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Paris, France
July 2-5, 2006

By Geoffrey Montgomery

Paths to complexity

"The basic question is: how does matter become complex?" said Nobel Laureate Jean-Marie Lehn (Université Louis Pasteur, Strasbourg, France) in the opening plenary lecture of the Sixth Human Frontier Science Program Awardees Meeting, held at the legendary Institut Pasteur in Paris on July 3-5, 2006. "How is it that the universe has generated organisms, including organisms that can ask the questions we are asking here at this meeting? Of course the answer to this question is self-organization. That's obvious. But 'self-organization' does not tell us how it happens. Science is not just about giving a word to a process. It is about explaining what this process is." Understanding the self-organizing "paths to complexity" operating within living cells, said Lehn, "is the ultimate frontier for the Human Frontier Science Program", which supports international research collaborations across traditional disciplinary boundaries.

This report reviews a representative sample of HFSP awardee presentations from the Paris meeting that provided new insights into biological complexity and self-organization across the spectrum of the life sciences:

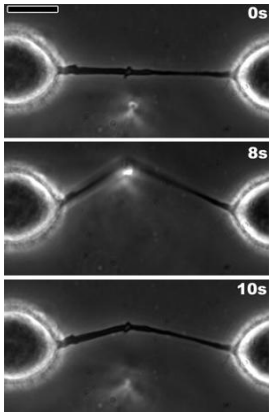
- the self-organizing cytoskeletal assemblies that enable complex cells to divide;
- the morphogen molecules that shape developing embryos and which biomedical researchers are trying to harness for regenerative medicine;
- the molecular genetic intricacies by which plants organize their growth patterns in response to light;
- a new molecular foundation for understanding how certain organisms' movements are guided by the earth's magnetic field; crucial new molecular connections between the morphogenesis of mitochondria and the 'self-deorganizing' process of programmed cell death;
- sensitive new methods for visualizing protein interactions in living cells;
- new perspectives on the functional architecture and self-organizing powers of the neuronal synapses
- and the assembly and infection process of a paradigmatic model system in molecular biology, the T4 bacteriophage, in an emerging textbook example of what Lehn has called "supramolecular chemistry, a broad new area at the interface of chemistry and biology, involving self-organization processes that make use of molecular recognition to control and direct the spontaneous formation of functional architectures of higher complexity."

The Paris meeting, which commenced with welcoming remarks from the President of the Institut Pasteur, Alice Dautry, who originally studied physics, also featured scientific discussions that ranged beyond the normal disciplinary boundaries of the lecture hall, including: an early evening wine and cheese poster session on Monday; a Tuesday dinner cruise on the Seine in glorious early summer weather; and a Wednesday farewell banquet in the regal rooms of the French Senate, where renowned neurobiologist Jean-Pierre Changeux gave a special after-dinner talk on the relationship between art and the functional organization of the human brain, amidst occasional cheers and gasps from the city outside as France played Portugal in the World Cup semi-finals.

Unravelling the Dynamic Complexity of Cell Division

The exquisitely choreographed stages of cell division, centering on the process by which sister chromosomes are pulled apart by spindle fibers into two daughter cells, have entranced biologists since the late 19th century. Molecular analysis has shown these mitotic spindle fibers to be composed of microtubules whose tubulin subunits assemble and disassemble at each cell division, and which are associated with many different proteins such as kinesin motors. "The mitotic spindle can be described as a self-organizing system," said Tarun Kapoor (The Rockefeller University, New York, USA), "in which multiple agents"—in this case protein molecules---"follow behavioral rules locally, based on local information, rather than following some master plan or leader." The first two scientific presentations of the Paris meeting provided striking evidence of just how remarkable the self-organizing behavior of the spindle's multiple molecular agents may be.

In the first talk, **Michel Bornens** (Institut Curie-CNRS, Paris) described his grant team's ongoing exploration of a startling discovery first reported by the Bornens laboratory in 2001 (Science 291:1550-3) involving the role of centrioles in controlling the final stages of cell division (cytokinesis). "The main goal of our project is to provide a quantitative description of how mechanical constraints are integrated during late cytokinesis in mammalian cells to allow daughter cells to physically separate from each other and to resume cell cycle progression." During the final stages of cell division, the two daughter cells remain tethered by an "intercellular bridge" of cytoplasm containing the remains of spindle microtubules.

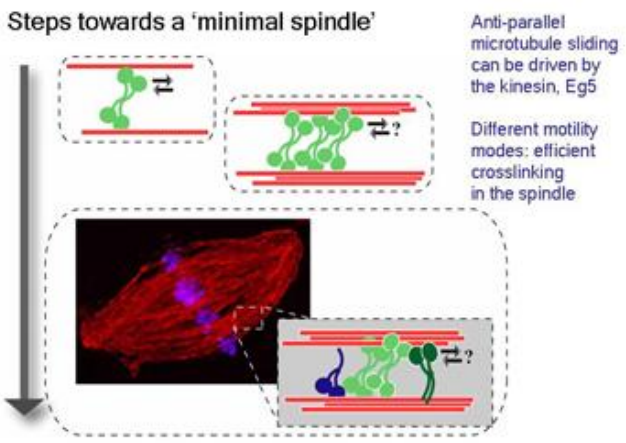


Working in cultured cells, the Bornens laboratory found that a centriole from one daughter cell (normally situated within the microtubule-organizing centrosome, which itself lies at one pole of the bipolar spindle) moves into this intercellular bridge, causing it to narrow. When the centriole moves back into the cell body, this intercellular bridge breaks, completing cytokinesis. If the centrioles in one daughter cell are laser-ablated, the intercellular bridge remains suspended for up to ten hours, only to snap back into its sister cell to make an abnormal fused cell with two nuclei. This strange migratory behavior of the centriole may act as a "checkpoint" at the final stages of cell division, analogous to earlier, well-characterized checkpoints monitoring the fidelity of DNA replication and spindle-attachment to chromosomes. "Our working model is that the centriole-dependent exit of cytokinesis allows animal cells to integrate spatial information"—about the presence of neighboring cells and the adhesive quality of its environment—"into the decision of whether or not to complete cell division."

Because adhesion appears to be a critical parameter for this decision, Bornens' team adapted micro-contact print techniques to imprint different patterns of fibronectin on glass slides and then monitor cell behavior by phase-contrast and epifluorescence video microscopy. This led to another surprising discovery about the early nature of mitosis: the pattern of the fibronectin extracellular matrix critically influenced the orientation of the cell division axis (*Nature Cell Biology* 7: 947-53 (2005)). Bornens also described his team's analysis of the mechanical properties of the intercellular bridge, as well as their formulation of a working physical model for the forces controlling spindle orientation and the cell division axis.

