ISTITUTO DI ANALISI DEI SISTEMI ED INFORMATICA
CONSIGLIO NAZIONALE DELLE RICERCHE

A. Bertuzzi, A. Gandolfi

A MODEL FOR ESTIMATING CELL KINETIC PARAMETERS OF EXPERIMENTAL TUMOURS STUDIED BY BROMODEOXYURIDINE LABELLING AND FLOW CYTOMETRY

R. 516 Dicembre 1999

Alessandro Bertuzzi – Istituto di Analisi dei Sistemi ed Informatica del CNR, viale Manzoni 30 - 00185 Roma, Italy. Email : bertuzzi@iasi.rm.cnr.it.

Alberto Gandolfi – Istituto di Analisi dei Sistemi ed Informatica del CNR, viale Manzoni 30 - 00185 Roma, Italy. Email : gandolfi@iasi.rm.cnr.it.

This work was partially supported by the Progetto Strategico CNR "Metodi e Modelli Matematici nello Studio dei Fenomeni Biologici".

ISSN: 1128–3378

Collana dei Rapporti
dell'Istituto di Analisi dei Sistemi ed Informatica, CNR

viale Manzoni 30, 00185 ROMA, Italy

tel. ++39-06-77161
fax ++39-06-7716461
email: iasi@iasi.rm.cnr.it
URL: http://www.iasi.rm.cnr.it

Abstract

A mathematical model that describes the time evolution of the DNA-BrdUrd distribution, as measured in experimental tumours after labelling with BrdUrd, is proposed in this paper. Cell-to-cell heterogeneity within the tumour of the rate of progression across cell cycle phases is taken into account, and a strict correlation between the durations of S and G2M phases is assumed. The model relates observable quantities to the parameters that characterize the cell population kinetics (such as the potential doubling time, T_{pot}), and appears thus to be suitable for parameter estimation. Simulations that illustrate the dependence of model response on the kinetic parameters are presented.

Key words: Cell kinetics, age-structured populations, DNA-BrdUrd flow cytometry, solid tumours.

1. Introduction

The kinetics of proliferating cells both in cultures and in experimental tumours is usually investigated by labelling a cohort of cells and observing the time evolution of well defined subpopulations of labelled cells. We recall here that proliferating cells move along a cycle composed by four phases called G1, S (the phase of DNA replication), G2, and M (the phase of mitosis). An early method largely used for the study of cell kinetics was based on the incorporation of ^{3}H-thymidine into S phase cells, followed by autoradiography. This method, which is rather laborious and involves a radioactive compound, is currently replaced by the technique based on the incorporation of the label bromodeoxyuridine (BrdUrd) (Dolbeare *et al.*, 1995) and flow cytometry. Because BrdUrd is an halogenated analogue of thymidine, if cells are surrounded by a medium containing BrdUrd the cells in active DNA synthesis incorporate BrdUrd into the newly synthesized DNA and become thus labelled.

We give a short description of the *in vivo* pulse labelling procedure used for the study of experimental tumours. A bolus of BrdUrd is delivered to identical animals bearing a tumour implanted under the same condition. At different times after the injection, the animals are sacrified and serial specimens of the tumour are obtained. The tissue of each specimen is disaggregated to have a cell suspension, the collected cells are permeabilized, and cell DNA is partially denatured to expose the incorporated BrdUrd molecules. The cells are reacted with an anti-BrdUrd monoclonal antibody directly or indirectly labelled with fluorescein isothiocyanate (FITC, that emits green fluorescence), and DNA is stained with a DNA-specific dye, typically propidium iodide (PI, that emits red fluorescence). A sample of the dual-stained cells (10,000 to 30,000 cells) is finally processed by a flow cytometer. In this instrument the cells are forced to flow in a single file under a laser beam that excites the fluorescences, and the measurements of the intensities of green and red fluorescence of each cell are accumulated to form a bivariate DNA-BrdUrd histogram. In this way, a time sequence of DNA-BrdUrd histograms showing the time evolution of the subpopulations of labelled and unlabelled cells is obtained.

Mathematical expressions for quantities related to the evolution of DNA-BrdUrd histograms have been derived assuming that there is no cell-to-cell variability of transit times in S and G2M phases (White and Meistrich, 1986; White *et al.*, 1990; Bertuzzi *et al.*, 1997). This assumption, that appears to be reasonable for cell cultures, is no longer adequate when data from *in vivo* experimental tumours have to be analyzed. An essential aspect of cell proliferation in tumours is indeed its heterogeneity, the proliferating cells exhibiting a large variability of the cell cycle phase transit times and then of the cycle time. This proliferative heterogeneity has been related to the presence of subpopulations that are different on genetic basis as well as to the different conditions of nutrition and oxygenation of the cells within the tumour. Mathematical and simulation models that predict the evolution of DNA-BrdUrd distributions assuming independently distributed phase transit times have been developed by Yanagisawa *et al.* (1985), Ubezio (1990), and Baisch *et al.* (1995).

In the present paper we propose a mathematical model that assumes cell-to-cell variability of phase transit times and a strict correlation between the durations of S and G2M phases. This feature of the model is in accordance with the view that cells display different rates of progression across the cycle because of the different microenvironmental conditions experienced by the cells. Thus, a slow cell exhibiting a long S phase is likely to have a long G2M phase. No specific assumption on the progression in G1 phase is required if data are analyzed at time-points at which cells born by division of labelled cells have not yet entered S phase. The model, relating observable quantities to few parameters that characterize the cell kinetics, would allow

their estimation from experimental data through a best fitting procedure. The estimation of parameters of cell kinetics in tumours is an important step for assessing therapeutic regimens designed on the basis of optimal control theory (Swierniak *et al.*, 1996).

