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Validation of mitotic harvesting method with human cervical carcinoma HeLa cells expressing fluorescent ubiquitination-based cell cycle indicators for radiation research

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ABSTRACT

The cell cycle is a series of events in the process of one cell giving rise to two daughter cells. The mitotic harvesting method, established by Terasima and Tolmach in the 1960s, causes minimal physiological stress on the cells and achieves a high degree of cell cycle synchrony by collecting only mitotic cells from a cultured cell system. The purpose of the present study is to validate the versatility of the mitotic harvesting method using human cervical cell line HeLa cells expressing Fluorescent Ubiquitination-based Cell Cycle Indicators (FUCCI) and to estimate the cell cycle-dependent changes in radiosensitivity in HeLa–FUCCI cells. The image analysis showed that cell cycle synchrony was maintained for at least 24 hours after mitotic cell collection. Also, the clonogenic assay demonstrated changes in radiosensitivity that were cell cycle dependent. These results indicate that the mitotic harvesting method using FUCCI-expressing cells has high versatility in the field of radiation cell biology.

INTRODUCTION

In 1951, Howard and Pelc, who developed the autoradiographic technique (Pelc 1947), experimentally demonstrated that cells have a period of DNA synthesis (known today as the S phase) during the division cycle (Howard and Pelc 1951), which led to the establishment of the concept of the cell cycle. Cell cycle synchrony in culture cells is an experimental technique that has supported significant progress to date in this field. The mitotic harvesting method, which uses the loss of adhesion associated with mitotic cell rounding, was established by Terasima and Tolmach in the 1960s (Terasima and Tolmach 1963) and led to their historic discovery that radiosensitivity changes in a manner that is cell cycle dependent (Terasima and Tolmach 1961). The greatest advantage of this method is that it causes minimal physiological damage to cells, unlike methods that use reagents that inhibit cell metabolism. Furthermore, this technique is easily reproducible in the lab.

Fluorescent Ubiquitination-based Cell Cycle Indicators (FUCCI) form a fluorescent probe that enables real-time observation of cell cycle progression in living cells (Sakaue-Sawano *et al.* 2008). Geminin is ubiquitinated during the M and G1 phases and degraded by the proteasome, while Cdc10 dependent transcript 1 (Cdt1) is ubiquitinated during the S and G2 phases and degraded by proteasome. By combining Geminin expressed in the S/G2 phases and Cdt1 expressed in the G1 phase with green fluorescent protein (monomeric Azami-Green1 (mAG1)) and red fluorescent protein (monomeric Kusabira-Orange2 (mKO2)), respectively, FUCCI can visualize the cell cycle phases at different fluorescent wavelengths. This imaging method enables the exploration of the spatiotemporal patterns of cell cycle

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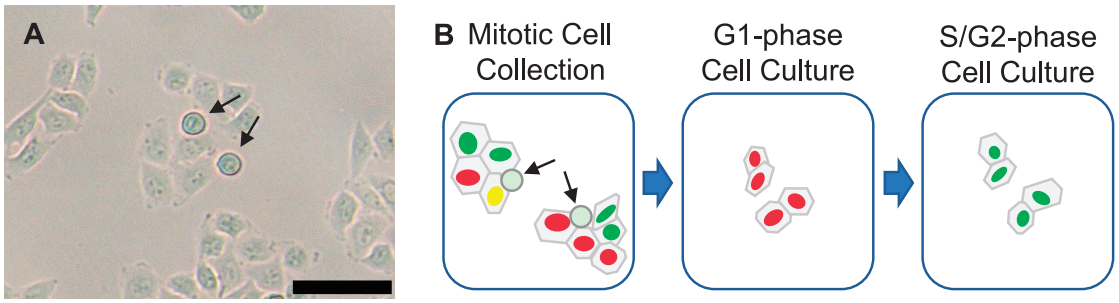


Fig. 1 Overview of the mitotic harvesting method. (**A**) Optical image of mitotic rounding cells (arrows). The scale bar indicates 50 μm. (**B**) Procedures for the mitotic harvesting method. Only the mitotic cells (arrows) in the cultured HeLa–FUCCI cell population are collected to obtain a synchronized cell cycle population. In a new dish, the cell nuclei change from red to green under fluorescence microscopy as the cell cycle progresses from the G1 phase to the S/G2 phases.

dynamics during structural and behavioral changes in cultured cells (Sakaue-Sawano and Miyawaki 2014).

