Comparison of Different Fluorescence Fluctuation Methods for their Use in FRET Assays: Monitoring a Protease Reaction

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Abstract: We compare the accuracy of a variety of Fluorescence Fluctuation Spectroscopy (FFS) methods for the study of Förster Resonance Energy Transfer (FRET) assays. As an example, the cleavage of a doubly labeled, FRET-active peptide substrate by the protease Trypsin is monitored and analyzed using methods based on fluorescence intensity, Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Intensity Distribution Analysis (FIDA). The presented fluorescence data are compared to High-Pressure Liquid Chromatography (HPLC) data obtained from the same assay. The HPLC analysis discloses general disadvantages of the FRET approach, such as incomplete labeling and the need for aliquots. However, the simultaneous use of two photon detectors monitoring the fluorescence signal of both labels significantly improves the analysis. In particular, the two global analysis tools Two-Dimensional Fluorescence Intensity Distribution Analysis (2D-FIDA) and Two-Color Global Fluorescence Correlation Spectroscopy (2CG-FCS) highlight the potential of a combination of FFS and FRET. While conventional FIDA and FCS auto- or cross-correlation analysis leaves the user with drawbacks inherent in two-color and FRET applications, these effects are overcome by the global analysis on the molecular level. Furthermore, it is advantageous to analyze the unnormalized as opposed to the normalized correlation data when combining any fluorescence correlation method with FRET, since the analysis of the unnormalized data introduces more accuracy and is less sensitive to the experimental drawbacks.

Key Words: Fluorescence Fluctuation Spectroscopy, Fluorescence Correlation Spectroscopy, Fluorescence Intensity Distribution Analysis, Förster Resonance Energy Transfer, Confocal Microscopy, Protease Reaction.

INTRODUCTION

The number of biological assays based on fluorescence detection has significantly increased in recent years. The superior sensitivity to environmental properties and as well as its multidimensionality, i.e., its ability to provide various simultaneous readouts (e.g., intensity, anisotropy, lifetime, spectral characteristics) have promoted fluorescence readouts to their current status as one of the most important tools in life sciences. The vast increase of attractiveness is also based on the ever-growing range of fluorescence measurement techniques [1], one of which is Förster Resonance Energy Transfer (FRET) [2]. FRET makes use of two fluorescence labels, a donor and an acceptor. Excitation of the donor results in a transfer of its energy to the acceptor. Characteristics such as the spectral overlap, the orientation and the distance of the two dyes determine the efficiency of the energy transfer and thus the amount of fluorescence emitted by the second dye. As a consequence, FRET provides a sensitive tool for measuring distance changes in a range of 1 to 10 nm that are caused by biological activities involving conformational changes or binding and cleavage reactions. A huge number of biological assays have been created based on the principle of FRET [3-9].

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However, several experimental effects have to be considered, which might result in the misinterpretation of FRET data. Variations in concentration due to photobleaching or dilution steps affect the overall fluorescence signal. Alterations in the fluorescence characteristics of the donor, caused by environmental changes such as quenching reactions, sensitize the fluorescence signal of the acceptor in the same manner as changes in distance or orientation. Furthermore, FRET most often constitutes labels with different excitation and emission wavelengths. The simultaneous use of two fluorescence colors introduces cross-talk, which results from the overlap of the fluorescence spectra of the different fluorescence labels applied. Fluorescence detected for the acceptor might not only be evoked by the energy transfer, but also by its direct excitation or by contributions of fluorescence from the donor “bleeding” into the acceptor detection channel. Several methods have been developed to circumvent these drawbacks [10-17]. Spectrally resolved detection of each dye’s contribution to the total amount of fluorescence tackles the cross-talk problem. Fluorescence readouts other than intensity, such as fluorescence lifetime or anisotropy, are independent of the fluorophore concentration and can distinguish fluorescence changes evoked by variation in the energy transfer efficiency or by the donor characteristics. Furthermore, the improvements in the field of confocal microscopy enabled the direct observation of single fluorescing molecules. Due to their substantial sensitivity and selectivity, biochemical applications increasingly apply FRET-based single-molecule experiments [6, 7, 18-24], ultimately refined.
in the simultaneous detection and analysis of multiple fluorescence parameters [25]. Alternating FRET and direct laser excitation of the acceptor introduces further accuracy and variability to the analysis of single-molecule FRET measurements. In special it allows for the exclusion of cross-talk artifacts and the analysis of subpopulation [23].

Another method, which is based on the detection of fluorescence signals from a low amount of fluorescent molecules, is the analysis of fluctuations in fluorescence. These fluctuations carry valuable information on the dynamics of the molecule under observation [26]. The first method based on Fluorescence Fluctuation Spectroscopy (FFS) was Fluorescence Correlation Spectroscopy (FCS) in 1972 [27], which analyzes the temporal fluctuations by calculating the auto-correlation function on the time axis. Chen et al. and Kask et al. introduced Photon Counting Histogram (PCH) analysis or Fluorescence Intensity Distribution Analysis (FIDA), which calculates the statistics on the fluctuation amplitude (i.e., number of photons) [28, 29]. While FCS resolves components with different diffusion coefficients, FIDA achieves molecular resolution according to the different values of molecular fluorescence brightness (photons counts per molecule). Thus, both analysis methods enable the distinction and quantification of different fluorescent species of a sample based on molecular characteristics. The introduction of Fluorescence Cross-Correlation Spectroscopy (FCCS) [30, 31] and Two-Dimensional FIDA (2D-FIDA) [32] or Dual-Color PCH [33] strongly increased the potentials of both methods. This improvement is based on the expansion into a second dimension, namely the simultaneous detection of different colors or polarization of fluorescence, and provides for coincident information. Consequently, FCCS [34-45] and 2D-FIDA [32, 46-50] have achieved remarkably results on various assays ranging from binding events to protease cleavage reactions. In particular, 2D-FIDA has several times been introduced into industrial application such as High Throughput Drug Screening (HTS) [48, 49, 51, 52].

FIDA and FCS studies can thus also be based on dual-color labels and detection and incorporate the use of FRET. Not only FRET itself but also cross-talk and concentration effects significantly influence FIDA and FCS data. Some intensive research in this field, which highlights the challenges involved when studying FRET by using fluorescence correlation data, has already been carried out so far [38, 53-57]. As an example, Kohl et al. [57] used a combined FCS and FRET analysis to study the proteolytic cleavage of a peptide labeled with two fluorescent proteins. A single wavelength was used to simultaneously evoke fluorescence emission of both labels by two-photon excitation. The influence of FRET on the FCCS amplitude was studied in detail and the protease reaction accurately resolved by introducing FRET-correction factors. Horn and Verkman [56] investigated the influence of FRET on two-color FCS and FCCS in detail when studying reversible bimolecular reactions between two fluorescently labeled reactants. Optimal energy transfer efficiencies have been elucidated for different reaction rates. Widengren et al. [55] introduced a concept for how the influence of FRET on correlation data can expose intramolecular dye-label distances and states. The introduction of Two-Color Global Fluorescence Correlation Spectroscopy (2CG-FCS), which simultaneously analyses all correlation curves on a global basis, has demonstrated improvements on this field [58].

This study compares the potentials and drawbacks of different fluorescence methods with respect to FRET assays. We concentrate on the analysis of FRET using total fluorescence intensity and the FFS tools FIDA, 2D-FIDA, FCS, FCCS, and 2CG-FCS. The use of other parameters such as fluorescence lifetime or anisotropy, which also are prominent analysis tools for FRET analysis, is not included in this study. In particular, the cleavage of a doubly labeled, FRET-active peptide substrate by the protease Trypsin is monitored. The biochemical activity is determined by each of these methods and the validity of the resulting kinetic constants as well as the accuracy is discussed. Following previous work on FCS and FCCS [56, 57], the influence of different FRET efficiencies is analyzed. In addition, special emphasis is put on the capability to correct for non-ideal experimental conditions such as sample-to-sample variations in substrate concentration or fluorescence cross-talk between the two detection channels. In addition to the FRET efficiency, the influence of a difference in the fluorescence emission and detection efficiency of both labels on the results obtained is stretched. Some of the methods are shown to be advantageous, and their benefits are discussed. For the correlation analysis methods, a special focus is provided for the use of normalized, as usually applied, and unnormalized correlation data. Furthermore, the Trypsin activity as determined by the combination of FCS and FRET is discussed based on results obtained by concomitant High-Pressure Liquid Chromatography (HPLC) studies.

MATERIALS AND METHODS

Trypsin

The presented assay investigates the activity of the Trypsin enzyme. As a member of a large and diverse family of serine proteases, it plays an essential role in digestion, blood clotting, and cell differentiation, and as a regulator through the activation of precursor proteins [59, 60]. A peptide substrate (NH-Gly-Pro-Ala-Lys-Leu-Ala-Ile-Gly-Lys-CONH₂) is labeled with Rhodamine Green (RhGr, Molecular Probes, Eugene, OR) and MR121 (Roche Diagnostics, Mannheim, Germany) at the N- and C-terminus, respectively. The peptide was synthesized on solid phase using standard Fmoc-protected coupling procedures. Fluorescent dyes were attached on solid phase after cleavage of the N-terminal Fmoc group (RhGr) and deprotection of a selectively cleavable side chain protective group (Mtt, 4-Methyltrityl) at the C-terminal lysine residue (MR121), respectively. After acidic cleavage of the crude labeled peptide from the resin with TFA (TriFluoroacetic Acid) (TFA 95%, 2.5% water, 2.5% TIS (TriIsopropylSilane)) the substrate was evaporated to dryness and subsequently purified by RP-HPLC (Reversed-Phase High-Pressure Liquid Chromatography). Purity and identity of the obtained prod-
uct was confirmed by analysis with LC/MS (Liquid Chromatography coupled to a Mass Spectrometer). A purity of above 95% was obtained as determined by product absorption at 500 and 650 nm.

Addition of Trypsin to the peptide results in the cleavage of the substrate at Lys-Leu and the formation of two separate fragments.

\[
\text{RhGr-NH-Gly-Pro-Ala-Lys-ConH}_2 - \text{MR121}
\]

\[
\text{RhGr-NH-Gly-Pro-Ala-Lys-COOH} \quad + \quad \text{H-Leu-Ala-Ile-Gly-Lys-ConH}_2 - \text{MR121}
\]

Two buffers are applied, incubation (50 mM HEPES (4-(2-HydroxyEthyl)Piperazine-1-EthaneSulfonic acid) pH 8, 100 mM NaCl, 10 mM CaCl\(_2\) and 0.05% Pluronic) and analysis buffer (5 mM EDTA (EthyleneDiamineTetraAcetic acid), 0.5% SDS (Sodium Dodecyl Sulfate), 10 mM Tris/HCl pH 7.8). The incubation buffer is used for the cleavage reaction at 37°C applying a Trypsin concentration of 75 nM and a substrate concentration of 2.5 \(\mu\)M. The cleavage reaction is stopped after 3 to 60 minutes of incubation time. Aliquots are withdrawn and 1000-fold diluted into the analysis buffer, yielding different fractions of cleaved peptide for analysis. The relatively high concentration of SDS in the analysis buffer is necessary to generate a sufficient FRET signal. In addition, this level of concentration will favor the formation of detergent micelles. The substrate concentration is applied far below the concentration isomerization or binding and cleavage reactions.

