Seeing the Invisible:

Understanding Structure to Understand Disease

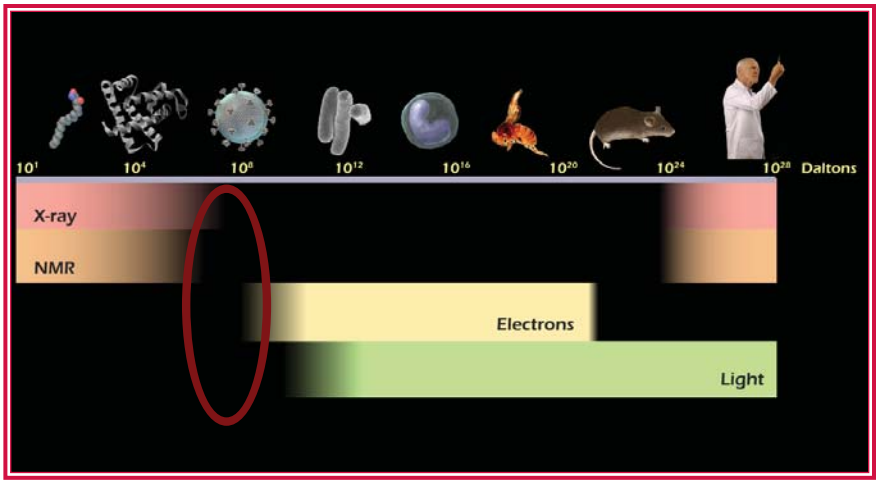
Vision is the art of seeing what is invisible to others. – Jonathan Swift

The submicroscopic world is yielding its secrets as new technologies probe further into its depths. "Seeing" this world is more than an exercise in intellectual curiosity: it is critical to understanding basic biology and the diseases that are rooted in subcellular space. Scientists in the Biophysics Section and Unit, in CCR's Laboratory of Cell Biology, are developing technologies with ever keener eyesight, capable of offering amazing glimpses into the nanometer-sized world of macromolecular complexes. These efforts go beyond simple technological advances, drawing on the collaborative expertise of biochemists, geneticists, physiologists, and computational modelers both at NCI and in the external scientific community.

Bridging the Gap

"Although genetics, physiology, and biochemistry are important tools for understanding and predicting the behavior of living cells and small organisms, we need to understand structure to complete the picture," said Sriram Subramaniam, Ph.D., Head of the Biophysics Section. "Our goal is to interpret cells at the level of molecular resolution."

Ever since Anton von Leeuwenhoek first saw bacteria through his finely ground glass lenses in 1676, visual technologies have steadily improved to the point of seeing even small viruses and cellular organelles. At the same time, X-ray diffraction (aka X-ray crystallography) and nuclear magnetic resonance have allowed us to "see" the structure of individual molecules, even ones with fairly complex structures. Yet there remains a significant—and critical—visual gap between individual molecules and subcellular organelles and organisms (Figure 1). This gap is where the action happens (i.e., the space where viruses perform their destructive function or where disease breaks down normal biological processes). And it is precisely on this gap where Subramaniam and his colleagues have trained their technological sights.



(*Image: Sriram Subramaniam*, CCR)

Figure 1: The Subramaniam laboratory aims to illuminate the gap between X-rays and electrons, where chemistry becomes biology.

The laboratory has woven studies in three interrelated, yet diverse areas—bacterial chemotaxis, HIV structure and infection, and development of new technologies for cancer imaging—in "a combination which you could only do at CCR," said Subramaniam. These studies are providing the basis for pursuing an entirely new way to understand and treat many different diseases, including HIV and cancer.

