Energy transfer between fluorescein isothiocyanate and propidium iodide – a problem in the estimation of T_{pot} with the bromodeoxyuridine–DNA flow cytometry technique?

Maria C. Johansson [a,*], Bo Baldetorp [a] and Stina M. Oredsson [b]

[a] *The Jubileum Institute, Department of Oncology, University Hospital, S-221 85 Lund, Sweden*
[b] *Department of Animal Physiology, University of Lund, S-223 62 Lund, Sweden*

Received 8 March 1999
Accepted 23 October 1999

Energy transfer in flow cytometry can occur when two fluorochromes are bound in close proximity (generally within 100 Å) and the emission spectrum of one fluorochrome overlaps significantly with the excitation spectrum of the other. The latter criterium is fullfilled for the fluorochromes fluorescein isothiocyanate and propidium iodide and also the former when they, e.g., are used in bromodeoxyuridine – DNA flow cytometry methods. In the present growth kinetic study using this method, we show that energy transfer does take place between fluorescein isothiocyanate and propidium iodide which results in a detected increase in DNA content with 2–3%. Despite the erroneous increase in the obtained DNA content values, this does not seem to have any influence on the calculation of DNA synthesis time and potential doubling time where the DNA content, based on the relative movement principle of the labelled cells, is used.

Keywords: Energy transfer, FITC, propidium iodide, flow cytometry, bromodeoxyuridine, DNA synthesis time, potential doubling time

*Corresponding author: Tel.: +46 46 17 75 71; Fax: +46 46 14 73 27; E-mail: maria.johansson@onk.lu.se.

1. Introduction

Bromodeoxyuridine (BrdUrd) is a thymidine analog that is used in various applications in the study of cell proliferation, including the elucidation of growth kinetic parameters [3,6,12,16,22]. In one application, cells *in vitro* or *in vivo* are pulse-labelled with BrdUrd. The cells are then either fixed directly after labelling or after a period of continued proliferation in an environment free of BrdUrd. In order to analyse the incorporated BrdUrd, it is labelled with primary monoclonal BrdUrd antibodies and secondary fluorescein isothiocyanate (FITC)-conjugated antibodies. In order to determine the cell cycle position of unlabelled and of BrdUrd-labelled cells, the total cell population is then stained with the DNA intercalating dye propidium iodide (PI). Since BrdUrd can only be detected in single-stranded DNA and PI can only bind to double-stranded DNA, the DNA is subjected to partial denaturation before the staining procedure. Thus, part of the DNA will be single-stranded and part of it double-stranded. The cells are analysed by flow cytometry (FCM), most often using an argon ion laser with excitation line at 488 nm by which both fluorochromes are excitable. The maximum emission of FITC fluorescence is at 520 nm, with a range of 480–600 nm and that of PI is at 630 nm, with a range of 550–700 nm [14]. The quantity of emitted FITC and PI fluorescence is proportional to the amount of incorporated BrdUrd and cellular DNA contents, respectively. In addition to being excited at 488 nm, PI has an excitation maximum at 520 nm [14] which is within the emission range of the FITC fluorescence and corresponds to its maximum emission. Thus, in any experiment using the combination of the

Analytical Cellular Pathology 19 (1999) 91–98
ISSN 0921-8912 / $8.00 © 1999, IOS Press. All rights reserved

fluorochromes PI and FITC, the latter may contribute to the excitation of the former, a phenomenon which is called energy transfer [4,11]. Energy transfer can occur when the separation between the two molecules is within 100 Å and can be observed as a loss of characteristic fluorescence from the donor molecule and an increase in the characteristic fluorescence of the acceptor molecule [4,7]. There are other conditions that have to be fulfilled for energy transfer to occur, e.g., the steric orientation of the dyes [11,18]. Considering the fact that both FITC and PI are bound to DNA when applied to detect incorporated BrdUrd and DNA content, there is a probability that both the distance and steric considerations are fulfilled for energy transfer to occur.

In the present investigation, we show that energy transfer indeed takes place between FITC and PI when using the BrdUrd/FCM method to study growth kinetic parameters. We discuss the impact of this on the interpretation of growth kinetic data, since energy transfer between FITC and PI would result in an increase in observed PI fluorescence. The consequence of that would be that BrdUrd-labelled cells containing FITC molecules as a means of localizing BrdUrd, would seemingly have a higher DNA content than unlabelled cells. An important step in the measurement of the DNA synthesis time (T_S) and potential doubling time (T_{pot}) with the BrdUrd/FCM method is the estimation of relative movement (RM), i.e., the increase in DNA content, of the BrdUrd-labelled cells a certain time after BrdUrd labelling. RM is used in the formulas to calculate T_S, which in turn is included in the expressions of T_{pot}. Thus, the aim was to study whether the energy transfer phenomenon has any impact on the T_{pot} measurement.

