Microirradiation techniques in radiobiological research

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The aim of this work is to review the uses of laser microirradiation and ion microbeam techniques within the scope of radiobiological research. Laser microirradiation techniques can be used for many different purposes. In a specific condition, through the use of pulsed lasers, cell lysis can be produced for subsequent separation of different analytes. Microsurgery allows for the identification and isolation of tissue sections, single cells and subcellular components, using different types of lasers. The generation of different types of DNA damage, via this type of microirradiation, allows for the investigation of DNA dynamics. Ion microbeams are important tools in radiobiological research. There are only a limited number of facilities worldwide where radiobiological experiments can be performed. In the beginning, research was mostly focused on the bystander effect. Nowadays, with more sophisticated molecular and cellular biological techniques, ion microirradiation is used to unravel molecular processes in the field of radiobiology. These include DNA repair protein kinetics or chromatin modifications at the site of DNA damage. With the increasing relevance of charged particles in tumour therapy and new concepts on how to generate them, ion microbeam facilities are able to address unresolved questions concerning particle tumour therapy.

In this period, the main research focus using ion microbeams was the evaluation of the impact of very small irradiation doses, down to one particle traversal, within a cell population (reviewed in Matsumoto et al. 2009). This aimed to answer an important basic question for the radiation protection of people who were occasionally exposed or at risk of exposure and for the classification of the risk caused by natural radiation (ICRP 2007).

Due to recent developments in molecular biology, the investigative spectra have broadened. A milestone in radiobiological research is the possibility of visualizing DNA double strand breaks (DSB) (Zirkle and Bloom 1953). However, it took until the late 1990s to develop the technology for controlled and precise ion microirradiation devices. These were able to control both the target area to irradiate a certain cell compartment and the number of ions applied there.

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1. Introduction

Even prior to the discovery of the structure of DNA in 1953 by Watson and Crick, it was clearly evident that the nucleus is more radiosensitive than the cytoplasm. This was supported by work conducted, mainly with alpha particles, in which cells were specifically shielded so that only a certain part of the cell was irradiated. These early experiments demonstrated the researchers’ interest in irradiating defined parts of cells. In the 1950s, these assays were further improved by the introduction of specialized microirradiation devices (Zirkle and Bloom 1953). However, it took until the late 1990s to develop the technology for controlled and precise ion microirradiation devices. These were able to control both the target area to irradiate a certain cell compartment and the number of ions applied there.

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Due to recent developments in molecular biology, the investigative spectra have broadened. A milestone in radiobiological research is the possibility of visualizing DNA double strand breaks (DSB), by staining the phosphorylated histone variant H2AX (γ-H2AX) (Rogakou et al. 1999), or by fluorescently tagging proteins (Chalfie et al. 1994) which accumulate at DSB for live cell imaging. These techniques allow the following of molecular processes such as the

Keywords. Chromosomal aberration; DNA damage; ion microbeam; laser; microdissection; microirradiation

http://www.ias.ac.in/jbiosci

J. Biosci. 40(3), September 2015, 629–643, © Indian Academy of Sciences

Published online: 27 June 2015
kinetics of protein accumulation, DNA-damage-induced chromatin alterations, spatial and temporal motion of DSB, and the targeted irradiation of subcellular structures like organelles or chromatin domains, which places them into the current focus of research.

In addition, high-energy ions are also used in tumour therapy (Jiang 2012). Previously, most of the knowledge as to the effect of high-energy particles in biological probes was generated by mathematical simulations. The immense progress in the advancement of cellular and molecular biology techniques has raised the possibility to better understand the molecular consequences of high-energy particle irradiation of cells and (artificial) tissues.

Laser microirradiation has been used to produce cellular damage for over 40 years. Local irradiation of cells with a focused ultraviolet (UV) microbeam is a well-established tool in experimental cell research and radiation biology (Berns 1978; Cremer and Cremer 1986). More recently, laser microbeams have been used in an increasing number of applications such as microirradiation of selected nucleolar genetic regions, laser microdissection of mitotic and cytoplasmic organelles (Berns et al. 1981), to cut between the two sister kinetochores on bioriented prometaphase chromosomes (Khodjakov et al. 1997a), micromanipulation of submicrometer cellular structures, and even that of individual DNA molecules (Greulich and Piłarczyk 1998; Greulich et al. 2000). Laser cell interaction mechanisms can be complex, involving photothermal, photochemical and photomechanical processes. The contributions of these processes to the laser–cell interactions depend on the power, wavelength, pulse duration, energy and beam diameter (Vogel and Venugopalan 2003; Vogel et al. 2005).

Laser microirradiation has been used for different purposes such as cell lysis, microdissection or microsurgery of subcellular structures and to induce DNA damage. The types of lasers and configurations are summarized in table 1. In addition, figure 1 shows a schematic of a standard laser beam configuration. These aspects will be discussed in further details in the following sections.

