

# Space and time in the universe of the cell nucleus after ionizing radiation attacks: a comparison of cancer and non-cancer cell response <sup>†</sup>

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**Abstract:** DNA double-strand breaks (DSBs) are known to be the most serious lesions in irradiated cells. Several protein pathways exist for repair. The mechanisms by which cells determine a specific pathway for repair remain poorly understood. DSB induction and repair can be spatiotemporally monitored by ionizing radiation-induced foci (IRIFs) and the formation of repair complexes. IRIF analyses revealed that DSB formation, repair and misrepair are strongly dependent on the radiation characteristics and the microarchitecture of the chromatin environment. However, the IRIF nanoarchitecture remains unknown, as does its impact on the decision-making process and follow-up protein recruitment. New insights into the relationship between the physical properties of radiation, environmental chromatin architecture, IRIF architecture, and DSB repair mechanisms are presented using single molecule localization microscopy. Ripley distance statistics and persistent homology calculations have shifted our ability to analyze chromatin and IRIF architectures from imaging to topology and structure calculations. We discuss these approaches for cancer treatment relevant irradiation processes on selected cell systems and consider whether this “structuromics” can enhance our knowledge about radiation response.

**Keywords:** ionizing radiation, DNA damage foci, DNA repair, super-resolution localization microscopy, chromatin nano-architecture, spatiotemporal repair protein organization, molecular cluster analysis, molecular topologies

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## 1. Introduction

Application of ionizing radiation has an increasing impact on bio-medical research, and cancer diagnosis and treatment. Nevertheless, there are a lot of open questions concerning the understanding of radiation DNA damaging processes and repair mechanisms within the light of radio-sensitivity and thus, individualized medical applications (for review [1]). The three-dimensional architecture of genomes on the micro-, meso- and nano-scale acts in combination with epigenetic modifications as an important player of gene regulation and, consequently,

fundamental biological processes such as DNA damage response and repair (for review [2]). In this context, chromatin and protein nano-probing and super-resolution microscopy are powerful methods for topological analyses of genomic targets in native chromatin and repair protein complexes in single cells at resolutions of single antibodies, proteins, histones, short DNA segments, etc. [3].

