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Biophysics at the Cutting Edge: A Report from the 55th Annual Meeting of the Biophysical Society

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The 55th Annual Meeting of the Biophysical Society held at Baltimore in early March was attended by over 6000 scientists from over 20 different nations. The diversity in nationality was matched by the diversity of science presented at the conference. Over the course of 5 days, more than 30 symposia and over 3000 short platform sessions and posters presented a stunning array of biophysical science. To convey all that was presented is obviously impractical. This article will attempt to cover some of the more cutting-edge research, especially on those topics that are related to chemical biology. Also, rather than describing the talks in chronological order as they happened, the article is arranged under various categories based on broad research topics. Emphasis is also on various techniques, though the biological insights gained by each method are discussed briefly.

BIOPHYSICISTS LIKE TO FRET, ONE MOLECULE AT A TIME

Walk into a symposium or platform talk at the Biophysical Society annual meetings, and there is a good possibility that Förster resonance energy transfer (FRET) is the technique being employed. This year celebrates the 65th year since the phenomenon, involving non-radiative energy transfer between two dipoles, was first described by Theodore Förster.[1] Excellent reviews on FRET can be found elsewhere,[2−4] but very briefly, the technique is used as a molecular ruler that determines distance between two points in a biological system (in the range of 1−10 nm), each labeled with a different fluorescent probe. In typical FRET experiments to determine the energy transfer efficiency and hence the distance, investigators use only one of three measurable parameters: intensity of the fluorescent probe, their lifetimes, or polarization. However, **Dr. Claus Seidel** and co-workers, during a talk at the **Molecular Biophysics Subgroup** symposium, demonstrated how using all three FRET parameters together in a correlated manner through two-dimensional histograms, a method they call multiparameter fluorescence detection (MFD), provides richer information and reduces many of the errors associated with these studies. Additionally, he showed that application of another method developed in their lab, photon distribution analysis (PDA), eliminates the contribution of photon noise and allows one to fit FRET distributions to multiple states,[5] leading to more detailed information about the conformational distribution of the biological system.[6] For usually used long dye linkers, he described a straightforward procedure that allows for very high accuracy of FRET-based structure determination through proper consideration of the position distribution of the dye and of linker dynamics.[7]

These FRET experiments described by Dr. Seidel were conducted at a single molecule level, *i.e.*, information was derived by observing a large number of pairs of fluorescent probes on single molecules. Such single molecule (or single molecular assembly) studies offer information on distribution of molecular states (such as conformation) that are lost in ensemble measurements. They also allow observation of how single dynamic trajectories evolve in time. While single molecules had been detected before, *e.g.*, ion channels, through the electrophysiology techniques invented by Neher and Skarmann,[8] the ability to directly observe single molecules by fluorescence was originally demonstrated in Shimon Weiss' laboratory around 1996.[9,10] It was then further developed for application in biological systems by various groups, including that of Nobel Prize winner and current U.S. Energy Secretary, Stephen Chu (who incidentally also spoke at the meeting about some of the science being conducted in his lab as well as the application of biophysics to address energy issues facing the world). Biophysical Society meetings since the late 1990s and early 2000s have been abuzz with single molecule fluorescence techniques, with applications mainly in nucleic acids or nucleic acid−proteins interaction[11] (due to the obvious simplicity of working with such molecules, especially in terms of labeling and immobilizing them). However, in recent years, membrane proteins and ion channels are being increasingly investigated by this method.

In this context, **Dr. Scott Blanchard**, speaking at the **Membrane Biophysics Subgroup** symposium, presented some interesting dynamic data for the LeuT transporter protein using single-molecule or single-pair FRET (smFRET) imaging performed as part of a sustained collaboration with Jonathan Javitch's group at Columbia University and Harel Weinstein and Lei Shi at Weill Cornell. Understanding the structural changes in these bacterial cation-coupled transporters is important since they are homologues[12] of the pharmaceutically important human Na^+-coupled neurotransmitter symporters that facilitate the reuptake of neurotransmitters from the synapse using the potential gradient of Na^+.[13] These proteins are believed to function through an "alternate access" model[14] whereby the protein can exist either in an inward facing or outward facing conformation with the substrates binding the face accessible to either side of the membrane. Substrate binding lowers the energy barrier through allosteric interactions, allowing conformational switching between the two states. The crystal structure of this protein was solved by Eric Gouaux's group

