Nanoscale distribution of nuclear sites analyzed by superresolution STED-ICCS

M. Oneto, L. Scipioni, M.J. Sarmiento, I. Cainero, S. Pelicci, L. Furia, P.G. Pelicci, G.I. Dellino, P. Bianchini, M. Faretta, E. Gratton, A. Diaspro, L. Lanzaò

KEYWORDS: image cross-correlation spectroscopy (ICCS) – Stimulated Emission Depletion (STED) – super-resolution – nuclear sites – replication – transcription – colocalization – local ICCS

2 Abstract

3 Deciphering the spatiotemporal coordination between nuclear functions is important to
4 understand its role in the maintenance of human genome. In this context, superresolution
5 microscopy has gained considerable interest as it can be used to probe the spatial organization of
6 functional sites in intact single cell nuclei in the 20-250 nm range. Among the methods that quantify
7 colocalization from multicolor images, image cross-correlation spectroscopy (ICCS) offers several
8 advantages, namely it does not require a pre-segmentation of the image into objects and can be used
9 to detect dynamic interactions. However, the combination of ICCS with super-resolution
10 microscopy has not been explored yet.

11 Here we combine dual color stimulated emission depletion (STED) nanoscopy with ICCS
12 (STED-ICCS) to quantify the nanoscale distribution of functional nuclear sites. We show that
13 STED-ICCS provides not only a value of colocalized fraction but also the characteristic
14 distances associated to correlated nuclear sites. As a validation, we quantify the nanoscale spatial
15 distribution of three different pairs of functional nuclear sites in MCF10A cells. As expected,
16 transcription foci and a transcriptionally repressive histone marker (H3K9me3) are not
17 correlated. Conversely, nascent DNA replication foci and the Proliferating cell nuclear antigen
18 (PCNA) protein have a high level of proximity and are correlated at a nanometer distance which
19 is close to the limit of our experimental approach. Finally, transcription foci are found at a
20 distance of 130 nm from replication foci, indicating a spatial segregation at the nanoscale.
21 Overall, our data demonstrate that STED-ICCS can be a powerful tool for the analysis of nanoscale
22 distribution of functional sites in the nucleus.

23 Statement of significance

24 Several methods are available to quantify the proximity of two labeled molecules from dual color
25 images. Among them, image cross-correlation spectroscopy (ICCS) is attractive as it does not
26 require a pre-segmentation of the image into objects and can be used to detect dynamic interactions.

27 Here, we combine for the first time ICCS with superresolution stimulated emission depletion
28 (STED) microscopy (STED-ICCS) to quantify the spatial distribution of functional sites in the
29 nucleus. Our results show that STED-ICCS, in addition to quantifying the colocalized fraction,
30 detects characteristic nanometer distances associated to correlated nuclear sites. This work shows
31 that STED-ICCS can be a powerful tool to quantify the nanoscale distribution of functional sites in
32 the nucleus.

33

34 **Introduction**

35 Healthy genome regulation and maintenance rely on the proper spatiotemporal coordination
 36 between several nuclear functions. Alterations in chromatin organization are often linked to human
 37 diseases (1). An example is represented by DNA transcription and replication, two fundamental
 38 genomic processes that can potentially compete for the same DNA template. Replication and
 39 transcription both occur within the highly packed chromatin environment and must be tightly
 40 coordinated in time and space to avoid interference and generation of DNA damage (2). Alterations
 41 in the coordination of replication and transcription represent a major source of genomic instability,
 42 a hallmark of cancer (3). Therefore, deciphering the coordination between nuclear functions at a
 43 high spatial and temporal resolution is important to understand its role in health and disease.
 44 Genome-scale sequencing methods provide high-resolution maps of spatiotemporal regulation of
 45 genomic processes (4, 5). However, they do not provide any information on spatial localization and
 46 lack single cell resolution. In this context, optical imaging methods are emerging as complementary
 47 tools to investigate genome organization and structure in intact single cell nuclei (6).

48 The simplest approach to study spatial organization of two labeled molecules consists in the
 49 analysis of dual color images for the presence of colocalized signal, i.e. signal that overlaps within
 50 the two detection channels. Colocalization is a measurement of the co-distribution of two probes at
 51 a spatial scale defined by the resolution of the optical microscope. In conventional optical
 52 microscopy, this spatial scale is limited by diffraction to about 250 nm. For this reason, alternative
 53 strategies have been developed to investigate cellular processes at the nanoscale. For instance, a
 54 popular method to probe molecular distances in the nanometer range is Forster Resonance Energy
 55 Transfer (FRET) (7-9) but, unfortunately, FRET is not sensitive when distances are larger than
 56 about 10 nm. Similarly, *in situ* Proximity Ligation Assay (PLA) is a powerful method to visualize
 57 proximity of two labeled species but its sensitivity does not exceed 40 nm (10, 11). This scenario
 58 changed dramatically with the introduction of superresolution microscopy (also called nanoscopy),

namely the ensemble of microscopy techniques providing optical resolution below the diffraction limit (12). For instance, in single molecule localization microscopy (SMLM), the fluorophores are sequentially switched on and off and localized with high precision on each frame, resulting in images with a resolution down to ~20 nm (13-15). In stimulated emission depletion (STED) microscopy the fluorophores are selectively switched off at the periphery of the diffraction-limited detection spot by a second, doughnut-shaped laser beam, producing an immediate improvement of spatial resolution down to ~50 nm (16). Thus, superresolution can be used to probe spatial organization in the 20-250 nm range, which corresponds to the range of higher order organization of chromatin in the nucleus. This nanoscale spatial organization can be studied in a ‘static’ way, from images of fixed cells or in a ‘dynamic’ way, from acquisition of superresolution data in live cells.

Methods that provide a quantitative measure of colocalization from static multicolor superresolution images can be divided in two major groups: object-based methods, which perform a segmentation of the image into objects prior to analyzing their relative spatial distributions, and pixel-based methods, which extract correlation coefficients from pixel intensities (17, 18). In general, object-based analysis can be performed on any type of superresolution image as long as the target objects are well resolved and identified. In particular, object-based methods are often the methods of choice in SMLM, where the acquired data are already segmented into a list of x y coordinates of individual molecules (19-23). In principle, object-based analysis provides a full description of the spatial distribution of the two labeled species: in fact, knowledge of the objects coordinates allows (i) mapping the locations of the specimen with a higher level of proximity and (ii) performing a statistical analysis of the relative distance between the particles. On the other hand, a great advantage of pixel-based methods is that they do not require a pre-segmentation of the images into objects but rely on the calculation of coefficients from the pixel intensity values (24, 25). Pixel-based methods are routinely applied to quantify spatial distribution in multicolor superresolution images (26-28).

