PULSE5

users' manual

Fluorescence Decay Analysis
by
Quantified Maximum Entropy Method

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1. Read me first

The following are quick notes to enable you to set up and run the PULSE5 program as supplied to you. It is essential that this document is read first.

The software is supplied on a PC diskette in DOS standard format. The files on the disk contain the following :

Executable codes on diskett:

FLAME5.exe :Auto-decompressed executable code of pulse5.exe
GRAPH.exe :Auto-decompressed executable code for three graphics applications* Graphdis.exe, Graphdev.exe Graphcrv.exe Graphdec.exe (see page 5 and readme.1st file on diskett)
Graphics for lifetime distribution display on screen

Demo files

Test-1.go: Input parameter file for running the example as a demo. It contains all the necessary responses to the questions asked by the program and the required filenames. We detail how to modify this for your specific analysis below.

excit.fl, test-1.im : Files containing data used in our demo example.

test-1.log: Output result file from demo data analysis

test-1.dis: Output file containing τ and $h(\tau)$ distribution for further use and plot.

test-1.crv: Output file containing data , fitting values, residuals and autocorrelations.

test-1.dec: Output file containing fluorescence decay corresponding to each peak in the lifetime distribution.

test-1.tab: Output file containing a table of the parameters of the $h(\tau)$ distribution

*not supported by MSL

2. Brief theoretical background

A detailed introduction to the Quantified Maximum Entropy Method for time-resolved fluorescence data analysis is given in Methods in Enzymology (1994) Vol. 240 chapter 1, pages 262-311.
The program inverts fluorescence decay data measured from either vertically and horizontally polarised components or at the magic angle to produce a spectrum of decay times.

With an exciting flash of vertically polarised light having a finite experimental width E(t) the measured parallel I_vertical (I_{vv}) and perpendicular I_horizontal (I_{vh}) components of the emitted fluorescence decay are:

$$Ivv = \frac{1}{3} E(t) \otimes \left\{ \int_0^\infty \int_0^\infty \int_{-0.2}^{0.4} g\,(t\,,q\,,A)\; e^{-t/t} \left(1 + 2A\; e^{-t/q} \right) dt\;\, dq\;\, dA \right\} \qquad (1)$$

$$Ivh = \frac{1}{3} E(t) \otimes \left\{ \int_0^\infty \int_0^\infty \int_{-0.2}^{0.4} g\,(t\,,q\,,A)\; e^{-t/t} \left(1 - A\; e^{-t/q} \right) dt\;\, dq\;\, dA \right\} \qquad (2)$$

where $g(t, q, A)$ are the number of fluorophore with fluorescence decay τ, rotational correlation time θ and initial anisotropy A which is related to the angle between absorption and emission dipole moments. Symbol ⊗ denotes the convolution with time.

Total fluorescence decay

The fluorescence decay is obtained by summing the two polarised components:

$$T(t) = Ivv(t) + 2gIvv(t) = E_l(t) \otimes \int_0^\infty h(t)\; e^{-t/t}\; dt \qquad (4)$$

where $h(t)$ is the distribution of fluorescence lifetimes given by:

$$h(t) = \int_0^\infty \int_{-0.2}^{0.4} A\, g\,(t\,,q\,,A)\; dq\;\, dA \qquad (5)$$

and g is a correction factor for the response of the optics and the detection of the vertically and horizontally polarised emission (if any).

If we are only interested in the fluorescence decay, is usually measured in one data curve by setting the emission side polariser at the "magic" angle of 54.75° and then

$$T(t) = E_l(t) \otimes \int_0^\infty h(t)\; e^{-t/t}\; dt \qquad (6)$$

Maximum Entropy

Maximum Entropy is an optimal criterion for reconstructing lifetime distribution $h(t)$ from imperfect data. The general formulation of the entropy for a fluorescence kinetics is only dependent of $h(\tau)$:

$$S = \int_0^\infty h(t) - m(t) - h(t) \log \frac{h(t)}{m(t)} dt \qquad (7)$$

In this expression, m is a measure, usually taken to be flat in logarithmic space (see reference below for the reason why), which quantifies the relative importance of the various "pixels".
S measures the deviation of the distribution $h(t)$ from this measure, attaining its global maximum of zero when $h(t)$ is equal to m(τ). Because S is maximised by distributions which are as close as possible to the uniform and featureless (in log. space) measure m, maximum entropy uniquely gives the most probable reconstruction: there must be evidence in the data for any structure seen in a maximum entropy reconstruction. Suitably normalised, S is also minus the information content of $h(t)$, so that maximum entropy affords a uniquely comprehensible reconstruction, having only that minimum of information which is required to fit the data.

The program set up a statistic which measures the misfit between the actual (noisy) data D_k and the calculated data T_k which would be observed (in the absence of noise) if the actual distribution were correctly represented by the particular numbers h_i.
It is usual to use the normalised chi-squared value as a fit statistic.

$$c^2 = \frac{1}{M} \sum_{k=1}^{M} \frac{D_k - T_k}{s_k^2}$$

where σ_k is the standard deviation of the kth datum
On average, each datum should lie about one standard deviation from its calculated value, so that the normalised residuals should have unit variance and c^2 should be close to unity.
However, very many distributions remain which can fit an incomplete or noisy data set to the correct precision and very close value of c^2, each of which is consistent with the data, and most of which tend to be alarmingly irregular. The maximum entropy principle is appropriate to distinguish among these in a consistent way. One selects that single consistent distribution h(τ) which has greatest entropy (or, more strictly, greatest generalised cross-entropy) leading to the best posterior probability distribution. Statistics from a Gaussian approximation around this optimum solution allows to calculate error bars on some parameters of interest: peak surface, peak width and peak position ..

In general, the distribution being sought is represented by set of "pixels" numbers h_i (i=1,2,...N) proportional to the numbers of fluorescing centres with decay time τ_i. These numbers are to be inferred from the observed data D_k (k=1,2,...M). It is clear that the digitalisation of the fluorescence decay in M data points (in linear space) as well as the lifetime domain in N points may influence the final shape of the recovered lifetime distribution. It is recommend to use a large value of N, particularly if the lifetime domain to explore spans over several decades.

3a. How to run the program step by step

First open a DOS window from Windows95 or Windows 3.xx session. Move to the current directory where the analysis should take place.

Decompress automatically the applications in type succesively:

FLAME5

which gives you uncompressed PULSE5.EXE application file and then type

GRAPH

for decommpression of Graphdis.exe,Graphdev.exe Graphcrv.exe, Graphdec.exe application files. Now you are in position to run PULSE5.EXE

The program asks a number of questions, so that it can set up the options you require. These can be typed in response to the questions if running in interactive mode (see page 8)or they can be edited in as parameter files if running directly or in batch mode (see below). As we expect that users will mainly run it directly or in batch mode once they have become familiar with the system, we have set up the questions with this in mind. Thus the program always asks the same number of questions in the same order even if as a result of a previous question the result is meaningless.

The beginner can run the program interactively and discover step by step the questions asked by the program. The explanations and responses to the questions are given below. The output should be similar to the example output enclosed (from PC with DOS operating system). However because of different accuracy's in different machines minor numbers may be slightly different. These may accumulate as the iterations proceed but will not affect the final result as the program will iterate to the unique global maximum of entropy. It may take a few more or less iterations to converge however.

