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PERSPECTIVE

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Single-molecule fluorescence microscopy for imaging chemical reactions: Recent progress and future opportunities for advancing polymer systems

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Abstract

Single-molecule fluorescence (smFL) imaging techniques have evolved greatly over the past two decades to encompass the ability to monitor chemical reactions, providing unique advantages of non-invasive sample preparation and characterization, labeling specificity, and high spatial and temporal resolutions. This work summarizes the recent progress in this important area by first providing a brief overview of different smFL techniques, including their common optical setups and working principles. We then introduce recent developments of smFL to characterize various model chemical reaction systems, such as biochemical synthesis, catalyzed systems, and nanomaterial assembly. Furthermore, several representative areas of using smFL to understand polymer reactions are discussed, including understanding interfacial phenomenon and polymerization kinetics, as well as characterizing electrochemical reactions. We also highlight the outlook of this exciting field and potential opportunities for further development and application of smFL to enable advances in polymer chemistry and physics.

KEYWORDS

advanced characterization, dynamics, in situ, optical microscopy, polymer chemistry, single-molecule imaging

1 | BACKGROUND

Quantitatively understanding reaction mechanisms and kinetics is fundamentally important for enabling advances in material synthesis, which often relies on the development and application of advanced characterization techniques. Throughout the past decades, the capability of monitoring and understanding chemical reactions has significantly evolved from observing visual color changes of reaction solutions with the naked eye to single-molecule level material characterization.[1–3] While a variety of spectroscopic-based methods have played a central role in understanding reaction kinetics and mechanisms, these techniques often produce ensemble information that contains a collection of process and molecule averages.[4–6] Noteworthily, single-molecule imaging methods can provide a unique advantage of in situ reaction monitoring, revealing individual pathways of different molecules, and elucidating important knowledge about system heterogeneities which might be difficult to obtain from using spectroscopic techniques.[6–9] Single-molecule fluorescence (smFL) imaging, particularly, received significant interest due to its high spatial resolution, noninvasive sample preparation, and characterization, as well as broad applicability to a wide variety of systems, including but not limited to, catalysis,[10–12] electrochemistry,[13–15] and interfacial science.[13,16–18]

In the infancy of single-molecule techniques, Collinson and Wightman observed individual chemical reaction events using single photon detection for chemiluminescent reactions through laser-induced fluorescence (LIF).[4] This early work allowed molecular-level resolution characterization by limiting the volume of the chemiluminescent reaction between 9,10-diphenylanthracene and acetonitrile to the surface of an electrode. The resulting excitation was probed by pulsing the electrode and monitoring photon emission. While the first detection in this work by Collinson and Wightman was an ensemble average, results at the single-molecule level were obtained with high temporal resolution and the isolation of a cathodic pulse event. Early experiments using LIF for reaction monitoring were performed in a solid state, and extending this method to solution state has been challenging. While LIF is not a widely used technique for monitoring chemical reactions at the single-molecule level, this work was foundational for the development of total internal reflection fluorescence for smFL, as well as many more advanced single-molecule localization microscopy (SMLM) techniques.

There are several emerging areas where smFL-based methods have already been employed to understand chemical reactions of larger subsets of molecules in real space and time. Example application domains include areas of biomaterials,[19–21] polymers,[22–24] catalysis,[12,25,26] electrochemical,[27,28] and interfacial systems,[29–31] with continuous advances in technique developments to enable its use for examining reaction kinetics, interaction dynamics, reaction mechanism, and equilibria. We note that applications of smFL imaging in polymer systems can range from studying protein dynamics,[32–35] water removal during drying of polymer coatings,[36–38] and polymer thermodynamic transition and relaxation behaviors.[24,39,40] There are still many research opportunities in the field of monitoring polymer chain growth as well as other polymer chemistry processes through smFL imaging. Technique development could allow more precise characterization of these reactions on the single-molecule level, potentially advancing the fundamental understanding of polymer physics and chemistry in its second century.

We acknowledge that there are other methods of obtaining single-molecule resolution for monitoring chemical reactions, and all have their own subsets of applications. These methods include, but are not limited to, scanning tunneling microscopy (STM),[41,42] atomic force microscopy (AFM),[43,44] surface plasmon resonance microscopy (SPRM),[45] Raman microscopy,[46–48] photothermal microscopy (PTM),[49–51] and transmission electron microscopy (TEM).[44,52] Additionally, there are several applications of imaging single-molecule radical reactions in real-time, such as using bond-resolved scanning tunneling microscopy (BTSTM) with functionalized tips,[53] and non-contact atomic force microscopy (ncAFM). In other areas of interest, the assembly of nanomaterials is often imaged on a single-molecule level through magnetic force microscopy (MFM) and STM.[54,55] There are several spectroscopic methods which, when paired with imaging, illustrate a more complete understanding of mechanistic and kinetic information. The scope of this perspective is primarily focused on the applications of smFL imaging to reaction monitoring; the reader is encouraged to explore other methodologies for obtaining single-molecule resolution with a variety of microscopy and spectroscopy techniques as referenced.[37,56–60]

In this article, we present a perspective on the developments in smFL imaging of chemical reaction kinetics and dynamics comprised of methodology and synthetic methods which are associated with polymer science. Compared with understanding general polymer properties through fluorescence microscopic and spectroscopic methods,[61] the research field of examining polymerizations and reactions of polymers in real-time and space with these tools is less developed. The goal of this perspective is to summarize the recent progress of employing smFL techniques to examine a broader scope of reactions that encompass polymer chemistry, while hopefully encouraging the expanded use of this robust toolbox for the advancement of scientific knowledge, such as understanding molecular-level reaction heterogeneity.

2 | BRIEF INTRODUCTION TO CONVENTIONAL SINGLE-MOLECULE FLUORESCENCE IMAGING METHODS

2.1 | Fluorescence microscopy techniques

Going beyond the optical diffraction limit with conventional fluorescence imaging techniques has been a challenge in the past. Due to the optical diffraction limit associated with visible light wavelengths, distinct fluorophore molecules within a few hundred nanometers are very hard to distinguish. Specifically, the diffraction limit for fluorescence molecules is characterized by the Abbe diffraction limit (d) which is dependent on the excitation wavelength (λ) and the numerical aperture of the objective (NA), as shown in Equation 1.[62]

$$d = \frac{\lambda}{2NA}, \quad (1)$$

This limit can be observed in the point spread function (PSF), which is the diffraction pattern of the light

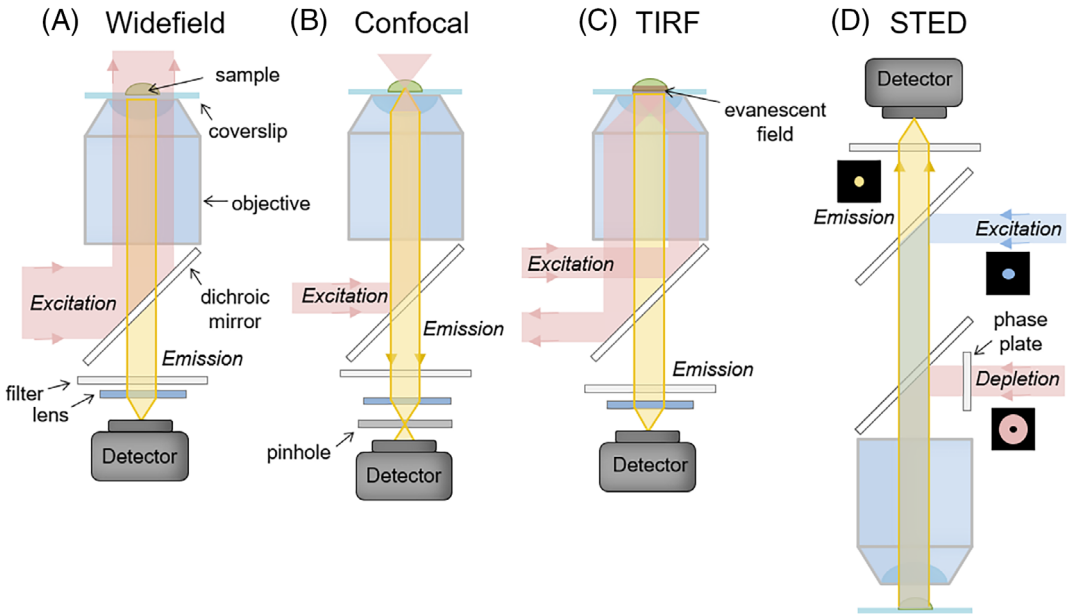

FIGURE 1 Simplified setup of (A) widefield, (B) confocal, (C) TIRF, and (D) STED fluorescence microscopes.

emitted from a single fluorophore. In a fluorescence image, the diffraction patterns can be used to further locate the fluorophore molecules by fitting the intensities as a function of space.[62] This method can be used to localize molecules at a much higher precision compared to the diffraction limit, typically in the range of tens of nanometers; however, to obtain single-molecule resolution, the density of the molecules must be sufficiently low so their PSFs are separated beyond the Rayleigh limit. While there have been many methods developed to further increase resolutions and get down to the single-molecule level, which will be discussed further,[27] the fluorescence microscope setup is also critical for characterizations. Therefore, confocal, total internal reflection fluorescence (TIRF), and stimulated emission depletion (STED) microscopy will be briefly introduced first.[63–65]

One of the most common optical setups for FL imaging is widefield (also called epifluorescence).[66] This technique involves the sample being excited by a collimated beam of light (Figure 1A), with the advantages of easy setup and being broadly adaptable to different materials and fluorophore systems. However, one drawback associated with this method is that light from fluorophores above and below the focal plane can interfere with the in-focus light, causing the images to appear blurry. Alternatively, confocal microscopy can be used to increase the imaging contrast by utilizing focused light and pinholes to block the out-of-focus light (above and below the focal plane) so that it does not make it to the detector (Figure 1B). Because a smaller area of the sample is excited and imaged, to obtain an image of comparable size to that from a widefield setup, the sample can be scanned in the x, y, and z-dimensions, while allowing an attainable resolution up to 180 nm laterally and 500 nm in the z-dimension.[67,68]

TIRF has a special optical setup that can be used to increase the signal-to-noise ratio over widefield imaging, which is frequently used for single-molecule imaging of chemical and biochemical reactions because it restricts excitation to a sample with a defined thickness to eliminate any out-of-focus excitation.[69,70] This is accomplished through the total internal reflection of the excitation light to create an evanescent wave of the same wavelength (Figure 1C), which is caused by a refractive index (RI) mismatch where the light first passes through the high RI material (typically a coverslip or sample holder) followed by a low RI sample. The combination of two materials with different RIs creates a critical angle for total internal reflectance to occur. This critical angle is derived from Snell's law (Equation 2) where n is refractive index, θ_1 is the incident angle, and θ_2 is the refracted angle.

$$n_1 \sin\theta_1 = n_2 \sin\theta_2, \tag{2}$$

The critical angle can be achieved from prism or objective-based methods.[70] The electromagnetic field created from the light at these conditions can then excite fluorophores within a sample.[69] Evanescent waves typically penetrate <200 nm into the sample, which is much thinner than an optical slice from a confocal microscope, and this relatively small penetration depth is what allows the higher signal-to-noise ratio where instead of having to block out-of-focus light, only a small portion of the sample is illuminated/excited.[71]

