Nanostructure of DNA repair foci revealed by superresolution microscopy

Dmitri Sisario,†,‡ Simon Memmel,†,‡ Sören Doose,*, Julia Neubauer,‡ Heiko Zimmermann,‡,§,* Michael Flentje,‡ Cholpon S. Djuzenova,‡ Markus Sauer,*, and Vladimir L. Sukhorukov†,‡,*

*Department of Biotechnology and Biophysics, University of Würzburg, Würzburg, Germany; †Department of Radiation Oncology, University Hospital of Würzburg, Würzburg, Germany; ‡Fraunhofer Institute for Biomedical Engineering (IBMT), Sulzbach, Germany; §Molekulare und Zelluläre Biotechnologie/Nanotechnologie, Universität des Saarlandes, Saarbrücken, Germany; and †Marine Sciences, Universidad Católica del Norte, Antofagasta/Coquimbo, Chile

ABSTRACT: Induction of DNA double-strand breaks (DSBs) by ionizing radiation leads to formation of micrometer-sized DNA-repair foci, whose organization on the nanometer-scale remains unknown because of the diffraction limit (~200 nm) of conventional microscopy. Here, we applied diffraction-unlimited, direct stochastic optical-reconstruction microscopy (dSTORM) with a lateral resolution of ~20 nm to analyze the focal nanostructure of the DSB marker histone γH2AX and the DNA-repair protein kinase (DNA-PK) in irradiated glioblastoma multiforme cells. Although standard confocal microscopy revealed substantial colocalization of immunostained γH2AX and DNA-PK, in our dSTORM images, the 2 proteins showed very little (if any) colocalization despite their close spatial proximity. We also found that γH2AX foci consisted of distinct circular subunits (“nanofoci”) with a diameter of ~45 nm, whereas DNA-PK displayed a diffuse, intrafocal distribution. We conclude that γH2AX nanofoci represent the elementary, structural units of DSB repair foci, that is, individual γH2AX-containing nucleosomes. dSTORM-based γH2AX nanofoci counting and distance measurements between nanofoci provided quantitative information on the total amount of chromatin involved in DSB repair as well as on the number and longitudinal distribution of γH2AX-containing nucleosomes in a chromatin fiber. We thus estimate that a single focus involves between ~0.6 and ~1.1 Mbp of chromatin, depending on radiation treatment. Because of their ability to unravel the nanostructure of DSB-repair foci, dSTORM and related single-molecule localization nanoscopy methods will likely emerge as powerful tools in biology and medicine to elucidate the effects of DNA damaging agents in cells.—Sisario, D., Memmel, S., Doose, S., Neubauer, J., Zimmermann, H., Flentje, M., Djuzenova, C. S., Sauer, M., Sukhorukov, V. L. Nanostructure of DNA repair foci revealed by superresolution microscopy. FASEB J. 32, 6469–6477 (2018). www.fasebj.org

KEY WORDS: double-strand breaks · dSTORM · γH2AX · DNA-PK · ionizing radiation

Double-strand breaks (DSBs) are the main type of DNA damage responsible for killing tumor cells by ionizing radiation (IR) and other DNA-damaging agents (1). Even a single unrepaired DNA DSB can be sufficient to cause cell death (2). Ever since the discovery that DSBs induce phosphorylation of histone H2AX at Ser139 to γH2AX (3), immunofluorescence assays with antibodies recognizing γH2AX have become the gold standard for DSB detection in radiation biology (4–7). The amount of H2AX phosphorylated upon induction of a DSB has been estimated to involve a large chromatin region of up to 2 Mbp or thousands of nucleosomes (8), which leads to the formation of microscopically visible γH2AX foci, with diameters of 0.5–1 μm at the sites of DSBs (1, 9, 10).

Phosphorylation of H2AX triggers the recruitment of various DNA-damage repair (DDR) proteins to the damaged sites, where those proteins also form discrete nuclear foci that usually colocalize with γH2AX foci (11, 12). Despite numerous studies regarding the involvement of γH2AX in DNA repair, the nanoscale organization of γH2AX and DDR proteins within IR-induced foci remains largely unknown because of the resolution limit of conventional fluorescence microscopy. At present, there are only a few reported studies using superresolution fluorescence microscopy to explore the nanoscale organization

ABBREVIATIONS: 2D, 2-dimensional; 3D, 3-dimensional; CGM, complete growth medium; CLSM, confocal laser scanning microscopy; DDR, DNA damage repair; DNA-PK, DNA protein kinase; DNA-PKcs, DNA protein kinase catalytic subunit; DSB, DNA double-strand break; dSTORM, direct stochastic optical-reconstruction microscopy; EM, electron microscopy; FWHM, full width at half maximum; GBM, glioblastoma multiforme; IR, ionizing radiation; NHEJ, nonhomologous end-joining; NND, nearest-neighbor-distance; PTEN, phosphatase and tensin homolog; ROI, region of interest

1 These authors contributed equally to this work.
2 Correspondence: Department of Biotechnology and Biophysics, University of Würzburg, Am Hubland, 97074 Würzburg, Germany. E-mail: sukhorukov@biozentrum.uni-wuerzburg.de
doi: 10.1096/fj.201701435
This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.
of DSB repair foci (13–16). Those studies revealed that DSB foci consist of several subfoci with a lateral diameter ranging between ~100 and 200 nm.

However, the organization of γH2AX foci below a spatial resolution of 100 nm remains largely unclear. In eukaryotic cells, DNA is packed with histone proteins (H2A, H2B, etc.) into regularly spaced chromatin subunits called nucleosomes (17). Given that H2AX, as a variant of H2A, is a core nucleosomal histone, improving the resolution below 100 nm seems crucial for the visualization of individual γH2AX-containing nucleosomes at DSB sites. Therefore, in this study, we resolved the nanostructure of radiation-induced γH2AX foci in 2 human glioblastoma multiforme (GBM) cell lines, DK-MG and SNB19, by direct stochastic optical reconstruction microscopy (dSTORM) (18). This type of single-molecule localization microscopy enables a lateral resolution of ~20 nm.