84 A method to study interactions in a ‘dynamic’ way is Fluorescence Cross-Correlation
85 Spectroscopy (FCCS), the dual channel version of Fluorescence Correlation Spectroscopy (FCS)
86 (29). In FCCS, the fraction of interacting particles is extracted from the analysis of temporal
87 intensity fluctuations originating from changes in fluorophore concentration within a small
88 observation volume, typically defined by the focal spot of a confocal microscope (30, 31). Both
89 FCS and FCCS have been applied to study the mobility and interactions of molecules in the nucleus
90 (32-34). Interestingly, the very same formalism of FCS and FCCS can be applied to the analysis of
91 the spatial intensity fluctuations found in images. Image Correlation Spectroscopy (ICS) and Image
92 Cross-Correlation Spectroscopy (ICCS) are the spatial variants of FCS and FCCS, respectively
93 (35). Notably, ICCS can be used as a pixel-based method to analyze the spatial distribution in static
94 images as well (36). Thus, ICCS appears as an extremely versatile method that can offer several
95 advantages compared to other analysis approaches, namely it does not require a pre-segmentation of
96 the image into objects and can be used to detect dynamic interactions (37-39). However, despite this
97 potential, the combination of ICCS with superresolution microscopy has not been fully explored
98 yet.

99 Here, we show that the combination of dual color STED nanoscopy with ICCS (STED-ICCS)
100 can be used to quantify the relative nanoscale spatial distribution of distinct nuclear foci. In
101 particular, we show that STED-ICCS can provide, to some extent, some of the attractive features
102 of an object-based analysis but without requiring a pre-segmentation of the super-resolved
103 image. In fact, we are able to (i) map the locations within nuclei with a higher level of proximity
104 and (ii) determine if the particles are correlated at a certain nanoscale distance. First, the analysis
105 is tested on simulated images of nuclear foci at variable density and compared with an object-
106 based analysis performed on the same simulated datasets. Then, the STED-ICCS analysis is
107 tested on dual color STED images of model samples based on DNA origami bearing green and
108 red fluorophores located at a characteristic distance of 20 nm or 100 nm. These data demonstrate

that STED-ICCS can provide nanoscale information on the distance between correlated particles. Finally, to validate the STED-ICCS method on a biological sample, we quantify the relative nanoscale spatial distribution of three different pairs of nuclear sites in MCF10A cells. As expected, transcription foci and a transcriptionally repressive histone marker (H3K9me3) show the minimum level of colocalization and random relative distance distribution. Conversely, nascent DNA replication foci and the Proliferating cell nuclear antigen (PCNA) protein have a high level of proximity and are correlated at a nanometer distance which is close to the limit of our experimental approach. Notably, nascent DNA replication foci and transcription foci are found to be partially correlated but at a distance of ~100 nm, indicating that the two functional sites are spatially segregated at the nanoscale. Overall, these data demonstrate that STED-ICCS can be a powerful tool for the analysis of relative nanoscale spatial distribution of functional sites in the nucleus.

Results

Simulations: STED-ICCS is a robust method to characterize relative spatial distribution at high densities of foci

To compare STED-ICCS with object-based analysis, we simulated dual color images of nuclear foci at variable densities (Fig.1). In each channel, the nuclear foci were simulated as N point-like particles distributed in a 16 μm wide circular region (40) and convoluted with a Gaussian Point Spread Function (PSF) with a Full Width Half Maximum (FWHM) of 3 pixels, corresponding to 120 nm. We simulated distributions of foci randomly distributed in each channel (uncorrelated), with a fraction $f_{\text{coloc}}=0.25$ of foci colocalizing in the two channels (colocalized) and with a fraction $f_{\text{coloc}}=0.25$ of foci colocalizing only in a specific sub-portion of the image (colocalized in a zone). The total number of foci in each channel was varied from $N=100$ to $N=10000$. These

simulations are intended to test the analysis workflow and not as a model of the more complex distributions and patterns that can be found in real samples.

At low density, the foci are easily localized in the XY plane and the relative distance between every center in one channel and every center in the other channel, can be calculated from their center positions (Fig. 1a) according to the localization-based approach. The resulting relative distance distribution (RDD) has a linearly growing trend for the uncorrelated sample, as expected from purely geometrical considerations (Fig.1b). For the other two samples in Fig.1a (colocalized and colocalized in a zone), the RDD shows an additional peak at a distance zero, corresponding to the fraction of colocalized foci, superimposed with the linear trend due to the random distribution (Fig.1b). At higher densities of foci (Fig.1c), the colocalized fraction retrieved with the object-based analysis (Fig.1f, triangles) deviates from the simulated value ($f_{\text{coloc}}=0.25$). In particular, a deviation of 20% from the expected value is obtained when the number of foci is $N=500$ (Fig.1f). For comparison, considering a FWHM value typical of diffraction-limited microscopy, $\text{FWHM}_{\text{conf}}=250$ nm, a deviation of 20% is obtained already when the number of foci is $N=115$. This is due to the lower accuracy in determining the center positions of the foci during the segmentation process.

On the other hand, ICCS is able to determine the colocalized fraction independently of the density of foci (Fig.1g). In ICCS, the colocalized fraction f_{ICCS} is extracted from the fitting of the image auto- and cross-correlation functions (41, 42). The amplitude of the cross-correlation function is zero for the uncorrelated sample (Fig.1d, top), whilst is positive for both colocalized samples (Fig.1d, mid and bottom). In the range of foci densities explored here, the value of colocalized fraction retrieved by ICCS is consistent with the simulated value (Fig.1g), in keeping with previous studies (36). Thus, STED-ICCS can be used to determine the fraction of cross-correlated particles also when the density of foci is too high for non-super-resolved methods or, equivalently, when the improvement of spatial resolution is not sufficient to resolve the foci.

158 An apparent disadvantage of ICCS, in comparison with object-based approaches, is that it
 159 provides only an average description of the properties of the sample in the region analyzed. In
 160 fact, the two correlated samples shown in Fig.1c (colocalized and colocalized in a zone) produce
 161 almost identical correlation functions (Fig.1d) despite a very different colocalization pattern.
 162 Thus, ICCS can be used to extract a value of the colocalized fraction at high density of foci but
 163 does not specify where the foci are colocalized. To partially overcome this limitation, we
 164 performed local ICCS analysis, i.e. ICCS performed on small sub-regions of the image
 165 (Fig.1e,h). It has been previously shown that, if the spatial correlation function is calculated
 166 locally, one can get maps of physical parameters such as protein velocity (43), particle size (44)
 167 or diffusion coefficient of a probe (45, 46). Local ICCS can be used to generate a map of the
 168 value of the colocalized fraction extracted by fitting the local auto- and cross-correlation
 169 functions (Fig.1e). The local ICCS maps show that the two correlated samples (colocalized and
 170 colocalized in a zone) have a very different pattern, despite containing the same total fraction of
 171 colocalized foci. The spatial resolution of the local ICCS map depends on the size of the sub-
 172 image employed for the local analysis. For instance, in the example of Fig.1e, the size of the sub-
 173 image was set to 69 pixels.

174 **Measurements on model systems: STED-ICCS detects particles correlated at a distance.**

175 Another limitation of STED-ICCS, when compared to object-based analysis, is that it cannot be
 176 used to perform a complete statistical analysis of the relative distance between the particles. This
 177 feature of object-based analysis is particularly useful to detect characteristic nanometer distances
 178 associated to inter-molecular complexes (20). Here we aim to show that, even if it is not able to
 179 provide a full statistical analysis, STED-ICCS can provide information on the average distance
 180 between correlated particles.

