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REVIEW

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# Imaging of protein movement induced by chromosomal breakage: tiny 'local' lesions pose great 'global' challenges

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**Abstract** Interruption of chromosomal integrity by DNA double-strand breaks (DSBs) causes a major threat to genomic stability. Despite tremendous progress in understanding the genetic and biochemical aspects of DSB-induced genome surveillance and repair mechanisms, little is known about organization of these molecular pathways in space and time. Here, we outline the key spatio-temporal problems associated with DSBs and focus on the imaging approaches to visualize the dynamics of DSB-induced responses in mammalian cells. We delineate benefits and limitations of these assays and highlight the key recent discoveries where live microscopy provided unprecedented insights into how cells defend themselves against genome-destabilizing effects of DNA damage.

## Introduction

Double-strand breaks (DSBs) are the deadliest DNA lesions resulting from the exposure to ionizing radiation, radiomimetic drugs, and also as an unavoidable consequence of stochastic errors during DNA replication (Shiloh 2003). Thus, even without any increased exposure to genotoxic stress from the environment, every actively proliferating cell experiences one or several DSBs during each cell cycle, and all these lesions must be faithfully repaired before a cell attempts to divide. Should the safeguard mechanisms fail, cells with unrepaired DSBs are destined to die or, in rare but for the organism much more dangerous cases, to survive with unstable genomes that in turn may give rise to serious diseases such as cancer (Kastan and Bartek 2004; Lukas et al. 2004b; Shiloh 2003). The key conceptual advancements covering the DSB-induced genome surveillance pathways (so-called 'checkpoints') and repair mechanisms have been recently discussed in depth on meetings (Shiloh and Lehmann 2004) and in a number of reviews (Bartek et al. 2004; Kastan and Bartek 2004; Nyberg et al. 2002; Shiloh 2003; Zhou and Elledge 2000). Our main intention here is to highlight one emerging, yet critically important, issue that remains somewhat underrepresented—namely, how the DSB-induced molecular pathways operate in space and time in their physiological environment (the nucleus of a living mammalian cell). We first define the main spatio-temporal 'problems' faced by the cell nucleus exposed to DSB-generating insults and then focus on a systematic overview of the experimental means that are currently available to visualize the key molecular events behind the DNA damage recognition, processing and repair. Each of these assays will be discussed in light of the key conceptual advancement it helped elucidate and linked to references where the more extensive technical details can be found. Finally, we conclude by a brief outline of the basic experimental requirements and controls that should accompany every attempt to study DNA damage response in live cells thereby facilitating direct exchange of the data obtained by different laboratories.

## Spatio-temporal challenges posed by DSBs

Let us start with defining the 'hurdles' in space and time that a cell must overcome to restore the DSB-disrupted chromosomal integrity. First of all, DSBs must be rapidly sensed by specific nuclear factors to timely initiate the correct type of signaling and repair programs (Petrini and Stracker 2003). And already here, at this most proximal step of the DSB response, cells face some formidable spatio-temporal problems. How are DSBs recognized? Does it happen by 'scanning' the genome by chromatin and/or replication fork binding factors? Or are DSBs sensed by a seemingly 'simple' yet potentially very ef-

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ficient mechanism including random collision with freely diffusing nuclear factors with inherently high affinity to broken DNA ends? Indeed, the latter scenario would not be unprecedented—the pioneering work by Houtsmuller et al. (1999) showed that the delivery of the nucleotide excision repair (NER) factors to the UV-damaged nucleotides occurs exactly in this way. Moreover, to bring yet another level of complexity, Bakkenist and Kastan (2003) proposed that DSB-induced local changes in chromatin topology are necessary and sufficient to instantly activate the bulk of nuclear ATM kinase, the key upstream trigger of DSB-induced signaling cascade. The intriguing (some would say, provocative) aspect of this model is that the initial steps of DBS signaling might not require physical interaction of its key upstream component with the primary DNA lesions at all (Bakkenist and Kastan 2004; Kastan and Bartek 2004).

Additional spatio-temporal problems arise when we realize that all major groups of proteins involved in various steps of DSB response (sensors, nucleases involved in processing of the DNA ends, signaling components, repair factors, various adaptor/mediator proteins) avidly associate with DSBs (Shiloh 2003). Cytologically, this is manifested by accumulation of proteins in microscopically discernible foci. However, although we have known these structures for quite a while, and we often use them as the key criterion that a given protein might be somehow involved in the DSB response, surprisingly little is known about what is the prime purpose for this massive protein concentration at the DSB-containing sites of the genome. Specifically, how do all these proteins (often large in size or potentially assembled in multicomponent complexes) organize themselves at the focal, spatially restricted DSB sites? How do they manage to coordinate the access to DSBs so that the most needed activity at a given stage of DSB metabolism prevails? Is there a strict timetable for their arrival and departure from the lesion as it emerges from the recent studies in yeast (Lisby et al. 2004), or do they interact with damaged chromosomes in some sort of a competitive fashion? Furthermore, are the spatio-temporal aspects of DSB-induced protein redistribution conserved throughout evolution or has the increased complexity of mammalian genomes added new ‘dances’ to the ‘choreography’ of the DNA damage response (Lukas and Bartek 2004)?

