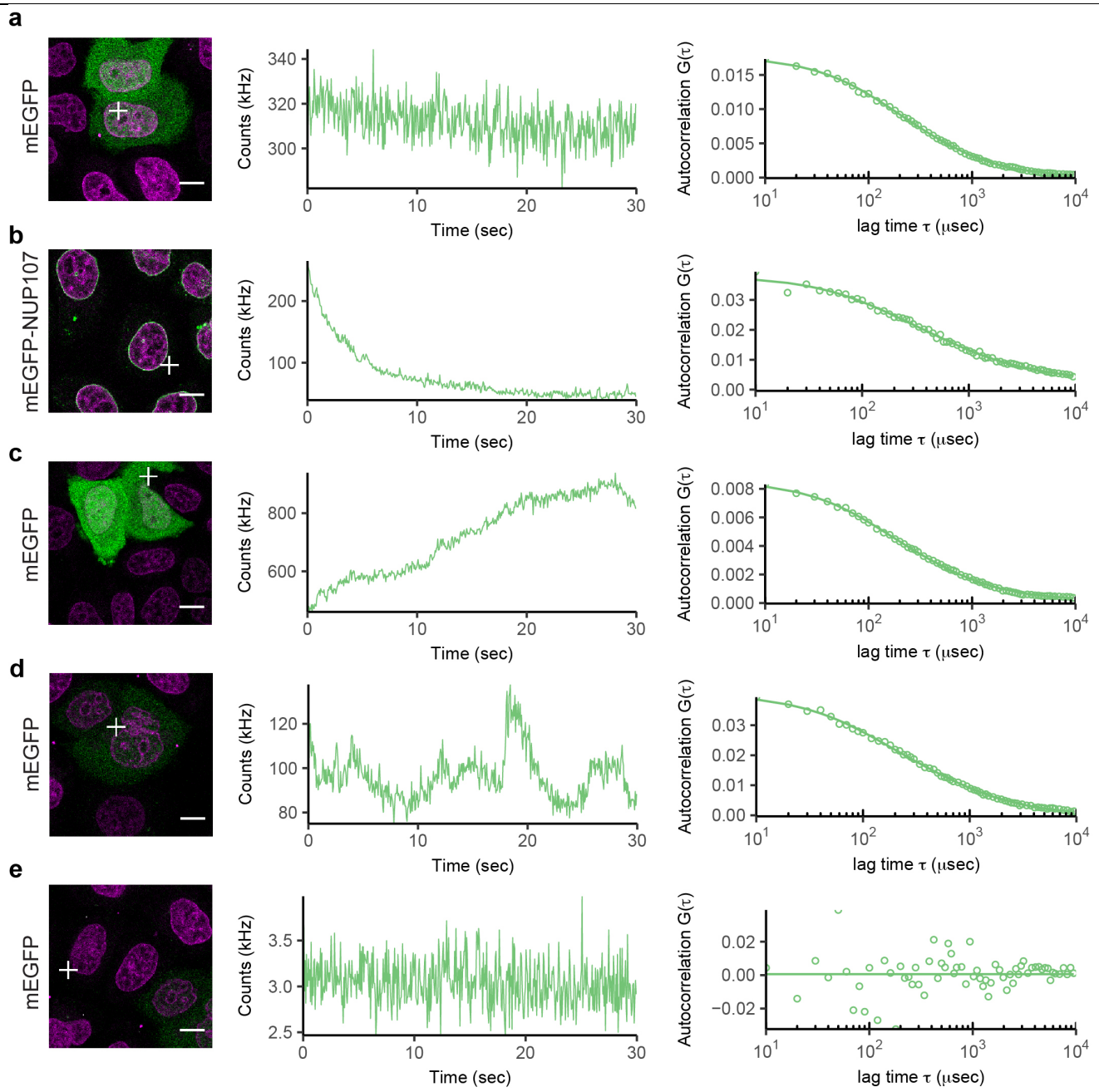

f

Trace	χ2	R2	Bleach	Visual counts	Visual ACF
a	0.845	0.999	1.021	good	good
b	0.303	0.997	**2.449**	**decrease**	good
c	0.362	0.999	**0.768**	**increase**	good
d	**1.260**	0.999	1.136	**large fluctuations**	good
e	1.051	**-0.052**	1.017	good	**large fluctuations**

