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Parameterless detection of liquid–liquid interfaces with sub-micron resolution in single-molecule localization microscopy

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GRAPHICAL ABSTRACT

A new method using a maximum likelihood estimator (MLE) is able to detect the location of soft interfaces, such as the oil–water interface, with high precision in single-molecule localization microscopy (SMLM) experiments. The method remains effective even if the interface contains, but is not saturated with, particles.

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Hypothesis: Knowing the exact location of soft interfaces, such as between water and oil, is essential to the study of nanoscale wetting phenomena. Recently, iPAINT was used to visualize soft interfaces in situ with minimal invasiveness, but computing the exact location of the interface remains challenging. We propose a new method to determine the interface with high accuracy. By modelling the localizations as points generated by two homogeneous Poisson processes, the exact location of the interface can be determined using a maximum likelihood estimator (MLE).

Experiments: An MLE was constructed to estimate the location of the interface based on the discontinuity in localization density at the interface. To test the MLE, we collected experimental data through iPAINT experiments of oil–water interfaces and generated simulated data using the Monte Carlo method.

Findings: Simulations show that the interface given by the MLE rapidly converges to the true interface location. The error of the MLE drops below the experimental localization precision. Furthermore, we show that the MLE remains accurate even if the field-of-view is reduced or when one or more particles are on the interface within the field-of-view. This work provides a key step towards the in situ, sub-micron characterization of (nanoparticle-laden) interfaces with minimal invasiveness.

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1. Introduction

Soft interfaces are ubiquitous in both production processes and products like foods, cosmetics, paints, and medicines (e.g., aerosols, emulsions). Their properties, such as composition and geometry, are essential to maintain structural integrity, enhance colloidal stability, and safeguard performance. Yet, soft interfaces, such as those between two immiscible liquids, remain difficult to characterize due to their dynamic character and susceptibility to perturbations [1,2]. In-depth characterization is further complicated in the presence of small stabilizers, such as nanoparticles, since their impact on interfacial properties is profound and multifactorial, so that small differences in particle properties can have a marked impact on interfacial properties [3–5]. A reliable method capable of accurately probing interfaces, both pristine and decorated with stabilizers, is thus essential to achieve a comprehensive understanding of the form and function of soft interfaces and to reverse engineer the junctions between liquid–liquid interfaces to meet application-specific requirements.

Over the past decades, numerous studies set out to address this challenge aiming to advance our fundamental understanding of particle-based stabilization of liquid–liquid interfaces [6] and to facilitate their utilization for e.g. drug delivery [7,8], vaccine efficacy [9], emulsification [10–13], and scaffolding of materials [14–16]. The properties of the interface and the particles are typically determined in separate experiments. For example, spinning drop tensiometry and surface shear rheometry are used to measure interfacial tension, surface shear elasticity, and surface shear viscosity [17], whilst particle morphology [13,16,19], surface charge, roughness [10,3], and size (distributions) [4] are determined by ex-situ small-angle X-ray scattering (SAXS) [20,21], atomic force microscopy (AFM) [22,23], and/or electron microscopy (EM) experiments [24,20,25]. Complemented with direct and quantitative imaging of in-plane dynamics, migration, and structuration of (predominantly) large colloidal particles of several microns in diameter, this revealed spatiotemporal variations masked by ensemble-averaging and established the foundations for quantitative relations between interfacial structuring and mechanics. Soft glassy-like mechanical responses [17], buckling transitions [5], and pathways to create two-dimensional crystals were elucidated [26,27].

Despite these efforts, the origin of variations and apparent inconsistencies in nanoscale wetting remained largely unexplained and debated. Since contact line pinning, line tension effects, as well as intraparticle and interparticle heterogeneities [28] were suspected culprits, alternative approaches were developed to also probe the behavior of nanoparticles at liquid–liquid interfaces at the single particle level [29–31]. Imaging tools amenable to micron-sized particles offered experimental evidence for hitherto elusive phenomena, such as contact line pinning and activated hopping [32]. Exploiting novel high-resolution techniques, such as the gel-trapping technique (GTT) [33–35] and freeze-fracture hopping [32], exploiting novel high-resolution techniques, such as contact line pinning and activated hopping, is thus essential to achieve a comprehensive understanding of the form and function of soft interfaces and to reverse engineer the junctions between liquid–liquid interfaces to meet application-specific requirements.

