Supplementary Figure 1

Impact of signal photon count on the localization precision of SIMFLUX in comparison to standard SMLM for different background levels.

(a-b) Average lateral localization precision of SMLM and SIMFLUX fits using a Gaussian PSF model on ground truth data simulated with (a) Gaussian PSF (spot width $\sigma_{\text{PSF}} = \lambda/4NA$) and (b) vectorial PSF (see Methods), as a function of signal photon count for different background levels. The performance (full, coloured lines) is at the CRLB (black dashed lines and data symbols) except for SIMFLUX Gaussian PSF fitting on vectorial PSF ground truth simulations with very high signal-to-background-ratio (SBR), indicating a sensitivity to PSF model mismatch there. (c-d) Improvement factor of SIMFLUX localization precision over SMLM localization precision ($= \Delta x_{\text{SMLM}}/\Delta x_{\text{SIMFLUX}}$) for fitting with a Gaussian PSF model on ground truth data simulated with (c) Gaussian PSF and (d) vectorial PSF. Gaussian PSF fitting works reliably for realistic signal photon counts (up to about $10^4$), with a need for more sophisticated PSF models only arising for extremely bright emitters. Simulation parameters as described in Methods.
Impact of signal photon count on the photon count estimation of SIMFLUX in comparison to standard SMLM.

(a-b) Relative error of the estimated signal photon count of SMLM and SIMFLUX fitting with a Gaussian PSF on ground truth simulated with (a) Gaussian (spot width $\sigma_{PSF} = \lambda/4NA$) and (b) vectorial PSFs (see Methods) as a function of signal photon count for different background levels. showing a perfect fit for Gaussian PSF fitting on Gaussian PSFs and a $\sim30\%$ underestimation for a correct PSF model. (c-d) Similarly, relative error of the estimated background photons per pixel, indicating an overestimation for high signal-to-background ratio. The erroneous signal photon count estimation has an impact of the localization precision of SIMFLUX at high SBR, as shown in Supplementary Figure 1. Simulation parameters as in Methods.
Impact of background photon count on localization precision of SIMFLUX in comparison to standard SMLM.

(a) Average lateral localization precision of SMLM and SIMFLUX fitting with a Gaussian PSF model on vectorial PSF generated ground truth data as a function of background photons per pixel for three modulation depths. The performance is at the CRLB in the range of realistic background levels for the considered modulation depths. (b) Improvement of SIMFLUX over SMLM localization precision, showing a small impact of background on the improvement factor. The same relative impact of background on precision as in standard SMLM implies that SIMFLUX can be used under the same experimental conditions as SMLM.
Supplementary Figure 4

Assessment of illumination pattern modulation estimation on localization precision in SIMFLUX.

(a) Average lateral localization precision in SIMFLUX as a function of an (incorrectly) assumed modulation depth in the SIMFLUX fit routine for different actual values of the modulation. For comparison the localization precision for SMLM on the summed simulation frames is plotted. Spots are simulated with 6000 signal photons and 30 background photons per pixel. (b) Improvement in SIMFLUX localization precision over SMLM localization precision as a function of an assumed modulation depth in the SIMFLUX fit routine for different actual values of the modulation, indicating that the modulation depth must be above about 0.9 for a ~2x improvement and must be known with a precision of about 0.05 for optimum fit results from comparing the green and black lines around 0.9.
Supplementary Figure 5

Impact of illumination pattern phase errors on localization precision and bias of SIMFLUX.

(a) Average lateral localization bias of SIMFLUX and localization precision of SIMFLUX over standard SMLM for three modulation depths as a function of the standard deviation of random illumination pattern phase jitter, modelled by a Gaussian distribution. (b) Similarly; for a constant phase shift due to a potential bias in the phase estimation. The plots indicate a tolerable phase jitter of about 2 deg.
Impact of emitter position in the global phase pattern on localization precision in SIMFLUX.

Simulated emitters with ground truth (a) x-positions fixed in the excitation pattern and (b) y-positions randomized in the excitation pattern over half the pitch. (a-b) SIMFLUX localization precision for different modulation depths as a function emitter position in the excitation pattern; the performance is at the CRLB. (c-d) Improvement of SIMFLUX over SMLM localization precision. (e-f) Normalized excitation patterns for perfect modulation in the (e) x-direction in which the x-coordinates are fixed and (f) y-direction where the y-coordinates are randomly distributed. The plots show the position dependent localization precision of SIMFLUX within the global phase pattern and the average localization precision. The best precision is achieved for an emitter position at the center of the illumination pattern maximum and worst for emitters located in a minimum. This is in line with theory (Supplementary Note and Supplementary Fig. 7), and deviates from MINFLUX because of the imperfect modulation depth.
Supplementary Figure 7

Impact of modulation depth and global phase on zero-background CRLB of the localization precision.

(a) The improvement in localization precision of SIMFLUX over standard SMLM for different numbers of phase steps $K$ and global phase of the molecule (measure for the position of the molecule from the intensity minimum of one of the patterns), as a function of modulation depth $m$, as well as the global phase averaged improvement factor. (b) The improvement in localization precision of SIMFLUX over standard SMLM for different numbers of phase steps $K$ and modulation as a function of global phase. The plots indicate a steep dependence on $m$ close to $m = 1$, in particular for the global phase where one of the images is acquired when the molecule is at the illumination pattern minimum. This agrees with the numerical simulations shown in Supplementary Fig. 6. The performance is worst for these cases in case the modulation is imperfect. For a larger number of phase steps, the variations in CRLB as a function of global phase strongly reduce, making the method more robust. In the computations we take a pitch to spot width ratio $p/\sigma = 2$. 
Supplementary Figure 8

ROI instance and illumination pattern retrieval.

