Supplementary Video Captions

Supplementary Video 1. Comparison of subdiffractive beads with different fusion schemes. Left: single-view; Middle: arithmetic fusion; Right: joint deconvolution. The 3D projections are rotated with respect to the Y axis. See also Fig. 2.

Supplementary Video 2. Comparison between SDCM and diSPIM on GFP-EB3 microtubules in live human umbilical vein endothelial cells. Top row: SDCM; Bottom row: diSPIM. SDCM and diSPIM images have similar initial SNR and were taken at equivalent illumination dose, but diSPIM enables collection of 3x more volumes, 3.2x more planes per volume, and 7.6-fold less photobleaching. Note that in SDCM, the cell is significantly bleached after 1 minute. See also Fig. 3.

Supplementary Video 3. Comparison of 3D GFP-EB3 microtubule dynamics in human umbilical vein endothelial cells of different thickness and in different cellular environments with diSPIM. All cells were sampled at 15 volumes per minute over the entire 5 minute imaging duration. Maximum intensity XY and ZY views are shown. The clarity of MT tips and the stability of the fluorescence signal enable reliable microtubule tracking in 4D. See also Fig. 4.

Supplementary Video 4. SDCM volumetric time series of GFP-histones in live, BV24 nematode embryos. Volumes were sampled every minute, at 30 planes/volume. The embryo develops normally under these illumination conditions, yet resolution and image quality in the axial view are poor. Maximum intensity XY and ZY views are shown. Note that this video was compiled from two datasets.

Supplementary Video 5. Comparative iSPIM and diSPIM volumetric time series of GFP-histones in a live BV24 nematode embryo from the 4 cell stage up to hatching. Volumes were sampled every minute, at 50 planes per volume with 1 µm inter-plane spacing. Embryos develop normally under these conditions, and diSPIM offers significantly increased resolution (especially axially) compared to iSPIM (where nuclei appear distorted in the axial view). Maximum intensity XY and ZY views are shown.

Supplementary Video 6. Comparison between iSPIM and diSPIM, when visualizing neuronal processes in developing embryo. Volumes were sampled every 15 seconds, at 100 planes per volume per view with 0.5 µm inter-plane spacing. Maximum intensity XY and ZY views are shown.

Supplementary Video 7. Comparison between iSPIM and diSPIM, highlighting differences in a single volume with GFP-labeled AIY neurons. The 3D projections are rotated with respect to the Y axis.
Supplementary Video 8. DiSPIM enables visualization of AIY outgrowth processes in a live DCR553 nematode embryo. A higher contrast level was used to highlight AIY neurites in the bottom row. Volumes were sampled every 20 seconds during 5 hours of embryogenesis, at 100 planes per volume per view with 0.5 μm inter-plane spacing. Maximum intensity XY and ZY views are shown.
Supplementary Note 1

Optimal fusion of multiple volumetric views is an essential processing step in any multiview light sheet microscope. In this manuscript, we present a new joint deconvolution algorithm that produces isotropic resolution. Although others\textsuperscript{1,2} have reported similar algorithms, they are not identical to our method. For dual-view volumes $View_A$ and $View_B$, the previous method is summarized as follows:

$$Estimate_0 = (View_A + View_B) / 2$$

For $i = 1, 2, \ldots, N$ (i.e., iteration number)

$$Estimate_i = Estimate_{i-1} \times \left\{ Blur_A \left( \frac{View_A}{Blur_A(Estimate_{i-1})} \right) + Blur_B \left( \frac{View_B}{Blur_B(Estimate_{i-1})} \right) \right\} / 2$$

End

Where $Blur()$ indicates convolution with the PSF. We note that a new estimate is obtained by averaging the estimates from each view. On the contrary, our joint deconvolution algorithm is described as:

$$Estimate_0 = (View_A + View_B) / 2$$

For $i = 1, 2, \ldots, N$ (i.e., iteration number)

$$Estimate_A = Estimate_{i-1} \times Blur_A \left( \frac{View_A}{Blur_A(Estimate_{i-1})} \right)$$

$$Estimate_B = Estimate_A \times Blur_B \left( \frac{View_B}{Blur_B(Estimate_A)} \right)$$

End

We use the estimate from $View_A$ to calculate the estimate for $View_B$; then the estimate from $View_B$ is used to calculate the new estimate for $View_A$, and this process is iterated until we achieve convergence. As a result, our joint deconvolution method converges significantly faster than the previous algorithm (Supplementary Fig. 3A).

