Options of flow cytometric three-colour DNA measurements to quantitate EGFR in subpopulations of human bladder cancer

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Received 22 August 1995; revised 2 May 1996; accepted 9 May 1996

Abstract

Flow cytometric multi-parameter analysis has proven to be a powerful tool to characterize subpopulations of cell suspensions, and is applied routinely in hematology. However, in studies of cancer where there is interest in defining phenotypic markers in conjunction with DNA content, this method has hardly been applied [6]. Our objective was to develop a methodology that extends previous investigations on relative and absolute quantitation of the epidermal growth factor receptor (EGFR) in dual parameter analysis in vitro on human bladder cancer cell lines [3]. In order to quantitate EGFR content in tumours and to relate it to DNA content, tumour selection, DNA-content, and EGFR-content measurements should be carried out simultaneously. Different fluorescent dyes were used to optimize DNA assessment and antibody staining, using a single laser instrument as a practical approach for clinical routine. In vitro cultures were used to validate the quality of tumour cell selection and antigen quantitation. Therefore, two urothelial tumour cell lines — lowly and highly differentiated — were incubated under different conditions: monolayer (ML), three-dimensional multi-cellular spheroids (MCS) and cocultures (COCU) with the fibroblast cell line N1 were investigated and EGFR quantitation was related to S-phase fraction (SPF). Accurate determination of instrument settings allows simultaneous three-colour analysis with DNA assessment. Tumor cell selection based on staining with phycoerythrine (R-PE) against a highly expressed urothelial glycoprotein, detected with the antibody Uro5 or against cytokeratin appeared to be possible in FL2, using the fluorochrome combination fluorescein-isothiocyanate (FITC), R-PE and propidiumiodide (PI). Using this staining protocol, relative and absolute EGFR quantitation

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(quantum simply cellular beads) is shown to be accurate, when FITC is used for EGFR staining and measured in the green fluorescence channel (FL1). Using this colour combination EGFR content and SPF of tumour cells were compared in different growth states, and could be monitored reliably. In spite of a higher emission spectrum of 7-aminoactinomycin-D (7-AAD)\[10\], this DNA stain provided no advantage over PI. Broad coefficients of variation (CV) were found when intact cells were stained, thus hindering accurate assessment of ploidy and S-phase fraction. Similarly, Syto-13, a DNA dye detected in FL1, could not be optimized for multi-parameter measurements. Although the emission maximum is at 520 nm, the spectrum is too wide to compensate fluorescence overlap in FL2 or FL3. Quantum Red (QR), used as a streptavidin conjugate in FL3, could not be combined with two other colours for DNA staining, since sufficient compensation was not obtainable when an argon ion laser is used. The coculture model allows verification of tumour cell selection and discrimination. The high differentiated tumour cell line RT4 shows an unambiguous correlation between EGFR content and S-phase fraction. The low differentiated tumour cell line J82 presents a similar pattern of post-transcriptional EGFR regulation with respect to culture condition, however, the S-phase fraction is basically unaffected.

**Keywords:** Multi-parameter flow cytometry; DNA measurements; FITC; PE; PI; 7-AAD; Syto-13; Quantum-Red; LDS-751; Quantum beads

1. Introduction

Dual-parameter DNA analysis was suggested by the DNA consensus meeting held in autumn 1992 to improve precision of determination of proliferative fractions and to identify aneuploid subpopulations more easily\[30\]. The basic idea was to characterize a cell population of interest by using additional markers as cytokeratin antibodies for positive detection of epithelial cells or leucocyte common antigen for exclusion of contaminating diploid inflammatory cells\[36\].

While there is an increasing effort to standardize this methodology for clinical applications, further efforts appear necessary to extend experimental work to define and quantitate tumour subpopulations, as in routine hematology\[7,12,17\]. When a multiparameter staining technique is used we have excellent options to define tumour subpopulations and correlate data to invasive behavior or recurrences of the tumours. Oncogene expression can be related to the proliferative state or ploidy\[16\]. There is an increasing demand to simultaneously detect receptor molecules or proliferation associated cyclins as well as important regulatory molecules of their signal-transduction pathway.

