Transforming Separation Science with Single-Molecule Methods

William Calabrase,[†] Logan D. C. Bishop,[‡] Chayan Dutta,[‡] Anastasiia Misiura,[‡] Christy F. Landes,[‡§⊥]* Lydia Kisley[†Δ]*

† *Department of Physics, Case Western Reserve University, Cleveland, OH 44106*

‡ *Department of Chemistry, Rice University, Houston, TX 77251*

§ *Department of Electrical and Computer Engineering, Rice University, Houston, TX 77251*

⊥ *Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX 77251*

Δ *Department of Chemistry, Case Western Reserve University, Cleveland, OH 44106*

AUTHOR INFORMATION:

*C. F. L. Email: cflandes@rice.edu, Phone: (713)-348-4232;

*L. K. Email: lydia.kisley@case,edu, Phone: (216)-368-2118

ABSTRACT

Empirical optimization of the multiscale parameters underlying chromatographic and membrane separations leads to enormous resource waste and production costs. A bottom-up approach to understand the physical phenomena underlying challenges in separations is possible with single-molecule observations of solute-stationary phase interactions. We outline single-molecule fluorescence techniques that can identify key interactions under ambient conditions. Next, we describe how studying increasingly complex samples heighten the relevance of single-molecule results to industrial applications. Finally, we illustrate how separation methods that have not been studied at the single-molecule scale can be advanced, using chiral chromatography as an example case. We hope new research directions based on a molecular approach to separations will emerge based on the ideas, technologies, and open scientific questions presented in this perspective.

INTRODUCTION

Liquid chromatography and membrane separations rely on the differential mass transfer of analytes pulled through a solid stationary phase by a liquid mobile phase. Conventional industrial grade separations are performed by empirical tuning of multiscale parameters, wasting analyte, reagents, time, and energy.[3] Separation depends on three major analyte phenomena: mobile/stationary phase exchange, adsorption/desorption kinetics at the stationary phase, and anomalous diffusion in complex environments.[4–7] These fundamental physicochemical, molecular level interactions of analytes within the stationary and mobile phase lead to the transport, retention, and selection properties at the analytical- and industrial-scale (**Figure 1**).

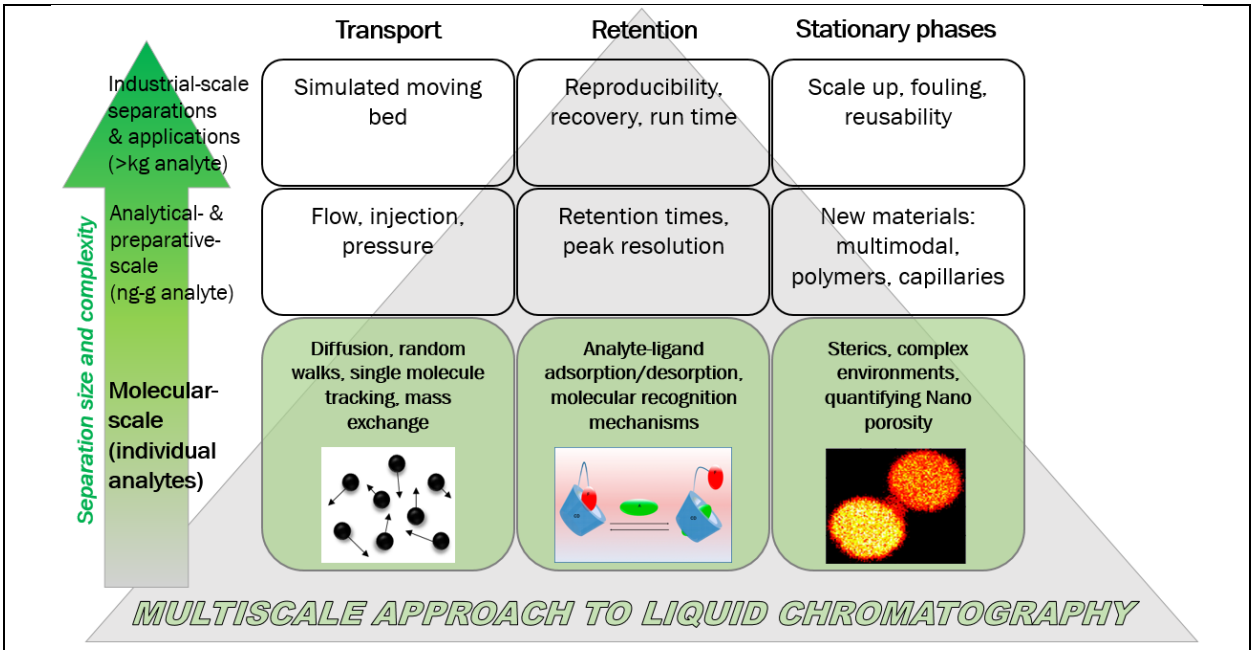


Figure 1. Connecting single-molecule results to key performance features of analytical- and industrial-scale separations. Properties that are accessible *via* single-molecule fluorescence microscopy scale up to become the important features in large-scale applications. By column, left-to-right: 1) Resolving molecular diffusion *via* single-molecule tracking will help determine important factors of analyte transport in bulk solutions. 2) Identifying how individual analytes interact with stationary phases (*e.g.* host-guest type molecular recognition, pictured)[1] can provide high-resolution information on how to optimize industrial-scale retention to improve analyte recovery, shorten separation times, and overall run time. Adapted with permission from ref.[1]. Copyright 2013 American Chemical Society. 3) Mapping the nanostructure of materials (*e.g.* quantifying nanoporosity in silica beads, picture)[2] can determine which stationary phases are most appropriate for separations at all scales. Adapted with permission from ref.[2]. Copyright 2010 American Chemical Society.

Studying separations from the bottom-up — one molecule at a time — can identify rare events that lead to the failure of challenging, high-purity separations (**Figure 1**). Heterogeneous interactions in the kinetic regime can lead to tailing, broadening, and peak overlap, resulting in low selectivity and recovery of desired analytes. The consequences from impure separations that result from rare populations can be catastrophic. For example, in chiral separations, the inability to purify enantiomers and prevent their interconversion led to the infamous thalidomide tragedy in the 1960's where fetal malformation occurred for pregnant women who used the racemic drug mixture as a morning sickness treatment.[8] In radiochemical separations, high purity is required to avoid hazardous radioactive wastes and to provide high-quality spectral signatures used in radiopharmaceutical imaging and treatment, nuclear energy, and nuclear forensic applications.[9–11] Oftentimes multiple, sequential separation steps are needed to obtain such rigorous purities due to low selectivity of similar isotopes. This results in separations being involved in 75% of the processing of rare earth elements: from the starting raw materials to the manufacturing and production to the end-of-use waste management.[10]

Single-molecule and super-resolution microscopy allows for the visualization of separations at a molecular level that could address challenges recently identified by the National Academies of Science, Engineering, and Medicine. In the summer of 2019, the National Academies Press released *A Research Agenda for Transforming Separation Science.*[12] The report proposes a range of unanswered fundamental science questions, identifies a need to measure analyte dynamics in realistic, complex environments, and encourages the use of new methods to understand the intrinsically non-equilibrium chemical processes that occur during separations to develop more efficient materials. Developments in single-molecule and super-resolution enable experimental

observation of non-equilibrium interactions that drive separations, offering an avenue to radically advance the separations field.

