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vLUME: 3D virtual reality for single-molecule localization microscopy

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vLUME is a virtual reality software package designed to render large three-dimensional single-molecule localization microscopy datasets. vLUME features include visualization, segmentation, bespoke analysis of complex local geometries and exporting features. vLUME can perform complex analysis on real three-dimensional biological samples that would otherwise be impossible by using regular flat-screen visualization programs.

Super-resolution microscopy based on three-dimensional single-molecule localization microscopy (3D-SMLM) is now well established^{1,2}, and its widespread adoption has led to the development of more than 36 software packages dedicated to quantitative evaluation of the spatial and temporal detection of fluorophore photoswitching³. While the initial emphasis in the 3D-SMLM field has clearly been on improving resolution and data quality, there is now a marked absence of 3D visualization approaches that enable the straightforward, high-fidelity exploration of this type of data. Inspired by the horological phosphorescence points that illuminate watch-faces in the dark, we present vLUME (visualization of the local universe in a micro environment, pronounced ‘volume’), an immersive virtual reality (VR)-based visualization software package purposefully designed to render large 3D-SMLM datasets. It is free for academic use. vLUME enables robust visualization, segmentation, annotation and quantification of millions of fluorescence puncta from any 3D-SMLM technique. vLUME has an intuitive user interface and is compatible with all commercial gaming VR hardware (Oculus Rift/Rift S and HTC Vive/Vive Pro; Supplementary Video 1). Although other microscopy data (that is, confocal) visualization tools have previously explored VR technology using volumetric representations^{4,5}, vLUME has been specifically and purposefully created for SMLM. It accelerates the analysis of highly complex 3D point-cloud data and the rapid identification of defects that are otherwise neglected in global quality metrics. (A comparison with other VR and non-VR tools can be found in Supplementary Table 1.)

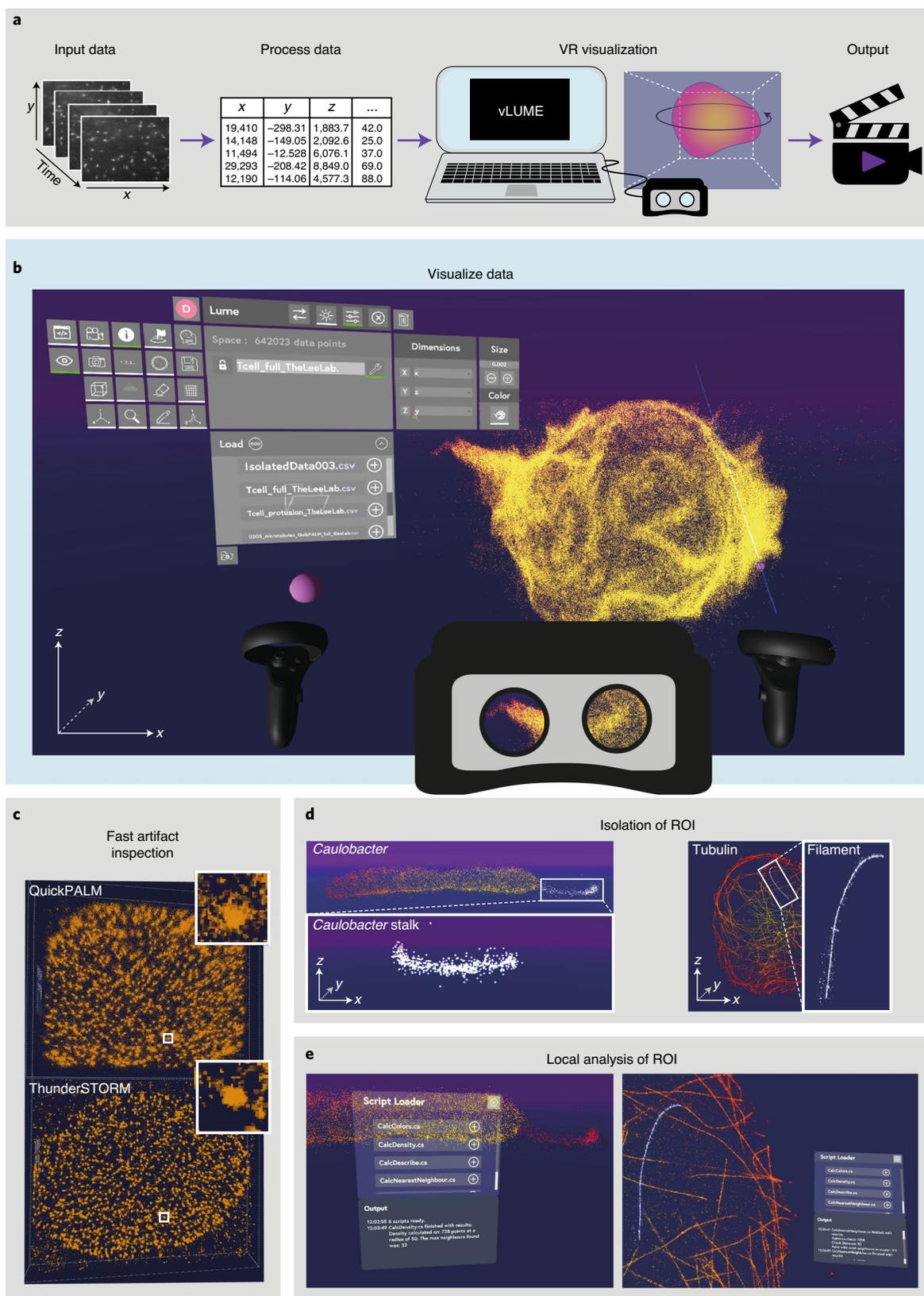
vLUME is a point-cloud based 3D-SMLM data visualization tool able to render all pointillism-based multidimensional datasets. It differs from other 3D tools for 3D-SMLM visualization such as ViSP⁶ by providing a complete VR interactive environment and intuitive interface for life scientists, dedicated to data visualization, segmentation and analysis. Users load multidimensional particle-list datasets into vLUME (.csv files; Fig. 1a), such as those generated by commonly used 3D-SMLM software^{7,8}. This allows users to comprehend the spatial and temporal relation between

