DEVELOPMENT OF WINDOWS BASED SOFTWARE TO ANALYZE FLUORESCENCE DECAY WITH TIME-CORRELATED SINGLE PHOTON COUNTING (TCSPC) SETUP

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Abstract

A VUV spectroscopic facility for studies in photophysics and photochemistry is being set up at INDUS-I synchrotron source, CAT, Indore. For this purpose, a data acquisition system based on time-correlated single photon counting method is being developed for fluorescence lifetime measurement. To estimate fluorescence lifetime from the data collected with this system, a Windows based program has been developed using Visual Basic 5.0. It uses instrument response function (IRF) and observed decay curve and estimates parameters of single exponential decay by least square analysis and Marquardt method as convergence mechanism. Estimation of parameters was performed using data collected with a commercial setup. Goodness of fit was judged by evaluating χ_R^2, weighted residuals and autocorrelation function. Performance is compared with two commercial software packages and found to be satisfactory.

Development of Windows based software to analyze fluorescence decay with time-correlated single photon counting (TCSPC) setup

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I INTRODUCTION

Time resolved measurements are widely used for fluorescence spectroscopy. Measurement techniques may be broadly divided into two categories, namely time-domain and frequency-domain methods. In time-domain or pulse fluorometry, the sample is excited with a pulse of light of width much shorter than the decay time τ of the sample. In frequency domain or phase modulation method, the sample is excited with intensity-modulated light; typically sine wave modulation is employed. Since the source to be used by the setup reported here is synchrotron radiation, which is a pulsed type source, time domain analysis is preferred. Among various methods available for time-domain analysis, TCSPC is in wide spread use [1] mainly because of:

- its good dynamic range (limited by size of each memory location of the multichannel analyzer) compared to streak camera,
- known distribution (Poisson distribution)
- high sensitivity – down to single photon level
- easy background subtraction and
- easier instrumentation.

Hence, TCSPC was selected as experimental setup for fluorescence lifetime studies with photophysics beamline of INDUS-I synchrotron radiation. Windows based software has been developed using Visual Basic 5.0 for analysis of fluorescence decay data. Though similar software is commercially available, this program has been developed as a part of the control and data acquisition software being developed for photophysics beamline. In this report, description of the software and results obtained have been presented. Performance comparison of the software with two commercial packages is also given in this report.

II PRINCIPLE OF TCSPC

Block schematic of the TCSPC setup is shown in Fig. 1. Photon counting starts with the excitation pulse, which excites the sample and initiates charging of a capacitor in the time-to-amplitude converter (TAC). The photon emitted from the sample reaches the detector to stop charging of the capacitor. The charge stored in the capacitor is proportional to the arrival time of the photon with respect to excitation pulse.
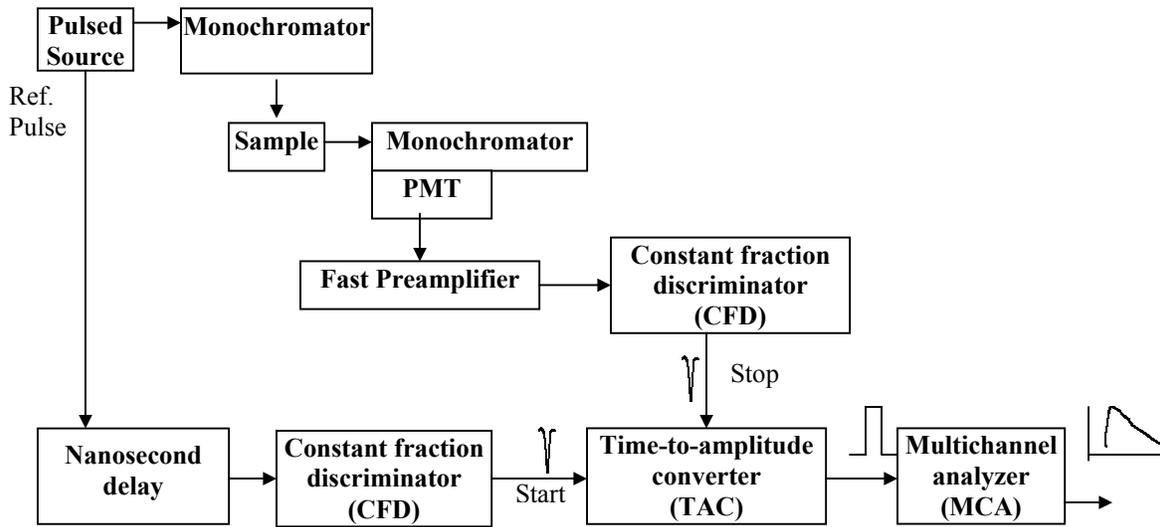

