Time-resolved fluorescence spectroscopy

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List of abbreviations

ADC	analogue-to-digital converter
CCD	charge-coupled device
CFD	constant fraction discriminator
CPS	counts per second
CW	continuous wave
DIRF	dynamic instrumental response function
FWHM	full width at half maximum
MCP	micro-channel plate
PMT	photomultiplier tube
S/N-ratio	signal-to-noise ratio
TAC	time-to-amplitude converter
TCPC	time-correlated photon counting

1. General introduction spectroscopy

All mechanisms in living matter of nature start at a molecular scale. Examples are the entering of a living cell by a virus or a seed into an egg-cell, the development of plant seeds upon contact with water and nutrient molecules, the response of a molecular photosynthesis system upon the absorption of a photon, the complex mechanisms of biochemical reactions involving enzymes, etc. Thus for a clear understanding of the working mechanisms of biological and natural systems and the eventual extrapolation of that knowledge to (bio) technological and medical applications, it is crucial to study and understand the underlying principles and properties of molecular systems.

Because of the small (nanometer) scale, the direct observation of the peculiarities of molecules in their environment is never a straightforward matter. Even the strongest optical microscope is not able to give any direct information concerning molecular behaviour. Therefor in the field of molecular research spectroscopy is used as an analytical tool and rather complicated setups are required in these studies. Then the molecules under study are subjected to continuous and/or alternating magnetic fields and/or light. The response of the molecular system to that treatment is detected and analysed. From the results a molecular model is proposed, that must confirm with the spectroscopic observations. Complementary conclusions can be drawn and the model can be refined by the variation of external parameters such as temperature, pressure, molecular concentration, type of environment or solvent, etc. Depending on the applied stimuli (magnetic fields or light etc.), spectroscopic techniques are divided in magnetic resonance techniques [Carrington and McLaughlan 1967] and optical spectroscopy [Steinfeld 1974].

1.1 Time-resolved fluorescence spectroscopy

In this paper a specific optical spectroscopic technique is subject for study: time-resolved fluorescence spectroscopy. When molecules are treated with light, several things may happen. The light may be reflected, refracted, scattered or absorbed. Reflection and refraction of light, as known from optical elements as lenses, prisms and mirrors, are conceptions from the field of optics and, although they are applied in optical spectroscopy, they are of different analytical importance to optical spectroscopy as the absorption and the scattering of light.

There are two essentially different ways of scattering; elastic and inelastic. When scattering occurs in an elastic way, the energy of the photons after scattering is unaffected. In that case the scattering is called Rayleigh scattering [Rayleigh 1912] when the scattering object is small (< 1/10) compared to the wavelength of the light and is called Mie scattering [Gustav Mie 1908] when the photons are scattered by objects larger than that. The scattering occurs in an inelastic way, if the energy of the scattered photons is changed due to the interaction of the photons with the internal dynamics of molecules. In this case the scattering is called Raman scattering [Morris and Wallan 1979].

When molecules absorb light, the energy of the photons may bring the molecules in an excited state and/or may be converted to heat. The probability of light absorption by a substance is dependent on the wavelength of the light, as given by the absorption spectrum of the substance. In Figure 1 an absorption spectrum of chlorophyll is given. From Figure 1 it can be seen that out of the visible wavelength range of 400-700 nm, the blue (400-450 nm) and red part (550-650 nm) are highly absorbing, whereas the green part (500 nm) is not. That is the reason why plants look green. From the white solar spectrum the blue and red parts are absorbed and only the green part is scattered towards the observer. The role of spectroscopic research here can be the search for the origin of the two absorption bands, to describe the mechanisms of molecular energy transfer and the role of all molecular components involved in that mechanism. That knowledge can for instance be used to apply the same efficient energy conversion mechanisms from natural to artificial systems.

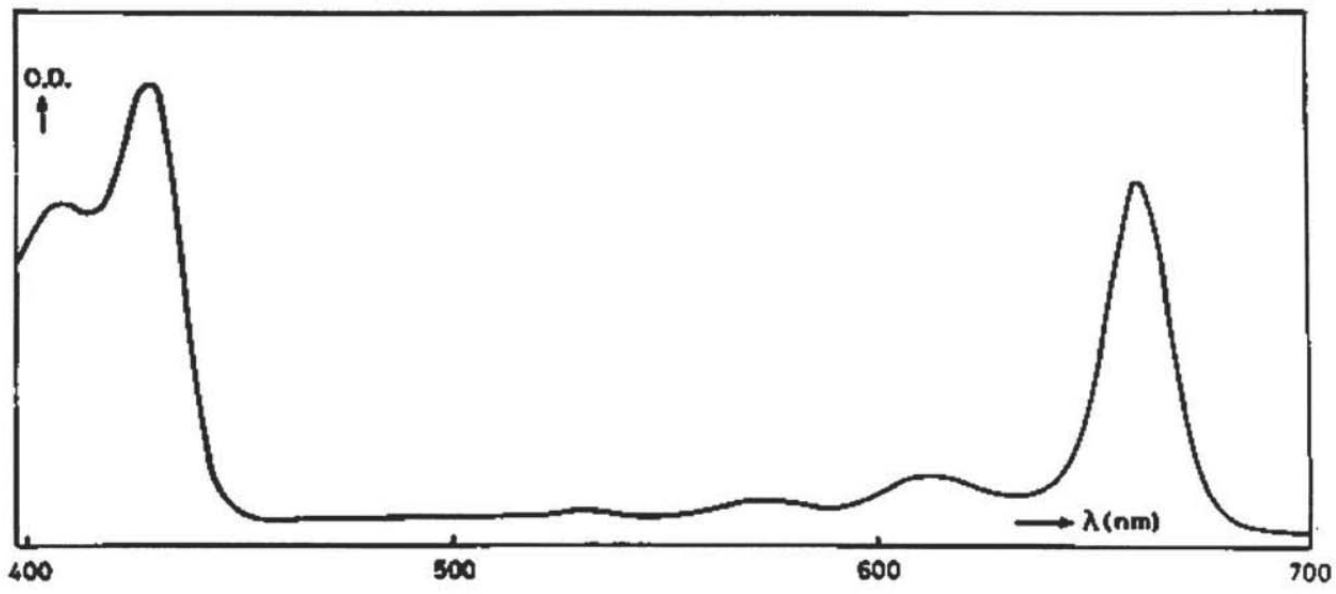

Figure 1 Room temperature absorption spectrum of dilute Chlorophyll Q in diethylether.

When a photon has brought a molecule into an excited state, different things may happen. There may be a radiation-less decay to the ground state by the release of heat. Or after a short delay time (nanosecond time scale) a photon of slightly lower energy may be emitted, called fluorescence emission [Stokes 1852, Lewschin 1925, Perrin 1929]. Or, caused by accidental changes of the electron spin configuration of the molecule, a different state may be initiated: a triplet state. From this state (after a much longer delay time at a millisecond time scale) a photon of even lower energy may be emitted, called phosphorescence emission [Becquerel 1896, McGlynn et al. 1969, Zahlan 1967]. From an ensemble of molecules the emission of fluorescence and phosphorescence photons is in arbitrary directions. From the terminology applied here above, it can be read that probability and quantum statistics play an important role in this field of research.

1.2 A short historical perspective

The oldest known reference to the phenomenon of fluorescence dates from the 16th century. The Spanish physician Nicolas Bautista Monardes (ea. 1512-1588) noticed the reddish fluorescence emission from an extract of plant material that he intended to use for medical purposes. In Seville a number of books of his hand were published (on botany and medical applications of plants), that were translated in several languages. It is not surprising that the fluorescence emission of chlorophyll was the first to be recognized because the red fluorescence emission can be initiated by excitation with blue light, far separated in the wavelength spectrum. Although unambiguous references are not easy to find, phosphorescence (Greek: light bearing) was discovered earlier because the long decay time of the phenomenon made it easier to notice.

For a better understanding of fluorescence properties a more detailed knowledge of the properties of light was essential at that time. Around the year 1666 the British scientist I. Newton (1642-1727) demonstrated that a beam of white light was a mixture out of which beams of coloured light could be separated. Then it lasted until the 19th century, when Sir G.G. Stokes (1819-1903) named the phenomenon of fluorescence referring to the mineral crystal of fluospar (Latin: fluo = to flow or to yield, spar = rock). He explained the mechanisms of fluorescence and absorption [Stokes 1852] on the basis of the wave theory of light [Stokes 1849, 1854]. The work of M. Faraday (1791-1867) and J.C. Maxwell (1831-1879), who included light within his electromagnetic theory, allowed a more detailed understanding of the properties of fluorescence mechanisms.

The introduction of quantum mechanical methods by M.K.E. Planck (1858-1947) around the year 1900, in the mathematical description of the interaction between electromagnetic radiation and matter, was an important contribution to the modern developments in fluorescence spectroscopy. The first specific experimental studies on molecular fluorescence spectroscopy (mainly on fluorescein in solution) date from the twenties [Wawilow 1922, Lewschin 1925, Perrin, 1926 and 1929, S.J. Wawilow 1927, E. Gaviola 1927, Jablonski 1935]. All these experiments were performed with the human eye as a detector. With the advent of electron multiplication vacuum tubes in the thirties [Farnsworth 1934, Iams and Salzberg 1935, Zworykin et al 1936, Weiss 1936, Kluge et al. 1937, Rajchman 1940], more sensitive and accurate measurements of light intensity and dynamics became possible. With these new tools the molecular spectroscopy was further developed in the following decades [Weber 1956, Brody 1957, Jablonski 1960, Bennet 1960].

The theoretical and prospective pioneering of Albert Einstein (1879-1922) was an essential condition for the realization of lasers in the sixties [Maiman 1960] and of the mode-locking and synchronously pumping of lasers in the sixties [DiDomenico 1964, Deutsch 1965, McDuff & Harris 1967, Dienes 1974]. This created possibilities for experimental research in the (sub) picosecond time domain and the discovery of many interesting molecular details with fluorescence dynamics spectroscopy [van Hoek and Visser 1987 and references therein]. Now the field of fluorescence research is really very broad and many applications are developed in a variety of scientific fields [Cundall and Dale 1983, Lakowicz 1983, Lakowicz 1991-1994]. That is undoubtedly not only because the pure scientific curiosity Is stimulated, but now also the (bio) technological applications of molecular spectroscopy are fully recognized now.

