

Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comments for Author:

Within the manuscript 'Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet' the authors addressed challenges in 3D single-molecule localization microscopy (SMLM) of thick samples, like high fluorescence background, long acquisition times, and chromatic errors in multi-color imaging. To tackle these challenges, the authors developed soTILT3D, an approach that integrates a range of technologies to generate a steerable light sheet using a single objective, bead-based drift correction with a novel microfluidic system with reflective optics. Data processing was done with a customized integration of existing 3D SMLM software and active drift correction.

Noteworthy results:

The novelty of this paper comprises the optical setup that generates a dithered steerable light sheet and the custom microfluidic chamber containing a mirror to reflected the beam. The main advantage of this system over other existing single-objective light sheets is that the light sheet beam is tilted only slightly towards the imaging plane. The type of construction enables imaging in close proximity to the coverslip while solution exchange is easily possible. The manuscript provides a detailed description to re-build soTILT3D, including the fabrication of the reflective microfluidic chamber.

In addition to these novelties, the authors integrate several already published advanced imaging and processing methods, including the double helix PSF shaping, the localization software DECODE for multi-emitter fitting active drift control and the drift correction using fluorescent beads. . All relevant sources are cited within the manuscript. In general, the manuscript is well written, the methods are described in great detail to enable reproduction of the data and re-building of the novel system. The quality of the manuscript suffers from a few issues (s. below).

General issues:

Most quantifications are based on single experiments (or N is not indicated), most graphs do not contain error bars and the main conclusions are not based on statistic tests. Particularly, more evidence is needed for the claim that soTILT3D offers enhanced speed and improved resolution in comparison to other available techniques. More specific issues are mentioned below:

Specific issues:

Background Reduction: The illumination scheme of soTILT3D largely enhances the signal-to-background ratios when compared to widefield-epi illumination but not in comparison to other light sheet systems (e.g. the 'TILT3D' which published by the same research group in 2018, which require two objectives). The authors should comment on that.

Imaging Speed Improvement:

The authors enhanced the speed of single molecule localization experiments (here DNA-PAINT) by employing higher imager concentrations. To address the challenge of overlapping emitters in those dense samples, they employed the recently published deep-learning-based multi-emitter fitting method known as 'DECODE.' The authors conducted a speed comparison between high-density data localized with DECODE and non-overlapping data processed using the localization software EASY-DHSP. The study revealed an approximate 8-fold increase in processing speed with an 8-fold rise in imager concentration, which is to be expected. The study would benefit much more from robust data illustrating the precise threshold at which multi-emitter fitting becomes essential as imager density increases. This transition could be quantified by comparing the resolution of single molecules with both algorithms across varying imager concentrations.

Resolution Enhancement:

The improved signal-to-back levels that light sheet illumination offers in soTILT3D leads to an improvement of the localization precision compared to Epi illumination (as expected by any light sheet approach). Unfortunately, Fig2c does not state how often this experiment was repeated and whether this improvement is statistically significant.

The experimental resolution of approximately 30 nm laterally and 40 nm axially has been reached in other double helix 3D SMLM approaches (see reference 55). It would be good if the authors could comment on the resolution that comparable approaches reach.

The improved resolution that DECODE achieves in dense probes, as compared to Easy-DHSP in sparse samples lacks substantiation through statistical tests on the FRC. Notably, Figure 3C lacks error bars, raising concerns regarding whether the quantification is derived solely from the single image in Figure 3a. A similar concern applies to the resolution in the multi-color approach of whole-cell imaging presented in Figure 5e.

Microfluidics and Multi-Target Imaging:

The authors offer a detailed description of a novel microfluidics system with reflective optics which indeed is an improvement, since the angle of the light sheet offers an illumination precisely within the image plane. In contrast to similar approaches the microfluidic chamber design allows to perform solution exchange and thus offers the possibility to do multicolour exchange PAINT experiments. From Figure S9 a channel-specific crosstalk of ~3-18% (post-to-pre-wash ratio) can be calculated. A high crosstalk of up to 18% would be unacceptable for precise multi-color experiments. Please comment on this high crosstalk and how that compares to the crosstalk of similar approaches in the field. Are there ways to reduce this crosstalk?

Drift control:

The authors used a rather complex two-step drift control, consisting of a real-time correction of the stage, based on transmission images of polystyrene beads and post-processing based on fluorescent beads. The additional path for transmission images adds additional complexity to the system. It would be beneficial if the authors could comment why they did not use the same type of fiducials for real-time and post-acquisition drift correction. To justify this two-step approach, a quantification of the efficiency of both single steps in drift correction would be necessary.

Data processing:

Some of the data was filtered to remove weak localizations. Additionally, 'spurious localizations' were removed from the images. This denoising might hide potential crosstalk. Please, provide also unfiltered data for each image in the supplement or main figures. Ideally the legends would indicate, which images were filtered. Are the quantifications in 3c and 5e based on raw unfiltered data? If not, please present also the FRC statistics without filtering.

Other issues:

The methods section lack information about the docking/imager sequences. Either the sequence or order number would be required to reproduce the assay.

Summary:

The work described in the paper appears to be of significant importance to the field of super-resolution microscopy and cell biology on the nano-scale. It presents a comprehensive platform, soTILT3D, that addresses several challenges in single-molecule super-resolution imaging of whole cells in 3D. This work provides detailed instructions for the integration of several advanced but already published aspects together with novel aspects. The work introduces one major novelty, which is the custom design of the reflective microfluidic chamber that allows fluid exchange and light sheet illumination in the imaging plane close to the coverslip. This approach makes further post-processing steps that would also compromise x/y resolution obsolete. However, in the current form the manuscript does not meet the expected standards in the imaging field, due to the issues mentioned above, especially the missing statistics and the use of highly filtered images. I therefore recommend a major revision addressing those issues before publication.

Reviewer #2

(Remarks to the Author)

Advanced Single-Objective Tilted Light Sheet Microscopy with Microfluidics for 3D Super-Resolution Imaging in Cells by Saliba et al.

Saliba and colleagues introduce an innovative microscopy technique for high-resolution, three-dimensional imaging of mammalian cell cultures. Their system incorporates a polydimethylsiloxane (PDMS) microfluidic device with a reflective metalized sidewall, enabling illumination at a 12-degree tilt from the focal plane. This setup facilitates comprehensive scanning in three optical dimensions, along with light-sheet pivoting to minimize shadows. The illumination's numerical

aperture is confined to a 39-degree half-angle due to the micromirror-based illumination method. The microfluidic channel measures 700 microns in width, 100 microns in height, and 1 cm in length. The system uniquely offers simultaneous light-sheet and diascope illumination, with the latter providing real-time feedback for drift correction. For enhanced resolution, the captured light is divided into two pathways, each passing through a double helix phase mask at a Fourier plane before reaching a single EMCCD for detection. The paper is thoroughly detailed and well-composed, effectively presenting the methods and implications of this novel imaging technology. While the manuscript may not break new ground in terms of innovation, its high-quality execution and clarity make it a valuable contribution to the field. Therefore, after a thorough review, I recommend considering this paper for publication, contingent upon addressing the following key points.

1. All Z localizations were scaled by a factor of 0.75. There is no indication that this correction value was empirically measured. If not, it seems appropriate given the sensitivity of the method to refractive index mismatches. While the full theoretical analysis of the shift is complex, an approximation can also be found in DOI 10.1038/s41596-020-00459-0. The correction should be done on a per-wavelength basis.
2. While the localization data is available upon request, it would be preferable for both the raw data and localizations to be made accessible on Zenodo, which permits uploads of approximately 50GB. In this case, a persistent DOI could be assigned to the data and included in the manuscript.
3. From Figure 1C, there appears to be a significant reduction in fluorescence at the limits of the double helix range, potentially impacting localization precision. Do the authors utilize localization events from the entire 2-micron range, or are localizations below a certain precision threshold omitted?
4. In the statement 'several LS approaches involving the use of separate objectives for illumination and detection...', the authors refer to oblique plane microscopes. These microscopes utilize a single objective for both illumination and detection of photons. Furthermore, many of the cited manuscripts do provide excellent optical sectioning in adherent cells. Therefore, this statement appears to be somewhat exaggerated. I recommend a careful review of each citation and necessary adjustments to the statement. Additionally, the omission of Wes Legant's 2016 Nature Methods paper should be addressed.
5. In the absence of the double helix phase mask, what is the Strehl ratio of your imaging system at a typical distance from the coverslip? The refractive index mismatch seems likely to result in a significant amount of spherical aberration. Indeed, even for the illumination beam, which has a lower numerical aperture, substantial spherical aberration is evident in Figure S2.
6. What is the apparent cause of the discrepancy between the average localization precision and the FRC resolution estimates? Could an alternative source of drift, beyond stage drift (which is corrected for), be contributing? Alternatively, is the FRC also indicating issues related to the density of labeling?
7. In Figure 3, it appears that the variables are confounded. Is the improvement in localization a result of the software alone, or a combination of the software and the imaging system? Specifically, what is the localization rate for the widefield mode, widefield + DECODE, light-sheet mode, and light-sheet mode + DECODE?
8. Certain drawbacks of this approach are not adequately addressed. For instance, the sample must be positioned close to the reflective surface. If it is too far away, imaging becomes unfeasible. This limitation reduces the throughput of the method.
9. The methods section lacks details on the software used for microscope control and data acquisition.
10. Critically, the main text states that the beam's thickness is 1.1 microns, yet Supplementary Figure 2 indicates a thickness of 2.1 microns.

Reviewer #3
Attached

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The article "Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet" has seen significant improvements compared to the initially submitted version. The authors have added substantial new material that convincingly supports their claims.

Specific Points:

Background Reduction:

The authors now provide additional examples demonstrating the superior signal-to-background ratio (SBR) of soTILT3D compared to epi-illumination. In their rebuttal letter, they highlighted this improvement over TILT3D by referring to the less pronounced increase of TILT3D vs. epi. However, it would strengthen the manuscript to include this comparison directly in the main text. This would again highlight the advance enhance the explanation that "soTILT3D employs a single, high-NA objective, resulting in a thinner light sheet suited for challenging samples with high background fluorescence" as a key technical advancement.

Imaging Speed Improvement:

The authors present convincing data showing that DECODE outperforms EASY-DHPSF in terms of both resolution and the number of localizations. Interestingly, EASY-DHPSF performance already declines at imager concentrations above 25 pM, which is quite low compared to other DNA-PAINT techniques (typically ~1 nM). The manuscript would benefit from a discussion on whether the need for low imager concentrations is an intrinsic limitation of soTILT3D.

Resolution Enhancement:

Compelling data from technical replicates on FRC quantification comparing DECODE and EASY-DHPSF (Fig. S9) has been added. The authors have also added repeated measurements demonstrating the improved localization precision due to background reduction when using the light sheet versus epi. In their rebuttal letter, the authors compare the soTILT3D resolution of 8 nm lateral and 12 nm axial to Pavani et al. (PNAS 2009), who achieved 10 nm lateral and 20 nm axial using the double-helix PSF. Including this comparison directly in the manuscript would help contextualize the resolution achievements.

Crosstalk:

The authors present clear evidence showing that crosstalk is limited to 2-3%, which is indeed negligible.

Drift Control:

The explanation of the two-step drift control process is clear, pragmatic, and easy to follow.

Data Processing:

An important update is the inclusion of raw, unfiltered data. Notably, the data already appears robust without denoising.

Summary:

In conclusion, this paper introduces a comprehensive platform for 3D super-resolution microscopy, combining novel and existing methods. With the new data and revisions, it provides a thorough technical characterization of the system, making it highly relevant to the field. The authors have addressed all my concerns, updated the figures and text, and provided additional replicates and data. With these changes, the manuscript now meets the expected standards in the imaging field. I highly recommend this excellent article for publication.

Reviewer #2**(Remarks to the Author)**

Thank you very much for providing such detailed and organized responses. Overall, I am happy with the revisions and feel as if the paper is greatly strengthened. The only statement that I am reluctant to accept is the response to query 8 for reviewer #2 regarding the drawbacks of the approach. In principle, the ETL can drive ~50 microns from the sidewall. Practically, this would be accompanied by a significant amount of spherical aberrations that would degrade the performance of the light-sheet. Also, it seems unlikely that one would be able to image close to the coverslip if the beam is ~50 microns away from the sidewall, as the unfocused, converging portion of the beam may interact with the coverslip itself far from the focus. Otherwise, I believe the manuscript is ready for publication.

Reviewer #3**(Remarks to the Author)****General Comments:**

I would like to highly congratulate the authors for their outstanding work. They have thoroughly addressed all of my previous comments and conducted major additional experiments, which further strengthen the manuscript. They have also provided relevant quantification and included stunning images that significantly enhance the overall presentation. This is an excellent piece of research that is now, in my view, ready for publication. I would also strongly encourage the authors to consider submitting their work for the journal's cover selection, as the visual elements presented in the manuscript are truly exceptional.

Minor points : While the authors have highlighted the live-cell imaging compatibility of the soTILT3D platform in the discussion, it would be beneficial to mention that in its current configuration, the system may not yet support the cultivation and maintenance of live cells for more than a few days, nor is it currently compatible with testing libraries of compounds. This could be a limitation, particularly for laboratories focused on long-term studies, such as developmental biology or drug screening. Addressing this point would help clarify potential challenges related to ease of use for these applications.

Additionally, I would like to suggest moving Supplementary Figure 13 to the main manuscript as Figure 6, or alternatively, using it to replace Figure 5, which could then be included in the supplementary materials. This change would better highlight

the significance of the data presented in Supplementary Figure 13, especially since Figure 1 mentions that cell aggregates can be imaged

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Below, we provide responses (in red) to each of the questions/comments (in black italic), with our additions to the text indicated (in blue).

Reviewer #1

Comments for Author:

Within the manuscript 'Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet' the authors addressed challenges in 3D single-molecule localization microscopy (SMLM) of thick samples, like high fluorescence background, long acquisition times, and chromatic errors in multi-color imaging. To tackle these challenges, the authors developed soTILT3D, an approach that integrates a range of technologies to generate a steerable light sheet using a single objective, bead-based drift correction with a novel microfluidic system with reflective optics. Data processing was done with a customized integration of existing 3D SMLM software and active drift correction.

We thank the reviewer for providing detailed comments and suggestions for improving the manuscript.

Noteworthy results:

The novelty of this paper comprises the optical setup that generates a dithered steerable light sheet and the custom microfluidic chamber containing a mirror to reflected the beam. The main advantage of this system over other existing single-objective light sheets is that the light sheet beam is tilted only slightly towards the imaging plane. The type of construction enables imaging in close proximity to the coverslip while solution exchange is easily possible. The manuscript provides a detailed description to re-build soTILT3D, including the fabrication of the reflective microfluidic chamber.

In addition to these novelties, the authors integrate several already published advanced imaging and processing methods, including the double helix PSF shaping, the localization software DECODE for multi-emitter fitting active drift control and the drift correction using fluorescent beads. All relevant sources are cited within the manuscript. In general, the manuscript is well written, the methods are described in great detail to enable reproduction of the data and re-building of the novel system. The quality of the manuscript suffers from a few issues (s. below).

We thank the reviewer for their highly positive comments about the novelty and benefits of our system.

General issues:

Most quantifications are based on single experiments (or N is not indicated), most graphs do not contain error bars and the main conclusions are not based on statistic tests. Particularly, more evidence is needed for the claim that soTILT3D offers enhanced speed and improved resolution in comparison to other available techniques. More specific issues are mentioned below:

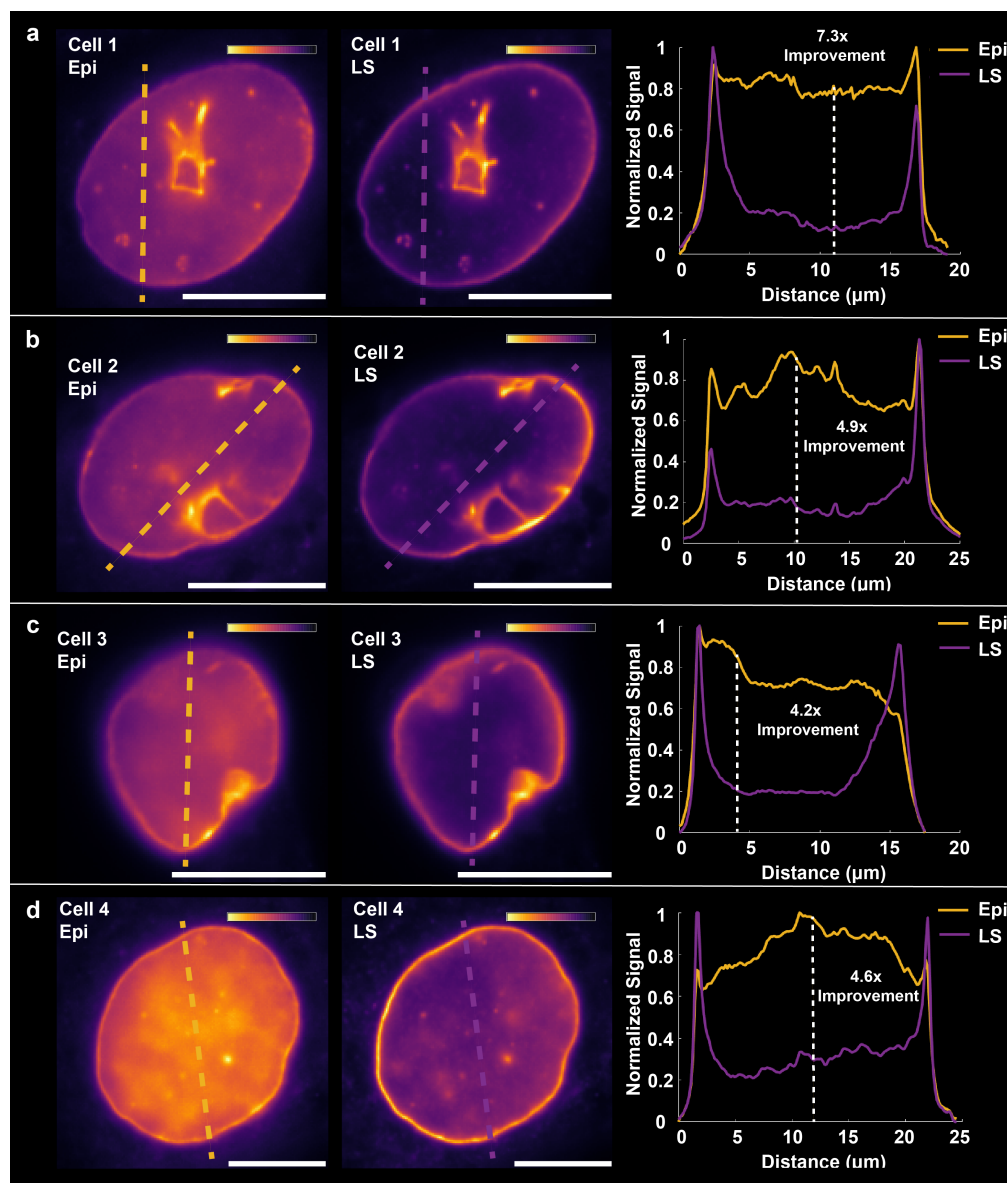
We thank the reviewer for these comments and we have addressed them point by point in the responses below.

Specific issues:

Background Reduction: The illumination scheme of soTILT3D largely enhances the signal-to-background ratios when compared to widefield-epi illumination but not in comparison to other light sheet systems (e.g. the 'TILT3D' which published by the same research group in 2018, which require two objectives). The authors should comment on that.

Since soTILT3D is based on a single, high numerical aperture objective for illumination, the thickness of the light sheet can be made much thinner than previous designs where a dual-objective system with a lower numerical aperture was used. SoTILT3D was here used with a beam waist radius of 1.1 μm compared to the 2.1 μm beam waist radius achievable with the TILT3D system. The TILT3D system was reported to yield a roughly 2x SBR improvement for diffraction-limited imaging and up to 5x SBR improvement for single-molecule imaging, whereas the soTILT3D system achieves > 4x SBR improvement for diffraction-limited imaging and > 6x SBR improvement for single-molecule imaging. Due to the inherent variability between samples such as cell thickness and labeling density, the numbers for the SBR can vary between measurements and studies. For single-molecule demonstrations we show that soTILT3D achieves comparable performance despite utilizing DNA-PAINT which is subject to much higher background as well as higher labeling densities. Ultimately, the optical sectioning capability will depend on the thickness of the light sheet, which is why soTILT3D can handle more challenging imaging conditions than TILT3D.

To demonstrate that soTILT3D can achieve even better sectioning than the TILT3D system, we provide more examples of nuclei sectioned with the soTILT3D light sheet versus epi-illumination in a new [Supplementary Fig. 5](#).



Supplementary Fig. 5. Diffraction-limited images of lamin B1 excited with epi- or light sheet (LS) illumination. Graphs show line scans demonstrating the consistent signal-to-background ratio (SBR) improvement with LS compared to epi-illumination. SBR improvement is variable between samples, ranging from >4x to >7x. Scale bars 10 μm . The colorbars show intensity normalized independently for each image.

We have adapted the text in the *SoTILT3D improves the localization precision of 3D single-molecule imaging* section in the *Results* to include this new supplementary figure:

Using the LS improves the SBR **over 4x** for diffraction-limited imaging (Fig. 2a, [Supplementary Fig. 5](#)) and over 6x for single-molecule imaging (Fig. 2b), as demonstrated for imaging of lamin B1 in U2OS cells.

