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

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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

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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 4×10^4 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_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 0.5×10^5 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_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 μl of medium at a concentration of 3×10^3 tumour cells and 6×10^3 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 (= 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 (1×10^6/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_{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 1
Fluorochromes, tested for DNA-three-parameter analysis on a single laser flow cytometer

FL1	FL2	FL3
FITC	R-PE	PI
FITC	R-PE	7-AAD
Syto-13	R-PE	QR
FITC	PI	QR
FITC	R-PE	LDS-751

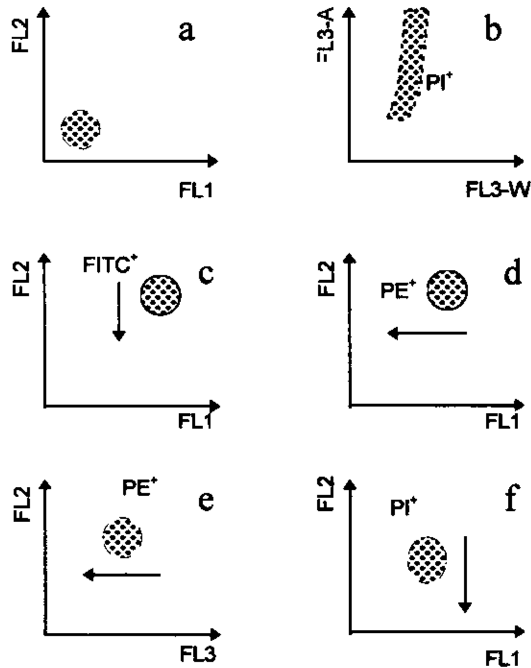

Fig. 1. Panel a: Autofluorescent cells, adjustment of FL1 and FL2 PMT voltages. Panel b: PI^+ cells, adjustment of FL3 PMT voltage. Panel c: $FITC^+$ cells, determination of FL2-%FL1 compensation. Panel d: PE^+ cells, determination of FL1-%FL2 compensation. Panel e: PE^+ cells, determination of FL3-%FL2 compensation. Panel f: PI^+ cells, determination of FL2-%FL3 compensation.

rochromes 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 form 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, 4095, 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 1×10^4 events of samples stained with one dye and 2×10^4 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 × 10^6/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 × 10^6/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.

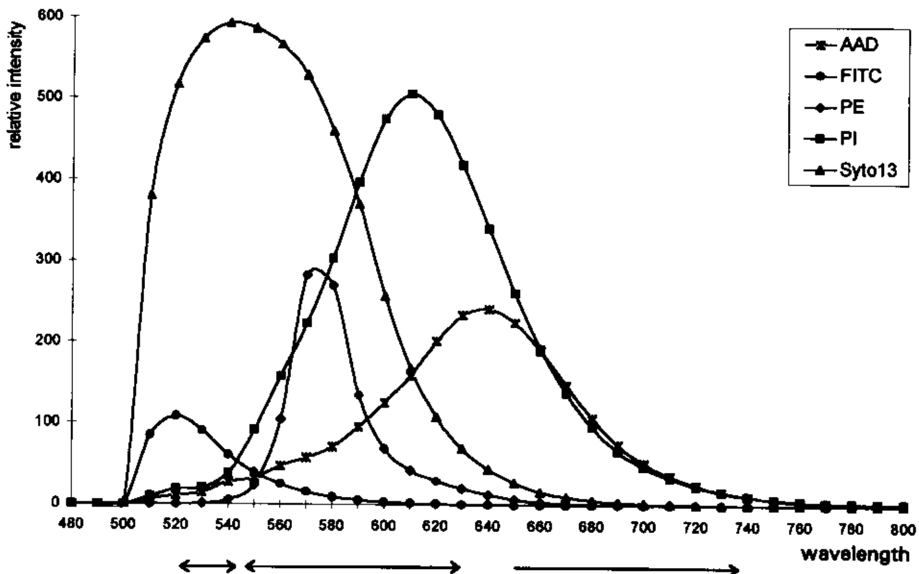

Fig. 2. Emission spectra and their relative fluorescence intensity occurring in 1 × 10^6/ml CaSki cells. The spectral emission of five tested fluorochromes is depicted and related the three fluorescence detectors of the FACScan. The difficulty to compensate the spill over is apparent. Arrows reflect 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 FACScan