181 In STED-ICCS, the shape of the cross-correlation function is dependent upon the distance d
 182 between the two probes and the effective spatial resolution of the STED microscope in the two

183 channels. For two single spots located at distance d along a given direction, if the single channel
 184 2D auto-correlation functions have width w_{11} and w_{22} , the corresponding 2D cross-correlation
 185 function has a width equal to $w_{cc} = ((w_{11}^2 + w_{22}^2)/2)^{1/2}$ and is shifted from the origin of an amount d
 186 along the same direction (47). In most real cases, however, the particles are found at all possible
 187 orientations with respect to each other. This has two effects on the cross-correlation function,
 188 namely a reduction of its amplitude and an increase of the value of its width compared to the
 189 value w_{cc} , expected for perfectly coaligned spots. The broadening of the cross-correlation
 190 function can be evaluated as $\Delta w = w_{12} - w_{cc}$. Importantly, Δw is a parameter sensitive only to the
 191 average distance between correlated particles, whilst the amplitude-related parameter f_{ICCS} is
 192 sensitive also to the relative amount of correlated particles. Thus, the Δw can be used to estimate
 193 the distance associated to the correlated particles. To actually convert values of Δw into values of
 194 distance, we performed simulations of particles correlated at variable distances and measured the
 195 broadening of the cross-correlation function (Supplementary Fig. S1).

196 To demonstrate this effect, we performed STED-ICCS on optical nanorulers, i.e. DNA origami
 197 structures designed to contain the same two fluorophores used in our experiments (Chromeo-488
 198 and Atto-532) at a well-defined distance of either $d=20\text{nm}$ or $d=100$ (Fig.2a,b). First, we
 199 characterized the samples by object-based analysis. The spatial resolution achieved in these
 200 STED images was $\sim 95\text{ nm}$ in the green channel, and $\sim 130\text{ nm}$ in the red channel, as determined
 201 from the FWHM of line profiles (Supplementary Fig. S2). Considering an average number of
 202 photons detected per spot per channel $N \sim 30$ and a 10% background noise level, this translates to
 203 an estimated localization precision $\sigma_G \sim 13\text{ nm}$ and $\sigma_R \sim 20\text{ nm}$ in the green and red channels,
 204 respectively (48). This propagates to an expected uncertainty $(\sigma_G^2 + \sigma_R^2)^{1/2} \sim 24\text{ nm}$ in the
 205 estimation of the relative distance d . The distributions of distances obtained from the object-
 206 based analysis of STED images of optical nanorulers are reported in (Fig.2c,d). They contain a
 207 peak superimposed to a linear growing component. The distance peak value found in the relative

distance distribution (RDD) of the 100-nm nanorulers was $d_{100\text{nm}}=100\pm 25$ nm (mean $\pm$ s.d.), in keeping with the expected value. These data indicate that, at the imaging conditions of this sample, we can measure distances with an accuracy $\sigma_d\sim 25$ nm. This sets a lower limit to the distances that can be detected with our experimental setup. In line with this, the distance peak value found in the RDD of the 20-nm nanorulers was $d_{20\text{nm}}=40\pm 30$ nm.

Then we performed STED-ICCS on the same samples. As expected, STED-ICCS detected a positive cross-correlation for both samples (Fig.2e,f), with the colocalized fraction being higher in the 20-nm nanorulers ($f_{\text{ICCS}}=1.04\pm 0.3$, mean $\pm$ s.d.) than in the 100-nm nanorulers ($f_{\text{ICCS}}=0.66\pm 0.11$, mean $\pm$ s.d.) (Fig.2g). More interestingly, the parameter Δw , related to the broadening of the cross-correlation function (Fig.2h), was higher in the 100-nm nanorulers ($\Delta w=1.2\pm 0.2$ pixels, mean $\pm$ s.d.) than in the 20-nm ones ($\Delta w=0.15\pm 0.04$ pixels, mean $\pm$ s.d.). According to the simulations, the measured values of Δw correspond to the distance values $d_{\text{ICCS}}=99\pm 8$ nm, for the 100-nm nanorulers, and $d_{\text{ICCS}}=35\pm 5$ nm, for the 20-nm nanorulers, which are in agreement with the peak values found with object-based analysis (Fig.2i). Note that the smaller uncertainty in the value of d_{ICCS} is due to the fact that each STED-ICCS measurement is performed on an image containing several tens of nanorulers whilst, in the object-based analysis, the distance is measured on each identified pair of objects.

Thus, the relative broadening of the STED-ICCS cross-correlation function can provide nanoscale information on the distance between particles that are not distributed randomly.

Measurements in cell nuclei: STED-ICCS quantifies the relative nanoscale distribution of functional nuclear sites

As validation of our STED-ICCS method, we characterized the nanoscale spatial distribution of three different pairs of functional nuclear sites in the diploid mammary epithelium cells MCF10A. We compared the nanoscale spatial distribution of i) transcription foci (labeled through incorporation of the nucleotide analogue BrU) *versus* a transcriptionally repressive histone marker

(H3K9me3) (Fig.3a), ii) nascent DNA replication foci (labeled through incorporation of the nucleotide analogue EdU) *versus* the replication machinery protein proliferating cell nuclear antigen (PCNA), during early S phase (Fig.3b), iii) transcription foci (BrU) *versus* nascent DNA replication foci (EdU), during early S phase (Fig.3c). The STED-ICCS analysis was compared with an object-based analysis performed on the same dataset.

Representative dual color STED images of the three samples are reported in Fig.3. For each image are shown the map of localized spots along with the calculated RDD, the image auto- and cross-correlation functions and the map of the colocalized fraction obtained by local ICCS (Fig.3). Overall, there was an agreement between the colocalized fraction extracted by the object-based analysis, f_{obj} , and that extracted by STED-ICCS, f_{ICCS} , for the different samples (Supplementary Fig. S3). Differences in the absolute values (f_{ICCS} was systematically higher than f_{obj}) are probably related to the specific settings of each analysis. For instance, in the object-based analysis, an intensity threshold value was set manually for each image whilst, in the ICCS analysis, we did not perform any background subtraction. Notably, the local ICCS maps can be used to visualize, in STED-ICCS, the regions of the nuclei with a higher level of proximity, similarly to what can be done with object-based analysis (Fig.3). The average co-localization fractions retrieved from the STED-ICCS analysis are reported in Fig.4c. Transcription foci and the transcriptionally repressive histone marker H3K9me3 show the minimum level of colocalization ($f_{ICCS}=0.02\pm0.05$, mean $\pm$ s.d., n=17 cells), in keeping with the evidence that H3K9me3 is an epigenetic marker of repressive heterochromatin, replicating during late-S phase and not associated with active transcription (49). This result is confirmed by object-based analysis, where transcription and H3K9me3 show a random-like RDD (Fig.4a,b). Conversely, DNA replication foci and the PCNA protein have the maximum level of colocalization ($f_{ICCS}=1.01\pm0.16$, mean $\pm$ s.d., n=19 cells), in agreement with the role of the PCNA protein in orchestrating the replication process (50). Transcription foci and DNA replication foci in early-S phase cells exhibit an intermediate level of colocalization

258 ($f_{ICCS}=0.36\pm0.09$, mean $\pm$ s.d., n=22 cells). However, this analysis does not show if this difference in
259 the level of colocalization is due to a different distribution at the nanoscale.

