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The shape of the radiation dose response for DNA double-strand break induction and repair

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Abstract

DNA double-strand breaks are among the most deleterious lesions induced by ionising radiation. A range of inter-connected cellular response mechanisms has evolved to enable their efficient repair and thus protect the cell from the harmful consequences of un- or mis-repaired breaks which may include early effects such as cell killing and associated acute toxicities and late effects such as cancer. A number of studies suggest that the induction and repair of double-strand breaks may not always occur linearly with ionising radiation dose. Here we have aimed to identify and discuss some of the biological and methodological factors that can potentially modify the shape of the dose response curve obtained for these endpoints using the most common assays for double-strand breaks, pulsed-field gel electrophoresis and microscopic scoring of radiation-induced foci.

Keywords: DNA double-strand break, Ionising radiation, Pulsed-field gel electrophoresis, Gamma-H2AX, Dose response, Low dose

Introduction

The topic of radiation risk to health, particularly at low-doses, i.e. a few to a few tens of millisievert or milligray in the case of sparsely ionising radiation, remains important, owing largely to the increasing (yet ever more effective) use of radiation in medical diagnosis, interventional radiology and also the treatment of cancers. For many years, our knowledge of both the health effects of and the molecular and cellular responses to ionising radiation exposure has been limited to the high dose range, above 100 mSv, due to a lack of sufficiently large and well controlled cohorts for epidemiological studies on one hand, and a lack of experimental tools for assessing low dose responses on the other hand. Based on the available evidence, a linear no-threshold model was generally assumed for cancer risk [1,2]. Over the past decade, however, considerable progress has been made, as illustrated by recent studies such as those on cancer risk associated with paediatric computed tomography (CT) scanning [3] and natural background radiation [4] which help reduce uncertainties about the shape of the dose response curve for cancer at low doses. In addition to cancer, cardiovascular disease has

recently been identified as a potentially equally important contributor to radiation mortality [5]. Large funding programmes dedicated to experimental research into low dose effects and underlying mechanisms, such as the U.S. Department of Energy Low Dose program (http://lowdose.energy.gov/) and the previous (RISK-RAD, NOTE) and current EU initiatives DOREMI and MELODI (http://www.melodi-online.eu/) have supported the introduction of sensitive assays and bio-markers which provide new insights into the cellular and molecular responses at low doses [6].

Chromosomal DNA is the most important cellular target damaged by exposure to ionising radiation. Radiation-induced DNA lesions include abasic sites, oxidised bases and sugars, strand breaks and cross-links within or between the complementary DNA strands or between DNA and surrounding proteins. Importantly, radiation causes clusters of such lesions along the track of the ionising particle [7,8]. It is this ability to produce 'locally multiply damaged sites' containing two or more lesions within 1–2 helical turns of DNA [9], which distinguishes ionising radiation from the numerous other genotoxic agents that we encounter in our daily lives. One important clustered lesion is the DNA double-strand break (DSB). As it affects both complementary DNA strands, it is much harder to repair than any single-stranded lesions which can utilise the

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complementary sequence on the opposite strand as a template to ensure correct and efficient repair. In the following sections we highlight recent findings that may have a bearing on the shape of the dose response for DSB induction, signalling and repair and review methodological limitations. We concentrate on quantitative questions – numerous recent reviews have addressed the biochemical aspects of DNA damage signalling and repair (e.g. [10-13]) which are therefore not covered here. Also, the focus of this article is mainly on sparsely ionising radiations such as X- or gamma-rays; see [14] for a recent review of the DNA damage response to densely ionising radiation.

DNA double-strand break induction by ionising radiation

Strand breaks are among the most highly studied DNA lesions induced by ionising radiation. This is partly because of their important contribution to the toxic, mutagenic, clastogenic and carcinogenic effects of radiation, but may also be explained by the availability of a wide range of detection and quantification methods for these particular lesions.

Ionising radiation-induced DNA strand breaks form following attack of the sugar phosphate backbone either by direct DNA radical production or by radicals formed through water radiolysis in the vicinity of the DNA (indirect effect). DSB form when two such nicks are present in opposite DNA strands within one or two helical turns. They seem to result mainly from the attack of multiple radical hits rather than the transfer of one radical between strands [15]. However, recent electron paramagnetic resonance spectroscopy results suggest that most DSB may not be derived from trappable radical pairs [16]. In contrast to DSB induction by H_2O_2 which shows a strong quadratic response at high concentrations [17] due to the interaction of independently produced radicals in DSB induction, radiation induces DSB by radicals originating from the same radiation track, and therefore linearly with dose, at least for doses up to several hundred gray [18].