2. Laser microirradiation techniques

2.1 Cell lysis

Laser microirradiation for cell lysis has been used for achieving the separation of analytes derived from individual cells. The laser can disrupt a portion of the membrane by positioning a microbeam within a subcellular region. Depending on the laser parameters used, the contents of either single or multiple cells are collected in a capillary (Li et al. 2001). This technique leads to rapid cell lysis and content collection. Thus, this technique is advantageous in many practical applications, especially if the release of stored contents, such as enzymes, within the cell compartments needs to be avoided. It is also possible to lyse the whole cell using a focused pulse which generates a cavitation bubble that disrupts the membrane (Rau et al. 2006). Rau et al. (2006) successfully performed such a cell lysis of potorous rat kidney epithelial (PtK2) cells. This was achieved by the delivery of aneodymium-doped yttrium aluminium garnet (Nd:YAG) pulsed laser microbeam (6 ns, 532 nm) using a 40×, 0.8 numerical aperture (NA) objective located 10 μm above the monolayer of cells. After the laser pulse delivery (0.5 ns to 50 μs), cell lysis underwent the processes of plasma formation, shock wave propagation and cavitation bubble formation, expansion and collapse. These authors’ results revealed a detailed visualization of the cellular damage, and showed that the cavitation bubble expansion was the primary mechanism leading to cellular lysis. Therefore, these systems can be used to study and predict the dynamic shear processes which are experienced by adherent cells, like those within a tissue, due to the displacement of fluid produced by cavitation bubble expansion. In addition the contribution of the cavitation bubble to the cell injury process can be observed.

Of note, the cells surrounding the point of the bubble collapse were deformed in a direction away from the centre of the bubble (Rau et al. 2006). The optical set-up for the purpose of cell lysis can be based in a vertically polarized microbeam of a Nd:YAG laser with the same wavelength (532 nm), but different pulse duration (750 ps). This configuration was used by Lai et al. (2008) to induce cell injury, followed by the electrophoretic separation of cellular analytes in a microfluidic device. Another system, based on the same type of pulsed laser microirradiation (532 nm, 540 ps), was used to irradiate non-adherent murine bone marrow-derived pro-B (BAF) cells. The rapid lysis obtained from this type of laser irradiation in microfluidic channels can be used in lab-on-a-chip applications, in order to make cell contents available for subsequent analysis, separation and/or the purification of cellular structures (Quinto-Su et al. 2008).

When a pulsed laser beam is focused on to a sufficiently small diameter, a localized plasma can be created, which in turn produces an outwardly moving propagation shock wave and an expanding cavitation bubble (Noack et al. 1998).

2.2 Microdissection or microsurgery

Over 40 years ago, it was demonstrated that a microsecond-pulsed argon ion laser could be focused on to a diffraction-limited spot for the subcellular microsurgery of chromosomes (Berns et al. 1969).

Microlaser dissection systems, combining the microscope, laser cutting and isolation beam technology, permit the identification and isolation of tissue sections, single cells and subcellular components (Schindler 1998). In addition,
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Nd:YAG, neodymium-doped yttrium aluminium garnet; PALM, positioning ablation laser microbeam; UV, ultraviolet; BrdU, 5-bromo-2′-deoxyuridine.
the combination of subcellular laser ablation, along with the use of gene fusion products, permits the visualization and alteration of cellular structures that are below the resolution of the light microscope (Khodjakov et al. 1997b).

The use of highly focused laser irradiation to alter and manipulate living tissues for applications in molecular and cellular investigations is becoming more pervasive. Nowadays, this technology permits the dissection of cellular structures, cytoskeletal portions and chromosomes. Also, microbeam laser irradiation can be used in order to deliver genes or biomolecules directly into cells by optoporation or optoinjection.

Venugopalan et al. (2002) investigated the use of Nd:YAG laser pulses (1064 nm or 532 nm) for microsurgery and micromanipulation. They determined that plasma formation was the primary mechanism of tissue injury. Using light microscopy, human metaphase chromosomes or subchromosomal regions can be selected for UV-laser ablation (Positioning Ablation Laser Microbeam (PALM) microlaser system, 337.1 nm, pulse width <4 ns). By atomic force microscope manipulation, disruption of the molecules for dissection and for the mechanical isolation of chromosomes, without any physical contact, is possible. This provides a useful technique for the generation of genetic probes, by microdissection, for high-resolution cytogenetic analysis (Stark et al. 2003). This easy access method can reduce the risk of contaminations from unwanted chromosome fragments, while providing finer dissection and faster recovery of the chromosomes without damaging the DNA (Shim et al. 2007).

Botvinick et al. (2004) described the use of picoseconds Nd:YAG laser pulses (80 ps, 76 MHz) in combination with two tubulin fusion proteins, to cut microtubules in mitotic and interphase cells. Using microtubule fluorescence as a guide, they demonstrated that the nature of damage appears to be different. This depended on either the use of enhanced yellow fluorescent protein or enhanced cyan fluorescent protein constructs, suggesting different photon interaction mechanisms. These authors concluded that the cutting of microtubules can be controlled by the combination of laser microbeam irradiation with fluorescent fusion proteins.

