Review

Systematic overview on the most widespread techniques for inducing and visualizing the DNA double-strand breaks

Ivett Berzsenyi [1], Vasiliki Pantazi [1], Barbara N. Borsos, Tibor Pankotai *

Institute of Pathology, Albert Szent-Györgyi Medical School, University of Szeged, 1 Állomás Street H-6725, Szeged, Hungary

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ABSTRACT

DNA double-strand breaks (DSBs) are one of the most frequent causes of initiating cancerous malformations, therefore, to reduce the risk, cells have developed sophisticated DNA repair mechanisms. These pathways ensure proper cellular function and genome integrity. However, any alteration or malfunction during DNA repair can influence cellular homeostasis, as improper recognition of the DNA damage or dysregulation of the repair process can lead to genome instability. Several powerful methods have been established to extend our current knowledge in the field of DNA repair. For this reason, in this review, we focus on the methods used to study DSB repair, and we summarize the advantages and disadvantages of the most commonly used techniques currently available for the site-specific induction of DSBs and the subsequent tracking of the repair processes in human cells. We highlight methods that are suitable for site-specific DSB induction (by restriction endonucleases, CRISPR-mediated DSB induction and laser microirradiation) as well as approaches [e.g., fluorescence-, confocal- and super-resolution microscopy, chromatin immunoprecipitation (ChIP), DSB-labeling and sequencing techniques] to visualize and follow the kinetics of DSB repair.

1. DNA damage response

The nucleotide sequence of the DNA encodes the genetic information responsible for all the biological processes of every living organism and thus, requires high fidelity between and during cell divisions. However, genotoxic stresses arising either endogenously (e.g., metabolic by-products) or exogenously (e.g., UV light or irradiation) constantly threaten the genome integrity and can give rise to a variety of DNA lesions. These impairments are among the leading causes of human cancerous diseases. It is worth mentioning that endogenous sources in human cells can also trigger genetic or even immune response variability during antibody production as part of the programmed developmental processes without leading to a detrimental outcome [1]. DNA damage response (DDR) can lead to the activation of the appropriate pathway for resolution of DNA damage [2]. For instance, replication errors are repaired by the mismatch repair pathway, whereas abasic sites, single-strand breaks and 8-oxoguanine (8-oxoG) are mainly removed by base-excision repair [3–5]. UV-induced photoproducts [e.g., cyclo-butane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs)] and bulky adducts [e.g., polycyclic aromatic hydrocarbons (PAH)] are recognized by nucleotide-excision repair (NER), which can be separated into two main sub-pathways: (I) global genomic NER (GG-NER), and (II) transcription-coupled NER (TC-NER), which are activated according to the presence of the stalled RNA polymerase II (RNAPII) [6]. Helix distorting lesions initiate the recruitment of TC-NER factors, which recognize the stalled RNAPII and participate in its backtracking or removal. On the other hand, GG-NER is activated by CPDs and does not require the presence of the stalled RNAPII. Nonetheless, the downstream steps are the same in both sub-pathways [7].

Undoubtedly, DSBs are the most hazardous DNA lesions for the cells. If left unrepaired or erroneously repaired, they can result in chromosomal translocations and genomic instability and subsequently, lead to tumorigenesis or cell death. Therefore, DNA double-strand break repair (DSBR) is an intricate process that can activate distinct pathways depending on the cell-cycle phase and the chromatin environment [8–11]. To this end, based on the presence of a sister chromatid—and thus the cell cycle phase—that can serve as a template for repair, DSBR is dominated by two major pathways: (I) non-homologous end joining

(NHEJ), which is active throughout the cell cycle, is a faster but error-prone pathway in which the two DNA ends re-ligate with no or minimal sequence-homology, and (II) homologous recombination (HR) which is mostly an error-free pathway, active in the S- and G2-phase and requires more extended resection and a RAD51-mediated sequence-homology search between the broken DNA end and the homologous sequence [12]. At this point, it is worth mentioning that alternative DSBR pathways with distinct activating mechanisms from the major ones have been further described but are considered highly mutagenic. Briefly, these are as follows: (I) microhomology-mediated end joining (MMEJ) [also referred to as alternative end-joining (alt-EJ)] which is based on the previously resected microhomologous sequences flanking the broken DNA ends and their ligation in a KU70/80- and RAD51-independent way, (II) single-strand annealing (SSA), which takes place when a DSB is generated between flanking homologous sequences, which are mainly repetitive regions, and (III) microhomology-mediated template switching which occurs when a free single-stranded DNA 3′ end anneals with minimal homology (2–4 nt) to ectopic sequences and then returns to the original one that has suffered the DSB [13,14].

In response to DNA damage, a signaling cascade orchestrated by several proteins recognizes the DNA lesions, locally amplifies the signal and globally triggers cell cycle arrest and DNA repair [9,11]. Following the formation of DSBs, DNA Damage Response (DDR) is activated, which includes the recruitment and extensive spreading of initiator key players around the lesions, contributing to the formation of a repair focus. Phosphorylation of the histone variant H2AX at Serine 139 (referred to as γH2AX) is a hallmark in the early signaling cascade of DDR, which is mainly mediated by the Ataxia Telangiectasia Mutated (ATM) [15]. Thus, this preliminary event acts as a signaling scaffold for the recruitment of additional repair factors. Although it is an extensively studied field, the precise role and fine-tune regulation of the repair factors still remain elusive. For instance, the role of γH2AX is still puzzling since recent data have uncovered its stronger correlation with DNA replication in post-UV-irradiated S-phase cells than with DNA damage under the same conditions [16]. Nevertheless, this should not devalue its role in the DDR but rather encourage future research activities.

Owing to the great importance of DNA repair-related cellular processes, several methods have been developed to induce and visualize the DDR. In the current review, we employ a comprehensive approach and extensively discuss these techniques, expounding on advantages as well as the disadvantages of each. Here, we summarize the most widely used tools for studying DSBs, including techniques for their induction, subsequent visualization and quantification of their repair kinetics. Accordingly, to highlight the most applied methods of site-directed DSBs induction, we focus on the site-specific endonucleases, clustered regularly interspaced palindromic repeats (CRISPR)-mediated assays, and laser microirradiation. Furthermore, we emphasize special methods, including fluorescence, confocal and super-resolution microscopy, chromatin immunoprecipitation (ChIP), and DSB-labeling and sequencing, by which the kinetics of the repair process can be efficiently monitored.

2. Techniques used to induce DSBs

2.1. Site-specific nucleases

The desired type of DNA damage can be generated via numerous ways in various biological systems (i.e., yeast or human cells), triggering either site-specific or random distribution of DNA damage. Anti-tumor agents [such as neocarzinostatin (NCS), phleomycin, bleomycin], γ-irradiation and UV played a significant role in the improvement of chemotherapy, and many of them (bleomycin, phleomycin, 5′fluorouracil, doxorubicin, etc.) are still used as part of standardized chemotherapy regimen because they can induce large numbers of random DNA breaks at non-predictive genomic positions in cancer cells.

To investigate scientific claims regarding DNA repair, further approaches that utilize endonucleases, such as AsiSI, I-PpoI or I-SceI, as well as laser striping, inducing DNA breaks at known genomic positions, have been mainly used so far [17–20]. The endonuclease-based method provides a unique possibility to induce site-specific DNA damage at a known genomic locus, while laser microirradiation induces targeted and localized laser tracks, thereby generating DSB foci in a small number of cells.