The ratio of SSB to DSB yields produced by sparsely ionising radiation is commonly estimated to be on the order of 25–40, based on the detection of relaxed circular vs. linear plasmid DNA in agarose gels following irradiation of supercoiled circular plasmids. A recent study, however, which has utilised a direct end-labelling approach for SSB detection, suggests that the true SSB yield may be 10 fold higher than previously assumed [19]. The inability of the conventional plasmid agarose gel assay to detect additional strand breaks in the presence of one break (which should occur frequently, given the clustered distribution of ionisation events for radiation) may explain the lower yields reported in previous studies. Once confirmed, this finding may have implications for the relative risk attributed to radiation-induced SSB and DSB.

Up until about a decade ago it was impossible to study DSB induction at radiation doses of relevance in occupational or radiodiagnostic settings, due to the very limited sensitivity of the DSB detection methods available, such as neutral filter elution or pulsed-field gel electrophoresis (PFGE), which all measure DSB indirectly through the associated decrease in average molecular weight or length of chromosomal DNA and in general require doses of at least several gray to detect any significant effect, though a detection limit of less than 1 Gy was reported by one group for an optimised PFGE-based assay [20].

Over the past 15 years, radiation-induced foci (RIF), each representing hundreds to thousands of individual proteins involved in the DNA damage response which accumulate in the vicinity of a DSB [21,22], have been established as surrogate markers for DSB [23]. The most widely utilised markers include 1) the phosphorylated histone variant gamma-H2AX [24-26], 2) the autophosphorylated DNA damage kinase ATM-pS1981 [27] and 3) the mediator protein 53BP1 which may play an important role in chromatin remodelling at the break site [28]. Immunofluorescence microscopy enables the spatial localisation and quantification – manually or by image analysis software – of individual RIF, each thought to represent one or more DSB. More recently, live cell imaging of cells expressing fluorescent fusion proteins that are recruited to the sites of DSB has enabled detailed studies of the spatio-temporal dynamics of RIF [29,30].

Electrophoretic DNA double-strand break assays

Although initial DSB induction by ionising radiation can conceptually be expected to occur linearly with dose, there are number of factors that can affect DSB measurements by any of the above assays. Figure 1 illustrates some of the different classes of lesions that may affect dose response or time course relationships obtained with PFGE or foci assays for DSB induction and repair. Note that their relative contributions in individual studies may differ considerably from the values shown here, depending on a wide range of experimental parameters.

For PFGE-based assays, mechanical shearing and nuclease attack can contribute to DNA fragmentation that is independent of radiation exposure. This 'noise' limits the dose range available for investigation. Excessive DNA degradation can be minimised, but not eliminated, by embedding cells into low gelling temperature agarose, incubation of samples in chelating agents to inhibit nucleases prior to lysis and strict avoidance of any risk of contamination of samples with DNAse.

Measurement of the fraction of DNA released into the gel reveals a sigmoid dose response and requires accurate molecular weight analysis of fragment distributions and calibration with I-125 for absolute quantification of DSBs [31]. The use of rarely cutting restriction enzymes
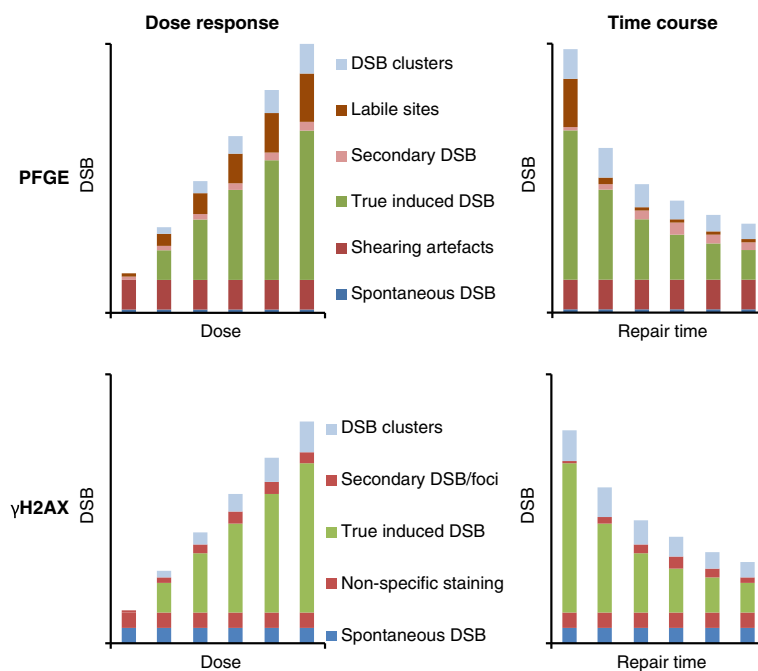
Figure 1 Schematic dose response and time course for different classes of signals that contribute to DSB measurements. *Top*: pulsed-field gel electrophoresis (PFGE). *Bottom*: fluorescence microscopic gamma-H2AX foci scoring. Note that the graphs are for illustrative purposes only. The values shown should not be taken as representative of the 'typical' contributions as they depend on numerous experimental factors.

