ARTEFACT AND DISTORTION SOURCES IN TIME CORRELATED SINGLE PHOTON COUNTING

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ABSTRACT

Sources for artefacts and distortions in data from a time correlated single photon counting spectrometer are described and illustrated. Methods are provided to avoid these instumental problems and to correct for the residual distortions.

1. INTRODUCTION

In time correlated single photon counting (TCPC) a straightforward determination of fluorescence decay times is an exception rather than the rule. Mostly an extensive computation technique $^{1-6}$ must be used to correct the results for the limited time resolution of the measurement system. For that purpose also the response function $f_{\Delta}(t)$ of the system to a "delta"-function (laser pulse or lamp flash) must be recorded. Because of the wide dynamic range of data from TCPC, (up to 5 decades of intensity vertical and several thousands of channels for the time scale), deconvolution and fit procedures can be applied with rather high accuracy.

Reliable results from these procedures require a high grade of reproducibility of the measurement system, reproducibility in producing data and distortions in that data. The first improvement in the reliability of the results is to optimize the equipment to give minimum distortions of the experimental data (high time resolution, good linearity, no background, etc.). The next step is to enhance the signal to noise ratio in the experimental data 6 . The last thing to achieve is a computational correction of the residual distortions.

2. EQUIPMENT

For the excitation of the sample we used a mode-locked continuous wave (cw) argon ion laser (CR 18 UV 7,8) or a synchronously pumped dye laser (modified CR 590), in some cases combined with frequency doubling techniques 9 . Pulses were supplied of up to some nanojoules per pulse and about 100 ps full width at half maximum (FWHM) for the UV and visible lines of the ion laser and about 5 ps FWHM for the dye laser output. For decreasing the 76 MHz repetition rate of mode-locked pulses a pulse picker was used at maximum repetition rate of 300 kHz 10 .

Most times our (biological) samples were in 10 mm pathlength fluorescence cuvets. In addition to room temperature experiments also liquid nitrogen or helium cryostats could be used. The fluorescence was detected at an angle of 90° with respect to the exciting beam and fluorescence was separated from the excitation light by using cut-off filters, interference filters or a dual 0.25 m monochromator. In the case of fluorescence anisotropy measurements a 90° rotatable polarization analyzer could be placed in the detection pathway.

Depending on the detection wavelength we used different photomultiplier tubes (PMT's). The first experiments^{7,8} were carried out using a Philips XP 2020 PMT. Now we routinely use a variant of that tube with an S20 photocathode, the PM 2254B. For the very red and near infra-red detection wavelengths we used a Varian VPM 152A.10Z, having sensitivity up to 1150 nm. Because of the lower gain of that tube a fast amplifier (Ortec 574) was placed between the tube and the constant fraction discriminator (CFD, Ortec 934 or Canberra 1428A).

The reference timing signal was normally obtained from the pulse picker driver 10, the mode-locker driver 7 or from a photo-diode monitoring the excitation pulses. A 100 MHz discriminator (Ortec 436) was used to generate the fast negative logic pulses as normally used with nuclear instrumentation modules (NIM). The time to amplitude converter (TAC) was an Ortec 547 or a Canberra 2043, the latter also containing a single channel analyzer (SCA) for the analogue TAC output pulses. The latter TAC was used for the recording of time resolved spectra, counting the SCA output pulses with the multichannel analyzer (MCA) in multichannel scaling (MCS) mode.

For the calibration of the timescale an Ortec 462 module was used. The analogue-to-digital converter (ADC), MCS module and MCA came from Nuclear Data (ND570, ND88-0583 and ND66). The keyboard of the MCA was used to control the communication and data transfer to the university computer system (DEC10 or VAX785). For data analysis a package of Fortran 77 algorithms was available 13.

3. SURVEY OF EXPERIMENTAL PARAMETERS

In order to achieve optimum time resolution and reliability many parameters have to be checked. Here we provide a check-list

of essential parts with respect to time-resolved experiments, encompassing the whole measurement system. A common theme consists of the importance of reproducibility, requiring a stable mechanical and thermal environment.

3.1 MODE-LOCKING AND SYNCHRONOUSE PUMPING

The argon ion laser was acousto-optically mode-locked. References are given as examples for the description of theory $^{14-16}$ and practice of mode-locking in the UV 17,18 and visible region 19,20 . The acousto-optic (AO) crystal is driven by a synchronizer controlled (Marconi TF2173) voltage controlled oscillator system with temperature controller for the crystal (Harris Corporation).

