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PERSPECTIVE

SPECIAL ISSUE
IMAGING CELL ARCHITECTURE AND DYNAMICS

Back to the future – 20 years of progress and developments in photonic microscopy and biological imaging

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ABSTRACT

In 2023, the ImaBio consortium (imabio-cnrs.fr), an interdisciplinary life microscopy research group at the Centre National de la Recherche Scientifique, celebrated its 20th anniversary. ImaBio contributes to the biological imaging community through organization of MiFoBio conferences, which are interdisciplinary conferences featuring lectures and hands-on workshops that attract specialists from around the world. MiFoBio conferences provide the community with an opportunity to reflect on the evolution of the field, and the 2023 event offered retrospective talks discussing the past 20 years of topics in microscopy, including imaging of multicellular assemblies, image analysis, quantification of molecular motions and interactions within cells, advancements in fluorescent labels, and laser technology for multiphoton and label-free imaging of thick biological samples. In this Perspective, we compile summaries of these presentations overviewing 20 years of advancements in a specific area of microscopy, each of which concludes with a brief look towards the future. The full presentations are available on the ImaBio YouTube channel (youtube.com/@gdrimabio5724).

KEY WORDS: Artificial intelligence, Biological imaging, Microscopy, Nanoscopy, Quantitative imaging

Twenty years of imaging multicellular assemblies

Gaëlle Recher

Exploring recent advances in microscopy for imaging multicellular assemblies is both exciting and challenging. Two-dimensional (2D) cell cultures are easily amenable to most imaging modalities, but imaging three-dimensional (3D) multicellular structures requires more specific, often cumbersome imaging approaches because of increased tissue thickness and consequent light diffusion. Here, we describe a journey into the past 20 years of imaging 'natural' multicellular objects (embryos) and 'manufactured' ones (such as cell assemblies and organoids).

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As the 20th century ended, technological progress brought significant advances. For example, Marvin Minsky's combination of lasers and a scanning microscope provided the first device capable of optical sectioning (Amos and White, 2003; Conchello and Lichtman, 2005; Minsky, 1957). This technology was further enhanced by the advent of genetically encoded fluorescent proteins (Lippincott-Schwartz and Patterson, 2003; Prasher, 1995). These imaging methods were preferentially employed in embryos and slices of tissue such as kidney or brain (Amos and White, 2003). Previously, live and real-time embryo development had mostly been observed using brightfield imaging, which, although offering a comprehensive overview of developmental processes, did not provide in-depth information (Karlstrom and Kane, 1996). Developmental biologists were thus eager to acquire multiscale data from the molecular to the tissue level (Blanchard and Adams, 2011), but depending on the morphogenetic process of interest, they often had to compromise. Achieving ideal conditions for a single imaging parameter (such as high spatial resolution, high temporal resolution, low signal-to-noise ratio or maximum observation length) typically occurs at the expense of another (Keller, 2013).

The multiphoton microscope, derived from the confocal microscope, represented another significant leap forward (Denk et al., 1990). Although multiphoton microscopy (MPM) was not as widely adopted as expected, likely because of the cost of pulsed infrared lasers, it nevertheless facilitated imaging of live embryo morphogenesis by localizing excitation and using infrared wavelengths to image thick specimens while preventing out-of-focus photobleaching (Zipfel et al., 2003). Another feature of MPM is the ability to monitor harmonic signals that rely on the intrinsic organization of matter at the molecular scale. For example, repetitive polymeric molecules like myosin or collagen generate a second harmonic signal (second-harmonic generation, SHG) (Campagnola et al., 2002; Plotnikov et al., 2006; Tiaho et al., 2007), and interfaces between aqueous medium and lipids can produce a third harmonic signal (third-harmonic generation, THG) (Mahou et al., 2011; Oron et al., 2004; Sun et al., 2004), although this non-linear microscopy technique requires careful use to avoid photodamage to the sample (Debarre et al., 2014; Picot et al., 2018; Recher et al., 2011). When, as a PhD student, I read an article from Nadine Peyriéras and Emmanuel Beaurepaire using this approach to image zebrafish embryos (Olivier et al., 2010), not only was I stunned by the aesthetic beauty of this method, but I also realized that it could unlock new insights into morphogenetic cell behaviors.

