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1 **Nanometric axial localization of single fluorescent molecules**
2 **with modulated excitation.**

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10
11 **Abstract**

12
13 Distance measurements are commonly performed by a phase detection based on a lock-in strategy. Super-
14 resolution fluorescence microscopy is still striving to perform axial localization but through entirely
15 different strategies. Here we show that an illumination modulation approach can achieve nanometric axial
16 localization precision without compromising on the acquisition time, emitter density or lateral localization
17 precision. The excitation pattern is obtained by shifting tilted interference fringes. The molecular
18 localizations are performed by measuring the relative phase between each fluorophore response and the
19 reference modulated excitation pattern. We designed a fast demodulation scheme compatible with the
20 short emission duration of single emitters. This modulated localization (ModLoc) microscopy offers a
21 typical axial localization precision of 6.8 nm over the entire field of view and the axial capture range.
22 Furthermore, the interfering pattern being robust to optical aberrations, a nearly uniform axial localization
23 precision enables imaging biological samples up to several micrometers in depth.

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27 Fluorescence microscopy is currently the most widespread technique for cell imaging. It has now achieved
28 the nanometer scale with the advent of super-resolution techniques such as single molecule localization
29 microscopy (SMLM)¹⁻⁴. However, axial localization is still an ongoing central issue⁵. The symmetry of the
30 microscopes and their optical optimization favor the transverse direction compared to the axial one.
31 Several strategies have been developed in SMLM based on point spread function (PSF) handling like multi-
32 focused techniques⁶⁻⁸, PSF engineering⁹⁻¹² or supercritical angle fluorescence¹³⁻¹⁵. Alternative techniques
33 retrieve axial localization based on the coherence of a single emitter fluorescence, like interferometric
34 PALM (iPALM)^{16,17}, 4Pi single marker switching nanoscopy (4Pi-SMSN)^{18,19} or Self-Interference (Selfi)²⁰. The
35 axial localization is often performed at the expense of the lateral one, reducing the working depth^{12,21} or
36 the field of view²⁰. Most techniques optimized in the focal plane quickly degrade with defocus. In addition,
37 their performances are hindered by the optical aberrations within the sample beyond a few micron depths
38 unless adaptive optics^{19,22} or more robust fluorescence self-interference strategies²⁰ are used.

39 In the case for coherent light, interferometry offers unmatched sensitivity for distance measurements by
40 detecting the relative phase between two electromagnetic wave²³. This phase is often obtained by
41 modulation using a standard lock-in detection. This strategy which has been used in coherent microscopy
42 configurations to obtain nanometric axial localization²⁴⁻²⁶, cannot be implemented directly for the non-
43 coherent fluorescence emission. However, localization by lock-in detection can be adapted to amplitude
44 modulation like in Light Detection and Ranging (LIDAR) or Time of Flight (TOF) cameras²⁷. Since the
45 response of the fluorophores is linear in intensity (providing sufficiently low excitation), it is possible to
46 retrieve the phase of the fluorescence signal emitted by a single emitter with a lock-in detection using
47 time-modulated structured illumination as a reference. This idea has been used for the transverse
48 direction in the early days of single emitter tracking to probe myosin V stepping mechanism²⁸ with gold
49 nanobeads²⁹. The illumination is obtained by a shifting interference pattern and the detection performed
50 by a fast mono-detector. Recently, this strategy has been revived to single molecule transverse localization
51 for SMLM^{30,31}. However, the lock-in detection using a slow camera is performed sequentially and excludes
52 a significant proportion of the emitters due to their limited emission time.

53 Here, we propose to extend the lock-in localization strategy to the longitudinal dimension using a tilted
 54 illumination pattern combined with a fast demodulation scheme. This modulation localization (ModLoc)
 55 being robust to aberrations and efficient in terms of photon budget is therefore particularly well adapted
 56 in the axial direction. It can operate with a standard single objective configuration. We show that the
 57 localization precision remains uniform (~ 6.8 nm) over the whole capture range and allows imaging up to
 58 several tens of microns within biological samples.

59 Principle

60 The illumination pattern is composed of tilted fringes produced by two interfering monochromatic plane
 61 waves with angular frequency ω and wavelength λ_{exc} (Fig. 1a). The axial localization is obtained by a
 62 combined analysis of the phase of the modulated fluorescent signal produced by the shifting structured
 63 illumination and of the lateral localization obtained by standard centroid fitting of the PSF. The illumination
 64 pattern in the xz -plane is fully characterized spatially by the angle θ between the fringes and the axial
 65 direction, and the fringe spatial period Λ_z resulting from the angle between the two interfering incident
 66 beams α (Fig. 1b). The fringes are shifted at the angular frequency Ω by dephasing the relative optical
 67 phases between the two excitation beams. The knowledge of the illumination pattern enables the
 68 localization of a fluorophore in the direction of the shifting fringes by measuring its temporal phase ϕ
 69 (modulo 2π , i.e. the fringe spatial period Λ_z). The axial localization z is given by

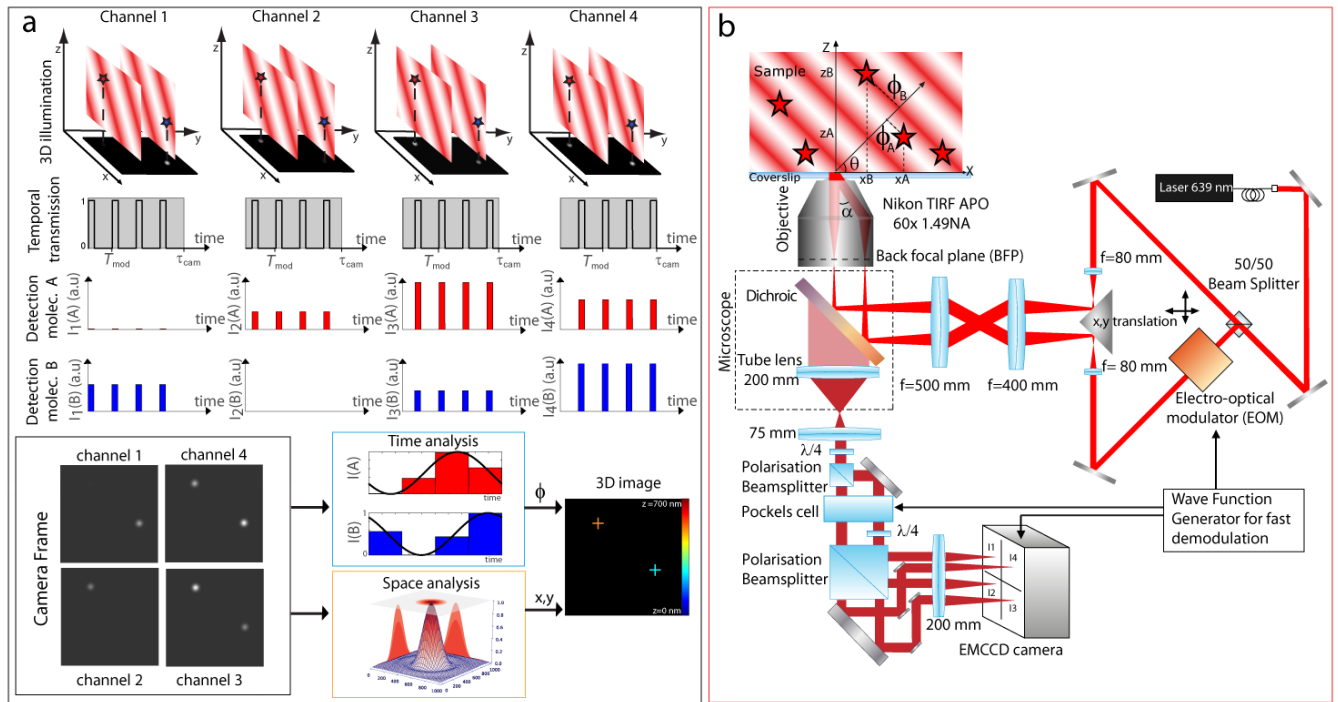
$$70 \quad z = \frac{\lambda_{\text{exc}} \phi}{4\pi n \sin\left(\frac{\alpha}{2}\right) \sin(\theta)} - \frac{x}{\tan(\theta)} \quad (\text{eq1})$$

71 with n the refractive index in the sample. The axial standard deviation σ_{ModLoc} can be written as

$$72 \quad \sigma_{\text{ModLoc}} = \frac{1}{\sin \theta \sqrt{N}} \sqrt{\frac{\lambda_{\text{exc}}^2}{16\pi^2 n^2 \sin^2\left(\frac{\alpha}{2}\right)} + \sigma_{\text{PSF}}^2 \cos^2 \theta} \quad (\text{eq2})$$

73 with N the number of detected photons and σ_{PSF} the PSF standard deviation (Supplementary Note 1).

