Parameter-free resolution estimation based on decorrelation analysis

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10   *Abstract*

11   **Super-resolution microscopy opened diverse novel research directions by overcoming the classical**

12   **resolution limit. Revealing structures beyond the diffraction limit was made possible by exploiting the**

13   **fluorescent emission of individual fluorophores. Involving sample properties to apply these techniques**

14   **entails a redefinition of the resolution parameter. Here, we propose a new method for assessing the**

15   **resolution of individual super-resolved images based on image partial phase auto-correlation. The**

16   **novel algorithm is model-free and does not require any user-defined parameters. We demonstrate its**

17   **performance on a wide variety of imaging modalities, including diffraction-limited techniques. Finally,**

18   **we show how our method can be used to optimize image acquisition and post-processing in super-**

19   **resolution microscopy.**

20

Introduction

22   Over the past decades, the field of microscopy was enriched with a broad range of novel imaging

23   methods, providing unprecedented insights into sub-cellular structures[1,2]. When designing a microscopy

24   experiment, one has to select an appropriate imaging modality taking into account the required spatial

25   and temporal resolution. The image quality greatly varies among different techniques and is influenced

26   by sample properties. Image formation for all microscopy techniques (coherent or incoherent,

27   diffraction-limited or super-resolution imaging) can be modelled as the convolution of a ground-truth

28   object with the specific point-spread function plus various method-related noise contributions. In Fourier

29   space, the object spectrum is multiplied by the transfer function of the system. The shape of the transfer

30   function depends on the imaging method employed, but common to all techniques is image low-pass

31   filtering, characterized by a cut-off frequency. This spatial frequency limit already known to Abbe[3] is

32   generally expressed as $k_c = NA\frac{2\pi}{\lambda}$, where NA is the sine of the maximum collection angle multiplied by

33   the refractive index and λ the illumination central wavelength, and corresponds to the resolution in

34   coherent imaging.

35   Super-resolution techniques overcome the diffraction limit by exploiting specific fluorophore

36   properties such as stimulated emission or temporal fluctuations. Therefore, image resolution needs to

37   be reconsidered by taking into account the fluorescent properties of the sample to establish a novel

38   resolution measure for super-resolved imaging[4]. Ideally, this resolution criterion should work on a single

39   image, be independent of the imaging method, have no user-dependent settings and be compatible with

40   classical resolution. Such an estimator of resolution is of particular interest for autonomous adaptive

41   microscopes[5,6] that require robust tools to automatically achieve and maintain optimal performance in

42   long-term imaging of biological samples.

43   In 1982, van Heel[7] and Saxton[8] independently proposed the use of Fourier Ring Correlation (FRC)

44   using two independent images of the same object for resolution estimation of electron microscopy

45   images. The concept was quickly expanded to 3D via Fourier Shell Correlation by Harauz[9] and several

46   threshold concepts have been proposed (0.5, 0.143[10], 2σ[8,11], SSNR[12]) to extract a resolution measure.

47   Later, Banterle[13] and Nieuwenhuizen[14] independently extended, reconsidered and applied the method

48   to assess the resolution of single-molecule localization microscopy (SMLM) images. Similar to electron

49   microscopy, their method requires two stochastically-independent images of the same object and

50   estimates the resolution by computing cross-correlations of Fourier space rings. The cut-off frequency is

51   defined as the spatial frequency where the so-called FRC curve drops below a fixed value equal to 0.143.

52   In SMLM, the two image realizations are typically accomplished by splitting the image series, i.e.

53   the localizations, into two distinct subsets. In general, it can be achieved for any imaging technique[15] by

54   acquiring two consecutive images under the same conditions. However, maintaining these conditions

55   may be difficult due to, for example, bleaching or temporal fluctuations of the fluorescence signal. This

56   is especially pertinent in live-cell imaging and significantly challenges the stationarity assumption of FRC.

57   Here, we propose a new method able to estimate the resolution based merely on an individual

58   image without any further requirement or a priori knowledge. The algorithm expects only a non-

59   saturated, bandwidth-limited signal with adequate spatial sampling. This novel estimator is based on

60   partial phase correlation and does not rely on any user-defined parameters. The algorithm is fast, uses

61   only linear operations and enables the real-time objective assessment of image resolution and Signal to

62   Noise Ratio (SNR). We successfully applied our new approach on a variety of microscopy data sets,

63   ranging from widefield imaging to SMLM and STED microscopy. We show that our estimator can also be

64   used to optimize image resolution, both during experiments and throughout data post-processing.

65

66   *Method*

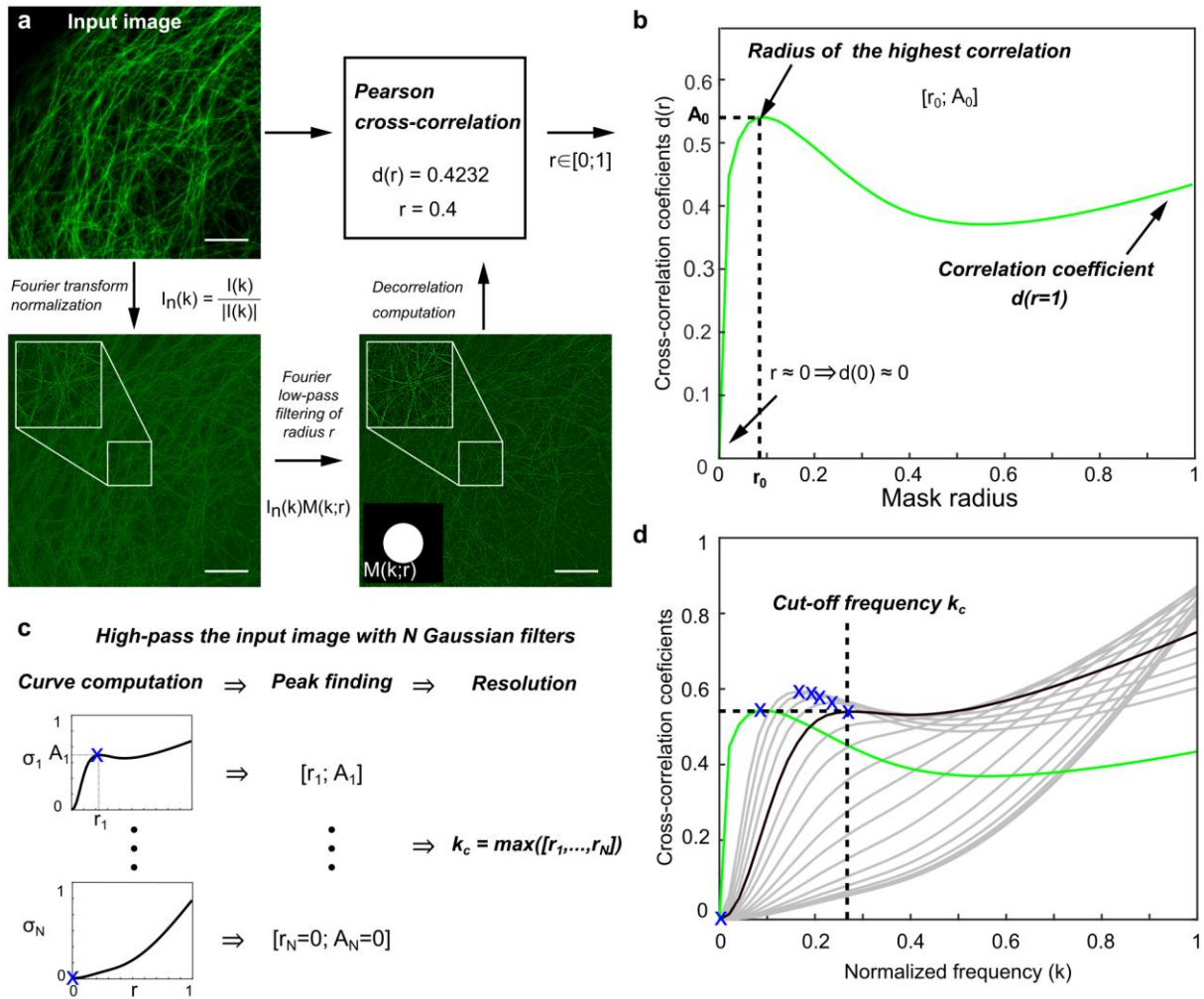67   **Decorrelation analysis**

68   To achieve objective threshold-free resolution estimation, we introduce a processing method

69   termed decorrelation analysis. The main algorithm is divided into two steps. First, the Fourier transform

