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Discrimination of Normal and Cancer Cells From Autofluorescence Spectra by Laser Excitation

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Photodynamic diagnosis and therapy (PDD & PDT) of cancer use generally photosensitizers for the discrimination of normal and cancer cells. In contrast we have studied the autofluorescence *spectra of normal and cancer cells of rat using a tunable optical parametric oscillator (OPO) laser system as a light excitation source. WKA rat fibroblast (normal cell) and H-*ras *oncogene-transfected rat fibroblast (cancer cell) were used in this study. The samples were mounted in a quartz cuvette. The absorption peaks were observed at about 205 nm for both normal and cancer cells. On the other hand, the specific emission peaks were observed at 450 nm and 455 nm for normal and cancer cells, respectively, whereas the autofluorescence intensity of cancer cells was 2.5 times larger than that of normal cells. These changes are attributed to changes in proteins in the cancer cell and to the increase of nuclei. This suggests the possibility that the autofluorescence of cancer cell can be used to diagnose a human malignant tumor, so that it is concluded that normal and cancer cells can be discriminated by means of autofluorescence analysis.*

Keywords Autofluorescence spectrum; H-*ras* oncogene-transfected fibroblast; NADH; rat fibroblast

Abbreviations:

D-MEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HpD	hematoporphyrin derivative
NAD	nicotinamide adenine nucleotide
NADH	nicotinamide adenine dinucleotide
OPO	optical parametric oscillator
PBS	phosphoric acid buffer solution
PDD	photodynamic diagnosis
PDT	photodynamic therapy

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1. Introduction

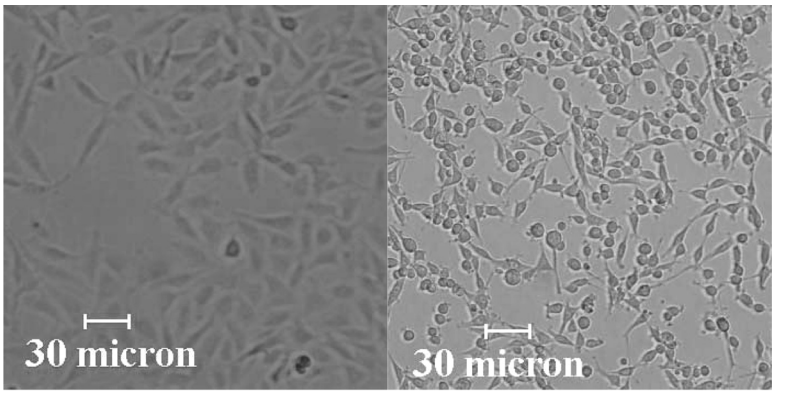
Recently in the field of cancer diagnosis and therapy the photodynamic processes, so called photodynamic diagnosis (PDD) and photodynamic therapy (PDT), have been developed rigorously. In PDD and PDT photosensitizers play an important role and the selection of photosensitizers becomes one of the key factors. The hematoporphyrin derivative (HpD) was developed by Lipson *et al*. in 1960 from the hydrochloric hematoporphyrin treated with acetic acid and sulfuric acid [1], since then the research of localized diagnosis and treatment of a tumor has been advanced enormously [2]. However the dosage of HpD causes side reactions such as photodermatosis under the daylight because its lifetime is quite long and remained in the skin for long time after dosage. NPe6 (Mono-L-aspartyl chlorin e6, ME2906) was developed as a second generation photosensitizer recently [3], and named Talaporfin by World Health Organization (WHO) in 2003. Talaporfin is approved to use to an early lung cancer (a stadium term or I term lung cancer) clinically by Ministry of Health, Labor and Welfare in Japan now.