2. Materials and methods

2.1. Materials

Growth medium components were purchased from Biochrom (Berlin, Germany) and tissue culture plastics from Nunc (Roskilde, Denmark). 5-Bromo-2′-deoxyuridine (BrdUrd) and PI were purchased from Sigma (St Louis, MO, USA). Monoclonal mouse anti-BrdUrd antibodies (M 744), FITC-conjugated rabbit anti-mouse antibodies (F 313), and biotin-conjugated rabbit anti-mouse antibodies (E 413) were purchased from Dakopatts (Glostrup, Denmark).

2.2. Cultured cell lines, bromodeoxyuridine labelling, and experimental designs

Chinese hamster ovary (CHO) cells, V79 hamster fibroblast cells, MCF-7 and SK-BR-3 human breast adenocarcinoma cell lines were maintained in serial passages in RPMI 1640 medium containing 10% foetal calf serum (FCS) and antibiotics (penicillin and streptomycin). Insulin (10 μg/ml) was also added to the MCF-7 growth medium. CHO and V79 cells were subcultured twice a week, while MCF-7 and SK-BR-3 cells were sub-cultured once a week. The cultures were incubated at 37°C in a water-saturated atmosphere consisting of air with 5% CO_2.

For the experiments, a number of replicate cultures, consisting of plateau phase cells seeded at a low density, were set up. When the cells reached exponential growth, BrdUrd (1 mM dissolved in phosphate-buffered saline) was added to achieve a final concentration of 5 μM. Labelling and subsequent cell handling were carried out under subdued light. After 30 min of incubation at 37°C, the BrdUrd containing medium was aspirated, the cells were rinsed twice with medium (37°C) containing 0.5% FCS (CHO, V79) or 1% FCS (MCF-7, SK-BR-3). Finally, growth medium (37°C) of the respective cell line was added and the cultures were further post-incubated at 37°C. In order to evaluate the effect of different BrdUrd concentrations, an experiment was designed in which CHO cells were labelled with 1, 5, 10, 20, or 50 μM BrdUrd for 30 min. At various times after labelling (sampling times), cultures were trypsinized, the cell numbers determined, and the cells pelleted at 500*g* for 5 min at 4°C. The pelleted cells were fixed by resuspension in ice-cold 70% ethanol to a final concentration of $1-2 \times 10^6$ cells/ml and the samples were stored at –20°C until analysis. Some cultures were fixed directly after labelling. For some applications, the entire population of CHO cells was labelled for 48 hours with 5 μM BrdUrd before fixation.

2.3. Preparation of cells for simultaneous flow cytometry-mediated quantification of DNA content and incorporated bromodeoxyuridine

The method was originally described by Dolbeare et al. [5] and further developed and modified by Schutte et al. [13] and van Erp et al. [19]. The preparation procedure has been described in detail previously [9]. Briefly, after washing the cells, the DNA of the nuclei was partly denatured and then the nuclei

were incubated with monoclonal anti-BrdUrd antibodies (diluted 1 : 10) followed by incubation with FITC-conjugated anti-mouse antibodies (diluted 1 : 20). Finally, nuclear isolation medium containing 10 μg/ml PI [17] was added for staining of the nuclear DNA.

In order to more accurately determine the DNA content of various cell populations, PI-stained nuclei from a human diploid tumour, used as an internal DNA reference, was added to the PI- and FITC-stained nuclei before FCM analysis in some experiments.

In order to evaluate whether there was an interaction between FITC and PI, the secondary biotin-conjugated anti-mouse antibody (diluted 1 : 200) was used in some experiments. This antibody is identical to the FITC-conjugated one, with the exception of the conjugate.

In one experiment, the BrdUrd-labelled nuclei were not stained with PI before FCM analysis and thus only the green fluorescence was measured. After FCM analysis, the nuclei were stained with PI and run through the FCM again to study the impact of PI on the green fluorescence signal.

