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Authors
Chiolo, Irene
Georgescu, Walter
Tang, Jonathan
et al.

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Nuclear dynamics of radiation-induced foci in euchromatin and heterochromatin

Irene Chiolo[1]*, Jonathan Tang[2,3], Walter Georgescu[2], Sylvain V. Costes[2,3]*

1. University of Southern California, 1050 Childs Way, Los Angeles, CA
2. Lawrence Berkeley National Laboratory, 717 Potter Street, Berkeley, CA
3. Exogen Biotechnology Inc., Berkeley, CA

* To whom the correspondence should be addressed: chiolo@usc.edu, svcostes@lbl.gov

Abstract

Repair of double strand breaks (DSBs) is essential for cell survival and genome integrity. While much is known about the molecular mechanisms involved in DSB repair and checkpoint activation, the roles of nuclear dynamics of radiation-induced foci (RIF) in DNA repair are just beginning to emerge. Here, we summarize results from recent studies that point to distinct features of these dynamics in two different chromatin environments: heterochromatin and euchromatin. We also discuss how nuclear architecture and chromatin components might control these dynamics, and the need of novel quantification methods for a better description and interpretation of these phenomena. These studies are expected to provide new biomarkers for radiation risk and new strategies for cancer detection and treatment.

Introduction

DSBs are the most deleterious DNA lesions. Even a single DSB can result in cell death or genomic instability [1-5], thus contributing to carcinogenesis [6]. Over the past fifteen years, the ability to follow DSB repair with imaging approaches has revolutionized our understanding of the repair process and its regulation in space and time [7]. Within seconds to minutes following ionizing radiation (IR), repair and checkpoint proteins are recruited to DSB sites, leading to the formation of RIF [8-14]. The number of RIF formed during the DSB response is routinely used to assess the amount of DNA damage and repair kinetics, as previously reviewed [15-19]. While initial studies suggested the tendency of repair sites to occupy relatively fixed positions in the nucleus [20, 21], more recent studies revealed that RIF explore the nuclear space during repair [22-28]. Most RIF motion is constrained and sub-diffusive [21, 27-29], but long-range directional motions have also been observed [21, 24-26]. Whether these dynamics are triggered predominantly in certain cell types and cell cycle phases is still unclear. Additionally, local and global chromatin changes contribute to these dynamics [25, 26, 30, 31], and the coordination of chromatin dynamics with repair progression is critical for accurate homologous recombination (HR) repair [22, 24-26, 28]. In this review, we will summarize our current understanding of RIF dynamics in the context of chromatin in various biological models. We will also discuss how these dynamics make quantification and modeling of the damage response challenging.

Non-uniform distribution of RIF suggests relocalization of heterochromatic repair sites

A great deal of evidence supports the assertion that RIF are non-randomly distributed in the nucleus. We previously showed that two DSB sensors, γH2AX and 53BP1, preferentially form RIF at the periphery of high-density DNA DAPI staining in human mammary and skin cells,

within one hour following IR [11, 13]. An independent study reached the same conclusion after observing that 53BP1 RIF patterns along densely ionizing tracks differed significantly from theoretical expectations, assuming a simple model of homogenous chromatin distribution [32]. Studies in mouse and Drosophila cells, where distinct DAPI-bright domains correspond to pericentromeric heterochromatin, revealed that this non-uniform distribution reflects exclusion of RIF from heterochromatin, at least at >60 minutes after damage induction (Figure 1A, 1C and [25, 26, 33, 34 , 35]). Importantly, this observation has been confirmed using other heterochromatin marks, such as H3K9me2me3 and the heterochromatin protein HP1 [25, 33, 35, 36]. One possible explanation for lower RIF levels in heterochromatin is better scavenging of radiation-induced radicals in these regions [18]. Radical scavengers are important factors to consider because 58% of low-LET (Linear Energy Transfer) ionizing radiation damage is generated by radicals [37]. However, a similar exclusion of RIF from heterochromatin occurs also when radical-induced DSBs are a minority, such as after exposure to densely ionizing particles, suggesting that other mechanisms are involved [13, 38, 39]. As we will discuss in this review, one main factor responsible for this non-uniform distribution is likely to be the dynamic relocalization of heterochromatic RIF to the euchromatic space during repair.

DSBs trigger 3D chromatin dynamics during homologous recombination repair

Chromatin undergoes continuous, local, and rapid motions within the nucleus even in the absence of DNA damage. These motions occur throughout different cell cycle stages, are typically non-directional, sub-diffusive, and sensitive to ATP levels [40-46], and are likely to reflect rapid opening and closing of the chromatin by remodeling complexes [31, 47]. Interestingly, the radius of the volume explored (0.5–0.7 μm) is very similar in different model systems, regardless of nuclear size [40-46]. This suggests the existence of similar constraints to motion, likely imposed by a combination of chromatin compaction, anchoring of chromosomal portions to the nuclear architecture (including centromeres and telomeres), and a nucleoplasm that behaves like a viscoelastic media that opposes movement (reviewed in [47]).

While chromatin motions are predominantly constrained in a small nuclear volume, the presence of DSBs induces a dramatic increase in the mobility of damaged sites. This response has been observed in yeast [27, 28], Drosophila [25], and mammalian cells [29], indicating conservation throughout evolution. Two elegant studies in yeast showed that a single DSB is sufficient to trigger a 3-10 fold increase in the nuclear volume explored by the damaged site during HR repair, and that this movement occurs by 'random walk' [27, 28]. The motion of repair sites in human cells is similar to those described in yeast. Human RIF explore a nuclear volume 1.7 times larger than undamaged sites, and RIF movements are consistent with a random walk [29]. It has been suggested that exploration of a larger nuclear volume facilitates HR by increasing the probability of associations between homologous chromosomes and strand invasion of homologous sequences. In fact, studies in yeast show that donor sequences and damaged templates tend to cluster together [22, 27], and defective movement correlates with defective repair [28]. Additionally, repair progression (indicated by formation of Rad52 foci) correlates with a drastic reduction in movement [27, 28], suggesting that after the homologous template has been found, DNA molecules are immobilized to facilitate strand invasion. These results point out that different phases of the same repair process might require changes in mobility of the repair site, from highly movable sites during homology search to relatively fixed sites during strand invasion.

