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Technical report:

An image analysis technique for detection of radiation-induced DNA fragmentation after CHEF electrophoresis

M. ROSEMANN†‡, B. KANON†, A. W. T. KONINGS† and H. H. KAMPINGA†*

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Abstract. CHEF-electrophoresis was used as a technique to detect radiation-induced DNA breakage with special emphasis to biological relevant X-ray doses (0–10 Gy). Fluorescence detection of DNA-fragments using a sensitive image analysis system was directly compared with conventional scintillation counting of ^{3}H-thymidine prelabelled DNA in HeLa S3 cells. It is shown that the image analysis-based fluorescence detection of fragmented DNA after ionizing radiation is as sensitive and reproducible as detection using radioactively prelabelled cells without the putative shortcomings of fluorescence detection methods described earlier (Blöcher and Kuhni 1990). Therefore, the image analysis-based detection of radiation-induced DNA fragmentation after CHEF electrophoresis seems to be the most reliable method for applications to non-cycling cells and biopsy material.

1. Introduction

The pulsed field gel electrophoresis in its different modifications found a broad application in biochemistry and genetics due to its sensitivity for the analysis of DNA molecues with length up to 10 Mbp. Thus, it became possible to study radiation-induced DNA fragmentation due to double-strand breakage in the dose range used for cell survival experiments (Elia *et al.* 1991). Detection of DNA fragmentation is usually done by slicing the gel and measuring the radiolabelled DNA extracted from the plug (DNA_{extr}) (Stamato and Denko 1990, Elia *et al.* 1991). In order to measure DNA fragmentation in non-dividing cells and, eventually, in biopsies of patient material, where radiolabelling can not be performed, fluorescence detection using UV-microscopy coupled to a photomultiplier was developed (Blöcher 1990, Blöcher and Kuhni 1990). This method, however, is hampered by the limited focus range of the microscope, leading to underestimations of the amount of DNA (Blöcher and Kuhni 1990). In the present study, fluorescence detection of ethidium bromide-stained DNA was done using a sensitive silicon intensified tube (SIT) camera. Following pulsed field gel-electrophoresis, quantification of DNA fragmentation was performed by the aid of a computer-based image analysis system. Measurements were directly compared with subsequent radioactive detection of the fraction extracted ^{3}H-thymidine prelabelled DNA extracted from the plugs.

* Author for correspondence.
† Department of Radiobiology, State University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.
‡ Radiotherapeutic Research Unit, Institute for Cancer Research, Sutton, Surrey SM2 5NG, UK.