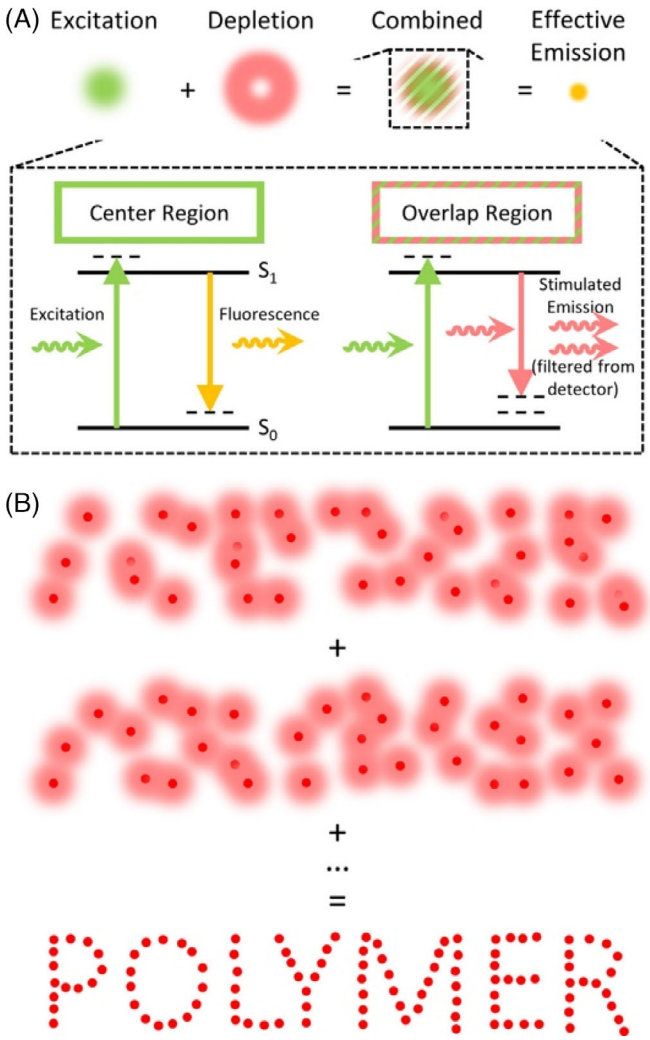

FIGURE 2 (A) Jablonski diagram of STED and (B) SMLM methods reconstruct super-resolution images by superimposing images with a small number of emitters fluorescing in each frame. *Source*: Adapted with permission from Reference 100. Copyright© 2020, American Chemical Society.

The last setup introduced in this section is STED,[72] a key technique to enable super-resolution imaging, which is based on the RESOLFT (reversible saturable/switchable optical fluorescence transitions) concept and initially applied for understanding fixed cell structures.[73] In general, STED involves the use of two beams: a focused excitation beam and a red-shifted doughnut-shaped STED/depletion beam to improve the resolution (Figure 1D and 2A). This system relies on the ability to spatially control the fluorophore emission behaviors through exciting with a laser pulse followed by the depletion laser pulse that brings the exposed fluorophores to a ground state almost instantaneously.[73] The doughnut-shaped point spread function of the depletion beam features a zero intensity at its center. Fluorophores at this location do not undergo depletion. At distances further away from the node, fluorophores experience an increasing intensity of depletion light. Above a threshold depletion intensity, the fluorophores emit negligible fluorescence light, and the effective point spread function is thus restricted to an area much smaller than the PSF of the excitation light, providing the ability to achieve high spatial resolution (typically in the range of 10s of nanometers) through STED.[72]

2.2 | Single-molecule localization microscopy

SMLM techniques address the diffraction limit challenge by precisely locating individual molecules based upon their diffraction patterns, which results in super-resolution images.[62,74] Single-particle tracking (SPT), a predecessor to SMLM, can also provide information below the diffraction limit.[75–77] However, in SPT, the fluorophores are designed to emit persistently, and a series of frames are recorded (movie) where fluorophore positions in each frame are localized from their PSF. The trajectories of the fluorophores are then mapped out with a particle tracking algorithm and can be further analyzed through correlation functions (e.g., van Hove function or mean-square displacement).[78] While SPT is conventionally used to obtain dynamic information (including non-Gaussian dynamics), SPT can still yield information related to structure, particularly with characterization of pores/channels in the sample.[79–81] In contrast, SMLM methods rely on fluorophores that can be turned ON/OFF, and super-resolution images are constructed by taking a series of images where the fluorophore positions are localized in each frame and then superimposed. Various types of fluorophores can be utilized including photoswitchable,[82–86] photoactivatable,[33,87–91] photoconvertible,[92–94] spontaneously blinking dyes,[95–97] and temporarily binding dyes.[62,98,99] In reaction monitoring, the most frequently used fluorophores are the first two, which will be discussed further in the following section.

Photoactivated localization microscopy (PALM), fluorescence photoactivated localization microscopy (fPALM), and stochastic optical reconstruction microscopy (STORM) are all types of SMLM which have been used for single-molecule characterization. STORM utilizes fluorophores which can be photoswitchable in the presence of surrounding buffer, and it relies on only a selected number of excited fluorophores for each image.[62] By only having a small number of emitted fluorophores in each frame, the position of these spatially separated molecules can be further localized from their PSFs.[33] The super-resolution image result is a reconstruction of a series of images in

succession (Figure 2B). Ultimately, this technique can localize and resolve large numbers of photoswitchable fluorophores to obtain single-molecule resolution, where multiple fluorophores may be attached to a single-host molecule.[62,101] PALM, typically utilizing TIRF, and fPALM, with confocal microscopy, can be used to image individual intracellular photoactivated fluorescent proteins.[33] PALM/fPALM is similar to STORM in that the result is a reconstructed series of images, however, the collection method and fluorophore type might be different. For PALM methods, the images are collected through a sequence of activation and collection/bleaching steps using different lasers to achieve super-resolution; only a small number of the fluorescent molecules are activated at any point in time.

Fluorescence and electron microscopy can be combined via correlative light and electron microscopy (CLEM) to obtain the best features of both types of microscopies; for fluorescence, this is the specificity from fluorescent tagging, and for electron, the high resolution (~1 nm).[102–104] One potential limitation of CLEM has been the resolution gap due to the differences in their resolution from the fluorescence diffraction limit. However, with the development of these SMLM and super-resolution optical microscopy techniques, the resolution gap can be significantly reduced and make super-resolution CLEM (srCLEM) possible. Specifically, STORM/SEM,[105,106] PALM/TEM,[107,108] STORM/TEM,[106,109,110] and PALM/SEM[111,112] have all been demonstrated. Additionally, many alternative optical imaging methods have been developed to achieve high resolution down to the nanometer-scale, including expansion microscopy,[113,114] DNA-Paint,[98,99] minimal photon flux (MINFLUX),[115,116] and most recently, Ångström-resolution fluorescence microscopy.[117] These exciting developments have not only allowed fluorescence imaging to break through the diffraction limit through single-particle tracking but also have the current capability of producing high spatial or temporal resolution images. Further advances in these techniques have the potential to couple spatial and temporal resolution in resolved fluorescent images.

2.3 | Förster resonance energy transfer

Single-molecule Förster/fluorescence resonance energy transfer (smFRET) allows the imaging of individual fluorescent molecules with a unique capability of characterizing inter- and intramolecular distances within nanomaterials, polymers, and metallic clusters.[62,71] FRET is a process that involves energy transfer between two fluorophores, a donor and acceptor, when they are in close proximity of ~1–10 nm.[118–120] Specifically, the process works by exciting a donor molecule with light and the excited state energy can be non-radiatively transferred to the acceptor (i.e., the acceptor quenches the donor). This energy transfer process can be quantified by the FRET efficiency (E) and the Förster distance (R_o), a value characteristic to each FRET pair. E can be used to directly calculate the distance between the donor and acceptor molecules (r), as described by the following equation:

$$E = \left(\frac{R_0}{R_0 + r}\right)^6, \tag{3}$$

FRET measurements are often utilized for kinetics studies as they can be extended for obtaining information regarding the dynamics of the system and polymer chain motion over time.[121–123] In general, FRET is often paired with single-molecule fluorescence imaging techniques to collect more information about how the donor and acceptor pair evolves in real-time and space, reflecting the dynamics and structure changes of their host molecules. In some cases, these techniques involve fluorescence lifetime imaging microscopy (FLIM), which characterize the lifetime of single-molecules to create high-contrast images distinguishing between different quenching kinetics.[35,118] When multiple donor-acceptor pairs are imaged in this manner, multicolor smFRET is achieved with colors dependent on the excitation/emission of the pairs. In this technique, the donor dye traditionally utilized in FRET may be transferred to multiple acceptors leading to measures of partial fluorescence for each possible pair (Figure 3). Therefore, other excitation methods and coupled programs such as alternating laser excitation (ALEX) are typically used to separate the absorbances from multiple acceptors.[35,124] These measurements typically produce images with high spatial (nanoscale) and temporal resolutions, and thus, FLIM-FRET is a useful tool for obtaining an image of an array of donors and acceptors.[118] More details about smFRET imaging techniques can be found in other excellent reviews.[125–127]

2.4 | Emerging techniques

Fluorescence techniques such as multicolor smFRET,[125] TIRF, and LIF have been employed to probe a variety of material processes and have the potential to monitor individual chemical reaction events kinetically and mechanistically.[4] In addition to these well-established, conventional single-molecule fluorescence imaging techniques, new advances have pushed the capabilities of these instruments through coupling imaging with other techniques, designing new software for data processing, and instrumentation improvement. As a push for

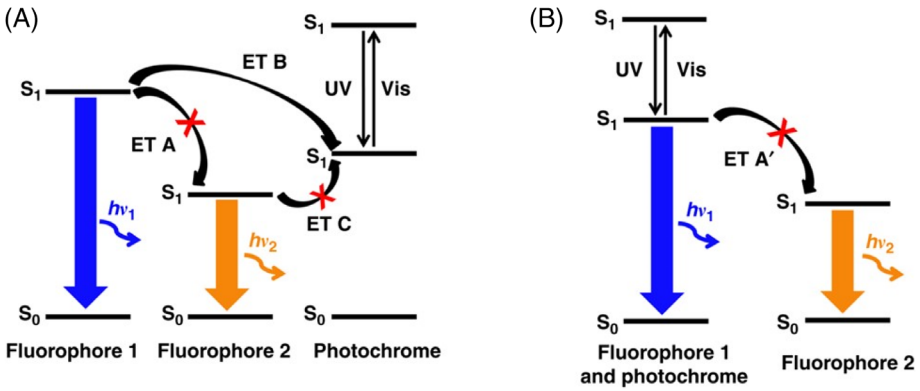

FIGURE 3 Jablonski diagrams of energy transfer (ET) and light irradiation mechanisms within (A) three-component color-specific photoswitching systems and (B) two-component color-specific photoswitching systems which can be applied to smFRET and other multicolor techniques. *Source*: Reprinted with permission from Reference 128. Copyright© 2019, Springer Nature.