The field of DNA repair has been tremendously revolutionized by using methods capable of inducing site-specific DNA damage. While certain DNA damaging agents, such as irradiation or the radiomimetic drug NCS, generate a high number of DNA breaks at unpredicted genomic positions, endonucleases cleave DNA at annotated genomic sites. The repertoire has been rapidly expanded with the establishment of various cell-based endonuclease-expressing model systems. The site-specific DSB induction was pioneered in *Saccharomyces cerevisiae,* where the expression of HO endonuclease, which recognizes a 22 bp sequence in the *MAT* locus, was controlled by a galactose-inducible promoter [21]. This possibility opened new roads for researchers and further applications in mammalian cells by introducing the HO recognition sequence into other genomes, such as *Schizosaccharomyces pombe* or mammalian cells [22]. Nonetheless, this strategy was expanded by the use of nucleases, such as I-PpoI or AsiSI, that do not require exogenous sequence introduction into systems other than *Saccharomyces cerevisiae* and tedious experimental procedures. For this reason, nowadays, the most commonly used endonucleases to induce locus-specific DSBs are AsiSI, I-PpoI, HO and I-SceI [17–20]. These enzymes are originally encoded by a transposable element and cut DNA mostly within intergenic or intronic sequences. AsiSI has an 8-base pair (bp) recognition sequence and is able to efficiently cut genomic DNA at approximately 200 sites, specifically identified by this enzyme, while I-PpoI, which is considered as a meganuclease, targets DNA within a semi-palindromic 15 bp sequence at approximately 30 sites which comprise 10 % of all its recognition sites (200–300 sites) in the genome and mainly found in ribosomal genes [17–19,23–25]. Many laboratories reported that I-PpoI was not able to efficiently cut every genomic region harboring its cutting site due to either recognition sequence degeneration or their inaccessibility caused by heterochromatinization [17,26,27]. On the other hand, HO and I-SceI are considered as unique cutters with an 18 bp recognition sequence that most of the times has to be integrated into the human genome by transgenes [20,28]. These endonucleases have been extensively used to study the formation and kinetics of repairing individual DSBs in yeast, mouse, and human systems via imaging and biochemical tools [26,29–31].

Endonuclease-based systems have several advantages and disadvantages. Although they induce site-specific DSBs, which are advantageous for microscopic techniques or chromatin immunoprecipitation, the homing endonucleases can induce only a limited amount of DSBs, which are not enough for large-scale experiments, such as mass-spectrometry, unless a large amount of material is used. However, they can be extremely useful in DSB-labeling and sequencing technologies (described below). Nevertheless, these systems provide the opportunity to follow the kinetics and exact outcome of repair mechanisms and pursue the steps of activated DNA repair pathways. Furthermore, various constructs are available in which the lacR-I-SceI system or estrogen receptor (ER)-fused endonucleases allow the generation of inducible DSBs that can be followed in real-time in living cells; this approach has revealed the order of the recruitment of DDR proteins for the resolution of DSBs [32]. Induction of endonuclease-based site-specific breaks is a unique tool used to study chromatin structural changes at the single nucleosome level in euchromatic or heterochromatic regions, as well as to follow the spreading of DDR proteins surrounding a break [33]. Apart from the low number of DSBs induced by endonucleases, several other disadvantages have been noted: (I) the variable time delay in the expression and translocation of these enzymes to the nucleus largely affects the induction time and the repair kinetics

of DSBs, and (II) the induction of persistent breaks due to the constitutive presence of the nuclease. Furthermore, as HO and I-SceI produce a single break and I-PpoI induces several breaks, but mainly in ribosomal DNA, the study and comparison of DSB repair in different genomic positions are quite challenging. The most elegant AsiSI system, which is based on the directed expression of the AsiSI homing endonuclease fused with an estrogen receptor, has been established in Gaëlle Legube's laboratory [18]. The estrogen receptor keeps the endonuclease in the cytoplasm, and upon 4-hydroxytamoxifen (4-OHT) treatment, it translocates into the nucleus and triggers the formation of DSBs. This construct also encodes an auxin-inducible degron protein, which leads to the degradation of the endonuclease following auxin treatment (Fig. 1A). However, the endonuclease-mediated DSB inducible systems are very sensitive and therefore require tight regulation to eliminate unwanted leakiness of the system, e.g., induction of DSBs under physiological conditions due to unintended nuclear endonuclease translocation in the presence of steroid traces. In order to keep the DSB induction under control, the cells need to be maintained in phenol red-free cell culture medium supplemented with steroid-free serum, as both of these compounds can activate ER-AsiSI translocation from the cytoplasm to the nucleus and the unwanted induction of DSBs. Moreover, they can lead to the rejection of the endonuclease from the cell because the cell populations losing the endonuclease target can gain a selective advantage over the AsiSI responsive cells containing the ER-AsiSI construct.

It should also be noted that zinc-finger nucleases (ZNFs) and transcription activator-like effector nucleases (TALENs) were among the first techniques developed to induce site-specific DSBs at any given genomic position. These programmable nuclease-based systems opened new horizons for the researchers, but their site recognition and cutting efficiency was low and challenging in the design process; each different DNA target demanded tedious molecular cloning experiments. For this reason, recently, the necessity of DSB induction at any genomic position avoiding the limitations of the aforementioned techniques has led to the application of the CRISPR-associated (Cas9) system in the field of DNA repair. Although CRISPR has only been used heavily for gene correction, it has already exhibited the capacity to induce site-specific DSBs in the genome, beneficial for the subsequent study of repair kinetics using biochemical techniques [34].

The CRISPR system is a structural component of the microbial adaptive immune system mainly found in bacteria and archaea [35]. The CRISPR system, together with the Cas proteins, provide a powerful protection from viruses and morbific plasmids. The array consists of genes that produce the Cas proteins, regulatory elements and a cluster of unique sequences (spacers) interrupted by repeats of various lengths. Among the different Cas proteins, Cas1 and Cas2 nucleases are the most conserved ones of the CRISPR system and are able to bind to the foreign DNA, cut it and insert a fragment of this DNA (protospacer sequence) into the host genome in a site-specific way, which can later serve as a basis for identifying a new infection from the same virus protecting the host organism [36]. For this, a significant role is attributed to the protospacer adjacent motif (PAM) sequence of the invader, which is necessary for the protospacers (usually adjacent to the PAM sequence) to be properly recognized and targeted by the Cas1/Cas2 complex. The complex later serves to excise the protospacer and integrate it as a new spacer into the CRISPR array, usually downstream of the regulatory elements and upstream of the spacers of the array, thereby creating a chronological tree of infections. Together with the CRISPR RNA (crRNA), Cas proteins are being produced to help guide the crRNA (later processed into mature crRNA), unwinding the foreign DNA, cleaving it and subsequently creating DNA double-strand breaks (DSBs) with blunt ends, 5'- or 3'- overhangs [36].

