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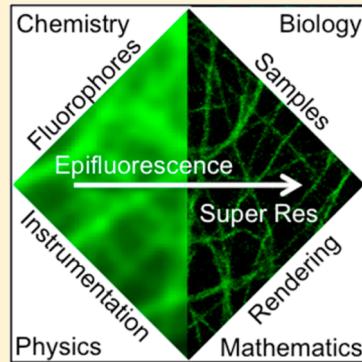
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## Super-Resolution Single-Molecule Localization Microscopy: Tricks of the Trade

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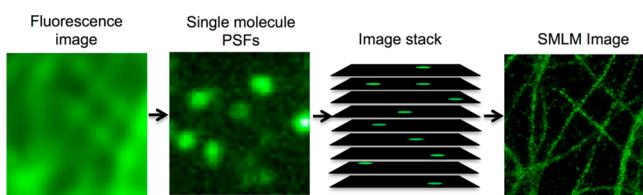
**ABSTRACT:** Application of single-molecule fluorescence detection has led to the development of light microscopy techniques that make it possible to study fluorescent samples at spatial resolutions significantly improved upon the diffraction limit of light. The biological and materials science applications of these “super-resolution” microscopy methods are vast, causing current demand for them to be high. However, implementation, execution, and interpretation of these techniques, particularly involving biological samples, require a broad interdisciplinary skillset, not often found in a single laboratory. Those already used to interdisciplinary work as well as navigating communication and collaboration between more pure forms of physics, chemistry, and biology are well-positioned to spearhead such efforts. In this Perspective, we describe various aspects of single-molecule super-resolution imaging, discussing, in particular, the role that physical chemistry has so far played in its development and establishment. We also highlight a selection of some of the remarkable recent research achievements in this vibrant field.



The detection of fluorescence emission from single molecules was first achieved in 1989<sup>1</sup> at cryogenic temperatures and at room temperature in 1993.<sup>2</sup> It was not until 2006, however, that the ability to image and inspect the emissions of single molecules was successfully and elegantly applied to achieve visualization of subdiffraction structures.<sup>3–5</sup> A number of so-called super-resolution methodologies founded on single-molecule detection, (d)STORM, (f)PALM, SPDM, GSDIM, and so forth,<sup>6</sup>—collectively referred to as single-molecule localization microscopy (SMLM)—have since been developed. Many reviews (too numerous to list here) covering all aspects of SMLM are available in the literature, and we refer the reader to a recent selection<sup>6–9</sup> as a starting point. The underlying theory and principles of SMLM are remarkably straightforward and are summarized Figure 1. The central key to achieving super-resolution imaging by single-molecule fluorescence detection is that point spread function (PSF) emissions of single

fluorophores are isolated temporally by (photo)physical and/or (photo)chemical means. This is so that the vast majority of fluorophores are temporarily nonfluorescent to the extent that most of the PSFs of those that are emitting are spatially well-separated from each other. An isolated PSF can be fitted, usually with a two-dimensional Gaussian function, so that the location of the emitter is determined to within several nanometers. By doing this many thousands of times with different subsets of emissive fluorophores, a map of the localization coordinates of the fluorophores is plotted, which can then be rendered (“reconstructed”) into an “image”. Application of single-molecule detection for super-resolution fluorescence microscopy does not require significantly more complex hardware than what is routinely used in many laboratories for experimental detection of single molecules by wide-field fluorescence microscopy. All that is then needed to perform SMLM is a means of time-based PSF separation. This has been achieved in a number of ways including photoactivatable proteins,<sup>3,4</sup> FRET activation,<sup>10</sup> photochemically achieved dark states,<sup>11</sup> and target-association triggered emissive or photoconverted states.<sup>12,13</sup>

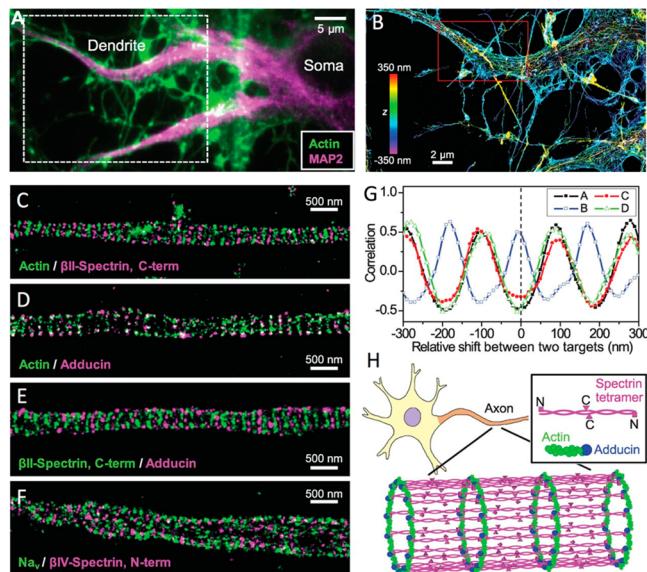
The applications of SMLM have thus far been predominately to biological systems or components thereof and have typically been aimed at elucidating the structure or distribution, often as a means to determine the mechanisms of action of subcellular constituents. An excellent example of such an application is the recent elucidation of the architecture of spectrin and actin in neural axons accomplished in the Zhuang laboratories as summarized in Figure 2.<sup>14</sup> In this work, both fixed and live cell 3D SMLM were undertaken along with extensive image analysis to detect the subdiffraction periodic arrangements of several