Even though the resolution of single-molecule localization microscopy is less than that of electron microscopy (EM), these techniques, combined with antibody staining, fluorescent proteins, and/or chemical tags have the overall advantage over EM in terms of greater labeling efficiency, as well as the possibility to imaging cellular structures at the molecular level in an aqueous environment. dSTORM has already been successfully applied for superresolution imaging of chromosomal DNA and the core histone protein H2B (19–21).

Although γH2AX is currently the DSB biomarker of choice and is widely used to monitor DSB formation and repair (22–24), there is growing evidence in the literature that γH2AX foci are not an absolute marker of DSBS (25–30). Therefore, in addition to the histone γH2AX, we also analyzed the foci of the DSB-repair protein kinase (DNA-PK) catalytic subunit (DNA-PKcs). Phosphorylated on S2056, DNA-PKcs accumulates at, and regulates repair of, DSBs of high complexity, such as those caused by IR routinely used in radiation therapy (31). As a major component of nonhomologous end-joining (NHEJ) (32), DNA-PKcs, alone or as part of a multiprotein complex, has been reported to protect and align broken ends of DNA (33–35). Because of its interactions with other NHEJ and non-NHEJ proteins, DNA-PKcs may also act as a scaffold protein to facilitate the localization of DNA-repair proteins to the site of DSBs (36). Unlike several other important DNA-repair proteins, such as, Ku70/80, 53BP1, Rad51, among others (12, 14, 16), DNA-PK has not yet been analyzed by superresolution microscopy.

MATERIALS AND METHODS

Cell culture

Human glioblastoma cell lines, DK-MG and SNB19, were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and routinely cultured in complete growth medium (CGM) under standard conditions (5% CO2, 37°C). The CGM contained DMEM (MilliporeSigma, Burlington, MA, USA) supplemented with 10% fetal bovine serum. Both cell lines were used at low (<15) passages after thawing and were authenticated on the basis of morphology, expression of phosphatase and tensin homolog (PTEN) and p53, and growth curve analyses, and they were regularly tested for Mycoplasma (Mycopalert; Lonza, Basel, Switzerland).

X-ray irradiation

Cells were seeded at 500 μl medium per well in 8-well Lab-Tek Chamber Slides (Thermo Fisher Scientific, Waltham, MA, USA) 1 d before irradiation. Irradiation was performed at room temperature with a 6-MV linear accelerator (Siemens, Munich, Germany) at a dose rate of 2 Gy/min. After irradiation, cells were kept in CGM for the indicated time until harvest.

Immunostaining

At 2 different times (30 min and 6 h) after irradiation, cells were washed in prewarmed (37°C) PBS and fixed for 10 min at room temperature with a PBS solution containing 4% paraformaldehyde (methanol free). Thereafter, the cells were washed 3 times in PBS for 5 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Following permeabilization, cells were washed 3 times in PBS for 5 min and then treated with blocking buffer containing 5% bovine serum albumin (MilliporeSigma) in PBS, for 30 min. Subsequently, samples were incubated with 1:1500 diluted mouse γH2AX antibodies and 1:400 diluted rabbit DNA-PKcs antibodies in blocking buffer for 1 h. After 3 washing steps (each 10 min) in a PBS washing buffer containing 0.1% Tween 20, cells were stained by incubating them with Alexa Fluor 647 F(ab)2 fragments of goat anti-rabbit IgG and Alexa Fluor 532 goat anti-mouse IgG (both diluted 1:400 in blocking buffer) for 1 h. Finally, cells were washed 3 times with PBS washing buffer. Cells were stored overnight at 4°C in PBS before measurement. The primary antibodies used were polyclonal rabbit anti-phospho-DNA-PKcs (S2056) antibody (0.4 mg/ml; Abcam, Cambridge, United Kingdom) and monoclonal (clone JBW301) mouse anti-phospho-histone H2AX (Ser139) antibody (1 mg/ml; MilliporeSigma). Secondary antibodies used were Alexa Fluor 532-conjugated goat anti-mouse IgG and Alexa Fluor 647–conjugated goat anti-rabbit IgG F(ab’)2 fragments. In reversed-staining control experiments, we used Alexa Fluor 532–conjugated goat anti-mouse IgG F(ab’)2 fragments as secondary antibodies.

Confocal laser scanning microscopy

Confocal fluorescence images were acquired with an LSM 700 microscope (Carl Zeiss, Oberkochen, Germany). Automatic 3-dimensional (3D) γH2AX foci counting was implemented with an in-house–developed script for ImageJ (v.1.49; National Institutes of Health, Bethesda, MD, USA) with 3D confocal laser scanning microscopy (CLSM) image stacks. Cell nuclei and foci segmentation was performed with a series of Auto Local Thresholds and Watershed algorithms (ImageJ plugin) on the normalized images corresponding to the full width at half maximum (FWHM), either of the nuclei background or the foci signal. Data analysis was performed with the Particle Analyzer (ImageJ plugin).

dSTORM

dSTORM uses photoswitchable fluorescent dyes, which can be transferred to a reversible dark state with a lifetime ranging between ~100 ms and a few seconds. A sparse subset of fluorophores is then spontaneously (stochastically) reactivated, allowing their precise localization. Photoswitching is based on

6470 Vol. 32 December 2018 The FASEB Journal • www.fasebj.org SISARIO ET AL.
thiol-mediated reduction of fluorescent dyes to a non-fluorescent dark state after excitation. Repetitive cycles of activation, localization, and deactivation enable a temporal separation of fluorophores from which a spatially superresolved image can be reconstructed. Reversible photoswitching of Alexa Fluor 647 and Alexa Fluor 532 was performed in a photoswitching buffer containing 100 mM mercaptoethylamine (MilliporeSigma) in PBS at pH ~8.0. The experimental setup for dSTORM was previously described in detail in van de Linde et al. (37). Measurements were performed in 8-well II chambered cover glasses (Sarstedt, Nümbrecht, Germany). For data processing and image reconstruction, the open access software for single-molecule-based localization microscopy, rapidSTORM 3.2, was used as previously described by Wolter et al. (38).