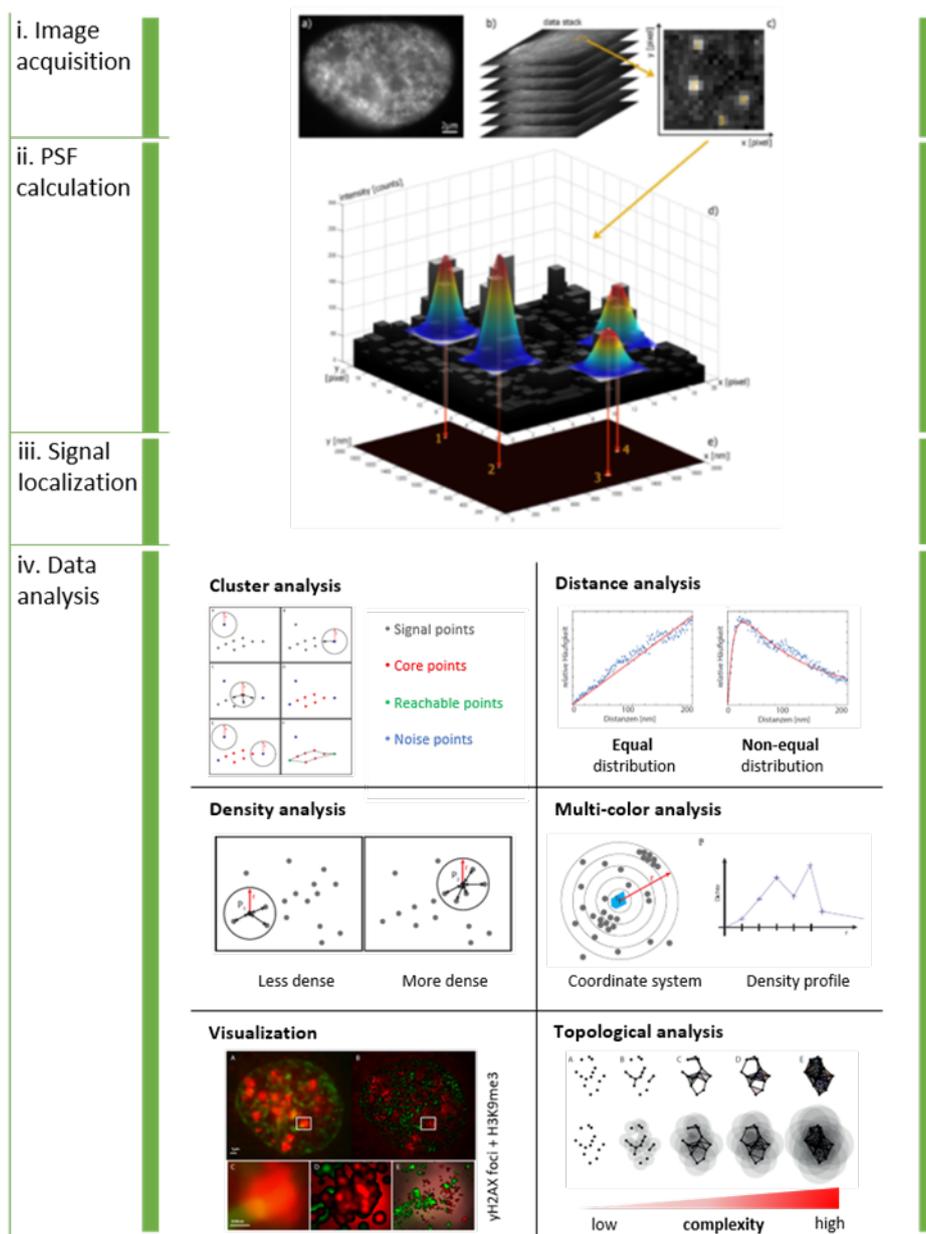
So far only little is known [4,5] about the impact of chromatin architecture on DNA double strand break (DSB) induction and repair pathway selection at individual damage sites. How does a cell nucleus manage DSBs and re-organize the chromatin to generate functionally intact repair units? Is there a radio-sensitivity related difference in this reaction? In order to answer these questions, we have investigated spatial and topological parameters of chromatin and repair foci during a time period of repair to highlight key aspects related to these questions. Nano-probing of radiation-induced chromatin damage sites and the recruited DNA repair proteins in combination with super-resolution Single Molecule Localization Microscopy (SMLM) [6-8] are powerful methods for geometric and topological analyses of these structures in single cells and single DSB sites and, thus, to study mechanisms of their formation and repair pathway regulation (Figure 1). In the following study we have analyzed the nano-architecture and -dynamics of DNA damage markers, chromatin markers, and DNA repair protein complexes in 3D-conserved nuclei of different cell types after exposure to various types (low-LET, high-LET;  $\alpha$ -,  $\beta$ -particles) and doses of ionizing radiation.

## 2. Spatial analysis of repair cluster formation after low-LET photon irradiation

We used variable tools for such investigations based on image-free high-precision SMLM, nano-scaled molecule distribution analyses, appropriate metrics following Ripley's distance frequencies and cluster formation analyses [9], as well as topological quantifications employing persistence homology calculations [10]. In low linear energy transfer (LET) X irradiated HeLa cell nuclei, DSB-induced  $\gamma$ H2AX foci being visible by standard fluorescence microscopy were subdivided in 3-4 equally sized (diameter about 400 nm), functionally relevant fluorescence tag-containing clusters. The amount of clusters was increasing with radiation dose (0 – 2 Gy) and decreasing with repair time (0.5 h – 8 h). This behavior was corresponding to the detected number of  $\gamma$ H2AX antibody labelling tags as seen by SMLM nanoscopy [9].

Persistence homologies of components and holes can be calculated as a measure of topology of repair clusters and be compared for their similarities [10]. The data obtain for HeLa cells and SkBr3 breast cancer cells suggested topological similarities in repair cluster formation associated to heterochromatin, indicating a well-defined, non-random molecule topology at given time points during repair. As to the size of the  $\gamma$ H2AX clusters, there was a persistent similarity independent of repair time. As long as detectable clusters were found, size and topology seem to be maintained.

