How the Molecule Number Is Correctly Quantified in Two-Color Fluorescence Cross-Correlation Spectroscopy: Corrections for Cross-Talk and Quenching in Experiments

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Abstract: Fluorescence correlation spectroscopy (FCS) and two-color fluorescence cross-correlation spectroscopy (FCCS) are among the cutting-edge technologies for measuring molecule numbers at the single-molecule level in liquid phases. Yet, even after single molecule technologies caught up with theory, the techniques remained tools only for specialists able to navigate the formulas that give meaning to their observations. This original article aims at the derivations of relevant and useful quantification of molecule numbers for researchers with more diverse backgrounds who have begun probing questions previously unanswerable, except on the level of the molecule.

The quantitation depends on the exact conditions of measurement. To some extent these are arbitrary, so that standard procedures are necessary in for valid comparisons of measurements among different data sets. To agree on and specify such procedures is one of the further aims here.

No matter what fluorophores, which have, of course, to meet photophysical and photochemical requirements for FCS/FCCS, and optical setups/devices are used, the primary measurement signal arises from fluctuations of the mean molecule number in a confocal femtoliter or smaller probe region. Since FCS/FCCS relies on fluorescence emission measurements of rare events, one is looking for small signals on essentially zero background. Optical separation by FCCS setups is usually defined in terms of cross-talk and cross-excitation/cross-emission, respectively, which can be calculated and minimized by the experimenter from readily measurable quantities of the absorption/emission scenario for single labels and multiple labels *n* and *m* bound to or incorporated into the two-color molecules. Furthermore, this article derives relevant formulas for the quantification of molecule numbers under different experimental conditions with substantial quenching of the two-color molecules such as single labels and multiple labels *n* and *m* bound to or incorporated into the two-color molecules, high-density labeling of two-color molecules with multiple *n* green labels and one red label. Here, we summarize and extend the formulas to make them more generally applicable.

Key Words: two-color fluorescence cross-correlation spectroscopy, dual-color fluorescence cross-correlation spectroscopy, fluorescence correlation spectroscopy, molecule number, cross-talk, cross-emission, bleed-through, spilling-over, cross-excitation, quenching, quantitative formulas.

INTRODUCTION

Dual-color fluorescence cross-correlation spectroscopy (FCCS), also called two-color fluorescence cross-correlation in the literature [1-8], is an extension of standard fluorescence correlation spectroscopy (FCS) where instead of one, two colors are simultaneously excited by two excitation lasers at different wavelengths [9-11]. In FCCS the spontaneous fluorescence fluctuations, which are stochastic, of two differentially labeled and spectrally distinguishable molecules in a small illuminated volume element are measured and compared to each other, in contrast to an autocorrelation measurement where the fluorescence signal in time is compared with itself. Thus, we observe the fluorescence emitted from molecules labeled differentially with green and red-emitting fluorescence dyes. If the green molecules (N_g) and the red molecules (N_r) are not interacting with each other, then each molecule species has its own autocorrelation function. Since the molecules move independently from each other, there will be no specific cross-correlation signal. If, however, the green molecules are bound to or incorporated into the red molecules, then those pairs of molecules (N_{gr}) will move together and the correlated reactants will generate a two-color cross-correlation signal.

There are some general intrinsic problems and limitations associated with cross-correlation experiments. One of them results from cross-talk within the detection channels. Green light migrating to the red channel and red light migrating to the green channel result in a phenomenon called cross-talk (cross-emission), bleed-through or spilling-over. In addition, excitation of either dye by the non-corresponding laser lines can also create cross-talk (cross-excitation), although for energetic reasons cross-excitation of the blue dye by the red laser is negligible in most cases (Scheme **1**). Cross-emission of the red dye into the green channel can often be completely suppressed by choosing appropriate emission filters that hardly compromise the signal strength in the green channel, since emission spectra are steep at the rising edge (Scheme **2**). In contrast, cross-excitation of the red dye by the blue laser is unavoidable. Due to the broad emission spectra there are hardly any filters available that can completely block the

