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# From Molecules to Classrooms: A Comprehensive Guide to Single-Molecule Localization Microscopy

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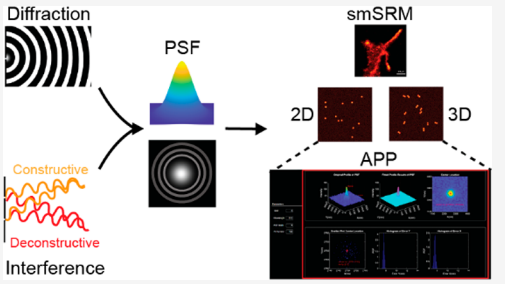
**ABSTRACT:** Single-molecule localization microscopy (SMLM) has revolutionized our ability to visualize cellular structures, offering unprecedented detail. However, the intricate biophysical principles that underlie SMLM can be daunting for newcomers, particularly undergraduate and graduate students. To address this challenge, we introduce the fundamental concepts of SMLM, providing a solid theoretical foundation. In addition, we have developed an intuitive graphical interface APP that simplifies these core concepts and makes them more accessible for students. This APP clarifies how super-resolved images are fitted and highlights the crucial factors that determine image quality. Our approach deepens students' understanding of SMLM by combining theoretical instruction with practical learning. This development equips them with the skills to carry out single-molecule super-resolved experiments and explore the microscopic world beyond the diffraction limit.




**KEYWORDS:** *Image Formation, Point Spread Function, Spatial Resolution, Wave Optics, Image Quantification*



Single-molecule localization microscopy (SMLM), or single-molecule super-resolution microscopy, is an interdisciplinary technique transforming our understanding of the microscopic world.[1,2] This powerful tool enables the investigations of protein behaviors and functions at the cellular level[3−7] and elucidation of molecular dynamics within catalysts or the catalyst−enzyme interface.[8−10] SMLM provides nanoscale insights into material structures, helping surface preparation,[11,12] revealing critical defects at the atomic level,[13] and offering real-time observation of intricate self-assembly processes.[14,15] Recent integration with electrochemical analyses further allows capture of ionic current signals, fluorescence images, optical spectra, and photocurrent intensity in the nanopore systems.[16,17] However, SMLM can be difficult for undergraduate and graduate students to comprehend due to its reliance on knowledge of wave optics, instrumentation, and software development. We developed a comprehensive teaching approach to bridge this educational gap that combines theoretical instruction with practical learning. We introduce students to the fundamental concepts of image formation in fluorescence microscopy[18−20] and provide them with a homemade graphic user interface (GUI) application (hereafter referred to as the "APP"). This APP is designed to demonstrate digital data collection, image quantification, and validation, aspects that are often overlooked in the literature.

Several groups have developed hands-on activities to help students understand single-molecule microscopy. For instance, Zimmermann and colleagues use a wide-field microscopy approach where students can observe fluorescing single molecules in real-time,[21] while Hu and colleagues introduce a nanopore technique for understanding single molecule behaviors.[17] Harbron and Barbara designed an undergraduate analytical chemistry experiment where students can investigate the spatial distribution of molecules from the Poisson distribution.[22] There has been an increasing interest in hands-on learning for the interdisciplinary concepts of SMLM, including Varra and colleagues' hands-on microscopy experiment that allows students to assemble a fluorescence microscope and learn image processing and analysis procedures.[23] Heo and colleagues developed an optical system for students to learn the concept of light diffraction and single-molecule localization.[24] Moerner and colleagues created an open-source GUI that processes single molecule double-helix images to display super-resolved three-dimensional reconstructed images.[25] However, these resources often overlook key concepts essential to thoroughly understanding SMLM. To address this, we have designed our homemade APP to cover these areas and help students improve their understanding of SMLM.

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Several key concepts are first introduced to prepare students to use the APP. We delve into the details of the principles of fluorescence microscopy, the role of the objective and tube lens in image formation, and the localization precision of SMLM. Besides providing a comprehensive overview of the principles of fluorescence microscopy, including diffraction, interference, point spread function, and resolution limitation, we also discuss the importance of the Abbe Sine Condition and the Rayleigh criterion for resolution, which are fundamental guidelines for designing optical systems.