**Addition of Trypsin to the peptide results in the cleavage of the substrate at Lys-Leu and the formation of two separate fragments.**

\[
\text{RhGr-NH-Gly-Pro-Ala-Lys-Leu-Ala-Ile-Gly-Lys-CONH}_2 - \text{MR121}
\]

**Comparison of Different Fluorescence Fluctuation Methods**

**Förster Resonance Energy Transfer (FRET) Assay**

One of the most sensitive methods for the detection of proteolytic activity is Förster Resonance Energy Transfer (FRET). FRET analyses the extent of an energy transfer between two dye molecules, a donor, \(D\), and an acceptor, \(A\), via a dipole-dipole interaction to gain information on the distance and orientation of both dyes. Upon excitation of the donor, \(D \rightarrow D^*\), its energy is transferred to the acceptor dye, leading to a quench of the donor fluorescence and without direct excitation to the formation of acceptor fluorescence, \(D^* + A \rightarrow D + A^*\). The efficiency, \(E\), of the energy transfer depends on the spatial distance, \(r\), of donor and acceptor.

\[
E = \frac{1}{1 + (r / R_0)^6}
\]

The Förster radius, \(R_0\), is characteristic for a specific donor acceptor dye pair and lies in the range of \(\approx 1\) to \(10\) nm for the dyes used [3-9]. The characteristic sixth power dependence on the spatial distance, \(r\), makes FRET such a sensitive tool for many biological reactions including conformational changes or binding and cleavage reactions.

The present peptide substrate, which is labeled at the N- and C-terminus with a donor and an acceptor dye, respectively, provides for an ideal FRET conjugate. Cleavage of the conjugate by Trypsin will interrupt the FRET signal and hence the kinetic of the reaction can be monitored by an increase in donor fluorescence intensity and a concurrent decrease in acceptor fluorescence intensity. RhGr serves as the donor and MR121 as the acceptor. Their absorption as well as the corresponding fluorescence emission spectra are shown in Fig. (1). The Förster radius of \(R_0 = 5\) nm is comparable to the size of the peptide. Incomplete labeling or an inactive acceptor will introduce FRET inactivity to the uncleaved substrate, which has to be taken into account.

**Optical FRET Equipment**

A single pinhole confocal fluorescence microscope (EVOTEC FCS+plus spectrometer, Evotec Technologies, Hamburg, Germany) is used as the detection system. Its optical scheme has been described in detail previously [32]. Continuous wave laser light from a 488 nm CW Argon Ion laser (JDS-Uniphase, 2014-25MLVW, CA 95131) is used for direct excitation of the donor dye and for FRET signal generation. The exciting light is focused onto the sample by a water-immersion objective lens (U-APO / 340, 40x, NA 1.15, Olympus Optical Co., Ltd., Tokyo, Japan). Fluorescent light is collected by the same objective and spectrally separated by the use of a dichroic beam splitter (centered at 565 nm) and interference filters (535 DF45 and 670 DF40, Omega® Optical, Inc., Brattleboro, Vermont). This enables to monitor green and red fluorescence of the donor and acceptor simultaneously on two different detectors (SPCM-AQ-131, Perkin-Elmer Optoelectronics, Fremont, California).
nia), defined as the green and red detection channel, respectively (see Fig. (1)).

The fluorescence signal is processed online and stored simultaneously as fluorescence intensity, FIDA and 2D-FIDA (40 µs time window), as well as FCS and FCCS (50 ns time resolution) data. The radius of the monitored sample volume is \( r_0 = 0.6 \mu \text{m} \), yielding diffusion times for simple organic dye molecules such as Rhodamine Green (RhGr) of \( t_0 = 160 \mu \text{s} \) in aqueous solution. Measurements are performed with a focal peak laser irradiance of 90 kW/cm\(^2\), corresponding to a power of 0.5 mW at the sample. Measurement time is ten seconds and statistical significance is induced by a 10-fold repetition of each sample prepared. At the applied irradiances, the background signal according to Rayleigh and Raman scattering and detector dark counts amounts to 1 kHz for the green and 0.2 kHz for the red detection channel, respectively.

**High-Pressure Liquid Chromatography (HPLC)**

HPLC studies are carried out on a Waters 2690 Separations module connected to a Jetstream 2 plus column oven, a Waters 996 photo diode array detector, a Waters FD 474 fluorescence detector, and a Micromass ZMD mass spectrometer (Waters GmbH, Eschborn, Germany).

The following chromatographic conditions are used: column: Waters Xterra MS C18, 2.5 µm, 30 × 2.1 mm with Phenomenex SecurityGuard Guard Cartridge and Phenomenex C18 ODS, 4 × 2 mm guard column (Phenomenex, Aschaffenburg, Germany); eluent: (A) H\(_2\)O / 0.1 % HCO\(_2\)H, (B) Methanol / 0.1 % HCO\(_2\)H; gradient: time (%A / %B), 0.00 min (98 / 2), 5.00 min (2 / 98), 5.5 min (2 / 98); flow rate: 0.8 ml / min; column temperature: 40°C; sample temperature: 4°C; injection volume: 20 µl; gradient: time (%A / %B), 0.00 min (90 / 10), 5.00 min (2 / 98), 3.75 min (2 / 98), 8.00 min (90 / 10); MS (mass spectrometer) detection: ESI+/ESI- scan mode, mass range 150 – 850 Da; UV/VIS detection: 200 nm to 798 nm, resolution 1.2 nm, 1 spectra per second.

**Trypsin Assay Characteristics**

**Michaelis-Menten Equation**

The enzymatic reaction of Trypsin can be described by the Michaelis-Menten equation, which combines the initial velocity of the cleavage reaction, i.e., the initial decrease of uncleaved substrate concentration, \( v \), the maximal velocity, \( V_{\text{max}} \), and the Michaelis constant, \( K_M \), of the enzymatic reaction [62].

\[
\frac{ds_1}{dt} = V_{\text{max}} \frac{s_1}{K_M + s_1} = V_{\text{max}} \frac{s_1}{K_M}
\]  

(3a)

The substrate concentration of 2.5 µM applied during incubation with Trypsin lies far below the \( K_m \) of 60 µM as previously reported for a Lys-Leu motive [61]. Thus, the cleavage of the substrate due to Trypsin follows that of an apparent first-order reaction and can be approximated by an exponential decrease over incubation time, \( t \).

\[
[s_1](t) = [(s_1)(0) - [s_1](\infty)] \exp(-t/t_0) + [s_1](\infty)
\]  

(3b)

The substrate concentration at time zero, \([s_1](0)\), equals the applied concentration of 2.5 µM, while \([s_1](\infty)\) accounts for potentially uncleaved substrate at infinite incubation time, \( t = \infty \). The experimentally determinable time constant, \( t_0 \), characterizes the Trypsin activity and allows for the calculation of \( V_{\text{max}} = \frac{t_0}{K_M} \). From \( V_{\text{max}} \) and the instituted Trypsin concentration of 75 nM, one can calculate the kinetic rate constant, \( k_{\text{cat}} \), of the enzymatic reaction.

\[
k_{\text{cat}} = V_{\text{max}} / 75 \text{ nM}
\]  

(3c)

**Trypsin FRET Assay**

The Trypsin assay is characterized by two species, i.e. uncleaved substrate carrying both dye labels, and cleaved substrate fragments, solely tagged by donor or acceptor. Their respective molar concentrations, \([s_1]\) and \([s_2]\) (unit M), are a monitor for the Trypsin activity (compare Eq. (3b)). In principle, the difference in FRET activity between cleaved and uncleaved substrate renders a readout to determine \([s_1]\) and \([s_2]\). Thus, we consider two species in our FRET assay, FRET-active and –inactive substrate, denoted by the index “on” and “off”, respectively. Their concentrations are determined by FFS methods in terms of mean numbers of particles in the confocal detection volume, \( c_{\text{on}} \) and \( c_{\text{off}} \). The mean number of particles, \( c_i \) (no unit), of a certain species \( i \) is interrelated with the molar concentration, \([s_i]/V_{\text{eff}}\), through the effective confocal detection volume, \( V_{\text{eff}} \).

– The FRET-active substrate emerges from a close vicinity of active donor and acceptor and is characteristic of the uncleaved peptide. It exhibits quenched donor and high acceptor fluorescence. Furthermore, due to the buffer conditions (in particular, the high SDS concentration applied) FRET activity may also be present for the cleaved substrate. The high concentration of SDS used within the analysis buffer is necessary to generate sufficient FRET signal. On the other hand, it produces substrate-enriched detergent micelles (as shown below). The cleaved peptide residing in close vicinity within the same detergent micelle may still exhibit FRET activity.

– An active donor but no acceptor in its vicinity causes the FRET-inactive species. This is either due to the cleaved substrate or an inactive acceptor. FRET inactivity causes unquenched donor and no acceptor fluorescence.

– Potential other species, e.g., those consisting of an active acceptor and an inactive donor or an inactive donor and an active acceptor, are practically invisible and not observed. Furthermore, recent work reports photodestruction-intermediate states of an acceptor, which are non-emitting but still able to quench the fluorescence of the donor at a very short distance scale [63]. Such a species would render quenched donor and no acceptor fluorescence, but is not observed in this assay.

Due to the characteristic FRET activity of the uncleaved substrate, cleavage by Trypsin leads to a decrease in the
concentration of the FRET-active species, $c_{\text{on}}$, and a subsequent rise in the concentration of the FRET-inactive species, $c_{\text{off}}$. According to Eq. (3b), the change of these concentrations with incubation time, $t$, is described by the exponential constant time, $t_0$.

$$c_{\text{on}}(t) = c_{\text{on}}^0 \exp(-t/t_0) + c_{\text{on}}^\infty$$

$$c_{\text{off}}(t) = c_{\text{tot}} - c_{\text{on}}(t) = (c_{\text{tot}} - c_{\text{on}}^\infty) - c_{\text{on}}^0 \exp(-t/t_0)$$

$c_{\text{tot}} = c_{\text{on}} + c_{\text{off}}$: total concentration of fluorescent / observable substrate (constant over incubation time, $t$); $c_{\text{on}}^\infty$: concentration of FRET-active species at infinite incubation time $t = \infty$; $c_{\text{on}}^0 + c_{\text{on}}^\infty$: concentration of FRET-active species at time $t = 0$, i.e., before incubation with Trypsin. $c_{\text{on}}^0$ and $c_{\text{on}}^\infty$ are given by the total substrate concentration, which is assumed to remain constant over time and thus equals the substrate concentration $c_i(0)$ at time zero, the potential uncleaved concentration $c_i(\infty)$ at infinite incubation time, and the fractions $f_{1\text{on}}$ and $f_{2\text{on}}$ of FRET-active uncleaved and FRET-active cleaved substrate, respectively; $c_{\text{on}}^0 = (f_{1\text{on}} - f_{2\text{on}})$ $c_i(0)$ and $c_{\text{on}}^\infty = (f_{1\text{on}} - f_{2\text{on}})$ $c_i(\infty) + f_{2\text{on}} c_i(0)$.

**Fluorescence Brightness**

In this experiment, each of the two species is characterized by the respective fluorescence emission of the donor or acceptor dye detected in the green or red detection channel. The detected fluorescence count-rate per single particle, denoted as molecular fluorescence brightness, $q$, can be approximated by the fluorescence detection efficiency, $\Psi$, the quantum yield of fluorescence, $\Phi$, and the excitation rate constant, $k_{\text{exc}} = \sigma_{\text{exc}} I_{\text{exc}} (I_{\text{exc}} = \text{excitation irradiance in W/cm}^2$ and $\sigma_{\text{exc}} (\lambda) = \text{absorption cross section at the excitation wavelength } \lambda \text{ in cm}^2/\text{J})$; $q = \Psi \Phi k_{\text{exc}}$ (neglecting saturation of the emission). A pair of brightness, $q^G$ and $q^R$, one for each detection channel, characterizes each species, $i = \text{on or off}$. The index “G” denotes the green and the index “R” the red detection channel. These brightness values can be expressed by the FRET efficiency, $E$ (Eq. (2)). Furthermore, emission and excitation cross-talks have to be taken into account. Emission cross-talk is due to the detection of RhGr fluorescence in the red acceptor detection channel and excitation cross-talk caused by direct excitation of the acceptor MR121 (compare Fig. (1)).