74



75

Figure 1 | Modulated localization (ModLoc) principle and experimental implementation. **a**, Fast demodulation is based on 4 time channels: for each channel the xz tilted illumination pattern is in quadrature relative to one another. The 4 time channels have complementary transmission lasting a quarter of a modulation period T_{mod} , repeated during multiple modulation cycles within the total acquisition time τ_{cam} . Illustration of the detected intensities in the four different channels for two molecules (A and B) is represented. These 4 channels are acquired simultaneously on the camera. A centroid fitting of the PSF gives access to the lateral x, y localization for each molecule. The phase ϕ of their modulated emission is retrieved from the relative PSF intensity in each channel. The combination of the two information leads to a 3D image. **b**, Implementation of ModLoc microscopy: The tilted illumination pattern ($\lambda_{\text{exc}} = 639 \text{ nm}$) is obtained by the interference of two beams focused in the back focal plane of the 1.49 NA objective with tunable off-axis distance to control the interference period and the fringe angle θ in the sample. The time modulation frequency is performed by shifting the relative phase between the two excitation beams using an electro-optic modulator (EOM). The lock-in demodulation is performed thanks to a dedicated detection module based on polarization sorting: a $\lambda/4$ plate, followed by a polarization beamsplitter, a Pockels cell, a $\lambda/4$ plate in one of the path shift the polarization state to obtain 4 complementary channel after a last polarization beamsplitter. The initial fluorescence image is steered into a set of 4 detection channels with complementary time varying transmission performed by a Pockels cell and synchronized with the EOM. These 4 channels are imaged simultaneously on the EMCCD camera. The modulation/demodulation frequency, typically between 600 Hz to 1200 Hz, is chosen to be compatible with the short ON-time of the emitters typically averaging over 30 to 60 modulation cycles.

For the high Numerical Aperture (NA) objectives used in SMLM, the axial modulation period Λ_z is of the order of λ_{exc} . The ultimate limit for a single objective excitation configuration is given by $\theta = \alpha/2 = 45^\circ$ and results in a quasi-isoprecision of $\sigma_{\text{ModLoc}} \approx 1.1 \sigma_x$, σ_x being the standard deviation in the lateral direction x . In this configuration however, Λ_z being smaller than the objective capture range, unwrapping strategies are needed to extract the axial information (Supplementary Fig.1). In the following to avoid phase wrapping, Λ_z is set to obtain a unique solution between the measured phase and the axial emitter

100

101 position. This preserves a straightforward observation over the whole depth of focus but reduces the axial
 102 localization compared to the optimal configuration.

103 The modulation frequency must be compatible with the limited ON-time emission of the molecules. The
 104 emitters have an ON-time with a Poissonian statistic which depends on the illumination intensity. To fulfill
 105 time sparsity and localize most of the molecules in a single shot, τ_{ON} must satisfy $\tau_{\text{ON}} < \tau_{\text{cam}}$, τ_{cam} being
 106 the minimum exposure time of the camera to optimize the acquisition time. For example, we measured
 107 for our experiments $\tau_{\text{ON}} = 15$ ms for AF647 observed in (d)STORM (Supplementary Fig. 2). The
 108 modulation period $T_{\text{mod}} = 2\pi/\Omega$ must satisfy $T_{\text{mod}} \ll \tau_{\text{ON}}$ to retrieve the phase for the most of the
 109 emitters (Supplementary Fig. 3). Typically, frequencies satisfying an average molecular ON-time lasting at
 110 least a few tens of modulation cycles are optimal (Supplementary Fig. 4) which hindered the sequential
 111 acquisition strategy. Figure 1a shows the principle of the fast lock-in detection strategy which must be
 112 developed to perform the demodulation.

113 The lock-in detection is performed with 4 time channels with complementary transmissions lasting a
 114 quarter of a modulation period T_{mod} (Fig. 1a). Alternative transmission functions are possible, but they
 115 should be complementary to avoid any photon loss. Each channel is associated to an illumination pattern
 116 in quadrature relative to one another and imaged into a specific subarray of the camera. Thanks to sparsity,
 117 the fluorescence intensity I_q transmitted into channel q by each fluorophore after integration by the
 118 camera can be measured and the phase deduced by $\phi = \frac{1}{2\pi} \text{atan} \left(\frac{I_4 - I_2}{I_3 - I_1} \right)$. With the lateral position obtained
 119 from centroid fitting of the PSF, the axial position of the fluorophore can be deduced using eq (1).

120

121 **Experimental setup**

122 Figure 1b shows the experimental implementation of the ModLoc microscope (Methods and
 123 Supplementary Fig. 1-10 for details). The tilted illumination pattern is obtained by the interference of the
 124 two parallel beams focused in the back focal plane of the high NA objective. The 639 nm laser beam is
 125 coupled through a polarizing fiber and divided in two symmetrical beam paths. Their off-axis distance can

126 be adjusted using a knife-edge mirror mounted on an x,y translation or independently using mirrors placed
127 after (not shown in Fig.1b). The interference fringe pattern is shifted with an electro-optic modulator
128 (EOM) placed in one of the beams to tune their relative phase. The fast lock-in demodulation is performed
129 with a specific scheme based on polarization sorting. The initial fluorescence image is sorted into 4
130 detection channels with complementary time varying transmission synchronized with the excitation
131 modulation (Supplementary Note 2 and Supplementary Fig. 5). The initial image is first split in two
132 complementary orthogonal polarization channels. The modulated transmission is obtained using a Pockels
133 cell in each path and splitting them into two complementary channels using a polarizing cube. To shift a
134 modulation in quadrature, a quarter waveplate is introduced in one of the path after the Pockels cell.
135 Standard registration code is used to associate the four intensity values and positions (Supplementary
136 Fig.6). Typically, the modulation frequency is set between 600 Hz and 1200 Hz for an acquisition rate of
137 the camera of 20 Hz. 30 to 60 modulation cycles are observed for each frame, allowing the detection of
138 most emitters even with short ON-time. As for classical SMLM imaging, 10000 to 20000 images are typically
139 acquired to reconstruct the final super-resolved image. Standard Direct Cross Correlation³² algorithm are
140 applied to retrieve drifts in all directions (Methods).

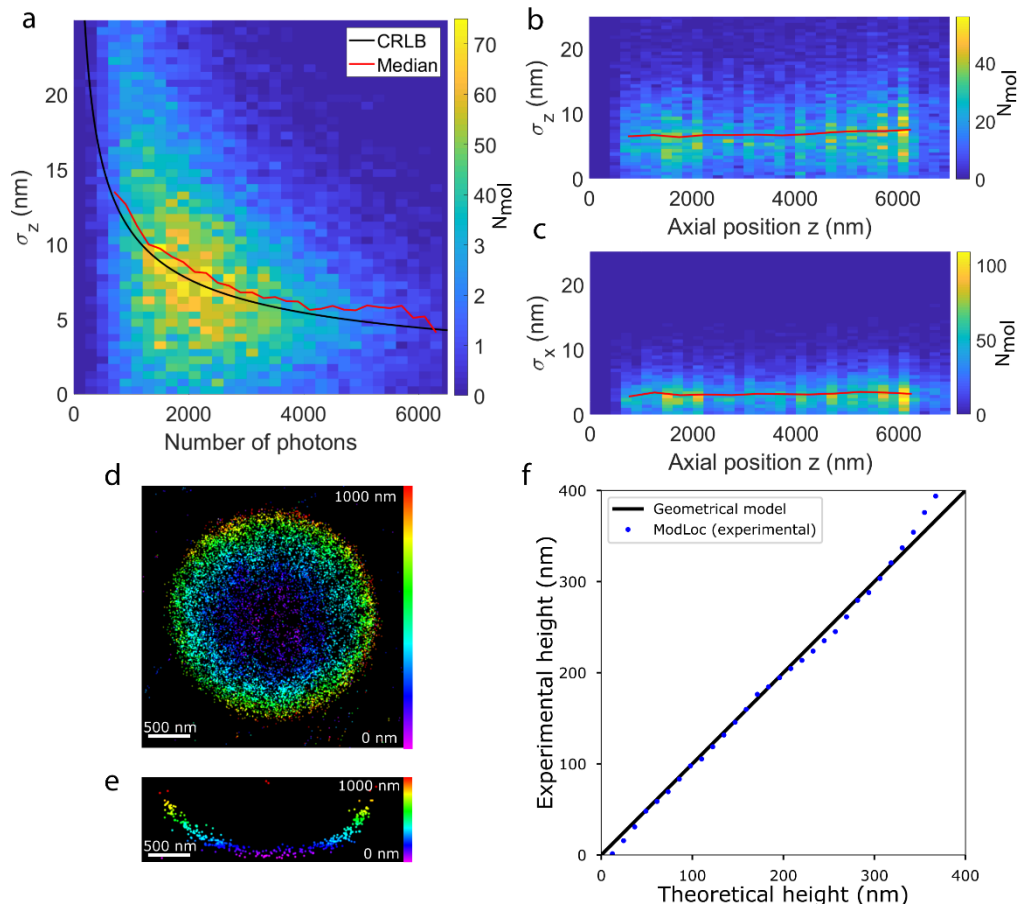
141 To avoid phase wrapping and optimize axial localization, Λ_z is set to approximately 1 μm (Supplementary
142 Fig. 1 and Notes 1) is obtained with $\alpha = 42^\circ$ and $\theta = 44^\circ$. This configuration with expected axial standard
143 deviation $\sigma_{\text{ModLoc}} \approx 2.5 \sigma_x$ is used to image from the coverslip to several tens of microns in depth
144 without unwrapping (Supplementary Note 3 and Supplementary Fig. 1, 7, 8, 9 and 10).