Finally you edit three parameter files containing all answers to question and you re-run the program in batch mode. An example is given in files:test-1.go, test-1.prm and memsys.prm listed in pages 6 and 7. These files contain respectively the parameters for individual decay analysis, for the instrument set-up and experimental condition and the MaxEnt options. Characters after the /...n/ label in the lines of data are ignored by the operating system (the program does not know they are there). They are thus an useful way of reminding the user of the content of the appropriate question. The label /...n/ contains the line number n of the current question and it refers to the corresponding explanations in "To Run interactively the program" in page 8.

3b. How to run directly the program

Using your favorite word editor, prepare the three input files as described in page 6 and 7. Then let start the program either in typing directly :

pulse5 <test-1.go

or in typing first :

pulse5

an header will appears on the CRT coming with the first question, then enter :

test-1.go

The iterates can be stopped at any time by pressing the **ESC or F3** keys, then follows instructions given on the graphics display to exit or to continue.

3c. How to run the program in batch mode

Just prepare a file (for example *dummy.bat)* containing the single line instruction as described above(pulse5 <test-1.go).
Mind to set the graphic flag value to 0 at line /22/ in the *.go files.

4. Input Files:

The following three files are an example of responses requested by the program for data analysis and supplied to the user for demonstration.

The first parameter file is set up for the analysis of the data curve recorded as file "test-1.im"

File: test-1.go

```
test-exp.prm     / 1/ Filename for instrument set up and methods
test-1.log       / 2/ Filename of the analysis listing
test-1.im        / 3/ Filename of the I-Magic or I_Vertical decay curve
bid.dat          / 4/ Filename of the I_Horizontal decay curve
excit.fl         / 5/ Filename of the FIRST Flash or Reference decay curve
bid.dat          / 6/ Filename of the SECOND flash or Reference decay curve
bid.dat          / 7/ Filename of the BLANK_Magic or BLANK_Vertical decay curve
bid.dat          / 8/ Filename of the BLANK_Horizontal decay curve
7-August-1998 DEMO test: PULSE5.EXE Mock data convolved with excit.fl file
Four exponentials; taus: 0.05 0.15 0.30 0.8ns / Alpha's: 0.40 0.35 0.20 0.05
(NO Correlation time)
 0.015  1.5   150      /12a/To_Minimum, To_Maximum, Number of lifetimes
 0.     0.    0         /../ To_Minimum, To_Maximum, Number of lifetimes
 0                      /13/ Addition of Scattered light : 0= No , 1= Yes
 0                      /14/ Addition of a Backgroung channel : 0= No , 1= Yes
 0. 0. 0. 0. 0.         /15/ Extra 5 single exponentials (outside of the To domains)
 701, 1945, 1961, 2020  /16/ Four channel # for Fluo. decay and Backgrd domains
 1937                   /17/ Fluo. decay chl number from where the CHI-SQUARE starts
 0.0    1               /18/ Excitation shift in channel unit, Flag for convolution type
 1                      /19/ First analysis (with a flat distribution):1=Yes, 0:Rerun
 0.                     /20/ Level of flat map: neg. or =0 ==> Calculated / >0.= Given
 0                      /21/ Flag for the PRIOR MODEL of the distribution
 1   1                  /22/ Graphics, Intermediate results after N: <N= No , >N= Yes
 -0.5  -0.10  -0.02     /23/ 1st_Rate   2d_Rate   Third Rate
 1                    /24/ Automatic peak-finding? :  1 = Yes, 0 = No
 0 0                  /25/ Upper and lower peak marker numbers (manual peak analysis)
```

The second parameter file contains all options about the instrument configuration and the experimental methods:

File: test-1.prm

02-10-95 / exp-1/ date of the experiments: DD-MM-YY
0.004 / exp-2/ Nanosecond/channel value in your MultiChannel Analyser
1 1 A / exp-3/ Flag to reverse curves: 0=No 1=Yes; Compression factor
1 0.0 / exp-4/ Flag Excitation polariser; G_factor:I_ver./I_hor. response
0.0 / exp-5/ Coefficient for BLANK_Magic or BLANK_Vertical curve
0.0 / exp-6/ Coefficient for BLANK_Horizontal curve
0. / exp-7/ Lifetime of the reference (in ns)
0 / exp-8/ Flag for average of 2 excitations
4. / exp-9/ Excitation pulse frequency in Mhz
-2. 0. /exp-10/ Background and its error (I_Magic or I_Vertical decay curve)
-2. 0. /exp-11/ Background and its error (I_Horizontal decay curve)
-2. 0. /exp-12/ Background and its error (FIRST Flash or Reference curve)
-2. 0. /exp-13/ Background and its error (SECOND Flash or Ref._Horizontal)
-2. 0. /exp-14/ Background and its error (BLANK_Magic or BLANK_Vert. curve)
-2. 0. /exp-15/ Background and its error (BLANK_Horizontal decay curve)
0 /exp-16/ Flag for entry of data 1./variance: 0=calculated, 1=given

The last parameter file contains all MemSys control variables which are rarely changed:

File: memsys.prm

500 /memsys-1/ Maximum number of Iterates
30 /memsys-2/ Number of iterates between plots on screen
1 1 /memsys-3/ Method options
10 /memsys-4/ LEVEL of diagnostic output
1.0 /memsys-5/ Aim
0.10 /memsys-6/ Tolerance
0 /memsys-7/ Number of samples for movies
4321 /memsys-8/ Iseed
1 /memsys-9/ Nrand
0 /memsys-10/ VESA-defined mode supported
1 / memsys-11/ Flag for graphics on screen, 0=No 1=Yes

To run <u>interactively</u> the program PULSE5

Notational Convention: *Italics* indicate questions asked by the program
Bold indicates the answers to the question

Load your program as usual.

Preliminary Question:

- -

Please enter the FILENAME of analysis PARAMETERS
to run the program in "batch" mode

OR enter TWO blank characters for running the program interactively

- -

R: This question asks the name of the first parameter file containing the answers to the questions for the selected analysis.
===> If you wish to run the program automatically, please enter the filename, up to 12 characters in the format *.xxx (no more than three characters after the point).
For demo supplied: **test-1.go**

===> If you wish to run the program interactively, please enter two blank or dash characters.

