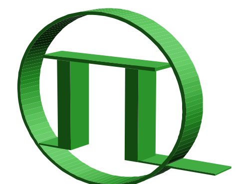
Application note



FRET analysis with pulsed interleaved excitation using the MicroTime 200

Benedikt Kraemer, PicoQuant GmbH
Felix Koberling, PicoQuant GmbH
Martin Roos, RKI, Berlin
Steffen Rüttinger, PTB, Berlin, March 2005

Introduction

Förster Resonance Energy Transfer (FRET) describes a process in which energy from and excited molecule (Donor) is transferred to a second molecule (Acceptor), which may fluoresce. As the amount of energy transferred is sensitive to the distance between these two molecules, this technique is used to measure intermolecular distances on a nanometer scale and has consequently found a broad range of applications, e.g. in binding studies and protein folding investigations [1-3]. However, the analysis of FRET measurements is often complicated by the fact that not all molecules under study are necessarily marked with both donor and acceptor molecule, but often lack e.g. the acceptor molecule. Additional complications arise from multiple labeled species, giving not only one but multiple FRET processes. A differentiation between these single species is therefore desirable, but effectively only possible on the single molecule level.
One approach is based on the pulsed interleaved excitation (PIE) of both, the acceptor and the donor molecule. Briefly, PIE is used to excite the acceptor dye independently of the FRET process and to prove its existence via fluorescence. This technique allows to differentiate a FRET molecule, even with a very low FRET efficiency, from a molecule with an absent or non–fluorescing acceptor. Such incomplete FRET molecules lead to a zero efficiency peak [1] in the FRET efficiency histogram which can easily be identified and removed.

This application note describes the use of the confocal fluorescence microscope MicroTime 200 [4] for single molecule FRET studies using PIE. In order to demonstrate this capability a set of FRET pairs with different polyproline spacers was synthesized. These peptides have been established as reference molecules for FRET both in ensemble [5] and single molecule experiments [6]. The presented measurements show results for a peptide containing 12 polyproline residues. N-terminal glycine and C-terminal cysteine is attached to permit labeling of opposite ends with Alexa Fluor 647 and Alexa Fluor 555 (Molecular Probes, Eugene, USA) as acceptor and donor chromophores (Alexa Fluor 647-Gly-$(Pro)_{12}$-Cys-maleimide-Alexa Fluor 555). The length of the polyproline 12 spacer accounts to around 4 nm, a value near the calculated Förster radius of 5 nm [7] of the FRET pair. Fig. 1 shows a molecular model of a FRET pair with a polyproline 12 spacer and two Alexa Fluor dyes.