145

146 **Results**

147 We first characterized ModLoc axial and lateral localization precisions at various depths. To evaluate the
148 intrinsic precision, we performed repeated localizations on single emitters (Atto647N) with DNA-PAINT
149 labelling whose long activation times (>100 ms) allow a repeated localization over consecutive frames
150 (see Methods). Fig. 2a shows the very good agreement between the experimental data and the theoretical
151 limits (Cramèr Rao Lower Bound, CRLB) as a function of detected photons (Supplementary Fig. 11 and 12,

152 and note 1). For a typical number of photons in (d)STORM experiment of ~ 2500 photons, ModLoc achieves
 153 an axial localization precision of about 7.4 nm. Fig. 2b and 2c show that the axial and the lateral localization
 154 precisions remain approximately constant over the first seven microns in depth with median values of 6.8
 155 ± 0.4 nm and 3.1 ± 0.2 nm respectively.



156

157 **Figure 2 | Performances of ModLoc.** The experimental localization precisions are obtained from the standard
 158 deviation of the localization distribution for single molecular emitters detected on several successive frames
 159 (ATTO647N in DNA-PAINT). **a**, Histogram of the axial localization precision of ModLoc as a function of the number of
 160 detected photons. The red curve is the experimental median and the black curve is the Cramèr-Rao lower bounds
 161 (CRLB). **b** and **c** Histogram of the localization precision in the axial and lateral direction respectively acquired at various
 162 depths up to $7 \mu\text{m}$. Their associated median values (red lines). The data are taken from the same experiment as in **a**
 163 with a number of detected photons per emitter between 1800 and 6500 and a median photon value of 2800 **d**, 3D
 164 super resolved image of a 3 micrometers diameter sphere labelled with AF647 fluorescent probes conjugated to
 165 streptavidin, the capture range limits the observation to the bottom of the sphere close to the coverslip. **e**, Sphere
 166 xz cross section shows the geometric profile of the sphere. **f**, Comparison of the experimental heights of different single
 167 molecules detected and the theoretical heights calculated from the sphere geometrical model. The black curve
 168 represents the expected tendency and median values of ModLoc are represented by blue dots.

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170 We performed complementary experiments using 40 nm diameter fluorescent nanobeads for which the
 171 emission level is controlled^{15,16,20} (see Supplementary Fig. 13). The localization precisions are also in very

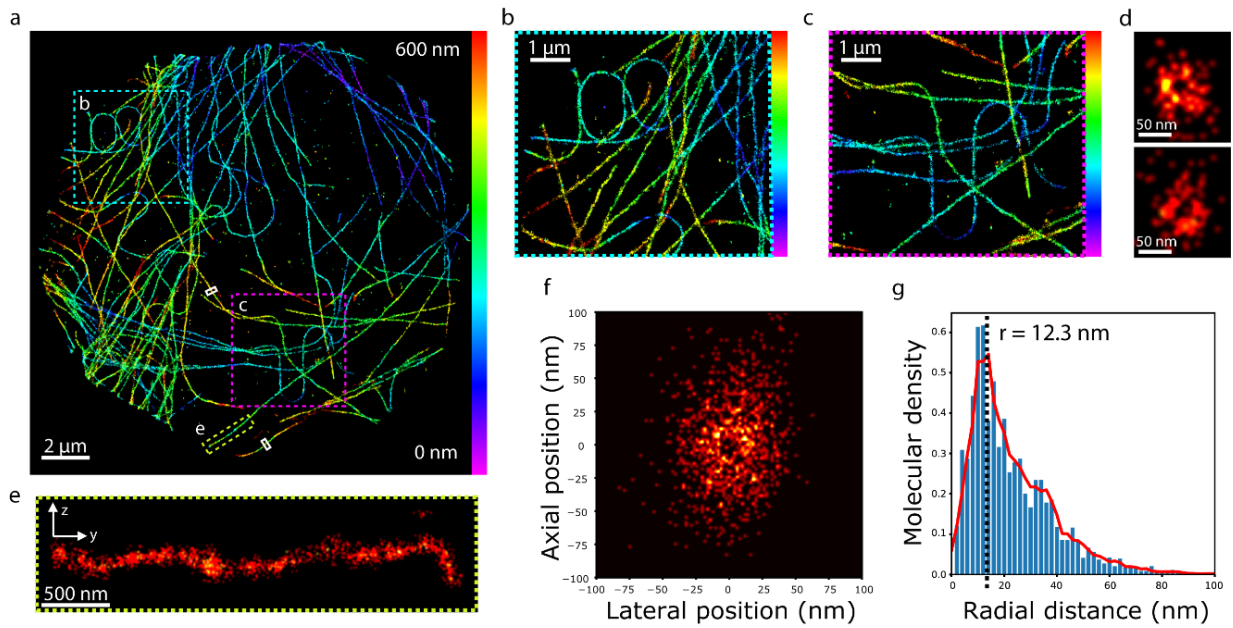
172 good agreement with CRLB calculations and the lateral and axial localization precisions are approximately
173 constant over the first seven microns in depth (with 3.4 ± 1.2 nm and 7.6 ± 2.1 nm). Unlike most PSF
174 shaping methods, the lateral precision is not compromised on the axial one. In addition, modeling the
175 influence of photon noise per pixel resulting from an axial defocusing confirm the experimental
176 observations of a nearly constant localization precision of ModLoc. This is in sound contrast with
177 astigmatism performances which degrade rapidly with defocusing (Supplementary Fig. 14 and 15).

178 The accuracy, representing the discrepancy between the measured position and the real one, is another
179 important parameter in SMLM. This parameter is evaluated from images of 3 μ m diameter microspheres
180 labeled with Alexa Fluor 647 imaged in (d)STORM (Methods)(Fig. 2d) and their associated xz projection
181 (Fig. 2e). The measured axial positions of different localized fluorescent probes coincide with their
182 theoretical positions on the microsphere (Fig. 2f). These findings prove that ModLoc is a real asset for 3D
183 single molecule imaging especially for in depth imaging.

184

185 We now focus on ModLoc performances on biological structures observed in (d)STORM (Methods). COS7
186 cells are imaged in the first 600 nm close to the coverslip with a standard labeling of tubulin to visualize
187 their microtubule network (Fig. 3a-3c). The precision of ModLoc enables visualizing the microtubule
188 cylindrical structure (Fig. 3d). Direct visualization of their hollownesses is impeded by the labelling density
189 and the uncertainty in the microtubule orientations (Fig. 3e), however statistically accumulation over
190 several microtubule sections at various depths (see Fig. 3f and Supplementary Fig. 16) yields a radial
191 density profile which exhibits a central dip with a radius of ~ 12.3 nm as expected from the microtubule
192 structure^{33,34} (Fig. 3g). The tail at higher radii originates from the distance distribution introduced by the
193 labelling.

