The changing face of microscopy: Celebrating 15 Years of in**focus**

Remember these?: *The world of mobile phones looked much like this in 2006. The first iPhone (left, middle) came out the following year.*

Image courtesy: Rayson Tan on Unsplash

...It's March 2006 and the first ever issue of infocus **is hot off the presses. Facebook is about to open up its social network to the world – but no one will be using an iPhone to update their status for another year. The word 'twitter' merely relates to birdsong, and any zooming being done is strictly within the realms of speed and scale. In politics, President George W Bush is midway through his second term of office in the US, while the likes of Tony Blair, Jacques Chirac and Silvio Berlusconi still hold sway across Europe. But the winds of change are strengthening around the globe and even beyond; yes, the solar system is about to lose a planet, with poor old Pluto set for a downgrading...**

Out in the cold: *Pluto fans were dealt a hammer-blow in 2006, when the International Astronomical Union controversially downgraded it to 'dwarf planet' status.*

Image courtesy: https://www.goodfreephotos.com

For the birds: *In January 2006, the only tweeting that mattered to anyone was the restful sound of birdsong.*

Image courtesy: Tom Bradley on Unsplash

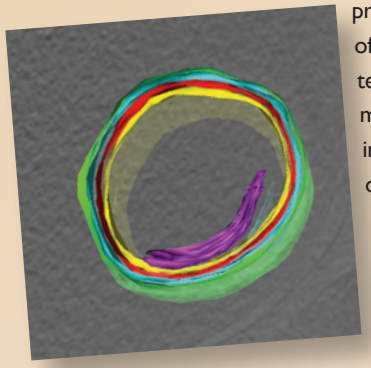
Fast forward 15 years and the world – including that of microscopy - is a very different place. Here, our infocus **Editorial Board Members highlight some of the most game-changing advances in microscopy since the first issue in 2006 – and explain how they have transformed research across various different fields.**

Political figures: US President George W. Bush and Italian Prime Minister Silvio Berlusconi were both serving their second terms of office when in**focus** was first issued in January 2006.

Image courtesy: Public Domain

Leandro Lemgruber – in**focus** Deputy Scientific Editor and Electron Microscopy representative

In the last 15 years, the field of Electron Microscopy observed a fast advance of two major developments – Cryo-Electron Microscopy (cryo-EM) and volume Electron Microscopy (vEM). Cryo-EM is the rapid-freezing of your sample (eg virus particles, ribosomes, proteins) and observation of this material at low temperature in the microscope, obtaining images of the structure or molecule of interest.



3D model of a secondary plastid in Plasmodium berghei, known as Apicoplast. This organelle was reconstructed using cryo-electron tomography.

Breakthroughs in hardware and software for image analysis have enabled the field to grow from observation of blobs (average resolution of 15 angstrom) to high-res 3D structure (now the average resolution is 2 angstroms), leading to the recent Nobel Prize in Chemistry being awarded to the frontrunners in the field. vEM is an amalgamation of different techniques (eg Focused Ion Beam SEM; serial block face SEM, array tomography, among others) to study the three-dimensional structure of different samples.

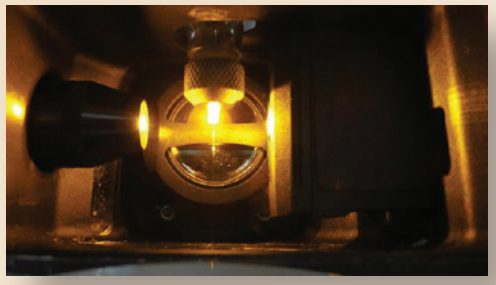
Due to crucial technological improvements in sample preparation, better SEM detectors, and in software for image acquisition and analysis, vEM rapidly expanded from Neurobiology and Connectomics to other areas in biomedical research – like infection and immunity, cancer, developmental biology, plant science, clinical research – where researchers require imaging complex biological processes in cells, tissues or organisms at a high-resolution in 3D.

Ian Titley – in**focus** Flow Cytometry representative

The flow cytometer, perhaps in common with the compound microscope and the internal combustion engine is, in its basic design, relatively unchanged to this day - a testament to the ingenuity of the originators of these devices. It is therefore the case that development of the technology subsequent to its inception is more "evolution than revolution".