The purpose of this classic study was to validate the versatility of the mitotic harvesting method using human cervical cancer cell line HeLa cells that express FUCCI (Sakaue-Sawano *et al.* 2008), as well as to examine changes in radiation sensitivity that are cell cycle dependent. Real-time fluorescence microscopy using FUCCI-expressing cells enables easy tracking of changes in cell cycle synchrony rates in a cell population over time. Also, clonogenic assay has been useful for assessing cell survival after irradiation (Franken *et al.* 2006). In the present study, these methods were used to determine the utility of the mitotic harvesting method from today's radiobiological perspective.

MATERIALS AND METHODS

Cell culture. A subline of the HeLa–FUCCI cells, RCB2812 HeLa.S–FUCCI (Sakaue-Sawano *et al.* 2008), was provided by the RIKEN BioResource Center in Japan. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA, USA), which contained 10% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, USA) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA), in a humidified incubator maintained at 37°C in an atmosphere of 95% air and 5% CO_2.

Mitotic harvesting method. As previously described (Terasima and Tolmach 1961; Terasima and Tolmach 1963), the collection of mitotic rounding cells, which makes up a few percent of the cultured cell population, allows for culturing while maintaining a high level of cell cycle synchronization (Fig. 1). Cultured animal cells that normally grow attached to a surface often round up and adhere much less firmly to the surface during mitosis, and we can take advantage of this attenuated binding to selectively harvest the mitotic cells from randomly dividing populations of animal cells (Terasima and Tolmach 1963). After replacing the medium, the bottom of the 150-mm diameter dish in which the cells were cultured was gently tapped, and mitotic cells adhering thinly to the bottom were suspended once in the new medium. The cells were then cultured in 12-mm glasss-bottomed dishes (IWAKI, Sayama-shi, Japan) for observation.

Imaging analysis. We used an All-in-One BZ-9000 Fluorescence Microscope (Keyence, Osaka, Japan) with excitation and emission wavelengths of 470 nm and 535 nm (green) and 540 nm and 605 nm (red), respectively. Under a fluorescence microscope, the nuclei of cells in the G1 phase are red, and those in the S/G2 phases are green. The early S phase, when the expression of both overlaps, is yellow. We observed more than 120 cells each hour, as well as the non-irradiated (control) cells from at least independent experiments on different days. The statistical significance of the cell cycle distributions was determined using the chi-square test as $P < 0.05$.

Irradiation setting. Cells were irradiated using a 150 kVp X-ray through a 1.0-mm aluminum filter using an X-ray generator (MBR-1520R-4; Hitachi Power Solutions, Ibaraki, Japan) at 1.83 Gy/min. The absorbed dose in the air was monitored using an ionization chamber placed next to the sample during irradiation.

Clonogenic assay. We used 60-mm culture dishes (SPL Life Sciences, Pocheon-si, South Korea) for a clonogenic assay. The cells were plated and allowed

to adhere for more than 8 hours. Following irradiation at room temperature, the cultures were incubated until colonies (≥ 50 cells) formed and were then stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany).

Colonies were counted visually. Plating efficiency (PE) was calculated as the number of colonies/number of cells, and the surviving fraction (SF) was calculated as the Experimental PE/Control PE. The linear-quadratic (LQ) modeling of cell survival demonstrates total lethal lesions comprising those produced by (1) a single hit (linearly related to dose) and (2) two hits (quadratically related to dose) (Steel and Peacock 1989). The cell survival curves were fitted using the LQ model, which defines two components of cell killing: αD, which is proportional to the dose, and βD^2, which is proportional to dose^2. The α/β component for irradiation was calculated using the equation $SF = \exp(-\alpha D - \beta D^2)$.

