# *In-vitro* diagnosis of human uterine malignancy using $N_2$ laser-induced autofluorescence spectroscopy

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**Results of an *in-vitro* study of $N_2$ laser excited autofluorescence from cancerous and adjoining normal human uterine tissues are presented. Discrimination functions based on multivariate linear regression analysis of the spectra could discriminate between the two tissue types with sensitivity and specificity values towards cancer of 100% when the cancerous site exhibited red fluorescence band and around 90% when cancerous site did not show red fluorescence.**

LASER-INDUCED fluorescence (LIF) from native tissues (autofluorescence) is being actively investigated for its potential use in cancer diagnostics[1]. This promising technique offers several important advantages like a very high intrinsic sensitivity, suitability for detecting small superficial tumours not detectable by other techniques such as X-ray diagnostics and the use of non-ionizing radiation which avoids the risks associated with ionizing radiation. Further, the diagnostics can be done in near real time and *in situ* without any tissue removal. Moreover, tissue diagnostics by this technique can be easily automated, facilitating use by less skilled personnel and mass screening. Extensive studies have therefore been carried out using human tissues, removed at surgery or at biopsy, and induced tumours in animals to evaluate the potential of this technique for discriminating cancerous as well as precancerous tissues from normal. The results have been very encouraging and are motivating considerable efforts to realize the *in vivo* diagnostic potential of the technique[2,3].

In this article we present results of our *in vitro* studies on $N_2$ laser excited autofluorescence from pathologically confirmed cancerous and adjoining normal human uterine tissues. A step wise multivariate linear regression (MVLR) analysis was used to quantify the observed spectral differences and form a discrimination function on the basis of which the tissue could be classified as normal or cancerous. Sensitivity and specificity values towards cancer of 100% were obtained when the cancerous site exhibited red fluorescence band and around 90% for cases where the cancerous site did not show any red fluorescence.

## Experimental procedure

Pathologically characterized full thickness tissue samples were obtained from Choithram Hospital and Research Centre, Indore, from six patients with uterine cancer, immediately after resection at surgery or at biopsy. The tissue samples were stored in ice until study. The spectroscopic experiments were performed within 4 to 24 h of tissue removal. For the experiments the specimens were thawed to room temperature, kept moist with buffered saline solution (pH 7.4) and mounted on a rectangular quartz slide for LIF studies paying attention to the orientation of the sample.

LIF spectra of the tissue samples were recorded using the experimental set-up shown in Figure 1. The excitation source used was a home-built pulsed $N_2$ laser emitting 7 ns pulses with a repetition rate of 10 Hz and a pulse energy of 200 μJ. The laser beam was coupled to a quartz optical fiber (core diameter 400 μm) via a dichroic mirror which reflected $N_2$ laser radiation and transmitted longer wavelength fluorescence output. Typical energies delivered to a tissue sample were 40–50 μJ. No sample photobleaching was observed at these energies. The power of the $N_2$ laser was monitored by a beam-splitter-photodiode combination and the fluorescence from the tissue kept in contact with the fiber was collected by the same fiber and imaged on the entrance slit of a scanning monochromator. The wavelength dispersed light at the exit slit of the monochromator was detected by a photomultiplier tube (Hamamatsu R406) detector. A microprocessor-based system developed in-house was

