

# Labeling of Antibodies with Cy3-, Cy3.5-, Cy5-, and Cy5.5-monofunctional Dyes at Defined Dye/Protein Ratios

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## Abstract

In our preceding article [Gruber et al. (2000) *Bioconjugate Chem.*, 11, 696-704.] the fluorescence signal per labeled antibody has been related to the number of bound Cy dye per protein molecule. Unexpectedly, the optimal dye/protein ratio strongly depends on the type of Cy dye. Optimal fluorescence is found at 6 to 8 Cy3 or Cy3.5, at 4 to 5 Cy5.5, and 2 to 3 Cy5 labels per antibody. The present technical note describes adjustment of the desired dye/IgG ratios by variation of protein concentration, dye concentration, and pH in the labeling reaction. Together, therefore, these two studies allow to aim at optimal fluorescence of labeled antibody by choosing proper labeling conditions.

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## INTRODUCTION

Due to their intense fluorescence and low hydrophobicity, Cy dyes<sup>1</sup> are popular labels for fluorescence microscopy (Wessendorf and Brelje, 1992). In addition, their high photostability renders them useful for single molecule fluorescence detection (Harms et al., 1999; Sako et al., 2000; Schütz et al., 2000a; Schütz et al., 2000b).

In most applications of labeled antibodies a maximum of fluorescence yield is desired. It is important to note that the fluorescence of Cy dye-labeled antibodies is not a simple function of dye/protein ratio. In fact, a dramatic discrepancy has been found between Cy3 or Cy3.5, on the one hand, and Cy5 or Cy5.5, on the other hand (Gruber et al., 2000). The fluorescence yield of Cy3 and Cy3.5 was raised by a factor of ~3 when one such fluorophore was covalently bound to a protein molecule. At higher numbers of labels per protein, the fluorescence yield per label was reduced by resonance energy transfer, the latter dominating

over the anomalous fluorescence enhancement at >6 Cy3 or Cy3.5 labels per IgG molecule.

The role of label/protein ratio was very different, and much more critical, in case of Cy5 monofunctional dye: (i) No indication of an anomalous fluorescence enhancement was seen at low Cy5/IgG ratio. (ii) Antibodies with >6 Cy5 labels were almost nonfluorescent, due to anomalously strong quenching. As a result, a narrower "optimum" was seen at 2-3 Cy5 labels per IgG molecule. Finally, the properties of Cy5.5 labels were found to be intermediate between Cy3 and Cy5.

Our previous study also described how to modify the manufacturer's standard procedure to arrive at a certain number of covalently bound Cy dye per protein. Unfortunately these data became obsolete after completion of our study because a major change took place in the production of Cy dyes. The succinimidyl ester fraction was raised from about 50-60% (Gruber et al., 1997) to well over 90%. At the same time it was not known whether, and to which extent, the total amount of Cy dye per vial ("to label 1 mg of protein") had been reduced to compensate for the higher succinimidyl ester percentage. The reviewers of the previous study, therefore, suggested to repeat the same experiments with new batches of Cy dyes and to present the updated results on the adjustment of Cy

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<sup>1</sup> **Abbreviations:** DMSO, dimethylsulfoxide; IgG, goat immunoglobulin G. For the full names of Cy dyes see Supplementary Information to Gruber et al. (2000), available free of charge via the Internet at <http://pubs.acs.org>.

dyes/antibody ratio in a separate technical note. In addition, the role of reaction pH and of higher IgG concentrations was also investigated to provide more information relevant to the practice of antibody labeling.

## EXPERIMENTAL PROCEDURES

**Materials.** Analytical grade materials were used as long as they were commercially available. Cy3-, Cy3.5-, Cy5-, and Cy5.5-monofunctional dye (with the lot numbers 164004, 981141, 169575, and 147897, respectively), as well as PD-10 columns were obtained from Amersham Pharmacia Biotech. Goat IgG was purchased from Sigma.

**Buffers.** Buffer A (pH 7.5) contained 100 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA (pH adjusted with NaOH). Buffer B (pH 8.3) contained 100 mM NaCl and 35 mM H<sub>3</sub>BO<sub>3</sub> (pH adjusted with NaOH). Buffer C (pH 8.8) contained 100 mM NaCl and 35 mM H<sub>3</sub>BO<sub>3</sub> (pH adjusted with NaOH). Buffer D (pH 9.3) contained 150 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9.3 adjusted with H<sub>3</sub>PO<sub>4</sub>).

