Varying label density allows artifact-free analysis of membrane-protein nanoclusters

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We present a method to robustly discriminate clustered from randomly distributed molecules detected with techniques based on single-molecule localization microscopy, such as PALM and STORM. The approach is based on deliberate variation of labeling density, such as titration of fluorescent antibody, combined with quantitative cluster analysis, and it thereby circumvents the problem of cluster artifacts generated by overcounting of blinking fluorophores. The method was used to analyze nanocluster formation in resting and activated immune cells.

Several strategies have been developed to achieve nanoscopic resolution in light microscopy. Among them, single-molecule-localization-based methods including photoactivated localization microscopy (PALM) and (direct) stochastic reconstruction microscopy ((d)STORM) have become increasingly popular1. The techniques rely on fluorophores that can be stochastically switched between a dark off-state and a fluorescent on-state under conditions where only a marginal number of fluorophores is in the on-state at any given time. Typically, stacks of thousands of images are recorded from a sample that is chemically fixed to preclude motion of the fluorophores during data acquisition. Finally, single-molecule positions are determined2 and localization maps of the sampled molecules are reconstructed. Currently, a number of image-processing algorithms are available to identify and characterize clusters of single-molecule localizations3–7.

In the field of membrane biology, PALM and (d)STORM have been widely used, providing maps of plasma-membrane constituents or associated proteins in unprecedented detail1,3,4–12. One remarkable finding has been the observation of nanoscale clustering of virtually any membrane protein8. Recently, however, notes of caution have been raised over the fact that multiple observations of single fluorophores also lead to clustered localizations and may closely resemble clustered molecules3,5,13–16, thereby impairing direct conclusions from the observed localization clusters about the presence of protein clusters. The main problem arises from the stochastic blinking behavior of organic fluorophores and fluorescent proteins.

Several researchers have approached this problem by a posteriori methods. Annibale et al. proposed to combine events that are assumed to be caused by multiple observations of the same fluorophore3,14. This approach, however, is hampered by the difficulty of discriminating overcounting of the same dye molecule from observations of different colocalized dye molecules, especially when long-lived dark states of fluorophores are present3, and this difficulty leads to erroneous interpretation of the data as clustered. On the other hand, too-rigorous elimination of potential multiple observations hinders the detection of true clusters. Another method applies pair (auto-) correlation analysis (PCA) to address the problem of overcounting5. A PCA curve ideally consists of two more-or-less well-separated components: a short decay corresponding to multiple observations of the same dye molecules within the localization precision and a long decay reporting the size of true protein clusters. Provided that all molecules are immobilized, PCA is robust against heterogeneities in the dye-blinking statistics. Chemical fixation protocols, however, are often unable to completely immobilize the molecular components of cellular samples17, particularly in view of the long recording times (tens of minutes) required for obtaining super-resolution images. In addition, the method reaches its limits when protein clusters are not significantly larger than the spread of localizations obtained from a single blinking fluorophore.

In contrast to previous approaches based on postprocessing, we tried to find experimental criteria to distinguish random from clustered distributions of molecules, which are insensitive to the blinking statistics of the fluorophores used and their residual mobility. We reasoned that varying the labeling density would lead to characteristic changes in the localization maps obtained. For homogeneous protein distributions the relative area covered by apparent clusters resulting from fluorophore blinking will increase steadily with an increasing degree of labeling. Concurrently, the density of localizations per cluster will remain constant. Conversely, if molecules are clustered, the relative area will saturate, and the density of localizations per cluster will be proportional to the degree of labeling.

In order to test our predictions, we simulated localization maps for randomly distributed (Fig. 1a) and clustered (Fig. 1b) molecules at varying densities. Each molecule was allowed to blink stochastically, yielding an average of seven observations per molecule that were distributed around each molecular position with a localization error of 40 nm. Clusters were simulated by randomly distributing varying numbers of molecules within
Figure 1 | Effect of label-density variation on randomly distributed versus clustered molecules. (a,b) Simulations of increasing numbers of randomly distributed (a) and clustered molecules (b), each yielding an average of seven localizations per molecule (red); the calculated cluster masks are shown in white. For clustered distributions we assumed cluster radii of 50 nm and 3 clusters per µm². We simulated localization densities of ~50 locs µm⁻² (left), ~500 locs µm⁻² (center) and ~1,000 locs µm⁻² (right). Scale bars, 1 µm. Cartoons illustrate the outcome of label titration in each scenario. (c) Quantification of the relative (rel.) clustered area per image (η) and the density of localizations per clustered area (ρ); the plots show characteristic changes of η and ρ with increasing numbers of localizations (left and center) as well as a characteristic η dependence of p/p₀ (right). p₀, which is used for normalization of p/η plots, is highlighted by a red circle (center). The red line indicates the reference curve for a random distribution.