Light-sheet microscopy (LSM) is an enticing alternative choice for 3D live imaging (Huisken and Stainier, 2009; Huisken et al., 2004) because in contrast to MPM, it minimises photodamage and photobleaching outside the imaging plane. LSM has gained popularity due to its orthogonal configuration, enabling fast imaging and optical sectioning with high versatility (Olarte et al.,

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2018). However, managing the massive amounts of data it generates requires significant technical knowledge and infrastructure for data storage and analysis (Amat et al., 2015). Because LSM was developed by physicists, simpler specimens such as zebrafish embryos and larvae or *Drosophila* pupa were initially the preferred samples for demonstrating proof of principle (Huisken and Stainier, 2009). Later, LSM was applied in more challenging mammalian embryos (de Medeiros et al., 2016). Successful imaging of post-implantation mouse embryos has now been achieved (McDole et al., 2018), even during the technically challenging time window of the peri-implantation stage (Ichikawa et al., 2022).

As these imaging systems were maturing, 3D cell biology systems such as organoids, spheroids, tumoroids, organs on chips and 3D-printed tissues also emerged (Garreta et al., 2021; Ingber, 2018; Lancaster and Knoblich, 2014; Lancaster et al., 2013; Mandrycky et al., 2016; Ozbolat and Hospodiuk, 2016; Sato and Clevers, 2013; Simian and Bissell, 2017; Xu et al., 2005). These systems aim to partially reproduce morphogenetic behaviors as well as some histological and functional features of native tissues. As such, they are a popular alternative to model organisms, especially in the context of the 3Rs (replacement, reduction and refinement) principles for animal research. To image these systems, cell biologists have faced the same difficulties as developmental biologists, but with an additional challenge. Unlike intact embryos, mammalian cells require a culture medium supplemented with serum and must be grown at 37°C in the presence of 5% CO_2. This makes long-term imaging more challenging, as the medium needs to be renewed while avoiding contamination. Nonetheless, advancements such as capturing the live development of intestinal organoids have been achieved, providing insights into stem cell niches and the formation of villi-like structures (Serra et al., 2019). Other groups have gone further by coupling transcriptomics with imaging to screen cancer organoids and decipher the key role of immune cells in combating cancer cells (Dekkers et al., 2023).

Despite the success of MPM and LSM, multiphoton microscopes and light-sheet microscopes are expensive to purchase and maintain, making them barely affordable for an average research group. Nevertheless, they can be accessible through shared microscopy platforms. Research groups for whom microscopy is essential should aim to find a balance between using shared state-of-the-art microscopes and purchasing their own less sophisticated but inexpensive and tunable systems. Recent advances in relatively low-cost optical sectioning microscopy systems, based on add-ons that can be plugged into an existing setup, are particularly exciting. These add-ons (from brands such as Aurox, Confocal.nl, CrestOptics and others) advantageously supplement the offerings from historical microscopy stakeholders (such as Yokogawa, Nikon, Evident, Leica and Zeiss). A striking example is the spinning-disk (also known as Nipkow disk) confocal microscope, where the use of LEDs instead of lasers and the incorporation of more sensitive cameras have made these microscopes more accessible. These systems provide a different and complementary option to those available on traditional microscopy platforms.

Overall, these recent technical breakthroughs in photonics and optical physics are gaining in technical readiness level and, thus, are increasingly available to biologists. Nevertheless, these tools must be supplemented with new methods for preparing biological samples, such as optical clearing, bright fluorescent reporters or mounting techniques that enable the recovery of the imaged sample for further analysis, such as proteomics or transcriptomics.