The APP introduces students to the key concept that determines the resolution of light microscopy: the diffraction-limited spot or point spread function (PSF). It shows how the PSF can be fitted with a two-dimensional Gaussian function to localize the centroid of the PSF with super-resolution. The APP also illustrates the impact of noise and wavelength on the localization precision. Moreover, it allows students to visualize the PSF of point light sources of various sizes and understand the concept of the optical diffraction limit. It also enables students to simulate PSF with assigned signal-to-noise levels and light source wavelengths, highlighting how noise and wavelength affect the localization precision. Finally, the APP shows the process of generating super-resolution reconstructed images from single-molecule fluorescence images. This activity introduces students to key SMLM concepts typically scattered throughout various lectures. We aim to enhance students' understanding of SMLM and equip them with the necessary skills to explore the microscopic world beyond the diffraction limit.

## ■ ACTIVITY DESIGN

### Course Information and Place in the Chemistry Curriculum

Within the realm of Chemistry, SMLM's precision and superior analytical prowess position it as an ideal subject for Analytical Chemistry. This is particularly true when students tackle the challenges of deciphering complex mixtures or navigating the intricate world of cellular environments. Shifting our focus to the Physics Department, the emphasis of SMLM on cutting-edge optical techniques makes it a standout topic for courses in Optics and Photonics. Here, students can witness the real-world implications of light's behavior and its myriad properties. Moreover, those diving into Instrumentation and Experimental Physics will find the introduction of SMLM to be a rewarding hands-on encounter with contemporary experimental methods.

While the course is designed for students with a foundational understanding of general chemistry and basic physics, it does not assume prior in-depth knowledge about microscopy, the diffraction limit of light, or advanced light−matter interactions. This three-week activity (two 1.5 h lectures per week) was implemented on a team-taught graduate level course, Fundamental Chemical Analysis (CHEM 6333), with typical enrollments of 10−20 students. This course aims to provide an overview and operational principles of major components commonly found in the instruments used in Physical and Analytical Chemistry research. The design of the activity begins with lectures related to image formation in the microscope and principles to achieve SMLM (week 1). Detailed explanation of the working principle of the APP were provided in the week 2 lecture. Students are actively engaged with the APP to explore practical effects, such as how varying wavelengths and Signal-to-Noise Ratios (SNR) influence localization precision as well as the framework to reconstruct 2D and 3D reconstructed images (Week 3). This hands-on approach ensures students not only grasp the theory but also gain practical insights into concepts such as diffraction, interference, and the intrinsic resolution limitations in microscopy.

### Guidance on Classroom Use

**Week 1: Image Formation and Principles to Achieve SMLM (Supporting Information 1).** The classroom sessions provide a comprehensive exploration of the intricacies of microscopy, with an emphasis on SMLM (Figure 1). The curriculum begins by examining the fundamental structure of microscopes, highlighting the advantages of the modern infinity-corrected system. This design ensures greater adaptability and superior image quality. Further exploration into ray tracing differentiates between paraxial and oblique rays. For a

