Technical Note

FCS Solutions Slide

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Introduction

State of the art confocal microscopes such as the PicoQuant MicroTime 200 (MT200) offer diffraction limited spatial resolution, highest (single molecule) sensitivity and picosecond fluorescence lifetime measurement accuracy. For developers, manufacturers, as well as users it is mandatory to assign values to these qualities. For the user it is often not easy to ascertain that the instrument is properly aligned as a large number of factors influence resolution and sensitivity. A simple to use testing procedure based on a reference sample to be deployed on a day-to-day fashion is therefore highly desirable. The FCS Solutions Slides are designed to help users in adjusting the pinhole and correction collar as well as to determine the molecular brightness as a measure for the total instrument sensitivity.

Sample Design

The FCS Solutions Slide contains four hermetically sealed sample chambers covered with cover glasses. They are filled with an aqueous buffer solution including a mixture of different Atto488 and Atto655 concentrations ranging from 10^{-6} M to 10^{-10} M. Small air bubbles inside each chamber provide a pressure cushion ensuring a long lifetime of the slide. The different concentrations allow for a wide range of measurement tasks ranging from coarse adjustments over measurements of the molecular brightness to real single molecule measurements. Both dyes can be easily separated either by their spectral properties (see fig. 3) or by their different fluorescence lifetime (Atto488: 3.8ns, Atto655: 1.8ns). Their high molecular brightness permits fluorescence collection at very low light intensities and/or misaligned systems.

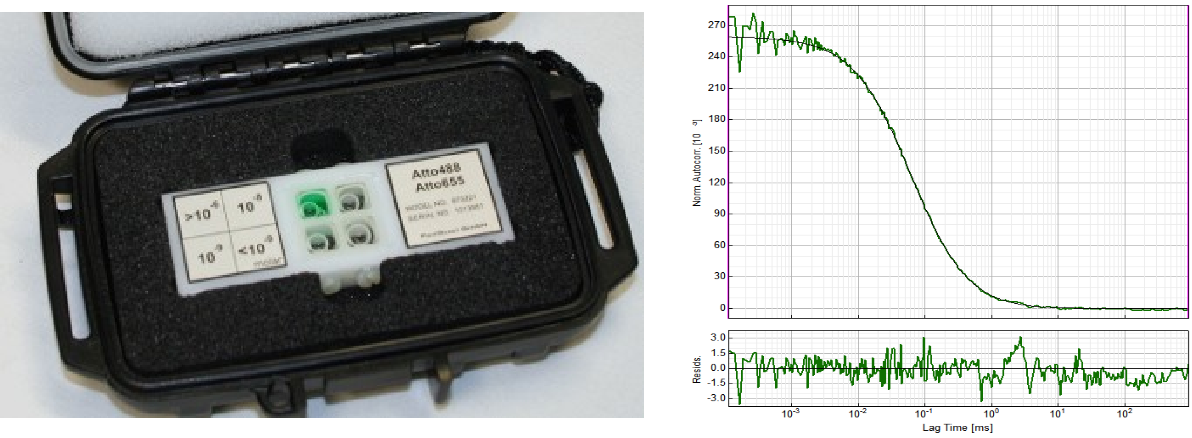


Fig. 1: *FCS Solutions Slide in special transport box. The Slide has 4 chambers containing different concentrations of an Atto488 and Atto655 mixture in aqueous buffer (left). Typical FCS curve and fit (single component, 3D diffusion, no triplet) of the nanomolar (10^{-9}M) solution, acquired within 10 seconds using the MicoTime 200 (right).*

	Upper left	Upper right	Lower left	Lower right
Atto488 chamber label and concentration range in mol/liter	$>10^{-6}$	10^{-8} $(1\times10^{-8} \ldots 10\times10^{-8})$	10^{-9} $(1\times10^{-9} \ldots 10\times10^{-9})$	$<10^{-9}$
Atto655 chamber label and concentration range in mol/liter	$>10^{-6}$	10^{-8} $(1\times10^{-8} \ldots 10\times10^{-8})$	10^{-9} $(1\times10^{-9} \ldots 10\times10^{-9})$	$<10^{-9}$

