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ORIGINAL PAPER

Measurement Conditions for Flow Cytometry Analyses of Cell Lines from Urological Carcinomas

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Abstract Prerequisites for successful flow cytometry investigations are specific antibodies labeled with appropriate fluorochromes and negligible autofluorescence of the untreated cells at the wavelength of interest. The aim of this study was (a) to characterize frequently used urological carcinoma cell lines with regard to their autofluorescence properties, (b) to demonstrate the autofluorescence as a serious interfering factor on FACS analysis of urological carcinoma cell lines and (c) to suggest an alternative to avoid interfering autofluorescence. Twenty-one cell lines originating from prostate carcinoma, renal cell carcinoma and bladder cancer were included in this study. The various cell lines were read on a flow cytometer in comparison to human erythrocytes as cells with low fluorescence intensity. Urological cell lines show a high autofluorescence when flow cytometry analyses are performed at the frequently used excitation wavelengths at 405 and 488 nm. At excitation wavelength of 633 nm, this problem was reduced and most of the cell lines (14/21) were without autofluorescence at the emission wavelength of 785 nm. In addition, with a spectrofluorometer three exemplary cell lysates were investigated. The above observations were confirmed. The dye APC-Cy7 is one suitable fluorochrome for successful investigation under these measurement conditions.

Keywords Autofluorescence · Cell lines · Flow cytometry · Urological carcinomas

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Introduction

The flow cytometry is a universally recognized tool for the detection of surface antigens in immunology [1–3]. New research fields are differentiation of apoptosis and necrosis, cell cycle analysis and the characterization of physiological state of cells. Moreover, this method is extensively applied in the field of tumor biology [4, 5]. Common applications are evaluation of allergic disease in humans, automated cell counting, measurement of cell proliferation and staining of intracellular cytokine or other intracellular targets [6]. Prerequisites for successful investigations of surface markers are specific antibodies labeled with appropriate fluorochromes and negligible autofluorescence of the untreated cells at the wavelength of interest. The autofluorescence comes from the biological components within the cell and varies with the cell type. In addition to porphyrins, biochemical substances such as tryptophan

Table 1 Examined cell lines of urological carcinomas in this study

Renal cell carcinoma		Prostate carcinoma		Urinary bladder carcinoma	
Cell line	Culture medium	Cell line	Culture medium	Cell line	Culture medium
786-0	RPMI 1640	BPH-1	RPMI 1640	HCV-29	RPMI 1640
A 498	RPMI 1640	Du-145	RPMI 1640	HT-1,376	RPMI 1640
A 704	RPMI 1640	LNCaP	RPMI 1640	J-82	RPMI 1640
ACHN	MEM	PC-3	RPMI 1640	RT-4	RPMI 1640
Caki-1	McCoy's	22 RV-1	RPMI 1640	RT-112	RPMI 1640
Caki-2	McCoy's			SCa-BER	RPMI 1640
HK-2	K-SFM			UM-UC-3	RPMI 1640
SN-12	RPMI 1640				
SW839	McCoy's				

and other aromatic ring systems are excited to fluorescence by ultraviolet light [7]. The excitation maxima of NADH and flavins are located at the near ultraviolet and blue spectral range, respectively. Especially flavins with wide range emission spectra contribute to background fluorescence [8]. The greatest part of the autofluorescence arises in the connective tissue by collagen and elastic fibers. The tissue autofluorescence depends on tissue structure, metabolic activities and morphologic aspects [9]. Frequently, this native fluorescence is used for minimal invasive diagnosis, for example for the detection of urinary bladder tumors [10, 11]. In the same way, the various cells in the blood stream are characterized by different intensities of autofluorescence. Lymphocytes exhibit the least autofluorescence of white blood cells, whereas monocytes and granulocytes have the most. The autofluorescence of unstained lymphocytes is equivalent to approximately 1,000 fluorescein molecules per cell. Therefore, the lowest limit of cellular detection is controlled by the sample itself [12]. The literature does not contain any information about possible intracellular interfering influences while the examination of permanent cell lines. Hence, it was necessary to create a systematic experimental overview.

The aim of this study was (a) to characterize frequently used urological carcinoma cell lines with regard to their autofluorescence properties, (b) to demonstrate the autofluorescence as a serious interfering factor on FACS analysis of urological carcinoma cell lines and (c) to suggest an alternative to avoid interfering autofluorescence.