The CRISPR-Cas system has two main classes divided into several types and subtypes [36]. Recently the CRISPR-Cas9 that belongs in Class II and Type II has been adopted and effectively used in various genome editing approaches. In Type II systems, crRNA and trans-activating RNA (tracrRNA) recruit Cas9 and together they form a complex (crRN A-T racrRNA-Cas9) that is primed for interference first scanning for PAM
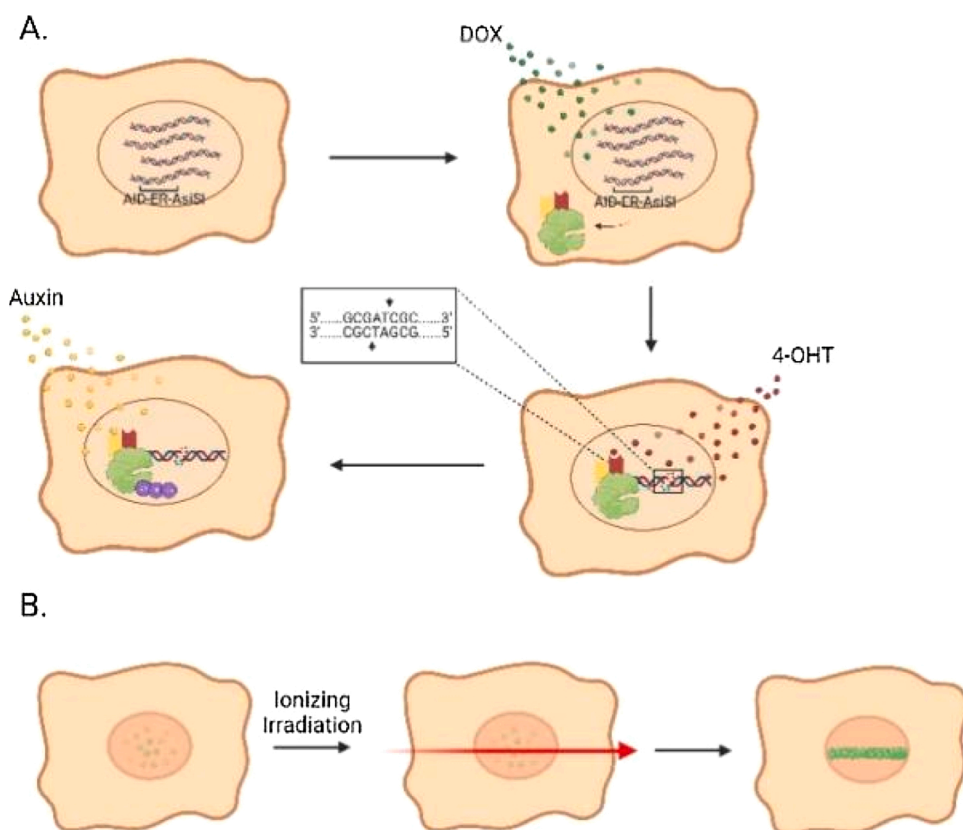
Fig. 1. A: Schematic representation of DIvA (DSB Induction via AsiSI) cell line engineered in the laboratory of Gaëlle Legube, stably expressing the AID-ER-AsiSI. Upon doxycycline (DOX) addition, the protein is expressed and sequesters in the cytoplasm. 4-OHT addition induces the translocation of the AID-ER-AsiSI into the nucleus, where it sequence-specifically cleaves the DNA. Auxin treatment leads to the ubiquitylation and degradation of the protein. B: DNA damage response upon ionizing radiation recruits GFP-tagged DNA repair proteins at the damaged foci in the nucleus.

sequence and subsequently recognizing a 10–20 nucleotide complementary region of the foreign DNA. For research purposes, the complex crRN A-T racrRNA has been engineered to form a single guide RNA (sgRNA) [36]. The genome editing technology is an accurate and cost-effective method to turn off and on genes. Besides CRISPR/Cas9, other gene editing possibilities, such as ZFNs and TALENs, are also being exploited as it was mentioned above [37]. However, for genetic manipulation, the CRISPR-Cas9 is the most commonly used and implemented system due to its specificity and effectiveness, and there are numerous online platforms that enable researchers to specifically design a sgRNA.

Cas nucleases can induce several different DNA ends after break induction, which can result in the activation of different DDR pathways. For instance, Cas9 produces blunt ends, whereas Cas12a produces sticky ends [38]. DSBs can be induced in functionally and structurally diverse chromatin regions. Euchromatin is mildly packed and transcriptionally active region, whereas heterochromatin has a massive compact transcriptionally inactive structure that is hard to access. In the latter case, damaged DNA is difficult to access, resulting in decreased DNA repair speed [39]. Furthermore, the long lifetime of Cas9 lengthens the repair process of the induced DSBs even more. It has been suggested that Cas9 endonuclease remains tied on the substrate DNA after break induction, restraining the recognition of the break by DDR proteins and impeding the DSB repair [40,41]. Another limitation, which needs to be considered in CRISPR-Cas9 systems, is the off-target effects which could be bypassed by engineered Cas9 proteins, sensitive detection methods and improved delivery systems [35,42]. Numerous orthologs of Cas effector proteins enable regular genome editing in a site-specific fashion by generating DSBs and introducing the desired modifications into the genome [36,37]. For instance, as in mammalian cells, NHEJ is the dominant way of repairing broken ends in an error-prone manner, harnessing CRISPR-Cas9 to generate a DSB within a specified locus ultimately leads to gene knock-out. On the other hand, HR has high fidelity when repairing DSBs, but it has a low incidence, and therefore researchers usually deliver an exogenous DNA template together with the CRISPR system to promote HR repair.

Endonuclease-based site-specific DSBs are advantageous tools. They can be applied to determine whether a candidate protein is recruited to DSBs and to follow the binding of the repair proteins in either a euchromatin or heterochromatin milieu. Moreover, it also gives information about how far these factors can spread from the break site. The application of chromatin immunoprecipitation combined with endonuclease-based DSB induction also provides evidence whether a repair protein is recruited in the close vicinity of a DNA break or spreads along the chromatin surrounding the DSBs. Nevertheless, as the number of breaks induced by an endonuclease is low, this method is not appropriate for investigating checkpoint-kinase activation as a response to DNA damage.

2.2. Laser microirradiation

A major advantage of the microscopy-based laser methodology is the combined approach, wherein integrated confocal microscopy can be used to visualize the formation of DNA breaks and follow the repair kinetics in real-time. Several various laser wavelengths have been used so far, including UV (240–410 nm), green (two-photon, 520–570 nm) and Near Infra-Red (NIR or three-photon, 750–800 nm) lasers. Additionally, pre-treatment with photosensitizers, such as halogenated nucleotide analogues [Bromodeoxyuridine (BrdU), 5′-iodo-2-deoxyuridine (IdU)] or dyes bound to the DNA minor groove (Hoechst 33258, Hoechst 33342) of the examined cells is required prior to UVA irradiation to allow the energy absorption necessary for inducing DSBs. BrdU is used as a nucleoside source, and it can be incorporated into the DNA in cultured cells in the S phase of the cell cycle. To this end, UVA (absorbed poorly by DNA) can photoactivate BrdU and result in the production of neighboring single-strand breaks (SSBs), which can subsequently give

rise to DSBs [43]. The combination of BrdU-UVA requires ten-times fold less energy to induce γH2AX foci formation than the sole UVA [44]. On the other hand, the DNA-bound dyes (Hoechst 33258, Hoechst 33342) can get activated upon UVA irradiation and inflict the production of DSBs. However, Hoechst 33342, which preferentially binds to A-T rich regions at 405 nm wavelength, is reported to produce not only DSBs but also residual cyclobutane pyrimidine dimers (CPDs), making the study of DSB repair pathways problematic. On the other hand, though these two dyes are chemically similar, Hoechst 33258 is mainly localized in the cytoplasm and only a small amount can enter the nucleus. This led to the application of combined pre-sensitization using BrdU/Hoechst followed by UVA treatment which was reported to resemble the lethal response of UVB and UVC microirradiation. Even in this case, the mutagenicity of Hoechst dyes prompted the development of a thiol derivative of BrdU (referred to as SBrdU), which can lead to fatal outcome in response to UVA without the need to use an additional chromophore [45]. However, novel damage-specific techniques which do not require either BrdU or Hoechst dye incorporation for pre-sensitization are still under development.