Figure S1

Supplementary Figure 1

Quality control of FCS traces

(a) Typical trace that passes the quality control (QC) according to the parameters shown in f. The QC is based on thresholds applied to fitting parameters such as the sum of squared residuals χ^2, the coefficient of variation R^2, and properties of the photon counts traces (e.g. Bleach coefficient, as calculated by *FA*). A visual inspection of the photon counts and autocorrelation traces can also be used for quality control. Scale bar 10 µm. (b) FCS measurement point is at the boundary of the cytoplasmic compartment causing a decreasing drift in photon counts. (c) FCS measurement point is at the boundary of the cytoplasmic compartment causing an increasing drift in photon counts. (d) FCS measurement point is at the boundary between a dim and a brighter cell causing large fluctuations in the photon counts. (e) FCS measurement point is in a cell that does not express a fluorescent protein. (f) Table of parameters used for the QC. The measurements in b and c do not pass the QC according to the bleach parameter. The measurement in d does not pass the QC according to the χ^2 value. The measurement in e does not pass the QC according to the R^2 value. None of the traces in b-e pass the visual inspection. The thresholds used were $\chi^2 < 1.2$, $R^2 > 0.9$, $0.8 <$ Bleach < 1.2. The thresholds can be interactively set in the *FCSCalibration* software (**Supplementary Software 4**).

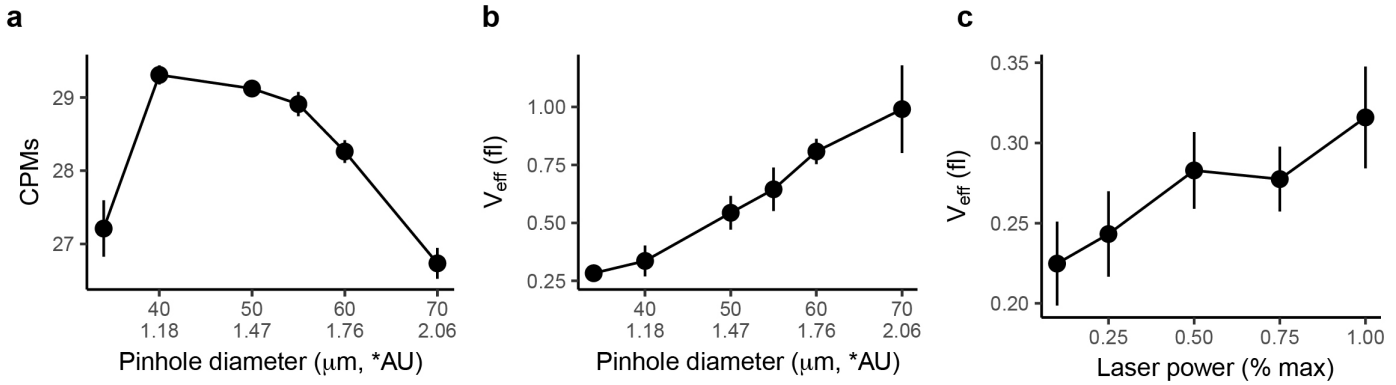

Figure S2

Supplementary Figure 2

CPM and effective volume dependency on pinhole diameter and laser power

(a) Counts per molecule (CPM) as function of the pinhole diameter for Alexa488 in water. (b) Effective confocal focal volume estimated as described in the supplement (**Supplementary note 1-2** and **Box 2**) as function of the pinhole diameter for Alexa488. (c) Effective confocal focal volume as function of the excitation laser (Argon 488 nm). The pinhole was 34 µm.