Using the localization data generated with rapidSTORM, we performed cluster analysis with an in-house developed Mathematica (Wolfram, Champaign, IL, USA)-based localization analyzer designed for computation of Ripley’s K function (39), as shown in Eq. 1:

\[ K(r) = \frac{1}{n} \sum_i \sum_j N_i(d_{ij}) \frac{1}{\lambda} \]

where \( r \) is the radius of the observed area, \( n \) is the total number of localizations within the observation area, \( d_{ij} \) is the distance between 2 localizations \( i \) and \( j \), \( N_i \) is an indicator function that is equal to 1 if the distance \( d_{ij} < r \), and 0 otherwise, and \( \lambda \) is a weighting factor correcting for the average density of the total observation area. Ripley’s K function can be linearized to Eq. 2:

\[ L(r) = \sqrt{\frac{K(r)}{\pi}} \]

Then, \( r \) is subtracted so that homogeneous, Poisson-distributed localizations lead to \( H(r) = 0 \) for all \( r \), as shown in Eq. 3:

\[ H(r) = L(r) - r \]

The maximum of Ripley’s H function lies in the range between the cluster radius and the diameter, thus providing an estimate for the average cluster size. To identify clusters on the micrometer and nanometer scales, we calculated Ripley’s H function for 2 sets of regions of interest (ROIs). Large ROIs with a diameter of 15 μm contained whole cell nuclei, whereas small ROIs with a diameter of 2 μm included only single DSB repair foci.

**RESULTS**

Although derived from the same tumor entity, the 2 glioblastoma multiforme (GBM) cell lines used in this study, DK-MG and SNB19, differ markedly in the mutational status of the most prominent tumor suppressors PTEN and p53 (40). Both genes are wild type in DK-MG cells, whereas in the SNB19 cell line, both genes are mutated. In a series of studies (40), we have previously shown that the 2 cell lines differ greatly in their morphologic appearance, invasiveness, and migratory behavior (41), as well as in their response to the pharmacologic inhibition of PI3K and mechanistic target of rapamycin (mTOR) in terms of migration (42). These findings prompted us to analyze whether the 2 cell lines also display differences in the micro- and nanoscale organization of radiation-induced DSB repair foci.

**Confocal microscopy of γH2AX and DNA-PK foci**

To assess DSB repair foci in GBM cells, DK-MG and SNB19 cells were immunolabeled for γH2AX and DNA-PK at 2 different times after irradiation and were analyzed with CLSM. Figure 1A, B shows typical CLSM images of DSB foci in the nuclei of a DK-MG cell observed 30 min after irradiation with 2 Gy. For CLSM images of SNB19 cells, see Supplemental Fig. S1. The foci of both proteins appear as bright, fluorescent spots distributed randomly over a dim background of the respective proteins. Moreover, CLSM reveals substantial colocalization of DNA-PK with γH2AX foci (Fig. 1C and Supplemental Fig. S1C). However, not all γH2AX foci contained DNA-PK, which is particularly evident from the merged CLSM images (Fig. 1C and Supplemental Fig. S1C). As pointed out elsewhere (30), low-level phosphorylation of H2AX not associated with DSB-repair factors may be caused by DNA lesions other than DSBs.

Intensity quantification through these foci also revealed that γH2AX and DNA-PK peaks were very similar regarding their position, shape, and relative intensity (Fig. 1D). This result corroborates the findings that many DDR proteins colocalize with γH2AX in DSB-repair foci examined by diffraction-limited fluorescence microscopy (9, 11, 12).

Computer-assisted foci counting from 3D-CLSM image stacks revealed substantial amounts of γH2AX and DNA-PK foci not only in irradiated but also in nonirradiated samples of both GBM lines (Fig. 1E). Even without being exposed to IR, both DK-MG and SNB19 cell lines displayed 19.6 ± 2.6 (means ± SD) and 11.1 ± 1.7 γH2AX foci per nucleus, respectively (Fig. 1E; 0 Gy). These numbers are well within the range reported for spontaneous γH2AX foci formation caused by replication-associated breaks in a variety of cancer cell lines (43).

Thirty minutes after irradiation with 2 Gy, the amount of γH2AX foci increased to 25.4 ± 2.3 per DK-MG and 24.9 ± 2.2 per SNB19 cell (Fig. 1E). As expected, 6 h after exposure to IR the foci numbers decreased to 19.6 ± 4.0 and 16.4 ± 2.2 foci per cell in DK-MG and SNB19 lines, respectively, because of DSB repair. Interestingly, along with the observed decay in the γH2AX foci number/nucleus, there was a significant increase in the mean foci volumes in irradiated DK-MG cells from 0.45 to 0.62 μm³, detected 30 min and 6 h postirradiation, respectively (Fig. 1F, black bars). This was also observed in SNB19 cells, but to a lesser extent compared with DK-MG cells (Fig. 1F, gray bars). We next analyzed the nanostructure of DSB repair foci by dSTORM.