Without pre-sensitization, a high-dose of laser irradiation is required to induce DSBs and detect the recruitment of repair factors, which can often lead to complete destruction of the chromatin structure at the chosen genomic locus [46]. However, this phenomenon is not usually observed in general DDR processes and can easily be misinterpreted. From this point of view, two- and three-photon lasers provide a solution for minimizing unwanted excitation outside of the focal area, which also yields an effective improvement in resolution. Furthermore, these laser systems have been used by different laboratories to induce spatial localization of sequence-specific DNA damage. Specifically, laser-induced two-photon excitation in combination with psoralen-modified triplex-forming oligonucleotides (psoTFOs) can direct the spatially targeted induction of interstrand crosslinks (ICLs), which may give rise to DSBs [47]. Moreover, laser microirradiation applied in cells expressing photoactivable green fluorescent protein (PA-GFP)-tagged repair proteins enables time-lapse microscopy and photobleaching techniques [Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Loss in Photobleaching (FLIP), Förster (or Fluorescence) Resonance Energy Transfer (FRET)] to be performed allowing molecular interaction and protein-protein co-localization to be investigated at a distance of 8−10 nm or less (Fig. 1B) [48]. This method also allows monitoring the dynamic changes of the chromatin structure following DNA damage. Furthermore, the function and the expression levels of all GFP-fusion proteins should be compared to their endogenous counterparts, as kinetic data rely on experimental systems that involve GFP-fusion proteins. During the application of this method, the researcher must also consider that the laser parameters – wavelength, pulse length, pulse frequency, variable "zoom" of the microscope/laser system, exposure time and energy – largely affect the type and number of lesions induced by the beam, and consequently determine the cellular response. Thus, these parameters have to be stringently determined for each experiment and well-described for the readers in order to be able to compare and reproduce the results and make reliable conclusions. According to this, in 2009, Kong and colleagues investigated the different DNA damage responses by comparing low and high dose UVA (with or without BrdU pre-sensitization), green and NIR lasers [44]. Furthermore, a growing body of literature has examined the differences in DNA lesion types produced by different laser systems, which showed that oxidized DNA bases (shown by 8-oxoG detection) are observed only under high dose UVA laser, whereas base cross-linking (shown by CPDs and 6−4PP detections) and SSBs (shown by PARP-1, XRCC1 and FEN1 detection) can be induced by nanosecond UVA, picosecond (and nanosecond in case of SSBs) green and femtosecond NIR laser [44,49]. On the other hand, the degree of aberrant DSB responses seems to be higher using high dose UVA (without pre-sensitization) than in pre-sensitized low-dose UVA, while they are also reported to be produced under green and NIR lasers (shown by KU detection). Additional studies

demonstrated that DSBs (shown by γH2AX, NBS1 and RAD50 detection) can be induced efficiently at even higher wavelengths, such as 800, 1035, and 1050 nm [44,50]. It is important to note that microirradiation is the most commonly used method to target specific compartments within individual cells and visualize repair events in unique cells (Fig. 1B). FRAP is suitable for revealing whether a candidate protein is recruited to DNA lesions and helps to determine the exact time-point of the recruitment after damage induction and to follow the interaction kinetics between repair proteins and damaged DNA. However, microirradiation is inconvenient for checkpoint-kinase activation studies or ChIP-seq methods in cell populations, as the laser is not site-specific and can irradiate only a small fraction of cells simultaneously.

Particle microirradiation (emitted by the decay of radioactive atoms, such as uranium, thorium, actinium, radium, polonium etc.) is a highly understudied method due to its technical difficulties and cost. Although α-particles have greater ionization ability, due to their large mass, they cannot penetrate e.g. flasks, medium, tissues, etc., properly, making their application in cell culture-related studies more challenging. To make radiobiological studies possible in mammalian cells, an α-particle irradiation exposure apparatus was developed [51]. However, α-particle irradiation is large enough to cause severe health problems that correlate with intricate DDR [52]. DSBs are the predominant outcomes of α-particle irradiation [or high-LET (linear energy transfer)]. Bannik and collegues have recently reported that the exposure of different tumor cell lines with Ra-223 resulted in time- and dose-dependent increases in DSB foci formation that were induced by the α-particle component of Ra-223 [53]. The complexity and the potency of the DNA damage caused by high-LET irradiation differs significantly from the damage derived from low-LET irradiation (X- and γ-rays) which causes more 'simplified' and more efficiently repaired DNA damage, as well as more precisely re-ligated DSBs [54–56]. Due to this, particle irradiation has led to its implementation into cancer therapy as unlike laser microirradiation, α-particle irradiation is not delivered via laser beams but rather systematically in conjugation with other agents to target cancer cells.

3. Techniques used to study DSBs

3.1. Microscopy-based visualization of DNA repair

3.1.1. Epi-fluorescence microscopy

Fluorescence microscopy is the most widely used approach to visualize DNA repair foci. To follow the recruitment of repair proteins, two methods are used for visualizing the protein of interest (I) by using specific primary antibodies combined with fluorophore-conjugated secondary antibody detection, or (II) by fusing the desired protein with fluorescent proteins (FPs) [e.g., Green Fluorescent Protein (GFP)] [57]. While antibody-based detection is mainly applied in fixed cells, FPs allow their monitoring by time-lapse microscopy in living cells. For detection of endogenous proteins, a primary antibody against the desired protein is necessary, while FP detection requires the overexpression of the protein of interest. However, anti-FP antibodies should also be applied to detect the fusion protein if the fluorescent signal is not strong enough. For immunodetection of the desired protein, the major steps are as follows: formaldehyde fixation, permeabilization, blocking of non-specific binding of primary antibodies, then primary and subsequently fluorophore-conjugated secondary antibody staining (Fig. 2). Two types of immunostaining can be used. In the case of direct immunofluorescence, specific primary antibodies are conjugated with a fluorophore, whereas additional fluorophore-conjugated secondary antibodies are needed for the indirect immunofluorescence detection. Both methods have advantages and disadvantages. (I) The direct method
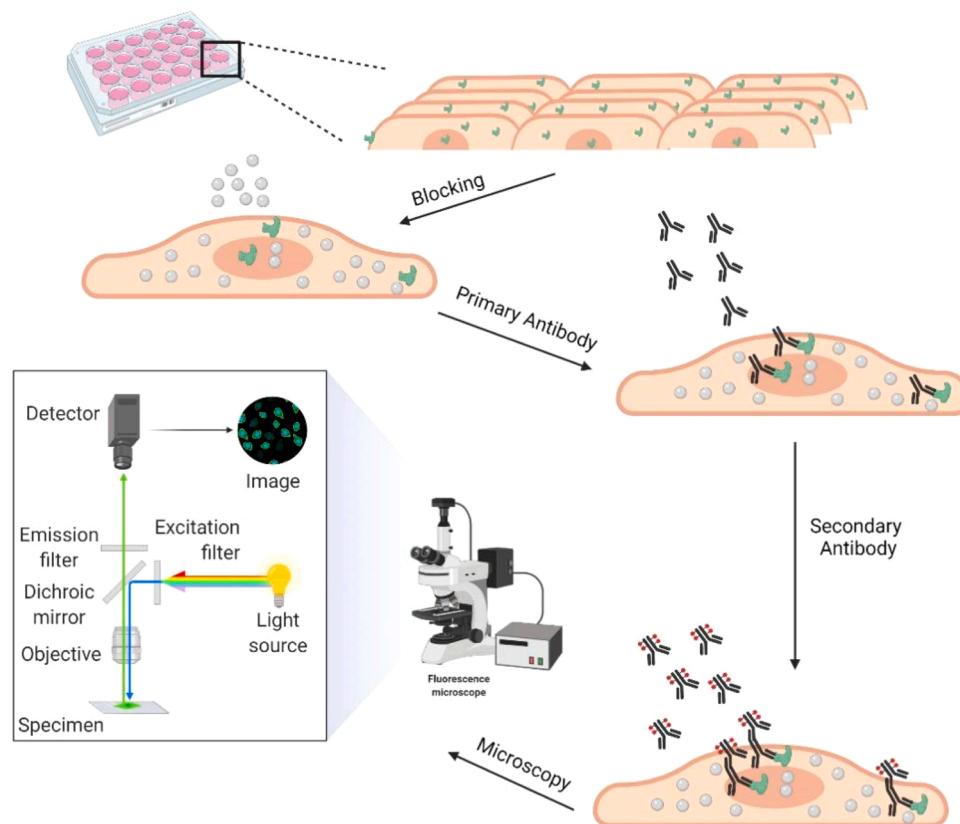
Fig. 2. Indirect Immunofluorescence Microscopy. Following blocking the unspecific regions (indicated with grey bubbles), specific primary antibodies are used to bind the protein of interest (labeled with green). Secondary antibodies raised in different species than the primary ones and conjugated with specific fluorophores are then used to bind the primary antibody and give a specific emission wavelength under excitation in a fluorescence microscope.

