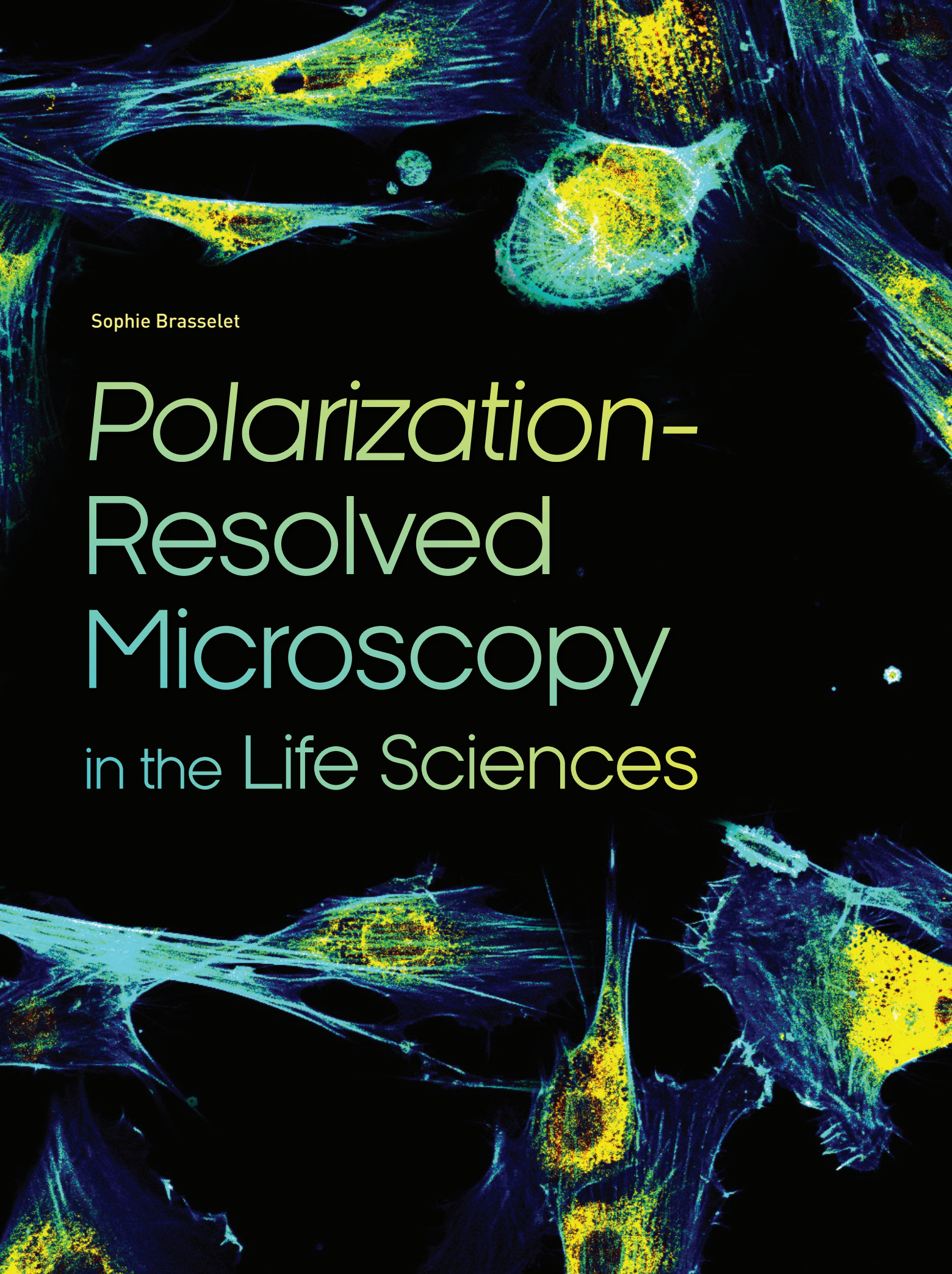