(a) Single-molecule image (11×11 pixels of size 65 nm) over 6 subsequent frames, with estimated signal photon counts, showing the frame-to-frame variation in emission intensity caused by the shifting and rotating illumination pattern. (b) Retrieved illumination pattern with position of the molecule with respect to the pattern indicated. (c) Expected and actual signal photon count, showing a good match, within the margins of shot noise induced variations. (d) Fit of the sinusoidal illumination pattern through the entire set of localizations. The point clouds show the estimated ratio of signal photon count to total photon count ("normalized intensity") over the $K = 3$ phase steps per pattern orientation as a function of $x$ and $y$-position mapped into a single period of the illumination pattern. The pattern phases are estimated with a precision equal to 0.64, 0.59, 0.68, 0.24, 0.55, and 0.41 deg (order pertaining to patterns as shown in second row), which we determine by splitting the entire dataset in 10 bins, repeating the phase estimation for the 10 bins, and computing the standard deviation over the estimated phases (normalized by $\sqrt{10}$).
Supplementary Figure 9

Precision and CRLB as a function of global phase.

(a) Measured precision in $x$ and $y$ for 424 analysed clusters of localizations for the 80 nm nanoruler dataset of Fig. 2, in comparison to the average CRLB over the localizations within each cluster. The clusters correspond to the binding sites of the nanorulers, the precision is quantified by the standard deviation of the set of localizations within each cluster. The total set of localizations has a median emitter intensity of 1175 photons, cumulative over the 6 frames, and a background of 10.7 photons/pixel, cumulative over the 6 frames. (b) Measured precision and CRLB as a function of the $x$-coordinate, where the $x$-values are mapped into a single period of the illumination pattern (the "global phase"). The median of the distribution of precision values is 4.8 nm for SIMFLUX and 7.3 nm for conventional SMLM, indicating an improvement factor of 1.5. The performance is not on par with the CRLB, probably due to a residual drift of around 4 nm over the full dataset. (c,d) Same as (a,b), for a simulated full-FOV dataset (signal photon count 1291, background/pixel 8.7), indicating a precision improvement factor of 2.2, and a performance on par with the CRLB.
Supplementary Figure 10

Precision and CRLB for SIMFLUX on 80 nm nanorulers with K=4 phase steps.

(a) Measured precision in x and y for 279 analysed clusters of localizations, in comparison to the average CRLB over the localizations within each cluster. (b) Measured precision and CRLB as a function of the x-coordinate, where the x-values are mapped into a single period of the illumination pattern (the "global phase"). The total set of localizations has a median emitter intensity of 1551 photons, cumulative over the 8 frames, and a background of 14.5 photons/pixel, cumulative over the 8 frames. (c,d) Distribution of precision values, with median 4.1 nm for SIMFLUX and 6.6 nm for conventional SMLM (average over x and y), indicating an improvement factor of 1.5. (e-h) Histograms of the total set of localizations, with average FWHM equal to 16.0 nm (SMLM) and 9.9 nm (SIMFLUX), indicating an improvement factor of 1.6. Comparison to the similar plots for K=3 phase steps in Supplementary Fig. 9 indicates a comparable precision and a somewhat larger improvement factor. The data was acquired at 70 fps, the modulation error filter was set to 0.012, and the spot detection threshold at 6. No repeated experiments were done with K = 4 phase steps.
Supplementary Figure 11

Additional DNA-origami grid results.

(a) Conventional SMLM and (b) SIMFLUX images of selected instances of the 20 nm DNA-origami grid structures. The SMLM and SIMFLUX images are based on the same image acquisitions. Imperfect labelling prevents all 4×3 binding sites to be visible. The grid structure is not resolved in SMLM, but clearly distinguishable in SIMFLUX. Two independent imaging experiments were done with similar outcome.
Supplementary Figure 12

Analysis of intensity variations during the fluorophore on-time for PAINT and STORM imaging.

The analysis is based on measurements in which the excitation patterns were not shifted. Spots were selected that are on for at least 10 frames, have a fitted spot intensity of at least 200 photons in each frame, and have an average spot intensity between 500 and 2000 photons (PAINT) or between 300 and 800 photons (STORM), over all on-frames. The PSF sigma was fitted on the sum of all frames of the emitter, and spots with a PSF sigma < 1.7 or > 2 pixels were filtered out, which also filters out any ROIs with overlapping emitters. (a) Examples of the first 10 frames of 6 filtered emitters for PAINT imaging. (b) To quantify variance in spot intensity on different timescales, we compute the 2-point unbiased variances of the spot intensities at frame $t$ and $t+T$, and plot the means of these\

\[
\frac{1}{L-1} \sum_{t=1}^{L-1} (N_t - N_{t+T})^2 / 2
\]

where $L$ is the number of frames for a spot, and $N_t$ the spot intensity at time $t$ in the histogram ($n = 3586$, dots indicate the mean). The results for PAINT indicate an error level that is ~27% above the expected CRLB, and increases slightly with time separation $T$ (by less than 3%). (c) The CRLB of estimating spot intensities for a background noise of 8 photons/pixel (matching that of the DNA paint measurement), showing a CRLB that is not simply $\sqrt{N}$, but substantially higher. This is related to the photon count underestimation of Gaussian PSF fitting (the estimate $N < N_{true}$ while the actual shot noise is $\sim \sqrt{N_{true}}$). This behaviour is known and explained before. (d) Examples of the first 10 frames of 6 filtered emitters for STORM imaging. (e) Spot intensity variation as a function of duration $T$ for STORM. The results indicate an error level that is ~125% above the expected CRLB, and varies with time separation $T$ by less than 12% ($n = 4534$, dots indicate the mean). (f) Simulation of the impact of intrinsic intensity noise on the improvement factor of SIMFLUX over SMLM. No repeated experiments were done.
Supplementary Figure 13

Impact of global phase on zero-background CRLB of the localization precision for a reduced phase scan range.