Here, we detail the current potential to use single-molecule fluorescence microscopy to understand the mobile/stationary phase exchange, adsorption/desorption kinetics, and anomalous diffusion in complex environments that occur in separations. We describe how new optical imaging technologies that use multicolored, three-dimensional, and expanded temporal capabilities can address open questions on the structure, stoichiometries, orientation, diffusion, and temporal changes in separations. We discuss how the study of more complex samples could relate single-molecule samples to column conditions in industrial settings. Finally, as a demonstrative example, we present how chiral chromatography, a technique that has not been studied at the single-molecule scale, could be advanced with single-molecule methods. The technologies and open research questions in this perspective are meant to inspire new research directions and collaborative opportunities among analytical chemists, spectroscopists, and separation scientists to drive improvements in separations from a molecular perspective.

SINGLE-MOLECULE IMAGING AND BOTTOM-UP SEPARATIONS

Why use single-molecule imaging techniques?

Single-molecule fluorescence microscopy, an optical imaging technique, is useful to understand the spatiotemporal nanoscale heterogeneity and dynamics that occur in separations (**Figure 2**). Individual fluorescently-labeled molecules are detected using a fluorescence microscope that has high sensitivity due to a laser excitation source, a high numerical aperture objective, high efficiency optical filtering, and high quantum efficiency detectors. By observing one molecule at a time, subpopulations that may be as low as less than one percent of the overall

total molecular population that lead to peak broadening and asymmetry in chromatography are resolved. Such rare events would otherwise be hidden in conventional ensemble experiments. Millisecond time resolutions and sub-diffraction limited, three-dimensional spatial resolutions at ~10 nm are realized *in situ*.[13–16]

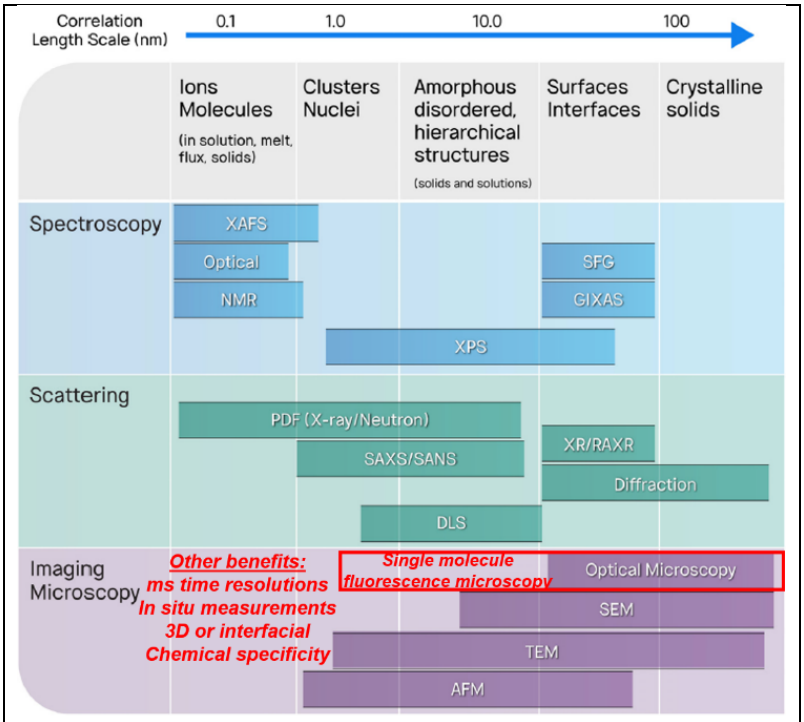


Figure 2. Single-molecule fluorescence microscopy complements the capabilities of other techniques used to study separations at length scales spanning subatomic distances to the bulk. (Red) The spatial capabilities of single-molecule fluorescence microscopy span from ~1's of nm using single-molecule FRET to ~10's of nm in three-dimensions using super-resolution imaging, to 10-100's of microns with wide field imaging. Please refer to the main text for technique abbreviations. Adapted with permission from ref[12]. Copyright 2019 The National Academies Press. Republisher is a for-profit organization.

Single-molecule fluorescence microscopy has detected and quantified the causes of common problems in chromatography and membrane separations.[5,17] Past findings include that defect silanol sites lead to rare (~1%), long-time adsorption of various analytes in reverse phase chromatography;[18–22] the balance of short- and long-range forces and surface polishing influence the behavior of biomolecules at the surface in capillary electrophoresis, normal phase and capillary liquid chromatography;[23–26] the sterics of the porous hydrogel stationary phase supports causes heterogeneity in ion-exchange chromatography;[27–29] and the relationships between analyte, surface, and solution chemistry are important in determining tortuosity, fouling, and adsorption in polymeric membrane separations.[30–32] Prior single-molecule studies have been carried out with

pM – nM concentrations of analyte so individual molecules can be localized and tracked. These low concentrations are relevant to kinetic tailing where rare, heterogeneous adsorption events that lead to peak asymmetry can be observed,[33] but below concentrations where column overloading that leads to thermodynamic tailing occurs. For more details on the results of previous single-molecule studies of separations, we refer the reader to prior review articles.[5,17,18]

Spectroscopic, scattering, and imaging techniques can also provide atomic to nano- to micrometer length scale information of materials used in separations. These were highlighted in the National Academies of Press report (**Figure 2**).[12] Spectroscopic methods using x-rays (x-ray fluorescence, absorption, photoelectron, and glancing incident absorption spectroscopies, [XRF, XAS, XPS, GIXAS]) obtain elemental composition information and atomic structural information down to molecular length scales of 0.6 nm. Nuclear magnetic resonance (NMR) spectroscopy provides information on the chemical functionalities of analytes, diffusion, and can even be used as a chromatographic detector, while sum frequency generation (SFG) and ellipsometry can determine electronic, vibrational, or structural dynamics selectively at surfaces. Scattering techniques such as dynamic light scattering (DLS), small-angle x-ray scattering (SAXS), small-angle neutron scattering (SANS), and x-ray reflectivity/resonant anomalous x-ray reflectivity (XR/RAXR) probe structural ordering and morphologies from atom–atom interactions (based on pair distribution functions [PDF]) to crystalline, macromolecular and aggregate structures. Finally, imaging techniques such as transmission and scanning electron microscopies (TEM, SEM) and atomic force microscopies (AFM) can provide sub-nanometer information about the structure of stationary phases.

The capabilities of single-molecule fluorescence microscopy complement the above-discussed methods. Fluorescence microscopy can be performed under ambient conditions relevant

126 to liquid separations, compared to techniques which require vacuum conditions (SEM, TEM).
127 Three-dimensional imaging can track dynamics within the bulk of porous stationary phases,
128 compared to scanning probe techniques that are limited to the interface (AFM). Fluorescence
129 microscopy achieves chemical specificity with targeted labeling so that analyte dynamics can be
130 visualized directly, compared to the stationary phase focus of many scattering and spectroscopy
131 techniques. While labeling allows for high certainty about which analytes are observed,
132 fluorophore selection and tagging strategies must be carefully considered to not interfere with
133 anticipated interfacial forces that drive adsorption. For small molecule organic separations, the
134 fluorophore is often strategically selected to be a model analyte based on size, charge, or
135 hydrophobicity, while in biomolecule separations, the protein terminus or end of a nucleic acid is
136 labeled to decrease possible interference with biomolecule function. Control experiments, such as
137 measuring structural[34] and functional changes[32] of the proteins due to the tagged fluorophores, are
138 required to understand the effects of dye labeling. Single-molecule fluorescence microscopy can
139 also be performed on accessible instrumentation. Many biophysics and medical microscopy core
140 facilities have confocal, wide field, and stimulated emission depletion (STED) microscopes
141 available. Construction of an economic homebuilt super-resolution microscopy setup can even be
142 achieved for < $30,000.[35] In contrast, select x-ray and neutron techniques require access and travel
143 to synchrotrons.

