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

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The ability to tag a protein at its endogenous locus with a fluorescent protein (FP) enables the quantitative understanding of protein dynamics at the physiological level. Genome editing technology has now made this powerful approach routinely applicable to mammalian cells and many other model systems, opening up the possibility to systematically and quantitatively map the cellular proteome in four dimensions. 3D time-lapse confocal microscopy (4D imaging) is an essential tool to investigate spatial and temporal protein dynamics, however it lacks the required quantitative power to make absolute and comparable measurements required for systems analysis. Fluorescence correlation spectroscopy (FCS) on the other hand provides quantitative proteomic and biophysical parameters such as protein concentration, hydrodynamic radius and oligomerization but lacks the ability for high-throughput application in 4D spatial and temporal imaging. Here, we present an automated experimental and computational workflow that integrates both methods and delivers quantitative 4D imaging data in high throughput. Our protocol describes the automated acquisition of FCS and imaging data of human cells. This data is processed to yield a calibration curve relating the fluorescence intensities of image voxels to absolute protein abundance. The calibration curve allows the conversion of the arbitrary fluorescence intensities to protein amounts for all voxels of 4D (3D plus time) imaging stacks. With our workflow the user can acquire and analyze hundreds of FCS-calibrated image series to map their proteins of interest in four dimensions.

INTRODUCTION

Why FCS-calibrated fluorescence imaging?

Tagging of proteins with a fluorescent marker is an essential tool to study the localization, function, dynamics and interactions of proteins in living cells. With the advantage of genome editing technologies such as CRISPR/Cas9 [1-3], it is now possible to engineer almost any higher eukaryotic cell to homozygously express the fluorescent fusion protein of interest (POI) at its physiological levels (see also related Nature Protocol, Koch et al.). Genome editing in combination with absolute quantitative fluorescence microscopy extends proteomics methods, typically restricted to large cell populations at specific time points, to single cell dynamic proteomics of living cells.

Modern confocal microscope detectors show a linear dependency of fluorophore concentrations and fluorescence intensities within several orders of magnitudes. To perform absolute quantitative imaging and convert relative fluorescence intensities to absolute physical quantities, such as the concentration of the fluorophore bearing protein, one can determine the calibration parameters of this linear dependency. To estimate the fluorophore concentrations temporal and spatial fluctuations of diffusing species can be used [4-6]. In this protocol we use single point confocal fluorescence correlation spectroscopy (FCS). FCS is a biophysical single molecule technique that allows to measure concentrations and diffusion coefficients of fluorescently labeled molecules [6-9]. This method is well suited for low to medium abundant proteins, a situation often observed at endogenous expression levels. Furthermore, most of the commercially available confocal microscopes can be equipped with the necessary detectors and hardware to perform FCS. In this protocol, FCS with 3D confocal time-lapse imaging is combined to estimate the calibration parameters and convert fluorescence intensities to concentrations in living cells. Such an approach has been previously used to estimate protein numbers on bulk chromatin [10,11], on chromosome boundaries [12], and large multi-protein complexes [13]. This robust technique makes FCS-calibrated quantitative imaging easily available on most confocal systems.

In this protocol, we offer custom software packages and a step-by-step guide based on a Zeiss LSM confocal microscope with FCS capability as a widely used commercial system. The data acquisition software packages (**Supplementary software 1-3**) simplify the repeated cycles of imaging and FCS measurements required for FCS-calibrated imaging. In combination with the provided online image analysis tool, data acquisition can be completely automated [14-16]. This

allows, without human supervision and in high-throughput, to automatically select cells with the optimal protein expression level and morphology and acquire images and FCS measurements. The data analysis software package (**Supplementary software 4**) is used to automatically extract the fluorescence intensities at FCS measurement points, compute protein concentrations and the FCS calibration parameters, and convert fluorescence image intensities to concentrations and protein numbers.

Fluorescence correlation spectroscopy

For a general introduction we refer to reviews and protocols describing FCS and dual color fluorescence cross-correlation spectroscopy FCCS (e.g. [5-9]). Here we provide only a short and practically oriented introduction to the topic (Fig. 1). FCS measures the fluctuations of fluorescent molecules in a sub-femtoliter observation volume of a focused laser beam. The intensity changes caused by single molecules moving in and out of the observation volume over time are recorded (Fig. 1a-b). The fluctuations depend on (*i*) the concentration of fluorophores, (*ii*) their mobility, (*iii*) their photophysics, and (*iv*), to a lesser extent, the properties of the detector. To analyze the fluctuations, the autocorrelation function (ACF) $G(\tau)$ is computed from the intensity time trace $I(t) = \langle I \rangle + \delta I(t)$

$$G(\tau) = \frac{\langle \delta I(t) * \delta I(t+\tau) \rangle}{\langle I \rangle^2}. \quad (1)$$

The bracket $\langle \cdot \rangle$ indicate time-averaged quantities and $\delta I(t)$ the mean fluctuation of the time-averaged intensity $\langle I \rangle$. The amplitude of $G(\tau)$ is inversely proportional to the number of particles in the observation volume N (Fig. 1c). The decay time τ_D of $G(\tau)$ gives the characteristic time a fluorophore remains in the observation volume, and consequently, is a direct measure of the fluorophore diffusion. These parameters can be extracted by fitting the ACF to a physical diffusion model (see Supplementary material). To compute the concentration of the fluorophore at the FCS measurement point, the number of molecules N is divided by an effective confocal volume V_{eff}, typically below 1 fl, measured for the used microscopy setup.

Since the amplitude of $G(\tau)$ decreases with the number of particles, the sensitivity of the method decreases for high concentrations of the fluorophore. For a fluorophore such as GFP concentrations up to 1-2 µM can be measured. At very low fluorophore concentrations, the method is impaired by scattering and background fluorescence. Nevertheless, by using

appropriate corrections for the background, GFP concentrations as low as a few pM can be measured. This limitation does not apply to the imaging of fluorescence intensity where the time integrated detector signal is used. In this case concentrations above the µM range can be measured as long as the detector is not saturated. A dynamic range of pM to µM concentrations means that over 70% of the proteome of a human cell line is accessible to this method [17].

Overview of the procedure

The procedure is summarized in Figure 2. The described method uses single color FCS to obtain one calibration curve for one type of fluorescent protein (FP). For the sample preparation it is convenient to use a multi-well coverslip glass bottom chamber containing separate wells with cells expressing the fluorescently tagged POI (mFP-POI), cells expressing a monomeric form of the fluorescent tag alone (mFP), non-expressing wild type cells (WT), and a droplet of a reference fluorescent dye with a known diffusion coefficient. The fluorescent dye is used to adjust the microscope and compute the effective confocal volume V_{eff}. Cells without a fluorescent tag are used to obtain the background photon counts, for correcting the FCS derived concentrations, and background image fluorescence intensity. Cells expressing the fluorescent tag alone are used to calibrate the monomeric fluorophore and estimate whether the POI oligomerizes. Furthermore, they provide a large dynamic range of concentrations and fluorescence intensities at different expression levels for robust estimation of the calibration parameters.

First the high NA water immersion objective is adjusted to the thickness of the cover slip glass by turning the correction ring (Fig. 2a). An optimal adjustment can be assessed by a maximal counts per molecule (CPM) and a thin reflection line at the immersion water glass interface in a XZ scan. Then repeated FCS measurements of the reference dye are acquired and the ACF is computed (Fig. 2b). The dye ACFs are fitted with a physical diffusion model to calculate the effective focal volume V_{eff}

$$V_{eff} = \pi^{\frac{3}{2}} w_0^3 \kappa, \tag{2}$$

where w_0 is the lateral radius and κ the ratio of axial to lateral radius of the focal volume. The fitting of the model to the ACF data and calculation of the effective confocal volume is performed in *FCSFitM* (**Supplementary software 4**).

Next, the microscope settings, such as pixel-dwell time, laser intensity, and detector gain are carefully chosen for imaging the mFP-POI. These imaging settings must be kept the same for all images that need to be quantitatively analyzed and compared.

The data for the calibration curve is obtained by placing 2-4 FCS measurement points at specific locations in the cell, typically at the two largest compartments, namely nucleus and cytoplasm. Then an image is acquired with the previously determined imaging settings, followed by the FCS measurements and storage of the image pixel coordinates of the FCS measurements for further processing. This procedure is repeated for several cells and the three cell lines WT, mFP, and mFP-POI (Fig. 2c). To simplify this workflow and link the images with the FCS measurements we provide macros for the ZEN software. With *FCSRunner* (**Supplementary software 1**) the user manually selects cells and FCS measurement points. For each cell, the macro acquires an image, FCS data, and stores position metadata. Using *MyPiC* (**Supplementary software 2**) and *Automated FCS* (**Supplementary software 3**) data acquisition can be fully automated. Cells are automatically selected, according to their morphology and protein expression level, imaged and measured using FCS.

The ACFs are fitted to physical models of diffusion to obtain the number of molecules N (see **Supplementary methods**). A bleach and background corrected number of molecules N_c is computed to yield a concentration C at the FCS measurement point

$$C = \frac{N_c}{N_A V_{eff}}, \tag{3}$$

where N_A is the Avogadro constant. These steps can be performed using *FCSFitM* (**Supplementary software 4**). At the same FCS measurement point the image fluorescence intensity I is calculated in a small region of interest (ROI) (*FCSImageBrowser*, **Supplementary software 4**). Finally, the data is quality controlled to correct for poor fits, measurements at the border of a compartment or on immobile structures (e.g. a nucleoporin on the nuclear envelope) (Fig. 2d and Fig. S1). The concentrations are then plotted against the fluorescence intensity and fitted with a linear relationship (Fig. 2e)

$$C = (I - I_b)k. \tag{4}$$

I_b is the mean background fluorescent intensity estimated from WT cells and k is the calibration factor. The last steps can be performed using the application *FCSCalibration* (**Supplementary software 4).**

Given that the laser intensity remains stable and that the imaging conditions are not changed, 3D stacks and 4D movies can now be acquired from cells in the multi-well plate without additional FCS calibration measurements. The image pixel fluorescence intensities I_p are converted to concentrations C_p using Eq. (4) and to protein number per pixel (Fig. 3a) using the physical size of the 3D voxels, characterized by the sampling Δx and Δy and the Z-slice interval Δz,

$$N_p = N_A \, C_p \, \Delta x \Delta y \Delta z. \tag{5}$$

Fluorescence intensities conversion is automatically performed using the ImageJ plugin *FCSCalibrate* (**Supplementary software 4**). See section "Considerations for quantification" for further details on quantifying protein numbers on cellular structures.

Quantification of FCS-calibrated images

The calibrated images can be used to obtain a precise estimate of the protein distribution in specific cellular compartments, organelles or structures. For that purpose additional fluorescent reference markers of the structure of interest imaged in an independent fluorescent channel are required (see also “Considerations for quantification”). For instance, to separate proteins on the nuclear envelope from proteins in the nucleus and cytoplasm a nuclear or DNA marker can be used. Figure 3 shows an example for the quantification of the protein density on the nuclear envelope. A cell line expressing NUP107, a nucleoporin which is a constitutive part of the nuclear pore complex (NPC), endogenously tagged with mEGFP has been used. Based on a DNA marker the nucleus was segmented in 3D to extract the localization of the nuclear envelope. For further processing the equatorial plane of the nucleus has been used (Fig. 3b) and the mean fluorescence intensity in the nuclear rim has been extracted. Depending on imaging settings and microscope, the value of the arbitrary fluorescent intensity units on the nuclear envelope can be significantly different (Fig. 3c). However, after conversion to absolute protein numbers using a separate calibration curve for each microscope, comparable results between the different imaging systems are obtained (Fig. 3d). The quantification of NUP107 using this method yielded a protein

density at the nuclear envelope that is in good agreement with the density predicted from the NUP107 stochiometry (32 proteins/NPC, [18]) and the NPC density in interphase (10.66 ± 2.69 NPCs/µm^2, [19]) (Fig. 3d, gray shaded area).