This system gives rise to short term (ms) phase noise on the radio frequency (RF) drive frequency (38 MHz) and for critical applications such as synchronously pumping the dye laser, a synthesizer source (Schomandl ND60M) is available. Good modelocking requires the modulation frequency of the crystal and the round trip frequency of light in the cavity to be exactly equal.

The optical modulation depth required for proper mode-locking is dependent on the optical gain at the appropriate laser line. The effective gain can be influenced by changing the discharge current in the plasma tube and the reflectance of the output coupler. The amount of optical modulation of the AO crystal is controlled by the RF drive power and is dependent on the position of the drive frequency within the resonance band of the crystal, some tens of kHz wide. The resonance frequency of the crystal is controlled by the temperature, the higher the temperature, the higher the resonance frequency.

Next to the temperature setting of the controller, the effective temperature in the crystal is dependent on the amount of RF dissipation, ambient temperature (thermal isolation) and also the optical power in the cavity. A dynamic equilibrium can be attained by choosing the resonance frequency of the crystal somewhat higher than the mode-lock frequency. A minor increase in temperature, thus a shift of the resonance band to higher frequency, will result in less dissipation of the RF power supplied, and therefore in stabilization of the effective temperature. Only a part of the maximum possible modulation at given RF power is used because the crystal is driven off-resonance.

In reference 21 a method is described where the crystal is kept in-resonance by a feedback loop, monitoring the RF power reflected by the crystal and regulating the effective temperature by supplying the proper amount of RF power. Synchronously pumping^{9,22,23} requires the cavity lengths of pump and dye laser to be exactly equal. In reference 24 a description is given of the effects of cavity length mismatch. Another important part of

running the dye laser is the stability of the dye jet (mechanical vibrations, pressure fluctuations and minor air bubbles in the dye stream). In references 25 and 26 the realization of high quality jet streams is described.

Improper mode-locking and synchronouse pumping will result in PULSE BROADENING and the occurrence of AFTER PULSES, leading to DISTORTION of the recorded pulse response and decay functions. Mechanical instabilities (floor vibrations, pressure shocks arising from the cooling water supply, dye jet) give rise to PULSE JITTER and PULSE ENERGY FLUCTUATIONS. We monitor the laser outputs using high speed photodiodes²⁷.

For good results a passively air damped table 10 and a regulated air cooling system, stable within 1°C are essential. As described in reference 28, DEGRADATION of the mode-locking process can be initiated by partial or diffuse reflection of laser light into the cavity. In that case the feedback percentage and the delay of the optical path outside the cavity are important parameters.

3.2 PULSE PICKING

With pulse picking the extinction ratio of suppressed to transmitted pulse intensities must be as high as possible because poor suppression gives DISTORTION of the decay profiles. Of course deconvolution can recover the proper decay times but that requires complete reproducibility. And, in case of measurements of biological samples, a minimum amount of excitation energy should be used. When 1 out of every 1000 mode-locked pulses is picked with a contrast ratio of 1:250, only a quarter of the excitation energy will be used effectively.

Normally we use an electro-optical modulator in dual-pass 10 , resulting in an extinction ratio of better than 1:100,000. Cavity dumping gives much worse extinction ratios. The temperature sensitivity of properties of the modulator requires the ambient temperature to be stable within 1°C.

3.3 REFERENCE TIMING SIGNAL

This signal must be an exact and constant timing for the instant of excitation of the sample. The best way to achieve this is to split-off part of the exciting beam to impinge on a solid state photodiode and use a CFD to generate a fast NIM pulse for the TAC. This can cause problems when exciting in the blue or UV part of the spectrum, where solid state detectors exhibit low sensitivity. In that case a fast wired side-window PMT 29 , with only part of all dynodes used, is an alternative solution.

With respect to the latter solution it should be noted that the effects of transit time jitter of photon responses in the reference PMT are much less important than in the case of the single photon detecting main photomultiplier (see 3.6). When detecting laser pulses multiphoton, the final output pulse of the reference PMT will correspond to an average of transit times of photon events. The accuracy of the correlation in time between the peaks of the final output pulse and the laser pulse will depend on the number of photons detected per laser pulse.

Every jitter, drift or walk in the reference chain will cause BROADENING of the pulse response function of the equipment and therefore decrease the final time resolution. For convenience we can use a reference timing signal from the modulator driver that introduces some extra broadening and small and slow whills in time (see 6).

dependent transit time and discrimination levels. When discriminating multi amplitude pulses (like from the PMT anode but also by detecting laser pulses) always a CFD should be used. The best way to detect constant amplitude pulses is discriminating at half of the maximum amplitude, in most cases the steepest part, so that minor level fluctuations introduce minimum time shift. The slow drift of discriminator transit times directly decreases the accuracy of the experiment.