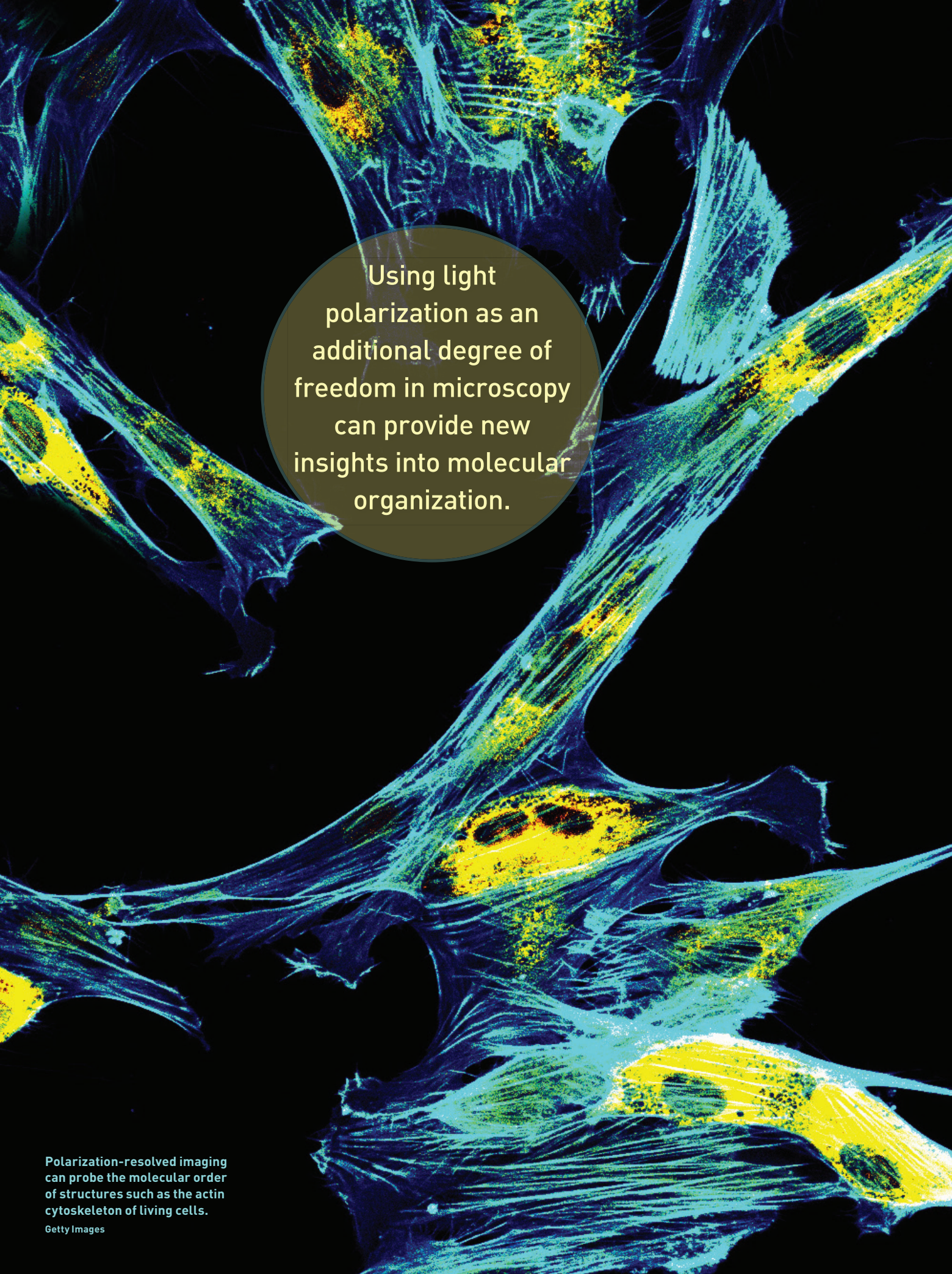