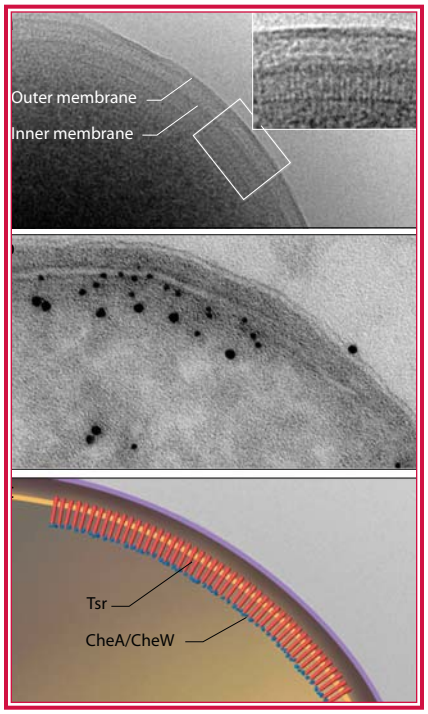
What the three share in common is a combination of powerful new software algorithms married to ever more precise electron microscopy (EM) techniques (see "Penetrating Vision: The Next Generation of Observational Tools").

Bacterial Chemotaxis: How Do Cells Respond to Their Environment?

Despite their small size, bacteria are not passive participants in their environmental niche. Motility is essential to their survival, as they need to sense both the good stuff (e.g., food) and the bad stuff (e.g., harmful chemicals) and move appropriately, a behavioral trait known as chemotaxis. This process is mediated by the binding of external molecules to so-called bacterial "chemotaxis receptors," which in turn set off an internal signaling pathway of molecules that ultimately revs the motors that drive the bacterial flagella. Understanding the spatial and temporal structure of chemotaxis is important not just for the sake of appreciating bacterial movement, but also because cellular sensing of environmental cues is a common evolutionary trait, from bacteria through human immune cells. Joining structural information to what is known about the genetics, physiology, and biochemistry of bacterial chemotaxis apparati should reveal basic principles about the machinery of cell signaling.

(*Photo: Feinstein Kean Healthcare*)

Collaborations like those between Subramaniam and Jacqueline Milne, Ph.D. (left), are helping researchers develop the complex visualization and computation tools to solve "simple" structural problems like how surface receptors physically transduce external signals to a cell's chemotactic machinery.



(*Image: Sriram Subramaniam*, CCR)

Figure 2: The physical relationships of the *E. coli* proteins CheA and CheW—which help drive the bacterium's chemotactic response—were only recently brought to light through the use of electron microscopy techniques developed in the Subramaniam laboratory. Tsr is an additional component of the *E. coli* chemotaxis mechanisim.

A great deal of work has been done in understanding the structures of individual components of the chemotaxis machine in the common bacteria *Escherichia coli* (*E. coli*), but how they assemble and work has been a mystery. Subramaniam and his colleagues have employed the visualization technology they have developed to make amazing progress in revealing the chemotactic machinery's structure in intact *E. coli*. A series of manuscripts over the past two years, the most recent published in the March 6, 2007, issue of the *Proceedings of the National Academy of Sciences*, describes a cluster of receptors in an extended lattice that is dependent on the interactions of two critical *E. coli* signaling proteins, CheA and CheW (Figure 2).

Working together with Jacqueline Milne, Ph.D., in CCR's Laboratory of Cell Biology, the scientists are now focusing their attention on looking at how these complexes transduce environmental signals at the level of molecular structures (i.e., how the individual receptors work in the context of their location within the entire chemotaxis structure). This "simple" structural problem requires continual technological improvement in visualization and computational tools, as well as ongoing collaboration of the computational modelers and technologists with the biochemists and physiologists within and outside of the laboratory.