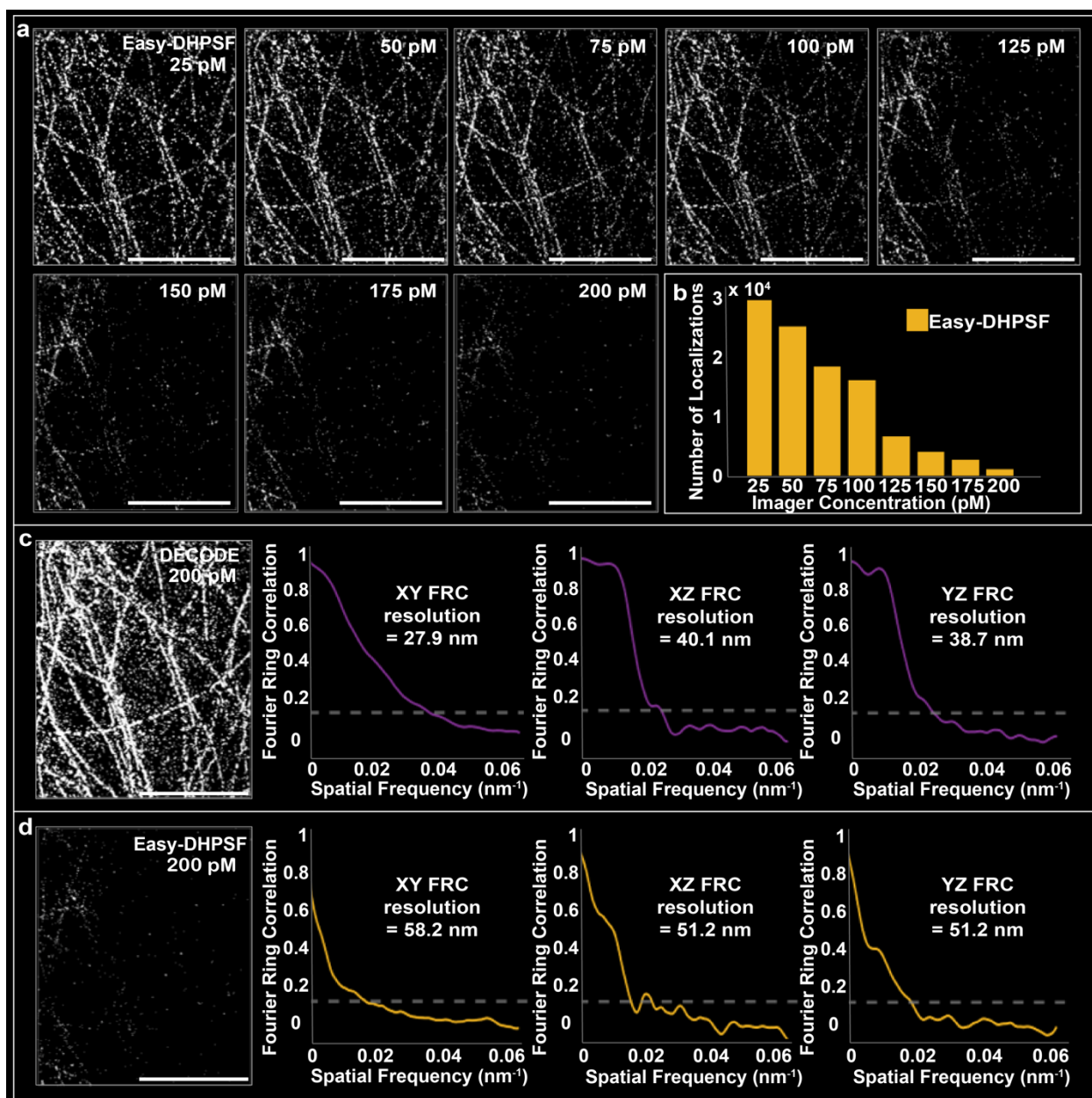
We have also added the following sentence to the *Discussion* to clarify the improved sectioning capability of soTILT3D compared to TILT3D:

In contrast to our earlier tilted LS design that utilized two objectives¹⁵, soTILT3D employs a single, high-NA objective. This configuration enables the formation of a significantly thinner LS, making it well-suited for challenging samples that exhibit higher background fluorescence.

Imaging Speed Improvement:

The authors enhanced the speed of single molecule localization experiments (here DNA-PAINT) by employing higher imager concentrations. To address the challenge of overlapping emitters in those dense samples, they employed the recently published deep-learning-based multi-emitter fitting method known as 'DECODE.' The authors conducted a speed comparison between high-density data localized with DECODE and non-overlapping data processed using the localization software EASY-DHFSP. The study revealed an approximate 8-fold increase in processing speed with an 8-fold rise in imager concentration, which is to be expected. The study would benefit much more from robust data illustrating the precise threshold at which multi-emitter fitting becomes essential as imager density increases. This transition could be quantified by comparing the resolution of single molecules with both algorithms across varying imager concentrations.

We thank the reviewer for this comment. We have added a new supplementary figure (Supplementary Fig. 8a,b) showing how the number of localizations drastically decreases after 25 pM with increasing imager strand density when analyzed with Easy-DHPSF. By 200 pM, the concentration at which the high-density imaging was performed (Fig. 3a,b), EasyDHPSF only detected 1,222 localizations compared to 108,254 detected with DECODE for the same data set (Supplementary Fig. 8c,d). This demonstrates the requirement of using neural net based analysis for high emitter density imaging. We have also in this figure quantified the resulting FRC resolution values (Supplementary Fig. 8c,d) achieved when analyzing the dense emitter concentration sample using the two approaches which shows the resolution degradation when using Easy-DHPSF for such samples.



Supplementary Fig. 8. Density breaking point for 3D single-molecule super-resolution imaging analysis with Easy-DHPSF, and comparison of the number of localizations and the resolution at overlapping-emitter densities when analyzing with Easy-DHPSF and DECODE. **a** 3D single-molecule super-resolution reconstructions of microtubules imaged with increasing imager strand concentrations and analyzed with Easy-DHPSF, simulated by summing frames from a sparse single-molecule data set, demonstrating the loss of localizations as the emitter density increases. A total of 25,000 frames after summation were analyzed for each concentration. Scale bars 5 μm . **b** Quantification of the number of localizations from the images in **a**, demonstrating a steady decrease in detected emitters after 25 pM when analyzed with Easy-DHPSF. **c** Reconstruction of the 200 pM imager strand concentration microtubule data set from **a**, when analyzed with DECODE, and resulting Fourier ring correlation (FRC) resolution analysis in the xy, xz, and yz planes, respectively. **d** Reconstruction of the 200 pM imager strand concentration

microtubule data set from a, analyzed with Easy-DHPSF, and resulting FRC resolution analysis in the xy, xz, and yz planes, respectively. For the same data set, DECODE detected 108,254 localizations, while EasyDHPSF detected 1,222 localizations. The FRC curves demonstrate improved resolution when analyzing with DECODE compared to Easy-DHPSF. Scale bars 5 μm .

We have also added the following in the *SoTILT3D improves the speed of 3D single-molecule imaging* section in the *Results* section of the manuscript:

The speed improvement of soTILT3D together with deep learning analysis was quantified by 3D single-molecule SR imaging of microtubules in U2OS cells.

First, the approach was benchmarked by analysis of the same fluorescent bead data and single-molecule data with Easy-DHPSF^{69,70}, an open-source least-squares fitting-based analysis software, and DECODE, which resulted in comparable localization precisions (Supplementary Fig. 7).

Then, the density breaking point at which 3D single-molecule data could no longer be localized in Easy-DHPSF due to overlapping emitters and at which analysis with DECODE becomes necessary was quantified (Supplementary Fig. 8). The number of detected localizations with Easy-DHPSF was found to decrease after 0.025 nM as the emitter concentration increased (Supplementary Fig. 8a,b). The high-density data was localized with a DECODE model which was trained on the 0.025 nM microtubule data set and converged to a Jaccard Index of 0.69 after 1000 epochs. After 25,000 frames at an imager strand concentration of 0.2 nM, the high-density regime, only 1,222 emitters were localized with Easy-DHPSF compared to 108,254 localized with DECODE (Supplementary Fig. 8c,d). The resulting Easy-DHPSF resolution values were also found to be degraded in comparison to those in the DECODE case (Supplementary Fig. 8c,d).

Next, high density data was acquired with a 0.2 nM concentration of imager strands while our standard concentration data for non-overlapping emitters was acquired at 0.025 nM (Fig. 3a,b). The non-overlapping single-molecule data was localized with Easy-DHPSF and the high-density data was localized with DECODE. This resulted in a more than ten-fold increase in possible imaging speed when using DECODE in conjunction with LS illumination (Fig. 3a,b).

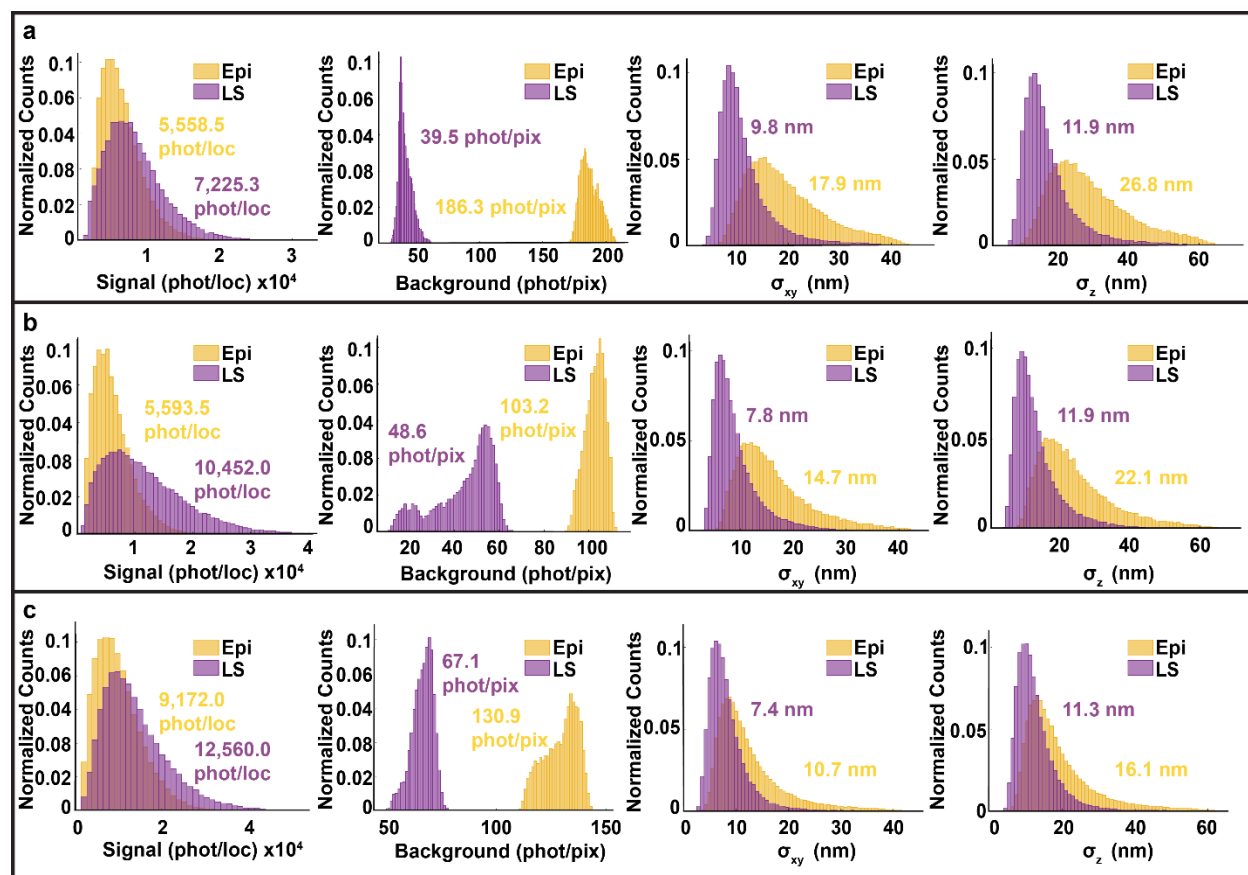
We have also updated the *Data analysis* section in *Methods* accordingly:

For microtubule data sets quantifying the density breaking point of Easy-DHPSF and the resulting resolution of high-density data when analyzed with DECODE compared to Easy-DHPSF (Supplementary Fig. 8), reconstructions were filtered to remove localizations with localization precisions greater than 30 nm in xy and greater than 40 nm in z.

Resolution Enhancement:

The improved signal-to-back levels that light sheet illumination offers in soTILT3D leads to an improvement of the localization precision compared to Epi illumination (as expected by any light sheet approach). Unfortunately, Fig2c does not state how often this experiment was repeated and whether this improvement is statistically significant.

We thank the reviewer for this comment. We have added a new Supplementary Fig. 6 showing three technical replicates, all showing consistent results in which the localization precision is improved with light sheet illumination.



Supplementary Fig. 6. Three technical replicates of the 3D single-molecule super-resolution imaging of lamin B1 shown in Figure 2c. a-c Epi-illumination and light sheet (LS) illumination imaging over 10,000 frames of three different cells labeled for lamin B1 demonstrating consistent improvements in the localization precision due to background reduction when using LS illumination (purple) compared to epi-illumination (yellow). The median values of the distributions are indicated for each histogram.

Additionally, we have added the following sentences to the *SoTILT3D improves the localization precision of 3D single-molecule imaging* section in the *Results* section of the manuscript regarding the replicates that also clarifies that the data shown in Fig. 2c is a representative example:

Single-molecule DNA-PAINT data of lamin B1 in U2OS cells was acquired in 3D using the DH-PSF with epi- or LS illumination to assess the performance of the soTILT3D platform in terms of localization precision (Fig. 2c, Supplementary Fig. 6). In a representative example (Fig. 2c), while roughly matching the signal intensities from the individual molecules with median values of 8,400 photons/localization for epi- and 11,400 photons/localization for LS illumination, the fluorescence background was drastically reduced when using LS illumination and resulted in median background values of 125 photons/pixel for epi- compared to 64 photons/pixel for LS illumination. This resulted in improvements in lateral and axial localization precisions, with median values for epi-illumination of 11.2 nm in xy and 16.9 nm in z and median values for LS illumination of 7.8 nm in xy and 11.8 nm in z (Fig. 2c). The median lateral and axial localization precisions across three technical replicates were found to be 17.9 nm, 14.7 nm, and 10.7 nm in xy and 26.8 nm, 22.1 nm,

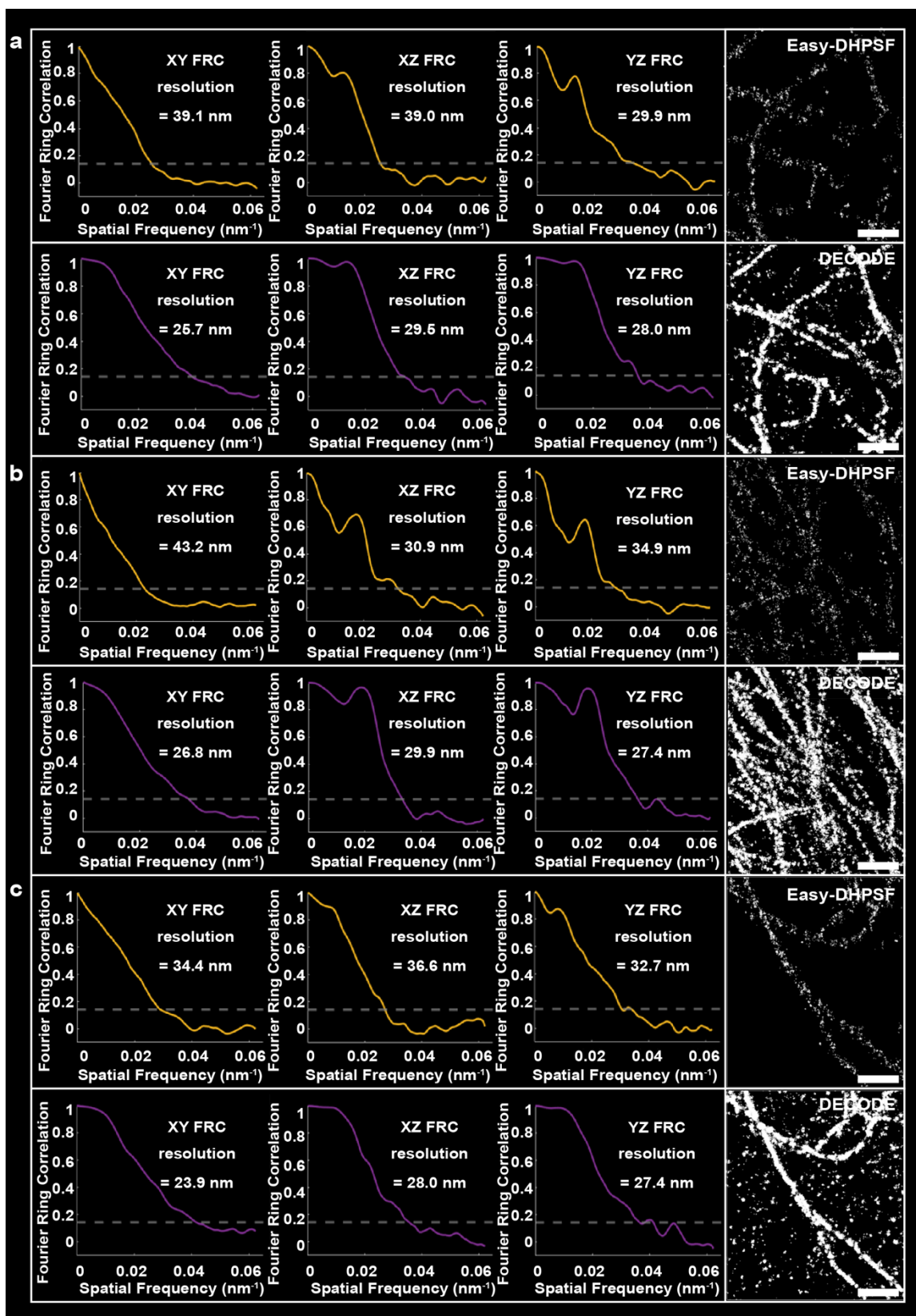
and 16.1 in z for epi-illumination, while the median values for LS illumination were 9.8 nm, 7.8 nm, and 7.4 nm in xy and 11.9 nm, 11.9 nm, and 11.3 nm in z (Supplementary Fig. 6).

The experimental resolution of approximately 30 nm laterally and 40 nm axially has been reached in other double helix 3D SMLM approaches (see reference 55). It would be good if the authors could comment on the resolution that comparable approaches reach.

In original reference 55 (Pavani *et al.*, PNAS 2009), the authors report localization precisions as low as 10 nm laterally and 20 nm axially for isolated fluorescent molecules with 500 ms exposure times using the double helix PSF. However, they do not report on the resulting resolution. Here, we achieve localization precisions down to 8 nm laterally and 12 nm axially with the double helix PSF when imaging inside cells with 100 ms exposure times. One paper that reports on resolution directly through Fourier Shell Correlation is provided in reference 11 (Chung *et al.* Nat. Methods, 2022), which achieves a resolution of just over 40 nm using a 4pi microscope (which roughly doubles the number of detected photons) and the astigmatic PSF which has a much shorter axial range of less than 1 micron compared to the double helix PSF used here with an axial range close to 3 microns. In comparison, in this work we achieve whole nucleus resolution below 40 nm in all dimensions. To our knowledge, other similar approaches utilizing the double helix PSF do not report resolution values.

The improved resolution that DECODE achieves in dense probes, as compared to Easy-DHPSF in sparse samples lacks substantiation through statistical tests on the FRC. Notably, Figure 3C lacks error bars, raising concerns regarding whether the quantification is derived solely from the single image in Figure 3a. A similar concern applies to the resolution in the multi-color approach of whole-cell imaging presented in Figure 5e.

We thank the reviewer for this comment. We have added a new [Supplementary Fig. 9](#) with statistics of three additional samples prepared and imaged across multiple different days demonstrating reproducibility in the FRC measurements in Figure 3c.



Supplementary Fig. 9. Three technical replicates of the 3D single-molecule super-resolution imaging of microtubules shown in Figure 3c,d. a-c Consistent resolution improvement shown

by Fourier ring correlation (FRC) analysis in the xy, xz, and yz planes, respectively, for three samples of 3D single-molecule super-resolution light sheet imaging of microtubules imaged at an imager strand concentration of 0.025 nM and analyzed with Easy-DHPSF (yellow) and an imager strand concentration of 0.2 nM and analyzed with DECODE (purple). Scale bars 1 μm .