Chamber labels and concentration ranges of the Atto488 and Atto655 aqueous solutions in the different chambers of the FCS Solutions Slide

Typical Alignment and Measurement Tasks

Adjustment of the Confocal Pinhole

The adjustment of the confocal pinhole is a very crucial step in the alignment procedures of the optical beam path. Generally speaking, the detection volume (as determined by the pinhole position) must overlap with the excitation volume (as determined by the laser focus). Alignment of the confocal pinhole is achieved by maximizing the fluorescence signal of a micro-molar dye solution included in the FCS Solutions Slide – the principal steps to perform the alignment are:

1. Select a large pinhole size and place the FCS Solutions Slide on top of microscope.
2. Focus approximately 20 µm (5 µm for oil immersion objectives) deep into the chamber that contains the solution with the highest concentration (>10^{-6} M).
3. Monitor the fluorescence signal intensity and adjust the pinhole position until you reach the maximum intensity.
4. Decrease the pinhole size and repeat the procedure until the alignment is satisfactory for the smallest available pinhole.

A more detailed description on how to perform this alignment at the MicroTime 200 can be found in the appendix of this technical note.

Correction Collar Setting

Water immersion objectives are designed for imaging in water on top of a cover glass. The cover glass has a different index of refraction than water (1.5 instead of 1.3), which must be corrected for, depending on the cover glass thickness. For this purpose water immersion objectives feature a dial which allows to correct for the currently used cover glass thickness. This is crucial for achieving optimal performance, as artifacts will become apparent if the wrong cover glass correction is selected. The scale of the correction collar dial can, however, deviate slightly from its imprinted values. This deviation can be measured using the FCS Solutions Slide – the principal steps to perform the correction are:

1. Place the FCS Solutions Slide on top of microscope and focus approximately 20 µm (5 µm for oil immersion objectives) deep into the chamber that contains the solution with the highest concentration (>10^{-6} M).
2. Adjust the laser intensity until the signal count rate is around 5×10^{5} counts per second.
3. Monitor the signal intensity and adjust the correction collar until a maximum intensity has been reached.

An alternative description on how to perform this alignment on the MicroTime 200 can be found in the appendix to this technical note.

Determination of the Molecular Brightness: a Benchmark Measurement

Molecular brightness, that is the count rate per molecule and excitation power, is a well suited parameter for comparison of instrument performance and quality of adjustment. If the system is not optimally aligned, the excitation and/or the detection will be less efficient and the molecular brightness will drop.
Regular measurement of the molecular brightness of a specific sample is practical for checking the alignment of a confocal microscope and enables to compare measurements performed on different days and/or by different operators.

In essence, the measurement of the molecular brightness is a FCS measurement. The average number of molecules in the confocal volume is obtained from the amplitude of the FCS curve. The lower the number of molecules in the confocal volume the higher is the correlation amplitude. Therefore the number of molecules contributing to a certain detected signal can be extracted by fitting a diffusion model to the FCS curve. Having obtained the correct number of molecules (N), the molecular brightness (mB) can be calculated as:

$$mB = \frac{count\ rate}{N \times excitation\ power}$$

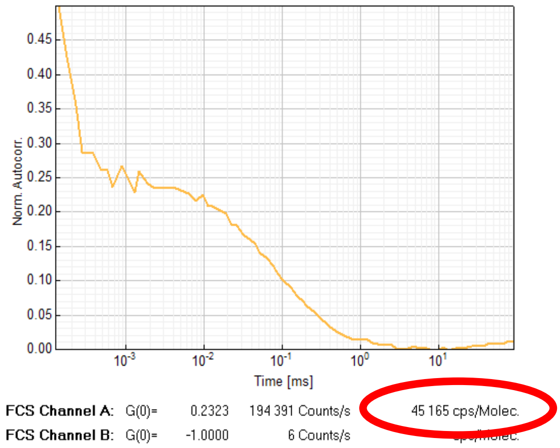
The necessary steps to determine the molecular brightness are:

1. Select a small pinhole size (e.g. 50 µm)
2. Perform a FCS measurement using the 10^{-8} M or lower concentrated solution
3. Use the amplitude of the FCS curve to calculate the average number of molecules in the confocal volume ($N=1/G(\tau=0)$).
4. Calculate the molecular brightness according to the above formula taking excitation laser power and average signal count rate into account.