and probing of individual restriction fragments bypasses these complications and allows direct quantification of DSB yields in specific regions of the genome [32,33]. However, as both these PFGE approaches measure the electrophoretic migration of DNA fragments in the size range of hundreds to thousands of kilo base pairs (to achieve the highest assay sensitivity), they fail to detect clustered DSB which produce smaller fragments and therefore underestimate the total yield of DSB, especially for densely ionising radiations. Separate electrophoretic runs are required to resolve small and large fragments and thereby determine DSB yields more accurately, albeit only at very high doses [34].

Replication forks can cause DNA molecules to be trapped in the agarose matrix, resulting in reduced mobility and associated underestimation of DNA breakage. Caution should therefore be used when interpreting PFGE results obtained with proliferating cells containing a significant S phase fraction (Figure 2; [35]).

In addition to inducing prompt DNA strand breaks, ionising radiation also induces heat- or alkaline-labile sites that are repaired by non-DSB pathways in the cell but can be converted into DSB during cell lysis and may contribute ~30% of all DSB measured using PFGE. Optimised 'cold' lysis and electrophoresis protocols have been established to eliminate these artefacts [36].
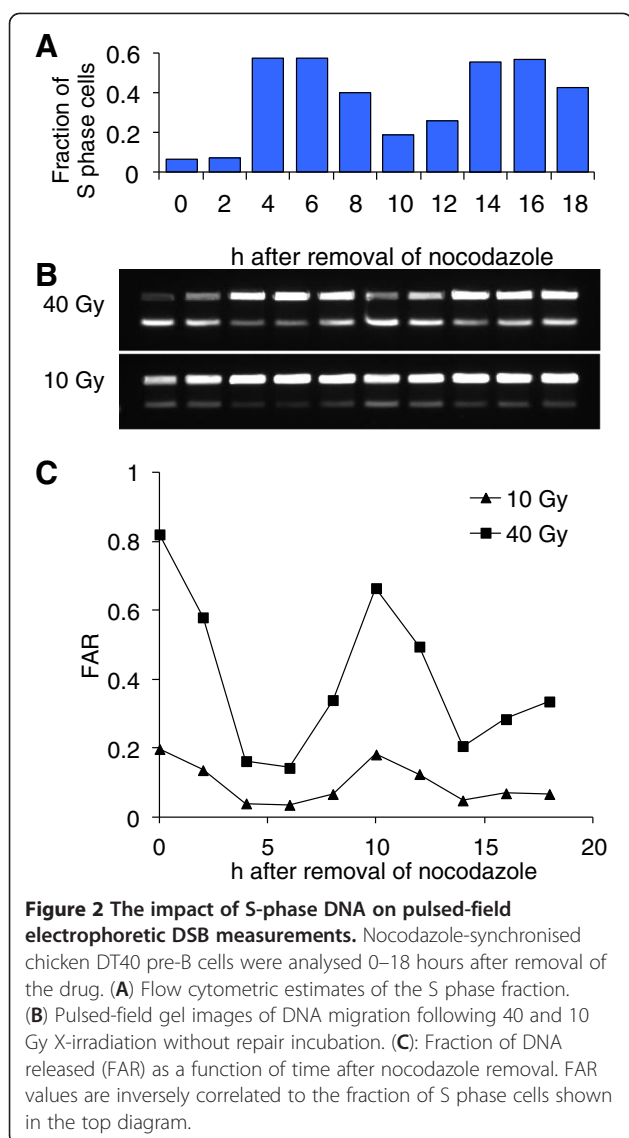
The high doses commonly used for PFGE studies may induce cell death in some cell types, potentially causing secondary DSB induction that may increase with repair time, and subsequent cell loss. Treatment with caspase inhibitors may help identify and control confounding apoptotic effects [37].