	PMT voltages	Gain
FSC	E-01	lg 1.0
SSC	183	lg 1.0
FL1	481	lg 1.4
FL2	329	lg 1.1
FL3	345	lin 1.1

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 FACScan. 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 MFIs of FL1 fluorescence, and did not change any quantitative 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 FACScan

Compensation settings	
FL1-%FL2	2
FL2-%FL1	6
FL2-%FL3	92.5
FL3-%FL2	6

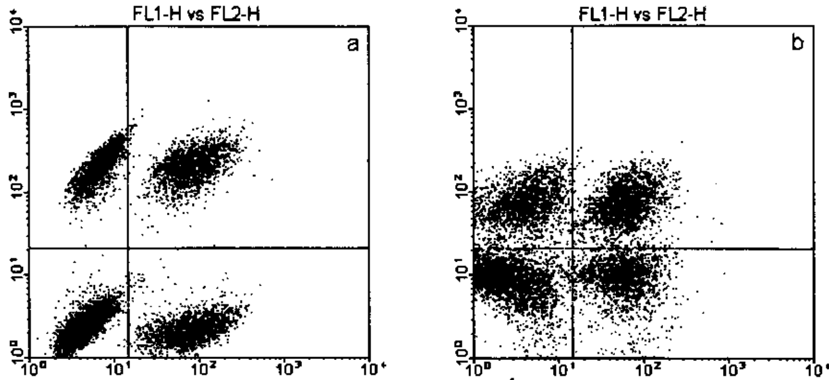

Fig. 3. (a) FL1 (FITC) vs. FL2 (R-PE) signals of differently stained and mixed RT4 cells. Panel a: without PI, panel b: with PI. The presence of the DNA dye PI does not basically affect the FL1 signals. In particular, the ratio of green positive cells (measured in FL1) compared with the isotype control 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-

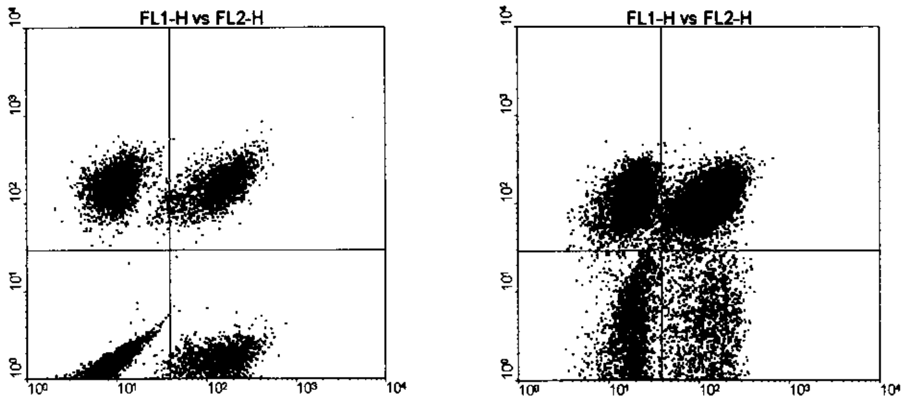

Fig. 4. (a) FL1 (FITC) vs. FL2 (R-PE) signals of differently stained and mixed RT4 cells. Panel a: without 7-AAD, panel b: with 7-AAD. The presence of the DNA dye 7-AAD does not basically affect the FL1 signals. In particular, the ratio of green positive cells (measured in FL1) compared with the isotype control is approximately the same in the absence *and* presence of 7-AAD. However, in the presence of 7-AAD the ratio of the MFIs of R-PE$^+$ and R-PE$^-$ cells (detected in FL2) are distinctively altered.