Using laser microdissection systems, specific cell populations from tissue sections can be cut out and isolated. And it is even possible to now identify mutations within single cells (Van Dijk et al. 2003). For example, when using this technique, premalignant cells can be morphologically distinguished, isolated and analysed from malignant ones. Furthermore, the PALM Nitrogen (337nm) microlaser improves the chances of obtaining informative, single-source DNA profiles from somatic cellular mixtures. In this way, Anoruo et al. (2007) investigated the use of this type of laser microirradiation to identify and isolate trace amounts of cells from mixtures of blood and saliva. The authors compared its efficiency with current protocols and found out that this system permits the isolation of cells from other cells and/or environmental debris. Therefore, it is possible to remove the need for DNA extraction, quantification and the analysis of profiles; making it potentially useful in the field of Forensic Science. In addition, current studies focus on the use of laser microbeam microdissection in combination with cDNA microarray techniques in order to analyse the genome-wide expression profile of breast cancers (Shimo et al. 2008).

2.3 DNA damage

It is well known that DNA is constantly under the protection of several biochemical pathways. The genome can be damaged naturally and spontaneously by a wide variety of physical and chemical agents, such as ionizing radiation, mutagenic, clastogenic or genotoxic agents and free radicals. It has been demonstrated that ultraviolet A (UVA) light irradiation at a fluence of 0.2–8 kJ/m² induces the production of both single (S-) and double strand breaks (DSB) after sensitizing the nuclear DNA via of 5-bromo-2′-deoxyuridine (BrdU) incorporation and staining with Hoechst 33258 (Limoli and Ward 1993). This technique was recently combined with laser-UVA-microirradiation to study the recruitment of DNA-repair proteins at damaged chromatin sites. The sensitization by the laser-UVA-microirradiation system
allows for the fast selection and precise irradiation of 1–2.5 μm within nuclear DNA.

One recently studied damage marker was the spatiotemporal dynamics of Poly(ADP-ribose) polymerase 1 (PARP1) recruitment to DNA damage sites (Haince et al. 2008). The authors found that this enzyme is rapidly activated by DNA DSBs and enters, in cooperation with the DNA repair/recombination 11 protein (MRE11) and the nijmegen breakage syndrome 1 protein (NBS1), into the surroundings of the DNA lesion. The PARP family is implicated in a wide range of biochemical mechanisms such as: DNA repair, transcription, mitotic segregation, telomere homeostasis, and cell death. PARP1 is a molecular sensor of SSB, while PARP2 is implicated in DNA damage, which involves redundant, but complementary functions, in genome stability (Wang et al. 1997; Ame et al. 1999; Menissier de Murcia et al. 2003). In this way, Mortusewicz et al. (2007) used a 405 nm UV diode laser to irradiate cells previously sensitized with either BrdU (10 μg/mL, 24–48 h) or with Hoechst 33258 (10 μg/mL, 10 min). These authors generated DNA lesions at preselected 1 μm sites to investigate the dynamics of both PARP1 and PARP2 recruitment by microirradiation within 1 s. They observed that both PARPs are recruited to DNA damage points, however, with different kinetics and roles. A rapid accumulation of the green fluorescent protein (GFP)-PARP1 (generated by the transfection of enhanced green fluorescent protein (EGFP) combined with Homo sapiens PARP (hPARP) in apEGFP-C3-hPARP-1 vector was observed at these damaged points. In addition, they found a large difference in DNA damage in relation to the sensitizing agent that was used. A greater DNA damage was observed in cells sensitized with Hoechst 33258, rather than with BrdU, due to the efficiency in the absorption of energy at the used laser wavelength used. A massive recruitment of GFP-PARP2 was also found in nucleoli in response to heavy DNA damage by microirradiation.

Another damage marker that has been investigated is the recombinase protein (Rad51), which is involved in the homologous recombination DSB repair mechanism. UVA laser microirradiation (364 nm, 3 pulses of 5.28 μs) in BrdU-sensitized cells (0.3 μM, 20 h; Hoechst 33258, 2 μg/mL) induced DSB at nuclear DNA, and led to the finding of an accumulation of Rad51 at these damaged sites (Walter et al. 2003). These authors reported that for minute energies (1 nJ), the fractions of cells showing Rad51 recruitment is very small. The number of cells with Rad51 accumulation rises in a dose dependent manner up to a maximum at 100 nJ. Conversely, using high energies (500 nJ), the fraction of cells with Rad51 accumulation decreases. The authors suggest this decrease is due to the tremendous amount of DNA damage, which results in DNA repair pathways not requiring Rad51 to be activated, or that the viability of the irradiated cells is affected. In addition, it is possible to change the type of DNA damage caused by this laser by changing the sensitization method (Walter et al. 2003).