Then the program asks the following questions. These are printed below with an explanation of the response and suggested values from the test-1.go file supplied with the package:

Q: / 1/ *Please enter the Filename for the instrument parameters*
and additional experimental conditions
R: The length of the filename is maximum of 12 characters
For demo supplied: **test-1.prm**

Q: / 2/ *Enter the Filename of the analysis listing*
R: The length of the filename is maximum of 12 characters. The first eight characters will be automatically imposed as the main part of the filenames *.dis and *.crv , *.tab for final outputs (see below).
For demo supplied: **test-1.log**

Q: / 3/ *Enter the Filename of the I_Magic or I_Vertical decay curve*
R: The length of the filename is maximum of 12 characters
For demo supplied: **test-1.im**

Q: / 4/ *Enter the Filename of the I_Horizontal decay curve*
R: The length of the filename is maximum of 12 characters
For demo supplied: **bid.dat**

Q: / 5/ *Enter the Filename of the FIRST Flash (or Reference decay curve)*
R: The length of the filename is maximum of 12 characters
For demo supplied: **excit.fl**

Q: / 6/ *Enter the Filename of the SECOND Flash (or Reference decay curve)*
R: The length of the filename is maximum of 12 characters
For demo supplied: **bid.dat**

Q: / 7/ *Enter the Filename of the BLANK_Magic or BLANK_Vertical decay curve*
R: The length of the filename is maximum of 12 characters
For demo supplied: **bid.dat**

Q: / 8/ *Enter the Filename of the BLANK_Horizontal decay curve*
R: The length of the filename is maximum of 12 characters
For demo supplied: **bid.dat**

Q: / 9/ *Please give 3 lines of text for identification*
and comments on the data (80 characters max/line)
R: A text line is 80 character length maximum.
For demo supplied:
7-August-1998 DEMO test: PULSE5.EXE Mock data convolved with excit.fl file
Four exponentials; taus: 0.05 0.15 0.30 0.8ns / Alpha's: 0.40 0.35 0.20 0.05
(NO Correlation time)

Q: /12../ *Enter Tau_Minimum, Tau_Maximum, Number of lifetimes*
-if the number of lifetimes is set positive then LOG scaling
-if it is set negative then scaling is LINEAR
R: "Tau" means τ . These are the lower and upper limits of lifetime domain (in nanosecond) in use to delimit the range of decay times to be considered by the program. The lifetime values will be spaced logarithmically or linearly between these limits. An entry of a negative value is a flag to select linear scaling (the absolute value is used in the program).

Once the number of lifetimes is sufficiently large to capture all the structure in the data, then increasing it will not affect the shape of the result (and will merely increase the computing time approximately linearly). Thus it is recommended to have the number of points large enough for the digital resolution required.
Values for demo supplied: **0.015 1.5 150**

Q: /12../ *Enter Tau_Minimum, Tau_Maximum, Number of lifetimes*
-if the number of lifetimes is set positive then LOG scaling
-if it is set negative then scaling is LINEAR
R: Five lifetime domains can be defined as far as the total number of lifetimes does not exceed the limit of 200 set in the program.*
Values for demo supplied: **0.0 0.0 0**

Q: /13/ Enter a flag for addition of SCATTERED LIGHT
0 = No scattered light
1 = Yes

R: Excitation light may be scattered directly into the detector if the filters are not perfect or if Raman scattering may occur. Scattered light is equivalent to a null emission lifetime. Therefore, if a scattering correction has to be determined, enter 1, if not enter 0. The amount of excitation light in the data is assumed to be "positive". If the response is 1, the first pixel in the recovered distribution (in the result file *.dis) contains the amount of scattering in the data.
Value for demo supplied: **0**

Q: /14/ *Enter a flag for addition of BACKGROUND*
0 = No background added
1 = Yes

R: If the background has been wrongly determined, the program can correct for this. The background is equivalent to an infinite lifetime t_{∞}. Respond 0 for no correction allowed. Respond 1 for more background only (the extra amount required will be the value of the last channel in the spectrum of τ).
Value for demo supplied: **0**

Q: /15/ *Addition of up to five SINGLE EXPONENTIAL,*
(outside of the already set lifetime domains).
Please enter FIVE values which are, either:
positive for the selected lifetime values (ns)
or null if not in use

R : Where the sample contains compound having a single exponential fluorescence decay and if the corresponding lifetimes are already precisely known, then the program needs these values as a prior information in order to supply the best reconstruction of lifetime distribution allowed by the data. For example that is recommended in studying a mixture of free and bound dye system.
Values for demo supplied: **0.0 0.0 0.0 0.0 0.0**

Q: /exp-1/ *Enter the date of measurements (DD-MM-YY)*

R: This date is used for identification of the test-1 file.
for demo supplied: **02-10-95**

