DeltaVision™ Localization Microscopy System

DeltaVision Localization Microscopy System is an advanced imaging option for the DeltaVision Elite and DeltaVision OMX™ platforms. It employs total internal reflection fluorescence (TIRF) imaging in combination with laser activation control to acquire frames of fluorophore images. These images are analyzed with our exclusive Dense Stochastic Sampling Imaging (DSSI) algorithm, which uses multiple-Gaussian fitting to find the locations of fluorophores within a sample; the fluorophore locations are used to reconstruct a super-resolution image (Fig 1).

- Provides 2-D resolution between 20 and 50 nm (requires appropriate sample preparation, labeling density, and localization precision)
- Is compatible with photoactivation, photoconversion, and photoswitching fluorophore systems
- Uses a DSSI algorithm that can resolve overlapping signals from fluorophores spaced closer than the diffraction limit in dense fields, thereby enabling localization at higher densities

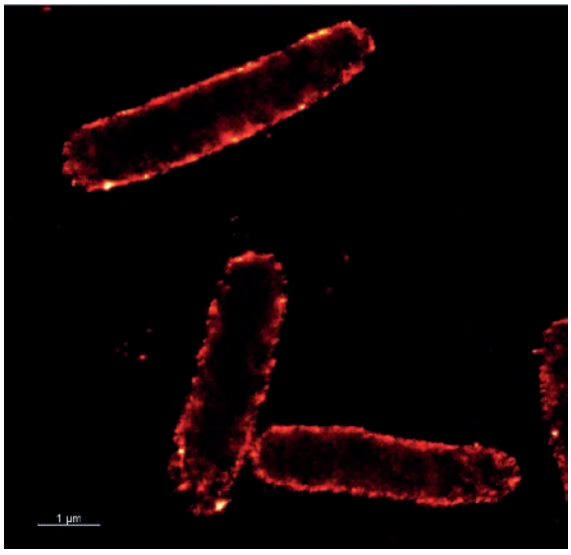


Fig 1. A reconstructed image of *E. coli* labeled with Alexa Fluor™ 647 probe. A DeltaVision OMX System was used for imaging with a 60× 1.49NA TIRF lens. A pco.edge scientific complementary metal oxide semiconductor (sCMOS) camera captured 10 000 frames (127 392 localizations).

Applications of localization microscopy

Localization microscopy is a powerful technique for cell biologists, particularly for research involving:

- Co-localization studies
- Structural organization
- Protein organization, distribution, and abundance
- Receptor organization and structure

To learn more about localization microscopy, we recommend the review articles listed in the *References* section (1, 2).

DeltaVision Localization Microscopy System

The DeltaVision Localization Microscopy System consists of two key elements:

- A flexible, multiwavelength, TIRF optical system with a beam concentrator for power density control
- softWoRx™ acquisition control and image reconstruction software utilizing GE Healthcare Life Sciences' DSSI algorithm

The TIRF optical module allows illumination control to be restricted to a very narrow plane adjacent to the coverslip, which maximizes the signal-to-noise collected from the fluorophores. The TIRF optics module includes a beam concentrator that increases power density to the sample to enhance the on/off switching rates of the fluorophores.

The DeltaVision Localization Microscopy optical system provides:

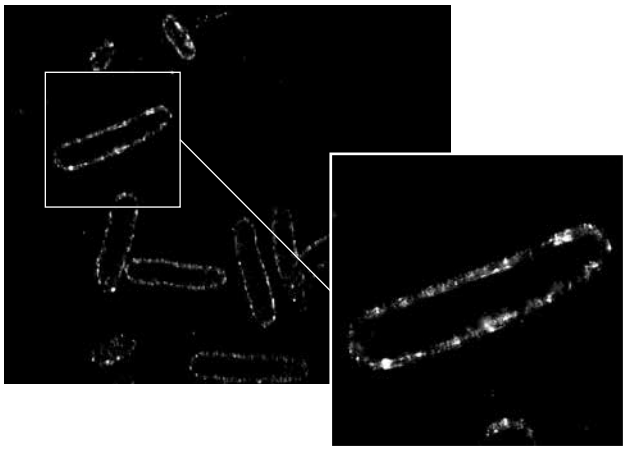
- Flexible laser selection: 405 nm, 488 nm, 568 nm, and 640 nm (or 642 nm depending on the system)
- High-power lasers that combine with a beam concentrator for high-power density at the sample
- Analysis algorithms that are optimized for different camera types (i.e., charge-coupled device [CCD], electronic multiplying charge-coupled device [EMCCD], sCMOS)

The DeltaVision Localization Microscopy System's software controls the laser power, TIRF penetration depth, camera operation, and acquisition sequencing. The final super-resolution reconstruction is generated by analysis of the acquired data using the DSSI algorithm, which localizes fluorophore positions and renders the reconstruction. A camera calibration tool enables translation of "Analog to Digital Units" (ADU) to absolute numbers of photoelectrons, which are then converted to numbers of photons emitted.