144 We believe there is strong future potential for studying separations using single-molecule
145 fluorescence microscopy. We complement the National Academies Press report by highlighting
146 the potentials of optical imaging techniques, with emphasis on new technologies that have yet to

be applied to separations (**Figure 3**). Creative sample preparations and microscopy hardware arrangements that have been developed by the biophysics and catalytic single-molecule communities should be applied to separations to provide new information on the structure, stoichiometries, orientation, diffusion, and temporal changes in separations.

Multicolored imaging to understand analyte structure and ligand stoichiometries:

Thus far, single-molecule imaging of separations have studied the adsorption and diffusion of analytes labeled with a single fluorophore. By using multiple dye labels that have different wavelengths of emission on either the analyte(s) or stationary phase, single-molecule fluorescence resonance energy transfer (FRET) and alternating laser excitation (ALEX)

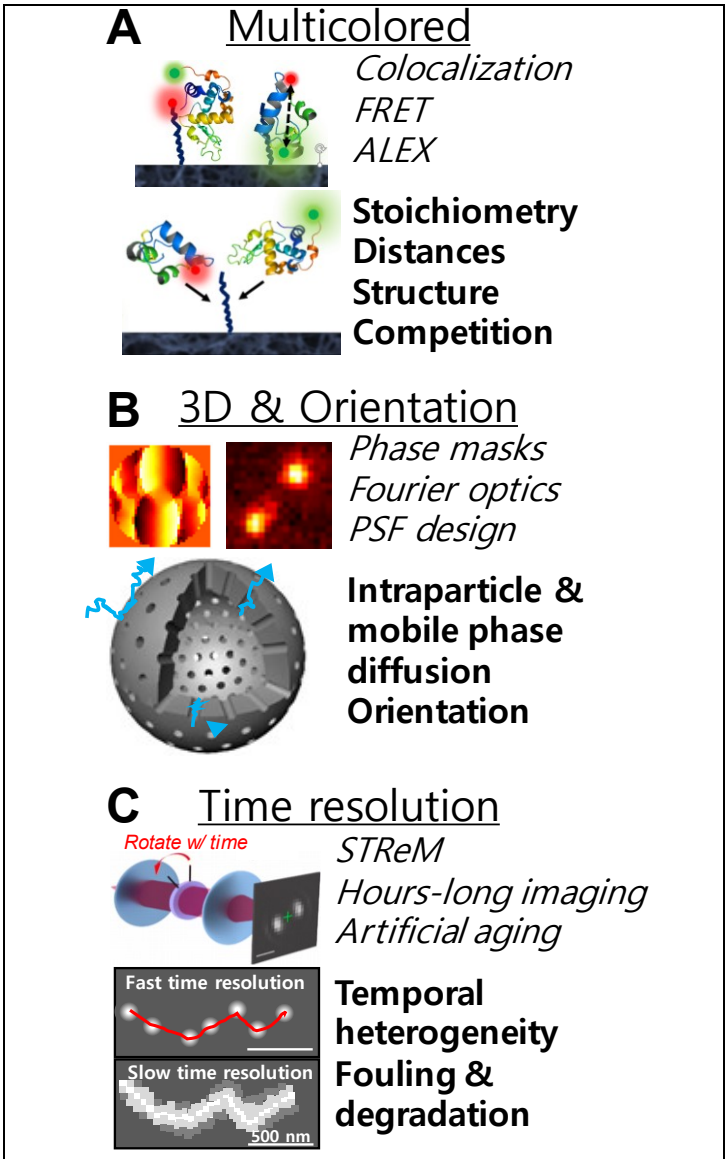


Figure 3. Single-molecule fluorescence techniques to apply to separations to resolve new phenomenon. (Italics) Advanced single-molecule fluorescence instrumentation and analysis techniques that have yet to be applied to separations can (bold) quantify new molecular dynamics. Using (A) multiple, spectrally-separated fluorophores and (B) designing new point spread functions (PSFs) for Fourier-plane imaging of three-dimensional location and orientation of individual molecules. Adapted with permission from ref[36]. Copyright 2019 Optical Society Publishing. (C) Measuring at shorter (<10 ms) and longer (hr-day) time scales with STReM or without motion blur. (C, bottom) Simulated moving particle measured with fast time resolution, enabling localization of distinct steps in the trajectory vs. slow time resolution, where localizations are hidden by motion blur. Adapted with permission from ref[37]. Copyright 2018 American Chemical Society.

analysis could be employed. FRET and ALEX resolve structural changes in analytes, stoichiometries between analytes and adsorption sites, and competition between analytes (**Figure 3A**).

Single-molecule FRET could provide information on the dynamic structural changes in biomolecules at separation interfaces. FRET quantifies the ~1-10 nm proximity between two fluorophores based on distance-dependent non-radiative energy transfer from a higher-energy "donor" fluorophore to a lower-energy "acceptor" fluorophore. Since this length scale is similar to the size of many biomolecules, FRET is often used to understand folding and conformational fluctuations. The donor and acceptor are placed at strategic locations on a biomolecule based on anticipated motions and unfolding distances that do not interfere with the function of the biomolecule. While the two fluorophore requirement for FRET limits the applicability of the technique for small analyte, organic molecule separations, single-molecule FRET can answer important questions regarding biomolecule structure and separation material function.

Single-molecule FRET studies show promise in answering fundamental science questions like those posed in the National Academies Press report. Studies of model proteins at polymer brush and silica interfaces with single-molecule FRET have shown that proteins diffuse and search in a folded state before unfolding and adsorbing at distinct surface sites for longer times.[38,39] Taking inspiration from these works, single-molecule FRET applied to separations could answer questions such as: Does the stationary phase disrupt the structure of the analyte causing it to unfold? How does the folded structure of the analyte correlate to its residence adsorption time at the surface? Does the analyte change structure, such as refolding, immediately before desorbing?

ALEX can allow for direct imaging of the arrangement of both the heterogeneous stationary phase and of unbound analyte dynamics, providing stoichiometric information beyond the usual

applications of single-molecule FRET.[40] In ALEX, an excitation scheme that alternates direct excitation of the donor fluorophore with direct excitation of the acceptor fluorophore at rates of 10-100's of kHz is used. FRET labels could be placed on separate molecules, such as tagging the ligand with an "acceptor" fluorophore and the analyte with the "donor" dye label (or vice versa). The fluorescently-labeled analytes and ligands can then be sorted based on the number and type of fluorophores detected using ALEX. Three types of information can be determined by ALEX: 1) the number of and spatial distribution of acceptor-labeled ligands observed with direct acceptor excitation; 2) the number of adsorbed analytes and the distance between the analyte and ligands as indicated by FRET acceptor emission with donor excitation; and 3) the number of analytes and their non-specific adsorption dynamics with direct donor excitation of the donor-labeled analyte.

