

Through the High-Tech Looking Glass

Science begins with observation; scientists have made telescopes to examine things farther away than the eye can see and microscopes to examine things invisible to human vision. Since Robert Hooke in the 17th century used the first microscope to document the existence of living cells, advances in cell biology have been tied to ever more innovative tools for visualizing and analyzing the microscopic world. CCR scientists continue to creatively expand the boundaries of observation to answer longstanding and diverse questions about the inner workings of cells.

Illuminating Discovery

When most people hear the word genome, they think about long sequences of nucleic acids strung together in a code that is read by each cell in the body. Some sequences predict eye color and some predispose to disease. But, long before its composition was known, the genome was observed as a physical structure occupying three-dimensional space inside the nucleus of every cell. Whether and how the spatial arrangement of the genome influences its function was a matter of speculation. Now we know that the positions of individual chromosomes and regions of the genome in the nuclear space are not random and their position is directly linked to their function.

Since his first year as a Postdoctoral Fellow at the Cold Spring Harbor Laboratory, Tom Misteli, Ph.D., now a Senior Investigator in CCR's Laboratory of Receptor Biology and Gene Expression, has wanted to know what cellular factors determine where a gene is positioned in the cell nucleus. It is one of those questions in science that is fundamentally important, but the technology was simply



(Photo: R. Bicer)

Gianluca Pegoraro, Ph.D., Sigal Shachar, Ph.D., and Tom Misteli, Ph.D., in the lab

not available to answer it—until recently. A few years ago, Misteli was instrumental in setting up the CCR High-Throughput Imaging Facility (HiTIF). The HiTIF combines state-of-the-art microscopy with the ability to automate sample handling and imaging using plates containing wells to simultaneously carry out 384 individual experiments.

“Imaging is traditionally fairly descriptive and based on a

candidate approach,” said Misteli. “You might examine your favorite protein in a particular biological situation and then perturb that system with a drug or genetic manipulation. I was interested in whether you could use imaging as an unbiased discovery tool.

“The concept is simple—you knock out every single gene in the genome and use an assay to figure out which one of the 20,000 genes

affects your favorite process. The ability to image a very large number of samples makes this now possible,” Misteli explained.

While the concept might be simple, the execution requires the right equipment: microscopes not generally available at research institutions, robotic pipette systems for sample handling, and, equally critical, a bioinformatics group to automate the analysis of terabytes of data. “Getting the images is easy,” said Misteli. “But to teach a computer to find the structures that you are interested in—and find them accurately—that is the challenge.”

In the August 2015 issue of *Cell*, Misteli, his Postdoctoral Fellow Sigal Shachar, Ph.D., and Gianluca Pegoraro, Ph.D., Head of HiTIF, published the results of their first screen for factors controlling genome organization. Using fluorescence *in situ* hybridization (FISH) to monitor the proper positioning of a representative set of functionally diverse genomic loci, the researchers used RNA silencing to identify 50 cellular factors required for proper positioning.

“The total imaging time for that experiment was about 27 days, and we analyzed 3.5 million data points,” said Misteli.

This approach, which Misteli calls Deep Imaging, is not only powerful for large-scale screening efforts, but is also equally powerful for examining rare events. “You can either take your 384-well plate and do a different experiment in every well by adding a different compound, or you can do the same experiment 384 times and look for very rare events in your very large dataset,” said Misteli.

Using Deep Imaging, Misteli and his Postdoctoral Fellow, Vassilis Roukos, Ph.D., reported in *Science* in 2013 the visualization of the formation of a chromosome translocation in living cells for the first time. Chromosomal translocations, in which a segment of one chromosome is inappropriately joined to another during replication, are a hallmark of cancer cells. They are also exceedingly rare.

High-throughput imaging will also form a cornerstone of a large consortium of investigators throughout the U.S. and Europe, including Misteli, which was recently funded as part of the 4D Nucleome program, a new NIH Common Fund initiative to map the genome space and time as an extension of the Human Genome Project.

Illuminating Detail

“Centrosomes are mysterious,” said Jadranka Loncarek, Ph.D., Tenure-Track Investigator in CCR’s Laboratory of Protein Dynamics and Signaling. “Once you start studying them, you just fall in love.”

