

Anomalous Fluorescence Enhancement of Cy3 and Cy3.5 versus Anomalous Fluorescence Loss of Cy5 and Cy7 upon Covalent Linking to IgG and Noncovalent Binding to Avidin

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This study provides a critical examination of protein labeling with Cy3, Cy5, and other Cy dyes. Two alternate situations were tested. (i) Antibodies were covalently labeled with Cy dye succinimidyl ester at various fluorophore/protein ratios and the fluorescence of the labeled antibodies was compared to that of free Cy dye. (ii) Fluorescent biotin derivatives were synthesized by derivatizing ethylenediamine with one biotin and one Cy3 (or Cy5) residue. The fluorescence properties of these biotin–Cy dye conjugates were examined at all ligand/(strept)avidin ratios ($0 \leq n \leq 4$). The results showed an astounding discrepancy between Cy3 and Cy5: Cy3-labeled antibodies fluoresced very well, even at high Cy3/protein ratios, and the same applied to (strept)avidin with up to four bound biotin–Cy3 conjugates. In contrast, antibodies with six covalently bound Cy5 labels (obtained with the recommended procedure) were almost nonfluorescent, only at 2–3 Cy5 labels/IgG some moderate fluorescence was obtained. By analogy, the biotin–Cy3 conjugate fluoresced intensely, even at high ligand/avidin ratio, in contrast to the weakly fluorescing biotin–Cy5 conjugate. Three mechanisms are responsible for the discrepancy between Cy3 and Cy5. (i) Attachment of Cy3 to a protein's surface causes an anomalous enhancement in fluorescence (by 2–3-fold) while no enhancement occurs with Cy5. (ii) Mutual quenching of IgG-bound Cy dyes by resonance energy transfer is much more pronounced for Cy5 labels than for Cy3. (iii) In IgG with six bound Cy5 labels, about one-third of the labels adopt a nonfluorescent state which is characterized by a large UV–vis absorption maximum at 600 nm instead of at 650 nm. Cy3.5 was found to mimic the properties of Cy3, while Cy7, and to some extent also Cy5.5, were similar to Cy5. In conclusion the Cy dye series is divided into two groups: Antibodies with multiple Cy3 or Cy3.5 labels yield bright fluorescence while extensive quenching occurs in antibodies labeled with Cy5 and Cy7.

INTRODUCTION

Immunofluorescence microscopy has been, and still is, an indispensable tool of cell biology and histology. It provides for specific visualization of one or two particular target proteins in live or fixed cells while all other biomolecules remain invisible, except for unavoidable autofluorescence of biomatter (Aubin, 1979; Benson et al., 1979; Marelus, 1995; Wolfbeis and Leiner, 1985). Usually the fluorescent labels are coupled to the lysine residues of the target-specific antibodies in a statistical way, and high fluorophore/antibody ratios are generally preferred for two purposes: (i) to maximize the fluorescence signal from each antibody molecule and (ii) to minimize the fraction of unlabeled or too weakly labeled antibodies in the total pool. Beyond some optimal fluorophore/antibody ratio, however, the fluorescence signal per antibody will decrease, rather than increase, due to resonance energy transfer between fluorophores on one protein molecule (Steinberg, 1971).

The parameters of optimal labeling are well documented for fluorescein, TMR, Texas Red, as well as the Bodipy and Alexa dye series (Goding, 1976; Haugland, 1995; Haugland, 1996). In contrast, no explicit information of that kind is available on the Cy dye series. This is unfortunate because Cy3 and Cy5 are most promising labels for single molecule fluorescence microscopy (SM-FM),¹ Cy3 because of exceptional brilliance (Mujumdar et al., 1993; Wessendorf and Brelje, 1992) and outstanding photostability (G. S. Harms, unpublished results) and Cy5 (or Cy7) because of excitation at wavelengths above

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¹ Abbreviations: Biotin-4-Cy, biotin-4-Cy3 and/or biotin-4-Cy5; biotin-4-Cy3 and *biotin-4-Cy5* denote the structures shown in Scheme 1; biotin-4-fluorescein, 5-(and 6)-[2-(biotinoyl)-aminoethylaminocarbonyl]-fluorescein; BSA, bovine serum albumin; Cy3-PE, Cy3-labeled phosphatidylethanolamine; DIEA, *N,N*-diisopropyl-*N*-ethylamine; DMF, dimethylformamide; EGF, epidermal growth factor; HgTx, hongotoxin; HgTx-A19C, mutant of HgTx in which alanine-19 is replaced by cysteine; Ig, bovine gamma globulins; IgG, goat immunoglobulin G; Ig(G), bovine Ig or goat IgG; MS, mass spectrometry; NHS, *N*-hydroxysuccinimide; PEG, poly(ethylene glycol); RT, room temperature (22–25 °C); SbTMU, *O*-(*N*-succinimidyl)-*N,N,N,N*-bis-(tetramethylene)-uronium hexafluorophosphate; SDS, sodium dodecyl sulfate; (strept)avidin, avidin and/or streptavidin; SMFM, single molecule fluorescence microscopy; TMR, tetramethylrhodamine. See Supporting Information for abbreviations of Cy dyes.

600 nm where autofluorescence of biomatter is much reduced (Aubin, 1979; Benson et al., 1979; Marelus, 1995; Wolfbeis and Leiner, 1985).

In the present study, the parameters of antibody labeling with Cy dyes were critically examined. To our great surprise the Cy dye series was found to be divided into two groups with widely different fluorescence properties, imposing limitations on the use of Cy5 and Cy7 for immunofluorescence microscopy, especially in SMFM.

EXPERIMENTAL PROCEDURES

Materials. Analytical-grade materials were used as long as they were commercially available. Cy3-, Cy3.5-, Cy5-, and Cy5.5-monofunctional dye, as well as Cy7-bifunctional dye, QAE Sephadex A-25, and PD-10 columns were obtained from Amersham Pharmacia Biotech. Bovine Ig (γ globulins), goat IgG, BSA, *d*-biotin, avidin, and streptavidin were purchased from Sigma. LiChroprep gel was obtained from Merck.

Buffers. Buffer A contained 100 mM NaCl, 50 mM NaH_2PO_4 , and 1 mM EDTA (pH 7.5 adjusted with NaOH). Buffer B was a 3/1 mixture of buffer A and 2 M NaCl. Buffer C contained 150 mM Na_2CO_3 (pH 9.3 adjusted with H_3PO_4). Buffer D contained 150 mM NaCl and 5 mM NaH_2PO_4 (pH 7.4 adjusted with NaOH).

