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Correlative imaging for polymer science

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Abstract
The characterization of polymeric materials is key towards the understanding of structure–activity relations and therefore for the rational design of novel and improved materials for a myriad of applications. Many microscopy techniques are currently used, with electron microscopy, fluorescence microscopy, and atomic force microscopy being the most relevant. In this perspective paper, we discuss the use of correlative imaging, that is, the combination of multiple imaging methodologies on the same sample, in the field of polymeric materials. This innovative approach is emerging as a powerful tool to unveil the structure and functional properties of biological and synthetic structures. Here we discuss the possibilities of correlative imaging and highlight their potential to answer open questions in polymer science.

KEYWORDS
atomic force microscopy, correlative imaging, electron microscopy, material characterization, super-resolution microscopy

1 | INTRODUCTION

Ever since their invention in the late 16th century, microscopes have been used to document the observations of micrometer-sized biological objects such as bacteria, cells, and muscle fibers. Since then, microscopy techniques have been one of the major approaches to study not only biological objects but also synthetic materials on a wide range of spatiotemporal scales, expanding visualization into the microscopic and even nanoscopic regimes.1

As a natural extension of unaided eyes, light microscopy (LM), electron microscopy (EM), and atomic force
microscopy (AFM) are currently in widespread use to characterize the structure and properties of polymeric materials, and this field progressed with an impressive speed in the last decade making available a variety of novel methods for materials scientists.

Dutch spectacle makers Zaccharias Janssen and Hans Lipperhey are noted as the first men to develop the concept of the compound microscope, at that time a tube with several lenses. Later in the 16th century, Antoni van Leeuwenhoek perfected the manufacturing of lenses allowing him to investigate living microorganisms but also fabrics in great detail, being probably the first application of microscopy to materials science.

Fluorescence microscopy is a pivotal approach due to its accessibility, high signal-to-background ratio, chemical specificity and multicolor ability. While

**BOX 1**  **Brief description of the principles of the imaging methods described.**

SRM (general): super-resolution microscopy (SRM) is a series of optical microscopy techniques that features resolutions higher than those in conventional optical microscopy limited by diffraction of light.

STED: stimulated emission depletion (STED) microscopy is a deterministic super-resolution fluorescence microscopy technique that realizes diffraction-unlimited resolution by shrinking the illumination area/volume through selective deactivation of fluorescent molecules.

SMLM (PAINT, STORM, PALM): single molecule localization microscopy (SMLM) consists of a series of stochastic single-molecule fluorescence techniques that realize diffraction-unlimited resolution by repeatedly imaging and localizing a small random subset of fluorescent molecules. Points accumulation for imaging in nanoscale topography (PAINT) is a subfamily of SMLM that achieves stochastic repeated imaging of single molecules by molecular adsorption/absorption and subsequent photobleaching/desorption. Stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM) utilize sequential activation and localization of photoswitchable and photoactivatable fluorophores to create high resolution images.

SIM: structured illumination microscopy (SIM) is a type of super-resolution fluorescence microscopy that is based on the excitation of the sample with a high spatial frequency pattern of light (Moiré fringes). Subsequent computational reconstruction provides a doubled resolution compared to Abbe’s diffraction limit.

TEM and cryoTEM: transmission electron microscopy (TEM) is a microscopy technique based on the transmission of an electron beam through a sample to form an image. cryoTEM is a method that facilitates the investigation of samples in a liquid and uses rapid cooling of thin liquid layers for preparation of vitrified specimens that then can be imaged at cryogenic conditions in TEM.

SEM: scanning electron microscopy (SEM) is a microscopy technique based on scanning an electron beam across the sample surface to form an image based on the detection of secondary electrons or back-scattered electrons.

AFM: atomic force microscopy (AFM) is a type of scanning probe microscopy (SPM) with high resolution on the order of a nanometer. AFM is based on gathering morphological information by scanning a mechanical probe across a sample surface and recording its height corresponding to a constant probe-sample interaction.

Soft X-ray microscopy: an imaging technique with a spatial resolution in between that of optical and electron microscopy wherein contrast between chemically distinct components in a specimen arises from differences in transmission of soft X-rays (2.34–4.4 nm) in the so-called water window.