Sophie Brasselet

Polarization-Resolved Microscopy in the Life Sciences



Using light polarization as an additional degree of freedom in microscopy can provide new insights into molecular organization.

Polarization-resolved imaging can probe the molecular order of structures such as the actin cytoskeleton of living cells.

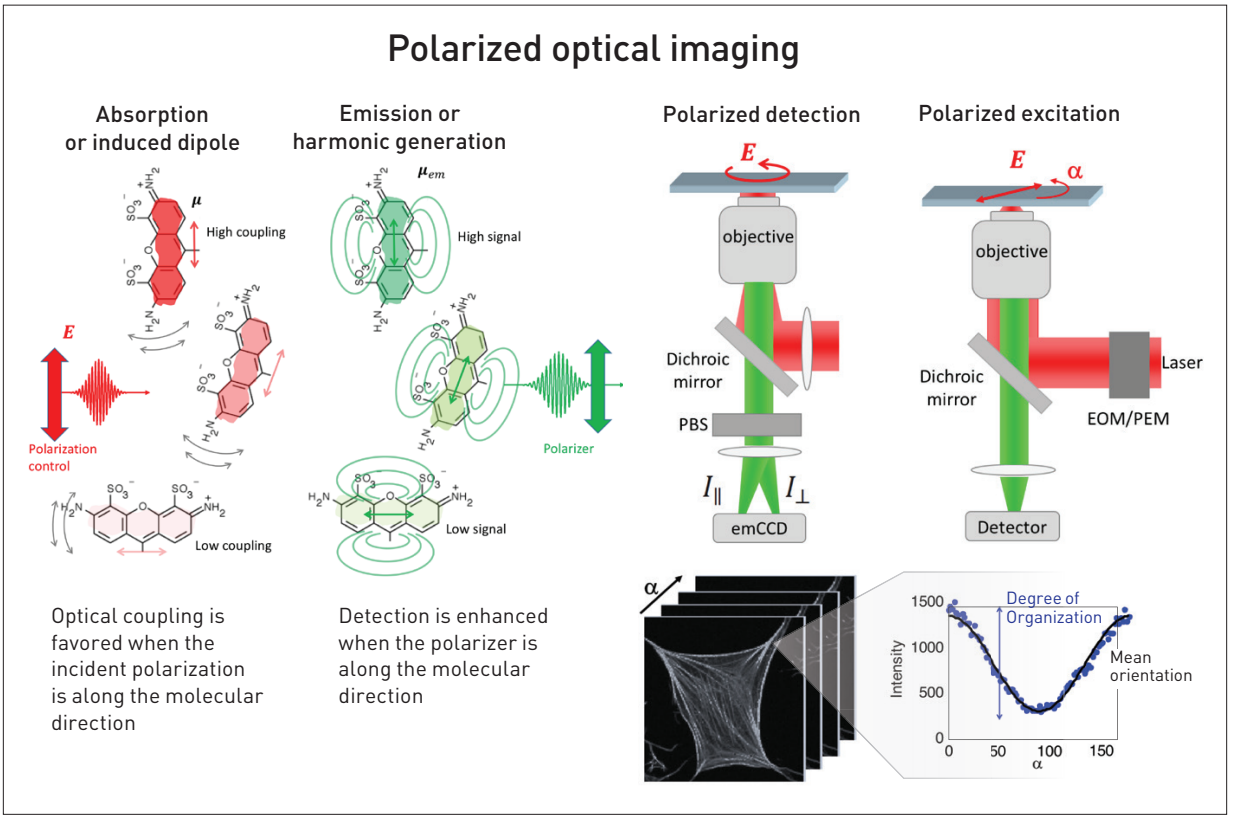
Getty Images

Optical microscopy offers invaluable access to biomolecular processes at sub-micron and even single-molecule scales. It occupies a unique position between techniques such as magnetic resonance imaging and ultrasound, which provide deep penetration but low spatial resolution, and others such as electron microscopy and X-ray diffraction, which yield sub-nanometer resolution but cannot image live organisms. Optical microscopes can image live organisms and tissues at relatively high spatial resolution (around 200 nm), large fields of view (up to millimeters) and reasonable penetration depths (a few hundreds of microns). And optics can specifically address biomolecules such as proteins, DNA and lipids in real time with single-molecule sensitivity using fluorescence labelling, or even via label-free imaging by taking advantage of nonlinear optical effects in tissues.

