1. Frampton, G.M. *et al. Nat. Biotechnol.* **31**, 1023–1031 (2013).
2. Conti, R.M. *et al. J. Clin. Oncol.* **31**, 1134–1139 (2013).
3. Gargis, A.S. *et al. Nat. Biotechnol.* **30**, 1033–1036 (2012).
4. Rehm, H.L. *et al. Genet. Med.* **15**, 733–747 (2013).
5. CAP Laboratory Accreditation Program. *Molecular Pathology Checklist* http://www.cap.org/apps/docs/laboratory_accreditation/checklists/new/molecular_pathology_checklist.pdf (College of American Pathologists, 2013).
6. Bose, R. *et al. Cancer Discov.* **3**, 224–237 (2013).
7. Krzyzanowska, M.K. *J. Clin. Oncol.* **31**, 1125–1127 (2013).
8. Alberts, S.R. *et al. J. Am. Med. Assoc.* **307**, 1383–1393 (2012).
9. Garber, K. *J. Natl. Cancer Inst.* **103**, 84–86 (2011).
10. Simon, R. & Roychowdhury, S. *Nat. Rev. Drug Discov.* **12**, 358–369 (2013).

# Two views on light sheets

Carl G Ebeling & Erik M Jorgensen

**A dual-view light-sheet microscope combines isotropic spatial resolution with high speed and minimal phototoxicity.**

The confocal microscope, long the dominant imaging technique in cell biology, is approaching obsolescence, soon to be superseded by a new generation of optical microscopes. The approach most likely to take its place is selective plane illumination microscopy (SPIM), a technique that delivers lower doses of light to the sample and achieves faster image acquisition compared with confocal systems. The major drawback of conventional SPIM is its poor axial resolution: whereas optical sections in confocal microscopes are 800-nm thick, those in light-sheet microscopes are 2–6 µm. In this issue, Wu *et al.*[1] describe a new dual-view SPIM instrument capable of providing 330-nm resolution, not only along the lateral axes (*x* and *y* planes) but also along the optical axis (*z* plane). The key insight is to use two orthogonal objectives that alternate in a duty cycle between excitation and detection as they scan through the sample. Light-sheet microscopy now stands to deliver upon its initial promise—rapid imaging of living organisms with low photo-dosage and high spatial resolution in three dimensions.

In traditional epifluorescence microscopy, a column of light excites fluorescent molecules above and below the focal plane in the specimen (**Fig. 1a**). The out-of-focus light generates a blurry image without detail, and absorption of light by biological tissue leads to phototoxicity and photobleaching. Confocal microscopy eliminates out-of-focus light by masking it with a pinhole before detection, greatly improving the axial resolution of the image. However, laser light is absorbed throughout the sample, causing phototoxicity and photobleaching. The basic problem is that the excitation beam overlaps the detection path. In addition, the slow acquisition rates preclude imaging of fast events in living specimens.

In SPIM, by contrast, the specimen is illuminated by an orthogonal light sheet, thereby eliminating out-of-focus light in the detection path (**Fig. 1b**)[2]. The resulting image is sharp, and the specimen does not absorb light outside the focal plane. But light-sheet microscopy is hampered by poor axial resolution. Creating the orthogonal light path requires multiple objectives in close proximity to the sample and hence to each other. High numerical aperture objectives would generate thinner light sheets on the excitation side and better resolution on the detection side, but because of their bulky diameters and short working distances, it is not feasible to use them. Lower numerical aperture objectives with longer working distances are used instead. This weakness has led to a search for improved SPIM microscope designs and the creation of many SPIM spin-offs.

A common approach to overcome poor axial resolution is to image the sample from multiple viewpoints and then computationally fuse the data into a single composite image. This concept was first implemented by rotating the sample so that the *z* axis becomes the new *x* axis[3]. To mitigate scattering effects and to increase simultaneous coverage of the sample, a second strategy has been to add another set of excitation and detection pathways[4,5]. While still rotating the sample, opposing excitation objectives illuminate the sample from opposite sides, and two detection objectives orthogonal to the excitation plane collect the fluorescence emission. However, the downside to this technique is increased light exposure and phototoxicity.

Rotating the sample has its own drawbacks, such as prolonging acquisition time, but the advantages of imaging the same volume from multiple vantage points is an increase in resolution. With the proper post-analysis software, the best spatial information from each image is preserved, whereby the poor axial resolution in one orientation is replaced with the higher lateral resolution from the second orientation. In short, multiview acquisition begins to solve the axial resolution problem. Previous implementations of multiple viewpoint acquisition systems[3–5] require anywhere from 4 to 36 different acquisitions of the same volume, unfortunately offsetting the inherent advantages of the low photo-dosage of conventional light-sheet microscopy. Moreover, multiview methods have not yet demonstrated true isotropic resolution (i.e., the same resolution in all spatial directions).