is much faster, and cross-reactivity between secondary antibodies can be avoided. However, it is an expensive process, and it can reduce the efficiency of highly abundant signals. (II) The indirect method is much cheaper and more sensitive because more secondary antibodies can bind to one primary antibody, amplifying the signal. Although this method can result in a high background, as secondary antibodies can react with the endogenous immunoglobulins and can also crossreact with each other.

To visualize the protein of interest, several fluorophores have been used with different wavelengths. Fluorophores are characterized based on their absorption and emission properties, which also influence their combinatorial usage. The specific fluorophores selected for microscopy irradiation often suffer from photobleaching, which depends on the characteristics of the laser beam and high-power density, which increase the emission intensity up to the point of dye saturation. Therefore, balancing fluorophore saturation with laser light intensity levels is critical in achieving the optimal signal-to-noise ratio for microscopy. Although the subset of fluorophores that are used in confocal microscopy is being rapidly expanded, the combination of these probes is still limited. When combining these dyes, the absorption and emission properties must be considered, as well as the complete separation of the excitation wavelengths between different dyes. Fluorescent dyes with an emission wavelength of 488 nm (green), 568 nm (red), and 647 nm (far red) can be easily separated from each other; therefore, these are the most commonly combined antibodies in the co-immunostaining methods. The Alexa Fluor dyes are sulfonated rhodamine derivatives, which display high photostability and substantial solubility in water, denoting that they can be used for both live and fixed cell preparations. Another group of dyes are the cyanine dyes (Cy2, Cy3, Cy5, Cy7), which function based on the partially saturated indole nitrogen heterocyclic core, with two aromatic units connected via a polyalkene bridge of varying carbon numbers.

3.1.2. Super-resolution microscopy

The resolution in conventional microscopic methods refers to the separation of distinct positions in which the fluorophore gives a detectable signal. Conventional fluorescence microscopy techniques have specific resolution limitations. It is caused by the wavelength of light being used while the maximal point distance that can be resolved by this microscopic method is approximately 300 nm for visible light on x-y axis. Adding the 3rd dimension is technically more challenging since the focal spot dissociation minimum is approximately 500 nm in the z axial direction. To overcome the limitations of light microscopy, several newly developed microscopic methods can be applied. These methods are called super-resolution microscopy or nanoscopy. The key step is to render the molecules detectable in conventional confocal microscopy in a short period of time, which results in unique detection of the signal obtained from the same diffraction region. Theoretically, by using this method, every fluorophore-conjugated secondary-primary antibody-protein complex can be individually detected [58]. Aside from the above mentioned Alexa and Cy dyes, different wavelengths of ATTO, Dylight and Dyamics fluorescent dyes, which all possess high stability to photobleaching and reduced background signal, can also be effectively applied in STORM microscopy even in combinatorial use [58].

The most commonly used method, which gives a maximum two-fold increase in resolution compared to the conventional microscopy, uses specific deconvolution approaches called structured illumination microscopy (SIM). Apart from the resolution restraint, the major advantage of the technique is that this method can be applied to perform live-cell imaging. Additionally, this technique can also be used during laser microirradiation by combining it with photo-switchable fluorescent-tagged proteins to monitor the structural changes of the chromatin surrounding the DSBs. The maximum distance, which can be efficiently resolved, is 100 nm [59,60].

Another super-resolution method is the stimulated emission depletion (STED) microscopy, which is often combined with specific light used to initiate transitions on a switchable (ON and OFF state) fluorophore. Similar to SIM, this technique also requires mathematical models to reconstruct the final image from sets of diffraction-limited images. For STED, the resolution minimum is achieved by a specific doughnut-shaped light focus. Using STED, a lateral resolution of 70–90 nm can be achieved, while an additional advantage is that it can be also combined with live-cell imaging. Furthermore, it can be used to perform dual-color STED detection on biological samples (Fig. 3) [61–63].

The methods which use photo-activated localization microscopy/ stochastic optical reconstruction microscopy (PALM/STORM) stochastically establish the ON state at the single-molecule level, so that only a single fluorophore within a distance larger than the diffraction limit is able to emit. It must be considered that most applications of the PALM/ STORM concept rely on fluorophore blinking in the presence of excitation light, as in the method termed ground state depletion with individual molecule return. By using this method, approximately 10 nm resolution can be achieved in 2D axis, while the use of high numerical aperture objective lenses enables 3D application (with 50 nm resolution for z-axis) (Fig. 3). For quantitative purposes, the PALM/ STORM is the direct method to obtain single-molecule data acquisition during DSB repair, while the limitation of the STORM is that it can be applied only on formaldehyde- or methanol-fixed samples, which can generate artificial DSBs [64–66].

The number of research articles targeting DSB repair by using super-resolution microscopy is increasing and providing new details regarding DNA repair at a single cell and locus-specific level. These are stimulating ideas for novel approaches that will help improve the resolution of conventional microscopy.
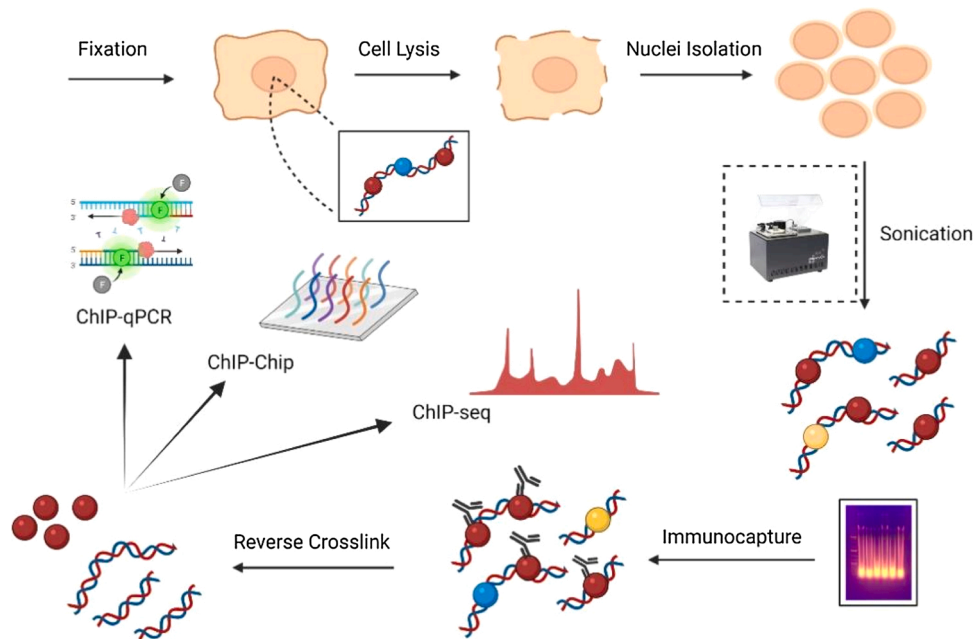
3.2. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a widely used technique to identify and elucidate differential binding patterns of proteins bound to DNA. To reveal the occupancy of a desired DNA repair protein around the DSB, a specific antibody is used to immunoprecipitate the protein of interest from the chromatin preparation together with the DNA region to which it has bound (Fig. 4) [67]. Furthermore, ChIP has been commonly applied to map the presence of specific histone post-translational modifications induced by DSBs at a given genomic locus. Although it provides information about the enrichment of a protein at a single locus or even at a single nucleosome, ChIP analysis offers a comprehensive insight into the kinetics of repair in a diverse cell population. The mapping of the profile changes of a protein of interest can be followed by ChIP-qPCR, ChIP-Chip or ChIP-seq. While ChIP-qPCR requires specific primers to detect the desired protein bound to a particular gene region, the high-throughput ChIP-seq is suitable for monitoring the occupancy of the protein of interest in a genome-wide scale.