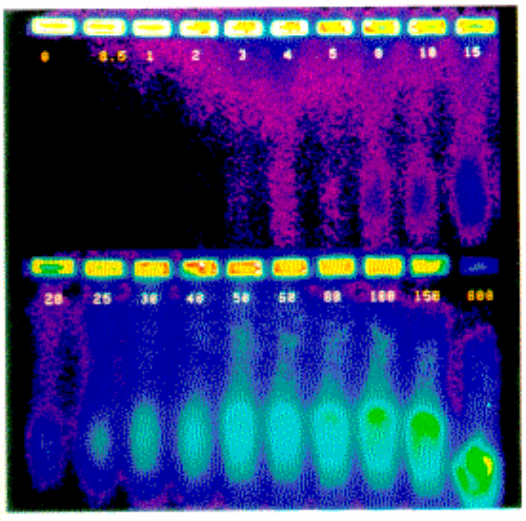
2. Materials and methods

2.1. *Cell culture and irradiation*

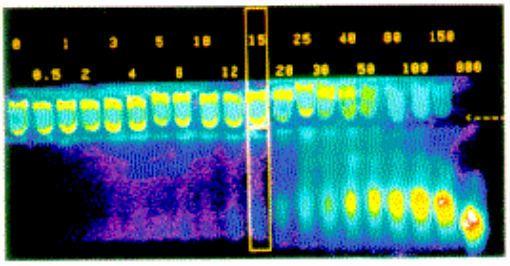
HeLa-S3 cells (doubling time around 26 h) were grown in suspension in Jokliks-medium with 10% foetal bovine serum (FBS) and antibiotics. DNA labelling was carried out by adding 0·5 μCi/ml 3^2H-TdR and 0·016 μM/ml TdR to the exponentially-growing cell culture. After 48 h, the medium was renewed and the cells chased with 0·016 μM/ml TdR for 2 h. Cells at a concentration of 10^7/ml were irradiated in suspension at 0°C. Irradiation was carried out using a Phillips–Mueller MG300 X-ray machine operating at 200 kV and 15 mA filtered with 0·5 mm AI and 0·5 mm Cu (dose rate of 6 Gy/min).

2.2. *Preparation of DNA for gel electrophoresis*

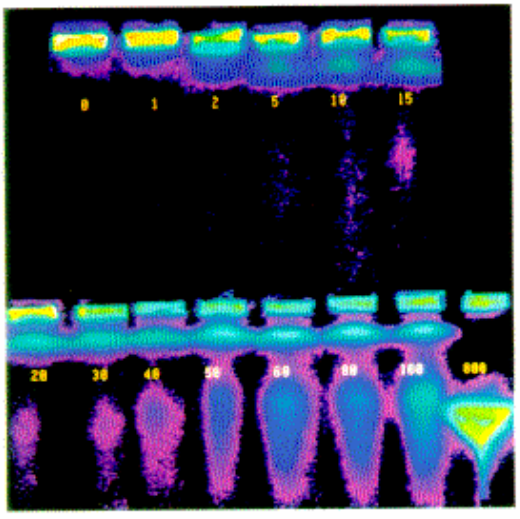
Immediately after irradiation, 200 μl of the cell suspension was mixed with an equal amount of 1% low melting agarose (Bio-Rad) in phosphate-buffered saline (PBS) at 37°C. Aliquots (40 μl) of this mixture were poured into the moulds of a Bio-Rad plug former. After 15-min gelation at 4°C, the plugs (1·5 × 4·5 × 6 mm) were put in lysis solution (2% Sarkosyl, 1 mg/ml proteinase K and 500 mM EDTA). After lysis (2 h on ice plus 16 h at 37°C), the plugs were washed twice with PBS buffer and treated for 2 h with 0·1 mg/ml RNase A at 20°C. The



(a)



(b)



(c)

plugs were inserted into the wells of a 0·5% agarose gel (Bio-Rad Chromosomal Grade) and sealed with a thin layer of agarose. The CHEF run was carried out using a Bio-Rad DRII system (field angle 120°) in TBE buffer (45mM Tris–Base, 45 mM Boric acid, 2 mM EDTA-buffer. pH = 8·2) at 18°C. The following pulse programs were used: $T_{pulse} = 75$ min., $T_{run} = 25$ h, E = 40 V (hereafter referred to as 1-day-run); or $T_{pulse} = 60$ min., $T_{run} = 90$ h, E = 45 V (hereafter referred to as 4-day-run).

2.3. *Analysis of DNA gel patterns: fluorescence detection*

After electrophoresis, the gel was stained for 12 h in TBE buffer containing 400 μg/l ethidium bromide and subsequently destained in TBE buffer for 4 h. The plugs were removed from the wells and placed horizontally on top of the gel some millimetres away from the corresponding lane. The gel was transilluminated with 312-nm UV-light (inhomogeneous light excitation of the transilluminator limits the use of it to a restricted area only) and analysed using a sensitive silicon intensified tube (SIT-66) camera (DAGE, MTI Inc.) connected to a digital signal, remote control processor (DSP100: DAGE) for enhancement of the signal-to-noice ratio by image accumulation and to allow accurate gain- and blacklevel setting. The latter were held constant throughout the entire measurement. For the calculation of the DNA-related fluorescence signals, a commercial image analysis system (Cue-2, Olympus) was used, which allows the quantification of the fluorescence signal over defined subareas of the gel. For plug and extracted DNA, subareas of the same size were defined (areas indicated in Figure 1b) and integrated fluorescent intensities (IFI) over these

