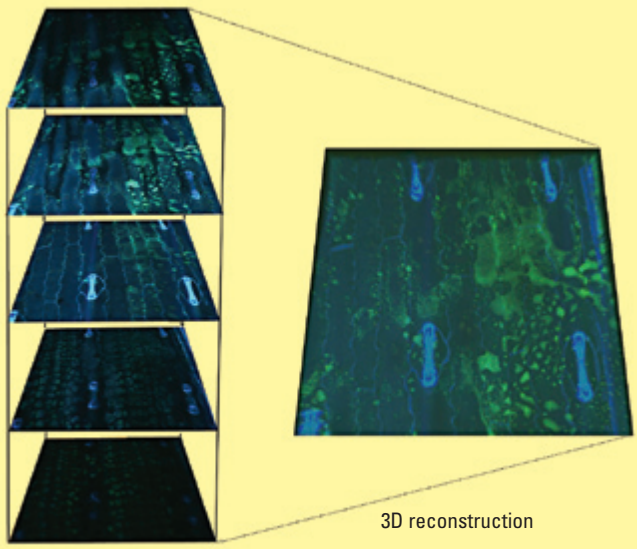
TPEM is a form of laser-scanning microscopy (*2*, *3*). It uses a pulsed-scanning laser to excite fluorophores within a sample. This creates fluorescence that is detected and used to produce an image. The sample is only excited at the focal point, where two low-energy photons simultaneously combine on a femtosecond timescale and in submicrometer volume. This produces a single, thin (e.g., <0.4 μm) optical section; a series of sections can then be made through the sample, by systematically adjusting the position of the focal point. Multiple optical sections can be generated and combined to give a 3D image (see Figure 1).

TPEM has traditionally been used in the medical and plant sciences, where it relies on the use of fluorescent probes, dyes, or markers, along with the real-time visualization of dye-marked constituents, to reveal the dynamics of interacting cells (*2*, *3*). TPEM has been widely used in combination with enhanced green–red fluorescent protein transgenic organisms (*4*, *5*), including mice embryos, to look at whole-tissue function in living systems. Examples are studies on neural activity deep within living brain tissues as well as lymphocyte mobility and antigen responses within intact lymph nodes (*4*–*6*). The technique can also be used to monitor specific cellular processes, including gene expressions and protein folding (*7*), or for tagging cellular organelles, including nuclei (*8*), plastids, mitochondria, and endoplasmic reticula (ER) (*9*). Specific fluorescent dyes and markers are commonly used with plants and animals to identify cellular structures, for instance, nucleic acid tagging for chromosome or DNA visualization using 4,6-diamidino-2-phenylindole hydrochloride (*10*) or propidium iodide for cell walls, particularly those of *Arabidopsis* roots (*11*), and carbocyanine dye for labeling ER and mitochondria in plant cells. Numerous specific fluorescent dyes also have been developed to

FIGURE 1

3D reconstruction

The schematic shows how multiple optical sections made through a sample can be combined to produce a 3D reconstruction. A fungicide (green) is depicted at the surface of a maize leaf (blue).



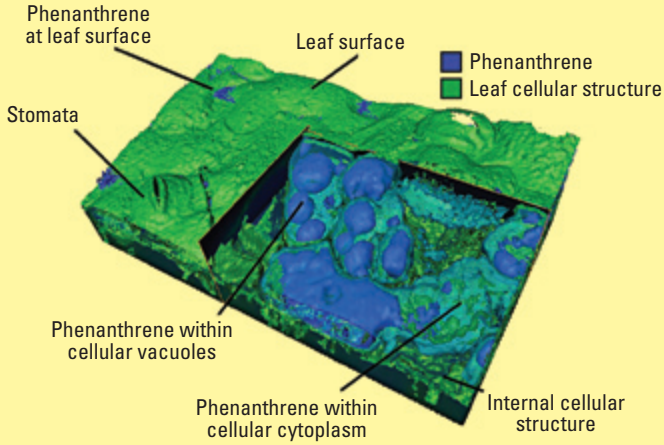
EDWARD WILD AND JOHN DENT

visualize changes in the free concentration of cellular ions (e.g., Ca^{2+}, H^{+}, Mg^{2+}, K^{+}, Cl^{-}, Zn^{2+}) within living cells; these include Indo-1, which is used for looking at changes in cellular Ca^{2+} concentrations (*12*, *13*). Traditionally, organic compounds with fluorescent properties have been widely used. However, new, more-specific compounds, including water-soluble quantum dots recently developed for use as fluorescent probes, are becoming increasingly common when bright fluorescence is required from very low level laser excitation (*14*).