**Figure 1.** A simple schematic illustration of the overarching theory of SMLM. In the first panel (from the left), the diffraction-limited epifluorescence image in which all fluorophores are emitting photons is shown. For SMLM, the majority of fluorophores are switched to a dark state so that isolated, spatially separate, single-molecule emissions are imaged (panel 2). Accumulation of thousands of frames of random subsets of single-molecule emissions (panel 3) and fitting of the PSFs to yield single-molecule localizations generates the final super-resolution image (panel 4).

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**Figure 2.** Recent work from the laboratory of STORM inventor Professor Xiaowei Zhuang, who investigated the subdiffraction structure, distribution, and mechanism of various molecules within neuronal axons. This included the architecture of actin, spectrin, the actin capping protein adducin, and sodium channels and found previously unknown periodic structures. (A) Conventional fluorescence image of actin (green) and the dendritic marker, MAP2 (magenta). (B) Magnified area of (A) with actin stain localized and rendered in 3D. (C–F) Actin, spectrin (both C and N terminals), adducin, and sodium channels are immunostained. (C) Alternating actin rings and C-terminus spectrin localizations. (D) Co-localized actin and adducin. (E) Alternating adducin and C-terminus spectrin localizations. (F) Alternating areas rich in sodium channels and N-terminal spectrin localizations. (G) Co-localized/alternating periodicity between actin and the  $\beta$ II-spectrin C terminus [black], between actin and adducin [blue], between adducin and the  $\beta$ II-spectrin C terminus [red], and between sodium channels and the  $\beta$ IV-spectrin N terminus [green]. (H) Determined model for the cortical cytoskeleton in axons. Adapted from Xu et al.<sup>14</sup> Reprinted with permission from AAAS.

structural proteins and then to deduce their interactive functions. Of course, there is also ample potential for SMLM to be used to elucidate structure and distribution characteristics from nonbiological materials such as in the subdiffraction characterization of plasmonic structures<sup>15</sup> and in subdiffraction mapping of chromatographic characteristics such as adsorption and diffusion on surfaces.<sup>16</sup> On the other hand, the development of the technique itself exists largely within the domains shared by physical chemistry with other scientific fields; cameras and other detectors, lasers, light sources, and optomechanical items are developed by engineers and physicists, software for analysis is devised by mathematicians and computer scientists, development and characterization of new fluorophores is a collaborative effort involving synthetic (bio)chemists and (bio)physical chemists, who are also the prime movers along with molecular and cell biologists in the preparation of fluorophore labeled samples. Moreover, overarching knowledge of molecular and cellular biology is required to pose questions answerable by SMLM, contextualize the hypotheses, and interpret the results. Undoubtedly, in-depth, comprehensive knowledge of all aspects of SMLM by a single person is, and will continue to be, rare because it would have to encompass all of the aforementioned already quite broad fields.

Instead, SMLM has to be approached and respected as a highly collaborative research area and recognized as one that is already yielding ground-breaking discoveries.

Seemingly allowing individuals to bypass the need for extensive collaboration and instead use a “black box” approach to the hardware, software, and even fluorophore selection and manipulation aspects of SMLM, several commercial instruments are now available. However, examination of the literature shows that up to this point in time, the majority of high-impact SMLM research is being conducted using home-built systems, most often in the large laboratories or the progeny laboratories thereof from which SMLM emerged—research groups already well versed and appreciative of the necessity for interdisciplinary work and collaboration.

The purpose of this Perspective is to detail the necessary and central role of interdisciplinary science in the successful use of SMLM with particular attention paid to those aspects that demonstrate how ideally suited physical chemists are to playing key roles in implementation of SMLM. We do not attempt to present a comprehensive review of SMLM or the wider field of super-resolution fluorescence microscopy. Many others have done this extensively, and the reader is referred to a selection of recent reviews.<sup>6,17</sup> Rather, we focus on how an interdisciplinary approach maximizes success in applying SMLM by examining each of the following facets: hardware, software, benchmarking, sample preparation, and fluorophores. We detail how interdisciplinary knowledge and skillsets, whether in the individual or within the research group and collaborators, are easily transferable to SMLM implementation and research, as well as highlight the areas in which physical chemists can quickly contribute to the forefront of SMLM. We will also discuss some of the persistent difficulties encountered and showcase some recent exemplary applications of SMLM techniques.

