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Inverse-Fluorescence Cross-Correlation Spectroscopy

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Inverse-fluorescence correlation spectroscopy (iFCS) was recently introduced as an alternative version of FCS that does not require labeling of the analyzed particles or biomolecules. In iFCS, the signal from a medium surrounding the particles is analyzed, as opposed to a signal from the studied particles themselves. As unlabeled particles diffuse through the detection volume, they displace a fraction of the fluorescent medium, causing transient dips in the detected signal which give information about the mobility and concentration of the analyzed particles. Here inverse-fluorescence cross-correlation spectroscopy (iFCCS) is introduced as an extension of iFCS. In iFCCS, labeled particles/biomolecules are analyzed and their fluorescence signal is cross-correlated with the signal from the surrounding medium. When labeled particles are analyzed, a direct estimate of the volume of the particles is obtained or, alternatively, an estimate of the size of the detection volume. Another possibility is to analyze the interaction of small, labeled molecules with unlabeled particles, resulting in cross-correlation as an indication of binding, even though only one binding partner is labeled. This also enables accurate estimation of the degree of labeling, since the amounts of labeled and unlabeled particles are estimated independently.

Fluorescence correlation spectroscopy (FCS) analyzes diffusing dye-labeled biomolecules that emit fluorescence bursts as they traverse a diffraction limited open detection volume. Autocorrelation of the fluorescence signal gives information about concentrations (~nanomolar) and molecular sizes and about any dynamic process that fluctuates between states of different fluorescence brightness. After the theory and first experiments had been presented by Magde, Elson, and Webb[1,2] in the early 70s, FCS had its real breakthrough in the early 90s due to a significantly improved signal-to-noise ratio and has since then become an important tool in biophysics and cell biology, in academia as well as in industry.[3−8]

FCS can be used for analysis of binding between biomolecules by measuring the reduced mobility of a small, dye-labeled, ligand upon binding to, e.g., a larger protein. An alternative assay for binding is fluorescence cross-correlation spectroscopy, FCCS, which detects the correlated movement of two binding partners labeled with fluorescent markers of different colors. While FCCS is a more sensitive assay for binding than FCS, it has the downside that both binding partners must be labeled. This requirement is time-consuming and may lead to perturbation of the analyzed biomolecules.[9]

Recently an alternative version of FCS was presented, called inverse-FCS (iFCS), where the signal from a medium surrounding the analyzed particles is detected, as opposed to a signal from the analyzed particles themselves.[10] As a particle/biomolecule diffuses through the detection volume, a fraction of the signal-generating medium will be displaced, thereby creating a transient dip in the detected signal. Autocorrelation of the detected fluctuations gives information about concentrations and diffusion times of particles, and potentially of biomolecules, without any labeling required.

In iFCCS, the signal from labeled particles is cross-correlated with the signal from the surrounding medium, which results in two interesting possibilities: Either prelabeled particles can be analyzed, in which case cross-correlation gives information about the size-ratio between a particle and the detection volume. This allows either estimation of the volume of the analyzed particles by precalibrating the detection volume or, alternatively, estimation of the size of the detection volume by measuring on particles of known size. The second possibility is to analyze the interaction of small, dye-labeled ligands to larger, unlabeled, particles. Binding between ligands and particles results in anticorrelation between the medium-signal and that from the labeled particles. This is a very sensitive indication of binding, which still allows the larger binding partner to remain unlabeled (Figure 1). Moreover the fraction of ligand-carrying particles can be determined accurately, since the amount of unlabeled and labeled particles are estimated independently.

