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1 **QCM: real-time quantitative quality control of single-molecule localization**
2 **microscopy acquisitions**

3
4 Short Title: Real-time quantitative quality control of SMLM data

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17
18 **Abstract**

19 Single molecule localization microscopy (SMLM) has revolutionized the understanding of
20 cellular organization by reconstructing informative images with quantifiable spatial
21 distributions of molecules far beyond the optical diffraction limit. Much effort has been devoted
22 to optimizing localization accuracy. Among them, assessing the quality of SMLM data in real-
23 time, rather than after lengthy post-acquisition analysis, represent a computational challenge.

24 Here, we overcome this difficulty by implementing an innovative mathematical approach to
25 drastically reduce the computational analysis of particle localization. We have therefore
26 designed the Quality Control Map (QCM) workflow to process data at a much higher rate than
27 that limited by the frequency required by current cameras. Moreover, QCM requires no
28 parameters other than the PSF radius characteristic of the optical system and only a GPU card
29 to reach its computational speed. Thus, QCM is robust and adaptable to any type of input data.
30 Finally, the QCM off-line mode can be used to evaluate synthetic or previously acquired data,
31 and as a tool for teaching the basic concepts of the SMLM approach.

32
33 **Teaser**

34 QCM, a parameter-free algorithm, calculates indicators for instant feedback on single-
35 molecule localization precision experiments

36 INTRODUCTION

37 In system biology, the combination of “omic” approaches can benefit significantly from Smart
38 Microscopy (SM) to bridge the gap between cellular events and organism-level phenomena,
39 enabling the unravelling of complex biological networks (1). By providing key spatio-temporal
40 observables, photonic microscopy has become the cornerstone of scientific research in biology,
41 to which SM is giving it a new technological breath (2). Innovative SM approach combines
42 cutting-edge hardware, sophisticated software and powerful algorithms to facilitate the use of
43 increasingly complex microscope modalities. As anticipated, SM offers the possibility of
44 combine imaging procedures thanks to automated data acquisition in a single experiment, in a
45 simplified and reproducible way. As the amount of information increases, approaches based on
46 real-time analysis or machine learning algorithms enable acquisition parameters to be adjusted
47 on the fly (3) and/or large quantities of data to be processed to identify patterns, anomalies and
48 subtle changes, ultimately enabling autonomous decision-making or rapid and accurate
49 diagnosis (4). Thus, next-generation microscopes are poised to assist humans in automating the
50 acquisition and analysis of data in regions of interest driven by specific events. To this purpose,
51 it is necessary to provide real-time feedback to adjust parameters, optimize imaging conditions
52 and dynamically explore samples.

53 This requirement is particularly relevant to photonic microscopy approaches based on single-
54 molecule localization microscopy (SMLM), which has revolutionized the understanding of
55 cellular organization by reconstructing informative images at the nanoscale (5-8). SMLM
56 observations can inherently produce well-resolved images from which biologically relevant
57 information can be determined such as the nanostructure and stoichiometry of macromolecular
58 complexes (9), provided that the SMLM data production process is properly mastered to resolve
59 a given biological question (10, 11). In this respect, many efforts have been made to optimize
60 not only the sample preparation (unbiased fixation, labeling procedures, etc.) (12-16), but also
61 the acquisition modalities (laser power, camera integration time, stabilized optical systems etc.)
62 (17-20) or the design of dedicated quantitative analytical methods (21-23).

63 The overall process of generating SMLM data, which includes image acquisition, handling and
64 analysis, is time-consuming, and results are highly dependent on the quality of the data acquired
65 to achieve a given localization accuracy and to avoid misleading interpretations. Therefore, a
66 computational challenge is to estimate this localization accuracy before rather than after data
67 acquisition to save time and avoid losing valuable samples (Fig. 1a). Still, most of software
68 packages ensure robust quantitative *a posteriori* analysis (see for review (24, 25)), assuming
69 that the data have been recorded appropriately for reconstructing super-resolution images, given
70 that no further adjustment or correction of the acquisition parameters can be made like NanoJ-
71 SQUIRREL (26) or SuperStructure (27). For instance, the former provides a quantitative
72 assessment of SMLM results by generating a quantitative evaluation of super-resolution images
73 to help experimenters optimize imaging parameters; this approach is based on comparing
74 diffraction-limited images and super-resolution equivalents.

75 Consequently, analytical tools for assessing the quality and robustness of SMLM data at any
76 time such as those enabling *a priori* quantitative control of data, are therefore in high demand
77 from a broad community of cell biologists (Fig. 1a). The aim is to carry out analyses in real
78 time in order to adjust the acquisition parameters for optimal data recording. This approach
79 should make it possible to avoid time-consuming and unnecessary data acquisition, when a
80 posteriori analysis will reveal only poor-quality and misleading data. Some strategies have
81 implemented new computational strategies to speed up image acquisition or processing (28,
82 29). Computer architecture design is another means of achieving high computing performance
83 (26, 28, 30-33). For instance, QC-STORM (30), a GPU-based software package, performs real-
84 time image processing and generates a list of particle localization, but lacks precise
85 quantification, relies only on indicators on the full dataset and provides only histograms.
86 Another method computes the Fourier Ring Correlation measurement in real-time (34).

87 Alternatively, hardware developments have been implemented to compute multi-emitter fitting
88 in real-time (32).

89 Considering that the expected localization accuracy is directly dependent on two parameters -
90 the signal-to-noise ratio (SNR) and the particle density per frame (D_{frame}) - we have
91 implemented the Quality-Control Maps (QCM), a parameter-free algorithm that represents a
92 major advance in the SMLM field and extends the SPT and SMLM algorithms previously
93 developed (35, 36). Here, by implement an original mathematical approach, we were been able
94 to harness the computing power of conventional computers to carry out the analysis of
95 2048×2048 pixels images at a rate of over 100 frames/second, which is sufficient for real-time
96 analysis.

97 The special feature of QCM is that it displays in real-time quantitative maps and histograms of
98 local (zoomed-in areas) and global (full frame) of a set of indicators to assess the quality of
99 SMLM data in an easily understandable way thanks to its color coding. These include the PSF
100 size in xy , and xyz positions, signal-to-noise ratio (SNR), background, intensity, and precision
101 of localization. It should be noted that another major advance of QCM relies on the SNR (in
102 dB) as the most relevant contrast parameter for summarizing expected achievable precision;
103 indeed, the root mean square of the localization precision when expressed by SNR depends
104 weakly on noise model or density/frame (see in Mailfert et al. (35) the Materials and Methods
105 section and Fig. S1).

106 Thus, the workflow of the Quality-Control Maps (QCM) software has been designed to conduct
107 real-time analysis of data, providing users with key observables in the decision-making process.
108 If the results do not meet predetermined criteria such as a given accuracy of molecular
109 localization, users can intervene on the setup, ensuring optimal acquisitions in line with the
110 FAIR principles (37). Used prior to acquisition (Fig. 1b, left panel), QCM primarily saves time,
111 improves data relevance and reduces data storage requirements. In addition, the use of QCM
112 for post-acquisition data analysis provides a standardized tool for educational purposes or for
113 peer review of data (Fig. 1b, right panel).

114

115 RESULTS

116 *The QCM heuristic*

117 The QCM algorithm is divided into two main modules, Ultra-Fast Unsupervised Localization
118 (UFUL) and Quality Control (QC) (Fig. 2). The first one is based on an innovative
119 mathematical approach designed to drastically reduce the computational steps involved in
120 particle localization analysis. As a result, UFUL performs the particle detection/localization
121 steps at a much faster rate than the frame rate acquisition by standard cameras used in SMLM.
122 The second module then uses the UFUL results to estimate the relevant SMLM indicators in
123 real-time, and displays their histogram distributions and map representations.

124 Classically, single particles localization involves a detection step and then an estimation at high
125 resolution (*i.e.* sub-pixel) of the particle position in the (i, j) plane and the size of the point
126 spread function (PSF) on the i and j axes (36). To solve this problem, the regular procedure is
127 based on a maximum likelihood estimator (MLE) or a minimum mean square error (MMSE)
128 estimator. The main objective is to avoid using estimators on a region of interest (ROI) devoid
129 of particles.

130 Within this framework, we have previously provided mathematical developments optimizing
131 the detection step by implementing a generalized likelihood ratio test (GLRT) at known
132 background (35), which means that the mean m and variance σ^2 of the background are known.
133 This test is an effective unsupervised detection tool whose threshold is set by the probability of
134 false alarm (*PFA*) (38-40); it is primarily designed as a detector in a ROI with a working
135 window of dimension ω , for the absence (H_0 hypothesis) or presence (H_1 hypothesis) of a

136 particle. The GLRT can also be conceived as an estimator since, under the H_1 hypothesis
137 assumption, it builds the intensity image using an estimator in the sense of the MMSE estimator,
138 which is close to the Cramer-Rao Bounds (CRB). As such, the GLRT performs an adaptive
139 filter to carry out the estimation of the intensity as previously described (see for instance the
140 Supplementary Note 2 in (35)). However, its application as initially conceived cannot handle
141 the real-time data flow of SMLM acquisitions.

