

1 **Quantification of DNA damage induced γ H2AX focus formation**
2 **via super-resolution dSTORM localization microscopy**

3

4

5 Dániel Varga^{2,#}, Hajnalka Majoros^{1,#}, Zsuzsanna Újfaludi¹, Miklós Erdélyi^{2,*} and Tibor
6 Pankotai^{1,*}

7

8 ¹ University of Szeged, Department of Biochemistry and Molecular Biology Genome
9 Integrity and DNA Repair Group

10 ² University of Szeged, Department of Optics and Quantum Electronics

11

12 Running title: Quantitative super-resolution measurement of γ H2AX foci

13

14 [#] These authors contributed equally to this work

15

16 * Corresponding authors, E-mail: pankotai@bio.u-szeged.hu, erdelyim@physx.u-szeged.hu

17

18 **SUMMARY**

19 In eukaryotic cells, each process, in which DNA is involved, should take place in the context
20 of chromatin structure. DNA double-strand breaks (DSBs) are one of the most deleterious
21 damages often leading to chromosomal rearrangement. In response to environmental stresses,
22 cells have developed repair mechanisms to eliminate the DSBs. Upon DSB induction, several
23 factors play roles in chromatin relaxation by catalysing the appropriate histone
24 posttranslational modification (PTM) steps, therefore promoting the access of the repair
25 factors to the DSBs. Among these PTMs, the phosphorylation of the histone variant H2AX at
26 its Ser139 residue (also known as γ H2AX) could be observed at the break sites. The structure
27 of γ H2AX focus has to be organized during the repair as it contributes to accessibility of
28 specific repair proteins to the damaged site. Our aim was to develop a quantitative approach
29 to analyse the morphology of individual repair foci by super-resolution dSTORM microscopy
30 to gain insight into genome organization in DNA repair. We have established a specific
31 dSTORM measurement process by developing a new analytical algorithm for gaining
32 quantitative information about chromatin morphology and repair foci topology at individual
33 γ H2AX enriched repair focus. By this method we quantified unique repair foci to show the
34 average distribution of γ H2AX clusters. By monitoring γ H2AX signal, we could reach 20 nm
35 spatial resolution and resolve a single DNA damage spot, which allow us to identify different
36 chromatin sub-clusters around the break site. Additionally, based on our new analysis method,
37 we were able to show the number of nucleosomes in each sub-cluster that could allow us to
38 define the possible chromatin structure and the nucleosome density around the break sites.
39 This method is the first demonstration of a single-cell based quantitative measurement of a
40 discrete repair focus, which could provide new opportunities to categorize spatial organization
41 of dot patterns by parametric determination of topological similarity.

42

43 **Keywords:**

44 DNA repair, dSTORM, super-resolution microscopy, γ H2AX, DNA repair foci, histone
45 quantification, DNA damage response, chromatin sub-cluster, chromatin

46

47

48

49 INTRODUCTION

50 The DNA in the nucleus is constantly targeted by different damaging agents deriving from
51 both endogenous and exogenous sources. DNA double-strand breaks (DSBs) are the most
52 deleterious lesions, therefore they have to be repaired as quickly and efficiently as it is
53 possible to prevent chromosomal loss and translocation. Since DSBs affect DNA integrity
54 simultaneously with the recruitment of early DNA repair factors, a DNA damage response
55 (DDR) is activated in the cells, which can arrest the cell cycle ^{1,2}. For efficient DDR
56 activation, different DSB sensors are required to activate chromatin reorganization and
57 recruitment of downstream repair proteins that can eventually accomplish the efficient repair
58 process ³. Recent studies have already shown that DNA damage can lead to immediate
59 chromatin relaxation around the site of the damage ⁴⁻⁶. One of the first steps of DSB induced
60 chromatin reorganization is the phosphorylation of the histone variant H2AX at its S139
61 residue (called γ H2AX) in the proximity of the damaged site ^{4,5,7,8}. The γ H2AX enriched
62 chromosomal locus, considered as repair focus, marks the damage site to initiate the
63 recruitment of further repair proteins required for the process and performance of the repair
64 ^{9,10}. Several factors, such as cell cycle state, functional activity of genes, break position along
65 the DNA sequence, temporal state of DNA compaction, number of simultaneously occurring
66 DSBs, etc. have been known to influence this process, thereby assigning the fate of the cell
67 ^{11,12}. The γ H2AX signal detection is regularly used to visualize and quantify the extent of
68 DSBs and to follow the DNA repair kinetics. Several techniques have been applied to follow
69 the changes in γ H2AX signal intensity. Chromatin immunoprecipitation studies have revealed
70 that the γ H2AX signal shows asymmetrical distribution around the damage site with lower
71 density at the transcribed regions ¹³⁻²⁰. It has been already shown that both the genome
72 topology and chromatin state are crucial for the organization of the recruitment of repair
73 proteins. A recent study based on chromosome conformation capture experiments has
74 highlighted the complexity of genome re-organization, including megabase range associations
75 between certain chromosomal regions as well as smaller genomic interactions, which involve
76 kilobase-length DNA segments ^{21,22}. Additionally, optical methods, such as conventional
77 confocal microscopy have also been regularly used for mapping the spatial distribution of
78 DSBs. Due to the spatial resolution of these methods, the DDR signal can be detected in
79 maximum 300 nm resolution. By using these techniques, it was shown that the γ H2AX signal
80 distributes up to a megabase around the damaged site ⁸ generating DNA repair foci with a
81 typical feature size of half a micron, which is just above the resolution limit of traditional

82 fluorescence microscopes. High resolution imaging based datasets of DSB structures have
83 already been published and demonstrated via single molecule localization methods (SMLM)
84 ²³⁻²⁸, structured illumination microscopy (SIM) ^{26,29,30}, and stimulated emission depletion
85 (STED) ²⁹⁻³¹ super-resolution methods. These images can be further evaluated by using cluster
86 analysis, and both the spatial distribution and the geometrical parameters of foci and even
87 nano-foci can be determined ^{32,33}. Single molecule localization methods (SMLM), such as
88 dSTORM, provide the highest spatial resolution among optical methods and open the way for
89 imaging biological structures in the sub-20 nm regime ³⁴⁻³⁶. SMLM determines the positions
90 of individual molecules, which are used to create the final image ³⁷⁻³⁹. Such an image
91 registration and processing method is especially appropriate for quantitative evaluation.
92 Although dSTORM separates the sub-domains of the repair focus, quantitative evaluation of
93 the images has been still challenging because the number of detected localizations
94 ($N_{localizations}$) generated by a single-labelled histone has been still unknown. Therefore, for
95 quantitative analysis, the number of localizations per labelled histone has to be statistically
96 determined.

97 Here we provide insight into γ H2AX distribution in nanometre resolution by using super-
98 resolution dSTORM microscopy technique applied either on U2OS cells exposed to
99 neocarzinostatin treatment or on AsiSI endonuclease-expressing D1vA cells ^{18,40}. By
100 surpassing the limitation of classical confocal microscopy, super-resolution dSTORM
101 microscopy possesses high prospecting capacity, which allows us to enlarge complex
102 structures at γ H2AX-covered chromatin regions in 20 nm resolution. With this technique we
103 measured the DDR profiles of several genomic regions and gained temporal, functional and
104 structural insights into the damaged chromatin units evolved during DSB repair. By means of
105 dSTORM, in accord with already published data, we observed that the number of γ H2AX foci
106 increased following DSB induction ^{41,42}. In addition, we demonstrated that the sizes of these
107 foci were extended under DSB formation and we could also provide a higher resolution of the
108 foci spatial organization using our new statistical approaches. This article preconcerts the
109 nano-scale organization of the repair foci, which could highlight the spatial localization of the
110 sub-domain structure and quantitative measurements of these repair centres.

111

112 **RESULTS**

113 **The experimental system and the determination of the parameters used in dSTORM**

114 The typical size of a DNA repair focus is about half micron, just above the resolution limit of
115 traditional fluorescence microscopes. However, at high density of the DNA breaks when
116 individual foci merge and form larger blobs the traditional imaging methods cannot be
117 utilized. In such cases optical super-resolution microscopy is required to distinguish
118 individual foci and reveal their sub-structures containing 20-60 nm nano-foci⁴³. In order to
119 prove this, we generated DNA DSBs by applying neocarzinostatin and 4-OHT treatment to
120 U2OS and D1vA cells, respectively^{14,40}. We quantified the DSB triggered γ H2AX foci
121 formation 2 hours following the break induction by labelling H2AX S139 phosphorylated
122 sites with fluorophore-conjugated antibody. We generated traditional EPI fluorescent and high
123 resolution dSTORM images from nuclei of non-treated and treated cells (Fig 1 A vs B). By
124 performing dSTORM, we observed an increase in the number and distribution of γ H2AX foci
125 following DSB induction. In higher magnification of individual foci, we could identify sub-
126 structures that we used in our quantitative analysis (Fig 1 C).