Fig. 1. Block schematic of single photon counting setup

The TAC output pulse height, corresponding to the photon arrival time is, then, converted by multichannel analyzer (MCA) to a channel number and one count is added to that channel. This process is repeated until count at peak channel reaches to 10^4-10^5. Summing over many pulses, the MCA builds up a probability histogram of counts vs. time channels. Since the time channel represents to the photon arrival time with respect to excitation pulse, the histogram provides the intensity decay of the sample.

III THEORY

Time-resolved fluorescence data are moderately complex and in general, cannot be analyzed using graphical methods. Since the mid-1970s many methods have been

proposed for analysis of TCSPC data. These include nonlinear least-squares analysis[2-4], method of moments [5], Laplace transformation [6-8] the maximum entropy method[9], Prony's method[10], sine transform[11], phase plane[12] and analytical methods[13]. Perhaps the most widely used deconvolution technique for TCSPC data is least-square curve-fitting[2]. Sometimes it is called reconvolution method since, the aim here is to obtain estimates of parameter values, which have the highest probability of being correct by repeated convolution and comparison of data. As Lackowitz[1] mentioned, least square analysis provides the best estimates for parameter values if the data satisfy a reasonable set of assumptions, which are as follows:

- all the experimental uncertainty is in the dependent variable(y-axis)
- the uncertainties in the dependent variable are distributed like a Gaussian, centered on the correct value
- there are no systematic errors in either the dependent (y-axis) or independent (x-axis) variables
- the assumed fitting function is the correct mathematical description of the system (Incorrect models yield incorrect parameters)
- the data points are all independent observations and
- there are a sufficient number of data points so that the parameters are over-determined.

Since these assumptions generally hold for TCSPC, least square estimation is the preferred method of analysis.

IIIA Least square curve fitting

Measured data from single photon counting setup consists of a source profile *L(t)* and an observed decay curve *N(t)*. *L(t)* represents the instrument response function i.e. response of the instrument to a zero-lifetime sample. *N(t)* represents a convolution of the *L(t)* with the impulse response function *I(t)*, also known as intensity decay law. The concept of convolution can be expressed as follows. Each excitation pulse *L(t)* can be considered as a series of δ-functions *L(t_j)* with different amplitudes. Therefore, each *L(t_j)* is assumed to excite an impulse response at *t_j* and its effect that remains at *t* is

$$N_j(t) = L(t_j)I(t-t_j)\Delta t \qquad\qquad (t > t_j) \qquad (1)$$

where Δt is the channel width.

