nature research

Corresponding author(s): Axel Munk

Last updated by author(s): March 17, 2021

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The Abberior STED microscope was controlled by Imspector software version 0.14.13919, as distributed by the microscope manufacturer.
Data analysis	The data analysis and all algorithms are carefully described in in Section B and C in the paper and code can be found on https://github.com/ctameling/OTC and on Zenodo https://doi.org/10.5281/zenodo.4553632. To enhance computation we have used CPLEX v12.6.3.0, which is free for academic purposes.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data are avilable on zenodo https://doi.org/10.5281/zenodo.4553856 and https://github.com/ctameling/OTC . There is no restriction on data availability

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For the manually selected image section the sample size is n=10. For the randomly selected image sections the sample size is at least 100. Depending on the number of images being available it is precisly n=102 for figure 5, n=105, 100, 100, 100 for figure 6, n=102 for figure 7 and n=100 for suppl. fig 7. All corresponding confidence bands provide statistical accuracy with prob =0.95.
Data exclusions	No data have been excluded
Replication	For simulated data the number of replication is n= 100 pairs of images (random point setting) , for the remaining structures, 5 pairs, respectively. For STED/confocal images no replications have been performed as we do not extrapolate from the given measurements/images.
Randomization	does not apply as there were no different treatment groups.
Blinding	does not apply as there were no different treatment groups.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Anti-GFP [3E6] Mouse, Thermo Fisher Scientific, A11120, Lot: 1859591 Anti-Tom40, Rabbit, Peter Rehling, Georg-August-University Göttingen, Germany Anti-Mic60/ IMMT, Rabbit, Proteintech, 10179-1-AP, Lot: 2 Anti-Tom20, Rabbit, Santa Cruz Biotechnology, sc-11415, Lot: G0811 Anti-Tom20, Mouse, BD Bioscience 612278, Lot: 6210812 Anti-Mic27, Rabbit, SIGMA-ALDRICH, HPA000612, Lot: A96569 Anti-ATPB, Mouse, abcam, ab5432, Lot:GR3177231-2 Anti-GFP [JL-8] Mouse, Clontech/Takara (former BD Biosciences),632381, Lot: A5033481 Anti-Porin [16G9E6BC4], Mouse, MitoSciences (abcam), MSA08(ab110326), Lot: FKASHK Alexa Fluor® 594 goat anti-rabbit IgG (H+L), Thermo Fisher Scientific, A11037, Lot: 1777945 Alexa Fluor™ 594 goat anti-mouse IgG (H+L), Thermo Fisher Scientific, A11032, Lot: 1826426 AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson Immuno Research, Code: 111-005-144, Lot: 127874 Was coupled to Abberior STAR RED (NHS carbonate), Abberior,Cat. No. 1-0101-011-3, Lot: 2001201 SN AffiniPure Sheep Anti-Mouse IgG (H+L), Jackson Immuno Research, Code: 515-005-062, Lot: 128442 Was coupled to Abberior STAR RED (NHS carbonate), Abberior,Cat. No. 1-0101-011-3, Lot: 2001201 SN Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), Jackson Immuno Research, Code Number: 115-035-062, Lot: 121006
Validation	Anti-GFP [3E6], Mouse, Thermo Fisher Scientific, A11120: According to the manufacturer, the monoclonal antibody was used in 54 publications for immunofluorescence detection of GFP, e.g. by Xu et al., Elife (2017). Anti-Tom40, Rabbit, Peter Rehling, Georg-August-University Göttingen, Germany: The polyclonal antibody was used by Melin et al., Mol Cell Biol, 34, 18, 3473-3485, (2014). Anti-Mic60, Rabbit, Proteintech, 10179-1-AP: According to the manufacturer, the polyclonal antibody specificity is knock-out/ knock-

down validated. A specific mitochondrial labeling in immunofluorescence application is validated by the manufacturer.
Anti-Tom20, Rabbit, Santa Cruz Biotechnology, sc-11415: According to the manufacturer the polyclonal antibody was used in 145 publications, e.g. by Große et al., EMBO J 37,10, (2016).
Anti-Tom20, Mouse, BD Bioscience, 612278: According to the manufacturer, the monoclonal antibody was used in 3 publications, e. g. by Abe et al., Cell, 100,5, 551-560, (2000).
Anti-Mic27, Rabbit, SIGMA-ALDRICH, HPA000612: According to the manufacturer, the polyclonal antibody was used in 3 publications, e. g. by Weber et al., PloS one, 8(5):e63683. doi: 10.1371, (2013).
Anti-ATPB, Mouse, abcam, ab5432: According to the manufacturer, the monoclonal antibody was used in 13 publications, e. g. by Jans et al., Proc Natl Acad Sci U S A 110:8936-41 (2013).
Anti-GFP [JL-8] Mouse, Clontech/Takara (former BD Biosciences),632381: The monoclonal antibody was used e. g. by Lorenz et al., Nat Methods 3, 205–210 (2006).
Anti-Porin [16G9E6BC4], Mouse, MitoSciences (abcam), MSA08(ab110326): According to the manufacturer, the monoclonal antibody was used in 26 publications, e. g. by Niemi et al., Nat Commun 10:3197 (2019).
Comment to Anti-Mic60 antibody, Proteintech: According to the manufacturer, antibody reactivity was tested positive in human and mouse and cited for positive reactivity in Human, Mouse, Rat and Hamster. In this study, the antibody was exclusively used for human cell lines. Additionally antibody specificity was verified by WB and IF in Stephan et al., EMBOJ 2020.
Alexa Fluor® 594 goat anti-rabbit IgG (H+L), Thermo Fisher Scientific, A11037: According to the manufacturer the polyclonal antibody was used in 275 studies, e.g. by Kim et al., Nature Communications 2019.
Alexa Fluor™ 594 goat anti-rabbit IgG (H+L), Thermo Fisher Scientific, A11032: According to the manufacturer the polyclonal antibody was used in 285 studies, e.g. by Clarke et al., Molecular Cell 2017.
AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson Immuno Research, Code: 111-005-144: According to the manufacturer the polyclonal antibody was used in 13 studies, e.g. by Zhang et al., Nature Methods 2020.
AffiniPure Sheep Anti-Mouse IgG (H+L), Jackson Immuno Research, Code: 515-005-062: According to the manufacturer, the polyclonal antibody was used at least in 1 study, Dittmar et al., The Journal of Investigative Dermatology 1999.
Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), Jackson Immuno Research, Code Number: 115-035-062: According to the manufacturer the polyclonal antibody was used in 183 studies, e.g. by Vershinin et al., Scientific Reports 2020.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	HDFa cells: ATCC, Manassas, VA, USA. U-2 OS: ECACC, Porton Down, Salisbury, UK. Yeast strains were generated as described in the materials and methods section.
Authentication	Mammalian cells were checked for morphology by light microscopy. Tagging of yeast cells was verified by PCR, sequencing and Western-blotting.
Mycoplasma contamination	Mammalian cell cultures were regularly tested for mycoplasma contamination by PCR (Food-PA 1051, 2008-11, SYNLAB Analytics & Services Germany GmbH, Jena, Germany) and certified to be mycoplasma negative. Additionally, stainings with DAPI against DNA were performed regularly, leading to negative results concerning extranuclear DNA.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study