Notably, these studies characterize repair dynamics when repair involves the homologous chromosome, not the sister chromatid [22, 27, 28]. If increased dynamics is required to facilitate the identification of homologous sequences on distant chromosomes, it is not expected to occur when the sister chromatid is used or when other pathways prevail, such as non-homologous end joining (NHEJ) or single-strand annealing. Further studies are required to clarify this point. In support of this idea, a single DSB that can be repaired by NHEJ does not undergo extensive movements in mammalian cells [20], and RNAi depletion of NHEJ components results in mobilization of this site [20]. An interesting exception is represented by unprotected telomeres in mouse cells, which resemble DSBs and are mobilized during NHEJ, likely to promote rejoining with other telomeres [48]. As a result of the positional stability of DSBs repaired by NHEJ, we would expect less RIF motion in G1 cells, but a thorough characterization of RIF dynamics throughout the cell cycle is still lacking. Krawczyk et al. observed less RIF motion in S-phase relative to G1 and G2 in human cells [29], which might reflect a general reduction in chromatin mobility during replication [43]. Importantly, these chromatin motions are not observed in the presence of other types of DNA damage [28], supporting the idea that increased exploration of the nuclear space is specifically important for DSB repair.

Interestingly, non-damaged ectopic sites in yeast also acquire more mobility in the presence of DSBs [27]. This mobilization is less extensive relative to the damaged site [27], and might reflect global chromatin relaxation in the presence of DSBs. Global chromatin relaxation in response to local damage has been described in Drosophila and mammalian cells [25, 30]. While this response has not been detected in other studies [28, 29], and its significance is still unclear, it has been suggested that it might promote DSB repair by facilitating chromatin accessibility and mobility [25, 30, 35]. Chromatin relaxation might be particularly important for sites that are otherwise difficult to mobilize, such as heterochromatic DSBs [25, 35], as we will discuss in the next section.

Altogether these results suggest that chromatin dynamics play important roles during HR repair of DSBs. Future studies are required to clarify the impact of different cell cycle phases and cell types on the extent of these dynamics, and their role in DSB repair.

Nuclear dynamics of heterochromatic RIF are characterized by directional motion

Our studies in Drosophila cells suggest that the movement of heterochromatic RIF might be different than a random walk. Here we define heterochromatin as the pericentromeric regions of the genome that are highly enriched in repeated DNAs (satellite repeats, transposons and scrambled repeats), and 'silent' epigenetic marks (H3K9me2, H3K9me3) and associated proteins (*e.g.,* HP1) [49, 50]. Heterochromatic DNA repeats are very abundant in multicellular eukaryotes, including mammalian cells and Drosophila, where they occupy about 30% of the genome, but they are absent in budding yeast. DSBs in heterochromatin are particularly dangerous because repeated sequences are prone to recombine with homologous repeats on non-homologous chromosomes, potentially resulting in translocations, formation of acentric and dicentric chromosomes, and genome instability [51]. Yet, HR is the preferred mechanism of DSB repair in heterochromatin in both Drosophila and mammalian cells [25, 52], and dynamic relocalization of heterochromatic repair centers seems to play a critical role in preventing aberrant recombination [25].

The concentration of all pericentromeric regions in one subnuclear domain in Drosophila facilitates the cytological analysis of RIF positioning relative to this compartment [25, 50]. Time-lapse analysis of fly cells showed that heterochromatic DSBs are initially retained in the

heterochromatin domain, where they cluster in large RIF that occasionally split and fuse for about 10 minutes after IR [25]. This behavior suggests the existence of strong forces maintaining the proximity of heterochromatic sequences in this initial phase of the response, likely mediated by heterochromatin proteins. In fact, RNAi of HP1a (the heterochromatic-specific isoform of HP1 in flies) results in relaxation of the heterochromatin domain [25]. Similar RIF clusters have been observed in other contexts, and will be better described later. As repair progresses, the entire heterochromatin domain expands [25], while heterochromatic RIF relocalize to outside the heterochromatin domain [25, 53], and frequently split into smaller foci [25]. Importantly, these two phases of the motion (constrained movements inside the heterochromatin domain and directional motion to outside the domain) correspond to distinct phases of HR repair progression, as judged by RIF formation. Early repair steps (DSB detection and resection) occur within the heterochromatin domain, while later repair steps (Rad51 focus formation and strand invasion) occur after relocalization to the euchromatic space [25]. Relocalization of heterochromatic DSBs to the euchromatic space has also been observed in mammalian cells ([26] and Figure 1). This movement occurs within the first 30 min after IR [25, 26], and is the most likely explanation for the exclusion of RIF from the heterochromatic domain at later time points [25, 26, 33-35].

A direct comparison between the motion of euchromatic and heterochromatic RIF in the same cell type is still lacking, but the similarity between yeast chromatin and the euchromatic domain of multicellular eukaryotes suggests that the motion of euchromatic RIF might occur through random walk (Figure 2). This is, in fact, the prevalent RIF motion observed in human cells [29]. Conversely, heterochromatic RIF feature directional motion, from inside to outside the heterochromatin domain [25, 26, 53]. Interestingly, similar directional movements characterize other repeated DNAs in response to DSBs. In budding yeast, damaged rDNA sites move to outside the nucleolus [24], and damaged telomeres relocalize to the nuclear periphery [54, 55]. This suggests that damaged sites undergo either directional movement or random walk depending on the purpose of the movement. Single copy sequences are repaired by recombination with the sister chromatid or the homologous chromosome. When the sister chromatid is not available, search for the homologous chromosome requires exploration of a larger nuclear volume and occurs by random walk. Conversely, repeated sequences are risk ectopic recombination if they randomly wander in the nuclear space. In this case, directional movement to specific locations in the nucleus might be critical to isolate repeated sequences from the bulk of similar repeats on non-homologous chromosomes before strand invasion, while promoting recombination with the sister chromatid or the homologous chromosome [25]. In fact, defective relocalization of repeated DNAs during DSB repair results in ectopic exchanges and genome instability [24, 25]. This model predicts that homologous templates for repair relocalize to outside the heterochromatic domain together with the damaged site, and further studies are required to test this hypothesis. Importantly, no separation of sister-chromatids has been observed when damaged rDNA move to outside the nucleolus in yeast [24], suggesting that in this case the homologous template for repair relocalizes together with the broken DNA. Cohesins, which are recruited to regions near the DSB sites during repair [56, 57], are likely to play a critical role in maintaining sister chromatid cohesion during RIF relocalization. In Drosophila, homologous pairing occurs throughout interphase, providing alternative homologous templates for HR repair [58, 59]. This might explain why proteins recruited after resection form RIF in Drosophila heterochromatin, not just in S and G2 phases, but also in G1 [25]. While HR is the preferred pathway to repair heterochromatin, a role for NHEJ in G1 mouse cells has also been suggested [35]. Whether RIF dynamics play a role in this context is unknown.