Here iFCCS is demonstrated by proof-of-principle measurements on fluorescent microspheres. Preliminary iFCS- and iFCCS-measurements in reduced detection volumes indicate that analysis

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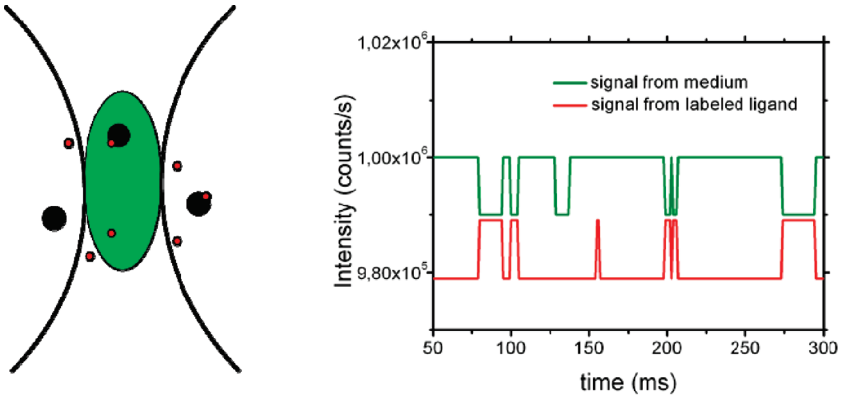

Figure 1. Principle of a possible iFCCS-measurement. Complexes of small, dye-labeled ligands and larger particles/biomolecules generate coinciding spikes in the two detection channels as they diffuse through the detection volume. Negative spikes in the medium-channel (green) coincide with positive spikes in the ligand-channel (red), indicating binding.

of considerably smaller particles and even protein molecules is possible. Even further enhancement of the sensitivity should be possible by using alternative photodetectors that allow higher count rates than the presently used APDs.[10]

THEORY

The theory of FCS[1,2] and of FCCS[11] has been described previously. In inverse-FCS, the dependence of the autocorrelation function and of the cross-correlation function on particle mobility is the same as for standard FCS. The particle concentration dependence however is different in iFCS,[10] as well as in iFCCS. The cross-correlation function is defined as

$$G_{cc}(\tau) = \frac{\langle I_g(t) I_r(t+\tau)\rangle}{\langle I_g\rangle\langle I_r\rangle} \tag{1}$$

from which the amplitude is given as

$$G_{cc}(0) - 1 = \frac{\langle \delta I_g(0)\, \delta I_r(0)\rangle}{\langle I_g\rangle\langle I_r\rangle} \tag{2}$$

In iFCCS, the detected fluorescence intensity is $I_g = I_{g,tot}(1 - N_{pg}V_{qg})$ for the green channel and $I_r = Q_p N_{pr} + (I_{ct} + I_{ligand})(1 - N_{pr}V_{qr})$ for the red channel in an iFCCS-measurement using green medium-molecules and red-labeled ligands. Here $I_{g,tot}$ is the average fluorescence intensity from the medium when the detection volume is void of particles, N_{pg} and N_{pr} are the average number of particles in the green and the red detection volumes, respectively, $V_{qg} = (V_{part}/V_g)$ and $V_{qr} = (V_{part}/V_r)$ where V_{part} is the volume of a particle and V_g and V_r are the sizes of the green and the red detection volumes, respectively, Q_p is the fluorescence intensity per particle (red channel), I_{ct} is the intensity in the red channel originating from cross-talk from green fluorescence, and I_{ligand} is the total intensity from unbound ligands in the red detection volume V_r. Furthermore $N_{pr} = N_{pg}(V_r/V_g)$ and $V_{qr} = V_{qg}(V_g/V_r)$. The above expressions together with eq 2 give at hand that the particle concentration dependence is given by

$$G_{cc}(0) - 1 = \frac{-\left[Q_p - (I_{ct} + I_{ligand})V_{qg}\frac{V_g}{V_r}\right]N_{pg}V_{qg}\sqrt{\frac{V_r}{V_g}}}{(I_{ct} + I_{ligand})(1 - N_{pg}V_{qg})^2 + N_{pg}Q_p\frac{V_r}{V_g}(1 - N_{pg}V_{qg})} \tag{3}$$

The cross-talk CT, defined as the fraction of the total count rate in the green channel that is detected in the red channel, can be determined independently from the emission spectrum of the green dye used for the medium, together with the emission filter-set for green and the red channels. Thus $I_{ct} = I_g$CT, where I_g is the total count rate detected in the green channel. I_{ligand} and Q_p can be estimated by first determining Q_{ligand} (the fluorescence intensity per ligand-molecule in the red channel) and the diffusion time $\tau_{D,ligand}$ from a separate, independent standard FCS-measurement and using these estimations in a subsequent measurement of ligands and ligand-binding particles with standard FCS.