**Labeling of IgG with Cy Dyes.** In the standard procedure, the contents of 1 vial ("to label 1 mg of protein") of Cy3-, Cy3.5-, Cy5-, or Cy5.5-monofunctional dye was dissolved in 50  $\mu$ L of DMSO. Goat IgG was dissolved in buffer B, buffer C, or buffer D, typically at 1 mg/mL protein concentration (Figures 1 and 2), unless stated otherwise (Figure 3). 10  $\mu$ L of dye/DMSO mixture was pipetted into 200  $\mu$ L IgG solution under slow vortexing. After 30 min incubation at 25°C in the dark the reaction was terminated by freezing in liquid nitrogen. Storage was at -25°C. For separation of unbound dye, 300  $\mu$ L of 100 mM NaH<sub>2</sub>PO<sub>4</sub> (to suppress further labeling after thawing) was added to a frozen sample and the sample was incubated in a 25°C water bath until thawed. The sample was loaded on a PD-10 column (10 mL bed of Sephadex G-25M) which had been pre-equilibrated in buffer A. After washing the column with buffer A (2  $\times$  1 mL) the labeled protein was eluted by adding 2 mL of water to the column top. Another 10 mL of water was added to elute all unbound dye, and the column was regenerated with 20 mL of buffer A.

Negligible traces of dye remained bound to the column. One column was used for one coherent series of data (typically 6 data points, see Figure 1), applying samples with little dye first and concentrated samples last. The IgG and bound Cy dye contents of the protein peak was measured as described below. All procedures were carried out in dim light.

Higher Cy dye concentrations than stated above were achieved by dissolving 2 vials of Cy dye in 50  $\mu$ L of DMSO. Lower Cy dye concentrations were adjusted by preparing more dilute dye/DMSO stock solutions. In any case, however, 10  $\mu$ L of dye/DMSO stock solution was added to 200  $\mu$ L of IgG solution (see above). In parallel, a 10  $\mu$ L aliquot of the dye/DMSO stock solution was diluted with buffer A to determine the amount of dye offered in the labeling reaction.

**Measurement of IgG and Dye Concentrations.** The cyanine dye concentrations were determined from the visible light absorption spectrum, using the published molar extinction coefficients ( $\epsilon_{550} = 150000 \text{ M}^{-1} \text{ cm}^{-1}$  for Cy3,  $\epsilon_{581} = 150000 \text{ M}^{-1} \text{ cm}^{-1}$  for Cy3.5,  $\epsilon_{650} = 250000 \text{ M}^{-1} \text{ cm}^{-1}$  for Cy5, and  $\epsilon_{678} = 250000 \text{ M}^{-1} \text{ cm}^{-1}$  for Cy5.5; Mujumdar, 1993). The protein concentrations in the IgG stock solutions and in the void peaks after gel filtration were also determined from the UV-vis spectrum ( $A_{280}^{1\%} = 14$ ;  $\epsilon_{280} = 210000 \text{ M}^{-1} \text{ cm}^{-1}$  according to the manufacturer's data sheet), taking into account the absorbance contribution from the covalently bound Cy dye at 280 nm ( $0.08 \times A_{550}$  for Cy3,  $0.24 \times A_{581}$  for Cy3.5,  $0.05 \times A_{650}$  for Cy5, and  $0.18 \times A_{678}$  for Cy5.5, according to the manufacturer's instructions).

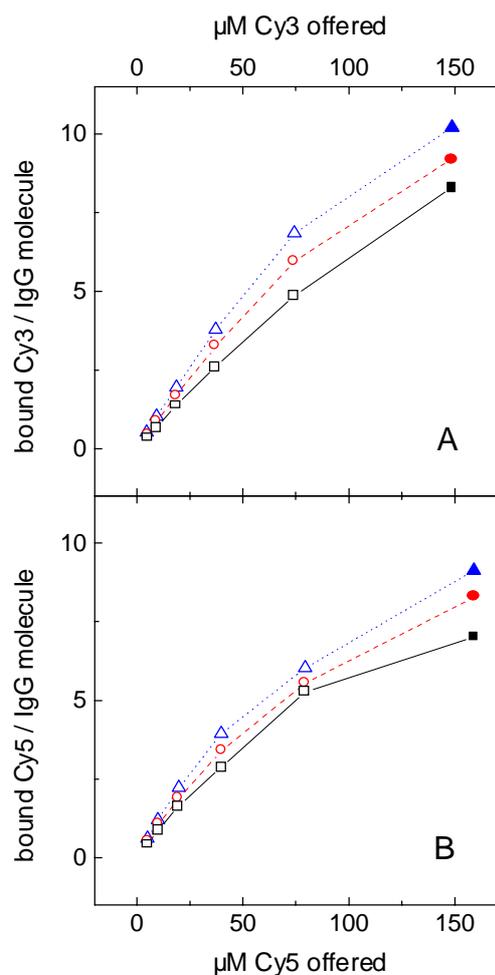
## RESULTS

Cy3-, Cy3.5-, Cy5-, and Cy5.5-monofunctional dye are commercially supplied as pre-dried portion in reaction vials which are sealed in dry atmosphere to prevent hydrolysis during storage. One portion was found to contain 150 nmol of Cy3, 125 nmol of Cy3.5, 160 nmol of Cy5, or 50 nmol of Cy5.5, as measured by UV-vis absorption (see Experimental Procedures). According to the lot analysis certificates, the succinimidyl ester

