Dual-view plane illumination microscopy for rapid and spatially isotropic imaging

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INTRODUCTION

Light sheet fluorescence microscopy (LSFM)

with a low-magnification objective mounted in the epifluorescence port of the inverted microscope, and it is translated to the focus of each objective. This geometry is well adapted for imaging cultured cells and horizontally mounted tissues, avoiding the difficulty of mounting such samples in a vertical sample holder[17]. More recently, we modified iSPIM to capture a second specimen view, by alternating excitation and detection between the two objectives. The resulting 'diSPIM' provides isotropic spatial resolution (down to 330 nm) at high speed (200 images per s, 0.5 s for a dual-view 50-plane volume)[11].

Overview of the procedure

Here we describe the step-by-step construction of a diSPIM entirely from commercially available parts (Fig. 1). Unlike our previous implementation[11], which used free-space optics to couple in laser excitation to the sample, this design is compatible with fiber-coupled laser excitation. Such fiber-based excitation facilitates alignment, making the device compatible with a broad array of commercial laser excitation sources, and it assures collinearity among different excitation wavelengths. Furthermore, instead of controlling multiple galvanometric mirrors, compact scan-heads (one for each specimen view) are used to generate and sweep the excitation sheets through the sample, also aiding in system alignment. Data acquisition software for generating and sweeping the light sheets, controlling and synchronizing objective piezos, and recording images from scientific complementary metal-oxide semiconductor (sCMOS) cameras is also provided.

After describing diSPIM construction, we present controls for verifying the spatial resolution of the system and the proper alignment of the light sheets. Next, we describe the use of diSPIM in performing 3D imaging of *C. elegans* embryos throughout 14 h of development, and imaging of whole cells over ~30 min. The same protocol can be adapted for 4D studies of other samples of approximately similar dimensions ($50 \times 50 \times 50$ μm^3). We conclude with the postprocessing (registration and image fusion and deconvolution) operations necessary to produce data sets with isotropic resolution.

Limitations

To obtain the best diSPIM data, it is necessary to obtain high-quality specimen views from each objective lens. Furthermore, the objectives must provide faithful but complementary measurements of the same object. If the object moves during dual-view acquisition (motion blur), if one view provides noticeably inferior image quality (owing to depth-dependent scattering or aberrations that preferentially degrade that view), or if the two views are poorly aligned, the fused reconstruction may display artifacts (Supplementary Note 1, SF1 and Supplementary Data 1). In extreme cases, the registration algorithm may be unable to correctly align the two views owing to a low degree of similarity between the views. Although we prefer dual-view acquisition owing to the isotropic resolution it provides, we note that single-view operation (iSPIM[5]) is at least twice as fast and may be preferred if acquisition speed is of paramount importance. Single-view operation also obviates imaging artifacts that arise because of the registration or fusion process, and it is fully compatible with this protocol.

We have enjoyed the most success when imaging small (~50–60 μm), transparent samples (single cells, a few layers of cells or *C. elegans* embryos; Supplementary Videos 1 and 2), wherein the 3D dynamics of interest occur no faster than several volumes per s. Larger samples may be accommodated by using lower- magnification objectives (10× or 20× lenses) and/or by widening the width of the light sheet (by further stopping down the iris)—although we do not cover this explicitly in this protocol, our design is flexible enough to accommodate such changes (Supplementary Table 1). For especially large, scattering samples (such as *D. melanogaster* embryos), two-photon excitation may provide better performance than conventional single-photon excitation[18]. In this case, modifications to our system might include fibers appropriate for the high pulsed intensities generated by femtosecond laser sources, optical components that are better adapted for near-IR wavelengths and alternate methods of shuttering or modulating the beam intensity. Implementing confocal slit detection[19] in each arm would also reduce background in scattering samples. Finally, we note that alternative LSFM implementations that use bidirectional illumination[18] and detection[7,8] may offer better sample coverage in large specimens than the implementation we describe here.

Experimental design

Fiber-coupled laser excitation—The laser excitation in this design is coupled to the diSPIM module by two optical fibers, one for each arm of the microscope. Many commercial options exist for single-mode fiber-coupled laser excitation (for example, the Nikon MLC 400B), providing compact assemblies with a range of wavelengths and powers (~10 mW per laser line at the output of each fiber is more than sufficient for most fluorescent samples). If you are obtaining a commercial solution, the two requirements are: (i) the end of the fiber should have an FC/PC (or FC/APC) connector, for coupling into the diSPIM excitation scanner; and (ii) it should be capable of rapidly modulating the power and switch between laser lines at high speed (ms or faster).

A cheaper, but potentially more time-consuming, option is to construct your own laser launch from component parts. Although the step-by-step construction of such an assembly is

outside the scope of this paper, we provide a schematic and photograph of our custom-built diSPIM launch (Supplementary Note 1, SF2). In this setup, we use an acoustooptic tunable filter (AOTF) to modulate 488-nm (for exciting GFP and similar fluorophores) and 561-nm (for exciting mCherry and similar fluorophores) laser wavelengths, combining the two lines before splitting the beams into two optical fibers. The maximum power at the fiber output (before insertion into the diSPIM scanners) in this design is 20 mW (488-nm excitation) and 10 mW (561-nm excitation).