points comprising a 3D structure. In time-lapse data, 3D reconstructions update for each time-point under user control. vLUME is built with the industry-standard cross-platform UnityEngine, providing a high-performance rendering framework that scales with future advances in graphics performance.

vLUME has four key features:

- (1) Data exploration and comparison. The configurable user interface allows researchers, without need for programming, to seamlessly switch back and forth from a global view of the entire captured sample to detailed nanoscale views of molecular elements in any arbitrary orientation, faster than with conventional flat screens⁹. Doing so allows the easy local selection of data for further analysis (Supplementary Videos 2 and 3). The software can be used to leverage the human capacity to quickly interpret local features in these data, such as global and local artifacts (Fig. 1b), that are more difficult to trace by automated software without the ground truth being known¹⁰. In addition, it is easy to quickly evaluate and compare different processing software side by side, such as, for example, QuickPALM versus ThunderSTORM (Fig. 1c). We include example datasets with different sample types, using various SMLM-based super-resolution methods and from different international super-resolution laboratories to demonstrate its broad applicability (Fig. 1b–e and Methods).
- (2) Extracting 3D regions of interest (ROI) from complex datasets. Complex biological interactions occur in intricate 3D geometries, with the evaluation of interaction data often requiring the extraction and analysis of specific subselections of a dataset. To demonstrate this capacity of vLUME, we carried out complex segmentation tasks where users needed to identify and select small local features (tens to hundreds of localizations) in data of large dimensions (millions of localizations, Fig. 1d). A single microtubule can be easily extracted from a complex 3D tangle of microtubules within a eukaryotic cell (Fig. 1d, right). This process can be performed in less than 1 min and the ROI exported for further analysis (Methods). Once uploaded, these data subsets can be scaled, highlighted, colored and selected in three dimensions via VR controls (Supplementary Video 4).
- (3) Custom analysis of user-defined subregions. Quantitative bioimaging not only relies on high-quality images but quantitative evaluation using bespoke code. Recognizing this, we included a user-definable script interpreter written in the multiparadigm language C# (Methods). These data can be easily evaluated to

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give the user instant quantitative feedback about the specific subregion of their dataset (Fig. 1e). We have included four widely used analyses (Methods section “Scripts in C# for local analysis”): Ripley’s K function^{11,12}, nearest neighbor^{13,14} (Supplementary Video 5), local density, and longest and shortest

distances. It is our hope to nucleate communities to create, evaluate and share new scripts with each other to enable quantitative SMLM imaging.

