RS-FISH: precise, interactive, fast, and scalable FISH spot detection

Fluorescent in-situ hybridization (FISH)-based methods extract spatially resolved genetic and epigenetic information from biological samples by detecting fluorescent spots in microscopy images, an often challenging task. We present Radial Symmetry-FISH (RS-FISH), an accurate, fast, and user-friendly software for spot detection in two- and three-dimensional images. RS-FISH offers interactive parameter tuning and readily scales to large datasets and image volumes of cleared or expanded samples using distributed processing on workstations, clusters, or the cloud. RS-FISH maintains high detection accuracy and low localization error across a wide range of signal-to-noise ratios, a key feature for single-molecule FISH, spatial transcriptomics, or spatial genomics applications.
compared with the axial ($z$) dimensions (Fig. 1d and Methods). Third, the computation speed of RS allowed us to combine RS with robust outlier removal using random sample consensus (RS-RANSAC) to identify sets of image gradients that support the same ellipsoid object given a 3D spots. Second, we extended RS to support axis-aligned, ellipsoid objects without the need for scaling the image (21), enabling RS-FISH to account for typical anisotropy in 3D microscopy datasets that results from different pixel sizes and point spread functions in the lateral ($x$, $y$) dimensions (10 µm).

Fluorophore 1

Fluorophore 2

Fluorophore 3

Fluorophore 4

Fig. 1 | RS-FISH accurately detects fluorescent spots. a, Illustration depicting single fluorescent spot detection using RS-based RANSAC. Left, gradients (blue lines) calculated in a local patch around a DoG-detected location (red square) for RS fitting. Middle, intensity gradients that agree on a common center point (green gradients, green dot) given a defined error (green dotted circle) are identified using RANSAC outlier removal, and rejected gradients are plotted in white. Using all gradients would lead to a different center point (blue). Right, the final RS-FISH center spot (pink dot with black cross) is computed by intersecting all green (inlier) gradients. b, Detecting two close spots using multi-consensus RANSAC. Both points are detected as a single DoG-spot owing to a high noise level. Multi-consensus RANSAC identified two independent spots visualized as yellow and pink sets of pixels (that is, gradients). c, Single $z$ slice through the 3D image of a $C.\text{elegans}$ larva expressing $\text{lea-1}$ mRNA (smFISH labeling). Red circles highlight the RS-FISH-detected spots, and the encircled area is shown as an $x$-slice below. Images are representative of four experimental replicates. d, To correctly detect spots in anisotropic images, a global scale factor estimated from the data is computed. The example image shows a mouse embryonic stem cell labeled by smFISH for $\text{Cdx2}$ mRNA. e, RS-FISH detections can be exported as result table or CSV file or transferred to the ROI manager, and can be overlayed onto data for inspection using Fiji or BigDataViewer. The example image shows a max projection of five $z$-slices of a $\text{Drosophila}$ brain with smFISH labeled for $\text{Pura}$ mRNA. f, OligoFISSEQ-labeled $\text{PGP1f}$ cells using barcodes with four different fluorophores showing one round of labeling. RS-FISH detected spots are labeled in four different colors. Images by Nguyen et al. (3). g, RS-FISH scales to large datasets, shown for 4,010 mixed-stage $C.\text{elegans}$ embryos with $\text{mdh-1}$ mRNA smFISH labeling. h, Large N image volumes, like the EASI-FISH 148-GB lightsheet image of a tissue section of the lateral hypothalamus (data by Wang et al. (13)), were analyzed with the Apache Spark version of RS-FISH.
To validate and benchmark RS-FISH, we performed quantitative comparisons against FISH-quant14, Big-FISH15, AIRLOCALIZE17, Starfish16, and DeepBlink19 using (1) simulated smFISH images with varying noise levels to assess detection performance, (2) simulated images of spot pairs that are close to one another to assess performance on dense datasets, (3) real smFISH Caenorhabditis elegans embryo datasets for runtime measurements, (4) real smFISH cell datasets with varying noise levels, and (5) large lightsheet datasets13. We show that RS-FISH is on par with the best methods in terms of detection performance. Notably, it provides high detection accuracy and low localization error (Fig. 2a–c and Supplementary Fig. SN4.1–SN7.2) while running 3.8–7.1 times faster than established methods (Fig. 2d and Supplementary Notes). We additionally compare localization error and detection accuracy across different noise levels (Fig. 2e,f). RS-FISH shows superior detection accuracy, especially in the presence of very high noise. The localization error is very good in low-noise scenarios and slightly increases for higher noise levels, which is partially explained by having to localize more spots that other methods do not detect. We provide example images of each noise class tested in Figure 2e,f as guidance for users to estimate the expected localization quality. We highlight that RS-FISH can easily be parallelized on the cloud by running smFISH extraction on 4,010 C. elegans image stacks (~100 GB in total) in 18 minutes on AWS at the cost of US$18.35 in June 2021 (Fig. 1g). Importantly, RS-FISH is currently the only method that can be directly applied to large volumes (Fig. 1h and Supplementary Video 1). Processing a reconstructed 148-GB lightsheet image stack took 32 CPU hours (~1 hour on a modern workstation). In comparison, a complex wrapping software for distributing AIRLOCALIZE, specifically developed for the expansion-assisted iterative FISH (EASI-FISH) project to run on the HHMI Janelia cluster, required significant development effort and took 156 CPU hours to finish the same task13.