**dSTORM images and cluster analysis of γH2AX and DNA-PK**

Two-color dSTORM analysis was performed on foci containing both γH2AX and DNA-PK. In sharp contrast to diffraction-limited CLSM images, in which γH2AX and DNA-PK foci appear as uniform fluorescent spots (Fig. 1), the foci exhibit a highly inhomogeneous staining in dSTORM images of both DK-MG (Fig. 2A–C) and SNB19...
cell lines (Supplemental Fig. S2A–C). As is clearly seen in the expanded dSTORM images (Fig. 2B and Supplemental Fig. S2B), a γH2AX focus consists of discrete, spatially separated subunits with a diameter of 40–50 nm (Fig. 2F and Supplemental Fig. S2F), denoted hereafter as nanofoci. A further important finding is that the staining patterns of γH2AX and DNA-PK in dSTORM images display little (if any) colocalization, as is evident from the merged image (Fig. 2D and Supplemental Fig. S2D) and the intensity profiles of both proteins (Fig. 2E and Supplemental Fig. S2E).

Next, we assessed the diameter of the γH2AX nanofoci and the distribution of nearest-neighbor-distances (NNDs) between them. Evaluation of the cross-sections of individual γH2AX nanofoci in both cell lines yielded normally distributed diameters with similar means ± SD of 44.4 ± 9.9 nm (DK-MG cells, Fig. 2F) and 46.0 ± 9.2 nm (SNB19 cells, Supplemental Fig. S2F). In both cell lines, the distribution of the NNDs between nanofoci within micrometer-sized γH2AX foci approaches a lognormal distribution, with a median value of about 85 nm (Fig. 2G and Supplemental Fig. S2G).

In addition to the γH2AX nanofoci distributed randomly over the focus area (Fig. 2B and Supplemental Fig. S2B), dSTORM also revealed distinct chain-like structures of nanofoci with a characteristic beads-on-a-string–like appearance (Fig. 2H and Supplemental Fig. S2H). These strings usually contained up to 8–10 nanofoci/chain with a mean distance of about 95 nm between adjacent nanofoci (Fig. 2H and Supplemental Fig. S2H). Their curved shape is consistent with the idea that these γH2AX-labeled chains represent higher-order chromatin structures known as chromatin loops, implicated, among others, in DSB induction and repair (44). A recent study demonstrated that γH2AX-decorated chromatin loops appear in 3D-stimulated-illumination microscopy and stimulated emission depletion images as subfoci with diameters of ~200 nm, whose clusters correspond to conventional γH2AX foci (13). Interestingly, in EM images, immunogold-labeled γH2AX also appears as string-like clusters containing several gold beads/chain (45).

To gain further quantitative insight into the distribution and clustering of γH2AX and DNA-PK, we calculated Ripley’s H-function \( H(r) \) (46) for 2 sets of ROIs using the dSTORM localization data of DK-MG (Fig. 3) and SNB19 cells (Supplemental Fig. S3). The first set of ROIs with a diameter of ~10–15 μm contained whole cell nuclei (large ROIs), as indicated by the white outline in Fig. 3A, B, illustrating a representative dSTORM image of a DK-MG cell. The second set of ROIs, with a diameter of 2 μm,
included only single foci (small ROIs), such as shown in Fig. 3C, D. The magnitude and location of the maximum of Ripley’s $H$ function ($r_{\text{max}}$) are related to cluster density and size, respectively (46). More precisely, $r_{\text{max}}$ is located between the cluster radius and diameter.

Judging from the $H(r)$ graphs calculated for large ROIs (Fig. 3E), γH2AX cluster density significantly increases 30 min after irradiation (red line), whereas the cluster size ($r_{\text{max}} \approx 0.5 \mu m$) remains nearly unchanged compared with the nonirradiated control (green line). With increasing repair time (6 h), γH2AX clusters in DK-MG cells increase in size ($r_{\text{max}} \approx 0.6 \mu m$) but not in density (Fig. 3E, blue line). Qualitatively similar results were obtained for SNB19 cells (Supplemental Fig. S3). The increase in γH2AX cluster size with increasing repair time (Fig. 3C) agrees well with the increase of γH2AX foci volume detected by CLSM (Fig. 1F). The $r_{\text{max}}$ of DNA-PK clusters also shows the largest value 6 h after exposure to IR (Fig. 3F, blue line).

Within small ROIs, only the $H(r)$ function of γH2AX (Fig. 3G and Supplemental Fig. S3G), but not that of DNA-PK (Fig. 3H and Supplemental Fig. S3H) shows a local maximum $r_{\text{max}}$ of ~45 nm in both cell lines, independent of radiation treatment and repair time after irradiation (30 min and 6 h, red and blue lines, respectively). This value ($r_{\text{max}} \approx 45$ nm) agrees strikingly well with the diameter of nanofoci (FWHM ~45 nm) determined from their intensity profiles (Fig. 2F and Supplemental Fig. S2F).

In contrast to γH2AX, the $H(r)$ of DNA-PK monotonically increases within the small ROIs (Fig. 3H and Supplemental Fig. S3H). The diameter of γH2AX nanofoci was determined from the distribution of the FWHM values extracted from the cross-sectional profiles of individual nanofoci ($N = 150$). The red line is a Gaussian fit with a mean FWHM of 44.4 nm and a standard deviation of 9.9 nm. The right-skewed histogram of the NNDs between nanofoci ($N = 270$) within γH2AX focal approximated with a lognormal fit (red line), shows a mean and a median value of 100.1 and 85.9 nm, respectively. $H$ Examples of the beads on a string–like arrangements of γH2AX nanofoci. The $r_{\text{max}}$ of DNA-PK clusters also shows the largest value 6 h after exposure to IR (Fig. 3F, blue line).
Supplemental Fig. S3), apparently because of a more diffuse and irregular distribution of that protein at the nanoscale. At earlier times after irradiation (5, 10, and 20 min; data not shown), the H(r) functions of both proteins were similar to those obtained at 30 min and 6 h after irradiation (Fig. 3G, H and Supplemental Fig. S3G, H). This suggests that γH2AX (but not DNA-PK) forms discrete nanofoci at all examined repair times after irradiation.