- (4) Exporting movies for publications and presentations. As well as allowing customization of data for presentation

Fig. 1 | Features of vLUME. **a**, vLUME rapidly takes large, multidimensional point-cloud datasets from 2D visualization into an immersive 3D VR environment through a systematic workflow: (1) multidimensional, SMLM image stacks are processed with any standard fitting algorithms providing multiparameter outputs as .csv files. (2) The resultant datasets can then be loaded directly into the vLUME software and instantly visualized in VR. (3) By anchoring at user-defined waypoints around these data, a smoothly interpolated fly-through video can be created and exported, providing the user with a tool to effectively communicate their discoveries. **b**, vLUME facilitates the 3D VR visualization of millions of localizations, demonstrated by the super-resolved membrane of a T cell. The accessible interface enables the user to customize the visual appearance of the localization. The T cell is $\sim 10\ \mu\text{m}$ in diameter and has an isotropic resolution of $\sim 24\ \text{nm}$ (FSA). **c**, Comparative inspection of artifacts introduced into data by localization fitting tools can be quickly performed. This is visualized by comparing localizations of nuclear pore complexes fitted with both QuickPALM and ThunderSTORM algorithms. The nuclear pore complex is $\sim 100\ \text{nm}$ in diameter, and the nuclear membrane $\sim 20\ \mu\text{m}$ in diameter. Data are taken over a $\sim 200\ \text{nm}$ axial range in *z*. **d**, Selection and isolation of nanoscale, complex biological features can be easily achieved by the user. As examples, the stalk of a *Caulobacter crescentus* bacterium (left) and a filament of a tubulin network (right) were isolated and a ROI saved for further analysis. The microtubule tangle (right) shows a region of $\sim 20 \times 30\ \mu\text{m}$ (and about $500\ \text{nm}$ in depth). The diameter of a single microtubule is $\sim 40\ \text{nm}$. **e**, The ROI can then be analyzed to instantly quantify desired properties using bespoke C# scripts (Ripley's K, local density plots, nearest neighbors and any others).

purposes, vLUME also allows custom waypoints (user angle, pitch and yaw) to be defined simply in the VR environment (Fig. 1a) to automatically generate a 'fly-through' video (Supplementary Video 6) to allow researchers to articulate their scientific discoveries.

In summary, vLUME provides a new immersive environment for exploring and analyzing 3D-SMLM data. It enables imaging scientists with any level of expertise to make straightforward analytical sense of what is often highly complex 3D data. We include a case study of the periodic submembrane scaffold along axons of cultured neurons, made of actin rings regularly spaced every $190\ \text{nm}$ (ref. ¹⁵). vLUME is used to segment, annotate and analyze complex subregions, including spectrins assembled in the axonal periodic scaffold, submembrane spectrin not organized as rings in cell body and dendrites, cytosolic spectrin, and residual nonspecific labeling outside the cell (Supplementary Video 7). Full documentation and software for vLUME are included in the Methods.

We envisage that users will use vLUME to share and explore existing and new emergent datasets and that the ability to interact with SMLM data in this way may inspire new methods, tools and discoveries. Future directions could include the incorporation of a multi-user tool for numerous users to use vLUME within the same environment, as well as the incorporation of advanced computation imaging tools such as focused training methods for machine learning.

Online content

Any methods, additional references, Nature Research 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/s41592-020-0962-1>.

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Methods

SMLM biological samples included with vLUME. In the installation package of vLUME, we include six biological datasets (12 files) from four different super-resolution microscopy laboratories obtained with a variety of different SMLM methods. The processed datasets (localization files) are saved in the comma separate values (.csv) file format and can be opened as point clouds from the virtual environment of vLUME. They can be found in the `\vLume_Data\StreamingAssets` folder of the vLUME installation directory (manual).