2. The cell population model

We consider the cell population as composed by a compartment of cells in G1 phase or in a quiescent state with the same DNA content of cells in G1 (compartment 1) and by cells in S and G2M phases (compartment 2). We assume that there is cell-to-cell variability of transit times in both compartments. More specifically, cells display different rates of progression across the cycle and we assume that transition of cells to different progression rates only can occur at mitosis or before the entry into S phase. Transitions to different progression rates are to be postulated to make it possible the asynchronous exponential growth (AEG) of the population, preventing the fastest cells to prevail in the growth. Therefore we take the view of compartment 2 as subdivided in subpopulations each characterized by the time T required by cells to progress from the entry in S to mitosis. For brevity, we say that a cell of compartment 2 is of class T if it belongs to such a subpopulation. Moreover, all cells of class T have a transit time in S, $T_S(T)$, that is a deterministic function of T. Similarly for the transit time in G2M, $T_{G2\,M}(T)$, being $T = T_S(T) + T_{G2\,M}(T)$. We do not consider cells possibly arrested in S or G2M, however these cells are to some degree represented by the slowest cells in these phases. Although cells having slower progression rates are likely to be more affected by death, according to the hypothesis that slower progression is related to worse microenvironment conditions, for simplicity and for keeping small the number of parameters we will take a common rate constant μ of cell loss in the population. The cell population is assumed to be in the AEG condition.

Considering T as a continuous variable with values from T_{min} to T_{max}, the population of cells in S and G2M phases can be described by a density function $n(a,t,T)$, such that $n(a,t,T)dadT$ is the number of cells with age between a and $a+da$ at time t in the subpopulation requiring a time between T and $T+dT$ to traverse S+G2M. The age a is counted from cell entry into S phase and thus $a \in [0,T]$. According to the above assumptions, $n(a,t,T)$ satisfies the continuity equation

$$\frac{\partial}{\partial t} n(a,t,T) + \frac{\partial}{\partial a} n(a,t,T) = -\mu n(a,t,T)\,, \quad a \in (0,T) \tag{1}$$

$$n(0,t,T) = g_1(t,T)\,, \tag{2}$$

where $g_1(t,T)dT$ is the number of cells that enter the subpopulations of classes between T and $T+dT$ in the unit time at time t.

Let a_1 be the cell age in compartment 1. We can define the density $n_1(a_1,t,T)$, such that $n_1(a_1,t,T)da_1dT$ is the number of cells with age between a_1 and a_1+da_1, *generated* by cells that have completed S+G2M phases in a time between T and $T+dT$. If T_{1min} is the minimal time required by cells to progress from mitosis to the entry in S, for ages $a_1 < T_{1min}$ the continuity equation for n_1 can be written without specifying the law of exit from compartment 1, obtaining

$$\frac{\partial}{\partial t} n_1(a_1,t,T) + \frac{\partial}{\partial a_1} n_1(a_1,t,T) = -\mu n_1(a_1,t,T)\,, \quad a_1 \in (0,T_{1min}) \tag{3}$$

$$n_1(0,t,T) = 2n(T,t,T)\,. \tag{4}$$

In the AEG condition, it is $n(a,t,T)=\bar{n}(a,T)\mathrm{e}^{\alpha t}$ and $g_1(t,T)=\bar{g}_1(T)\mathrm{e}^{\alpha t}$, where α denotes the growth rate constant of the population, so it it easily seen that

$$n(a,t,T)=\bar{g}_1(T)\mathrm{e}^{-\beta a}\mathrm{e}^{\alpha t} \tag{5}$$

with $\beta=\alpha+\mu$. The mitotic rate density $g(t,T)$, *i.e.* the density with respect to T of the number of cells that undergo division in the unit time at time t, is given by

$$g(t,T)=n(T,t,T)=\bar{g}_1(T)\mathrm{e}^{-\beta T}\mathrm{e}^{\alpha t}\,. \tag{6}$$

By defining in the population in asynchronous exponential growth the frequency function p_T of T as $p_T(T)=g(t,T)/\int g(t,T)\,dT$, we have

$$p_T(T)=\frac{\bar{g}_1(T)\mathrm{e}^{-\beta T}}{\int_{T_{min}}^{T_{max}}\bar{g}_1(T)\mathrm{e}^{-\beta T}\,dT}\,. \tag{7}$$

Similarly, from (3) and (4) it can be easily seen that

$$n_1(a_1,t,T)=2\bar{g}_1(T)\mathrm{e}^{-\beta T}\mathrm{e}^{-\beta a_1}\mathrm{e}^{\alpha t} \tag{8}$$

The balance equation for the total number of cells of the population, N, can be written as

$$\dot{N}(t)=\int_{T_{min}}^{T_{max}} g(t,T)\,dT-\mu N(t)\,. \tag{9}$$

In asynchronous exponential growth it is $N(t)=\bar{N}\mathrm{e}^{\alpha t}$ and thus from Eqs. (6) and (9) we have the following expression for the rate constant of cell production K_p, defined as the ratio between the number of cells that undergo division in the unit time and the number of cells of the population (Steel, 1977):

$$K_p=\frac{1}{\bar{N}}\int_{T_{min}}^{T_{max}}\bar{g}_1(T)\mathrm{e}^{-\beta T}\,dT=\beta\,. \tag{10}$$

The rate constant of cell production defines the so-called potential doubling time of the cell population, $T_{pot}=\ln 2/K_p$ (Steel, 1977). From Eqs. (7) and (10) we have

$$\frac{\bar{g}_1(T)}{\bar{N}}=\beta p_T(T)\mathrm{e}^{\beta T}\,. \tag{11}$$

Thus the fractional density of cells in S+G2M, $\nu(a,T)=n(a,t,T)/N(t)$, is given by

$$\nu(a,T)=\beta p_T(T)\mathrm{e}^{\beta T}\mathrm{e}^{-\beta a}\,, \tag{12}$$

and the fractional density $\nu_1(a_1,T)=n_1(a_1,t,T)/N(t)$ is given by

$$\nu_1(a_1,T)=2\beta p_T(T)\mathrm{e}^{-\beta a_1}\,,\quad a_1\in[0,T_{1min}]\,. \tag{13}$$

Although the cell age density in compartment 1 is not completely described, the previous equations allow us to express the fraction f_1 of cells in compartment 1 in terms of β and of the

distribution of T. Denoting by $N_1(t)$ the number of cells in compartment 1, we can write the following balance equation

$$\dot{N}_1(t) = 2\int_{T_{min}}^{T_{max}} g(t,T)\,dT - \int_{T_{min}}^{T_{max}} g_1(t,T)\,dT - \mu N_1(t)\,. \tag{14}$$

In the asynchronous exponential growth, recalling that $N_1(t) = f_1 N(t)$, we have

$$\beta f_1 = \frac{2}{\bar{N}}\int_{T_{min}}^{T_{max}} \bar{g}_1(T)\mathrm{e}^{-\beta T}\,dT - \frac{1}{\bar{N}}\int_{T_{min}}^{T_{max}} \bar{g}_1(T)\,dT\,. \tag{15}$$

By expressing $\bar{g}_1(T)$ in terms of $p_T(T)$ by means of (7), it follows

$$\beta f_1 = \frac{1}{\bar{N}}\int_{T_{min}}^{T_{max}} \bar{g}_1(T)\mathrm{e}^{-\beta T}\,dT\left(2 - \int_{T_{min}}^{T_{max}} p_T(T)\mathrm{e}^{\beta T}\,dT\right), \tag{16}$$

and taking into account Eq. (10) we obtain

$$f_1 = 2 - \int_{T_{min}}^{T_{max}} p_T(T)\mathrm{e}^{\beta T}\,dT\,. \tag{17}$$

Similar expressions for the fractions in S and G2M can be written by integrating the density $\nu(a,T)$ between suitable limits.