Measurement of Cy Dye Concentrations. The molar extinction coefficients of the Cy dyes in methanolic solution are known ($\epsilon_{550} = 150\,000\text{ M}^{-1}\text{ cm}^{-1}$ for Cy3, $\epsilon_{581} = 150\,000\text{ M}^{-1}\text{ cm}^{-1}$ for Cy3.5, $\epsilon_{650} = 250\,000\text{ M}^{-1}\text{ cm}^{-1}$ for Cy5, $\epsilon_{678} = 250\,000\text{ M}^{-1}\text{ cm}^{-1}$ for Cy5.5, and $\epsilon_{750} = 250\,000\text{ M}^{-1}\text{ cm}^{-1}$, for Cy7, according to the manufacturer's data sheets). Here it was found that the absorption changed very little when the Cy dyes were dissolved in aqueous buffer. Covalent attachment of the Cy dyes to protein was also seen to have little effect, except for a small bathochromic shift (see Figure 7 and Figures 8A and 9A in the Supporting Information). Unless stated otherwise, all Cy dye concentrations (including those of biotin-4-Cy3 and biotin-4-Cy5) were calculated from the absorbance values at λ_{max} , assuming the above cited molar extinction coefficients.

Covalent Labeling of Proteins with Cy Dyes. Goat IgG, bovine Ig, or BSA were reacted with Cy3-, Cy3.5-, Cy5-, or Cy5.5-monofunctional dye, as well as with Cy7-bifunctional dye, either according to the manufacturer's recommendations (solid symbols in Figures 1–3) or at lower dye/protein ratios (open symbols in Figures 1–3). One portion of Cy dye ("to label 1 mg of protein") was dissolved in 50 μL of DMSO, and from this stock solution a series of dye/DMSO solutions was prepared which corresponded to a consecutive dilution by a factor of 2 in each dilution step (as an exception, the first two dilution steps were by a factor of 4 in the Cy5 series in Figure 2). Labeling was started by mixing 0.2 mL of protein (1 mg/mL in buffer C) with 10 μL of dye/DMSO solution and vortexing was repeated after 10 and 20 min. After 30 min reaction time at RT (with mixing after 10 and 20 min) the protein with bound Cy dye was separated from unreacted Cy dye by gel filtration in buffer A [same protocol as used for avidin (see Supporting Information)]. The protein concentrations in the void peak were calculated from A_{280} ($\epsilon_{280} = 210\,000\text{ M}^{-1}\text{ cm}^{-1}$ for goat IgG according to the manufacturer's data sheet; $\epsilon_{280} = 174\,000\text{ M}^{-1}\text{ cm}^{-1}$ for bovine Ig, determined experimentally; $\epsilon_{280} = 44\,300\text{ M}^{-1}\text{ cm}^{-1}$ for BSA, Elgersma et al., 1990), taking into account the absorbance contribution from covalently bound Cy dye at 280 nm ($0.08 \times A_{550}$ for Cy3, $0.24 \times A_{580}$

for Cy3.5, $0.05 \times A_{650}$ for Cy5, and $0.18 \times A_{680}$ for Cy5.5, according to the manufacturer's instructions; $0.11 \times A_{750}$ for Cy7, determined from the absorption spectrum of Cy7-bifunctional dye in buffer A). The Cy dye concentrations in the protein peaks were measured by absorbance as described above.

Synthesis of Biotin-4-Cy3 and Biotin-4-Cy5 (see Scheme 1 and Supporting Information). All operations were carried out under dim light, and light was further excluded by wrapping flasks and glass columns with aluminum foil. Cy3-monofunctional dye (1.4 μmol) or Cy5-monofunctional dye (1.0 μmol) was dissolved in 1 mL of DMF containing 8.4 mg SbTMU (20.4 μmol , Bannwarth and Knorr, 1991), and 5 μL neat DIEA (57 μmol) was added. After 40 min of stirring at RT this mixture was added to dry biotin-NH-CH₂-CH₂-NH₃⁺ Cl[−] [prepared according to Garlick and Giese (1988)]. Another 20 μL of neat DIEA (230 μmol) was added and stirring was continued under argon for 1 h at RT. The solvent was removed and the dry residue sequentially chromatographed on QAE Sephadex A-25 (Gruber et al., 1997), on LiChroprep gel (Gruber et al., 2000), and on an HPLC C-18 column (Vydac no. 218TP510) as described in the Supporting Information. The compounds were found to be pure by HPLC and by electrospray MS, and the correct molecular structures were proven by NMR spectroscopy (see Supporting Information).

Fluorescence Measurements of Cy-Dye-Labeled Immunoglobulins and of Biotin-4-Cy3 and Biotin-4-Cy5 in Absence and Presence of (Strept)avidin. All measurements were done in buffer A at 25 °C. Cy dye-labeled goat IgG or bovine Ig were consistently diluted to 50 nM Cy label concentration (according to absorbance at λ_{max} , see above). In the control experiments, the fluorescence of commercial Cy3- and Cy5-monofunctional dye or Cy7-bifunctional dye was also measured at 50 nM chromophore concentration.

For characterization of biotin-4-Cy3 and biotin-4-Cy5, 2 mL of 40 nM avidin [freshly prepared from the standardized 1.75 μM avidin stock solution (see Supporting Information)] or 2 mL 40 nM streptavidin [freshly prepared from the standardized 3.03 μM stock solution (see Supporting Information)] were stirred in the cuvette and 2 μL volumes of biotin-4-Cy3 [12.3 μM stock solution (see Supporting Information)] or biotin-4-Cy5 [16.7 μM stock solution (see Supporting Information)] were successively added from a Hamilton syringe at 3 min intervals and Cy dye fluorescence at the end of each 3 min interval was recorded (see Figure 4).

RESULTS

Fluorescence Properties of Cy3-, Cy3.5-, Cy5-, Cy5.5-, and Cy7-Labeled Proteins. All samples of labeled protein were diluted to the same Cy label concentration (50 nM according to Cy dye absorption) and the fluorescence signals were compared to the fluorescence intensities of 50 nM free Cy dye in buffer (see Figures 1A, 2A, and 3A). At the lowest numbers of covalently bound Cy labels per Ig(G), many protein molecules were actually unlabeled and most of the labeled protein molecules carried a single Cy dye only. Extrapolation to 0 labels/Ig(G), therefore, gave the fluorescence of exactly mono-labeled Ig(G) in comparison to free fluorophore in buffer. As can be seen in Figures 1A, 2A, and 3A, Ig(G) molecules with a single Cy5 or Cy5.5 label (extrapolation of the dashed lines to $x = 0$) or with a single Cy7 label (extrapolation of the dotted line in Figure 2A to $x = 0$) had almost the same fluorescence