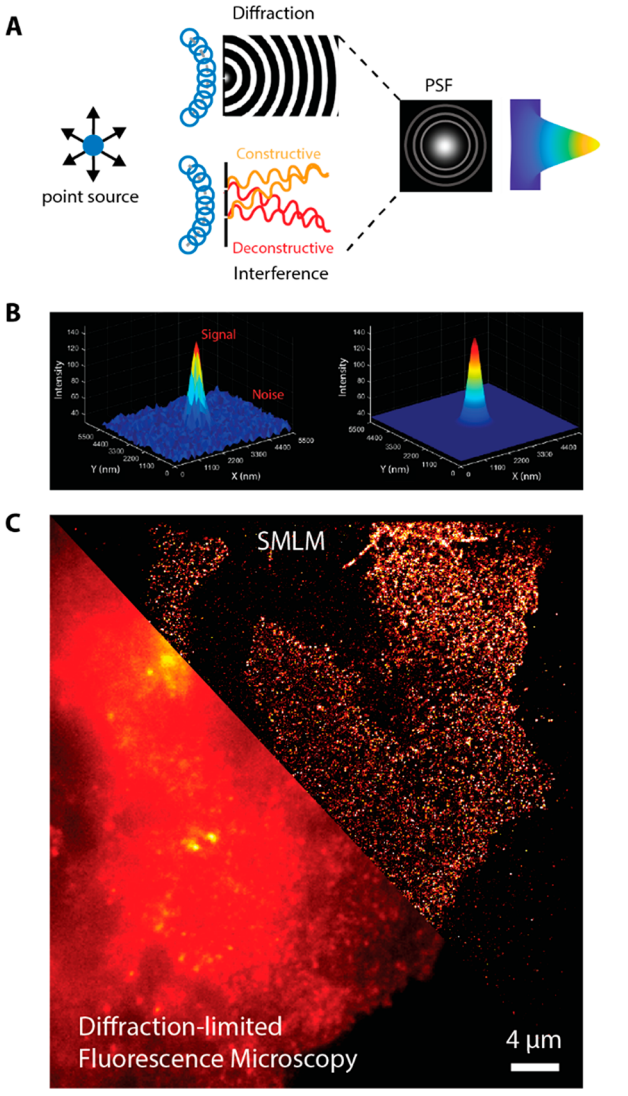


**Figure 1.** Working principles of SMLM. (A) Formation of PSF through interference. (B) Obtaining the centroid of PSF via a 2D Gaussian Fitting. (C) Example of SMLM. Fused image of the diffraction-limited fluorescence image and SMLM image. The feature represents the copper transporter protein tagged with mEos4c fluorescent protein distributed in a corner of the green monkey kidney cell bottom surface.


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J. Chem. Educ. XXXX, XXX, XXX−XXX

comprehensive understanding of these concepts, we delve deeper into the Abbe Sine Condition, showcasing its role as a pivotal principle for minimizing optical aberrations (Supporting Information 1, section 1). As the course progresses, the interaction between light and lenses is dissected, elucidating concepts such as diffraction and interference. We further elaborate on the Rayleigh criterion in the Supporting Information, emphasizing its significance in defining resolution boundaries (Supporting Information 1, section 2). Central to achieving the desired resolution in SMLM is the choice of fluorophores. Their unique properties, including brightness and stability, are instrumental in realizing the full potential of SMLM. We have incorporated a discussion on fluorophores suitable for SMLM imaging, emphasizing their pivotal role in SMLM (Supporting Information 1, section 3). This leads to the heart of microscopy: the point spread function and inherent resolution limitations. SMLM emerges as a technique where precision takes precedence, demonstrating the art of pinpointing the essence of observed objects (Supporting Information 1, section 4). The curriculum does not remain confined to two dimensions; it ventures into the third dimension with PSF engineering, including astigmatic PSF generated by a cylindrical lens and advanced methods like the Double Helix Point Spread Function (Supporting Information 1, section 5). Concluding the exploration, various factors influencing microscopic resolution are discussed, from optical component nuances to illumination techniques and the critical principle of Nyquist sampling (Supporting Information 1, section 6). This comprehensive approach ensures that students receive a robust foundation in microscopy, positioning them for future advanced research and applications.

**Week 2: Working Principle of the APP (Supporting Information 2).** The SMLM APP (Figure 2) emphasizes transparency in its coding logic, ensuring that students fully understand its inner workings rather than perceiving it as a mere "black box". The APP's intricate processes of simulating and fitting the PSF provide essential insights into SMLM. Structured into three distinct tabs, the APP offers targeted learning experiences. The first tab provides an interactive exploration of the influence of different wavelengths and SNR on localization precision. Subsequently, the second tab elucidates the reconstruction process from individual molecular PSFs, while the third tab delves into the nuances of Double Helix-PSF (DH-PSF) image reconstruction. This systematic approach ensures that users not only navigate the APP efficiently but also grasp the foundational principles and algorithms, fostering a deeper understanding of SMLM.