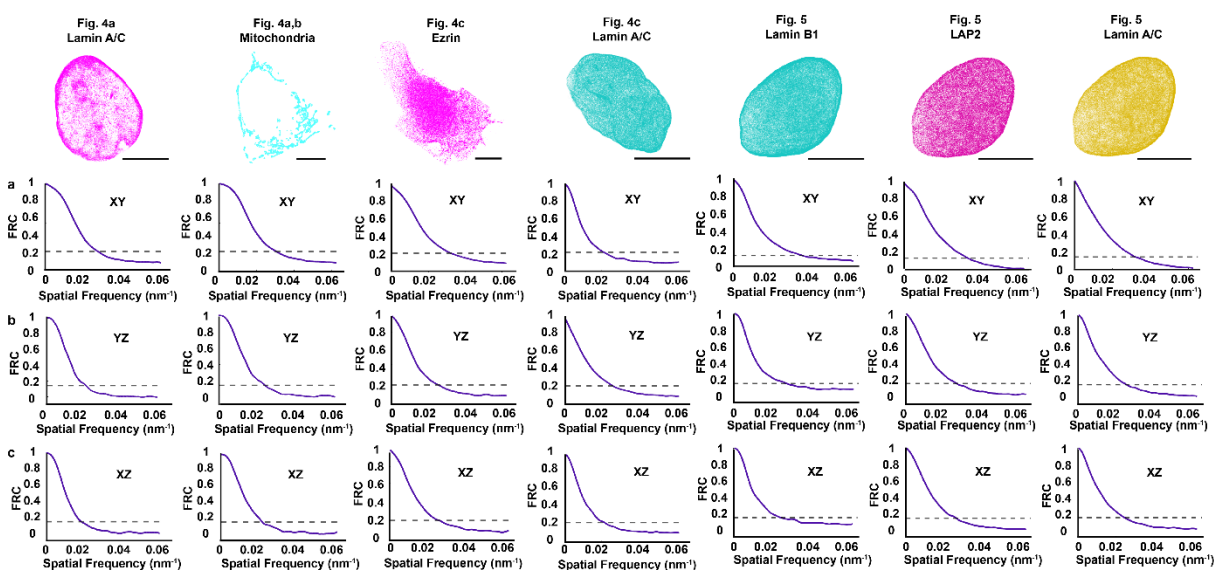
We have updated the text in the *SoTILT3D improves the speed of 3D single-molecule imaging* section in the *Results* section of the manuscript accordingly to include the new data.

The achieved resolutions after 50,000 frames were estimated using FRC calculations in the xy, yz, and xz planes, resulting in resolutions of 35.4/38.6/36.9 nm and 28.4/32.1/33.0 nm for the Easy-DHPSF data set and the DECODE data set, respectively, in the representative example shown in Fig. 3c,d, demonstrating overall improved resolution when using DECODE for the same number of frames. The mean resolution across three technical replicates (Supplementary Fig. 9) was 38.9 ± 2.1 nm, 35.5 ± 2.0 nm, and 32.5 ± 1.2 nm for the Easy-DHPSF data sets and 25.5 ± 0.7 nm, 29.1 ± 0.5 nm, and 27.6 ± 0.2 nm for the DECODE data sets (reported as mean $\pm$ standard error of the mean) in the xy, xz, and yz planes, respectively.

We have also clarified in the figure caption that the data in Fig. 3c,d is a representative example:

c Fourier ring correlation (FRC) curves in the xy, yz, and xz planes after 50,000 frames for the Easy-DHPSF-analyzed data and **d**, the DECODE-analyzed data for the representative example shown in a, demonstrating improved resolution when using DECODE.

The data added demonstrates reproducibility across replicates. Updated Supplementary Figure 11 (now extended to also include new reconstructions of ezrin and lamin A/C shown in revised Fig. 4) demonstrates, together with Figure 5, the robustness in the resulting FRCs across multiple different cellular structures.



Supplementary Fig. 11. Statistics for double-helix PSF localization data used for the 3D super-resolution reconstructions of lamin A/C, mitochondria, and ezrin in Fig. 4 and of lamin B1, LAP2, and lamin A/C in Fig. 5. a Fourier ring correlation (FRC) for the xy plane, **b**

FRC for the yz plane, and c FRC for the xz plane. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4a,b were 35.0/45.7/51.5 nm for lamin A/C and 32.7/40.2/44.9 nm for mitochondria. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4c were 31.0/39.2/38.2 nm for ezrin and 38.0/46.3/47.2 nm for lamin A/C. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 5 were 27.9/36.1/39.9 nm for lamin B1, 30.2/37.6/38.0 nm for LAP2, and 31.3/38.9/40.6 nm for lamin A/C. Scale bars 10 μ m.

We have added the following sentence to the *Whole-cell multi-target 3D single-molecule super-resolution imaging with soTILT3D* section in the *Results* section of the manuscript:

Taken together, the resolution from the FRC analyses were found to be 32.3 ± 1.2 nm, 40.6 ± 1.4 nm, and 42.9 ± 1.8 nm (reported as mean $\pm$ standard error of the mean for seven structures, see Supplementary Fig. 11) in the xy, yz, xz plane, respectively, for all targets, demonstrating robust performance across multiple different cellular structures.

Microfluidics and Multi-Target Imaging:

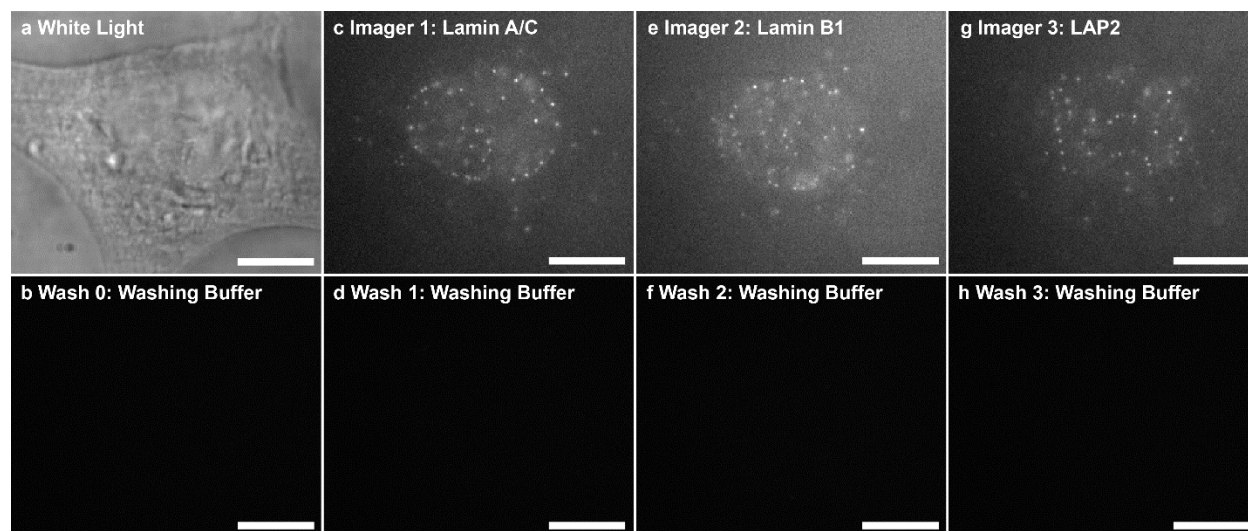
The authors offer a detailed description of a novel microfluidics system with reflective optics which indeed is an improvement, since the angle of the light sheet offers an illumination precisely within the image plane. In contrast to similar approaches the microfluidic chamber design allows to perform solution exchange and thus offers the possibility to do multicolour exchange PAINT experiments.

We thank the reviewer for these very positive comments about our microfluidic system.

From Figure S9 a channel-specific crosstalk of ~3-18% (post-to-pre-wash ratio) can be calculated. A high crosstalk of up to 18% would be unacceptable for precise multi-color experiments. Please comment on this high crosstalk and how that compares to the crosstalk of similar approaches in the field. Are there ways to reduce this crosstalk?

The nonspecific localizations after washes were relatively constant between targets whereas the number of specific localizations varied for each target depending on labeling density. We acquired new data where we better matched the labeling densities across the different samples and acquired more statistics to more accurately show the crosstalk for the different probes.

We have updated Supplementary Fig. 16 with this new data and updated the caption accordingly with the new values. This new data with better matched labeling densities across targets showed a crosstalk of ~ 2-3% between different probes, making it acceptable for multi-target experiments.



Supplementary Fig. 16. DNA-PAINT imaging controls. 1,000 frames for each target were acquired in 2D with the same imaging settings and analyzed in ThunderSTORM with the same settings and filters. **a** White light image of a cell labeled for lamin A/C, lamin B1, and LAP2 and **b** wash 0 with washing buffer which yielded 33 localizations. Single-molecule data **c** of 0.1 nM imager strand concentration for lamin A/C, which yielded 27,838 localizations, **d** after wash 1 with washing buffer, which yielded 750 localizations, **e** of 0.04 nM imager strand concentration for lamin B1, which yielded 22,276 localizations, **f** after wash 2 with washing buffer, which yielded 639 localizations, **g** of 0.1 nM imager strand concentration for LAP2, which yielded 27,028 localizations, and **h** after wash 3 with washing buffer, which yielded 575 localizations. Scale bars 10 μm .

Drift control:

The authors used a rather complex two-step drift control, consisting of a real-time correction of the stage, based on transmission images of polystyrene beads and post-processing based on fluorescent beads. The additional path for transmission images adds additional complexity to the system. It would be beneficial if the authors could comment why they did not use the same type of fiducials for real-time and post-acquisition drift correction. To justify this two-step approach, a quantification of the efficiency of both single steps in drift correction would be necessary.

As the reviewer points out, real-time localization of a fluorescent fiducial bead is an alternative to transmission-based active-stabilization drift correction. However, this would require algorithm development for real-time fitting and hardware feedback control. The extra pathway for transmission-based drift correction only required the addition of a few additional optics and a simple CMOS camera and could be implemented with the already available Micromanager plugin. Therefore, we went with this option.

We have added a discussion about this in the manuscript *Discussion* section.

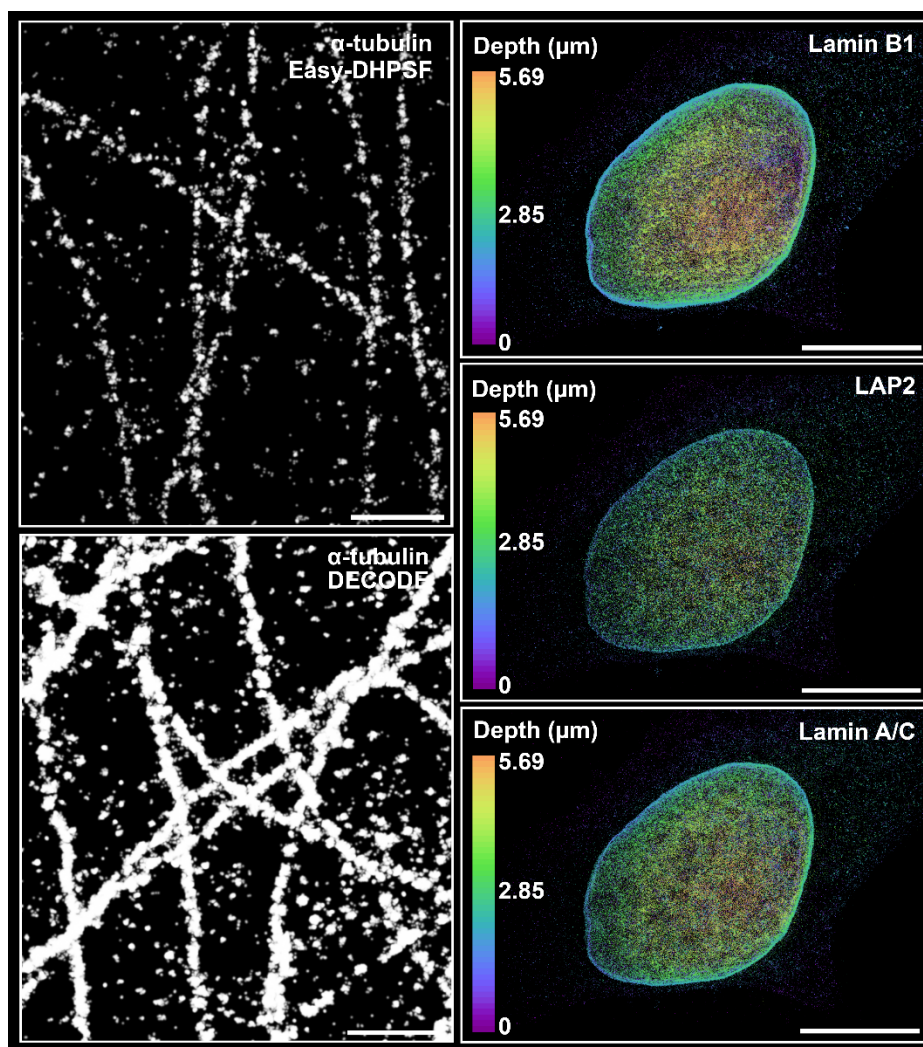
Transmission-based imaging of polystyrene beads was used for real-time stabilization to keep the sample within the field of view and within the same LS slice during long-term imaging. This approach is based on an open-source plugin for easy implementation¹³. The fluorescent fiducial beads were then used to correct any residual drift in post-processing. An alternative approach is

using real-time localization of the fluorescent bead together with real-time feedback to the stage for active stabilization during imaging¹⁵.

Data processing:

Some of the data was filtered to remove weak localizations. Additionally, 'spurious localizations' were removed from the images. This denoising might hide potential crosstalk. Please, provide also unfiltered data for each image in the supplement or main figures. Ideally the legends would indicate, which images were filtered. Are the quantifications in 3c and 5e based on raw unfiltered data? If not, please present also the FRC statistics without filtering.

We thank the reviewer for this comment. We have added a new **Supplementary Fig. 19** which includes the reconstructions without filtering for spurious localizations for Figures 3 and 5, and have included their corresponding FRC statistics.



Supplementary Fig. 19. Reconstructions for double-helix PSF localization data used for the 3D super-resolution reconstructions of microtubules in Fig. 3a and of lamin B1, LAP2, and

lamin A/C in Fig. 5 without filtering of spurious localizations. The FRC resolutions in xy/yz/xz for the reconstructions of microtubules in Fig. 3a when including spurious localizations were 36.1/41.8/42.2 nm for the Easy-DHPSF analyzed sample and 29.8/37.2/42.2 nm for the DECODE analyzed sample. Scale bars 1 μ m. The FRC resolutions in xy/yz/xz nm for the reconstructions in Fig. 5 when including spurious localizations were 22.5/29.7/29.0 nm for lamin B1, 31.8/39.5/41.4 nm for LAP2, and 32.7/40.2/43.6 nm for lamin A/C. Scale bars 10 μ m.

We refer to this figure in the *Data analysis* section in *Methods*.

For Easy-DHPSF versus DECODE comparison data of microtubules (Fig. 3a and Supplementary Fig. 9), localizations with a localization precision greater than 20 nm in xy or greater than 30 nm in z were filtered out. The data sets were also filtered for 20-nearest neighbors with a denoise range of 0.01 to 10 to remove spurious localizations. For reconstructions and FRC resolution values without filtering of spurious localizations, please see Supplementary Figure 19.

Whole-cell lamin B1, lamin A/C, and LAP2 data sets (Figs. 5a,b) were filtered to remove localizations with localization precision greater than 30 nm in xy and greater than 150 in z, and for 10-nearest neighbors with a denoise range of 0.01 to 10 to remove spurious localizations. Each slice was also filtered to remove localizations with z values greater than 1 μ m (the upper limit of our 2- μ m DH-PSF range). For reconstructions and FRC resolution values without filtering of spurious localizations, please see Supplementary Figure 19.

We have further addressed this comment by modifying Figure 4 in the manuscript so that all reconstructions are rendered and shown without denoising, and we have updated the caption accordingly with additional text regarding crosstalk.

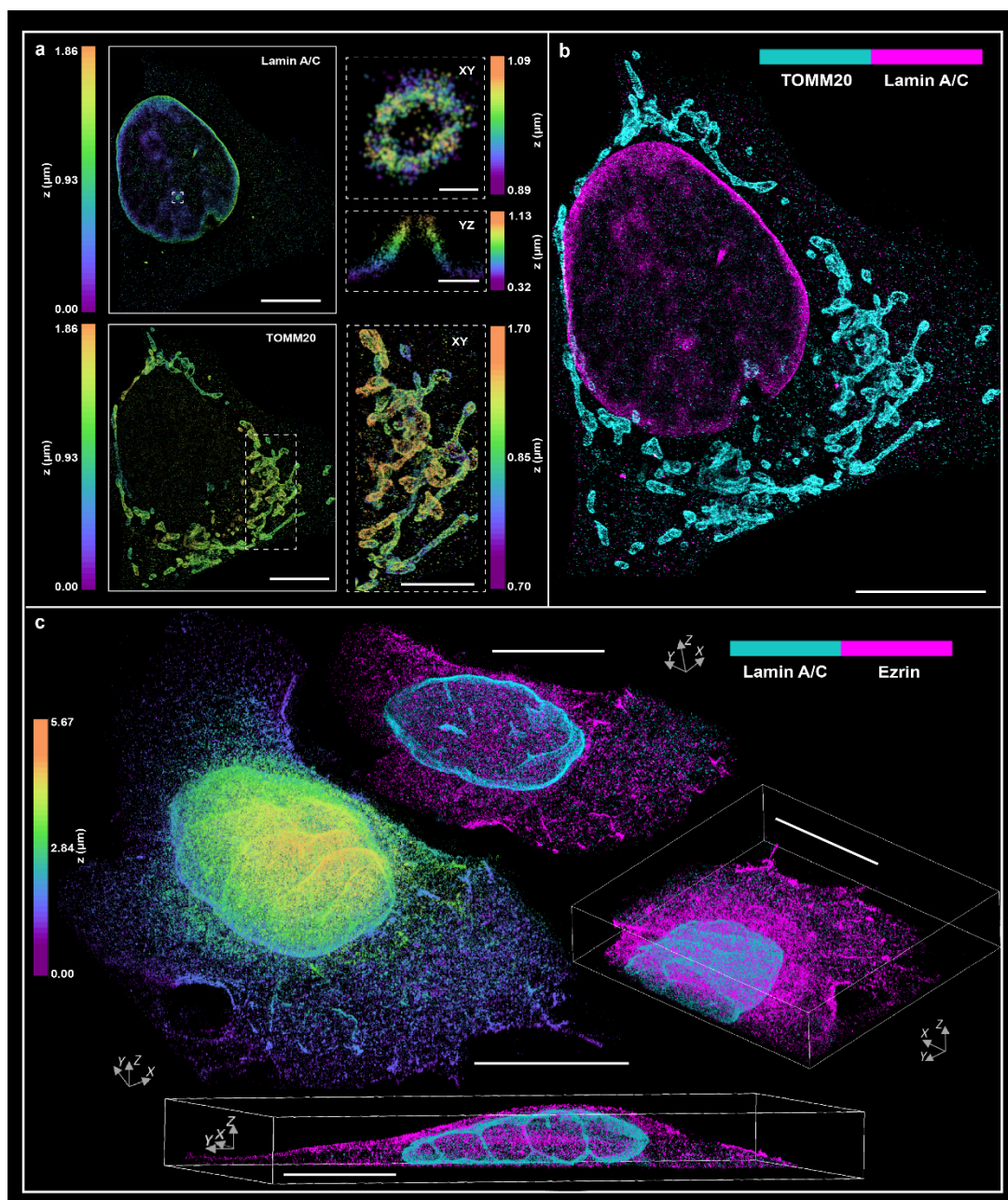


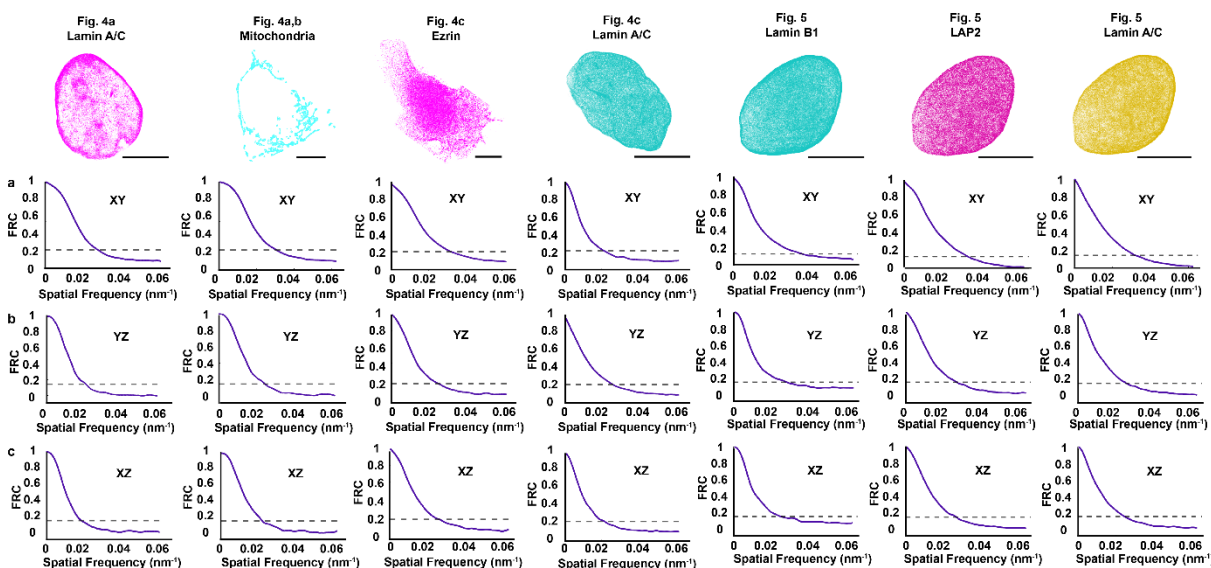
Fig. 4. Two-target 3D high-density whole-cell single-molecule super-resolution (SR) imaging with soTILT3D. **a** 3D SR reconstruction of separately rendered lamin A/C and mitochondria (TOMM20) in the same U2OS cell, revealing no notable crosstalk between the two targets. Scale bars 10 μm . Inset panels for lamin A/C shown for a 200 nm thick z-slice in the xy plane and for a 150 nm thick x-slice in the yz plane resolved the nanoscale architecture of a nuclear lamina channel. Xy inset scale bar 200 nm and yz inset scale bar 500 nm. Inset panel for mitochondria shows details of the mitochondrial network. Mitochondrial inset scale bar 5 μm . **b** A merge of the 3D SR reconstructions of lamin A/C and mitochondria shown in a. Scale bar 10 μm . **c** 3D whole-cell SR reconstruction of lamin A/C and ezrin. The left panel shows a merged reconstruction of both targets, colored by depth. The top panel shows a 1.2 μm thick z-slice colored by probe. The right panel shows the bottom right half of the reconstruction in the left panel. The bottom panel shows a 2.5 μm thick slice through the center of the cell. Scale bars 10 μm .