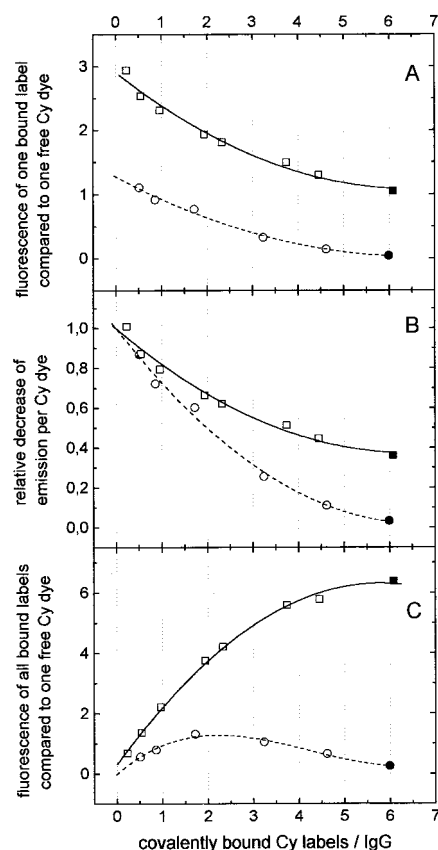


Figure 1. Covalent labeling of goat IgG with Cy3 (squares)- or Cy5-monofunctional dye (circles). IgG (1 mg/mL in buffer C) was reacted for 30 min at RT with amine-reactive Cy dye, either according to the manufacturer's instructions (solid symbols) or at lower dye/protein ratio (open symbols) and IgG with covalently bound Cy dye was separated from unreacted Cy dye by gel filtration. (A) IgG with covalently bound Cy dye was diluted to 50 nM Cy3 or Cy5 concentration (according to absorbance at 550 or 650 nm), and the fluorescence yield of IgG-bound label was compared to that of 50 nM free Cy3- or Cy5-monofunctional dye, respectively. Cy3 or Cy5 were excited at 530 or 630 nm, and the emission was measured at 565 or 665 nm, respectively. (B) Progressive fluorescence decrease of IgG-bound Cy3 (squares) or Cy5 labels (circles). The ordinate values from panel A were divided by the extrapolated intensities at 0 labels/protein in panel A. (C) The cumulative fluorescence yield of one labeled IgG molecule was calculated by multiplying the average fluorescence yield of one IgG-bound fluorophore (ordinate values in panel A) by the average number of fluorophores per IgG (see Experimental Procedures). The solid symbols in (A–C) indicate results obtained by the standard procedure.

yield as free Cy5, Cy5.5, or Cy7, respectively. Higher numbers of Cy5 or Cy7 labels, however, led to such a rapid drop in fluorescence (dashed and dotted lines in Figures 1A and 2A) that even the cumulative fluorescence of all Ig(G)-bound labels together started to drop beyond 3 fluorophores/protein (dashed and dotted lines in Figures 1C and 2B). It is concluded that, in practice, antibody molecules should carry no more than 2–4 Cy5 or Cy7 labels which is far less than obtained with the recommended procedure (solid circles and triangles in Figures 1 and 2). A more favorable result was obtained with Cy5.5. While, in general, Cy5.5 (circles in Figure 3) followed a similar pattern as Cy5 (circles in Figures 1 and 2), much less quenching occurred in antibodies labeled with ~5 Cy5.5 labels/protein molecule (solid circle in Figure 3B), thus the stoichiometry of Cy5.5 over IgG is less critical, and higher levels of fluorescence per antibody can be achieved with Cy5.5 than with Cy5.

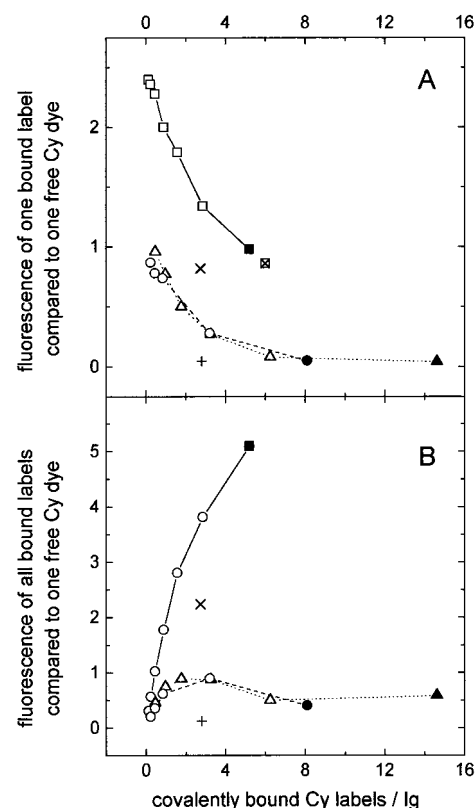


Figure 2. Covalent labeling of bovine Ig with Cy3 (squares), Cy5 (circles), and Cy7 (triangles). Ig (1 mg/mL in buffer C) was reacted for 30 min at RT with amine-reactive Cy dye, either according to the manufacturer's instructions (solid symbols) or at lower dye/protein ratio (open symbols) and Ig with covalently bound Cy dye was separated from unreacted Cy dye by gel filtration. (A) Gel-filtered Ig with covalently bound Cy dye was diluted to 50 nM Cy3, Cy5, or Cy7 concentration, and the fluorescence yield of Ig-bound label was compared to that of 50 nM free Cy3, Cy5, and Cy7, respectively. Excitation was at 530, 630, or 730 nm and the emission was measured at 570, 670, or 770 nm, respectively. (B) The cumulative fluorescence yield of one labeled Ig molecule was calculated by multiplying the average fluorescence yield of one Ig-bound fluorophore (ordinate values in panel A) by the average number of fluorophores per Ig (see Experimental Procedures). The solid symbols in (A and B) indicate results obtained with bovine Ig by the standard procedure. Labeling of BSA (at 1 mg/mL) with Cy3 (×) or Cy5 (+) by the standard procedure is also shown in (A and B).

To our great surprise, extremely bright fluorescence was seen in Cy3- and Cy3.5-labeled antibodies. At high fluorophore/antibody ratio (see solid squares in Figures 1C, 2B, and 3B) each individual Cy3 or Cy3.5 label fluoresced with the same average intensity as one free Cy3 or Cy3.5 dye in buffer (see solid squares in Figures 1A, 2A, and 3A). At lower numbers of Cy dye per Ig(G) the fluorescence per Cy3 or Cy3.5 was even higher (solid lines in Figures 1A, 2A, and 3A). Extrapolation to 0 labels/protein indicated that goat IgG with a single Cy3 label was three times more fluorescent (solid line in Figure 1A), bovine Ig with a single Cy3 label was 2.5 times more fluorescent (solid line in Figure 2A) than free Cy3 in buffer, and a similar enhancement in fluorescence was seen at low Cy3.5 numbers per IgG (solid line in Figure 3A).

