NOVÝ SOFTWARE VYUŽÍVAJÍCÍ UMĚLÉ NEURONOVÉ SÍTĚ A HLUBOKÉ STROJOVÉ UČENÍ PRO AUTOMATICKOU KVANTIFIKACI RADIAČNÍHO POŠKOZENÍ DNA A TRIÁŽ OZÁŘENÝCH

NEW SOFTWARE BASED ON ARTIFICIAL NEURAL NETWORKS AND DEEP MACHINE LEARNING FOR AUTOMATIC QUANTIFICATION OF DNA RADIATION DAMAGE AND TRIAGE OF IRRADIATED POPULATION

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Abstract

Rapid classification of irradiated victims in the framework of mass radiation events in the absence of accurate dosimetric measurements relies on biological markers. We present a new software, DeepFoci, based on artificial neural networks and deep learning, which we developed for fast and fully automatic detection of DNA double-strand breaks (DSBs), manifesting in cells as so-called radiation-induced foci (IRIFs). DSBs represent the most severe type of radiation damage to DNA, and IRIF is therefore an important biodosimetric marker. The software enables not only fast and fully automatic quantification of IRIF and therefore estimation of absorbed radiation dose, but also analysis of micromorphological parameters of IRIF. This makes it possible to differentiate exposure to radiation with low and high linear energy transfer (LET), and possibly also combat chemical agents generating DSBs.

Key words: *mass radiation accidents, biodosimetry, new software for radiation victim triage, artificial neural networks, ionizing radiation-induced foci (IRIF) quantification and morphological analysis*

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1. INTRODUCTION AND MOTIVATION

1.1 Biodosimetry based on ionizing radiation-induced (repair) foci for mass radiation accidents

In the case of a mass radiation accident, a terrorist attack using a dirty bomb or the military use of nuclear weapons, a large number of residents can be expected to be exposed to various doses of ionizing radiation. As part of medical intervention, it is therefore first necessary to categorize irradiated persons according to the absorbed dose of radiation, and therefore also the required medical care and subsequent prognosis. In the case of mass radiation accidents, in contrast to minor accidents mostly limited to professional radiation workers, we cannot rely on accurate dose measurements from personal dosimeters, which makes biodosimetry (based on the analysis of the victims' biological material) a fundamental approach to estimate the dose absorbed by individual persons [1],[2].

The most sensitive target for damage by ionizing radiation is the cell nucleus, more precisely the DNA molecule (genome) located within this organelle. Various signs of genome damage thus represent important biodosimetric markers. The most serious type of genome damage is DNA double-strand breaks (DSBs), potentially leading to structural rearrangements of chromosomes (chromosomal aberrations) [3]. Ionizing radiation is the most effective inducer of DSB. Although cells can effectively repair DSBs to a certain extent, since this repair is very complicated, a certain fraction of DSBs is repaired erroneously or remains unrepaired. As a result of misrepaired and persistent DSBs, the aforementioned chromosomal aberrations (especially dicentric and circular chromosomes) and micronuclei occur. Both these types of DNA damage appear already after exposure to very low doses of ionizing radiation and represent well-established biomarkers [2],[3]. However, chromosomal aberrations and micronuclei have certain disadvantages from a biodosimetric point of view - a) their analysis is generally possible only after the cells have passed through mitosis, which requires time- and laboratory-intensive cell cultivation, b) the number of chromosomal aberrations and micronuclei is affected not only by the absorbed dose of radiation, but also by the effectiveness of repair mechanisms, which may depend on many factors.
The quantification of so-called repair foci (ionizing radiation-induced foci, IRIF) [4], which are formed by focal accumulations of epigenetic changes and repair proteins at DSB sites, appears to be more advantageous. γH2AX foci appear as the result of histone H2AX phosphorylation on serine 139 in a chromatin region of about 2 Mb around each DSB [5]. These foci create binding platforms for the subsequent binding of the entire sequence of repair proteins involved in biochemical DSB repair pathways [6]. Foci of γH2AX and some repair proteins are formed at DSB sites almost immediately (in minutes) after irradiation and persist in cell nuclei until DSB repair is accomplished. Depending on the dose and type of radiation, it is thus possible to detect these foci for a period of 1 day to several days after irradiation [7],[8], which is sufficient time to take biological samples from irradiated victims even in the case of mass radiation events (except of extensive use of nuclear weapons). The important advantage of γH2AX quantification is the enormous sensitivity and robustness of the method; 1 Gy of gamma radiation produces approximately 35 γH2AX foci in the cell nucleus, and irradiated samples can be distinguished from non-irradiated samples up to a dose of 10 mGy [9]. The dependence of the number of generated γH2AX foci on the dose of ionizing radiation is usually given in a wide range of doses (mGy -> Gy) as linear [10]–[12] or linear quadratic [13]. The type of dose dependence can be