We also updated the text in the *Whole-cell multi-target 3D single-molecule super-resolution imaging with soTILT3D* section of the *Results* accordingly:

First, 3D two-target imaging was demonstrated by imaging of lamin A/C and mitochondria in U2OS cells, clearly resolving nuclear lamina channels and the intricate mitochondrial network (Fig. 4a,b). The resulting FRC resolutions in the xy/yz/xz planes were found to be 35.0/45.7/51.5 nm for lamin A/C and 32.7/40.2/44.9 nm for mitochondria (Supplementary Fig. 11).

Next, 3D two-target imaging of whole cells was demonstrated by imaging of membrane-bound protein ezrin and lamin A/C in U2OS cells, where the entire axial profile of the cell was reconstructed (Fig. 4c). The resulting FRC resolutions in the xy/yz/xz planes were found to be 31.0/39.2/38.2 nm for ezrin and 38.0/46.3/47.2 nm for lamin A/C (Supplementary Fig. 11).

Furthermore, we have updated Supplementary Figure 11 and the corresponding caption to reflect the FRC values of the reconstructions without denoising in Figure 4.



Supplementary Fig. 11. Statistics for double-helix PSF localization data used for the 3D super-resolution reconstructions of lamin A/C, mitochondria, and ezrin in Fig. 4 and of lamin B1, LAP2, and lamin A/C in Fig. 5. a Fourier ring correlation (FRC) for the xy plane, **b** FRC for the yz plane, and **c** FRC for the xz plane. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4a,b were 35.0/45.7/51.5 nm for lamin A/C and 32.7/40.2/44.9 nm for mitochondria. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4c were 31.0/39.2/38.2 nm for ezrin and 38.0/46.3/47.2 nm for lamin A/C. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 5 were 27.9/36.1/39.9 nm for lamin B1, 30.2/37.6/38.0 nm for LAP2, and 31.3/38.9/40.6 nm for lamin A/C. Scale bars 10 μ m.

Other issues:

The methods section lack information about the docking/imager sequences. Either the sequence or order number would be required to reproduce the assay.

We thank the reviewer for pointing out that this information was missing. The docking and imager strands are ordered through Massive Photonics, which do not provide information about the specific sequences, but we have added order numbers for the three-target DNA-PAINT kits used which includes all docking and imager strands so that the order may be reproduced.

Summary:

The work described in the paper appears to be of significant importance to the field of super-resolution microscopy and cell biology on the nano-scale. It presents a comprehensive platform, soTILT3D, that addresses several challenges in single-molecule super-resolution imaging of whole cells in 3D. This work provides detailed instructions for the integration of several advanced but already published aspects together with novel aspects. The work introduces one major novelty, which is the custom design of the reflective microfluidic chamber that allows fluid exchange and light sheet illumination in the imaging plane close to the coverslip. This approach makes further post-processing steps that would also compromise x/y resolution obsolete. However, in the current form the manuscript does not meet the expected standards in the imaging field, due to the issues mentioned above, especially the missing statistics and the use of highly filtered images. I therefore recommend a major revision addressing those issues before publication.

We once again thank the reviewer for their highly positive comments about the significance and novelty of our platform and for their helpful feedback on how to further improve our manuscript. We have addressed all reviewer comments in the point-by-point responses above.

Reviewer #2

Advanced Single-Objective Tilted Light Sheet Microscopy with Microfluidics for 3D Super-Resolution Imaging in Cells by Saliba et al.

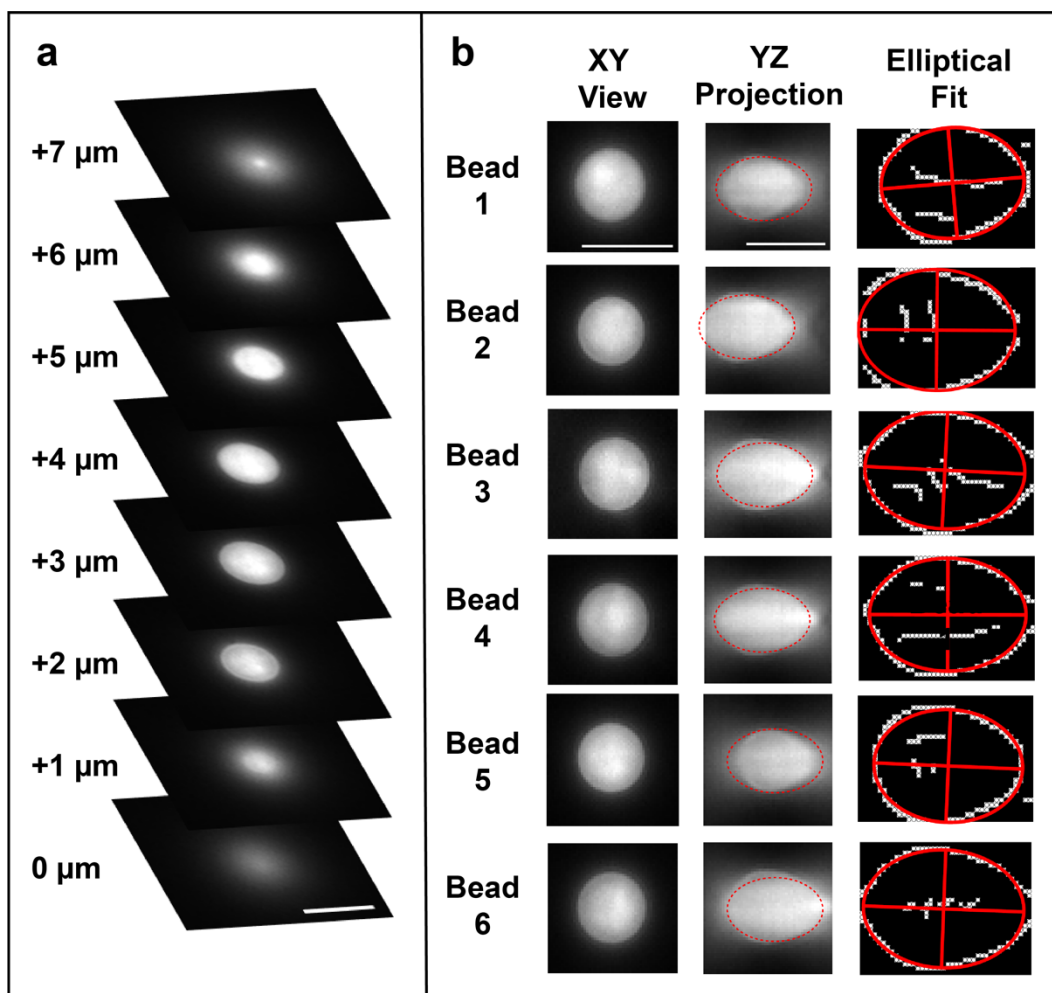
Saliba and colleagues introduce an innovative microscopy technique for high-resolution, three-dimensional imaging of mammalian cell cultures. Their system incorporates a polydimethylsiloxane (PDMS) microfluidic device with a reflective metalized sidewall, enabling illumination at a 12-degree tilt from the focal plane. This setup facilitates comprehensive scanning in three optical dimensions, along with light-sheet pivoting to minimize shadows. The illumination's numerical aperture is confined to a 39-degree half-angle due to the micromirror-based illumination method. The microfluidic channel measures 700 microns in width, 100 microns in height, and 1 cm in length. The system uniquely offers simultaneous light-sheet and diascope illumination, with the latter providing real-time feedback for drift correction. For enhanced resolution, the captured light is divided into two pathways, each passing through a double helix phase mask at a Fourier plane before reaching a single EMCCD for detection. The paper is thoroughly detailed and well-composed, effectively presenting the methods and implications of this novel imaging technology. While the manuscript may not break new ground in terms of innovation, its high-quality execution and clarity make it a valuable contribution to the field. Therefore, after a thorough review, I recommend considering this paper for publication, contingent upon addressing the following key points.

We thank the reviewer for their careful review and for their highly positive comments about our manuscript.

1. All Z localizations were scaled by a factor of 0.75. There is no indication that this correction value was empirically measured. If not, it seems appropriate given the sensitivity of the method to refractive index mismatches. While the full theoretical analysis of the shift is complex, an approximation can also be found in DOI 10.1038/s41596-020-00459-0. The correction should be done on a per-wavelength basis.

We thank the reviewer for pointing this out. The 0.75 factor which was used to scale all z localizations was experimentally determined by fitting an ellipse to the yz projection of 4 μm fluorescent beads. We have added a new [Supplementary Fig. 18](#) to clarify how this axial compression factor was determined and refer to it in the *Data analysis section in Methods*:

To account for index mismatch between the glass coverslip and the sample, all z localizations were scaled by a factor of 0.75^{55,79} ([Supplementary Fig. 18](#)).



Supplementary Fig. 18. Empirical measurements of the axial compression factor. **a** Example images of a z stack of Bead 4 in b. In total, six separate 4 μm beads were imaged. Scale bar 5 μm . **b** An ellipse was fit to the YZ projection of each bead and the axial compression factor was determined by taking the ratio of the minor and major axes of the fitted ellipse. This yielded a compression factor of 0.75 ± 0.02 (mean $\pm$ standard deviation for the six beads). Scale bars 5 μm .

We have also added the following text to the *Data analysis* section in the *Methods* to address this point:

The z compression factor of 0.75 was experimentally determined using 4 μm fluorescent beads (Fluospheres Sulfate Microspheres 4.0 μm (580/605), Invitrogen). Beads were diluted at a concentration of 1:1000 in PBS before being left to dry and adhere in an 8-well ibidi chamber (80827, Ibidi GmbH) overnight in an oven at 45°C. After adhesion, the beads were imaged in 1X PBS. Z-scans of 6 beads were performed that were each 8 μm in total height with 100 nm steps using 560 nm epi-illumination at $\sim 95 \text{ W/cm}^2$ with a calibrated EM gain of 182 and an exposure time of 50 ms. After the z-scan was performed, an ellipse was fit to the orthogonal yz projection of each bead and the ratio between the major and minor axes of the ellipse was used to determine the experimental compression factor⁸⁰.

2. *While the localization data is available upon request, it would be preferable for both the raw data and localizations to be made accessible on Zenodo, which permits uploads of approximately 50GB. In this case, a persistent DOI could be assigned to the data and included in the manuscript.*

We thank the reviewer for this suggestion. The raw image data is about 600 GB in size, however, we have made all reconstruction localizations available on Zenodo at the following DOI: [10.5281/zenodo.12786066](https://doi.org/10.5281/zenodo.12786066).

We have also added a statement about this in the *Data availability* section:

The single-molecule localizations generated and analyzed during the current study are available from the corresponding author upon request and from [Zenodo](https://doi.org/10.5281/zenodo.12786066) at the following DOI: [10.5281/zenodo.12786066](https://doi.org/10.5281/zenodo.12786066).

3. *From Figure 1C, there appears to be a significant reduction in fluorescence at the limits of the double helix range, potentially impacting localization precision. Do the authors utilize localization events from the entire 2-micron range, or are localizations below a certain precision threshold omitted?*

The reviewer is indeed correct in their observation that the fluorescence signal starts to decrease at the ends of the double helix PSF range. The used short range double helix PSF has a specified range of roughly 3 microns, but to preserve good precision, we limit the effective use to a 2 micron range.

We have added the following to specify this in the *Optical platform* section in the *Methods*:

This was achieved using a dielectric DH phase mask (DH-1 phase mask, 590 nm with diameter of 2.367 mm, Double-Helix Optics, LLC) in the green channel for single-molecule data collection over a 2- μm axial range and a DH phase mask (DH-12 phase mask, 670 nm with diameter of 2.484 mm, Double-Helix Optics, LLC) in the red channel for bead localizations and drift correction over a 12- μm axial range. Beyond these ranges, the fluorescence signal of the PSFs becomes dimmer and the rotation rates slow down relative to axial position change.

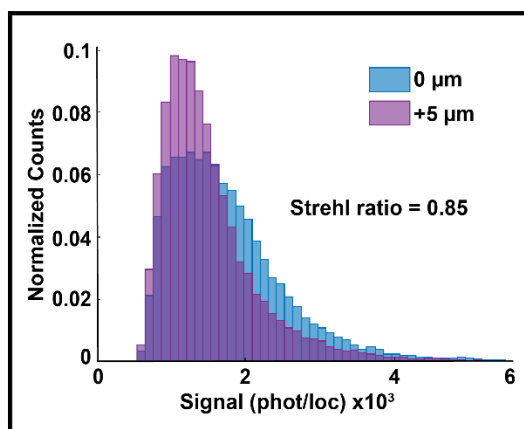
4. In the statement 'several LS approaches involving the use of separate objectives for illumination and detection...', the authors refer to oblique plane microscopes. These microscopes utilize a single objective for both illumination and detection of photons. Furthermore, many of the cited manuscripts do provide excellent optical sectioning in adherent cells. Therefore, this statement appears to be somewhat exaggerated. I recommend a careful review of each citation and necessary adjustments to the statement. Additionally, the omission of Wes Legant's 2016 Nature Methods paper should be addressed.

We thank the reviewer for pointing this out. We have modified the specified statement as stated below to more clearly point out the differences between soTILT3D and previous works. Additionally, we have added Wesley Legant's 2016 Nature Methods paper to the *Introduction*.

Several LS approaches involving the use of separate objectives for illumination and detection have been designed for single-molecule imaging in cells. However, they suffer from optical complexity, low numerical aperture (NA) objectives, steric hindrance, the inability to optically section adherent cells, incompatibility with microfluidic systems, or drift between the objectives^{14,19–30}. Previous single-objective LS designs have been demonstrated to reduce the complexity and limitations of multi-objective systems, but they have been limited by beam thickness¹⁷, the need for beam scanning^{31,32}, and limited effective NA in the detection path and the requirement for post-processing due to an illumination beam that is not aligned to the detection axis^{33–37}. Our approach based on a fully steerable single-objective tilted LS together with engineered PSFs solves all issues above and enables the use of a single high-NA objective lens for LS focusing and high photon collection efficiency without steric hindrance or relative drift, optical sectioning of entire adherent cells cultured on regular coverslips, and easy combination with microfluidic chips while avoiding the LS aberrations that occur when imaging through microfluidic chip walls^{25,33,38–41}. When imaging above the coverslip, in contrast to other single-objective LS methods^{33–37}, soTILT3D enables 2D imaging parallel to the image plane and enables 3D imaging down to the coverslip without the need to rotate the image plane.

5. In the absence of the double helix phase mask, what is the Strehl ratio of your imaging system at a typical distance from the coverslip? The refractive index mismatch seems likely to result in a significant amount of spherical aberration. Indeed, even for the illumination beam, which has a lower numerical aperture, substantial spherical aberration is evident in Figure S2.

We thank the reviewer for this comment. We have added a new [Supplementary Fig. 3](#) where we have experimentally determined the Strehl ratio of our system to be 0.85 at 5 μm from the coverslip, thereby demonstrating that spherical aberrations, while present, do not severely degrade the performance of our system.



Supplementary Fig. 3. Strehl ratio experimental determination. Single-molecule localization signal histograms from 3,000 frames of single-molecule data of nuclear lamina protein lamin B1 acquired at 0 μm and 5 μm above the coverslip. By comparing the median localization signal at 5 μm (1,355 photons per localization) to the median localization signal at 0 μm (1,603 photons per localization), the Strehl ratio was determined to be 0.85.

We have updated the manuscript to include this information in the *SoTILT3D design and performance* section in the *Results*:

The resulting LS is 1.1 μm thick (1/e² beam waist radius) (Fig. 1c, Supplementary Fig. 2f), 74.7 μm wide (1/e² diameter) (Supplementary Fig. 2g), and has a confocal parameter of 18.0 μm (1/e²) (Supplementary Fig. 2h), and these parameters can easily be tuned by the choice of lenses (see Methods). The Strehl ratio of our system at 5 μm from the coverslip was determined to be 0.85 (Supplementary Fig. 3).

We have included the sample preparation corresponding to this experiment under the *Sample Preparation* section:

For single-molecule imaging comparing epi- and LS illumination, and for determining the Strehl ratio of our system,...

Additionally, we have added under the *Imaging Procedure and Settings* section in *Methods*:

Single-molecule images were acquired to compare epi- and LS illumination SBR and localization precision (Fig. 2b,c, Supplementary Fig. 6), to determine the Strehl ratio of our system (Supplementary Fig. 3), to compare the speed and resolution when using Easy-DHPSF or DECODE for analysis (Fig. 3, Supplementary Figs. 8 and 9), and for epi- versus LS illumination resolution analysis in the high-density regime with DECODE (Supplementary Fig. 10). For the epi- versus LS illumination localization precision comparisons of lamin B1, the 560 nm laser was used at $\sim 580 \text{ W/cm}^2$ for epi-illumination and at $\sim 800 \text{ W/cm}^2$ for LS illumination using an exposure time of 100 ms to match the binding kinetics of the imager strands. 50,000 frames were acquired. To determine the Strehl ratio of our system, lamin B1 in U2OS cells was imaged with the 560 nm laser at $\sim 800 \text{ W/cm}^2$ first at 0 μm and then at 5 μm above the coverslip. 3,000 frames at a 100 ms

exposure time were acquired at each height and the resulting median photon counts were compared.

We also have added under the *Data Analysis* section in *Methods*:

The 2D data acquired for Strehl ratio determination was filtered to remove localizations with sigma values, the width of the Gaussian that fit the localizations, greater than 300 nm (Supplementary Fig. 3).

We have also added the following sentences into the *Discussion* section to detail ways to further reduce index mismatch-induced aberrations:

Additionally, refractive index mismatch-induced aberrations such as spherical aberrations can be reduced in future studies by the use of a silicone oil immersion detection objective for better matching with the refractive index of the sample. Adaptive optics can also be implemented as an addition to the 4f setup to mitigate the effects of setup- and sample- induced aberrations, including spherical aberrations.

6. *What is the apparent cause of the discrepancy between the average localization precision and the FRC resolution estimates? Could an alternative source of drift, beyond stage drift (which is corrected for), be contributing? Alternatively, is the FRC also indicating issues related to the density of labeling?*

We thank the reviewer for these questions. The resolution of a reconstruction will be improved both by the quantity and precision of the localizations, and will be reduced by e.g. residual sources of drift. As our drift correction approach works well over time, we do not expect residual drift to be a major factor separating the localization precision values and the FRC value. However, because the FRC is dependent on the number of localizations, it is also subject to parameters such as labeling density and number of frames. Therefore, discrepancy between the localization precision and the FRC is to be expected, and we typically do see improved FRC with increasing number of frames/localizations.

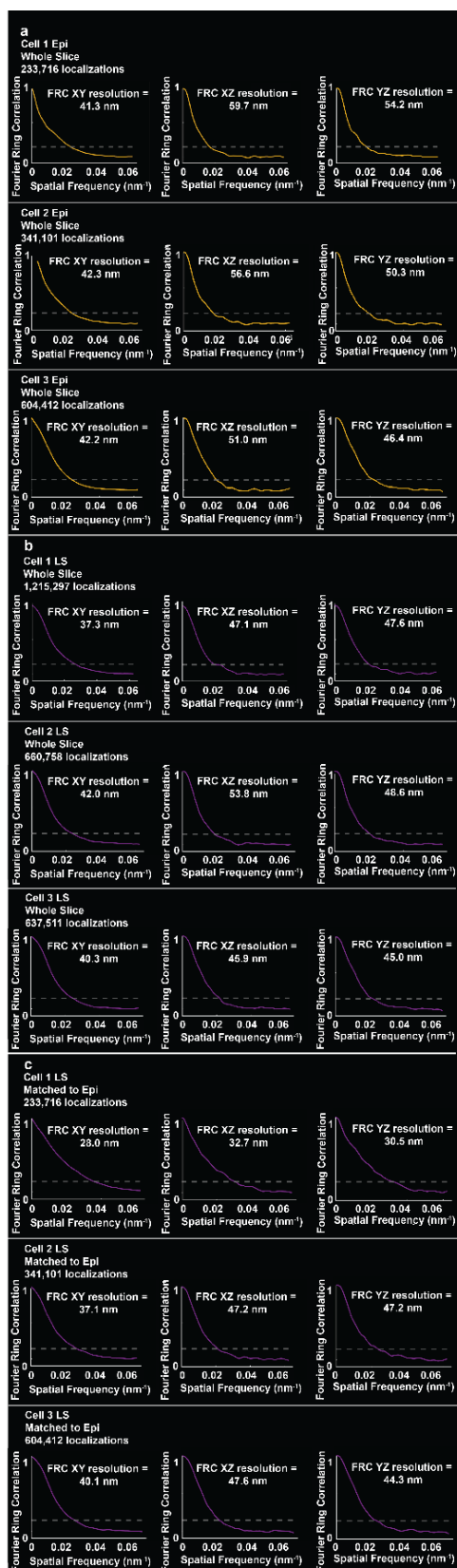
We have added a sentence in the *Data analysis* section to clarify and address these points:

The FRC resolution will typically improve with the number of localizations until the point it becomes limited by the localization precision and residual drift.