142 To overcome this difficulty, we have rewritten the mathematical operations of the GLRT
143 detector at known background to considerably accelerate the computational steps, without
144 impacting its robustness (see Materials and Methods, for details). Practically, this implies to
145 evaluate first the mean \hat{m} and variance $\hat{\sigma}^2$ of the background as in (35). Then, the GLRT assess
146 the presence of a signal at each pixel and when a signal is detected, the estimator searches the
147 sub-pixel positions of the PSF. UFUL computes $\hat{\alpha}_{\hat{m}}$ as the expression of the intensity $\hat{\alpha}$ minus
148 the image background \hat{m} . Three separable convolutions are processed for each pixel to estimate
149 the background, its variance, and the signal intensity $\hat{\alpha}$, respectively. It is then possible to
150 estimate the sub-pixel position of each detected particle and the size of its PSF. To do this, the
151 PSF is modeled by a Gaussian function from which UFUL uses a logarithm of the intensity $\hat{\alpha}$
152 to derive literal expressions for estimating for each particle, its PSF radii r_i, r_j and sub-pixel
153 coordinates i_0, j_0 on axes i and j , respectively.

154 We test the UFUL performances to ascertain that the analyses coincide with the MMSE
155 estimation, with respect to the variances in positions. This was done on realistic synthetic data,
156 *i.e.* on data close to the levels of noise, signal, PSF size, etc. that are typically the ones observed
157 on experimental SMLM. We report that UFUL overlap those of an MMSE estimator; both being
158 close on the CRB. For a PFA of 10^{-6} , the detection probability $PD \approx 100\%$ for any $SNR >$
159 20 dB (Supplementary Text, Fig. S1 and S2).

160 Moreover, UFUL provides the estimation of r_0 on both i and j axes with the estimator $\hat{r}_0 =$
161 $(\hat{r}_i + \hat{r}_j)/2$ regardless of the PSF size and the working window. Consequently, when image
162 acquisitions are performed with an astigmatic lens (41), the axial PSF of a particle is distorted
163 in i and j axes of the focal plane as a function of the particle's position on the optical axis,
164 enabling it to be localized in 3D (Supplementary Text, Fig. S3). Under these conditions, the
165 size of the working window ω for the GLRT detector set at $8 \times 8, 10 \times 10, 12 \times 12, 14 \times 14,$ or
166 16×16 pixels, depends on r_0 , the size of the PSF.

167 ***UFUL computation rate performance***

168 We evaluated the UFUL performances on synthetic datasets generated at a given density of
169 particle per frame and for different image sizes (Fig. 3). The analyses were obtained on a
170 computer with the option of processing data with CPU (central processing unit) or GPU
171 (graphical processing units) processors (see Material & Methods for the specifications).

172 The performances are expressed in number of frames analyzed per second (Fig. 3a). The
173 computation times correspond to the analysis of 16-bit raw images stored in the PC RAM, from
174 which the detection/estimation process returns a list of particles with position, size of 2D or 3D
175 PSF astigmatism, intensity, SNR, noise level, and position errors in the PC RAM. This time
176 mainly results from the one used by the GLRT detector and therefore, for D_{frame} values ranging
177 from 0 to 0.2 part/ $\mu\text{m}^2/\text{frame}$, the number of particles has hardly any impact on the performance
178 of detection/estimations steps (Fig. 3a).

179 The UFUL module incorporates a computing segment specifically designed to optimize
180 computation time to achieve a data flow of over 5 GB/s, enabling it to perform data analysis at
181 a rate well above the acquisition performance of a standard SMLM camera, *i.e.*, ≈ 100 fps for
182 2048×2048 -pixel images. Indeed, with GPU, the analysis rate for particle detection and
183 position estimation reaches over 10,000 fps for 256×256 -pixel images and up to ≈ 800 fps for
184 2048×2048 -pixel images (Fig. 3a). Furthermore, GPU computing increases the number of
185 particles analyzed per second by a factor up to 10 compared to CPU computing on large images

186 (Fig. 3b). For the GPU, images of size equal to or greater than 512×512 pixels present a similar
187 number of particles analyzed per second, unlike the CPU where the cache miss is significant.

188 Overall, the GPU speeds up analysis considerably, with a more noticeable difference on large
189 images compared to the CPU. It should be noted, however, that while the UFUL computation
190 rate with a GPU is well above the frame rate of SMLM camera, performance with a CPU
191 remains above this threshold for image up to 1024×1024 -pixels (Fig. 3a). As a result, the
192 second module of QCM can process the output of UFUL results and display the relevant quality
193 control indicators in real time.

194 ***QC module and the QCM interface***

195 The QC module relies on UFUL results for the image background, particle positions, intensity
196 and PSF size. It evaluates in real-time the key quality control indicators - D_{frame} , SNR and
197 precision of localization parameters – and displays using histograms and maps. Thus, we set up
198 a graphical interface for easy, real-time evaluation of QCM analysis results (Fig. 4 and
199 Supplementary Video 1). In the opening panel, QCM requires no parameterization other than
200 the following physical parameters (Fig. 4a):

- 201 - The characteristic PSF radius (r_0) of the optical system, a physical characteristic
202 inherent in the optical system for a given excitation wavelength and numerical aperture
203 of the objective. This value must be expressed in pixels. As part of internal quality
204 control, QCM displays the r_0 histogram evaluated during data acquisition.
- 205 - The binning and exposure time parameters of the camera.

206 QCM displays in real-time the histograms of the PSF radius (r_0), D_{frame} , SNR and precision
207 parameters. Analyses are visualized on the last 50, 500 or full stack of images; it is also relevant
208 for specific applications to estimate these indicators on a zoomed region of interest (Fig. 4b and
209 Supplementary Video 2). Moreover, when a biological question requires achieving a given
210 SMLM precision (e.g., dimensionality or count of macromolecular complexes, inter-distances
211 between macromolecular structures, etc.), QCM offers the option of displaying the density-
212 SNR space diagram in real-time allowing standardized evaluation of experimental data. Other
213 options allow to display the images captured by the camera, the SMLM image reconstructed at
214 the time being, or those of quality control indicators (background, D_{frame} , SNR, and precision
215 parameters) (Fig. 4c).

216 ***Assessment of the robustness of QCM analyses***

217 To assess the robustness of quality control indicators calculated by QCM, we collect stacks of
218 2,000 images of DNA origami nanorulers as nanoscopic benchmark structures (42). The SMLM
219 DNA-PAINT imaging technique is used to assess the metrological traceability of nanorulers
220 with marks 80 nm apart. The data acquired at different laser powers and camera integration
221 times were analyzed in real-time by QCM. Each initial acquisition is short, around one minute
222 per condition, but long enough to display informative SNR and D_{frame} histograms for deciding
223 whether or not to continue data acquisition. QCM results were compared with those obtained
224 on the same dataset using UNLOC and GATTAnalysis as post-acquisition analysis tools
225 (Fig. 5). As illustrated, the images reconstructed by UNLOC provides a qualitative estimate of
226 the nanorulers while GATTAnalysis evaluation is based on three parameters, the pass ratio, i.e.
227 the percentage of good spots, the mark-to-mark distance in nm and the fraction of nanorulers at
228 a precision threshold better than 20 nm. For example, under acquisition at a laser power of
229 37 mW and a camera integration time of 36 ms, the histograms of D_{frame} and SNR provided by
230 QCM peak at 1.3 particles/ $\mu\text{m}^2/\text{frame}$ and 22.4 dB, respectively. Under such conditions, we
231 cannot expect to ensure robust SMLM resolution, as assessed by post-acquisition analyses,
232 where only 12% of nanorulers achieve accuracy better than 20 nm. A go/no-go decision based
233 on the SNR and D_{frame} histogram distribution provided by QCM analyses on a small number of
234 frames is in good agreement with the results of post-acquisition analyses provided by the
235 UNLOC or GATTAnalysis algorithms. Therefore, QCM enables instant adjustment of camera

236 integration time and/or laser power to find the optimal acquisition parameters for achieving the
237 highest possible localization accuracy on the samples, before starting the SMLM data
238 acquisition.

239 ***QCM-optimized SMLM acquisition on biological samples***

240 Next, we test QCM for imaging biological samples using different SMLM methods, to
241 investigate the robustness of the displayed key parameters, SNR and D_{frame} histograms, before
242 starting data recording. To do this, we compared the QCM results obtained on the first stack of
243 2,000 images with those obtained by post-analysis with UNLOC on the whole recorded dataset
244 (up to 50,000 frames).

245 Cellular expression of β -tubulin and the nuclear pore protein Nup133 was imaged by dSTORM
246 SMLM (Fig. 6). After chemical fixation, cells were incubated with primary antibodies before
247 staining detection with fluorescently-labelled secondary antibodies. Samples were imaged in
248 freshly prepared dSTORM buffer, and laser power and camera integration time were adjusted
249 to image β -tubulin in COS-7 cells and Nup133 in HeLa cells respectively. Since QCM quality
250 control on given imaging conditions prior to acquisition can be based on a few hundred frames,
251 it is fast enough to avoid distorting the recording of a whole dataset due to lengthy adjustment
252 procedures (e.g., due to photobleaching or dSTORM buffer deterioration) (Supplementary
253 Video 3). For example, the initial QCM analyses of β -tubulin imaging in COS-7 cells were
254 carried out on just 2,000 frames displaying informative and robust SNR and D_{frame} histograms
255 (Fig. 6a). Besides, the QCM analyses can be operated for the entire duration of the data
256 recording, so that the mean values of indicators are tracked over time, enabling their stability
257 or inconsistency to be assessed, for instance in the event of focal plane loss (Fig.4b). Finally,
258 QCM and UNLOC analyses carried out on the same number of frames show that 35% and 49%
259 of detected signals have an estimated precision greater than or equal to 20 nm for β -tubulin and
260 Nup 133, respectively (Fig. 6b).

