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

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An approach based on track-structure calculations has been developed to take account of artefacts 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 labeling 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 a 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 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. In this work, the new approach is presented, as well as its benchmark with available data on X-ray-induced foci. The extension to the case of high linear energy transfer (LET) particles with more complicated track-structures will be the subject of future studies.

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% CO₂ and cultured in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% 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, 10⁵ cells in 0.5 ml medium were plated in each well of a 4-chamber CultureSlide flasks (Falcon). Cells were not synchronized.

Irradiation Setup

X-ray irradiations have been carried out using the Westinghouse Coronado X-ray machine (225 kVp, 1 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 IX70 (IX-ILL 100 LH) 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 a developed macro written with the software ImageJ⁽⁴⁾. Fragmented or pleomorphic cells were excluded from evaluation. Figure 1a shows an example of TSA cells irradiated with 1 Gy X-rays, 30 min post-irradiation, as observed with the Olympus microscope. The macro is based on common image processing steps, as background removal, histogram-based binarization (black/white to distinguish objects from the background) and watershed segmentation (Figure 1b). Regions of interest (ROIs) for foci are recognized by setting thresholds on morphological parameters as the circularity, the size and the intensity (Figure 1c). The macro has been optimized to recognize all the events that are unequivocally at focus, with halos from foci in different focal planes

often removed by means of the thresholding step. The macro is semi-automatic, to allow the user to refine any upstream step before final focirecognition; thresholds on the morphological parameters are kept fixed.

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- (spherical) and fibroblast-like (ellipsoidal nuclei) cells (G0/G1 state), starting from an atom by atom description of the DNA, up to higher levels of condensation (up to chromosome domains). TSA cells display, once cultured, roundish nuclei, which justifies the adoption of the lymphocytic cell nucleus for their software reproduction. The difference between murine and human chromosomal content is neglected for simplicity, under the assumption that geometrical factors have a major role in the limitations of the scoring technique under investigation in this work. The nucleus has been implemented as 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. The aim is to recognize the detectable foci that might be present in the single image that has been acquired with the optical conventional microscope. In the development phase, the macro has been calibrated by comparing the results with foci yields scored ‘by eye’ by the experimenter for different conditions (doses). Nevertheless, the macro can be adjusted for specific purposes, e.g. less strict criteria can be adopted to recognize as foci objects in the

Figure 1. (a) TSA cell irradiated with 1 Gy X-rays and fixed 30 min post-irradiation, visualized with a wide-field Olympus microscope (60X). (b) Background removal and binarization; (c) ROIs selected as foci according to thresholds on morphological parameters in the ImageJ macro.

background planes, in an attempt to trace back overall foci yields in the whole cell, as far this is possible. Instead, for this purpose we here resort to foci quantification, backed up by a modelling approach that aims at explaining the results (underestimation), starting from the clustering of neighboring lesions and from the microscope technical properties. Results for X-ray-induced foci at two time-points (30 min and 24 h) are given in terms of extra yields (Δ Foci) with respect to the sham condition, as in Figure 2a. Experimental data are average values for cells in different phases of the cell cycle, and also possibly showing some heterogeneity in their genomic content due to their cancerous nature. Thirty minutes post-irradiation, Δ Foci has been found to be 6.80 for 1 Gy, 9.93 for 2 Gy and 13.33 for 5 Gy (sham values were found of ~ 3 foci), values relatively low with respect to what found in literature⁽⁸⁾. As expected, quantification at the highest doses (5 Gy) is difficult. 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, Figure

2a 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, Δ Foci 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 artefacts 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 Figure 2b, 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, due to the close proximity of different DSBs within the lesion and therefore the phosphorylation of more H2AX sites. While at early time-points the linear increase might be due to spatial clustering of damages, IRIF are easily distinguishable 24h post-exposure and their superimposition is unlikely.

Figure 2. 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 24h post-irradiation. Results are shown as mean of at least three independent experiments $\pm$ SEM.

Modelling Approach for γ -H2AX Foci Induction

Considering all the limitations in the experimental scoring of IRIF discussed above, an 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 artefacts.

The approach consists in the following steps:

- at first, the biophysical track-structure code PARTRAC is used to obtain the 3D spatial distribution of DSBs following X-ray irradiation of the simplified cell model. The underlying assumption is

that a single DSB will be enough to determine the onset of a focus;

- only DSBs found in a *slice thickness* Δz , centered in the middle of the nucleus, are kept for further analysis. This is done to account for the selection imposed by the depth of field when detecting foci at the microscope;
- DSBs found in the selected slice are projected onto the x - y plane, to further correlate the calculations with the yields of γ -H2AX IRIF from 2D pictures;
- finally, a recursive algorithm searches for DSBs that fall within a certain resolution parameter, referred to as *clustering radius* r , and merges them in a single focus.

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 $\approx 0.3 \mu\text{m}$.

To benchmark the code and tune its parameters, calculation results were compared with data on IRIF from X-rays at the early (30 min) time-point. In Figure 3, 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 estimates of foci. Predicted foci yields are given for different combinations of the two parameters (see legend), overall showing the dependence of calculation results on the code parameters. 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 Figure 3, most parameter combinations recreate the saturation in the foci yield, meaning that the algorithm is able to reproduce this general behavior. Few parameter combinations seem to closely reproduce experimental data: a clustering radius of 1 μm and thicknesses of 0.75–1 μm seem to best reproduce the presented dataset. Figure 4 reports the trends for the calculated foci size, simply defined as the area of a circular focus, whose center is in the geometrical centroid of the group of DSBs clustered in a single focus, and whose radius is the distance from the centroid to the farthest DSB in the cluster. Again, $r = 1 \mu\text{m}$, $\Delta z = 0.75 \mu\text{m}$ give the closest predictions to experimental data, besides reproducing the increasing trend, though more realistic definitions of the area have to be implemented for further comparison. With this combination of model parameters, the average number of DSB per focus in the selected slice varies from 1 to 1.5 DSBs/focus. Experimental observations on the number of foci reproduced by this approach can be related to the predicted total damage in the whole nuclear volume. In our calculations the total number of DSBs is as follows: 45.3, 96.2 and 230.3 DSBs for 1, 2 and 5 Gy, respectively, showing an increasing linear trend as a function of the dose built on the expected value of ~ 40 DSBs/Gy.

Figure 3. Comparison of experimental $\Delta\text{Foci}/\text{cell}$ (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 .

Figure 4. Comparison of average foci size from experimental data (squares, black) and calculations (differently coloured and dotted curves). See details in the text for the definition of size adopted in the calculations. The slice thickness Δz and the clustering radius r are expressed in μm .

CONCLUSIONS

This study describes an 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 planar 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 initially benchmarked with low-LET radiation with IRIF data from not-synchronized murine cancer cells. The application of the approach to human cell lines, characterized in their cell cycle phase, and the extension to high-LET particles are currently being studied. The dense distribution of DSBs following high-LET radiation would result in an even more pronounced clustering of damages in fewer foci (higher number of DSBs/focus), usually with streak-like morphologies. This approach has the potential to deliver results for an actual quantification of damage (e.g. DSB contents) behind experimentally detected foci, which is essential when the comparison of the effectiveness of different radiation qualities is carried out with this assay. To this aim, further refinements and the implementation in 3D will be needed (and are indeed currently under development) for highly clustered lesions distributed along the particle track, possibly coupled to experimental data obtained with confocal microscopy, so that the total amount of foci in the whole nuclear volume can be predicted and benchmarked. This will finally provide a reliable tool for predictions of γ -H2AX foci and their characteristics due to all kinds of ionizing radiation.

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