**Week 3: Applications of APP (Supporting Information 3).** To deepen the understanding of SMLM concepts, a specialized APP has been introduced, offering a hands-on learning experience with fundamental principles. The APP is divided into three essential modules (Figures 2 and 3).

*Localization of Single-Molecule PSF.* This section showcases how SMLM pinpoints precise positions from PSFs. Using the APP, students can generate simulated spots with exact center locations, fit them, and observe their distributions. The Gaussian fitting algorithm sheds light on the PSF's attributes, such as center location, intensity, and background noise. Results from this module highlight the slight variations in localization precision from the true center.

*Effects of Wavelength and SNR on Localization Precision.* This module delves into the nuanced interplay of light's wavelength and its impact on localization precision. By simulating PSF results across varied wavelengths, we show the influence of shorter wavelengths on improved precision becomes evident. Furthermore, the APP provides simulations of images with different SNRs and wavelengths, illuminating the combined effects of these parameters on the localization precision. It becomes clear that a myriad of factors, from photon counts to light wavelength, collectively shape the precision of the molecular positions.

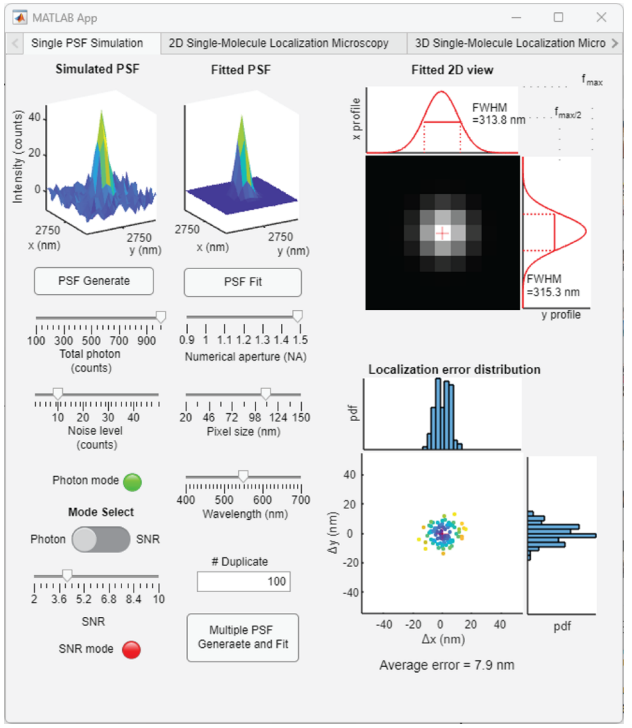


**Figure 2.** SMLM APP interface. The three tabs provide direct visualization of single-molecule fluorescence images and the working principles of SMLM. Displayed here is Tab 1, designed to enable students to explore the effects of various factors, such as photon count, noise levels, signal-to-noise ratio (SNR), wavelength, pixel size, and numerical aperture, on localization precision.

*Reconstructing 2D Super-Resolved Images from Localizations.* This exercise offers an insightful exploration of the SMLM process. Within the APP, students simulate a 2D movie, tracking the progressive buildup of single-molecule localizations. They adjust molecule activation densities to understand the impact on localization accuracy and image quality. This hands-on experience highlights the critical role of appropriate activation rates in achieving high-quality SMLM images, sometimes outweighing the influence of factors like wavelength and SNR. The APP now provides a reconstructed image function within Tab 2, allowing direct comparison of the fluorescence images pre- and post-application of the super-resolution algorithm. The reconstruction uses the localized points, convoluted with a 2D PSF reflective of localization precision, to showcase the enhanced resolution.

*Extraction 3D Localizations beyond Diffraction Limit.* Venturing into the 3D territory, this segment demonstrates the process of crafting 3D super-resolved images. Incorporating this APP into classroom settings provides an invaluable tool for visualizing and engaging with SMLM concepts. Through interactive simulations, abstract theories come to life, reinforcing foundational knowledge and setting the stage for advanced exploration in the field.