FIGURE 2

Spinach leaf exposed to phenanthrene

A 3D reconstruction of a spinach leaf after 12 days of exposure to an atmosphere contaminated with phenanthrene. Phenanthrene can be observed within the cuticle at the leaf surface. Where the leaf surface has been cut away, phenanthrene can be observed within the cellular cytoplasm and vacuoles of the epidermal cells. This image was generated from 255 separate optical sections made through a living leaf.



The use of fluorescent probes can be problematic if knowledge of chemical-specific behavior within a living system is needed, because applying fluorescent dyes, probes, or markers alters the natural state of the chemical and sample and modifies the way it behaves. Tagged or labeled molecules inevitably will behave differently from nontagged ones. The recent breakthrough with TPEM-AF was to combine TPEM with the natural autofluorescence of the living matrix and compound of interest to visualize their interactions in an unmodified state. This has enabled xenobiotics and plant cellular structures—such as the leaf cuticle, cell wall, and chloroplast—to be identified simultaneously. Each chemical or plant component has unique molecular arrangements that yield distinct fluorescence signals detectable at specific wavelengths. Chemicals that fluoresce can be identified separately from plant cellular structures at specific wavelengths and superimposed or integrated into a single image. For the first time, it is possible to "see" xenobiotics within living cells or associated with subcellular structures within whole, living, unmodified plants. Figure 1 illustrates this approach by depicting a fungicide at the surface of a maize leaf.

Practical considerations

TPEM-AF can be used as a purely qualitative tool to see just a chemical's location. However, it is often useful to have an indication of the mass of the target compound visualized in the sample and to assess the amounts freely dispersed or aggregated. This can be done if known amounts of the compound are added to the experimental system or spiked onto control microscope slides. A calibration of the intensity of the fluorescence signal with compound mass can then be performed, so that the mass present in samples can be estimated. The technique benefits from being extremely sensitive; fluorescing PAHs have been detected in plants in the picogram range, for example, close to ambient levels.

The sample matrix may give a background signal at a particular wavelength that is so high that the S/N at that wavelength hampers detection. Nonetheless, if the fluorescence spectra of the compound of interest and the matrix are scanned, nonoptimal wavelengths can be selected to give a clear distinction between them. This still allows routine detection and imaging. However, one practical constraint concerns the depth of laser penetration and visualization. This is on the order of hundreds of micrometers and depends on the sample type. For example, when living leaves or roots are studied, imaging can be done through multiple cellular layers from the surface, but perhaps not through older or thicker materials. Another limitation concerns sample matrices with variable autofluorescence. For example, soil organic matter gives such intense fluorescence across a broad range of wavelengths that distinguishing a target molecule above the background may not be possible.

PAHs in vegetation

Our TPEM-AF work started with a broad interest in the role of vegetation in the global cycling of persistent organic pollutants (POPs). Several studies pointed to the role of vegetation in the POPs cycle—scavenging airborne compounds, influencing their air–surface exchange, storage, compound degradation, and phytoremediation (*1*). However, underlying these processes are fundamental uncertainties about compound uptake kinetics by plants, whether uptake is air-side- or plant-side-limited, and plants' storage locations and capacities. For modeling purposes, POPs are usually assumed to be distributed in plant lipids, dominated by the surface cuticle and wax layers (*1*). Models that assume one, two, or more compartments in leaves have all been applied, indicating mechanistic uncertainties remain. We reasoned that if there were a way to locate compounds in leaves, we would be able to better understand whether a deposited compound could readily be reemitted to the atmosphere, photodegraded, or metabolized. However, the only approach available at the time seemed to be simple dissolution of the leaf—by dipping it in solvents or harsher extractions—or isolation of individual plant parts, such

as the cuticle, by enzymolysis, for spiking studies. These methodologies would substantially alter the plant constituents.