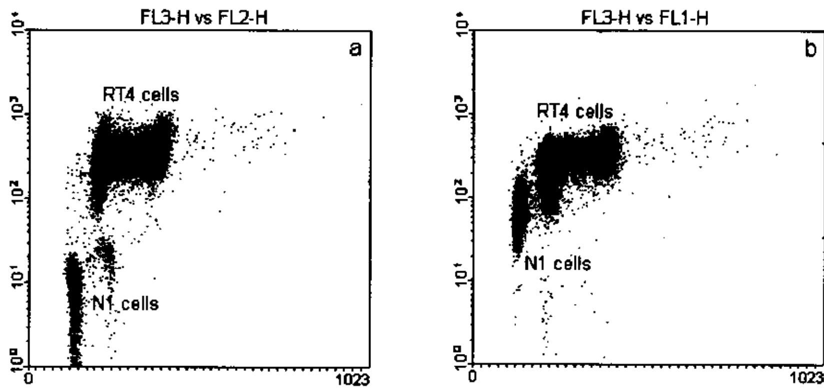

Fig. 5. (a) Mixture of RT4 and NI cells, measured under conditions of three parameter instrument settings. Panel a: FL3-H (PI) vs. FL2 (R-PE) signals: only the tetraploid RT4 cells are Uro5$^+$. Panel b: FL3-H (PI) vs. FL1 (FITC) signals: both cell lines express the EGFR.

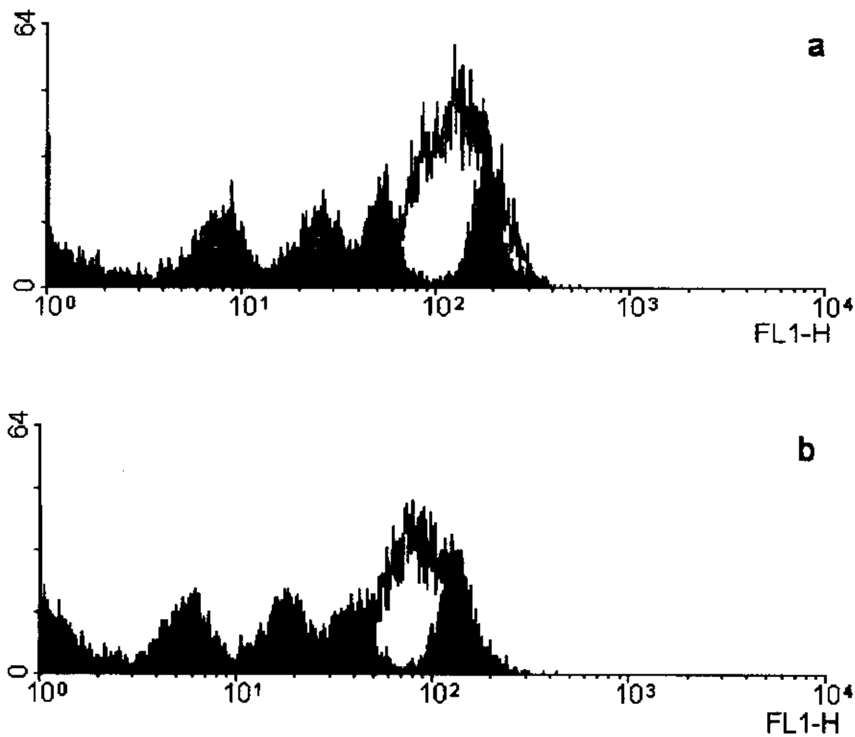

Fig. 6. Indirect immunofluorescence staining with FITC of quantum beads and RT4 cells against the EGFR. FL1-histograms of measurements in absence (panel a) and presence (panel b) of PI. Both measurements result in identical EGFR quantities.