Twenty years of image analysis boosted by artificial intelligence

Daniel Sage

Over the past two decades, imaging has exerted a significant impact on cell biology research. The imaging of living cells through optical microscopy has become common practice for biologists, both for visually validating hypotheses and increasingly for driving scientific discoveries. The rapid evolution of microscopy has enabled image acquisition at unprecedented resolutions, speeds and depths. Digital microscopy images represent a massive amount of data (Ouyang and Zimmer, 2017), requiring the automated extraction of quantitative information such as morphometric measurements, spatial aggregations, interactions, diffusion dynamics, protein expression and more. In this context, fluorescence microscopy has been fundamental in allowing the direct visualization of specific phenotypes in living cells.

In biology, images are often multidimensional, encompassing the three spatial dimensions (*xyz*), the temporal dimension (*t*) and multiple fluorescence channels (*c*). Processing these '5D' bioimages necessitates a consistent suite of new analysis methods, which has shaped a distinct new discipline known as bioimage informatics (Myers, 2012). To address their specific needs, researchers have developed a range of open-source tools, often as plug-ins for imaging software platforms. One such platform, ImageJ/FIJI (Schneider et al., 2012), has played a particularly important role in uniting the community around common tools. This has helped establish intense collaboration within organizations such as the Network of European BioImage Analysts (NEUBIAS) and ImaBio (including through MiFoBio conferences) and has contributed to the recognition of the distinct profession of bioimage analysts, who are experts in managing and analyzing bioimage data and developing new bioimaging tools.

In addition to image acquisition and data archiving, bioimaging involves three phases: image reconstruction, image processing and image analysis. Image reconstruction, which aims to construct an image based on measurements from various sources and the physical modeling of the acquisition system, exemplifies the deep integration between microscopy and computer science. Super-resolution modalities in microscopy, such as structured illumination microscopy (SIM) and single-molecule localization microscopy (SMLM), rely on these image reconstruction algorithms. Image processing is tailored to microscopy data, taking advantage of its multidimensional nature, and allows for the restoration of weak and transient signals through tasks such as denoising, deconvolution, photobleaching correction, axial information restoration, drift correction and inter-modality correlation.

In contrast, image analysis, which translates all pixels in an image into objects of interest, is largely specific to the type of sample. The tasks of image analysis include feature detection, object segmentation, object tracking, quantification and classification of cells. The most common task is segmentation, which splits the image into different defined regions in order to study objects of interest like nuclei, dendrites or microtubules. Over the past 20 years, the landscape of segmentation algorithms and image analysis more broadly has undergone complete transformation. Currently, two paradigms exist that are often seen as opposing yet complementary. First, model-based methods are used when explicit models of objects of interest are available (including contours of objects, intensities, patterns or visual textures, among others). These methods, based on mathematical models, are conveniently controllable and interpretable. Second, data-driven deep learning (DL) methods rely on supervised training of algorithms from large amounts of example image data.

The data-driven approach has rapidly advanced with the development of highly parallel processors called graphics processing units (GPUs) that are capable of training deep convolutional neural networks (CNNs) with millions of optimized parameters. Since 2015, which marked the emergence of CNN architectures well suited to learning complex and hierarchical representations of images, such as U-Net (Ronneberger et al., 2015), bioimaging has now transitioned into the realm of artificial intelligence (AI). Although DL enables the solving of complex data problems, it raises several challenges. First, the training of algorithms requires extensive computer resources and energy consumption, which are sometimes disproportionate to the intended goal. Second, the training dataset must also contain an exhaustive and unbiased distribution of case studies. Finally, DL functions are difficult to explain or interpret because they consist of multiple layers of millions of interconnected 'neurons', making it almost impossible to trace how decisions are made. These models often operate like a 'black box' (Meijering, 2020).

In the quest for ready-to-use solutions, pre-trained models [such as Cellpose (Stringer et al., 2021), StarDist (Schmidt et al., 2018) and BioImage Model Zoo (Ouyang et al., 2022 preprint)] are highly sought after and widely used for bioimage segmentation. However, these models have been trained for specific tasks on individual microscopy modalities. Recently, foundational models [for example, the Segment Anything model (Kirillov et al., 2023)] have been trained on very large datasets of natural images without explicit direct biological relevance and have shown potential for generalized application in bioimage segmentation tasks. Although these pre-trained models are easy to deploy, it is highly recommended to fine-tune them with specific data to avoid the common pitfall of dataset shift, which occurs when using a model to analyze images outside the training data distribution (Uhlmann et al., 2022). The increasing use of AI in bioimaging highlights the crucial importance of human-verified image databases. Such large, representative and unbiased training datasets must be rigorously curated, as they will form the new backbone of image analysis.