These observations suggest that HR repair of repeated sequences is characterized by directional motion of repair sites to the euchromatic space, in flies, humans and budding yeast. The coordination of RIF relocalization with repair progression is critical to prevent ectopic recombination and genome instability.

Mechanisms responsible for chromatin dynamics of RIF in heterochromatin and euchromatin

The distinct features of DSB-induced motions in heterochromatin and euchromatin suggest that they are controlled by different mechanisms. Directional movements of heterochromatic DSBs are potentially promoted by active mechanisms, such as nuclear actin and myosins that are responsible for the directional movement of chromosome loci during transcription [60, 61] and chromosome territory repositioning [62]. Interestingly, actin is abundant in the nucleus and a direct role for actin in DSB repair has recently been suggested [63, 64]. Alternatively, global and/or local decondensation of heterochromatin could be sufficient to provide more freedom for RIF movement, which might then proceed via a random walk in the nuclear space. Global heterochromatin expansion occurs during RIF relocalization [25], and has been observed in both mouse and Drosophila cells [25, 65-67]. Additionally, local heterochromatin relaxation is suggested by the loss of HP1a at heterochromatic DSBs in Drosophila [25], and DNA decondensation of mouse heterochromatin traversed by low energy ions [26]. After heterochromatic DSBs have relocalized to the euchromatic space, their return to the heterochromatin domain could be temporarily prevented by the formation of physical barriers, such as by re-establishing the compaction of undamaged sequences. Partial compaction of the heterochromatin domain has indeed been observed immediately after RIF relocalization [25]. Additionally, anchoring mechanisms might retain RIF to nuclear architecture components, restricting their ability to move back into the heterochromatin. Similar mechanisms participate in DSB repair of persistent damage and telomeric lesions in *S. cerevisiae*, where anchoring to the nuclear periphery requires nuclear pore components and inner nuclear membrane proteins [54, 55, 68, 69]. Relocalization of persistent DSBs to the nuclear pore facilitates the restart of HR repair mediated by enzymatic activities associated with nuclear pore components [70]. Similar compartmentalization of nuclear activities could potentially tether heterochromatic DSBs to nuclear architecture components while reactivating HR progression. Notably, Drosophila and mammalian cells feature a much larger nucleus and a more complex nuclear architecture than yeast cells, including lamins, lamin-associated proteins, and nucleoskeleton components, which can provide additional anchoring systems for heterochromatic RIF at both the nuclear periphery and the nuclear interior. For example, PML bodies seem to promote the progression of certain HR processes in mammalian cells [69, 71], similar to nuclear pores in yeast [69]. A more detailed description of the motion of heterochromatic RIF is required to understand whether active forces are involved, and to identify which architecture components contribute to RIF relocalization. Understanding these processes and their role in DSB repair are among the most important open questions in the field.

Despite the differences between spatial and temporal dynamics of repair for single-copy versus repeated sequences, studies so far have revealed surprising similarities in some of the mechanisms that control these dynamics (Figure 2). Specifically, DSB resection seems to play a critical role in mobilizing repair sites in both Drosophila heterochromatin and in yeast [25, 27, 70]. One interesting possibility is that resection promotes these dynamics by inducing the activation of the checkpoint kinase ATR, which, in turn, would trigger chromatin relaxation and

RIF mobilization. Notably, while euchromatin is in principle more 'open' and easier to mobilize, an early response to DSBs is local chromatin silencing and compaction. Transcribed genes are turned off in the proximity of DSBs [72], and HP1 variants are transiently recruited to the site of the break in mammalian cells [67, 73]Additionally, cohesins become enriched at DSBs [56, 57, 74], potentially contributing to local chromatin 'stiffness'. This suggests that chromatin compaction might be the initial state in both euchromatin and heterochromatin responding to DSBs. Contrary to the prevalent view that chromatin compaction and silencing create a repressive environment for repair, this state seems to promote initial steps of checkpoint and repair. In fact, HP1 recruitment to euchromatic DSBs facilitates HR repair progression in mammalian cells [67, 75, 76], and cohesins promote repair in yeast and mammalian cells [74, 77]. Histone modifications associated with chromatin silencing (including H3K9me and H4K20me) are also required for DSB signaling, checkpoint and repair [78, 79]. These discoveries might explain why early phases of DSB detection and repair proceed faster in heterochromatin than in euchromatin in Drosophila [25] and mammalian cells [26]. Chromatin 'stiffness' might also represent an advantage for sister chromatid exchange (SCE), where reduced mobility of the locus is likely to facilitate interactions with the template during strand invasion. On the other hand, reduced mobility would inhibit pathways that require the mobilization of repair sites, such as HR repair of repeated sequences or HR repair with the homologous chromosome. In these cases, resection-mediated checkpoint activation might be essential to induce global and local chromatin relaxation, and RIF mobilization. In fact, global heterochromatin expansion in fly cells requires both resection and ATR activation [25], and defects in this pathway affect relocalization of repair foci to the euchromatic space. In yeast, increased chromatin dynamics during repair relies on the ATR homologue Mec1 [28] and the chromatin remodeler Ino80 [31], which is targeted by checkpoint activation in response to damage [80]. Interestingly, the hypothesis that a strong resection signal is required to trigger chromatin mobility would also explain why little mobility is observed in the presence of a single DSB in mammalian cells, particularly when NHEJ is utilized for repair [20], and when the repair pathways involved do not rely on resection [28].

Another common feature between the mechanisms controlling the dynamics of repair of single-copy and repeated sequences is that the progression of repair plays an important role in DSB relocalization [25, 27, 28]. Rad51 and Rad54 are required for heterochromatic RIF relocalization to the euchromatic space in Drosophila [25], and for increasing RIF dynamics in yeast cells [27, 28]. Similarly, Rad52 and Rad51 play a role in the relocalization of DSBs to the nuclear periphery in yeast [55]. The reason why repair progression is required for repair dynamics is still unclear, but may reflect a requirement for strand invasion to ensure tethering outside the heterochromatin domain.

In summary, dynamic movement of repair sites is a common behavior during HR repair of euchromatin and heterochromatin, although directional motions might be specifically involved in preventing ectopic recombination among repeated DNAs. Global and local chromatin relaxation is likely involved in promoting these dynamics, perhaps in concert with nuclear motor components, checkpoint kinases, and repair activities. Understanding the molecular mechanisms involved is one of the most important challenge for future studies.