**Hardware.** As noted above, the hardware requirements for a basic SMLM setup are essentially no more than what is needed for an epifluorescence widefield microscope: the mounting of diode lasers, basic optomechanics for alignment and excitation/emission spectral control, expansion and focusing of the beams, a microscope frame (or equivalent optomechanics) with high NA objective, and a good camera for detection. We have recently published the assemblage procedure for a three-color excitation, two-channel detection system<sup>18</sup> and noted some key considerations for such systems. One is to ensure vibrational isolation and stability of samples in order to minimize drift because drift artifacts will manifest with movement of even a few nanometers. Another key consideration is incorporation of an illumination pathway that can be adjusted during experiments to a “quasi-TIRF” configuration. This allows further flexibility of important parameters, in particular, the excitation power density in different axial planes of the sample. Quasi-TIRF illumination can readily be achieved by directing the excitation beam onto the back focal plane of an appropriate objective, off-center but still parallel to the optical axis. This angles the excitation light into an almost sheet-like illumination pattern and leads to brighter single-molecule signals as well as lower background fluorescence.<sup>19</sup>

A home-built setup can be implemented at a cost of less than \$U.S. 100K, with the majority of that spent on a state-of-the-art electron multiplying charge coupled device (EM-CCD). Recently, Holm et al.<sup>20</sup> critically examined all aspects of instrumentation with a view to reduce cost and have detailed an in-house assembled system capable of SMLM measurements with only an approximately 1.4× trade-off in localization precision

## Application of single-molecule detection for super-resolution fluorescence microscopy does not require significantly more complex hardware than what is routinely used in many labs.

compared to high-end setups for a cost of only \$U.S. ~25K. This opens the way for introduction of SMLM at an undergraduate level or as a teaching/training instrument in any biofocused laboratory and is in contrast to the commercial SMLM setups that currently retail for between \$U.S. ~400 and 800K.

Clearly, there is a financial advantage to in-house assembly of SMLM setups using off-the-shelf components, and that this can be achieved without extensive prior experience makes this a highly attractive approach. Perhaps an even greater advantage in developing in-house SMLM setups is the ability to continue to modify and add in various functionalities; one camera setups can easily be upgraded to two, further excitation lasers added, pulsed and/or cycling excitation schemes developed, additional microscopic or spectroscopic detection methods established, as well as various illumination paths allowing for simultaneously accessible epifluorescent, TIRF, Q-TIRF, and confocal alignments. Advances on the original design of SMLM setups have also allowed implementation of imaging in 3D, usually via biplane or astigmatic routes<sup>6</sup> although other methods are gaining popularity,<sup>21</sup> and this is another readily incorporated upgrade for a home-built system. The design, build, and modification of the setup also stimulates interdisciplinary discussion and learning by directly exposing those responsible for the hardware setup to the questions and samples being measured, as well as the scientists (primarily biologists) providing the samples and posing the questions to the hands-on use of the equipment. Such interactions and cross-disciplinary exposure also aids in optimizing the parameters required for matching samples with measurements and analysis.

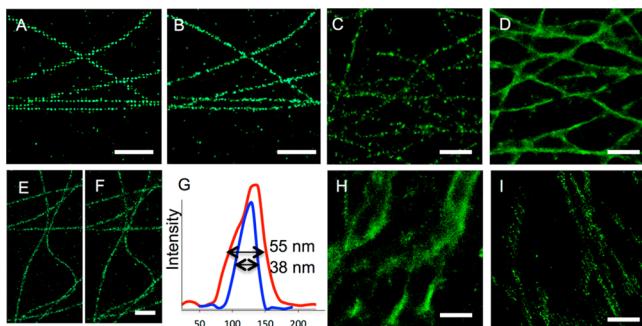
In summary, the hardware side of SMLM is very approachable to an experienced physical chemist or chemical physicist and is considerably less complicated than the equipment assembly required to conduct other super-resolution techniques such as stimulated emission depletion (STED) microscopy or structured illumination microscopy (SIM). Indeed, it is also more straightforward than other fluorescence spectroscopic methods such as fluorescence correlation spectroscopy or ultrafast laser spectroscopies such as “pump–probe” transient absorption or fluorescence upconversion. Furthermore, when extensive microscopy and optics skills are present in a research group, the opportunity is available to move quickly to the front of the field and develop advanced systems such as the recently published improvement to 3D-STORM using a self-bending PSF.<sup>22</sup>

**Software.** A key requirement for SMLM is the ability to acquire and process tens to hundreds of thousands of images and then select and fit the PSFs to single-molecule emission patterns. This adds up to millions or tens of millions of fitted localizations using a predetermined function that is complex enough to model emissions reliably in order to locate the underlying molecule to within a few nanometers of its actual position. In most cases, a two-dimensional Gaussian is used because it is considered to offer a favorable trade-off between

