EMCCD-based spectrally resolved fluorescence correlation spectroscopy

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Abstract: We present an implementation of fluorescence correlation spectroscopy with spectrally resolved detection based on a combined commercial confocal laser scanning/fluorescence correlation spectroscopy microscope. We have replaced the conventional detection scheme by a prism-based spectrometer and an electron-multiplying charge-coupled device camera used to record the photons. This allows us to read out more than 80,000 full spectra per second with a signal-to-noise ratio and a quantum efficiency high enough to allow single photon counting. We can identify up to four spectrally different quantum dots in vitro and demonstrate that spectrally resolved detection can be used to characterize photophysical properties of fluorophores by measuring the spectral dependence of quantum dot fluorescence emission intermittence. Moreover, we can confirm intracellular cross-correlation results as acquired with a conventional setup and show that spectral flexibility can help to optimize the choice of the detection windows.

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References and links


1. Introduction

Fluorescence correlation spectroscopy (FCS) is a method originally developed in the 1970s [1–4] that allows to measure diffusion properties, concentrations and interaction properties of fluorescently labelled biomolecules as well as photophysical properties of fluorophores in vitro and in vivo [5–8]. Alongside the development of lasers, detectors and confocal laser scanning microscopes (CLSM) that now permit three-dimensional fluorescence imaging with
diffraction-limited resolution and (close to) single molecule sensitivity, FCS has likewise gained increasing popularity with a growing number of applications in recent years.

FCS gives access to single molecule properties yet averaged over a larger number of molecules and events. Primarily, it employs thermal concentration fluctuations in equilibrium to determine diffusion properties and concentrations. The focus of a confocal laser illumination and fluorescence detection system such as that of a CLSM defines a small observation volume that is fixed at a position of interest. Due to their diffusion, fluorescently labelled molecules can enter and leave the focus, resulting in signal fluctuations at the detector. The average lengths and amplitudes of the fluctuations are determined by a temporal autocorrelation analysis, resulting in the mean number of molecules and their mean dwell time in the observation volume as basic readouts [8]. Appropriate biophysical models for the sources of fluctuations allow, for example, the quantification of the concentrations and diffusion coefficients and the discrimination of small free proteins from large complexes to which they are bound. Besides diffusion, other sources of fluctuations are photophysical effects such as molecular blinking or fluorescence intermittence [9].

To distinguish a bound from a free fraction of mobile molecules with FCS it is essential that the fractions differ substantially in the diffusion coefficients so that the formation for example of dimers of molecules of the same size can hardly be detected. Therefore, dual color fluorescence cross-correlation spectroscopy (FCCS) was conceived [10, 11]. Here, potential binding partners are labelled with spectrally different fluorophores. From the cross-correlation of the signals of corresponding detection channels, one can identify co-diffusion of the binding partners and thus extract quantitative interaction as well as diffusion parameters.

This concept was extended recently to more than two detection channels using conventional dichroic mirrors and filters, prisms or gratings as dispersive elements as well as combinations or arrays of avalanche photodiodes (APDs) or multichannel photomultiplier tube (PMT)/multichannel plate (MCP) combinations as detectors [12–15]. The combination of dichroic mirrors and filters can be implemented in a straightforward manner; however, it reduces the overall transmission of the setup and the number of simultaneously recordable detection channels is limited. Spectroscopic gratings can be optimized in such a way that they feature linear dispersion and a high spectral resolving power. Nevertheless, higher diffraction orders reduce the effective transmission even when they are suppressed. Prisms, on the other hand, show a high transmission but suffer potentially from nonlinear dispersion and a lower resolving power.

In most FCS setups, APDs are used as fluorescence detectors due to the high quantum efficiency of up to 60% in the visible range and the very low dark signal and high signal-to-noise ratio (SNR). However, recently, the electron-multiplying charge-coupled device (EMCCD) cameras have been developed that feature a quantum efficiency of up to 90% in back-illuminated configuration and comparable dark signal and SNR. In addition to conventional imaging applications, EMCCD cameras can be used for FCS provided they can be read out fast enough to sample the processes of interest [16, 17], offering a large array of thousands of point detectors that can be used for spatially resolved FCS in various configurations [18–22].

To extend the spectral flexibility of a commercial CLSM-based FCS setup we have implemented a prism-based spectrometer with an EMCCD camera for fluorescence detection for FCS. We show here that the camera allows to acquire FCS data and that the spectral resolution matches the requirements for multichannel FCS and dual–color FCCS. We present the successful application of this setup to identify different diffusing species in vitro and in vivo, to study photophysical properties of fluorophores with good spectral and temporal
resolution and to measure molecular interactions in living cells with spectrally optimized settings.