1.3 Experimental potentialities with time resolved fluorescence research

Molecular interactions, molecular energy transfer, molecular environment (solvent) dynamics and structural fluctuations of molecules have a direct influence on the fluorescence and phosphorescence properties of molecules. From a careful observation and analysis of the fluorescence emission, a detailed description of these processes can be drawn. By a systematic variation of external parameters as temperature, pressure, molar concentration, solvent, etc., selective experiments can be carried out. The study of the dynamics of fluorescence emission in particular, provides a powerful tool and an extra dimension for the interpretation of fluorescence data. In Figure 2 some examples have been schematized of excited state processes, which influence the fluorescence dynamics.

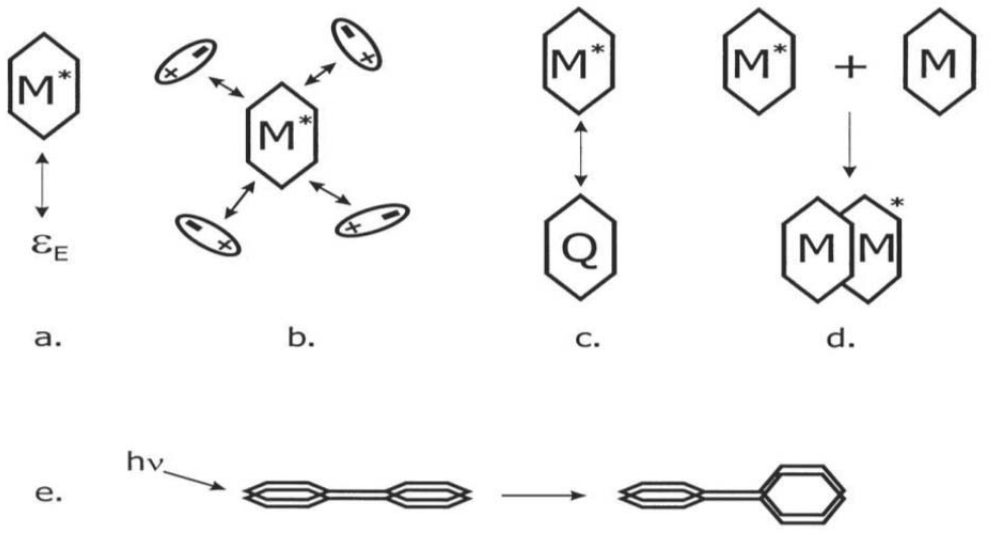

Figure 2 Examples of excited state processes that influence fluorescence dynamics. The fluorescence decay time of a chromophore can be influenced by different causes. For instance, by the dielectric properties ε_E of the environment (a.), the polarity interactions of the excited molecule with the environment (b.), the chromophore can interact with a quencher molecule (c.), excimer formation can take place (d.), or the excited molecule may undergo conformational changes upon excitation, and again that may influence the fluorescence dynamics. All these different molecular effects can be studied by a careful registration and analysis of fluorescence dynamics.

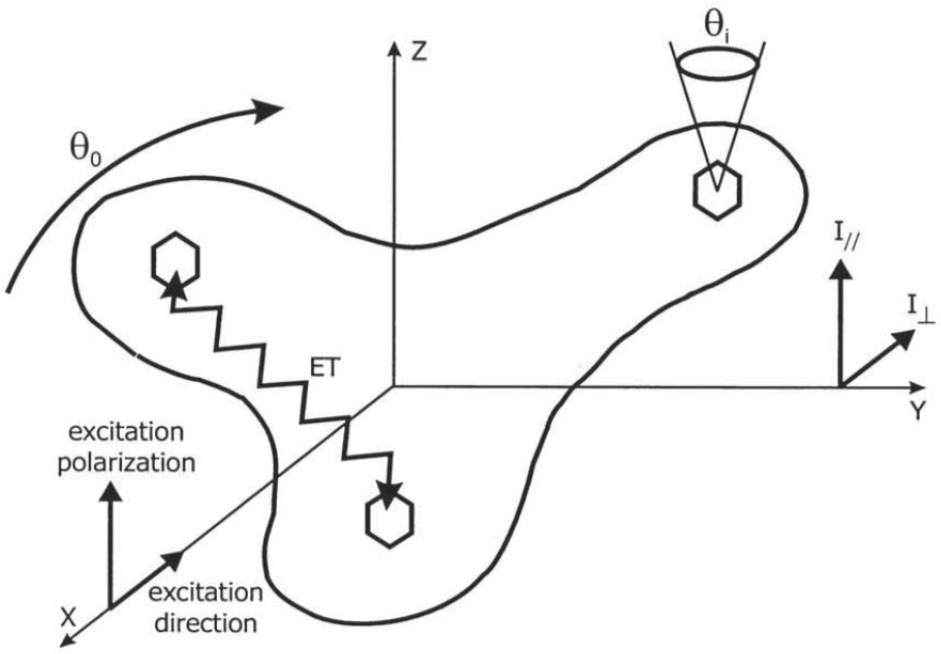

Figure 3 Fluorescence anisotropy is measured by the determination of the (normalized) difference between the time-resolved fluorescence emission that is polarized parallel ($I_{//}$) and perpendicular ($I_\perp$) with respect to the polarization direction of the excitation light. Fluorescence anisotropy can have been initiated by local flexibility (Θ_i) of the part of the molecule where the chromophore is situated, as well as by overall movement (Θ_o) of the complete molecule. Next to that, internal energy transfer (ET) can initiate fluorescence anisotropy. By changing the experimental parameters as sample temperature or the wavelength of excitation and/or detection, the contributions of the different origins of fluorescence anisotropy can be separated.

An advantage of the application of fluorescence spectroscopy for studies on biological materials is the high sensitivity in combination with the very specific information content. Even the tiniest amount of (fluorescence) light, the single photon, can be detected. And in proper experimental conditions, there will not be any background of photons from other origins, which may blur the detected fluorescence light. The fluorescence photon carries information on i) the photon energy, ii) the polarization direction and iii) the timing moment, which can be used to elucidate details of molecular processes. In some advanced optical spectroscopic techniques, such as Coherent Anti-Stokes Raman Scattering [Levenson 1977], even the direction of propagation of the emitted photon contains information. So when measuring not only the spectral properties of the fluorescence emission, but simultaneously the polarization properties and the timing moment as well (relative to the excitation moment or in a cross-correlation procedure), optimum use is made of the information content of the photon.

In addition to this, a large variety of natural and artificial molecular fluorescence probes is available which can be used to probe and investigate a broad range of molecular phenomena [Beddard and West 1981, Haugland 1996]. These probes can be chosen to be sensitive to a variety of parameters as, for instance, the presence and vicinity of specific molecules as oxygen, calcium, etc. These fluorescent molecular probes can be incorporated into macromolecules or self-aggregating molecular systems such as mono- bi- and multi-layers, artificial interfaces or in micelles. The whole system can then be effectively studied using time-resolved fluorescence spectroscopy.

Another important reason to use fluorescence techniques for probing molecular mechanisms is, that these molecular processes take place at the same time scale as the phenomenon of fluorescence itself. This aspect can be illustrated using the Einstein relation:

$$<\Delta x^2> = 2Dt \qquad (1)$$

stating that the mean square root molecular displacement Δx is related to the translational diffusion coefficient D and the traveling time t. If we substitute a time of 100 ns (order of magnitude of the time constant of the fluorescence decay of pyrene) and a diffusion constant $D = 3 \times 10^{12}$ m^2 /s (lateral diffusion over a bilayer membrane) the displacement ($<\Delta x^2>^{½}$) is 0.5 nm which is a significant microscopic distance over the surface of such membrane. This relatively simple example may illustrate that fluorescence spectroscopy is a sensitive method to register dynamic events in biological systems.

The information retrieved from molecules can be even more specific, when the influence of their environment can be modulated, minimized or well described. Alternatively, minimizing the number of molecules under study can reduce the heterogeneity of the spectroscopic data (and thus the complexity). Examples are: i) the loading of single molecules in a host matrix with silent spectroscopic properties, ii) using an ultra-small experimental volume by applying evanescent-field, near-field or multi-photon excitation, or iii) decreasing the concentration of molecules in solution or other matrices. Increasing the specificity of information from spectroscopic measurements by decreasing the size of the molecular system stops of course at the level where the functional behaviour of that system is lost. The application of site- selective excitation, or the freezing of thermal movements are other methods for obtaining more specific spectroscopic information. One should keep in mind, however, that it is not always realistic to cool biological samples down to cryogenic temperatures, because their properties might be drastically altered and results cannot be directly extrapolated to the situation at physiological temperatures.

2. Different detection methods

The short (nanosecond) time scale of fluorescence decay implies the necessity of complicated equipment for the detection of the dynamic properties of that phenomenon. Next to a variety of hybrid techniques, there are six basically different detection techniques to retrieve data of the fluorescence dynamics of a sample; 1) time-correlated photon counting (TCPC) [W.R. Ware 1971], 2) phase-fluorometry [Spencer and Weber 1969], 3) streak camera [Freiberg and Saari 1983, Staerk et al. 1996], 4) pump-probe techniques [Hallidy and Topp 1977, Beddard et al. 1981, Ruggiero et al. 1990], 5) boxcar sampling techniques and 6) direct detection with an analogue-to-digital converter. This paper is devoted in particular to the TCPC detection technique, although other detection techniques are discussed in short as well. Their main advantages and disadvantages will be discussed here because it is worthwhile for a better conspectus of the following chapters.