In our own recent work dealing with the detection of the EGFR on bladder cancer cell lines (a molecule significantly involved in bladder cancer development and progression\[21–24,31,33\]) it could be shown that double staining of the EGFR and DNA allowed reliable relative and absolute quantitation of the receptor, when the data were compared to Scatchard analysis\[3,4\]. Double staining in a tumour, however, only allowed discrimination between diploid and non-diploid cells, and therefore it seemed necessary to identify the cells as urothelial cells as well. This can be achieved by panurothelial antibodies as Uro5\[8,14\] or by fairly urothelial-selective antibodies against cytokeratins\[36\].
A three-colour DNA staining with a urothelium specific antibody, an antibody against the EGFR, and different stains for DNA were established in our laboratory. Since the single laser flow cytometer is the most frequently used clinical device, and best standardized for clinical applications, we adapted the choice of fluorescent stains for a single laser (argon ion) device. In contrast to three-colour staining with antibodies, simultaneous DNA measurements require the inclusion of a linear measurement.

Possible ways of sufficient compensation, as well as limits of quantitative measurements, due to the required compensation settings and data obtained with different (DNA-) fluorochromes available are presented.

As a standardized test system two- and three-dimensional in vitro cultures were used to validate the method and were extended to heterologous tumour-cell fibroblast cocultures (COCU) to mimic solid tumours.

2. Material and methods

2.1. Cell lines/cell culture

The well differentiated clonal bladder carcinoma cell line RT4 [19,27] was used to examine three-parameter staining. This cell line has a receptor content of 4 \times 10^6 EGFR/cell in ML culture [3] and shows strong positive staining with the antibody Uro5 [14]. CaSki cells are originally derived from a cervical carcinoma specimen and are known to express a high amount of EGFR [9,13,18].

Both cell lines were cultured under standard tissue culture conditions in an incubator with 5% CO\textsubscript{2}. The medium used was from Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco) with 10% fetal calf serum (FCS, Seromed), 2 nM L-glutamin, 1 nM sodium pyruvate, and penicillin/streptomycin (100 mg/ml, all from Gibco). For experiments, cells were seeded at a density of 0.5 \times 10^5 cells/T75 tissue flask as monolayer cultures (ML). Medium was changed every other day. Cells were harvested with 0.02% trypsin/EDTA in exponential growth phase to avoid EGFR down-regulation due to cell–cell contact [18].

Further, a Uro5 negative normal fibroblast cell line (N1), derived from the adult skin of a healthy donor [29], was cultured in DMEM in an incubator with 8% CO\textsubscript{2} atmosphere.

For MCS cultures, the liquid overlay culture technique was applied: tumour cells or fibroblast cells were suspended from exponentially growing ML cultures, and seeded into 96-well plates onto solid agarose in 200 \mu l of medium at a concentration of 3 \times 10^3 tumour cells and 6 \times 10^3 fibroblast cells. After 3 days of static incubation, single MCS had formed (1/well), and were subsequently cocultured. A single cell suspension of MCS cells was also produced by the trypsin/EDTA reaction and mechanical dissociation (by an Eppendorf pipette) was carried out in addition. The first measurement of mono MCS and cocultured MCS (COCU) was performed after 3 days of coculture (\approx 5 days single MCS and 3 days COCU), the second measurement after 8 days of coculture. ML cells were measured after 3 days of exponential growth.
2.2. Immunocytochemistry

Single cell suspensions were prepared from ML cells and washed with cold phosphate buffered saline (PBS). For EGFR staining cells were incubated with the antibody clone A3007 subclass IgG1 (CIS, Langen-Hess, Germany) and for Uro5 staining with the Uro5 antibody, subclass IgG2b (Signet, Bad Homburg, Germany). Subclass specific rabbit anti-mouse IgG1 and IgG2b antibodies, labeled with R-PE or FITC, respectively, for simultaneous EGFR and Uro5 staining, were used and obtained from Southern Biotechnology Associates (SBA, Birmingham, USA). Following immunocytochemical staining, cells were washed in PBS and postfixed for 1 h with 70% methanol at room temperature for DNA staining.