127

128 **Spatial distribution of DSB foci within cells**

129 In contrast to traditional optical microscope images, at which the separation of individual foci
130 is a great challenge and their size can only be quantified by their intensity values, cluster-
131 analysis dSTORM images pave the way for quantitative evaluation. Quantitative functions of
132 DSB foci, such as their spatial density variation and their area distribution were evaluated by
133 means of 2D density-based spatial cluster analysis (DBSCAN). This algorithm requires two
134 input parameters: a minimum number of points that forms a cluster (N_{core}) and the maximum
135 distance between two adjacent points (ϵ)⁴⁴. For the elimination of non-specific labelling and
136 imprecise, out-of-focus localization, N_{core} and ϵ were set to 8 and 50 nm during the
137 simulations, respectively. Figure 2 A, C and E represent a typical dSTORM super-resolved
138 image of non-treated (NT) and treated (T) (with NCS and 4-OHT) U2OS and D1vA cells. The
139 cluster-analysis images of the selected cells are also shown (Figure 2 B, D and F). In order to
140 efficiently reveal the DSB distribution pattern inside the nucleus, the cluster analysis module
141 was implemented into our rainSTORM localization software (for details see Methods).

142 The algorithm isolated and quantified each DSB focus based on their area and spatial
143 distribution inside the nucleus. While Figure 2 A-F show single but typical nuclei, Figure G-L
144 depict the evaluation results of several, 5 untreated, 4 treated U2OS and 6 treated D1vA
145 nuclei, respectively. By using the algorithm, discrete DSB foci were analysed by quantifying

146 the γ H2AX tag-pair distances. These defined foci are different from those detected by
147 standard microscopy showing the sub-structures of molecular arrangements (Figure 2 B, D
148 and F).

149 It was reported that in euchromatic milieu DNA breaks could be repaired more effectively
150 since these breaks do not need to be repositioned outside of the heterochromatic domain for
151 the successful repair⁴⁵. In order to detect the distribution of the γ H2AX enriched repair foci
152 within the nucleus we applied cluster recognition, and we compared their position to the
153 centre of the nucleus determined by a computer algorithm. This position was used as a central
154 point to plot individual repair foci by modelling their localization as a circular shell. The
155 spatial density of the clusters shows a non-linear distribution (grey dots and trend lines in
156 Figure 2 G, H and I). Following DSB induction, in comparison to the control nuclei, we could
157 detect almost 3-times more foci formation in the treated U2OS and DlvA cells (grey dots and
158 trend lines in Figure 2 H and I vs G), respectively. The amount of the measured density
159 increases from the periphery towards the centre of the nucleus due to the different chromatin
160 organization. For further quantitative analysis of the clusters, we sorted them into two classes
161 based on their size (indicated in red and blue in Figure 2 G-I). While in the nuclei of untreated
162 cells, both populations have similar and regular density distribution, in the treated cells the
163 number of larger clusters ($>5,000 \text{ nm}^2$) was found to be 2 times more than the number of the
164 small ($<5,000 \text{ nm}^2$) ones (Figure 2 H-I red vs. blue lines). Therefore, the larger clusters could
165 be appeared upon DNA damage induction and it could be differed from the foci induced by
166 endogenous DNA damages.

167 Finally, for deep evaluation, several individual foci of each treatment category were chosen.
168 For that, the sizes of clusters associated with the DSB foci were categorized by their area, and
169 their distribution was presented in histograms shown on Figure 2 J, K and L. The measured
170 distributions could be fitted with lognormal curves³⁷. In control cells the expected area of the
171 calculated surface was found to be $2,950 \text{ nm}^2$, and this value was only slightly changed in the
172 treated U2OS and DlvA cells ($2,850 \text{ nm}^2$ and $3,150 \text{ nm}^2$). However, the mean values of the
173 calculated distributions were increased with 18% ($8,750 \text{ nm}^2$) and 55% ($11,550 \text{ nm}^2$) in the
174 treated cells compared to the untreated ones ($7,450 \text{ nm}^2$), since the normalized occurrence of
175 the large-sized clusters was enriched following DSB induction. The presented data reveal that
176 this algorithm can also be used to separate the γ H2AX background, i.e. endogenous versus
177 DSB induced signals (Figure 2 J vs. K and L). These data suggest that DSBs induced by either

178 neocarzinostatin or 4-OHT could result in elevated γ H2AX enriched foci both in number and
179 size.

180 The evaluation of spatial distribution of DSB foci is based on the cluster analysis of the raw
181 dSTORM images, in which the pixel value represents the number of the accepted
182 localizations. However, the number of localizations ($N_{localizations}$) generated by a single labelled
183 histone strongly depends on the lifetime of the fluorescence ON state ($N_{lifetime}$), the labelling
184 density ($N_{labelling}$) and the number of reactivation circles of the applied dye molecules
185 ($N_{activation}$). Due to multiple localizations, the accepted ones belonging to the same target
186 molecule form a cluster, the size of which depends on the localization precision.

$$N_{localizations} = N_{lifetime} \times N_{labelling} \times N_{activation}$$

187 Segmentation and quantitative evaluation of individual blobs are required to determine the
188 response function of dSTORM imaging, in other words the number of localizations belonging
189 to a single labelled histone molecule. Being aware of this response function the size and
190 spatial distribution of the captured foci can be statistically evaluated.

191 *Trajectory fitting of individual blinking events ($N_{lifetime}$)*

192 In dSTORM the fluorescence dye molecules are stochastically switched between their OFF
193 (no fluorescence), ON (fluorescence) and bleached states. The occupation of these states can
194 be controlled by a special switching buffer and data acquisition (laser power etc.) parameters
195 ⁴⁶. The lifetime of the ON state strongly depends on the biological sample and the local
196 chemical environment ⁴⁷. Ideally, the lifetime of the ON state is in accordance with the
197 exposure time, and the captured photons emitted by a single dye molecule can be visualized
198 on a single image frame. However, the detector is not triggered, and the lifetime of ON state
199 is not constant. As a result, the same dye molecule can be captured on sequential frames and
200 the trajectory length of a single emitted fluorescence shows an exponential decay (Figure 3
201 A). A trajectory fitting module was built into the rainSTORM localization software that can
202 realign these sections⁴⁸. This resulted in less but more precise detection of localizations
203 (Figure 3 B). Labelling density, buffer condition and image acquisition parameters were set to
204 minimize the possibility of spatial and temporal overlap of individual PSFs (Point Spread
205 Function), hence single Gaussian fitting could be used throughout this work. Figure 3 shows
206 the dSTORM (Figure 3 C and D) and the cluster analysis images (Figure 3 E and F) of a focus
207 before and after trajectory fitting, respectively. The trajectory fitted image reveals more

208 structural details, and consequently provides a more appropriate data source for cluster
209 analysis (Figure 3 D and F).

210 ***Number of blinking per individual nano-foci ($N_{labelling} \times N_{activation}$)***

211 Generally, during immunostaining techniques, proteins are recognized by primary and
212 fluorophore-conjugated secondary antibodies (Figure 4 A). However, the number of
213 molecules, taking part in the labelling procedure and then in super-resolution imaging,
214 strongly depends on the actual biological sample (number of epitopes etc.) and the local
215 environment (pH, permeability etc.). During our measurements, due to the sterical hindrance
216 of the nucleosomes, a single primary antibody can bind to the target γ H2AX molecule. In
217 addition, our measurements also support already published data that the connection between
218 the primary and secondary antibody is not equal (i.e. IgG), since the 2nd antibody could
219 recognize two epitope surfaces on the first antibody binding ⁴⁹. Consequently, a single
220 γ H2AX molecule is labelled by one primary and one or two secondary antibodies. The
221 number of dye molecules per secondary antibody was set to four based on consultations with
222 the manufacturer. In conclusion, we used a model in which a single target histone molecule is
223 labelled either by 4 or 8 fluorescent dye molecules, therefore the number of dye molecules per
224 γ H2AX molecule is

225
$$N_{labelling} = N_{primary} \times N_{secondary} \times N_{dye} ,$$

226 where $N_{primary}=1$, $N_{secondary}=1$ or 2 and $N_{dye}=4$. The real ratio of γ H2AX labelled by 4 or 8
227 fluorescent dye molecules could be determined by means of cluster analysis (DBSCAN). To
228 eliminate larger clusters belonging to multiple γ H2AX foci, N and ϵ were set to 5 and 25 nm
229 during the simulations, respectively. After further filtering steps, only the small clusters
230 (area<5000 nm²) were evaluated by providing us a high likelihood that all the accepted
231 clusters were associated with the footprint of a single γ H2AX nano-focus. These clusters are
232 represented with dark blue colour in Figure 4 D. The histograms of localizations per nano-
233 focus were depicted by using four different image stack sizes and were fitted with a
234 theoretical curve (Figure 4 E). This curve is a linear combination of the two distributions
235 representing the cases of 4 and 8 dyes/ γ H2AX. Based on the weight of the two components,
236 the ratio of γ H2AX molecules labelled with a single or two secondary antibodies can be
237 determined ⁴⁹.