On the other hand the fluorescence from the early stage of cancer tissus was observed under the photoirradiation of specific wavelength without the excitation of photosensitizers. This fluorescence is called *autofluorescence*. Autofluorescence studies of human tissues *in vivo* and *ex vivo* include the cervix [4], bladder [5], lung [6], breast [7], esophagus [8], aorta [9], stomach [10], uterus [11], skin [12], and colon [13]. However, the elucidation of the mechanism of indigenous fluorescence is not carried out as yet. The excitation condition to the cellular level is limited so far, and hence the ultraviolet excitations of the cells are required. In this paper, the specific fluorescence spectra of a normal cell and a cancer cell were measured using ultra-violet pulsed laser as a first step. We could find a clear difference in the fluorescence spectra between a normal cell and a cancer cell.

2. Materials and Methods

2.1. *Cells and Cell Culture*

WKA rat normal fibroblast (WFB) and H-*ras* oncogene-transfected rat fibroblast (W31) were used in this study, where WFB is a normal cell and W31 is a cancer cell (Fig. 1). Cells were cultured in the cell line of Dulbecco's modified Eagle's medium (D-MEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). Cell lines were maintained at 37°C in a 100% humidified 5% CO_2 atmosphere. It is noted that cells used in this study are not the primary but cultured many times, so that they should be called 'cell lines', but for simplicity we use 'cells' hereafter. Cells were routinely grown in 80 cm^2 collagen coated tissue culture flask. They were harvested after reaching 80–100% confluency by detachment reagent of 0.05% trypsin, pelleted for 5 min at 1500 rpm in a refrigerated centrifuge, and then resuspended in 15 ml culture medium. The number of cells was counted in a hemocytometer slide using a microscope. The density of WFB normal cells and W31 cancer cells used in the following experiments were counted as about $350(\pm 25) \times 10^5$ cell/ml. Nicotinamide adenine dinucleotide (NADH: ORIENTAL YEAST) was dissolved in the phosphoric acid buffer solution (PBS), which was used as a standard sample of fluorescence measurement. The concentration of NADH was the 2.5×10^{-3} M.



(a) (b)

Figure 1. WFB normal cells (left) and W31 rat H-*ras* oncogene-transfected fibroblast cells (right).

2.2. *Measurement Method*

The excitation light of OPO wavelength variable solid laser (wavelength: 300 to 380 nm, 450 to 650 nm, pulse width: 7 ns, and power: 150 μJ/pulse, repeat frequency: 10 Hz, VIBRANT, OPOTEK) was used for measurement of the autofluorescence spectra of WFB cells and W31 cancer cells. The autofluorescence of each cell under excitation was filtered by the bandpass filter, cutting off only excitation light. The photonic multi-channel analyzer (C7473, Hamamatsu Photonics) was used for detection of autofluorescence. The PBS solution of the cell pellet was put into the quartz cuvette, and each measurement was carried out under the conditions of a room temperature and 60% or less of humidity. Ultraviolet and visible spectrophotometer (UV-2400PC, Shimadzu) was used for absorption spectrum. The measurements were scanned in the range of 190–1100 nm.

3. Results and Discussion

As shown in Figure 1, W31 rat H-*ras* oncogene-transfected fibroblast cells (cancer cells) become smaller than the original WFB normal cells, and the morphology changes slightly by the cancerization. The scale bar is 30 micrometers. Autofluorescence spectra of WFB normal cells and W31 cancer cells under the excitation wavelength of 330, 340, 350, and 360 nm are shown in Figures 2 and 3, respectively. The autofluorescence intesity of excitation wavelength 350 nm is the highest among four excitations. Comparison of the autofluorescence spectrum under 350 nm excitation of WFB normal cells and W31 cancer cells is shown in Figure 4. Measurements of WFB normal cells and W31 cancer cells were carried out 5 times respectively. It should be noted that the maximum of autofluorescence of W31 cancer cells is located at 455 nm and is redshifted by 5 nm compared to that of WFB cells (450 nm). The autofluorescence intensity of cancer cells was 2.5 times larger than that of normal cells. These changes are attributed to changes in proteins in the cancer cell and to the increase of nuclei. It is considered that the endogenous substance which participates in fluorescence is NADH. That is the peak appeared in wavelength of 260 nm