The fluorescence of Cy dye-labeled BSA followed the same general pattern as observed with Ig(G). BSA with 2.7 bound Cy3 labels fluoresced quite well (× in Figure 2) while BSA with 2.8 Cy5 labels was almost nonfluorescent (+ in Figure 2). The lower fluorescence of Cy3-BSA (× in Figure 2A) in comparison to Cy3-Ig (squares

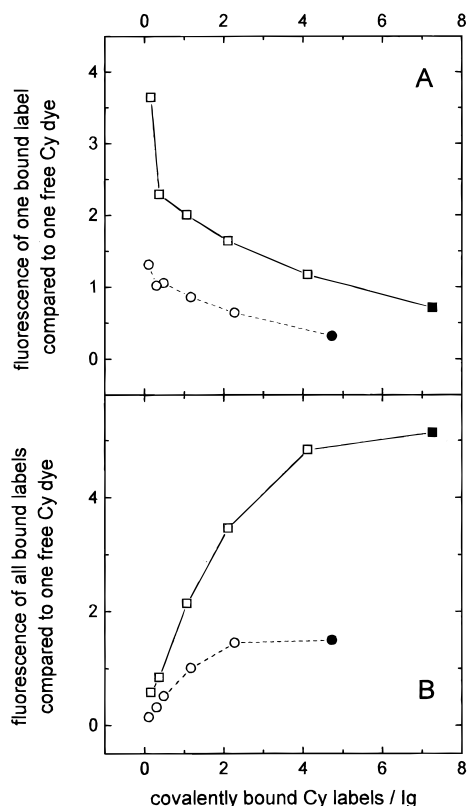


Figure 3. Covalent labeling of goat IgG with Cy3.5- (squares) or Cy5.5-monofunctional dye (circles). IgG (1 mg/mL in buffer C) was reacted for 30 min at RT with amine-reactive Cy dye, either according to the manufacturer's instructions (solid symbols) or at lower dye/protein ratio (open symbols) and IgG with covalently bound Cy dye was separated from unreacted Cy dye by gel filtration. (A) IgG with covalently bound Cy dye was diluted to 50 nM Cy3.5 or Cy5.5 label concentration (according to absorbance at 580 or 680 nm), and the fluorescence yield of IgG-bound label was compared to that of 50 nM free Cy3.5- or Cy5.5-monofunctional dye, respectively. Cy3.5 or Cy5.5 were excited at 560 or 655 nm, and the emission was measured at 600 or 700 nm, respectively. (B) The cumulative fluorescence yield of one labeled IgG molecule was calculated by multiplying the average fluorescence yield of one IgG-bound fluorophore (ordinate values in panel A) by the average number of fluorophores per IgG (see Experimental Procedures). The solid symbols in (A and B) indicate results obtained by the standard procedure.

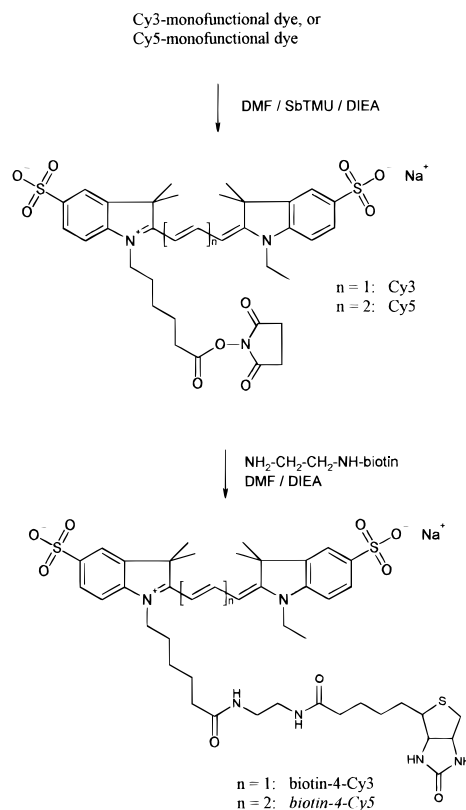
in Figure 2A) was largely explained by the different size of the two proteins. BSA with 2.7 Cy3 labels was to be compared to Ig having the same average mutual distance of Cy3 labels on the protein. Taking into account the elongated structures of both BSA (Andrade et al., 1992) and IgG, the average Cy3–Cy3 distance should roughly correlate with the mass ratios of Cy3 over protein. In other words, BSA with 2.7 bound Cy3 was expected to have similar fluorescence as Ig with 6 bound Cy3 labels. The corresponding data point was generated by extrapolation (crossed square in Figure 2A) and indeed the calculated fluorescence yield almost coincided with the observed fluorescence of BSA with the same Cy3/protein mass ratio (\times in Figure 2A). These data indicate that the dramatic difference between Cy3 on one hand and Cy5 (or Cy7) on the other was not a peculiarity of antibody labeling but a general consequence of statistical protein labeling with amine-reactive Cy dyes.

In the following, it was tested whether the anomalous enhancement of Cy3 fluorescence would also occur when Cy3 was noncovalently linked to the surface of (strept)-avidin via biotin anchors. As described below, the answer

to this question was indeed positive, giving rise to discovery of the first small biotin derivative with strong fluorescence in the (strept)avidin-bound state (Gruber et al., 1997, 1998; Kada et al., 1999).

Synthesis and Characterization of Biotin-4-Cy3 and Biotin-4-Cy5. Biotin-4-Cy3 and biotin-4-Cy5 were prepared by the same route as previously described for biotin-4-fluorescein (Kada et al., 1999), except that an excess of biotin-NH-CH₂-CH₂-NH₂ had to be reacted with a small amount of Cy dye, due to the high cost of the latter. The large excess of biotin-NH-CH₂-CH₂-NH₂ and of the other reagents was efficiently removed by a combination of anion exchange (Gruber et al., 1997) and reversed phase chromatography (Gruber et al., 2000). Complete activation of commercial Cy dye with SbTMU (step 1 in Scheme 1) and the essentially complete Cy dye

Scheme 1. Syntheses of Biotin-4-Cy3 and Biotin-4-Cy5 from Commercial Cy3- and Cy5-Monofunctional Dye



coupling to biotin-NH-CH₂-CH₂-NH₂ (step 2 in Scheme 1) guaranteed that every Cy dye was linked to a biotin moiety. Residual traces of free Cy dye were easily removed because binding to QAE-Sepharose was tighter (Gruber et al., 1997) and binding to LiChroprep gel was less tight (Gruber et al., 2000) than observed with the products.