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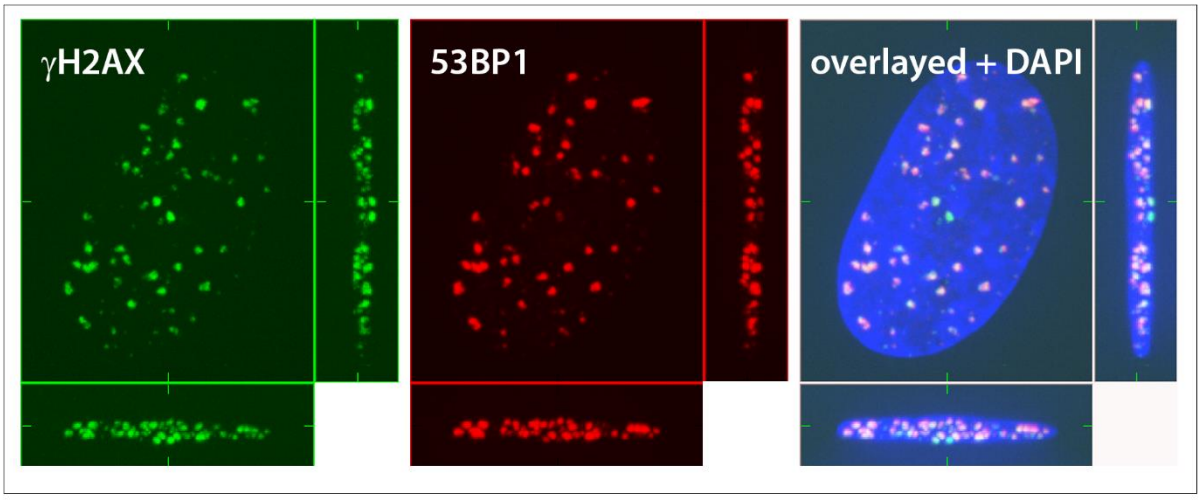
influenced by, for example, the dose rate [13]. Tommasiono demonstrated a linear increase in the γH2AX signal up to a dose of approx. 10 to 20 Gy [14], which is more than sufficient from the point of view of patient triage, since roughly 4 Gy of photon radiation represents the $LD_{50/30}$ dose in humans and doses above 10 Gy are already associated with the neurovascular form of acute radiation syndrome (ARS) and certain death [15],[16].

Other advantages of the biodosimetric approach based on the quantification of DSB repair foci include the possibility of further refining the estimate of the absorbed dose from the number of γH2AX foci using the co-detection of γH2AX foci with the foci of one of the repair proteins [17],[18]. In this regard, the 53BP1 protein is particularly interesting as it creates foci with a size and morphology very similar to γH2AX foci. Crucially, the 53BP1 protein binds to all DSBs, regardless of the repair mechanism activated at the particular genomic site to repair them. Moreover, the kinetics of 53BP1 foci formation and their persistence in the nucleus corresponds to that of γH2AX foci. Thanks to this, most 53BP1 and γH2AX foci significantly colocalize with each other, thus confirming each other's identity [4] (**Fig. 1**). As our results show, the number of γH2AX foci colocalizing with 53BP1 foci gradually increases up to about 0.5 to 1 h after irradiation [4],[18]. This fact must be taken into account in situations (research, space flights, clinical biodosimetry) where samples for biodosimetry can be taken already in a short time interval after irradiation. However, in the case of mass radiation events, the shortest time between irradiation and sampling can be expected to be in the order of a few (4 to 8) hours.