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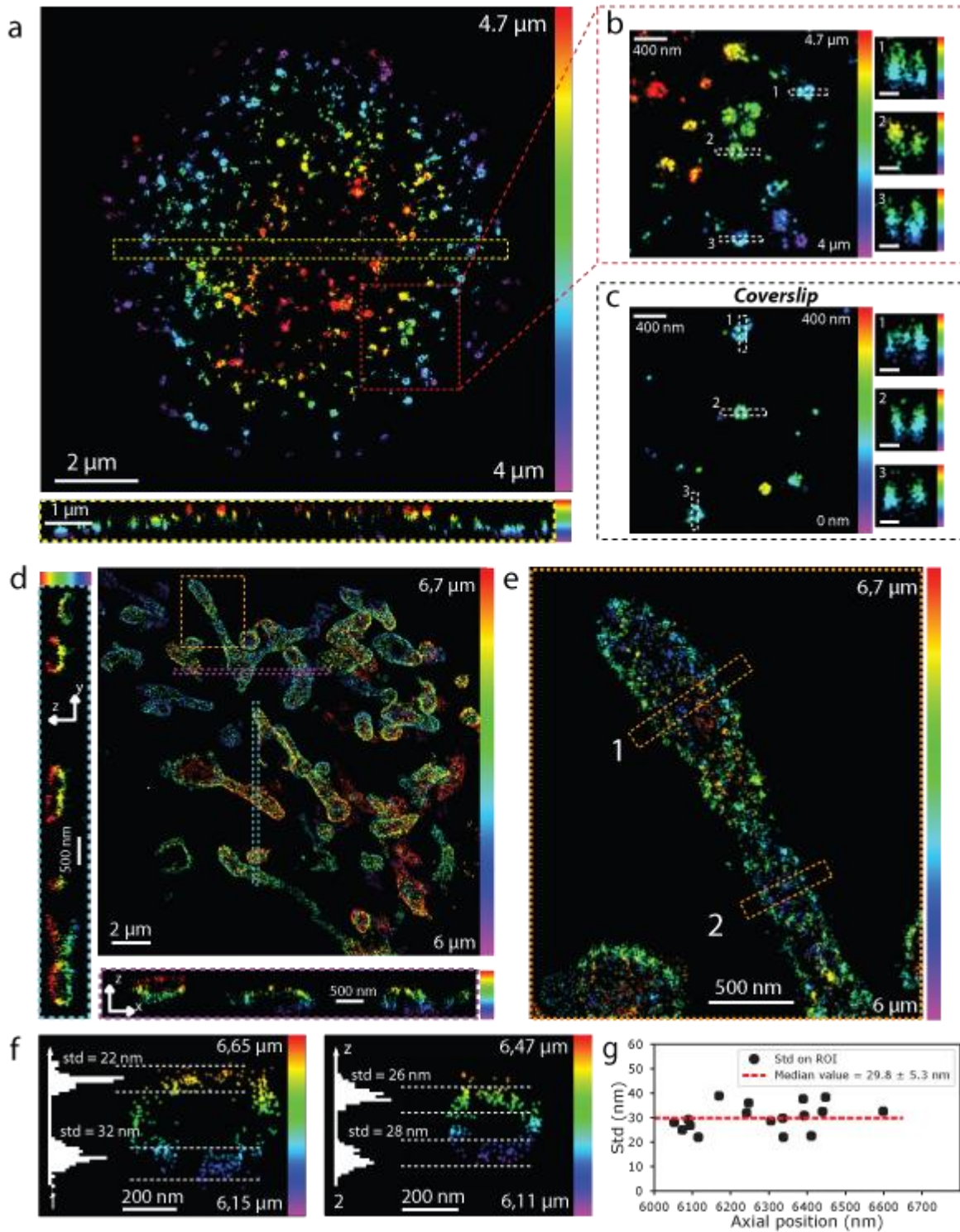
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196 **Figure 3 | 3D imaging of microtubules network in the first 600 nm in COS7 cell in (d)STORM.** **a-** Full 3D imaging of
 197 tubulin in cos7 cell at the coverslip labeled with AF 647 fluorescent probes, **b** zoom on the cyan ROI of **a**. **c** zoom on
 198 the pink ROI in **a**. **d** Different yz cross sections highlighted by white ROI in **a**, which evidenced the cylindrical structure
 199 microtubules. **e** zoom on the yellow ROI of **a**, yz cross section of a microtubule. **f** Density map of detected fluorescent
 200 molecules for 7 different microtubules located at various depths within the capture range. **g** radial molecular density
 201 of **f** exhibiting a central dip with a radius of ~ 12.3 nm.

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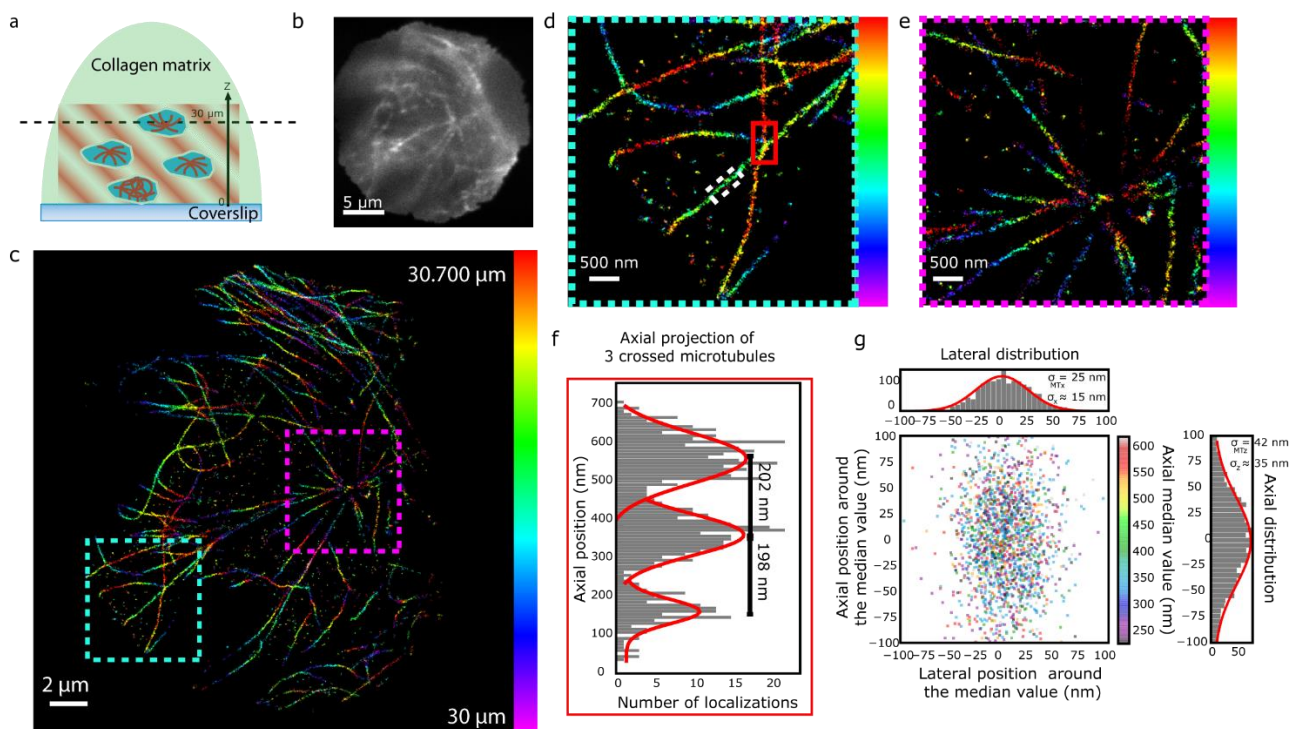
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204 We now study the performances of ModLoc at several micron depths in (d)STORM (Fig. 2f). Figure 4a
 205 shows clathrin pits repartition in COS7 cells labeled with AF 647 at a depth of 4 microns revealing the
 206 presence of the underlying nucleus. The yz cross sections of the clathrin pits (Fig. 4b) show morphologies
 207 similar to the one obtained near the coverslip (Fig. 4c) confirming the constant axial localization precision
 208 of ModLoc. Figures 4d-4g show mitochondria at 6 microns depth in COS7 cells. The uniformity of the
 209 localization precision is investigated by measurements of the thickness of mitochondria membranes at
 210 different depths (see Supplementary Figure 17). Results show that the measured thicknesses are
 211 unaffected by the axial position with a median value of 29.8 ± 5.3 nm. This value agrees with the reported
 212 values of mitochondria membranes ~ 7.5 nm measured in cryotomography³⁵) and primary and secondary
 213 antibodies sizes (~ 20 nm) as well as measurements with other super-resolution techniques³⁶.