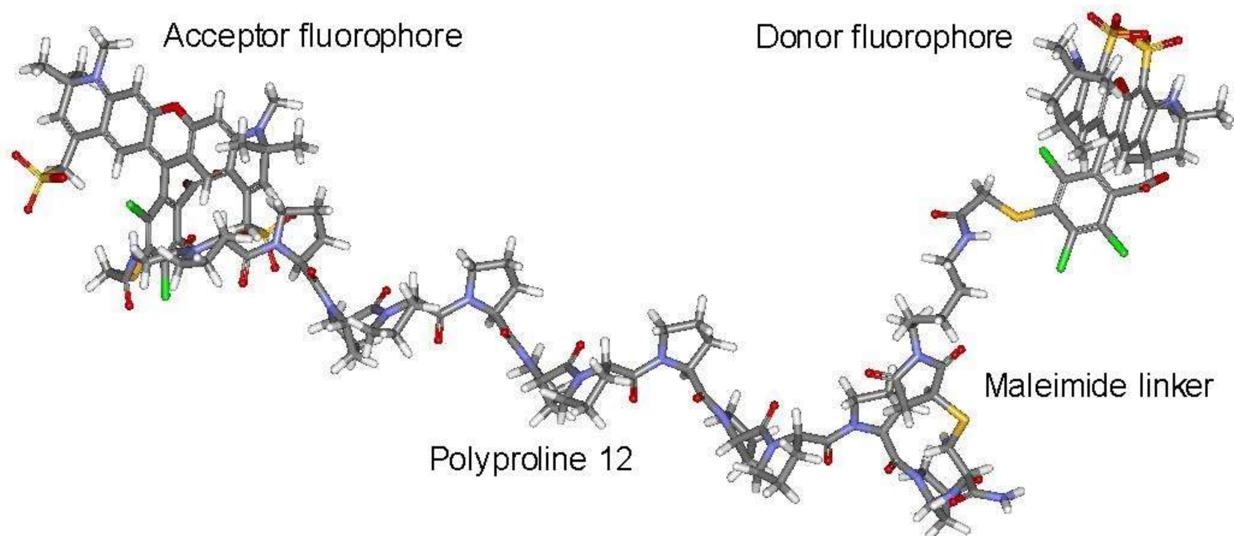


Fig.1: Molecular model of a polyproline 12 peptide and maleimide linker with two Alexa Fluor dyes

Principle of PIE FRET

PIE-FRET can be used to identify FRET molecules with a non-fluorescing or absent acceptor molecule. Two lasers are chosen with suitable wavelengths for the excitation of both the donor and acceptor molecule. The laser pulses are delayed with respect to each other to yield a pulse sequence with interleaved pulses. In our setup the fluorescence after excitation with the red laser is visible in the early time window and that of the green laser in the late time window (see fig. 2). Each diagram shows in the upper part the fluorescence decay measured in the donor detection channel and in the lower part the decay detected in the acceptor channel along with a schematic representation of the laser pulse.

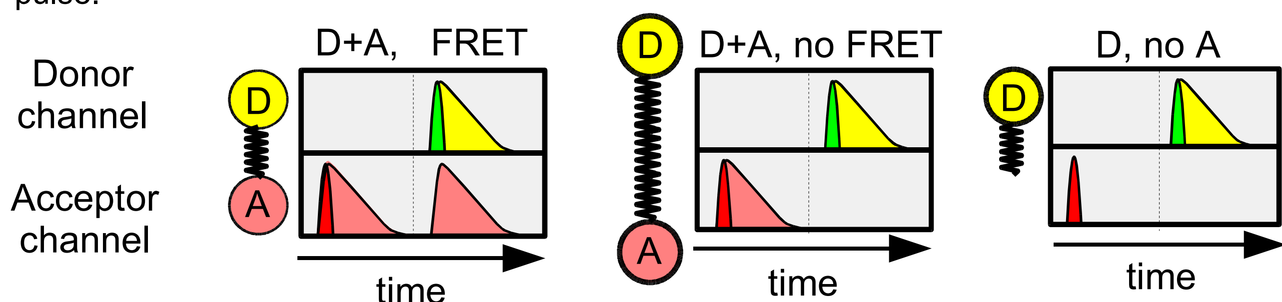


Fig.2: Schematic PIE–FRET results for a close FRET pair (left), a well separated FRET pair (middle) and donor molecule lacking a FRET acceptor (right).

The left part of the image schematically shows the desired FRET pair under study: Excitation with the red laser only leads to observable fluorescence from the acceptor molecule in the early time window, while on the other hand excitation of the donor molecule with the green pulse leads to fluorescence from the donor molecule and also from the acceptor molecule due to FRET in the late time window. If the distance between the donor and the acceptor molecule is too large for an efficient FRET process, like schematically shown in the middle of Fig.2, one can only observe fluorescence from each excited fluorophore after direct excitation. Finally, if the molecule lacks the acceptor

molecule completely, there will consequently be no emission at all after a red excitation in the early time window, as shown on the right. In that way incomplete FRET-molecules without a fluorescing acceptor (or as well donor fluorophore) are identified. This information can be used to obtain useful information for the calculation of correction parameters (e.g. for direct excitation or bleed through) and to eliminate the zero efficiency peak.

This method works as well using alternating pulses having longer pulse widths in the microsecond range as was demonstrated from the group of Shimon Weiss using cw-lasers with electro-optical modulators [9]. However, this technique does not allow for fluorescence lifetime analysis and complicates fluorescence correlation spectroscopy of the different fluorescent molecules due to the low number of excitation cycles in the time period in which molecules diffuse through the excitation volume.