Microscope hardware—

MATERIALS

REAGENTS

For verifying diSPIM alignment and system performance

- Alexa Fluor 488 fluorescent streptavidin (Invitrogen, cat. no. S-32354)

- Yellow-green fluorescent beads, 100 nm (Invitrogen, cat. no. F8803)

For *C. elegans* embryo imaging

- Gravid young adult *C. elegans*, strain BV24 [ltIs44 [pie-1p-mCherry:: PH(PLC1delta1) + unc-119(+)]; zuIs178 [(his-72 1 kb::HIS-72::GFP); unc-119(+)] V], as described in Wu *et al.*[5]

- Poly-L-lysine (Sigma-Aldrich, cat. no. P1524)

- Methyl cellulose (Sigma-Aldrich, cat. no. H7509-25G)

- M9 buffer, see Stiernagle[21]

For cellular imaging

- DMEM with high glucose (4.5 g/l), supplemented with GlutaMAX and sodium pyruvate (Life Sciences, cat. no. 10569-069)

- DMEM without glucose, glutamine, pyruvate and phenol red (Life Technologies, cat. no. A14430-01)

- Glucose solution, 45% (wt/vol) (Corning, cat. no. 25-037-CI)

- Sodium pyruvate, 100 mM (Life Technologies, cat. no. 11360088)

- GlutaMAX supplement (Life Technologies, cat. no. 35050061)

- Sodium bicarbonate, 7.5% (wt/vol) solution (Life Technologies, cat. no. 25080094)

- HyClone FBS, defined (Thermo Scientific, cat. no. SH30070.03)

- HEPES 1 M sterile-filtered solution, pH 7.0–7.6 (Sigma, cat. no. H0887)

- TurboFect transfection reagent (Thermo Scientific, cat. no. R0531)

- Tissue culture dish, 100 mm, BD PureCoat amine (BD, cat. no. 354732)

- Hydrochloric acid (HCl), 37% (wt/vol) solution (Sigma, cat. no. 320331)

- Ethanol, 99.5% (vol/vol; Sigma, cat. no. 459844)

- GFP-tagged H-Ras plasmid[22] (GenBank accession no. AF493916)

- Transformed human lung fibroblasts[22]

- Glass coverslips, no. 1.5, 24 × 50 mm (VWR International, cat. no. 48393-241)

EQUIPMENT

For diSPIM system

- DiSPIM acquisition and data processing computer workstation (HP, cat. no. Z820 with features and accessories described in Supplementary Table 2). This computer should also include a solid-state RAID drive, with specifications equivalent or similar to the following: Corsair Neutron Series GTX, solid state drive—240 GB—

quantity 4, clamping fork for 1.5-inch post, cat. no. PF-175; quantity 2, 1/2-inch diameter (length 4 inches) post holder, cat. no. PH4; quantity 4, 1/2-inch diameter (length 4 inches) stainless steel optical post, cat. no. TR4)

- Optical table with isolators, 4 feet × 6 feet × 12 inches (TMC, cat. nos. 784-651-02DR and 14-416-34)

- Metric and imperial ball-drivers and hex keys for bolting and unbolting optomechanical components (Thorlabs, cat. nos. TC2, TC3/M)

For testing equipment

-

- Dissecting microscope with 10×–50× zoom range and fluorescence capability (for example, Leica, cat. no. MZ10F)

- Platinum wire, 0.5-mm diameter (Sigma-Aldrich, cat. no. 267201)

- Pasteur pipette (Corning/Sigma-Aldrich, cat. no. CLS7095D5X)

REAGENT SETUP

M9 buffer—M9 buffer for imaging worm embryos is prepared as described in Stiernagle[21]. M9 buffer can be stored at 23 °C for 6–8 weeks.

Poly-L-lysine—Poly-L-lysine is used for sticking the embryos to glass coverslips. We use a working concentration of 1 mg/ml Poly-L-lysine dissolved in Milli-Q or similarly clean water. For long-term storage, prepare 5–10-ml aliquots and store them at −20 °C. Once thawed, the aliquots can be stored at room temperature (23 °C) indefinitely.

Methyl cellulose—Methyl cellulose is used for cutting the worms in order to release the embryos. Prepare a 1% (wt/vol) solution in M9 buffer (we usually prepare 50-ml batches containing 0.5 g of methyl cellulose). Methyl cellulose must be stirred in warm M9 until it is dissolved. Once prepared, this solution can be stored at room temperature for up to 6 months.

Preparation of coverslips with fluorescent bead layer—Fluorescent beads are convenient resolution targets, and they are useful in aligning and testing the diSPIM. Pipette 50 μl of poly-L-lysine onto a cleaned coverslip, by smearing it across the central region. After letting the solution dry for 5 min and pipetting off excess poly-L-lysine, add 50 μl of 2,000×-diluted 100-nm yellow-green bead solution. Allow the beads to dry completely, and wash them two or three times with water. Coverslips can be stored indefinitely at room temperature.

Dye solution—Dye solution is useful to visualize the fluorescent emissions from the light sheets during diSPIM alignment. We usually start by diluting Alexa Fluor 488 streptavidin conjugate 1,000× in water (this solution can be stored at room temperature over a period of weeks), adjusting the concentration as necessary during imaging.

