

Supporting Information: Combining fluorescence fluctuations and photobleaching to quantify surface density

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Summary: Theoretical derivation of occupancy probabilities and its consequences for possible m values (S1); Results of experiments with 20 nm fluorescent beads and corresponding analysis(S2).

S1. Theoretical derivation of occupancy probabilities

When the number of labels borne by an entity can only take 3 non-nil values, its distribution depends on 2 independent parameters, because of the normalization. This means that for a measured parameter $S_{\sigma m}$ (that equates $\sigma^2/m + m - 1$), any given possible value of m fully determines the 3 occupancy probabilities. The fact that these probabilities are to be between 0 and 1 implies limited ranges of values for m . We now exploit this simple mathematical

framework in the particular case of 1, 2 and 3 labels, but it can be easily extended to any distribution with 3 non-nil occupancy probabilities (more generally, when the distribution of the number of labels depends on 2 independent parameters, whatever they are, this induces constraints on m that depend on the measured value of $S_{\sigma m}$).

Let us now consider the mean value, m , the standard deviation, σ and the normalisation of the distribution. These are given by:

$$\begin{aligned}
 m &= p_1 + 2p_2 + 3p_3 \\
 \sigma^2 &= (1 - m)^2 p_1 + (2 - m)^2 p_2 + (3 - m)^2 p_3 \\
 p_1 + p_2 + p_3 &= 1
 \end{aligned}
 \tag{S1}$$

These equations can be easily transformed to express p_1 , p_2 and p_3 as functions of m and σ :

$$\begin{aligned}
 p_1 &= 3 + m(S_{\sigma m}/2 - 2) \\
 p_2 &= -3 + m(3 - S_{\sigma m}) \\
 p_3 &= 1 + m(S_{\sigma m}/2 - 1)
 \end{aligned}
 \tag{S2}$$

By writing that each of these probability occupancies is between 0 and 1, one obtains the corresponding constraints on m as a function of $S_{\sigma m}$:

$$\begin{aligned}
 \frac{4}{4 - S_{\sigma m}} &\leq m \leq \frac{6}{4 - S_{\sigma m}} \\
 \frac{3}{3 - S_{\sigma m}} &\leq m \leq \frac{4}{3 - S_{\sigma m}} \\
 0 &\leq m \leq \frac{2}{2 - S_{\sigma m}}
 \end{aligned}
 \tag{S3}$$

We show in Fig. S1 the series of $m(S_{\sigma m})$ curves giving these upper and lower lim-

its. Henceforth, the common region where all the constraints are simultaneously satisfied (hatched area) is defined by:

$$\frac{3}{3 - S_{\sigma m}} \leq m \leq \frac{2}{2 - S_{\sigma m}} \quad \text{for} \quad 0 \leq S_{\sigma m} \leq 1 \quad (\text{S4})$$

$$\frac{3}{3 - S_{\sigma m}} \leq m \leq \frac{6}{4 - S_{\sigma m}} \quad \text{for} \quad 1 \leq S_{\sigma m} \leq 2 \quad (\text{S5})$$

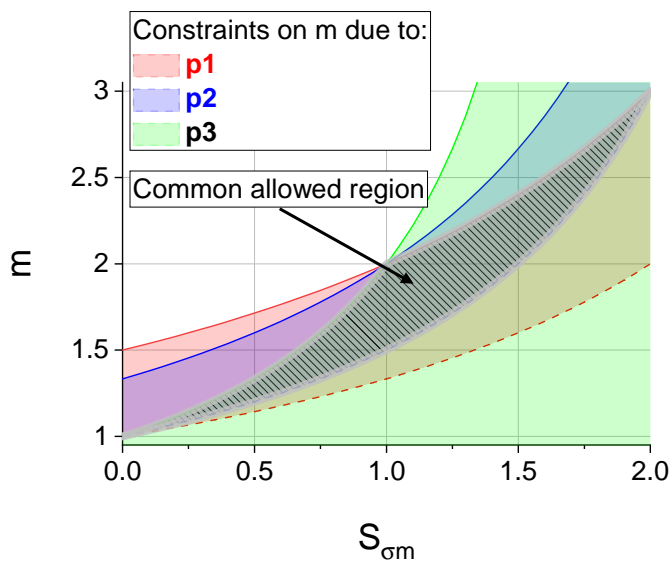


Figure S1: Limits of the possible values of m . For each value of $S_{\sigma m}$, the occupancy probabilities for 1, 2 and 3 labels (p_1 (red zone), p_2 (blue) and p_3 (green)) are to be between 0 and 1, which implies corresponding minimum and maximum values of m . The common zone, allowed for any of the occupancy probabilities, is hatched and surrounded in gray.