Materials and methods

Cell lines

Cell lines originating from prostate carcinoma, renal cell carcinoma and bladder cancer representing the three most frequent urologic carcinomas were included in this study (Table 1). Cells purchased from American Type Culture Collection, Manassas, VA (ATCC) and from German Collection of Microorganism and Cell Culture, Braunschweig (DSMZ) were cultured as described in the manufacturer's instructions. All cell lines were maintained in a humid chamber at 37°C and 5% CO_2. Tissue culture media were obtained from Invitrogen GmbH (Karlsruhe, Germany) and fetal calf serum from PAA Laboratories GmbH (Pasching, Austria).

The cells were used untreated and treated with 2% formaldehyde (FA) in PBS for flow cytometry. The FA was washed out after treatment.

Freshly prepared human erythrocytes [13] as cells with low autofluorescence [12] served as negative reference.

Table 2 FACSCanto™ II configuration (abbreviation in brackets)

Excitation laser wavelength [nm][a]	Emission maxima [nm][a]	Common used fluorochrome (Abbreviation)
405	452	Pacific Blue
	491	Protein of Anemonia Majano (AmCyan)
488	519	Fluoresceine isothiocyanate (FITC)
	578	Phycoerythrine (PE)
	695	Peridinin-chlorophyll-carbocyanin 5.5 (PerCP-Cy5.5)
	785	Phycoerythrine-carbocyanin 7 (PE-Cy7)
633	660	Allophycocyanin (APC)
	785	Allophycocyanin-carbocyanin 7 (APC-Cy7)

[a] The mention of wavelengths corresponds with the Clinical Research Product Catalog of BD Biosciences, 2009, Heidelberg, Germany.

The human breast adenocarcinoma cell line MCF-7 was additionally used as comparative cell line to the urological carcinoma cell lines.

Flow cytometry and data analysis

Samples were read on a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA). The device was equipped with three air-cooled lasers. The configuration of FACS-Canto™ II allowed an excitation at 405, 488 and 633 nm and fluorescence detection in eight channels. The data were analyzed by using FACS-DiVa™ software (BD Biosciences, San Jose, CA). The parameter "5,000" was selected with the device function "threshold" to exclude cell debris. Fluorescence intensities of the various cell lines were assessed in relation to the above mentioned human erythrocytes as negative reference. A higher fluorescence intensity than the reference (erythrocytes) was considered as autofluorescence. Typical instrument configuration is described in Table 2. Each cell sample contained 1.5–2× 10^6 vital or fixed cells and was diluted in 0.5 ml phosphate buffered saline (PBS) before analyzed.

Microscopy and fluorescence spectra

The cells were harvested with a trypsin/EDTA-solution according to manufacturer's instructions. The cell suspension was washed with PBS and than the native cells were checked with a confocal laser scanning microscope (CLSM, ZEISS LSM 510 system with Axiovert microscope, Carl Zeiss Jena GmbH, Jena, Germany) equipped with argon, helium laser set. Two emission spectra (525–792 nm and 707–792 nm) were measured at the excitation of 488 and 633 nm, respectively. The fluorescence spectra from cell lysates were obtained using the spectrofluorometer "SPEX Fluorolog 1680" according to