It is also important to realize that the potentially deleterious effects of DSBs spread far beyond the primary DNA lesions (Bartek et al. 2004; Shiloh 2003). First and foremost, generation of a DSB leads to immediate posttranslational histone modifications of relatively large regions of the neighboring chromatin. According to the original estimates of Bonner and colleagues, DSB-induced phosphorylation of histone H2AX may span up to 1 Mbp around the primary DSB lesion (Paull et al. 2000; Rogakou et al. 1999). The question is, which mechanisms determine the extent of these regions? In other words, how do cells limit spreading the DSB-associated chromatin modifications to otherwise undamaged parts of the genome? Such question is by no means trivial because DSB-induced chromatin modifications alter the physiological epigenetic marks on histones. Moreover, just like the damaged DNA strands, these chromatin modifications (often referred to as the histone code, see Jenuwein and Allis 2001 for review) must be restored before a cell attempts to divide and pass all its information to the subsequent generations (Koundrioukoff et al. 2004).

Finally, due to its seriousness, DSB response is inextricably coupled to the fundamental processes in the entire nucleus such as the cell-cycle checkpoints that transiently delay cell-cycle progression (to gain time for repair) and/or adjustment of the gene-expression program (to provide sufficient amount of repair factors and/or to foster cell-cycle arrest in case of a more complicated DNA lesion) (Lukas et al. 2004b). Since many effectors of these vital processes associate with relatively immobile structures determining the cell-cycle progression (such as origins of DNA replication) and/or the pace of the DSB-responsive gene expression (for instance, promoters of the p53-regulated genes), there must be a mechanism to rapidly export the ‘DSB alert’ in a form of active and highly mobile messenger(s). In summary, without directly addressing the above questions in living cells, our knowledge about the increasingly complex genome surveillance network remains incomplete. So, what experimental tools do we have to approach this task?

## Ionizing radiation: nuclear foci pave the way

The traditional means to induce DSBs include ionizing radiation and/or radiomimetic drugs (Shiloh 2003). Indeed, a careful combination of such ionizing radiation-induced foci (IRIF) with dynamic photobleaching approaches in live cells provided important insights into some basic principles of how the repair proteins interact with the DSB sites. A milestone in this type of experimental approach is represented by the work of Essers et al. (2002) who demonstrated that the members of the Rad52 epistasis group (mammalian components of the homologous recombination repair machinery) are highly mobile proteins that form functional holoenzymes only in the context of the DSB lesions. Moreover, while Rad51 was found as a relatively stable IRIF component, Rad52 and Rad54 underwent a dynamic exchange between IRIF and the undamaged nucleoplasm (Essers et al. 2002). This is very reminiscent of what has been originally described for the NER repair proteins (Houtsmuller et al. 1999; see above), and it has important conceptual ramifications. First, the components of the DSB regulatory network appear to rapidly diffuse throughout the nucleus in a form of individual proteins and/or small protein complexes (this means that given the size of an average mammalian cell nucleus, these freely diffusing proteins can be at any place in just a few seconds). Only after encountering the DNA breaks, these factors become transiently immobilized and assemble into high-order functional units (so-called ‘assembly on the spot’). Such a mechanism allows efficient recognition of

DNA damage by means of a random collision of rapidly diffusing proteins with the aberrant DNA structures. Second, the rapid exchange of the repair proteins (Essers et al. 2002) and other, more proximal DSB regulators (Lukas et al. 2003) generates the much needed opportunity for a flexible access of various enzymatic activities to DSBs. This, in turn, allows dynamic interaction patterns dictated by the specific DNA an/or chromatin intermediates generated during the distinct stages of the repair process. In broader terms, the above-outlined mobility patterns of DNA damage regulators follow the general principles of the cell nucleus as a highly dynamic organelle where proteins freely diffuse in an energy-independent manner to search for high-affinity binding sites (Misteli 2005; Phair et al. 2004b).