In the first step of the ChIP procedure, chromatin is crosslinked via formaldehyde fixation, which is subsequently quenched with glycine. Then the cells are lysed, nuclei are isolated, and the chromatin is sheared either by sonication or nuclease treatment. Once appropriate chromatin size is achieved (300–1000 bp), specific antibodies against the proteins of interest can be used to pull down the DNA regions to which those proteins have bound. Next, the cross-linking is reversed, and the proteins are digested by proteinase K, so the captured regions can be further analyzed by qPCR, microarray analysis or next-generation sequencing (NGS) (Fig. 4). However, it is important to note that there are different types of ChIP: (I) X-ChIP uses chemically (e.g., formaldehyde) or UV crosslinked chromatin fragmented by sonication, while (II) N-ChIP uses native chromatin prepared by nuclease (e.g., MNase) digestion. Although fixation can improve the stability of the protein/DNA complex, it can also decrease the binding efficiency of the antibody. Therefore, if the desired protein is known to bind DNA with high affinity (e.g., MeCP2, histones, histone post-translational modifications), N-ChIP is recommended. For the fixation step, formaldehyde, UV light or cisplatin can be used. Nevertheless, each method requires an
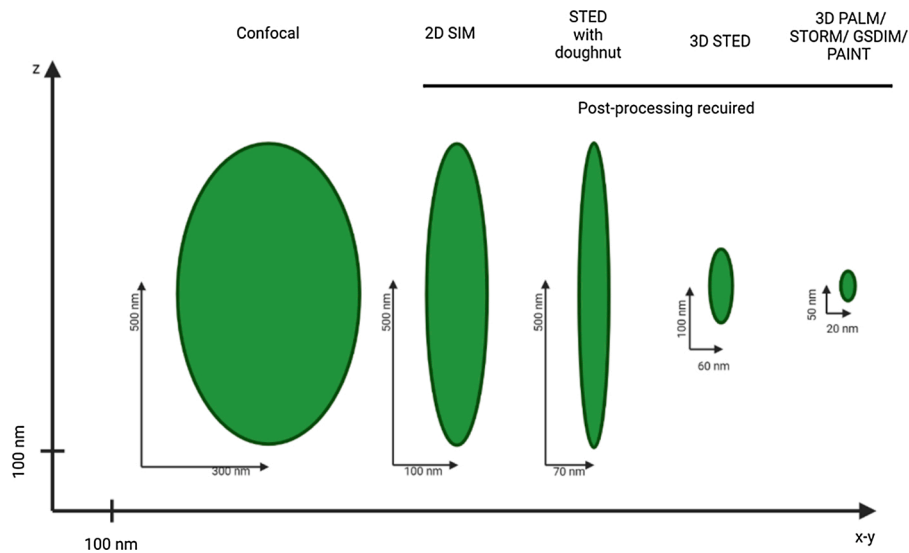
Fig. 3. The resolution limits of the super-resolution microscopy methods. Comparison of routine levels of 3D resolution obtained using different microscopy techniques for cellular imaging is depicted. The ellipsoids indicate the lateral (x, y) and axial (z) resolution levels of the methods.

Fig. 4. Chromatin Immunoprecipitation overview. Subsequently to fixation, cell lysis and nuclei isolation, chromatin is sheared to an approximately 500 bp fragment size. Following immunocapturing with specific antibodies recognizing the protein of interest (labeled with red bubbles), the cross-linking of the protein-DNA complexes is reversed, and DNA fragments are isolated and subjected to either qPCR, DNA hybridization (chip) or sequencing.

optimization step to set the appropriate conditions for fixation. Under-fixation can lead to the failure of stabilizing the interaction between protein/DNA complexes, while over-fixation can cause artificial positive data during the detection, which arises from cross-linking between distant protein complexes. Furthermore, MNase digestion and chromatin shearing are critical steps since during MNase treatment nucleosomes can be rearranged and thus modify the results. Over-fragmentation during chromatin shearing can disrupt the nucleosome-DNA interactions, while under-fragmentation can prevent the high quality of peak resolution. With careful optimization of the protocol and applying the appropriate controls, ChIP can be a very useful approach to investigate DNA-protein interactions on a genome-wide scale both *in vitro* and *in vivo*. Moreover, techniques, such as OxiDIP-Seq, have been developed in combination with ChIP to immunoprecipitate single-stranded DNA with a highly efficient antibody against 8-oxo-7,8-dihy-dro-2′-deoxyguanosine (8-oxodG) to monitor the distribution of oxidative stress-related DNA damage with high-throughput sequencing. Nevertheless, approaches, such as Cleavage Under Targets and Tagmentation (Cut&Tag) and Cleavage Under Targets and Release Using Nuclease (Cut&Run), that have been recently designed, facilitate chromatin profiling by requiring much less working time (1–2 days), fewer input cell numbers and lower sequencing depth. These characteristics make them suitable for providing high-quality data at a low cost [68, 69]. Briefly, Cut&Run uses a recombinant ProteinA/G-MNase endonuclease to target the DNA locations that contain protein-antibody complexes that are to be studied and cleaves the DNA sequence from both sides flanking the protein of interest providing specificity to the method and limiting the target site incorporation in the sequencing dataset.

Subsequently, the MNase-digested fragments are released from the nucleus and subjected to sequencing, producing less background noise compared to ChIP. On the other hand, Cut&Tag which is a variation of Cut&Run uses Protein A-T n5 recombinant transposase loaded with adapters, targets the DNA sequences bound with the protein-antibody complexes and inserts the adapters. Next-generation sequencing libraries can thus be generated using primers hybridizing to the adapter sequences.

3.3. DSB-labeling and sequencing technologies

Chromatin immunoprecipitation revolutionized the field of DNA repair when it was coupled with sequencing techniques (ChIP-seq), providing genome-wide profiling of DNA damage for the first time [70]. In ChIP-seq, DSBs are not directly labeled *in situ*; DNA-protein complexes are pulled down, chromatin is reverse crosslinked and purified fragments previously bound to a specific protein are sequenced. This can provide a bias in the genome-wide profile of DSBs as not all of them are exclusively characterized by the same bound proteins. Nevertheless, being among the first approaches to provide genome-wide data for DSBs, ChIP-seq has been utilized by many laboratories providing significant information for DNA-protein interactions during DSB repair [23].

In 2011, a novel method, integrase-defective lentiviral vector (IDLV)-mediated DNA break capture, was developed for profiling DSBs in a genome-wide scale [71]. First, cells are transduced with the linear double-stranded DNA of IDLV. The NHEJ machinery of the cell results in ligation of IDLV with broken DNA strands. Afterwards, the IDLV-tagged broken ends are enriched by linear amplification-mediated (LAM) PCR and are sequenced [72]. However, this method excludes the detection of those DSBs repaired by pathways other than NHEJ. In addition, the efficiency of transduction can vary, which may become highly challenging when using primary cells or tissues. Additional methods, apart from IDLV-mediated DNA break capture, are also based on NHEJ repair to tag the sites of DSBs and quantify their frequency, such as GUIDE-seq and

high-throughput genome-wide translocation sequencing (HTGTS) (Fig. 5) [73–75].