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J. Chem. Educ. XXXX, XXX, XXX−XXX

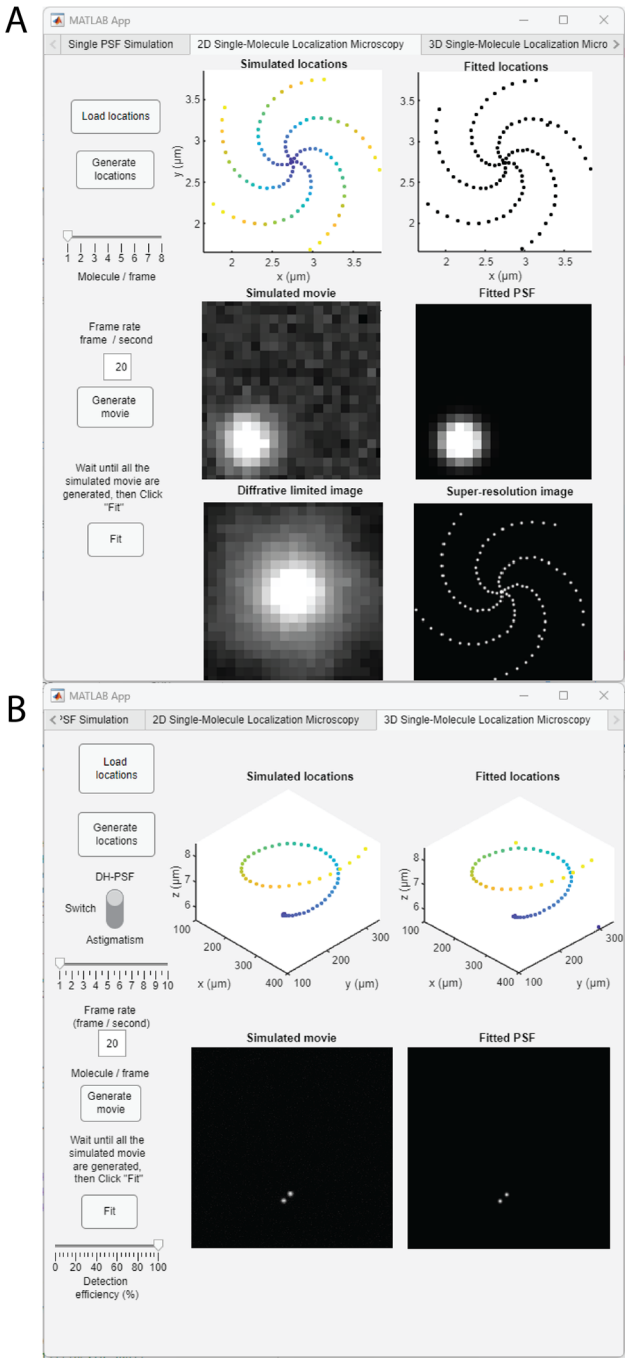


**Figure 3.** SMLM APP content for 2D and 3D SMLM. (A) In Tab 2, users can simulate single-molecule fluorescence images, fit these simulated images, and generate plots for accumulated locations, effectively demonstrating the principles of SMLM in a two-dimensional (2D) framework. (B) Same as (A) but for the 3D SMLM. The PSF is depicted as a double-helix pattern, and the $z$ information is encoded into the angle of the two lobes.

## ■ ASSESSMENT

The introduction of the APP into our curriculum was intended to deepen students' understanding of SMLM and its foundational concepts. To streamline the course objectives and enhance the learning experience, we have added Supporting Information 4, which includes a thorough lecture schedule, tailored questions for evaluating student knowledge before and after the course, and a survey aimed at assessing the APP's impact on SMLM education.

### Pretest–Post-test Analysis

The incorporation of the APP into the academic program aimed to enhance students' comprehension of SMLM and its underlying principles. To provide clarity on the objectives of this curriculum, we have outlined key learning outcomes that set clear expectations for students. Students are expected to delve into the fundamental structures of microscopes, understanding the nuances of various designs with a focus on the modern infinity-corrected system renowned for its adaptability and exceptional image quality (Table 1 Domain 1). They should become familiar with essential optical principles, highlighting the Abbe Sine Condition and the Rayleigh criterion. Emphasis is placed on the distinctive capabilities of Single-Molecule Localization Microscopy (SMLM), showcasing its prowess in achieving super-resolution imaging (Table 1 Domain 2). Furthermore, students engage with the complex dynamics of the Point Spread Function (PSF) and its implications in localization precision, exploring concepts such as the Cramér-Rao lower bound and the importance of Gaussian fitting in SMLM (Table 1 Domain 3). With these clear outcomes in mind, both pretests and post-tests were conducted, specifically tailored to gauge students' understanding of essential SMLM principles.