Finally, multicolored single-molecule spectroscopy could determine the mechanism of competitive interactions in separations by placing dye labels on two separate analytes. Due to the focus on fundamental interfacial processes in separations, previous single-molecule reports have focused on the dynamics of a single type of analyte. Yet, if an analyte is already isolated, a separation would not be needed! In reality, all separations are performed in a complex environment with multiple analytes competing for adsorption sites. While we have previously studied a multicomponent sample, only one analyte was labeled, requiring the dynamics of the other unlabeled analyte to be inferred.[41] If labels were placed on each analyte in future work, their dynamics could be directly imaged to answer questions such as: Are some binding sites preferred by one analyte compared to the other? How do the kinetics differ at the same binding site with different analytes? Can one analyte displace the other? Studying more complex, multicomponent (>2) samples closer to real column conditions should be pursued.

Three-dimensional imaging to understand realistic mass-transfer dynamics:

Three-dimensional single-molecule imaging could resolve mass-transfer dynamics of analytes through separation materials in a new axial dimension: the z-position of a molecule (**Figure 3B**). Local, nanoscale differences in concentrations based on heterogeneous pore size, surface chemistry, and confinement effects drive the function of separation materials. Single-molecule tracking of diffusion and localization of adsorption in separation materials has characterized local differences in one- and two-dimensions,[7,26,42,43] but chromatography columns and membranes are three-dimensional on larger scales: chromatographic beads with diameters of 1-10's μm or polymeric membranes ~100 μm thick.

Three-dimensional single-molecule imaging is achieved through hardware modifications to typical single-molecule microscopy setups. Introduction of an optical astigmatism encodes the axial position of individual molecules in the orientation of the abberated emission pattern.[14] More advanced setups using phase masks in the Fourier domain produce engineered point spread functions such as double-helix (**Figure 3B**),[44] tetrapod,[45] tri-spot,[46] and stretching-lobe,[36] patterns that can determine three-dimensional molecular position at high precisions of ~10's of nm through up to 20 μm thick samples.[47] Alternatively, hardware changes with light sheet excitation[48] or detection with multiplane collection geometries[49] using multiple detectors placed at different focal planes can image distinct axial locations in the sample.

If applied to separation science, three-dimensional single-molecule microscopy could resolve the local fluid mechanics through three-dimensional stationary phase materials, informing the relationship between nanoscale separation, material structure, and actual function.[50–52] Characterizing intraparticle diffusion in three dimensions informs the relationship between stationary phase structure and mass transfer resistance, a driving force of analyte separation and source for elution profile artifacts.[53,54] Increasing the yield and reliability of molecular separations

require proper characterization of the mass transfer resistance process and its control in three dimensions. Recent studies of protein interactions on stimuli-responsive porous polymers have demonstrated the dominance of confined diffusion of proteins on porous structures and the ability to actively control molecular desorption processes at a single-molecule level.[55] Three-dimensional tracking[56] combined with super-resolution diffusion imaging[57] could be implemented to understand the relative distribution of adsorption/desorption and anomalous diffusion through active porous materials and measure the effect on mass transfer resistance. Controlling the physicochemistry of the porous stationary phase will increase the macroscale efficiency of molecular separations.

Expanding temporal resolutions to both shorter and longer times:

New capabilities in hardware and analysis of single-molecule techniques will advance the temporal capabilities of analyzing individual molecules in separations (**Figure 3C**). Molecular dynamics monitored using single-molecule fluorescence imaging are conventionally observed from 10's of milliseconds to minute time scales. This temporal range has monitored diffusion over micron distances and adsorption kinetics,[58] but fails to resolve faster dynamics such as conformational changes, intermediate formation, and electron transfer reactions, along with longer dynamics such as fouling, degradation, formation of defects and unwanted products, and aging of stationary phases.[12] Therefore, expanding the temporal capabilities of single-molecule fluorescence microscopy to both shorter and longer scales should be pursued.

Faster time scales can be achieved by applying recent hardware advancements to separation samples. Newer camera technology with back illuminated scientific CMOS can detect single-molecules at a frame rate of ~80 Hz with better detection quantum yields than EMCCD detectors. We have also improved the temporal resolution of single-molecule measurements below the

integration time of the camera, achieving "super temporal-resolved microscopy (STReM)" by using a rotating phase mask (**Figure 3C**).[59] Traditionally, processes occurring faster than the collection time of an individual frame are integrated and appear as identical emission patterns. In STReM, a double-helix phase mask is rotated at a rate that is synchronized to have a 180 degree rotation during the integration time of an individual frame so that the orientation and arc shape of a double-helix point spread function in the image indicates the arrival time, residence time, and desorption time of the molecule. STReM achieved improvements in temporal resolution by a factor of 20, from 100 to 5 milliseconds. Further integrating stroboscopic illumination[60] or pump-probe techniques with single-molecule fluorescence microscopy could access millisecond to sub-millisecond processes in reactions. Faster time scales are currently not accessible even with state-of-the art techniques. However, single-molecule methods are ideal for identifying rare events (on the order of millisecond or sub-millisecond) occurring on the adsorbent surfaces during analyte separations, which are the source for kinetic tailing.[61] Quantifying these rare event populations and the respective time constants are crucial for specifically designed stationary phases for optimal separations.

Longer time scale studies can understand the temporal changes that occur during the lifetime of industrially-relevant separation materials. The National Academies Press report identified understanding the robustness of separation materials over time as one of two major themes to pursue in the future research agenda of separation science.[12] Separation materials are used on a temporal scale ranging from days to even months or years. Over that time, hydrolysis, fouling, and aggregation can irreversibly change the chemical and physical properties of stationary phases. In microscopy, measuring longer time scales presents challenges with sample drift and data storage, but super-resolution measurements with ~14 hour collection times at 2 Hz frame rates have been

previously demonstrated.[62] Long collection time measurements require accurate compensation for microscope stage-drift and can be achieved by a feedback control system with fiducial markers.[63,64] Possible observations could include fluctuations in the local activity of adsorption sites due to blocking or desorption of analytes or fouling agents. Studying materials that have been used realistically or have undergone accelerated aging will increase the relevance of the stationary phases studied. Measurements at hour- or day-long time scales could quantify aging, fouling, and passivation that take place over the lifetime of the use of separation materials.

STUDYING COMPLEX SINGLE-MOLECULE SAMPLES MORE RELEVANT TO REALISTIC, INDUSTRIAL INTERESTS

Future single-molecule work in separations should continue to advance the complexity of samples. Many of the single-molecule spectroscopic studies of chromatographic interfaces have focused on model systems: materials simplified to a few components to better match ideal behavior, but that are far from the conditions for their intended use. Similar to how the single-molecule biophysics community studies living cells in their natural complexity,[65] more industrially-realistic, multicomponent separation systems should be studied in the future. Increasing complexity will present new experimental challenges in preparing samples with low enough background for single-molecule detection, but realistic systems will speed development of predictive models for analyte-stationary phase interactions. Observations at the single-molecule scale will resolve rare phenomena that drive macroscale engineering problems, refining commercial applications through reduction of optimization time and increase in retained product. As stated in the National Academies Press report, "[t]he ability to understand and design separation

systems for complex mixtures under various realistic conditions will be a turning point for the separations community and is a key to transforming separation science."[12]

Modeling and measuring realistic stationary and mobile phases at higher concentrations of analytes is crucial to understanding the driving forces of separation and harnessing those forces for optimal separation system design. Prior single-molecule studies[17,18,21,22,24] used very low (pM-nM) concentrations of labeled analytes in pristine solutions comprised of ultrapure solvents and buffer salts. While this is beneficial for studying fundamental interactions, separations occur in multicomponent mixtures with different species ranging from highly dilute to very concentrated over the course of a column. Imaging an analyte in the presence of cellular lysate (100's of mg/mL or ~ mM concentrations of macromolecules)[66] would present a challenging, complex mobile phase relevant to biologic pharmaceuticals produced by recombinant-expression. Frequently-used organic mobile phases, such as acetonitrile or methanol, could be used as more dyes compatible with organic solvents are developed.[67] Microfluidics and solvent mixing could be incorporated to monitor dynamics that occur during gradient elutions. Higher concentrations of analytes could be studied by mixing labeled and unlabeled molecules[41,68] or utilizing unique sample hardware geometries that limit the excitation to a small volume.[69,70] High concentration samples could observe thermodynamic tailing caused by overloading, in addition to the established studies of kinetic tailing at low concentrations.[61] Further complex conditions include incorporating high pressures,[71] temperatures, and oxidative/corrosive chemicals that are used in more extreme separations such as (ultra) high-performance liquid chromatography.