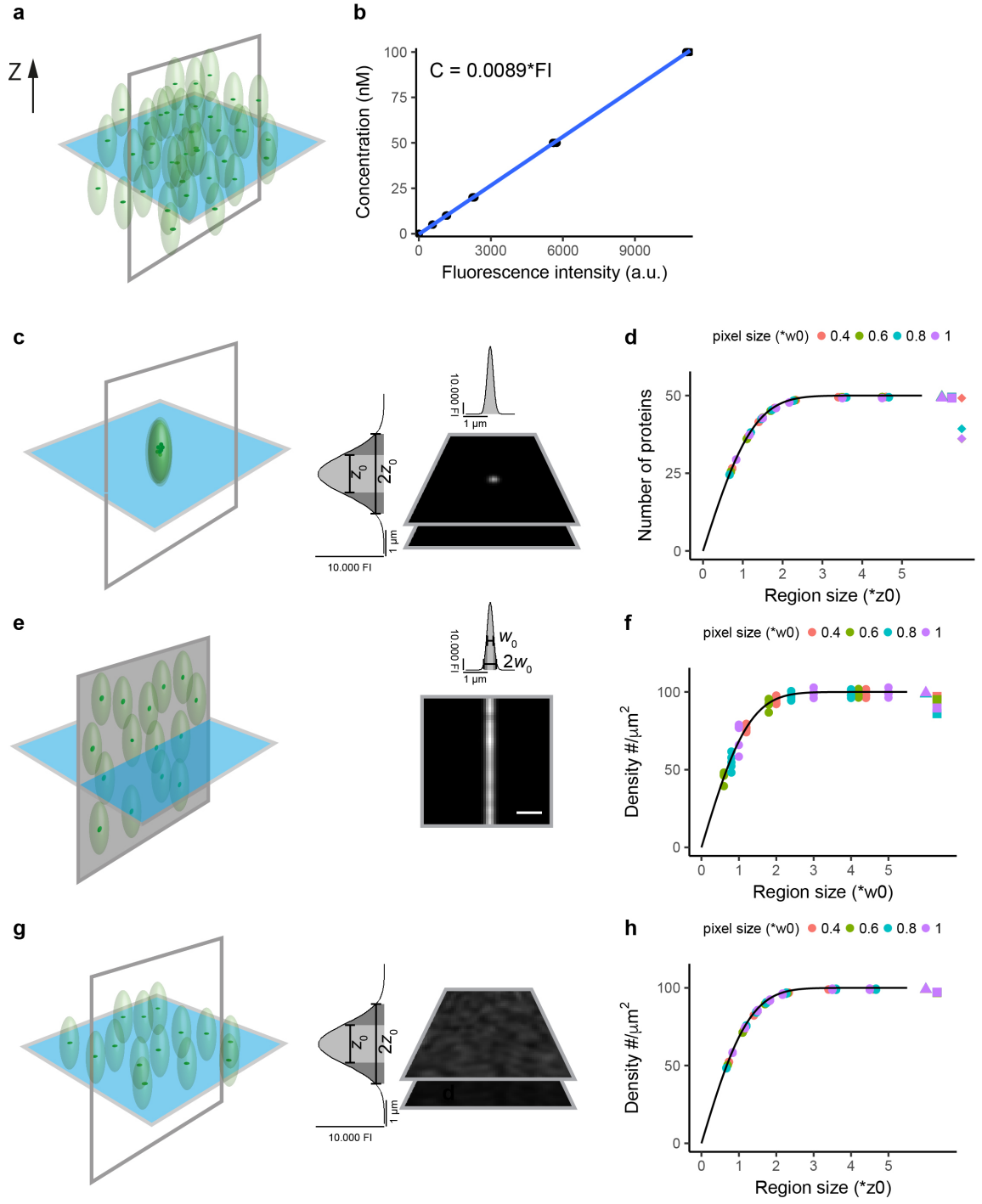

Figure S3

Supplementary Figure 3

Simulated FCS-calibrated imaging

(a) Illustration of a homogeneous fluorophore distribution. (b) The slope of the fluorophore concentration as function of pixel

