Artefacts Caused by Non-Specific Binding of Fluorescent Dyes in Single Molecule Experiments

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Introduction

Single-molecule fluorescence imaging techniques are becoming popular tools for probing the structure and dynamic properties of living matter. The success of these techniques is dependent on detecting the weak fluorescence signal above background noise, and dealing with the single molecule effects of bleaching and blinking. For this reason, dyes are often selected for single molecule experiments based on their photophysical characteristics, such as quantum yield, photostability, and resistance to blinking. However, in an earlier publication we demonstrated that another important factor for single molecule experiments in cells is the propensity of dyes to bind non-specifically to the substrate on which the cells are cultured[1]. This non-specific binding results in a large number of immobile fluorescent molecules, skewing the results of any analysis of molecular mobility in cells. Here we describe the results of a systematic study of many dyes, and demonstrate that dye chemistry has a strong influence on the propensity of dye-protein conjugates to adhere non-specifically to the substrate[2].

Overview of dye chemistry

Many fluorescent probes are available for single molecule work. The characteristics of the dyes we tested are summarized in Table 1.

Dye	Mean single molecule photon detection rate (s^{-1})	Apparent photobleaching time constant (s)	net charge pH 7.4[a]	logD at pH 7.4[a]
Alexa Fluor 488	1164±181	15.1±0.2	-3.94	-10.48
Bodipy FL	2586±393	21.3±0.6	0	-1.99
CF488	1200±213	17.8±0.4	-3	-8.83
Fluorescein	2436±350	1.4±0.1	-1.9	-1.30
Alexa Fluor 546	2697±230	24.0±0.1	-3.41	-2.53
Atto 565	2850±535	14.5±0.2	0	-0.83
CF568	1042±194	40.7±0.5	-3	-3.74
Cy3	986±198	25.3±0.2	0	+3.03
Rhodamine Red C2	3268±453	8.3±0.1	-0.99	+1.53
TMR6	832±223	7.5±0.1	0	-5.6
Atto 647N	3290±231	36.0±0.2	+0.61	+1.96
CF633	851±170	16.4±0.1	-2	-5.44
CF640R	1084±202	37.7±0.2	-3	-10.29

Table 1. Summary of dye characteristics. [a]Calculated from structures using "Marvin Sketch" software (Chemaxon). Structures of CF dyes are unavailable but charge and logD were calculated by the manufacturer, using the same method. Bold lines indicate divisions between groups of dyes excited at different wavelengths, as follows: Top four dyes, 491 nm; middle six dyes, 561 nm, bottom three dyes, 638 nm. Laser flux exiting the objective was 3.2 µW/µm2 at 491 and 561 nm, 3.4 µW/µm2 at 638 nm.

The first two columns in the table are photophysical characteristics as measured in the single molecule TIRF microscopy station on the *Octopus* facility. In both cases, higher numbers are better indicating higher brightness and greater resistance to photobleaching, respectively. The second two columns show two dye properties that we considered might affect non-specific binding, charge and hydrophobicity, the latter being measured by the distribution coefficient, LogD, that describes the propensity of the dyes to prefer polar or non-polar solvents. Dyes with negative LogD values are hydrophilic, those with positive LogD values hydrophobic.

Methods

We assessed the non-specific binding of dyes by tracking the motion in cultured T47D cells of anti-EGFR affibody labelled with a range of dyes. The underlying principle is that affibody that binds to EGFR receptor in the plasma membrane of the cells will be mobile, but affibody binding non-specifically to the glass substrate will be immobile. A full description of the methods used can be found in Zanetti-Domingues *et al* (2013)[2]. Briefly, Anti-EGFR Affibody (Abcam) was labelled at a single cysteine residue in a 1:1 stoichiometry following the manufacturer's instructions with the following maleimide dyes: Alexa 488, Alexa546, Tetramethylrhodamine-6, Bodipy FL, and Rhodamine Red C2 (Molecular Probes -Invitrogen), Cy3 (GE Healthcare), CF488A, CF568, CF633 and CF640R (Biotium), Atto565 and Atto647N (AttoTec), and Fluorescein-5 (Sigma Aldrich). Cells were labelled with fluorescent affibody at a concentration of 4 nM for 15 minutes at 37 ºC. Cells were then rinsed and imaged using a Zeiss Axiovert TIRF microscope at the *Octopus* facility[3], using wavelengths of 491 nm, 561 nm, or 639 nm as appropriate for the dye being studied. Single-molecule time-series data were analysed using custom software[4], giving mean square displacement values for tracks of individual fluorescent molecules. These were used to calculate the instantaneous diffusion coefficient (*D*), a measure of molecular mobility.

Results

We investigated the possibility that the propensity of a fluorescent dye for non-specific binding was related to either its net charge or its hydrophobicity (logD). Figure 1 shows a plot of diffusion coefficient versus net charge (A) and logD (B). The data show a strong correlation between logD and dye conjugate mobility (R^2 0.75), but only a weak correlation between net charge and mobility (R^2 0.2). This indicates that dye hydrophobicity is a strong indicator of a dye's propensity for non-specific binding. As an independent confirmation that dye hydrophobicity is correlated with non-specific binding, we also measured directly the density of conjugate binding to substrate for selected dyes. PEG-BSA nanogel treated glass substrates were exposed to dye conjugates and the number of fluorescent spots remaining after washing was counted. These data are plotted in Fig. 2C, which shows a strong correlation between

logD and spot density, confirming the association between hydrophobicity and non-specific dye binding to the substrate.