260 To get insight on the relative nanoscale distribution of the correlated functional sites, we analyzed
261 the broadening of the cross-correlation function, Δw (Fig.4d), and compared it with the relative
262 distance distribution (RDD) derived from the object-based analysis (Fig.4a,b). The RDD of DNA
263 replication foci and the PCNA protein revealed a peak distance of 55 nm ($d_{PCNA-EdU}=55\pm34$ nm,
264 mean $\pm$ s.d., n=19 cells). STED-ICCS detected a broadening $\Delta w_{PCNA-EdU}=0.4\pm0.4$ pixels
265 (mean $\pm$ s.d., n=19 cells) corresponding to a distance value $d_{PCNA-EdU}=58\pm40$ nm. The values of
266 distance detected by the two methods are very close to the limit of our experimental approach, as
267 determined with the 20-nm optical nanorulers ($d_{obj}=40$ nm and $d_{ICCS}=35$ nm). A higher level of
268 inaccuracy might also come from the use of primary and secondary antibodies. Moreover, we
269 analyzed only single optical sections that represent only a projection of the distribution of
270 nuclear foci in three dimensions. This could result in underestimation of the recovered distance
271 values. Besides these technical considerations, we should also take into account that the EdU
272 signal labels the DNA sequences that were replicating during the previous 20 minutes and that
273 PCNA, not only tethers polymerases to DNA for during replication, but also participates in non-
274 replicative DNA synthesis events, such as those occurring during DNA repair, and other cell
275 functions that extend well beyond DNA synthesis.

276 The RDD of transcription and DNA replication foci in early-S phase cells exhibited a quasi-random
277 relative distance distribution (Fig.4b). After subtraction of the random component, the RDD showed
278 a small peak indicating a positive correlation at a distance of $d_{BrU-EdU}=100\pm50$ nm (mean $\pm$ s.d.,
279 n=22 cells) (Fig.4b). Interestingly, STED-ICCS detected a broadening $\Delta w_{BrU-EdU}=2.3\pm1.0$ pixels
280 (mean $\pm$ s.d., n=22 cells) corresponding to a distance value $d_{BrU-EdU}=138\pm35$ nm (Fig.4b,d,e).
281 Thus, STED-ICCS was able to reveal a correlation between these nuclear sites in the ~100-nm
282 range, similarly to the object-based analysis. The non-random distribution in the BrU-EdU sample

is consistent with the cell population which was analyzed; during early-S phase, replicating DNA is mainly euchromatic and gene-rich. Most of the genes contained within these regions are generally expressed during early S-phase, and importantly, although their transcription is time-controlled in order to avoid transcription-replication clashes, it is still very close to ongoing replication, not only in space, but also in time, both prior and after DNA synthesis. Thus, in early-S phase, transcription and replication operate on the same portion of the genome, thus explaining proximity of the corresponding BrU and EdU signals (51). In summary, STED-ICCS detected a small but positive correlation between replication and transcription (quantified by the parameter f_{ICCS}) but indicated spatial segregation at the nanoscale (quantified by the parameter d_{ICCS}), in keeping with a long-standing model of genome organization suggesting spatial segregation between replication and transcription (2, 51).

Discussion

In this work, we have explored the use of super-resolution STED imaging, combined with image cross-correlation spectroscopy (ICCS), to investigate the relative nanoscale spatial distribution of nuclear foci. There are three main technical points that characterize STED-ICCS. The first important aspect is that, being based on the calculation and fit of the image spatial correlation functions, STED-ICCS does not require a pre-segmentation of the images into objects. To illustrate the consequences of this point, we have performed ICCS and object-based analysis on simulated data of point-like particles at different density/spatial resolution. These simulations indicated that ICCS could be applied even at high densities of foci, whilst the object-based analysis was affected by a decreasing accuracy in the pre-segmentation process when the spatial resolution was not sufficient to resolve the foci. A second technical aspect is that in STED-ICCS the colocalized fraction is estimated from the amplitude of the image cross- and auto-correlation functions, calculated over the image. Compared to object-based analysis, this calculation does not specify where the foci are colocalized. In this respect, we have shown that a local STED-

308 ICCS analysis can be used to map the value of the colocalization coefficient across the sample
 309 and partially compensate for this limitation. The third important aspect is related to the detection
 310 of characteristic correlation distances. In the object-based approaches, it is possible to perform a
 311 complete statistical analysis of the relative distance between the particles and detect
 312 characteristic nanometer distances associated to inter-molecular complexes (20). In this respect,
 313 we have shown that the broadening of cross-correlation function in STED-ICCS can also provide
 314 quantitative information on the nanoscale distance between correlated particles.

315 To validate our approach, we performed STED-ICCS on dual color STED images of model and
 316 biological samples and compared the results with an object-based analysis performed on the
 317 same datasets. In particular, the object-based quantification was presented in terms of a relative
 318 distance distribution (RDD), representing the histogram the distance values between particles.
 319 However, we expect that any other type of quantitative analysis applied to the list of coordinates
 320 of the positions of the foci, such as radial distribution functions or Ripley's functions (20, 21),
 321 would give similar results. We quantified by STED-ICCS the relative nanoscale distribution of
 322 three pairs of functional nuclear sites. Notably, STED-ICCS was able to detect not only a value
 323 of colocalized fraction but also the characteristic correlation distance associated to correlated
 324 nuclear sites. In particular, PCNA was found in close association with EdU-labeled replication
 325 foci, with a detected distance of ~50 nm, very close to the experimental limit of the analysis. On
 326 the other hand, transcription foci were found at a distance of 130 nm from EdU-labeled
 327 replication foci, indicating a spatial segregation at the nanoscale despite both transcription and
 328 replication taking place on the same portion on the genome during the early S phase of the cell
 329 cycle. Overall, there was a good agreement between STED-ICCS and the object-based analysis,
 330 demonstrating that, even without requiring a pre-segmentation, STED-ICCS can provide some of
 331 the most attractive features of an object-based analysis.

332 An important point to discuss is how general is the applicability of our method. We have shown
 333 experimental data obtained on model and fixed biological samples by dual color STED imaging.
 334 In particular, in the conditions of our experiments, we achieved a lateral resolution in the order
 335 of ~100 nm. The same type of nanoscale ICCS analysis could be applied to other types of ‘static’
 336 superresolution images, including images obtained with SMLM. In the case of SMLM, a
 337 segmentation of the data is already available making object-based co-localization analysis the
 338 method of choice. Nevertheless, we believe that the ICCS analysis could be useful as an
 339 independent cross-validation of the results obtained with object-based approaches. Even more
 340 interesting appears the application of our method to the analysis of ‘dynamic’ superresolution
 341 images, for which an object-based analysis is less straightforward. In our data, a spatial
 342 resolution of the order of ~100 nm was sufficient to characterize the relative nanoscale spatial
 343 distribution of the three pairs of functional nuclear sites investigated in our samples. The same
 344 spatial resolution could be achieved, in principle, with many live-cell superresolution imaging
 345 approaches. For instance, Structured illumination Microscopy (SIM), and its point-scanning
 346 equivalent Image Scanning Microscopy (ISM), are superresolution techniques compatible with
 347 live cell imaging, even if their resolution improvement is limited to a factor of ~2 (12). The same
 348 STED nanoscopy includes several variants developed to reduce the STED beam intensity and its
 349 potentially photo-damaging effects (40, 52, 53). Thus, we expect that our ICCS formalism could
 350 be applied to live cell super-resolved images obtained, for instance, by dual color STED- or
 351 SIM-based setups. In this perspective, we believe that our work could be useful to establish, in
 352 the near future, a new type of dynamic analyses that cannot be obtained in other static
 353 superresolution techniques.

