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Full Length Research Paper

An optimized colony forming assay for low-dose-radiation cell survival measurement

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The aim of this study is to develop a simple and reliable method to quantify the cell survival of low-dose irradiations. Two crucial factors were considered, the same number of cells plated in each flask and an appropriate interval between cell plating and irradiation. For the former, we optimized cell harvest with trypsin, diluted cells in one container, and directly seeded cells on the bottom of flasks in a low density before irradiation. Reproducible plating efficiency was obtained. For the latter, we plated cells on the bottom of flasks and then monitored the processing of attachment, cell cycle variations, and the plating efficiency after exposure to 20 cGy of X-rays. The results showed that a period of 4.5 h to 7.5 h after plating was suitable for further treatment. In order to confirm the reliability and feasibility of our method, we also measured the survival curves of these M059K and M059J glioma cell lines by following the optimized protocol and obtained consistent results reported by others with cell sorting system. In conclusion, we successfully developed a reliable and simple way to measure the survival fractions of human cells exposed to low dose irradiation, which might be helpful for the studies on low-dose radiation biology.

Keyword: Colony forming assay; Low-density cell survival; Low-dose hyper-radiosensitivity

INTRODUCTION

The phenomenon that cells die from excessive sensitivity to very low doses of irradiation (<20 cGy) is defined as low-dose hyper-radiosensitivity (HRS). An increase in cell survival response is induced as radiation dose increases and this subtle change in radiosensitivity over the 20–70 cGy dose range is defined as 'increased radioresistance' (IRR) (Marples et al., 2004). HRS is of applicative significance in clinical tumor radiotherapy thus it draws a lot of attention. However, the mechanisms underlying HRS/IRR remains controversial. Short (2003) reported that irradiated G2 phase cells exhibit more distinctive HRS than irradiated G1 or S phase cells and took for granted that G2 phase cells with little amount of DNA damage will proceed to mitosis and lead to cell death, while increased DNA damage induced by higher doses causes G2 arrest and allow time for cells to repair. Joiner (2001) proposed that there is a putative damage-sensing threshold and the injury produced by larger doses is above the threshold for triggering faster and more efficient DNA repair while that induced by low doses under the threshold could not be recognized and consequently resulted in extra cell killing. Obviously,

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further studies are required to reveal the underlying mechanisms.

A bottleneck problem in HRS/IRR studies is to precisely measure the survival of cells exposed to low doses. Conventionally, the cell survival of *in vitro* cultured mammalian cells exposed to radiation dose less than 1 Gy is extrapolated from those exposed to radiation doses higher than 1 Gy and assessed by using clonogenic assay (Puck and Marcus, 1956). Nevertheless, this method lacks accuracy and usually overestimates survival level due to the uncertainty in the number of cells plated in each culture dish and the random errors in cell counting, dilution and plating procedures (Bedford and Griggs, 1975; Boag, 1975).

Two main strategies have been proposed to overcome this problem (Palcic et al., 1983; Palcic and Jaggi, 1986; Spadinger and Palcic, 1992; Spadinger and Palcic, 1993; Spadinger et al., 1989; Spadinger et al, 1990; Wouters and Skarsgard, 1994). One is to use the cell sorting system to plate each flask with precisely known number of cells into each dish (Skarsgard et al., 1991; Wouters and Skarsgard, 1994) and the other is to use a Dynamic Microscopic Imaging Processing Scanner (DMIPS) cell analyzer (Palcic and Jaggi, 1986), which can locate and record the positions of the plated cells and track them individually to determine colony formation. These two experimental approaches have the advantage of substantially reducing the errors associated with uncertainties in the number of cells plated but both of them require expensive facilities. In addition, cell sorter might conceivably count cell ghosts and large cell fragments as cells (Lambin et al., 1993). Also, long procedure or exposure to laser pulse might result in extra stresses to cells. Obviously, an alternative is in demand to broaden low-dose survival analysis.

In this study, we developed an easy and reliable method of survival assay for low-dose irradiation by slightly modifying the routine colony-forming assay. We tested the feasibility of this method with NNF-16 cells and further confirmed its reliability with human glioma cell lines, M059K and M059J, whose radiosensitivity to low-dose radiation has already been well studied by other groups. Our optimized colony-forming assay has made it possible to examine cellular radiosensitivity to doses less than 1Gy with sufficient accuracy and provided the opportunity independent of expensive facilities to elucidate the underlying mechanisms of HRS/IRR.

