CHAPTER 2

MATERIALS AND METHODS

The present chapter deals with the experimental materials and methods which were used for the measurement of steady state fluorescence spectroscopic techniques such as emission, excitation, synchronous and fluorescence lifetime measurements of different fluorophores present in the urine samples and were explained. The method of collection of urine samples from both the healthy subjects and cancer patients with different etiologies and stages are given in this chapter. The theory behind the statistical methods used in the discrimination of abnormal from normal subjects is also discussed.

2.1 SAMPLE COLLECTION

First voided morning urine samples were collected in a sterile container from cancer patients of different etiologies and stages who are admitted for treatment in various hospitals in Chennai and from healthy volunteers. Cancer samples were collected from patients suffering from both oral and cervical cancer. Both healthy and abnormal cases are selected in such a way that they were free from other abnormalities like diabetes, jaundice and bacterial infections. Urine samples were stored in the refrigerator before fluorescence measurements. The raw samples without any preprocessing were examined before 48 hours of collection after thawing it to room temperature.
2.2 CLASSIFICATION OF SAMPLES

Cancer may be classified according to the site of origin as oral, cervix, breast cancer etc., and according to the severity of cancer as grade I, II, III and IV. Criteria for the individual grades vary with each form of neoplasia. Staging of cancer is based on the size or extent of the primary (main) tumor and whether it has spread to other areas of the body. The TNM system is one of the most widely used cancer staging systems. This system has been accepted by the Union for International Cancer Control and the American Joint Committee of Cancer.

Most medical facilities use the TNM system as their main method for cancer reporting. The TNM system is based on the size and/or extent (reach) of the primary tumor (T), the amount of spread to nearby lymph nodes (N), and the presence of metastasis (M) or secondary tumors formed by the spread of cancer cells to other parts of the body. A number is added to each letter to indicate the size and/or extent of the primary tumor and the degree of cancer spread.

In this context, the samples were classified and analyzed with the following five categories.

2.2.1 Normal versus Cancer Patients

In this category, all the first voided raw urine samples which were obtained from both oral cancer and cervical cancer patients were considered as cancer subjects and were compared with normal groups.
2.2.2 **Normal versus Early Stage Cancer Patients**

In the present study TNM classified stages I and II were considered as early stage and were compared with normal groups.

2.2.3 **Normal versus Advanced Stage Cancer Patients**

The TNM classified stages III and IV were considered as advanced stage of cancer and were compared with normal groups.

2.2.4 **Normal versus Oral Cancer Patients**

In this type of classification, the samples which were drawn from the patients those who are suffering from oral cancer were subjected to fluorescence spectral measurements and they were compared with normal subjects.

2.2.5 **Normal versus Cervical Cancer Patients**

Here, the samples which were drawn from the patients those who are suffering from cervical cancer were subjected to spectral measurements and they were compared with urine of normal female subjects.

2.3 **INSTRUMENTATION FOR FLUORESCENCE MEASUREMENTS**

2.3.1 **Experimental Arrangement for Steady State Fluorescence Measurements**

The steady state fluorescence emission, excitation and synchronous luminescence spectroscopic characterization were measured using a
commercially available spectrofluorometer Fluoromax-2 (ISA JOBIN YVON-SPEX, Edison, New Jersey, USA). The excitation source (150 W Ozone free Xenon arc lamp) coupled to the monochromator delivers light to the sample spot at a desired wavelength and the fluorescence emission from the sample is collected by another monochromator connected to a photomultiplier tube (R928P, Hamamatsu, Japan).

The gratings in the excitation and emission monochromators have a groove density of 1200 grooves/mm and are blazed at 330 nm and 500 nm respectively. The collected signal is transferred to the PC through an RS232 interface. The data were processed by Windows based data acquisition program – DataMax software powered by GRAMS/386. The schematic diagram of spectrofluorometer is shown in Figure 2.1.

![Figure 2.1 Schematic of the instrument setup for fluorescence measurements](image-url)
The urine samples of normal subjects and cancer patients were placed in a four side polished quartz cuvette and excited from one side and the fluorescence spectra was measured. The instrument was turned on 30 min before the measurements were taken in order to improve stability and reduce noise.