Figure 2 shows example *D* data for four of the dyes, Alexa 488 (high average *D*), CF633 (moderate average *D*), and Alexa 546 and Atto 647N (low average *D*). These data confirm that lower average *D* values are associated with a high fraction of immobile fluorescent molecules, shown by the relative size of the peak centered on a zero diffusion coefficient. The higher the mobility of the dye, the smaller the fraction of spots in the zero diffusion coefficient peak. This confirms that *D* is a good measure of mobility and hence the propensity of a dye to bind to the substrate.

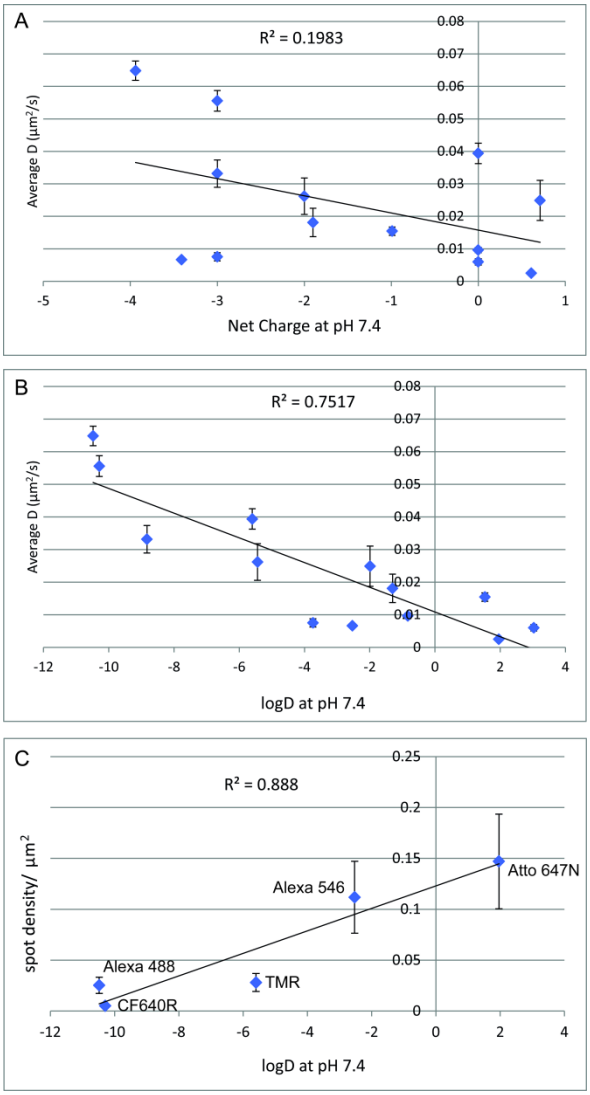

Figure 1. Effect of logD and charge on affibody conjugate mobility. Plots of mean instantaneous *D* fit for different anti-EGFR Affibody conjugates vs charge at pH 7.4 (A), and logD (B). C) Plot of spot density for selected anti-EGFR Affibody conjugates vs charge at logD. Each datapoint corresponds to mean ± SEM of at least 10 independent areas. Lines show linear regression fit to the data, R^2 values indicating goodness of fit.

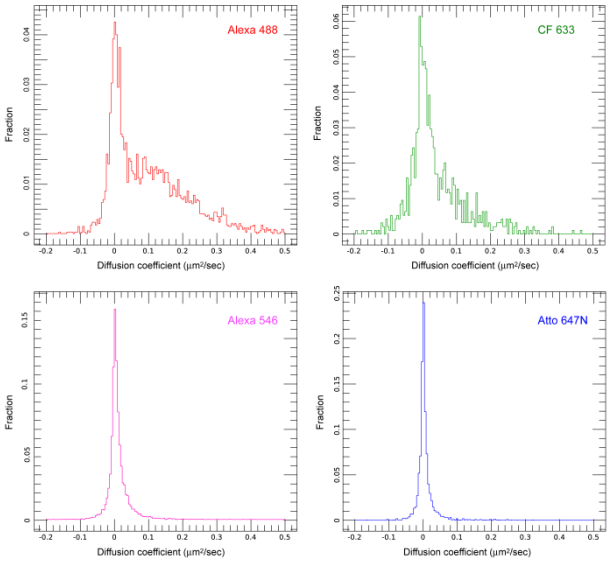

Figure 2. Plots of distributions of mean instantaneous D fits for affibody-dye conjugates. Dyes selected to represent high (Alexa 488), moderate (CF 633), low (Alexa 546), and very low (Atto 647N) spot mobility.

Conclusions

Our results show that non-specific binding of dye conjugates to the substrate is a significant effect, highly variable between dyes. It is therefore important to consider this in addition to photophysical characteristics such as quantum yield, photostability, and resistance to blinking when selecting a dye. We have demonstrated that hydrophobicity is the major determinant of the propensity of a dye for binding to the substrate. We therefore suggest that hydrophilic dyes (strongly negative logD) with good photophysical characteristics should be selected in the first instance. Of the dyes we have examined, Alexa 488 appears to be the dye of choice for excitation with blue light, TMR for green, and CF640R for red.

Acknowledgements

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References

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