CHIRAL CHROMATOGRAPHY – EXPANDING THE TYPES OF SEPARATIONS STUDIED AT THE SINGLE-MOLECULE LEVEL

Among the types of separations that could benefit from a single-analyte understanding is chiral chromatography. Chiral chromatography is challenging due to the identical size, surface charge, and hydrophobicity of the enantiomers being separated (**Figure 4A**). The difference in enantiomer efficacy can lead to drastically different drug prices for racemic mixtures vs. enantiomerically-pure drugs. For example, the enantiopure asthma inhaler treatment levalbuterol costs five times more than its racemic mixture albuterol (**Figure 4A**).[74] Improving the time and material cost to separate enantiomers could reduce the expenses of producing bioactive drugs.

Single-molecule spectroscopy is suited to identify the molecular recognition mechanisms in chiral chromatography. At the molecular level, chiral chromatography relies on multimodal recognition between the analyte and stationary phase in a geometrically-dependent "three-point interaction model"[75] in the simplest description. The formation of transient diastereomeric complexes is driven by a combination of non-covalent hydrogen bonds, ionic, ion-dipole, dipole-

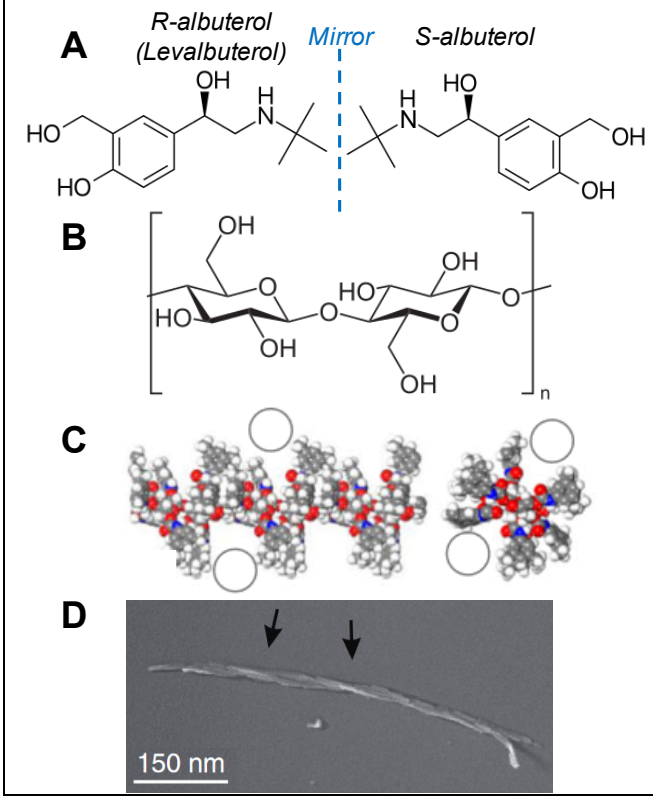


Figure 4. Chiral chromatography separates enantiomers with cellulose stationary phases. (A) Example of a commercial chiral drug, albuterol, used for treatment of asthma. The enantiopure sample of R-albuterol costs five times as much as the racemic mixture. (B-D) Chirality is present at different length scales in cellulose stationary phases. (B) Dimer structure of cellulose monomer. (C) Views of cellulose tris(3,5-dimethylphenylcarbamate) chain (left) perpendicular to the chain axis and (right) top-down the chain axis where circles indicate position of chiral grooves. Adapted with permission from ref.[72]. Copyright 2012 Wiley Online Library. (D) Cellulose chains self-assemble into nano-, micro- and even macrofibers that have chiral properties. Cryo-SEM image of nanocellulose fibrils where arrows indicate observed contrast indicative of a right-hand twist. Adapted with permission from ref[73]. Copyright 2015 Nature Publishing Group.

342 dipole, van der Waals, or π-π interactions. The three-dimensional spatial arrangement of one
343 enantiomer will only allow for these multiple interactions to line up for an "ideal fit" with the
344 stationary phase. Single-molecule imaging of achiral dyes conjugated to chiral molecules could
345 quantify the different adsorption kinetics that occur with ideal and non-ideal fits and answer open
346 questions such as: Do both enantiomers have kinetic heterogeneity present when the wrong steric
347 fit occurs and only one or two of the required three-points are formed? Using a point spread
348 function that changes based on the orientation of a molecule (**Figure 3B**), does the rotation or
349 conformational adaptation of the analyte take place to form the desired geometric arrangement?
350 Do non-stereoselective adsorption sites exist?[76] How many are there, where are they located at,
351 and how do the kinetics of non-stereoselective sites contribute? By monitoring $>10^5$ individual
352 interactions, what is the percent contribution of the different ideal, non-ideal, and non-
353 stereoselective kinetic populations? Can both ideal and non-ideal adsorption kinetics be observed
354 for an enantiopure analyte that is studied under conditions that cause interconversion?[77]

355 Single-molecule spectroscopy could quantify the heterogeneous nano- to microscale lengths
356 relevant to the stationary phase in chiral chromatography. Commonly used cellulose-based
357 stationary phases contain chirality on hierarchal length scales: from the Angstrom-level of
358 individual monomer cellulose sugars (**Figure 4B**) to the polymerization of chiral chains (**Figure**
359 **4C**) and finally the self-assembly of chains into supramolecular chiral fibrils on nano-, micro-, and
360 even macro-length scales (**Figure 4D**). Previous work on the molecular recognition mechanisms
361 of cellulose stationary phases have focused on the individual cellulose unit and its chemical
362 functionalization.[72] But longer-length scale chirality could also play a role, especially in the
363 separation of biomolecules.[78] Super-resolution imaging could spatially resolve which chiral length
364 scale is important based on the distances between adsorption sites. Further, derivatives of cellulose

have been observed in ensemble studies to have reduced separation resolutions due to hypothesized heterogeneous chemical substitution and poor ordering of the supramolecular cellulose structure.[79,80] Single-molecule imaging could directly visualize if certain areas of the cellulose are inactive due to heterogeneity in ligand functionalization or crystallinity.