70   of the image is computed after a standard edge apodization to suppress high-frequency artefacts. The

71   Fourier transform is normalized as $I_n(\boldsymbol{k}) = \frac{I(\boldsymbol{k})}{|I(\boldsymbol{k})|}$. The input image $I(\boldsymbol{k})$ and its normalized version $I_n(\boldsymbol{k})$

72   are then cross-correlated in Fourier space using Pearson correlation and condensed in a single value

73   between 0 and 1 (Fig. 1a). Second, the operation is repeated, but the normalized Fourier transform is

74   additionally filtered by a binary circular mask of radius $r \in [0,1]$ expressed in normalized frequencies

75   (Fig. 1b). By repeating the calculation, we compute $d(r)$ which is expressed as

$$d(r) = \frac{\iint Re\{I(\boldsymbol{k})I_n^*(\boldsymbol{k})M(\boldsymbol{k};r)\}\,dk_x dk_y}{\sqrt{\iint |I(\boldsymbol{k})|^2 dk_x dk_y \iint |I_n(\boldsymbol{k})M(\boldsymbol{k};r)|^2 dk_x dk_y}}, \tag{1}$$

76   where $\boldsymbol{k} = [k_x, k_y]$ denotes Fourier space coordinates, $I(\boldsymbol{k})$ the Fourier transform of the input image,

77    $I_n(\boldsymbol{k})$ the normalized Fourier transform and $M(\boldsymbol{k};r)$ the binary mask of radius r. $\iint$ is the double integral

78   over k_x and k_y. For a detailed mathematical derivation and additional considerations, see

79   Supplementary Material Section 1.

80   The core idea of the method is that by normalizing the Fourier transform of the input image, we balance

81   the signal and noise contributions while the information of the object structure is preserved in the phase

82   (the phase is responsible for organizing the constructive and destructive interferences of the complex

83   exponentials to form the image, the amplitude plays only a minor role in this process). Taking a radius of

84   the binary mask equal to 1 allows the extraction of the correlation value related to the original ratio of

85   signal and noise. If we consider an image containing only white noise, we see that $d(r = 1) \approx 1$, since

86   the white noise power spectrum is constant by definition (the normalization does not affect the signal).

87   If we add a bandwidth-limited signal to the image, the correlation value for $r = 1$ will decrease (the

88   normalization now has a direct effect on the added signal and thus $I_n(\boldsymbol{k})$ only partially correlates with

89    $I(\boldsymbol{k})$).

90   By decreasing the radius of the mask ($r < 1$), we progressively remove the noise contribution but

91   preserve the signal due to its bandwidth-limited nature. If the image contains only noise, the cross-

92

98   correlation value will decrease linearly as a function of radius r. If we now add a signal, the decorrelation

99   function $d(r)$ will exhibit a local maximum of amplitude A_0 that indicates the spatial frequency r_0 of best

100   noise rejection and signal preservation ratio. Restricting the mask further removes more signal than

101   noise, therefore reducing the correlation below A_0 until it drops to 0 for $r = 0$. The position r_0 of the

102   local maximum is therefore directly related to the spatial frequency distribution of the image and its

103   amplitude A_0 is positively correlated with the image Signal-to-Noise Ratio (see Supplementary Fig. S1 for

104   detailed plots of decorrelation function behaviour with respect to different noise statistics, aberrated

105   transfer functions, various cut-off frequencies, various SNRs and high-pass filtering strength). For a

106   detailed description of the algorithm, see Supplementary Material Section 1.1.

107   While being related to the spatial frequency content of the image, the position of the maximum

108   does not directly indicate the resolution of the image. The input image is then subjected to a total of N_g

109   high-pass filters (spanning the range from weak to very strong filtering) in order to attenuate the energy

110   of the low frequencies. For each filtered image, a decorrelation function is computed and the peak

111   position r_i and amplitude A_i are extracted, generating a set of $[r_i, A_i]$ pairs (Fig. 1c). If the high-pass

112   filtering removes too much signal, the decorrelation function will not exhibit a local maximum and the

113   peak position and amplitude will both be set to 0. We investigated two different strategies for the

114   resolution estimation: selecting the highest frequency peak or selecting the peak corresponding to the

115   highest geometric mean $\left(GM = \sqrt{r_i A_i} \right)$ (giving the same weight to the position and amplitude). After

116   processing several simulations and microscopy images taken from different imaging modalities including

117   Confocal, STED, SIM, SOFI and PAINT, we observed that in all cases but confocal images, both criteria

118   provide identical estimates of resolution (see Supplementary Material Section 1.2). We consequently

119   define the resolution estimate as

$$k_c = \max\left[r_0, \dots, r_{N_g} \right] \tag{2}$$

120   which corresponds to the local maximum of highest frequency (Fig. 1d). The resolution is then

121    $resolution = \frac{2*pixel\ size}{k_c}$, where k_c is expressed in normalized frequencies. By computing the resolution

122   with a varying sampling of $d(r)$ and a varying number of high-pass filtering N_g, we confirm the

123   robustness of the algorithm and estimate the precision of the algorithm to be about ± 1 to 3nm,

124   independently of the type of image (see Supplementary Material Fig. S2). We confirmed, via simulations

125   of point emitters, MTFs, rings and crossing lines, that our resolution estimate depends linearly on the

126   frequency support of the image (see Supplementary Material Section 2.1, 2.2 and 2.3) and that the

127   amplitude of the local maximum A_0 before any filtering is directly correlated with the image SNR.

128   Instead of searching for the frequency at which the transfer function vanishes (which can only

129   be measured in the absence of noise), we estimate the highest frequency from the local maxima of the

130   decorrelation functions, enabling parameter-free image resolution estimation. The presented method

131   does not estimate the theoretical resolution as stated by Abbe, but rather the highest frequency with

132   high enough signal with respect to the noise. It provides a rapid and objective way to quantify the

133   frequency content of a single image without any user-defined parameter.
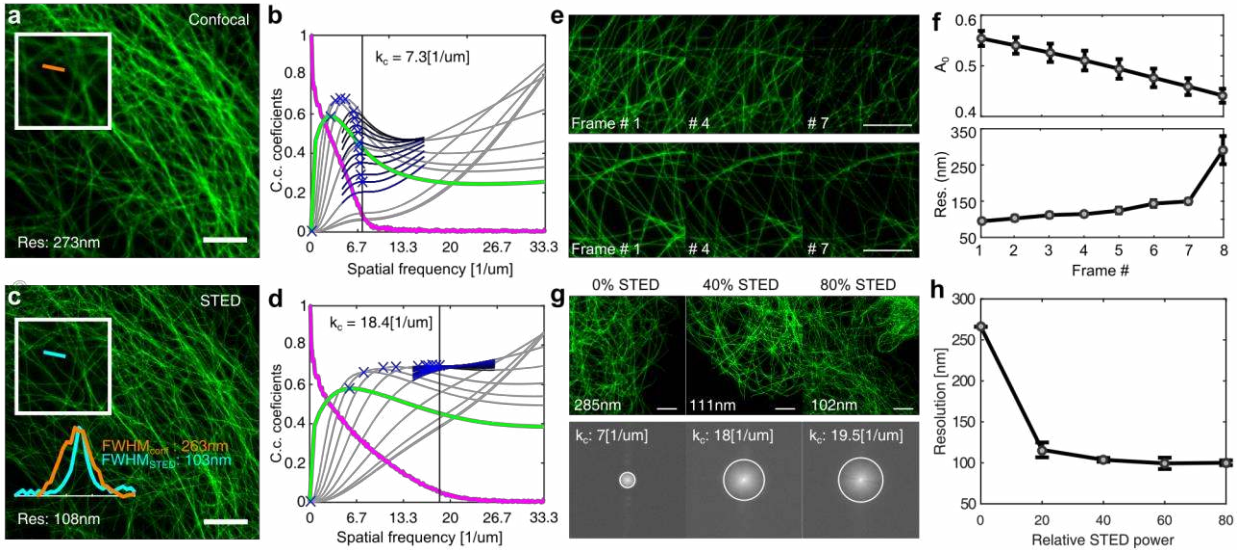
134   *Results*