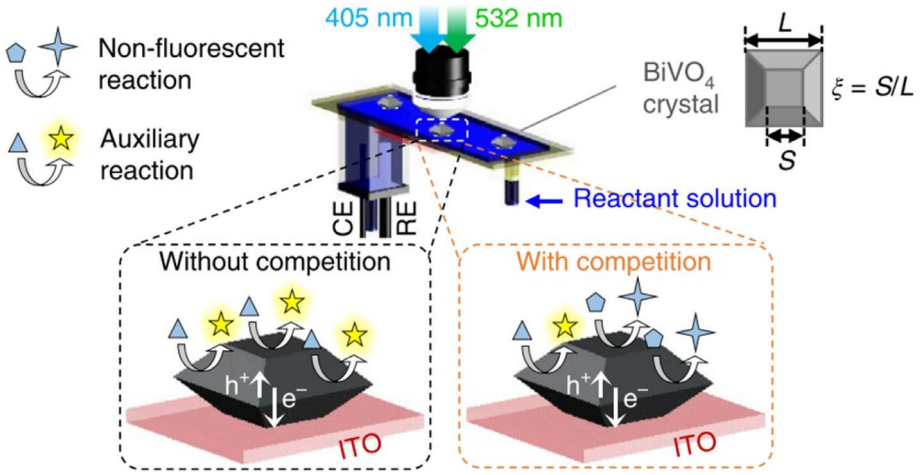

FIGURE 4 Competition reactions monitored by COMPEITS widefield microscopy technique (counter electrode CE and reference electrode RE) on an ITO-coated surface stage and a bismuth vanadate crystal with shape parameter ξ. *Source*: Adapted with permission from Reference 134. Copyright© 2019, Nature.

reaction monitoring capabilities drives these advances, in situ techniques become more desirable.[129,130] Several methods have already been implemented for catalytic and electrochemical reaction monitoring. Some examples of these techniques are fluorescence-enabled electrochemical microscopy and competition-enabled imaging technique with super-resolution methods.[131–133]

In a recent study, Mao et al. demonstrated the capabilities of a new imaging technique for catalytic studies, which is referred as competition-enabled imaging technique (COMPEITS).[134] This tool allowed for the imaging of non-fluorescent surface reactions with a competition component where both fluorescent and non-fluorescent molecules compete to bind to a catalyst which traditionally propels a fluorescent reaction (Figure 4). This technique can be generalizable to other catalytic reactions and was first applied to photoelectrocatalysis of a bismuth vanadate catalyst and the photo-electrocatalytic oxidation of hydroquinone. Through this work, the authors obtained a series of images by the combined fluorescence of resorufin and the absorption of quinone, indicating the potential applications in catalysis and nanotechnology and confirmed non-fluorescent, unlabeled molecules could be imaged in high-resolution (single-molecule level). These images were ultimately obtained by monitoring adsorption affinities of fluorescent and nonfluorescent reactants which permitted the isolation of size and shape information for image reconstruction. Additionally, the COMPEITS method has been utilized in the electrochemical space to observe binding capabilities to indium titanium oxide (ITO) electrodes, and in this work the authors suggested that this technique may have further applicability in characterizing surface chemistry.

Diving further into the electrochemical methods, fluorescence-enabled electrochemical microscopy (FEEM) was introduced for coupling a redox reaction with a fluorogenic reaction on a bipolar electrode.[36,135,136] FEEM works with a fluorogenic indicator, such as dihydroresorufin or resazurin, which is attached to a molecule involved in a non-fluorogenic redox process to image the immobilized system on a bipolar electrode. When first introduced, FEEM was able to observe minute changes in inconsistent concentration profiles of redox reactions.[136] Through the development of FEEM, this group established its capabilities for detecting reduction reactions to concentrations as low as 100 μM.[135] Over the past decade, this technique has been used in a wide variety of electrochemical systems.[137–139] The potential for new electrochemical

techniques to emerge in single-molecule imaging represents an opportunity for an expansion into the polymer science space and to monitor a wider variety of systems.

3 | GENERAL REACTION STUDIES WITH SINGLE-MOLECULE FLUORESCENCE IMAGING

3.1 | Biochemical synthesis and quantification

Monitoring biochemical reaction is a prevailing field in single-molecule fluorescence and encompasses imaging of protein, antibody, and organelle functions in living systems.[32] Most of the work to date is centered around molecule counting and other quantitative techniques, and the emergence of new tools to monitor biological reactions is pushing the boundaries in other fields such as electrochemistry,[140] polymer science,[19,141,142] catalysis,[143,144] and interfacial reaction monitoring.[145–147] For example, single-molecule photobleaching microscopy (smPM) and SMLM are often employed for imaging proteins.[146] Biological smFL regularly utilizes fluorescent antibody tags or proteins, such as green fluorescent protein (GFP), and a PALM setup. These techniques can quantitatively characterize cellular activity or organelle functions. The most common technique in biochemical studies is smPM where a fluorophore's quantized drop in intensity upon photobleaching can be imaged with TIRF techniques.[32,148] However, irreversible photobleaching at the single-molecule level presents biological challenges for characterizing how a reaction proceeds as it can hinder activity of living systems. A study from Knight et al. used TIRF in vitro to analyze membrane lipids and targeting proteins in specified pH domains of well-defined systems, isolating interactions, and dissociations involved in the docking mechanism.[147] Ultimately, this work led to the discovery of electrostatic searching, dissociation, and rebinding events in the membrane docking mechanism (Figure 5) which prompted other mechanistic studies into biochemical events. Several other studies have expanded upon this work and utilized methods such as statistical deconvolutions and SMLM to quantitatively image and count individual GFPs.[32,145,146,149,150] Through this, it became a common practice to use single-molecule fluorescence to determine protein stoichiometry of fluorescent species.[145,150] While this is a quantitative set of studies which do not directly image chemical reactions, protein counting, and similar techniques may be used to quantify reactions kinetics and yield. Quantitative smFL imaging may also be further extended to encompass synthetic polymer and small molecule reactions.

3.2 | Catalytic synthesis

The field of catalytic imaging includes works of monitoring and/or observing reaction intermediates with fluorescence and connecting this information to structure-reactivity relationships between catalyst and substrate. Additionally, an emphasis in the field relies on deriving changes in catalytic activity throughout the course of a reaction. Feng et al. determined the role of lithium chloride in the reaction of alkyl iodides and zinc powder to form soluble organozinc reagents.[151] In this work, wide-field epifluorescence microscopy was used to produce real-time confocal images of lithium chloride activity throughout the course of the reaction. With the attachment of a boron dipyrromethene (BODIPY) fluorophore to an organoiodide molecule, nonuniform intermediates caused by oxidative addition reactions were successfully imaged on the surface of the zinc powder by varying the structure of the organic component (Figure 6). These intermediates were present in the lithium-chloride assisted production of organozinc species. This study also determined the selectivity of alkylation and arylation steps in the formation of the organozinc reagents and suggested lithium chloride can act as a solubility promoter for the reaction.[11,151] Several other studies have elucidated different catalytic intermediates due to surface free energy stabilization and charge stability in redox chemistries.[26,152]

Organometallic reaction intermediate characterization has also expanded to encompass some aspects of surface chemistry and localized reaction heterogeneity, often through deciphering reactivity of the initiation step of pyridine-enhanced pre-catalyst, preparation, stabilization and initiation (PEPPSI) of palladium catalysts which are frequently utilized in coupling reactions.[25,26] This can be accomplished as a fluorescently labeled ligand detaches from a catalyst site upon the initiation step of the mechanism; thus, it is possible to elucidate the kinetics and dynamics of the system through imaging since fluorescence intensity is lost upon reaction initiation.[25] In a recent study, TIRF methods were used for this purpose which resulted in images of heterogeneous kinetics of the initiation step. Using this technique, Ng et al. hypothesized the broader impact on the bulk kinetics of the system by coupling imaging and computational studies of BODIPY catalysts. Heterogeneity observed with single-molecule techniques of organometallic reactions with respect to base concentration implied relationships between inactive catalysts and decreased reaction rates in bulk.[25] Specifically,

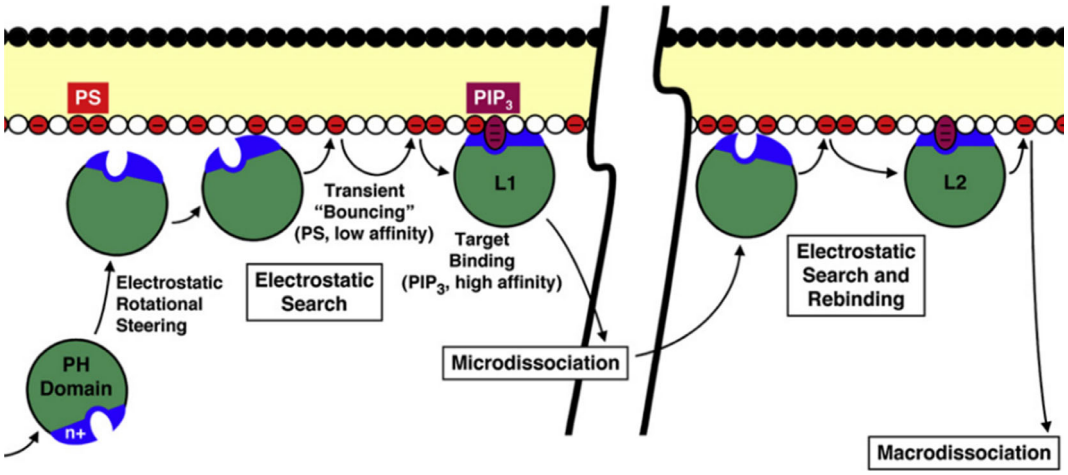

FIGURE 5 Proposed mechanism of the docking reaction of PIP_3-binding pH domains to a polystyrene (PS) membrane through electrostatic search and binding followed by micro-dissociation; then, a separate electrostatic search, rebinding, and macro-dissociation. *Source*: Reprinted with permission from Reference 147. Copyright© 2009, Elsevier.