2.3.1.1 Native Fluorescence emission spectral measurements

The steady state fluorescence emission spectra of first voided raw urine samples of normal human subjects and cancer patients were measured at different excitation wavelengths viz. 280, 350, 405 and 450 nm.

2.3.1.2 Native Fluorescence excitation spectral measurements

The steady state fluorescence excitation spectra of urine samples were obtained for different emission wavelengths viz., 390 nm, 450 nm and 520 nm. During the fluorescence excitation spectral measurements, the desired peak emission wavelength is fixed for the emission monochromator and the corresponding fluorescence excitation intensity range to be recorded in the excitation monochromator.

2.3.1.3 Synchronous Luminescence spectral measurements

The synchronous luminescence spectroscopic (SLS) measurements of raw urine samples were also measured. During data acquisition, the excitation and the emission monochromators were simultaneously scanned with a constant wavelength difference between them and the SL signal was collected in the range 250 to 600 nm. The SL spectra were measured at different $\Delta\lambda$ values. Among all the $\Delta\lambda$ values, $\Delta\lambda$ =20 nm showed better profile and was fixed as constant for all measurements.
For all the steady state fluorescence measurements, the excitation and the emission slit widths were set at 5 nm. The acquisition interval and the integration time were maintained as 1 nm and 0.1 seconds respectively.

2.3.2 Experimental arrangement for Time Resolved fluorescence Measurements

Lifetime measurements was made using Time Correlated Single Photon Counting System (TCSPC, HORIBA JOBIN YUVON IBH, UK) with a fast response red sensitive PMT (Hamamatsu Photonics, Japan) detector. The excitation source used in the experiments was light emitting diode laser of wavelength 460 nm and the decay was measured at 520 nm. The fluorescence emission was collected at 90 degree from the path of the light source. The electrical signal was amplified by a TB-02 pulse amplifier (Horiba) fed to the constant fraction discriminator (CFD, Phillips, The Netherlands). The first detected photon was used as a start signal by a time-to-amplitude converter (TAC), and the excitation pulse triggered the stop signal. The multichannel analyzer (MCA) recorded repetitive start-stop signals from the TAC and generated a histogram of photons as a function of time-calibrated channels. The instrument response function was obtained using a Rayleigh scatterer of Ludox-40 (40 wt. % suspension in water, Sigma-Aldrich) in a quartz cuvette at 460 nm excitation. Decay analysis software (DAS6 v6.0, Horiba) was used to extract the lifetime components. The goodness of fit was judged by chi-square values as well as visual observations of fitted line and residuals.

2.4 STATISTICAL ANALYSIS OF SPECTRAL DATA

The steady state and time resolved fluorescence spectral characteristics of urine of normal and diseased subjects measured in this study
were analyzed in detail using statistical methods. For this purpose, stepwise linear discriminant analysis (LDA) and principal components analysis (PCA) based linear discriminant analysis was adopted to estimate the specificity and sensitivity of the present technique, in discriminating normal from diseased subjects and the best of the two methods was discussed.

The discriminant analyses were performed for different combinations of the experimental groups studied. The details of the statistical analysis of the spectral data are given below.

2.4.1 Preprocessing of Spectral Data

Detailed statistical analysis of the fluorescence spectral data was carried out by the following three primary steps:

i) Normalization of each fluorescence spectrum

ii) Identifying characteristic spectral features of each experimental group and introduction of different ratio parameters

iii) Development of a classification algorithm using stepwise multiple linear discriminant analysis, by including the ratio parameters as input variables.

2.4.1.1 Normalization

Each fluorescence spectrum was normalized by dividing the fluorescence intensity at each emission, excitation and synchronous wavelength by the corresponding peak intensity value of the spectrum. Normalizing a fluorescence spectrum removes absolute intensity information
and the main advantage of utilizing normalized spectrum is that fluorescence intensity need not to be recorded in calibrated units (Ramanjuam et al 1996a).

2.4.1.2 Introducing ratio variables

Average spectra were generated for each experimental group and the corresponding difference spectra were computed at each excitation / emission and synchronous wavelength, by subtracting the average spectrum of the diseased group (oral/cervical cancer) from that of normal subjects. The excitation / emission and synchronous wavelengths at which characteristic spectral differences are observed between the normal and diseased groups were identified. In order to quantify the observed spectral differences and to estimate the diagnostic potentiality of the present technique, different ratio variables were introduced for discriminant analysis at each excitation / emission and synchronous wavelength. These variables were calculated by using fluorescence intensities at those excitation / emission and synchronous wavelengths, which represent characteristic spectral features of different experimental groups studied.