Arguably the key advantages of flow cytometry when compared to more conventional fluorescence microscopy are speed of single particle throughput and the ability to collect the output simultaneously from many fluorophores from each particle in turn. An example of the power and utility of this approach is in the analysis of human immunological subsets of white cells from blood and other samples. By labelling the sample with multiple fluorescent tagged antibodies, each one with a different colour, data from many thousands (or indeed millions) of cells can be collected in minutes.

In 2006 ten colour flow cytometry was the 'state of the art'. To this date the constant upgrading of both equipment, and fluorophore design - much of which is now synthetic - has been the story of the last 15 years. This has now blossomed into recent reports of



Laser and flow stream intersect on a Beckman Coulter Astrios EQ flow sorter.

more than 40 colour marker panels, and this really matters.

The canonical example of this is the study and dissection of immune cell subsets. Capability to analyse, at the single cell level, the nuances of T, B, NK and antigen presenting cell population dynamics in the recent coronavirus pandemic, has proved invaluable in understanding the progress and biology of the disease.

Similarly, the interplay between solid tumours and communication with, and evasion of, the immune system cell subsets has provided valuable insights that have led to greater understanding and the identification of treatment candidates for these difficult and deadly conditions. The improvement of existing and development of new fluorophore markers along with high parameter "conventional" and "full spectrum" instruments signals a bright future.

Rebecca Higginson – in**focus** representative for Engineering and Physical Sciences; and **Mark Jepson**, Loughborough University

One of the most significant developments for Materials Science and Engineering over the past 15 years has been in Focused Ion Beam (FIB) microscopes. In particular, the combination of an ion beam with a scanning electron microscope opened up a world of possibilities through the combined milling capability of the ion beam and imaging of the electron beam.

The FIB allows the 2D structure of the polished samples to be milled to reveal 3D detail through successive cross-sectioning and imaging or to produce trenches to create Transmission Electron Microscope (TEM) samples from highly site-specific regions of a sample, which can then be thinned to electron transparency using the ion beam.

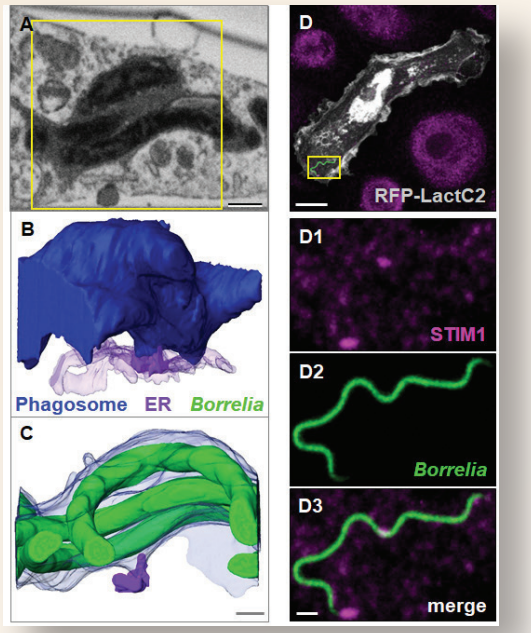
Those who have spent many hours electro-polishing thin foils for TEM analysis, only to find no electron transparent region, or that they did not contain the desired feature, will watch with envy as a site-specific sample can be taken and thinned for TEM analysis with comparative ease in a FIB system.

The relatively gentle approach of the ion beam (compared to mechanical methods of sample production) makes it possible to produce cross sections and make TEM foils from previously difficult or impossible to produce samples, for example coatings, oxides, complex composites, and electronic devices.

Through the use of cryo-FIB, cross-sections of soft materials or biomaterials can also be produced realising the site-specific preparation benefit across the full range of materials. We are now able to examine grain boundaries, phases, and interfaces in more detail and to discover new mechanisms to increase our understanding of engineering materials and their behaviour.