Conclusion

Confocal volume and molecular brightness depend mainly on the setup configuration, i.e. detector type, filters, objective etc. and measurement conditions (e.g. laser intensity, distance between focus and cover glass, correction collar setting). The optimum performance of a given setup can nicely be monitored with the FCS Solutions Slide. Any decline of alignment will result in a decrease of the molecular brightness making the slides an easy tool for following and maintaining the system's performance. On the MT200 a rough estimate of the count rate per molecule is readily available from the FCS Online Preview (see fig. 2). To get the molecular brightness this value only needs to be divided by the laser power.

A more detailed description on how to perform this measurement on the MT200 can be found in the appendix to this technical note.



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FCS Channel A:  G(0)=   0.2323   194 391 Counts/s        45 165 cps/Molec.
FCS Channel B:  G(0)=  -1.0000         6 Counts/s               cps/Molec.
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Fig. 2: The SymPhoTime FCS-Measurement preview makes the determination of the molecular brightness easy.

Appendix

Spectral Properties and recommended Filters

The FCS Solutions Slide includes different concentrations of an Atto488 and Atto655 solution in aqueous buffer. The excitation wavelength and emission filters for these dyes should be chosen in a way to avoid spectral cross-talk between Atto488 and Atto655. Recommended excitation wavelengths and emission filters are summarized in following table, excitation and emission spectra are shown in Fig. 3.

Dye	Excitation laser / wavelength	Detection spectral range / filter	Fluores-cence Lifetime
Atto488	LDH 470 or LDH 485 470 to 488 nm	(520±20) nm HQ520/40	3.8 ns
Atto655	LDH 635 or LDH 640 635 – 640 nm	(690±35) nm HQ690/70	1.8 ns

Table 1: Summary of spectral and lifetime properties of the used dyes in the FCS Solutions Slide.

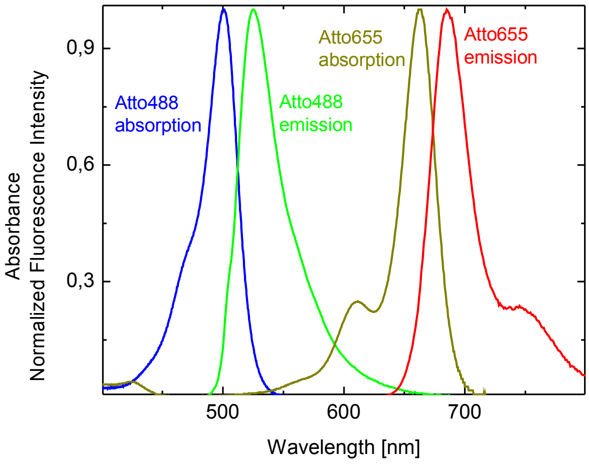


Fig. 3: Excitation and emission spectra of Atto655 and Atto488

Detailed Procedures on the MicroTime 200

Adjustment of the Confocal Pinhole

1. Start with the largest pinhole (150µm).
2. Put the immersion medium on the objective and place the FCS Solutions Slide on top of the objective, use the chamber with the highest concentration (> 10^{-6} M solution) and target the upper side of the cover slide. This can be easily achieved by watching the camera image during focusing.
3. Be aware that the bottom side of the cover glass also generates a back-reflection image on the camera. Then, locate the upper surface, which is approximately one and ¾ of full turn of the Olympus IX microscope focusing knob (approx. 160 µm) away from the bottom.
4. Raise the objective so that the focus is more than 20 µm inside the dye solution (if you are using an oil immersion objective raise the objective by only 5 µm).
5. Install a suitable fluorescence filter in front of SPAD 2. Start with strongly attenuated excitation light and guide all fluorescence light to SPAD 2 by opening the appropriate shutter and inserting the 100% mirror in the beam splitting tower.
6. Start the count rate monitoring using the SymPhoTime oscilloscope window. Set the binning for the data acquisition to 30 ms (see fig. 4).
7. Adjust the laser intensity until you reach approx. 5×10^5 cps signal rate.
8. Adjust the pinhole position to reach the maximum intensity. Be careful not to over illuminate the SPAD detector. Decrease the laser intensity if necessary.
9. When done with the 150 µm pinhole, close the detector shutter and install the appropriate, smaller pinhole (typically the 50 µm pinhole). Open the shutter again and repeat the pinhole alignment to reach the maximum intensity.