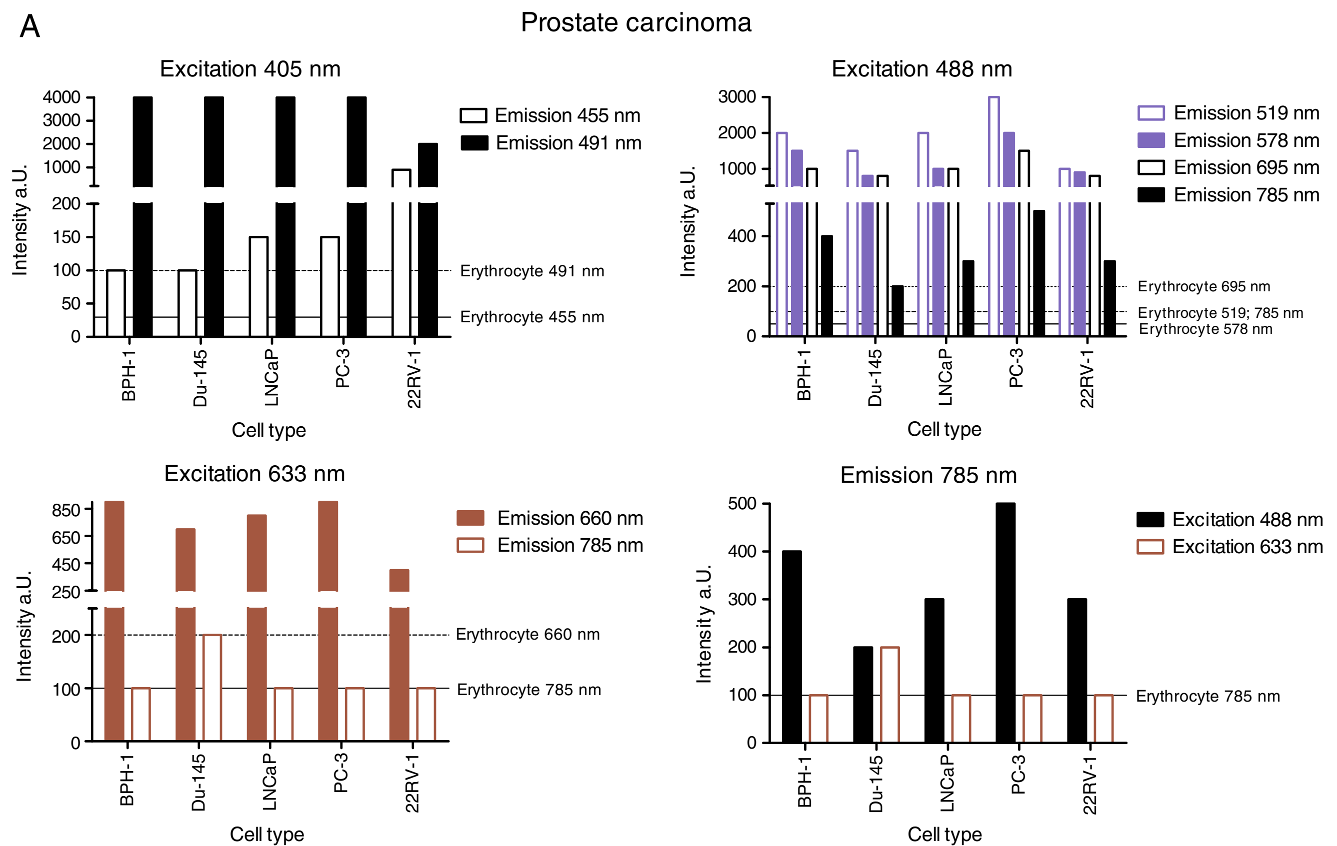

Fig. 1 Autofluorescence of cell lines from (**a**) prostate carcinoma, (**b**) renal cell carcinoma, (**c**) urinary bladder carcinoma. For each tumor entity three column charts show the autofluorescence intensity of the investigated cell lines at three excitation wavelength (405, 488 and 633 nm). The fourth column chart for each entity combines the autofluorescence emission at 785 nm with two used excitation wavelengths. The *horizontal lines* mark the fluorescence intensity of erythrocytes at various emission wavelengths as limit for the determination of autofluorescence