"We are examining the micromechanics of the cell division apparatus, with a focus on how the mitotic spindle assembles, and how our cells segregate chromosomes without making any mistakes," said Grant Team primary investigator **Tarun Kapoor** during the Paris meeting's second talk. Kapoor showed Fluorescent Speckle Microscopy movies of spindle dynamics during mitosis, which showed a bidirectional flow of labeled tubulin subunits at the local level. "So the question is, maybe what we are seeing is the movement of two microtubule filaments [sliding] relative to each other" in the bipolar spindle. Kapoor's team has chosen to "approach the complex problem" of cell division micromechanics by a "simplification strategy of using purified components to see if we can recapitulate in the test tube what we see using these high-resolution imaging methods." As one central molecular component, Kapoor focused on a kinesin motor protein called Eg5. Mitotic spindles fail to form if Eg5 is inhibited by the drug Monastrol or RNAi; moreover, Eg5 has a "very curious structure." Unlike classical kinesin motors, which have only two heads attached to a stalk-like region, Eg5 has two heads on each end of this stalk, forming a dumbbell-like structure that could be well-adapted to binding simultaneously to two different microtubules of opposite orientations in the bipolar spindle.

Reconstituting bi-directional microtubule movement *in vitro* presented a number of challenges. To begin with, the two microtubules that needed to slide past each other were chemically identical. Team member **Christof Schmidt**, a physicist, now at the University of Goettingen in Germany, developed methods for treating microtubules with finely tuned hydrophobic polymer brushes that enabled one microtubule to stick to a surface and the other to be bound only when a Eg5 motor was present; the two microtubules could be differentially visualized by red and green fluorescent protein. The team also controlled microtubule orientation so that a red and green microtubule, coupled by an Eg5 motor, had their plus-ends pointed in opposite directions, as in the bipolar mitotic spindle. Using this simplified system, they discovered that "Eg5 has the remarkable capability of simultaneously moving at ~20nm sec^{-1} towards the plus-ends of each of the two microtubules it crosslinks. For anti-parallel microtubules, this results in relative sliding at ~40 nm sec^{-1}, comparable to spindle pole separation rates *in vivo"* (*Nature* 435: 114-8 (2005). Moreover, they discovered strong evidence of a second mode of diffusive motion, not dependent on ATP, which may be responsible for "recruiting microtubules into bundles" that can then be slid apart by the ATP-dependent processive "walking" of the Eg5 motor.



"We have recapitulated one aspect of the very complex dynamics of this mitotic spindle, which is essentially an organizational state of the whole cytoplasm, and holds thousands of proteins," Kapoor concluded as he showed a slide entitled 'Steps towards a 'minimal spindle'. "The goal now is to build the complexity of the system, adding more microtubules, and measuring forces [using such physical methods as optical tweezers], and then mixing in other motor molecules that we can purify and that are known to be involved in the function and assembly of the bipolar

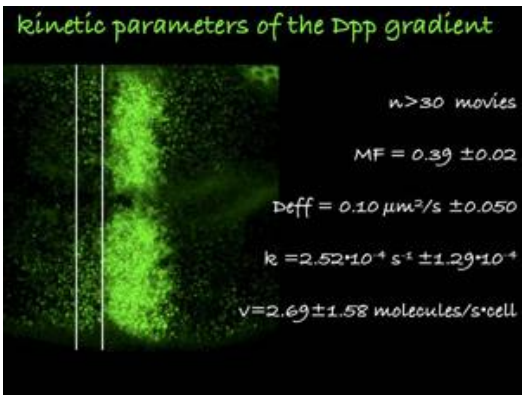
mitotic spindle. And we are especially curious to understand how you would regulate mechanically or post-translationally the switching between" Eg5's diffusive cross-linking of microtubule filaments and its processive walking along them.

Analyzing the Movement of "Form-Generating Substances"

Over the past decade, the developing wing of the Drosophila fruit fly has become one of biology's premier systems for studying the inner workings of morphogens, the "form-generating substances" postulated in 1952 by the brilliant mathematician Alan Turing. In his 1969 "French Flag Model," Lewis Wolpert elaborated upon the morphogen concept, proposing that morphogens act by diffusing from a source in the embryo to form a long-range concentration gradient. Cells then read their relative position along this morphogen gradient by expressing different genes at discrete concentration thresholds. In development, Wolpert said, "the genome provides the choices, but position is the chooser."

"The question we have been addressing, " said Grant Team leader **Marcos González-Gaitán** (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany; now at the University of Geneva), "is how a morphogen actually moves from the source into the target tissue to form a gradient that is stable and robust." Studies by González-Gaitán's laboratory have indicated that the movement of the classic Dpp morphogenen in the Drosophila wing cannot be accounted for by simple extracellular diffusion. Instead, it seems that Dpp protein is internalized by a receiving cell and then re-secreted through a Dynamin-dependent endocytic pathway. Normally, Dpp forms a long-range gradient extending some 30 cell diameters; but if target cells are mutant for the Dynamin protein, this gradient extends to only 2 cells. "To understand the relative contribution of diffusion and endocytosis (also called "planar transcytosis") in forming the Dpp morphogen gradient", said González-Gaitán, "we need to have high-resolution quantitative data" in developmental space and time.

In particular, González-Gaitán described experiments using FRAP (Fluorescent Recovery After Photobleaching) to measure the four key parameters of the Dpp gradient, which his lab has nick-named "the Tetrad: the diffusion coefficent [of Dpp protein], degradation rate, production rate, and mobile fraction." (recently published in Science 315: 521-5, 2007) In FRAP, green fluorescent-labeled Dpp protein is bleached by a laser in a small area of target tissue; as unbleached Dpp diffuses into this area, the fluorescence signals "recover;" the greater the diffusion coefficient, for instance, the faster the recovery rate. González-Gaitán showed how FRAP movies have allowed quite reliable measurement of the four kinetic parameters of the Dpp gradient.



These values "do not reveal much about the molecular and cellular mechanisms of Dpp gradient formation in the system, but the FRAP set-up serves as a quantitative assay that allows us to use mutants and drugs" to target different possible components making up the Dpp gradient. As a first step, for instance, Gonzalez-Gaitin showed results of FRAP experiments utilizing a temperature-sensitive Dynamin allele, which

arrests the spread of Dpp across the target field, implying that endocytosis is indeed required for Dpp morphogen formation.

However, González-Gaitán also showed FRAP experiments involving the Wingless morphogen which indicate that dynamin-dependent endocytosis is not essential for the spreading of this morphogen. Other members of the Grant team are using sophisticated image-analysis to examine gradients of the zebrafish-equivalent of the Dpp morphogen, which seems to behave quite differently from its homologue in flies. Team Member **Sharad Ramanathan** (Harvard University, USA**),** a physicist, is using "fluctuation theory" to quantify morphogen diffusion in zebrafish embryos, the same fluctuation theory that Einstein applied to Brownian motion in 1905 to prove the existence of atoms. "What we see is quite different from what Marco is seeing in Drosophila," said Ramanathan in a conversation. It seems that nature has used the same basic morphogen molecule in different ways during animal evolution. "Systems biology [involves] quantitative measurement, modeling, reconstruction, and theory," the noted cell biologist Marc Kirschner wrote recently (*Cell* 121: 503-4 (2005)). Yet "systems biology is not a branch of physics but differs from physics in that the primary task is to understand how biology generates variation. No such imperative to create variation exists in the physical world. It is a new principle that Darwin understood and upon which all life hinges."