261 As discussed in the Supplementary Text and Fig. S4, the detection/estimation achieved by
262 UFUL is primarily designed for image analysis under low density conditions, i.e. D_{frame} less than
263 ≈ 0.2 part./ $\mu\text{m}^2/\text{frame}$. To overcome this limitation, a density evaluation calibration has been
264 integrated so that the algorithm returns realistic density values. But the fine analysis of raw data
265 in most cases requires post-acquisition analysis with a dedicated algorithm based on heuristic
266 for reliable particle localization at variable local density. Nevertheless, the analysis obtained
267 with QCM give a very good estimate of those obtained with a dedicated algorithm such as
268 UNLOC (Fig. 6b).

269 Among other SMLM methods, DNA-PAINT, based on the transient association of a
270 fluorescently-labeled probe with a target molecule, has become particularly popular due to the
271 ability to adjust experimental conditions to the expression level of the proteins of interest being
272 visualized. The signal detection is mediated by pairing a docking oligonucleotide coupled to a
273 target probe that recognize a protein of interest with an imager, i.e., a fluorescently labelled
274 complementary oligonucleotide freely diffusing in the buffer (43). This method relies on the
275 concentration of imager to control the density of transient docker/imager hybridization per
276 frame enabling a stochastic detection of the protein of interest by recoding fluorescence signals.

277 For two-color DNA-PAINT experiments (Fig. 7), we used an automated workflow system to
278 deliver sequentially into a channel slide, the respective imagers to detect in HeLa cells the
279 mitochondrial 20 kDa outer membrane protein TOM20 and the major building block of
280 microtubules α -tubulin (see Materials and Methods). For acquisitions at appropriate D_{frame} , the
281 imager concentrations were pre-adjusted using QCM over just 500 frames, i.e. an acquisition
282 time of 18 s, as shown in Fig. 7b to define the conditions required for TOM20 protein imaging.
283 QCM analyses were performed at three successive imager concentrations on the same sample
284 preparation. At 1.5 nM, the precision of localization was significantly impaired. At 0.3 nM, the
285 QCM returns significant intracellular variability in D_{frame} and SNR values, with poorly resolved

286 area as illustrated in the insert. At a 10-fold lower imager concentration, i.e. 30 pM, QCM
287 returns adequate precision of TOM20 localization for any intracellular area. The relevance of
288 the QCM analyses obtained on a few hundred images is demonstrated by the comparison with
289 the reconstructed images obtained with UNLOC on complete datasets. We thus detected the
290 distribution of TOM20 and α -tubulin in HeLa cells. Consequently, the efficiency of QCM
291 makes it possible to adjust experimental conditions in real time for optimal DNA-PAINT-based
292 multicolor sequential localization of multiple cellular components such as TOM20 and α -
293 tubulin, as illustrated by the integrated-Gaussian reconstructed images from the post-processed
294 UNLOC analyses (Fig. 7b). Thus, QCM is a key asset for unlocking the power of the multicolor
295 DNA-PAINT SMLM approaches.

296

297 **DISCUSSION**

298 In line with FAIR principles (37), the emergence of smart microscopes where tools such as
299 QCM enable quantitative data analysis provide effective feedback for real-time readjustment of
300 key parameters (44). Overall, the interactive QCM capability encourages adaptive
301 experimentation and reduces trial and error cycles, especially with biological samples to which
302 access is limited.

303 As compared over currently available software solutions (30, 32-34, 45-47), QCM is parameter-
304 free software package, requiring no parameters other than those characterizing the optical
305 system; non-expert users can therefore easily operate it. Overall, QCM features an optimized
306 software interface and display with easy-to-evaluate color-coded maps and histograms
307 generated in real-time. This instantaneous quantitative information enables parameters to be
308 adjusted, imaging conditions to be optimized and sub-regions of interest in the sample to be
309 explored dynamically. Assessing such quality control of raw SMLM data at the earliest steps
310 of acquisition enables an acceptance or rejection decision to be made on the basis of just a few
311 hundred images, and thus optimizes the amount of data to be acquired, stored and analyzed for
312 proper quantification of relevant observables.

313 It should be noted that the overall computation rate currently achievable here in real-time is
314 mainly ensured by the UFUL module, which is based on a one-Gaussian fitting hypothesis, i.e.,
315 for low D_{frame} value, ideally below 1.0 particles/ $\mu\text{m}^2/\text{frame}$. It is therefore advisable to perform
316 post-processing analyses of the recorded SMLM data, and to use dedicated algorithms to
317 quantify any effective non-uniformly distributed molecules (21, 23). In this framework, we
318 previously implemented UNLOC, a parameter-free algorithm approaching the Cramér-Rao
319 bound for particles at high-density per frame and without any prior knowledge of their intensity
320 (35).

321 We would like to underline that the QCM off-line mode offers invaluable possibilities to be
322 used with post-acquisition SMLM data. We further stress that this mode is perfectly suited to
323 carrying out standardized studies with no a priori assumptions on reusable SMLM raw metadata
324 or during the review process of publications including SMLM data. QCM is also of general
325 interest for teaching basic SMLM concepts to a wide audience. Overall, QCM can be seamlessly
326 integrated into the workflows of homemade or commercial systems and cloud-based data
327 analysis frameworks.

328 At present, we have succeeded in analyzing 2048×2048 pixels images at a rate of over 100
329 frames/second, a rate fast enough to explore dynamic processes in living samples. However, if
330 it is possible to record data at a faster acquisition rate - for example, by focusing on a small ROI
331 - we might face an intrinsic limitation of the SMLM technique due to the fact that the number
332 of photons collected will be limiting at some point. Alternatives such as the promising event-
333 based vision sensor-based imaging method (48) for *in vivo* imaging pave the way for a very
334 promising paradigm shift in cell biology by giving access to a new quantitative set of relevant
335 observables.

336

337 MATERIALS and METHODS

338 Ultra-Fast Unsupervised Localization (UFUL)

339 This section describes the mathematical basis of the Ultra-Fast Localization (UFUL)
340 conception.

341 *Computational optimization of the GLRT detection*

342 The detection step is based on previously mathematical developments (35, 36). In summary,
343 when a particle is present, the PSF is modeled by a Gaussian $g_{p=\{i,j\}}(i_0, j_0, r_i, r_j)$ centered in
344 $(i_0, j_0) \in \mathbb{R}^2$ and of dimension r_i and r_j :

$$g_p(i_0, j_0, r_i, r_j) = \frac{1}{\sqrt{\pi r_i r_j}} \exp\left(-\frac{(i - i_0)^2}{2 r_i^2} - \frac{(j - j_0)^2}{2 r_j^2}\right) \quad \text{Eq. 1}$$

345 where the constant of normalization is such that $\iint g^2 = 1$.

346 The detection theory cannot estimate at the same time the value of the parameters (i_0, j_0, r_i, r_j)
347 and the presence or absence of a particle (49). For the GLRT detector, the PSF is in the center
348 of the window and $r_i = r_j = r$ is known. Thus, the GLRT assesses the presence of a signal at
349 each pixel such that $(i_0, j_0) = (i_n, j_n)|_{n \in \mathbb{N}}$. When a signal is detected, the estimator searches in
350 (i_n, j_n) the sub-pixel positions (i_0, j_0) of the PSF. For a PSF in a window, it is easier to write
351 $(i_n, j_n) = (0, 0)$ for simplification purposes.

352 For a GLRT at known background (35), the mean m and variance σ^2 of the background are
353 known. This detector is based on the two H_0 and H_1 hypotheses, both perturbed by independent
354 identically distributed additive Gaussian noise. For H_0 in the working window ω , the signals at
355 pixel $p = \{i, j\}$ are the sum of background m and noise n_p of variance σ^2 :

$$H_0: x_p = m + n_p \quad \text{Eq. 2}$$

356 The H_1 hypothesis has a Gaussian centered in the window that is modeled by:

$$H_1: x_p = \alpha g_p(0, 0, r, r) + m + n_p \quad \text{Eq. 3}$$

357 where α is the particle intensity.

358 Let L_0 be log-likelihood of the H_0 hypothesis:

$$L_0 = -\frac{N}{2} \log(2\pi\sigma^2) - \frac{1}{2\sigma^2} \sum_{p \in \omega} (x_p - m)^2 \quad \text{Eq. 4}$$

359 where N is the size of the window ω .