Emily Eden – in**focus** Life Sciences Representative; and **Stefan Linder** - RMS LS Committee Member

The last 15 years have witnessed remarkable advances in the microscopy approaches available to life-sciences researchers. We have chosen to focus on the impact of 3D-electron microscopy (EM) on the rapidly developing field of membrane contact sites (MCS) biology. Membranes of closely apposed organelles at MCS are tethered between five and forty nanometres apart. This distance can only be accurately resolved by EM, which is widely considered the 'gold standard' for



Borrelia-containing phagosomes are associated with STIM1-positive ER contact sites. (A) SEM image of a phagosome containing GFP-expressing Borrelia burgdorferi spirochete, with associated ER. Yellow box indicates area volume-rendered in (B,C). (B,C) Rendering of phagosome, with phagosome surface in blue, internalized Borrelia cell in green, and ER in magenta, with (B) showing all ER parts associated with phagosome fully coloured, and (C) showing only membrane contact site (MCS) fully coloured, and the more distal parts transparent. Scale bar: 0.2 µm. (D) Immunofluorescence micrographs of primary macrophages expressing RFP-LactC2, a reporter for phosphatidylserine, for visualization of phagosomes (white), incubated with GFP-expressing borreliae (D2) and stained for MCS marker STIM1 using specific antibody (C1), with merge of all three channels (D3). Yellow box in (D) indicates detail areas shown in (D1-D3). Scale bars: 10 µm for (D), 2 µm for (D1-D3). FIB/SEM performed in the lab of Gerhard Wanner (Munich, GER), and IF in the lab of Stefan Linder (Hamburg, GER). See also (Klose et al., Journal of Cell Science, 2021; https://pubmed.ncbi.nlm.nih.gov/33380490).

MCS identification. However, the volume of sample that can be imaged by conventional EM is limited by the penetration depth range of the electron beam. Manually imaging cells through a series of sections, circumvents this limitation, but is extremely laborious and sections are often damaged during handling.

The development of technology for automated acquisition of serial images, coupled with advances in acquisition rates and computational power, has revolutionised volume EM. Automated serial sectioning with a diamond knife (serial block-face EM (SBEM)), or serial milling with a focused beam of high-energy gallium ions (focused-ion beam scanning EM (FIB-SEM)), removes sequential layers from the block-face, with each newly exposed surface imaged by integrated SEM. For a non-destructive alternative, automated tape-collecting ultramicrotome SEM (ATUM-SEM), collects serial sections on a tape for SEM imaging.

Combining light and electron microscopy modalities adds further information, enabling visualisation of fluorescent proteins in the context of the cellular ultrastructure (e.g. correlative FIB-SEM shown on previous page). The ground-breaking development of cryo-EM technology removes issues of poor fluorescence retention and potential membrane shrinkage associated with chemical fixation by imaging frozen hydrated samples. By correlating cryo FIB-SEM with light microscopy, multiple fluorescent proteins can be visualised within close-to-native state nanoscale cellular landscapes.

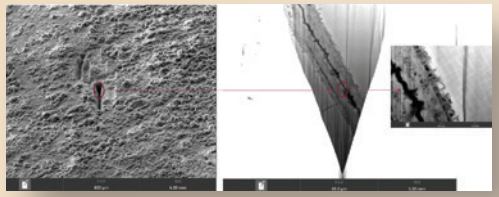
Together 3D-EM technologies have challenged the view of organelles as isolated compartments, revealing a dynamic network of organelles, constantly communicating through coordinated interactions at MCS.

Maadhav Kothari – in**focus** Board Member and Materials Scientist

Since 2006 advancements in field ionisation beam milling and co-purpose scanning electron microscopy (FIB-SEM) of materials has become prevalent within materials science communities.

The use of various techniques such as FIB 3D tomography whereby a user can develop a 3D model of cross sectioned material, precise deposition of protective layers such as carbon and platinum to protect against damage from an ionising beam are now commonplace.

Further advancements for purpose specific needs have resulted in an expanding area of FIB-SEM. One could use He ion gas in order to obtain advanced resolution (gas field ionisation sources GFIS), rapid



A corroded C-ring Ni-superalloy inconel material which has been tested under salted conditions aimed to mimic aerospace gas turbine corrosion conditions.
Critically, it is difficult to inspect cracks through CT scanning as the resolution is not good enough (typically in the 50-20 µm range). It is impossible to inspect crack growth without sectioning the material, which can cease study of the corrosion. Therefore, it is important to develop new methods using FIB-SEM milling to detect crack growth, salt composition and depth.
Here a very narrow trench has been 'dug' in an area identified to have a mound-like scale. It has been possible to follow the crack and further using a tilting stage it is possible to identify corrosion products within the crack using EDS. Further work can be done by creating a lameler for further inspection using a TEM.

area and volume milling using plasma (inductively coupled plasma ICP), rapid cross sectioning using laser milling (laser cooled low temperature ion sources LoTIS) as an alternative to typical liquid Gallium ion milling.