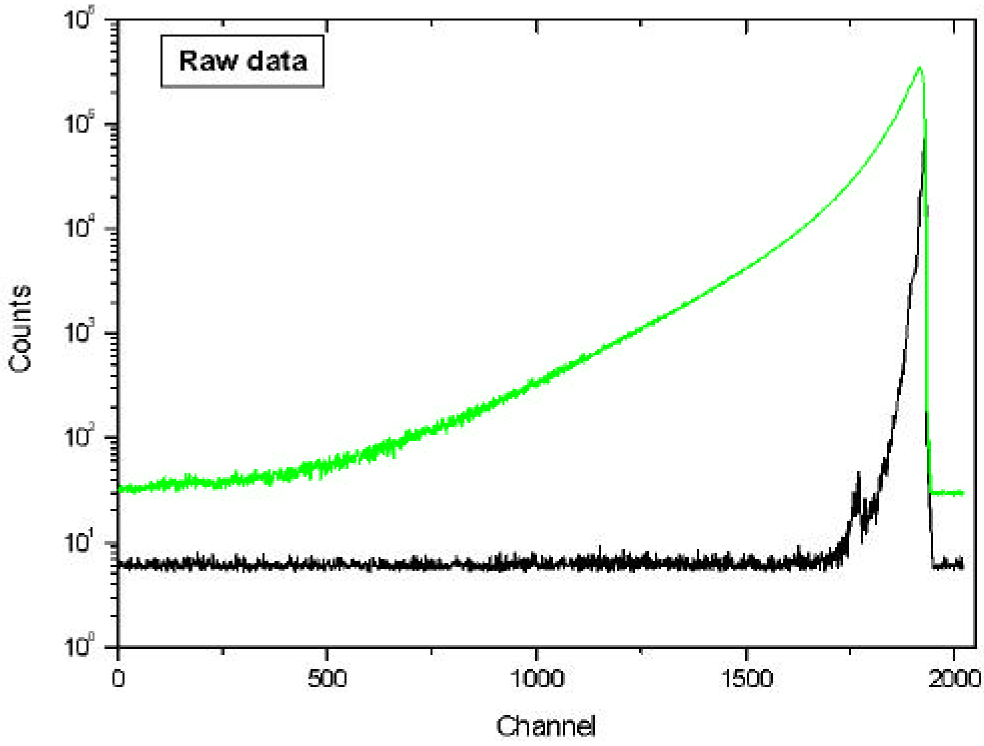

Figure 1a

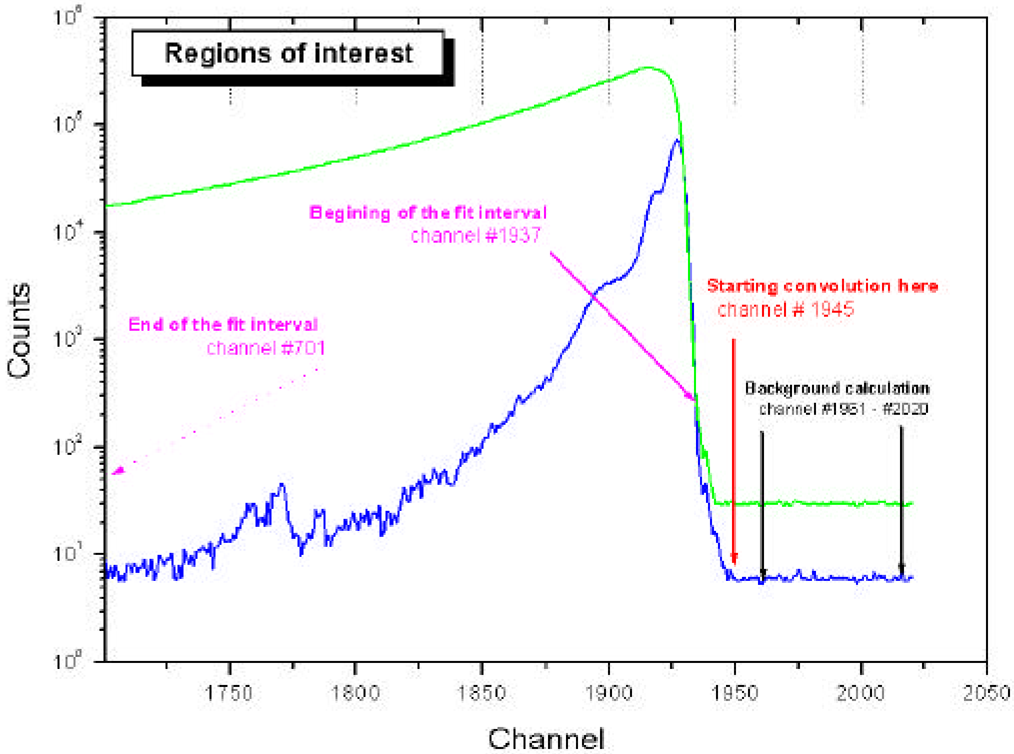

Figure 1b

Q: /exp-2/ *Enter the nanosecond/channel value of your Mutichannel Analyzer*
R: This is the number of nanosecond between successive data channels in the MCA..
Value for demo supplied: **0.004**

Q: /exp-3/ *Enter a flag for reversed data curve: 0 = No, 1 = Yes*
AND
a value for a data compression (if needed): 1, 2 or 3
AND a Letter coding for data format
R: The letter E code for the "Edimburg" data format: the 20 first lines of comment and then the data in a single value colomn. Other data foramt are available.
Value for demo supplied: **1 1 A**

Q: /16/ *Give the channel numbers which delimit Fluorescence decay*
AND Background domains
R: These are the markers (channel number) which delimit the useful fluorescence decay and markers which define the zone where an average background can be calculated. The background markers must be given even if the backgrounds (and its error bar) are subsequently imposed by the user. In this later case the background marker positions will be ignored. See figure 1b for color code.
Values for demo supplied: **701 1945 1961 2020**

Q: /17/ *Indicate the channel number in the leading edge*
of the fluorescence decay from where the CHI-SQUARE starts
to be calculated
Value for demo supplied: **1937**

Q: /exp-4/ *Enter a flag for the excitation polarisation*
AND give a G_Factor value for the I_horizontal decay
Flag=0 for Unpolarised excitation
Flag=1 for Vertically polarised excitation
G_factor= 0. for I_magic fluorescence decay
R: Flag should be 0 or 1. If not, it is set automatically to 1.
The G-factor corrects for the relative transmission and detection of the vertically and horizontally polarised light. If it is set zero the program will assume that the data correspond to a fluorescence measured at the magic angle or fully depolarised.
Values for demo file supplied: **1 0.000**

Q: /exp-5/ *Enter the coefficient for the Blank_magic (Blank_vertical) curve*
R: This is the multiplication factor by which the measured blank fluorescence must be multiplied before addition to the vertically polarised component (or I_magic) data. If no blank was used or detected this factor should be set to zero.
(A **negative** coefficient should be given for a blank **subtraction**).
Value for demo supplied: **0.0**

Q: /exp-6/ *Enter the coefficient for the Blank_horizontal curve*
R: see question /exp-5/

Value for demo supplied: **0.0**

Q: /exp-7/ *Give the lifetime of the Reference (in ns) if measured*
instead of the true excitation profile.
Enter 0.0 for a direct excitation flash measurement

R: Respond zero if the flash was measured directly. Give the lifetime of the known référence chemical if a fluorescence decay is used instead.

Value for demo supplied: **0.0**

Q: /exp-8/ *Enter a flag for the use of flash/reference curves*
flag=0 for only one curve in use
flag=1 for a mean of two curves
flag=2 for a sum of the two polarised reference decays

Value for demo supplied: **0**

Q: /exp-9/ *Enter the frequency of the excitation pulse (in Mhz)*
R: This is the repeat frequency of the excitation flash. It is necessary to ensure that any breakthrough of decay from a previous flash is correctly added to the signal from subsequent flashes.
Value for demo supplied: **4.0**

Q: /18/ *Enter a time shift for the excitation profile (in channel unit)*
AND a flag for convolution type
R: Sometimes a shift in the origin occurs between the measurement of the flash and the fluorescence. The program can correct for such a known flash shift. Enter the shift (positive or negative in channel unit. By adjusting step by step the shift value an improvements in the randomness of the first residuals, i.e. around the peak of the flash, may occur with a significant reduction in the final chi-squared. This parameter is generally very difficult to adjust without any additional information from a specific experiment.
The convolution type should always set to 1 (reserved for future developments)
Value for demo supplied: **0.0** **1**

Q: / exp-10/ *Give the background and its error for the I-Magic or I_Vertical decay curve:*
R: Enter the background (dark noise) in this decay together with an estimate of the accuracy of this guess. If the background is given as a negative number the program will calculate an averaged background and its accuracy between the marker limits given above at question 16.
Value for demo supplied: **-2.00 0.0**

Q: / exp-11/ *Give the background and its error for the I_Horizontal decay curve:*
Value for demo supplied: **-2.00 0.0**

Q: / exp-12/ *Give the background and its error for*
the FIRST Flash (or Reference decay curve):
Value for demo supplied: **-2.00 0.0**

Q: / exp-13/ *Give the background and its error for*
the SECOND Flash (or Reference decay curve):
Value for demo supplied: **-2.00 0.0**

Q: / exp-14/ *Give the background and its error for*
the BLANK_Magic or BLANK_Vertical decay curve:
Value for demo supplied: **-2.00 0.0**

Q: / exp-15/ *Give the background and its error for*
the BLANK_Horizontal decay curve:
Value for demo supplied: **-2.00 0.0**

Q: / exp-16/ *Enter a flag for calculated or given accuracies on data*
(reciprocal standard deviations)
flag=0 for calculated values
flag=1 for given values in file "nameiv".acc

R: The accuracies on time-correlated photon counting can be simply calculated from the Gaussian approximation of the Poisson statistics providing the counts in a channel exceed 20.

Recently it appears that the statistics is a compound statistics if the counts per channel are strongly correlated (ref. 1 and ref. 2). The user can either ask the program to calculate the accuarcies (inverse of the standard deviations) or to enter these accuracies from a separated calculation. The corresponding input file should have the same root filename as the decay filename with "acc" as an extension name (for example from the demo file: mem-deca.acc).

Value for demo supplied: **0**

Q: /19/ *Is the analysis starting from the beginning*
or continuing from a previous run ?
Enter 1 for starting from the beginning
Enter 0 for continuing with a distribution saved from a previous run

R: If a run has stopped after the imposed number of iterations (see memsys.prm file) without termination to the most probable solution (which is reached for an OMEGA value close to 1.0), the calculation can be continued from the previous result for additional iterates, preventing the need to repeat initial calculations. That often happens if the rates (see question 23 below) are too small and slow down the progress on the entropy trajectory. The user must reply 0. Then the files *.are and *.sav allocated to the previous partial result are automatically read in.