2.1 Time-correlated photon counting

When applying the TCPC method [O'Connor and Phillips 1984], short light pulses excite the sample and the (often exponential) decay of the fluorescence emission is directly detected by registration of the

fluorescence photon density as a function of time. The method is based on the repetitive measurement of the delay of fluorescence photons relative to the moment of sample excitation (in modern practice often a laser pulse of relatively negligible duration). In this sense, a decay of fluorescence emission is not seen here as a graph describing the fluorescence light intensity as a function of time, but as a histogram of the probability for the detection of fluorescence photons as a function of time. Upon one exciting light pulse, the relative delay of a fluorescence photon is measured and stored in a multichannel memory (see Figure 4). That process is repeated many times, until from the statistical distribution of the fluorescence photons in time, a histogram is build-up of sufficient accuracy.

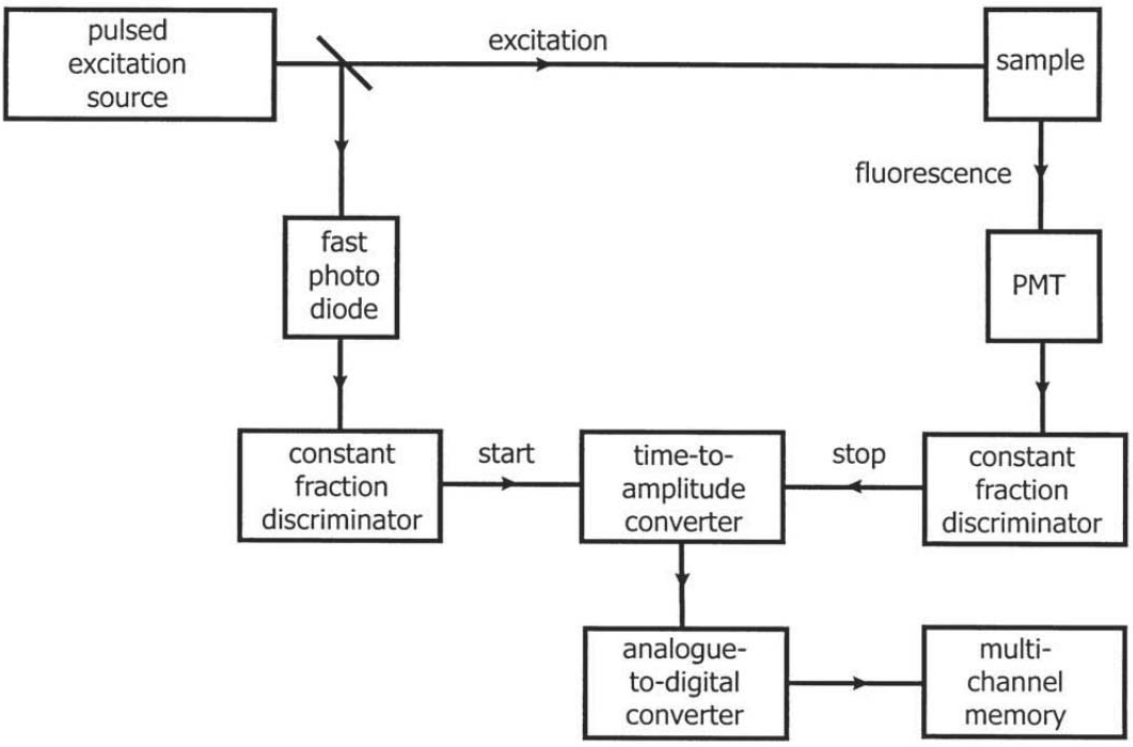

Figure 4 Block diagram of a start-stop arrangement. A picosecond light pulse excites the sample and simultaneously starts the time-to-amplitude converter (TAC) via a fast photodiode and a constant fraction discriminator. Fluorescence photons detected by the PMT stop the TAC after constant fraction discrimination. The TAC converts the time difference between start and stop pulses into a pulse height. That analogue output pulse is analysed by the analogue-to-digital converter and the result is stored in a memory. This sequence is repeated until sufficient accuracy is obtained.

The principle of the TCPC technique was originally developed in the field of nuclear physics. There it was used to determine the fluorescence dynamics of (liquid) scintillators. The development took place as well at the Argonne National Laboratories [Bollinger and Thomas 1961], as at the Commissariat a l' Energie Atomique in Paris [Koechlin dissertation 1961]. With this method the (multiphoton) light pulse of the scintillator

was used to start the timing measurement and the to single photon intensity level reduced light of the same light pulse was used to stop the timing measurement. Different types of radiation were applied at a time resolution of down to the sub-nanosecond time scale [Koechlin and Raviart 1964]. It took some time before the TCPC was recognized as a powerful tool for molecular spectroscopy in the field of (bio) chemistry and (bio) physics [Birks and Munro 1967, Schuyler & Isenberg 1971].

In TCPC for the detection of fluorescence photons most times a photomultiplier (PMT) is used [Hamamatsu 1994]. That is a vacuum electron tube capable to generate an avalanche of electrons (current pulse) as a response to one photoelectron. In the PMT the photon is first converted to a photoelectron (at a certain probability) by the photoelectric effect at the photocathode. Then multiplication of electrons is attained by secondary emission of electrons upon collisions of electrostatically accelerated electrons in a dynode structure or in microchannels (up to 106 to 1010 electrons per photoelectron). Using fast electronics, the current pulse output of the PMT can be used as an electronic signal for detecting the presence and timing of photons, with an overall timing accuracy of down to a few tens of picoseconds [Bebelaar 1986].

The electron multiplication number of a PMT is not a fixed factor. In the first place the gain depends on the acceleration voltages between the different electrodes. When these voltages become higher, the acceleration and also the final kinetic energy of the electrons will become higher. That will result in a higher probability for the emission of secondary electrons, so a higher overall gain. At increasing high voltage over the PMT, the electron gain increases exponentially [Hamamatsu 1994]. Next to that, there is a gain distribution over all processed photoelectrons, due to i) the statistical properties of the phenomenon of secondary emission and ii) minor deviations in the paths of the different electrons. So even at a fixed high voltage across the PMT, the single photoelectron output pulses of the PMT vary in amplitude. These differences in gain can easily be a factor of 100 to 10,000, depending on the type of PMT [Hamamatsu 1994].

When not the single-photon output pulses of a PMT are counted, but for instance the average current output is measured after integration with a low-pass filter, these gain variations will result in extra noise. That kind of detection method is only useful when a high photon rate must be detected, so that these gain variations will be averaged out. In all other cases, and also with TCPC, the PMT is used in single photon mode, where the gain variations will not influence the accuracy of fluorescence signal intensity, because the single photon responses out of the PMT are after discrimination further processed as unity pulses.

Another advantage of the TCPC method is, that the electronic bandwidth of the PMT does not directly influence the accuracy of the timing measurement. That is because in principle only the time of arrival of the current pulse at the PMT output is measured. Of course the accuracy of the timing measurement will be increased when that pulse has a shorter rise time. But even more important is in this case that the transit time of the PMT, the time difference between photon arrival and current pulse output, is reproducible. The spread in this transit time is in practice the limiting factor of the time resolution in TCPC. The best timing resolution can be obtained by the application of proximity focussed micro-channel plate (MCP) PMT's [Hamamatsu 1994]. A dynamic instrumental response function (DIRF, see 4.2) of a few tens of picoseconds full width at half maximum (FWHM) can be awaited then [Bebelaar 1986].

The dark current of the PMT solely limits the dynamic range of the TCPC detection technique. A practical value for that is for instance 100 counts per second (CPS). From literature it is known that these dark counts are not quite Poisson distributed in time [Coates 1972, Raouf et al. 1986]. But even then, when a measurement is performed at a repetition rate of exciting light pulses of 1 MHz and the time scale is 10 ns divided over 1024 channels (< 10 ps per channel), the probability for dark counts in a channel will be less than (10 ps / 1 µs) x 100 CPS = 10^3 CPS. So in a practical TCPC experiment the dynamic range is seldom limited by the detection part. Then the background luminescence of the solvents or other artificial luminescence sources are the next factors that will limit the dynamic range. Therefore for sample preparation always fluorescence grade solvents should be used and very pure water must be used for the buffer solutions. And even then, the background luminescence intensity should always be measured and, if applicable, be subtracted from the sample fluorescence intensity.

With the standard TCPC equipment only one fluorescence photon can be detected upon exciting the sample with one pulse of light. When the energy per exciting light pulses is increased, also the probability increases of the emission of more than one fluorescence photon per excitation pulse. And although all photons contain essential statistical information, the detection equipment can process only the firstly arrived photon. In this way a distortion of the detected photon distribution is caused, called pile-up distortion [Harris and Sellinger 1979]. So the energy of the excitation pulses should be low enough to yield a negligible probability for the detection of more than one photon per excitation pulse, and that is at the condition where the frequency of detected photons is lower than 5 % of the frequency of excitation pulses.

Next to this pile-up anomaly, the dynamic range of with the TCPC technique is essentially linear. To increase the accuracy of a measurement with the TCPC technique, not the energy per exciting light pulse should be increased, but the experimental time and/or the repetition rate of exciting light pulses. Several methods exist to handle the detection of more than one photon per excitation pulse; experimentally with special electronics or afterwards with mathematical methods.

2.2 Phase fluorometry

Using the phase method, the sample is excited using a repetitive signal (sinusoidal modulated light intensity or repetitive short light pulses). The fluorescence light will then be modulated with the same frequency of modulation f_m, but, depending on the decay time of fluorescence emission, aberrations of phase delay and modulation depth will occur. The fluorescence decay law is retrieved from measuring the phase delay and modulation depth of the fluorescence signal, relative to the modulation phase and modulation depth of the excitation light. For observing reasonably measurable phase shifts from fluorescence decays with time constants in the sub-nanosecond range, modulation frequencies of up to the GHz range are required. In that frequency range the phase shift and modulation depth of the signals cannot be directly detected, as at low frequencies. So then in a (super-heterodyne) frequency mixing process the modulation frequency of the fluorescence light f_m is mixed with an artificial demodulation frequency f_d to observe a difference frequency $f_\Delta = f_m - f_d$ (see Figure 5). The demodulation frequency f_d is chosen in such a way that the difference frequency f_Δ is relatively small, so that the phase and modulation parameters can be determined with relatively simple equipment. The modulation frequency can be up to hundreds of MHz and f6 can be down to a few Hz.

