Application Note #2001

Webinar Recap: Single-Molecule Localization Microscopy—Beyond the Coverslip

Traditional microscopy methods have been used for imaging biological organization but are limited to a lateral resolution of ~200-300 nanometers. Single-molecule localization, a super-resolution microscopy approach, allows for high-resolution imaging of specifically labeled biological samples at a resolution of 20 nanometers laterally. This webinar recap summarizes the presentation given by Lauren Gagnon, Applications Scientist for Bruker's super-resolution microscopy solutions. Presented here are the advantages and principles of single-molecule localization. Also, the unique benefits of the Vutara top-hat illumination and bi-plane technology, as well as the multiplexing capabilities supported by the microfluidics unit, are discussed. Finally, examples of imaging a variety of sample types and popular applications of single-molecule localization microscopy are presented.

Why use single-molecule localization microscopy?

Biological systems exist across a wide range of sizes. Light microscopy, particularly fluorescence microscopy, has been a useful tool for visualizing biological organization. However, traditional methods, such as widefield and confocal, are unable to resolve features below 200 nanometers due to Abbe's diffraction limit. While other microscopy methods, such as electron microscopy, allow for high-resolution imaging below the optical diffraction limit, it is difficult to label specific, targeted structures within the sample. This presents a challenge to certain research questions because cells are highly organized well below the diffraction limit and cellular structure often informs function. To visualize specific structures in nanoscale detail, a variety of super-resolution approaches have been developed. One of the most useful super-resolution approaches is single-molecule localization microscopy (SMLM). SMLM uniquely allows for imaging at a resolution down to ~20 nm laterally of specifically labeled biological structures. SMLM enables visualization of a range of sample types, such as single cells, cell colonies, tissues, and even whole organisms in nanoscale detail.

Principles of single-molecule localization

In a diffraction-limited volume that is densely labeled with fluorescent molecules, conventional light microscopy acquires an image that is an unresolved blur of fluorescence, regardless of the underlying structure (Figure 1). This is due to image collection of all molecules

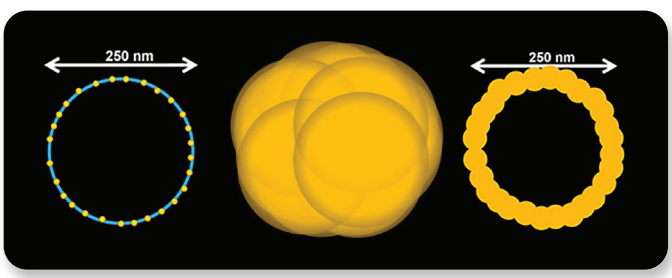

Figure 1. Single-molecule localization principles. Example of the sample to be imaged in a diffraction limited volume (left), image of the diffraction limited volume with traditional microscopy methods (middle), image of diffraction limited volume with SMLM (right).

in the frame at one time that are within 200 nm of each other. This is not the case with SMLM. With SMLM, data from individual molecules are collected separately by stochastically exciting individual molecules within the diffraction-limited volume at different time points. Essentially, the fluorophores are turned on and off one at a time so that the center position of each molecule can be recorded during image acquisition (Figure 1). When the fluorescent "on" state is optically controlled, only a small subset of molecules is on at a given time. This results in point spread functions (PSF) that are sparse enough across a single camera frame so that they can be isolated individually. After identifying individual PSFs, localization algorithms are performed on each PSF. This allows for the extraction of the below-diffraction-limit spatial information on the location of each molecule. The super-resolution image is a result of compiling all the localized information from each PSF (Figure 2).

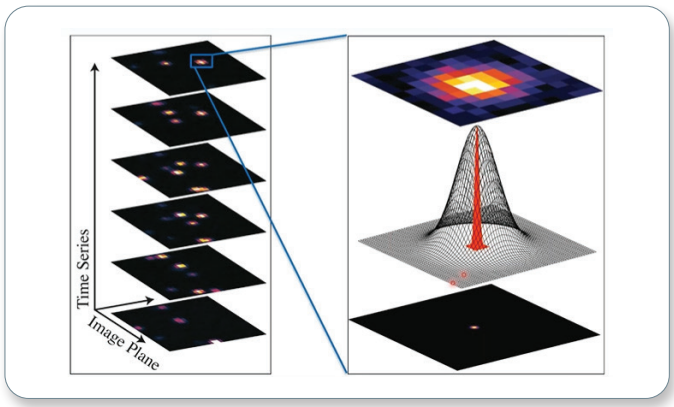

Figure 2. Image acquisitions of single molecules over time with SMLM. Each molecule is imaged stochastically within an imaging plane over time. The point spread function data are collected and used to localize the center of each individual molecule.