Motivated to investigate, in a minimally invasive manner, the properties of liquid–liquid interfaces and their relation to intraparticle and interparticle variations in surface charge, surface roughness, morphology, and particle size, the single-molecule localization microscopy (SMLM) tool interface Point Accumulation for Imaging in Nanoscale Topography (iPAINT) was recently developed [37]. Advantageously, this approach allows to resolve both liquid–liquid and particle–liquid interfaces, without vitrification or gelation of either liquids. Instead, the relevant interfaces are located due to differences in partitioning of soluble, fluorescent dyes, which are added in nanomolar concentrations. ‘Interfacial staining’ is typically achieved in iPAINT in a non-covalent, transient, and non-specific manner due to uneven partitioning in the immiscible liquids and interfacial adsorption of soluble, fluorescent probes. Fitting the point spread function of the individual fluorescent probes then results in high-precision localizations. And differences in the affinity of the probes for both the bulk liquids and the interfaces present within the sample result in differences in probe density and concomitant single-molecule localizations across the interfaces. This allows to identify and discriminate between the liquid–liquid and solid–liquid interfaces present and to reconstruct a high-resolution, pointillistic image of the sample. The suitability of iPAINT to characterize soft interfaces in situ, was demonstrated in a series of papers focusing on the wetting properties of individual, (sub-)micron-sized particles at oil–water interfaces and particle-induced interfacial deformations [38,4].

One major advantage of iPAINT to image soft interfaces is that it requires low probe concentrations and does not require sample vitrification nor fixation, making this approach minimally invasive. However, as a consequence of the variable affinity of the soluble probes for different interfaces, the localization density depends heavily on the chemical composition of the sample and the imaging settings. Consequently, an accurate, versatile, and non-laborious determination of the location of the interface is challenging. The typical approach is to use density-based thresholds to identify various features [38–42]. However, choosing an appropriate density threshold is non-trivial and different users can end up choosing different thresholds. To combat this issue, thresholds are typically determined by averaging over a large number of similar samples. However, this in turn ignores inter-sample variability. The identification of interfaces can therefore be improved by removing the dependency on user-defined thresholds or inter-sample averages. Automated and parameterless detection methods could therefore improve and standardize the detection of interfaces in SMLM tools like iPAINT.

In this work, we propose a maximum likelihood estimator (MLE) to detect the in situ location of fluid–fluid interfaces in SMLM experiments. Assuming that localizations are distributed according to a Poisson process, this method is able to detect the interface 1) on a sample-by-sample basis, 2) based solely on a discontinuity in the density of localizations, 3) with a resolution of several to tens of nanometers, and 4) without any input parameters. The accuracy of the method is quantified using Monte Carlo simulations that represent SMLM experiments. The robustness of the method is also investigated using experimental samples and reduced data sets. Finally, the method is extended and tested such that the presence of particles at the interface does not influence the detected interface location. Enabling the accurate localization of interfaces in situ, this new data analysis method for iPAINT provides an essential tool to study wetting phenomena at the nanoscale.

2. Methods

2.1. Imaging of the oil–water interface with single-molecule localization microscopy (SMLM)

Sample preparation: A liquid–liquid interface between water and 2-octanol was selected as an exemplary soft interface for our purposes. It was created from a pure 2-octanol phase and a water phase in which the dyes were solubilized. Specifically, the water
phase consisted of MilliQ quality water, 10^{-7} M of dodecylamine, and 10^{-7} M of either CAGE-552 or CAGE-635 (both Abberior\textsuperscript{\textregistered}, NHS ester) as imaging agent.

To prevent migration of the interface during the experiment, an air-tight sample chamber was created using either double-sided tape or an imaging spacer with a well (thickness \sim 100 \mu m). The spacer was placed on the microscopy slide, after which a 15 \mu L droplet of water and a 15 \mu L droplet of oil were placed next to each other. The sample chamber was then closed off with a Menzel Gläser cover glass (no. 1.5, 24x24 mm, thickness 170 \mu m). An illustration of the experimental setup can be seen in Fig. 1 of Aloï et al. \cite{38}, except that our setup did not contain any particles.

**Acquisition:** SMLM experiments were performed on a NIKON Eclipse Ti-E N-STORM system with \sim 17.9 mW (lambda = 405 nm), \sim 55.2 mW (lambda = 561 nm), and \sim 125 mW (lambda = 647 nm) lasers and a quad-band pass dichroic filter (97335 Nikon). The system was configured for total internal reflection fluorescence (TIRF) imaging and equipped with a 100x Apo TIRF oil immersion objective (NA. 1.49). All acquisitions were performed over a 255x255 pixel region using an EMCCD camera (ixon3, Andor, 160 nm pixel size) with 40 to 50 ms per frame. Total imaging times were 5 min or less.