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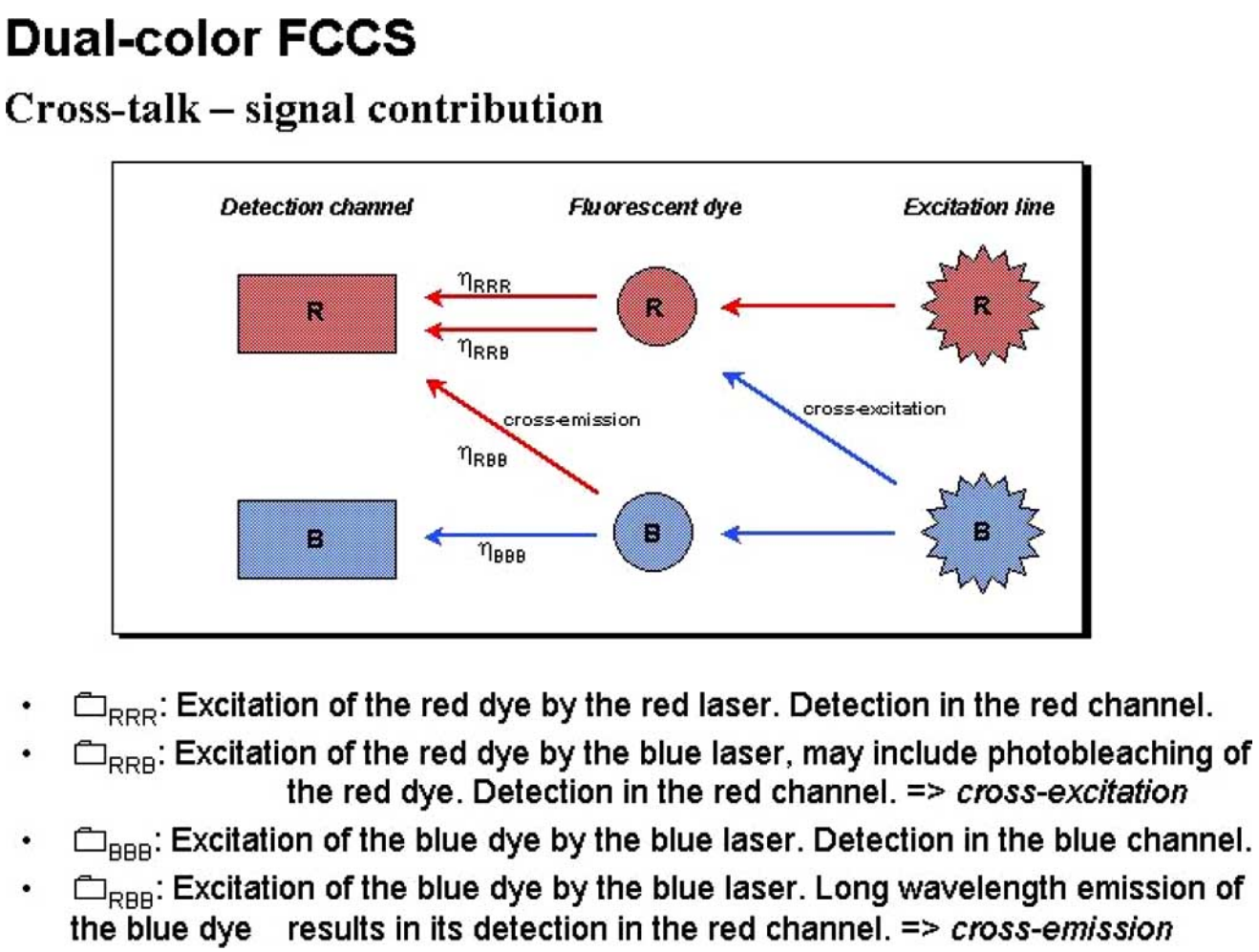


Scheme 1. The scheme was provided by Carl Zeiss GmbH, Jena, Germany.

green emission spill over into the red channel without incurring significant losses in the red channel signal.

The extent of cross-talk depends upon various experimental conditions that can be modified by the researcher. Additional considerations concern the brightness of small molecules and how well the molecules work under two-color excitation conditions. Yet another issue that has to be dealt with properly is the quantum yield differences between free and bound fluorescent molecules.