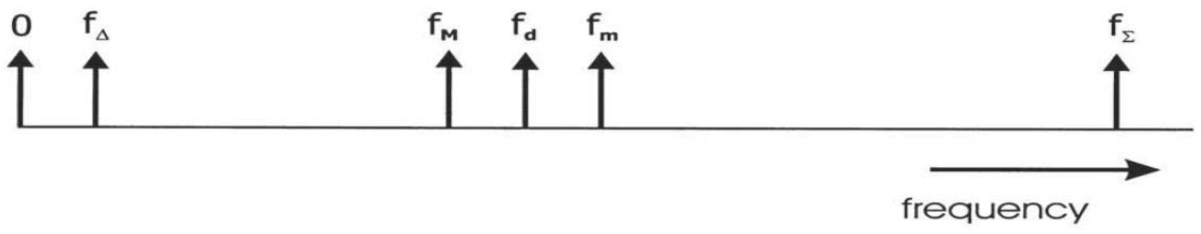

Figure 5 Positions of the modulation frequency f_m, the demodulation frequency f_d, the difference frequency f_Δ, the sum frequency f_Σ and the mirror frequency f_M in the frequency spectrum (see text).

Different methods exist for the realization of this frequency mixing. In one method, the frequency mixing is carried out by gain modulation of the PMT detecting the fluorescence light. Then the demodulation frequency f_d is fed to one of the early dynodes of the PMT [Schurer et al. 1976], or a specific modulation electrode [Wang et al. 1989, Williamson et al. 1993]. With another method, the electronic current of the fluorescence signal, as retrieved from the PMT output, is electronically mixed with f_d [Laczko et all, 1990]. The goal of these methods is always to attain an electronic bandwidth of detection that is as high as possible, to obtain good accuracy for fast decay components. In the dynode-type PMT, the obtained electronic bandwidth is higher at the first dynodes (up to the GHz region) than at the later dynodes. In the MCP-PMT this gain modulation future is not yet available, but the initial bandwidth (multi-GHz) is already much higher than in case of dynode-type PMT's. For imaging applications MCP image amplifiers are available with a gain modulation option for frequencies up to 300 MHz [Gadella et al. 1997].

With the super-heterodyne frequency mixing methods, sum ($f_\Sigma = f_m + f_d$) and difference ($f_\Delta = f_m - f_d$) frequencies of f_m and f_d are generated, and signal at the difference frequency f_Δ is normally used for determination of the relative phase and modulation depth of the fluorescence light. But, the frequency f_Δ is not only the difference frequency between f_m and f_d ($f_m = f_d + f_\Delta$, see Figure 5). Also signal (or; noise) at the (mirror) frequency of $f_M = f_d - f_\Delta$ will after mixing result in noise at the difference frequency f_Δ (the difference of frequencies $f_d - f_M = f_d - (f_d - f_\Delta) = f_\Delta$). Furthermore, all amplifiers and mixers that are used in this way, exhibit a certain noise level and that will not be negligible. And, with this phase sensitive detection technique, the statistical fluctuation of the gain of single photoelectrons in the PMT, as present in its output current, is detected as noise as well. All this extra noise, as compared to the unity detection of the presence and timing of photons, results in an essentially worse signal-to-noise (S/N) ratio and dynamic range for the phase method versus the TCPC method. And that will be in particular the case, when only a low rate of fluorescence photons can be detected. Therefore, starting at identical rates of fluorescence photons, a decrease of the S/N ratio of at least a factor 2 to 10 can be awaited when the phase method is applied. So, as compared to the phase method, the TCPC method will always need less excitation light to observe data from fluorescence dynamics at identical accuracy.

An interesting aspect of phase fluorometry is the method of modulation of the excitation light. In the early phase fluorometers the light of a continuous lamp was modulated using an electro-optic Pockels cell [Hauser and Heidt 1975] or an acoustic grating in a fluid tank [Schurer et al. 1976]. A disadvantage of that

method was that the modulation depth of the excitation light was maximum a few tens of percent. That means that a greater part of the light that excited (and bleached and heated) the sample, did not contain any information of the modulation frequency. That part of the light only resulted in needless bleaching of the sample and extra noise in the phase and modulation measurements [Gadella et al. 1997].

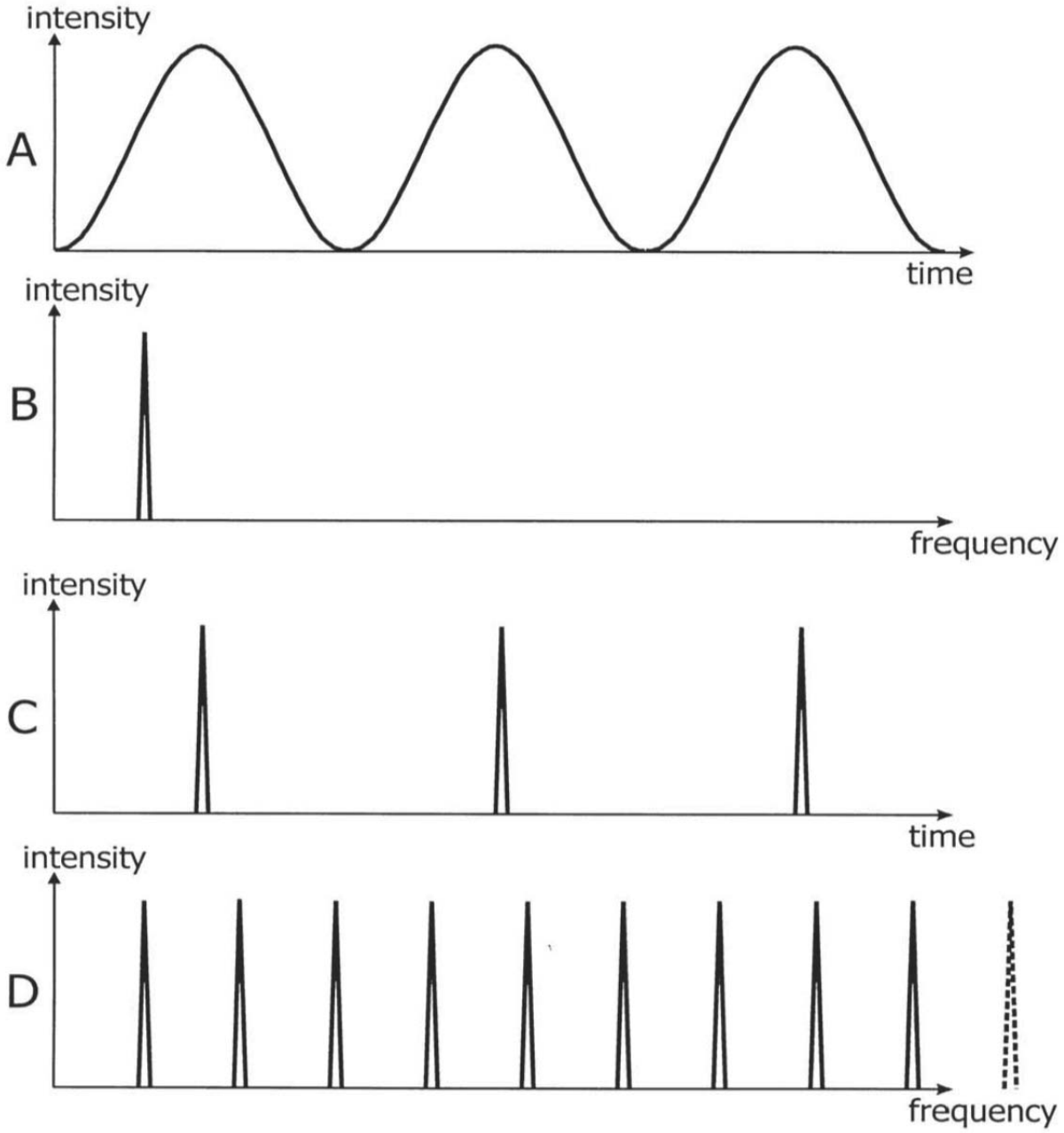

Figure 6 Caricatures of two different examples of modulated excitation light for phase fluorometry. In picture A the light is sinusoidally modulated at a 100% modulation depth. Picture B gives the Fourier transform of picture A and so that describes the frequency spectrum of picture A, showing that here there is only one frequency component. In picture C the time spectrum of a mode-locked loser is depicted, with short pulses at the same repetition frequency as in picture A. Picture D gives the frequency spectrum of picture C, showing a wide range of frequency components that all con be used for phase and modulation depth analysis.

For optimum efficiency of the excitation, measurements require a high ratio of accuracy of the experimental results, versus applied excitation light energy. Nowadays for excitation often mode-locked CW lasers are applied, supplying a high repetition rate (MHz) of picosecond light pulses. That method has several advantages. Sinusoidally modulated light at up to 100 % modulation depth (se Figure 6 A+B) contains per definition only information at the first harmonic of that modulation frequency. At a similar content of number of photons, light from a mode-locked laser can be applied (se Figure 6 C+D). That light contains, next to the same information as the sinusoidally modulated light (the first harmonic of the repetition frequency of light pulses), a wide range of harmonic frequencies of that repetition frequency. The signals at all those harmonics can be used to determine phase and modulation information of the fluorescence light at these frequencies simultaneously. And, the phase of the signal frequency is most accurately described when the signal has the shape of a steady train of pulses.

Nevertheless, with the presently applied detection electronics (phase-sensitive detectors), only limited number (often only one) of detection frequencies can be simultaneously processed [Clays 1989, Bright 1990]. But for a complete description of the fluorescence dynamics, information is required of the phase and the modulation depth at all frequencies available in the fluorescence signal, in particular when the decay law is more complicated than just single exponential. That means that parameters of phase and modulation depth must be sequentially being determined for all relevant frequencies and that is of course a waste of information and time. From a mathematical point of view, the timing signal of a single fluorescence photon can be used to determine the phases and modulation depths at all signal frequencies simultaneously, so there is no theoretical limitation. And when equipment will come available where the timing information of the fluorescence photons can be directly used to generate phase and modulation information at all frequencies (applying timing correlation of the fluorescence photons and avoiding the introduction of mixing or amplification noise), both methods will have identical sensitivity.