MATERIALS AND METHODS

Cell culture

Human primary fibroblast NNF-16 cells were grown in a 1:1 mixed medium of MCDB (Sigma, St. Louis, MO) and Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing sodium bicarbonate and 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, Kansas, USA) in T75 culture flasks. The cells were then incubated in a humidified incubator maintained at 37 ^{o}C in an atmosphere of 95% air and 5% CO_2. Human glioma cell lines, M059K and M059J, were maintained in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1 mM glutamine. Antibiotics were not used in all the cell cultures. The cells were ascertained free of mycoplasma by periodic testing (Bionique, Saranac Lake, NY, USA).

Irradiation

X-ray irradiation was performed with a RT-100 Philips generator operated under 100 kVp and 8 mA with 1.7 mm Al filter. The dose rate was 8.69 cGy/min and all irradiations were carried out at room temperature in dark. The radiation doses were 0, 0.05, 0.1, 0.2, 0.3, 0.5, 1, and 2 Gy for NNF-16 and 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.75, 0.85, 1, 2, and 3 Gy for M059K and M059J.

Colony formation assay

Cells from one confluent stock were harvested with trypsin and resuspended in the medium complemented with 10% FBS. Cell concentration was determined with hemocytometer and at least 300 cells were counted for each sample. Cells were diluted with pre-warmed medium and the same amount of cells was plated in each T25 flask (200 cells/flask), which provided 10-100 colonies per flask. Flasks were incubated for 14 days, fixed with 70% ethanol for 10 min, and then stained with 1% Crystal Violet. Colonies containing more than 50 cells

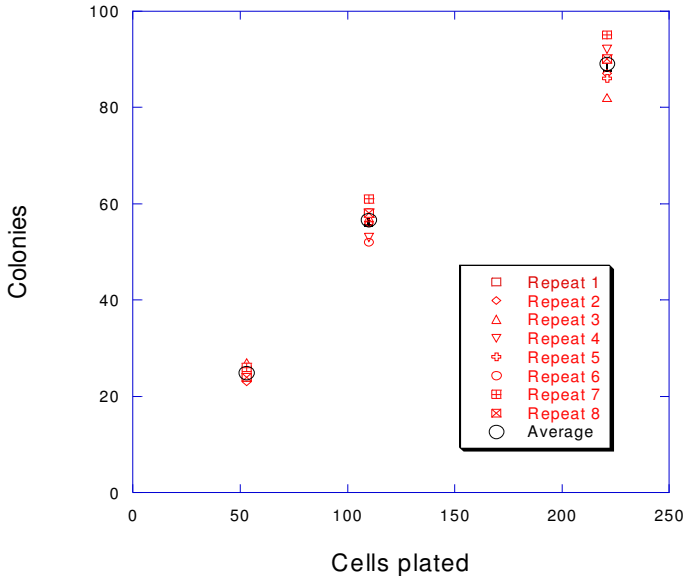

Figure 1. Variation of NNF-16 plating efficiency among 8 flasks. Three dilutions were conducted for flasks pre-seeded with 53, 110, and 221 cells. The colonies per flask were 24.9 ± 0.5, 56.6 ± 1.0, 89.0 ± 1.4, respectively.

were identified as survivors.

Cell cycle assay

Cells were collected by trypsin treatment and then fixed in 70% ethanol for 24 h at -20 °C. The fixed cells were washed twice with PBS and incubated in propidium iodide (PI)/RNase staining buffer (BD Biosciences, San Jose, CA) for 30 min at room temperature. Flow cytometric analysis was carried out using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using ModFit LT software version 2.0 (Verity Software House, Topsham, ME).

Statistical analysis

All the experiments were independently repeated at least three times and the data were presented as mean ± standard error (SEM).