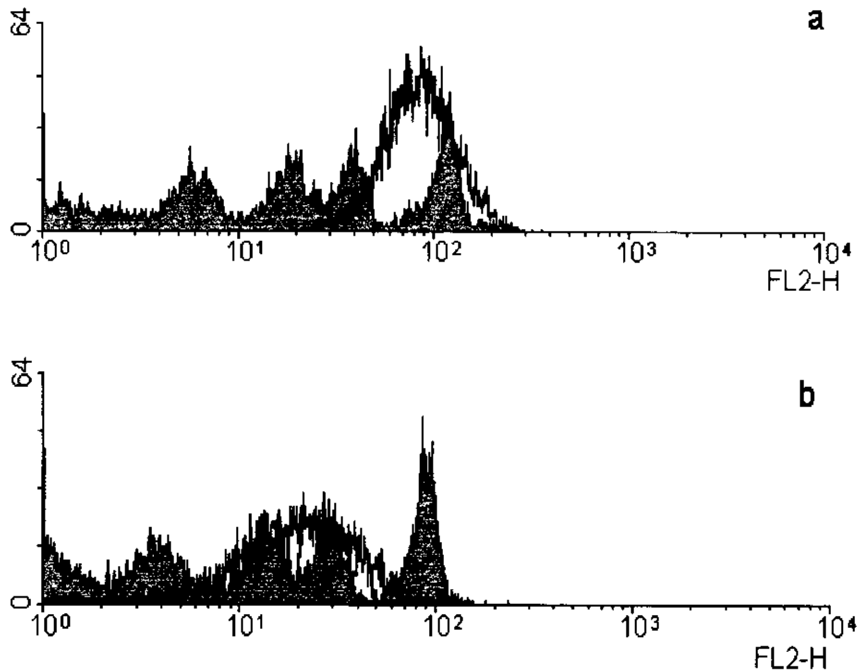

Fig. 7. Indirect immunoflourescence staining of quantum-beads with R-PE and RT4 cells against the EGFR. FL2 histograms of measurements in absence (panel a) and presence (panel b) of PI. Absolute EGFR quantities deviate from the original count when simultaneous PI staining is carried out.

pendence 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-

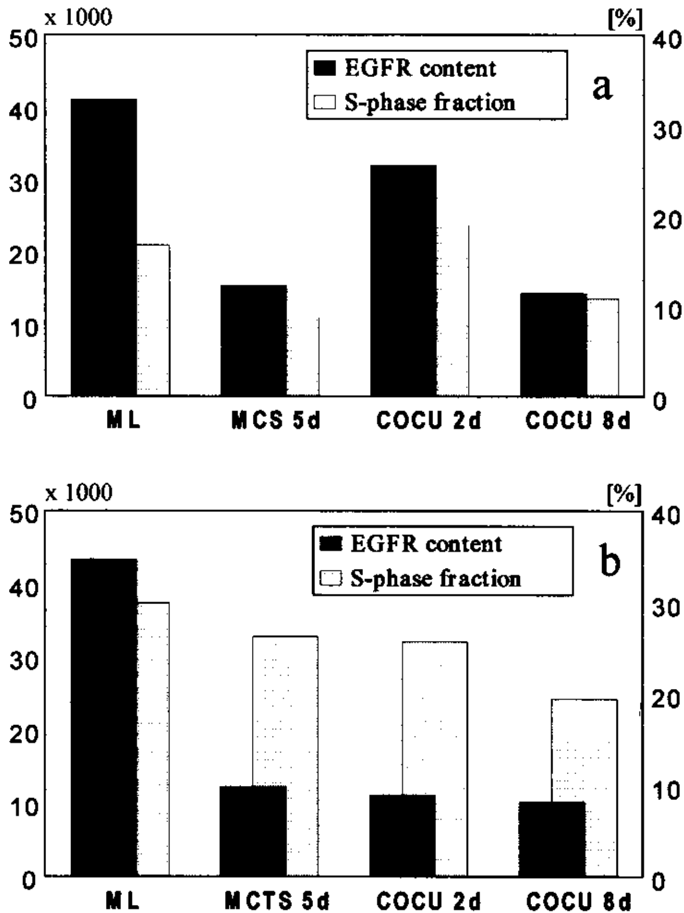

Fig. 8. (a,b) Panel a: EGFR content (left y-axis) and SPF (right y-axis) of the well differentiated cell line RT4. SPF correlated with EGFR expression for all culture conditions. Panel b: EGFR content (left y-axis) and SPF (right y-axis) of the low differentiated cell line J82. SPF of tumour cells did 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 (1×10^6) 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 FACScan 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 FACStar^{Plus} (Becton Dickinson) (data not shown) with replaceable filter combinations. Other groups have already used the combination FITC, PE and PI on a FACStar^{Plus} [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 fixation, and it is also known that binding and fluorescence properties of 7-AAD may be especially sensitive to chromatic 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.