**FRET-active - red detection channel**

$$q^R = \Psi^R \Phi^R k_{\text{exc}R}$$

**FRET-active - green detection channel**

$$q^G = \Psi^G \Phi^G k_{\text{exc}G}$$

**FRET-inactive - red detection channel**

$$d^R_{\text{off}} = \Psi^R \Phi^R k_{\text{exc}R}$$

**FRET-inactive - green detection channel**

$$d^G_{\text{off}} = \Psi^G \Phi^G k_{\text{exc}G}$$

**FRET-inactive - red detection channel**

$$d^R_{\text{off}} = \Psi^R \Phi^R k_{\text{exc}R}$$

$$= K_{q^G}q^G$$

$$= K_{q^R}q^R$$

$\Psi^R$, $\Phi^R$, and $k_{\text{exc}R}$ denote the fluorescence detection efficiency of the donor (D) or acceptor (A) in the green (G) or red (R) detection channel, respectively; $\Phi^R_{D/A}$: fluorescence quantum yield of the donor alone (D), of the donor in the presence of FRET (DA) ($E = 1 - \Phi^R_{DA}/\Phi^R_{D}$), or of the acceptor (A); $k_{\text{exc}D/excA}$ is the ratio of fluorescence detection efficiencies throughout the two dyes, $C_{\text{exc}} \approx 1.5$ % and $C_{\text{exc}} \approx 1$ % (compare Fig. (1)). Besides being characteristic for each fluorescent species present in the sample, the brightness parameters intrinsically carry information of non-ideal experimental conditions such as cross-talk ($C_{\text{exc}}$) or different fluorescence detection efficiencies of donor and acceptor ($C_{\text{DA}}$). Taking the brightness values as an experimental measure thus enables to take these non-idealities into account and to subsequently correct for them.

**ANALYSIS METHODS**

**Fluorescence Intensity**

The detected fluorescence count-rate or intensity, $F$, scales with the fluorescence brightness, $q$, and the concentration, $c$; $F = c q$. The intensities detected in the green, $F_G$, and red, $F_R$, detection channel should therefore directly depend on the concentration of each fluorescent species present in this assay, $c_{\text{on}}$ and $c_{\text{off}}$. The fluorescence intensity is capable of monitoring the Trypsin activity, and the time course of the fluorescence intensity is described by an exponential growth or decay, respectively.

$$F_G = c_{\text{off}}q^G_{\text{off}} + c_{\text{on}}q^G_{\text{on}} = B + A \exp(-t/t_0)$$

with $A = -E c_{\text{on}}q^G_{\text{on}}$ and $B = (c_{\text{on}}q^G_{\text{on}}) q^G_{\text{off}}$.$\phi$

$$F_R = c_{\text{off}}q^R_{\text{off}} + c_{\text{on}}q^R_{\text{on}} = B + A \exp(-t/t_0)$$
with \( A = (K_2 - K_1) c_{on} q_{off}^G, \) \( B = (K_1 c_{on} + (K_2 - K_1) c_{on}) q_{off}^G. \)

In the case of no cross-talk, \( F_G \) remains unchanged, while \( F_R \) changes with \( A = C_{off} E c_{on} q_{off}^G \) and \( B = C_{off} E c_{on} q_{off}^G. \)

**Fluorescence Intensity Distribution Analysis (FIDA)**

**FIDA**

FIDA is based on the collection of photon count numbers, \( n \), recorded in time intervals of fixed duration and uses this information to assemble a count number histogram, \( P(n) \). A theoretical probability distribution of photon count numbers is fit to this histogram, yielding specific fluorescence brightness values, \( q_i \), and molecular concentrations, \( c_i \), for all different species, \( i \), in the sample. The overall detected signal, \( I_G \), is split up accordingly. Fluorescent compounds of a sample as well as the background signal, \( B_G \) and \( B_R \), can be resolved on the molecular level, as long as these components display different values for molecular fluorescence brightness, such as FRET-active and -inactive species.

\[
I_G = \sum q_i^G + B_G = c_{on} q_{on}^G + c_{off} q_{off}^G + B_G
\]

\[
I_R = \sum q_i^R + B_R = c_{on} q_{on}^R + c_{off} q_{off}^R + B_R
\]

In contrast, the direct fluorescence intensity readout, \( F \), averages over all compounds without separating into brightness and concentration (compare Eq. (6)). FIDA yields a more statistical reliability for biological assays, since compounds are differentiated and background signals, originating in cross-talk or autofluorescing compounds, are accounted for. A detailed description of FIDA is given elsewhere [28, 29, 64].

**2D-FIDA**

Two-Dimensional FIDA (2D-FIDA) is the expansion of FIDA towards a two-detector set-up (e.g., two-color detection as in this case, or a set-up for two different polarizations). It is based on fitting a joint distribution of photon count numbers, \( P(n_G, n_R) \), with \( n_G \) and \( n_R \) denoting the photon count numbers recorded per time interval of fixed duration on two detectors monitoring different colors as in this assay (G = green and R = red). A theoretical distribution is fit to this two-dimensional histogram, yielding molecular concentrations, \( c_i \), and a pair, \( q_i^G \) and \( q_i^R \), of specific fluorescence brightness values of the first (green) and second (red) detection channel for all different fluorescent species, \( i \), of the sample.

\[
\begin{pmatrix}
I_G \\
I_R
\end{pmatrix} = \sum c_i \begin{pmatrix}
q_i^G \\
q_i^R
\end{pmatrix} + \begin{pmatrix}
B_G \\
B_R
\end{pmatrix} = c_{on} \begin{pmatrix}
q_{on}^G \\
q_{on}^R
\end{pmatrix} + c_{off} \begin{pmatrix}
q_{off}^G \\
q_{off}^R
\end{pmatrix} + \begin{pmatrix}
B_G \\
B_R
\end{pmatrix}
\]

Compared to usual FIDA, the two-dimensionality introduces additional accuracy, because different species are characterized and distinguished by an increasing number of fluorescence parameters, i.e., a pair of brightness values. A detailed description of 2D-FIDA is given elsewhere [32, 64].

**Fluorescence Correlation Spectroscopy (FCS)**

**Unnormalized FCS**

In contrast to FIDA, FCS analyzes the temporal behavior of the fluctuating signal intensities by calculating the autocorrelation functions, \( G^G_{FCS}(t_c) \) or \( G^R_{FCS}(t_c) \), separately for each detection channel or by calculating the cross-correlation function, \( G^G_{FCCS}(t_c) \), between both channels (Fluorescence Cross-Correlation Spectroscopy, FCCS).

\[
G^G_{FCS}(t_c) = \langle \delta I_{GGR}(t) \delta I_{GGR}(t + t_c) \rangle
= \langle I_{GGR}(t) I_{GGR}(t + t_c) \rangle - \langle I_{GGR}(t) \rangle \langle I_{GGR}(t) \rangle
= \langle I_{G}(t) I_{R}(t + t_c) \rangle - \langle I_{G}(t) \rangle \langle I_{R}(t) \rangle
\]

\[ t_c \text{: correlation time; } I_{GGR}(t_c) \text{: signal intensity in the green (G) or red (R) detection channel monitored at measurement time, } t_c \text{; } \langle \cdot \rangle \text{: averaging over measurement time, } t_c ; \delta I_{GGR}(t) = I_{GGR}(t) - \langle I_{GGR}(t) \rangle ; \text{fluctuation of the signal at measurement time, } t_c. \]

The correlation functions decay with time constants characteristic of the diffusion times and triplet populations of the different fluorescing molecules, \( i \), passing the confocal volume. The amplitudes are a measure of the respective concentrations, \( c_i \), and fluorescence contributions. These contributions are given by the specific brightness of fluorescence, \( q_i^G \) and \( q_i^R \), in the green or red detection channel, respectively [31, 58, 65].

\[
G^G_{FCS}(t_c) = c_{on} q_{on}^G D_{on}^G(t_c) + c_{off} q_{off}^G D_{off}^G(t_c)
\]

\[
G^R_{FCS}(t_c) = c_{on} q_{on}^R D_{on}^R(t_c) + c_{off} q_{off}^R D_{off}^R(t_c)
\]

\[
G^G_{FCCS}(t_c) = c_{on} q_{on}^G q_{on}^R D_{on}^G D_{on}^R(t_c) + c_{off} q_{off}^G q_{off}^R D_{off}^G D_{off}^R(t_c)
\]

\[
D^X(t_c) = \frac{1}{1 + t_c/\rho_i^X} \left[ \frac{1}{1 + AR_X^2 t_c / \rho_i^X} + \frac{T_i^X}{1 - T_i^X} \exp(-t_c / \mu_i^X) \right]
\]

\( \rho_i^X \text{: average residence time of a molecule in the detection volume, characterized by the molecule’s diffusion properties, } T_i^X \text{: probability of triplet population, } \mu_i^X \text{: triplet correlation time of species, } i \text{ (on or off), and } AR_X \text{: axis ratio of lateral and axial focal expansion as monitored by the green auto-correlation (} X = G \text{), the red auto-correlation (} X = R \text{), and by the cross-correlation (} X = FCCS \text{) function.} \)

In contrast to equations given in some previous publications [31, 65], the triplet term is added to the diffusion term, since the population of the triplet occurs on a much faster time scale (\( \mu_i^X = 1 \) - 2 \( \mu s \)) than the average diffusion time (\( \rho_i^X > 150 \mu s \)), as has also been reported in previous publications.

**Normalized FCS**

Normalization yields more familiar expressions of the auto- and cross-correlation functions [66, 67].
\[ G_{\text{FCS}}^{G}(t) = \frac{\langle I_{G}(t') I_{G}(t'+t_c) \rangle}{\langle I_{G}(t') \rangle^2} = 1 - \frac{\langle \delta I_{G}(t') \delta I_{G}(t'+t_c) \rangle}{\langle I_{G}(t') \rangle^2} \]

\[ G_{\text{FCS}}^{R}(t) = \frac{\langle I_{R}(t') I_{R}(t'+t_c) \rangle}{\langle I_{R}(t') \rangle^2} = 1 - \frac{\langle \delta I_{R}(t') \delta I_{R}(t'+t_c) \rangle}{\langle I_{R}(t') \rangle^2} \]

The decay as well as the amplitude of the normalized correlation functions, \( G_{\text{FCS}}^{G}(t_c) \), \( G_{\text{FCS}}^{G}(t_c) \), and \( G_{\text{FCS}}^{R}(t_c) \), regarding diffusion as well as triplet kinetics of the FRET-active and -inactive species, are expressed in a similar manner as in Eq. (10) [31, 65].

\[ G_{\text{FCS}}^{G}(t_c) = \frac{c_{\text{on}} q_{\text{on}}^G}{F_{G}^2} D_{\text{on}}^{G}(t_c) + \frac{c_{\text{off}} q_{\text{off}}^G}{F_{G}^2} D_{\text{off}}^{G}(t_c) \]  
\[ G_{\text{FCS}}^{R}(t_c) = \frac{c_{\text{on}} q_{\text{on}}^R}{F_{R}^2} D_{\text{on}}^{R}(t_c) + \frac{c_{\text{off}} q_{\text{off}}^R}{F_{R}^2} D_{\text{off}}^{R}(t_c) \]

The normalization is thus performed by the total fluorescence count rates, \( F_{G} \) and \( F_{R} \) (compare Eq. (6)), and results in the well-known relationship, \( G_{\text{FCS}}(t_c) = 1/c D(t_c) \), for a single species.