fluorescence intensity gives the calibration coefficient. Simulated PSF is a 3D Gaussian (Eq. S1). The size of the PSF is characterized by its e^2 decay w_0 and z_0 in XY and Z direction, respectively. Here and in all subsequent panels background and detector noise are not simulated. (c) Simulated Z-stack of a point source (50 fluorophores). (d) Using the calibration coefficient and Eqs. S15, S16, and S19 the total number of proteins in each plane is calculated. Several planes along the Z-direction are summed (circles). The expected protein number is obtained when a large region along Z is considered (>2*z0). Simulated images have a pixel-size of 0.4, 0.6, 0.8, 1 times w_0 in all directions (different colors). Triangles give the result when all pixels are considered (Eq. S19). Squares give the approximation using the integral of the PSF along Z (Eq. S21). Diamonds give the approximation when the integral of the whole PSF and the pixel with the highest intensity is used (Eq. S23). (e) Simulated fluorophores distributed in a XZ plane (density of 100 fluorophores/μm^2). Scale bar 10 μm. (f) Protein density is computed using Eqs. S15 and S24. The expected density is obtained for a region width along the X direction of > 2*w_0. Triangles give the result when all pixels are considered. Squares give the approximation when the integral of the PSF along X is used (Eq. S26). (g) Simulated fluorophores distributed in a XY plane (density of 100 fluorophores/μm^2). (h) The average protein density is summed for planes along Z (Eqs. S15 and S17). The expected density is obtained for a region width > 2*z_0. Triangles give the result when all pixels are considered. Squares give the approximation when the integral of the PSF along Z is used (Eq. S29). Each fluorophore has a simulated intensity of 1000 (a.u.), the simulated PSF is characterized by w_0 = 250 nm, z0 = 1500 nm. The black lines give the theoretically expected result after integration of the Gaussian function $N = N_e \mathrm{erf}\left(\frac{Rs}{\sqrt{2}}\right)$ where N_e is the expected protein number/density (50 in d, and 100 in f and h) and R_s is the region size in unit of the PSF characteristic size.

Supplementary information: Quantitative mapping of fluorescently tagged cellular proteins using FCS-calibrated four dimensional imaging

Antonio Z. Politi[1], *Yin Cai*[1,2], *Nike Walther*[1], *M. Julius Hossain*[1], *Birgit Koch*[1,3], *Malte Wachsmuth*[1,4], *Jan Ellenberg*[1,*]

[1]EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

[2]Current address: Roche Diagnostics, Waiblingen, Germany

[3]Current address: Max Planck Institute for Medical Research, Heidelberg, Germany

[4]Current address: Luxendo GmbH, Heidelberg, Germany

[*]Correspondence should be addressed to JE, jan.ellenberg@embl.de

Supplementary Tables

Software tool	Software requirements	Operating system	Description	Source
FCSRunner Supplementary Software 1	ZEN black edition ≥ 2010	Windows	VBA ZEN macro to perform combined FCS and image acquisition. Install on data acquisition computer.	*https://git.embl.de/grp-ellenberg/fcsrunner*
MyPiC Supplementary Software 2	ZEN black edition ≥ 2010	Windows	VBA ZEN macro to perform complex imaging and FCS pipelines. Automated data acquisition in combination with AutomtedFCS plugin. Install on data acquisition computer.	*https://git.embl.de/grp-ellenberg/mypic*
Automated FCS Supplementary Software 3	FiJi	Windows (Linux, Mac OSX)	A FiJi plugin that interacts with MyPiC and allows among other things for unsupervised FCS data acquisition. Install on data acquisition computer.	*https://git.embl.de/grp-ellenberg/adaptive_feedback_mic_fiji* directory: *automatedfcs*
FCSFitM Supplementary Software 4	Matlab version ≥ R2014a Toolboxes: Optimization, Statistics Fluctuation Analyzer 4G	Windows, Linux, Mac OSX	Matlab workflow to batch fit FCS data, compute effective focal volumes, and concentrations. As input data the software uses the correlation curves generated by Fluctuation Analyzer 4G.	*https://git.embl.de/grp-ellenberg/fcsanalyze* directory: *matlab/FCSFitM*
setup_FCSFitM_web.exe Supplementary Software 4	Fluctuation Analyzer 4G	Windows 64-bit	The compiled version of FCSFitM for Windows.	
FCSImageBrowser Supplementary Software 4	FiJi	Windows, Linux, Mac OSX	FiJi plugin to visualize images and FCS positions and extract fluorescence intensities.	*https://git.embl.de/grp-ellenberg/fcsanalyze* directory: *fiji*
FCSCalibration Supplementary Software 4	R (≥3.3.2) RStudio (recommended) the installation of package fcsresfun will force the installtion of missing packages		R program to interactively compute FCS calibration parameters	*https://git.embl.de/grp-ellenberg/fcsanalyze* directory: *R/FCSCalibration* *R/fcsresfun*
setup_FCSCalibration.exe Supplementary Software 4	Fluctuation Analyzer 4G	Windows 64- bit	Bundled version of FCSCalibration.	
FCSCalibrateImage Supplementary Software 4	FiJi	Windows, Linux, Mac OSX	FiJi plugin to convert fluorescence intensities to concentrations and protein numbers using the FCS calibration parameters	*https://git.embl.de/grp-ellenberg/fcsanalyze* directory: *fiji*

Supplementary Table 1: Software packages for FCS-calibrated imaging.