This has seen a rapid expansion in microstructure analysis, 3D tomography in macro through to nano scale dimensions. Coupled with typical analytical techniques such as electron x-ray energy dispersive spectrometry (EDS) or electron backscatter diffraction (EBSD) 3D and fundamental material analysis can be both rapid, functional and industrially relevant.

Further hardware and software advancements in detectors from well known manufacturers such as Oxford Instruments and EDAX have enabled analysis time to be cut down encouraging both academics and industry to develop advanced failure analysis techniques for high value and precision alloys. The future of FIB-SEM continues to grow rapidly and the increased market of energy materials such as fuel-cell, battery anode/cathode materials and the development of quantum computing hardware will result in continuous development in the area over the next 10 years.

Laura Fumagalli – in**focus** representative for AFM and SPM

Scanning probe microscopy (SPM) has transformed science and technology, driving the nanotechnology revolution since its introduction in 1982. SPM has enabled scientists to see and manipulate matter on the atomic scale with relatively simple instrumentation, thus becoming an essential tool for research in both Life and Materials sciences.

Since its early years, SPM has developed into various microscopies with the unparalleled ability to image samples under various environmental conditions (vacuum, air and liquid) and determine materials properties. In the past 15 years, the family of SPM has continued to make significant advances. Continued progress has been made towards higher resolution, as atomic force microscopy (AFM) has shown to provide images of single molecules with sub-molecular lateral resolution. Examples include the chemical structure of individual molecules at low temperature and biomolecules such as DNA and proteins in near physiological environments. Impressive progress has been made by the SPM community to go beyond structural information and probe molecular dynamics and function on the atomic scale.

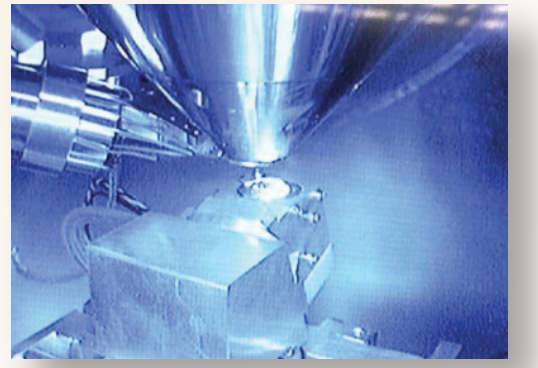
High-speed AFM (HS-AFM) has allowed filming dynamical processes of biomolecules in liquid at unprecedented spatiotemporal resolution, even occurring in live cells. Once accessible only to few groups, the instrumentation required for HS-AFM is now commercially available, thus an increasing use of HS-AFM is expected in the upcoming years. The ability to acquire data at high speed has also led to the development of three-dimensional AFM (3D-AFM), which enables to atomically resolve 3D information of surfaces within reasonable imaging times. As an important example, 3D-AFM has revealed the molecular arrangement of interfacial water near solid surfaces and molecules.

Furthermore, great progress has been made in the measurement of local materials properties and their quantitative analysis, in particular electronic, dielectric and optical properties, based on spectroscopic and force-distance approach curves. Such progress combined with the capability of fast data acquisition and processing has opened the door to "big data" analysis based on machine-learning techniques, which are currently being explored. By integrating 3D and functional experimental data with complex theoretical simulations, the SPM technology promises to provide quantitative multifunctional and multidimensional information in the near future, which should lead to a better understanding of matter on the atomic scale and new scientific discoveries.

Rhiannon Heard – in**focus** Early Career representative

Looking back over the last 15 years of microscopy, scientists and industry specialists have explored the idea of not just using microscopy as a static imaging tool, but one that can capture dynamic processes in real time.

These experiments are referred to as "in situ" microscopy. This idea has been supported by studies that have focussed on developing supporting techniques and technologies to facilitate experimentation within an electron microscope. In the physical and life sciences, these advancements have facilitated the capturing of chemical, mechanical and biological processes by relating changes on the microstructural and atomic scale to the overall system kinetics.



An in situ heating stage within a Scanning Electron Microscope.

In particular, the initial development of specialist sample holders for Transmission Electron Microscopy (TEM), where the sample is enclosed in a reactive environment between two electron transparent membranes, led to significant improvement in observation of biological complexes in their natural environment.