Figure 1: Ionizing radiation-induced (repair) foci visualized by immunofluorescence confocal microscopy at 8 h after irradiation of spatially (3D) fixed normal human skin fibroblasts with 8 Gy of gamma rays (^{60}Co, D=1 Gy/min). Colocalization of two IRIF markers, γH2AX and 53BP1, is shown in all three planes. Blue color (right panel): cell nucleus (chromatin) labeled with DAPI. Magnification 100x.



The possibility of parallel quantification of multiple types of repair foci—e.g., γH2AX, 53BP1, RAD51 and others—is essential for research in the field of radiation damage and DNA repair. Co-detection of γH2AX and 53BP1 repair foci would be important also in pre-therapeutic

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analysis of tumor sensitivity to radiotherapy, which can be predicted to emerge as part of future personalized medicine. There, it would be necessary to quantify repair foci directly in tumor cells taken from patients and irradiated *in vitro*. Different tumor cells often create variable repair foci, which might be irregular, diffuse, and potentially accompanied by a pronounced pannuclear signal background [19]. These characteristics of repair foci significantly complicate their correct identification [4], which can be significantly improved by the co-detection of two mutually colocalizing DSB markers (repair foci) [17].

On the other hand, in case of mass radiation events, the minimally invasive collection and analysis of normal peripheral blood lymphocytes is the most advantageous. Lymphocytes form well-defined repair foci after irradiation and are blocked in the G_0 phase of the cell cycle. These features contribute to the homogeneity of repair foci parameters as well as sharper distribution of focus numbers per radiation dose and nucleus. Therefore, biodosimetry with lymphocytes would rely on the simple detection of γH2AX foci as the only DSB marker. The simultaneous detection of other repair foci would significantly and unnecessarily increase the price of the analysis.

1.2 Problems with automated software detection of IRIFs and analysis of large microscopy image datasets

We can detect and quantify fluorescently labeled γH2AX (or repair proteins) in two practically relevant ways – using flow microscopy and/or immunofluorescence (confocal) microscopy [20]. Quantification of the γH2AX (or other repair protein) signal provides the advantage of high automation of the process with the possibility of analyzing a huge number of cells. Microscopic detection then offers the possibility of evaluating the micromorphological parameters of repair foci and the character of their topological distribution in the cell nucleus. For biodosimetry, these parameters can provide important information about the type of incident radiation (radiation with low vs. high linear energy transfer, LET) (**Fig. 2**) [18], [20]; in clinical applications, micromorphological parameters of repair foci could be useful to understand DNA repair and genetic instability in different tumor cells [6], [19]. Microscopy of repair foci formed by γH2AX and various repair proteins is then a unique and absolutely irreplaceable method in radiobiological research, where it allows studying the processes of radiation damage and DNA repair in real time in single spatially fixed or even living cells, at the sites of individual DSBs [21]–[23]. It should be mentioned that there are also approaches combining analysis through flow cytometry and microscopy, though, with only low image resolution.

The detection and analysis of microscopically visualized repair foci is surprisingly problematic even in today's age of advanced software [4]. Conventional thresholding-based detection methods for repair foci fail completely, forcing thus evaluators to count foci manually. This is enormously demanding on experience and time, and it is therefore not possible to consider biodosimetric applications in larger scale situations. In addition, a direct comparison of results between laboratories is not practically possible [4], and all information about reparation foci, apart from their number, remains unextracted.

