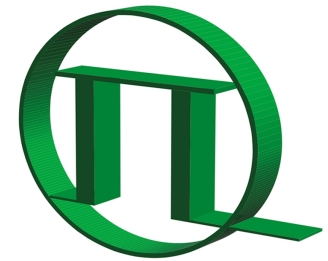
Fluorescence Lifetime Correlation Spectroscopy (FLCS) - A Powerful Tool for Measuring Diffusion, Concentrations and Interactions

Volker Buschmann, Peter Kapusta, Felix Koberling, Marcelle König, Benedikt Krämer, Steffen Rüttinger, Sebastian Tannert, Manoel Veiga, Rainer Erdmann, Uwe Ortmann



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Webtalk February 2014

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Your PicoQuant team

Fluorescence Correlation Spectroscopy (FCS) in Chemistry and Biology

Study of dynamic chemical or biomolecular processes causing fluctuations in the fluorescence intensity:

Exact determination of concentrations
- Molecular diagnostics
- Monitoring reaction kinetics

Molecular interactions
- Protein-protein interactions
- Association / dissociation processes
- Surface adsorption / desorption
- Complex formation, stoichiometry
- Formation of micelles (size, heterogeneity)
- Polymerization through monitoring viscosity

Diffusion behavior
- Folding / unfolding of proteins
- Lipid dynamics in model and cell membranes
- Hydrodynamic radius of complexes like molecules with their solvation shell

Molecular dynamics
- Conformational dynamics
- Binding kinetics
- Protonation dynamics / equilibrium
- FRET

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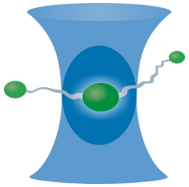
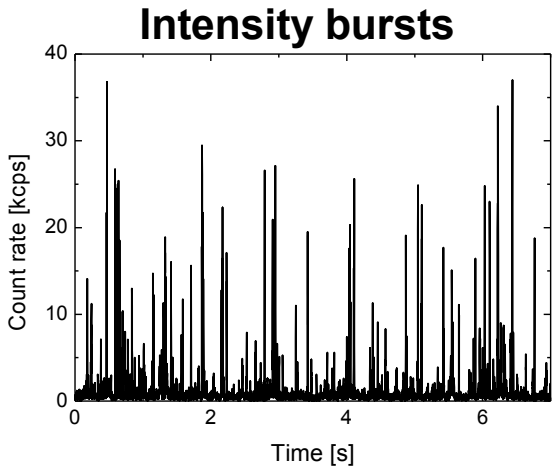
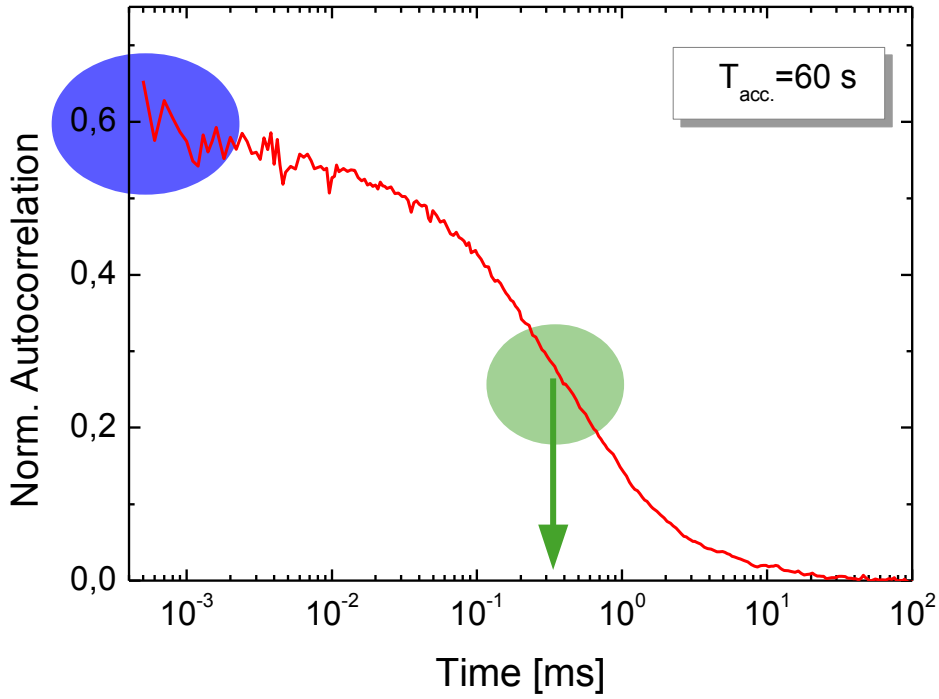
Principle of Fluorescence Correlation Spectroscopy



Diffusion of a single molecule through the laser focus





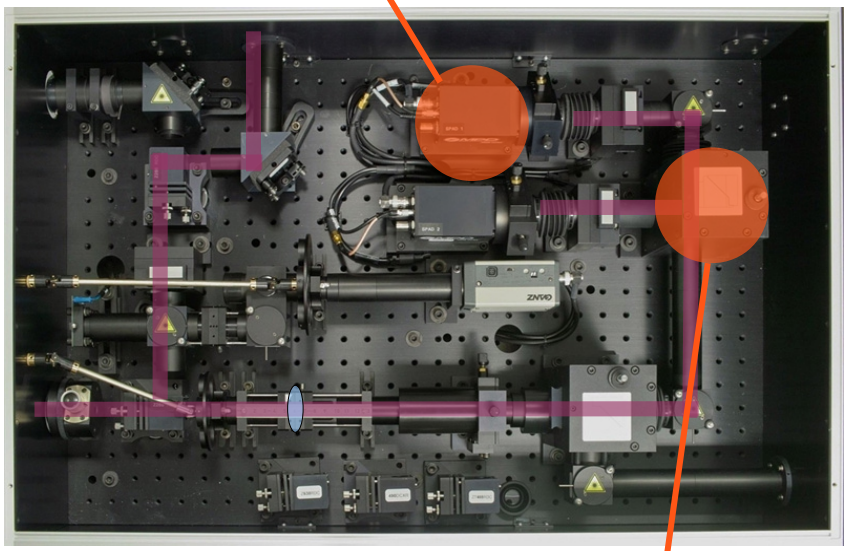
$$G(\tau)=\frac{\langle \delta F(t)\cdot \delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}=\frac{1}{V_{eff}\langle C\rangle}\cdot\frac{1}{1+\frac{\tau}{\tau_D}}\cdot\frac{1}{\sqrt{1+\kappa^2\cdot\frac{\tau}{\tau_D}}}$$

G(τ) = Autocorrelation function
τ = Correlation time
F(t) = Fluorescence at time t
V_{eff} = Effective volume
⟨C⟩ = Concentration
τ_D = Diffusion time
κ^2 = z/xy – Focus extention

Curve fitting:
number of molecules in the focal volume → (+ focus size) → molecule concentration
diffusion time through focus → (+ focus size) → diffusion coefficient