2.3. Flow cytometry

In Table 1 all tested fluorochromes are listed and assigned to the corresponding emission filter combination for detection:

All measurements were performed on a standard FACScan flow cytometer equipped with an argon laser (Becton Dickinson, Heidelberg, Germany). Instrument settings were determined for each dye combination individually. Three fluorochromes have been used for simultaneous three-colour analysis, thus the signals from all three PMTs have to be compensated individually with respect to each fluorochrome.

A protocol was established to determine instrument settings of the FACScan because instrument settings (PMT-voltages and compensations) determined with fluorescent micro-beads as used in hematology were not transferable to measurements of bladder tumour cells.

Instead of microbeads, the cervix carcinoma cell line CaSki [13] was used. This cell line gives strong fluorescence signals for each individual fluorescence dye and thus was found suitable to calibrate compensation settings [11,15].

EGFR of CaSki cells (1 × 10^5/ml) were stained by an indirect immunochemical method under saturating conditions, using either R-PE, or FITC. Alternatively, a three-step technique with rabbit anti-mouse F_\text{ab} fragments (Dakopatts, Hamburg, Germany) and QR-conjugated streptavidine (Sigma-Biosciences, St. Louis, USA) was carried out. DNA was stained with PI, 7-AAD, LDS-751 (Sigma-Biosciences, St. Louis, USA) or Syto-13, (Molecular Probes, Eugene Oregon, USA). DNA fluo-

<table>
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<th>FL3</th>
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<td>FITC</td>
<td>R-PE</td>
<td>PI</td>
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<tr>
<td>FITC</td>
<td>R-PE</td>
<td>7-AAD</td>
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<td>Syto-13</td>
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<td>QR</td>
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<tr>
<td>FITC</td>
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<td>QR</td>
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<tr>
<td>FITC</td>
<td>R-PE</td>
<td>LDS-751</td>
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Fig. 1. Panel a: Autofluorescent cells, adjustment of FL1 and FL2 PMT voltages. Panel b: PI* cells, adjustment of FL3 PMT voltage. Panel c: FITC* cells, determination of FL2-%FL1 compensation. Panel d: PE* cells, determination of FL1-%FL2 compensation. Panel e: PE* cells, determination of FL3-%FL2 compensation. Panel f: PI* cells, determination of FL2-%FL3 compensation.

Fluorochromes were applied in the following concentrations: PI: 50µg/ml, 7-AAD: 25µg/ml, Syto-13: 10 µmol/ml, and LDS-751 in a range from 1 to 100 µg/ml.

An example of the individual steps of the protocol for achieving correct instrument settings for the combination FITC, R-PE, and PI is given in Fig. 1. Other compensation protocols for other dye combinations were performed in an analogous manner.

First, PMT voltages of FL1 and FL2 were adjusted by using unstained cells. Autofluorescence of unstained cells should appear under FL1 and FL2 and serve as negative fluorescence levels for later measurements. Next, FL3 voltage was determined using PI stained cells. Voltage was adjusted so that signals of pulse processors allowed doublet discrimination.

To determine compensation settings in percentages, FL1 has to be compensated for cross-over fluorescence from the orange fluorochrome, FL2 for cross-over fluorescence from both the green and red fluorochromes and FL3 for cross-over fluorescence from the orange fluorochrome. FITC stained CaSki cells were measured to adjust the FL2-%FL1 compensation. R-PE positive cells are used to adjust FL1-%FL2 as well as FL3-%FL2 compensation. Finally the emission of PI carrying cells
was checked. This dye has a very broad emission spectrum. It is only possible to correct the spectral spill-over into the second channel by adjusting FL2-%FL3.