It is possible to add further restrictions regarding the distribution of the number of labels. For instance, concerning the SA_v-Alex molecules the specifications of which indicate an average of 2 labels per molecule, it is reasonable to discard the cases where $p_3 > p_2$, which leads to $m > \frac{1}{1 - \frac{3}{8}S_{\sigma m}}$.

Finally, it is also interesting to ask what one would expect if one assumed that the entities could carry 2, 3 or 4 labels. A derivation analogous to that leading to the above Eq. S1 to S3 gives:

$$\begin{aligned}
\frac{8}{5 - S_{\sigma m}} &\leq m \leq \frac{9}{5 - S_{\sigma m}} \\
\frac{10}{6 - S_{\sigma m}} &\leq m \leq \frac{12}{6 - S_{\sigma m}} \\
\frac{4}{4 - S_{\sigma m}} &\leq m \leq \frac{6}{4 - S_{\sigma m}}
\end{aligned}
\tag{S6}$$

S2. Experiments with 20 nm fluorescent beads

We performed experiments by plating 20 nm red fluorescent polystyrene beads (FluoSpheres™ Carboxylate Modified Microspheres, F8786, Invitrogen) on a glass substrate (Lab-Tek™II Chambered Coverglass, Nunc) that was previously treated with O_2 plasma, covered by poly(l-lysine) and then washed. After a few hours of incubation, the surface was rinsed with miliQ water to remove unbound beads. 9 different zones of $25.7 \times 25.7 \mu m^2$ were imaged, using the same acquisition and analysis protocol as the one described in the main part of the manuscript.

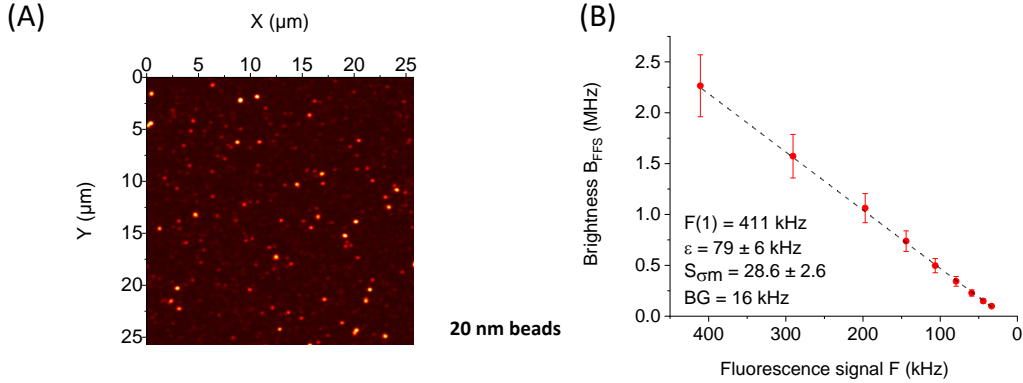


Figure S2: Analysis of 20 nm fluorescent beads deposited on a glass surface. (A) Initial image (before photobleaching) of one of the 9 zones ($25.7 \times 25.7 \mu m^2$); (B) Corresponding brightness decay, the slope of which, $S_{\sigma m}$, is many tens of times larger than the one measured with SAV-Alex or bAtto molecules.

We see in Fig. S2 an image of one zone of beads and the corresponding photobleaching decay, the different zones showing the same trend. The striking property is the typical value of the slope, $S_{\sigma m}$, of a few tens, that is much larger than the one obtained with SAV-Alex

or bAtto molecules. Exploratory single particle detection, on images acquired at sufficiently low surface concentration, has confirmed that the bead intensity distribution is very broad, as one can guess according to Fig. S2(A). Assuming that the distribution of the number of labels follows a Poisson law, we can deduce that the mean number of fluorophores per bead is also of the order of a few tens (since in this case $m = S_{\sigma m}$), in agreement with the rough specifications given by the manufacturer.

We nevertheless stress the fact that the output parameters of the brightness decay fit might be very sensitive to the background. The reason relies in the very small final value of the brightness (that of the last measured point), relatively to the initial (unbleached) brightness. Depending upon the value found or fixed for the background, the estimated single label brightness can vary a lot and so can the slope $S_{\sigma m}$.

Finally, this data show that pbFFS can in principle be applied to particles with a high degree of labelling, although, to be reliable, measurements should be performed with a high dynamic range and a carefully controlled background.