MATERIALS AND METHODS

All measurements were performed on a Zeiss Confocor 2. For the measurements on fluospheres, simultaneous excitation using 458 and 543 nm was used. For the binding-measurements, only 488 nm excitation was used. The 200 nm fluospheres (540/560 carboxylate modified fluospheres, Invitrogen) were measured in 20 mM Tris, 75 mM KCl, pH 8.6, containing 100 μM alexa 488 carboxylic acid (Invitrogen) and 0.025% Triton X-100. The streptavidin coated polystyrene microspheres (320 nm diameter, Bangs Laboratories) were dissolved in the same buffer as described above but containing 2.5 μM alexa 488 carboxylic acid and 0.13% Triton X-100. R-Phycoerythrin-biotin and R-Phycoerythrin (both from Invitrogen), both with emission maximum at 575 nm, were used as the ligand and control, respectively. Emission filter BP475-525 for the alexa 488-emission and emission filter BP585-615 for the fluosphere-emission were used. For the binding-measurements emission filter BP505-530 were used for the alexa 488 emission and BP560-615 for the RPE-emission.

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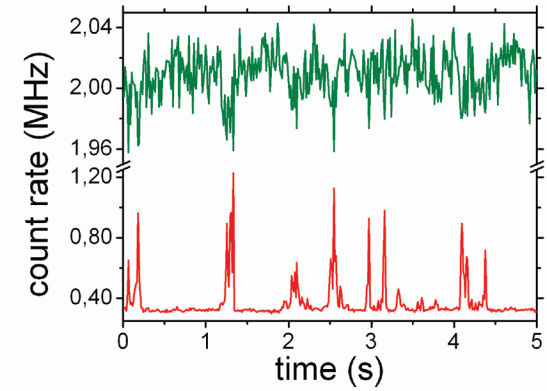


Figure 2. Fluorescence intensity traces from 200 nm diameter fluospheres (560 nm emission maximum) diffusing through the confocal volume in a solution containing 100 μM alexa 488 (517 nm emission maximum). Transiting fluospheres cause negative spikes in the green channel that coincide with positive spikes in the red channel.

RESULTS AND DISCUSSION

To verify the concept of iFCCS, measurements were performed on fluorescently labeled (emission maximum 560 nm) polystyrene beads of 200 nm diameter, in a buffer containing 100 μM alexa 488 (emission maximum 517 nm). The transit of a bead through the detection volume simultaneously generates a negative spike in the green channel and a positive spike in the red channel (Figure 2).

Cross-correlation curves were measured for six different concentrations of the 200 nm fluospheres. As expected, the curves display anticorrelation between the two detection channels, and the amplitude of the curves, $G_{CC}(0) - 1$, becomes more negative with increasing particle concentration (Figure 3a).

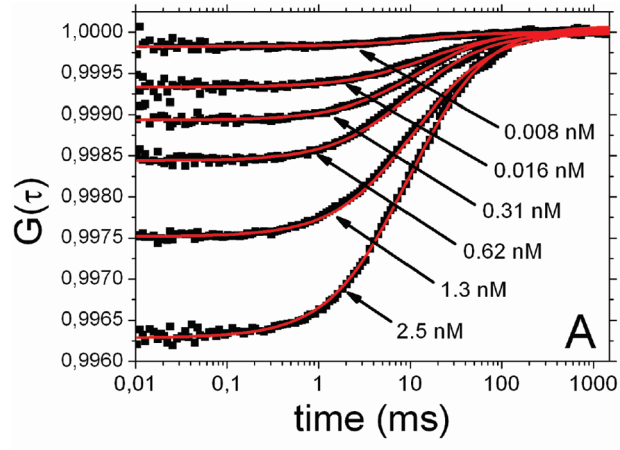
The dependence of $G_{cc}(0) - 1$ on particle concentration is a result of cross-talk between the green and the red detection channels (I_{ct}) and/or the presence of free ligands (I_{ligand}, see eq 3). In the measurements on fluospheres, no free ligands were present ($I_{ligand} = 0$ in eq 3) and for such a situation eq 3 reduces to

