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MODELLING γ-H2AX FOCI INDUCTION TO MIMIC LIMITATIONS IN THE SCORING TECHNIQUE

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An *ab-initio* approach based on track-structure calculations has been developed to take account of artifacts occurring during γ-H2AX foci detection in 2D images of samples analyzed through immunocytochemistry. The need of this works stems from the observed saturation in foci yields measured after X-ray doses higher than few grays, hindering an unambiguous quantification of DNA damage and of radiation effectiveness. The proposed modelling approach allows to simulate the observer's point of view for foci scoring, mimicking the selection of a slice Δz of the cell nucleus due to the microscope depth of field, and applying a clustering algorithm to group together damages within a resolution parameter *r*. Calculation results were benchmarked with experimental measurements at an early time-point for mouse breast cancer cells, irradiated with X-ray doses in the range 0 – 5 Gy. The model is able to reproduce the saturation in experimental data.

INTRODUCTION

Radiation quality influences how energy depositions are distributed in cell nuclei and therefore the pattern of lesions in exposed targets, determining the complexity of the overall insult. It has been demonstrated that double-strand breaks (DSBs) are likely to cause the loss of genetic integrity, triggering either cell death pathways or oncogenic transformations[(1)]. Common detection techniques rely on the labelling of repair proteins recruited at the DSB site by means of fluorescent probes, whose signal is then recorded *e.g.* through flow cytometry or immunocytochemistry (ICC). In particular, many studies focused on the molecular mechanisms regulating the phosphorylation of the histone H2AX (giving rise to the so-called γ-H2AX focus), and on the dependence of the repair kinetics on the spatial distribution of induced lesions and their complexity. A saturation of γ-H2AX signals was reported by several authors[(2,3)], where the maximum value of the recorded quantity is reached at doses depending on the detection assay.

In this study, a characterization of the induction of γ-H2AX ionizing radiation induced foci (IRIF) following exposure to X-rays is presented. 2D images, taken after ICC treatment, were analyzed by means of an *ad hoc* semi-automated macro written with the software ImageJ[(4)]. The yield of foci and parameters on their "morphology" (intensity and size) have been extracted as indicators to quantify the damage, along with its complexity and/or clustering in space, characteristics that are intimately related to the radiation biological effectiveness. Furthermore, a novel *ab-initio* Monte Carlo approach has been developed to simulate the induction of IRIF, with the aim to shed light on the real content of damage imparted by different radiation qualities, to overcome the limitations related to the detection technique and image acquisition. The approach has been initially benchmarked with available data on X-ray-induced foci, but the extension to the case of high linear energy transfer (LET) particles with more complicated track-structures is possible.

MATERIALS AND METHODS

Cell cultures and reagents

TSA mouse breast cancer cells (courtesy of Dr. E. B. Golden)were grown at 37°C in a humidified atmosphere with 5% CO_2 and cultured in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 12.5% Fetal Bovine Serum (FBS, Gibco) in T75 flasks (Falcon) up to confluence. Cells between passages 1 and 11 were used for experiments. The day before X-ray irradiation, 10^5 cells in 0.5ml medium were plated in each well of a 4-chamber CultureSlide flasks (Falcon).

Irradiation setup

X-ray irradiations have been carried out using the Westinghouse Coronado X-ray machine (225 kVp, 1

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mm Al and 0.5 mm Cu filters in the beamline) available at the Radiological Research Accelerator Facility (RARAF), Columbia University. The photon beam is emitted perpendicularly to the biological samples. Dosimetry was performed by means of the Accu-Dose+ (Radcal) ionization chamber, to guarantee a dose-rate of ≈ 1.1 Gy/min; doses of 0, 1, 2 and 5 Gy have been chosen for the experiments.