The correct structures of biotin-4-Cy3 and biotin-4-Cy5 were proven by NMR and electrospray MS (see Supporting Information). In both cases, binding studies with avidin showed that 100% of the colored material was specifically bound by avidin (see Supporting Information), i.e., every Cy dye was chemically linked to a biotin group via an ethylenediamine spacer (see Scheme 1). The molar concentrations of biotin-4-Cy3 and biotin-4-Cy5 were also accurately measured by quantitative assessment of binding site occupation in standardized avidin and strept-avidin samples, using endogeneous tryptophane fluorescence in (strept)avidin to monitor ligand binding (see Supporting Information).

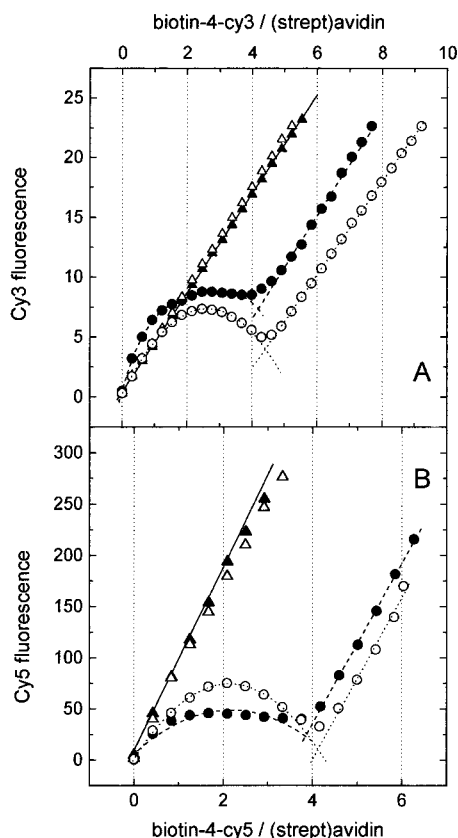


Figure 4. Fluorescence of biotin-4-Cy3 and *biotin-4-Cy5* in the presence (circles) or absence (solid lines) of avidin or streptavidin. Avidin (40 nM, solid circles) or streptavidin (40 nM, open circles) was titrated with increasing amounts of biotin-4-Cy3 (panel A) or *biotin-4-Cy5* (panel B) while monitoring Cy3 fluorescence (excitation at 530 nm, emission at 570 nm) or Cy5 fluorescence (excitation at 630 nm, emission at 665 nm), respectively. In the presence of 8 μ M *d*-biotin neither avidin (solid triangles) nor streptavidin (open triangles) had an effect on the fluorescence of biotin-4-Cy3 or *biotin-4-Cy5*. For the sake of clarity, only the linear fits (solid lines) through the control data points (absence of avidin and streptavidin) are shown.

Fluorescence of Biotin-4-Cy3 and *Biotin-4-Cy5* before/after Binding to Avidin and Streptavidin.

(Strept)avidin samples with known *functional* concentrations (see Supporting Information) (=biotin-binding sites/4) were titrated with biotin-4-Cy3 or *biotin-4-Cy5* working solutions. The *functional* concentrations (=biotin group concentration) of the latter were also known from prior standardization (see Supporting Information). Cy dye fluorescence was measured during cumulative titrations and plotted versus known ligand/protein ratios (see Figure 4). In the absence of (strept)avidin (solid lines) or when (strept)avidin had been pre-blocked with *d*-biotin (triangles) the addition of biotin-4-Cy3 or of *biotin-4-Cy5* gave rise to a steep linear increase in Cy dye fluorescence, as expected for absence of interaction with (strept)avidin.

When functional avidin or streptavidin was titrated with *biotin-4-Cy5* (solid or open circles in Figure 4B, respectively) a similar profile was obtained as with all previously tested short biotin-fluorophore conjugates (Gruber et al., 1997; Gruber et al., 1998; Kada et al., 1999): Between 0 and 2 ligands/(strept)avidin the fluorescence signal showed a weak rise, whereas further addition of *biotin-4-Cy5* caused a *decrease* in fluorescence, minimal fluorescence occurring at 4 ligands/(strept)avidin tetramer. The steep linear rise at >4 ligands/protein was parallel to the control series (solid line and triangles in Figure 4B) and evidently derived from the intense

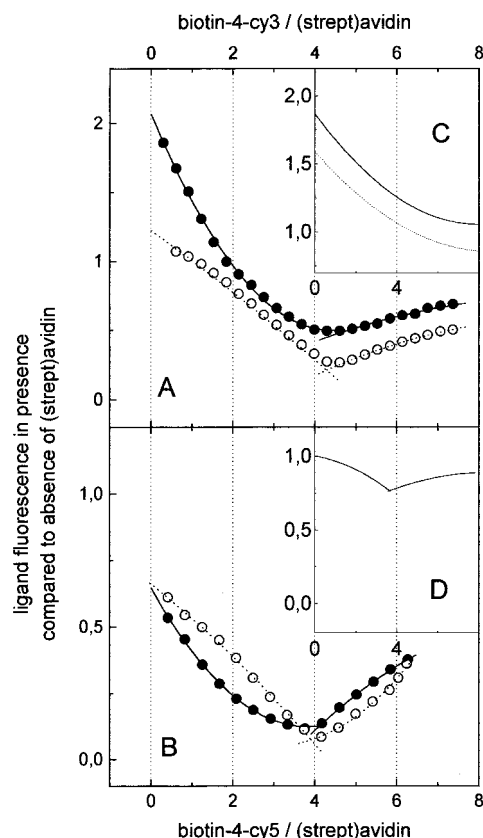


Figure 5. Persistence of biotin-4-Cy3 fluorescence (A) and *biotin-4-Cy5* fluorescence (B) after binding to avidin (solid circles) or streptavidin (open circles). The data were calculated by dividing fluorescence in the presence of (strept)avidin (circles in Figure 4) by fluorescence in absence of protein (solid lines in Figure 4). For comparison, the effect of avidin (solid lines) and streptavidin (dotted line) upon the fluorescence of a biotin-PEG₈₀₀-Cy3 conjugate (C) and a biotin-PEG₁₉₀₀-Cy5 conjugate (D) is shown in the insets; the data are taken from Gruber et al. (1996).

fluorescence of unbound ligands which were in excess. The relative extent of (strept)avidin-induced fluorescence quenching is shown in Figure 5B. At the smallest ligand/protein ratio a large fraction of (strept)avidin molecules carried no ligand at all and most of the remaining (strept)avidins were occupied by a single *biotin-4-Cy5* molecule. Extrapolation to 0 ligands/(strept)avidin in Figure 4B obviously gave an estimate for the fluorescence of (strept)avidin tetramers with a single bound *biotin-4-Cy5* molecule (~65% in comparison to the fluorescence of unbound *biotin-4-Cy5*, see Figure 5B). In 4:1 complexes with (strept)avidin, however, *biotin-4-Cy5* fluorescence was quenched by ~90% (see Figure 5B). The loss in *biotin-4-Cy5* fluorescence at high ligand/protein ratio resembled the loss in Cy5 fluorescence observed at high Cy5/IgG ratios (circles in Figures 1A and 2A); the similarity was even more pronounced when the cumulative fluorescence yields were plotted for different ratios of *biotin-4-Cy5* over (strept)avidin (compare Figure 6B with the circle series in Figure 1C). In both cases the optimal fluorescence yield was seen at ~2 bound Cy5 labels/protein.