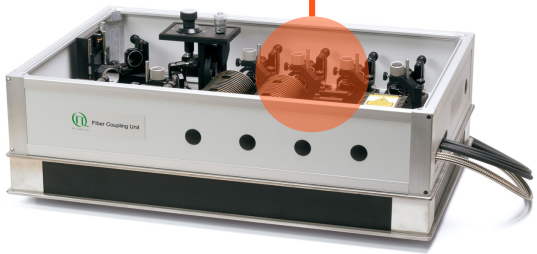
Limitations of FCS with cw Excitation

SPAD afterpulsing adds a fluctuating component in the µs regime



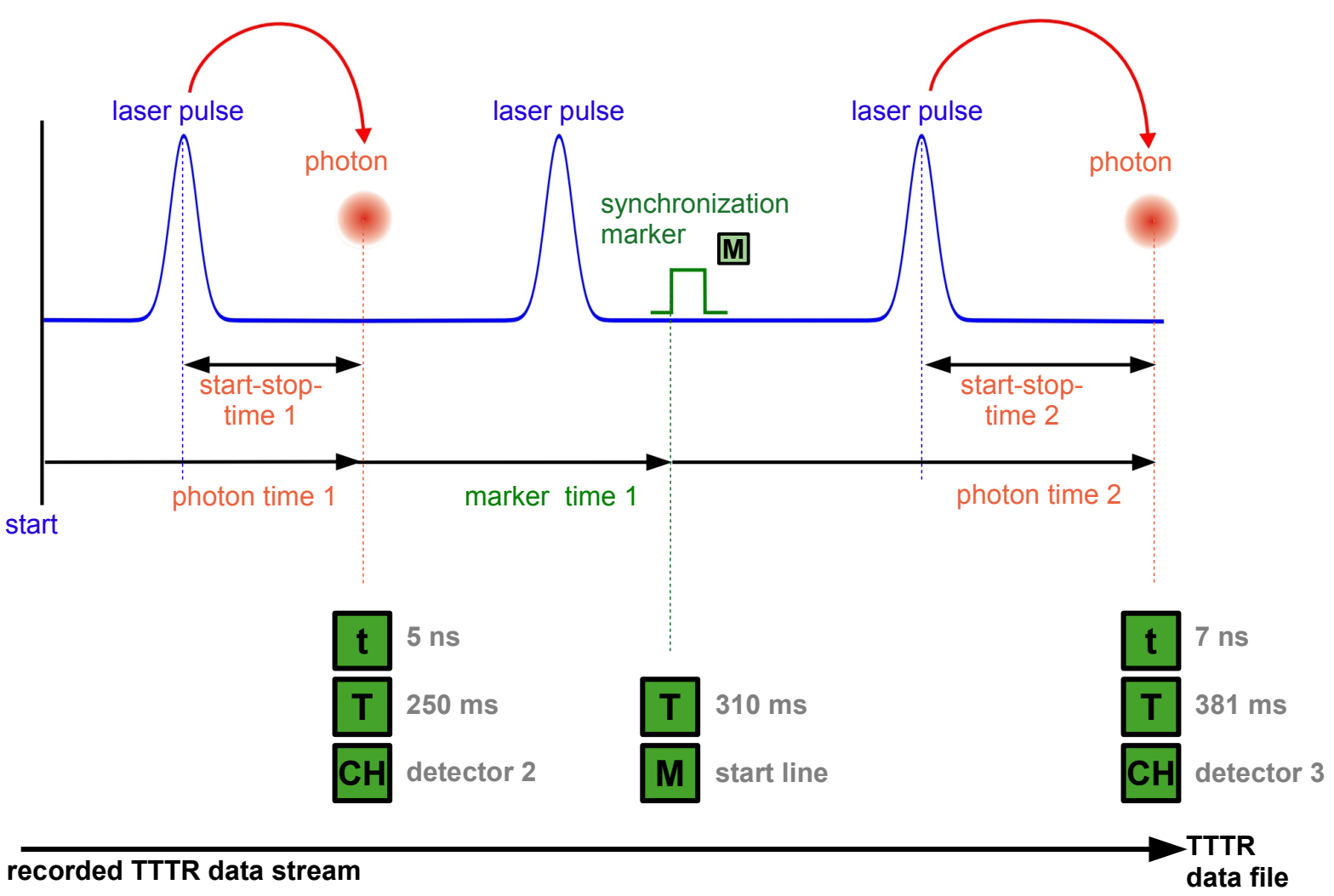
Autofluorescence + background
in the same spectral region
➔ change the extracted amplitude
= concentration

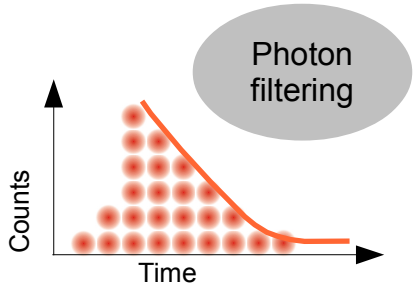
FCCS with dual colour excitation:
➔ **different excitation volumes**
and perhaps positions
➔ difficult to be calibrated and to be described with a fit function

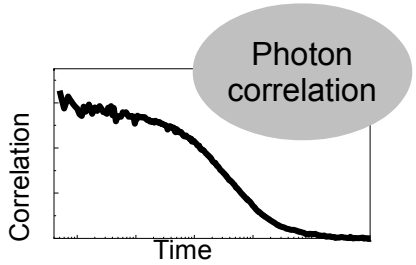


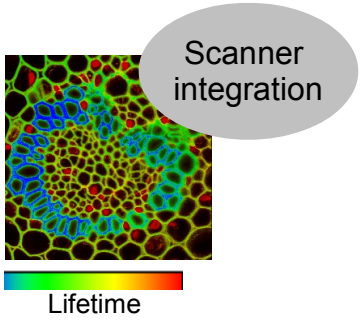
Dual colour FCCS:
spectral crosstalk
➔ adds a wrong cross correlation