354

355 **Materials and Methods**

356 *Cell culture*

Human mammary epithelial cells MCF10A were grown in DMEM (Merck KGaA, Darmstadt, Germany):Ham's F12K (Thermofisher Scientific, Waltham, MA, USA) medium (1:1) containing 5% horse serum, 1% penicillin/streptomycin, 2 mM L-glutamine, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 50 ng/mL cholera toxin (all from Merck KGaA) and 20 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA), at 37 °C in 5% CO₂. For fluorescence microscopy measurements, cells were seeded on glass coverslips coated with 0.5% (w/v) gelatin (Sigma Chemical Co.) and cultured for 18h in growth medium. Cells were incubated for 20 min with the synthetic nucleotides 5-ethynyl-2'-deoxyuridine 10 µM, EdU (Thermofisher Scientific) and 5-Bromouridine 10 mM, BrU (Sigma Chemical Co.) to allow labelling of replication and transcription, respectively. Upon nucleotide incorporation, cells were washed with PBS and fixed with 4% paraformaldehyde (w/v) for 10 min at room temperature.

Sample preparation

For immunostaining, MCF10A cells were permeabilized with 0.1% (v/v) Triton X-100 in blocking bugger (BB), composed of 5% w/v bovine serum albumin (BSA) in PBS, for 1h at room temperature. To recognize nascent DNA (transcription) and RNA (replication) filaments, incorporated BrU and EdU were labeled, respectively. For BrU detection, cells were incubated overnight at 4 °C with a primary rabbit antibody anti-BrdU (Rockland Immunochemicals Inc., Limerick, PA, USA) diluted at 1:1000 in BB, followed by three 15 min rinses in BB. EdU incorporation was detected using the Click-iT EdU imaging kit (Thermo Fisher Scientific) according to the manufacturer instructions but replacing the kit's azide-Alexa488 with Azide-PEG3-biotin-conjugated (Merck) at a 1:500 dilution to allow the subsequent immunostaining. After the click reaction, cells were washed with PBS and incubated with the primary mouse antibody anti-biotin (Merck) diluted at 1:1000 in BB, for 1h at room temperature. To detect PCNA and EdU, incorporated EDU nucleotides were conjugated with biotin-azide using the Click-iT EdU imaging kit as previously described. Samples were then incubated with primary antibodies anti-biotin (1:1000 dilution in BB) and anti-PCNA in rabbit IgG (Santa Cruz Biotechnology Inc. Dallas, Texas,

USA) (1:500 dilution in BB) at the same time for 1h at room temperature. To detect transcriptionally active and repressed chromatin, we labeled BrU and Histone 3 lysine 9 trimethylated (H3K9me3), respectively. The cells were simultaneously incubated with primary antibody anti-BrdU as previously described and anti-H3K9me3 in mouse IgG (Abcam, Cambridge, UK) at a 1:800 dilution, overnight at 4 °C in BB.

After three 15 min rinses in BB, cells were incubated with secondary antibodies (1:200 dilution) for 1h at room temperature in BB, upon which cells were washed with BB three times for 15 min. The secondary antibodies were Atto532 and Chromeo488 conjugated with goat anti-mouse IgG (Rockland Immunochemicals Inc.) and anti-rabbit IgG (Abcam, Cambridge, UK), respectively. All samples were washed with PBS and water before mounting with Mowiol (Sigma).

DNA origami structures containing the two fluorophores Chromeo488 and Atto532 at a well-defined distance of either $d=20$ nm or $d=100$ nm were purchased from GATTAquant (custom nanorulers, GATTAquant, Germany).

Experiments

All imaging experiments were performed on a Leica TCS SP5 gated-STED microscope, using an HCX PL APO 100x 100/1.40/0.70 Oil immersion objective lens (Leica Microsystems, Mannheim, Germany). Emission depletion was accomplished with a 592 nm STED laser.

Excitation was provided by a white laser at the desired wavelength for each sample. For imaging of MCF10A nuclei, Chromeo488 was excited at 470 nm and its fluorescence emission detected at 480-530 nm, with 1-10 ns time gating using a Hybrid detector (Leica Microsystem). Atto532 excitation was performed at 532 nm and the emission collected between 545-580 nm by a Hybrid detector, with time gating of 2.5-6 ns. The two channels were acquired in line-sequential mode and the excitation and depletion power were adjusted separately for each channel. 512×512 pixel images were acquired with a pixel size of 40 nm. Similar settings were used for imaging the 100 nm and 20 nm nanorulers.

408 *Simulations*

409 Dual color images of nuclear foci were simulated using MATLAB. For each channel, the object
 410 consisted in a variable number N of point-like emitters distributed randomly inside a circular
 411 area with a diameter of 16 μm (40). The images were made by 512×512 pixels with a pixel size
 412 of 40 nm. The maximum total number of photons detected from a single pixel position from a
 413 single particle was set to $S=40$. For each channel, the object was convolved with a gaussian Point
 414 Spread Function (PSF) with Full Width Half Maximum (FWHM) of 120 nm and a uniform
 415 background level $B=3$ was added within the circular region. Finally, the resulting images were
 416 corrupted by Poisson noise. To simulate the uncorrelated sample, the foci were distributed
 417 randomly in both channels. To simulate a partially colocalized sample, a number $N/4$ of foci in
 418 the second channel was set to have the same coordinates of $N/4$ foci in the first channel. To
 419 simulate the sample colocalized in a zone, the colocalized foci were forced to be in a specific
 420 quarter of the circular area. The total number of foci in each channel was varied from $N=100$ to
 421 $N=10000$.

422 *Image cross-correlation spectroscopy (ICCS) and local ICCS*

423 The ICCS analysis was performed in MATLAB using a custom code. The 2D image correlation
 424 functions were calculated as:

$$425 \quad G_{ij}(\delta_x, \delta_y) = \frac{\langle I_i(x,y)I_j(x+\delta_x,y+\delta_y) \rangle}{\langle I_i(x,y) \rangle \langle I_j(x,y) \rangle} - 1 \quad (1)$$

426 where $I_1(x,y)$ and $I_2(x,y)$ are the images in the first and the second channel, respectively, and the
 427 angle brackets indicate averaging over all the selected pixels of the image. The two autocorrelation
 428 functions were obtained by setting $i=j=1$ and $i=j=2$, respectively, whereas the cross-correlation
 429 function was obtained by setting $i=1$ and $j=2$. The numerator in Eq.(1) was calculated by a 2D-FFT
 430 (Fast Fourier Transform) algorithm. Before calculation, a Region of Interest (ROI) corresponding to
 431 the nucleus was defined and all the pixels outside this ROI were assigned an intensity value equal to

the average value inside ROI, as reported previously (41). This step is useful to minimize the effects of nuclear borders on the correlation functions.

The 2D correlation functions were then converted into radial 1D correlation functions $G_{ij}(\delta_r)$ by performing an angular mean, as described previously (44). The resulting radial correlation functions were then fitted to a gaussian model:

$$G_{ij}(\delta_r) = G_{\infty} + G_{ij}(0)\exp(-\delta_r^2/w_{ij}^2) \quad (2)$$

in order to extract the amplitude parameters $G_{ij}(0)$ and the width parameters w_{ij} .