135   To demonstrate the validity and broad applicability of the method, we processed nanorulers data

136   provided by GATTAquant (courtesy of P. Tinnefeld and J. Schmied). DNA origami nanorulers emerged as

137   a platform for reliable performance evaluation across different super-resolution modalities. Their

138   adaptability is due to the fact that one can design these self-assembled nanostructures by placing a

139   defined number of fluorescent dye molecules in precise geometries[16]. The resolutions estimated by our

140   algorithm are smaller than the mark-to-mark distances of the nanorulers, which corroborates the fact

141   that they are resolved in all imaging modalities (see Supplementary Material Section 3). After establishing

142   the legitimacy of our method on the DNA origami samples, we extended our analysis to various

143   diffraction-limited (see Supplementary Material Section 4 for bright-field imaging) and super-resolution

144   microscopy images of biological structures, presented in the following. By imaging a z-stack of fluorescent

145   beads, we validated that our resolution estimate fits the expected resolution well. Our estimator

146   provides a unique tool to assess the alignment of an optical setup or the performance of a microscope

147   objective lens using a single experimental image (see Supplementary Material Section 5). All the

148   presented results have been processed using custom Matlab code (source code publicly available at

149   https://github.com/Ades91/ImDecorr.git). For ease of use, the algorithm has also been implemented in

150   Java and is available as an open source ImageJ[17] plugin (see Supplementary Material Section 6).

151   **Confocal/STED**

152   We started with confocal[18] and Stimulated Emission Depletion (STED) microscopy[19,20], both

153   point-scanning techniques that can be realized on the same setup thus allowing the transition from

154   diffraction-limited to super-resolution imaging. STED microscopy is a super-resolution method that uses

155   confocal illumination to excite the fluorophore and a donut-shaped depletion beam to de-excite (via

156   stimulated emission) most of the surrounding fluorophores prior to fluorescence emission. The

157   resolution of STED microscopy for a given fluorophore is dependent on the spatial and temporal co-

158   alignment of the two beams[21], the shape, and the quality of the depletion beam and its power[22].

159   Using a commercial STED microscope (Leica TCS SP8 3D STED), typically available in state-of-the-

160   art imaging facilities, we imaged the microtubule network of COS-7 cells immuno-labelled with Abberior

161   Star635P, both in confocal and STED mode (pulsed fluorescence excitation at 635nm and pulsed STED

162   depletion laser at 775nm). Fig. 2a-d show the resulting images and their corresponding decorrelation

163   analysis. Throughout the manuscript, we used the following convention: the green line and the grey lines

164   are the original decorrelation functions prior to high-pass filtering and post-high-pass filtering; the blue

165   to black lines are the decorrelation functions with refined mask radius and high-pass filtering range; the

178 blue crosses indicate all the local maxima; the magenta line is the normalized radial-averaged log of the

179 absolute value of image Fourier transform. The image resolution is indicated as a black vertical line. As

180 expected, all the decorrelation functions exhibit a local maximum, with STED showing a 2.52-fold

181 resolution improvement over confocal imaging. We plotted in Fig. 2c the manually selected line profile

182 of a microtubule cross-section, a method typically used to estimate the resolution. The measured

183 FWHMs are in good agreement with our estimates.

184   Fig. 2e and f show the result of the analysis performed on a time series of eight consecutive STED

185 images (each STED image is the average of 8 scans; acquisition time per STED image of 15 sec) and a total

186 of four sequences (only two shown here). As expected, repeated imaging of the same structure gradually

187 bleaches the fluorophores, progressively degrading the SNR as indicated by the parameter A_0 . We also

188 observe a consistent deterioration of the resolution until the 7th frame, where we reach a resolution

189   close to the one observed in the confocal image. The bleaching is so strong that the structure is no longer

190   continuous. The proposed method confirms the expected degradation of image resolution and provides

191   a quantitative estimation of the image SNR and resolution. Fig. 2g and h illustrate how our resolution

192   estimation can be used to optimize STED imaging. Imaging under several STED illumination powers was

193   performed, always adjusting the excitation power to maintain the optimal dynamic range of the image

194   to avoid noise-limitation of STED resolution[15] (for a total of 5 images per STED power, pixel size 40 nm).

195   We see that using 20% of STED laser power has a significant improvement on the resolution (about 2.4

196   fold) but doubling the power only decreases the resolution by a factor of 1.1 as expected due to the non-

197   linear behaviour of STED. Further increases in STED power do not lead to significant changes in

198   resolution, possibly due to a misalignment of the excitation and STED beam, imperfect "zero" of the STED

199   doughnut as well as excess photo-bleaching and background induced by the STED beam[20]. We conducted

200   additional experiments, investigating further acquisition parameters such as the STED delay, pixel size

201   and comparing the performance of different dyes to choose the optimal label (see Supplementary

202   Material Section 7). STED microscopy critically depends on photophysics that can also be exploited to

203   increase the resolution (lifetime[21], photostability[23], spectra[24], etc.). For pulsed STED experiments, the

204   best resolution is reached when the depletion pulses immediately follow the excitation pulse. We used

205   our resolution estimate as a readout to adjust the delay between the pulses in the Leica system. Figure

206   S14c shows a drastic improvement in resolution at about $\Delta t = 1800$ps. Previously, an indirect strategy

207   based on minimizing the remaining fluorescence intensity[25] or an FRC resolution estimate was used[15].

208   Our algorithm provides a direct and straightforward estimation of image resolution that can be used to

209   objectively find the best acquisition settings and optimize sample preparation, including choice of dye,

210   based on a single image of the sample of interest without imposing additional requirements on the data

211   acquisition scheme. In principle, it should as well be possible to use our resolution estimate to tune the

212   microscope alignment, e.g. to adjust the overlap of STED donut with confocal excitation spot.

213 **WF/SIM**

214   STED has also been used for high-resolution live-cell imaging, but care should be taken to avoid

215 sample damage due to high-depletion laser powers by special imaging procedures[20]. A super-resolution

216 method that is widely used for imaging dynamics of living cells is Structured Illumination Microscopy[26–28]

217 (SIM). SIM aims at improving the lateral and axial resolution by multiple imaging of the sample with high-

218 frequency illumination patterns[29]. The theoretical resolution improvement of SIM is linked to the

219 frequency of the illumination. In practice, SIM resolution depends on the pattern modulation contrast,

220 refractive index mismatch and local distortion of the pattern[30]. Fig. 3a shows the analysis of a pseudo

221 widefield image, obtained by averaging the raw SIM sequence of actin filaments in U2OS cells[31] labelled

222 with Phalloidin-Atto488 (obtained on a Delta-Vision|OMX v4, courtesy of T. Huser). A resolution of about

223 253 nm is estimated by decorrelation analysis (cut-off of $7.9[1/um]$, the pixel size of 80 nm; Fig. 3b). Fig.

224 3c shows the SIM reconstruction (see Supplementary Material Section 8 for the details of the

225 reconstruction) of Fig. 3a and its corresponding decorrelation analysis (cut-off of $12.4[1/um]$, the pixel

226 size of 45.8 nm; Fig. 3d). We measure a resolution improvement of about 1.56. Measuring the position

227 of the illumination peaks in the Fourier transform of the raw data (4.75 and $9.5[1/um]$ for the first and

228 second diffraction order respectively), provides a way to estimate the theoretically expected resolution

229 improvement. We observe that the contribution of the first diffraction order with the wide-field

230 resolution $\left(\frac{7.9+4.75}{7.9} \approx 1.6\right)$ fits well with our estimation, indicating that the information encoded in the

231 second diffraction order is not sufficiently contrasted. The use of more advanced reconstruction

232 algorithms may improve this result.