3.4 BACKGROUND EMISSION

For checking the contribution of emission of impurities (buffer), we always make a record of the sample without the experimental chromophore (probe) at an excitation power and time (dead time corrected) equal to that used for measuring the sample. At low optical density (≤ 0.1) of the sample at excitation and detection wavelengths the contribution of the "blank" can simply be subtracted from the data of the sample. At higher optical densities the data of the "blank" must be multiplied first by a certain factor 30 .

In this way also a correction is made for detected excitation light arising from insufficient emission filtering. At extremely low chromophore concentration (nM) and low fluorescence yield the ratio between emission and scattered light can be too low to have sufficient filtering from simple cut-off filters.

Even when focussing the beam of a mode-locked cw laser, the photon density is too low to introduce multiple photon effects that can disturb the measurements. In some cases a Raman signal can be detected as a fast component in the luminescence decay³¹. We have found Raman effects working with some porphyrin compounds. It can easily be recognized in emission wavelength selective experiments (see 5) or by shifting the excitation wavelength several nanometers.

3.5 DETECTION WAVELENGTH SELECTION

The processing of the fluorescence beam of the sample requires more attention than the thin colinear and coherent laser beam. All pathlength differences of different fractions of light in a beam will give a DECREASE of TIME RESOLUTION. Even high throughput optics, which efficiently collect the fluorescence light, will lead to a great difference of pathlengths of different fractions of light. The 1 cm pathlength in the cuvet will already introduce a time spread of some 50 ps.

The important conditions are the minimum concentration of chromophores, the maximum possible excitation energy and the maximum possible experimental time, also in connection with the long term stability of the equipment. When using higher concentrations, the exciting spot surface can be decreased at constant yield of detected fluorescence. With a simple thin lens an unprepared laser beam can be focussed to an exciting spot of some tens of microns diameter (see also 6). The fluorescence from a smaller spot can be collimated efficiently with a smaller aperture condenser lens, giving rise to smaller pathlength differences.

We usually work with biological samples, measuring fluorescence over a concentration range down to the nanomolar region and therefore had to increase the sensitivity at the cost of some time resolution. That proved to be a reasonable solution since the time resolution is mainly limited by the PMT.

When using cut-off or interference filters for fluorescence filtering one should be aware that the filter in the emission light path will introduce an extra time delay caused by the refractive index of the filters, resulting in an experimental SHIFT IN TIME (some picoseconds per filter). This also pertains to neutral density filters used to attenuate the exciting beam to proper intensity. The shift in time can be experimentally recovered by placing an uncoated (or anti-reflection coated) substrate, with equal (extra) time delay at the concerning wavelength, in case the filter is not used. Introducing a shift factor in the computer analysis is another way to correct for this effect.

We have built a compact and large aperture (38 mm) detection scheme for fluorescence anisotropy measurements, using filters for fluorescence selection. With a completely light-tight housing a high (many thousands per second) and steady yield of photons was found from some interference filters from Balzers (mainly in the UV and blue) when the coloured glass side of the filters was placed into the direction of the PMT. Turning the interference section of the filter into that direction almost eliminated the photon rate. First we thought of scintillation effects, but it appeared to be emission with lifetimes up to half an hour. Of course, fluorescing and phosphorescing filters were known to us³²

but we were not prepared for emission due to excitation with ambient light before placing the filter in the housing.

Using a grating monochromator for wavelength selection an extra DECREASE of TIME RESOLUTION is caused by the diffraction and interference process from the grating 29 . At high spectral purity and resolution (double) spectrometers, the broadening of $f_{\Delta}(t)$ can be up to some hundreds of picoseconds. With the rotation axis of the grating(s) in the middle of the front of the grating(s) a change of wavelength setting will introduce a minor change of the broadening of $f_{\Delta}(t)$, the mean transit time will remain constant. For experiments requiring both high spectral purity and high time resolution a dual spectrometer in subtractive dispersion can be used 34 .

From grating imperfections or irregular reflections or scattering inside the monochromator GHOSTS can appear, for instance the transmission of excitation light at wavelength settings quite different from the wavelength of the exciting laser line. The tandemized 2×0.25 m monochromator (Jarrel Ash 82-410) of our system shows a ghost at 660 nm at about 10^{-6} intensity upon excitation at 457.9 nm, bandwidth close in conformity with the slit setting. In the fluorescence decay the ghost appears as an instrumental response, superimposed on the decay but shifted in time due to transit time differences. A suitable cut-off filter will be appropriate. In measurements of fluorescence anisotropy the dramatic dependency of the transmission efficiency on the polarization direction (and the wavelength) of the detection light should be taken into account.