circles with a radius of 50 nm, which corresponded to the size of published nanoclusters. Notably, irrespective of whether randomly distributed or clustered molecules were simulated, all images displayed heterogeneities in the localization density, which were also reflected by characteristic peaks in Ripley’s K function (Supplementary Fig. 1). Our method is based on the quantitative characterization of these apparent clusters at different labeling densities. Binary cluster masks were established from thresholded localization-density maps based on the recorded or simulated single-molecule positions and the known localization errors (Fig. 1a,b; see also Online Methods and Supplementary Fig. 2).

For each image, we counted the number of total localizations per µm² (locs µm⁻²), the relative area coverage by the cluster masks (η) and the average density of localizations within the apparent clusters (ρ). Both η and ρ showed a characteristic difference in their dependence on locs µm⁻² for randomly distributed versus truly clustered molecules (Fig. 1c). We defined the intersection of the density curves with the y-axis as p₀; hence, ρ₀ × σ²π gives an estimate of the number of counted localizations per molecule, with σ denoting the localization precision. The difference between clustered and random distributions becomes even more pronounced when plotting the normalized density p/ρ₀ against η: for randomly distributed molecules a rather horizontal line can be observed, whereas true clustering yields a strong increase in p/ρ₀.

To validate the robustness of our approach, we varied several parameters in our simulations. First, we ensured that the selected threshold for the mask algorithm and the degree of overcounting had no effect on the results of the analysis; the degree of overcounting accounts for multiple reappearances of single dye molecules and for the presence of multiple chromophores per labeling antibody. For both randomly distributed and clustered data, we obtained robust curves after normalization (Supplementary Fig. 3a,b; see Supplementary Fig. 3c–f for curves that were not normalized). Also, residual mobility of the randomly distributed proteins had no effect on the shape of the obtained curves (Supplementary Fig. 4a,b). As discussed above, repeated photocycling of the same molecule generate pseudoclusters in super-resolution images, which are difficult to discriminate from true clusters. We thus simulated the worst-case scenario, in which a small proportion of randomly distributed molecules is virtually not photobleachable and thus generates substantially more localizations per molecule than average (Supplementary Fig. 4c,d). Also, under these circumstances, the overall shape of the curve remained unchanged. The additional spread in the p/ρ₀ ratios for small values of η can be ascribed to fluctuations in the total number of nonbleachable molecules per localization map. Taken together, we could use the obtained η-dependence of p/ρ₀ for randomly distributed localizations as a reference curve for subsequent plots (red line plotted in Fig. 1c).

We next evaluated the effect of different numbers of true clusters per image (Supplementary Fig. 4e) and of different cluster sizes (Supplementary Fig. 4f). From this it became evident that our approach robustly detects a broad range of cluster scenarios; only high numbers and large sizes of clusters are difficult to identify, as the images converge to a homogeneous distribution of localizations. Next, we emulated the effect of the presence of nonclustered molecules or of unspecifically bound labels by adding randomly distributed blinking molecules to localization maps of clustered molecules. The presence of large numbers of background signals shifted the obtained curves toward the reference for randomly distributed molecules (Supplementary Fig. 4g). In summary, our method yields clear differences between random and clustered distributions of molecules over a broad range of cluster parameters.

We further assessed the sensitivity of our method in detecting small oligomers. To this end, we simulated pentamers at a density of 50 molecules per µm² (i.e., 10 pentamers per µm²). We further included free monomers at varying concentrations up to equal densities as background. To mimic the label-titration experiment, we calculated images with increasing numbers of dye molecules assigned to each oligomer. As expected, increasing background levels of unclustered molecules reduced the sensitivity (Supplementary Fig. 4h). Still, our simulations showed that pentamers can be readily detected at all simulated background levels.