233   So far, we only considered the global resolution, i.e. averaged over the whole image and in all

234 directions. In order to account for non-isotropic resolution, we subdivide the Fourier space in sectors and

235 compute the cut-off frequency as a function of the direction. This is referred to as sectorial resolution

236 (see Supplementary Material Section 9). Fig. 3e shows a different pseudo widefield and SIM

237 reconstruction of the mitochondria network[31] in U2OS cells (measured on a Zeiss Elyra S1, courtesy of

238 M. Sauer, resolution gain of 1.73). Fig.3f displays the Fourier transform of the SIM reconstruction,

239 overlapped with the sectorial resolution (solid white line) and the average resolution (dashed white
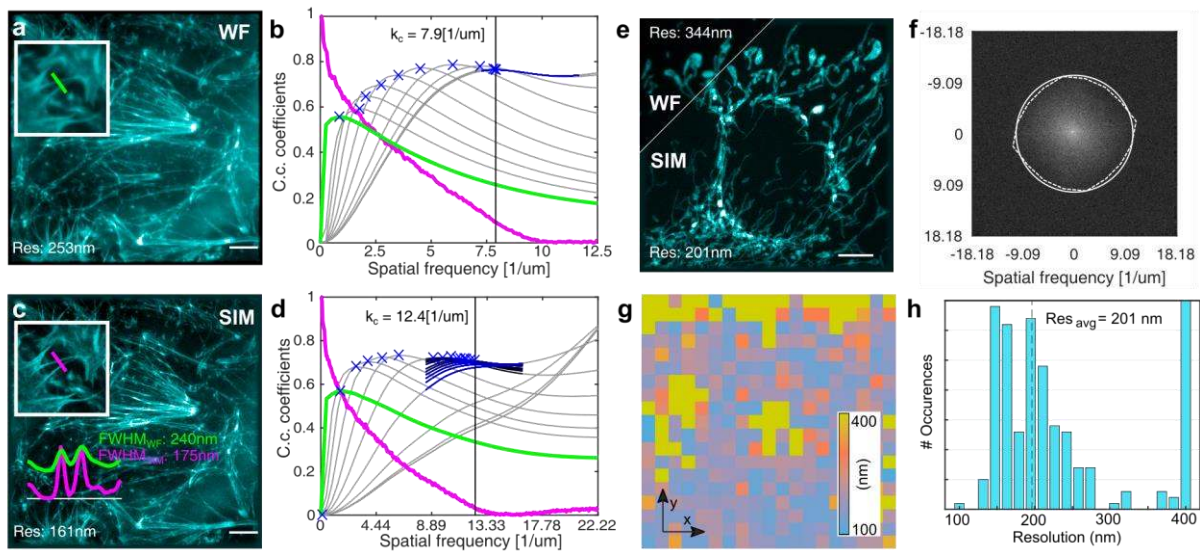
240

241 *Figure 3: Widefield and SIM (a) Pseudo widefield image of the actin network in fixed U2OS cells labeled with phalloidin-Atto 488*
242 *(Courtesy of T. Huser). (b) Corresponding decorrelation analysis. (c) SIM reconstruction of (a) with selected cross-sections. (d)*
243 *Corresponding decorrelation analysis. (e) Pseudo widefield and SIM reconstruction of mitochondria network in U2OS cells labeled*
244 *with mitotracker (Courtesy of M. Sauer). (f) Sectorial resolution estimation (dashed white line) and average resolution (solid*
245 *white circle). (g) Local resolution estimate of (e). (h) Histogram of local resolution shown in (g). Scale bar, 5 µm. Image acquisition*
246 *and sample details are provided in Table S1.*

247 circle). Finally, by subdividing the image into smaller tiles (as was done for FRC in[14]) (70x70 pixels with an

248 overlap of 20 pixels), we can estimate the resolution over the whole field-of-view and reveal local

249 variations in resolution (Fig. 3g). Due to the very weak signal in the top and center part of Fig.3e,

250 corresponding sub-regions have very large resolution values. In order to preserve the dynamic range, all

251 resolutions larger than 400nm were set to 400nm. Furthermore, the resolution map can be plotted as a

252 histogram of resolutions (Fig. 3h), providing another perspective of the image. We also see that the

253 average resolution is approximatively the median of all local resolutions.

254 **SOFI/Deconvolution**

13

255   Sub-diffraction imaging can also be achieved by analysing a time series of stochastically blinking

256   emitters. Stochastic Optical Fluctuation Imaging[32,33] (SOFI) achieves super-resolution by computing high-

257   order spatiotemporal cumulants. SOFI processing is of interest to analyse because it predicts a resolution

258   improvement of $1/\sqrt{n}$, where n is the correlation order and $1/n$ after deconvolution and brightness

259   linearization. It provides an ideal test case for our resolution estimator.

260   Fig. 4a shows the results of SOFI analysis (up to 6th order) of MEF cells expressing paxillin labelled

261   with mEos234 (courtesy of H. Deschout). Fig. 4b displays the results of the decorrelation analysis, where

262   the raw cumulants follow the theoretical resolution improvement up to 70 nm for 6th order linearized

263   SOFI, indicating good blinking statistics. The deviation observed for the widefield (average of the whole

264   sequence, here denoted as SOFI 1) and 2nd order SOFI can be attributed to sub-optimal out-of-focus

265   light rejection. Similarly, the linearized SOFI cumulants, obtained by 10 iterations of Lucy-Ridcharson

266   deconvolution and taking the nth root of the SOFI image, follow a similar trend close to the theoretical

267   value. The deconvolution operation by itself constitutes an interesting case study for our algorithm. We

268   show (see Supplementary Material Section 10) that the resolution can be set to any value, as a function

269   of the input point-spread function and number of iterations of the deconvolution. It is the duty of the

270   user to ensure that the resolution is not enhanced beyond the limit supported by the microscopy

271   method. Our algorithm can thus be used to quantify deconvolution strength but not the validity of the

272   deconvolution.

273   To minimize bleaching and drift artefacts, practical SOFI processing is achieved by first

274   subdividing the whole acquisition into sub-sequences, computing SOFI and averaging the results of the

275   sub-sequences[35,36]. By using our decorrelation analysis over the resulting SOFI image for various sub-

276   sequences lengths, we are able to identify the optimal resolution-SNR sub-sequence length for the given

277   input data. Fig. 4c shows the results of such an analysis (sub-sequence length ranging from 50 to 4000