Remark 1. The preceding equations can easily be extended to the case in which the rate constant of cell loss from compartment 1, μ_1, is different from the rate constant μ_2 of cell loss from compartment 2. Different values for μ_1 and μ_2 could be related to the presence of a large amount of quiescent cells in compartment 1. Following the same lines as above, Eqs. (10), (13), and (17) become respectively

$$K_p = \alpha + \mu_2(1 - f_1) + \mu_1 f_1\,, \tag{18}$$

$$\nu_1(a_1,T) = 2K_p p_T(T)\mathrm{e}^{-\beta_1 a_1}\,, \quad a_1 \in [0, T_{1min}]\,, \tag{19}$$

where $\beta_1 = \alpha + \mu_1$, and

$$f_1 = \frac{K_p}{\beta_1}(2 - \int_{T_{min}}^{T_{max}} p_T(T)\mathrm{e}^{\beta_2 T}\,dT)\,, \tag{20}$$

where $\beta_2 = \alpha + \mu_2$.

Remark 2. The theory of this section does not provide a dynamic model for the evolution of the cell population, since the rate of exit from compartment 1 is not prescribed and thus the growth rate α cannot be calculated. The equations given describe the age structure in AEG of the cell subpopulations that determine the evolution of the quantities measured in a labelling experiment within a time horizon not too large (as it will be specified in the next section).

3. BrdUrd labelling

After the BrdUrd injection into the animal bearing the tumour (the injection be delivered at time $t=0$), the label concentration in the fluids surrounding tumour cells changes with the time and eventually decreases exponentially. Although some experimental evidences suggest that the concentration decay is rapid, so that the labelling is often considered as a true impulsive labelling, we will take into account for more generality a continuous labelling in conditions of variable concentration of the label. Recalling that a cell having age a in S+G2M at time t has entered S phase at time $t-a$, we can write for the amount $b(a,t,T)$ of BrdUrd incorporated at time t in the DNA of a cell of class T having age a, the following equation:

$$b(a,t,T) = \int_{t-a}^{t} \zeta(s+a-t,s,T)\, ds\,, \tag{21}$$

where $\zeta(a,t,T)$ is the rate at which BrdUrd is incorporated into a cell of class T with age a at time t.

Since the BrdUrd molecules rapidly traverse the cell membrane, we assume that ζ is a function of the extracellular BrdUrd concentration $c(t)$ and of the DNA synthesis rate (amount of DNA synthesized in the unit time). Experimental *in vitro* data (Kriss and Revesz, 1961) have shown a saturable behaviour of the amount of incorporated BrdUrd with respect to the extracellular BrdUrd concentration. Because the initial value of the BrdUrd concentration in mice could be greater than the saturating concentration, we assume for ζ the following expression

$$\zeta(a,t,T) = q\, v(a,T) \frac{c(t)}{\bar{c}+c(t)} \tag{22}$$

where $v(a,T)$ is the rate of DNA synthesis of a cell of class T with age a, and q and $\bar{c}$ are constants depending on cellular parameters. Note that $c(t)$ is equal to 0 for $t<0$.

Let the DNA content of cells in G1 and G2M be equal to 1 and 2, respectively. We assume that cells characterized by different values of T have DNA synthesis rates that exhibit the same pattern across S phase. We have chosen for $v(a,T)$ the following expression which allows to represent increasing or decreasing rates:

$$v(a,T) = \begin{cases} \dfrac{1}{T_S(T)} - \dfrac{k}{T_S(T)^2}(T_S(T)-2a)\,, & 0 \le a \le T_S(T) \\ 0 & T_S(T) < a \le T \end{cases} \tag{23}$$

with $k \in (-1,1)$ (Bertuzzi *et al.*, 1995). If the DNA synthesis rate were constant across S phase, $v(a,T)$ would be equal to $1/T_S(T)$ for $a \in [0, T_S(T)]$.

For times larger than the minimum duration of G2M phase, division of labelled cells occurs and a subpopulation of labelled divided cells arises. The amount b_1 of BrdUrd present in a cell of compartment 1 of age a_1 at time t, generated by a cell of class T, is then given by

$$b_1(a_1,t,T) = \frac{1}{2}\, b(T, t-a_1, T)\,. \tag{24}$$

Note that it is $b_1 = 0$ if $t-a_1 < T_{G2M}(T)$. Therefore the fraction $f_1^l(t)$ of labelled cells in compartment 1 at time t is equal to

$$f_1^l(t) = \int_{T_{min}}^{T_{max}} \int_{0}^{\max[0,\, t-T_{G2M}(T)]} \nu_1(a_1,T)\, da_1 dT \tag{25}$$

for $0 \le t \le T_{1min} + \min(T_{G2M}(T))$. This limitation is required since ν_1 is defined by Eq. (13) only for $a_1 \le T_{1min}$.