RESULTS

Optimization of cell plating procedure

In order to recognize the tiny difference in survival fractions induced by low-dose radiation, minimizing the variance in cell number plated in a series of flasks is crucial. However, it is impossible to plate absolutely the same number of cells each time into each dish. In this study, we developed several tips to make it as accurate as possible. First, optimize the cell harvest procedure to achieve maximal and stable plating efficiency of each cell line. The sensitivity of each cell line to trypsin is different. Using an appropriate type and concentration of trypsin, pre-warming the enzyme and shortening the treatment time are helpful to achieve good separation of individual cells. Strong pipetting might be helpful to segregate the cells but probably harmful to cellular viability and plating efficiency. Second, make final dilutions of cell suspension before seeding cells into flasks. The total amount of cells required for one dilution was diluted in one container. In the preliminary experiments, three dilutions were performed (Figure 1). Sixty mL of cell suspension was added to the first bottle to obtain a final concentration of 53 cells / 6 mL, second one 110 cells / 6 mL, and third one with 221 cells / 6 mL. Six mL of suspension was placed into each flask, which theoretically resulted in 53, 110 and 221 cells/flask, respectively. Slowly vortex the bottle while pipetting the cell suspension into flasks. Third, avoid the contact of the cell suspension to other inner surfaces of the flask since cells in the suspension can easily attach to dry surface which results in additional loss of cells. To achieve this goal, we lay down the flask

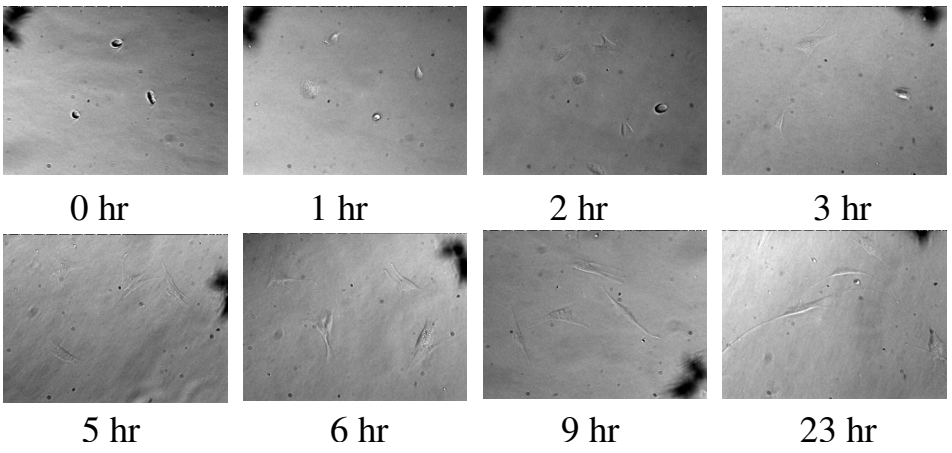

Figure 2. Photos of NNF-16 human fibroblast cells at various time points after plated.

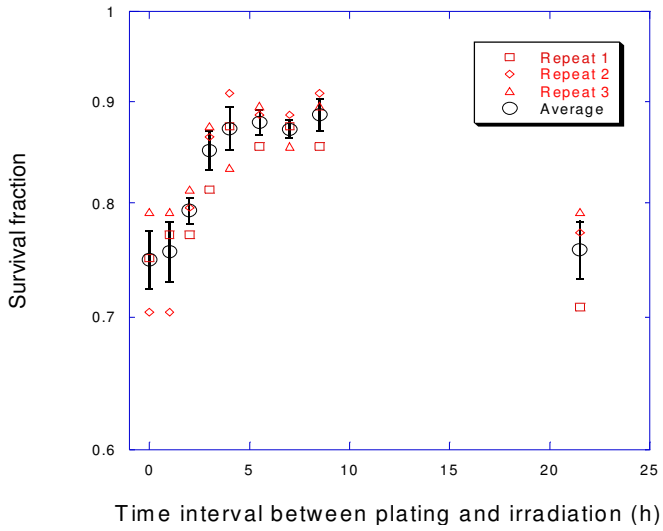

Figure 3. Survival of NNF-16 cells exposed to 20 cGy of X-rays. The same amount of cells was seeded into T25 flasks following optimized protocol and exposed to 20 cGy of X-rays. Error bars indicate the standard error of the mean (SEM) for 3 independent experiments

while moving the cell suspension into it. Colonies obtained in the flasks pre-seeded with 53, 110, 221 NNF-16 cells were 24.9 ± 0.5, 56.6 ± 1.0, 89.0 ± 1.4, respectively.

Optimization of the interval between cell seeding and irradiation

The interval between cell plating and irradiation plays vital role in determining plating efficiency. After plated, cells were monitored under microscope. As shown in Figure 2, most of the cells adhered to the flask surface in 1 h and became flattened in 3 h. Four hours later, changes in cell morphology were observed. Cells stretched out filopodia-like protrusions. In the following several hours, some of the protrusions became slimmer and longer. Cell division was observed 20 h later, which implies that additional treatment such as ionizing radiation is no more applicable, otherwise no reliable results can be obtained.