3.6 PHOTOMULTIPLIER TUBE

The high gain and the low dark current of the PMT create the possibility to detect individual photons. In modern tubes the thermionic emission of electrons from the cathode is the only serious contribution to the dark current. That emission can be drastically decreased by cooling the tube³⁵. Sometimes³⁶ a higher dark count rate of the PMT due to higher cathode temperature is accepted as a compromise with the blue shift of the long wavelength edge (1150 nm, Varian VPM152) of the quantum efficiency characteristic, which occurs at lower cathode temperatures.

Transit time fluctuations of single photon events in the electron multiplier give rise to BROADENING and DISTORTION of the pulse response function $f_{\Delta}(t)$ of the TCPC equipment. However with fixed voltages at the electrodes these broadening statistics are completely reproducible (in contrast to microchannel plate (MCP) multipliers at different photon rates 37). When a well stabilized high voltage power supply (low ripple) and a reliable dynode chain are used, only moisture deposited at the socket can lessen the stability, particularly in case of a cooled PMT housing.

Another important property of the PMT is the TRANSIT TIME DEPENDENCE on the PART of the CATHODE that is illuminated. Sometimes this dependence is specified by the manufacturer 38 . In general, side-on PMT's show greater place dependency of the transit time than end-on tubes. The focussing of the light onto a small part of the photocathode will enhance the time resolution. When a pulse response function $f_{\Delta}(t)$ is measured, the projection of the image of scattered light at the photocathode should be similar to the projection of the fluorescence emission (see 6).

The Varian VPM 152A.10Z tube we used for the near infra-red emissions showed an extreme transit time dependence on the place of cathode illumination. Focussing on a small part (some tens of microns) of the 5 mm diameter InGaAsP cathode the transit time jitter was some 100 ps. Scanning the spot across the cathode, the average transit time varied about 1 ns. Illumination of the whole cathode gave a pulse response function of about 1 ns width with a shape dependent on the intensity distribution at the cathode surface. That is an unwelcome property for obtaining a proper $f_{\Delta}(t)$ for deconvolution purposes.

Another factor of influence in time resolved measurements is the WAVELENGTH DEPENDENCE of the TRANSIT TIME, since the pulse response function $f_{\Delta}(t)$ is recorded at the excitation wavelength while the fluorescence decay is determined at a different wavelength. Both the total transit time and the width and shape of $f_{\Delta}(t)$ can change with wavelength.

In general the width of $f_{\Delta}(t)$ will decrease at longer wavelengths because of the more uniform start conditions of photoelectrons at the cathode³⁹. Similar kinetic effects will influence the total transit time at different wavelengths. Particularly in the UV region of the spectrum these effects will become larger. There is no linear relation between transit time change and wavelength, as suggested in reference 40.

The main factors influencing wavelength dependent properties are the photocathode material and the extraction field at the cathode. With a given tube the changes can be minimized by using the maximum possible voltage between cathode and first dynode, sometimes a focussing grid is built in between.

The XP 2020 and PM 2254B show an AFTERPULSE at about 13 ns after the main peak. That is due to the ionization of residual gasses in the tube, in this case probably $\mathrm{He^+}$, 41 . The probability for the occurrence of this effect is (on the timescale of the experiment) constant and completely reproducible at fixed voltages at the electrodes of the PMT. In the experimental TCPC data the afterpulse is manifest as a second peak in the pulse, response function $f_{\Delta}(t)$ and also in the fluorescence decay of short lifetime materials. The shape of $f_{\Delta}(t)$ appeared to be almost independent of the (single) discrimination level of the CFD. The width of the delayed peak is somewhat larger than the main peak because of the badly defined timing of the ionization process. With the Varian PMT we did not find any afterpulse.

For the PM 2254B we use a PMT socket assembly (Products for Research, customer specified), on which provisions have been made for tunability of the voltages at the second dynode and the focussing grid in the cathode to first dynode region. The total voltage across the voltage divider is kept at -2900 V. By adjusting the voltages at the first few electrodes of the PMT the ratio of the pulse and afterpulse amplitude can be varied between 200:1 to 1000:1, the shape of the peaks can be considerably changed, the number of afterpulses can be extended and even a prepulse can be produced. Normally we adjust for optimum FWHM and shape of $f_{\Delta}(t)$. At very different wavelengths like 300 and 600 nm the settings for optimum adjustments are also different.