Having tested our method on simulated data, we further evaluated our approach in a synthetic experimental setting. We used
Figure 2 | Label-density variation of randomly distributed and clustered proteins on synthetic surfaces. Streptavidin was adsorbed to glass surfaces either randomly (a) or as 200-nm-sized clusters via microcontact printing (b) and incubated with biotinylated murine IgG. The images show a titration series where the biotinylated IgG was detected via an AF647-conjugated IgG-specific antibody at the indicated concentrations. From reconstructed (d)STORM localization maps $\rho$ and $\eta$ values were calculated for each image to generate normalized $\rho/\rho_0$ plots (graphs at bottom right). 10 or more images were recorded per titration step; each data point represents a single image. The red lines indicate the reference curve for a random distribution. Scale bars, 1 μm.

microcontact printing to produce circular clusters of IgG arranged in periodic patterns on glass surfaces (~200-nm diameter, ~6 clusters per μm$^2$); interspaces were passivated with bovine serum albumin (BSA). To mimic random protein distributions, we immobilized the biotinylated primary IgG via streptavidin randomly on the glass surface. In both cases, an AF647-conjugated secondary antibody was titrated to probe for clustering. Imaging conditions, particularly laser settings, were kept constant for recording the titration series. The plot of $\rho/\rho_0$ versus $\eta$ showed clear differences between the clustered scenario and the random distribution (Fig. 2).

Finally, we applied our method in cell biological settings. Clathrin-coated pits (CCPs) represent a well-established example of protein clusters in the plasma membrane. We stained CCPs in fixed Jurkat cells with different concentrations of an AF647-labeled clathrin-heavy-chain (clathrin HC)-specific antibody (Supplementary Fig. 5a), thereby varying the degree of clathrin labeling between ~5% and ~85% (Supplementary Fig. 6a). Indeed, results were characteristic of clustered molecules (Fig. 3a).

To test our method on membrane-protein nanodomains, we analyzed the clustering of LFA-1, an integrin specific to immune cells, which was reported to cluster upon activation. In this case, too, we found pronounced deviation from a random distribution (Fig. 3b and Supplementary Fig. 6b). As negative control, we confirmed previous PALM data that glycosylphosphatidylinositol (GPI)-anchored mGFP does not form substantial clusters in the plasma membrane (Fig. 3c and Supplementary Fig. 6c). In this case, we titrated AF647-labeled GFP–Trap to detect the mGFP–GPI.

The major asset of our method, however, is its robustness against the erroneous detection of nanoclusters due to overcounting. Specifically, several reports have proposed the existence of nanoscopic clusters of signaling components in T cells. One example relates to Lck, the key kinase responsible for early T cell signaling, which was found to be clustered in domains with diameters of 100 nm. Hence, we used our method to revisit Lck nanoclustering on the T cell plasma membrane. At first inspection, dSTORM data for endogenous Lck labeled with an AF647-conjugated antibody might seem indicative of a nonrandom protein distribution (Supplementary Fig. 5b). Label-titration analysis, however, yielded results consistent with a homogeneous protein distribution, and so did not support the presence of Lck nanoclusters (Fig. 3d and Supplementary Fig. 6d).

If nanocluster formation does not depend directly on the protein of interest, our method is also compatible with PALM if we utilize

Figure 3 | Cluster analysis of different proteins in the cellular plasma membrane. Label-density variation for different membrane proteins yielded characteristic normalized $\rho/\rho_0$ curves for each protein. (a–d) Cells were stained at different label concentrations and imaged via dSTORM: Jurkat T cells were labeled with anti-clathrin-HC–AF647 (a) or anti-LFA-1–AF647 (b). CHO cells expressing GPI–mGFP were labeled with GFP–Trap–AF647 (c). Jurkat T cells were labeled with anti-Lck–AF647 (d). (e,f) PALM was carried out at different expression levels of GPI–mEos3.2 in CHO cells (e) or Lck–mEos3.2 in JCaM1.6 T cells (f). Each data point represents a single cell; up to five independent experiments were pooled for each graph. The red lines indicate the reference curve for a random distribution. Cartoons depict the topology of each sampled protein and the site of labeling.
natural variations in the expression levels between individual cells. For this, we analyzed the clustering behavior of ectopically overexpressed GPI-anchored fluorescent protein mEOS3.2 and an mEOS3.2 chimera of Lck. Consistent with the dSTORM experiments, we found considerable heterogeneity in the single-molecule localizations (Supplementary Fig. 7), but no clustering of the two constructs (Fig. 3e,f). In the case of Lck, T cell activation via antibody-coated surfaces also did not affect the random distribution (Supplementary Fig. 8). Finally, we reconstructed the putative diffraction-limited image from a Lck–mEOS3.2 super-resolution image and compared it with the according diffusion-limited protein distribution obtained by direct antibody labeling of Lck–mEOS3.2 on the same cell; strikingly, there was no similarity between the two images (Supplementary Fig. 7), demonstrating the extent of the problem.