EQUIPMENT SETUP

Worm-pick creation—Worm picks for gathering and moving gravid *C. elegans* are made by inserting a 32–38-mm platinum wire into the thin end of a glass Pasteur pipette, so that roughly 20 mm of the wire protrudes from the pipette. The end of the pipette is briefly heated over an ethanol lamp to fuse the glass to the platinum wire. After fusing, pliers are used to flatten the end of the platinum wire, thus increasing the available surface area for picking worms. Note that this procedure is covered in great detail in Murray and Bao[23].

Mounting coverslips into the imaging chamber—The samples in the following procedure need to be mounted on cleaned (as described previously[24]) rectangular coverslips that are then placed into the steel imaging chamber. This procedure usually takes 5–10 min,

9| Install the excitation laser launch. If you are using a commercial unit, determine how the laser lines can be controlled with analog voltages. Make the necessary electronic connections to provide analog voltages to the commercial laser launch from the DAQ cards. If you are using a home-built laser launch, control the lasers using the AOTF. Connect the AOTF controller to the DAQ cards with BNC cables. Verify that all laser lines can be shuttered and that their intensity can be varied using the AOTF controller.

! CAUTION To avoid serious injury when using lasers, always follow recommended safety procedures. We recommend the use of laser warning signs and safety equipment (such as laser goggles) in line with requirements set by institutional, local and national safety standards.

10| Connect and test the bottom camera using the manufacturer-supplied camera control software.

Setting up the diSPIM frame and installing the lower imaging path   TIMING 4–6 h

11| Bolt the RAMM frame to the optical table.

12| Attach the bottom LS-50 stage.

13| Attach the mirror cube to the LS-50 stage.

14| Insert emission filters (488-nm long-pass and 561-nm notch filters) into the dichroic filter cube, and then attach the dichroic filter cube to the mirror cube.

15| Slide the tube lens through the bracket and lock it onto the dichroic filter cube.

16| Attach the bottom camera to the tube lens using the c-mount connection.

17| Attach the 10× objective to the objective adaptor on the LS-50 stage

the stage. Make sure that the marked surface is facing up. Turn on the room lights and focus the bottom objective on the coverslip. Locate the black mark on the coverslip and position it in the center of the FOV of the bottom camera. Focus the bottom objective on the black mark using visual feedback from the camera (focusing at the edge of the mark may be easier than focusing in the center; use exposure time: 300 ms). Record the *XY* position of the stage and the position of the bottom objective displayed on the stage controller.

Aligning the excitation scanners   TIMING 3–4 h

21| Connect the fiber output from the laser launch to each scanner. Turn on the 488-nm laser output and project both beams onto a wall or board, which is at least 1 m away from the scanner.

22| Ensure centration of beams on each MEMS mirror (Supplementary Fig. 1). If the beams are not centered, use the MEMS mirror adjusters (the four screws holding each MEMS mirror) to improve centration.

23| Once the beams are centered, open and close the iris and monitor the output beams. Close the iris so that the MEMS mirror boundary is not visible in the beam profile. (This ensures that the beam diameter is not bigger than the MEMS mirror.)

   CRITICAL STEP Do not close the iris completely, as this affects the light sheet thickness (Supplementary Table 1) and reduces excitation power.

24| Switch off the 488-nm laser, attach the tube lens to the scanner and mount both scanner assemblies on an optical table using tube lens brackets and posts (Supplementary Fig. 1). Switch on the 488-nm laser and examine the collimation of each beam. The beams should not markedly change their diameters (i.e., they should be well collimated) over a distance of at least 5 m, and they should emerge from the center of the tube lenses (using a circular target is helpful in checking centration after the tube lenses; Supplementary Fig. 1). If the beam diameter noticeably grows or shrinks over this distance, the beam collimation state must be adjusted by translating the position of the collimation mirror (Fig. 1d). Once both beams are collimated, remove the tube lens and check the centering of each beam on its MEMS mirror again (Step 22). If the beam is not centered, repeat Steps 22–24 and recheck the collimation. Repeat this process until both beams are well centered and collimated.

Assembling the optics in dichroic filter cube holders and attaching them to excitation scanners   TIMING 1 h

25| Remove the top plate of the dichroic filter cube holder along with the dichroic filter cube (refer to Supplementary Note 1, SF5). Release the dichroic filter cube by loosening the screw on the top plate.

26| Open the dichroic filter cube and slide in the dichroic mirror.

CRITICAL STEP Make sure that the reflecting surface of the dichroic mirror faces the laser input. Do not overtighten the screws holding the dichroic mirror in place, as this will cause optical aberrations.

27| Insert the emission filters (488-nm long-pass and 561-nm notch filters) on the transmission side of each dichroic filter cube. Reattach the dichroic filter cube to

38| Connect the Tiger controller inputs to the DAQ cards (BNC extension boards) using BNC cables, as described in Supplementary Note 2.

▲ **CRITICAL STEP** Unplug the power cable of the Tiger controller before connecting the inputs to the DAQ card.