With the emergence of faster image analysis methods, it is also now possible to control image acquisition while monitoring the evolution of observed phenotypes in real time. For example, real-time adaptation of spatial resolution or frame rate allows for selective imaging of a sample only where useful information can be obtained, thereby limiting phototoxicity. This technology, termed 'Smart Microscopy', is beginning to open up additional new areas of development in image analysis (Carpenter et al., 2023).

Over the past two decades, substantial advancements in hardware and software have enabled the resolution of complex multidimensional image analysis problems in microscopy, thereby contributing to the advancement of biological research. More recently, developments in AI have drastically transformed the field, particularly for the most complex bioimage analysis tasks. However, the deployment of AI systems introduces challenges and risks, emphasizing the critical importance of carefully curated datasets to ensure reliable models.

Twenty years of imaging molecular motions and interactions in cells

Cyril Favard

Living is moving. Biology can be thought of as a parallel multiprocessing system consisting of molecules moving and interacting in different locations of the cell to achieve processes such as signal transduction, homeostasis and gene expression. Deciphering and quantifying these motions and interactions directly *in situ* can be achieved using microscopy. Monitoring molecular motions and interactions in living cells experienced a renewal in the early 2000s with the development of chimeric GFP proteins and advanced, user-friendly fluorescence microscopy-based imaging. However, most biophysical techniques for quantifying molecular motions and interactions were developed 30 to 50 years before this renaissance. Fluorescence recovery after photobleaching (FRAP), which is perhaps the most famous imaging technique in cell biology; fluorescence correlation spectroscopy (FCS); and single-particle (molecule) tracking (SPT) are currently the principal microscopic techniques used to quantify molecular dynamics in their biological context. FRAP and FCS were developed in the mid-1970s in the lab of W. W. Webb (Axelrod et al., 1976; Magde et al., 1974), whereas SPT emerged in the late 1980s in the Hollenbeck and Sheetz labs. However, the concept of and first experiments using correlation spectroscopy and SPT under a microscope had already been developed around the beginning of the 20th century (Nordlund, 1914; Svedberg and Inouye, 1911). At this time, theories dealing with reactions, interaction processes and molecular motions were being developed by Einstein, Langevin, Perrin and Smoluchowski – theories that are still extensively used today to describe and quantify these processes in cells. Even so, analytically describing motions in the complex and heterogeneous environment of the cell remains challenging.

Understanding molecular dynamics in crowded environments continues to be a playground for theoretical physicists (Hoefling and Franosch, 2013; Waigh and Korabel, 2023; Woringer et al., 2020). These complex motions are characterized by a non-linear relationship between space and time, and correctly identifying and quantifying these relationships requires experimentally monitoring them over large spatiotemporal scales. Whereas SPT directly allows sampling of spatial heterogeneities in molecular motions, FCS and FRAP were initially conceived to be performed at a single position, preventing detailed spatiotemporal analysis. To address this issue, development of FCS and FRAP before 2010 focused on enabling multiscale spatial sampling in one or two dimensions, either through line scanning (a high-speed scan of a line in the sample, followed by temporal auto-correlation of each pixel in the line separately), spot waist variation (shaping of the point spread function to illuminate successively larger regions of the sample at the same position) or, more recently, imaging FCS (2D simultaneous recording of fluorescence intensity using a high-speed camera, followed by temporal auto-correlation of each pixel, allowing, for example, diffusion maps of the whole cell) (Bag and Wohland, 2014; Salomé et al., 1998; Wawrezinieck et al., 2005). Recent advancements in these fields are tightly connected to the advent of super-resolution optical microscopy, which allows monitoring of molecular motions below the diffraction limit. Super-resolution optical microscopy has enabled real-time tracking of multiple particles with SPT and, in the case of FCS, improved multiscale spatial resolution (from 30 nm to micrometers) and temporal resolution (from microseconds to hundreds of milliseconds) (Eggeling et al., 2009; Manley et al., 2008).