360 Let L_1 be the generalized log-likelihood of H_1 hypothesis:

$$L_1 = -\frac{N}{2} \log(2\pi\sigma^2) - \frac{1}{2\sigma^2} \sum_{p \in \omega} (x_p - \alpha g_p(0, 0, r, r) - m)^2 \quad \text{Eq. 5}$$

361 The estimated intensity is given by:

$$\hat{\alpha} = \frac{\sum_{p \in \omega} g_p(x_p - m)}{\sum_{p \in \omega} g_p^2} \quad \text{Eq. 6}$$

362 Thus, for a test based on the detection theory (49), the H_0 hypothesis is rejected with a
363 probability of false alarm $PFA \in]0, 1]$ if:

$$2(L_1 - L_0) > \text{Inv } \chi^2(1 - PFA, 1) \quad \text{Eq. 7}$$

364 with $\text{Inv } \chi^2(1 - PFA, 1)$ the inverse law of χ^2 with one degree of freedom. Thus, this test
 365 discriminates that, for a given PFA , the window contains either noise alone or a particle of
 366 $SNR > 20 \text{ dB}$, with a detection probability $PD \approx 100\%$ (49).

367 Here, we rewrite the GLRT expression to significantly optimize the computation time but
 368 without simplifying the robustness of the mathematical model.

369 Let $S_{PFA} = \text{Inv } \chi^2(1 - PFA, 1)$ be the detection threshold, we can write the GLRT for a given
 370 pixel as:

$$2(L_1 - L_0) > S_{PFA} \quad \text{Eq. 8}$$

371

$$2 \left[\left(-\frac{1}{2\sigma^2} \sum_{p \in \omega} (\bar{x} - \hat{\alpha} g_p(0,0, r, r))^2 \right) - \left(-\frac{1}{2\sigma^2} \sum_{p \in \omega} (\bar{x})^2 \right) \right] > S_{PFA} \quad \text{Eq. 9}$$

372 with $\bar{x} = x_p - m$.

373 Thus,

$$\frac{\hat{\alpha}^2}{\sigma^2} > \frac{S_{PFA}}{\sum_{p \in \omega} g_p^2} \quad \text{Eq. 10}$$

374 This requires first estimating the background mean \hat{m} and variance $\hat{\sigma}^2$ as previously described
 375 (see in (35) the note S6 in Supporting Material.). In practice, they are estimated once every 50
 376 frames.

377 This test can therefore be performed for all pixels of a given frame. Computing the left term of
 378 Eq. 10 simply as a convolution (Eq. 6) provides the corresponding image of the $\hat{\alpha}$ and GLRT
 379 values of the pixels. When the test is true in the region of interest (ROI), it corresponds to a
 380 particle defined as a single pixel or as a set of pixels for bright ones, from which a list of detected
 381 particles with an integer pixel value is established.

382 ***Estimation of the particle localization***

383 Once the particles are detected, the objective is to determine their subpixel localization, *i.e.*, at
 384 which the signal intensity $\hat{\alpha}$ is maximum. Two computational methods are classically
 385 implemented:

- 386 • The ones based on an algorithm that performs oversampling of the $\hat{\alpha}$ image are
 387 computationally expensive and cannot estimate the r_i, r_j radii of the PSF;
- 388 • The others based on iterative fitting computation to estimate r_i, r_j, i_0, j_0 are also time
 389 consuming.

390 Here, we demonstrate that a third alternative is possible to determine the position of the particles
 391 and their radius with sub-pixel accuracy while guaranteeing an ultra-fast computational speed,
 392 meaning at a speed higher than that of image acquisition.

393 The PSF is modeled by a Gaussian and the algorithm is using the logarithm of $\hat{\alpha}$ to obtain a
 394 quadratic expression. This enables a literal expression from which to derive the estimation of
 395 r_i, r_j, i_0, j_0 , corresponding to the PSF sizes and sub-pixel coordinates of each particle,
 396 respectively.

397 As such, the current expression of $\hat{\alpha}$ needs to be rewritten to provide a fast and efficient estimate
 398 of these parameters. By replacing m by its estimated value \hat{m} , we obtain:

$$\hat{\alpha}_{\hat{m}} = \sum_{p \in \omega} (x_p - \hat{m}) g_p(0,0,r,r) \quad \text{Eq. 11}$$

399 However, $\hat{\alpha}_{\hat{m}}$ does not correspond to the minimal mean square error (MMSE) estimator that is
 400 expected from the solution given by $\hat{\alpha}$. It is still possible to obtain a variance that coincides
 401 with that of the MMSE on the coordinates. When the H_1 hypothesis is true (Eq. 3), we rewrite
 402 $\hat{\alpha}_{\hat{m}}$ as:

$$\hat{\alpha}_{\hat{m}} = \alpha \sum_{p \in \omega} g_p(i_0, j_0, r_i, r_j) g_p(0,0,r,r) + \sum_{p \in \omega} g_p(n_p + (m - \hat{m})) \quad \text{Eq. 12}$$

403 The second term $\sum_{p \in \omega} g_p(n_p + (m - \hat{m}))$ is a noise processed by a matched filter, but $(m -$
 404 $\hat{m})$, the noise term on the estimate of m , implied that $\hat{\alpha}_{\hat{m}}$ is a good approximation of a MMSE
 405 filter.

406 *Ultra-fast estimation of PSF dimensions and particle sub-pixel positions*

407 We first detail the expression for estimating the intensity $\hat{\alpha}_{\hat{m}}$ and the PSF sizes with logarithms.
 408 The characteristic PSF sizes are r_i, r_j and i_0, j_0 are the sub-pixel positions of the particle.
 409 Moreover, PSF images modeled by Gaussians, filtered by a Gaussian kernel generate
 410 Gaussians. Thus, the intensity estimate is equal to:

$$\hat{\alpha}_{\hat{m}} = \alpha \sum_{p \in \omega} g_p(i_0, j_0, r_i, r_j) g_p(0,0,r,r) = \alpha k_q(i_0, j_0, r_i, r_j) \quad \text{Eq. 13}$$

411 with $k_{q=(i,j)}(i_0, j_0, r_i, r_j) = \frac{2r_i r_j}{\sqrt{r_i^2 + r^2} \sqrt{r_j^2 + r^2}} \exp\left(-\frac{(i-i_0)^2}{2(r_i^2 + r^2)}\right) \exp\left(-\frac{(j-j_0)^2}{2(r_j^2 + r^2)}\right)$.

412 The discrete second derivative on the i -axis of the logarithm of $\hat{\alpha}_{\hat{m}}$ calculated at the positions
 413 $(i_n, j_n) \in \mathbb{N}^2$ of the detected particles is:

$$\log \hat{\alpha}_{\hat{m}}(i_n + 1, j_n) - 2 \log \hat{\alpha}_{\hat{m}}(i_n, j_n) + \log \hat{\alpha}_{\hat{m}}(i_n - 1, j_n) = -\frac{1}{r_i^2 + r^2} \quad \text{Eq. 14}$$

414 Thus, the estimator of the PSF sizes is:

$$\hat{r}_i^2 = -\frac{1}{\log \hat{\alpha}_{\hat{m}}(i_n + 1, j_n) - 2 \log \hat{\alpha}_{\hat{m}}(i_n, j_n) + \log \hat{\alpha}_{\hat{m}}(i_n - 1, j_n)} - r^2 \quad \text{Eq. 15}$$

415 Furthermore, the discrete first derivative on the i -axis of the logarithm of $\hat{\alpha}_{\hat{m}}$:

$$\frac{1}{2} (\log \hat{\alpha}_{\hat{m}}(i_n + 1, j_n) - \log \hat{\alpha}_{\hat{m}}(i_n - 1, j_n)) = \frac{i_0 - i_n}{\hat{r}_i^2 + r^2} \quad \text{Eq. 16}$$

416 Then the estimator of i_0 is:

$$\hat{i}_0 = i_n + \frac{1}{2} (\log \hat{\alpha}_{\hat{m}}(i_n + 1, j_n) - \log \hat{\alpha}_{\hat{m}}(i_n - 1, j_n)) (\hat{r}_i^2 + r^2) \quad \text{Eq. 17}$$

417 Similarly, for the j -axis, the estimators are:

$$\hat{r}_j^2 = -\frac{1}{\log \hat{\alpha}_{\hat{m}}(i_n, j_n + 1) - 2 \log \hat{\alpha}_{\hat{m}}(i_n, j_n) + \log \hat{\alpha}_{\hat{m}}(i_n, j_n - 1)} - r^2 \quad \text{Eq. 18}$$

418 and

$$\hat{j}_0 = j_n + \frac{1}{2} (\log \hat{\alpha}_{\hat{m}}(i_n, j_n + 1) - \log \hat{\alpha}_{\hat{m}}(i_n, j_n - 1)) (\hat{r}_j^2 + r^2) \quad \text{Eq. 19}$$

419 Thus, by computing only five logarithms of the image $\hat{\alpha}_{\hat{m}}$, *i.e.*, $\log \hat{\alpha}_{\hat{m}}(i_n, j_n)$, $\log \hat{\alpha}_{\hat{m}}(i_n +$
420 $1, j_n)$, $\log \hat{\alpha}_{\hat{m}}(i_n - 1, j_n)$, $\log \hat{\alpha}_{\hat{m}}(i_n, j_n + 1)$ and $\log \hat{\alpha}_{\hat{m}}(i_n, j_n - 1)$, we can estimate all the
421 parameters $(\hat{i}_0, \hat{j}_0, \hat{r}_i, \hat{r}_j)$ for the size and sub-pixel localization of a particle.

