Supplementary Information (SI) for Nanoscale.

Supplementary information
nanoFeatures

Cristina Izquierdo-Lozano, Niels van Noort, Marrit M.E. Tholen, Stijn Van Veen, Francesca Grisoni and Lorenzo Albertazzi

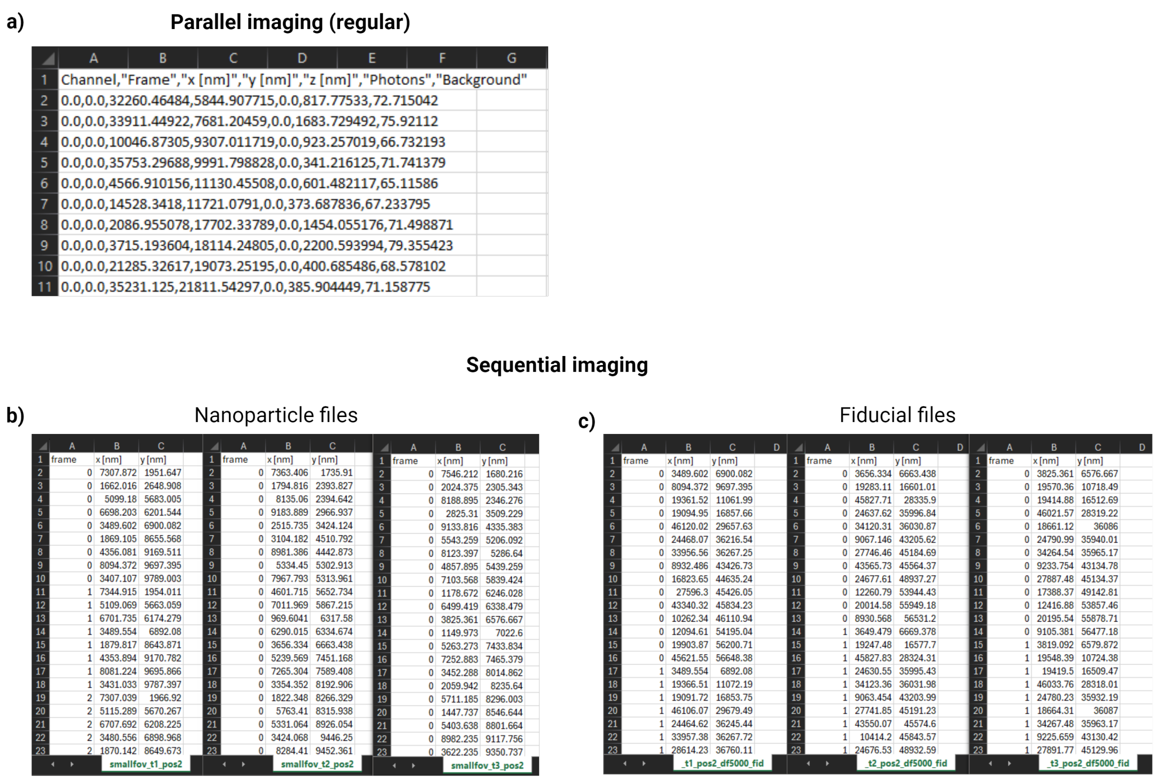

Figure 1: Raw data example of nanoparticles imaged in super-resolution microscopy (DNA-PAINT), **a)** all color channels imaged in the same acquisition and **b)** three different color channels imaged sequentially, which has to include **c)** the corresponding files containing the fiducial localizations.

Table 1: Description of the input parameters required by *nanoFeatures*.

Parameter	Description
Filters tab	
Input file	Files(s) to be analyzed by *nanoFeatures*.
Input type	Microscope or software the files were obtained from. Current options are Nikon (N-STORM), Oxford Nanoimager (ONI) and ThunderSTORM (ImageJ plugin).
Channel alignment	Checkbox to align the different channel colors in the case of exchange PAINT (sequentially imaging each color instead of simultaneously). This filter is based on fiducials, then the user would need to first input the different files (one per color) and then nanoFeatures will ask to input the files containing the fiducial localizations. This filter doesn't admit batch analysis.