TEM holders have since been enhanced to support imaging of chemical reactions and dynamic material processes; improving understanding of micro and nano-scale interactions. Additionally, the development of in situ Scanning Electron Microscopy (SEM) has led to micro-observations of bulk sample experiments including phase, precipitate and grain evolution during changes in temperature, pressure and under loading. It is anticipated that this exciting, new area will continue to develop over the next decade with improvements in micro-testing stages and detector imaging speeds furthering investigations into innovative microscopy applications.

Susan Cox – in**focus** representative for Light Microscopy

Breaking in two papers in 2006, localisation microscopy (also known as PALM or STORM) brought super-resolution into the hands of anyone with a widefield microscope, a powerful laser, and a willingness to tear things apart. With achieved resolutions as low as 20nm, this method allowed visualisation of never-before-seen structures, like the actin ring structures in axons.

Localisation microscopy relies on fluorophores switching between emitting and non-emitting states, taking many thousands of frames. In each frame only a few fluorophores are emitting light, and they are computationally localised. The positions of all the fluorophores in all the images is then plotted in a new, super-resolution image. Commercialised by a number of companies, localisation microscopy systems are now present in most large facilities. Recent combinations with MINFLUX, expansion microscopy and lattice light sheet have improved the resolution and expanded the size of the volume that can be imaged.

Of course, as with many techniques, ultimately what localisation microscopy has done is bring up new shortcomings to overcome: the distance between the fluorophore and the molecule being labelled, or the challenge of quantification when not all fluorophores behave the same. I can't wait to see what new biology is discovered as people push back at these new frontiers.

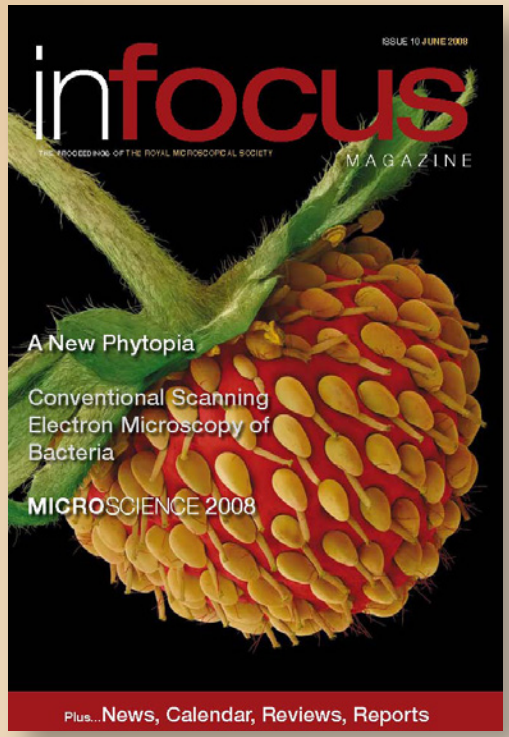
Cover stories

March 2006: Glassy flames

This eye-catching cover image won First Prize in the Light Microscopy Materials Science category of the RMS Micrograph Competition in 2004. Submitted by Professor Josef Spacek (Charles University Medical Faculty, Czech Republic) and entitled 'Glassy Flames', the image is a microrelief of an occasional acrylate mounting medium artifact (Prim. magnification 30x).

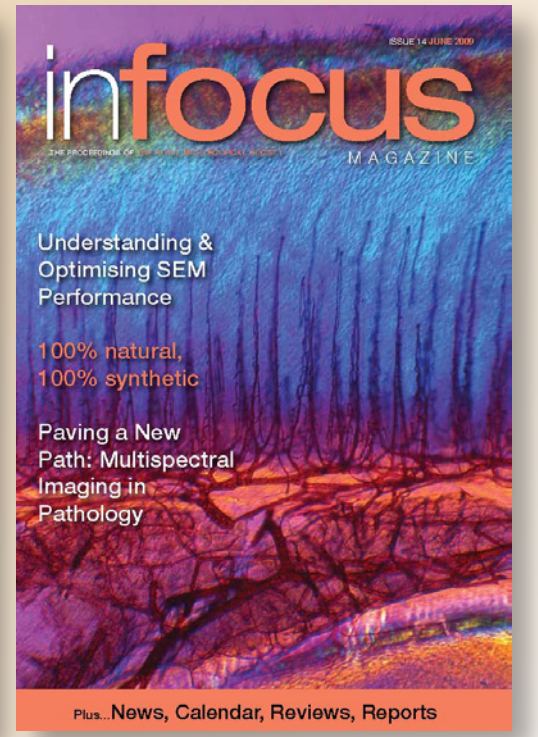
December 2007: White Wine

This image was also an entry in the 2004 Royal Microscopical Society Micrograph Competition. Submitted by Mario Schmied, it shows the tartar of a typical Styrian white wine (Welschriesling) (AuPd coated) (SEM Gemini DSM 982, 5kV, 0.4 nA). Another tipple – Martini Rosso – would feature on the front cover 13 years later (see p66).