## Targeting DSBs to predefined nuclear compartments

Despite their undisputable potential, IRIF (as an experimental system) have also some severe limitations for real-time imaging. This is largely because the 'conventional' IRIF (that is, DSBs generated by ionizing radiation) are scattered randomly throughout the genome (we cannot influence when and where in the nucleus such a 'focus' appears), and it is difficult to follow the individual foci in real time in the same cell. What further complicates a clear-cut discrimination of a bona fide 'IRIF' is the fact that even the intact nucleus often contains a limited number of 'foci' representing physiological nuclear structures (telomeres, PML bodies, active replicons, etc.) where at least some DSB regulators naturally reside even without any obvious DSB-generating insult (Lombard and Guarente 2000; Mirzoeva and Petrini 2001, 2003; Wu et al. 2000, 2003; Yang et al. 2002; Zhu et al. 2000). To overcome these inherent IRIF limitations, a number of laboratories set out to develop assays that would allow a more defined compartmentalization of the nucleus to the regions that contain DSBs and those with undamaged nucleoplasm (Table 1). The first successful attempt to generate local areas with DSBs in mammalian cells, and link those with redistribution of established DSB regulators, was achieved by Nelms et al. (1998). They approached this task by irradiating mammalian cells by synchrotron-generated ultrasoft X-rays through a golden-coated grid resulting in DSB areas restricted only to the 'unshielded' parts of the nucleus. These experiments provided the first evidence that the Mre11–Rad50–Nbs1 (MRN) nuclease complex rapidly redistributes to the DBS sites and that the damaged chromosomes remain relatively fixed in their intranuclear position during the repair. Despite subsequent work using more refined assays to introduce local DSBs did reveal some limited movement of DSBs (see below), the Nelms et al.'s (1998) study provided the much needed impetus to explore the mobility of DSBs (and redistribution of their regulators) in a system based on defined DSB-containing and DSB-free areas in the same nucleus.

**Table 1** Means to generate DSBs in defined spatially restricted nuclear volumes

| Technique | Reference |
|---|---|
| Irradiation with ultrasoft X-rays through a shielding grid | (Nelms et al. 1998) |
| Cleavage of rare restriction sites by endonucleases | (Melo et al. 2001)<br>(Lisby et al. 2003)<br>(Jasin 1996) |
| Microirradiation with alpha particles | (Aten et al. 2004) |
| Microirradiation with energetic heavy ions | (Jakob et al. 2002)<br>(Jakob et al. 2003)<br>(Hauptner et al. 2004) |
| Microirradiation with UV-A lasers of cells sensitized by halogenated thymidine analogs and/or DNA intercalating dyes | (Rogakou et al. 1999)<br>(Tashiro et al. 2000)<br>(Walter et al. 2003)[a]<br>(Lukas et al. 2003)<br>(Celeste et al. 2003)<br>(Fernandez-Capetillo et al. 2004)<br>(Lukas et al. 2004a)[a]<br>(Bradshaw et al. 2005)<br>(Bekker-Jensen et al. 2005)[a] |
| Microirradiation with two-photon laser of cells sensitized by DNA intercalating dyes | (Bradshaw et al. 2005)[a] |
| Microirradiation with UV-A laser in non-sensitized cells | (Lan et al. 2004) |
| Microirradiation with Nd:YAG laser | (Kim et al. 2002a)<br>(Mikhailov et al. 2002) |

[a]Available in integrated workstations allowing continuous and interactive measurements of DSB-induced protein redistribution in vivo

## Endonuclease-mediated 'clean cuts': a lesson learned from yeast

Another approach is represented by a powerful genetic assay developed originally in yeast and based on introducing into the genome a rare restriction site coupled with conditional expression of the corresponding endonuclease. The key advantage of this system is that it generates 'clean' DSBs without additional types of DNA modifications (such as single-strand breaks, oxidative base modification, etc.) that inevitably accompany most of other DSB-generating insults (including IR). Melo et al. (2001) successfully used this approach in yeast to demonstrate that simultaneous and mutually independent accumulation of distinct protein complexes at the DSB sites is required to fully activate the DSB-induced genome surveillance program. More recently, Lisby et al. (2003) further improved this assay by making the endonuclease-inducible DSBs directly tractable in living cells (also in this case, the

authors used *Saccharomyces cerevisiae* as the model organism). This has been achieved by flanking the restriction sites by Tet or Lac repressor cassettes combined with expression of the fluorescently tagged Tet- and/or Lac-binding fusion proteins. Thanks to this elegant system, the authors were able to demonstrate that multiple DSB sites, generated at distinct genomic loci, could be assembled into shared repair centers. This is an important observation suggesting that generation of a sufficient threshold of enzymatic activities associated with recombinational type of DSB repair requires clustering of these factors into higher-order structures (so-called 'repair factories') (Lisby et al. 2003, 2004). Conceptually similar conclusion has been reached by Aten et al. (2004) in mammalian cells (see below), and the existence of the repair centers is broadly consistent with the recent findings that other crucial DNA transactions (such as simultaneous transcription of spatially proximal genes) can also cluster into centers (Osborne et al. 2004). Such a clustering of multiple sites of DNA repair or promoter regions (even from different chromosomes) would require relative long-range movement of chromatin loops beyond their otherwise constrained chromosome territories as recently suggested by Cremer and Cremer (2001) and Mahy et al. (2002).

