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Fluorescence spectroscopy for detection of malignancy: *H-ras* overexpressing fibroblasts as a model

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Abstract

Autofluorescence from intracellular chromophores upon illumination of cells by monochromatic light has been studied towards the development of novel noninvasive and sensitive technology for the early detection of cancer. To investigate the relationship between biochemical and morphological changes underlying malignant disease and resulting fluorescence spectra, an in vitro model system of a paired normal and malignant murine fibroblasts cell lines, differing in cancer-associated *H-ras* expression was employed. A comparison of fluorescence excitation and emission spectra of proliferative cells revealed that fluorescence intensity of malignant cells was significantly less than that of normal cells upon excitation at 290 nm. Fluorescence of both cell lines decreased with decreasing cell concentration, but at each concentration, normal cells had higher fluorescence intensity than malignant cells. Similar differences between the cell lines were observed when brought to quiescence or at stationary phase. Results suggested that the chromophore contributing most significantly to these spectra is tryptophan and its moieties in proteins. This model system demonstrates the specific contribution of *H-ras* to subcellular chromophores, resulting in a significant difference in their autofluorescence intensity, and implies the potential use of the technique for cancer detection. This model system is potent for analysis of the

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contribution of other oncogenes and their combinations towards spectral detection of cancer.

Keywords: Endogenous fluorescence; Optical diagnosis; Cancer; Fibroblasts; *H-ras*

1. Introduction

Early tumor localization has a major effect on the tumor treatment procedures and the prognosis of the patients. In recent years, ultraviolet and visible spectroscopy has been studied towards the development of novel, noninvasive and sensitive real-time technologies that could identify malignancy in situ [1]. Among these methods, the use of autofluorescence for early detection of cancer relies on the ability to differentiate between normal and malignant cells and tissues, based on endogenous fluorescence from intracellular chromophores upon illumination of cells by a monochromatic light source [2–7].

Many studies employed laser-induced fluorescence (LIF) parallel to conventional means to detect cancer in vivo. These included in vivo scanning of various tissue and body areas, analysis of dissected suspected malignant tissues, and various malignant cell lines [1–13]. Occasionally, results were compared with histopathology. LIF was studied by applying various sources and wavelengths ranging from the UV region (280 nm) to visible light. Excitation and emission wavelengths scanned were in conjunction with the accumulated information about endogenous tissue fluorescent chromophores. Among these, NADH, elastin, collagen, flavins, and aromatic amino acids [8,14–17]. Other expected difficulties originated from absorption of light by nonfluorescent chromophores, such as hemoglobin [1]. In addition, to augment native fluorescence, exogenous photosensitizers tending to concentrate in malignant tissues were administered to patients [3,4].

Diagnostic algorithms were developed, which could classify tissue as normal or diseased, based on fluorescence emission spectra grasped from empirical in vivo studies [18]. However, the underlying biochemical and morphological changes that occur in disease are as yet partially understood, and relevance of tissue properties to the resulting spectra has not been fully utilized to optimize the diagnostic potential of fluorescent spectroscopy. This goal was approached by investigating in vitro models such as frozen–thawed tissue sections (e.g. Ref. [19]), or by using short-term cultures such as cervical normal tissue [13], and cervical epithelial cancer cells [10]. Among the limitations of these model systems are the lack of blood perfusion in both types, loss of metabolic chromophores such as NADH and flavins in frozen sections [7], and lack of appropriate genetic resemblance in the cultured cells [13].

The goal of this study was to employ an alternative, well defined in vitro model of normal and malignant murine fibroblasts cell lines, differing in the level of expression of the cancer-associated *H-ras* oncogene [20–22]. Overexpression of *ras* and *ras* mutations have been described in various human tumors. The highest incidences were found in oral or head and neck squamous cell carcinoma, lung cancer, colon cancer, thyroid tumors, myeloid leukemia and in pancreatic carcinoma [23–27], and as well as in preceding the onset of neoplasia in experimental models of carcinogenesis [28], suggest-

ing its early role in neoplasia. In this model system, LIF excitation and emission spectra were characterized and parameters that affect detection of malignancy such as the cell concentration, cellular viability and proliferative activity were determined. The results strengthen the power of LIF to differentiate between normal and abnormal cells, and offer a potent model system to further study spectral detection of malignancy.