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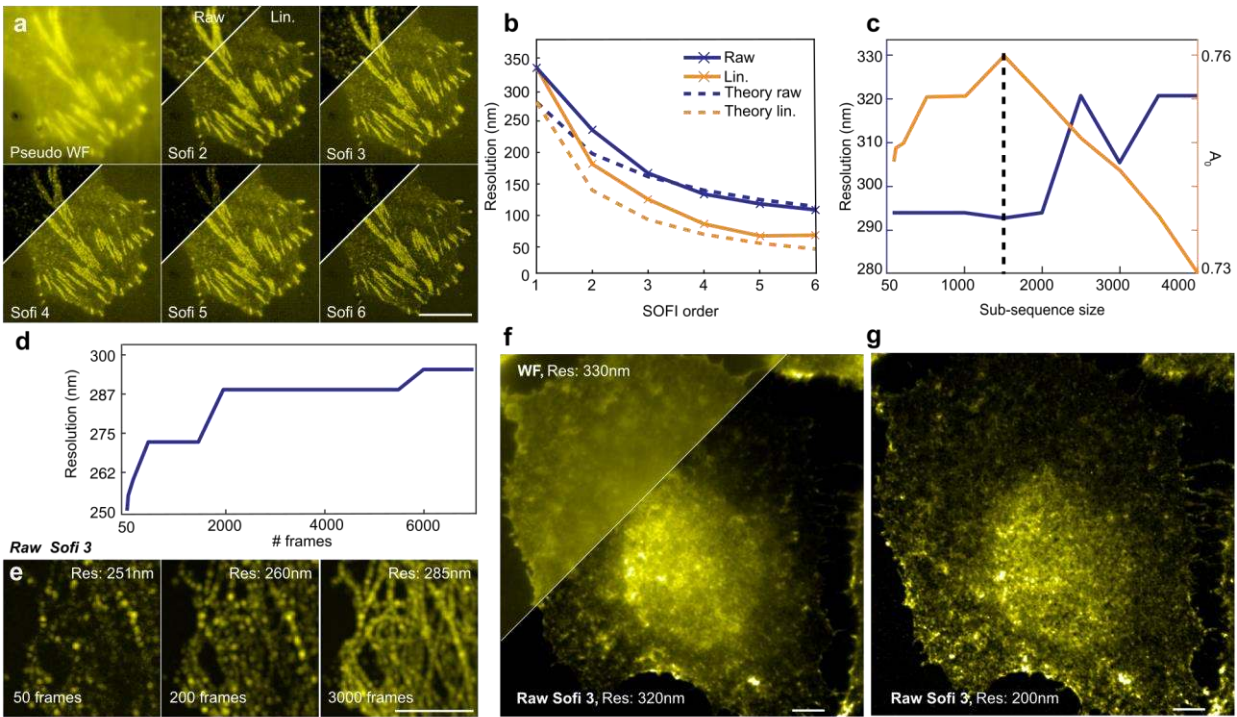
Figure 4: SOFI (a) Raw SOFI and Linearized SOFI images of focal adhesions in MEF cells expressing paxillin-mEos2 (courtesy of H. Deschout) shown up to order 6. (b) Estimated and theoretical resolution vs the SOFI order. (c-e) Cumulant analysis of microtubules in HeLa cells immunolabeled with Alexa Fluor 647. (c) Resolution and SNR estimate vs the sub-sequence size used for SOFI processing. (d) Resolution estimate vs number of frames. (e) Raw SOFI 3 images for 50, 200 and 3000 frames. (f) Un-optimized raw SOFI image of fixed HeLa cells labeled with wheat germ agglutinin-Atto 565 using "default" processing parameters. (g) Same images as (f) after optimization of the resolution. Scale bar, 5 μm. Image acquisition and sample details are provided in Table S1.
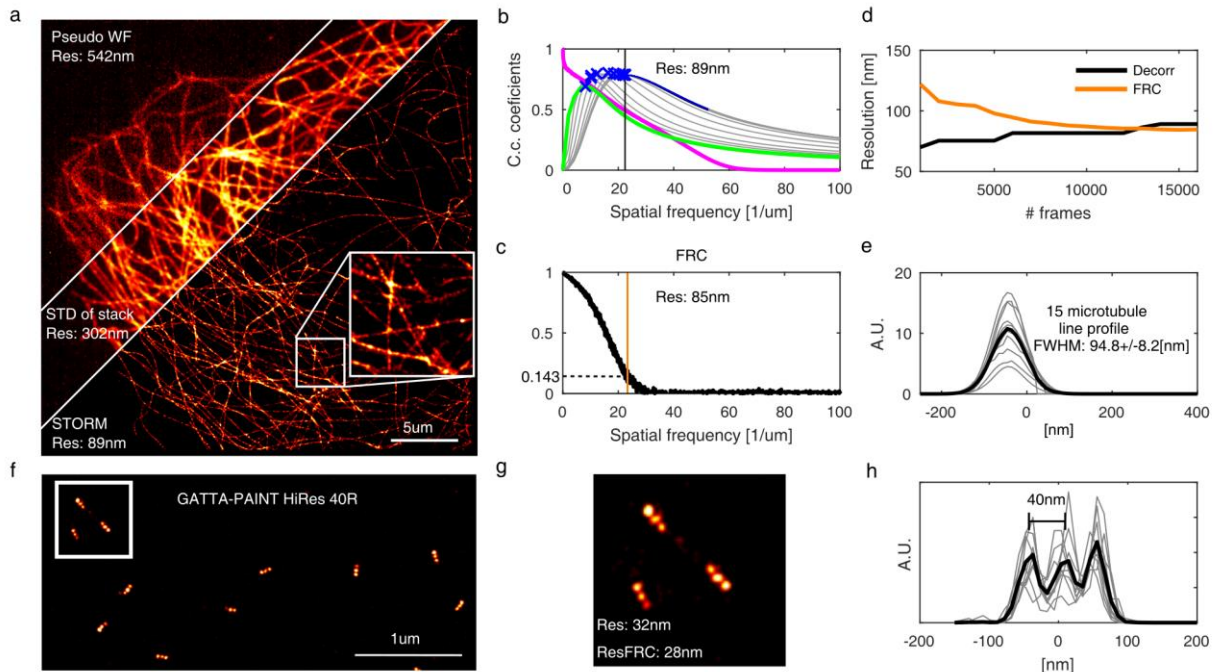
frames), performed on a sequence of blinking Alexa647 targeting microtubules of HeLa cell (images shown in Supplementary Material Section 8, Fig. S6a, 8000 frames in total). For this data, we found an optimal sub-sequence length of 1500 frames.

Fig. 4d and 4e demonstrate how the total number of frames used to compute SOFI impacts the resolution in HeLa-cell microtubules labelled with Alexa 647. We see that 50 frames already allow the computation of a 3rd order SOFI image. However, due to the blinking kinetics, many more frames are required to properly resolve the underlying structure. We also see that the resolution is slightly better when the image consists only of sparse point-like structures. This is due to the fact that our algorithm estimates the resolution from a single image. Using only 50 frames, the algorithm sees a sparse distribution of high-frequency dots. As we include more frames in the analysis, a larger-scale structure

15

296   containing more low frequencies starts to emerge, leading to a slightly modified resolution estimate.

297   Finally, Fig. 4f shows a raw 3rd order SOFI image of HeLa cells stained with wheat germ agglutinin-Alexa

298   488, processed with default parameters (first 1000 frames removed and sub-sequence length of 500

299   frames). Fig. 4g shows the same image, after optimization of the sub-sequence length and the number

300   of frames to be removed at the beginning and the end of the acquired data to obtain the best resolution

301   (1800 first frames removed, subsequence length of 1000, no frames removed at the end of the

302   sequence). The optimized processing procedure results in a 1.6-fold improvement of resolution

303   compared to the starting image.

304   **Localization microscopy**

305   Finally, we applied our method on Single Molecule Localization Microscopy[37–39] (SMLM) data. In this case,

306   super-resolution is achieved by the individual localization of a subset of sparsely and stochastically

307   blinking emitters in successive image frames. By fitting the emission point-spread functions, single- and

308   multiple-emitter positions can be determined with nanometric accuracy[40,41]. To estimate the resolution,

309   our method requires a rendered image. This is typically realized by replacing a filtered set of localization

310   events by a 2D Gaussian of standard deviation equal to the localization uncertainty. The filtering step

311   consists of rejecting unphysical or poor localization events. We validated, using simulations, that our

312   algorithm is also able to correctly estimate the resolution of a localization-based image (see

313   Supplementary Material, Section 11 and Fig. S17). Fig. 5a shows a STORM image of immuno-labelled

314   microtubules (Abberior Flip 565, 16000 frames, localized using single-emitter least square fitting in

315   ThunderSTORM[42] with default parameters). Each localization was rendered (in Matlab, the pixel size of

316   10 nm) as a normalized Gaussian with standard deviation equal to the localization uncertainty.

325   With self-blinking dyes[43,44], it is not possible to take a widefield image. We thus obtained a pseudo

326   widefield (WF) and standard deviation (STD) image by computing the temporal average and standard

327   deviation of all the frames. We estimate a resolution of 542nm for the pseudo widefield, due to the low

328   SNR of the image and a resolution of 302nm for the STD image. Finally, we estimate a resolution of 89nm

329   for the STORM image. Fig. 5b and c display the corresponding decorrelation resolution estimate and FRC

330   curve, respectively (obtained by splitting the localizations in two odd and even localization subsets to

331   generate two independent realizations). Both estimates agree on the resolution, with FRC (estimated

332   resolution of 85nm) being slightly more optimistic, which is consistent with its behaviour observed in

333   simulations and reported recently by Marsh et al[40] .