Automatic segmentation of repair foci using software is particularly problematic due to the enormous variability of these structures, *de facto* in all their parameters [4]. In particular, there

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are large differences in the size and fluorescence intensity of repair foci both between and within samples. These differences appear, for instance, due to the different post-irradiation period at the time of sample fixation, the labeling method, the cell cycle distribution of samples, etc. Most of the available software procedures therefore use the so-called adaptive thresholding and/or image standardization [4]. However, these approaches cause the sensitivity of the detection of repair foci to be significantly affected by the focus number in the nucleus. Moreover, it is not possible to set the detection parameters universally in such a way that it is possible to correctly detect and segment all potential focus types – small foci observed at early times after exposure, fully developed large foci, late persistent or decaying foci, and foci associated with replication stress/genetic instability in S-phase cells. It is therefore necessary to set the parameters of detection and segmentation specifically for individual samples, which not only complicates the comparison of samples with each other, but also only partially solves the mentioned problems. Even within one sample, there is considerable heterogeneity of cells and therefore of repair foci. In the case of analysis of peripheral blood lymphocytes, the heterogeneity of cells is relatively small due to their blocking in the G_0 phase of the cell cycle.

A fundamental problem for current detection and segmentation algorithms is also represented by cells without repair foci or with a low number of them. In these cases, due to image standardization and automatic thresholding, significant false positive detection of foci often occurs. This is a serious problem for biodosimetry, because in most situations one can expect samples with a small number of foci per cell due to the combination of relatively low radiation doses received and later post-irradiation times of sample examination (in the order of hours post-irradiation). Unexposed people or people exposed to low doses of radiation could thus be considered more significantly exposed.

2. RESULTS AND DISCUSSION

The goal of our research presented in this article was to check whether the current problems with repair focus detection, resulting from biological and technical variability of repair focus labeling, can be bridged with the use of deep machine learning. The knowledge has been used to develop software for fast, objective (unbiased), and fully automated detection and morphological characterization of repair foci in large datasets of confocal (3D) microscopy data. The result is the creation of DeepFoci software, which represents an algorithm based on U-Net convolutional neuronal network, which is described in detail in a recent publication by Vičar et al. (2021) [4], including training datasets, software training procedure and its practical application to analyze very difficult samples. Currently, DeepFoci has been trained and tested for the detection and segmentation of γH2AX, 53BP1 and RAD51 foci in several types of normal or cancer human cells exposed to different doses (0.5 to 8 Gy) of photon radiation, specifically, γ-rays from a ^{60}Co source (D=1 Gy/min). However, the applicability should in principle be the same for other sources of γ-rays or X-rays of different energies, because all types of photon radiation create spatially separated DSBs and thus repair foci (in contrast to the so-called repair protein "tracks" in cells exposed to high-LET particles) (**Fig. 2**). The datasets used for DeepFoci training and testing contained a large number of cells, which were fixed at a wide range of post-irradiation times (from 30 min to 24 h post-irradiation) and imaged in 3D by confocal microscopy. The software was able to detect and segment the repair foci of all the tested DSB

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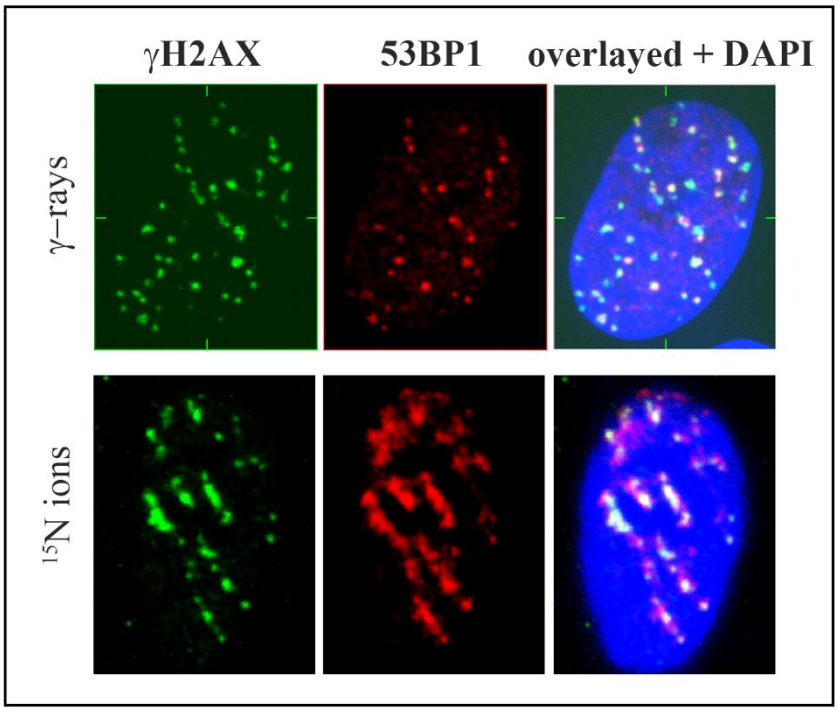
markers (γH2AX, 53BP1 and RAD51) with high accuracy, approaching that achieved by manual analysis by experienced raters. Even under these difficult circumstances, DeepFoci has demonstrated high accuracy, reliability, reproducibility, and robustness even in difficult cases where the datasets contained cells with a small number of foci, biologically highly variable samples or samples of technically poor quality as they may appear during analyzes in practice.