2.4.1.3 Student’s t-test

Mean and standard error values of all the ratio variables were calculated for each group of experimental subjects. A two tailed Student’s t-test was performed to determine the level of significance (p value) with which each ratio variable and principal components discriminates diseased subjects from normal. For most of the analysis, only those ratio variables and principal components (PC’s) with p < 0.05 were chosen. The less significant variables were removed during the discriminant analysis.
2.4.1.4 Development of classification algorithm

Stepwise multiple linear discriminant analysis and PCA based LDA were performed using SPSS / PC+ 17.0. The discriminant analysis used a partial F-test (F to enter 3.84; F to remove 2.71) and a stepwise method to sequentially incorporate the input variables into a Fisher’s linear discriminant function. Stepwise discriminant analysis performed across n groups would generally result in the coefficients of n Fisher’s linear discriminant (or) classification functions (one for each group) and (n-1) canonical discriminant functions. The classification function of each group could discriminate only that group from the rest of the groups in the analysis. Thus, when more than two groups are to be classified with respect to each other, it is preferred to use the canonical discriminant functions for classification, instead of the classification functions. This would reduce the complexity of the analysis.

To check the reliability of the analysis in the present study, leave-one out cross validation (LOOCV) was used. In this procedure, discriminant scores of one particular case were eliminated and discriminant analysis was used to form a classification algorithm using the remaining samples. The resulting algorithm was then used to classify the excluded case. This process was repeated for each one of the cases. This process known as leave-one out cross validation (LOOCV) or jackknife cross validation, provides optimal use of a small data set to validate the performance of a decision surface without bias (Green & Carroll 1978).

2.4.2 Principal Component Based Linear Discriminant Analysis

Principal Component Analysis (PCA) is a transformation used for representing high dimensional data into fewer dimensions such that maximum information about the data is present in the transformed space. It decomposes
spectral data into a small number of independent variations called factors and contributions of these factors to each spectrum are called scores. Since PCA is often used in identifying trends and patterns in data, supervised linear discriminant analysis (LDA) was also employed. Here, all the steps adopted in LDA are same for PCA except normalization and introducing ratio variables. Instead of that, the principal components were used as input variables after subjecting them to students t-test.

A brief introduction and overview of the discriminant analysis procedure is given in the following section.

2.4.3 Discriminant Analysis

Discriminant Analysis (DA) is an object-oriented multivariate technique and it is used to analyze the differences between the groups and/or provide us with a means to assign (classify) any case into the groups which it most closely resembles. DA includes a set of response variables and one or more grouping or nominally scaled variables. This analysis assumes that there exist two or more groups which differ on several variables and that those variables can be measured at the interval or ratio level. There is no limit to the types of variables that can be employed, but problems with interpretation may result (Klecka 1980). A researcher may be willing to make choices about the variables that will be involved in an analysis.

Stepwise method can be used for this purpose and it will select a subset of variables from the original set and also determine the relative importance of the set of variables even if no variables are too eliminated (Huberty 1989). Several researchers Huberty (1989), Huberty (1994), and Thompson (1989) have noted the common use of stepwise analyses. According to Thompson (1989), "stepwise analytic methods may be among
the most popular research practices employed in both substantive and validity research." However, some of these same researchers as well as others have advanced strong arguments against the use of stepwise methodologies.

2.4.3.1 Key terms and concepts

Discriminating variables are the independent variables, also called predictors. The criterion variable is the dependent variable, which is the object of classification efforts. Discriminant function, also called a canonical root, is a latent variable which is created as a linear combination of discriminating (independent) variables, such that

\[ L = b_1x_1 + b_2x_2 + \ldots + b_nx_n + c, \]

where the b's are discriminant coefficients, the x's are discriminating variables, and c is a constant. This is analogous to multiple regression, but the b's are discriminant coefficients which maximize the distance between the means of the criterion (dependent) variable. Note that the foregoing assumes that the discriminant function is estimated using ordinary least-squares, the traditional method, but there is also a version involving maximum likelihood estimation.