The phase method can be very effectively applied when the average fluorescence lifetime of a sample (or a series of samples) must be determined in a short time span [Li and McGown, 1996]. In that case there are no mathematical procedures in data analysis required. When there is a sufficient high S/N ratio, an average fluorescence lifetime can simply be read from the phase shift of the fluorescence signal. That may very much speed up the experimental procedure. Another important fact is, that in phase fluorometry the S/N ratio per experimental time can be simply increased by increasing the intensity of the excitation light (until

reaching the maximum detection rating of the fluorescence detector or damaging the sample). That last possibility cannot be applied with the TCPC technique because of the limitation due to pile-up distortion [Harris and Sellinger 1979].

2.3 Streak camera

When a streak camera is applied for the detection of fluorescence decays, a single light pulse or a train of short light pulses is used for excitation of the sample. Then the streak camera, a fast opto-electric vacuum detection tube, directly gathers the fluorescence light. The presence as well as the timing information of the fluorescence photons is detected. With this technique the registration of the wavelength dependency of the fluorescence light can be simultaneously included in the same experiment. Depending on the type of streak camera, time resolutions down to the range of a few hundreds of femtoseconds can be observed.

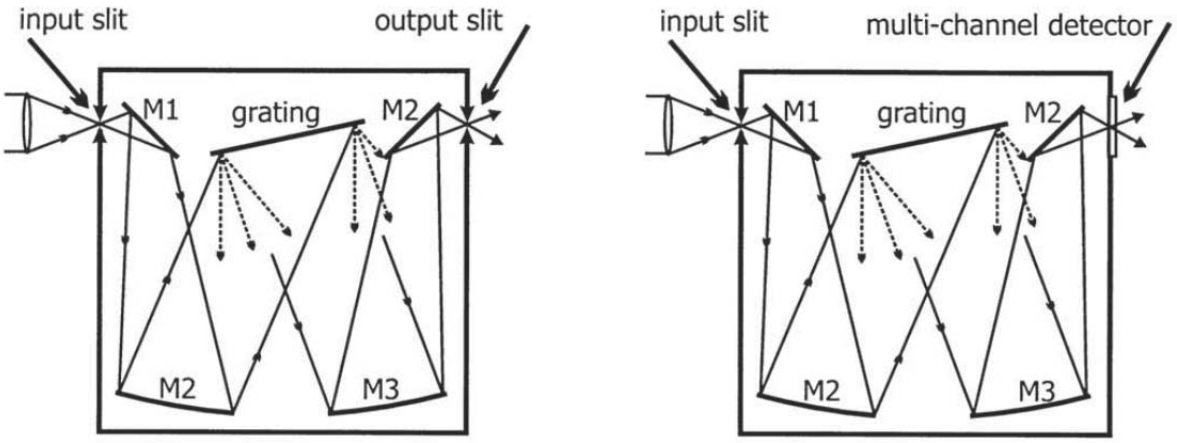

Figure 7 Schematic pictures of a monochromator and a polychromator with gratings as the dispersing elements. The light to be analysed is focused on the input slit. That light is then deflected by a 45° mirror Ml towards a collimating mirror M2, which creates a parallel beam of light towards the grating. The light is scattered then in all horizontal directions due to the grooves in the grating. These grooves are perpendicular to the plane of the drawing and at constant mutual distances of the same order of magnitude as the wavelength of the light. Part of the light is scattered in the direction of the focusing mirror M3 and is then reflected towards M4 and the output section. There a range of images of the input slit is projected in the plane of the output slit, gradually distributed in order of wavelength. In case of a monochromator configuration the output section is equipped with a slit for the selection of the desired wavelength band. In case of a polychromator configuration the output light can be focussed on a multi-channel light detector.

Dispersion techniques, both in the time and in the wavelength domain, are powerful tools in optical spectroscopy. Examples of wavelength dispersers are the monochromator and the polychromator (see Figure 7), where the input radiation is spatially spread, in order of wavelength and in a well-described way. The dispersing element can be a prism or a grating. This wavelength dispersion is used for the performance of spectral analysis of the fluorescence emission. The throughput efficiency of a wavelength disperser depends on the maximum input (and output) angle of the device and on the transmission and/or reflection properties of the applied optical components. Fluorescence photons from a sample are emitted in random directions of propagation. So the wider the maximum input angle of the disperser, the more fluorescence photons can be catched. But that requires a more complicated design and more expensive optics. In practice a ratio of optics diameter to the focus distance in the disperser of 1:3 is already a high value. In that case only a sub-promille part of the actual number of emitted photons will be detected.

The extent of resolution of the dispersion is, next to the dispersive power of the disperser, given by the size of the spot of input light. The size of that spot is normally limited and reduced by an input slit. For that reason the fluorescing spot should have dimensions comparable to the size of the input slit of the disperser and, depending on the required resolution and the dispersion power of the device, that can be down to a width of a few tens of microns. A large dimension of the fluorescing sample will result in high loss of light. Furthermore it can be shown that the reduction of the size of the focussed spot of fluorescence light at the input slit of the disperser, by applying an optical component with shorter focus length, will result in an increase of the output angle. That on its turn will result in loss of light due to the limited input angle of the disperser as shown above. The conclusion must be that the application of a disperser will in general result in a less efficient use of the available fluorescence light.

For the generation of dispersion in the time domain, a streak camera is used. That is a vacuum electron tube where, like in a PMT, the photons to be processed are first converted to photoelectrons by a photocathode. Then those electrons are also accelerated (in the Y-direction, see Figure 8) by an electric field but, unlike in the PMT, now a time dispersion is initiated by bending the electron pathway with a linearly sweeping electric field, perpendicular (Z-direction) to the direction of the accelerating electric field. Then the sweep of accelerated electrons hits a phosphor screen; the early ones at one side, the late ones at the other side. The luminescence of the phosphor can be imaged on a light detector, so that the temporal information of the original (fluorescence) photons can be described. The light detector that reads the image from the

phosphor screen can be a relatively slow device, as for instance a camera with a charge-coupled device (CCD) detector. Streak cameras can have time resolutions of down to a few hundreds of femtoseconds [Guido and Novokhatsky 1995, Hamamatsu 1997].

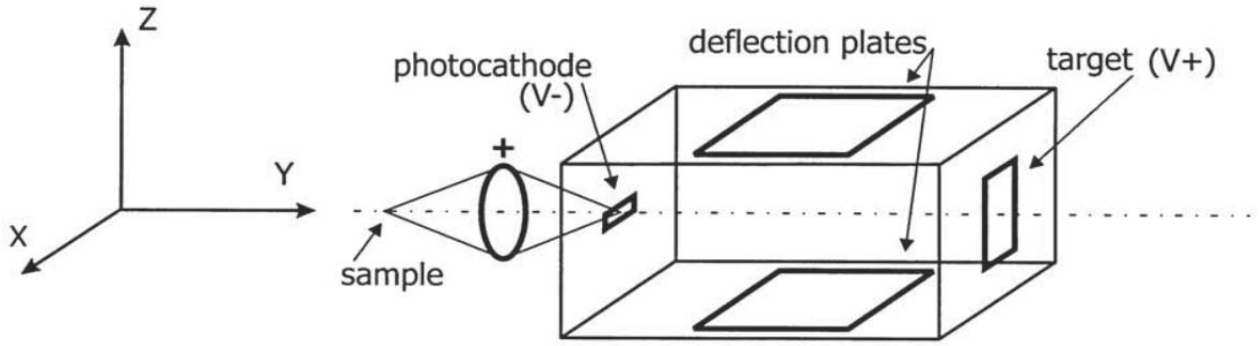

Figure 8 Schematic representation of a streak camera. The light of the fast fluorescence decay of the sample is focused on the photocathode of the streak tube. The photoelectrons created in this way, are accelerated towards the target by the (constant) high voltage between these two electrodes. During the travel of the electrons in the tube the voltage between the two deflection plates is linearly ramped. That causes the electrons to bend their pathway. The early electrons will hit the target at one side, the late ones at the other side. The target may consist of a phosphor screen, eventually equipped with a MCP in front to increase the electron yield. The luminescence of the screen can be read with a slow scan CCD camera.

Between the bending section and the phosphor screen a MCP can be placed, where the (accelerated and bent) photoelectrons are multiplied then. The detection sensitivity of this type of temporal disperser is at single photon level, because one photoelectron from the photocathode can, after the amplification in the MCP, easily ben discriminated at a pixel of the CCD camera to originate one (photon) count there. A great advantage of this method is, that the dynamic range as well as the accuracy is enhanced in this way. In t:he situation without MCP, the luminescence of the phosphor is the result of the bombardment of the phosphor with electrons accelerated by the electric field between photocathode and target (see Figure 8). At a fixed accelerating voltage the yield of the luminescence per photoelectron is not constant, because of statistical fluctuations. That results in noise in excess to photon noise [Saleh et al. 1983, Brown 1984, Arnoldus and Nienhuis 1986], which becomes relatively more obvious when only a low number of photons can be detected as for instance in the tail of the fluorescence decay. When a MCP is placed in between, the packages of light from the phosphor screen, that originate from amplified single photoelectrons, can be discriminated at the

CCD camera to unity pulses, so that all gain fluctuations are eliminated. The dynamic range of the detection part is only limited then by the dark current of the photocathode and the CCD pixels. The dark current of the cathode is very low because the photocathode surface that corresponds with one pixel at the CCD camera is very small (some square micrometers). The dark current of the CCD is also negligible in most cases, also because of the small size and because the detector can be cooled down to achieve that.

