

Estimation of the Dividing Fraction and Potential Doubling Time of Tumors Using Cytochalasin B¹

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ABSTRACT

We devised a new *in vitro* method to estimate the proportion of dividing cells and the potential doubling time (T_{pot}) of tumors using the same technique as with the cytokinesis-block micronucleus assay. The usefulness of this methodology was confirmed by comparing the data with those obtained by flow cytometry after bromodeoxyuridine (BrdUrd) incorporation. Xenografted human and murine tumors were excised 0.5–8 h after BrdUrd injection and disaggregated to single cells. A portion of these cells was then plated in dishes to which 1 or 2 $\mu\text{g/ml}$ cytochalasin B were added. These concentrations of cytochalasin B blocked cytokinesis but not karyokinesis with the result that cells became multinucleate after mitoses. At every 12 or 24 h of culture, the proportion of multinucleate cells and the total number of nuclei and cells were scored. The remaining cells were analyzed with a flow cytometer and the BrdUrd-labeling index and T_{pot} were determined. In all 8 tumor lines studied, the proportion of multinucleate cells reached a plateau within 3–7 days of culture, and we therefore defined the dividing fraction as the plateau value. The dividing fraction ranged between 33 and 98% and clearly tended to be high in rapidly growing tumors. A significant correlation was seen between the dividing fraction and BrdUrd-labeling index ($r = 0.74$, $P < 0.001$). The increase in the average number of nuclei per cell also tended to be higher in rapidly growing tumors. The T_{pot} was estimated as the time for this nucleus/cell ratio to reach 2.0. In 7 of 8 tumor lines, T_{pot} values estimated by this method compared reasonably with those estimated by the BrdUrd method. Therefore, this simple technique, originally developed for radiosensitivity prediction, would also seem to be useful in estimating tumor proliferative activity.

INTRODUCTION

Several methods are used to estimate the proliferative activity of tumors. The S-phase fraction or LI^3 is currently measured by flow cytometry (1, 2) and the growth fraction is now estimated by detecting particular substances present only in proliferating cells using monoclonal antibodies or autoradiography techniques (3–6). Generally, tumors with a high S-phase or growth fraction are associated with a poor prognosis (7–9). The T_{pot} which takes the growth fraction but not the cell loss factor into account (10) is considered to represent repopulation rates during and after radiotherapy better than the pretreatment volume doubling time (11, 12). Tumors with a short T_{pot} are less likely to respond to conventional radiotherapy and are considered to be good candidates for accelerated fractionation (11–13). The T_{pot} can be measured using flow cytometry following *in vivo* labeling of tumors with BrdUrd (14–17), and clinical trials in which patients are selected for accelerated fractionation by this method are now in progress (18).

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³ The abbreviations used are: LI, labeling index; BrdUrd, bromodeoxyuridine; T_{pot} , potential doubling time; CB, cytochalasin B; MNC, multinucleate cells; DF, dividing fraction; PBS, phosphate-buffered saline; RM, relative movement; T_s , DNA synthetic period, N/C, nuclei/cell; HP, Harding-Passey.

In our previous investigation, we established a cytokinesis-block micronucleus assay in experimental tumor systems for the prediction of radiosensitivity (19). However, it also appeared possible with this technique to predict the proliferative activity of tumors. This assay uses appropriate concentrations of CB to block cytoplasmic (but not nuclear) division (20), so that cells that have undergone mitoses are easily recognizable by their multinucleate appearance. The proportion of MNC was found to reach a plateau after certain culture periods and appeared to be high in rapidly growing tumors. Moreover, since the number of nuclei increases while the cell number remains constant (assuming that the cytokinesis-blocking action of CB is perfect), it seems possible to estimate the time for the number of nuclei to double. Given certain conditions, this time may well be similar to the T_{pot} .

In this study, therefore, the maximum proportion of MNC, the DF, and the T_{pot} of 8 experimental tumor lines were estimated using this technique, and the results were compared with the BrdUrd-LI and T_{pot} measured by flow cytometry to evaluate the usefulness and validity of our method.