We developed RS-FISH based on a generic derivation of 3D RS for anisotropic objects that is efficiently implemented using imglib2, Fiji, and Spark. RS-FISH runs as a Fiji plugin, allowing interactive parameter adjustment and result verification on small and large images, making the task of correctly detecting diffraction-limited spots in microscopy images as accessible as possible. Processing speed is significantly improved and similar localization performance to established methods is achieved. RS-FISH is simple to install and run through Fiji, additionally providing macro-recording functionality to automate FISH spot detection easily. Our efficient block-based implementation allows easy single-molecule spot detection in large datasets or big volumes using local processing, clusters, or the cloud. Importantly, although we have demonstrated RS-FISH’s utility using only a 148-GB dataset, there is no conceptual limit that prohibits RS-FISH from being executed on significantly larger volumes well into the petabyte range. RS-FISH is an accurate, easy-to-use, versatile, and scalable tool that makes FISH spot detection on small and especially large datasets amenable to
**Brief Communication**

**Detection accuracy as $F_1$**

- RS-FISH: 0.996
- Big-FISH: 0.974
- FISH-quant: 0.996
- AIRLOCALIZE: 0.980
- Starfish: 0.994
- deepBlink: 0.961

**Localization error**

- RS-FISH: 0.362
- Big-FISH: 0.638
- FISH-quant: 0.251
- AIRLOCALIZE: 0.202
- Starfish: 0.560
- deepBlink: 0.649

**Average localization error**

- RS-FISH: 0.996
- FISH-quant: 0.974
- AIRLOCALIZE: 0.986
- Starfish: 0.202
- deepBlink: 0.560

**Processing speed**

- RS-FISH: 0.721
- Big-FISH: 2.605
- FISH-quant: 5.154
- AIRLOCALIZE: 3.189
- Starfish: 3.377
- deepBlink: 3.865

**Influence of noise on detection accuracy**

- $F_1$ decreases as image noise increases.

**Influence of noise on the localization error**

-Localization error increases with image noise.

**Counts**

- RS-FISH: 13,155
- Big-FISH: 12,858
- FISH-quant: 13,149
- AIRLOCALIZE: 12,849
- Starfish: 12,858
- deepBlink: 12,972

**Time (seconds)**

- RS-FISH: 0.721
- Big-FISH: 2.605
- FISH-quant: 5.154
- AIRLOCALIZE: 3.189
- Starfish: 3.377
- deepBlink: 3.865
researchers and whose functionality extends to the dynamically growing fields of spatial transcriptomics and spatial genomics.

**Online content**

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-022-01669-y.

