

Multiphoton imaging in Arthritis Research

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Abstract

Rheumatoid arthritis (RA) is a prevalent autoimmune disease which primarily affects the joints of the hands and feet, however, it is also associated with significant co-morbidities including cardiovascular disease and cancer. Optical imaging technique is a major approach employed in *in vivo* RA research due to its advantages over other bio-imaging modalities such as MRI, radiography and ultrasound, specifically its sensitivity and cellular resolution. However, key limitations remain with this technology, particularly the limited depth of imaging and problems due to tissue autofluorescence. This review will focus on rapid advances in illumination sources such as pulsed infrared lasers for multiphoton imaging and near-infrared fluorescent (NIRF) probes that promise to overcome these obstacles, enabling analysis of cellular and molecular events *in vivo* in a non, or minimally, invasive manner.

Introduction

Intravital imaging is an essential technique to investigate the spatial and dynamic behaviour of complex biological systems in a physiological environment *in vivo*. Generally, *in vivo* optical imaging

approaches only permit resolution and detection of single cells at the surface of a tissue. This is due to the inherent light scattering and absorbing properties of tissue components and pigments. While the use of transparent specimens can overcome these problems (1), multiphoton imaging is the technology of choice for live intravital imaging of mammalian cell biology in intact tissue or *in vivo*.

Rheumatoid arthritis (RA) is a debilitating chronic autoimmune disease currently estimated to affect as much as one per cent of the population, therefore posing a significant socio-economic burden (2). The pathogenesis of RA has been extensively reviewed in detail elsewhere and is well established as an immune-mediated disease (2-4). RA predominantly affects the joints, particularly those of the hands, feet and cervical spine, and is characterised by persistent synovitis. There is considerable immune cell infiltrate in the synovial membrane which is responsible for the production of pro-inflammatory cytokines, particularly tumour necrosis factor (TNF)- α and interleukin (IL)-6 within the joint (3). This inflammatory environment promotes cartilage and bone degradation leading to impaired joint function and arthralgia (5). The development of effective therapeutics including disease-modifying

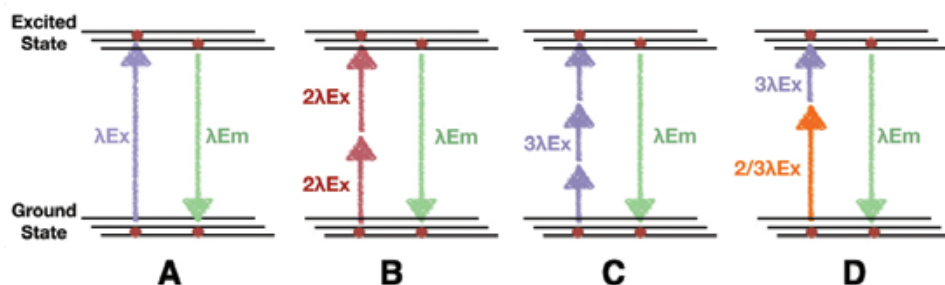


Figure 1: Different modes of fluorophore excitation used in optical imaging. Jablonski diagrams demonstrating different photon absorptions leading to fluorophore excitation. In each case emitted light photons have the same wavelength. (A) single photon excitation, (B) two photon excitation, (C) three photon excitation and (D) photon mixing.