These advantages have carved out a place for optical imaging in the study of fundamental stochastic interactions in cell biology, and have opened up ways to produce super-resolution images with spatial resolutions down to 10–20 nm. Optics is also compatible with functional studies, using techniques such as genetic modifications, mechanical perturbations and electrical transient recording in neurons, allowing researchers to understand key biomolecular processes behind important questions in immunology, morphogenesis and neuroscience—and helping point the way to potential solutions for pathologies such as cancer, neurodegeneration and genetic disorders.

Beyond the attributes listed above, however, optics possesses another degree of freedom not accessible in other methods: light polarization. The direction of the oscillating electric field emerging from the light source (for linearly polarized light) can easily be controlled using polarizers, modulated using electro-optic modulators (EOMs) or photo-elastic modulators (PEMs). Whatever the optical process used—be it fluorescence, reflectance, optical coherence tomography (OCT), coherent nonlinear optics or something else—the strength of optical signals generated from illuminated molecules is fundamentally sensitive to the relative orientation between the molecules and the polarization of light.

This makes it possible to envision using light not only to image biomolecules, but to flesh out the details of their orientation and organization. That kind of information, usually absent even from high-resolution optical imaging, can have relevance in understanding and treating disorders ranging from neurodegenerative diseases to



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Polarization-resolved imaging allows the study of non-isotropic organization, revealing how molecules are oriented in cells and tissues.

cancer. This article looks at the state of the art for polarization-resolved optical imaging in biomedicine.

Measuring molecular order

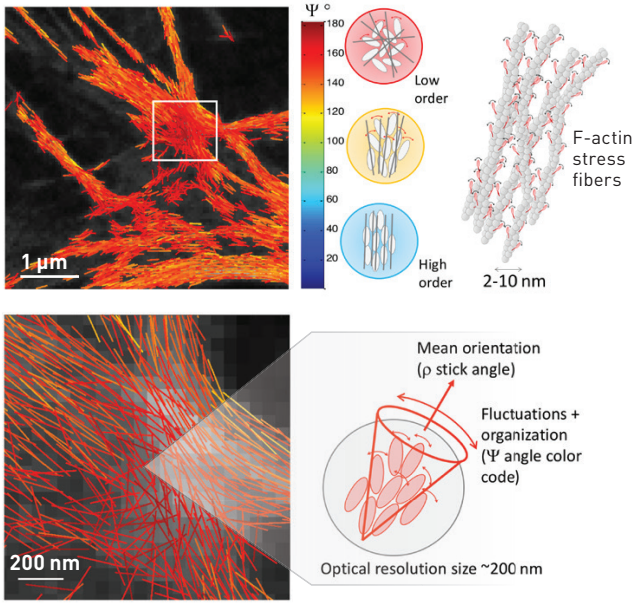
Biochemists and biologists have used polarization, in the form of fluorescence anisotropy, in research since the 1950s. By illuminating and detecting, with polarized light, fluorophore-labeled proteins and molecules in viscous solutions, these scientists could measure the steady-state ratio between the rotational diffusion time of the fluorophores and their fluorescence lifetime, and thereby decipher the rotational dynamics in large, complex systems.

It is only decades later, with recent progress in optical microscopy, that polarized optical imaging has emerged as a way to access molecular orientation at the sub-micron scale. Such imaging requires full control of the polarization of the incident light, the detected light or both. With an appropriate optical setup, however, that can be achieved not only in fluorescence imaging but also in label-free nonlinear coherent imaging such as second-harmonic generation (SHG), third-harmonic generation (THG), and coherent Raman scattering.

In particular, polarization-resolved imaging allows the study of non-isotropic organization, revealing how molecules are oriented in cells and tissues. This is expressed via a quantity called molecular order—the distribution of angles explored by the probed molecules over time and space, defined by a mean orientation and an angular fluctuation aperture. The molecular order essentially gives an average over the space and time scales of the imaging system—for an optical microscope, around 200 nm and between several tens of microseconds to a second—of many molecules undergoing natural angular fluctuations. Within that window, molecular order can range from very small angular fluctuations (very high order, as in a crystal) to a completely isotropic distribution (very low order).