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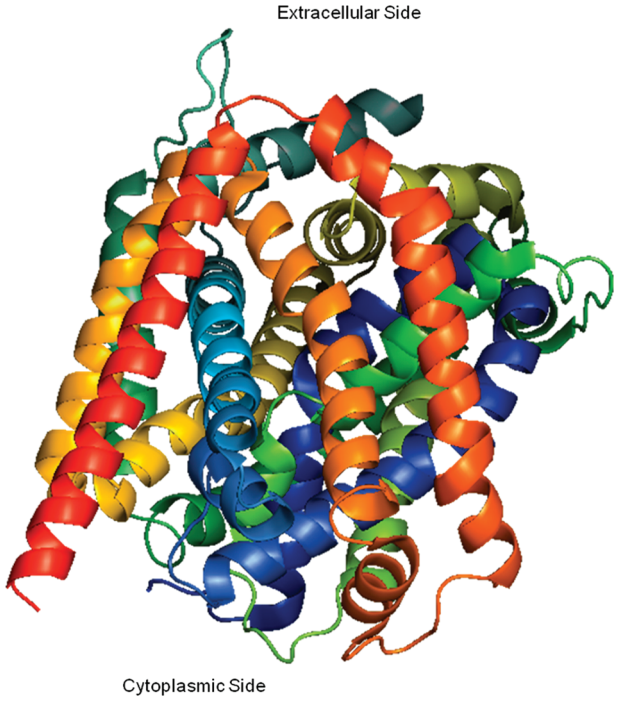

Figure 1. Outward facing conformation of LeuT as solved by Yamashita et al.[15] Image generated from PDB coordinates 2A65 using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

in 2005 [15] (Figure 1), but the nature of the conformational rearrangements during function remains unresolved.

The team was able to visualize this rearrangement directly using the smFRET approach by labeling two sites in the protein and observing FRET under different ionic conditions and in the presence of substrates and inhibitors. The initial challenge was to find the appropriate sites for fluorescent labeling. Many sites were tested, and ultimately pairs of residues on the intracellular side of the protein were identified that when labeled led to preserved transport activity and allowed detection of motion. As opposed to earlier smFRET studies on membrane proteins, Blanchard's group was also able to adjust conditions appropriately so that detergent-solubilized LeuT proteins immobilized on a passivated glass surface[16] could be imaged over long observation periods (up to 15 min). Spontaneous changes in FRET efficiency observed during such long recordings were shown through molecular dynamics simulations undertaken by Drs. Harel Weinstein and Lei Shi to correspond to inward and outward facing conformational states of the protein and found to correspond in time scales to those of the transport process. Key mutations in the gating regions of LeuT were shown to increase the probability of the protein residing in either inward or outward facing conformations. Consistent with the observed FRET dynamics reporting directly on the transport mechanism, the substrate alanine was shown to lower the activation barrier for conformational transitions between inward and outward facing confromations, markedly accelerating the rates of FRET dynamics observed. Finally, the measurements also showed that inhibitors that compete for the substrate binding site stochastically shut off the alanine-induced dynamics, arresting the protein in an outward facing conformation as anticipated from previous structural investigations.[17,18]

These single-molecule studies, described in a paper published in the April 24 issue of *Nature*, are possibly the most direct visualizations of the dynamic mechanism of transporter function under various conditions, where earlier evidence was available only indirectly through biochemical studies or kinetic assays or through ensemble EPR spectroscopy. An emerging story from this work is a possible role for a "second substrate binding site" located within the extracellular vestibule of LeuT that appears to allosterically regulate the transport mechanism. In future studies, the team aims to obtain information about the timing of specific molecular motions in LeuT dynamics underlying the transport process, using multicolor smFRET, where two or more pairs of FRET probes can be imaged simultaneously. Dr. Harel Weinstein also spoke about the LeuT transporter during the **New and Notable** symposium about the use of these experimental data in combination with MD simulations to provide a complete description of the transport pathway and mechanism.