2.4. Flow cytometric analysis

The FCM analysis and handling of the data have previously been described in detail [9]. Two different flow cytometers were used, either an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ, USA) equipped with a 15 mW air-cooled argon-ion laser, or a Cytofluorograph System 50-H (Ortho Instruments, Westwood, MA, USA) equipped with a 4 W argon-ion laser (output, 200 mW) (Lexel Corp, Palo Alto, CA, USA). The excitation line of 488 nm was used for both flow cytometers. PI fluorescence was separated from FITC fluorescence by a 560 nm dichroic mirror. The FITC fluorescence was quantified in the wavelength interval of 515–545 nm defined by a bandpass filter. The PI fluorescence beyond 600 nm (Cytofluorograph System 50-H) respective 620 nm (Ortho Cytoron Absolute) was limited by a lowpass filter. Otherwise the filter settings were the same. The computerized analysis of stored data was performed either with Multi 2D® and MultiCycle® software programs (Phoenix Flow Systems, San Diego, CA, USA) (Ortho Cytoron Absolute flow cytometer) or with Model 2140, DG MP 200 (Ortho Instruments and Data General, Westboro, MA, USA) (Cytofluorograph System 50-H). The obtained fluorescence signals were the same irrespective of which flow cytometer was used.

Because of the partial spectral overlap between the PI and FITC fluorescence emissions (PI into FITC), a proportional reduction of the PI fluorescence measured in the FITC fluorescence optics was done electronically. The FITC fluorescence into the PI fluorescence was blocked by the 600 and 620 nm lowpass filters in the PI fluorescence optics for respective flow cytometer.

2.5. Interpretation and obtainable parameters of flow cytometry-mediated data

The interpretation of FCM data has previously been described in detail [1,9].

In some experiments, narrow windows were set in the middle of the DNA distribution of the G_1 and G_2 phases [8], in order to estimate the fraction of BrdUrd-labelled cells in the middle of each phase (Fig. 1).

In order to investigate if energy transfer could affect growth kinetic parameters, T_S and T_{pot} were calculated.

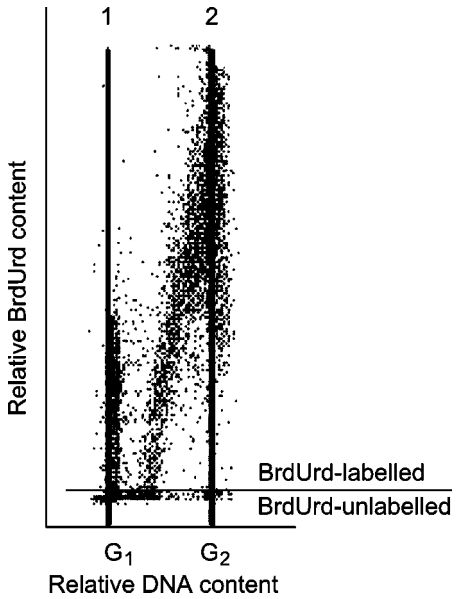

Fig. 1. Cytogram with mid-G_1 (Region 1) and mid-G_2 (Region 2) phase windows, which were set after determination of the mean DNA contents of G_1 and G_2 phase cells in the corresponding DNA histogram of the total cell population. CHO cells were labelled with 5 μM BrdUrd for 30 min and then allowed to progress through the cell cycle for 4 hours before fixation and processing to detect BrdUrd and DNA contents with FCM. The BrdUrd-labelled cells in G_1 and G_2 phases are seen to the right of the windows in the respective phase. Note that the BrdUrd-labelled divided cells in G_1 phase, appearing as a narrow and vertical cluster, does not lean to the right (i.e., show any increase in DNA content) with increasing BrdUrd content. This implies that no leakage of FITC into the PI detector occurs.

The formulas by Johansson et al. [8] to estimate RM and T_S (T_{SJ}) were used. T_{pot} was calculated according to Steel [15] and LI according to Johansson et al. [10].

3. Results

In an experiment studying the fraction of BrdUrd-labelled cells in mid-G_1 and mid-G_2 phases, we serendipitously found that the peaks representing unlabelled and BrdUrd-labelled cells in G_1 phase did not coincide with respect to relative mean DNA content. To study the fraction of BrdUrd-labelled cells in mid-G_1 and mid-G_2 phases, narrow windows were set manually in the BrdUrd cytogram (Fig. 1), the positions of which were determined in the DNA histogram of the total cell population. We consistently observed that the G_1 and G_2 phase windows were found to the left of the BrdUrd-labelled cells in G_1 phase, as well as to left of the BrdUrd-labelled cells in G_2 phase. The implication of this is that BrdUrd-labelled cells seemed to have a higher relative DNA content than unlabelled cells.

We then incubated CHO cells for 48 hours with 5 μM BrdUrd to obtain a cell population where all cells were labelled. These cells were firstly not subjected to the denaturation procedure but directly stained with PI. Comparing the DNA histograms of those cells with a population of unlabelled cells, there was no difference in the relative DNA content of G_1 phase cells of unlabelled and BrdUrd-labelled populations (not shown). Neither was there any difference in the DNA content of the same cell populations when they were subjected to denaturation before staining with PI without the antibodies (not shown).