(a) Improvement in localization precision of SIMFLUX over standard SMLM as a function of global phase for four different modulation values for a scan range $R = p/5$, with $p$ the pattern pitch. (b) Same for a scan range $R = p/25$. In the computations we take a pitch to spot width ratio $p/\sigma = 2$. The plots show a huge improvement factor when the molecule is placed within the range of positions where the illumination pattern minimum is scanned. Interestingly, close to the $K$ illumination pattern minima the improvement factor experiences a dip. This is in line with the performance for the full scan range $R = p$, shown in Supplementary Figs. 6 and 7.
**Supplementary Figure 14** | SIMFLUX setup. Laser 640 nm, F1 excitation filter, L1 fiber coupling lens, SMF polarization maintaining single mode fiber, L2 fiber collimation lens, HWP zero order half wave plate 633 nm, QWP zero order quarter waveplate 633 nm, LP glan-laser linear polarizer, PC Pockels cell, M1-4 aluminium steering mirrors, PBS polarizing beam splitter, G1,2 binary phase gratings mounted on piezo stages, L3 75 mm relay lens, SF spatial filter, L4 350 mm relay lens, L5 180 mm relay lens, Objective 1.49 NA TIRF, XYZ Piezo Stage 100x100x100 nm travel range piezo stage, DM dichroic long pass mirror, F2 emission filter, TL tube lens, Camera sCMOS Hamamatsu Orca Flash 4.0 V2.
Supplementary Figure 15 | Modulation estimation. (a) Cropped image of a bead in a 10×10 pixel ROI at three phases that roughly correspond to the frame number on the x axis of the x modulation plot. (b,c) Measured brightness of a 20 nm bead as a function of camera frame where for each frame the phase grating is shifted by 40 nm, out of a 8.496 µm period, in the grating plane with the piezos. ROIs of size 11×11 pixels were automatically segmented from a 26 µm field of view, a background that was slowly changing due to photobleaching was fit with a linear decay curve and subtracted, and their summed ADU (minus the background) was fit with a sinusoidal curve with an $R^2 > 0.98$ for both directions. (d,e) Estimated modulation contrast values from 184 ROIs over the 26 µm field of view. Outliers at low contrast values (10-20% with $m < 0.75$) are attributed to regions with multiple beads or to false segmentations giving largely noisy regions. No repeated experiments were done.
Supplementary Figure 16 | Pattern pitch calibration. (a) 600x600 pixel cropped region of interest from localized high-density fluorophores illuminated with a periodic pattern, and Fourier transform of the image for the x-oriented pattern. (b) Same for the y-oriented pattern. The estimated pitch is displayed in the image, and agrees well with the expected value 219.9 nm (see Methods). No repeated experiments were done.
Supplementary Figure 17 | DNA origami structures. (a) 2×2 square with 40 nm spacing between binding sites. (b) 4×3 grid with 20 nm spacing. Not all strands/binding sites are present in the image, however, due to a limited incorporation efficiency.
Supplementary Figure 18 | Timing chart. DT digital trigger, GE global exposure, P1, P2 piezos 1,2, PC Pockels cell. The digital trigger for the acquisition (DT) is a digital TTL generator that causes a global exposure (GE) event to occur on the camera. The timings between the DT trigger (10 ms and 14 ms can be adjusted). GE is high when all pixels experience the same amount of exposure. Only acquiring images and illuminating the sample during the global exposure ensures that there is an even flux of photons across the field of view and that there is no cross-talk between our two imaging arms. The falling edge of the GE sequence triggers an Arduino to cycle through a three bit digital output that controls the two translation piezos (P1 and P2) and the Pockels cell (PC). The piezoelectric stages P1 and P2 are set to translate a by a user defined amount every other DT high trigger in between global exposure events, ensuring that the piezo gratings are not moving during their acquisition period. Each piezo mounted grating moves while the opposite grating is illuminated and imaged, thus maximizing imaging speed. The Pockels cell switches between the s and p beam paths by inducing a half wave voltage every DT high trigger event. To ensure that illumination from the s polarized arm does not appear in images from the p polarized arm, and vice versa, the laser was pulsed to only illuminate during the camera global exposure.
Supplementary Figure 19 | Simulation of SIMFLUX images for a full field of view (FOV). (a) SIMFLUX reconstruction of a simulated filamentous, microtubule-like structure (size FOV 4.2 µm). (b) SMLM and SIMFLUX reconstruction of a zoom-in on a dense area indicated as a turquoise box in (a). (c) Histogram of lateral localization errors in (a) for SIMFLUX and SMLM, resulting in a standard deviation of 8.3 nm and 4.1 nm, respectively. (d) SIMFLUX reconstruction of line-pair objects with decreasing separation distance. (e) Histograms of projected localizations along four different line-pair objects in (d). For well separated objects the estimated line FWHM is 2.6 nm (true), 10.7 nm (SMLM), 5.6 nm (SIMFLUX). (f) Line pair contrast ratio= 1 − valley / max(peak), showing better resolution of SIMFLUX, and (g) error in the estimated peak distance of SMLM and SIMFLUX as a function of the separation distance of the two line objects, showing a near zero bias. (h) Fraction of false negative and false positives rates for SIMFLUX localizations as a function of the modulation filter threshold. The reconstructions in (a) and (d) are generated with a modulation filter threshold of 0.03.
Supplementary Figure 20 | Data processing pipeline for pattern estimation and SIMFLUX localization. **(a)** The raw frames from the camera are converted to photon counts, and summed in blocks of 6 frames. The conventional localization microscopy pipeline is then used to perform spot detection and 2D Gaussian localization. **(b)** Filtering of the localizations. The signal intensity (photon count) on the first and the last frame of an on-event is estimated using a 2D Gaussian fit, during which the molecule $x, y$-position is kept fixed. Molecules for which the signal photon count on the first or last frame is lower than a predetermined threshold $N_{\text{min}}$ are rejected. **(c)** SIMFLUX localization. The 6 ROI frames are fitted to a model that uses both the illumination pattern information and the 2D spot center, resulting in a higher precision. **(d)** Initial estimates for the illumination pattern pitch and angles are found by locating peaks in the Fourier domain of the rendered standard SMLM image. The SMLM localizations are rendered into a super resolution image with a zoom factor of 6 compared to the camera pixel size. A 2D Fourier transform is then used with zero padding to zoom in on the peak. The peak is then fitted with a 1D quadratic fit in both $q_x$ and $q_y$-directions to calculate the subpixel peak position. **(e)** Using the estimated spatial frequency vectors $\tilde{q}_i$, intensities $N_i^{\text{pk}}$ and SMLM localizations $(x, y)$, the phases $\psi_{ik}$, modulation depths $m_i$ and relative intensities $\eta_i$ of the $x$ and $y$-pattern are computed using a least square fit as described in the methods section. **(f)** The pitch and phase offset is refined by doing a least squares line fit on the difference between the SMLM and SIMFLUX localizations in both modulation orientations. A nonzero slope of this line will correspond to an error in the pitch, and a nonzero offset will correspond to a bias shared between all phases of the modulation orientation.
Supplementary Figure 21 | Deviation between SIMFLUX and SMLM localizations over the FOV. (a,b) Difference between the SIMFLUX and conventional SMLM position estimate for 419 analysed clusters of localizations, projected on the x-oriented and y-oriented pattern directions. The data is averaged over all localizations within each cluster. (c,d) show the cross-sections of (a,b). The plots show no systematic error over the FOV, indicating that the iterative procedure of estimating the pitches and orientations of the illumination patterns converges to a uniform description of the sinusoidal illumination pattern. The rms value of the SIMFLUX-SMLM bias over the full dataset is 8.2 nm (x) and 7.5 nm (y), on the order of the localization uncertainty. The rms value over the median values of the clusters is 5.0 nm (x) and 4.2 nm (y).
**Supplementary Table.** Number of localization events used in statistical evaluation of localization precision.