238 Additionally, this ratio also depends on the duration of the measurement, since
239 photobleaching plays a central role in the model. In dSTORM technique dye molecules can be
240 switched ON and OFF several times before they are finally bleached. It was shown that in a
241 three-state switching model ⁴⁷ the number of switching circles follows Poisson and
242 geometrical distributions in short ($k_{bl}t \ll 1$) and long ($k_{bl}t \gg 1$) data acquisition times,
243 respectively ⁴⁷. However, the typical number of switching circles ($N_{activation}$) has been already
244 published ⁵⁰, it should be determined more specifically, since it strongly depends on the
245 sample, the buffer conditions and the data acquisition parameters. Based on the evaluation of
246 the fitted curves (Figure 4 E) it can be realized that a measurement time longer than 500 s
247 (>20,000 image frame with 30 ms exposure time) was required for adequate statistical data
248 analysis. This stoichiometric evaluation proved that under the measurement conditions detailed
249 above on average 20 localizations belonged to a single γ H2AX molecule, i.e. the response
250 function of the system was found to be 20 localizations/target molecule.

251 **Quantitative analysis of single DBS foci**

252 Based on the statistically given response function we could determine the number of labelled
253 γ H2AX in the individual foci both in the untreated and in the treated cells. dSTORM images
254 and their cluster maps of three randomly selected cells with three typical foci are represented
255 in Figure 5 (A-I). In the treated cells, the density of DSB foci was found to be 2.8- and 2.2-
256 times higher compared to the untreated U2OS control cells (Figure 5 D and G vs. A). In those
257 cells, in which DSBs were induced, an increased number of γ H2AX localization could be
258 observed within the DSB focus compared to control cells. The histograms obtained from the
259 quantitative measurements are shown on Figure 5 M, N and O. The distribution of cluster
260 sizes based on their γ H2AX values (Figure 2 J, K and L) follows the same kinetic.
261 Consequently, the number of γ H2AX molecules within a cluster is linearly proportional to its
262 area in untreated ($397 \pm 7 \gamma H2AX/nm^2$), treated U2OS ($427 \pm 3 \gamma H2AX/nm^2$), and treated DivA
263 ($412 \pm 4 \gamma H2AX/nm^2$) cells shown on Figure 5 J, K and L, respectively.

264 The major advantage of our algorithm is that it allows the schematic representation of the
265 individual localization of repair foci and we could apply a topological analysis of the captured
266 images. By using the parameters (localization, primary and secondary antibody number,
267 fluorophores, etc.) determined in our measurements, we could mathematically analyse the
268 topology of DNA repair clusters within a focus. Each blinking event was measured, quantified
269 and following the calculations the number of the independent γ H2AX positions were plotted
270 into two-dimensional complexes (Figure 5 A-I). In the representation process each point

271 which localized in proximity ($N=8$ and $\epsilon=50$ nm) were considered to belong to the same
272 cluster. The described plots of each condition (U2OS control, NCS treated U2OS and 4-OHT
273 treated D1vA cells) are shown in Figure 5 C, 5 F and 5 I. These characterizations allow a
274 compact and illustrative visualization of specific sub-structures. Based on this plot we could
275 tag the point structures with barcodes, which provides novel possibilities to analyse and
276 categorize the number of γ H2AX clustered sub-domain structures in cell nuclei (Figures 5 J-
277 L). These representations demonstrate that the endogenous and induced DSBs are covered by
278 approximately 10-50 H2AX S139 phosphorylated histones, which implies an approximately
279 20-40 kb DNA region (Figures 5 M-O)^{51,52}.

280

281 **DISCUSSION**

282 DNA double-strand breaks are one of the most harmful DNA damages since the dsDNA
283 strand loses its integrity and the improper association of these broken DNA strands could lead
284 to chromosomal rearrangements. During DSB repair, the chromatin structure is rearranged
285 and H2AX S139 phosphorylation rapidly appears around the damage sites^{5,6,24}. These steps
286 allow the efficient recruitment of the repair factors to the damaged DNA regions and
287 implicate in the choice between the DNA repair pathways. For examining the DSB-induced
288 chromatin changes, confocal microscopy-based techniques are used in most of the studies,
289 although in the last few years high-throughput chromosome conformation capture technique
290 (4C) and single cell microscopy were utilized to gain detailed insights about the protein
291 interactions and cascades involved in the different repair pathways^{31,53-57}. A more detailed
292 overview has raised more questions, which could be answered only at a single-cell level: how
293 the different DNA repair pathways are chosen and how individual repair proteins are
294 regulated to access the DNA repair site. Answering these questions requires a better
295 resolution, most favourably in a single molecule detection level deeply into the mechanical
296 organization of the orchestrated repair focus. For a single molecule detection, the 200-300 nm
297 resolution, which is the limitation of the conventional microscopy would not provide
298 sufficiently detailed image resolution. Recently G. Legube's laboratory has published detailed
299 information about the chromatin organization of DNA repair centres by using 4C⁵³. However,
300 the limitation of the technique is that it shows the average of a given focus by combining the
301 data obtained from a large population of cells. Recent applications of electron-microscopy and
302 super-resolution light microscopy^{24,30,31,55-57} have demonstrated that it is feasible to study
303 single molecular arrangements within a repair focus. By improving the resolution of
304 microscopy and data evaluation of structures in meso- and nano-scale level, the searching for

305 the best-suited analysis parameters and potentially useful classification criteria of repair foci
306 and damaged chromatin sites have become to be important.

307 In this study we addressed the nano-scale resolution of a single repair focus by quantitative
308 dSTORM technique in order to reveal the structure of γ H2AX containing foci within the
309 nuclear environment. For this, we quantified numerous parameters, such as the number of
310 fluorophores, primary and secondary antibodies, which could bind to a single target molecule,
311 etc. and we applied these parameters to evaluate the images by using dSTORM based image
312 processing. By this unconventional procedure we provided 20 nm resolution imaging
313 followed by cluster analysis of various repair loci. However, the quantitative dSTORM
314 technique has been used for studying cellular events, such as cytoskeleton formation in the
315 cytoplasm, in our study it has been utilized for the first time to study cellular events in the
316 nucleus. By the data we obtained from our quantitative measurements it is the first
317 demonstration of the deep structure of a DNA repair focus, at which a single nucleosome
318 resolution has been obtained together with the γ H2AX sub-domain cluster organization. Our
319 data suggest a looping mechanism, in which approximately twenty S139 phosphorylated
320 H2AX histones are included within a single chromatin sub-domain, which are localized
321 within an approximately 40-50 kb DNA region^{51,52}.

322 Additionally, another important finding of our study is that a single repair focus contains
323 approximately 10 units of γ H2AX enriched sub-cluster. However, we could not determine
324 whether it is a single DSB or several broken DNA regions are associated in one focus. Since
325 γ H2AX clusters spatially distribute in the nuclear space according to a pattern that is
326 dependent on the progression of DDR. This pattern recapitulates the previously described
327 repair kinetics, underlying an euchromatin-to-heterochromatin repair trend since it was shown
328 that heterochromatin regions require further structural remodelling before specific DNA
329 repair proteins could access to those regions. These data highlight another mechanism, in
330 which the complex DNA breaks could be associated in repair centres for efficient DNA
331 repair. This question could be answered in the future by using our quantitative dSTORM
332 method.

333 In conclusion, we could show that dSTORM is the most adequate tool for deep investigation
334 of DNA double-strand break induced repair focus formation. We believe that nowadays this is
335 the most appropriate procedure for quantitative analyses of the structural changes of a single
336 repair focus in individual cells at nano-scale resolution. The measurements and the procedure
337 we applied in our study allow ultra-resolution insights into structures and architectures,

338 offering new perspectives for further understanding the mechanisms of chromatin function in
339 DNA repair.

