

Calibration-Free, Nanometer-Scale Single-Molecule Localization with MINFLUX and RASTMIN via Direct Minimum Fitting

Fernando Daniel Stefani

`fernando.stefani@cibion.conicet.gov.ar`

Centro de Investigaciones en Bionanociencias (CIBION-CONICET)

Lucía Lopez

National Scientific and Technical Research Council

Florencia Edorna

Centro de Investigaciones en Bionanociencias (CIBION-CONICET)

Alan Szalai

Ludwig-Maximilians-Universität <https://orcid.org/0000-0002-3795-3230>

Andrés Zelcer

Centro de Investigaciones en Bionanociencias (CIBION-CONICET)

Article

Keywords:

Posted Date: October 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10932088/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: There is no conflict of interest

Calibration-Free, Nanometer-Scale Single-Molecule Localization with MINFLUX and RASTMIN via Direct Minimum Fitting

Lucía F. Lopez¹, Florencia Edorna^{1,2}, Alan M. Szalai¹, Andrés Zelcer¹, Fernando D. Stefani^{1,2*}

¹ Centro de Investigaciones en Bionanociencias (CIBION), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2390, C1425FQD Ciudad Autónoma de Buenos Aires, Argentina

² Departamento de Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Güiraldes 2620, C1428EHA Ciudad Autónoma de Buenos Aires, Argentina

*fernando.stefani@df.uba.ar

Abstract

Ultraprecise super-resolution fluorescence microscopy techniques such as MINFLUX and RASTMIN achieve single-digit nanometer-scale localization precision of single molecules by probing them with a minimum of light. Their standard analysis pipelines rely on maximum likelihood estimation (MLE) combined with prior calibration of the excitation profile, imposing significant experimental complexity and sensitivity to imperfections. Here, we introduce a calibration-free localization framework for these methods based on direct fitting of photon-position arrays using physically motivated point spread function models. Through Monte Carlo simulations, we show that direct fitting—particularly with a donut-based model—achieves localization precision approaching the Cramér–Rao lower bound and closely matches MLE performance over a wide range of photon counts. Experimental validation using RASTMIN measurements on single emitters confirms that, at ≥ 1000 detected photons, calibration-free fitting yields localization precision indistinguishable from MLE while maintaining nanometer-scale accuracy. We further demonstrate that incorporating modest model flexibility enables robustness against realistic optical aberrations and accurate reconstruction of spatial patterns. These results establish direct fitting as a simple and robust alternative to calibration-dependent approaches, providing a more practical route toward high-precision photon-efficient single-molecule localization using minima of light.

Introduction

Super-resolution fluorescence microscopy has revolutionized optical imaging by overcoming the diffraction limit and enabling the visualization of biological and synthetic structures at the nanometer scale¹. Landmark methods such as stimulated emission depletion (STED) microscopy and single-molecule localization microscopy (SMLM) have provided complementary strategies, either by spatially controlling fluorescence emission or by computationally localizing individual emitters with high precision. Among these approaches, SMLM techniques—including STORM² and DNA-PAINT³—have become particularly widespread due to their ability to reconstruct super-resolved images from sequential localization of single emitters by fitting the emission pattern of individual fluorophores to a model of the point spread function (PSF). The achievable localization precision is fundamentally limited by photon statistics and is commonly described by the Cramér–Rao lower bound (CRLB), which has guided the development of optimal estimation and analysis strategies.^{4–6}

A major advance in this direction was the introduction of MINFLUX nanoscopy,⁷ which localizes emitters by probing them with an excitation field featuring an intensity minimum. By placing the emitter near this minimum, MINFLUX dramatically increases the information content per photon, achieving nanometer and even sub-nanometer localization precision with orders of magnitude fewer photons than conventional centroid-based approaches. This concept has subsequently been extended^{8,9} and generalized in methods such as RASTMIN,¹⁰ which replace sparse excitation patterns with raster-scanned minima, preserving near-optimal precision while simplifying implementation on standard confocal or multiphoton microscopes¹¹. Together, these approaches represent a paradigm shift toward photon-efficient, coordinate-targeted localization at the molecular scale.

Despite these advances, the analysis pipelines used in MINFLUX and related techniques to estimate the emitter position remain strongly dependent on prior calibration of the excitation field. In particular, maximum likelihood estimation (MLE)—the standard approach for localization¹²—relies on high-fidelity reference measurements of the PSF to achieve optimal performance. Such calibration procedures require high signal-to-noise ratio measurements with sub-nanometer sampling and are sensitive to experimental imperfections, misalignments and drift. This calibration burden constitutes a significant practical limitation, imposing periodic recalibrations especially for dynamic experiments or long acquisitions.

In parallel, camera-based SMLM has long relied on direct fitting of the measured signal to analytical PSF models as a simple and robust alternative to calibration-intensive approaches. However, translating this paradigm to MINFLUX-type modalities has not been straightforward, as the associated estimation problem has traditionally favored likelihood-based formulations.

Notably, the emergence of RASTMIN—where the measurement naturally forms an image of the excitation minimum—raises the question of whether direct fitting strategies can be extended to coordinate-targeted localization methods.

Here, we address this question by introducing and systematically evaluating a calibration-free localization framework based on direct fitting of photon-position arrays in MINFLUX and RASTMIN. We show that, despite the absence of prior calibration, simple physically motivated PSF models can recover emitter positions with nanometer-scale precision and low bias, approaching the performance of MLE under realistic conditions. By bridging concepts from camera-based localization and coordinate-targeted nanoscopy, this work provides a timely route toward simpler, more robust, and widely accessible implementations of molecular-scale fluorescence microscopy.

Results

MINFLUX and RASTMIN localization pipelines.

Figure 1A shows the measurement pipeline of 2D MINFLUX and RASTMIN. In both cases, the localization measurement involves measuring the fluorescence of a single emitter (molecule) as it is excited with an excitation field $I(\mathbf{r})$ comprising an intensity minimum, over a series of K positions $\{\mathbf{r}_i\}_{i=1}^K = [\mathbf{r}_1, \mathbf{r}_2, \dots, \mathbf{r}_K]$ near the target emitter (molecule). The series of positions is usually called the excitation beam pattern (EBP). Here MINFLUX and RASTMIN differ. MINFLUX uses EBPs with small number of positions, e.g. a central point plus a triangular arrangement ($K = 4$)^{7,8,13}, a central point plus an hexagonal arrangement ($K = 7$)¹⁴, or two pairs of points along perpendicular directions ($K = 4$)¹⁵. In contrast, RASTMIN employs a grid of equidistant measurement points just like the standard image acquisition in any scanning microscope. In both cases the output of a measurement is an array of number of detected photons $\{n_i\}_{i=1}^K = [n_1, n_2, \dots, n_K]$ corresponding to each measurement position of the EBP.