2. Experimental setup

2.1 Hardware

The setup is based on the commercial CLSM/FCS system Leica SP2 AOBS FCS2 (Leica Microsystems, Mannheim, Germany) where the externally arranged FCS detection unit comprising two band-pass filters (500-550 nm, 607-683 nm), a dichroic mirror (560 nm) and two fiber-coupled APDs SPCM-AQR-14 (Perkin-Elmer Optoelectronics, Vaudeuil, Canada) is replaced, see Fig. 1A. Instead, the light enters a Pellin Broca prism (CVI/Korea Electro Optics, Bucheon, Korea) as collimated beam. The resulting spectrum is focused onto the pixels of a SamBa SE-34 EMCCD camera (Sensovation, Stockach, Germany) using a 6.3 mm focal length achromatic lens (Thorlabs/Jinsung Instruments, Daejeon, Korea) such that the Airy radius of the diffraction-limited focal spot matches the pixel size of 7.4 µm and the spectral range of 400-800 nm is distributed over 80 pixels very close to the storage area of the chip. The unused part of the chip is devoid of any illumination. Due to the nonlinear dispersion of the prism the effective resolution ranges from 2 nm/pixel at 400 nm via 5 nm/pixel at 530 nm through to 13 nm/pixel at 800 nm.

The CLSM system is equipped with an AOTF-based beam splitter (AOBS) which allows flexible combinations of laser lines that are also used for spectral calibration. For all experiments, an HCX Plan Apo CS 63×/1.2 NA water immersion objective lens (Leica) was used and the diameter of the detection pinhole was set to the size of 1 Airy disk. In vivo experiments were carried out at 37°C using an incubation chamber enclosing the microscope stage and body (EMBL workshops).

2.2 Software

The EMCCD camera is operated by a software written in our laboratory in Visual C++ (Microsoft Germany, Unterschleißheim, Germany) based on a modified camera firmware that allows the readout of a flexible area ranging from fractions of a single line to the full frame,
effectively limited only by the pixel readout clock of 10 MHz (see Fig. 1B). For this application, we read out 80 pixels of a single line, from which averaged dark signal recordings were subtracted for background correction post acquisition. The camera was operated at a nominal charge multiplication gain (CMG) of 30 with up to 84,000 lines/second or 84 kHz (depending on the chosen integration time), resulting in wavelength-time images (Fig. 1C). Pixel values were converted to photon numbers, could be binned into spectral detection windows and were extracted as intensity time traces that were then subject to a temporal auto- \((k = l)\) and cross-correlation analysis \((k \neq l)\) according to Eq. (1):

\[
G_{kl}(\tau) = \left\langle \delta F_k(t) \delta F_l(t + \tau) \right\rangle / \left\langle F_k(t) \right\rangle \left\langle F_l(t) \right\rangle, \quad \delta F_k(t) = F_k(t) - \left\langle F_k(t) \right\rangle.
\]  

Auto- and cross-correlation functions (ACF/CCF) were computed directly based on a multiple-tau algorithm \([23]\) with 10 lag time points per linear section, doubling of the lag time increment between the sections and the longest lag time limited to 5% of the total acquisition time. ACFs/CCFs were fitted using Origin (OriginLab, Northhampton/MA, USA) based on the Levenberg-Marquardt algorithm with the standard model function for free or anomalous two-component diffusion and blinking \([24]\)

\[
G(\tau) = \frac{1}{N} \left[ f_1 \left( \frac{\tau}{\tau_{\text{diff},1}} \right)^{\alpha_1} + f_2 \left( \frac{\tau}{\tau_{\text{diff},2}} \right)^{\alpha_2} \right]^{1/2} \left[ 1 + \frac{1}{\kappa^2} \left( \frac{\tau}{\tau_{\text{diff},1}} \right)^{\alpha_1} \right]^{1/2} \left[ 1 + \frac{1}{\kappa^2} \left( \frac{\tau}{\tau_{\text{diff},2}} \right)^{\alpha_2} \right]^{1/2} \left[ 1 + \theta \exp \left( -\frac{\tau}{\tau_{\text{blink}}} \right) \right].
\]  