Following Light's Path in Plants

Some eighty-five years ago, biologists discovered that seasonal changes in day-length trigger flowering in many plants. In 1936, Erwin Bünning hypothesized that plants use an oscillating circadian clock to measure day-length time. Two presentations at the Paris meeting offered new insights into the molecular nature of this convergence between light signals and circadian oscillators, as well as into the fundamental puzzle of how light signals control gene expression.

Young Investigator Grant Team member **Julin Maloof** (University of California, Davis, USA) showed how the tips of dark-reared seedlings snake upwards instead of budding petals. **"**Seedlings that are growing in the dark become very elongated, because the plant believes that it is underneath the soil surface and it needs to find the soil surface to begin photosynthetic growth." Conversely, light in the red and far-red portion of the spectrum inhibit seedling elongation. Molecular genetic studies of the *Arabidopsis* mustard-weed have shown that plants use two photoreceptors, phytochromeA (phyA) to sense red and phyB to sense far-red light. Maloof described his team's discovery of two genes which apparently act to coordinate light signals and a plant's circadian clock. The plant's internal circadian clock, intensively studied in recent years, controls the transcription of these two genes, while light signals mediated by phyA and phyB degrade their encoded proteins. Maloof also described the team's investigation of natural allelic variations in phytochrome genes which might underlie how plants adapt to different light environments.

A plant "knows" that it is being shaded by other plants, for instance, when its phytochrome signaling pathway detects a low ratio of red to far-red light, explained HFSP Long-Term Fellow **Andreas Hiltbrunner** (Institut für Biologie II/Botanik, Freiburg, Germany). Indeed, microarray analysis has shown that 17% of the genes in the *Arabidopsis* genome are regulated by red and far-red light signals; both phyA and phyB phytochromes move to the nucleus upon exposure to light. Yet both phytochromes lack nuclear localization signals; how then is their activity transmitted

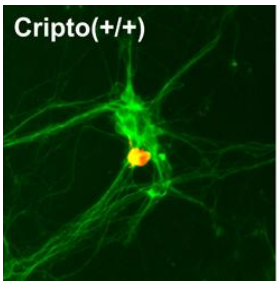
to the genome? Hiltbrunner focused his attention on two phytochrome-signalling mutants, *fhy-1* and *FHL.* He showed that "fhy-1 FHL RNAi knock-down seedlings are insensitive to far-red light," equivalent in phenotype to *phyA* null mutants, thus indicating that in their absence phyA phytochrome function is abolished. Hiltbrunner discovered that both fhy-1 and FHL physically interact with the phyA phytochrome in a light-dependent fashion; this interaction depends on a highly homologous 36 amino acid C-terminal domain on the FHY1 and FHL proteins, which also possess a nuclear localization signal; upon light exposure, all three molecules co-localize to mysterious nuclear structures called "nuclear speckles. "We therefore identify the first component[s] required for light-regulated phytochrome nuclear accumulation," Hiltbrunner concluded in a paper in *Current Biology*. (15: 2125-30 (2005); see *Plant and Cell Physiology* August 2006 issue for FHL studies).

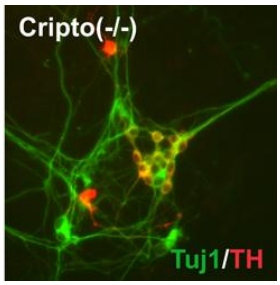
Harnessing Basic Biology to Improve Possible Embryonic Stem Cell Therapies for Parkinson's Disease

"Stem cells are very much in the news for both political and scientific reasons," Vincenzo Pirrotta and Maarten van Lohuizen write recently in *Current Opinion in Genetics and Development (*16: 443-5 (2006)). "....What are the mechanisms that maintain [stem cell] pluripotency, and what happens as stem cells differentiate are questions that involve key issues in development, gene regulation, and genome structure and organization in the nucleus." These fundamental questions must be addressed, these researchers write, if stem cells are to realize their "much touted but somewhat vaguely glimpsed potential to become extraordinarily valuable tools for therapeutic purposes." On the second morning of the Paris meeting, Long-Term Fellow **Clare Parish (**Karolinksa Institute, Sweden) reported significant progress towards this goal.

Parish explained that the two primary features that make embryonic stem cells so well-suited for cell replacement therapies are also their major limitation. Embryonic stem cells self-renew; yet this same unlimited proliferative potential requires "that we be able to regulate the growth of this tissue in order to prevent tumor formation." Embryonic stem cells have the ability to differentiate into multiple lineages; yet this requires that "we be able to specify their differentiation so that they generate a select population of cells."

Parish described her efforts to use embryonic stem cells to generate dopaminergic neurons equivalent to those that degenerate in the midbrain of patients with Parkinson's disease. In order both to prevent tumor formation and spur dopaminergic neural differentiation, she has focused her attention on a protein called Cripto, a co-receptor in the Nodal signaling pathway and a key regulator of neural induction. Mice lacking the Cripto gene die during gastrulation, with a high proportion of their cells transformed to anterior neuroectoderm. Moreover, Cripto is overexpressed in a many types of cancer, and antibodies against Cripto protein can suppress tumor growth. These and other studies suggested to Parish and her collaborators that Cripto protein activity may function in normal embryonic cells to repress neural differentiation and promote proliferation; could these functions be reversed if the Cripto gene was knocked out of embryonic stem cells used for Parkinson's disease cell replacement therapy?



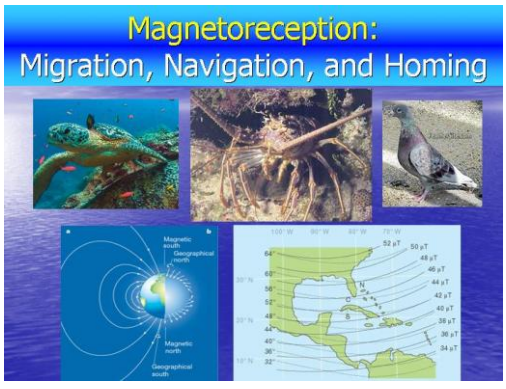


Parish showed that, in rat cell culture, some 70% of Cripto null (-/-) embryonic cells differentiate into cells that express neural markers; this percentage could be increased to 100%--include a significant proportion "that showed the classic bipolar morphology of midbrain dopaminergic cells"--if she added two morphogens (FGF8 and Shh) which normally act in the embryonic ventral midbrain to create dopaminergic cells.