Features of the software include:

- Independent control of focus, activation lasers, and reporter laser
- Acquisition monitor to provide near real-time feedback on progression of acquisition and monitoring of blinking rates and sample state
- Drift correction tools to compensate for lateral stage drift during acquisition (Fig 2)

(A) **No drift correction**



(B) **Drift correction applied**

Fig 2. DeltaVision Localization Microscopy System drift correction algorithms are used to account for lateral stage movement during data acquisition.

Dense Stochastic Sampling Imaging

Many localization microscopy algorithms require that the fluorophores be separated by at least a few pixels to be included in the final super-resolution reconstruction (Fig 3, left). DSSI is a multiple-Gaussian-fitting algorithm that enables fluorophore localization of overlapping signals from fluorophores spaced closer than the diffraction limit (Fig 3, right).

The reconstruction process generates a table with the following data:

- X-Y locations of detected fluorophores
- Localization precision associated with each fluorophore's position
- Number of photons emitted by the fluorophore

These data are used to reconstruct an image in which each fluorophore's location is plotted either as a single (x, y) coordinate or as a Gaussian peak with the width given by the localization precision of the fluorophore (i.e., the uncertainty of the fluorophore's position). The resulting data can also be exported for analysis with other visualization tools (not included).

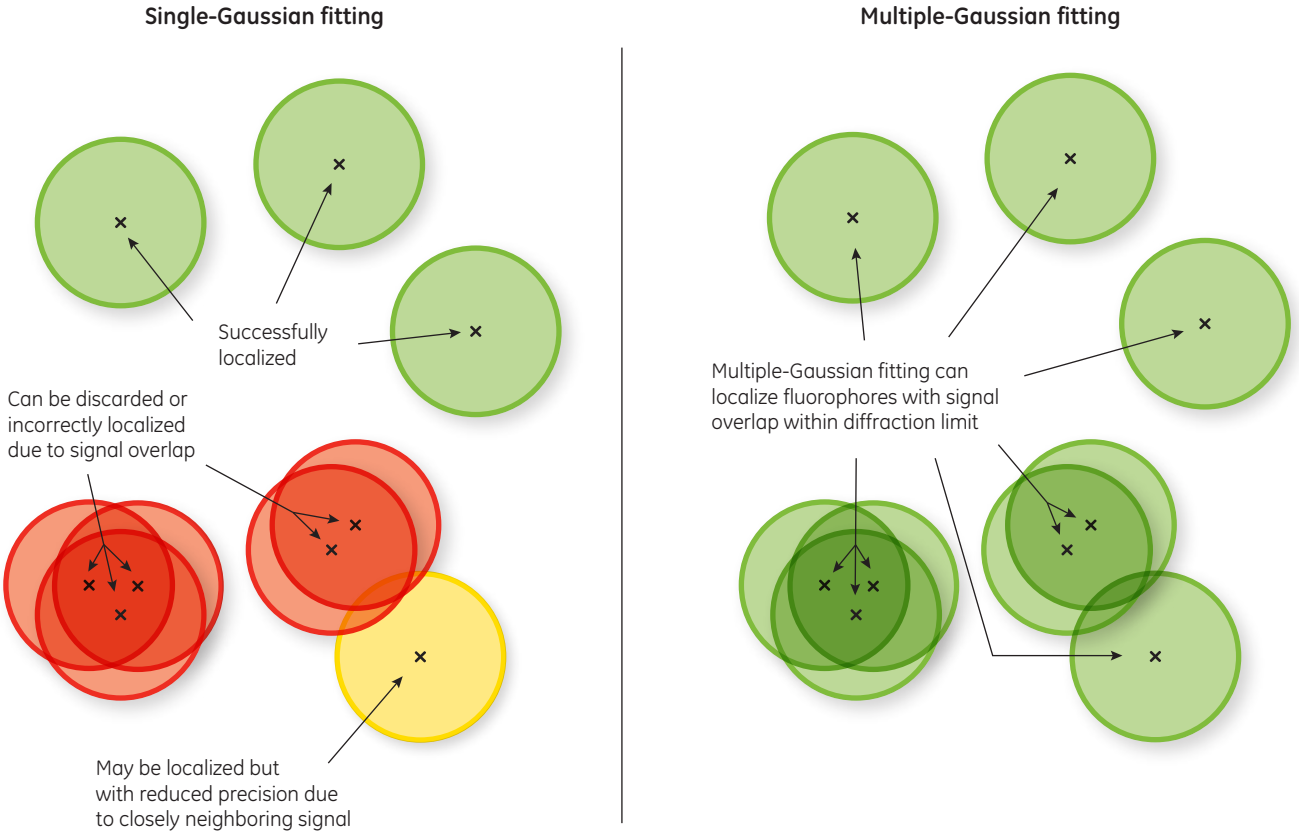


Fig 3. Illustration of the advantages to using DSSI, a multiple-Gaussian-fitting algorithm, when presented with densely overlapping signals.

Overview of localization microscopy

Localization microscopy determines the position of fluorophores in a sample through a combination of imaging and mathematical modeling. When viewed with a microscope, the signal from any sub-diffraction-limit object (e.g., a single fluorescent molecule) will be blurred because of diffraction, thus causing the object to appear larger than its actual size.

Though the image of a sub-diffraction-limit object is blurred, it is possible to locate the position of the object very precisely as long as it is well separated in space, and sufficient photons have been collected. However, in many biological systems, it can be difficult to distinguish individual fluorophores because labeled proteins are often very densely packed. To overcome this obstacle, localization microscopy techniques rely on the random activation of only a subset of fluorophores at a time, making it possible to determine the position of each fluorophore with high precision.