4. Time course of the DNA-BrdUrd fluorescence distribution

Let y be the intensity of red (PI) fluorescence of a cell measured by the flow cytometer. The quantity y depends on the amount of fluorochrome bound to DNA, and then on the DNA content of the cell. Let z be the measured intensity of green (FITC) fluorescence, related to the amount of BrdUrd incorporated into the cell. Because of various instrumental and staining sources of variability, the values of y and z of cells having equal DNA content x and BrdUrd content b will be different, and it appears reasonable to consider y and z as two independent random variables. The dispersion of the measured values can be represented by defining a conditional probability density function (pdf) $P_y(y|x)$ for the red fluorescence intensity y of a cell with DNA content x ($x \in [1,2]$), and a conditional pdf $P_z(z|b,x)$ for the green fluorescence z of a cell with BrdUrd content b and DNA content x. The pdf $P_y(y|x)$ has been assumed as usual of the form:

$$P_y(y|x) = \frac{1}{\sqrt{2\pi}\sigma_y(x)} \exp\big[-\frac{(y-m(x))^2}{2\sigma_y(x)^2}\big], \tag{26}$$

i.e. as a gaussian distribution with mean $m(x)$ and standard deviation (SD) $\sigma_y(x)$. The fluorescence mean is approximately proportional to x and a more accurate representation is $m(x) = y_1 + (y_2 - y_1)(x-1)$, where y_1 and y_2 are the mean red fluorescences of G1 and G2M cells, respectively. Since the SD of PI fluorescence increases with the DNA content and the coefficient of variation is sometimes not constant, we have assumed $\sigma_y(x) = \sigma_1 x^r$, with $r \geq 0$, where σ_1 is the SD of the fluorescence of cells having $x = 1$. A similar probability density can be defined for z:

$$P_z(z|b,x) = \frac{1}{\sqrt{2\pi}\sigma_z(b,x)} \exp\big[-\frac{(z-d(x)-hb)^2}{2\sigma_z(b,x)^2}\big], \tag{27}$$

where the mean value of z is given by the sum of the contribution due to incorporated BrdUrd, hb (h being a constant depending on the experimental procedure), and of an aspecific contribution due to cell autofluorescence. Since the autofluorescence appears to increase with cell mass, and so with the position of the cell in the cycle, we have assumed $d(x) = z_1 + (z_2 - z_1)(x-1)$, where z_1 and z_2 are the mean green fluorescences of unlabelled cells in G1 and G2M, respectively. For the SD of green fluorescence we have simply assumed a constant coefficient of variation CV_z, so it is $\sigma_z(b,x) = CV_z(d(x) + hb)$.

Denoting by $\rho(y,z,t)$ the joint pdf of y and z at time t, we have

$$\rho(y,z,t) = \rho_1^{ul}(y,z,t) + \rho_1^{l}(y,z,t) + \rho_2(y,z,t) \tag{28}$$

where ρ_1^{ul} and ρ_1^{l} are the contributions of unlabelled ($b_1 = 0$) and, respectively, labelled ($b_1 > 0$) cells in compartment 1, and ρ_2 is the contribution of cells in compartment 2. Thus we can write, for $0 \leq t \leq T_{1min} + \min(T_{G2M}(T))$, the following equations:

$$\rho_1^{ul}(y,z,t) = P_y(y|1) P_z(z|0,1)(f_1 - f_1^l(t)) \tag{29}$$

$$\rho_1^{l}(y,z,t) = P_y(y|1) \int_{T_{min}}^{T_{max}} \int_0^{\max[0,t-T_{G2M}(T)]} P_z(z|b_1(a_1,t,T),1)\nu_1(a_1,T)\, da_1 dT \tag{30}$$

$$\rho_2(y,z,t) = \int_{T_{min}}^{T_{max}} \int_0^{T} P_y(y|x(a,T)) P_z(z|b(a,t,T),x(a,T))\nu(a,T)\, da dT \tag{31}$$

where f_1 and $f_1^l(t)$ are given by Eqs. (17) and (25), $b(a,t,T)$ and $b_1(a,t,T)$ are given by Eqs. (21) and (24), and according to Eq. (23) the DNA content $x(a,T)$ of a cell of class T with age a is given by

$$x(a,T) = \begin{cases} 1 + \dfrac{a}{T_S(T)} - \dfrac{k}{T_S(T)^2}(a(T_S(T)-a)), & 0 \le a \le T_S(T) \\ 2 & T_S(T) < a \le T. \end{cases} \tag{32}$$

We note that the distribution of green fluorescence z for a given value of y depends, in the present model, both on the instrumental and staining sources of variability and on the presence of cells that have different BrdUrd content because of the different rates of progression across S phase.

In an experimental DNA-BrdUrd histogram, the cells are identified as labelled (or positive with respect to BrdUrd) if the measured intensity of green fluorescence exceeds a threshold value fixed by the experimenter according to the level of autofluorescence. With a similar gating on the red fluorescence, the labelled cells are in practice identified as labelled undivided and labelled divided, and the fractions of these cells are actually measured. The distribution $\rho(y,z,t)$ allows us to predict the measured fractions and their time evolution after using the above thresholds to integrate the theoretical distribution. Let $\bar{z}$ be the threshold value of the green fluorescence and $\bar{y}$ be the threshold value of the red fluorescence that separates the undivided from the divided cells. The measured fraction of labelled undivided cells, $f_{ud}^+(t)$, is given by

$$f_{ud}^+(t) = \int_{\bar{y}}^{\infty} \int_{\bar{z}}^{\infty} \rho(y,z,t)\, dzdy, \tag{33}$$

and the measured fraction of labelled divided cells, $f_d^+(t)$, is given by

$$f_d^+(t) = \int_{y_{min}}^{\bar{y}} \int_{\bar{z}}^{\infty} \rho(y,z,t)\, dzdy, \tag{34}$$

where $y_{min} < y_1$ is a reasonable lower limit for the red fluorescence intensity of viable cells. When labelled divided cells are not present, $\bar{y}$ is set to y_{min}. We observe that, in a condition of impulsive labelling or of "ideal" continuous labelling (*i.e.*, all cells entering S phase are immediately detectable as labelled), the above fractions are well approximated by their theoretical values that can be obtained from the cell population model, and that are related by a rather simple equation expressing the conservation of labelled cells at division. Equations (33) and (34) encompass instead more general situations in which label concentration is decreasing with time and thus the consideration of a detectability threshold becomes relevant. Another useful quantity is the relative movement RM of labelled undivided cells at time t, defined as the increment of the mean red fluorescence of positive undivided cells with respect to the mean red fluorescence of G1 cells (Begg *et al.*, 1985). The relative movement can be computed as

$$RM(t) = \frac{1}{y_2 - y_1}\left(\frac{1}{f_{ud}^+(t)} \int_{\bar{y}}^{\infty} \int_{\bar{z}}^{\infty} y\rho(y,z,t)\, dzdy - y_1\right). \tag{35}$$