In contrast to the biotin-Cy5 conjugate, biotin-4-Cy3 excelled with bright fluorescence even after binding to avidin and streptavidin (see Figures 4A, 5A, and 6A). In the case of avidin the anomalous fluorescence increase reached a factor of ~2 when a single biotin-4-Cy3 molecule was bound per tetramer (extrapolation of the solid line in Figure 5A to 0 ligands/avidin) while this

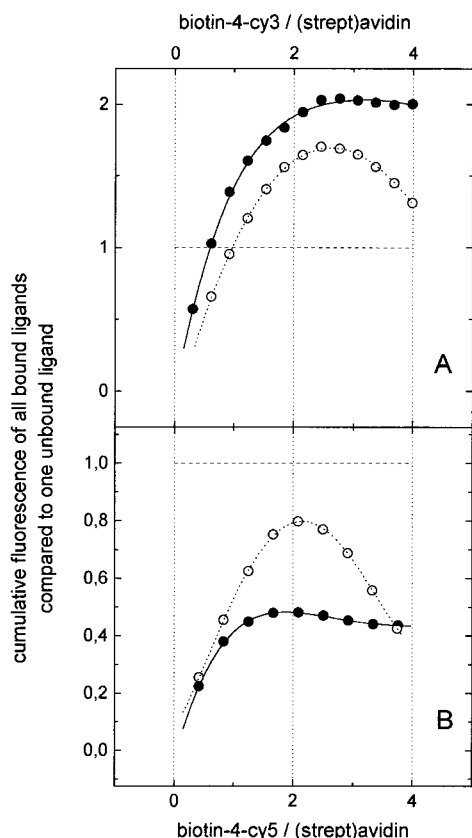


Figure 6. Total Cy3 or Cy5 fluorescence from one avidin (solid circles) or streptavidin molecule (open circles) with $0 \leq n \leq 4$ bound biotin-4-Cy3 (A) or *biotin-4-Cy5* (B), normalized to the fluorescence of one free biotin-4-Cy3 or *biotin-4-Cy5* molecule, respectively. These cumulative fluorescence yields were calculated by multiplying the average fluorescence yield of one (strept)avidin-bound ligand (ordinate values in Figure 5A and Figure 5B) by the average number of bound ligands (abscissa values in Figure 5A and Figure 5B), respectively.

effect was small in case of streptavidin (extrapolation of the dotted line in Figure 5A to 0 ligands/streptavidin). Admittedly some Cy3 quenching did occur at >2 ligands/(strept)avidin (Figure 5A) but the cumulative fluorescence at $2 \leq n \leq 4$ ligands/protein was always larger than that of one free biotin-4-Cy3 (Figure 6A), with a plateau at 2-fold fluorescence in the case of avidin (solid circles). Comparison with the square series in the Figures 1C and 2B reveals that Cy3 fluorescence saturated much faster at high ligand/avidin ratios than at comparable Cy3/Ig-(G) ratios. Nevertheless the remarkable superiority of Cy3 over Cy5 (compare squares with circles in Figures 1C and 2B) was retained when switching from covalent Cy dye labeling to noncovalent binding of short biotin-Cy dye conjugates to (strept)avidin (compare Figure 6A with Figure 6B).

Absorption and Fluorescence Spectra of Cy5 Labels at High Ratios of Label/Protein. One reason for the brilliant fluorescence of Cy3-labeled antibodies (and of avidin-bound biotin-4-Cy3) has already been identified: Binding of one Cy3 label to a protein's surface generally caused an anomalous fluorescence increase by a factor of 3 (Figure 1A), 2.5 (Figure 2A), or 2 (solid line in Figure 5A). However, this was not the only reason proteins with a large number of Cy3 labels were much more fluorescent than proteins with the same number of Cy5 labels (see Figures 1C, 2B, and 6). Figure 1B demonstrates that Cy5 fluorescence suffers from much stronger "quenching" at high fluorophore/protein ratio than does Cy3. This effect was most pronounced in the

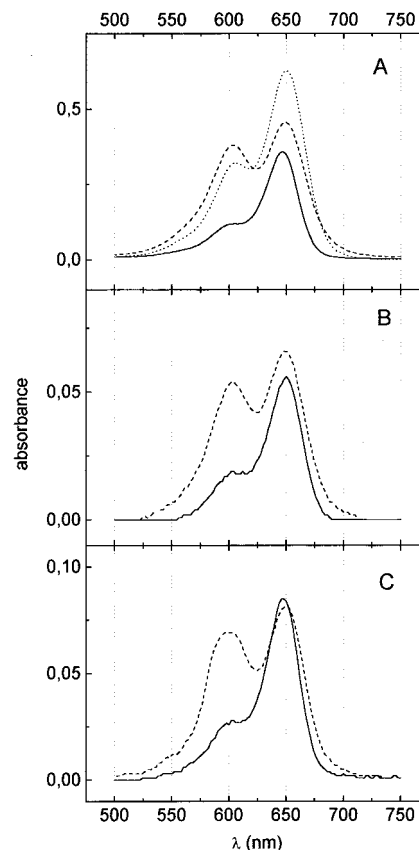


Figure 7. Absorption spectra of free and protein-bound Cy5-labels. (A) Goat IgG with 6.0 bound Cy5 labels/protein in absence (dashed line) and in the presence of 1% SDS (dotted line). The spectrum of free Cy5-monofunctional dye is shown for comparison (solid line). (B) Goat IgG with 6.0 bound Cy5 labels (same sample as in panel A) has been diluted with 9 volumes of buffer A (dashed line). For comparison, goat IgG with 0.5 bound Cy5 labels/protein is shown (solid line). (C) Free *biotin-4-Cy5* (0.29 μ M, solid line) is compared with an equimolar mixture of *biotin-4-Cy5* and avidin (both 0.5 μ M, dashed line).

case of covalently labeled IgG (see Figure 1B). Attachment of 6 Cy5/IgG caused a 97% (!) drop in fluorescence yield per Cy5 label (open circles) while 36% of initial fluorescence per Cy3 label was retained in IgG with 6 Cy3 labels (solid squares). A similar pattern was observed with avidin and streptavidin, i.e., quenching of biotin-4-Cy3 was consistently weaker than of *biotin-4-Cy5* (not shown).