Imaging molecular interactions using fluorescence microscopy often utilizes the non-radiative resonant energy transfer (RET) effect between two fluorophores: namely, a donor and an acceptor. On average, RET occurs over distances below 10 nm. This phenomenon, although initially observed by Perrin in 1910, was first analytically described in 1947 by Förster and later applied in biology by the group of G. Weber. Förster RET (FRET) imaging in cells began to be used at the end of the 20th century. Many different

approaches have been developed, from measuring changes in fluorescence intensities – direct but less precise – to monitoring changes in emission anisotropy – highly complex but more precise (Jares-Erijman and Jovin, 2003). Amongst these, the most popular approach used in biological labs today is imaging fluorescence lifetime changes directly in biological samples (termed fluorescence lifetime imaging microscopy or FLIM). FLIM involves measuring the time lag between the absorption of a photon by a fluorophore and the detection of the fluorescent emitted photon. This time lag is characteristic of both the fluorophore and its immediate environment and does not depend on its concentration, in contrast to intensity-based imaging. In particular, the (spectral) phasor plot approach, which allows the direct identification of FRET in a simplified manner, represents one of several significant advances achieved by the team of E. Gratton (Malacrida et al., 2021).

Recent advances in the field are strongly linked to the appearance of single-photon avalanche diode (SPAD) array detectors, which enable fluorescence lifetime measurements of single or a few molecules in a nanoscopic mode (Koenderink et al., 2022). Single-molecule FRET (sm-FRET) was initially proposed by the group of S. Weiss in 1996 (Ha et al., 1996); however, sm-FRET imaging in living cells has only recently become possible, opening new avenues for understanding molecular interactions in their biological context and native environment.

Thanks to the significant technical advancements of the past 20 years, the observation of dynamics and interactions of (single) molecules now generates a massive amount of data that must be recorded and, sometimes simultaneously, analyzed. AI accelerates this process, either by restricting the amount of data collected or by facilitating parameter extraction from the data (see 'Twenty years of image analysis boosted by artificial intelligence' above). One of the most exciting challenges for the next 20 years is to bridge experimental measurements of single-molecule interactions and dynamics in living cells with (coarse-grained) molecular dynamics simulations, based on the experimentally obtained atomic structures of the molecules involved (Quast and Margeat, 2021). This is now becoming possible thanks to the growing overlap of temporal and spatial resolution accessible in experimental and computational approaches.

Twenty years of fluorescent proteins

Marie Erard

Fluorescent proteins (FPs) are genetically encoded fluorophores derived from marine organisms (Heim et al., 1994). Over the past 25 years, FPs have become indispensable tools for investigating biological mechanisms, not only in basic cell biology but also in drug discovery, preclinical research, microbiology and biotechnology. This remarkable and versatile toolkit has continuously evolved, expanding from a limited color palette to include a wide range of FP emission and excitation spectra (Lambert, 2019).