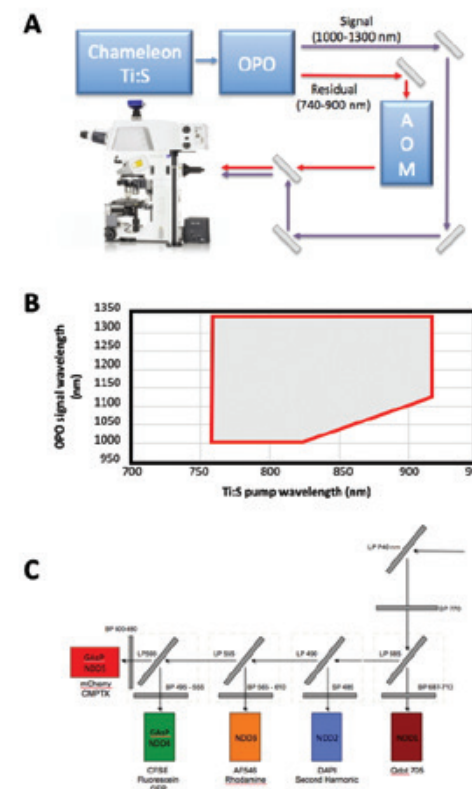


Figure 2: Example of a two beam, multichannel MPLSM system. (A) a solid-state, tuneable, pre-compensated Titanium: sapphire laser system (4W, Chameleon Ultra II, Coherent Laser Group) coupled to an Optical Parametric Oscillator (Chameleon MPX; Coherent) to generate (B) fully tuneable, two beam laser excitation. (C) This is routed into a laser scanning microscope (LSM 7 Multiphoton, Carl Zeiss, Germany) equipped with appropriate combinations of objectives, filters and photomultiplier tubes that provide a system capable of simultaneously imaging five fluorescence channels.

antirheumatic drugs (DMARDs) and various biologics, particularly anti-TNF- α (6) have been facilitated by the availability of ex vivo synovial tissue from the clinic as well as the availability of animal models of disease such as collagen-induced arthritis (CIA) for *in vivo* studies. However, it is broadly accepted that the disease cure will be dependent on earlier intervention strategies to treat the disease before symptoms become apparent. This requires an understanding of the very early stages in disease development to identify targets for drug discovery as well as companion biomarkers to indicate when and which drugs are most likely to be effective.

RA research has greatly benefited from innovations

in optical imaging, and intravital imaging clearly has an important role to play in understanding disease. In particular, these approaches are revealing the spatial and dynamic behaviour of key cell populations in the initiation and development of disease within immunological tissues and affected joint environments *in vivo*. The purpose of this article is to describe the current status of intravital imaging studies in RA and introduce some of the technical developments in optics that have enabled this work. We will also discuss some future developments in imaging that may be applicable to enable better and more revealing studies in disease development.

Multiphoton Microscopy

Traditional fluorescence microscopy uses external light of a suitable wavelength to excite a fluorophore to a higher energy state. The consequent decay causes emission of photons at a longer wavelength which can then be captured, typically using either charge coupled devices (CCD) or complementary metal oxide semiconductors (CMOS) (7). This relies on a linear interaction where one photon is absorbed by the fluorophore, resulting in the emission of a single photon (Figure 1). In contrast, multiphoton imaging is based on a non-linear effect where two or more photons of lower energy than that of single photon excitation are absorbed by the fluorophore near-simultaneously ($<10^{-15}$ seconds) (8). The probability of this near-simultaneous absorption event is poor and can only be achieved when there is particularly high photon flux in the range of $10^{20} - 10^{30}$ photons/cm²s (9). However, the delivery of high-powered laser light has the potential to irreversibly damage the target under examination. This can be overcome using pulsed lasers, for example, Titanium/Sapphire lasers (Ti:S), which generate short pulses of high-intensity laser light approximately every 10ns, therefore achieving high temporally limited, photon fluxes with low average power, minimising damage to the target. Due to the low probability of two-photon absorption, excitation is therefore spatially limited to the focal point of the beam. This avoids light