Silhouette metric	Checkbox to calculate the Silhouette coefficient for each cluster found by DBSCAN. This metric can be used while optimizing the parameters and measures how well the nanoparticles are clustered. The closer to 1 the better. The calculation of this metric is computationally expensive if the images are too dense.
qPAINT	Checkbox to perform the qPAINT analysis. That is, to calculate the actual number of molecular targets based on the number of localizations and binding kinetics.
Parameters tab	
DBSCAN clustering	
Scanning diameter	Diameter, in nanometers, used by DBSCAN to follow the density of points. Generally, it is the same as the size of the nanoparticles in the image. However, if these are too dense, it is better to set a smaller diameter.
Minimum points	Minimum number of localizations that have to be contained in the scanning diameter to be considered a full cluster or part of a bigger cluster. Relates to the nanoparticle's density.
Filtering	
Maximum aspect ratio	Maximum ellipticity of the nanoparticles, being 1 a perfect sphere.
Desired aspect ratio	Theoretical shape of the nanoparticles being analyzed.
Min. inter-cluster distance	Minimum separation, in nanometers, between nanoparticles. Generally, at least the same distance as their diameter.
Maximum points	Maximum number of points for a cluster to be considered a nanoparticle.
Particle size check	
Radius low limit	Lowest radius size, in nanometers, for a cluster to be considered a nanoparticle.
Radius high limit	Highest radius size, in nanometers, for a cluster to be considered a nanoparticle..
Radius threshold	Fraction of the localizations within the radius. Sometimes some isolated localizations in a cluster might be further away from the actual radius and would be better to exclude them.
Fiducial alignment	
Maximum inter-fiducial distance	Maximum separation, in nanometers, for fiducials in different channels to be considered the same. Avoids mismatching.
qPAINT tab	
Options	
Filter non-specific clusters	Checkbox to filter out clusters that are not present for at least 50% of the imaging time.
Initial frames cut	Number of frames to cut from the beginning of the imaging movie.
Frames threshold to merge	Allowed gap in between binding events for them to be still considered consecutive.
Number of channels	Number of laser channels (colors) present in the analyzed images.
Exposure time	Acquisition's exposure time, in milliseconds.
Give information per channel	
(k_{on})	Association constant for each docking-imager pair, determined experimentally.
(C_i)	Imager concentration.
Graphs tab	
Loc/NP	Number of localizations per nanoparticle histogram.
NP diam	Nanoparticle's diameter (size) histogram.
Diam/locs	Nanoparticle's localizations compared to their size scatterplot.
NP Td	True dark time per nanoparticle histogram.

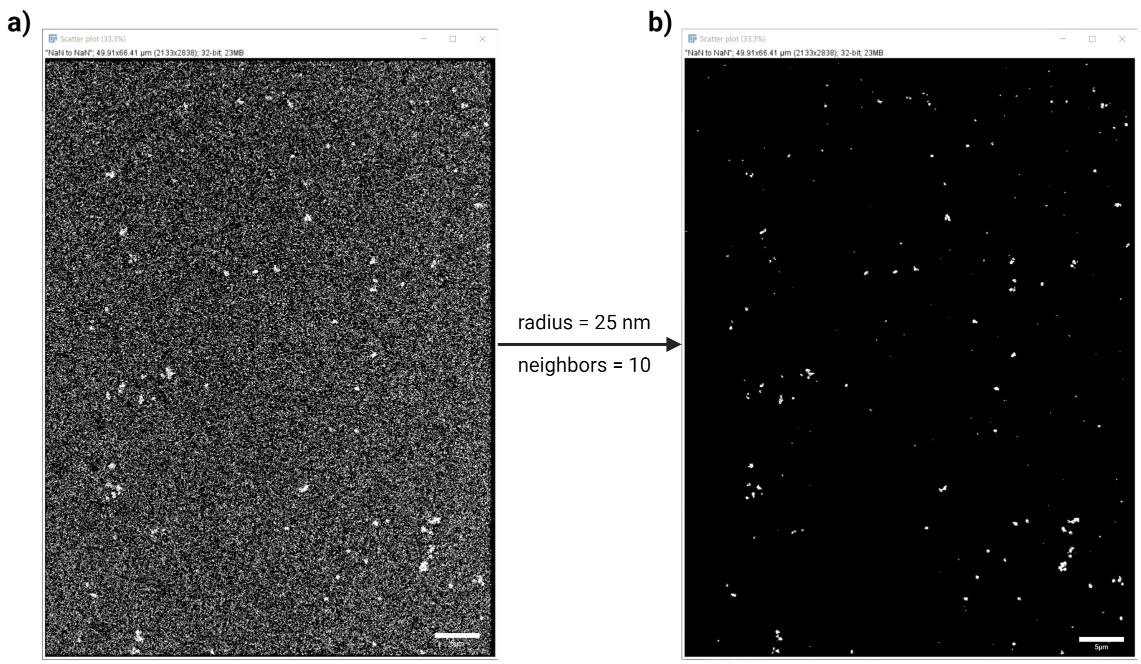

Figure 2: Before and after example of a density-filtered super-resolution microscopy image. In this case, an image of 300 nm nanoparticles was filtered by density with a radius of 25 nm and 10 minimum number of neighbors. Scale bar: 5µm.