Value for demo supplied: **0**

Q: /20/ *Setting up the default value of the initial flat distribution.*
Enter a value,
positive : the default level is imposed at this value
null : the level is calculated from the decay and flash surfaces
*negative X: the calculated default level is then multiplied by 10**X*
If continuing an analysis, please enter any value.

Value for demo supplied: **0.0**

Q: /21/ *If starting from the beginning,*
do you want to use a PRIOR MODEL of distribution ?
Enter: 0= No , 1= Yes
- If yes, the filename of the model distribution
should be model.dis (see PULSE5 users" manual)
- If no , the model is assumed to be flat and set
at the same level as the starting flat distribution

R: The MaxEnt formalism allows the user to test whether there is evidence in the data for a deviation from some expected (model) distribution of lifetimes. (This model might be, for example, either expected from theory or measured at some different physical conditions).

Usually there is no model and the expectation is set to be flat over the expected range of solution at the default level and reply 0.

Otherwise the user should respond 1 and the file model.dis containing the expected distribution of lifetimes is automatically read in from the file model.dis The scaling in τ should identical to those given in question 12, see above).

Value for demo supplied**:** **0**

Q: /memsys-1/ *Enter the maximum number of iterates for the calculation*

R: The automatic convergence criteria are very powerful, therefore if more than 500 iterations are run or the program does not converge within 500 iterations, check for an error in the input parameters (check also the *.log output file), a fault in the required lifetime domain or an error in the data format or the presence of large systematic errors (check the residuals in the *.crv file).

Value for demo supplied: **500**

Q: /memsys-2/ *Enter the number of iterates between graphics update*

R: The indicates to the program to plot on screen the current and previous lifetime distribution h(τ). An other plot can be display for the chi-square and Omega progress along the iterations (see question 22 below)

Value for demo supplied: **30**

Q: /22/ *Enter two flags for graphics updates*
of the progress of the calculation : 1=yes, 0=no
First flag for the distribution h(tau)
Second flag for chi-square and omega progress

R: This enables two graphics pages to be displayed: first the intermediate h(τ) distributions and second the chi-square and Omega progress. That may be of a particular interest if the selected rates for driving the convergence are not appropriate i.e. too low or much too high.

Value for demo supplied: **1 1**

Q: /23/ *Enter Rate1, Rate2 and Rate3*
[if the entry is 0. 0. 0. then the default values
are set to -0.5 -0.15 -0.05]

R: Rate controls the change in the distribution which is allowed in each iterate. Small values ensure that the MaxEnt trajectory is closely followed but the computation time can be long. Using higher values allows the MaxEnt algorithm more freedom and although the computation may be dramatically faster but there is an increased risk of the program diverging from the MaxEnt trajectory ,not converging or losing its way. The symptoms are that the program goes to the maximum of allowed iterates with Test, Entropy, Omega, Chi-square diagnostics bouncing and failing to converge or in extreme cases losing its way and getting very high values.

Similarly, setting rate negative provides further freedom and shorter computation times but does not enhance the risk of convergence not being reached.

Although the chance of the program not converging is small, the setting of rate id dependent on the quality of data presented to MaxEnt. Therefore, where time is important, the user should explore how much the rate setting may be "abused" for the types of data being processed.

In general, the large rate Rate1 will enable the program to initially converge rapidly during few tens of iterates and the rate is lowered as OMEGA reaches 0.1. Then the rate can be lowered at the Rate2 value till OMEGA reaches value of 0.80 and at this stage of the trajectory the rate is automatically set to the Rate3 value.
Value for demo supplied: **-0.5 - 0.10 - 0.02**

Q: /memsys-3/ *Enter the two selected MaxEnt options*
R: Enter two integer values for setting METHOD options:
The first sets the stopping criterion:
1 for the classic maximum entropy
2 for the classic automatic, with noise scaling
If there are no large systematic errors in the data and if the error bars on data are precisely known, the option 1 is recommended. The program should automatically terminate successfully at OMEGA=1.0 and chi-square at the lowest value allowed by the data, ideally equal to 1. However the presence of large systematic errors will prevent this target from being reached, in which case we recommend the user to test the automatic noise scaling, option 2 .
The second value sets the type of entropy:
1 for the standard entropy
2 for the positive /negative entropy
Value for demo supplied: **1 1**

Q: /memsys-4/ *Enter the level of diagnostic outputs*
R: Enter a value for setting the diagnostic type
Value for demo supplied: **10**

Q: /memsys-5/ *Aim value*
R: This value ought to be set to 1.0 . Setting aim to a larger value forces the algorithm to proceed less down the trajectory towards fitting the data less closely.
Value for demo supplied: **1.0**

Q: /memsys-6/ *Tolerance*
R: This value is a user-defined tolerance which control the precision of the MaxEnt calculations. For most of fluorescence decay data set, the default value is set to 0.1
Value for demo file supplied: **0.1**

Q: /memsys-7/ *movies*
R: This parameter should be set to zero
Value for demo file supplied: **0**

Q: /memsys-8/ *seed*
R: That is the seed for the internal pseudo-random number generator.
Value for demo file supplied: **4321**

Q: /memsys-9/ *Nrand*
R: That is the number of random vectors to use to calculate the evidence.

It is normally set to 1
Value for demo file supplied: **1**

Q: /memsys-10/ *VESA-defined mode supported*
R: Most graphics have several modes available which define the resolution and number of colors. In most of cases the program detect automatically the video hardware to be supported (enter 0 value). If not you can select the VESA-defined mode corresponding to your hardware. For example, enter 16 for a resolution 640 by 350 and 16 colours.
Value for demo file supplied: **0**

Q: /24/ *Automatic peak-finding?, 0 = No , 1 = Yes*
R: In entering a zero value, the user can impose the lower and upper limits of peaks to analyse in the recovered distribution. If this option for the imposed selection of peaks (0 value) is given, then the program asks for the lower and upper limits of each selected peak. For example, this option may be useful in case of a soft shoulder in a broad peak which cannot be detected by the automatic search peak.
Value for demo supplied : **1**

Q: /25/ *Please enter lower and upper peak markers.*
0,0 ==> exit of peak analysis routine.
R: The two markers of a region of interest (ROI) are given in each of the next lines.
Please enter 0 0 if there is no more ROI to consider. The number of ROI in the distribution is arbitrarily limited to 20.
Recommended value for demo supplied : **0 0**

4. Output Files:

The program produces a number of results and diagnostic on the analysis.