2. Materials and methods

2.1. Cell lines

Balb/c 3T3 murine fibroblasts were transfected with the 6.6-Kbp BamHI fragment containing *H-ras* oncogene from the human T24 bladder carcinoma [29], cloned into the BamHI site of selectable plasmid pSV2neo [20]. The resultant clone 98/6 showed a high tumorigenicity in Balb/c mice, and a transformed morphology in vitro, and is designated as "malignant". Control cells (98/1) that were transfected only with the vector plasmid were not tumorigenic and showed normal morphology in vitro, and are designated as "normal" [20,22].

2.2. Growth conditions and assurance of cell physiology

Cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), glutamine (2 mM), and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml, and gentamycin, 50 μg/ml). Growth conditions were 37 C, 95% humidity and 8% CO_2.

Cells were maintained at pre-confluence and their morphology was routinely inspected. Growth curves of the two cell lines were repeatedly determined, using proliferation parameters such as total and viable cell number determined microscopically. Average protein content and chromosomal content of proliferating cells was determined using the BioRad kit, and chromosomal analysis was analyzed using standard cytological methods at the Institute of Genetics, Soroka University Medical Center. For cell cycle analysis, and assurance of quiescence, cells were collected following FCS starvation, washed, fixed with ethanol 70%, and analyzed following propidium iodide staining, as described by Morasca and Erba [30], using a Coulter flow cytometer (model EPICS XL-MCL; Coulter, Miami, FL) and System II program.

Spectral analysis of proliferating cells was performed with pre-confluent cells (80% coverage of the tissue culture dishes). Spectral analysis of stationary phase cells was determined 48 h post confluence. Cells were brought to quiescence by 48-h starvation with 0.01% FCS (98/6) or 0.025% FCS (98/1), beginning 48 h following seeding.

2.3. Light-induced fluorescence (LIF)

For LIF analysis, cells were collected from the plates by trypsinization, at the desired stage of growth, washed with ice-cold phosphate buffered saline (PBS), and suspended in PBS at the desired concentration. Viability was determined using the standard dye exclusion method. Spectral analysis was performed, while stirring the cells, within less than 3 h after they were harvested. To eliminate quenching or artifacts, growth medium

and trypsin lacking phenol red and antibiotics were used in the final stages of preparation.

Excitation and emission spectra were measured using SLM-Aminco spectrofluorometer. The instrument consists of a 175-W ozone-free xenon lamp, two monochromators with concave holographic gratings for excitation and emission, and a photomultiplier tube under computer control. A beam splitter at the excitation path reflects about 4% of the incident light, to be measured as a reference channel. The resultant fluorescence was in arbitrary units (AU).

Measurements were made in 1-nm increments over the range of excitation from 200 nm, and emission intensity was measured each 1 nm up to 500 nm, with band pass set to 4 mm. Preliminary experiments revealed that maximal fluorescence was emitted at 330 nm. This corresponded to application of excitation at 280–300 nm. Accordingly, excitation and emission spectra were determined for fibroblast suspensions while in PBS, with excitation wavelengths of 290 nm, and maximal emission of 330 nm. Excitation spectra were then scanned from 200 to 310 nm, and emission spectra were scanned from 300 to 450 nm. Comparison of cell cultures was performed within each session of measurements and under identical instrument settings. Coefficient of variation of repeated scans of the same sample ranged from 0.3% to 4%.

3. Results

3.1. Effect of H-ras on fluorescence spectra of proliferating cells

Maximal emission of autofluorescence of malignant 98/6 and normal 98/1 cell lines was observed at 330 nm following excitation of cell suspensions at wavelengths of 290 nm. Comparison of excitation spectra (scanned from 200 to 310 nm; e.g. Fig. 1), and emission spectra (scanned from 300 to 450 nm; e.g. Fig. 2) revealed that malignant cells