FPs are characterized by a barrel-shaped structure formed by eleven β-sheet strands. The chromophore results from the autocatalytic cyclization of three residues on a central helix embedded within the barrel structure. FPs exhibit considerable plasticity, as evidenced by the rapid expansion of available colors within the first few years of their introduction, which was achieved through mutation of residues within or in the vicinity of the chromophore (for example, see Shaner et al., 2004). However, the development of near-infrared (NIR) FPs is limited by their chromophore chemistry. To overcome this fundamental limit, another family of proteins – bacterial phytochrome photoreceptors – have emerged as an alternative for NIR probes and biosensors (Chernov et al., 2017; Tang et al., 2021); however, their popularity remains lower than that of FPs, likely due to their relatively modest brightness and the requirement for a mandatory cofactor, biliverdin (the NIR chomophore), with limited bioavailability (Frei et al., 2024). Important developments in FPs over the years include the design of FPs tailored for specific applications and the creation of increasingly selective, sensitive and rapid FP-based biosensors for the accurate detection of almost any cellular function (Greenwald et al., 2018), from the detection of variations in the concentration of ions such as Ca^{2+}, Zn^{2+}, Cl^{-} or H^{+} (Wu et al., 2022), to the study of cell signaling (Frei et al., 2024) and redox processes (Pedre, 2024), or the monitoring of physical parameters as mechanical forces (Fischer et al., 2021). FPs and FP-based biosensors are compatible with a variety of imaging techniques, from widefield video-microscopy to advanced nanoscopy (Frei et al., 2024; Nienhaus and Nienhaus, 2022), enabling detailed spatial and dynamic analysis of molecular events in living cells.

The fluorescence emission of any dye is strongly influenced by its environment, which is why so many fluorescence-based sensors have been developed (Valeur, 2002). FPs are no exception to this rule, and, as optical reporters, they must have known and controlled optical, physical and chemical behavior in complex media such as living cells or tissues. For example, significant efforts have been made to limit the pH sensitivity of FPs, so that they can be employed in acidic cell compartments (Bousmah et al., 2021). Most importantly, the sensitivity of detection in any microscopy experiment is directly determined by the total number of photons that can be collected per fluorophore. This property is governed by the intrinsic molecular brightness of the FP, its ability to efficiently mature its chromophore and its long-term photostability under illumination (Gadella et al., 2023). Advanced imaging and biosensor design require FPs to exhibit the highest possible performance in all these aspects simultaneously. After developing efficient strategies for bright and fast-maturing FPs, photostability remains the next technical bottleneck in optimization. To address this, we might find inspiration from newly discovered FPs such as the highly bright and photostable StayGold, although the mechanism underlying its high photostability remains unknown (Goedhart and Gadella, 2024). Going forward, we anticipate the development of an even wider palette of high-performing FPs optimized for use in a diverse range of cellular compartments and physiological conditions.

Twenty years of small-molecule fluorophores

Luke D. Lavis

At the time of the first MiFoBio conference, 20 years ago, the future of small-molecule fluorophores – synthetic dyes designed by chemists – did not look particularly bright. Chemical dyes had dominated the field of fluorescence imaging for decades as a result of their widespread use in immunofluorescence (Coons and Kaplan, 1950) and the development of optimized commercial dye labels such as the CyDyes (Mujumdar et al., 1993), Alexa Fluor dyes (Johnson, 2010; Panchuk-Voloshina et al., 1999) and ATTO dyes (Arden-Jacob et al., 2001). The field of imaging was rapidly moving towards live-cell applications, however, driven by advances in microscopy techniques and the discovery of FPs (Rodriguez et al., 2017). Although chemists had developed some useful cellular stains and indicators, their functionality could be straightforwardly replicated with genetically encoded fluorophores. Moreover, FPs could be expressed in defined cell types, facilitating the imaging of sparse and specific cells *in vivo*. It seemed that small-molecule dyes

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would soon be eclipsed by other types of fluorophores (Lavis, 2017, 2021).

However, 20 years later, small-molecule dyes remain a key topic at meetings like MiFoBio and beyond. These compounds are an integral part of modern biological imaging, and their continued development represents a dynamic and growing field at the intersection of chemistry and microscopy. So, what turned the tide? The answer is twofold. First, the development of genetically encoded self-labeling tags – proteins that react specifically and irreversibly with small-molecule fluorophore ligands – such as SNAP-tag (Keppler et al., 2003; Lukinavičius et al., 2015) and HaloTag (Cook et al., 2023; Los et al., 2008) was instrumental. These labeling strategies combined the subcellular precision of genetically encoded tools with the diversity of small-molecule fluorophores. Such systems have become important components of the imaging toolbox, complementing the existing and expanding palette of FPs (Rodriguez et al., 2017), particularly in the red region of the spectrum. Self-labeling tags can also serve as scaffolds for indicators, where the improved photostability of the bound fluorophore ligand allows for functional imaging experiments requiring high intensity, such as voltage measurements (Abdelfattah et al., 2019), or facilitates access to long-wavelength sensors for multicolor imaging (Deo et al., 2021; Farrants et al., 2024).