Given sufficient iterations, we find that both algorithms converge to the same isotropic resolution, but in the examples in our paper we use only 10 iterations. We note that the previous algorithm provides a significantly anisotropic PSF given this number of iterations. Images processed with our algorithm thus display a qualitative and quantitative improvement relative to previous methods, assuming the parameters we chose in our paper (Supplementary Fig. 3B).
References


Supplementary Fig. 1. Excitation Optics (A, B) and acquisition cycle (C) for diSPIM. (A, B) Axial (A) and Lateral (B) views of optics used for diSPIM excitation: 488 nm and 561 nm lasers are combined via a dichroic mirror (DC). Both lasers are directed through half wave plates and an AOTF for power control and shuttering. Lens pairs (L1 and L2, L3 and L4, and L5 or L6 and OBJ A or OBJ B), each in a 4f configuration, reimage the surface of GALVO1 onto the sample plane. Rotating GALVO1 thus rotates the beam at the sample plane (purple arrows in (B)), mitigating striping artifacts that occur in diSPIM. Rotations of GALVO2 (placed at the front focal plane of L1 and reimaged onto the back focal planes of OBJ A and OBJ B) laterally translate the beam at the sample plane (red arrows in (B)), thus forming a light sheet. Rotating
GALVO3 (placed at the front focal plane of L3 and reimaged onto the back focal plane of OBJ A and OBJ B) translates the light sheet in the axial direction (red arrows in (A), enabling rapid volumetric excitation. A beamsplitting cube (BS) and liquid-crystal shutters (SHUTTER) provide independent control of illumination in each iSPIM arm. Not shown for clarity: periscopes that elevate the beam from the optical table surface into the iSPIM excitation objectives, additional mirrors for beam alignment. Note that the figure is not to scale, and that views (A) and (B) are rotated 90 degrees with respect to each other. Also note that in (A, B), the illumination is shown propagating through both objectives simultaneously, but in reality imaging is performed sequentially and illumination and detection alternate between objectives, as in acquisition cycle (C): Volumetric View$_A$ is collected by axially scanning the light sheet produced by OBJ B (by rotating GALVO 3) while synchronously translating OBJ A (via its PIEZO mount) to maintain focus. Next, volumetric View$_B$ is collected by axially scanning the light sheet produced by OBJ A (also by rotating GALVO 3) and synchronously translating OBJ B (via its PIEZO) to maintain focus. This cycle is repeated until 4D imaging is complete. Volumetric View$_A$ and View$_B$ are then combined with our multiview deconvolution algorithm, as described in the text.
Supplementary Fig. 2. Overview of data processing. One of the two perpendicular views (View B in this figure) is rotated 90 degrees to place it in coarse alignment with View A. This simple alignment is inadequate, as overlaying the two volumes results in obvious registration artifacts (blue arrows in images). Instead, intensity-based registration and subsequent transformation operations are applied to all volumes in the time series, resulting in minimal registration artifacts when volumes are overlaid. A simple arithmetic fusion is used as an initial estimate for the joint deconvolution operation that produces isotropic resolution. Right hand image column is a higher magnification view of the rectangular boxed regions in the preceding column. See text for further details.
Supplementary Fig. 3. Improved convergence of our joint deconvolution algorithm compared to previous algorithms. (A) Lateral (black) and axial (red) FWHM values as a function of iteration number, as measured on 10 100 nm yellow green fluorescent beads. Both our method and others\(^1\) converge to an isotropic resolution of \(~330\) nm given sufficient iterations, however our method converges significantly faster. (B) Comparison between the same methods as in (A), but after only 10 iterations and on H2B-labeled chromosomes in a dividing nucleus, within a live nematode embryo. Our method offers better axial resolution (red arrows).
Supplementary Fig. 4. Performance of our joint deconvolution method under widefield illumination conditions. Widefield illumination was simulated by rapidly scanning the light sheet within over a 150 µm thick volume. Even in this case, our method recovers ~330 nm isotropic resolution within 30 iterations.
Supplementary Fig. 5. Hybrid rolling/global shutter mode. In rolling shutter mode, camera rows are read out sequentially. Although all lines are exposed for the same period (e.g. $t_{\text{firstline}} = t_{\text{lastline}}$), the sequential nature of readout can introduce a significant temporal delay between first and last lines ($t_{\text{readout}}$). We circumvent this problem by turning our light sheet illumination on after the last line is reset (done reading out) and turning our illumination off immediately before the first line is read out. This results in effective global exposure time $t_{\text{global}}$. We use a DAQ card to externally trigger the camera, and the ‘exposure output’ signal from the camera to control the AOTF and thus our illumination.