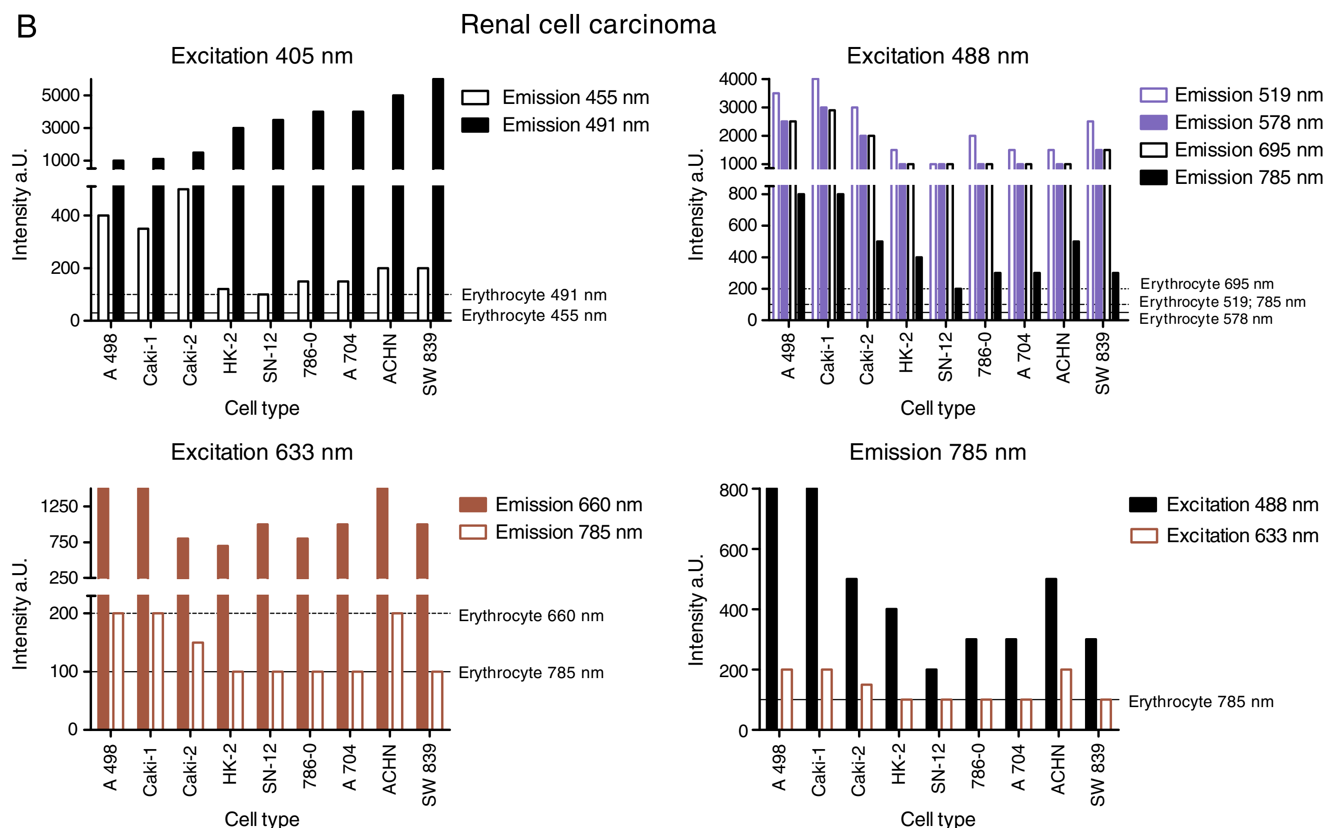

Fig. 1 (continued)

Heck [14] and Schädel [15]. The cell lysates were prepared in a 50 mM Tris-buffer according to the method of Zigrino [16].

Results

In flow cytometry, human erythrocytes showing low fluorescence intensities in all commonly used excitation wavelengths are generally used as reference cells. Therefore, if the fluorescence intensity of the examined urological cell lines was higher than the fluorescence of erythrocytes, the carcinoma cell line was estimated to have autofluorescence. Detailed results are summarized in Fig. 1.

All 21 urological cell lines showed a high autofluorescence both at excitations of 405 and 488 nm. The fluorescence intensity was higher than for normal human erythrocytes at all emission maxima (452; 491; 519; 578; 695; 785 nm).

Different results were observed at the excitation wavelength of 633 nm. Whereas all urological cell lines showed a distinct autofluorescence at the 660 nm (e.g. APC) emission peak, only seven cell lines revealed an autofluorescence at the 785 nm (e.g. APC-Cy7) emission peak. Thus, the flow cytometry investigation of the most cell lines from urological carcinomas was only feasible using the excitation at 633 nm and the emission peak at 785 nm.

The treatment with FA did not significantly change the autofluorescence of the cells (data not shown).

To confirm the observed autofluorescence, cell lysates from three cell lines were exemplarily investigated using confocal laser scanning microscope and spectrofluorometry. For these measurements, platelet cells were used as negative reference. These cells were used as references as they are always available for laboratory work and their autofluorescence is low [17]. The renal carcinoma cell line Caki-2 represented one example of the human urological carcinoma cell lines. Simultaneous measurement of MCF7 cells, a breast carcinoma cell line, allowed a comparison with another tumor entity. The confocal laser scanning microscope showed image of granularly autofluorescence in the cytoplasm of Caki-2 cells, Fig. 2.