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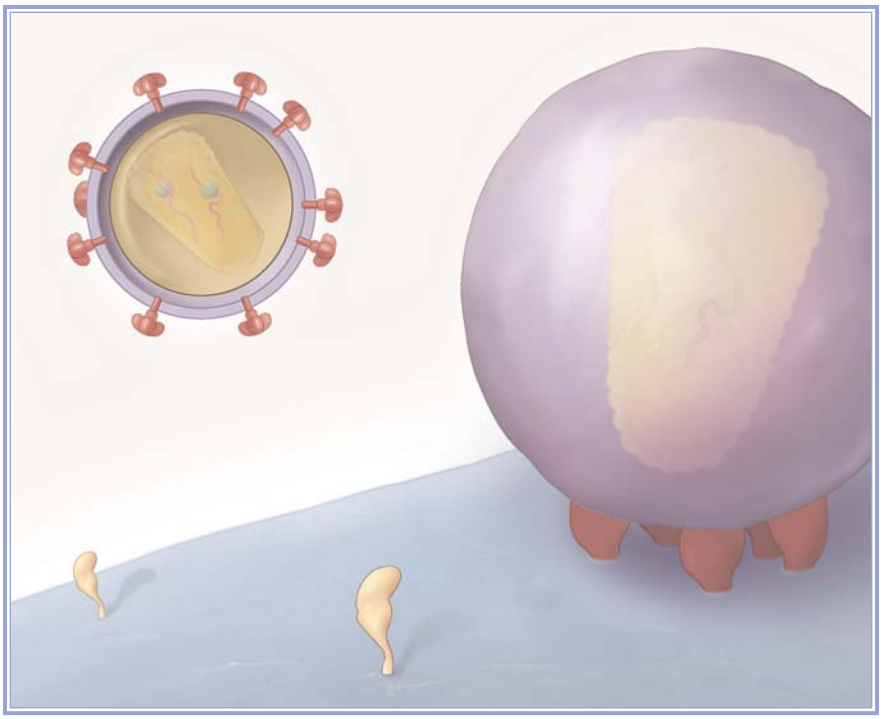
A Good Look at a Viral Foe

HIV's devastating ability to evade the immune system's attempts to control or remove it is primarily linked to its structure and how it interacts with its host's cells. This trait is largely a function of one specific HIV protein (gp120) interacting with a specific target cell receptor (CD4), with subsequent viral and host proteins getting involved to allow infection following the initial binding. It is known that some antibodies and certain drugs can interrupt this binding, but not always effectively. Understanding the actual structure of this interaction (and the subsequent series of events) could lead to more effective ways to prevent it.

In the fall of 2004, the CCR scientists began research aimed at visualizing the series of structures involved in HIV infection. Their interests included understanding the actual conformation and distribution of the gp120 proteins on the viral surface, the interaction of different kinds of antibodies with those proteins, the binding of gp120 to CD4, and the functional structure of the infection machinery. Over the past year, research into each of these issues has begun to yield new—and surprising—information about HIV and its molecular interchange with its host target cell.

One particularly striking recent finding is the actual physical structure of the viral coat proteins. Although there are atomic models of gp120 in both its CD4-bound and -unbound states, there is still significant uncertainty about what the actual surface proteins look like in their native state and how they are distributed around the viral coat. It is thought that the gp120 spikes on the viral coat are composed of three gp120 molecules in a "trimer" formation. However, retroviruses such as HIV are more heterogeneous in their structure than other virus types, which undoubtedly contributes to their success at eluding immune responses. Thus, it is critical to uncover the actual structure, both for the sake of developing potential new and effective drugs and for understanding the immune responses to HIV that need to be elicited by a vaccine.



(*Image: Sriram Subramaniam*, CCR)

Figure 3: The HIV "entry claw," a unique structure the virus uses to grasp cells it seeks to infect. The claw was discovered using 3D software tools for electron tomography developed in the Subramaniam lab.

To address this question, Subramaniam and his colleagues first developed new computational tools that can rigorously classify three-dimensional structure data derived from electron cryo-tomography techniques. Bringing these tools to bear on the viral coat proteins, the scientists have found a unique feature—an "entry claw"—that HIV uses to grasp onto and infect its host (Figure 3). These findings, published in the May, 2007, issue of PLoS *Pathogens*, open up new pathways to understanding HIV and thus will have a major impact on future drug and vaccine discovery efforts.

Ongoing work on HIV-host interactions is focused on the molecular machinery by which HIV actually gains access to the cell, how neutralizing antibodies exert their effects, and the structural mechanisms of action of current experimental HIV drugs that are believed to inhibit viral entry.

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Building on the Foundation

Although the Subramaniam laboratory chose bacterial chemotaxis and HIV structure as the initial problems to focus on in concert with developing the necessary tools,

the goal is the refinement and application of these powerful new techniques to many areas of biology and disease research. Current projects in the lab include cancer cell imaging at resolutions approaching 20 nm (about 15 times better than can be achieved with current confocal techniques), structural analysis of the mechanism of melanosome transfer in the skin (which is related to the development of skin cancer), nanoparticle detection and 3D tissue localization, building further automation into EM workflow (thereby allowing relatively high-throughput of samples and collection and interpretation of data), and the adaptation of these techniques for use in pre-clinical and clinical diagnostics. In one exciting recent advance, the lab has developed techniques for imaging entire cancer cells and visualizing how various internal organelles such as mitochondria are organized—a major step toward defining some of the structural hallmarks of cancer at the cellular level.