The focal plane is placed at the cover slip as this is where the probe molecules are (temporarily) immobilized. Both fluorescent probes, CAGE-552 and CAGE-635, are uncaged with the 405 nm UV laser. CAGE-552 is excited with the 561 nm laser; for excitation of CAGE-635 the 647 nm laser is used. Laser powers and inclination (or TIRF) angle were tuned on a case-by-case basis to optimize the fluorescence (TIRF) imaging and equipped with a 100x Apo TIRF oil immersion objective (NA. 1.49). All acquisitions were performed over a 255x255 pixel region using an EMCCD camera (ixon3, Andor, 160 nm pixel size) with 40 to 50 ms per frame. Total imaging times were 5 min or less.

**Analysis:** Acquisitions were analyzed using Detection of Molecules (DoM) Utrecht with an initial standard deviation of 1.6 pixels \cite{43,44}. DoM was found to provide the best image quality in the presence of highly non-uniform background signals as is the case in our experiments. This large difference in background lighting is due to the high resp. low solubility of the dyes in water and octanol. All subsequent analysis and numerical methods were performed using MATLAB R2019b (Natick, Massachusetts: The MathWorks Inc.). Experimental data are always shown in blue within figures.

**Data reduction:** Artificial data sets with reduced localization density were generated from the original experimental data sets to test the robustness of the MLE interface-estimation method; specifically how much the interface location changes with respect to the original location depending on the fraction of localizations that has been discarded. Two scenarios were examined. In the first scenario, the MLE was applied to a smaller region around the putative interface (representative for e.g. interface detection on a 5x5 \mu m zoom instead of the entire 30x30 \mu m field of view). In the second scenario, the MLE was applied after a certain fraction of the localizations was randomly removed (emulating a sample with the same interface location but fewer localizations). Because of the randomness involved in the second scenario, this procedure was repeated 100 times. Additionally, we also tested the robustness of the MLE in the presence of hypothetical particles at the interface. This was done by cutting one or more circular regions from the data before applying the MLE. This last procedure is identical for simulation and experiment and is described in more detail in the section on the Monte Carlo simulations.

2.2. Poisson processes with an interface as a model for the localizations

Before developing a mathematical method for interface detection, we define an appropriate statistical model to describe the localizations around an interface. This mathematical approach serves two goals. First, the statistical model creates an abstraction that allows us to frame the boundary estimation problem into a mathematical question. This leads to the right field within mathematics and statistics, whereof results and methods can be employed to find an efficient estimation procedure for the boundary. Second, based on the statistical model, a large variety of data sets can be generated with Monte Carlo simulations and used to test the effectiveness of the proposed estimation method.

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**Fig. 1.** MLE interface-estimation detects simulated linear interfaces with high accuracies beyond the maximum localization precision of \sim 5 nm. a) the normalized L1 error of the estimated interface, given by the MLE, with respect to the true interface, b-e) two examples of the simulations at N = 20 000 (b, c) and N = 5 000 (d, e), showing the full field of view (b and d) and the band of 1 \mu m centered about the interface (c and e), respectively. Note that the simulated data shown is only a representative example; the estimated interfaces in panels b-e originate from five independent simulations.
The model is based on the following premises. First, the localizations resulting from single-molecule localization microscopy can be regarded as a stochastic point process, i.e. a process that places points in a given area according to some stochastic mechanism. Secondly, it is observed that the localizations in each fluid are very homogeneously distributed, albeit with different densities. The localizations in the imaging area are thus modelled as a combination of two independent homogeneous Poisson processes [45] with different densities $\lambda_1$ and $\lambda_2$, respectively for the left and right side of the interface. This combination in itself is again a Poisson process with an interface. The expected number of points $N$ in a given area $A$ equals $N = \lambda_1 A_1 + \lambda_2 A_2$, where $A_1$ and $A_2$ are the areas of the parts of $A$ that are left and right of the interface, respectively. The actual number of points in $A$ is random, and distributed according to a Poisson distribution with mean $N$. Together with the fact that the number of points in disjoint regions $A$ and $B$ are independent, this completely defines the Poisson process. More details on the Poisson process can be found in the Supplementary material. For the analysis, we assume that $\lambda_1 \neq \lambda_2$ and that there is no accumulation of localizations on the interface itself. For experiments, this translates into the requirement that the fluorescent probes are distributed unequally in the two phases and do not accumulate on the interface itself.