To rule out the possibility that the observed clustered distribution of γH2AX was due to sample preparation or labeling methods used, we performed a series of control experiments, including variation of antibody concentration and reversed staining (Alexa Fluor 647 vs. 532) experiments (Supplemental Figs. S4 and S5, respectively). The clustered distribution of γH2AX was observed in both tested GBM cell lines and was independent of the dye and antibody concentrations used. Thus, increasing antibody concentration from 0.67 to 10 μg/ml did not affect the size of γH2AX nanoclusters, as indicated by the unchanged position of the maximum (rmax ≈ 40–50 nm) of Ripley’s H function (Supplemental Fig. S4A). This indicates that nanofoci are not a product of sparse labeling. In contrast, to γH2AX, DNA-PK shows a more diffuse staining pattern with no local H(r) maxima within the indicated distances r < 100 nm, regardless of antibody concentration (0.26–4 μg/ml; Supplemental Fig. S4B). The results of reversed staining experiments confirmed both the presence of γH2AX nanoclusters (rmax ≈ 45 nm) and the lack of distinct DNA-PK clustering at distances smaller than 100 nm (Supplemental Fig. S5).

DISCUSSION

Because H2AX is a core histone, the clusters of nanofoci revealed by dSTORM (Figs. 2B and 3C) likely represent the arrangement of γH2AX-decorated nucleosomes within DSB foci. This conclusion is supported by the following lines of evidence:

1. Taking into account the sizes of primary and secondary IgG antibodies, with a size of ~15 nm and a dSTORM localization precision of 12 nm (Supplemental Fig. S6), the size of nanofoci (FWHM ~ 45 nm, Fig. 2F and Supplemental Fig. S2F) is well within the order of the size of a nucleosome. Accordingly, our data suggest that each nanofocus contains only 1 γH2AX molecule, which is in line with the relatively small fraction of H2AX per nucleosome found in mammalian cells (1).

2. We commonly observed distances of ~100 nm between adjacent γH2AX nanofoci. This is confirmed not only by the NND-histograms over the randomly scattered nanofoci (Fig. 2G and Supplemental Fig. S2G) but also by distance measurements along the beads on a string–like structures (Fig. 2H and Supplemental Fig. S2H). In eukaryotic cells, 147 bp of DNA are wrapped around an
octamer of core histones into an 11-nm diameter DNA-core nucleosome particle (47). Separated by 20–75 bp of DNA, nucleosomes are wound in solenoids of 6 nucleosomes/turn forming the 30-nm chromatin fiber, comprising 5–6 nucleosomes/11 nm of fiber length (48, 49). In mammalian cells, only 1 in 5 nucleosomes contains H2AX (1), which amounts to 1 nonphosphorylated, H2AX-containing nucleosome for every 11 nm of the chromatin fiber.

Interestingly, the distance of 11 nm accounts for only about one-tenth of the mean spacing (~100 nm) that we observed between adjacent γH2AX nanofoci in our dSTORM images (Fig. 2G, H and Supplemental Fig. S2G, H). Therefore, we conclude that only ~10% of H2AX is phosphorylated to γH2AX within DSB repair foci. This finding agrees well with the estimates of a previous study by Pilch et al. (1), in which a ratio of about 1 γH2AX molecule for every 50 nucleosomes was determined, roughly equal to ~10,000 bp of DNA or ~100 nm of the chromatin fiber. Taken together, dSTORM images of γH2AX (Figs 2 and 3 and Supplemental Figs S2–S5) allow the visualization of the elementary structural units of DSB foci, that is, the individual γH2AX-containing nucleosomes.

Although confined to 2 dimensions, the dSTORM data presented here can be used to estimate the total amount of chromatin involved in a repair focus. The mean number of nanofoci/foci visible in 2-dimensional (2D) dSTORM images (N2D) of DK-MG cells were found to be ~16, ~20, and ~23 in nonirradiated samples and in irradiated cells with short and long repair times, respectively (Supplemental Fig. S7A–C). Similar results were obtained for SNB19 cells (Supplemental Fig. S7D–F). The corresponding numbers of γH2AX nanofoci (focus volume) (N3D ≈ N2D3/2) in DK-MG cells can thus be estimated to be ~64, ~89, and ~110, respectively. This increase in nanofoci counts with increasing repair time is in agreement with the increase of foci volume detected by CLSM (Fig. 1F) and the rmax increase of H(r) in our dSTORM-based cluster analysis of large ROIs (Fig. 3E). Considering that the distance between γH2AX-labeled nucleosomes was found to be ~100 nm or 104 bp of DNA, these numbers of nucleosomes correspond to ~0.6–1.1 Mbp of chromatin/mean DSB repair focus over which H2AX phosphorylation spreads.

An interesting finding derived from the dSTORM-based cluster analysis of large ROIs (i.e., whole cell nucleus) is that, with increasing repair time from 30 min to 6 h, both γH2AX and DNA-PK clusters increase in size in both tested cell lines (blue vs. red lines in Fig. 3E, F and Supplemental Fig. S3E, F), which also agrees well with the increase of foci volume detected by CLSM (Fig. 1F). As pointed out elsewhere (31), phospho-DNA-PKcs has been reported to localize to DSBs of high complexity. Because clearance of complex DSBs can be expected to be slower than of non-complex breaks, the ratio of complex to less-complex DSBs can be expected to be larger in cells with longer repair times. As a result, an increased portion of the remaining complex breaks would lead to an increased mean size/volume of the DSB foci at longer repair times after irradiation. Interestingly, an increase in foci size with time after irradiation has also been reported for various repair proteins, such as, BRCA1/53BP1, Rad51, and Mre11, during homologous recombination (50, 51). According to van Veelen et al. (51), a possible explanation for this could be that persistent, that is, complex, DSBs are difficult to repair, which might result in an accumulation of more DNA repair proteins at the break site and, thus, larger foci.