422 Then, in addition to calculate $\hat{\alpha}_{\hat{m}}(\hat{i}_0, \hat{j}_0, \hat{r}_i, \hat{r}_j)$ and the mean square error (MSE) to determine
423 the SNR and variance of the error of the positions, it is necessary to calculate at the sub-pixel
424 position, $\hat{\alpha}_{\hat{m}}$ as well as the MSE on the error on these corresponding positions.

425

426 **Evaluation of the algorithm performances**

427 All evaluations to compare the mathematical models or validate the algorithms were performed
428 as shown on simulated images generated at a given SNR, PSF size, particle density/frame or
429 image sizes. The codes used to generate these datasets are available on request from the authors.

430 For the evaluation of the CPU/GPU UFUL computation rate performances, the analyses were
431 obtained with the following computer configuration: DELL Precision 7740 laptop; Central
432 Processing Unit (CPU): E-2286M, 64 GB RAM; Graphics Processing Unit (GPU): NVIDIA
433 Quadro RTX4000M. For CPU computations, the code is compiled in C for Matlab (MEX),
434 using Advanced Vector Extensions (AVX) for 8-float 32-bit (single) operations to handle
435 parallel computations. For GPU computations, the code is compiled with CUDA for Matlab
436 (MEX-CUDA). The computation times correspond to the analysis of 16-bit RAW images stored
437 in the PC RAM, from which the detection/estimation process provides the list of particles
438 (position, size of 2D or 3D PSF astigmatism, measured intensity, SNR, noise level, position
439 error) in the PC RAM.

440 Simulations used to demonstrate the real-time performances of QCM will be available under
441 an approved open source license at the time of journal publication.

442

443 **Software and code**

444 QCM is a multi-thread application developed on C/CUDA code on a LINUX platform (LINUX
445 Ubuntu 20.04.2 LTS). It requires a CUDA toolkit for NVIDIA GPU. Two modes are available:
446 a virtual mode for post-acquisition data evaluation that only requires a Graphic Programmable
447 Unit (GPU) and an acquisition mode requiring in addition a PCO Edge 4.2 CLHS sCMOS
448 camera. The data acquisition has been prioritized to offer the maximum frame rate of the camera
449 (*i.e.*, ≈ 100 frames per second). All other processes are running in parallel on specific and
450 dedicated threads.

451 A QCM package is freely available online (see Supplementary Materials) for academic and
452 nonprofit users. It includes a user guide, a set of experimental and synthetic data, and videos
453 illustrating the visualization and quantification of observations.

454

455 **Data acquisition and analysis**

456 All acquisitions were made using total internal reflection fluorescence (TIRF) illumination on
457 a custom-built system based on an inverted microscope (Nikon, TE2000-U) as previously
458 described (35) with a CFI Apo TIRF 100 \times NA 1.49 oil immersion objective (Nikon), a
459 Argon/Krypton multiline laser (Innova 70C-Spectrum, Coherent Inc.), an axial drift correction
460 by the autofocus module, except that the images are acquired with a PCO Edge 4.2 Camera link
461 High Speed (CLHS) scientific Complementary Metal-Oxide-Semiconductor (sCMOS) camera
462 on a LINUX platform. The microscope was controlled with a homemade Labview v.2021
463 (National Instruments) code, while the present homemade QCM code was used to acquire the
464 data.

465 During acquisitions, data are evaluated in real-time with QCM to set appropriate acquisition
466 conditions. Post-acquisition data analyses were performed with UNLOC (35) in high density
467 mode with a high spatial frequency variation of background, a reconnection process with one
468 Off-state lifetime frame and an integrated Gaussian rendering process after drift correction by
469 correlation and without data filtering.

470 The raw experimental data illustrating the QCM performances are available on request from
471 the authors.

472

473 **Reagents and sample preparations for experimental data**

474 Quantitative experiments were performed with DNA-origami with GATTA-PAINT HiRes 80R
475 nanorulers and the results evaluated using GATTAnalysis v1.5 software (GATTaquant).

476 COS-7 cells (ATCC CRL 1651TM) and HeLa cells (ATCC CCL-2TM) were grown in DMEM
477 (Gibco) supplemented with 10% bovine fetal serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM
478 sodium pyruvate (Gibco), and 1% penicillin/streptomycin (Gibco).

479 For the dSTORM experiments, cells were plated on coverslips N° 1.5 of 18 mm diameter
480 (Marienfeld GmbH, #0117580) and incubated at 37°C, 10% CO₂ for 48 h before staining
481 procedures. Cells were washed twice with pre-warmed (37°C) phosphate-buffered saline (PBS)
482 before being fixed with 4% PFA into PBS for 15 min at room temperature (RT) than washed
483 with PBS and treated with 50 mM NaBH₄ for 10 min to reduce background fluorescence and
484 finally washed with PBS. Fixed cells were permeabilized with 0.3% TritonTMX-100 in PBS, for
485 30 min, then washed 3 times in PBS, and saturated with 3% bovine serum albumin (BSA) in
486 PBS, for 45 min to reduce unspecific labeling. Nuclear pore complex protein Nup133 or β -
487 tubulin were labeled overnight at 4°C with rabbit anti-Nup133 antibody (Abcam, #ab155990)
488 and mouse anti-human β -tubulin mAb (Sigma-Aldrich, #05-661-I), respectively. Cells were
489 then washed 5 times with 1% BSA in PBS before being incubated 30 min at room temperature
490 with Alexa Fluor 647 AffiniPureTM Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch
491 Europe Ltd., #111-605-003) for Nup133 labeling and Alexa FluorTM 647-conjugated F(ab')₂-
492 goat anti-mouse IgG (H+L) (ThermoFisher Scientific, #A-21237) for β -tubulin. After 5 washes
493 with 1% BSA in PBS, cells were fixed again with 2% PFA, PBS for 5 min. Finally, after 3
494 washes in PBS, samples were mounted in depression slide with freshly prepared dSTORM
495 buffer (50mM Tris, 50 mM NaCl a pH 8.0 supplemented with 50 mM cysteamine). Sealed
496 samples with Twinsil® Speed 22 (Picodent, #1300 1002) were ready to be imaged.

497 For the two-color DNA-PAINT experiments, the HeLaCells_Tubulin_Tom20 smart samples
498 (Abbelight, France) were prepared in μ -slide VI 0.5 (IBIDI GmbH, #80607) using the
499 microfluidic system Smart Flow (Abbelight, France) and the Smart Staining Kit instruction
500 (Abbelight, France). Next, instructions for the DNA-PAINT kit (Massive Photonics GmbH,
501 #MASSIVE-sdAB-FAST 1-PLEX) with anti-mouse and anti-rabbit nanobodies were applied
502 to recognize the mouse anti- α -tubulin mAb (Sigma-Aldrich, #T6188) and rabbit recombinant
503 anti-TOM20 mAb (abcam, #ab232589) antibodies, respectively. The α -tubulin and
504 mitochondria were detected in a unique imaging buffer with a mix of imager 1 (Cy3b) at a final
505 concentration of 0.5 nM, and imager 2 (ATTO 655) at the specified concentration, respectively.
506 Sequential acquisition was performed with a stack of 50,000 frames recorded at 514 nm
507 (200 mW) using a 525/50 filter for α -tubulin and a stack of 20,000 images was recorded at 647
508 nm (155 mW) using a 710/75 filter for TOM20.

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651

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665

666 **Author contributions:** N.B., S.M., and D.M. conceived the project. N.B. and S.M. developed
667 the algorithm, M.D. and R.F. performed the experiments to test extensively the software. D.M.
668 supervised this work and prepared the original draft with the support of N.B. and S.M. All
669 authors revised and edited the manuscript.

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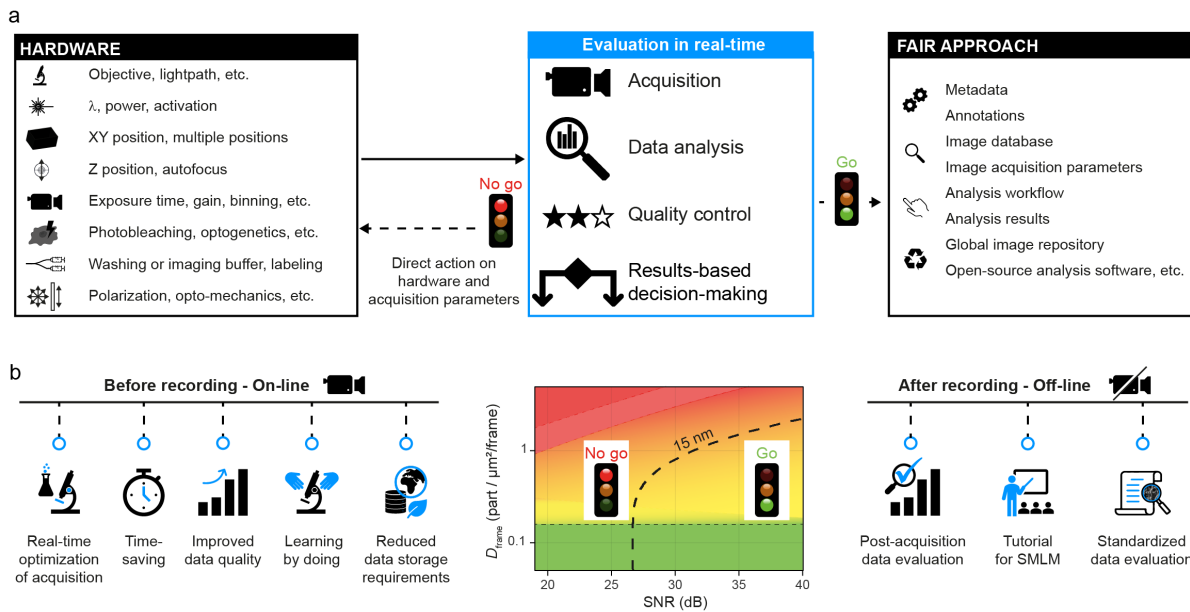
671 **Competing interests:** R.F. is now employee of the Carl Zeiss SAS-France company. The other
672 authors declare no competing interests.