Figure 1. DNA fragmentation in HeLa S3 cells after X-irradiation of gels analysed by the image analyser after various running protocols (1 versus 4 days) and gel preparations (with or without plug removal). Photographs are from multiple images made from the (same) gel after analysis. Electrophoresis direction is from top to bottom. Increasing fluorescent light intensitities are depicted here by different colours (in the order dark purple, light purple, dark blue, light blue, green, yellow, red, white) each covering an fluorescence intensity range of 32 bit. Radiation doses are indicated in Gy. (a) 1-day run; (b) 1-day run with removed plugs. The same gel as in Figure 1a but with the plugs removed from their original place (arrow) in the wells and put flat on top of the gel. Boxes indicate the defined areas (the same must be used for plug and extracted DNA) over which integrated Fluorescent Intensities (IFI) were measured; (c) 4-day run.

areas were measured. Colour illustrations in this paper have been composed on the image analyser from multiple images made from the (same) gel after analysis.

2.5. *Analysis of DNA gel patterns: radioactive detection*

Immediately after recording the fluorescent picture, the removed plugs and corresponding lanes were dissolved in 0·5 ml 2M HCl in a microwave oven at about 98°C, neutralized with 0·5 ml 2M NaOH and mixed with 12 ml Hydro Luma (Lumac, Belgium). Radioactive counting was carried out using a Packard Tri-Carb 2660 liquid scintillation counter with quench correction.

3. Results

3.1. *DNA-fragmentation: 1-day CHEF-run*

Figure 1a shows a typical fluorescent image for a 1-day CHEF run for DNA fragmentation after X-irradiation. Increasing fluorescent light intensities are depicted here by different colours each covering an fluorescence intensity range of 32 bit. Apparently fragmentation can be seen for doses as low as 0·5 Gy. There are, however, some difficulties in the straightforward quantification of this DNA-pattern. It is obvious that, for the samples irradiated with low doses, the fluorescent signals from the plugs overtuned the camera (white areas), a compromise which has to be made in order to achieve a high detection sensitivity. Second, there is no clear distinction between well- and extracted DNA; this can be attributed to fragmented DNA with low mobility (Rosemann *et al.* 1993, for further details) and/or putative fluorescent light scattering from the well-DNA into the lanes. To overcome these problems the plugs were physically separated from the wells and put horizontally on top of the gel (Figure 1b). This led to an alteration of the fluorescent intensity compared with that of the plugs at their original position in the wells. The reduction of the fluorescence light was about 30%, which was shown to be due to the absorbance of the UV-excitation light by the gel. This effect was reproducible and thus appropriate corrections could be made when measurements were done with removed plugs. As can be seen (Figure 1b), the fluorescence distribution in the plugs is not uniform. This could not be attributed to differences in staining and must be due to inhomogeneous DNA distribution in the plug. The extent of this effect was rather different for different experiments but had no effect on the measurements of the fraction of extracted DNA. Finally, in control experiments it was confirmed that the fluorescent signal emitted by the ethidium bromide labelled DNA is not altered by irradiating the cellular DNA before labelling with doses up to 150 Gy. For DNA concentrations between 15 and 300 μg/ml (corresponding to cell densities in the plugs ranging from 5×10^5 to 10^7 cells/ml) a linear dependence of the measured fluorescent signal was found (no quenching effects, high linear dependence of the camera signal from the emitted fluorescent light). So DNA_{extr} can be quantified by measurement of IFI of the fluorescent light intensities over the lane area relating it to the overall fluorescence in the lane plus the plug according to the formula:

$$\%DNA_{extr} = \frac{IFI_{lane}}{(IFI_{removed\ plug} \times 1{\cdot}3‡) + IFI_{lane}} \times 100\%.$$

The corresponding dose response curve for $\%DNA_{extr}$ is shown in Figure 2 (closed circles). Subsequent radioactive detection of the physically-removed plug and the corresponding lane from the same gel (measuring the DNA in exactly the same area as the fluorescence method) yields a dose response curve identical to that found for the fluorescence method (Figure 2). Both detection methods reveal an increase in the $\%DNA_{extr}$ of $2\% \pm 0{\cdot}3\%$ per Gy in the lowest dose range (0–10 Gy), which is steeper than the related value in the higher dose range of $0{\cdot}95\% \pm 0{\cdot}25\%$ per Gy (20–60 Gy) (Rosemann *et al.* 1993 for further details on the shape of the induction curve).