Immunocytochemistry and image acquisition

Cells were fixed 30 min and 24 h post-irradiation with 2% paraformaldehyde (Electron Microscopic Sciences) in phosphate buffered saline (PBS, Gibco) at room temperature (RT) for 20 min. Permeabilization was carried out at -20°C with 100% methanol for 20 min. Cells were washed thrice for 5 min and blocked against non-specific binding for 15 min in 0.2% bovine serum albumin (BSA, Invitrogen) in PBS at RT. Incubation with 1:500 anti-phospho-histone H2AX Rb primary antibody (Cell Signaling Technology) in 0.2% BSA/PBS was performed for 1 h, at RT. Afterwards, cells were washed as previously described, and incubation with 1:1000 of anti-rabbit goat IgG Alexa Fluor 555 conjugated secondary antibody (Invitrogen) in 0.2% BSA/PBS was carried out for 45 min, at RT. After washing, samples were mounted and nuclei simultaneously counterstained with VectaShield Mounting Medium with DAPI (Vector Laboratories, Inc.). An Olympus fluorescent microscope (magnification of 60X) has been used to acquire several fields, to analyze at least 200 - 250 cells per slide. Each 2D picture was analyzed with an *ad hoc* developed macro written with the software ImageJ[4]. Fragmented or pleomorphic cells were excluded from evaluation.

Track-structure simulations: PARTRAC

The biophysical track-structure code PARTRAC[5] has been used to carry out simulations of DNA damages (*i.e.* DSBs) after X-ray exposure. It provides a fully implemented model of the human genome for lymphocyte-like cells (G0/G1 state), starting from an atom by atom description of the DNA, up to higher levels of condensation (up to chromosome domains). The difference between murine and human chromosomal content are neglected for simplicity. A simplified software reproduction of a TSA cell has been implemented: the nucleus is a sphere of 5 µm radius, while the cytoplasm is a box of 14 µm along both x and y axes, and of 10.2 µm along the z axis, both centered at the origin. Photons have been generated randomly from a plane, positioned at the bottom surface of the cytoplasm, perpendicularly to the cell. The broad energy beam from an Xstrahl-200 machine, peaked at 220 kVp, has been implemented. Calculations are stopped when fixed doses (1, 2 and 5 Gy) are delivered to the nucleus. Sixty-four runs have been performed for all calculations, to have statistically significant results. Uncertainties are given as standard deviations among results for different runs.

RESULTS AND DISCUSSION

Experimental data on γ-H2AX foci induction

Manual foci counting is known to be a time-consuming technique, and semi- or fully-automated analysis approaches have started to be developed for a more high-throughput assessment[6,7]. For this work, an ImageJ macro has been optimized for foci recognition but also to extract parameters of interest such as the size and foci fluorescence intensity.

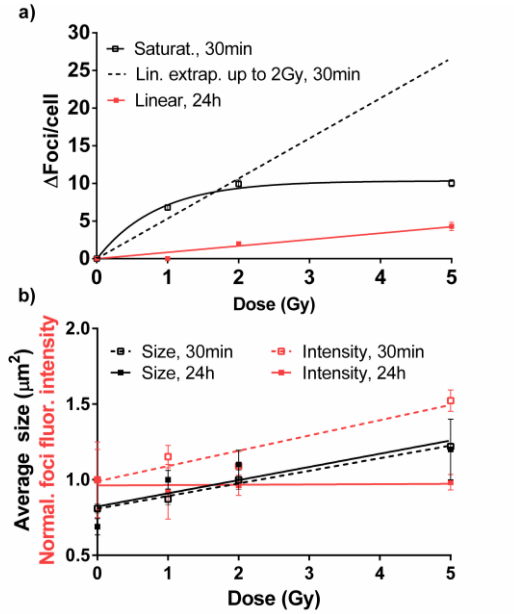

Figure 1: Experimental data concerning a) the extra yield of foci and b) their average size (µm^2) and foci intensity (normalized to the value of the sham) following exposure with X-rays, 30 min and 24 h post-irradiation. Results are shown as mean of at least 3 independent experiments ± SEM.