More detailed information can be obtained from the experimental data, for instance the distribution of positive cells with respect to the intensity of red fluorescence, and the mean green fluorescence of positive cells as a function of red fluorescence intensity. The fractional density of positive undivided cells with respect to y, $p_{ud}^+(y,t)$, can be computed as

$$p_{ud}^+(y,t) = \int_{\bar{z}}^{\infty} \rho(y,z,t)\, dz, \quad y > \bar{y} \tag{36}$$

and the mean green fluorescence of positive undivided cells as a function of y, $\langle z\rangle^+_{ud}(y,t)$, as

$$\langle z\rangle^+_{ud}(y,t) = \frac{\int_{\bar z}^{\infty} z\rho(y,z,t)\,dz}{p^+_{ud}(y,t)}\,, \quad y > \bar y\,. \tag{37}$$

The above functions of time, that represent observable quantities and are the outputs of the model, depend on the parameters and functions that characterize the kinetics of the tumour cell population and the process of labelling and measurement. In order to have a parametric identification problem, we have chosen to represent the BrdUrd concentration $c(t)$ by a decreasing exponential function with time constant τ

$$c(t) = c_0 \mathrm{e}^{-t/\tau}\,, \tag{38}$$

and the distribution $p_T(T)$ by the expression

$$p_T(T) = \frac{\varepsilon}{(1-\mathrm{e}^{-\varepsilon})(T_{max}-T_{min})} \exp\left[-\varepsilon\frac{T-T_{min}}{T_{max}-T_{min}}\right], \tag{39}$$

for $T_{min} \le T \le T_{max}$ and by zero elsewhere. This form permits skewed distributions and a uniform distribution as $\varepsilon \to 0$. Concerning the function $T_S(T)$, we have chosen for simplicity $T_S(T) = \gamma T$, $\gamma \in (0,1)$, so the durations of phases S and G2M are uniformly affected by the lengthening of T. Thus the outputs will depend on the following tumor kinetic parameters: β (or, equivalently, T_{pot} as usual in the biological literature), T_{min}, T_{max}, ε, γ, k, and on the following parameters of labelling and measurement: τ, the ratio $\bar c/c_0$, the product hq, z_1, z_2, σ_1, r, CV_z.

We note that the parameters z_1, z_2, σ_1, r, and CV_z can be estimated from the bivariate histogram using the software currently available with the flow cytometer. For this purpose, a histogram measured immediately after the BrdUrd administration is particularly useful, because the subpopulations of unlabelled cells with DNA content equal to 1 and 2 can be easily recognized. A value to the ratio $\bar c/c_0$ can be assigned assuming for $\bar c$ the value ($\bar c = 4\,\mu$M) deduced from the data of Kriss and Revesz (1961), and for c_0 the ratio of BrdUrd dose to the distribution volume of the animal (estimated as 20 ml in mice). The product hq appears to be highly variable among histograms and cannot be accurately estimated *a priori*. An approximate value can be obtained on the basis of the measured mean green fluorescence of labelled cells and using a guess of the mean synthesis time and of the ratio $c(t)/(\bar c + c(t))$. However, when the value of hq is much larger than the threshold $\bar z$, its influence on the computed value of the positive cell fractions is reduced and thus a precise determination of this product is not necessary. Moreover, we note that hq appears as a multiplicative factor of the mean green fluorescence and thus it can be disregarded if the evolution of $\langle z\rangle^+_{ud}(y,t)$ is considered after suitable normalization.

Having assigned values to the parameters of labelling and measurement (except the time constant τ), the kinetic parameters of the tumour (and τ) can be estimated by fitting the theoretical expressions of Eqs. (36), (37) and (34) (f^+_{ud} and RM are indeed functions of $p^+_{ud}(\cdot)$) to the time sequences of measured data. The mean and variance of the distribution of T can then be computed from the estimated values of T_{min}, T_{max}, and ε. Although a formal study of parameter identifiability has not been done and it appears to be very complex, numerical simulations have shown that changes in the parameter values significantly affect the observed data. Some of these simulations are reported in the following section.

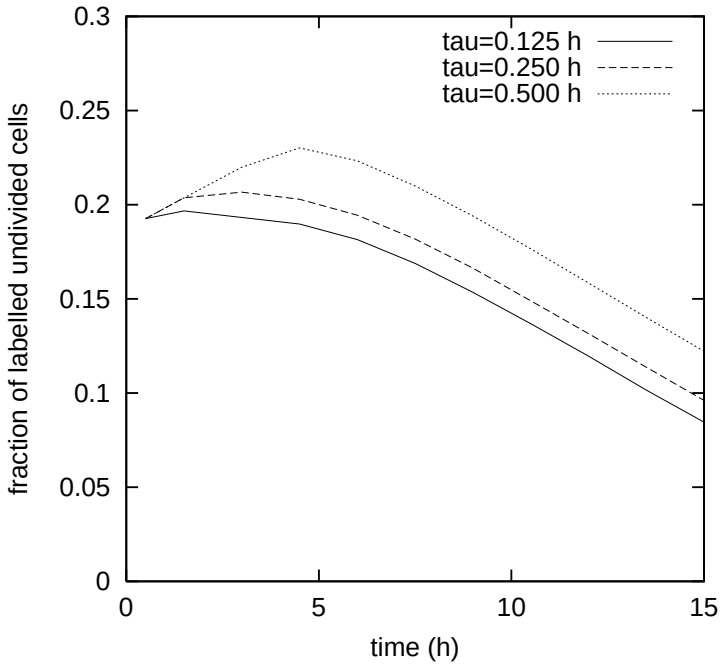

Fig. 1. Time course of the fraction of labelled undivided cells, f_{ud}^{+}, for different values of τ. The values of the other parameters are given in the text.