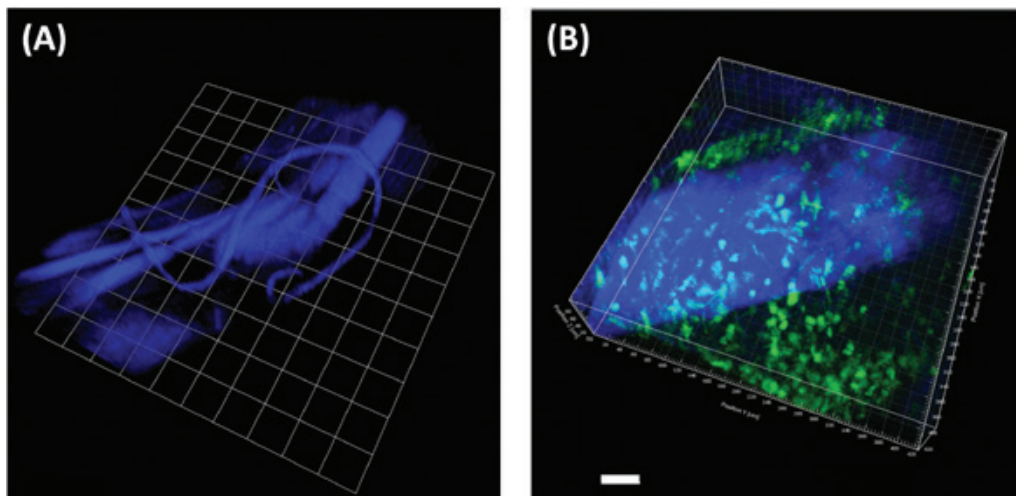


Figure 3: MPLSM of arthritic joints. Murine ankles were imaged in real time from a medial aspect, with anaesthetised mice being transferred to a warmed saline bath prior to imaging. (A) Second harmonic generation (SHG) localised tendons to allow anatomical localisation and orientation; grid size 365 μm . (B) Visualisation of tendons in this way aids localisation to entheses when imaging for inflammatory infiltrate as seen in CX3CR1-GFP reporter mice during collagen-antibody-induced-arthritis (CAIA). Scale bar 50 μm .

absorption above and below the plane of interest, meaning there is no out of focus signal, negating the requirement for the pinhole required for example, in confocal microscopy. Thus, the point of excitation can be raster scanned through the tissue of interest with any emitted light collected, via the objective lens. While these features of multiphoton microscopy produce advantages in terms of sensitivity, this approach is additionally unrivalled in its ability to resolve cells and fluorescent molecules deep inside tissues through two properties. Firstly, the absorption of NIR light is lower in tissue pigments compared with conventional single photon illumination in the optical window (10). Secondly, NIR illumination used in multiphoton imaging is intrinsically less scattered by tissue components than shorter wavelengths of light (11). Combined, these effects mean that while conventional imaging is limited to roughly 50 microns below the sample surface (12), multiphoton imaging can resolve objects at much greater depths, around 0.5 to 1 mm.

Following photon absorption and therefore the excitation of the fluorophore, relaxation of electrons causes emission of a photon with more energy than that of the incident photons, unlike that seen in single photon absorption where the resulting photons have lower energy (Figure 1). Thus, while multiphoton

imaging overcomes problems with excitation of fluorescent probes in deep tissue, emitted light is subject to the same absorption and scattering limitations as seen in conventional single photon imaging. Fluorescent probes with emission in the NIR window can overcome these issues, however, this requires much longer excitation wavelengths. These can be produced via a number of approaches, most popularly by coupling an Optical Parametric Oscillator (OPO) to an existing Ti:S multiphoton laser to extend the excitation wavelengths, typically up to ~ 1300 nm. This combination can allow simultaneous imaging on both the Ti:S and OPO wavelengths, and increases the range of excitation wavelengths available to image a greater range of fluorescent species (Figure 2). An additional benefit occurs if the two sources have their pulses synchronised, allowing 'photon mixing' of the two different wavelengths to produce a third excitation wavelength (Figure 1D; (13)). While longer wavelength excitation does increase absorption of light by water, a second NIR window in the 1,300-1,700 nm region has been shown where this effect is minimised. The second NIR window has been exploited by fluorescent imaging using three-photon excitation (Figure 1C) with wavelengths around 1300 nm, permitting brain imaging through the intact skull *in vivo* (14).