The two main strategies for controlling the "on" state of fluorophores are (1) the use of photoactivatable or photoswitchable fluorescent proteins and (2) the use of organic dyes, which through a series of light-driven reduction reactions, bind a side group to the fluorophore, thereby rendering it non-fluorescent. The use of organic dyes for SMLM is the more widely used strategy of the two. An alternative to strategies that rely on photoswitching of organic dyes to generate blinking is a technique called points accumulation in nanoscale topography (PAINT). With PAINT, fluorophores are bound only for short times to the structure of interest. In DNA-PAINT, single-stranded DNA labeled with an organic dye binds and unbinds to oligos that are attached to specific structures within a cell. This allows for imaging of the specific structure to which it is bound.

What sets the Vutara apart from other SMLM systems?

The optical design of the Vutara takes localization one step further by using bi-plane imaging. Bi-plane imaging, or imaging of two focal planes simultaneously, allows for the collection of three-dimensional data by localizing the x, y, and z position of each molecule. This technique is unique from other commercial SMLM systems that use PSF engineering to get z-dimensional information and are much less conducive to imaging deeper in samples. Other commercial systems are limited to either using TIRF or HILO imaging with complicated three-dimensional calibration, which can only achieve imaging depths of around 5 microns. In contrast, the Vutara can easily image 50 microns from the coverslip with a standard calibration. With this depth, a user can image a wide range of samples in three-dimensions from singe cells and cell cultures to cell tissues and whole model organisms like *Drosophila* or *C. elegans*.

In addition to bi-plane technology, the Vutara uses top-hat illumination, which enables even illumination over the whole field of view; that is, every part of the field of view is usable for data analysis. With conventional Gaussian optics, there will be an area of the field of view with usable data and an area with unusable data that is not useful for image analysis (Figure 3). In contrast, with Vutara's top-hat illumination the whole field of view is illuminated evenly, resulting in uniform data collection across the entire imaging region. The benefits of this technique are consistent results across the field of view and reliable images, which is critical for quantitative data analysis.

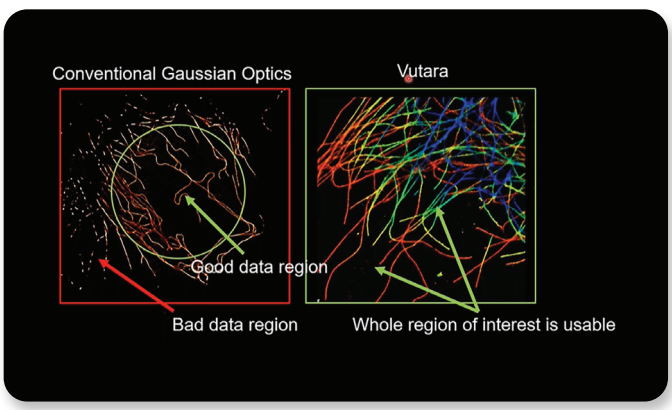

Figure 3. Vutara's top-hat illumination technology. By illuminating the entire field of view, the Vutara enables quality imaging for the entire plane, resulting in acquisitions suitable for quantitative analysis.

The Vutara is also uniquely well-suited for multiplex imaging, or imaging of multiple specific biological targets. This is useful for many applications, including DNA-PAINT and genomic imaging among others. Multiplex imaging is supported by Bruker's fully integrated fluidics system that makes imaging sequentially with multiple probes simple. The fluidics system is fully integrated into Bruker's

SRX software and was designed specifically for SMLM imaging (Figure 4). Each system has four large reservoirs for high-volume reagents, like imaging buffers and washes, and 15 smaller reservoirs for precious labeling reagents. The smaller reservoirs may be swapped out during the experiment, making imaging greater than 15 targets possible. The user interface is intuitive and gives the experimenter full control over fluidic sequences and parameters. The system can be used as a standalone with existing microscopes or as a fully integrated system into the acquisition process using the Vutara with SRX software.