FCS settings

For FCS the estimation of the effective confocal volume is particularly important since it is used to scale all measurements. The effective confocal volume is estimated by measuring a sufficiently diluted solution of a fluorophore with a known diffusion constant (5-50 nM, [20]) and carefully mounting the sample to avoid any non-planarity of the cover slip with the objective lens (see "Reagent setup/Cellular samples"). The laser intensity is adjusted sufficiently high to obtain good CPMs, as this will lead to a good signal to noise ratio, but also sufficiently low to avoid photobleaching, photodamage and saturation effects during the FCS measurement. A good practice is first to set the fluorescence intensity for the biological sample expressing the FP only to obtain CPMs from 2 to 4 kHz with low photobleaching. The choice of the pinhole size is a tradeoff between detected signal, sensitivity, and the quality of the fits. Larger pinholes cause a decrease in CPM leading to a decrease in the quality of the fit. This lowers the precision of the diffusion time estimate, a parameter affecting the precision of the effective confocal volume estimate. We found that pinhole sizes from 1 to 1.6 Airy Units (AU) gave reliable confocal volume estimates without impairing the quality of the fits (Fig. S2a and [21]). Since the size of the effective confocal volume depends on the laser power and pinhole size (Fig. S2b-c), the same parameters used for the FCS POI measurements must also be used for the reference dye.

Imaging settings

We distinguish between images that are used to compute the FCS calibration curve and images where we apply the image calibration. To define the parameters of the calibration curve, single plane images at single time-points are best suited. Saturation of the image should be avoided, as this will preclude quantification above an upper limit. Furthermore photobleaching during the acquisition of the calibration images should be minimal. To compute an approximation of the protein numbers in a measurement point, the method uses the 3D pixel dimensions of the imaging system. Consequently, images that need to be quantified must be 3D (XYZ) or 4D (XYZT) image stacks. In time-lapse movies, photobleaching at later time points should be corrected if necessary. The spatial sampling must be high enough in order to resolve the structure of interest. For

structures that are close to the size of the point spread function (PSF) or non-isotropic, such as membranes, a spatial sampling close to the Nyquist criterion should be used. The imaging pinhole needs to be adjusted similarly to the FCS measurement and small enough for good confocal sectioning (also 1-1.6 AU).

Considerations for quantification

To quantify the total number of molecules in the whole cell or in specific subcellular compartments or structures, image processing is required to define discrete volumes. To obtain the protein amount in the whole cell, the protein number for each pixel in the cell can be added up. Precise results require a marker that allows for segmentation of the cell surface. An easy solution to this problem is to use fluorophores that stain the extracellular culture media and cannot penetrate the cellular membrane, such as fluorophores linked to a bulky molecule (e.g. high molecular weight dextran or IgG). In addition, a nuclear or DNA marker is required to define protein numbers in the nucleus/chromatin and in the cytoplasm. This marker is used to segment the chromatin mass and sum the intensity of pixels within the chromatin volume. It should be noted that reference markers, enabling cellular or subcellular image segmentation, must be coupled to fluorophores that do not interfere with the FP tagged to the POI used for FCS calibration. Fluorophores that work in good combination with the commonly used FPs mEGFP and mCherry are provided in the section "Reagents Setup, Markers to aid image segmentation" and **Table 1**.

The quantification of total protein numbers on structures that are within the size of the PSF, such as large multiprotein complexesor membranes require particular care [12] [13]. As shown in the supplement the fluorescent signal needs to be integrated throughout the structure in 3D (Fig. S3). A diffraction-limited structure requires integration of the signal in a 3D volume of the size of the PSF (Fig. S3c-d). If the size of the PSF is known, one can approximate the total number of proteins from a subset of pixels (Fig. S3c-d, squares and diamonds). Furthermore, spatial symmetry properties of the structure of interest can be used. For example, in the equatorial plane of the nucleus the nuclear membrane can be assumed to be locally isotropic in Z. In this case to obtain the density of proteins on the nuclear membrane, it is enough to estimate the protein number per unit length on a sufficiently broad nuclear rim at the equatorial plane (Fig. 3 and Fig. S3f).

MATERIALS

EQUIPMENT

Hardware requirements

- Scanning confocal microscope with fluorescence correlation setup. The detectors for fluorescence correlation must work in photon counting mode. For imaging the detectors must be in a linear response range (see EQUIPMENT SETUP)
- Temperature control for long time imaging of living cells
- A high numerical aperture water-immersion objective (see EQUIPMENT SETUP)
- Observation chamber with coverglass bottom suitable for cell culture with at least 4 separate wells. Imaging plates 96CG, glass bottom (zell-kontakt, cat. # 5241-20), 4 to 8 well IBIDI (IBIDI, cat. # 80427, 80827), 4 to 8 well LabTek (Thermo Fisher, cat. # 155382, 155409, 155383, 155411) both #1 or #1.5 glass thickness can be used
- For long time imaging an objective immersion micro dispenser system should be used. Leica provides a commercial water immersion micro dispenser.
- 15 cm Nunc dishes for cell culture (D9054 Sigma)
- PD-10 Sephadex desalting columns (optional, GE Healthcare, cat. #52130800)
- Slide A-Lyzer 10.000 MWCO (optional, Thermo Fisher, cat. # 66810)
- Viva spin 30.000 MWCO (optional, Sigma-Aldrich, cat. # Z614041-25EA)
- 0.22µm Filters
- Workstation with Windows 7 or higher

Software requirements

- Microscope software licensing for FCS data acquisition
- For Zeiss microscopes, ZEN black edition (version higher or equal to ZEN 2010) to run the VBA macros (**Supplementary Software 1** and **2**)
- FiJi installation ([22], [23]) on analysis computer
- For high-throughput FCS and imaging data acquisition with adaptive feedback (25|-37|), FiJi on the computer with the ZEN software controlling the microscope

- Fluctuation Analyzer 4G (FA, [16]) on the analysis computer to compute corrected autocorrelation traces from the raw photon counting data
- The workflow for FCS fitting (*FCSFitM*, **Supplementary Software 4**) is a MATLAB tool compiled for Windows. This does not require a MATLAB installation. To run the source code on a different operating system, MATLAB (R2014a or later, The MathWorks, Inc., Natick, Massachusetts, United States) with the toolboxes optimization and statistics needs to be installed
- The data analysis tool *FCSCalibration* (**Supplementary Software 4)** is a R tool with a *shiny* graphical user interface ([24], [25]). In the packaged version for Windows all required dependencies are installed. To directly use the source code, a R installation is required
- All custom software packages are provided as **Supplementary Software 1–4**. An overview of software requirements is provided in **Supplementary Table 1** with links to the most recent source

REAGENTS

Cell lines

- Cell line of interest with the POI labelled with a FP. Preferably endogenously tagged cell line. Here we show the data for a HeLa Kyoto cell line with mEGFP-NUP107 (endogenously tagged using a zinc finger nuclease) [19]

  CRITICAL The FP must be appropriate for the available laser lines and filters. For this protocol mEGFP and mCherry can be used as FPs
- Cell line of interest as wild type clone. Here we used a HeLa Kyoto cell line

Mammalian cell culture

- High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, cat. # 41965-039)
- Fetal Bovine Serum (FBS, Life Technologies, cat. #.10270106),
- 10000 units/ml Penicillin–Streptomycin (Life Technologies, cat. # 15140122)
- 200 mM L-Glutamine (Life Technologies, cat. # 25030081)
- PBS (see "REAGENTS SETUP")

- 0.05% Trypsin (Life Technologies, cat. # 25300054)

Live cell imaging

- Cell culture buffers suitable for imaging. These should be non-fluorescent and have a suitable refractive index. For the protocol presented here, we used CO_2-independent imaging medium without phenol red (Gibco, cat. # ME080051L1)

Confocal volume calibration

- Bright and photostable dyes that match the spectral properties of the FP with known diffusion coefficients are required (see "REAGENTS SETUP"). For example Alexa488 (Thermo Fisher, NHS ester, cat. # A20000), Alexa568 (Thermo Fisher, NHS ester, cat. # A20003) or Atto488 (Atto-Tec, NHS ester, cat. # AD 488-31)

Transfection

- Plasmid expressing monomeric form of the FP (e.g. mEGFP)
- Opti-MEM® I Reduced Serum Medium, GlutaMAX™ Supplement (Life Technologies, cat. #. 51985-026)
- Fugene6 (Promega, cat. # E2691)

Cellular markers (optional)

- SiR-DNA (also known as SiR-Hoechst) (SPIROCHROME, cat. #. SC007).
- Hoechst-33342 (Thermo Fisher, cat. # 62249)
- Dextran, Amino, 500,000 MW (Life Technologies, cat. # D-7144)
- Dy481XL-NHS-Ester (Dyomics, cat. # 481XL-01)
- Atto430 LS NHS Ester (Molecular Probes, cat. # AD-430LS-31)
- Molecular Probes® DMSO (Thermo Fisher, cat. # D12345)

Other reagents

- Pure ethanol for cleaning the objective
- Lens cleaning tissue (e.g. Whatman)
- Silicon grease

EQUIPMENT SETUP

Detectors

For FCS on Zeiss LSM confocal systems GaAsP or APD detectors (Confocor-3 system or in combination with a PicoQuant upgrade kit) can be used. For Leica microscopes the HyD SMD detectors or APD detectors can be used. The protocol and software is based on Zeiss LSM microscopes (LSM780, LSM880) using ZEN black edition and an inverted Axio Observer. In particular the VBA macros only work with ZEN black edition. For imaging we tested GaAsP detectors (LSM780 and LSM880) and the AiryScan detector (LSM880).

Microscope objective

Use a water objective with a high numerical aperture and the best chromatic correction as recommended for FCS. On Zeiss systems use C-apochromat Zeiss UV-VIS-IR 40x 1.2 NA, specially selected for FCS (421767-9971-711). Prior to objective purchase we recommend verifying the PSF and confocal volume, using fluorescent dyes (e.g. Alexa488 and Alexa568) and fluorescent beads (TetraSpeks microspheres, 100nm, Thermo Fischer, cat. # T7279). Use pure water as immersion media as this yields the best optical properties. Oil immersion media that match the refractive index of water only do this at a specific temperature (typically 23 °C). Thusdue to refractive index mismatch the results with oil immersion media may not have the same quality as the results obtained with water as immersion media.

SOFTWARE SETUP

The analysis and acquisition software is listed in **Supplementary Table 1.** For the installation of the custom packages (**Supplementary software 1-4**) follow the instructions included in the software documentation. The user guides can also be found on the Wiki pages of the *git* source directories. Install all required software prior to starting the experiment.

Installing Fluctuation Analyzer 4G (FA)

Download the software from *www.ellenberg.embl.de/software/* [16]. On the data analysis computer with a Windows operating system unpack the software and run the setup.

Installing FiJi

Download the latest version of FiJi from *https://fiji.sc/* and install the software on the data analysis computer. For the adaptive feedback pipeline install the software on the computer that runs the software controlling the microscope.

Installing R (optional)

In case the *FCSCalibration* software packaged for Windows packaged is not used (**Supplementary software 4**), you need to install the R software. Download *R* (https://cran.r-project.org/) or *RStudio* (*https://www.rstudio.com/*) and install the software on the data analysis computer.

REAGENTS SETUP

HeLa growth medium High Glucose DMEM supplemented with 10 % FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-Glutamine. Supplemented DMEM medium is abbreviated as complete DMEM.

Imaging medium CO_2 independent medium without phenol red supplemented with 10% FBS, 2 mM L-Glutamine, 1 mM sodium pyruvate. Eventually add glucose to achieve a final glucose concentration as in the complete DMEM (4.5 g/liter). Filter through a 0.22 µM membrane to clear the medium of precipitants.

PBS, 1X Prepare 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 in ddH_2O. Adjust the pH to 7.4 with HCl and autoclave.

Cellular samples

For FCS-calibrated imaging three different cell line samples have to be seeded together in a multi-well glass-bottom chamber:

(1) To measure the background fluorescence and background counts the cell line of interest is needed as a wild type clone (WT), i.e. not expressing recombinant FPs. Alternatively,

measurements in solution or culture media can be taken. However, this typically underestimates the background by about 10%.

(2) To obtain concentration and fluorescence intensity measurements that span a range of up to 2 µM, cells expressing the FP alone are needed. These cells can be generated by transiently transfecting WT cells with a plasmid containing the monomeric form of the FP used for tagging of the POI.

(3) To achieve physiological expression of the POI, genome edited cells (ZNF, CRISPR/Cas9) expressing the POI tagged with FP are preferred (see related Nature Protocol, Koch et al.).