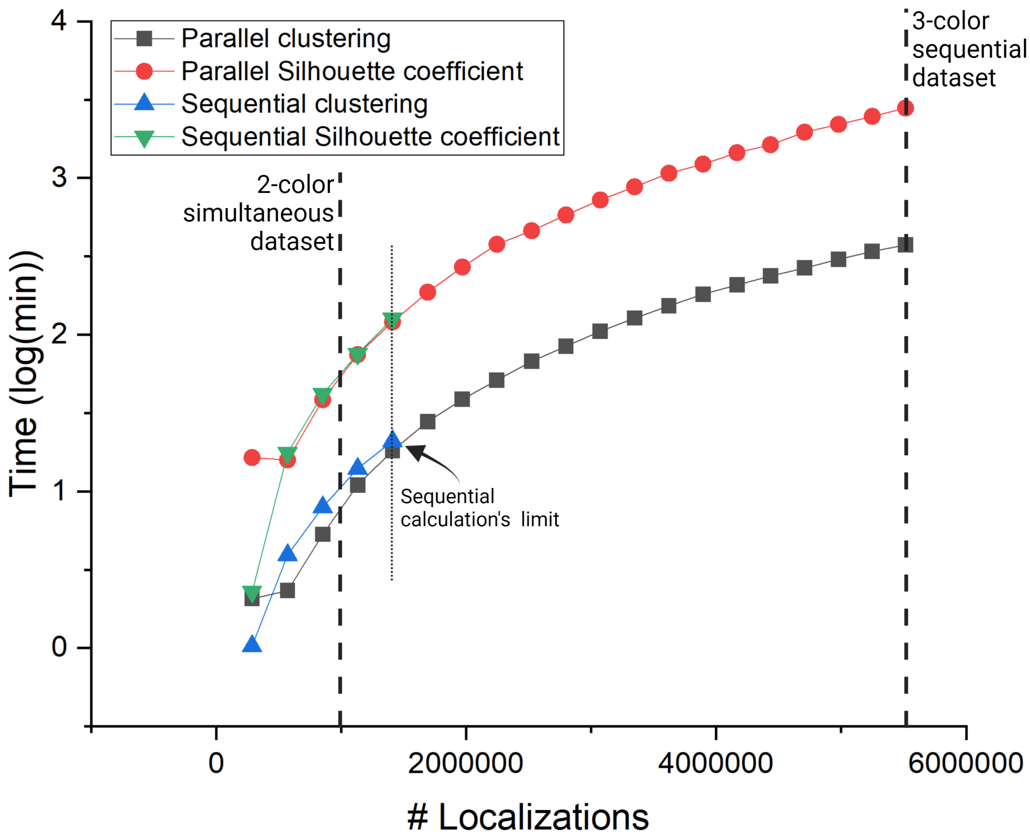

Figure 3: Time analysis of the *nanoFeatures* execution, comparing between parallel and sequential execution of the nine sections and with or without the Silhouette analysis. Note that sequential analysis is only apparent for the lower number of localizations due to rapidly increasing computational costs and memory requirements, resulting in the execution breaking.