Thereafter we proceeded to compare the unlabelled and BrdUrd-labelled cells in a cell population that had been pulse-labelled with BrdUrd for 30 min, i.e., the population contained both unlabelled and BrdUrd-labelled cells. The cells were prepared for the detection of DNA and BrdUrd contents with PI and with FITC. The DNA histograms of unlabelled and BrdUrd-labelled cells were compared concentrating on the positions of, i.e. the mean DNA value of, G_1 phase cells (Fig. 2), since this is the cell cycle phase from which the most reliable data can be obtained from a statistical point of view. A ratio between the peak values of BrdUrd-labelled and unlabelled G_1 phase cells was calculated for the cell lines CHO, V79, MCF-7, and SK-BR-3. In all samples, it was found that the BrdUrd-labelled G_1 phase cells were found to have a slightly higher DNA content than the unlabelled cells, giving

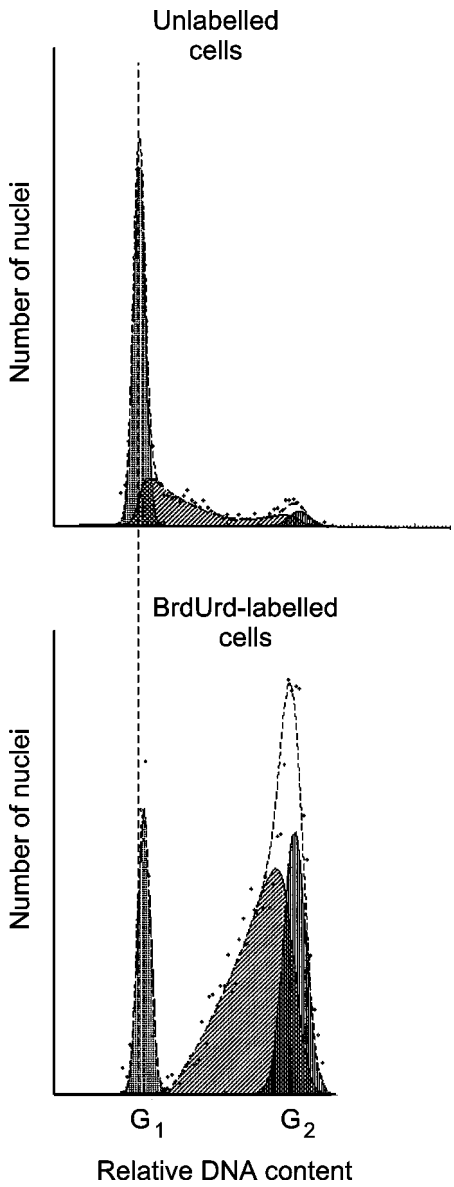

Fig. 2. DNA histograms of unlabelled and BrdUrd-labelled cells from one cell population of MCF-7 cells. MCF-7 cells were labelled with 5 μM BrdUrd for 30 min and then allowed to progress through the cell cycle for 8 hours before fixation and processing to detect BrdUrd and DNA contents with FCM. Unlabelled and BrdUrd-labelled cells were distinguished in the DNA versus BrdUrd cytogram before each cell population was displayed as a histogram. The line is drawn through the mean DNA content of unlabelled G_1 phase cells. The ratio between the mean DNA content of BrdUrd-labelled G_1 phase cells and that of unlabelled G_1 phase cells was 1.03 ± 0.001 ($n = 62$).

a quota of 1.03 ± 0.001 (mean ± standard deviation (SD), $n = 62$). The quota was the same in CHO cells incubated with 1, 5, 10, 20, or 50 μM BrdUrd.

To test whether the increase in relative DNA content in BrdUrd-labelled cells was due to energy transfer, we compared the relative DNA contents of BrdUrd-labelled G_1 phase cells that after the incubation with the primary BrdUrd antibody were incubated either with the secondary FITC-conjugated antibody or with a secondary biotin-conjugated antibody (Fig. 3). In these experiments we included separately PI-stained human diploid tumour cells as an internal DNA reference. The results show that there is a shift in the G_1 peak to a higher relative DNA content in cells that were incubated with FITC-conjugated antibodies, in relation to those that were incubated with biotin-conjugated antibodies. In this experiment too, the ratio was 1.03, when comparing mean DNA contents of FITC-conjugated and biotin-conjugated G_1 phase cells, which is equivalent to the ratio between the mean DNA contents of BrdUrd-labelled and unlabelled G_1 phase cells.