Experimental details

The acceptor was excited using a picosecond pulsed diode laser with a wavelength of 638 nm and the donor molecule was excited using a PicoTA laser emitting at 532 nm. Narrow band clean-up filters ensured that no parasitic light reached the sample. The repetition frequency of each laser was set to 40 MHz. To realize the pulsed interleaved excitation with a pulse train of alternating colors the 532 nm laser was electronically delayed by 12.5 ns with respect to the 638 nm laser. A dual band dichroic reflecting 532 nm and 638 nm guided the light to a high numerical aperture apochromatic objective (60x, NA 1.2, water immersion, Olympus), which finally focussed the light to a confocal volume of 1.1 femtoliter (resp. 1.7 femtoliter) for excitation with 532 nm (resp. 638 nm) and detection at (575±15) nm (resp. (685±35) nm). The size of the confocal detection volume was measured using Tetra Spec beads (Molecular Probes) with a diameter of 100 nm immobilized on a cover glass. Fluorescence from excited molecules was collected with the same objective and focused onto a 50 µm diameter pinhole to enable confocal detection. The donor and acceptor emission were separated using a dichroic longpass filter with a dividing edge at 640 nm. Bandpass filters are used to eliminate the respective excitation wavelength and to minimise spectral crosstalk. The fluorescence was detected with two avalanche photodiodes (SPCM–AQR–14, Perkin Elmer Inc.) using the method of time-correlated single photon counting (TCSPC) with the TimeHarp 200 board. The data was stored in the Time-Tagged Time-Resolved Mode (TTTR), allowing to record every detected photon with its individual timing and detection channel information which is the basis for the following analysis [8]. The presented measurement was performed 10 µm deep in the solution with a total acquisition time of 40 minutes.

Results

The MicroTime200 software offers several possibilities to display and analyze the acquired TTTR raw data. For a first visualisation the single photon data of the acceptor channel was summed up in bins of 1 ms, resulting in the time trace shown in the upper part of Fig. 3.