'Inelastic collisions' result in the formation of RIF clusters during DSB repair

As mentioned above, RIF can merge into clusters during DSB repair (Figure 3). These clusters often split and reform, suggesting that they result from random collisions associated with

Brownian chromatin motions [21, 25, 36, 65]. However, RIF clusters can also remain merged for an extended period of time [13, 23, 36, 81]. Inelastic collisions leading to clustering suggest the existence of forces that maintain associations between repair sites, likely mediated by bridging proteins. These bridges might be formed by silencing components, such as HP1 ([25], discussed in this review), or checkpoint and repair proteins, such as ATM and Mre11 [23]. Interestingly, proteins involved in late steps of DSB repair do not seem to be required for RIF clustering, suggesting that the progression of repair is not required for cluster formation or maintenance [23].

RIF clustering has been observed in many independent studies [13, 21, 23, 25, 29, 36, 65, 81], and its meaning and consequences remain mostly speculative. One interesting possibility is that RIF clustering could have different functions and consequences in different cell types, cell cycle phases, and chromatin environments. RIF clustering is potentially dangerous, because the presence of DSBs in close proximity can trigger NHEJ-mediated translocations. In fact, DSB clustering in human blood cells has been proposed as a mechanism for chromosomal rearrangements observed after exposure to densely ionizing radiation [82]. Interestingly, RIF clusters that remain permanently associated take much longer to be resolved, and get brighter and larger over time (Figure 3). It is tempting to speculate that persistent clusters represent repair sites that ultimately undergo ectopic exchanges. The potential long-term impact of RIF clustering will be further discussed in this review.

Different studies also indicate that the phenomenon of RIF clustering is enhanced by high doses of IR. When adherent human cells are exposed to increasingly higher local doses of ions, with LET ranging from 150 to 14,300 keV/µm, the concentration of RIF remains ~1 RIF/µm along individual ion tracks [83]. This suggests that clustering increases as amounts of damage increases. Similarly, we observed a reduction in the number of RIF/Gy at increasing X-ray doses [84]. Thus, it is possible that RIF clustering occurs rarely in physiological conditions [36, 65], but becomes a frequent event after acute local doses of IR, such as during cancer therapy. On the other hand, RIF clustering might be common in sub-nuclear domains characterized by more constrained motions, such as heterochromatin [25]. While RIF clustering is a potential source of genome instability, it might also have a positive role in certain repair contexts. In heterochromatin, for example, the temporary formation of RIF clusters might increase the local concentration of checkpoint and repair components to promote the damage response. The cell cycle dependence of RIF clustering is also poorly studied. For example, temporary RIF clusters observed in Drosophila heterochromatin are associated with resected DSBs, suggesting that they form during HR [25]. Conversely, mammalian cells traversed by α-particles form RIF clusters mostly in G1, suggesting a role during NHEJ [23].

Altogether, the discovery of different RIF motions, including clustering, random walk, and directional motions, motivate a revision of the concept of ‘repair centers’. This term was initially coined to propose the existence of a physical location in the nucleus where DSBs converge for repair [22, 85, 86], and the existence of these centers is still controversial. However, ‘repair centers’ are more likely to represent RIF that are initially built at the site of the break by the recruitment of repair components to the DSB and subsequent coalescence, rather than stable nuclear sites dedicated to repair. Independently formed RIFs could eventually coalesce as a consequence of their proximity to each other or the need strand invade homologous sequences on distant chromosomes. In addition, some types of damage might trigger directional motions to complete repair in specialized ‘centers’ located far away from the original site of damage, such as DSBs in heterochromatin and other repeated sequences.

Challenges in quantification and modeling of RIF kinetic data

The number of RIF at different time points after IR is frequently used as an indirect measurement of DNA repair kinetics. In this section, we will discuss mathematical models that have been developed to interpret RIF kinetics, and their current limitations in dealing with various confounding factors, including stochasticity of DSB detection by damage sensors, optical limits associated with RIF identification, and RIF dynamics.

Most studies measure RIF only at discrete time points in fixed specimens after the induction of damage. As a result, RIF that have already been resolved or that have not yet been produced are not counted, and the total number of damage sites is underestimated. This is illustrated in Figure 4, showing a time-lapse analysis of a human cell expressing 53BP1-GFP. In this example, a total of three RIF are produced over a 4-hour time course (cumulative counts), but at each time point, only two RIF are visible (observable counts). Because observable counts are the only possible measurement in fixed specimens, live cell imaging is currently the only method to compute the total number of RIF produced by IR. However, time lapse imaging has its own set of challenges, including the need to limit analysis to a lower number of cells and time points, utilization of tagged proteins that might potentially modify cell behavior, and difficulties in monitoring some DNA break sensors (e.g. γH2AX).

Ideally, one would like to combine the statistical power of fixed specimens with the accuracy of RIF counting provided by live cell imaging. To address this need, we monitored the repair response in live studies on a small number of cells to generate mathematical models that identify the parameters needed to interpret the results of fixed cells ([84] and equations 1 and 2 in Figure 4C). This approach allows to estimates cumulative counts (which would require live imaging), based on the observable counts obtained in fixed specimens The model predicts the total number of RIF (α) by correcting for the average rate of RIF formation (k_1) and the average rate of RIF resolution (k_2). One limitation of this model is that it does not account for differences in RIF rates of formation and resolution (in the example shown in Figure 4, RIF#1 persists for much longer time than RIF#2 and RIF#3). To overcome this limitation, Foray and colleagues have introduced a mathematical model (equation 3 in Figure 4C) that associates a repair probability unique to each DSB [87]. This approach yields more accurate repair rates and repair probabilities for the population of DSBs using Euler's Gamma function. However, this method does not predict the cumulated number of RIF throughout the whole kinetic, and can only fit the monotonically decreasing portion of the observable RIFs curve (dotted dark curve in Figure 4C).

RIF clustering represents an additional challenge for interpreting RIF kinetics. Clusters are made of multiple RIFs, each of which contributes to the overall signal observed. Typically, the presence of multiple RIFs results in a brighter signal, which is detected faster and persists longer. For example, γH2AX RIF are brighter after exposure to high-LET than they are after exposure to X-rays [11]; similar results have also been observed for Mdc1, Rad52 and 53BP1 [39, 84, 88]. We initially thought this was because a stronger response is necessary to repair more complex breaks. However, 53BP1 protein diffusion measurements in time-lapse studies revealed that 53BP1 proteins are not recruited to RIF faster after high-LET [39, 88]. This suggests that RIF are formed at the same rate independent of dose or break complexity, and that clustering is instead responsible for brighter, larger and earlier appearance of RIF at higher doses of IR.