Another application of smFRET was presented by **Dr. David Rueda** during the **Future of Biophysics** symposium, on the investigation of the yeast spliceosome assembly. Rueda in close collaboration with Pacific Biosciences has been interested primarily in the dynamics of the U2/U6 snRNA. His group has previously published on the dynamics of the protein-free immobilized U2/U6 complex that suggests the complex acts as a Mg^{2+}-dependent conformational switch.[19] However, they want to extend their studies to observe these dynamics in a full spliceosome. Hence the group labeled U6 with a donor−acceptor pair (Cy3/Cy5) to observe the smFRET changes in the presence of the full complex. Since background fluorescence turns out to be an issue when full cell extracts are used, they used a zero-mode waveguide (ZMW) system (developed at Cornell[20] and currently used by Pacific Biosciences for next generation DNA sequencing[21]). ZMWs are metallic holes whose diameter is smaller than the wavelength of light. Upon laser illumination directed at ZMWs, evanescent waves are produced that excite fluorophores within a tiny volume (in the range of a few tens of zeptoliters!). The restricted volumes allows near physiological concentration of the reacting molecules to be imaged in single-molecule modes. This work is in progress, but the group could observe complicated dynamics from smFRET studies of the cellular extract. To make sense of the dynamics, the data was analyzed by hidden Markov modeling and classified into three categories: Class I molecules exhibit similar dynamics to the protein-free U2/U6 snRNA and may correspond to active spliceosomes, Class II events that behave differently at different times (*i.e.*, initially low FRET then fast transitions), and Class III, which included events that could be classified in any of the categories. To determine structural states represented by each category, they resorted to mutations, *e.g.*, a mutation that blocks the second splicing step resulted in FRET states similar to Class II and hence this category could represent assemblies that get stuck at that stage. One drawback of this current study is that spliceosomal dissociation cannot be distinguished from dye photobleaching. The group plans to overcome this issue by adding an additional probe, possibly a molecular beacon that binds only to the splicing product.

That fluorescence is not the only method for detecting single molecule dynamics was demonstrated by **Dr. Keiichi Torimitsu** during a **Membrane Biophysics Subgroup** symposium. Interestingly Dr. Torimitsu works in the research division of NTT, a telecommunications company in Japan whose eventual research goal is to develop biomimetics such as artificial synapses that can

be used to monitor, support, or even control neuronal activity. Such a nanobio interface seemed to be one of the themes of this conference, and some other talks in this context will be mentioned later. However, research described in Dr. Torimitsu's presentation focused on their current work involving investigation of conformational changes in neuronal receptors using fast scanning atomic force microscopy (AFM). In this method, a nanomechanical probe is used to scan biomolecules so as to be able to observe their topography in aqueous solutions. Using AFM, the group could observe real-time conformation changes in a number of receptors important for neuronal activity, *e.g.*, an ATP-induced monomorer to trimer transition in P2X receptors and movement of the N-terminal domain in ionotropic glumatate receptors (iGlu-R).[22,23] Dr. Torimitsu stated that one of the applications of this detection system could be for pharmacokinetic measurements by rapidly observing structural changes in control *versus* agonist-induced proteins. As such, judging by the number of other posters and presentations, AFM is becoming popular as a single-molecule observation tool. With the advent of the fast scanning technologies, AFM has overcome the method's earlier temporal resolution limits, thus allowing visualization of dynamics in addition to structural features. The allure of AFM is that it can image conformational changes in a physiological state, *i.e.*, lipid membranes in buffered medium without having to label biomolecules.

INTRACELLULAR GPS

The methods described so far visualized single proteins *in vitro*, but there is obviously a great deal of interest in observing behavior of biomolecules in their natural environment, *i.e.*, living cells. Observation of real-time dynamics of single molecules or molecular complexes *in vivo* can shed light on cellular processes and how they are regulated. For example, **Dr. Diane Lidke**, winner of this year's Margaret Oakley Dayhoff Award, presented a talk during the **Awards Symposium** on combining quantum dots (QDs) with fluorescence microscopy for tracking receptor proteins to investigate their oligomerization dynamics and the role of cytoskeletal domains in regulation.