SAXS: small-angle X-ray scattering (SAXS) is a technique used for materials characterization to a.o. obtain structural information on length scales of 1–100 nm (e.g., particle size, shape, crystallinity) through an analysis of the pattern of monochromatic X-rays scattered towards small angles (usually 0.1–10°).

Raman microspectroscopy: Raman microspectroscopy is a microspectroscopic technique that relies on inelastic scattering of photons, or Raman scattering, which is a reporter of a molecule’s chemical fingerprint. Raman microspectroscopy uses a diffraction-limited optical system to collect both spatial and spectral information across a sample, achieving imaging with chemical identification.

Laser speckle imaging: laser speckle imaging is an imaging technique that is used to visualize dynamics within materials, can be applied to turbid materials, and accesses a wide range of timescales. Speckle patterns arising from interference of monochromatic laser light are recorded and the fluctuations herein analyzed to quantify motion within the specimen in a spatially resolved manner.

WANG ET AL.
diffraction limits the resolution of traditional light-based microscopes to a few hundred nanometers, scientists have recently made exciting breakthroughs to bypass this limit. Following pioneering work of single-molecule imaging in the 90s 2 a series of super-resolution microscopy (SRM) or nanoscopy methods have been developed with a resolution typically one order of magnitude higher than the diffraction limit. 3

Despite advances in SRM, polymeric materials often contain structures <20 nm which requires access to even higher resolution approaches. This can be achieved by employing electron beams (EM) to image the sample, or by using atomically sharp tips that scan the sample topography (AFM). EM extends the optical diffraction limit by using electrons as an illumination source which typically exhibit a wavelength > 1000 times smaller than light. However, for polymeric materials the electron beam sensitivity sets the limit in achievable resolution. Recent developments in cryo-EM and liquid EM now allow imaging of non-stained materials in native conditions and/or of dynamic phenomena with nanometer resolution.

AFM employs nanometer-sized tips for sample surface scanning. With potentially atomic level resolution AFM is broadly used in materials science to probe the morphology of materials and coatings. However, the technique has always been limited by slow speed and poor chemical specificity. Recent developments in high-speed AFM dramatically improve time resolution making it possible to visualize dynamic phenomena. 4 Moreover, using dedicated instrumentation sensing mechanical forces, electrostatic and molecular properties expand the possibilities of AFM to new dimensions. 5

Looking at the whole field of imaging it is clear how every technique has its own strength and weaknesses, and the different methods are highly complementary. The strengths of optical microscopy happen to be the weaknesses of EM and AFM and vice versa (Box 1).

This has led to the development of a toolbox of correlative approaches that exploit the strengths of each individual technique. In correlative imaging the same sample is visualized with two or more techniques and the images for the same field of view are superposed (Figure 1). This allows to look at the same object from multiple angles, to obtain a more comprehensive and informative “picture” than any of the individual tools can offer, trying to obtain “the best of all worlds.” Correlative microscopy (CM) is in its formative years but the potential benefits for materials characterization are tremendous. This perspective paper discusses the impact of correlative imaging on polymer material research and offers an introductory guide to select the most suitable (combination of) techniques to address some of the most pressing open questions in the field of polymer science.

1.1 | State of the art in correlative imaging

Given the many methods currently available, a large number of correlative combinations are possible and desirable. Herein, we will mainly focus our discussion on two combinations, EM with SRM and AFM with SRM, because of their complementarity and major impact in the field (Figure 2).

Optical microscopy correlated with EM has shown great potential to bridge the resolution gap and has proven to be a powerful correlative microscopy (CM) technique for biological samples. Several timely and exhaustive review articles have appeared on the technical aspects of CM that we will only summarize here. 6 Correlative light electron microscopy (CLEM) started early from the 60s to 90s as an emerging technique to visualize subcellular content in the same cell. The technical issue of the big resolution gap between the two methods was resolved later by the earliest efforts of super-resolution correlative light-electron microscopy (SR-CLEM) was achieved by Betzig et al. in 2006, in which PALM images of intracellular FP-tagged proteins were correlated with EM images in the same cryosectioned mitochondria matrix. 7 Since then, super-resolution correlative microscopy has been applied extensively to resolve the ultrastructure of cellular samples. Both single-molecule localization microscopies (SMLM) 8 and STED 9 have been correlated with EM for specific protein and cellular content localization.