A system utilizing a rare cutting endonuclease to induce DSBs has also been generated in mammalian cells (Jasin 1996) and proved invaluable to elucidate mechanisms of DSB repair by homologous recombination. So far, however, these mammalian studies have been primarily based on biochemical and/or cell population readouts (the latter involving for instance flow cytometry). Perhaps the main reason why this powerful system has not yet been fully exploited by real-time microscopy is that the cleavage kinetics by the inducible endonucleases is relatively slow —in yeast, it may take up to 30 min to see significant protein accumulation at the DSB sites (Melo et al. 2001). This in turn complicates direct imaging of the immediate protein rearrangements generated by acute disruption of the DNA integrity.

## Charged particles

The next group of assays to generate local DSBs is based on focal irradiation of cell nuclei by charged particles or heavy ions. Several approaches have been successfully tested and indeed produced intriguing results. First, Aten et al. (2004) irradiated mammalian nuclei by alpha particles to introduce linear tracks of DSBs. By this approach, the authors detected movement and clustering of the DSB-containing chromosomal domains, provided evidence for the involvement of the MRN complex in this process and proposed that it may explain the origin of chromosomal translocations. In a broader perspective, these findings support and extend the concept of the dynamic DSB repair centers proposed by Lisby et al. (2003, 2004) in yeast (see above).

Second, Jakob et al. (2002, 2003) designed a system capable of producing local DSBs by subjecting defined nuclear volumes to low-energy bismuth and carbon ions. These authors found that while some proteins involved in DSB repair by homologous recombination (MRN complex) avidly accumulate in the ion-exposed nuclear trajectories, DNA-PK (the key component of the alternative repair mechanism—non-homologous end joining) does not. In addition, the same group detected rapid accumulation of p21(CDKN1A) at the ion-generated DSB sites suggesting that this potent inhibitor of cyclin-dependent kinases (CDKs) and a PCNA regulator directly contributes to some aspects of DSB signaling and/or repair (Jakob et al. 2002). Finally, by comparison of the expected and observed patterns of protein clustering along the ion trajectories, these authors proposed that compaction and/or confined movement of chromatin contribute to the protein concentration in the DSB-containing chromosomal loci (Jakob et al. 2003). Indeed, a recent study by Hauptner et al. (2004), using another means to deliver energetic gold ions to focal subnuclear areas (the so-called ion microprobe SNAKE), reported frequent splitting of Rad51-containing fluorescent foci after single-ion irradiation. Thus, together with the above studies (Aten et al. 2004; Jakob et al. 2003), this observation provides yet another piece of evidence in support of small-scale chromatin movements at the sites of chromosomal breakage.

## Microlaser technologies

Although the above achievements are undoubtedly important, the relative drawback of the underlying approaches is that they require extremely specialized technology that is not readily available in most laboratories. Luckily, the recent development in laser microdissection provides an alternative solution that becomes more affordable and begins to generate important insights into the biology of the DSB response. These techniques are based on a relatively old finding by Limoli and Ward (1993) that DNA presensitized by low levels of halogenated thymidine analogs (BrdU, IdU) and/or DNA intercalating dyes (Hoechst 33258) becomes hypersensitive to the light within the UV-A spectrum. In essence, it is possible to adjust the UV-A dose to a range that does not cause detectable level of the 'classical' DNA lesions produced by UV-B and/or UV-C. At the same time, a collision of focused laser beam within an appropriate UV-A spectrum (wavelengths between 337 and 390 nm have been successfully used) with BrdU/IdU-containing DNA generates a photochemical reaction that is sufficient to induce DNA breakage (Fig. 1).

Two pioneers in developing and applying microlaser technology to cell and chromosomal biology are Michael Berns and Thomas Cremer. In an impressive series of papers published over more than three decades, Berns et al. (1969, 1971) and Berns and Floyd (1971) set the basic paradigms of cellular responses to chromosomal lesions generated by a broad spectrum of lasers. More recently, Tashiro et al. (2000) combined a pulsed UV-A laser ($\lambda$=337 nm) with BrdU presensitization to show that the Rad51 repair protein preferentially associates with post-