The criteria for FPs in FCS are the same as for fluorescent imaging: (*i*) high molecular brightness, (*ii*) fast maturation time, (*iii*) minimal oligomerization, (*iv*) high photo stability, (*v*) spectral match with available laser lines and filters. The method presented here can only estimate the number of FPs; therefore if the maturation time of the fluorescent tag is of the same order as the life-time of the POI, the total number of proteins will be significantly underestimated. Monomeric EGFP and monomeric EYFP or other bright green/yellow FPs are well suited for FCS-calibrated imaging. mCherry is suitable as red FP.

The objective as well as the bottom of the sample should be carefully cleaned and mounted to avoid any non-planarity between objective and cover-slip as this leads to significant aberrations in the PSF. Planarity can be tested using the glass reflection in a XZ scanning mode, with the FCS objective at the smallest zoom or with a lower magnification objective. One should observe a horizontal straight line with negligible deviation in Z (<0.1 degrees tilting) (Fig. 2a).

Fluorescent dyes for calibration

To characterize the confocal volume of excitation and detection we use a fluorescent reference dye in aqueous solution. The criteria for the fluorescent dye is the obtainable molecular brightness, spectral match with available laser lines and filters, spectral match with the FP of interest, and a known diffusion coefficient. To investigate proteins tagged with green/yellow FPs (e.g. GFP, YFP), Alexa488 is a suitable dye. For proteins tagged with a red FP (e.g. mCherry), Alexa568 is a suitable dye. To compute the parameter of the confocal volume we use the diffusion coefficients of the fluorescent dye. The estimated diffusion coefficient of Alexa488 and Alexa568 at 27 ˚C is D_{A488} = 365 ± 20µm^2/sec and D_{A568} = 410 ± 40 µm^2/sec (M. Wachsmuth personal communication and [26]) yielding at 37 ˚C a mean of D_{A488} = 464.23 µm^2/sec and D_{A568} =

521.46 μm^2/sec (Supplementary Eq. S7). Diffusion coefficients for additional fluorescent dyes can be found in [27].

Markers to aid image segmentation

Cellular compartments which are useful to be stained for image segmentation and further quantification are for example the nucleus/DNA and the cytoplasm. Negative staining of the whole cell using culture media dyes can be used as proxy for the cytoplasm. The difference between the whole cell and the DNA yields the cytoplasmic compartment. The fluorophore used for staining, in particular for inner cell structures, should not cross-talk into the channel of the POI used for quantitative imaging. For mEGFP a suitable DNA counterstain is SiR-DNA in combination with a 633 nm laser [28]. Concentrations as low as 50 nM SiR-DNA give sufficient signal in HeLa Kyoto (2 h incubation) and are well tolerated by living cells. When excited with 561 nm SiR-DNA has a minor cross-talk into the mCherry channel. This makes SiR-DNA less suitable in combination with a protein tagged with mCherry and a detection window up to 650nm. In combination with mCherry, a small molecule DNA staining alternative is the vital dye Hoechst 33342. To minimize toxicity, incubate cells for 10 min with 10 nM Hoechst. Nevertheless, the UV excitation with 405 nm can cause considerable photodamage. Instead of a chemical stain, fluorescently labelled histone 2B (H2B) can be used as an alternative nucleus/chromatin marker. In combination with mEGFP a suitable tag for H2B is mCherry, with mCherry, mCerulian or mEGFP are suitable tags for H2B in combination with mCherry.

To segment the cell boundary, a fluorescent marker in the culture media can be used. This negative stain of the cell boundary has the advantage of being easily segmentable and the fluorescent dye can be adapted to the combination of DNA stain and protein of interest fluorescent probe. A suitable marker should not penetrate into the cells. For instance dyes coupled to large molecular weight dextran (>= 500 kDa) or IgG can be used. A suitable dye in combination with mEGFP and SiR-DNA is DY481XL. This large stokes-shift dye can be excited together with mEGFP at 488 nm but emits in the red spectral range. A suitable dye in combination with mCherry for the POI and H2B-mCerulian is Atto430LS. Atto430LS is a large stokes-shift dye that can be excited at 458nm together with mCerulian and emits in the green range. More combinations are summarized in **Table 1**.

The protocol to generate dextran labeled with a fluorescent dye such as Dy481XL-NHS-Ester is described below:

i. Dissolve 125mg Dextran, Amino, 500 000 MW, in 6.25ml of 0.2 molar $NaHCO_3$ (pH 8.0-8.3, freshly prepared)
ii. Mix well by stirring
iii. Dissolve 10mg Dy481XL-NHS-Ester (Dyomics 481XL-01) in 0.7 ml of DMSO
iv. Add Dy481XL directly into the dextran solution as quickly as possible since it is not stable
v. Mix at RT, stirring with ~500 rpm for 60 min
vi. Use 3x PD-10 Sephadex desalting columns to separate unreacted Dy481XL following the suppliers manual and eluate with 3.5 ml water per column
vii. Dialysis against water (MonoQ) using Slide A-Lyzer 10000 MWCO against water (sterile MonoQ) at 4ºC whereby exchange the water after ~2h. Dialysis was performed over night
viii. Concentrate labeled Dextran using Viva Spin 30.000 PES as described in the suppliers manual
ix. Measure the amount at the wavelength of 515 nm and calculate the concentration of the fluorescently labeled Dextran using the following equation

$$\text{Concentration} = \frac{Absorbance}{extinction * pathlength} \quad (6)$$

PROCEDURE

Sample preparation TIMING 1h hands-on, 1d waiting time, 15 min hands-on

1| For imaging and FCS, a cell density of 50-70% confluence is recommended. Seed the cells on a glass bottom multi-well plate one day before the experiment. Prior to seeding, wash each well twice with PBS. For an 8 well LabTek chamber seed 1.6-2.0x10^4 HeLa Kyoto cells into 300 µl DMEM complete growth medium per well. Seed cells expressing the fluorescently tagged POI in one well and WT cells in two separate wells. One well should be left empty for the reference FCS measurement with a fluorescent dye.

2| Grow cells in DMEM complete growth medium at 37 ºC, 5% CO_2.

3| *Transfection of HeLa Kyoto cells with a plasmid for the FP 6 h after seading*

Transfection can be carried out using FuGENE reagent and the following protocol

(i) Mix 50 µl Optimem with 1 µl FuGENE6 in an Eppendorf tube.

(ii) Incubate for 5 min at room temperature (RT).

(iii) Add 300 ng of plasmid DNA and mix well.

(iv) Incubate the transfection mixture for 15 min at RT.

(v) Add 25-50 µl of transfection complex to one well with seeded WT cells.

CRITICAL Dispense the transfection mix to avoid detaching of cells or contamination of neighboring wells. The amount of cDNA, transfection reagent or the time between transfection and imaging needs to be adapted. The goal is to achieve rather low expression levels of the FP in WT cells, which should be in the expression level range of the mFP-POI.

4| On the next day, prior to imaging, change the medium to a phenol red free imaging medium. Use a CO_2 independent medium if your microscope incubator does not allow for CO_2 perfusion or if the CO_2 perfusion impairs the FCS measurements.

5| For the automated acquisition of FCS data and imaging data (steps 25|-37|), staining of the DNA (e.g. using SiR-DNA) is required. Add SiR-DNA to achieve a final concentration of 50-100 nM. Incubate for 2 h at 37 ^{o}C (without CO_2) until incorporation of SiR-DNA is complete In the meanwhile proceed with steps 6| to 20|.

? TROUBLESHOOTING

6| Add any further fluorescent markers for the cell boundary at this step, if required.

7| Place a 20-50 µl drop of the diluted fluorescent reference dye (5-50 nM) in the empty chamber well.

8| To minimize liquid evaporation during the measurement, seal the multi-well plate chamber with silicon grease or use a gas permeable foil suitable for cell culture when using CO_2 independent or CO_2 dependent imaging media, respectively. This step is recommended in case the humidity level is not sufficiently maintained in the microscope incubation chamber during the measurement.

Microscope setup and effective confocal volume measurement TIMING 1-2h

9| Before preparing the sample for imaging (steps 4| to 8|), turn on the microscope, microscope incubator and all required lasers. Allow the confocal microscope system and the incubation chamber to equilibrate and stabilize. Depending on the setup, this can take over one hour.

10| Select the beam path and emission filter/range for FCS and sample imaging, achieving the highest match between the both. Select the emission filters to get highest emission signal but smallest (in the ideal case no) crosstalk between the individual fluorescence channels. Additional fluorescent channels may be needed for cellular markers (**Table 1**). The settings for the green (mEGFP) and red (mCherry) FPs as well as the fluorescent dyes Alexa488/Alexa568 are shown in **Table 2**. The emission range can be increased if only one fluorescent marker is present. Note that the protocol uses single color FCS to derive a calibration curve for a FP.

11| Clean objective and glass bottom plate with lens cleaning tissue rinsed with pure ethanol.
CAUTION The objective can be damaged if pressure is applied to the lens.

12| Make a rough adjustment of the water objective correction collar to match the glass thickness based on manufacturer's specification (#1 corresponds to 130-160 um, #1.5 corresponds to 160-190 µm).

13| Place a drop of pure water on the objective and mount the sample.

14| Check correct and planar mounting of the sample using the glass reflection. If the reflection of the glass is not planar, adjust your sample or the sample holder accordingly.

15| Wait for the system to equilibrate (~30 min).

16| Meanwhile, create the folder structure to save the calibration data

Your_Experiment_Folder\
    Calibration\
        dye\ contains the FCS measurements of the fluorescent dye
        WT\ contains the images and FCS measurements of the WT cells
        mFP\ contains the images and FCS measurements of the cells expressing the monomeric FP
        POI\ contains the images and FCS measurements of the cells expressing the POI tagged with the FP

17| *Pinhole, beam collimator and objective correction collar adjustments*.

(i) Focus the objective on the well with the fluorescent reference dye.

(ii) Set the pinhole size to 1-1.6 AU.

(iii) Press the count rate tab. In the ZEN software the count rate window displays average photon counts and average counts per molecule (CPM). The latter is calculated from the photon counts divided by an estimated number of molecules.

(iv) Find the interface between glass and fluorescent solution. This is the Z position where the count rate and the total photon count suddenly increase.

(v) Move 30 µm above the glass-surface inside the fluorescent solution.

(vi) Select the laser power to achieve a CPM above 3 kHz. The final laser power will be set using the FP as reference (step 18|). Close the count rate window.

(vii) When using Zeiss LSM systems, perform both coarse and fine pinhole adjustments in X and Y for the channel of interest.

(viii) Press the count rate tab and turn the correction collar to achieve the maximal count rate. Repeat step **Error! Reference source not found.**. Verify that this position corresponds to a maximal CPM.

18| *Set FCS laser power using the POI*

(i) Focus the objective in the well containing the cells expressing the POI.

(ii) Acquire a fluorescent image.

(iii) Place a FCS measurement point in a region of the cell where the protein is expected to be low abundant, freely diffusing, homogeneously distributed and free of aggregates (e.g. fluorescent aggregates in juxtanuclear regions). When cells exhibit variable expression levels, it is recommended to preferentially measure cells with a low expression level of the mFP-POI.

(iv) Press the count rate tab and change the laser power to achieve a CPM >= 1 kHz whereby the total counts should not exceed 1000 kHz. For the case the protein expression level is too high and the desired detection parameters cannot be achieved see **Table 3** for troubleshooting.

(v) Perform a FCS measurement over 30 sec to assessing the photobleaching.

CAUTION The APD and GaAsP detectors are very sensitive and can be damaged if too much light is used. Be careful not to exceed the maximal detection range. Stop acquisition if the detector shutter closes.

? TROUBLESHOOTING

19| *Set imaging conditions using the POI*

These imaging settings will be used to compute the FCS calibration parameters (step 44|)

(i) Set the pinhole size and laser power as close as possible to the values used for the FCS measurements (steps 17|-18|).

(ii) Set the pixel size, pixel dwell, and the zoom to be suitable and optimal for your sample.

(iii) Set the detector gain value so that saturation is avoided. Saturation can be tested by analyzing the intensity histogram or visualized using the range indicator.

(iv) Set the imaging conditions to acquire a single plane (2D images and no time-lapse). This is the image used to derive the FCS calibration parameters. For 3D imaging (XYZ stacks) use an uneven number of sections in order to always associate the central Z plane with the FCS measurements. Save the settings as a user-defined configuration in ZEN.