We used PAHs as the target analytes for the initial TPEM-AF work. They fluoresce strongly—indeed, fluorescence spectroscopy is an accepted and popular form of conventional analysis for PAHs. Initial work characterized different plant species and optimized plant autofluorescence procedures. Use of low laser intensities meant that plants were not damaged, and chemical uptake and exposure could be monitored in the same specimens over many days or weeks. Experiments were conducted on the rates of migration of surface-applied compounds into treated leaves (*15*). Information on rates of penetration into the leaf is vital for assessment of uptake kinetics and likely equilibrium status (and is an important parameter in determining herbicide and fungicide efficacy).

TPEM-AF showed that PAHs did not just stay in leaf-surface lipids. They migrated into the inner leaf and—in some species—accumulated within the aqueous milieu of the cell vacuole (*15*, *16*). Compound fate was influenced strongly by location; it would be relatively protected from photodegradation if it could reach the leaf subsurface (*17*). Figure 2 highlights the level of detail possible with the technique. It clearly shows that phenanthrene is distributed in different portions of the leaf. A particularly fascinating observation was that the compound clusters in the surface waxes and also becomes focused before metabolic degradation in parts of the root (*18*). Most importantly, different plants store and process PAHs in different ways (*16*). Insights into the fundamental mechanisms involved may help improve phytoremediation strategies and lead to better models of compounds in vegetation. Other examples of compound imaging in plants are available on our website (www.lec.lancs.ac.uk/ccm/research/visualisation).

Practical challenges in using PPPs

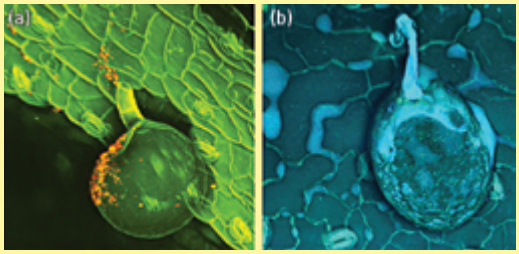
Knowledge of how and where chemicals move into plants and their sites of storage, metabolism, and action is fundamental to the efficient and targeted use of plant protection products (PPPs)—a generic term for fungicides, herbicides, insecticides, and so on. Simultaneous visualization of the crop, the pest (e.g., a fungus), and the active chemical ingredient clearly would be a major breakthrough for TPEM-AF. This could lead to more targeted, mechanistically based PPP use.

The active ingredients of PPPs are usually mixed into a formulation (containing surfactants, for example) intended to help deliver the product efficiently to the target. However, this mixing can be a rather black art. We wondered whether TPEM-AF could help make this a more systematic science. To evaluate the use of AF for such compounds, we screened a range of active ingredients and proprietary products. The success rate in detecting such compounds was 60%. As Figure 3 illustrates, crops, their fungal infection, and their complex interactions with contact and systemic fungicides can be visualized simultaneously. Differences in the efficacy of systemic and contact fungicides have been observed directly, and the influence of fungicide formulation in delivering the active ingredient to the site of action has also been shown. In short, these techniques may help improve PPP development, efficacy, and management. However, one major challenge is that—whereas PAHs are model fluorescing compounds—many PPPs are not usually monitored in this way.

FIGURE 3

Simultaneous visualization

Fungal infection and fungicide activity at the surface of living spinach leaves are visualized simultaneously in 3D 2 hours after application. (a) Contact fungicide (red) interacts with a leaf and fungal spore simultaneously. (b) Systemic fungicide (blue) interacts with a fungal spore and leaf simultaneously.