Supplementary Fig. 6. Acquisition control scheme. Control for side ‘A’: blue. Control for side ‘B’: red. Control for both sides: black. DAQ: Data Acquisition; PIEZO: piezoelectric objective positioner. The counters in our DAQ cards provide pulse trains to trigger the cameras (a, c). The external output of the cameras (b, d) is digitally combined (e) to control the AOTF blank line, enabling the laser illumination to be synchronized with the rolling shutter mode of the cameras. The AOTF intensity is further controlled by a step function provided by a DAQ analog output channel (f) to modulate the laser intensity at different imaging planes. Two DAQ digital outputs (g & h) are used to control the optical shutters for sequential excitation/acquisition of the two perpendicular views. Two analog triangle waveforms (i, j) are used to control GALVO 1 (for anti-stripping) and GALVO 2 (for light sheet creation), respectively. A step function (k) is used to control GALVO 3 (for axially scanning the light sheet within the sample); another two step functions (l, m) are used to translate PIEZOs A and B (for moving diSPIM objective lenses), respectively. See the DiSPIM Data Acquisition section of Methods for more details.
Supplementary Fig. 7. Rolling shutter mode provides lower readout noise and fewer hot pixels than global shutter mode. Top row: background comparison with no illumination. Note the greater background and hot pixels in global vs. rolling shutter mode. Bottom row: single plane in GFP-histone nematode embryo. Rolling shutter mode enables data collection with better SNR (defined as the ratio of average intensity in the red box to standard deviation of intensity values in an identically-sized background region outside the embryo).
Supplementary Fig. 8. Hybrid rolling/global shutter mode removes artifacts present in rolling shutter mode. (A) Hybrid rolling/global shutter mode, illumination synchronized to readout cycle of camera. In this mode, laser beam scanning (which is driven by DAQ analog output to GALVO2) and laser shuttering (controlled by AOTF blanking input) are triggered when the central row starts to read out, i.e., when all camera rows are exposing. This results in an identical effective exposure time for each row of camera pixels (in the schematic, all rows are exposed for two sweeps of GALVO2), and an artifact-free image. (B) Rolling shutter mode with unsynchronized illumination. In this default mode of camera operation, the start of laser scanning and laser shuttering occur when the first lines are read out (i.e., the edges of the field of view). Thus, different lines are exposed at different times, causing spatial nonuniformity in the image. For example, camera rows in zones ‘a’, ‘b’ and ‘c’ receive successively less signal during each readout cycle, as they are exposed four, three, and two times respectively. Note that the camera readout front starts from the edges of the field of view and proceeds towards the center in both (A) and (B).
Supplementary Fig. 9. Dual-view iSPIM retains imaging improvement despite coarse axial sampling. (A) Maximum intensity projections and (B) single imaging planes from GFP-labeled histones in a live nematode embryo. Lateral (YX) and axial (YZ) views are presented. Single-view volumes demonstrate poor axial resolution and optical sectioning regardless of Nyquist (0.1625 µm, left-most column) or coarser (1 µm, right-most column) axial sampling between image planes. Processed dual-view volumes (middle columns) recover isotropic resolution and demonstrate improved optical sectioning, displaying only minor image degradation with coarse axial sampling.
Supplementary Fig. 10. Motion blur artifacts in nematode embryogenesis. (A) Exemplary imaging planes from single view iSPIM volume, ~1 hour after the onset of twitching. Nuclei appear clearly resolved. (B) Planes from the same dataset, but at a later timepoint. Motion in later planes (red arrows) blurs nuclear positions, making them hard to distinguish. (C) Quantifying the degree of motion revealed an increase in blurred volumes in the post-twitching regime.
Supplementary Fig. 11. Comparisons in nuclear imaging after muscular twitching. diSPIM, iSPIM, and SDCM projections (left, middle, and right columns) at 11:16 hpf. Bottom two rows: higher magnification views of the yellow boxed regions in top rows. Note the increased resolution and SNR in diSPIM images. See also Supplementary Videos 4-5 and Fig. 5.
Supplementary Fig. 12. Comparison in neurite imaging between SDCM and diSPIM. SDCM (top) resolves AIY neurites in lateral (XY) views, but neurites are obscured in axial (ZY) views because of poor axial resolution. diSPIM resolves neurites in both views, enabling measurements of 3D neurite length. Right hand columns: higher magnification views of the yellow boxed regions at left.
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**Supplementary Table 1. Resolution comparison between different fusion schemes.** Mean and standard deviation FWHM values for 10 100 nm yellow green fluorescent beads are compared, as determined from single-view, fusion, or deconvolved datasets. See also Fig. 2, Supplementary Fig. 3, and Supplementary Video 1.