MATERIALS AND METHODS

Animals and Tumors. Four human tumor xenografts in NMRI nude mice (MeWo, melanoma; PECA4451, squamous cell carcinoma; PECA4197, squamous cell carcinoma; L5, leiomyosarcoma) and four murine tumors, EMT6 sarcoma in BALB/c, SCCVII carcinoma in C3H/He, HP melanoma in BALB/c, and O771 adenocarcinoma in C57BL mice were used. The characteristics of the tumors and tumor transplantation procedures were described previously (19). Briefly, a piece of tumor (3–4 mm in diameter and 1 mm thick) was inoculated s.c. into the right flank of mice ages 9–11 weeks. Female mice were used predominantly although males were also used, and the sex of the mice did not seem to influence the results. Tumor size was measured with a caliper every other day to estimate the volume doubling time of each tumor. Experiments were carried out when the tumor size reached approximately 500 mm³.

Culture Medium and Treatment of Culture Dishes. Eagle's minimum essential medium supplemented with 20% fetal calf serum and gentamicin sulfate (0.2 mg/ml) and preheated to 37°C was used throughout the experiments. Standard culture dishes (20 cm²) were used except with the O771 tumor line, for which the same dishes were precoated with fibronectin (5 $\mu\text{g/cm}^2$) to improve cellular attachment.

Experimental Procedures. BrdUrd (100 mg/kg) dissolved in physiological saline was injected i.p. into all the tumor-bearing mice. Tumors were excised 0.5 to 8 h later. The following procedures were the same as employed in the previous study (19). Briefly, the tumors were disaggregated to single cells by treating them with collagenase/dispase (1 mg/ml) for 1 h. More than 90% of the cells were judged as viable by a trypan blue dye exclusion test. A portion of the cells from individual tumors was plated in multiple dishes (3 \times 10⁵ cells/dish and 2 dishes/point whenever the cell yield allowed it) and CB dissolved in dimethyl sulfoxide was added immediately. The CB concentrations used were 1 $\mu\text{g/ml}$ for the PECA4197, L5, and SCCVII tumors and 2 $\mu\text{g/ml}$ for the other tumors as determined in the previous study (19). In preliminary experiments, the cell number per dish was monitored after plating and no significant increase in the cell number was seen after addition of these concentrations of CB in any tumor cell line, suggesting that cytokinesis was effectively blocked. Cultures were terminated every 12

or 24 h over durations of 4 to 8 days, and the cells were fixed with 1% glutaraldehyde in phosphate buffer, treated with 5 N HCl for 20 min, and stained with Schiff's reagent for 1 h. The number of respective cells with different numbers of nuclei (mononucleate, binucleate, trinucleate, etc.) was scored under a microscope at $\times 1000$. The remaining single cells were fixed with 96% ethanol for flow cytometric analysis.

BrdUrd/DNA Staining and Flow Cytometry. About 2×10^6 cells were incubated with 0.5% pepsin in 0.055 N HCl solution for 10 min at 37°C, washed with physiological saline, and incubated with 2 N HCl for 20 min at room temperature. After one washing with physiological saline and then with PBS/0.05% Tween 20, the cells were treated with the same solution plus anti-BrdUrd antibody (Becton Dickinson, Heidelberg, Germany; 20:1) for 30 min at 4°C in the dark. They were then washed with PBS/0.05% Tween 20/1% bovine serum albumin and incubated with the same solution containing fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (DuPont, Dreieich, Germany; 100:1) for 30 min at 4°C in the dark. After a washing with PBS/Tween 20/bovine serum albumin, the cells were resuspended in 1 ml PBS, to which 100 μ l propidium iodide were then added, and their fluorescence was determined by flow cytometry.

All samples were analyzed with a FACScan system equipped with an argon ion laser operating at 488 nm and 300 MW and Cell-Fit software (Becton-Dickinson, Heidelberg, Germany). Log green fluorescence (fluorescein isothiocyanate) was measured through a 530 nm band-pass filter and red fluorescence (propidium iodide) through a 650 nm long-pass filter. In each specimen 10^4 cells were analyzed. The DNA index of each tumor line was determined as described previously (21).