She then tested the therapeutic potential of these cells in rats with a Parkinson's-like syndrome induced by depletion of dopaminergic midbrain cells. The rats displayed significant anatomical and behavioral recovery. Moreover, none of her Cripto null (-/-) grafts yielded tumors. "One of the greatest tribulations when you're working with embryonic stem cells is the risk of tumor formation, and this was one of the most interesting findings in our study," said Parish. "Within our wild-type [Cripto (+/+)] grafts we saw 100 percent tumor formation. And there were times when these tumors were quite small, but in other cases they extended all the way through forebrain, midbrain and hindbrain." As Parish concluded in *Stem Cells* (23: 471-76 (2005)): "The use of knockout ES [embryonic stem] cells that can generate dopamine cells while eliminating tumor risk holds enormous potential for cell replacement therapy in Parkinson's disease."

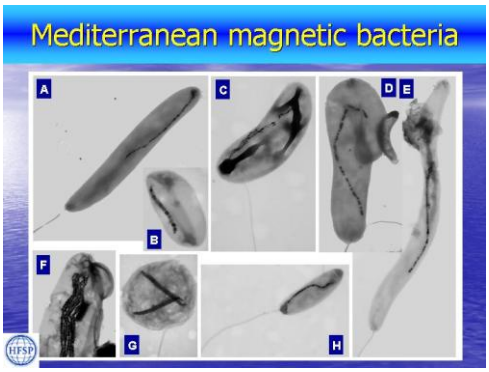
More recently, Parish said, she has used more sophisticated protocols, in which embryonic stem cells are exposed to various morphogens at different time points, to increase the yield of dopaminergic cells and induce full behavioral recovery in Parkinson's rats eight weeks after transplantation. "But unfortunately we also began to see tumor formation," said Parish. "So we can see there is a fine line between optimal behavioral recovery and tumor formation, and this will require teasing apart." In a conversation over coffee after her talk, Parish said that she planned to continue this "teasing apart" of the molecular factors needed to safely control stem cell differentiation when she returns to her native Australia. She would like to investigate, for instance, whether one could modulate "signaling pathways to push axonal guidance in these dopaminergic cells."

New Molecular Insights into Magnetoreception



"During attempts to isolate *Spirochaeta plicatilis* from marine marsh muds, I observed microorganisms which rapidly migrated toward one side of drops of the mud transferred to microscope slides," Woods Hole biologist Richard Blakemore wrote in 1975 (*Science* 190: 377-9). "I presumed this to be a phototactic response toward light from a northwest laboratory window." But as Blakemore experimented with different light sources and conditions, he found no change: these bacterial cells kept quickly migrating to the same side of their mud drops. Then, however, he began waving small magnets near the microscope slide, altering the direction in which the bacteria themselves moved. "It was experimentally confirmed that the migration of the bacteria was, indeed, directed by the earth's magnetic field." Yet until very recently, the molecular basis of these magnetotactic bacteria's remarkable behavior remained largely unknown.

Turtles, homing pigeons and salmon are all capable of deriving “positional information from the earth’s geomagnetic field,” said HFSP Grant team leader **Long-Fei Wu** (IBSM-CNRS), Marseille, France). “How they do this is one of the biggest mysteries of behavioral science.” Wu showed chains of magnetite extracted from the head of salmon, which look rather similar to the chains of magnetite-containing “magnetosomes” found in magnetotactic bacteria of the Mediterranean sea.



Magnetosomes are membrane-bound organelles, 35-150 nm in size, with species-specific morphologies. “Most importantly, all the particle are aligned to form a chain. Why do they do so?” Wu showed calculations indicating that the typical size of these chains—15-20 magnetosomes—are just sufficient in length to align in the earth’s magnetic field, like the needle of a compass. “It has been reported that magnetotactic bacteria isolated from the Northern hemisphere swim in parallel with the geomagnetic field; in contrast, those isolated from the Southern hemisphere swim anti-parallel with the geomagnetic field. By doing so all swim down down in the water towards conditions and food sources favorable to their growth. ”

Recent studies by other researchers strongly suggest that magnetosome chains are organized by binding to linear filaments made of a bacterial actin-like protein called MamK. (*Science* 311: 242-5 (2006). In MamK deletion mutants, magnetosome chains fail to form. “So the function of this filament might serve as a track for positioning and intracellular trafficking of magnetosomes,” said Wu, “similar to what happens to organelles in eukaryotic cells”—a rather remarkable feature of prokaryotic architecture, given the classical view of bacteria as devoid of both cytoskeleton and intracellular organelles. Wu’s team has studied different features of magnetosomes, including the mechanism of protein targeting to them and the sub-organelle distribution of specific proteins. At the Paris meeting, Wu described new studies related to the biogenesis and function of the MamK filaments. Wu’s team expressed the MamK protein in E. coli (a non-magnetotactic bacteria) and showed that MamK filaments assemble from multiple nucleation sites, with the end of an existing filament seeming to form the nucleation site for a new filament in a daughter cell rather than dividing in the middle.

Wu concluded by outlining the team’s long-term interest in understanding how such a MamK-aligned magnetosome chain “converts magnetic torque into a biochemical signal” that then somehow feeds into the signaling pathway and machinery that guides bacterial chemotaxis.

During a panel discussion, Dan Kiehart (Duke University) commented on the relevance of such studies into the strange cellular phenomena of magnetosomes: “One form of interdisciplinarity lives in the fact that there have been scientists who have been interested in a lot of very esoteric things in biology. And that kind of interest in a wide variety of organisms is what drives many biologists, and should be capitalized on”. He told the story of Professor O. Shimomura’s long-time fascination with the strange bioluminescence of a jellyfish living off the coast of Washington: a fascination that led to the discovery of green fluorescent protein, whose use has utterly revolutionized bio-imaging during the last decade. “.” Indeed, magnetosomes already have attracted

great attention from biotechnological researchers.

Keeping Mitochondria in Shape: A Matter of Cellular Life and Death

Cells that are stressed or damaged—whose DNA has been irremediably damaged during replication, for instance—are programmed to commit suicide, a process known as apoptosis. Oddly, this intrinsic pathway to programmed cell death converges on an electron carrier protein (cytochrome *c*)in an organelle(the mitochondria)generally considered the central engine of eukaryotic life: for it is the inner membranes or "cristae" of mitochrondria that house the respiratory enzyme chains that convert food into life-sustaining energy. Yet over the past fifteen years, researchers have discovered that cell death-promoting signals cause the release of cytochrome *c* from the mitochondria outer membrane into the cytosol, to which cytochrome *c* binds and activates the adaptor protein Apaf-1, setting in motion a cell death-inducing proteolytic cascade.