Plasma membrane of a whole T cell. This shows the large depth-of-focus data of the plasma membrane of a whole Jurkat T cell imaged in three dimensions using the double helix point spread function (DH-PSF) (imaging done at the Lee laboratory, Cambridge, UK). The cell membrane was labeled using fluorescently tagged wheat germ agglutinin using PAINT, similarly to work previously described¹⁶. Each cell was imaged as series of 4- μ m optical slices and reconstructed using overlapping fiducial alignment to create a whole cell of \sim 10 μ m with a resolution of \sim 22 nm (confirmed by Fourier shell correlation)¹⁷. Localization data were processed using easy-DH-PSF (10.1038/protex.2013.026). Figure 1b shows the T cell within the virtual environment. The .csv file contains three columns: *x*, *y* and *z* positions (nm) (filename `Tcell_full_TheLeeLab.csv`).

Microtubules in U-2 OS cells. The microtubules in U-2 OS cells were imaged with astigmatic dSTORM^{3,18} at the Reis laboratory in Heidelberg, Germany. The microtubules were labeled with anti- α -tubulin primary and Alexa Fluor 647-tagged secondary antibodies. The single emitters were processed using ThunderSTORM^{3,8} (Fig. 1d,e, right). The comma-delimited file contains five columns: frame number, *x*, *y* and *z* positions (nm) and intensity (photons). These data are openly available³ (filename `U2OS_microtubules_ThunderSTORM_full_RiesLab.csv`).

The same raw data were used again (`U2OS_microtubules_ThunderSTORM_full_RiesLab.csv`), but processed using QuickPALM^{3,7}. The comma-delimited file contains five columns: frame number, *x*, *y* and *z* positions (nm) and intensity (photons) (filename `U2OS_microtubules_QuickPALM_full_RiesLab.csv`).

A single microtubule obtained from a subset selection from `U2OS_microtubules_ThunderSTORM_full_RiesLab.csv` using vLUME (Fig. 1d, right and Supplementary Video 2). The comma-delimited file contains five columns: frame number, *x* position, *y* position and *z* position (nm) and intensity (photons) (filename `U2OS_microtubules_ThunderSTORM_single_RiesLab.csv`).

Nuclear pore complexes (NPCs) in U-2 OS cells. The NPC in U-2 OS cells was imaged using astigmatic dSTORM^{3,18} at the Ries laboratory. The NPCs were labeled using NUP107-SNAP-BG tagged with AF647. The single emitters were processed using ThunderSTORM^{3,8} (Fig. 1c, lower). The comma-delimited file contains five columns: frame number, *x*, *y* and *z* positions (nm) and intensity (photons). These data are openly available³ (filename `U2OS_NPC_ThunderSTORM_RiesLab.csv`).

The same raw data for NPCs in U-2 OS cells are shown in `U2OS_NPC_ThunderSTORM_RiesLab.csv`, but were processed using QuickPALM^{3,7} (Fig. 1c, upper). The comma-delimited file contains five columns: frame number, *x* position, *y* position and *z* position (nm) and intensity (photons). These data are openly available³ (filename `U2OS_NPC_QuickPALM_RiesLab.csv`).

Caulobacter crescentus bacteria. *C. crescentus* bacteria were imaged in three dimensions using the DH-PSF in the Moerner laboratory, Stanford, United States. Colonies of the wild type (CB15N) strain of *C. crescentus* were grown overnight in a manner previously described² (Fig. 1d,e left). The bacteria were covalently labeled with *N*-hydroxysuccinimide-functionalized rhodamine spirolactam via a coupling reaction with the exposed amines on the bacteria surface² and imaged in three dimensions using the DH-PSF and processed using bespoke software. The comma-delimited file contains three columns: *x*, *y* and *z* positions (nm) (filename `CaulobacterC_2_R09_MoernerLab.csv`).

A single *C. crescentus* bacterium showing the stalk, imaged in three dimensions using the DH-PSF. The comma-delimited file contains three columns: *x*, *y* and *z* positions (nm) (`CaulobacterC_2_R23_MoernerLab.csv`).

Stalk of a *C. crescentus* bacterium obtained using the data isolation tool in vLUME from dataset `CaulobacterC_2_R09_MoernerLab.csv` (Supplementary Video 3). The comma-delimited file contains three columns: *x*, *y* and *z* positions (nm) (`CaulobacterC_2_R09_stalk_MoernerLab.csv`).