Overall the combination of EM and SRM is one of the most used but its full potential has still to be explored and innovations in this fields are maturing such as the development of CLEM in cryogenic conditions. 10, 11

SRM–AFM was also first successfully applied to biological samples. Harke et al. employed STED-correlated AFM to successfully localize 40 nm fluorescent beads and cellular structures. 12 This method was further expanded for a variety of samples such as labeled amyloid fiber aggregates, and revealed possible artifacts of fluorescence imaging caused by dye interactions with biomolecules. 13 Also, TIRF-based SMLM was correlated with AFM with an integrated SRM–AFM setup used to probe single DNA protein complexes. 14

1.2 | From biology to materials: Pioneering applications of correlative imaging to synthetic structures

In recent years, research fields like materials science and nanotechnology have also adopted CM to study synthetic material systems. Standalone optical techniques have
already been employed to study synthetic nanomaterials such as nano- and micro-particles, polymers, films and self-assembled materials, showing structure and dynamics on unprecedented length scales. This highlights the impact of nanometer spatial resolution in the study of synthetic materials and has sparked the application of correlative imaging methods that combine an even higher spatial resolution of EM and AFM with the chemical specificity of optical microscopy. Beuwer et al. demonstrated a wide-field fluorescence measurement of fluorescent supramolecular polymer correlated with liquid AFM, showing that mechanical properties of single fibers can be determined thanks to nanometric resolution of the correlated AFM. With a similar approach, Taylor et al. demonstrated DNA-PAINT optical nanoscopy correlated with AFM on single gold nanoparticles while

FIGURE 1 Schematic representation of the correlative imaging process

FIGURE 2 Correlative SRM imaging with a collection of techniques, (A) PALM correlated with EM with scale bar 1 μm. From Reference 7, reproduced with permission from AAAS; (B) SMLM correlative with cryoEM. From Reference 10, redistributed under Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CC BY-NC-ND); (C) STED correlated with confocal and AFM. From Reference 12, redistributed under the terms of the Creative Commons Attribution 2.0 International License.
reaching 5 nm localization precision of single DNA docking strands.17

2 | FOLLOWING THE LIFE CYCLE OF A POLYMER WITH CORRELATIVE IMAGING

In the following sections we will discuss the potential of correlative microscopy to contribute answering open questions in the field of polymer science. In this framework we will follow the life cycle of a polymeric material discussing the information that can be gained with CM along these key steps. Figure 1 shows schematically the steps we tackle from the design of a material to its final fate. For each one the most suitable CM combination is discussed, and the potential outcomes discussed (Figure 3).

2.1 | Assembly and processing

The first stage in the life cycle of a polymer material after synthesis is its (multistep) assembly into primary particles of varying shape and size. These building blocks may subsequently be transformed, assembled or further processed downstream to generate functional materials with complex multiscale morphologies. This first step is, therefore, critical as it largely determines the morphology and properties of the material.18 Following the (self-)assembly of polymers into aggregates can be followed by scattering approaches that provide ensemble properties directly in solution at sub-ms timescales and at mesoscopic to nanoscopic length scales. Equally applicable are spatially resolved imaging techniques that provide access to single-particle properties such as dispersity in shape, size, and sometimes composition/functionalization.

To date, most imaging studies have utilized (cryogenic) transmission electron microscopy (spectro) for nanoscale imaging of polymeric assemblies in a near-native vitrified environment.19 Alternatively, super-resolution optical microscopy (SRM) and fluorescence microscopy (FM) that image gently immobilized or spatially confined structures in liquid environments may be employed, nevertheless, at resolutions of tens and hundreds of nanometers, respectively.20

These reports illustrate the pros and cons of the different tools, and concomitantly the benefits of their combined application, that are able to follow a material “live” but with less resolution (FM) or at the ensemble level (scattering) and measurements that provide a wealth of information but in near-static conditions (EM and SRM).

An interesting possibility here is to use correlative live-fixed imaging: that is, first monitor assembly and processing with a liquid-based “live” technique (e.g. rheo-scattering or fluorescence microscopy) followed by in situ fixation or vitrification that allows to perform a second imaging round with higher resolution on the same exact field of view to zoom in into a specific structure during the evolution of its assembly.