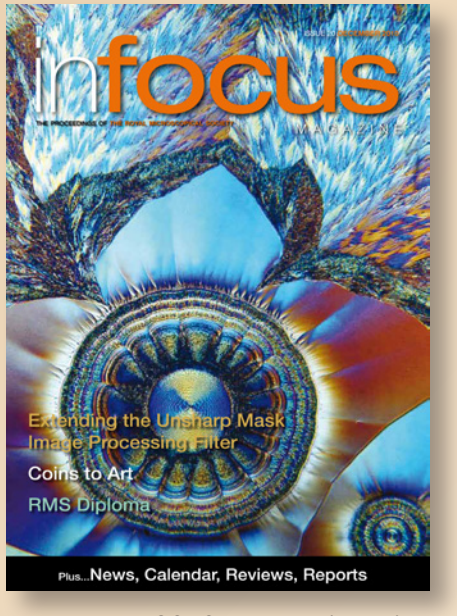
June 2008: Fragaria x ananassa. Garden strawberry

This image was submitted by RMS Ambassador and regular infocus contributor Rob Kesseler. It was part of an article showcasing some of Rob's work, published in the same issue. Rob is a visual artist and Professor of Ceramic Art & Design at Central Saint Martins College of Art & Design. Within a very diverse range of outputs, his work examines the natural world and its place within contemporary society through the interrelationship between the Arts and Sciences.



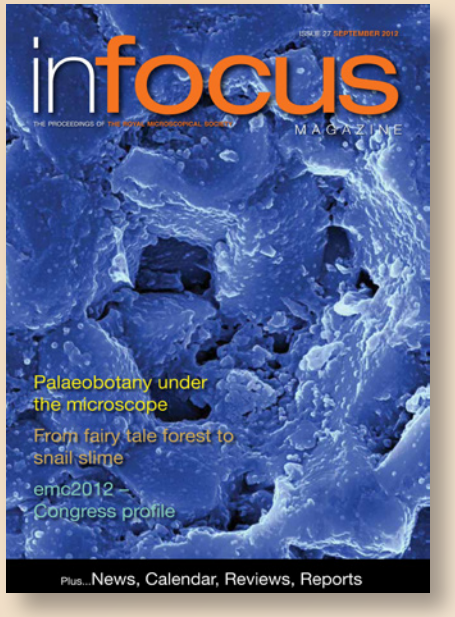
June 2009: Hen's Tongue

The cover image for this issue was a winning entry in the 2008 RMS Micrograph Competition, submitted by Steve Lowry. Not quite as rare as hen's teeth; the image is instead of the bird's tongue.



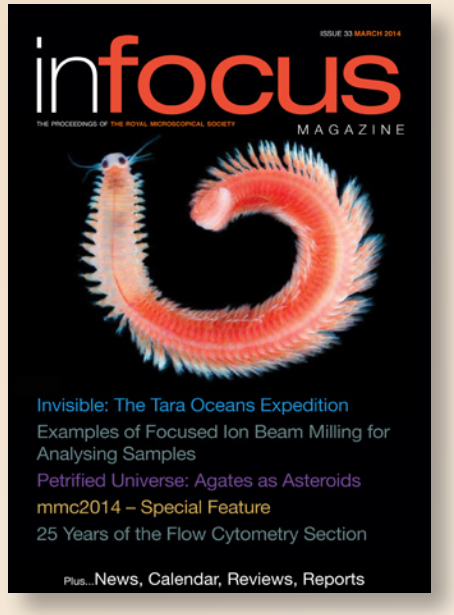
December 2010: Ascorbic Acid M

This kaleidoscopic and (to continue the avian theme) somewhat feathery image by Michael Much, won Second Prize in the Light Microscopy – Materials category of the RMS International Micrograph Competition 2010. No peacocks involved though – it in fact shows ascorbic acid crystals photographed by cross-polarisation.