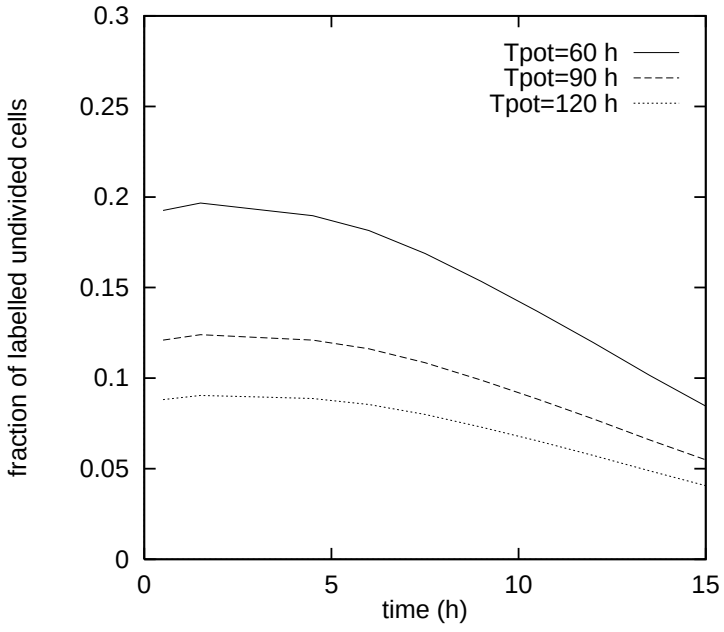

Fig. 2. Time course of the fraction of labelled undivided cells, f_{ud}^{+}, for different values of T_{pot}.

5. Numerical results

Simulations of the model have been performed by taking for the measurement parameters the following values: $\bar{c}/c_0 = 0.16$, $hq = 10^5$, $z_1 = 10$, $z_2 = 20$, $\bar{z} = 70$, $y_1 = 1$, $y_2 = 2$, $\bar{y} = 1.08$, $\sigma_1 = 0.04$,

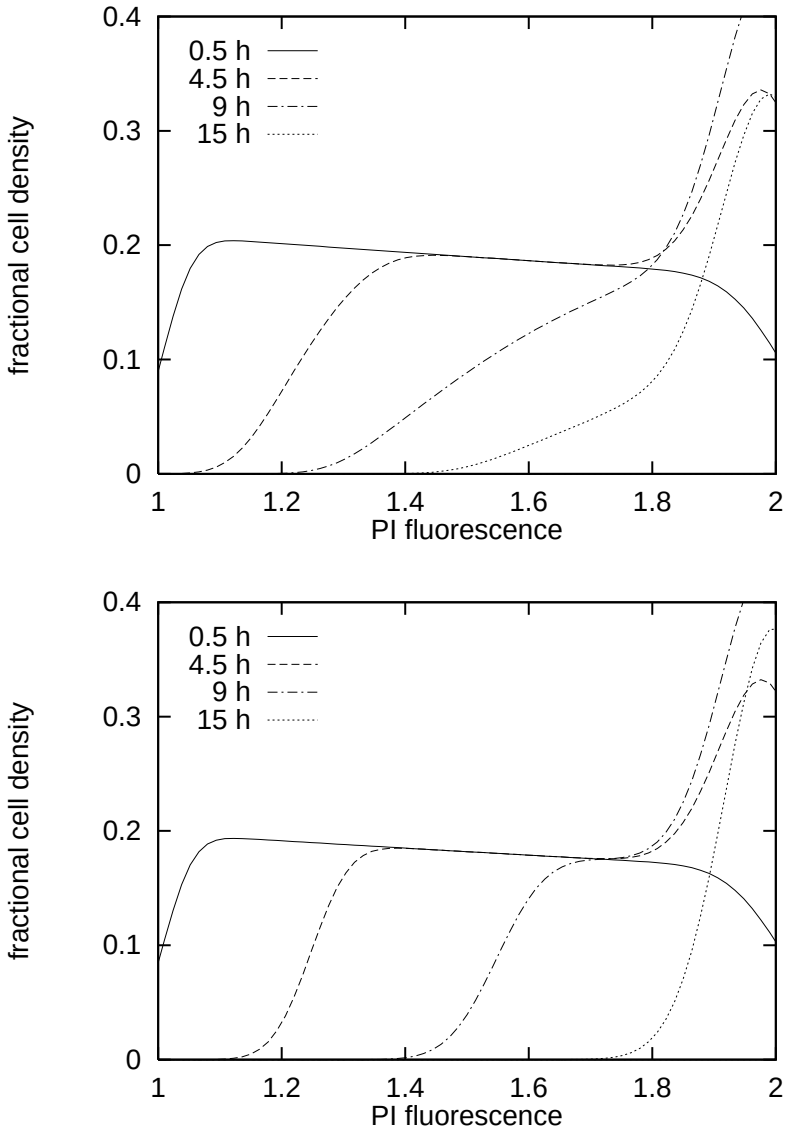

Fig. 3. Fractional density of positive undivided cells, $p^{+}_{ud}(y)$, at different times after label injection. $T_{min} = 13.0$ h and $T_{max} = 41.0$ h (upper panel); $T_{min} = 20.3$ h and $T_{max} = 22.3$ h (lower panel). The two distributions of T have the same mean (20.9 h).

$r = 1$, $CV_z = 0.2$. Moreover, we assumed the following reference values for τ and the kinetic parameters: $\tau = 0.125$ h, $T_{pot} = 60$ h, $T_{min} = 13$ h, $T_{max} = 41$ h, $\varepsilon = 3$, $\gamma = 0.7$, and $k = 0$. In this case the distribution of T is skewed to the right, with mean 20.9 h and SD 6.5 h, and the DNA synthesis rate is constant across S phase.

The BrdUrd pharmacokinetics mainly affects the fraction of positive undivided cells. In the case of true impulsive labelling, the number and the fraction of labelled cells cannot increase with the time, whereas an increase occurs in the case of continuous labelling also when label concentration is decreasing. This effect is shown in Fig. 1: with the reference value of τ the labelling is almost impulsive, whereas with larger values evident deviations from impulsive labelling occur.