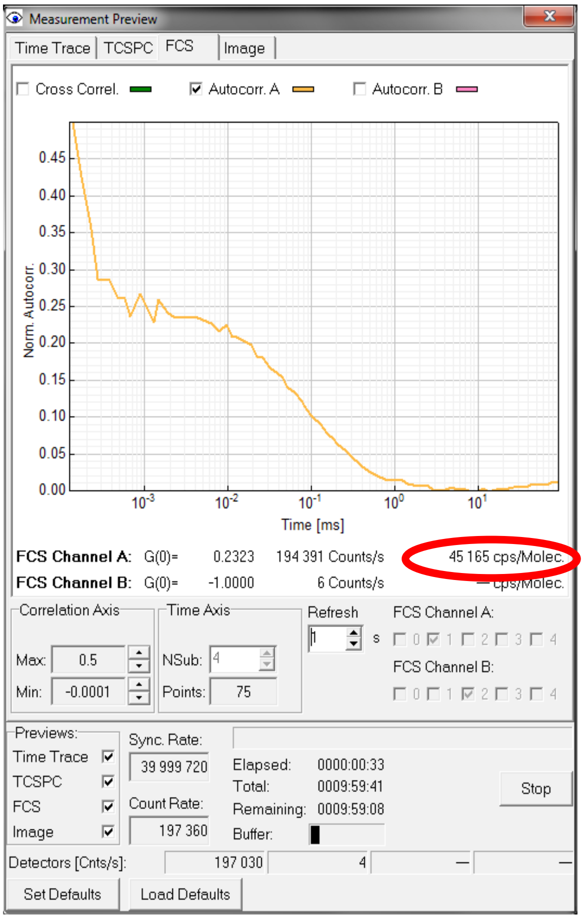
Correction collar setting

a) using the fluorescence intensity

1. Place the FCS Solutions Slide on top of microscope and focus approximately 20 µm (5 µm for oil immersion objectives) deep into the chamber that contains the highest concentration (>10^{-6} M) solution
2. Adjust the laser intensity so that the signal count rate is around 5×10^5 cps
3. Start the count rate monitoring using the SymPhoTime oscilloscope window. Set the binning for the data acquisition to 30 ms.
4. Adjust the correction collar to reach the maximum intensity.
5. By comparing the found optimal correction setting and the cover glass thickness of the FCS Solutions Sample (can be found on the box of the sample) the deviation of the scale imprinted on the objective and the real setting can be estimated.

b) using the count rate per molecule

1. Place the FCS Solutions Slide on top of microscope and focus approximately 20 µm (5 µm for oil immersion objectives) deep into the chamber that contains the nanomolar (10^{-9} M) solution
2. Start a point measurement in "Test-mode", which will continuously measure with a selectable refresh interval.
3. Monitor the count rate per molecule using the "FCS" tab in the "Measurement Preview".
4. Adjust the laser power until a maximum cps/molecule is reached (see fig. 5, marked in red).
5. Now adjust the correction collar until a maximum cps/molecule is reached.
6. By comparing the found optimal correction setting and the cover glass thickness of the FCS Solutions Sample (can be found on the box of the sample) the deviation of the scale imprinted on the objective and the real setting can be estimated.



☐ Cross Correl. ☑ Autocorr. A ☐ Autocorr. B

FCS Channel A: G(0)= 0.2323 194 391 Counts/s 45 165 cps/Molec.

FCS Channel B: G(0)= -1.0000 6 Counts/s — cps/Molec.