Second, photon-hungry super-resolution microscopy has driven renewed interest in chemical dyes. A common theme among the variety of super-resolution techniques is the trade-off between the numbers of excited states or emitted photons and resolution. SMLM (Betzig et al., 2006; Lelek et al., 2021) uses numerous excitation and emission cycles to determine the location of individual molecules with high precision; SIM (Heintzmann and Huser, 2017) harvests data from several images to create a super-resolution image; and stimulated emission depletion (STED) microscopy (Vicidomini et al., 2018) intentionally depletes excited states, restricting emitting molecules to a sub-diffraction-limited spot. In these contexts, the improved photon yield and photostability of single-molecule fluorophores have proven invaluable, leading to discoveries such as the membrane-associated periodic skeleton in neurons (Sigal et al., 2018).

Rather than fading away, chemical dyes have seen new advances in the past two decades. Looking forward, the future appears bright. Chemists have developed general strategies to enhance the properties of dyes, resulting in new generations of fluorophores (Grimm and Lavis, 2022). Probe developers are now borrowing concepts from medicinal chemistry to develop compounds that are not only bright and photostable but also capable of efficiently traversing cellular membranes and entering tissues and animals (Bucevičius et al., 2023; Grimm et al., 2020). Attendees of future MiFoBio meetings will undoubtedly discuss new experiments, methods and data in which small-molecule fluorophores continue to play an important role in generating images that help us unravel the complexities of biological systems.

Twenty years of multiphoton lasers

Hervé Rigneault

Recent years have seen MPM emerge as a powerful tool for visualizing cellular machinery and tissue architecture. MPM techniques, using lasers with tunable wavelengths to enable excitation of a large variety of fluorescent dyes, generate contrast from the interaction of short laser pulses with cells and tissues. MPM uses infrared lasers that can penetrate deeper into tissue samples than visible lasers, therefore providing deep tissue imaging at depths of ∼300 µm to ∼1 mm. Multiphoton absorption is efficient only at the laser focus, leading to intrinsic optical sectioning. Several multiphoton processes have been applied in biological imaging, including not only multiphoton fluorescence but also harmonic generation imaging and coherent anti-Stokes Raman scattering (CARS). Two-photon excitation fluorescence (2PEF) was first demonstrated in 1990 using a dye laser (Denk et al., 1990) and allowed for unprecedented penetration depth (hundreds of micrometers), as compared to that of conventional one-photon confocal fluorescence microscopy, as well as *z*-sectioning capability. Three-photon excitation fluorescence (3PEF) offers even greater penetration depth (∼1 mm) by using longer wavelengths (1.3 µm and 1.7 µm) for excitation (Maiti et al., 1997).

Harmonic processes (such as SHG and THG) generate higher frequencies of light due to the light–matter interactions produced with short laser pulses. SHG was first demonstrated in 1961 (Franken et al., 1961) and is used to visualize non-centrosymmetric (e.g. chiral or polar) molecular structures in tissues, such as collagen and muscle fibers. THG is similar to SHG and occurs in samples where there is a change in the refractive index, such as at water–lipid interfaces in tissues. THG imaging was first demonstrated in 1997 (Barad et al., 1997) and has been used to visualize lipids, myelin and cell membranes, as well as bones and other mineralized tissue interfaces.

CARS is another MPM technique suitable for visualizing chemical bonds in biological tissues (Müller and Zumbusch, 2007; Rigneault and Berto, 2018) without the need for fluorescent dyes. CARS imaging was first reported in 1982 using gas lasers (Duncan et al., 1982), and Zumbusch and colleagues subsequently demonstrated CARS imaging with solid-state lasers in 1999 (Zumbusch et al., 1999). Unlike 2PEF, 3PEF and SHG, which can be activated with a single laser beam, CARS requires two picosecond laser beams that are spatially and temporally synchronized at the sample plane such that the difference in their optical frequencies matches the molecular vibrational resonance of the sample.