**Two-Color Global FCS Analysis (2CG-FCS)**

The analysis of the normalized or unnormalized FCS or FCCS data alone does not allow for a direct determination of the concentrations, \( c_{\text{on}} \) and \( c_{\text{off}} \), but only of the respective amplitudes, \( q_{\text{on}}^2 \) and \( q_{\text{off}}^2 \). To directly extract the concentration as well as the brightness values for each species, the information from all three correlation functions must be used in a way recently introduced by Two-Color Global FCS Analysis (2CG-FCS) [58]. In 2CG-FCS, the information present in all three simultaneously recorded correlation data (two auto- and one cross-correlation curve) is combined by a global analysis. All data, \( G_{\text{FCS}}^{G}(t_c) \), \( G_{\text{FCS}}^{R}(t_c) \), and \( G_{\text{FCCS}}(t_c) \), are fitted simultaneously using Eq. (10) or (12), respectively, applying the two concentrations, \( c_{\text{on/off}} \), and the four brightness parameters, \( d_{\text{on/off}} \), as global variables. As outlined before, this global analysis tool offers a significant increase in accuracy of FCS and FCCS analysis in FRET applications [58].

**Saturation and Bleaching Effects**

For an analysis method based on fluorescence photon statistics, the influence of saturation and photobleaching has to be considered. The excitation and thus the fluorescence emission are saturated towards high excitation irradiance, especially in the case of an extensive triplet population. In a confocal microscope, this effect leads to a distortion of the excitation profile. While triplet population is directly accounted for in the FCS theory (compare Eq. (10) or (12)), extensive saturation leads to a systematic change in the correlation curve, yielding an overestimated diffusion time and concentration value [66, 68-70]. In FIDA, extensive triplet population or saturation will also lead to a wrong estimate of brightness and concentration values [64, 71]. Photobleaching accounts for the enhanced reactivity of the excited fluorophore, thereby irreversibly losing its ability to fluoresce. Occurring from the excited state, the probability to do so increases with a rise in the excitation irradiance. In addition to a decrease in focal concentration, photobleaching renders underestimated diffusion (contrary to the saturation effect) and decreased brightness values [66, 72, 73]. However, a decrease of the overall fluorophore concentration in the sample is not present, since photobleaching is confined to the focal volume, which is much smaller (below 10^{-12} liter) than the sample volume (about 10^{-3} liter). In this experiment, an excessive influence of saturation or photobleaching is circumvented by selecting a rather low excitation irradiance (90 kW/cm^2, see Ref. [73]). Furthermore, the deviation due to these effects will be consistent for all instances of FCS- or FIDA-based analysis and all measurements, since the excitation irradiance, which is the causing factor, is kept constant. Thus, it should extend no influence on the present FRET assay.

**RESULTS**

**1. Enzymatic Reactivity – HPLC Data**

HPLC is used to give an independent estimate of the enzymatic reactivity of Trypsin and a control of the results rendered by the FRET assay. HPLC analysis is performed directly in the incubation buffer and does not demand any aliquot or dilution steps into the analysis buffer as needed for FFS. With HPLC, the amount of uncleaved and cleaved substrate in the same incubation buffer can be directly determined over incubation time, \( t \) (Fig. (2)). According to Eq. (3b), the corresponding time courses can be described by an exponential decay or growth with the time constant, \( t_0 = 6.4 \pm 0.5 \) min. Furthermore, no uncleaved substrate is present at infinite incubation time, \( t = \infty \) \( (\delta I_1(\infty) = 0) \). The Trypsin activity seems to be complete.

**2. Enzymatic Reactivity – FRET Assay**

For FRET analysis, samples differing in Trypsin incubation times from 0 to 60 minutes are prepared in the incubation buffer. The cleavage reaction is stopped and aliquots are withdrawn and 1000-fold diluted into the analysis buffer, yielding different fractions of cleaved peptide for simultaneous fluorescence intensity, FIDA, 2D-FIDA, FCS, FCCS, and 2CG-FCS analysis. Each of the samples is measured 10 times rendering the statistical precision of each method. In contrast, other causes for experimental artifacts such as sample preparation errors are outlined in sample-to-sample variations and render the accuracy of each method to observe the Trypsin activity despite of these artifacts. A background signal of 1 kHz and 0.5 kHz in the green and red detection channel, respectively, arises due to solvent scattering. The background signal is intrinsically accounted for FIDA and 2D-FIDA (compare Eqs. (7) and (8)), subtracted from the total signal intensity for fluorescence intensity analysis, and
neglected in the case of any FCS-based analysis due to the high signal-to-background ratio present (20:1 at minimum).

**Fluorescence Intensity**

The fluorescence intensity is the most direct and straightforward of all fluorescence parameters and can be monitored in the two detection channels (averaged over the measurement time). Fig. 3A shows the time-dependent detected fluorescence intensity in the green, $F_G(t)$ (black squares), and red detection channel, $F_R(t)$ (open circles). The decrease of the red (MR121) fluorescence and the increase of the green (RhGr) fluorescence clearly indicate the trypsin cleavage activity, i.e., a significant drop in FRET-active substrate and concurrent rise in FRET-inactive substrate. A fit of Eq. (6) to the data results in time constants of $t_0 = 11 \pm 5$ min ($F_G$) and $7.2 \pm 0.2$ min ($F_R$).

**FIDA**

An obvious advantage of FIDA is its molecular resolution. FIDA enables to distinguish and quantify different fluorescent species of the sample on the basis of their molecular fluorescence brightness. For FIDA, this operation is performed for one of the detection channels, respectively, thus using a single molecular fluorescence brightness parameter. The background signal caused by solvent scattering or impurities is included by an additional component (compare Eq. (7)). Cross-talk is expressed within the brightness values (Eq. (5)).

**Green Detection Channel**

A close examination of the fluorescence data in the green detection channel from control experiments of samples with solely uncleaved ($t = 0$ min) and with maximum cleaved substrate ($t = 60$ min) by FIDA reveals only one single fluorescent species with a brightness of $q_{0G}^G = 57 \pm 1$ kHz. The respective concentration values, $c_{off}$, determined from a one-species fit with the brightness fixed to the above value are plotted in Fig. 3B. An exponential fit to the data results in a cleavage time of $t_0 = 11 \pm 5$ min (Eq. (4)). The rise in concentration with Trypsin incubation time as well as the high brightness value reveals that only the FRET-inactive substrate is resolved by FIDA in the green detection channel.

**Red Detection Channel**

FIDA applied to the fluorescence data in the red detection channel bears two species with two different brightness values, FRET-active substrate ($q_{on}^R = 35 \pm 1$ kHz) and FRET-inactive substrate ($q_{off}^R = 1.6 \pm 0.4$ kHz). The respective concentration values of a two-species fit with the brightness fixed to these values yield the time course of the FRET-active and FRET-inactive concentration, $c_{on}$ and $c_{off}$ as plotted in Fig. 3C. A fit according to Eq. (4) results in the time constants of $t_0 = 7.8 \pm 0.2$ min and 13 $\pm$ 2 min, respectively.

**2D-FIDA**

In 2D-FIDA, all fluorescent species of a sample can be characterized and distinguished by two parameters, i.e., brightness in the green and red detection channel. 2D-FIDA has proven to be a valuable tool for two-color fluorescence experiment [32, 64]. A major advantage arises from the fact that experimental artifacts can be corrected for. For example, any background signal due to solvent scattering or impurities is accounted for by an additional component (compare Eq. (8)). Cross-talk is expressed within the brightness values (Eq. (5)). Therefore, 2D-FIDA holds as an excellent analysis tool for the present FRET assay.

From control measurements on samples with pure uncleaved ($t = 0$ min) and with maximum cleaved substrate ($t = 60$ min), two pairs of brightness values are determined for the species present, (i) $q_{on}^G = 1.3 \pm 0.2$ kHz and $q_{off}^R = 36.3$
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Fig. (3). Trypsin activity monitored by the different fluorescence methods plotted against incubation time, $t$, and exponential fits ($B + A \exp(-t/t_0)$) (gray line) to the data. (A) Fluorescence intensity in the green, $F_G$ (black squares), and red detection channel, $F_R$ (open dots); $B = 148 \pm 4$, $A = -28 \pm 7$, $t_0 = 11 \pm 5$ min ($F_G$) and $B = 11.1 \pm 0.3$, $A = 47.8 \pm 0.6$, $t_0 = 7.2 \pm 0.2$ min ($F_R$). (B) and (C) FIDA in the green and red detection channel: concentration of FRET-inactive, $c_{\text{off}}$ (black squares), and FRET-active substrate, $c_{\text{on}}$ (open dots); $B = 2.6 \pm 0.1$, $A = -0.5 \pm 0.1$, $t_0 = 11 \pm 5$ min ($c_{\text{off}}$, FIDA - green), $B = 3.5 \pm 0.2$, $A = -3.5 \pm 0.2$, $t_0 = 13 \pm 2$ min ($c_{\text{off}}$, FIDA - red), and $B = 0.15 \pm 0.01$, $A = 1.52 \pm 0.02$, $t_0 = 7.8 \pm 0.2$ min ($c_{\text{on}}$, FIDA - red). (D) 2D-FIDA: $c_{\text{off}}$ (black squares) and $c_{\text{on}}$ (open dots) and total substrate concentration, $c_{\text{tot}} = c_{\text{off}} + c_{\text{on}}$ (cross); $B = 2.6 \pm 0.1$, $A = -0.5 \pm 0.1$, $t_0 = 11 \pm 5$ min ($c_{\text{off}}$ and $c_{\text{on}}$) and $B = 0.22 \pm 0.01$, $A = 1.3 \pm 0.02$, $t_0 = 7.2 \pm 0.2$ min ($c_{\text{on}}$). (E) and (F) Unnormalized and normalized FCS: amplitude, $G_{\text{FCS}}(0)$, and inverse amplitude, $G_{\text{FCS}}^{-1}(0)$, observed in the green (black squares) and red detection channel (open dots); $B = 8100 \pm 220$, $A = -1100 \pm 470$, $t_0 = 8 \pm 7$ min ($G_{\text{FCS}}^G(0)$), $B = 190 \pm 10$, $A = 1850 \pm 20$, $t_0 = 7.5 \pm 0.2$ min ($G_{\text{FCS}}^R(0)$). $B = 2.7 \pm 0.1$, $A = -0.7 \pm 0.1$, $t_0 = 15 \pm 6$ min ($G_{\text{FCS}}^{-1}(0)$), and $B = 0.64 \pm 0.01$, $A = 1.05 \pm 0.03$, $t_0 = 4.9 \pm 0.3$ min ($G_{\text{FCS}}^{-1}(0)$). (G) and (H) Unnormalized and normalized FCCS: amplitudes, $G_{\text{FCCS}}(0)$ and $G_{\text{FCCS}}^{-1}(0)$; $B = 0.105 \pm 0.006$, $A = -0.078 \pm 0.007$, $t_0 = 21 \pm 4$ min ($G_{\text{FCCS}}^{-1}(0)$). An exponential fit to the unnormalized data ($G_{\text{FCCS}}(0)$) does not give any reliable results. (I) 2CG-FCS of unnormalized correlation data: $c_{\text{off}}$ (black squares) and $c_{\text{on}}$ (open dots); $B = 2.3 \pm 0.1$, $A = -0.3 \pm 0.1$, $t_0 = 8.4 \pm 6.7$ min ($c_{\text{off}}$) and $B = 0.14 \pm 0.01$, $A = 1.39 \pm 0.02$, $t_0 = 7.5 \pm 0.2$ min ($c_{\text{on}}$).
± 0.4 kHz, and (ii) $q_{	ext{off}}^G = 58 ± 1$ kHz and $q_{	ext{off}}^R = 1.2 ± 0.1$ kHz. FRET is observed by a decrease in green and a concurrent increase in red brightness. A two-component analysis of the 2D-FIDA data with the brightness values fixed to the values given yields a time course of the corresponding concentrations, $c_{\text{on}}$ and $c_{\text{off}}$, as shown in Fig. (3D). A fit to the data using Eq. (4) yields values of $t_0 = 11 ± 5$ min ($c_{\text{off}}$) and $7.2 ± 0.2$ min ($c_{\text{on}}$).