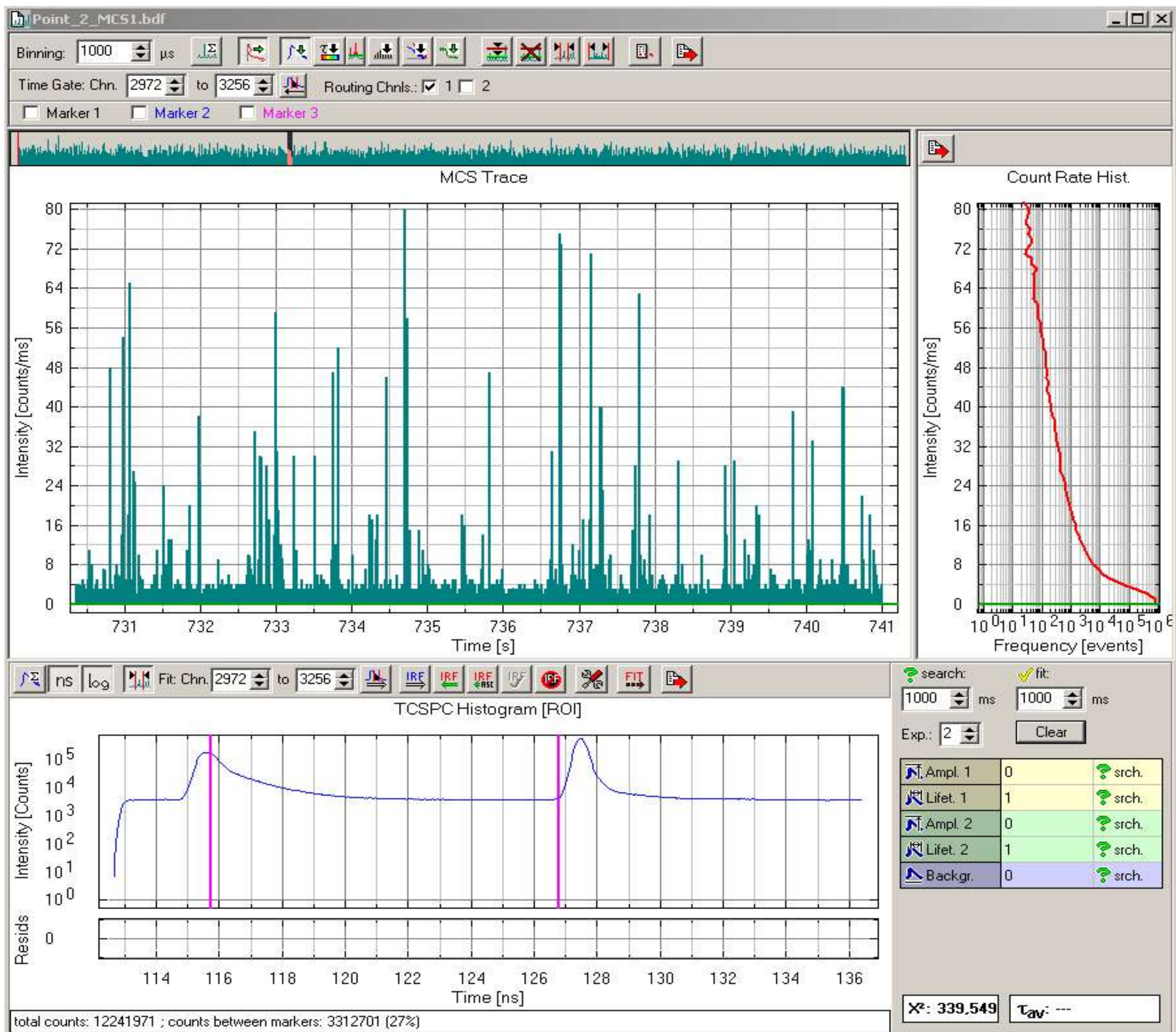


Fig. 3: MCS trace, count rate and TCSPC histograms for the acceptor molecules (routing channel 1), calculated from the TTTR raw database.

Additionally, the overall TCSPC histogram can also be calculated from the TTTR file. The corresponding result is shown in the lower part of Fig. 2. In the TCSPC histogram two fluorescence decays can be observed: The first decay originates from direct excitation of the acceptor fluorophores with the 638 nm laser, the second after a time delay of 12.5 ns is the result of FRET - excitation of the acceptor after donor excitation with the 532 nm PicoTA.

The next step in a conventional FRET analysis would be to identify single photon bursts and to use this data to calculate e.g. the FRET efficiency. However, the result of such a procedure can be falsified by molecules lacking the acceptor dye [3]. For an exact result these molecules therefore need to be sorted out using the results of the PIE-FRET measurements. As this is of course only possible on the single molecule level, one has to make sure that only a single molecule was present in the excitation volume during the measurement. This is most elegantly done using Fluorescence Correlation Spectroscopy. Due to the very flexible TTTR measurement mode, this information can easily be extracted from the measurement data. In this case a temporal window which corresponds only to the fluorescence from the acceptor after excitation at 638 nm (116 ns to 127 ns –

vertical markers in the TCSPC histogram) was chosen to calculate the time-gated autocorrelation function. The result of this calculation is shown in Fig. 4. The increasing correlation at lag times in the microsecond range is due to the afterpulsing of the SPAD detector. The G(0) value of this curve accounts to 7.7, leading to a concentration of acceptor molecules in the detection volume of 0.08 molecules per femtoliter and to a particle number in the detection volume of 1/7.7 = 0.13. This low number indicates a high probability that only one molecule at a time was present in the measurement volume, which is a prerequisite for the further single pair FRET (spFRET) analysis.