RESULTS AND DISCUSSION

Cell cycle synchronization of HeLa–FUCCI cells

After mitotic cell collection, it took at least 8 hours for many cells to adhere to the bottom of the dish, and cell cycle progression was observed in live cells. In fact, as Fig. 2A shows, fluorescence microscopy images 8 and 14 hours after mitotic collection showed clear cell cycle synchronization.

Fig. 2B shows the color-coded percentages of the cell population after mitotic collection. The red peak was at 6 hours, and the green peak was at 14 hours. These color-coded percentages differed significantly between the control and synchronized groups. At 6 hours, as noted above, there were still many cells that were not adherent. Clear M-phase cells appeared after 16 hours, and the doubling time was approximately 18 to 20 hours. Although the cell cycle synchronization was maintained for at least 24 hours, after 44 hours, more than twice the doubling time, there was no significant difference in the color percentage compared to the control group.

The observation results confirmed that a certain degree of cell cycle synchronization was maintained during the first cycle of the cell cycle. In HeLa cells, cell–cell interactions that synchronize the cell cycle in a cell population have not been confirmed, and according to a previous study, radiation-induced bystander effects on the cell cycle have not been detected (Fukunaga *et al.* 2021a). Thus, the cell cycle of each individual cell may proceed independently, which may disrupt the cell cycle synchrony of the population, with, or without irradiation.

This method has many technical limitations. For example, as pointed out previously, there is diversity in the length of the G1 phase of each cell after mitotic harvesting; thus, cell cycle synchronization often breaks down after one cycle (Shimono *et al.* 2020). Also, there is no one-to-one correspondence between color and cell cycle, as red cells can be regarded as being in the G1 phase only, while green cells contain a mixture of S phases and G2 phases. Thus, it is not possible to demarcate all processes of the cell cycle by color. However, the advantage of easily confirming cell cycle synchrony with fluorescence microscopy would be greater than the disadvantages of these technical limitations.

Cell cycle dependence of radiosensitivity

Relative cellular radiosensitivities were investigated in the clonogenic assay at 0 hours, 8 hours, and 14 hours after mitotic cell collection and over a range of doses ranging from 0.5–8 Gy (Fig. 3A). The cell survival curves of the groups were fitted using LQ modeling. The summary data of the survival fractions indicated that the 14-hour group demonstrated more radioresistance and that the 8-hour and 0-hour groups demonstrated more radiosensitivity. Fig. 3B shows the PE and α/β components calculated from the respective survival curves. These results demonstrate cell cycle-dependent changes in radiosensitivity in HeLa–FUCCI cells. To the best of our knowledge, the present study is first to demonstrate cell cycle-dependent survival responses in HeLa–FUCCI cells following irradiation, so our findings are expected to contribute to future radiation research.

Although a previous study using time-lapse fluorescence imaging reported that HeLa–FUCCI cells became radioresistant from the early to late G1 phase and rapidly became radiosensitive in the early S phase (Shimono *et al.* 2020), the technical limitations of the present study did not enable us to confirm reproducibility. The validation of cell cycle-dependent radiosensitivity, especially in the S phase and G2 phase, remains an issue to be addressed. Further studies using cell lines other than HeLa cells and various irradiation conditions, such as microbeam radiotherapy (Fukunaga *et al.* 2021b) and ultrahigh dose-rate FLASH radiotherapy microbeams (Friedl *et al.* 2022), are essential to increase understanding of the mechanism of the cell cycle dependence of radiosensitivity.