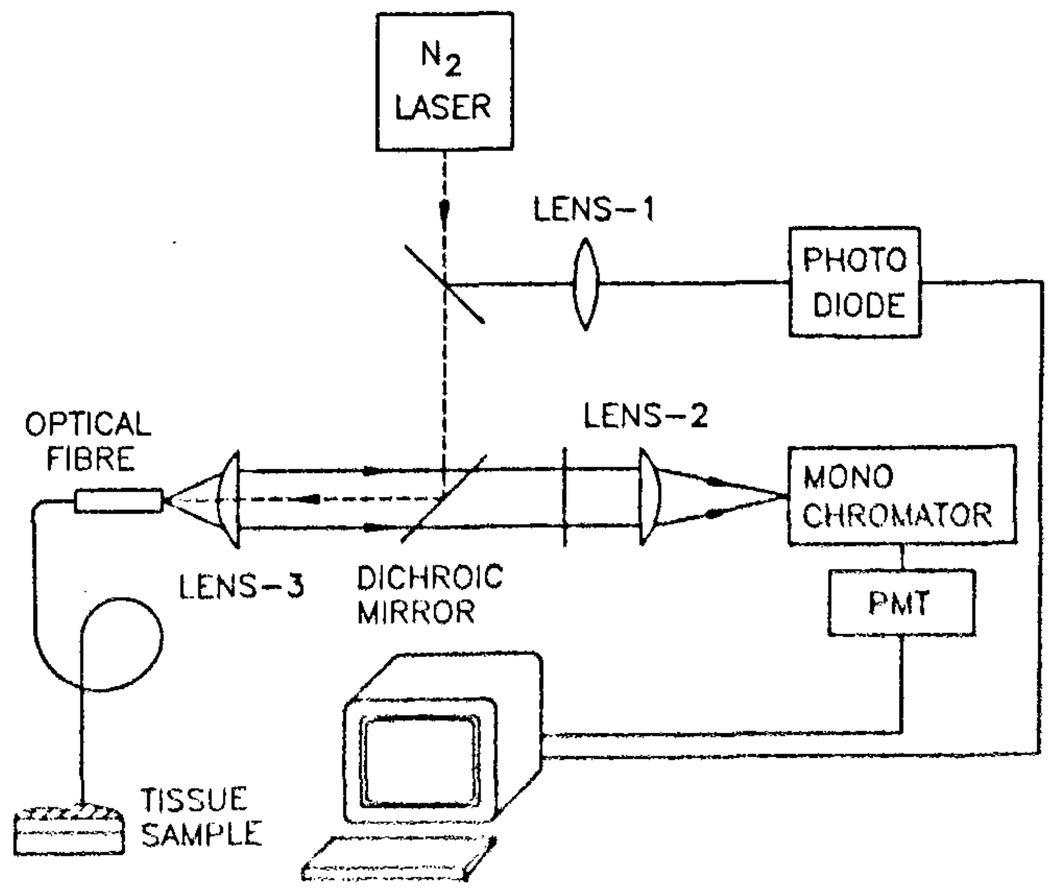


**Figure 1.** Schematic diagram of the experimental set-up for autofluorescence spectroscopy of tissues.

used for on-line monitoring of the excitation power and the fluorescence spectral data.

The fluorescence spectra were recorded from a total of 220 sites, of which 130 sites were from histopathologically confirmed malignant tissues and the remaining 90 were from surrounding normal tissues. A single spectrum was obtained per site and on an average 15–20 spectra were recorded from both the cancerous and normal part of the tissue sample from each of the six patients.

## Results and discussions

### *Autofluorescence spectra*

The mean autofluorescence spectra from the uterine tissues are shown in Figure 2 without correction for detection system spectral response. All $N_2$ laser-excited autofluorescence spectra (from both cancerous and normal uterine tissue) were characterized, in general, by two major emission bands, one relatively narrower short wavelength band peaking at about 390 nm and the other comparatively broader long wavelength band peaking around 460 nm. Some malignant tissue spectra also showed a pronounced red emission band with peak in the region 590–660 nm. This red band was not observed in any of the normal uterine tissue samples investigated by us.

The observed fluorescence spectrum is a superposition of the spectra of a number of fluorescing substances present in the tissue. The spectra are also influenced by the absorption and scattering of both the excitation light and the fluorescence emission from the tissue. The presence of blood in the tissue is known to strongly affect the recorded fluorescence spectra by its strong absorption bands in the range 380–660 nm (ref. 4). The fluorophores responsible for the 390 nm band are believed to be structural proteins (collagen and elastin). The broad 460 nm band is believed to be due to the fluorescence from the co-enzyme NADH/NADPH, pyridoxal-5-phosphate, flavins, etc. with maximum contribution from NADH[1]. The red emission band can be ascribed to the endogenous porphyrins which are selectively retained in the rapidly dividing cells and may be considered as strong indicator of malignancy[1,5]. From Figure 2, the spectral intensity distribution of the fluorescence emission from normal and cancerous tissues are seen to be different even when the red band is not present in the cancerous tissue spectra.