In summary, both our mathematical kinetic correction for RIF formation and resolution, and the Foray model assuming unique RIF repair constant, have distinct advantages that can be combined for a better description of RIF kinetics in fixed specimens. However, the complexity of

RIF dynamics, including RIF clustering, constrained Brownian motions, and directional motions will require models that incorporate measurements of spatio-temporal dynamics. Monte Carlo models are promising methods to simulate RIF spatial movement under various conditions [13, 89] as they do not suffer the mathematical limitations of deterministic approaches. We therefore suggest integrating these stochastic methods into deterministic kinetic models to provide a more accurate interpretation of RIF experimental data.

Use of RIF spatio-temporal properties as biomarkers for repair defects and radiation risk

We propose that RIF kinetics (formation and resolution of RIF) and dynamics (3D movement of RIF in the nuclear space, including clustering) are properties that can be used to identify repair defects in individuals, and to evaluate risk factors for diseases. For example, we mentioned how persistent RIF clusters might represent DSB misrejoining. If this is confirmed, quantification of persistent clusters could be used as an assay to predict the probability of translocations, which typically results in genomic instability and cell death.

We previously estimated that DSBs located less than ~1.2 μm from each other tend to cluster within the same RIF [13, 84]. This range is in good agreement with the theoretical distances at which two DSBs trigger chromosomal rearrangements [90, 91]. In addition, clustered RIF tend to be more persistent than isolated RIF (Figure 3), suggesting that DSBs associated with RIF clusters are more difficult to repair. In agreement, recent modeling work suggested that DSBs in close proximity have a higher probability of inducing cell death than isolated DSBs [92, 93]. In this model, experimental radiation survival curves can be fitted accurately if one assumes that two DSBs located in the same large chromatin region (a unit of chromatin that spans several megabases, called a 'Giant Loop', [94, 95]) have a higher probability of inducing cell death than two DSBs on distinct Giant Loops [96]. This model could be generalized to predict the impact of ionizing radiation with different spatial dosimetry. For example, high-LET ions, which deposit high amounts of energy along their trajectory, would be more efficient at inducing cell death not only because they produce more complex fragments, but also because they induce more DSBs in close proximity to each other. In support of this prediction, increasing the LET of an α particle without changing the fluence of the beam does not increase the total number of aberrations per track traversal, and instead increases the ratio of complex to total aberrations [97]. In other words, because the number of DSBs in close proximity increases with higher LET (for LET>100 keV/μm), each RIF would consist of more DSBs, thereby increasing the probability of complex chromosomal rearrangements. High resolution imaging of high-LET tracks in combination with Monte Carlo simulation supported this idea by confirming the presence of multiple DSBs within each RIF [98].

To summarize, individuals with either deficient DNA repair or strong RIF clustering phenotype would show persistent RIF in comparison to normal individuals. Consequently, we hypothesize that persistent RIF can be used as a biomarker for long-term risk associated with DNA repair defects and radiation exposure, such as increased cell-death, chromosomal aberration, and increased cancer risk. In agreement with this hypothesis, primary lymphocytes from radiation sensitive mice with known defects in DNA repair (e.g. Balb/C and SCID mice) have RIF repair kinetics 1.5 to 6 times slower than resistant strains such as C57Bl/6J (repair rates inferred from Rube and colleagues [99]). Similarly, ATM defects that result in persistent RIF [35, 84] are associated with radiation-induced carcinogenesis in mice [100], and increased toxicity from radiotherapy in ATM heterozygous patients [101, 102]. Additionally, DNA repair deficiencies are considered risk factors for both acute radiation toxicity and cancer,

independently of the type of radiation [103-107]. Importantly, functional RIF kinetic assays may be sensitive enough to detect subtle genetic differences between individuals that would be hard to detect with more classic genomic methodologies, such as SNP arrays. For example, recent studies on primary human fibroblasts, derived from 25 apparently healthy individuals and 10 patients with DNA repair-deficient syndromes, have shown a wide variation in RIF levels and kinetics between individuals [108]. Our mathematical interpretation of RIF kinetics establishes novel metric that can characterize such subtle differences. This approach may help identify genes that have not been previously implicated in DNA repair and individuals that are sensitive or cancer-prone in response to radiation exposure.

Heterochromatin epigenetic changes are also emerging as biomarkers of radiation and cancer risk. Profound changes in the level and distribution of HP1 have been observed during cancer development [109, 110], and desilencing of heterochromatic repeats occurs at early phases of tumorigenesis [111, 112]. As we discussed previously, epigenetic changes can potentially contribute to tumorigenesis by affecting the stability of repeated sequences through deregulation of RIF dynamics. Lowering the levels of HP1a and other silencing components in Drosophila results in increased spontaneous damage, defective relocalization of heterochromatic DSBs to the euchromatic space, and aberrant recombination among repeated sequences [25, 113, 114]. Similarly, loss of silencing components results in repair defects in mouse cells [35]. Thus, epigenetic changes have a high potential to influence the risk of DSB misrejoining by deregulating RIF dynamics and HR repair. This correlation between heterochromatin marks and repair could also be exploited to develop new strategies for cancer therapy. For example, it has been shown that growing human breast cells in 3D results in higher chromatin compaction and silencing marks [115]. When breast cancer cells are reverted to a normal phenotype in models of tumor reversion, and are subsequently grown in 3D cultures, they show more efficient DSB repair, lower chromosome aberration, and better resistance to radiation or bleomycin exposures [116]. Addressing whether these phenotypes are derived from epigenetic reorganization and reestablishment of efficient DSB dynamics during repair remains critical for considering epigenetic changes as an opportunity for novel anticancer strategies.