Two-channel image of microtubules and clathrin in COS cells. Two-channel images of microtubules and clathrin-coated pits in COS cells were imaged using astigmatism and with DNA-PAINT¹⁹ at the Leterrier laboratory, Marseille, France. Briefly, cells were labeled with rabbit anti-clathrin light chain and mouse anti- α -tubulin antibodies, and secondary antibodies coupled to DNA-PAINT docking strands (Ultivue). The cell was imaged on a commercial N-STORM microscope (Nikon) in buffer containing imaging strands coupled to far-red (clathrin) and red (microtubule) fluorophores by alternating frames. Single emitters were detected using the N-STORM software (Nikon) and processed using ThunderSTORM.

The first channel is labeled for microtubules (anti- α -tubulin), imaged in three dimensions (Extended Data Fig. 1, purple). The file contains six columns: frame

number, *x*, *y* and *z* positions (nm), lateral uncertainty (nm) and axial uncertainty (nm) (filename `COS_microtubules(ch1)_LeterrierLab.csv`).

The second channel is labeled for clathrin pits (anti-clathrin light chain), imaged in three dimensions (Extended Data Fig. 1, green). The file contains six columns: frame number, *x*, *y* and *z* positions (nm), lateral uncertainty (nm) and axial uncertainty (nm) (filename `COS_clathrin(ch2)_LeterrierLab.csv`).

Spectrin scaffold in neurons. Mature neurons (35 d in culture) were fixed and labeled with an anti- β 2-spectrin primary antibody, then an antimouse secondary antibody coupled to Alexa Fluor 647, and imaged using STORM²⁰ at the Leterrier laboratory. Imaging was performed on a commercial N-STORM microscope (Nikon) in Smart Buffer Kit (Abbelight). Single emitters were detected using the N-STORM software (Nikon) and processed using ThunderSTORM. The file contains six columns: frame number, *x*, *y* and *z* positions (nm), lateral uncertainty (nm) and axial uncertainty (nm) (filename `Spectrin_neurons_LeterrierLab.csv`).

Large datasets visualization. vLUME can easily render large point-cloud datasets in real time (manual). As an example, the `T_cell_full_TheLeeLab.csv` file has \sim 650,000 single-molecule localizations and opens up in \sim 15 s on a Gigabyte Aero 15 laptop with an Intel i7 (eighth generation) CPU, an nVIDIA Geforce RTX 2080 GPU and 32 GB of RAM. It keeps a constant refresh rate of \sim 80–90 Hz while navigating the VR environment, which is the industry standard to avoid motion sickness. Roughly 2 million localizations can be rendered without a significant drop in performance using the same system. This performance scales up linearly with the graphics processing power of the system.

Arbitrary selection and isolation of a ROI in large complicated datasets.

Arbitrary selection and isolation of a local ROI is one of the tasks where VR provides an advantage over flat screens thanks to its intuitive navigation⁹. We performed the selection of a stalk in a *C. crescentus* bacterium and isolated its dataset (sample `CaulobacterC_2_R09_stalk_MoernerLab.csv`) (Fig. 1d left and Supplementary Video 3) and a single microtubule from a complex tangle (sample `U2OS_microtubules_ThunderSTORM_single_RiesLab.csv`) (Fig. 1d right and Supplementary Video 2) in less than 1 min (manual). Using the selection feature also enables the application of bespoke C# scripts to perform analyses in any desired region.

Scripts in C# for local analysis. An important feature of vLUME is the ability to perform local analysis on a subregion of the point-cloud data (ROI). C# scripts can be programmed to perform any custom analysis once uploaded to the folder `\vLume_Data\StreamingAssets\Scripts` within the vLUME installation directory (.cs file extension). Further information can be found on how to program, share and use the scripts as plugins in the next two sections and in the software manual.

We include four useful functions as .cs scripts for the vLUME interpreter that can be used for local cloud data-point analysis and that are often used in SMLM. These can be applied by selecting a ROI and toggling to the scripting menu (see the software manual). Note that, depending on the complexity of the script, a busy indicator may be seen (out of the virtual environment) when the script is called, which is normal. Also, be aware that some operations require more than the available memory, particularly in large datasets (for example, density plots with millions of points that need to compute every single distance from one point to another).

We include four scripts; however, it is our hope that other groups will develop and share their own.