Bundled packages are found on the Nature Protocols site. In source the link to the *git* repositories hosting the most recent source code is given. Recent bundled packages for **Supplementary Software 1-3** are found in *https://git.embl.de/grp-ellenberg/* followed by *fcsrunner/tags*, *mypic/tags*, and *adaptive_feedback_mic_fiji/tags*, respectively. Recent bundled version of Fluctuation Analyzer 4G[1] and **Supplementary Software 4** can be downloaded from *https://www-ellenberg.embl.de/resources/data-analysis*.

Parameter name	Meaning
N	Number of molecules in confocal volume
τ_{D_i}	Diffusion time of *i*-th component
α_i	Anomalous diffusion coefficient of *i*-th component
$f_1, f_2 = 1 - f_1$	Fraction of each component
θ_T	Fraction of fluorophore in a non-fluorescent state
τ_T	Relaxation time of the non-fluorescent state
$\kappa = z_0/w_0$	Structural parameter

Supplementary Table 2: Parameters of the diffusion models. Parameters of the models to fit the ACF of the protein and fluorescent dye (Eqs. (S4)-(S5)).

Supplementary Note 1: Effective confocal volume

To compute the effective confocal volume we approximate the PSF by a 3D Gaussian

$$PSF(x,y,z) = \exp\left(-2\frac{(x^2+y^2)}{w_0^2} - 2\frac{z^2}{z_0^2}\right). \quad (S1)$$

The parameters w_0 and z_0 characterize the e^2 decay length of the PSF. We also define the structural parameter as the ratio $\kappa = z_0/w_0$. The effective confocal volume for FCS [2] is given by

$$V_{eff} = \frac{(\int PSF\, dV)^2}{\int PSF^2 dV} = \pi^{\frac{3}{2}} w_0^3 \kappa \quad (S2)$$

This is larger than the confocal volume given by

$$V_{conf} = \int PSF\, dV = \left(\frac{\pi}{2}\right)^{\frac{3}{2}} w_0^3 \kappa = \frac{V_{eff}}{2^{\frac{3}{2}}} \quad (S3)$$

Supplementary Note 2: Fitting of the ACFs

For fitting of the ACFs of the fluorescent proteins we assume a 3D Gaussian focal volume (Eq. (S1)) and use a two component anomalous diffusion model with fluorescent protein-like blinking [1]

$$G(\tau) = \frac{\left(1 - \theta_T + \theta_T \exp\left(-\frac{\tau}{\tau_T}\right)\right)}{N} \sum_{i=1,2} f_i \left(1 + \left(\frac{\tau}{\tau_{D_i}}\right)^{\alpha_i}\right)^{-1} \left(1 + \kappa^{-2}\left(\frac{\tau}{\tau_{D_i}}\right)^{\alpha_i}\right)^{-0.5} \quad (S4)$$

In the software *FCSFitM* (**Supplementary software 4**) the data is fitted using the MATLAB routine *lsqnonlin*. The meaning of the parameters is listed in **Table S2**. As expected for the number of proteins *N* we obtain only minor differences (less than 2%) between a one component ($f_1 = 1$) or a two component model.

For the fluorescent dye in solution we use a single component non-anomalous diffusion model with triplet-like blinking

$$G(\tau) = \frac{\left(1 + \frac{\theta_T}{1-\theta_T}\exp\left(-\frac{\tau}{\tau_T}\right)\right)}{N}\left(1 + \left(\frac{\tau}{\tau_D}\right)\right)^{-1}\left(1 + \kappa^{-2}\left(\frac{\tau}{\tau_D}\right)\right)^{-1/2}. \quad (S5)$$