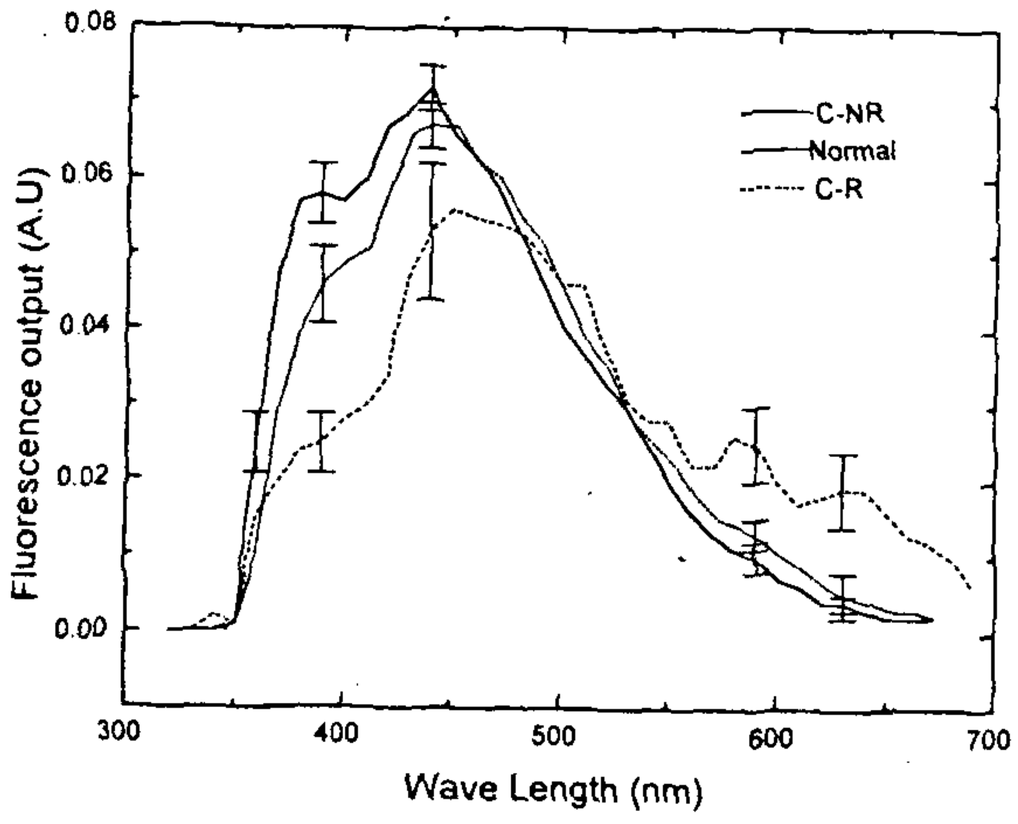


Figure 2. Mean spectra from normal (N) and cancerous sites with red fluorescence (C–R) and with no red fluorescence (C–NR). Normal and C–NR spectra are average of spectra from 45 sites, and C–R spectrum is the average of spectra from 25 sites.

