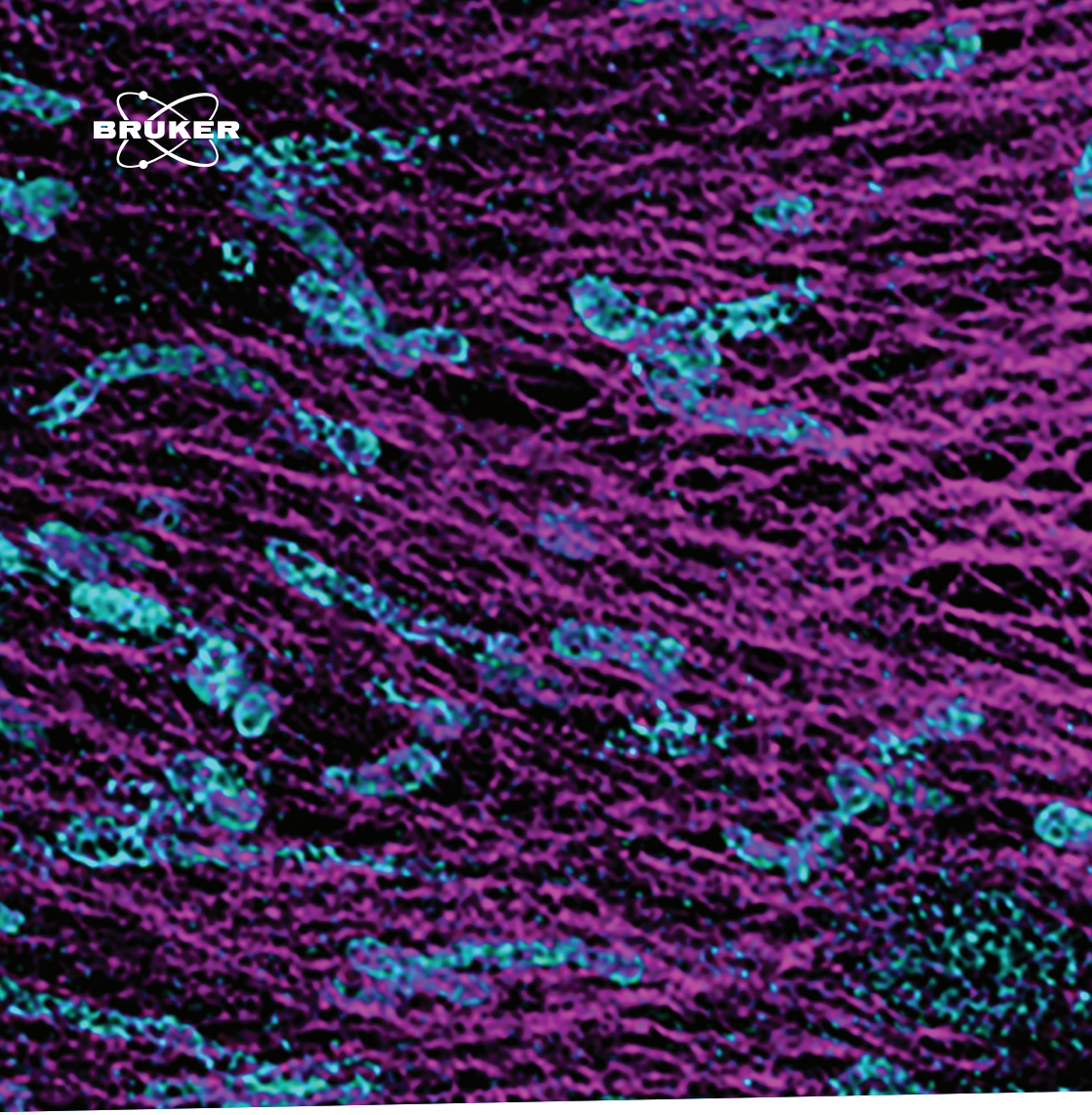


FLUORESCENCE MICROSCOPY

Super-Resolution Microscopy

See Biology in Nanoscale Detail

Table of Contents

Acknowledgments

Portions of this book were sourced with data and images originally published by Bruker customers. We thank them for their support and excellent research.

Cover Image:

Multiplexed image with DNA-PAINT. Alpha-tubulin is labeled in magenta and mitochondria in cyan.

Introduction

Since the early days of their invention, microscopes have been at the forefront of many biological discoveries and scientific advances, such as the first description of a cell,[1] as well as the discovery of bacteria,[2] cholera, and anthrax.[3] Over the years, microscope technology has become increasingly more powerful, allowing scientists to look at the world at a level previously unimaginable. The light microscope is the most common microscope found in the laboratory; it works using visible light and a system of lenses to generate a magnified image of an object. Despite advances, light microscopy still suffers from certain limitations, especially in terms of its imaging resolution. Light microscopes are confined to Abbe's optical diffraction limit, which limits imaging resolution to 200-300 nm laterally. This limitation presents a challenge across different research fields—including but not limited to neuroscience, cell biology, genomics, and virology—because there are many interesting structures and processes that fall below the 200-300 nm diffraction limit. Alternatively, electron microscopy (EM) can be used for imaging down to 0.1–0.2 nm laterally, but it is limited by its inability to image specifically labeled molecules.

Super-resolution microscopy methods have been created to overcome some of the key challenges associated with light microscopy. These techniques can surpass the diffraction limit and generate higher resolution images of biological specimens. Super-resolution microscopy enables highest-resolution imaging of biological samples, down to 20 nm laterally, of fluorescently labeled molecules—effectively bridging the gap between conventional and EM imaging. Super-resolution microscopy supports research questions that require nanoscale imaging with a lateral resolution of 20–200 nm of molecular distribution, quantification, colocalization of proteins, and particle tracking.

Currently, there are two main approaches of super-resolution microscopy that allow for imaging beyond the diffraction limit of light. One approach is spatial coordination via patterned illumination to differentially modulate the fluorescence emission of molecules within a diffraction-limited volume, which allows for separate detection of molecules within that volume. Popular techniques that fall under this approach are stimulated emission depletion (STED) and structured illumination microscopy (SIM). The second approach, and arguably the one best-suited for cell biology research questions, is single-molecule localization (SMLM). SMLM achieves the separation of molecules by stochastically exciting individual molecules within the diffraction-limited volume at different time

Microscopy Method	Lateral Resolution	Counting of Molecules	Specific Labeling
Super-Resolution	~20 nm	YES	YES
Cryo EM	~0.1–0.2 nm	YES	Limited
Confocal	~200–300 nm	NO	YES
Widefield	~200–300 nm	NO	YES

TABLE 1.

Comparison of microscopy methods in terms of lateral resolution, quantitative capabilities, and specific labeling ability.

points. Fluorescent labeling strategies for SMLM include the use of fluorescent proteins and organic dyes. Compared to STED and SIM, SMLM has the highest resolution.

This guide begins by introducing the principles and advantages of super-resolution microscopy (SRM) for life sciences research. It discusses the problem that SRM solves—imaging specifically labeled molecules beyond the optical diffraction limit—while providing an explanation of the common approaches to achieving beyond diffraction-limited imaging, including localization and labeling techniques. This guide will focus primarily on the single-molecule localization microscopy (SMLM) method of super-resolution. It then expands on the transcendent applications of SRM across life sciences disciplines, including its current utilization in neuroscience, cell biology, genomics, and virology. Finally, this guide explores the most recent and cutting-edge advancements related to SRM—including its expanding potential when paired with biplane technology, top-hat illumination, and microfluidics—and discusses future trends in super-resolution microscopy research.

Principles and techniques

Approaches to super-resolution 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 200–300 nanometers due to Abbe's diffraction limit—or the optical diffraction limit of light. While other microscopy methods, such as electron microscopy, allow for high-resolution, sub-nanometer imaging below the optical diffraction limit, specific labeling of target structures with fluorophores is challenging. This presents a challenge to researchers because cells are highly organized well below the diffraction limit and research questions may require specific labeling to investigate.

To visualize specific structures and processes in nanoscale detail, a variety of super-resolution approaches have been developed. Currently, there are two main approaches of super-resolution microscopy that allow for imaging beyond the diffraction limit of light. One approach is spatial coordination via patterned illumination to differentially modulate the fluorescence emission of molecules within a diffraction-limited volume, which allows for separate detection of molecules within that volume. Two popular techniques that fall under this approach are stimulated emission depletion (STED) and structured illumination microscopy (SIM).

The second approach is single-molecule localization (SMLM). SMLM achieves the separation of molecules by stochastically exciting individual molecules within the diffraction-limited volume at different time points. Fluorescent labeling strategies for SMLM include the fluorescent proteins and organic dyes. Compared to STED and SIM, SMLM has the highest lateral resolution or down to 20 nm. SMLM can be utilized to image an unlimited number of specific proteins.