A characteristic of energy transfer is that the fluorescence of the donor molecule decreases as the fluorescence of the acceptor molecule increases. In order to test this, FITC-labelled nuclei were analyzed in the flow cytometer before and after staining with PI. The results showed that the green fluorescence signal decreased about 6 times after staining with PI. This net green fluorescence corresponds very well to any BrdUrd experiment in general.

When studying growth kinetics, a number of formulas exist to calculate T_S and T_{pot}. In the calculation of these parameters, the mean relative DNA contents of G_1, G_2, and BrdUrd-labelled S and $G_2 + M$ phase cells are included. It is possible, that the increase in DNA content caused by energy transfer may affect the outcome of T_S and T_{pot}. In order to partly exclude such a possibility, we compared T_S using the mean relative DNA content of the G_1 phase cells of the total cell population, of the unlabelled cells, and of the BrdUrd-labelled cells (Table 1). We found no difference in T_S and T_{pot}.

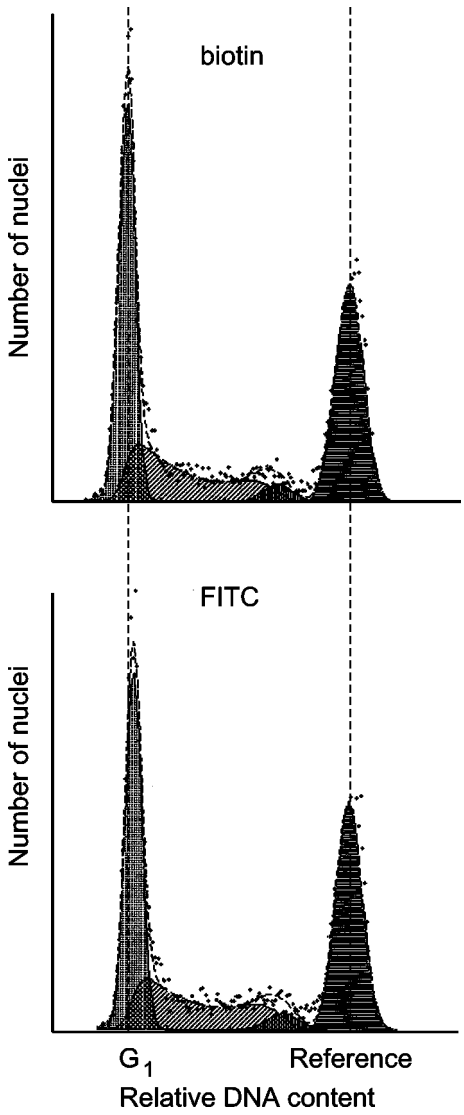

Fig. 3. Comparison of using a secondary biotin-conjugated antibody and a secondary FITC-conjugated antibody for the detection of incorporated BrdUrd in CHO cells where all cells were labelled with BrdUrd. The peaks to the right are G_1 peaks of human diploid cells added as an internal DNA reference. The left line is drawn through the mean DNA content of biotin-labelled G_1 phase cells. The ratio between the mean DNA contents of the G_1 peak of the human cells, and that of the G_1 peak of the CHO cells was 2.405 and 2.379 with biotin- and FITC-conjugated antibodies, respectively. The ratio between the mean DNA content of FITC-labelled and biotin-labelled G_1 phase cells was 1.03 ($n = 8$).

4. Discussion

We have been using the BrdUrd–DNA FCM method to study growth kinetics of experimental tumours and cells in culture for almost a decade. When evaluating DNA histograms of unlabelled and BrdUrd-labelled cells, obtained after detection of DNA and BrdUrd contents, we have been confounded by the fact that there seemed to be a difference in DNA content, based on the PI fluorescence, between these two populations although they were part of the same total cell popu-

Table 1
DNA synthesis time (T_{SJ}*) and potential doubling time (T_{pot}**) in MCF-7 cells calculated with the mean DNA content of G_1 phase cells obtained in different ways

	Total[1] ($n = 21$)		BrdUrd-labelled[2] ($n = 21$)		Unlabelled[3] ($n = 15$)	
	T_{SJ}*	T_{pot}**	T_{SJ}*	T_{pot}**	T_{SJ}*	T_{pot}**
Mean ± SD	17.5 ± 1.2	39.6 ± 3.8	17.6 ± 1.2	39.9 ± 3.7	17.1 ± 1.0	38.5 ± 3.2