Time-Tagged Time-Resolved (TTTR) Single Photon Detection Allows for FLCS

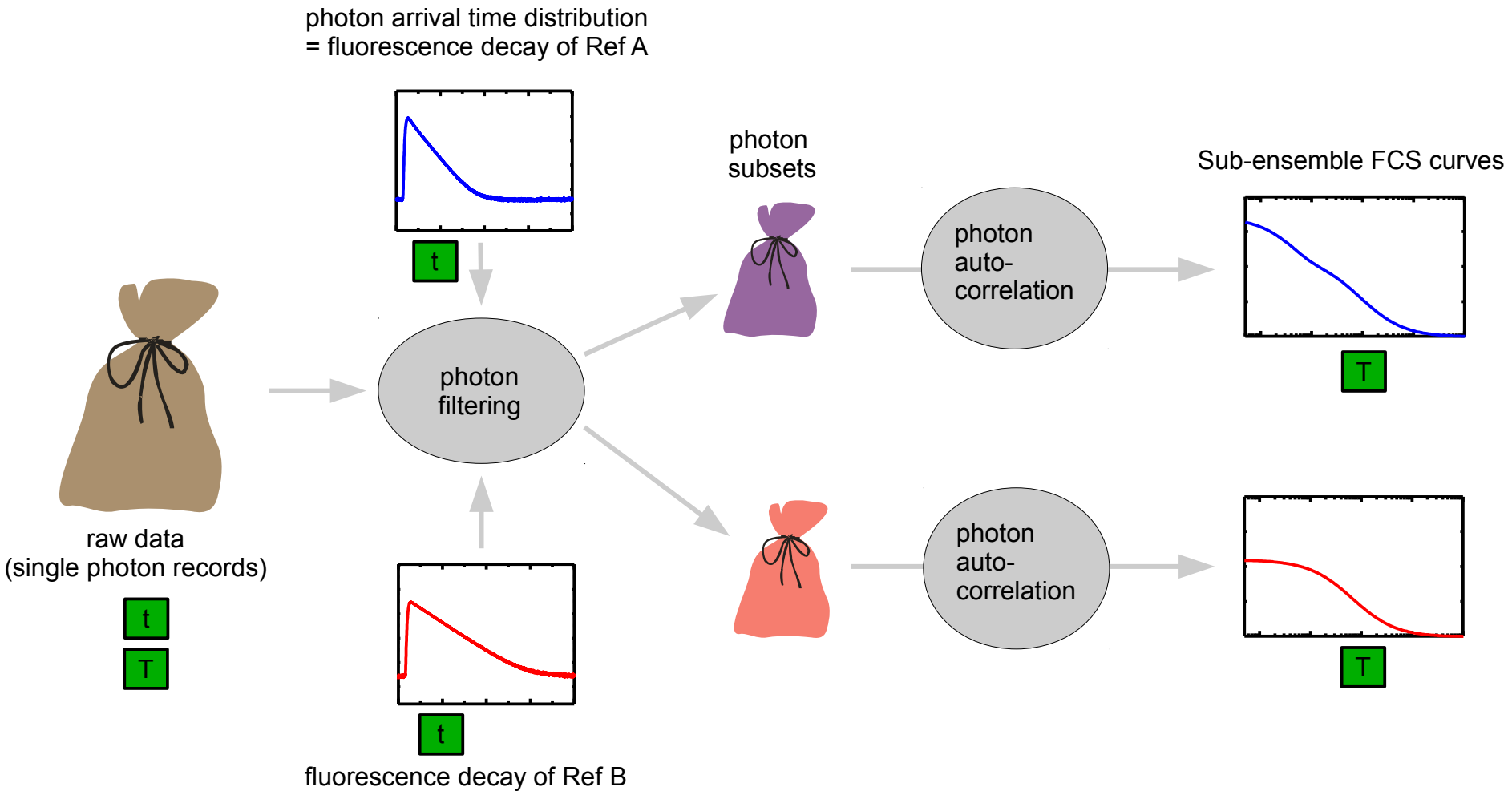






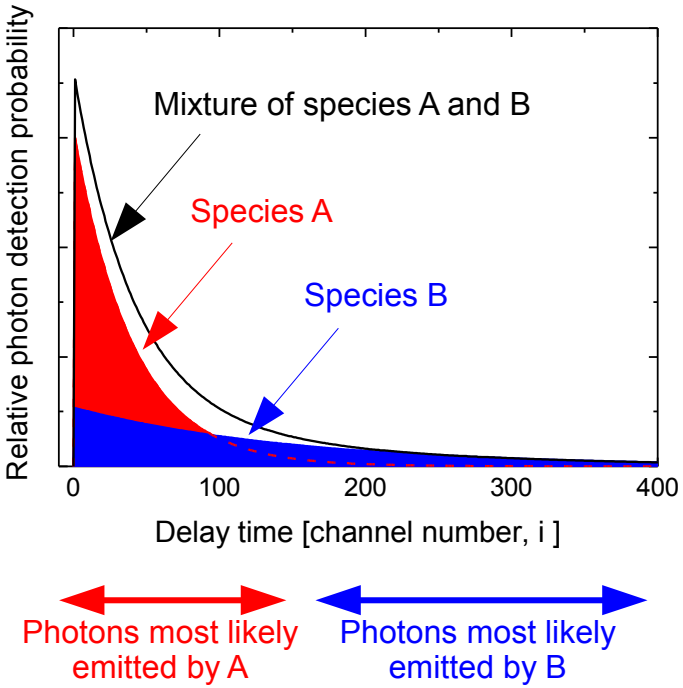


FLCS Principle

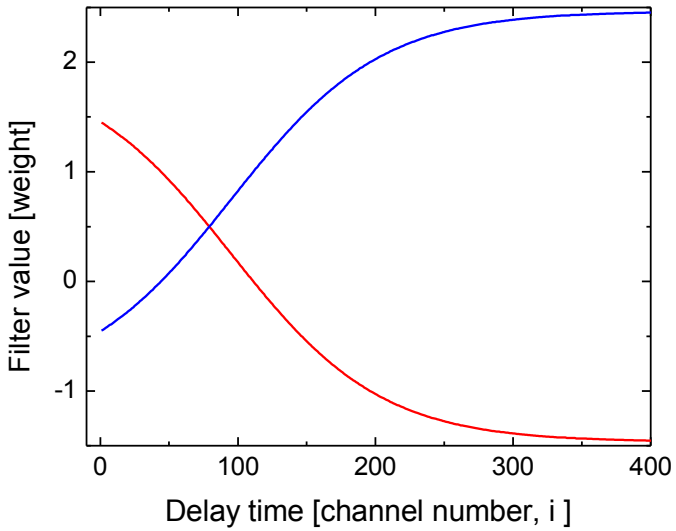


Principles of FLCS

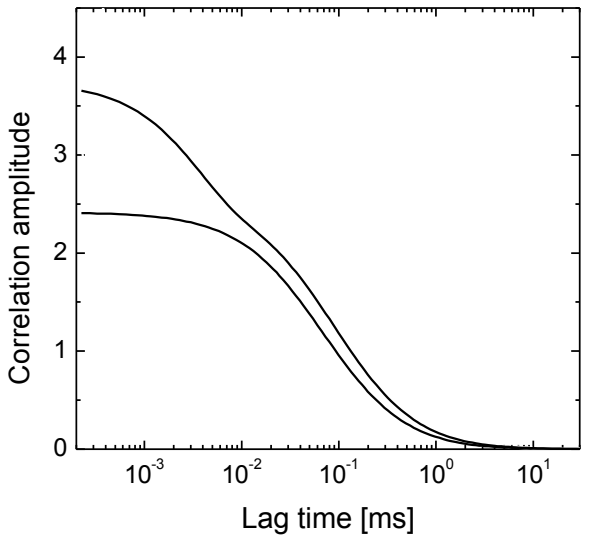
Fluorescence lifetime histograms



Filter functions (“weights”)



Separated FCS curves for species A and B

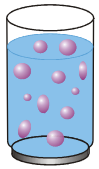


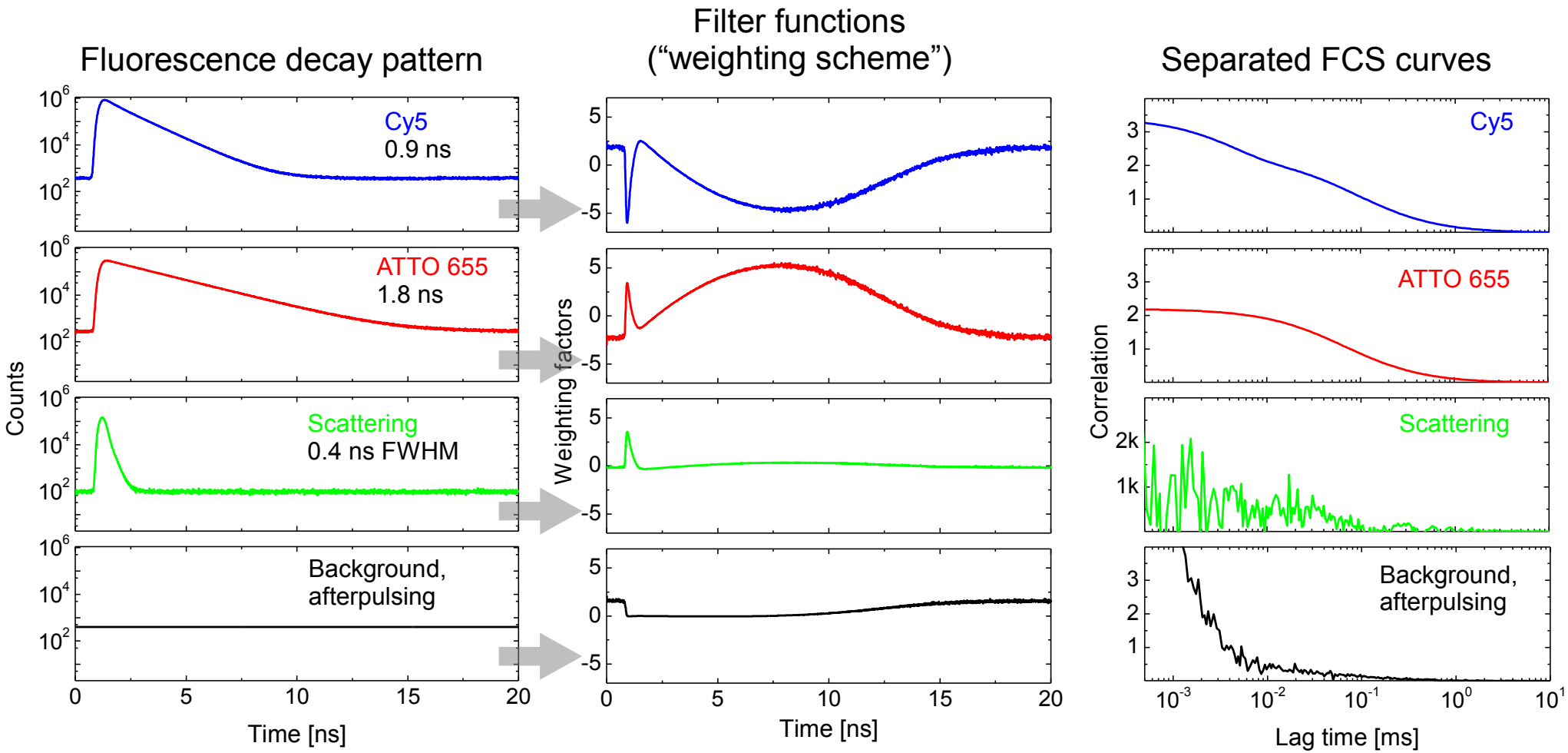
➡ **Separated FCS curves for different species**

- Removal of background
- Removal of afterpulsing
- More accurate results for concentrations and diffusion constants
- Separation of different fluorescent species

(1) Separation of Different Fluorescent Species with FLCS: Unmixing of 4 Components

Equimolar mixture of Cy5 and ATTO 655:

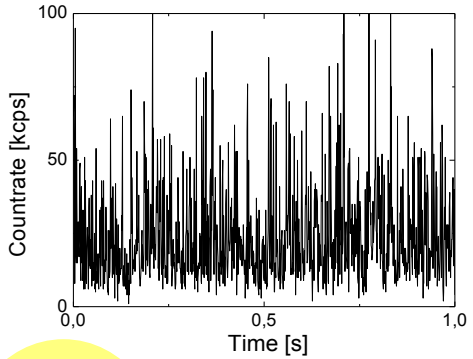


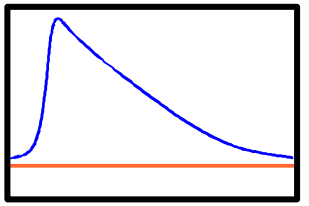


Graphs courtesy of Steffen Ruettinger, former member of PTB, Berlin, Germany

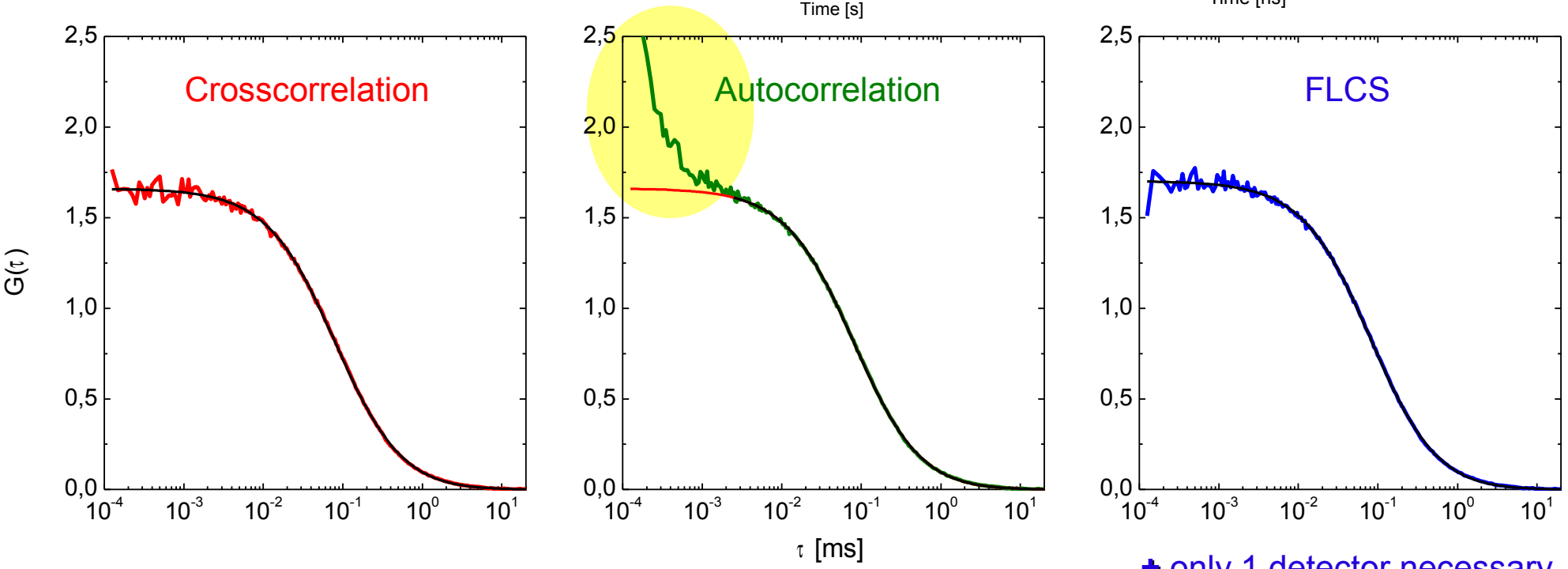
(2) FLCS to Eliminate Detector Afterpulsing

high fluorophore concentration
(ATTO 655 in water **c = 1 nM)**
low background contribution





