

Supplementary information *nanoFeatures*

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a) Parallel imaging (regular)

	A	B	C	D	E	F	G
1	Channel,"Frame","x [nm]","y [nm]","z [nm]","Photons","Background"						
2	0.0,0.0,32260.46484,5844.907715,0.0,817.77533,72.715042						
3	0.0,0.0,33911.44922,7681.20459,0.0,1683.729492,75.921112						
4	0.0,0.0,10046.87305,9307.011719,0.0,923.257019,66.732193						
5	0.0,0.0,35753.29688,9991.798828,0.0,341.216125,71.741379						
6	0.0,0.0,4566.910156,11130.45508,0.0,601.482117,65.11586						
7	0.0,0.0,14528.3418,11721.0791,0.0,373.687836,67.233795						
8	0.0,0.0,2086.955078,17702.33789,0.0,1454.055176,71.498871						
9	0.0,0.0,3715.193604,18114.24805,0.0,2200.593994,79.355423						
10	0.0,0.0,21285.32617,19073.25195,0.0,400.685486,68.578102						
11	0.0,0.0,35231.125,21811.54297,0.0,385.904449,71.158775						

Sequential imaging

b) Nanoparticle files

	A	B	C		A	B	C		A	B	C	
1	frame	x [nm]	y [nm]		1	frame	x [nm]	y [nm]	2	frame	x [nm]	y [nm]
2	0	7307.872	1951.647		2	0	7363.406	1735.91	3	0	7546.212	1680.216
3	0	1662.016	2648.908		3	0	1794.816	2393.827	4	0	2024.375	2305.343
4	0	5099.18	5683.005		4	0	8135.06	2094.642	5	0	8188.895	2346.276
5	0	6698.203	6201.544		5	0	9183.889	2966.937	6	0	2825.91	3599.229
6	0	3489.602	6900.082		6	0	2515.735	3424.124	7	0	9133.816	4335.383
7	0	1869.105	8658.568		7	0	3104.182	4510.792	8	0	8543.259	5206.092
8	0	4356.081	9169.511		8	0	8981.396	4442.873	9	0	8123.397	5286.64
9	0	8094.372	9697.395		9	0	5334.45	5302.913	10	0	4857.895	5439.259
10	0	3407.107	9789.003		10	0	7967.793	5313.961	11	0	7103.568	5839.424
11	1	7344.915	1954.011		11	0	4601.715	5652.734	12	0	1178.672	6246.028
12	1	5109.069	5663.059		12	0	7011.969	5867.215	13	0	6499.419	6338.479
13	1	6701.735	6174.279		13	0	969.6041	6317.58	14	0	3825.361	6576.667
14	1	3489.554	6892.08		14	0	6290.015	6334.674	15	0	1149.973	7022.6
15	1	1879.817	8643.871		15	0	3656.334	6963.438	16	0	5263.273	7433.834
16	1	4353.894	9170.762		16	0	3239.669	7431.168	17	0	7252.885	7465.379
17	1	8081.224	9636.866		17	0	7265.304	7889.408	18	0	3452.288	8014.862
18	1	3431.033	9787.397		18	0	3354.352	8192.906	19	0	2059.942	8235.64
19	2	7307.039	1966.92		19	0	1822.348	8266.329	20	0	5711.185	8296.003
20	2	5115.289	5670.267		20	0	5763.41	8315.938	21	0	1447.737	8546.644
21	2	6707.692	6208.225		21	0	5331.064	8926.054	22	0	5403.638	8901.664
22	2	3480.556	6898.968		22	0	3424.068	9446.25	23	0	6882.235	9117.756
23	2	1870.142	8649.673		23	0	8284.41	9452.361	24	0	3622.235	9350.737