Our cluster analysis of the large ROIs also revealed that judging from the rmax values the clusters of DNA-PK (i.e., 0.49 and 0.61 μm for 30 min and 6 h repair times, respectively) were smaller than the corresponding rmax values for γH2AX clusters (0.52 and 0.68 μm; Fig. 3E, F). This finding agrees well with the results of a spatial colocalization study (52), in which it was shown that phospho-DNA-PK covers only part of the total area of γH2AX foci on the micrometer scale.

On the nanometer scale (i.e., small ROIs), in sharp contrast to the histone γH2AX, firmly anchored in the chromatin fiber, the mobile, nonhistone DNA-PK, which is recruited to the focus by the Ku70/80 heterodimer (53), does not form discrete nanofoci but displays a diffuse distribution of variably sized, irregular subunits (Figs 2 and 3 and Supplemental Figs S2 and S3). The absence of discrete DNA-PK nanofoci could be due to an autophosphorylation-induced dissociation of DNA-PKcs from DNA-bound Ku (54, 55). Furthermore, Uematsu et al. (53) demonstrated that DNA-PKcs is not present at DNA ends as a rigid complex, but that there is a dynamic exchange between DNA-bound and free proteins. Unbound DNA-PKcs can be expected to be randomly distributed near the break, which would explain why no distinct clustering of this repair protein was observed on the nanometer scale (i.e., within small ROIs), as opposed to the micrometer scale (large ROIs).

However, the above conclusions and quantitative estimates, especially regarding the apparent lack of colocalization of γH2AX and DNA-PK, are based on the assumption that most γH2AX molecules in the chromatin fiber and the DNA-PK molecules within the foci were accessible to the antibody and were immunostained. Even though we conducted a series of control experiments (Supplemental Figs S4 and S5), we cannot exclude the possibility that some molecules of these proteins were not visualized in our dSTORM images because chromatin is a highly compacted and dense DNA–protein complex. Yet, there is a large body of evidence in the literature indicating that in response to DSB, compact chromatin surrounding the DSB site undergoes rapid relaxation and decondensation (56). This DSB-mediated chromatin relaxation allows an effective detection of the damaged site and also enables access for repair proteins (57, 58). Accordingly, the DSB-induced decondensation of chromatin may enhance not only the accessibility for DSB repair proteins but also facilitate the accessibility of γH2AX and DNA-PK for the antibodies.

Our conclusions regarding γH2AX nanofoci are reinforced by the results of Pilch et al. (1) obtained by experimental approaches different from our study. As already mentioned, based on the observed distance of ~100 nm between adjacent γH2AX nanofoci visible in dSTORM images (Fig. 2G, H), we concluded that only 10% of H2AX is phosphorylated to γH2AX within DSB repair foci.
results are corroborated by the finding of Pilch et al. (1) that only ~1 of 10 H2AX molecules is phosphorylated to γH2AX upon irradiation and the spacing between adjacent γH2AX nucleosomes was estimated to be ~100 nm [see Fig. 2E in Pilch et al. (1)]. The striking agreement of our results with those reported by Pilch et al. (1) is consistent with the assumption that in our experiments most γH2AX moieties were accessible to immunostaining.

Our finding that conventionally detected γH2AX foci consist of discrete subunits is further corroborated by the results of recent studies employing superresolution microscopy (13, 15). The main achievement of our study is the direct visualization of previously unobserved γH2AX-decorated nucleosomes as elementary structural units of DSB repair foci. γH2AX nucleosomes appear in dSTORM images as distinct nanofoci with a mean diameter of ~45 nm. We thus identified nanofoci that are ≥2- to 4-fold smaller compared with γH2AX nanostructures reported so far (13, 15). Other key findings of the present study include dSTORM-based nanofoci counting and distance measurements between nanofoci, providing quantitative information on the total amount of chromatin involved in DSB repair as well as on the number and longitudinal distribution of γH2AX-containing nucleosomes in a chromatin fiber. Moreover, although allowing only lateral resolution, implementation of 2-color dSTORM already revealed a lack of colocalization of γH2AX and the DSB repair factor DNA-PK, despite their close spatial proximity. In the future, 3D-dSTORM (59) will likely provide further insights into the spatial arrangement of DSB foci.

In summary, because of its diffraction-unlimited resolution, dSTORM enables the study of the fine structure of DSB repair foci in great detail with a resolution well below 100 nm. Such deeper insights into the organization of DSB repair complexes are highly important both for basic research and especially for radiation oncology, in which DNA DSBs are the critical lesions induced by irradiation to activate cell-death pathways. Therefore, understanding the fine focal structure and interactions of γH2AX and DNA repair proteins may reveal novel therapeutic targets involving the disruption of DSB repair foci, which, in turn, could improve the efficacy of tumor treatment.

AUTHOR CONTRIBUTIONS

J. Neubauer, H. Zimmermann, M. Flentje, C. S. Djuzenova, M. Sauer, and V. L. Sukhorukov conceived the project; D. Sisario, S. Memmel, C. S. Djuzenova, and V. L. Sukhorukov designed the experiments; D. Sisario and S. Memmel performed the experiments; D. Sisario, S. Memmel, S. Doose, and V. L. Sukhorukov performed data analyses; and D. Sisario, S. Doose, M. Flentje, C. S. Djuzenova, M. Sauer, and V. L. Sukhorukov wrote the manuscript, with inputs from J. Neubauer and H. Zimmermann.