SPAD
afterpulsing



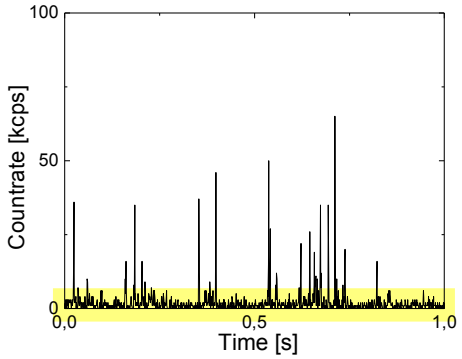
+ not influenced by detector afterpulsing

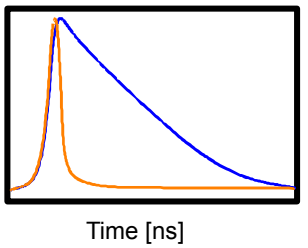
+ only 1 detector necessary
- afterpulsing

+ only 1 detector necessary
+ not influenced by detector afterpulsing

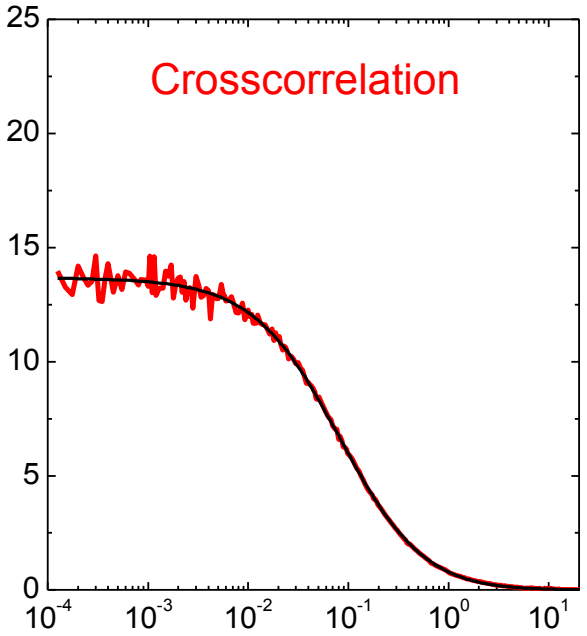
(3) FLCS to Extract Accurate Concentrations

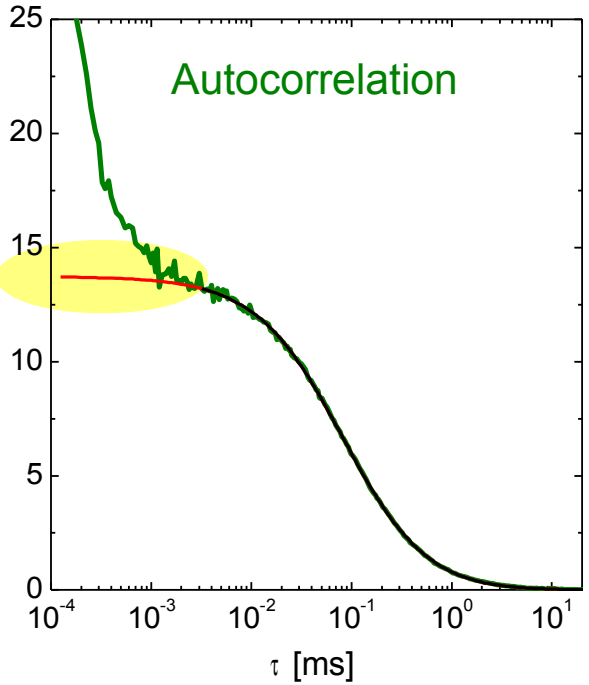
low fluorophore concentration
(ATTO 655 in water **c = 0.05 nM**)
strong background contribution

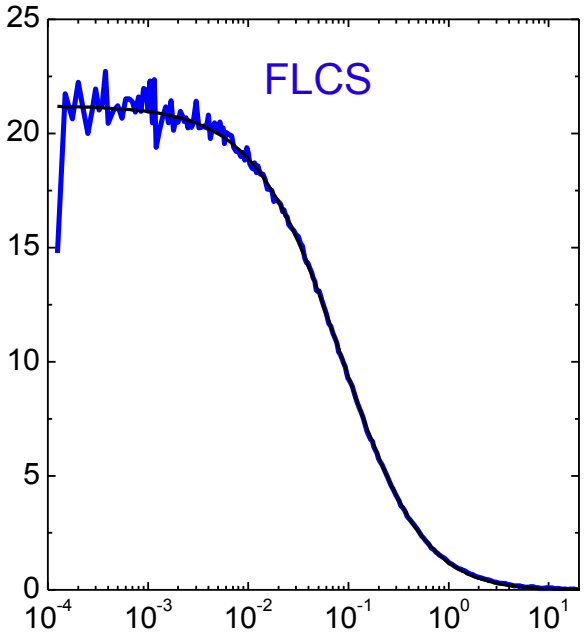




Scattered excitation light

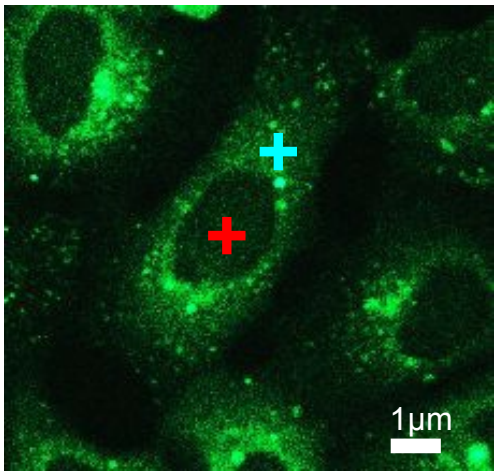




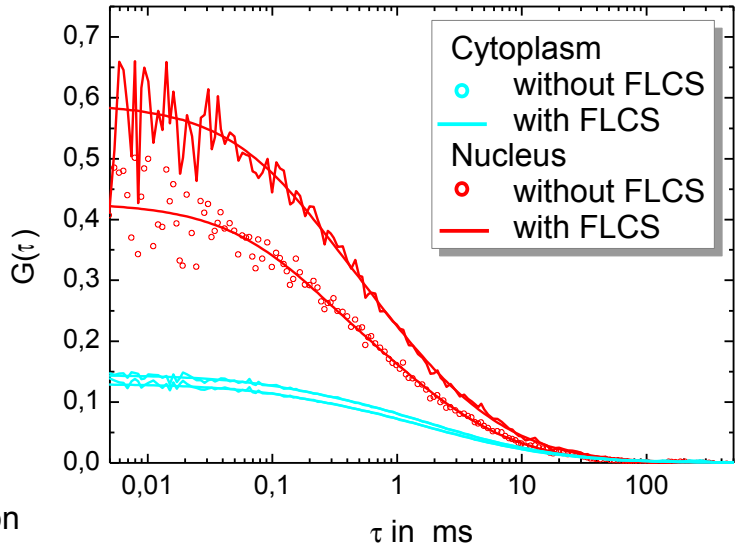


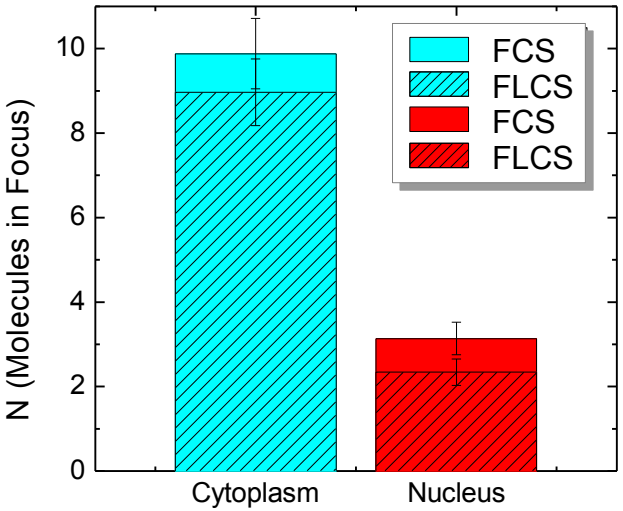
✚ <N> background corrected
→ accurate concentration

Concentration Measurements *in vivo*: GFP-tagged Proteins in Living Cells (Example)



ER293-cells with constant expression of GFP-Ago2





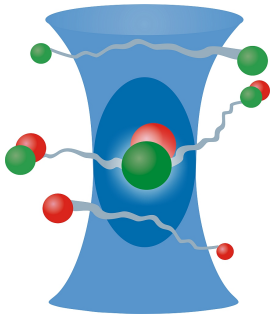
Excitation: 470 nm, 32 MHz
Emission: 520/40 band-pass filter
Objective: C-Apochromat 40x 1.2 W
10 curves with 30 s acquisition time are averaged
LSM Upgrade Kit

- Concentration of GFP-Ago2 can be determined in different cellular compartments.
- FLCS removes background contributions.