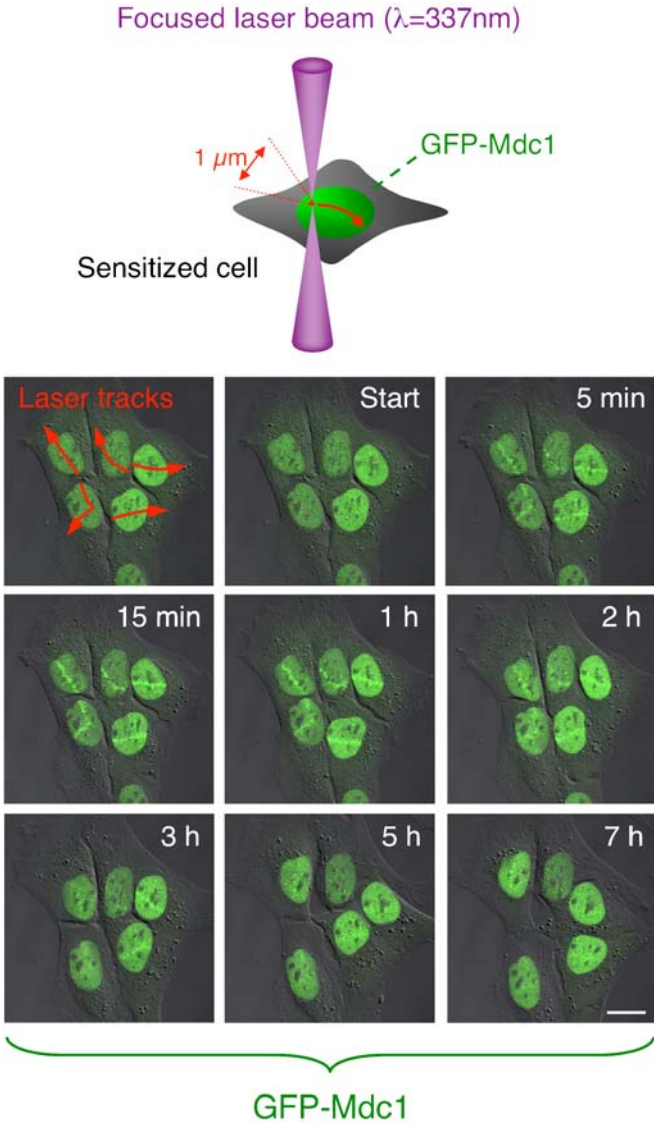


**Fig. 1** Example of spatio-temporal redistribution of the Mdc1 checkpoint mediator in and out of the laser-generated DSB sites. A derivative of the human osteosarcoma cell line (U-2-OS) stably expressing physiological levels of GFP-Mdc1 was sensitized with BrdU (10 μM; 20 h) and subjected to microirradiation with pulsed UV-A laser ($\lambda$=337 nm) to generate linear tracks of DSBs (*upper panel*; the *red arrow* indicates the laser movement during microirradiation). To minimize the extent of DNA damage to the degree compatible with efficient repair, the laser output was set to the minimum energy that was still able to produce local DSBs. According to our comparisons of the dynamics of protein redistribution and/or the persistence of γ-H2AX-modified chromatin in laser-microirradiated cells with those exposed to ionizing radiation (in the latter case, 1 Gy generates approximately 40 DSBs), we estimate that each laser track contains approximately 100–200 DSBs. The microirradiated field (*bottom panel*) was subject to time-lapse recording spanning the entire duration of the DNA damage-induced response starting from the initial assembly of GFP-Mdc1 in the DSB-containing nuclear tracks (clearly discernible at the 5-min time point) and culminating with its dissociation back to the nucleoplasm after completion of DNA repair (completed between 3 and 5 h after microirradiation). The selected images illustrating the key DSB-associated events are presented as an overlay of fluorescence and differential interference contrast (*DIC*) channels. Further technical details could be found in Lukas et al. (2003, 2004a). Scale bar= 10 μm

replicative chromatin, thereby defining a window of opportunity for the recombinational repair of DSBs. The latter study also provided a valuable biophysical analysis of the laser-induced DNA damage including calculation of the extent of DNA single- and double-strand breaks in the microirradiated nuclear tracks. Most recently, Walter et al. (2003) further developed this assay by generating a semi-automatic system for generation of spatially restricted DSBs by a UV-A laser ($\lambda$=364 nm) integrated directly in the widely used, commercially available laser-scanning microscope. Although not directly linked to DNA damage, it is undoubtedly worth mentioning the contribution made by Cremer et al. (1982) in closely related areas of nuclear biology—using a UV microlaser, Cremer et al. designed ingenious experiments showing for the first time that each chromosome occupies a distinct nuclear territory.