REFERENCES

1. Pilch, D. R., Sedelnikova, O. A., Redon, C., Celeste, A., Nussenzweig, A. A., and Bonner, W. M. (2005) Characteristics of γH2AX foci at DNA double-strand breaks sites. Biochem. Cell Biol. 81, 123–129

2. Takahashi, A., and Ohnishi, T. (2005) Does γH2AX foci formation depend on the presence of DNA double strand breaks? Cancer Lett. 229, 171–179

3. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. 273, 5898–5908

4. Fernandez-Capetillo, O., Lee, A., Nussenzweig, M., and Nussenzweig, A. (2004) H2AX: the histone guardian of the genome. DNA Repair (Amst.) 3, 950–967

5. Kroeber, J., Wenger, B., Schwegler, M., Daniel, C., Schmidt, M., Djuzenova, C. S., Polat, B., Flentje, M., Fietkau, R., and Distel, L. V. (2015) Distinct increased outliers among 136 rectal cancer patients assessed by γH2AX. Radiother. Oncol. 10, 36

6. Lobachovsky, P., Leong, T., Dalv, P., Smith, J., Best, N., Tomaszewski, J., Thompson, E. R., Li, N., Campbell, I. G., Martin, R. F., and Martin, O. A. (2016) Compromised DNA repair as a basis for identification of cancer radiotherapy patients with extreme radiosensitivity. Cancer Lett. 383, 212–219

7. Deniz, M., Kaufmann, J., Stahl, A., Gundelach, T., Jani, W., Hoffmann, I., Keimling, M., Hampp, S., Ihle, M., and Wiesmüller, L. (2016) In vitro model for DNA double-strand break repair analysis in breast cancer reveals cell type-specific associations with age and prognosis. FASEB J. 30, 3786–3799

8. Rogakou, E. P., Boom, C., Redon, C., and Bonner, W. M. (1999) Megabase chromatin domains involved in DNA double-strand breaks in vivo. J. Cell Biol. 146, 935–946

9. Sak, A., and Stuschke, M. (2010) Use of γH2AX and other biomarkers of double-strand breaks during radiotherapy. Semin. Radiat. Oncol. 20, 223–231

10. Bewersdorf, J., Bennett, B. T., and Knight, K. L. (2006) H2AX chromatin structures and their response to DNA damage revealed by 4Pi microscopy. Proc. Natl. Acad. Sci. USA 103, 18117–18122

11. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr. Biol. 10, 886–895

12. Nakamura, A. J., Rao, V. A., Pommier, Y., and Bonner, W. M. (2010) The complexity of phosphorylated H2AX foci formation and DNA repair assembly at DNA double-strand breaks. Cell Cycle 9, 389–397

13. Natale, F., Rapp, A., Yu, W., Maier, A., Harz, H., Scholl, A., Gruhl, S., Anton, T., Hör, D., Chen, W., Durante, M., Taucher-Scholz, G., Leonhardt, H., and Cardoso, M. C. (2017) Identification of the elementary structural units of the DNA damage response. Nat. Commun. 8, 15760

14. Britton, S., Coates, J., and Jackson, S. P. (2013) A new method for high-resolution imaging of Ku foci to decipher mechanisms of DNA double-strand break repair. J. Cell Biol. 202, 579–595

15. Lopez Perez, R., Best, G., Nicolay, N. H., Greubel, C., Rossberger, S., Reindl, J., Dollinger, G., Weber, K.-J., Cremer, C., and Huber, P. E. (2016) Superresolution light microscopy shows nanostructure of carbon ion radiation-induced DNA double-strand break repair foci. FASEB J. 30, 2767–2777

16. Reindl, J., Girst, S., Walsh, D. W. M., Greubel, C., Schwarz, B., Siebenborn, C., Drexler, G. A., Friedl, A. A., and Dollinger, G. (2017) Chromatin organization revealed by nanostructured imaging of irradiation induced γH2AX, 53BP1 and Rad51 foci. Sci. Rep. 7, 40616

17. Rhodes, D. (1997) Chromatin structure: the nucleosome core all wrapped up. Nature 389, 231, 233

18. Heilemann, M., van de Linde, S., Schüttelpelz, M., Kasper, R., Seefeldt, B., Mukherjee, A., Tinnefeld, P., and Sauer, M. (2008) Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. Annu. Rev. Chem. Int. Ed. Engl. 47, 6172–6176

19. Zessin, P. J. M., Finan, K., and Heilemann, M. (2012) Super-resolution fluorescence imaging of chromosomal DNA. J. Struct. Biol. 177, 344–348

20. Wombacher, R., Heidbreder, M., van de Linde, S., Sheetz, M. P., Heilemann, M., Cornish, W. V., and Sauer, M. (2010) Live-cell super-resolution imaging with trimethoprim conjugates. Nat. Methods 7, 717–719

21. Klein, T., Löschberger, A., Propper, S., Wolter, S., van de Linde, S., and Sauer, M. (2011) Live-cell dSTORM with SNAP-tag fusion proteins. Nat. Methods 8, 7–9

22. Streltova, M. P., Solovejov, L. V., and Tomilin, N. V. (2010) Mechanism of elimination of phosphorylated histone H2AX from chromatin after repair of DNA double-strand breaks. Mutat. Res. 685, 54–60

23. Mah, L.-J., Orłowski, C., Ververis, K., Vasireddy, R. S., El-Osta, A., and Karagiannis, T. C. (2011) Evaluation of the efficacy of
radiation-modifying compounds using γH2AX as a molecular marker of DNA double-strand breaks. Genome Integr. 2, 3

24. Vignard, J., Mirey, G., and Salles, B. (2013) Ionizing-radiation-induced DNA double-strand breaks: a direct and indirect lighting up. Radiat. Oncol. 108, 362–369

