Supplemental Materials
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Supplementary Material

Synthetic and genetic dimers as quantification ruler for single-molecule counting with PALM

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Supplementary Note: Guide for quantitative PALM analysis

Image acquisition

1) Place the sample on the microscope stage of a widefield setup equipped with TIRF illumination and an electron multiplying charge-coupled device (EMCCD) camera. Adjust the height of the objective such that the immersion oil touches the sample. In the case of cell measurements, use bright-field illumination to find the right plane via the eye piece of the microscope.

2) Start acquisition software (e.g. camera software or µManager). Set the exposure time to 100 ms, frame transfer on and the frame number to 100,000 frames (measurement will be stopped before reaching 100,000 frames; ensures recording of all emission events). The EM gain has to be adjusted such that an optimal signal-to-noise ratio is obtained. In the present study, the EM gain was set to 200, which in the case of the EMCCD camera used in this study (iXon Ultra X-10971, Andor) translates to a real gain of 200.

3) Position a region of interest (ROI) in the field of view.
   a) In the case of cells, search cells with bright-field illumination. The plane is adjusted such that the basal membrane is in focus. Switch off the bright-field illumination.
   b) In the case of single-molecule surfaces, adjust the right plane with 561 or 568 nm illumination. Switch the laser off and move the sample to a new region that was not illuminated with laser light.

4) Start data acquisition (prior to switching on the lasers to ensure the detection of all blinking events). The adjustable TIRF mirror should be positioned such that the sample is not illuminated (maximum distance of the excitation beam from the centrum of the back focal plane of the objective).

5) Switch on excitation laser (561 or 568 nm). For all fluorescent proteins (mEos2, mEos3.2, mMaple3, Dendra2, PAmCherry2) used so far for quantitative PALM, suitable irradiation intensities are about 0.2 kW/cm². Move the laser beam slowly back towards the center of the objective’s back focal plane using the TIRF mirror, such that only a small area on one edge of the ROI is illuminated. Fine tune the
focus by adjusting the z-position of the sample, and then adjust TIRF illumination for the whole field of view.

6) Slowly increase the intensity of the UV laser (405 nm) depending on the number of simultaneously emitting fluorophores. Take care that point spread functions (PSFs) do not overlap. UV irradiation intensities typically vary between 0-38 mW/cm².

7) Acquire data until there are no further blinking events observed in the case of dimers and higher oligomers, because fluorescent proteins are activated stochastically. Stop data acquisition.

8) For sufficient statistics, independent samples should be prepared and measured on different days.

Quantitative PALM analysis

(A) Single-molecule localization and reconstruction of super-resolved qPALM images

- Generate super-resolved images with the rapidSTORM software (Wolter et al., 2012) and store as .png files. The physical pixel size, the PSF full width half maximum (FWHM) and the intensity threshold have to be adjusted user-defined.

  - Determine the physical pixel size from the camera pixel size and the magnification of the objective.

  - Determine the PSF FWHM using the respective output module implemented in rapidSTORM.

  - Select the intensity threshold such that background signal is subtracted but not the signal of emitting fluorophores. In our case, an intensity threshold of 63 photons has proven itself.

  - In the output options domain, set the “Extent of histogram normalization” in the image display sub-item to zero.

  - Select “Output only Malk fields (x,y,z,t,I)” in the sub-item “Localizations file”.

  - Start localization analysis by pressing the “Run” button. A localization file is generated and an image is reconstructed from all single-molecule localizations.
• After the analysis is finished, adjust the intensity cutoff such that the maximal “Key” (in ADC units) displayed beside the reconstructed super-resolved image is about 3-10 k. The intensity cutoff is set such that the full dynamic range of pixel intensities is reflected.

• Save the super-resolution image by pressing “Save current image”.

• If a measurement was split into separate files, analyze all files as described above and merge afterwards:
  - Select the “Minimal” tool under “Job” > “Replay”.
  - Choose the number of input channels and load the text files into the channels (file type “Localization files”).
  - Set the “Join inputs on” item to “In time”.
  - Add the output modules “Expression filter”, “Image display”, and “Localizations file” in the respective order and adjust as described before.
  - Define a “Output file basename”.
  - Run the task and set the intensity cutoff accordingly.