The main kinetic parameter, T_{pot}, largely affects the time course of f^{+}_{ud}. When T_{pot} increases, keeping the other parameters unchanged, the initial value of the positive fraction is decreased

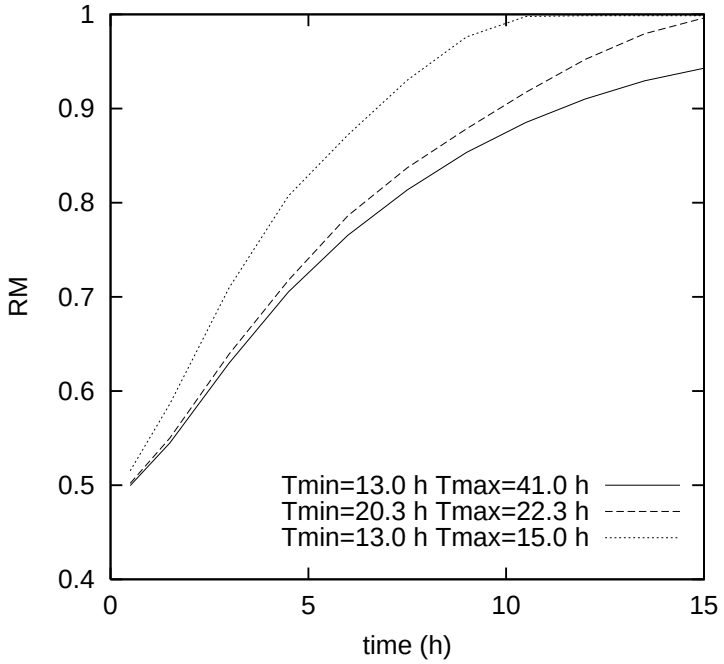

Fig. 4. Time course of the relative movement of labelled undivided cells, RM, for different values of T_{min} and T_{max}.

(because the fraction of cells in S phase becomes smaller) and also the slope of the descending branch decreases (Fig. 2).

When the mean value and the SD of T change, the progression of the labelled cells across S+G2M is affected, and this is shown by the function $p^+_{ud}(y,t)$ and by the derived quantity $RM(t)$. Figure 3 reports the evolution of the fractional density of positive undivided cells of the reference case (upper panel) and of a case in which the mean T is the same whereas the SD of T is very small (SD=0.5 h), thus representing a population with negligible cell-to-cell variability. The slowing of progression in S caused by the dispersion of T is evidenced by the relative movement as seen in Fig. 4, where the effect of a shortening of the mean value of T, with SD unchanged, is also depicted. Figure 5 shows the evolution of $p^+_{ud}(y,t)$ for two distributions of T with the same mean and SD, one skewed to the right (upper panel) and the other skewed to the left (lower panel). The differences in the pattern, that can be seen in the figure and that also are present in the time evolution of $\langle z \rangle^+_{ud}(y)$ (not shown), might however be hardly detectable in the experimental data. This result suggests that a reliable estimation procedure could provide only the mean value and the SD of the transit time T (unless highly accurate measurements are available), so that a reasonable choice for $p_T(T)$ could be a simple flat distribution.

The relative duration of S and G2M phases, as expressed by the parameter γ, influences the time of appearance of labelled divided cells and thus the time behaviour of the fraction of positive divided cells (Fig. 6). The pattern of the mean green fluorescence $\langle z \rangle^+_{ud}(y)$ at a time close to the time of BrdUrd injection reflects the pattern of the DNA synthesis rate across S phase. The effect of different values of k is clearly shown in Fig. 7 (where the mean green fluorescence is normalized to its integral on the interval from 0.8 to 1.2), in which the cases of constant, increasing and decreasing rate are considered.

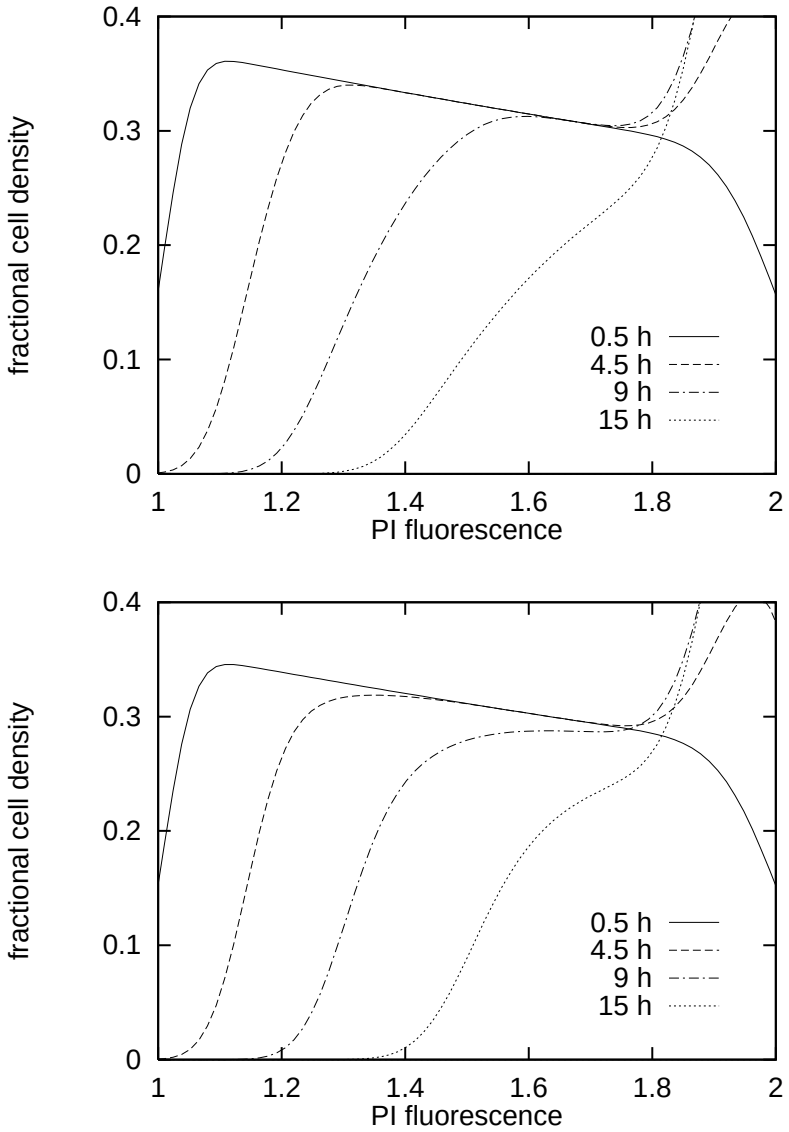

Fig. 5. Fractional density of positive undivided cells, $p^{+}_{ud}(y)$, at different times after label injection. $T_{min}=20.6$ h, $T_{max}=54.6$ h and $\varepsilon=2$ (upper panel); $T_{min}=10.0$ h, $T_{max}=44.0$ h and $\varepsilon=-2$ (lower panel). The two distributions of T have the same mean (32.3 h) and the same SD (8.7 h).