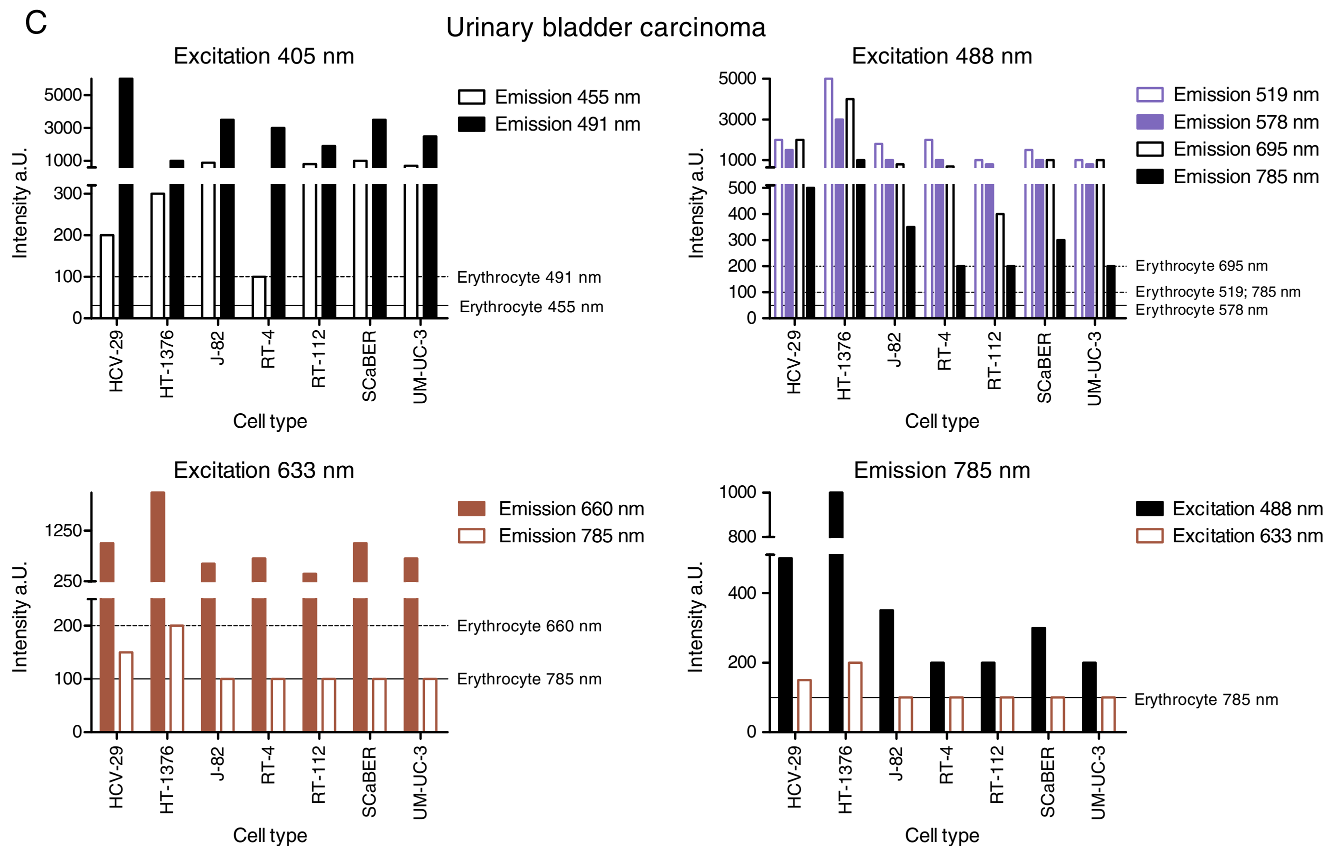

Fig. 1 (continued)

Emission spectra (525–792 nm and 707–792 nm) showed also a high autofluorescence with an excitation wavelength at 488 and 633 nm (data not shown). The spectrofluorometric measurements resulted in emission spectrum of Caki-2 cells of high signal intensity between 500 and 600 nm with an excitation wavelength at 405 and 488 nm. The excitation at 633 nm caused no signal in the emission spectrum, Fig. 3. The breast carcinoma cell line MCF7 developed clear lower signal intensity than Caki-2 cells. The reference platelet cells developed the lowest fluorescence signal in comparison to both carcinoma cell types.

Discussion

The autofluorescence is a fluorescent signal generated by the cells themselves without any specific labeling. Inherent fluorescence is evoked by high concentrations of fluorophores. The responsibility for this autofluorescence in living tissues is caused by intracellular molecules such as elastin, collagen, tryptophan, flavins and porphyrins. Köhler and Fromter [18] identified mitochondria-rich cells as source of autofluorescence. Cancer cells have a very high energy demand due to their high metabolic activity resulting in a general high content of NADH and FADH. The excitation maxima of NADH and flavins are located at the near ultraviolet and blue spectral range, respectively. Especially flavins with wide range emission spectra contribute to background fluorescence. Native cellular fluorescence characteristics are used to differentiate between normal and malignant tissue in colon, lung, cervix, esophagus, skin, urinary bladder and head-neck carcinoma [19–21]. The autofluorescence bronchoscopy is a practical way of identifying early stage lung cancer [22, 23]. Bellini [24] showed with renal cell carcinoma bearing mice that an increased fluorescence at ~635 nm correlated with tumor areas while normal kidney samples did not show any characteristic fluorescence band.

