Abschlussarbeit im Masterstudiengang Applied and Engineering Physics

Double-Strand Break Distributions along high-LET Particle Tracks in Human HeLa Cells

Doppelstrangbruch-Verteilungen entlang von hoch-LET Teilchenspuren in menschlichen HeLa Zellen

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Abstract

Ionizing radiation finds widespread application in cancer treatment because it induces DNA double-strand breaks (DSB), which are causal to the killing of tumor cells and are ultimately required for a patient’s recovery. Based on its clinical relevance, it is of great importance to study the influence of radiation quality on the number of induced DSB. In previous experiments, the number of observed damage sites in the cell for low-LET X-rays matched well with the number of DSB predicted by simulations. However, for high-LET ionizing radiation, a saturation in the number of damage sites was observed that is less than the predicted number of DSB. The cause of this saturation is attributed to the method of visualizing DSB. It is performed by imaging the distribution of proteins like 53BP1 and γH2AX, which are involved in DSB-signalling and form 1 µm-sized ionizing radiation induced foci (IRIF) at these sites. Due to a decreased spacing between consecutive DSB with increasing LET, single DSB can no longer be resolved within the IRIF. The proteins KU70/80 and DNA-PKcs might be a promising alternative to these conventional damage markers, since one copy each binds to the end of double-stranded DNA immediately after damage induction. Thus, for these proteins significantly smaller IRIF are expected, which might allow the visualization of single DSB.

The aim of this work was to count every DSB that is induced by high-LET ionizing radiation in human HeLa cells. For this, the IRIF-formation of DNA-PKcs and KU70/80 was tested and examined. Induction of DSB was achieved by irradiation with α-particles and small angle irradiation at the ion microprobe SNAKE with lithium and carbon ions. Visualization of the target proteins’ distribution in the cell was accomplished through the method of indirect secondary immunofluorescence staining and imaging was performed with the help of a super-resolution STED-microscope.

In the experiments, no IRIF-formation was detected for primary antibodies specifically targeting KU80 and DNA-PKcs after α-irradiation. The abundant presence of 400000 proteins of each type masked the signal of single proteins bound to double-stranded
DNA, indicating that the proteins are not suitable for the counting of single DSB in their indistinguishable collective natural state. DNA-PKcs bound to DSB reportedly undergo phosphorylation at Thr2609, which leads to the dissociation from the DSB. Although this way the expected strong localisation at sites of DSB is lost, it allows for the discrimination of DNA-PKcs proteins that are not involved in damage response. Based on these findings in the relevant literature, the experiments were carried out and IRIF-formation for a primary antibody specific to phosphorylated DNA-PKcs was tested positive after α-particle irradiation. Furthermore, particle tracks were visible after lithium and carbon ion irradiation for samples fixed 2, 3 and 5 minutes post-irradiation. Evaluation of 30 particle tracks for each time point yielded an average number of $2.5 \pm 0.4$ IRIF per micron after 2 minutes, which increased to $3.2 \pm 0.6$ IRIF per micron 5 minutes after irradiation for lithium ions with LET $= 116 \pm 10 \text{ keV} \mu\text{m}$. For carbon ions with LET $= 500 \pm 80 \text{ keV} \mu\text{m}$, the number of observed IRIF increased from $4.1 \pm 0.6$ per micron to $4.5 \pm 0.7$ per micron from 2 to 5 minutes after irradiation. The increase for both ion types can be attributed to a delayed accumulation of protein to a fraction of DSB, which become accessible by changes in the conformation of heterochromatin at later times. PARTRAC simulations predict $2.7 \pm 0.4$ DSB per micron for lithium and $10.2 \pm 2.2$ DSB per micron for carbon ions. The number of observed IRIF for lithium ions exceeded the number from linear scaling of low-LET X-rays and matched well with the predicted number from PARTRAC. However, the observed number of IRIF for carbon ions was only half the number of the predicted DSB by PARTRAC. Thus, it is concluded that the goal of counting single DSB for high-LET irradiation can only be partly fulfilled: up to LET $= 116 \pm 10 \text{ keV} \mu\text{m}$, the average spacing between DSB can be resolved by the IRIF-size with diameters from $188 \pm 36 \text{ nm}$ to $205 \pm 49 \text{ nm}$. However, at LET $= 500 \pm 80 \text{ keV} \mu\text{m}$, the decreased average spacing between consecutive DSB can no longer be resolved and is exceeded by the minimal observed IRIF-diameter of $178 \pm 40 \text{ nm}$. PARTRAC takes into account that DSB in close vicinity may not be resolvable and provides a reduced number of observable IRIF, which is derived by assigning all DSB within 150 nm to one observable cluster. This results in a predicted number of $3.3 \pm 0.3$ observable IRIF per micron for carbon ions, which is matched and partly exceeded by the actual observed number of IRIF per micron. This indicates that the experimental results of this thesis are compatible with PARTRAC simulations.
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Chapter 1

Introduction

Ionizing radiation is both a curse and a blessing for mankind. Day by day, every human being is exposed to ionizing radiation from artificial and natural sources without protection. Radon, the main contributor to radiation burden, is incorporated into the human organism by breathing air and consuming food and it constantly damages cells [1]. The most serious threat for a cell are damages to its genetic information, which is encoded in the DNA. Disruptions in the DNA, so called double-strand breaks (DSB), that are repaired erroneously result in cell death (apoptosis) or mutations. A small fraction of these mutations can eventually lead to the formation of cancer, so it can be stated that erroneously repaired DSB pose a lethal health risk for humans [2]. With increasing life expectancy, the risk of developing cancer even increases [3]. The beneficial nature of ionizing radiation for humans becomes clear when considering the fact that the most applied treatment of cancer employs ionizing radiation [4]. The large-scale induction of DNA DSB triggers the death of the cancer cells and it is supposed that it is the interaction of the large number of DSB and their resulting complexity, which determines the survival of the cell [5, 6]. Because the life saving feature of ionizing radiation takes place on the cellular level, it is of great interest and importance to study the damage induction of ionizing radiation to the DNA and its dependence on radiation quality. In the biomedical approach, which aims at assessing the influence of radiation quality, cells are irradiated in a Petri dish and a colony test is performed. This way, the surviving fraction of cells can be compared with regard to different radiation qualities [7]. However, this method does not provide information on the distribution or number of DSB on the cellular level. A method that allows for an assessment of the number of DSB that are induced by ionizing radiation is the pulsed-field gel electrophoresis assay. In this method, DNA fragments caused by
DSB are extracted from $10^3$ to $10^6$ cells, sorted by their size and counted \[8\]. The disadvantage of this method is that high doses in the order of ~100 Gy are required in order to obtain a high number of short DNA-fragments \[9, 10\]. Furthermore, the averaging of information over a large set of cells and the loss of spatial information on the distribution of DSB on the single cell level render this method useless for the present work.

A method that has proven suitable for the examination of the spatial DSB-distribution and the observation of the effects of ionizing radiation on the single cell level is high-resolution microscopy \[11\]. DSB can only be observed indirectly by visualizing the distributions of proteins involved in their repair. 53BP1 and $\gamma$H2AX are proteins that reliably indicate DSB by accumulation or being phosphorylated in the vicinity of DSB. These accumulation sites of proteins are termed \textit{ionizing radiation induced foci (IRIF)}. The linear energy transfer (LET) describes the amount of energy that is transferred from ionizing radiation to its surrounding. It is expected that the number of induced DSB scales with increasing LET. However, the number of IRIF that is derived from scaling values from low-LET X-rays has not match the number of observed IRIF for high-LET irradiation in previous research \[12\]. Fewer repair regions were observed than expected. This circumstance is attributed to the minimal size of these repair regions. For low-LET irradiation, the number of induced DSB is low and each IRIF co-localizes with a DSB. However, at high-LET irradiation, the spacing between consecutive DSB is reduced and cannot be resolved any more by the large size of the IRIF. Thus, the repair region indicated by one IRIF harbours more than one DSB. This situation is sketched in figure 1.1 for the low- and high-LET regime.

It is the goal of this thesis to further push the limits in the detection of DSB as well as the counting of every single DSB that is induced by ionizing radiation in the high-LET regime. Two promising proteins for fulfilling this aim are KU70/80 and DNA-PKcs. Both proteins are the first ones to be recruited to DSB after their induction, thus allowing insight into the initial damage distribution before the onset of repair. In contrast to 53BP1 and $\gamma$H2AX, they are directly recruited to the loose DNA-ends and only one protein each is bound at one double-stranded end. Thus, it is believed that the accumulation of KU70/80 and DNA-PKcs forms small IRIF that enable the counting of single DSB, as sketched in figure 1.1. Visual KU70/80 and DNA-PKcs accumulation has already been reported in the literature for high intensity lasers focussed to a spot. However, the number of DSB in the laser focus has been estimated
For low-LET irradiation, the number of observed repair-regions matches the predicted number of induced DSB. However, in the high-LET regime, less repair-regions are observed than the number of expected DSB. The KU70/80 and DNA-PKcs proteins are thought to form smaller IRIF than conventional damage-repair proteins like γH2AX and 53BP1 because of their direct localization at double stranded DNA-ends with only one protein attached at each end. Thus, they are deemed suitable for the goal of this work to resolve and count single DSB that are induced by high-LET radiation.

at \( \sim 3000 \) and protein accumulation was saturated, excluding the resolution of single DSB [13]. Another approach by researchers comprised the extraction of any unbound KU70/80 protein from the nucleus in order to artificially increase contrast at sites of DSB, which falsifies the protein distribution [14] and is thus not recommended for this thesis. For the examination of the IRIF-formation of KU70/80 and DNA-PKcs in this work, human HeLa cells were irradiated with α-particles in Neubiberg. Small angle cell irradiation with lithium and carbon ions was performed at the ion microprobe SNAKE at the Munich Tandem Accelerator and allowed mapping of ion trajectories in the x-y plane for microscopy. The irradiated cells were fixed and imaged by super-resolution STED-microscopy [15]. STED provides the resolution that is necessary for evaluating single ion trajectories and assessing the number of DSB that are induced. For this, the number of IRIF in one particle track was counted and normalized for its length. The results were tested for validation by comparing them to the predicted number of DSB created by these ions, derived from the Monte Carlo based simulation code PARTRAC. In chapter 2, the basics of ionizing radiation, its mechanism of DSB-induction and the subsequent repair process with the proteins involved will be elucidated. Furthermore, the diffraction limit and the theory of super-resolution STED-microscopy will be introduced. Chapter 3 will deal with the materials and methods employed in this project, starting with the irradiation geometries and further involving sample preparation, visualization through indirect immunofluorescence labelling and the STED-imaging setup.
The necessary optimization process of fluorescence dyes will be followed by the method for the evaluation of the number of IRIF and the derivation of DSB predictions from PARTRAC. The examination of the IRIF-formation of KU70/80 and DNA-PKcs and the results from small-angle irradiation will be presented and discussed in chapter 4. The thesis will close with an outlook for further improvements and alternatives in chapter 5.
Chapter 2

Fundamentals of DSB-induction through ionizing radiation and microscopy

This chapter will explain the physical background for the interaction of ionizing radiation with matter and elucidate the mechanism by which biological damage in the form of double-strand breaks is induced. This is followed by an introduction to the proteins that were examined in this thesis and the subsequent NHEJ repair process. The chapter will finish with the theoretical background on super-resolution STED-microscopy, which is employed for the visualization of double-strand breaks.

2.1 Interaction of ionizing radiation with matter

The following sections will engage with the physical stage of damage induction by ionizing radiation, the stopping processes of ions in matter and the relevant quantities describing this processes, namely linear energy transfer and dose.

2.1.1 Ionizing radiation

Ionizing radiation can be any particle or electromagnetic wave with sufficient energy to ionize a target atom by removing one or more electrons from the target atom’s attractive potential [16]. It is represented by $20 \pm 3\text{ MeV}$ lithium, $27 \pm 8\text{ MeV}$ carbon ions and $2.3 \pm 0.2\text{ MeV}$ $\alpha$-particles in this work and the ionization process is depicted in figure 2.1. For an amount of energy transferred from the ion to the target that
Figure 2.1: Representation of the excitation and ionization process in Helium. Black spheres denote electrons on their respective orbitals, blue and red the protons and neutrons in the nucleus. If the transferred energy of ionizing particles matches the energy gap between two atomic orbitals, the electron is excited from the ground state to the higher orbital. For transferred energy values exceeding the energy gap to the highest orbit, the electron can overcome the nucleus’ attractive potential and is removed from the atom, leaving it ionized. Any surplus of energy higher than the ionization energy is passed on to the emitted electron’s kinetic energy.

is smaller than the ionization energy, the target atom’s electron is excited from the ground state to a higher electronic state. If the transferred energy is sufficient to overcome the target atom’s attractive potential, the electron is removed and the atom is ionized. The ionization process implies a loss of kinetic energy and results in slowing down the moving ions, protons and electrons. Repeated collisions with the target atoms successively slow down the ion until its remaining kinetic energy is no longer sufficient to further ionize target atoms and it finally thermalizes [17]. Any surplus of transferred energy in the ionization process is converted to kinetic energy of the emitted electron. For high transferred kinetic energies, the emitted electron itself is able to induce further ionization events and is called a secondary or $\delta$-electron [18]. A single high-energetic particle produces a whole cascade of secondary electrons on its path through the target. The trajectories of ions form a nearly straight path through the target volume and consequently the energy they lose is deposited locally. Secondary electrons are scattered to higher angles because of their low mass [18], thus depositing their energy away from the track center formed by the passing ion.

2.1.2 Stopping processes of ionizing radiation in matter

The stopping of particles in matter is promoted by two distinct processes that dominate each in different regimes of particle energy. The first process is referred to as electronic
stopping and is due to inelastic collisions and Coulombic interaction of the moving particle with the bound electrons in the target volume. The result is the ionization of the target atoms and the slowing down of the ion [18]. This stopping mechanism is predominant for particle energies in the order of MeV.

The second factor that contributes to the stopping arises from elastic interaction of the moving particle with the nuclei of the target atoms and is referred to as nuclear stopping. The heavier the particle, the greater the power of nuclear stopping. Elastic interaction implies that no excitation or ionization takes place, only the particle’s trajectory changes [19]. Nuclear stopping peaks at energies of around ~ 1 keV per nucleon and is thus only predominant for low kinetic energies of the particle. The contributions of nuclear, electronic and total stopping power for an α-particle in water with kinetic energies up to 2.3 MeV are depicted in figure 2.2.

For a high velocity particle with a given starting kinetic energy, the contribution of
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Table 2.1: Comparison of nuclear and electronic stopping and particle range in water for α-particles, lithium and carbon ions with indicated kinetic energies. Data was calculated with SRIM [21].