The term $(t-t_j)$ appears because the impulse response is started at $t = t_j$. The measured decay at t i.e. $N(t)$ is the sum of the impulse responses $N_j(t)$ created by all the individual $L(t_j)$'s occurring until t,

$$N(t) = \sum_{t_j=0}^{t} L(t_j)I(t - t_j)\Delta t \tag{2}$$

Since data are discrete, Eqn.2 can be rewritten in terms of channel number as

$$N(i) = \sum_{j=0}^{i} L(j)I(i - j)\Delta t \tag{3}$$

where i and j are channel numbers. When the counts in the decay curve have reached a suitable precision, the number of counts in each channel i.e. $N(i)$, follows a Poisson distribution with a standard deviation, σ_i, given by

$$\sigma_i = \sqrt{N(i)} \tag{4}$$

Like any least square analysis, one start with a model, which is assumed to describe the data. In the present work, we have considered single exponential decay i.e.

$$I(t) = a_1 e^{-t/a_2} \tag{5}$$

where a_1 is amplitude and a_2 is the lifetime of the decay. Since $L(t)$ and $N(t)$ are recorded at different wavelength, light of different energies striking the photo-cathode of photomultiplier causes the consequent electron bunches traveling down the tube to have different electron distributions. Hence anode pulse shapes vary with the energy of the incident photons. When these pulses are processed in a timing discriminator, this instrument sees the different shapes merely as differently sloped rising edges, which leads to a shift in the time at the output of the discriminator. To correct this wavelength effect, the zero time of the instrument response function i.e. the time at which excitation occurs should be shifted. Therefore another parameter a_3 has been used to take care of this time shift. The fourth parameter named a_4 has been used for background correction. The Eqn.4 therefore takes the form of:

$$N(i) = \sum_{j=0}^{i} L(j + a_3)\left[a_1 e^{-(i-j)/a_2}\right] + a_4 \tag{6}$$

To obtain the parameter (a) values, which provide the best match between the observed data and the computed decay, the goodness-of-fit parameter χ^2 as shown in Eqn.7 is minimized.

$$\chi^2 = \sum_{k=n_1}^{n_2} \frac{1}{\sigma_k^2}[N(k) - N_c(k)]^2 = \sum_{k=n_1}^{n_2} \frac{[N(k) - N_c(k)]^2}{N(k)} \tag{7}$$

where n_1 and n_2 lower and upper channel number of the range for which χ^2 is evaluated

$N_c(k)$ is computed decay using Eqn.6 and assumed parameter values

$N(k)$ is the observed decay.

In TCSPC, the number of data points is typically much larger than the number of parameters. Hence for random errors and the correct model, χ^2 is expected to be approximately equal to the number of data points (channels). The values of a are varied until χ^2 is minimum, which occurs when $N(k)$ and $N_c(k)$ are most closely matched. A variety of general methods are available for selecting how a are changed after each iteration. In the software reported here, Marquardt [14] method was followed.

The fitting function $N_c(t_k)$ can be linearized in a Taylor series in the fitting parameters i.e.

$$N_c(k) = N_c^0(k) + \sum_{j=1}^{l} \frac{\partial N_c^0(k)}{\partial a_j} \delta a_j \tag{8}$$

where l is number of parameters to be estimated and $N_c^0(k)$ is initially evaluated with initial estimation of a and for subsequent iterations, it holds the most recent evaluated $N_c(k)$.

Substituting $N_c(k)$ in Eqn.7

$$\chi^2 = \sum_{k=n_1}^{n_2} \frac{\left[N(k) - N_c^0(k) - \sum_{j=1}^{l} \frac{\partial N_c^0(k)}{\partial a_j} \delta a_j\right]^2}{N(k)} \tag{9}$$

The condition for a minimum,

$$\frac{\partial \chi^2}{\partial(\partial a_j)} = 0 \tag{10}$$

leads to equation of the form

$$\beta = \delta \, a.\alpha \tag{11}$$

$$\text{where} \quad \beta_k = \sum_{j=1}^{l} (\delta a_j \alpha_{jk}) \qquad\qquad k = n_1 \ldots n_2 \qquad\qquad (12)$$

$$\text{and} \quad \alpha_{jk} = \sum_{i=n_1}^{n_2} \frac{1}{N(i)} \left[\frac{\partial N(i)}{\partial a_j} \times \frac{\partial N(i)}{\partial a_k} \right] \qquad\qquad j=1 \ldots l \qquad (13)$$