Our results show that the natural autofluorescence of urological tumor tissues exists also in permanent cell cultures

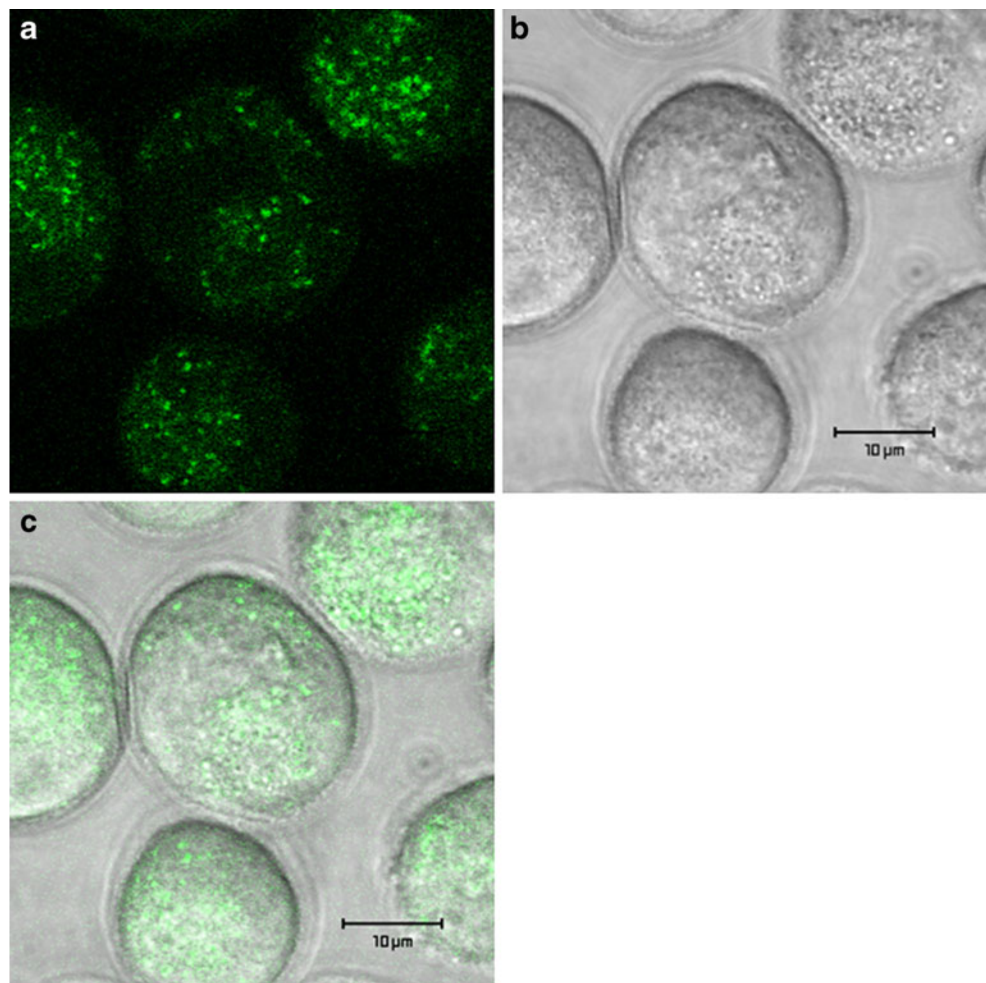

Fig. 2 Confocal laser scanning microscope micrograph of Caki-2 cells. (**a**) Fluorescence image recorded at 488 nm excitation and LP505 emission filter, (**b**) Image in transmission mode and (**c**) an overlay of (**a**) and (**b**)

of these tissues. On the one hand, significant differences between normal and tumor-bearing tissues in autofluorescence are helpful in the clinical diagnosis of tumors. On the other hand, autofluorescence makes it problematic to perform flow cytometry analysis in permanent urological carcinoma cell lines at 488 and 405 nm excitation since this signal covers the fluorescence generated by many standard fluorescence dyes. Such dyes are for example FITC, PE, PerCP-Cy5.5, PE-Cy7, Pacific Blue or AmCyan. These dyes are emitting light at 519, 578, 695, 785, 452 or 491 nm. The fluorescence intensity of cells at the above mentioned emission peaks is greater than the intensity of normally used reference cells. The best result was seen at excitation wavelength at 633 nm and emission wavelength at 785 nm using, for example, the fluorochrome APC-Cy7 since the majority of cell lines (14/21) were without autofluorescence under these measurement conditions.

As changes of fluorescence intensity were observed in leukocytes following staining and fixation [25], we also investigated the autofluorescence intensity in FA pre-treated cells. However, that procedure did not change the autofluorescence and was therefore no solution for this problem. Furthermore, the level of autofluorescence was different to permanent carcinoma cell lines from various tumor entities.

In conclusion, we have demonstrated that urological cell lines show a high autofluorescence when flow cytometry analyses are performed at the frequently used excitation wavelengths of 405 and 488 nm. At excitation wavelength of 633 nm, this problem was reduced and most of the cell lines (14/21) were without autofluorescence at the emission wavelength of 785 nm. The use of the dye APC-Cy7 would be a suitable fluorochrome for successful investigation under these measurement conditions. There is the essential conclusion that before flow cytometry analyses are performed for any tumor markers, it is necessary to check the level of autofluorescence of the cells of interest.