In order to study the protein dynamics involved in the repair process of DNA damage generated by UVA laser microirradiation, Mortusewicz et al. (2005) investigated the role of DNA methyltransferases at DNA repair sites. The authors irradiated cells that were sensitized with BrdU for 20 h, and then exposed them to a pulsed N2 laser (337 nm, 8 nJ per pulse, 30 pulses). Alternatively, they used a diode laser (405 nm, 50 mW, 1 s). These systems permit a high resolution by the irradiation of 1 μm within the nucleus via the use of a coupled confocal microscope. Under the mentioned conditions, thymidine dimers and DSBs are generated. These authors observed that the histone variant H2AX becomes phosphorylated (γ-H2AX) within a few minutes after DNA DSB induction. An accumulation of the red fluorescent protein-tagged proliferating cell nuclear antigen (RFP-PCNA) and green fluorescent protein-tagged DNA methyltransferase (GFP-Dnmt1), colocalizing with γ-H2AX was also found. In contrast, two de novo methyltransferases (Dnmt3a, Dnmt3b), which were GFP-tagged, were found bleached at the microirradiated sites. This indicates that the de novo methyltransferases are not recruited to the sites of DNA damage induction.

Later, Mortusewicz et al. (2006) irradiated human HeLa cells to investigate the involvement and recruitment of DNA Ligases at DNA repair sites. These authors introduced DNA lesions, in BrdU-sensitized cells, by means of a 405 nm diode laser coupled to a Leica TCS SP2/AOBS confocal laser scanning microscope to irradiate spots of 1 μm for 1 s. This showed that microirradiation generates a mixture of different types of DNA damage, where different repair pathways, mediated by either DNA ligase I or III, are involved. DNA Ligase I is implicated in long patch base excision repair and nucleotide excision repair, whereas DNA Ligase III is implicated in short patch base excision repair and SSB repair. Among the alternatives found at microirradiated sites, these authors detected phosphorylated histone variant H2AX (γ-H2AX), and poly(ADP)-Ribose, which is generated by PARP at SSB.

Similar experimental systems that consist of a confocal laser scanning microscope and pulsed UVA laser (365 nm) can produce different types of DNA damage, within the nucleus, in a laser dose-dependent manner. When the laser light penetrates with 0.75 μJ per single pulse, it produces SSB, and yet when the energy is 2.5 μJ per pulse, both SSB and DSB are generated (Lan et al. 2004; Nakajima et al. 2006). The authors found that the E3 ubiquitin ligase (RAD18) accumulates at the sites where the laser light induces SSB, in the same way as x-rays do. In addition, Negishi et al. (2007) assayed the effects of UVA laser (364 nm) on different strains of oxidative stress sensitive Drosophila larvae, and compared them with those caused by
x-ray irradiation. They measured the amount of 8-hydroxydeoxyguanosine (8-OHdG) as an indicator of the oxidative DNA damage, and found that laser light irradiation causes significant oxidative DNA damage in this model organism. In contrast, the authors did not find an increase in 8-OHdG after x-ray exposure.

Kim et al. (2007) studied how cells reacted to DNA damage when using a second harmonic (532 nm) pulsed Nd:YAG laser. These authors reported the recruitment of factors involved in both, the non-homologous end-joining and the homologous recombination pathways, to repair the induced damage, suggesting the induction of DNA DSBs. They also observed the recruitment of the sister chromatid cohesin complex to the damaged point in the DNA.

3. Ion microbeams

3.1 General considerations

Most of the radiobiological microbeam facilities are located at ion accelerators for physics research, so they must be adapted to their environment in order to meet certain requirements. For example, the ion beam, which is transported in a vacuum, due to the relative short range of ions in air, has to leave the vacuum prior to hitting the cells. This problem is solved by sealing the beam transport line with very thin foils (several tenths of a micrometer thickness) which are glued on to the beam exit nozzle. Another example is the orientation of the beam transport line. In the case of a vertical beam line, exposure of the cells to the beam is easily achieved by just placing a suitable culture vessel on top of the beam exit window. In the case of a horizontal beam line, special cell culture vessels have to be designed, which must avoid media leakage in order to prevent the cells from drying. In addition, several technical devices need to be closely arranged to the sample and beam exit nozzle, such as a heating system to maintain a suitable temperature for the cells, an ion detection system and a microscope, just to name a few. Taken together, this leads to individual ways for people to solve the technical problems with which they face. In general, ion microbeams have to fulfill some basic criteria in order to perform a proper radiobiological experiment:

- A stable ion beam with a beam size in the micro- or sub-micrometer range has to be generated. This can be achieved by using collimators, which narrows the beam to the micrometer range. While narrowing the beam with a collimator is less costly and not too complex technically, the beam spot size is broadened due to ion scattering. Smaller beam spots in the sub-micrometer range can be generated by using ion-optical focusing.
- The focal plane of the beam spot needs to be placed outside of the vacuum of the beam transport line.
- An efficient ion detection system is necessary. This records the number of ions applied to the biological sample and requires a sufficiently fast triggering system, which shuts down the beam when the desired number of ions is achieved.
- An imaging system to choose an appropriate area of a sample or even single cells. In the case of single cell irradiation, software to record the coordinates of the target cells is necessary. If the imaging system is adequately equipped, live cell imaging after irradiation is possible.
- The ability to irradiate multiple positions of a sample quickly and accurately. This can be achieved either by moving the sample holder, the microscope stage, or by deflecting the beam with electric or magnetic fields.
- Cell culture conditions surrounding the cell sample during the irradiation and the subsequent live cell observation.