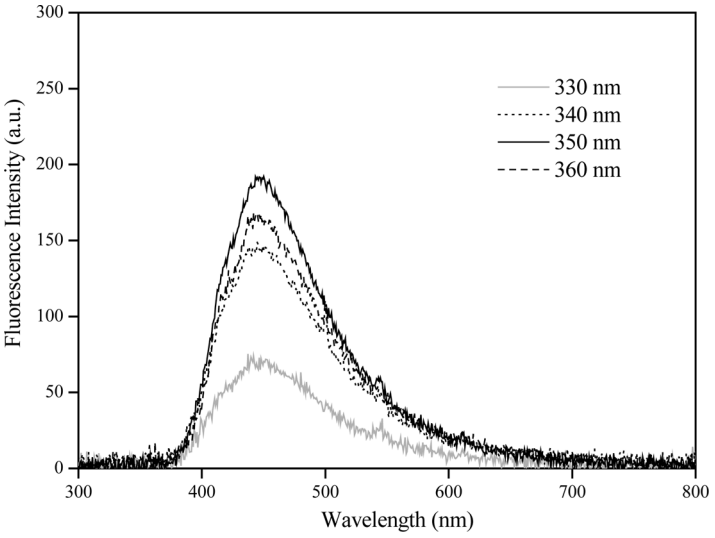


Figure 2. Autofluorescence spectra of WFB cells. The excitation wavelengths are 360 nm (broken line), 350 nm (solid line), 340 nm (dotted line), and 330 nm (gray line).

in the absorption spectrum is attributable to the nucleotide in a basic skeleton, whereas NADH absorbs the ultraviolet light near 350 nm (Fig. 5). Measurement of NADH was performed only once because reproducibility was quite good.

On the other hand, although a possibility of the red fluorescence by a porphyrin derivative in a living body tissue is strongly implied in the *in vivo* experiment, there is no existence of the autofluorescence in a red range. Therefore it is suggested that

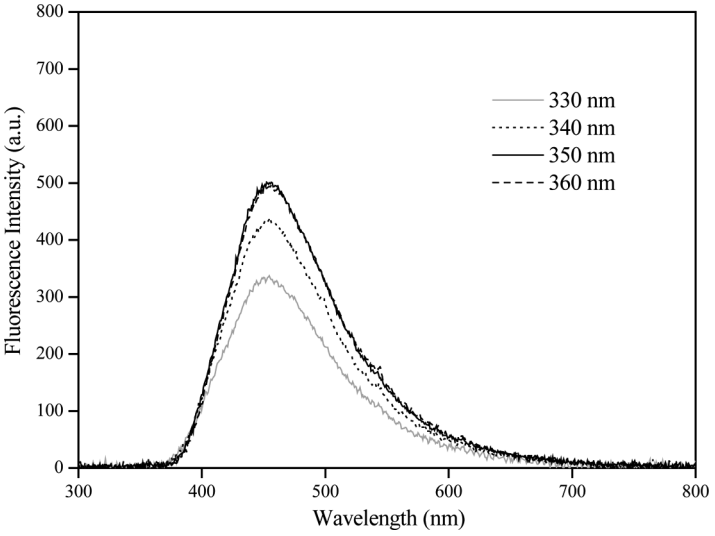


Figure 3. Autofluorescence spectra of W31 rat H-*ras* oncogene-transfected fibroblast cells. The excitation wavelengths are 360 nm (broken line), 350 nm (solid line), 340 nm (dotted line), and 330 nm (gray line).