As an HFSP Long-Term Postdoctoral Fellow in the late Stanley Korsmeyer's lab, Career Development Awardee **Luca Scorrano** (Dulbeco-Telethon Institute, Padova, Italy) made a pioneering discovery about the nature of this mitochondrial release of cytochrome *c* (*Developmental Cell* 2: 55-67 (2002)). Most of the cytochrome *c* in mitochondria is sequestered in intra-cristae pockets of the mitochondrial inner membrane, pockets separated from the inner membrane space by a bottleneck "tight junction." Scorrano discovered that a death-inducing signal causes cristae to become remodeled, opening the neck of this tight junction and mobilizing cytochrome *c* for release into the cytosol (see below).

Since making this intracellular discovery as a post-doctoral fellow, Scorrano told his Paris audience, "my interest has always been to understand the molecular mechanism that regulates this process."

While most textbooks depict mitochrondria as fixed, sausage-shaped organelles, said Scorrano, "mitochondria are in fact very dynamic organelles, they are continuously fusing and dividing, and these processes are controlled by what I like to call 'mitochrondrial-shaping' proteins." In his own lab in Padova, Scorrano's group focused their attention on the only 'mitochondrial-shaping protein' known to be associated with the inner membrane: a dynamin –related protein called OPA-1. Two papers published in *Cell* the week after the Paris meeting (*Cell* 126: 163-175; 177-189) chronicle the remarkable molecular detective story that ensued.

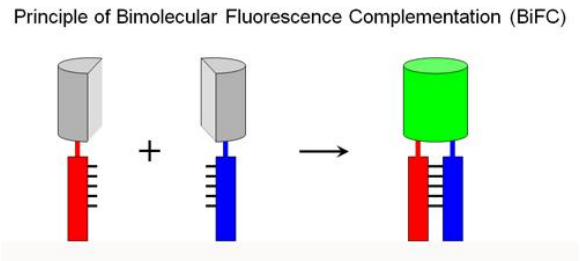
Scorrano and his colleagues found that wild-type OPA-1 prevents apoptosis by inhibiting the release of cytochrome *c;* moreover, it does so by preventing the cristae remodeling process he had discovered as a post-doctoral fellow. Conversely, mutant forms of OPA-1 that cause dominant optic atrophy disease (the origin of the OPA name) fail to inhibit apoptosis in response to death-inducing signal. "We then turned to a biochemical approach, checking the sublocalization of OPA1" in mitochondria, Scorrano recounted, and found that while most OPA-1 was in the inner membrane, a small amount was in the inner membrane space. "This was rather surprising for us, and we thought it might be important." Indeed, Scorrano and his colleagues realized that the yeast homologue to the mitochrondrial-shaping OPA-1 existed in two different isoforms whose processing depended on a so-called rhomboid protease. This yeast-guided detective trail led Scorrano to the mammalian homologue of this rhomboid protease, a protein called PARL. Scorrano showed converging evidence that "PARL and OPA-1 participate in the same pathways that regulates apoptosis," and presented

a working hypothesis for how they do so - a working hypothesis that his Padova laboratory is avidly pursuing.

Visualization of Protein Interactions in Living Cells

"We tend to give proteins names that attribute to them a particular function," said Grant Team leader **Tom Kerppola** (HHMI, University of Michigan, Medical School, USA). "But proteins are not really autonomous agents." Regulatory proteins, for instance "function as interchangeable modules in networks where the signals that provide the information that modulate cellular functions are, at each stage, interpreted and encoded by protein complexes. …Cells respond to their environment by altering protein interactions."

Kerppola has developed the new method of Bimolecular Fluorescence Complementation (BiFC) to visualize such specific partnering between proteins in living cells. In BiFC, fragments of fluorescent proteins—fragments which are non-fluorescent themselves--are fused to proteins which may interact *in vivo* (for recent review by Kerppola, see *Nature Review Molecual Cell Biology* 7: 449-456). If these potential interaction partners do in fact form complexes, the reassembly of the fluorescent protein fragments will provide a visual report of this interaction. The method is highly sensitive, enabling the identification of subcellular sites of protein interactions in their native environment, without the need for structural information about the protein complex. However, the temporal resolution of protein dynamics is still limited, because of the delay in the fluorescent signal upon complex formation, and more importantly, because the bimolecular complex formation tends to be 'trapped' by fluorescent-fragment reassembly—"and this is something we are constantly working on trying to overcome."



Kerppola focused his presentation on his collaboration with HFSP co-investigator **Lars-Gunnar Larsson (**Swedish University of Agricultural Sciences, Uppsala, Sweden) on the Myc-Mad-Max transcription factor network. In this network, Max interactions with members of the Myc protein family typically drive cell proliferation; while the mutually exclusive interactions between Max and members of the Mad family generally drive cell differentiation or quiescence.

"We think that most proteins are sticky by nature, they are designed to interact with lots of other proteins, and that the specificity of interactions is driven by competition," said Kerppola. In the case of Max, "we have this balancing act, if you will, of who will win between Myc and Mad family members when both groups are expressed in the same cell."

Using multicolor BiFC analysis with different engineered-variant fluorescent protein fragments, Kerppola showed how his team could visualize multiple complexes in the same cell (showing, intriguingly, that different complexes localized to different nuclear compartments) and quantify the relative efficiencies of dimerization between Max, Myc and Mad proteins. They found that there are no simple rules for complex formation. "Very closely related members of the same family that have intrinsic binding affinities of the same order, have very different efficiencies of competition within the cell. At this stage we don't really know what the determinants of those differences are, but we're

very interested in finding out how signaling that leads to alternative cell fates shifts the competitive balance between these alternative protein complexes."

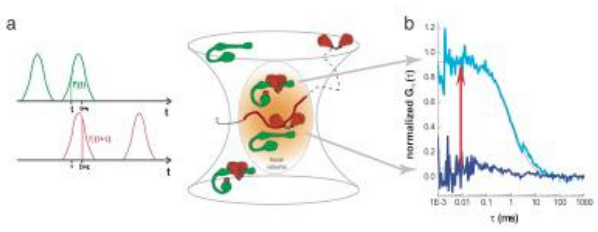
Grant Team member and biophysicist **Petra Schwille** (Dresden University of Technology, Germany) then described her collaboration with neurobiologist **Neal Waxham** (The University of Texas Medical School at Houston, USA) in monitoring binding between calmodulin (CaM) and Ca^{2+}/CaM-dependent protein kinase II (CaMKII)—an interaction known to be crucial for synaptic plasticity, learning and memory.

"Life is based on dynamics and interactions," Schwille's opening slide emphasized, and she has pioneered the development of a highly sensitive new method, Two-Photon Cross-Correlation Spectroscopy (TPCCS, for analyzing ineractions between the "dynamic manifold of cellular proteins." (For a recent review by Schwille, see *Nature Methods* 3: 83-9 (2006)). What "we want to do is see not only whether protein A interacts with protein B, but also to do quantitative biochemistry, to get stoichiometric data in the living cell in the very spot where it happens."