3.2 Low-dose experiments

In 1992, the first report dealing with what is known as the radiation induced bystander effect was published by Nagasawa and Little. They irradiated cells with a broad beam, using a low fluence of alpha-particles to traverse only 0.1–1% of the cells. The subsequent analysis of sister chromatid exchanges revealed a frequency of 30–50%, much higher than that which was predicted from the number of cells actually traversed by a particle (Nagasawa and Little 1992). The bystander cells show radiation induced stress, reduced survival, increased programmed cell death (apoptosis), increased mutation and transformation rates, and an elevated genomic instability. The communication from the irradiated cell to the non-irradiated cells can be done via two mechanisms. One possibility is the direct communication from cell to cell via gap-junctions. The other one is the secretion of signal transducers into the media, where they diffuse and act over a large distance (reviewed in Prise et al. 2009). Although these experiments can be performed without an ion microbeam by adjusting the particle fluence of a broad beam (Nagasawa and Little 1992), an ion microbeam is advantageous. Individually selected cells can be irradiated with a defined number of ions per cell. In addition, with appropriate experimental planning, the irradiated cells can be excluded in the analysis of the bystander cells. Recent experimental data implies that even cytoplasmatic traversal of particles can induce a bystander response (Shao et al. 2004; reviewed in Zhou et al. 2009). Several reports even point to the involvement of the mitochondria in the signalling pathways of the bystander response, induced by both nuclear as well as cytoplasmatic irradiation (Tartier et al. 2007; Zhou et al. 2008; Chen et al. 2008).
The increasing number of recent reports, which show responses in tissues and in vivo in mice (Singh et al. 2011; Rastogi et al. 2012), supports the clinical and radioprotection relevance of the bystander effect. Also, the inheritance of radiation induced epigenetic marks over cell generations has been reported (Tammenga and Kovalchuk 2011; Ihnyskyy and Kovalchuk 2011). All in all, the opinion that non-targeted radiation response needs to be taken more into account, with respect to tumour therapy and radiation protection (Hei et al. 2011; Mancuso et al. 2012), is rising.

3.3 Analysis of molecular processes at sites of DNA damage

A great advantage of the use of microbeams is that the site of irradiation is known. This was shown in a series of laser microbeam experiments, which led to the identification of numerous proteins accumulating at the damage site, forming so called ionizing radiation induced foci (IRIF). These proteins play a role in damage recognition, signal transduction and repair, or are involved in further cellular processes like apoptosis, cell cycle regulation, transcriptional regulation or chromatin modification (reviewed in Bekker-Jensen and Mailand 2010).

However, not all of these proteins which form foci after laser irradiation, will form foci after high LET ion irradiation (Nagy and Soutoglou 2009; Splinter et al. 2010; Seiler et al. 2011; Suzuki et al. 2011; Drexler et al. 2015). Whether this is due to different damage spectra or due to a higher amount of damage induced by laser microirradiation still remains unclear.

The protein accumulation can be visualized using immunofluorescent techniques or live cell imaging. For live cell imaging, the protein of interest needs to be fluorescently tagged via genetic engineering, e.g. by fusion to GFP. Immunofluorescent techniques possess the disadvantage that cells need to be fixed, thus only revealing a snapshot of a process. Live cell imaging eliminates this disadvantage and can provide the real time resolution of a process, but contains other pitfalls, such as phototoxicity (Yamagata et al. 2012).

Ion Microbeam experiments are used to analyse the kinetics of protein recruitment to the sites of damage and the persistence of induced foci. Additionally, investigation of a potential dose dependency of the recorded kinetics is possible by applying different numbers of ions per point or per nucleus. Also, a possible LET dependency for the obtained kinetics can be determined by applying ions with different LET. As a consequence, recruitment kinetics for different damage spectra, namely complex and clustered DSBs, as well as spatial isolated DSBs, can be analysed. It was shown that the accumulation kinetics of MDC1 and Rad52 is LET dependent, when applying comparable doses of 43 MeV carbon ions (LET = 370 keV/μm, one ion per point) or 20 MeV protons (2.6 keV/μm, 128 protons per point). In contrast, 53BP1 exhibits LET-independent recruitment kinetics. The authors suggest a model where recruitment of MDC1, as well as Rad52, is dependent on the damage density or complexity, whereas 53BP1 accumulation is independent of these factors (Hable et al. 2012).