Estimation of the BrdUrd-LI and T_{pot} . According to the method of Begg *et al.* (14) and Wilson *et al.* (15), appropriate windows were placed on the cytograms with red versus green fluorescence around the unlabeled G_1 , S, and G_2 -M regions and the labeled G_1 and non- G_1 regions. The mean fluorescence of each window was then calculated. The BrdUrd-LI was defined as the percentage of labeled cells after halving the number of labeled cells lying in the G_1 position. The RM of the labeled cells was calculated from the equation

$$RM = \frac{F_L - F_{G_1}}{F_{G_2-M} - F_{G_1}}$$

where F_L , F_{G_1} , and F_{G_2-M} are the mean red fluorescence values of labeled cells in the non- G_1 region, unlabeled G_1 cells, and unlabeled G_2 -M cells, respectively. The DNA synthetic period, T_s , was calculated from

$$T_s = \frac{t \times 0.5}{RM - 0.5}$$

where t is the time between BrdUrd injection and tumor excision. The T_{pot} was estimated by the formula

$$\frac{0.8 \times T_s}{BrdUrd - LI}$$

For aneuploid tumors, the G_1 region of the diploid population was excluded from the analysis.

RESULTS

Changes in the Proportion of MNC as a Function of Culture Duration. The percentage of MNC increased until 72–168 h and then reached a plateau in all the tumor lines (Fig. 1). Therefore the DF was defined as the plateau level and was calculated from the average percentage of MNC at the last two points (*e.g.*, at 72 and 96 h for the EMT6, SCCVII, and O771 tumors).

Correlation between the DF and BrdUrd-LI. The individual and average values of the DF and BrdUrd-LI are shown in Fig. 2 and Table 1, respectively. The DF ranged between 33 and 98% and tended to be high in rapidly growing tumors. The correlation coefficient between the individual values of DF and

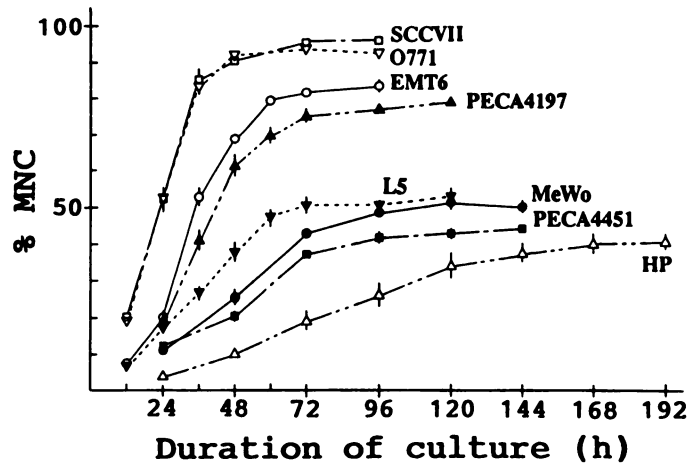


Fig. 1. Changes in the proportion of multinucleate cells as a function of culture duration. Each point represents the mean \pm SE (bars) of 8 or 9 tumors.

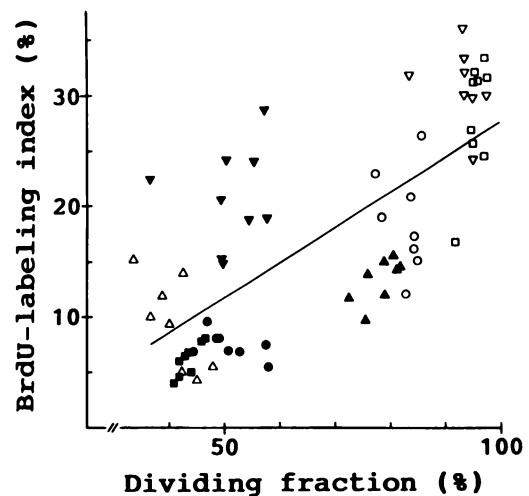


Fig. 2. Correlation between the dividing fraction and BrdUrd-labeling index. The symbols indicating each tumor line are the same as used in the other figures.

BrdUrd-LI was 0.74 ($P < 0.001$) and 0.86 ($P < 0.01$) for the respective average values.