Fluorescent Probes for Arthritis Imaging

Non-Specific Fluorescent Dyes

In arthritic joints, the inflammatory microenvironment results in increased vascular permeability and hyperaemia of blood vessels, therefore causing unspecific dyes to accumulate at the inflamed joints through the enhanced permeability and retention (EPR) effect, a concept first described in tumours (15, 16). While non-specific fluorescent dyes such as Indocyanine green (ICG; (17)) and 1,1'-bis-[4-sulfobutyl] indotricarbocyanine-5,5'-dicarboxylic acid digluconate monosodium salt (SIDAG; (18, 19)) have been widely used in whole-body imaging to identify inflammation, in intravital microscopy

intravenous injection of non-specific fluorescent dyes are most often used as *in vivo* contrast agents to identify blood vessels. This is useful for studies of extravasation as well as using blood vessel structure for tissue registration (20).

Targeted probes

In addition to detecting the general presence of rheumatoid arthritis, as seen when using unspecific dyes, highly specific probes can be used to carry out fluorescence imaging, therefore, allowing for more detailed information to be obtained on the cellular and molecular events occurring within the joint such as assessing receptor expression and distribution (7). Targeted probes used for optical imaging are designed to be highly specific

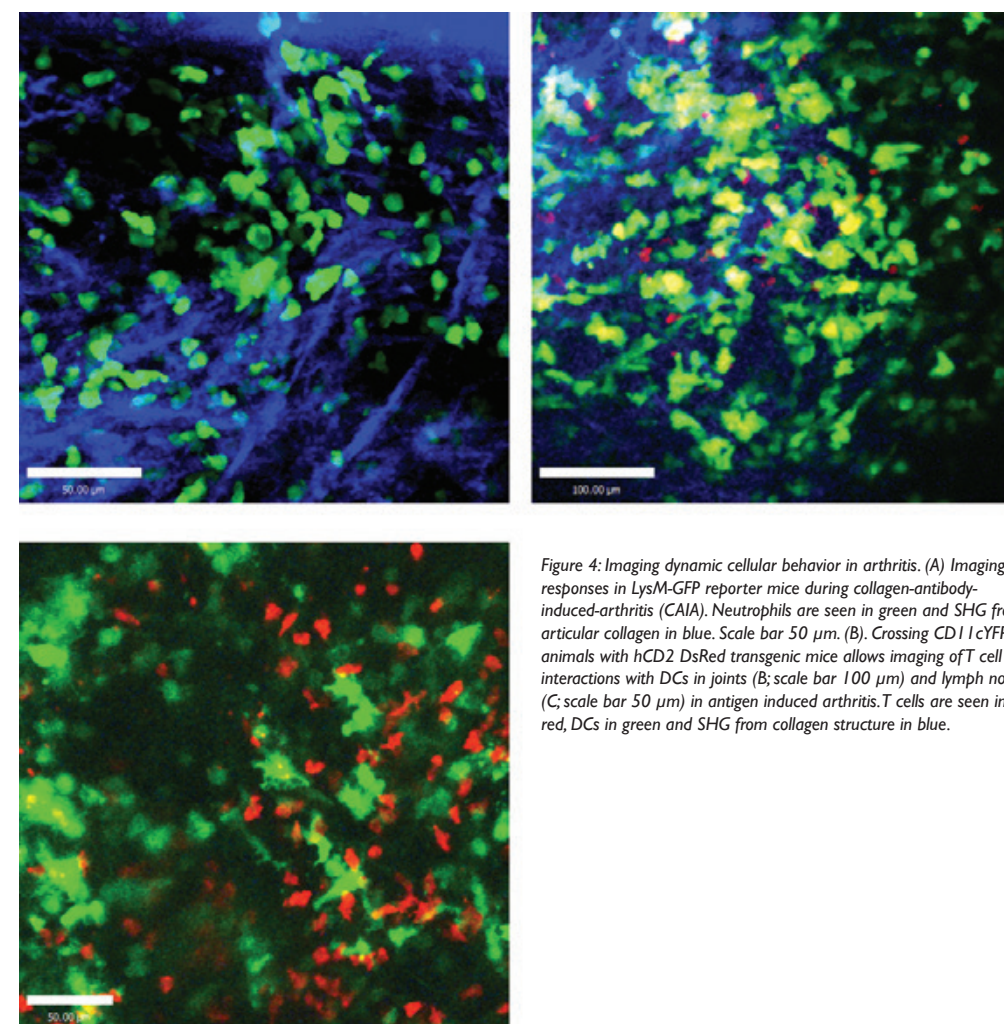


Figure 4: Imaging dynamic cellular behavior in arthritis. (A) Imaging responses in LysM-GFP reporter mice during collagen-antibody-induced-arthritis (CAIA). Neutrophils are seen in green and SHG from articular collagen in blue. Scale bar 50 μm . (B). Crossing CD11cYFP animals with hCD2 DsRed transgenic mice allows imaging of T cell interactions with DCs in joints (B; scale bar 100 μm) and lymph nodes (C; scale bar 50 μm) in antigen induced arthritis. T cells are seen in red, DCs in green and SHG from collagen structure in blue.

to a particular target, this is usually achieved by conjugating a fluorophore to a monoclonal antibody specific to a molecular target within a rheumatic joint (21). Despite their high sensitivity and ease of development, monoclonal antibodies may bind non-specifically via their Fc (fragment crystallisable) domains and their large size, approximately 160kDa kilodaltons (kDa), correlates with slower clearance, resulting in a high background signal (22).