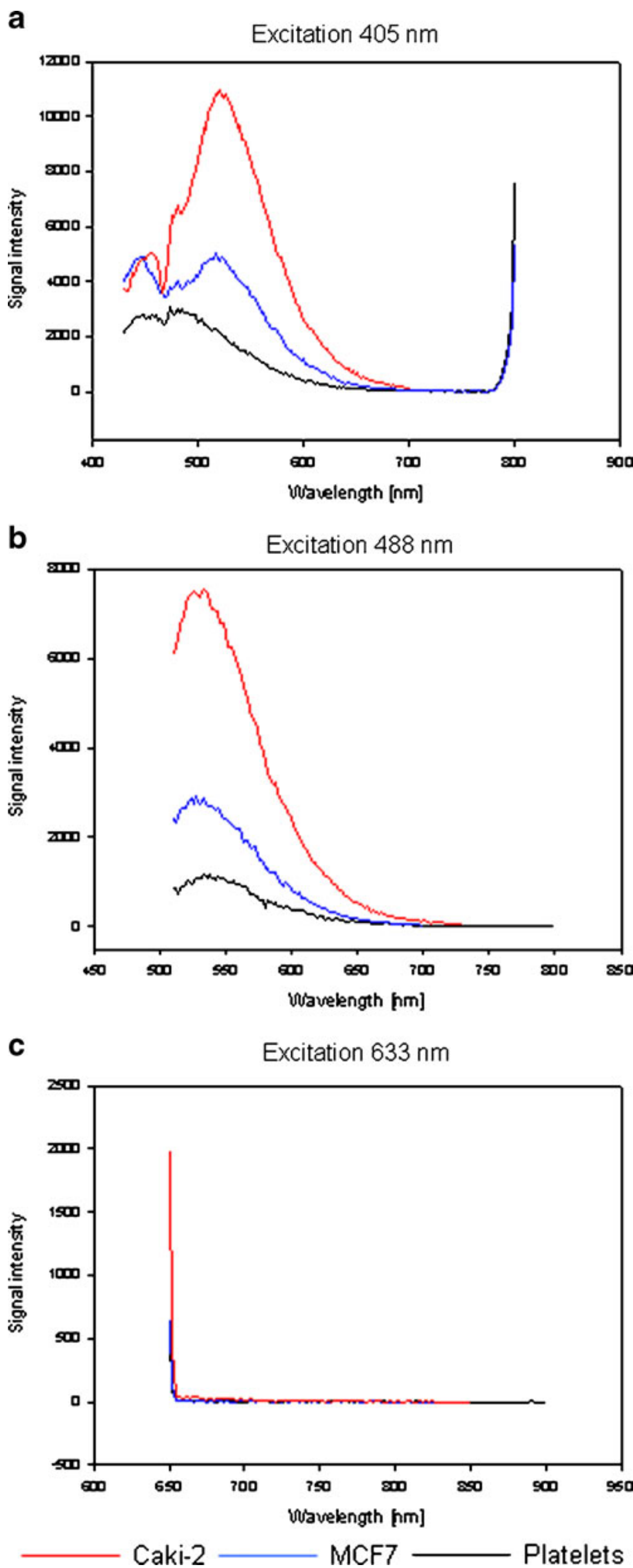

Fig. 3 Emission spectra of carcinoma cell lines Caki-2 and MCF7 in comparison to platelet cells. The measurements were performed at the excitation wavelength of (**a**) 405 nm, (**b**) 488 nm and (**c**) 633 nm using spectrofluorometer "SPEX fluorolog II"