CRITICAL The pinhole size should be adjusted to achieve a good confocal sectioning for the structure of interest. The imaging settings determined in this step should be kept for further 3D or 4D imaging (step 24|) and calculating concentrations and protein numbers using the FCS calibration curve.

? TROUBLESHOOTING

20| *Effective confocal volume measurement*

(i) Move to the well containing the fluorescent reference dye.

(ii) Focus 30 µm above the glass surface inside the fluorescent solution.

(iii) Place the FCS measurement point in the center of the field of view.

(iv) Use FCS settings identical to the settings for the POI (step 18|).

(v) Set the measurement time to 30 sec and 6 repetitions.

(vi) Start the measurement.

(vii) Save the data with the raw data for further processing to the *Calibration/dye* folder (see Troubleshooting table).

CRITICAL It is essential that the laser power and pinhole size are the same as determined in 18|. *Fluctuation Analyzer* (*FA*) computes a bleach-corrected ACF using the raw counting data. Thus it needs to be ensured that this data is saved. For Zeiss LSM select in the Maintain/Confocor options `save raw data during measurement`. In the saving directory you should find files of type raw.

? TROUBLESHOOTING

Manual acquisition TIMING 0.5h setup, 2h measurement

21| *Background measurements (5-10 cells)*

Aim is to estimate the photon counts offset for calculating the background correction for the estimated concentrations of the FP (step 39| and 41|). The fluorescent images are used to calculate the fluorescence intensity background (step 42|).

(i) Start the VBA macro *FCSRunner* (**Supplementary Software 1**).

(ii) Specify the output folder as the *Calibration*/*WT* folder.

(iii) Define the number of measurement points per cell. By default each point is associated to the cellular compartment nucleus/chromatin or cytoplasm. For each compartment at least one point should be measured per cell.

(iv) Focus in the well with WT cells.

(v) Search for cells.

(vi) Set the FCS and imaging settings according to the settings determined in 18| and 19|, 30 sec one repetition.

(vii) Start the live mode and focus in XYZ to the cell of interest; add points to *FCSRunner* for the different compartments.

(viii) Repeat steps (v)-(vii) for up to 5 cells.

(ix) Press `Image and FCS` in the *FCSRunner* VBA macro to start the acquisition. Wait for the acquisition to finish.

(x) Delete the positions in *FCSRunner* and repeat steps (v)-(ix) if more cells are needed.

The VBA macro will automatically acquire and save images, FCS measurements, and FCS measurement coordinates at each cell position. Consult the software documentation for further details on *FCSRunner*.

CRITICAL When adding FCS points to the same object/cell, do not move the XY stage or change the focus.

? TROUBLESHOOTING

22| *Measurement of the mFP for the FCS calibration curve (10-20 cells)*

(i) Specify the output folder *Calibration*/*mFP* in *FCSRunner*.

(ii) Remove previous stage positions in the *FCSRunner*.

(iii) Move the stage to the well containing the cells expressing the monomeric FP construct (mEGFP in this protocol).

(iv) Search for cells exhibiting low mFP expression levels.

(v) Switch to the FCS and imaging parameters determined in 18| and 19|, respectively, 30 sec one repetition.

(vi) Verify that the image does not saturate and that the total photon counts are low (< 1000 kHz).

(vii) Start the live mode and focus in XYZ to the cell of interest; add measurement points to *FCSRunner* for the different compartments.

(viii) Repeat steps **Error! Reference source not found.**-(vii) for up to 5 cells.

(ix) Press `Image and FCS` in the *FCSRunner* VBA macro to start the acquisition. Wait for the acquisition to finish.

(x) Delete the positions in *FCSRunner* and repeat steps **Error! Reference source not found.**-(ix) to acquire more cells (up to 20).

CRITICAL The APD and GaAsP detectors are sensitive detectors. The protein expression level needs to be low enough in order to avoid saturation of the imaging intensities and exceeding of the FCS counts beyond 1000 kHz. Make sure that the imaging settings correspond to the imaging settings for the protein of interest. When adding FCS points to a cell do not move the XY stage or the focus.

? TROUBLESHOOTING

23| *Measurement of the fluorescently tagged POI (10-20 cells)*

(i) Specify the output folder *Calibration*/*POI* in the *FCSRunner*.

(ii) Remove previous stage positions in *FCSRunner*.

(iii) Move the stage to the well containing the cells expressing the fluorescently tagged POI.

(iv) Search for cells that express the fluorescently tagged POI.

(v) Switch to the FCS and imaging parameters determined in 18| and 19|, respectively, 30 sec one repetition.

(vi) Verify that the image does not saturate and that the photon counts are low (< 1000 kHz). For proteins tagged at their endogenous locus the expression levels are typically homogeneous within the population. In this case this step can be omitted.

(vii) Start the live mode and focus in XYZ to the cell of interest; add measurement points to *FCSRunner* for the different compartments.

(viii) Repeat steps (iv)-(vii) for up to 5 cells.

(ix) Press `Image and FCS` in the *FCSRunner* VBA macro to start the acquisition. Wait for the acquisition to finish.

(x) Delete the positions in *FCSRunner* and repeat steps (iv)-**Error! Reference source not found.** to acquire more cells (up to 20).

CRITICAL Avoid acquiring FCS data in positions where the protein is bound to immobile structures. Indication of a large stable fraction is a strong bleaching during the FCS data acquisition.

? TROUBLESHOOTING

24| *Additional imaging (Optional)*

Perform additional imaging using the same detector and laser settings as specified in 19|. The fluorescence intensity in these images can be converted to concentrations and protein number using the calibration parameters (see step 45|).

CRITICAL To convert fluorescence intensities to protein numbers the image needs to be a 3D (XYZ) or 4D (XYZT) stack.

Automated adaptive feedback acquisition (optional) TIMING 1.5h setup (first time only), 2h unattended acquisition

This procedure is an alternative to the manual data acquisition (21|-23|). In combination with online image analysis this procedure performs focus drift correction, cell detection, imaging and FCS measurements without user supervision. This yields reproducible measurements and a higher throughput. The procedure requires a fluorescent marker to identify cells. It is optimal to use a DNA marker but any other fluorescent marker can be used as long as the image segmentation workflow is able to detect it. Three different imaging settings are used: An autofocus XZ scan to detect the cover glass reflection, a low resolution imaging of the fluorescent marker and mFP-POI, and a high-resolution imaging of the fluorescent marker and mFP-POI. The software *MyPiC* (**Supplementary software 2**) and *Automated FCS* (**Supplementary**

software 3) are used for this part of the protocol. Refer to the corresponding manuals for a detailed description. After the first setup settings can be reused. This reduces the setup time to less than 10 min.

25| *Load the MyPiC ZEN Macro* (**Supplementary software 2**)

(i) Start the macro.

(ii) Click on *Saving* and specify the output directory to *Calibration* directory (see 16|).

(iii) Click on the *JobSetter* button.

26| *Start the Automated FCS Fiji plugin* (**Supplementary software 3**)

Start FiJi and go to *Plugins* > *EMBL* > *Automated FCS*.

27| *High resolution imaging settings*

These imaging settings are used for computing the FCS calibration curve and determine the FCS measurement points using image analysis.

(i) Add an imaging channel for the cellular marker to the settings from 19|.

(ii) Click the + button on the *JobSetter* to add the imaging job to *MyPiC* and name the job HR (high-resolution).

28| *Low-resolution imaging settings*

These imaging settings are used to automatically detect cells to be imaged and measured with FCS.

(i) Change the zoom settings in 27| to acquire a large field of view.

(ii) Adjust pixel size and dwell time to achieve fast acquisition. The quality of the image should be good enough to allow for segmentation of the cellular marker by *Automated FCS*. Use a Z stack if this improves the segmentation of the cellular marker.

(iii) Click the + button in the *JobSetter* to add the imaging job to *MyPiC* and name the job LR (low resolution).

29| *Autofocus imaging settings*

To speed up the acquisition and minimize the hardware load it is recommended to keep the same light path settings as 28|.

(i) Change the settings in 28| to XZ line-scanning and reflection mode (see also the software manual for **Supplementary software 2**).

(ii) Choose a laser line that is not reflected by the MBS but detected with the current detector settings. For example use the Argon 514nm laser line with the MBS 488/561/633 and GFP detection range.

(iii) Set the laser power and gain to achieve a visible reflection without saturating the detector.

(iv) Set the number of stacks to cover 20-80 µm with a 100-500 nm Z step size. A small Z step size gives higher precision but requires a longer acquisition time in the absence of a piezo Z stage.

(v) Verify that the imaging yields a thin bright line.

(vi) Click the + button in the *JobSetter* to add this imaging job to *MyPiC* and name the job AF (Autofocus).

CRITICAL With the recommended MBS and laser settings the transmitted light can be high and damage the detector. The user should start with a low laser light and gain and then adjust it accordingly.

30| *FCS settings*

(i) Set in ZEN the FCS settings for the mFP-POI (see also 18|).

(ii) Use one repetition and 30 sec measurement time.

(iii) In the *JobSetter* click on the FCS tab.

(iv) Load the FCS settings from by clicking on the + button and name the job POIFCS. ZEN will prompt to also save the light path settings.

31| *Tasks of the Default Pipeline*

(i) Click on the *Default* button in *MyPiC*.

(ii) Click the + button and add the AF-job as first task to the default pipeline with a double click.

(iii) Set *Process Image/Tracking* of the first task to *Center of mass (thr)* and click on *TrackZ*. At each position and repetition *MyPiC* computes the fluorescence center of mass of the upper 20% of the signal and updates the Z position accordingly.

(iv) Click the + button of *MyPiC* and add the LR job as second task to the default pipeline with a double click.

(v) Test the default pipeline by pressing the play button in the *Pipeline Default tasks* frame. Adjust the value of the Z offset for the LR-job to achieve the necessary imaging position from the cover glass. Acquire a final test image.

(vi) Set *Process Image/Tracking* of the second task to *Online Image Analysis*. At every position and repetition *MyPiC* acquires first the autofocus image and second the low resolution image. Then *MyPiC* waits for a command from the *Automated FCS* FiJi plugin (33|).

32| *Tasks of the Trigger1 pipeline*

(i) Press the *Trigger1* button in *MyPiC*.

(ii) Press the + button and add the AF job as first task to the Trigger1 pipeline with a double click.

(iii) Set *Process Image/Tracking* of the first task to *Center of mass (thr)* and click on *TrackZ*.

(iv) Press the + button in *MyPiC* and add the HR job as second task to the Trigger1 pipeline with a double click.

(v) Test the Trigger1 pipeline by pressing the play button in the *Pipeline Trigger1 tasks* frame. Adjust the value of the Z offset for the HR job to achieve the necessary imaging position from the cover glass. Acquire a final test image.

(vi) Set *Process Image/Tracking* of the second task to *Online Image Analysis*.

(vii) Press the + button of *MyPiC* and add the POIFCS job as third task to the Trigger1 pipeline.

33| *Analysis settings to detect the cell of interest in the default pipeline*

Please refer to the manual of *Automated FCS* (**Supplementary Software 3**) for a detailed explanation of the options.

(i) In the FiJi macro *Automated FCS* click on *Parameter Setup*.

(ii) Change *Pipeline* to *Default* to process data from the default pipeline of *MyPiC*.

(iii) Change *Task* to *2* and *Command* to *trigger1*. If a cell of interest is found in the low resolution image, the Trigger1 pipeline is started at the position of the cell of interest.

(iv) Specify the channel that contains the image of the cellular marker in *Main seg. Channel.*

(v) Test the detection of cells by clicking *Run on file*. The image from 31|(v) is stored in the directory *Test* with the name DE_2_W0001_P0001_T0001.lsm|czi. If objects of interest are detected, an image with the processing results will be generated.

(vi) Change the *Seg. method* and area size limits and repeat step (v) until single cells are reliably detected. If required, acquire additional images as in 31|(v) and repeat steps (v)-(vi).

(vii) In *Channel intensity filter1* specify the channel of the cellular marker and set a lower and upper value for the mean FI. This avoids cells with an abnormally high fluorescence intensity of the cellular marker (e.g. apoptotic cells). Check the results by repeating step (v). If required, acquire additional images as in 31|(v) and repeat steps (v) and (vi) .