To obtain the emitter position, a separate measurement is necessary to characterize the excitation field $I(\mathbf{r})$, which is typically achieved by scanning $I(\mathbf{r})$ over a sub-diffraction-sized fluorescent bead. This reference image of the point spread function (PSF) is used to compute a probability map for different molecular positions, which together with $\{\mathbf{r}_i, n_i\}_{i=1}^K$ and a measured signal-to-background ratio (SBR) enables determining the most probable position of the emitter through maximum likelihood estimation (MLE, Figure 1B)¹⁶. More details on the MLE pipeline for RASTMIN are shown in Figure S1 of the Supplementary Information. In order to achieve nanometric localization precision with this methodology, the reference image of

$I(\mathbf{r})$ must be measured with high definition (nanometric pixel sizes) and high signal-to-noise ratio (SNR).

The maximum localization precision achievable is given by the CRLB, which scales with the size of the EBP (L) and inversely with the square root of the total number of detected photons ($N = \sum_{i=1}^K n_i$)^{7,16}. Both MINFLUX and RASTMIN achieve comparable theoretical performance (Figure 1C), and their predicted precisions have been consistently confirmed experimentally^{7,9,10}.

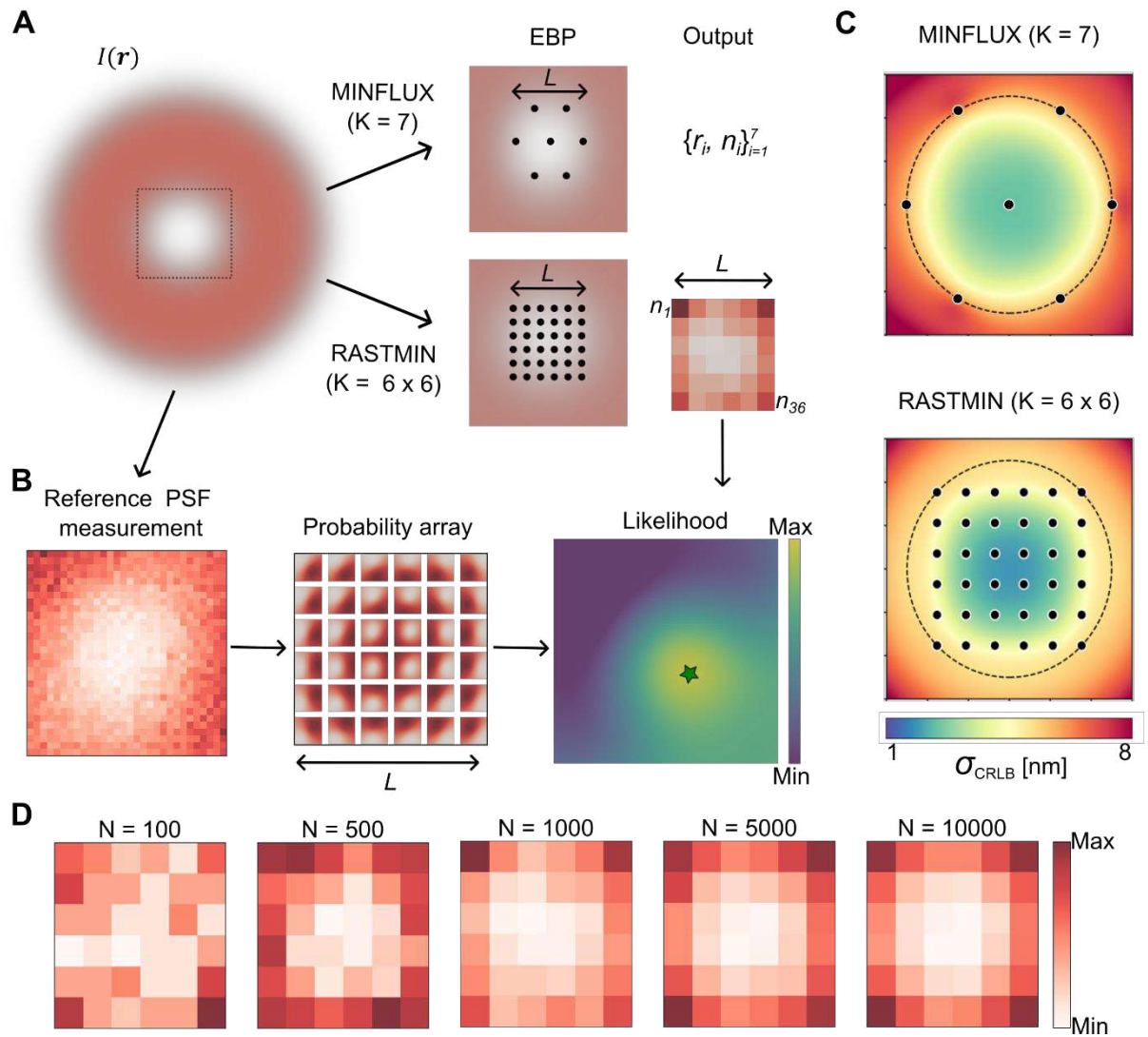


Figure 1: A) MINFLUX and RASTMIN measurement pipelines for single molecule localization. An excitation field $I(\mathbf{r})$ featuring an intensity minimum sequentially moved over a series of positions $\{\mathbf{r}_i\}_{i=1}^K$ that define the excitation beam pattern (EBP) of size L . Examples shown of MINFLUX with $K = 7$ and RASTMIN with $K = 6 \times 6$. The output is an array of photon counts corresponding to each

measurement position of the EBP $\{\mathbf{r}_i, n_i\}_{i=1}^K$. In the case of RASTMIN, this takes the form of an image of size $L \times L$ of the minimum $I(\mathbf{r})$. **B) MLE of the molecular position for RASTMIN.** A reference measurement of $I(\mathbf{r})$ is used to construct a probability array of photon detection for different molecular positions, which is used together with $\{\mathbf{r}_i, n_i\}_{i=1}^K$ to localize the emitter in the position where the likelihood function is maximum (green star in the shown example). **C) MINFLUX and RASTMIN performances.** Cramér-Rao lower bound (CRLB) of the localization precision (σ_{CRLB}) of MINFLUX ($K = 7$) and RASTMIN ($K = 6 \times 6$) as a function of emitter position for $L = 100$ nm, $SBR = 4$ and $N = 500$. **D) RASTMIN output data examples.** Example simulated RASTMIN images (6×6 pixels) for $L = 100$, $SBR = 10$, and $N = 100, 500, 1000, 5000$ and 10000 photons.

Single molecule localization by direct fit

In camera-based single molecule localization methods, determining the molecular position by fitting the molecular signal to the PSF has emerged naturally. MINFLUX, by contrast, was developed as a kind of triangulation method, for which the MLE approach was more suited. The emergence of RASTMIN as an equivalently precise alternative to MINFLUX called our attention towards the position estimation pipeline. In RASTMIN, the measurement output $\{\mathbf{r}_i, n_i\}_{i=1}^K$ forms an image of the minimum of $I(\mathbf{r})$; an image of the PSF, just like in camera-based methods. Figure 1D shows simulated RASTMIN frames for different photon counts (N) with $L = 100$ nm, $SBR = 10$ and $K = 6 \times 6$ pixels. As N increases, the position of the intensity minimum becomes visually more apparent and statistically better defined.