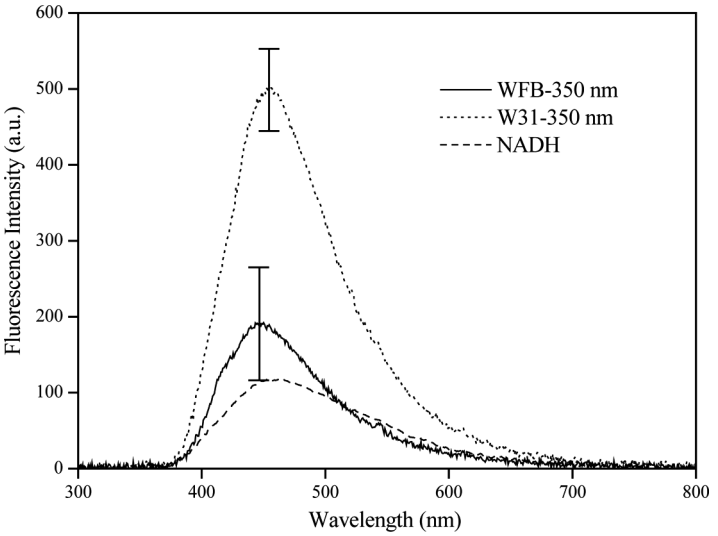


Figure 4. Autofluorescence spectra of WFB cells (dotted line) and W31 cancer cells (solid line) under the excitation at 350 nm. The broken line indicates the fluorescence of NADH.

a porphyrin derivative does not exist in a cell. Depending on the excitation wavelengths, the intensity becomes the largest at 350 nm excitation, and the autofluorescence intensity of WFB cells and W31 cancer cells determines the most suitable excitation wavelength. This is in good agreement with the absorption band of NADH. It is considered that the quantity of NADH increases by the cell cancerization and so the W31 cancer cells show the stronger autofluorescence than

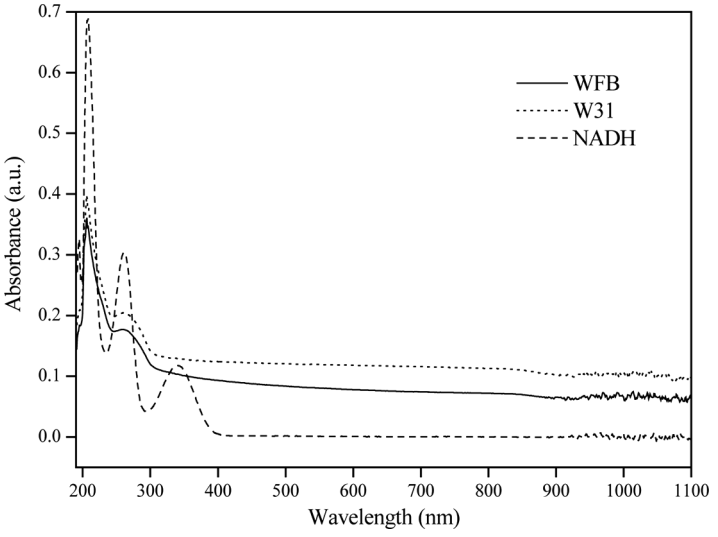


Figure 5. Absorption spectra of WFB cells (dotted line) and W31 cancer cell (solid line). The broken line indicates the absorption spectrum of NADH.

the WFB normal cells. The fibroblast in the living body produces NADH which is a reduction type of a nicotinamide adenine nucleotide (NAD) coenzyme. This tendency of autofluorescence increase is in good agreement with the case of tumor malignancy [14].

4. Conclusion

In this study, autofluorescence spectra measurement of WFB normal cells and W31 cancer cells was carried out, and the useful difference was extracted for the cancer discrimination. In the cellular level, the enhancement of autofluorescence intensity was confirmed in the tumor tissue. Since NADH is in connection with ribonucleic acid and phosphorylation protein of cellular cancerization, the increase of autofluorescence intensity in the W31 cancer cells shows a good agreement with this tendency. The autofluorescence life time of WFB normal cells and W31 cancer cells are under investigation. In future, it is necessary to solve specification of the cell organella which generates autofluorescence.

Although the ultraviolet radiation has a bad influence on a human organism when cytological diagnosis is performed out of the body, it can be used as excitation light. As for the application to the cytodiagnosis of cancer, the autofluorescence from the cells will give us a lot of information and useful in future.

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