Finally, we would like to contribute some points to perspectives on future applications and development of the mitotic harvesting method in the clinical oncology field. Unlike cell cycle synchronization

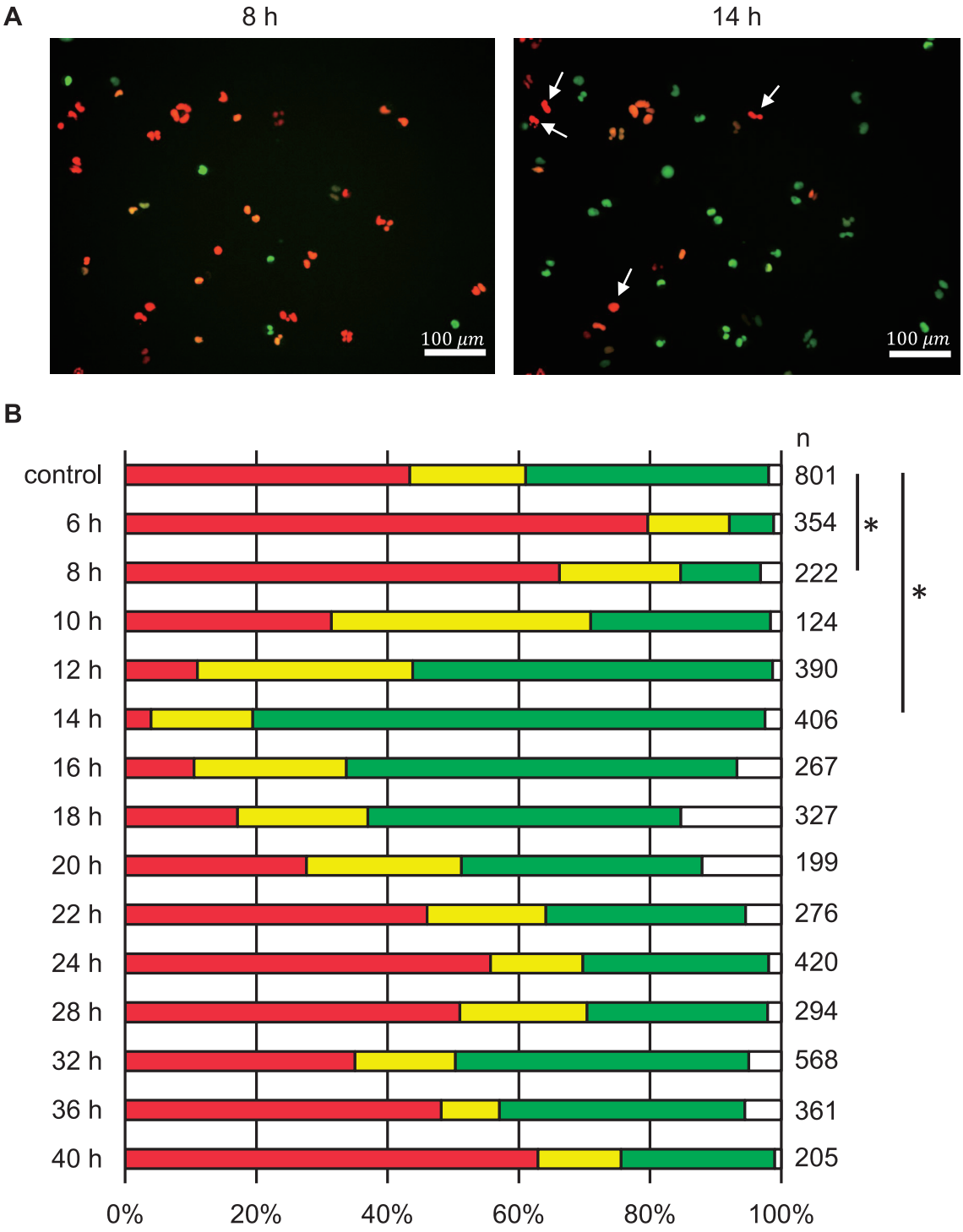
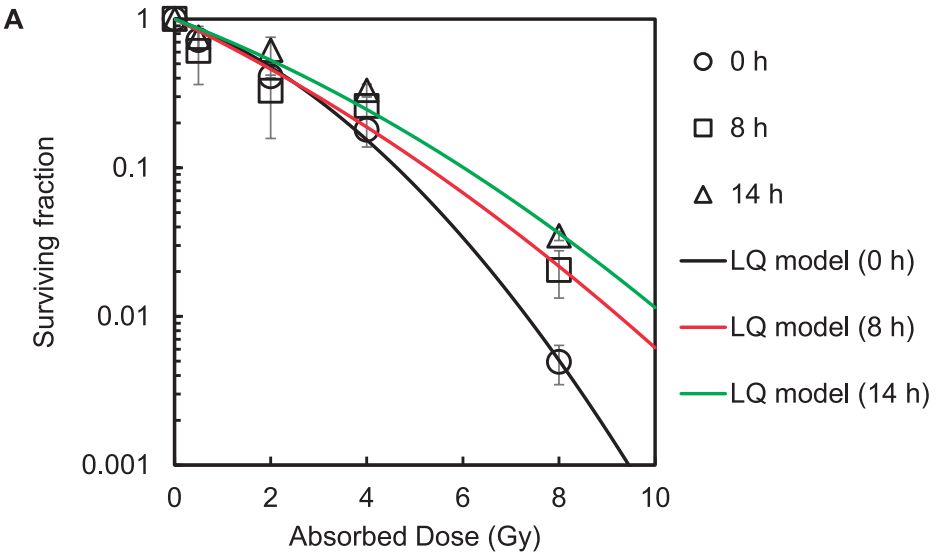