Foci-based assays for DNA double-strand break analysis

A similar set of factors can also modulate DSB yields determined with RIF-based assays (Figure 1). Spontaneous DSB/foci levels have been observed to be much lower for non-cycling cells such as quiescent lymphocytes or tissues with a low turnover than for rapidly dividing cells and tissues. This effect is assumed to reflect replication-associated DNA breakage, with DSBs being carried over into subsequent cell cycle stages [38].

Artifactual foci formation in the absence of a DSB can be caused by non-specific staining or aggregate formation of the primary or secondary antibody. Gamma-H2AX antibodies may bind to and form foci at parts of the endoplasmic reticulum and/or Golgi vesicles (Scherthan, personal communication). Careful optimisation of staining conditions and close monitoring of the antibody performance are required in order to obtain consistent results. Staining artefacts can typically be distinguished from 'true' foci based on their different morphology. Subtle differences can, however, be lost in maximum projection images, so 'live' imaging of slides may be preferable.

Figure 2 The impact of S-phase DNA on pulsed-field electrophoretic DSB measurements. Nocodazole-synchronised chicken DT40 pre-B cells were analysed 0–18 hours after removal of the drug. (**A**) Flow cytometric estimates of the S phase fraction. (**B**) Pulsed-field gel images of DNA migration following 40 and 10 Gy X-irradiation without repair incubation. (**C**): Fraction of DNA released (FAR) as a function of time after nocodazole removal. FAR values are inversely correlated to the fraction of S phase cells shown in the top diagram.

Absolute yields of DSB per unit radiation dose change linearly with the DNA content, as observed for foci frequencies in cell lines with different DNA content [39]. Also, cells in late S/G2 were shown to form almost twice as many foci compared to G1 when exposed to the same radiation dose [38,40], though foci morphology and signal to noise ratios differ in different cell cycle phases, complicating such a comparison.

Scoring of foci relies on setting threshold criteria for foci size, signal intensity and overall morphology of spots to distinguish 'true' foci from gamma-H2AX 'speckles' (which may form at the sites of transcription 'bubbles'), antibody aggregates and non-specific binding of the antibody to other targets. Co-localisation of gamma-H2AX foci with 53BP1 is generally assumed to reflect true DSB [41,42] and double immunostaining for two foci-forming markers can therefore be used to validate the signal (Figure 3). Still, it has to be noted that very high spontaneous levels of apparently 'real' foci do seem to occur occasionally and it is not currently clear whether these really always reflect the DSB [21].

As foci form as a biological response to DNA damage, a period of at least several minutes post exposure is required before they can be microscopically detected; the exact minimum delay depends on the individual signal to noise level which varies between cell types and is also affected by experimental protocols and reagents used for foci immunostaining [24]. Consequently, some radiation-induced DSB may already have been repaired before the 'initial' foci yield can be determined. Similarly to PFGE, RIF assays fail to detect clustered DSB, as multiple DSB within $<\sim0.5$ µm would only be scored as one focus. In addition to DSB clustering caused by clustered ionisation events along the particle track, movement of multiple break ends (as far apart as 1–2 µm) into 'repair centres' [29] may introduce a second layer of clustering facilitated by an active biological response after physical damage induction. The latter effect may result in lower foci yields (per unit dose) at high compared to low doses. These effects could therefore contribute to an underestimation of DSB yields, based on foci counts, and to deviation from dose linearity.

Secondary gamma-H2AX foci have been reported in irradiated and bystander cells in association with replication stallage [45-47] and transcription activity [48]. Different types of secondary gamma-H2AX signals in UV-irradiated cells were recently reviewed [49], including weak and strong pan-nuclear gamma-H2AX induction in association with nucleotide excision repair and S phase apoptosis, respectively. Early apoptotic DNA breakage can also give rise to foci patterns that may sometimes be scored as residual radiation-induced foci, despite their distinct morphology [50]. As already mentioned for PFGE studies, apoptotic DSB can be identified and controlled using caspase inhibitors when analysing foci kinetics.

Current automated and manual foci scoring methods tend to underestimate RIF yields at high damage levels, thereby causing a deviation from the linear dose response relationship towards saturation. Using the highest possible optical resolution, manual, rather than automated scoring and scoring of gamma-H2AX rather than 53BP1 foci at high damage levels (see Figure 3; also discussed in [41]) may help minimise this effect.