An emerging focus in research is the alteration of the chromatin modifications at the site of a DSB. It has become evident that the chromatin status not only affects transcription, but all processes requiring interaction of proteins with the DNA. Thus, an opened chromatin conformation, like that in replication and DNA repair is of importance. Over the last few years, several reports have described the changes of the post translational modifications of histones as a consequence of ionizing radiation (reviewed in Friedl et al. 2012). In the case of so called ‘global changes’, the alterations are not only close to the DNA damage site, but span a large portion of the genome. This allows for the possibility to detect the relevant histone modification by Western blotting (Tjeertes et al. 2009; Seiler et al. 2011). In that case, variation of the histone modifications can be easily addressed using non focused sparsely ionising radiation. The situation becomes more complex when a more detailed analysis of the local changes is performed. One strategy is to perform CHIP (chromatin immunoprecipitation), a high-resolution analysis, which requires site specific DSB induction. However, with current irradiation techniques, this is not feasible. Another strategy used to address local alterations of histone modifications at the site of DNA damage is to perform immunostaining after damage induction. In this case, a damage marker, e.g. γ-H2AX, and the histone modification of interest are visualized in a time course experiment and the local changes of the post transcriptional histone modification are analysed. For this, irradiation should be carried out in an easily recognizable damage pattern, to distinguish the induced damage from spontaneous ones (figure 2), making ion microirradiation a perfect tool for this purpose (Seiler et al. 2011). Although laser microirradiation also leaves an obvious damage pattern, the poorly characterized load and types of DNA damage of laser systems is a major disadvantage. In addition, high laser energies may lead to unspecific chromatin alterations.

3.4 Sequential irradiation

The opportunity to perform cell irradiation in a defined pattern enables researchers to target a cell twice with a time interval in between the irradiations. By choosing an intelligent pattern design, it is possible to discriminate between the sequential irradiations, and to analyse separately the cellular response to each irradiation. By sequential irradiation, it was discovered that for certain DNA damage response proteins (e.g. 53BP1, Rad51), foci formation is strongly reduced in
pre-irradiated cells (figure 3). This emphasizes the role of binding and the turnover characteristics of DNA repair proteins (Greubel et al. 2008a, b).

3.5 Analysis of the spatial and temporal processes during chromosomal aberration formation

So far, the temporal and spatial processes during chromosomal aberration formation, where error prone ligation of DSB-ends occur, are unclear. To date, two contradictory models are used to describe the scenario. The ‘contact first’ model predicts that chromosomal aberration formation is restricted in a spatial manner. Only chromatin fibres close to each other each containing a DSB, can lead to miss re-joining of the wrong ends. The ‘breakage first’ model assumes mobile DSB-ends, moving large distances, before interacting with each other. The breakage first model is supported by the observation that damaged chromatin domains, identified via indirect immuno-fluorescence, seem to form clusters after a small angle alpha irradiation of cultured cells (Athen et al. 2004). They reported highly mobile damaged chromatin domains, moving several micrometers within 3 h. In our own work, where ions were applied in a so called ‘line pattern’ on the targeted cells (Hauptner et al. 2006; Hable et al. 2006). Rather, we identified diffusion as the driving force for damaged chromatin movement (Hauptner et al. 2006; Hable et al. 2006). Rather, we identified diffusion as the driving force for damaged chromatin movement (Du et al. 2011), excluding the directed movement of the damaged chromatin to repair centres. This is in line with live cell observations, where GFP-tagged repair proteins were used to visualize the motion of the damaged chromatin. Here only (sub-) diffusion was identified as the driving force for the motion of the damaged chromatin domains (Jakob et al. 2009; Girst et al. 2013). No tendency for clustering could be observed, which favours the ‘contact first’ hypothesis.

3.6 Targeted irradiation of cellular substructures

Owing to the success in achieving a high targeting accuracy with ion-optical focused microbeams, targeted irradiations of...
cell organelles is possible. This allows studying the impact of ionizing radiation on these structures. Targeting much smaller structures, like fluorophore tagged chromatin domains, approximately 0.5 μm in size, is desired, but the current targeting accuracy is only in the range of 1.0 to 1.5 μm (reviewed in Gerardi 2009). Thus, successful targeting of such small structures can only occur by chance and is not yet routine. To achieve less than 1.0 μm targeting accuracy might be difficult, because it depends not only on the technical set-up, like the beam localization accuracy, but also on the target definition accuracy or the accuracy of the beam scanning unit as well. Also the biological processes, such as intracellular as well as cellular movement, during the time interval of target definition and irradiation, needs to be taken into account.

Targeting larger structures like nucleoli (Siebenwirth et al. 2015) or heterochromatin centers (Jakob et al. 2011) has already been successful performed.