A study carried out by Hansch et al in 2004 (23) was one of the first to use NIR fluorescence imaging to identify antigen-induced experimental arthritis. To achieve this, they intravenously injected anti-F4/80 monoclonal antibodies labelled with Cy5.5 to target F4/80⁺ macrophages, a major cell population present in rheumatic joints. Similarly, synovitis could also be identified in various models of experimental arthritis using a folate-targeted NIR probe which binds activated synovial macrophages, this also was validated using histology, microscopy and immunohistochemistry (24). Fab fragments are an alternative to monoclonal antibodies, which consist of only the antigen binding region of the antibody. They can also be used to minimise unspecific binding and increase target specificity (25). More recently, there have been developments in the use of nanobodies or single-domain antibodies for use either as therapeutics or probes for imaging. Nanobodies accumulate in the tissue faster, have higher stability and their small size means they are subject to a high clearance rate, thus reducing toxicity. However, this high clearance rate also makes it more challenging to successfully obtain images of the joint and may hinder efficient binding to all epitopes available. This topic has been analysed and discussed in detail by Chakravarty and colleagues (26).

Intelligent or bio-responsive probes are non-fluorescent until modified by cellular enzymes. This is typically achieved by conjugating a fluorophore to a quencher via an enzyme sensitive peptide sequence (27, 28). The quencher reduces the

fluorescence intensity of the fluorophore, typically by Förster resonance energy transfer (FRET) from the fluorophore whilst in its bound state. For the fluorophore to emit light, cellular enzymes must cleave the peptide sequence thus freeing the fluorophore from the quencher. Typically, disease-associated enzymes such as cathepsins (29) and matrix metalloproteases (30, 31) are targeted by these approaches. While these intelligent probes have been demonstrated to measure arthritis progression using whole-body imaging in animal models, they are less useful for cellular resolution and are therefore outside the scope of the current review.

Endogenous reporters

Multiphoton microscopy can make use of non-linear optical properties of structural proteins in the joint, such as fibrillar collagen resulting in second harmonic generation (SHG). SHG, or frequency doubling, arises as two photons of light are combined during interaction with fibrillar structures in the tissue, producing a photon of light with half the wavelength. This acts as a particularly useful contrast agent when imaging joints, providing clear anatomical structures for image localisation and registration (Figure 3). As SHG is dependent on the fibrillar features of collagen, changes in collagen structure arising from various disease processes, such as inflammation can also be analysed (32).