In summary, our method complements current experimental strategies to characterize protein organization at the nanoscale. It can detect nanoclustering over a broad range of parameters found in cells. Importantly, the approach is insensitive to common imaging artifacts inherent to single-molecule-localization-based super-resolution techniques.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
F.B., K.L., A.M.A. and K.S. performed experiments; F.B. analyzed the data; A.M.A. and K.L. wrote the code for the analytical methods; A.M.A. wrote the code for the simulations; M.F. implemented Ripley’s K analysis; H.S. and J.W. provided cell lines and materials; F.B., A.M.A. and G.J.S. developed the analytical method and conceived the simulations; F.B. and G.J.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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**Online Methods**

**Cell culture, DNA constructs, antibodies and reagents.** Jurkat E6-1 T cells and Chinese Hamster Ovary (CHO) cells were from the American Type Culture Collection. Lck-deficient JCaM1.6 T cells were from the European Collection of Authenticated Cell Cultures. All cell lines were regularly tested to exclude *Mycoplasma* contamination. Jurkat cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich), CHO cells in DMEM/HAM's F-12 medium (Lonza); media were supplemented with 10% FBS, 2 mM L-glutamine, 1,000 μM penicillin–streptomycin (all from Sigma-Aldrich) and cells were grown in a humidified atmosphere at 37 °C and 5% CO2. For microscopy, we used an imaging buffer consisting of HBSS (Lonza) supplemented with 2% FBS. Fluorescent proteins (mEOS3.2, mGFP) were fused to the C terminus of Lck20 or to the N terminus of the GPI-anchor signal. OKT3; catalog number SAB4700041; 10^1:10 serial dilutions were used. CD3ε antibodies were from Sigma-Aldrich if not noted otherwise. Sample preparation. For imaging, cells were seeded on surface-coated LabTek chamber slides in imaging buffer at 37 °C for 5–10 min. Surfaces were prepared by incubating slides with 50 μg ml^-1^ fibronectin for 30 min at 21 °C. To activate Jurkat cells, slides were coated with 10 μg ml^-1^ anti-CD3ε for 2 h at 37 °C. For experiments under activating conditions, cells were incubated on anti-CD3ε-coated glass slides for 10 min at 37 °C. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min at 21 °C. For antibody staining, cells were permeabilized with 0.1% (wt/vol) Triton X-100 for 10 min at 21 °C and unspecific binding sites were blocked by incubation with blocking buffer consisting of HBSS containing 3% BSA (wt/vol) for 30 min at 21 °C. Samples were incubated with antibodies diluted in blocking buffer at varying concentrations for 2 h at 21 °C. Finally, cells were washed with HBSS and fixed again with 4% PFA for 10 min at 21 °C to avoid unbinding of antibodies during dSTORM measurements.

**Soft lithography.** Microstructured surfaces were made following a protocol adapted from Schwarzenbacher et al. Polydimethylsiloxane (PDMS)-based polymers with 200 nm pillars (EV Group) were incubated with 50 μg ml^-1^ streptavidin in PBS for 15 min and dried with N2. Immediately after drying, the stamp was placed onto a plasma-cleaned glass coverslip (Menzel Gläser, Cover Slips #1) and incubated for 60 min. After removal of the stamp, the coverslip was incubated with biotinylated mouse IgG for 15 min at a concentration of 10 μg ml^-1^ in PBS with 1% BSA (wt/vol) and washed extensively with PBS. Finally, AF647-conjugated goat anti-mouse antibody was titrated at different concentrations (1 μg ml^-1^ and 4 sequential 1:10 serial dilutions were used), incubated for 15 min and washed with PBS. All steps were performed at 21 °C.