334   Fig. 5d shows how our method and FRC resolution vary as a function of the number of frames.

335   We observe a drastic difference in the predicted resolution between the two methods only up to 5000

336 frames. This is due to the fact that FRC requires two images instead of one. For a low number of frames,

337 the localization events are too sparse to produce significant correlations, leading to a large resolution

338 estimate. On the other hand, our method only considers a single image, which is constituted of sparse

339 Gaussians with no apparent structure but a very good SNR (since there is no noise in a rendered

340 localization image). This leads to a very optimistic resolution estimate with the localization uncertainty

341 as a lower bound. As we increase the number of frames, the two random subsets of localization events

342 start to correlate and the FRC resolution estimate starts to decrease. Similarly, as we include more

343 localization events, a larger-scale structure (hence containing low spatial frequencies) starts to emerge.

344 Consequently, our resolution estimate increases as the structure is built up. We finally observe that both

345 methods converge at approximately the same speed at around 12000 frames, with FRC estimating a

346 slightly better resolution[45]. Fig 5e shows a total of 15 microtubules cross-sections randomly selected over

347 Fig. 5a. The apparent average microtubule diameter is about 95nm, which is consistent with the

348 resolution estimate and the secondary immunostaining, which increases the apparent microtubule

349 diameter by 10-30nm[46,47].

350   Fig. 5f shows a rendered image of GATTAquant HiRes 40R nanorulers[48] (courtesy of P. Tinnefeld

351 and J. Schmied). Fig. 5g shows a closeup image of three molecules showing that the three point-source

352 spaced by 40nm can be resolved. Our algorithm estimates a resolution of 32nm while FRC estimates a

353 resolution of 28nm, again being slightly more optimistic than our method. Fig. 5h shows a total of 10 line

354 profiles of individual molecules (grey lines) and their average (solid black line).

355   Finally, it has been shown that multiple blinking events can severely impact the FRC resolution

356 estimate by introducing spurious correlations[14]. While this effect can in principle be mitigated, it requires

357 an accurate estimation of the underlying blinking statistics[49]. We show (see Supplementary Material Fig.

358 S18) that our resolution estimate is independent on the probability of multiple blinking event, as our

359 method does not require any assumption on the blinking kinetics.

Discussion

361   We proposed a new method for parameter-free resolution and SNR estimation of a single

362   microscopy image. We were able to circumvent the need for a threshold by introducing a new form of

363   partial phase correlation. In principle, our method can be applied to any imaging technique, including

364   electron microscopy, atomic force microscopy, X-ray tomography and live-cell imaging. Here, we

365   demonstrated its broad applicability by applying it to various types of microscopy images, ranging from

366   bright field to single-molecule localization microscopy images. By processing nanoruler data, we showed

367   that the method can be used to quantitatively assess the resolution which enabled an optimization of

368   imaging parameters, post-processing and reconstruction of acquired data. Our approach provides a new

369   and objective way to quantify the effective resolution in super-resolution microscopy. The extension of

370   our method to 3D would require a reformulation of our algorithm in the spherical coordinate.

371   By developing an open-source ImageJ plug-in, we enable the use of our method to non-image

372   processing specialist. We provide a unique and novel approach for resolution estimation based on the

373   analysis of a single image that can be used for image processing optimization and image reconstruction

374   comparison. We envision that our new resolution estimate represents a powerful tool for on-the-fly

375   microscopy setup characterization and optimization as well as for automated microscopes. We hope that

376   our method will be adopted by the ever-growing microscopy community as an everyday tool, helping

377   them to achieve high-quality research.

378   **Materials and methods**

379   *Cell culture*

380   HeLa and COS-7 cells were cultured at 37 °C and 5 % CO_2 using DMEM high glucose with pyruvate (4.5 g

381   l^{-1} glucose, with GlutaMAX^{TM} supplement) supplemented with 10 % fetal bovine serum and 1 x penicillin-

382   streptomycin (all gibco$^®$, Thermo Fisher Scientific).

383   *Cell fixation and immunostaining*

384   Cells were seeded in Lab-tek® II chambered cover slides (nunc) or on 18 mm high-precision No. 1.5

385   borosilicate coverslips (Marienfeld) in 12 well plates (Thermo Fisher Scientific) 1-2 days before fixation

386   in DMEM (see cell culture) or DMEM high glucose w/o phenol red (4.5 g l^{-1} glucose) supplemented with

387   4 mM L-gluthamine, 10 % fetal bovine serum and 1x penicillin-streptomycin (all gibco®, Thermo Fisher

388   Scientific).

389   *HeLa cells:*

390   Cells were washed twice in pre-warmed buffer (microtubule stabilizing buffer (MTSB): 100 mM PIPES pH

391   6.8, 2mM $MgCl_2$, 5 mM EGTA or PBS for wheat germ agglutinin (WGA) staining), followed by application

392   of pre-warmed fixation buffer (3.7 % paraformaldehyde (PFA), 0.2 % Triton X-100 in MTSB or 3.7 %

393   paraformaldehyde (PFA) in PBS for wheat germ agglutinin (WGA) staining) for 15 min at room

394   temperature (RT). Cells were then washed three times for 5 min each with 1 x PBS and stored in 50 %

395   glycerol in 1 x PBS at 4 °C or the immunostaining protocol was continued to prepare samples for

396   fluorescence imaging. Fixed and permeabilized cells were blocked with 3 % BSA in 1 x PBS and 0.05 %

397   Triton X-100 for 60 min at RT or overnight at 4 °C.

398   Cells fixed without permeabilization were stained with 5 ng ml^{-1} WGA-Atto 565 for 10min followed by

399   three times 5 min washes with 1 x PBS. The blocked samples with prior permeabilization were

400   immediately incubated with a mix of primary anti-tubulin antibody (1 mg ml^{-1} DM1a mouse monoclonal

401   (ab80779) 1:150 dilution, Abcam) in antibody incubation buffer for 60 min at RT (AIB: 1 % BSA in 1 x PBS

402   and 0.05 % Triton X-100). Cells were then washed three times for 5 min each with AIB, followed by

403   incubation with donkey anti-mouse-Alexa Fluor 647 antibody (0.005 mg ml^{-1} Invitrogen) for 60 min at RT.

404   This and all subsequent steps were performed in the dark. All cells were again washed three times for 5

405 min each with AIB and incubated for 15 min post-fixation with 2 % PFA in 1 x PBS followed by three 5 min

406 washes with PBS. Cells were imaged immediately or stored in 50 % glycerol in 1 x PBS at 4 °C until imaging.

407 **COS-7 cells:**

408 The protocol is similar as described previously by Chazeau et al.[50]. Cells were washed twice in pre-

409 warmed DMEM w/o phenol red (see cell culture) following 90s incubation with extraction buffer

410 (microtubule stabilizing buffer 2 (MTSB2: 80 mM PIPES, 7 mM MgCl2, 1 mM EGTA, 150mM NaCl, 5mM

411 D-glucose adjust pH to 6.8 using KOH) with freshly added 0.3 % Triton X-100 (AppliChem) and 0.25%

412 glutaraldehyde (stock solution 50% electron microscopy grade, Electron Microscopy Sciences).

413 Immediately afterwards, pre-warmed 4 % paraformaldehyde (PFA) in PBS was incubated for 15 min at

414 room temperature (RT). Cells were then washed three times for 5 min each with 1 x PBS and stored in

415 50 % glycerol in 1 x PBS at 4 °C or the immunostaining protocol was continued. Next, a freshly prepared

416 solution of 10mM NaBH4 in 1x PBS was incubated on the cells for 7 minutes followed by one quick wash

417 in 1xPBS, and two washes 10min 1xPBS on an orbital shaker. Cells were permeabilized in PBS with 0.25

418 % Triton X-100 for 7min followed by blocking with blocking buffer (BB: 2% (w/v) BSA, 10mM glycine,

419 50mM ammonium chloride NH$_4$Cl in PBS pH 7.4 for 60 min at RT or overnight at 4 °C.