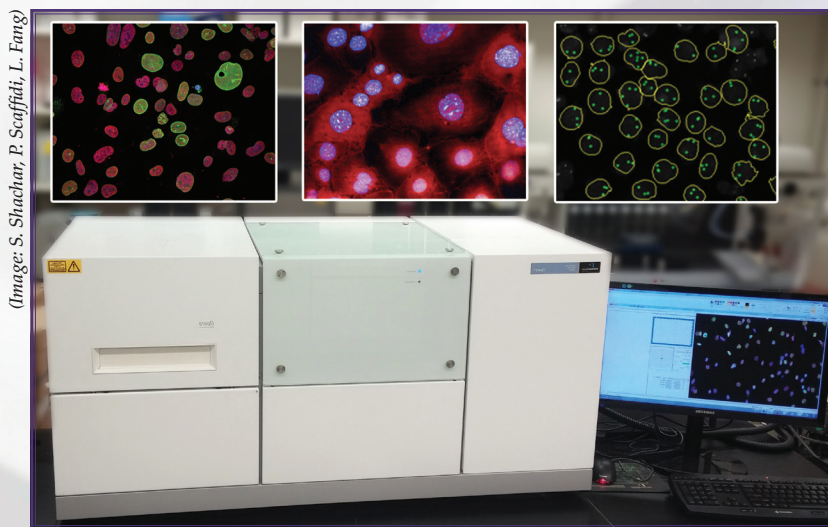
The centrosome is a complex organelle that regulates the cell division cycle. It comprises a stable core structure of a single or duplicated centriole at the center of a highly structured matrix of pericentriolar material. The centrosome is also a dynamic organelle that changes in volume during the cell cycle, and is associated with about 70–80 reliably identified core proteins and many more transiently associated ones.

“The centrosome doesn’t have a clear boundary,” said Loncarek. “It’s an open platform for protein interactions that changes constantly during the cell cycle.”

Centrosomes play critical roles in cell division; however, their own duplication processes are unusual and poorly understood. Normally, centrioles only duplicate once during the cell cycle, forming a mother–daughter pair that remains in close, orthogonal proximity. However, under certain conditions, centrioles can move away from one another and reduplicate.

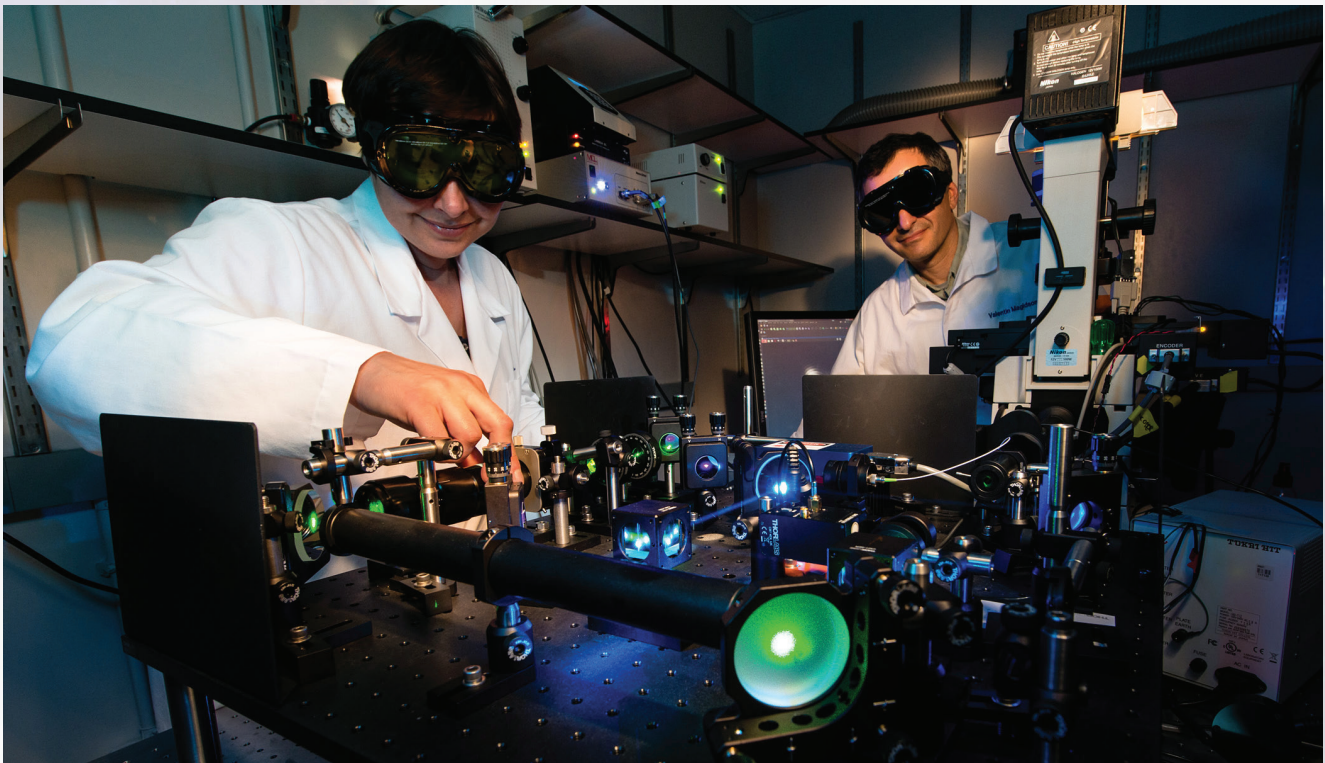
But understanding centrosomes duplication is more than just a matter of academic curiosity: it turns out that most tumors have aberrations, either in the structure or number, of these organelles. Since the degree of centrosome aberrations (which in turn rests on the regulation of centriole duplication) correlates with tumor aggressiveness, the mechanisms that allow for incorrect regulation of centrosome formation are of broad interest.