**FCS**

FCS enables to distinguish different species of a sample on basis of their diffusion time through the confocal detection volume, i.e., their masses. As outlined in Eqs. (10) and (12), the respective analysis of normalized or unnormalized auto-correlation data of the green or red detection channel further considers triplet population as well as concentration and fluorescence brightness of each species. FCS is therefore in principle a useful measure for biological assays resulting in a change in mass, such as binding events or cleavage reactions as studied in this case.

At first normalized and unnormalized auto-correlation data recorded in the green and red detection channel of samples with pure uncleaved ($t = 0$ min) and with maximum cleaved substrate ($t = 60$ min) are fitted using Eqs. (10) and (12). The diffusion times, $\bar{\rho}_{\text{on}}^G = \bar{\rho}_{\text{off}}^G = \bar{\rho}_{\text{on}}^R = \bar{\rho}_{\text{off}}^R = 590 ± 40$ µs, and triplet parameters, $T_{\text{on}}^G = T_{\text{off}}^G = 0.16 ± 0.01$, $T_{\text{on}}^R = T_{\text{off}}^R = 0.21 ± 0.06$, $\mu_{\text{on}}^G = \mu_{\text{off}}^G = 1.6 ± 0.3$ µs, and $\mu_{\text{on}}^R = \mu_{\text{off}}^R = 1.7 ± 0.8$ µs, are, however, indistinguishable for FRET-active and -inactive substrate, regardless of a choice of green or red detection channel, or normalized and unnormalized FCS data. The only parameter left that changes over incubation time is the amplitude of the auto-correlation curves, $C_{\text{FCS}}^G(0)$ and $C_{\text{FCS}}^R(0)$, or $\bar{C}_{\text{FCS}}^G(0)$ and $\bar{C}_{\text{FCS}}^R(0)$, as plotted in Fig. (3E) and (3F) (note that for reasons outlined later the reciprocal amplitudes are plotted for the normalized data). These values are obtained by fitting Eqs. (10) and (12) to the respective FCS data with the diffusion times, $\bar{\rho}_{\text{on}}^G$, and triplet parameters, $T_{\text{on}}^G$ and $\mu_{\text{on}}^G$, fixed to the above values, and the axis ratio fixed to $AR_X = 3.5$, as determined from FCS measurements on pure dye. Using the identity of the diffusion and triplet terms, $D_X(t_i)$, and the expressions of the brightness values of Eq. (5), the amplitudes can be expressed on basis of the characteristic time constant, $t_0$, of the enzymatic reaction, the concentration of total and FRET-active substrate, $c_{\text{tot}}$, $c_{\text{on}}^G$, and $c_{\text{on}}^R$, and experimental (cross-talk) factors, $K_1$, $K_2$, and $K = K_1/K_2$.

**Unnormalized Amplitudes**

\[
C_{\text{FCS}}^X(0) = A_X \exp(-t / t_0) + B_X
\]  
with \[
A_X = -(2E - E^2) c_{\text{on}}^G / q_{\text{off}}^G \]

\[
B_X = c_{\text{on}}^G (2E - E^2) c_{\text{on}}^R / q_{\text{off}}^G
\]

\[
A_R = (K_1^2 - K_2^2) c_{\text{on}}^R q_{\text{off}}^G \]

\[
A_R = [K_1^2 c_{\text{on}} (K_2^2 - K_1^2) c_{\text{on}}^R] / q_{\text{off}}^G
\]

An exponential fit to this equation to the unnormalized FCS amplitudes results in $t_0 = 8 ± 7$ min for the data of the green ($G_{\text{FCS}}^G(0)$) and $t_0 = 7.5 ± 0.2$ min for the data of the red detection channel ($C_{\text{FCS}}^R(0)$).

**Normalized Amplitudes**

\[
\bar{G}_X^G(0) = \frac{1}{c_{\text{tot}}} \left[ \bar{A}_X \exp(-t / t_0) + \bar{B}_X \right]^2
\]

with \[
\bar{A}_X = -(2E - E^2) c_{\text{on}}^G / c_{\text{tot}}
\]

\[
\bar{B}_X = 1 - 2E c_{\text{on}}^G / c_{\text{tot}}
\]

\[
\bar{A}_X = (1 - K_2^2) c_{\text{on}}^R / c_{\text{tot}}
\]

\[
\bar{B}_X = K_1(1 - K_2^2) c_{\text{on}}^R / c_{\text{tot}}
\]

\[
\bar{A}_X = 1 - E c_{\text{on}}^G / c_{\text{tot}}
\]

The theoretical description of the normalized data recorded in the green detection channel ($\bar{G}_{\text{FCS}}^G(0)$) can be simplified in case of a complete energy transfer, $E = 1$ (as in this experiment).

\[
\bar{G}_{\text{FCS}}^G(0)^{-1} = c_{\text{off}}^G(t) = c_{\text{on}}^G(0) - c_{\text{on}}^G(0) \exp(-t / t_0)
\]

Thus the reciprocal value of the correlation amplitude directly expresses the concentration of FRET-inactive substrate. The fit of this simplified equation to the data leads to a cleavage time of $t_0 = 15 ± 6$ min.

Due to the high number of unknown parameters, the normalized FCS amplitude, $\bar{G}_{\text{FCS}}^G(0)$, cannot reliably be described using Eq. (14a). A simplification can be introduced by neglecting cross-talk ($K = 0$) and assuming approximately the same fluorescence quantum yield and detection efficiency of the donor and the acceptor dye in the respective detection channel ($c_{\text{on}}^R = 1$. With $E = 1$, the inverse amplitude then directly follows the concentration of FRET-active species.

\[
\bar{G}_{\text{FCS}}^R(0)^{-1} = c_{\text{on}}^R(t) = c_{\text{on}}^R(0) \exp(-t / t_0) + c_{\text{on}}^R
\]

The resulting fit renders a time constant of $t_0 = 4.9 ± 0.3$ min.

**FCCS**

In contrast to the FCS auto-correlation analysis, the cross-correlation data intrinsically contains information from both channels. The most important feature of FCCS is its capability to directly detect coinciding fluorescence signal from both detection channels, which makes it an ideal tool for two-color assays including FRET.
As for FCS, the analysis of the cross-correlation data of samples with pure uncleaved \((t = 0\) min) and with maximum cleaved substrate \((t = 60\) min) reveals indistinguishable diffusion times of FRET-active and -inactive substrate, \(\rho_{\text{FC}} = \rho_{\text{off}} = 610 \pm 120\, \mu s\). A triplet term is not present since the triplet population kinetics of RHGR (green detection channel) and MR121 (red detection channel) are hardly correlated. Nevertheless, the FCCS amplitude contains enough information about coinciding green and red fluorescence, in principle only present in the case of FRET activity. FCCS theory predicts an increase of the amplitude of the normalized cross-correlation curve with increased coinciding signal [31]. Figs. (3G) and (3H) show the course of the normalized and unnormalized FCCS amplitude with incubation time. The data are obtained from a fit of Eqs. (10c) and (12c) to the FCCS data with decay parameters fixed to values known from control measurements \(\rho_{\text{FC}} = 600\, \mu s, \tau_{\text{FC}} = 0\, \mu s\). Due to the indistinguishable diffusion parameters, the theoretical expressions of the FCCS amplitudes follow the approach of the respective FCCS amplitudes.

**Unnormalized Amplitudes**

\[
G_{\text{FC}}(0) = A \exp(-t/\tau_0) + B
\]  
with \(A = ((1-E)K_2-K_1)c_{\text{on}}^2 q_{\text{off}}^2\) and \(B = (K_1c_{\text{off}} + ((1-E)K_2-K_1))c_{\text{on}}^2 q_{\text{off}}^2\).

The course of the unnormalized FCCS amplitude with incubation time (Fig. (3G)) cannot reliably be described by this exponential function.

**Normalized Amplitudes**

In most examples of two-color FCCS analysis, the normalized amplitude is directly used as a measure for coinciding signal, i.e., the concentration of two-color labeled species. The normalized FCCS amplitude can in this case be described by the Eq. (16).

\[
\tilde{G}_{\text{FC}}(0) = \frac{1}{c_{\text{int}}} \frac{A \exp(-t/\tau_0) + B}{C \left[\exp(-t/\tau_0)\right]^2 + D \exp(-t/\tau_0) + E}
\]  
with \(A = (1-E)K_2c_{\text{on}}^2 / c_{\text{tot}}\), \(B = (1-E)K_2c_{\text{on}}^2 / c_{\text{tot}} + K_1\), \(C = (KE-E)c_{\text{on}}^2 / c_{\text{tot}}^2\), \(D = (1-K-E)c_{\text{on}}^2 / c_{\text{tot}}\), \(E = Kc_{\text{on}}^2 / c_{\text{tot}}^2(1-K-E)c_{\text{on}}^2 / c_{\text{tot}}\).

This expression is however too complex to reliably fit the observed normalized FCCS amplitudes (Fig. (3H)). A simple exponential fit to the data renders a value of \(\tau_0 = 21 \pm 4\) min.

**Two-Color Global Fluorescence Correlation Spectroscopy (2CG-FCS)**

2CG-FCS combines all the features of both auto- as well as the cross-correlation curves. The combined analysis of all three curves should enhance the accuracy of two-color correlation analysis, as has recently been introduced [58].

In 2CG-FCS, all parameters that are not subject to global fitting are determined from single auto- and cross-correlation data. This concerns the diffusion times, triplet parameters, and axis ratios. Their values are discussed in the respective FCCS and FC chapters. As outlined above, the diffusion times as well as the triplet parameters are indistinguishable for FRET-active and -inactive substrate. 2CG-FCS is applied to the control measurements of samples with pure uncleaved \((t = 0\) min) and with maximum cleaved substrate \((t = 60\) min) with the diffusion times, triplet parameters, and axis ratios fixed to the values determined from the separate auto- and cross-correlation curves, \(\rho_{\text{on/off}}^X = 600\, \mu s, \tau_{\text{on/off}}^X = 0.16, \tau_{\text{on/off}}^R = 0.2\, \mu s, \rho_{\text{on/off}}^G = 0\, \mu s, \rho_{\text{on/off}} = 1.7\, \mu s, \rho_{\text{on/off}}^R = 1.7\, \mu s, \rho_{\text{on/off}}^G = 1.7\, \mu s, \rho_{\text{on/off}} = 3.5\). As for 2D-FIDA, a pair of brightness values is determined for each species. Applying 2CG-FCS to the normalized correlation data yields \(q_{\text{on}}^G = 1.5 \pm 0.2\, \text{kHz}, q_{\text{off}}^R = 36.3 \pm 0.2\, \text{kHz}, q_{\text{off}}^G = 58.7 \pm 0.4\, \text{kHz}, \) and \(\rho_{\text{on/off}}^R = 1.3 \pm 0.1\, \text{kHz}\). These values agree very well with those obtained by 2D-FIDA. However, the brightness values cannot be established using the normalized data. For further fitting, all brightness parameters are fixed to the above values.

A two-component global analysis of all unnormalized correlation data reveals the time course of the concentrations, \(c_{\text{on}}\) and \(c_{\text{off}}\), as shown in Fig. (3I). A fit of Eq. (4) to the data results in the time constants of \(\tau_0 = 7.5 \pm 0.2\, \text{min}\) \((c_{\text{on}})\) and \(\tau_0 = 8.4 \pm 6.7\, \text{min}\) \((c_{\text{off}})\). 2CG-FCS applied to the normalized correlation data is not capable of the determination of \(c_{\text{on}}\) and \(c_{\text{off}}\), since the according brightness parameters cannot be established.