CONCLUSIONS – AN INVITATION TO JOIN THE MOLECULAR SEPARATIONS SCIENCE COMMUNITY

In conclusion, we show that single-molecule and super-resolution microscopy methods provide new and exciting ways to characterize the fundamental properties of analyte-stationary phase interactions in the context of liquid separations. Bottom-up, single-molecule studies of interactions in complex environments addresses central themes in the National Academies Press report[12] on the future of separation science. The methods highlighted here present new avenues for detecting rare interactions that stymie better separations. Overall, we hope the technologies and open questions presented in this perspective inspire new directions and exchange of ideas between those in the broad field of separation science (analytical chemists, spectroscopists, chemical engineers, computational/theorists, *etc.*). Only then will we achieve the molecular understanding needed to drive improvements in the performance of separation processes.

ACKNOWLEDGEMENTS

W.C. and L.K. thank the Case Western Reserve University College of Arts and Sciences for financial support. C.D., A.M., and C.F.L. thank the National Science Foundation (Grant no. CHE-1808382) and the Welch Foundation (Grant no. C-1787), L.D.C.B. acknowledges that this material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program (Grant 1842494). We also thank Prof. Christine Duval for helpful discussion.

REFERENCES

(1) Szente, L.; Szemán, J. Cyclodextrins in Analytical Chemistry: Host–Guest Type Molecular Recognition. *Anal. Chem.* **2013**, *85*, 8024–8030.

(2) Skvortsova, Y. A.; Freeney, R. M.; Zhong, Z.; Geng, M. L. Tissue Phantoms Constructed with Hydrophobic Nanoporous Silica Particles. *Anal. Chem.* **2010**, *82*, 6712–6716.

(3) Avorn, J. The $2.6 Billion Pill - Methodologic and Policy Considerations. *N. Engl. J. Med.* **2015**, *372*, 1877–1879.

(4) Bishop, L. D. C.; Landes, C. F. From a Protein's Perspective: Elution at the Single-Molecule Level. *Acc. Chem. Res.* **2018**, *51*, 2247–2254.

(5) Moringo, N. A.; Shen, H.; Bishop, L. D. C.; Wang, W.; Landes, C. F. Enhancing Analytical Separations Using Super-Resolution Microscopy. *Annu. Rev. Phys. Chem. Annu. Rev. Phys. Chem* **2018**, *69*, 353–375.

(6) Kisley, L. Single Molecule Spectroscopy at Interfaces. In *Spectroscopy and Dynamics of Single Molecules*; Elsevier, 2019; pp 117–161.

(7) Higgins, D. A.; Park, S. C.; Tran-Ba, K.-H.; Ito, T. Single-Molecule Investigations of Morphology and Mass Transport Dynamics in Nanostructured Materials. *Annu. Rev. Anal. Chem.* **2015**, *8*, 193–216.

(8) Rekoske, J. E. Chiral Separations. *AIChE J.* **2001**, *47*, 2–5.

(9) Florek, J.; Giret, S.; Juère, E.; Larivière, D.; Kleitz, F. Functionalization of Mesoporous Materials for Lanthanide and Actinide Extraction. *Dalton Transactions*. Royal Society of Chemistry September 27, 2016, pp 14832–14854.

(10) Bond, A. H.; Rogers, R. D.; Dietz, M. L. Nuclear Separations for Radiopharmacy: The Need for Improved Separations to Meet Future Research and Clinical Demands. *Ind. Eng. Chem. Res.* **2000**, *39*, 3130–3134.

(11) Yu, M.; Renner, J. N.; Duval, C. E. A Lysine-Modified Polyethersulfone (PES) Membrane for the Recovery of Lanthanides. *Front. Chem.* **2020**, *8*, 512.

(12) National Academies of Sciences, E. and M. *A Research Agenda for Transforming Separation Science*; National Academies Press: Washington, D.C., 2019.

(13) Betzig, E.; Patterson, G. H.; Sougrat, R.; Lindwasser, O. W.; Olenych, S.; Bonifacino, J. S.; Davidson, M. W.; Lippincott-Schwartz, J.; Hess, H. F. Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* **2006**, *313*, 1642–1645.

(14) Huang, B.; Wang, W.; Bates, M.; Zhuang, X. Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy. *Science* **2008**, *319*, 810–813.

(15) Dertinger, T.; Colyer, R.; Iyer, G.; Weiss, S.; Enderlein, J. Fast, Background-Free, 3D Super-Resolution Optical Fluctuation Imaging (SOFI). *Proc. Natl. Acad. Sci.* **2009**, *106*, 22287–22292.

(16) Hell, S. W.; Wichmann, J. Breaking the Diffraction Resolution Limit by Stimulated Emission: Stimulated-Emission-Depletion Fluorescence Microscopy. *Opt. Lett.* **1994**, *19*, 780–782.

(17) Kisley, L.; Landes, C. F. Molecular Approaches to Chromatography Using Single Molecule Spectroscopy. *Anal. Chem.* **2015**, *87*, 83–98.

(18) Wirth, M. J.; Legg, M. A. Single-Molecule Probing of Adsorption and Diffusion on Silica Surfaces. *Annu. Rev. Phys. Chem.* **2007**, *58*, 489–510.

(19) Kang, S. H.; Shortreed, M. R.; Yeung, E. S. Real-Time Dynamics of Single-DNA Molecules Undergoing Adsorption and Desorption at Liquid−Solid Interfaces. *Anal. Chem.* **2001**, *73*, 1091–1099.

(20) Zhong, Z.; Lowry, M.; Wang, G.; Geng, L. Probing Strong Adsorption of Solute onto C18-Silica Gel by Fluorescence Correlation Imaging and Single-Molecule Spectroscopy under RPLC Conditions. *Anal. Chem.* **2005**, *77*, 2303–2310.

(21) Cooper, J. T.; Peterson, E. M.; Harris, J. M. Fluorescence Imaging of Single-Molecule Retention Trajectories in Reversed-Phase Chromatographic Particles. *Anal. Chem.* **2013**, *85*, 9363–9370.

(22) Mabry, J. N.; Skaug, M. J.; Schwartz, D. K. Single-Molecule Insights into Retention at a Reversed-