The discriminant score, also called the DA score, is the value resulting from applying a discriminant function formula to the data for a given case. The Z score is the discriminant score for standardized data. Cutoff: If the discriminant score of the function is less than or equal to the cutoff, the case is classed as 0, otherwise it is classed as 1. When group sizes are equal, the cutoff is the mean of the two centroids (for two-group DA). If the groups are unequal, the cutoff is the weighted mean.

Unstandardized discriminant coefficients are used in the formula for making the classifications in DA, as b coefficients are used in regression in making predictions. The product of the unstandardized coefficients with the
observations yields the discriminant scores. Standardized discriminant coefficients are used to compare the relative importance of the independent variables, as beta weights are used in regression. The group centroid is the mean value for the discriminant scores for a given category of the dependent. Two-group discriminant analysis has two centroids, one for each group. There is one discriminant function for 2-group discriminant analysis, but for higher order DA, the number of functions (each with its own cut-off value) is the lesser of \( (g - 1) \), where \( g \) is the number of groups. Each discriminant function is orthogonal to the others.

2.4.3.2 Tests of significance

Wilk’s lambda is used in DA, such that the smaller the lambda for an independent variable, the more that variable contributes to the discriminant function. Lambda varies from 0 to 1, with 0 meaning that group means differ (thus the more the variable differentiates the groups), and 1 meaning all group means are the same. The F test of Wilk’s lambda shows which variables’ contributions are significant. Wilk’s lambda is sometimes called the U statistic. Wilk’s lambda is also used in a second context of discriminant analysis, to test the significance of the discriminant function as a whole.

2.4.3.3 Measuring strength of relationships

The classification table also called confusion, assignment, or prediction matrix or table, is used to assess the performance of DA. This is simply a table in which the rows are the observed categories of the dependent and the columns are the predicted categories of the dependents. When prediction is perfect, all cases will lie on the diagonal. The percentage of cases on the diagonal is the percentage of correct classifications. This percentage is called the hit ratio. Mahalanobis D-Square and Rao’s V are two other indexes
of the extent to which the discriminant functions discriminate between criterion groups. Squared canonical correlation, $R_c^2$, is the percent of variation in the dependent discriminated by the set of independents in DA.

2.4.3.4 Interpreting the discriminant functions

The structure matrix table shows the correlations of each variable with each discriminant function. These simple Pearson correlations are called structure coefficients or correlations or discriminant loadings. When the dependent has more than two categories there will be more than one discriminant function. In that case, there will be multiple columns in the table, one for each function. The correlations then serve like factor loadings in factor analysis, that is, by identifying the largest absolute correlations associated with each discriminant function the researcher gains insight into how to name each function.

2.4.3.5 Structure coefficients vs. standardized discriminant function coefficients

The standardized discriminant function coefficients indicate the partial contribution of each variable to the discriminant function(s), controlling for other independents entered in the equation. The structure coefficients indicate the simple correlations between the variables and the discriminant function or functions. The structure coefficients should be used to assign meaningful labels to the discriminant functions. The standardized discriminant function coefficients should be used to assess each independent variable's unique contribution to the discriminant function.
2.4.3.6 Measure of discriminatory power

One set of measures of the discriminatory power or accuracy of a diagnostic algorithm is the sensitivity and specificity. The sensitivity indicates the percentage of correctly classified positive (diseased) cases. Sensitivity does not take into account false positive cases. In contrast, specificity represents the percentage of correctly predicted negative (non-diseased) cases. By summing the

\[
\text{Sensitivity} = \frac{TP}{TP + FN}
\]

\[
\text{Specificity} = \frac{TN}{TN + FP}
\]

number of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) results for the classification of the final point across all the \(n\) samples, the sensitivity and specificity can be calculated according to the following equations:

\[
\text{Accuracy} = \frac{TP + TN}{TP + TN + FP + FN}
\]

True positive (TN): Diseased cases correctly diagnosed as diseased
False positive (FP): Healthy people incorrectly identified as diseased
True negative (TN): Healthy people correctly identified as healthy
False negative (FN): Diseased people incorrectly identified as healthy

A perfect separation, as could be achieved for two non-overlapping groups, would have a sensitivity and specificity of 100% and 100%, respectively.