**Summary and Conclusion**

**Comparison to HPLC data – Problems Inherent to Fluorescence Analysis**

Fig. (4A) depicts the time constants, \(\tau_0\), derived from the different analysis methods. Compared to HPLC data, especially the results obtained for the green detection channel and for the concentration values \(c_{\text{off}}\) (FRET-inactive species, which is predominant for the green detection channel), show strong discrepancies as well as high standard deviations. These high standard deviations result from an inaccurate fit to the data. Possible reasons for these discrepancies are outlined in Figs. (4B) and (4C). Fig. (4B) depicts the statistical precision of each method, which is the standard deviation of each single measurement, whereas Fig. (4C) depicts the accuracy of each method. The accuracy denotes the standard deviation from one measurement to another, i.e., it demonstrates how the data analysis is biased by sample-to-sample variations. Variations from sample to sample exceed the standard deviation of the single measurements by a factor of approximately two. These variations are due to variation in the total fluorescent substrate concentration, \(c_{\text{int}}\) (Fig. (3D), exemplarly determined for 2D-FIDA), which in principle should remain constant. This makes it reasonable to assume that these variations are due to sample handling errors such
as misdispensing of the aliquots rather than imprecise data analysis. This assumption is further supported by the fact that these variations are not present when directly analyzing the Trypsin activity in the incubation buffer as done for HPLC. Such experimental artifacts lead to imprecise and biased values of $t_0$ and a demand for analysis methods that are independent of $c_{in}$. For the present fluorescence analysis methods the bias introduced by the sample-to-sample variations can be accounted for by calculating the ratio of observed values from both detection channels or of the concentration values, $c_{off}(c_{in}+c_{off})$, as will be outlined in the next chapter.

Several other issues become obvious, when comparing the HPLC data with the respective data obtained by fluorescence analysis.

- The amount of cleaved substrate as detected by HPLC is below 10% at time point $t = 0$ (Fig. (2B)). In the context of the FRET assay, one would thus expect almost 100% FRET-active substrate and hardly any FRET-inactive species at this time point ($c_{off}(t = 0) \approx 0$). However, $c_{off}(t = 0)$ already amounts to $\approx 2$ (compare e.g. Fig. (3D)), i.e., about 65% of all original fluorescent uncleaved substrates are FRET-inactive. In a previous study of the same assay type, this finding has been attributed to an inactive or missing acceptor label [58].

- A residual FRET-active substrate concentration of $c_{on} \approx 0.2$ is observed at infinite incubation time (compare e.g. Fig. (3D)). This is contrary to the observation resulting from HPLC, which detects no residual uncleaved substrate (Fig. (2A)). This may be explained by substrate-enriched detergent micelles formed by the relatively high concentration of SDS (0.5%) in the analysis buffer of the aliquot. The relative high SDS concentration is necessary to generate a sufficient FRET signal. Cleaved peptide residing in close vicinity within the same detergent micelle might still exhibit a significant degree of FRET activity. This becomes obvious by the finding of a significantly enlarged diffusion time of the substrate in the presence of SDS ($\rho_{on/off} = 610 \mu s$), compared to the absence of SDS ($\rho_{on/off} = 280 \mu s$) as measured by FCS.

### Comparison of the Different Fluorescence Methods

Fig. (4). Results obtained for the different analysis methods; (A) time constant, $t_0$, resulting from an exponential fit to the data (compare Fig. (3)) (the gray line depicts the value resulting from HPLC data), (B) statistical precision given by the relative standard deviation (s. dev.) of a single measurement, and (C) accuracy given by the sample-to-sample deviations (s. dev.). The accuracy is determined by the average relative deviation of the measurement points from the exponential fit (compare Fig. (3)). The synonyms given are: “green” and “red”, green and red detection channel, respectively; “off” and “on”, concentration of FRET-inactive and -active substrates, $c_{off}$ and $c_{on}$, respectively; “un.” and “norm.”; unnormalized and normalized correlation data.

Other issues become prominent, when comparing the sets of fluorescence data to each other.

- The statistical precision as well as the accuracy is slightly better for the FIDA-based methods than for the FCS-based methods. This is not surprising, insasmuch as the different species of the present assay are distinguished by their respective brightness. Brightness analysis is, however, based on fluorescence amplitude analysis, which is favored for FIDA-based methods [58]. An assay featuring a change in diffusion time would display the capabilities of correlation data-based analysis.

- The brightness values determined by the different methods, FIDA, 2D-FIDA and 2CG-FCS, agree with each other very well. This consistence shows that such an analysis tool as 2CG-FCS, which is solely based on correlation analysis, can also be employed for amplitude analysis. This finding offers potentials for a combined global analysis of both correlation and intensity distribution data in order to enhance precision and accuracy of FFS analysis.

- Using the determined brightness values, $q_{on}^G$ and $q_{off}^G$, of the donor on FRET-active and -inactive substrate, a FRET-efficiency of $E = (1-q_{on}^G / q_{off}^G) = 0.99 \pm 0.001$ is determined. Thus, the donor fluorescence is completely quenched in the case of FRET activity. The brightness parameters, $q_{on}^G$, $q_{off}^G$, $q_{off}^R$, and $q_{on}^R$, together with Eq. (5)
yield a result of $K_1 = 0.021 \pm 0.002$ and $K_2 = 0.63 \pm 0.01$ (thus $K = K_1 / K_2 = 0.033 \pm 0.003$). Together with the amount of excitation cross-talk, $C_{exc} = k_{excA}/k_{excD} = \sigma_{excA} (488 \text{ nm}) / \sigma_{excD} (488 \text{ nm}) = 0.015 \pm 0.002$, determined from the absorption spectra of Fig. (1), these values give a ratio of fluorescence detection efficiencies, $C_{AD} = 0.62 \pm 0.09$, and an emission cross-talk, $C_{p} = 0.012 \pm 0.003$ (compare Eq. (5)). The value of $C_p$ calculated in this way agrees very well with that estimated from the fluorescence emission spectra of Fig. (1), $C_p \approx 0.01$. As expected from the constituted donor-acceptor dye pair, the amount of cross-talk is rather low. Nevertheless, even these low values can introduce some bias into the results, especially on the correlation data, as will be outlined below. Furthermore, the biasing influence of different values of $C_{AD}$ and $E$ will be discussed.

- FIDA applied to only one channel exhibits several drawbacks. Only the FRET-inactive species is observed using FIDA in the green detection channel. The FRET-active substrate is not detectable due to the complete quench of the green donor fluorescence (i.e., a FRET efficiency of $E \approx 1$). A normalization using the ratio $c_{off}(c_{on} + c_{off})$ is thus not applicable in this case and the variations of the substrate concentration from sample to sample cannot be corrected. When comparing the concentration values obtained for FIDA in the red detection channel (Fig. (3C)) to the corresponding data from FIDA in the green channel (Fig. (3B)), 2D-FIDA (Fig. (3D)), or 2CG-FCS (Fig. (3I)), one observes a bias in the concentration values of the FRET-inactive species, $c_{off}$. The corresponding brightness, $q_{off}^k = 1.6 \text{ kHz}$, in the red detection channel is a very low value. The precision and accuracy of the concentration parameter established by FIDA is highly dependent on the according brightness, i.e., the amount of photons detected for the species, and very insensitive when only slightly above the background as in the present experiment [74]. Thus the erroneous determination of $c_{off}$ solely applying FIDA in the red detection channel can most probably be attributed to the extremely low corresponding brightness value. Other reasons such as a high degree of triplet population, fluorescence saturation, or photobleaching can be excluded, because the FRET activity as well as the very low brightness value excludes a high excitation efficiency of the acceptor and thus a high triplet population, saturation level, or photobleaching yield. Furthermore, these effects should bias the determination of $c_{off}$ by the other methods 2D-FIDA or 2CG-FCS in the same way.

- The resulting values of $t_0$ obtained by the FCS-based methods are much more biased for the case of normalized data. It seems that the analysis of unnormalized correlation data in conjunction with FRET is more reliable. As an example, the theoretical description of the normalized amplitudes in the case of auto-correlation analysis is much more complex (compare Eqs. (13) and (14a)). A reliable fit to the observed normalized auto-correlation amplitudes even demands approximations (see Fig. (3F)).

In the case of 2CG-FCS, the brightness and concentration values can only be determined for the unnormalized correlation data. The appearance of the global brightness parameters as well as of the determinable concentration values in the denominator of the theoretical expressions used for fitting appear to influence the accuracy of the global analysis of the normalized correlation data (compare Eq. (12)). The analysis of the present FRET assay using FCCS is almost impossible due to reasons that will be outlined below.

3. Normalization Procedures for Independence from the Total Substrate Concentration

The sample-to-sample variations in total substrate concentration probably evoked by misdispensing of the aliquots demand for concentration-independent methods of analysis. The information contained in the fluorescence data from a single detection channel or in the concentration value of FRET-active or -inactive species alone obviously seems to be insufficient to correct for these effects. An easy way to increase the information content of the analysis and thus to correct for such variations is to express the information from the green and red detection channel or from the concentration of FRET-active and -inactive species simultaneously.

Concentration Ratio – 2D-FIDA and 2CG-FCS

Since the variations from sample to sample are most probably caused by variations in the total substrate concentration, they should be accounted for by calculating the ratio $c_{on}/(c_{on} + c_{off})$. Following Eq. (4) this is given by an exponential decay characterized by the same time constant, $t_0$, and the normalized concentrations, $c_{on}^0/c_{tot}$ and $c_{off}^0/c_{tot}$.

$$\frac{c_{on}(t)}{c_{on}(t)+c_{off}(t)} = \frac{c_{on}^0}{c_{tot}} \exp(-t/t_0) + \frac{c_{on}^\infty}{c_{tot}}$$ (17)

This calculation is in principle possible for all methods that are capable of directly resolving the concentrations, $c_{on}$ and $c_{off}$, i.e., FIDA, 2D-FIDA, and 2CG-FCS. However, we perform this calculation only for 2D-FIDA and for 2CG-FCS of unnormalized correlation data, since these are the only methods able to yield reliable estimates for $c_{on}$ and $c_{off}$ as outlined in the previous chapter (compare Fig. (3)). The corresponding time courses are shown in Fig. (5A) resulting in time constants of $t_0 = 9.2 \pm 0.3$ and $9.5 \pm 0.4$ min from a fit of Eq. (17) to the data.

Fluorescence Intensity Ratio Analysis (FIRA)

A straightforward way to account for the errors induced by the sample-to-sample fluctuations in fluorescence intensity analysis is to calculate the ratio, $F_R/(F_G+F_R)$, of the fluorescence intensities monitored in both detection channels as shown in Fig. (5B) over incubation time, $t$. Following the expressions of the single fluorescence intensities of Eq. (6), the ratio, $F_R/(F_G+F_R)$, is given by Eq. (18a).

$$\frac{F_R}{F_G+F_R} = \frac{A \exp(-t/t_0)+B}{C \exp(-t/t_0)+D}$$ (18a)
with \( A = (1-K) c_{on}^\infty /c_{tot}, \ B = K+(1-K) c_{on}^\infty /c_{tot}, \)
\( C = (1-K-E) c_{on}^\infty /c_{tot}, \ D = 1+K+(1-K-E) c_{on}^\infty /c_{tot}, \) and \( K = K_1/K_2. \)

The data of Fig. (5B) can, however, not reliably be described by Eq. (18a), since too many unknown parameters are variable and the pre-factor \( C \) of the denominator is expected to be rather small in this particular case \((1-K \approx 0.97 \Rightarrow E \approx 0.99).\) A simplified expression becomes feasible when the absence of cross-talk \((C_\Psi = C_{exc} = K \approx 0)\) and approximately the same value for the fluorescence detection and the quantum yield of donor and acceptor fluorescence \((C_{AD} \approx 1)\) are assumed.

\[
\frac{F_R}{F_G} = E \frac{c_{on}^\infty}{c_{tot}} + E \frac{c_{on}^\infty}{c_{tot}} \exp(-t/t_0) \quad (18b)
\]

A simple exponential fit (Eq. (18b)) to the data leads to the cleavage time of \( t_0 = 8.5 \pm 0.3 \) min.