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References

[1] Bauer KD, Duque RE, Shankey TV, eds. Clinical flow cytometry — principals and applications. Williams & Wilkins, Baltimore, 1992.

[2] Begg AC, Hofland I.Cell Kinetic Analysis of mixed populations using three-colour fluorescence flow cytometry. Cytometry 1991;12:445–454.

[3] Brockhoff G, Hofstädter F, Knüchel R. Flow cytometric detection and quantitation of the epidermal growth factor receptor in comparison to Scatchard Analysis in human bladder carcinoma cell lines. Cytometry 1994;17:75–83.

[4] Brotherick I, Lennard TWJ, Wilkinson SE, Cook WS, Angus B, Shenton BK. Flow cytometric method for the measurement of epidermal growth factor receptor and comparison with the radio ligand binding assay. Cytometry 1994;16:262–269.

[5] Corver WE, Cornelisse CJ, Fleuren GJ. Simultaneous measurement of two cellular antigens and DNA using fluorescein-isothiocyanate, R-phycoerythrin, and propidium iodide on a standard FACScan. Cytometry 1994;15:117–128.

[6] Dent GA, Leglise MC, Pryzwansky KB, Ross DW. Simultaneous paired analysis by flow cytometry of surface markers, cytoplasmic antigens, or oncogene expression with DNA content. Cytometry 1989;10:192–198.

[7] Festin R, Björkland A, Töttermann H. Single laser flow cytometric detection of lymphocytes binding three antibodies labelled with fluorescein, phycoerythrin and a novel tandem fluorochrome conjugate. J Immunol Methods 1990;126:69–78.

[8] Fradet Y, Cordon-Cardo C, Whitmore WF, Melamed MR Jr., Old LJ. Cell surface antigens of human bladder tumours: Definition of tumour subsets by monoclonal antibodies and correlation with growth characteristics. Cancer Res 1986;46:5183–5188.

[9] Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP. In vitro cultivation of cell lines derived from a series of solid tumours. J Natl Cancer Inst 1973;51:1417–1421.

[10] Gill JE, Jotz MM, Young SG, Modest EJ, Sengupta SK. 7-Amino-actinomycin-D as a cytochemical probe. J Histochem Cytochem 1975;23(11):793–799.

[11] Givan AL. Flow Cytometry, First Principals, Chapter 5: Seeing the Light: Lasers, Fluorochromes, and Filters. Wiley-Liss, Inc., New York, 1992.

[12] Gruber R, Reiter C, Riethmüller G. Triple immunofluoresence flow cytometry, using whole blood, of $CD4^+$ and $CD8^+$ lymphocytes expressing CD45RO and CD45RA. J Immunol Methods 1993;163:173–179.

[13] Knüchel R, Keng P, Hofstädter F, Langmuir V, Sutherland RM, Penney DP. Differentiation patterns in two- and three-dimensional culture systems carcinoma cell lines. Am J Pathol 1990;137:725–736.

[14] Knüchel R, Meckl S, Masters JRW, Hofstädter F. Verteilungsmuster Urothel-assoziierter Glycoproteine an in vitro Kulturen humaner Harnblasenkarzinomzellinien. Verh Deutsch Ges Pathol 1993;77:256–259.

[15] Lanier LL, Recktenwald DJ. Multicolour immunfluorescence and flow cytometry. Methods: A Companion to Methods in Enzymology 1991;2(3):192–199.