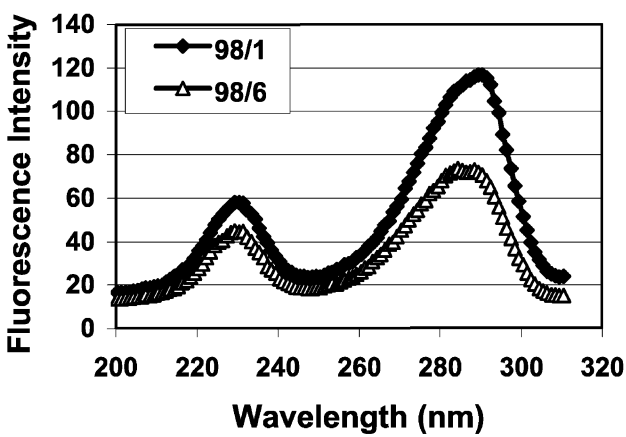

Fig. 1. Effect of *H-ras* on excitation spectra of proliferating cells. Proliferating normal 98/1 and malignant 98/6 cells were harvested from tissue culture plates, suspended in PBS and were measured at emission wavelength of 330 nm while stirring. Excitation spectra were scanned using SLM-Aminco spectrofluorimeter as detailed in Section 2. Depicted are spectra obtained for 500,000 cells/ml.

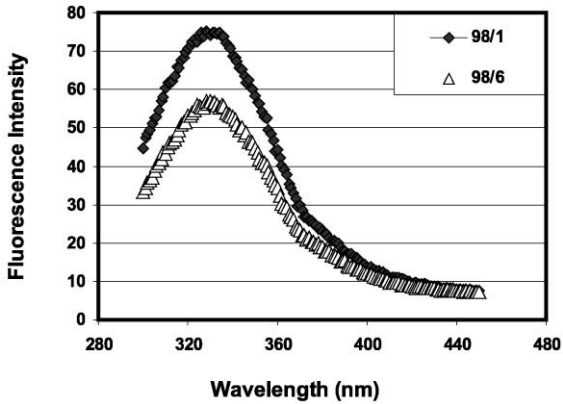

Fig. 2. Effect of *H-ras* on emission spectra of proliferating cells. Proliferating normal 98/1 and malignant 98/6 cells were harvested and treated as described for Fig. 1, and their emission spectra were recorded following excitation at 290 nm. Depicted are spectra obtained for 500,000 cells/ml.

(98/6) had a lower fluorescence intensity than normal cells (98/1). In an attempt to quantify these spectra, comparison was performed within experiments obtained on the same day under the same setting of the spectrofluorometer, while examining a constant cell concentration (5×10^5/ml), at a comparable proliferative physiological state. In a summary of 10 independent experiments (Table 1), for both cell lines, excitation spectra comprised of two peaks: one with maximal fluorescence intensity at a shorter wavelength of 229–230 nm ("S peak"), and one with a higher maximal intensity at a longer wavelength of 285–288 nm ("L peak"). The ratio of maximal fluorescence intensity at the L/S peaks was 2.17 ± 0.6 for 98/1 (ranging from 1.35 to 3.7) and was significantly lower for 98/6 (1.70 ± 0.44, ranging from 1.15 to 2.54; $p < 0.025$). Between the cell lines, maximal fluorescence intensity in the L peak was higher for 98/1 than for 98/6 (by 1.43 ± 0.27-fold; average of 10 experiments), while it was higher by only 1.12 ± 0.10-fold for the S peak. A similar difference between the cell lines was obtained for emission spectra in these experiments (Table 1). A higher maximal emission fluores-

Table 1
Effect of *H-ras* on excitation and emission spectra of proliferating cells

Cell line	Parameter	Excitation spectra	Emission spectra
98/1	S peak wavelength (nm)	229.8 ± 0.9	332.8 ± 2.0
	L peak wavelength (nm)	288.6 ± 2.1	
	relative fluorescence (L/S)	2.17 ± 0.63	
98/6	S peak wavelength (nm)	229.0 ± 0.9	332.8 ± 3.4
	L peak wavelength (nm)	285.2 ± 2.9	
	relative fluorescence (L/S)	1.70 ± 0.44	
98/1 vs. 98/6	fluorescence in S peak	1.12 ± 0.10	1.42 ± 0.27
	fluorescence in L peak	1.43 ± 0.27	

The table depicts results of 10 independent experiments. S peak = peak of fluorescence at shorter wavelength (nm). L peak = peak of fluorescence at longer wavelength.

cence intensity was recorded for 98/1 than for 98/6 (by 1.42 ± 0.27-fold) at the same wavelength (332.8 ± 2.0 nm for 98/1; 332.8 ± 3.4 for 98/6), following excitation at 290 nm.