where \(N\) is the average number of molecules in the focus, \(f_i\) the amplitude fraction of the first component, \(\tau_{\text{diff},i} = w_0^2 / (4D_i) \propto R_i\) and \(\alpha_i\) the diffusion correlation time and the anomaly parameter, respectively, of component \(i\) with its diffusion coefficient \(D_i\) and its radius of gyration \(R_i\), and \(\kappa = z_0 / w_0\) the structure parameter defined by the axial and the lateral \(1/e^2\) radius \(z_0\) and \(w_0\) of the focal volume. \(\theta = T / (1 - T)\) accounts for molecules in a nonfluorescent state: when at any given time an average fraction \(T\) (or an average number \(N\cdot T\)) is in such a dark state with a typical lifetime of \(\tau_{\text{blink}}\), the amplitude of the correlation function is increased accordingly. The normalized cross-correlation amplitude is defined as the ratio of the cross- and the geometrically averaged autocorrelation amplitudes as obtained from the fits of Eq. (2) to the data and is computed as

\[
\text{ratioG} = G_{12}(0) \times \left( \frac{G_{11}(0) G_{22}(0)}{G_{12}(0)} \right)^{1/2}.
\]  

This is a convenient representation of the cross-correlation amplitude that is directly related to parameters describing molecular interactions such as the average fraction of labelled molecules integrated into a multi-component complex \([25]\).

3. Sample preparation

MCF7 cells either co-expressing EGFP and mRFP or expressing a fusion protein including a single EGFP and two mRFP copies coupled with a short peptide containing a caspase-3 recognition site \([26]\) were used as negative and positive control, respectively, for two-color cross-correlation measurements. For \textit{in vivo} experiments, cells were seeded in 8 well Nunc LabTek chambered coverglasses (Thermo Fischer Scientific, Langenselbold, Germany) with phenol red-free RPMI medium. For \textit{in vitro} experiments, Alexa 488 and quantum dots (QDs) with emission maxima at 525, 565, 605 and 655 nm referred to as QD 525, 565, 605, and 655, respectively (Invitrogen Germany, Karlsruhe, Germany), were dissolved in water and
dispensed in 8 well LabTek chambered coverglasses, too, at the concentrations as given below.

4. Characterization of the setup

4.1 Camera characterization

EMCCD cameras are in general suitable for FCS experiments [27] so that we also wanted to confirm this for the camera used here. The photons falling onto a camera pixel generate with a certain probability, the quantum efficiency, photo-electrons (e–) in this pixel. Their number is converted by the camera electronics into pixel gray values given in analog-to-digital units (ADU). In order to transform pixel values in a camera image back to counted photo-electrons the corresponding conversion factor $f_{\text{conv}}$ must be determined. The number of photo-electrons results from a counting process and thus obeys Poisson statistics so that its average equals its variance. Thus, for homogeneous illumination the standard deviation of the pixel values $\sigma_{\text{ADU}}$ as averaged over the whole image depends on the average pixel value $\mu_{\text{ADU}}$, the conversion factor and the readout noise $\sigma_{\text{RO}}$ according to [28] as shown in Eq. (4):

$$\sigma_{\text{ADU}}^2 = f_{\text{conv}}^{-1} \mu_{\text{ADU}} + f_{\text{conv}}^2 \sigma_{\text{RO}}^2. \quad (4)$$

We recorded the detector signal at homogeneous low light illumination for a range of integration times at a CMG of 30. The slope of the pixel value variance as plotted against the average pixel value for all integration times yielded a conversion factor of 0.052 e–/ADU.

Next, we determined the readout noise by acquiring pairs of images without illumination at minimized integration time (bias frames). In order to remove fixed pattern noise, difference images were computed. Dividing their standard deviation by $2^{1/2}$ gave an RMS readout noise of 0.6 e–, i.e., small enough for single photon counting and FCS.

Variations of the detection efficiency across the detector were analyzed using the homogenous illumination scheme mentioned above. Intensity profiles from the pixels along the line used for spectral signal acquisition showed that pixel value variations remained below 1% of the pixel values, so that we did not apply any further correction for this.

Replacing the prism in the optical path with the bandpass filter used for APD-based measurements allowed us to measure the average fluorescence intensity as given in counts per second (CPS) of a 50 nM Alexa 488 solution using the EMCCD camera. It was about two-fold smaller then the intensity recorded with the APDs under otherwise identical conditions at different laser powers, in agreement with the difference in quantum yield of 30% (EMCCD) compared to 60% (APD) as specified by the respective manufacturers.