The corresponding files have a common root name with the "*.log" filename which is given by the user (for the test supplied **test-1.log)** but they have specific extension names.

test-1.log file contains information on the data : integral, peak position, average lifetime etc... It recall the lifetime domain parameters and the MaxEnt options in use. It provides diagnostics on the progress of the maximum entropy trajectory. The final distribution and the corresponding residuals are recorded along with a peak search results. Finally the error bars on the peak parameters are also given.

test-1.dis file contains six columns corresponding respectively to:
-1 the "pixel" values
-2 the lifetimes in nanosecond
-3 the pre-exponential factors h(τ)
-4 the peak label of the recovered distribution
-5 the % normalised pre-exponential factors h(τ) ; the scattered light content is not included

$$f_i = \frac{h_i}{\sum_{i=1}^{i=N} h_i} *100.$$

-6 the percentage f_i to the steady-state fluorescence intensity for each lifetime.

$$f_i = \frac{h_i}{\sum_{i=1}^{i=N} h_i}$$

In case of scattered light option, the corresponding lifetime should normally be zero and the X-plot of lifetime in log scale may be difficult. The corresponding lifetime is artificially set to one tenth of the MCA channel value in nanosecond and set the corresponding "pixel" value to zero. In the same way the background positive option comes with a pseudo lifetime of ten times the greatest lifetime set in the distribution.

test-1.tab file is a table which summarise the peak search in the distribution. The associated error bars are also given. This file may be directed directly to the printer.

test-1.crv file consist of six columns corresponding respectively to:

1) the channel number or time in picoseconde (entire value)
2) the flash intensity
3) the fluorescence intensity (data) after background subtraction.
4) the calculated fluorescence decay
5) the residuals
6) the autocorrelations values

test-1.dec file consist of 7 columns corresponding respectively to:

1) the channel number
2) the flash intensity
3) the fluorescence intensity (data) after background subtraction.
4) the calculated fluorescence decay corresponding to the peak number 1 (τ_1=0.051)
5) the calculated fluorescence decay corresponding to the peak number 2 (τ_2=0.156)
6) the calculated fluorescence decay corresponding to the peak number3 (τ_3 =0.302)
7) the calculated fluorescence decay corresponding to the peak number3 (τ_4 =0.799)

and so on

test-1.are and **test-1.sav** are the files which contain all the areas and internal parameters saved within the computer after the last iteration.

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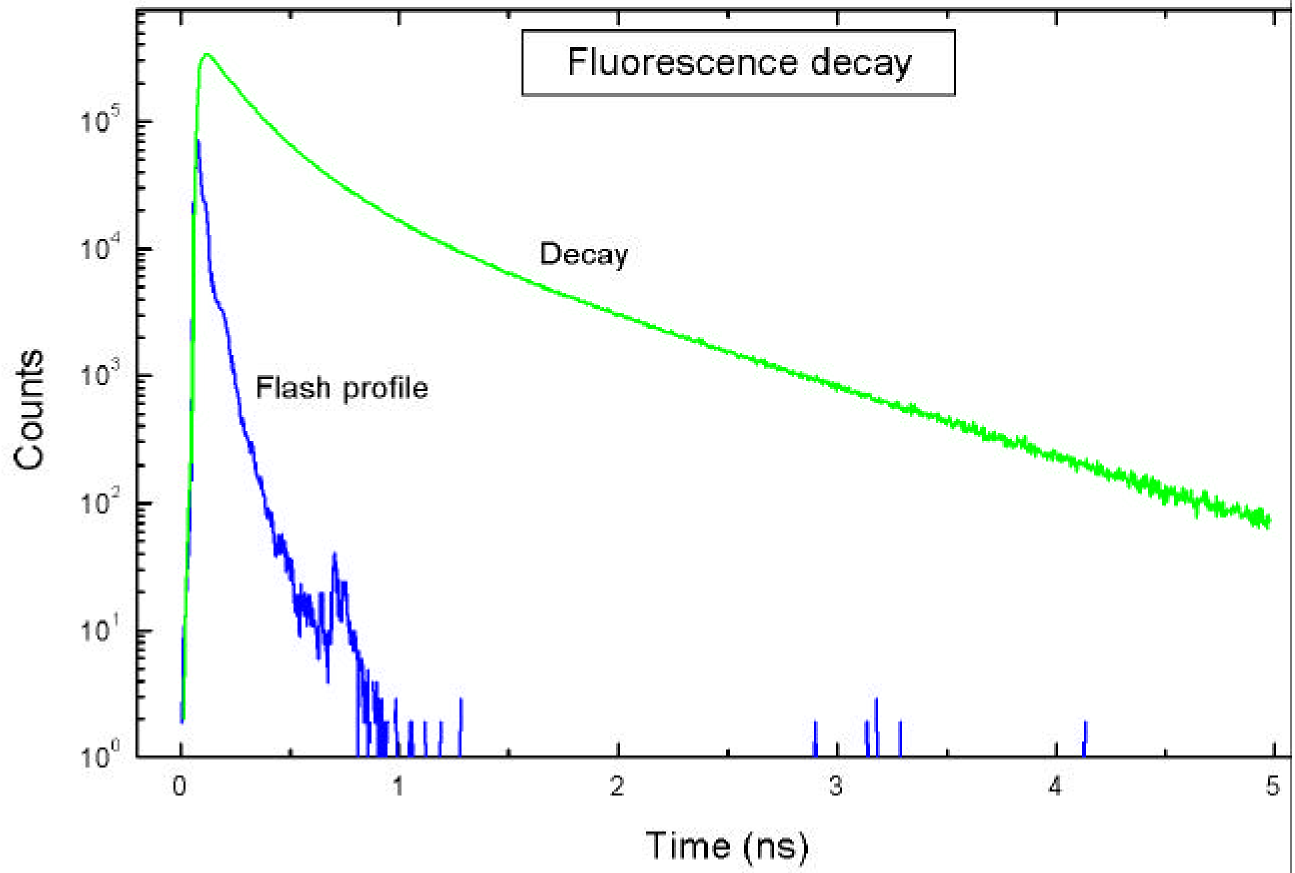
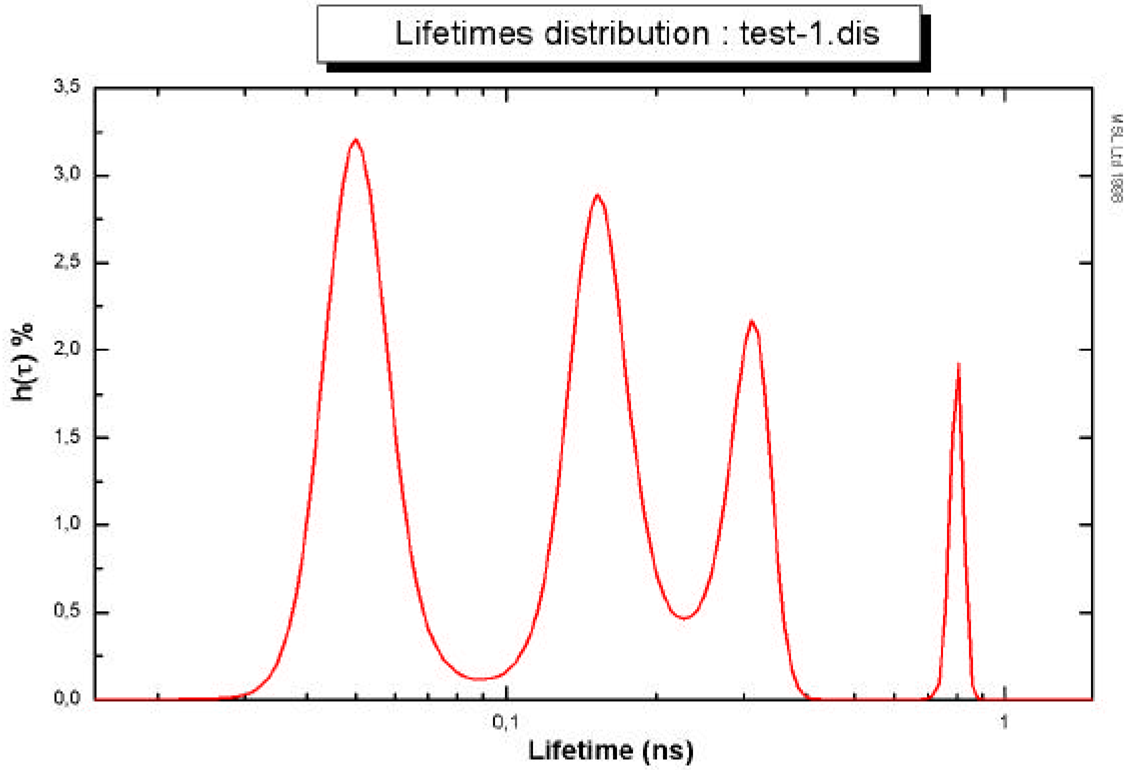
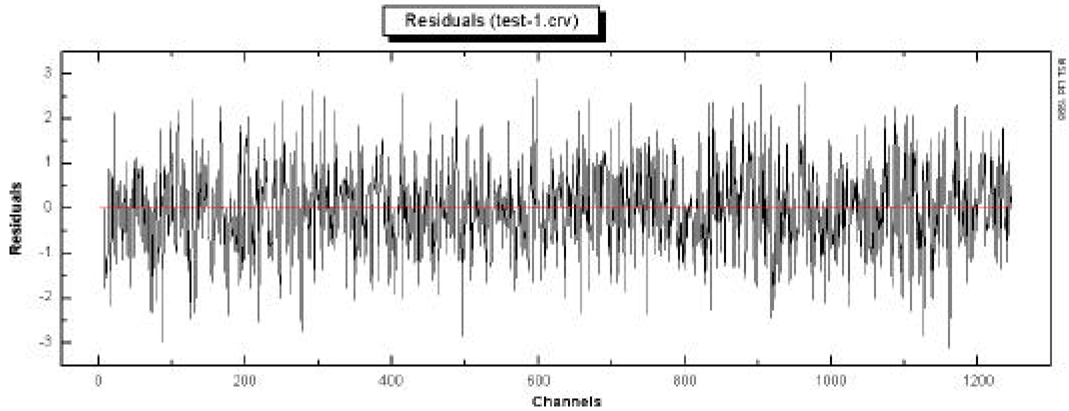
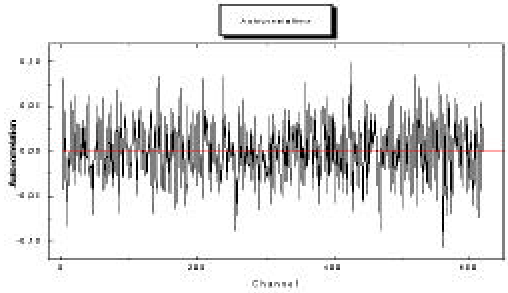