Results for X-ray-induced foci at two time-points (30 min and 24 h) are given in terms of extra yields (ΔFoci) with respect to the sham condition, as in Fig.1 a). Thirty minutes post-irradiation, ΔFoci has been found to be 6.80 for 1 Gy, 9.93 for 2 Gy and 10.03 for 5 Gy, values relatively low with respect to what found in literature[8]. However, it has to be recalled that non-confocal microscopy has been resorted to, and only a single picture has been acquired for each field: the visualization of all foci in the nucleus is therefore hampered by the microscope depth of field.

Concerning the dose-response, Fig.1 a) shows that the extra yield of foci at 24 h is linear with increasing dose, while for the early time-point, linearity might hold only up to 2 Gy. At increasing doses for the early time-point, ΔFoci tends to reach a constant plateau. For this reason, a fit function of the type:

$$\Delta\text{Foci}(D) = b * (1 - e^{-c*D}) \quad (1)$$

has been adopted to better reproduce the saturation, with *b* and *c* free parameters of the fit.

Linear fits have been applied in many studies to X-ray-induced foci yields at low doses[9]. The saturation following photon irradiation was instead assessed in the same dose range of this study when ICC is performed for the scoring[2], while higher saturation thresholds were observed when the overall fluorescence intensity from the whole nucleus is recorded by means of flow cytometry[3]. However, since this behavior does not seem to have any physical explanation, it has to be assumed that artifacts related to the detection technique hinder a real quantification of IRIF after relatively high doses of X-rays.

Although the number of foci saturates as a function of the dose, dose-dependent differences in the morphological characteristics of γ-H2AX IRIF were evident. In Fig. 1 b) the average size and intensity (normalized to sham values) of single foci are reported as a function of the dose, and it can be observed that both quantities increase linearly. This suggests that poor resolution of the technique might conceal significant pieces of information about IRIF. Size and intensity are good indicators of either the overlap of different events close in space, or of increasing damage complexity. While at early time-points the linear increase might be due to spatial clustering of damages, IRIF are easily distinguishable 24 h post-exposure and their superimposition is unlikely.

Modelling approach for γ-H2AX foci induction

Considering all the limitations in the experimental scoring of IRIF discussed above, an *ab-initio* approach based on Monte Carlo track-structure simulations has been proposed to predict the induction of this endpoint starting from radiation-induced DNA damages and their distribution in space. The aim of this approach is to reproduce not only the damage, but the final observer's point of view, therefore including imaging artifacts.

At first, the biophysical track-structure code PARTRAC was used to obtain the 3D spatial distribution of DSBs following X-ray irradiation of the simplified cell model. The initial assumption is that a single DSB is enough to determine the onset of a focus. For the purpose of this study, DSBs have to be projected on the x-y plane, to further correlate the calculations with the yields of γ-H2AX IRIF from 2D pictures. Furthermore, the projection needs not to be done for the whole nucleus, but only for a *slice thickness Δz*, centered in the middle of the nucleus, to account for the selection imposed by the depth of field when detecting foci at the microscope. Finally, a recursive algorithm was developed to cluster projected DSBs within a certain resolution parameter, referred to as *clustering radius r*.

The values of the slice thickness *Δz* and clustering radius *r* are to be tuned to reproduce experimental data, with initial realistic guess values chosen based on physical considerations:

- for *Δz*, the range 0.5 - 1 μm has been explored, close to values for the depth of field of microscopes at magnifications of, respectively 60 - 40X.
- for *r*, a minimum value of 0.5 μm was selected: the smallest signal considered as a focus in sham samples has a radius (circular foci) of ≈ 0.3 μm.