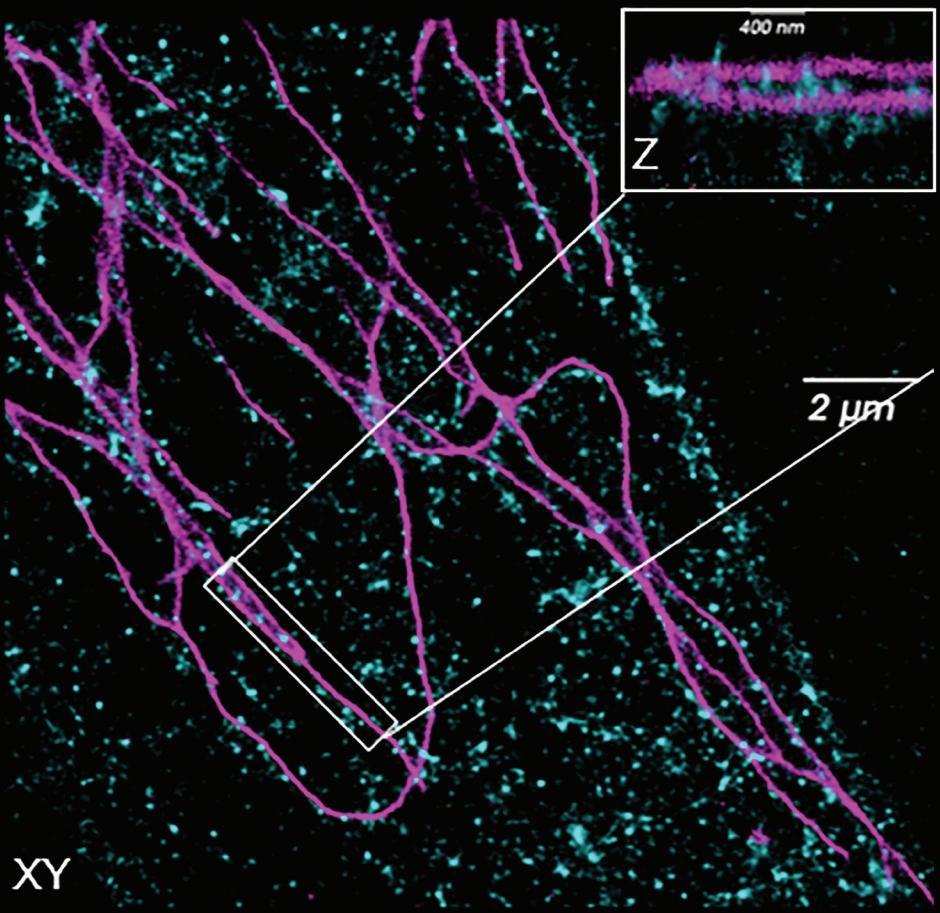


FIGURE 1.

Protein BSC1 Cells, Target: Alpha tubulin (AF647) and Microtubule associated protein (CF568).

Principles of SMLM

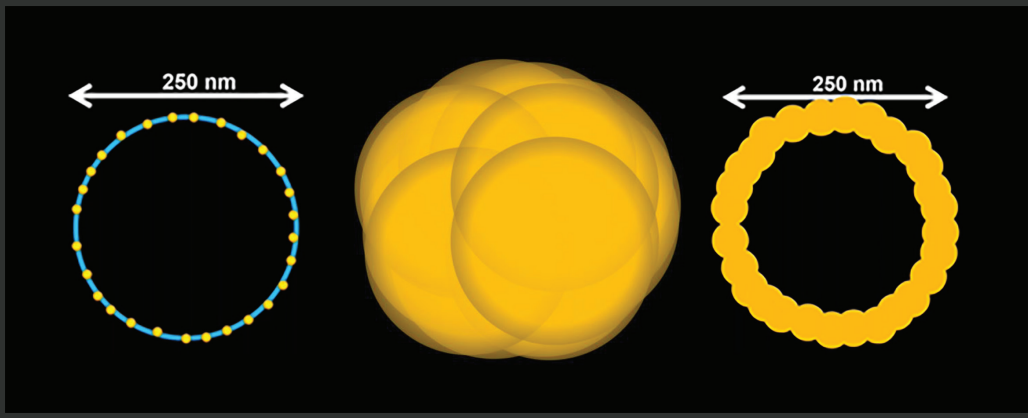
In a diffraction-limited volume that is densely labeled with fluorescent molecules, conventional light microscopy acquires an image that is an unresolved blur of fluorescence, regardless of the underlying structure. This is due to image collection of all molecules in the frame at one time that are within 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 (see Figure 2). 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 can be performed on each PSF. This allows for the extraction of 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 (see Figure 3).

SMLM fluorescent labeling techniques

The two main strategies for controlling the "on" state of fluorophores, or achieving stochastic blinking of molecules, are (1) the use of fluorescent proteins or (2) the use of organic fluorescent dyes. When using fluorescent protein labeling, proteins can be photoactivatable (where fluorescence is turned on/off with bleaching), or photoconvertible (where the emission spectra of the protein is switched). Photoactivated localization microscopy (PALM) is an example of photoactivatable fluorescence. When using fluorescent dyes, fluorescence is controlled by either (1) light-induced chemical reactions or (2) the fluorescent binding of dye to a specific cellular target. Stochastic optical reconstruction microscopy (STORM) and direct-STORM (dSTORM) are examples of light-induced chemical reactions that result in blinking. Points accumulation for imaging in nanoscale topography (PAINT) is an example of transient dye binding to a specific cellular target.

FIGURE 2.

Example of a 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).



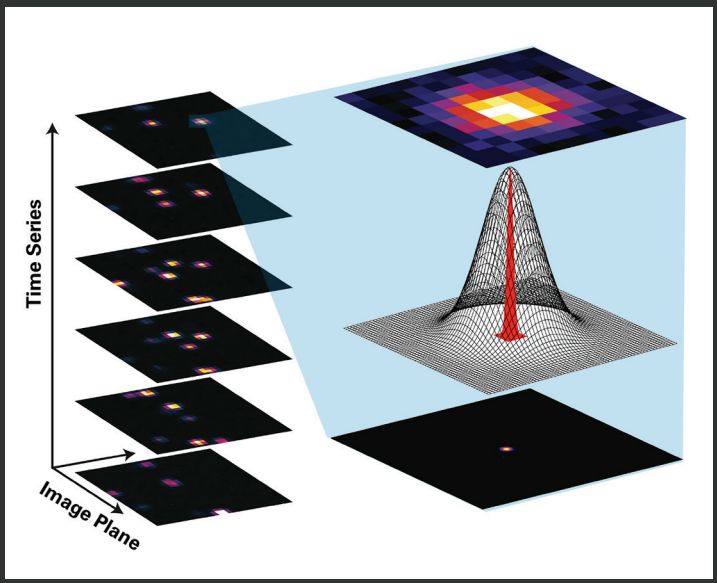


FIGURE 3.

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.

PALM

Photoactivated localization microscopy (PALM) uses photoactivatable fluorescence proteins (e.g., paGFP, mEOS2) that can be used for endogenous labeling. These proteins are switched on with one wavelength and imaged and then switched off with another. The main advantage of PALM, compared to STORM, is that it allows for labeling at the endogenous protein level. PALM provides a single localization event per molecule, making it well-suited for stoichiometric applications, as compared to other techniques like STORM.

STORM/dSTORM

Both STORM and dSTORM techniques rely on the use of photoactivatable or photoswitchtable dyes for sample illumination with different wavelengths. STORM uses active switching, while dSTORM uses stochastic blinking. The advantage of STORM/dSTORM over PALM is that it can achieve higher resolution imaging, given that PALM relies on a limited number of photons being emitted from the endogenous protein that is subject to photobleaching.

PAINT/DNA-PAINT

Points accumulation for imaging in nanoscale topography (PAINT) is perhaps the most useful and flexible technique for the cell biologist. This technique utilizes fluorophores bound to oligos that float in a buffer solution and are designed to target specific nucelotide regions attached to the sample. Instead of using a laser for photoswitching, transient binding and immobilization of a dye (e.g., Nile red) out of the buffer and onto the targeted structure provide the on-off mechanism. This allows imaging of an unlimited number of structures of interest within the sample. This makes PAINT easier and more efficient to implement than other SMLM methods. The PAINT technique does not result in photobleaching, and it provides the highest spatial resolution compared to other techniques. A recent advancement of this technique, DNA-PAINT, allows transient binding of fluorescent-labeled oligos to complementary strands attached to the protein of interest. For more on DNA-PAINT, see the multiplexing case study on page 28.

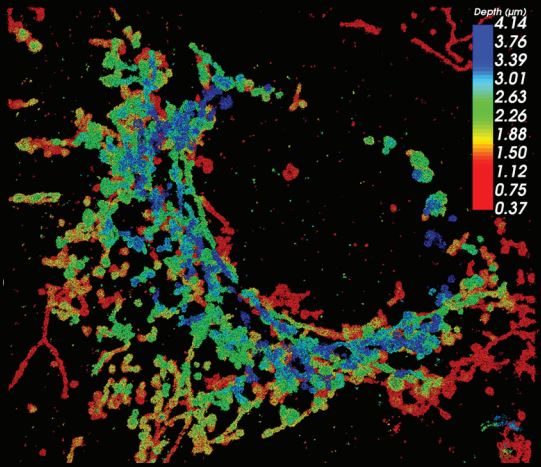


FIGURE 4.

Mitochondria stained for TOM20 with Alexa 647, imaged with SMLM (dSTORM).