Loncarek and her colleagues wanted to know what was happening at an ultrastructural level to relieve the duplication block.



(Image: S. Shachar, P. Scaffidi, L. Fang)

High-throughput microscopes enable interrogation of a wide spectrum of intricate cellular features.



(Photo: R. Frederickson)

Jadranka Loncarek, Ph.D., and Valentin Magidson, Ph.D., preparing for laser microsurgery.

Centrioles are electron-dense structures of approximately 0.5 micrometers in length, and they can be easily observed through transmission electron microscopy. However, in order for those observations to be meaningful, they need to be matched with observations of the cell at the light microscope level.

Loncarek uses a method called correlative light and electron microscopy (CLEM). “It is not easy,” said Loncarek. “First, you study the cell under the light microscope, and then you have to find that same cell among thousands after all the preparation necessary for electron microscopy. It’s a very error-prone technique. You need to develop expertise in both light and electron microscopy to bridge the gap.”

After almost two years of troubleshooting, Loncarek and her Postdoctoral Fellow, Dong Kong, Ph.D., have overcome the technical hurdles to making CLEM a routine and reliable part of their

research strategy. Using this technique, Loncarek’s team found that beyond a critical distance of only 80 nanometers between mother and daughter centrioles, the mother centriole can undergo reduplication. Moreover, contrary to widely held beliefs, they showed that the orthogonal positioning was not important for blocking duplication. Their findings were published in *Nature Communications* in August 2015.

“We would really like to understand this distancing process between mother and daughter, such that the latter slowly walks away until the mother doesn’t feel it any more as an inhibitory element for its reduplication. We are designing experiments to study the molecular mechanism behind this process,” said Loncarek.

Loncarek and her team are now working with super-resolution microscopy to study the organization of proteins around the centriole. Stochastic optical reconstruction

microscopy (STORM) relies on fluorescent probes that switch rapidly between light and dark states. A single snapshot shows only a small subset of fluorescent spots, whose centers pinpoint position with high accuracy. A final composite image, therefore, has higher resolution than an image in which all loci were simultaneously labeled.

“STORM allows us to use mathematical algorithms to reconstruct images with up to a 10-nanometer resolution. However, the sample preparation is complicated, and only certain dyes can be used,” said Loncarek. “A lot of people are working to develop better probes we can use in live cells and then in super-resolution microscopy. The current great challenge is to find a probe that maintains sufficient fluorescence, even after processing, for electron microscopy for direct comparison of protein localization with ultrastructure.”