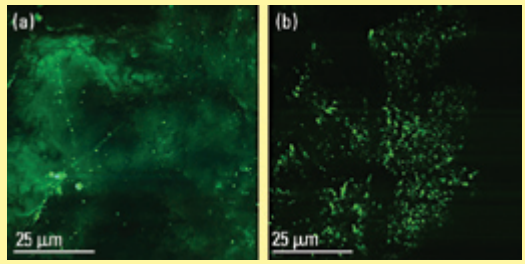
Migration and distribution of chemicals within materials

During our work on PAHs in plants, we made a fascinating observation: the chemicals appeared to form in clusters in the surface waxes. This goes against the way in which their distribution is usually considered and modeled. Nonpolar organics are generally assumed to disperse evenly throughout lipids (*19*). Initially, we hypothesized that such clustering may reflect variations in compound affinity for certain structural or compositional differences in the plant cuticles. After all, cuticles are not homogeneous structures. We decided to investigate this further, treating homogenized pure waxes with PAHs and monitoring them over time. In fact, these studies show compounds clustering with themselves, or with other similarly structured compounds, typically over periods of days or weeks (Figure 4). What are the implications if clusters occur in biota, particularly in target organs? What mechanisms are responsible? Should models be modified to account for these phenomena in plants and perhaps in other waxlike phases? For example, passive sampling techniques used for nonpolar organics in air and water rely on phase partitioning for preconcentration (e.g., solid-phase microextraction polymer-coated techniques [*20*, *21*]) and ignore intra- or intermolecular interactions. Clearly, TPEM-AF is a valuable tool in helping to elucidate these unexpected observations. Other practical examples of where TPEM-AF may be useful for looking inside media include compound distributions on solid phases (e.g., pharmaceuticals on powders and delivery media; diffusion

FIGURE 4

Chemical clustering within a wax phase over time

A chemical (green) shows (a) relatively diffuse distribution 1 hour after addition of molten wax to a slide and (b) more clustering after 25 days.



of compounds, including sunscreens and cosmetics, through skin; and compound diffusion in and out of food packaging materials).

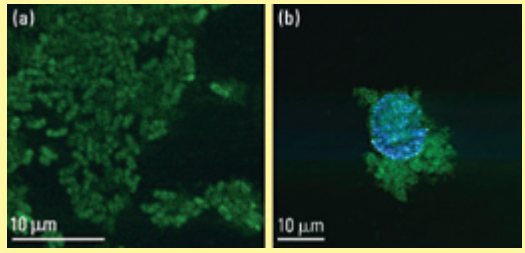
Visualizing bacteria and their interactions with chemicals

Recent research has centered on visualizing the in situ behavior of compounds in environmental systems and with bacteria. We anticipate potential applications to study pollutant degradation, biofilm formation, and pre- and postproduction food contamination. Figure 5 gives an example in which in situ degradation of phenanthrene by *Pseudomonas* was visualized, with the bacteria forming aggregates and biofilms, in real time (www.lec.lancs.ac.uk/ccm/research/visualisation).

FIGURE 5

Degradation of phenanthrene

(a) *Pseudomonas* bacteria (green) (b) forming around a crystal of phenanthrene after 5 days.



These examples provide a brief glimpse of the potential applications for this technique, showing how technology designed for applications within one discipline can have far-reaching implications and applications across other fields.

Edward Wild is a research associate at the Centre for Chemicals Management, Lancaster University (U.K.). Kevin C. Jones is a professor and director of the center. Address correspondence about this article to Wild at e.wild@lancaster.ac.uk.

Acknowledgments

We are grateful to John Dent for his excellent technical support and to the U.K. Natural Environment Research Council for its financial support through a Ph.D. studentship to Wild and for a Follow-on Fund research grant (NE/C506999/1). Chris Paynter and Kirk Semple kindly provided *Pseudomonas* strains.

References

(1) Barber, J. L.; et al. Current Issues and Uncertainties in the Measurement and Modelling of Air–Vegetation Exchange and Within-Plant Processing of POPs. *Environ. Pollut.* **2004**, *128*, 99–138.

(2) Piston, D. W. Imaging Living Cells and Tissues by Two-Photon Excitation Microscopy. *Trends Cell Biol.* **1999**, *9*, 66–69.