The examination framework encompassed three distinct domains (Table 1).

**Microscope Setup and Optics.** Focused on the optical foundations of SMLM, this domain assessed students' comprehension of concepts such as the infinity-corrected system and ray tracing principles.

**Fundamental Principle of SMLM.** This segment explored the core tenets of SMLM, evaluating students' knowledge about its unique ability to achieve super-resolution imaging and the principles that set it apart from other microscopy techniques.

**PSF and Localization Precision.** This section delved into the intricacies of the PSF and its relationship with localization precision. Key concepts included the Cramér-Rao lower bound formula, the role of Gaussian fitting in the SMLM, and the significance of the double helix phase mask in the DH-PSF method.

The pretest was meticulously crafted to determine students' initial knowledge of core SMLM principles. Questions covered a range of topics, from the capabilities of microscopy techniques to the intricacies of the Cramér-Rao lower bound formula. Analysis of the outcomes revealed areas of difficulty, particularly concerning the dominant noise sources in SMLM, with a mere 25% accuracy rate (Figure 4A, Q2). Similarly, only a third of the respondents correctly addressed questions about the double helix phase mask and astigmatism imaging (Figure 4A, Q8 and Q9). These insights proved invaluable, guiding instructors on which topics required further elaboration during subsequent lectures and APP sessions.

The post-test, designed to be more challenging, tested students to apply their knowledge rather than rely on mere recollection. Despite this heightened complexity, the outcomes were promising. All students displayed comprehensive understanding of modern microscopy setups, particularly the infinity-corrected system and the Double Helix Point Spread Function (Figure 4A, Q9). Nonetheless, a challenging area remained:


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J. Chem. Educ. XXXX, XXX, XXX−XXX

**Table 1. Pretest and Post-test Questions in Three SMLM Domains**

| Domains | Pretest Questions | Post-test Questions |
|---|---|---|
| Microscope Setup and Optics[3,26,27] | Which microscopy technique is known for its ability to achieve imaging beyond the diffraction limit of light? | Which type of rays strike the lens at an angle, rather than running parallel to the optical axis? |
| | Which is a dominant source of noise in SMLM? | What does the Fourier plane in an optical system represent? |
| | What does the Nyquist criterion ensure in microscopy? | What issue can the traditional setup's fixed optical path length lead to? |
| Fundamental Principle of SMLM[1,28−30] | In SMLM, what function is typically utilized to fit the PSF? | What is the central bright area of the Airy pattern called? |
| | Why is it essential to perform Gaussian fitting in SMLM? | What is the primary factor that determines the spatial resolution of a microscope at the tube-lens-focusing step? |
| | The Cramér-Rao lower bound formula is associated with which aspect of SMLM? | Which criterion states that two equally bright points can be distinguished if the peak of the intensity distribution of one coincides with the first minimum of the other? |
| PSF and Localization Precision[31−35] | Which parameter influences the localization precision as indicated by the Cramér-Rao lower bound formula? | In SMLM, how is the localization precision of a spot determined? |
| | Astigmatism imaging introduces a cylindrical lens to produce what kind of PSF? | Which factor does NOT directly impact the localization precision in SMLM? |
| | What is the primary role of the double helix phase mask in the DH-PSF method? | What do the two lobes of the Double Helix Point Spread Function (DH-PSF) represent? |
| | How does PSF engineering enhance three-dimensional SMLM imaging? | Which criterion ensures proper sampling in microscopy? |