QDs are semiconductor material nanocrystals that possess a number of desirable fluorescent properties, the most important in this context being very high photostability. This allows the tracking of particles labeled with QDs over a long period of time without photobleaching.[24] Dr. Lidke, while a postdoc at Tom Jovin's laboratory at the Max Plank Institute, was among the pioneers in using QD-labeled proteins for *in vivo* tracking. Back in 2004, they employed QD-labeled epidermal growth factor (EGF) ligands to study receptor endocytosis and were the first to demonstrate retrograde transport of EGF receptors (EGFR, erb1) along cellular filopodia as a precursor to endocytosis.[25,26] That study showed that dimer formation was sufficient for starting retrograde transport, but the question remained whether the kinase domain of the receptor or its extracellular domain drives dimer formation. In recent investigations, by using EGF labeled with two different QDs emitting at different wavelengths, Dr. Lidke's own research group was able to follow the motions of EGFR monomers and formation of dimers. Analyzing the microscopy data with a novel three-state Hidden Markov Model allowed them to extract information on the dynamics of the dimer formation process. Three different receptor mobility states were observed: most mobile in a free state, and reduced mobility in a confined or the dimer state. Transient dimer interactions were observed in the absence of ligand. Importantly, they determined that the formation of a stable dimer required EGF binding and that inhibition of the kinase domain did not change dimerization kinetics (unpublished results, personal communication).

While Dr. Lidke's talk featured particle tracking in 2D, **Dr. William Moerner** spoke at the **New and Notable** symposium about single molecule tracking in 3D. His group has achieved this by developing a method called double-helix point spread function (DH-PSF).[27] Essentially, by using additional lenses and a spatial light modulator, they generate PSFs that have two lobes instead of one and the angle between the lobes provides information about the axial position of the light emitter. An application of this technique was demonstrated with mRNA movement in budding yeasts. The mRNA coding for Arg3, each containing 36 GFP molecules (labeled by engineering loops in the gene from a bacteriophage that binds to its corresponding coat protein and then coexpressing the proteins with GFP) for tracking was observed in live yeast cells. A 2D image of the movement seemingly exhibited a confined motion; however, when the 3D motion was visualized, the mRNA was observed to be much more dynamic. Additionally, analysis of the tracking provided quantitative information on the nature of movement of the mRNA, found to consist of diffusive, Brownian, and confined components.[28]

HIGH RESOLUTION CRYSTAL STRUCTURES ARE STATIC, OR ARE THEY?

It has been a quarter of a century[29] since the first membrane protein was successfully crystallized, a fact that was commemorated at this meeting with a "25 years of membrane protein structure" symposium (not directly covered in this article). While the rate of solving high-resolution structures of these difficult to crystallize proteins has grown exponentially in the past decade, it is still a non-trivial challenge. At the time of writing there are only 279 unique membrane protein structures in the database, representing a little over 0.5% of solved protein structures (source: http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html).

In absence of high-resolution structures, scientists often rely on hydrophobicity scales to model the topology of membrane proteins, particularly the trans-membrane (TM) section. This allows for further hypothesis testing using other biochemical and biophysical techniques, leading to mechanistic insights. However, as **Dr. Karen Fleming** pointed out during her talk at the **New and Notable** symposium, most current hydrophobicity scales are not based on a full length protein inserting into real lipid membranes. Her lab is approaching the problem by determining the free energy of folding of the bacterial outer membrane protein OmpIa into large unilamelar vesicles (LUVs). Specifically, the free energy is measured for a set of mutations where the residue A210, which points out into the bilayer and is located in the middle of the bilayer when the protein is inserted, is replaced by each of the other 19 amino acids. As expected, Phe is found to be the most hydrophobic, while lysine is the most hydrophilic, but interestingly, His is more hydrophilic than Arg by this scale. This scale, based on a real protein and real lipid bilayers, was found to be able to predict rhodopsin transmembrane regions and therefore is not limited to β-barrel proteins (unpublished results, personal communication).

While hydrophobicity scales are simple and useful, for gaining mechanistic insights, an atomistic model based on well-resolved crystals is invaluable. At the same time, such structures are static

and require further biophysical experiments (such as the smFRET or AFM experiments described earlier) to understand protein dynamics. But is it possible to figure out alternate conformations or the mechanistic pathways when the crystal structure of only one form of the protein is available? **Dr. Lucy Forrest**, speaking at the **Future of Biophysics** symposium, demonstrated how structural symmetry within membrane transporters offered clues on the mechanism. Forrest showed that in a majority of the secondary transporter high resolution structures solved so far, the trans-membrane regions have topological motifs that are repeated but pseudosymmetrically inverted with respect to each other. For example, LeuT, discussed earlier in terms of the alternate access model, has an inverted repeat topology between TM1-5 and TM6-10. Separating the repeats and rotating them by 180° and superimposing leads to structures that were related but distinctly different. In particular, TM1-2 and TM6-7 differed by a 30° rotation. This gap is possibly the pathway for the substrates to bind to the transporter from one side.