3.2. Effect of cell concentration on fluorescence spectra

An important factor in the early detection of cancer is tumor size. In analogy, in the current in vitro model system, excitation and emission spectra were compared for cell concentrations ranging from 0.2×10^6 to 2×10^6 cells/ml. For both cell lines, fluorescence of either spectrum decreased with decreasing cell concentration, but at each concentration, fluorescence intensity of the normal 98/1 cells was higher than the malignant 98/6 cells. Plotting the maximal excitation fluorescence intensity (L peak) vs. cell concentration revealed a strain-specific linearity (Fig. 3). A similar linearity was obtained when plotting the maximal emission fluorescence intensity vs. cell concentration (Fig. 4).

Repeating experiments varied in fluorescence intensity, but the finding that fluorescence intensity of the malignant cells was significantly less than normal cells remained the same. Notably, the maximal fluorescence of the excitation scans was red-shifted gradually for both cell types, with increasing cell concentration. For example, the fluorescence peak of the excitation spectra following excitation of 2×10^5 cells/ml was recorded at 288 nm, while that of 2×10^6 cells/ml was shifted to 293 nm. This shift in wavelength is believed to be due to multiple light scattering from cells, arising from Rayleigh scattering.

3.3. Effect of physiological state on fluorescence spectra

An important issue in optical biopsy is conceivably the ability to detect malignant cells at their early stage, or even at quiescence [9]. Thus, further parameters studied were

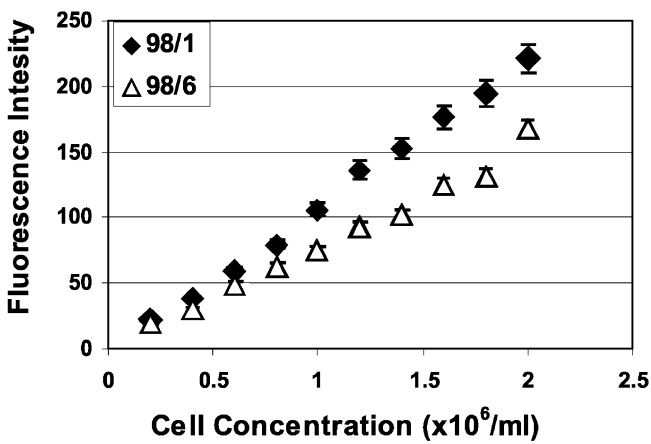

Fig. 3. Effect of cell concentration on excitation spectra of normal and malignant cells. Excitation spectra were determined as described for Fig. 1 for proliferating cells suspended at concentrations varying from 200,000 to 2,000,000 cells/ml. Depicted are the average maximal values of fluorescence intensities at the peak of longer wavelength (L peak), obtained within one experiment with standard deviations of repeated scanning.

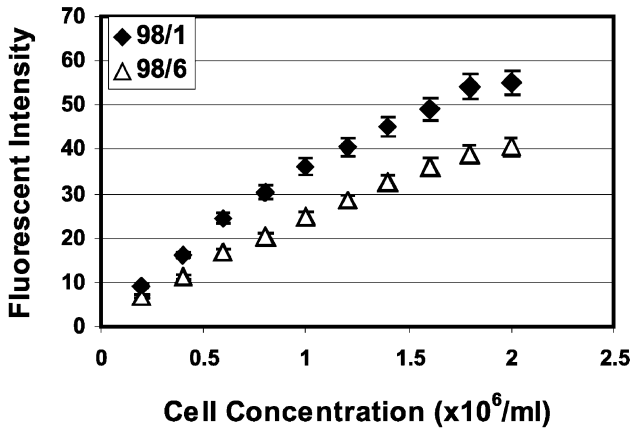

Fig. 4. Effect of cell concentration on emission spectra of normal and malignant cells. Emission spectra were determined as described for Fig. 2 for proliferating cells suspended at concentrations varying from 200,000 to 2,000,000 cells/ml. Depicted are the average maximal values of fluorescence intensities at the peak wavelength (330 nm), obtained within one experiment with standard deviations of repeated scanning.

the effects of cellular viability and the physiological state of the cells. Stationary phase cells, which stopped dividing and were 2 days post-confluence, and cells brought to quiescence by prolonged FCS starvation were compared to their proliferative controls. Excitation and emission spectra of each cell line were comparable among all physiological states. This is reflected in the ratio between maximal fluorescence at excitation (Table 2). Thus, the fluorescence intensity of normal 98/1 cells was higher than for malignant 98/6 upon excitation, irrespective of the physiological state.