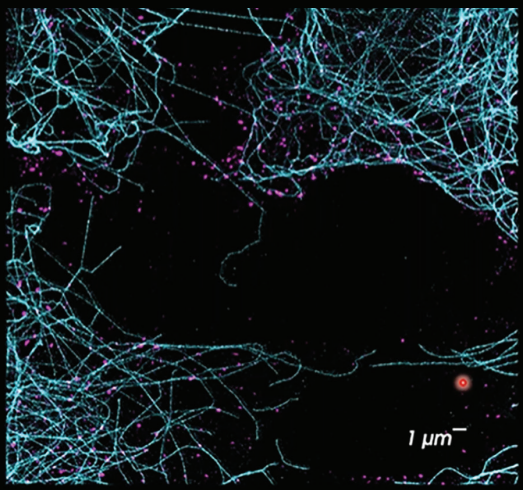
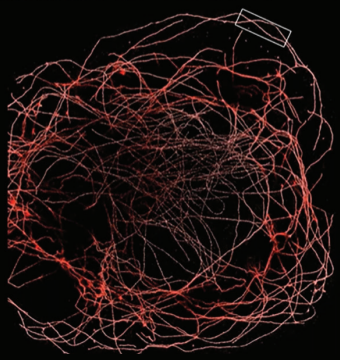
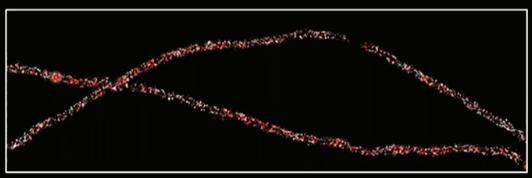


FIGURE 5.

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 (lower right and left). A two-color, multiplexed image of labeled microtubules and clathrin imaged with the Vutara Super-Resolution Microscope (upper left).





Applications

Super-resolution microscopy is a powerful tool for research questions that require imaging of specifically labeled molecules at a resolution of 20-200 nm laterally. In particular, SMLM supports imaging of a range of biological sample types, such as single cells, cell colonies, tissues, and even whole organisms in nanoscale detail. Fixed or live samples can be imaged with SMLM, although fixed sample imaging is more common. This technique supports nanoscale investigation of research questions regarding molecular quantification, distribution, colocalization, and particle tracking. The applications of SMLM transcend research disciplines, and it currently is being utilized for exciting neuroscience, cell biology, genomics, virology research and other areas.

Neuroscience

Imaging neurons in nanoscale detail beyond the optical diffraction limit opens doors in neuroscience research. SMLM can be used to answer complex questions regarding neurological and psychiatric disorders. Answers to these questions can lead to the development of innovative strategies to prevent the clinical manifestation of devastating conditions.[4]

The high resolution provided by SMLM can produce images of extremely small neurological structures. Perhaps one of the most interesting components of the brain is the synapse—the junction essential to the transmission of signals in the brain. Super-resolution microscopy is required to visualize these structures that are approximately 20-40 nm wide. SMLM is ideal for the study of neuronal structures and processes, such as neural development, neural circuit assembly synaptic formation synaptic transmission, and synaptic proteins.

One such example is the use of SMLM to label the synapses in *Caenorhabditis elegans*. Scientists were able to study and understand the relationship between the membrane-bound calcium sensor and endosomes in endocytosis (see Figure 6). Only SMLM can distinguish these domains, since the synaptic structures themselves are smaller than the diffraction limit of light. SMLM has also been used to determine the spatial distribution of the vesicular monoamine transporter 2 (VMAT2) pre- and post-drug treatment in rat brain tissue sections.

FIGURE 6.

The synaptic labeling of *C. elegans*. Three markers present in this image: yellow (Skylan-s Active zone marker), Magenta (HaloTag::JF646 Calcium Channel 1) and Cyan (SNAPf::JF549 Calcium Channel 2).
Image courtesy of Sean Merrill and Dr. Erik Jorgensen, University of Utah.

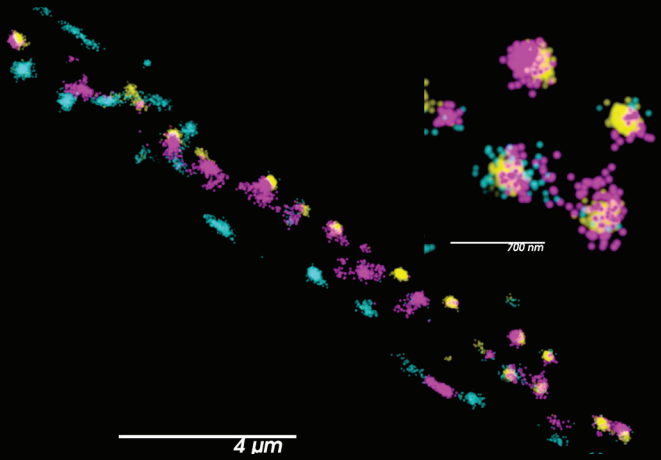
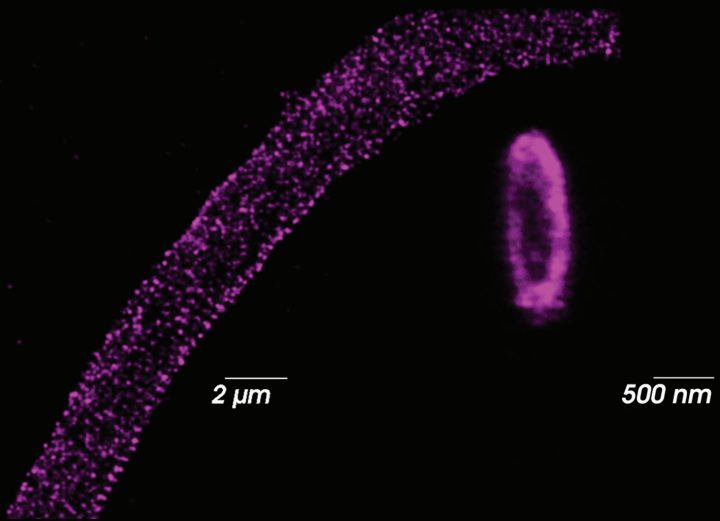


FIGURE 7.

Cross section of a single Beta Spectrin ring: 190 nm repeating structures Rat primary hippocampal neuron axon. Beta2-spectrin (AF647).
Image courtesy Yu-Mei Huang, Rasband Lab, Baylor College of Medicine (Houston, TX, USA).



Developmental and cell biology

Disease modeling and the development of future treatment resources relies on the fundamental knowledge gained from exploring the inner workings of cells, along with understanding the intricacies of animal and plant development. Biological imaging produced by SMLM can be used to track individual biomolecules or observe biological processes. Research applications in developmental and cell biology that can be achieved with SMLM include studies on molecular quantification, molecular distribution, colocalization of proteins, and motion models and particle tracking.

"Structure informs function" is a key paradigm in biology. However, many interesting cellular structures—such as substructures of most organelles and macromolecular machines, channels, and receptors—are smaller than the optical diffraction limit of ~200–300 nm, and therefore require super-resolution microscopy for imaging specifically labeled structures at this scale. See Table 2 for some examples of structures that can be uniquely and specifically imaged with SMLM.

Structure	Size
Diffusion channel of nuclear pore complex	40–50 nm in length
Nucleoid in mitochondria	~100 nm in diameter
Microtubules	24 nm in thickness
Ribosome	~20–30 nm in diameter
Bacterial heat shock protein DegP	20 nm in diameter
Cylindrical pyrenoid tubules in the chloroplast	107 ±26 nm in diameter
Endocytic pits in mouse cells	86 ±2.4 nm in diameter
Tubular segments of cristae in rat liver mitochondria	30–40 nm in diameter
Plasma membrane caveolae	50–100 nm in diameter
Hook of the bacterial flagellum	~55 nm in length

TABLE 2.

Examples of cellular structures that are strong candidates to be imaged with SMLM. Nanoscale substructures and their dimensions are from 'The database of Useful Biological Numbers'. Link: https://bionumbers.hms.harvard.edu/search.aspx