2.2 | Structure and properties

First and foremost, microscopy techniques are employed to visualize and quantify the structural properties of
materials of interest. Yet, many of these tools offer further information beyond size and morphology can be obtained by correlating different imaging modalities. AFM can be used to map mechanical or electrical properties of materials by the use of suitable cantilevers. Fluorescence-based techniques benefit from the environmental sensitivity of dyes, that is, dyes whose emission spectrum depends on the physicochemical properties of their surroundings. This provides detailed information on local hydrophobicity, viscosity, and electric potential among others. This allows to go beyond a mere structural analysis towards a more functional characterization of materials. This is particularly important taking into consideration that many polymeric materials are highly heterogenous meaning that these properties vary significantly in space and time. Microscopy can unveil maps of such functional heterogeneity and thereby contribute to the understanding and rationalization of performance. In this framework the goal is to measure, spatiotemporally resolved, with the highest possible resolution, the largest possible number of properties to achieve a comprehensive morphological and functional analysis. Correlative imaging can play a pivotal role here as individual techniques can provide access to a limited subset of properties, while the combination of multiple techniques will allow the study of multiparametric heterogeneity of materials.

2.3 | Dynamics

The intrinsic dynamics of polymer materials spans orders of magnitude in timescales from the notoriously slow reconfigurations in glassy matrices to the extremely fast exchange of labile protons between hydrogen-bond donors and acceptors in solution. The daunting challenge that this poses is the necessity to monitor structure, dynamics and function in an integrative manner across a broad range of length- and timescales. No single tool can deliver such a comprehensive picture. Therefore, it is critical to correlate information from various experimental techniques including microscopy, scattering and spectroscopy, often complemented with molecular dynamics (MD) simulations, kinetic modeling and quantitative theoretical frameworks to deepen the insights further.

Combination of SRM and modeling has proved to be successful to highlight the building block reconfiguration of supramolecular polymers thanks to the specific labeling of fluorescence techniques, and these methods are now more widely applied in the polymer arena.

Laser speckle imaging tools offer a unique vista into dynamics in turbid media ranging from fracture mechanics in soft elastomers, the drying of waterborne paints and the self-healing of engineering materials to subcutaneous blood perfusion and microvascular remodeling.

The development and implementation of correlative tools within these proposed techniques capable of resolving with high spatiotemporal resolution and chemical specificity local (nanoscopic) variations in dynamics (sub-ms) and micromechanics (pN) of thick and turbid materials throughout their entire lifespan would realize a step-change in our understanding of the processes involved in the material evolution in time and enable the rational design of materials which self-report on and self-heal these often ill-controlled and undesirable effects.

2.4 | Material interactions

After synthesis and assembly, the resulting material has to perform its function in a specific environment. Biomaterials will have to be implanted or administered in the human body, energy harvesting materials will be integrated into solar panels and electronic materials will perform their function inside specific devices. In all these situations it is crucial to study the interactions of the materials with the surrounding environment and understand the time-evolution of the material in operando and upon aging. A typical example are the interactions of biomaterials with biomolecules, cells and tissues. Once injected materials immediately interact with their biological environment, binding biomolecules and changing due to the stress exerted by the surroundings (e.g. change in pH, blood flow, presence of hydrophobic elements). Fluorescence and super-resolution microscopy are used extensively to probe these events due to their minimal invasiveness and multicolor ability (both the materials and the partner biomolecules can be labeled in different colors). However, the specific labeling of fluorescence, necessary to distinguish the molecule of interest in the complex matrix, poses also a disadvantage as the overall structural background (e.g., the ultrastructure of the cell or tissue) is lost. Here correlative imaging can be of great help by combining fluorescence-based methods with techniques able to provide an overall map of the setting of the interactions such as EM, AFM, and soft X-ray microscopy.