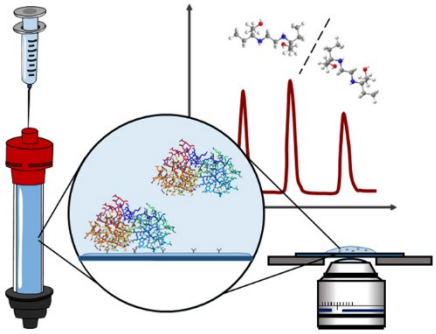
Phase Chromatographic Interface. *Anal. Chem.* **2014**, *86*, 9451–9458.
(23) Xu, X.-H. N.; Yeung, E. S. Long-Range Electrostatic Trapping of Single-Protein Molecules at a Liquid-Solid Interface. *Science* **1998**, *281*, 1650–1653.
(24) Kang, S. H.; Yeung, E. S. Dynamics of Single-Protein Molecules at a Liquid/Solid Interface: Implications in Capillary Electrophoresis and Chromatography. *Anal. Chem.* **2002**, *74*, 6334–6339.
(25) Isailovic, S.; Li, H.-W.; Yeung, E. S. Adsorption of Single DNA Molecules at the Water/Fused-Silica Interface. *J. Chromatogr. A* **2007**, *1150*, 259–266.
(26) Kisley, L.; Poongavanam, M.-V.; Kourentzi, K.; Willson, R. C.; Landes, C. F. PH-Dependence of Single-Protein Adsorption and Diffusion at a Liquid Chromatographic Interface. *J. Sep. Sci.* **2016**, *39*, 682–688.
(27) Kisley, L.; Chen, J.; Mansur, A. P.; Shuang, B.; Kourentzi, K.; Poongavanam, M.-V.; Chen, W.-H.; Dhamane, S.; Willson, R. C.; Landes, C. F. Unified Superresolution Experiments and Stochastic Theory Provide Mechanistic Insight into Protein Ion-Exchange Adsorptive Separations. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 2075–2080.
(28) Kisley, L.; Chen, J.; Mansur, A. P.; Dominguez-Medina, S.; Kulla, E.; Kang, M. K.; Shuang, B.; Kourentzi, K.; Poongavanam, M. V.; Dhamane, S.; Willson, R. C.; Landes, C. F. High Ionic Strength Narrows the Population of Sites Participating in Protein Ion-Exchange Adsorption: A Single-Molecule Study. *J. Chromatogr. A* **2014**, *1343*, 135–142.
(29) Daniels, C. R.; Kisley, L.; Kim, H.; Chen, W.-H.; Poongavanam, M.-V.; Reznik, C.; Kourentzi, K.; Willson, R. C.; Landes, C. F. Fluorescence Correlation Spectroscopy Study of Protein Transport and Dynamic Interactions with Clustered-Charge Peptide Adsorbents. *J. Mol. Recognit.* **2012**, *25*, 435–442.
(30) Cai, Y.; Schwartz, D. K. Mapping the Functional Tortuosity and Spatiotemporal Heterogeneity of Porous Polymer Membranes with Super-Resolution Nanoparticle Tracking. *ACS Appl. Mater. Interfaces* **2017**, *9*, 43258–43266.
(31) Langdon, B. B.; Kastantin, M.; Schwartz, D. K. Surface Chemistry Influences Interfacial Fibrinogen Self-Association. *Biomacromolecules* **2015**, *16*, 3201–3208.
(32) Moringo, N. A.; Bishop, L. D. C.; Shen, H.; Misiura, A.; Carrejo, N. C.; Baiyasi, R.; Wang, W.; Ye, F.; Robinson, J. T.; Landes, C. F. A Mechanistic Examination of Salting out in Protein-Polymer Membrane Interactions. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 22938–22945.
(33) Bishop, L. D. C.; Misiura, A.; Moringo, N. A.; Landes, C. F. Unraveling Peak Asymmetry in Chromatography through Stochastic Theory Powered Monte Carlo Simulations. *J. Chromatogr. A* **2020**, *1625*, 461323.
(34) Moringo, N. A.; Shen, H.; Tauzin, L. J.; Wang, W.; Landes, C. F. Polymer Free Volume Effects on Protein Dynamics in Polystyrene Revealed by Single-Molecule Spectroscopy. *Langmuir* **2020**, *36*, 2330–2338.
(35) Ma, H.; Liu, Y. Super-Resolution Localization Microscopy: Toward High Throughput, High Quality, and Low Cost. *APL Photonics* **2020**, *5*, 060902.
(36) Wang, W.; Ye, F.; Shen, H.; Moringo, N. A.; Dutta, C.; Robinson, J. T.; Landes, C. F. Generalized Method to Design Phase Masks for 3D Super-Resolution Microscopy. *Opt. Express* **2019**, *27*, 3799.
(37) Wang, W.; Shen, H.; Moringo, N. A.; Carrejo, N. C.; Ye, F.; Robinson, J. T.; Landes, C. F. Super-Temporal Resolved Microscopy Reveals Multistep Desorption Kinetics of α-Lactalbumin from Nylon. *Langmuir* **2018**, *34*, 6697–6702.
(38) Weltz, J. S.; Schwartz, D. K.; Kaar, J. L. Surface-Mediated Protein Unfolding as a Search Process for Denaturing Sites. *ACS Nano* **2016**, *10*, 730–738.
(39) Faulón Marruecos, D.; Schwartz, D. K.; Kaar, J. L. Impact of Surface Interactions on Protein Conformation. *Curr. Opin. Colloid Interface Sci.* **2018**, *38*, 45–55.
(40) Kapanidis, A. N.; Laurence, T. A.; Lee, N. K.; Margeat, E.; Kong, X.; Weiss, S. Alternating-Laser Excitation of Single Molecules. *Acc. Chem. Res.* **2005**, *38*, 523–533.
(41) Kisley, L.; Patil, U.; Dhamane, S.; Kourentzi, K.; Tauzin, L. J.; Willson, R. C.; Landes, C. F. Competitive Multicomponent Anion Exchange Adsorption of Proteins at the Single Molecule Level.

Analyst **2017**, *142*, 3127–3131.
(42) Langdon, B. B.; Mirhossaini, R. B.; Mabry, J. N.; Sriram, I.; Lajmi, A.; Zhang, Y.; Rojas, O. J.; Schwartz, D. K. Single-Molecule Resolution of Protein Dynamics on Polymeric Membrane Surfaces: The Roles of Spatial and Population Heterogeneity. *ACS Appl. Mater. Interfaces* **2015**, *7*, 3607–3617.
(43) Robben, K. C.; Tran-Ba, K. H.; Ito, T.; Higgins, D. A. Trajectory-Profile-Guided Single Molecule Tracking for Assignment of One-Dimensional Diffusion Trajectories. *Anal. Chem.* **2014**, *86*, 10820–10827.
(44) Thompson, M. A.; Lew, M. D.; Badieirostami, M.; Moerner, W. E. Localizing and Tracking Single Nanoscale Emitters in Three Dimensions with High Spatiotemporal Resolution Using a Double-Helix Point Spread Function. *Nano Lett.* **2009**, *10*, 211–218.
(45) Shechtman, Y.; Sahl, S. J.; Backer, A. S.; Moerner, W. E. Optimal Point Spread Function Design for 3D Imaging. *Phys. Rev. Lett.* **2014**, *113*, 133902.
(46) Zhang, O.; Lu, J.; Ding, T.; Lew, M. D. Imaging the Three-Dimensional Orientation and Rotational Mobility of Fluorescent Emitters Using the Tri-Spot Point Spread Function. *Appl. Phys. Lett.* **2018**, *113*, 031103.
(47) Shechtman, Y.; Weiss, L. E.; Backer, A. S.; Sahl, S. J.; Moerner, W. E. Precise Three-Dimensional Scan-Free Multiple-Particle Tracking over Large Axial Ranges with Tetrapod Point Spread Functions. *Nano Lett.* **2015**, *15*, 4194–4199.
(48) Tokunaga, M.; Imamoto, N.; Sakata-Sogawa, K. Highly Inclined Thin Illumination Enables Clear Single-Molecule Imaging in Cells. *Nat. Methods* **2008**, *5*, 159–161.
(49) Ram, S.; Kim, D.; Ober, R. J.; Ward, E. S. 3D Single Molecule Tracking with Multifocal Plane Microscopy Reveals Rapid Intercellular Transferrin Transport at Epithelial Cell Barriers. *Biophys. J.* **2012**, *103*, 1594–1603.
(50) Hlushkou, D.; Gritti, F.; Daneyko, A.; Guiochon, G.; Tallarek, U. How Microscopic Characteristics of the Adsorption Kinetics Impact Macroscale Transport in Chromatographic Beds. *J. Phys. Chem. C* **2013**, *117*, 22974–22985.
(51) Hlushkou, D.; Gritti, F.; Guiochon, G.; Seidel-Morgenstern, A.; Tallarek, U. Effect of Adsorption on Solute Dispersion: A Microscopic Stochastic Approach. *Anal. Chem.* **2014**, *86*, 4463–4470.
(52) Gritti, F.; Hochstrasser, J.; Svidrytski, A.; Hlushkou, D.; Tallarek, U. Morphology-Transport Relationships in Liquid Chromatography: Application to Method Development in Size Exclusion Chromatography. *J. Chromatogr. A* **2020**, *1620*, 460991.
(53) Van Deemter, J. J.; Zuiderweg, F. J.; Klinkenberg, A. van. Longitudinal Diffusion and Resistance to Mass Transfer as Causes of Nonideality in Chromatography. *Chem. Eng. Sci.* **1956**, *5*, 271–289.
(54) Giddings, J. C. *Unified Separation Science*; Wiley: New York, 1991.
(55) Dutta, C.; Bishop, L. D. C.; Zepeda O, J.; Chatterjee, S.; Flatebo, C.; Landes, C. F. Imaging Switchable Protein Interactions with an Active Porous Polymer Support. *J. Phys. Chem. B* **2020**, *124*, 4412–4420.
(56) Zhong, Y.; Wang, G. Three-Dimensional Single Particle Tracking and Its Applications in Confined Environments. *Annu. Rev. Anal. Chem.* **2020**, *13*, 381–403.
(57) Kisley, L.; Brunetti, R.; Tauzin, L. J.; Shuang, B.; Yi, X.; Kirkeminde, A. W.; Higgins, D. A.; Weiss, S.; Landes, C. F. Characterization of Porous Materials by Fluorescence Correlation Spectroscopy Super-Resolution Optical Fluctuation Imaging. *ACS Nano* **2015**, *9*, 9158–9166.
(58) Armstrong, M.; Hess, H.; Tsitkov, S. Statistical Inference in Single Molecule Measurements of Protein Adsorption. In *Nanoscale Imaging, Sensing, and Actuation for Biomedical Applications XV*; Cartwright, A. N., Nicolau, D. V., Fixler, D., Eds.; SPIE, 2018; Vol. 10506, p 46.
(59) Wang, W.; Shen, H.; Shuang, B.; Hoener, B. S.; Tauzin, L. J.; Moringo, N. A.; Kelly, K. F.; Landes, C. F. Super Temporal-Resolved Microscopy (STReM). *J. Phys. Chem. Lett.* **2016**, *7*, 4524–4529.
(60) Xiang, L.; Chen, K.; Yan, R.; Li, W.; Xu, K. Single-Molecule Displacement Mapping Unveils Nanoscale Heterogeneities in Intracellular Diffusivity. *Nat. Methods* **2020**, *17*, 524–530.
(61) Giddings, J. C. Kinetic Origin of Tailing in Chromatography. *Anal. Chem.* **1963**, *35*, 1999–2002.