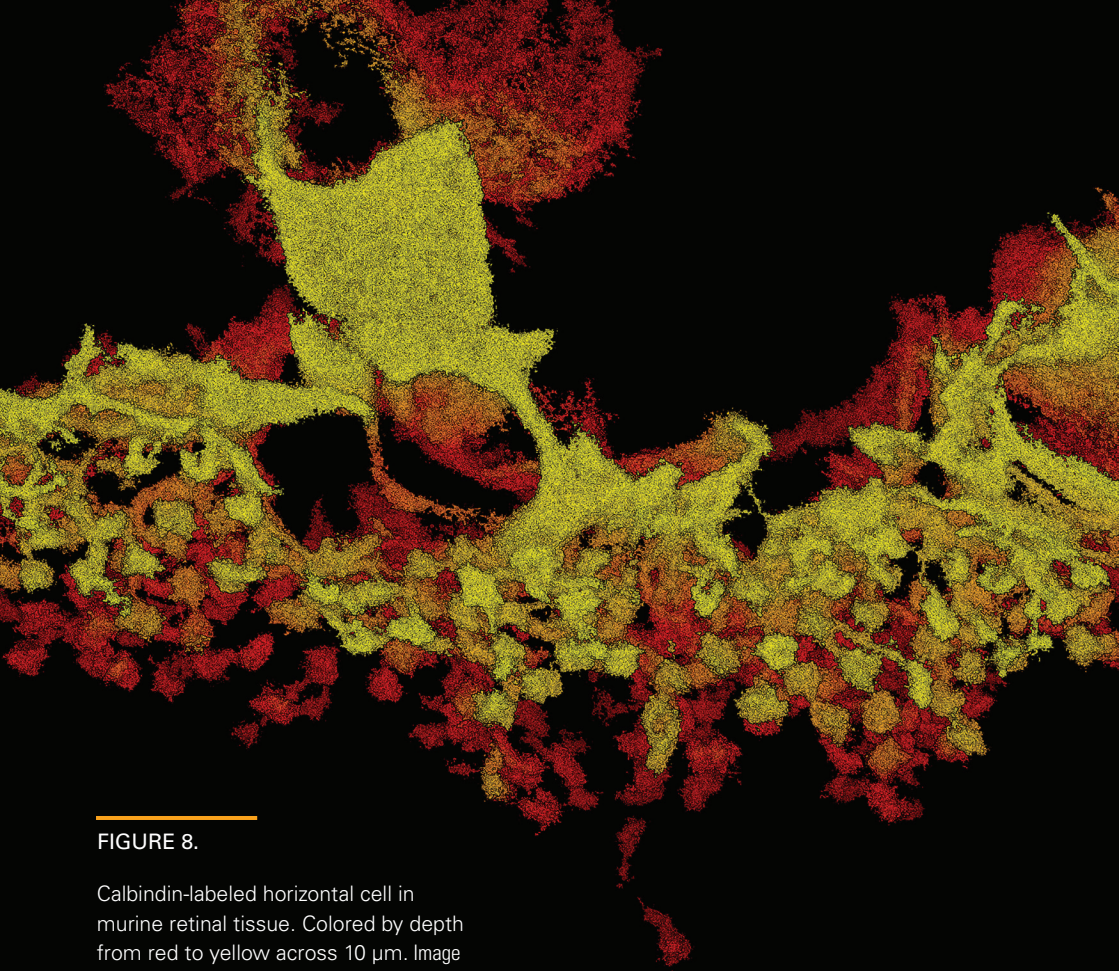


FIGURE 8.

Calbindin-labeled horizontal cell in murine retinal tissue. Colored by depth from red to yellow across 10 µm. Image courtesy of Nicholas Albrecht and Melanie Samuel from the Samuel lab at Baylor College of Medicine (Houston, TX, USA).

Genomics

In genomics, SMLM can be used to study the functional organization of the genome. The organization of the genome within the nucleus can provide a lot of information on cellular pathology; in particular, it can help distinguish between pathological and non-pathological states. Understanding these differences can help facilitate research toward future medical diagnoses and treatment. However, work within this field has been hampered by the resolution limits of conventional light microscopy. By enabling the generation of highly detailed images using fluorescent methods, super-resolution approaches such as SMLM have overcome these limitations.

Chromosomes are a collection of proteins and DNA that store genetic information. The three-dimensional (3D) organization of chromosomes regulates the expression of genes. Since the function of a chromosome depends on its 3D structure, it is important to image chromosomes, as pathology can cause differences in their structure, resulting in differences in gene expression. Using SMLM, a 3D image of a chromosome can be generated using specially designed oligonucleotides to label chromosomes. The number of targets imaged is limited by the number of probes that can be labeled. Therefore, combining SMLM with techniques such as microfluidics, allows more targets to be sequentially labeled (see Figure 9).

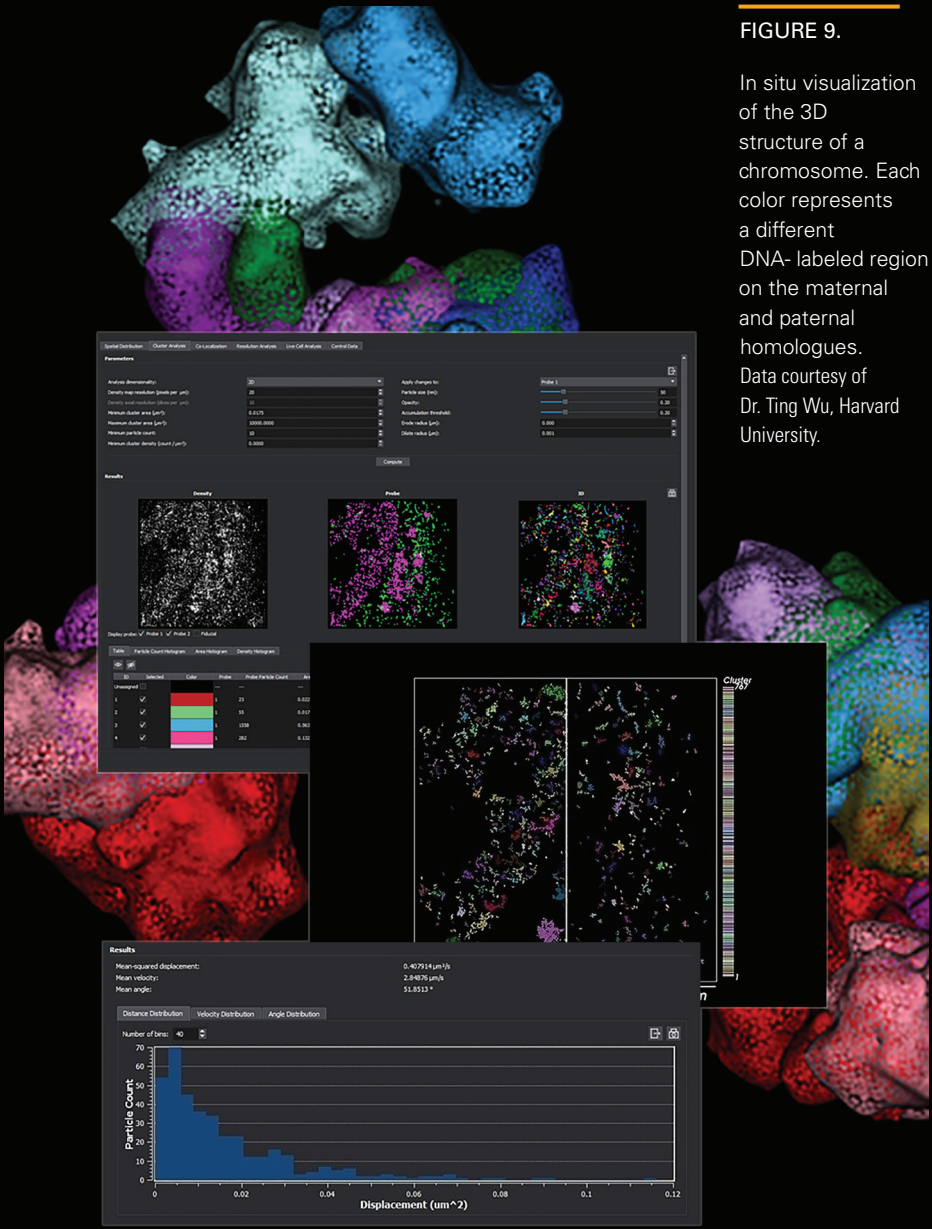


FIGURE 9.

In situ visualization of the 3D structure of a chromosome. Each color represents a different DNA- labeled region on the maternal and paternal homologues. Data courtesy of Dr. Ting Wu, Harvard University.

CASE STUDY:

Exploring genome structure and gene expression using super-resolution microscopy

A critical step in the central dogma of biology, the flow of genetic information within a biological system, is the transcription of DNA into RNA. RNA transcripts serve certain functions within a cell, and these functions can be influenced by a variety of factors. Guy Nir, Ph.D., Assistant Professor in the Department of Biochemistry and Molecular Biology at the University of Texas Medical Branch, leads a research program that focuses on how genome structures can shape transcriptional regulation in different biological systems, utilizing super-resolution microscopy as a main technology for investigation. His lab studies this phenomenon at the single-cell level, enabling simultaneous detection of gene expression and structure, which is critical for determining their relationship. This relationship between genome structure and function can be explored in a variety of biological systems, lending to several interesting research efforts transcending disciplines. Dr. Nir uses SMLM to address questions including, but not limited to, viral infection rate efficiencies, translocation influences on chromosome structure in cancer, and fundamental questions like how transcription influences genome organization.