The afterpulse of the PMT both distorts the fluorescence and anisotropy decay and influences the recording of time resolved emission spectra (see 5), comparable to the effects of a poor extinction ratio of the pulse picker or a badly shaped exciting light pulse. The use of a deconvolution procedure will recover the proper decay times but the presence of an afterpulse will, next to a wide $f_{\Delta}(t)$, obstruct the use of a large dynamic range in the time region just after the exciting pulse. In that case the accuracy of the determination of the short decay times will be decreased. Best temporal results are obtained from MCP photomultipliers $^{37,42-43}$, but also using the much cheaper standard side-window PMT's like the Hamamatsu R 928 quite satisfactory results have been published 40,44 .

3.7 ELECTRONICS

For the discrimination of single photon response pulses from the anode of the PMT a CFD is used. In spite of high level electronic development this device will introduce WALK ($\leq\pm$ 150 ps at a 200:1 dynamic range, Ortec 934), resulting in a broadening of $f_{\Delta}(t)$. Using a CFD with upper and lower discrimination levels not only non-photon events can be eliminated, but also double photon events can be rejected when a PMT is used with sufficient single photon pulse resolution 45 . That acts as pile-up rejection (see 3.8) for the case where two (or more) photon events are hardly separated in time, less than the width of a single photon response pulse at the input of the CFD. As far as we know the smallest walk specified from a commercially available CFD is \pm 25 ps at a 100:1 dynamic range (46 , Phillips Scientific model 714, a model optimized for discriminating the fast anode pulses of a MCP PMT).

An electromagnetic interference signal at the input of the CFD in addition to the single photon response pulses from the anode of the PMT will disturb the normal discrimination statistics. The interference will act as a time dependent change of the discrimination level. When such an interference signal or

transient is synchronous with the repetition rate of the exciting light pulses, this disturbance will be correlated to the statistical process of the repeating TAC cycles to be recorded and result in DISTORTION of the data. The result will be distortion of the recorded decay profiles as quasi from non-linearity of the time scale.

The cable between the anode of the PMT and the CFD or preamplifier should be as short as possible to minimize external radiation to the inputs. Another simple and effective way is to increase the distance between PMT (and CFD) and electronic or electromagnetic signals that are synchronous with the repetition rate of the exciting light pulses such as signals from the modelocker driver, pulse-picker driver and reference timing signal.

For the positioning of the experimental time window into the TAC range we used variable time delays (Ortec 425) and fixed 100 ns coaxial 500 cable. When the fast negative NIM signals are delayed in this way, it is important to have a continuous 500 delay line. A mismatch will introduce partial reflections and so distortions of the very fast NIM pulses. A stationary mismatch does not yield problems, but when it is caused by a poor connection or switch (contact cleaner), it can introduce amplitude and therefore trigger jitter, which results in broadening of $f_{\Delta}(t)$. With poor quality cable sometimes just touching the roll results in some shift (tens of ps). An oscilloscope with a bandwidth of 350 MHz or more is an indispensable tool when using this kind of equipment.

With the TAC the time difference between start and stop NIM pulse is determined and an analogue pulse is produced with an amplitude proportional to the measured time difference. Depending on the experimental conditions the TAC can be started with a pulse from the reference line or from the photomultiplier chain (normal or inverted configuration).

In reference 46 arguments are given for the OPTIMIZATION of the data-acquisition rates with a sub-microsecond decay time spectrometer. Most arguments are general and apply to sub-nanosecond spectrometers as well. Because of the high percentage (>95%) non-stopped start-stop sequences (see 3.8) a lot of "busy"-time of the TAC is wasted when starting the TAC with the reference line (excitation pulse repetition rate). Thus with repetition times of the excitation pulses shorter than the busy time of the TAC, as usual with mode-locked cw lasers, an inverted configuration should be used.

The LINEARITY of the TAC and simultaneously of the ADC can be checked using random start and stop rates or a reference timer (Ortec 462^{37}). With the modules we used (see 2) the linearity was always sufficient, distortions due to interference and noise were always dominant in the experimental condition. When using stop pulses at a repetition rate of 50 to 100 MHz internally in the TAC the linearity can be disturbed due to electromagnetic cross-

talk from the very fast NIM input(s) to the analogue electronic circuitry, also resulting in oscillation-like distortions of the decay profiles. Such a high frequent stop rate is used in case of the inverted configuration and using a mode-locked or synchronously pumped cw laser or synchrotron source for excitation. In references 48 and 49 circuits are described for decreasing the stop rate to be equal to the start rate.