Figure 3 clearly shows that the period of 4.5 h to 8.5 h after plating is appropriate for additional treatment such as radiation. The same amount of cells was exposed to 20 cGy of X-rays after plated in T25 flasks. The plating efficiency was highly reduced by X-ray irradiation when

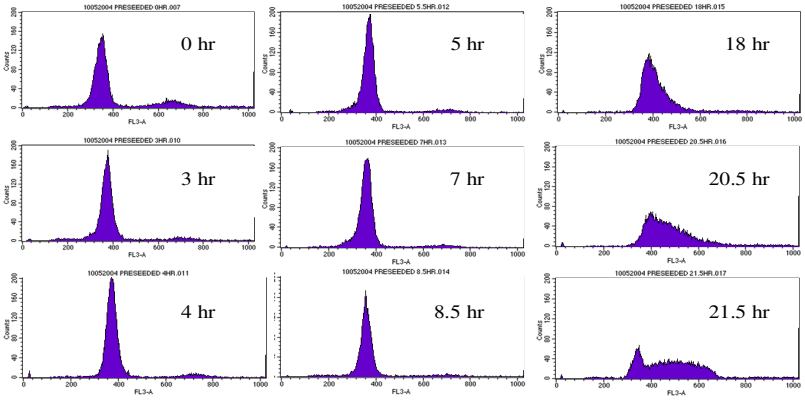

Figure 4 A

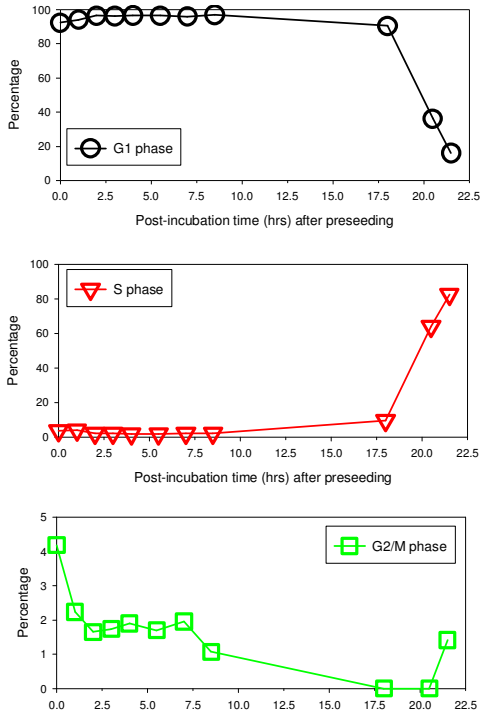

Figure 4 B

Figure 4. Cell cycle distribution analyzed with flow cytometer at various time points after pre-seeding. Panel A: Flow cytometry profiles; Panel B: Cell cycle distribution in NNF-16 cells.

cells were exposed right after plating. The highest and stable plating efficiency was obtained between the intervals of 4.5 h to 8.5 h. The plating efficiency diminished when the interval was too long, such as 21.5 h. Therefore, the intervals of 4.5 h to 8.5 h might be the best period to treat the cells with radiation. During this period, cells have accomplished the attachment and conformation change without division.

To confirm this conclusion, variations in cell cycle were analyzed with flow cytometer and the results were shown in Figure 4. All the cells were from one confluent stock so that more than 90% cells were in G1 phase and less than 5% cells stayed in G2/M phase. Cells were plated by following the optimized procedure. As shown in Figure 4, G2/M phase cells at a proportion of 1.6% remained unchanged from 2 h to 7.5 h and more than 95% cells stably stayed in G1 phase. 18 h later, part of the cells entered S phase. As shown in Figure 4B, the fraction of S phase cells increased linearly and reached to 85% at 21.5 h. G2 phase cells began to accumulate after 20 h.