DeepFoci can work in two analysis modes. In the first case, it uses the colocalization of two independent DSB markers, for instance γH2AX and 53BP1, displayed in different color channels for the detection and segmentation of repair foci. In the alternative mode, the software detects repair foci in individual color channels separately, regardless of colocalization. Both approaches have their biological and practical justification. In the first approach, the software achieves the best values of all analysis parameters. From a biological point of view, only actively repairing DSB foci are detected in this way. It is therefore possible to study how individual repair proteins mutually interact in post-irradiation time, at different places of the cell nucleus (e.g., in sparse vs. condensed chromatin, etc.), in different types of normal and tumor cells, in cells exposed to different types of ionizing radiation, etc. It is also possible to distinguish γH2AX foci at actively repaired DSBs from apoptotic foci. All this information is important for research and clinical applications, but for biodosimetry in the case of mass radiation situations, the simultaneous staining of two DSB markers could be considered a significant and relatively unjustified increase in costs. The analysis of repair foci based on the colocalization of γH2AX with 53BP1 is also not feasible for samples taken in short times after irradiation (approx. up to 1 h); though this situation does not seem realistic in the case of radiation accidents of larger scale.

Figure 2: Different topological distribution of γH2AX and 53BP1 repair foci in spatially (3D) fixed normal human skin fibroblasts irradiated either with 1.5 Gy of gamma rays (^{60}Co, D=1 Gy/min) (top line) or ^{15}N ions (LET=181.4 keV/μm, E=13.1 MeV/n, 10° angle between the cell monolayer



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and ion beam). X-Y projections of 3D confocal microscopy images are shown. Cell fixation at 4 h post-irradiation. Blue color (right panel): cell nucleus (chromatin) labeled with DAPI. Magnification 100x.

For the biodosimetry of mass radiation events but also some research applications, an algorithm was therefore introduced that works with individual DSB markers independently. It means, the repair foci in two color channels are detected separately. With this procedure, the detection accuracy is slightly lower compared to the procedure based on the colocalization of two DSB markers; nevertheless, it is still very acceptable and enables the rapid detection of a large number of samples. It should be emphasized here that confocal microscopy of cells in 3D at the required resolution (magnification 60x to 100x) represents a relatively time-consuming (in the order of hours) procedure, so the necessity to scan only 2 color channels (cell nucleus and one DSB marker) instead of three channels (cell nucleus + 2 DSB markers) not only makes the analysis of repair foci significantly cheaper but also faster. In theory, ordinary (2D) immunofluorescence microscopy can also be used instead of confocal (3D) microscopy to fasten the process. However, in this case (not tested by our software), mutual overlap of repair foci in the 2D projection may result in an underestimation of irradiation [18], especially after the exposure to higher radiation doses. As the biodosimetry of mass radiation events uses lymphocytes collected from peripheral blood, which form well-defined repair foci and have a low background of γH2AX, the analysis based only on this single marker can be used with high accuracy. Biodosimetry based on γH2AX only is also possible due to the fact that these foci are formed at all DSBs, regardless of the mechanism of their repair. Moreover, DSBs start to appear in cells very soon (within minutes) after irradiation and persist there until the time when all DSBs had been repaired.