Concluding remarks

Altogether these studies suggest the existence of opposing forces that work at DSB sites, involved in compaction and relaxation of the chromatin, motion and tethering. The balance between these activities is likely carefully modulated to promote different aspects of the repair response in heterochromatin and euchromatin, and both checkpoint and repair proteins seem to be required to regulate these transitions. The existence of nuclear architecture components that provide anchoring structures for chromatin [117] raises the questions about the potential roles of these components in constraining RIF motion and what generates the forces required for mobilizing chromatin during DSB repair. Additionally, it will be important to determine how the repair response interacts with structural and motor components to promote RIF dynamics. The roles of pre-existing and newly established epigenetic changes in these processes are also mostly uncharacterized and little is known about the various chromatin-dependent repair mechanisms as a function of cell cycle progression. Finally, important challenges for future studies include developing better quantification methods and mathematical models to describe RIF motions, and identifying targets and regulators of different aspects of these dynamics. Deregulation of RIF motions is likely to trigger genome instability and promote tumorigenesis. Thus, understanding the correlation between RIF dynamics, chromatin and nuclear architecture is expected to

promote the development of new biomarkers for radiation sensitivity and early cancer detection, as well as novel strategies for cancer therapy.

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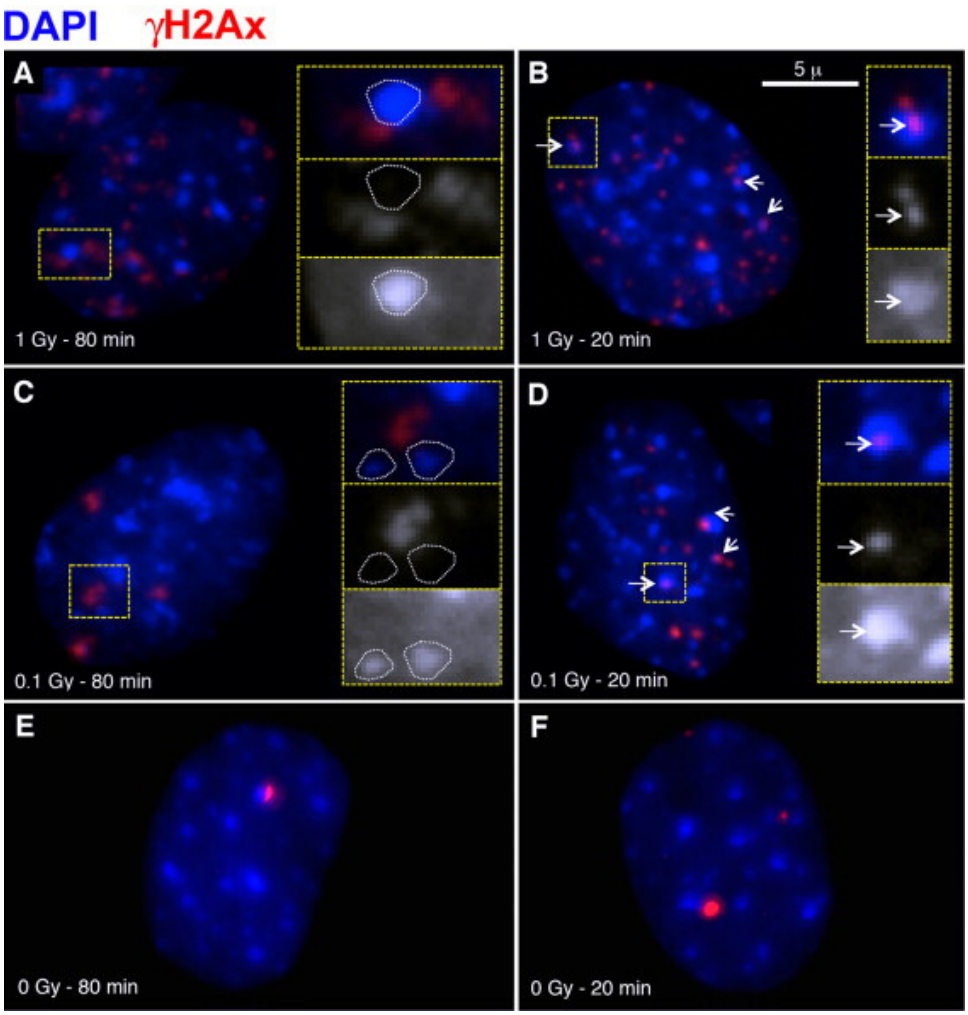

Fig. 1. γH2AX RIF move from heterochromatin to euchromatin in mouse cells. Immunofluorescence analysis [11] of Balb/C mouse cells show that γH2AX RIF (red) are mostly excluded from the heterochromatic domains (DAPI-bright regions, with DAPI in blue) at late time points after IR (80 min; A and C). Conversely, γH2AX RIF are observed inside the heterochromatin domains at early time-points after IR (20 min; B and D). This behavior is observed at high doses (1 Gy; A and B) and low does (0.1 Gy; C and D) of X-rays. Untreated cells are also shown as a reference for the two experiments (E and F). Images were acquired using a Zeiss Plan-Apochromat 40× dry objective (NA of 0.95) on the AxioObserver Z1 (Carl Zeiss, Jena, 16 Germany). Images are maximum intensity projections of seven Z-stacks taken at 0.75 μm of distance from each other.