420 The blocked samples were immediately incubated with primary anti-tubulin antibody (clone B-5-1-2

421 ascites fluid 1:100-1:200 dilution, Sigma-Aldrich) in BB for 60 min at RT. Cells were then washed three

422 times for 5 min each with BB, followed by incubation with either donkey anti-mouse-Alexa Fluor 647

423 antibody for SOFI imaging (donkey anti-mouse (H+L) highly cross-adsorbed at 0.005 mg ml^{-1} Invitrogen),

424 donkey anti-mouse-AbberiorFlip565 for SMLM imaging (preparation see below at 1:200 dilution) or goat

425 anti-mouse-AbberiorStar635P (at 0.005-0.01 mg ml^{-1} Abberior), goat anti-mouse-Atto594 (at 0.0025-

426 0.005 mg ml^{-1} Atto-tec), donkey anti-mouse-Alexa Fluor 594 antibody (donkey anti-mouse (H+L) highly

427 cross-adsorbed at 0.005 mg ml^{-1} Invitrogen)or donkey anti-mouse-biotin (Biotin-SP (long spacer)

428   AffiniPure Donkey Anti-Mouse IgG (H+L), at 1:200 Jackson ImmunoResearch) for STED imaging for 60 min

429   at RT. This and all subsequent steps were performed in the dark. All cells were again washed three times

430   for 5 min each with AIB and incubated for 15 min post-fixation with 2 % PFA in 1 x PBS followed by three

431   5 min washes with PBS. Cells with biotinylated secondary antibody were additionally incubated with

432   streptavidin-Atto490LS (at 0.01 mg ml^{-1} Atto-tec) in PBS for 30min followed by 3 washes for 5 min in PBS

433   before post-fixation. Cells were imaged immediately or stored in 50 % glycerol in 1 x PBS at 4 °C until

434   SOFI or SMLM imaging. For STED microscopy, cells were mounted on a coverglass slide (Thermo Fisher

435   Scientific) using Mowiol-DABCO (preparation see below) and allowed to harden for at least 24h at RT.

436   Cells were imaged within 1 week of sample preparation.

437   ***Preparation of labeled proteins***

438   2 mg ml^{-1} donkey anti-mouse (H+L) highly cross-adsorbed antibody (Invitrogen) was incubated with

439   AbberiorFlip565-NHS (Abberior) and 2 mg ml^{-1} WGA (Vector Labs) was incubated with Atto565-NHS

440   esther (Atto-tec) at a ratio of 1: 6 for 1h at RT while shaking with the pH raised to 8.3 using sodium

441   bicarbonate. The mixture was purified using illustra NAP Columns (GE Healthcare) according to

442   manufacturer's instructions and eluted with slightly acidic PBS to recover the labeled antibody at neutral

443   pH. The protein concentration was estimated by absorption spectrometry to <1.5 mg ml^{-1} donkey anti-

444   mouse AbberiorFlip565 and 0.5 mg ml^{-1} WGA-Atto565.

445   ***Imaging buffer and embedding medium***

446   The samples for SOFI using Alexa Fluor dyes were imagined in a 50 mM Tris-Hcl pH 8.0, 10 mM NaCl

447   buffer containing an enzymatic oxygen scavenging system (2.5 mM protocatechuic acid (PCA) and 50 nM

448   Protocatechuate- 3,4-Dioxygenase from Pseudomonas Sp. (PCD) with >3 Units m g^{-1}) and a thiol (2-

449   Mercaptoethylamine). The thiol and a stock solution of 100 mM PCA in water, pH adjusted to 9.0 with

450   NaOH, were always prepared fresh. PCD was aliquoted at a concentration of 10 µM in storage buffer

451 (100 mM Tris-HCl pH 8.0, 50 % glycerol, 50 mM KCl, 1 mM EDTA) at -20 °C and thawn immediately before

452 use. The samples for SMLM using Abberior Flip 565 were imaged in 1x PBS. Mowiol-DABCO for STED

453 embedding was prepared as described in the manufacturer protocol (Roth Gebrauchsanweisung Mowiol

454 488). Aliquots were kept at -20C and thawn immediately before use.

455 *Microscope setups*

456 *Widefield, SOFI and SMLM*

457 Data for Fig. 4e-g, Fig. 5, Fig. S3a-d and Fig. S7a were acquired on a standard widefield custom build

458 microscope. A total of 4 illumination laser lines (405nm 200 mW Roithner; 488nm 200mW Toptica;

459 561nm 350mW Quantum laser; 635nm 1W Roithner) are collimated, expanded and combined with

460 dichroic filters. The beams are then cropped with a rectangular aperture of approx. 7.2 mm placed in the

461 conjugated object plane, resulting in a 120x120 µm field of view. The beams are then focused with an

462 achromatic lens (f = 200mm) and reflected by a 3mm thick Quad Line Beamsplitter (R405/488/561/635;

463 Semrock) in the back focal plane of the objective (Nikon 60x/1.27NA SR water immersion). The

464 fluorescence signal is focused on the camera (Orca Flash 4.0; Hamamatsu) with a 200mm achromatic

465 lens. The sample position is controlled in X and Y by a Scan-plus IM 120x80 (Marzheuser) and in Z by a

466 Nano-Z piezo nanopositioner (Mad City Labs). All acquisitions were performed using Micromanager. The

467 laser intensities used in the experiments can be found in Table S1.

468 *Confocal and STED*

469 Confocal and STED microscopy was performed at the EPFL bioimaging and optics platform (BIOP) using a

470 Leica SP8 STED 3X. The setup consists in a Leica DMi 8 inverted microscope body equipped with a white

471 light laser (470-670nm) and a Leica HC PL APO 100X/1.40 oil objective for STED. For STED imaging we

472 used the 775nm pulsed depletion laser and detected the fluorescence on HyD detectors. 100% 775nm

473 laser power corresponds to 403mW, 100% 633nm laser power corresponds to 1.12mW, 100% 488nm

474 laser power corresponds to 0.33mW and 100% 520nm laser power corresponds to 0.43mW (power

475 measurements were performed after the objective by the BIOP). The laser powers and other acquisition

476 parameters used in the experiments can be found in Table S1.

477 ***Data processing***

478 The algorithm is implemented in MATLAB (Mathworks) and ImageJ. All the codes are available upon

479 request.

480 ***Acknowledgments***

489 ***Author contributions***

490 A.D. proposed and developed the method, processed all the presented data, wrote the Matlab and Java

491 code. K.S.G. prepared all the cells and performed measurements. A.R. supervised the research. A.D.

492 wrote the manuscript with comments of all co-authors at all stages.

493 **Data and materials availability**

494 All data needed to evaluate the conclusions in the paper are present in the paper and/or the

495 Supplementary Materials. Additional data related to this paper may be requested from the authors.

496   ***Bibliography***

497   1.   Sahl, S. J., Hell, S. W. & Jakobs, S. Fluorescence nanoscopy in cell biology. *Nat. Rev. Mol. Cell Biol.*

498   **18,** 685–701 (2017).

499   2.   Sigal, Y. M., Zhou, R. & Zhuang, X. Visualizing and discovering cellular structures with super-

500   resolution microscopy. *Science* **361,** 880–887 (2018).

501   3.   Abbe, E. Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. *Arch. für*

502   *Mikroskopische Anat.* **9,** 413–418 (1873).

503   4.   Sheppard, C. J. R. Resolution and super-resolution. *Microsc. Res. Tech.* **80,** 590–598 (2017).

504   5.   Power, R. M. & Huisken, J. Adaptable, illumination patterning light sheet microscopy. *Sci. Rep.* **8,**

505   1–11 (2018).

506   6.   Štefko, M., Ottino, B., Douglass, K. M. & Manley, S. Autonomous illumination control for

507   localization microscopy. *Opt. Express* **26,** 30882–30900 (2018).

508   7.   Heel, M. Van. Similarity measures between images. *Ultramicroscopy* **21,** 95–100 (1987).

509   8.   Saxton, W. & Baumeister, W. The correlation averaging of a regularly arranged bacterial cell

510   envelope protein. *J. Microsc.* **127,** 127--138 (1982).

511   9.   Harauz, G. & van Heel, M. Exact filters for general geometry three dimensional reconstruction.

512   *Optik* **78,** 146–156 (1986).

513   10.   Rosenthal, P. B. & Henderson, R. Optimal determination of particle orientation, absolute hand,

514   and contrast loss in single-particle electron cryomicroscopy. *J. Mol. Biol.* **333,** 721–745 (2003).