Fig. 2 Cell cycle synchronization of HeLa–FUCCI cells. (**A**) Fluorescence microscopy images of HeLa–FUCCI cells at 8 hours (left) and 14 hours (right) after mitotic cell collection. Cells in the G1 phase at 14 hours are shown in red (arrows). The scale bars indicate 100 μm. (**B**) Summary graph of fluorescent color distributions in HeLa–FUCCI cells in the control and cell cycle synchronization groups. The color of the bars indicates the color of the cells. The * denotes $P < 0.001$ (chi-square test).



B

	Plating Efficiency	α	β	α/β
0 h	0.059 ± 0.007	0.275 ± 0.065	0.048 ± 0.009	5.693 ± 1.725
8 h	0.048 ± 0.012	0.358 ± 0.132	0.015 ± 0.018	23.59 ± 29.75
14 h	0.066 ± 0.007	0.165 ± 0.047	0.032 ± 0.007	5.221 ± 1.853

Fig. 3 Cell cycle dependence of radiosensitivity. (**A**) Surviving fraction of HeLa–FUCCI cells collected at 0, 8, and 14 hours after mitotic cell collection plotted against radiation doses ranging from 0.5–8 Gy. (**B**) Summary data from LQ modeling of the survival curves for the cells collected at 0, 8, and 14 hours after mitotic cell collection.

approaches that use reagents that inhibit cell metabolism, the mitotic harvesting method causes minimal physiological stress to cells and, therefore, is suitable for studying responses after radiation exposure. Some anticancer drugs work in a manner that is cell cycle dependent. Radiobiological exploration using this method will significantly increase the accuracy of predictions regarding treatment outcomes when such drugs are combined with fractionated radiotherapy.

In conclusion, the combination of the mitotic harvesting method and the FUCCI probe remains a useful cell cycle synchronization approach to cell biology research, even today. Furthermore, the results of this study experimentally demonstrated a change in radiosensitivity in HeLa–FUCCI cells that was cell cycle dependent. Further investigations using other cell lines and irradiation settings are expected to increase the understanding of the cell cycle dependence of radiosensitivity.

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CONFLICTS OF INTEREST

The authors have declared that there is no conflict of interest.

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