25. Pospelov, T. V., Demidenko, Z. N., Bukreeva, E. I., Pospelov, V. A., Gadkov, A. V., and Blagosklonny, M. V. (2009) Pseudo-DNA damage response in senescent cells. Cell Cycle 8, 4112–4118

26. Pankotai, T., Hoffbeck, A. S., Boumendil, C., and Soutoglou, E. (2009) DNA damage response in the absence of DNA lesions continued... Cell Cycle 8, 4025–4026

27. McManus, K. J., and Hendzel, M. J. (2005) ATM-dependent DNA damage-independent mitotic phosphorylation of H2AX in normally growing mammalian cells. Mol. Biol. Cell 16, 5013–5025

28. De Feraudy, S., Revet, I., Bezrukovko, V., Feeney, L., and Cleaver, J. E. (2010) A minority of foci or pan-nuclear apoptotic staining of γH2AX in the S phase after UV damage contain DNA double-strand breaks. Proc. Natl. Acad. Sci. USA 107, 6870–6875

29. Cleaver, J. E., Feeney, L., and Revet, I. (2011) Phosphorylated H2AX is not an unambiguous marker for DNA double-strand breaks. Cell Cycle 10, 3223–3224

30. Rybak, P., Huang, A., Bujnowicz, L., Bernas, T., Berta, K., Zarębski, M., Darzyńkiewicz, Z., and Dobrucki, J. (2010) Low level phosphorylation of histone H2AX on serine 139 (γH2AX) is not associated with DNA double-strand breaks. Oncotarget 1, 9557–9567

31. Reynolds, P., Anderson, J. A., Harper, J. V., Hill, M. A., Botchway, S. W., Parker, A. W., and O’Neill, P. (2012) The dynamics of Ku70/80 and DNA-PKcs at DSBs induced by ionizing radiation is dependent on the complexity of damage. Nucleic Acids Res. 40, 10821–10831

32. Neal, J. A., Sugimura-Marangos, S., VanderVere-Carozza, P., Wagner, M., Turchi, J., Lees-Miller, S. P., Junop, M. S., and Meek, K. (2014) Unraveling the complexities of DNA-dependent protein kinase autophosphorylation. Mol. Cell. Biol. 34, 2162–2175

33. Cui, X., Yu, Y., Gupta, S., Cho, Y-M., Lees-Miller, S. P., and Meek, K. (2005) Autophosphorylation of DNA-dependent protein kinase regulates DNA end processing and may also alter double-strand break repair pathway choice. Mol. Cell. Biol. 25, 10842–10852

34. Jiang, W., Crowe, J. L., Liu, X., Nakajima, S., Wang, Y., Li, C., Lee, B. J., Dubois, R. L., Liu, C., Yu, X., Lan, L., and Zha, S. (2015) Differential phosphorylation of DNA-PKcs regulates the interplay between end-processing and end-ligation during nonhomologous end-joining. Mol. Cell 58, 172–185

35. Jette, N., and Lees-Miller, S. P. (2015) The DNA-dependent protein kinase: a multifunctional protein kinase with roles in DNA double strand break repair and mitosis. Prog. Biophys. Mol. Biol. 117, 194–205

36. Collis, S. J., DeWeese, T. L., Jeggo, P. A., and Parker, A. R. (2005) The life and death of DNA-PK. Oncogene 24, 949–961

37. Van de Linde, S., Aufmkolk, S., Franke, C., Sauer, M., and van de Rakt, M. W. M., Theil, A. F., Esers, J., and Kanaar, R. (2005) Analysis of ionizing radiation-induced foci of DNA damage repair proteins. Mutat. Res. 574, 22–33

38. Asaihambay, U., Uematsu, N., Chatterjee, A., Story, M. D., Burma, S., and Chen, D. J. (2008) Repair of HZE-particle-induced DNA double-strand breaks in normal human fibroblasts. Radiat. Res. 169, 437–446

39. Uematsu, N., Weterings, E., Yano, K., Morotomi-Yano, K., Jakob, B., Taucher-Scholz, G., Mari, P.-O., van Gent, D. C., Chen, B. P. C., and Chen, D. J. (2007) Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. J. Cell Biol. 177, 219–229

40. Block, W. D., Yu, Y., Merkle, D., Gifford, J. L., Ding, Q., Meek, K., and Lees-Miller, S. P. (2004) Autophosphorylation-dependent remodeling of the DNA-dependent protein kinase catalytic subunit regulates ligation of DNA ends. Nucleic Acids Res. 32, 4353–4357

41. Chan, D. W., and Lees-Miller, S. P. (1996) The DNA-dependent protein kinase is inactivated by autophosphorylation of the catalytic subunit. J. Biol. Chem. 271, 8936–8941

42. Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D. C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006) Chromatin relaxation in response to DNA double-strand breaks is mediated by a novel ATM- and KAP-1-dependent pathway. Nat. Cell. Biol. 8, 870–876

43. Timinszky, G., Till, S., Hassa, P. O., Hothorn, M., Kustatscher, G., Nijmegen, B., Colombelli, J., Altmeyer, M., Stelzer, E. H. K., Scheifetz, K., Hottiger, M. O., and Ladurner, A. G. (2009) A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. Nat. Struct. Mol. Biol. 16, 925–929

44. Khurana, S., Kruhlak, M. J., Kim, J., Tran, A. D., Liu, J., Nysawer, K., Shi, L., Jhaiba, P., Sung, M.-H., Hakim, O., and Oberdoerffer, P. (2014) A macrodomain variant links dynamic chromatin compaction to BRCA1-dependent genome maintenance. Cell Rep. 8, 1049–1062

45. Franke, C., Sauer, M., and van de Linde, S. (2017) Photometry unlocks 3D information from 2D localization microscopy data. Nat. Methods 14, 41–44

Received for publication December 4, 2017. Accepted for publication May 21, 2018.