Another important impetus for introducing laser technology to DNA damage and repair fields came from Rogakou et al. (1999). Rogakou et al. (1999) generated DSBs by another UV-A laser ($\lambda$=390 nm) to show that phosphorylation of histone H2AX (γ-H2AX) rapidly follows DSB generation in sizable parts of the DSB-flanking chromatin. They also showed that the repair factors Rad50, Rad51, and Brca1 undergo a sequential assembly at the DSB sites where they co-localize with γ-H2AX. Using a similar technology (a 337-nm laser line), Celeste et al. (2003) demonstrated that the interaction of DNA damage regulators with the UV-A-generated DSBs proceeds in a bimodal fashion. Thus, while the initial migration of proteins to the DNA breaks is independent of H2AX phosphorylation, the retention of these factors at the DSB sites strictly requires the presence of γ-H2AX in distinct chromosomal areas that the authors nicely term as the DSB-surrounding 'chromatin microenvironment'. Fernandez-Capetillo et al. (2004) also successfully used the laser technology to uncover a novel DSB-induced chromatin modification—namely, phosphorylation of histone H2B on Ser14. Finally, by a similar approach (a 390-nm laser line) and by implementing microirradiation by the two-photon laser ($\lambda$=790 nm) of Hoechst 33258-sensitized cells, Bradshaw et al. (2005) observed early accumulation of the telomere-binding protein TRF2 around the photo-induced DSBs. Apart from the intriguing observation that a genuine telomere regulator may also participate in general response to broken DNA ends, the somewhat unexpected aspect of this work is that TRF2 migration to the DSB sites appeared to be extremely rapid and apparently independent of any of the key upstream DSB regulators including the MRN complex, so far the 'hottest' candidate for the DSB sensor (Petrini and Stracker 2003).

Our own laboratory has been systematically using the UV-A laser technology to study the mechanisms that modulate mammalian cell-cycle progression (so-called 'checkpoints') in response to DNA damage. Here, the focal illumination of defined nuclear volumes with a pulsed UV-A microlaser ($\lambda$=337 nm) of BrdU-sensitized cells proved instrumental to identify two distinct modes of protein interactions with DSBs (Lukas et al. 2003). Specifically, one class of proteins (represented by the Nbs1

component of the MRN complex) becomes concentrated at and around DSBs through a dynamic exchange between the damaged sites and the surrounding nucleoplasm, similar to what has been described by Essers et al. (2002) for some of the DSB repair factors. On the contrary, a smaller group of proteins (a class represented by the Chk2 and Chk1 kinases) becomes only very transiently associated with DSB from where (after phosphorylation by ATM) they rapidly distribute in their active forms throughout the nucleus (Lukas et al. 2003; and our unpublished results). The latter finding set a precedent for a 'DSB messenger' capable of a rapid functional connection of the focal DNA breaks with the checkpoint effectors anywhere in the nucleus.

More recently, we managed to couple the DSB-generating microlaser with the laser-scanning confocal microscope and spectral detector into an integrated imaging unit allowing continuous monitoring of even the earliest (in a range of a few seconds) protein assembly events at the acutely generated DSBs (Lukas et al. 2004a; Bekker-Jensen et al. 2005). As this unit allows also interactive photobleaching and adjustable time-lapse recording, such a system provides a versatile research tool to study the dynamics of protein trafficking in and out of the DSB sites throughout the entire checkpoint response (spanning the DSB detection, signaling, repair, recovery and resumption of cell-cycle progression). We have used this technology to strengthen the notion that the ATM phosphorylation events are generally restricted to the DSB sites and provided evidence that these types of enzyme–substrate interactions are typically very transient and do not manifest as cytologically detectable protein accumulation (in other words, ATM–substrate interaction does not require formation of IRIF). Instead, we showed that retention of Nbs1 at the DSB sites requires a 'mediator' protein (Mdc1) that is more stably integrated throughout the DSB-flanking chromatin microenvironment and whose main function might be to help concentrate the 'activated ' MRN complex in the 'legitimate' nuclear regions (that is, the DSB sites) and prevent its dispersal to the 'illegitimate', damage-free nuclear compartments (Lukas et al. 2004a). Importantly, because checkpoints, by definition, should provide time for repair and then allow resumption of cell-cycle progression, the above conclusions have been reached under experimental conditions when that extent of local damage was compatible with dissociation of the DSB signaling and repair factors after the DNA repair (see Fig. 1 as an example), followed by a productive division of a sizable fraction of the microirradiated cells (Bekker-Jensen et al. 2005).