We thus explored the possibility of obtaining the molecular position by fitting $\{\mathbf{r}_i, n_i\}_{i=1}^K$ to a model of the PSF. In contrast to Gaussian models typically used in camera-based single molecule localization, here we need PSF models comprising a minimum. We tested two models, a parabola and a donut shaped beam whose 2D intensity profiles are represented by:

$$I_{Parabola}(x, y) = A \left(\frac{(x-x_E)^2}{2\sigma^2} + \frac{(y-y_E)^2}{2\sigma^2} \right) + B \quad (1)$$

$$I_{Donut}(x, y) = A 4e \ln 2 \left(\frac{(x-x_E)^2 + (y-y_E)^2}{d^2} \right) \exp \left(-4 \ln 2 \frac{(x-x_E)^2 + (y-y_E)^2}{d^2} \right) + B \quad (2)$$

where A is the amplitude, B is the background offset, $(x_E, y_E) = \mathbf{r}_E$ are the molecular coordinates which are the key fitting parameters for localization, d is related to the full width at half maximum ($fwhm$) of the donut, and σ is the parabola curvature. More details of the fitting functions and parameter bounds can be found in the Methods section.

Comparison of calibration-free direct fit and MLE localizations

To evaluate the performance of the proposed calibration-free method, we used Monte-Carlo generated RASTMIN and MINFLUX data with experimentally realistic parameter values. The simulated data was then analyzed with the two proposed models for calibration-free fitting and the localization results were compared both to the ground truth and to the results of the MLE approach, which is known to asymptotically reach the theoretical best performance achievable (CRLB). We considered a donut-shaped focus (I_{Donut} , $fwhm = 360$ nm) and an EBP size of $L = 100$ nm. For RASTMIN, the EBP was a square with $K = 6 \times 6$, whereas for MINFLUX it was a 7-exposure EBP (hexagon plus center). A single emitter was placed in every point of a square grid of size $L = 100$ nm with 1 nm spacing. This simulated area directly matches the spatial extent of the EBPs for both RASTMIN and MINFLUX. For each emitter position, we generated Poisson distributed photon counts over a uniform background to deliver a given SBR and total photon counts N . The resulting photon array was then fitted using two different PSF models (I_{Donut} , $I_{Parabola}$) and analyzed with the MLE approach using the same PSF that was employed to generate the simulated data. Performance was evaluated by computing (i) the localization precision as the standard deviation of the estimated coordinates, $\sigma = \sqrt{\sigma_x^2 + \sigma_y^2}$, and (ii) the accuracy as the 2D bias between the average localization position $(\hat{x}, \hat{y})$ and the true emitter position (x_E, y_E) , $Bias = \sqrt{(\hat{x} - x_E)^2 + (\hat{y} - y_E)^2}$. Maps of σ and $Bias$ were computed for a range of N values.

Figure 2A shows the precision (σ) and accuracy ($Bias$) maps for RASTMIN and MINFLUX for $N = 1000$ and $SBR = 10$ obtained using the MLE and direct fit approaches. As expected, the MLE approaches the theoretical maximum precision limit of the CRLB. For the used parameters this means $\sigma = 1.4 - 2$ nm for emitters in the central region of the EBP, both for MINFLUX and RASTMIN. Also consistent with previous reports^{10,16}, RASTMIN delivers a better precision than MINFLUX to localize emitters in the outer area of the EBP. Remarkably, similar localization precisions are achieved for emitters in the central region of the EBP simply by fitting the photon-position arrays with I_{Donut} . For emitters outside the EBP center, performance degrades reaching values of $\sigma \sim 8$ nm at the corners, providing a less uniform precision map compared to the MLE approach. Regarding accuracy, the MLE shows minimal bias ($< 1 - 2$ nm) across all the evaluated area, as theoretically expected. Fitting the photon counts with I_{Donut} results in a slight increase in the bias especially towards the edges of the probed area, reaching up to $\sim 3 - 4$ nm at the corners. Precision and accuracy maps obtained after fitting the simulated RASTMIN and MINFLUX data with $I_{Parabola}$ are shown in Figure S2 of the Supplementary Information.

The performance of the localization pipelines as a function of the number of acquired photons (N) is illustrated in Figure 2B. The curves represent the average values of σ ($\bar{\sigma}$) and $Bias$ ($\overline{Bias}$) computed over a central square area (75% of the simulated area, indicated with a dotted line in the MLE precision and accuracy maps for RASTMIN in Figure 2A) as a function of N , obtained with the MLE approach, fitting with I_{Donut} and fitting with $I_{Parabola}$. In the case of the average precision ($\bar{\sigma}$), we include in the plots the corresponding average CRLB over the same region for comparison. The difference between the MLE or fitting approaches and the CRLB values is also plotted, $\Delta\bar{\sigma} = \bar{\sigma} - \bar{\sigma}_{CRLB}$ for precision and the difference between the fitting approaches and MLE $\Delta\overline{Bias} = \overline{Bias}_{fit} - \overline{Bias}_{MLE}$ for accuracy.

For both precision and accuracy, the performance obtained when fitting with I_{Donut} systematically outperforms the $I_{Parabola}$ model for both the RASTMIN and MINFLUX configurations. Regarding the average localization precision ($\bar{\sigma}$) in RASTMIN, the I_{Donut} fit is only marginally inferior to the MLE benchmark — $< 0.5 \text{ nm}$ — within the intermediate photon range ($100 < N < 1000$). Beyond $N = 1000$ photons, the precision of both estimators becomes virtually identical and reaches the CRLB as N increases. For MINFLUX, this discrepancy is even more negligible; the I_{Donut} fitting approach yields a localization precision indistinguishable from the MLE standard from $N = 100$ photons onwards. The average accuracy ($\overline{Bias}$) shows a more pronounced difference between the fitting approaches and the MLE benchmark than precision does, although the absolute bias values remain small in all cases (sub-nanometer to a few nanometers across the entire photon range explored). For both RASTMIN and MINFLUX, the donut fit and the parabola fit exhibit a larger $\overline{Bias}$ than MLE. The performance for both fitting estimators improves up to $N > 100$, where both estimators find a plateau ($\sim 0.7 - 0.8 \text{ nm}$ for the donut fit, $\sim 1.5 - 2 \text{ nm}$ for the parabola fit). In contrast, the MLE bias continues to decrease monotonically. Among the two fitting models, the donut fit consistently achieves lower average bias than the parabola fit across the full photon range and for both geometries, reflecting its closer correspondence to the true excitation PSF shape and, correspondingly, smaller edge effects. The difference panels ($\Delta\overline{Bias}$) confirm that the donut fit remains closer to the MLE benchmark than the parabola fit, with the difference settling around $\sim 0.5 \text{ nm}$ for the donut fit and $\sim 1.3 \text{ nm}$ for the parabola fit.