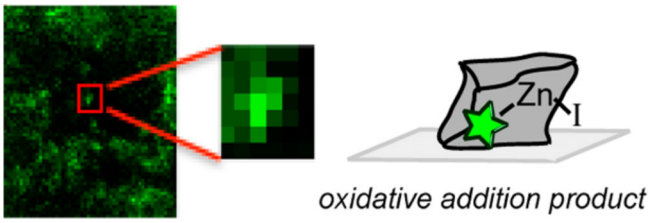

FIGURE 6 Images of oxidative addition surface product formed in the presence of LiCl. *Source*: Reprinted with permission from Reference 151. Copyright© 2017, American Chemical Society.

surface heterogeneities were connected to the localized variations in inorganic catalysis. Further expansion of these studies into polymer tethers for catalysts was suggested as a future opportunity for additional studies, and several of these works were performed to fill the gap in polymer initiation via catalysis.[26,153–156]

3.3 | Nanomaterials synthesis

Fluorescence-based characterization methods and single-molecule localization have been used to study the activity of nanomaterials by understanding their emission intensities and spectra.[71] Fluorescent nanomaterials can provide several advantages for smFL imaging, including their generally large window for high absorption cross-section, photostability, and structural dependent fluorescence spectra.[71] Recently, this field has expanded to single particle fluorescence imaging of perovskite quantum dots,[157–159] up-conversion nanomaterials,[160–162] and larger plasmonic metal nanoparticles.[163–166] However, limitations in this field prevent the isolation of activity independent of local nano-photonic environment.[71] Many studies imaging interactions between these materials rely on TIRF and confocal microscopies to obtain high-resolution images, while several mechanisms for monitoring reactions of nanomaterials utilize these structures as vessels to contain reactions in smaller localized volumes such as polymer formation.[167,168] Studies have been performed in the intermittency domain to determine the state of fluorophore activity, which could correspond to reaction activity of some nanomaterials and reactors.[169] While there are few studies into the roles of nanomaterials for chemical synthesis using smFL imaging,[166,170–172] future work could illuminate opportunities in this field to determine structure–property relationships within reactions.

There have been attempts to utilize single-molecule and single-particle fluorescence imaging techniques to characterize the assembly and synthesis of nanomaterials.[173] Combining these tools with computational methods provides explanation to synthetic phenomena which can be related to other systems.[174] In situ SMLM techniques with multiple donor-acceptor pairs (for FRET) have been used to image the kinetics of co-micelle assembly (Figure 3).[128,174] For example, Robin et al. prepared block copolymers with a dithiomaleimide (DMT) fluorophore in either the core or the shell and determined the emissive properties of each assembly which were measured with FLIM-FRET.[175] Through this work, it was found that shell-labeled systems have a faster fluorescence decay rate due to potential quenching imposed by collisions, but the core-labeled systems emitted brighter ($\Phi_f = 17\%$) due to the protection of the fluorophore from the shell. Another study from Kim et al. used color-specific color switching through an excited-state intramolecular proton transfer (ESIPT).[128] In this study, 100% switching efficiency was achieved by implementing 3,3′-(perfluorocyclopent-1-ene-1,2-diyl)bis (2-ethyl-benzo[b]thiophene 1,1-dioxide) (DBTEO) and

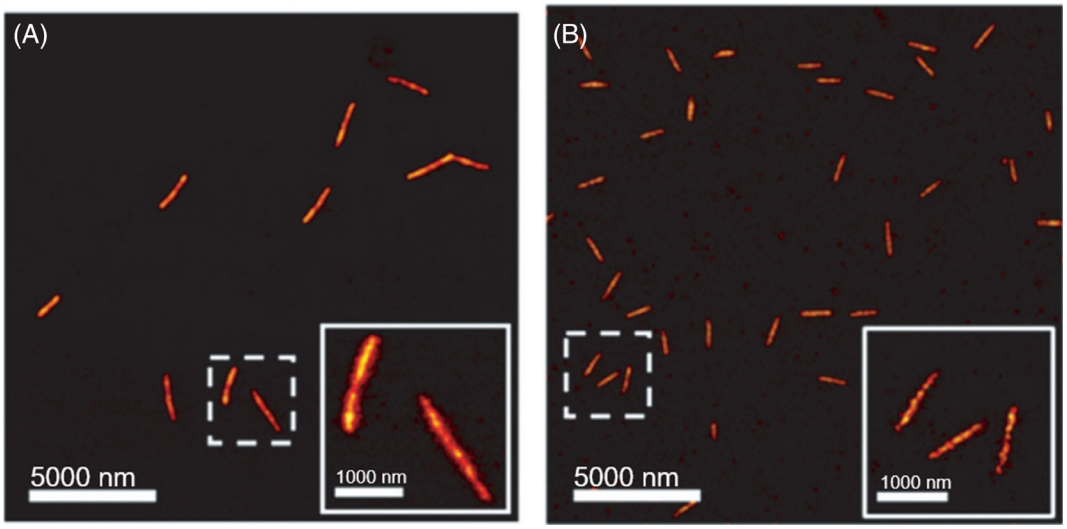

FIGURE 7 STED images of cylindrical micelles with (A) fluorescent STAR635 dye and (B) non-fluorescent CAGE635 dye. *Source*: Adapted with permission from Reference 174. Copyright© 2015, Wiley.

3-(1-phenyl-1H-phenathro[9,10-d]imidazole-2-yl)napthalen-2-ol (HPNIC) dual-color fluorescence nanoparticles, which show promise for fluorescence imaging in biological sciences.

Other methods such as employing structured illumination microscopy (SIM) and utilizing aggregation induced emission (AIE) dyes with STED have been applied to bio-based nanomaterial synthesis studies of materials like cylindrical micelles and nanoplatelets (Figure 7).[174,176–178] Particularly, AIE has been utilized by Lei et al. to investigate the assembly and stimuli response of 2D block copolymer nanoplatelets and found the fluorescence emission intensity to increase nonlinearly with the surface area of the nanoplatelet.[179] In this work, single-molecule fluorescence images of AIEgenic materials indicated a cooperative mechanism which restricts the intramolecular motion upon assembly of nanoplatelets, and the fluorescence of these systems appeared to be solvent-dependent. Ultimately, the conclusions of this study suggested an elevated solvophobicity of the system leading to a 2D geometry. Future work to investigate self-assembly behavior of complex nanomaterial systems with single-molecule and single-particle fluorescence could provide new mechanistic information about associated nanotechnologies.

4 | SINGLE-MOLECULE FLUORESCENCE IMAGING POLYMER REACTIONS

4.1 | General polymerizations

There are several comprehensive studies of leveraging the power of fluorescence imaging and spectroscopy to examine polymer diffusion dynamics,[180–186] chain folding behaviors,[61,184,187–189] glass transition behaviors,[61,190–194] and assembly kinetics.[18,55,86,174,195] Compared to these established research areas, smFL techniques for monitoring polymerizations and/or reactions of polymers is an emerging yet rapidly developing field. Several criteria exist for implementing smFL methods to characterize chemical reactions associated with polymer materials. For example, target polymers must be solubilized prior to imaging which implies a need for extremely low concentrations to dissolve high molecular weight species.[196] Additionally, understandings of polymers with single-molecule techniques are often limited to studies of dilute polymer solutions to prevent intramolecular backbiting, excessive intermolecular interactions, and solubility issues.

There has been extensive work in visualizing single-molecule events such as the photochemical binding of two dendrimers and constricting polymer growth to a nanoreactor,[23,167,197] but in situ studies were relatively limited until the introduction of STORM and PALM. In an early study of stepwise polymer growth with smFL, Shin et al. developed a nanoreactor of (mercaptoethyl)-ether to examine the growth of polysulfides by separating individual reactants and monitoring conductance (Figure 8).[23] In this work, individual reactions of a single polymer chain growth were imaged from the mean lifetimes derived from each image; this suggests protein pores with attached polymer chains could be used in future studies to monitor stochastic fluctuations of macromolecules. Additional studies have been performed using magnetic tweezers to elucidate chain growth dynamics.[196,198] Specifically, Baral et al. studied the polymerization dynamics of polyacetylene, a conjugated

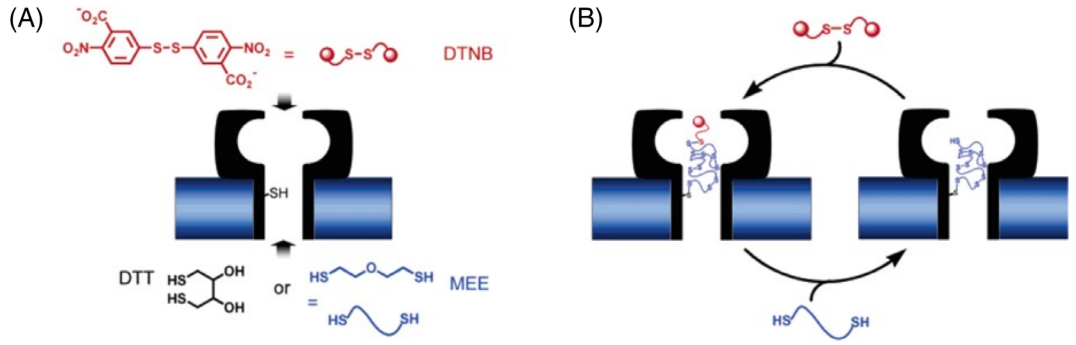

FIGURE 8 (A) Separated 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), and (mercaptoethyl)-ether (MEE) within a sequence-defined nanoreactor containing a staphylococcal α-hemolysin (αHL) pore with cis and trans defined chambers and (B) polymer growth within the nanoreactor in the presence of MEE. *Source*: Reprinted with permission from Reference 23. Copyright© 2005, American Chemical Society.

polymer, with magnetic tweezer measurements and bright field transmission images.[196] The results of this study indicated the formation of previously undetected non-equilibrium conformational entanglements resembling hairballs upon monomer addition despite the structurally stiff conjugation in the chain backbone. Additionally, a comparison of conjugated and non-conjugated analogues also indicated conformational differences and kinetic discrepancies in favor of the formation of longer conjugated chains and densely entangled, nonconjugated polymer.[196] However, the use of smFL has still been limited as it relates to monitoring polymer growth in this respect.

Ueda et al. recently employed SPT epifluorescence method to monitor photopolymerizations of liquid crystal monomer on a heated stage,[22] which resulting polymers are frequently used for production of optoelectronic and photonic devices.[22,199,200] In this work, spatially patterned, unpolarized light was used to initiate the photopolymerization of 4-[(6-acryloyloxy)hxyloxy]-4′-cyanobiphenyl (A6CB) in toluene over an indium doped titanium oxide substrate, and some portions of the material were covered in photomask to present a control for comparison.[22] The resulting images from a polarization microscope in transmission mode were used to deduce the kinetics of the system. This work suggests the formation of highly directional flow as the liquid crystal polymerization proceeds. Through single-particle fluorescence imaging, the physical alignment of liquid crystals was observed which revealed a flow-induced process. This knowledge could be used in future studies to mechanistically examine polymer alignment in films as well as other commercial products.

Overall, smFL imaging of polymerizations is still an emerging field. Researchers have successfully used fluorescence techniques to image polymer interactions governed by diffusion behaviors,[123,185,186,201,202] the glass-rubbery transition,[190,193,203,204] and polymer solution properties.[7,186] Catalyzed by these results and successes, SMLM and STED techniques could be utilized further to derive more information about chain conformation and reactivity through the course of a polymerization reaction via trajectory imaging. Current spectroscopic work at the single-molecule level focuses on bridging the knowledge gap of single polymer reactions.[8,182,205–207] As an example, Park et al. created a new spectroscopic technique to sense localized hydrogen bonding character.[56] In this work, Nile blue dye was implemented as a sensor to reveal differences in local hydrogen environments under a variety of solvent conditions. Another study from Wöll et al. examined radical polymerizations of styrene using single-molecule spectroscopy and widefield fluorescence microscopy.[57] The results of this study indicated bulk radical polymerizations could be tracked with fluorescence techniques. Additionally, with variations in cross-linker content and initiating species, differences in fluorescent probe dynamics could be detected and linked to diffusion phenomena. Wöll et al. also suggested an expansion of this work to encompass the formation of nanocomposites and interpenetrating networks. Generally, studies of these systems with imaging techniques may provide a deeper understanding of results produced from spectroscopic techniques, while obtaining heterogeneity information.