→ more accurate concentration determinations

Courtesy of M. Gärtner, J. Mütze, P. Schwille, TU Dresden, Germany
see also: T. Ohrt, J. Mütze et al., Nucl. Acids Res. 2008

Studying Molecular Interactions with Fluorescence Cross Correlation Spectroscopy (FCCS)

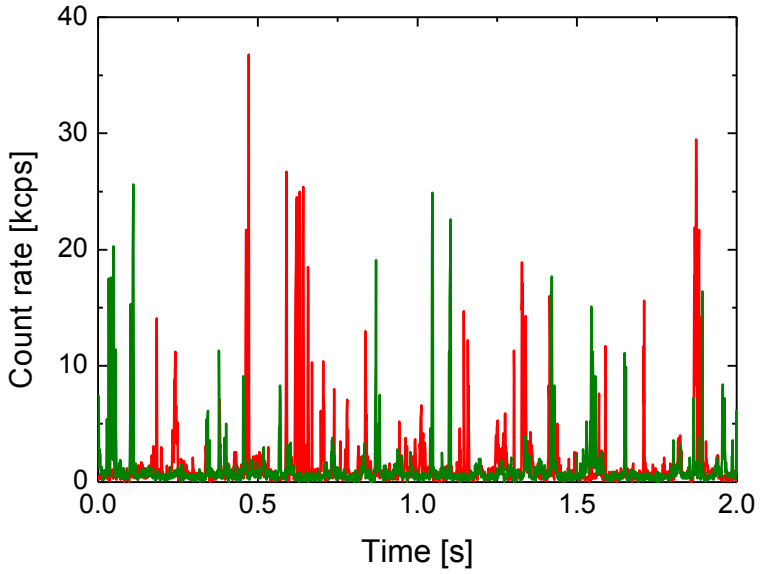


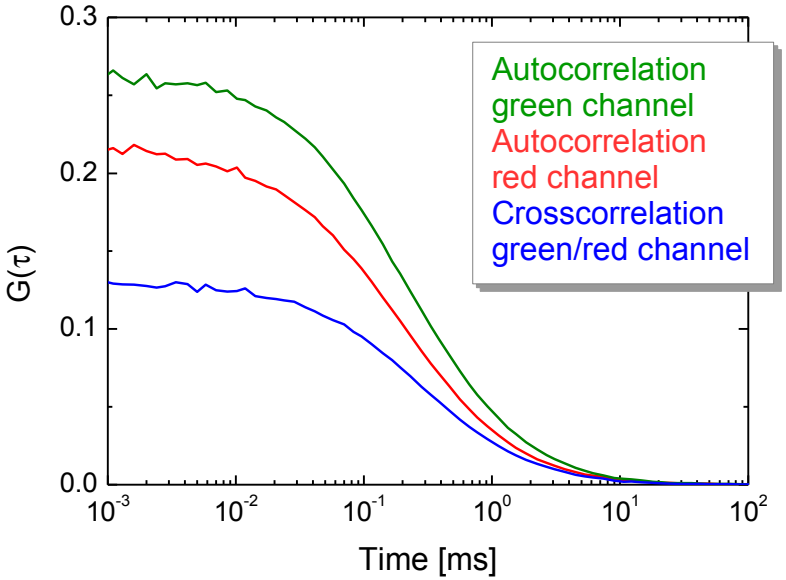
diffusion of molecules with
2 fluorescent labels through
the laser focus

$$\langle C_{gr} \rangle = \frac{G_x(0)}{G_g(0) \cdot G_r(0) \cdot V_{eff}}$$

$\langle C_{gr} \rangle$	=	concentration double labeled species
$G_g(0)$	=	Amplitude autocorrelation green channel
$G_r(0)$	=	Amplitude autocorrelation red channel
$G_x(0)$	=	Amplitude Crosscorrelation
V_{eff}	=	effective volume

Intensity bursts in two spectral channels

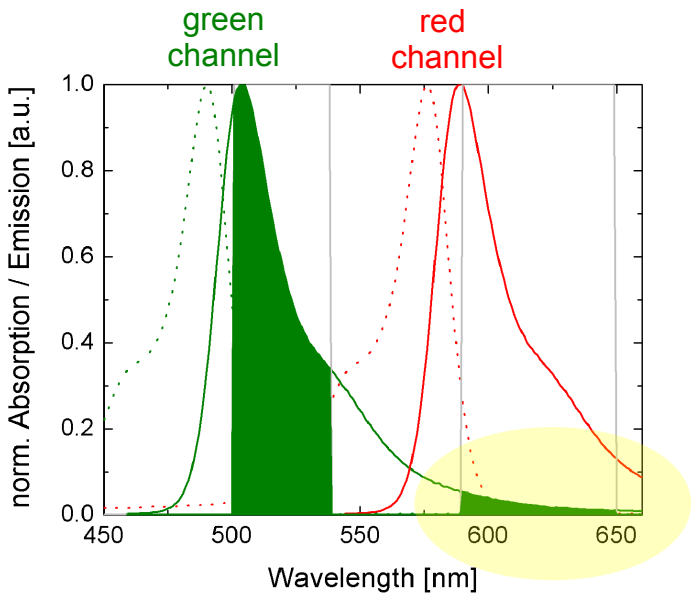




Autocorrelations green channel / red channel
→ molecule concentration green / red molecules

Crosscorrelation
→ concentration of double labeled species

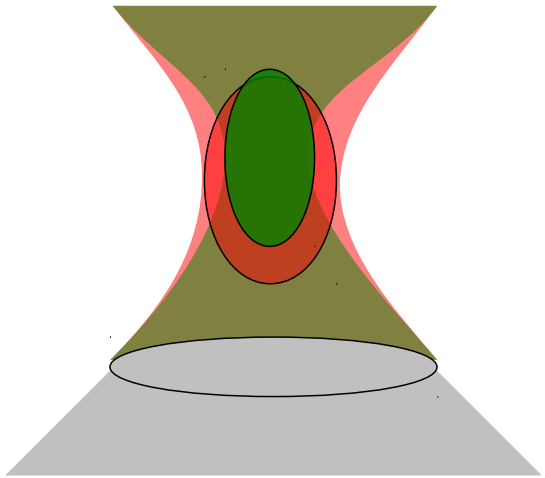
Limitations of Dual Color FCCS: Spectral Crosstalk and Excitation Volumes



- Emission Spectra are usually broader than the *spectral window* of the detection channels.
- Especially the green dye's emission spectrum reaching into the red dye's detection window results in spectral bleedthrough.

→ Spectral bleedthrough causes a *false positive* crosscorrelation.

→ Necessary is a *negative control.*



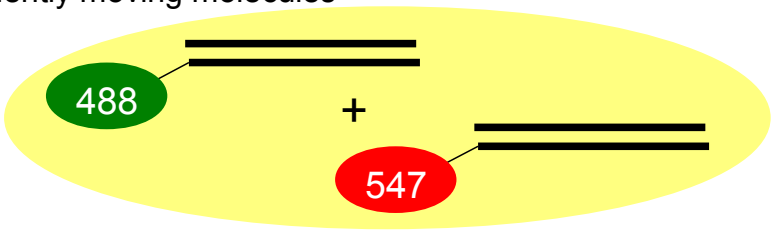
- Focus size depends on the *excitation wavelength.*
- Additionally, imperfections of the optics & alignment can cause a *non – perfect overlay* of the different colors.

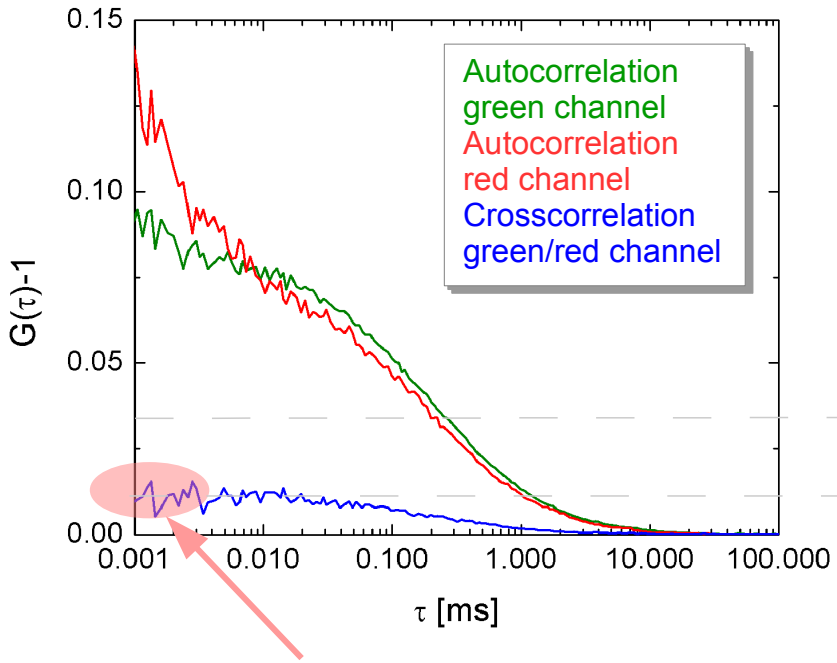
→ The overlapping (effective) volume needs to be *calibrated.*

→ Necessary is a *positive control*.