The amplitude parameters were used to calculate the coefficients of colocalizations M_1 and M_2 (36):

$$M_1 = G_{12}(0)/G_{22}(0) \quad (3)$$

$$M_2 = G_{12}(0)/G_{11}(0) \quad (4)$$

The colocalized fraction f_{ICCS} was then calculated as the arithmetic mean of the two coefficients M_1 and M_2 . The broadening of the cross-correlation function with respect to the corresponding auto-correlation function has been evaluated with the parameter $\Delta w = w_{12} - w_{\text{cc}}$, where $w_{\text{cc}} = ((w_{11}^2 + w_{22}^2)/2)^{1/2}$.

To discard bad cross-correlation function fits, we excluded i) fits with a chi-square value 50 times larger than the chi-square value of the auto-correlation function fit; ii) fits with a width w_{12} too different compared to the average autocorrelation function widths ($r_w < 0.5$ or $r_w > 2$, where $r_w = w_{12}/(w_{11}w_{22})^{1/2}$); iii) fits with a negative offset G_{∞} ($G_{\infty} < -0.2 G_{12}(0)$). In all these cases, we set $f_{\text{ICCS}} = 0$.

The local ICCS analysis consisted in performing ICCS iteratively on small, 69×69 pixels wide, sub-regions of the full-size image. For each sub-region, the local auto- and cross-correlation function were calculated and the parameters $G_{ij}(0)$, w_{ij} and f_{ICCS} were extracted as described for ICCS analysis. In addition to the conditions described above, we excluded local auto-correlation fits with an amplitude 10-times larger than the amplitude of the global autocorrelation function and local cross-correlation fits with an amplitude 2-times larger than the amplitude of the local autocorrelation function. The calculation was limited to $N_{\text{samp}} = 100$ sampling sub-regions

centered on N_{samp} pixels uniformly distributed over the image. Then the resulting values were interpolated to produce a map of the same size of the full-size image.

Object-based analysis and relative distance distribution (RDD)

The central coordinates of the foci in each channel were obtained using the JaCoP plugin (54) in ImageJ (55). For each channel, an intensity threshold was set manually. The minimum size of the particles was set to 2 pixels. The algorithm provided the coordinates of all the localized particles in each channel and the values of distance from each particle in the first channel from all the particles in the second channel. In the simulations, particles were considered colocalized if their distance was lower than 50 nm. In the biological samples, particles were considered colocalized if their distance was lower than 140 nm. All the values of distance were used to build the relative distance distribution (RDD) histogram. To estimate the random component in the cumulative RDD histogram, a linear fit of the data through the origin was performed in the range 250-500 nm. This linear component was then subtracted from the data to obtain a RDD without the random component.

Author Contributions. LL, AD, EG, MF, GID, PGP designed research; MO, LS, MJS, LF, PB performed research; LL and LS wrote software; LL, MO, LS, MJS, IC, SP analyzed data; LL wrote the paper with input from all the authors.

Acknowledgements. LL, MO and MJS were supported by Fondazione Cariplo and Associazione Italiana per la Ricerca sul Cancro (AIRC) through Trideo (Transforming Ideas in Oncological Research) Grant number 17215. The research leading to these results has also received funding from AIRC under MFAG 2018 - ID. 21931 project – P.I. Lanzaò Luca.

482 References

- 483 1. Misteli, T. 2010. Higher-order genome organization in human disease. *Cold Spring Harb Perspect*
484 *Biol* 2:a000794.
- 485 2. Wei, X., J. Samarabandu, R. S. Devdhar, A. J. Siegel, R. Acharya, and R. Berezney. 1998. Segregation
486 of transcription and replication sites into higher order domains. *Science* 281:1502-1506.
- 487 3. Garcia-Muse, T., and A. Aguilera. 2016. Transcription-replication conflicts: how they occur and how
488 they are resolved. *Nat Rev Mol Cell Biol* 17:553-563.
- 489 4. Dellino, G. I., and P. G. Pelicci. 2014. Next-generation sequencing and DNA replication in human
490 cells: the future has arrived. *Future Oncol* 10:683-693.
- 491 5. Macheret, M., and T. D. Halazonetis. 2018. Intragenic origins due to short G1 phases underlie
492 oncogene-induced DNA replication stress. *Nature* 555:112-116.
- 493 6. Bintu, B., L. J. Mateo, J. H. Su, N. A. Sinnott-Armstrong, M. Parker, S. Kinrot, K. Yamaya, A. N.
494 Boettiger, and X. Zhuang. 2018. Super-resolution chromatin tracing reveals domains and
495 cooperative interactions in single cells. *Science* 362.
- 496 7. Giral, H., L. Lanzano, Y. Caldas, J. Blaine, J. W. Verlander, T. Lei, E. Gratton, and M. Levi. 2011. Role
497 of PDZK1 protein in apical membrane expression of renal sodium-coupled phosphate transporters.
498 *J Biol Chem* 286:15032-15042.
- 499 8. Sekar, R. B., and A. Periasamy. 2003. Fluorescence resonance energy transfer (FRET) microscopy
500 imaging of live cell protein localizations. *J Cell Biol* 160:629-633.
- 501 9. Pelicci, S., A. Diaspro, and L. Lanzano. 2019. Chromatin nanoscale compaction in live cells visualized
502 by acceptor-to-donor ratio corrected Forster resonance energy transfer between DNA dyes. *J*
503 *Biophotonics*:e201900164.
- 504 10. Furia, L., P. G. Pelicci, and M. Faretta. 2013. A computational platform for robotized fluorescence
505 microscopy (II): DNA damage, replication, checkpoint activation, and cell cycle progression by high-
506 content high-resolution multiparameter image-cytometry. *Cytometry A* 83:344-355.
- 507 11. Lonn, P., and U. Landegren. 2017. Close Encounters - Probing Proximal Proteins in Live or Fixed
508 Cells. *Trends Biochem Sci* 42:504-515.
- 509 12. Schermelleh, L., A. Ferrand, T. Huser, C. Eggeling, M. Sauer, O. Biehlmaier, and G. P. C. Drummen.
510 2019. Super-resolution microscopy demystified. *Nat Cell Biol* 21:72-84.
- 511 13. Betzig, E., G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W.
512 Davidson, J. Lippincott-Schwartz, and H. F. Hess. 2006. Imaging intracellular fluorescent proteins at
513 nanometer resolution. *Science* 313:1642-1645.
- 514 14. Hess, S. T., T. P. Girirajan, and M. D. Mason. 2006. Ultra-high resolution imaging by fluorescence
515 photoactivation localization microscopy. *Biophys J* 91:4258-4272.
- 516 15. Rust, M. J., M. Bates, and X. Zhuang. 2006. Sub-diffraction-limit imaging by stochastic optical
517 reconstruction microscopy (STORM). *Nature methods* 3:793-795.
- 518 16. Hell, S. W., and J. Wichmann. 1994. Breaking the diffraction resolution limit by stimulated emission:
519 stimulated-emission-depletion fluorescence microscopy. *Opt Lett* 19:780-782.
- 520 17. Arena, E. T., C. T. Rueden, M. C. Hiner, S. Wang, M. Yuan, and K. W. Eliceiri. 2017. Quantitating the
521 cell: turning images into numbers with ImageJ. *Wiley Interdiscip Rev Dev Biol* 6.
- 522 18. Lagache, T., N. Sauvonnnet, L. Danglot, and J. C. Olivo-Marin. 2015. Statistical analysis of molecule
523 colocalization in bioimaging. *Cytometry A* 87:568-579.
- 524 19. Andronov, L., I. Orlov, Y. Lutz, J. L. Vonesch, and B. P. Klaholz. 2016. ClusterViSu, a method for
525 clustering of protein complexes by Voronoi tessellation in super-resolution microscopy. *Sci Rep*
526 6:24084.
- 527 20. Caetano, F. A., B. S. Dirk, J. H. Tam, P. C. Cavanagh, M. Goiko, S. S. Ferguson, S. H. Pasternak, J. D.
528 Dikeakos, J. R. de Bruyn, and B. Heit. 2015. MliSR: Molecular Interactions in Super-Resolution
529 Imaging Enables the Analysis of Protein Interactions, Dynamics and Formation of Multi-protein
530 Structures. *PLoS Comput Biol* 11:e1004634.
- 531 21. Rossy, J., E. Cohen, K. Gaus, and D. M. Owen. 2014. Method for co-cluster analysis in multichannel
532 single-molecule localisation data. *Histochem Cell Biol* 141:605-612.