340

341 ***AUTHOR CONTRIBUTIONS***

342 Conceived the project and designed the experiments: T.P., M.E., H.M., D.V., Zs.U.,

343 Performed the experiments: H.M., D.V., Analysed the data: H.M., D.V., Zs.U., T.P., M.E.

344 Contributed reagents/ materials/ analysis tools: M.E., T.P. Wrote the paper: T.P., M.E., H.M.,
345 D.V., Zs.U.

346

347 ***DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST***

348 No potential conflicts of interest were disclosed.

349

350 ***FUNDING***

351 This research was supported by a grant from National Research, Development and Innovation
352 Office grants GINOP-2.3.2-15-2016-00020, GINOP-2.3.2-15-2016-00036, the Hungarian
353 Brain Research Program (2017-1.2.1-NKP-2017-00002), the EU-funded Hungarian Grant
354 EFOP-3.6.1-16-2016-00008, and the Tempus Foundation

355

356 ***FIGURE LEGENDS***

357

358 **Figure 1.** Traditional EPI fluorescence (A) and dSTORM super-resolved (B) images of nuclei
359 of nontreated (NT) and treated (via NCS and 4-OHT) U2OS and DlvA cells, respectively.
360 Magnified dSTORM images (C) of the selected individual foci. Scale bar: 1 μm (B) and 200
361 nm (C)

362

363 **Figure 2.** dSTORM (A, C, and E) and cluster-analysis (B, D, and F) images of untreated (A,
364 B) and treated U2OS (C, D) and DlvA (E, F) cells. The average cluster density functions are
365 indicated in grey (G, H and I). The density function calculated for small (<5000 nm²) and
366 large (>5000 nm²) clusters are also shown in blue and red, respectively. Comparative
367 histograms of the area distribution of cluster sizes are presented for untreated (J) NCS treated
368 (K) and DlvA cells (L)

369

370 **Figure 3.** The histogram represents the lifetime of the ON state (A). Trajectory fitting precise
371 localizations (orange in B) and the original distribution (blue in B) are represented in B. 2D

372 original (C) and trajectory fitted dSTORM image (D) of the same focus, respectively. Scale
373 bar is 200 nm. Graphical representation of cluster analysis of a focus before (E) and after (F)
374 trajectory fitting, respectively. The different colours indicate different sub-clusters within a
375 γ H2AX cluster.

376

377 **Figure 4.** Schematic representation of the binding of first and secondary antibodies to a
378 γ H2AX (A) and their frame indexes are shown (B). Super-resolved dSTORM image before
379 (C) and after (D) cluster analysis. Images taken during dSTORM (C) and the clusters
380 containing γ H2AX molecules were selected via cluster analysis (D) and their histogram was
381 applied to determine the ratio of labelling via 4 and 8 dye molecules (E) and the response
382 function. Scale bar represents 1 micron.

383

384 **Figure 5.** Super-resolved dSTORM images of the entire nuclei of untreated (A) and treated
385 U2OS (D) and DivA (G) cells. Three typical foci were selected (B, E and H) and cluster
386 analysed (C, F, and I). The number of γ H2AX as function of the area of the cluster is depicted
387 and fitted by a linear curve (J, K, and L) based on evaluation of 5 untreated, 4 treated U2OS
388 and 6 treated DivA cells. Histograms of the γ H2AX number/cluster (M, N and O) using the
389 same data show a similar distribution as the area distribution.

390 **MATERIALS AND METHODS**

391 **Trajectory fitting algorithms.** The exposure time in localization microscopy is matched to
392 the ON state lifetime of individual molecules. However, due to the stochastic feature of the
393 blinking process, a single fluorescent molecule is typically captured in several sequential
394 frames. Trajectory fitting is an inbuilt algorithm in the rainSTORM localization software that
395 links together photons emitted by the same dye molecule. Localizations on sequential frames
396 which are closer to each other than a preliminary defined *Acceptance Radius* are assumed to
397 belong to the same fluorescence dye molecule. As a result, the code calculates the weighted
398 localization coordinates taking into consideration the captured photon numbers. Therefore, the
399 higher the localization precision, the higher the weight factor⁵⁸.

400

401 **Determination of cluster density.** A Matlab code was written to determine the spatial
402 density of clusters inside the nuclei using localization data provided by rainSTORM. First the
403 selected nucleus was segmented with a simple and irregular $N_{polygon}$ -sided ($N_{polygon} \approx 100$)
404 polygon using the sum image of the captured frames. The centre of the polygon⁵⁹ was
405 calculated and connected to all the vertices of the polygon, and all these lines were segmented

406 into ten equal parts (nine division points). In the next step, ten polygons were formed by the
407 n^{th} division points of each line. Clusters inside the i^{th} , but outside the $(i-1)^{\text{th}}$ polygons were
408 counted and the normalized area cluster density was calculated in each.

409

410 **Implementation of 2D/3D DBSCAN into rainSTORM.** A DBSCAN based cluster analysis
411 module was implemented into the rainSTORM program. After the reconstruction of the high
412 resolution (SupRes) image the user can select a region using the box tracking tool, and set the
413 two cluster analysis parameters (N_{core} , ϵ). The program plots and saves data for further
414 evaluation and visualization. Larger areas (entire nuclei etc.) can also be selected, but the code
415 automatically segments them into smaller regions to avoid computation fails. After cluster
416 analysis is performed for all sub-regions, the code saves the merged data.

417

418 **Experimental determination of bleach rate.** The number of cumulative localizations
419 ($N_{cumulative}$) as a function of time follows an exponential curve the decay of which is
420 proportional to the bleach rate (k_{bl}):

$$421 \quad N_{cumulative} = N_0(1 - e^{-k_{bl}t}),$$

422 where N_0 is the average number of switching cycles of the fluorophore. The two parameters
423 (k_{bl} , N_0) were determined by fitting the theoretical curve to the measured data.

424 **Statistics of N_{dye} independently switching fluorophores.** Fluorescent switching was
425 described by a three-state (ON, OFF and bleached) model. The probability of detecting n
426 blinking of N_{dye} fluorophores is

$$427 \quad P_{N_{dye}}(n) = \sum_{x_1+x_2+\dots+x_N=n} P_1(x_1)P_1(x_2) \dots P_1(x_{N_{dye}}),$$

428 where x_i gives the blinking number of the i -th molecule and $P_1(m)$ is the probability of m
429 blinking of a single fluorophore⁴⁷. Due to the assumption that single γ H2AX molecules were
430 labelled by 4 or 8 fluorophores, the overall probability was given as the linear combination of
431 the probabilities

$$432 \quad P_{4,8}(m) = a_1 \cdot P_4(m_1) + a_2 \cdot P_8(m_2),$$

433 where $m_1+m_2=m$ gives the blinking number, and the ratio of a_1 and a_2 parameters can be
434 determined by fitting. (see Supplementary Note 1).

435 **Cell lines, media and culture conditions**

436 DiVA cells were cultured at 37 °C in DMEM (Dulbecco's Modified Eagle Medium 4.5 g/l
437 glucose, supplemented with L-pyruvate; Lonza,) supplemented with 10 % foetal bovine serum
438 (Lonza), 4 mM L-Glutamine (Sigma-Aldrich), 1 mM puromycin (Gibco) and 1 % antibiotic
439 (Lonza). U2OS osteosarcoma cells were cultured at 37 °C in DMEM (Dulbecco's Modified
440 Eagle Medium; Lonza) supplemented with 10 % foetal bovine serum (Lonza), 4 mM L-
441 Glutamine (Sigma-Aldrich) and 1 % antibiotic (Sigma-Aldrich).

442 Both cell lines were grown under standard conditions.

443 U2OS cell line was purchased from ATCC, DiVA cells were provided by G. Legube. All
444 experimental protocols were approved by the guidelines of the University of Szeged and the
445 Medical Research Council.

446

447 **Neocarzinostatin (NCS) treatment**

448 U2OS cells were treated with 5 ng/ml concentration of neocarzinostatin and incubated for 15
449 minutes. Following the treatment, cells were washed with PBS (phosphate-buffered saline)
450 and incubated in cultured medium for 2 hours.

451

452 **4-hydroxytamoxifen (4-OHT) treatment**

453 DiVA cells were treated with 1 µM concentration of 4-OHT and incubated for 2 hours for the
454 nuclear transport of AsiSI endonuclease. Following the treatment, cells were washed with
455 PBS and then were immunostained.