3.2. *DNA fragmentation: 4-day run*

For some applications it may be difficult to (physically) remove the plugs from the wells after the electrophoresis run. Longer run times of the CHEF electrophoresis can be used to enhance the separation between different molecular weights of the extracted DNA. As can be seen in the record of the image analyser (Figure 1c), a 4-day run results in a better discrimination between well and (slowly migrating) extracted DNA. Thus, fluorescence detection of the extracted DNA can be carried out without removal of the plugs. Counting all the flourescence intensities beyond 2 mm from the plugs as extracted DNA yields a dose response curve for DNA_{extr}, as depicted in Figure 3 (closed circles). The yield for DNA_{extr} increases with $2{\cdot}5\% \pm 0{\cdot}2\%$ per Gy

‡This factor has to be determined for each individual gel, since it depends on agarose concentration and gel thickness.

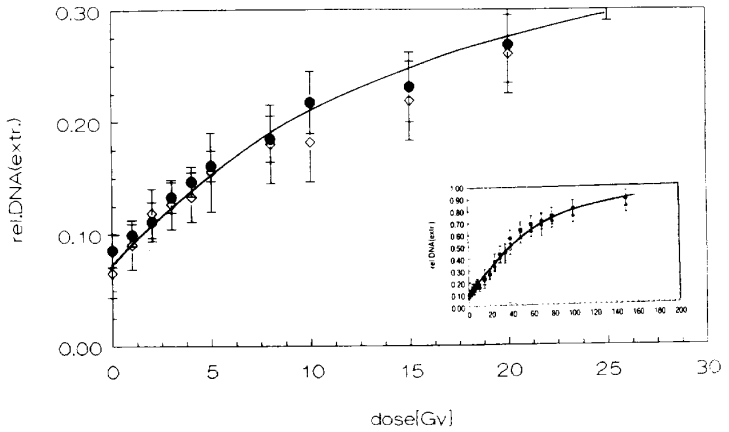


Figure 2. Relative amount of extracted DNA from HeLa S3 cells after different X-ray doses: comparison of fluorescent and radioactive detection (1-day run). The $\%DNA_{extr}$ was detected after a 1-day CHEF run using the fluorescence based image analysis method (circle) or using radioactive detection (diamond). Removed plugs were put on top of the gel some millimetres away off the lane and analysed as described in §3. Lanes and plugs from the same gel were subsequently counted for ^{3}H TdR (DNA) radioactivity. Data from at least three independent experiments are presented: error bars represent 95% confidence ranges (the insert shows the curve for doses up to 150 Gy).

in the low dose range (0–10 Gy) and with 0·5% ±0·1% per Gy in the higher dose range (20–60 Gy). For the subsequent radioactive detection, the plugs were cut out together with the first 2 mm of the lane to measure the same area as with the fluorescence method. The curve for DNA_{extr} is depicted in Figure 3 (diamonds). The slope for the dose response in this case is 2·6% ±0·2 Gy (0–10 Gy) and 0·75% ±0·15 per Gy (20–60 Gy).

4. Discussion

The fluorescence detection of fragmented DNA was calibrated here for the first time by systematic comparison with radioactive DNA detection. The aim was the development of a system which is (1) as sensitive as the radioactive method without the necessity of prelabelling, (2) highly reproducible, and (3) not hampered by shortcomings of the optical system and the light detection as in the case with fluorescence microscopy (Blöcher and Kuhni 1990) or film development.