- 533 22. Xu, J., H. Ma, J. Jin, S. Uttam, R. Fu, Y. Huang, and Y. Liu. 2018. Super-Resolution Imaging of Higher-
534 Order Chromatin Structures at Different Epigenomic States in Single Mammalian Cells. *Cell Rep*
535 24:873-882.
- 536 23. Levet, F., G. Julien, R. Galland, C. Butler, A. Beghin, A. Chazeau, P. Hoess, J. Ries, G. Giannone, and J.
537 B. Sibarita. 2019. A tessellation-based colocalization analysis approach for single-molecule
538 localization microscopy. *Nat Commun* 10:2379.
- 539 24. Adler, J., and I. Parmryd. 2010. Quantifying colocalization by correlation: the Pearson correlation
540 coefficient is superior to the Mander's overlap coefficient. *Cytometry A* 77:733-742.
- 541 25. MANDERS, E. M. M., F. J. VERBEEK, and J. A. ATEN. 1993. Measurement of co-localization of objects
542 in dual-colour confocal images. *Journal of Microscopy* 169:375-382.
- 543 26. Galiani, S., D. Waithe, K. Reglinski, L. D. Cruz-Zaragoza, E. Garcia, M. P. Clausen, W. Schliebs, R.
544 Erdmann, and C. Eggeling. 2016. Super-resolution Microscopy Reveals Compartmentalization of
545 Peroxisomal Membrane Proteins. *J Biol Chem* 291:16948-16962.
- 546 27. Neumann, D., J. Buckers, L. Kastrup, S. W. Hell, and S. Jakobs. 2010. Two-color STED microscopy
547 reveals different degrees of colocalization between hexokinase-I and the three human VDAC
548 isoforms. *PMC Biophys* 3:4.
- 549 28. Reisinger, E., C. Bresee, J. Neef, R. Nair, K. Reuter, A. Bulankina, R. Nouvian, M. Koch, J. Buckers, L.
550 Kastrup, I. Roux, C. Petit, S. W. Hell, N. Brose, J. S. Rhee, S. Kugler, J. V. Brigande, and T. Moser.
551 2011. Probing the functional equivalence of otoferlin and synaptotagmin 1 in exocytosis. *J Neurosci*
552 31:4886-4895.
- 553 29. Magde, D., E. Elson, and W. W. Webb. 1972. Thermodynamic Fluctuations in a Reacting System-
554 Measurement by Fluorescence Correlation Spectroscopy. *Physical Review Letters* 29:705-708.
- 555 30. Bacia, K., and P. Schwille. 2007. Practical guidelines for dual-color fluorescence cross-correlation
556 spectroscopy. *Nat Protoc* 2:2842-2856.
- 557 31. Foo, Y. H., N. Naredi-Rainer, D. C. Lamb, S. Ahmed, and T. Wohland. 2012. Factors affecting the
558 quantification of biomolecular interactions by fluorescence cross-correlation spectroscopy. *Biophys*
559 *J* 102:1174-1183.
- 560 32. Di Bona, M., M. A. Mancini, D. Mazza, G. Vicidomini, A. Diaspro, and L. Lanzano. 2019. Measuring
561 Mobility in Chromatin by Intensity-Sorted FCS. *Biophys J* 116:987-999.
- 562 33. Wachsmuth, M., T. A. Knoch, and K. Rippe. 2016. Dynamic properties of independent chromatin
563 domains measured by correlation spectroscopy in living cells. *Epigenetics Chromatin* 9:57.
- 564 34. Langowski, J. 2017. Single plane illumination microscopy as a tool for studying nucleome dynamics.
565 *Methods* 123:3-10.
- 566 35. Wiseman, P. W. 2015. Image correlation spectroscopy: principles and applications. *Cold Spring Harb*
567 *Protoc* 2015:336-348.
- 568 36. Comeau, J. W., S. Costantino, and P. W. Wiseman. 2006. A guide to accurate fluorescence
569 microscopy colocalization measurements. *Biophys J* 91:4611-4622.
- 570 37. Digman, M. A., P. W. Wiseman, A. R. Horwitz, and E. Gratton. 2009. Detecting protein complexes in
571 living cells from laser scanning confocal image sequences by the cross correlation raster image
572 spectroscopy method. *Biophys J* 96:707-716.
- 573 38. Semrau, S., L. Holtzer, M. Gonzalez-Gaitan, and T. Schmidt. 2011. Quantification of biological
574 interactions with particle image cross-correlation spectroscopy (PICCS). *Biophys J* 100:1810-1818.
- 575 39. Gao, X., P. Gao, B. Prunsche, K. Nienhaus, and G. U. Nienhaus. 2018. Pulsed interleaved excitation-
576 based line-scanning spatial correlation spectroscopy (PIE-lsSCS). *Sci Rep* 8:16722.
- 577 40. Sarmiento, M. J., M. Oneto, S. Pelicci, L. Pesce, L. Scipioni, M. Faretta, L. Furia, G. I. Dellino, P. G.
578 Pelicci, P. Bianchini, A. Diaspro, and L. Lanzano. 2018. Exploiting the tunability of stimulated
579 emission depletion microscopy for super-resolution imaging of nuclear structures. *Nat Commun*
580 9:3415.
- 581 41. Comeau, J. W., D. L. Kolin, and P. W. Wiseman. 2008. Accurate measurements of protein
582 interactions in cells via improved spatial image cross-correlation spectroscopy. *Mol Biosyst* 4:672-
583 685.
- 584 42. Wu, Y., M. Eghbali, J. Ou, R. Lu, L. Toro, and E. Stefani. 2010. Quantitative determination of spatial
585 protein-protein correlations in fluorescence confocal microscopy. *Biophys J* 98:493-504.