Fluorescence decay
Decay
Flash profile
Counts
Time (ns)
10^5
10^4
10^3
10^2
10^1
10^0
0
1
2
3
4
5



Lifetimes distribution : test-1.dis
h(τ) %
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,1
1
Lifetime (ns)


Residuals (test-1.crv)
Residuals
Channels
3
2
1
0
-1
-2
-3
0
200
400
600
800
1000
1200



```
Filename: test-1.log              3/12/1998  at  7h34

          *******************************************************
          *                                                     *
          *                                                     *
          *     PPPP   U   U   L       SSS   EEEEE    55555     *
          *     P   P  U   U   L      S      E        5         *
          *     PPPP   U   U   L       SS    EEEE     5555      *
          *     P      U   U   L         S   E            5     *
          *     P       UUU    LLLLL  SSS    EEEEE     555      *
          *                                                     *
          *                                                     *
          *                                                     *
          *              Pulsed Fluorescence Analysis           *
          *                           by                        *
          *               Quantified Maximum Entropy            *
          *                                                     *
          *                                                     *
          *                                                     *
          *                                                     *
          *  J-C Brochon & MSL                                  *
          *  Version 1.21 , Winter 1997                         *
          *  Copyright (C) 1995,1996,1997                       *
          *******************************************************

          < Unauthorized access to this program is forbidden >


 7-August-1998 DEMO test: PULSE5.EXE Mock data convolved with excit.fl file
 Four exponentials; taus: 0.05 0.15 0.30 0.8ns / Alpha's: 0.40 0.35 0.20 0.05
 (NO Correlation time)

...............................................................................



               ****   LIFETIME DOMAIN 1   ****

     Smallest lifetime .......................   0.015 ns
     Largest  lifetime .......................   1.500 ns
     Number of lifetimes .....................150
     Log scaling

     No Scattered light channel in use
     No Background channel in use
     No additional single lifetime channel
     Total number of parameters .............. 150


     ****    DATA MEASUREMENT PARAMETERS    ****

     Analysis assuming ...........  0.0040 ns/channel
     The following M.C.A. channels were used to
     delimit fluorescence and background data :
      N1 Fluorescence channel ..................  701
      N2 Fluorescence channel .................. 1945
      N-CHI2 limited at channel ................ 1937
      Number of data points set "unmeasured" in
      the fluorescence leading edge was ........    8

      N1 Background channel .................... 1961
```

```
 N2 Background channel .................... 2020
Excitation was vertically polarised.
Magic angle fluorescence decay analysed.

"Blank" solution were NOT measured.
```

```
     ****  EXCITATION PARAMETERS ****

Frequency of the excitation ...........  4.000 MHz
Convolution calculated with "n" excitation curves.
Convolution calculated with true excitation data.
 with a polynomial approximation of degree 1
Flash (Reference) curve was NOT shifted.


     ****          DATA           ****

I_vertical or I_magic fluorescence ... test-1.im
Peak channel in M.C.A. was ...........  1915
The corresponding peak value was ..... 342042.
The total counts between markers was 25868098.
The background level was automatically calculated:
 This background was ................   0.00
 The corresponding error was .........   0.00


First FLASH or Reference decay ....... excit.fl
Peak channel in M.C.A. was ...........  1927
The corresponding peak value was .....  71251.
The total counts between markers was   759031.
The background level was automatically calculated:
 This background was ................   0.07
 The corresponding error was .........   0.03

Total SAMPLE fluorescence decay:
Peak channel ........................  1915
The corresponding peak value ......... 342042.

CURVE INTEGRALS:
 Excitation .........................   0.7589480E+06
 Fluorescence  ......................   0.2586810E+08
Mean lifetime .......................   0.338 ns
Default level * N_tau ............... 0.305738E+06
The estimated default level.......... 0.203825E+04
The total switched out data points are    8
Starting analysis from a flat map:
 The estimated default level was .....   2038.2515
Constant default level used as a model


Maximum vector sizes:
 Convolution time domain (data) ...... 4000
 Lifetimes+Scatter+Background ........  202


MEM parameters were:
Method  ===   1  1
Level   ===   10
Aim     ===   1.00E+00
Default ===   2.0383E+03
Rate1   === -0.50 up to Omega =0.10
Rate2   === -0.10 up to Omega =0.75
Rate3   === -0.02

++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
  The 150 lifetimes of the distribution are:
  0.015   0.015   0.016   0.016   0.017   0.018   0.018   0.019   0.019   0.020
  0.020   0.021   0.022   0.022   0.023   0.024   0.025   0.025   0.026   0.027
```

```
  0.028   0.029   0.030   0.031   0.031   0.032   0.034   0.035   0.036   0.037
  0.038   0.039   0.040   0.042   0.043   0.044   0.046   0.047   0.049   0.050
  0.052   0.053   0.055   0.057   0.058   0.060   0.062   0.064   0.066   0.068
  0.070   0.073   0.075   0.077   0.080   0.082   0.085   0.087   0.090   0.093
  0.096   0.099   0.102   0.105   0.108   0.112   0.115   0.119   0.123   0.127
  0.131   0.135   0.139   0.143   0.148   0.152   0.157   0.162   0.167   0.172
  0.178   0.183   0.189   0.195   0.201   0.208   0.214   0.221   0.228   0.235
  0.242   0.250   0.258   0.266   0.274   0.283   0.292   0.301   0.310   0.320
  0.330   0.340   0.351   0.362   0.373   0.385   0.397   0.410   0.422   0.436
  0.449   0.463   0.478   0.493   0.509   0.524   0.541   0.558   0.575   0.593