Wu *et al.*[1] offer a solution to many of the issues plaguing light-sheet microscopy. They show that their dual-view, inverted SPIM (diSPIM) setup achieves isotropic resolution with minimal phototoxicity while keeping the sample immobile and mounted by conventional methods. The enabling innovation is the use of a duty cycle whereby the two objectives alternate in rapid sequence between excitation and detection (**Fig. 1c**). Each volume is therefore imaged only twice, and from orthogonal vantage points. The two volumes are then combined computationally into a single, isotropic image by a fast, joint-deconvolution algorithm (**Fig. 1d**). Together with fast, scientific-grade, complementary metal oxide semiconductor cameras, an imaging readout speed of 200 Hz is achieved, yielding a volumetric acquisition time of 2 Hz, which is about 10 times faster than what has been demonstrated with the fastest comparable methods. The method also makes selective plane illumination more practical because standard sample preparations, namely a specimen on a coverslip, can be used, as opposed to the time-consuming agar-immersion methods of sample preparation that SPIM instruments generally require. This has the further advantage of limiting the bench-to-instrument time, which is critical in studies looking at rapid dynamic processes.

The authors show that the high acquisition speed, low phototoxicity and isotropic resolution of their diSPIM setup allow the imaging of biological processes that would have been difficult to observe with more conventional

*Carl G. Ebeling is at the Department of Biology, Howard Hughes Medical Institute, University of Utah, Salt Lake City, Utah, USA, and the Department of Physics, University of Utah, Salt Lake City, Utah, USA, and Erik M. Jorgensen is at the Department of Biology, Howard Hughes Medical Institute, University of Utah, Salt Lake City, Utah, USA.*
*e-mail: jorgensen@biology.utah.edu*