That range can be important for biological and biomedical study. Many biomolecular assemblies, such as lipid membranes, chromatin, and protein aggregates and filaments like microtubules and F-actin, are fundamentally organized; a read-out of molecular order can suggest potential problems in that organization. Orientation also plays an important role in the efficiency of fundamental biological processes, such as the generation of forces in cells and muscle tissues and the efficiency of signaling processes by protein clusters.

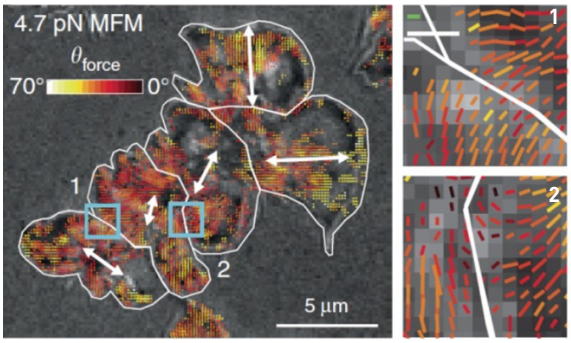
A few approaches exist for revealing molecular orientation, including X-ray or electron paramagnetic resonance (EPR) spectroscopies, which do not permit live biological imaging. This makes polarized microscopy a unique tool to address still-unsolved questions, such as the role of the orchestrated interaction between cytoskeleton proteins in biomechanics, with consequences in morphogenesis, muscles disorders and tumor proliferation; the role of conformational changes



Fluorescence polarization imaging

Polarization-resolved fluorescence microscopy reveals information that is not present in a pure intensity image. Each pixel is encoded with a color representing the molecular order within each focus position, e.g., the mean orientation and averaged angular extent of fluorescent molecules attached to actin fibers.

Data from Institut Fresnel (courtesy of C.V. Rimoli and M. Mavrakis)



Mapping molecular forces

In one example, fluorescence polarization microscopy, using fluorescent molecules as "tension sensors," helped to detail the magnitude and direction of piconewton molecular forces at the adhesion surfaces between blood platelets—information that could illuminate studies of blood coagulation and disorders related to it.

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of membrane proteins in cell signaling and adhesion; and the role of the conformation of DNA and RNA in transcription processes. The following sections provide some examples of how polarization-resolved techniques, applied to fluorescence and label-free microscopy, are casting light on those questions.

Fluorescence: Revealing protein organization

Polarized fluorescence microscopy rests on the intrinsic dependence of fluorescence on the orientation of both the fluorescent molecules and the light polarization. Fluorescence is a two-step process involving absorption followed by emission. Absorption efficiency is highest when the incident-light polarization lies in the direction of the fluorophore's absorption dipole moment—roughly, along the direction of oscillation of its bound electrons within its conjugated backbone. Emission efficiency is driven by the fluorescence quantum yield, with the emission polarized along the field radiated by the emission dipole, which also relates to the molecular structure.

Molecular organization can thus be probed either at the absorption level, by tuning the incident polarization, or at the emission level, by projecting the emission beam on different polarization states. Typically four polarizations (incident or detected) are required to retrieve, per pixel in an image, the two parameters that define molecular order—mean orientation and angular range of fluctuations—within the 200-nm spatial resolution.

Many recent studies have used polarized fluorescence imaging, with tuning of the incident polarization, to retrieve molecular organization. Such studies, using fluorescent lipid probes as reporters, allow quantitative assessment of lipid order in cell membranes, which encompasses not only local packing effects due to lipid compositions but also sub-diffraction morphological effects due to local, nm-scale modifications in the membrane. Such membrane organization in living organisms can open a window to complex phenomena, such as during the fusion between cells in immunological processes. Researchers have also probed the orientation of membrane proteins in living organisms, using, for example, the green fluorescent protein (GFP), fused to the protein of interest at specific points where the angular fluctuations are minimized.