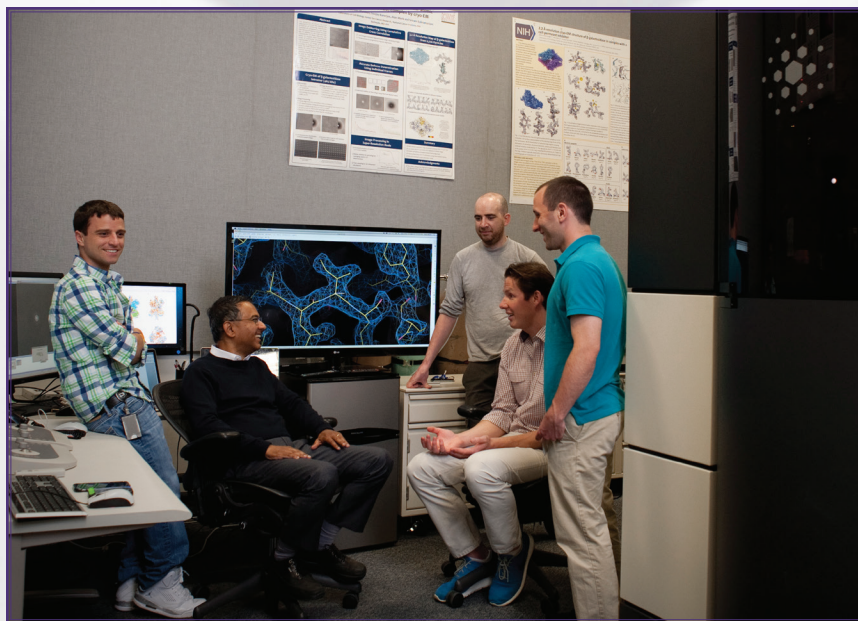
Illuminating Molecules

“Why shouldn’t it be possible to look at the 3D structure of a protein like you look at a cell through a microscope?” asked Sriram Subramaniam, Ph.D., Senior Investigator in CCR’s Laboratory of Cell Biology. Subramaniam has spent more than a decade bridging the technological gaps that stand in the way of solving interesting biological problems, using high-resolution electron microscopy at the interface between structural biology and cell biology. (See Box: Center for Molecular Microscopy).

Most recently, his laboratory has focused on cryo-electron microscopy as a means to access the structure of proteins at atomic resolution, while suspended in solution. Many biologically important proteins have not been amenable to classical techniques of structural biology because of their size or their conformational heterogeneity.

Given recent advances in electron detectors, it is now possible to take images of individual molecules at higher definition than ever before using a transmission electron microscope. Because electron beams are damaging over time, each particle is only viewed very briefly, leaving a grainy image. However, by averaging images of tens of thousands of particles, it is possible to computationally create a three-dimensional picture of the particle. It is now even possible to categorize these images, deriving separate three-dimensional structures for the particle in several different conformations at the same time.

Subramaniam and his colleagues have used this technique to study mechanisms of ion-channel gating and enzymatic regulation. Earlier this year, they described, in *Science*, the structure of the bacterial enzyme β -galactosidase in complex with an inhibitor at a resolution of close to 2 Å. In addition, the Subramaniam



(Photo: R. Baer)

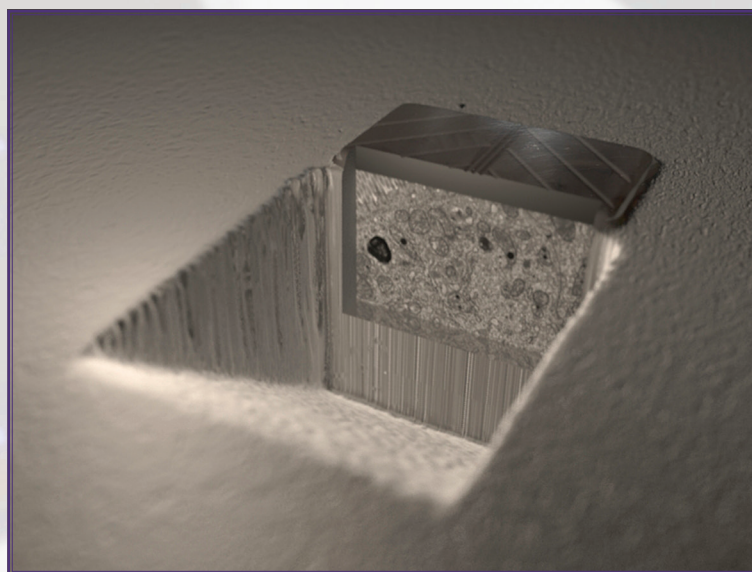
Sriram Subramaniam and members of his team discussing the Krios microscope with FEI Company representatives. Left to right: Alan Merk, Sriram Subramaniam, Ph.D., Kieran Moynihan, Gijs Janssen, and Joseph Darling, Ph.D.

lab has a new manuscript in press in *Cell*, which describes new mechanistic insights into a 200kDa ion channel, one of the smallest membrane proteins yet studied by cryo-electron microscopy.