| on-time [frames] | Fig. 2l SMLM | Fig. 2m SIMFLUX | Fig. 3f SMLM | Fig. 3f SIMFLUX | Fig. 3m SMLM | Fig. 3m SIMFLUX |
|------------------|--------------|----------------|--------------|----------------|--------------|----------------|
| 6                | 3181         | 6358           | 257182       | 176153         | 403842       | 337795         |
| 12               | 1330         | 2634           | 32003        | 11904          | 78047        | 94413          |
| 18               | 882          | 1551           | 5313         | 2218           | 41324        | 42336          |
| 24               | 584          | 984            | 1302         | 640            | 25897        | 22034          |
| 30               | 483          | 685            | 441          | 255            | 17890        | 12738          |
| 36               | 399          | 494            | 185          | 126            | 12785        | 7867           |
| 42               | 340          | 434            | 90           | 66             | 9481         | 4877           |
| 48               | 269          | 319            | 71           | 38             | 7292         | 3212           |
| 54               | 247          | 234            | 36           | 26             | 5748         | 2169           |
| 60               | 219          | 195            | 27           | 11             | 4448         | 1466           |
|                  |              |                |              |                |              |                |
| Number of unlinked localizations | 60239 | 51955 | 383863 | 236009 | 1555821 | 1003499 |
| Number of linked localizations | 9621 | 14849 | 296737 | 191500 | 628754 | 532722 |
Supplementary Note

1. Image formation model

We have a sequence of \(k = 1, 2, ..., K\) illuminations for orientations \(l = 1, 2, ..., L\) with a harmonic intensity profile \(P(\phi)\) as a function of pattern phase \(\phi\) that is displaced according to phase offsets \(\psi_{lk}\) such that:

\[
\sum_{l=1}^{L} \sum_{k=1}^{K} P(\phi_{lk}(\vec{r})) = 1
\]  

(1)

with the phase:

\[
\phi_{lk}(\vec{r}) = 2\pi \hat{q}_l \cdot \vec{r} - \psi_{lk}
\]

(2)

Here the spatial frequency vectors are:

\[
\hat{q}_l = (q_{lx}, q_{ly}) = \frac{1}{p} (\cos \beta_l, \sin \beta_l)
\]

(3)

where \(p\) is the pattern pitch, \(\beta_l = \pi l/L + \beta_0\), with \(\beta_0\) a global angular offset. The Point Spread Function (PSF) is \(h(\vec{r})\), and is assumed to be a Gaussian:

\[
h(\vec{r}) = \frac{1}{2\pi \sigma^2} \exp \left( -\frac{\vec{r}^2}{2\sigma^2} \right)
\]

(4)

with \(\sigma\) the spot width. The expected photon count on pixel \(j\) is:

\[
\mu_j^l = NP(\phi_{lk}(\vec{r}_0)) h(\vec{r}_j - \vec{r}_0)
\]

(5)

with \(\vec{r}_0\) the emitter position and \(N\) the total photon count. We will consider a sinusoidal illumination pattern:

\[
P(\phi) = \frac{1}{LK} (1 + m \cos \phi)
\]

(6)

with \(m\) the modulation. For a perfect modulation \(m = 1\), we find:

\[
P(\phi) = \frac{1}{LK} (1 + \cos \phi) = \frac{2}{LK} \cos((\phi/2)^2)
\]

(7)

The phases of the different illumination patterns are assumed to be equidistant. For a full \(2\pi\) phase scan this implies:

\[
\psi_{lk} = 2\pi (k - 1)/K + \chi_l
\]

(8)

where \(\chi_l\) is the phase offset of the patterns in direction \(\hat{q}_l\). We then find that:

\[
\phi_{lk}(\vec{r}_0) = \xi_l(\vec{r}_0) - 2\pi (k - 1)/K
\]

(9)

with the global phase \(\xi_l(\vec{r}_0)\) of the molecule with respect to the phase offset of the illumination patterns in direction \(\hat{q}_l\) defined by:

\[
\xi_l(\vec{r}_0) = 2\pi \hat{q}_l \cdot \vec{r}_0 - \chi_l
\]

(10)

Note that the use of equidistant phases over the full \(2\pi\) phase range ensures that the normalization condition Equation 1 is automatically satisfied. This condition requires that the number of phase steps \(K \geq 2\). It turns out, however, that for \(K = 2\) and global phase \(\xi_l(\vec{r}_0) = 0\) or \(\xi_l(\vec{r}_0) = \pi\) (molecule at maximum and minimum of pattern for the \(K = 2\) images) there is no improvement over conventional localization. For that reason \(K \geq 3\) is used in practice. In the following, we will use this model to derive the Fisher-matrix and Cramér-Rao Lower Bound (CRLB) for the estimation of the position of the molecule.