Correlation Axis: Max: 0.5 Min: -0.0001

Time Axis: NSub: 4 Points: 75

Refresh 1 s

FCS Channel A: ☐ 0 ☑ 1 ☐ 2 ☐ 3 ☐ 4

FCS Channel B: ☐ 0 ☐ 1 ☑ 2 ☐ 3 ☐ 4

Previews: Time Trace ☑ TCSPC ☑ FCS ☑ Image ☑

Sync. Rate: 39 999 720

Count Rate: 197 360

Elapsed: 0000:00:33

Total: 0009:59:41

Remaining: 0009:59:08

Buffer:

Detectors [Cnts/s]: 197 030 4 — —

Fig. 4: FCS Measurement Preview for the 10^{-9} M chamber of the FCS Solutions Slide with an optimized correction collar setting. The focus was set 20 µm above the cover glass, excitation at 638 nm, emission bandpass: HQ690/70 (i.e. the count rate per molecule of Atto655 was monitored).

Molecular Brightness

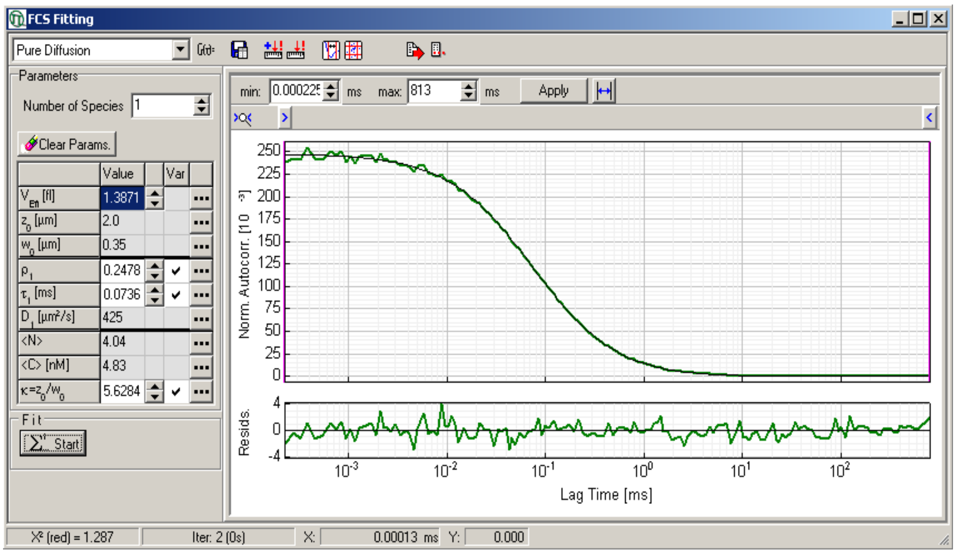
1. Depending on size of the confocal volume of your system you can use either the 10^{-8} M, 10^{-9} M, or $<10^{-9}$ M chamber. For the standard MicroTime 200 configuration with 60x 1.2 NA water immersion or 100x 1.45 NA oil immersion objective and pinhole size of 50 µm we recommend to use the 10^{-9} M chamber.
2. Target the upper surface of the cover glass. Then set the focus (axial) z-position roughly 20 µm above the glass/solution interface for water immersion objectives or 5 µm above the interface for oil immersion objectives.
3. In order to avoid bleaching and saturation, the excitation intensity should be kept low. For a typical setup we recommend approximately 15 µW in front of the objective. Alternatively, you can find the optimal laser power by measuring a saturation curve (dependence of fluorescence count rate on the excitation intensity) of your dye. The intensity should be chosen to be in the range where the count rate depends linearly on the excitation power.
4. When using cw excitation we recommend splitting the signal onto two detectors by using a 50:50 beam splitter cube. By cross-correlation analysis you can then avoid artifacts caused by detector afterpulsing. When using pulsed excitation, choose the repetition rate according to the fluorescence lifetimes, that is 20 MHz for Atto488 and 40 MHz for Atto655. Using pulsed excitation is more convenient, because detector afterpulsing artifacts can be eliminated using the simplest FLCS analysis. Please refer to our application note "*FLCS analysis using SymPhoTime*" (available at http://www.picoquant.com/appnotes.htm). Another advantage of FLCS is that it enables you to get the correct molecular brightness value individually for each detector.
5. Start a Point Measurement. Depending on the desired statistics / curve flatness, acquisition times from 20 seconds to 5 minutes are recommended.
6. Enabling (selecting) online correlation in the "Measurement Preview" window, you can watch the correlation curve building up during the measurement. A rough estimate of the counts per molecule is already displayed at the right below the FCS curve (see fig. 5). For a quick assessment, neglecting µs kinetics and detector after pulsing artifacts, this value is sufficient.