These advances in MPM have been closely linked to the development of ultra-fast lasers capable of delivering pulses with sufficiently high peak power to activate the multiphoton processes. The first demonstration of mode-locked titanium-doped sapphire (Ti:sapphire) lasers, which are capable of producing femtosecond pulses, in 1992 (Curley et al., 1992) was crucial for the development of MPM. Since then, other solid-state laser media, such as Nd:glass (Au et al., 1997), Yb:glass (Hönninger et al., 1998), Yb:tungstate (Druon et al., 2004) and Cr:forsterite (Seas et al., 1992), have been developed. These sources have enabled the dramatic growth of multiphoton and harmonic-generation imaging (Denk et al., 1994; Svoboda et al., 1999; Theer et al., 2003). Standard Ti:sapphire lasers are tunable in the range 700–1000 nm, with pulse energies of 20–30 nJ, pulse durations under 100 fs, repetition rates of ∼100 MHz and average powers of 2 W. Moreover, solid-state lasers can be coupled with optical parametric oscillators (OPOs) to further extend the operating wavelength range for THG and 3PEF imaging (Horton et al., 2013), as first demonstrated in 1993 by T. F. Albrecht and colleagues (Albrecht et al., 1993).

Fiber laser systems (using optical fiber doped with rare-earth elements) have also become particularly suitable for MPM, starting from the late 1990s, with applications in multiharmonic (Millard et al., 1999) and multiphoton fluorescence imaging. Fiber lasers offer several advantages: a reduced need for precise optical alignment, lower repetition rates (∼40 MHz) compared to those of Ti:sapphire lasers (∼80 Mhz) and higher power (Xu and Wise, 2013). For a fixed laser power, a lower repetition rate corresponds to

higher peak intensity and therefore a higher multiphoton signal. However, short-pulse fiber lasers are less tunable than solid-state lasers. This limitation can be addressed by combining fiber lasers with optical parametric amplifiers (OPAs) and OPOs (Cerullo and De Silvestri, 2003). OPAs operate at lower repetition rates (typically 1–100 kHz) and require high-intensity pump lasers, whereas OPOs can be used with small-scale femtosecond pump lasers. These small-scale lasers provide pulses at high repetition rates (100 MHz) (Giordmaine and Miller, 1965; Mak et al., 1992), which is beneficial for detecting weak signals through modulation transfer techniques (De la Cadena et al., 2022) such as stimulated Raman scattering, a process similar to CARS. However, OPAs are easy to operate and offer higher-energy pulses and broader tunability compared to OPOs.

Until recently, most laser systems for MPM operated with high repetition rates (10–100 MHz) and OPOs with pulse energies in the nanojoule range. However, recent advances in 3PEF for neuroscience applications have driven the development of systems with low repetition rates (100 kHz–2 MHz) and pulse energies in the microjoule range for deep tissue imaging (Ouzounov et al., 2017). These systems use high-power Yb lasers to pump OPAs, which can provide several watts of power (10–100 W) at repetition rates of hundreds of kilohertz in the 700 nm–1.7 µm wavelength range (Guesmi et al., 2018). Although most of these laser technologies have primarily been used in point-scanning non-linear optical microscopes, recent developments suggest that OPA-based high-power laser systems are suitable for widefield MPM with extended fields of view and higher frame rates (Fantuzzi et al., 2023).

Today, MPM is used in many life science fields, mostly to image deep inside tissues. The associated laser technology is still complex and expensive; however, it is expected that further advancements in laser technology, such as fiber lasers, will decrease the complexity and cost of MPM systems and will continue to drive the future adoption of MPM.

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Competing interests
Luke D. Lavis is a scientific cofounder, consultant and shareholder of Eikon Theraputics, and is an inventor on patents and patent applications describing fluorescent molecules. All other authors declare no competing or financial interests.

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