Figure 2.3: Longitudinal range straggling (left) and radial distances to the initial trajectory (right) for 27 MeV carbon ions. Both datasets consist of 100000 individual ion trajectories that were binned for presentation [21].

nuclear stopping is only \( \sim 1\% \) compared to the electronic stopping. This is reflected in the characteristic data in table 2.1 for the stopping of α-particles, lithium and carbon ions in water. It is only at the end of the particle’s track, when its kinetic energy is very low, that nuclear stopping exceeds electronic stopping and the originally straight trajectory becomes curved [18]. Due to the statistical nature of the particle’s interaction with matter, its range is subjected to fluctuations. The range is distributed around an average value (listed in table 2.1), as can be seen for 27 MeV carbon ions in 2.3. The predominant influence of nuclear stopping towards the end of the particle track causes an angular deflection from the trajectory, resulting also in a radial distribution of the final ion position from the track center.
2.1.3 Linear energy transfer and dose

The energy lost by the particle during its stopping process that is locally transferred to the matter is measured by the linear energy transfer (LET). The LET is closely related to the stopping power. However, for particles causing secondary electrons with high range, a significant fraction of the particle’s lost energy is transported away from the track center and the locally deposited energy is smaller than the stopping power. For the ion energies used in this work, the range of secondary electrons is limited and all the energy lost is deposited locally within the cell. Thus, the LET is given by the average energy $\Delta E$ lost per unit path length $\Delta x$ by continuous electronic stopping processes of an ionizing particle and is measured in units of $\text{keV} \mu\text{m}$ \[7, 22\]:

$$\text{LET} = \frac{\Delta E}{\Delta x} \tag{2.1}$$

The LET is a function of the kinetic energy and can be approximated by the Bethe-Bloch Formula for non-relativistic charged particles,

$$-\frac{dE}{dx} \approx 4\pi Z_T \rho \frac{Z_P^2 e^4}{m_e v^2} \ln \left(\frac{2 m_e v^2}{I}\right) \tag{2.2}$$

where $\rho$ and $I$ denote the target material’s density and average ionization potential, while $Z_T$ and $A$ are the materials charge and atomic number respectively \[23\]. The particle’s attributes are represented in its electric charge $Z_P$ and velocity $v$, while $m_e$ and $e$ are the electron mass and charge. The formula has a lower limit of validity, since it requires a constant particle charge. At low kinetic energies the particle’s charge state changes because its velocity becomes comparable to those of atomic electrons, which are described by the Bohr velocity $v_b$ \[24\]. For ions with small charges this lower limit is around 0.5 MeV and with increasing charge scales with $v_b \propto Z_P^{2/3}$ \[22, 23\]. The energy loss scales quadratic with the particle’s charge and drops inversely to the square of the velocity for high kinetic energies, as it can be seen in figure 2.2. When the particle is slower, there is more interaction time with electrons and thus the number of ionizations and the LET is higher \[18\]. Transferring this dependence to the spatial energy deposition along the ion track, this implies that the maximum energy transfer peaks just before the ion stops. In this work the LET is considered as constant as the HeLa cell thickness is 5 to 15 $\mu\text{m}$ \[25\], depending on the irradiation geometry. Figure 2.4 illustrates exemplary for $27 \pm 8$ MeV carbon ions that the LET varies only little
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Figure 2.4: Dependence of the nuclear and electronic stopping on the traversed path length for a fixed starting kinetic energy. Depicted for the stopping of a $27 \pm 8 \text{MeV}$ carbon ion in water, based on the simulation of 100000 particle trajectories [21]. The corresponding particle energy at $0 \mu m$ is $35 \text{MeV}$. It is apparent, that the nuclear stopping is negligible for most of the stopping process and that the LET is properly described by the electronic stopping. Furthermore, on the length scale of the effective thickness of HeLa cells, which is $5 - 15 \mu m$ (depending on the irradiation geometry), the LET can be considered constant. The LET value is rather subjected to fluctuations, due to the large distribution of kinetic energies of $27 \pm 8 \text{MeV}$ for the carbon ions, which is caused by the irradiation geometry (cf. section 3.1.1.1 in chapter 3). This results in an average LET value of $500 \pm 80 \text{keV} \mu m^{-1}$ for the carbon ions.

on this length scale. It is rather the distribution of the ions' kinetic energy, which is caused by the irradiation geometry, that leads to a variation of the LET on the cellular level (cf. section 3.1.1.1 in chapter 3).

The energy lost by the particle, characterized by its LET, is not only deposited locally in the particle track but is also transported to its surroundings (the so-called penumbra) by secondary electrons. The physical quantity measuring the average amount of energy


\[ 
\Delta E \text{ deposited in a unit mass } \Delta m \text{ is the dose } D, \text{ defined as} 
\]

\[ 
D = \frac{\Delta E}{\Delta m} 
\]

(2.3)

in units of Gray, while 1 Gy = 1 J/kg \cite{19}. The absorbed dose depends both on the target material and the particle. It scales linear with the LET and the number of absorbed particles. The relation between the dose and LET for a given fluence \( \Phi \) [\( \mu m^{-2} \)] of particles of the same type with known density of the target material \( \rho \) can be calculated as:

\[ 
D = \frac{\Phi \cdot LET}{\rho}, 
\]

(2.4)

The fluence of 2.3 MeV particles from the \( \alpha \)-radiation source is \( \Phi = 4.7 \cdot 10^{-2} \mu m^{-2} \) for a radiation exposure time of 10 minutes. This corresponds to a dose in water of \( D = 1.2 \) Gy. The dose is a macroscopic quantity that describes the average absorbed energy on a larger scale. On the microscopic level of the single particle tracks that are examined in this thesis the dose is a function of space. The dose exhibits a radial decay perpendicular to the ion incidence that scales roughly with \( r^{-2} \). It is caused by scattered \( \delta \)-electrons that transport energy away from the track center. The range of these secondary electrons depends on the kinetic energy transferred by the incident particle and is in the order of nanometers to micrometers. The maximum distance to the ion trajectory can be estimated by

\[ 
\max = 0.0616 \mu m \left( \frac{MeV}{u} \right)^{-1.7} \cdot \left( \frac{E_A}{u} \right)^{1.7}, 
\]

(2.5)

where \( \frac{E_A}{u} \) denotes the kinetic Energy per nucleon of the ion \cite{26}. According to this equation, secondary electrons of 20 MeV \( \approx 2.9 \) MeV/u lithium ions have a maximum range of around 370 nm and 250 nm for 27 MeV \( \approx 2.3 \) MeV/u carbon ions. While different types of ions at the same LET lead to the same physical energy deposition, the difference in specific kinetic energy leads to a deviation of their radial dose distributions. This has to be kept in mind for the biological effects of ionizing radiation. For the heavy lithium and carbon ions used in this work, the difference in biological damage in comparison to other heavy ions at the same LET is negligible. However, the deviation in biological effect is pronounced more heavily if heavy ions are compared to light ions or \( \alpha \)-particles at the same LET \cite{27}.
Figure 2.5: Simple representation of the DNA double-strand. Neighbouring nucleotides are connected to each other by covalent bonds of the sugar and the phosphate group, forming the backbone. Each of the bases binds only to one kind of the other bases through hydrogen bonds, connecting the two single DNA strands and forming the double helix. One turn of the double helix harbours 10 to 10.5 base pairs.

2.2 DSB-induction and NHEJ repair-pathway

In this section, the biological background and the process of double-strand break induction by ionizing radiation will be explained. First, the structure and higher order organization of the DNA will be presented, followed by the process of double-strand induction through ionizing radiation and its dependence on radiation quality. The non-homologous end-joining repair-pathway will be elucidated with focus on the proteins examined in this work.

2.2.1 Structure and organization of the DNA

The structure of deoxyribonucleic acid (DNA) was first revealed by J. Watson and F. Crick in 1953 [28]. On the smallest scale, DNA consists of a sequence of nucleotides that encodes the genetic information. Each nucleotide is made up of the sugar deoxyribose, a phosphate group and one out of the four different bases cytosine, guanine, adenine and thymine. Consecutive nucleotides form a covalent bond between the sugar of one nucleotide and the phosphate group of the next nucleotide, resulting in a nucleotide chain. While the backbone of the chain is made up of repetitive building blocks, it is the unique sequence of the linked bases that encodes the genetic information. The four bases form two sets of compatible bases, called complementary base pairs. Within one base pair, the bases bind to each other through hydrogen bonds [29]. The compatible bases are cytosine with guanine and adenine with thymine. DNA consists of two
nucleotide chains, with complementary base sequence, that form a double-strand [30], as illustrated in figure 2.5.

The two single-strands revolve around each other in a distance of 2 nm and exhibit a double helical structure [31]. Successive base pairs are 0.34 nm apart and shifted by an angle of 36°, giving rise to the helical form of the DNA double-strand [28]. The DNA strand is folded to structures of higher orders in the nucleus of the cell, as depicted in figure 2.6. The DNA double helix is wrapped around proteins called histones in a cylindrical fashion and the resulting structure is termed nucleosom. These nucleosomes are the building blocks of another higher order organization of the DNA into chromatin fibres with a diameter of around 30 nm [32]. The chromatin fibres finally make up the 46 chromosomes in human cells, containing the entirety of the $6 \cdot 10^9$ bps that encode the genetic information. The total length of the chromatin fibres is $\sim 5.5$ cm. From this it is estimated that the volume of the nucleus occupied by DNA is only 5.5 % [25].

![Figure 2.6: 3-dimensional higher order organization of the DNA. 10 to 10.5 base pairs of the DNA double-strand equal one revolution of the double helix. The DNA double helix itself is wrapped around histones, forming nucleosomes. The nucleosomes are organized in 30 nm thick chromatin fibres. Adapted from [30].](image-url)
2.2.2 DSB-induction through ionizing radiation

Exposing cells to ionizing radiation can lead to programmed cell death (apoptosis), non-proliferating cells and necrosis, or mutations that turn into cancer in the long term [2]. It was discovered that the crucial target for ionizing radiation in cells is the DNA. While the effects of ionizing radiation can also render proteins dysfunctional and damage structures in the cell, it is breakages in the backbone of the DNA double-strand that determine the survival of the cell. Ionizing radiation induces single-strand breaks (SSB) and double-strand breaks (DSB) to DNA through ruptures in the sugar-phosphate backbone. These ruptures are caused by two distinguishable mechanisms. A direct damage to the backbone is induced, if the ion or secondary electron trajectory passes within a small distance to the backbone and directly ionizes it. The indirect damage arises from the ionization of the water molecules surrounding the DNA. These form OH\(^{-}\) radicals, which can diffuse and ionize the DNA strand by oxidation [7].

The two main lesions caused by backbone ruptures are single-strand breaks and double-strand breaks, as characterized in figure 2.7. A SSB is induced when only one backbone strand is affected by the rupture while the complementary strand stays intact. Even if the backbone ruptures multiply on one strand and base pairs are lost, SSB are easy to repair since the complementary base is still present and the information can be retrieved for repair. A DSB occurs when both strands are ruptured at the same base pair or within ~10 bp [33], leading to a total break of the DNA double-strand.

Depending on the LET, DSB are not necessarily produced blunt-ended, meaning that several bases might be detached from one strand, leaving the complementary strand single-stranded. If two DSB are produced in close vicinity, the small DNA fragment that is created might be omitted by the repair-process and the double-strand
is joined despite the fragment is missing, which leads to the creation of defective and altered DNA. Ionizing radiation creates a variety of combinations of SSB and DSB, with several levels of complexity that require different approaches in repair and preprocessing before ligation of the broken ends. The most lethal damage is posed by complex or clustered DSB, which are increasingly induced by high-LET particles, as these are particularly hard to repair [6]. DSB are categorized as complex, when two or more DSB are induced within 30 bp on the double helix [34]. The contribution from direct and indirect effects to the total number of SSB and DSB depends on the LET. For low-LET X-rays, the dominant effect for the creation of DSB is the indirect effect, which is responsible for around 65 percent of total DSB [27]. With increasing LET, however, the importance of the indirect effect decreases [35]. Additionally, the cell-cycle-dependent chromatin structure influences the contribution of these two individual effects: If the DNA is strongly compacted, a part of it is shielded from free radicals and the share of indirect effect is reduced compared to direct effects [7]. It is known from low-LET X-Rays that the combination of direct and indirect effects induces on average 1000 SSB [36] and 35 DSB per Gray and cell [37] over a wide range of doses. However, there is a discrepancy in the number of DSB produced in the high-LET regime compared to the number of DSB obtained by linearly scaling these low-LET X-rays values. Using linear scaling, $3.8 \pm 0.6$ DSB per micron would be anticipated for carbon ions (LET $= 500 \pm 80 \text{ keV} \text{µm}^{-1}$) and $1.0 \pm 0.1$ DSB per micron for lithium ions (LET $= 116 \pm \text{ keV} \text{µm}^{-1}$) [25]. However, it is already known that this linear scaling is not applicable for high-LET ionizing radiation, due to the influence of radiation quality on DSB-induction.

### 2.2.3 Influence of radiation quality on DSB-induction

With respect to its biological impact, ionizing radiation is divided into two regimes dependent on its LET: sparsely and densely ionizing, or low- and high-LET respectively. The transition is set to an LET-value of around $10 \text{ keV} \text{µm}^{-1}$ [38]. The difference is illustrated in figure 2.8 for two particles with the same specific kinetic energy but LET-values in the sparsely (upper track) and densely ionizing (lower track) region. A comparison of the ionization density of the tracks on the microscopic scale demonstrates that the gaps between single ionization events for low-LET radiation start to vanish and that a quasi-continuous ionization of the target along the particle trajectory
Figure 2.8: PARTRAC track simulation of a proton (upper track) and a oxygen ion (lower track) with the same kinetic energy of 6.25 MeV/u but different LET-values in the sparsely and densely ionizing regime respectively. DNA double-strands are overlayn to illustrate the difference of sparsely and densely ionizing radiation. The gaps visible between single ionization events in the proton track do not exist int the oxygen track, due to the high and quasi-continuous ionization density. For the DNA, this implies the most definite induction of one or even more DSB in the latter case, while for the sparsely ionizing proton there is a significant probability that the DNA gets spared from damage. Additional to the track center, the particle’s energy is deposited in the so-called penumbra by $\delta$-electrons. Adapted from [39].

is achieved in the high-LET regime [25]. A significant fraction of the particle energy is deposited in the penumbra around the track core by $\delta$-electrons. The impact of the categorization into sparsely and densely ionizing radiation on the DNA becomes clear when visually overlaying a DNA double-strand to the track of a densely ionizing particle. The ionization density for a sparsely ionizing particle is low and when crossing path with the DNA, only one or even no DSB are induced. By contrast, for high-LET particles the ionization density is in the order of nanometers or smaller and leads to the creation of most probably several DSB upon interaction with DNA. This categorization allows an explanation of the mismatch between the number of DSB derived by linear scaling and the actual number of DSB. Since the number of DSB as well as the number of SSB scales with LET, there is an increased probability that two SSB can be found within 10 bp on the double-strand and create an additional DSB. This gives rise to a quadratic component of DSB-induction, which exceeds the number of DSB anticipated from linear scaling in the high-LET regime [22]. Figure 2.9 compares the
2.2 DSB-induction and NHEJ repair-pathway

Figure 2.9: Comparison of DSB-yields (DSB/µm) obtained from linear scaling (left column) and simulated by PARTRAC (column in center). Column on the right represents data from the middle, in which DSB occurring within a distance of 150 nm are counted as one DSB-cluster. This accounts for the inability to resolve single DSB when the spacing between consecutive DSB is too small. Data was provided mainly for α-particles and oxygen ions for a variety of kinetic energies. Black lines represent pure simulations, while red indicates data from experiments made on this issue. Blue and green areas correspond to the range of the number of DSB per micron for lithium and carbon ions that were interpolated from the existing data. Adapted from [39].

The data is mainly limited to α-particles and oxygen ions for a variety of kinetic energies. The arrows and corresponding numbers spanning from the left column to the...
Table 2.2: Number of DSB per micron for simple linear scaling from low-LET X-rays and from simulations of PARTRAC. The simulated data takes into account the enhanced effectiveness of DSB-induction for high-LET radiation. By counting all DSB occurring within 150 nm as one DSB-cluster in the PARTRAC simulation, the number of observable IRIF in microscopy can be estimated.

<table>
<thead>
<tr>
<th>Ion</th>
<th>LET [keV/µm]</th>
<th>Linear scaling</th>
<th>PARTRAC</th>
<th>PARTRAC cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium</td>
<td>116 ± 10</td>
<td>1.0 ± 0.1</td>
<td>2.7 ± 0.4</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Carbon</td>
<td>500 ± 80</td>
<td>3.8 ± 0.6</td>
<td>10.2 ± 2.2</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

middle indicate the enhancement factor in DSB-induction compared to linear scaling. Black lines indicate pure simulations, while red lines represent experimental data. The green and blue areas correspond to the respective range of numbers for carbon (LET = 500 ± 80 keV/µm) and lithium ions (LET = 116 ± 10 keV/µm). Since these values for carbon and lithium ions were not covered in the original data, they were acquired by interpolation in this work, as described in chapter 3, section 3.5. This interpolation suggests 2.7 ± 0.4 DSB per micron for lithium and 10.2 ± 2.2 DSB per micron for carbon ions. It is expected that these values are too high and the DSB-spacing too dense to be able to resolve single DSB by microscopy. For an estimation on how many IRIF might be observable, DSB from PARTRAC simulations occurring within 150 nm from each other are counted as one DSB-cluster. This leads to reduced values compared to the total number of DSB per micron and the numbers of predicted DSB-cluster per micron are presented in the column to the right in figure 2.9. The numbers on the lines, which connect the predicted number of DSB per micron with the corresponding number of DSB-clusters, indicate the ratio of the two values. The number of DSB-cluster per micron given by PARTRAC is 1.6 ± 0.1 for lithium and 3.3 ± 0.3 for carbon ions. Table 2.2 summarizes the values from linear scaling and the PARTRAC simulations for the total number of DSB and DSB-cluster for both ion types.

### 2.2.4 NHEJ repair-pathway and proteins

Immediately after the induction of DSB in the cell, a versatile biological machinery starts to respond: DSB repair. Depending on the cell cycle phase, there are two distinct repair-pathways available for the repair of DSB. Non-homologous end-joining (NHEJ) is available in all cell-cycle phases [40], while homologous recombination (HR) is only available after the duplication of the DNA and before mitosis. HR relies on the presence
of the sister chromatid for the repair of the broken DSB. Since missing segments in the damaged double-strand of DNA are still available in the sister chromatid, repair without errors and deletions is possible. NHEJ is more prone to errors, because no information about the original DNA sequence is available and DSB-ends might be joined, while short strands of DNA are missing [32]. KU70/80 and DNA-PKcs, the proteins examined for IRIF formation in this work, are involved in the NHEJ repair-pathway.