7. *In Figure 3, it appears that the variables are confounded. Is the improvement in localization a result of the software alone, or a combination of the software and the imaging system? Specifically, what is the localization rate for the widefield mode, widefield + DECODE, light-sheet mode, and light-sheet mode + DECODE?*

We thank the reviewer for pointing this out. We have added a new Supplementary Figure 10 where we have performed 3D super-resolution imaging in the high-density regime of three individual cells with both epi-illumination and light sheet illumination and compared the resulting FRC resolution values and number of detected localizations when analyzed with DECODE. When using

light sheet as the illumination modality, DECODE detected more emitters in all three data sets and the resulting resolutions were improved compared to when the same cell was imaged with epi-illumination and analyzed with DECODE. This demonstrates that although DECODE is very much necessary for imaging in this high-density regime (as shown in [new Supplementary Fig. 8](#), please see the response to Reviewer 1 comment on *Imaging Speed Improvement*) the light sheet enables an improvement in resolution and number of detected emitters when using DECODE and therefore results in an increase in acquisition speed.



Supplementary Fig. 10. Fourier ring correlation (FRC) curves for light sheet (LS) compared to epi-illumination for 3D high-density single-molecule super-resolution imaging of lamin

A/C. a FRC in the xy, xz, and yz planes, respectively, for the reconstruction of a whole 3D slice imaged with epi-illumination for three different cells. The FRC resolutions in xy/xz/yz are 41.3/59.7/54.2 nm from 233,716 localizations for cell 1, 42.3/56.6/50.3 nm from 341,101 localizations for cell 2, and 42.2/51.0/46.4 nm from 604,412 localizations for cell 3. **b** FRC in the xy, xz, and yz planes, respectively, for the reconstruction of the same whole 3D slice imaged with LS illumination for the same three cells as in a. The FRC resolutions in xy/xz/yz are 37.3/47.1/47.6 nm from 1,215,297 localizations for cell 1, 42.0/53.8/48.6 nm from 660,758 localizations for cell 2, and 40.3/45.9/45.0 nm from 637,511 localizations for cell 3, demonstrating improved number of localizations and resolution for LS compared to epi-illumination. **c** FRC in the xy, xz, and yz planes, respectively, for the reconstruction of a whole 3D slice imaged with LS illumination after the localizations with the best localization precisions are kept to match the number of localizations acquired with epi-illumination for the three different cells in a. The FRC resolutions in xy/xz/yz are 28.0/32.7/30.5 nm for cell 1, 37.1/47.2/47.2 nm for cell 2, and 40.1/47.6/44.3 nm for cell 3, demonstrating improved resolution over epi-illumination even when the numbers of localizations are matched. All data was filtered for localization precision less than 30 nm in xy. A high imager strand concentration of 0.1 nM was used for all single-molecule acquisitions. 10,000 frames were acquired for each data set and analyzed using DECODE.

We have modified the manuscript to clarify this point with the following statement in the *SoTILT3D improves the speed of 3D single-molecule imaging* section in the *Results*:

Finally, 3D high-density single-molecule super-resolution imaging of three separate cells labeled for lamin A/C revealed an increase in the number of localizations detected by DECODE and thus in the acquisition speed, as well as an improvement in the resolution when using LS as the illumination modality compared to epi-illumination (Supplementary Fig. 10). The mean resolution for three technical replicates in the xy, xz, and yz planes when imaged with epi-illumination was 41.9 ± 0.4 nm, 55.8 ± 3.6 nm, and 50.3 ± 1.8 nm, while the mean resolution when imaged with LS-illumination was 39.9 ± 1.9 nm, 48.9 ± 3.5 nm, and 47.1 ± 1.5 nm (reported as mean $\pm$ standard error of the mean, Supplementary Fig. 10a,b). The number of localizations detected for the three technical replicates when imaged with epi-illumination was 233,716, 341,101, and 604,412 for cell 1, cell 2, and cell 3 respectively, while the number of localizations when imaged with LS-illumination was 1,215,297, 660,758, and 637,511 for cell 1, cell 2, and cell 3 respectively (Supplementary Fig. 10a,b). Next, the LS-illuminated data sets were filtered for localization precision to match the number of localizations acquired in the epi-illuminated data sets (Supplementary Fig. 10c). The resulting resolution values in the xy, xz, and yz planes were 35.1 ± 3.0 nm, 42.5 ± 4.0 nm, 40.7 ± 4.2 nm (reported as mean $\pm$ standard error of the mean) for cell 1, cell 2, and cell 3 respectively. This demonstrates the necessity of the soTILT3D approach to enable imaging in the high-density regime at increased speeds and with improved resolution.

We have also updated the *Sample preparation* section accordingly:

For resolution analysis comparing epi- versus LS illumination in the high-density regime with DECODE (Supplementary Fig. 10), U2OS cells were labeled with mouse anti-lamin A/C (sc-376248, Santa Cruz Biotechnology) primary antibodies at a dilution of 1:100 in 1% (w/v) BSA for two hours. Cells were then washed three times with 0.1% (v/v) Triton X-100 in PBS with a three-minute incubation period during each wash before being labeled with donkey anti-mouse

oligonucleotide-conjugated secondary antibodies (Massive Photonics, order number AB2401012) at a dilution of 1:100 in antibody incubation buffer (Massive Photonics) for one hour. Cells were then washed three times with 1X washing buffer (Massive Photonics) in nanopure water, three times with 0.2% (v/v) Triton X-100 in PBS, and once in imaging buffer (500 mM NaCl in PBS, pH 8) before imaging. Finally, complimentary oligonucleotide-Cy3B dye conjugates (Massive Photonics, order number AB2401012) diluted in imaging buffer were flowed in at concentrations of 0.1 nM for high-density imaging.

Additionally, we have updated the information in the *Imaging procedure and settings* section:

Single-molecule images were acquired to compare epi- and LS illumination SBR and localization precision (Fig. 2b,c, Supplementary Fig. 6), to determine the Strehl ratio of our system (Supplementary Fig. 3), to compare the speed and resolution when using Easy-DHPSF or DECODE for analysis (Fig. 3, Supplementary Figs. 8 and 9) and for epi- versus LS illumination resolution analysis in the high-density regime with DECODE (Supplementary Fig. 10). For the epi- versus LS illumination localization precision comparisons of lamin B1, the 560 nm laser was used at ~ 580 W/cm² for epi-illumination and at ~ 800 W/cm² for LS illumination using an exposure time of 100 ms to match the binding kinetics of the imager strands. 50,000 frames were acquired. To determine the Strehl ratio of our system, lamin B1 in U2OS cells was imaged with the 560 nm laser at ~ 800 W/cm² first at 0 μ m and then at 5 μ m above the coverslip. 3,000 frames at a 100 ms exposure time were acquired at each height and the resulting median photon counts were compared. For the speed and resolution comparison of microtubules, the 560 nm laser was used at ~ 800 W/cm², the exposure time was set to 100 ms, and 100,000 frames were acquired. For the resolution analysis of lamin A/C comparing epi- versus LS illumination with DECODE in the high emitter density regime, three cells were imaged with the 560 nm laser at ~ 470 W/cm² for epi-illumination and ~ 450 W/cm² for LS illumination at an exposure time of 100 ms. 10,000 frames were acquired for each data set and the resulting number of localizations and resolution values in the xy, xz, and yz planes were determined.

We have also updated the information in the *Data analysis* section:

For high-density single-molecule LS and epi-illumination comparison data of lamin A/C (Supplementary Fig. 10), all data was filtered to remove localizations with localization precision greater than 30 nm.

8. *Certain drawbacks of this approach are not adequately addressed. For instance, the sample must be positioned close to the reflective surface. If it is too far away, imaging becomes unfeasible. This limitation reduces the throughput of the method.*

We thank the reviewer for their feedback. Due to the light sheet steering elements such as two galvanometric mirrors and a tunable lens, the light sheet can be positioned to enable imaging of cells at a distance of ~ 0 μ m to ~ 50 μ m from the side wall. Given that the EMCCD FOV is ~ 50 μ m, our light sheet can cover the entire visible FOV. Additionally, the microfluidic inserts are printed to be ~ 4 mm in length, enabling numerous cell options during imaging. Stronger tunable lenses would enable imaging even further from the side wall in the future.

We have added a sentence to the *Methods* section to clarify this:

The beam was then reduced and collimated once again AC254-150-A-ML, $f = 150$ mm, Thorlabs; AC254-075-A-ML, $f = 75$ mm, Thorlabs) and focused in one dimension onto a tunable lens (EL-3-10-VIS-26D-FPS, Optotune; EL-E-4 Electrical Lens Driver, Optotune) conjugated to the back focal plane of the objective lens for focal steering of up to 50 μm in the sample plane.

We have also added a sentence to the *Discussion* section about future improvement opportunities:

New fluorophore developments, including self-quenched DNA-PAINT probes⁷⁶, and tunable lenses with stronger defocusing abilities, will further improve the performance of soTILT3D imaging in the future.

9. *The methods section lacks details on the software used for microscope control and data acquisition.*

We thank the reviewer for pointing this out. We have included details about the software used for microscope control and data acquisition in the *Optical Platform* section of the *Methods*:

This allowed for active stabilization of the system using the standard PSF of non-fluorescent 3 μm polystyrene beads (C37484, Invitrogen) in both lateral and axial dimensions using an open-source active stabilization ImageJ/Micromanager Plugin¹³ termed Feedback Focus Lock (Supplementary Fig. 14), which relied on a fine adjustment xyz piezoelectric translation stage (OPH-PINANO-XYZ, Physik Instrumente) to correct for drift while acquiring single-molecule data.

For fluorescence imaging, diffraction-limited and single-molecule data was acquired with the Andor Solis EMCCD imaging software.

10. *Critically, the main text states that the beam's thickness is 1.1 microns, yet Supplementary Figure 2 indicates a thickness of 2.1 microns.*

We apologize for the confusion. The main text states the beam waist radius as 1.1 μm , representing the half thickness, whereas the supplementary text refers to the beam diameter as 2.1 μm , representing the full thickness. We have added the following statement in the caption of Supplementary Figure 2 to clarify this point:

f The thin end of the LS imaged in a fluorescent solution and corresponding line scan (yellow) and Gaussian fit (purple) are shown in the graph, demonstrating a thickness ($1/e^2$ diameter) of the LS of 2.1 μm , corresponding to a beam waist radius of ~ 1.1 μm .

Reviewer #3 (Remarks to the Author):

The amalgamation of optics, PSF engineering, microfabrication, and deep learning methods presented in this paper represents a remarkable achievement. While individual components of these techniques have been explored in prior research, the innovative aspect of this study lies in the seamless integration and synergy achieved by combining these approaches.

We thank the reviewer for their highly positive comments about the inventive combination and synergy of approaches in our system.

However, it is important to note that certain arguments presented in the article may not hold validity or need to be significantly attenuated due to their prior publication. Furthermore, there is a glaring lack of precise benchmarking in the study, and a notable absence of innovative results that have not been previously achieved. Addressing these deficiencies is crucial to strengthen the scientific contribution of the paper and ensure its distinctiveness in the context of existing research.

We thank the reviewer for providing detailed comments and suggestions for making the distinction between our work and previously published works clearer.

Consequently, I cannot recommend the publication of this paper in its current form unless substantial revisions are undertaken, addressing at least the major concerns raised herein :

We thank the reviewer for their feedback and have addressed all points raised below:

Main text:

1-The utilization of other single-objective Light Sheet Microscopy (LSM) techniques for objects of varying sizes, or the application of multifocus techniques in the Light Sheet configuration with DNA-PAINT, has already been documented in earlier studies. Therefore, these specific points have to be reconsidered and reevaluated in the context of the existing body of literature (V-LSFM, soSPIM, multifocus techniques, SOLEIL, ...).

Furthermore, and rather surprisingly, the authors make no reference to the paper published in PNAS in 2014 titled 'Whole-cell, multicolor superresolution imaging using volumetric multifocus microscopy' by Hajj et al. Even though the DNA-PAINT technique was not utilized in this 2014 paper, the outcomes of their volumetric multifocus microscopy (V-MFM) combined with STORM/PALM techniques (similar super-resolution techniques as DNA-PAINT) bear substantial resemblance and should not be disregarded by the authors, as indicated by the proximity in methodology and even the title. This omission needs to be precisely addressed for the sake of clarity, to establish the distinctiveness and originality of the current work, and to provide fair comparisons with existing literature.

We thank the reviewer for this comment with respect to comparing and distinguishing our work from other techniques. Our system is distinct from multifocus techniques in that we employ PSF engineering to bypass the need for beam splitters and consequent split of photons in the emission pathway. Furthermore, with respect to V-LSFM in particular, in contrast to soTILT3D, the sample holder requires elevation of the sample above the coverslip and does not permit active solution perfusion throughout imaging. Additionally, V-LSFM reports a ~2x worse localization precision laterally and a ~5x worse localization precision axially for the same sample type, lamin B1 in a U2OS cell, using the same imaging modality, DNA-PAINT. Throughout our *Introduction*, we already provide distinctions with respect to our work and soSPIM and SOLEIL.

We have added the following sentences in the *Introduction* of the manuscript discussing multifocus techniques, referencing the Hajj et al. V-MFM and V-LSFM work:

In contrast to other 3D single-molecule methods, such as multifocus microscopy, PSF engineering bypasses the need for beam splitters and consequent split of photons in the emission pathway while still offering a relatively thick axial range of several microns^{47,63}.

Previously developed single-objective LS systems have utilized various reflection mechanisms such as a micromirror⁴², an AFM cantilever mirror⁴³, pyramidal microcavities⁴⁴, a glass prism⁴⁵, and a microfluidic chamber with metalized side walls⁴⁶ to redirect the LS into the sample. However, these systems use imaging chambers that do not offer design flexibility^{21,42–44,46,47}, active solution exchange^{21,42–44,47}, or compatibility with epi-illumination and transmission microscopy^{42,46}.

2-The authors discuss the customization capability of their microdevices, yet they only present a single size and type of object (isolated cells).

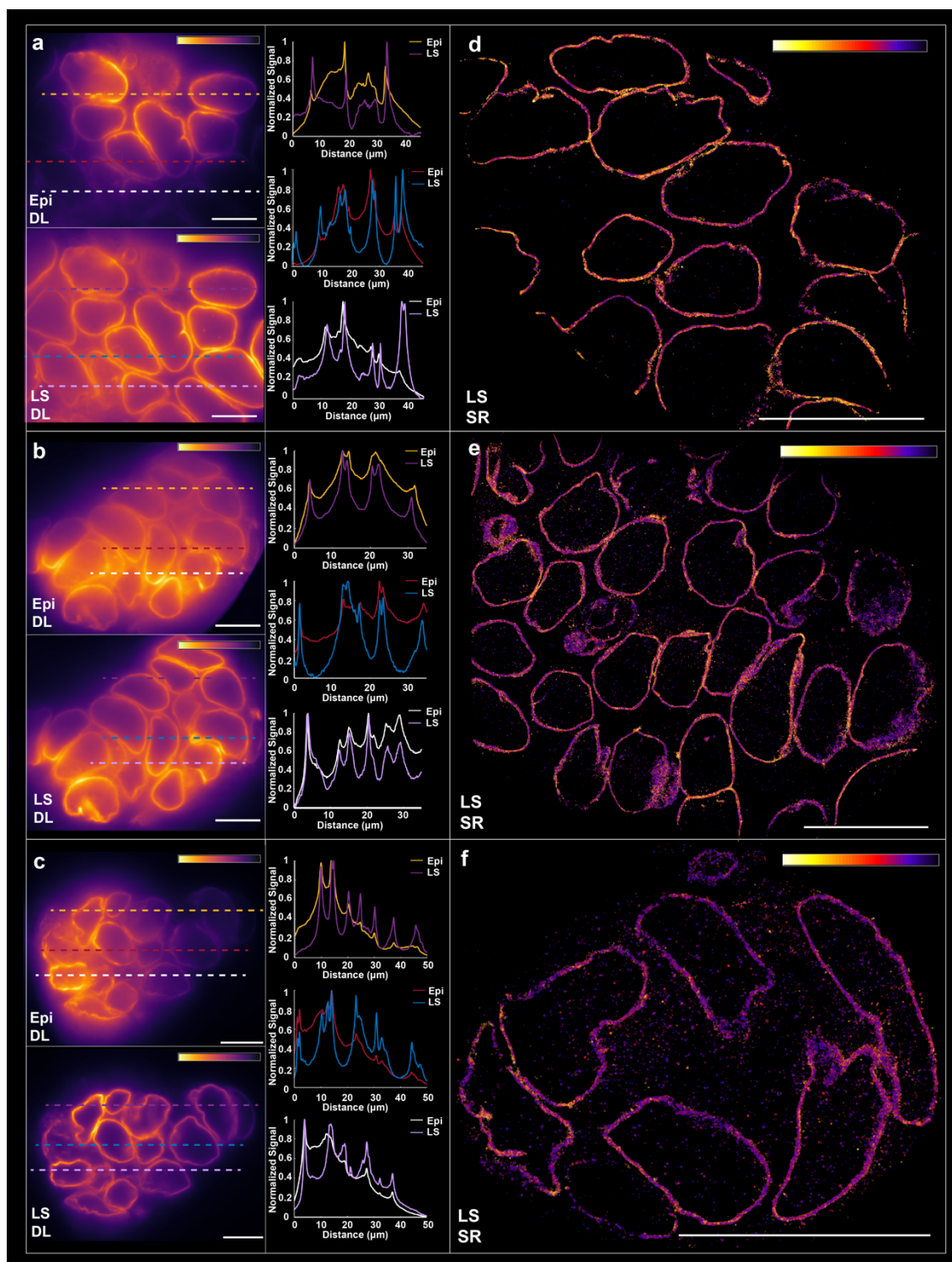
We thank the reviewer for this comment on the versatility of the microfluidic system. Our microfluidic system is customizable both in its overall dimensions and reflection angle of the light sheet. To demonstrate the flexibility of the microfluidic channel design, we have now, in addition to featuring isolated cell imaging in microchannels which are 700 μm wide, 100 μm tall, and 1 cm long, added imaging of stem cell aggregates in microchannels which are 2 mm wide, 250 μm tall, and 1 cm long (new Supplementary Fig. 13).

We have added the following sentence to the *Introduction* highlighting these points:

We further demonstrate soTILT3D for single-molecule SR imaging of lamin B1 throughout stem cell aggregates using direct stochastic optical reconstruction microscopy (dSTORM)^{55,68}, demonstrating applicability and versatility beyond isolated cell samples.

Additionally, we have added the following sentence to the *Results* section under *Whole-cell multi-target 3D single-molecule super-resolution imaging with soTILT3D*:

Finally, super-resolution imaging of a broader array of biological samples was demonstrated by imaging of lamin B1 in stem cell aggregates using dSTORM (Supplementary Fig. 13). Line scans of diffraction-limited images acquired using epi- and LS illumination revealed up to a 4x improvement in SBR when using LS illumination (Supplementary Fig. 13a-c) and 2D SR images of lamin B1 resolved details of the nuclear architecture throughout the aggregates (Supplementary Fig. 13d-f).



Supplementary Fig. 13. Diffraction-limited (DL) and super-resolution (SR) imaging of stem cell aggregates. a-c Line scans across three different stem cell aggregates cultured in the microfluidic chips and imaged with epi-illumination (top) and light sheet (LS) illumination (bottom), demonstrating the consistent background reduction capabilities of soTILT3D, even when imaging larger samples. Scale bars 10 μm. d-f Single-molecule super-resolution imaging of three different stem cell aggregates cultured in the microfluidic chips and imaged with LS

illumination demonstrating the versatility of soTILT3D for imaging diverse biological samples and the adaptability of the microfluidic device to enable sample culturing and imaging across different scales. Scale bars 20 μm . The colorbars show intensity normalized independently for each image.

We have added the following section to the *Methods*:

Human embryonic stem cell line

The human embryonic stem cell line used in this study (ESI-017, courtesy of the Warmflash lab at Rice University) was originally purchased from ESIBIO. Karyotype and genomic integrity checks were done by the manufacturer. Pluripotency (OCT4+, SOX2+, NANOG+) was regularly monitored prior to and during the study. The cell line was tested regularly for mycoplasma and found negative.

All experiments were performed with a human embryonic stem cell line that was previously established and is in the NIH registry. Experiments conform to the ISSCR guidelines found at <https://www.isscr.org/policy/guidelines-for-stem-cell-research-and-clinical-translation>.

We have updated the *Cell Culture* section in the *Methods* to include this addition:

Human pluripotent stem cells (ESI-017, seeded and fixed in the Warmflash lab at Rice University), were seeded into the microfluidic chips 4 hours before fixation and incubated at 37°C and 5% carbon dioxide (Thermo Scientific Heracell 150i CO2 Incubator, 51-032-871, Fisher Scientific) in mTeSR complete cell culture medium (courtesy of the Warmflash lab at Rice University).