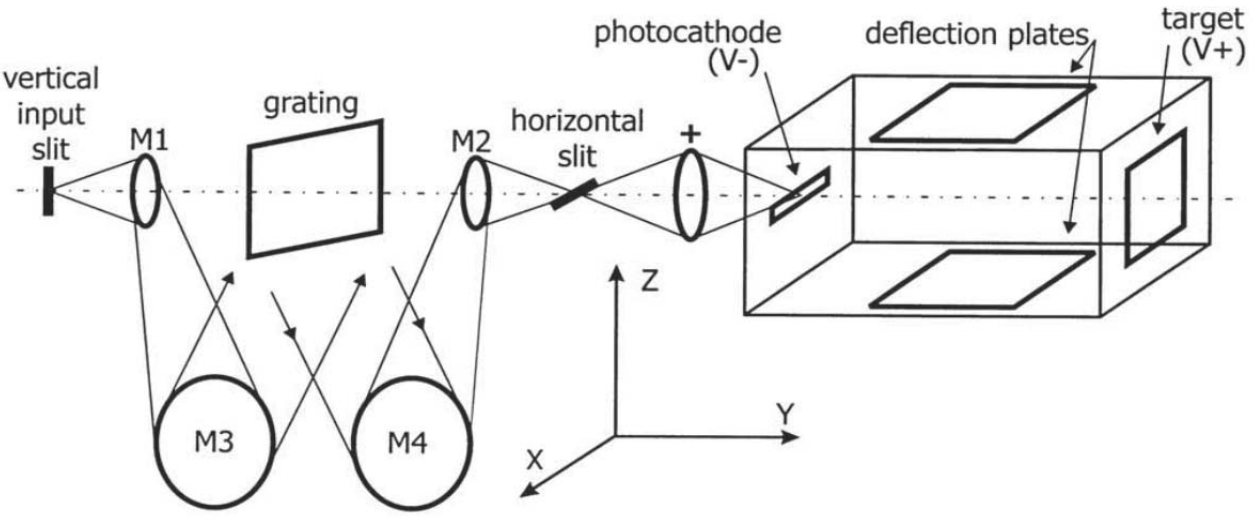

Figure 9 Schematic representation of a polychromatic streak camera. Here the fluorescence light spot is focused on the input slit of a polychromator. At the photocathode of the streak tube the fluorescence light is spectrally dispersed in X direction. At the target of the streak tube the temporal dispersion is added in Z direction. Because the wavelength dispersion is in a direction (X) perpendicular to the direction of temporal dispersion (Z), the slits are crossed, so that the size of the fluorescing volume should preferably be small in these dimensions (see text).

The time dispersion as realized with a streak camera can be combined with a simultaneous wavelength dispersion of the fluorescence light. In that case the wavelength dispersion is first generated (in the X-direction, see Figure 9) by a polychromator. The wavelength dispersed fluorescence light is projected then on the input window (cathode) of the streak camera as a line, where over the length of that line, the different wavelengths are gradually distributed. From this line, the streak tube generates the time dispersion in the Z-direction as described above. At the output screen of the streak tube, from one experiment a three-dimensional data set is created (intensity versus wavelength and time). Because the original fluorescence light spot is dispersed in X and in Z direction, the size of that spot should be very small, leading to high excitation power densities in the sample. In spite of the given disadvantages this technique is powerful because in one

experiment the complete spectral dynamics of fluorescence emission can be simultaneously described. In other cases the fluorescence dynamics at different wavelengths must sequentially be detected and that will not only require more experimental time, it is also a waste of data because in every measurement only a part of the available information is used. So also a multiple of excitation energy must be finally used in these cases.

2.4 Pump-probe techniques

There is a wide variety of different and dedicated methods in pump-probe optical spectroscopy. They are applied for the time-resolved measurement of absorption and emission and also for more advanced experiments [Wiersma 1994]. They all have in common that the sample is treated with two (or more) different (trains of) synchronous ultra-short optical pulses from mode-locked lasers. One laser beam is used to create (or pump) a certain state in the sample. The result of that excitation is then probed with the second beam, by a gradual shifting in time of the probe pulses relative to the pump pulses. In this way the temporal profile of the effect of the pump pulses on the sample is explored. The time resolution of this kind of experiments is in principle limited only by the temporal width of the optical pump and probe pulses. That is very attractive, because optical pulses with temporal widths of down to a few tens of femtoseconds can be easily generated and that gives this technique by far the highest time resolution that is available to study fluorescence dynamics.

When pump-probe techniques are applied for the detection of fluorescence dynamics, the light pulses of a focussed pump beam generate fluorescence decay profiles. The emitted fluorescence light is then mixed in frequency with light pulses of the probe beam in an optically nonlinear crystal (se Figure 10). Light at the sum frequency (UV) of probe beam and fluorescence light is then detected, sometimes in a photon counting mode in a last attempt to increase the detection efficiency somewhat.

The efficiency of the frequency mixing process in the nonlinear crystal is in general linearly dependent on the input powers [Terhune et al. 1963, Lengyel 1966, Kleinman 1972]. That does not only highly limit the dynamic range (to between 1 and 2 decades) of the experiment but makes also the required excitation power density in the sample extremely high as compared with the other detection techniques for fluorescence dynamics. Another disadvantage of the pump-probe technique is that the temporal profile of the fluorescence decay must be sequentially scanned. That means that also in this case only a small part of the information is

detected of the amount that is available every time. So with this type of experiment not only all detection wavelengths must be sequentially scanned but also the temporal profile. In this sense the pump-probe technique has a very low ratio of information content versus applied excitation energy.

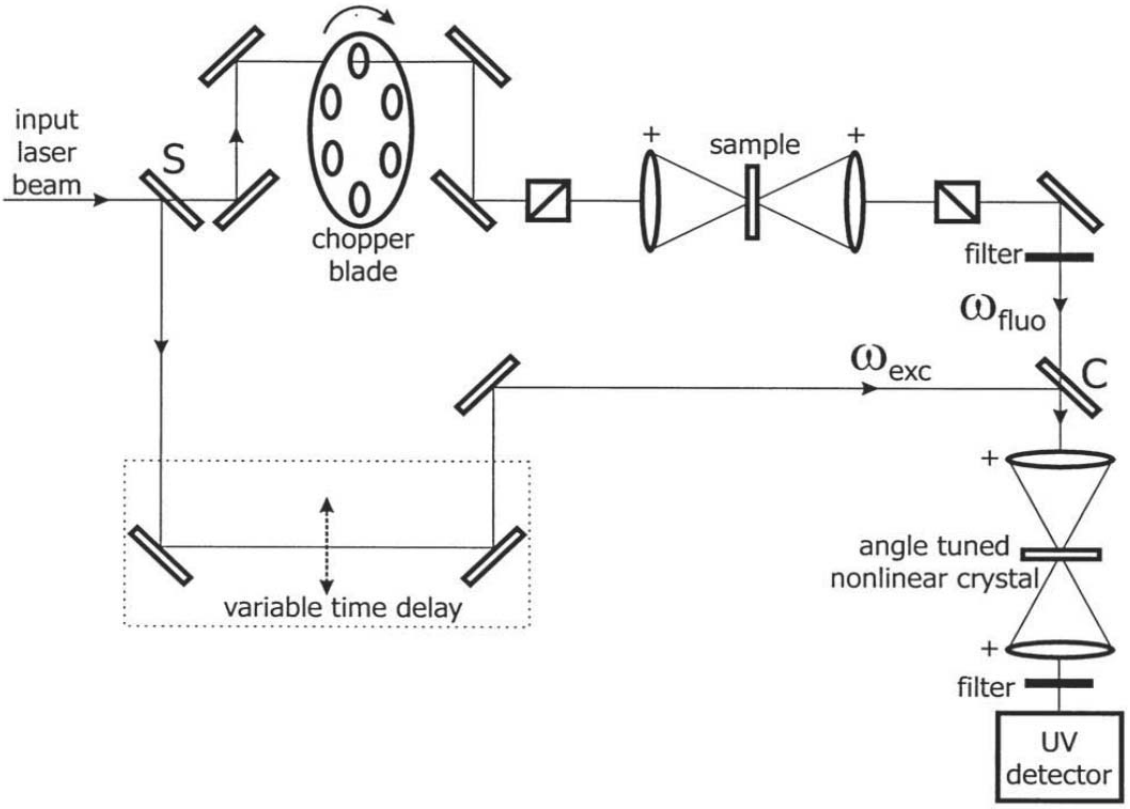

Figure 10 Schematic diagram of a basic pump-probe setup for fluorescence dynamics measurement. The excitation beam of ultra-short light pulses is first split (S) in two. The beam exciting the sample is modulated with a chopper blade at a frequency in the kHz range. The fluorescence emission (ω_{fluo}) is then combined (C) with the other part of the excitation beam (ω_{exc}) and mixed in frequency in a nonlinear crystal. The light at the sum frequency (UV) is then wavelength filtered and converted to an electronic signal by a UV detector. That signal is then used as an input signal for a phase sensitive detector, where the kHz reference signal is taken from the modulation speed of the chopper blade. By gradual changing of the variable time delay, the temporal profile of the fluorescence decay can be probed.

2.5 Boxcar sampling techniques

Although this is an old fashioned method, it is discussed here as well to show the relative value of the technique. When the boxcar sampling technique is applied, the experimental sample is excited with a train of short light pulses (se Figure 11) and after wavelength selection with a filter or monochromator the fluorescence emission is detected with a PMT. The temporal profile of the output signal of the PMT is then sequentially

sampled in time with an electronic gate that shifts gradually along the time axis. This technique is also one of the most inefficient of all detection techniques for fluorescence dynamics, with respect to both required excitation energy and experimental time. In the first place the spectral information must be sequentially detected, the noise of statistical gain fluctuations of photoelectrons is contained in the signal and also the time spectrum is sequentially scanned. The fact that also the temporal resolution is low as compared to the pump-probe method, is the reason that this technique is seldom applied anymore.