It is critical to note that localization microscopy requires the use of special fluorophores that switch from an "on" state to an "off" state. Close attention to sample preparation technique is required to achieve optimal results since each detected fluorophore contributes to the final image, even if it is not associated with the object or structure of interest.

The spatial resolution of the resulting reconstruction is no longer limited by diffraction; instead, it is limited by the density of the labeling and the localization precision of each fluorophore. Under optimal conditions, the resulting images can achieve lateral resolutions of less than 50 nm.

Photoactivation, photoconversion, and photoswitching fluorophore labeling strategies for localization microscopy are compared in Table 1.

Table 1. Comparison of fluorophore labeling strategies for localization microscopy

	Photoactivation	Photoconversion	Photoswitching
How it works	Uses fluorescent probes that can be turned on when exposed to particular light wavelengths	Involves a shift in wavelength characteristics in response to light wavelengths	Leverages blinking characteristics of certain fluorescent dyes that transition between "on" and "off" states
Acquisition control	Continuous or pulsed activation, if desired, followed by continuous or pulsed excitation		
Scan time	Ranges from tens of minutes to hours. Typically requires thousands to tens of thousands of frames to generate a single super-resolution reconstruction		
Considerations	Genetic engineering can be required to construct clones expressing appropriate fluorescent proteins Fluorescent proteins are generally dimmer and less photostable than organic dyes but have the advantage of being directly tagged to the protein of interest Although organic reactive dyes and antibodies can be more readily available, they might require labeling and optimizing of dye ratios In many cases, organic dyes require special imaging buffers to achieve desired "on/off" switching kinetics. The microenvironment of the cell can affect these switching rates. Multicolor imaging can require different environmental conditions for different fluorophores		

Example images captured with DeltaVision Localization Microscopy System

The DeltaVision Localization Microscopy System was used to generate images of tubulin (Fig 4) and EGF receptors (Fig 5).

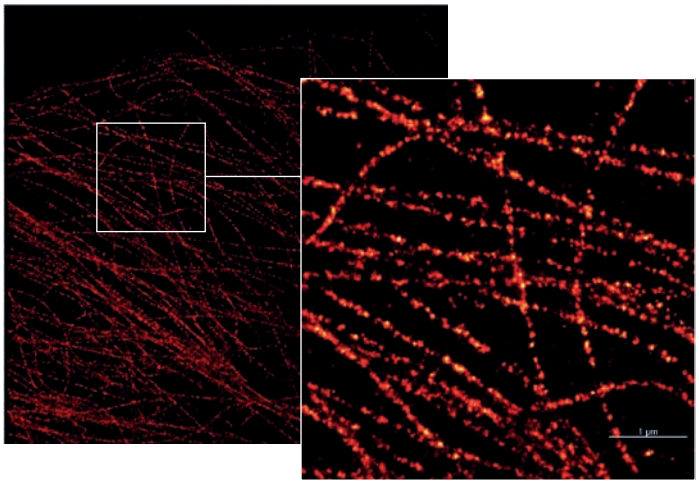


Fig 4. Tubulin network imaged by the DeltaVision Localization Microscopy System. The right image shows detail of microtubules in the highlighted area of the left image. The system acquired 5000 frames and detected approximately 148 311 localizations. The secondary antibody was labeled with Alexa Fluor 647. Images were acquired on the DeltaVision OMX system with the 60× 1.49NA lens and a pco.edge sCMOS camera.