The image formation model must be amended in case a non-zero background and/or a non-zero pixel size is taken into account. The expected photon count on pixel \(j\) then becomes:

\[
\mu_j^l = NP(\phi_{lk}(\vec{r}_0)) E(\vec{r}_j - \vec{r}_0) + b/LK
\]

(11)
with $b$ the cumulative background over $L \times K$ frames. The background is assumed to be uniform over the Region Of Interest (ROI), and constant from frame-to-frame. The integration of the PSF over the pixel area gives the factor:

$$E(\vec{r} - \vec{r}_0) = \int_{\vec{r} \in A_j} d^2r \ h(\vec{r} - \vec{r}_0)$$

(12)

with $A_j$ the $a \times a$ sized area of pixel $j$. For the Gaussian PSF this results in:

$$E(\vec{r} - \vec{r}_0) = \frac{1}{4} \left[ \text{erf} \left( \frac{x_j - x_0 + a/2}{\sqrt{2}\sigma} \right) - \text{erf} \left( \frac{x_j - x_0 - a/2}{\sqrt{2}\sigma} \right) \right] \left[ \text{erf} \left( \frac{y_j - y_0 + a/2}{\sqrt{2}\sigma} \right) - \text{erf} \left( \frac{y_j - y_0 - a/2}{\sqrt{2}\sigma} \right) \right]$$

(13)

Simple analytical results for the CRLB cannot be obtained in this more general case, and we must resort to fully numerical simulations.

In the numerical analysis of our experimental results we also need to take into account that the modulation depth and the overall intensity of the illumination patterns can vary with the direction of the illumination pattern. In that case the illumination pattern for orientation $l$ changes to:

$$P_l(\varphi) = \frac{\eta_l}{K} (1 + m_l \cos \varphi)$$

(14)

where $\eta_l$ is the relative intensity factor normalized as $\sum_l \eta_l = 1$, nominally $\eta_l = 1/L$. The overall normalization condition changes to:

$$\sum_{l=1}^{L} \sum_{k=1}^{K} P_l(\varphi_{lk}(\vec{r})) = 1$$

(15)

and the expected photon count on pixel $j$ changes to:

$$\mu_{jk} = N P_l(\varphi_{lk}(\vec{r}_0)) E(\vec{r} - \vec{r}_0) + \eta_l b / K$$

(16)

where the relative intensity also affects the background (that should scale with illumination intensity too).

2. Log-likelihood and derivatives

The mixed shot-noise and readout noise log-likelihood is:

$$\log L = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \left( \left( n_{jk}^{\text{lk}} + \sigma_{\text{rn}}^2 \right) \log \left( \mu_{jk}^{\text{lk}} + \sigma_{\text{rn}}^2 \right) - \left( \mu_{jk}^{\text{lk}} + \sigma_{\text{rn}}^2 \right) - \Gamma \left( n_{jk}^{\text{lk}} + \sigma_{\text{rn}}^2 + 1 \right) \right)$$

(17)

with $n_{jk}^{\text{lk}}$ the actual detected photon count on pixel $j$ for image $lk$, where $\sigma_{\text{rn}}$ is the root mean square (rms) readout noise, where $\Gamma(x) = \int_0^{\infty} dt \ t^{x-1} \exp(-t)$ is the Gamma-function, and where the sum is over the $M$ pixels of the ROI. In the numerical implementation of the MLE problem the parameters $\theta = [x_0, y_0, N, b]$ are estimated. This is done using the Levenberg-Marquardt routine based on the derivatives of the log-likelihood:

$$\frac{\partial \log L}{\partial \theta_r} = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \left( \frac{n_{jk}^{\text{lk}} - \mu_{jk}^{\text{lk}}}{\mu_{jk}^{\text{lk}} + \sigma_{\text{rn}}^2} \right) \frac{\partial \mu_{jk}^{\text{lk}}}{\partial \theta_r}$$

(18)

The relevant derivatives of the expected photon count on pixel $j$ for image $lk$ are:

$$\frac{\partial \mu_{jk}^{\text{lk}}}{\partial x_0} = N P_l(\varphi_{lk}(\vec{r}_0)) \frac{\partial E(\vec{r} - \vec{r}_0)}{\partial x_0} + N \frac{\partial P_l(\varphi_{lk}(\vec{r}_0))}{\partial x_0} E(\vec{r} - \vec{r}_0)$$

(19a)
\[
\frac{\partial \mu_j^{lk}}{\partial y_0} = N P_l(\varphi_{lk}(\vec{r}_0)) \frac{\partial E(\vec{r}_j - \vec{r}_0)}{\partial y_0} + N \frac{\partial P_l(\varphi_{lk}(\vec{r}_0))}{\partial y_0} E(\vec{r}_j - \vec{r}_0)
\]
(19b)
\[
\frac{\partial \mu_j^{lk}}{\partial \sigma} = N P_l(\varphi_{lk}(\vec{r}_0)) \frac{\partial E(\vec{r}_j - \vec{r}_0)}{\partial \sigma}
\]
(19c)
\[
\frac{\partial \mu_j^{lk}}{\partial N} = P_l(\varphi_{lk}(\vec{r}_0)) E(\vec{r}_j - \vec{r}_0)
\]
(19d)
\[
\frac{\partial \mu_j^{lk}}{\partial b} = \eta_l
\]
(19e)