(viii) In *Channel intensity filter2* specify the channel of the FP and set a lower and upper value for the mean fluorescence intensity. The lower value should be set so that cells not expressing the FP are avoided. The upper value ensures non-saturation in the high resolution image and the FCS measurements. Test the settings by repeating step (v). If required, acquire additional images of cells expressing the monomeric FP and endogenously tagged POI and repeat steps (v), (vii), and (viii).

(ix) Specify in *Number of particles* the maximal number of cells to process: use *4*.

(x) Specify in *Pick particle* how the cells should be chose: use *random*.

? TROUBLESHOOTING

34| *Analysis settings to specify the points for FCS measurements*

(i) In the *FiJi* macro *Automated FCS* click on *Parameter Setup*.

(ii) Change *Pipeline* to *Trigger1* to process data from the Trigger1 pipeline of *MyPiC*.

(iii) Change *Task* to *2* and *Command* to *setFcsPos*. If a cell of interest is found in the high resolution image, FCS position coordinates are computed and passed to *MyPiC* to start FCS measurements.

(iv) Specify the channel that contains the image of the cellular marker in *Main seg. Channel.*

(v) Test the detection of cells by clicking *Run on file* in *Parameter Setup*. The image from 32|(v) is stored in the directory *Test* with the name TR1_2_W0001_P0001_T0001.lsm|czi. If objects of interest are detected, an image of the processing results will be generated.

(vi) Change the *Seg. method* and area size limits and repeat step 33|(v) until single cells are reliably detected. If required, acquire additional images as in 32|(v) and repeat steps (v)-(vi)

(vii) In *Channel intensity filter1* specify the channel of the cellular marker and set a lower and upper value for the mean FI. This avoids cells with an abnormally high fluorescence intensities of the cellular marker (e.g. apoptotic cells). Check the results by repeating step (v). If required acquire additional images as in 32|(v) and repeat steps (v) and (vii).

(viii) In *Channel intensity filter2* specify the channel of the FP and set a lower and upper value for the mean fluorescence intensity. The lower value should be set so that cells that not expressing the FP are avoided. The upper value ensures non-saturation in the high resolution image and the FCS measurements. Test the settings by repeating step (v). If required acquire additional images of cells expressing the monomeric FP and endogenously tagged POI as in 32|(v) and repeat steps (v), (vii), and (viii).

(ix) Specify the number of FCS measurement points and the pixel distance from the boundary (*FCS pts. region1.2* and *# oper. (erode <0, dilate >0)*, respectively). Use at least one point inside (nucleus) and one point outside (cytoplasm) of the segmented cell marker.

(x) Test the positioning of the FCS measurement points by repeating step (v). If necessary, adjust the pixel distance to ensure correct placement of the FCS measurement points.

? TROUBLESHOOTING

35| *Automated measurement of the FP (10-20 cells)*

(i) In *MyPiC* specify the output directory to *Calibration/mFP*.

(ii) Press *Default Positions*, click on *Multiple* and mark 3-10 positions in the mFP well. To improve the throughput manually select positions where several cells in the field of view express the mFP construct.

(iii) In *Automated FCS* set the *Directory to monitor* to *Calibration/mFP*.

(iv) Press start in *Automated FCS* and *MyPiC*.

(v) When the acquisition is finished press *Stop* in *Automated FCS*.

CRITICAL When this step is performed for the first time the user should supervise the acquisition to ensure that the cells obtained from the analysis express the FP. Furthermore, images must not show saturation and the FCS counts should be below 1000 kHz. See also troubleshooting on how to adjust the settings in *Automated FCS*.

? TROUBLESHOOTING

36| *Automated measurement of the fluorescently tagged POI (10-20 cells)*

(i) In *MyPiC* specify the output directory to *Calibration/POI*.

(ii) Press *Default Positions*, delete previous positions and mark 3-10 positions in the POI well. To improve the throughput, manually select positions where several cells in the field of view express the fluorescently labeled POI.

(iii) In *Automated FCS* specify the current directory to *Calibration/POI*.

(iv) Press start in *Automated FCS* and *MyPiC*.

(v) When the acquisition is finished press *Stop* in *Automated FCS*.

CRITICAL Same considerations as for step 35| apply.

? TROUBLESHOOTING

37| *Automated background measurements* (5-10 cells)

(i) In *MyPiC* specify the output directory to *Calibration/WT*.

(ii) Press *Default Positions*, delete previous positions and mark 2-3 positions in the WT well.

(iii) In *Automated FCS* set the current directory to *Calibration/WT*.

(iv) Change *Channel intensity filter2* to *None* in order to not limit to the fluorescence intensity of the FP channel.

(v) Press start in *Automated FCS* and start in *MyPiC*.

(vi) When the acquisition is finished press *Stop* in *Automated FCS.*

Data Processing TIMING 10 min setup, 0.5-1h of unattended computation (depending on the measurement), 30 min quality control, 0.5-1h image processing

The software *FA* can be used for fitting the auto-correlation data to physical models (see manual in the **Supplementary software 4**). This allows more freedom in the parameter choice for the advanced users. The software *FCSFitM* uses predefined settings that have been shown to achieve reliable results.

38| *Start the tool FCSFitM (**Supplementary software 4**)*

See the manual of the software for detailed explanations.

39| *Compute the background photon counts with FCSFitM*

(i) Change to tab *FCS background* in *FCSFitM*.

(ii) Specify the directory *Calibration/WT*.

(iii) Press *SelectAll* and *Compute* to calculate the average photon counts for all measurements. For the background value no distinction is made between measurements in the nucleus and the cytoplasm.

(iv) Write down the counts of Ch1 (or Ch2). This will be needed in step 41|.

CRITICAL In this and all subsequent analysis steps Ch1 indicates the channel with the lowest wave length (e.g. GFP, Alexa488) and Ch2 the channel with the higher wave length (e.g. mCherry, Alexa568). If APDs are used the ZEN notation of Ch2 and Ch1 corresponds in the analysis to Ch1 and Ch2, respectively.

? TROUBLESHOOTING

40| *Effective confocal volume estimation from the fluorescent dye using FCSFitM*

(i) Change to tab *Fit Dye*.

(ii) Specify the diffusion coefficient of the fluorescent dye used for Ch1 or Ch2. The default values are for Alexa488 and Alexa568 at 37 ˚C. See also **Supplementary material** and Eq. S7.

(iii) Specify the directory that contains the fluorescent dye data (*Calibration/dye*).

(iv) Press *SelectAll* and *Compute.* Wait until the computation is finished. The program computes a best estimate for w_0 and κ by performing repeated rounds of parameter optimization. Finally a mean effective confocal volume V_{eff} is computed using Eq. S2.

(v) Check that results have been saved in *Calibration/dye/focalVolume.txt* and *Calibration/dye/optimisedFit.*

If the ACF data is generated from the raw data using *FA* then *FCSFitM* will use it and save the fitted traces in a *FA* compatible format.

41| *Compute correlations and corrections using Fluctuation Analyzer*

(i) Start *FA*.

(ii) Specify the raw data format *Zeiss/ZEN (*.zen)*

(iii) Specify the import settings according to the used detectors. See also the manual for the **Supplementary software 4**.

(iv) Select the path to *Calibration* and click *Include subdirectories*.

(v) Add all measurements and name the session *2c* for later reuse.

(vi) Press *Check files* and wait for all files being processed.

(vii) In *Modify and correlate* set the base frequency to 100.000 Hz (i.e. 10 µs time interval). Press *Calculate all* and wait for the end of the computation.

(viii) In the *Intensity corrections* tab enter the background values obtained from 39| in the *Offset Ch1* text element.

(ix) Press *Apply to* and *Calculate All*. Wait for the computation to finish.

(x) Perform a visual quality check of the data according to the requirements in supplementary Fig. S1 and annotate traces that do not pass the QC with a 'x'. Parameter-based QC are applied at step 44|.

(xi) In the *Save, export and report* tab press *Save all* and the *FA format* buttons. This saves the data to the tab delimited result table *Calibration/2c.res* (see also documentation to **Supplementary software 4**).

CRITICAL To maintain the channel convention (see 39|) when loading data acquired using APDs on a Zeiss Confocor you may need to swap the order of the channels (see also documentation to **Supplementary software 4**).

42| *Extract fluorescence intensities at the FCS measurement points*

(i) Start *FiJi*.

(ii) Start the macro *Plugins* > *EMBL* > *FCSImageBrowser*.

(iii) Press *Load .res file* and load the result table created in 41|.

(iv) Click on *Report* to generate the table *2c.fint*. The table contains the fluorescence intensities in ROIs of different sizes at the FCS measurement points.

(v) If images have been acquired using the adaptive feedback pipeline, perform an additional visual QC of the images. Omit points located at the cell boundary. Annotate the FCS points that do not pass the visual QC of the images with a 'x' in Fluctuation Analyzer. Save the modified results to a table (step 41|(xi)).

43| *Fit the ACFs functions using FCSFitM*

(i) Press *Add res file* and load the result table from 41| into *FCSFitM*.

(ii) Press *Run fit* to perform fits to the autocorrelation data.

(iii) The program generates two separate tables, *1c_opt.res* and *2c_opt.res* for one- and two-component anomalous diffusion models, respectively.

FCSFitM uses the data from 40| and 42|. The generated tables summarize the fit parameters, the concentrations, and fluorescence intensities at the FCS measurement points (see documentation of **Supplementary software 4**). The ACF fit results can be viewed in FA using the session name *1c_opt* or *2c_opt* (*1c_opt_w* and *2c_opt_w* if a weighted fit is used).

? TROUBLESHOOTING

44| *Compute FCS calibration curve using FCSCalibration*

All data to compute the calibration curve are stored in the tables generated in step 43|. The R application *FCSCalibration* provides a graphical interface to further quality control the data and automatically computes the calibration curve.

(i) Start the *FCSCalibration* and specify the directory containing the *2c_opt.res* (43|).

(ii) If required, change the name of *Dye*, *POI*, and *mFP* entries to match the name of the directories of the current experiment.

(iii) Choose the ROI size and the compartment (nucleus, cytoplasm or all) to use.

(iv) Choose the filtering parameters by investigating their distribution. Typically the listed default parameters work for most applications.

(v) Press *Report* to save the calibration data to the folder *Results/Calibration*.

? TROUBLESHOOTING

45| *Convert fluorescence intensities to concentrations and protein numbers*

For the linear transformation of the fluorescence intensities to a concentration map the user only needs the parameters of the FCS calibration curve. To convert concentrations into number of proteins the voxel volume is required, which is stored in the metadata. Conversion in protein number per pixel is only performed for 3D and 4D data. The FiJi plugin *FCSCalibrateImage* (**Supplementary software 4**) reads the image, the metadata, and the calibration data to generate a calibrated image for the channel of interest.

TIMING

Steps 1|-8|: Sample preparation: 1 h hands-on, 1 d waiting time, 15 min hands-on

Steps 9|-20|: Microscope setup and effective confocal volume measurement: 1 h to 2 h depending on the experience

Steps 21|-24|: Manual acquisition: 0.5 h setup, 2 h measurement. The time can extend if additional images to calibrate are acquired

Steps 25|-37|: Automated adaptive feedback acquisition: 1.5 h setup (first time only) and 2 h unattended acquisitions. This is an alternative to manual acquisition (steps 21|-24|). When settings are reused, the setup time reduces to less than 10 min

Steps 38|-45|: Data Processing: 10 min setup, 0.5-1 h of unattended computation (depending on the number of measurements), 30 min quality control, 0.5-1 h image processing

ANTICIPATED RESULTS

The workflow provides a calibration coefficient for converting fluorescence intensity data to protein concentrations and numbers. The coefficient needs to be calculated for every experiment. Depending on the stability of the system, its value may remain similar over time. In combination with further image analysis steps using image analysis software such as MATLAB or ImageJ the stoichiometry of the protein of interest on cellular structures can be computed (Fig. 3). The adaptive feedback imaging workflow can also be used to acquire additional FCS measurements and 4D image movies in high-throughput. The FCS measurements can be further used to study the biophysical properties of the POI (diffusion coefficient, aggregation). In our laboratory we used this method to generate FCS-calibrated 4D image movies of cells during mitosis.