Thus, the camera is sensitive enough for FCS experiments as shown previously [19].

4.2 Spectral calibration

The CLSM is equipped with 8 discrete laser lines. Setting the beam splitter to reflection for all laser lines allowed us to use the signal reflected from a coverglass to calibrate the wavelength assignment of the pixels, see Fig. 2A. The focusing of the achromatic lens was optimized for the 514 nm line that showed an FWHM of 1.7 pixels, which was two-fold larger for 405 and 633 nm due to remaining chromatic errors. The prism was rotated in such a way that 90° deflection was obtained for 514 nm.

Next, we prepared a solution of a mixture of the 4 QD species mentioned above at 50 nM concentration each as well as solutions of each species separately at 50 nM. Figure 2B shows the corresponding time-integrated spectra of the solutions excited at 488 nm where all QD species have a reasonable excitation efficiency. It is known that the excitation quantum yield of QDs at 488 nm increases with increasing emission wavelength maximum and correspondingly with
increasing QD crystal size [29] resulting in the different peak heights of the emission maxima in the mixture. We could fit a sum of 4 Gaussian curves to the intensity spectrum, which corresponded in width and position to the fits of single Gaussian curves to the spectra of the separate solutions.

4.3 Comparison of FCS measurements acquired with the EMCCD and with APDs

We used a 20 nM solution of Alexa 488 and a 25 nM solution of QD 655 in order to compare FCS measurements taken with the EMCCD-based spectrometer and with the APD-based filter setup. For Alexa 488 we chose a spectral range of 500-551 nm whereas for QD 655 we chose 608-682 nm in order to match the properties of the filters used. Figure 3A and B show that the ACFs are very similar for the two setups with the major difference that the time resolution of the EMCCD is limited to 12 µs. From a single component free diffusion fit (i.e. \( \alpha = 1 \) and \( f_1 = 1 \)) of Eq. (2) to the data very similar diffusion correlation times could be obtained, showing that we get substantially the same results with both setups.

In order to assess the concentration range accessible with the setup we acquired FCS data from a concentration series of QD 655 covering 1-50 nM with constant laser intensity. The number of molecules in the focal volume \( N \) resulting from the fits of Eq. (2) to the data showed an almost linear dependence on the concentration, see Fig. 3C, supported by a fit of a power law to a double-logarithmic representation of the data that yielded a slope of 1.06±0.04. Only for relatively small concentrations below 2 nM we observed a deviation from linearity due to an increasing influence of the background signal.
5. Spectrally resolved FCS measurements

5.1 FCS of a mixture of QDs

From full spectral intensity time traces acquired in the equimolar mixture solution, see Fig. 2B, channel intensity time traces were extracted by binning over the wavelength windows 507-540 nm, 546-582 nm, 588-625 nm, and 633-696 nm corresponding to the FWHM of the emission profiles of the single QD species. The channel intensity time traces were then subject to an autocorrelation analysis, see Fig. 4A. From an integration of the emission spectra of the single QD species over the channel windows weighted with the peak intensities in the mixture (Fig. 2B), the cross-talk of the different species into channels assigned to the other species could be determined: the signal in the first window was composed to 85% of QD 525 emission and to 15% of QD 565 emission; in the second channel, the contributions were 9% QD 525, 83% QD 565, 8% QD 605; in the third channel, 4% QD 565, 79% QD 605, 17% QD 655; and in the fourth channel, 6% QD 605; 94% QD 655. Since in an ACF, different species are weighted with the square of the brightness per particle, the cross-talk-induced contributions to the ACFs amounted to not more than \((0.17/0.79)^2 \approx 5\%\). Therefore, fits with a single freely diffusive component and an exponential blinking contribution, i.e. \(\alpha = 1\) and \(f_1 = 1\) in Eq. (2), were justified and resulted in diffusion correlation times that increased with increasing maximum emission wavelength, see Fig. 4B. For an estimation of the expected spectral dependence of the diffusion correlation times we used the radii of gyration of 6, 7, 8 and 9 nm (values provided by the manufacturer) for QD 525, 565, 605 and 655, respectively, including core, shell and polymer coating of the QDs. We estimated the additional chromatic contribution based on the fact that a constant pinhole size was used for the complete spectrum and on the assumption that the focal radius \(w_0\) depends on the illumination and the detection wavelengths \(\lambda_{ill/det}\) according to

\[ w_0^2 \propto \lambda_{ill}^2 + \lambda_{det}^2 \quad \text{so that} \quad \tau_{\text{diff}} \propto \left(1 + \frac{\lambda_{\text{ill}}^2}{\lambda_{\text{ill}}} \right) R, \quad N \propto \left(1 + \frac{\lambda_{\text{ill}}^2}{\lambda_{\text{ill}}} \right)^{3/2} \tag{5} \]