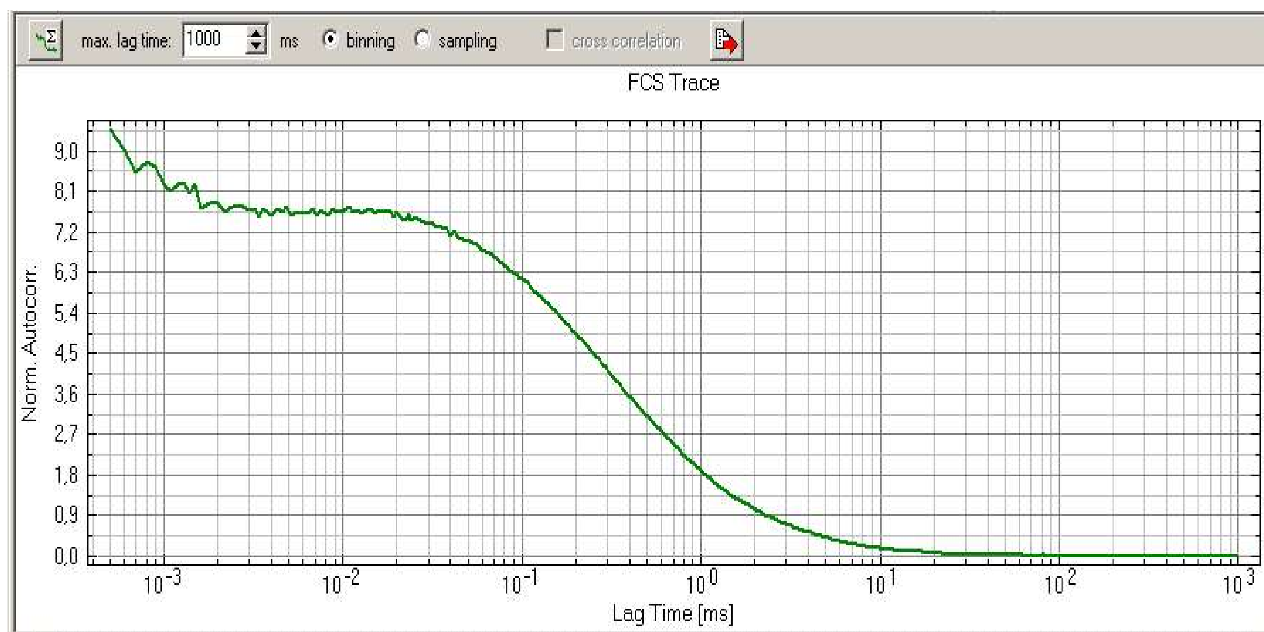


Fig. 4: Time-gated FCS analysis for the acceptor fluorescence after red excitation. The curve corresponds to a G(0) value of 7.7.

For the spFRET analysis the MT200 software is first used to identify all photons which belong to every individual detected FRET event [3]. Then the overall number of photons in each photon burst is sorted according to the detection channel (donor, acceptor) and timegate (red excitation, green excitation) and each exported via the burst export feature for further detailed analysis using third party software.
The FRET efficiencies E are calculated and histogrammed according to equation 1, with n_D and n_A being the detected numbers of photons in the bursts for the donor and acceptor channel respectively.

$$E = \frac{n_A}{n_A + \gamma\ n_D} \qquad (1)$$

Different quantum yields of the two chromophores and detection efficiencies in the two detection channels are accounted by γ. To demonstrate just the effect of the PIE analysis we set γ to 1 and did not take into account the bleed-through of the donor emission into the detector channel and the direct excitation of the acceptor. This leads to an overestimation of the calculated FRET efficiencies.
To demonstrate the effect of PIE, a FRET efficiency histogram was first calculated using all identified photon bursts, i.e. molecules lacking the acceptor dye were also considered. The resulting FRET efficiency histogram is shown in figure 5 (left) and is dominated by a maximum close to an efficiency of 0.9. The width of the distribution is mainly due to shotnoise [6]. Additionally, a broad distribution with low FRET efficiencies, the zero

efficiency peak, can also be observed. This peak is most likely due to molecules lacking the acceptor dye. By using the information of the PIE-FRET measurement it is now possible to sort out these photon events and to recalculate the FRET efficiency histogram. The result of this calculation is shown in fig. 5 (right)