Equation (S5) is fitted to the ACF curves for the fluorescent dye to obtain the diffusion time τ_D and the structural parameter κ. The width of the focal volume is then given by

$$w_0 = 2\sqrt{D_{dye}\tau_D}. \quad (S6)$$

Here D_{dye} is the diffusion coefficient for the fluorescent dye. The diffusion coefficient changes as a function of the temperature and needs to be corrected according to

$$D_{dye}(T) = D_{dye}(T_{ref})\frac{(T + 273.15)\,\eta(T_{ref})}{(T_{ref} + 273.15)\eta(T)}. \quad (S7)$$

In Eq. (S6) all temperatures are in grad Celsius and T_{ref} is a reference temperature for which the value of the diffusion coefficient is available. The $\eta(T)$ is the dynamic viscosity at T. For water we have $\eta(27\,^{\circ}C)$ = 0.8509 mPa sec and $\eta(37\,^{\circ}C)$ = 0.6913 mPa sec (http://www.viscopedia.com/) yielding for the reference dyes Alexa488 and Alexa568 a mean diffusion coefficient of 463.23 µm^2/sec and 521.46 µm^2/sec, respectively.

Supplementary Note 3: Correction for background and photobleaching

From fitting equation (S4) to the ACF of the fluorescent protein we extract the protein number N. The protein number needs to be corrected for photobleaching and background using the coefficients computed in *Fluctuation Analyzer* [1]

$$N_c = N * Total\ Ch1|2. \quad (S8)$$

For low photon counts the computed bleach correction is less reliable. We found that for counts lower than twice the background counts in WT cells it is better to solely correct for the background

$$N_c = N * BG\ Ch1|2. \qquad (S9)$$

The correction parameters $Total\ Ch1$ and *BG Ch*1 as well as $Total\ Ch2$ and *BG Ch*2 for the FCS Channels 1 and 2, respectively are found in the result table from Fluctuation Analyzer. The concentration is calculated from the corrected number of proteins N_c according to

$$C_c = N_c/(V_{eff}\ N_A). \qquad (S10)$$

The effective volume V_{eff} is computed from Eq. (S2) using the previously estimated values κ and w_0. The computation of the corrected concentration is performed by *FCSFitM* and *FCSCalibration*.

Supplementary Note 4: Estimate the number of proteins on small structures

The fluorescence intensity generated by a point-source of one fluorophore is defined by

$$I(x,y,z) = I_0\ PSF(x,y,z) + I_b \qquad (S11)$$

where $PSF(x,y,z)$ is the confocal observation profile for the imaging settings, I_0 the fluorescence intensity characterizing a single fluorophore, and I_b the background intensity. We denote the imaging volume by

$$V_{\text{conf}} = \int PSF\ dV. \qquad (S12)$$

For FCS measurements performed within a volume larger than the PSF, we can assume a homogeneous distribution of fluorophores in space $I(x,y,z) = I$. For a linear detector the fluorescence intensity scales with the concentration *C* according to

$$I = I_0 C\ N_A V_{\text{conf}} + I_b. \qquad (S13)$$

The concentration C is the concentration obtained from the FCS measurement and N_A the Avogadro constant. We thus obtain the linear relationship

$$C = (I - I_b)k \qquad (S14)$$

between concentration and fluorescence intensity with the calibration factor $k = \frac{1}{I_0 N_A V_{conf}}$. An example calibration curve for simulated fluorophore distributions is shown in Supplementary Fig. 3 a-b. Relation (S14) holds true at every pixel with index j

$$C_j = \left(I_j - I_b\right)k. \qquad (S15)$$

We can approximate the number of molecules at each pixel with

$$N_j = N_A\ C_j\ \Delta x\ \Delta y\ \Delta z. \qquad (S16)$$

The parameters Δx, Δy and Δz characterize the pixel resolution in the 3 dimensions.

Equation (S16) can be applied at every pixel. To estimate the total number of proteins on a structure it is necessary to sum the protein number for all pixels in the structure of interest. For structures within the size of the PSF the signal must be integrated in 3D so that a large portion of the PSF is included. Simulations show that the estimation precision is directly proportional to the fraction of PSF included in the integration (Supplementary Fig. 3). Over 95% of the signal is accounted for when the region considered is more than twice the e^2 decay of the PSF (~ 2/3 of the Airy disc diameter). If the size of the imaging PSF is known, approximations can be used to compute the signal from fewer pixels. For instance, a 3D Gaussian approximation of the PSF is obtained from the FCS measurement of the reference dye. These results can be used for the imaging PSF if the same imaging parameters are used (laser power and pinhole size). Below we provide some examples as a guideline.