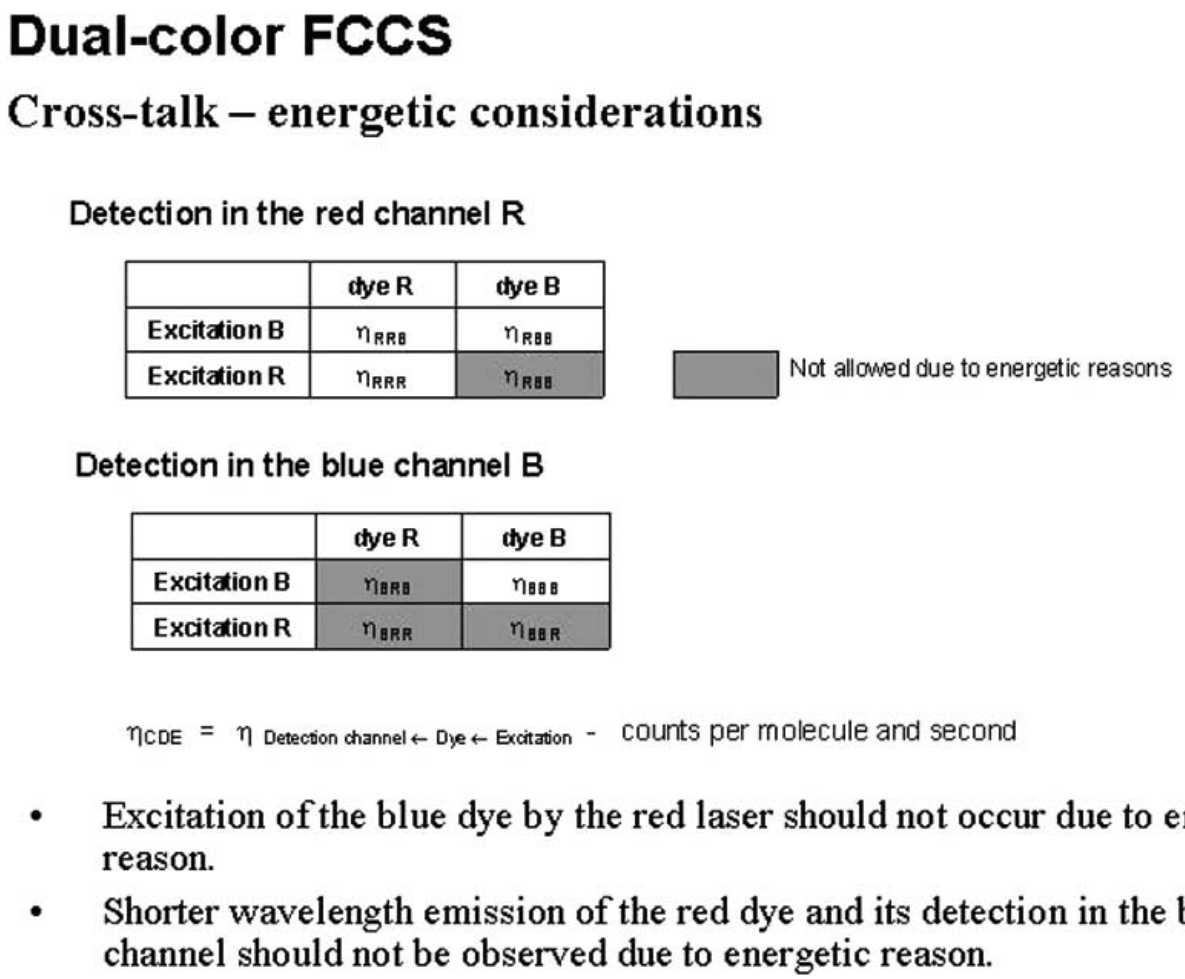
Modern diagnostic assays are extremely sensitive and generally capable of detecting a small number of specific molecules [6]. Since experiments may need to quantify molecule numbers below 10 in single phases such as solutions or membranes [7, 8], the main focus of the original article is on practical applications of the quantitation at the 'single-molecule level'. The experimental conditions considered here enable us to provide universal procedures for quantifying the absolute numbers of two-color molecules in cases of two-color excitation. The basic mathematical background for correcting cross-talk and quenching in two-color fluorescence cross-correlation spectroscopy have been published previously [1-5]. For detailed discussion and experimental verification of the formulas, we refer to these papers, where we dealt with certain specific molecular biology experiments. Here, we summarize and extend the formulas to make them more generally applicable.