39| Open 'diSPIM Control.vi' (Supplementary Note 2) and select parameter adjustment mode. Enter the following parameters: Time/Plane, 1,000; Z Step Size, 0; Planes/Volume, 0; Light Sheet Width, 0; no. of Volumes, 1; Time Point Delay, 0; SPIM arm A and B Z-Galvo offset, 2.5 V; Y-Galvo offset, 2.5 V; Objective offset, 5 V; Z-Galvo and Piezo Conversion factor, 0. Here 'X, Y and Z ' are local coordinates that refer to the individual excitation, defined in Supplementary Note 2.

40| Switch on the Tiger controller and run 'diSPIM Control.vi'. This will bring the objective piezos to the center of their range by providing an offset voltage of 5 V. It will also move the MEMS mirror to the zero position in both arms.

▲ **CRITICAL STEP** It is prudent to monitor the output of the piezos using an oscilloscope. Connect the 'sensor out' signal from the piezo card to the oscilloscope, and ensure that the sensor readout displayed on the oscilloscope matches the voltage applied to the piezo. If inconsistencies are observed, contact ASI.

41| Bring the diSPIM module down (toward the coverslip) using the stage controller and ensure that the objectives do not touch the coverslip.

▲ **CRITICAL STEP** Move the diSPIM module in small (~10 μm) steps to ensure that the objectives do not hit the side walls of the chamber.

42| Stop moving the diSPIM module toward the coverslip once the intersection of the two beams touches the coverslip surface (visual inspection is enough at this point).

43| If the intersection of beams is laterally offset from the black mark, adjust the position of the CDZ-1000 stage (Supplementary Note 1, SF9) until the beams intersect each other on the black mark. Record the F axis position from the stage controller.

44| Move the diSPIM module as far away from the coverslip as possible (~35 mm), remove the imaging chamber, replace the marked coverslip with a fluorescent bead sample and place the imaging chamber back in the diSPIM. Bring the diSPIM module toward the coverslip at the F axis position recorded in Step 43, r97oller.

46| If the spots do not overlap, attempt to bring them closer using the objective adjuster and by moving the diSPIM module toward or away from the coverslip (in 5–10-μm steps). Both spots should be as focused as possible, as viewed in the bottom camera, and they should overlap completely (as shown in Supplementary Fig. 2). Record the *F* axis position once the spots overlap and the focus has been optimized.

    CRITICAL STEP Do not attempt to focus by using the lower objective; the goal is to bring the spots to the focal position determined in Step 20.

47| Once the spots in the bottom camera overlap, refocus the bottom objective and compare their relative sizes (if the spots are very bright and saturate the camera pixels, reduce the laser power). If they are not of the same size, repeat Steps 33 and 34 until the spots are equal in size (the difference should not be >20%).

48| Expect to iterate Steps 33–47 until the two spots are separated by no more than 50 μm, as viewed in the bottom camera, and they appear to be of the same size. This might take several hours.

Assembling and positioning the SPIM cameras   TIMING 4–5 h

49| Bring the diSPIM module to the *F* axis position recorded in Step 46.

50| Attach 65-mm spacer tubes to each dichroic filter cube holder (Supplementary Note 1, SF10).

51| Attach the mirror cube holders to the spacer tubes so that the circular opening in the cube points toward the back of the microscope.

52| Attach the tube lenses to the SPIM cameras.

53| Place the SPIM cameras in a horizontal position so that the bottom plate faces down, toward the optical table (the bottom plate of each Flash 4.0 camera has four threaded holes that are a convenient reference point, Supplementary Note 1, SF11).

54| Mount the tube lenses into the brackets and mount the brackets onto variable height posts (Supplementary Note 1, SF3; posts are assembled by screwing post holders to mounting posts (P4-1.5-inch to PH4)). Adjust the height of the posts until the top surface of the tube lens bracket is ~50 cm above the surface of the optical table.

55| Position the camera assemblies until the tube lens is in line with the mirror cube (Supplementary Note 1, SF6) and the separation between tube lens and mirror cube is ~ 3 mm. Bolt the camera assemblies to the optical table using clamping forks (PF175).

56| Slowly move the diSPIM module away from the coverslip (~5 mm), making sure that the mirror cubes do not touch the tube lenses.

57| Rotate the SPIM arm A camera (along with the tube lens) 45° clockwise and the SPIM arm B camera 45° counterclockwise (looking from the back of the

diSPIM system; Supplementary Note 1, SF11). Visual alignment as in Supplementary Note 1, SF11 is sufficient.

58| Bring the diSPIM module back to the position in Step 46.

59| Switch on both SPIM cameras and open the Hamamatsu HCIMG software. Set the exposure time to 100 ms, use the entire FOV (2,048 × 2,048 pixels) and set the cameras to 'auto intensity' mode. Switch on the 488-nm laser and block the laser input to arm B. Run 'diSPIM Control.vi' using the parameters entered in Step 39. Examine the fluorescence in the SPIM camera B: you should see a horizontal pencil beam (Supplementary Fig. 3). If the beam is substantially tilted, adjust the dichroic mirror of SPIM arm A until the beam is horizontal.