2.2.4.1 Non-homologous end-joining

The following description of the NHEJ pathway will focus on the early repair stage, which involves KU70/80 and DNA-PKcs, and is sketched in figure 2.10. After the induction of a DSB through ionizing radiation, KU70/80 binds to double-stranded DNA-ends and subsequently recruits DNA-PKcs. Both the binding of KU70/80 and recruitment of DNA-PKcs occurs within seconds of damage induction [13]. Upon the binding of DNA-PKcs at the DNA-termini, KU70/80 is translocated inwards on the double-stranded DNA-end [41]. The presence of double-stranded DNA-ends is a prerequisite for the interaction of KU70/80 with DNA-PKcs [40]. The complex consisting of KU70/80, DNA-PKcs and DNA, is referred to as DNA-PK. This complex blocks the joining of the DSB (ligation) until single-stranded overhang at the DNA-termini is removed through nucleases and blunt ends are created, which are required for the ligation of the DSB in NHEJ [42]. When two broken DNA-ends with DNA-PK complex are in close proximity, a synaptic complex of these DNA-PK is formed, which leads to the bridging and stabilization of the DSB [41]. This concentration is necessary for a successful ligation through proteins that are recruited by KU70/80 and DNA-PKcs [42, 43]. Once ligation is completed, the repair-complex disassociates and KU70/80 and DNA-PKcs dislocate from the repaired DSB. Note that the single-stranded overhangs, which are visible in figure 2.10 before binding of KU70/80, are removed by end processing and are missing in the final repaired DNA-segment, leading to a loss of genetic information.

2.2.4.2 DSB damage marker and repair proteins

The DSB that are induced by ionizing radiation cannot be observed directly, but have to be visualized indirectly through appropriate staining of proteins that are involved
Figure 2.10: Repair-pathway of non-homologous end-joining. KU70/80 binds to the DSB and recruits DNA-PKcs to form a complex called DNA-PK. Two DNA-PK can form a synaptic complex if double-stranded DNA-ends are in close proximity and this way stabilize the DSB by holding the broken ends together. This synaptic complex eases the end processing and ligation steps required for successful DSB repair.
in their repair. After the introduction of the established DSB markers $\gamma$H2AX and 53BP1, the early repair factors KU70/80 and DNA-PKcs, which were examined in this project, and their interplay will be presented in more detail.

### 2.2.4.2.1 $\gamma$H2AX

In section 2.2.1 of this chapter, histones were introduced as cylindrical proteins, which are involved in the higher order organization of the DNA. Since the DNA double helix is wound around histones, their spatial distribution is closely related. Histones consist of several subunits, one of them is the histone H2A. In a fraction of histones, H2A is replaced by its variant H2AX. Upon the induction of a DSB through ionizing radiation, H2AX is phosphorylated at Ser139 and is named $\gamma$H2AX in this state [44]. Several protein kinases are known to phosphorylate H2AX, among which there are DNA-PKcs [45], ATM [46] and ATR [47]. The phosphorylation of H2AX takes place in a surrounding of several megabasepairs of the DSB. This leads to 1 $\mu$m-sized observable IRIF when visualizing $\gamma$H2AX through indirect fluorescence labelling [48]. For this reason, $\gamma$H2AX is used for the recognition of DSB sites in the cell nucleus.

### 2.2.4.2.2 53BP1

Another damage marker for DSB is 53BP1 (p53 binding protein 1). In contrast to $\gamma$H2AX, it does not co-localize with DNA in its natural state. Instead, it participates in the signal transduction and mediation of DSB repair and prevents the onset of homologous recombination [49, 50]. 53BP1 is thought to be hyperphosphorylated by ATM after the formation of DSB in the cell [51] and accumulates in large number of up to $\sim$ 1000 molecules in the vicinity of DSB [52]. 53BP1 was found to co-localize and bind to $\gamma$H2AX at DSB [53, 54], which makes 53BP1 a reliable DSB marker. However, 53BP1 exhibits a partial non-co-localisation with $\gamma$H2AX in its fine structure [Reindl et al, to be published].

### 2.2.4.2.3 KU70/80

In contrast to DNA damage markers like $\gamma$H2AX and 53BP1, which are phosphorylated in a large region around a DSB or recruited in large numbers respectively, KU70/80 is thought to allow a better localization of DSB. This assumption is based
on the findings that out of the total number of 400000 [55] to 500000 [56] proteins in the nucleus only one or two KU70/80 are bound at each side of a DSB [14, 41]. Another advantage of this protein might be its role in the early detection of DSB and its fast accumulation within seconds [57, 58], which gives insight into unprocessed DSB. KU70/80 is a heterodimer, consisting of the subunits KU70 and KU80 with an approximate molecular weight in kilodaltons given by the numbers in their names. The protein exhibits a circular shape with an attached flexible arm, formed by the C-Terminal-Region of KU80 [59, 60]. The circular domain of the heterodimer acts as DNA-binding channel and it possesses a high affinity for double-stranded DNA-ends [42], which is the result of direct interaction with the phosphate groups of the DNA backbone. This allows binding to the DSB independent of the DNA base sequence [41]. Despite the high affinity for DSB, KU70/80 is not permanently bound to the DNA but in dynamic exchange with free and unbound KU70/80 [58]. The flexible arm is responsible for the interaction and enhanced recruitment of DNA-PKcs to the damage site and is further thought to be required for DNA-PKcs kinase activation [13, 59, 61]. The presence of KU70/80 at sites of DSB is essential for the accumulation of ligases such as XRCC4 [58].

2.2.4.2.4 DNA-PKcs

Because of its KU70/80 dependent recruitment to DSB, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) is also thought of to closely localize with DSB. The polypeptide of over 4000 amino acids [60] with a molecular weight of ~ 470 kD [61] plays a crucial role in DSB repair, since cell lines lacking its expression show increased radiosensitivity and decreased DSB rejoining ability [62–64]. Like KU70/80, it is expressed in large numbers of ~ 400000 proteins per cell [59] and is also recruited to DSB within seconds [13]. The longitudinal extension of the protein is reported to ~ 14 nm with a thickness of 7 to 10 nm [60, 65]. The protein’s N-terminal section is formed in a pincer-shaped structure, which acts as the double-stranded DNA-binding channel and promotes its interaction with the flexible arm of KU80 [42, 59]. A crown-structure, which represents the C-terminal-region of DNA-PKcs and harbours the domain responsible for kinase activity, sits on top of this binding channel. This domain is not accessible in the unbound state of DNA-PKcs. It is known that after its binding to DNA, a conformational change is induced that exposes catalytic groups and ATP binding pockets in the crown-structure, and consequently initialises
DNA-PKcs kinase activity \cite{42, 59, 66}. However, it is not clear yet if the activation can take place at one single end of the DSB, or if the two ends of the DSB have to be in close proximity and whether a synaptic complex of two DNA-PK is required \cite{42}. The kinase activity targets a number of different substrates \cite{58, 61}, but it has been shown that the phosphorylation of these substrates can be performed by other proteins than DNA-PKcs. Impairing of its kinase activity, however, results in the loss of DSB repair ability, showing that DNA-PKcs plays a major and complex role in the NHEJ pathway \cite{60}. The best-known substrate for phosphorylation is DNA-PKcs itself. While 40 phosphorylation sites are known, the most prominent one is a cluster of sites at threonine 2609, which is located in the vicinity of the crown-structure \cite{42}. Upon its phosphorylation, the molecule undergoes a large conformational change, which increases the width of its DNA-binding channel and promotes its disassociation from KU70/80 and the DNA \cite{59}, while additionally deactivating its kinase activity \cite{67}. Cell lines in which this cluster was substituted and thus could not be phosphorylated, showed a prolonged dwelling time at DSB and blocking of repair, indicating that the release of DNA-PKcs from DSB is crucial for successful end processing \cite{13, 64}. The Thr2609 phosphorylation cluster is not only targeted by the protein itself, but also by ATM and ATR \cite{42}. Additionally to its dislocation from DSB, the phosphorylation at Thr2609 is important for the activation and recruitment of DNA-end processing enzymes like artemis \cite{68}. It has been shown that phosphatases are able to reverse the phosphorylation status of DNA-PKcs \cite{67}.

\subsection*{2.2.4.2.5 Interplay of KU70/80 and DNA-PKcs at DSB}

It is expected that the phosphorylation status of DNA-PKcs plays a major role in the progression of DSB repair. After the recognition and binding to the DSB by Ku70/80, it recruits unphosphorylated DNA-PKcs and forms the DNA-PK complex. In this unphosphorylated state, DNA-PKcs is in constant association and dissociation equilibrium at the double-stranded DNA with surrounding DNA-PKcs, even though at a slow rate \cite{13}. Thus, for most of the time the DNA-termini are blocked by the DNA-PK complex and ligation and processing of the DSB is inhibited. It is only when the DNA-ends are in close proximity, that a synaptic complex is formed by two DNA-PK at adjacent sides of the DSB \cite{58}. The dimerisation of two DNA-PK complexes at different sides of the DSB has been observed \cite{59, 61} and it is thought to function in concentrating and stabilizing the two loose DNA-ends for ligation. The
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Figure 2.11: Synaptic complex of two DNA-PK molecules at a DSB. Each DNA-PK complex consists of one KU70/80 heterodimer (red), one end of the ruptured DNA (yellow) and one DNA-PKcs (remaining colours). The C-terminal region of KU80 allows interaction and positioning of the DNA-PKcs molecule on the adjacent side of the DSB, juxtaposing the loose DNA-ends. In this geometry, trans-autophosphorylation and dissociation from the DSB of DNA-PKcs is promoted. From [61].

constellation of the synaptic DNA-PK complex and its bridging property is illustrated in figure 2.11. It has been shown that the flexible arm of KU80 is long enough and is able to interact with DNA-PKcs bound at the opposite side of the DSB, allowing to juxtapose the DNA-ends [59, 60]. However, as long as DNA-PKcs is bound to the ends of the DSB, the access for consecutive repair proteins is blocked [58]. At some point in time before this arrangement, the kinase activity of DNA-PKcs is established [42]. The close proximity enables trans-autophosphorylation of the two DNA-PKcs molecules at Thr2609 [13, 59, 60], which results in their dissociation from the DSB and a loss of kinase activity [60, 64]. As noted earlier, phosphorylation can also be induced through repair factors as ATM and ATR. It is proposed by [13] that the conformational change in the phosphorylated form results in a change of the exchange dynamics of bound and unbound DNA-PKcs at DSB-ends: While the time between the unbinding and binding of a new DNA-Pkcs is unaffected by phosphorylation, the dwelling time at the DSB is reduced, thus effectively reducing competition of other repair factors and allowing them to process the DSB.
2.3 Super-resolution fluorescence microscopy

The biological response, which includes the accumulation of repair protein after induction of DSB through ionizing radiation, is not observable directly, but has to be stained with fluorescence dyes for visualization. Fluorescence microscopy allows the imaging of cellular structures, which are not visible in conventional microscopy, due to low contrast. Confocal microscopy expands fluorescence microscopy to the third dimension, providing the means for the acquisition of three dimensional stacks in z-direction. However, both techniques underlie the diffraction limit for visible light. A technique that overcomes this limit and substantially increases resolution is STED. The Nobel Prize in Chemistry 2014, which was awarded to its inventor Stefan Hell [15], underlines the significance of STED for high-resolution microscopy.

2.3.1 Limits of optical resolution

Before introducing super-resolution microscopy, it is essential to understand the limits in resolution that are implied by the diffraction limit of light. The imaging of a small point-like light source through an optical system leads to an alteration of the signal in the image plane. The function that describes the transformation from the object to the image plane is called point-spread-function (PSF). From the imaging process depicted in figure 2.12 it can be seen that the PSF for a point, which is imaged by a round aperture, is described by an Airy function with radial declining maxima and minima in intensity [69].

The broadness of this distribution is caused by the diffraction of light at the round aperture and limits the optical resolution. Each feature in the object plane gets distorted by this PSF and the resulting image is a sum of the single PSF. With decreasing distance of neighbouring features, the sum of intensity no longer allows to distinguish these features. The resolution of an imaging system is defined as the minimal distance of distinct features in the object plane, that can still be distinguished in the image plane. Abbe discovered that for the resolution of small objects the major and the first minor maxima of the intensity distribution are of importance and thus limit the resolution. Furthermore, the resolution depends on the wavelength of the light as well as the numerical aperture NA, which defines the maximum amount of light than can
Figure 2.12: First row: The imaging process from the object to the image plane is described through the point-spread-function. It describes the response of the imaging system to a point-like light-source. For diffraction at a round aperture, the PSF is described through an Airy function that creates declining maxima and minima of intensity (coloured) in the image plane. These minor maxima result in diffraction rings around the object in the image plane (corresponding top-view on the right). Second row: 2D intensity distribution for two point-like light-sources (red and blue) in close proximity and top view for the observed image on the right. The final image is the sum (black line) of the two PSF applied to every point in the object plane. With decreasing distance, the influence of diffraction no longer allows the separation of the two points by the sum of their intensity. The distance chosen between the two points represents a special case, upon which the resolution criterion of Rayleigh is based. According to Rayleigh, two points are resolvable when the intensity maximum of the first point coincides with the first intensity minimum of the second point. For any distance shorter than this, the points can no longer be resolved.
be collected by the imaging system and leads to the following equation [70]:

\[ d = \frac{\lambda}{NA} \]  

(2.6)

According to Abbe, features smaller than \( d \) can no longer be resolved through systems limited by diffraction. For the fluorescence dye Abberior Star 440SXP, which was used in this work, the maximum emission wavelength is 511 nm and imaging was performed with an oil objective with \( NA = 1.4 \). Thus, the maximal achievable resolution for this dye is 365 nm under consideration of the Abbe criterion. Abbe utilizes the distance from the intensity maximum to the first minor maximum for his definition of the resolution. There are also approaches that consider the sum of the intensity of two single points in order to define the resolution. The resolution criterion of Rayleigh is based on the assumption that two points are resolvable if the intensity maximum of the first point coincides with the first intensity minimum of the second point, as illustrated in figure 2.12. Points that are closer than this distance can no longer be resolved. This criterion modifies the resolution equation of Abbe with a factor of 0.61 and thus implicates a higher resolution. Consequently, the maximum diffraction-limited resolution for Abberior Star 440SXP is 223 nm. For the resolution of smaller structures, the diffraction limit needs to be circumvented.

### 2.3.2 Fluorescence microscopy

Fluorescence microscopy allows high contrast visualization of structures that are not visible in conventional microscopy due to the low contrast. The high contrast in fluorescence microscopy is achieved by specific labelling of desired structures. Structures that are not labelled do not fluoresce and hence a high contrast is provided.

The principle of fluorescence microscopy is based on the excitation of fluorescence molecules by light. Upon the absorption of a photon, the fluorescence molecule is excited from the electronic ground state \( S_0 \) to a higher energetic state \( S_{\geq 0} \) (figure 2.13 a)). For photon energies exceeding the energy difference of the fluorescence molecules electronic ground and excited state, additional rotational and vibrational states are excited. Thus, fluorescence dyes exhibit a continuous spectrum, rather than one absorption line. The molecule relaxes radiationless from these additional excited states into the main excited state in the order of picoseconds. The consecutive relaxation to the ground state through the emission of light occurs on the timescale of nanoseconds.
Figure 2.13: a) Principle of fluorescence. A fluorescence molecule in the ground state can be excited to a higher electronic state by a photon. Vibrational and rotational states allow for a continuum of photons to be absorbed, indicated by the blue arrow heads. Relaxation to the corresponding excited electronic state occurs in the order of picoseconds. The molecule relaxes to the ground state by the emission of fluorescence light with higher wavelength in the order of nanoseconds. Occupation of vibrational and rotational states of the ground state creates an emission continuum. b) Absorption and emission spectra for the fluorescence dye Abberior Star 635P. The Stokes-shift is apparent in different positions of the maxima of the spectra. Excitation is ideally performed at the maximum absorption cross-section of the fluorescence dye, which is 635 nm for the Abberior Star 635P. Maximum in emission occurs at 651 nm.
and is known as fluorescence [71]. For the relaxation to the ground state $S_0$, a variety of vibrational and rotational energy levels are available, creating a continuous emission spectrum. A representative absorption (dark blue, 635 nm) and emission spectrum (light blue, maximum 651 nm) is shown for the fluorescence dye Abberior Star635P in figure 2.13 b).