More recently, polarized fluorescence microscopy has been applied to study the organization of filaments in cells and tissues—addressing, in particular, the still poorly understood process by which these proteins affect changes in cell shape. By revealing the organization of septin during cell division in yeast cells, for example, the technique helped solve a long-standing controversy on cell dynamics not visible using other techniques.

Polarized fluorescence microscopy has also revealed high-order organization of actin filaments (F-actin) in the early development in *Drosophila* (fruit fly) embryos, as well as during the formation of the sarcomeres in the pupae muscle, offering a new way to observe tissue organization that is considerably less invasive than EM. And polarized fluorescence has recently emerged as a tool for measuring forces mediated by proteins, as demonstrated using tension sensor labels attached to intregrin, a protein responsive to forces at the adhesion sites of cells.

Several challenges remain open in polarized fluorescence microscopy. One is that, strictly speaking, the molecular organization reported is that of the fluorescent label, attached to the protein of interest using a linker. For that reason, intrinsic angular fluctuations of the fluorophore exist that are generally not known and that prevent obtaining absolute values of protein orientations—instead, measurements are performed relative to a reference control sample. Second, molecular organization is probed in the sample plane, in which the incident polarization is controlled. The measured order is therefore "projected" in the sample plane, which limits the study of 3-D geometries. New

Polarized super-resolution microscopy has been used to probe the organization of structures in DNA and in amyloid proteins, which are thought to contribute to neurodegenerative diseases.

methods are under development to achieve polarization control in 3-D. For instance, a phase plate that imparts a radial polarization in the excitation (or emission) path of a microscope can create (or detect) a longitudinal polarization at the focus of a high-numerical-aperture objective, enabling the access to molecular orientation along the longitudinal direction.

Super-resolution: Quantitative structural imaging

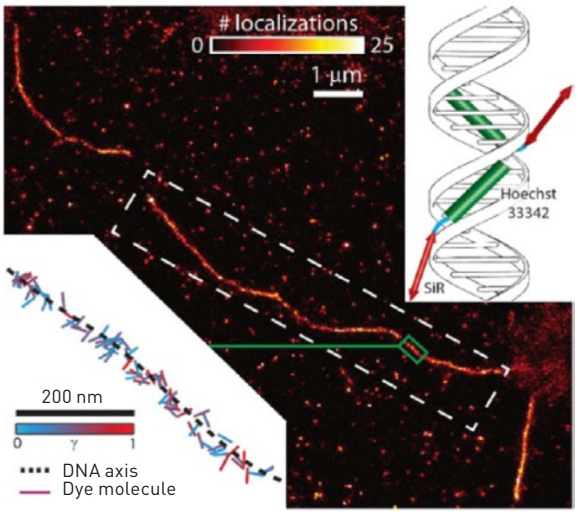
One way to address intrinsic angular fluctuations of fluorophores is to measure them by imaging single molecules, which is possible using sensitive cameras. Polarized single-molecule signals provide access to the angular range explored by the molecules—the "wobbling angle"—and their mean orientation during the measurement's integration time. Such molecular wobbling is usually hidden in ensemble measurements; quantifying it molecule by molecule allows one to correct those intrinsic fluctuations and retrieve the protein's real structural organization. Such a measurement can be achieved by rotating the incident polarization, but that requires the ability to measure a single molecule for a long period, a challenging undertaking.

A more appropriate technique is to control fluorescence polarization at the *emission* stage rather than at the excitation stage. A few recent studies have used polarization beam splitters to divide the fluorescence image of many single molecules into different states of polarizations. Tapping single molecules in this way offers interesting capabilities for super-resolution imaging based on stochastic localization, provided the molecules switch between on (fluorescent) and off (non-fluorescent) states. This principle forms the basis of super-resolution imaging methods such as stochastic optical reconstruction microscopy (STORM) and photoactivation light microscopy (PALM).

Super-resolution imaging today offers 3-D spatial resolution down to tens of nanometers. Accessing the orientation of single molecules in addition to their location can give this exquisite technique another dimension: it not only provides a high-resolution estimate of the spatial morphology of molecular assemblies, but also enables unique insight into the way molecules are organized within these assemblies—in a way that is not biased by their wobbling. This opens access to invaluable new pieces of information on the conformation of protein assemblies.