Thus distortions (non-linearities) can be initiated in different parts of the electronics. The global shapes are similar; decaying sinusoidal waveforms like resonances in passive networks (cables), excited by the fast NIM pulses or interference. The final distortion as found on the measured fluorescence decay can be affected and sometimes even almost eliminated by varying delays in different parts of the electronics.

For checking the LINEARITY of the <u>total</u> detection system a (known) long decay time can be recorded and the deviation curve can be observed or, even better, the instrumental response function $f_C(t)$ to a CONTINUUM of uncorrelated photons can be recorded \overline{b} 0. For that purpose we block the laser beam to the sample housing and slightly open the shutter to the PMT to have photons of the room light at a rate as in the normal experimental condition. Especially when using laser pulses to trigger the reference line care should be taken that no laser light can be detected by the PMT in this measurement. The shape of this instumental response function $f_C(t)$ should be a straight line in the ideal case. In practice the oscillation like distortions can be observed. That curve can also be used to correct for distortions \overline{b} 0 (see 4).

The TIME RESOLUTION of the electronics can be checked using two outputs of a dual fast NIM output discriminator (triggered by some signal generator) to trigger both signal and reference line of the start-stop equipment. In that case a time resolution of for instance some 10 ps should be obtained. This set-up creates the possibility to check easily the influence of temperature on the different parts, the quality of the delay line connections or switches and dependency of the instrumental response on different start-stop rates. (Particularly the very slow drift of transit times is important for the reproducibility, see 6)

The performance of the CFD can be checked by simulating a pulse (shape) as normally obtained from the PMT anode, and using an attenuator with constant or at least with known deviations of the transit time at different attenuation positions. In the ideal case the transit time of the CFD should be constant at different attenuations of the input pulses.

When arranging start- stop equipment, the electronics should be capable of handling the signal rates 47 , as indirectly supplied by the excitation equipment (see 3.8). The maximum rate of the CFD (up to 100 MHz, Ortec 934) must be much higher than the fluorescence photon rate. Of course the occurrence of only one

photon per excitation flash will be arranged (3.8), but per infinitesimal time slice of the experimental time window photons are Poisson distributed over the subsequent start-stop cycles. The dead-time of the TAC (Canberra 2043 $\geqslant 1\mu$ s, Ortec 457 $\geqslant 4\mu$ s) and the conversion time of ADC, and MCA, (depending on chosen number of channels up to 10 μ s) are the next limitations for the data rate but these will only introduce a decrease of data handling efficiency.

3.8 PILE-UP

TCPC equipment can process only the timing information of one emission photon per excitation pulse. Emission photons following to the first emission photon after the excitation pulse carry essential statistical information but cannot be processed. To prevent distortion due to the occurrence of more than one emission photon per excitation pulse (pulse pile-up 51) the frequency of emission photons is experimentally chosen to be low with respect to the frequency of excitation pulses.

When there is a great variance of energy per excitation pulse, the correlation between pile-up distortion and the ratio of emission photon and excitation pulse frequency is more difficult to predict. Major sources of fluctuations of the energy of the light pulses are described in 3.1. Our laser system and detection optics are placed on a passively air damped table 10 and much attention was given to the dye jet properties. The resulting amplitude instability is about \pm 10 %, which hardly influences the pile-up statistics.

We use a ratio of emission photon versus excitation pulse frequency of 5%, resulting in a pile-up distortion negligible with respect to the noise amplitude at several tens of thousands of counts in the peak of the detected decay curves. This number 5% is just a compromise between pile-up distortion and speed of data collection and when very accurate experimental data are required this percentage should be chosen lower. Although the pile-up and other dist ortions are not simple comparable to the Poissonian noise, the accuracy of the decay curves should also be increased then by collecting more counts per channel to really improve accuracy.

There are several methods for rejecting multiple photon start-stop sequences in an electronic way 45,52 , allowing higher rates of emission photons (see also 3.7). Until now we did not use them because the time of some minutes required to record a fluorescence decay curve can be considered quite satisfactory.

For the determination of the maximum photon frequency to prevent pulse pile-up only the part of the photon counts falling within the experimental time window of the TAC is of value. So when a high DARK COUNT RATE of the PMT is observed, only the

expectancy of the dark current in counts per second, multiplied by the quotient of TAC time window and experimental pulse interval time, must be taken into account for the contribution of that $(random^{53})$ dark counts to the pile-up mechanism. In practice the mean photon rate can then be much higher than 5 % of the experimental pulse rate.