673

674 **Data and materials availability:** The simulated and experimental datasets that illustrated the
675 findings of this study are available from the corresponding authors upon request.

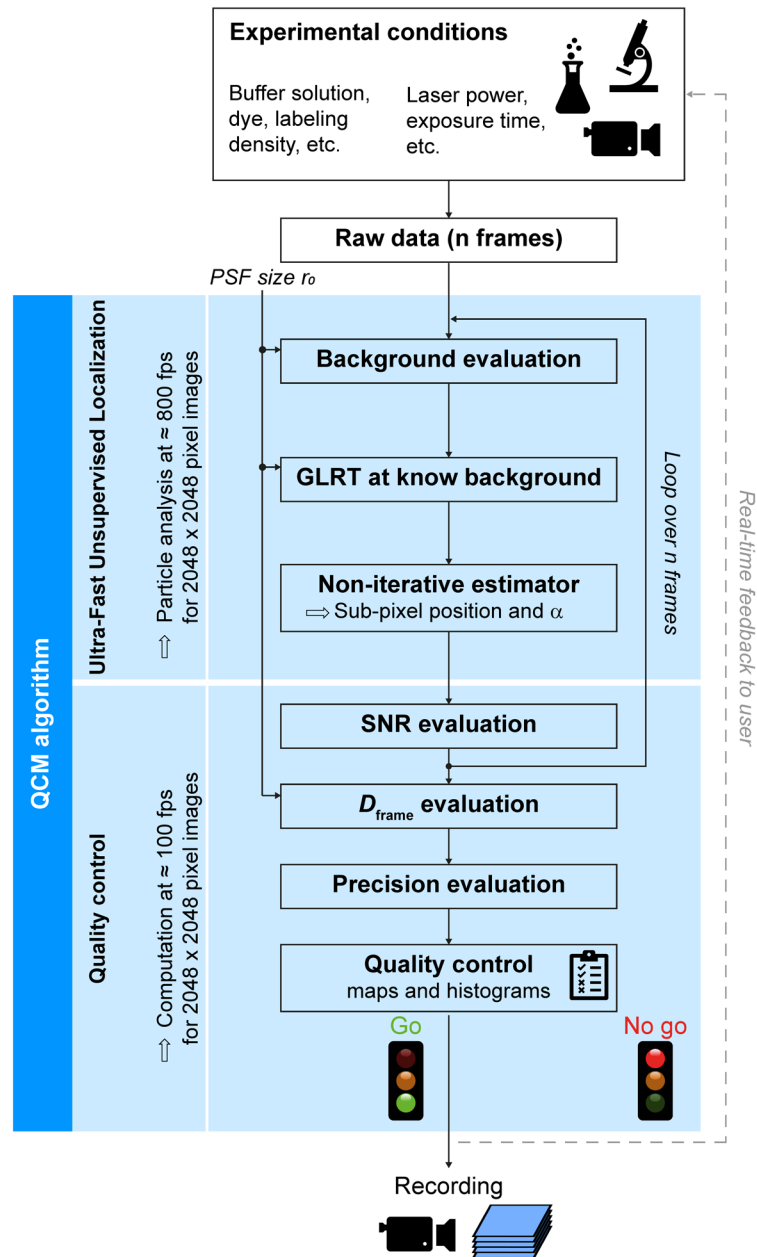
676 The QCM code is protected by the certificate Inter Deposit Digital Number (IDDN):
677 IDDN.FR.001.510001.000.S.C.2022.000.31235 issued by the Agency for the Protection of
678 Programs. The package is freely available online (see Supplementary Information) for
679 academic and nonprofit users. It includes a user guide, a set of experimental and synthetic data,
680 and videos illustrating the visualization and quantification of observations.
681

682 **FIGURES and LEGENDS**



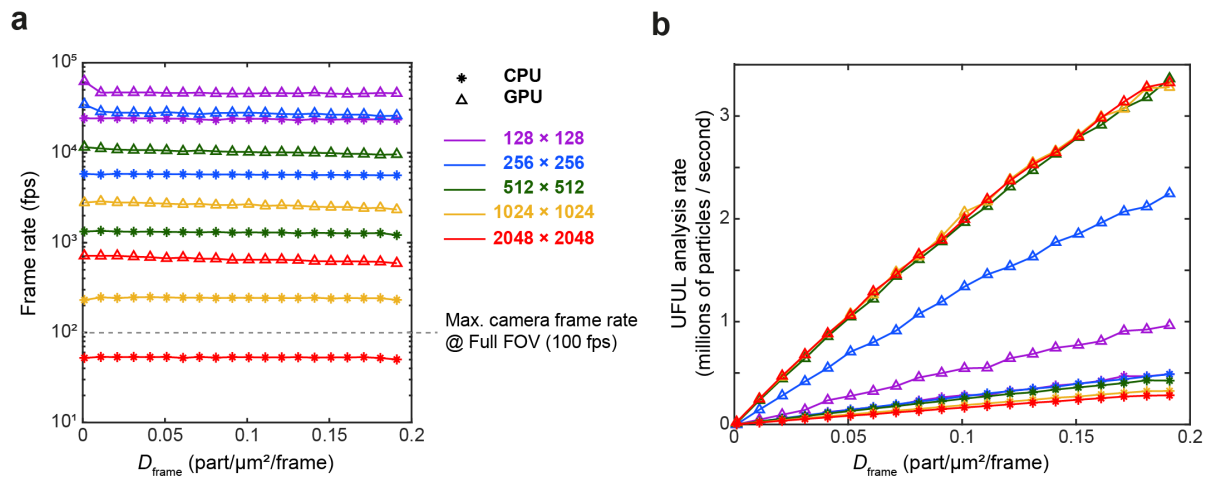
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684 **Fig. 1. A need for comprehensive quality control tools for SMLM acquisitions.** (a) Smart
685 microscopy guidelines aim to integrate quality control tools from the earliest steps of the SMLM
686 acquisition process up to post-process analysis. (b) The density-SNR space diagram (middle
687 panel) summarizes the expected localization accuracy as a function of the two key indicators,
688 SNR and D_{frame} . The black dashed line marks the limit for achieving an overall particle
689 localization accuracy of e.g. 15 nm (35). The on-line mode of QCM processes these key
690 indicators in real-time, providing the instant feedback needed to optimize acquisition
691 parameters prior to data recording (left panel). In addition, the use of the off-line mode in
692 post-acquisition data analysis provides a tool for standardized data review or for teaching
693 SMLM methods (right panel).



694

695 **Fig. 2. QCM workflow.** The QCM algorithm extends from the initial setting of microscope
696 parameters to the decision whether or not to record SMLM data. It combines (1) the Ultra-Fast
697 Unsupervised Localization (UFUL) algorithm to perform the particle detection/localization
698 steps at a rate of ≈ 800 fps for 2048×2048 -pixel images, i.e. at a speed higher than that of
699 image acquisition by current SMLM cameras, with (2) the Quality Control (QC) module for
700 real-time estimation of indicators: D_{frame} , SNR, and localization accuracy. Acquisition
701 parameters that pass quality control criteria are used to start recording data.