515   11.   Orlova, E. V. *et al.* Structure of keyhole limpet hemocyanin type 1 (KLH1) at 15 Å resolution by

516   electron cryomicroscopy and angular reconstitution. *J. Mol. Biol.* **271,** 417–437 (1997).

517   12.   Unser, M., Trus, B. L. & Steven, A. C. A new resolution criterion based on spectral signal-to-noise

518   ratio. *Ultramicroscopy* **23,** 39–52 (1987).

519   13.   Banterle, N., Bui, K. H., Lemke, E. A. & Beck, M. Fourier ring correlation as a resolution criterion

520   for super-resolution microscopy. *J. Struct. Biol.* **183,** 363–367 (2013).

521   14.   Nieuwenhuizen, R. P. J. *et al.* Measuring image resolution in optical nanoscopy. *Nat. Methods*

522   **10,** 557–562 (2013).

523   15.   Tortarolo, G., Castello, M., Diaspro, A., Koho, S. & Vicidomini, G. Evaluating image resolution in

524   stimulated emission depletion microscopy. *Optica* **5,** 32 (2018).

525   16.   Raab, M. *et al.* Using DNA origami nanorulers as traceable distance measurement standards and

526   nanoscopic benchmark structures. *Sci. Rep.* **8,** 1780 (2018).

527   17.   Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. Image processing with ImageJ. *Biophotonics Int.*

528   **11,** 36–42 (2004).

529   18.   Minsky, M. Memoir on inventing the confocal scanning microscope. *Scanning* **10,** 128–138

530   (1988).

531   19.   Hell, S. W. & Wichmann, J. Breaking the diffraction resolution limit by stimulated emission:

532   stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **19,** 780 (1994).

533   20.   Vicidomini, G., Bianchini, P. & Diaspro, A. STED super-resolved microscopy. *Nat. Methods* **15,**

534   173–182 (2018).

535   21.   Tortarolo, G., Sun, Y., Teng, W., Ishitsuka, Y. & Vicidomini, G. Photon-separation to enhance the

536   spatial resolution of pulsed STED microscopy. *Nanoscale* **11,** 1754–1761 (2019).

537   22.   Westphal, V. & Hell, S. W. Nanoscale resolution in the focal plane of an optical microscope.

538   *Phys. Rev. Lett.* **94,** 1–4 (2005).

539   23.   Oracz, J., Westphal, V., Radzewicz, C., Sahl, S. J. & Hell, S. W. Photobleaching in STED nanoscopy

540   and its dependence on the photon flux applied for reversible silencing of the fluorophore. *Sci.*

541   *Rep.* **7,** 11354 (2017).

542   24.   Vicidomini, G., Moneron, G., Eggeling, C., Rittweger, E. & Hell, S. W. STED with wavelengths

543   closer to the emission maximum. *Opt. Express* **20,** 5225–5236 (2012).

544   25.   Galiani, S. *et al.* Strategies to maximize the performance of a STED microscope. *Opt. Express* **20,**

545   7362–7374 (2012).

546   26.   Heintzmann, R. & Cremer, C. G. *Laterally modulated excitation microscopy: improvement of*

547   *resolution by using a diffraction grating*. (International Society for Optics and Photonics, 1999).

548   27.   Frohn, J. T. Super-resolution fluorescence microscopy by structured ligth illumination. (ETH

549   Zürich, 2000).

550   28.   Gustafsson, M. G. L. Surpassing the lateral resolution limit by a factor of two using structured

551   illumination microscopy. *J. Microsc.* **198,** 82–87 (2000).

552   29.   Heintzmann, R. & Huser, T. Super-Resolution Structured Illumination Microscopy. *Chem. Rev.*

553   **117,** 13890–13908 (2017).

554   30.   Demmerle, J. *et al.* Strategic and practical guidelines for successful structured illumination

555   microscopy. *Nat. Protoc.* **12,** 988–1010 (2017).

556   31.   Müller, M., Mönkemöller, V., Hennig, S., Hübner, W. & Huser, T. Open-source image

557   reconstruction of super-resolution structured illumination microscopy data in ImageJ. *Nat.*

558   *Commun.* **7,** 1–6 (2016).

559   32.   Dertinger, T., Colyer, R., Iyer, G., Weiss, S. & Enderlein, J. Fast, background-free, 3D super-

560   resolution optical fluctuation imaging (SOFI). *Proc. Natl. Acad. Sci. U. S. A.* **106,** 22287–92 (2009).

561   33.   Dertinger, T., Colyer, R., Vogel, R., Enderlein, J. & Weiss, S. Achieving increased resolution and

562   more pixels with Super-resolution Optical Fluctuation Imaging (SOFI). *Opt. Express* **18,** 18875–

563   18885 (2010).

564   34.   Deschout, H. *et al.* Complementarity of PALM and SOFI for super-resolution live-cell imaging of

565   focal adhesions. *Nat. Commun.* **7,** 13693 (2016).

566   35.   Geissbuehler, S. *et al.* Mapping molecular statistics with balanced super-resolution optical

567   fluctuation imaging (bSOFI). *Opt. Nanoscopy* **1,** 4 (2012).

568   36.   Peeters, Y. *et al.* Correcting for photodestruction in super-resolution optical fluctuation imaging.

569   *Sci. Rep.* **7,** 10470 (2017).

570   37.   Rust, M. J., Bates, M. & Zhuang, X. W. Sub-diffraction-limit imaging by stochastic optical

571   reconstruction microscopy (STORM). *Nat Methods* **3,** 793–795 (2006).

572   38.   Betzig, E. *et al.* Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **313,**

573   1642–5 (2006).

574   39.   Sauer, M. & Heilemann, M. Single-Molecule Localization Microscopy in Eukaryotes. *Chem. Rev.*

575   **117,** 7478–7509 (2017).

576   40.   Marsh, R. J. *et al.* Artifact-free high-density localization microscopy analysis. *Nat. Methods* **15,**

577   689 (2018).

578   41.   Legant, W. R. *et al.* High-density three-dimensional localization microscopy across large

579   volumes. *Nat. Methods* **13,** 359–365 (2016).

580   42.   Ovesny, M., Krizek, P., Borkovec, J., Svindrych, Z. & Hagen, G. M. ThunderSTORM : a

581   comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging.

582   *Bioinformatics* **30,** 2389–2390 (2014).

583   43.   Fölling, J. *et al.* Photochromic Rhodamines Provide Nanoscopy with Optical Sectioning **.

584   *Angew. Chemie - Int. Ed.* **46,** 6266–6270 (2007).

585   44.   Bossi, M. *et al.* Multicolor Far-Field Fluorescence Nanoscopy through Isolated Detection of

586   Distinct Molecular Species. *Nano Lett.* **8,** 2463–2468 (2008).

587   45.   Lambert, T. J. & Waters, J. C. Navigating challenges in the application of superresolution

588   microscopy. *J. Cell Biol.* **216,** 53–63 (2016).

589   46.   Mikhaylova, M. *et al.* Resolving bundled microtubules using anti-tubulin nanobodies. *Nat.*

590   *Commun.* **6,** 1–7 (2015).

591   47.   Pleiner, T., Bates, M. & Görlich, D. A toolbox of anti – mouse and anti – rabbit IgG secondary

592   nanobodies. *J. Cell Biol.* **217,** 1143–1154 (2017).

593   48.   Schmied, J. J. *et al.* Fluorescence and super-resolution standards based on DNA origami Flaws in

594   evaluation schemes for pair- input computational predictions. *Nat. Methods* **9,** 1133–1134

595   (2012).

596   49.   Annibale, P., Vanni, S., Scarselli, M., Rothlisberger, U. & Radenovic, A. Quantitative Photo

597   Activated Localization Microscopy : Unraveling the Effects of Photoblinking. *PLoS One* **6,** e22678

598   (2011).

599   50.   Chazeau, A., Katrukha, E. A., Hoogenraad, C. C. & Kapitein, L. C. Studying neuronal microtubule

600   organization and microtubule-associated proteins using single molecule localization microscopy.

601   *Methods Cell Biol.* **131,** 127–149 (2016).

602