These differences in the spectra of cancerous and normal tissues may arise due to biochemical or morphological changes in the tissues as they undergo transition from normal to cancerous condition. The present understanding of these is not very satisfactory. Non-cancerous mucous membrane and carcinoma usually show a variable amount of structural cell proteins which may be reflected in the fluorescence intensity of the 390 nm band[5]. Similarly the 460 m band will be strongly influenced by changes in the concentration or in the binding properties of the co-enzyme NADH which is located in the mitochondrial membrane system and is involved in numerous enzymatic reactions. The oxidation–reduction equilibrium for NADH-$NAD^+$ is believed to be different in normal and cancerous cells[4]. Since NADH is strongly fluorescent but $NAD^+$ is not, this will also contribute to a change in 460 nm band intensity. Although several researchers have reported a general decrease in blue-green fluorescence from cancerous tissues[1,3], we observe this decrease (see Figure 2) in the fluorescence from only those cancerous sites which gave red fluorescence band. Since porphyrins responsible for red fluorescence have absorption band over 350–450 nm with peak intensity at around 400 nm (ref. 6), this observation suggests that reabsorption of blue fluorescence may be a reason for the observed decrease in fluorescence.

### *Discrimination analysis*

In order to establish whether the observed differences in the spectra of normal and cancerous tissues are statistically significant and to facilitate diagnosis, the spectral differences were quantified by forming a discrimination score for characterizing normal or cancerous tissue. This score can be based on values of the fluorescence intensities at specific wavelengths and/or the ratios of intensities at specific wavelength pairs or wavelength bands chosen appropriately to maximize the differences in values for the tissue types to be discriminated. Instead of intensity, the ratio of intensities was used by us since the absolute intensity can get affected by several unavoidable factors like the nature

of contact between tissue and fiber, variations in excitation and collection geometry, etc.

For the purpose of discrimination the malignant tissue spectra were grouped into two categories. The first category (C–R) comprised of cancerous tissue spectra in which red emission band was present and the second category (C–NR) comprised of cancerous tissue spectra wherein the red emission band was not present. A total of six intensity ratios were derived from each of the LIF spectra for use as input parameters for the discrimination function. Two of the parameters used were the ratio of intensities at peaks of the 460 nm (BG) and 390 nm (VB) bands $R_p$ (BG/VB) and the ratio of integrated intensities over the wavelength bands 420–580 nm (BG) and 360–415 nm (VB) $R_{BG/VB}$. These were chosen because the relative collagen and NADH fluorescence components are seen to be different for cancerous and noncancerous tissues (Figure 2). For the same reason, for tissues showing red fluorescence bands, the ratio of integrated intensities over the bands 580–680 nm (R) and 360–415 nm (VB) $R_{R/VB}$ was also used as a parameter. Further, in order to take care of the likely interference from blood absorption, the ratio of intensities at three wavelength pairs of equal blood absorption was also used as input parameters for the discrimination functions.

A step-wise MVLR analysis[7] was used for maximizing the discrimination between the malignant and normal tissue spectra, where these six ratio variables were utilized as input data. Of the six input parameters, $R_{BG/VB}$ and $R_{R/VB}$ were found to be the most significant for discriminating normal from C–NR and C–R respectively. Two parameters ($R_{450/550}$ and $R_{510/590}$) corresponding to the ratio of intensities at wavelength pairs of equal blood absorption made positive contribution to discrimination and the remaining two parameters ($R_p$(BG/VB) and $R_{440/380}$) either did not contribute or reduced discrimination. These two were, therefore, removed from the MVLR analysis. The discrimination function derived by analysing the whole set of parameters for the spectra of C–NR and normal uterine tissues had the following form:

$$\mathrm{DF1} = -4.559 + 2.1870 \times (R_{BG/VB}) + 1.2394 \times (R_{R/VB}) + 0.0911 \times (R_{450/550}) - 0.5577 \times (R_{510/590}).$$

The mean scores ± standard deviation of DF1 were $-1.114 \pm 1.059$ and $1.114 \pm 0.921$ for C–NR and normal uterine tissues respectively. The cut-off point for discrimination was taken as the average of the mean scores for normal and C–NR. This correctly identified 80 out of 90 malignant sites and 79 out of 90 normal sites investigated.

Similarly the MVLR analysis for discrimination between the C–R and the normal uterine tissue spectra led to the following discrimination function:

$$\mathrm{DF2} = -2.5185 + 0.8975 \times (R_{BG/VB}) + 1.3900 \times (R_{R/VB}) - 0.5264 \times (R_{450/550}) - 0.3154 \times (R_{510/590}).$$

The mean scores ± standard deviation of DF2 were $1.927 \pm 1.162$ and $-1.927 \pm 0.612$ for C–R and normal tissues respectively. The cut-off value chosen was zero and this correctly identified all of the malignant and normal sites investigated.