Notwithstanding all the potential technical caveats in measuring radiation-induced DSB, and the complex biological processes that may result in secondary DSB formation, as highlighted in the above paragraphs, DSB appear to be induced linearly with radiation dose for a wide range of radiation types and doses. At low doses, however, the situation is less clear. Specifically, supralinear foci induction in lymphocytes from paediatric patients was reported recently for both *in vivo* and ex vivo exposures to diagnostic X-ray
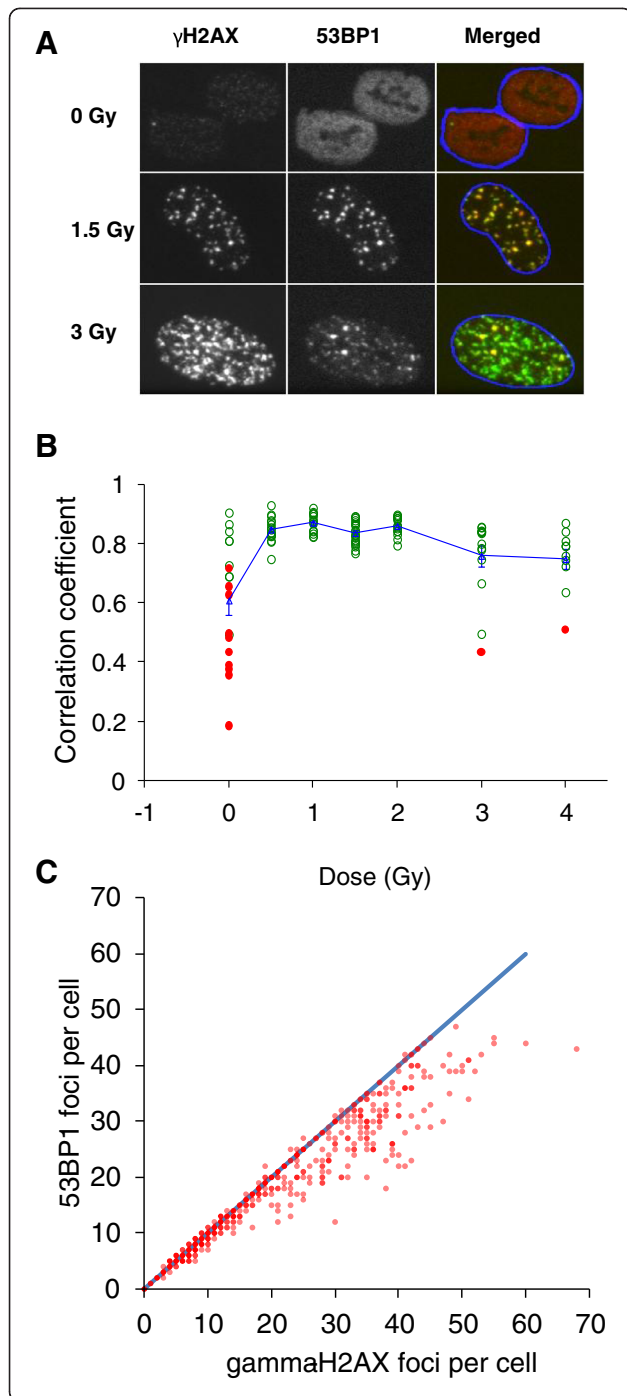
Figure 3 gamma-H2AX and 53BP1 foci induction by X-rays.
(**A**) Immunofluorescence microscopy images were taken at 0.5h following X-irradiation of normal human fibroblasts. Each image is 20 μm wide. In the merged images 53BP1 is red, gamma-H2AX green and the nuclear margins are shown in blue. Co-localising foci appear yellow or orange. (**B**) Colocalisation analysis of gamma-H2AX and 53BP1 foci. Pearson's correlation coefficients were calculated as described in [43]. A value of one represents total co-localisation. The significance of correlation coefficients was determined for individual cells using Costes' spatial statistics approach [44]. Each point represents one cell. Filled red circles: non-significant, open green circles: significant correlation. Blue triangles, connected by blue line: mean correlation coefficient; error bars are standard errors from the analysis of 10–20 cells for each dose. (**C**) Gamma-H2AX versus 53BP1 foci count per cell, manually scored in the same 1,000 double-immunostained cells following exposure to a range of X-ray doses. Each data point corresponds to one cell. Shading of data point symbols reflects the number of coinciding points. The blue line indicates a 1:1 ratio.

doses [51]. As this effect was more pronounced when whole blood was irradiated rather than isolated lymphocytes [52], bystander-type effects were suggested as a possible explanation. However, Beels and colleagues observed this effect only for low dose X- but not gamma-rays. The clustering of adjacent foci into repair centres, as reported in [29,52], may offer an alternative explanation. Overall, there is still controversy over the shape of the dose response for foci induction at low doses, due to a diminishing signal to noise ratio, lack of sensitive assays to confirm RIF data and the need for larger data sets to obtain conclusive results at doses in the milligray range.