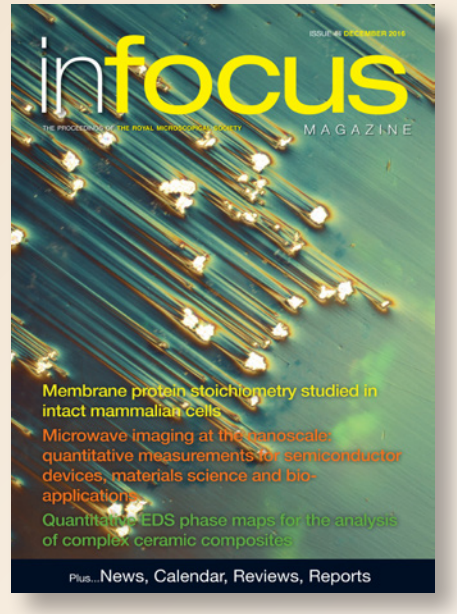
September 2012: Acetabular cup – wear and tear

This craggy image might resemble some extra-terrestrial landscape, but Amit Rana's intriguing SEM effort actually depicts the surface of an acetabular cup, taken from a retrieved alumina hip joint. It shows severe wear and fracture of the surface with the presence of a high degree of debris, generated through wear, trapped within the rough surface.



March 2014: Platynereis sp. (Lophotrochozoa)

This proud little creature was captured by Eric Roettinger during the Tara Oceans expedition of 2009-2012. During sexual reproduction, the normally benthic, crawling polychaete annelid worms become pelagic, and swim freely in the water column. This specimen was caught during a nightly plankton tow, in the lagoon of Gambier Islands (French Polynesia) - one of the stop-overs of the world-wide Tara oceans expedition.

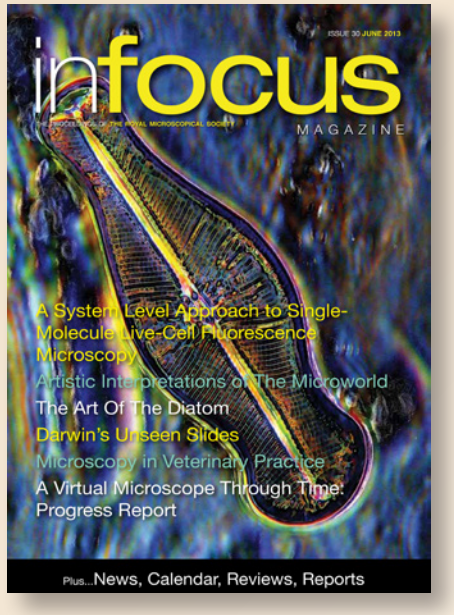


December 2016: Pyrite Meteorite Shower in Australian Opal

This meteoric image by Nathan Renfro was produced using Nomarski differential interference contrast. It shows numerous pyrite crystals breaking the surface of an Australian opal. Polishing lines, or drag lines, resulting from the differential hardness of the two materials, help to give an imaginative impression of a meteorite shower in a night sky. Horizontal field of view 1.24mm

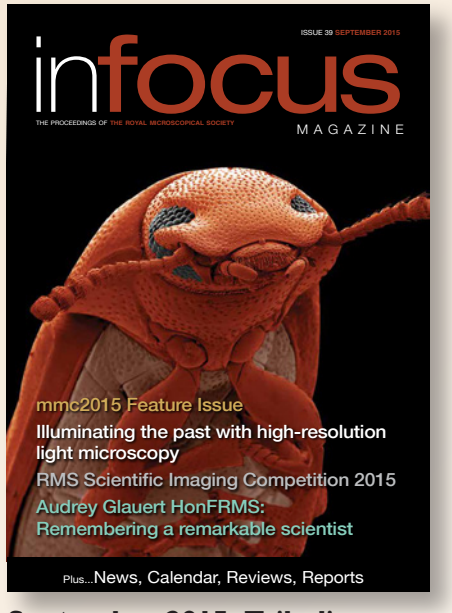
March 2011: Soap Film

Karl E. Deckart kept it clean with his cover image for March 2011. Yes, you're looking at a micrograph of a soap film through a light microscope. It was part of a series of images included in an article from the same issue, which explored Karl's technique for capturing such images.