c) Fiducial files

	A	B	C	D		A	B	C	D		A	B	C	D
1	frame	x [nm]	y [nm]		1	frame	x [nm]	y [nm]		1	frame	x [nm]	y [nm]	
2	0	3489.602	6900.082		2	0	3656.334	6663.438		2	0	3825.361	6576.667	
3	0	8094.372	9697.395		3	0	19283.11	16601.01		3	0	19570.36	10718.49	
4	0	19361.52	11061.99		4	0	45827.71	28335.9		4	0	19414.88	16512.69	
5	0	19094.95	16837.66		5	0	24637.62	35996.84		5	0	46021.57	28319.22	
6	0	46120.02	29657.63		6	0	34120.31	36030.87		6	0	18661.12	36086	
7	0	24468.07	36216.54		7	0	9067.146	43205.62		7	0	24790.99	35940.01	
8	0	33956.56	36267.25		8	0	27746.46	45184.69		8	0	34264.54	35965.17	
9	0	8932.486	43426.73		9	0	43565.73	45564.37		9	0	9233.754	43134.78	
10	0	16823.65	46035.24		10	0	24677.61	48937.27		10	0	27887.48	43134.37	
11	0	27596.3	45426.05		11	0	12260.79	53944.43		11	0	17388.37	49142.81	
12	0	43340.32	45834.23		12	0	20014.58	55849.18		12	0	12416.88	53857.46	
13	0	10262.34	46110.94		13	0	8930.568	56531.2		13	0	20195.54	55878.71	
14	0	12094.61	54195.04		14	1	3649.479	6669.378		14	0	9105.381	56477.18	
15	0	19903.87	56200.71		15	1	19247.48	16577.7		15	1	3819.092	6579.872	
16	0	45621.55	56648.38		16	1	45827.83	28324.31		16	1	19548.39	10724.38	
17	1	3489.554	6892.08		17	1	24630.55	35995.43		17	1	19419.5	16509.47	
18	1	19366.51	11072.19		18	1	34123.36	36031.98		18	1	46033.76	28318.01	
19	1	19091.72	16883.75		19	1	9063.454	43203.99		19	1	24780.23	35932.19	
20	1	46106.07	29679.49		20	1	27741.85	45191.23		20	1	18664.31	36087	
21	1	24464.62	36245.44		21	1	43550.07	45574.6		21	1	34267.48	35963.17	
22	1	33957.38	36267.72		22	1	10414.2	45843.57		22	1	9225.659	43130.42	
23	1	28614.23	36760.11		23	1	24676.53	48932.59		23	1	27891.77	45129.96	

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	File(s) to be analyzed by <i>nanoFeatures</i> .
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 <i>nanoFeatures</i> 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	
<i>DBSCAN clustering</i>	
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.
<i>Filtering</i>	
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.
<i>Particle size check</i>	
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.
<i>Fiducial alignment</i>	
Maximum inter-fiducial distance	Maximum separation, in nanometers, for fiducials in different channels to be considered the same. Avoids mismatching.
qPAINT tab	
<i>Options</i>	
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.
<i>Give information per channel</i>	
(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.

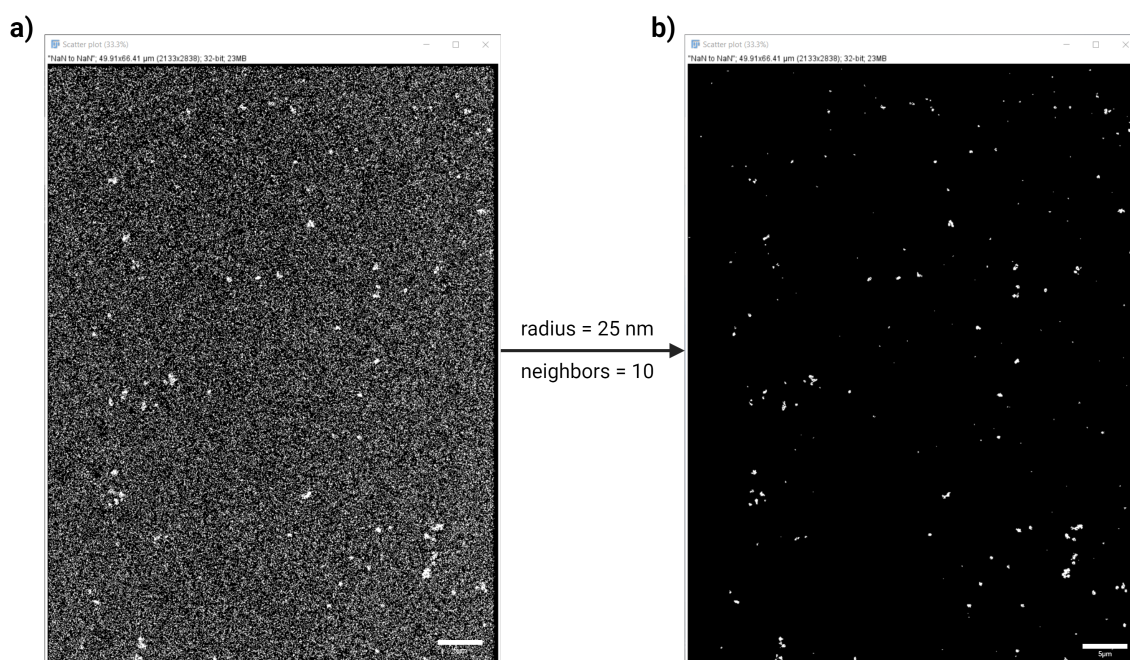


Figure 2: Before and after example of a density-filtered super-resolution microscopy image. In this case, an image of 300 nm nanoparticles was filtered by density with a radius of 25 nm and 10 minimum number of neighbors. Scale bar: 5 μm.