Increase in the N/C Ratio with Culture Duration. The average number of nuclei per cell tended to increase with the culture duration faster in rapid than in slow growing tumors (Fig. 3). Under ideal conditions, this curve should follow the equation

$$N/C = DF \times 2^t + (1 - DF)$$

where t is the culture duration and A is a constant ($1/A =$ average cell cycle time, the former and latter terms represent dividing and nondividing populations, respectively). It is assumed that in the absence of CB, the total cell number doubles *in vitro* during the time required for the N/C ratio to reach 2.0, provided that CB does not influence the cell cycle time or mitotic rate. Therefore, the time would be similar to the T_{pot} if the cell cycle time and mitotic rate in these early *in vitro* phases are similar to those *in vivo*. However, some delay appeared to exist before mitoses commenced and the actual curves were not always exponential; in some tumors the N/C ratio never reached 2.0. Therefore the T_{pot} was estimated as shown in Fig. 4. A few data points before the rollover of the original curve were chosen by eye and fitted by an exponential curve ($y = B2^{Cx}$, where B and C are constants) and the T_{pot} was calculated from

Table 1 Cell kinetics data for the eight tumor lines studied

Tumor	DNA index	DF (%)	BrdUrd-LI (%)	T_s (h)	T_{pot} (days)		Volume doubling time (days)
					BrdUrd method	CB method	
MeWo	1.6	51.0 ± 4.8 ^a	7.5 ± 1.2	11.2 ± 1.3	4.8 ± 0.6	3.9 ± 0.4	6.2 ± 0.9
PECA 4451	1.6	43.4 ± 2.0	6.1 ± 1.5	12.6 ± 2.9	7.9 ± 3.3	4.5 ± 0.4	7.8 ± 1.2
PECA 4197	1.0	78.3 ± 3.2	13.5 ± 2.0	6.5 ± 1.5	1.7 ± 0.3	2.0 ± 0.3	2.7 ± 0.3
L5	1.5	51.2 ± 6.5	20.8 ± 4.5	19.3 ± 9.8	3.0 ± 1.2	3.9 ± 0.7	3.4 ± 0.4
EMT6	1.7	82.9 ± 3.1	18.7 ± 4.6	8.8 ± 2.1	1.5 ± 0.2	1.7 ± 0.1	2.2 ± 0.3
SCCVII	1.8	95.8 ± 1.8	28.1 ± 5.3	9.9 ± 2.1	1.3 ± 0.4	1.5 ± 0.2	1.9 ± 0.2
HP	2.1	40.8 ± 4.7	9.5 ± 4.2	10.7 ± 2.2	4.4 ± 2.6	9.5 ± 1.9	5.4 ± 1.6
O771	1.0	93.4 ± 4.1	30.7 ± 3.4	7.5 ± 1.6	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.1

^a All values (except for the DNA index) represent the mean ± SD.

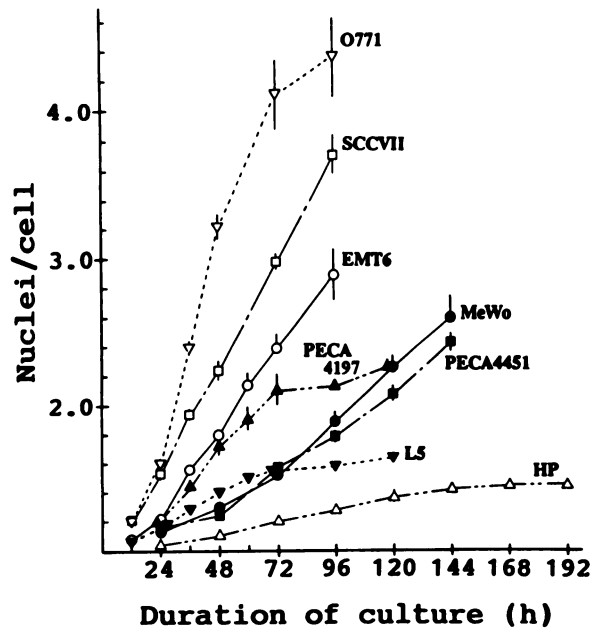


Fig. 3. Changes in the average number of nuclei per cell as a function of culture duration. Each point represents the mean ± SE (bars) of 8 or 9 tumors.