THEORY

The measured, normalized two-color (dual-color) fluorescence cross-correlation amplitude is given by

$$G(0) - 1 = \tilde{G}(0) = \frac{1}{N}. \quad (1)$$

We consider cases where excess non-bound labels (e.g., free dyes) contribute to the correlation function due to cross-talk. Hence, the measured two-color cross-correlated curve is evaluated for a superposition of two correlation functions: (i) the cross-correlation term resulting from two-color molecules, and (ii) an 'autocorrelation' term showing a shorter 'diffusion time' and resulting from cross-talk by the free dyes and single-color molecules. The fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$ follows the rules for calculating the number of two-color molecules N_{gr} from the amplitude of the cross-correlated curve $\frac{1}{N}$ [1, 2, 3]. The



Scheme 2. The scheme was provided by Carl Zeiss GmbH, Jena, Germany.

amplitude of the measured two-color cross-correlated curve depends on the total number of fluorescence-emitting molecules in the green and red volume elements as well as in the overlapping volume element (cross-correlated volume). Thus the total number of red-emitting molecules N_r exhibiting no cross-correlation and determined in red autocorrelation mode, and the two-color cross-correlated value N have to be corrected for the size of the green volume element by multiplying with $1/\frac{V_r}{V_g}$ and $1/\frac{V_{gr}}{V_g}$, respectively, as exemplified in ref. [1], and Table **2**. The determined volume elements are, for example, V_g = 0.328 fl (green volume element), V_r = 0.452 fl (red volume element) and V_{gr} = 0.389 fl (two-color cross-correlated volume element).

I. Correction in Cases without Substantial Quenching of the Two-Color Molecules

I.A. Single Labels Bound to or Incorporated into the Two-Color Molecule

Let us consider the measured absorption/emission scenario summarized in Table **1**. Here, we defined the absorption/emission parameters $Q_{\lambda excitation}^{\lambda emission}$: Q_B^G , Q_R^R and the cross-talk parameters $Q_{\lambda excitation}^{\lambda emission}$: Q_B^R , Q_R^G in the same way as in ref. [1].

We expand Eqn.(1) by the factor $N\ Q^2$

$$\tilde{G}(0) = \frac{N\ Q^2}{[N\ Q]\ [N\ Q]} \ . \qquad (2)$$

The way we look at Eqn. (2) is

$$\tilde{G}(0) = \frac{\overbrace{N\ Q^2}^{stands\ for\ excitation}}{\underbrace{[N\ Q]}_{stands\ for\ green\ emission}\ \underbrace{[N\ Q]}_{stands\ for\ red\ emission}} \ . \qquad (3)$$

For the total number of molecules we can write

$$N = N_g + N_r + N_{gr} \ . \qquad (4)$$

Combining Eqns. (3) and (4) with the absorption/emission scenario of Table **1** yields, for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$,

Table 1. Measured Experimental Values (an Example) for Defining the Relevant Parameters to Quantify the Cross-Talk in Two-Color Fluorescence Cross-Correlation Spectroscopy. Reproduced in a Slightly Modified form from ref. [1]

Dye	λ_{ex} (nm)	λ_{em} (nm)	$T_{\lambda_{ex}}^{\lambda_{em}}$ (x 10^3) [absolute photon counts per molecule and per second]	$Q_{\lambda_{ex}}^{\lambda_{em}}$ [relative photon counts per molecule and per second, *dimensionless*]
Rhodamine-Green	488	540	67	$Q_{Blue}^{Green} = \frac{T_{Blue}^{Green}}{T_{Blue}^{Green}} = 1.0$
	488	685	3	$Q_{Blue}^{Red} = \frac{T_{Blue}^{Red}}{T_{Blue}^{Green}} = 0.045$
Cy5-dCTP	633	685	147	$Q_{Red}^{Red} = \frac{T_{Red}^{Red}}{T_{Blue}^{Green}} = 2.194$
	633	540	0	$Q_{Red}^{Green} = \frac{T_{Red}^{Green}}{T_{Blue}^{Green}}$ 0.0

$$\tilde{G}(0) = \frac{N_g Q_B^G Q_B^R + N_r Q_R^R Q_R^G + N_{gr}\left(Q_B^G Q_R^R + Q_B^G Q_B^R + Q_R^R Q_R^G\right)}{\left[N_g Q_B^G + N_r Q_R^G + N_{gr}\left(Q_B^G + Q_R^G\right)\right]\left[N_g Q_B^R + N_r Q_R^R + N_{gr}\left(Q_R^R + Q_B^R\right)\right]} . \quad (5)$$