456

457 **Immunocytochemistry**

458 Cells were washed with PBS then incubated with CSK buffer 3 times for 3 minutes and once
459 for half minute [10 mM Hepes pH 7.0 (Sigma-Aldrich), 100 mM sucrose (Sigma-Aldrich), 3
460 mM MgCl₂ (Sigma-Aldrich), 0.7 % Triton X-100 (Sigma-Aldrich), 0.3 mg/ml RNase A
461 (Roche)]. Cells were washed twice with PBS, then fixed with 4 % formaldehyde (Sigma-
462 Aldrich) for 10 minutes. Cells were permeabilized with 0.2 % Triton X-100/PBS for 5
463 minutes. After washing steps, cells were blocked with 5 % BSA (Sigma-Aldrich) in PBST
464 [0.1 % Tween 20 (Sigma-Aldrich) in PBS], supplemented with GAR HRP antibody in 1:200
465 dilution for 20 minutes. Cells were washed with PBST, then incubated with primary
466 antibodies diluted in 1 % BSA/PBST: anti-γH2AX (Abcam, ab2893) in 1:400 dilution. After
467 washing steps, the following secondary antibody was used: GAR Alexa 647 (Abcam,
468 ab150091) in 1:1500 dilution. After several washing steps with PBST the experiments were

469 conducted after the addition of imaging buffer, which is an aqueous solution diluted in PBS
470 containing an enzymatic oxygen scavenging system GluOx (2,000 U/ml glucose-oxidase
471 (Sigma-Aldrich), 40,000 U/ml catalase (Sigma-Aldrich), 25 mM potassium chloride (Sigma-
472 Aldrich), 22 mM tris(hydroxymethyl)aminomethane (Sigma-Aldrich), 4 mM tris(2-
473 carboxyethyl)phosphine (TCEP) (Sigma-Aldrich)) with 4 % (w/v) glucose (Sigma-Aldrich)
474 and 100 mM β -mercaptoethylamine (MEA) (Sigma-Aldrich). The final pH was set to 6.0-8.5.
475 60-62

476

477 **dSTORM microscopy**

478 We used a Nikon Eclipse Ti-E frame with a Nikon CFI Apochromat TIRF objective (NA
479 1.49, 100 \times magnification, oil immersion) for imaging. EPI-fluorescent illumination was
480 applied at excitation wavelength of 647 nm (2RU-VFL-P-300-647-B1, 300 mW, MPB
481 Communications Ltd.). A filter set from Semrock was used in the microscope (Di03-
482 R405/488/561/635-t1-25x36BrightLine[®] quad-edge quad-edge super-resolution / TIRF
483 dichroic beamsplitter and FF01-446/523/600/677-25BrightLine[®] quad-band bandpass filter).
484 An Andor iXon3 DU897 EMCCD camera was used for image acquisition (pixel size: 16 μ m)
485 with the following acquisition parameters: 30 ms exposure time, EM gain of 100, temperature
486 of -75 $^{\circ}$ C

487 **SUPPLEMENTARY INFORMATIONS**

488

489 **Supplementary Note 1: Switching statistics of multiple labelling**

490

491 In a three-state switching model, the time-dependent probability of m switching circles of a
492 single fluorescent dye molecule is [2016_Nieuwenhuizen]:

$$P_m^1 = (1 - b)^m \frac{r^m}{m!} \exp(-r) + b(1 - b)^{m-1} \sum_{n=m}^{\infty} \frac{r^n}{n!} \exp(-r), \quad (1)$$

493 where parameters r and b depend on the k_{sw} switching and k_{bl} effective bleaching rates as
494 $r=k_{sw}t$ and $b=k_{bl}/k_{sw}$.

495 In practice, using immunohistochemical procedures, several fluorescence dye molecules label
496 the target molecule and their common switching pattern provides the detected signal. The
497 number of fluorescence dye molecules depends on the stoichiometry of the labelling.
498 Therefore, it is essential to determine the overall probability of m switching circles of N

499 independent dye molecules (P_m^N). It can be given as the sum of probabilities of all the
 500 possible cases when N molecules generate m switching circles:

$$P_m^N = \sum_{x_1+x_2+\dots+x_N=m} P_{x_1}^1 \cdot P_{x_2}^1 \cdot \dots \cdot P_{x_N}^1 \quad (2)$$

501 where x_1 , x_2 , and x_N mark the number of switching circles can be associated to the 1st, 2nd and
 502 nth dye molecules, respectively.

503 As an example, let us assume that only 2 independent dye molecules label the target molecule
 504 and provide m switching circles. In other words, the total number of switching circles is m but
 505 we do not know how many switching circles belong to each dye molecules. If the first one
 506 was detected x_1 time, the second one must be detected $x_2=m-x_1$ times and the overall
 507 probability can be given as the sum of all the possible cases ($P_{x_1}^1 \cdot P_{x_2}^1 = P_{x_1,x_2}$):

$$P_m^2 = \sum_{x_1+x_2=m} P_{x_1}^1 \cdot P_{x_2}^1 \quad (3)$$

508 In general, all the possible cases can be calculated and can be arranged in a matrix form. In
 509 this representation the sum of elements of the mth minor diagonal gives the overall probability
 510 of m switching circles generated by two dye molecules. It can be shown that after a critical
 511 cluster size the larger that matrix (the larger the possible number of switching circles), the
 512 smaller the sum of the minor diagonal elements (smaller the probability of the effective
 513 switching circles). The sums of the minor diagonal elements form a vector and give the
 514 probability distribution of the switching circles.

P_0,0 P_0,1 P_0,2 P_0,3 ... P_0,m

P_1,0 P_1,1 P_1,2 ... P_1,m-
1

P_2,0 P_2,1

P_3,0

... P_m-
1,1

P_m,0

515 **Table S1:** Probabilities of all the possible switching cases are arranged in a matrix form.

516 The method can be generalized further and the probability of m switching circles generated by
517 N molecules can be calculated.

518 It is worth to note that the calculation can be simplified by dividing the N number of dye
519 molecules into two independent but known populations (e.g. K and $N-K$) with number of
520 switching circles of i and $m-i$, respectively.

$$P_m^N = \sum_{i=0}^m P_i^K \cdot P_{m-i}^{N-K}. \quad (4)$$

521

522 Supplementary Note 2: Validation of 2D analysis

523

524 In this paper all the results and conclusions are based on the evaluation of 2D dSTORM
525 measurements. However, the DSB foci inside the nucleus have a 3D spatial distribution.
526 Therefore, the applicability of the used 2D data analysis requires a validation process. A test
527 sample simulator software (TestSTORM^{63,64}) was used to generate the ground truth model
528 and comparative 2D and 3D evaluations were performed on the reconstructed super-resolved
529 images. Images were evaluated from the same aspect (number of clusters, mean number of
530 cluster elements etc.) as they were studied in the main text.

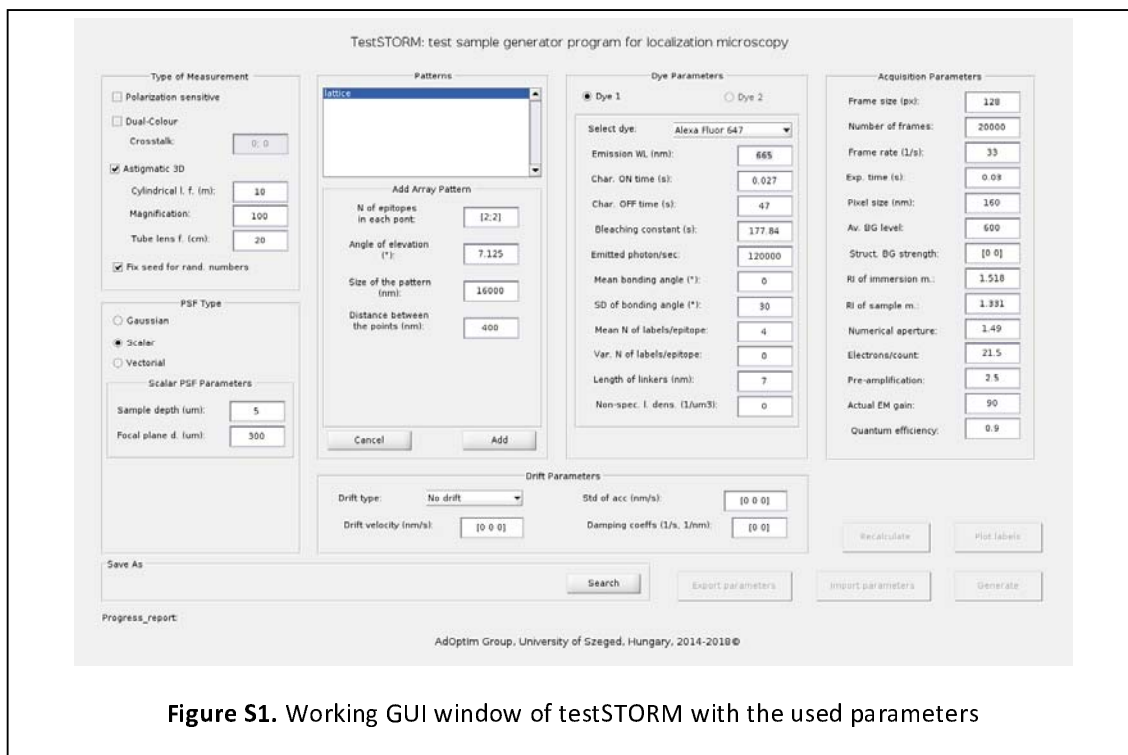


Figure S1. Working GUI window of testSTORM with the used parameters

531

532 **Simulation parameters** were matched to the experimental parameters. The most important
533 parameters are depicted in Figure S1, which shows the working GUI window of the
534 TestSTORM code.