Fluorescent Proteins

Since the cloning of Green Fluorescent Protein (GFP) in 1992 (33) and its initial use in tracking gene expression as a fusion protein, the palette of currently available fluorescent proteins (FP) and biological applications has evolved significantly. By genetically encoding FPs, cell lineage and functional responsiveness can be visualised in complex systems. FP expression driven by cell lineage-specific promoters in transgenic mice can be applied to establishing the spatiotemporal dynamics of pathologically relevant cellular recruitment and interactions. Several of these have been particularly

useful in determining the dynamics of cellular processes directly in the inflamed joint. The LysM-EGFP reporter strain, a knock-in of the enhanced GFP gene into the murine lysozyme M locus (34), allows visualisation of neutrophil and monocyte recruitment into the joint (35) (Figure 4A). Mice co-expressing DsRed and EYFP under the control of the human CD2 and murine CD11c promoter respectively, have also allowed visualisation of T cell interactions with dendritic cells within the articular joint (36) and reactive lymph node (Figure 4 B, C). Although not currently used in arthritis models, Col2.3-ECFP and tartrate-resistant acid phosphatase (TRAP)-tdTomato reporter mice are allowing the visualisation of osteoblast and osteoclast interactions during bone remodelling (37), and will no doubt be valuable to future preclinical arthropathy studies.

While cell lineage reporters are extremely useful in visualising cellular dynamics, FPs can also be employed to detect changes in functional activity. Constructs where FP expression is induced in response to specific signalling cascades such as NFAT (Nuclear Factor of Activated T cells) and Nr4a1 (nuclear receptor subfamily 4, group A member 1) promoter-driven GFP reporter constructs (38-40), have been used effectively in studying adaptive immune responses and in detection of T cell receptor stimulation, while numerous cytokine reporter mouse strains exist that indicate functional activity of those T cells (41, 42) e.g. YETI and GREAT (IFN γ -YFP), IL-10^{EYFP}, IL-17-RFP. Subcellular localisation and/or intracellular redistribution of fluorescently tagged proteins can result from cellular responses to external stimuli and indicate changes in functional activity. Dephosphorylation of NFAT reveals a nuclear translocation sequence, resulting in its transport to the nucleus, facilitating its transcription factor activity. Using NFAT-GFP fusion proteins, this nuclear localisation can be imaged and used as a readout of cellular responses, being of particular relevance to T cell activation (43). Redistribution of fluorescent

fusion proteins is also proving informative in studying bone remodelling processes. GFP fused with the $\alpha 3$ subunit of the V-type H⁺-ATPase pump not only allows identification of mature osteoclasts but redistribution of the GFP-fusion protein to membranes in contact with bone identifies cells actively involved in the resorption process (37).

Biosensors such as genetically encoded calcium indicators reveal information relating to active signalling processes within cells. These have been particularly valuable in studies of the nervous system, allowing imaging of functional responsiveness to exogenous stimuli. Similarly, such changes in intracellular calcium availability in immune systems cells is indicative of responsiveness to their environment e.g. calcium flux seen during T cell activation. Proteins such as “cameleons” are chimeric molecules consisting of blue or green fluorescent protein, calmodulin (CaM), CaM-binding domain of myosin light chain kinase (M13) and a green or yellow fluorescent protein. Upon binding of Ca²⁺, CaM is able to bind the M13 component of the chimeric protein, with the resulting molecule being more compact, facilitating Förster/fluorescence energy transfer (FRET) from the shorter to longer wavelength fluorescent protein (e.g. ECFP to EYFP) (44). Fuelled by efforts to improve upon these original cameleons, modern permutations include single FP sensors and even photoactivatable variants (45).

It should be highlighted, however, that most of the fluorescent probes employed in multiphoton microscopy have only been optimised for their intended use in single photon microscopy. Many of these reporters have particularly low two-photon absorption cross sections of < 50 GM and therefore highlight an area where new probe development specifically for multiphoton microscopy could yield significant improvements (46).