2.3. Simulated data obtained through the Monte Carlo method

Monte Carlo simulations were performed to emulate SMLM experiments on pristine liquid–liquid interfaces and generate simulated data sets for a systematic assessment of the performance of the interface-detection method. To this end, an interface was created by generating two regions with a different localization density within a 30x30 $\mu$m domain; being a localization-dense region left of $x = 15$ $\mu$m and a localization-sparse region right of $x = 15$. The input for the simulations is the mean number of localizations in the entire sample $N$ and the mean relative density $\rho = \frac{\lambda_1 A_1}{\lambda_2 A_2} = \frac{N_{A_1}}{N_{A_2}}$. The localizations are then generated using two homogeneous Poisson processes (see Supplementary material for more details). These Monte Carlo simulations thus represent SMLM experiments with a single liquid–liquid interface at $x = 15$. The estimated interface location can then be compared to the true interface location. All simulations were repeated 100 times using independent copies to produce reliable estimates for the mean and standard deviation of the estimation error. The domain of 30x30 $\mu$m was considered for demonstration purposes. However, because the estimation method (proposed in the next section) is scale-invariant the results apply to all length scales. The only independent parameters are $N$ and $\rho$. 

Another set of Monte Carlo simulations was performed to emulate SMLM experiments on liquid–liquid interfaces in the presence of nanoparticles. To this end, either one or three circular regions were permanently deleted from the simulated data before the use of any interface-detection method. These regions represent data that is not taken into account during the interface detection because data in these regions has been affected by the presence of a (hypothetical) particle located at the interface. These simulations were performed using $N = 20\,000$ (before deletion) and $\rho = 5$, and as before, repeated 100 times using independent copies. The current work considers the size and location of the particles as known. In the one-particle simulations, the single, circular cut was centered at the interface $x = 15$ $\mu$m with $y = 15$ $\mu$m; i.e. corresponding to a particle which is not preferentially wet by either water or octanol. In the three-particle simulations, the three circular cuts were centered at the interface $x = 15$ $\mu$m and equally spaced with $y = \{5, 12, 25\}$ $\mu$m. Both the one- and three-particle simulations were performed for particles of varying size using radii of the circular cuts, $R = \{100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000, 5000\}$ nm. Simulation data are always shown in orange within figures.

2.4. Estimating the location of the interface using the Maximum Likelihood Estimator (MLE)

Development of MLE approach: Having defined an appropriate statistical model for the localizations around an interface in terms of a Poisson process, an effective estimation method can be developed. To this end we follow the Maximum Likelihood Estimator (MLE) approach as documented by Chernoff and Rubin [46]. Note that the overall density $\rho$ depends on the two different localization densities, $\lambda_1$ and $\lambda_2$, and the position of the interface. The interface is assumed to be straight, so that it is completely characterized by two points $\bar{a}_0$ and $\bar{b}_0$ at which it intersects with the boundaries of the image area. The boundary detection problem is now reduced to the problem of estimating the points $\bar{a}_0$ and $\bar{b}_0$.

Consider a realization of the point process according to the statistical model and suppose for a moment that the densities $\lambda_1$ and $\lambda_2$ are known. Then, any choice of the coordinates $\bar{a}$ and $\bar{b}$ defines a candidate model for the observed localizations. One can then compute the probability that the observed localizations are in fact generated by this candidate model. The resulting value is called the likelihood of the model given the selected coordinates $\bar{a}$ and $\bar{b}$.

We will describe in broad terms how this MLE procedure works and refer the reader to the Supplementary material for further details.

Given that we have a total of $N$ localizations $x_N = \{x_1, \ldots, x_N\}$, let $A_1(\bar{a}, \bar{b})$ and $A_2(\bar{a}, \bar{b})$ denote, respectively, the areas to the left and right of the boundary defined by $\bar{a}$ and $\bar{b}$. In addition, let $N_1(\bar{a}, \bar{b})$ be the number of localizations in $A_1(\bar{a}, \bar{b})$. Then the likelihood function is given by

$$L(\bar{a}, \bar{b}; x_N) = \left(\frac{N_1}{N}\right)^{N_1}(\bar{a}, \bar{b})^{N_1}(\bar{a}, \bar{b})^{N_2}, \quad (1)$$

This can be interpreted as the probability that the data $x_N$ was generated by the model with a boundary determined by $\bar{a}$ and $\bar{b}$. The Maximum Likelihood Estimator (MLE) for $\bar{a}$ and $\bar{b}$ are those coordinates $\bar{a}$ and $\bar{b}$ for which the corresponding likelihood in Eq. (1) is maximal. In practice, since the likelihoods are often very small numbers, one usually works with the logarithm of the likelihood, called the log-likelihood

$$\ell(\bar{a}, \bar{b}; x_N) = N_1(\bar{a}, \bar{b}) \ln \lambda_1 + N_2(\bar{a}, \bar{b}) \ln \lambda_2 - N \ln N. \quad (2)$$

Note that, since $N$ is known, we need only to maximize the first two terms in Eq. (2).