The necessity for nucleotide resolution of DSBs mapped throughout the genome in a direct DSB-labeling fashion led to the development of *in situ* breaks labeling, enrichment on streptavadin and next-generation sequencing techniques (BLESS) [76]. Streptavidin biotinylated linkers are used to label DSBs *in situ,* avoiding capturing non-relevant DSBs. BLESS was used to map the AsiSI cutting sites, characterizing the transcriptional status of the desired DNA regions surrounding the break sites [24]. Nevertheless, BLESS has several limitations, such as requiring a substantial amount of cells and sequential rounds of PCR amplifications, due to adapter ligation steps which are known to cause a bias in the read output.

For this reason, additional methods that are based on the BLESS approach, such as DSBCapture and END-seq, were subsequently developed to improve and eliminate the limitations of BLESS [77,78]. END-seq employs agarose plugs to protect cells from artificial break induction during the fixation step that takes place in the BLESS protocol and performs hairpin adapter ligation, which precedes A-tailing of DNA ends [77]. A-tailing is also used in DSBCapture, but without the process of embedding cells in agarose plugs. During DSBCapture protocol, the library amplification steps are reduced by using modified P5 Illumina adapters, thereby accelerating and facilitating the sequencing process [78]. Several reports have exploited the capacity of END-seq to quantify the frequency of DNA end resection, unraveling antagonistic roles during HR with potential therapeutic relevance [79].

Another variation of BLESS, immobilized-BLESS (i-BLESS), was established in 2018 and was optimized for yeast systems and enabled the use of agarose beads instead of agarose plugs (as in case of END-seq) to immobilize the cells (Fig. 5) [80]. This method has the advantage of better reagent diffusion inside the cell-containing beads demonstrating even higher sensitivity than END-seq detecting one I-SceI break site in 100,000 cells. However, similar to the other techniques, blunting of DSB sites during sequencing library preparation results in information loss of
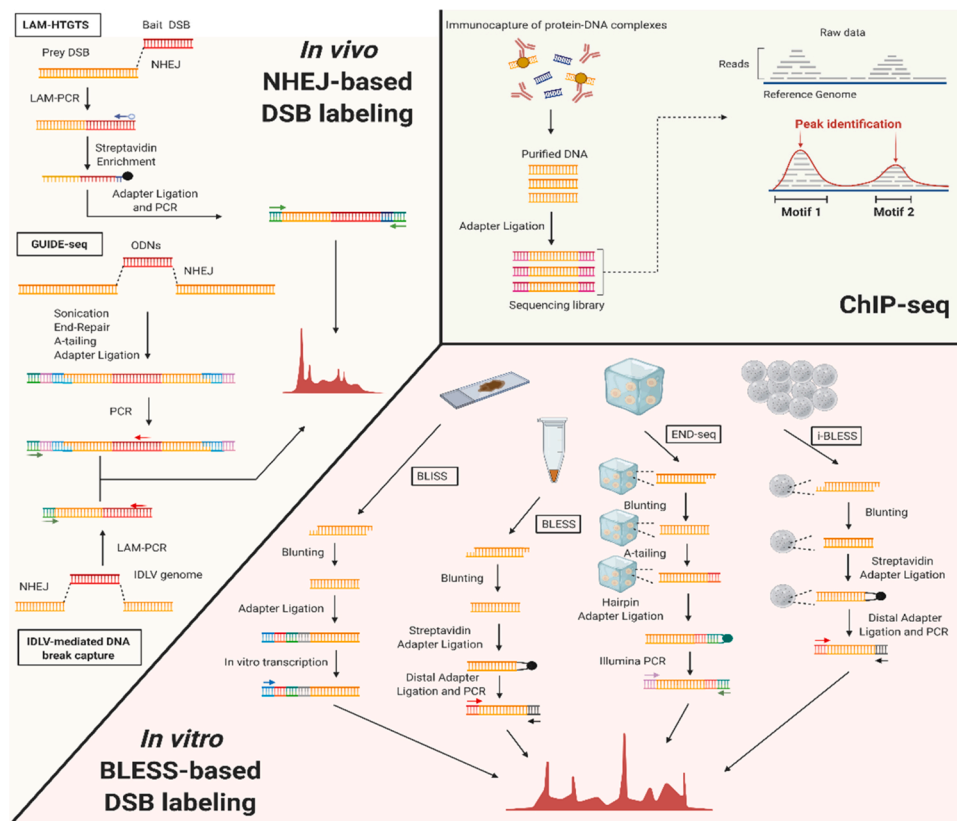
Fig. 5. Genome-wide DSB-capture and sequencing techniques. *Left panel*: in vivo techniques that are based on the NHEJ machinery of the host to incorrectly incorporate a bait double-stranded DNA at the break site. *Right upper panel*: ChIP-seq overview. Sequencing library is prepared from reads derived from immunoprecipitated reverse crosslinked protein-DNA complexes. *Right lower panel*: in vitro BLESS-based technologies that use method-specific sample preparation, DSBs processing and labeling. Colored arrows: annealed primers, arrows with an empty circle at the end: biotinylated primers, ODNs: double-stranded oligodeoxynucleotides.

the exact nucleotide position of the DSB [81].

An elaboration of the BLESS technique is called Breaks Labeling *in situ* and Sequencing (BLISS) [82], in which DSBs can be directly labeled in fixed cells or tissue samples, substantially decreasing the initial input material. In addition, instead of biotinylated adapters, oligonucleotide adapters containing T7 promoter sequence were used, which further reduced the stringency regarding the cell amount and enabled linear amplification by *in vitro* transcription of the labeled DSBs, thereby increasing the specificity of this method (Fig. 5). These adapters also contained a short sequence of random nucleotides termed as Unique Molecular Identifier (UMI) and a sample-specific barcode sequence for multiplexing and semi-quantitative measurements. Scalability was one of the limitations governing the previous techniques and was elegantly addressed in BLISS technology by using multi-well plates to perform the protocol. Evaluation of relative frequencies was also substituted with exact quantification of DSBs per cell, which gave a great advantage to BLISS over other similar technologies. However, retrieving high-quality data requires significantly complex computational work since the UMIs are diverse among samples and in different sequencing depths [82]. It is worth mentioning that a further advancement of BLISS method, called suspension-BLISS (sBLISS), has been published recently, which is compatible with any cell type maintained in suspension, providing further scalability and flexibility [83].

Eventually, a computational approach that can be coupled with most of the DSB detection and sequencing techniques will be introduced. Quantitative DSB sequencing (qDSB-seq) guarantees to overcome the limitation of relative DSB frequencies detected by other techniques, providing precise genomic coordinates as well as absolute frequencies of DSBs per cell [84]. The method relies on inducing spike-in DSBs by rare restriction endonucleases among the studied DSBs that are labeled by BLESS, i-BLESS, END-seq, or BLISS, etc. The novelty of this technique is the ability of normalizing the studied DSBs induced either globally or locus-specifically to the spike-in DSBs, whereas other methods usually normalize by the total number of reads, thereby obscuring data of global DSB induction [81].