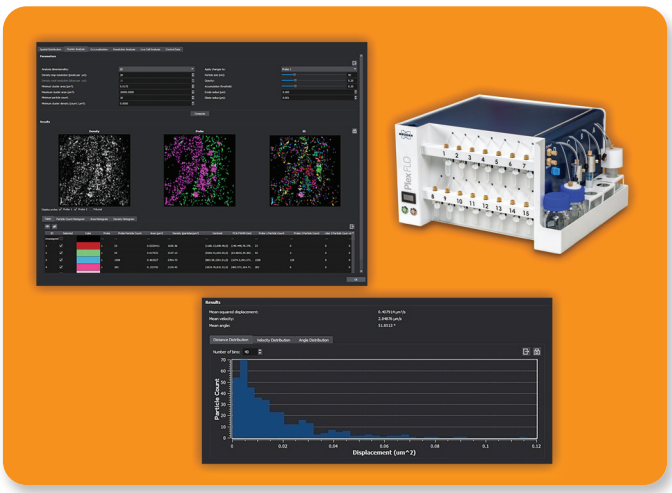

Figure 4. Microfluidics system and SRX software. The microfluidics system (right) and SRX software (left and bottom) can be used with the Vutara to support multiplexed, specific imaging and subsequent data analysis.

Examples of imaging a variety of sample types with the Vutara

The bi-plane and top-hat illumination technology of the Vutara supports imaging of a variety of sample types ranging from single cells to whole model organisms. Fixed or live samples can be imaged, although fixed-sample imaging is more common. Here are some examples of how SMLM is currently being used to image different sample types:

Cell lines, cell cultures, and tissue sections. Due to its optical design, the Vutara can perform three-dimensional multicolor super-resolution imaging of cells and cell cultures. Presented here is an example of the mitochondria of multiple cells colored by each individual cell (Figure 5). Additionally, the Vutara is the only commercially available single-molecule system that combines wide-field illumination and bi-plane localization, making it uniquely qualified to image z-stacks in tissue sections. Because the Vutara does not use PSF engineering, it can easily acquire this three-dimensional information deep within the tissue section.

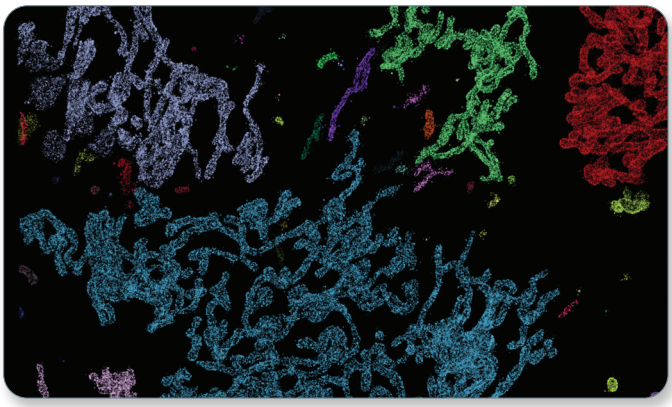

Figure 5. Mitochondria of multiple cell lines colored by individual cell.

Whole model organisms. The Vutara is also capable of imaging whole model organisms. Using the Vutara, whole mount, cleared *Drosophila melanogaster* larvae were imaged ~30 microns from the coverslip (Figure 6).

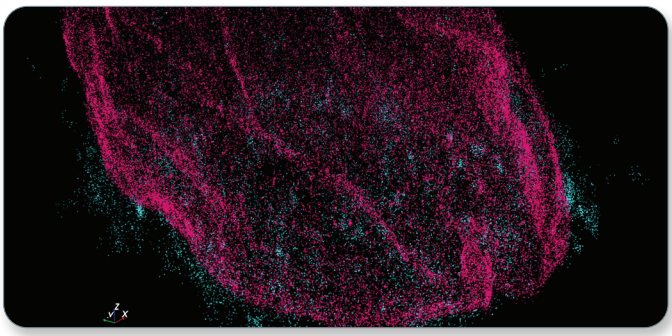

Figure 6. Whole mount, fixed D. melanogaster imaging. A whole mount Drosophila larva imaged with labels for nuclear cytoskeleton (magenta) and frizzled receptor (cyan) imaged at a depth of 30 microns.

Multiplex imaging applications

The Vutara is uniquely well-suited for multiplex imaging, which is necessary for applications such as DNA-PAINT and genomic imaging. Multiplex imaging is possible with Bruker's fully integrated fluidics solution that makes imaging sequentially with multiple probes simple.