Calibration Measurements using Dual Color FCCS (without Lifetime Analysis)

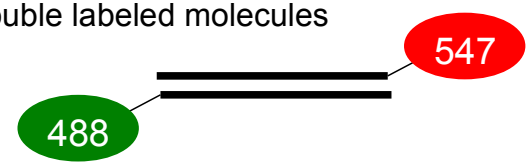
independently moving molecules

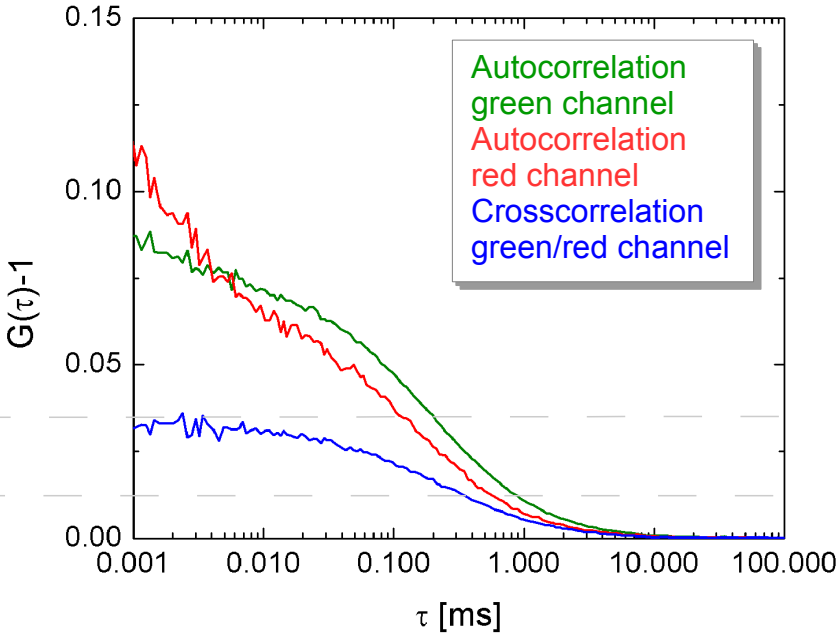




False positive crosscorrelation
due to spectral bleedthrough

double labeled molecules



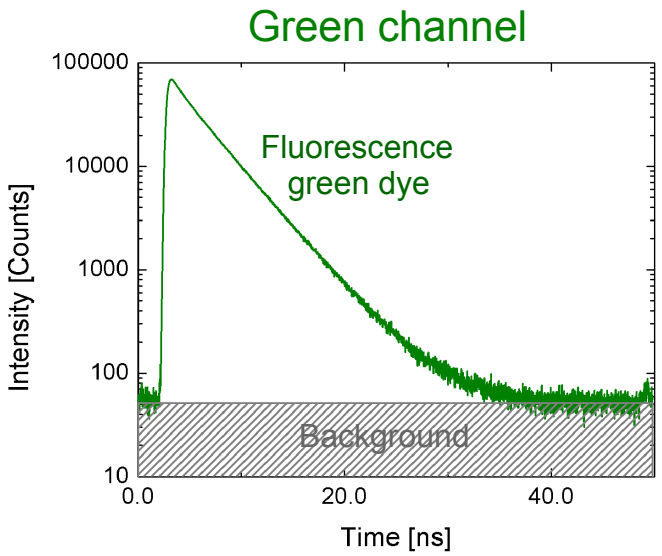


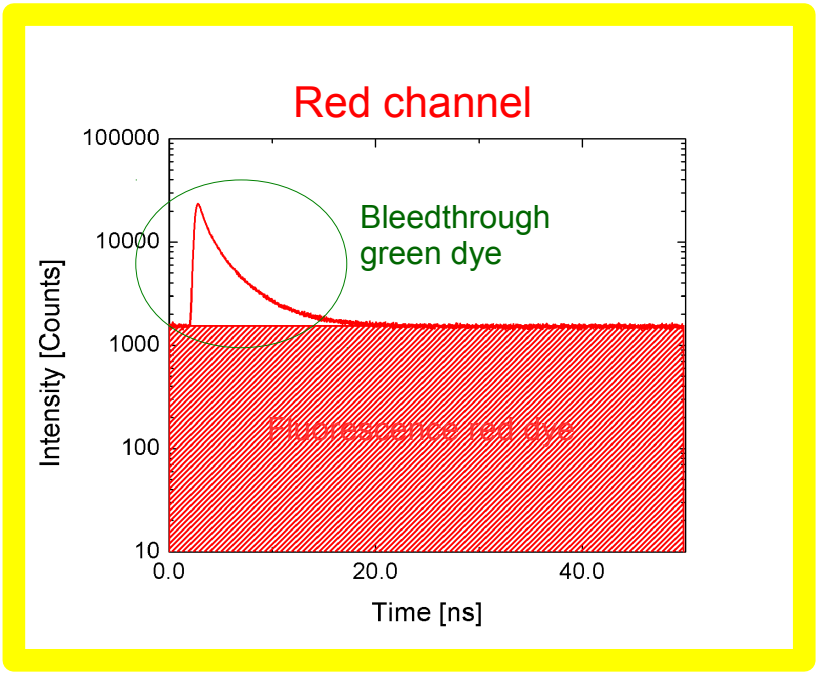
Sample: In vitro fluorescence crosscorrelation standard;
(FCCS standard) 488-543 nm from IBA
Data acquired with: LSM Upgrade Kit
$\lambda_{Exc\ 488\text{-}dye}$: 485 nm, 20 MHz, $\lambda_{Exc\ 547\text{-}dye}$: 559 nm, cw
UPLAPO 60x, NA 1.2
Emission bandpass: Det. 2: HQ520/40, Det. 1: HQ620/60
DM488/559/635 + 570 nm dichroic

Sample courtesy of IBA, see:
http://www.iba-lifesciences.com/FCCS_Standards_Products.html

FLCCS: Fluorescence Decay based Crosstalk Identification

- *Idea:* Excite the samples with a *pulsed 485 nm* and a *cw 559 nm* laser.
- The fluorescence decay pattern in respect to the laser pulse is very distinct in both channels:





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Emission bandpass: Det. 2: HQ520/40, Det. 1: HQ620/60
DM488/559/635 + 570 nm dichroic

Sample courtesy of IBA, see:
http://www.iba-lifesciences.com/FCCS_Standards_Products.html

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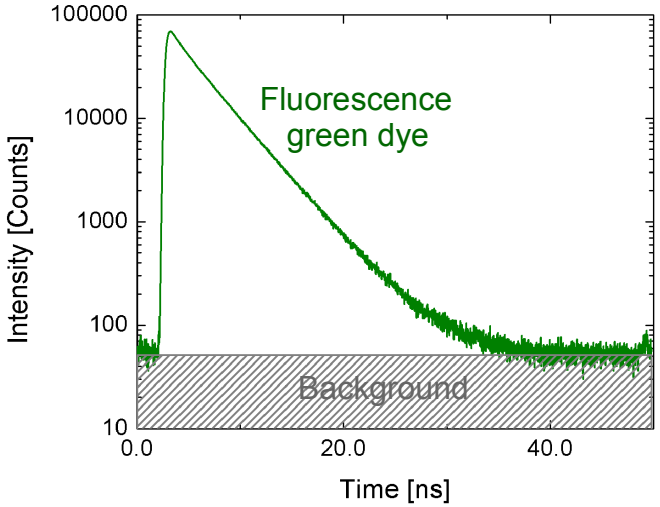
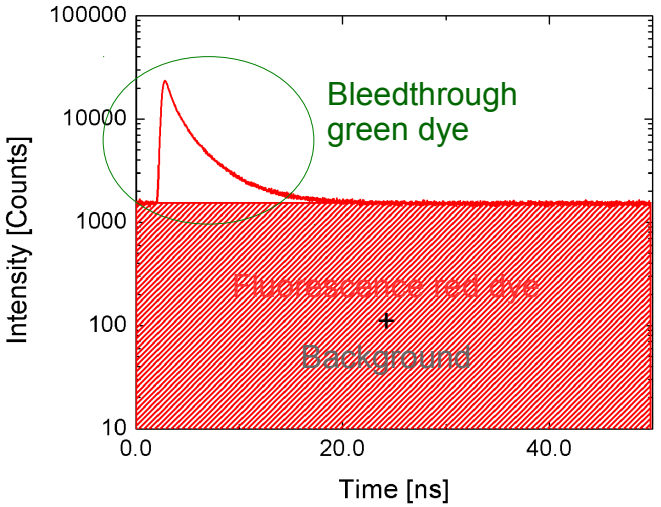
FLCCS: Fluorescence Decay based Crosstalk Removal