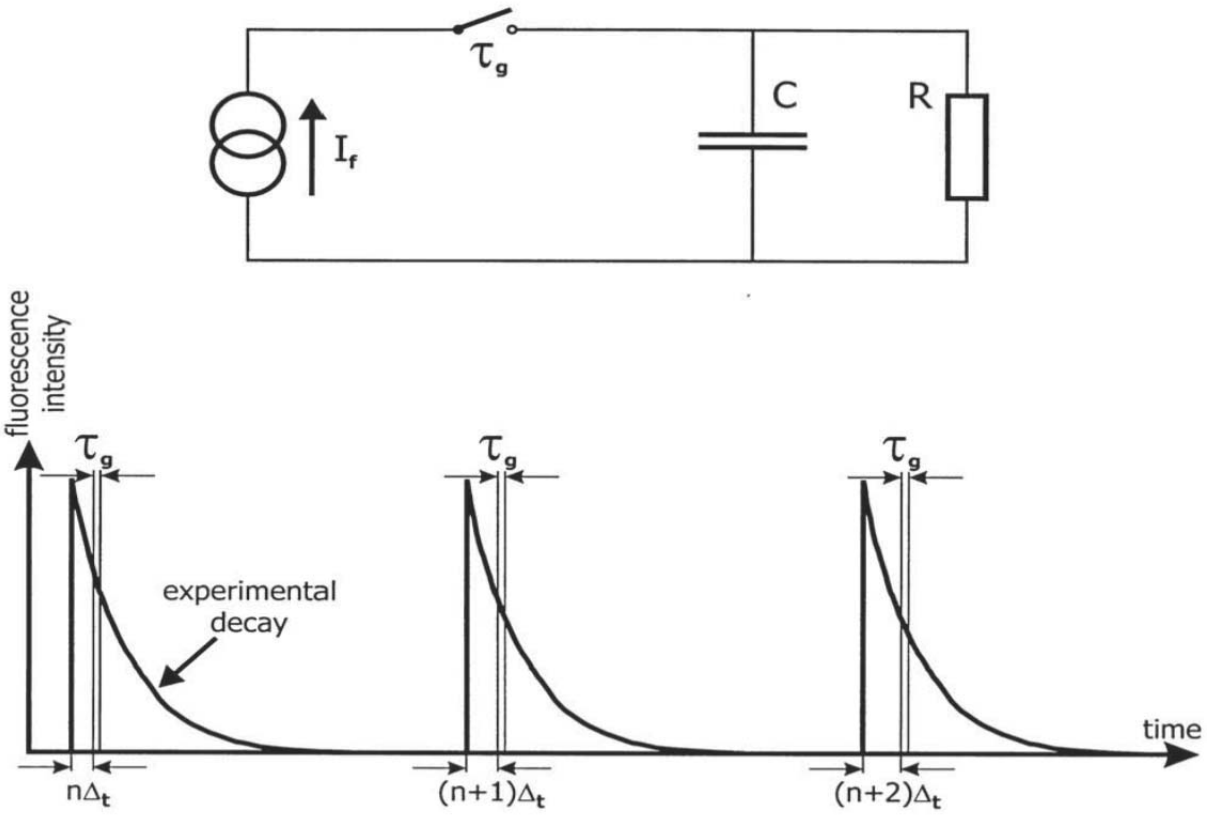

Figure 11 Schematic diagram and timing diagram of a boxcar integrator. Fluorescence decays are generated every time that an exciting light pulse hits the sample. The PMT that detects the fluorescence light intensity is the signal source I_f in the schematic diagram. An electronic switch connects the signal source temporally with the integrating pair R and C, so sampling the output signal of the PMT. As illustrated in the timing diagram, the switch is closed during a fixed gating time (τ_g) and the delay of that gating time (τ_d) relative to the exciting light pulse is slightly increased (Δ_t) every next excitation pulse. In this way the fast phenomenon of the fluorescence decay is converted to a slowly varying signal at the integrating pair, that can be easily detected.

The dynamic range of this technique is, next to the dark current of the PMT, limited by the noise figure of the electronic gate circuitry. In practice with sub-nanosecond electronics an initial dynamic range in the order of two decades can be awaited. Furthermore as well the rise time and the transit time fluctuations of single photoelectron responses at the output of the PMT contribute to time uncertainty in the detection chain. Next to the pulse width of the exciting light pulses, the time resolution of this detection technique is limited by the temporal characteristics of the PMT (rise times down to the sub-nanoseconds [Hamamatsu 1984] and the applied electronic gate (gate widths down to a few tens of picoseconds, [EG&G 1997]. In a few specific cases it can still be useful to apply this technique [Alsins et al. 1982].

2.6 Direct detection

Thanks to modern electronic technology, analogue-to-digital converters (ADC's) with digitization speeds of up to the gigasamples per second are available nowadays. So fluorescence decays with multi-nanosecond decay times can now be digitized directly, but of course still this method is not useful for decay times faster than the nanosecond range, also because of the sub-nanosecond response times of the PMT. And because with this method the output current of the (multi-photon) fluorescence detecting PMT is digitized, part of the disadvantages of the boxcar sampling method are applicable here as well. The dynamic range of the currently available ADC's is limited to some 7 to 8 bits, but the final dynamic range can of course be further increased by the application of digital signal averaging techniques.

An alternative way that will provide full advantage of all information available with the fluorescence photon statistics is to perform a (cross) correlation in time of fluorescence photons upon excitation with a modulated light source as for instance a mode-locked CW laser. But the resolving of photon correlations on a picosecond time scale requires new electronic techniques with (sub) picosecond time resolution because this will directly limit the overall time resolution. Furthermore the photon density in time must be sufficient low to be able to distinguish the separate photon events (as with the TCPC method). As well for these interesting applications as for TCPC the development of new electronic techniques in fast correlation and fast photon detectors with low-transit time jitter is of essential importance.

3. Requirements for measurements on biological materials and experimental limitations

The measurements of spectroscopic properties of biological materials require rather specific conditions. From most of them the properties are largely changed when they are subjected to a high intensity of light. Natural proteins in solution like chlorophyll proteins, flavo-proteins, bioluminescent proteins and model systems for photosynthesis (as well in solution as on artificial substrates), are easily even destroyed when high power light is used for excitation. So it is of major importance to use for excitation of the sample an intensity level as low as possible. That is next to the requirement for the exclusion of the probability to generate unwanted molecular energetic states like the (long living) triplet state, that can easily mystify the results of fluorescence experiments. And for the performance of an experiment of unimpeachable quality, any pretence of experiment-induced artificial results must be avoided or clearly disproved. So always an amount of excitation light should be used as low as possible and that can only be realized when the detection equipment uses the full information content of the emitted fluorescence.

Both the streak camera and the pump-probe methods require the use of very small excitation volumes. The volume should be at the size of a focussed laser beam (down to a few microns in diameter). As a consequence of that, the energy density of excitation light in the sample is high. Most materials will not withstand that treatment without being (partly) destroyed [Cornet et al. 1982], and with these spectroscopic techniques the sample is then rotated or pumped through in a sample replenishing system [Hirsch et al. 1976]. After the measurements the absorption spectrum of the sample is compared with the spectrum recorded before the measurements to check the degree of degradation of the sample. But still these methods always require highest attention to the aspect of excitation light induced sample degradation.

In case of the streak camera the disadvantage of the high density of excitation energy is primarily due to the requirement of a small size fluorescing spot because that directly determines the temporal (and wavelength) resolution of the streak tube. In case of the pump-probe method the small excitation volume is also due to the requirement for concentration of the fluorescence to yield a high power density in the nonlinear crystal. Also with the TCPC method there are limitations to the size of the excitation volume, although the impact is smaller because of different reasons. The limitation in time resolution is here only limited by the sample excitation length times the local speed of light and the temporal resolution of this method is already initially lower.

Next to that, the comparisons of the TCPC method with phase fluorometry, the boxcar and the direct ADC detection method learn that in almost all cases the TCPC method has superior properties. That means that when not the determination of decay times of less than a few picoseconds is required, TCPC should be preferably applied.

4 Instrumental requirements for TCPC

Here the requirements for TCPC equipment are presented and discussed, to be used as a short introduction to the many instrumental aspects and detailed considerations for the experimental conditions. In [van Hoek & Visser, 1985] a broad range of artefact and distortion sources in TCPC is discussed.

4.1 Excitation sources

In time-correlated photon counting the procedure of the registration of the delay of fluorescence photons with respect to the excitation moment must be repeated many times, to attain high accuracy of the detected photon statistics. So preferably an excitation source should be applied with a high repetition rate {MHz range) of picosecond light pulses. The increasing of the rate of excitation pulses will reduce the experimental time, required to obtain a certain accuracy of the measurement results. Ideal light sources for that purpose are mode-locked or synchronously pumped continuous wave (CW) lasers. In some cases the application of synchrotron or storage ring radiation [Munro 1983, Munro & Martin 1991] is a good choice because of the wide tunability range of the excitation wavelength. But with these equipment other specific problems arise like linearity distortions from electromagnetic interference of the ring and also the experimental time resolution is often limited then by the pulse width of the source to the sub-nanosecond region [Munro and Martin 1991, Couprie et al. 1994, 60 ps FWHM].

It is also important that the pulse-to-pulse reproducibility is very good, in particular of the pulse energy, Normally the experiment is arranged in such a way that less than 5 % of the exciting light pulses results in a detected photon, to prevent pulse pile-up distortion [Harris and Sellinger 1979]. A strong pulse will of course have a higher probability to cause the detection of more than one fluorescence photon, as compared to a weak pulse. So when there is a wide distribution in pulse energy in a pulse train, the probability for multi-photon detection per exciting light pulse, cannot be applied as an average. Therefore a train of exciting light

pulses with a very small energy distribution should be applied. And of course also the temporal shape of the pulses should be reproducible as well.

The excitation sources in early TCPC setups were nanosecond flash lamps [Malmberg 1957, Brody 1957, Birch and Imhof 1981]. Disadvantages of these lamps were the low energy of the light flashes, the long duration (ns) of the flashes, the relatively low repetition rate (up to a few hundreds of kHz) and the poor pulse-to-pulse reproducibility. In particular when measurements take place over a many hours period, changes may occur in the lamp (electrode distance, internal pressure, gas decomposition, pulse tail etc.), causing a slow change of the temporal shapes of the respective flashes and of course that yields a very serious limitation of the accuracy of the measurements. Also the electro-magnetic interference radiated by the first types of lamps gave rise to serious source of linearity distortions in the measurements.