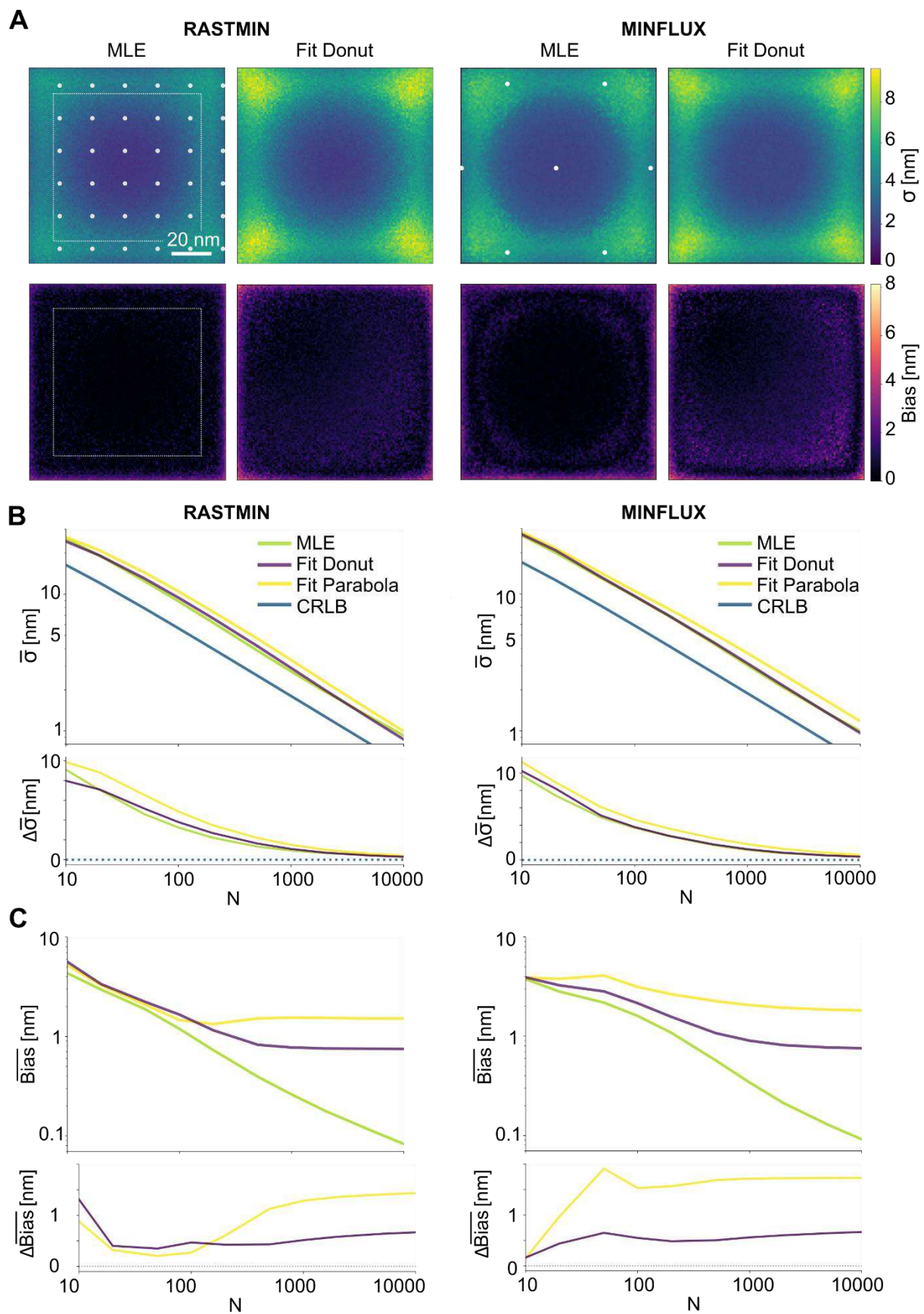


Figure 2: A) Localization precision and accuracy in RASTMIN and MINFLUX simulated experiments using MLE and the fitting approach. Localization precision (σ) and accuracy ($Bias$)

maps for the MLE and the fitting method using I_{Donut} . Simulation parameters: $L = 100\text{ nm}$, $K = 6 \times 6$ for RASTMIN and $K = 7$ for MINFLUX, $N = 1000$, $SBR = 10$, $grid\ step = 1\text{ nm}$. Scale bar: 20 nm (applies to all maps). White dots indicate the EBP. **B) Localization precision performance as a function of detected photons.** Average localization precision $\bar{\sigma}$ obtained with MLE, fitting with I_{Donut} or $I_{Parabola}$ and average CRLB (75% square central region of the simulated area, indicated in the RASTMIN MLE map of Figure 2A) as a function of N for $SBR = 10$ (log scale). Lower panels display $\Delta\bar{\sigma} = \bar{\sigma} - \bar{\sigma}_{CRLB}$, the dotted line indicates $\bar{\sigma} = \bar{\sigma}_{CRLB}$. **C) Accuracy performance as a function of detected photons.** Average bias (75% square central region of simulated area) as a function of N for $SBR = 10$ (log scale). Lower panels display $\Delta\overline{Bias} = \overline{Bias}_{fit} - \overline{Bias}_{MLE}$.

Experimental validation

The performance of the calibration-free localization framework beyond ideal theoretical conditions was evaluated on both experimental RASTMIN measurements of single fluorophores and simulated MINFLUX acquisitions generated via SimuFLUX¹⁷. Real-world experiments introduce the challenge of imperfections in the intensity distribution of $I(\mathbf{r})$, which are contemplated in the MLE but not in the direct fit approach.

First, we validated the pipeline using experimental RASTMIN measurements ($K = 6 \times 6$, $L = 100\text{ nm}$) of single ATTO 647N molecules attached to a DNA origami structures. Exemplary RASTMIN frames for different photon counts (N) are shown in Figure 3A. Sample preparation details and DNA origami design are provided in Methods and Supplementary Information, respectively. Molecular coordinates were estimated using (i) an experimentally calibrated MLE estimator, (ii) a calibration-free direct fit using the donut model of the PSF (Eq. 2), and (iii) a calibration-free direct fit using an anisotropic donut model of the PSF (Eq. 2 with different values of d for the x and y directions and an optional rotation angle, see Methods for more detail). The latter modification of the donut model was introduced to account for imperfections of the intensity distribution of the experimental illumination field; real donut-shaped foci often exhibit asymmetries due to optical aberrations, imperfect alignment of the vortex phase plate, astigmatism or mixed polarization states¹⁸. The localization precision σ was estimated by calculating the standard deviation of consecutive localizations of the same molecule. Figure 3B shows the experimental $\sigma(N)$ obtained with the three methods, and the theoretically expected precision (σ_{CRLB}). Additionally, the shaded area represents the maximum precision expected ($\sigma_{Total} = \sqrt{\sigma_{CRLB}^2 + \sigma_{DC}^2}$) considering the drift correction precision ($\sigma_{DC} = 1 - 2\text{ nm}$). Consistent with theoretical predictions, MLE achieves a localization precision closest to the theoretical limit of the CRLB. However, fitting the RASTMIN data with I_{Donut} yields localization precisions that are only slightly lower – or even indistinguishable from the MLE –

for $N \geq 1000$. Interestingly, the anisotropic donut model yields slightly worse precision than its symmetric counterpart at low photon counts ($N = 300$ and $N = 500$), while for $N \geq 1000$ both fitting models perform comparably. At high ($N \geq 1000$) photon counts, the differences between MLE and both fitting models become negligible. This behavior can be explained by the trade-off between model complexity and estimation stability. The anisotropic donut model introduces two additional free parameters. At low N , the photon budget is insufficient to constrain these additional degrees of freedom reliably, leading to increased variance in the position estimate. As N increases, the additional parameters become well-determined, and the precision gap closes.