Green channel



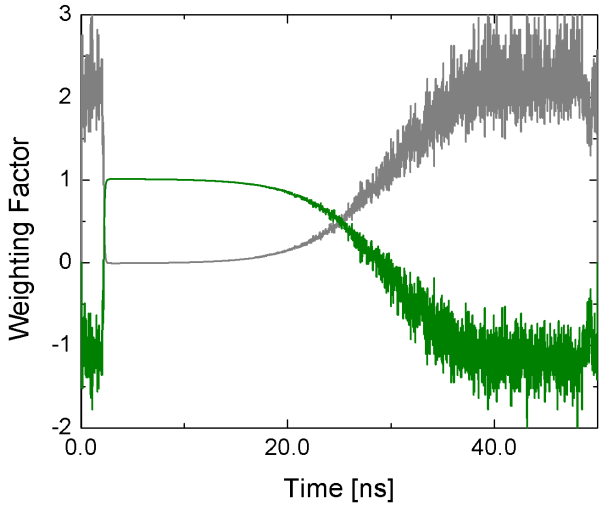
Red channel

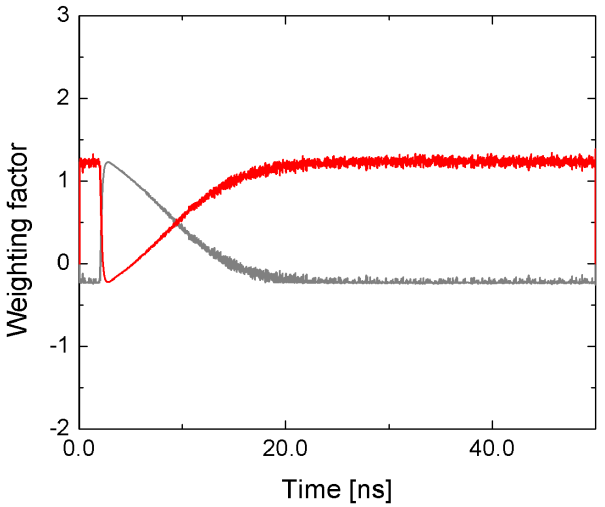


From decay pattern

calculate

time – dependent
weighting factors
(FLCS filter functions)
for each spectral channel

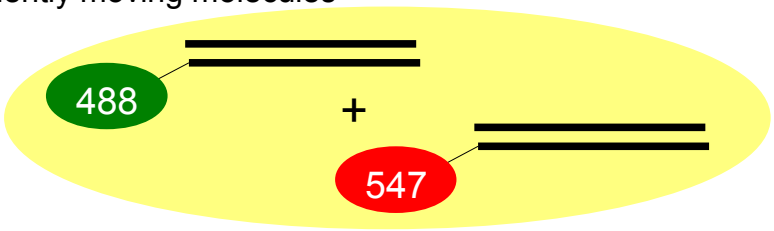


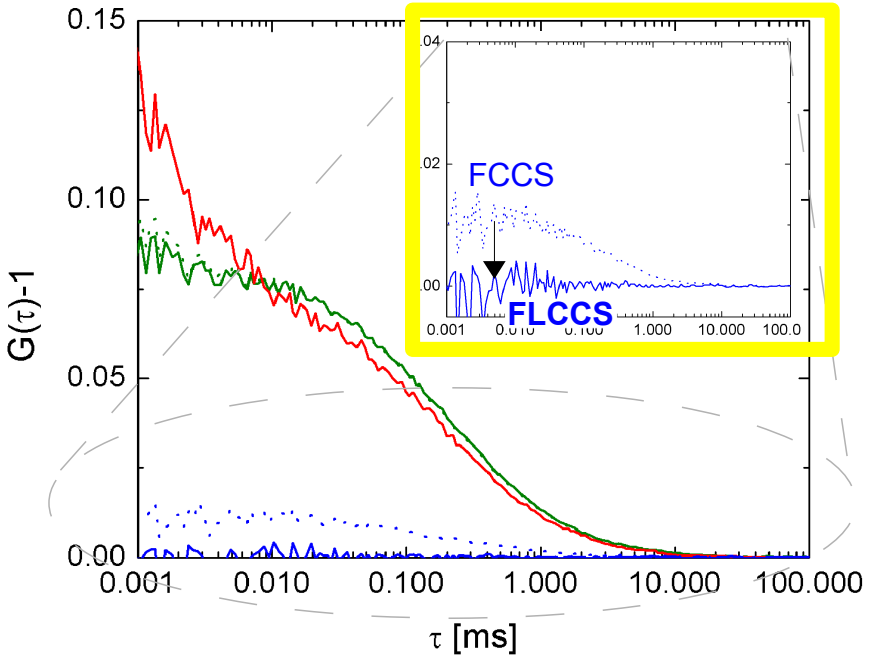


Sample courtesy of IBA, see: http://www.iba-lifesciences.com/FCCS_Standards_Products.html

FLCCS: Results for False Positive Crosstalk Removal

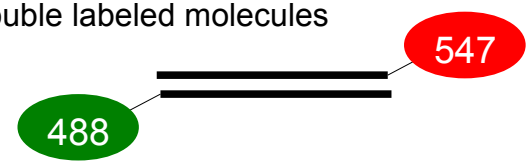
independently moving molecules

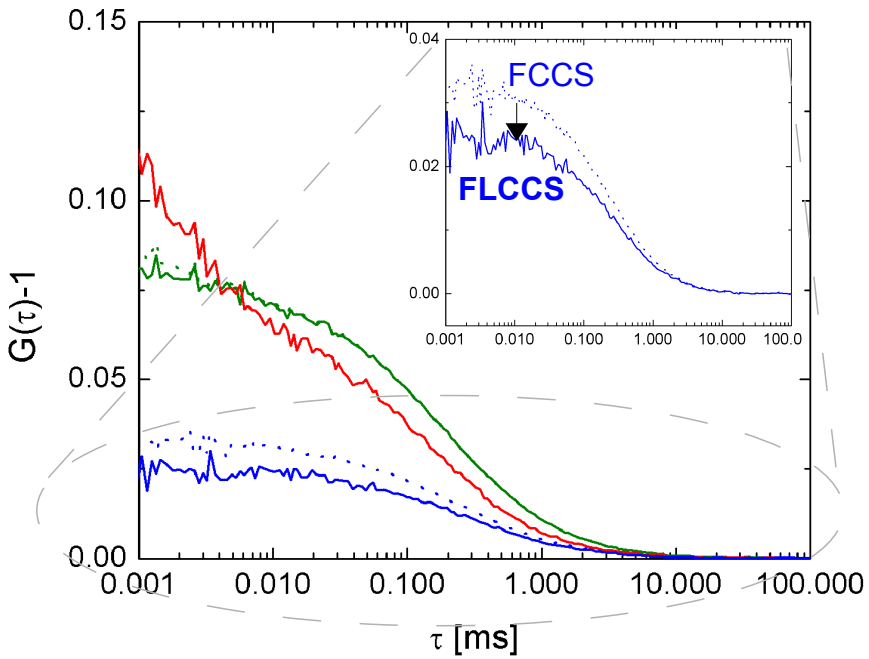




Sample:
In vitro fluorescence crosscorrelation standard;
(FCCS standard) 488-543 nm from IBA
Data acquired with: LSM Upgrade Kit
$\lambda_{Exc\ 488\text{-}dye}$: 485 nm, 20 MHz, $\lambda_{Exc\ 547\text{-}dye}$: 559 nm, cw