2.4. Flow cytometric EGFR quantitation

To quantify average receptor content/cell a set of microbeads with five defined quantities of antigenic sites (Quantum Simply Cellular, Flow Cytometry Standard Europe, FCSE, Leiden, Netherlands) was added to cell samples and stained together as one suspension. Before the set of microbeads was added, saturating conditions for immunochemical staining of these particles were determined by evaluating a saturation curve. Bead data were collected over FSC/SSC live gating into a separated file. Data evaluation of microbead signals was performed by drawing a linear calibration curve of the mean fluorescence intensity (MFI) for each bead population against the corresponding number of binding sites. Binding sites of the batch used were 0, 4093, 15 493, 35 340, and 134 309 per population. The number of antibodies bound to the cells were calculated by comparing the average fluorescence intensity of the cell population with the calibration curve. Autofluorescent cells represent the detection limit due to the instrument settings and conditions. Values of mean fluorescence were converted into number of antibodies bound per cell after normalized subtraction of the isotype control from EGFR-stained cells. Finally, the correct EGFR calculation requires the subtraction of the difference of the MFI between control cells and MFI of the blank beads, since the fluorescence level of control cells was found to be higher than that of unlabeled beads.

All data were gathered and evaluated with the Lysis software (Becton Dickinson). A minimum of $1 \times 10^4$ events of samples stained with one dye and $2 \times 10^4$ events of mixed cell populations were collected. Cells without DNA staining were gated over the FSC vs. SSC signal, doublet elimination of DNA fluorescent cells was done with the use of pulse area and width.

Two different measurements for testing instrument settings were carried out routinely. Equal quantities of four differently stained cell suspensions of RT4 cells were mixed: RT4 stained with FITC anti-EGFR, stained with R-PE anti Uro5, cells which carry both dyes, and control cells without any dye. The other sample consisted of the same four populations which were stained with the DNA dye PI in addition. Measurements revealed possible spectral overlap into neighboring channels.

In addition, a mixture of N1 fibroblasts and RT4 cells was measured to validate the possibility of tumour cell selection by Uro5 staining.

2.5. Determination of S-phase fraction (SPF)

Cell cycle analysis was calculated with Multi-Plus Software (Phoenix Flow Systems, Tucson, USA) after gating of Uro5 positive tumour cells initially. Then, cell cycle phases were determined using the analysis model, with debris and aggregates exclusion.

2.6. Fluorescence spectroscopy

To determine the emission spectra of the fluorochromes FITC, R-PE, PI, Syto-13,
and 7-AAD, fluorimetric measurements were made with a Hitachi F-2000 fluorescence-spectrophotometer (Colora Messtechnik, Lorch, Germany). CaSki cells were suspended in PBS (1 × 10⁶/ml), and stained as described in Section 2.2. To simulate the situation at the flow-cytometer, we used cells to determine emission spectra, because some of the dyes used are only fluorescent if incorporated in cells (e.g. PI has another emission spectrum when intercalated in nucleic acids). The wavelengths of dye excitation and emission were selected by a monochrome mirror. Slit setting was 10 nm, both for excitation and emission. The excitation wavelength was 488 nm and fluorescence was recorded using quartz cuvettes with a 10-mm lightpath. Fluorochrome-emission was recorded in 10-nm steps.

3. Results

The emission spectra of the fluorochromes FITC, R-PE, PI, Syto-13, and 7-AAD of stained CaSki cells (1 × 10⁶/ml) were measured by fluorescence spectrometry (Fig. 2.) These spectra are related to the standard filter sets of the FACScan instrument. QR can not be shown, since this fluorochrome is not excitable by a xenon lamp.

Evaluation of all fluorochromes in the three-parameter setting mentioned above resulted in the combination of FITC, R-PE, and PI as the most practical methods.

Fig. 2. Emission spectra and their relative fluorescence intensity occurring in 1 × 10⁶/ml CaSki cells. The spectral emission of five tested fluorochromes is depicted and related the three fluorescence detectors of the FACScan. The difficulty to compensate the spill over is apparent. Arrows reflect the filter combination of the FACScan: FL1: BP 535/15 nm, FL2: BP 585/42 nm, FL3: LP 650 nm. BP = bandpass filter; LP = longpass filter.
Table 2
Instrument settings for a DNA-three-parameter analysis with FITC, R-PE, and PI for the single laser flow cytometer FACSscan

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<td>FSC</td>
<td>E-01 lg 1.0</td>
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<tr>
<td>SSC</td>
<td>183 lg 1.0</td>
</tr>
<tr>
<td>FL1</td>
<td>481 lg 1.4</td>
</tr>
<tr>
<td>FL2</td>
<td>329 lg 1.1</td>
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<tr>
<td>FL3</td>
<td>345 lin 1.1</td>
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PMT voltages and compensation settings had to be adjusted sequentially, and the resulting values are summarized in Tables 2 and 3.