$$G_{cc}(0) - 1 = \frac{-\left[Q_p - I_{ct}V_{qg}\frac{V_g}{V_r}\right]N_{pg}V_{qg}\sqrt{\frac{V_r}{V_g}}}{I_{ct}(1 - N_{pg}V_{qg})^2 + N_{pg}Q_p\frac{V_r}{V_g}(1 - N_{pg}V_{qg})} \quad (4)$$

The amplitudes of the experimentally obtained iFCCS-curves (Figure 3a) agree with the predictions from eq 4 (Figure 3b). The slight deviations from the prediction of eq 4 are mainly caused by variations in particle brightness Q_p between different measurements and by variations in cross-talk I_{ct} due to differences in I_g between measurements. Especially for measurements at low particle concentrations, Q_p will vary between measurements, due to the low number of transiting events per measurement, which causes variations in $G_{cc}(0) - 1$.

Since eq 4 takes into account cross-talk (I_{ct}), it is still dependent on N_{pg}. In the case of both $I_{ligand} = 0$ and $I_{ct} = 0$ however, eq 4 reduces to

$$G_{cc}(0) - 1 = \frac{-V_{qg}\sqrt{\frac{V_g}{V_r}}}{(1 - N_{pg}V_{qg})} \quad (5)$$



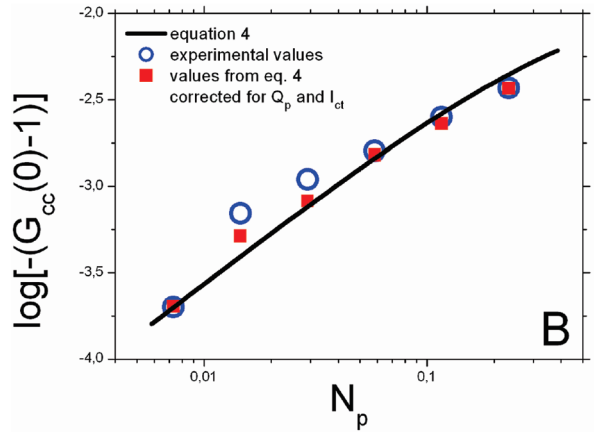


Figure 3. (a) iFCCS-curves of red fluospheres of 200 nm diameter measured at six different concentrations. The fluospheres were dissolved in a medium containing 100 μM alexa 488. Because of the presence of cross-talk from the green (medium-) channel to the red (fluosphere-) channel, the amplitude of $G_{cc}(\tau)$ is dependent on particle concentration. The 2.5 nM curve was measured for 2 min, the other curves for 5 min. The curves were fitted to a one diffusion component-model allowing the amplitude to be negative. (b) The amplitude of $G_{cc}(0) - 1$ versus the average number of particles in the detection volume N_p. The N_p values are taken as dilutions by a factor 2 in each step, from a starting-concentration of $N_p = 0.23$ calculated using eq 4. Black line: theoretical prediction of $G_{cc}(0) - 1$ from eq 4, where the mean values of Q_p and I_{ct} from all measurements was used. Open circles (blue): experimental values of $G_{cc}(0) - 1$ from Figure 3a. Filled squares (red): theoretical predictions from eq 4 corrected for Q_p and I_{ct} which were estimated from the individual measurements.