The relaxation of the vibrational and rotational states causes an increase of the photon wavelength from absorbed to emitted photon, called Stokes-shift. This circumstance is exploited in fluorescence microscopes, as it allows the separation of excitation light and fluorescence light by spectral filtering [72]. For a bright fluorescence signal, a high fluorescence quantum yield is desired for the dye. The fluorescence quantum yield is the ratio of emitted to absorbed photons by a dye and its value is $Q_E < 1$ [73]. The quantum yield of Abberior Star 635P is $Q_E = 0.92$ and it mirrors in bright signals in images. The architecture of a conventional fluorescence microscope (FM) is sketched in figure 2.14 a). In the simplest implementation of a fluorescence microscope, the light is provided by a lamp with continuous spectrum, and the excitation wavelength is chosen by an excitation filter. The excitation filter exhibits selective transmission for a narrow wavelength band desired for excitation, and absorbs the rest of the spectrum. The brightest fluorescence signal is achieved by excitation at the maximum absorption cross-section of the dye. The excitation light is reflected by a dichroic mirror and guided to the focal plane through a condensor. The fluorescence signal from the specimen and the reflected excitation light are collected by the objective and separated by the dichroic mirror, since the dichroic mirror is transmissive for the longer wavelengths of the fluorescence light and reflective for the excitation wavelength. An emission filter further purifies the transmitted beam and the pure fluorescence light can then be observed through an ocular or camera [74].

### 2.3.3 Confocal microscopy

Confocal microscopy expands fluorescence microscopy to the third dimension and enhances contrast. Instead of a regular light source, confocal microscopy employs a laser for fluorophore excitation. In contrast to a conventional fluorescence microscope, where the whole sample is illuminated, the laser is focused to a tight spot, thus reducing the illuminated volume on the sample (cf. figure 2.14 b)). This reduces the signal contributions from out-of-focus planes that would blur the image. Image acquisition is
Figure 2.14: a) Setup of a conventional fluorescence microscope. The desired excitation wavelength for the fluorophore is transmitted by the excitation filter, while it blocks the remaining continuum from the light source. The excitation light is focused on the sample plane and induces fluorescence in the specimen. Fluorescence and reflected excitation light are collected by the objective. While the excitation light is discriminated by the dichroic mirror and the emission filter, the fluorescence light is transmitted and imaged by a camera. b) Difference in the illumination of the sample of a conventional fluorescence microscope (FM) and a confocal microscope. The whole sample is illuminated and imaged at the same time in the case of the FM. In the confocal microscope, a laser is focused to a small spot, reducing image blurring from out-of-focus planes. The image is acquired one pixel at a time, by rastering the laser spot in the x-y plane and detecting the fluorescence signal for each pixel.
performed step by step, by scanning the focused laser spot in the x-y image plane and detecting the fluorescence signal for each image coordinate. A photomultiplier is mandatory for confocal microscopy, since the image is acquired step by step and cannot be viewed in real time. One crucial additional component in the beam path of a confocal microscope allows the acquisition of three dimensional image stacks: An aperture pinhole is located between the emission filter and the photomultiplier. Fluorescence light from the focal plane passes through the small opening in the aperture pinhole and is measured by the detector. By contrast, the signal from planes below and above the focal plane is mapped to the periphery from the aperture pinhole and blocked so it does not reach the detector. This discrimination of the out-of-focus signal enables imaging of three dimensional stacks. After the regular two dimensional image is acquired, the focal depth is changed and image acquisition is repeated [75]. Confocal microscopy significantly increases the axial resolution for thick samples compared to conventional FM by discrimination of the out-of-focus signal. For thin samples, the out-of-focus signal is sparsely present and thus conventional FM is at no disadvantage. By illuminating a small volume rather than the whole sample, a higher contrast is achieved. While confocal microscopy also increases the lateral resolution, it is still subjected to the diffraction limit.

### 2.3.4 Principle of STED-microscopy

A technique that overcomes the diffraction limit and substantially increases the resolution is STED (Stimulated Emission Depletion). Stimulated Emission is the forced relaxation of excited fluorescence molecules to the ground state. This process is depicted in figure 2.15 a).

In the diagram it can be seen that the fluorescence molecule is excited by a photon (blue). Excited rotational and vibrational states relax to the first excited state (red). From there they can regularly fluoresce to the ground state through the emission of a photon with higher wavelength (green). If the excited fluorescence molecule is illuminated with a higher STED-wavelength before the regular fluorescence occurs, it is forced to stimulated emission (purple). The molecule relaxes to the ground state and emits a photon with the same wavelength as the incident STED-photon. A STED microscope is based on a confocal microscope and induces stimulated emission through a depletion laser (STED-Laser), that is added to the beam path (figure 2.15 b). In
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Figure 2.15:  
a) Principle of stimulated emission. The excited fluorophore can relax to the ground state through regular fluorescence. When the dye is illuminated by a photon of longer wavelength (STED-wavelength), before fluorescence has occurred, it is forced to relax by the emission of a photon of the same wavelength. 

b) For the implementation of stimulated emission into a confocal microscope, a second depletion laser is added to the beam path that is overlain to the excitation laser in the image plane. 

c) Intensity profiles of the excitation and STED-laser in the image plane. The Gaussian beam of the excitation laser is superimposed by the doughnut-shaped STED-beam. The result is an effective fluorescence spot (green) that is not limited by diffraction.

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order to make use of stimulated emission, the Gaussian shaped excitation beam is overlain by the STED-beam in the image plane (figure 2.15 c)). Both the excitation beam and the STED-beam are limited by diffraction. The STED-beam exhibits a doughnut-shaped intensity profile with a minimum in the middle [76]. The excited fluorophores are relaxed to the ground state by the STED-beam. It is only in the middle of the fluorescence spot, where the depletion intensity has a minimum, that the majority of fluorescence molecules is unaffected by stimulated emission. In this central spot, fluorescence only occurs through spontaneous emission [15]. Since the wavelength of the stimulated emission is linked to the STED-laser wavelength, it can be filtered and separated from the spontaneous emission. What remains is a fluorescence spot that is not limited by diffraction. The turning-off of the fluorescence in the periphery of the illumination spot leads to an increase in lateral resolution:

\[
d = \frac{\lambda}{2 \ NA \sqrt{1 + \frac{I_{\text{STED}}}{I_{\text{Sat.}}}}} \tag{2.7}
\]

with the Numerical Aperture NA of the objective, wavelength \(\lambda\) of the STED-laser and the the maximum of the STED-intensity \(I_{\text{STED}}\) [77]. \(I_{\text{Sat.}}\) denotes the saturation intensity and is defined as the intensity at which the probability for fluorescence emission is reduced to 50 percent [78]. By increasing the STED-intensity, the central spot is narrowed down and the resolution is increased. However, the STED-intensity cannot be increased arbitrarily in biological systems. First, heat induction can lead to damage or destruction of the biological sample. Second and limiting, high laser intensities induce reactive oxygen species (ROS) in the sample, which react with the fluorescence dyes [79]. The dyes undergo an irreversible structural change and lose their ability to fluoresce. This phenomenon is referred to as photobleaching [80]. For the setup used in this thesis, a resolution of \(\sim 100 \ \text{nm}\) is achievable [81]. In addition to the STED-laser intensity, there are other factors that have to be considered for the combination of STED-wavelength and fluorescence dyes. The STED-wavelength has to be sufficiently apart from the excitation wavelength and the fluorescence dye in use should exhibit a low absorption cross-section for the STED-wavelength in order not to get excited further. It is even more important that the emission spectrum of the dye exhibits an overlap with the STED-wavelength. Otherwise, no depletion by stimulated emission is possible. These requirements are exemplarily illustrated for the fluorescence dye Abberior Star 635P in figure 2.16. The dye exhibits no absorption at the STED-wavelength.
Figure 2.16: Emission spectrum (light blue) with detection range (grey area), excitation (dark blue) and STED-wavelength (orange) for Abberior Star 635P. The STED-wavelength should lie outside the absorption spectrum of the dye, but has to overlap with the emission spectrum to allow for stimulated emission.

of 775 nm, but sufficient emission to allow for stimulated emission. It additionally makes a wide detection range possible. These properties make this dye perfect for STED-microscopy.
Chapter 3

Material and Methods

In the first part of this chapter, the cell irradiation geometries, the methods of sample fixation and antibody-labelling will be explained. In the second part, the STED-microscopy setup will be presented, followed by the methods for the processing of acquired images: Spectral dye separation and deconvolution are required to retrieve the original image of the fluorescence distribution within the cell. This will be completed by a summary the optimization process of the fluorescence dyes employed in this work. The chapter ends with the extraction of the number of DSB for high-LET ionizing radiation from PARTRAC data and an explanation of the experimental evaluation of the number of IRIF per micron.

3.1 Cell irradiation

In the present experiments, the irradiation for the induction of DSB in cells was performed with high-LET lithium and carbon ions at the ion microprobe SNAKE at the 14 MeV Tandem Accelerator in Garching and with α-particles in Neubiberg.

3.1.1 Small angle irradiation at the ion microprobe SNAKE

The ion microprobe SNAKE (supraconducting Nanoscope for Applied nuclear (german: Kern) physics Experiments) provides a setup for small angle irradiation of cell samples. The ion beams are generated by the 14 MeV Tandem Accelerator of the Maier-Leibnitz-Laboratorium, which is capable of accelerating heavy ions to high velocities, with LET-values ranging from a few keV per micron (protons) to thousands of keV per micron (gold ions) [12]. In the course of this work, cell irradiation was
performed with 33 MeV lithium and 55 MeV carbon ions.

3.1.1.1 Experimental setup

The layout of the SNAKE setup is sketched in figure 3.1. Single negatively charged ions from an ion source are accelerated by the positive terminal voltage in the Tandem accelerator [82]. Upon passage of the carbon stripper foil in the middle, electrons are stripped from the ions [83]. The positive ions are then accelerated a second time by the terminal voltage. A 90° deflection magnet is used to filter the incoming ions by their kinetic energy and to select the ions with the desired energy, as they leave the magnet at 90°. Ions that are faster or slower possess a higher or lower radius of curvature respectively, causing them to leave the magnet at other angles than 90° and being absorbed.

The micro slit object of the ion microprobe SNAKE is positioned at the focal plane of
the magnet and reduces the beam diameter. Another set of micro slits further crops the beam and limits the divergence, thus increasing the brilliance [84, 85]. The experiment slits are opened for the irradiation to create a ~ 4 mm × 4 mm field at the beam exit nozzle. Up to the 7.5 µm thick exit window made from Kapton, the whole beam line is evacuated and this way minimizes energetic losses. A microscope carries the small angle sample-mounting and a particle detector, which is located in the microscope’s objective barrel and is used to measure particle rates.

The irradiation geometry employed in the experiments is based on the work of J. Seel [86] and is sketched in the magnification in figure 3.1. The sample mounting allows inclined positioning of the cover slip in an angle of 9° to the particle incidence in front of the beam exit window (left, dotted line). The advantage of this setup is a redistribution of the deposited energy and induced DSB in the x-y plane, which offers a better resolution for microscopy than in z-direction [75]. Because of the thickness of the cover slip, ions are stopped and particle count rates cannot be determined during irradiation, but have to be measured after the sample is removed from the beam line. The average dose induced at the cell layer can be estimated for each sample from the count rates measured after the sample irradiation. Starting from the transition of the vacuum to the Kapton exit window (7.5 µm), the ions’ kinetic energy is further reduced by 2 to 3 cm of air between the exit window and the upper and lower boundaries of the cover slip, and an amount of 5 to 10 µm cell culture medium, which is still present on the cell layer. The cell culture medium layer transforms to an effective thickness of 33 to 65 µm for the ions due to the inclined plane. Based on these values, the energy of 55 MeV carbon ions is reduced to 27 ± 8 MeV (LET = 500 ± 80 keV µm) for this irradiation setup. The 33 MeV lithium ions are slowed down to 20 ± 3 MeV with a corresponding LET of 116 ± 10 keV µm [21]. For the calculation of the irradiation duration, the fluence rate is measured once before the experiment. Starting from this rate, the irradiation duration necessary to produce a predetermined number of particle hits is calculated. The irradiation duration is the same for all samples, and by measuring the fluence rate after the irradiation of each sample, the actual particle fluence for the sample can be estimated.

For the carbon ion irradiation in this project, the beam size at the exit window was 3.9 mm × 3.9 mm and a fluence rate of 110 kHz was measured before the experiment. A total number of 500000 ions was set as goal, implicating an irradiation duration of 5 seconds for each sample. The average fluence rate for 18 samples after irradiation was
104 ± 14 kHz. Taking into account the uncertainty of the LET, this leads to an average applied dose of 2.8 ± 0.8 Gy for the carbon irradiation.

A total of 480000 particle hits were targeted for the irradiation with lithium ions and the fluence rate before the experiment was measured as ~ 105 kHz, corresponding to an irradiation duration of 5 seconds. The average fluence rate after sample irradiation was determined as 108 ± 8 kHz. Consequently, an average dose of 0.7 ± 0.1 Gy was deposited per sample. The observed variation of the dose is not of great interest, since the goal of this work was the examination of single ion tracks. The irradiated area on the cover slip, where particle tracks can be observed, depends on the beam size and transforms to ~ 4 mm × 22 mm.

3.1.1.2 Cell irradiation procedure

200000 human HeLa cells were seeded on a coverslip with a thickness of 170.00 ± 0.05 µm and area of 22 mm × 22 mm for each sample and incubated in cell culture medium at 37°C the day before the experiment. During this time, the cells were able to adhere to the coverslip. Before the irradiation, the cells were taken out of the warm cell culture medium and the remaining fluid was soaked off carefully with a paper towel in order to reduce the slowing down of the ions. After the irradiation, the cells were put back in the 37°C cell culture medium until the fixation process.

3.1.2 α-particle source

The α-particle irradiation source is a compact device delivering α-particles in the high-LET-regime, allowing the examination of IRIF-formation of the proteins under investigation. Irradiation incidence is perpendicular to the coverslip. The device was designed by Ross and Kellerer [87] and will be described in detail in the next section. The fluence of the source was determined in this work and enables an estimation of the deposited dose.

3.1.2.1 Setup and cell irradiation

The α-particles are produced by the decay of americium 241 with an activity of 0.37 GBq. 85 % of the α-particles possess an energy of 5.49 MeV. The americium is shaped in a thin disc with a rotational axis and is properly shielded by the steel case of the device, schematically shown in figure 3.2. The rotation of the americium source
### 3.1 Cell irradiation

**Figure 3.2:** $\alpha$-irradiation device delivering high-LET particles, used for tests on antibodies. The collimator system restricts the angles of the particles from the americium source that arrive in the sample chamber. The americium source is turned to ensure a homogeneous irradiation and, additionally, the collimator system is set to circular motion without rotation. The shutter disc allows defined turning on and off of irradiation in the sample chamber. The space between the 2.5 $\mu$m thin Mylar exit window and the source is filled with helium to reduce energetic losses. The coverslip with the adhered cells is placed upside-down on a rubber band on the exit window. Setup according to [87] with further adjustments.

At 5 Hz counters inhomogeneities during irradiation. A collimator system, consisting of hexagonally arranged adjacent cylinders, is placed above the Americium source. The collimator reduces the angles of the particles accepted for irradiation at the sample stage to 12° to the normal and consequently limits the energy range. Two wobble axes set the collimator system to a circular motion without rotation to ensure a uniform fluence. A computer-controlled shutter disc between the collimator and the exit window allows for defined irradiation of the samples by switching the source on and off in a controlled manner [87]. The exit window is made of a 2.5 $\mu$m thick Mylar foil that is thin enough not to stop the particles. The space between the source and the exit window is filled with helium at normal pressure to reduce energy losses of the particles to 20% compared to those in air [87]. Cell irradiation takes place in the subsequent sample chamber. The cover slip with the adhered cells is placed upside-down on a bar spacer with a distance of 3 mm to the exit window. Due to self-absorption in the
Helium atmosphere, the energy of the α-particles is reduced to 4 MeV before they pass the exit window [87]. The energy is further reduced upon the passage of the Mylar foil, traversing 3 mm of air and 5 to 10 µm of cell culture medium. The particle energy at the cell layer is 2.3 ± 0.2 MeV, with a LET-value of 152 ± 8 keV/µm [21].

While the compact α-device allows for convenient irradiation in the high-LET regime, the particle flux is low. Consequently, an irradiation time of 10 minutes is required for a significant number of particle hits that allow for the identification of irradiated cells. The sample chamber was cooled down to 4 to 8°C by placing cooling elements on the outside, in order to slow down cellular processes, transport and diffusion during the long time of irradiation. Samples were incubated in medium with HEPES for about 1 minute before irradiation, in order to maintain the pH in the absence of CO₂ during irradiation. The medium was also cooled to assist suppression of cellular activity. Small bowls filled with water were placed in the sample chamber to establish a saturated H₂O atmosphere that kept the cells from drying out during irradiation. After 10 minutes of irradiation, the samples were put back in 37°C cell culture medium to restore normal cellular activity and they were stored in the incubator until the fixation process.