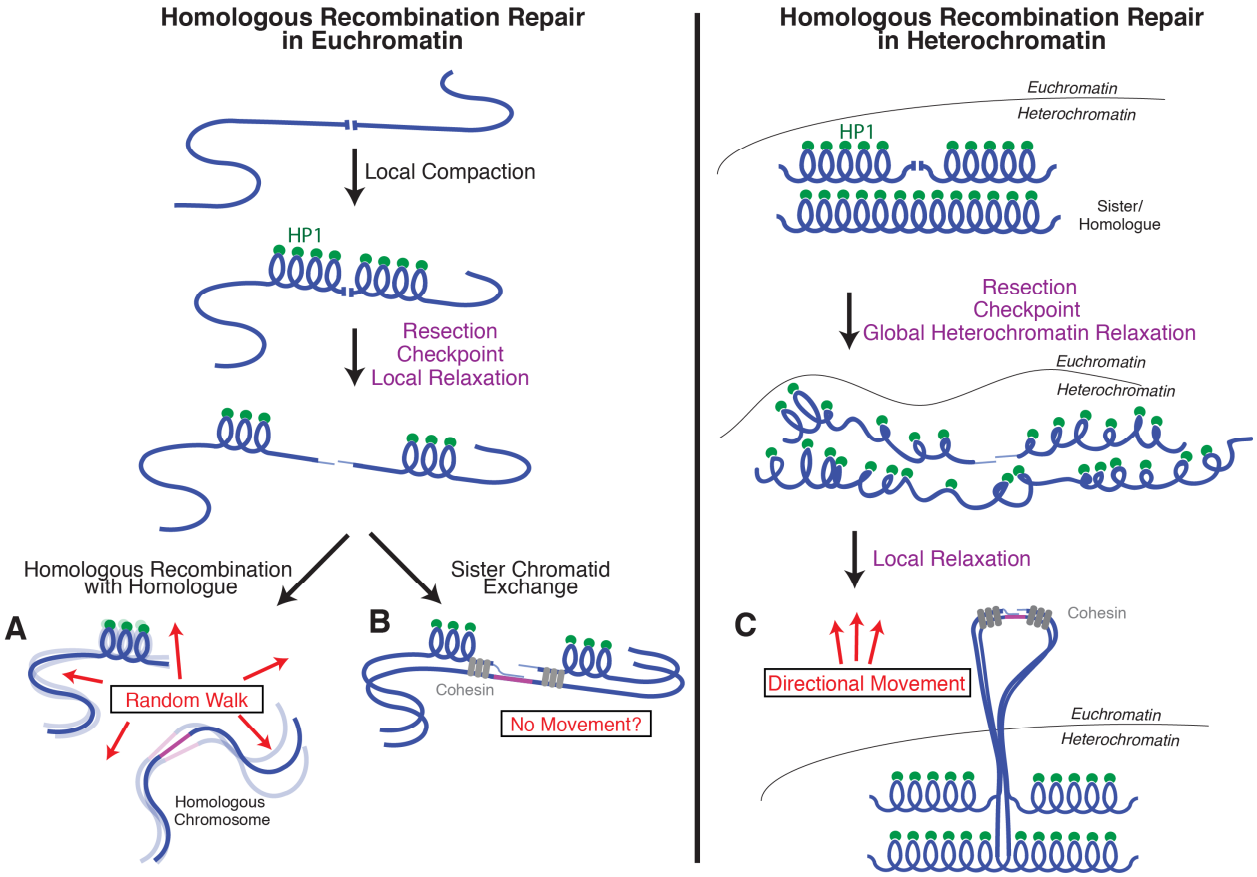

Fig. 2. Model for HR repair of DSBs in euchromatin and heterochromatin. We hypothesize that HR repair is characterized by different dynamics in euchromatin (left) and heterochromatin (right). Euchromatin is mostly composed of single-copy sequences that can easily be repaired by HR. (A) When the sister chromatid is not readily available, single copy sequences explore the nuclear volume by random walk until they find the homologous chromosome, then reduction of the movement facilitates strand invasion. (B) When the sister chromatid is available, no movement is required to complete HR. Local chromatin 'stiffness' generated by the recruitment of silencing marks (HP1) and cohesins might facilitate initial steps of sister chromatin exchange. (C) Heterochromatic repeats are potentially at risk of ectopic recombination if they remain in the heterochromatin domain, where there is a high concentration of homologous sequences present on non-homologous chromosomes. Thus, directional movement to the euchromatic domain would promote strand invasion and completion of HR using templates on sister chromatids or homologs, which would not result in detrimental translocations. Other repeated DNAs (rDNA and telomeres) feature similar directional movements during DSB repair. On the right, snapshots from a time-lapse experiment with Drosophila cells show the relocalization of heterochromatic RIF (ATRIP foci that formed inside the HP1a domain) to the euchromatic space [25] (scale bar = 1 μm. Minutes indicate the time after IR exposure). After relocalization, cohesins would still facilitate repair by maintaining the association of the damaged DNA with the sister chromatid. Alternatively, homologous chromosomes might be used as templates for repair.Notably, despite the differences between random walk in euchromatin and directional motions in heterochromatin, the mobilization of repair sites might involve similar mechanisms, including resection, checkpoint and local and global chromatin relaxation (see details in the main text).