So far, the general assumption is that the localization densities are known in both phases, which is not always the case. The MLE can nonetheless overcome density-dependent estimations by including appropriate estimates for both densities. From Eq. (2) it follows that the MLE $\lambda_1$ for $\lambda_1$ is given by $N_1(\bar{a}, \bar{b})/|A|$. This expression is obtained by using the relation $\rho = \frac{N_{A_1}}{N_{A_2}}$ to write $\lambda_2$ in terms of $\lambda_1$, solving the equation $\partial \ell/\partial \lambda_1 = 0$ to get the expression for $\lambda_1$, and using the formula for $\rho$ to get $\lambda_2$. Plugging this back into Eq. (2), the maximum likelihood optimization can now be executed with the log-likelihood function.
The resulting estimates for the boundary coordinates are thus the values $\hat{a}$ and $\hat{b}$ that maximize Eq. (3).

It was proven that this MLE approach for estimating the coordinates $\overrightarrow{a}$ and $\overrightarrow{b}$ for this model is consistent, meaning that the estimators $\hat{a}$ and $\hat{b}$ become more accurate as the number of localizations $N$ created by the model tends to infinity [46]. Remarkably, the convergence speed is known to be linear in the inverse of the number of localizations, which is extremely fast compared to conventional methods (which often converge as the inverse of the square root of $N$) [46]. Such high convergence speeds arise since the density of the overall Poisson process is discontinuous at the interface if $\lambda_1 \neq \lambda_2$. Moreover, the MLE considers samples on an individual basis and is essentially parameterless, i.e. users do not need to define any type of density threshold to be able to determine the interface location.

**Implementation of MLE approach for interface detection:** To apply the MLE approach in practice, the likelihood for all possible choices of coordinates $\overrightarrow{a}$ and $\overrightarrow{b}$ need to be computed. Since this is computationally expensive, two line-segments on the border of the imaging area are divided into $K$ intervals of equal size. The boundaries of these intervals then yield $K + 1$ possible coordinate sets for $\overrightarrow{a}$ and $\overrightarrow{b}$ which are used to compute the MLE in Eq. (3). This discretization means that the computational cost of the method scales quadratically with the desired accuracy, but it is still linear in the number of localizations. In this work the interval size is chosen to be 2.5 nm. Since this is below the precision of a single localization, this discretization of the image area does not significantly influence the precision of the estimation method. Another concern might be that soft interfaces itself are not discrete, causing a smearing of the localizations near the interface. However, the interferential width of liquid–liquid interfaces is typically less than 5 nm for two liquids consisting of small molecules such as water and octanol.

If a sample contains not only two liquids but also other objects, then localizations around these objects, such as particles, may be distributed differently than in the rest of the sample. To avoid that this affects the interface location, all localizations within a certain region can be removed before applying the MLE. The MLE can be adjusted to take into account that a region is now missing from the data. Because the density is zero for the removed region, the MLE does not change and only the values of $A_i$ and $\tilde{\lambda}_i$ have to be adjusted. This means that the area and localizations within the removed regions are not included when computing the values of $A_i$ and $\tilde{\lambda}_i$. A comparison of the MLE with and without this adjustment is given in the “Results and discussion” section. A link to the MATLAB source code of the MLE implementation can be found in the Supplementary information.

**Tests of effectiveness of the proposed estimation method:** We assess the performance of the MLE using the L1 loss function, either with respect to the true interface location (in case of a simulation) or a previous estimate of the interface location (in case of an experiment). The L1 loss function is defined as

$$L_1 = \frac{1}{2} \int_{-\infty}^{\infty} |x - \hat{x}| \, dy,$$

where $x$ is the true $x$ location of the interface and $\hat{x}$ its estimate. The L1 loss function is normalized by $S$, the length of the interface, so that the resulting loss function is the average error per unit length of interface. In other words, the L1 loss function gives the average distance between the real interface and the estimated interface in nanometers.