Marquardt [14] has developed an algorithm by which the minimum in χ^2 may be rapidly attained through simultaneous variation of each parameter. This is obtained by increasing the diagonal terms of the curvature matrix α by a factor λ. As per the algorithm, Eqn.11 can be rewritten as

$$\beta = \delta a.A \qquad\qquad (14)$$

$$\text{where} \quad A_{jk} = \alpha_{jk}(1+\lambda) \qquad \text{for } j = k$$

$$= \alpha_{jk} \qquad \text{for } j \neq k$$

λ initially set to relatively large and is reduced as the minimum is approached [2].

$$\delta a = \beta. A^{-1} = \beta. B \qquad\qquad (15)$$

$$\text{or} \quad \delta a_j = \sum_{k=1}^{l} \beta_k B_{jk} \qquad\qquad (16)$$

New χ^2 is then computed with $a_j + \delta a_j$ and compared to old χ^2. This whole process is repeated until required precision has arrived.

It is not convenient to interpret the values of χ^2 because χ^2 depends on the number of data points. For this reason one uses the value of reduced χ^2,

$$\chi_R^2 = \frac{\chi^2}{n - l} = \frac{\chi^2}{\nu} \qquad\qquad (17)$$

where $n = n_2 - n_1 + 1$ is the number of data points, l is the number of floating parameters and $\nu = n - l$ is the degrees of freedom. If only random errors contribute to χ_R^2, then this value is expected to be near unity. This can be understood with reference to Eqn.8, where each data point is expected to contribute σ_k^2 to χ^2, which is in turn normalized by σ_k^2. If the model does not fit the data, then χ_R^2 will be significantly larger than unity. The probability of obtaining a value of χ_R^2 because of random errors for various number of data points can be found in standard mathematical tables of the χ_R^2 distribution [2]. A model can be accepted if probability exceeds 5% [1].

IIIB Goodness of fit

Apart from $\chi_R{}^2$, the goodness of fit can be visually inspected using weighted residuals and autocorrelation function. The weighted residuals in channel i is calculated using the equation

$$r(i) = \frac{N(i) - N_c(i)}{\sqrt{N(i)}} \tag{18}$$

Residuals from successful fits when plotted against channel number should be randomly distributed about zero.

To check randomness of scattering of the experimental points auto-correlation function $C(i)$ of the residuals $r(k)$ and $r(k+i)$ as shown in Eqn.19 is used.

$$C(i) = \frac{\dfrac{1}{m}\sum_{k=1}^{m} r(k) \times r(k+i)}{\dfrac{1}{n}\sum_{k=1}^{n} r^2(k)} \tag{19}$$

The calculation is usually extended to test for correlations across half of the data channels i.e. $m = n/2$. Therefore autocorrelation plots have half as many data points as the original set. For a good fit, the autocorrelation function shows high frequency low amplitude oscillations about zero when plotted against i [3].