The factor $(V_g/V_r)^{1/2}$ is needed to give an estimate of the size-ratio between the green and the red detection volumes. As long as $N_{pg}V_{qg} \ll 1$, eq 5 is approximately independent of N_{pg} and roughly equals $-V_{qg}(V_g/V_r)^{1/2}$. As an example, if 200 nm fluospheres in a diffraction-limited detection volume of 0.17 fL would be used, $N_{pg} = 1$ results in $(1 - N_{pg}V_{qg}) = 0.98$. For smaller particles $(1 - N_{pg}V_{qg})$ is even closer to unity. An approximate estimation of $V_{qg} = (V_{part}/V_g)$ and of N_{pg} is sufficient for proving that $(1 - N_{pg}V_{qg}) \approx 1$ is valid. V_g can be estimated from measurement on a dye with a known diffusion coefficient and usually some preknowledge exists about the particles/biomolecules to be analyzed from which a rough estimate of V_{part} can be made. An estimate of N_{pg} is given from the standard FCS-curve obtained as part of the iFCCS-measurement. Thus, given that $(1 - N_{pg}V_{qg}) \approx 1$ can be

assumed, eq 5 will give an estimate of $-V_{qg}(V_g/V_r)^{1/2}$. Hence, if the size of the fluorescent spheres is known, an estimate of V_g is obtained. With such a calibration of V_g, which may also be performed by measurement of a dye with known diffusion coefficient, V_{qg} obtained from a subsequent iFCCS-measurement on particles of unknown size will give a direct estimate of the average volume V_{part} of the measured particles.

The standard procedure for calibrating the FCS detection volume is to estimate the $1/e^2$-radius ω by measuring the diffusion time of a dye with known diffusion coefficient, from which the volume is calculated using $V = \pi^{3/2}\omega^3 S$, where $S = z/\omega$ is the structure parameter and z is the half-height of the detection volume. However, in FCS the measured diffusion times are relatively weakly influenced by z, wherefore it is often difficult to estimate S. Furthermore, viscosity and temperature affect the diffusion coefficient of the calibration dye and must therefore be known or controlled. In contrast, with iFCCS and by use of eq 5 an estimate of V_g is obtained from the size ratio between the sphere and the detection volume which is independent of viscosity and temperature. In addition, estimations of detection volumes for which no analytical expressions describe their shape[12] will benefit from the approach described here.

The factor $(V_g/V_r)^{1/2}$ appears because two-color cross-correlation measurements are performed with different sizes of the green and the red detection volumes. However a single laser line is sufficient, if measurements can be carried out on red-labeled particles that can be sufficiently excited with the same green laser line as is used for exciting the medium. With the use of pinholes of the same size for the two detection channels, an estimate of V_{qg} and thus of V_{part} or V_g can be obtained without requiring estimation of (V_g/V_r).

Since cross-talk was present in our measurements on fluospheres (Figure 3a), eq 5 could not be applied. However the detection volume can still be estimated by applying an approximation of eq 4. Since $I_{ct}V_{qg}(V_g/V_r) \ll Q_p$ and since $V_{qg} = V_{part}/V_g \approx 0.03$ and $N_{pg} < 0.1$ imply that $(1 - N_{pg}V_{qg}) \approx 1$ (compare to eq 4), a valid approximation of eq 4 is

$$G_{cc}(0) - 1 = \frac{-Q_p N_{pg} V_{qg}\sqrt{\frac{V_r}{V_g}}}{I_{ct} + Q_p N_{pg}\frac{V_r}{V_g}} \tag{6}$$