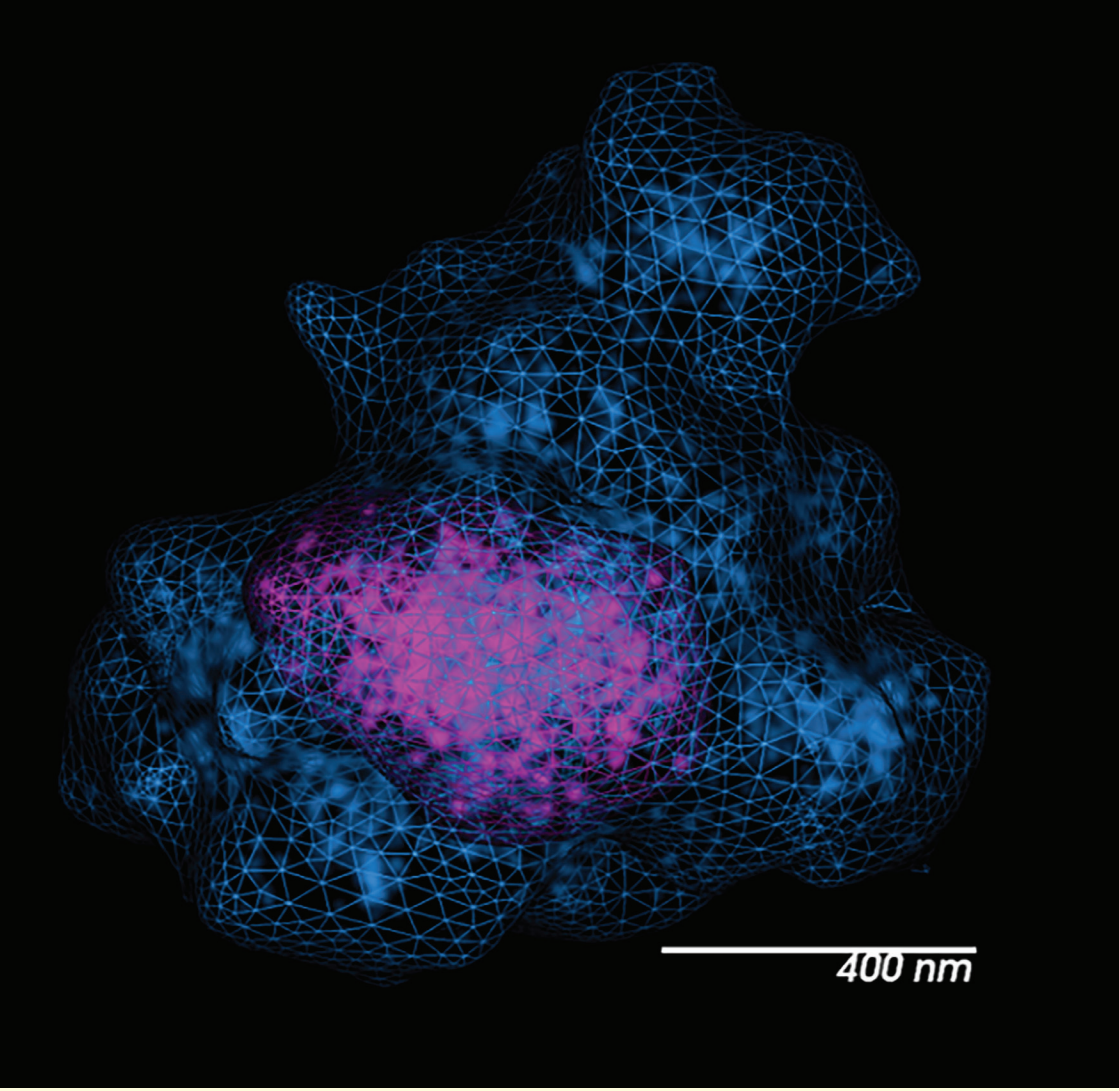


FIGURE 10.

Chromosome 19 topologically associating domain (TAD, in magenta) within a compartment (in blue). Data courtesy of Dr. Nir and Dr. Ting Wu, Harvard University.

Virology

Viruses are small infectious agents that invade living cells using their chemical machinery to survive and reproduce. Some viral infections can cause devastating symptoms and may lead to the death of the host. An important stage in viral pathology involves the transfer of the viral genome to the host cell after the virus has attached itself to a cell membrane. This process can occur via many different cellular pathways.[5] A better insight into virus-host interactions, particle structure, and pathology will therefore help with virus identification and the development of treatments. Since viral particles are typically smaller than the diffraction limit of light (<200 nm), viral research can be difficult. However, SMLM has become a critical means for the study of viral particles, capable of resolving structural details and locating components within the cellular machinery.[6] In the last few years, SMLM has been used successfully to study early infectivity and replication of enveloped viruses,[7] the relationship between a therapeutic antibody on respiratory syncytial viral infections,[8] and the discovery of the critical role that actin cytoskeleton plays on viral infectivity.[5]

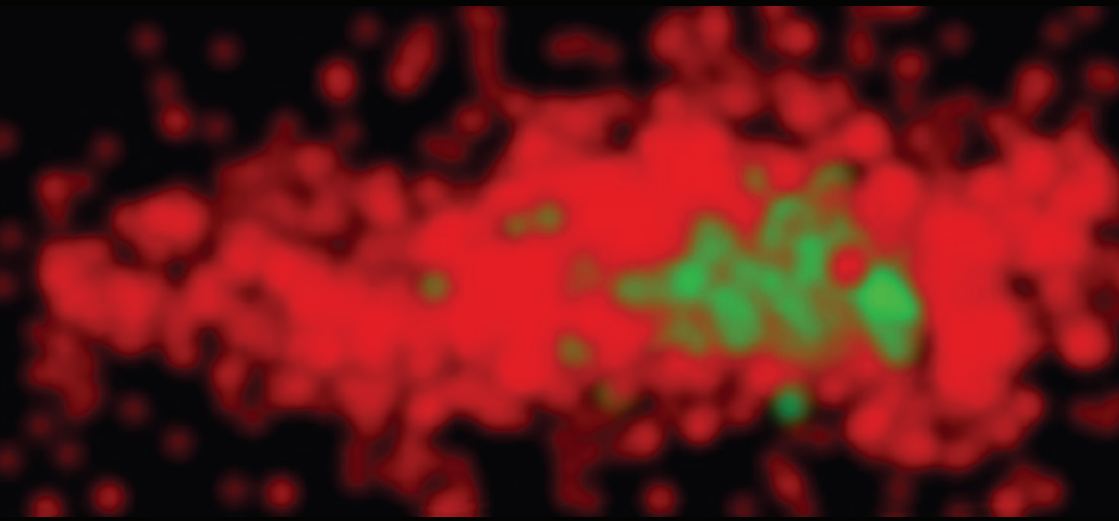


FIGURE 11.

Vesicular stomatitis virus particle.
Red – VSV-G protein tagged with
Alexa Fluor 647.

Cancer biology

Cancer research and treatment has advanced greatly over the last few decades. However, it is an extremely complex and heterogenous disease, and there are still many unknowns to be answered. Preclinical and clinical research on potential drugs is needed to determine potential toxicities, confirm efficacies, and establish any off-target effects. Imaging technologies can be employed to study the effects of treatments in preclinical models and uncover key tumor signaling and progression mechanisms.[9] Furthermore, genomic imaging is a powerful tool used to study cancer biology as structural changes within the genome are associated with the heterogeneity of cancers.[10]

SMLM has been used to open avenues in cancer research to achieve a better understanding of spatial organization and treatment responses to therapeutic agents, at single-cell level in normal and cancer cells. An increase of knowledge in this scientific field can eventually offer new potentials for individualized medicine.[11]

Cardiology

In the US, approximately 647,000 people die from cardiovascular disease per year.[12] Therefore, cardiology is an important branch of medicine and involves the study and treatment of disorders of the heart and blood vessels. Imaging technologies can be used to provide detailed insights into the cardiovascular system, driving the development of diagnostic and treatment strategies. The high resolution offered by SMLM has helped solve complex questions within cardiology research.

Forty percent of patients suffering from heart failure develop delays in ventricular electrical activation, which can result in ventricular dyssynchrony–the difference in timing or synchronization of ventricle contraction—and ultimately death.[12] However, if detected, dyssynchronous heart failure can be treated with cardiac resynchronization therapy (CRT). This therapy can resynchronize the ventricular mechanical and electrical activity, reducing mortality in patients. In an elegant study using SMLM to investigate the sarcomeric organization pre- and post-CRT treatment, α-actinin was used as a marker to reveal the sarcomeric structures (Figure 13).[13]

FIGURE 12.

A routine three-color experiment using a Vutara Super-Resolution Microscope to image U2OS cells.
Image courtesy of Leremy Colf and Dr. Wes Sundquist, University of Utah.

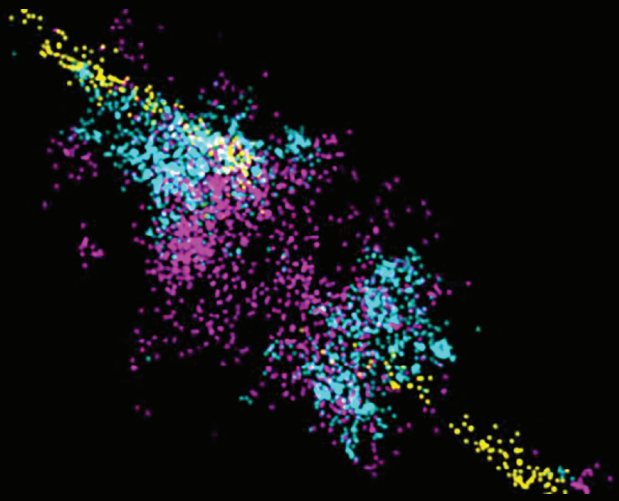
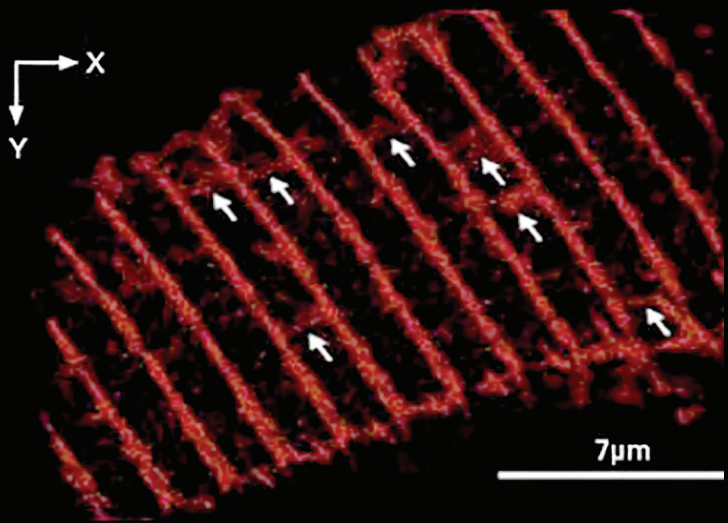


FIGURE 13.