[16] Li BDL, Bauer KD, Carney WP, Duda RB. Detection of c-erB-2 oncoprotein expression in breast tissue by multiparameter flow cytometry. J Surg Res 1993;54:179–188.
[17] Mandy FF, Bergeron M, Recktenwald D, Izaguirre CA. A simultaneous three-colour T cell subsets analysis with single laser flow cytometers using T cell gating protocol — comparison with conventional two colour immunophenotyping method. J Immunol Methods 1992;156:151–62.
[18] Mansbridge JN, Knüchel R, Knapp AM, Sutherland RM. Importance of tyrosine phosphatases in the effects of cell-cell contact and microenvironments on EGF-stimulated tyrosine phosphorylation. J Cell Phys 1992;151:433–442.
[19] Masters JRW, Hepburn PJ, Walker L, Highman WJ, Trejdosiewicz LK, Povey S, Parkar M, Hill BT, Riddle PR, Franks LM. Tissue culture model of transitional cell carcinoma: characterization of 22 human urothelial cell lines. Cancer Res 1986;46:3630–3636.
[20] Melamed MR, Lindmo T, Mendelsohn ML. Flow Cytometry and Sorting, 2nd edition. Wiley-Liss, Inc., New York, 1990.
[21] Messing EM. Clinical implications of the expression of epidermal growth factor receptors in human transitional cell carcinoma. Cancer Res 1990;50:2530–2537.
[22] Messing EM. Growth factors and bladder cancer: Clinical implications of the interactions between growth factors and their urothelial receptors. Sem Surg Oncol 1992;8:285–292.
[23] Neal DE, Marsh C, Bennett MK, Abel PD, Hall RR, Sarnsbury JRC, Harris AL. Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumours. Lancet 1985;1:366–368.
[24] Neal DE, Sharples L, Smith K, Fennelly J, Hall RR, Harris AL. The epidermal growth factor receptor and the prognosis of bladder cancer. Cancer 1990;65:1619–1625.
[25] Rabinovitch PS, Torres RM, Engel D. Simultaneous cell cycle analysis and two-colour surface immunofluorescence using 7-amino-actinomycin D and single laser excitation: applications to study of cell activation and the cell cycle of murine LY-1 B cells. J Immunol 1986;136(8):2769–2775.
[26] Ramaekers FCS, Huysmanns A, Moesker O, Schaart G, Herman C, Voiijs P. Cytokeratin expression during neoplastic progression of human transitional cell carcinoma as detected by a monoclonal and a polyclonal antibody. Lab Invest 1985;52:31–38.
[27] Rigby CC, Franks LM. A human tissue culture cell line from a transitional cell tumour of the urinary bladder: growth, chromosome pattern, and ultrastructure. Br J Cancer 1970;24:746–754.
[28] Sasaki DT, Dumas SE, Engelmann EG. Discrimination of viable and non-viable cells using propidium iodide in two colour immunfluorescence. Cytometry 1987;8:413–420.
[29] Schuster U, Büttner R, Hofstädter F, Knüchel R. A heterologous coculture system to study interaction between human bladder cancer cells and fibroblasts. J Urol 1994;151:1707–1711.
[30] Shankey TV, Rabinovitch PS, Bagwell B, Bauer KD, Duque RE, Hedley DW, Mayall BH, Wheeless L. Guidelines of implementation of clinical DNA cytometry: Cytometry 1993;14:472–477.
[31] Smith K, Fennely JA, Neal DE, Hall RR, Harris AL. Characterisation and quantitation of the epidermal growth factor receptor in invasive and superficial bladder tumours. Cancer Res 1989;49:5810–5815.
[32] Stewart CC. Multiparameter analysis of leucocytes by flow cytometry. In: Darzynkiewicz Z, Crissman HA, eds. Methods Cell Biol 1990;33:427–450.
[33] Stoschek CM, King LE Jr. Role of epidermal growth factor in carcinogenesis. Cancer Res 1986;46:1030–1037.
[34] Terstappen LWMM, Shah VO, Conrad MP, Recktenwald D, Loken MR. Discrimination between damaged and intact cells in fixed flow cytometric samples. Cytometry 1988;9:477–484.
[35] Toba K, Winton EF, Bray RA. Improved staining method for the simultaneous flow cytofluorometric analysis of DNA content, S-phase fraction, and surface phenotype using single laser instrumentation. Cytometry 1992;13:60–67.
[36] Zarbo RJ, Visscher DW, Crissmann JD. Two-colour multiparametric method for flow cytometric DNA analysis of carcinomas using staining for cytokeratin and leucocyte-common antigen. Anal Quant Cytol Histol 1989;11:391–402.