* T_{SJ}, T_S calculated according to Johansson et al. [8].
** $T_{pot} = T_{SJ}/LI_J$, LI_J calculated according to Johansson et al. [10].
[1] The mean DNA content of the G_1 phase cells obtained from the total cell population.
[2] The mean DNA content of the G_1 phase cells obtained from the BrdUrd-labelled cell population.
[3] The mean DNA content of the G_1 phase cells obtained from the unlabelled cell population.

lation. The BrdUrd-labelled cells always had a DNA content that seemed to be greater than the unlabelled cells after DNA denaturation and BrdUrd detection using FITC-conjugated secondary antibodies. We have observed this phenomenon in all cell lines we have worked with. We envisaged several causes for this observation, including energy transfer, and we will discuss those below.

Before doing so, it should be mentioned that a discussion on a change in DNA content may be based on the entire cell population and/or on cells in a selected cell cycle phase. We have mainly based our comparative studies on cells in the G_1 phase, simply because that is the phase where the most reproducible and accurate results may be obtained, using either manual or computer-based region settings. In some experiments we have included an internal DNA reference that was not affected by the preparation procedure to ascertain that the differences found were indeed not caused by the preparation.

The incorporated BrdUrd molecule as such could have a positive impact on the binding of PI, somehow promoting the binding of more PI molecules. This was not the case since the G_1 DNA content of a cell population totally labelled with BrdUrd was the same as that of a totally unlabelled cell population, when stained with PI without denaturation. The incorporated BrdUrd molecules could affect DNA denaturation in a negative manner, resulting in a slightly lower degree of DNA denaturation in BrdUrd-labelled cells than in unlabelled cells. This was not the case either, since the G_1 DNA content of a cell population totally labelled with BrdUrd was the same as a totally unlabelled cell population when stained with PI after the DNA denaturation step. The presence of primary and secondary antibodies could promote the binding of more PI molecules. The fact that the use of the secondary biotin-conjugated antibody did not result in an increase in the DNA content rules out this possibility. Neither was there a change in DNA content when only the primary antibody was used. Thus, it is our notion that the difference in DNA content seen in unlabelled and BrdUrd-labelled cells, where BrdUrd is detected with the use of FITC-conjugated antibodies and DNA with PI, is caused by energy transfer. In addition to the increase in PI-derived red fluorescence, we found that the FITC-derived green fluorescence decreased, which also is characteristic of energy transfer. Notably, the decrease in the green fluorescence signal was much larger than the increase in the red fluorescence signal. There may be other physical factors than energy transfer involved in this phenomenon, e.g., bleaching and/or quenching as described by Shapiro [14].

Although the increase in DNA content was persistent in several cell lines, it was not of a large magnitude. The ratio between the mean DNA content of BrdUrd-labelled G_1 phase cells and that of unlabelled G_1 phase cells, where BrdUrd is detected with the use of FITC-conjugated antibodies and DNA with PI, was 1.03 ± 0.001. In an attempt to see whether this increase was dependent on the amount of incorporated BrdUrd, we labelled CHO cells with BrdUrd concentrations ranging from 1–50 μM. There was no difference in the magnitude of the shift in the mean DNA content of BrdUrd-labelled G_1 phase cells relative to that of unlabelled G_1 phase cells (not shown). These results may imply that even if there is an increase in the amount of incorporated BrdUrd molecules, only a maximum amount of them impart a positioning of the secondary FITC-conjugated antibody with respect to distance and steric orientation so that an interaction with PI can occur. Presumably that occurs around the boundaries of native and denatured DNA.

Although our discussion has been based on the G_1 phase cells, it is our assumption that a shift also takes place in the BrdUrd-labelled S and G_2 phase cells, when BrdUrd is detected with the use of FITC-conjugated antibodies and DNA with PI. It is possible

to measure the DNA content of unlabelled and BrdUrd-labelled G_2 phase cells although with a lower degree of accuracy and reproducibility than can be done with the G_1 phase cells. However, it is not possible to measure the mean DNA content of a population of BrdUrd-labelled cells moving through S phase by other means than by first detecting them with antibodies towards BrdUrd.

It should be mentioned that the observed effect of an increase in the DNA content of BrdUrd-labelled cells subjected to denaturation and labelled with the FITC antibodies and PI could also be the result of spectral overlap. However, we have taken careful measures to prevent the spectral overlap of FITC fluorescence leaking into the PI detector, by the optical filter settings as described in Section 2. A means to detect that no further compensation for spectral overlap is needed is that the cluster of BrdUrd-labelled divided cells in G_1 as they appear in the cytogram would be leaning to the right, the more FITC the higher DNA content. In addition, the coefficient of variation of the G_1 peak would be increased. We see neither of these phenomena, thus indicating that there is no leakage of FITC fluorescence into the PI detector (Fig. 1).