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Figure 4 | 3D imaging of biological samples with ModLoc in (d)STORM. **a**, Top: full 3D imaging of clathrin in cos7 cell at 4 microns in depth with heavy chain labeled with AF 647 fluorescent probes. Bottom: xz cross section of the yellow ROI. The z bar color is defined in the same interval as in the top. **b**, Left: zoom on the red ROI of **a**. Right: different xz and yz cross sections of the white ROI (scale 100 nm). **c**, Left: 3D image of clathrin in cos7 at the coverslip with heavy chain labeled with AF 647 fluorescent probes. Right: different xz and yz cross sections of the white ROI (scale 100 nm). **d**, 3D image of mitochondria in cos7 cell at 6 microns in depth with Tom22 labelled with AF647, 2 cross sections of ROIs are shown at left and at the bottom of the full field image. The colorbar range is the same for the 3 images. **e**, Top: zoom on the orange ROI of **d**. **f**, XZ cross sections at different positions along one mitochondria (zone 1 and 2 in **e**), with axial histograms showing the membrane thickness at the top and the bottom of the mitochondria **g**, Standard deviation of different histograms for membrane thickness measured at different depths showing uniform z precision over the whole capture range.



226

227 **Figure 5 | 3D imaging of COS 7 cell microtubule grown in collagen matrix in(d)STORM .** a, Schematic representation of COS 7
 228 grown in a collagen matrix. b, Wide field image of COS 7 cell tubulin at 30 microns in depth. c, 3D super resolved imaged of wide
 229 field represented in b. d, Zoom on the blue ROI in c. e, Zoom on the purple ROI in c. f, Axial cross section of 3 microtubules (red
 230 ROI in c) at different depths in the capture range. g, Scatter plot represents projection of different microtubules (supplementary
 231 Figure 18 for ROI positions) along around 500 nm for each. The median axial position of each microtubule in encoded with the
 232 color bar and lateral and axial distribution are represented with histograms. Weighted standard deviation of each histogram σ_{MTx}
 233 ~ 25 nm and $\sigma_{MTz} \sim 42$ nm gives an approximation of ModLoc localization precision $\sigma_x \sim 15$ nm and $\sigma_z \sim 35$ nm (Supplementary Figure
 234 19).

235 Performances of ModLoc are evaluated under harsh conditions at even greater depths and in the presence
 236 of strong optical aberrations. To perform observations at different depths, we use COS7 cells cultured in a
 237 collagen matrix with standard labeling of tubulin to visualize their microtubule network (see Fig. 5a). The
 238 wide-field image of the microtubule network at 30 μ m depth (taking into account the focal shift³⁷ as the
 239 microscope z-display indicated 50 μ m) clearly shows the incidence of the aberrations and scattering
 240 induced by the collagen matrix (Fig. 5b). The axial information can still be accurately retrieved at such large
 241 depths as shown by the super-resolved 3D reconstructed image in Fig. 5c and the close-ups in Fig. 5d-5e.
 242 An axial projection of 3 microtubules (Fig. 5f) shows that they are easily distinguishable when set 200 nm
 243 apart. Figure 5g presents the fluorophore localization in the cross section of 10 microtubules at different
 244 (color coded) depths within the capture range. The localizations are evenly distributed along the lateral
 245 and axial directions confirming the steady performance of ModLoc within the capture range. The
 246 hollowness cannot be observed in such harsh conditions but the lateral and axial distributions have a

247 Gaussian shape related to the radial fluorophore distribution profile around the microtubule (see Fig. 5g,
248 Supplementary Fig. 18 and 19). Measurements gives a lateral localization precision of about 15 nm and an
249 axial precision of around 35 nm. The strong scattering properties of the collagen matrix impacts the
250 localization precision in all directions.

251 **Discussion**

252 ModLoc technique is easy to implement with a single objective configuration and offer a uniform axial
253 localization precision at various depths. It takes full advantage of the robustness of the excitation pattern
254 throughout the whole capture range compared to PSF-based techniques. ModLoc is well suited for deep
255 biological samples imaging as demonstrated by images of the cytoskeleton and of various organelles up to
256 30 μm .

257 ModLoc implementation could still be improved by modifying the excitation or the detection. The fringes
258 pattern period could be reduced by using two counter propagating beams, either by using a reflecting
259 mirror or in a 4Pi geometry which has the further advantage of doubling the collection efficiency¹⁶. In this
260 case, the axial and lateral localizations become independent and an ultimate axial localization precision of
261 $\sigma_{\text{ModLoc}} \approx 0.5 \sigma_x$ (see eq. 1 with $\alpha/2 = \theta = 90^\circ$). The lock-in detection could be based alternatively on
262 moving mirrors which, although limited in frequency and potential source of mechanical drift³⁸, would be
263 polarization independent and enhance the contrast.

264 iPALM such as ModLoc is based on a lock-in detection scheme to localize single emitters. While in iPALM
265 the measured phase is that of the emitted electromagnetic field whose self-interference gives the position
266 of the emitter, in ModLoc the phase is that of the amplitude modulation of the fluorescence emission
267 which is related to the structured illumination pattern. The iPALM fundamental precision limit, like that
268 of ModLoc, results from the shot noise of the molecular fluorescence signal and the spatial period of the
269 modulation. With an equal spatial period of $\lambda/2$ (with counter-propagating beam configuration) and the
270 same number of collected photons, both techniques would approximately give the same localization
271 precision. Creating an interference illumination pattern is however much less challenging than achieving
272 the interference of a single fluorophore. In addition, phase wrapping and distortion of the PSF when

273 imaging deeper in the sample restricted iPALM to thin samples and observations close to the coverslip,
274 deeper imaging was made possible only with adaptive optics¹⁹.

275 Both MINFLUX³⁹ technique and ModLoc are based on a structured illumination to localize the molecule. In
276 the former case, the estimator and the illumination pattern in the shape of a doughnut have been
277 optimized to reach unmatched precision for a specific point, albeit breaking the translational invariance⁴⁰.
278 ModLoc retains the translational invariance and is therefore designed to handle full field imaging.

279 ModLoc performances at depth shows that it is robust to optical aberrations. It mainly relies on the
280 robustness of the structured illumination obtained from the interference of two plane waves regarding
281 spherical aberrations. In the case of coherent illumination, the effect of aberrations is limited to a global
282 phase shift as shown in standard Structured Illumination Microscopy^{41,42}. The phase detection of ModLoc
283 depends only on the relative number photons in each channel. Contrary to PSF engineering techniques,
284 this phase detection is independent of the PSF shape and thus robust to optical aberrations.

285 Taking advantage of the 3D imaging capability of ModLoc to decipher multiple proteins organization would
286 be a real asset. Several multiplexing strategies are fully compatible with ModLoc and could be readily
287 implemented, like sequential labeling approaches^{43,44} or by demixing red dyes with a dichroic beam-
288 splitter inserted at the detection⁴⁵ or salvaging photons discarded by the dichroic⁴⁶. Multiplexing strategy
289 based on the excitation modulation of lasers at different frequencies⁴⁷ could be adapted to enable
290 simultaneous multicolor imaging.

291 Standard amplitude modulation LIDAR and TOF cameras use an electronic driven modulation of the
292 excitation beam scattered by an object to obtain localization²⁷. This technique couples spatial and
293 temporal modulations and limit the spatial periodicity of the excitation to typically a few meters. The
294 ModLoc structured illumination by interferences and its independent temporal shift enables a decoupling
295 of the spatial and temporal frequencies and a spatial period down to the micrometer range. This could
296 find potential applications in LIDAR and TOF camera to reach really high localization precision
297 (Supplementary Fig. 20).

298 The generic concept of ModLoc based on lock-in detection is well suited to perform fast multiple frequency
299 demodulation without compromising on the acquisition time nor discarding ON-time emitters, should it
300 be for multiple direction localization or for additional information at the single molecule level, such as the
301 molecular orientation or the local chemical environment through the fluorescence lifetime measurement.