The mean value of the ratio parameters for each tissue type was also calculated and discrimination power of these in discriminating normal and malignant tissue spectra was tested by applying Student's *t*-test. Table 1 shows the mean value ± standard deviation of the intensity ratios

**Table 1.** Mean value ± standard deviation of the statistically significant discrimination parameters for uterine tissues

| Discrimination parameter | Normal | C–NR | C–R |
|---|---|---|---|
| $R_{BG/VB}$ | $3.049 \pm 0.378$ | $2.213 \pm 0.436$ | $4.183 \pm 0.629$ |
| $R_{510/590}$ | $3.016 \pm 0.697$ | $3.694 \pm 0.835$ | $2.036 \pm 0.333$ |
| $R_{R/VB}$ | $0.324 \pm 0.092$* | $0.302 \pm 0.100$ | $1.620 \pm 0.725$ |

*The parameter $R_{R/VB}$ is significant for discriminating N from C–R but not significant for discriminating N from C–NR.

**Table 2.** Sensitivity, specificity, predictive value positive (PVP) and predictive value negative (PVN) towards cancerous uterine tissue using MVLR score, $R_{BG/VB}$ and $R_{R/VB}$

| Discrimination goal | Diagnostic parameter | Sensitivity (%) | Specificity (%) | PVP (%) | PVN (%) |
|---|---|---|---|---|---|
| N from C–NR | MVLR score | 88.9 | 87.8 | 87.9 | 88.8 |
| N from C–R | (four-variable) | 100 | 100 | 100 | 100 |
| N from C–NR | $R_{BG/VB}$ | 86.7 | 87.8 | 87.6 | 86.8 |
| N from C–R | | 87.8 | 97.8 | 94.6 | 94.6 |
| N from C–R | $R_{R/VB}$ | 100 | 100 | 100 | 100 |

Sensitivity and specificity towards cancerous tissue are defined as the conditional probability of predicting cancerous tissue as cancerous and the conditional probability of predicting non-cancerous tissue as non-cancerous respectively. The predictive value positive term is the conditional probability that the predicted cancerous tissue is truly cancerous and the predictive value negative term is the conditional probability that predicted non-cancerous tissue is truly non-cancerous.