- 586 43. Hebert, B., S. Costantino, and P. W. Wiseman. 2005. Spatiotemporal image correlation
587 spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO
588 cells. *Biophys J* 88:3601-3614.
- 589 44. Scipioni, L., E. Gratton, A. Diaspro, and L. Lanzano. 2016. Phasor Analysis of Local ICS Detects
590 Heterogeneity in Size and Number of Intracellular Vesicles. *Biophys J* 111:619-629.
- 591 45. Scipioni, L., M. Di Bona, G. Vicidomini, A. Diaspro, and L. Lanzano. 2018. Local raster image
592 correlation spectroscopy generates high-resolution intracellular diffusion maps. *Commun Biol* 1:10.
- 593 46. Hendrix, J., T. Dekens, W. Schrimpf, and D. C. Lamb. 2016. Arbitrary-Region Raster Image
594 Correlation Spectroscopy. *Biophys J* 111:1785-1796.
- 595 47. Sancataldo, G., L. Scipioni, T. Ravasenga, L. Lanzanò, A. Diaspro, A. Barberis, and M. Duocastella.
596 2017. Three-dimensional multiple-particle tracking with nanometric precision over tunable axial
597 ranges. *Optica* 4:367-373.
- 598 48. Thompson, R. E., D. R. Larson, and W. W. Webb. 2002. Precise nanometer localization analysis for
599 individual fluorescent probes. *Biophys J* 82:2775-2783.
- 600 49. Bannister, A. J., and T. Kouzarides. 2011. Regulation of chromatin by histone modifications. *Cell Res*
601 21:381-395.
- 602 50. Choe, K. N., and G. L. Moldovan. 2017. Forging Ahead through Darkness: PCNA, Still the Principal
603 Conductor at the Replication Fork. *Mol Cell* 65:380-392.
- 604 51. Malyavantham, K. S., S. Bhattacharya, W. D. Alonso, R. Acharya, and R. Berezney. 2008. Spatio-
605 temporal dynamics of replication and transcription sites in the mammalian cell nucleus.
606 *Chromosoma* 117:553-567.
- 607 52. Lanzano, L., I. Coto Hernandez, M. Castello, E. Gratton, A. Diaspro, and G. Vicidomini. 2015.
608 Encoding and decoding spatio-temporal information for super-resolution microscopy. *Nat Commun*
609 6:6701.
- 610 53. Vicidomini, G., G. Moneron, K. Y. Han, V. Westphal, H. Ta, M. Reuss, J. Engelhardt, C. Eggeling, and
611 S. W. Hell. 2011. Sharper low-power STED nanoscopy by time gating. *Nature methods* 8:571-573.
- 612 54. Bolte, S., and F. P. Cordelieres. 2006. A guided tour into subcellular colocalization analysis in light
613 microscopy. *J Microsc* 224:213-232.
- 614 55. Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image
615 analysis. *Nature methods* 9:671-675.

616

617

618 Figure Legends

619 **Fig.1 Comparison between STED-ICCS and object-based analysis on simulated data.** (a)
620 Simulated dual color images of nuclear foci at low density (total number of foci, $N=100$),
621 assuming a random distribution of foci in each channel (uncorrelated), a 25% of foci
622 colocalization in the two channels (colocalized) and a 25% of foci colocalizing only in a specific
623 sub-portion of the image (colocalized in a zone), indicated by the dashed contour. The data were
624 simulated with a PSF size of FWHM=120 nm. (b) Relative distance distributions (RDD)
625 obtained by object-based analysis of the simulated images. (c) Simulated dual color images of
626 nuclear foci at high density (total number of foci, $N=2000$). (d) Spatial correlation functions of
627 the images shown in (c). Shown are the cross-correlation function (black triangles) and the single
628 channel auto-correlation functions (magenta dots and cyan squares) along with the corresponding
629 fits (solid lines). (e) Local ICCS maps of the images shown in (c). The colormap represents the
630 value of parameter f_{ICCS} calculated on a moving subregion of 69×69 pixels. (f) Colocalized
631 fraction extracted from object-based analysis of the simulated data as a function of the number of
632 foci N . The dashed red line shows the trend for the colocalized sample when the PSF size is
633 FWHM=250nm. (g) Colocalized fraction extracted by ICCS analysis of the simulated data as a
634 function of the number of foci. (h) The value of f_{ICCS} extracted from the local ICCS map inside
635 (in) and outside (out) the colocalization zone for the ‘colocalized in a zone’ sample, compared
636 with the value of f_{ICCS} in the ‘colocalized’ sample.

637 **Fig.2 STED-ICCS of model samples.** (a,b) Schematic drawing and representative dual color
638 STED images of the optical nanorulers. These model systems consist of Chromeo-488 and Atto-
639 532 fluorophores located at a fixed distance of 20 nm (a) and 100 nm (b). The shaded circles
640 schematically represent the PSF. Scale bar 1 μ m. (c,d) Object-based analysis for the 20-nm (c)
641 and 100-nm (d) nanorulers. Shown are the relative distance distribution histograms (RDD)
642 before (top) and after (bottom) subtraction of the uncorrelated random component. Solid red
643 lines are Gaussian fits of the data ($d_{20nm}=40 \pm 30$ nm, $d_{100nm}=100 \pm 25$ nm, mean $\pm$ s.d.). (e,f) Raw
644 (top) and normalized (bottom) correlation functions of the representative images shown in (a,b).
645 Shown are the cross-correlation function (black triangles) and the red (red square) and green
646 (green circles) channel auto-correlation functions along with the corresponding fits (solid lines).
647 (g) Colocalized fraction extracted from STED-ICCS analysis. (h) Cross-correlation function
648 broadening obtained from STED-ICCS. (i) Values of distances determined by object-based
649 analysis and STED-ICCS.

650 **Fig.3 Analysis of nuclear foci by STED-ICCS and object-based localization.** Analysis of
651 representative STED images of MCF10A cells acquired upon labelling of (a) BrU (green) and
652 H3K9me3 (red), (b) PCNA (green) and EdU (red), (c) BrU (green) and EdU (red). Shown are
653 (from left to right) the dual color STED image, the positions of the colocalized (cyan) and non-
654 colocalized (red and green) foci recovered by particle localization, the map of the colocalized
655 fraction recovered by local ICCS, the RDD histogram and the spatial correlation functions
656 recovered by ICCS. The ICCS plot shows the cross-correlation function (black triangles) and the
657 red (red squares) and green (green circles) channel auto-correlation functions along with the
658 corresponding fits (solid lines).

659 **Fig.4 Relative nanoscale spatial distribution of the investigated nuclear sites in MCF10A**
660 **cells.** (a,b) Cumulative results of the object-based analysis. Shown are the relative distance
661 distribution histograms (RDD) before (a) and after (b) subtraction of the uncorrelated random

662 component. Solid red lines are Gaussian fits of the data (PCNA-EdU: $d=55\pm34$ nm, mean $\pm$ s.d.,
 663 $n=19$ cells; BrU-EdU: $d=100\pm50$ nm, mean $\pm$ s.d., $n=22$ cells). The dashed red lines indicate the
 664 standard deviation of the data in the range 250-500 nm after subtraction of a linear fit of the
 665 uncorrelated component. (c,d) Cumulative results of the STED-ICCS analysis. (c) Colocalized
 666 fraction extracted from STED-ICCS analysis. (d) Cross-correlation function broadening
 667 extracted from STED-ICCS. (e) Values of distances extracted by object-based analysis and
 668 STED-ICCS for the correlated samples.

669

a **b** **c** **d** **e**