IV SOFTWARE DETAILS

The Windows based software has been developed using Visual Basic 5.0. The steps followed in the software are explained using flowchart as shown in Fig.2. Visual Basic has been preferred to develop the software because creation of user interface is easier with this. The screen is divided into three parts, e.g. parameters, plot and results as shown in Fig.3. In parameters section, name of data files, channel range, zero shift limit can be set by user. Calibration factor i.e. ns per channel can be set using CALIBRATE button which displays an input dialog window to accept TAC range in ns and channel range setting in MCA. Plot section plots channel number vs. source profile (blue), observed decay profile (green) and fitted curve (red) in one graph and channel number vs. weighted residuals in another graph. Results section displays initial estimation of lifetime
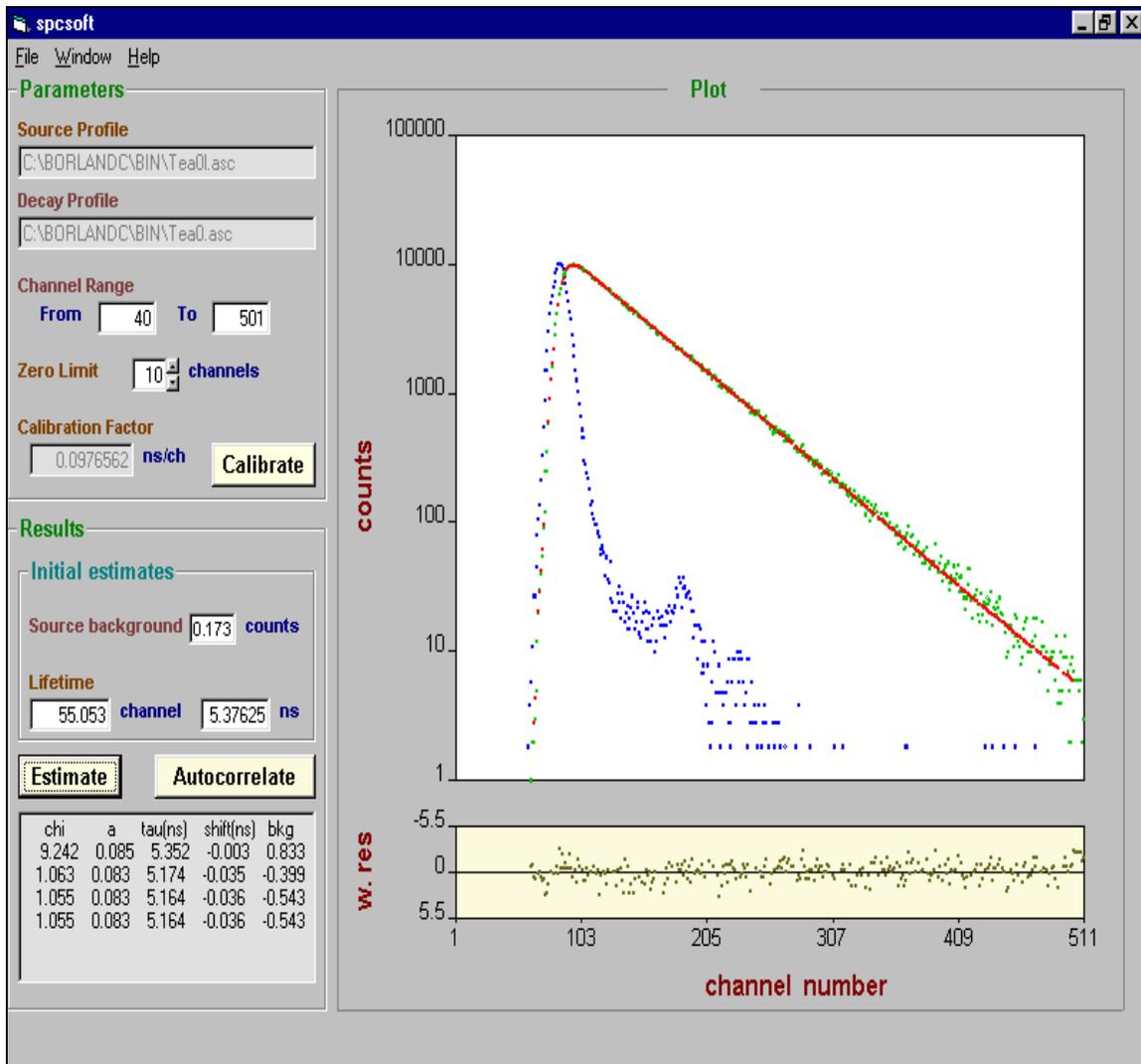
Fig.3. Users Interface of the software