Aside from the above described methods, recently several additional technologies, have come into the spotlight of the DNA repair field though with limited resolution. One of these is BREAK-seq which was utilized in yeast systems and employed direct biotin labeling of DNA ends with dATPs limiting the detection to 5′ overhangs of DSBs [85]. Similar examples of low resolution DSB-labeling techniques are the damaged DNA Immunoprecipitation (dDIP) method and its variation, the Double-Strand Break Immunocapture (DBrIC) during which an immunoprecipitation step with anti-biotin antibody follows the fragmentation and end-labeling with DBrIC applying radiolabeled nucleotides for DSB quantification as well [85–87]. On the other hand, more robust techniques with high resolution at a single cell level include Cas9-digested whole-genome sequencing (Digenome-seq) which was developed to identify potential Cas9-induced DSBs and its off-target sites, as well as coverage-normalized cross-correlation sequencing (CNCC-seq), which is the first technique providing genome-wide analysis of DSB end structures, providing an even higher resolution [88,89].

4. Discussion

Various biochemical and microscopic methods have expanded our knowledge in DNA repair. In this review, we summarize methods suitable for DSB induction and studying the molecular events at DSBs and their surrounding regions. (Supplementary table 1. and 2.) Since DSBs constitute a hallmark in cancer formation, it is of high interest to mimic the tumor microenvironment in order to be able to study and elucidate the mechanisms governing cancer formation, relapse and metastasis. For this reason, the induction of DSBs in various cell lines and in several ways has been the main target in the field of DNA repair and tumorigenesis. Many DSB inducing agents have been described so far having different modes of action in the DSB formation (e.g., actinomycin D,

NCS, phleomycin, etc.). Most of these agents are antibiotics isolated from *Streptomyces* species with profound anti-tumor characteristics. Many of these DSB forming compounds are applied in chemotherapy, mainly in combinatorial use due to their severe side-effects. However, in cancer research, these antineoplastic agents are suitable, convenient, and easily applicable, and it is up to the researcher to determine the appropriate agent to be used for the experimental setup and the research interest. For instance, actinomycin D causes the induction of DSBs through the stabilization of the DNA-topoisomerase I complex and the subsequent transcription elongation block, and it is usually used in transcription-related studies [90]. On the other hand, NCS is a chromoprotein anti-tumor antibiotic comprising of an unstable protein part tightly bound to a labile non-protein chromophore component able to cleave double-stranded DNA at random sites in the genome. Moreover, the methods used to induce DSBs (endonucleases, CRISPR-Cas9, laser microirradiation) should be considered carefully because they can produce various chemistries of DSBs (blunt ends, 3′- and 5′-overhangs) that might activate different DDR pathways. UV laser microirradiation, for example, induces multifarious DNA lesions (SSBs, DSBs, mutations) while the polarity of DSBs can bear either 3′-overhang, 5′-overhang or blunt ends which are recognized by different sets of enzymes for appropriate end processing. Free radicals that arise from ionizing irradiation usually lead to non-canonical DSBs carrying nucleoside 5′-aldahyde or 3′-phosphoglycolaldahyde, formyl-phosphate or 3′-keto-2′-deoxynucleotide termini, which are referred to as blocked DSBs that are not able to re-ligate and need extensive processing [91]. On the other hand, endonucleases inflict the production of DSBs with homogenous chemistries and non-blocked DNA ends.

Given the necessity of examining DNA repair in a site-specific manner and at the nucleotide level, the effects of DSBs have brought the locus-specific endonucleases (I-SceI, I-PpoI, HO, AsiSI, CRISPR-Cas9) into practice. However, the apparent advantage of this technique does not come without limitations. Some of the drawbacks are as follows: (I) the restricted number of DSBs that can be induced, which can underestimate the DDR response, (II) the cutting efficiency of the endonuclease, which can be further diminished if the cutting site is located in relatively inaccessible DNA regions (e.g., heterochromatin), (III) the tedious process of producing stable cell lines expressing the desired endonuclease, and (IV) the transfection efficiency in case of transiently transfected experimental systems. In addition, the prolonged presence of the endonuclease in the nucleus makes the measurement of repair kinetics challenging, and for this reason, the DIvA cell line generated in Gaëlle Legube's laboratory utilizes AsiSI restriction endonuclease fused with an auxin-inducible degron system [18]. If all these aspects have been properly addressed and optimized, the use of endonucleases can be a powerful tool for the researchers. Over the years, many laboratories have benefited from this approach when combined with chromatin immunoprecipitation, sequencing or microscopy techniques (extensively addressed here). Using endonuclease-based techniques, it has become possible to directly characterize DNA repair factors recruited to the endonuclease-mediated DSB site as well as histone post-translational modifications around the break site. Furthermore, a preference of specific DSB repair pathways could be elucidated according to the genomic locations, the transcription status and the chromatin landscape flanking the DSB [33]. Moreover, the triumph of CRISPR-Cas9 systems, their modifications, and improvements enabled the induction of DSBs at any desired genomic location and together with the restriction endonuclease-mediated DSB induction systems they promise to broaden our knowledge even more in the field of DNA repair and the biological responses to DSBs that potentially lead to carcinogenesis.

Super-resolution microscopy is a relatively novel technique that has revolutionized the DNA repair field since it enabled the nanoscale resolution of DSB-related studies of the chromatin structure for the first-time as well as the architectural features of DDR. Furthermore, since this technique takes the 3D structure of the cell nuclei into account,

protein-protein co-localization could be more precisely exposed even at a nucleosomal level. Using microscopy-based techniques, three criteria need to be considered: (I) the specificity of the antibody has to be validated by siRNA knock-down, (II) the overexpression of an epitope-tagged protein, though beneficial, can lead to the generation of artificial datasets due to the differences with the endogenous protein level and the subsequent molecular responses, (III) upon designing an experiment, the selection of specific controls is critical for comparison with the examined samples in order to gain valuable information from each experiment. It is also recommended that data obtained via microscopic methods be validated by biochemical methods (e.g., ChIP).

ChIP, as well as DSB-labeling and sequencing techniques described above, all constitute remarkable methods, yet tedious experimental approaches, to isolate and analyze genomic locations that suffer a DSB, quantify their frequency and elucidate the molecular repair pathways. Applying those techniques can potentially provide a deeper understanding of the location and the reason of the DSB formation, which is involved in carcinogenesis, various diseases and syndromes or even aging. Deciphering a link between the DNA sequence and the DNA damage may potentially lead to the prevention of genotoxicity or disease progression, and may even result in biomarker production.

The increasing repertoire of biochemical and microscopic techniques used in many laboratories to study DNA repair has created novel opportunities to characterize and monitor protein dynamics. The knowledge obtained by using the methods described in this review is crucial as each technique holds both advantages and disadvantages. Although some can provide single-cell and discrete locus detection, the spatial resolution limits the quality of the acquired data. Usually, to characterize the localization of repair proteins at DNA lesions, overexpression of the epitope-tagged protein is combined with conventional microscopy-based live-cell imaging. Although a better understanding of the recruitment of DNA repair proteins to the lesion site can be obtained, these methods have limited usage when trying to decipher the kinetics of the DNA repair. In contrast, ChIP-based technologies combined with next-generation sequencing provide a unique tool able to demonstrate the linear distribution of DNA repair proteins. Additionally, recent improvements, like chromosome conformation capture (3C, 4C, 5C) methods, which are able to reveal interactions between chromatin regions where DNA repair takes place, open new avenues to investigate some aspects of the DDR that, hitherto, have been only addressed *in vitro* on naked DNA or reconstituted chromatin. Nevertheless, with these techniques only a small cell population can be examined and therefore the obtained data represent only a snapshot of the current status of the DNA repair process at a specific genomic locus.

In conclusion, a combination of the biochemical and super-resolution microscopic tools is necessary to properly understand the specific function of the desired repair factors and to identify their targets in the complex nuclear architecture.

Author contributions

Wrote the paper: I.B., V.P., B.N.B., T.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mrrev.2021.108397.

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