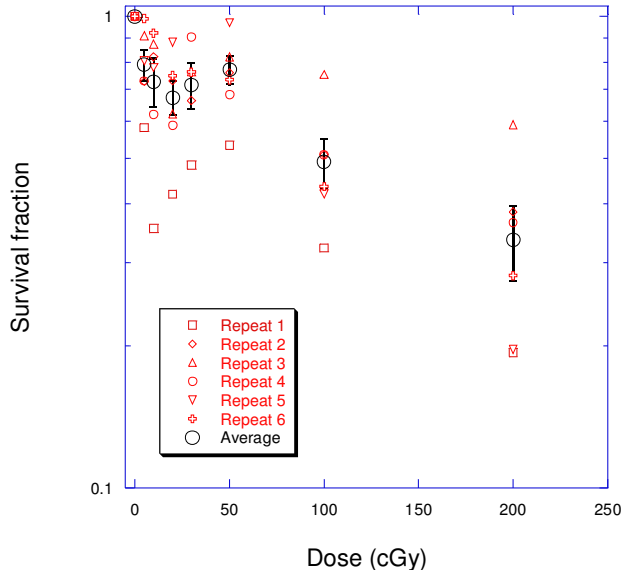

Figure 5. Lethal effect of X-rays on NNF-16 cells. The same amount of NNF cells were plated in a series of flasks following optimized protocol and then submitted to irradiation with X-rays during a period of 4.5 to 7.5 h. Error bars indicate the standard error of the mean (SEM) for 6 independent experiments.

Survival curves obtained with optimized method

To test the feasibility of this method, the same amount of NNF cells were plated in a series of flasks, and then submitted to irradiation with X-rays 4.5 to 7.5 h after plating. As shown in Figure 5, survival fraction reduced rapidly as dose increased to 20 cGy. But as doses increased higher, survival fraction increased and reached the maximum value at 50 cGy and decreased again.

It has been reported that human glioma cell line M059K exhibited low-dose hyper-radiosensitivity and induced radioresistance (HRS/IRR) while glioma cell line M059J did not (Wykes et al., 2006). Thus, we used these two cell lines to confirm the reliability of the method we developed here. M059K and M059J cells were irradiated with X-rays after a post-plating incubation of 4.5 h to 7.5 h. As shown in Figure 6, M059J cells lost their viability drastically and its survival fractions decreased sharply with the increasing irradiation doses while M059K cells showed hyper-radiosensitivity and induced radioresistance (HRS/IRR) at 20 cGy and 30-60 cGy, respectively.

DISCUSSION

In the present study, we slightly modified the routine colony-forming assay, optimized its operating protocol, and successfully fulfilled its application in accurate measurement of clonogenic survival fraction induced by very low-dose radiation. There are several tips for this method, including best condition of trypsin treatment, one dilution in one bottle for all samples, direct cell seeding on the bottom of each flask, and the appropriate period of 4.5 h to 8.5 h after plating for additional treatment. The first three tips are to minimize the difference in the cell number plated in each flask. Figure 1 demonstrates that reproductive colony formation can be achieved by following these rules. The last but not the least tip is to Figureure out the best period for radiation. The cross section of a cell varies during attachment to the surface of flask and morphological change. In the period of 5 h to 9 h after plated, cells accomplished the attachment and conformation change but did not start to divide yet (Figure 2). The plating efficiency was very stable during the period of 4.5 h to 8.5 h after plating (Figure 3). Cell cycle distribution also provided a clue since more than 95% of NNF-16 cells stably stayed in G1 phase and remained unchanged from 2 h to 7.5 h (Figure 4). Therefore, we set the interval between cell plating and irradiation as 4.5 h to 7.5 h for NNF-16 cells. Basing on our own experience, neither of these tips can be ignored to successfully use this method for low-dose survival assay.

Mammalian cell lines exhibit different sensitivity as a result of different cell cycle distribution when exposed to