Therefore by taking the outward facing crystal structure of LeuT (Figure 1) and swapping the conformations of the repeats through molecular modeling, a putative structure could be found for the inward facing conformation.[30] The validity of this model was confirmed with experimental studies that measured accessibility of certain sites predicted by the model. Additionally, the inward structure bore resemblance to a related transporter MhpI that was later crystallized in the inward facing conformation. By comparing the inward *versus* outward facing structures, they could hypothesize which particular motions of the TM helices were involved in the inward to outward transition of the protein, leading to a "rocking bundle" hypothesis for the conformational mechanism, where the structural change occurs by the rigid body movement of a four helix bundle.[31]

This invert repeat hypothesis and modeling also lead to an inward structure based on the outward conformation crystal for the glutamate transporter homologue, GltPh.[32] This method works for other transporters families too, *e.g.*, for lactose permease (Lac Y), a member of the Major Facilitator Superfamily (MFS), an outward facing structure was proposed on the basis of the original inward facing confirmation of the crystal structure.[33] In both cases, the modeled structure was confirmed by accessibility studies, cross-linking, or similarity with other crystal structures. Dr. Forrest concluded with speculations on whether the inverted topology could be the basis of a general mechanism for the alternate access mechanism of transporter proteins. The repeats possibly provide a mechanism for generating two degenerate symmetry-related conformations for one protein; by having two almost symmetrical but inverted structures, the protein can adopt two conformations of almost similar energies, but each facing the opposite side.

Moving away from transporters, one of the most visually appealing and compelling storylines during the meeting was the talk by **Lawrence Lee** during the **New and Notable** symposium on deciphering of flagellar motion of bacteria, the basis of bacterial chemotaxis. Bacterial motion is usually a smooth forward run, but every so often, on environmental cues, they tumble for few hundred milliseconds allowing change in the direction of motion. Both the run and tumble motions are carried out by a complicated protein motor that is almost 11 MDa large and involves multiple copies of ~13 proteins! This system rotates the bacterial flagellar filaments simultaneously in counterclockwise directions at rates greater than 100,000 rpm; when the flagella separate due to switching of rotational direction, the bacterium tumbles. Lee and colleagues from Dr. Daniela Stock's laboratory solved the structure of one of the proteins in this assembly, FliG, which is involved in generating the rotational torque, switching of direction, and coupling of the torque to the flagellar filament. On the basis of the structure and mutational studies, they identified putative dynamic regions and sites for polymerization (there are 34 copies of the protein in the assembly), leading to a model of the complete torque generating assembly. This work is much better conveyed by looking at structural images and particularly by viewing the movies that are part of the Supporting Information for their recent publication.[34]

These last two talks demonstrate that while crystal structures by themselves are static, combining them with mutational and computation studies can lead to dynamic information.

IT'S A NANO, NANO WORLD

There were quite a few Platform talks and posters that used the term "nanobiology", involving the convergence of nanotechnology and nanomaterials with biology. Since most proteins or biomolecular assemblies are on the scale on 1 nm to 1 μm, developing materials that interact at that scale either for sensing or control, is an important area of focus. The two talks on these topics by **Drs. Bianxiao Cui** and **Charles Lieber** as part of the Bioengineering symposium were possibly the most futuristic. Cui spoke of developing and utilizing vertical nanopillars of controllable lengths (~700 nm) and diameter (~150 nm) that can be used as optical or electrical probes, as well as structural elements. As optical probes they can be used for evanescent wave microscopy within cells. Current evanescent wave methods (which use light that only propagates a few nanometers into a material so as to avoid background fluorescence) such as TIRF or ZMW are limited to studying cell surfaces only. For investigating most processes within the cell, either confocal or wide-field is utilized. However, these transparent quartz nanopillars could be utilized for detailed location-specific investigations in the cells with little background fluorescence. Cui showed preliminary data with experiments where the end of the nanopillars functionalized with QD could be imaged at single molecule levels over a background of unlinked QD floating around (though only for concentration of background QDs below 100 μM). They also succeeded in growing PC12 cells on glass nanopillars showing that cells were healthy (SEM images indicated that the pillars were possibly inside the cytoplasm). When the cells expressed GFP and the pillars were coated with anti-GFP antibodies, distinct spots could be observed at the pillar positions with little background. This indicated that the pillars could be functionalized and perhaps even used as recruitment or signaling sites in addition to imaging.[35]