60| Unblock the laser input to arm B and you should see a new fluorescence spot caused by epifluorescence close to the pencil beam (in Step 59). If you do not

FOV, as determined by the *Y* offset (defined in Supplementary Note 1, SF12). If the beams are not centered, adjust the mirror cube using the fine adjustment screws on the top plate of the mirror cube (Supplementary Note 1, SF10) until SPIM beams appear centered in both camera FOVs.

CRITICAL STEP The *Y* offset is crucial because the Flash 4.0 camera acquires images in rolling shutter mode, and the speed of acquisition thus depends upon the location of the FOV in the camera chip. The value of *Y* offset depends on the region of interest (ROI) size. For example, for a 500×500 pixel FOV, the *Y* offset should be 774 pixels and the beam should appear in the middle of the FOV (~1,024 pixels; Supplementary Note 1, SF12).

72| Run 'diSPIM Control.vi' in parameter adjustment mode for 1,000 volumes using the parameters recorded in Step 70. As long as the .vi file is running, two vertical lines corresponding to the SPIM beam and epifluorescence beam should be visible in SPIM cameras.

73| Examine the vertical lines in the SPIM cameras using the HCIMG software, and record the *X* offset position. If the *Y* offset is lower or higher than 774 (for example, if using a 500×500 pixel FOV), adjust the beam position using the fine adjustment screws on the top plate of the mirror cube (Supplementary Note 1, SF10).

74| Stop 'diSPIM Control.vi' and record all front panel parameters and SPIM camera offset values. Examine both beams using the bottom camera. Save an image that records the position of the beams in the bottom camera. Future samples should be moved using the *XY* stage until they are located at this beam position, thus ensuring that the light sheets excite the sample and that the sample lies in the FOV of both SPIM cameras.

Imaging a fluorescent bead layer to measure the PSF and beam thickness   TIMING 1 d

75| *Setting image acquisition parameters for optimal image quality (Steps 75–81).* Place a coverslip with a fluorescent bead layer sample in the sample chamber. Fill the chamber with water and place it on the *XY* stage. Bring the diSPIM module close to the coverslip (F axis position used in Step 74).

76| Select the scouting mode in 'diSPIM Control.vi'. Enter *Z*-Galvo offset, *Y*-Galvo offset and Piezo offset as specified in Step 74. Enter Time/Plane, 5 ms; *Z* Step Size, 0.5 μm; no. of Planes/Volume, 80; Light Sheet Width, 60 μm; no. of Volumes, 1; Time Point Delay, 0; GFP laser Intensity, 5; *Z*-Galvo Conversion factor, 0.013 V; Piezo Conversion factor, 0.05 V.

77| Acquire a single volume by running 'diSPIM Control.vi'.

78| Open up both stacks of tiff images in ImageJ (i.e., corresponding to each view).

79| Examine the maximum intensity projection and individual planes in the volume. Ideally, the beads should appear as shown in Supplementary Figure 7 (middle)

adjust the focus of the collection objectives as follows: set the *Z*-galvo conversion factor in the 'diSPIM Control.vi' to zero, ensuring that the light sheet is stationary (i.e., is not scanned in the *Z* direction). Acquire 1 volume. Examine the stack of tiff images by opening the stack in ImageJ; the image should go from out-of-focus to in focus, and then leave focus again (Supplementary Data 3). Next, find the frame at which the beads are in best focus. If the collection objective is in focus, then this frame should be in the middle of the stack. If the focused frame number is more than half the total number of frames, reduce the

85| Subtract the average background (background image taken in Step 83) from this stack (Process -> Image calculator).

86| Select a bead from this stack (draw a rectangle around the bead, ~14 pixels on a side) and duplicate it in ImageJ (Image   duplicate).

87| Clear the scale (Analyze   Set scale   click to remove scale).

88| Set the Z-scale to 3.07 (0.5/0.1625, i.e., the step size divided by the pixel size (Image   Scale)), so that the voxel size is isotropic (0.1625 μm in each dimension).

89| Reslice this image from the top (Image   Stack   Reslice).

90| Draw a horizontal line across the center of the bead, and measure the lateral intensity profile of the bead.

Draw a vertical line across the center of the bead and measure the axial intensity profile for the bead (Analyze   Plot profile). From these intensity profiles, record the full-width at half-maximum (FWHM) values in pixels and convert to μm by multiplying by 0.1625. These values should be close to 0.5 μm (lateral) and 1.7 μm (axial) (as in Fig. 2c, Supplementary Table 4 and Supplementary Data 5 (SPE6

series of measurements described above, repeat the same procedure for the other SPIM arm. Example results are displayed in Figure 2 and Supplementary Note 1, SF13. The expected light sheet thickness should be close to ~2 μm at the beam waist.

? TROUBLESHOOTING

Imaging cells or *C. elegans* embryos   TIMING 1–5 d

96| Once the system is assembled and characterized, *C. elegans* embryos (option A) or cultured cells (option B) can be imaged.