Other dye combinations tested, as listed above, were not suitable for any DNA-multi-parameter analysis on a standard FACSscan. These fluorochromes will be discussed below.

Double staining with an indirect fluorescence protocol against Uro5 and EGFR was carried out simultaneously, and showed no cross-reactivity of immunoglobulins, as was proven by single staining with exchange of second antibodies.

In double-stained cells FL1 was chosen for FITC and FL2 for R-PE (Fig. 3a). The addition of PI to these double-stained cells did not critically affect the MFI s of FL1 fluorescence, and did not change any quantiative results in the subpopulations in the different quadrants (Fig. 3b). Addition of the DNA dye 7-AAD results in similar histograms (Fig. 4). This was tested in monocultures of tumour cells (Figs. 3a,b) and could also be reproduced when mixed cultures of fibroblasts and tumour cells were tested (Figs. 5a,b). Fibroblasts and tetraploid bladder tumour cells can be distinguished in FL2 (Fig. 5a) and express similar amounts of EGFR (Fig. 5b).

Looking at absolute quantitation of fluorescence intensities, i.e. receptor numbers, beads were stained simultaneously with RT4 cells in a three-parameter staining protocol. Figs. 6a,b show that quantitation of FL1-fluorescence allows reliable quantitation of EGFR fluorescence after PI staining, since relative positions of cells are

Table 3
Compensation settings for a DNA-three-parameter analysis with FITC, R-PE, and PI for the single laser flow cytometer FACSscan

<table>
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<th>Compensation settings</th>
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<td>FL1-FL2</td>
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<td>FL2-FL1</td>
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<td>FL2-FL3</td>
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<td>FL3-FL2</td>
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<tr>
<td>2</td>
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<td>6</td>
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<tr>
<td>92.5</td>
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Fig. 3. (a) FL1 (FITC) vs. FL2 (R-PE) signals of differently stained and mixed RT4 cells. Panel a: without PI, panel b: with PI. The presence of the DNA dye PI does not basically affect the FL1 signals. In particular, the ratio of green positive cells (measured in FL1) compared with the isotype control is approximately the same in the absence and presence of PI. However, in the presence of PI the MFI of R-PE⁺ cells (detected in FL2) are dramatically decreased.

unaltered (Figs. 6a, b). However, similar quantitations are not possible in FL2 (see Fig. 7a, b), because in presence of PI the FL2 signal is artificially reduced.

Fig. 8a and 8b show the results of determination of EGFR content and SPF of RT4 and J82 under culture conditions as described in Materials and methods. The cell line RT4 showed good correlation of EGFR content and S-phase fraction in de-

Fig. 4. (a) FL1 (FITC) vs. FL2 (R-PE) signals of differently stained and mixed RT4 cells. Panel a: without 7-AAD, panel b: with 7-AAD. The presence of the DNA dye 7-AAD does not basically affect the FL1 signals. In particular, the ratio of green positive cells (measured in FL1) compared with the isotype control is approximately the same in the absence and presence of 7-AAD. However, in the presence of 7-AAD the ratio of the MFI of R-PE⁺ and R-PE⁻ cells (detected in FL2) are distinctively altered.
Fig. 5. (a) Mixture of RT4 and N1 cells, measured under conditions of three parameter instrument settings. Panel a: FL3-H (PI) vs. FL2 (R-PE) signals: only the tetraploid RT4 cells are Uro5⁺. Panel b: FL3-H (PI) vs. FL1 (FITC) signals: both cell lines express the EGFR.

Fig. 6. Indirect immunofluorescence staining with FITC of quantum beads and RT4 cells against the EGFR. FL1-histograms of measurements in absence (panel a) and presence (panel b) of PI. Both measurements result in identical EGFR quantities.
Fig. 7. Indirect immunofluorescence staining of quantum-beads with R-PE and RT4 cells against the EGFR. FL2 histograms of measurements in absence (panel a) and presence (panel b) of PI. Absolute EGFR quantities deviate from the original count when simultaneous PI staining is carried out.

dependence of in vitro growth: three-dimensional growth as MCS causes down-regulation of EGFR. In young (3 days) COCU higher receptor numbers were found in comparison to mono-MCS, while 8-day-old COCU showed an EGFR content similar to that of mono-MCS. In general, SPF correlated with EGFR expression for all growth states.