The moderate quenching of Cy3 at higher degrees of protein labeling (squares in Figure 1B) was comparable to quenching in fluorescein- or TMR-labeled proteins (Goding, 1976; Haugland, 1995). Resonance energy transfer between the fluorophores on the identical protein molecule is known to be the main mechanism of quenching in this case (Steinberg, 1971). The anomalous extent of Cy5 quenching (circles in Figure 1B), however, was caused by an additional mechanism besides resonance energy transfer: At high degrees of Cy5 labeling the regular absorption maximum at ~ 650 nm was reduced, an additional absorption maximum at ~ 600 nm was observed (see dashed spectra in Figure 7), and the latter derived from a nonfluorescent state of Cy5 (see Figures 8 and 9 in the Supporting Information).

The normal absorption spectrum of free Cy5 dye is shown in Figure 7A (solid trace). Almost the same spectrum was obtained for IgG with 0.5 bound Cy5 labels (solid trace in Figure 7B) and for free *biotin-4-Cy5* (solid trace in Figure 7C). IgG with 6 bound Cy5 labels,

however, gave an absorption spectrum with two similar absorption maxima at ~ 600 nm and at ~ 650 nm (dashed trace in Figure 7A), and the same profile was obtained after 10-fold dilution with buffer (dashed trace in Figure 7B). The anomalous effect was largely reversed when SDS was added at a final concentration of 1% (dotted trace in Figure 7A), i.e., when the protein was partially unfolded. This process took several minutes at RT and it did not occur in 0.1% SDS.

Further characterization of Cy5-labeled IgG and of avidin-bound *biotin-4-Cy5* gave the following results (see Supporting Information). (i) The spectra in Figure 7 were linear composites of a single absorption peak at 600 nm plus the normal spectrum of Cy5 ($\lambda_{\text{max}} = 650$ nm). (ii) The 600 nm absorption peak corresponded to a completely nonfluorescent state of protein-bound Cy5 residues. (iii) The nonfluorescent state was caused by intramolecular Cy5–Cy5 label interactions only and not by Cy5–protein interaction.

Upon the basis of these findings, it was possible to assess the relative contribution of the nonfluorescent state to fluorescence reduction at high Cy5/protein ratios. Figure 7A shows that the normal absorption peak at 650 nm was increased by a factor of 1.4 when IgG with 6 Cy5 labels (dashed trace) was treated with 1% SDS (dotted trace). This means that $\sim 30\%$ of all Cy5 labels had been in the nonfluorescent state before the addition of SDS, while 70% of all IgG-bound Cy5 labels had been in their normal condition, i.e., in a potentially fluorescent state. Nevertheless the actual extent of Cy5 quenching was as high as 97% (open circle at 6 Cy5/IgG in Figure 1B). From this follows that quenching of Cy5 fluorescence in highly Cy5-labeled proteins is mostly due to resonance energy transfer, while only a minor fraction of bound Cy5 labels adopts a nonfluorescent state, causing some additional reduction in fluorescence yield.

DISCUSSION

The present study provides a critical examination of protein labeling with Cy dyes. The results showed an astounding discrepancy between Cy3 and Cy3.5 on one hand and Cy5 and Cy7 on the other: Cy3- and Cy3.5-labeled antibodies fluoresced very well, even at high fluorophore/protein ratios (see Figures 1C, 2B, and 3B), whereas Cy5- and Cy7-labeled antibodies showed weak fluorescence, with a critical "optimum" at 2–3 labels/protein (see Figures 1C and 2B).

The exceptional fluorescence of Cy3- and Cy3.5-labeled antibodies and the noncritical role of fluorophore/protein ratio derived from fortunate coincidence of two effects: (i) covalent attachment of Cy3 or Cy3.5 labels to antibodies generally resulted in nearly 3-fold fluorescence enhancement (extrapolation of the solid lines in Figures 1A, 2A, and 3A to low fluorophore/protein ratio) which did not occur with the other Cy dyes, and (ii) mutual intramolecular quenching between Cy3 (or Cy3.5) labels by resonance energy transfer was much weaker than between the other Cy dyes (see Figure 1B, 2B, and 3B).

Fortunately the anomalous increase in Cy3 fluorescence was also observed when a very short biotin–Cy3 conjugate was noncovalently bound to (strept)avidin (see Figure 5A) and high fluorescence was still observed when up to four biotin-4-Cy3 were bound per (strept)avidin tetramer (see Figure 6A). Thus, biotin-4-Cy3 was discovered to be the first *small* fluorescent biotin derivative with strong fluorescence in the (strept)avidin-bound state. All other previously tested fluorescent biotin derivatives had suffered from strong quenching in the

bound state unless the fluorophores were linked to biotin via long PEG spacers (Gruber et al., 1997, 1998; Marek et al., 1997; Kada et al., 1999). In conclusion, biotin-4-Cy3 appears well suited for specific and intense postlabeling of immobile (strept)avidin molecules and subsequent fluorescence detection.

The findings of the present study are particularly relevant to SMFM where detection of single probe molecules depends on fluorophores with high fluorescence yield, high photostability, and long excitation wavelength. Cy5, Cy5.5, and Cy7 can be excited at > 600 nm where the autofluorescence background of live cells is lower by orders of magnitude as compared to the excitation range of TMR, Cy3, and Cy3.5 (Aubin, 1979; Benson et al., 1979; Marelus, 1995; Wolfbeis and Leiner, 1985). In fact, a Cy5-labeled phospholipid (Cy5-PE; Schütz et al., 1999; Schütz et al., 2000a) and a 1:1 conjugate of Cy5 and hongotoxin (Gruber et al., 2000; Schütz et al., 2000b) were the first single molecules visualized by far-field microscopy in live cells. Nevertheless Cy3-labeled antibodies are also expected to be useful for SMFM on live cells because (i) the cumulative signal from 5 to 6 Cy3 labels/protein is very high (see Figures 1C and 2B), (ii) the fluorescence signal (counts/ms) of a single Cy3 (Cy3-PE in a lipid membrane; G. Harms, unpublished results) is about 5-fold stronger than that of TMR–PE or Cy5-PE, and (iii) Cy3-PE proved to be 2–3-fold more stable to photobleaching than TMR–PE or Cy5-PE (G. Harms, unpublished results). Meanwhile single copies of a 1:1 EGF–Cy3 conjugate have also been observed on living cells (Sako et al., 2000), thus antibodies labeled with 6 Cy3 residues should be even easier to resolve despite the autofluorescence background.