2.5 | Fate

The last stage in the life cycle of a polymer is its degradation. As loss of functional, structural and chemical properties are usually coinciding long-term processes, correlative analysis needs to be carried out over periods of days, months, or even years. This poses a great
challenge to material characterization. Of high societal importance are at present, nano- and sub-μ-plastic for which a methodological gap exists\(^28\) which we believe can be filled by correlative imaging and spectroscopic analysis. For instance, by using closed graphene liquid cells in combination with fluorescent dyes\(^29\) that can detect the release of specific components, efficient screening of (rare) events, first utilizing FM followed by EM of the prior identified positions, has come into reach. Furthermore, it is our ambition that such analysis approaches may also lead to methods for the controlled break down of polymeric materials by chemical and physical triggers into primary building blocks to facilitate the transition towards a circular economy.

3 | TECHNICAL CHALLENGES

Here we discuss the current technical limitations of CM and the improvements that will be necessary for a broad application in polymer science. The main challenge concerning correlative analysis of the polymer life cycle is the sample preparation. It is necessary to devise a sample preparation that is compatible with both the imaging techniques and at the same time preserves the native state of the material. This is challenging as most techniques require heavy sample manipulation such as freezing or fixation. Here polymer scientists can build on recently developed workflows from the field of life sciences for cryo correlative light and electron microscopy (cryo-CLEM)\(^1\); correlative cryo-fluorescence and cryo-soft X-ray tomography\(^26\) or correlative cryo-fluorescence, cryo-FIB-SEM and cryo-electron tomography.\(^30\) However, as cryogenic preservation results in a static sample, in addition to time-resolved vitrification also rapid vitrification of specific samples states as monitored by fluorescence microscopy using transparent high-pressure freezing sample holders is expected to be essential to resolve dynamics and rare events. In the field of biomaterials novel fixation procedures that better preserve cell and materials structures are also needed. Finally, studies of the polymer life cycle would significantly benefit from a generic platform, that is, sample enclosures, holders, and equipment stages, that enable liquid-phase and cryogenic analysis using the broadest range of characterization techniques such as TEM, SRM, SAXS, SXM, Raman, and others. It has to be noted that the sample preparation for fluorescence and super-resolution imaging involves sample labeling with dyes and may result in material perturbation. In this framework the correlation of fluorescence with label-free techniques is of crucial importance to assess the impact of labeling on the polymer of interest.

Another relevant issue is the necessity to verify the findings of imaging methods with ensemble tools that enable adequate sampling of all relevant states with high signal-to-noise levels. Individual single-molecule/particle techniques have already led to unprecedented insights into hitherto masked correlations between structure and function and we anticipate that correlative imaging modalities will accelerate these discoveries. As an example, SRM was recently employed to quantify intra- and interparticle heterogeneities in the surface composition of nano- and microparticles with major implications for their interactions, assembly, motility and consequentially, their application in coatings, sensing, and nanomedicine. However, every microscopy method is vulnerable for introducing bias (i.e., imaging only a non-representative part of the sample) and has to be verified with ensemble methods. Correlative approaches involving spectromicroscopy will be instrumental to relate highly localized variations in the structure, composition, hydrophobicity, viscosity, and micromechanics of regions of interest to the overall material properties and performance.

Notably, most of the examples of advanced characterizations discussed in this paper are related to discrete particle-like assemblies. This is not surprising as bulk materials pose a challenge to many imaging methods due to their large size, thickness and scattering. Despite this, there are reports of application of imaging methods to bulk polymer materials such as poly-methyl methacrylate films.\(^31\) Extending the palette of techniques able to visualize the structure of bulk materials will greatly extend the potential of CM.

Another important issue to discuss is the presence of thermal drift, particularly in cryoEM-SRM correlative imaging. Such drift may result in loss of resolution and difficulties in image correlation. However, in the last years several solutions have been proposed including the use of more stable TEM grids and protocols to minimize laser irradiation.\(^32\)

Lastly, we have to consider that polymeric materials have often a hierarchical structure that spans a large range of length- and time scales, from atomic level to bulk and from microseconds to days. Correlative imaging has the potential to sample several of these scales combining techniques with temporal and spatial resolutions. However, this poses two main challenges. It is necessary to avoid big gaps in resolution of the techniques to allow for a reliable correlation of the information. Moreover, imaging large fields of view is challenging for many methods that are intrinsically slow and low throughput (e.g., EM and SRM). In this framework we envision that automation of the imaging procedure will be crucial to achieve atomic/molecular resolution on large fields of view bridging the multiple spatial scales of polymeric materials.
4 | CONCLUSION AND OUTLOOK