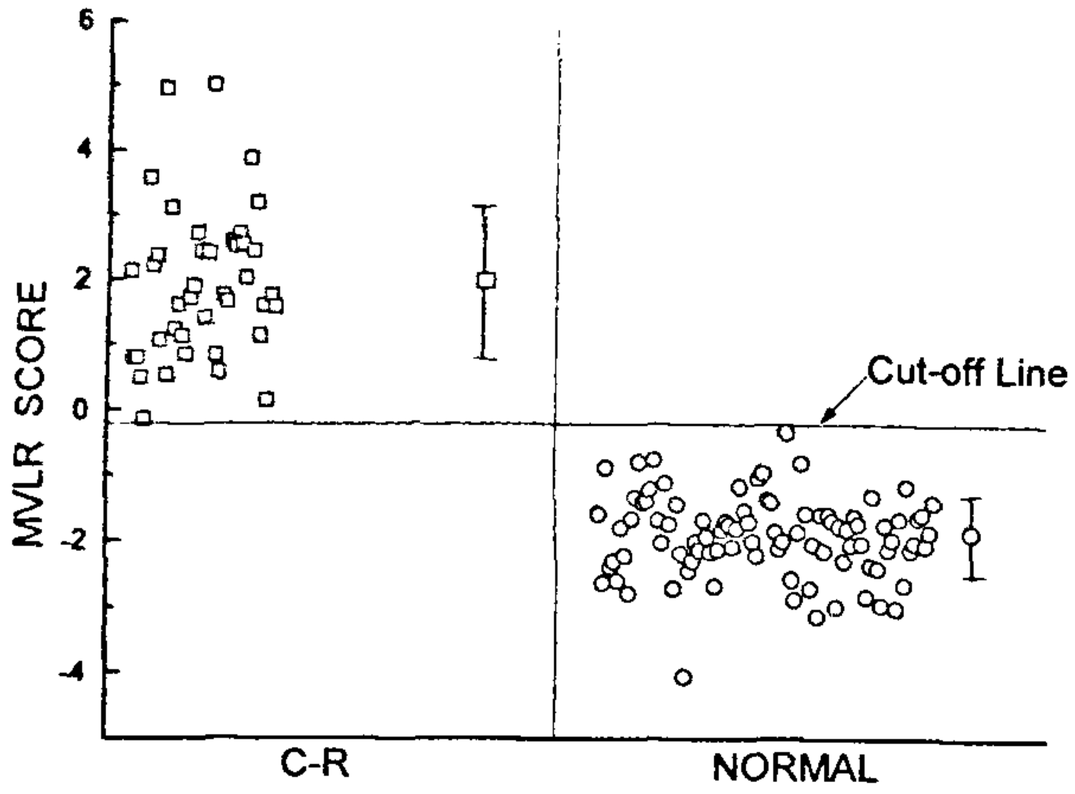


**Figure 3.** Scatter plot of the four-variable MVLR score from normal (N) and cancerous sites without red fluorescence (C–NR). The bar shows mean value ± standard deviation.