4.2 | Biopolymer systems

In situ characterization of protein reactions and other biomacromolecule systems is a relatively well-established area.[208] Initial challenges associated with single-chain polymer imaging in applications of proteins, enzymes, cells, and organelles include limited dye lifetime due to irreversible photobleaching and hindered resolution due to high-intensity photo-fluctuations resulting from the

excited triplet state[208,209]; these have previously hindered the development for determination of kinetics and dynamics of polymer systems. Significant efforts have been focused on addressing these challenges, including through the addition of oxygen scavengers such as glucose oxidase and/or a buffer to quench the triplet state.[208,210] Most experimental methods for imaging protein dynamics employ TIRF microscopy and/or a combination of photoactivated and photoswitchable fluorophores. For example, Baranova et al. successfully used both on FtsZ, a GTPase with a structure similar to tubulin, to derive an effective rate constant for the polymerization of the protein.[208] Specifically, the initiation kinetics of the Z-ring, an important structure for cytokinesis, were investigated by imaging the process with protein labeling and TIRF. The results of this study indicated similar assays may allow for the study of polymerization and depolymerization of membrane-bound structures which could be further extended to elucidate kinetic relationships between mechanistic information and architecture for other protein reactions. Another example in this area is the exploration of meiotic spindles and active measure of the polymerization dynamics of tubulin in the system.[211] Needleman et al. used confocal fluorescence microscopy to obtain images of tubulin growth, and the results indicated the process is consistent with a biased random walk model. This study also indicated a local increase in nucleation phenomena which produced a higher density of microtubules. Building on these seminal studies, future investigations can use smFL to study intercellular reaction kinetics which is particularly relevant as multiple studies have examined membrane protein reactions pertaining to cellular function.[34,142,147] Additionally, future work in the field may highlight the importance of local aggregation and concentration changes and associated effects on biological processes.

Understanding protein dynamics and biopolymers has been an area of interest in smFL imaging, while recent studies of protein imaging have focused on the mechanistic characterization of these systems via SPT.[34,35] Typically, imaging studies of proteins with single-molecule techniques have been performed on reconstituted solutions in vitro with FRET.[35] smFRET has been used to track unimodal systems, and with the introduction of multicomponent systems, like proteins with multiple donor-acceptor pairs, the use of multicolor smFRET is often implemented to gain additional information associated with complex reaction pathways. A previous study utilized these techniques to accrue images of the heat shock protein 90 (Hsp90) (Figure 9) which undergoes multiple reactions for signaling, gene regulation, and homeostasis.[35] The results indicated upwards of 1000 unique transitions in the protein and suggested the Hsp90 establishes a thermodynamic equilibrium and changes conformation accordingly. Another study from Fan et al. investigated the electron-transfer kinetics of individual nucleic acids and revealed heterogeneities within the system which were sequence-dependent.[212] This work resulted in the discovery of an mRNA point mutation in human glioma model culture cells which suggests single-molecule fluorescence applications may be extended to encompass diagnostic medicine and clinical pathology applications. Additionally, several model systems of proteins and enzymes, such as endonucleases, polymerases, and lipases, involved in biological processes like DNA replication have been characterized with similar methods (i.e., single-molecule localization, confocal microscopy, and TIRF) to provide insight into kinetic mechanisms.[19,142,213–215] As an example, Sobhy et al. utilized FRET and confocal microscopy and found that flap endonuclease 1 (FEN1) blends DNA molecules in accordance with a diffusion-limited model. While DNA sequencing is not within the scope of this perspective, there are multiple studies and reviews discussing the applications of single-molecule techniques to image these processes.[34,141,216,217] Additionally, significant work has been performed with fluorescence microscopy to image structures pertaining to RNA and DNA function such as ribosomes, transcriptase, and spliceosomes.[217]

Other dynamic studies of proteins have been performed with different single-molecule techniques, but the use of multicolor smFRET and smFL imaging techniques represents significant promise for understanding complex biological systems.[33,34] Since early applications of smFL are in the field of biochemistry and living systems, there are several pathways for utilizing this technology to observe other synthetic and/or material systems. For example, Fan et al. tested the redox switching of organic fluorophores by coupling TIRF with a three-electrode electrochemical cell and observed the emissive and redox properties of a photoswitchable fluorophore,[62] conjugated to bovine serum albumin (BSA).[6] In this study, fluorescence emission intensity was minimized upon reduction and maximized upon oxidation, allowing imaging molecules switching between different states. From this relationship, it was found that fluorescence intensity depended upon the pH value of the system due to disturbances in the local proton/electron environment.

Enzymatic single-molecule imaging is also a growing field of catalysis research, focusing on understanding biochemical processes at a molecular level, and the application of TIRF to these systems has been developed. Within the past decade, more efforts have been made to dynamically study enzymes like lipase,[214] DNA polymerase,[20] and endonuclease.[215] To investigate these biocatalysts or enzymes, surface immobilization or entrapment is necessary to obtain high-quality images.[216] For example, an

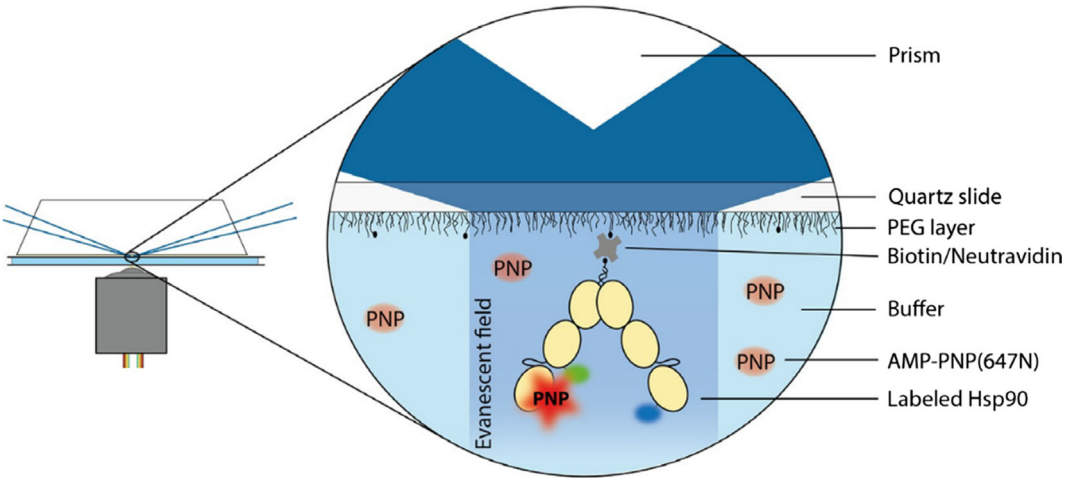

FIGURE 9 Schematic of HSP90 imaging setup. *Source*: Reprinted with permission from Reference 35. Copyright© 2016, Elsevier Inc.

early study from Tokunaga et al. used TIRF in an epi-fluorescence configuration to image ATPase analogues during associative and dissociative mechanisms.[218] In this study, clear fluorescence ON/OFF signals were obtained corresponding to individual enzymatic reactions on the surface of a coverslip. Alternatively, other imaging studies have revealed stochastic fluctuations in enzyme activity in situ.[144,216] A study in 2017 attempted to image the dynamic interactions between DNA Polymerase III holoenzyme (Pol III*) and Escherichia coli replisome, which is responsible for the replication process of DNA.[20] The imaging results indicated an exchange mechanism between Pol III* and the *E. coli* replisome which was explained with concentration-dependent dynamic network interactions between proteins and DNA (Figure 10). Additionally, other studies relating the sequencing of DNA have been focused on the single-molecule level to unravel different aspects of the sequencing process, uncovering mechanistic information on the single-molecule level.[141,215,216] We note that enzymatic studies continue to be a large part of the single-molecule imaging research, and future studies into how catalyzed biological processes work on a single-molecule level could provide further insight into these interesting and complicated systems.

4.3 | Catalyzed polymerizations

A large area of interest in smFL imaging techniques lies in catalyzed reactions, while most of the current work focuses on small molecule imagining and understanding the kinetics. Interest in this area stems from reaction phase environments of homogeneous and heterogeneous catalytic systems, which affect the reactivity of the catalyst site, and thus the kinetics of entire reaction.[219] Studying these reactions mechanistically through smFL techniques shows a unique opportunity for enabling advances in other polymerization systems, including atom transfer radical polymerization (ATRP), ring-opening metathesis polymerization (ROMP), and catalyst transfer polycondensation (CTP). To date, the general understanding of catalyst activity and kinetics on a molecular level through imaging has been limited due to the complexity of the local catalyst environment, which is potentially imposed by segmental motion of sufficient molecular weight chains, solvation discrepancies, and the fact that a molecular weight distribution can be obtained from chain growth processes.[220]

There is a distinct set of criteria for monitoring catalytic reactions with smFL techniques. Specifically, a catalyst must be surface immobilized, adequately fluorescently labeled to provide structural information, and be stable in ambient conditions for substantial time periods.[25] Despite the criteria for smFL imaging, it is used in favor of other single-molecule imaging techniques and is slowly emerging as a critical method for characterizing catalytic activity, especially as it pertains to polymerizations. Differences arise in monitoring catalytic reactions particularly in solution environments, and in situ monitoring of catalysis is a relatively underexplored field, while we note smFL has advantages of high spatial and temporal resolution as well as the ability to selectively label different domains to distinguish between different reaction pathways.[44,221,222] It is worth noting that several other techniques can monitor catalytic chemical reactions in situ, including TEM and transmission x-ray microscopy (TXM) as well as scanning probe microscopes like AFM and STM,[44,223,224] but these techniques could have their own limitations in comparison to SMFL. For