fitting complexity and resultant precision without too high of a demand on computing power. Various other functions have been applied, qualified, and recently reviewed.<sup>23</sup> Initial SMLM measurements were quite time-consuming due to read-out sensitivity and rate; however, current computing power allows for fast USB readout and write rates from top-end cameras. Routinely, 10–20 000 frames are recorded at a frame rate of 50–100 Hz, allowing for acquisition in well under 5 min. By acquiring fewer frames, dynamic processes in live cells have been measured,<sup>24</sup> and new advances in sCMOS camera technology promise even faster acquisitions.<sup>25</sup> Some software, such as that used in the authors' laboratories (Andor Solis for Andor Ixon Ultra EM-CCDs), allows direct spooling of frames into a TIFF-stack file that can then begin to be processed midmeasurement. However, the analysis of SMLM data is not straightforward. Even assuming a predetermined nonfluctuating PSF size, as many SMLM solving programs do, the processing and fitting of millions of PSFs alone is computationally demanding. More complicated calculations must also be made to determine and deal with irregular PSFs such as those from out-of-plane fluorophores or from two proximate fluorophores emitting at once. Parameters are also needed to instigate thresholds for PSF fitting and a multitude of other considerations. Fortunately, there are several freely available software suites,<sup>23,26</sup> of which the authors are most familiar with rapidSTORM.<sup>27</sup>

As this software (and others) is openly available, continuously updated, and widely applicable, the ability to use it in a straightforward and intuitive manner might make it seem like there is little need for significant appreciation of the coding and mathematics that allow the software to function. However, the ability to appraise critically the relationship between the raw data and the rendered image, as well as appreciate the many parameters that can be manipulated to change the image, is important for obtaining the best-possible image and for avoiding software-based artifacts. These artifacts can be very subtle, such as introducing unwanted bias to which PSFs are localized successfully but can also occur if a simple parameter such as PSF size or pixel size is incorrectly specified as shown in Figure 3. Simply misidentifying the projected pixel size (as shown in Figure 3E–G) can result in erroneously good localization precisions and spatial resolutions in images that appear legitimate in all other ways, making this artifact difficult to identify without independent analysis of the raw data.

Sufficient mathematical expertise also helps enable comprehension and communication within a research group regarding the important differences between spatial resolution and localization precision as well as the relationship between these two important parameters. Because of the unreal nature of the SMLM image, which in reality is simply a plot of a list of single-molecule location coordinates, a method for exact determination of localization precision is yet to be agreed upon.<sup>28</sup> Despite the ongoing debate among mathematicians and physicists, SMLM papers continue to be published with “precisions” and “resolutions” reported without qualification and, in some cases, erroneously as interchangeable values. As a general rule, the localization precision can be reasonably estimated based on the number and distribution of emitted photons. Translating that localization precision into an absolute spatial resolution is extremely difficult due to a number of complicating factors. First, Nyquist's sampling rule must be met, which states that in order to achieve a spatial resolution of  $x$  nm within a two-dimensional image, the labeling density of the target structure



**Figure 3.** Common SMLM artifacts due to errors in acquisition and image rendering. (A,B) Same area of microtubules (MTs) rendered using an incorrect PSF value (A) demonstrating the crosshatch pattern indicative of this and (B) with the correct PSF value. (C) Discontinuity rendered when too few frames are taken and an insufficient number of molecules are localized. (D) Blurring artifact that results from incorrect center-fitting of overlapping PSFs. (E,F) MTs rendered with the correct imaged pixel size (E) and with an overestimated pixel size (F). Little difference in seen in the images, but the resolved width of the MTs and the determined spatial resolutions of the image are significantly different with a correct input pixel size yielding a cross section of 55 nm (red trace in (G)) and the incorrect pixel size yielding a cross section of 38 nm (blue trace in (G)). (H) Blurring caused by axial drift or significant fluorescence from out-of-plane fluorophores. (I) Smearing caused by lateral drift. Scale bars are all 1  $\mu$ m.

must be such that there are at least two labels every  $x$  nm. Other factors to be considered are the difference between accuracy and precision, the possibility of energy-transfer effects, the size of the target itself, and the assumptions made when applying Rayleigh's criterion to a list of single-molecule coordinates.

Appreciation of the difficulty in definitively stating spatial resolutions for experiments or images is very important in interpretation of results and also implies that spatial resolution figures quoted generally for a given technique or microscope have little real meaning. Correct identification of the significance of co-localizations, distribution patterns, and separation distances is strongly dependent on the actual resolving power, and if these are not well-determined, incorrect interpretation can ensue. One useful approach that errs on the side of caution is to state spatial resolutions as "better than" any level of discernible detail observed. For example, if two microtubules (MTs) side-by-side can be resolved at half-height well apart from each other with a distance of only 50 nm between them, then the spatial resolution of the image can be said to be as good as, if not better than, 50 nm. Defining the resolution as such engenders robustness in further deductions about the image. Standardization of analytical techniques by erring on the side of caution and exercising good understanding of the reasons for such caution allows for stronger conclusions to follow.