fractions were 97.7%, 70.0%, 96.2%, or 77.3%, respectively. The manufacturer recommends to dissolve 1 mg of antibody (6.7 nmol) in 1 mL of buffer (pH 9.3) and to react for 30 min with 1 pre-dried portion of Cy dye. We chose to pre-dissolve the Cy dye in little DMSO in order to adjust higher or lower Cy dye concentrations as compared to the standard protocol. It is safe to assume that no hydrolysis of succinimidyl ester groups occurred in DMSO while preparing a series of Cy dye stock solutions.

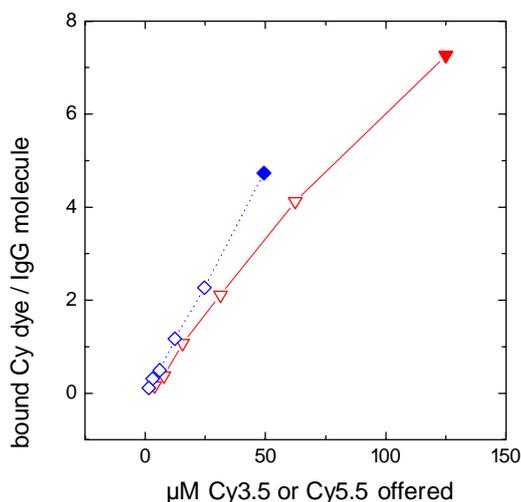
Figure 1 shows the numbers of bound Cy3/IgG (panel A) and Cy5/IgG (panel B) obtained at a constant IgG concentration of 1 mg/mL. The solid square in each panel resulted from conditions which were analogous to the recommended procedure. These numbers are only 10-20% higher than seen with the Cy dye batches purchased before the major production change (Gruber et al., 2000). From this follows that the manufacturer has largely compensated for the much higher succinimidyl ester contents by the appropriate reduction in the total amount of Cy dye per pre-dried portion.

The favorable results at lower pH values (see circles and triangles in Figure 1) came as a surprise because in the original article on Cy dyes pH 9.3 had been recommended to achieve fast enough labeling kinetics (Mujumdar et al., 1993). Nevertheless the observation of higher bound dye/protein ratios at lower pH can easily be rationalized by competition between the "labeling reaction" and hydrolysis. At pH 9.3, the standard procedure (solid squares in Figure 1A) gave 8.3 Cy3 labels/IgG, i.e. 56 out of 150 nmol Cy3 was bound to 6.7 nmol of IgG, while certainly the rest was largely hydrolyzed at pH 9.3 (Mujumdar et al., 1993). At pH 8.3 [OH<sup>-</sup>], and therefore also the rate of hydrolysis, will be 10-fold lower as compared to pH 9.3. Assuming an effective pK<sub>a</sub> of 9.8 for a very reactive lysine residue, a shift from pH 9.3 to pH 8.3 will reduce the concentration of deprotonated lysines by a factor of 7.8 only, thus the reaction of lysine groups with Cy dye will get a relative advantage over hydrolysis at pH 8.3. This explains why 68 out of 150 nmol Cy3 were bound to 6.7 nmol of IgG at pH 8.3 (solid triangle in Figure 1A), as compared to 56 nmol bound Cy3 labels at pH 9.3 (solid square in Figure 1A).



**Figure 1:** Labeling of IgG at 1 mg/mL protein concentration with Cy3- (A) and Cy5-monofunctional dye (B). The pH values in the reaction mixtures were 9.3 (squares), 8.8 (circles), or 8.3 (triangles). The solid squares correspond to the manufacturer's standard procedure (addition of 1 mg protein in 1 mL buffer to 1 vial Cy dye). The solid circles and triangles derived from analogous conditions, except for the lower pH values.

The results for Cy3.5 at pH 9.3 (triangles in Figure 2) were comparable to those for Cy3 (squares in Figure 1). Labeling of IgG with Cy5.5, however, appeared significantly more effective, as is obvious from the higher Cy5.5/protein ratios obtained under equal labeling conditions (diamonds in Figure 2). This explains why the manufacturer provides smaller pre-dried portions of Cy5.5 "to label 1 mg protein".