Subramaniam has now turned his attention to cancer, studying

complexes of small molecules with cancer targets, with the goal of influencing drug design at an early phase.

“There is a lot of information we can obtain about the interaction of drugs with the molecules they target,” said Subramaniam. “So far,



(Image: D. Bliss, NIH, and S. Subramaniam, CCR)

A resin-embedded cell is excavated with an ion beam, then imaged with a scanning electron beam. Its surface is abraded with the ion beam and again imaged in an iterative process. Layer by layer, images are collected and then aligned to form a 3D volume. Here, the exposed face of the specimen is shown at the back of a trench created by the ion beam. A protective carbon pad overlays the embedded cell where future imaging will take place.

“It is now possible to take high-definition images of individual particles—that have been trapped in a frozen solution—with a transmission electron microscope.”

structural biology has been useful for snapshots, but if you could get the same information in a more physiological context and bound to its molecular partners, that information would be much more incisive in making choices about which drugs might work.”

Illuminating the Future

“We are fortunate to work in an institute that supports not just the application, but also the development of new technologies,” said Misteli. “Given the large

investments involved, very few academic institutions have developed the imaging resources we now use routinely.”

Loncarek noted that the investment is not only in equipment, but also in personnel time. “We combine biochemistry with high-end imaging. We spend a lot of time training to become experts in both.”

Ultimately, the investment pays off many times over, as scientific progress is intimately tied to technology development. For example, demand for access to the

Center for Molecular Microscopy, which builds on technologies developed in Subramaniam’s laboratory, already exceeds capacity. “The center is a way for other CCR investigators to benefit from the investment made in my program,” said Subramaniam.

To learn more about Dr. Misteli’s research, please visit his CCR website at <http://1.usa.gov/1NdIq0T>.

To learn more about Dr. Loncarek’s research, please visit her CCR website at <http://1.usa.gov/1Nrjw3>.

To learn more about Dr. Subramaniam’s research, please visit his lab’s website at <http://electron.nci.nih.gov>.

Center for Molecular Microscopy

Ten years ago, Sriram Subramaniam, Ph.D., described four classes of problems that could be solved by advances in imaging:

1. Is it possible to distinguish cell architectures, for example in cancer, by quickly determining and comparing three-dimensional structures of whole cells?
2. How do we describe how receptors and signaling assemblies change in a concerted way, in response to external signals?
3. Can we determine the structures of proteins and other entities, such as HIV, that cannot be crystallized?
4. Why can’t we just look at the structure of a protein in different conformations?

Over the next 10 years, his laboratory implemented new approaches, including focused ion beam scanning electron microscopy,

whole-cell tomography, and cryo-electron microscopy, to address these questions.

“Two years ago, I saw an inflection point in the field where some of the techniques we were developing would go from niche applications in specialized laboratories to being more widespread. The Center for Molecular Microscopy (CMM) is an experiment to transition technology from my laboratory into a collaborative environment, which lets others within CCR experience and eventually adopt the technology,” said Subramaniam.

CMM is staffed with dedicated scientists, many of whom trained with Subramaniam, who work with CCR scientists on their research questions.

“It’s not like a core facility with a confocal microscope or a gene sequencer. Its goal is to enhance the

research that others are doing on interesting biological problems by providing a unique technological perspective,” he explained.

Proof that this approach can yield rich dividends is already evident. Two publications recently resulted from collaborations between CMM staff and research groups at the NIH: the three-dimensional structure of mitochondrial networks in muscle was published in the July 30, 2015, issue of *Nature*, and the structure of bacterial spores was published in the April 9, 2015, issue of *Nature Communications*.

To learn more about the Center for Molecular Microscopy, please visit its website at <https://cmm.nci.nih.gov>.