MRE11 clusters analyzed in fibroblasts and MCF-7 breast cancer cells were embedded into the corresponding  $\gamma$ H2AX clusters. With a diameter of about 200 nm, the MRE 11 clusters were considerably smaller than the  $\gamma$ H2AX clusters (diameter of 400 nm). Again the cluster sizes of the repair proteins were independent of the repair time after radiation exposure and seem to be also independent of the cell type [11]. In fibroblasts irradiated with a dose of 2 Gy,  $\gamma$ H2AX clusters showed a high averaged topological similarity for persistent homologies within the first 3 hours after irradiation. After 2 hours the averaged similarity of MRE11 clusters considerably increased and maintained until 48 hours post irradiation (Hahn et al., manuscript in preparation). In contrast randomly occurring  $\gamma$ H2AX and MRE11 clusters in the non-irradiated control cells did not show any significant similarity.



**Figure 1.** General workflow of SMLM and data analysis: Serial images are acquired from the same region of interest (i). The point-spread function of each blinking molecule in each single image frame is Gaussian fitted to estimate the intensity maximum (ii), which represents the idealized lateral coordinates of the signal (iii). The result is a data table containing the coordinates of all detected signal points. The matrix representation of data allows mathematical and statistical analysis of clustering, distance distributions, signal densities, multi-color signal distributions, enhanced visualization and topology (iv). Note: This figures and figure legend were originally published under CC BY license in [1].

### 3. Spatial analysis of repair cluster formation within $\alpha$ -particle tracks and $\beta$ -particle damage sites

SMLM in combination with Ripley cluster analysis of single molecule signal-point density regions of DSB repair markers was applied to investigate the nano-structure of DNA damage foci tracks in leukocytes exposed in-solution to Ra-223 decay products [12]. Alpha-particle induced single DNA damage tracks in chromatin were efficiently outlined by  $\gamma$ H2AX that formed large foci composed of numerous 60–80 nm sized tag-clusters. The tracks contained 60–70 % of all  $\gamma$ H2AX point signals in a nucleus, while less than 30 % of 53BP1, MRE11 or p-ATM signals were located

inside  $\gamma$ H2AX damage tracks. 53BP1 formed clusters whose tags were interspersed with the  $\gamma$ H2AX cluster tags, indicating that both proteins are part of the larger chromatin domain around DSB DNA damage. MRE11 and p-ATM tags, on the other hand, formed focal nano-clusters of about 20 nm peak size embedded into the  $\gamma$ H2AX clusters. On average about 12 ( $\pm$  9) MRE11 nanoclusters were found within the  $\gamma$ H2AX-marked alpha-particle track. In cell nuclei where the alpha-particle track did obviously not completely cross the nucleus, the amount of 53BP1 and MRE11 clusters was increasing at the end of the track; thereby the signal ratio of 53BP1/ $\gamma$ H2AX and MRE11/ $\gamma$ H2AX, respectively, remained constant.

The nano-structure of DNA damage foci of Lu-177 in-solution exposed leukocytes was also analyzed (Scherthan and Hausmann, unpublished). Preliminary results indicated an average cluster radius of about 300 nm for all types of repair-associated protein clusters analyzed ( $\gamma$ H2AX, MRE11, 53BP1). However the signal density in the clusters was highly variable; an issue that is under further investigation.

#### 4. Spatial analysis of repair cluster formation within damage tracks of high LET $^{15}\text{N}$ ions

SMLM has been successfully applied to study DNA repair and cluster formation of  $\gamma$ H2AX and 53BP1 molecules under high-LET radiation condition [13]. 53BP1 foci were investigated in differently radio-resistant cell types, the moderately radio-resistant neonatal human dermal fibroblast cell line (NHDF) and highly radio-resistant U87 glioblastoma cell line [14]. Specimens of both cell types were exposed to high-LET  $^{15}\text{N}$ -ion radiation of doses of 1.3 Gy ( $10^\circ$  irradiation scheme) and 4.0 Gy ( $90^\circ$  irradiation scheme) at the particle irradiation facility of the Joint Institute for Nuclear Research, Dubna, Russia.