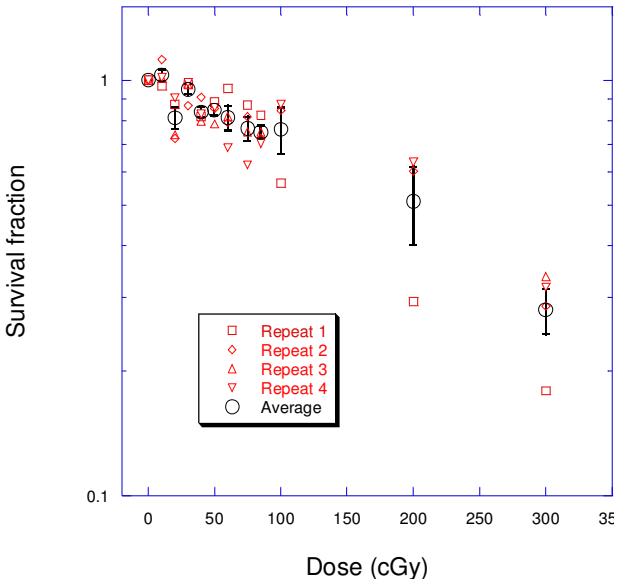

Figure 6 A

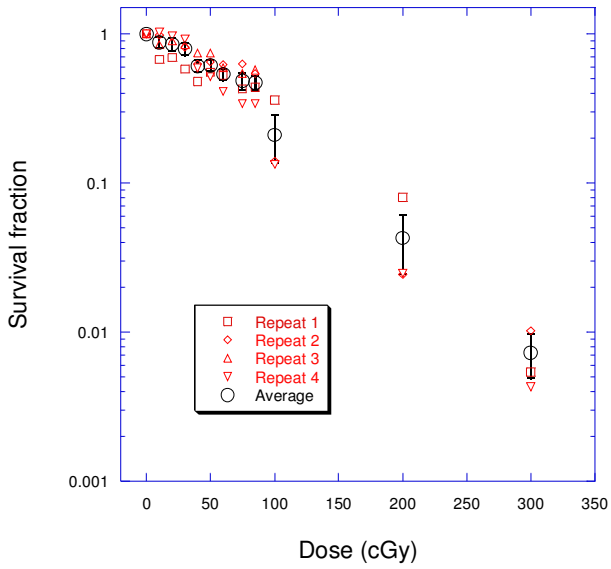

Figure 6 B

Figure 6. Survival of M059K and M059J cells exposed to X-rays. M059K and M059J cells were plated into flasks following optimized protocol and irradiated with X-rays after a post-plating incubation of 4.5 h to 7.5 h. Panel A: Survival curve of M059K cells; Panel B: Survival curve of M059J cells. Error bars indicate the standard error of the mean (SEM) for 4 independent experiments.

radiation (Short et al., 2003) since DNA repair ability and fidelity vary with cell-cycle phase (Sinclair, 1972) and G2 phase cells are the most sensitive to irradiation (Dewey et al., 1972; Iliakis and Nusse, 1983; Short et al., 2003). Thus, ensuring that the cell populations are synchronous is also a critical element in this survival assay. Deducing from the cell cycle data, after plating the cells, a period of 2 h to 7.5 h is properly appropriate for irradiation because the cells are synchronized in G1 phase while the amount of G2 phase cells is less than 2% and quite stable (Figure 4). The highest and stable plating efficiency was achieved when cells were exposed to irradiation during

this period (Figure 3). G2 phase cells began to accumulate after 20 h and the reduced plating efficiency was observed at 21.5 h (Figure 4B).

The plating efficiency was highly reduced by X-ray irradiation when cells were exposed right after plating (see Figure 3). One possibility is that radiation weakened the cells from attaching to flasks. The plating efficiency gradually increased when the interval between cell plating and irradiation increased, probably due to more and more cells attached before irradiation. Summarily, an interval of 4.5 to 7.5 h between plating and irradiation is appropriate for this low-cell-density survival assay. In this period, cells attach to the flask, accomplish the conformation change, accumulate in G1 phase, and consequently lead to stable plating efficiency. Some groups reported that HRS existed in whole cell population (Marples and Joiner, 1993) or G2 phase cell (Marples, 2004). Meanwhile, HRS has also been demonstrated in G1 phase cells (Short et al., 1999). Our method detected HRS in NNF-16 cells which were synchronized in G1 phase.

M059K and M059J were used to confirm the reliability and reproducibility of this method since the low-dose survival assay has been carried out by other groups using a flow cytometry-based clonogenic survival assay (Wykes et al., 2006). Cells were plated in a series of flasks as described, 4.5 to 7.5 h later submitted for irradiation with X-rays. The results showed that M059K exhibited HRS/IRR while M059J cells did not, which is consistent with other reports (Wykes et al., 2006). These results provide direct evidence that the modified colony-forming assay we presented here is reliable and reproducible. It is an easy and feasible method for the low-dose studies.

When using any other cell lines, the suitable interval between plating and radiation has to be determined due to the difference of cell lines in the speed of attaching to flasks and morphological change. Basing on our experience in using M059K and M059J cell lines, checking the cell morphology and cell cycle distribution at several time-points between 4.5 h and 7.5 h after plating should be enough for Figuring out the interval between cell plating and radiation.

CONCLUSION

In this paper, we successfully developed an easy and reliable method for low-dose survival assay by modifying the routine colony-forming assay and optimized its protocol. Plating the same number of cells into each flask and an interval of 4.5 h to 7.5 h between plating and irradiation are crucial for this assay. No special equipments are required so that this method would be very helpful for studies on low-dose radiation.

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