Schwille labeled the calcium-binding calmodulin protein with a red fluorescent protein, and CaMKII with green fluorescent protein. These two fluorescence spectral signals are split by a dichroic mirror and then cross-correlated. The cross-correlation signal is a quantitative measure of the dynamic co-localization between the two molecules within a focal volume about the size of an E. coli cell.

"But there's another big challenge, in that it's not a one-to-one binding between calmodulin and CamKinase," said Schwille. CamKII forms a holoenzyme that can bind up to twelve calmodulin molecules, and thus exists in a number of molecular states depending on local calcium concentrations—a biochemical property central to its unique role in synapses. Schwille and her colleagues had to develop a new experimental and theoretical framework to relate the red-green brightness signals to the probability distribution of the number of bound calmodulin molecules--a framework that "can be re-derived and apply to other biochemical systems with different binding stoichiometries and labeling schemes".

By using TPCCS to analyze CaM/CamKII complexes under a number of physiologically relevant conditions, they demonstrated, "for the first time, the full analytical power of this ultra-sensitive technique in living cells" (*Biophysical J.* 88: 4319-36, 2005). However, these pioneering studies of *in vivo* quantitative biochemistry were conducted in non-neural cells. "So now the challenge is to transfer this technique to neurons" and synapses, a challenge Schwille continues to pursue in collaboration with Waxham beyond the period of their HFSP program grant.

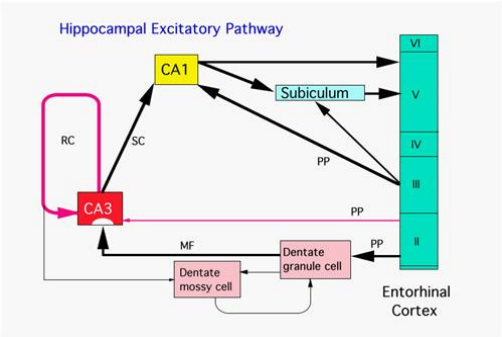


On the final day of the Paris meeting, four speakers presented a remarkable overview of the many interwoven mysteries of the molecular organization of synapses, including a *tour de force* plenary lecture on the distinct functions of the same "memory molecule" in different brain regions.

The second plenary speaker, **Susumu Tonegawa** (The Picower Institure, MIT, USA) initiated a revolution in neuroscience in 1992 by creating "knock-out" mice in which the CaMKII gene was deleted, and then used behavioral tests to demonstrate that these mutant mice were impaired in their ability to learn and remember information about their spatial environment (Science 257: 206-11).

Then in 1996 (in parallel with studies in Eric Kandel's laboratory at Columbia) Tonegawa initiated a second-wave revolution through his studies of another crucial "memory gene"—the gene encoding the NMDA receptor. The NMDA receptor responds to neurotransmitter signals by channeling in the Ca^{2+} ions that bind to calmodulin and CamKII, giving the synapse a long-lasting biochemical memory of neural activation which can be transmitted both to the genome and to other synaptic proteins. Tonegawa combined Cre/*lox* recombinant DNA methodology with knowledge of how specific DNA regulatory sequences of the CaMKII gene specifically directed expression in the so-called CA1 region of the hippocampus, a structure known to be critical for the formation of long-term memories in both humans and mice Through these intricate genetic manipulations, Tonegawa's laboratory deleted the NMDA receptor gene specifically from CA1, so that "NMDA receptor function is impaired specifically in CA1 pyramidal cell, but not anywhere else in the entire brain," Tonegawa explained. Tonegawa's group could then study both the physiological and behavioral consequences of this brain-specific "memory gene" deletion (*Cell* 87: 1327-38 (1996)).

"Memory is the vehicle by which you have a sense of self," said Tonegawa (who received the 1987 Nobel Prize for discovering the molecular genetic basis of antibody diversity). It is memory that allows you retain your history "of interactions with other people and your environment. Memory defines who you are." Tonegawa said that a characteristic question of a patient with advanced Alzheimer's disease is: "Could you please remind me who I am?" Of course, such issues of selfhood and personal identity cannot be addressed in mice. But Tonegawa demonstrated how one can study—at levels ranging from molecules to synapses to brain systems—how it is that a mouse learns and remembers "where I am in space."



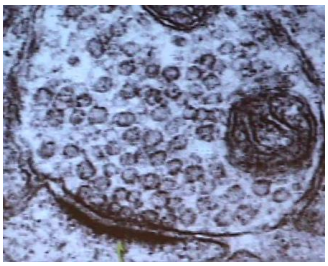
Tonegawa described work in his laboratory linking three distinct forms of spatial memory to three distinct stages of neural processing in the hippocampus. First, by deleting the NMDA receptor gene in CA1 hippocampal cells in the experiment described above, Tonegawa's group showed that mice could no longer learn and remember the location of a safety platform in a Morris water maze. This behavioral defect was correlated with the inability of their CA1 cells to undergo long-term potentiation (LTP), a form of synaptic strengthening. For humans, this CA1-NMDA deficit would be equivalent to being unable to learn and remember the location of an assigned parking space amid a sea of cars.

The second form of memory described by Tonegawa is called "pattern completion". Suppose that your memory of your assigned parking space depended on seeing a nearby lamp-post and the relative angle of three buildings in the horizon. Now suppose three of these four spatial cues were removed—as they were for mice swimming in the Morris water maze. Control mice were still able to find the platform—to complete the spatial pattern and match it to the pattern in memory. However, mice who had their NMDA receptor gene specifically deleted in the so-called CA3 regions of the hippocampus, which provides the major input to CA1 neurons, were impaired in this "pattern completion" task. Indeed, in 1970, the computational neuroscientist David Marr had predicted that specific circuitry features of CA3—in particular, its pattern of recurrent connections synapsing back upon itself—made it well suited for performing "pattern completion" memory tasks. "And in real life," said Tonegawa, "you don't have the entire set of cues for recalling a memory. You have limited cues. And this is where the mutant animal has a problem."

Finally, imagine your parking space is no longer fixed, but shifts from day to day. "Your memory of where you parked your car yesterday and the day before can interfere with your memory of where you parked your car today," said Tonegawa. Tonegawa showed that, at least in mice, keeping these memories distinct—a task called "memory separation"—appears to rely on cells in the hippocampus's dentate gyrus (the primary input for the CA3 region). Deleting the NMDA receptor gene specifically from these cells impairs a mouse's ability to distinguish between similar but distinct spatial environments.

"We saw that in the hippocampus, if you knock out the same gene in three different brain areas---CA1, CA3, dentate gyrus-- they exhibit very different kinds of deficits in terms of learning and memory. That is because the circuitries [of these brain regions] are different, they have different cell densities and connections." His laboratory uses molecular genetic methodology, but at the same time it conducts highly interdisciplinary research involving many collaborations with other laboratories with expertise in particular neural systems and behaviors. "Systems biology is becoming very popular," Tonegawa concluded, "and in the brain, you definitely have to study the system and the circuitry if you want to understand what this complex organ is doing."