(B) Tracking and tracing of the merged localization files

• Load the merged localization file into the “Minimal” tool of rapidSTORM (“Job” > “Replay” > “Minimal”).

• Add the output modules “Expression filter”, “Track emissions”, “Trace filter”, “Image display”, and “Localizations file” and adjust them as follows.
  - In the “Expression filter” menu, set the “Number of expressions” to 2 and the “Value to assign to” to “sigmaposx” for the first and “sigmaposy” for the second one with an “Expression to assign from” of 30 nm for both values.
  - Implement a “Distance threshold” of 3 into the “Track emissions” menu.
  - In the “Trace filter” menu, set the “Minimum number of emissions per trace” to 3. In the trace script, all localizations that last only one frame are rejected to effectively subtract background signal.
- Adjust “Image display” and “Localizations file” as described before.

- Run the analysis.

- After the analysis is finished, adjust the intensity cutoff as before.

- Save the tracked and traced super-resolution image.

(C) Generation of a localization image with the LAMA software

- Load the tracked and traced localization file into the LAMA software (Malkusch and Heilemann, 2016) via the “Browse” button in the “Input” tab.

- In the main menu, start the auto detection of ROIs and set x,y and t values to zero; pixel size and integration time are specified in the “Setup” section.

- Set the desired pixel size in the “Image” sub-section of the “Visualize” menu to 10 nm.

- Check the box “Choose maximum manually” with the respective value set to 255 and check “Compute intensity based image”.

- Generate the image by pressing “Compute”. In the newly generated image, the number of tracked and traced localizations are converted into fluorescent emissions. Hence, the number of fluorescent events can be determined from this image for blinking analysis.

(D) Spot selection and extracting the number of fluorescent events

- Load the super-resolved image, the tracked and traced image as well as the LAMA image into the Fiji software (Schindelin et al., 2012).

- Adjust the brightness of the LAMA image to values between 0 and 3.

- Stack images (Images > Stacks > Images to stack; “copy (top-left)” method).

- Select spots with rectangular ROIs in the super-resolved image and save them to the ROI manager. The following criteria are applied for spot selection
  - characteristic size (minimum area of 4 pixels; maximum diameter of 10 pixels)
- a relatively high intensity
- round shape
- clear separation from neighboring spots

• Use the LAMA image to measure the number of fluorescent events: Load the LAMA image again separately into Fiji and measure the intensity of the ROIs (activate “Set measurements” > “Integrated density”).

• Save the text file with the respective intensities per ROI for further analysis. The intensity of a single ROI corresponds to its number of fluorescent events.

**(E) Analysis of blinking histograms**

• Import the number of fluorescent events for all analyzed ROIs into OriginPro2017G (or another data analysis program)

• Perform a frequency analysis with a binning of 1.

• Subtract 1 from the number of fluorescent events to obtain the blinking number, i.e. 1 fluorescent event corresponds to 0 blinking events.

• Plot the frequency against the number of blinking events.

• Fit the blinking distribution with the hypergeometric function derived from EquationS1(Hummer et al., 2016) with \( p \) (bleaching probability) and \( q \) (fraction of undetected molecules).

\[
B_n(n) = \sum_{k=n}^{m} \binom{m}{k} (\frac{n}{k}) q^{m-k} (1-q)^k p^{n-k} (1-p)^n-k
\]  
\( (S1) \)

• For pure monomer, dimer, or monomer-dimer \((f = \text{monomer fraction})\) populations use the following fit functions (Equation S2-S4).