Correlative imaging for polymer science is in its infancy but due to the enormous potential for material characterization we envision that CM will become an important tool in the field. This requires several key developments. Simpler, automated and broadly applicable correlative workflow are necessary to promote the wide adoption of these methods. In this framework the design of microscopes able to perform multiple modalities or a sample holder that can be simply moved from one instrument to another are crucial developments. Moreover, polymeric samples are often thick, colored/highly scattering, multicomponent and hierarchical and will need tailored (preparation) methods that are currently not available. With these advancements correlative methods will transform from niche techniques adopted only by dedicated specialists and enthusiasts to a widely applicable cornerstone of polymer characterization capable of providing a comprehensive “3D” picture. Such a “3D” picture provides 3D structural information and temporal changes thereof (t), but also unveils multicomponent chemical information such as hydrophoby, charge or surface chemistry of the material that dictates its interactions with the local environment and thus its function.

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Yuyang Wang obtained his PhD in biophysics for his work on single-molecule fluorescence and nanoplasmonics at Eindhoven University of Technology (TU/e). From 2020 he continued his research in biophysics at TU/e after being awarded a joint postdoctoral fellowship between Institute for Complex Molecular Systems (ICMS) and Institute for Bioengineering of Catalonia (IBEC). His current research interest focuses on optical super-resolution microscopy (SRM), correlative light-electron microscopy (CLEM) and nanoplasmonics-based optical sensing.

Heiner Friedrich is assistant professor at the Laboratory of Physical Chemistry at the Department of Chemical Engineering of TU Eindhoven. His research focusses on understanding and controlling chemical and physical processes relevant to the creation of multiscale functional networks from polymeric and inorganic colloidal building blocks. To create truly hierarchical morphologies, he combines bottom-up self-organization and top-down (additive) manufacturing approaches. Furthermore, he is active in the development of quantitative (cryo-, in-situ, electron, and correlative) microscopy approaches and leads the Center for Multiscale Electron Microscopy.

Ilja Voets studied Molecular Sciences at Wageningen University & Research, where she obtained her PhD (cum laude) in 2008 at the Laboratory of Physical Chemistry and Colloid Science under supervision of dr. Arie de Keizer and prof. Martien A. Cohen Stuart. Her PhD thesis focused on micellization in dilute aqueous solutions of two oppositely charge double hydrophilic block copolymers. From 2008 to 2011 Voets was a postdoctoral researcher at the Aldolph Merkle Institute of the University of Fribourg (Switzerland). In 2011 she started as assistant professor in Physical Chemistry at Eindhoven University of Technology (TU/e), at the Department of Chemical Engineering and Chemistry and the Institute for Complex Molecular Systems. Since 2018 she leads the TU/e Laboratory of Self-Organizing Soft Matter as a full professor.

Peter Zijlstra is an associate professor of Molecular Plasmonics in the research group Molecular Biosensing at Eindhoven University of Technology. He received his PhD in 2009 working on the spectroscopy of single plasmonic nanoparticles with applications in optical data storage. His research currently focuses on single-molecule sensing using fluorescent and label-free optical approaches. To develop novel sensing concepts he employs a combination of correlative microscopy and numerical modeling to characterize sensing materials based on particles and nanostructured materials.

Lorenzo Albertazzi obtained a MSc in Chemistry (2007) and a PhD in Biophysics (2011) from Scuola Normale Superiore (Pisa, Italy). He then joined Eindhoven University of Technology (TU/e, The Netherlands) as postdoctoral researcher. In 2013, he was awarded a fellowship of the Institute for Complex Molecular Systems (ICMS) at TU/e, and in 2014 he became a NWO/VENI fellow (2014). In 2015, he moved to Barcelona (Spain) to the Institute of Bioengineering of Catalonia (IBEC) to start the “Nanoscopy for Nanomedicine” group that he currently leads. In 2017, he received an ERC starting grant and in 2018 he was appointed Associate Professor at the TU/e department of Biomedical Engineering, with the research group Molecular Biosensing.