3.7 Simulation of new approaches for tumour therapy

Recent developments in laser technologies allows for the acceleration of charged particles by pulsed high-energy lasers (Beyreuther et al. 2010; Bin et al. 2012). Since particle therapy using conventional ion accelerator facilities is highly costly and space consuming, the vision to use laser accelerated particles for therapy in order to lower the financial and spatial requirements has emerged. The laser pulses generate a very dense particle bunch in the atto- to femtosecond range when they hit special target foils. Due to the different particle energies released from the foil, and thus due to different speeds, the ion bunch is widened over the time of flight until it reaches the target. The time extensions of the ion bunch is only up to the nanosecond range, resulting in a dose rate of >10⁹ Gy/s. However, when compared to a conventional accelerated particle beam, the particle fluence is still dense. A conventional particle beam generates the same dose continuously within the millisecond range, resulting in a dose rate of ~30 Gy/s. The higher dose rate of the laser accelerated particle beam might lead to a reduction in the yield of hydroxyl radicals, when two proton tracks are tightly overlapped in space and time. As a consequence, the relative biological effectiveness (RBE) might be decreased (Kreipl et al. 2009). Additionally, the possible influence of the dose rate on the RBE is still under debate (Fourkal et al. 2011). A series of reports have addressed this issue by irradiating cell systems and tissue models with a specially modified proton microbeam. The same doses of 20 MeV protons were administered either as a continuous or pulsed proton beam and several endpoints were analysed. These experiments concluded, that there is no significant difference in the RBE of continuous or pulsed applied protons (Dollinger et al. 2009; Auer et al. 2011; Schmid et al. 2009, 2010; Greubel et al. 2011), for the endpoints that were investigated, including clonogenic cell survival. The same conclusion was drawn by Doria and co-workers in experiments which investigated the survival of V-79 hamster cells after irradiation with laser-accelerated protons in the MeV range, and the comparison of the obtained RBE value with data in literature (Doria et al. 2012).

4. X-ray microbeams

4.1 Soft x-ray and ultrasoft x-ray microbeams

Ultrasoft x-rays, with energies of 280–500 eV, will be absorbed in the initial layers of a tissue, and thus radiobiological
experiments are restricted to single cell layer probes. However, soft x-rays, with higher energies (up to 5 keV), will penetrate tissues deeper, allowing radiobiological experiments with tissues or tissue models to be performed (Harken et al. 2011).

Soft and ultrasoft x-ray microbeams achieve a beamspot size ranging from below one up to several micrometers, depending on the technique used to focus the x-rays. By using zone plates for focusing a beamspot, a diameter below one micrometer is possible (Folkard et al. 2001). These x-ray microbeams are able to target single cells in a sample or to discriminate between irradiation of the nucleus or cytoplasm. On the other hand, by using mesh filters in the focusing procedure, the geometric pattern of the irradiation applied to the cell sample and the beam spot size is adjustable (van Oven et al. 2009). Since this is a technique where a broad beam is shielded to obtain a certain irradiation geometry, the targeting of certain subcellular structures is often not intended.

Research conducted with x-ray microbeams often addresses the low dose region of the dose response curve, including causes and effects of the bystander effect, as well as the LET dependence of these non-targeted effects (Folkard et al. 2001; Tomita et al. 2013). Molecular processes at sites of ionizing radiation induced DSB are investigated using immunofluorescent techniques and real time live-cell imaging as well (van Oven et al. 2009; Schettino et al. 2011; Reynolds et al. 2012).

### 4.2 Synchrotron-generated microbeams

Synchrotron generated microbeams are arrays of collimated, planar and quasi parallel microbeams of x-rays in the kilovoltage energy range. A single beam of this array is typically 25–50 μm wide and is separated by centre-to-centre distances of 200–400 μm from the next one (figure 4a). The whole array is applied in a single session in a scanning mode. The resulting dose profile is illustrated in figure 4b. At the peak zones, very high doses are deposed, exceeding 100 Gy, whereas in the valley regions only a very low dose is delivered.

Due to the competitive and limited beam times available at the few worldwide operating synchrotron facilities, only a few pioneering experiments regarding the radiation therapy of brain tumours have been performed (reviewed in Grotzer et al. 2015). Still, conventional radiotherapy remains poorly effective when targeting malignant brain tumours, because the most frequent brain tumours are aggressive and resistant to radiotherapy (Ricard et al. 2012, Beygi et al. 2013). For synchrotron generated microbeams several independent teams have consistently reported that single fraction microbeam radiation therapy (MRT) in several small animal model organisms yields a larger therapeutic index than a single dose of broad beam radiotherapy (Bräuer-Krisch et al. 2010; Crobie et al. 2010; Dilmanian et al. 2001, 2003; Laissue et al. 2001). Interestingly the skin tolerates the relatively high doses delivered by the microbeam array well (Priyadarshika et al. 2011). The radiobiological mechanisms of MRT, which lead to the radio-resistance of the skin on the one hand, and to radiosensitization of the tumour tissue on the other hand, are not yet understood. Morphological differences between the tumour-specific vasculature and the normal vasculature in the brain are a plausible hypothesis, substantiated with data, to explain this feature (van der Sanden et al. 2010; Sabatasso et al. 2011). Additionally, a role of the bystander effect, spreading damage signals from the high dose peaks to the low doses valley in the tumour tissue has been proposed (Fernandez-Palomo et al. 2015).

### 5. Limitations of these techniques

A deeper understanding of laser-cell interaction is necessary to use these techniques for new applications in research and medical practice. Many factors have to be controlled such as: irradiation wavelength, pulse duration, pulse energy, focus angle, and beam diameter. Nd:YAG lasers are an attractive option due to easy operation and configuration at low cost (Rau et al. 2006).