Additionally, we have added the following sentences to the *Methods* section under *Sample preparation* related to the stem cell imaging:

After culturing the U2OS cells inside the microfluidic chip for 24 hours, or 4 hours in the case of the stem cells, the cells were fixed and immunolabeled inside of the microfluidic chip. For fixation, cells were first washed three times in PBS (SH3025601, Fisher Scientific), where each wash represents a complete fluid exchange in the microfluidic chip, fixed for 20 min in chilled 4% formaldehyde solution made by dissolving paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS, washed once more in PBS, and incubated with 10 mM ammonium chloride (Sigma-Aldrich) in PBS for 10 minutes. The cells were then permeabilized with three washing steps with 0.2% (v/v) Triton X-100 (Sigma-Aldrich) in PBS with a five-minute incubation period between each wash, and blocked with 3% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 hour in the case of the U2OS cells, and in 10% (v/v) donkey serum (ab7475, Abcam) in PBS for 1 hour in the case of the stem cells.

For diffraction-limited and dSTORM super-resolution imaging of stem cell aggregates, stem cells were labeled with rabbit anti-lamin B1 primary antibodies (ab16048, Abcam) using a 1:1,000 dilution in 10% (v/v) donkey serum in PBS for two hours. Cells were then washed three times with 0.1% (v/v) Triton X-100 in PBS with a three-minute incubation period during each wash before being labeled with donkey anti-rabbit secondary antibodies conjugated with dye CF568 (20098-1, Biotium) at a 1:100 dilution in 10% (v/v) donkey serum in PBS for one hour. Cells were then washed five times in 0.1% (v/v) Triton X-100 in PBS. An oxygen scavenging

buffer containing 100 mM Tris-HCl, 10% (w/v) glucose, 2 μ l/ml catalase, and 560 μ g/ml glucose oxidase was added during imaging to reduce photobleaching, and this buffer was supplemented with 286 mM β -mercaptoethanol (BME) for dSTORM imaging.

We have added the following sentences to the *Methods* section under *Imaging procedure and settings*:

For diffraction-limited imaging of stem cell aggregates comparing epi- and LS illumination (Supplementary Fig. 13), cells were imaged with the 560 nm laser at ~ 0.2 -4 W/cm² for epi-illumination and ~ 0.2 -5 W/cm² for LS illumination at an exposure time of 50 ms (Supplementary Fig. 13a-c). For dSTORM imaging of stem cell aggregates (Supplementary Fig. 13d-f), cells were imaged with the 560 nm laser at ~ 2 kW/cm² using LS illumination with an exposure time of 50 ms for 10,000 frames (Supplementary Fig. 13d,e) and 7,500 frames (Supplementary Fig. 13f). Blinking buffer was flowed through the microfluidic channel with the pressure-based flow control pump set to a pressure of 345 mbar, which corresponded to a calibrated flow rate of 58 μ L/min.

We have added the following sentences to the *Methods* section under *Data analysis*:

The 2D super-resolution stem cell aggregate data sets were filtered to remove localizations with photon counts greater than 5,000 (Supplementary Fig. 13 d-f).

Furthermore, we show the tunability of the LS angle by showing both individual cell and stem cell aggregate single-molecule super-resolution reconstructions acquired with a reflection angle of 12°, and LS images acquired with a reflection angle of 0°. We have added new panels to Fig. 1, showcasing the different chip designs used in this work.

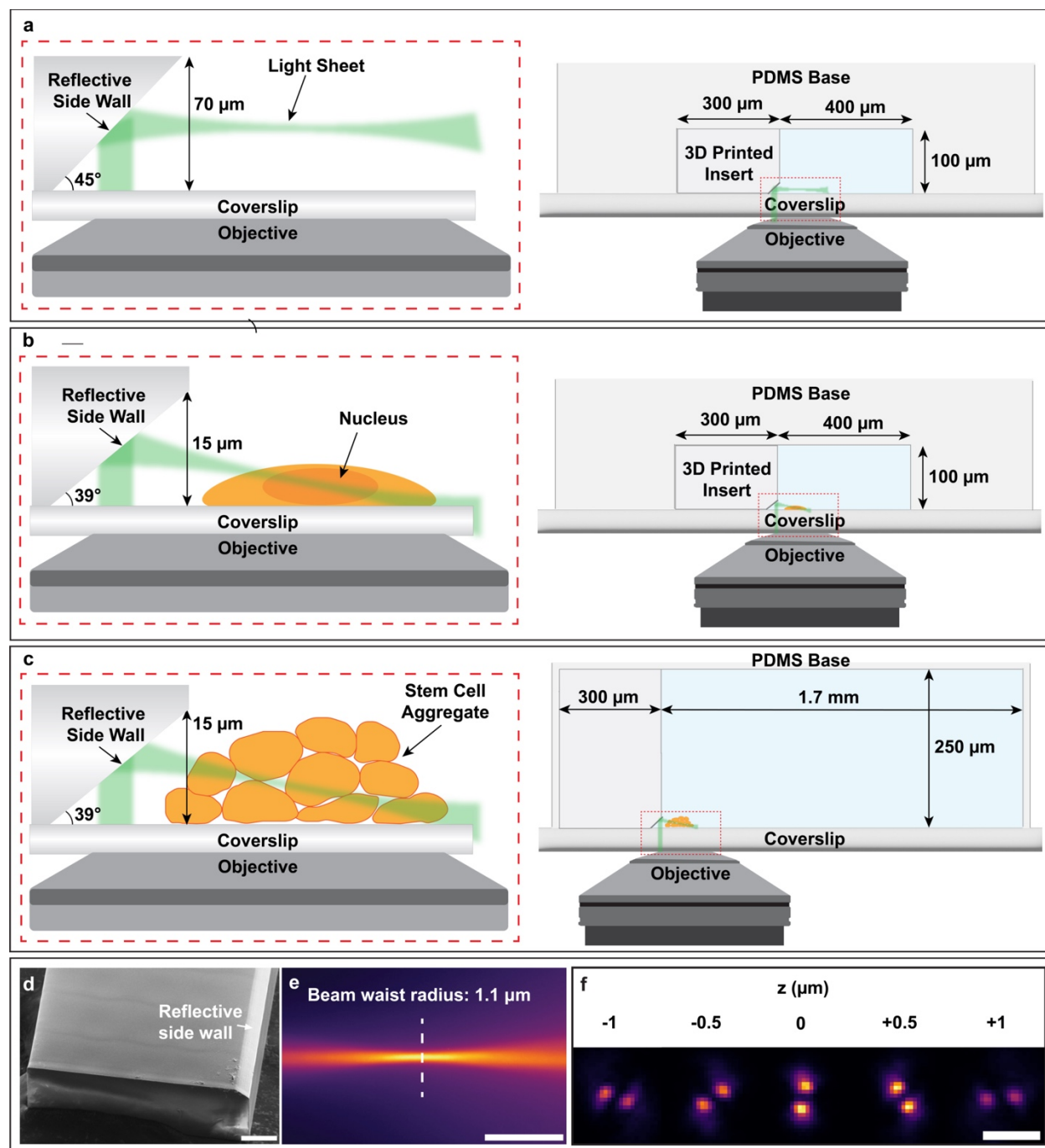


Fig 1. Illustration and characterization of the soTILT3D approach. **a-c** SoTILT3D combines a microfluidic chip that has a nanoprinted insert with metalized side wall with a single-objective tilted light sheet (LS) and is compatible with epi- and transmission illumination. The geometry and size of the insert and the microfluidic chip can be flexibly adjusted for different targets. Schematics not to scale. **d** Scanning electron micrograph of the microfluidic chip insert design shown in b. Scale bar 50 μm. **e** The thin end of the LS imaged in a fluorescent solution. Scale bar 10 μm. **f** Fluorescent beads imaged with the 2 μm axial range double helix point spread function (DH-PSF) at the indicated positions. Scale bar 2 μm.

We have modified the following sentence in the *Discussion* section of the manuscript highlighting the demonstration of the customizability of the chip:

The flexibility of the 3D nanoprinted insert geometry along with the wide range of design options for the PDMS channels allow for easy adaptability and implementation for various applications, as demonstrated by imaging of both isolated cells and stem cell aggregates in microchannels of different dimensions.

Additionally, have added the following sentences to the *Methods* section in the *Fabrication process for microfluidic chips with reflective angled side walls* section regarding the fabrication of the microchannels of different dimensions and reflective inserts of different angles:

An overview of the various chip dimensions and modifications used throughout this work can be found in Fig. 1.

For imaging throughout stem cells, inserts were printed to be 260 μm tall, 300 μm wide, and 4 mm long. In both cases, the side wall had an angle of 39° and a height of 15 μm to allow for a reflected LS with a 12° tilt. For imaging the LS in fluorescent solution, inserts were printed to be 105 μm tall, 300 μm wide, and 2.5 mm long with an angle of 45° and a height of 70 μm to allow for a reflected LS with no tilt for characterization purposes. A mirror with a height of 70 μm was used to allow for a greater mirror surface area in order to image both the width and thickness of the LS by rotating the cylindrical lens.

SU8 molds with a 250 μm height for stem cell imaging were prepared by spin-coating approximately 2 mL of the photoresist onto a silicon wafer first to 500 rpm at 100 rpm/second acceleration, then held at 500 rpm for 10 seconds, then ramped to 1500 rpm at an acceleration of 300 rpm/second and held at this speed for 30 seconds. The molds were then heated on a hot plate at 65°C for 2 hours followed by 95°C for 4 hours. A photomask with channels that were 2 mm wide and 1 cm long was applied to the photoresist-coated silicon wafers which were then exposed to UV light under vacuum with an 18 W light source for 45 seconds. The molds were then heated again on a hot plate at 65°C for 1 minute followed by 95°C for 30 minutes. The molds were then placed in a beaker with approximately 20 mL of SU8 developer and gently swirled for 20 minutes to dissolve residual untreated photoresist.

Additionally, we have added clarifying statements to sentences in the *Methods* section in the *Fabrication process for microfluidic chips with reflective angled side walls* section regarding the dimensions of the microchannels:

SU8 molds with a 100 μm height for isolated cell imaging and LS characterization were prepared by spin-coating (WS-650Mz-23NPPB, Laurell Technologies) approximately 2 mL of the photoresist onto a silicon wafer first to 500 revolutions per minute (rpm) at 100 rpm/second acceleration, then held at 500 rpm for 10 seconds, then ramped to 3000 rpm at an acceleration of 300 rpm/second and held at this speed for 30 seconds. The molds were then heated on a hot plate at 65°C for 10 minutes followed by 95°C for 30 minutes. A custom film photomask (Micro Lithography Services Ltd) with channels that were 700 μm wide and 1 cm long designed in AutoCAD (Autodesk) was applied to the photoresist-coated silicon wafers which were then

exposed to UV light under vacuum (140010, Vacuum UV-Exposure Box, Gie-Tec GmbH) with an 18 W light source for 27 seconds.

Meanwhile, other single-objective LSM (such as soSPIM) techniques have already covered a wide range of object sizes in super-resolution imaging, including but not limited to Drosophila specimens, organoids, and cell aggregates (Galland et al. 2015, and then Beghin et al. 2022 for organoids). Therefore, the authors must diversify their experimental scope to encompass a broader array of biological samples, demonstrating the versatility and adaptability of their microdevice across various scales and biological contexts.

We thank the reviewer for this comment with regards to related works. In these works (Galland et al. 2015, Beghin et al. 2022), high- but not super-resolution capabilities are demonstrated for larger biological specimens using light sheet imaging. We have added a new Supplementary Fig. 13 (please see response to comment 2) demonstrating our system's applicability for a more diverse array of larger biological specimens by imaging stem cell aggregates. Moreover, we demonstrate single-molecule super-resolution imaging of lamin B1 in stem cell aggregates using single-objective light sheet illumination and dSTORM. Beyond this demonstration, our system offers unique benefits with respect to biological context as it enables active perfusion of different solutions through the microfluidic chip, allowing for a wider range of studies with respect to extracellular environmental control.

We have added the following sentence to the *Introduction* highlighting these points:

We further demonstrate soTILT3D for single-molecule SR imaging of lamin B1 throughout stem cell aggregates using direct stochastic optical reconstruction microscopy (dSTORM)^{55,68}, demonstrating applicability and versatility beyond isolated cell samples.

Additionally, we have added the following sentence to the *Whole-cell multi-target 3D single-molecule super-resolution imaging with soTILT3D* section in the *Results* section:

Finally, super-resolution imaging of a broader array of biological samples was demonstrated by imaging of lamin B1 in stem cell aggregates using dSTORM (Supplementary Fig. 13). Line scans of diffraction-limited images acquired using epi- and LS illumination revealed up to a 4x improvement in SBR when using LS illumination (Supplementary Fig. 13a-c) and 2D SR images of lamin B1 resolved details of the nuclear architecture throughout the aggregates (Supplementary Fig. 13d-f).

3-The argument that the microchannel facilitates sample rinsing and marker switching using DNA-PAINT is not compelling, as other techniques employing open coverslips also offer these capabilities. Therefore, this feature may not be a unique selling point of the presented microdevice. The authors should reconsider this point or provide a more specific advantage of their microfluidic system compared to other methods in terms of sample manipulation and marker exchange.

We thank the reviewer for this comment and the suggestions for better discussing the advantages of our microdevice. In contrast to typical open-top systems, our device offers automation of the solution exchange, facilitating convenient multi-solution imaging over extended times without the

user having to manually perform each solution exchange. In addition, manual fluid exchange in open-top systems often comes with the risk of moving or perturbing the sample. For techniques such as Exchange-PAINT, it is technically challenging to wash the sample without severely offsetting the field of view and inducing unnecessary drift on an open-top system. This issue is avoided with our design. Furthermore, the microfluidic channel, in addition to being a chamber for solution exchange, also functions as an optical element enabling reflection of the single-objective light sheet into the sample.

In addition to sample rinsing and marker switching, the use of microchannels allows for active perfusion of different solutions, allowing for a wider range of studies with respect to extracellular environmental control. Furthermore, given that the microfluidic channel is a constantly replenished fluid reservoir, the sample is protected from unnecessary heat damage. Finally, fluid flow was found to increase observed localizations for DNA-PAINT applications, thus improving image quality, as reported by Rames et al. 2023. Thus, our microfluidic channel is unique relative to previous works (Galland et al. 2015, Meddens et al. 2016, Beghin et al. 2022) in that it simultaneously 1) allows for automated exchange of solutions without the need for manual handling, 2) allows for active perfusion of various fluids throughout imaging which improves the quality of DNA-PAINT imaging, and 3) incorporates microoptics into microchannels in a way that offers easy design customization as well as flexibility for combination with light sheet illumination.

We have added the following sentence to the *Introduction* of the manuscript highlighting these benefits of the microchannel:

Beyond design flexibility, our microfluidic channel, through its active perfusion compatibility, eliminates the need for manual exchange of solutions which potentially offsets the field of view being imaged and induces drift, reduces thermal damage to the sample by removing heat via constant flow, and increases the number and quality of localizations for DNA-PAINT applications⁴⁵.

4-The authors introduce a specific tilt angle (12 degrees) without providing a rationale for this particular choice. Moreover, if the purpose of employing a particular angle is to visualize adherent cell structures close to the coverslip, none of the proposed markers or results substantiate this claim. It would have been pertinent for the authors to incorporate membrane or peri-membrane markers (such as integrins or actin) to demonstrate the effectiveness of their microdevice in imaging close-to-surface adherent cell structures.

We thank the reviewer for this comment and suggestions for better motivating the selected tilt angle. As the light sheet is formed by a Gaussian beam, it will be diverging away from focus where imaging is performed. Bringing a horizontal light sheet (0° tilt angle) close to the coverslip will therefore induce scattering, aberrations, and reflections that degrade the light sheet's optical sectioning capability. The tilt of the light sheet is introduced to allow light sheet sectioning of the sample all the way down to the coverslip while avoiding these effects. A tilt angle of 12° was chosen as it is slightly larger than the ~10° convergence angle of our light sheet focusing so that the light sheet does not interact with the coverslip until it reaches its focus.

We have added the following sentence to the *SoTILT3D design and performance* section in the *Results* section of the manuscript, clarifying the choice of tilt angle:

The tilt angle of 12° was chosen to be slightly larger than the $\sim 10^\circ$ LS convergence angle, allowing for imaging all the way down to the coverslip without inducing scattering, aberrations, and reflections that would degrade the optical sectioning performance of the LS.

We have also added new data to an additional panel c to Fig. 4 demonstrating whole membrane imaging of membrane-bound cytoskeleton linker protein ezrin, illustrating the soTILT3D imaging capabilities of adherent cell structures at the coverslip.

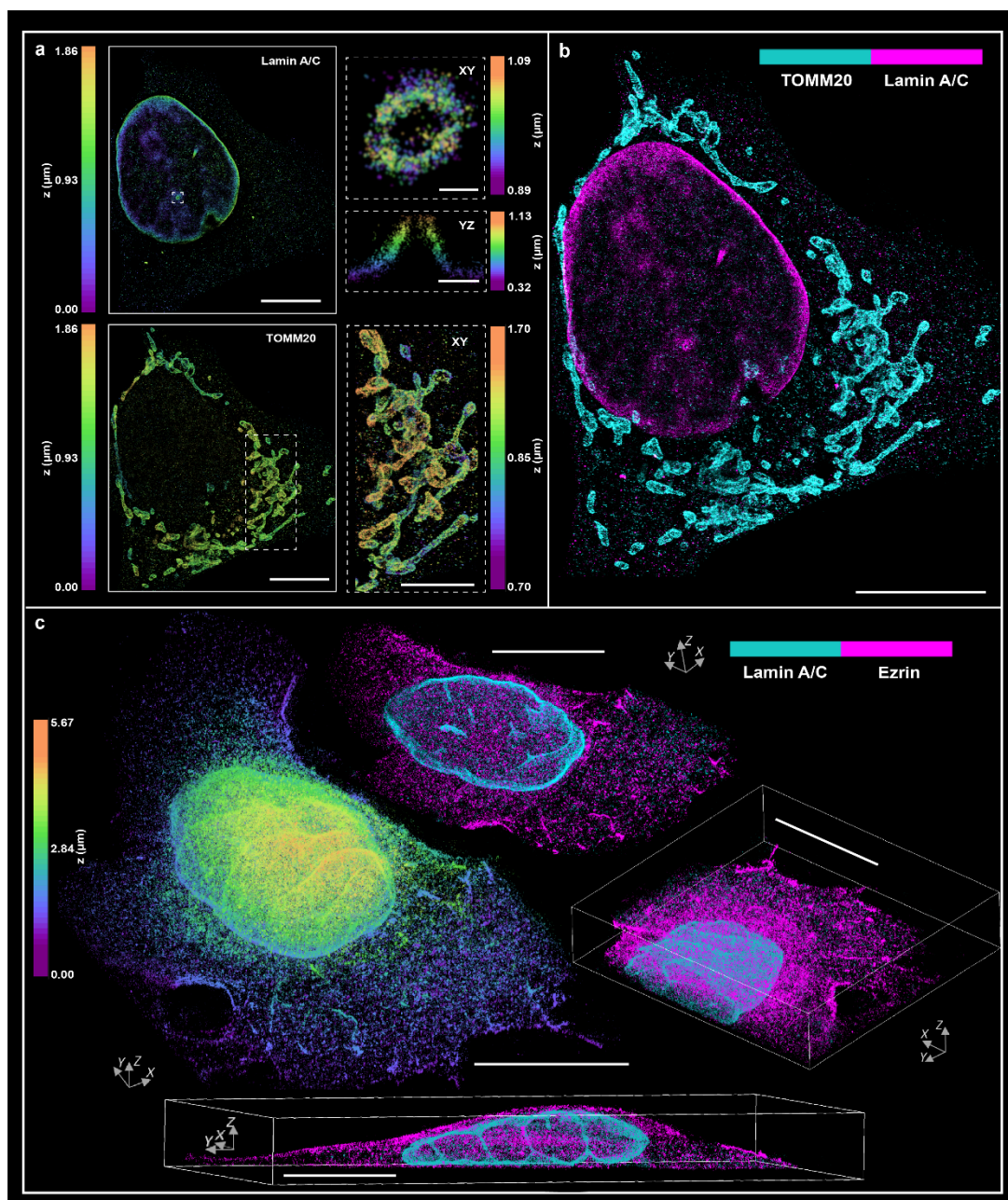
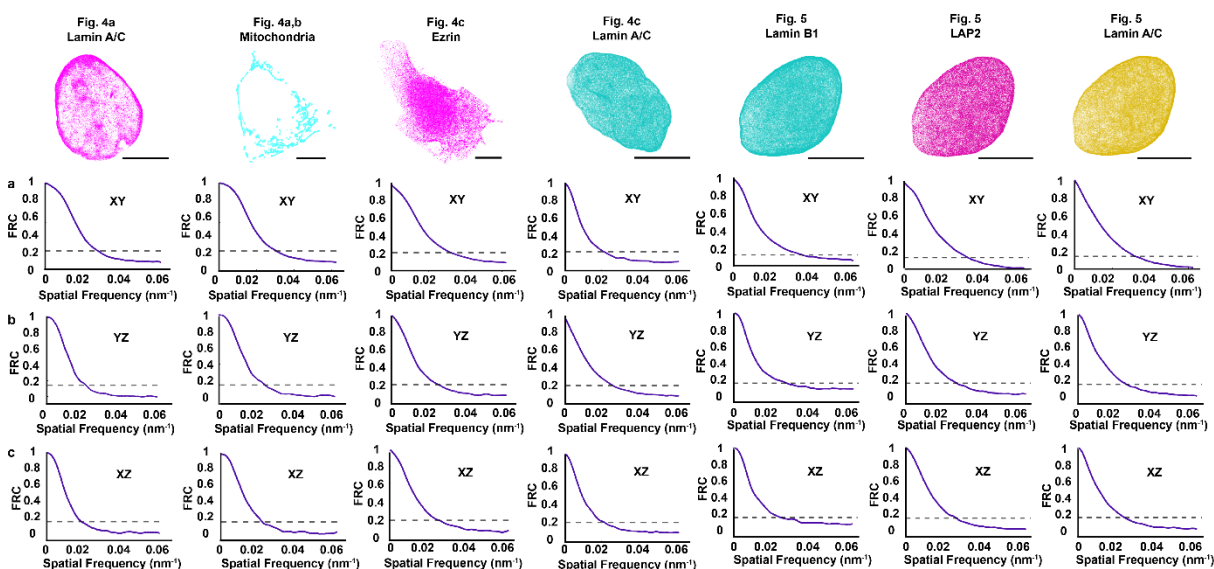


Fig. 4. Two-target 3D high-density whole-cell single-molecule super-resolution (SR) imaging with soTILT3D. a 3D SR reconstruction of separately rendered lamin A/C and mitochondria (TOMM20) in the same U2OS cell, revealing no notable crosstalk between the two targets. Scale bars 10 μm . Inset panels for lamin A/C shown for a 200 nm thick z-slice in the xy plane and for a 150 nm thick x-slice in the yz plane resolved the nanoscale architecture of a nuclear lamina channel. Xy inset scale bar 200 nm and yz inset scale bar 500 nm. Inset panel for mitochondria shows details of the mitochondrial network. Mitochondrial inset scale bar 5 μm . **b** A merge of the 3D SR reconstructions of lamin A/C and mitochondria shown in a. Scale bar 10 μm . **c** 3D whole-cell SR reconstruction of lamin A/C and ezrin. The left panel shows a merged reconstruction of both targets, colored by depth. The top panel shows a 1.2 μm thick z-slice colored by probe. The right panel shows the bottom right half of the reconstruction in the left panel. The bottom panel shows a 2.5 μm thick slice through the center of the cell. Scale bars 10 μm .