The anomalous fluorescence enhancement of protein-bound Cy3 labels can tentatively be explained by simple assumptions. The fluorescence quantum yield of Cy3 is known to depend on solvent polarity and especially on viscosity; in ethanol Cy3 is ~ 2 and in glycerol ~ 10 times more fluorescent than it is in water (Mujumdar et al., 1993). If we assume that proteins contain surface patches with moderate Cy3-affinity then we expect two consequences: (i) intact Cy3 succinimidyl ester should preadsorb toward these sites and subsequently the activated hexanoic acid side chain of Cy3 should couple to some lysine residue that is within reach, and (ii) the so-coupled Cy3 residue will further stay adsorbed to the same patch most of the time, and the fluorescence of Cy3 will be enhanced by the low polarity of the site (as seen in ethanol), as well as by the reduction in conformational mobility of Cy3 (as observed in glycerol). The same concept also accounts for the enhancement of biotin-4-Cy3 fluorescence upon binding to avidin (see Figure 5A, solid circles). Obviously, the $-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CO}-(\text{CH}_2)_5-$ spacer (see Scheme 1) between the avidin-bound biotin residue and the chromophore of Cy3 allows the latter to reach a "Cy3-philic" patch on avidin. Strept-avidin does not have such a site within reach of a bound biotin-4-Cy3 (see Figure 5A, open circles). Yet strept-avidin can also enhance Cy3 fluorescence when a biotin-PEG₈₀₀–Cy3 conjugate is bound (Figure 5C, dotted line), i.e., the "Cy3-philic" patches on streptavidin are far away from the biotin-binding sites and only the PEG₈₀₀ spacer gives the Cy3 label sufficient tether to reach it.

When a large protein is labeled with Cy3 succinimidyl ester the abundance of lysine residues provides for a high probability that Cy3 gets coupled to a surface patch which enhances Cy3 fluorescence. In contrast, there should be little chance for favorable Cy3-protein interaction if a small protein/peptide with a single free cysteine residue

is labeled with a thiol-reactive Cy3 derivative. To check this point we actually labeled a cysteine mutant of hongo toxin (HgTx-A19C; Schütz et al., 2000a) with a maleimide derivative of Cy3 (prepared as described for Cy5, Gruber et al., 2000). At 50 nM concentration this Cy3-HgTx conjugate showed only 55% of the fluorescence of 50 nM Cy3 succinimidyl ester or of 50 nM Cy3-maleimide derivative (data not shown). This finding, and the results for streptavidin-bound biotin-4-Cy3 (Figure 5A, open circles) proved that the anomalous enhancement of Cy3 fluorescence indeed depends on protein surface patches with *particular* properties and is not a general consequence whenever Cy3 is linked to the surface of a protein.

In contrast to Cy3, the fluorescence of Cy5 labels was neither enhanced nor much reduced when linked to a protein with a 1:1 stoichiometry. This was deduced from the relative fluorescence yield at low Cy5/antibody ratios (circles in Figures 1A and 2A) and from the observation that the 1:1 conjugate of HgTx-A19C with Cy5 showed the same fluorescence yield as free Cy5 succinimidyl ester (Gruber et al., 2000). On the other hand, the linking of ≥ 2 Cy5 labels to a single protein molecule was found to cause severe "quenching" (see circles in Figures 1A, 2A, and 5B).

The minor reason for low fluorescence at high Cy5/antibody ratio was appearance of a nonfluorescent state of Cy5 which showed a unique absorption spectrum, with a single absorption peak at 600 nm (see dashed lines in Figure 7). Interestingly, the fraction of Cy5 labels in the nonfluorescent state was linearly dependent on Cy5/antibody ratio, ranging from exactly 0% at the extrapolated number of 0.0 Cy5/antibody to 30% at 6 Cy5 labels/antibody molecule (see Figure 10A in the Supporting Information). From this dependence on Cy5/protein ratio, we conclude that (i) the nonfluorescent state can only be caused by a second Cy5 label which is located on the same protein molecule and (ii) the spatial distribution of Cy5 labels over the antibody surface must be far from random. In case of random distribution there would be no chance for Cy5–Cy5 interaction at very low Cy5/antibody ratios (e.g., at 0.1 or 0.2 Cy5/protein): even in the rare case of an antibody molecule with two Cy5 labels the average Cy5–Cy5 distance would be too large for direct Cy5–Cy5 contact and too large also for dipole–dipole interaction through space (dependent on r^{-6}). We therefore propose that the nonfluorescent state of protein-bound Cy5 derives from a Cy5 dimer, the two hexanoic acid side chains being covalently linked to properly spaced lysine residues on the protein. In aqueous solution Cy5 analogues lacking the sulfonate residues are well-known for dimer formation (Mujumdar et al., 1993), thus if positively charged side chains on the protein happen to compensate for the negative charges of the sulfonate groups (see Scheme 1), such a Cy5 dimer may well be stabilized to the extent that even in 1% SDS it takes minutes to break the Cy5–Cy5 and the Cy5–protein interactions (see Figure 7A). We suspect that the first Cy5 preadsorbs to such a positively charged site on the protein, covalently binds to a lysine, subsequently attracts a second Cy5 succinimidyl ester, and the latter also gets anchored to another lysine the position of which permits to retain the tight dimer geometry. If such an optimally positioned second lysine residue is not within reach, then the adsorbed second Cy5 succinimidyl ester molecule would still have a high chance for covalent reaction with some nearby lysine residue; the strict dimer could no longer persist but, due to the short Cy5–Cy5 distance, there should be strong resonance energy trans-

fer between the two neighboring Cy5 residues, resulting in strong fluorescence quenching. In other words, the anomalously strong fluorescence quenching at high Cy5/antibody ratios (see circles in Figures 1A and 2A) seems to derive from "catalysis" of Cy5 attachment by already protein-bound Cy5 labels, leading to clusters of Cy5 even at rather low Cy5/protein ratios. As expected, this problem was much reduced in case of Cy5.5 the chromophore of which carries four, rather than two sulfonate groups; in comparison to Cy5, less quenching (solid circle in Figure 3A) and higher cumulative fluorescence (solid circle in Figure 3B) was seen at five Cy5.5 labels/antibody.

We are aware that the above presented hypotheses for anomalous enhancement of Cy3 fluorescence and anomalous quenching of Cy5 fluorescence are not yet proven by the available data but the concepts provide a first basis for discussion and for further strategies to get more fluorescence yield from Cy dye-labeled probes, needed to simplify single molecule studies in living cells and other systems.

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Supporting Information Available: Experimental details and supporting results (Table 1, Figures 8–14). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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