Laser-based lysis produces a rapid dispersion and dilution of small molecules such as small proteins, ATP, glucose, amino acids and Mg$^{2+}$. Thus, cellular reactions that require these will be slower and finish in milliseconds. Therefore, these molecules are not available for analysis or manipulation (Quinto-Su et al. 2008). The imaging systems have insufficient spatial resolution to visualize the effects of cavitation dynamics on some tissues (Rau et al. 2006). Additionally, one important limitation is the inability to clearly visualize and then manipulate the target structures, which are usually below the resolution of the light microscope. This problem has been resolved in some applications of microsurgery by the combination of laser pulses with fusion proteins (Botvinick et al. 2004).

Conventional microdissection systems result in the generation of genetic damage, are time consuming and labour-intensive work, and require an experienced person with high-skilled techniques (Shim et al. 2007). In addition, laser UV microirradiation for microdissection is a tedious procedure. However, the use of laser systems based on a confocal laser scanning microscope allows for easy handling with modest costs (Walter et al. 2003). The lasers used in a combination with fluorescent dyes in the sensitization of cells are limited by the amount of dye which is bound to the DNA, especially if it is below the level of the fluorescence detection system (Liang et al. 2000).
The major limitation of the devices for the microirradiation of cells with ionizing radiation is their limited availability within the scientific community. Worldwide, there are only a limited number of synchrotron or focused ion beam facilities available. This is due to the size requirements of these facilities and the tremendous costs for building and maintaining them in operation. Additionally, each ion microbeam facility is limited in the ion spectra and energies that it can provide. Furthermore, due to the limited beam time availability experiments should be well planned. Experimental procedures or certain necessary hardware should be well tested before the experiment, and whenever possible without the use of the operating beamline.

Compared to synchrotron or ion microbeam facilities, devices for microirradiation with soft/ultrasoft x-rays are tabletop devices. However, setting up such a device requires sophisticated and costly equipment (Walter et al. 2003), as well as an advanced knowledge in x-ray physics. Additionally, the local laws of radioprotection must be adhered to and structural alterations may be required to fulfill them.

Nevertheless, scientists with the intention to perform microbeam experiments with ionizing radiation are encouraged to look out for a fruitful collaboration with their colleagues who already have access to a functionally operating microbeam.

6. Conclusions

Laser microirradiation techniques can be used for cell lysis, microsurgery and to induce DNA damage. Cell lysis by laser permits the subsequent separation of molecules from individual cells. Microsurgery, via the use of laser beams, combines the microscope with laser cutting and isolation technology in the identification and isolation of tissue sections, single cells and subcellular components, such as cytoskeletal portions and chromosomes. The generation of different types of DNA damage within the nucleus can be produced and preselected in a laser dose-dependent manner at small sites to investigate DNA dynamics. Different types of lasers have been used for this with a wide range of configurations. It is important to mention here, the combination of laser beam with the confocal laser scanning microscope and the sensitization of cells with BrdU or Hoechst.

Ion-microbeam facilities for radiobiological experiments are highly complex technical devices and were originally
developed to study the bystander-effect. A large proportion of the research conducted there, as well as with ultrashot/soft x-ray microbeams, is still dedicated to the understanding of low dose effects and the bystander effect. Due to the improved molecular biology techniques, the research focus for the non-targeted effects has shifted from investigating the consequences on the cellular level to unravelling the molecular processes mediating the bystander response. This will lead to a better risk assessment for radiation protection purposes and may also have a clinical impact on radiation tumour therapy. Additionally, ion-microbeams are versatile tools to further address radiobiological questions, which require the knowledge of the location of the radiation-induced damage, like spatially restricted molecular changes in the vicinity of radiation-induced DSB, and the analysis of their consequences. Furthermore, ion-microbeams and ultrashot/soft x-ray microbeams will contribute to the understanding of LET-dependent reactions of irradiated cells and, more importantly, tissues. Thus, taken together, microirradiation techniques of cells with ionizing radiation will improve the understanding of the molecular processes induced within cells and tissues. This in turn will have consequences for various aspects: Due to the better knowledge of radiation-induced tumour development after low-dose irradiation, development of more sophisticated concepts for radioprotection will be possible. Better understanding of radiation-induced signalling cascades may also lead to the identification of possible targets for the specific radiosensitization of the tumour tissue. The temporal dissection of these cascades may lead to improved irradiation regimes in radiation therapy. Thus, even by elucidating mainly basic mechanisms at the moment, there is great potential to generate clinically relevant knowledge.

This has been most impressively demonstrated by synchrotron microbeam irradiation, presenting convincing data in several small animal model organisms. The next step will be to show the advanced effects of MRT when compared to radiotherapy using broad beams in larger animals, before starting clinical trials with human patients. Due to the promising reports of the MRT so far and in order to accelerate research in that field, first experiments were conducted to show how the characteristic dose distribution of the MRT can be mimicked (Zlobinskaya et al. 2013; Hadsell et al. 2014).

Acknowledgements

The authors thank Steven J Smith for carefully reading the manuscript. GAD acknowledges the financial support of the DFG Cluster of Excellence: Munich-Centre for Advanced Photonics (MAP).

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MS received 23 January 2015; accepted 19 May 2015

Corresponding editor: Veena K Parmaik

J. Biosci. 40(3), September 2015