### Two-Color Ratio FCS (TCR-FCS)

An easy way to increase the information content of FCS analysis is to use the results from the green and red detection channel simultaneously by investigating the ratio of the amplitudes (or their reciprocals) of both sets of FCS data, \( G_{RCS}^R(0)/(G_{RCS}^G(0) + G_{RCS}^R(0)) \) and \( G_{RCS}^R(0)/(G_{RCS}^G(0) + G_{RCS}^R(0)) \) (triangles); \( B = 0.021 \pm 0.002, A = 0.20 \pm 0.005, t_0 = 8.2 \pm 0.3 \) min (\( G_{RCS}^R(0)/(G_{RCS}^G(0) + G_{RCS}^R(0)) \)) and \( B = 0.19 \pm 0.004, A = 0.26 \pm 0.01, t_0 = 7.1 \pm 0.5 \) min (\( G_{RCS}^R(0)/(G_{RCS}^G(0) + G_{RCS}^R(0)) \)).
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Measurement, which is rendered by approximately the same value of accuracy and statistical precision (compare Table (1)). All of the given normalization procedures seem to be sufficient for compensating the sample-to-sample variations and are essential to account for the bias introduced by variations in total substrate concentration due to, e.g., misdispensing of the aliquots. However, the values determined for \( t_0 \) are still higher than those obtained for HPLC (\( t_0 = 6.4 \pm 0.5 \) min, compare Table (1)). In fact, they deviate even more than those values obtained for \( c_{\text{tot}} \) or for the red detection channel (\( \approx 7 \) to 7.5 min, compare Fig. (4A)). However, those values are not reliable, owing to the bias introduced by the low degree of accuracy. Furthermore, the values obtained for FIRA or TCR-FCS are based on assumptions of zero cross-talk or of a ratio of fluorescence detection efficiencies of unity, which do not represent the actual experimental conditions. In the following, we will show that these assumptions lead to biased results, even in the case of a very low cross-talk of \( \approx 1 \% \) as present in this experiment. Thus, it appears that the results obtained for 2D-FIDA and 2CG-FCS (\( t_0 = 9.2 \pm 0.5 \) and 9.5 \( \pm 0.4 \) min) are the most reliable values from fluorescence measurements.

Applying Eq. (3), the kinetic rate constant, \( k_{\text{cat}} \), of the enzymatic reaction is determined to be \((1.5 \pm 0.1) \text{ s}^{-1}\) for 2D-FIDA or 2CG-FCS and \((2.1 \pm 0.2) \text{ s}^{-1}\) for the HPLC-based analysis. These values reside within the very low range of expected values of \( k_{\text{cat}} = 0.8 \text{ to } 40 \text{ s}^{-1}\) as reported by Kurth et al. [75]. This may be explained by the fact that the applied substrate concentration \((2.5 \mu M)\) lies far below the \( K_M \) of \( \approx 60 \mu M\) for a Lys-Leu motive [61]. The slight discrepancy between the values of \( k_{\text{cat}} \) obtained by fluorescence and HPLC analysis may be explained by the presence of the substrate-enriched detergent micelles. In the present experimental approach, the Trypsin activity is characterized by the change of FRET-active substrate concentration over incubation time. However, this concentration becomes increasingly overestimated as the amount of cleaved peptide grows, which still exhibits almost 100 % FRET activity due to a close vicinity within the same detergent micelle. In this as-

Table (1) lists all time constants and the degree of precision and of accuracy resulting from those FFS methods that allow for a correction algorithm of the sample-to-sample variations. 2D-FIDA and 2CG-FCS (on unnormalized data) enable the calculation of the fraction of FRET-active species over incubation time (Fig. (5A)). In contrast, a ratio of the fluorescence intensities or the FCS amplitudes determined for both detection channels can be calculated for Fluorescence Intensity Ratio (FIRA) (Fig. (5B)) or Two-Color Ratio FCS (TCR-FCS) analysis (Figs. (5C) and (5D)), respectively. Most obviously, the resulting sample-to-sample noise of all methods equals the standard deviation of a single measurement, which is rendered by approximately the same value of accuracy and statistical precision (compare Table (1)). All of the given normalization procedures seem to be sufficient for compensating the sample-to-sample variations and are essential to account for the bias introduced by variations in total substrate concentration due to, e.g., misdispensing of the aliquots. However, the values determined for \( t_0 \) are still higher than those obtained for HPLC (\( t_0 = 6.4 \pm 0.5 \) min, compare Table (1)). In fact, they deviate even more than those values obtained for \( c_{\text{tot}} \) or for the red detection channel (\( \approx 7 \) to 7.5 min, compare Fig. (4A)). However, those values are not reliable, owing to the bias introduced by the low degree of accuracy. Furthermore, the values obtained for FIRA or TCR-FCS are based on assumptions of zero cross-talk or of a ratio of fluorescence detection efficiencies of unity, which do not represent the actual experimental conditions. In the following, we will show that these assumptions lead to biased results, even in the case of a very low cross-talk of \( \approx 1 \% \) as present in this experiment. Thus, it appears that the results obtained for 2D-FIDA and 2CG-FCS (\( t_0 = 9.2 \pm 0.5 \) and 9.5 \( \pm 0.4 \) min) are the most reliable values from fluorescence measurements.

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Table 1. Time Constant \( t_0 \), Precision, Accuracy, Dynamic Range and \( Z' \)-Factor for the Different Fluorescence Analysis Methods Including a Correction Procedure for Sample-to-Sample Variations

<table>
<thead>
<tr>
<th>Analysis Method</th>
<th>( t_0 ) [min]</th>
<th>Precision</th>
<th>Accuracy</th>
<th>Dynamic Range</th>
<th>( Z' )-Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>6.4 ± 0.5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2D-FIDA</td>
<td>9.2 ± 0.3</td>
<td>3</td>
<td>3.2</td>
<td>0.35</td>
<td>0.97</td>
</tr>
<tr>
<td>2CG-FCS un</td>
<td>9.5 ± 0.4</td>
<td>5.7</td>
<td>5.2</td>
<td>0.37</td>
<td>0.95</td>
</tr>
<tr>
<td>FIRA</td>
<td>8.5 ± 0.3</td>
<td>2.4</td>
<td>2.5</td>
<td>0.25</td>
<td>0.97</td>
</tr>
<tr>
<td>TCR-FCS un</td>
<td>8.2 ± 0.5</td>
<td>3</td>
<td>2.7</td>
<td>0.2</td>
<td>0.96</td>
</tr>
<tr>
<td>TCR-FCS n</td>
<td>7.1 ± 0.5</td>
<td>3</td>
<td>3.3</td>
<td>0.25</td>
<td>0.92</td>
</tr>
</tbody>
</table>

The time constants, \( t_0 \), results from an exponential fit to the data (compare Fig. (5)), the statistical precision is given by the relative standard deviation of a single measurement, the accuracy denotes the standard deviation from one measurement to another (i.e., sample-to-sample variations) as determined by the average relative deviation of the measurement points from the exponential fit, the dynamic range marks the difference between the data recorded at incubation time \( t = 0 \) min and at incubation time \( t = 60 \) min, and the \( Z' \)-factor is determined according to Eq. (20). The synonyms given are: “un” and “n”, unnormalized and normalized correlation data.
say, the high concentration of SDS detergent is necessary to generate a sufficient FRET signal. A redesign of the peptide substrate may render the high SDS concentration redundant.

Instead of the relative standard deviation of a single measurement listed as precision in Table (1), the \( Z' \)-factor is used to compare the statistical precision of the various analysis methods. The usage of this factor is widespread in High Throughput Screening (HTS), since it reflects both the assay signal dynamic range (Table (1)) and the standard deviation associated with the measurement and analysis of the according method [76]. With \( s_{t=0} \) and \( s_{t=60} \) being the readout values of each analysis method (e.g., \( c_{ad}(c_{on}+c_{ad}) \)) at the time point \( t = 0 \) min and \( t = 60 \) min and denoting \( \sigma(s) \) as the standard deviation of the according readout, the \( Z' \)-factor is a dimensionless statistical characteristic.

\[
Z' = 1 - \frac{3\sigma(s_{t=0}) + 3\sigma(s_{t=60})}{|s_{t=0} - s_{t=60}|} \tag{20}
\]

Its maximum value is 1. The \( Z' \)-factor depends on the measurement time, as \( \sigma(s) \) is inversely proportional to the square root of the measurement time. Any screening application is rated sufficiently robust if \( Z' > 0.5 \).

The maximum degree of statistical precision is achieved by 2D-FIDA with \( Z' = 0.97 \) close to unity. This is not surprising, since 2D-FIDA has already proven a valuable tool in several drug-screening experiments [48, 49, 51, 52]. With \( Z' \)-values of 0.92 to 0.97 the precision of FIRA, TCR-FCS or 2CG-FCS seems equally appropriate for such experiments. However, it has to be noted that an analysis applying FIRA or TCR-FCS do not allow for a correction of potential background signal by autofluorescent drug compounds, which is a common artifact in High-Throughput Screening using a fluorescence readout [48, 49]. Such a correction procedure demands for analysis tools capable of molecular resolution of fluorescent species expressing different brightness values, as offered by 2D-FIDA or 2CG-FCS [48, 49].

Overall, the analysis of the unnormalized correlated data has in all cases been found to achieve a higher degree of precision than that of the normalized correlation data.

### 4. Influence by Experimental Factors

The following chapter outlines the influence of the cross-talk and of different values of the energy transfer efficiency or of the ratio of detection efficiencies of donor and acceptor on the results obtained by the different FFS methods. Here, it is demonstrated that all methods but 2D-FIDA and 2CG-FCS need to be handled carefully even with low amounts of cross-talk.

### FIDA, 2D-FIDA and 2CG-FCS

All of these methods enable a molecular resolution of different fluorescent species based on their specific brightness parameters. According to Eq. (5), different values of cross-talk \( (C_{exc} \text{ and } C_p) \), energy transfer \( (E) \), or ratio of fluorescence detection efficiencies \( (C_{AD}) \) directly influence the brightness values of FRET-active and inactive species, \( q_{in}^G \), \( q_{in}^R \), \( q_{off}^G \), and \( q_{off}^R \). These brightness parameters are intrinsically employed to distinguish between species and thus directly account for any experimental condition, \( C_{exc} \), \( C_p \), \( E \), and \( C_{AD} \). However, as shown in this example, an excessively low brightness value of \( q < 2 \text{ kHz} \) severely biases the results obtained for single FIDA on the green or red detection channel, since the according concentration value cannot be reliably determined. These analysis methods would favor a FRET assay constituting an energy transfer efficiency below 100%, which would render brightness values of donor and acceptor above this critical threshold. On the other hand, the FFS methods 2D-FIDA and 2CG-FCS readily offer a precise and accurate analysis for the given experimental conditions. It is obvious that a global FFS analysis of the data recorded simultaneously in both detection channels is advantageous or even essential for the analysis of FRET assays.

### Fluorescence Intensity Ratio Analysis (FIRA)

The data obtained for FIRA can only be described by a simple exponential fit (Eq. (18b)) assuming the absence of cross-talk and the presence of about the same fluorescence detection efficiency of donor and acceptor, \( C_{AD} = 1 \) (compare Fig. (5B)). The accurate description of the fluorescence intensity ratio (Eq. (18a)) is too complex to be used within a reliable fit.