Polarized super-resolution microscopy has been used in this way to probe the organization of structures in DNA and in amyloid proteins, which are important protein aggregates thought to contribute to neurodegenerative diseases. Current work is focusing on taking the picture further, and on the possibility of retrieving, from polarized single-molecule images, their still-missing 3-D tilt angle of orientation, based on a careful analysis of the way the shape of a single molecule's point-spread function deforms depending on this 3-D angle.

DNA orientation imaging



Deciphering DNA dynamics

Using beam splitters or electro-optic modulators (EOMs) to sample polarization of emitted light from individual dye molecules, serving as tags on biomolecules, allows orientation measurements at the single-molecule level. The method has permitted the study of nanoscale deformations DNA filaments *in vitro*—a study with potential relevance to drug development.

A.S. Backer et al., Optica **3**, 659 (2016)

Nonlinear label-free imaging: Early-stage diagnosis

Since the late 1990s, nonlinear optics has offered unique capabilities for microscopy at depths of up to a few hundreds of microns in complex biological media, where visible light is absorbed or scattered. Nonlinear microscopy uses near-infrared excitation wavelengths and pulsed excitation regimes, which provide high efficiencies for label-free imaging. Commonly, these studies use Ti:sapphire laser oscillators with roughly 100-fs high-peak-power pulses and 80-MHz repetition rates.

A typical nonlinear microscope uses a point-scanning excitation modality with galvanometric mirrors, and enjoys the strong advantage of natural "depth sectioning" due to the nonlinear dependence on the incident intensity. Nonlinear coherent optical processes originate from induced dipoles created by the nonlinear interaction of light with matter, which creates radiating dipoles that are in phase with the incident excitation field. This process is inherently polarization dependent—and thus opens the prospect of polarization-resolved imaging in these nonlinear microscopy systems.

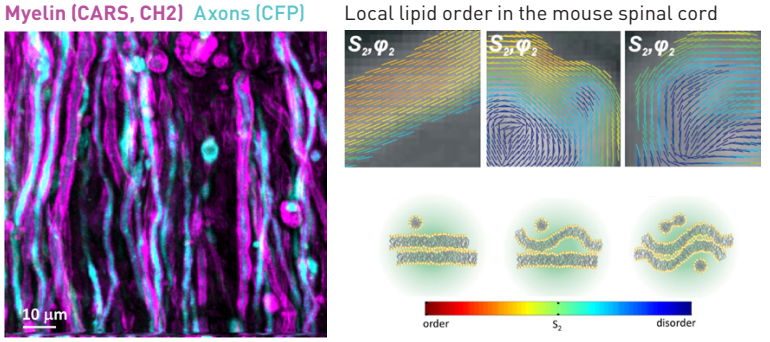
One such nonlinear process, second-harmonic generation (SHG), results from the excitation of a system at the fundamental optical frequency, ω, into a scattering at the double frequency, 2ω. In biological tissues, coherent SHG occurs in organized structures such as tissues rich in collagen I, skeletal muscles and microtubules. SHG imaging and its polarized version are today exploited as a functional contrast for diagnosing pathologies in particular cancers, for the study of aging, or for understanding of the mechanics of conjunctive tissues in which stiffness strongly depends on collagen content and structure.

A promising label-free nonlinear coherent imaging method for future diagnostics is polarized coherent Raman scattering. In particular, coherent anti-Stokes Raman scattering (CARS) allows vibration bonds of molecules in cells and tissues to be monitored in real time and with high chemical specificity, and permits chemical species in biological samples to be distinguished at sub-second timescales.

CARS relies on the sample's illumination by two synchronized lasers at the Stokes (ω_s) and pump (ω_p) frequencies, which results in a nonlinear induced anti-Stokes radiation (ω_{as}) that's enhanced at the resonance condition, $\omega_p - \omega_s = \Omega_r$. The resulting signal is strongly sensitive to incident-light polarization when the probed vibration bonds are correctly oriented. That makes CARS an interesting probe for the molecular orientation inside a focal spot typically 300–500 nm in size.