302

303 **Methods**

304 **Optical setup.** A complete schematic of the optical setup used is presented in Figure 1a. We used a Nikon
305 Eclipse Ti inverted microscope with a Nikon Perfect Focus System. The dichroic and filters in the
306 microscope cube are from Semrock (Di03-R635-t1-25x36 and BLP01-635R-25). The excitation was
307 performed using a 639-nm laser (Genesis MX 639, 1W, Coherent), couple to a single-mode optical fiber
308 maintaining polarization. The laser beam was separated in 2 paths with a 50/50 cube beamsplitter (BS010,
309 Thorlabs) and recombined in the sample through a Nikon APO TIRF x60 1.49 NA oil immersion objective
310 lens. The fluorescence was collected through the same objective lens, sent in the demodulation module
311 and recorded on four quadrants of a 512x512-pixel EMCCD camera (iXon3, Andor). The camera was placed
312 at the focal plane of the module of magnification 2.6 in order to get an optical pixel size of 100 nm. The
313 phase modulation at the excitation is performed by an electro optical modulator (EOM) (EO-PM-NR-C1,
314 Thorlabs), while the phase sorting in the detection path is performed by a Pockels cell (CF1043-20SG-
315 500/700, Fast pulse) controlled by a high voltage power amplifier (10/10B-HS, TREK). The synchronization
316 was provided thanks to a 4-channel generator that triggers the EOM, the Pockels cell and the camera.

317 **Experimental demodulation process.** Sampling of the temporal modulated intensity is performed by a
318 homemade demodulation module composed of polarizing components and an active Pockels cell placed
319 before the camera. The module creates 4 different channels accessible to the fluorescent signal and each
320 channel is acquired in a subarray on the camera. The state change of the Pockels cell allow to modify the
321 active channel of the fluorescence intensity and is synchronized with the excitation pattern position in
322 order to collect the fluorescence of in each subarray. The fluorescence transmission for each state of the
323 Pockels cell is described in Supplementary Note 1 and Supplementary Figure 5.

324 **Calibration samples preparation.** To obtain the localization precisions displayed in Supplementary figure
325 13, we prepared a sample of 40-nm diameter dark red fluorescent nanobeads (10720, Thermo Fisher)
326 randomly distributed on fixed and non-labeled COS7 cells (fixation with 4%PFA). This operation allows us
327 to obtain a sparse sample with isolated fluorescent nanobeads at different randomly heights. This sample
328 also allows us to be closer to experimental conditions of 3D single molecule imaging. We added 25 μL of a
329 10^{-5} diluted stock solution in 1 ml of Phosphate Buffer Saline (PBS) and we wait 15 minutes before
330 observation for the beads to deposit.

331 The calibration of the tilt angle described Supplementary Note 1 and Supplementary Figure 10 was
332 performed by using coated biotin microspheres (Kisker Biotech, PC-B-3.0) labeled with AF647-streptavidin
333 conjugated fluorophores⁴⁸. We prepared a solution containing 500 μL of water, 500 μL of PBS, 30 μL of
334 stock microspheres solution and 1 μL of streptavidin-functionalized AF647 (Life Technologies, S21374). The
335 solution was centrifuged during 30 minutes at 10 krpm and the liquid phase was removed and replaced
336 with 100 μL of PBS. Then, we vortexed the solution in order to dissolved the deposit. We added 1 ml of
337 PBS to 50 μL of the final solution on the coverslip and we waited 1 hour before starting the acquisitions.

338 The influence of the modulation frequency on the precision of single molecule events (Supplementary
339 figure 4), was evaluated following the previous protocol adapted for DNA-PAINT imaging. Streptavidin
340 coated microspheres (SpheroTech SVP60-5) were conjugated with biotin DNA strand (Biotin-
341 TTATACATCTA, Metabion). A solution containing 500 μL of water, 500 μL of PBS, 30 μL of stock
342 microspheres solution and 1 μL of biotin-DNA Strand (100 μM) was centrifuged during 30 minutes at 10
343 krpm and the liquid phase was removed and replaced with 100 μL of buffer (Imaging buffer, Massive
344 Photonics). We added 1 ml of buffer to 50 μL of the final solution on the coverslip and we waited 1 hour
345 before starting the acquisitions. 0.4 nM of DNA-Strand imager labelled with ATTO647N was added to the
346 solution (CTAGATGTAT-ATTO647N Metabion).

347 Pattern excitation was calibrated by imaging single fluorescent fluorophores at the coverslip. The sample
348 is obtained by diluting 1 μL of stock AF647 florescent probe solution in 500 μL of dSTORM buffer (Abbelight
349 dSTORM buffer). Calibration process are developed in Supplementary Note 1 et Supplementary Fig. 10.

350 **Biological samples preparation.**

351 *Cell culture*

352 COS-7 cells were grown in DMEM with 10% FBS, 1% L-glutamin and 1% penicillin/streptomycin (Life
353 Technologies) at 37°C and 5% CO₂ in a cell culture incubator. For U2OS culture, DMEM was replaced by
354 McCoy's 5A medium. Several days later, they could be plated at low confluence on cleaned round 25 mm
355 diameter high resolution# 1.5 glass coverslips (Marienfield, VWR).

356 *Cell fixation for Tubulin imaging*

357 After 24 hours, the cells were washed three times with PHEM solution (60 mM PIPES, 25 mM HEPES, 5 mM
358 EGTA and 2 mM Mg acetate adjusted to pH 6.9 with 1M KOH) and fixed for 20 min in 4% PFA, 0.02%
359 glutaraldehyde and 0.5% Triton. They were then washed 3 times in PBS (Invitrogen, 003000). Up to this
360 fixation step, all chemical reagents were pre-warmed at 37°C. The cells were permeabilized 10 min with
361 PBS + 0.1% Triton X-100 before to be reduced 10 min with NaBH₄.

362 *Cell fixation for Clathrin and Mitochondria imaging*

363 The cells were washed three times with PHEM solution (60 mM PIPES, 25 mM HEPES, 5 mM EGTA and 2
364 mM Mg acetate adjusted to pH 6.9 with 1M KOH) and fixed for 10 min in 4% PFA. They were then washed
365 3 times in PBS (Invitrogen, 003000). Up to this fixation step, all chemical reagents were pre-warmed at
366 37°C. The cells were reduced 7 min with 50 mM NH₄Cl.

367 *U2OS Cell labelling for DNA-PAINT imaging*

368 Saturation step was performed by washing in PBS three times before being blocked 30 min in PBS + 3%
369 BSA + 0.5% Triton. The cells were incubated 1 hour at 37°C with 1:300 mouse anti-Clathrin Heavy Chain
370 antibody (Sigma Aldrich, C1860) in PBS + 1% BSA. This was followed by three washing steps in PBS +3%
371 BSA, incubation during 1h at 37° C with 1:300 anti-mouse combined to DNA-PAINT strand (Massive
372 Photonics) diluted in antibody buffer (Massive Photonics). Three more washes with washing buffer

373 (Massive Photonics) was performed before imaging with a concentration of 0.2 nm of DNA-Strand imager
374 labelled with ATTO647N (CTAGATGTAT-ATTO647N Metabion). No post-fixation was performed

375 *COS7 Cell labeling for tubulin imaging in dSTORM*

376 Saturation step was performed by washing in PBS three times before being blocked 15 min in PBS + 1%
377 BSA. The cells were incubated 1 hour at 37°C with 1:300 mouse anti- α -tubulin antibody (Sigma Aldrich,
378 T6199) in PBS + 1% BSA. This was followed by three washing steps in PBS +1% BSA, incubation during 45
379 min at 37° C with 1:300 goat anti-mouse AF647 antibody (Life Technologies, A21237) diluted in PBS 1%
380 BSA and three more washes in PBS. A post-fixation step was performed using PBS with 3.6% formaldehyde
381 during 15 min. The cells were washed in PBS three times and then reduced during 10 min with 50 mM
382 NH₄Cl (Sigma Aldrich, 254134), followed by three additional washes in PBS.

383 *COS7 Cell labeling for Clathrin imaging in dSTORM*

384 Saturation step was performed by washing in PBS three times before being blocked 15 min in PBS + 3%
385 BSA + 0.5% Triton. The cells were incubated 1 hour at 37°C with 1:300 mouse anti-Clathrin Heavy Chain
386 antibody (Sigma Aldrich, C1860) in PBS + 3% BSA. This was followed by three washing steps in PBS +3%
387 BSA, incubation during 45 min at 37° C with 1:300 goat anti-mouse AF647 antibody (Life Technologies,
388 A21237) diluted in PBS 1% BSA and three more washes in PBS. A post-fixation step was performed using
389 PBS with 3.6% formaldehyde during 15 min. The cells were washed in PBS three times and then reduced
390 during 10 min with 50 mM NH₄Cl (Sigma Aldrich, 254134), followed by three additional washes in PBS.