We have also updated Supplementary Fig. 11 to include this new data set.



Supplementary Fig. 11. Statistics for double-helix PSF localization data used for the 3D super-resolution reconstructions of lamin A/C, mitochondria, and ezrin in Fig. 4 and of lamin B1, LAP2, and lamin A/C in Fig. 5. a Fourier ring correlation (FRC) for the xy plane, **b** FRC for the yz plane, and **c** FRC for the xz plane. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4a,b were 35.0/45.7/51.5 nm for lamin A/C and 32.7/40.2/44.9 nm for mitochondria. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 4c were 31.0/39.2/38.2 nm for ezrin and 38.0/46.3/47.2 nm for lamin A/C. The FRC resolutions in xy/yz/xz for the reconstructions in Fig. 5 were 27.9/36.1/39.9 nm for lamin B1, 30.2/37.6/38.0 nm for LAP2, and 31.3/38.9/40.6 nm for lamin A/C. Scale bars 10 μm .

We have added the following sentences to the *Results* in the section *Whole-cell multi-target 3D single-molecule super-resolution imaging with soTILT3D* discussing the whole-cell imaging capabilities:

Next, 3D two-target imaging of whole cells was demonstrated by imaging of membrane-bound protein ezrin and lamin A/C in U2OS cells, where the entire axial profile of the cell was

reconstructed (Fig. 4c). The resulting FRC resolutions in the xy/yz/xz planes were found to be 31.0/39.2/38.2 nm for ezrin and 38.0/46.3/47.2 nm for lamin A/C (Supplementary Fig. 11).

We have also modified a sentence in the *Introduction* to further reflect this data:

Finally, we demonstrate the performance of soTILT3D for multi-target 3D single-molecule whole-cell SR imaging of nuclear lamina proteins, mitochondria, and plasma membrane protein ezrin and quantify the relative distances between nuclear lamina proteins lamin B1, lamin A/C, and lamina associated protein 2 (LAP2).

Additionally, we have added the following sentences to the *Methods* section under *Sample preparation*:

For two-target imaging of ezrin and lamin A/C, U2OS cells were labeled with rabbit anti-ezrin (ab40839, Abcam) and mouse anti-lamin A/C primary antibodies (sc-376248, Santa Cruz Biotechnology) at a dilution of 1:50 and 1:100, respectively, in 1% (w/v) BSA, 5% (v/v) salmon sperm ssDNA (ab229278, Abcam), and 10% donkey serum (ab7475, Abcam) in PBS for two hours. Cells were then washed three times with 0.1% (v/v) Triton X-100 in PBS with a three-minute incubation period during each wash before being labeled with donkey anti-rabbit and donkey anti-mouse oligonucleotide-conjugated secondary antibodies (Massive Photonics, order number AB2401012) at a dilution of 1:100 in antibody incubation buffer (Massive Photonics) for one hour. Cells were then washed three times with 1X washing buffer (Massive Photonics) in nanopure water, three times with 0.2% (v/v) Triton X-100 in PBS, and once in imaging buffer (500 mM NaCl in PBS, pH 8) before imaging. Next, 0.2 μ m 660/680 nm fiducial beads at a dilution of 1:100,000 in imaging buffer were flowed in for drift correction and allowed to settle for 5 minutes. Finally, complimentary oligonucleotide-Cy3B dye conjugates (Massive Photonics, order number AB2401012) diluted in imaging buffer were flowed in at concentrations of 0.08 nM for ezrin and 0.08 nM for lamin A/C sequentially for the two targets during imaging.

We have added the following sentences to the *Methods* section under *Imaging procedure and settings*:

For two-target single-molecule imaging of ezrin and lamin A/C (Fig. 4c), 20,000 frames of ezrin and 10,000 frames of lamin A/C were acquired at each slice using the 560 nm laser at ~ 550 W/cm² and an exposure time of 100 ms. For fiducial bead drift correction, 0.2 μ m 660/680 nm fiducial beads were excited using 642 nm epi-illumination at ~ 3 W/cm². To combat fiducial bead saturation, a 1.0 ND filter was placed in the red channel of the emission path. Imager strands for each target and washing buffer between targets were flowed through the microfluidic channel with the pressure-based flow control pump set to a pressure of 345 mbar, which corresponded to a calibrated flow rate of 58 μ L/min. After imaging, dark frames were acquired with the lasers turned off and camera shutters closed and z-scan calibration stacks of the 12- μ m axial range DH-PSF in the red channel and the 2- μ m axial range DH-PSF in the green channel were acquired with fiducial beads (T7280, TetraSpeck, 0.2 μ m, Invitrogen) spin coated in 1% (w/w) PVA in nanopure water on a coverslip. Registration images of both channels were acquired using the standard PSF with fiducial beads (T7280, TetraSpeck, 0.2 μ m, Invitrogen) spin coated in 1% (w/w) PVA in nanopure water on a coverslip.

We have added the following sentences to the *Methods* section under *Data analysis*:

The ezrin and lamin A/C reconstructions (Fig. 4c) were filtered to remove localizations with localization precisions greater than 50 nm in xy and greater than 100 nm in z.

5-Using nuclear and mitochondrial markers does not justify the use of the term "whole cells" as these structures are highly localized and do not extend throughout the cellular volume. The authors should consider employing markers that truly encompass the entirety of the cell to validate their claim accurately.

We thank the reviewer for this comment and suggestions for better demonstrating the capabilities of the soTILT3D system for whole-cell imaging. Please see our response to comment 4.

6-Additionally, the comparison made with epifluorescence illumination is not valid and should be removed, as it is not a technique of choice used for SMLM imaging. The authors should use comparable methods and consider conducting a more comprehensive study, incorporating techniques such as Hilo/TIRF illumination.

We thank the reviewer for this comment relating to relevant illumination techniques in the SMLM field. We think that wide-field epi-fluorescence illumination is a valid illumination method for SMLM imaging, as demonstrated through its use in many other SMLM publications (e.g. Hajj et al. *PNAS*. 2014, V-MFM; Nehme et al. *Nat. Methods*. 2020; Liao et al. *PNAS*. 2015; Wertz et al. *Nano Lett.* 2015; Gahlmann et al. *Nano Lett.* 2013; Diekmann et al. *Nat. Methods*. 2020). Furthermore, related light sheet works in the field (e.g. Galgani et al. 2022; V-LSFM) solely compare SBR ratios from light sheet illumination to epi-fluorescence illumination. Compared to our thin light sheet, HILO beams tend to be relatively thick and do thus not provide as good optical sectioning. TIRF illumination, while useful for imaging at the coverslip, is not an option for imaging at depth, as needed for whole-cell imaging. Further comparisons between light sheet and HILO illumination have been explored in other works (e.g., Hung et al., 2022; SOLEIL), which discuss HILO's limitations, including a reduced field of view and a limitation to thin specimens due to aberrations.

We have added the following sentence in the *Introduction* of the manuscript to further clarify these points:

LS illumination offers distinct benefits over other illumination modalities such as total internal reflection fluorescence (TIRF) or highly inclined and laminated optical sheet (HILO)¹⁷ and variable-angle epifluorescence microscopy (VAEM)¹⁸ illumination for imaging throughout thick samples due to its ability to illuminate with a thin LS beam and optically section the sample well above the coverslip.

7-Furthermore, the comparisons should include a comprehensive benchmark analysis, considering factors such as acquisition time, reconstruction time, and the training duration of the utilized neural networks, such as DECODE. This holistic evaluation is necessary to provide an

accurate and insightful understanding of the proposed methodology's efficiency and practical applicability in comparison to existing techniques.

We thank the reviewer for this comment relating to relevant details of the methodology used in the manuscript. We have added a [Supplementary Table 1](#) mentioning these details with respect to acquisition time, reconstruction time, and the training duration of DECODE.

Supplementary Table 1. Acquisition and reconstruction times for 3D super-resolution (SR) reconstructions. Acquisition and reconstruction times for all 3D SR reconstructions. Fitting for reconstruction was performed using a DECODE model that took ~12 hours to train. The exposure time was 100 ms for all targets.

	Number of frames per slice	Number of slices	Total acquisition time (minutes)	Reconstruction time (minutes)
Fig. 4a,b Lamin A/C	50,000	1	~80	~25-50
Fig. 4a,b Mitochondria	50,000	1	~80	~25-50
Fig. 4c Lamin A/C	10,000	4	~70	~20-40
Fig. 4c Ezrin	20,000	4	~130	~40-80
Fig. 5 Lamin B1	10,000	8	~130	~40-80
Fig. 5 Lamin A/C	10,000	8	~130	~40-80
Fig. 5 LAP2	10,000	8	~130	~40-80

We have added the following sentences to the *Data analysis* section in *Methods* referencing this table to address this point:

Details related to acquisition time and reconstruction time for each 3D SR reconstruction can be found in Supplementary Table 1. The DECODE model used to fit all data took ~12 hours to train with a NVIDIA GeForce RTX 2060 12GB graphics processing unit on a Windows 10 64-bit operating system.

Figures:

Figure 1 lacks essential details and fails to provide adequate information about the microfabrication device, the fabrication method, object sizes, and the distance from the coverslip. To enhance the clarity and comprehensiveness of the study, it is imperative for the authors to include these crucial details in Figure 1. A detailed schematic or diagram, accompanied by explanations in the figure caption, would significantly improve the readers' understanding of the experimental setup and methodology.

We thank the reviewer for this comment relating to the details of the microfluidic chip. Detailed schematics regarding the fabrication scheme can be found in Supplementary Fig. 15 (previously Supplementary Fig. 8) and the fabrication details and designs are described in the *Fabrication process for microfluidic chips with reflective angled side walls* section in *Methods*. We have now added new panels to Fig. 1 featuring notated cross-sections of the microfluidic channels used for the various measurements featured throughout the work to better improve the reader's understanding of the system and its flexibility in design. Cells are cultured directly on the coverslip and thus do not need to be elevated above the coverslip.

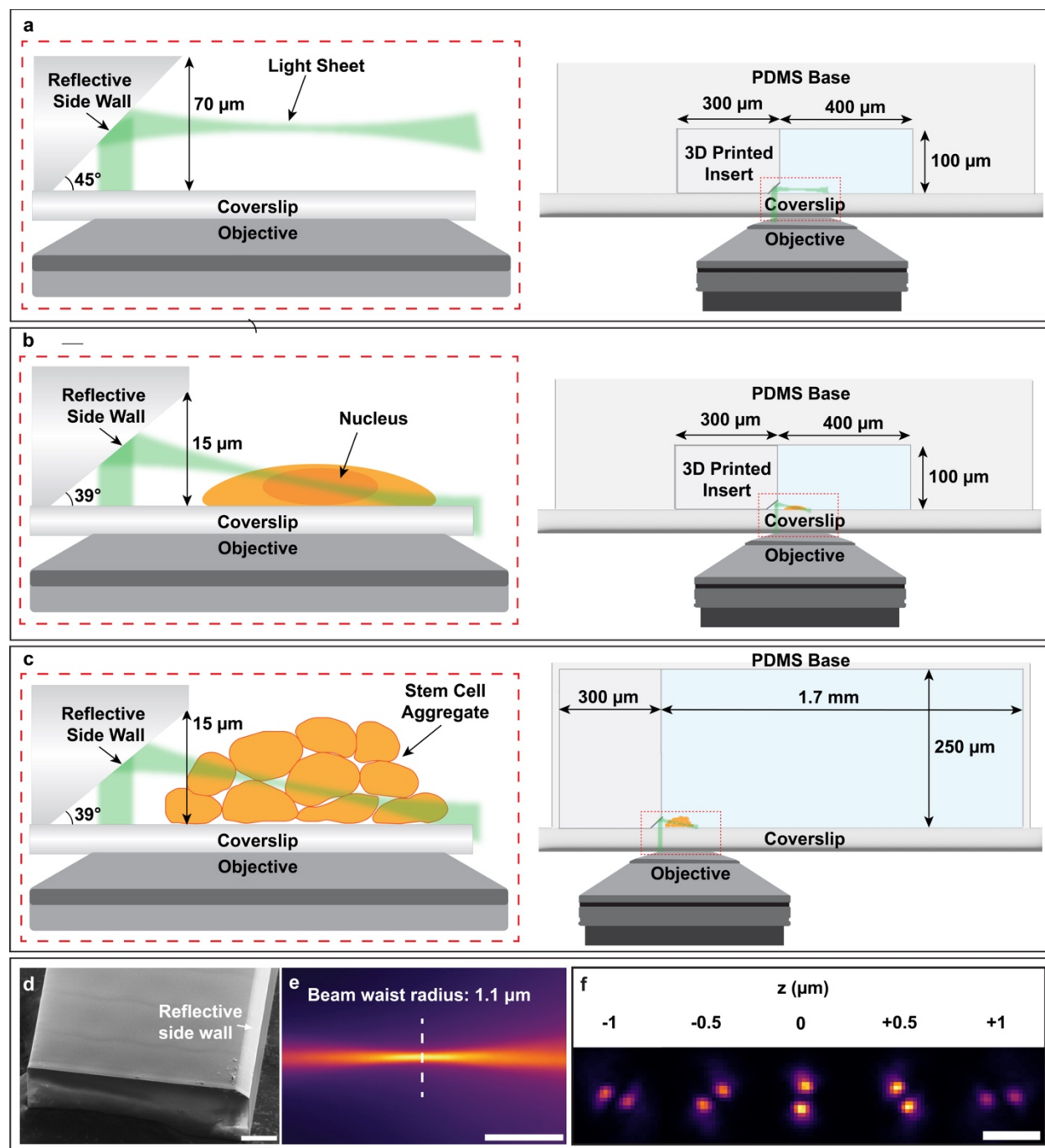


Fig 1. Illustration and characterization of the soTILT3D approach. **a-c** SoTILT3D combines a microfluidic chip that has a nanoprinted insert with metalized side wall with a single-objective tilted light sheet (LS) and is compatible with epi- and transmission illumination. The geometry and size of the insert and the microfluidic chip can be flexibly adjusted for different targets. Schematics not to scale. **d** Scanning electron micrograph of the microfluidic chip insert design shown in b. Scale bar 50 μm. **e** The thin end of the LS imaged in a fluorescent solution. Scale bar 10 μm. **f** Fluorescent beads imaged with the 2 μm axial range double helix point spread function (DH-PSF) at the indicated positions. Scale bar 2 μm.

We have added the following sentences in the *Fabrication process for microfluidic chips with reflective angled side walls* section in *Methods* to reference this new figure:

An overview of the various chip dimensions and modifications used throughout this work can be found in Fig. 1.

We have also added in the caption of Figure 1:

“Schematics not to scale.”

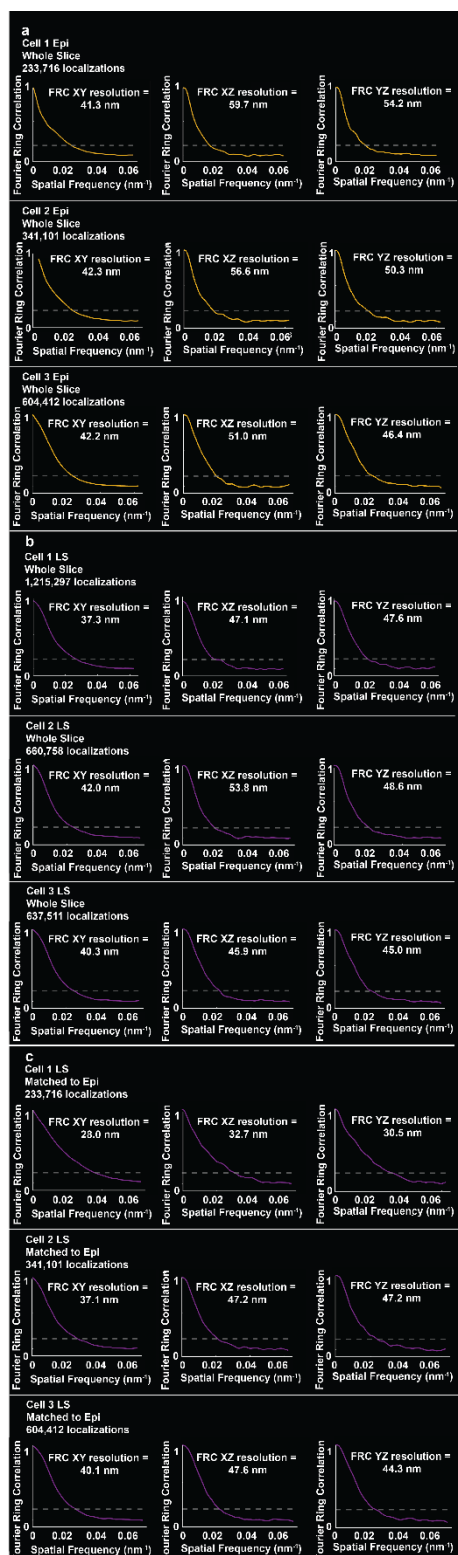
Figure 2 should be removed, and relevant comparisons should be provided (see point 6).

We thank the reviewer for this comment. We have addressed this concern in our response to comment 6.

Figures 3 and 4 could be combined for a more cohesive presentation. Currently, Figure 3 primarily demonstrates the utility of DECODE (which has been previously published once again, Speiser et al. 2021) rather than showcasing the unique advantages of soTILT3D combined with DECODE. The key contributions of soTILT3D with DECODE are better represented in Figure 4. However, Figure 4 lacks essential information regarding acquisition time and reconstruction, which are vital for a comprehensive understanding of the technique's efficiency. Including these details, along with a clearer distinction between the contributions of soTILT3D and DECODE, is mandatory for the clarity and impact of the paper.

We thank the reviewer for this comment regarding the distinct advantages of the soTILT3D system relative to DECODE. We have addressed concerns related to acquisition and reconstruction time in our response to comment 7.

Regarding better distinguishing the advantages that the soTILT3D system offers independently of DECODE, we have added a new Supplementary Figure 10 where we perform 3D SR imaging of three nuclei using a dense emitter concentration for both epi-illumination and light sheet illumination both analyzed with DECODE. We demonstrate that the soTILT3D system improves the number of localizations and the resolution, demonstrating that light sheet illumination yields more localizations of higher quality in the dense emitter regime, thus enabling faster acquisition speed.