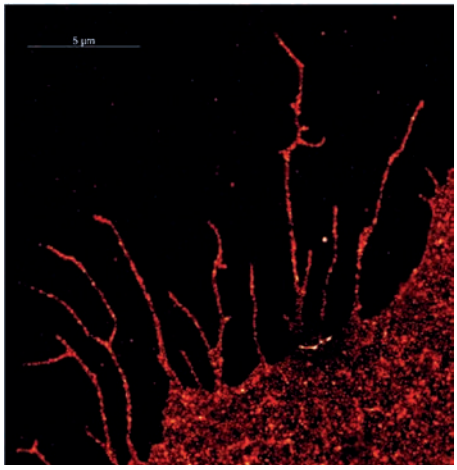


Fig 5. mEOS2-labeled EGF receptor imaged using the DeltaVision Localization Microscopy System. The system acquired 5000 frames, and approximately 203 150 localizations were detected. The image was acquired on the DeltaVision Elite system with the 60× 1.49NA lens and a pco.edge sCMOS camera.

Technical details for use of the DeltaVision Localization Microscopy System

	DeltaVision Elite	DeltaVision OMX
2-D localization precision	~ 20 nm Localization precision is dependent on the number of photons collected	
2-D lateral resolution	< 50 nm Requires appropriate sample preparation and labeling density Achievable under optimal conditions	
Fluorophore system	Photoactivation, photoconversion, photoswitching	
Lasers available	405 nm, 100 mW 488 nm, 100 mW 568 nm, 150 mW 640 nm, 100 mW Note that the X4 laser chassis can support up to 4 lasers	405 nm, 100 mW 488 nm, 100 mW 568 nm, 100 mW 642 nm, 110 mW Note that the DeltaVision OMX system can support up to 6 lasers
Laser intensity control	Direct laser power control	Direct laser control and neutral density (ND) filters for additional attenuation
Laser shuttering (open/close time)	5 ms fast electronic shutters	2 ms fast electronic shutters or 200 µs high-speed galvanometer shutters
Activation laser control	Pulse duration and laser intensity adjustable during acquisition	Laser intensity adjustable during acquisition Pulse duration fixed for experiment
Cameras supported	CoolSNAP™ HQ2 CCD Evolve™ EMCCD pco.edge sCMOS	pco.edge sCMOS Evolve EMCCD
Field of view	Maximum 512 × 512 pixels	
Exposure times (representative; depends on dye and imaging conditions)	Organic dyes: ~ 20 to 50 ms Fluorophores: ~ 50 to 100 ms	
Supported camera and TIRF objectives	CoolSNAP HQ2: 60× objective pco.edge sCMOS: 60× objective Evolve EMCCD: 60×, 100× objectives	pco.edge sCMOS: 60× objective Evolve EMCCD: 60×, 100× objectives
Pixel size (+/- 5 nm)	CoolSNAP HQ2: 108 nm at 60× objective pco.edge sCMOS: 108 nm at 60× objective Evolve EMCCD: 133 nm at 60× + 2× Optivar Evolve EMCCD: 80 nm at 100× + 2× Optivar	pco.edge sCMOS: 82 nm at 60× objective Evolve EMCCD: 133 nm at 60× objective Evolve EMCCD: 80 nm at 100× objective

References

1. Manley, S. *et al.* A starter kit for point-localization super-resolution imaging. *Curr. Opin. Chem. Biol.* **15**, 813–821 (2011).
2. McEvoy, A. L. *et al.* Q&A: Single-molecule localization microscopy for biological imaging. *BMC Biol.* **8**,106 (2010).

For local office contact information, visit
www.gelifesciences.com/contact

www.gelifesciences.com/deltavision

GE Healthcare UK Limited
Amersham Place
Little Chalfont
Buckinghamshire, HP7 9NA
UK

imagination at work

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe, GmbH
Munzinger Strasse 5
D-79111 Freiburg
Germany

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Japan Corporation
Sanken Bldg., 3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan

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