**References**

1. Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A., & Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Publ. Group 5, 877–879 (2008).
2. Femino, A. M., Fay, F. S., Fogarty, K. & Singer, R. H. Visualization of single RNA transcripts in situ. Science 280, 585–590 (1998).
3. Nguyen, H. Q. et al. 3D mapping and accelerated super-resolution imaging of the human genome using in situ sequencing. Nat. Methods 17, 822–832 (2020).
4. Beliveau, B. J. et al. Versatile design and synthesis platform for visualizing genomes with Oligopaint FISH probes. Proc. Natl Acad. Sci. 109, 21301–21306 (2012).
5. Wang, F. et al. RNAscope a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagnostics 14, 22–29 (2012).
6. Shah, S. et al. Single-molecule RNA detection at depth by hybridization chain reaction and tissue hydrogel embedding and clearing. Development 143, 2862–2867 (2016).
7. Codeluppi, S. et al. Spatial organization of the somatosensory cortex revealed by osmFISH. Nat. Methods 5, 932–935 (2018).
8. Shah, S., Lubeck, E., Zhou, W. & Cai, L. In situ transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus. Neuron 92, 342–357 (2016).
9. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. Science 348, aaaa090 (2015).
10. Eng, C.-H. L. et al. Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH. Nature 568, 235–239 (2019).
11. Friedich, D. et al. Stochastic transcription in the p53-mediated response to DNA damage is modulated by burst frequency. Mol. Syst. Biol. 15, e9068 (2019).
12. Wheat, J. C. et al. Single-molecule imaging of transcription dynamics in somatic stem cells. Nature 583, 431–436 (2020).
13. Wang, Y. et al. EASI-FISH for thick tissue defines lateral hypothalamic spatio-molecular organization. Cell 184, 6361–6377 (2021).
14. Mueller, F. et al. FISH-quant: automatic counting of transcripts in 3D FISH images. Nat. Methods 10, 277–278 (2013).
15. Eichenberger, B. T., Zhan, Y., Rempfler, M., Giorgetti, L. & Chao, J. A. deepBlink: threshold-independent detection and localization of diffraction-limited spots. Nucleic Acids Res. 49, 7292–7297 (2021).
16. Axelrod, S. et al. Starfish: open source image based transcriptomics and proteomics tools http://github.com/spacetx/starfish (2018).
17. Lionnet, T. et al. A transgenic mouse for in vivo detection of endogenous labeled mRNA. Nat. Methods 8, 165–170 (2011).
18. Imbert, A. et al. FISH-quant v2: a scalable and modular tool for smFISH image analysis. RNA 28, 786–795 (2022).
19. Parthasarathy, R. Rapid, accurate particle tracking by calculation of radial symmetry centers. Nat. Methods 9, 724–726 (2012).
20. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
21. Liu, S.-L. et al. Fast and high-accuracy localization for three-dimensional single-particle tracking. Sci. Rep. 3, 2462 (2013).
22. Fischler, M. A. & Bolles, R. C. Random sample consensus: A paradigm for model fitting with applications to image analysis and automated cartography. Commun. ACM 24, 381–395 (1981).
23. Lindeberg, T. Image matching using generalized scale-space interest points. J. Math. Imaging Vis. 52, 3–36 (2015).
24. Pietzsch, T., Preibisch, S., Tomancak, P. & Saalfeld, S. ImgLib2—generic image processing in Java. Bioinformatics 28, 3009–3011 (2012).
25. Saalfeld, S., Fetter, R., Cardona, A. & Tomancak, P. Elastic volume reconstruction from series of ultra-thin microscopy sections. Nat. Methods 9, 717–720 (2012).
26. Pietzsch, T., Saalfeld, S., Preibisch, S. & Tomancak, P. BigDataViewer: visualization and processing for large image data sets. Nat. Methods 12, 481–483 (2015).
27. Thompson, R. E., Larson, D. R. & Webb, W. W. Precise nanometer localization analysis for individual fluorescent probes. Biophys. J. 82, 2775–2783 (2002).
Methods

\(n\)-dimensional derivation of Radial Symmetry localization

The goal of RS is to accurately localize a bright, circular spot \(p\), with sub-pixel accuracy. In noise-free data, image gradients \(\nabla I(p)\) at locations \(p\) point towards the center of the spot and intersect in that single point \(p\) (Fig. 1a), thus computing the intersection point solves the problem of accurate localization. In realistic images that contain noise, these gradients do not intersect, therefore computing \(p\) constitutes an optimization problem that RS solved using least-squares minimization of the distances \(d^n\) between the common intersection point \(p\), and all gradients \(\nabla I(p)\) (Supplementary Fig SN1.1).