(a_2) and source background (B_l) and least square method based estimation of four parameters (a) along
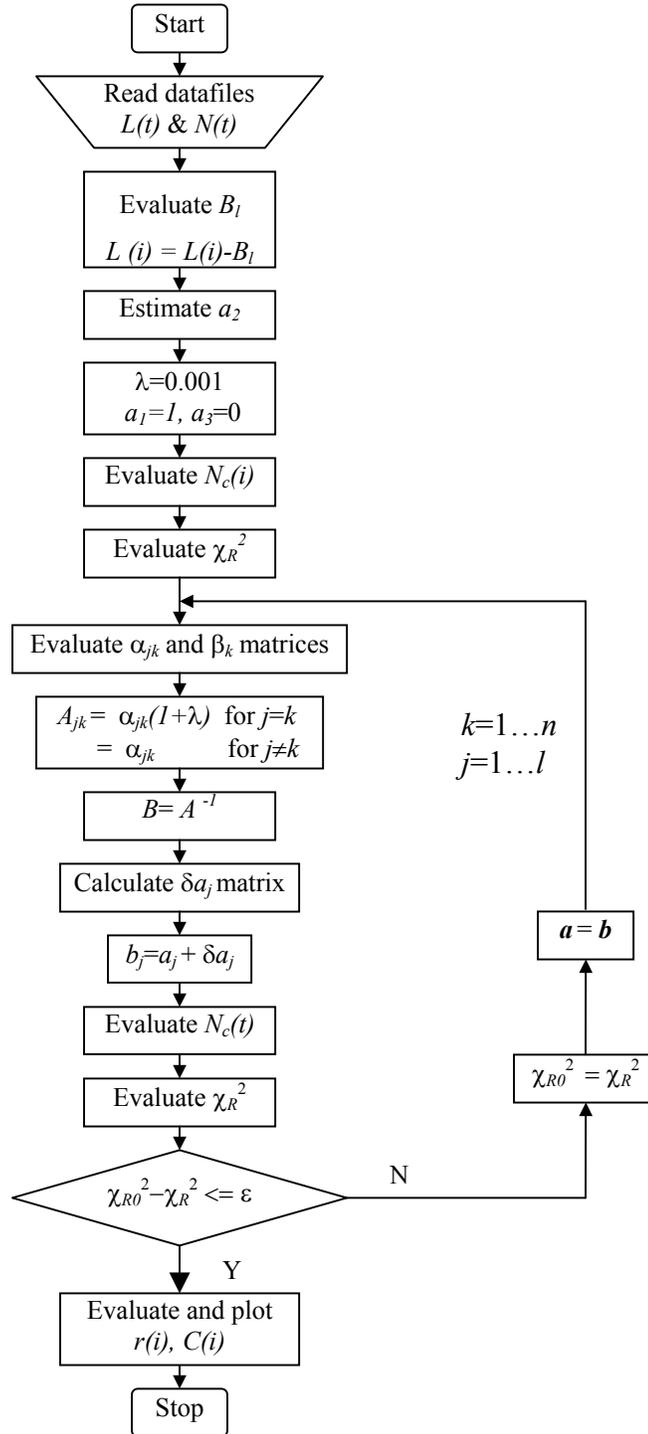

Fig.2. Flowchart of the software

with $\chi_R{}^2$. To evaluate background, a line is fitted with average value of counts. The number of points above and below this line is counted. If there are fewer points above the line than below, they are considered peaks and discarded. The process is then repeated until number points above the line is greater than or equal to those below the line. The final average value is used as background and subtracted from data. For initial estimation of a_2, the software takes 128 points ($\frac{1}{4}$th of total number of points) from peak channel of *N(i)* and evaluates natural logarithm of corresponding counts. Linear fitting of those values yields the slope, inverse of which gives rough estimated a_2. ESTIMATE button, in this section, starts estimation of a_j's. After estimation is over, values estimated are displayed. To check goodness of fit visually, AUTOCORRELATE button should be pressed to see the plot of correlation vs. channel number plot as shown in Fig. 4.