In our experiments we are not using the maximum laser effect which may imply that the FITC and PI fluorochromes are not maximally excited thus permitting the additional excitation of PI by the FITC emission fluorescence. By using a higher laser effect, the risk of energy transfer may diminish. However, commercial flow cytometers are not equipped with high effect lasers, of technical and economic reasons. Furthermore, the current laser power used is sufficient to produce excitation, resulting in fluorescence light intensities which are easily detected by the optics.

In the calculations of T_{SJ}, the RM of the moving cohort of BrdUrd-labelled cells is used [8]. In the calculation of RM, the mean DNA contents of BrdUrd-labelled S and G_2+ M, G_1, and G_2 phase cells are used [2]. The mean DNA contents of G_1 and G_2 phase cells are usually determined in the DNA histogram of the total cell population while that of the BrdUrd-labelled cells is determined in the DNA histogram of BrdUrd-labelled cells. In the present study, we have used the relative mean DNA contents of G_1 phase cells obtained in the DNA histogram of the total cell population and in the DNA histograms of unlabelled cells and of BrdUrd-labelled cells, for the calculations of T_{SJ}. Fortunately, the differences in mean relative G_1 values were not reflected in the obtained T_{SJ} values. However, the greatest source of error due to energy transfer is probably in the determination of the mean DNA content of BrdUrd-labelled cells in S and G_2+ M phases. When the mean DNA contents of G_1 and G_2 phase cells are determined in the DNA histogram of the total cell population, it will be influenced by the mean DNA contents of unlabelled cells and BrdUrd-labelled cells in G_1 and G_2 phases, and also by the relative numbers of unlabelled and labelled cells. Presumably, the mean DNA contents of G_1 and G_2 phase cells of the total cell population will not deviate significantly from either the mean DNA contents of G_1 and G_2 of the unlabelled or labelled cells. On the other hand, the mean DNA content of the BrdUrd-labelled cell population is only based on a population where energy transfer may occur. This may imply that T_{SJ} [8] and any other formula to calculate T_S [2,20,21] where the mean DNA content of the BrdUrd-labelled S phase cells is used, underestimates T_S, which will result in an underestimation of T_{pot}. We are currently trying to theoretically solve the degree of underestimation that might take place. A means to solve this experimentally would be to use fluorochromes with excitation and emission spectra that do not overlap.

In conclusion, our data show that energy transfer takes place between FITC and PI when a secondary FITC-conjugated antibody is used to detect incorporated BrdUrd and PI is used to stain total cellular DNA. This reasoning does not only hold for the detection of BrdUrd and DNA, but in any application using these two fluorochromes bound in close proximity. Fortunately, energy transfer between FITC and PI in the BrdUrd/FCM method does not seem to have any impact on the calculation of T_S and T_{pot}.

Acknowledgements

Supported by grants from the Crafoord Foundation, the Swedish Natural Science Research Council, the Royal Physiographical Society, the Mrs Berta Kamprad Cancer Foundation, the John and Augusta Persson Foundation for Medical Scientific Research, the University Hospital of Lund Foundation, the Gunnar, Arvid and Elisabeth Nilsson Cancer Foundation, the IngaBritt and Arne Lundberg Research Foundation, the Medical Faculty, University of Lund, the Swedish Cancer Society, and the Swedish Society for Medical Research.

We thank Professor Dick Killander for valuable discussions. Eva Henriksson is acknowledged for help with figures.

References

[1] B. Baldetorp, M. Dalberg, U. Holst and G. Lindgren, Statistical evaluation of cell kinetic data from DNA flow cytometry (FCM) by the EM algorithm, *Cytometry* **10** (1989), 695–705.

[2] A.C. Begg, N.J. McNally, D.C. Shrieve and H. Kärcher, A method to measure the duration of DNA synthesis and the potential doubling time from a single sample, *Cytometry* **6** (1985), 620–626.

[3] A.C. Begg, L. Moonen, I. Hofland, M. Dessing and H. Bartelink, Human tumour cell kinetics using a monoclonal antibody against iododeoxyuridine: Intratumour sampling variations, *Radiother. Oncol.* **11** (1988), 337–347.

[4] H.A. Crissman and J.A. Steinkamp, Cytochemical techniques for multivariate analysis of DNA and other cellular constituents, in: *Flow Cytometry and Sorting*, M.R. Melamed, T. Lindmo and M.L. Mendelsohn, eds, 2nd edn, Wiley-Liss, Inc., New York, 1990, pp. 227–247.