Solving eq 6 for V_{qg} and replacing V_{qg} by (V_{part}/V_g) gives

$$V_g = \frac{-Q_p N_{pg} V_{part}\sqrt{\frac{V_r}{V_g}}}{(G_{cc}(0) - 1)\left(I_{ct} + Q_p N_{pg}\frac{V_r}{V_g}\right)} = \frac{-(I_r - I_{ct})V_{part}\sqrt{\frac{V_g}{V_r}}}{(G_{cc}(0) - 1)I_r} \tag{7}$$

where the total intensity in the red channel $I_r = I_{ct} + Q_p N_{pg}(V_r/V_g)$. The factor $(V_g/V_r)^{1/2}$ (eq 7) is needed to give an estimate of the size ratio between V_g and V_r. By comparison of the diffusion time of RPE measured with 458 nm excitation with the diffusion time of RPE measured with 543 nm excitation, using the same pinhole settings as were used in the iFCCS-measurements on fluospheres, $(V_g/V_r)^{1/2}$ was estimated to 0.66 ($V_r/V_g = 2.28$). The curves (Figure 3a) together with eq 7 give the values 0.159, 0.169, 0.166, 0.176, 0.155, and 0.250 fL, respectively, for V_g. Since the clearly deviating value 0.250 fL is derived from the measurement on the most dilute sample (less than 10 transits of fluospheres per minute), we assume here that it is nonrepresentative. The remaining values give

$$V_g = 0.165 \pm 0.008\ \text{fL}$$

where the error is the standard deviation. For comparison we estimated the detection volume using alexa 488 ($D = 414\ \mu m^2/s$, from $D = 435\ \mu m^2/s$[13] and corrected for our room temperature of 22.5 °C) with a measured diffusion time of 16.8 μs (458 nm excitation was used) and a mean structure parameter $S = 5.9$, using $V = \pi^{3/2}\omega^3 S$. This gives $V_g = 0.153 \pm 0.02$ fL which agrees very well with the estimation above from iFCCS on fluospheres. The traditional estimation gives a larger standard deviation which is the result of uncertainty in the estimated structure parameter S. It should be pointed out that our measurements were carried out using a diffraction limited detection volume, in which case the structure parameter S usually can be estimated. For larger detection volumes however, with defocused laser excitation and using pinholes of $\geq 100\ \mu$m diameter, a value of S can often not be obtained. The fact that different iFCCS-measurements give so similar values for the size of the detection volume means that, conversely, if the size of the detection volume is estimated beforehand, then the volume of the analyzed particles or biomolecules can be estimated very precisely.

To test the ability of iFCCS to detect the interaction between a small dye-labeled ligand and a large binding partner, nonfluorescent streptavidin coated polystyrene beads were mixed with biotin-tagged R-Phycoerythrin (RPE-biotin). Again alexa 488 was used as signal-generating medium molecule. Due to the large Stokes shift of RPE, the emission spectra of RPE and alexa 488 could be separated even though a single 488 nm laser line was used for excitation of both fluorophores, as for example has been used by Hwang et al.[14] The nonfluorescent spheres become fluorescent upon binding to RPE-biotin, causing the negative spikes in the medium-channel to coincide with positive spikes in the RPE-channel. Streptavidin-coated spheres were measured in the presence of 60 nM RPE as the control (Figure 4A) and in the presence of 15, 30, or 60 nM of RPE-biotin (Figure 4B–D). iFCCS-measurements were initiated 2–3 min after mixing. Positive spikes in the red channel that coincided with negative spikes in the green channel were observed (Figure 4B–D), and the frequency of coinciding spike-pairs increased with increasing RPE-biotin concentration.

The fraction of negative spikes that coincide with a positive spike is a direct estimate of the fraction of streptavidinized beads that carry an RPE-biotin label. One way of determining this fraction