### 3. Results and discussion

The aim of this work is to develop an iPAINT method that detects the location of liquid–liquid interfaces in situ with minimal invasiveness. iPAINT achieves this by using low concentrations of a small, freely-diffusing, fluorescent probe. However, the resulting localization densities strongly depend on the details of the individual experiment and determination of the interface location by e.g. density thresholding is challenging. We therefore developed a mathematical method based on the Maximum Likelihood Estimator (MLE) approach to estimate the location of an interface based on distinctly different dye concentrations in the immiscible bulk phases. Building upon the assumption that the single molecule localizations are distributed according to a homogeneous Poisson process, the MLE method optimizes a log-likelihood function to obtain the most likely location of the interface. Advantageously, the MLE method detects the interface location on a sample-by-sample basis and requires no predetermined density threshold. This allows the potential investigation of inter-sample differences and reduces biases caused by manual density thresholding.

Next, the accuracy of the MLE method to estimate the location of a soft interface and its suitability for use in super-resolution microscopy were assessed using simulated datasets with a vertical interface. To this end, several data sets were generated by Monte Carlo simulations with selected variations in the total number of localizations $R = (5000, 10000, 20000, 40000, 80000, 160000, 320000, 640000)$ and in relative densities $\rho = (2.5, 10, 20)$. The developed MLE method was applied to estimate the interface location in these datasets and the L1 loss function of the estimated interface was computed to assess MLE performance. To determine the convergence speed of the MLE and to judge whether convergence is sufficiently high to accurately estimate the interface location, we first investigated how the resulting values of the L1 loss function depend on $N$ and $\rho$ (Fig. 1a1). Increasing either $N$ or $\rho$ rapidly improves the accuracy. Notably, the L1 loss function appears to be proportional to $\frac{1}{N}$ and $\frac{1}{\rho}$ (Fig. S3 and S4). Fitting these dependencies of the L1 loss function results in

$$L_1 = 1.511 \times 10^6 \left( \frac{1}{N} \right) \left( 2.491 \times 10^{-2} + \frac{1}{\rho} \right).$$

which describes the mean L1 loss function of the MLE for a given sample with an adjusted R-squared of 0.999. This confirms that the MLE method converges with a speed proportional to $\frac{1}{N}$ and $\frac{1}{\rho}$, which is indeed remarkably fast compared to conventional methods that typically converge proportional to $\frac{1}{\sqrt{N}}$ and even faster in terms of the relative density $\rho$. Inspection of representative examples with either 5 000 or 20 000 localizations and a relative density of $\rho = \frac{10}{2} = 10$ showed that the MLE indeed accurately detects the interface within a 30x30 µM region (Fig. 1b and 1d, middle column). Zooming in further shows that the MLE has almost fully converged for these samples (Fig. 1c and 1e, right column). In fact, the accuracy of the MLE in these examples is around 5 nm (Fig. 1a, Eq. (3)). Estimating the interface location at lower density contrasts, e.g. $\rho = 2$, is of limited relevance because values of $\rho$ observed in experiment are typically higher than 10. Nonetheless, simulations at $\rho = 2$ show that the accuracy decreases, as would be expected given Fig. 1a and Eq. (5), but convergence remains excellent nonetheless (Fig. S5). Furthermore, changing
the orientation of the interface has little effect on the accuracy of the method (Fig. S6). The aforementioned observations from the simulations thus imply that the proposed MLE method accurately estimates the interface location without needing any input parameters.

Next, we cross-validated the algorithm against experimental data to confirm the validity of the MLE method and its applicability to experimental data. Therefore, we performed iPAINT on a sample with 2-octanol and water to create a soft interface and reconstructed the corresponding interface (Fig. 2, Fig. S1). Consistent with the simulations, the MLE was able to determine the interface with high accuracy in the experimental dataset. Again, upon re-determination of the interface for a small region of interest, effectively reducing the number of localizations (N = 872, ~3% of the total) and changing the density contrast (ρ = 12), our results show that the original estimate of the interface changes little and remains stable (Fig. 2b,c). Similarly, data reduction by stochastic removal of localizations from the initial experiment showed that the MLE accurately estimates the interface even at low localization densities (Fig. 2c). Deleting almost a third (28%) of the total number of localizations resulted in no significant change in interface location (L1 < 10 nm). Noticeable change in the interface location did occur when the interface was determined using exclusively a 5x5 μm region instead of the full 30x30 μm sample because only ~3% of the total number of localizations was used. Compared to the simulation results, the L1 increases slightly faster for experimental data. This could result from the experimental data having slight deviations with respect to a completely homogeneous Poisson process. Nonetheless, our results show that the MLE can achieve highly accurate estimations of the location of straight interfaces in iPAINT data sets.