Several groups used laser illumination to generate local DSBs without any concomitant presensitization of DNA. This has been described for both UV-A ($\lambda$=365 nm; Lan et al. 2004) and non-UV, pulsed Nd:YAG ($\lambda$=532 nm) lasers (Kim et al. 2002a). The obvious advantage of such approach is that it eliminates the concerns about the background DNA damage potentially caused by the prolonged exposure of cells to UV-sensitizers. The potential disadvantages include that the techniques using the UV-A laser in non-sensitized cells require higher energy to induce DSBs, and this may lead to generally broader spectrum of more dense chromosomal lesions and increases the risk for damage of other structural component of the nucleus. Kim et al. (2002a) combined the latter approach with immunofluorescence of fixed cells to show that the Smc1 component of mammalian cohesin (the protein assembly known to hold together the nascent sister chromatids) progressively accumulates in the DSB-containing nuclear areas in S/G2 phases of the cell cycle and in an Mre11-dependent manner. This is an interesting result suggesting that in addition to the previously described phosphorylation of Smc1 by the ATM kinase (Kim et al. 2002b; Yazdi et al. 2002), the mechanism that loads cohesin onto the acute sites of DNA damage, and the resulting local chromatid cohesion, may facilitate DNA repair and promote survival of cells exposed to DSB-generating genotoxic stress. Indeed, recent genetic experiments in yeast demonstrating de novo loading of cohesin components on the endonuclease-generated DSB sites are consistent with this scenario and provide important evidence for the evolutionarily conserved role of chromatid cohesion in maintaining the genomic integrity (Strom et al. 2004; Unal et al. 2004). It would be very informative to extend these studies by directly testing the kinetics of cohesin assembly and exchange at and around the DSB sites.

Another exciting application of the Nd:YAG laser technology can be found in a number of publications from Khodjakov et al. (2000). These authors routinely use the 532-nm pulsed laser for intracellular 'microsurgery' or ablation of cellular organelles such as centrosomes (Khodjakov et al. 2000). Recently, they also applied this technique to address an intriguing question in the DNA damage filed, namely, how do cells respond to DSBs after chromosomal condensation and irreversible commitment to undergo mitosis (Mikhailov et al. 2002). Although it has been known that mitotic cells exposed to DNA damage can activate ATM, it was not clear whether and how the ATM-controlled checkpoint signaling contributes to the mitotic delay. Mikhailov et al. (2002) performed the 'laser microsurgery' on condensed mitotic chromosomes by exposing them to pulsed green laser light ($\lambda$=532 nm). Surprisingly, these experiments revealed that the laser-generated DSBs delayed mitosis only when they disrupted the kinetochores thereby impairing proper attachment of the mitotic spindle. This, in turn, activated the mitotic spindle checkpoint, a mechanism that delays metaphase to anaphase progression completely independent of the 'canonical' DSB-induced and ATM-driven pathways that delay cell-cycle progression during interphase (Mikhailov et al. 2002).

## Future challenges and standards for advanced imaging of DSB responses

In conclusion, the above examples illustrate the potential of diverse imaging systems to study protein redistribution in and out of the spatially restricted DSBs. Each of the described techniques (Table 1) has its advantages and disadvantages, and it is therefore desirable that all these

approaches further develop to complement each other in providing a clearer picture of how the DSB-activated molecular machines operate in space and time. We feel that this particular area of research is now facing a transition when more and more laboratories are starting to use these approaches as a useful complementation of various biochemical and genetic experimental systems. We would therefore like to conclude this review by a brief outline of the most essential experimental requirements and controls. We hope that this may help set the standards for these assays in the future and facilitate direct comparison of the results obtained in different laboratories.

First of all, the future of these approaches clearly lies in constructing integrated systems allowing real-time and interactive measurements of the DSB-induced protein trafficking instantly after DSB generation and then throughout the DNA repair process. Several such units already exist (Bekker-Jensen et al. 2005; Bradshaw et al. 2005; Lukas et al. 2004a; Walter et al. 2003), and they will undoubtedly further develop in the near future. The key features of these technologies to look for include highly sensitive detectors (cameras) capable of minimizing the duration and intensity of cell illumination during repetitive image acquisition. It is important to realize that mammalian cells are extremely sensitive to light (any kind of light—not only that used for excitation of the commonly used fluorophores). Excessive illumination during long time-lapse recordings can arrest cell-cycle progression and trigger stress responses that can impair/modify the kinetics of protein interaction with DSBs. In fact, combination of the integrated imaging approaches with sensitive detectors is central to fully exploit the potential of these technologies.

Second, the real-time imaging is inevitably based on coupling the component of the DSB regulatory network with various GFP spectral variants. Here, a particular attention should be paid to the fact that overexpression of many DSB regulators may severely disrupt the natural equilibrium and exchange rate of these proteins at the DSB sites and thereby undermine the dynamics of DSB signaling and repair processes (this applies not only to transient transfections but also for various inducible expression systems). In practical terms, the most relevant results will be obtained with cell lines stably expressing physiological (or subphysiological) levels of the given GFP-tagged DSB regulator. Only these models provide a reasonable chance that the 'labeled' proteins are fully tolerated by the host cell, and that they become fully integrated into the natural DSB network. In case of high toxicity of even a slight excess of a given DSB factor, the endogenous counterpart should be reduced by RNA interference (RNAi). Ideally, mammalian cell researchers should learn from smaller eukaryotes where the GFP fusions are genomic thereby eliminating any adverse effect of overexpression. In principle, the contemporary mouse genetics combined with bacterial artificial chromosome rescue technology should be powerful enough to allow in-frame knock-in of the GFP coding sequence into physiological genomic loci to allow imaging of purely endogenous DSB regulators. The GFP-tagged fusions must be thoroughly tested for their functionality such as the productive and stoichiometric interaction with its physiological partners, the ability to undergo the DSB-induced posttranslational modifications and, whenever applicable, for their capability to rescue known defects caused by mutations of the respective genes in various human genetic syndromes.