The effect of cellular viability on excitation spectra was determined during prolonged measurement sessions of 1.5–4 h after harvesting. Viability of normal 98/1 decreased within this period more than malignant 98/6, resulting in 14% vs. 4% dead cells, respectively. Maximal fluorescence intensity of excitation spectra decreased by 18.5% for 98/1, and by 2% for 98/6, thus reflecting the change in viable cells in suspension. However, the ratio between maximal fluorescence at the L peaks of 98/1 to 98/6 was 1.57 ± 0.1, similar to the ratio for proliferating viable cells.

Table 2
Effect of *H-ras* on excitation and emission spectra of resting cells

Cell line and physiological state	Relative maximal fluorescence intensity at	
	Excitation spectra	Emission spectra
Stationary vs. proliferative 98/1	1.05 ± 0.11	1.07 ± 0.10
Stationary vs. proliferative 98/6	1.00 ± 0.07	1.14 ± 0.12
Quiescent vs. proliferative 98/1	0.96 ± 0.01	0.97 ± 0.20
Quiescent vs. proliferative 98/6	0.98 ± 0.04	0.93 ± 0.05

The table summarizes results of three independent experiments of each physiological state. Depicted are the average ratios ± standard deviations of maximal fluorescence of resting cells vs. their proliferative controls, derived from the prominent peak at longer wavelength of excitation spectra and the emission spectra.

Taken together, the results suggest that autofluorescence of malignant cells, harboring *H-ras* originating from a human sarcoma, may be differentiated spectrally from that of their normal controls under a large variety of conditions.

3.4. Putative endogenous fluorophores

In this model system, maximal emission fluorescence was at 330 nm, corresponding to the application of excitation at wavelengths ranging from 280 to 300 nm. This observation led to the hypothesis that tryptophan, with a maximal excitation at 280 nm and emission at 350 nm, may be the putative endogenous fluorophore (i.e. fluorescing chromophore), which contributes the most to these spectral differentiations, as suggested in other model systems [15–17]. Examination of excitation and emission spectra of tryptophan, scanned in the spectrofluorimeter under the same conditions as the cell suspensions, revealed linearity between maximal fluorescent intensity of both excitation (L peak) and emission (Fig. 5). While tryptophan is the fluorophore that best matches the excitation/emission spectra derived in this study, it is probable that the spectra obtained for the cells are a summation of the contribution of other fluorophores as well [6,14–17].

4. Discussion

The goal of this study was to examine in vitro the possible application of spectral noninvasive techniques to differentiate between malignant and normal cells, using a well-defined paired murine fibroblast, differing in the level of expression of the cancer-associated *H-ras* oncogene [20–22]. Specifically, endogenous fluorescence upon excitation at 290 nm and emission at 330 nm, as well as their excitation and emission spectra were determined. A large variety of experimental conditions were tested, including variations in cell concentration, in physiological conditions (proliferative, stationary and quiescent), and in viability. Under all conditions, fluorescence intensity of the normal 98/1 cell line was higher than the malignant 98/6, harboring *H-ras* from human bladder carcinoma. In analogy to these experiments, it is envisioned that the malignant cells may be detected when embedded as small clusters in the tissue, and even at quiescence, or at an early stage of cancer. This will be due to their autofluorescence which differs from that of the surrounding normal cells. Our concurrent finding that these cells may be differentiated at the single cell level, based on microscopic FTIR analysis (Jagannathan et al., in press, [31]) further substantiate the potency of noninvasive spectral technologies for early detection of malignancy.

The described significant differences between the cell lines, with normal cells having a higher fluorescent intensity than malignant cells, are comparable in general to reports of in vitro-induced fluorescence in other cell types, in dissected tumors and tissues, as well as in vivo. The differences among these reports are in the extents of relative fluorescence between healthy and malignant preparations, ranging from 2- to 10-folds, and the varying selection of excitation/emission wavelength ranges, type of cells, and other experimental conditions [1–17]. These variations also led to different conclusions

considering the identity of fluorophores contributing to the spectral characteristics of the examined preparations. However, none employed cells that were genetically identical—an important issue that is encountered in vivo in variations of the extent of spectral differences between healthy and malignant tissues among patients (summarized in Ref. [1]). Unique to this study is the use of the paired transfectants, constructed to differ genetically only in one gene, *H-ras*, a key enzyme in cancer-associated gene.