  0.612   0.631   0.651   0.672   0.693   0.714   0.737   0.760   0.784   0.808
  0.834   0.860   0.887   0.915   0.944   0.973   1.004   1.035   1.068   1.101
  1.136   1.171   1.208   1.246   1.285   1.326   1.367   1.410   1.454   1.500
++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++

  Sum of contents of spectrum ........ 0.6045164E+06
  Iterations executed , ISTAT ........   30 / 500     001011
  Current ENTROPY ,ISTAT ............ -0.355700E+06  001011
  Current CHI2, OMEGA ...............  0.105149E+01  0.229091E-01

  Sum of contents of spectrum ........ 0.6010486E+06
  Iterations executed , ISTAT ........   60 / 500     001010
  Current ENTROPY ,ISTAT ............ -0.563222E+06  001010
  Current CHI2, OMEGA ...............  0.101304E+01  0.611090E+00

  Sum of contents of spectrum ........ 0.6008844E+06
  Iterations executed , ISTAT ........   90 / 500     001001
  Current ENTROPY ,ISTAT ............ -0.639250E+06  001001
  Current CHI2, OMEGA ...............  0.101182E+01  0.985292E+00

  Sum of contents of spectrum ........ 0.6009516E+06
  Iterations executed , ISTAT ........   98 / 500     000000
  ENTROPY, ISTAT: Final Values ....... -0.639250E+06  000000
  CHI2, OMEGA  ......................  0.101242E+01  0.103041E+01 (x_def= 0.)


                    MEM DISTRIBUTION OF EXPONENTIALS

           0.17          0.20          0.25          0.32          0.40
           0.51          0.65          0.84          1.10          1.46
           1.95          2.63          3.59          4.96          6.92
           9.78         13.96         20.17         29.46         43.46
          64.71         97.09        146.57        222.14        337.17
         511.11        771.27       1154.58       1708.06       2487.37
        3550.92       4948.76       6705.57       8799.34      11141.47
       13567.65      15846.08      17712.05      18918.74      19298.79
       18804.24      17522.09      15646.52      13429.87      11124.64
        8937.30       7002.55       5386.17       4095.45       3101.22
        2356.66       1811.31       1418.73       1140.47        947.27
         817.64        736.96        696.07        690.59        720.25
         789.08        906.03       1086.11       1352.13       1736.72
        2283.08       3045.56       4083.07       5448.69       7166.44
        9203.25      11440.75      13663.36      15581.99      16895.69
       17379.33      16956.73      15728.55      13931.97      11865.02
        9799.39       7931.21       6365.29       5128.12       4200.92
        3539.94       3103.26       2854.45       2774.20       2857.80
        3118.55       3590.36       4324.52       5379.69       6797.69
        8550.63      10466.04      12151.93      13038.74      12599.90
       10706.89       7830.38       4849.97       2514.67       1087.87
         394.15        121.27         32.36          7.73          1.72
           0.37          0.08          0.02          0.01          0.00
           0.00          0.00          0.00          0.00          0.00
```

```
        0.00          0.02          0.13          0.97          8.68
       77.19        582.65       3068.68       9035.64      11568.76
     4906.48        511.04          9.57          0.02          0.00
        0.00          0.00          0.00          0.00          0.00
        0.00          0.00          0.00          0.00          0.00
        0.00          0.00          0.00          0.00          0.00

  First residuals are:
    0.0000        0.0000        0.0000        0.0000        0.0000
    0.0000        0.0000        0.0000        -1.779        -1.133
    -1.548       -0.5742       -0.2616        0.8916        0.7741
   -0.5975        -2.157        0.6653        0.3289       -0.6638
     2.146       -0.8602        -1.236       -0.8713        0.3960
    0.3783       -0.3919        0.4866        0.6107        0.1895
    0.2526        -1.140       -0.2822        0.5484       -0.7186E-01
    -1.044       -0.1663         1.030       -0.5301        0.5670E-01

  Top of the flash/Ref. is at channel   19

  Top value in the lifetime distribution = 0.19299E+05
  Then the level of discrimination for peak search is set to: 0.19299E+01


++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
Sum of h(tau)=.600952E+06      + or - 0.810E+03  ==>    0.13 %

   Tau_mean     =   1.7230E-01    + or - 2.252E-04  ==>   0.131 %

++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++


                        ********************
                        *  PEAK POSITIONS  *
                        ********************

     Left marker  11      Right marker 59     Top position 40
     Left marker  60      Right marker 89     Top position 76
     Left marker  90      Right marker109     Top position 99
     Left marker 125      Right marker133     Top position130



                        *** PEAK NUMBER= 1 ***

     Total flux = 0.2438E+06   + or -  1.583E+04   ==>    6.49 %
     Centroid   = 5.1457E-02   + or -  2.555E-03   ==>    4.96 %
     Dispersion = 9.1914E-03   + or -  4.878E-03   ==>    53.07 %
-----------------------------------------------------------------------


                        *** PEAK NUMBER= 2 ***

     Total flux = 0.2170E+06   + or -  2.619E+04   ==>   12.07 %
     Centroid   = 1.5601E-01   + or -  9.282E-03   ==>    5.95 %
     Dispersion = 2.6412E-02   + or -  1.204E-02   ==>    45.57 %
-----------------------------------------------------------------------


                        *** PEAK NUMBER= 3 ***

     Total flux = 0.1104E+06   + or -  2.216E+04   ==>   20.07 %
     Centroid   = 3.0208E-01   + or -  1.535E-02   ==>    5.08 %
```

```
     Dispersion = 3.2168E-02   + or -  1.749E-02   ==>    54.37 %
----------------------------------------------------------------------


                         *** PEAK NUMBER= 4 ***

     Total flux = 0.2977E+05   + or -  9.835E+02   ==>    3.30 %
     Centroid   = 7.9937E-01   + or -  7.786E-03   ==>    0.97 %
     Dispersion = 2.4996E-02   + or -  3.235E-02   ==>   129.43 %
----------------------------------------------------------------------
                       ===  Elapsed time:   1mn 35sec  ===
```