We extend RS to 3D similar to Liu et al.\(^{21}\) and additionally describe how to generalize the derivation to the \(n\)-dimensional case. To achieve this, we replace the Roberts cross operator with separable convolution for image gradient \(\nabla I(p)\) computation, and we use vector algebra to compute the intersection point \(p\) of image gradients. The derivations are shown in detail in Supplementary Fig SN1.1 and Supplementary Notes.

Radial Symmetry for axis-aligned ellipsoid (non-radial) objects

Diffraction-limited spots in 3D microscopy images are usually not spherical but show a scaling in the axial (z) dimension compared with the lateral (xy) dimensions. Previous solutions suggested scaling the image in order to be able to detect spots using RS\(^{1}\). This can be impractical for large datasets, and it might affect localization quality, as the image intensities need to be interpolated for scaling. Here, we extend the RS derivation to directly compute the intersection point \(p\), from anisotropic images by applying a scale vector \(s\) to spot locations \(p\) and applying the inverse scale vector \(s^{-1}\) to the image gradients \(\nabla I(p)\). Although we derive the case specifically for 3D, it can be straightforwardly applied to higher dimensions. The derivation is shown in detail in the Supplementary Notes.

RS-FISH supports a global scale factor (called anisotropy factor) for the entire dataset that compensates for anisotropy of the axial (z) dimension, which can be computed from an image containing diffraction-limited spots (Supplementary Notes).

Radial Symmetry Random Sample Consensus

RS localization is implemented as a fast, closed-form solution, and it is therefore feasible to combine it with robust outlier removal. We use RANSAC\(^{10}\) to identify the maximal number of gradients \(\nabla I(p)\) that support the same center point \(p\), given a maximal distance error \(\varepsilon\), so that all \(d^n < \varepsilon\).

To achieve this, RANSAC randomly chooses the minimal number of gradients (that is, two gradients) from the set of all gradients (candidate gradients) to compute the center point and tests how many other gradients fall within the defined error threshold \(\varepsilon\). This process is repeated until the maximal set of gradients is identified (inlier gradients) and the final center point \(p\) is computed using all inlier gradients. This allows RS-FISH to exclude artifact pixels and to differentiate close-by spots.

The number of gradients that are computed for each spot is defined by the support region radius, which can be selected as one of the RANSAC parameters. By default, we propose a radius of 3 pixels, which corresponds to a \(7 \times 7 \times 7\) pixel patch, resulting in 216 gradients for the 3D case. These settings are reasonable choices for acquisition parameters typically used for smFISH images (500–700 nm emission, \(\times 63\) oil detection objective, EMCCD or SCMOs camera with \(-10\) μm pixels, corresponding to a \(-159\)-nm lateral pixel size in the sample plane), where the pixel patch comfortably covers the central peak of the point spread function (PSF). Importantly, the radius should be adjusted to the respective acquisition settings so that an area that is approximately twice the size of the central peak of the PSF is entirely covered to ensure that all gradients that point towards the center of each spot are included in the localization.

To identify and locate close-by points, RS-FISH runs a multi-consensus RANSAC. Here, RANSAC is run multiple times on the same set of candidate gradients. After each successful run that identifies a set of inliers, the inliers are removed from the set of candidate gradients, and RANSAC tries to identify another set of inliers (Fig. 1b). This process is iterated until no other set of inliers (corresponding to a FISH spot) can be found in the local neighborhood of each DoG spot. To not detect random noise, the minimal number of inliers required for a spot can be adjusted (typically around 30).