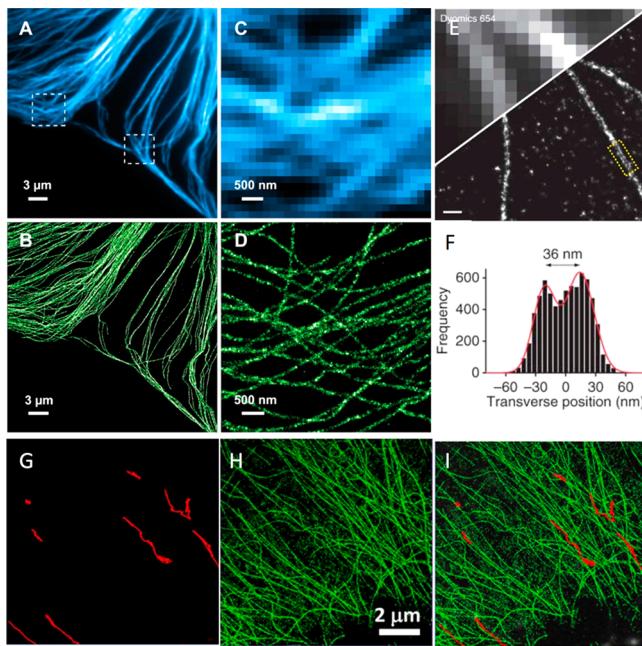
Currently, there is very little standardization or validation of SMLM data and rendered images due mostly to the newness of the technique and the fact that images are not true images at all. A striking comparison is with current practices in place for publication of X-ray crystallography data, which require that entire raw data sets are uploaded and made openly available, along with solving parameters. Assessment of data and images is therefore another area of SMLM where interdisciplinary in-house expertise capable of appreciating the nature of both the sample and the rendered image, as well as comprehending the parameters within the software, is crucial.

**Benchmarking.** The ability to visualize detail with an order of magnitude improved spatial resolution inevitably forces an order of magnitude improved detection of both biologically native and artifactual structure; indeed, wherever a fluorophore resides within a sample, whether biologically relevant or not, it will be localized and considered equal to all other fluorophores. Due to all of these differences, care should be taken when comparing SMLM and confocal images, so that acquired images can be considered prescriptive of biological reality. It is crucial that SMLM microscopes and their software, as well as sample preparation protocols and fluorophore photophysics, be benchmarked to demonstrate high-quality subdiffraction imaging of known structures prior to application to unknown samples. Benchmarking is also useful in determining actual achievable localization precision on a particular setup with a particular fluorophore, as well as for identifying any hardware/software artifacts such as lateral or axial drift.

The ability to visualize detail with an order of magnitude improved spatial resolution inevitably forces an order of magnitude improved detection of both biologically native and artifactual structure.

Nonbiological targets can be used for this, for example: fluorophore-labeled silica spheres of predetermined size or stretched or so-called origami DNA.<sup>29,30</sup> Biological targets within fixed cells can also be used with many of the initial proof-of-principle SMLM experiments targeting the cytoskeletal MT architecture because of the well-defined cylindrical polymeric structure (internal diameter = 24 nm). Upon addition of antibodies, the "width" of a MT increases to ~40–50 nm, while rendering as a SMLM image further adds width due to the uncertainty in fluorophore localization; thus, the expected imaged width is approximately 60–80 nm, depending on the localization precision and the size of the antibodies.<sup>31</sup> Moreover, because 2D SMLM images are projections of the three-dimensional shape, MTs should image with discernibly less intense centers<sup>32</sup> because there are fewer labels per unit area "along the top" of a MT than "down the sides" once projected into 2D, as shown in Figure 4. Detection of the less intense center-line of MTs demonstrates resolvable intensity changes over ~15–20 nm and indicates a very robust setup in which all aspects of hardware, software, sample preparation, and fluorophore blinking are working well. MTs can be prepared using straightforward fixation protocols for adherent cell lines, as well as assembled from tubulin monomers in vitro and labeled with commercially available fluorescent antibodies, making them an ideal biological target for benchmarking.

The nuclear pore complex (NPC) is another well-documented cellular target for benchmarking<sup>33,34</sup> (Figure 5). Several thousand individual NPCs exist on the nuclear membrane of vertebrate cells, each comprising more than 450 individual proteins assembled into a complex system that facilitates the transport of proteins across the membrane. It is particularly useful for benchmarking because it is approximately 120 nm in diameter and labeling of gp120, one of the integral proteins within the complex, can yield both the eight-fold symmetry and the 41 nm diameter of the central pore channel, as has previously been achieved using SMLM.<sup>33</sup>



**Figure 4.** MTs are an ideal biological target for benchmarking as well as new SMLM research. (A–D) Epifluorescence and SMLM images of MTs at high magnification showing the improved spatial resolution achieved. (Adapted from Bates et al.<sup>31</sup> Reprinted with permission from AAAS.) (E,F) Under optimal SMLM conditions, well-stained MTs when rendered in two dimensions, display a characteristic less intense center. (F) Average intensity cross section taken from the boxed area in (E). Scale bar = 250 nm. (Adapted from Dempsey et al.<sup>32</sup> with permission from Macmillan Publishers Ltd, copyright 2011.) (G,H) Innovative correlative live cell epifluorescence/SMLM experiments of Balint et al.<sup>33</sup> used to monitor lysosome cargo trafficking on MT networks. (G) Live cell, diffraction-limited single-particle tracking pathways of several lysosomes. After acquisition, the sample was fixed and immunostained for tubulin to obtain the subdiffraction cytoskeleton architecture shown in (H). Overlay of these two images allows analysis of the pathways taken and demonstrated the hindrances caused by MT intersections that are eventually overcome with high fidelity.