Multiplying numerator and denominator with $\frac{1}{Q_B^G \; Q_R^R}$ gives the final expression as found in ref. [1] for $Q_R^G = 0$

$$\tilde{G}(0) = \frac{N_g \left(\frac{Q_B^R}{Q_R^R}\right) + N_{gr}\left(1 + \frac{Q_B^R}{Q_R^R}\right)}{\left[N_g + N_{gr}\right]\left[N_r + N_g \left(\frac{Q_B^R}{Q_R^R}\right) + N_{gr}\left(1 + \frac{Q_B^R}{Q_R^R}\right)\right]} \quad (6)$$

Under the experimental conditions of the optical set-up $\frac{Q_B^R}{Q_R^R} << 1$, *and* with $N_g, N_r \approx N_{gr}$, we obtain directly from Eqn. (6) the very practical and well-known approximation for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$ [1]

$$\frac{1}{N} = \frac{N_{gr}}{\left[N_g + N_{gr}\right]\left[N_r + N_{gr}\right]} . \quad (7)$$

We have to emphasize that Eqn. (7) only applies for experimental situations in which the dimensionless values of the relative photon counts per molecules and per second Q_B^R and Q_R^R are optically adjusted as given, for example, in Table **1**. N_g and N_r were measured by means of a two-component green and red autocorrelation, respectively, in single-color excitation mode.

In the extreme case $N_g, N_r >> N_{gr}$ it follows from Eqn. (7) that

$$\frac{1}{N} = \frac{N_{gr}}{\left[N_g\right]\left[N_r\right]} , \quad (8)$$

where N_{gr} is the number of cross-correlated molecules showing both labels and N_g , N_r are the numbers of molecules exhibiting no correlation in simultaneous two-color excitation.

In the ideal case where $N_g, N_r << N_{gr}$ and thus A_{gr} = 1 we have

$$\tilde{G}(0) = \frac{1}{N} = \frac{1}{N_{gr}} \tag{9}$$

I.B. Multiple Labels* n *and* m *Bound to or Incorporated into the Two-Color Molecules

The number of green-emitting labels of the two-color molecule is *n*. The number of red-emitting labels of the two-color molecule is *m*. Starting again with Eqns. (3) and (4), the following physical relationship holds for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$

$$\tilde{G}(0) = \frac{\overbrace{\underbrace{\overbrace{N_g Q_B^G}^{\text{green molecules}} + \overbrace{N_r Q_R^G}^{\text{portion of red molecules with green cross-talk}} + N_{gr}\left(\overbrace{nQ_B^G}^{\text{without cross-talk}} + \overbrace{mQ_R^G}^{\text{with green cross-talk}}\right)}^{\text{portion of two-color molecules}}}^{\text{green emission}} \quad \overbrace{\underbrace{\overbrace{N_g Q_B^R}^{\text{portion of green molecules with red cross-talk}} + \overbrace{N_r Q_R^R}^{\text{red molecules}} + N_{gr}\left(\overbrace{mQ_R^R}^{\text{without cross-talk}} + \overbrace{nQ_B^R}^{\text{with red cross-talk}}\right)}^{\text{portion of two-color molecules}}}^{\text{red emission}}}{\overbrace{\underbrace{N_g Q_B^R + N_r Q_R^R + N_{gr}\left(\overbrace{nQ_B^G mQ_R^R}^{\text{blue excitation of green molecules with red cross-talk}} + \overbrace{nQ_B^G nQ_B^R}^{\text{red excitation of red molecules with green cross-talk}} + \overbrace{mQ_R^R mQ_R^G}^{\text{portion without cross-talk}}\right)}^{\text{blue/red excitation of two-color molecules}}}^{\text{portion with red cross-talk}} + \overbrace{}^{\text{portion with green cross-talk}}} \tag{10}$$