Fig. (6A) depicts how the experimental conditions, cross-talk \( (C_{exc} \text{ and } C_p) \), difference in fluorescence efficiency of donor and acceptor \( (C_{AD}) \), and energy transfer efficiency \( (E) \), alter the results obtained for the dynamic range of the time course as well as the results obtained from an exponential approximation of the time course of the Trypsin activity. Using Eq. (18a), the time course, as monitored by \( F_R/(F_G+F_R) \), is simulated for \( t_0 = 9.2 \text{ min} \) and different values of \( C_{exc} \) and \( C_p \), \( C_{AD} \), and \( E \). Both the dynamic range and exponential decay time, \( t_0 \), are determined from a simple exponential fit to the simulated data. As expected, the highest dynamic range is attainable with the least amount of cross-talk \( (C_{exc} \approx C_p \approx 0) \), the highest energy transfer \( (E = 1) \), and coinciding fluorescence detection efficiencies of donor and acceptor \( (C_{AD} = 1) \). However, presuming a sufficient dynamic range, a completely unbiased time constant, \( t_0 \), is only achievable with \( C_{AD} = 1 \) and minimized cross-talk, \( C_{exc} = C_p \approx 0 \), as already outlined in Eq. (18b). The time constant and dynamic range predicted for the present experimental conditions, \( t_0 = 8.4 \text{ min} \) and range = 0.27 (black dots Fig. (3B)), coincide very well with the observed values of \( t_0 = 8.5 \pm 0.3 \text{ min} \) and range = 0.25 ± 0.1.

The present FRET assay shows that monitoring the fluorescence light intensity simultaneously in two detection channels is advantageous and in this case even essential for the correct analysis of FRET or two-color assays. However, the results will be biased due to excitation and emission cross-talk as well as different fluorescence detection efficiencies of donor and acceptor, which is a common problem in any two-color experiment. This calls for an exact analysis.
Comparison of Different Fluorescence Fluctuation Methods

Fig. (6). Dependence of the results of the Trypsin activity obtained by different fluorescence methods on different experimental factors, cross talk, (C_{exc} and C_{qu}, gray line), ratio of fluorescence detection efficiency of donor and acceptor dye, (C_{AD}, black line), and FRET efficiency (E, open dots). For each fluorescence method, the according data are simulated over incubation time using the corresponding equations. Plotted are the dynamic range of the time course (denoted as range on the y axis, lower panel) and the time constant $t_0$ (upper panel), resulting from an exponential fit ($B + A \exp(-t/t_0)$) to the simulated data. The instituted parameters are, $t_0 = 9.2$ min (dashed line), $C_{c/m}^\infty / C_{c/m} = 0.36$, $C_{c/m}^\infty / C_{c/m} = 0.075$, and if not varied $C_{exc} = C_{qu} = 0.015$ (for "E" and "C_{AD}" labelled curves) or $C_{exc} = C_{qu} = 0$ (for "$E^*" and "C_{AD}^*" labelled curves), $C_{AD} = 0.62$, and $E = 1$ (for FIRA and TCR-FCS) or $E = 0.5$ (for FCCS). The black dot represents the present experimental conditions ($C_{exc} = 0.012$, $C_{qu} = 0.015$, $C_{AD} = 0.62$, and $E = 1$). (A) FIRA; fluorescence intensity ratio $F_{FR} / (F_{FG} + F_{FR})$ applying Eq. (18a). (B) and (C) TCR-FCS; ratios of unnormalized, $G_{FCR}^{R}(0) / G_{FCR}^{G}(0) + G_{FCR}^{R}(0)$, and normalized FCS amplitudes, $\overline{G_{FCR}}^{R}(0)^{-1} / (\overline{G_{FCR}}^{R}(0)^{-1} + \overline{G_{FCR}}^{G}(0)^{-1})$, applying Eqs. (13) and (14a), respectively. (D) and (E) FCCS; unnormalized, $G_{FCCS}(0)$, and normalized FCCS amplitudes, $\overline{G_{FCCS}}(0)$, applying Eqs. (15) and (16), respectively. (E) Exemplary FCCS data of un-cleaved substrate with solely 488 nm excitation (open dots) and of a RhGr and MR121 labelled double 62-base-pair DNA strand with combined 488 nm and 633 nm excitation and no FRET occurrence (black line - 95 kW/cm² irradiance at 488 nm and 110 kW/cm² irradiance at 633 nm). The data are normalized to the value of one. The noise in the data is determined by the amount of coincidence signal (i.e., simultaneous occurrence of red and green fluorescence from the same molecule). In the case of un-cleaved substrate, the RhGr fluorescence is completely quenched by FRET ($E = 1$). Thus, the coincidence signal is solely due to cross talk signal, which is rather weak and leads to noisy FCCS data. In contrast, simultaneous excitation by 488 nm and 633 nm without FRET as in the case of the DNA strand leads to the simultaneous appearance of RhGr and MR121 fluorescence and thus to a higher level of coinciding signal.
using Eq. (18a), which however might be problematic due to the rather high number of unknown parameters.

Two-Color Ratio FCS (TCR-FCS)

The most accurate FCS analysis is performed by calculating the ratio of the amplitudes (or their reciprocal) of the unnormalized or normalized correlation data recorded in the two detection channels, as done for TCR-FCS. However, once again the data obtained for TCR-FCS of unnormalized or normalized correlation amplitudes can only be described by a simple exponential fit (Eq. (19)) assuming the absence of cross-talk, a transfer efficiency of about 100 %, and a ratio of the fluorescence detection efficiencies of donor and acceptor of close to unity, $C_{AD} = 1$ (compare Figs. (5C) and (5D)).

Figs. (6B) and (6C) further outline the influence of cross-talk to the TCR-FCS data. The time course of the Trypsin activity, as monitored by $G_{FCR}^D(0)/(G_{FCR}^D(0) + G_{FCR}(0))$ and $G_{FCR}^R(0)^{-1}/(G_{FCR}^R(0)^{-1} + G_{FCR}(0)^{-1})$, is simulated for $t_0 = 9.2$ min and different values of $C_{exc}$ and $C_{yr}$, $C_{AD}$, and $E$ using Eqs. (13) and (14a), and their dynamic range and exponential decay time, $t_0$, are determined by a simple exponential fit to the data. Presuming a sufficiently large dynamic range > 0.1, the best approximation of the time constant, $t_0$, is in both cases obtained for the least cross-talk ($C_{exc} = C_{yr} \approx 0$), the highest energy transfer ($E = 1$), and coinciding fluorescence detection efficiencies of donor and acceptor ($C_{AD} = 1$). A similar message has already been made for the fluorescence intensity analysis (compare Fig. (6A)). At first glance, none of the methods seems to be advantageous. However, while the time constant, $t_0$, and dynamic range (as predicted by the simulation) coincide with the experimental findings for the unnormalized FCS analysis ($t_0 = 7.8$ min and range = 0.2 compared to $t_0 = 8.2 \pm 0.3$ min and range = 0.20 $\pm 0.003$), this is not quite the case for the normalized FCS analysis ($t_0 = 8.8$ min and range = 0.32 compared to $t_0 = 7.1 \pm 0.5$ min and range = 0.25 $\pm 0.01$). The resulting dynamic range and time constant not only seem to be influenced by the mentioned experimental factors, but also by the statistical precision of the respective analysis method. The absolute standard deviation of the normalized data is on average about 2.5-times larger compared to the unnormalized FCS or fluorescence intensity analysis as outlined by the respective Z’-values (compare Table (1) and error bars in Fig. (5)).

In conclusion, although TCR-FCS is able to account for the sample-to-sample variations, the resulting time constants are still biased due to non-ideal experimental conditions, such as cross-talk or different fluorescence efficiencies of donor and acceptor. The optimized experimental conditions arise at high energy transfer efficiencies up to 100 %. The analysis of the unnormalized data yields more precise results.

FCCS

Although commonly advertised as an ideal analysis tool for two-color and FRET assays, the precision and accuracy of FCCS is worst for the present FRET assay. Thus, the influence of experimental parameters on the cross-correlation analysis is studied in more detail.

Unnormalized Amplitudes

The course of the unnormalized FCCS amplitude over incubation time (Fig. (3G)) cannot reliably be described by the simple exponential function of Eq. (15). Due to variations from sample to sample resulting from the rather low degree of precision and accuracy of this FCCS analysis, no clear tendency can be observed. To investigate the general capability of this analysis method for such FRET assays, the time course of the Trypsin activity, as monitored by $G_{FCR}(0)$, is simulated for $t_0 = 9.2$ min and different values of $C_{exc}$ and $C_{yr}$, $C_{AD}$, and $E$ (using Eq. (15)). Fig. (6D) plots the dynamic range determined by this simulation. In correspondence to Eq. (15), the time course always describes an exponential with the correct time constant, $t_0 = 9.2$ min. However, a sufficiently large dynamic range of > 400 is only obtained close to a value of $E = 0.5$, which is furthermore very influential with regards to the other experimental parameters. In particularly, the dependence on the amount of cross-talk is substantial; starting from 560, the absolute value of the dynamic range approaches zero for a cross-talk of about 10 to 20 %, but increases to above 1000 towards an extended cross-talk further along the line. A large amount of cross-talk leads to a good coinciding signal in both channels, which can be observed very efficiently by unnormalized FCCS. At the present experimental conditions, i.e., especially of $E = 1$ (black dot), the dynamic range is expected to be < 75, which is far below that of the sample-to-sample noise. FCCS is unfavorable in this particular case since the donor fluorescence is almost completely quenched for the FRET-active species and no fluorescence emission of the acceptor is present for the FRET-inactive species. Consequently, the coinciding signal in both detection channels is solely due to the (in this case) rather weak cross-talk, which leads to noisy FCCS data as indicated in Fig. (6E). In fact, the analysis of the unnormalized FCCS data favors a larger amount of cross-talk. The analysis could thus in principle be optimized by intentionally choosing non-ideal dye pairs and filters that cause spectral overlaps in the two detection channels. On the other hand, FRET systems, which exhibit less energy transfer efficiency and thus higher brightness values of the donor (e.g., $> 5$ kHz), have already yielded much better results for FCCS as the present ones [57]. This is also predicted in Fig. (6D).

Normalized Amplitudes

In most form of two-color FCCS analysis, the normalized amplitude is directly used as a measure for the coinciding signal, i.e., the concentration of two-color labeled species. The exact expression of this amplitude (Eq. (16)) is however too complex to reliably fit the observed normalized FCCS amplitudes, which can only be described by a simple exponential function rendering a strongly biased value of $t_0 = 21 \pm 4$ min (compare Fig. (3H)). To further elucidate the error made by this approximation, Eq. (16) is used to simulate the time course of the Trypsin activity observed by $G_{FCR}(0)$ for
This introduces additional dilution steps and fluorophore concentration, which is not applicable to enzymatic reactions. This introduces additional dilution steps and fluorophore concentration, which is not applicable to enzymatic reactions. The present FRET assay has to account for non-ideal experimental conditions such as sample-to-sample variations in the substrate concentration, fluorescence cross-talk between the detection channels, differences in fluorescence efficiency of donor and acceptor, or extreme values of the FRET efficiency. These non-ideal conditions are common to FRET or two-color fluorescence experiments and cannot be accounted for by methods incapable of molecular resolution, such as fluorescence intensity analysis, or which gather information only from one of the detection channel, such as usual FIDA. The high statistical precision and accuracy of 2D-FIDA and 2CG-FCS in resolving enzymatic reactions opens up the door to a wide range of biochemical applications, in particular high throughput drug discovery and molecular sorting. In particular, the capability of molecular resolution enables the exclusion of background signals, e.g., autofluorescence, a problem inherent to drug discovery [48, 51]. As shown previously [58], the use of beam scanning additionally increases the precision and accuracy and motivates the user to apply these methods even at measurement times below one to two seconds.

The non-ideal experimental conditions introduce drawbacks inherent in fluorescence auto-correlation (FCS) or cross-correlation (FCCS) analysis. Even the introduction of Two-Color Ratio FCS (TCR-FCS), which combines the results obtain from single FCS analysis in both detection channels, cannot circumvent this disadvantage. As outlined above, TCR-FCS as well as FCCS leads to biased results in a large variety of experimental conditions. This bias is reduced by the global analysis of all auto- and cross-correlation data recorded in the two detection channels, as performed for 2CG-FCS. For fluorescence auto- and in special cross-correlation spectroscopy of FRET signals it is generally advantageous to analyze the unnormalized correlation data instead of applying the normalized curves as usually done.

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Comparison of Different Fluorescence Fluctuation Methods


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