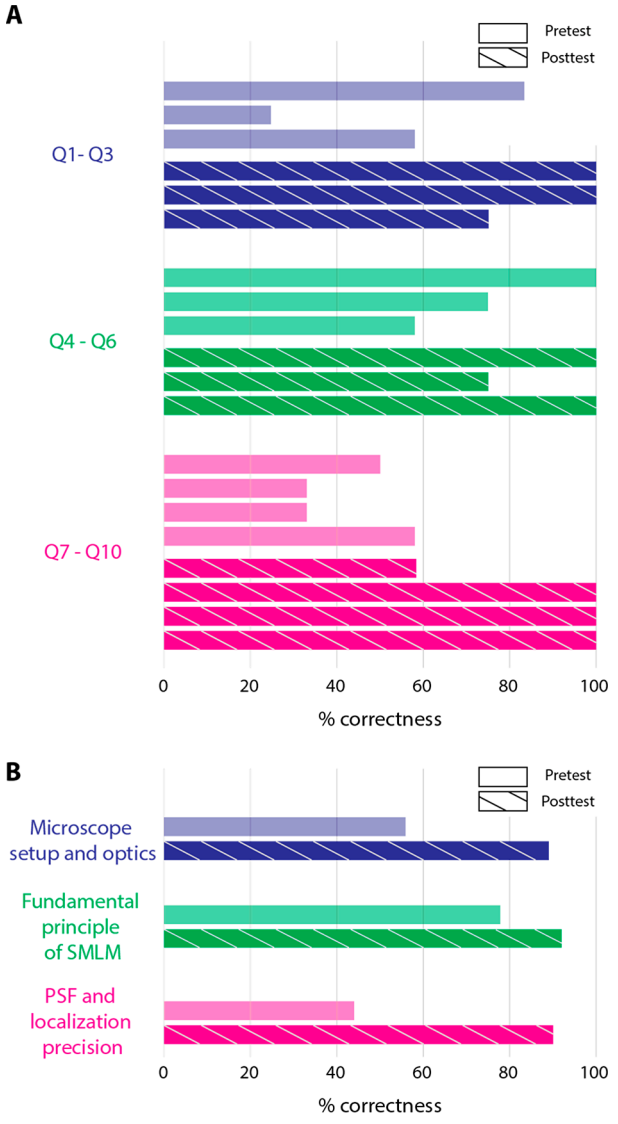


**Figure 4.** Pretest−post-test analysis. (A) Percent correctness results of individual questions listed in Table 1. (B) Percent correctness analysis in three different domains.

only 17% could precisely define how SMLM determines the localization precision of a spot.

Upon analysis of the pretest and post-test scores, we observed a notable enhancement in performance (Figure 4B). Specifically, the accuracy rates for domains 1, 2, and 3 improved by 30%, 15%, and 50%, respectively. These improvements underscore the instrumental role of the APP in bolstering students' comprehension of the material. Such progress affirms the effectiveness of the instructional methods adopted, with the APP playing a crucial role in addressing and bridging knowledge gaps.

To conclude, the strategic use of pretests and post-tests provided clarity on students' strengths and areas of improvement concerning SMLM. The data from the pretest informed educators on topics that required additional emphasis, while the post-test results, despite their demanding nature, exhibited substantial advancements in student comprehension. This progression highlights the combined success of lectures, APP demonstrations, and students' dedication to grasping SMLM.

## Survey Analysis

A comprehensive survey (Table 2) was conducted to gauge students' perceptions of the APP as a learning tool for SMLM. Feedback was overwhelmingly positive (Figure 5), underscoring the platform's educational value. Regarding interface navigation (Figure 5, S1), the APP garnered an average score of 4.58 out of 5, indicating that most users found the platform intuitive. The clarity of the SMLM explanations and demonstrations within the APP was also well-received, earning an average rating of 4.50 (Figure 5, S2). Notably, the tool's interactive elements, encompassing simulations and quizzes, received an applaudable average score of 4.83 (Figure 5, S3). This mirrored the sentiment that the APP enhanced SMLM comprehension, outperforming traditional teaching methods, as reflected in a similar score of 4.83 (Figure 5, S4).