DNA-PAINT. DNA-PAINT is a SMLM technique that works through the transient binding of a short dye-conjugated oligonucleotide imager strand to a target labeled with a complementary DNA strand (docking strand). Here, microtubules were imaged with the Vutara using oligo-labeled secondary antibodies from Massive Photonics, a company that creates probes specifically for DNA-PAINT imaging. A high-resolution image was acquired of the hollow feature of the microtubule, which was uniquely

supported with SMLM (Figure 7). The next example highlights the utility of multiplex labeling of specific structures in 3D, of microtubules and clathrin (Figure 7).

Genomic Imaging. Multiplex imaging strategies have been further extended to imaging the genome. As an example, maternal and paternal homologues of chromosome 19 were imaged using a technique called Oligo-STORM (Figure 8). The Vutara system is well-situated to performing these large genomic studies due to the integrated fluidics option, as well as the SRX software that is capable of analyzing these large genomic data sets.

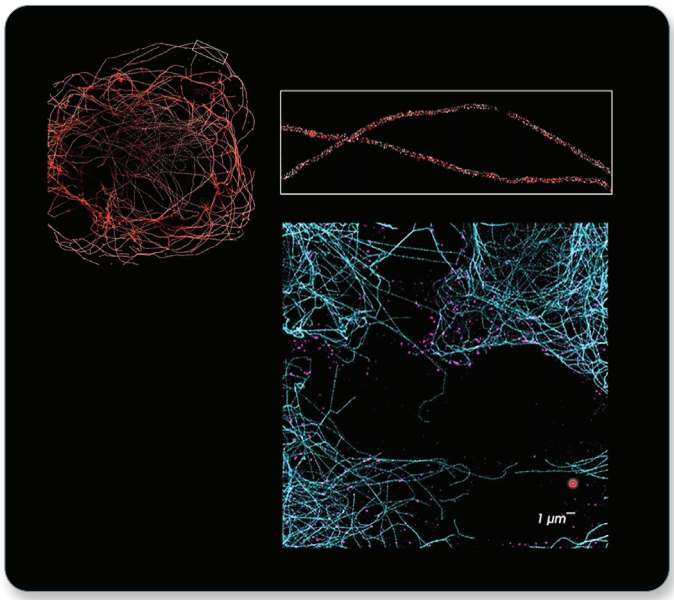

Figure 7. Single- and multi-colored DNA-PAINT images with SMLM. A single-colored DNA-PAINT image was collected of the structure of microtubules, detailing the hollow feature of the microtubules (topleft and right). A two-color, multiplexed image of labeled microtubules and clathrin imaged with the Vutara (bottom).

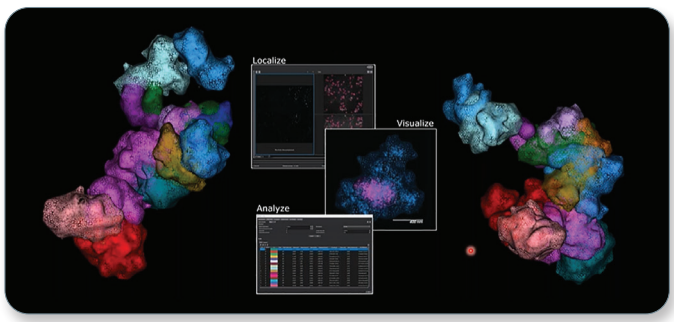

Figure 8. Maternal and paternal homologues of chromosome 19 acquired with the Vutara and associated microfluidics unit and SRX software.

Conclusion

With SMLM, a user can image specifically labeled structures at a resolution of 20 nanometers laterally, which is much higher resolution compared to traditional microscopy methods like widefield or confocal. The Vutara imaging platform with bi-plane technology offers a unique ability to image depths of entire cells and culture, greater than 30 microns in small organisms and tissues, and up to 100 microns in hydrogels. With Vutara's top-hat illumination, the entire field of view is illuminated to support high-quality data collection and quantitative analysis. There are many potential applications of SMLM to address research questions in fields such as cell biology, neurobiology, virology, genomics, developmental biology, cardiology, and more. Bruker's optional integration of fluidics and the rich software environment for acquisition and analysis makes single-molecule localization accessible to any application.

Resources

To view the webinar "Super-resolution Microscopy: Beyond the Coverslip" visit here

To learn more about Bruker's comprehensive suite of super-resolution microscopy solutions visit here.

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