(A) Imaging *C. elegans* embryos   TIMING 4–14 h

i. *Embryo isolation.* Add 20 μl of 1% (wt/vol) methyl cellulose solution to a square watch glass.

ii. By using the dissecting scope to visualize worms, use the platinum wire worm pick to transfer 5–8 gravid adult *C. elegans* from the worm plate to the drop of methyl cellulose. The adult *C. elegans* should be seen swimming in the methyl cellulose solution.

iii. Locate a swimming adult and use the scalpel to slice the adult worm in half. Embryos should be seen to spill from the two halves of the adult *C. elegans*.

iv. *Transferring embryos to the well chamber.* Place a coverslip into the imaging chamber and pipette 30–40 μl of poly-L-lysine into the center of the coverslip. Wait for ~5 min, and then fill up the chamber with fresh M9 buffer (7–8 ml; Supplementary Note 1, SF14).

v. Insert the capillary into one end of the aspirator tube (Equipment Setup). Insert a clean 20–200-μl plastic pipette tip into the other end of the aspirator tube. Make sure that only the clean 20–200-μl pipette tip enters the mouth when using the aspirator tube.

vi. Place the capillary into the methyl cellulose solution and gently suck several embryos into the capillary via gentle pressure.

vii. Transfer the tip of the capillary into the imaging chamber. Position the capillary tip so that embryos will fall into the central square of the coverslip.

viii.

xi. Use the bottom camera to locate an embryo to be imaged, with the room lights on.

xii. Bring the embryo to the *XY* stage position recorded in Step 74.

xiii. Open the LabVIEW program 'diSPIM Control.vi'.

xiv. Bring the diSPIM module to the *f* axis position recorded in Step 80 by selecting the diSPIM Module Control Mode in 'diSPIM Control.vi' and running 'diSPIM Control.vi'.

xv. Once the diSPIM module moves to this *F*-axis position, select 'scouting mode' and enter the parameters from Step 80, except set the *Z* Step Size to 1 μm and the number of Planes/Volume to 50.

xvi. Set the ROI of each SPIM camera as in Step 73.

xvii. Acquire one volume. Open the volume in ImageJ. Verify that the embryo is in the FOV. If the embryo is not in the FOV (either in one or both SPIM cameras), translate the *XY* stage slowly. After each move, acquire a volume and examine it in ImageJ to check the embryo position. The goal is to center the embryo in both SPIM cameras' FOVs.

xviiiEnsure that the light sheet is covering the entire embryo. If this is not the case, change the *Y*-galvo offset of the arm (in steps of 0.1 V/8 μm) causing incomplete illumination, until the entire embryo is illuminated (Supplementary Fig. 9 and Supplementary Video 3).

xix. Make sure that the image stack corresponding to each view covers the entire embryo (as shown in Supplementary Fig. 9 and Supplementary Video 3). If part of the embryo is located outside the imaging stack, move the diSPIM module up and down (in steps of 5 μm). Acquire an imaging volume each time the diSPIM module is moved, checking the embryo coverage. Stop when the embryo is contained within the stack. Open the stack and examine it in ImageJ.

CRITICAL STEP Do not change the *Z* offset of the MEMSoveTlume and8enStep not wate169.720071

? TROUBLESHOOTING

xxiii Run 'diSPIM Control.vi' in Image Acquisition Mode (GFP, 488 nm) with the following parameters for 14-h imaging: number of Volumes, 1,000; Time Point Delay, 60 s, and provide a file name. Note that we assume that imaging commences when the embryo is in the 4–8-cell stage.

xxiv Process the images according to the instructions in Steps 97–106. Figure 3 shows selected maximum intensity projections from the deconvolved data set (see Supplementary Data 7 for data relating to this figure); see also Supplementary Data 1.

(B) Imaging cells   TIMING 4–5 d

i. *Preparing the coverslips for cellular imaging.* Place rectangular glass coverslips in a glass beaker half-filled with distilled water, taking care to separate coverslips that are stuck together. Boil them for 10 min. Let the solution cool and occasionally agitate.

ii. Rinse the coverslips extensively with distilled water.

iii. Transfer the coverslips to a clean glass beaker with 1 M HCl solution. Place them in a fume hood and allow them to stand overnight.

iv. The next day, rinse them extensively with distilled water.

CRITICAL STEP

ix. Pipette 500 μl of plain DMEM in a sterile 1.5-ml tube. Add 5-10 μg of plasmid DNA (GFP-tagged H-Ras), and mix it well but gently.

    CRITICAL STEP Plasmid DNA should be of high purity to increase transfection efficiency and to minimize toxicity.

x. Add TurboFect liposomes reagent at a 2:1 or 3:1 liposome:DNA ratio and mix four or five times gently. Let it stand for 15–20 min.

xi. Distribute the transfection mix to the cells dropwise using a 1-ml pipette (the broad tip reduces the disruption of lipid-DNA complexes).

xii. Return the cells to the incubator (37 °C, 5% CO_2) overnight.

xiii. *Cellular imaging.* On the next day, insp psFukT2igeF0 10 Tf 71.12plls1of 1 0

volumes and time delay between volumes on the basis of your requirements (for the data in Fig. 4 and Supplementary Data 8, the number of volumes was 350 and the time delay 5 s).

xxv. Process the acquired images as described in Steps 97–106 below.

Data processing   TIMING 3–8 h

97| The data files output from each SPIM camera should be in multipage TIFF format—i.e., each TIFF file should contain an entire volume represented as a stack of individual planes (images). Separate all volume files into two folders, corresponding to the two SPIM cameras, as shown in Supplementary Note 1, SF16.