At given time points up to 24 h post irradiation, SMLM of fluorescently tagged 53BP1 molecules was performed and clusters were determined as sub-units of repair foci. The formation and relaxation of these clusters was studied during the repair period. The results revealed a higher ratio of 53BP1 proteins being recruited into clusters in NHDF cells (less radio-resistant) as compared to U87 cells (more radio-resistant) with a different starting situation and different levels of distribution of repair proteins prior to DNA damage induction. This relation of 53BP1 inside and outside the track clusters remained different for both cell types during the whole repair time observed. Hence, the 53BP1 ratio within and outside repair foci could be seen as a measure of the “just-in-time” availability of 53BP1 proteins but did not reflect the absolute number of 53BP1 proteins available. The speed of cluster formation and relaxation differed for the two cell types indicating the recruitment of the existing proteins in the cell nucleus (higher in U87 cells) rather than a de novo production. A certain number of the clusters remained persistent even longer than 24 h post-irradiation and the number of these remaining clusters varied for the cell lines; persistent foci/clusters could thus causatively be linked to the cell-type specific radio-resistance [13,14].

Appropriate metrics following Ripley’s distance frequencies and cluster formation analyses revealed equally sized clusters in NHDF cells during the repair period analyzed. For U87 cells, the cluster formation resulted in more variable clusters. Comparing the topology of repair clusters by persistence homology calculations suggested similarities of repair cluster formation along given particle tracks [15]. The resulting components and holes were compared and their similarity was calculated. For both cell types the similarity measure as being determined by the Jaccard index [10,15] was very high for the components ( $>0.9$  means high similarity), independent of the irradiation scheme ( $10^\circ$ ,  $90^\circ$ ). This indicates that the complex, ion-induced damages were marked with specific repair cluster setups. On the other hand, the similarity measure for the holes was below 0.5 for both cell types and irradiation schemes; thereby the similarity of holes was on average higher for NHDF than for U87 cells. In general it can be concluded that the normal (non-transformed), more radio-sensitive NHDF cells revealed higher topological similarity in 53BP1 clustering than the more radio-resistant U87 cancer cells [15].

## 5. Dose dependent ALU signaling

Multi-color nano-probing using specific antibodies for heterochromatin (H3K9me3) and specific oligonucleotides uniquely binding to the ALU consensus sequences (17-mer oligonucleotide) [7] and SMLM were applied to study pan-nuclear chromatin re-arrangements in the cell nuclei of SkBr3 breast cancer cells [6,16]. Counting of signals of dispersed genomic Alu sequences resulted in decreasing linear-quadratic dose-effect curves for low to higher dose ranges, which may be related to DNA breakage events preferentially in these regions. In the low dose range (< 500 mGy) a linear approximation was possible [6]; thereby the fit values of the curves were the same within the error ranges. In addition, the spatial distribution changes of anti-H3K9me3-labeled heterochromatin clusters indicated a dose-independent chromatin relaxation upon radiation exposure.

## 6. Conclusion

We demonstrated efficient applicability of SMLM to study radiation-induced damage and the associated repair protein recruitment to damage sites in space and time. The results revealed a cell type- and radiation type-specific nano-architecture of DNA damage foci with respect to  $\gamma$ H2AX, Mre11 or 53BP1 and their dynamic molecular rearrangements during repair processes. Correlations to radio-sensitivity were revealed after  $^{15}\text{N}$ -ion irradiation. SMLM studies thus contributed and will further contribute to the molecular understanding of cellular radiation responses at the sub-light microscopic chromatin levels, indicating that chromatin and repair focus nano-architecture might contribute to repair pathway decision. Showing how chromatin architecture around complex damage sites and repair focus nano-architecture may impact ongoing repair process, our studies pave the way to the molecular understanding of cellular radiation response and its regulation in cancer and non-cancer cells.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, M.H. and M.F.; methodology, M.H. and H.S.; software, M.H. and D.W.H.; investigation, C.N., H.H., R.W., H.S. and G.P.; resources, M.H., H.S., and M.F.; data curation, M.H., C.N., and G.P.; writing—original draft preparation, M.H.; writing—review and editing, M.F., H.S., D.W.H.; visualization, C.N. and R.W.; supervision, M.H., H.S., G.P., G.H., and M.F.; project administration, I.F. and M.H.; funding acquisition, M.H. and M.F.. All authors have read and agreed to the published version of the manuscript.

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