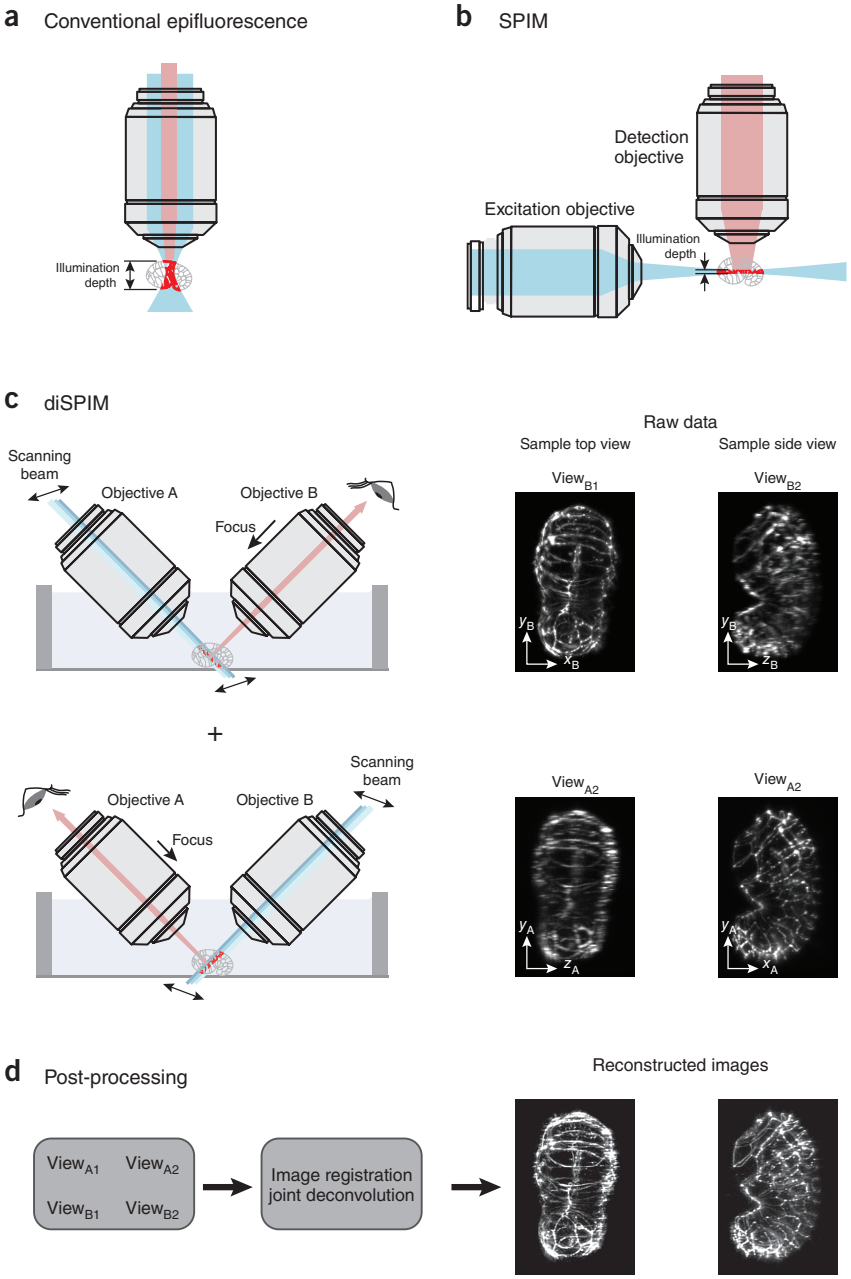


**Figure 1** Comparison of conventional confocal epifluorescence and light-sheet microscopy. (**a**) In an epifluorescence configuration, the same objective is used to illuminate the sample and to collect the emitted fluorescence signal; such a setup exposes the entire sample, regardless of the focal position of the objective, to excitation light for the entire recording duration. (**b**) In light-sheet microscopy, the excitation objective is positioned at 90° with respect to the emission objective. The excitation objective illuminates a thin light sheet, only exposing the sample in the focal plane of the emission objective to excitation light. Compared to conventional epifluorescence, the illumination depth and the overall phototoxicity are much lower. (**c**) In diSPIM[1], the two objectives are positioned above the sample, which is mounted on a coverslip (left). The two objectives operate in a duty cycle, alternating between excitation and detection. A focused beam is quickly scanned back and forth through the sample by the first excitation objective (objective A), generating a tightly confined light sheet. The light sheet is scanned through the sample from top to bottom. The emitted fluorescence is collected by the detection objective, creating a three-dimensional volume (right); the *xy* image is crisp, whereas the *z* axis is poorly resolved. The optical path is then reversed through objective B, and the process is repeated (below). A single plane is imaged in 5 ms, with 50 planes typically taken per volume; the duty cycle of the objectives requires 25 ms for switching. Thus, a sample volume of 70 × 70 × 50 µm can be fully recorded by both objectives in 0.5 s, ~10× faster than the fastest comparable method. (**d**) The resulting two image volumes, labeled here as the four projections, are then merged and jointly deconvolved into a single, final volume with isotropic resolution in the *x*, *y* and *z* dimensions.

microscopes or standard SPIM. They make use of the setup's high isotropic resolution and high speed to track microtubule dynamics in human umbilical vein endothelial cells in four dimensions. The low light dosage of the method allows long-term imaging of live samples, as illustrated by a 14-h recording of a developing *Caenorhabditis elegans* nematode, with minimal phototoxicity to the specimen. Finally, the unique combination of high resolution and low phototoxicity enables tracking of neuronal outgrowth during *C. elegans* development.

With all its advantages, diSPIM does not solve every problem associated with light-sheet microscopy. Thicker biological specimens (greater than ~250 µm, if the sample is opaque) pose a challenge owing to the large amount of light scattering in the sample: too much scattering degrades resolution and can pose problems for image reconstruction. This issue can be partly circumvented with multiple excitation and detection objectives that compensate for scattering in turbid samples[4–6] but come with a cost of higher photo-dosage. Systems employing Bessel beams have been constructed to take advantage of the inherently narrower central peak of such beams, theoretically yielding finer axial resolution than systems using Gaussian beams[7,8]. However, the larger side lobes of Bessel beams also create imaging artifacts and increase the amount of overall photodamage within the sample. Two-photon excitation methods eliminate most issues with out-of-focal-plane fluorescence but can still be phototoxic to the sample because of the high laser powers[9,10].

For dynamic studies, especially of relatively thin samples such as the small embryos of *C. elegans* or cultured cells, the dual-view implementation of Wu *et al.*[1] is likely to provide a workable benchmark solution for years to come. And your confocal microscope can be relegated to the broom closet.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Wu, Y. *et al. Nat. Biotechnol.* **31**, 1032–1038 (2013).
2. Keller, P.J., Schmidt, A.D., Wittbrodt, J. & Stelzer, E.H.K. *Science* **322**, 1065–1069 (2008).
3. Huisken, J., Swoger, J., del Bene, F., Wittbrodt, J. & Stelzer, E. *Science* **305**, 1007–1009 (2004).
4. Krzic, U., Gunther, S., Saunders, T.E., Streichan, S.J. & Hufnagel, L. *Nat. Methods* **9**, 730–733 (2012).
5. Tomer, R., Khairy, K., Amat, F. & Keller, P.J. *Nat. Methods* **9**, 755–763 (2012).
6. Huisken, J. & Stainier, D.Y.R. *Opt. Lett.* **32**, 2608–2610 (2007).
7. Planchon, T.A. *et al. Nat. Methods* **8**, 417–423 (2011).
8. Gao, L. *et al. Cell* **151**, 1370–1385 (2012).
9. Truong, T.V., Supatto, W., Koos, D.S., Choi, J.M. & Fraser, S.E. *Nat. Methods* **8**, 757–760 (2011).
10. Fahrbach, F.O., Gurchenkov, V., Alessandri, K., Nassoy, P. & Rohrbach, A. *Opt. Express* **21**, 13824–13839 (2013).