ACKNOWLEDGMENTS

We thank the mechanical and the electronics workshop of EMBL for custom hardware, the Advanced Light Microscopy Facility of EMBL for microscopy support and the Flow Cytometry Core Facility of EMBL for cell sorting. We gratefully acknowledge Bianca Nijmeijer, Stephanie Alexander and Arina Rybina for critically reading the manuscript. We thank Thomas Ohrt (Zeiss) for support in the hardware control. This work was supported by grants to J.E. from the European

Commission EU-FP7-Systems Microscopy NoE (Grant Agreement 258068), EU-FP7-MitoSys (Grant Agreement 241548) and iNEXT (Grant Agreement 653706), as well as by EMBL (A.Z.P., Y.C., N.W., M.J.H., B.K., M.W., J.E.). Y.C. and N.W. were supported by the EMBL International PhD Programme (EIPP).

Fluorescent protein for POI	**Dye/fluorescently tagged protein or nuclear landmark**	**Dye for cell boundary landmark**	**Remarks**
mEGFP Ex: 488 Em: 492-544	*SiR-DNA* Ex: 633 Em: 640-695	*Dy481XL-01* Ex: 488 Em: 640-695	
mCherry Ex: 561 Em: 588-690	*H2B-mCerulian* Ex: 458 Em: 458-492	*Atto430LS-31* Ex: 458 Em: 588-690	Small bleed-through of mCerulian in the Atto430LS-31 channel
mEGFP Ex: 488 Em: 492-544	*H2B-mCherry* Ex: 488 or 561 Em: 588-624	*Dy481XL-01* Ex: 488 Em: 625-695	Cross-talk between mCherry and Dy481XL-01 channel
mCherry Ex: 561 Em: 588-690	*Hoechst 33342* Ex: 405 Em: 410 - 480	*Alexa488* Ex: 488 Em: 492-544	Phototoxicity of 405 nm laser upon long-term imaging
mCherry Ex: 561 Em: 588-610 (small emission range to minimize bleed-through)	*SiR-DNA* Ex: 633 Em: 640-695	*Atto430LS-31* Ex: 458 Em: 588-690 or *Alexa488* Ex: 488 Em: 492-544	Bleed-through of SiR-DNA in the mCherry channel. This impairs the FCS measurements at low protein numbers

Table 1: Combination of fluorescent proteins and dyes that can be used for tagging the POI and staining cellular landmarks. The first 3 entries represent optimal combinations. For low expressing proteins the chromatin stain should not cross-talk into the mFP-POI channel. The emission range is given as a guideline for minimizing crosstalk. Ex: Excitation, Em: Emission.

Alexa-488/GFP settings	
FCS APD detectors	
Main beam splitter (MBS)	488, 488/561, 488/561/633
Second beam splitter (NFT)	NFT 565
Green channel emission filter (channel 2)	505-540, 505-550
FCS GaAsP detectors	
Main dichroic	488, 488/561, 488/561/633
ChS1	501-544
Zeiss LSM, Imaging detectors	
Main dichroic	488, 488/561, 488/561/633
Ch1/ChS1	492-544

Alexa-568/mCherry settings	
FCS APD detectors	
Main beam splitter (MBS)	488/561, 488/561/633
Second beam splitter (NFT)	NFT 565
Red channel emission filter (channel 1)	600-650
FCS GaAsP detectors	
Main beam splitter (MBS)	488/561, 488/561/633
ChS2	595-655
Zeiss LSM, Imaging detectors	
Main dichroic	488, 488/561, 488/561/633
Ch2/ChS2	595-655

Table 2: Imaging and FCS settings to be used for green and red fluorescent proteins and dyes. The settings are given for Zeiss LSM microscopes.

Step	Problem	Possible reason(s)	Solution
5|	Weak staining with SiR-DNA	Short incubation time. The uptake may depend on the cell line.	Increase the incubation time.
18|	High count rates (exceeding 500 kHz in FCS).	High laser power. High protein expression.	Decrease the laser power. Decrease the pinhole size. Find cells with a lower protein expression.
19|	Saturation of the image.	High protein expression. High laser power. High gain.	Find a cell with lower protein expression. Reduce the laser power and/or detector gain.
20|	No FCS raw data are saved.	*Maintain* options are not set properly.	Make sure *save raw data during measurement* is checked in the Maintain tab.
21|22|23|	Trend or large fluctuations in the photon counts leading to poor quality FCS measurements (e.g. Fig. S1).	Focus drift when acquiring multiple cells.	Acquire only 5 cells at a time that are close enough in space.
		Focus drift during acquisition.	Wait for the system to equilibrate before starting the experiment.
		FCS measurement point is close to cell boundary.	Optimize the Z position for the FCS measurement. Start a live FCS measurement and move the Z position to reach a maximal count rate. This indicates a point far from the cell boundary.
33|	*Automated FCS* does not detect cells from the low-resolution imaging.	Segmentation parameters are not properly set.	Change the segmentation parameters. Verify that the fluorescence limits match your sample and image bit depth. The default maximal fluorescence value is set for 8bit images.
		Cell(s) are close to the image boundary.	Increase image size.
		Poor imaging or staining conditions.	Change the imaging settings to get better imaging. Optimize the staining conditions for the cellular marker.
35|	Image saturation of the high-resolution image. Photon counts > 1000 kHz.	Parameters in *Automated FCS* are not set correctly.	Decrease the maximal value for the FI (*Max min intensity*) of the fluorescent protein.

	Imaged cells do not express the fluorescent protein.	Parameters in *Automated FCS* are not set correctly.	Increase the minimal value for the FI (*Min mean intensity*) of the fluorescent protein.
	Drift in the photon counts.	Imaging Z position may be too high or too low. This often occurs for points in the cytoplasm.	In *MyPiC* change the Z offset of the high resolution imaging (Trigger1, task 2). Use the cells expressing the mFP and manually vary the Z position and verify that the photon counts are maximized.
36|	No cells expressing the POI are automatically selected.	Low FI of the mFP-POI in the segmented area.	Decrease the minimal value for the fluorescence intensity (*Min intensity*).
39|40|	List remains empty.	GUI responsiveness.	After clicking *Select All* the files name should appear.
43|	Warning: "No focal volume found!..." .	*Fit Dye* has not been performed.	Perform the step *Fit Dye* or click on *Use local volume file* if the confocal volume has already been calculated. The file is stored as *focalVolume.txt* in the dye directory.
44|	High concentrations for low fluorescence values of WT measurements.	WT measurements have not been properly included.	For WT FCS measurements make sure that the number of molecules has been set to 0. This should occur automatically if the name of the WT folder is correctly specified in *FCSFitM*.
44|	No data is shown.	The filtering parameters are too strict.	Change or remove filtering parameters.
	Data strongly deviates from linear relationship.	Low protein concentration. Strong bleaching. High concentration.	Try to use only nucleus or cytoplasmic compartment. Set limits to the concentrations.
	POI data does not show the same linear relationship as mFP data.	Protein multimerization	Normalize the POI data using the mFP data.

Table 3: Troubleshooting table.

Figure legends

Figure 1 Principle of fluorescence correlation spectroscopy (FCS) for point confocal microscopes

(a) The excitation laser beam is positioned to a specific location of a cell. Fluorophores entering the confocal volume will be excited and the number of photons emitted are recorded. Shown are two cells expressing different levels of the fluorescent protein mEGFP.

(b) The fluctuations of the fluorophores diffusing in and out of the confocal volume cause fluctuations in the number of photons.

(c) Computing the self-similarity of the fluctuations in (b) yields auto-correlation functions (ACF). The amplitude of the ACF is directly proportional to the inverse of the number of molecules observed on average in the confocal volume.

Figure 2 Workflow for FCS-calibrated imaging

(a) Setup of the multi-well chamber with four different samples and optimization of the water objective correction for glass-thickness and sample mounting using a fluorescent dye and the reflection of the cover glass. dye: fluorescent dye to estimate the confocal volume, WT: WT cells, mFP: WT cells expressing the monomeric form of the FP, mFP-POI: cell expressing the fluorescently labeled POI.

(b) Computation of parameters of the focal volume using a fluorescent dye with known diffusion coefficient D_{dye} and similar spectral properties as the fluorescent protein. Fitting of the ACF to a physical model of diffusion yields the diffusion time τ_D and structural parameter κ. The two parameters are used to compute the focal radius w_0 (Eq. S6) and the effective volume V_{eff} (Eq. S4).

(c) Images and photon counts fluctuations are acquired for cells that do not express a fluorescent protein (WT), cells expressing the mFP alone (mEGFP), and cells expressing the tagged version of the POI, mFP-POI (mEGFP-NUP107). The image fluorescent intensity (FI) at the point of the FCS measurement is recorded.

(d) The ACF is fitted to a physical model to yield the number of molecules in the effective volume. WT concentrations are set to 0. After background and bleach correction concentrations

are computed using the V_{eff} estimated in (a-b) and the Avogadro constant N_A. Data is quality controlled with respect to the quality of the fit and the amount of photobleaching.

(e) Concentrations estimated from FCS and image fluorescent intensities are plotted against each other to obtain a calibration curve (black line).

Figure 3 Example of image quantification using FCS calibration

(a) The fluorescence intensity at each pixel, I_p, is converted to concentrations and protein numbers (for 3D and 4D images). The images are acquired with the same imaging parameters as the images used to compute the FCS calibration curve. Shown is data for a HeLa Kyoto cell line with endogenous NUP107 tagged N-terminally with mEGFP.

(b) The quantitative distribution of a POI can be derived by using markers for cellular structures. DNA stained with SiR-DNA is used to compute a 3D chromatin mask of the nucleus. In the equatorial plane a three pixel wide rim for the nuclear envelope is determined. Fluorescence intensities and protein numbers on the nuclear envelope can then be calculated.

(c) mEGFP-NUP107 average fluorescence intensity on the nuclear envelope. Data shows the results obtained on two different microscopes. The boxplots show median, interquartile range (IQR), and 1.5*IQR (whiskers), for n = 16-22 cells and 2 independent experiments on each microscope. System 1: LSM880, FCS and imaging using the 32 channel GaAsP detector. System2: LSM780, FCS using the APD detector (Confocor-3), imaging using the 32 channel GaAsP detector.

(d) Conversion of fluorescence intensity to protein numbers using the corresponding calibration curve obtained for each experiment and microscope system (not shown). The protein density on the nuclear envelope has been computed according to Eq. S24. The gray shadowed boxes show the expected protein numbers for NUP107 [18,19].

References

1 Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819-823, doi:10.1126/science.1231143 (2013).

2 Shen, B. *et al.* Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature methods* 11, 399-402, doi:10.1038/nmeth.2857 (2014).

3 Trevino, A. E. & Zhang, F. Genome Editing Using Cas9 Nickases. *Methods in Enzymology* 546, 161-174, doi:http://dx.doi.org/10.1016/B978-0-12-801185-0.00008-8 (2014).

4 Digman, M. A., Dalal, R., Horwitz, A. F. & Gratton, E. Mapping the Number of Molecules and Brightness in the Laser Scanning Microscope. *Biophysical Journal* 94, 2320-2332, doi:10.1529/biophysj.107.114645 (2008).

5 Krieger, J. W. *et al.* Imaging fluorescence (cross-) correlation spectroscopy in live cells and organisms. *Nat. Protocols* 10, 1948-1974, doi:10.1038/nprot.2015.100 http://www.nature.com/nprot/journal/v10/n12/abs/nprot.2015.100.html#supplementary-information (2015).

6 Bacia, K., Kim, S. A. & Schwille, P. Fluorescence cross-correlation spectroscopy in living cells. *Nature methods* 3, 83-89, doi:10.1038/nmeth822 (2006).

7 Bacia, K. & Schwille, P. Practical guidelines for dual-color fluorescence cross-correlation spectroscopy. *Nature protocols* 2, 2842-2856, doi:10.1038/nprot.2007.410 (2007).

8 Elson, Elliot L. Fluorescence Correlation Spectroscopy: Past, Present, Future. *Biophysical Journal* 101, 2855-2870, doi:10.1016/j.bpj.2011.11.012 (2011).

9 Digman, M. A. & Gratton, E. Lessons in Fluctuation Correlation Spectroscopy. *Annual review of physical chemistry* 62, 645-668, doi:10.1146/annurev-physchem-032210-103424 (2011).

10 Weidemann, T. *et al.* Counting Nucleosomes in Living Cells with a Combination of Fluorescence Correlation Spectroscopy and Confocal Imaging. *Journal of Molecular Biology* 334, 229-240, doi:http://dx.doi.org/10.1016/j.jmb.2003.08.063 (2003).