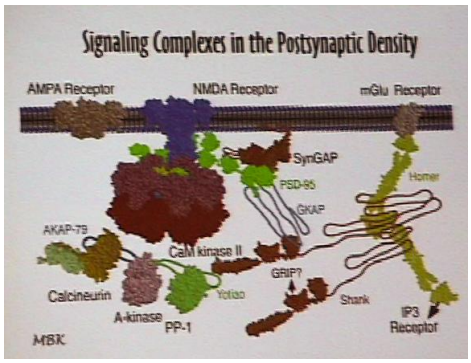
New Insights into the Functional Architecture of Synapses



"The structure that we're interested in visualizing and understanding is the post-synaptic density (PSD) on the post-synaptic side of excitatory synapses in the central nervous system," said Grant Team member Mary Kennedy (Caltech, USA), as she showed an electron micrograph of the object of her team's study.

"We know now that the post-synaptic density contains a very complex array of signaling enzymes that transduce signals through the NMDA receptor into changes in the size and strength of the synapse. These are complicated biochemical processes, and what's most fascinating about how this structure works as a whole is that it responds very delicately to the pattern and the amount of calcium that flows through the NMDA receptor." Small, infrequent pulses of calcium can shrink the synapse and reduce its strength; while larger, high-frequency pulses set off a signaling cascade that enlarges and strengthens the synapse.

Grant Team Principal Investigator Wolfgang Baumeister (Max Planck Institute for Biochemistry, Martinsried, Germany) has developed new computational methods for analyzing cryo-electron tomographic images of synapses (for initial studies of the synaptic cleft, see *Structure* 13: 423-34 (2005)). Grant Team member Andej Sali (University of California at San Francisco, USA), a world-leader in applying computational and bioinformatics methods to protein-structure determination, is creating models of post-synaptic density proteins, with the eventual aim of fitting these 3-D structures onto the cryo-EM density maps made by the Baumeister group. Finally, Kennedy's Caltech group is working to determine the structure of the cytosolic tails of the NMDA receptor (on subunits NR2A and NR2B) that are "extremely long—they have as many amino acids as the receptors themselves."



Thus far, Kennedy's attempts to express sufficient amounts of NMDA cytosolic tail proteins for crystallographic analysis have not yet worked, so she has collaborated with Sali's group in using "homology models" to fit the tail's primary sequence to known structures and rule-based structural motifs. Working in Sali's group, post-doctoral fellow Dmitry Korkin has developed a strategy called "comparative patch analysis" whereby patches of the PSD-95 protein known to be involved in protein-protein interactions in homologous proteins are mapped onto a model of the PSD-95 protein, and "then by a series of tests you can decide which is the most likely structure for the particular interaction being studied"—a method which has been validated by a number of criteria (Korkin et al. Plos Comput Biol 2: e153, 2006). "Our next step will be to test these models and try to differentiate them by biochemical probes of the recombinant protein—which in this case seems easier to do than getting the actual X-ray structure."

Getting a grip on the synapse

HFSP Career Development Awardee **Casper Hoogenraad**, who now heads his own laboratory at Erasmus Medical Center in the Netherlands, described the illuminating set of discoveries he made as Long-Term Fellow at MIT studying another of the post-synaptic density proteins displayed on Mary Kennedy's slide: a protein named GRIP, for Glutamate-Receptor Interacting Protein.

Originally isolated as a binding partner of the AMPA glutamate receptor, GRIP was found to bind many other proteins as well, suggesting it may play a general role as a "synaptic organizer" in addition to conveying AMPA receptors to the post-synaptic densities in dendritic spines. However, experimental dissections of GRIP's function lagged behind the construction of such hypotheses. Knocking-out the GRIP gene in mice resulted in embryonic lethality, preventing functional studies. Thus Hoogenraad adopted an RNAi "knock-down" approach in neural cell culture with striking results: the dendritic arbors of both developing and mature neurons were severely reduced in the absence of GRIP function. "This was basically the first experiment I did," said Hoogenraad. "There's hardly any branching of dendrites in this GRIP-minus neuron, hardly any complexity….We saw long primary dendrites but all the secondary dendrites were gone."

Previous investigators had shown that one of GRIP's binding partners was the KIF5 kinesin motor; it has been suspected that GRIP served as an "adaptor protein" by which kinesin transported AMPA receptors to the synapses via microtubules. But Hoogenraad's extensive tests of GRIP indicated a more general and integrated function. In particular, Hoogenraad used various RNAi and peptide constructs to define critical interaction sites between the GRIP protein, kinesin and the EphB2 ephrin tyrosine kinase receptor---already known to be crucial to dendritic spine morphogenesis. This has led to a model by which GRIP acts both as an adaptor protein steering EphB2 receptors to the synapse, and as a synaptic scaffold protein—a true synaptic organizer. Moreover, it is known that EphB2 receptors bind to the NMDA receptors central to synaptic plasticity. "Involvement of the NMDA receptor would allow for activity-dependent regulation of dendritic arbor development," write two commentators on Hoogenraad's *Nature Neuroscience* paper on GRIP (7: 906-915; 848-50 (2005). "The Hoogenraad *et al.* study suggests that GRIP1's integration of these [different] functions underlies a fundamental step in dendritic arborization."

Diffusion in Dendrites

Dendritic spines are bulbous, mushroom-shaped protrusions from dendritic shafts that serve as the primary targets of excitatory neural signaling in the central nervous system. "Typical spines have a thick head and a rather thin neck," explained Grant Team leader and theoretical neurobiologist **Erik De Schutter** (University of Antwerp, Belgium).

It is generally thought that a dendritic spine's thin neck serves to chemically compartmentalize it from the dendrite and neuron as a whole, especially by limiting calcium influx and outflux. Indeed, previous studies have shown that only very small quantities of calcium can escape from the spine into the dendritic shaft itself.

"Previous studies have focused on diffusion from spine to dendrite," said De Schutter. "What we want to look at comes from the opposite direction: What about diffusion from the dendrites into the spine?.... Surprisingly this is a question that has not been addressed in much detail until now." Grant Team member **George Augustine** (Duke University Medical Center, USA) used photolysis of caged fluorescein dextran (an inert substance) to monitor diffusion in Purkinje neurons,which have, on the same neuron, both spiny dendrites and "smooth dendrites" which lack spines—"so it's easy to do controls." Augustine then carried out parallel studies with IP_3, an important second messenger in neurons. Classical diffusion theory was able to describe diffusion rates of small molecules in smooth dendrites. "However, if you go to spiny dendrites, you see a very different story. We observe that there is very little spread of these molecules in the shafts of spiny dendrites**."** De Schutter's group has modeled this process using specially-designed software, and concluded it to be a case of "anomalous diffusion. Anomalous diffusion has been quite a focus in physics over the last ten to twenty years. It's something that occurs when you have a mechanism that transiently traps diffusing molecules." In this case, the trapping mechanism is the dendritic spine.

