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Corresponding author(s): Ricardo Henriques

☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

We aimed to acquire at least 5 data sets for each experimental data set and analysed these to validate repeatability of the algorithm. One representative data set was then chosen for display for each experiment.
The number of repeats performed for simulations was determined by simulation run-time.

2. Data exclusions

Describe any data exclusions.

No data were excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Repeatedly running the same image through the SQUIRREL software yielded near-identical error maps and quality metrics each time.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Randomization was not relevant as our manuscript presents an analytical tool for image analysis.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Blinding was not relevant as all data acquired for the experiments were analysed using the software described in this manuscript.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
☒	☐	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☒	☐	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☒	☐	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Our study presents a novel software package for quantifying super-resolution image quality; the software itself is fully described in the manuscript and supplementary information and a link is provided for downloading the software. All image analysis was performed using Fiji (ImageJ 1.51n), and where published plugins have been used for image reconstruction (QuickPALM (v1.1), ThunderSTORM (version dev-2016-09-10-b1), SRRF (version 1.13Stable1) the settings have been described in the Methods section. The PSF Generator (v1.0.0), SIMcheck (v1.0.0) and fairSIM (v1.0.2) plugins were used in the supplementary information. The SuReSim software package (v0.5.1) was used for simulations in Figure 1.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	No unique materials were used

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	Primary antibody for microtubule imaging in HeLa, CHO and COS cells: mouse monoclonal anti-alpha-tubulin, clone DM1A, Sigma catalog number T9026 (validation as per manufacturer's website: indirect immunofluorescence 1:500 using cultured chicken fibroblasts with cross-reactivity validated in human cell lines. Immunofluorescence images for hamster and human cell lines are available on the manufacturer's website). For COS cells an additional primary antibody was used: mouse monoclonal anti-alpha- tubulin, clone B-5-1-2, Sigma catalog number T5168 (validation as per manufacturer's website: indirect immunofluorescence 1:2000 using cultured human or chicken fibroblasts; cross-reactivity confirmed for African green monkey). Secondary antibody for microtubule imaging in HeLa, CHO and COS cells: goat anti-mouse IgG (H+L) highly cross-adsorbed Alexa Fluor 647, ThermoFisher Scientific catalog number A-21236. Primary antibody for clathrin-coated pit imaging in rat glial cells: rabbit polyclonal to clathrin heavy chain, Abcam catalog number Ab21679 (validation as per manufacturer's website: immunofluorescence at concentration of 1ug/ml; reacts with rat) Secondary antibody for clathrin-coated pit imaging in rat glial cells: anti-rabbit DNA-conjugated, part of Ultivue kit Ultivue-2 Anti-GFP nanobody for VACV lateral body imaging: GFP-Trap uncoupled protein, catalog number gt-250, Chromotek

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	HeLa cells were kindly provided by Prof Mark Marsh, UCL CHO cells were originally provided by Ira Mellman, Genentech COS cells were obtained from ATCC.
b. Describe the method of cell line authentication used.	Cell lines were not authenticated
c. Report whether the cell lines were tested for mycoplasma contamination.	Cell lines tested negative for mycoplasma
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Wistar Rat hippocampal neurons and glial cells were harvested from embryonic day 18 pups, following established guidelines of the European Animal Care and Use Committee (86/609/CEE) and approval from the local ethics committee (agreement D13-055-8).

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.