Although the implications for clinical application of these technological advances are astounding, at heart this is still a basic life sciences laboratory interested in answering fundamental questions about biology and disease. To that end, the Subramaniam lab is comprised of about 15 scientists—about half of whom are students—with a diversity of experience and expertise, including electrical engineering, physics, and biochemistry (see "Laboratory Visionaries"). These fields would not interact regularly in any other setting, but within Subramaniam's domain, they work well together. "I believe that integrating all these expertises is the only way to do this kind of research successfully," said Subramaniam, who himself has experience in very different scientific areas. "Only at a place like CCR can you build something like this group of people and give them the freedom to produce great things."

For more information and astounding peeks at the submicroscopic world, visit the lab's Web site: http://electron.nci.nih.gov.

Penetrating Vision:

The Next Generation of Observational Tools

Electron Cryo-Tomography

Although the first electron microscope (EM) was developed in 1931 by Max Knoll and Ernst Ruska at the Technical College in Berlin, it was not until 1968 that it became feasible to generate three-dimensional (EM) images, a technique known now as electron tomography. Such images are created by taking a single EM image of the specimen being studied, and then tilting it slightly to take another image. A series of these images can then be recombined into a three-dimensional structure using software designed specifically to handle such image data.

Initial attempts to image native biological specimens, uncompromised by fixation procedures, were not successful. This was largely because the harsh environment of the EM chamber (high vacuum and electron beam bombardment) was too much for fragile cells, which were usually torn apart before they could be imaged. Improvements in cryo-preservation techniques in the 1980s finally allowed some cells to survive the EM chamber long enough to provide useful images. This technique, known as electron cryo-tomography, was a breakthrough in imaging cellular structures in their native form.

Constant improvements in both the technology (cryo-preservation methods, low-dose electron beams, and more sensitive electron detectors/cameras) and in the analytical software that reconstructs the images into a 3-D model are pushing the resolving capabilities of electron cryo-tomography ever smaller, filling the gap between biophysical methods and more traditional electron microscopy.

Ion-Abrasion Scanning Electron Microscopy ("Dual Beam")

The National Library of Medicine's "Visible Human Project" is a computer-assisted virtual 3-D atlas of the human body. The Project makes it possible to examine a real body, either male or female, from almost any angle, in three dimensions. The data that feed this tool are a series of images taken of a donor body that was sliced from the top, a millimeter at a time (or less, for the female body), with a picture taken at each slice.

Ion-abrasion scanning electron microscopy (IA-SEM), called "dual beam" in laboratory shorthand, is based on the same basic principle as the Visible Human Project, though on a much smaller scale: In this case, the slicing is done by an electron beam followed by a scanning electron photograph after each slice. Described last July by Subramaniam and his colleagues in the *Journal of Structural Biology*, IA-SEM offers the resolution of subcellular organelles an order of magnitude greater than can be achieved by other techniques. In addition, it works on whole specimens, eliminating the need for generating thin sections of the specimen to use traditional EM approaches.

The FEI Company, a long-time maker and supplier of electron microscopy and related products, is collaborating with the Subramaniam lab in developing this new technology.

Laboratory Visionaries

(Photo: Rhoda Baer Photography)

Sriram Subramaniam, Ph.D., Senior Investigator

When Sriram Subramaniam came to CCR in 2000, NCI was actively looking to build its capabilities in high resolution imaging at scales that had not yet been attainable. Although he had experience in studying the structure of membrane proteins, including rhodopsin, Subramaniam did not have a great deal of experience in electron microscopy. "CCR took a huge risk," he said. "I was learning even as I was building these capabilities." By all accounts, that risk is paying off.

Subramaniam completed his doctorate at Stanford University in 1987, followed by postdoctoral work in the laboratory of H. Ghobind Khorana, Ph.D., at MIT. He then joined the faculty at the Johns Hopkins School of Medicine (where he is still a visiting associate professor) prior to coming to CCR—and back to bench work. "I never wanted to be a manager; I need to stay close to the science," said Subramaniam. "Other places I was writing grants while everyone else did the science. Here I can be at the bench, as well as mentor some amazingly talented students who will drive the future applications of this work."