Important for the application of this model system for the generation of detection, analysis and treatment algorithms is the volume of data related to the biochemical and morphological characteristics of these paired transfectants, and the ability to relate these to the *H-ras* gene and its tumorigenicity. *H-ras* transfection affected 98/6 cells in many aspects, including differential expression of many genes. The cell cycle of malignant 98/6 cells is shorter than that of the normal cells (22.5 vs. 32 h, respectively), and they are more resistant to serum starvation when brought to quiescence. 98/6 cells tend to spread less on tissue culture plates, suggesting a differential expression of adhesion molecules [20,22]. They express H-2K, a major histocompatibility complex type 1 molecule, much less than normal 98/1—an effect that is related to the ability of these cells to escape immune response and to their tumorigenicity [22]. 98/6 cells also express less EGF receptor molecules on their surface and do not respond to its mitogenic effect relative to 98/1 [21]. This was related to a differential expression and activity of the transcription factor AP-1 [32]. Finally, in a recent parallel study of these cell lines, using infrared (IR) absorption spectra, we have found that they also differ in the phosphate content and in the level of phospholipids. Both metabolites were higher for normal cells (Jagannathan et al., [31]).

As yet, it is difficult to isolate, quantify, and assign the contribution of each of these molecular factors to the spectral behavior of the cells. However, complementary findings

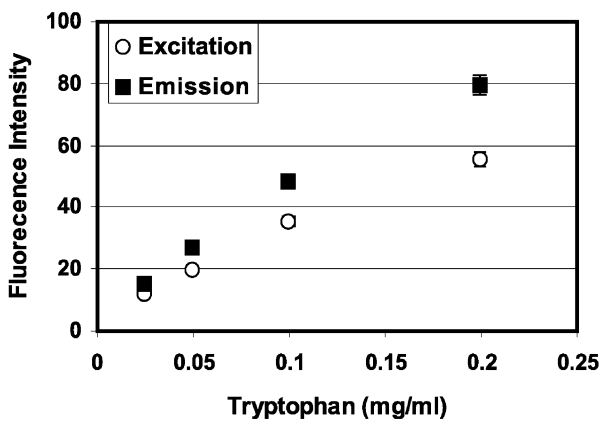

Fig. 5. Linearity of maximal fluorescence intensity of excitation and emission spectra with various tryptophan concentrations. Excitation and emission spectra of tryptophan, at various concentrations while in PBS, were scanned in the spectrofluorimeter under the same conditions as the cell suspensions. Depicted are the maximal values of fluorescence intensity at the longer wavelength peak (L peak) of excitation spectra and at the emission spectra.

might allude to the contribution of tryptophan and hypothetically to tryptophan in EGF receptor to autofluorescence in this model system. First, tryptophan residues in the extracellular domain of the EGF receptor were shown to be involved in EGF binding using circular dichroism and fluorescence spectroscopic techniques [33,34]. Second, 98/6 cells were shown previously to express less EGF receptor molecules on their surface [21], and now were shown to fluoresce less, being a system which was also sensitive to tryptophan concentrations (Fig. 5).

This study yields that the approach of using genetically engineered paired transfectants differing in specific cancer-related gene(s) is attractive and is expected to be powerful in investigating new detection technologies for the detection and treatment of cancer. Future model systems that may be developed in this line are the application of tumors in vivo by injecting these cell lines, or assay spectral differences between inducible transfectants, expressing or not expressing the *ras* protein. The multidisciplinary collaboration of biologists and physicists is instrumental in tailoring the model system and future application of spectral analysis.

5. Conclusions

The model system employed in this study demonstrates the specific contribution of *H-ras* to subcellular chromophores that yield a spectral differentiation between normal and malignant cell lines under various physiological conditions and commends its further application for investigation of optical biopsy and diagnosis. The results suggest that tryptophan is among endogenous fluorophores enabling this differentiation. This study indicates the potential use of the LIF technique to distinguish between normal and malignant fibroblasts based on the autofluorescence intensity using both emission scan and excitation scan measurements.