### 3.1.2.2 Determination of the fluence of the α-source

To estimate the dose induced at the cell layer and the number of particle hits per cell nucleus, it is necessary to determine the particle fluence of the α-source. The determination of the fluence was achieved by the irradiation of 200 µm thick plates of allyl diglycol carbonate (CR39) in the same geometric setup as the cell irradiation. CR-39 is a transparent polymer, whose chemical bonds break along the trajectory of a passing ionizing particle. Subsequent chemical etching ablates the ionized target molecules from the solid and results in the growth of cones on the surface [88]. These cones become visible in a light microscope by refraction and can be counted to determine the fluence.

While the concentration and temperature were kept constant throughout the experiments, a time series was done and an etching time of 30 minutes was found to be a good compromise between visibility of single hits and clustering of the cones of neighbouring hits. Etching at the time of 30 minutes was then performed for samples representing different radiation exposure times in the α-source. Irradiation times were 1, 3, 5, 7 and 10 minutes, while one reference sample was not irradiated at all (0 minutes). Sodiumhydroxid (NaOH) was diluted in distilled water to 6.3 molar and etching was performed in
3.1 Cell irradiation

Figure 3.3: Etched CR-39 plates that were irradiated for 1 minute (left), 3 minutes (middle) and 7 minutes (right). The second column shows clustered irradiated spots from the sample that was irradiated for 7 minutes (left), along with corresponding binary masks (right), used for object recognition and counting. These clusters are more reliably counted by eye.

a pre-heated waterbath with a constant measured temperature of 79 ± 1°C. The CR-39 plates were thoroughly rinsed with distilled water after the etching process. The Image acquisition of the plates was accomplished with an inverted light microscope (Axio Observer Z1, Zeiss GmBH) and a 40× objective (Plan Apochromat 40x/0.9 Korr Ph3 M27).

The non-irradiated reference sample showed no effect to etching and no cones were visible, while for the samples with long irradiation exposure strong clustering of single spots was characteristic. The samples irradiated 1, 3 and 7 minutes were selected for further evaluation and determination of the fluence and are presented in figure 3.3, along with typical clusters at long irradiation times.

Especially for the sample irradiated for 7 minutes, strong clustering is present (figure 3.3, second column). Because of the high number of particle hits per image, counting was automated. For this, a Python-based script was written that recognized and counted objects in the images, sorted the objects by size and then saved images of the recognized objects together with a mask. This allowed visual checking of the counted
result in about 5 minutes for one sample. Sorting by object size allowed a quick detection of objects with clustered hits by scrolling through the single pictures. Between 12 and 15 images were taken from each sample along the contour of a rectangle of ~ 8 mm × 2 mm. The fluence is derived by dividing the number of particle hits by the area given by the image dimensions of 223.3 µm × 167.3 µm. The fluence obtained through the counting of the particles is presented in figure 3.1.2.2.

Linear least-square fitting of the fluence at different times yields the flux J as the slope of the line: \( J = 4.68 \pm 0.03 \cdot 10^{-3} \text{min}^{-1} \mu\text{m}^{-2} \). Taking into account the uncertainty of the LET, the applied dose after 10 minutes of irradiation is 1.14 ± 0.07 Gy. The average maximum cross-section of the HeLa nucleus is \( \sim 140 \mu\text{m}^2 \) [25], which on average results in about 6.5 α-particle hits per nucleus. In order to check if a homogeneous fluence is achieved within one sample, the single values acquired from different positions on the sample were tested for a normal distribution. The statistical nature of radiation causes a fluctuation in the number of particles for a fixed area around an average value. The variation is described by a Poisson distribution that transforms to

Figure 3.4: Fluence vs. irradiation time, error bars representing standard deviation. A linear fit (red line) is used to determine the particle flux, which is given by the slope of the line.
Figure 3.5: Simulation of the irradiation of HeLa cells with α-particles for 10 minutes, based on the fluence determined from CR-39 plates. pDNA-PKcs (green) signal approximates cytoplasm plus nucleus and and 53BP1 (red) signal represents the nucleus. Random irradiation pattern is pictured as white dots. The fluctuations of the fluence on the microscopic level and varying cell cross-sections lead to a broad distribution of the number of particle hits per cell.

a Gaussian distribution for expectation values higher than 20 [89]. The average number of particle hits was 182 for one minute of irradiation and the variance in fluence should consequently be characterized by a Gaussian distribution. Testing for normal distribution confirmed that the variance of fluence for each sample was covered within the variance of a Gaussian normal distribution. Furthermore, spatial plotting of the fluence vs. image acquisition position did not show a significant increase or decrease in a particular direction. Thus, homogeneous irradiation and fluence on the large scale can be assumed for samples positioned above the middle of the exit window. However, on the microscopic level of the cell nucleus area, the fluence is subjected to major fluctuations. Furthermore, the varying cross-sectional area of HeLa cells gives rise to a broader distribution of the number of particle hits per cell, as depicted in figure 3.5.
3.2 Sample preparation and fluorescence labelling

Since proteins in cells are not visible per se, the method of fluorescence labelling through antibodies is necessary. Sample preparation was performed according to an established protocol and will be described according to [81, 90]. The incubation time after the irradiation was varied as a parameter in the experiments to allow visualization of different phases of DSB repair. Following the incubation, the samples were fixed with 2%-Paraformaldehyd (PFA) for 15 minutes and washed with Phosphate-buffered Saline (PBS). Consecutive application of 0.15%-Triton reduced the surface tension and eased penetration of the cell membrane for following reactants. Bovine Serum Albumin (BSA) and Glycine were diluted in PBS to form PBS+. Treatment with PBS+ blocked off any unspecific binding of the antibodies in the cell.

The distribution of the protein of interest in the cells was visualized through the method of indirect immunofluorescence staining through antibodies (cf. 3.6). A primary antibody possesses one specific binding site for the protein of interest and
3.2 Sample preparation and fluorescence labelling

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clonality</th>
<th>Manufacturer</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>γH2AX</td>
<td>mouse</td>
<td>Merck Millipore</td>
<td>05-636</td>
<td>1:350</td>
</tr>
<tr>
<td>53BP1</td>
<td>rabbit</td>
<td>Novus Biologicals</td>
<td>NB100-305</td>
<td>1:350</td>
</tr>
<tr>
<td>Ku80</td>
<td>rabbit</td>
<td>Cell Signal</td>
<td>2180P</td>
<td>1:400</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>rabbit</td>
<td>Cell Signal</td>
<td>4602P</td>
<td>1:400</td>
</tr>
<tr>
<td>pDNA-PKcs T2609</td>
<td>mouse</td>
<td>Abcam</td>
<td>ab18356</td>
<td>1:250</td>
</tr>
</tbody>
</table>

Table 3.1: List of primary antibodies with manufacturer information and dilution ratio.

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Clonality</th>
<th>Manufacturer</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromeo 505</td>
<td>mouse</td>
<td>Active Motif</td>
<td>15050</td>
<td>1:400</td>
</tr>
<tr>
<td>Chromeo 505</td>
<td>rabbit</td>
<td>Active Motif</td>
<td>15060</td>
<td>1:400</td>
</tr>
<tr>
<td>Abberior Star 440SXP</td>
<td>mouse</td>
<td>Abberior</td>
<td>20002-003-7</td>
<td>1:50</td>
</tr>
<tr>
<td>Abberior Star 440SXP</td>
<td>rabbit</td>
<td>Abberior</td>
<td>20012-003-4</td>
<td>1:50</td>
</tr>
<tr>
<td>Abberior Star 635P</td>
<td>mouse</td>
<td>Abberior</td>
<td>20002-007-5</td>
<td>1:250</td>
</tr>
<tr>
<td>Abberior Star 635P</td>
<td>rabbit</td>
<td>Abberior</td>
<td>20012-007-2</td>
<td>1:250</td>
</tr>
<tr>
<td>Oregon Green 488</td>
<td>mouse</td>
<td>Thermo Fischer</td>
<td>011033</td>
<td>1:250</td>
</tr>
<tr>
<td>Oregon Green 488</td>
<td>rabbit</td>
<td>Thermo Fischer</td>
<td>011033</td>
<td>1:250</td>
</tr>
</tbody>
</table>

Table 3.2: List of secondary antibodies (fluorophores) with manufacturer information and dilution ratio.

several universal binding sites for a matching secondary antibody. Fluorophores are coupled to the secondary antibody and thus allow indirect visualization of the protein distribution by fluorescence microscopy. Since one primary antibody is capable of binding several secondary antibodies, an amplification in signal is achieved. In order to label two different proteins in one sample, two matching sets of primary and secondary antibodies with different binding motifs are required. The antibodies for the targeted proteins in this work were derived from goats and contained either binding motifs for secondary antibodies derived from rabbits or mice. The primary and secondary antibodies utilized in this project are summarized in table 3.1 and 3.2 respectively.

The samples were covered with 95 µl of primary antibody, which was diluted in PBS+, and incubated over night at 4°C. Before the incubation of the secondary antibody, samples needed to be washed with PBS to remove remaining unbound primary antibodies from the cells. The application of Triton, PBS and PBS+ was repeated before the samples were covered in 95 µl of secondary antibodies diluted in PBS+. The incubation was performed for two hours at room temperature. The samples were first washed with PBS and then Triton was applied. Then the samples were washed with
PBS 5 times. This procedure ensured that unbound secondary antibodies were removed from the cells and only signal from bound antibodies is imaged in microscopy. Finally, remaining fluid was aspirated from the coverslip and the coverslip was joined with the object slide, using Prolong Gold as embedding medium. Prolong Gold was let dry light-protected for one day at room temperature and then the samples were stored at 4°C, ready for microscopy.

### 3.3 STED-microscopy setup and imaging

In this section, the STED-microscopy setup is introduced and the necessary processing steps of the raw-data images are explained.

#### 3.3.1 STED-microscopy setup

STED-microscopy was performed with a Leica TCS SP8 STED 3X. The microscope achieves dye excitation by a white light laser with a power in the order of 1 mW. The laser generates a spectral continuum from 470 nm to 670 nm and selection of wavelength is controlled by an acousto-optical tunable filter [91]. Two STED-lasers with wavelengths of 592 nm and 775 nm are available; they allow depletion of two spectrally separated dyes. The applicable power of the STED-lasers depends on the fluorescence dye stability and is in the range of 50 to 100 mW [81]. The microscope is capable of also increasing the resolution in axial direction by the application of a z-doughnut intensity profile. Since the intensity for z-improvement is coupled out of the regular x-y-STED-beam, a decrease in lateral resolution at a fixed intensity is the consequence. For the imaging of high-LET particle tracks, the improvement in z-direction was turned off, since it seemed that the fluorescence dyes were further excited by the STED-beams and contrast was lost in this geometry. In order to circumvent this observed effect, it would have been necessary to reduce the total STED-laser power to a small fraction of the laser power applicable when only imaging with x-y improvement. This would have resulted in almost no gain in total resolution and for this reason, imaging of particle tracks was performed with the whole laser power focussed on x-y-resolution improvement.

For each dye, the excitation laser power was adjusted to generate a high range of intensity in the image, while not saturating. A balance has to be found for the STED-intensity: for high-resolution imaging, a high laser intensity and power is desirable.
This, however, leads to fast photobleaching when taking several images in a z-stack. The STED-laser intensity was set to a value that kept dye bleaching minimal throughout one image stack. The emitted fluorescence light is collected by a 100x oil objective (Leica HCX PL APO 100x/1.4 Oil) and detected by a set of Hybrid Photo Detectors (HyD), which combine the technology of a regular photomultiplier tube with an avalanche photodiode. A two-step acceleration of generated photoelectrons with sharp pulses allows single-photon-counting [92]. The range of the wavelength detection can be adjusted in a range from ~350 nm to ~800 nm. The HyD allows temporal gating of the fluorescence detection to further increase the resolution: during the timecourse of the illumination of the STED-beam, the region in which spontaneous fluorescence occurs shrinks. By setting the HyD to a temporal offset in the order of nanoseconds during stimulated emission, fluorescence light from a smaller area is recorded [93]. This increase in resolution, however, competes with a decrease in the intensity of the fluorescence spot. A dye-dependant trade-off of resolution and intensity needs to be found. When the optimal parameters for excitation, depletion and detection were set, imaging could be performed. The sampling for the three-dimensional stacks was set to 40 nm × 40 nm in x-y, with a z-spacing of 160 nm.

### 3.3.2 Sample mounting

The samples were taken out of the refrigerator before imaging and given time to adapt to room temperature. The object slides were cleaned carefully, before they were covered in immersion oil (n = 1.52) and mounted to the microscope stage. A climate chamber set to 23°C is built around the microscope stage in order to minimize temperature-dependent shift during image acquisition. Irradiated cells were searched through the ocular while fluorescence excitation was performed with a mercury lamp. After moving the microscope stage, delaying imaging for a few minutes helped to stabilize the system and to reduce the drift.[81]

### 3.3.3 Processing of raw-data images

The raw data acquired from the microscope had to be further processed to enhance resolution and to remove noise. Spectral Dye Separation is necessary when more than one fluorescent dye is present in the sample with overlapping spectra, which is the case for the experiments conducted in this work. Deconvolution of the image data was used
to retrieve the original fluorescence distribution which is blurred in the acquired raw image by the point spread function of the imaging system.

### 3.3.3.1 Spectral dye separation

Since for the validation of IRIF-formation of DNA-PKcs and KU70/80 an established damage marker protein is necessary, the presence of two distinct fluorescence dyes in the cell sample was required. Spectral dye separation is used for dyes with overlapping spectra to remove signal components that leak from one dye channel to the other (crosstalk) [94]. Crosstalk is induced both for the excitation of dyes and fluorescence detection, as demonstrated for the fluorescence pair Chromeo 505 and Abberior Star 440SXP in figure 3.7.

While two STED-lasers were available, only one STED-wavelength could be used for this dye-combination. There is no overlap of the emission spectra of the two dyes with the 775 nm STED-laser. Both can only be depleted by the 592 nm STED-laser. The spectral proximity of Abberior Star 440SXP and Chromeo 505 leads to significant crosstalk. The excitation of Abberior Star 440SXP could not be performed at its maximum absorption cross section but at 470 nm, which is the lower limit of excitation through the white light laser. Chromeo 505 exhibits a non-zero absorption cross section at this wavelength, which leads to a wrong excitation of 52% (ratio of green and blue line at 470 nm excitation). Vice versa, excitation of the Chromeo 505 also excites the Abberior Star 440SXP. By not exciting Chromeo 505 at its absorption maximum but at 514 nm, the cross-excitation can be reduced to 7% for the Chromeo 505 channel. Additional cross-talk arises from the fluorescence detection. Because of the large overlap of the two emission spectra, detection in the respective channels inevitably leads to cross-detection of the wrong dye. Limiting the detection range for the Abberior Star 440SXP would lead to an extinction of the crosstalk in detection from Chromeo 505, but would significantly reduce Abberior Star 440SXP signal and is thus not a reasonable option. The total crosstalk from excitation and detection for this dye configuration is 5% for the Abberior Star 440SXP channel and 7% for Chromeo 505.

A dye combination that stands out for low crosstalk is Oregon Green 488 and Abberior Star 635P. Spectra are provided in the second row of figure 3.7. The absorption spectra are well separated. Thus, both dyes can be excited at their maximum absorption cross section, which is 501 nm for Oregon Green 488 and 635 nm for Abberior Star 635P. At the excitation wavelength of Oregon Green 488, Abberior Star 635P exhibits a local
3.3 STED-microscopy setup and imaging

Figure 3.7: Absorption (left) and emission spectra (right) for the fluorescence pair Chromeo 505 and Abberior Star 440SXP (first row) and the fluorescence pair Oregon Green 488 and Abberior Star 635P (second row). Excitation of Abberior Star 440SXP at 470 nm and Chromeo 505 at 514 nm leads to wrong excitation of the non-targeted dye respectively. The percentage of wrong excitation is given by the ratio of lines of the same colour. Due to the huge overlap of the emission spectra, cross-detection cannot be avoided. Detection ranges are represented by the grey areas. Both dyes are confined to the same depletion laser of 592 nm. For Oregon Green 488 and Abberior Star 635P, excitation maxima are well separated and a low cross-excitation is achieved. Emission spectra exhibit only a small overlap and thus detection crosstalk is minimal. Detection ranges are presented by coloured areas. The employed depletion wavelengths were 592 nm for Oregon Green 488 and 775 nm for Abberior Star 635P.
minimum plateau, minimizing cross-excitation. The other way round, the absorption of Oregon Green 488 at the excitation wavelength of 635 nm is zero and thus no cross-excitation is expected. The emission spectra are also well separated with maxima of emission at 526 nm and 654 nm. This required the use of both STED-lasers. Oregon Green was depleted at 592 nm and Abberior Star 635P at 775 nm. The small overlap in emission allowed for a wide detection range for both dyes: 640 nm to 795 nm for Abberior Star 635P and 505 nm to 560 nm for Oregon Green 488. Since emission data for Abberior Star 635 is not available below 600 nm, the upper detection limit for Oregon Green 488 was set to 560 nm. By doing this, the probability of cross-detection was reduced, while still obtaining a high fluorescence intensity.