Implementation details and limits

RS-FISH is implemented in Java using ImgLib2, the mpicbg framework, BigDataViewer, Fiji, and Apache Spark. The computation of RS is performed in blocks with a size of \(b\) for each dimension \(d\) (for example, \(256 \times 256 \times 128\) pixels) and requires an overlap of only 1 pixel in each dimension with neighboring blocks, thus the overhead \(a = 1 - \frac{b}{256}, \frac{b}{256}, \frac{b}{256}\) is minimal (for example, 1.5% for \(256 \times 256 \times 256\) blocks or 0.6% for \(1024 \times 1024 \times 1024\) blocks). When processing each block, the local process has access to the entire input image, which is either held in memory when running within Fiji or is lazy-loaded from blocked N5 datasets when running on large volumes using Apache Spark. Because the computation across blocks is embarrassingly parallel, computation time linearly scales with the dataset size. Thus, RS-FISH will run on very large volumes supported by N5 and ImgLib2. Owing to current limitations in Java arrays, the theoretical upper limit is \(2^{31} = 2,147,483,648\) blocks, with each block maximally containing \(2^{31} = 2,147,483,648\) pixels (for example 2048 \(\times\) 2048 \(\times\) 512 pixels). Given sufficient storage and compute resources, the limit for RS-FISH is thus 4,072 peta-pixels (4,072 petabytes at 8 bit, or 8,144 petabytes at 16 bit) taking into account the overhead, whereas every individual block locally processes only 2 gigapixels (\(2^{30} = 2,147,483,648\) pixels).

The code can be executed on an entire image as a single block for smaller images, or in many blocks multi-threaded or distributed using Apache Spark. It is important to note that RS-RANSAC uses random numbers to determine the final localization of each spot. We use fixed seeds to initialize each block; therefore, the results for a single block of the same size in the same image with the same parameters are constant. However, for blocks of different sizes (for example, single-threaded versus multi-threaded), the results will be slightly different, as the RANSAC-based localizations are not traversing the DoG maxima in the same order, and thus initialize and RANSAC differently. For practicality, the interactive Fiji mode runs only in single-threaded mode (although the DoG image is computed multi-threaded) to yield comparable results across different testing trials. Importantly, this applies only if the RANSAC mode is used for localization. Multi-threaded processing is available in the recordable advanced mode in the Fiji plugin, while the Apache Spark based distribution can be called from the corresponding RS-FISH-Spark repository.

Data simulation for assessing localization performance

To create ground-truth datasets for assessing localization performance, we generated images simulating diffraction-limited spots in the following way: \((x,y,z)\) spot positions were randomly assigned within the \(z\)-stack chosen dimensions, and each spot was assigned a brightness picked from a normal distribution. We computed the intensity \(I(x,y,z)\) generated by each spot as follows: we first computed the predicted average number of photons received by each pixel \(I_{\text{pred}}(x,y,z)\) computed using a gaussian distribution centered on the spot, with user-defined lateral and axial extensions. We then simulated the actual intensity collected at each pixel using a Poisson-distributed value with mean \(I_{\text{pred}}(x,y,z)\). We eventually added gaussian-distributed noise to each pixel of the image.

Code for generating the images with simulated diffraction-limited spots is available in the GitHub repository. There is also a folder included with the simulated data used in the parameter grid search.
Importantly, RS-FISH runs on such volumes natively and can easily be the workstation CPUs (according to https://www.cpubenchmark.net). Overall speed increase of ~5× generally agrees with our measurements in at 2.7 GHz and Intel Cascade Lake (Gold 6248 R) at 3.0 Ghz CPUs. The scripts and was executed on a mix of Intel SkyLake (Platinum 8168) 156 CPU hours was extracted from the cluster logs of the submission ten to specifically run only on the Janelia cluster. The compute time of of AIRLOCALIZE that was developed for the same project, as it is writing high detection accuracy and low localization error (Fig. 2a–c,e,f ) and Supplementary Fig. SN4.2d,e ). Additionally, RS-FISH is currently the only available tool that can be directly applied to large images, which we highlight using a 148-GB lightsheet image stack13 (Fig. 1h, Supplementary Video 1, and Supplementary Notes). The image size of the lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used for detection was 256 × 256 × 128 pixels. The detection of spots using lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used of AIRLOCALIZE that was developed for the same project, as it is writing high detection accuracy and low localization error (Fig. 2a–c,e,f ) and Supplementary Fig. SN4.2d,e ). Additionally, RS-FISH is currently the only available tool that can be directly applied to large images, which we highlight using a 148-GB lightsheet image stack13 (Fig. 1h, Supplementary Video 1, and Supplementary Notes). The image size of the lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used for detection was 256 × 256 × 128 pixels. The detection of spots using lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used of AIRLOCALIZE that was developed for the same project, as it is writing high detection accuracy and low localization error (Fig. 2a–c,e,f ) and Supplementary Fig. SN4.2d,e ). Additionally, RS-FISH is currently the only available tool that can be directly applied to large images, which we highlight using a 148-GB lightsheet image stack13 (Fig. 1h, Supplementary Video 1, and Supplementary Notes). The image size of the lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used for detection was 256 × 256 × 128 pixels. The detection of spots using lightsheet stack is 7,190 × 7,144 × 1,550 pixels, and the block size used.
Author contributions
The project was conceived by T. L. and S. P. The mathematical concepts and derivations were done by T. L. and S. P. The software was written by S. P., E. B., and M. Z. with contributions by K. K. and K. I. S. H. The documentation was written by L. B. and K. K. The simulated data were created by T. L. The benchmarking was performed by E. B., L. B., and L. E. Experiments and imaging were performed by L. B., N. M., B. K., and X. L., K. I. S. H., T. L., and S. P. supervised the project. L. B. and S. P. wrote the manuscript with input from all co-authors.