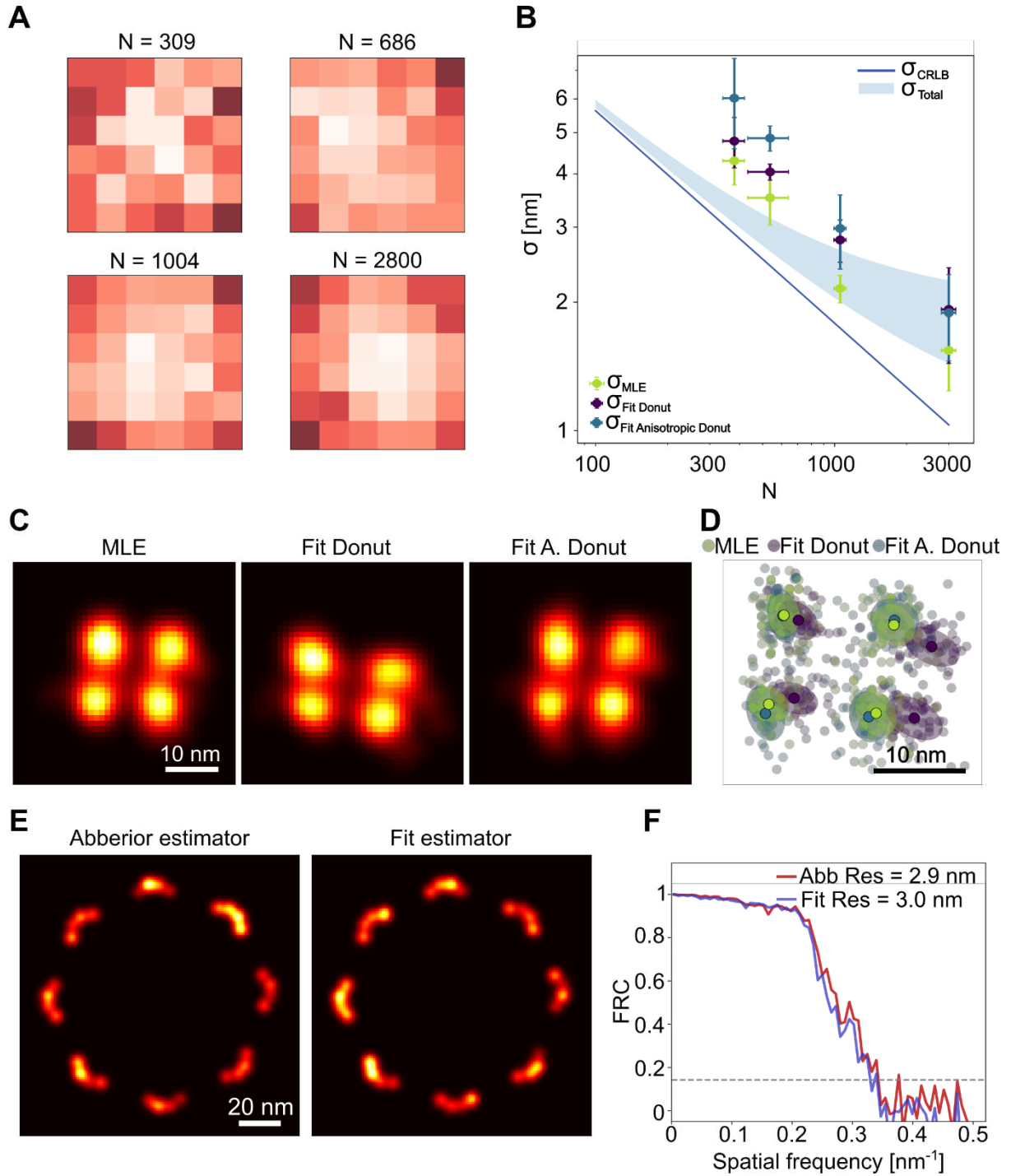


Figure 3: A) Exemplary RASTMIN frames. Images ($100 \text{ nm} \times 100 \text{ nm}$ frames) of a single ATTO 647N fluorophore immobilized on DNA origami structures, acquired with approximately 300, 600, 1000 and 3000 photons. **B) RASTMIN localization precision.** Localization precision σ as a function of N obtained in RASTMIN measurements using three analysis methods: MLE (green), isotropic donut fit (purple) and anisotropic donut fit (blue). For each N , three independent measurements were performed, each consisting of 15 consecutive localizations. The theoretical Cramér-Rao lower bound (σ_{CRLB} , solid blue line) is shown for comparison, along with the predicted total uncertainty for the experiments σ_{Total} (shaded blue area) accounting for drift correction precision $\sigma_{\text{DC}} = 1 - 2 \text{ nm}$. The CRLB was calculated

for the central $10\text{ nm} \times 10\text{ nm}$ region of the RASTMIN frame. **C) Localization results for an imaging experiment.** Gaussian-rendered images of the localizations obtained with MLE and the two calibration-free fitting approaches (isotropic and anisotropic donut). Scale bar: 10 nm. **D) Comparison between MLE and fitting approach for the imaging experiment.** Overlay of the 3 localization results rendered in C): MLE (green points), isotropic donut fit (purple) and anisotropic donut fit (blue). Covariance ellipses represent 1σ localization spread. Scale bar: 10 nm. **E-F) SimuFLUX validation.** Gaussian-rendered reconstructions of a simulated MINFLUX imaging experiment (SimuFLUX mimicking Abberior instrument acquisition) analyzed with the default estimator and with the calibration-free donut-fitting estimator. Both renders yield comparable spatial resolution as quantified by Fourier ring correlation (FRC).

To evaluate localization accuracy (bias), we performed RASTMIN imaging experiments. Using a piezoelectric stage and a custom-made stabilization system¹⁹, we sequentially displaced a single fluorescent molecule between four positions defining the vertices of an 11 nm square. Experimental details are provided in the Methods section. RASTMIN acquisitions were continuously performed during the controlled motion. Figure 3C-D shows the localizations obtained using the three methods, as a Gaussian-rendered image of the localizations obtained. The isotropic donut fit fails to preserve relative distances resulting in a distorted spatial pattern compared to the MLE localizations. In contrast, incorporating modest model flexibility via the anisotropic donut model recovers the correct geometry. This is more clearly shown in Figure 3D, where the localizations obtained with the 3 methods are overlaid.