V RESULT AND DISCUSSION

A test sample was run and data collected using a commercial system with 511 (n_c) channels was analyzed by the software developed for channel range of 40 (n_1) to 510 (n_2) and zero shift limit of 10 (z) channels. Total number of data points used for analysis is n_c-z-n_1+1 (511-10-40+1 = 462) or n_2 -n_1+1 (510-40+1 = 471) whichever is minimum. Initial estimation of B_l and a_2 are 0.173 counts and 5.376 ns respectively. Estimation of parameters at different iterations is shown in the Table 1.

Table 1. Parameter estimation at various iterations

Iterations	a_1	a_2(ns)	a_3(ns)	a_4	χ^2
0 (Initial values)	1	5.376	0	0	201378.934
1	0.085	5.352	-0.003	0.833	9.242
2	0.083	5.174	-0.035	-0.399	1.063
3	0.083	5.164	-0.036	-0.543	1.055
4	0.083	5.164	-0.036	-0.543	1.055

To find out the probability (P) of $\chi_R{}^2$ =1.055 due to random noise, a program following Bevington [2] was developed and for 462 data points P is found to be 20% which is quiet satisfactory. The plot of observed and fitted curves as well as weighted residuals with 4-parameter model is shown in Fig 3. Total time taken by this software is 7.47 sec, whereas

2-parameter (a_1, a_2) model takes 4.62 sec. Number of iterations remaining same, the 2-parameter model saves times at the cost of poor χ_R^2 of 1.769. a_1 and a_2 calculated with this model is 0.083 and 5.197 ns. Autocorrelation function plot of 4-parameter model (Fig. 4a) as well as 2-parameter model (Fig 4b) clearly indicates that the former one is more acceptable than the later one as explained in section IIIB.
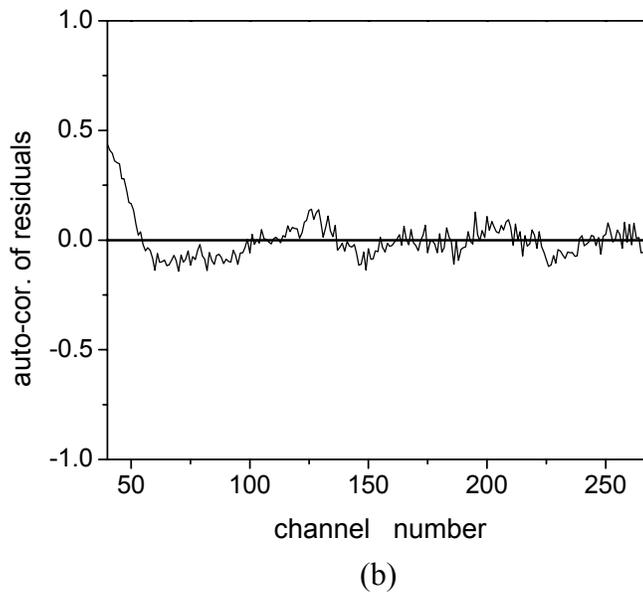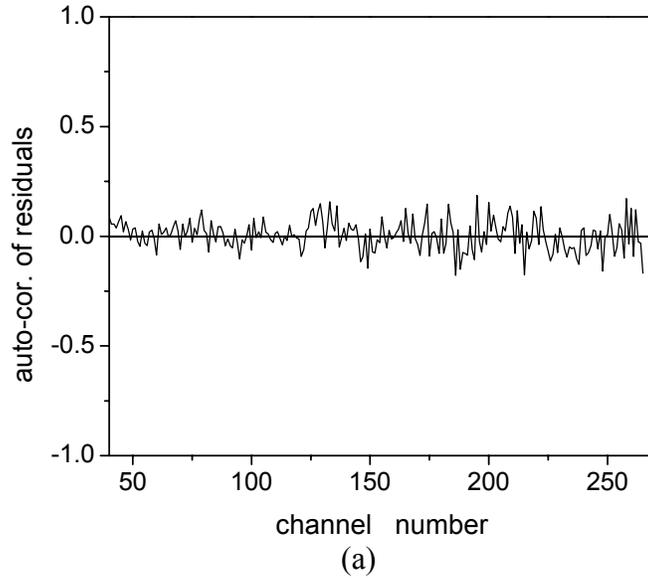