**Monomer fit:**

\[
B_n(n) = p (1-p)^n
\]  
\( (S2) \)

**Dimer fit:**

\[
B_n(n) = p (1-p)^{(1-f)n} (1-q) (1-q) + (1-p) q
\]  
\( (S3) \)
Monomer dimer linear combination fit:

\[ p_{\text{2dimer}}(n) = f \cdot p \cdot (1 - p)^n + (1 - f) \cdot p \cdot (1 - p)^{n-1} \cdot q \cdot (1 - q) + (1 - p)q \]  

(S4)
Figure S1. Photon count distribution of different fluorescent proteins. Photon counts of single emission events were calculated from intensity values and corrected with the conversion factor of the EMCCD camera. (A) Photon count distribution of mEos3.2 in PBS (left) and in 100 mM MEA in PBS (right). Mean photon counts of 1109 (N=128268 emission events, PBS) and 1333 photons (N=24244 emission events, 100 mM MEA) were determined. (B) Photon count distribution of mMaple3 in PBS (left) and in 100 mM MEA (right). The mean photon counts are 601 (N=11866 emission events, PBS) and 897 photons (N=18366 emission events, 100 mM MEA). (C) Photon count distribution of Dendra2 in PBS with a mean photon count of 533 photons (N=6096 emission events). (D) Photon count distribution of PAmCherry2 in PBS yielded a mean photon count of 644 photons (N=22015 emission events).
Figure S2. Blinking distribution of mEos3.2 determined on a commercial Nikon N-STORM microscope. Single-molecule surfaces of mEos3.2 were measured in PBS on a commercial Nikon N-STORM microscope. The frequency distribution of blinking events was fitted with a monomeric function (orange line) revealing a $p$-value of $0.30 \pm 0.01$ (N = 871 spots).
Figure S3. Theoretical analysis of oligomeric states by using monomeric mEos3.2 data. (A) Oligomeric histograms were generated by combining different amounts of mEos3.2 spots. The blinking distributions were fitted with linear combinations of monomer and dimer (left, dark-grey line); monomer, dimer and trimer (middle, grey line) and monomer, dimer, trimer and tetramer (right, light grey line) fit functions. The $\mu$-value was used from the monomeric mEos3.2 data ($\mu = 0.32$) and the $\sigma$-value was set to zero. The fractions of monomer, dimer, trimer and tetramer obtained from the mixed fit functions are shown in their corresponding color. (B) Histograms of theoretically mixed monomer/dimer populations. The theoretical dimer population was mixed with 30% monomer data to mimic incomplete and incorrect maturation of the fluorescent protein normally present in living cells. This new dimeric population revealed a $\sigma$-value of 0.33. Different ratios of the new dimer population and monomer data were mixed (dimer/monomer: 30/70, left; 50/50, middle; 70/30, right) and fitted with linear combinations of the monomer and dimer function with a $\mu$-value of 0.32 and $\sigma = 0.33$. 
Figure S4. Preparation of the 30 bp DNA linker and purification of the synthetic dimer.