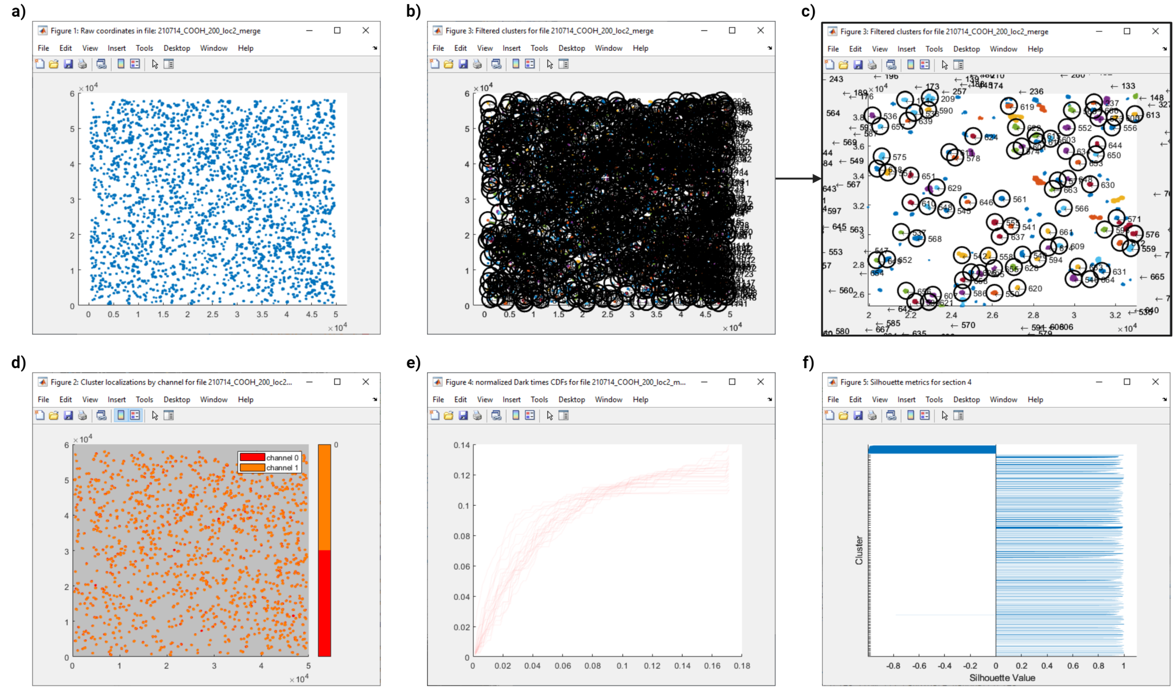

Figure 4: Example of *nanoFeatures* output figures. In this case, for DNA-PAINT dual-color 200nm nanoparticles. **a**) Raw coordinates, plotted directly from the localization list input by the user. **b)** Identified nanoparticles by DBSCAN, after going through the quality filters and **c)** the zoom in. Colored clusters are the ones identified by DBSCAN and the circled clusters are selected by the quality filters. **d)** Selected nanoparticles colored based on the channel each localization was found in. **e)** normalized Cumulative Distribution Function (CDF) of each nanoparticle's dark times. **f)** Silhouette metric for each of the identified clusters by DBSCAN, from -1 (not clustered) to 1 (perfectly separated cluster). Note that the -1 group corresponds to the background localizations, discarded by DBSCAN. Group ID (given by DBSCAN) can be found by clicking in any of the localizations colored in *nanoFeature*'s figure 3 (**b)** in this figure).

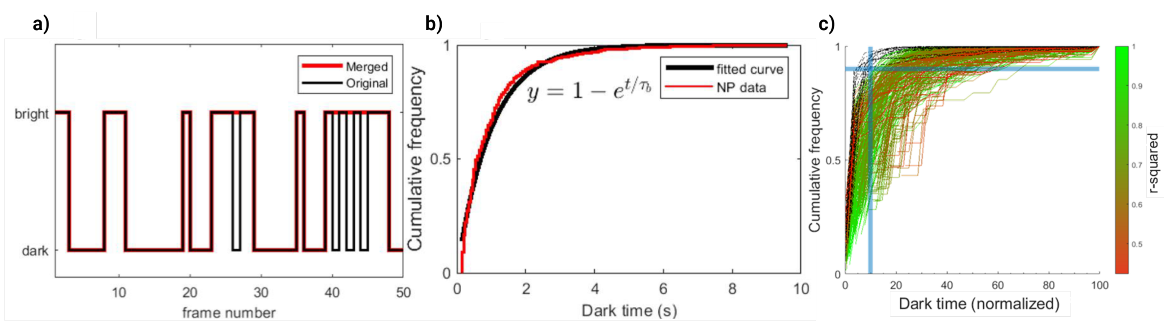

Figure 5: qPAINT filter **a)** The first 50 frames of a binary particle time trace (black). Consecutive binding events (when the particle is 'bright') with a gap of up to three frames are merged into a single event, resulting in the merged time trace shown in red. **b)** Example of the dark time CDF for a cluster (red) superimposed with its fitted curve (black), computed using equation 1. **c)** All normalized CDFs for a measurement, colored by their R-squared value from a bad fit (red) to a good fit (green). CDFs with an unexpected shape (black) that go above the threshold (blue cross) are filtered out.

Table 2: Description of the features obtained by *nanoFeatures*.