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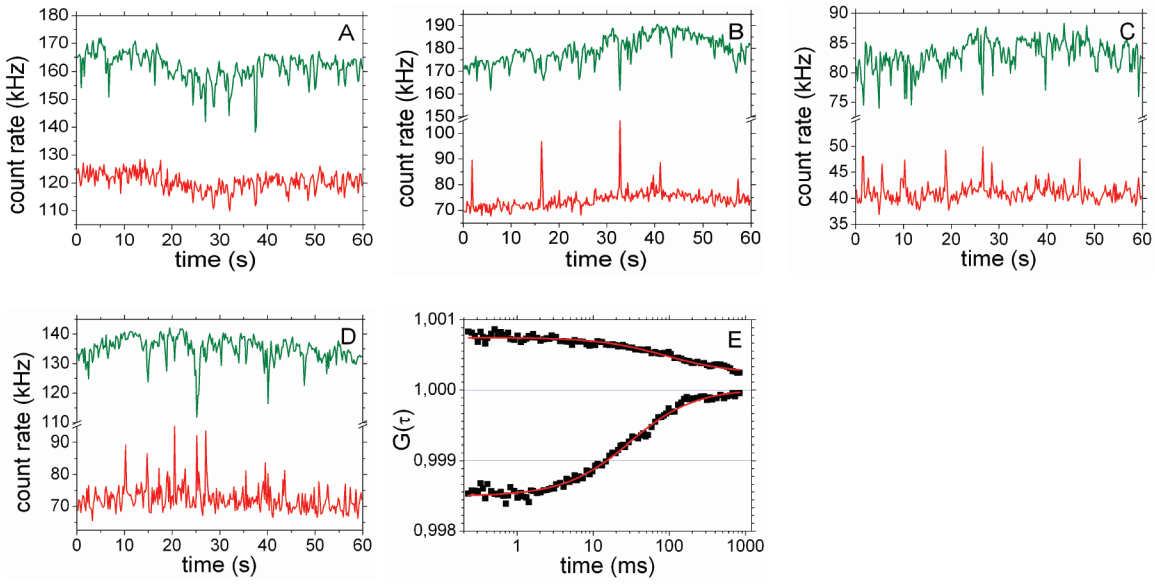

Figure 4. Binding of biotin-tagged R-Phycoerythrin (RPE-biotin), or of nontagged RPE, to streptavidin-coated microspheres. Parts A−D show 60 s intensity traces from both the surrounding medium (upper, green) and from RPE or RPE-biotin (lower, red): (A) 60 nM of non-tagged RPE, (B) 15 nM of biotin-tagged RPE, (C) 30 nM of biotin-tagged RPE, and (D) 60 nM of biotin-tagged RPE. The fraction of all negative spikes during a 300 s measurement that coincided with a positive spike was for (A) 8% (6/74), for (B) 30% (27/91), for (C) 41% (38/92), and for (D) 58% (62/106). (E) iFCCS-curves of streptavidin coated spheres in a solution of 60 nM non-tagged RPE (upper curve) and in a solution of 60 nM biotin-tagged RPE (lower curve). The lower curve shows anticorrelation, indicating binding between the RPE-biotin and the spheres. The upper curve shows no anticorrelation, indicating that non-tagged RPE does not bind to the spheres. The upper curve even shows positive correlation as a result of cross-talk from the medium-channel to the RPE-channel (see Figure 4A).

is to estimate the concentration of labeled beads from the amplitude of the component with longer diffusion time τ_{diff} of the red standard FCS-curve and compare this estimate with the total number of beads (labeled and unlabeled), obtained from the amplitude of the iFCS-curve.[10] The brightness of the labeled beads is however different from that of unbound ligands. For such a situation the true concentration of labeled beads can in principle still be obtained by separately estimating the brightness of unbound ligands. This however turned out to be difficult, likely due to the presence of free streptavidin molecules detached from the beads which, when bound to up to four RPE-biotin molecules, will influence the apparent brightness of unbound ligand. Another complication could be the presence of many binding sites per streptavidin-coated bead, which likely will give rise to a brightness-distribution among beads. If such a distribution is not considered it may affect the estimated bead concentration. We therefore instead counted the number of negative spikes in the green channel that coincided with a positive spike in the red channel. Since cross-talk is present from the green, medium signal to the red channel, the transits of beads not carrying any RPE result in negative spikes also in the red channel (Figure 4A). Therefore, when indeed an increase in intensity in the red channel is found to coincide with a negative spike in the green channel, this provides a sufficient criterion for the detection of a labeled bead.