Third, the various means to generate local DSB areas should be adjusted so that they do not saturate the natural cellular capacity to reach the cell-cycle stage where they can properly process DSBs, repair the DNA lesions and restore the epigenetic marks on the neighboring chromatin. In other words, the cells exposed to local DNA damage should survive the treatment and remain capable of resuming cell-cycle progression. Only such conditions allow direct comparison of these experiments with cellular responses to the 'natural' genotoxic stress from the outside environment and/or from the potentially toxic intracellular metabolic products. As mentioned above, several of the discussed techniques are apparently able to fulfill these criteria (see Fig. 1 for one example).

Fourth, the assays based on presensitization of DNA by halogenated thymidine analogs and/or DNA intercalating dyes must always provide controls showing that under the given experimental settings (and in the given cell type), these sensitization procedures do not activate DNA damage response per se. Again, there is evidence in the literature showing that careful titration (dose, timing) of the most commonly used presensitizers is achievable and capable of minimizing their potentially adverse affects (Lukas et al. 2003, 2004a; Rogakou et al. 1999; Tashiro et al. 2000). One emerging trend from these studies is that combination of halogenated thymidine analogs with Hoechst 33258 in the same experiment may lead to 'oversensitization' of cells thereby causing extensive scattering of the DNA damage and precluding accurate targeting of DSBs to spatially restricted subnuclear compartments. From our own experience, exposure of cells to moderate levels (10 μm) of BrdU for the restricted time period (20 h) is sufficient to increase the sensitivity to UV-A laser light ($\lambda$=337 nm) to an extent allowing generation of broad-range DSB densities without detectable 'background' damage outside the microirradiated parts of the nucleus.

Fifth, the field must urgently agree on some suitable and generally accepted internal standards to compare the kinetics of protein assembly and exchange at DSBs and the surrounding chromatin. Due to the inherent differences in various techniques to inflict local DSBs (see above), it is currently difficult (if not impossible) to compare these dynamic parameters from different laboratories. This is largely due to the fact that apart from the endonuclease-induced DNA breaks, virtually all the approaches described above share the ability to generate rather complex chromosomal damage including DSBs, DNA single-strand breaks and various base modifications (the latter being probably the most abundant type of damage). What discriminates the individual approaches is the relative proportion and local density of DNA alterations. Therefore, introducing a sufficiently sensitive surrogate compatible with real-time imaging would undoubtedly help estimate

how many of those lesions include DSBs. Based on our own experience (Lukas et al. 2004a), and the related work in yeast (Lisby et al. 2004), we would propose that the most suitable candidate for the earliest DSB-induced protein redistribution includes the components of the MRN complex. As mentioned above, MRN appears to arrive extremely rapidly at DSBs (a feature conserved from yeast to man), and it appears to have unique ability to directly interact with both DNA ends and the surrounding chromatin. A suitable standard for the delayed assembly events may then include components of the Rad52 epistasis groups of proteins (Essers et al. 2002; Lisby et al. 2003, 2004). Functional GFP versions of all the above-mentioned proteins (including the basic parameters of their intranuclear diffusion and exchange rates at DSBs) are available, and their integration to the in vivo experiments would greatly facilitate accurate interpretation of the kinetic behavior of the newly studied DSB regulators.

Finally, the emerging picture from the existing kinetic measurements of various DNA transactions (including the DSB response) strongly indicates that few (if any) of these processes operate in a strict on/off fashion. Instead, is seems that the quantitative aspects of these reactions and (sometimes subtle) adjustments of spatio-temporal dynamics of proteins and/or protein complexes dictate the pace and indeed the effectiveness of the genome surveillance programs. To extract meaningful quantitative data from the above-discussed experimental systems, the real-time imaging must be combined with an attempt to find the appropriate mathematical models that would characterize the kinetics of the intranuclear protein redistribution. Several powerful approaches have been outlined for the key nuclear metabolic events such as gene expression, chromatin compaction and DNA repair (Houtsmuller et al. 1999; Phair et al. 2004a,b), and the first attempts have been made directly in the DSB field (Essers et al. 2002; Lukas et al. 2004a). Further elaboration and standardizing of these approaches would greatly strengthen the power of the live-cell imaging to gain new and unprecedented insights into how cells defend themselves against the deadliest of all types of genetic alterations.

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