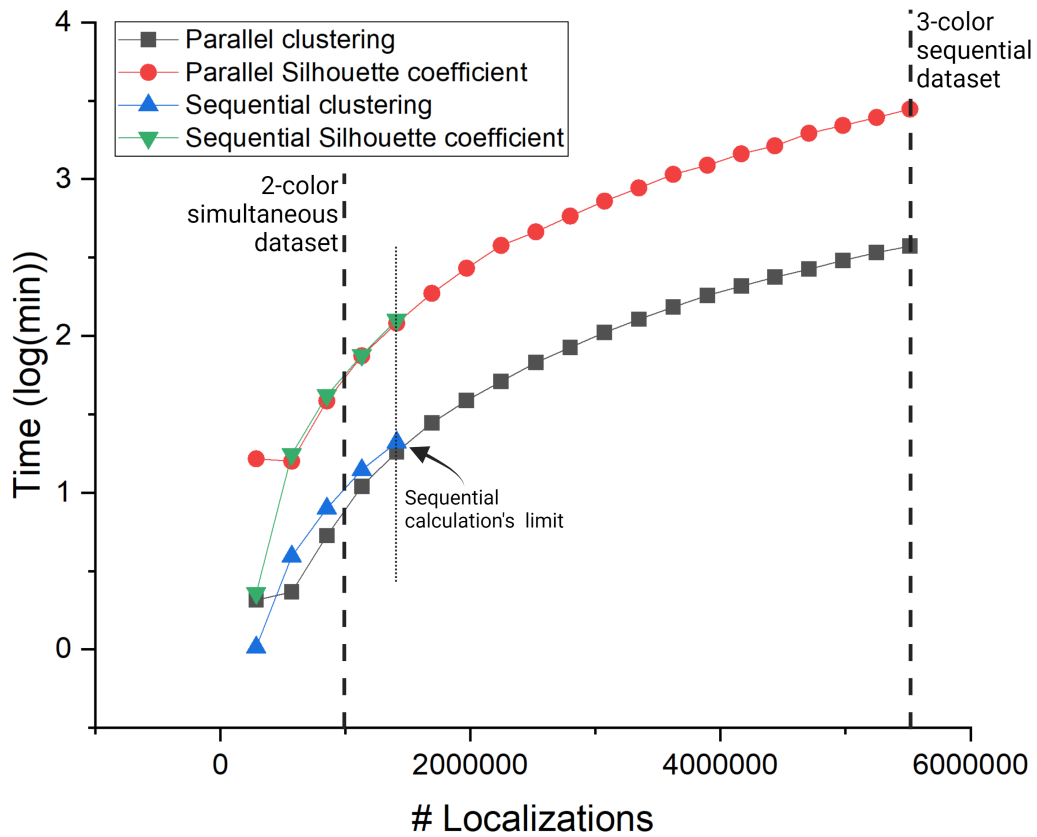


Figure 3: Time analysis of the *nanoFeatures* execution, comparing between parallel and sequential execution of the nine sections and with or without the Silhouette analysis. Note that sequential analysis is only apparent for the lower number of localizations due to rapidly increasing computational costs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