(3) Diaspro, A., Ed. *Confocal and Two-Photon Microscopy: Foundations, Applications and Advances*; Wiley-Liss: New York, 2002.

(4) Felter, L.; Amigorena, S. Brain Under Surveillance: The Microglia Patrol. *Science* **2005**, *309*, 392–393.

(5) Miller, M. J.; et al. Two-Photon Imaging of Lymphocyte Motility and Antigen Response in Intact Lymph Node. *Science* **2002**, *269*, 1869–1873.

(6) Bousso, P.; et al. Dynamics of Thymocyte-Stromal Cell Interactions Visualized by Two-Photon Microscopy. *Science* **2002**, *296*, 1876–1880.

(7) Tissen, R. Y. The Green Fluorescing Protein. *Ann. Rev. Biochem.* **1998**, *67*, 509–544.

(8) Chytilova, E.; Macas, J.; Galbraith, D. W. Green Fluorescing Protein Tagged to the Nucleus: A Transgenic Phenotype Useful for Studies in Plant Biology. *Ann. Bot.* **1999**, *83*, 645–654.

(9) Haseloff, J.; Siemering, K. R. The Use of GFP in Plants. In *Applications and Protocols*; Chalfie, M., Kain, S., Eds.; Wiley: New York, 2002; pp 191–220.

(10) Suzuki, T.; et al. DNA Staining for Fluorescence and Laser Confocal Microscopy. *J. Histochem. Cytochem.* **1997**, *45*, 49–53.

(11) Tirlapur, U. K.; König, K. Near-Infrared Femtosecond Laser Pulses as a Novel Non-Invasive Means for Dye-Permeation and 3D Imaging of Localized Dye-Coupling in the *Arabidopsis* Root Meristem. *Plant J.* **1999**, *20*, 363–370.

(12) Fricker, M. D.; et al. Fluorescence and Luminescence Techniques To Probe Ion Activities in Living Plant Cells. In *Fluorescence and Luminescent Probes*, 2nd ed. Mason, T. W., Ed.; Academic Press: New York, 1999; pp 569–596.

(13) Tirlapur, U. K.; König, K. Two-Photon Near-Infrared Femtosecond Laser Scanning Microscopy in Plant Biology. In *Confocal and Two-Photon Microscopy: Foundations, Applications and Advances*; Diaspro, A., Ed.; Wiley-Liss: New York, 2002; pp 449–468.

(14) Larson, D. R.; et al. Water-Soluble Quantum Dots for Multiphoton Fluorescence Imaging In Vivo. *Science* **2003**, *300*, 1434–1436.

(15) Wild, E.; et al. A Novel Analytical Approach for Visualizing and Tracking Organic Chemicals in Plants. *Environ. Sci. Technol.* **2004**, *38*, 4195–4199.

(16) Wild, E.; et al. Visualizing Air-To-Leaf Transfer and Within-Leaf Movement and Distribution of Phenanthrene: Further Studies Utilizing Two-Photon Excitation Microscopy. *Environ. Sci. Technol.* **2006**, *40*, 907–916.

(17) Wild, E.; et al. Real-Time Visualization and Quantification of PAH Photodegradation on and within Plant Leaves. *Environ. Sci. Technol.* **2005**, *39*, 268–273.

(18) Wild, E.; et al. Direct Observation of Organic Contaminant Uptake, Storage, and Metabolism within Plant Roots. *Environ. Sci. Technol.* **2005**, *39*, 3695–3702.

(19) Mackay, D. *Multimedia Environmental Models: The Fugacity Approach*, 2nd ed. CRC Press: Boca Raton, FL, 2001.

(20) Eisert, R.; Pawliszyn, J. New Trends in Solid-Phase Microextraction. *Crit. Rev. Anal. Chem.* **1997**, *27*, 103–135.

(21) Harner, T.; et al. Characterization of Polymer-Coated Glass as a Passive Air Sampler for Persistent Organic Pollutants. *Environ. Sci. Technol.* **2003**, *37*, 2486–2493.