Having tested the MLE in both simulation and experiment, we reconsidered whether the MLE method is sufficiently practical, i.e. whether the MLE can reach a meaningful accuracy for experimentally-achievable values of N and ρ. In the case of very bright fluorophores, the maximum precision of a single localization in SMLM experiments can reach ~5 nm. Fig. 1a and Eq. (5) illustrate how the accuracy depends on N and ρ, which also show that the MLE already reaches a mean accuracy of about 5 nm for (N, ρ) being either (20 000, 5) or (10 000, 10). Fig. 2 (and later also Fig. 4) demonstrates that these values of N and ρ can readily be achieved in SMLM experiments with an imaging time of 5 min. This illustrates not only that the MLE method is practical and highly feasible, but also that in experiment the precision of the individual localizations is more likely to be limiting than the accuracy of the MLE method.

Finally, we tested whether the MLE can still accurately locate the interface despite the presence of particles. In the following tests, we consider a number of hypothetical particles at the interface. The localizations corresponding to these particles were removed from the data set before applying the MLE method. MLE performance was then assessed for simulated data (Fig. 3) as well as experimental data (Fig. 4). We anticipate that this is helpful for the analysis of experimental data sets, in particular for large and non-spherical particles that significantly disturb the localization density in the vicinity of the interface and may induce interfacial undulations. In fact, the exclusion of data will inevitably affect the localization densities estimated by the MLE method, especially for larger particles. Therefore, an adjusted version of the MLE, which ignores the removed regions when estimating the localization densities, is also introduced and tested. This version of the MLE will be referred to as the particle-adjusted MLE.

Fig. 3a shows an example of a simulated data set with hypothetical particles, represented by superposed circular cuts, and the interface location as given by the unmodified and particle-adjusted MLE method. Inspection of the L1 loss function for simulated data in Fig. 3b shows that both MLEs remains highly accurate (L1 < 10 nm) as long as the particles are small (radii < 1100 nm) or only a single particle is present. However, the performance of the MLE deteriorates in the presence of multiple larger particles at the interface, especially for the unmodified MLE. Nevertheless, the particle-adjusted MLE strongly reduces the influence of the particles in all cases and gives a consistently superior performance. Furthermore, additional simulations show that our particle-adjusted MLE also performs well on highly covered interfaces (L1 < 20 nm for a coverage of 80%) and will therefore be suitable to study densely packed interfaces such as Pickering emulsions (Fig. S7 and S8). The proposed particle-adjusted MLE therefore indeed helps to prevent unwanted changes to the estimated interface location. This fortifies the robustness of the MLE method and also increases the range of particle sizes and numbers to which it can be used reliably.

Next, we examined the performance of the particle-adjusted MLE on an experimental data set with either one or three circular cuts superposed on the interface to represent the presence of adsorbed particles (Fig. 4). Again, we observe that the estimate is
Fig. 3. In simulated data, the particle-adjusted MLE accurately detects the interface location even when large circular cuts (radii $\approx 1000$ nm) are superposed on the data. The circular cuts are a representation of particles at the interface. a) Example simulated data where three circular cuts were superposed on the data before detecting the interface location (also shown) using either of the two MLE methods. b) The L1 error on simulated data for the unmodified MLE and the particle-adjusted MLE, both for a single as well as three circular cuts. All simulations had $N = 20,000$, $\rho = 5$, and were repeated 100 times.

Fig. 4. Removing one or multiple circular regions from the experimental data has negligible effect on the interface location given by the particle-adjusted MLE. The circular cuts are a representation of particles at the interface. The top row shows data for a single circular cut whereas the bottom row shows data for three circular cuts. Figures a) and d) show example data together with interface estimates for the original sample and the same sample with circular cuts applied before detection of the interface. The radii of the cuts or hypothetical particles in the examples are a) 2500 nm and b) 2000 nm. Figures b) and e) show the same sample for a smaller field of view. Figures c) and f) show the L1 error with respect to the interface of the original sample as function of the radius of the regions that are cut from the sample. Both the unmodified and particle-adjusted MLE are shown. This sample has $N = 9,208$ with $\rho = 16$ with CAGE-552 as imaging probe.
little affected by the presence of a single particle regardless of particle size (Fig. 4a-c). A slight reduction in performance (L1 < 5 nm) is observed for particle radii larger than ~3 μm. The particle-adjusted MLE performs better than the original MLE (for which L1 > 30 nm) for the larger particle sizes studied. Interestingly, the particle-adjusted MLE also performs well if several particles are present (Fig. 4d-f). Regardless of particle size, for radii as large as 5 μm the L1 remains < 5 nm. By contrast, the original MLE performs worse as particle sizes increase with L1 ~100 nm for three particles of 3 μm in radius. Note that such large, micron-sized particles can be detected in a straightforward manner by conventional microscopy and are therefore of lesser interest for imaging by super-resolution microscopy.