535 A tilted array pattern (lattice) was defined with the following parameters:

536 Depth inside the sample: $\text{Depth}_{\text{sample}} = 5 \mu\text{m}$

537 Refractive index of the sample: $n_{\text{sample}} = 1.331$

538 Axial range of the sample: $Z_{\text{range}}: (-1 \mu\text{m}, +1 \mu\text{m})$

539 Axial steps between the adjacent rows: $Z_{\text{step}} = 50 \text{ nm}$

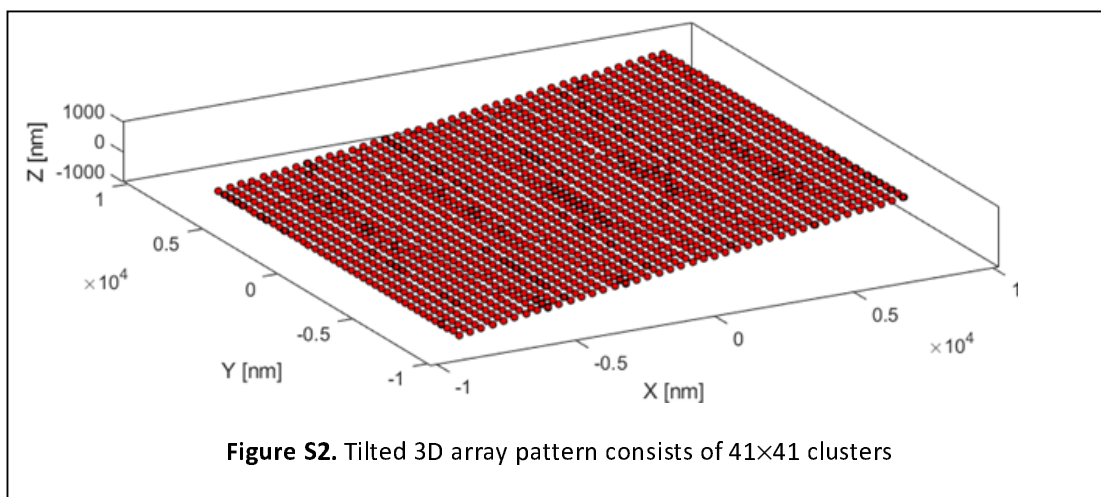
540 Distance between the elements (cluster) of the lattice: $d = 400 \text{ nm}$

541 Number of elements in a single column and row: $N_{\text{cluster}}/Z_{\text{plane}} = 41$

542 Number of dye molecules per cluster: 8 dye molecules/cluster

543 Length of the linker: 7 nm

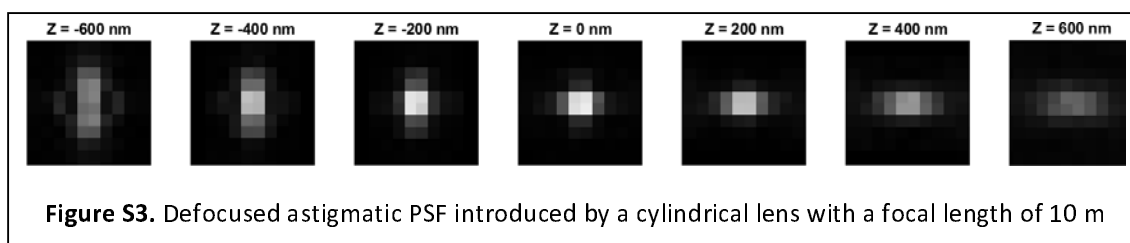
544



545

546 A scalar model based on the Pankajakshan-Gibson-Lanni model [2009_Pankajakshan] was
547 applied to calculate the PSF. During the 3D simulations, an additional cylindrical lens with a
548 focal length of 10 m was added to the optical system.

549



550

551 High resolution localization images were reconstructed and analysed via the rainSTORM
552 code⁶⁵ with the following key parameters:

553

554 Thompson precision limit: 25 nm

555 Applied acceptance radius during the trajectory fitting: $r_{\text{acceptance}} = 50$ nm

556 Residue threshold: 0.06

557 Lateral cluster analysis distance parameter: $\epsilon_{xy} = 50$ nm

558 Axial cluster analysis distance parameter: $\epsilon_z = 100$ nm

559 Minimum number of points in a single cluster: $N_{\text{core}}=8$

560

561 **Our simulation results** prove that 2D and 3D imaging provides identical DOF ranges, i.e.
562 dye molecules in the same axial range (~ 1 μm) can be associated with the accepted
563 localizations (see Figure S4-a). The slightly reduced number of identified clusters in the 3D
564 case ($\sim 7\%$) is caused by the asymmetry of the PSF. This difference does not affect the trend
565 of the evaluation but shows that 3D analysis requires a different calibration process. The mean
566 number of cluster elements (see Figure S4-b) shows an approx. 20% reduction in the 3D case
567 in contrast to the 2D one, and the simulations reveal a slight axial dependence in both cases.
568 During the evaluation this axial dependence was neglected, and an average value was applied.
569 Based on these simulation results one can state that 2D measurements (presented in the main
570 text of the paper) provide reliable data and results for the quantitative evaluations. However,
571 determination of 3D specific merit functions (volume of foci etc.) and features (structure of
572 foci etc.) requires 3D STORM imaging.

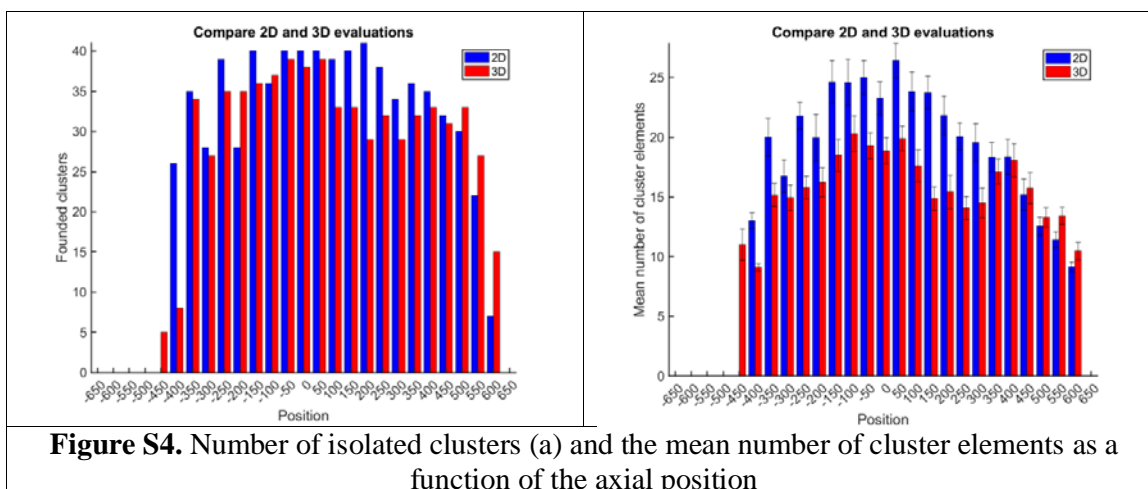


Figure S4. Number of isolated clusters (a) and the mean number of cluster elements as a function of the axial position