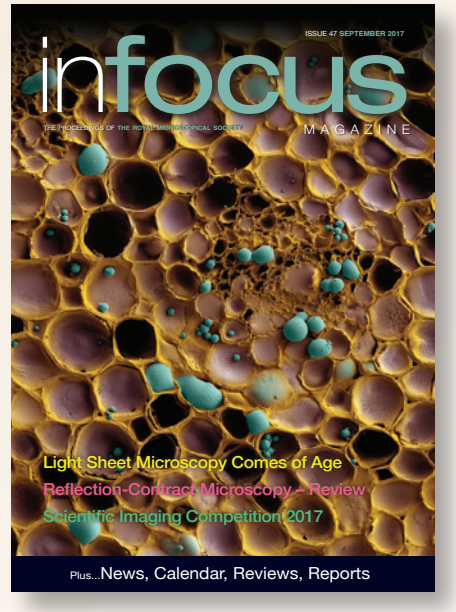
June 2013: Lips 2

'Other-worldly' might also be an apt description of this image, which made the front cover the following year. Provided by regular infocus contributor Winston Ingram, the pouting object is not the underside of an alien spacecraft, but an unidentified diatom on a 3x1 slide. It was taken from Winston's book "The Art of The Diatom" in which subjects from natural sciences are given an artistic interpretation. The microscope used was a Leitz Ortholux 11.



September 2015: Tribolium confusans

Another critter reared its head in 2015, courtesy of this fine effort by David Spears – submitted as an entry to the 2014 RMS Scientific Imaging Competition. Here, we have an Electron Microscopy image of a Confused Flour Beetle. The insect is infamous for causing economic damage by infesting stored flour and grain.

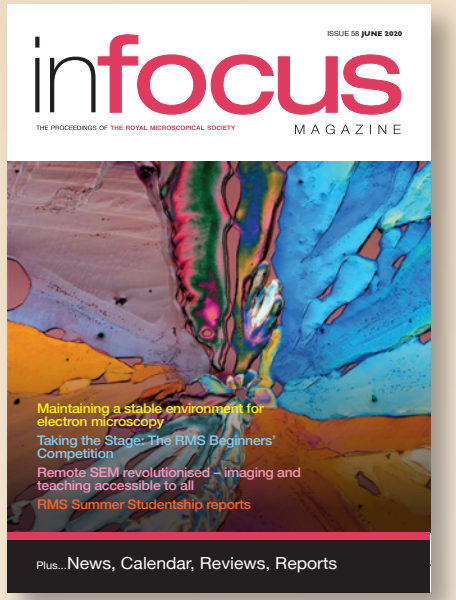


September 2017: Cryo Cross-section of Dracaena root

This honeycomb-like image by Martin Ciprian of TESCAN shows the root of a Draceana – popularly known as a dragon tree. Dracaena helps remove formaldehyde and is said to be one of the best plants for removing xylene and trichloroethylene. This is one of the reasons why scientists all over the world are interested in understanding its properties. Cryo-SEM techniques are used for studying the inner structure of the Madagascar dragon tree in order to help reveal the mysteries of this intriguing plant.

September 2018: Thymol

Are these the brush strokes of an abstract artist? – well, yes, metaphorically speaking. This image by Lawrence Wayne of Forensic Analytical Laboratories, Inc. was a shortlisted submission to the 2018 RMS Calendar Competition. The image shows Thymol recrystallised from melt, imaged with fully crossed polars using an Olympus BX51 polarised light microscope.



June 2020: Martini Rosso

Submitted by Bernardo Cesare, this is the polarised light photomicrograph of a crystallised drop of Martini Rosso, the Italian alcoholic beverage world renowned as an aperitif. Bernardo, who is better known for producing photomicrographs of rocks and minerals (go to page 20 of this issue), was inspired to capture this image by the work of the late Michael Davidson. He said: "After a conference where I showed Davidson's 'Bevshots' and my rock photomicrographs, a guy came to me and said, 'you should try some Italian drinks'. I did, and the result is what you see here." Width 2.7mm.

March 2019: Stinging nettle hair

Ouch! This image, by Alan Wood of the Quekett Microscopical Club, was a submission to the 2019 RMS Calendar Competition. Captured with an Olympus BH-2 microscope, it shows a hair on the stem of a stinging nettle – in exquisite, if slightly menacing detail.

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