Multiplying numerator and denominator with $\frac{1}{Q_B^G \; Q_R^R}$ yields

$$\tilde{G}(0) = \frac{N_g \frac{Q_B^R}{Q_R^R} + N_r \frac{Q_R^G}{Q_G^B} + N_{gr}\left(nm + n^2 \frac{Q_B^R}{Q_R^R} + m^2 \frac{Q_R^G}{Q_B^G}\right)}{\left[N_g + N_r \frac{Q_R^G}{Q_B^G} + N_{gr}\left(n + m \frac{Q_R^G}{Q_B^G}\right)\right]\left[N_{gr} \frac{Q_B^R}{Q_R^R} + N_r + N_{gr}\left(m + n \frac{Q_B^R}{Q_R^R}\right)\right]} . \tag{11}$$

Thus the final formula for setting-up $Q_R^G \quad 0$ found in ref. [2] reads

$$\tilde{G}(0) = \frac{N_g \frac{Q_B^R}{Q_R^R} + N_{gr}\left(n\left[m + n \frac{Q_B^R}{Q_R^R}\right]\right)}{\left[N_g + n \;\; N_{gr}\right]\left[N_r + N_g \frac{Q_B^R}{Q_R^R} + N_{gr}\left(m + n \frac{Q_B^R}{Q_R^R}\right)\right]} . \tag{12}$$

For the specified condition $n = m = 1$ in the generalized Eqn. (12), we immediately obtain the formula/case of Eqn. (6). We theoretically described the system in a representation where the fluorescence detection efficiencies $Q^{\lambda emission}_{\lambda excitation}$ at different excitation and emission wavelengths are coupled to the labels *n* and *m* [2]. It was experimentally shown in ref [2] that the amplitude *1/N* increases in proportion to the number of incorporated green (*n*) and red (*m*) labels under the condition of a constant level of N_g and N_r over N_{gr} as predicted by Eqn. (11). We called this behavior of the two-color fluorescence cross-correlation amplitude the signal amplification.

Under the experimental conditions of the optical set-up $\frac{Q^R_B}{Q^R_R} << 1$, *and* with $N_g, N_r \approx N_{gr}$, Eqn. (12) yields, for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$,

$$\frac{1}{N} = \frac{n \;\; m \;\; N_{gr}}{\left[N_g + n \;\; N_{gr}\right]\left[N_r + m \;\; N_{gr}\right]}. \qquad (13)$$

With $n = m = 1$, Eqn. (13) equals Eqn. (7). It should again be noted that Eqn. (13) only holds for experimental situations in which the dimensionless values of the relative photon counts per molecules and per second Q^R_B and Q^R_R are optically adjusted as given, for example, in Table **1**.

II. Correction in Cases with Substantial Quenching of the Two-Color Molecules

We now consider the absorption/emission scenario of Table **2** with quenching of the two-color molecules [4, 2]. We first introduce the parameters *R* that describe the relative quantum yield differences between bound (two-color molecules) and free labels (single-color molecules). *R* is defined as [2]

$$R_g = \frac{Q^G_B\left(bound \;\; species\right)}{Q^G_B\left(free \;\; species\right)}, \qquad (14)$$

$$R_r = \frac{Q^R_R\left(bound \;\; species\right)}{Q^R_R\left(free \;\; species\right)}, \qquad (15)$$

$$R \;\; = R_g \;\; R_r \;. \qquad (16)$$

Since the emission yield of a DNA-incorporated red nucleotide was the same as for the free non-incorporated red label, the relative emission yield becomes $R_r = \frac{Q^R_{R,\ DNA}}{Q^R_{R,\ free}} = \frac{\frac{323}{2}}{147.5} \;\; 1$. The parameter that describes the quenching effect upon incorporation of green nucleotides into the DNA is in terms of the relative fluorescence emission yield of a DNA-incorporated rhodamine-green nucleotide $R_g = \frac{Q^G_{B,\ DNA}}{Q^G_{B,\ free}} = \frac{\frac{15}{1}}{67.06} \approx 0.22$ for $n = 1$ and of a DNA-incorporated tetramethylrhodamine-nucleotide $R_g = \frac{Q^G_{B,\ DNA}}{Q^G_{B,\ free}} = \frac{\frac{46}{11}}{28.8} \approx 0.15$ for $n = 11$.