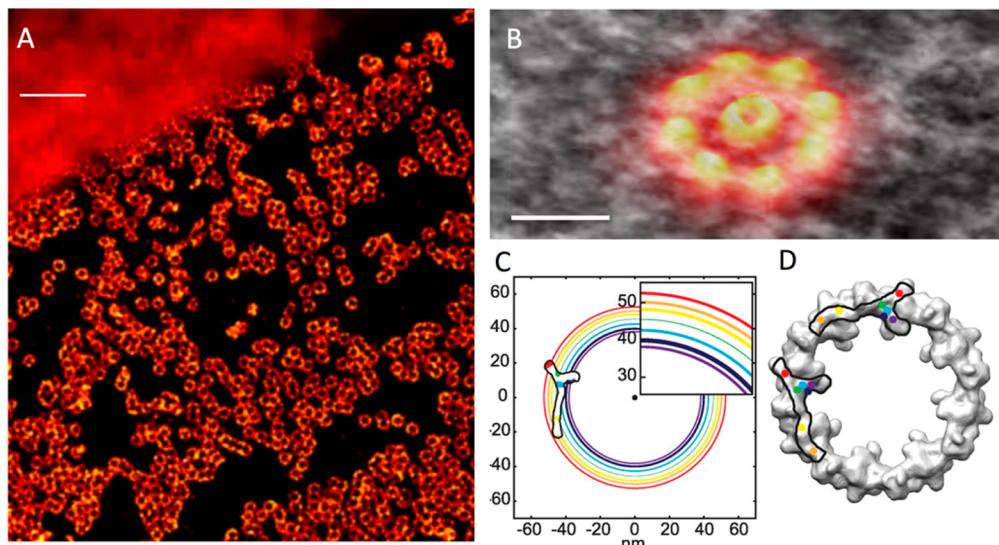
Beyond the scope of benchmarking, SMLM has been used to probe the subdiffraction structures and mechanisms of both MTs and NPCs, revealing new and exciting detail. Using correlative live cell STORM, the transport trajectories of cargos along individual MTs have been investigated and the impact of various cytoskeletal geometries quantitatively determined.<sup>35</sup> A previously undetected bundling of MTs in response to viral proteins has also been imaged.<sup>36</sup> Through particle averaging, the nanometer-precise orientation of the major building block of the NPC, Nup107–160, within the complex has also been revealed.<sup>34</sup> Moreover, NPCs are now being demonstrated as ideal for correlative SMLM–electron microscopy benchmarking experiments as they allow a direct overlay and any variations between the two methods to be determined.<sup>37,38</sup> Another recent example of correlative work combined atomic force microscopy with SMLM to image double-stranded DNA and highlighted the use of correlative techniques for both validation and probing new questions about biological samples.<sup>39</sup> Undoubtedly, correlative experiments are currently at the forefront of SMLM research, and physical chemists are well placed to make significant contributions to these.

**Sample Preparation.** A perhaps underdiscussed difficulty in conducting good SMLM research is the expertise and time

necessary for preparing fluorescently stained biological samples. This can generally be achieved by immunolabeling or via the coexpression of fluorescent proteins alongside the target molecules, as has been used for confocal imaging for many decades. Despite a long history of the successful use of both methodologies in order to obtain excellent, trustworthy confocal images, new and extensive development of these protocols is crucial for SMLM. This is primarily because the published sample protocols have only had to be robust enough to preserve cell structure at a diffraction-limited level; SMLM, of course, indiscriminately detects the structure and distribution of the target constituent at an impressive subdiffraction level whether it is biologically relevant or artificial. Protocols for SMLM experiments need to maintain ultrastructure down to ~10 nm spatial resolution as well as control for nonspecific and background fluorescence. Nonspecific staining will unavoidably result in single-molecule localizations of the target fluorophore in positions that are not biologically relevant, while heightened background fluorescence will inhibit the localization precision of the entire image.