In Figures 2 and 3, it can be seen that after both CHEF protocols (1- and 4-day run) the fluorescence detection is as sensitive as the radioactive method for the detection of DNA fragments after exposure to X-rays. Significant increases in the relative amount of extracted DNA are detected for doses as low as 1 Gy. In control experiments it could be shown that the SIT-camera has a strong linear signal output for fluorescence signals produced by DNA over a wide concentration range (data not shown). Since the long focus camera lens can detect the DNA over the whole gel depth, no gel drying has to be carried out as for the fluorescence microphotometry (Blöcher 1990).

Especially after the 1-day run, the data for both radioactive and fluorescence detection methods were almost identical. The longer CHEF run gave a slightly less good correlation between both detection methods at doses between 40 and 100 Gy (figure 3). It is clear that the extracted DNA after a 4-day run is distributed over a much longer distance in the gel than after the 1-day run (compare Figures 1b and c). This may contribute to a higher influence of impurities and higher background effects on the results obtained with the image analysis system.

Recently, Smeets *et al.* (1993) also developed an elegant and sensitive method for the measurement of DNA fragmentation in non-prelabelled cells. Using field inversion gel electrophoresis, fragmented DNA is collected on a filter inserted in the gel and hybridized with a human ^{32}P-labelled probe. In this method, however, equal loading for all samples is essential since the amount of DNA remaining in the plug is not measured and thus, unlike in our method, differences in sample loading might affect the outcome of the measurement. Furthermore, in a separate study (Rosemann *et al.* 1993), it was demonstrated that the separation between immobile DNA in the plugs and extracted DNA may drasti-

cally influence the shape of the dose-response curve in the low dose range. Removal of the plugs from the wells has to be performed (Figure 1a, b) in order to provide a reliable estimation of percentage of extracted DNA. The introduction of arbitrary limits (Blöcher 1990, Blöcher and Kuhni 1990, Smeets *et al.* 1993) may thus lead to an underestimation of the fraction of extracted DNA and thus may be erroneous when trying to quantify double-strand break-induced DNA fragmentation accurately (Rosemann *et al.* 1993 for further discussion).

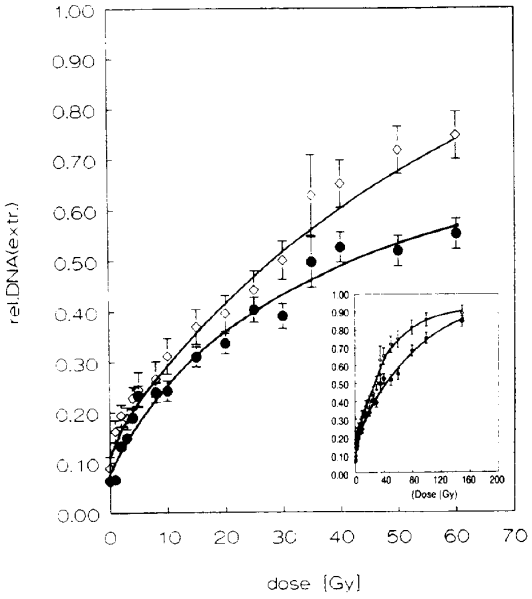


Figure 3. Relative amount of extracted DNA from HeLa S3 cells after different X-ray doses: comparison of fluorescent and radioactive detection (4-day run). The $\%DNA_{extr}$ was detected after a 4-day CHEF run using the fluorescence based image analysis method (circle) or using radioactive detection (diamond). For the detection using the fluorescence method, all signals 2 mm beyond the well were considered as DNA out of the well. For the subsequent radioactive detection, the plugs were cut out together with the first 2 mm of the lane and counted for ^3H TdR (DNA) radioactivity. Data from at least three independent experiments are presented: error bars represent 95% confidence ranges (the insert shows the curve for doses up to 150 Gy).

In summary it is shown that the application of a sensitive electronic camera connected to an image analysis system results in a sensitive and reproducible method (comparable with the radioactive method) for the detection of DNA fragments stained with ethidium bromide after ionizing radiation at biologically relevant doses, making the CHEF technique applicable to non-cycling cells and putatively to biopsy material.

Acknowledgements

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