The decrease in alpha-actinin transverse fibers in cardiomyocytes resulting from heart failure.
Data from Lichter et al., 2014.[14]



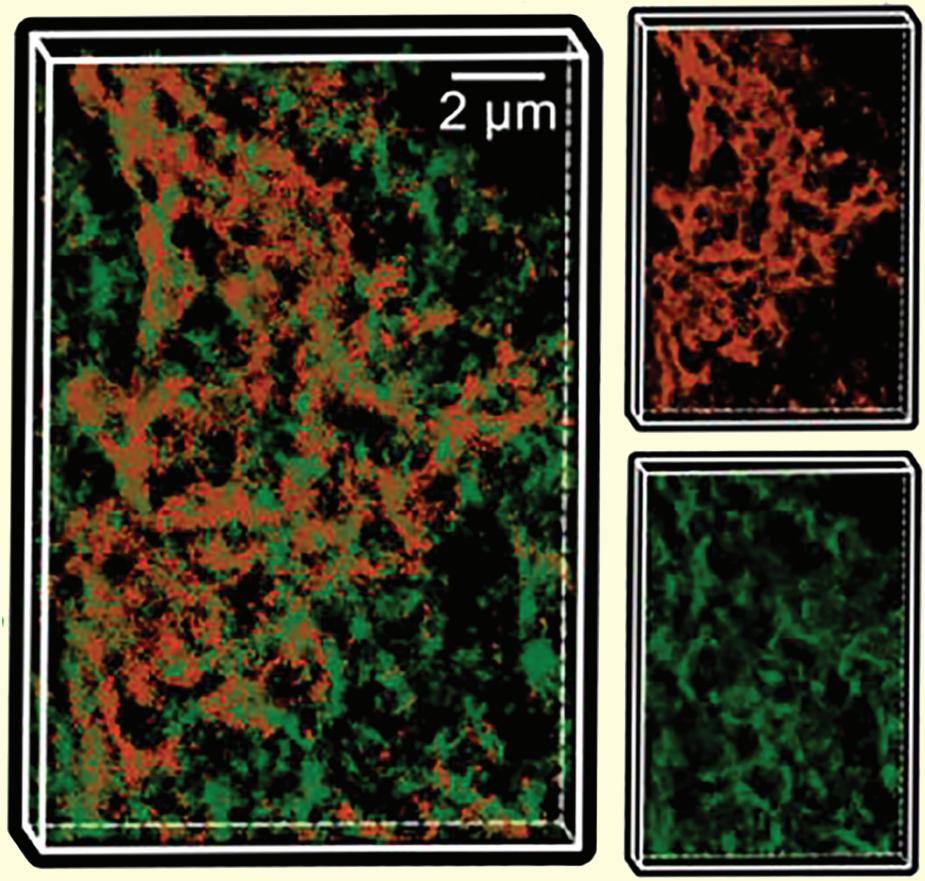
CASE STUDY:

STORM-based quantitative analysis of ion channel localization in failing human hearts

Rengasayee (Sai) Veeraraghavan, Ph.D., of The Ohio State University conducts research on the structural underpinnings of electrical signal propagation in the heart. By leveraging the high resolution of the Vutara Super-Resolution Microscope and taking advantage of structural fiducials identifiable via both light and electron microscopy, he developed indirect correlative light and electron microscopy (iCLEM) as a low-cost, high throughput imaging option with extensive quantitative capabilities. More specifically, he uses iCLEM to study the intercalated disk (ID), a specialized structure that affords electrical and mechanical coupling between muscle cells in the heart, and obtained the first-ever quantitative picture of the ID. This enabled the construction of realistic computational models of electrical signal propagation. By doing so, his lab is uncovering previously unappreciated structure-function relationships that determine the regularity of the heart's rhythm. These predictions, along with functional imaging studies of electrical signal spread in the heart, are providing the basis for the development of novel classes of anti-arrhythmic drugs.

FIGURE 14.

Point-cloud representation of sodium channels (green) and N-cadherin molecules (orange) distributed throughout an intercalated disc from a failing human heart.
Data courtesy of Dr. Veeraraghavan.



Recent Advancements

Super-resolution technology continues to advance rapidly, increasing its capabilities for ever more complex research investigation. Among the major recent advancements, Bruker has been delivering equipment and software for multiplexing with microfluidics, three-dimensional imaging with biplane technology, top-hat illumination, and FISH and smFISH methods.

Multiplexing

Multiplex imaging with super-resolution microscopy allows for imaging of multiple specific targets, which can be necessary depending on the research question. This technique is used in applications such as DNA-PAINT, OligoSTORM, refresh STORM, multiplexed antibody labeling, and chromatin tracing.

Applications are developing in localization microscopy to a degree in which the number of targets for imaging far outstrips the number of probes that can be chromatically separated. Sequential labeling with microfluidics allows a much larger number of targets to be addressed. Microfluidics units (such as Brukers's PlexFlo and PlexFlo96) can be used for unlimited sequential labeling to provide endless research possibilities.

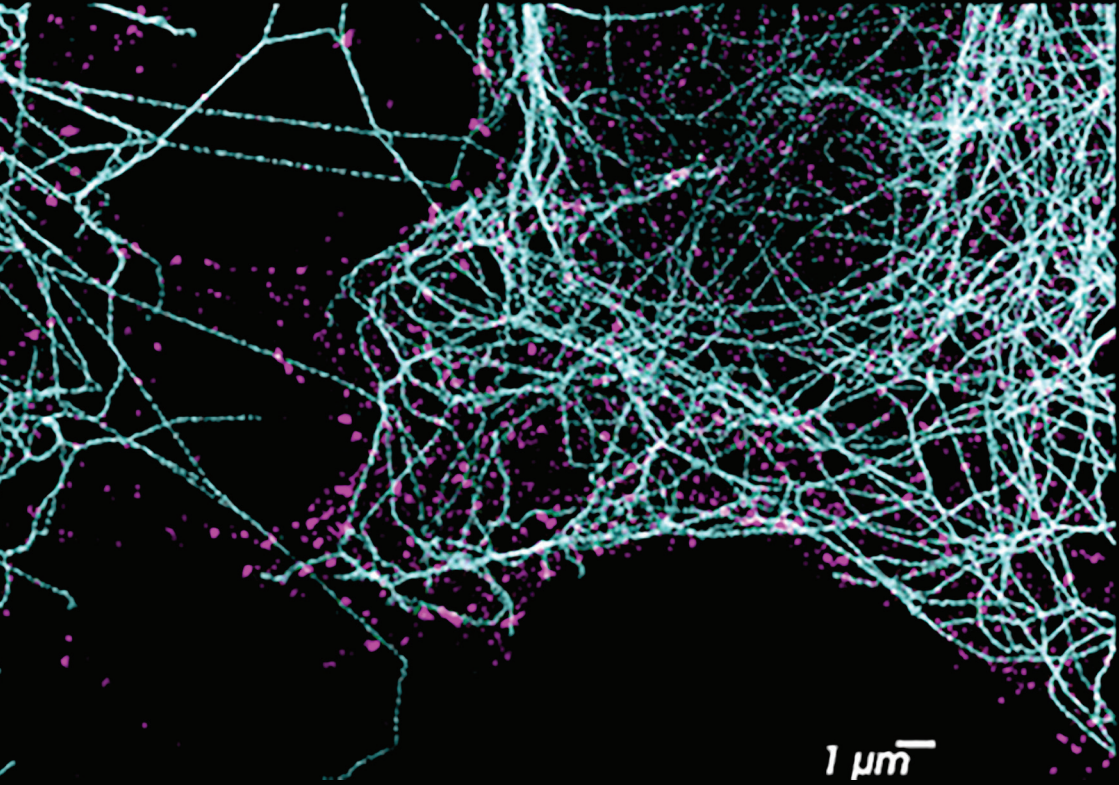


FIGURE 15.

Two-color DNA-PAINT experiment was performed on the Vutara Super-Resolution Microscope. Tubulin is labeled in cyan and clathrin in magenta.