98|

registration. If the sample does not move much during imaging, an early time point can be used to generate a registration matrix that is applicable to the entire time course. In such cases, we usually choose one of the first 20 time points for matrix generation. In samples in which substantial movement occurs during imaging, it is necessary to process the data in subgroups (we have found that subgroups consisting of ~100 time points work well), or even run registrations at each time point in the series by choosing 'All time registration'. In the latter case, users will only need to specify the directory in which to save the matrix for each time point.

103| After setting the registration parameters, define the range of angles used when transforming volume B into volume A via the registration procedure. Typically, a searching angle range from $-10°$ to $10°$, a coarse angle increment of $2°$–$3°$ and a fine-angle increment of $0.5°$–$1°$ results in an acceptable registration within 5–10 min.

104| Specify where the raw data are located (Supplementary Note 1, SF18). Images placed in the 'transform' directory will be rotated by $90°$ about the Y axis (as illustrated in Supplementary Note 1, SF19), transforming them into the coordinate system of the images inside the 'base' directory. Also specify the range of volumes that need to be transformed, fused and deconvolved. If this range is left empty, the plug-in will automatically select all volumes.

105| Set parameters in the 'Option' tab (Supplementary Note 1, SF20), including pixel size and the number of concurrent fusions to run (at most, the number of CPU cores on the machine). Users also need to set other options by selecting the 'Options' tab in the plug-in, as shown in Supplementary Note 1, SF20. In the 'algorithm options' (red box), set the image pixel size and the number of concurrent fusions, which depend on the number of CPU cores (e.g., users can input up to 12 if the computer has 12 CPU cores). If desired, specify whether to display or to save intermediate processing steps, such as maximum intensity projections or fusions based on the arithmetic or geometric mean between the two views. Finally, set the GPU-based deconvolution parameters including iteration number (at least 10), sigma values (in pixels) of the PSF characterizing each view (typically 3.5, 3.5 and 9.6 for X