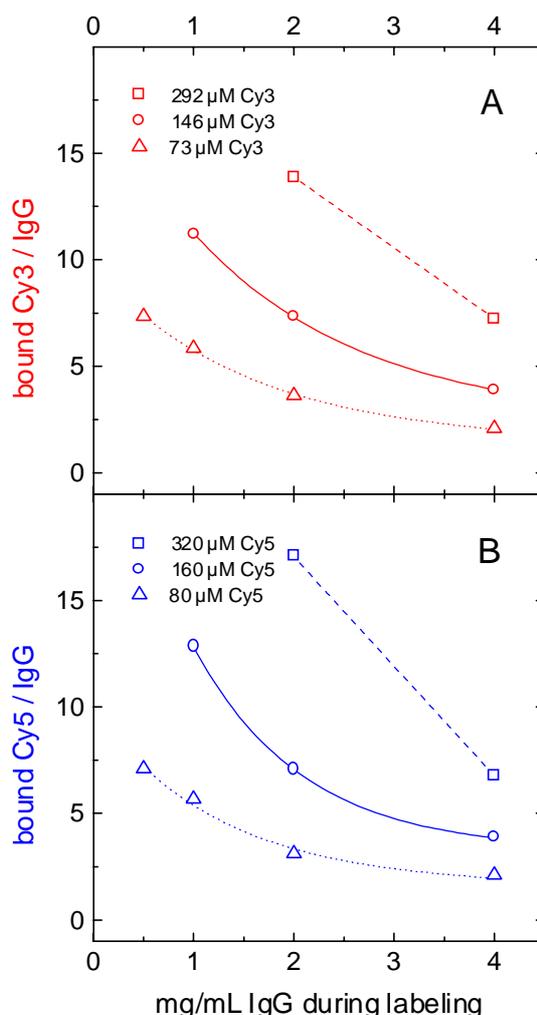


**Figure 2:** Labeling of IgG at 1 mg/mL protein concentration and pH 9.3 with Cy3.5-monofunctional dye (triangles) and Cy5.5-monofunctional dye (squares). The solid symbols correspond to the manufacturer's standard procedure (addition of 1 mg protein in 1 mL buffer to 1 vial Cy dye).

The role of protein concentration in combination with different Cy3 and Cy5 concentrations is shown in Figure 3. The nonlinear influence of both parameters on the number of bound fluorophores per protein obviously derived from shifts in the competition between protein labeling and hydrolysis of reactive dye. The reason is that the rate of protein-dye coupling is proportional to  $[\text{protein}] \times [\text{reactive dye}]$ , whereas the rate of hydrolysis solely depends on  $[\text{reactive dye}]$  at a given pH. The data in Figure 3 are relevant to the practice of antibody labeling because antibodies are usually stored at several mg/mL protein concentration, thus labeling at high IgG concentration will be much preferred.

## DISCUSSION

The object of this study was to characterize the parameters which allow to adjust a desired number of bound Cy dye per antibody molecule. Admittedly the dye/protein ratios in Figures 1-3 are *nominal* values because they are based on a simple approximation underlying the manufacturer's instructions: The



**Figure 3:** Variation of IgG concentration during labeling with Cy3- (A) or Cy5-monofunctional dye (B). The pH value was 8.3 in all experiments. The Cy dyes were from the same batches as stated in Figure 1.

absorption spectrum of any Cy dye is assumed to be the same, whether the dye is bound to protein or free in solution. Consequently the molar extinction coefficient ( $\epsilon$  at  $\lambda_{\text{max}}$ ) of the free dye was used to calculate the concentration of bound dye, and the manufacturer's correction factors were applied to subtract the bound dye contribution from the measured 280 nm absorbance before calculating the protein concentration (see Experimental Procedures). Unequivocal determination of the *true* number of bound Cy dye per protein would have been laborious, and for most practical purposes it is not necessary to know the *true* dye/protein ratio.

What is essential for immunofluorescence is to achieve intense fluorescence per labeled antibody while retaining specific binding of antigen.

In our preceding article (Gruber et al., 2000) the fluorescence per labeled antibody has also been related to *nominal* Cy dye/protein ratios, while the latter are now related to particular labeling conditions. Together, therefore, these two studies allow to aim at optimal fluorescence of labeled antibody by proper labeling conditions, taking into account the previously unknown discrepancy between Cy3 or Cy3.5, on the one hand, and Cy5 or Cy5.5, on the other hand.

In particular, it appears necessary to use 3-fold lower Cy5 concentrations or 4-fold higher IgG concentrations as compared to the manufacturer's procedure to avoid strong fluorescence quenching in Cy5-labeled IgG. In case of the other monofunctional Cy dyes the standard protocol can be used, bearing in mind that an upper limit of Cy dye/IgG will be obtained; these should not be exceeded, whereas labeling at somewhat lower Cy dye concentrations or higher IgG concentrations appears noncritical and will give quite similar effective fluorescence per labeled antibody.

#### ACKNOWLEDGEMENTS

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