Competing interests
The authors declare no competing interests.

Additional information

Supplementary information
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Correspondence and requests for materials
Correspondence and requests for materials should be addressed to Kyle I. S. Harrington, Timothée Lionnet or Stephan Preibisch.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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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: Micro-Manager (2.0.0), NIS Elements software, Applied Precision SoftWoRx

Data analysis: RS-FISH (0f6ab4a), FISH-quant (v3), Big-FISH (0.5.0), AIRLOCALIZE (1.6), Starfish (0.2.2), deepBlink (0.1.1), Fiji (2.3.0)

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All datasets used for benchmarking are available in the RS-FISH GitHub repository (https://github.com/PreibischLab/RS-FISH and https://github.com/timotheelionnet/simulated_spots_rsFISH), which includes simulations and 3D smFISH images of C. elegans embryos. The raw data underlying Fig. 1 are available at figshare: https://doi.org/10.6084/m9.figshare.21067342.v1; https://doi.org/10.6084/m9.figshare.21067366; https://doi.org/10.6084/m9.figshare.21067360.v1; https://doi.org/10.6084/m9.figshare.21067354; https://doi.org/10.6084/m9.figshare.21067369.v1; https://doi.org/10.6084/m9.figshare.21067372
### Life sciences study design

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

| Sample size | No measure were taken to estimate the sample sizes. However the sample size was limited by the computational capacity since we performed grid searches over the parameter space for each tool and analysis. The sample size was adequate as it illustrates the detection capabilities of RS-FISH compared to similar tools. The sample size of 50 images for localization performance were chosen to cover a reasonable range of simulated SNRs and point densities (each containing 30-300 points) - all can be inspected in the github repository. For the analysis of close points we created 720 images (each containing 30 points). For the benchmarks using real data we created 63 two-dimensional images with different noise levels. For speed measurements we chose 13 different samples of actual 3D image data stacks. We limited it to 13 since each tool needs to be manually tuned to yield similar numbers of points, which constitutes a major effort. |
| Data exclusions | Data in Supplementary Note 6 was excluded due to too low SNR (explained in the text). Otherwise no data was excluded. |
| Replication | Unless stated otherwise, experiments were performed once. The manuscript assers the performance of an image analysis software (mostly on simulated data) and not on variability of biological samples. |
| Randomization | The location of the points for the benchmarking datasets were chosen randomly. Data analysis was not randomized since this analysis is not affected by human bias. |
| Blinding | The benchmarking was performed and scored by computer algorithms after a grid search to find the best parameters for each tool. Since this analysis is not affected by human bias, no blinding was necessary. |

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|---------------------|-------------------------------------------------------------------|
| Authentication      | As cell lines were purchased directly from JCRB, visual inspection was used to confirm morphology was consistent with description and pictures provided by the vendor. |
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| Wild animals       | No wild animals were used in this study.                                                                                                    |
| Field-collected samples | No field-collected samples were used in this study.                                                                                           |
| Ethics oversight   | No ethical approval was required for Drosophila and C. elegans                                                                                  |

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