The repair focus detection algorithm based on the independent detection of individual DSB markers allows to compute colocalization between these markers (foci) during post-irradiation time, and thus study mutual interactions between different DSB repair proteins and/or DSB repair-associated epigenetic modifications of chromatin and their dynamics. This algorithm is thus necessary in research and applicable also in the situation where we study mutual interactions of repair proteins that create foci which do not mutually colocalize in 1:1 ratio. For example, the RAD51 protein is exclusively involved in the repair of DSBs by means of homologous recombination. Homologous recombination is used by cells to repair only a subfraction of DSB lesion and is limited to the S/G2 phase of the cell cycle. RAD51 foci can be thus detected only in some cells (when non-synchronized cell population is used) at some DSB sites, which makes RAD51 unsuitable for biodosimetry. On the other hand, in research, the mode of separate detection of individual DSB repair foci (here γH2AX and RAD51) allows to identify DSBs repaired by homologous recombination (HR) and non-homologous end-joining (NHEJ) and examine the formation, nuclear localization, and decay of NHEJ- and HR-associated repair foci. This information is necessary to understand the mechanisms of NHEJ and HR and how these processes are initiated at individual DSB lesions. It should be emphasized that DeepFoci has shown very good abilities to detect RAD51 foci, despite not being specifically trained for this repair protein. This underlines the universality (robustness) and high

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adaptability of the developed software, which can also be trained for the analysis of specific samples (cell type, repair protein, nature of microscopic images, etc.) if necessary.

Topological analysis of the distribution of repair foci in the cell nucleus also allows to distinguish low- and high-LET radiation exposure (**Fig. 2**). This option can be very useful especially in situations where exposure to mixed radiation fields can be expected, e.g., during a planned manned flight to Mars.

An important advantage of software analysis of repair foci, in addition to their fast automatic quantification, is the possibility of extracting a whole range of morphometric parameters characterizing these foci. Morphometric parameters of repair foci seem to be important not only for research on radiation DNA damage and repair [6]; we are currently investigating whether these parameters could also be used for biodosimetry to distinguish the exposure to ionizing radiation from the exposure to DSB-inducing chemical warfare agents (e.g., mustard gas).

3. CONCLUSIONS

DeepFoci is a newly developed robust and accurate software for the fully automatic detection and analysis of repair foci induced by ionizing radiation (IRIF) and possibly chemical warfare agents. The software is built on artificial neural networks and deep machine learning, offering high accuracy and adaptability. The problems with IRIF detection outlined in the previous paragraphs were circumvented by the introduction of a robust 3D segmentation technique based on the standard U-Net. The main modifications involve the application of erosion on segmentation masks and subsequent postprocessing of binary predictions, which lead to the correct separation of individual nuclei. DeepFoci also uses the same U-Net architecture for both nucleus segmentation and IRIF detection, which reduces its implementation complexity. In contrast to manual IRIF counting, the developed method is fast and automatic, and it provides the possibility to extract many other IRIF features in addition to the IRIF count. Compared to available automation approaches, DeepFoci is trainable and utilizes advanced deep-learning algorithms. This fundamental advantage leads to better results than those obtained by methods based on thresholding and maxima detection approaches, which approach the results achieved by experienced observers. The proposed method can be safely used for a wide range of radiation doses – at least in the interval of 0.5 to 8 Gy – without risk of detection accuracy saturation. Moreover, the proposed approach operates on 3D samples, which offers higher accuracy for biodosimetry and opportunity to follow DSB repair in space and time in research. The only other available 3D method is FocAn. However, it utilizes simple IRIF detection approaches and provides unsatisfactory results on our challenging datasets. DeepFoci thus allows to conduct complex research studies, effective medical triage (biodosimetry) in clinical radiotherapy or even in the events of mass-casualty radiological incidents. Significant advantage over manual analysis is the possibility to compare results between samples and laboratories.

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