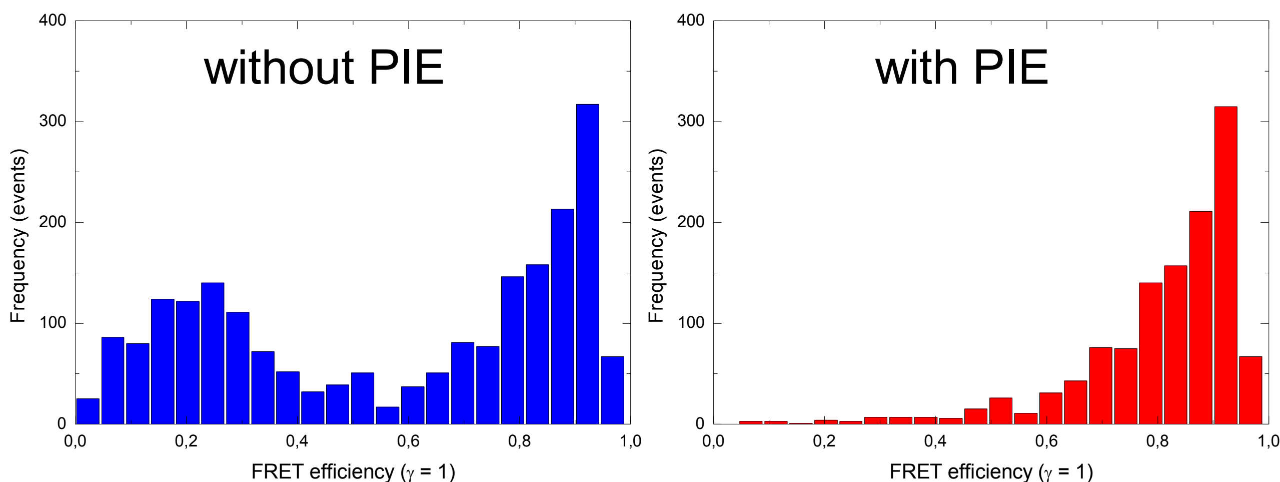


Fig. 5: FRET efficiencies taking all burst events into account (left) and using PIE, i.e. only events originating from FRET pairs with a fluorescing acceptor are displayed (right)

As expected the zero efficiency peak has vanished proving that it originated from FRET molecules with a missing or non fluorescing acceptor. The PIE FRET technique becomes even more advantageous for the study of larger FRET pair distances or distributions of distances leading to efficiency histograms where the two maxima do not form any more two distinct populations.

With PIE the three subpopulations donor only, acceptor only and complete FRET molecules can be distinguished and furthermore the concentrations of all three species can be derived using time-gated FCS after burst selection. Therefore it is in principle possible to deduce all important parameters necessary for the FRET efficiency calculation (γ, bleed through of the donor emission into the acceptor channel and direct excitation of the acceptor) from a single measurement of a mixture of all three subpopulations. This could be of importance under conditions where control measurements can not be performed easily like e.g. in living cells.

Conclusion

The presented results clearly show that the MicroTime 200 along with its software is an extremely useful tool to investigate molecular properties and dynamics of freely diffusing molecules. FRET measurements can be used to investigate the folding and dynamics of a wide range of biomolecules [10]. Pulsed interleaved excitation allows for an excellent separation of different dyes due to the dye-specific excitation wavelengths even for measurements performed in liquids (e.g. FCS). For studies resolving molecular distances in the nanometer scale FRET can be enhanced using PIE to eliminate contributions from incomplete FRET pairs with missing or non fluorescing acceptors. The method can be extended for excitation with more than two wavelengths for complete analysis of triple FRET or FRET in combination with the detection of other fluorescently labeled molecules.

Further Applications of PIE:

- Fluorescence Cross-Correlation (FCCS) experiments in the presence of FRET
- Enhanced sensitivity and elimination of crosstalk in FCS and FCCS experiments
- Simultaneous multi-color excitation with a single detection channel

Other general applications for FRET measurements include:

- Spatial distribution and assembly of protein complexes
- Receptor/ligand interactions
- Probing interactions of single molecules
- Structure and conformation of nucleic acids
- Detection of nucleic acid hybridization
- Primer-extension assays for detecting mutations
- Automated DNA sequencing
- Distribution and transport of lipids

Further reading

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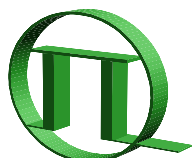

PicoQuant GmbH
Unternehmen für optoelektronische Forschung und Entwicklung
Rudower Chausse 29, 12489 Berlin, Germany
Telephone: +49 / (0)30 / 6392 6560
Fax: +49 / (0)30 / 6392 6561
e-mail: photonics@pq.fta-berlin.de
WWW: http://www.picoquant.com