This method has revealed high-molecular-organization CH_2 vibrational stretching bonds in lipids, providing considerable information on lipid order without any chemical label. Lipid-order imaging by polarization-resolved CARS has been used in mice to probe the architecture of myelin, a lipid-rich substance that forms dense multilayer sheaths around axons in the brain and spinal cord. This myelin architecture is crucial for structural integrity in the central and peripheral nervous system, with important consequences in neuropathology.

Recently, fast polarization modulation using EOMs, coupled with direct detection by demodulation of the signal on a per-pixel basis (using a lock-in amplifier), has sped up polarization-resolved CARS imaging considerably—to a rate of one molecular-order image per second. That speed could permit "live" CARS polarization imaging, a unique way to assess myelin organization *in vivo*. Other versions of CARS polarization sensitivity have used circularly polarized beams capable of measuring the level of molecular order in a single shot.



Polarization-resolved nonlinear label-free imaging

Coherent anti-Stokes Raman scattering (CARS) microscopy, a label-free imaging method, can reveal lipids—such as those present in the myelin sheath around axons in the spinal column—through sensitivity to their CH_2 vibrational bonds. Here, polarized CARS imaging, which can read out molecular orientations, reveals regions of damaged lipid order. P. Gasecka et al., Biophys. J. **113**, 1520 (2017)

A promising label-free nonlinear coherent imaging method for future diagnostics is polarized coherent Raman scattering.

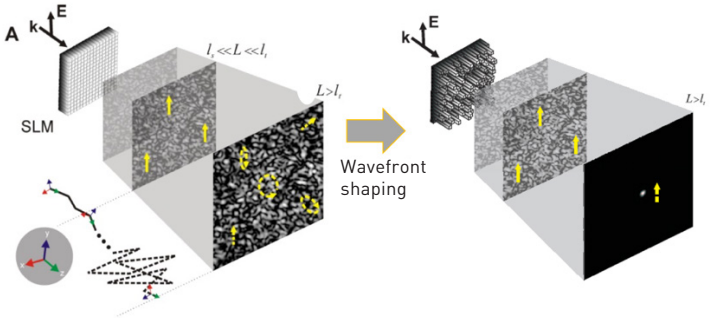
The advances outlined above demonstrate the unique potential for polarized nonlinear microscopy, and polarized CARS microscopy in particular, as a tool for investigating molecular order as a potential marker for early-stage damage and for quantitative diagnosis.

Toward polarized deep-tissue imaging?

Unfortunately, while polarization-resolved imaging has proved a valuable tool for assessing molecular order near the surface of samples, it is currently impossible in deep biological media. That's because light propagation through multiply scattering media, such as tissues hundreds of microns or more in thickness, inherently scrambles polarization states. After excitation of a well-defined polarization state, each successive scattering event in the thick tissue will rotate the propagation direction and therefore the polarization directions, inevitably leading to a random intensity pattern (speckle) containing multiple polarization states.

One possible solution may come from wavefront shaping (WS). By using spatial light modulators (SLM) to control the incident wavefront, WS can coherently control the speckle pattern generated from multiple scattering events, and thus can increase the energy density at targeted positions, building constructive interference locally. Recent research has shown that WS can reconstruct a focus through a scattering medium and allow recovery of the excitation beam's initial polarization state.

WS has revolutionized optical imaging in complex scattering media, allowing spatial and temporal refocusing of light through or inside scattering media through manipulation of the wavefront phase or amplitude. Further, it's been shown that using broadband source to perform WS can preserving the polarization state of the incident beam through a millimeter of biological tissue, via coherent gating inside the medium that preferentially selects short paths of photons in the propagation. This finding opens a potentially promising future for polarization-controlled processes in scattering media—and for zeroing in on molecular order in structures that lie deeper than we can access today. OPN



Polarization recovery in scattering samples

Wavefront shaping through scattering media can recover not only a focus but also the incident polarization of the light, a promising route to polarization nonlinear imaging at depth in scattering biological tissues.

H.B. De Aguiar et al., Sci. Adv. **3**, e1600743 (2017)

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