Spectral dye separation can get rid of this unwanted signal contribution from crosstalk, which comprises the original distribution of a dye pair. A $2 \times 2$ matrix is employed in this process and it subtracts the relative contributions for each pixel in the two channels. For the determination of the Matrix values, two samples were created that only contained one dye each. The samples got imaged in both channels with the same settings as for regular samples. In the ideal case, imaging in the channel of the not-labelled dye would lead to no intensity. The average intensity in an IRIF region was calculated for both channels. These two values for each of the two single stained samples make up the components of the matrix. The effect of the spectral dye separation is clarified in figure 3.3.3.1 for $\gamma$H2AX (Chromeo 505, red) and KU80 (Abberior Star 440SXP, green). In the raw-data images, it seems that KU80 accumulates within $\gamma$H2AX IRIF (cf. magnified boxes). After the spectral dye separation, it is apparent that this assumption is based on signal leakage from Chromeo 505 to Abberior Star 440SXP.

### 3.3.3.2 Deconvolution

Throughout the imaging process, the three-dimensional point-spread-functions of the imaging components lead to deviation from the imaged dye distribution to the original distribution in the sample. The dye distribution is folded with a total point-spread-function (PSF) of the imaging system, which degrades the resulting image. Confocal microscopy already reduces the contribution of out-of-focus planes in the imaging plane through an aperture pinhole. However, the acquired images are blurred and contain noise due to photon statistics [95]. Deconvolution aims at redistributing the imaged intensities to their original coordinates and removing noise, thus increasing the image
Figure 3.8: Spectral dye separation based on a decomposition matrix for γH2AX (Chromeo 505, red) and KU80 (Abberior Star 440SXP, green) fixed 15 minutes after α-particle irradiation. Signal contributions from crosstalk is removed throughout the dye separation process. While in the raw data KU80 seems to co-localize with γH2AX IRIF (see magnified yellow boxes), the intensity in the KU80 channel after the dye separation is reduced and is similar to the intensity outside of the γH2AX IRIF.
resolution and signal to noise ratio [96]. For the redistribution of the intensity, a 3D z-stack is required. Computational Deconvolution is performed with the software solution "Huygens Professional" (Scientific Volume Imaging, The Netherlands) [97]. The software estimates a total PSF based on the imaging parameters. Parameters for noise and signal to noise in the image can be adjusted manually to achieve artefact-free deconvolution. Based on this PSF, a maximum likelihood algorithm in Fourier-Space calculates the best fitting signal distribution that lead to the acquired input image. For both the lateral and axial resolution, a ~ 2-fold increase can be achieved [98]. Figure 3.9 illustrates the impact of deconvolution on a spectrally dye-separated image of DNA-PKcs after carbon irradiation, stained with Oregon Green 488. The overall noise is significantly removed and small structures in the carbon track are higher resolved.
3.4 Optimization of secondary antibodies

Since on average only one KU70/80 binds on each site of a DSB [14, 41] and KU70/80 recruits DNA-PKcs [13], it is supposed that the intensity of IRIF formed by those proteins is only slightly higher compared to the rest of the nucleus. Thus, it is crucial to employ a dye combination with minimal spectral leakage in order to not distort the protein distribution and to not falsely classify crosstalk as a KU70/80 or DNA-PKcs focus. Staining of only KU70/80 or DNA-PKcs would indeed eliminate crosstalk, but is not a valid option since for the classification of an IRIF, co-localization with a damage marker such as 53BP1 or $\gamma$H2AX is necessary. The fluorescence pair first employed for the visualization of KU70/80 and DNA-PKcs repair regions was the Chromeo 505 and Abberior Star 440SXP. Images of samples with either KU80 or DNA-PKcs and co-stained with $\gamma$H2AX damage marker exhibited strong crosstalk, as explained and discussed in section 3.3.3.1. Alleged IRIF in raw STED-images turned out to be induced by crosstalk after spectral dye separation, as demonstrated for KU80 in figure 3.3.3.1. A better signal purity can be achieved by employing a fluorescence pair with well-separated absorption and emission spectra. A suitable combination that matches these requirements is given by Oregon Green 488 and Abberior Star 635P. The overall low crosstalk is mirrored in two-channel STED-images from samples with only one fluorescence dye labelled in figure 3.10.

The first row represents a sample that was stained with goat anti-rabbit Abberior Star 635P only. The sample in the second row was only labelled with goat anti-rabbit Oregon Green 488. The primary antibody for both samples was goat anti-rabbit 53BP1 and fixation took place 10 minutes after irradiation with $\alpha$-particles. The left and right column represent the imaging channels with the same excitation and detection settings as for regular samples with two fluorescence dyes. Note that the image intensity of the unlabelled channels was significantly increased for visualization. While a maximum of 40 photons was counted for the brightest IRIF in the Abberior Star 635P labelled sample, the detected maximal number of photons in the corresponding Oregon Green channel (unlabelled) was 4. The outlines of the nucleus are visible in the unlabelled channel and a slight increase of leakage can be observed in IRIF-areas. Similar results were obtained for the sample only labelled with Oregon Green 488: a maximum of 35 photons was detected in the labelled channel, while in the unlabelled sample the number did not exceed 3. However, the average intensity of crosstalk is much lower for
**Figure 3.10:** Demonstration of total crosstalk for two samples, each stained with goat anti-rabbit 53BP1 primary antibody. Fixation was performed 10 minutes after irradiation with α-particles. While the sample in the first row was exclusively fluorescence labelled with Abberior Star 635P, the sample in the second row was stained with Oregon Green 488 only. Detection of fluorescence was acquired for both channels (left and right column). The intensity range of unlabelled samples was increased significantly for visualization. Overall, low crosstalk was achieved.
leakage from Oregon Green 488 to Abberior Star 635P than vice-versa. While the crosstalk for this fluorescence combination is smaller, it has one minor disadvantage compared to Chromeo 505 and Abberior Star 440SXP: For the latter, acquisition of z-stacks could be performed by imaging one plane in both dye-channels before moving to the next z-plane. Thus, shifts between the two stacks that are caused by changing the focal plane are minimized. However, for Abberior Star 635P and Oregon Green 488, first the complete stack for Abberior Star 635 and then the stack for Oregon Green 488 had to be recorded. This was necessary because subjection of Abberior Star 635P to the excitation- and STED-wavelength of Oregon Green 488 caused rapid photobleaching. While for exact pixel-wise co-localisation studies of both channels this might be a disadvantage, it is negligible for the scope of this work since the IRIF-size of 53BP1 and $\gamma$-H2AX is in the order of 1 $\mu$m [81]. Because of its low crosstalk and high fluorescence quantum yield, the dye combination of Oregon Green 488 and Abberior Star 635P was preferably chosen for the detection of low-intensity IRIF.

3.5 Determination of DSB-yield from PARTRAC data

The goal of this work, which was to experimentally count the total number of DSB induced through high-LET, is closely related to simulations of DSB-yield of PARTRAC (PARTicle TRACk) [27]. The Monte-Carlo-based simulation software of the Helmholtz Zentrum in Munich performs the prediction of DSB in two steps: simulation of particle tracks and consequent overlay to a three-dimensional model of the cell that contains the higher order spatial organization of the DNA. Particle tracks can be simulated with full damage cascades (ionization, excitation events etc.) for a variety of particles with kinetic energies ranging several orders of magnitude. In a physico-chemical stage, the intersection of these damage cascades with DNA is used to determine the number of DSB. Multiple repetition of the simulation yields the average number of DSB per unit path length. The simulated DSB-yields reflect the enhanced DSB-induction for high-LET irradiation and are higher than obtained from simple linear scaling. Since available data is limited for certain ion types with discrete kinetic energy spacing, the values for carbon and lithium ions had to be interpolated. The work of A. Hauptner [25] pools data for DSB-yields of oxygen ions and $\alpha$-particles for a variety of kinetic energies. For the determination of DSB-yield of carbon (LET = 500 $\pm$ 80 keV/\(\mu\)m) and
lithium ions (LET = 116 ± 10 keV_{\text{um}}), the values available for oxygen ions and α-particles with the same LET were considered. While the quantity of energy deposition per unit track length is the same for two particles with the same LET, it has to be kept in mind that the radial dose distribution depends on kinetic energy and thus varies (see section 2.1 and equation 2.5). The following interpolation process refers to figure 2.9 in section 2.2.3 of chapter 2. The LET-value of carbon ions is matched by 61 MeV to 102 MeV oxygen ions [21] and the number of DSB per micron that is derived by linear scaling is 3.8 ± 0.6 (green area, left column). The closest PARTRAC data available is from 65.5 MeV and 100 MeV oxygen ions, which exhibit an enhancement factor compared to linear scaling of 2.5 and 2.8 respectively. These enhancement factors were assumed to apply to carbon ions as well. The lower and upper limit for the number of DSB per micron were multiplied with these enhancement factors respectively. This results in a total number of 10.2 ± 2.2 DSB per micron (green area in column in center). In the work of A. Hauptner, it was considered that DSB which are generated in close proximity cannot be resolved and the number of observed IRIF might be smaller than the total number of DSB. This clustering of DSB was taken into account in order to estimate the number of visible IRIF by defining a cluster diameter of 150 nm. DSB from the PARTRAC simulations, which were closer together than this diameter, were counted as one DSB-cluster [25]. This lead to a prediction for the number of observable IRIF that is smaller than the number of induced DSB and is represented by a reduction factor between the column in the middle and the one to the right. The reduction factor for carbon ions was estimated by averaging the factors of the two closest available PARTRAC data points. The closest factors for the upper limit of the predicted number of DSB per micron for carbon ions are 4.2 (50 MeV oxygen ions) and 2.9 (100 MeV oxygen ions). This leads to an average reduction factor for the upper limit of 3.6. For the lower limit, these factors are given as 2.9 (100 MeV oxygen ions) and 3.2 (87.5 MeV nitrogen ions), resulting in an average reduction factor of 3.1. Consequently, the expected number of observable IRIF per micron due to DSB-clustering is 3.3 ± 0.3 for carbon ions (green area in right column).

The LET-value of lithium ions equals that of α-particles between 3 MeV and 4 MeV and 1.0 ± 0.1 DSB per micron are the result from linear scaling (blue area, first column). The closest simulated data points are given by a series of energies for α-particles from 10 MeV to 5 MeV with corresponding DSB-enhancement factors of 2.1 to 2.6. A tendency to an increase of the factor is apparent. Thus, enhancement factors of 2.6 to
2.8 are applied to the linearly scaled values and the resulting number of total DSB per micron is $2.7 \pm 0.4$ (blue area, second column). Similar to the enhancement factors, the reduction factors also increase from 1.3 to 1.6 for the $\alpha$-particles. Thus, reduction factors in the range of 1.6 and 1.8 were assumed for the lithium ions, which results in $1.6 \pm 0.1$ observable DSB-cluster per micron (blue area, third column). For the validation of the results of this thesis, both the total number of induced DSB and the expected number of observable IRIF will be considered for comparison.

### 3.6 Experimental IRIF-evaluation

Experimental determination of the number of induced IRIF per $\mu$m for lithium and carbon tracks was performed in ImageJ [99]. Counting was performed with the 'Foci Picker 3D' plugin, described in detail in [53]. The working principle of the 'Foci Picker 3D' is visualized in figure 3.11. It is based on the assumption that each focus is represented by exactly one maximum in intensity. The plugin searches for local maxima in the image and grows them until a local threshold of intensity above background intensity (red lines) is reached. The threshold is adjustable as input parameter and is set to 50% for the evaluations. The plugin returns a coloured mask and a list with recognized foci. The list provides useful information about focus-positions, volume, intensity range and projected area in three spatial dimensions. Setting a minimum intensity range for recognized foci allows the discrimination of protein accumulations in the cell that are not produced by IR (red circle). The returned mask is screened by eye and compared to the original image. Especially for very bright foci intensity accumulation in z-slices above and below the particle track is present that were not removed by deconvolution. These artefacts can be misinterpreted as foci by the plugin. Counting was performed in a conservative manner and foci in doubt were deleted from the list.
Figure 3.11: Working principle of the 'Foci Picker 3D' plugin. The diagram shows an intensity profile along the yellow line across the particle track. The mask at the bottom represents the recognized foci. Maxima in the image stack are grown until they reach an intensity threshold of 50%. The threshold is referenced above a local background (red lines in graph). The focus volume is the number of voxels within this threshold (coloured areas in graph). When two foci overlap and the intensity threshold is below the local intensity minimum, the growth in this direction is stopped (orange foci). By setting a minimum intensity range, foci can be discriminated (red circle). Note that foci that seem to be missing in the mask are assigned to a different z-plane and the corresponding intensity in the depicted plane is already below the threshold.
Chapter 4

Results and Discussion

This chapter will be concerned with the experiments that lead towards the goal of counting single DSB and their results. First, the results of the examination of IRIF-formation of KU70/80, DNA-PKcs and its phosphorylated form, pDNA-PKcs, will be summarized. This will be followed by the results of the small angle irradiation at the ion microprobe SNAKE. The next step is a comparison of the obtained number of IRIF per micron for high-LET lithium and carbon ions to the simulated DSB-yields by PARTRAC. Finally, the observations of the experiments will be discussed.

4.1 Examination of IRIF-formation for KU70/80, DNA-PKcs and pDNA-PKcs

With an optimized fluorescence pair at hand, the examination of IRIF-formation of DNA-PKcs and KU70/80 can be carried out. Both proteins are involved in the early detection and repair stage of DSB. Maximum accumulation at DSB created in focussed laser spots has been observed 3 minutes after damage induction in previous research [57, 58]. Based on this background, experiments with α-particles were conducted in this work. An incubation time series (fixation after irradiation of 0, 1, 2, 5, 10 and 15 minutes) was performed in this project for two sets of samples. One set was labelled with a primary antibody targeting KU80, in representation of the KU70/80 heterodimer. The second set was targeted by a primary antibody to DNA-PKcs. Both sample sets were co-stained with γH2AX for IRIF-detection. Representative STED-images for DNA-PKcs and KU80 are pictured in figure 4.1 with the indicated secondary antibodies Abberior Star 635P and Oregon Green 488.
Figure 4.1: Examination of IRIF-formation for DNA-PKcs (fixed 5 and 15 minutes after irradiation) and KU80 (fixed 15 minutes after post-irradiation) for α-irradiation. γH2AX co-staining for IRIF-classification. The small boxes on the right are magnifications of the yellow boxes. Intensity plots for both channels are taken along the yellow lines. No IRIF-formation is detectable for DNA-PKcs or KU80 in damage regions marked by γH2AX. DNA-PKcs rather forms irradiation-independent foci, while KU80 is distributed homogeneously throughout the nucleus. Switching of fluorescence dyes (compare first and second row) does not influence the protein distribution. Images are spectrally demixed but not deconvolved.
4.1 Examination of IRIF-formation for KU70/80, DNA-PKcs and pDNA-PKcs

Intensity plots slicing through γH2AX IRIF and the corresponding DNA-PKcs/KU80 channel are provided for the enlarged sections. Neither for KU80 nor for DNA-PKcs IRIF-formation was observed within the γH2AX IRIF regions for the whole observed time range. The intensity plots did not show any significant signal distribution within the IRIF, compared to the region outside the IRIF. In order to rule out influences of the fluorescence dyes, Oregon Green 488 and Abberior Star 635P were interchanged (first and second row), causing the same protein distribution. KU80 was distributed homogeneously throughout the nucleus. DNA-PKcs, however, accumulated in a large number of distinct spots independent of ionizing radiation. This implies that it is either also engaged in other processes in the cell than DSB-repair, or the protein tends to aggregate. One reason why no IRIF were observable for DNA-PKcs and KU80, although they take part in DSB repair, might be the huge numbers of proteins expressed in each cell, which is estimated as 400000 [55] to 500000 [56] for KU70/80 and 400000 for DNA-PKcs [59]. Based on the acquired images, it seems that the binding of only one of each of these proteins at double-stranded DNA-ends is lost in the intensity of the abundant free proteins. In order to be able to resolve single DSB, only proteins bound to DSB or those locally attracted by DSB should be labelled specifically. It has been reported that during the course of DSB repair, DNA-PKcs is phosphorylated at multiple sites around its Thr2609 amino acid [13, 42]. A primary antibody that only binds to the phosphorylated form consequently allows labelling of a subset of total DNA-PKcs. Further research on the IRIF-formation of KU70/80 was dismissed in this work and efforts were focused on the study of the antibody for the phosphorylated form of DNA-PKcs. In a further experiment at the α-source, an incubation time series (1, 3, 5, 10 min) was performed for the antibody that only binds to DNA-PKcs phosphorylated at Thr2609 (pDNA-PKcs). The according secondary antibody in this experiment was Oregon Green 488 (green channel in STED pictures). IRIF-validation was performed through primary antibodies to 53BP1, which was fluorescence labelled by Abberior Star 635P (red channel in STED images). Assignment of fluorescence dyes was chosen with regard to the theoretical higher achievable resolution of Oregon Green 488, due to its shorter emission wavelengths.