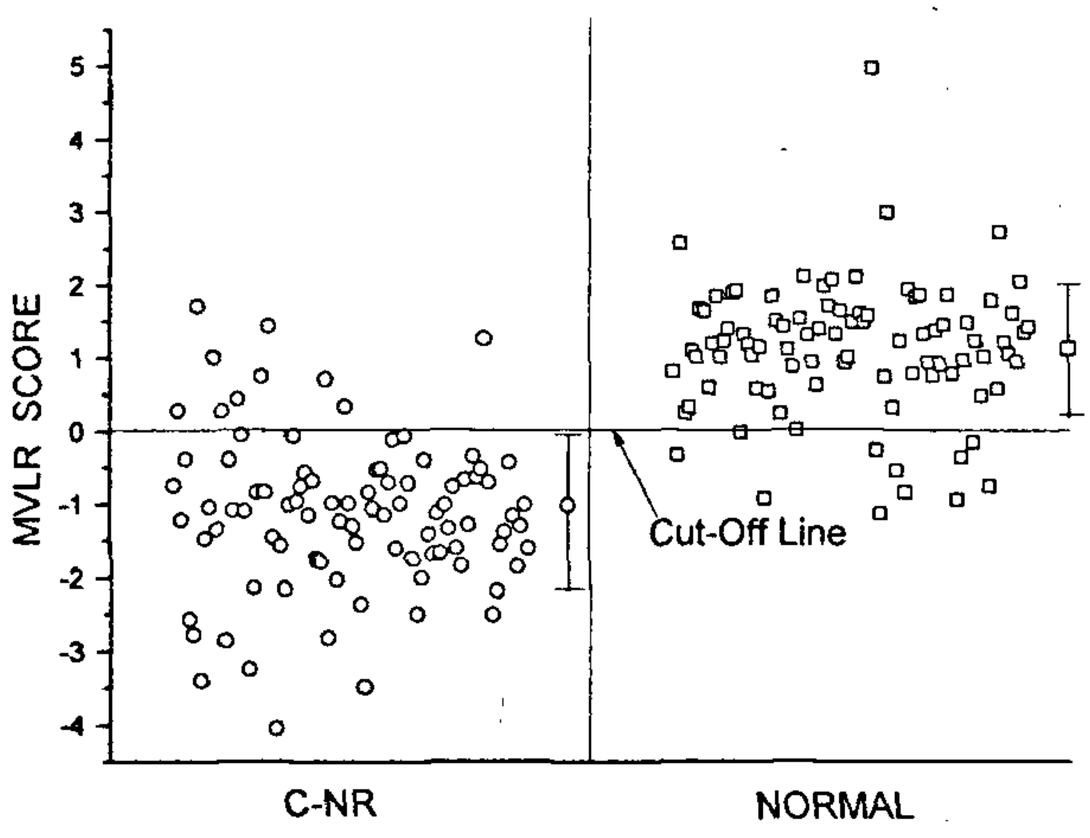


**Figure 4.** Scatter plot of the four-variable MVLR score from normal (N) and cancerous sites with red fluorescence (C–R). The bar shows mean value ± standard deviation.

which were found to be statistically different for normal and cancerous tissues to a $p$-value of 0.001. The predictive values of individual parameters and of MVLR score using the average of the mean values for cancerous and normal tissues as cut-off value are shown in Table 2, which lists the sensitivity, specificity, predictive value positive and predictive value negative towards uterine cancerous tissues with red fluorescence band (C–R) and without red fluorescence band (C–NR). Scatter plots for the values of the MVLR score for cancerous and normal uterine tissues are shown in Figures 3 and 4. The cent per cent predictive value obtained for discriminating cancerous tissue site with red fluorescence band from adjoining normal tissue is primarily due to the large difference in the value for the parameter $R_{R/VB}$ in the two cases. This large difference arises because the red porphyrin fluorescence in malignant tissue is accompanied by porphyrin absorption-induced decrease in the blue-green fluorescence as discussed earlier. No significant difference in the value of $R_{R/VB}$ for normal and C–NR samples was observed as would be expected. The same reabsorption of violet-blue fluorescence may account for the fact that the value for $R_{BG/VB}$ is much higher for C–R site as compared to normal whereas for C–NR the opposite holds true.

## Conclusion

Our *in vitro* studies on $N_2$ laser-excited autofluorescence spectra of human uterine tissues show that this technique can provide good discrimination between cancerous and adjoining normal tissue. Sensitivity and specificity towards cancer of 100% were obtained when the cancerous site exhibited red emission band and value of about 90% was achieved in general. The transportability of *in vitro* discrimination functions for *in vivo* studies is an important issue needing confirmation for clinical exploitation of the technique. Some recent studies[2,3] have shown that regression coefficients optimized for *in vitro* measurements give poorer results compared to the MVLR analysis of *in vivo* spectra. The *in vivo* studies are therefore now being planned.

1. See for example: Andersson-Engels, S., Berg, R., Johansson, J., Svanberg, S., Stenram, U. and Svanberg, K., in *Photodynamic Therapy: Basic Principles and Applications* (eds Henderson, B. W. and Dougherty, T. J.), Marcel Dekker, New York, 1992.
2. Schomacker, K. T., Frisoli, J. K., Compton, C. C., Flotte, T. J., Richter, J. M., Nishioka, N. S. and Deutsch, T. F., *Lasers Surgery Med.*, 1992, **12**, 63–78.
3. Ramanujam, N., Mitchell, M. F., Mahadevan, A., Warren, S., Thompson, S. and Richards-Kortum, R., *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 10193–10197.
4. Andersson-Engels, S., Johansson, J., Sternram, U., Svanberg, K. and Svanberg, S., *IEEE J. Quant. Electron.*, 1990, **26**, 2207–2217.
5. Marchesini, R., Brambilla, M., Pignoli, E., Bottrioli, G., Croce, A. C., Fante, M. D., Spinelli, P. and Palma, S. D., *J. Photochem. Photobiol. B: Biol.*, 1992, **14**, 219–230.
6. See for example Wijesekera, T. P. and Dolphin, D., in *Methods in Porphyrin Photosensitization* (ed. Kessel, D.), Plenum, New York, 1984, pp. 229–266.
7. See for example: Kleinbaum, D. G., Kupper, L. L. and Muller, K. E., *Applied Regression Analysis and other Multivariate Methods*, PWS-KENT Pulishing Company, 1988, pp. 560.

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