391 *COS 7 Cell labelling for Mitochondria imaging in dSTORM*

392 Saturation step was performed by washing in PBS three times before being blocked 15 min in PBS + 3%
393 BSA + 0.5% Triton. The cells were incubated 1 hour at 37°C with 1:300 mouse anti-TOM22 antibody (Sigma
394 Aldrich, T6319) in PBS + 3% BSA. This was followed by three washing steps in PBS +3% BSA, incubation
395 during 45 min at 37° C with 1:300 goat anti-mouse AF647 antibody (Life Technologies, A21237) diluted in
396 PBS 1% BSA and three more washes in PBS. A post-fixation step was performed using PBS with 3.6%

397 formaldehyde during 15 min. The cells were washed in PBS three times and then reduced during 10 min
398 with 50 mM NH₄Cl (Sigma Aldrich, 254134), followed by three additional washes in PBS.

399 *COS7 cells in collagen matrix*

400 COS-7 cells were grown in DMEM with 10% FBS, 1% L-glutamin and 1% penicillin/streptomycin (Life
401 Technologies) at 37°C and 5% CO₂ in a cell culture incubator. Upon reaching confluency, the cells in DMEM
402 were added to 80% collagen, 10% MEM and 5.83% neutralizing solution (RAFT3D Cell Culture Kit, LONZA).
403 The last step was done on ice at 4°C. Then, the cells were plated at $2,5 \cdot 10^{-5}$ cells per well on cleaned
404 round 25 mm diameter high resolution #1.5 glass coverslips (Marienfield, VWR). After 30 min at 37°C in
405 the incubator, the exceeding solution was removed and replaced by DMEM without phenol red with 10%
406 FBS, 1% L-glutamin and 1% penicillin/streptomycin. After 24 hours, we did the same steps as described
407 previously.

408 **Imaging modalities.** Biological experiments were performed mainly in (d)STORM conditions with a classical
409 dSTORM buffer (Smart Kit Abbelight), with a typical number of image acquisition between 10000 to 40000
410 images and an excitation of 2 kW.cm². Results presented in Figure 2a, 2b and 2c have been obtained in
411 DNA-PAINT, with a similar number of acquisition but an excitation power reduced to 0.4 kW.cm²

412 **Cramèr-Rao Lower Bounds.** Calculation are developed in Supplementary Note 1. The CRLB of ModLoc is
413 obtained from the classical CRLB of the lateral detection and the CRLB of the phase. Influence of the
414 number of photons and the background noise are represented in Supplementary Figure 11 and
415 Supplementary Figure 12. Comparison with astigmatism method are illustrated in Supplementary Figure
416 14 and Supplementary Figure 15.

417 **Data processing wokflow. All processing scripts are based on public available software.**

418 *Localization on raw data*

419 First, we remove the background signal on the 512x512-pixel raw frames by subtracting the temporal
420 median of the 10 previous and 10 next frames, pixel per pixel. Then, we detect the PSFs by using a wavelet
421 filtering⁴⁹ associated to a low intensity threshold and large spot width filtering parameters. This is

422 necessary due to the large disparities in fluorescence intensity obtained by modulation in the frame. We
423 measure the nanometric position by Gaussian fitting using the Gpufit maximum likelihood estimate Python
424 toolkit⁵⁰. The intensity is then obtained by a photon counting over a 1 μm x 1 μm around the center of the
425 point spread function.

426 The second step recombines the four intensities to obtain the phase information for each molecule. The
427 localization array is split in four parts corresponding to the four detection channels. Lateral positions
428 detected in other channels by Gaussian fitting are corrected by using affine transformations in order to
429 correct magnification, rotation and translation caused by misalignment of the detection paths. The top left
430 channel is used as a reference for this operation, and affine transformation parameters are obtained with
431 a simple registration algorithm (see supplementary fig. 6 for an estimation of registration errors). We then
432 merge the four localizations corresponding to each fluorophore and we apply transmission correction
433 coefficients on each intensity channel determined for the considered fluorescent probe. Transmission
434 coefficient are obtained by acquiring fluorescence in each channel without any modulation of the intensity,
435 at the beginning of the experiment. Finally, we calculate the phase from the four intensities for each
436 molecule. The axial position is obtained by using the x coordinates from the Gaussian fitting and the phase
437 measurements as:

$$438 \quad z = \frac{\Delta\phi}{2\pi\sin(\theta)} - \frac{x}{\tan(\theta)}$$

439 *Drift correction*

440 Drifts are corrected using an axial and lateral custom-made program based on a Direct Cross Correlation
441 (DCC) algorithm³². The localization list is divided into several temporal slices based on the frame numbers,
442 the reference slice being chosen as the first and the lateral drift correction is performed at first. The
443 transverse temporal drift is obtained by a 2D cross-correlation of 2 temporal stack. Thus, it is possible to
444 obtain a temporal drift curve whose sampling depends of the size of temporal Stack. When lateral drifts
445 are corrected, the operation is repeated along the axial direction. The localizations are binned in 3D images

446 with a voxel size typically set to 60x60x40 nm in x,y,z, and the slice size is generally 500 frames. This
447 operation can be repeated several times by changing the slice size in order to refine the precision.

448 **Localization precision estimation from repeated localizations on single emitters**

449 Localization precision plotted in figure 2 a-c were obtained by calculating the standard deviation on the
450 positions of localized molecules on successive frames. Molecules present on a radius less than 30 nm are
451 considered to be identical. To be considered, the molecule must be active on 4 successive frames at the
452 minimum and 10 successive frames at the maximum. The positions obtained on the first and last image
453 are not considered for the standard deviation calculations.

454 **Data availability**

455 Data are available upon reasonable request to the corresponding author.

456 **Code availability**

457 Processing code are based on already published solutions as described in the supplementary

458 **References**

- 459 1. Betzig, E. *et al.* Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* **313**,
460 1642–1645 (2006).
- 461 2. Hess, S. T., Girirajan, T. P. K. & Mason, M. D. Ultra-High Resolution Imaging by Fluorescence
462 Photoactivation Localization Microscopy. *Biophysical Journal* **91**, 4258–4272 (2006).
- 463 3. Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction
464 microscopy (STORM). *Nat Methods* **3**, 793–796 (2006).
- 465 4. Heilemann, M. *et al.* Subdiffraction-Resolution Fluorescence Imaging with Conventional Fluorescent
466 Probes. *Angewandte Chemie International Edition* **47**, 6172–6176 (2008).
- 467 5. von Diezmann, A., Shechtman, Y. & Moerner, W. E. Three-Dimensional Localization of Single
468 Molecules for Super-Resolution Imaging and Single-Particle Tracking. *Chemical Reviews* **117**, 7244–
469 7275 (2017).

- 470 6. Ram, S., Prabhat, P., Chao, J., Ward, E. S. & Ober, R. J. High Accuracy 3D Quantum Dot Tracking with
471 Multifocal Plane Microscopy for the Study of Fast Intracellular Dynamics in Live Cells. *Biophysical*
472 *Journal* **95**, 6025–6043 (2008).
- 473 7. Juette, M. F. *et al.* Three-dimensional sub-100 nm resolution fluorescence microscopy of thick
474 samples. *Nature methods* **5**, 527–9 (2008).
- 475 8. Hajj, B., El Beheiry, M., Izeddin, I., Darzacq, X. & Dahan, M. Accessing the third dimension in
476 localization-based super-resolution microscopy. *Phys. Chem. Chem. Phys.* **16**, 16340–16348 (2014).
- 477 9. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-Dimensional Super-Resolution Imaging by
478 Stochastic Optical Reconstruction Microscopy. *Science* **319**, 810–813 (2008).
- 479 10. Pavani, S. R. P. *et al.* Three-dimensional, single-molecule fluorescence imaging beyond the diffraction
480 limit by using a double-helix point spread function. *Proceedings of the National Academy of Sciences*
481 **106**, 2995–2999 (2009).
- 482 11. Shechtman, Y., Weiss, L. E., Backer, A. S., Sahl, S. J. & Moerner, W. E. Precise Three-Dimensional
483 Scan-Free Multiple-Particle Tracking over Large Axial Ranges with Tetrapod Point Spread Functions.
484 *Nano Letters* **15**, 4194–4199 (2015).
- 485 12. Badieirostami, M., Lew, M. D., Thompson, M. A. & Moerner, W. E. Three-dimensional localization
486 precision of the double-helix point spread function versus astigmatism and biplane. *Appl. Phys. Lett.*
487 **97**, 161103 (2010).
- 488 13. Bourg, N. *et al.* Direct optical nanoscopy with axially localized detection. *Nature Photonics* **9**, 587–
489 593 (2015).
- 490 14. Deschamps, J., Mund, M. & Ries, J. 3D superresolution microscopy by supercritical angle detection.
491 **11** (2014).
- 492 15. Cabriel, C. *et al.* Combining 3D single molecule localization strategies for reproducible bioimaging.
493 *Nat Commun* **10**, 1–10 (2019).
- 494 16. Shtengel, G. *et al.* Interferometric fluorescent super-resolution microscopy resolves 3D cellular
495 ultrastructure. *Proceedings of the National Academy of Sciences of the United States of America* **106**,
496 3125–3130 (2009).