double labeled molecules

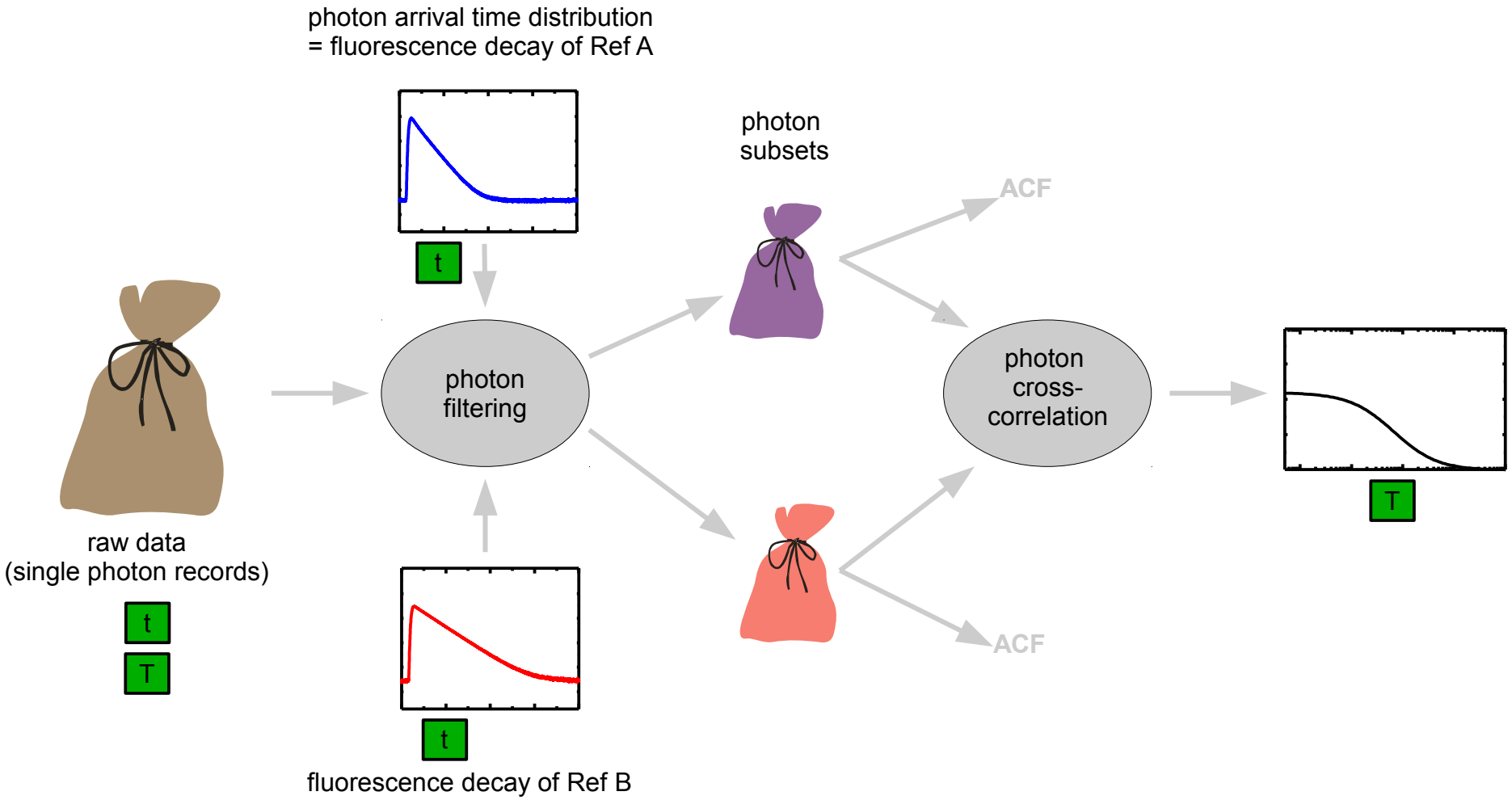




FLCCS almost *completely* removes false positive cross correlation caused by spectral bleedthrough

Sample courtesy of IBA, see:
http://www.iba-lifesciences.com/FCCS_Standards_Products.html

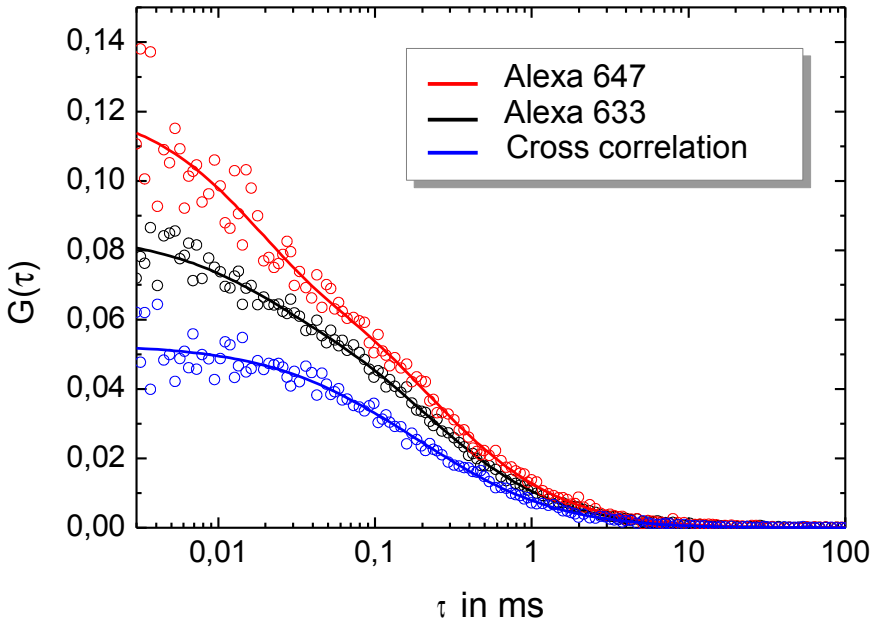
FLCCS Principle



Example for FLCCS: Cross Correlation between Two Spectrally Inseparable Dyes

Alexa 633
τ_{L2} = (3.13 ± 0.03) ns
Emission max: 647 nm

Alexa 647
τ_{L1} = (1.07 ± 0.01) ns
Emission max: 668 nm



Diffusion times:

- dsDNA + Alexa 633: (222.4 ± 6.3) µs
- dsDNA + Alexa 647: (215.1 ± 7.8) µs
- dsDNA + Alexa 633 + Alexa 647: (244.4 ± 7.8) µs

Crosscorrelation: (69.8 ± 2.0) %

single color excitation
single channel detection

Sample: double stranded DNA oligonucleotide labeled with Alexa 633 and Alexa 647
Acquisition time: 10 min
Excitation: 635 nm, 20 MHz, 3.21 µW
Emission: HQ685/70 band-pass filter
Objective: C-Apochromat 40x 1.2 W
LSM Upgrade Kit

Courtesy of M. Gärtner, P. Schwille, TU Dresden, Germany

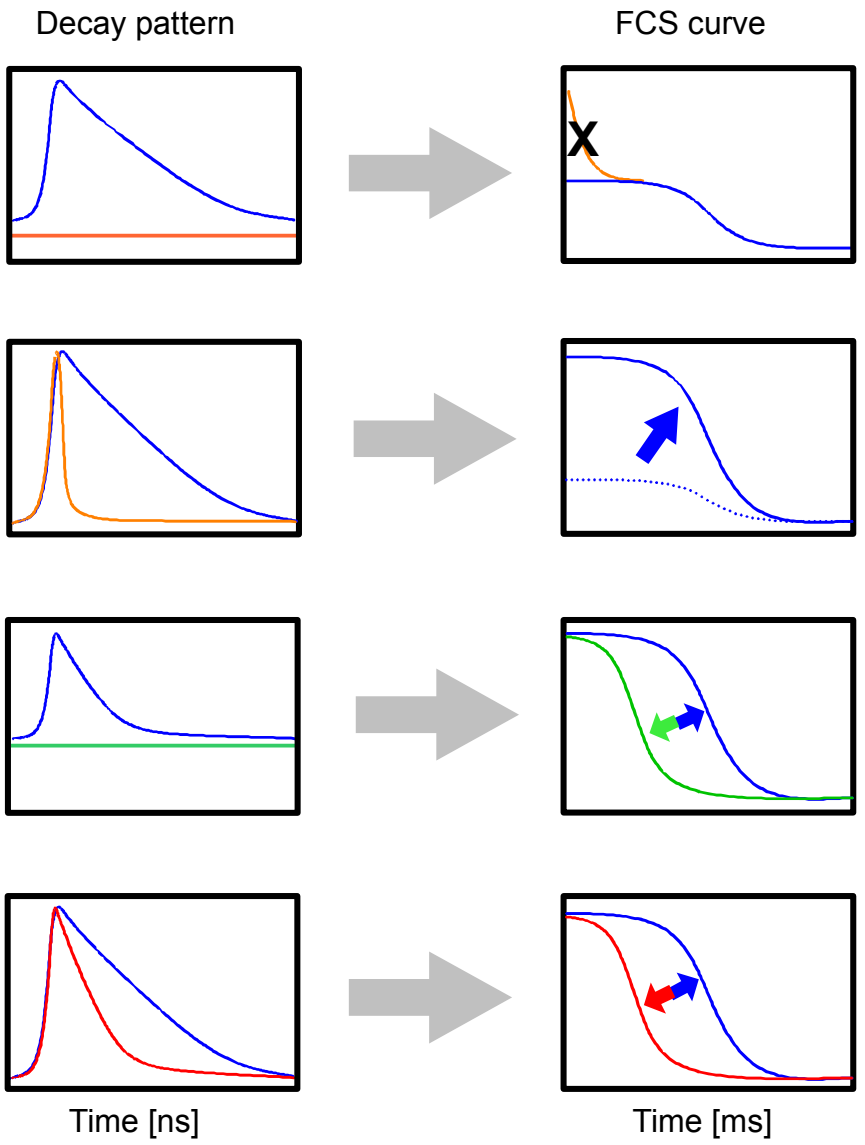
Summary: FLCS Applications

Remove SPAD afterpulsing
➔ In a single channel setup!

Remove scattered excitation light and parasitic fluorescence
➔ quantitative results

Separate pulsed and cw excitation

Separate species with different lifetimes
➔ but overlapping fluorescence spectra



Further Information

See specifications on our website:
http://www.picoquant.com/applications/category/life-science/fluorescence-lifetime-correlation-spectroscopy-flcs
and the application note
http://www.picoquant.com/images/uploads/page/files/7272/appnote_flcs.pdf

Check our website for training courses on FLIM, FCS and Time-Correlated Single Photon Counting: http://www.picoquant.com/events/workshops-and-courses

Share your experiences with the scientific community in the PicoQuant forum at:
http://forum.picoquant.com/

Please contact PicoQuant at info@picoquant.com for further information on:

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