The derivatives of the Gaussian PSF term \(E(\vec{r}_j - \vec{r}_0)\) are as in Smith et al.\(^2\). The derivatives of the illumination pattern factor \(P(\varphi_{lk}(\vec{r}_0))\) are:
\[
\frac{\partial P_l(\varphi_{lk}(\vec{r}_0))}{\partial x_0} = -\frac{2\pi q_{lx} \eta_l m_l}{K} \sin(\varphi_{lk}(\vec{r}_0))
\]
(20)
and similarly for the derivative with respect to \(y_0\). The Fisher-matrix can be computed according to:
\[
F_{rs} = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \frac{\partial \mu_j^{lk}}{\partial x_r} \frac{\partial \mu_j^{lk}}{\partial x_s} + \sigma_{rn}^2 \frac{\partial \mu_j^{lk}}{\partial \theta_r} \frac{\partial \mu_j^{lk}}{\partial \theta_s}
\]
(21)

The CRLB follows from the diagonal of the inverse of the Fisher-matrix. The impact of the very small readout noise of sCMOS cameras (typically \(\sigma_{rn} \approx 1\) e) is neglected in the further analysis.

3. Analytical approximation Fisher-matrix and CRLB

In order to make a theoretical assessment of the expected gain in localization precision we will develop an analytical approximation to the Fisher-matrix and CRLB. This can be done for the case of zero background, if we ignore the non-zero pixel size, and if we neglect the finite support of the Region Of Interest (ROI). The relevant Fisher matrix elements can be written as:
\[
F_{x_0x_0} = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \mu_j^{lk} \left( \frac{\partial \log \mu_j^{lk}}{\partial x_0} \right)^2
\]
(22a)
\[
F_{x_0y_0} = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \mu_j^{lk} \left( \frac{\partial \log \mu_j^{lk}}{\partial x_0} \right) \left( \frac{\partial \log \mu_j^{lk}}{\partial y_0} \right)
\]
(22b)
\[
F_{y_0y_0} = \sum_{l=1}^{L} \sum_{k=1}^{K} \sum_{j=1}^{M} \mu_j^{lk} \left( \frac{\partial \log \mu_j^{lk}}{\partial y_0} \right)^2
\]
(22c)

For the case with zero background and ignoring the non-zero pixel size and the direction dependence of the illumination intensity and modulation we have:
\[
\frac{\partial \log \mu_j^{lk}}{\partial \sigma} = \frac{\vec{r}_j - \vec{r}_0}{\sigma} + \frac{1}{P(\varphi_{lk}(\vec{r}_0))} \frac{\partial P(\varphi_{lk}(\vec{r}_0))}{\partial \sigma}
\]
(23)

Approximating the summation over the pixels of the ROI with an integration over the entire 2D image plane results in:
\[
F_{x_0x_0} \approx \frac{N}{\sigma^2} + \frac{4\pi^2 N}{L} \sum_{l=1}^{L} q_{lx}^2 Q(\xi_l(\vec{r}_0))
\]
(24a)
\[
F_{x_0y_0} \approx \frac{4\pi^2 N}{L} \sum_{l=1}^{L} q_{lx} q_{ly} Q(\xi_l(\vec{r}_0))
\]
(24b)
\[ F_{y_0y_0} \approx \frac{N}{\sigma^2} + \frac{4\pi^2 N}{L} \sum_{l=1}^{L} q_{ly}^2 Q(\xi_l(\vec{r}_0)) \]  

Here the function \( Q(\xi_l(\vec{r}_0)) \) is defined by:

\[ Q(\xi_l(\vec{r}_0)) = \frac{m^2}{K} \sum_{k=1}^{K} \sin(\varphi_{lk}(\vec{r}_0))^2 \frac{1}{1 + m \cos(\varphi_{lk}(\vec{r}_0))} \]  

(25)

4. Localization precision with perfect modulation

In case of perfect modulation \( m = 1 \), the function \( Q(\xi_l(\vec{r}_0)) \) simplifies to:

\[ Q(\xi_l(\vec{r}_0)) = \frac{1}{K} \sum_{k=1}^{K} \sin(\varphi_{lk}(\vec{r}_0)/2)^2 = \frac{1}{2} \]  

(26)

and is thus independent of position \( \vec{r}_0 \) and orientation \( l \). For \( L \geq 2 \) (at least 2 orientations) we find:

\[ \sum_{l=1}^{L} q_{lx}^2 = \frac{1}{p^2} \sum_{l=1}^{L} \cos \beta_l^2 = \frac{L}{2p^2} \]  

(27a)

\[ \sum_{l=1}^{L} q_{lx}q_{ly} = \frac{1}{p^2} \sum_{l=1}^{L} \cos \beta_l \sin \beta_l = 0 \]  

(27b)

\[ \sum_{l=1}^{L} q_{ly}^2 = \frac{1}{p^2} \sum_{l=1}^{L} \sin \beta_l^2 = \frac{L}{2p^2} \]  

(27c)

independent of the overall angle offset \( \beta_0 \). Substituting Equations 26 and 27 in Equations 24 results in the two diagonal Fisher-matrix elements being non-zero and equal to:

\[ F_{x_0x_0} = F_{y_0y_0} = \frac{N}{\sigma^2} + \frac{2\pi^2 N}{p^2} \]  

(28)

This gives an isotropic localization uncertainty:

\[ \Delta x_0 = \Delta y_0 = \frac{\sigma}{\sqrt{N} \sqrt{1 + 2\pi^2 \sigma^2 / p^2}} \]  

(29)

which improves over SMLM with a factor of around 2 depending on the pattern pitch \( p \) in relation to the spot width \( \sigma \).

5. Localization precision with imperfect modulation

In case \( m < 1 \) the precision will become worse. Moreover, there will be a (slight) dependence on the global phase \( \xi_l(\vec{r}_0) \) defined in Equation 10. This will make the localization precision non-uniform and anisotropic to some degree.