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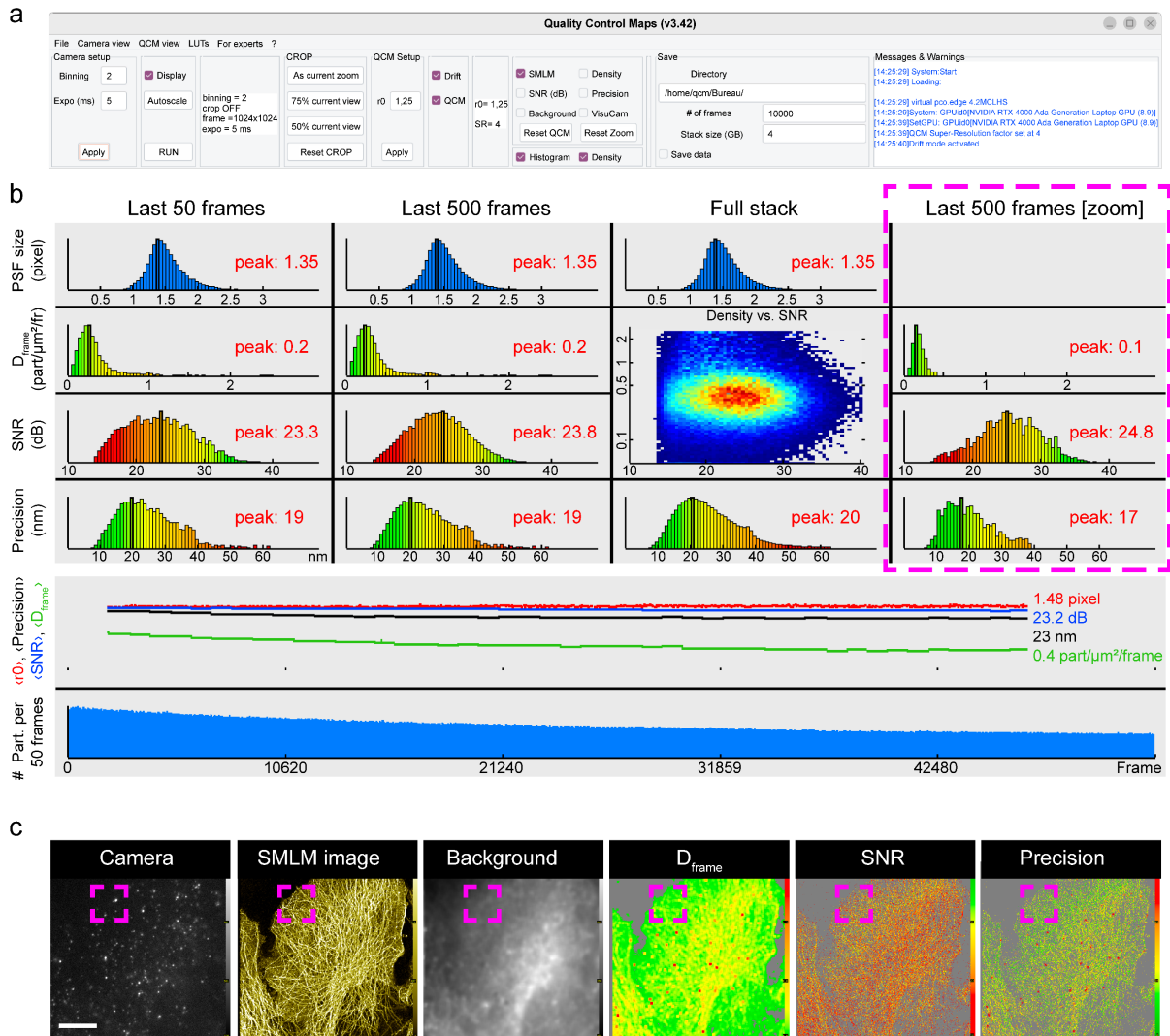
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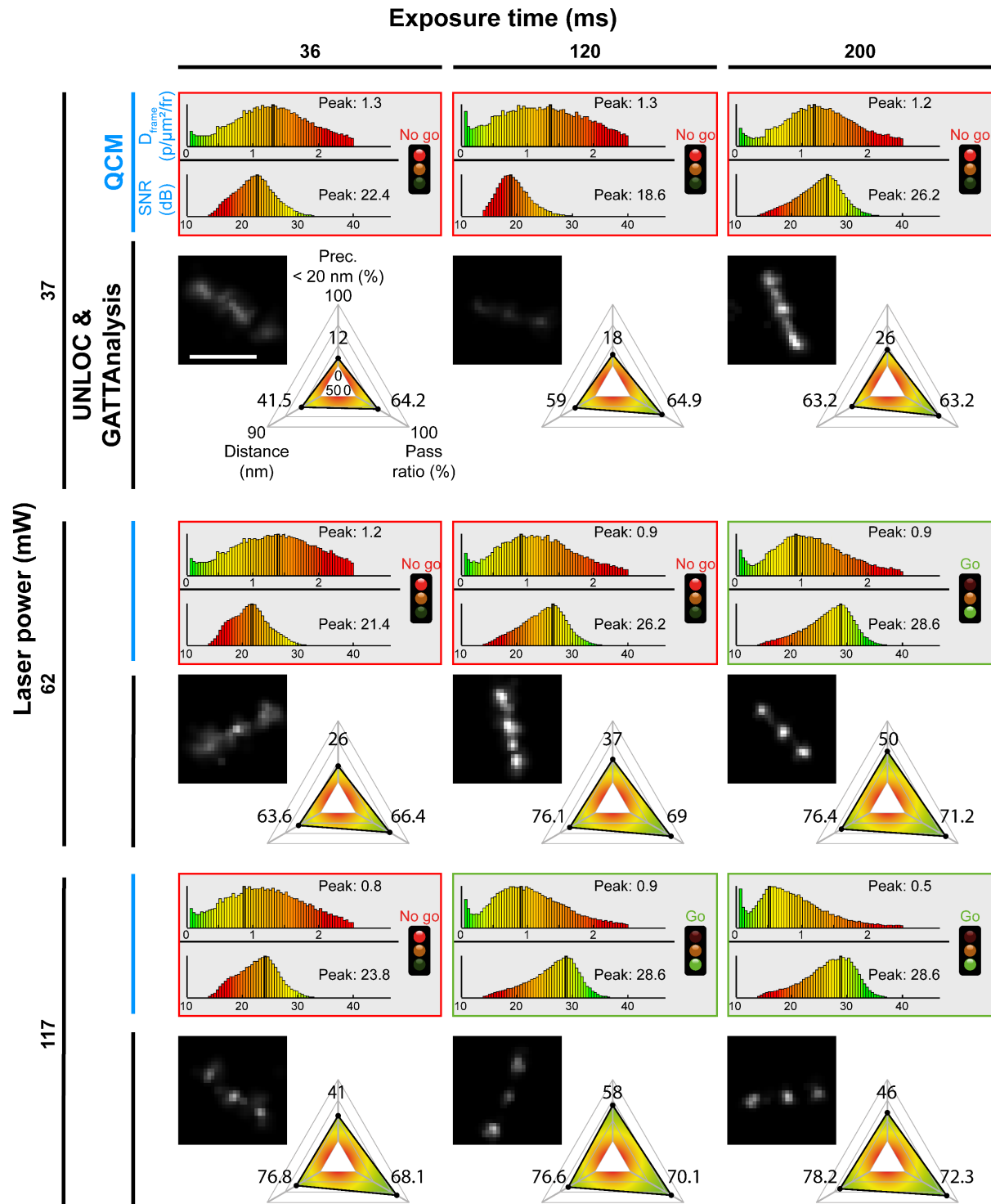
708

Fig. 3. Comparison of CPU and GPU UFUL computation rates as a function of D_{frame} and image sizes on realistic simulated data. (a) Number of frames analyzed per second (fps) based on given D_{frame} values for images ranging from 128×128 to 2048×2048 pixels. (b) UFUL analysis rate expressed as number of particles detected and estimated position per second as a function of D_{frame} for different image sizes.