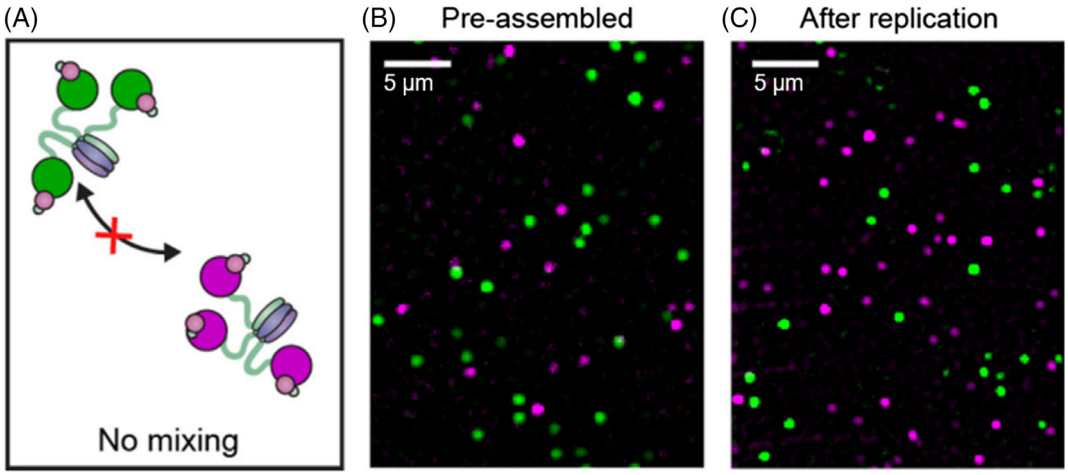

FIGURE 10 (A) The separately assembled red and green Pol III* (B) smFL images of red and green Pol III* complexes which are not co-localized prior to or (C) after DNA synthesis suggesting their cores do not exchange during the process. *Source*: Adapted with permission from Reference 20. Copyright© 2017, eLife.

example, STM requires the use of non-polar liquids unless a special coated tip is used, which might involve high cost. AFM is a surface measurement technique, which is also more suitable for crystallographic studies in solution environments, and in situ experiments have been often limited to contact modes with generally lower spatial resolution than smFL by an order of magnitude.[44]

In 2011, Esfandiari et al. reported mechanistic differences in homogeneously and heterogeneously catalyzed metathesis polymerizations using single particle fluorescence imaging,[222] and have examined several organometallic catalytic systems.[225] In a seminal work, they observed the early-stage polymerization of dicyclopentadiene via ROMP mechanisms with Grubbs II catalyst (Figure 11).[222] On the macroscale, polymerization occurs faster in proximity to solid masses of Grubbs II, but on the nanoscale, there were questions raised if this was a heterogenous or homogenous catalytic polymerization. To answer these fundamental questions, a combination of TIRF microscopy and epifluorescence was performed, and precipitating polymer with tagged fluorophores could visualize the colocalization or lack thereof. It was found that the polymerization of dicyclopentadiene with Grubbs II is a homogeneous process due to the lack of colocalization of poly(dicyclopentadiene) and sufficient solubility of Grubbs II. This study promotes the idea that heterogeneity of other catalyzed polymerizations may be characterized on a nanoscale to determine the relationship between bulk properties and single-molecule behaviors; a continuation of these types of studies would potentially provide more understanding to the scientific community as to how nanoscale polymerization kinetics and mechanisms differ from the bulk counterpart.

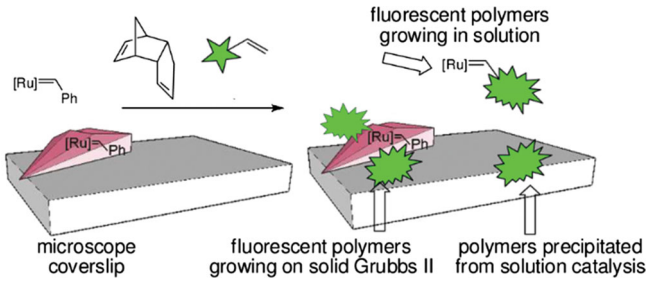

FIGURE 11 Fluorescent polymers of dicyclopentadiene on the surface of Grubbs II; these are tagged with a BODIPY dye which binds differently in homogeneous and heterogeneous processes. *Source*: Adapted with permission from Reference 222. Copyright© 2011, American Chemical Society.

In another study, Easter et al. utilized TIRF to image individual ruthenium catalysts-mediated polymerizations of norbornene via ROMP or enzyme metathesis to visualize single-turnover events by tagging a norbornene monomer with a BODIPY fluorophore.[219] The ability to image this reaction is attributed to rapid diffusion rates of norbornene monomer relative to the growing polymer chains, leading to a high resolution for spatial localization.[154,156,219,226] However, this technique relied on the ruthenium catalyst being in the active state, and the polymer could not be imaged otherwise.[219] These studies were eventually expanded to determine individual molecule kinetics of ruthenium catalyst-governed ROMP which varied from bulk concentration kinetics for the same reaction.[154–156] At a molecular level, catalysis kinetics vary depending on local environments, and at low concentrations of substrate, single monomer insertion events could be imaged within the precipitated polymer. Therefore, these methods of reaction monitoring have

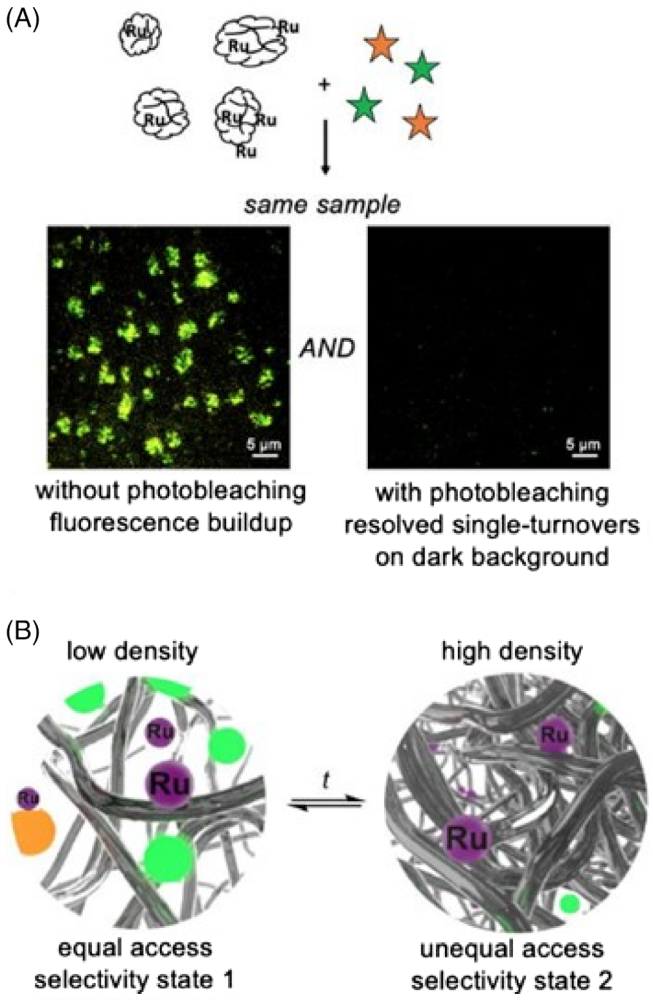

FIGURE 12 Schematic of (A) fluorescence buildup through control experiment in which irreversible incorporation of aggregates and photobleaching produced single-turnover resolution and (B) incorporation of aggregates occurs at different rates within the polymerization. *Source*: Reprinted with permission from Reference 220 Copyright© 2021, Wiley.

been limited to polymers of substantial molecular weight but not of oligomeric growth toward reaching entanglement molecular weight. Furthermore, the kinetic non-uniformity in polymerization catalysis was identified via smFL imaging of an isolated monomer insertion,[154,155] and this technique could potentially be applied to other systems to understand polymerizations and insertions in other systems which may not follow a ROMP mechanism.[156]

Chemo-selectivity of polymerization catalysts for ROMP has been a growing field since the reporting of molecular level kinetics of norbornene polymerizations, which led to successful imaging of individual catalyst selectivity by Garcia et al. (Figure 12).[220] Through the combined incorporation of irreversibly photobleaching fluorophores and aggregation phenomena, diffraction-limited fluorescence images were taken to count single-turnover events and obtain kinetic information; this data corresponded to chemo-selectivity of two different aggregate states. Specifically, a pair of fluorescent probes which could distinguish between multiple reactions was designed with the capability of spatiotemporally resolving catalyst selectivity between distinct reaction pathways. In turn, the favorable pathway for catalysis was determined from fluorescence of the individual probes resolved in the images. Looking forward, several scientific questions were posed at the end of this study as to how changes of chemoselectivity at the single molecular level could affect reaction behaviors on the macroscopic level such as polydispersity or molecular weight averages; a combination of chromatographic and single-molecule microscopic techniques may be implemented in future studies to address these questions.

There are several other catalytic systems which have been explored with single-molecule fluorescence techniques, such as outlining ligand exchange mechanisms, nanoparticle catalytic activity,[227–229] and catalytic turnover rates.[230] Additionally, as previously discussed, enzymatic reactions are a growing area as they pertain to cellular function.[143,144,216] Another major area of interest is the catalytic synthesis of conjugated polymers. While other techniques have been utilized to examine individual conjugated polymers, smFL and catalytic monitoring may provide information about electron transport,[231] intermolecular interactions,[232,233] and chain conformation in addition to capabilities of monitoring polymerizations in conjugation with other single-molecule characterization methods.[42,234,235] Additionally, this area of work in conjugated system for reaction monitoring may be expanded to non-catalyzed systems. Despite the challenges of imaging catalytic processes, surface immobilization and non-polar solution components make it possible to directly reveal these chemical phenomena. Though most of the current studies of imaging catalysis with polymers are rooted in biological applications like proteins and enzymes, these in situ studies provide a foundational framework for the development of methods to image other catalyzed polymerizations and polymer reactions. Recent progress in this area highlights the importance of single-molecule microscopy of catalysts for determining local heterogeneities in ensemble averaging measurements.[154–156,222] Imaging a wider variety of catalyzed polymerizations could expand the horizons of smFL by monitoring the activity of the individual catalysts and comparing that to the bulk kinetics of a reaction. Additionally, the development of new techniques, like COMPEITS shows

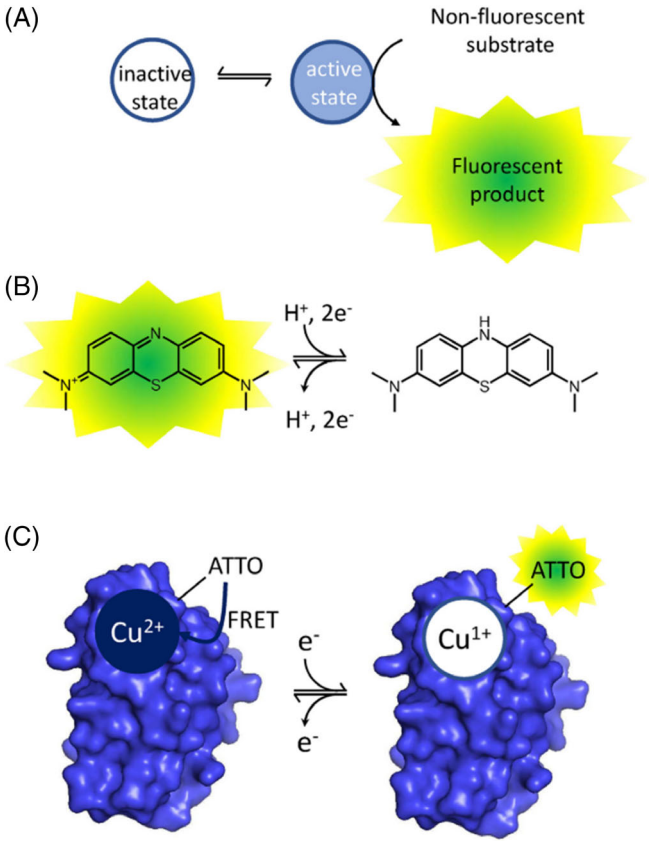

FIGURE 13 (A) Equilibrium between active and inactive catalyst states, and the generation of an imageable fluorescent product via side reaction. (B) Redox reaction in which an educt or product fluoresces. (C) Auxiliary label is quenched by a component of a redox reaction. *Source*: Reprinted with permission from Reference 27 Copyright© 2023, Elsevier.

significant promise for the visualization of a variety of catalytic polymerization processes.[134]

4.4 | Electrochemical and interfacial reactions

In the space of small molecule reaction monitoring, there are several other applications ranging from electrochemical studies to interfacial and membrane reactions for informing material design toward practical applications.[14,27,137,138,236,237] Many of these studies have expanded to encompass polymers and coatings for protective and electrochemical applications.[13,36] Single-molecule imaging of these phenomena provides an understanding of small-scale interactions between materials and interfaces. Imaging electrochemical reactions in solution via fluorescence isolates individual excitation events and the movement of protons and electrons. Through these fluorescence-based imaging techniques, it becomes possible to distinguish individual redox states, an important aspect of understanding electrochemical reactions.[27,138] While new techniques are still emerging, there is significant progress and promise for development in material science applications pertaining to polymer synthesis and characterization for electrochemical and interfacial studies relevant to several industries.