The low differentiated cell line J82 showed a similar pattern of EGFR regulation compared with the RT4 cell line. In general, three-dimensional in-vitro growth caused a down-regulation of EGFR content compared to ML cells. However, the SPF is basically unaffected by conditions of tumour cell growth. A slight decrease of SPF under three-dimensional culture conditions was observed.

4. Discussion

In this study different dye combinations were investigated for practicality of DNA-three-parameter analysis on a single laser flow cytometer. The aim is to perform an EGFR evaluation of human bladder cancer cells and simultaneous exclusion of stromal or inflammatory cells. For this purpose we chose Uro5 as an antibody against a panurothelial glycoprotein, known to be highly specific [8]. Stain-
Fig. 8. (a,b) Panel a: EGFR content (left y-axis) and SPF (right y-axis) of the well differentiated cell line RT4. SPF correlated with EGFR expression for all culture conditions. Panel b: EGFR content (left y-axis) and SPF (right y-axis) of the low differentiated cell line J82. SPF of tumour cells did not correlate with EGFR content.

ing of antibodies was carried out as an indirect method, providing high sensitivity of antigenic detection. The process of staining was significantly shortened by using different immunoglobulin subclasses as secondary antibodies, allowing simultaneous incubation of the two matching types of fluorescent-linked antibodies. As has been stated already by Corver et al. [5], no cross reactivity was found in our experiments, resulting in reliable specificity.

To adjust instrument settings, fluorescence beads were not sufficient for tumour cell measurements due to their low autofluorescence. Since compensation should be independent of the intensity of the fluorochrome [32], separately stained tumour
cells were used, which showed brighter staining than any bladder cancer cell lines tested. CaSki cells with abnormally high amounts of EGFR (1 $\times$ 10$^6$) were stained separately with one fluorochrome against the EGFR. These cells facilitate the determination of instrument settings. Unstained cells were used to adjust PMT voltages of FL1 and FL2. The settings remained constant within that protocol from day to day. To monitor correct compensation, single-stained controls can always be run in addition within each experimental group.

Best results were achieved with the dye combination FITC, R-PE, and PI. In agreement with Corver et al. [5], correct compensation of the spectral spill over of both PI and R-PE fluorescence into FL1 is not possible. It is easy to compensate the R-PE overlap into the green channel by adjusting FL1-%FL2, but increasing this value due to the PI spill over into FL1 would result in an over-compensation of the R-PE signal.

Cells without FITC staining, but with PE and PI staining, appear over-compensated in FL2, because of the PI spill over into FL1. Consequently, the MFI of positive orange-fluorescent cells is lower than the MFI of double-stained cells, i.e. stained with R-PE and PI. Our measurements demonstrate that the FL2 signal is not suitable for antigen assessment, but sufficient for cell--cell discrimination, provided that the marker-molecule is highly expressed. In this context, the choice of R-PE because of a high quantum yield [20] does not provide any advantage over FITC.

The green fluorescence is basically unaffected (Fig. 3) even in the presence of PI. Our results show that EGFR quantitation of tumour cells is possible without any limitations. Previously made quantitative flow measurements and ligand binding experiments yield the same EGFR content per cell [3,4].

The example of using cocultures of fibroblasts and tumour cells for three-parameter measurement resulted in reproducible data within changes in receptor expression and SPF could be demonstrated.

It was not possible to transfer the instrument settings published by Corver et al. [5] to our flow cytometer. Although this group also used a FACSscan instrument, we found only one combination of instrument settings for all PMT voltages and compensations, that give reproducible results in contrast to their findings of several options for instrument settings. All efforts to align instrument settings after changing one parameter (e.g. one PMT voltage) failed. Compensation settings are valid only for a particular combination of fluorochromes with a particular set of filters and mirrors and with particular voltages applied. In each case, instrument settings have to be determined individually for each flow cytometer as well as for each fluorescence-conjugated antibody. The emission spectrum of any fluorochrome varies in dependence of labeling density and requires individual compensation. Furthermore, because instrument settings are not transferable to other flow cytometers, they can only serve as a guideline for other groups to find out their correct settings.