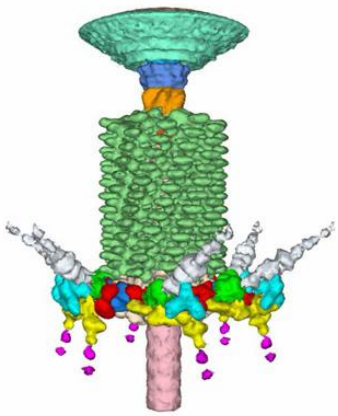
The observation that the diffusion of IP_3 is anomalous led De Schutter to ask whether this is true of other signaling molecules, and whether trapping by dendritic spines affects signaling cascades within them. These are just some of the questions his interdisciplinary team plans to pursue in future experiments and computational models.

"Mein Gott! They've got tails!" was the reaction of "kindly old Professor J.J. Bronfenbrenner, who had worked on bacteriophages for many years, …[as] he clapped the palm of his hand to his forehead" at the sight of the first electron micrographs of phage virus particles in the early 1940s [see Thomas Anderson's reminiscence in *Phage and the Origins of Molecular Biology*, Cold Spring Harbor Press, 1966 (expanded 1992), pp. 63-78)]. Initially, Bronfenbrenner and others thought phage tails to be "sperm-like structures" and "organs of locomotion." It would take a decade for the "phage group"—the vanguard international community of scientists led by physicist-turned-biologist Max Delbruck—to realize that this "tail" was the needle and syringe by which the phage virus injected DNA into E. coli cells.

In the Paris meeting's final presentation, Program Grant team Principal Investigator **Michael Rossman** (Purdue University, Indiana, USA) gave a *tour de force* demonstration of how studies of the T4 bacteriophage tail are leading to what commentators are calling a new "textbook example of combining X-ray and cryo-EM data to understand the structure, function and concerted transformation of a complicated molecular device" (*Current Opinion in Structural Biology*, 14: 120 (2004).

"Too complex to crystallize" was the heading of Rossman's schematic diagram of a T4 phage particle—a complexity challenge also presented by many of the higher-order macromolecular assemblies that carry out the intricately interlocking functions of eukaryotic cells.

To meet this challenge, Rossmann and his colleagues have been at the scientific frontiers of showing how "the combination of cyro-electron microscopy to study large biological assemblies at low resolution with crystallography to determine near atomic structures of assembly fragments is quickly expanding the horizon of structural biology" (*Structure* 13: 355-62 (2005)). Rossmann quickly reviewed how this approach has led to a "quasi-atomic" model of the T4 phage head--including, most recently, "the ATPase for the DNA packaging motor that provides the energy for packaging DNA into the head." Rossmann then focused his presentation on the "supramolecular metastable structure" of the phage's intricate tail.



The T4 tail consists of about twenty different proteins, many of whose crystal structures have been solved by Rossmann and his colleagues. The tail assembles itself from the foundation of a "baseplate" structure which "is a kind of nerve center for the virus," said Rossmann. "Extending from the baseplate are the long tail fibers, which recognize the host, and the short tail fibers, which eventually attach very firmly to the outside of the host and allow the tail to penetrate" the bacterial cell wall. Before this penetration can occur, the baseplate transforms its overall structure from a hexagonal dome to a six-point star-shape. The individual tail proteins do not change shape during contraction; rather, cryo-EM reconstruction maps comparing the un-contracted and contracted tail structure indicate that "what has happened when the virus infects a cell, and goes from having a baseplate being hexagonal-shaped to star-shaped, is that the different proteins, as rigid bodies, slide on top of each other." Moreover, it seems that this sliding is accompanied by a near full-turn rotation of the tail sheath and "head on top…which therefore acts as a kind of drill, so that the pin"—

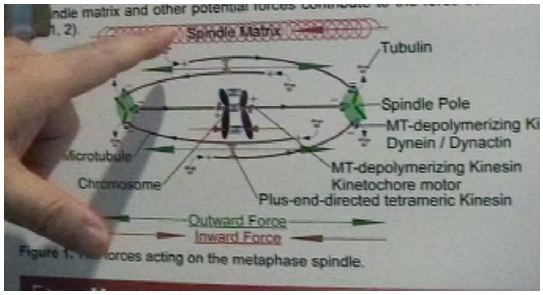
the ‘needle’ of the T4 injection machine made from a phage protein called gp5—“punches its way through the outer membrane of the host.”

Rossmann concluded his talk with a short but stunning animated movie (made by Purdue computer science students apparently, clearly inspired by the aesthetics of science fiction films – available on the lab web site) which illustrated this dynamic supramolecular process: A T4 phage particle (its special head vertex proteins artistically rendered as ‘eyes’) floats down through a dark watery suspension of bacteria; lands on a bacterial cell surface with insect-like legs; its legs firmly bound, the tail sheath contracts and rotates like a drill; the rotation driving its tail tube and accompanying lysozyme enzymes through the cell wall; the gp5 ‘pin’ protein falls off the end of the tail tube so that DNA can be injected into the inner space of the cell, where (not pictured in the movie) the head and tail proteins of some 100 new phage virus particles will be synthesized and assembled, then burst the cell open like a molecular cluster bomb, beginning the phage life cycle anew.

Conclusion

Rossmann’s talk on T4 phage structural dynamics was interwoven with references to the dynamic interchange of students and post-doctoral fellows between his own laboratory and the labs of HFSP co-investigators **Fumio Arisaka** (Tokyo Institute of Technology, Japan) and **Vadim Mesyanzhinov** (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia). “And having formed the HFSP group, other groups began to join us,” Rossmann said, employing a crystallographic metaphor for the growth of the HFSP project. “Once you have a nucleus, you can crystallize around it more and more collaborations.”

This dynamic interchange of ideas, experimental know-how, and most importantly, talented young scientists, was evident in many conversations with HFSP Awardees at the Paris meeting. **Shin’ichi Ishiwata**, for instance, whose laboratory in the physics department of Waseda University in Tokyo has long used vanguard biophysical methods to study muscle contraction, spoke during a poster session of his HFSP-supported collaboration with old friend, physicist Christoph Schmidt, and new friend, chemical biologist Tarun Kapoor, on biophysical aspects of cell division and spindle dynamics. “This is a real challenge,” said Ishiwata, pointing to the “delicate balance of forces” operating in the mitotic spindle.



“This is quite a complex system compared to muscle sarcomeres.” The HFSP grant enabled Ishiwata to send a post-doctoral fellow to Kapoor’s laboratory to learn how to use Xenopus frog extracts to study spindle dynamics. “I didn’t know anything about the egg, this system is a new experience for me,” said Ishiwata. “It has brought a new dimension to my lab, yet it’s a natural extension to the work we were doing in muscle. Much more complex, but to understand a self-organizing system like this, I think is suitable for the next target of our research.” Ishiwata laughed and concluded: “It is a natural progression, but maybe ten years of hard work.”