In a recent review, Jeuken et al. identified several potential methods for imaging the redox state of reactions at a single-molecule level using smFL.[27] One potential method involves tagging a reactant with a fluorescent probe in a side reaction to generate a fluorescent product (Figure 13A), but to our knowledge, this method has not yet been used in any other studies likely due to the difficulty associated with coupling redox states to fluorogenic reactions and the ability for the reactant to switch between active and inactive states.[27] Another potential method involves the use of a fluorescent reactant directly in a redox reaction without the need of an additional tag (Figure 13B).[27,238] A third method implements a fluorescent label that is not directly attached to a reactant; this label complexes or binds close to the center of the reaction (Figure 13C).[27] The optics (emission behaviors) of this label will change in accordance with the redox state of the reaction, but it does not participate as a substituent for the redox reaction. Fluorescent labeling has also been used for imaging electrochemical processes involving proteins and enzymes since the labels often do not disrupt molecular function and provide insight into the system's heterogeneity.[28] It is important to note that electrochemical and interfacial reactions of polymeric materials are important processes, which are highly relevant with various industrial and consumer products as coatings,[239,240] adhesives,[241,242] and semiconductors.[243–245] The understanding of chemical reactions of polymers at interfaces and in electrochemical cells continues to advance and examining these systems at the single-molecule level with fluorescence techniques furthers this understanding relevant to industry. As imaging technology becomes more available and accessible, interest and capability grow in characterizing commercially used polymer products and reactions, and further development in this area may lead to new discoveries or applications for already implemented products and technologies.

smFL imaging for monitoring electrochemical reaction events has been a popular research area over the past decade, in conjunction with the development of surface-enhanced Raman spectroscopy, scanning probe microscopy, catalytic amplification, and electrochemiluminescence microscopy.[236,246] Study of the electrocatalytic current, redox state, or charge transfer of an individual molecule could adequately probe a single electrochemical event.[28] In fluorescence-based

characterization methods, this is often monitored via photoblinking, and fluorescent molecules or labels are used which vary in intensity with the redox state.[28] There are still limits for what can be observed in electrochemical reactions since single electron transfer cannot currently be imaged with fluorescent techniques, but advances in single-molecule solution chemiluminescence and fluorescence may address the gaps for monitoring single electrochemical events.[28,246] Alternatively, Yu et al. reported the use of a scanning tunneling microscope-break junction for isolating the contribution of monomer sequencing on charge transport in conjugated, sequence-defined oligomers.[42] In this work, a 10-fold increase in conductance ($10^{-3.5}G_0$ as opposed to 10^{-4}–$10^{-5}G_0$) was observed in oligomers with specified monomer sequences containing imidazole and pyrrole groups. While this work does not utilize smFL imaging, the authors acknowledge the importance of characterizing sequence defined structures through microscopy and computation. Therefore, the potential introduction of smFL imaging into this area could inform the mechanism of monomer insertion on conjugated polymer geometry. Current limitations for monitoring electrochemical events on a single molecular level may arise from the lack of redox active fluorophores for labeling and the need for immobilization of fast-moving small molecules, which can be quenched by the electrode.[36]

Another important area of interest is the study of surface modification reactions of thermoplastics through ultraviolet light and ozone exposure since these reactions ultimately affect material properties.[29–31] Additionally, investigating the mechanisms and kinetics of these reactions is important for understanding solvent wetting and electrochemical properties. In a recent study, ONeil et al. have examined multiple thermoplastic surfaces functionalized with carboxylic acid groups using STORM and computational approaches.[31] Specifically, when poly(methyl methacrylate) (PMMA) and cyclic olefinic copolymer (COC) surfaces were modified with ultraviolet light and ozone or oxygen plasma, a heterogeneous distribution of carboxylic acid functional groups were imaged on the surface. High-resolution images were obtained as the fluorophores localized to the charged surfaces imposed by the carboxylic acid functionalities. Additionally, computational methods and nano-electrophoresis successfully isolated the charged species through a technique known as mobility matching,[31] and this work further suggests that coupling of these methods could be expanded to study phenomena like DNA sequencing.

With the growth of the adhesives and coatings industries, more single-molecule techniques can be employed to characterize industrial products to decipher mechanisms for application, drying, and adhesion.[38,239,240,247] In a recent work, Rueckel et al. investigated film formation mechanism and solvent transport of polyacrylate films with single-molecule fluorescence and a 1,2-bis [4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethylene (BSPOTPE) fluorescent dye, which was used due to its water sensitivity (Figure 14).[38] Through the combined effects of AIE and the non-fluorescent properties in moisture-rich environments, an inverted confocal laser-scanning microscope captured the localized movement of water as films dried. Acrylate films with grafted alkyl sulfonate chains were probed with BSPOTPE probes and dried at ambient conditions, resulting in an increase of BSPOTPE fluorescence intensity and a decrease in probe mobility with time due to the removal of moisture and reduced polymer chain mobility. This technique, though only applied to acrylate systems so far, may be relevant to other coatings systems to examine pot life and film formation at the single-molecule level for industrial-relevant applications.

4.5 | Other areas of single-molecule imaging heterogenous polymer systems

Overall, fluorescence microscopy is still an emerging method for imaging and characterizing polymerization phenomena, but there is current work in the field that examines the feasibility of probing other dynamic polymer properties such as diffusion and glass transition behaviors. These studies have the potential to propel the field into studying more complex polymer behaviors as they relate to reactions of polymeric species and polymerizations in general, with demonstrated spatial and temporal resolutions. Previous work has studied polymer properties with single-molecule imaging techniques to determine polymer melt/solution behavior, diffusive properties, and information pertaining to thermal transitions.[24,40,248–250] Recent literature has utilized advances in single-molecule imaging and spectroscopy to further understand how the glass transition and chain relaxation/reorientation of polymers progresses in real time and space.[39] As an example, Oba et al. examined reorientation of poly(methyl acrylate) films near the glass transition temperature with astigmatic fluorescence imaging, which mapped the axial positions of each molecule.[40] This work suggested there is not a clear relationship between film thickness and molecular relaxation and alluded to the presence of a surface immobility effect which prevents molecules at the interfaces of a material or film from diffusing at the innermost portion of a material. Additionally, Flier et al. observed the dynamics of perylene diimide (PDI) molecules within supported polystyrene thin films at elevated temperatures.[202] This was

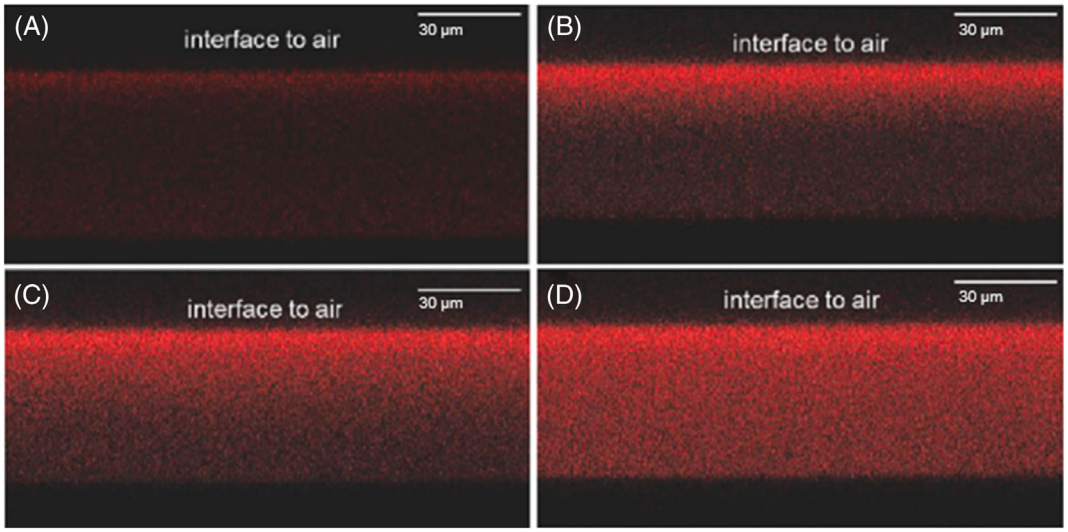

FIGURE 14 smFL images of BSPOTPE in drying poly(styrene-co-acrylate) films after (A) 7.5 s, (B) 20 s, (C) 30 s, and (D) 40 s. *Source*: Reprinted with permission from Reference 38. Copyright© 2023, Elsevier.

accomplished with epifluorescence and a separate coated heated stage to prevent damage to the optical components of the microscope. The results of this study were indicative of non-uniform structural dynamics of PDI near the glass transition temperature and a thickness dependence on polymer mobility above the glass transition temperature.

Several other studies have examined the glass transition phenomena of polymers as it relates to film thickness, including model systems of polystyrene and poly(methyl methacrylate).[40,248,249] For example, a study performed by Deres et al. investigated the 3D heterogeneity of monodisperse poly(n-butyl methacrylate) as polymer chains reoriented near the glass transition temperature with single-molecule defocused wide-field fluorescence microscopy to study polymer thin films and compare them to bulk measurements.[250] This work utilized stroboscopic excitation, which produced images depicting similar trajectories for individual molecules in thin films comparable to those of molecules in the bulk in proximity to the glass transition (Figure 15).[250] Glass transition relaxation phenomena have also been examined for understanding heterogeneity of segmental motion dynamics on a single-molecule scale. Additionally, Paeng et al. utilized a compact perylene dicarboximide probe to characterize the glass transition dynamics of individual polystyrene chains through monitoring molecular rotations with a linear dichroism technique.[24] The results of this study suggested the importance of probe selection for monitoring glass transition phenomena, and some dyes may not be able to indicate dynamic heterogeneity while polymer matrix experiences glass transition phenomena.