(a)

(b)

Fig 4. Plot of autocorrelation function of (a) 4-parameter model, (b) 2-parameter model

In this data set, peak channel for source profile is 86 and that for decay profile is 96. To decide the channel range for best fit, χ_R^2 vs. channel number is plotted as shown in Fig.5. It shows that for initial 70 (approx.) channels, the curve rises towards highest point. This is because at these channels counts are zero, i.e. there is no contribution of counts for these channels in χ_R^2 evaluation. But in the denominator of Eqn.17, effects of these channels are present. As the lower channel number of the range is reduced only degree of freedom increases, which decreases χ_R^2. It was observed that as the counts in channels starts increasing, χ_R^2 reaches its maximum, then starts reducing and finally fluctuates. It is found to be the best in between peak channels for source profile and decay profile. Setting lower channel beyond decay peak is improper as this may degrade estimation.
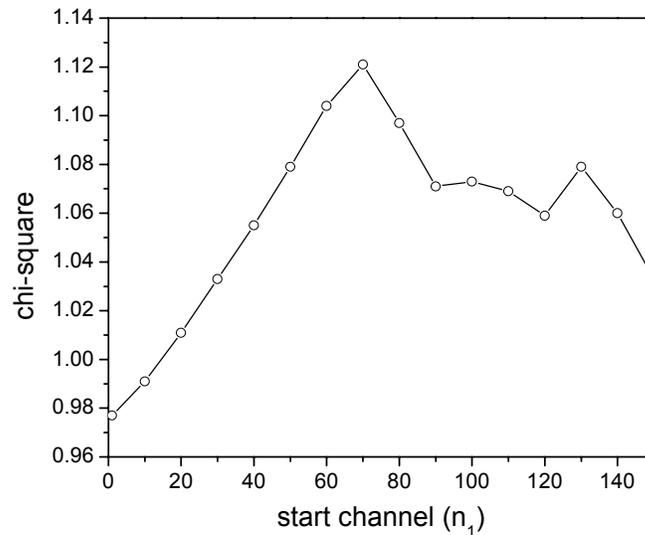

Fig.5. Plot of reduced chi-square vs. start channel number

Estimation has also been performed with two commercial software packages using same data set. The comparison of performance between two commercial software packages and the developed software are shown in the Table2. The result shows the performance of the software developed is comparable to the commercial one.

Table 2. Comparison of performance of developed and commercial softwares

Parameters	Commercial-1	Commercial-2	Developed
a_1	0.085	0.085	0.083
a_2 (ns)	5.172	5.176	5.164
a_3 (ns)	0.005	0.410	-0.037
a_4	-0.32	-0.26	-0.543
χ^2	1.165	1.151	1.056
Convergence method	Not given	Hybrid grid search algorithm	Marquardt algorithm

VI CONCLUSION

A Windows based program has been developed using Visual Basic 5.0 to estimate fluorescence lifetime using data collected with single photon counting. It needs instrument response function (IRF) and observed decay curve for this purpose. It uses least square analysis for estimation of parameters and Marquardt method as convergence mechanism. Estimation of parameters was performed using data collected with a commercial setup. χ_R^2 evaluated shows that probability of getting that value due to random noise is 20%, which is quiet satisfactory. Plot of channel number vs. weighted residuals and auto correlation function shows random fluctuation about zero. Performance is compared with two commercial software packages and found to be satisfactory.

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