11 Mahen, R. *et al.* Comparative assessment of fluorescent transgene methods for quantitative imaging in human cells. *Molecular biology of the cell* 25, 3610-3618, doi:10.1091/mbc.E14-06-1091 (2014).

12 Cuylen, S. *et al.* Ki-67 acts as a biological surfactant to disperse mitotic chromosomes. *Nature* 535, 308-312, doi:10.1038/nature18610 (2016).

13 Germier, T. *et al.* Real-time chromatin dynamics at the single gene level during transcription activation. *bioRxiv*, doi:10.1101/111179 (2017).

14 Conrad, C. *et al.* Micropilot: automation of fluorescence microscopy-based imaging for systems biology. *Nature methods* 8, 246-249, doi:10.1038/nmeth.1558 (2011).

15 Tischer, C., Hilsenstein, V., Hanson, K. & Pepperkok, R. Adaptive fluorescence microscopy by online feedback image analysis. *Methods in cell biology* 123, 489-503, doi:10.1016/B978-0-12-420138-5.00026-4 (2014).

16 **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).**

17 **Beck, M. *et al.* The quantitative proteome of a human cell line. *Molecular systems biology* 7, 549, doi:10.1038/msb.2011.82 (2011).**

18 **Ori, A. *et al.* Cell type-specific nuclear pores: a case in point for context-dependent stoichiometry of molecular machines. *Molecular systems biology* 9, 648, doi:10.1038/msb.2013.4 (2013).**

19 **Otsuka, S. *et al.* Nuclear pore assembly proceeds by an inside-out extrusion of the nuclear envelope. *eLife* 5, doi:10.7554/eLife.19071 (2016).**

20 **Rüttinger, S., Buschmann, V., Krämer, B., Erdmann, R., MacDonald, R., & Koberling, F. Comparison and accuracy of methods to determine the confocal volume for quantitative fluorescence correlation spectroscopy. *Journal of Microscopy* 232, 343–352, doi:https://doi.org/10.1111/j.1365-2818.2008.02105.x (2008).**

21 **Mütze, J., Ohrt, T. & Schwille, P. Fluorescence correlation spectroscopy in vivo. *Laser & Photonics Reviews* 5 (2011).**

22 **Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature methods* 9, 676-682, doi:10.1038/nmeth.2019 (2012).**

23 **Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular reproduction and development* 82, 518-529, doi:10.1002/mrd.22489 (2015).**

24 **R CoreTeam. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.* 2015).**

25 **Chang, W., Cheng, J., Allaire, J. J., Xie, Y. & McPherson, J. *Shiny: Web Application Framework for R. R package version 1.0.0. http://CRAN.R-project.org/package=shiny*, < http://CRAN.R-project.org/package=shiny> (2017).**

26 **Capoulade, J., Wachsmuth, M. & Hufnagel, L. Quantitative fluorescence imaging of protein diffusion and interaction in living cells. *Nature biotechnology* 29, 835–839, doi:http://doi.org/10.1038/nbt.1928 (2011).**

27 **Kaputsa, P. *Absolute diffusion coefficients: compilation of reference data for FCS calibration.*, <https://www.picoquant.com/scientific/technical-and-application-notes/category/technical_notes_techniques_and_methods> (2010).**

28 **Lukinavicius, G. *et al.* SiR-Hoechst is a far-red DNA stain for live-cell nanoscopy. *Nat Commun* 6, 8497, doi:10.1038/ncomms9497 (2015).**

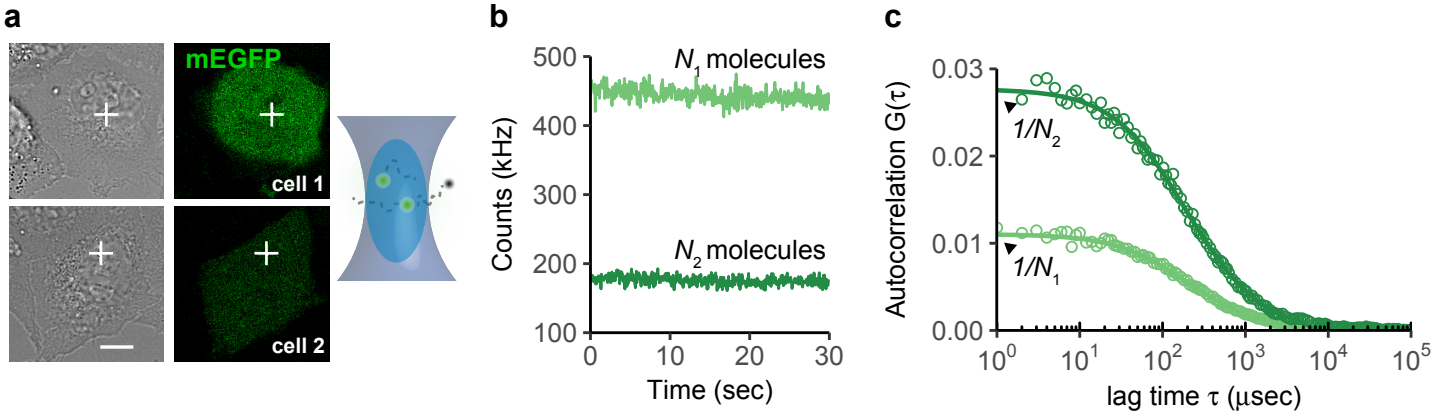
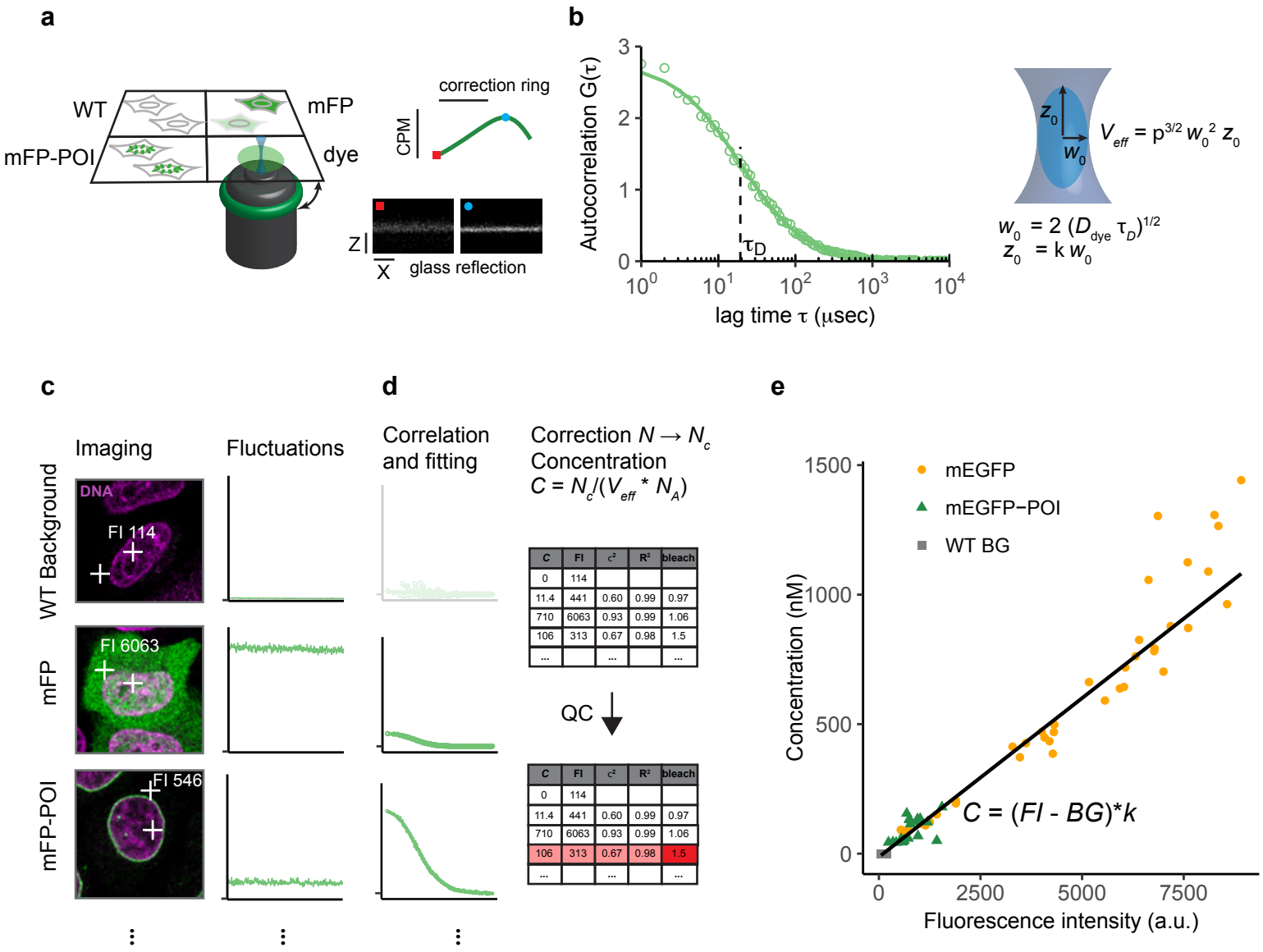

a
mEGFP
cell 1
cell 2
b
Counts (kHz)
500
400
300
200
100
N_1 molecules
N_2 molecules
0
10
20
30
Time (sec)
c
Autocorrelation G(τ)
0.03
0.02
0.01
0.00
$1/N_2$
$1/N_1$
10^0
10^1
10^2
10^3
10^4
10^5
lag time τ (μsec)

Figure 1



C	FI	c^2	R^2	bleach
0	114			
11.4	441	0.60	0.99	0.97
710	6063	0.93	0.99	1.06
106	313	0.67	0.98	1.5
...		...		...

C	FI	c^2	R^2	bleach
0	114			
11.4	441	0.60	0.99	0.97
710	6063	0.93	0.99	1.06
106	313	0.67	0.98	1.5
...		...		...