Moreover, several studies have examined solution properties and diffusion rates of polymers with complex architectures using smFL imaging and spectroscopy. For example, Habuchi et al. used epifluorescence microscopy to examine the diffusion of 4-arm and dicyclic 8-arm star polymers which varied the topological isomerism.[39] In this work, a centrally located perylene diimide fluorophore was attached to both topological isomers, and single molecule localization and tracking were performed to reveal the best-fit model for each isomer, suggesting polymer topology strongly affects its diffusion dynamics. Another study performed by Habuchi et al. examined the same diffusion characteristics of cyclic and linear poly(tetrahydrofuran)s using perylene diimide fluorophore.[251] In this work, epifluorescence of linear and ring poly(tetrahydrofuran) polymers of comparable molecular weights was performed to obtain trajectories of the individual polymers in toluene. This study elucidated specific diffusion coefficients for each polymer through spectroscopic techniques, which could expand in the future to encompass smFL imaging studies. Several other studies have examined the effects of polymer topology and isomerism on diffusion parameters,[76,252,253] and these studies have the potential to propel reaction monitoring research into diffusion-controlled processes, and diffusion-limited polymerization regimes, such as the Tromsdorff effect.

Additional studies of polymers have focused on imaging complex architectures to confirm polymer morphology and/or conformation. Chan et al. imaged polymer bottlebrush architectures in a polymer-rich environment.[254] Macroinitiator comprised of vinyl monomer was used to synthesize bottlebrush polymers with a grafting-from approach, and these materials were imaged with

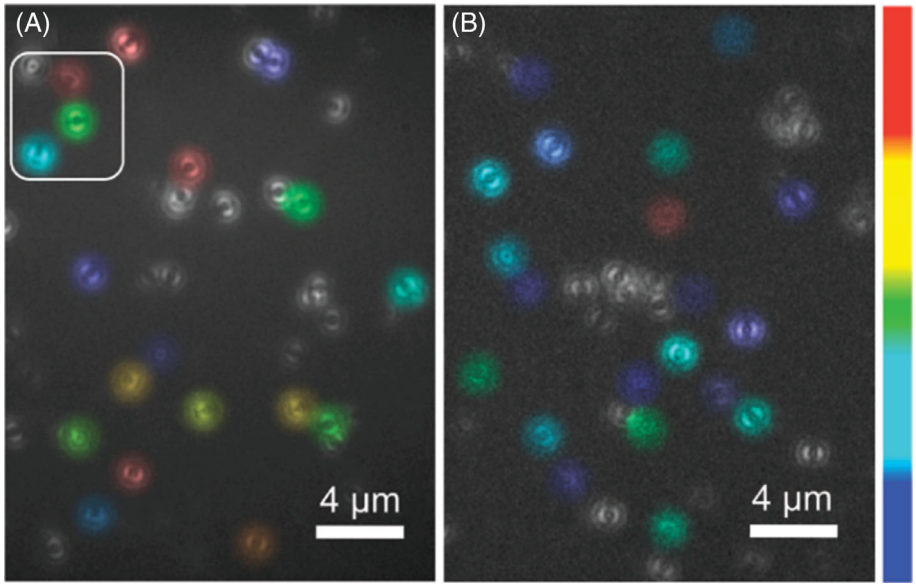

FIGURE 15 Spatial heterogeneity mapped via single-molecule defocused images where colors represent deviation from correlation times from average value for (A) 296 K and (B) 315 K. *Source*: Reprinted with permission from Reference 250. Copyright© 2011, American Chemical Society.

PALM which resolved chain conformation, an autophobic dewetting process, and a decreased persistence length for collapsing branches. This work resulted in skeletonized images of the bottlebrush polymers and traces from which conformational information could be obtained. A follow-up work from the same group reported the changes in directionality and orientation of bottlebrush polymers upon spin coating, elucidating how this process affects polymer single-chain morphology.[239] Additionally, imaging of these complex architectures has future potential to provide more information about self-assembly behaviors of bottlebrush polymers, and these studies could be expanded to hyperbranched, star, or dendrimer systems.

We acknowledge that there have been several applications of single-molecule fluorescence spectroscopy to monitor individual reactions for obtaining kinetics and dynamics information. In fact, other single-molecule spectroscopic techniques have been used to elucidate rate laws and mechanisms governing polymerizations and other phenomena associated with morphology and thermal transitions. For example, Wöll et al. used wide field microscopy and perylene derivative dyes to study the thermally initiated radical polymerization of styrene with and without crosslinkers.[57] The images obtained in this work followed a diverse range of conversions and depicted decreases in the mobility of polymer chains with increasing reaction conversions. While single-molecule spectroscopy of polymerizations defines the current state of the field, applications of single-molecule imaging could improve the outlook for determining mechanistic and heterogeneity information about a polymerization process. Several single-molecule studies in elucidating polymer morphology during reactions have been based upon spectroscopic techniques. In a recent study, Omagari et al. investigated free volume of a single polystyrene chain with a dual fluorescent flapping dopant.[60] This study determined the amount of time it takes to generate a large quantity of free volume in polymer chains when subject to short and long wavelengths, and they were able to reveal local environmental effects on this phenomenon. However, these spectroscopic techniques do not image/visualize the material under investigation. Moreover, single-molecule spectroscopic and computational techniques have also collectively probed the localized relaxations of polymers near glass transition temperatures and other polymerization kinetics which can be further extended to investigate dynamic environments and conformations of polymers upon incorporation of additional segments.[203,204,255]

5 | OUTLOOK

As of now, using smFL imaging for understanding polymer reactions is still an underdeveloped research area with growing interests where the majority of work focuses on catalysic systems and electrochemical interfaces. Other popular fields of studies in polymer research emphasize imaging of dynamic phenomena include understanding the glass transition temperature, diffusion, chain mobility, and conformation, which could prompt growth into reaction monitoring. While these studies do not directly investigate reaction mechanisms and kinetics, technique development could still benefit the ability to in situ characterize polymerization reactions, determining reaction kinetics and molecular dynamics throughout individual processes. Single-molecule

spectroscopy is already on its way to capturing mechanistic insights of chemical reactions, and imaging techniques could further this understanding for a wider variety of systems while providing complementary information. By coupling single-molecule fluorescence with other spectroscopic and microscopic techniques, it may be feasible to resolve individual proton and electron transfer events, which would provide more insight for reaction mechanisms in small molecule and polymer studies.

Morphological characterization with smFL techniques is a relevant research area,[254] and expanding these studies to encompass morphological changes within polymer reactions by coupling fluorescence techniques with other characterizations could justify processes relevant to copolymer synthesis.[256] Future studies may also examine self-assembly behaviors for topological complex polymers, such as star and bottlebrush architectures. Current work utilizes methods such as single-molecule magnetic tweezers to study in situ polymerizations of conjugated polymers, but this has not yet been expanded to single-molecule fluorescence.[196] We note the growing ability of laboratories to establish and customize their own instruments and the availability of commercially produced microscopes, smFL studies can be utilized in future work to elucidate kinetics and dynamics of self-assembly and synthetic steps.

Additionally, while the scope of solution electrochemical methods is currently mostly limited to chemiluminescence, the application of smFL techniques to surface immobilized electrochemical reactions presents the possibility of monitoring a broader scope of systems, including reactive polymer systems. When the ability of a probe to fluoresce is dependent on the redox state of a system, imaging of a redox process is possible and could be simplified to individual reaction events. Work has been performed to monitor the synthesis of simple conjugated polymer systems on a single-molecule level with non-fluorescent probes.[180,232,233] Development of new electrically responsive fluorescent probes could further broaden research and prompt growth of the field to include in situ monitoring of electrochemical processes. Moreover, with the ever-growing field of polymer adhesives and coatings, the benefits of imaging these systems on a single-molecule level during chemical process are apparent, which would allow informed material design. Advances in monitoring processes relevant to the coatings and adhesives industries have thus far allowed imaging of the drying processes of acrylates.[38] Expansions of this study to other latex systems or waterborne adhesives and coatings could contribute to the collective understanding of these processes at a single-molecule level. Other coatings reliant on crosslinking mechanisms and other dying processes involving volatile organic contents (VOCs) have yet to be imaged at high spatial resolution with smFL techniques, but the development of new probes may create opportunities in this field for fundamental mechanistic research for industry-important chemistries.[38,123,226,239] Understanding interfacial reactions and the importance of surface energy contributions to a transition state have been thoroughly studied, and additional work in this field may elucidate other transition states of interfacial reactions.

While there has been limited research in examining (de)polymerizations with smFL imaging, several small molecule studies have imaged the process of cleaving and forming new bonds. For example, Zhang et al. recently examined diffusion dynamics in Diels-Alder reactions and discovered fast diffusion behaviors consistent with a threshold phenomenon.[201] This work can be further leveraged to understand polymer reactions with dynamic and/or reversible bond formation and breaking. Additionally, techniques for monitoring proton and electron transfer mechanisms have been limited to spectroscopic techniques, but the coupling of these methods with microscopy might further elucidate reaction mechanisms. Electron and proton transport reactions have been extensively studied with special attention to protein reactions via single-molecule imaging. Krzemiński et al. coupled smFL imaging with electrochemical methods to probe the electron-transfer rates of the nitrite reductase interfacial reaction.[58] Moreover, there is an abundance of studies in the space of reaction monitoring at the single-molecule level which are composed of both spectroscopic and microscopic experiments. The ability to probe these reactions with fluorescent probes encourages the expansion to macromolecular systems which are applicable to obtain biological, industrial, and general scientific understanding for how reaction events occur on the molecular scale. Translation of these events to comparisons in bulk provide more information about the kinetics, catalytic efficiency, conformational changes, and general homogeneity of a system. The ability to image these events related to reaction efficacy, molecular transitions, and diffusion capabilities could explain more phenomena going beyond basic small molecule chemistry.

6 | CONCLUSIONS

In this perspective, we summarize smFL imaging methods and their utility for achieving high resolution and understanding polymer reactions in a plethora of applications. We first outline how single-molecule resolution is obtained with different fluorescence microscopy techniques and highlight how these techniques are applicable in discerning reaction intermediates, kinetic

phenomena, and polymer behaviors and properties. There are several emerging areas of research, and the development of smFL imaging methods will broaden the range of imageable materials. Many smFL imaging applications for understanding chemical reactions are discussed in this article, including characterizing catalytic synthesis processes, assembly of nanomaterials, and protein counting. In the field of polymer science, smFL imaging techniques, such as STORM, PALM, and single-particle tracking, are emerging tools to visualize polymerizations and reactions of polymers in real space and time. With the broad range of reaction pathways accessible in the polymer space, smFL imaging has the capacity to make broader impacts in studies of proteins, enzymes, membranes, coatings, adhesives, polymer catalysis, and fundamental polymer physics. Ultimately, smFL imaging has been applied to several catalyzed polymerizations, interfacial reactions, electrochemical applications, and biochemical reactions, while there is room for this field to grow for further elucidating the fundamental physics and chemistry of reactive polymer systems.

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