[5] F. Dolbeare, H. Gratzner, M.G. Pallavicini and J.W. Gray, Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine, *Proc. Natl. Acad. Sci. USA* **80** (1983), 5573–5577.

[6] J.O. Fredlund and S.M. Oredsson, Normal G1/S transition and prolonged S phase within one cell cycle after seeding cells in the presence of an ornithine decarboxylase inhibitor, *Cell Prolif.* **29** (1996), 457–465.

[7] J.H. Jett, R.A. Keller, J.C. Martin, D.C. Nguyen and G.C. Saunders, Ultrasensitive molecular-level flow cytometry, in: *Flow Cytometry and Sorting*, M.R. Melamed, T. Lindmo and M.L. Mendelsohn, eds, 2nd edn, Wiley-Liss, Inc., New York, 1990, pp. 381–396.

[8] M.C. Johansson, B. Baldetorp, P.O. Bendahl, R. Johansson and S.M. Oredsson, An improved mathematical method to estimate DNA synthesis time of bromodeoxyuridine-labelled cells, using FCM-derived data, *Cell Prolif.* **27** (1994), 475–488.

[9] M.C. Johansson, B. Baldetorp, P.-O. Bendahl, I.A. Fadeel and S.M. Oredsson, Comparison of mathematical formulas used for estimation of DNA synthesis time of bromodeoxyuridine-labelled cell populations with different proliferative characteristics, *Cell Prolif.* **29** (1996), 525–538.

[10] M.C. Johansson, R. Johansson, B. Baldetorp and S.M. Oredsson, Comparison of different labelling index formulae used on bromodeoxyuridine-flow cytometry data, *Cytometry* **32** (1998), 233–240.

[11] T.M. Jovin, Fluorescence polarization and energy transfer: theory and application, in: *Flow Cytometry and Sorting*, M.R. Melamed, P.F. Mullaney and M.L. Mendelsohn, eds, John Wiley & Sons, Inc., New York, 1979, pp. 137–165.

[12] A. Riccardi, M. Giordano, M. Danova, M. Girino, S. Brugnatelli, G. Ucci and G. Mazzini, Cell kinetics with *in vivo* bromodeoxyuridine and flow cytometry: clinical significance in acute non-lymphoblastic leukaemia, *Eur. J. Cancer* **27** (1991), 882–887.

[13] B. Schutte, M.M.J. Reynders, C.L.M.V.J. van Assche, P.S.G.J. Hupperets, F.T. Bosman and G.H. Blijham, An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry, *Cytometry* **8** (1987), 372–376.

[14] H.M. Shapiro, *Practical Flow Cytometry*, 2nd edn, A.R. Liss, Inc., New York, 1988.

[15] G.G. Steel, *Growth Kinetics of Tumours*, Clarendon Press, Oxford, 1977.

[16] N.H.A. Terry, M.L. Meistrich, L.D. Roubein, P.M. Lynch, R.A. Dubrow and T.A. Rich, Cellular kinetics in rectal cancer, *Br. J. Cancer* **72** (1995), 435–441.

[17] J.T. Thornthwaite, E.V. Sugarbaker and W.J. Temple, Preparation of tissues for DNA flow cytometric analysis, *Cytometry* **1** (1980), 229–237.

[18] A.S. Waggoner, Fluorescent probes for cytometry, in: *Flow Cytometry and Sorting*, M.R. Melamed, T. Lindmo and M.L. Mendelsohn, eds, 2nd edn, Wiley-Liss, Inc., New York, 1990, pp. 209–225.

[19] P.E.J. van Erp, P.P.T. Brons, J.B.M. Boezeman, G.J. de Jongh and F.W. Bauer, A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation, *Cytometry* **9** (1988), 627–630.

[20] R.A. White and M.L. Meistrich, A comment on "A method to measure the duration of DNA synthesis and the potential doubling time from a single sample", *Cytometry* **7** (1986), 486–490.

[21] R.A. White, N.H.A. Terry, M.L. Meistrich and D.P. Calkins, Improved method for computing potential doubling time from flow cytometric data, *Cytometry* **11** (1990), 314–317.

[22] G.D. Wilson, N.J. McNally, S. Dische, M.I. Saunders, C. Des Rochers, A.A. Lewis and M.H. Bennett, Measurement of cell kinetics in human tumours *in vivo* using bromodeoxyuridine incorporation and flow cytometry, *Br. J. Cancer.* **58** (1988), 423–431.