Subramaniam also notes that the work in his laboratory has benefited immensely from the close collaboration with Jacqueline Milne, Ph.D., who has pioneered work on studying large multiprotein assemblies using electron cryo-tomography. "CCR has been very supportive of this type of team science approach that has allowed us to focus intensively on difficult problems requiring interdisciplinary approaches," he said.

(Photo: Feinstein Kean Healthcare)

Adam Bennett, Graduate Student

Adam Bennett joined CCR's Biophysics Section in the fall of 2005 as an Oxford-Cambridge student in the NIH-University of Cambridge Graduate Partnerships Program (gpp.nih.gov). He earned a B.S. in chemistry from the University of Florida in 2004, and then went to the University of Cambridge (UK) on a Churchill Scholarship.

"From the age of ten, I was going to be an organic chemist," said Bennett, and his chemistry work focused on making optically pure drugs using enzymatic biological systems. However, he grew excited by the potential for the techniques being developed in the Subramaniam laboratory to define the molecular architecture and mechanisms of such systems.

Among his current projects is visualizing the mechanistic and structural basis of endosomal HIV-1 budding from macrophages.

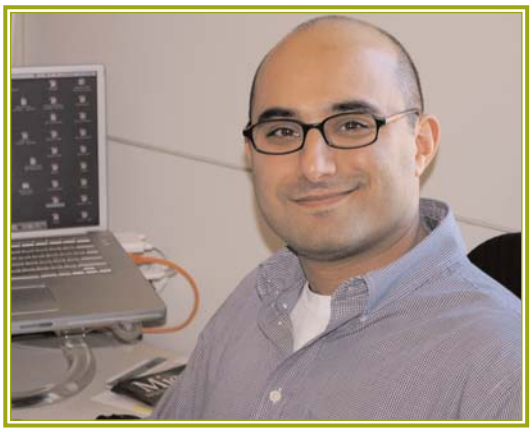
(Photo: Feinstein Kean Healthcare)

Sang Kim, Post-Baccalaureate Student

Sang Kim also joined CCR in the fall of 2005, after receiving a B.S. from the University of Maryland. His ultimate goal is medical school, but he decided to enroll in the two-year NIH post-baccalaureate program to gain greater insight into scientific method. "Medicine is not a static field," said Kim. "There are constant advances. I believe what I learn here will prepare me to understand and 'keep up' in the future."

Kim has been happily surprised by the amount and kind of work he is doing. He was particularly excited to get regular access to the new dual-beam electron microscope to perform important cancer-cell imaging studies. "They train you to become independent pretty quickly and encourage you to take the initiative," he said. "I think I've received a pretty good glimpse of how science works at its best."

Although leaving for medical school in the fall of 2007, Kim decided to forego the usual practice of taking the summer off before starting medical school: He stayed in the lab to get as much done as possible.



(Photo: Feinstein Kean Healthcare)

Cezar Khursigara, Ph.D., Postdoctoral Fellow

Cezar Khursigara did his doctoral work on *E. coli* membrane proteins using 3-D crystallography and biophysical techniques at McGill University in Montreal, Canada, and came to CCR looking for a different approach to bacterial proteins for his postdoctoral training. He had met Subramaniam at a Federation of American Societies for Experimental Biology (FASEB) meeting about eight months before he finished his degree and recalls being excited about the potential of electron cryo-tomography to elucidate the bacterial chemotaxis machinery. He contacted Subramaniam once he began his hunt for post-doctoral positions. "He remembered me, invited me to CCR to give a talk, and the rest is history," he said.

Since joining CCR, Khursigara has worked on a diverse collection of projects concerned with the structure of chemotaxis receptors and signaling complexes, including more biochemical and molecular approaches in addition to the cryo-tomography work. He is particularly struck by the diverse experience and expertise of the lab. "Sriram isn't big on position and title," said Khursigara. "If you have drive and ability, and he can provide the tools and environment to match it, then his attitude is, 'let's do this.'"