Ripley's K function. Ripley's K function (filename `CalcRipleysK.cs`) is a spatial point statistics analysis commonly used to evaluate clustering in SMLM data^{11,12}. The script computes the Euclidean distance from a series of pairwise points in a ROI and counts the number of neighbors from a single point to a moving 3D radius (defined by the user and prompted as `RegionSize` and `RegionStep`). The input units need to be the same as the dataset. The output of the script is the L function, the linearized and localized Ripley's K function¹², for every `RegionStep` from 0 to `RegionSize`. The script also outputs a .txt file (named after the type of analysis and the time it was performed) in `StreamingAssets\Scripts\Output` saving the number of points in the ROI, the volume, region size and the step value, then the computed L (radius), and finally the position of every single point within the ROI.

Note that the function is only taken within the ROI as an isolated region and does not take into account any points in the periphery. Be aware that every single distance from point to point is computed; therefore, large ROIs should be executed only in systems with large amounts of memory. The volume is computed as the smallest bounding box containing the whole dataset.

Nearest-neighbor plot. Nearest-neighbor plot (filename `CalcNearestNeighbour.cs`) is a widely used analysis tool in super-resolution^{13,14}. The user is prompted for the value of the `ThresholdRadius` (that must be inputted in the same units as the dataset), which will be used to compute the number of neighbors of every single point in the ROI up to the `ThresholdRadius`. The script then assigns a false color depending on that number. These numbers are normalized with reference to the maximum number of neighbors, red being the lower density and blue the highest

within a color gradient (note that the colors will be plotted on top of the selection). The script also outputs a .txt file (named after the type of analysis and the time it was performed) in StreamingAssets/Scripts/Output, saving the number of points of the ROI, the radius tested and the number of neighbors of every single point within the ROI together with its position.

As an example, the stalk of the *C. crescentus* bacterium (dataset CaulobacterC_2_R09_MoernerLab.csv) (Fig. 1e, left, Extended Data Fig. 2) and some NPCs (in the dataset U2OS_NPC_ThunderSTORM_RiesLab.csv) (Supplementary Video 5) were analyzed. For the stalk, we can clearly see that the number is larger in the core and lower in the surface, as expected (Extended Data Fig. 2).

Note that the function is only taken within the ROI as an isolated region and does not take into account any points in the periphery. Be aware that every single distance from point to point is computed; therefore, large ROIs should be executed only in systems with large amounts of memory.

Density. Calculate the density of points in ROI (filename CalcDensity.cs). We provide a very simple implementation that calculates the points within the ROI and divides this value by the volume of the ROI to create the localization density. The result is printed within vLUME as points/volume using the units of the .csv dataset.

Note that the volume is computed as the smallest bounded box that can contain the selected dataset, which is only an approximation for irregular volumes.

Maximum and minimum distances. Calculate the maximum and the minimum distances between points in ROI (filename CalcShortAndFarDistances.cs). For syntax purposes, we provide a very simple script that calculates the maximum and minimum distance between all the points within the ROI. The result is printed within vLUME using the units of the .csv dataset (Supplementary Video 5).

Note that the user should be aware that every single distance from point to point is computed; therefore, large ROIs should be executed only in systems with large amounts of memory.

Collaborating on the open-source C# plugins using GitHub. vLUME will, hopefully, nucleate communities of SMLM researchers by facilitating active collaboration, development of bespoke analysis features (open-source C# plugins) and the sharing of these tools through the two GitHub repositories below.

The first GitHub repository houses the base program builds (<https://github.com/lumevr/vLume/releases>), through which future revisions of vLUME will be released, with updated additional features and bug fixes. The community is able to suggest improvements and inform the developers of any bugs that they may encounter while running vLUME using the default issues report tab in GitHub.

Users can contribute to vLUME by uploading their own open-source code and communicate with other users through the second GitHub repository (https://github.com/lumevr/vLume_OpenSourcePlugins). In this area, contributors are able to create new branches and request pulls, to develop a subproject that can later be committed and merged to the main branch.

The four original scripts included with the first release of vLUME (see the section "Scripts in C# for local analysis") can be found in this repository as an initial starting point.

Application programming interface (API) to communicate with vLUME during runtime. The API enables scripts to communicate with the software runtime. It is initialized using the following libraries, which must be included at the start of scripts: using System; using System.Collections.Generic; using System.Collections; using System.Linq; using UnityEngine.