4.2 Sample requirements

In TCPC almost in all cases a deconvolution method is part of the data analysis procedure (see 4.7). That means that during the analysis of the experimental data, the temporal shape of the instrumental response curve is taken into account. Because the time resolution of the detection system is not infinitely faster than the fluorescence decay time, the experimental fluorescence decay curve will be distorted by the temporal shape of the DIRF of the experimental setup. The inclusion of the knowledge of the temporal shape of the DIRF in the data analysis procedure creates the possibility to enhance the final temporal resolution of the experiment. An essential condition for a flawless application of the deconvolution method is of course, that the applied DIRF is a perfect description of the temporal properties of the setup in exact the same situation as when the fluorescence decay is recorded. Important is the effective size of the fluorescence volume as compared with the volume used for the registration of the DIRF because that has direct consequences for the overall temporal resolution.

4.3 Fluorescence wavelength selection and reference method

For the selection of the wavelength band of fluorescence detection in TCPC experiments most times a filter or a monochromator is used. But also simultaneous multi-wavelength experiments with TCPC detection have been carried out as well [Courtney 1991, Ainbund 1992, Kelly et al. 1995, Mcloskey et al. 1996, Suhling et al. 1996]. Therefor special detection tubes were applied with multiple input and output channels, allowing the simultaneous detection of the timing of several photons in the same tube. So these different channels can

be used for the simultaneous detection of fluorescence at different wavelengths. A disadvantage so far of these methods is, that not a time resolution and accuracy can be obtained that is comparable with TCPC using a single MCP-PMT detection channel. But when for a specific measurement the optimum time resolution is not quite required, the multichannel detection method is very useful because then a multiple of the available information is used as compared to the case of single channel detection. So that leads to a more efficient use of the applied excitation light.

When a monochromator is used, there are some limiting requirements for the effectively detected fluorescing volume, as described in section 2.3. An advantage of the application of a monochromator in the detection chain is that a DIRF can be simply recorded by replacing the sample by a scattering object of the same size as the sample and then tuning the monochromator towards the excitation wavelength. Important items are the wavelength dependency of the DIRF and dependency of the DIRF on the part of the photocathode of the PMT that is excited.

In general the wavelength selection with filters can be performed at a much more efficient throughput of the fluorescence photons as compared with the application of a monochromator (see 2.3). When filters are used for the selection of the wavelength band of fluorescence detection in a kinetic fluorescence experiment, some other aspects must be considered. In that case the DIRF must be derived from the measurement of the fluorescence dynamics of a fast decaying single exponential reference compound or mimic.

4.4 Fluorescence polarization

A normal condition is the detection of the fluorescence at an angle of 90° with respect to the excitation direction, because in that situation the excitation light can be easily catched away from the detection channel. Also normally the sample is excited with vertically polarized light. Then the fluorescence is detected with an optical polarization analyser at magic angle in case of only fluorescence decay time measurements, or (simultaneously or sequentially) at a parallel (vertical) and perpendicular (horizontal) direction in case of fluorescence anisotropy measurements. In the present setup an optical polarizer is mounted at the front of the sample housing, further optimizing the polarization degree of the laser light. Scattered light from contamination at the surface of this polarizer will result in depolarization. So this polarizer should be well cleaned and at some distance from the sample to minimize scattered secondary light exciting the sample.

Another important item in polarized experiments is the maximum detection angle that can be used for an accurate registration of the parallel and perpendicular polarized fluorescence. In measurements of polarized emission a certain error will be introduced when the solid angle of detection is larger than infinitesimal small. But just to detect any photon, the detection angle should be larger than that. So also here there is a clear competition between (polarization) accuracy and available experimental time. In practice the degree of polarization of fluorescence is rather low (≤ 0.3), so a small increase of the detection angle will only result in a minor decrease of the accuracy of the polarization measurement. In the present setup an efficient optical lens scheme is applied (f/1.1), ideal for experiments with non-polarized emission. When fluorescence anisotropy measurements must be carried out, a diaphragm is inserted in the detection pathway. The maximum diaphragm opening that can be applied was experimentally determined. A series of measurements was carried out of parallel and perpendicularly polarized fluorescence emission from a sample with a high degree of polarization, at gradually increasing diaphragm opening. It was found that for an opening of f/4 the error in the degree of polarization is well below 1 %.

When filters are used for detection wavelength selection, the G-factor = 1. That means that the sensitivities of the detection channel for horizontally and vertically polarized light are equal. When a monochromator is used, the sensitivity may highly depend on (the wavelength and) the polarization direction of the detected light, in particular when holographic gratings are used. Then always a G-factor must be taken into account in the analysis of the experimental data for anisotropy measurements. That G-factor can be easily determined at the given detection wavelength from the measurement of the fluorescence decay of a small fluorescence probe molecule in non-viscous solution. The rotational correlation time will be very short, so that the intensities of vertically and horizontally polarized fluorescence in the tail of the decay are essentially equal. So when decays are measured of fluorescence polarized vertically and horizontally, from a numerical analysis of these intensities in the tail of the decays, the relative sensitivities at these polarization directions can be determined, leading to the G-factor.

4.5 Detection equipment

The sensitivity of a TCPC experiment is determined by the maximum (useful) available intensity of excitation light and the overall detection efficiency. When fluorescence dynamics measurements must be carried out at chromophore concentrations down to the nanomolar regions, a larger detection volume will increase the number of detected fluorescence photons. And in particular when large volumes have to be

measured, a PMT is an almost ideal detector because of the large size of the sensitive surface, measuring in the range of 1 cm^3, at maintained picosecond time resolution [Bebelaar 1986] and low dark counts. A disadvantage is the relatively low quantum efficiency (maximum some tens of percent), as compared to semiconductor light detectors (up to 90 %). But the high internal gain (10^6 - 10^{11}) of the PMT gives the possibility to detect single photons because the tube converts one photoelectron into a current pulse that can be directly detected by an electronic discriminator.

Another detector that can be used is the avalanche photodiode [Cova et al. 1981], in particular when active quenching circuits are applied [Cova et al. 1996]. The advantage can be the high quantum efficiency, particularly in the red region of the spectrum, but there are still some drawbacks to these devices. In the first place the sensitive surface is very small (down to a few µm) and furthermore the dark current can be rather high (from many kilo-cps down to 100 cps when cooled to 200K) [Robinson & Metscher 1987]. Already from this short description it can be read that only in case of extremely small fluorescing volumes the avalanche photodiode can be effectively used for TCPC.

4.6 Electronics

Start-stop electronics are available from different companies and still new types are developed, adapting to the high data throughput rates required in some new applications as particle-in-flight fluorescence decay time measurements on cells and combined fluctuation correlation and fluorescence decay time measurements. General requirements on these electronics are sensitivity, time resolution, reproducibility and the lack on temperature dependency.

With constant fraction discriminators (CFD) the "walk" is an important parameter. That is the time dispersion of the output pulse with respect to the position in time of the input pulse upon changing the input amplitude. A state-of- the-art specification is a walk of a few tens of ps at a 100: 1 dynamic range at the input. Also the dependency of the transit time of the CFD upon changing the temperature is an important parameter that directly influences the reproducibility of the measurements. Normally the data from a TCPC experiment are subject to a deconvolution procedure. A massive requirement for a smooth deconvolution Is that the DIRF (see 4.2) and unknown decay have no relative time shift. A practical value for the transit time of a CFD is a few to less than ten nanoseconds, the temperature dependency is + a few tens of picoseconds per degrees Centigrade. From a 50 Ω delay cable with a length resulting in 100 ns delay the temperature dependency of

the delay time is up to - 60 ps per degrees Centigrade for type 58 CU. The electronic time resolution of the CFD and time-to-amplitude converters (TAC) can be a few ps FWHM.

Important artefacts that should be avoided are non-linearities or distortions in the decay data. These distortions can be originated in different ways. The source of these distortions is always the pick-up in the detection electronics of electro-magnetic interference that is synchronous with the excitation rate. There are two different places in the setup where that interference can efficiently introduce distortions; the ramp generator of the TAC and the discrimination input circuit of the CFD. In the TAC a voltage ramp is generated by loading a capacitor with a constant current source. When a stop pulse occurs, the ramp generation is stopped (at voltage V_{stop}), and a voltage pulse is generated with fixed duration and voltage V_{stop} that is later analysed by an ADC. When the constant current source is disturbed by a fluctuating interference voltage, the generated ramp is no longer linear. When that interference is synchronous to the start rate, the fluorescence decay data will be distorted. Another place where distortions can be initiated is the discrimination circuitry of the CFD. In the CFD the (amplified) output voltage pulses from single photon responses at the PMT are discriminated. These pulses have fluctuating voltage amplitudes due to the statistical process of electron multiplication (see 2.1). The CFD will generate a logic output pulse when an input voltage pulse occurs that has a higher amplitude than a, normally, fixed discrimination voltage level. Next to that there is an electronic (constant fraction) circuit that controls the moment in time of generating that logic output pulse. Now suppose that there is not a fixed discrimination level but a voltage level that is modulated synchronously at the start rate of the experiment due to electromagnetic interference. Then the probability for the discrimination of a photon pulse from the PMT is changed in time with these modulations. That will introduce non-linearities or distortions in the decay data. Avoiding these distortions (shielding, separating interference source and detection) is a matter of trial and error and sometimes very time consuming, because when the interference is through air, already the moving of a person in the room might influence the effects of the interference.

4.7 Data analysis

The analysis of the experimental data is another important part of the measurement of fluorescence decays. Already in the late seventies there was a variety of techniques for the analysis of data from time-resolved fluorescence experiments [Cundall & Dale 1983]. Still there is a wide choice of available analysis

programs, from research laboratories and commercial companies. Also in our laboratory an analysis program was developed [Vos et al. 1987], and we found that an extended test of the performance of this kind of programs is very time consuming. Now it is preferred to buy the analysis programs [Brochon 1994] as well to cooperate with other research groups [Beechem et al. 1991, Novikov et al. 1999], also for the development of programs for special purposes. A common subject in the judgement of the accuracy of the analysis is the simulation of data. In particular in critical cases a parallel simulation of the experiment with comparable parameters should be executed to check the performance of the analysis. The variance on the determined amplitude and decay time parameters can then be described in detail.

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