(62) Dai, M.; Jungmann, R.; Yin, P. Optical Imaging of Individual Biomolecules in Densely Packed Clusters. *Nat. Nanotechnol.* **2016**, *11*, 798–807.
(63) Pertsinidis, A.; Zhang, Y.; Chu, S. Subnanometre Single-Molecule Localization, Registration and Distance Measurements. *Nature* **2010**, *466*, 647–651.
(64) Gustavsson, A. K.; Petrov, P. N.; Lee, M. Y.; Shechtman, Y.; Moerner, W. E. 3D Single-Molecule Super-Resolution Microscopy with a Tilted Light Sheet. *Nat. Commun.* **2018**, *9*, 1–8.
(65) Lord, S. J.; Lee, H. D.; Moerner, W. E. Single-Molecule Spectroscopy and Imaging of Biomolecules in Living Cells. *Anal. Chem.* **2010**, *82*, 2192–2203.
(66) Davis, C. M.; Deutsch, J.; Gruebele, M. An in Vitro Mimic of In-cell Solvation for Protein Folding Studies. *Protein Sci.* **2020**, *29*, 1046–1054.
(67) Aloi, A.; Vargas Jentzsch, A.; Vilanova, N.; Albertazzi, L.; Meijer, E. W.; Voets, I. K. Imaging Nanostructures by Single-Molecule Localization Microscopy in Organic Solvents. *J. Am. Chem. Soc.* **2016**, *138*, 2953–2956.
(68) McUmber, A. C.; Larson, N. R.; Randolph, T. W.; Schwartz, D. K. Molecular Trajectories Provide Signatures of Protein Clustering and Crowding at the Oil/Water Interface. *Langmuir* **2015**, *31*, 5882–5890.
(69) Crouch, G. M.; Han, D.; Bohn, P. W. Zero-Mode Waveguide Nanophotonic Structures for Single Molecule Characterization. *J. Phys. D. Appl. Phys.* **2018**, *51*, 193001.
(70) Goldschen-Ohm, M. P.; White, D. S.; Klenchin, V. A.; Chanda, B.; Goldsmith, R. H. Observing Single-Molecule Dynamics at Millimolar Concentrations. *Angew. Chemie Int. Ed.* **2017**, *56*, 2399–2402.
(71) Sung, H.-L.; Nesbitt, D. J. DNA Hairpin Hybridization under Extreme Pressures: A Single-Molecule FRET Study. *J. Phys. Chem. B* **2020**, *124*, 110–120.
(72) Kim, B.-H.; Lee, S. U.; Moon, D. C. Chiral Recognition of *N* -Phthaloyl, *N* -Tetrachlorophthaloyl, and *N* -Naphthaloyl α-Amino Acids and Their Esters on Polysaccharide-Derived Chiral Stationary Phases. *Chirality* **2012**, *24*, 1037–1046.
(73) Usov, I.; Nyström, G.; Adamcik, J.; Handschin, S.; Schütz, C.; Fall, A.; Bergström, L.; Mezzenga, R. Understanding Nanocellulose Chirality and Structure–Properties Relationship at the Single Fibril Level. *Nat. Commun.* **2015**, *6*, 7564.
(74) Asmus, M. J.; Hendeles, L. Levalbuterol Nebulizer Solution: Is It Worth Five Times the Cost of Albuterol? *Pharmacotherapy* **2000**, *20*, 123–129.
(75) Lämmerhofer, M. Chiral Recognition by Enantioselective Liquid Chromatography: Mechanisms and Modern Chiral Stationary Phases. *J. Chromatogr. A* **2010**, *1217*, 814–856.
(76) Lang, J. C.; Armstrong, D. W. Chiral Surfaces: The Many Faces of Chiral Recognition. *Curr. Opin. Colloid Interface Sci.* **2017**, *32*, 94–107.
(77) Sepsey, A.; Németh, D. R.; Németh, G.; Felinger, A. Rate Constant Determination of Interconverting Enantiomers by Chiral Chromatography Using a Stochastic Model. *J. Chromatogr. A* **2018**, *1564*, 155–162.
(78) Wallworth, D. Chiral Amino Acid and Peptide Separations – the Next Generation. *Chromatogr. Today* **2016**, *August/Sep*, 44–47.
(79) Francotte, E.; Wolf, R. M. Benzoyl Cellulose Beads in the Pure Polymeric Form as a New Powerful Sorbent for the Chromatographic Resolution of Racemates. *Chirality* **1991**, *3*, 43–55.
(80) Kasuya, N.; Nakashima, J.; Kubo, T.; Sawatari, A.; Habu, N. Chiral Discrimination with Regioselectively Substituted Cellulose Esters as Chiral Stationary Phases. *Chirality* **2000**, *12*, 670–674.

588 **FOR TABLE OF CONTENTS ONLY**

589



590