Although our current method was tested using straight interfaces populated by spherical particles, we predict that our approach is highly adaptable to curved interfaces and nonspherical particles (see Supplementary information for a more in-depth discussion). Furthermore, we stress that the MLE method can be applied to any interface that separates two chemically distinct regions, including solid–liquid and liquid–air interfaces. This is because the MLE method can be said to act according to a maximum ignorance principle, using only the contrast in localizations to determine the interface. As long as the fluorescent probe can be tuned to give different localization densities in the different phases, the MLE method is thus agnostic towards the chemistry of the sample. The versatility of the MLE method with respect to different types of interfaces, either with or without particles, make it a method that is broadly applicable to the detection of interfaces.

In sum, we reason that the (particle-adjusted) MLE is a highly accurate and parameterless method to determine the interface location for point cloud data in the absence and presence of one or more nanoparticles. In fact, the accuracy of the estimated interface location is more likely to be limited by the localization precision of the SMLM experiment than the accuracy of the MLE. The accuracy of the interface-estimate reduces when data is not distributed according to a perfectly homogeneous Poisson process. This is why higher values of N and ρ are needed to arrive at the same error in the interface estimates based on experimental data sets compared to data generated by Monte Carlo simulations. These values are readily attainable for real systems (e.g. Figs. 2 and 4) within 5 min of data acquisition. For sufficiently high values of N and ρ, e.g. N > 10 000 and ρ > 10, the location of the interface can be detected in a reliable manner even for rather small fields of view, which capture no more than a few microns of the liquid–liquid interface. The MLE method thus offers a new avenue to study wetting phenomena at the nanoscale by SMLM.

4. Conclusion

Estimating the location of a liquid–liquid interface is a recurring problem that previously required either invasive techniques [33–36] or averaging of samples [4,37,38], both of which reduce the accuracy of the interface location. This has hindered the detailed study of nanoscale wetting phenomena such as contact line pinning, line tension effects, and intra- and interparticle heterogeneities, all of which may have drastic effects on the stability of liquid–liquid interfaces [4,28]. The present work provides a new method for parameterless determination of the location of a straight interface in single-molecule localization microscopy (SMLM) experiments. By requiring only a single SMLM experiment as input, this new method avoids averaging of samples as well as invasive sample preparation techniques, such as fixation and vitrification. The developed method is based on a maximized likelihood estimator (MLE) and assumes a linear interface given by a discontinuity in the density of localizations which follow from a Poisson process. Importantly, this mathematical method abolishes the need for interfaces to be positively stained, which further minimizes the perturbation of the system due to sample manipulation for imaging. Monte Carlo simulations and SMLM experiments showed that the estimated location of the interface is accurate and robust. The MLE quickly approaches an accuracy that equates the localization precision, even for SMLM experiments of several minutes. This can be attributed to the surprisingly high convergence speed of the MLE, which is proportional to the inverse of the number of localizations (1/N) and the inverse square of the relative density of localizations (1/ρ²). After a correction to the original MLE, it is found that the method is not significantly affected by the presence of one or several particles at the interface. This makes the proposed method both a pragmatic and accurate tool to determine the location of soft interfaces in SMLM.

An interesting extension of the present methodology would be to combine the proposed algorithm with a method to simultaneously determine particle size, so as to directly interrogate the relation between contact angle and particle dimensions within one and the same sample. Moreover, a direct comparison of results of replicate experiments on e.g. different aliquots of the same stock is now possible, so that the impacts of sample manipulation, sample history, interparticle variations, batch-to-batch differences, and out-of-equilibrium phenomena such as (transient) pinning can be investigated. Studying the effects of surface asperities and particle morphology on nanoscale wetting and interface undulations is another intriguing prospect.

CRediT authorship contribution statement

Dingeman L.H. van der Haven: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. Roderick Prudent Tas: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. Pim van der Hoorn: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. Remco van der Hofstad: Conceptualization, Investigation, Supervision, Writing – review & editing. Ilja Karina Voets: Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article, including hyperlinks to the MATLAB source code, can be found online at https://doi.org/10.1016/j.jcis.2022.03.116.

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