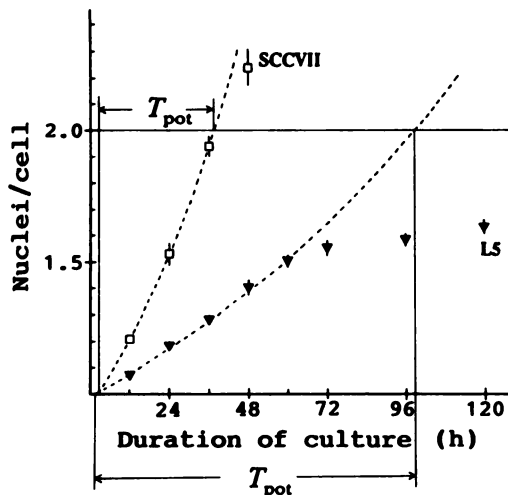


Fig. 4. Method of estimating the potential doubling time from changes in the nuclei/cell ratio.

$$(Time\ at\ N/C\ ratio = 2.0) - (time\ at\ N/C\ ratio = 1.0)$$

for the fitted curve. The individual and average T_{pot} values thus obtained are shown in Fig. 6 and Table 1, respectively.

Correlation between the Two T_{pot} Measurements. The RM for each tumor is shown in Fig. 5. In all tumors the RM values

appeared to be fitted by linear regression through an intercept of 0.5, rather than 0.6. Therefore,

$$T_s = \frac{t \times 0.5}{RM - 0.5}$$

was used to calculate the T_s . The average values of the T_s and T_{pot} are shown in Table 1 together with the volume doubling time. Fig. 6 shows the relationship between the T_{pot} values estimated by the CB method and by the BrdUrd method for individual tumors. In MeWo, PECA4197, L5, EMT6, SCCVII, and O771 tumors, the two measurements agreed fairly well.

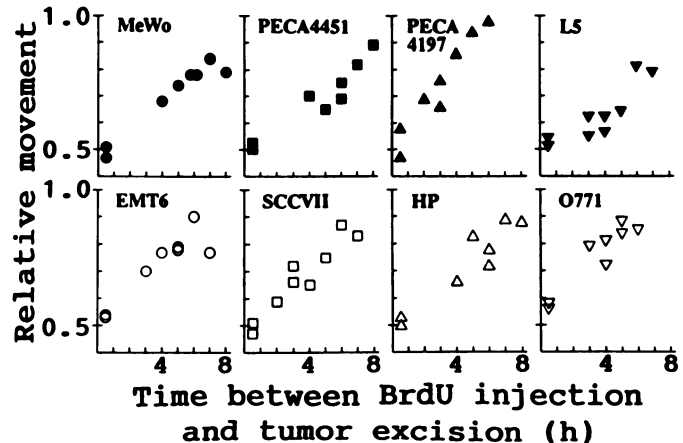


Fig. 5. The relative movement of BrdUrd-labeled cells as a function of the time between BrdUrd injection and tumor excision.

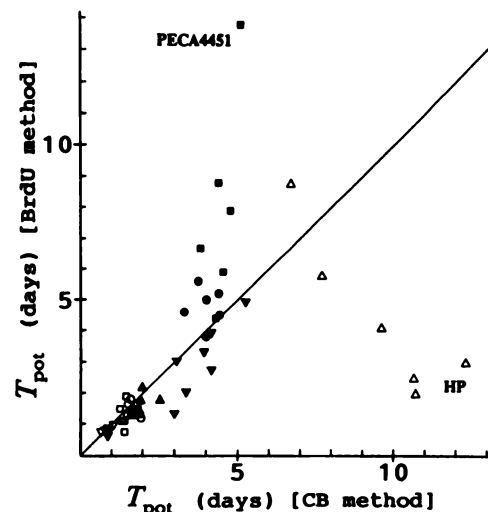


Fig. 6. Correlation between the T_{pot} estimated by the cytochalasin B method and that estimated by the BrdUrd method. The symbols indicating each tumor line are the same used in the other figures.

However, in HP and some of PECA4451 tumors, they did not agree. In light of the volume doubling time, the results of the BrdUrd method appeared more reasonable for the HP tumor but those of the CB method seemed more realistic for the PECA4451 tumor. The correlation coefficient of the individual T_{pot} values of all the tumors was 0.45 ($P < 0.01$) and 0.60 ($P > 0.05$) for the respective average values. When the HP and PECA4451 lines were excluded, these coefficients reached 0.89 ($P < 0.001$) and 0.92 ($P < 0.01$), respectively.