Repair of radiation-induced DSB
PFGE studies suggest fairly dose-independent kinetics of DSB rejoining which follow a biexponential decay, allowing repair half-times for a fast (of the order of 10–30 minutes) and a slow component (a few hours) to be calculated [53]. The biphasic nature of DSB repair kinetics has been associated with different repair pathways [53], the complexity of break ends [54,55] or surrounding chromatin structures [56], requiring additional processing or remodelling steps, respectively. It should be noted that most PFGE data were obtained using tens of gray of sparsely ionising radiation, and that pre-electrophoretic cell lysis likely converted radiation-induced heat/alkaline-labile sites into DSB, resulting in a larger fast component [36]. As repair of these labile sites does not seem to require functional end-joining, XRCC1 or poly(ADP-ribose) polymerase 1 [36,57], their biological significance remains unclear.

Dose-independent, biexponential kinetics are also widely observed using foci assays, but typically with longer half times; e.g. Horn et al. [41] reported 1.5 h and 1.5 days for the fast and slow component, respectively, following 0.5 – 4 Gy X-irradiation of human lymphocytes. Apart from the issue with heat/alkaline labile sites mentioned above, a

number of other factors may contribute to the delayed foci loss compared to PFGE-based DSB rejoining kinetics: 1) The formation of foci over several minutes following irradiation means that some rapidly repaired DSB may never be registered as foci. 2) Whilst a few publications have reported maximum foci yields within the first few minutes post exposure, followed by rapid loss with kinetics compatible with those reported for PFGE [58-60], most studies observed foci counts peaking later, at 0.5-1h post exposure, and reaching a lower maximum yield per unit dose, followed by a slower loss. As discussed in [24], these discrepancies may be explained by differences in the detectability of early, i.e. small, gamma-H2AX foci, depending on the signal-to-noise ratio of immunostained samples. The half times reported in [41] were obtained using 0.5 h post exposure as the earliest time point, thus missing out on a large part of the fast component measured in PFGE experiments. 3) Dose ranges available for DSB repair studies are one to three orders of magnitude lower for RIF compared to PFGE assays [58]. Consequently, a lack of induction of DSB repair or secondary DSB formation which may contribute significantly to overall DSB frequencies only at low doses, may result in apparently slower kinetics following low dose exposure, or in the long-term persistence of residual foci [58,61,62]. 4) It is not clear how closely RIF loss follows the resealing of DSB ends. Resolution of foci is facilitated by a number of protein phosphatases whose complex roles in the DNA damage response are not very well understood yet [63].

Neumaier et al. [29] reported slower foci loss at higher doses, in line with the hypothesis that multiple DSB may congregate into one shared 'repair center', represented by one gamma-H2AX focus, resulting in longer overall persistence of such a focus, until all breaks contained in it are repaired. This concept has some intriguing implications for the way chromosomal rearrangements arise. The crucial impact of spatial and temporal proximity of DSB on mis-rejoining of break ends had already been highlighted in previous experimental and modelling studies (reviewed in e.g. [64-70]). However, it was only more recent work using high resolution interphase in situ hybridization [71] and time lapse imaging of RIF [29] that has unveiled the considerable intermingling of chromosome territories and DSB, respectively, thus explaining the large interaction distances for DSB of 1–2 μm that had been estimated in the earlier modelling studies of the quadratic dose dependence of chromosome rearrangement formation at high doses of sparsely ionising radiation.

Conclusions

In conclusion, the seemingly simple task to determine the shape of the dose response curve for DSB induction and repair is, at closer inspection, associated with numerous technical and conceptual caveats and uncertainties that should be considered when interpreting any experimental data. New assays have considerably advanced our understanding of the way cells respond to radiation-induced damage. It is becoming increasingly clear that multiple biological processes, but also methodological factors, may cause the dose response to deviate from linearity. Estimating their impact on the effect of radiation at a tissue or organism level remains a major challenge. In this context extrapolation of results from single experimental studies to draw conclusions on the most appropriate dose–response model to use for the protection of populations against the health effects of ionising radiation may be seen as unwise and potentially misleading.

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