- 497 17. Wang, G., Hauver, J., Thomas, Z., Darst, S. A. & Pertsinidis, A. Single-Molecule Real-Time 3D Imaging
498 of the Transcription Cycle by Modulation Interferometry. *Cell* **167**, 1839-1852.e21 (2016).
- 499 18. Aquino, D. *et al.* Two-color nanoscopy of three-dimensional volumes by 4Pi detection of
500 stochastically switched fluorophores. *Nature Methods* **8**, 353–359 (2011).
- 501 19. Huang, F. *et al.* Ultra-High Resolution 3D Imaging of Whole Cells. *Cell* **166**, 1028–1040 (2016).
- 502 20. Bon, P. *et al.* Self-interference 3D super-resolution microscopy for deep tissue investigations. *Nature*
503 *Methods* **15**, 449 (2018).
- 504 21. von Diezmann, A., Shechtman, Y. & Moerner, W. E. Three-Dimensional Localization of Single
505 Molecules for Super-Resolution Imaging and Single-Particle Tracking. *Chem. Rev.* **117**, 7244–7275
506 (2017).
- 507 22. Burke, D., Patton, B., Huang, F., Bewersdorf, J. & Booth, M. J. Adaptive optics correction of
508 specimen-induced aberrations in single-molecule switching microscopy. *Optica* **2**, 177 (2015).
- 509 23. LIGO Scientific Collaboration and Virgo Collaboration *et al.* Observation of Gravitational Waves from
510 a Binary Black Hole Merger. *Phys. Rev. Lett.* **116**, 061102 (2016).
- 511 24. Zernike, F. How I Discovered Phase Contrast. *Science* **121**, 345–349 (1955).
- 512 25. Beaufort, E., Boccardi, A. C., Lebec, M., Blanchot, L. & Saint-Jalmes, H. Full-field optical coherence
513 microscopy. *Opt. Lett., OL* **23**, 244–246 (1998).
- 514 26. Taylor, R. W. *et al.* Interferometric scattering microscopy reveals microsecond nanoscopic protein
515 motion on a live cell membrane. *Nature Photonics* **13**, 480–487 (2019).
- 516 27. *TOF Range-Imaging Cameras*. (Springer-Verlag, 2013).
- 517 28. Cappello, G. *et al.* Myosin V stepping mechanism. *Proc Natl Acad Sci U S A* **104**, 15328–15333 (2007).
- 518 29. Busoni, L., Dornier, A., Viovy, J.-L., Prost, J. & Cappello, G. Fast subnanometer particle localization by
519 traveling-wave tracking. *Journal of Applied Physics* **98**, 064302 (2005).
- 520 30. Reymond, L. *et al.* SIMPLE: Structured illumination based point localization estimator with enhanced
521 precision. *Opt. Express, OE* **27**, 24578–24590 (2019).
- 522 31. Clossen, J. *et al.* Localization microscopy at doubled precision with patterned illumination. *bioRxiv*
523 554337 (2019) doi:10.1101/554337.

- 524 32. Wang, Y. *et al.* Localization events-based sample drift correction for localization microscopy with
525 redundant cross-correlation algorithm. *Optics Express* **22**, 15982 (2014).
- 526 33. Weber, K., Rathke, P. C. & Osborn, M. Cytoplasmic microtubular images in glutaraldehyde-fixed
527 tissue culture cells by electron microscopy and by immunofluorescence microscopy. *Proceedings of*
528 *the National Academy of Sciences* **75**, 1820–1824 (1978).
- 529 34. Zwettler, F. U. *et al.* Molecular resolution imaging by post-labeling expansion single-molecule
530 localization microscopy (Ex-SMLM). *Nature Communications* **11**, (2020).
- 531 35. Gold, V. A. M. *et al.* Visualizing active membrane protein complexes by electron cryotomography.
532 *Nature Communications* **5**, 4129 (2014).
- 533 36. Xu, F. *et al.* Three-dimensional nanoscopy of whole cells and tissues with in situ point spread
534 function retrieval. *Nature Methods* **17**, 531–540 (2020).
- 535 37. Bratton, B. P. & Shaevitz, J. W. Simple Experimental Methods for Determining the Apparent Focal
536 Shift in a Microscope System. *PLOS ONE* **10**, e0134616 (2015).
- 537 38. Gu, L. *et al.* Molecular resolution imaging by repetitive optical selective exposure. *Nat Methods* 1–5
538 (2019) doi:10.1038/s41592-019-0544-2.
- 539 39. Balzarotti, F. *et al.* Nanometer resolution imaging and tracking of fluorescent molecules with
540 minimal photon fluxes. *Science* **355**, 606–612 (2017).
- 541 40. Gwosch, K. C. *et al.* MINFLUX nanoscopy delivers multicolor nanometer 3D-resolution in (living) cells.
542 *bioRxiv* 734251 (2019) doi:10.1101/734251.
- 543 41. Arigovindan, M., Sedat, J. W. & Agard, D. A. Effect of depth dependent spherical aberrations in 3D
544 structured illumination microscopy. *Opt. Express, OE* **20**, 6527–6541 (2012).
- 545 42. Booth, M., Andrade, D., Burke, D., Patton, B. & Zurauskas, M. Aberrations and adaptive optics in
546 super-resolution microscopy. *Microscopy (Oxf)* **64**, 251–261 (2015).
- 547 43. Jungmann, R. *et al.* Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-
548 PAINT. *Nature Methods* **11**, 313–318 (2014).
- 549 44. Klevanski, M. *et al.* Automated highly multiplexed super-resolution imaging of protein nano-
550 architecture in cells and tissues. *Nat Commun* **11**, 1–11 (2020).

- 551 45. Lampe, A., Haucke, V., Sigrist, S. J., Heilemann, M. & Schmoranzer, J. Multi-colour direct STORM with
552 red emitting carbocyanines. *Biology of the Cell* **104**, 229–237 (2012).
- 553 46. Zhang, Y. *et al.* Nanoscale subcellular architecture revealed by multicolor three-dimensional salvaged
554 fluorescence imaging. *Nat Methods* **17**, 225–231 (2020).
- 555 47. Gómez-García, P. A., Garbacik, E. T., Otterstrom, J. J., Garcia-Parajo, M. F. & Lakadamyali, M.
556 Excitation-multiplexed multicolor superresolution imaging with fm-STORM and fm-DNA-PAINT. *PNAS*
557 **115**, 12991–12996 (2018).
- 558 48. Cabriel, C., Bourg, N., Dupuis, G. & Lévêque-Fort, S. Aberration-accounting calibration for 3D single-
559 molecule localization microscopy. *Opt. Lett., OL* **43**, 174–177 (2018).
- 560 49. Izeddin, I. *et al.* Wavelet analysis for single molecule localization microscopy. *Optics Express* **20**, 2081
561 (2012).
- 562 50. Przybylski, A., Thiel, B., Keller-Findeisen, J., Stock, B. & Bates, M. Gpufit: An open-source toolkit for
563 GPU-accelerated curve fitting. *Sci Rep* **7**, 1–9 (2017).

564

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573

574 **Author contributions**

575 P.J, C.C., N.B., C.P., E.F. and S.L.F. conceived the project. P.J. designed the optical setup, performed the
576 acquisitions, CRLB calculations and data analysis. P.J. and E.F. carried out simulations. N.B. developed the

577 (d)STORM buffer. N.B., C.C. and P.J. optimized the immunofluorescence protocol. P.J., C.C. and S.L.F
578 prepared the COS-7 and U2OS cells samples. M.B designed the 3D sample protocol. All authors
579 contributed to writing the manuscript.

580 **Competing financial interests**

581 The CNRS has deposited a patent FR3054321-A1 on the 25 July 2016 to protect this work, currently
582 under international extension. S.L.F, E.F. and N.B. are co-inventors.