[25] and using the value of QD 525 as reference. Measured and theoretically expected diffusion correlation times agreed well with the exception of QD 655. The spectral dependence cannot be explained with either the chromatic or the size dependence alone so that we are indeed able to distinguish the 4 species present in the same solution. Different properties of photophysical effects such as blinking may have affected the apparent diffusion correlation times because it can be difficult to distinguish diffusional and photophysical contributions to the fluctuations when they occur on similar time scales [30], thus leading to the observed deviation of theory and experiment especially for QD 655. This may result from underestimating the contribution of blinking, thus yielding an underestimation of the diffusion correlation time. Therefore, QD 655 was studied in more detail.

![Fig. 4. FCS of a mixture of QDs. (A) ACFs of QD 525 (blue), 565 (green), 605 (yellow), and 655 (red) based on time trace extracted from a full spectral intensity recording by binning over appropriate detection windows. (B) The resulting diffusion correlation times for the respective QDs as a function of the maximum emission wavelength (circles) and the spectral dependence as theoretically expected from size and chromatic effects (arrows).](image-url)
5.2 Spectrally resolved fluorescence intermittence of QD 655

In a next step, we restricted the FCS analysis to spectral intensity recordings of QD 655 in solution for a better assessment of the different contributions to the spectral dependence of the ACFs. We extracted time traces binned over ~17 nm windows centered at 625, 633, 641, 650, 658, 668, 677, and 686 nm and fitted the ACFs computed therefrom globally with linked blinking parameters and individual amplitudes and diffusion parameters using the same model as before in order to account for the expected chromatic size dependence of the focal volume, see Fig. 5A and B. In order to compare the outcome with the expected chromatic dependence, we extrapolated the diffusion correlation time and the number of molecules as described in the previous section and based on the values for 655 nm, i.e. the emission maximum. Whereas above 655 nm, the values followed the estimated behaviour, both the diffusion correlation time and the number of molecules were significantly smaller than the prediction for shorter wavelengths. This is indicative for higher populated and longer-lived non-fluorescent states because it can be difficult to distinguish diffusional from photophysical contributions when they occur on similar time scales when fitting correlation functions. Underestimating the fraction of molecules in a dark state can result in an artificial underestimation of the total number of molecules, and underestimating the lifetime of the dark states can result in an artificial underestimation of the diffusion correlation time. When fitting with all parameters kept independent, we obtained an intermediate result where $\tau_{\text{diff}}$ and $N$ showed a spectral dependence stronger than the prediction and also $\tau_{\text{blink}}$ and $\theta_{\text{blink}}$ varied with the wavelength. Therefore, we repeated the fitting with the number of molecules and diffusion correlation times following the estimated spectral dependence (solid lines in Fig. 5A and B) whereas the blinking correlation time and the fraction of non-fluorescent particles were free parameters, see Fig. 5C, D and E. Both parameters decreased with increasing wavelength and approached a plateau above 655 nm. That way we made use of the spectral resolution to obtain information that is not accessible with a conventional FCS setup.

This observation is in agreement with previous studies stating that fluorescence intermittence of QDs cannot be described sufficiently with single characteristic transition rates to and exponential ACFs of the occupation of non-fluorescent trap states [30–33]. Our observations suggest that excitation into electronic levels farther above the energy gap of the
semiconductor crystal (as indicated by the shorter emission wavelength) result not only in a higher energy of the photons created upon electron-hole recombination but also in a higher probability to occupy trap states that have in addition longer lifetimes. This will be subject of a more detailed study.

5.3 Spectrally resolved FCCS in living cells

To assess the usability of our setup for live cell FCS and FCCS experiments, we used MCF7 cells either expressing a fusion protein containing EGFP and two mRFP copies [26] or co-expressing EGFP and mRFP as positive and negative control, respectively, for FCCS, see Fig. 6A. Using the conventional APD-based filter setup we could confirm a pronounced cross-correlation for the positive control with ratio\(G = 0.44\pm0.04\) and the substantial absence of cross-correlation for the negative control with ratio\(G = 0.04\pm0.03\), see Fig. 6B. In general, ratio\(G\) is obtained from fits of the ACFs and the CCF and Eq. (3).