Cui went on to talk about similar nanopillars but made with platinum and designed into a chip for measurement of electrical signals from cells. Currently, such measurements are conducted by path-clamp methods that are invasive and low throughput and provide only short-term recordings. Alternative to patch-clamp readings are electrode arrays for externally measuring voltage drops across the membranes, but they have poor signal with low spatial regulation. The nanopillar chips seemed to overcome these issues. When cardiomyocyte cells were grown over them, the action potential could be measured with high accuracy. A second advantage of the platform is the ability to electroporate the cells by sending a voltage pulse through the pillars. Unlike electroporation in cuvettes, where ~200 V pulses are required to porate cells in suspensions, these nanoelectrodes could do it with

0.5−4 V pulses of less than 1 ms duration. An application of this was shown where cells were electroporated, loaded with channel blocking dyes, and resealed, and their action potential was recorded. By loading both Ca^{2+} and K^{+} channel blockers, the appropriate inhibitions in action potential could be obtained. This could lead to a high-throughput but long-term (cells are stable over 3 days) and non-invasive drug screening with high spatial resolution (unpublished results; personal communication).

Finally, Cui showed data where apparently neuronal processes prefer growing on the nanopillars, and hence by patterning, neuronal cell or axon growths can be guided. This work[36] allows noninvasive pinning of neuronal cells for studying them.

The nanobio interface theme was taken further by Charles Lieber, especially with respect to the electrical measurements described above. His research group has developed a two-dimensional array of semiconductor nanowires that could be interfaced with neuronal or cardiac cells that allowed multiplexed recordings with extremely high spatial and temporal resolution.[37−39] These arrays could be fabricated on transparent substrates as well, allowing for simultaneous optical and electrical recordings. The group is pushing the limit of extracellular recording by developing nanowires that can be sub-100 nm devices capable of resolving 250 ns, *i.e.*, recording devices approaching the spacing and size of ion channels themselves! However, since extracellular recordings are not ideal for probing details within a cell, they prepared 3D kinked nanowire probes. By coating the end of these kinks with phospholipids, the probes were observed to be taken inside cardiac cells and allowed measurement of intracellular action potentials.[40] In such recordings, all the detailed electrical recordings possible from patch-clamp measurement are available, plus additional features reflecting the nanoscale nature of the measurements. The fact that these are biomimetic and do not enter the cell through mechanical invasion gives them advantages over other intracellular probes. Lieber and colleagues are currently merging these kinked nanowire devices with macrostructures that can be manipulated in 3D in a manner similar to conventional electrophysiology tools. Additionally, the group is growing cardiac tissues on a networked nanowired array, which will be potentially implantable. Lieber ended his talk on a very futuristic note predicting that the continued development of such nanobio interface materials would eventually "blur the distinction between electronic devices and living cells and tissues."

CONCLUDING THOUGHTS

With a meeting as diverse as this, it is difficult to describe an underlying theme. Overall, "traditional" biophysical topics such as ion channel physiology and structure were well represented during the conference as usual. Rather than any major breakthroughs, these fields seem to be undergoing incremental progress, though nonetheless significant. Membrane transporters on the other hand, are generating a great deal of excitement, particularly due to the large number of high resolution crystal structures that have been solved in recent times. The availability of the structures is leading to computational approaches combined with biochemical methods such as mutagenesis to understand more about the structure−function relationships. In terms of biophysical methods, single-molecule techniques, both fluorescence and AFM are playing an important role by allowing observation of real-time dynamics. Among newer topics, nanobiology is starting to register on the landscape along with systems biology, with a full symposium dedicated to the latter topic for possibly the first time during a Biophysical Society annual meeting.

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