Having confirmed experimental validity on RASTMIN, we extended our evaluation to MINFLUX configurations mimicking a commercial instrument. Because raw photon-count data from commercial MINFLUX systems are generally inaccessible to users, we made use of SimuFLUX¹⁷, a software package designed to simulate realistic MINFLUX acquisitions following commercial hardware specifications (i.e., Abberior Instruments). We integrated our donut-fitting estimator into the SimuFLUX pipeline and evaluated it on a simulated 2D MINFLUX imaging experiment of a nuclear pore complex (NPC), a well-characterized reference structure widely used to benchmark super-resolution microscopy methods²⁰. Gaussian-rendered reconstructions obtained from both methods are shown in Figure 3E. To quantitatively compare the achieved spatial resolution, we performed Fourier ring correlation (FRC) analysis²¹ on both renders. The resolution values obtained were virtually identical (2.9 nm for the default commercial estimator versus 3.0 nm for our calibration-free fit). This confirms that a simple, calibration-free fitting workflow performs comparably to the commercial, calibration-dependent pipelines under realistic MINFLUX imaging conditions.

Discussion

In summary, we have introduced and validated a calibration-free localization strategy for MINFLUX and RASTMIN microscopy based on direct fitting of the measured photon-position arrays. Through simulations, we show that this approach—particularly when using a donut-based PSF model—achieves localization precision close to the theoretical optimum, approaching that of maximum likelihood estimation (MLE) over a broad range of photon counts. While small deviations arise at high photon numbers and near the edges of the excitation beam pattern, the localization bias remains limited to the nanometer scale.

Experimental validation using RASTMIN measurements on single emitters confirms these findings. Calibration-free fitting with a physically motivated PSF model yields a localization precision that becomes indistinguishable from MLE at experimentally relevant photon counts (≥ 1000 photons), while maintaining nanometer-scale accuracy. Importantly, incorporating modest model flexibility—such as an anisotropic donut description—enables robust performance under realistic optical imperfections, at the cost of slightly increased variance only at low photon numbers.

Beyond precision metrics, controlled displacement experiments reveal that naive symmetric models can introduce geometric distortions in reconstructed positions, whereas appropriately extended models faithfully recover relative spatial arrangements. This underscores that achieving high precision alone is insufficient; accurate structural reconstruction requires models capable of capturing deviations from ideal excitation fields. The ability to recover nanometer-scale spatial patterns without explicit calibration constitutes a key step toward simplifying MINFLUX- and RASTMIN-based measurements.

We extended our evaluation to simulated MINFLUX acquisitions generated with SimuFLUX, reproducing the acquisition scheme and hardware specifications of a commercial (Abberior) instrument. Using a simulated NPC as a realistic benchmark structure, we compared our calibration-free estimator against the manufacturer's default localization algorithm. Despite the general inaccessibility of raw photon-count data from commercial MINFLUX systems, this comparison showed that the two approaches achieve nearly identical spatial resolution. This result indicates that the benefits of calibration-free fitting demonstrated here for RASTMIN extend to MINFLUX implementations.

We note that the calibration-free localization does not extend to pulsed-interleaved MINFLUX (p-MINFLUX)^{9,22} or other implementations where multiple excitation beams are generated from physically separated optical paths. A calibration measurement is therefore strictly required to determine the excitation beam pattern (EBP) geometry. This distinction is not merely technical but defines the practical scope of our contribution: speed-critical applications

may still prefer p-MINFLUX with calibration, whereas simplicity and ease of implementation favor the calibration-free scanning-based architectures.

The practical benefits of the calibration-free method for the broader community are substantial. For example, RASTMIN can be implemented on a standard confocal microscope with minor modifications (a vortex phase plate and a basic active stabilization system)^{10,19}, and our direct fitting pipeline is easily implemented on any programming language and doesn't need reference measurements. This combination lowers the technical and computational barriers to achieving molecular-scale and even single-digit nanometer resolution, making MINFLUX-class localization accessible to laboratories without specialized optics or complex calibration routines. Furthermore, the real-time position estimation enabled by this approach opens the door to adaptive imaging strategies and closed-loop single-molecule tracking on conventional scanning microscopes. Future work may extend this framework to three-dimensional localization, adaptive excitation schemes, and real-time implementations, further advancing the accessibility and versatility of nanometer-scale fluorescence microscopy. We anticipate that this streamlined workflow will accelerate the adoption of photon-efficient structured illumination methods for applications in cell biology, biophysics, and nanotechnology.

Materials and methods

Simulated RASTMIN and MINFLUX experiments

Simulations were performed to compare the localization performance of maximum likelihood estimation (MLE) versus direct fitting approaches. A donut-shaped excitation PSF was generated according to:

$$I_{Donut} = A 4e \ln 2 \left(\frac{(x - x_E)^2 + (y - y_E)^2}{d^2} \right) \exp \left(-4 \ln 2 \frac{(x - x_E)^2 + (y - y_E)^2}{d^2} \right) + B$$

where A is the amplitude, B is the background offset, (x_E, y_E) are the emitter coordinates and $d = 1.2 \text{ fwhm}$ is related to the full width at half maximum (FWHM) of the donut (set to 360 nm). For RASTMIN simulations, a donut-shaped excitation point spread function (PSF) was raster-scanned across an $100 \text{ nm} \times 100 \text{ nm}$ FOV in a 6×6 pixel grid. For MINFLUX simulations, the same PSF was sequentially positioned at the $K = 7$ exposure pattern comprising a central point and six surrounding points arranged in a hexagon.

For each simulated emitter position (sampled on a 1 nm grid over the FOV), photon counts were generated from a Poisson distribution with expected value determined by the local excitation intensity, the total photon budget N (ranging from 10 to 10,000), and the signal-to-

background ratio (*SBR*, fixed at 10 unless otherwise specified). For each set of parameters, 100 independent measurements were averaged to compute localization precision and bias.

Localization was performed using three estimators: (i) MLE with full knowledge of the true PSF (benchmark), (ii) direct fitting with the donut model I_{Donut} , and (iii) direct fitting with a $I_{Parabola}$.

Precision was defined as the standard deviation of estimated positions $\sigma = \sqrt{\sigma_x^2 + \sigma_y^2}$, and bias as the Euclidean distance between the estimated and true emitter positions. All fitting parameters are listed in Supplementary Table S2.

Fitting functions

For the analysis of experimental RASTMIN data, three fitting models were implemented in Python using non-linear least squares (`scipy.optimize.curve_fit`).

Isotropic donut model:

$$I_{Donut}(x, y) = A 4e \ln 2 \left(\frac{(x - x_E)^2 + (y - y_E)^2}{d^2} \right) \exp \left(-4 \ln 2 \frac{(x - x_E)^2 + (y - y_E)^2}{d^2} \right) + B$$

Parabolic model:

$$I_{Parabola}(x, y) = A \left(\frac{(x - x_E)^2}{2 \sigma^2} + \frac{(y - y_E)^2}{2 \sigma^2} \right) + B$$

Anisotropic donut model (accounts for PSF asymmetries):

$$I_{Anisotropic\ Donut} = A 4e \ln 2 \left(\frac{x_{rot}^2}{d_x^2} + \frac{y_{rot}^2}{d_y^2} \right) \exp \left(-4 \ln 2 \left(\frac{x_{rot}^2}{d_x^2} + \frac{y_{rot}^2}{d_y^2} \right) \right) + B$$

where d_x and d_y are the donut widths along the rotated principal axes and θ is the rotation angle and:

$$x_{rot} = (x - x_E) \cos \theta + (y - y_E) \sin \theta$$

$$y_{rot} = -(x - x_E) \sin \theta + (y - y_E) \cos \theta$$

The fitting parameters used in the analysis are listed in Table S2.