After replacing the APD-based setup by the EMCCD-based spectrometer we repeated the experiments by reading complete spectral intensity traces. The data were binned either into two discrete channels at 504-540 nm (assigned to EGFP) and 617-696 nm (mRFP) or binned over ~16 nm into 6 spectral channels, three of which were assigned to EGFP and centered at 512, 521 and 530 nm, whereas the other three were assigned to mRFP and centered at 603, 618 and 633 nm. The cross-correlations obtained with the discrete channels that were similar to the properties of the filters used before were in good agreement with the APD-based measurements with ratio\(G = 0.39\pm0.03\) and 0.07±0.02 for the positive and the negative control, respectively, see Fig. 6C.

![Fig. 6. Spectrally resolved FCCS in living cells.](image-url)

Fig. 6. Spectrally resolved FCCS in living cells. (A) Confocal images of a positive control MCF7 cell showing the EGFP (green) and the mRFP (red) fluorescence and an overlay image (yellow), scale bar 20µm. (B) ACFs of the EGFP (green) and the mRFP fluorescence signal (red) as well as their CCF acquired in positive (pos.) and negative control (neg.) MCF7 cells with the APD-based filter setup. (C) The same as in (B), but acquired with the EMCCD-based spectrometer setup. (D) ratio\(G\) values determined for 3×3 spectral windows from data acquired in positive (pos.) and negative (neg.) control cells and their difference (pos.-neg.).
Then we determined the auto- and cross-correlations and the corresponding ratio $G$ values for all 9 combinations of the narrow band channels, see Fig. 6D. As expected, the closer the detection windows were, the higher was the cross-talk-induced cross-correlation in the negative control and the higher was also the overall cross-correlation in the positive control, and vice versa. The difference of the ratio $G$ values therefore defines the useful accessible dynamic range for measuring interactions which was maximal for the shortest wavelength windows for both fluorophores. Thus, for EGFP/mRFP-based FCCS experiments it is more important to match the emission maximum with the detection window than to reduce cross-talk but risk to lose signal at the same time.

6. Conclusion

Fluorescence correlation spectroscopy is a powerful tool now widely employed in biochemistry and cell biology to measure diffusion and interaction properties of biomolecules. We present here the successful implementation of an EMCCD- and prism-based spectrometer for spectrally resolved FCS that combines the advantages of a prism, i.e. high transmission and robust operation, with those of an EMCCD, i.e. a very large number of detectors (pixels) with high quantum efficiency, low SNR and μs time resolution. We could show that our setup allows to read out fluorescence spectra from 400 to 800 nm distributed over 80 pixels with a time resolution of 12 μs and single photon counting capability. The setup benefits from the high transmission of a prism. The major drawback of a prism, the nonlinear dispersion, is accounted for in the data treatment and does not affect the data because the requirements in terms of spectral resolution are rather relaxed. Attached to a commercial CLSM, we could use our setup in solution as well as in living specimen in combination with confocal microscopy. We could confirm that spectrally resolved FCS measurements of small organic fluorophores and of quantum dots in solution agree with data acquired with conventional APD-based FCS and that they allow to recover diffusion properties and concentrations as well as to identify and distinguish more than two components with different yet overlapping spectral and diffusion properties. From a spectrally resolved analysis of fluorescence intermittence of quantum dots emitting at 655 nm we obtained additional information that can help to further elucidate the underlying processes. In addition, we could show with the first EMCCD-based spectrally resolved FCCS experiments in living cells that this approach can be used to measure molecular interactions in vivo and that it provides the required flexibility to optimize experimental conditions.

As an outlook, spectrally resolved FCS should allow to measure the formation of higher-order complexes labelled with more than two fluorophores that may even have overlapping emission spectra. Due to the spectral flexibility, overlapping detection channels can be defined to optimize the fluorescence yield whereas in combination with interleaved excitation [34, 35], cross-talk could be reduced. Also for point-scanning microscopy, such a fast spectrometer could serve as spectral detector, and the use of back-illuminated EMCCD chips with up to three-fold higher quantum efficiency should increase the sensitivity significantly. Moreover, since EMCCD-based setups have been used for spatial FCS [18–22, 36], too, our study opens the path towards combined spatially and spectrally resolved FCS, i.e. spectrally optimized imaging of interactions, transport processes and molecular dynamics in living cells.

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