Recently, we have shown that structural and distribution artifacts that significantly impact SMLM images can be caused by even slight changes in fixative concentration, temperature, incubation time, permeabilization time, and antibody dilution, as well as the ordering of these steps and the inclusion (or not) of washing steps.<sup>40</sup> This necessitates careful consideration of all of these aspects. Furthermore, when these parameters are optimized, well-understood, and carefully applied, they become key to obtaining SMLM images with the necessary high level of fidelity between the native biological state and the rendered image that allows for breakthrough SMLM research to occur. This is generating renewed interest in fundamental research aimed at understanding the biochemical mechanisms of fixation such as the cross-linking effect of aldehydes and the precipitative dehydration of alcohols, as well as the effects of surfactants used for permeabilizing membranes. While such reactions are understood in simple systems, the full extent of the chemical interactions of these reagents in cells is largely still speculative. The role that incubation time, concentration, and temperature play in such fixation procedures can be considered as fundamental questions of reaction kinetics, allowing for a direct approach to optimization of these parameters in fixation protocols. True efforts to achieve this as well as to develop well-optimized and tested protocols for the preparation of specific cells and target structures for benchmarking and standardizing results are a crucial part of the ongoing development of SMLM and require extensive chemical and biological expertise. Importantly, any new cell type, target molecule, fluorophore, or antibody necessitates reoptimization of a pre-existing protocol. Understanding this is another facet of SMLM that is vital in achieving robust and trustworthy results.

The demonstrated extensive and complicated effects of fixation on a sample's subdiffraction ultrastructure, as well as the fact that SMLM images cannot be treated as equivalent to confocal images in that they are renderings of single-molecule coordinates, mean that the relationship between a fixed sample and the biological reality should always be carefully considered. To do so requires communication of the known and hypothesized biology as well as the underlying chemistry of the sample preparation and the effects of the measurement and the processing on the image. Due to the large and variable amount of chemistry that occurs with each preparative step including everything from the en masse cross-linking or precipitation

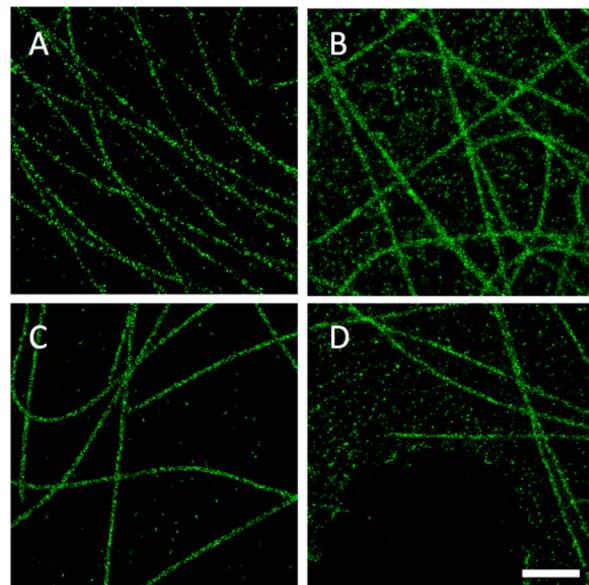


**Figure 5.** The NPC is another ideal biological target for benchmarking as well as novel research. (A) Immunostained gp210 proteins within the NPC using both epifluorescence (top left corner) and SMLM. Scale bar = 1  $\mu\text{m}$ . (Adapted from Löschberger et al.<sup>33</sup> Reprinted with permission from AAAS.) (B) The eight-fold symmetry of this protein can clearly be seen in the super-resolution image, which can be correlated directly with the EM image. Scale bar = 100 nm. (Adapted from Sauer.<sup>37</sup> Reprinted with permission from AAAS.) (C,D) By labeling various locales on the Nup107–160 subcomplex of the NPC and using particle averaging, the nanometer-precise arrangement of the proteins was also determined. (Adapted from Szymborska et al.<sup>34</sup> Reprinted with permission from AAAS.)

effects of fixatives to the subtle changes brought on by small changes in pH, the final sample is biochemically quite different from the biologically native state. The aim of sample fixation is to maintain the ultrastructure using these chemical changes while at the same time preserving the epitopes needed for fluorophore labeling. The success of a particular sample preparation in maintaining ultrastructure is usually assessed empirically through comparison with the known structure of cellular constituents from electron microscopy, confocal fluorescence, and, as a gold standard, live cell microscopy. However, electron microscopy is just as susceptible, if not more, to fixation artifacts, and confocal microscopy only allows matching of structures at a diffraction level. This is another reason why benchmarking of methodologies with well-tested structures such as MTs or NPCs (as discussed above) is ideal. Even with this type of benchmarking, careful attention to the chemical effects of fixation, and well-established structure from the literature, SMLM images can still deviate from what is expected and necessitate further consideration.

An example of such an occurrence is our recent observation<sup>40</sup> that by changing the point at which permeabilization is done, the level of localized apparent nonspecific dye can be drastically lowered in cells immunostained for tubulin, the monomeric unit of MTs, as shown in Figure 6. While prepermeabilization of cells allowed for a very clean polymeric architecture to be imaged using SMLM (Figure 6C), it was later realized that the “nonspecific” localizations were likely to be unpolymerized dimeric tubulin units. By permeabilizing after fixation, samples with higher nonspecific stain were acquired, and these are much closer to the biological reality (Figure 6D). Thus, by changing the preparation protocol, “cleaner” images could be obtained; these are, however, less indicative of the biological native state of the cells.

The immunolabeling of a sample postfixation can also cause artifacts such as over-staining, leading to enlarged features, or under-staining, which leads to discontinuous or absent features.