PSF characterization sample

1.5 thickness coverglasses with CoverWell™ Imaging Chambers (Grace Bio-Labs) were

treated with 2% Hellmanex™ for 30 min, washed (3×) with Milli-Q water and functionalized with a positively charged layer of Poly(diallyldimethylammonium chloride) (PDDA) Mw = 400,000–500,000 (Sigma-Aldrich), by incubating for 30 min a solution of PDDA (1mg/mL in 0.5M NaCl). Next, the chamber was rinsed with 3x Milli-Q water and incubated for 10 min with a solution (1:50 from original 1 OD stock) containing 200 nm spherical gold nanoparticles (Nanopartz) diluted in PBS 1X. Next, the chamber was rinsed 3x with PBS 1X and incubated for 10 min with 40nm Dark Red FluoSpheres Fluorescent Microspheres (Thermo Fisher Scientific). The beads solution was prepared directly by diluting the stock solution in PBS 1X (10^{-6} dilution from original stock) and was sonicated for 10 min just before being added to the chamber.

DNA origami sample

1.5 thickness coverglasses with CoverWell™ Imaging Chambers (Grace Bio-Labs) were treated with 2% Hellmanex™ for 30 min, washed (3×) with Milli-Q water and functionalized with BSA-biotin (1 mg/mL, 15 min incubation). Next, the chamber was washed (3x) with Milli-Q water and incubated for 15 min with Neutravidin (1 mg/mL). After washing (3x) with PBS 1X + MgCl₂ (12.5 mM), a solution containing 200 nm spherical gold nanoparticles (Nanopartz) diluted (1:50 from original 1 OD stock) in PBS 1X + MgCl₂ (12.5 mM) was incubated for 10 min. Next, the chamber was rinsed 3x with PBS 1X + MgCl₂ (12.5 mM) and incubated for 10 min with DNA origami samples in PBS 1X + MgCl₂ (12.5 mM) (DNA origami concentration = 50 pM). Next, chambers were washed (5×) with PBS 1X + MgCl₂ (12.5 mM) and filled with imaging buffer (TAE 1x –40 mM Tris, 20 mM acetic acid, 1 mM EDTA–, 2 mM Trolox/Troloxquinone, 12.5 mM MgCl₂, 3% glycerol, 1.25 mM KCl, 1% (w/v) D-(+)-glucose, 0.6 mg/mL glucose oxidase –Sigma-Aldrich– and 50 µg/mL catalase –Sigma-Aldrich–).

DNA origami structure design

Staples used to fold the origami are shown in Table S1.

RASTMIN setup

The RASTMIN technique was implemented in a custom-built confocal microscope with a beam scanner based on galvanometric mirrors and a piezoelectric stage for sample positioning (a complete description of the setup can be found in¹⁰). Excitation is performed using a linearly polarized pulsed laser at 642 nm (200 ps pulse width, PicoQuant LDH-P-C-640B) operating at 40 MHz repetition rate. A Glan-Thomson polarizer (Thorlabs) and a half-wave plate (AQWP05M-600, Thorlabs) were used to control the polarization as the beam is directed towards the spatial light modulator (SLM-100, Santeq) programmed to generate the donut-

shaped focus. Circular polarization was subsequently adjusted using a half-wave plate and a quarter-wave plate. The SLM was controlled using open-source Python code developed in ²³. The beam was directed to the scanning system, which consists of two lenses, two orthogonal galvanometric mirrors (Cambridge Technology, 6215H) (horizontal, x, and vertical, y), and one concave mirror. Driving voltages for the galvanometric mirrors were supplied by a linear-regulated power supply (Peaktech 6060) controlled by a DAQ board (National Instruments PCIe-6353), which was interfaced with the Inspector software. Light was collimated by the microscope tube lens and focused onto the sample with a 1.4 NA oil-immersion objective (Leica HCX PL APO 100×/1.40-0.70 Oil CS). The sample was mounted on a piezoelectric nanopositioning stage (Thorlabs NanoMax MAX311D/M with BPC303 controller).

Fluorescence emission was collected through the same scanning system, passed through a long-pass dichroic mirror (FF649-Di01-25x36, Semrock), an emission band-pass filter (ET700/75m, Chroma) and was focused onto an avalanche photodiode detector (SPCM-AQR-13, PerkinElmer Optoelectronics). Two notch filters (ZET647NF, Chroma and HQ655LP, Chroma) were placed in the emission path to block back-reflections of the excitation laser.

An active stabilization system was added as described in ¹⁹. Briefly, an 830nm IR laser (Lambda mini EVO, rgb-lasersystems) was combined with the excitation/fluorescence path using a dichroic mirror (T790SPXRXT, Chroma). The Z-focus and XY-stabilization paths were separated by a 50:50 polarizing beam splitter (CCM1-PBS251/M, Thorlabs). Both excitation paths were recombined by using a polarizing beam splitter (10FC16PB.5, Newport). A pellicle beam splitter (BP145B1, Thorlabs) separated the excitation light from scattered/reflected light, which was detected on a CMOS camera (UI-3060CP-M-GL Rev 2, IDS). An aperture in the detection path blocked off-axis reflected wide-field illumination.

RASTMIN measurement and MLE analysis

RASTMIN localizations were obtained as previously described in ¹⁰. The experimentally measured PSF was used as input for MLE to determine emitter positions. PSF reference images were acquired by averaging 16 images of a single fluorescent bead (*pixel size* = 10 nm, scanned area = $1.8 \times 1.8 \mu\text{m}^2$) with active stabilization enabled. The background signal required for the *SBR* calculation was determined by analyzing the photon counts detected after photobleaching of single molecules.

Localization precision measurement

With excitation power of 32 μW at the back focal plane (BFP) of the objective, fluorescence traces were acquired at different dwell times in order to achieve total detected photon counts of $N \approx 300, 600, 1000$ and 3000 photons. The *SBR* under these conditions was 5.7 ± 1.2 . The

reported localization precisions were calculated as the average standard deviation of consecutive localizations in both x and y directions. Emitter positions were estimated using three different approaches: (i) MLE, (ii) a fitting procedure using a donut-shaped excitation profile, and (iii) a fitting procedure using an anisotropic donut model that accounts for asymmetries in the excitation beam. Each data point in Figure 3B represents the mean precision from three independent single-molecule measurements. Error bars indicate the standard deviation of the precision (y-axis) and the number of photons (x-axis). The theoretical CRLB was calculated as the average CRLB over a $10 \times 10 \text{ nm}^2$ central region of the FOV.