At very high dark count rates the TAC should be gated with excitation event pulses to prevent a superfluous decrease of data collection efficiency. That is only useful when the experimental time window is shorter than the excitation pulse interval time. Finally the maximum pulse rate of the CFD must be sufficient to process all those (Poisson distributed 53) dark counts.

4. NON-LINEARITIES

We developed a method for the correction of non-linearities of the detection electronics⁵⁰. It is our experience that when the distortions are very intense, the correction is seldom perfect. When distortions originate from electromagnetic interference the effective external radiation can even be influenced by human motion near the electronics cabinet. It would be useful to correct for possible distortions but the best is to arrange a situation with negligible distortions.

The correction procedure consists of dividing all experimental data files by the instrumental response curve $f_C(t)$ to a continuum (see 3.7) which we call the correction curve, before the deconvolution procedure is started. But first the correction curve is smoothed somewhat. The time between distortion maxima and minima in the correction curve is mostly some nanoseconds, therefore when a time scale of some tens of picoseconds per channel is used, a smoothing procedure will not affect the results.

To preserve a constant integral of counts in the experimental data file, after the dividing procedure all dividends are multiplied by the mean content per channel of the correction curve. That average is calculated by integrating the count content of all channels of the correction curve and dividing that by the number of channels.

Normally the correction curve is recorded during the night following a day of measurements at one specific time scale. Suppose a repetition rate of the experimental excitation pulses f_r and a time scale of t_Δ (pico-) seconds per channel, then the collection ratio per channel CR_{Ch} for uncorrelated photons is $t_\Delta f_r.$ The photon rate for recording the correction curve is chosen similar to the experimental rate 0.05 f_r (see 3.8), so after t seconds the expectancy for the content per channel C_{Ch} will be:

$$C_{ch} = 0.05. f_r. CR_{ch}. t = 0.05 f_r^2. t_{\Delta}. t$$

In a practical case where for instance t_Δ = 50 ps/ch, f_Γ = 300 kHz and after a night of 14 hours an expectancy of about 9000 counts per channel can be found. Assessing this content one should keep in mind that with longer time scales the content, and so the accuracy, will increase and with shorter time scales an extended smoothing procedure can be applied because of the relatively slow fluctuating shape of the distortions.

When a high DARK COUNT RATE from the PMT is observed, <u>first</u> the correction of the distortions should be applied and after that a constant number of counts per channel C_d should be subtracted (see also⁵⁴) from the whole data set of the experimental decay curves according to:

$$C_d = CR_{ch}.f_d.t_e = f_r.t_{\Delta}.f_d.t_e$$
,

where t_e is the collection time of the data set of the experimental decay, if possible corrected for instrumental dead-time, and f_d is the dark count rate of the PMT.

Division of all experimental data files by the correction curve and particularly a high dark count rate will introduce extra noise in the final data sets and therefore give less accuracy. A proper dedicated correction procedure will keep the loss of accuracy at a minimum but making corections superfluous is of course the best way of proceeding.

That is illustrated in figure 1, showing the fluorescence decay of diphenylhexatriene (DPH) in unilamellar phosphatidylserine (PS) ves icles at 40°C. The concentration of DPH was 0.1 μM , the concentration of PS was 10 μM . The excitation wavelength was 363.5 nm and emission was selected via a 437 nm B20 interference filter from Balzers. The upper curves of weighted residuals and autocorrelation function of residuals are from the raw experimental data, fitted to a model decaying as a sum of two exponentials (τ_1 =3.07 ns, α_1 =0.13, τ_2 =9.21 ns, α_2 =0.87, X^2 =0.34, DW=0.28, number of zero crossings in the autocorrelation function =23). The lower curves of residuals and autocorrelation function are from data corrected for instrumental non-linearities as described above, using the same model $(\tau_1=4.69 \text{ ns}, \alpha_1=0.15,$ τ_2 =9.37, α_2 =0.85, X^2 =0.07, DW=1.40, number of zero crossings in the autocorrelation function =152). The lower most curves are the pulse response function and the experimental and calculated decay in case of corrected and uncorrected (2 decades vertical shift) data.

As can be seen the correction is not perfect but particularly in the autocorrelation function, the sensitivity to imperfect fits is drastically increased. Also in this case distortions would have been minimized experimentally.

FIGURE 1 Example correction for instrumental non-linearities (see text).

5. TIME RESOLVED SPECTRA

There are two essentially different methods to obtain time resolved emission spectra. One way of measuring is to detect decay profiles at a set of different emission wavelengths, another way is to detect emission spectra using photon counts occurring in a set of time slices out of the experimental time window.