In addition to negative spikes in the green channel without coinciding spikes in the red channel, which correspond to unlabeled beads, positive spikes in the red channel were observed that did not coincide with a negative spike in the green channel (Figure 4B−D). These are likely the result of free streptavidin molecules detached from the microspheres, able to bind up to four RPE-biotin molecules. Because of their limited volume, they generate however no negative spikes in the medium-channel.

The fraction of the negative spikes that coincided with a positive spike was 30% (27 of 91) at 15 nM RPE-biotin, 41% (38 of 92) at 30 nM RPE-biotin, and 58% (62 of 106) at 60 nM RPE-biotin. In a control measurement on 60 nM RPE without biotin mixed with streptavidin coated spheres, 8% (6 of 74) of the negative spikes coincided with a positive spike (Figure 4A).

In a parallel project not presented here, measurements in reduced detection volumes show that significantly smaller particles and even protein molecules can be analyzed by iFCS (Dr. Tor Sandén, personal communication). This will allow iFCCS to be significantly extended to applications beyond the proof-of-principle measurements shown in this work. iFCCS should enable direct estimation of the volume of protein molecules, and cross-correlation can be used as the indication of binding between ligands and unlabeled protein molecules. For example, in FCS-based assays for high-throughput screening (HTS), standard FCCS is not used because of its requirement that both binding partners must be labeled.[5] iFCCS has the potential to circumvent this requirement. Also, iFCS[10] could become an alternative to dynamic light scattering (DLS) for label-free analysis of proteins, with a similar lower limit of analyzable concentration of ~1 μM.[15]

Further enhancement of the sensitivity should be possible by using photodetectors that allow higher count rates than the presently used APDs. Simple photodiodes or PMTs in dc-mode both give a current as output and are capable of detecting count rates of >10^{13} Hz, about 10^6 times more than APDs. When such detectors are used, the minimum relative noise in the medium-

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signal that can be accomplished will be limited not by the photodetectors but by the amount of signal that can be generated from the medium.

An iFCCS-measurement on labeled particles gives a direct estimate of the labeled particles' average volume; however, the same measurement also gives an estimate of the particles diffusion coefficient from the measured diffusion time τ_{diff}. Since the diffusion coefficient is dependent not only on the size of particles but also on their shape, comparison between τ_{diff} and the estimated particle volume V_{part} should give information about the particles' shape.

Several other label-free analysis methods exist, for example, surface plasmon resonance,[16] stimulated Raman scattering microscopy,[17] dynamic light scattering (DLS),[18] and more recently deep-nulling interferometer microscopy.[19] The latter two are used for analysis in free solution, making them similar to iFCS and iFCCS. Label-free methods have obvious advantages. However, compared to label-dependent methods such as standard FCS and FCCS, they lack in specificity; samples should preferably contain only the analyzed particles/biomolecules of interest. Simultaneous analysis of labeled and unlabeled particles/biomolecules in a single measurement combine the strength of label-free and label-dependent methods. iFCCS offers such a simultaneous analysis, and in principle DLS and deep-nulling interferometer microscopy could also be combined with label-dependent methods such as standard FCS.

In summary, iFCCS makes two new analyses possible: First, labeled particles/biomolecules can be analyzed, which gives information about the size ratio between the analyzed particles/biomolecules and the detection volume. This ratio can be used to estimate the volume of the particles, without any assumptions about the particles' shape, or it can be used to estimate the size of the detection volume. Second, the interaction of small, labeled ligands with larger unlabeled particles/biomolecules can be studied. Binding between ligands and particles/biomolecules gives rise to anticorrelation, a sensitive indication of binding, without requiring labeling of the particle/biomolecule. Omitting labeling saves time, and the risk of perturbing the analyzed particles/biomolecules is avoided. Using iFCCS in such a binding-assay can also give accurate estimation of the degree of labeling of particles/biomolecules. This could be very useful for testing the success of post-translational labeling of proteins.

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