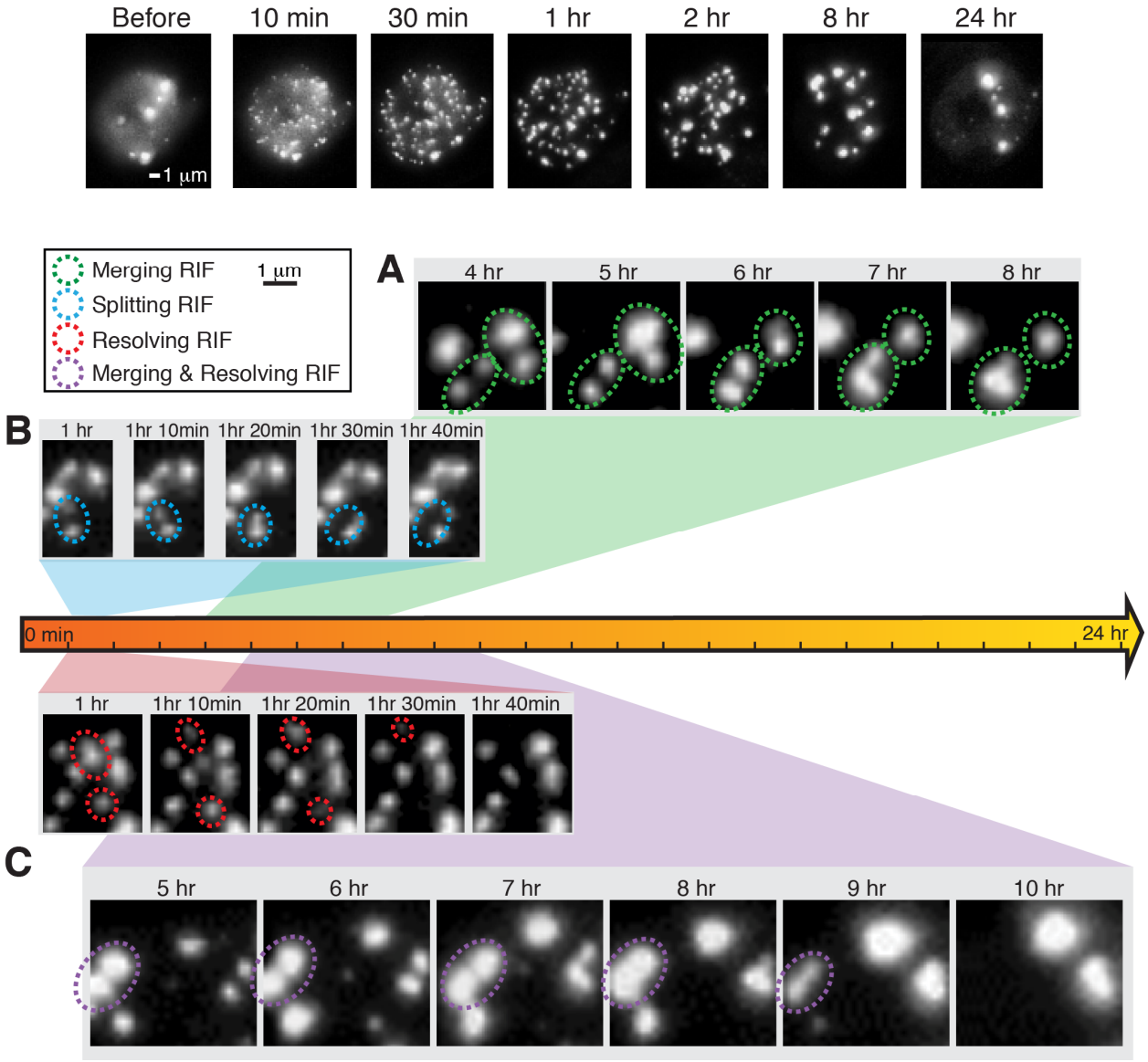

Fig. 3. RIF dynamics in live cell imaging shows elastic and inelastic collisions. Immortalized non-malignant MCF10A human breast cells expressing 53BP1-mCherry [84] were imaged at different time points after IR (2.3 Gy). Following time-lapse, Z-stacks were collapsed to a 2D image set using maximum intensity projection, and registered to correct for nuclear motions. RIF movements are highlighted in zoomed sub-panels that illustrate various collision scenarios where RIFs merge (inelastic collision, (A)), split (B) or simply resolve (C). Notably, merged RIFs get larger, brighter and take longer to get resolved.

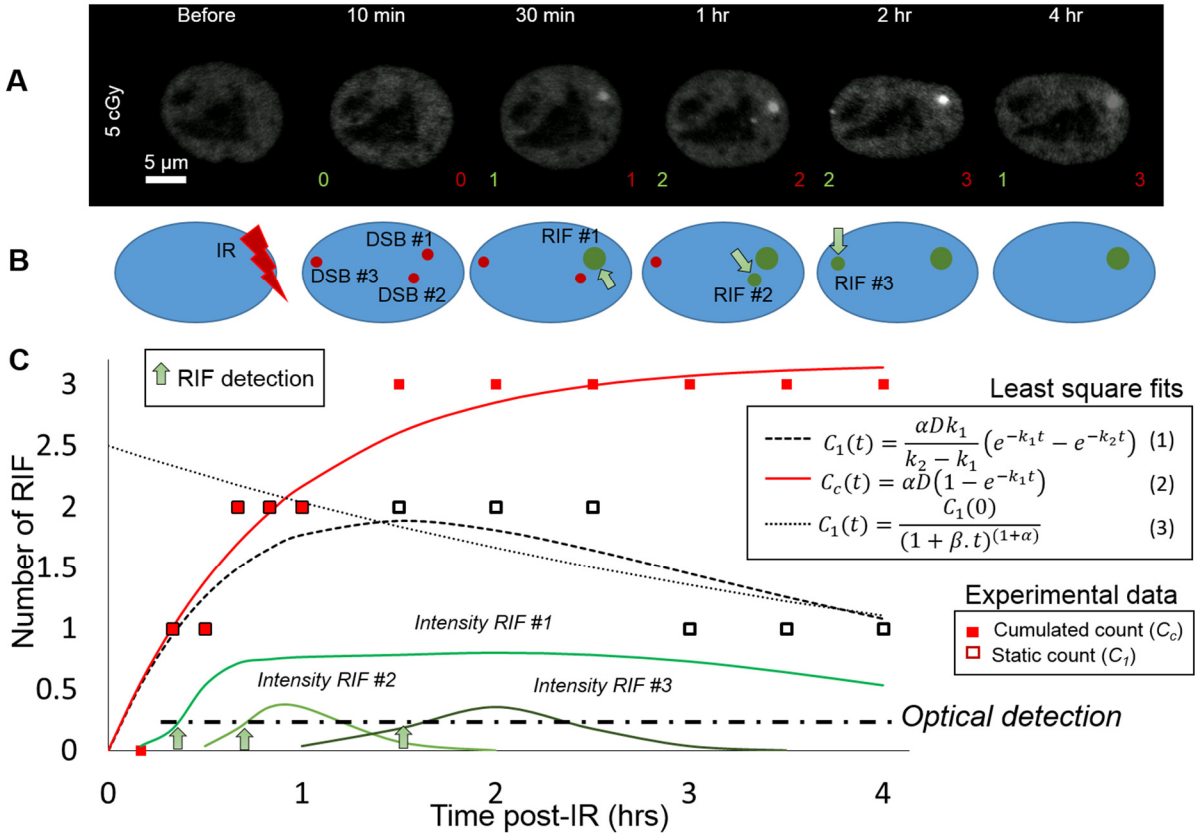

Fig. 4. The number of RIF visible at each time point is an underestimate of the total number of RIF formed over time. (A) Human fibrosarcoma HT1080 cells expressing 53BP1-GFP have been imaged at different time points after IR (5 cGy; [84]). (B) Cartoon depicting localization of DSBs (red circles) and the corresponding RIF (green circles) inside the nucleus (blue). This cartoon matches the experiment shown in panel A. Large green arrows indicate when DSB have led to a detectable RIF. (C) Quantification of RIF kinetics from panel A. Green curves in the lower part of the plot are the intensity profiles of each individual RIF as a function of time. The dotted-dash horizontal line indicates the intensity level above which RIF can be detected. Intersection of this line with intensity profiles indicates when RIF detection occurs (shown by large green arrows). The heterogeneity of repair rates can be observed, as RIF#1 is visible for much longer than RIF#2 and RIF#3. Observable RIF counts (black empty squares) and cumulative RIF counts (red full squares) can then be deduced from this detection process. Our mathematical model [84] provides a good prediction of both types of counts (Eq. (1) and dashed black line refer to observable counts, Eq. (2) and solid red line corresponds to cumulated counts). Parameters k1 and k2 are rates for RIF induction and resolution, respectively. α is the number of naked DSB/Gy before formation of RIF, and D is the dose delivered. In contrast, Foray et al. model [87] is only able to fit the repair part of one type of count (observable RIF, dotted black line). In this model, α and β are the shape and location parameters for the Gamma probability distribution function. All data were fitted using the non-linear least squares functionality of R (http://www.r-project.org/, minpack.lm package), which uses the Levenberg–Marquardt algorithm to find a minimum solution.

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