Highlighting the APP's impact, a significant 92% of participants recommended its use for prospective SMLM learners, culminating in an overall satisfaction score of 4.92 (Figure 5, S5 and S6). When probed about the APP's standout features, responses were diverse: while some appreciated the 3D fitting capabilities, others emphasized the value of visualizing the influence of parameters, such as wavelength and SNR, on localization precision. Direct visualization of


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J. Chem. Educ. XXXX, XXX, XXX−XXX

**Table 2. Survey to Evaluate the Effectiveness of APP for SMLM Comprehension**

| On a Scale of1−5, Give Scores for the Following Questions. (5) Strongly Agree; (4) Agree; (3) Neutral; (2) Disagree; (1) Strongly Disagree |
|---|
| S1. The APP's interface is user-friendly. |
| S2. The explanations and demonstrations of SMLM concepts within the APP are clear. |
| S3. The interactive elements of the APP (e.g., simulations) enhance your understanding of SMLM. |
| S4. The APP improved your understanding of SMLM compared to traditional teaching methods. |
| S5. You recommend the APP to peers or students studying SMLM in the future. |
| S6. Overall, you are satisfied with the APP as a learning tool for SMLM. |
| Free Response Questions |
| Which feature of the APP did you find most beneficial for grasping the key concepts of SMLM? |
| Were there any topics or concepts that you felt were not adequately covered or explained in the APP? If so, please specify. |
| Are there any additional features or topics you would like to see added to the APP? |
| Please suggest any modification of the APP to further improve your understanding of SMLM. |

simulated PSF images over time was another frequently mentioned advantage, elucidating the creation process of SMLM movies.

While the feedback was largely positive, areas for potential enhancement emerged. Concepts such as 3D visualization and the "$z$" value calculation were identified as needing further emphasis. Future addition recommendations included functionalities for $z$-value calculations from rotation angles, ensemble imaging comparisons, and the introduction of real experimental data for a more hands-on experience. In summary, the survey results accentuate the APP's pivotal role as an esteemed learning instrument for SMLM, while also spotlighting avenues for future refinements.

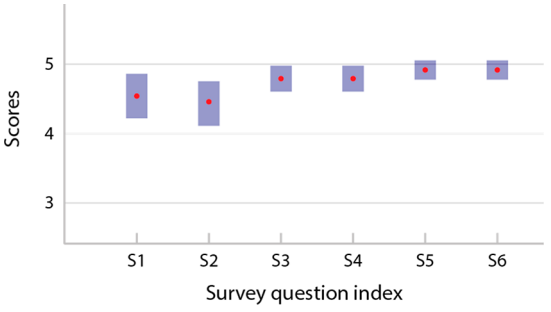


**Figure 5.** Survey results to evaluate the effectiveness of the APP for SMLM comprehension. The red dot and the box represent the average and standard deviation of the scores, respectively.

## CONCLUSION

SMLM can be challenging for undergraduate and graduate students due to its reliance on wave optics, instrumentation, and software development knowledge. To address this gap, we introduce key concepts in image formation in fluorescence microscopy and develop a homemade APP that illustrates digital data collection, image quantification, and validation that are often absent from the literature.

To address this, a comprehensive teaching strategy was introduced, blending traditional instruction with hands-on learning through a specialized APP. This methodology not only solidified foundational SMLM concepts but also offered students an interactive engagement platform. Feedback from participants highlighted the APP's effectiveness and value in enhancing their understanding. Constructive feedback also pointed to areas of potential refinement, ensuring that the tool remains relevant to evolving educational needs. Overall, this initiative represents a significant stride toward a more integrative and engaging approach to SMLM education, preparing learners to delve deeper into the world of advanced microscopy.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.3c00938.

Supporting Information 1: Scientific and Pedagogical Background of Single Molecule Localization Microscopy (PDF; DOCX)

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### Author Contributions

†G.Y., Y.Z., and A.A. contributed equally. All authors contributed to the scientific and pedagogical background of this research. Y.Z., A.A., and G.Y. developed the APP. K.N.B., W.C., and S.Y. tested the APP and provided feedback on lecture questions and survey designs. Y.Z., A.A., G.Y., and T.-Y.C. designed the figures and wrote/reviewed the paper.

### Notes

The authors declare no competing financial interest.

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