Figure 2

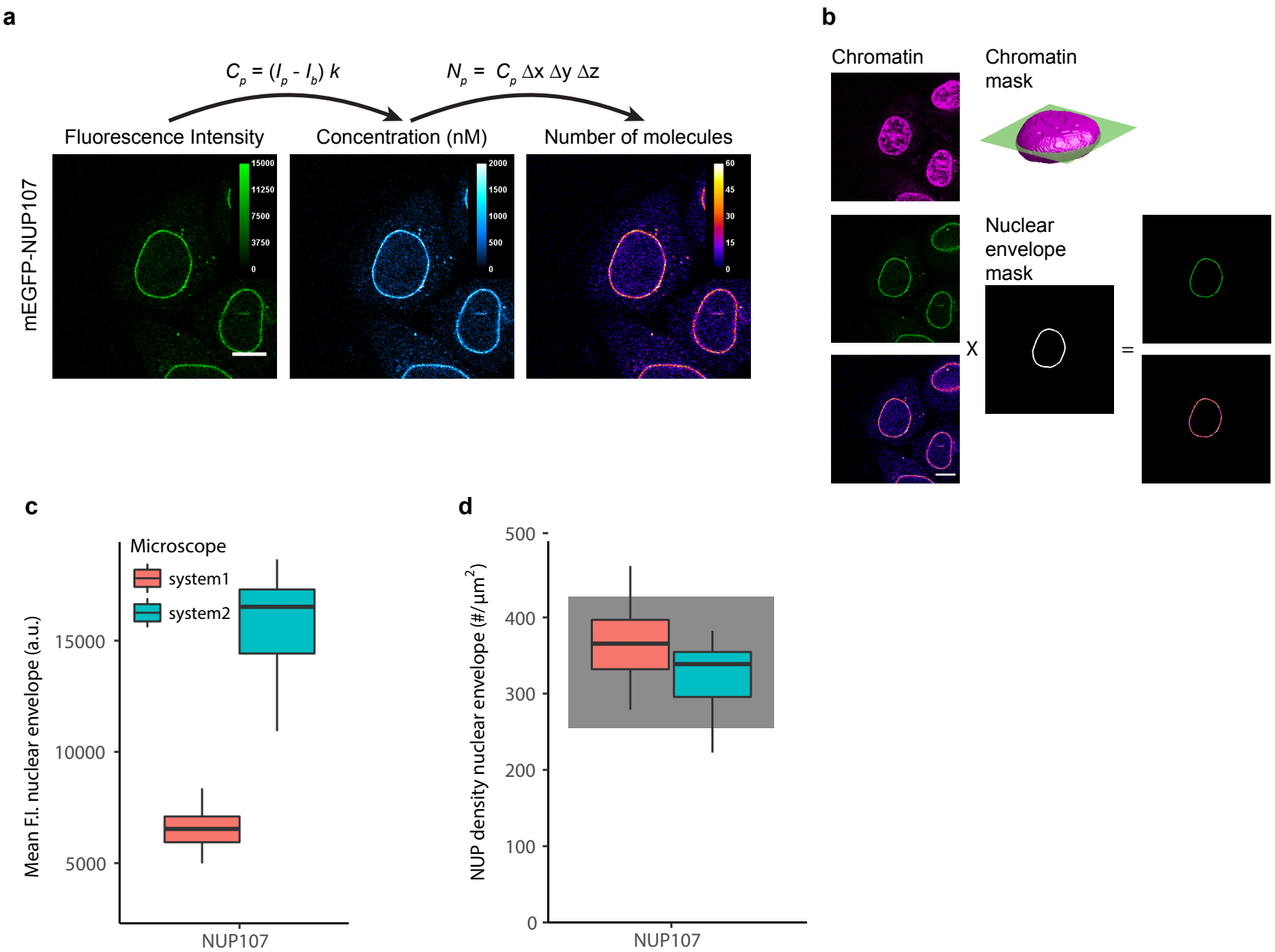
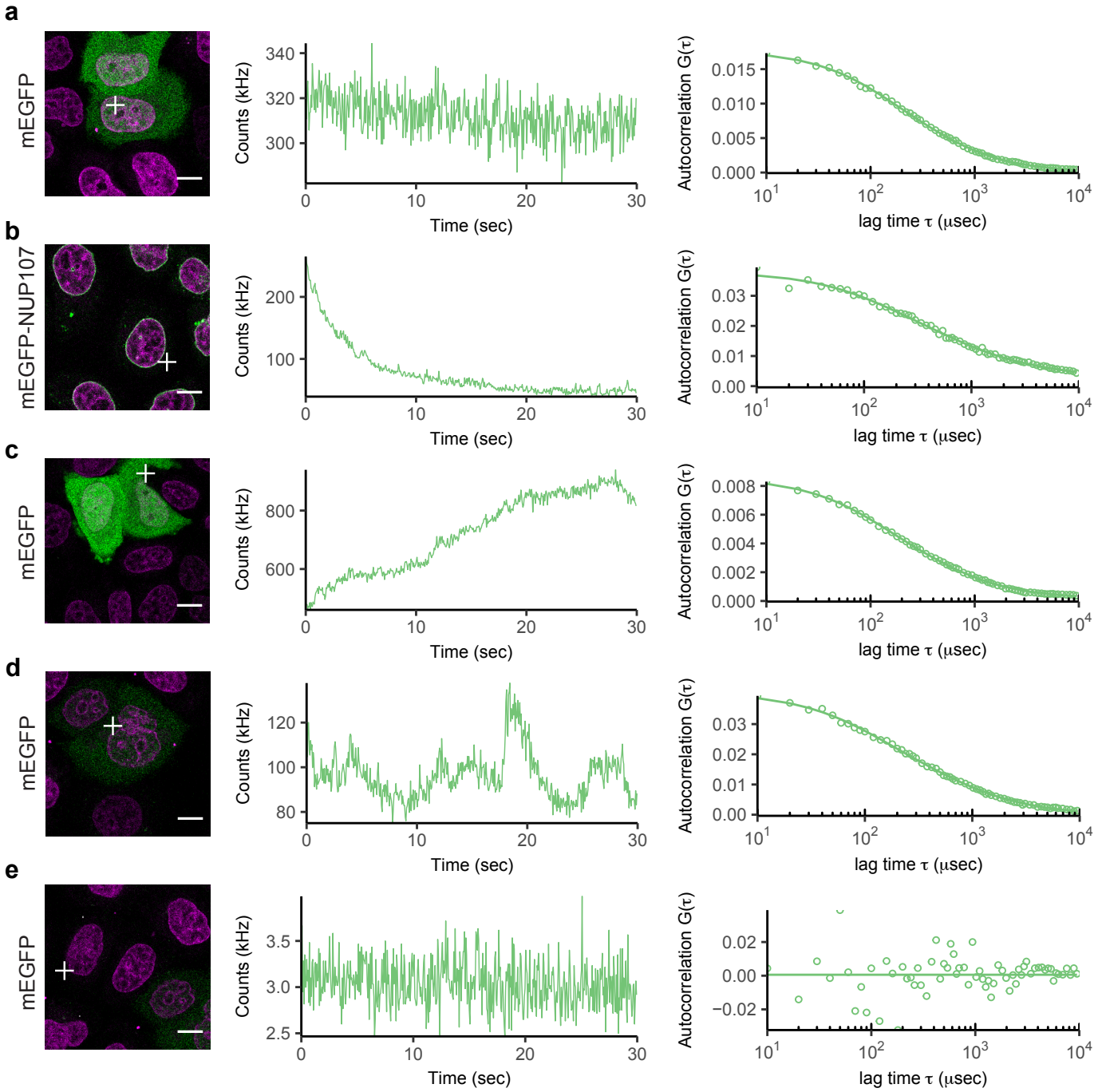

a
$C_p = (I_p - I_b)\ k$
$N_p = C_p\ \Delta x\ \Delta y\ \Delta z$
Fluorescence Intensity
Concentration (nM)
Number of molecules
mEGFP-NUP107
15000
11250
7500
3750
0
2000
1500
1000
500
0
60
45
30
15
0
b
Chromatin
Chromatin mask
Nuclear envelope mask
X
=
c
Microscope
system1
system2
Mean Fl. nuclear envelope (a.u.)
15000
10000
5000
NUP107
d
NUP density nuclear envelope (#/µm²)
500
400
300
200
100
0
NUP107

Figure 3



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

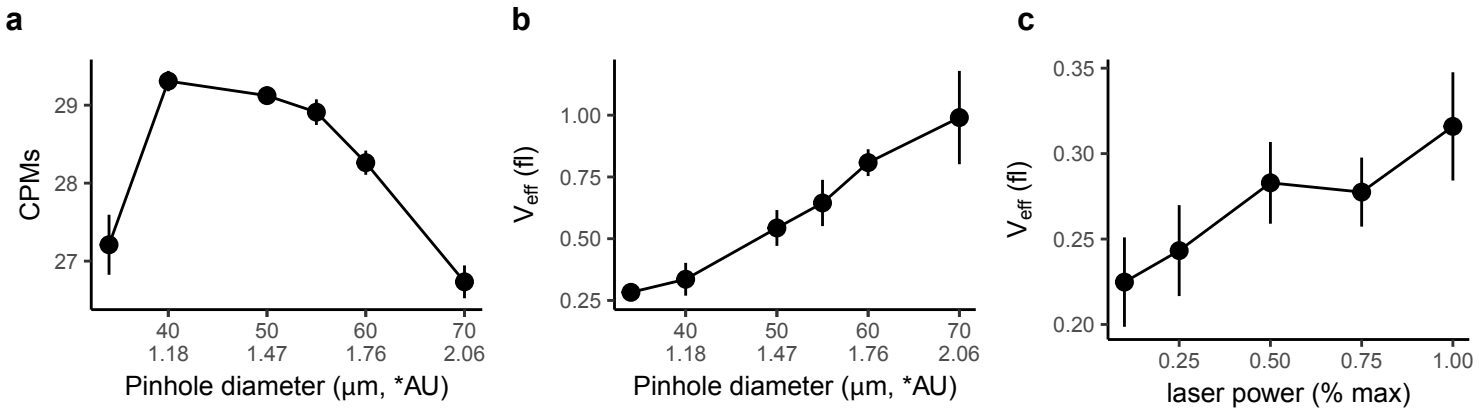

a
CPMs
29
28
27
40
1.18
50
1.47
60
1.76
70
2.06
Pinhole diameter (µm, *AU)
b
V_{eff} (fl)
1.00
0.75
0.50
0.25
40
1.18
50
1.47
60
1.76
70
2.06
Pinhole diameter (µm, *AU)
c
V_{eff} (fl)
0.35
0.30
0.25
0.20
0.25
0.50
0.75
1.00
laser power (% max)

Figure S2

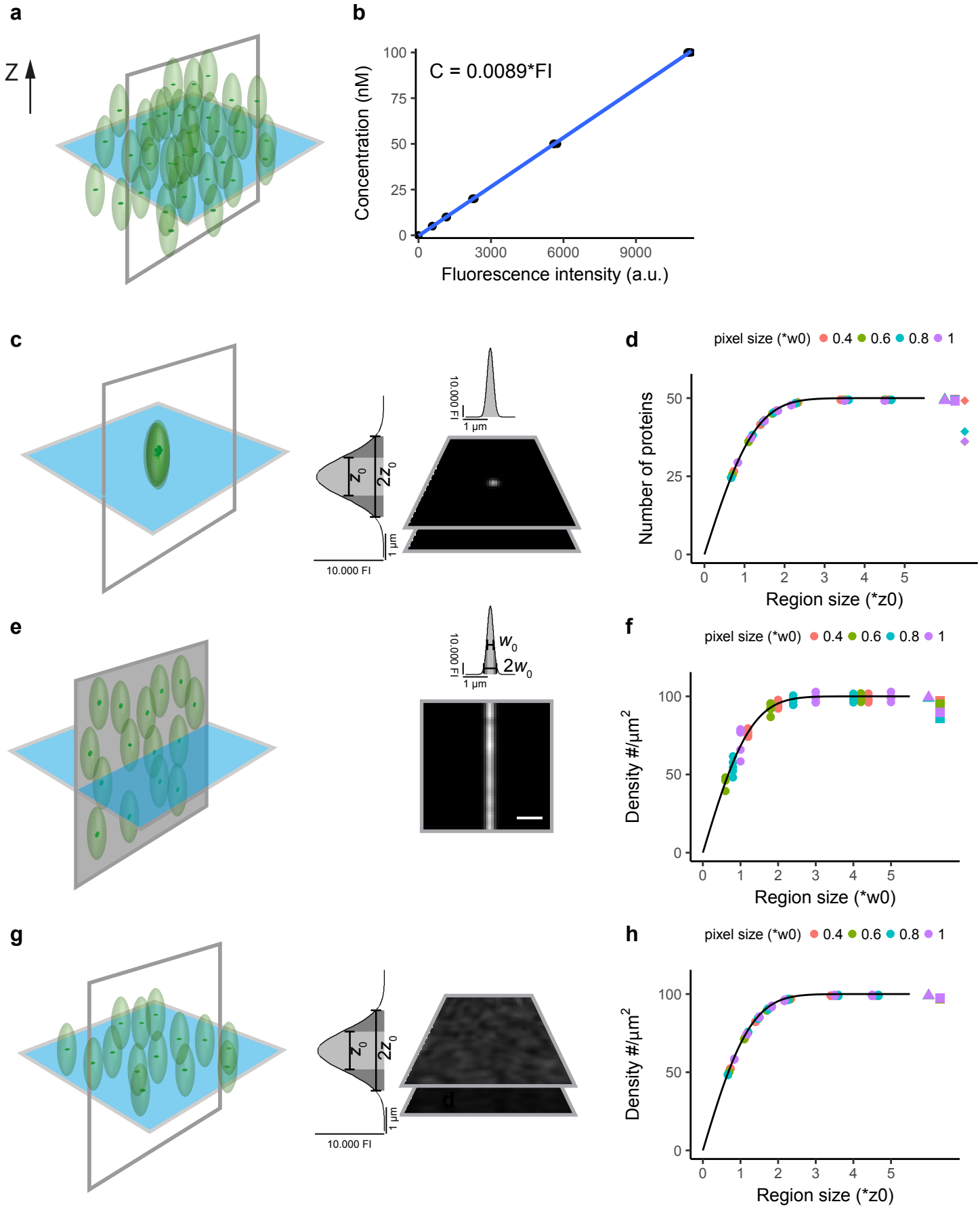

a
Z
b
C = 0.0089*FI
Concentration (nM)
Fluorescence intensity (a.u.)
c
10.000 FI
1 µm
d
pixel size (*w0) 0.4 0.6 0.8 1
Number of proteins
Region size (*z0)
e
f
Density #/µm²
Region size (*w0)
g
h

Figure S3