Supplementary Note 5: Total fluorophores in a point source

Consider *m* emitters concentrated at a point (Supplementary Fig. 3 c-d). The intensity is given by

$$I(x,y,z) = m\, I_0\, PSF(x,y,z) + I_b. \qquad (S17)$$

Using Eq. (S15) and after integration in 3D one obtains the expected value

$$N = \iiint N_A\, C\, dx\, dy\, dz = \frac{m\, I_0}{I_0} = m. \qquad (S18)$$

With (S15) and (S16) the integral is approximated by

$$N_c = \sum_j^{P_{XYZ}} N_j. \qquad (S19)$$

The sum is for all pixels that enclose the object of interest. When the parameters of the imaging PSF are known one can approximate Eq. (S18). Using the number of proteins in the plane of maximal intensity (here $z = 0$) one obtains

$$N_c = \sum_j^{P_{XY}} N_A\, C_j\, \Delta x\, \Delta y \int PSF(0,0,z)dz. \qquad (S20)$$

For a PSF approximated by a 3D Gaussian the Eq. (S20)

$$N_c = \sum_j^{P_{XY}} N_A\, C_j\, \Delta x\, \Delta y \sqrt{\frac{\pi}{2}} z_0\,. \qquad (S21)$$

The peak intensity from the point source can also be used

$$N_c = N_A \max(C_j) \iiint PSF(x,y,z)dx\, dy\, dz \qquad (S22)$$

For a PSF approximated by a 3D Gaussian Eq. (S22) reads

$$N_c = N_A \max(C_j) \left(\frac{\pi}{2}\right)^{\frac{3}{2}} w_0^3 \kappa \,. \qquad (S23)$$

The smaller the imaging pixel size the more precise is the result (Supplementary Fig. 3 d, $\leq 0.6\, w_0$).

Supplementary Note 6: Density of fluorophores on a membrane

For a homogeneous spatial density the region size to be considered for quantification decreases due to symmetry properties of the membrane.

For example, for a membrane extending in the XZ-plane and parallel to the Y-axis we only need to consider pixels in one Z-plane (Supplementary Fig. 3 g). The density of fluorophores, d_c, at a specific Y position is computed from the sum of pixels along the X direction in a region that enclose the fluorescence signal of the membrane (Supplementary Fig. 3 h, circles)

$$d_c = N_A \sum_j^{P_X} C_j \Delta x. \qquad (S24)$$

Using the peak intensity one obtains

$$d_c = N_A \max(C_j) \int PSF(x,y,z)dx \,. \qquad (S25)$$

For a PSF approximated by a 3D Gaussian Eq. (S25) reads (Supplementary Fig. 3 h, squares)

$$d_c = N_A \max(C_j) \left(\frac{\pi}{2}\right)^{\frac{1}{2}} w_0 \qquad (S26)$$

Similarly, the density d_c of fluorophores on a membrane extending in the XY plane can be computed from the pixels in the Z direction (Supplementary Fig. 3 e). We denote $\bar{C}_j$ the average concentration in a specific Z-plane. One obtains

$$d_c = N_A \sum_j^{P_Z} \bar{C}_j \, \Delta z. \qquad \text{(S27)}$$

Using the plane peak intensity we have

$$d_c = N_A \max(\bar{C}_j) \int PSF(x, y, z) dz. \qquad \text{(S28)}$$

For a PSF approximated by a 3D Gaussian Eq. (S28) reads

$$d_c = N_A \max(\bar{C}_j) \left(\frac{\pi}{2}\right)^{\frac{1}{2}} \kappa \, w_0 \, . \qquad \text{(S29)}$$

References

1 Wachsmuth, M. *et al.* High-throughput fluorescence correlation spectroscopy enables analysis of proteome dynamics in living cells. *Nature biotechnology* **33**, 384-389, doi:10.1038/nbt.3146 (2015).

2 Schwille, P. & Haustein, E. in *Biophysics Textbook Online* Vol. 1 1-62 (2001).