In order to analyse these results we will assume, for the sake of simplicity, that the illumination patterns are oriented along the the \( x \)-axis and \( y \)-axis. Following the same steps as above we then find that:

\[ \Delta x_0 = \frac{\sigma}{\sqrt{N} \sqrt{1 + 2\pi^2 Q(2\pi x_0 / p - \chi_1) \sigma^2 / p^2}} \]  

(30a)

\[ \Delta y_0 = \frac{\sigma}{\sqrt{N} \sqrt{1 + 2\pi^2 Q(2\pi y_0 / p - \chi_2) \sigma^2 / p^2}} \]  

(30b)
The average behaviour can be deduced by replacing the summation over the $K$ phase steps in Equation 25 by an integral over all phases:

$$Q(\xi_{i}(\vec{r}_{0})) \approx \frac{m^2}{2\pi} \int_{0}^{2\pi} d\varphi \frac{\sin \varphi^2}{1 + m \cos \varphi} = \frac{m^2}{1 + \sqrt{1 - m^2}} \equiv F(m)$$

(31)

Supplementary Figure 7 shows the effect of the dependence of the localization precision on the global phase. The worst case happens when one of the phases $\varphi_{ik}(\vec{r}_{0}) = \pi$ (emitter at minimum of illumination pattern for one of the frames). In case of an ideal modulation contrast $m = 1$ this is a perfectly dark fringe and this helps significantly to decrease the CRLB. In case of a non-ideal modulation contrast $m < 1$ there is no added value. For example, for $m = 0.95$ and $K = 3$ phase steps can vary between 1.6 and 2.3, with an average of 2.1 (taking a pitch to spot width ratio $p/\sigma = 2$). These variations decrease when the number of phase steps is increased, making the method more robust. For example, for $m = 0.95$ and $K = 4$, the improvement factor varies between 1.8 and 2.2, for $m = 0.95$ and $K = 5$, the improvement factor merely varies between 1.9 and 2.2. In practice, these variations are further mitigated by the non-zero background.

6. **Localization precision with reduced scan range**

The current results can be generalized by changing the translation range of pattern shifting from the pattern pitch $p$ to a smaller range $R$, similar as in MINFLUX. So, we take pattern phases:

$$\psi_{ik} = 2\pi(k - 1)R/Kp + \chi_{i}$$

(32)

In order to enforce the normalisation condition Equation 1 (keeping the parameter $N$ the number of detected photons) we must normalize the patterns with a factor:

$$G(\xi_{i}(\vec{r}_{0})) = \frac{1}{K} \sum_{k=1}^{K} \left(1 + m \cos(\varphi_{ik}(\vec{r}_{0}))\right) = 1 + m' \cos \left(\xi_{i}(\vec{r}_{0}) - \frac{\pi(K - 1)R}{Kp}\right)$$

(33)

with

$$m' = \frac{m \sin(\pi R/p)}{K \sin(\pi R/Kp)}$$

(34)

This normalization factor reaches a minimum when:

$$\xi_{i}(\vec{r}_{0}) = \frac{2\pi p}{K} + \left(\frac{K - 1}{2K}\right) R$$

(35)

which implies that half way the scan the illumination pattern intensity minimum coincides with the molecule. The expected photon count on pixel $j$ is:

$$\mu_{jk}^{i} = N \frac{P(\varphi_{ik}(\vec{r}_{0}))}{G(\xi_{i}(\vec{r}_{0}))} h(\vec{r}_{j} - \vec{r}_{0})$$

(36)

Taking the same steps as in the previous derivation results in a function $Q(\xi_{i}(\vec{r}_{0}))$ that is rather involved:

$$Q(\xi_{i}(\vec{r}_{0})) = \frac{1}{1 + m' \cos \left(\xi_{i}(\vec{r}_{0}) - \frac{\pi(K - 1)R}{Kp}\right)} \times$$

$$\frac{1}{K} \sum_{k=1}^{K} (1 + m \cos(\varphi_{ik}(\vec{r}_{0}))) \left[\frac{m \sin(\varphi_{ik}(\vec{r}_{0}))}{1 + m \cos(\varphi_{ik}(\vec{r}_{0}))} - \frac{m' \sin \left(\xi_{i}(\vec{r}_{0}) - \frac{\pi(K - 1)R}{Kp}\right)}{1 + m' \cos \left(\xi_{i}(\vec{r}_{0}) - \frac{\pi(K - 1)R}{Kp}\right)}\right]^2$$

(37)

and a localization precision still given by Equations 23. For the limiting case $R \ll p$ and perfect modulation $m = 1$ the function $Q(\xi_{i}(\vec{r}_{0}))$ is sharply peaked around the value $\xi_{i}(\vec{r}_{0}) = \pi$, indicating
that a small scan range, centred around the intensity minimum, results in a small localization precision, as in MINFLUX. At points in the FOV close to the crossing points between the intensity minimum lines of the patterns oriented along the $x$-axis and $y$-axis there will be a large improvement in precision, given that a constant photon count $N$ per localization event can be achieved. Supplementary Figure 13 shows that for a perfect modulation (and zero background) an in principle unlimited improvement over SMLM can be achieved by reducing $R$, in agreement with Balzarotti et al.\textsuperscript{3}. For an imperfect modulation, however, this is not the case. The improvement factor can reach values up to about 10, and has dips for global phase values corresponding to the molecule being at the minimum of one of the illumination patterns, similar to the case of a full scan range.

It is mentioned that this type of precision improvement (for a reduced phase scan range) can only be achieved for STORM type of photo-switching, where the typical number of photons per on-event is molecule specific and intensity independent, as the lower intensity near the illumination pattern minimum will reduce the number of photons per unit time, but at the same time make the molecular on-time longer. The major drawback in this case is that the longer on-time makes the on-off ratio unfavourable, more molecules per unit area will be in the on-state at any given moment in time. For PAINT type of photo-switching the on-off transition is diffusion driven, implying that the gain in localization precision per detected photon is cancelled by a reduction in the number of detected photons per on-event.