7. For a precise result, the average number of molecules in the focus, N, is calculated by fitting a model function to the FCS curve. Since Atto655 has only a negligible triplet fraction, it is possible to fit the simplest single species diffusion model (see fig. 6). For Atto488 the single species model with one additional triplet term is required.

8. Depending on whether you used FLCS or cross correlation, the average count rate is calculated by dividing the number of photon events by the measurement time. In case of FLCS us the effective numbers of photons displayed in the FCS window (see fig. 7). If you used cross correlation you can use the sum of the average count rate for each both saved in the annotations file.

9. Having obtained the correct N values, the molecular brightness (mB) can be calculated as:

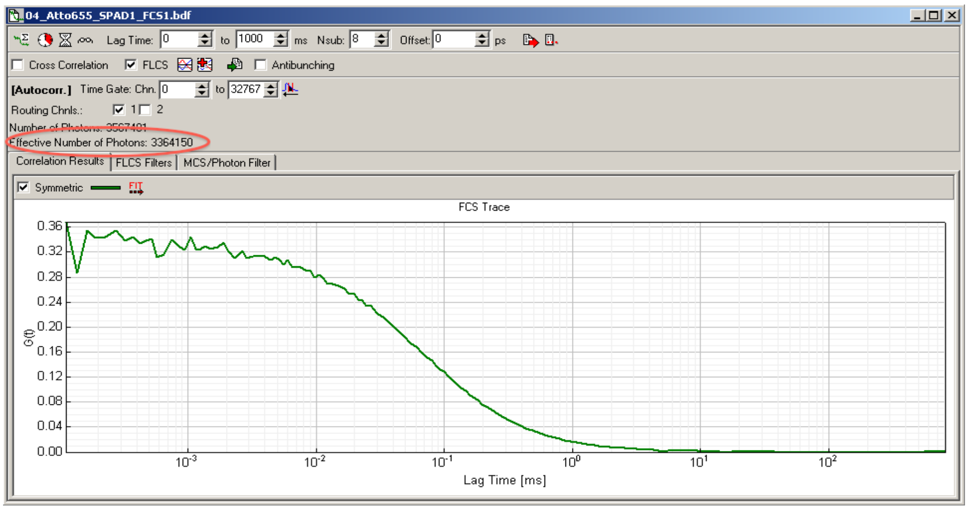
$$mB = \frac{countrate}{N \times excitation\ power}$$



	Value	Var
V_{Eff} [fl]	1.3871	
z_0 [µm]	2.0	
w_0 [µm]	0.35	
ρ_1	0.2478	✓
τ_1 [ms]	0.0736	✓
D_1 [µm²/s]	425	
<N>	4.04	
<C> [nM]	4.83	
$\kappa=z_0/w_0$	5.6284	✓

X^2 (red) = 1.287 Iter: 2 (0s) X: 0.00013 ms Y: 0.000

Fig. 6: FCS-Fitting Dialog of the SymPhoTime Software. The number of molecules in the confocal volume on average <N> is 4.



Lag Time: 0 to 1000 ms Nsub: 8 Offset: 0 ps

☐ Cross Correlation ☑ FLCS ☐ Antibunching

[Autocorr.] Time Gate: Chn. 0 to 32767

Routing Chnls.: ☑ 1 ☐ 2

Number of Photons: 3567401

Effective Number of Photons: 3364150

Fig 7: FCS window displaying the effective number of photons used to calculate the displayed FCS curve.