**Microscopy setup.** All experiments were performed on a modified Zeiss Axiovert 200 inverted microscope equipped with a 100× oil-immersion objective (Zeiss Apochromat NA1.46). The setup was equipped with a 640 nm diode laser (Toptica iBeam smart 200 mW), a 532 nm diode-pumped solid state (DPSS) laser (Spectra physics Millennia 6s) and a near UV light 405 nm ion laser (Coherent Innova 90C). Intensity modulation and timings were controlled either directly or with an acousto-optic modulator (AO) using custom-written Labview software. Laser lines were overlaid with an OIS Galaxy beam combiner (Coherent). Emission light was filtered using appropriate filter sets (Chroma) and recorded on an IXON DU 897-DV EM-CCD camera (Andor). Multicolor imaging was performed using an emission light splitter (Optosplit; Cairn Research) adapted to the spectral characteristics of the used fluorophores. Total internal reflection fluorescence (TIRF) illumination was achieved by shifting the excitation beam in parallel to the optical axis with a mirror mounted on a motorized movable table. For ratiometric Fura-2 imaging, we used a polychromatic Xenon light source combined with a monochromator (polychrome V; TILL photonics) that provided light at 340 nm and 380 nm.

**Ratiometric Ca^{2+} measurements.** T cell activation was quantified with Fura-2, AM. Cells were incubated with 5 μg ml^-1^ Fura-2, AM in supplemented RPMI medium for 15 min at 21 °C, washed twice in imaging buffer and kept on ice until imaging. For each experiment, cells were resuspended in imaging buffer at 5 × 10^5^ cells/ml and 5 μl were deposited close to the surface of an imaging chamber, which was mounted on the microscope at 21 °C. Image acquisition began immediately, recording 1,000 frames at 1 Hz. Image stacks were processed and analyzed using ImageJ.

**Quantitative antibody binding assay.** In order to quantify the degree of antibody binding, we labeled fixed and permeabilized cells with different concentrations of AF647-conjugated antibodies. Antibody binding was measured as mean fluorescence per pixel under TIRF illumination using the 640 nm laser line. For each dilution step 15–20 cells were imaged. Average background-corrected fluorescence values were fitted to the equation $[AB] = [B] × (A_{max})/(K_D + [B])$ assuming first-order binding.
where \([AB]\) denotes the surface density of bound antibody, \([B]\) is the antibody concentration in solution, \([A_{\text{max}}]\) stands for the fitted total surface density of antibody binding sites and \(K_d\) is the dissociation constant.

**Super-resolution microscopy and image reconstruction.** PALM experiments were carried out in imaging buffer. For excitation of mEOS3.2 we used the 532 nm laser line. The 405 nm laser constantly illuminated the sample in order to continuously switch new molecules. For dSTORM measurements, we used previously published switching buffer conditions optimized for AF647 (ref. 22): PBS (pH 7.4) was supplemented with 10% glucose, 0.5 mg ml\(^{-1}\) glucose oxidase, 40 µg ml\(^{-1}\) catalase and 50 mM cysteamine. In dSTORM experiments with AF647, the majority of fluorophores was first transferred into a nonfluorescent dark state using high-power 640 nm laser illumination. Then, single molecules were imaged at 640 nm excitation at lower power, keeping the 405 nm laser continuously on in order to switch molecules back to a fluorescent state. Both PALM and dSTORM images were recorded as stacks of 10,000 frames at 100 Hz. Stroboscopic illumination protocols were applied with 3 ms illumination time and 7 ms delay between consecutive images. Single-molecule signal localization and image reconstruction was carried out with the open-source ImageJ plugin ThunderSTORM\(^{24}\). Stringent post-processing parameters were chosen to discard signals with low localization precision. On average, we obtained localization errors of \(\sigma = 20\) nm for AF647 and \(\sigma = 30\) nm for mEOS3.2. Merging of localizations was performed with a grouping radius adjusted to the average localization precision of the respective fluorophores. If not specified otherwise, we used 50 frames off-time. No drift correction was applied. Ripley’s K analysis\(^{25}\) was carried out using custom-written Matlab code.

**Quantitative cluster analysis.** Clusters were identified using custom-written Matlab code (Supplementary Software and Supplementary Note). Each localization within a region of interest was represented by a 2D Gaussian function with fixed \(\sigma = 35\) nm, centered at the recorded position. By summing up the Gaussian peaks, localizations in close proximity to each other resulted in higher peaks than well-separated localizations (Supplementary Fig. 2a). Binary-cluster masks were obtained by applying a threshold (\(\text{thr} = 2.5\)) that was chosen after visual inspection of cluster masks at different thresholds (Supplementary Fig. 2c). The cluster masks were then used to classify localizations as clustered or not clustered. To reduce the overestimation of cluster sizes, 2D Gaussians were set to zero beyond a radius of \(2\sigma\) (Supplementary Fig. 2b). Finally, the total cluster area \((A_{\text{tot}})\) within the region of interest \((A)\), as well as the number of localizations inside \((\#_{\text{int}})\) and outside of clusters \((\#_{\text{out}})\), was determined. This allowed us to calculate the density of localizations per cluster area \((\rho = \#_{\text{int}}/A_{\text{tot}})\) and the relative clustered area per image \((\eta = A_{\text{tot}}/A)\).