**Figure 6.** Four common artifacts caused by incorrect sample preparation. (A) MTs that are understained causing gaps in the filamentous structure; (B) overstained MTs that have cross sections significantly larger than well-stained MTs. (C) shows a very clean image of MTs due to prepermeabilization of the sample to remove all cytosolic components, while (D) has more nonpolymerized localizations likely due to the presence of dimeric tubulin. Scale bar = 1  $\mu\text{m}$ . (Adapted from Whelan and Bell.<sup>40</sup>)

Similarly, expression of fluorescent proteins can change the biochemistry of a live cell and also generate artifacts. Because of this, alternate labeling techniques are being applied to SMLM experiments. These include intercalating membrane and DNA fluorophores as well as synthetic pathways for attachment of fluorophores onto biological targets such as azide–alkyne Huisgen cycloaddition click chemistry<sup>41</sup> and SNAP protein fusion tags.<sup>42</sup>

*Fluorophores and Blinking Mechanisms.* Like labeling methods for SMLM, the repertoire of fluorophores and mechanisms by which single emitters can be temporally separated is currently in a rapid state of expansion. Initially, SMLM was limited to a handful of specialized synthetic small-molecule-based FRET pairs or photoactivatable fluorescent proteins and this was a major limitation on uptake by the general scientific community. Recognition of the innate photoswitching of Alexa Fluor 647, an ideally bright and deep-red SMLM dye, in the presence of a reductant made SMLM much more accessible.<sup>11</sup> However, *d*STORM was originally criticized by some for being restricted to only a small subset of potential fluorophores even though by utilizing immunolabeling, an extensive range of targets are accessible with this single fluorophore. In the 6 years since, dozens of fluorophores capable of photoswitching in the presence of a reductant have been identified and well-characterized.<sup>32</sup> The buffering of these dyes in order to achieve ideal photoswitching is an ongoing area of research with different reductants investigated as well as oxygen removal mechanisms and reactivation pathways.<sup>43</sup> With every dye possessing unique photophysics, the potential for research on these aspects alone is vast. It is also extremely important because better fluorophores and switching mechanisms lead to better SMLM images. This is one area of SMLM research that can be conducted by physical chemists and chemical physicists without an immediate need for collaboration. The pre-existing ability to characterize fluorophores, namely, by determining the excited state and redox properties key to defining the fundamental off states required for SMLM, is already a well-established facet of many such laboratories.

Further to the development of new synthetic fluorophores,<sup>44</sup> various new photoactivatable, photoconvertible, or photoswitchable fluorescent proteins have also been identified and used for SMLM.<sup>6,45</sup> Moreover, various other mechanisms for detecting temporally separated single-molecule emissions have been published, most of which are based on changes in fluorescence upon association with a target molecule. Examples of this include the use of Nile Red for lipid bilayer detection,<sup>13</sup> intercalation-activated dyes for DNA detection,<sup>12</sup> and the use of diffusion-assisted FRET pairs to map structure in live cells.<sup>46</sup> SMLM that allows quantification of dyes and, by extension, target molecules is another burgeoning area with recent publications demonstrating careful control of fluorophore blinking and well thought out analysis to achieve such quantification.<sup>47,48</sup> The development of new fluorophores with photoswitching particularly suited to such methods is also expected to be an important area of future SMLM research. There is ample potential for discovery and development of both new photoswitchable fluorophores and mechanisms for SMLM that could potentially be used to probe currently inaccessible biological structures.

*Outlook and Future Directions.* At just over 8 years old, SMLM is still relatively new and holds immense potential for immediate and obvious applications in cellular and molecular biology, as well as in materials science. However, uptake of SMLM in laboratories not already dedicated to microscopy-based interdisciplinary research has been hampered by the broad collaborative demands necessary for ongoing use of the required hardware and software, as well as for the production of SMLM-appropriate samples and the analysis and interpretation of the resultant images. Physical chemists often work in highly collaborative environments, communicating with and between physicists and biologists while encompassing several niche areas

of interdisciplinary science. Such scientists are well-situated to facilitate implementation of SMLM research as well as the dialogues that are necessary to develop the technique in a holistic fashion.

**It is crucial that single-molecule localization microscopy (SMLM) microscopes and their software, as well as sample preparation protocols and fluorophore photophysics, be benchmarked.**

In this Perspective, we have stressed the necessity of benchmarking, protocol optimization, and cultivating an in-depth understanding of sample preparation, as well as fluorophore development and photophysical characterization and the role that interdisciplinary science must play in such endeavors. It is envisaged that SMLM will continue to operate as an interdisciplinary field with the best research conducted in the presence of various specialist scientists; however, it is also hoped that the technique will become more approachable. To do this, research into the chemistry of fixation and labeling is needed to develop more reproducible and successful protocols and optimization processes. Standardization methods and regulatory protocols for SMLM images also need to become commonplace in order to identify and avoid artifacts prior to publication. What is clear, however, is that the future is indeed bright for SMLM with implementation now readily approachable to many research groups with countless exciting open research questions waiting to be tackled.

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