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References

1. Zola H (2000) Immunological applications of flow cytometry. J Immunol Methods 243(1–2):1–2
2. De Rosa SC, Brenchley JM, Roederer M (2003) Beyond six colors: a new era in flow cytometry. Nat Med 9(1):112–117
3. Chattopadhyay PK, Yu J, Roederer M (2005) A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 11(10):1113–1117
4. Jennings CD, Foon KA (1997) Recent advances in flow cytometry: application to the diagnosis of hematologic malignancy. Blood 90(8):2863–2892
5. Gagner JP, Shamamian P (2007) Antigen expression profile in circulating endothelial progenitor cells. Nat Rev Cancer 7, doi:10.1038/nrc1971-cl
6. Moloney M, Shreffler WG (2008) Basic science for the practicing physician: flow cytometry and cell sorting. Ann Allergy Asthma & Immu 101(5):544–549
7. Harris DM, Werkhaven J (1987) Endogenous porphyrin fluorescence in tumors. Lasers Surg Med 7(6):467–472
8. Richards-Kortum R, Sevick-Muraca E (1996) Quantitative optical spectroscopy for tissue diagnosis. Annu Rev Phys Chem 47:555–606
9. Fryen A, Glanz H, Lohmann W, Dreyer T, Bohle RM (1997) Significance of autofluorescence for the optical demarcation of field cancerisation in the upper aerodigestive tract. Acta Otolaryngol 117(2):316–319
10. Kriegmair M, Zaak D, Knuechel R, Baumgartner R, Hofstetter A (1999) Photodynamic cystoscopy for detection of bladder tumors. Semin Laparosc Surg 6(2):100–103
11. Zaak D, Hungerhuber E, Schneede P, Stepp H, Frimberger D, Corvin S, Schmeller N, Kriegmair M, Hofstetter A, Knuechel R (2002) Role of 5-aminolevulinic acid in the detection of urothelial premalignant lesions. Cancer 95(6):1234–1238
12. Owens MA, Loken MR (1995) Flow cytometry principles for clinical laboratory practice. Wiley-Liss, Inc., New York
13. Baumler H, Neu B, Mitlohner R, Georgieva R, Meiselman HJ, Kiesewetter H (2001) Electrophoretic and aggregation behavior of bovine, horse and human red blood cells in plasma and in polymer solutions. Biorheology 38(1):39–51
14. Heck M, Schadel SA, Maretzki D, Bartl FJ, Ritter E, Palczewski K, Hofmann KP (2003) Signaling states of rhodopsin. Formation of the storage form, metarhodopsin III, from active metarhodopsin II. J Biol Chem 278(5):3162–3169
15. Schadel SA, Heck M, Maretzki D, Filipek S, Teller DC, Palczewski K, Hofmann KP (2003) Ligand channeling within a G-protein-coupled receptor. The entry and exit of retinals in native opsin. J Biol Chem 278(27):24896–24903
16. Zigrino P, Mauch C, Fox JW, Nischt R (2005) Adam-9 expression and regulation in human skin melanoma and melanoma cell lines. Int J Cancer 116(6):853–859
17. Stoya G, Klemm A, Baumann E, Vogelsang H, Ott U, Linss W, Stein G (2002) Determination of autofluorescence of red blood cells (RbCs) in uremic patients as a marker of oxidative damage. Clin Nephrol 58(3):198–204
18. Kohler M, Fromter E (1985) Identification of mitochondria-rich cells in unstained vital preparations of epithelia by autofluorescence. Pflugers Arch 403(1):47–49

19. Schantz SP, Savage HE, Sacks P, Alfano RR (1997) Native cellular fluorescence and its application to cancer prevention. Environ Health Perspect 105(Suppl 4):941–944
20. Klahr N (2003) Protoporphyrin IX Fluoreszenz nach topischer 5-Amonolävulinsäure (ALA)-Applikation: klinische Evaluation eines neuen Diagnoseverfahrens für Kopf-Hals-Karzinome. Philipps-Universität, Marburg.
21. Lippert BM, Gross U, Klein M, Kulkens C, Klahr N, Brossmann P, Teymoortash A, Ney M, Doss MO, Werner JA (2003) Excretion measurement of porphyrins and their precursors after topical administration of 5-aminolaevulinic acid for fluorescence endoscopy in head and neck cancer. Res Commun Mol Pathol Pharmacol 113–114:75–85
22. Lam S, Standish B, Baldwin C, McWilliams A, leRiche J, Gazdar A, Vitkin AI, Yang V, Ikeda N, MacAulay C (2008) In vivo optical coherence tomography imaging of preinvasive bronchial lesions. Clin Cancer Res 14(7):2006–2011
23. Lam B, Lam SY, Wong MP, Ooi CG, Fong DY, Lam DC, Lai AY, Tam CM, Pang CB, Ip MS, Lam WK (2009) Sputum cytology examination followed by autofluorescence bronchoscopy: a practical way of identifying early stage lung cancer in central airway. Lung Cancer 64(3):289–294
24. Bellini MH, Coutinho EL, Courrol LC, Rodrigues de Oliveira SF, Vieira Junior ND, Schor N (2008) Correlation between autofluorescence intensity and tumor area in mice bearing renal cell carcinoma. J Fluoresc 18(6):1163–1168
25. Stewart JC, Villasmil ML, Frampton MW (2007) Changes in fluorescence intensity of selected leukocyte surface markers following fixation. Cytometry A 71(6):379–385