CASE STUDY:

Multiplexing with DNA-PAINT technique

DNA-PAINT works through the transient binding of a short imaging oligonucleotide containing a fluorophore to a complementary oligonucleotide – called the docking strand – on the target of interest, such as an antibody, nanobody, aptamer, or suicide enzyme ligand. Super-resolution imaging with DNA-PAINT involves several steps. First, the sample is labeled with the "docking strand" through conventional techniques and prepared for imaging. For imaging, the sample is bathed in imaging buffer (typically PBS but can include oxygen scavengers) and a low (typically 0.1nm) concentration of imaging oligo complementary to the docking strand. The imaging oligo is typically 9–10 nucleotides in length and contains a fluorophore. Once in the imaging buffer, the sample can be imaged. The transient binding of the imaging strand to the docking strand stops the diffusion of the fluorophore, allowing it to be imaged on the camera. Since the sample is bathed in a large excess of constantly exchanging imaging strand, the target is essentially unbleachable, making it possible to batch-process many frames and extended Z-stacks. The nature of DNA-PAINT enables sequential labelling of multiple targets making multiplexed imaging easy, particularly when combined with an automated fluidic device.

DNA-PAINT has enormous potential for multiplexed imaging. Using orthogonal docking strands on different probes makes imaging an unlimited number of targets possible. In this case study, a multiplexed DNA-PAINT experiment was performed using a Vutara VXL microscope and microfluidics unit from Bruker. The multicolored images shown in Figure 16 are a result of specific labelling and imaging of four cellular targets, including F-actin, mitochondria, microtubules, and clathrin.

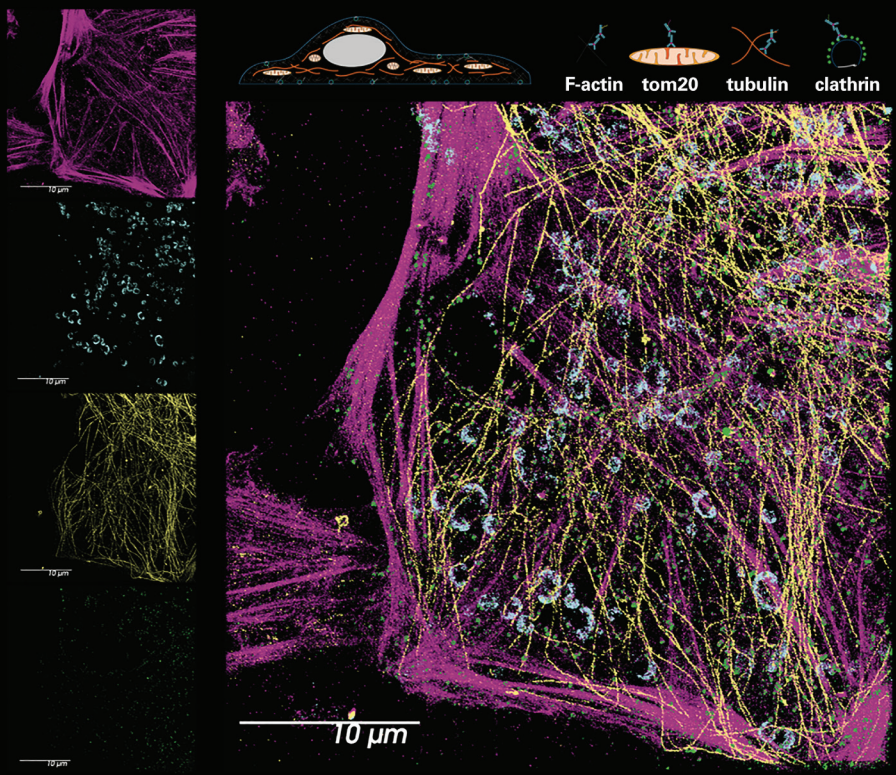


FIGURE 16.

DNA-PAINT multiplexed image. The images from each of the four targets (left) were acquired separately and combined automatically to create final image (right) in SRX software from Bruker. The image was colored by molecule, with F-actin in magenta, mitochondria in cyan, microtubules in yellow, and clathrin in green.

Three-dimensional imaging with biplane technology

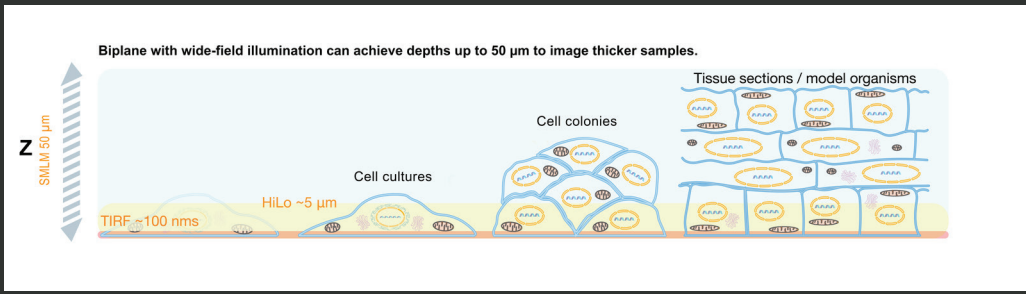
Biplane imaging, or imaging of two focal planes simultaneously (e.g., with Bruker's proprietary biplane technology), allows for the collection of 3D data by localizing the x, y, and z position of each molecule. This technique is unique from most commercial SMLM systems that use PSF engineering to get z-dimensional information and are much less conducive to imaging deeper in samples. Most commercial systems are limited to either using TIRF or HiLo imaging with complicated 3D calibration, which can only achieve imaging depths of around 5 µm (see Figure 17). In contrast, with biplane imaging, one can easily image 50 µm from the coverslip with a standard calibration. With this depth, a user can image a wide range of samples in 3D, from single cells and cell cultures to cell tissues and whole model organisms like Drosophila or *C. elegans*.

Top-hat illumination

Another advancement is the use of top-hat illumination, which enables illumination over the entire field of view so that every part of the field of view is usable for data analysis. With Gaussian beam profile, 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 (see Figure 18). In contrast, with 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 17.

Comparison of imaging depth with biplane technology versus HiLo or TIRF.



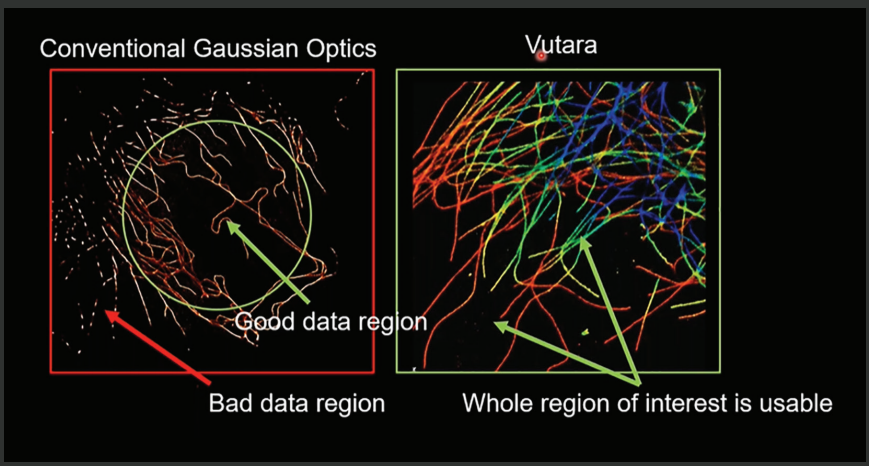


FIGURE 18.

Bad data acquisition without top-hat illumination (left) and quality data acquisition with top-hat illumination with Vutara (right). By illuminating the entire field of view, the Vutara enables quality imaging for the entire plane, resulting in acquisitions suitable for quantitative analysis.

FISH and smFISH

Fluorescence in situ hybridization (FISH) is a technique used for the visualization of DNA, either specific genes or portions of genes, within a cell. This technique works via the use of probes that hybridize to the desired genomic region of interest and are labeled with fluorescent dye that is visible with SMLM. FISH is a powerful tool to investigate genomics questions, as it supports chromatin tracing applications. Like FISH, single molecule fluorescence in situ hybridization (smFISH) is a cutting-edge technique for studying gene expression in single cells but differs in its ability to detect and count individual RNA molecules. With smFISH, RNA targets are imaged via the application of many short, labeled oligonucleotide probes. Both FISH and smFISH assays can be multiplexed to support experiments that require many probes for specific labeling. Both FISH and smFISH have transcendent applications across life sciences disciplines, such as genomics, cancer biology, neuroscience, and more. Specialized software (e.g., Bruker's SRX software) is required for the analysis of FISH and smFISH data.