It is apparent that pDNA-PKcs forms small IRIF in damage regions marked by 53BP1 in the observed time range (cf. figure 4.2). Overall, the IRIF are well established and developed at all times. However, some IRIF for cells fixed after one minute do not always show 53BP1 IRIF, even when pDNA-PKcs IRIF are present. This can
Figure 4.2: Time series from 1 to 10 minutes for the examination of IRIF-formation of pDNA-PKcs (green) after α-irradiation. 53BP1 (red) staining for IRIF-classification.
presumably be attributed to the later accumulation maximum of 53BP1 [100, 101]. For most of the time, the 53BP1 signal is sufficient to allow for statements about co-localization. It can be seen from the pictures that the majority of IRIF coincide in both channels. However, some 53BP1 IRIF do not contain pDNA-PKcs IRIF. This could be caused by DSB that had already been present before the irradiation. While 53BP1 can reside for several hours at DSB, pDNA-PKcs might already be gone. Thus, it is advised that the IRIF-formation of pDNA-PKcs is observed over a longer period of time in future experiments. Since the majority of IRIF coincide in both channels and pDNA-PKcs IRIF exhibit a high signal compared to the cellular level, it is concluded that the primary antibody to pDNA-PKcs is well suited for the examination of DSB-yield by high-LET particles in small angle irradiation at the SNAKE-setup.

4.2 High-LET particle tracks visualized through pDNA-PKcs

Small angle irradiation was performed with carbon (LET = 500 ± 80 keV/µm) and lithium ions (LET = 160 ± 10 keV/µm) and samples were stained with primary antibodies to pDNA-PKcs and 53BP1. Secondary antibodies were Oregon Green 488 for the first and Abberior Star 635P for the latter. Samples irradiated with carbon ions were fixed after times of 0, 0.5, 1, 2, 3, 5 and 10 minutes. While particle tracks were visible for samples representing 2, 3, 5 and 10 minutes (cf. figure 4.3), the samples fixed after 0, 0.5 and 1 minutes did not show an irradiation pattern. Thus, incubation times for lithium irradiation were set to 2, 3 and 5 minutes. Lithium particle tracks were both visible for 53BP1 and pDNA-PKcs staining (cf. figure 4.4).

For both the irradiations with carbon and lithium ions, the signal intensity of 53BP1 was partly low for samples fixed 2 minutes post irradiation because of its later accumulation maximum of around 15 minutes [100, 101]. In this early phase of repair, pDNA-PKcs stains the particle tracks reliably with a high ratio of signal from IRIF to signal of cellular protein. The particle tracks match well in both channels and magnifications reveal a fine-structure for 53BP1, which has already been studied in detail by J. Reindl [81]. The IRIF of pDNA-PKcs in the corresponding particle tracks appear more defined and exhibit a spherical shape. Overlapping IRIF can still partly be recognized as separated spheres. However, staining of 53BP1 provides important information on
Figure 4.3: Carbon ion particle tracks for increasing incubation times of 2, 3, 5 and 10 minutes. Visualized through staining of 53BP1 (red) and pDNA-PKcs (green). Merge of the two channels represents the co-localization of 53BP1 and pDNA-PKcs. Yellow rectangles are shown enlarged in the second row.
Figure 4.4: Lithium ion particle tracks for incubation times of 2, 3 and 5 minutes. Visualized through staining of 53BP1 (red) and pDNA-PKcs (green). Merge of the two channels represents the co-localization of 53BP1 and pDNA-PKcs. Yellow rectangles are shown enlarged in the second row.
the presence of nucleoli. They are perceptible as dark, circular regions within the nucleus and can barely be recognized through the staining of pDNA-PKcs. Particle tracks crossing the nucleoli show no IRIF-formation or staining of 53BP1. Although in a few cases IRIF-formation was visible for pDNA-PKcs, segments of particle tracks within nucleoli are not considered for the evaluation of particle tracks, since this might artificially reduce the number of IRIF per particle track length.

4.2.1 Temporal track structure development

While the visual density of observed IRIF is different for lithium and carbon ions, their distribution along the trajectories shows similar time dependence (compare figure 4.3 and 4.4). IRIF seem to be distributed statistically along the particle track for the samples fixed after 2 and 3 minutes. However, for some cells, 5 minutes after irradiation single IRIF seem to be arranged in IRIF-clusters of larger size, leaving bigger spacings along the trajectory without IRIF. This observation was made both for the lithium and carbon experiment and, additionally, tracks were evaluated from duplicate samples as well. This clustering of IRIF starting as early as 5 minutes after irradiation has already been reported for H2AX IRIF by [102]. It was proposed that this aggregation might be due to the movement of single IRIF to form clusters and an increase in the number of cells exhibiting this aggregation with time was reported. Another reason might be that a fraction of DSB is repaired quickly within 5 minutes and leaves behind dark regions, and thus the track appears to be aggregated. In the carbon sample fixed after 10 minutes some particle tracks also exhibited these IRIF-clusters. Whether these IRIF only appear to cluster visually or whether aggregation does take place indeed, was not further investigated and quantified in this work since the goal of this thesis was to count single DSB. Possible causes for an aggregation could be active transport during DSB repair or directed diffusion, but no statements can be made based on the static data present. It might be of interest to further examine this observation. Lice Cell Imaging would allow to study the temporal development of the track structure and influences from the fixation process could be ruled out.

4.2.2 IRIF cross-sectional area and diameter

The analysis of pDNA-PKcs IRIF within the particle tracks yields information about their average size. Because of the reduced resolution in z-direction, not the volume
but the cross-sectional area in the x-y-image plane is examined. The distribution of IRIF- cross-sectional area is visualized in figure 4.5 and mean values are listed in table 4.1. Assuming a spherical cross-section, the average IRIF-diameter is derived from the area.

<table>
<thead>
<tr>
<th>Ion</th>
<th>( t_{\text{inc}} ) [min]</th>
<th>( \Phi \text{ Area} \pm SD ) ([10^3 \text{nm}^2])</th>
<th>SEM [10^3 \text{nm}^2]]</th>
<th>( \Phi \text{ Diameter} \pm SD ) [nm]</th>
<th>SEM [nm]</th>
<th>IRIF #</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2</td>
<td>26.3 ± 11.7</td>
<td>2.2</td>
<td>178 ± 40</td>
<td>7</td>
<td>1036</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>31.8 ± 15.0</td>
<td>2.9</td>
<td>196 ± 47</td>
<td>9</td>
<td>1674</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>32.3 ± 16.2</td>
<td>3.4</td>
<td>196 ± 50</td>
<td>10</td>
<td>1464</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>29.5 ± 13.2</td>
<td>3.7</td>
<td>189 ± 43</td>
<td>12</td>
<td>807</td>
</tr>
<tr>
<td>Li</td>
<td>2</td>
<td>28.7 ± 11.1</td>
<td>2.1</td>
<td>188 ± 36</td>
<td>7</td>
<td>657</td>
</tr>
<tr>
<td>Li</td>
<td>3</td>
<td>28.7 ± 12.7</td>
<td>2.4</td>
<td>187 ± 41</td>
<td>8</td>
<td>662</td>
</tr>
<tr>
<td>Li</td>
<td>5</td>
<td>35.0 ± 16.7</td>
<td>3.2</td>
<td>205 ± 49</td>
<td>9</td>
<td>789</td>
</tr>
</tbody>
</table>

**Table 4.1:** Average x-y-cross-sectional area of pDNA-PKcs IRIF for carbon and lithium ions for increasing incubation time \( t_{\text{inc}} \). Corresponding average diameter assuming a spherical cross-section. SD: Standard deviation. The standard error from the mean is based on the number of independent cells, from which the individual tracks were evaluated from.

The overall distribution of the cross-sectional area looks similar for both ion types at all incubation times and it is apparent that the IRIF-size is subjected to large fluctuations. The average IRIF-diameter for samples irradiated with carbon ions rises from 178 ± 40 nm (SEM: 7 nm) at 2 minutes to 196 ± 50 nm (SEM: 10 nm) at 5 minutes and decreases to 189 ± 43 nm (SEM: 12 nm) 10 minutes after irradiation. For samples irradiated with lithium ions, an increase from 188 ± 36 nm (SEM: 7 nm) at 2 minutes to 205 ± 49 nm (SEM: 9 nm) at 5 minutes is observed for the diameter. Although between 662 and 1674 IRIF were evaluated for each time point, the standard error of the mean is based on the number of independent cells which is given in table 4.2. The width of the distribution is mirrored in the large standard error (SD) of the cross-sectional areas and diameters. The broadness could be explained by the mode of occurrence of IRIF. A fraction of IRIF exists isolated and thus their diameter is not influenced by the presence of neighbouring IRIF. However, neighbouring and overlapping IRIF that still can be separated by the ‘Foci Picker 3D’ might be assigned a reduced diameter compared to isolated IRIF due to the overlap. These might be responsible for the lower tail of the diameter distribution. Furthermore, overlapping IRIF that are indistinguishable to the ‘Foci Picker 3D’ might be counted as one and the assigned diameter corresponds to the width of several IRIF. These IRIF might be represented by the the upper tail of
Figure 4.5: Distribution of the x-y-cross-sectional area of IRIF for carbon ion (left) and lithium ion (right) irradiation. Increasing fixation time from top to bottom.
4.2 High-LET particle tracks visualized through pDNA-PKcs

the distribution. The varying distance between IRIF might be responsible for the wide
distribution of sizes.

A further evaluation, which assigns IRIF-diameters to the corresponding position in
the particle track, might allow for a more profound understanding of the origin of
the broad distribution. Within the SD, the values for lithium and carbon ions at
all incubation times are equal. A two-tailed t-test was performed and the differences
in average diameters were considered to be not significant. This indicates that the
IRIF are well established over the observed period of time and are suited for the
evaluation of the number of IRIF per micron. While in the time range covered in the
underlying experiments the diameter is constant, it might be of interest to expand the
examination of IRIF-sizes to further time points in order to obtain a proper temporal
description of the average IRIF-diameter. This could shine light on the dynamics of
IRIF-formation. Furthermore, smaller IRIF-diameters might be found that allow for a
even better separation of neighbouring IRIF.

4.2.3 Number of IRIF per unit path length

For the number of pDNA-PKcs IRIF per micron, the IRIF in the particle track were
counted and divided by the distance from the first to the last IRIF. 30 particle tracks
each were evaluated for the time points of 2, 3 and 5 minutes for both lithium and
carbon ions. Additionally, 20 tracks for the carbon sample that was fixed 10 minutes
after the irradiation were examined. Evaluation was performed as described in section
3.6. When more than one particle track was analysed from the same cell, both the
number of IRIF and track lengths were summed up each and one value was calculated,
before averaging with the remaining particle tracks. The average number of IRIF per
µm for carbon and lithium ions at different incubation times are listed in table 4.2, and
their distributions are displayed in figure 4.6. The SEM that is provided in the table
is based on the number of independent cells, from which the tracks were evaluated.

While particle tracks from lithium and carbon ions exhibit similar cross-sectional
areas and diameters of IRIF, the number of observed IRIF is different. The higher
LET of the carbon ions is mirrored in a higher average number of observed IRIF per
unit path length compared to the lithium ions. For carbon ions, the corresponding
values are $4.05 \pm 0.65$ (SEM: 0.12) at 2 minutes, $4.53 \pm 0.90$ (SEM: 0.18) at 3 minutes,
$4.47 \pm 0.72$ (SEM: 0.15) at 5 minutes and $4.16 \pm 0.56$ (SEM: 0.16) at 10 minutes
Figure 4.6: Distribution of the number of IRIF per micron for lithium and carbon ions with increasing incubation time. Data is based on 30 particle tracks for the incubation times of 2, 3 and 5 minutes and 20 tracks for the 10 minutes post-IR carbon samples.
4.2 High-LET particle tracks visualized through pDNA-PKcs

Table 4.2: Average number of detected IRIF per unit path length ± standard deviation (SD) for carbon and lithium ions at different incubation times \( t_{inc} \). The number of independent cells from which tracks were analysed is written in brackets. Standard error from the mean (SEM) is based on the number of independent cells.

<table>
<thead>
<tr>
<th>Ion</th>
<th>( t_{inc} ) [min]</th>
<th>IRIF/( \mu \text{m} \pm \text{SD} )</th>
<th>SEM</th>
<th># Cells (Tracks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2</td>
<td>4.05 ± 0.65</td>
<td>0.12</td>
<td>29 (30)</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>4.53 ± 0.90</td>
<td>0.18</td>
<td>26 (30)</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>4.47 ± 0.72</td>
<td>0.15</td>
<td>23 (30)</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>4.16 ± 0.56</td>
<td>0.16</td>
<td>13 (20)</td>
</tr>
<tr>
<td>Li</td>
<td>2</td>
<td>2.49 ± 0.44</td>
<td>0.08</td>
<td>28 (30)</td>
</tr>
<tr>
<td>Li</td>
<td>3</td>
<td>2.81 ± 0.47</td>
<td>0.09</td>
<td>28 (30)</td>
</tr>
<tr>
<td>Li</td>
<td>5</td>
<td>3.25 ± 0.60</td>
<td>0.12</td>
<td>27 (30)</td>
</tr>
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</table>

The number of IRIF per micron for lithium ions continuously rises from 2.49 ± 0.44 (SEM: 0.08) at 2 minutes to 2.81 ± 0.47 (SEM: 0.09) at 3 minutes and further to 3.25 ± 0.60 (SEM: 0.12) at 5 minutes post irradiation. The values for different incubation times of one ion type are equal within the SD. There might be two major contributions to the fluctuations around the average within one time point. The first might be due to different LET-values of the ions at the cell layer, caused by the individual path length and stopping of the ions in air and water. The second contribution to the fluctuation could arise from the spatial distribution of the DNA in the nucleus. As stated earlier, the volume occupied by DNA in HeLa cells is approximately 5.5% of the nucleus volume. Thus, the number of observed IRIF depends on the exact intersection of the particle trajectory with the DNA-molecule.

With regard to the SEM, the number of IRIF per micron differ for different time points of one ion type. Especially for the lithium ions, there seems to be an increase with the progression of time up to 5 minutes. The significance of this tendency was checked by a two-tailed t-test. For lithium ions the differences in all time points were considered significant. The p-value for the difference between 2 and 3 minutes is \( p = 0.0119 \) and the increase from 3 to 5 minutes was considered very significant \( (p = 0.0039) \). Furthermore, the increase from 2 to 5 minutes was even considered extremely significant \( (p < 0.0001) \). By contrast, the only significant difference for carbon ions could be confirmed for the rise from 2 to 3 minutes \( (p = 0.0274) \) and from 2 to 5 minutes with a p-value of 0.0319. Summarizing this evaluation, it can be stated that the strong increase is factual for the lithium ions, while the average values for carbon ions can be considered constant except for the average value at 2 minutes. The cause for the increase in the number of IRIF with time for the lithium
ions could be biological. The packaging of DNA in chromatin occurs in the subforms of euchromatin and heterochromatin, of which the latter is packed more densely [103]. Thus, when DSB are induced in heterochromatin, they are shielded from the access of repair proteins [104]. In the course of DSB repair, the heterochromatin unfolds and exposes the DSB, making them accessible for repair [103]. The increase in the number of IRIF for lithium ions could be attributed to a delayed accumulation of DNA-PKcs at these DSB. The reason why such an increase can only be partly observed for carbon ions might be that the number of observable IRIF saturates and that the increase of DSB is thus not resolvable. The saturation in the number of IRIF per micron might represent an upper limit for the resolution of single IRIF and any further increase in their number can no longer be resolved. It has to be considered that additional to the statistic fluctuations also systematic errors might be present that can hardly be quantified. However, it was tried to keep them minimal throughout all stages of the experiments. Although particle tracks for each time point were analysed from 2 to 3 different samples, only one experiment was conducted for each ion type and therefore it might be beneficial to repeat the experiments for representativity. During the experiments, it was emphasized to accurately match the intended incubation times and variation was kept below ~ 10 seconds. Furthermore, the counting of the IRIF was only performed by one person and in order to rule out influences of the experimenter it is advised that counting of the same particle tracks is performed by a second person. Especially for overlapping IRIF, it was hard to distinguish single IRIF and thus a certain error in counting has to be assumed. Another contribution to errors in counting was given by out-of-focus signal that could be mistakenly counted as IRIF and thus was treated with increased attention. In order to reduce these errors, counting was performed conservative and foci in doubt were dismissed. While it was up to the judgement of the experimenter to decide whether a foci is counted or not, great importance was attached to evaluating all tracks according to the same standard. To further eliminate errors in counting, all tracks were revised some time after their evaluation and the numbers adjusted if necessary.
4.3 Comparison of experimental results to PARTRAC data

The idea of this thesis was to visualize and count single DSB with high resolution. For this, the experimentally obtained number of IRIF per micron was compared to the predicted number of induced DSB per micron, derived from PARTRAC simulations as described in section 3.5.