Conclusion and Future Perspectives

The information regarding the cellular and

molecular events that occur within arthritic joints which can be gained from intravital microscopy is vast, with the major advantages being dynamic analysis with tissue context. To date, the majority of intravital microscopy studies have employed fluorescent proteins expressed under cell-specific reporters for studying cellular behaviour in arthritis. The fluorescent proteins employed in these imaging studies still employ the long-established GFP variants (CFP, YFP etc). To maximise light recovery, increase sensitivity and ultimately enable improved imaging at depth, there has been a concerted effort to develop NIR emitting fluorescent proteins. Such NIR FPs include far-red, GFP-like proteins, such as mCherry and mNeptune with emission peaks ranging from 610 to 650 nm. However, research with bacterial phytochrome photoreceptors (BphPs) are yielding probes emitting in the range of 670 to 720 nm (47). Such NIR emitters not only offer reduced tissue absorption and scattering, but will also complement existing GFP variants, further enabling multiplex studies. Development in pulsed lasers producing wavelengths in the 1000-1300 nm range are suitable partners to illuminate these NIR fluorescent probes using two, and sometimes three photon excitation. Despite these developments, fluorescence imaging in the joints of animal models with cellular resolution is limited to depths of around 0.5mm, which remains a significant limiting factor for both experimental animal imaging and the translation of fluorescence imaging to the clinic.

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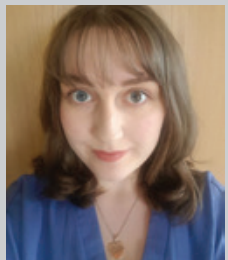
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From the President

As I begin my term, I first want to express my thanks and appreciation to Prof. Michelle Peckham for her wonderful leadership of RMS over the past three years, and to the RMS Council and our great RMS Staff for their amazing work and support.

Of course, our recent international microscopy congress, mmc2019, has been a major success due to outstanding efforts of our RMS members and staff as well as our EMAG colleagues. With an impressive international attendance of just under 1200 (excluding exhibitors), Europe's largest microscopy and microanalysis commercial exhibition, and an exciting, first-class scientific programme, we have become firmly and internationally established as a premier microscopy and microanalysis event! I was extremely pleased to hear many glowing compliments about this congress from attendees – this, of course, is solely due to the incredible efforts of our RMS Sections, Focused Interest Groups, our members, and our dedicated RMS Staff. The RMS Dinner at the impressively

restored Principle Hotel was a major social highlight of mmc 2019 – with a chance to catch up with many old friends and new friends – and even dance!

As I look ahead, one of the areas of special interest to me is engagement with our members, particularly our student and our early career members, as they are the future of our 180-year-old Society. Colum O'Leary, a member of Prof. Pete Nellist's and Prof. Angus Kirkland's research groups at Oxford, is working to establish a group within RMS to bring our current students and early career members together to establish relevant programming and activities of particular interest to the group. I am extremely pleased and delighted to support this effort.

Following on from Michelle's establishment of Focused Interest Groups as a mechanism for bringing together RMS members with a shared interest in activities that fall outside of current RMS Sections, I plan to encourage the formation of new FIGs in RMS, as this will not only advance our scientific and technical goals but address important topics of great relevance to our members. So, stay tuned for more information. Of course, if you already have specific areas you believe would benefit from developing a Focused Interest Group in RMS, please contact us!

Finally, it is with great sadness that I convey the news that two of our past RMS Presidents passed away recently. Prof. Chris Hawes, who was due to stand down as the Executive Honorary Secretary of the Society at the AGM on 3 July 2019, died on 4 July 2019. Chris was a vital member of the RMS, serving as President (2005-6) and Hon Sec until his death. Prof. John Garrett of King's College London was the 1980-1 RMS President and Chair of the RMS 150th Anniversary Committee in 1989. We mourn their passing, and are grateful for their service, accomplishments and friendship, and offer our condolences to their families.

Grace Burke, RMS President.