The first method, that was extensively described in early publications 55 , delivers complete and accurate results but is time consuming. The set of decay profiles is fitted to conform a model and the calculated (deconvoluted) curves can be used to present an undistorted (pulse width, after pulse, etc.) three-dimensional display of fluorescence intensity versus time and wavelength. The long experimental time requires a good long term stability of the equipment. Therefore an automated cycle with repeated registration of the pulse response function $f_{\Delta}(t)$ is advisable $^{37},^{55}$.

The second method gives quicker but less accurate results. A time slice is selected out of the experimental time window by discriminating the analogue output pulses of the TAC. That can be done with a SCA (see 2) or with the ADC (if equipped with upper and lower level discriminator). The procedure with the SCA (built-in, Canberra 2043) is preferable because of the short dead-time (1 μ s).

A disadvantage of this time slice method is that distortions in the normally recorded decay (pulse width, after pulse) also give rise to distortion of the time resolved spectra and in this case distortions cannot be simply recovered. Furthermore the available data is not efficiently used since only the discriminated part of the data is used to record a spectrum. That can be a very small part, depending on the ratio of the width of the time slice versus the total experimental time window.

6. PULSE RESPONSE FUNCTION

When deconvolution of the experimental data is required, the pulse response function $f_{\Delta}(t)$ must be representative for the condition of the equipment during the recording of the fluorescence decay. As described above there are several factors causing discrepancies, like deviant pathways and images of excitation and emission light (3.5, 3.6), wavelength dependent transit times (3.5, 3.6), place dependent temporal response (3.6) and a very slow and temperature sensitive variation of transit times and output levels (3.1, 3.3, 3.7).

The simplest way to obtain a $f_{\Delta}(t)$ is to replace the sample by a scattering solution of glycogen, Ludox, coffee creamer 56 or

a dispersion of $\mathrm{Al_20_3}^{40}$. In all cases only the exciting wavelength should be selected for detection (filter or monochromator) to prevent artificial pulse distortion due to fluorescence. Also the place and the shape of the image as projected on the photocathode should be similar (optical density at excitation wavelength identical in the two cases).

When photomultipliers are used with negligible place dependent temporal response like the XP 2020 or PM 2254B, it is even possible to "match" a pair of detectors to do simultaneously fluorescence and excitation registration $^{57-59}$. An elegant way for reaching that goal is described in reference 37, requiring a more sophisticated optical set-up.

We routinely use a small rod (approximately laserbeam diameter) rough grounded quartz for scattering, exploiting the same relatively insensitivity to place-dependent response. This simple method of registrating a $f_{\Delta}(t)$ will not always be satisfactory, particularly when a very accurate fit is required.

Temporal shifts of $f_{\Delta}(t)$ can be easily recovered by introducing a shift factor in computer analysis of the data. When that shift is drifting steadily, for instance due to thermal effects, also a broadening or smearing out of $f_{\Delta}(t)$ (during fluorescence measurement) should be anticipated. This kind of shift is more pronounced just after tuning all controls to reach the experimental condition (see 3.1, 3.2, 3.3, 3.7) and before starting the first experiment we always wait about half an hour for equilibration of the equipment.

We routinely take a record of $f_{\Delta}(t)$ before and after registering a fluorescence decay. For deconvolution a weighted average of these two $f_{\Delta}(t)$ curves is used. That gives a reasonable correction with shifts much smaller than the peak width of $f_{\Delta}(t)$.

Yet all kinds of changes in shape of $f_{\Delta}(t)$ due to wavelength effects (3.4, 3.5, 3.6) cannot be recovered in this way. For recovery of these effects a so called excitation pulse-shape mimic technique 60 , 61 can be used. In that method a synthetic $f_{\Delta}(t)$ is computed from a known short lived (preferable single) decay time 62 , detected at the same wavelength as the experimental decay to be deconvoluted. The best way to establish $f_{\Delta}(t)$ consists of application of the global analysis method to data of both known and unknown decay 63 .

When measuring non-liquid samples like for instance electrophoresis gels, in which case sample and scatter (or mimic) medium cannot be reproducibly interchanged, the fluorescing or scattering spot should be determined by the laserbeam, focussed to a spot smaller than the sample. In that case the chromophore concentration should be increased to keep an equal yield of detected fluorescence.

DISCUSSION

Of course this list of artefacts and distortion sources in TCPC will not be a complete one. We just tried to give an overview of experience that is relevant to this field. Another source of information for users of TCPC or starters in this field of spectroscopy is the book of O'Connor and Phillips 64 .

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