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References

[1] Bigio IJ, Mourant JR. Phys Med Biol 1997;42:803–14.
[2] Alfano RR, Das BB, Cleary J, Prudente R, Celmer EJ. Bull N Y Acad Med 1991;67:143–50.
[3] Andersson-Engles S, Elner A, Johanson J, Karlsson S-E, Salford LG, Stormbald L-G, et al. Lasers Med Sci 1989;6:415–24.
[4] Andersson-Engles S, Canti G, Cubeddu R, Ecker C, af Klinteberg C, Pifferi A, et al. Lasers Surg Med 2000;26:76–82.
[5] Svanberg K, Andersson-Engels S, Baert L, Bak-Jensen E, Berg R, Brun A, et al. SPIE Proc 1994; 2135:2–15.

[6] Gillenwater AM, Jacobs R, Richards-Kortum R. Head Neck 1998;20:556–62.
[7] Schomacker KT, Frisoli JK, Compton CC, Flotte TJ, Richter JM, Nishioka NS, et al. Lasers Surg Med 1992;12:63–78.
[8] Bigio IJ, Loree TR, Mourant JR, Shimada T, Story-Held K, Glickman RD, et al. SPIE Proc 1993; 2081:174–84.
[9] Zhang JC, Savage HE, Sacks PG, Delohery T, Alfano RR, Katz A, et al. Lasers Surg Med 1997;20:319–31.
[10] Heintzelman DL, Lotan R, Richards-Kortum R. Photochem Photobiol 2000;71:327–33.
[11] Fabian H, Wessel R, Jackson M, Schwartz A, Lasch P, Fichtner I, et al. SPIE Proc 1998;3257:13–23.
[12] Jackson M, Kim K, Tetteh J, Mansfield JR, Dolenko B, Somorjai RL, et al. SPIE Proc 1998;3257:24–34.
[13] Brookner CK, Follen M, Boiko I, Galvan J, Thomsen S, Malpica A, et al. Photochem Photobiol 2000;71:730–6.
[14] Young AR. Phys Med Biol 1997;42:789–802.
[15] Tang GC, Yang YL, Huang ZZ, Hua W, Zhou F, Cosloy S, et al. SPIE Proc 1995;2387:169–72.
[16] Yang Y, Katz A, Celmer EJ, Zurawska-Szczcepaniak M, Alfano RR. Photochem Photobiol 1997;66:518–22.
[17] Brancaleon L, Lin G, Kollias N. J Invest Dermatol 1999;113:977–82.
[18] Ramanujam N, Mitchell MF, Mahadevan A, Thomsen S, Malpica A, Wright T, et al. Lasers Surg Med 1996;19:46–62.
[19] Lohmann W, Mussman J, Lohmann C, Kunzel W. Naturwissenschaften 1989;96:125–7.
[20] Tainsky MA, Shamanski FL, Blair D, Vanda Woude G. Mol Cell Biol 1987;7:1280–4.
[21] Gopas J, Ono M, Princler G, Smith MR, Tainsky MA, Siddiqui MAQ, et al. Cell Mol Biol 1992;38:605–14.
[22] Ehrlich T, Wishniak O, Isakov N, Cohen O, Segal S, Rager-Zisman B, et al. Immunol Lett 1994;39:3–8.
[23] Barbacid M. Annu Rev Biochem 1987;56:779–827.
[24] Bos JL. Cancer Res 1989;49:4682–9.
[25] Scully C, Field JK, Tanzawa H. Oral Oncol 2000;36:311–27.
[26] Tockman MS. J Cell Biochem 1996;25:177–84.
[27] Lohr M, Maisonneuve P, Lowenfels AB. Int J Pancreatol 2000;27:93–103.
[28] Kumar R, Sukumar S, Barbacid M. Science 1990;248:1101–4.
[29] Santos E, Tronick SR, Aronson A, Pulciani S, Barbacid M. Nature 1982;298:343–7.
[30] Morasca L, Erba E. In: Freshney RI, editor. Animal Cell Culture: A Practical Approach. Oxford: IRL Press 1986:125–48.
[31] Jagannathan R, Salman A, Hammody Z, Cohen B, Gopas J, Grossman N, et al. J Biochem Biophys Meth 2001, in press.
[32] Okimoto T, Kohno K, Kuwano M, Gopas J, Kung H-F, Ono M. Oncogene 1996;12:1625–33.
[33] Greenfield C, Hiles I, Waterfield MD, Federwisch M, Wollmer A, Blundell TL, et al. EMBO J 1989;20:4115–23.
[34] Martin-Fernandez ML, Clarke DT, Tobin MJ, Jones GR. Cell Mol Biol 2000;46:1103–12.