7. Extension to 3D-localization

The reported experiments were done with a TIRF setup, a low background 2D imaging mode. Imaging deeper into a cellular sample would require a change to a HILO-type of illumination with an increased background. This will deteriorate the performance, but the localization precision relative to the zero background case is the same as for conventional SMLM (see Supplementary Figure 3). In this way 2D-slices of a 3D sample with around twofold improved in-plane localization precision can be generated.

Improvement in localization precision in 3D single-molecule localization can be realized in a number of ways. The imaging light path can be modified to incorporate the astigmatic\textsuperscript{4}, bifocal\textsuperscript{5}, multi-focal\textsuperscript{6}, double-helix\textsuperscript{7}, tetrapod\textsuperscript{8}, or any other method for 3D-localization that relies on modifying the imaging PSF. It can be expected that SIMFLUX is fully compatible with any of these methods, so that the lateral localization precision can still be improved compared to conventional SMLM, while keeping the axial localization precision at a comparable performance.

More exciting are extensions that offer the potential for improvement in localization precision in all spatial directions. This would require illumination patterns that not only depend on the lateral coordinates but also on the axial coordinate. The most straightforward way to do this is to alternate between periodic illumination patterns in the $x$, $y$, and $z$-directions. This results in a minimum of 9 distinct patterns, as at least three phase steps are needed per pattern orientation. The pitch of the interference pattern arising from counter propagating plane waves along the optical ($z$) axis is $\lambda / (2n)$, leading to a precision in the absence of background equal to:

\[
\Delta z_0 = \frac{\sqrt{3}\lambda}{4\pi n \sqrt{N}}
\]  

where the factor $\sqrt{3}$ occurs due to the division of photons over the three independent pattern directions. The lateral precision in the absence of background changes to:
\[ \Delta x_0 = \Delta y_0 = \frac{\sigma}{\sqrt{N} \sqrt{1 + 4\pi^2 \sigma^2 / 3p^2}} \]  

(39)

because of the same division of photons over the three independent pattern directions. An interesting choice would be to use only the illumination pattern in the \( z \)-direction and rely on standard camera based localization in the lateral directions. This would reduce the required number of images to just 3, and improve the axial precision to:

\[ \Delta z_0 = \frac{\lambda}{4\pi n \sqrt{N}} \]  

(40)

which is on par with interferometric axial localization in interferometric axial localization. In this way the axial localization precision can be improved over 3D techniques that rely on detecting spot shape, without compromising the lateral localization precision of standard 2D-localization. Of course, this comes at the expense of having to build and operate a 4\( \pi \)-setup.

Restricting the attention to more standard epi-illumination setups suggests the use of three-beam interference for creating a woodpile shaped illumination pattern, just as in 3D-SIM. The three beams travel along the optical axis and at angles \( \pm \alpha \) with the optical axis, and have a lateral pattern pitch \( p = \lambda_{ax} / n \sin \alpha \), and an axial pattern pitch \( p_{ax} = \lambda_{ax} / n(1 - \cos \alpha) \), with \( \lambda_{ax} \) the excitation wavelength and \( n \) the medium refractive index. The illumination pattern as a function of lateral pattern phase \( \varphi \) and axial pattern phase \( \theta \) is now:

\[ P(\varphi, \theta) = a[1 + m_1 \cos \theta \cos \varphi + m_2 \cos(2\varphi)] \]  

(41)

with the first and second order modulations \( m_1 \) and \( m_2 \), and where \( a \) is a normalization constant. The lateral pattern phase takes values \( \varphi_{lk}(\vec{r}) \) as a function of lateral position \( \vec{r} = (x, y) \) as defined in Equation 2 for \( k = 1, 2, ..., K \) lateral pattern phases and \( l = 1, 2, ..., L \) pattern orientations, and where the lateral spatial frequencies have magnitude \( |\vec{q}_l| = 1/p \). The axial pattern phase takes values:

\[ \theta_s(z) = 2\pi q_{\perp} z - \delta_s \]  

(42)

with the axial modulation spatial frequency \( q_{\perp} = 1/p_{ax} \), and where \( \delta_s \) is the axial phase offset. A normalization condition like Equation 1 requires that the number of lateral phase steps \( K \geq 3 \), but using at least 4 phase steps is likely more robust. With \( L = 2 \) orientations this seemingly gives a total of at least 8 patterns. What is not incorporated yet, however, is that the pattern must also be phase shifted in the axial direction, in order to create a dependence of photon count on the axial position of the molecule. Taking \( s = 1, 2, ..., S \) axial phase steps according to:

\[ \delta_s = 2\pi (s - 1)/S + \epsilon \]  

(43)

with \( \epsilon \) a constant phase offset, is sufficient for that purpose. The normalization condition Equation 1 now becomes:

\[ \sum_{s=1}^{S} \sum_{l=1}^{L} \sum_{k=1}^{K} P(\varphi_{lk}(\vec{r}), \theta_s(\vec{r})) = 1 \]  

(44)

and the normalization constant should then be \( a = 1/\(LKS\) \). The number of axial phase steps must be \( S \geq 3 \), as the axial dependence only contains a single harmonic. The requirements on lateral and axial phase steps implies a total \( L \times K \times S \) of at least 18 illumination patterns are needed during the time of a typical molecular on-event, an impractically large number. We speculate that the number of phase steps that is required can be substantially reduced by phase shifting in the lateral and axial direction simultaneously. The displacement of the illumination pattern would then be in a single diagonal direction compared to the lateral and axial directions of the woodpile shaped illumination pattern. This could be feasible with \( \sim \)kHz framerate cameras and illumination pattern generators. High camera frame rates are attainable for current sCMOS cameras if a limited set of lines is read out per
frame, ferroelectric LCOS devices could enable fast pattern switching. The multi-focus microscope has been shown to be compatible with single-molecule imaging, and the extension to 3D-SIM has already been demonstrated. This proven platform is possibly a promising route for exploring 3D SIMFLUX configurations in an epi-illumination setting.

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