573

574

575 **REFERENCES**

- 576 1 Ciccia, A. & Elledge, S. J. The DNA damage response: making it safe to play with knives. *Mol*
577 *Cell* **40**, 179-204, doi:10.1016/j.molcel.2010.09.019 (2010).
- 578 2 Jackson, S. P. & Bartek, J. The DNA-damage response in human biology and disease. *Nature*
579 **461**, 1071-1078, doi:10.1038/nature08467 (2009).
- 580 3 Blackford, A. N. & Jackson, S. P. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA
581 Damage Response. *Mol Cell* **66**, 801-817, doi:10.1016/j.molcel.2017.05.015 (2017).
- 582 4 Du, G. *et al.* Spatial dynamics of DNA damage response protein foci along the ion trajectory
583 of high-LET particles. *Radiat Res* **176**, 706-715 (2011).
- 584 5 Jakob, B. *et al.* DNA double-strand breaks in heterochromatin elicit fast repair protein
585 recruitment, histone H2AX phosphorylation and relocation to euchromatin. *Nucleic Acids Res*
586 **39**, 6489-6499, doi:10.1093/nar/gkr230 (2011).
- 587 6 Zhang, Y. *et al.* Radiation induced chromatin conformation changes analysed by fluorescent
588 localization microscopy, statistical physics, and graph theory. *PLoS One* **10**, e0128555,
589 doi:10.1371/journal.pone.0128555 (2015).
- 590 7 Lowndes, N. F. & Toh, G. W. DNA repair: the importance of phosphorylating histone H2AX.
591 *Curr Biol* **15**, R99-R102, doi:10.1016/j.cub.2005.01.029 (2005).
- 592 8 Turinetto, V. & Giachino, C. Multiple facets of histone variant H2AX: a DNA double-strand-
593 break marker with several biological functions. *Nucleic Acids Res* **43**, 2489-2498,
594 doi:10.1093/nar/gkv061 (2015).
- 595 9 Rogakou, E. P., Boon, C., Redon, C. & Bonner, W. M. Megabase chromatin domains involved
596 in DNA double-strand breaks in vivo. *J Cell Biol* **146**, 905-916 (1999).
- 597 10 Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S. & Bonner, W. M. DNA double-stranded
598 breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**, 5858-5868
599 (1998).
- 600 11 Ceccaldi, R., Rondinelli, B. & D'Andrea, A. D. Repair Pathway Choices and Consequences at
601 the Double-Strand Break. *Trends Cell Biol* **26**, 52-64, doi:10.1016/j.tcb.2015.07.009 (2016).
- 602 12 Falk, M. *et al.* Determining Omics spatiotemporal dimensions using exciting new nanoscopy
603 techniques to assess complex cell responses to DNA damage: part A--radiomics. *Crit Rev*
604 *Eukaryot Gene Expr* **24**, 205-223 (2014).
- 605 13 Berkovich, E., Monnat, R. J., Jr. & Kastan, M. B. Roles of ATM and NBS1 in chromatin
606 structure modulation and DNA double-strand break repair. *Nat Cell Biol* **9**, 683-690,
607 doi:10.1038/ncb1599 (2007).
- 608 14 Caron, P. *et al.* Cohesin protects genes against gammaH2AX Induced by DNA double-strand
609 breaks. *PLoS Genet* **8**, e1002460, doi:10.1371/journal.pgen.1002460 (2012).
- 610 15 Iacovoni, J. S. *et al.* High-resolution profiling of gammaH2AX around DNA double strand
611 breaks in the mammalian genome. *EMBO J* **29**, 1446-1457, doi:10.1038/emboj.2010.38
612 (2010).
- 613 16 Kim, J. A., Kruhlak, M., Dotiwala, F., Nussenzweig, A. & Haber, J. E. Heterochromatin is
614 refractory to gamma-H2AX modification in yeast and mammals. *J Cell Biol* **178**, 209-218,
615 doi:10.1083/jcb.200612031 (2007).
- 616 17 Meier, A. *et al.* Spreading of mammalian DNA-damage response factors studied by ChIP-chip
617 at damaged telomeres. *EMBO J* **26**, 2707-2718, doi:10.1038/sj.emboj.7601719 (2007).
- 618 18 Pankotai, T., Bonhomme, C., Chen, D. & Soutoglou, E. DNAPKcs-dependent arrest of RNA
619 polymerase II transcription in the presence of DNA breaks. *Nat Struct Mol Biol* **19**, 276-282,
620 doi:10.1038/nsmb.2224 (2012).
- 621 19 Savic, V., Sanborn, K. B., Orange, J. S. & Bassing, C. H. Chipping away at gamma-H2AX foci.
622 *Cell Cycle* **8**, 3285-3290, doi:10.4161/cc.8.20.9719 (2009).
- 623 20 Shroff, R. *et al.* Distribution and dynamics of chromatin modification induced by a defined
624 DNA double-strand break. *Curr Biol* **14**, 1703-1711, doi:10.1016/j.cub.2004.09.047 (2004).

- 625 21 Pope, B. D. *et al.* Topologically associating domains are stable units of replication-timing
626 regulation. *Nature* **515**, 402-405, doi:10.1038/nature13986 (2014).
- 627 22 Rao, S. S. *et al.* A 3D map of the human genome at kilobase resolution reveals principles of
628 chromatin looping. *Cell* **159**, 1665-1680, doi:10.1016/j.cell.2014.11.021 (2014).
- 629 23 Grant, M. J., Loftus, M. S., Stoja, A. P., Kedes, D. H. & Smith, M. M. Superresolution
630 microscopy reveals structural mechanisms driving the nanoarchitecture of a viral chromatin
631 tether. *Proc Natl Acad Sci U S A* **115**, 4992-4997, doi:10.1073/pnas.1721638115 (2018).
- 632 24 Hausmann, M. *et al.* Super-resolution localization microscopy of radiation-induced histone
633 H2AX-phosphorylation in relation to H3K9-trimethylation in HeLa cells. *Nanoscale* **10**, 4320-
634 4331, doi:10.1039/c7nr08145f (2018).
- 635 25 Krufczik, M. *et al.* Combining Low Temperature Fluorescence DNA-Hybridization,
636 Immunostaining, and Super-Resolution Localization Microscopy for Nano-Structure Analysis
637 of ALU Elements and Their Influence on Chromatin Structure. *Int J Mol Sci* **18**,
638 doi:10.3390/ijms18051005 (2017).
- 639 26 Lopez Perez, R. *et al.* Superresolution light microscopy shows nanostructure of carbon ion
640 radiation-induced DNA double-strand break repair foci. *FASEB J* **30**, 2767-2776,
641 doi:10.1096/fj.201500106R (2016).
- 642 27 Ma, H., Fu, R., Xu, J. & Liu, Y. A simple and cost-effective setup for super-resolution
643 localization microscopy. *Sci Rep* **7**, 1542, doi:10.1038/s41598-017-01606-6 (2017).
- 644 28 Prakash, K. *et al.* Superresolution imaging reveals structurally distinct periodic patterns of
645 chromatin along pachytene chromosomes. *Proc Natl Acad Sci U S A* **112**, 14635-14640,
646 doi:10.1073/pnas.1516928112 (2015).
- 647 29 Britton, S., Coates, J. & Jackson, S. P. A new method for high-resolution imaging of Ku foci to
648 decipher mechanisms of DNA double-strand break repair. *J Cell Biol* **202**, 579-595,
649 doi:10.1083/jcb.201303073 (2013).
- 650 30 Natale, F. *et al.* Identification of the elementary structural units of the DNA damage
651 response. *Nat Commun* **8**, 15760, doi:10.1038/ncomms15760 (2017).
- 652 31 Reindl, J. *et al.* Chromatin organization revealed by nanostructure of irradiation induced
653 gammaH2AX, 53BP1 and Rad51 foci. *Sci Rep* **7**, 40616, doi:10.1038/srep40616 (2017).
- 654 32 Bobkova, E. *et al.* Recruitment of 53BP1 Proteins for DNA Repair and Persistence of Repair
655 Clusters Differ for Cell Types as Detected by Single Molecule Localization Microscopy.
656 *International journal of molecular sciences* **19**, 3713 (2018).
- 657 33 Hofmann, A., Krufczik, M., Heermann, D. & Hausmann, M. Using persistent homology as a
658 new approach for super-resolution localization microscopy data analysis and classification of
659 γ H2AX foci/clusters. *International journal of molecular sciences* **19**, 2263 (2018).
- 660 34 Folling, J. *et al.* Fluorescence nanoscopy by ground-state depletion and single-molecule
661 return. *Nat Methods* **5**, 943-945, doi:10.1038/nmeth.1257 (2008).
- 662 35 Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical
663 reconstruction microscopy (STORM). *Nat Methods* **3**, 793-795, doi:10.1038/nmeth929
664 (2006).
- 665 36 Sauer, M. Localization microscopy coming of age: from concepts to biological impact. *J Cell*
666 *Sci* **126**, 3505-3513, doi:10.1242/jcs.123612 (2013).
- 667 37 Betzig, E. *et al.* Imaging intracellular fluorescent proteins at nanometer resolution. *Science*
668 **313**, 1642-1645, doi:10.1126/science.1127344 (2006).
- 669 38 Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by
670 stochastic optical reconstruction microscopy. *Science* **319**, 810-813,
671 doi:10.1126/science.1153529 (2008).
- 672 39 Moerner, W. E. Single-Molecule Spectroscopy, Imaging, and Photocontrol: Foundations for
673 Super-Resolution Microscopy (Nobel Lecture). *Angew Chem Int Ed Engl* **54**, 8067-8093,
674 doi:10.1002/anie.201501949 (2015).
- 675 40 Caron, P. *et al.* Non-redundant functions of ATM and DNA-PKcs in response to DNA double-
676 strand breaks. *Cell reports* **13**, 1598-1609 (2015).