Displacement experiment

Single ATTO 647N molecules were measured while their position was shifted by moving the piezoelectric stage in the lateral directions. Four discrete positions defining the vertices of an 11 nm square were sequentially imposed. Excitation power at the BFP was 32 μW . A total of 200 frames were acquired with an integration time of 216 ms per frame. Each frame consisted of a 6×6 pixels window covering a $100 \text{ nm} \times 100 \text{ nm}$ FOV. The emitter was maintained at each corner of the square for 50 ms. Under these conditions, the total detected photon counts per frame was 1868 and the *SBR* was 3.76. Emitter positions were estimated using the three methods described above.

MINFLUX simulation and estimator validation using SimuFLUX

To evaluate the performance of the calibration-free estimator under conditions representative of a commercial MINFLUX instrument, we modified the SimuFLUX simulation framework¹⁷ to incorporate our donut-fitting estimator as an alternative to its default localization algorithm. The modified codebase is available at https://github.com/azelcer/SimuFLUX/tree/use_fit_estimator. Briefly, the fitting-based estimator was implemented as a new function in `python/estimators/est_fit.py` and exposed through the estimator's module via `python/estimators/__init__.py`. The simulation pipeline (`python/simulators/sim_sequencefile_abberior.py`) was modified to import this estimator, which was then used in place of the default linearized least-squares estimator (`est_donutLSQ1_2D`) within `python/tools/get_abberior_pattern.py`. The modified pipeline was validated on SimuFLUX's example nuclear pore complex imaging experiment (`example6_Fluorophore_Collections`, adapted from the original example distributed with SimuFLUX), using the unmodified experiment configuration file (`python/examples/Imaging_2D.json`). Localization outputs from both the default and calibration-free estimators were recorded for subsequent rendering and analysis.

Gaussian rendering and resolution estimation

Super-resolved images were generated from the localization lists using Gaussian rendering, in which each localization was represented as a 2D Gaussian of fixed width ($\sigma = 2$ nm) centered on its estimated coordinates, evaluated on a grid with 1 nm pixel size. Spatial resolution was estimated by Fourier ring correlation (FRC) analysis²¹, computed independently on the renders obtained from the default and calibration-free estimators.

Data availability

Data is available from the first author upon request.

Acknowledgements

Giovanni Ferrari and Philip Tinnefeld are acknowledged for providing the DNA origami structures used in this work. Sebastián Giusti and Facundo Rouquaud are acknowledged for their help with sample preparation and measurements. F.D.S. acknowledges the support of the Max Planck Society. F.D.S. and A.M.S. acknowledge the Alexander von Humboldt Foundation. This work has been funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaria de Innovación, Ciencia y Tecnología (Red Federal de Microscopía de Super-resolución), and Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), projects PICT-2017-0870 (F.D.S.), and PICT-2021-01216 (F.D.S.).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

F.D.S. supervised the project. F.E., A.M.S and L.F.L. prepared samples and performed experiments. L.F.L. developed and wrote all the code for the simulations and analyzed the data. A.Z. adapted the SimuFLUX code and analyzed data. L.F.L. and F.D.S. wrote the manuscript with input from all the authors.

References

1. Hell, S. W. Nanoscopy with Focused Light (Nobel Lecture). *Angew. Chem. Int. Ed.* **54**, 8054–8066 (2015).
2. Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793–796 (2006).
3. Jungmann, R. *et al.* Single-Molecule Kinetics and Super-Resolution Microscopy by Fluorescence Imaging of Transient Binding on DNA Origami. *Nano Lett.* **10**, 4756–4761 (2010).
4. Thompson, R. E., Larson, D. R. & Webb, W. W. Precise Nanometer Localization Analysis for Individual Fluorescent Probes. *Biophys. J.* **82**, 2775–2783 (2002).
5. Ober, R. J., Ram, S. & Ward, E. S. Localization Accuracy in Single-Molecule Microscopy. *Biophys. J.* **86**, 1185–1200 (2004).
6. Mortensen, K. I., Churchman, L. S., Spudich, J. A. & Flyvbjerg, H. Optimized localization analysis for single-molecule tracking and super-resolution microscopy. *Nat. Methods* **7**, 377–381 (2010).
7. Balzarotti, F. *et al.* Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science* **355**, 606–612 (2017).
8. Gwosch, K. C. *et al.* MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells. *Nat. Methods* **17**, 217–224 (2020).
9. Masullo, L. A. *et al.* Pulsed Interleaved MINFLUX. *Nano Lett.* **21**, 840–846 (2021).
10. Masullo, L. A. *et al.* An alternative to MINFLUX that enables nanometer resolution in a confocal microscope. *Light Sci. Appl.* **11**, 199 (2022).
11. Masullo, L. A. & Stefani, F. D. Multiphoton single-molecule localization by sequential excitation with light minima. *Light Sci. Appl.* **11**, 70 (2022).
12. Smith, C. S., Joseph, N., Rieger, B. & Lidke, K. A. Fast, single-molecule localization that achieves theoretically minimum uncertainty. *Nat. Methods* **7**, 373–375 (2010).

13. Moosmayer, T. *et al.* MINFLUX fluorescence nanoscopy in biological tissue. *Proc. Natl. Acad. Sci.* **121**, e2422020121 (2024).
14. Deguchi, T. *et al.* Direct observation of motor protein stepping in living cells using MINFLUX. *Science* **379**, 1010–1015 (2023).
15. Wirth, J. O. *et al.* MINFLUX dissects the unimpeded walking of kinesin-1. *Science* **379**, 1004–1010 (2023).
16. Masullo, L. A., Lopez, L. F. & Stefani, F. D. A common framework for single-molecule localization using sequential structured illumination. *Biophys. Rep.* **2**, 100036 (2022).
17. Marin, Z. & Ries, J. Evaluating MINFLUX experimental performance in silico. *Nat. Commun.* **17**, 246 (2025).
18. Lopez, L. F. *et al.* Optimization and characterization of toroidal foci for super-resolution fluorescence microscopy: tutorial. *J. Opt. Soc. Am. B* **40**, C103 (2023).
19. Edorna, F. *et al.* Open-source sub-nanometer stabilization system for super-resolution fluorescence microscopy. *Light Sci. Appl.* **14**, 385 (2025).
20. Thevathasan, J. V. *et al.* Nuclear pores as versatile reference standards for quantitative superresolution microscopy. *Nat. Methods* **16**, 1045–1053 (2019).
21. Banterle, N., Bui, K. H., Lemke, E. A. & Beck, M. Fourier ring correlation as a resolution criterion for super-resolution microscopy. *J. Struct. Biol.* **183**, 363–367 (2013).
22. Cole, F. *et al.* Super-Resolved FRET and Co-Tracking in pMINFLUX. *Nat. Photonics* **18**, 478–484 (2024).
23. Antonello, J., Barbotin, A., Chong, E. Z., Rittscher, J. & Booth, M. J. Multi-scale sensorless adaptive optics: application to stimulated emission depletion microscopy. *Opt. Express* **28**, 16749 (2020).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [LopezetalSI.docx](#)