6. Concluding remarks

In this paper we have presented a mathematical model describing the time evolution of the DNA-BrdUrd fluorescence distribution as measured in cell samples from eperimental tumours, after the administration of the label to the animal as a single bolus. The model takes into account the proliferative heterogeneity of tumour cells and does not require specific assumptions on the progression through G1 phase. On the other hand, this generality is obtained by renouncing the possibility of describing the time evolution of the distribution beyond the entry in S of the labelled divided cells. The time interval in which our model holds appears adequate for the analysis of data from most labelling experiments (12-18 h). The application of the model for

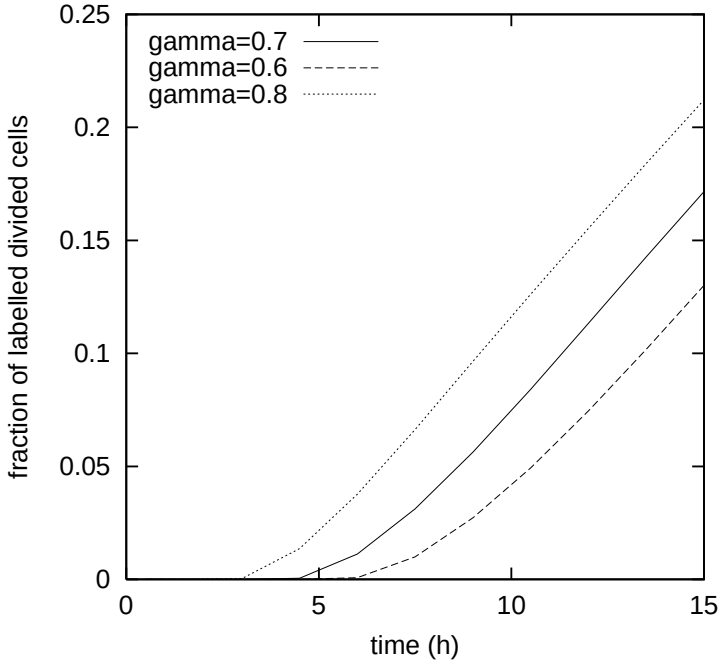

Fig. 6. Time course of the fraction of labelled divided cells, f_d^+, for different values of γ.

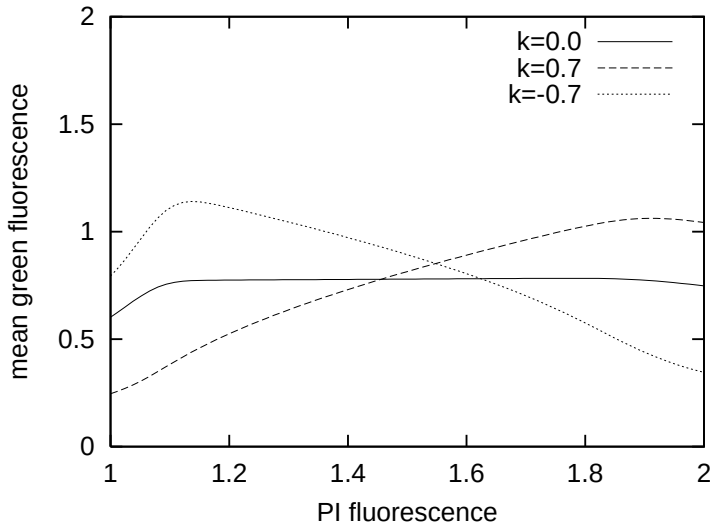

Fig. 7. Mean green fluorescence of labelled undivided cells $\langle z \rangle_{ud}^{+}(y)$, at $t = 0.5\,\mathrm{h}$, for different values of k.

estimating the kinetic parameters of a human ovarian cancer growing in mice is in progress.

Because the results of the numerical simulations suggest that the observable quantities are scarcely affected by moments of order higher than two of the distribution of transit time in S and G2M phases, the possibility of estimating the shape of the distribution of T needs careful consideration, and a more robust model could assume for T a uniform distribution. A simple extension of the model to the case of nonuniform cell loss can be obtained on the basis of equations (19) and (20). The observable quantities depend in this case on the kinetic parameters

K_p, β_1, and β_2, which are all equal to β in the case of uniform cell loss. Further investigations are required to assess the practical identifiability of these new parameters.

References

Baisch, H., U. Otto, U. Hatje and H. Fack (1995), Heterogeneous cell kinetics in tumors analyzed with a simulation model for bromodeoxyuridine single and multiple labeling, *Cytometry* 21, 52-61.

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

Bertuzzi, A., N. Del Grosso, A. Gandolfi, C. Sinisgalli and G. Starace (1995), Cell cycle analysis by the relative movement approach: effect of variability across S-phase of DNA synthesis rate, *Cell Prolif.* 28, 107-120.

Bertuzzi, A., A. Gandolfi, C. Sinisgalli and G. Starace (1997), Estimation of cell cycle kinetic parameters by flow cytometry. In *Advances in Mathematical Population Dynamics - Molecules, Cells and Man* (O. Arino, D. Axelrod and M. Kimmel, eds.), World Scientific, pp. 167-180.

Dolbeare, F. (1995), Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: historical perspectives, histochemical methods and cell kinetics, *Histochem. J.* 27, 339-369.

Kriss, J.P. and L. Revesz (1961), Quantitative studies of incorporation of exogenous thymidine and 5-bromodeoxyuridine into deoxyribonucleic acid of mammalian cells in vitro, *Cancer Res.* 21, 1141-1147.

Steel, G.G. (1977), *Growth Kinetics of Tumors: Cell Population Kinetics in Relation to the Growth and Treatment of Cancer*, Clarendon Press, Oxford.

Swierniak, A., A. Polanski and M. Kimmel (1996), Optimal control problems arising in cell-cycle-specific cancer chemotherapy, *Cell Prolif.* 29, 117-139.

Ubezio, P. (1990), Cell cycle simulation for flow cytometry, *Comp. Meth. Programs Biomed.* 31, 255-266.

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

White, R.A., N.H.A. Terry and M.L. Meistrich (1990), New methods for calculating kinetic properties of cells in vitro using pulse labelling with bromodeoxyuridine, *Cell Tissue Kinet.* 23, 561-573.

Yanagisawa M., F. Dolbeare, T. Todoroki and J.W. Gray (1985), Cell cycle analysis using numerical simulation of bivarite DNA/Bromodeoxyuridine distributions, *Cytometry* 6, 550-562.