(A) Synthesis and assembly of 30 bp dsDNA linker. Step 1: 5'-NHS-modified oligonucleotides were mixed with amine-functionalized trisNTA. After the reaction, the trisNTA-modified oligonucleotides were cleaved and deprotected by 32% ammonia. Step 2: Oligonucleotides were purified by IP-RP-C18 HPLC. Step 3: A complementary pair of oligonucleotides was mixed stoichiometrically and purified by IP-RP-C18 HPLC. Finally, the dsDNA-dimerization tools were loaded with Ni(II). (B) Schematic illustration of the 30 bp DNA linker. A pair of trisNTAs is separated by the rigid dsDNA linkers with a length of approximately 10 nm. The octahedral complex of the high-affinity His-tag/trisNTA interaction pair is illustrated in the zoom-in. POI: protein of interest. (C) Quality control of the 30 bp DNA linker. Native PAGE analysis of duplex formation of dsDNA linker in comparison to the corresponding trisNTA-ssDNA oligonucleotides. The low mobility shifts of dsDNA linker indicate the duplex formation (19% nPAGE, O’GeneRuler Ultra Low Range DNA ladder, SYBR Gold staining). (D) Chemically induced protein dimerization of 30 bp dsDNA linker analyzed by fluorescenceSEC. mEos3.2-His₆ (500 nM) was first analyzed alone (green line). 30 bp dsDNA linker (100 nM) were incubated with mEos3.2-His₆ (500 nM) (blue line) and applied on the column (200 Increase 3.2/300; flow: 0.075 mL/min). The buffer for the SEC contained 20 mM HEPES, 150 mM NaCl and 10 mM MgCl₂ pH 7.4.
Figure S5. The blinking properties of mEos3.2 and mMaple3 are affected in the presence of the reducing agent β-mercaptoethylamine. (A) Blinking distribution of monomeric mEos3.2 measured in 100 mM MEA, pH 7.8. The frequency diagram was fitted with a monomer fit (orange, solid line), reporting a bleaching parameter of $p = 0.17 \pm 0.01$ (N = 920 spots). For comparison, the monomeric fit function derived from the blinking histogram of mEos3.2 in PBS is shown as a reference (orange, dashed line) (B) Blinking distribution of monomeric mMaple3 measured in 100 mM MEA, pH 7.8. The frequency diagram was fitted with a monomer fit (blue, solid line), reporting a bleaching parameter of $p = 0.56 \pm 0.01$ (N = 858 spots). The monomeric fit function derived from the blinking histogram of mMaple3 in PBS is shown as a reference (blue, dashed line).
Figure S6. PALM images and blinking histograms of mEos3.2-tagged proteins in HeLa cells. HeLa cells were transfected with CD86-mEos3.2 or CTLA-4-mEos3.2, fixed, and imaged on a home-built microscope in TIRF mode. (A) Representative PALM image (left) and blinking distribution (right) of a CD86-mEos3.2-transfected cell. The blinking histogram was fitted with a monomer fit function and revealed a $p$-value of 0.27± 0.01 (N=1957 spots). (B) PALM image (left) and blinking distribution (right) of the fusion protein CTLA-4-mEos3.2. The obtained data was fitted with a dimer fit function ($p$ =0.27) and revealed a $q$-value of 0.39± 0.01 (N=1050 spots). The monomer fit function is shown as dashed line as a guide to the eye. All scale bars are 2 µm.
Supplementary Tables

Table S1: Photon budgets of different FPs in PBS and under reducing conditions. Single-molecule surfaces of fluorescent proteins were imaged with PALM and the number of photons per activation event was determined using the calibration factor of the camera. The mean photon budget with its standard error of the mean as well as the median are listed.

| FP                | Mean photon numbers | Median photon number |
|-------------------|---------------------|---------------------|
| mEos3.2           | 1109 ± 4            | 657                 |
| mEos3.2 + 100 mMMEA | 1333 ± 12           | 768                 |
| mMaple3           | 601 ± 11            | 374                 |
| mMaple3 + 100 mMMEA | 897 ± 14           | 394                 |
| Dendra2           | 533 ± 16            | 265                 |
| PAmCherry2        | 644 ± 8             | 455                 |
Table S2: Primer pairs used for PCR-based cloning of various constructs. Restriction enzyme-based cloning or Hot Fusion reactions were used to generate the desired plasmids. The forward and reverse primers for cloning the different constructs are listed as well as the cloning method and the backbone vectors.

| Cloning | Forward primer | Reverse primer | Vector | Cloning method/restriction sites |
|---------|----------------|----------------|--------|---------------------------------|
| mEos3.2 | GAAGGATCCATGAG TGCGATAGCCAG | GAAGAATTCTTATCT TCGTCTGGCATTCGAG | pRESET | BamHI; EcoRI |
| mMaple3 | GCAGGATCAGTCCGTGCGC | GGAAGAAATTCATCT CTGGCATTATGGAC | pRESET | BamHI; EcoRI |
| mEos3.2 dimer | TCTGGATTGCCTGACAAT GCCAGACGAGAAGAGG TGAGCGCTGTGCCTGGCT | TTAGCAGGGATCAAGCTTCGAAATTTCTATCTTCTG CTTGGATGTGTCAGGCAA | pRESET | HotFusion |
| CD86 mEos3.2 and CTLA4 mEos3.2 | GCCAGGTGGCTGGCAAC CCGGCCGCTACTATGGACTAGAGATACGTGGATAGCCAGCAT | CACACTGGATACGTTGCTATCT TATGCGGCCGCTTATCTGCTGTCTGGCATTCGAGG | pIRESpuro2 | HotFusion |

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