- An example of raw cellular data acquired from each arm and the resulting deconvolution (corresponding to Fig. 4 and Supplementary Data 8)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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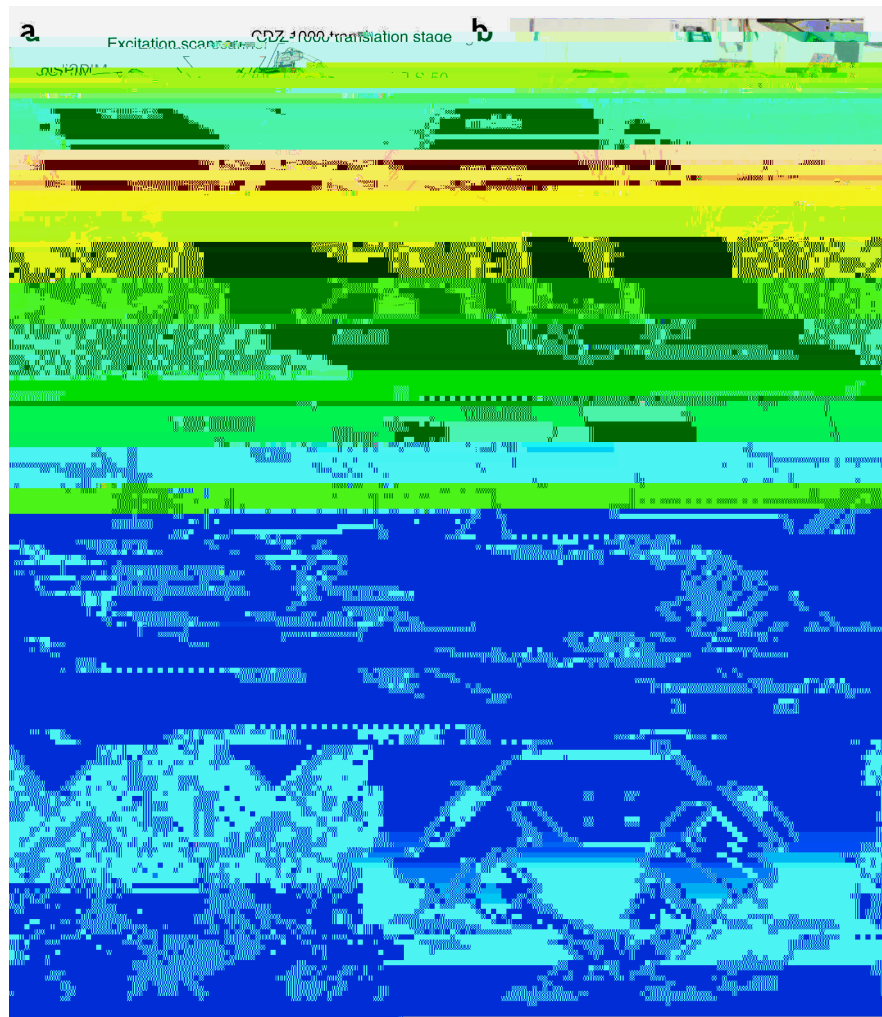
Figure 1.
DiSPIM with major subassemblies. (**a**) Perspective view of assembled diSPIM, with microscope frame, inverted microscope components, cameras and major diSPIM subassemblies indicated. 'DiSPIM module' refers to scanners, dichroic filter cubes, tube lenses, objectives, piezos and associated optomechanics that move as a single unit on an LS-50 translation stage. Refer to the MATERIALS section for further description of component parts. (**b**) Photograph to accompany **a**. (**c**) Higher-magnification view of excitation and detection subassemblies in the diSPIM module. Excitation (blue) and emission (red) along a single arm of the module are highlighted. (**d**) Detailed view of the excitation scanner, showing fiber input, mirrors and relay lenses. (**e**) Higher-magnification view of objectives, piezos and holders/adjusters. (
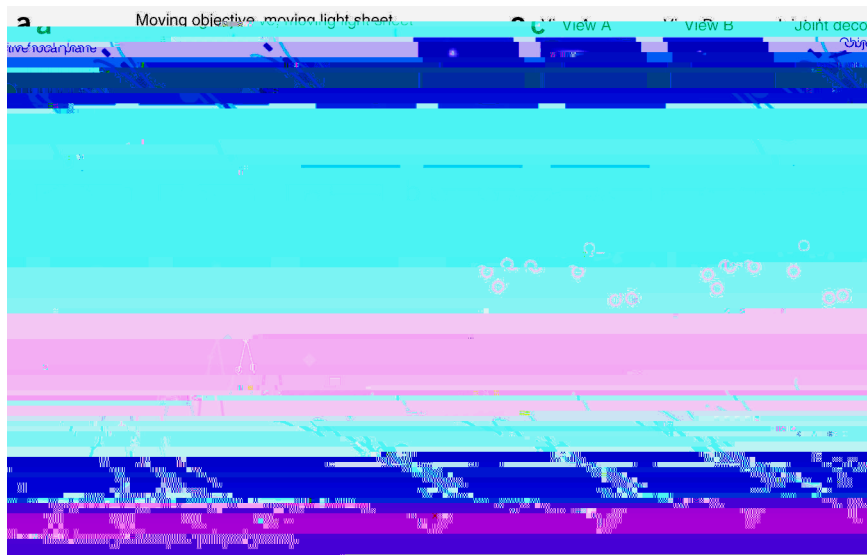
Figure 2.
Verifying system performance. (**a**) Measuring the system PSF across the FOV by collecting a *Z* stack on a fluorescent bead. Light sheet and the collection objective move in sync. (**b**) *XY* maximum intensity projections of beads, showing raw data from view A (left) and view B (right). Scale bar, 5 μm. (**c**) Higher-magnification view of beads within dashed rectangular region in **b**, highlighting the improvement in lateral and axial resolution after joint deconvolution procedure. Scale bar, 3 μm. See also Supplementary Table 4 with lateral and axial resolution from all beads marked with red circles in **b**. (**d**) Measuring the light sheet thickness. The collection objective remains focused on the fluorescent bead, integrating the fluorescence as the light sheet is scanned through the bead. (**e**) Light sheet thicknesses in arm A (blue diamonds) and arm B (red squares). Apparent light sheet thicknesses at this location are 2.1 μm (SPIM A) and 1.8 μm (SPIM B). Data were derived from the bead marked with the blue squares in **b**. See also Supplementary Note 1, SF13, showing the light sheet thickness measured at different locations across the FOV. Note that schematics **a** and **d** are not to scale; dimensions have been altered to improve image clarity. See also Supplementary Data 2–5.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
29	Beams are not intersecting	Scanner is not mounted properly	Remount the scanner and recheck beam intersection
		Dichroic mirror is not placed properly	Adjust the dichroic filter cube using fine alignment screws (shown in SF5 in Supplementary Note 1) until the beams intersect
60	Epifluorescence spot is not close to the pencil beam	Objectives are not focused at same spot	Adjust the position of the objectives coarsely (as in Step 46) until both focus at the same spot
		Y offset of the MEMS mirror (Y-galvo offset) is not optimal	Change the Y offset of the MEMS mirror (either arm or both arms) to bring the epifluorescence spot closer to the pencil beam
63	In the SPIM camera, only one line is visible	This signifies that both objectives are co-focused and well aligned	To test alignment, move the diSPIM module up or down (5–10 μm); two lines should be visible
65	Light sheet is tilted (shown in Supplementary Fig. 4)	Scanner does not make a 45° angle with respect to the optical table	Rotate the scanner that is creating the light sheet so that lines appear vertical in the bottom camera
80	Maximum intensity projection of beads image is blurry	Collection objective is not focused	Change the collection piezo offset
		Light sheet and collection objectives are not synchronized	Change the Z-galvo conversion factor
		Z-galvo offset is not optimal	Change the Z-galvo offset
		Light sheet is thicker than it should be	Measure the light sheet thickness
		Beads in the sample are aggregated	

Step	Problem	Possible reason	Solution
		Registration is not optimal	Check the transformed image and make sure that registration is good (examples are shown in Supplementary Note 1, SF1). Also consider breaking up the registration as described in Step 102
		X, Y, Z resolution parameters entered into processing software are incorrect	Check the X, Y and Z resolution parameters entered in MIPAV
		Deconvolution parameters are wrong	Check the deconvolution parameters in MIPAV