DISCUSSION

We defined a new index equal to the maximal proportion of MNC, DF, that can be used to measure proliferative activity without the use of flow cytometry or any special techniques. The percentage of MNC reached a plateau in all tumors and therefore the DF represents the proportion of cells undergoing mitoses *in vitro*. The DF tended to be high in rapidly growing tumors and a significant correlation was found between the DF and BrdUrd-LI. The average values for the 4 human tumor xenografts were between 43 and 78%, similar to the reported growth fractions of other xenografted human tumors measured by using the Ki67 antibody (22). It seems reasonable to assume that most of proliferating cells *in vivo* undergo mitoses *in vitro* whereas most of nonproliferating cells do not and that the DF is closely related to the growth fraction *in vivo*. Therefore it would be worthwhile to compare the DF and growth fraction in human tumors.

The BrdUrd method has been the only practical method currently used to estimate the T_{pot} . The method is reasonable and simple if BrdUrd (or iododeoxyuridine) administration and a flow cytometer are available, but in some countries (e.g., Japan), i.v. administration of these compounds is not permitted. Moreover, the obtained T_{pot} values are sometimes unrealistic especially when the LI is low, as seen in this study and as reported by others (17). It is also difficult with diploid or near-diploid tumors to distinguish tumor cells from normal cells in the DNA histogram. Because of the relatively large associated errors, some authors have recommended use of the method only to divide tumors into 2 groups, rapid or slow (16).

We devised a new estimation method for the T_{pot} . The idea is quite simple inasmuch as the value is obtained from the time required for the number of cell nuclei to double. The T_{pot} (as well as the DF) could be estimated similarly by the stathmokinetic method using Colcemid or other spindle poisons that arrest cells in metaphase (10, 23). However, with our CB method, cells that have undergone the second (or even further) mitosis can be identified and taken into account, which would be an advantage of this method. In order for this *in vitro* nuclear doubling time to be similar to the T_{pot} , the cell cycle time and mitotic rate should be similar in *in vivo* and early *in vitro* phases and should not be influenced by CB. The former is the common problem in applying cell kinetic methods *in vitro*. To determine whether or not these conditions existed, we compared the T_{pot} measured by our method with that measured by the BrdUrd method. We found that the latter requirement is sometimes not met, and indeed, CB can suppress the mitotic rate as seen typically in L5 and HP tumors. However, even in such cases, estimation of the T_{pot} was possible in L5 tumors from the initial portion of the curve. Overall, reasonably accurate T_{pot} estimation was possible in 7 of 8 tumor lines. In the HP tumor line, presumably CB suppressed the cell cycle progression or the tumor cells did not adapt to culture well, so that the T_{pot} value

became too large. Some human tumors are known to be difficult to proliferate in cultures and CB is considered never to quicken or increase the cell cycle time or mitotic rate. As a result, if a tumor is found to have a short T_{pot} by this method, this should constitute solid proof of rapid growth. This method should thus be especially useful in uncovering rapidly growing tumors. On the other hand, it might be difficult to judge whether the finding is true or not, if a tumor is found to be slow growing. This would be a limitation of the method.

In using this technique in primary human tumors, minor modifications of the method might be necessary such as the use of various disaggregating procedures depending on the tumor type (24) and the routine use of precoated culture plates (25). Preliminarily, we have found that our disaggregation method works on pancreatic and rectal cancers, with a cell yield of $3-8 \times 10^6/g$.⁴ Moreover, attention should be paid to the concentration of CB to be used. In order for the estimation of the DF and T_{pot} to be effective, the cytokinesis-blocking action of CB must be complete. This effect was confirmed in preliminary experiments in this study, but in future clinical investigations, it will be impossible to check this for each tumor. However, our results indicate that the concentration of CB optimal to yield the highest proportion of MNC almost completely blocks cytokinesis. Therefore, in clinical practice, it seems desirable to test 2 or more CB concentrations in order to determine the optimal one. Our previous work suggested the optimal concentration to most likely be 1 or 2 $\mu g/ml$ (19).

This assay requires relatively large tumor samples (probably more than 300 mg) for plating of cells in multiple dishes. Moreover, culture dishes must be serially removed from incubation every day for 1 week, making the assay labor intensive. Still, cell staining is not necessary immediately after fixation and fixed cells can be kept under humidified conditions until a sufficient number of dishes accumulate, allowing the efficient staining. Finally, it should be emphasized that such DF and T_{pot} data are obtained together with radiosensitivity data from the cytokinesis-block micronucleus assay. In other words, this can be a predictive assay of both radiosensitivity and proliferative activity. We have started using this assay in primary human tumors and have succeeded in most of tumors tested thus far.

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