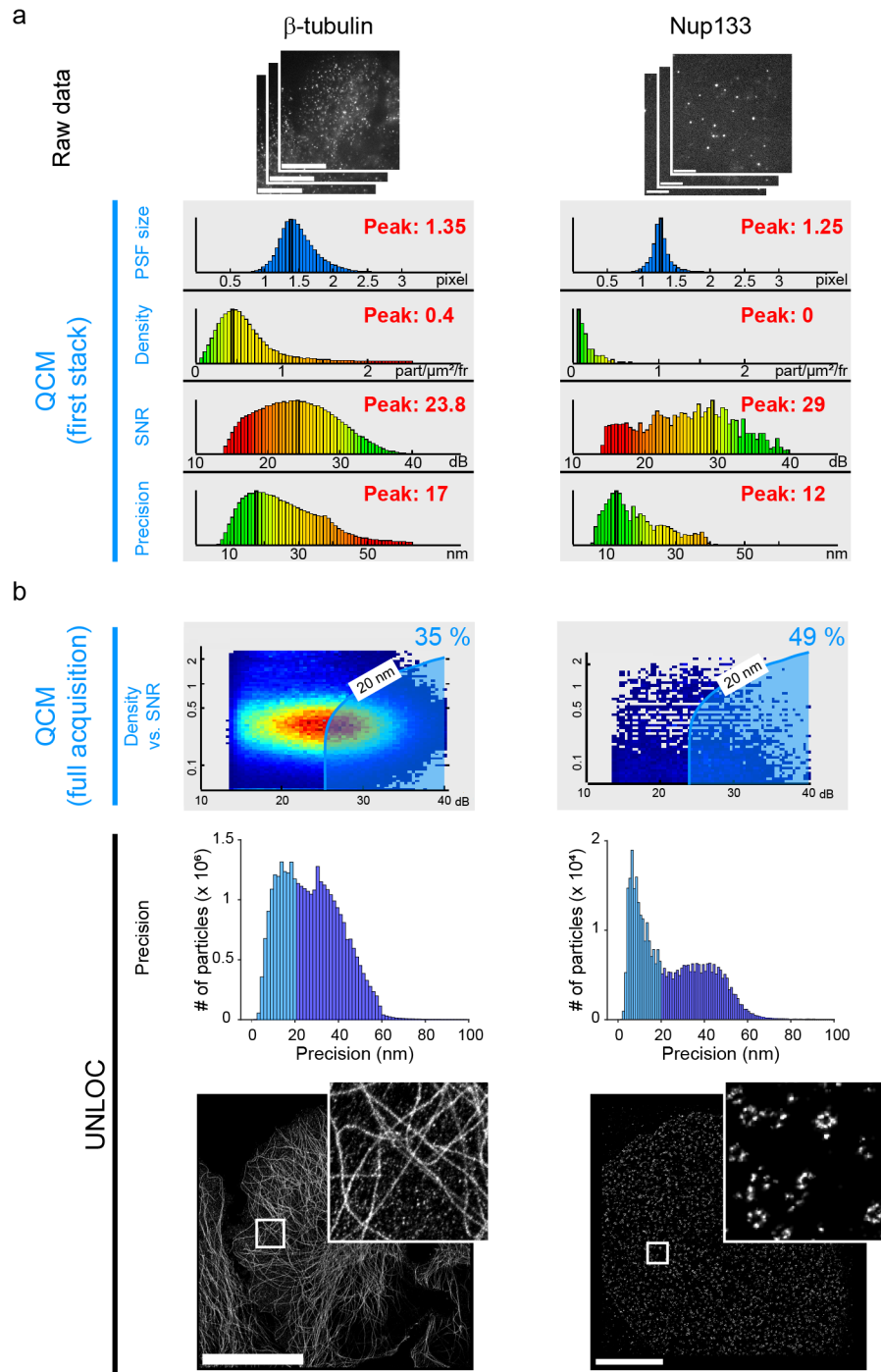
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710 **Fig. 4. General description of the QCM graphical interface.** dSTORM imaging of β -tubulin
 711 in COS-7. **(a)** Main functions from left to right: *camera setup*: binning and exposure time; *QCM*
 712 *setup*: setting the PSF size of the microscope; *Visualization & indicators*: histogram or map
 713 visualization options (see below **(b)** and **(c)**), selection of the QCM calculated parameters; *Save*:
 714 file and data acquisition saving options, and *messages & warnings*. **(b)** Real-time histograms
 715 of the PSF size r_0 (pixels), D_{frame} (particles/ $\mu\text{m}^2/\text{frame}$), SNR (dB), precision (nm), and the
 716 number of particles detected per image can be displayed for the last 50, 500, or cumulative full
 717 field of view (FoV) frames or the last 500 frames on a zoomed ROI. Indicator values are also
 718 traced over time to assess their stability. **(c)** Real-time QCM windows – *Camera*: shows in real-
 719 time the full FoV or zoomed area of a frame recorded by the camera; *SMLM image*: compilation
 720 of detected particle localizations; *Background*: background intensity; D_{frame} : color-coded D_{frame}
 721 values; *SNR*: color-coded SNR values; *Precision*: color-coded of the root mean square particle
 722 precision estimated from the combination of D_{frame} and SNR indicators. Scale bar: 20 μm and
 723 insert 5 μm on a side. For more details on the QCM display, see the user manual.



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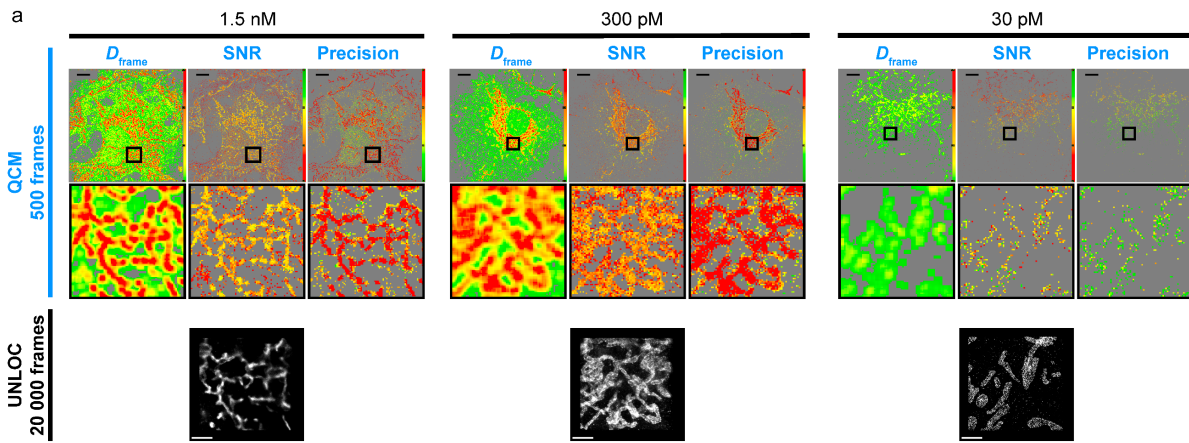
725 **Fig. 5. Validation of the robustness of QCM analyses.** Image stacks of 2,000 frames of DNA
726 origami 80 nm nanorulers were acquired at different camera integration times and laser powers.
727 Data analyzed by QCM (SNR and D_{frame} histograms) were compared with the color-coded
728 spider plots quantified by GATTAnalysis software and the UNLOC results (scale bars: 160 nm)
729 with data at a precision threshold better than 20 nm. The real-time go/no-go decisions based on
730 QCM analyses are in good agreement with the post-acquisition analyses.



731

732 **Fig. 6. QCM-optimized dSTORM acquisitions.** dSTORM imaging of β -tubulin and Nup133
733 labelling in COS-7 and HeLa cells, respectively. (a) QCM histograms and maps were computed
734 from the first 2,000 frames. (b) Post-acquisition analysis of the recorded raw data sets. The
735 density-SNR space diagrams displayed by QCM for β -tubulin and Nup133, reveal that 36%
736 and 49% of detected particles have a localization precision better than 20 nm, respectively.
737 UNLOC show the integrated Gaussian reconstructed images for particles with precision better
738 than 20 nm. Scale bars: 20 μm (inserts: 5 μm on a side) and 5 μm (insert: 1.5 μm on a side) for
739 β -tubulin and Nup133, respectively.

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Fig. 7. QCM-optimized acquisitions of two-color DNA-PAINT data. (a) Detection of TOM20 in HeLa cells by DNA-PAINT imaging on 500 frames. Real-time QCM analyses at different imager concentrations anticipates incorrect and inappropriate acquisitions based on poor D_{frame} , SNR, and precision indicator values at imager concentrations above 30 pM. Post-acquisition analyses validate the quality control observations, as evidenced by the shape of the mitochondrial network in the reconstructed images by UNLOC. Scale bars: 10 μm (in the inserts: 1 μm). (b) Reconstructed images from post-processed α -tubulin and TOM20 data with UNLOC. The concentrations of imagers for two-color DNA-PAINT acquisition with sequential fluid exchange were pre-adjusted with QCM. Scale bars: 20 μm (in the insert: 2 μm).

751 **SUPPLEMENTARY MATERIALS**

752
753 **Supplementary Materials for this manuscript include the following:**

- 754 - A supplementary text for validation of the UFUL module and information on QCM
755 software with figures S1 to S5.
756 - Movies S1 to S4

757
758 **Captions for movies S1 to S4**

759 **Movie S1 – QCM user interface**

760 QCM requires only the setting three physical parameters (camera binning, exposure time and
761 PSF size r_0). QCM in real-time quality control indicators in the form of histograms (SMLM
762 reconstruction, D_{frame} , SNR, Precision, and estimated PSF size r_0) and corresponding maps.

763 **Movie S2 - QCM performances on synthetic data**

764 QCM analysis is performed at 100 fps on 6,000 synthetic 2048×2048 pixel images with spatial
765 densities ranging from 0.005 to 1.5 part/ μm^2 /frame (see Suppl. Fig. 5). Histograms and maps
766 of key indicators are updated instantly when the zoomed window is dragged to another areas of
767 the image.

768 **Movie S3 - Adjustment of acquisition settings based on real-time QCM analyses**

769 Real-time QCM analysis of dSTORM acquisition parameters for β -tubulin imaging in COS-7
770 cells. Histograms and maps of key indicators are updated instantly when the zoomed window
771 is dragged within the image. This allows a close inspection of different ROIs to adjust
772 acquisition parameters in a few hundred images before starting acquisition.

773 **Movie S4 - QCM user interface for multi-color SMLM acquisition**

774 For multi-color SMLM acquisition, the QCM procedure is illustrated on a 256×256 pixel
775 synthetic image dataset. Simulated objects of immunoglobulin-like shape are encoded in three
776 particle types, which are sequentially simulated with parameters specified as follow:

- 777 - channel #1, 5 000 frames with PSF size $r_0 = 1.25 \text{ pixels}$, 20 ms exposure time, $D_{\text{frame}} =$
778 $0.3 \text{ part}/\mu\text{m}^2/\text{frame}$, SNR = 27 dB and a corresponding precision of 17 nm for red
779 fluorescent particles;
780 - channel #2, 10 000 frames with $r_0 = 1.15 \text{ pixels}$, 10 ms exposure time, $D_{\text{frame}} =$
781 $0.1 \text{ part}/\mu\text{m}^2/\text{frame}$, SNR = 30 dB and a corresponding precision of 13 nm for green
782 fluorescent particles;
783 - channel #3, 2 000 frames with $r_0 = 1.45 \text{ pixels}$, 15 ms exposure time, $D_{\text{frame}} =$
784 $0.2 \text{ part}/\mu\text{m}^2/\text{frame}$, SNR = 32 dB and a corresponding precision of 11 nm for blue
785 fluorescent particles.