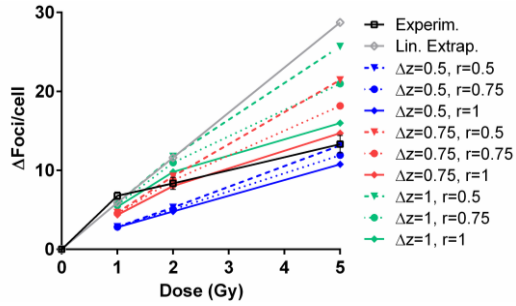

Figure 2: Comparison among experimental ΔFoci/cell (squares, black), linear extrapolation of data up to 2 Gy (rhombi, grey) and calculations (differently coloured and dotted). The slice thickness *Δz* and the clustering radius *r* are expressed in μm.

To benchmark the code and its parameters, calculation results were compared with data on IRIF from X-rays at the early (30 min) time-point. In Fig. 2, the black squares represent experimental results obtained by means of ICC, while the grey rhombi show the linear extrapolation of data up to 2 Gy, which can be considered as an upper limit for the estimates of foci. The differently coloured and dotted curves are the calculation results for different combinations of the two parameters (see legend).

At fixed radius, simulated yields increase as a function of the dose, since more and more DSBs are distributed in the cell nucleus. This remains valid until the density of damages is so high that many are grouped together in a single focus, causing the curve to bend towards a plateau. For the same reason, at fixed dose, less foci are expected for larger values of *r*. The "slicing" of the nucleus results instead in the removal of a fraction of DSBs, leading at the same time to a reduction in the number of foci, but also to a better chance of resolving close damages, especially for small *r*. The effect of reducing foci yields seems however to

be predominant, at least for X-ray induced IRIF. As it can be seen from Fig. 2, most parameter combinations recreate the saturation observed experimentally but a clustering radius of 1 µm and thicknesses of 0.75 – 1 µm seem to best reproduce the presented dataset. Fig. 3 reports the trends for the calculated foci size: a simplified definition of size is used, calculated as the area of a circular focus, with radius given by the distance from the centroid of clustered damages to the farthest DSB in the cluster.

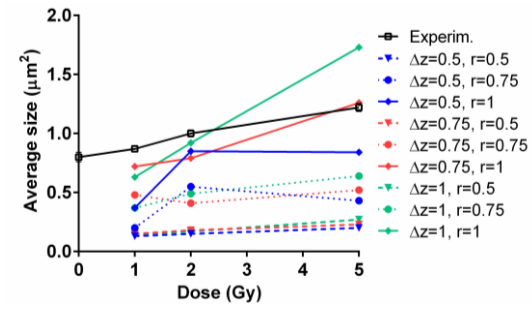

Figure 3: Comparison of average foci size from experimental data (squares, black) and calculations (differently coloured and dotted curves). The slice thickness *Δz* and the clustering radius *r* are expressed in µm.

CONCLUSIONS

This study describes an *ab-initio* approach based on track-structure calculations to predict the induction of γ-H2AX foci as a function of the radiation quality, taking into account limits of the detection technique when scoring is performed following ICC and 2D image acquisition. Track-structure calculations allowed to simulate the spatial distribution of DNA damages due to exposure to X-rays. The observer's point of view has been reproduced mimicking the selection of a slice *Δz* of the cell nucleus due to the depth of field of the microscope, and applying a clustering algorithm, where more DSBs are grouped together within a resolution parameter *r*. This is supported by experimental evidence that γ-H2AX IRIF conceal fine nanostructures directly related to single DSBs, due to poor resolution and scarce sensitivity of the antibodies[10]. The use of only two free parameters made it possible to reproduce the saturation observed in the dose-response for X-ray-induced foci yields. The following values seem to give the best agreement between predictions and experimental data: a slice thickness of 0.75 – 1 µm, and a clustering radius of 1 µm. The modelling approach has been benchmarked with low-LET radiation, and an extension to high-LET particles is currently being studied. Further refinements of the approach and the implementation in 3D will be needed for densely clustered lesions distributed along the particle track, thus finally providing a reliable tool for predictions of γ-H2AX foci and their characteristics due to all kinds of ionizing radiation.

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