The API is able to perform various functions in two sets of actions: get the manually selected segmented data, and return and push the results back to the VR environment. There are two more actions to view the progress of a script and/or abort it. The number of actions that can be performed can be expanded in future software releases. Please refer to the software manual for a detailed explanation of each of the functions.

Exporting figures and videos. Videos (in .h264 format) and images (in .jpg format) can be captured from the vLUME environment and used for publication or presentation purposes. The fly-through video of the T cell (Supplementary Video 6), the images of the two-channel microtubule-clathrin in COS cells (Extended Data Fig. 1) and the nearest neighbor plot of the isolated *C. crescentus* (Extended Data Fig. 2) were taken using these integrated features (manual). The .h264 videos can be opened and converted to other formats (that is, .mov or .avi for larger compression ratios) in many video players (for example, VLC).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All the experimental data used in the present communication can be found in the Supplementary Software.

Code availability

vLUME is available as Supplementary Software. Updated versions of the software can also be found at <https://github.com/lumevr/vLume/releases>, vLUME software for Windows (with manual, license, samples and scripts). Open-source plugins for vLUME and a forum for collaborative creation and improvement can be found at https://github.com/lumevr/vLume_OpenSourcePlugins.

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Author contributions

S.F.L., A.S. and A.K. conceived the initial idea of visualizing 3D SMLM data in VR. A.S. and A.K. wrote, developed and designed the software. A.S., A.H. and D.E.-F. cowrote the open-source C# scripts. A.H. and D.E.-F. beta-tested the software for SMLM applications. A.H., D.E.-F., L.-M.N., R.H. and S.F.L. cowrote the manuscript. A.R.C. and A.P. performed and analyzed the T cell experiments. A.M.S., S.J.D. and J.M. provided the cell samples and labeling methodology. C.L. provided the spectrin data. All authors edited and revised the manuscript.

Competing interests

A.S. and A.K. are cofounders of Lume VR Ltd, a company dedicated to creating image analysis software for life sciences and working with groups to tackle visualization and analysis problems. Lume VR Ltd. is providing a free for academic use licence of vLUME governed by Terms of Use that can be found in the Supplementary Software folder. All intellectual property rights in the vLUME software (not the user data), throughout the world, belong to Lume VR Ltd. The remaining authors declare no competing interests.

Additional information

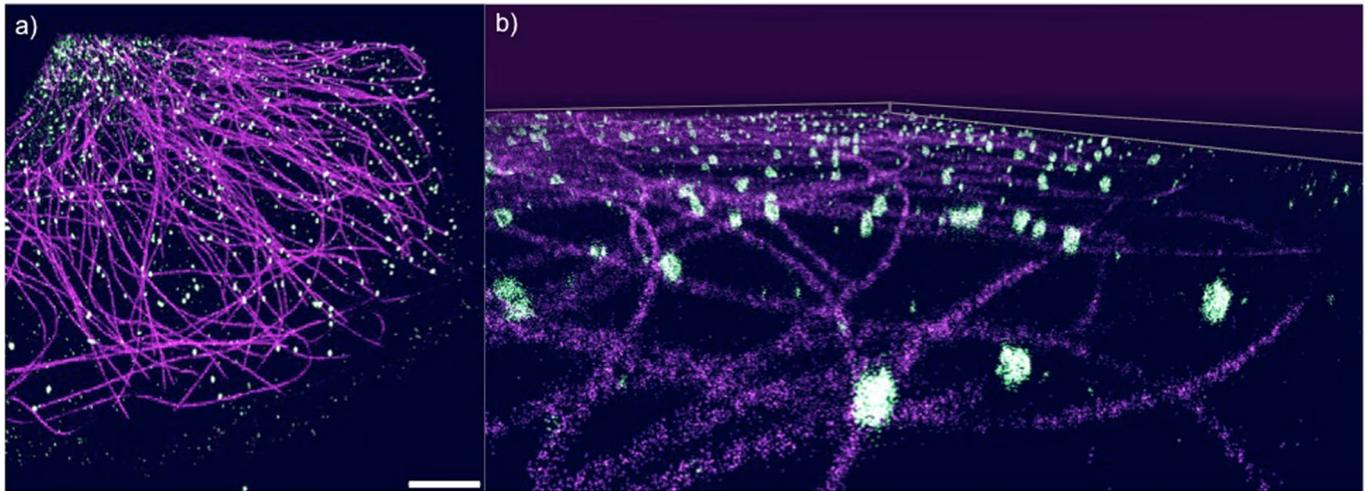
Extended data is available for this paper at <https://doi.org/10.1038/s41592-020-0962-1>.