Table 2. Comparison of the Average Green and Red-Emitted Fluorescence Intensities Expressed in Photon Counts per DNA Molecule and Per Second. Reproduced in a Slightly Modified form from ref. [2]

Fluorescent-labeled two-color DNAs (A) and (B) measured number of × tags which are incorporated per DNA molecule on average	Absolute photon counts per DNA molecule and per second (x 10^3) measured in single-color excitation mode
(A) Target amplification*	
1 × rhodamine-green	15.0
1 × Cy5	149.0
(B) Two-color FCS signal amplification	
11 × tetramethylrhodamine	46.0
2 × Cy5	323.0
Single-color DNA	
6 × tetramethylrhodamine**	27.6**

* The template DNA was single-stranded (ss) M13mp18+ DNA obtained from Pharmacia Biotech. The hybridization (binding) sites of the forward and reverse 23-mer primers to amplify the 217 bp DNA were at template positions 6154 and 6370, respectively (Földes-Papp et al., 1997 [5]). We used 42 nM 5'-tagged rhodamine-green and Cy5 amplification primers (see Rigler et al., 1998 [1]). The hot-start reaction steps with AmpliTaq Gold® DNA polymerase (Perkin Elmer) were performed at optimal temperatures and cycle profiles to ensure specificity in early cycles as well as higher product yield: in brief, for example, a preincubation step at 95°C for 12 min; amplification for 10 cycles of 30 sec at 95°C, 30 sec at 65°C and then 72 °C, 2 min at 72°C followed by 20 cycles of 30 sec at 95°C, 30 sec at 65°C (217 bp), respectively, and 2 min plus 20 cycle elongation for each successive cycle at 72°C. Finally, an elongation step was carried out for 7 min at 72°C. Afterwards the temperature was held at 4°C until the PCR product was used. In addition, all PCR reactions were carried out in the presence of 3 µg of calf thymus DNA as a control.

**DNA master sequence developed for the first time in Földes- Papp et al. (2001b) [4]. Total substitution of dTTP by tetramethylrhodamine-6-dUTP (Roche Diagnostics) in the labeling PCR.

II.A. Single Labels Bound to or Incorporated into the Two-Color Molecules

Under most experimental conditions, the fluorescence intensities of the two-color species N_{gr} are quenched by a factor *R* relative to the free labels [2] (see, for example, Table **2**). When there is one green label and one red label bound to or incorporated into the two-color species, Eqn. (6) becomes

$$\tilde{G}(0) = \frac{N_g \left(\frac{Q_B^R}{Q_R^R} \right) + N_{gr} \quad R \left(1 + \frac{Q_B^R}{Q_R^R} \right)}{\left[N_g + N_{gr} \quad R_g \right] \left[N_r + N_g \left(\frac{Q_B^R}{Q_R^R} \right) + N_{gr} \quad R_r \left(1 + \frac{Q_B^R}{Q_R^R} \right) \right]} \tag{17}$$

Since photons of the green-emitted signal (green fluorophores) can be detected in the red channel (cross-talk), Eqn. (17) also gives the relative contribution of the cross-talk in one direction from the green channel to the red channel on the amplitude of the two-color cross-correlated signal for $Q_R^G \quad 0$.

Under the experimental conditions of the optical set-up $\frac{Q_B^R}{Q_R^R} << 1$, *and* with $N_g, N_r \approx N_{gr}$, Eqn. (17) simplifies to

$$\frac{1}{N} = \frac{N_{gr} \quad R}{\left[N_g + N_{gr} \quad R_g \right] \left[N_r + N_{gr} \quad R_r \right]} \tag{18}$$

for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$.

II.B. Multiple Labels **n** *and* **m** *Bound to or Incorporated into the Two-Color Molecules*

For the situation where *n* green labels are bound to or incorporated into the same target, Eqn. (17) for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$ was expanded to [2]

Using formula (20) for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$ and $R_r \quad 1$, $R_g \quad [0.15, 0.22]$, the differences in the N_{gr} values were about 20% compared with the Eqn. (13), fixing $R_r = R_g = 1$ (ref. [2]).