**Simulation of randomly distributed molecules.** Molecules were distributed randomly as \(xy\) coordinates on a 12,800 x 12,800 nm sized image. Each molecule was assumed to yield multiple localizations because of blinking with an average of seven observations per molecule drawn from an exponential distribution. Localizations were scattered around the \(xy\) position of each molecule, following a 2D Gaussian probability distribution defined by \(\sigma = 40\) nm. To account for variations in the labeling density, we simulated different numbers of molecules over a broad range, keeping all other parameters constant. Random noise consisting of 300 randomly distributed single localizations was also included in each image. In Supplementary Figure 3, we varied the blinking statistics and the analysis threshold. In Supplementary Figure 4a,b, we tested the effect of residual diffusion of molecules during image acquisition. To simulate single-molecule trajectories, we assumed a diffusion coefficient \(D = 1.1 \times 10^{-7} \mu m^2 \text{s}^{-1}\) (determined experimentally for Lck–mEOS3.2 in fixed JCaM1.6 cells, not shown) as well as a specified image acquisition rate and total number of recorded frames. Localizations were then randomly distributed along each trajectory, using the blinking statistics and the localization errors as above. Finally, in Supplementary Figure 4c,d we included the indicated small fractions of molecules with different blinking statistics of 100 localizations per molecule on average (exponentially distributed).

**Determination of the standard curve for randomly distributed localizations and \(\rho_0\).** We simulated data for randomly distributed molecules under different conditions (variations in blinking statistics and thresholds, see Supplementary Fig. 3). Each data set could be fitted well with a polynomial of the form \(\rho = \rho_0(1 + \alpha \times \eta^\beta)\) with the constant coefficient \(\alpha = 1.4\) and the power \(\beta = 4\); \(\rho_0\) turned out to depend on various imaging and analysis parameters. We normalized the data sets with respect to \(\rho_0\), yielding the average reference curve for randomly distributed localizations \(\rho/\rho_0 = 1 + 1.4 \times \eta^4\). Clustered distributions could also be fitted well with this polynomial, albeit with different values for \(\alpha\) and \(\beta\). We hence used such fits for determining \(\rho_0\) in all simulations and experiments, which ultimately allowed for plotting \(\rho/\rho_0\) versus \(\eta\).

**Simulation of clustered molecules.** To simulate clusters, molecules were placed randomly according to a uniform distribution within circles around cluster centers, which themselves were distributed uniformly within the field of view. The number of molecules per cluster was varied following a normal distribution with an s.d. of 33% of the mean number of molecules per cluster. Additionally, we added molecules that were uniformly distributed over the whole field of view (25% of the total number of clustered molecules) in order to account for unspecific binders or monomeric blinking molecules. All simulated molecules followed the same blinking statistics (average of seven localizations per molecule). Random noise consisting of 300 uniformly distributed single localizations was further included in each image. In order to account for variations in the labeling density, we simulated different numbers of molecules per cluster over a broad range, keeping all other parameters constant.

**Simulation of pentamers.** Pentamers were simulated as randomly distributed 5-mer centers. Each pentamer center was filled with \(n\) molecules following a binomial distribution with the probability \(p\). Hence, \(p\) corresponds to the labeling efficiency in a titration experiment. As in the other simulations, each
molecule was assumed to yield multiple localizations because of blinking with an average of seven observations per molecule drawn from an exponential distribution. Random noise consisting of 300 randomly distributed single localizations was also included in each image. Additionally, we added different amounts of molecules that were uniformly distributed over the whole field of view in order to account for unspecific binders or monomeric blinking molecules. For all simulations of small oligomers, the total number of molecules was kept constant (50 µm⁻²), only p was varied.

**Code availability.** We provide the code used for data analysis as Matlab files. See Supplementary Software and Supplementary Note for details.

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