- 677 41 Kalousi, A. *et al.* The nuclear oncogene SET controls DNA repair by KAP1 and HP1 retention to
678 chromatin. *Cell Rep* **11**, 149-163, doi:10.1016/j.celrep.2015.03.005 (2015).
- 679 42 Lemaitre, C. *et al.* Nuclear position dictates DNA repair pathway choice. *Genes Dev* **28**, 2450-
680 2463, doi:10.1101/gad.248369.114 (2014).
- 681 43 Sisario, D. *et al.* Nanostructure of DNA repair foci revealed by superresolution microscopy.
682 *FASEB J*, fj201701435, doi:10.1096/fj.201701435 (2018).
- 683 44 Ester, M., Kriegel, H.-P., #246, Sander, r. & Xu, X. in *Proceedings of the Second International*
684 *Conference on Knowledge Discovery and Data Mining* 226-231 (AAAI Press, Portland,
685 Oregon, 1996).
- 686 45 Janssen, A. *et al.* A single double-strand break system reveals repair dynamics and
687 mechanisms in heterochromatin and euchromatin. *Genes Dev* **30**, 1645-1657,
688 doi:10.1101/gad.283028.116 (2016).
- 689 46 Nahidiazar, L., Agronskaia, A. V., Broertjes, J., van den Broek, B. & Jalink, K. Optimizing
690 Imaging Conditions for Demanding Multi-Color Super Resolution Localization Microscopy.
691 *PLoS One* **11**, e0158884, doi:10.1371/journal.pone.0158884 (2016).
- 692 47 Nieuwenhuizen, R. P. *et al.* Quantitative localization microscopy: effects of photophysics and
693 labeling stoichiometry. *PLoS One* **10**, e0127989, doi:10.1371/journal.pone.0127989 (2015).
- 694 48 Metcalf, D. J., Edwards, R., Kumarswami, N. & Knight, A. E. Test samples for optimizing
695 STORM super-resolution microscopy. *J Vis Exp*, doi:10.3791/50579 (2013).
- 696 49 Bauer, C. R. Labeling and use of monoclonal antibodies in immunofluorescence: protocols for
697 cytoskeletal and nuclear antigens. *Methods Mol Biol* **1131**, 543-548, doi:10.1007/978-1-
698 62703-992-5_34 (2014).
- 699 50 Dempsey, G. T., Vaughan, J. C., Chen, K. H., Bates, M. & Zhuang, X. Evaluation of fluorophores
700 for optimal performance in localization-based super-resolution imaging. *Nat Methods* **8**,
701 1027-1036, doi:10.1038/nmeth.1768 (2011).
- 702 51 Vian, L. *et al.* The Energetics and Physiological Impact of Cohesin Extrusion. *Cell* **173**, 1165-
703 1178 e1120, doi:10.1016/j.cell.2018.03.072 (2018).
- 704 52 Xiang, W. *et al.* Correlative live and super-resolution imaging reveals the dynamic structure of
705 replication domains. *J Cell Biol* **217**, 1973-1984, doi:10.1083/jcb.201709074 (2018).
- 706 53 Aymard, F. *et al.* Genome-wide mapping of long-range contacts unveils clustering of DNA
707 double-strand breaks at damaged active genes. *Nat Struct Mol Biol* **24**, 353-361,
708 doi:10.1038/nsmb.3387 (2017).
- 709 54 Bintu, B. *et al.* Super-resolution chromatin tracing reveals domains and cooperative
710 interactions in single cells. *Science* **362**, doi:10.1126/science.aau1783 (2018).
- 711 55 Bach, M. *et al.* Super-Resolution Localization Microscopy of gamma-H2AX and
712 Heterochromatin after Folate Deficiency. *Int J Mol Sci* **18**, doi:10.3390/ijms18081726 (2017).
- 713 56 Eberle, J. P. *et al.* Super-Resolution Microscopy Techniques and Their Potential for
714 Applications in Radiation Biophysics. *Methods Mol Biol* **1663**, 1-13, doi:10.1007/978-1-4939-
715 7265-4_1 (2017).
- 716 57 Eryilmaz, M. *et al.* Localization Microscopy Analyses of MRE11 Clusters in 3D-Conserved Cell
717 Nuclei of Different Cell Lines. *Cancers (Basel)* **10**, doi:10.3390/cancers10010025 (2018).
- 718 58 Group, A. O. I. *rainSTORM user guide*, <[http://titan.physx.u-](http://titan.physx.u-szeged.hu/~adoptim/?page_id=582%20?)
719 [szeged.hu/~adoptim/?page_id=582%20?](http://titan.physx.u-szeged.hu/~adoptim/?page_id=582%20?)> (2015).
- 720 59 Bose, P. & Toussaint, G. in *Proceedings of CG International '96*. 102-111.
- 721 60 Tinnefeld, P., Eggeling, C. & Hell, S. W. *Far-Field Optical Nanoscopy*. (2015).
- 722 61 Schafer, P., van de Linde, S., Lehmann, J., Sauer, M. & Doose, S. Methylene blue- and thiol-
723 based oxygen depletion for super-resolution imaging. *Anal Chem* **85**, 3393-3400,
724 doi:10.1021/ac400035k (2013).
- 725 62 van de Linde, S. *et al.* Direct stochastic optical reconstruction microscopy with standard
726 fluorescent probes. *Nat Protoc* **6**, 991-1009, doi:10.1038/nprot.2011.336 (2011).

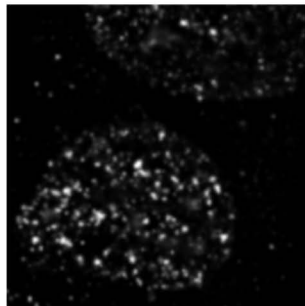
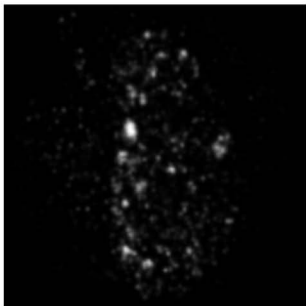
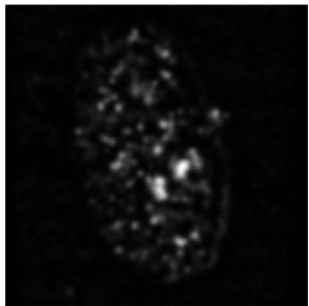
- 727 63 Novak, T., Gajdos, T., Sinko, J., Szabo, G. & Erdelyi, M. TestSTORM: Versatile simulator
728 software for multimodal super-resolution localization fluorescence microscopy. *Sci Rep* **7**,
729 951, doi:10.1038/s41598-017-01122-7 (2017).
- 730 64 Sinko, J. *et al.* TestSTORM: Simulator for optimizing sample labeling and image acquisition in
731 localization based super-resolution microscopy. *Biomed Opt Express* **5**, 778-787,
732 doi:10.1364/BOE.5.000778 (2014).
- 733 65 Rees, E. J., Erdelyi, M., Schierle, G. S. K., Knight, A. & Kaminski, C. F. Elements of image
734 processing in localization microscopy. *Journal of Optics* **15**, 094012, doi:10.1088/2040-
735 8978/15/9/094012 (2013).
- 736

NT

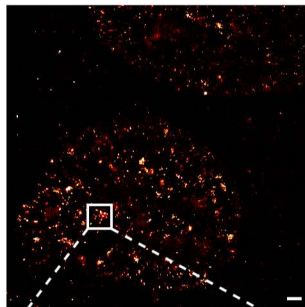
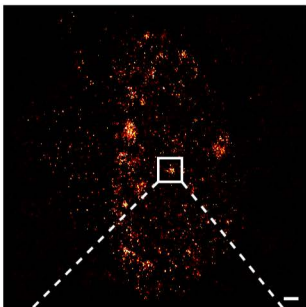
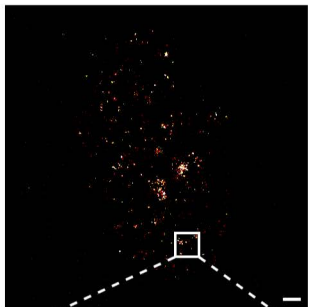
U2OS T

DivA T

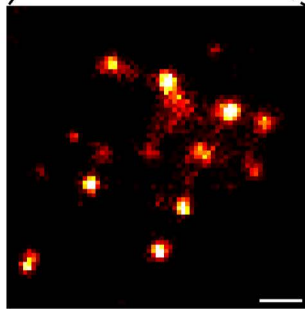
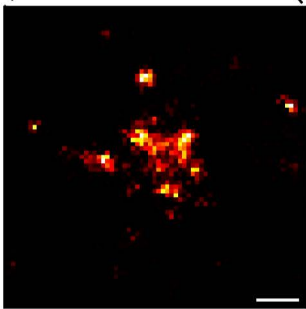
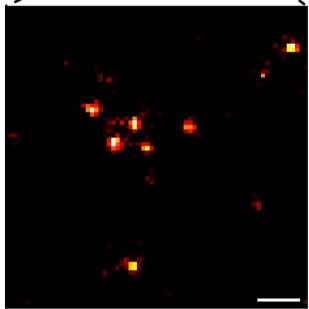
A



B

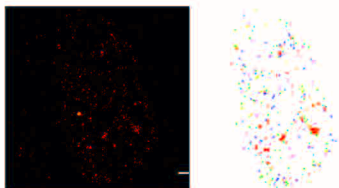


C



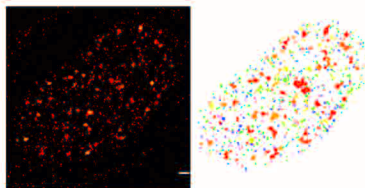
NT

A B



U2OS T

C D



DivA T

E F