II.C. High-Density Labeling of Two-Color Molecules with Multiple n Green Labels and one Red Label (m = 1)

We exemplify the special case of high-density labeled DNA in which one nucleotide is completely substituted by its green-fluorescent analogue, with $\bar{n} = 85$ and $m = 1$ [3]. Supposing that the heavily green-labeled molecule shows a dramatic effect of green fluorescence quenching of approximately 95% or more, one further useful procedure is to apply Eqn. (10) [3]. Instead of introducing the relative but uniform quantum yield difference *R* between bound (two-color molecule) and free labels (single-color molecules), we worked with $\tilde{Q}_B^G$ representing the quenching of green tags in the two-color molecule and with $\tilde{Q}_B^R$ standing for the quenched cross-talk from green to red [3]. The advantage of this is that Eqn. (10) for the fraction of the cross-correlation term A_{gr} with $0 < A_{gr} \leq 1$ can now be written as

$$\tilde{G}(0) = \frac{N_g \frac{Q_B^R}{Q_R^R} + N_{gr} \quad R \left[n \left(m + n \frac{Q_B^R}{Q_R^R} \right) \right]}{\left[N_g + nN_{gr} \quad R_g \right] \left[N_r + N_g \frac{Q_B^R}{Q_R^R} + N_{gr} \quad R_r \left(m + n \frac{Q_B^R}{Q_R^R} \right) \right]} . \tag{19}$$

In the case $\frac{Q_B^R}{Q_R^R} << 1$ and $n \quad \frac{Q_B^R}{Q_R^R} < 1$, we simplified to

$$\frac{1}{N} = \frac{n \quad m \quad N_{gr} \quad R}{\left[N_g + n \quad N_{gr} \quad R_g \right] \left[N_r + m \quad N_{gr} \quad R_r \right]} . \tag{20}$$

$$\tilde{G}(0) = \frac{N_g Q_B^G Q_B^R + N_r Q_R^R Q_R^G + N_{gr}\left(n\tilde{Q}_B^G m Q_R^R + n\tilde{Q}_B^G n\tilde{Q}_B^R + m Q_R^R m Q_R^G\right)}{\left[N_g Q_B^G + N_r Q_R^G + N_{gr}\left(n\tilde{Q}_B^G + m Q_R^G\right)\right]\left[N_g Q_B^R + N_r Q_R^R + N_{gr}\left(m Q_R^R + n\tilde{Q}_B^R\right)\right]} . \quad (21)$$

Multiplying numerator and denominator with $\frac{1}{Q_B^G \; Q_R^R}$ and setting-up $Q_R^G \quad 0$ yields

$$\tilde{G}(0) = \frac{N_g \frac{Q_B^R}{Q_R^R} + N_{gr}\left(n\frac{\tilde{Q}_B^G}{Q_B^G} m + n\frac{\tilde{Q}_B^R}{Q_R^R}\right)}{\left[N_g + N_{gr}\left(n\frac{\tilde{Q}_B^G}{Q_B^G}\right)\right]\left[N_r + N_g \frac{Q_B^R}{Q_R^R} + N_{gr}\left(m + n\frac{\tilde{Q}_B^R}{Q_R^R}\right)\right]} . \quad (22)$$

According to the experimental (realistic) case $\frac{Q_B^R}{Q_R^R} << 1$ in the optical set-up, what immediately follows for the fraction of the cross-correlation term A_{gr} with $\frac{\tilde{Q}_B^G}{Q_B^G} = \frac{\tilde{Q}_B^G}{1}$, $n\ \frac{\tilde{Q}_B^R}{Q_R^R} << 1$, and m = 1 (ref. [3]) is

$$\frac{1}{N} = \frac{N_{gr} \quad n\ \tilde{Q}_B^G}{\left[N_g + N_{gr} \quad n\ \tilde{Q}_B^G\right]\left[N_r + N_{gr}\right]} . \quad (23)$$

Taking into account the (measured) number of heavily green-labeled two-color molecules

$$\tilde{N}_{gr} = N_{gr} \quad n\ \tilde{Q}_B^G , \quad (24)$$

where N_{gr} is the numerical equivalent of two-color molecules in the case of negligible interactions (quenching) between the internal incorporated dyes and their surroundings, we obtain for the experimental case $N_{gr} << N_r$ [3]

$$\frac{1}{N} = \frac{\tilde{N}_{gr}}{\left[N_g + \tilde{N}_{gr}\right]\left[N_r\right]} . \quad (25)$$

But the solution of this is just

$$\tilde{N}_{gr} = \frac{N_g \quad N_r}{N - N_r} \quad (26)$$

Here N_g is the number of free green dye molecules, i.e. green dye nucleotides, and N_r is the number of free red dye molecules, i.e. red dye nucleotides [3]. $\tilde{Q}_B^G$ could be independently determined by green-color autocorrelation in single-color excitation mode [3].

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