Feature	**Description**
Diameter	The diameter of the cluster as determined by ellipse fit-ting from *nanoFeatures*.
Aspect ratio (shape)	The aspect ratio (starting at one) of the ellipse fitted over the cluster by *nanoFeatures*.
x-coordinate	The x-coordinate of the cluster center determined by *nanoFeatures*. This can be used for the reconstruction of cluster centers.
y-coordinate	The y-coordinate of the cluster center determined by nanoFeatures. This can be used for the reconstruction of cluster centers.
Cluster localizations	The total number of localizations in the cluster.
For each channel	
Channel localizations	The number of localizations of the cluster that are in the corresponding channel.
True mean dark time	The mean dark time of the localizations in the corresponding channel as determined by qPAINT analysis through CDF fitting.
R-squared	The goodness of fit of the CDF fitting expressed in R-squared.
Mean dark time	The mean value of the dark times in the corresponding channel calculated during qPAINT calculations.
Median dark time	The median value of the dark times in the corresponding channel calculated during qPAINT calculations.
SD dark time	The standard deviation of the dark times in the corresponding channel calculated during qPAINT calculations.
Mean bright time	The mean value of the bright times in the corresponding channel calculated during qPAINT calculations.
Median bright time	The median value of the bright times in the corresponding channel calculated during qPAINT calculations.
SD bright time	The standard deviation of the bright times in the corresponding channel calculated during qPAINT calculations.
Target count	The number of binding sites for the corresponding channel as determined by qPAINT calculations using the true mean dark time and user-set parameters for acquisition frame rate and association constant (k_{on}).

Table 3: Comparison of *nanoFeatures* to other available softwares to analyse super-resolution microscopy data. More open microscopy software packages (for SMLM and other techniques), can be found in this GitHub repository: `https://github.com/HohlbeinLab/OpenMicroscopy/blob/main/src/OM_Software.md` and `https://srm.epfl.ch/srm/software/index.html`

Software	**Input data**	**Output data**	**Visualization**	**Clustering**	**Analysis & features**
nanoFeatures	Localization files from diverse SMLM formats	Single-particle features (see supplementary Table 2)	Yes (scatter plots)	Yes (DBSCAN)	localization merging, quality control, image alignment, quantitative analysis, and qPAINT.
SMAP [1]	Image, metadata and localization files from diverse SMLM formats	Processed images and localization files, plus custom-made analysis outputs.	Yes (renders and scatter plots)	Yes (diverse)	Image post-processing (drift correction, localization merging...), rendering, ROI manager, and custom-made plugins (statistics, counting, tracking...)
Bayesian cluster identification in SMLM data [2]	Localization files from ThunderSTORM	Clustering proposals	Yes (scatter plots)	Yes (Bayesian)	—
PYME [3]	Localization files (csv)	custom-made analysis outputs.	Yes (3D renders)	Yes	Quality control, artifact correction, image reconstruction and quantitative analysis.
Mars [4]	Image formats	Processed images, features, and custom-made analysis outputs based on Fiji	Yes (renders)	No	Image processing, classification, filtering, and interactive data exploration.
ThunderSTORM [5]	Raw SMLM data	Processed localization files and images	Yes (images and scatter plots)	No	Raw data processing, post-processing, and visualization and simulations.
Picasso [6]	Raw SMLM data, localization files and metadata.	Processed images and localizations, and classification	Yes (renders)	Yes (diverse)	DNA-PAINT simulations, localize, filter and post-processing

References

[1] Jonas Ries. "SMAP: a modular super-resolution microscopy analysis platform for SMLM data". en. In: *Nature Methods* 17.9 (Sept. 2020). Number: 9 Publisher: Nature Publishing Group, pp. 870–872. ISSN: 1548-7105. DOI: 10.1038/s41592-020-0938-1.

[2] Patrick Rubin-Delanchy et al. "Bayesian cluster identification in single-molecule localization microscopy data". In: *Nature methods* 12.11 (2015), pp. 1072–1076.

[3] Zach Marin et al. "PYMEVisualize: an open-source tool for exploring 3D super-resolution data". In: *Nature methods* 18.6 (2021), pp. 582–584.

[4] Nadia M Huisjes et al. "Mars, a molecule archive suite for reproducible analysis and reporting of single-molecule properties from bioimages". In: *Elife* 11 (2022), e75899.

[5] Martin Ovesnỳ et al. "ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging". In: *Bioinformatics* 30.16 (2014), pp. 2389–2390.

[6] Joerg Schnitzbauer et al. "Super-resolution microscopy with DNA-PAINT". en. In: *Nature Protocols* 12.6 (June 2017). Number: 6 Publisher: Nature Publishing Group, pp. 1198–1228. ISSN: 1750-2799. DOI: 10.1038/nprot.2017.024. (Visited on 01/31/2024).