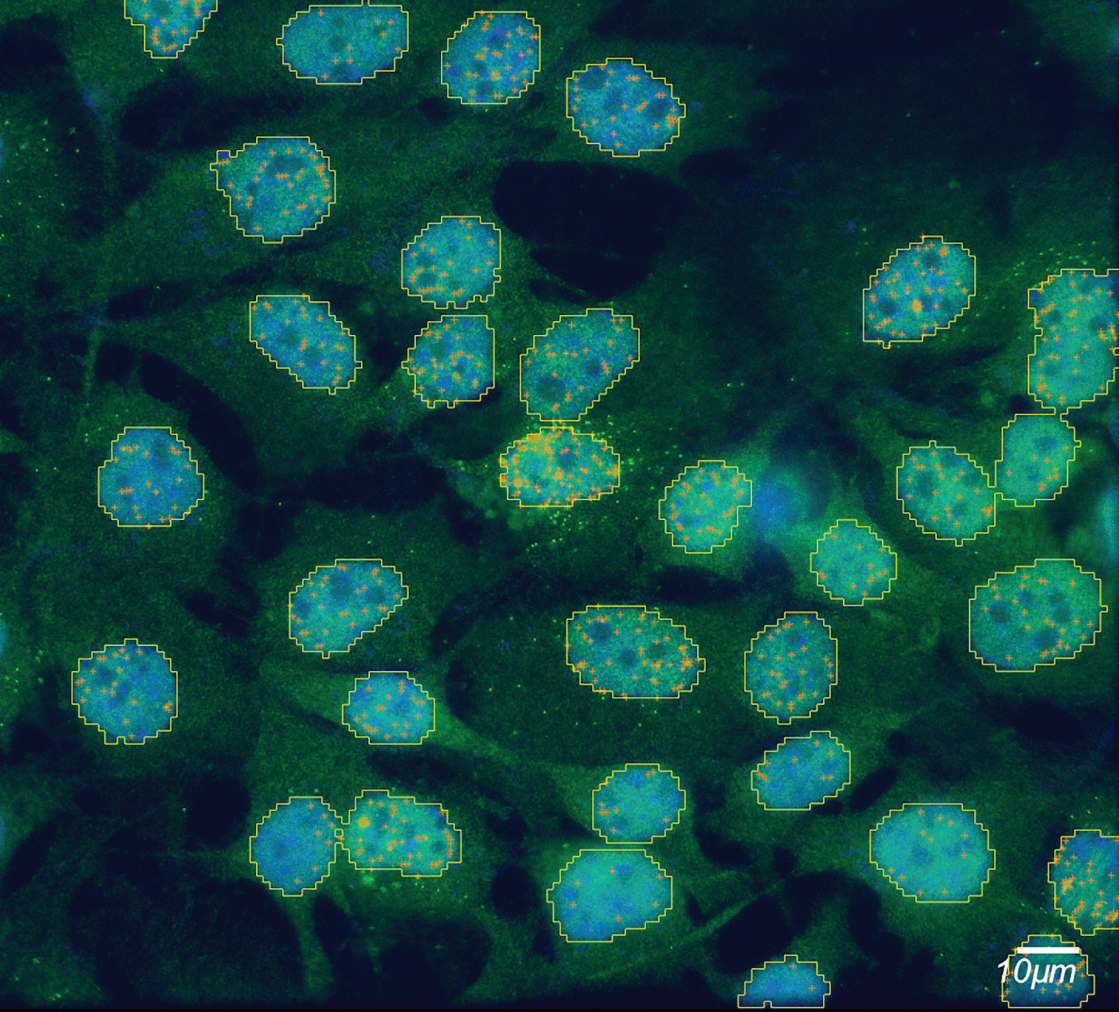


FIGURE 19.

SRX software interface during analysis of smFISH with open-source segmentation.

Future Trends

Live imaging

Biological imaging requires viewing dynamic structures within cells; either visualizing individual biomolecules in cells (particle tracking) or observing biological processes, such as membrane, organelle, or cytoskeletal movement. The ability to perform live imaging on a timescale that reflects the underlying biology is necessary for many biological research questions. With SMLM, live-cell and particle tracking applications are not only possible, but are becoming increasingly more specialized and revealing. For particle tracking experiments, samples can be labeled with organic dyes, proteins, or quantum dots and the single fluorophores can be imaged at high speeds. For example, the Vutara Super-Resolution Microscope uniquely enables particle tracking in three dimensions. In addition to particle tracking, live cellular imaging of larger cellular structures, such as organelles, is possible. This unique imaging modality allows researchers to collect dynamic super-resolution images of structures of interest, while also giving the precise three-dimensional position of each molecule over time if paired with biplane technology. SMLM systems that can perform multicolor single-molecule imaging and two-color experiments combining particle tracking and cellular imaging are achievable. For example, a two-color experiment in cells expressing tomm-20::HaloTag® supported particle tracking and cellular imaging together (see Figure 20). In this experiment, the tomm20::HaloTag® is labeled with a dense concentration of JF549® and a very sparse concentration of PA-JF646®. This allows the dense dye to be used as a contextual marker and enabled the reconstruction and localization of mitochondria over time. The second dye allowed the tracking of the diffusion of single molecules in the context of the mitochondria. Using this technique, one can monitor how the diffusion of proteins in a membrane changes as mitochondria traffic and undergo fusion or fission.

Correlative microscopy

While some research questions benefit from a single imaging method alone, there are many cases in which combining different microscopy methods is necessary. For example, fluorescence microscopy enables specific labeling of cells or cellular structures but is often diffraction limited, or in the case of super-resolution microscopy able to achieve lateral resolution of 20 nm. On the other hand, electron microscopy provides the highest resolution, down to less than 1 nm, of morphology but specific labeling is very challenging. Correlative microscopy, the combination of microscopy methods and correlation of the acquired data, provides the advantages of the two combined methods—in this case, the

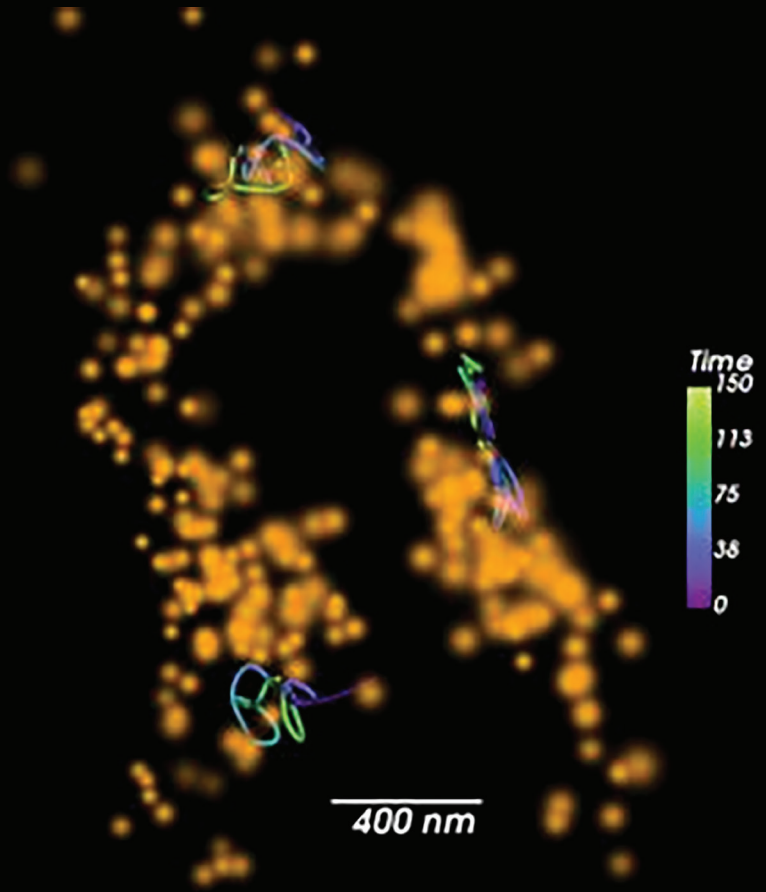


FIGURE 20.

Overlay of tomm20 diffusion streamlines and the structural image of mitochondria.

targeted labeling capability of fluorescence microscopy and the highest resolution of electron microscopy. The combination may also include confocal microscopy, atomic force microscopy, X-ray, MRI, and other imaging methods. Correlative microscopy, particularly with SMLM, enables richness and depth of data collection beyond what any single technique is able to deliver.

Imaging across the central dogma

As super-resolution technology evolves, so does the interest in questions and methods that transcend the central dogma of biology—the flow of genetic information from DNA to RNA to protein. The most technologically advanced SMLM systems, such as Vutara VXL, are well-suited for fundamental nanoscale spatial investigations in the genome, transcriptome, and proteome, delivering both nanoscale resolution imaging and quantitative analysis. Genomic investigations, such as chromatin tracing, can be performed easily with SMLM. RNA imaging and quantification can be performed in basic research experiments using smFISH software. Protein structures can be fluorescently labeled and imaged with SMLM and, with multiplexing, many proteins can be labeled at once. Future SMLM experiments will tie together these steps in the central dogma, enabling a more holistic view of biological processes.

Conclusion

Super-resolution microscopy enables imaging of specifically labeled specimens at a resolution beyond the diffraction limit of light, down to 20 nm laterally, effectively bridging the gap between traditional light microscopy and electron microscopy. There are several approaches to super-resolution microscopy; however, the highest resolution and most optimized approach for life science imaging is that of single-molecule localization (SMLM). When paired with additional advancements, such as biplane technology, top-hat illumination, microfluidics units for multiplexing, and analysis software, SMLM is a powerful tool for nanoscale investigations spanning life sciences disciplines of neuroscience, cell biology, cancer biology, and more. Future advancements in SMLM technology will continue to expand transformative research in these fields.

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Further Resources

Bruker's Vutara Super-Resolution Microscope:
https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/super-resolution-microscopes/vutara-vxl.html

Microfluidics Accessories:
https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/super-resolution-microscopes/microfluidics-unit.html

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