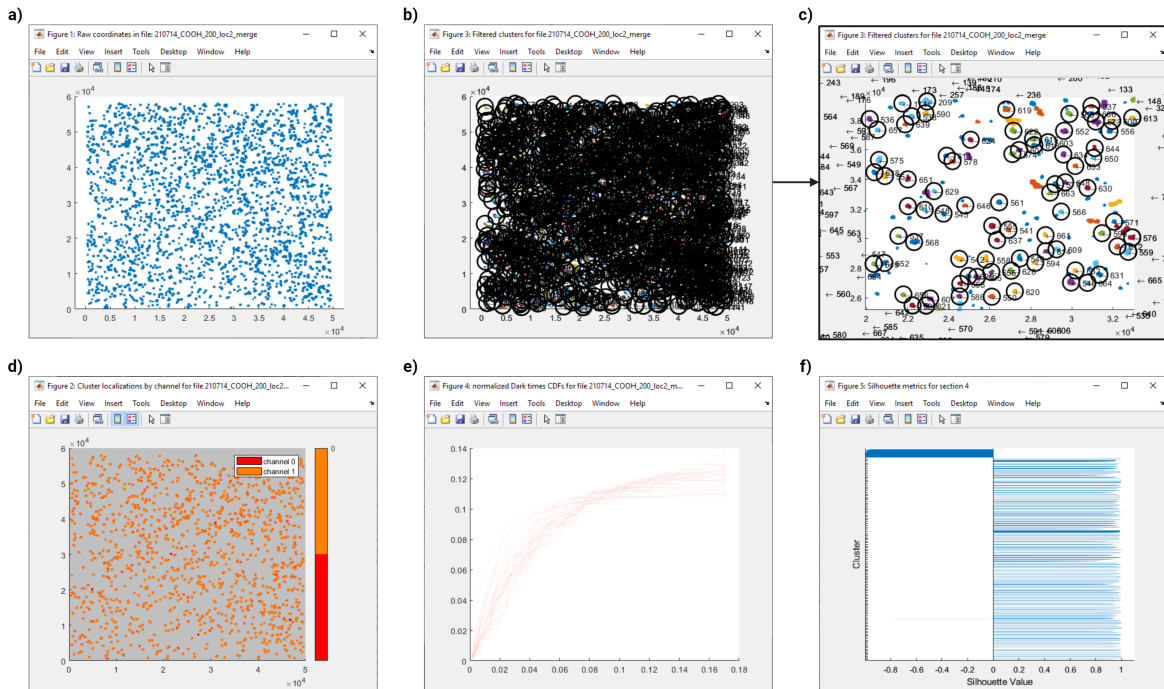


Figure 4: Example of *nanoFeatures* output figures. In this case, for DNA-PAINT dual-color 200nm nanoparticles. **a)** Raw coordinates, plotted directly from the localization list input by the user. **b)** Identified nanoparticles by DBSCAN, after going through the quality filters and **c)** the zoom 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).

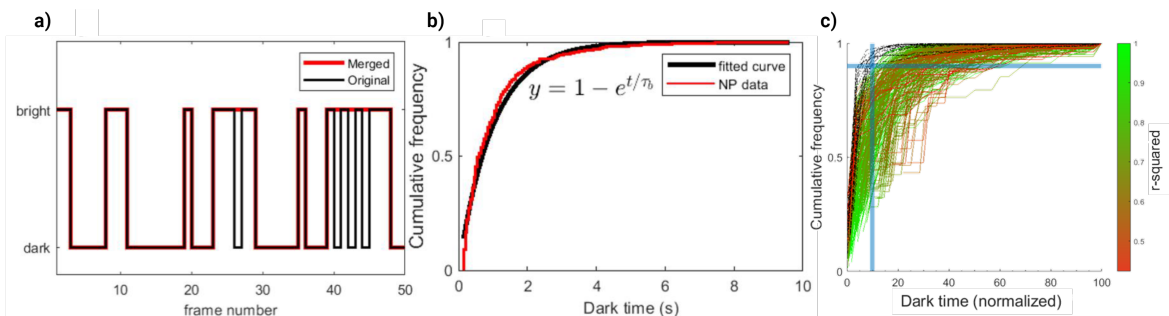


Figure 5: qPAINT filter **a)** The first 50 frames of a binary particle time trace (black). Consecutive binding events (when the particle is 'bright') with a gap of up to three frames are merged into a single event, resulting in the merged time trace shown in red. **b)** Example of the dark time CDF 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 <i>nanoFeatures</i> .
Aspect ratio (shape)	The aspect ratio (starting at one) of the ellipse fitted over the cluster by <i>nanoFeatures</i> .
x-coordinate	The x-coordinate of the cluster center determined by <i>nanoFeatures</i> . This can be used for the reconstruction of cluster centers.
y-coordinate	The y-coordinate of the cluster center determined by <i>nanoFeatures</i> . This can be used for the reconstruction of cluster centers.
Cluster localizations	The total number of localizations in the cluster.
<i>For each channel</i>	
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
<i>nanoFeatures</i>	Localization files from diverse SMLM formats	Single-particle features (see supplementary Table 2)	Yes (scatter plots)	Yes (DB-SCAN)	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 meta-data.	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.
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- [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).