4.3.1 Lithium ions

For 116 ± 10 keV lithium ions, the number of 2.7 ± 0.4 DSB per micron predicted by PARTRAC matches well with the experimentally observed number of IRIF per µm (2.49 ± 0.44 at 2 min, 2.81 ± 0.47 at 3 min, 3.25 ± 0.60 at 5 min) over the whole observed time range. This indicates that the algorithm of PARTRAC is well suited to predict the DSB-yield in this LET regime. By contrast, the predicted number of 1.6 ± 0.1 visible IRIF per micron due to DSB-clustering was not observed and was instead exceeded by the experimental results. Consequently, the predicted occurrence of DSB-clusters by PARTRAC has to be rejected for lithium ions. This mismatch can be attributed to the difference in the radial dose distribution for lithium ions and α-particles, from which the data was extrapolated. According to equation 2.5, the maximum distance of the secondary electrons to the track center for the lithium ions is 370 ± 94 nm and for 3 MeV to 4 MeV α-particles it is 50 ± 12 nm. Since the total dose is distributed within this distance, the spacing between DSB for lithium ions can be assumed larger compared to α-particles and thus all DSB are resolvable. Furthermore, the IRIF-size in the work of A. Hauptner was in the order of 1 µm and thus it is obvious that single DSB could not be resolved and the definition of observable 150 nm-sized DSB-clusters was required. The small pDNA-PKcs IRIF-diameters of 188 ± 36 nm (at 2 minutes) might crucially contribute to the resolution of all DSB, since still ~ 17% of IRIF are smaller than this cluster diameter. It can be concluded that for the given LET-range of the lithium ions, pDNA-PKcs IRIF reliably stain single DSB. The number of DSB per micron predicted by simulation matches well the observed number of IRIF. Furthermore, the number of 1.0 ± 0.1 DSB per unit path length obtained by linear scaling is clearly exceeded by the results presented here. These results thus underline the enhanced DSB-induction by high-LET irradiation. This indicates an enhancement factor for DSB-induction of 2.9 ± 0.4 for high-LET lithium ions. These promising results encourage and justify
the demand for PARTRAC simulations for lithium ions with \( \text{LET} = 116 \pm 10 \text{keV/\mu m} \) for a more accurate comparison.

### 4.3.2 Carbon ions

The simulations of PARTRAC for carbon ions (LET = 500 ± 80 keV/\mu m) seem to be compatible with the experimental results when comparing to the number of predicted DSB-cluster. The observed numbers of IRIF per micron (4.05 ± 0.65 at 2 min, 4.53 ± 0.90 at 3 min, 4.47 ± 0.72 at 5 min, 4.16 ± 0.56 at 10 min) do match and are partly higher than the number of 3.3 ± 0.3 DSB-clusters per micron within the SD. However, the predicted total number of 10.2 ± 2.2 DSB per micron does not match with the observed number of IRIF per micron. It has to be kept in mind that these numbers are derived by interpolating data from oxygen ions with the same LET and thus might not necessarily be applicable for carbon ions. The maximum range of secondary electrons from 61 MeV to 102 MeV oxygen ions is 1017 ± 418 nm, while for the carbon ions it is only 257 ± 123 nm. Since this represents a smaller penumbra around the core track, the effective interaction area of the track with the DNA might be reduced and could lead to a smaller number of DSB per micron for carbon ions compared to oxygen ions. In this case, the number of observed IRIF per micron in the experiments could represent the total number of DSB that is induced by carbon ions. This idea is further supported by experimental results given in figure 2.9. The number of DSB from linear scaling is approximately the same for 200 MeV oxygen ions and 70.8 MeV to 75 MeV carbon ions. PARTRAC predicts the same enhancement factors of 2.6 for carbon and oxygen ions. However, experimental results from 70.8 MeV carbon ions implied an enhancement factor of only 1.8, indicating that DSB-yields might not be comparable even at the same LET. Since experimental data for carbon ions in the LET range of 500 keV/\mu m is not available, it cannot be stated with certainty if the values for carbon ions are comparable to those of oxygen ions. This underlines the importance for the acquisition of PARTRAC data that is specifically simulated for the ions and LET values employed in this thesis.

If the simulated data for the total number of DSB for oxygen ions is correct and can be transferred to carbon ions, this would imply that each carbon IRIF contains 2.4 ± 0.6 DSB on average. In this case, the minimal average diameter of 178 ± 40 nm might be limiting for the resolution of single DSB. Since the average number of observed IRIF per micron is partly higher than the number of predicted DSB-cluster, it can
be concluded that the diameter of 150 nm, which is used for their classification, is set too high. Slightly more DSB are resolvable through pDNA-PKcs IRIF than predicted. The assumption of the limiting influence of the IRIF-diameter is supported by further PARTRAC simulations, which calculated the distribution of DNA fragment sizes after 2 Gy carbon irradiation [105]. The majority of fragment sizes ranges between 30 and 1000 bp. This corresponds to a maximum distance along the double-strand of 10 nm to 340 nm between two DSB. Higher order organization of DNA can significantly reduce the three-dimensional distance. With decreasing distance, neighbouring DSB are no longer resolvable through IRIF with diameters of 178 ± 40 nm and thus more than one DSB is contained in one IRIF.

Interestingly, the number of 3.8 ± 0.6 DSB per micron derived from linear scaling is by chance larger than the number of predicted DSB-cluster and is slightly smaller than the observed numbers of IRIF per micron. The average number of IRIF per micron over all observed times is 4.2 and consequently an enhancement factor of 1.13 is implied. When distributing the 3.8 ± 0.6 DSB per micron on a line according to a poisson distribution and subsequently applying the DSB-cluster diameter of 150 nm, the expected number of IRIF per micron would be smaller than the original number of DSB per micron from linear scaling. Thus, it can be expected that the observed number of IRIF per micron does not represent the number obtained from linear scaling and indeed mirrors the enhanced effectiveness of high-LET irradiation. For further comparisons and quantification of the enhancement in DSB-induction, simulated data for carbon ions at the given LET is required.

4.4 Comparison of findings with literature and research

This section will compare the observations about DNA-PKcs to previous findings in literature and research. It was previously found that DNA-PKcs is recruited to DSB within seconds of their induction and is phosphorylated at some point during DSB repair, which leads to its release from double-stranded DNA-ends [13]. The phosphorylation of Thr2609 was reported to be induced by repair proteins like ATM [68] or PP5 [106] and also by trans-autophosphorylation from two DNA-PKcs from opposing ends of one DSB [64]. A dynamic exchange of DSB-bound and unbound protein takes place in the proximity of DSB for both the unphosphorylated and
phosphorylated protein [13].

This matches well with the observations made throughout the experiments for this work. Despite the reports of the fast recruitment and binding of only one protein at each side of a DSB, no IRIF-formation for the pure DNA-PKcs antibody was detected in the experiments throughout the observed incubation times. This can be explained through the large number of 400000 [59] unbound proteins in the cell and it seems that the signal of one DNA-PKcs is lost in this background. This problem can be circumvented by staining phosphorylated DNA-PKcs. This way it is possible to visualize a subset of DNA-PKcs that co-localizes with DNA-damage regions stained by 53BP1 in ion trajectories. This underlines the fact that phosphorylation of DNA-PKcs is indeed the consequence of DSB response. Furthermore, IRIF-formation was not observed instantaneously. This might support the findings that phosphorylation leads to unbinding from the DSB and that a dynamic exchange of DNA-PKcs at DSB takes place: With increasing time, the repeated phosphorylation, unbinding of phosphorylated DNA-PKcs and binding of another new DNA-PKcs might lead to the accumulation of pDNA-PKcs in the vicinity of the DSB. There might be a threshold for the number of pDNA-PKcs required to allow for a detection of the fluorescence signal of the IRIF. The rise in the number of converted species with time could make this accumulated ‘cloud’ visible. Another indication that pDNA-PKcs is not strictly located at the DSB but in its surrounding, is the minimum average IRIF-diameter of at least 178 ± 40 nm. The smallest observed IRIF exhibited diameters of ~ 100 nm. To clarify, if these sizes can be caused by two single bound proteins and the corresponding fluorescence labelling, the maximum extension of bound DNA-PKcs with primary and secondary antibody labelling is estimated. The extensions of one DNA-PKcs molecule are stated with a longitudinal axis of 14 nm and a thickness of 7 to 10 nm [65]. Since two DNA-PKcs are bridging one DSB in the synaptic DNA-PK complex, their maximum end-to-end distance is 28 nm. The longest extension of one IgG antibody is ~ 16 nm [81, 107]. It is assumed for the estimation that binding sites for primary antibodies are at opposing ends of the DNA-PKcs dimer and that each primary antibody binds one secondary antibody. Then the maximum physically possible extension is 92 nm. Due to binding angles and configurations that are energetically more favourable, the average extension will be even less. The maximum 2-dimensional extension of 92 nm is significantly smaller than the average IRIF diameter of 178 ± 40 nm and comparable to the smallest
observed IRIF. Thus, this indicates to the presence of more than two pDNA-PKcs at each DSB on average, which are detached from the DSB and are not strongly localized.
Chapter 5

Conclusion and outlook

While only two DNA-PKcs and KU70/80 are reported to locate at a DSB and thus theoretically allow for high resolution counting of DSB, their signal is lost in the vast number of unbound protein. A distinct feature is necessary that allows for a discrimination of unbound protein and highlights proteins involved in DSB repair. The phosphorylated form of DNA-PKcs manifested itself as suitable for this goal. The study of the phosphorylation status of KU70/80 during DSB repair might also result in the observation of IRIF-formation, but was not examined in this work and might be subject to future research.

It has been shown in this work that phosphorylated DNA-PKcs in principle enables the counting of single DSB and reflects the enhanced induction of DSB of high-LET particles compared to linear scaling. For lithium ions (LET = 160 ± 10 $\text{keV}/\mu\text{m}$), the number of observed IRIF matches well with the number of predicted DSB by simulations of PARTRAC. On the contrary, DSB-clustering predicted by PARTRAC was not observed. The number of observed IRIF for carbon ions (LET = 500 ± 80 $\text{keV}/\mu\text{m}$) does not match the predicted number of DSB and is smaller by a factor of 2.4. It rather conforms with the predicted number of observable IRIF due to DSB-clustering and is even a bit higher. One possibility is that the predicted number of DSB per micron from oxygen ions with the same LET can not be transferred to carbon ions and the observed number of IRIF corresponds indeed to the number of induced DSB. The second possibility is that the assignment of single DSB to observed IRIF is no longer possible and each IRIF harbours more than one DSB, due to its finite size. For a further assessment on the number of DSB, simulated PARTRAC data specific to carbon and lithium ions with the given LET is required. For both ion types there was a trend towards an increase in the number of IRIF up to 5 minutes. This increase can be attributed to a delayed
accumulation of DNA-PKcs to DSB that are exposed by the unfolding of heterochromatin. This might indicate, that the number of induced DSB is best represented by IRIF 5 minutes after their creation. This recommendation is in contrast to the observed clustering of single IRIF for some cells, since this might result in an alteration of the initial DSB-distribution. The cause for this observation has still to be found and it cannot be excluded that it is due to the fixation process. While the IRIF-diameter was constant for the observed time points, it might be of interest to investigate the temporal development on a larger time scale. If smaller pDNA-PKcs IRIF were to be found, this might lead to a gain in resolution of DSB in close proximity. Especially the detection of single DSB in the LET regime of the carbon ions might benefit from an increased resolution. The significance of the observed tendency for an increase in the number of IRIF per micron could not be confirmed for all time points due to the limited number of independent cells. The current number of 30 tracks per time point is small for a biological experiment. It is advisable to evaluate a larger number of tracks and expand the experiment to include further time points for a proper sampling of the IRIF dynamics. Even with a helpful tool as the 'Foci Picker 3D' at hand, scoring of a single track with the according statistics and subsequent repetition of counting can take up to half an hour. For a large scale examination of particle tracks, further automatization and simplification of the control of the plugin is inevitable. Since the number of DSB were estimated from PARTRAC data for ions with the same LET but different kinetic energy, the difference in radial dose distribution is not taken into account. It would be of interest to examine if the radial dose distribution is mirrored in the radial IRIF-distribution. Since the evaluation of the number of IRIF with the 'Foci Picker 3D' plugin also returns the IRIF-coordinates as a by-product, these can be further evaluated at low additional effort. Fitting of the particle tracks, which are represented by the IRIF-coordinates, by orthogonal distance regression and determination of their distance to the track center can give an insight into the radial DSB-distribution. A first analysis of a few particle tracks indicated a broadening of the radial IRIF-distribution compared to the predicted initial radial DSB-distribution by [39]. Further detailed examination of particle tracks might give some indication of the dependence of the radial DSB-distribution on the specific kinetic energy.

A. Hauptner determined the number of 53BP1 IRIF with 0.8 per micron, both for 29 MeV lithium (LET = 86 keV_{\mu m}) and 24 MeV carbon ions (LET = 520 keV_{\mu m}) using a conventional fluorescence microscope [39]. Similar observations were made from other
research groups that determined 0.7 to 0.8 $\gamma$H2AX IRIF per micron after irradiation with 150 keV/$\mu$m iron ions [108]. The combination of high-resolution STED-microscopy and the pDNA-PKcs protein, which forms smaller IRIF, yields an enhanced number of IRIF by a factor of $\sim 3$ for lithium and $\sim 5$-fold improvement for carbon ions. However, the strong localization at DSB of DNA-PKcs is lost by the examination of the phosphorylated form. There are methods available that allow the extraction of unbound protein within the cell and thus increase contrast [14]. The localized KU70/80 and DNA-PKcs might be resolvable with this technique. However, for the examination of the cell in a state as natural as possible, this invasive technique might corrupt representativity. Focusing on the characterized phosphorylated DNA-PKcs, improvements are still to be made. The observations of the IRIF-formation are restricted to a few time points and thus an expansion is recommended in order to cover a larger range in time. In order to rule out influences of the fixation process on the IRIF-formation (cf. observation of IRIF-clustering), the implementation of a Live-Cell-Imaging (LCI) setup [109], which allows the extension of particle track evaluation to the fourth dimension, is recommended. The omission of the fixation process allows to examine the cells in their most natural state. Monitoring of the temporal development of IRIF-diameter and number could enable for a better separation of single DSB and thus might allow further advances in the counting of DSB induced through high-LET ions. However, the visualization needs to be revised for a LCI setup. The fluorescence dyes employed in this work are optimized for the acquisition of a single z-stack that leaves behind a bleached imaging area. Thus, recording of several z-stacks at the same region-of-interest with progressing time is not possible. This problem could be overcome by the use of fluorescence quantum dots. The semiconductor-based fluorophores possess a high quantum yield and photostability [110]. In the long run, dismissing the use of proteins for DSB-classification and replacing them might be the key to the resolution and counting of every single DSB, even of those in DSB-cluster. The intrinsic appearance of proteins in cells produces an additional signal that may not be affiliated with DSB repair. Even for pDNA-PKcs, his proportion of signal can not be dismissed, only optimized. Implantation of so-called DNA-probes (extrinsic DSB marker that only bind to double-stranded DNA-ends and thus strongly localize), might lead to a breakthrough in the resolution of single DSB.
Bibliography


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Statement

With the submission of this masters thesis I testify that I have written it independently and did not use other sources than the cited references.

Garching, October 10th 2016

Josef Huber