Supplementary Fig. 10. Fourier ring correlation (FRC) curves for light sheet (LS) compared to epi-illumination for 3D high-density single-molecule super-resolution imaging of lamin A/C. a FRC in the xy, xz, and yz planes, respectively, for the reconstruction of a whole 3D slice imaged with epi-illumination for three different cells. The FRC resolutions in xy/xz/yz are

41.3/59.7/54.2 nm from 233,716 localizations for cell 1, 42.3/56.6/50.3 nm from 341,101 localizations for cell 2, and 42.2/51.0/46.4 nm from 604,412 localizations for cell 3. **b** FRC in the xy, xz, and yz planes, respectively, for the reconstruction of the same whole 3D slice imaged with LS illumination for the same three cells as in a. The FRC resolutions in xy/xz/yz are 37.3/47.1/47.6 nm from 1,215,297 localizations for cell 1, 42.0/53.8/48.6 nm from 660,758 localizations for cell 2, and 40.3/45.9/45.0 nm from 637,511 localizations for cell 3, demonstrating improved number of localizations and resolution for LS compared to epi-illumination. **c** FRC in the xy, xz, and yz planes, respectively, for the reconstruction of a whole 3D slice imaged with LS illumination after the localizations with the best localization precisions are kept to match the number of localizations acquired with epi-illumination for the three different cells in a. The FRC resolutions in xy/xz/yz are 28.0/32.7/30.5 nm for cell 1, 37.1/47.2/47.2 nm for cell 2, and 40.1/47.6/44.3 nm for cell 3, demonstrating improved resolution over epi-illumination even when the numbers of localizations are matched. All data was filtered for localization precision less than 30 nm in xy. A high imager strand concentration of 0.1 nM was used for all single-molecule acquisitions. 10,000 frames were acquired for each data set and analyzed using DECODE.

We have modified the manuscript to clarify this point with the following statement in the *SoTILT3D improves the speed of 3D single-molecule imaging* section in the *Results*:

Finally, 3D high-density single-molecule super-resolution imaging of three separate cells labeled for lamin A/C revealed an increase in the number of localizations detected by DECODE and thus in the acquisition speed, as well as an improvement in the resolution when using LS as the illumination modality compared to epi-illumination (Supplementary Fig. 10). The mean resolution for three technical replicates in the xy, xz, and yz planes when imaged with epi-illumination was 41.9 ± 0.4 nm, 55.8 ± 3.6 nm, and 50.3 ± 1.8 nm, while the mean resolution when imaged with LS-illumination was 39.9 ± 1.9 nm, 48.9 ± 3.5 nm, and 47.1 ± 1.5 nm (reported as mean $\pm$ standard error of the mean, Supplementary Fig. 10a,b). The number of localizations detected for the three technical replicates when imaged with epi-illumination was 233,716, 341,101, and 604,412 for cell 1, cell 2, and cell 3 respectively, while the number of localizations when imaged with LS-illumination was 1,215,297, 660,758, and 637,511 for cell 1, cell 2, and cell 3 respectively (Supplementary Fig. 10a,b). Next, the LS-illuminated data sets were filtered for localization precision to match the number of localizations acquired in the epi-illuminated data sets (Supplementary Fig. 10c). The resulting resolution values in the xy, xz, and yz planes were 35.1 ± 3.0 nm, 42.5 ± 4.0 nm, 40.7 ± 4.2 nm (reported as mean $\pm$ standard error of the mean) for cell 1, cell 2, and cell 3 respectively. This demonstrates the necessity of the soTILT3D approach to enable imaging in the high-density regime at increased speeds and with improved resolution.

We have also updated the *Sample preparation* section in *Methods* accordingly:

For resolution analysis comparing epi- versus LS illumination in the high-density regime with DECODE (Supplementary Fig. 10), U2OS cells were labeled with mouse anti-lamin A/C (sc-376248, Santa Cruz Biotechnology) primary antibodies at a dilution of 1:100 in 1% (w/v) BSA for two hours. Cells were then washed three times with 0.1% (v/v) Triton X-100 in PBS with a three-minute incubation period during each wash before being labeled with donkey anti-mouse oligonucleotide-conjugated secondary antibodies (Massive Photonics, order number AB2401012) at a dilution of 1:100 in antibody incubation buffer (Massive Photonics) for one hour. Cells were

then washed three times with 1X washing buffer (Massive Photonics) in nanopure water, three times with 0.2% (v/v) Triton X-100 in PBS, and once in imaging buffer (500 mM NaCl in PBS, pH 8) before imaging. Finally, complimentary oligonucleotide-Cy3B dye conjugates (Massive Photonics, order number AB2401012) diluted in imaging buffer were flowed in at concentrations of 0.1 nM for high-density imaging.

Additionally, we have updated the information in the *Imaging procedure and settings* section:

Single-molecule images were acquired to compare epi- and LS illumination SBR and localization precision (Fig. 2b,c, Supplementary Fig. 6), to determine the Strehl ratio of our system (Supplementary Fig. 3), to compare the speed and resolution when using Easy-DHPSF or DECODE for analysis (Fig. 3, Supplementary Figs. 8 and 9) and for epi- versus LS illumination resolution analysis in the high-density regime with DECODE (Supplementary Fig. 10). For the epi- versus LS illumination localization precision comparisons of lamin B1, the 560 nm laser was used at ~ 580 W/cm² for epi-illumination and at ~ 800 W/cm² for LS illumination using an exposure time of 100 ms to match the binding kinetics of the imager strands. 50,000 frames were acquired. To determine the Strehl ratio of our system, lamin B1 in U2OS cells was imaged with the 560 nm laser at ~ 800 W/cm² first at 0 μ m and then at 5 μ m above the coverslip. 3,000 frames at a 100 ms exposure time were acquired at each height and the resulting median photon counts were compared. For the speed and resolution comparison of microtubules, the 560 nm laser was used at ~ 800 W/cm², the exposure time was set to 100 ms, and 100,000 frames were acquired. For the resolution analysis of lamin A/C comparing epi- versus LS illumination with DECODE in the high emitter density regime, three cells were imaged with the 560 nm laser at ~ 470 W/cm² for epi-illumination and ~ 450 W/cm² for LS illumination at an exposure time of 100 ms. 10,000 frames were acquired for each data set and the resulting number of localizations and resolution values in the xy, xz, and yz planes were determined.

We have also updated the information in the *Data analysis* section:

For high-density single-molecule LS and epi-illumination comparison data of lamin A/C (Supplementary Fig. 10), all data was filtered to remove localizations with localization precision greater than 30 nm.

Figure 5 illustrates not a 'whole cell' but rather a 'whole nuclei' multi-target 3D high-density single-molecule super-resolution (SR) imaging. Unfortunately, this representation does not adequately showcase the full capabilities of the soTILT3D with DECODE. Achieving 3D reconstruction with multi-stained nuclei does not inherently necessitate the utilization of a soTILT3D imaging system, unless the claimed reduction in acquisition time is indeed of an order of magnitude lower than other existing approaches. This aspect should be addressed to accurately assess the distinct advantages offered by the proposed methodology.

We thank the reviewer for this comment and suggestions for better demonstrating the capabilities of the soTILT3D system for whole-cell imaging. We have addressed this concern in our responses to comment 4.

Below, we provide responses (in red) to each of the questions/comments (in black italic), with our additions to the text indicated (in blue).

Reviewer #1

Comments for Author:

The article "Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet" has seen significant improvements compared to the initially submitted version. The authors have added substantial new material that convincingly supports their claims.

We thank the reviewer for their highly positive comments about the revisions to our manuscript and for providing helpful comments for improving our manuscript.

Specific Points:

Background Reduction:

The authors now provide additional examples demonstrating the superior signal-to-background ratio (SBR) of soTILT3D compared to epi-illumination. In their rebuttal letter, they highlighted this improvement over TILT3D by referring to the less pronounced increase of TILT3D vs. epi. However, it would strengthen the manuscript to include this comparison directly in the main text. This would again highlight the advance enhance the explanation that "soTILT3D employs a single, high-NA objective, resulting in a thinner light sheet suited for challenging samples with high background fluorescence" as a key technical advancement.

We thank the reviewer for this comment. We have added sentences to the text in the *Discussion* to emphasize this point:

In contrast to our earlier tilted LS design that utilized two objectives¹³, soTILT3D employs a single, high-NA objective. This configuration enables the formation of a significantly thinner LS, making it well-suited for challenging samples that exhibit higher background fluorescence. This is demonstrated by a >4x SBR improvement for diffraction-limited imaging and >6x SBR improvement for single-molecule imaging (Fig. 2a,b) in contrast to our previous tilted light sheet design¹³ that yielded a roughly 2x SBR improvement for diffraction-limited imaging and up to 5x SBR improvement for single-molecule imaging.

We have added sentences to the text in the *Introduction* to emphasize this point:

Since soTILT3D is based on a single, high NA objective for illumination, the thickness of the light sheet can be made much thinner than previous designs where a dual-objective system with a lower NA illumination objective was used¹³, ultimately enhancing the optical sectioning capabilities and enabling imaging of more challenging sample types.

Imaging Speed Improvement:

The authors present convincing data showing that DECODE outperforms EASY-DHPSF in terms of both resolution and the number of localizations. Interestingly, EASY-DHPSF performance already declines at imager concentrations above 25 pM, which is quite low compared to other DNA-PAINT techniques (typically ~1nM). The manuscript would benefit from a discussion on whether the need for low imager concentrations is an intrinsic limitation of soTILT3D.

We thank the reviewer for this comment. We have observed that with the introduction of flow through the microfluidic chip, as compared to a sample chamber without a flow, there is an increase in the number of localizations achieved in DNA-PAINT imaging. This has been corroborated by previous studies (M.J. Rames et. al., “Multiplexed and Millimeter-Scale Fluorescence Nanoscopy of Cells and Tissue Sections via Prism-Illumination and Microfluidics-Enhanced DNA-PAINT,” Chem. Biomed. Imaging 1(9), 817–830 (2023)) where the use of flow in a microfluidic chip was found to enhance image quality and enable a significant increase in localizations. Therefore, where we would generally use a concentration of ~1 nM in conventional imaging chambers without flow, in a microfluidic chip and with flow, we can achieve the same number of localizations at much lower concentrations. Thus, for the purpose of these experiments, we utilized DNA-PAINT imager concentrations in the 0.1 nM range, which when coupled with our light sheet, enabled single-molecule DNA-PAINT imaging in the overlapping emitter regime while maintaining a high signal-to-background ratio. The increase in number of localizations with a flow thus enables the use of a lower imager strand concentration, which is an intrinsic benefit of the soTILT3D system.

We have added a sentence to the *SoTILT3D improves the speed of 3D single-molecule imaging* section to emphasize this point:

Due to the flow in a microfluidic chip enabling a significant increase in localizations compared to when using a sample chamber without a flow⁴³, a suitable number of emitters per frame can be achieved with DNA-PAINT using a much lower imager strand concentration.

Resolution Enhancement:

Compelling data from technical replicates on FRC quantification comparing DECODE and EASY-DHPSF (Fig. S9) has been added. The authors have also added repeated measurements demonstrating the improved localization precision due to background reduction when using the light sheet versus epi. In their rebuttal letter, the authors compare the soTILT3D resolution of 8 nm lateral and 12 nm axial to Pavani et al. (PNAS 2009), who achieved 10 nm lateral and 20 nm axial using the double-helix PSF. Including this comparison directly in the manuscript would help contextualize the resolution achievements.

We thank the reviewer for recommending the replicate data to be added to the manuscript in their earlier review. As the imaging conditions were different in our manuscript compared to the work by Pavani *et al.* (they imaged bright fluorescent beads and single molecules in thin PMMA films rather than single molecules in cells), a direct comparison of the reported localization precision is difficult to accurately report. We have added a sentence to the *Introduction* section of the

manuscript to report our achieved localization precision and resolution using soTILT3D with the double-helix PSF:

Here, we achieve localization precision values as low as 8 nm laterally and 12 nm axially, and resolution values as low as 29 nm laterally and 36 nm axially for cellular imaging with the DH-PSF.

Crosstalk:

The authors present clear evidence showing that crosstalk is limited to 2-3%, which is indeed negligible.

We thank the reviewer for highlighting this point in their earlier review.

Drift Control:

The explanation of the two-step drift control process is clear, pragmatic, and easy to follow.

We thank the reviewer for emphasizing the importance of readability of this section of the manuscript.

Data Processing:

An important update is the inclusion of raw, unfiltered data. Notably, the data already appears robust without denoising.

We thank the reviewer for recommending us to show the data without denoising in their previous review. The data is indeed robust without denoising.

Summary:

In conclusion, this paper introduces a comprehensive platform for 3D super-resolution microscopy, combining novel and existing methods. With the new data and revisions, it provides a thorough technical characterization of the system, making it highly relevant to the field. The authors have addressed all my concerns, updated the figures and text, and provided additional replicates and data. With these changes, the manuscript now meets the expected standards in the imaging field. I highly recommend this excellent article for publication.

We thank the reviewer for this highly positive feedback on our manuscript.

Reviewer #2

Comments for Author:

Thank you very much for providing such detailed and organized responses. Overall, I am happy with the revisions and feel as if the paper is greatly strengthened. The only statement that I am

reluctant to accept is the response to query 8 for reviewer #2 regarding the drawbacks of the approach. In principle, the ETL can drive ~50 microns from the sidewall. Practically, this would be accompanied by a significant amount of spherical aberrations that would degrade the performance of the light-sheet. Also, it seems unlikely that one would be able to image close to the coverslip if the beam is ~50 microns away from the sidewall, as the unfocused, converging portion of the beam may interact with the coverslip itself far from the focus. Otherwise, I believe the manuscript is ready for publication.

We thank the reviewer for their positive comments about our manuscript. To minimize interactions with the coverslip, we used a tilt angle of 12° from the horizontal, which is slightly larger than the convergence angle of the beam. This tilt angle enables imaging all the way down to the coverslip. The divergence angle θ of the light sheet was estimated using $\lambda = 0.560 \mu\text{m}$ and the experimental beam thickness w in the equation $\theta = 2\lambda/\pi w$ and determined to be 10° .

Spherical aberrations of the light sheet will be more prevalent when imaging far from the sidewall. We have adapted the discussion about adaptive optics in the *Discussion* section of the manuscript to include this point:

Adaptive optics can also be implemented [in the illumination pathway and](#) as an addition to the $4f$ setup to mitigate the effects of setup- and sample-induced aberrations, including spherical aberrations [when imaging away from the coverslip or far from the side wall](#).

Reviewer #3

Comments for Author:

General Comments:

I would like to highly congratulate the authors for their outstanding work. They have thoroughly addressed all of my previous comments and conducted major additional experiments, which further strengthen the manuscript. They have also provided relevant quantification and included stunning images that significantly enhance the overall presentation. This is an excellent piece of research that is now, in my view, ready for publication. I would also strongly encourage the authors to consider submitting their work for the journal's cover selection, as the visual elements presented in the manuscript are truly exceptional.

We thank the reviewer for their highly positive comments about our manuscript and for providing helpful comments for improving our manuscript. We will submit options for consideration for the journal cover.

Minor points : While the authors have highlighted the live-cell imaging compatibility of the soTILT3D platform in the discussion, it would be beneficial to mention that in its current configuration, the system may not yet support the cultivation and maintenance of live cells for more than a few days, nor is it currently compatible with testing libraries of compounds. This

could be a limitation, particularly for laboratories focused on long-term studies, such as developmental biology or drug screening. Addressing this point would help clarify potential challenges related to ease of use for these applications.

We thank the reviewer for their constructive suggestions about clarifying the platform's compatibility with live-cell imaging and tracking. As it stands, cells have been cultured in the microfluidic chips for up to 48 hours before fixation and compounds can be tested in the chip with our pump system as long as they are compatible with the FEP (Fluorinated Ethylene Propylene) tubing and the PDMS microfluidic channel. We have added a sentence to the manuscript in the *Discussion* section to emphasize this point:

The biocompatibility of the insert and PDMS together with the gas permeability of PDMS makes the microfluidic channels suitable for live-cell studies. In fact, the cells in this study were cultured inside the chip *and live cells have been kept in the microfluidic channel for up to 48 hours*. Solution perfusion through the channel allows for precise control of the extracellular environment, *the introduction of compounds for drug screening applications*, and swift removal of heat or waste products with a constantly replenishing fluid reservoir.

Additionally, I would like to suggest moving Supplementary Figure 13 to the main manuscript as Figure 6, or alternatively, using it to replace Figure 5, which could then be included in the supplementary materials. This change would better highlight the significance of the data presented in Supplementary Figure 13, especially since Figure 1 mentions that cell aggregates can be imaged

As the focus of this manuscript was on improved imaging of individual mammalian cells, we have not included Supplementary Figure 13 in the main figures, but we highly thank the reviewer for emphasizing the significance of the added stem cell aggregate imaging data.

Whole-cell multi-target single-molecule super-resolution imaging in 3D with microfluidics and a single-objective tilted light sheet.

The amalgamation of optics, PSF engineering, microfabrication, and deep learning methods presented in this paper represents a remarkable achievement. While individual components of these techniques have been explored in prior research, the innovative aspect of this study lies in the seamless integration and synergy achieved by combining these approaches.

However, it is important to note that certain arguments presented in the article may not hold validity or need to be significantly attenuated due to their prior publication. Furthermore, there is a glaring lack of precise benchmarking in the study, and a notable absence of innovative results that have not been previously achieved. Addressing these deficiencies is crucial to strengthen the scientific contribution of the paper and ensure its distinctiveness in the context of existing research.

Consequently, I cannot recommend the publication of this paper in its current form unless substantial revisions are undertaken, addressing at least the major concerns raised herein :

Main text:

1-The utilization of other single-objective Light Sheet Microscopy (LSM) techniques for objects of varying sizes, or the application of multifocus techniques in the LightSheet configuration with DNA-PAINT, has already been documented in earlier studies. Therefore, these specific points have to be reconsidered and reevaluated in the context of the existing body of literature (V-LSFM, soSPIM, multifocus techniques, SOLEIL, ...).

Furthermore, and rather surprisingly, the authors make no reference to the paper published in PNAS in 2014 titled 'Whole-cell, multicolor superresolution imaging using volumetric multifocus microscopy' by Hajj et al. Even though the DNA-PAINT technique was not utilized in this 2014 paper, the outcomes of their volumetric multifocus microscopy (V-MFM) combined with STORM/PALM techniques (similar super-resolution techniques as DNA-PAINT) bear substantial resemblance and should not be disregarded by the authors, as indicated by the proximity in methodology and even the title. This omission needs to be precisely addressed for the sake of clarity, to establish the distinctiveness and originality of the current work, and to provide fair comparisons with existing literature.

2-The authors discuss the customization capability of their microdevices, yet they only present a single size and type of object (isolated cells). Meanwhile, other single-objective LSM (such as soSPIM) techniques have already covered a wide range of object sizes in super-resolution imaging, including but not limited to Drosophila specimens, organoids, and cell aggregates (Galland et al. 2015, and then Beghin et al. 2022 for organoids). Therefore, the authors must diversify their experimental scope to encompass a broader array of biological samples, demonstrating the versatility and adaptability of their microdevice across various scales and biological contexts.

3-The argument that the microchannel facilitates sample rinsing and marker switching using DNA-PAINT is not compelling, as other techniques employing open coverslips also offer these capabilities. Therefore, this feature may not be a unique selling point of the presented microdevice. The authors should reconsider this point or provide a more specific advantage of their microfluidic system compared to other methods in terms of sample manipulation and marker exchange.

4-The authors introduce a specific tilt angle (12 degrees) without providing a rationale for this particular choice. Moreover, if the purpose of employing a particular angle is to visualize adherent cell

structures close to the coverslip, none of the proposed markers or results substantiate this claim. It would have been pertinent for the authors to incorporate membrane or peri-membrane markers (such as integrins or actin) to demonstrate the effectiveness of their microdevice in imaging close-to-surface adherent cell structures.

5-Using nuclear and mitochondrial markers does not justify the use of the term "whole cells" as these structures are highly localized and do not extend throughout the cellular volume. The authors should consider employing markers that truly encompass the entirety of the cell to validate their claim accurately.

6-Additionally, the comparison made with epifluorescence illumination is not valid and should be removed, as it is not a technique of choice used for SMLM imaging. The authors should use comparable methods and consider conducting a more comprehensive study, incorporating techniques such as HiLo/TIRF illumination.

7-Furthermore, the comparisons should include a comprehensive benchmark analysis, considering factors such as acquisition time, reconstruction time, and the training duration of the utilized neural networks, such as DECODE. This holistic evaluation is necessary to provide an accurate and insightful understanding of the proposed methodology's efficiency and practical applicability in comparison to existing techniques.

Figures:

Figure 1 lacks essential details and fails to provide adequate information about the microfabrication device, the fabrication method, object sizes, and the distance from the coverslip. To enhance the clarity and comprehensiveness of the study, it is imperative for the authors to include these crucial details in Figure 1. A detailed schematic or diagram, accompanied by explanations in the figure caption, would significantly improve the readers' understanding of the experimental setup and methodology.

Figure 2 should be removed, and relevant comparisons should be provided (see point 6).

Figures 3 and 4 could be combined for a more cohesive presentation. Currently, Figure 3 primarily demonstrates the utility of DECODE (which has been previously published once again, Speiser et al. 2021) rather than showcasing the unique advantages of soTILT3D combined with DECODE. The key contributions of soTILT3D with DECODE are better represented in Figure 4. However, Figure 4 lacks essential information regarding acquisition time and reconstruction, which are vital for a comprehensive understanding of the technique's efficiency. Including these details, along with a clearer distinction between the contributions of soTILT3D and DECODE, is mandatory for the clarity and impact of the paper.

Figure 5 illustrates not a 'whole cell' but rather a 'whole nuclei' multi-target 3D high-density single-molecule super-resolution (SR) imaging. Unfortunately, this representation does not adequately showcase the full capabilities of the soTILT3D with DECODE. Achieving 3D reconstruction with multi-stained nuclei does not inherently necessitate the utilization of a soTILT3D imaging system, unless the claimed reduction in acquisition time is indeed of an order of magnitude lower than other existing approaches. This aspect should be addressed to accurately assess the distinct advantages offered by the proposed methodology.