As mentioned above there is no device for FL1-%FL3 correction and FL3-%FL1. Probably, this option would result in a significant improvement of the present situation. As an addition or alternative, the option of exchanging optical filters in instruments used for clinical routine may be helpful. Compensation parameters could be improved by using a 630/22 nm bandpass filter instead of a 650 longpass filter.
which can be shown on the more complex FACStarPlus (Becton Dickinson) (data not shown) with replaceable filter combinations. Other groups have already used the combination FITC, PE and PI on a FACStarPlus [2] or on an Ortho 50-h flow cytometer [28], but these machines cannot be recommended for routine clinical measurements. It would seem most advantageous for multiparameter-DNA measurements to develop a flow cytometer equipped with an UV-lamp and an argon-ion laser which is easy enough to use for routine application, i.e. provides easy calibration and shows stable instrument performance. The wide spectrum of obtainable fluorochromes for DNA measurements could be applied more easily.

Though less than PI, 7-AAD still shows spill over into the FL1 [10]. In spite of higher emission of 7-AAD in comparison with PI, no significant advantage could be noticed using this DNA probe (Fig. 4). Just as PI, 7-AAD altered the FL2 signals to wrong values measured on the FACScan flow cytometer.

Possibly, the big portion of cytoplasm of tumour cells in contrast to hematologic cells is responsible for the 7-AAD emission spectrum down to 520 nm, because calf thymocytes with a small cellbody show 7-AAD emission in the range from 570 up to 720 nm (data not shown). In agreement with other data [35], CVs of 7-AAD are higher than those of PI (data not shown). The comparatively big molecule causes variation of staining in dependence of fixaion, and it is also known that binding and fluorescence properties of 7-AAD may be especially sensitive to chromat conformation of the DNA [25].

The tandem conjugate QR revealed strong signals of EGFR staining and could easily be detected in FL3. It seems to be an excellent fluorescence stain when emission of high wavelength is required. However, in combination with the DNA dyes Syto-13 or PI, a three-parameter analysis on a FACScan is impossible. Syto-13 can be detected in all three PMTs with extreme intensity. The emission maximum is at 520 nm, but the spectral overlap of this fluorochrome is too intensive to allow adequate compensation. Furthermore, Syto-13 fluorescence could also be detected within the cytoplasm of cells. This nucleic acid stain is not suitable for flow cytometric analysis. Furthermore, when PI is detected in FL2, the spill over into FL3 cannot be compensated, even when QR is applied simultaneously.

In agreement with investigations of other groups [34] the nucleic acid fluorochrome LDS-751 has a suitable emission spectrum for the combination with FITC and R-PE resulting in little spectral interference. However, the DNA histograms were insufficient to determine ploidy or SPF. It also was not possible to achieve low enough CVs. Broad CVs hindered the discrimination of cell cycle phases. In spite of RNase digestion, LDS-751 was detectable, not only within the cell nucleus but also within the cytoplasm. In addition LDS-751 was incorporated in dependence of the cell type. Only differences in fluorescence intensity between single cells and small aggregates are sufficient to support the discrimination of singlets and doublets. Therefore, this stain cannot be integrated in a scheme for multi-parameter-DNA staining.

In conclusion, data have shown that three-parameter-DNA analysis for the definition of tumour subpopulations is possible with a single laser instrument, and can provide answers in tumour biology that are hard to obtain with any other method,
e.g. defining the EGFR content of urothelial tumour cells in dependence of ploidy in a mixed cell population. However, as mentioned above, the methodology can only be extended to routine clinical applications if further improvements either on the instrument or fluorochromes are made to make discrimination of cell populations as easy as in non-DNA-multiparameter measurements.

Acknowledgments

The authors thank Dr. Peter S Rabinovitch, Experimental Pathology, Seattle WA, for helpful advice.

References