Supplementary information is available for this paper at <https://doi.org/10.1038/s41592-020-0962-1>.

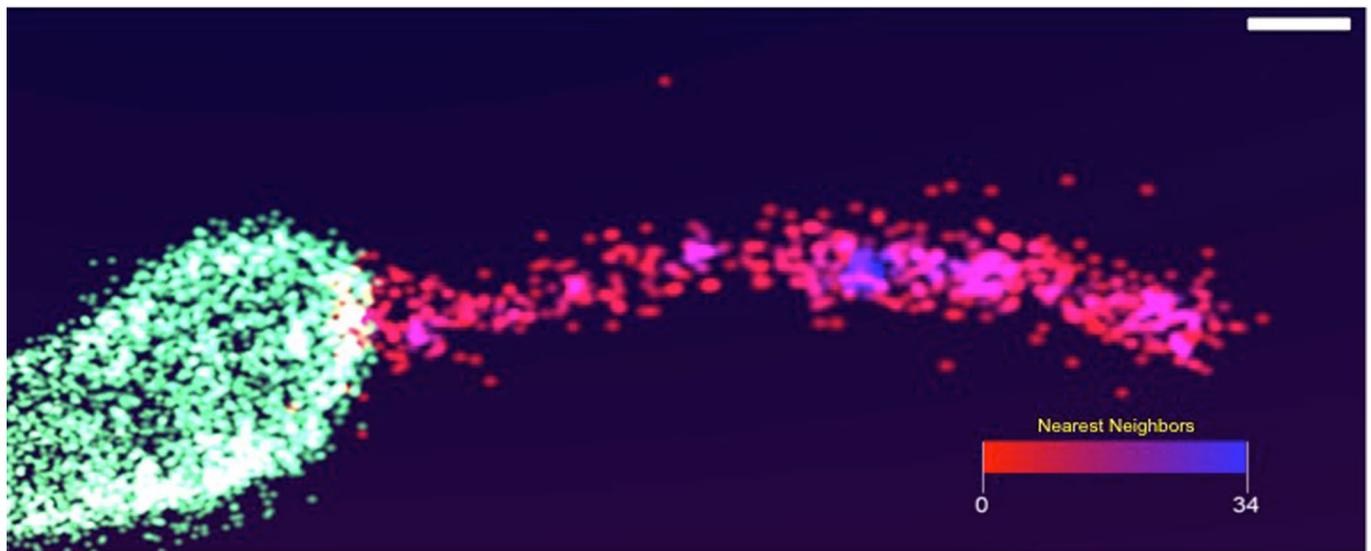
Correspondence and requests for materials should be addressed to S.F.L.

Peer review information Rita Strack was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | Microtubules and Clathrin in COS cells. Two-channel data of Microtubules (purple) and Clathrin (green) in COS cells from dataset 5. Scale bar is approximately 1 μm . **a**, A 'birds-eye' projection of the two channels in vLUME. **b**, The same data set from a different point of view closer to the ground to show the 3D nature of the data. To achieve this superposition the first channel has to be opened in vLUME and the color changed. Then the second channel also needs to be opened and changed in color. Subsequently with simple data translation of one of the datasets the two axes need to be overlapped (this task is very simple in VR, manual). The 3D nature of the oblique figure makes it difficult to render a scale bar.



Extended Data Fig. 2 | Nearest Neighbours plot on *Caulobacter* stalk. Nearest Neighbor plot using the C# script (CalcNearestNeighbour.cs) after selecting *Caulobacter crescentus*' stalk from dataset 4 of the Supplementary Information (Fig. 1d, e, left). The red to blue gradient of the image shows an increasing density of nearest neighbors within a radius of 50 nm (user defined). The color-gradient scale bar goes from 0 to 34 neighbors. Scale bar is approximately 200 nm.

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