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Dear Prof. Falcone,

This is a "Category B" Letter of Intent to perform experiments at the Linac Coherent Light Source, focused on the scientific goal of using the LCLS to determine structures of membrane proteins in two-dimensional crystals/arrays. The first part of the letter describes the scientific case; it also includes a brief description of some potential in-hutch instrumentation desired to perform experiments, and a list of references. The second part of the letter lists the team members.

Scientific case:

Membrane proteins are extremely important in biology, playing critical roles in a broad range of systems, including catastrophic diseases, neurobiology, photosynthetic energy conversion, and bioremediation. They constitute roughly 30% of the genes encoded by genomes, yet distinct intact integral-membrane-protein structures represent less than 0.1% of the structures deposited to the Protein Data Bank. Part of the reason for this paucity of membrane-protein structures is that they are notoriously difficult to crystallize in three dimensions for X-ray crystallography experiments. They have a natural tendency to arrange in two dimension, which renders them suitable for electron microscopy experiments. However, in both cases, crystallization of proteins often resembles alchemy more than science, and such experiments remain very difficult. Despite some impressive structures obtained in recent years via X-ray and EM experiments, new methods are needed.

We propose to determine structures of membrane proteins and other proteins in two-dimensional crystals or arrays near atomic resolution using the Linac Coherent Light Source (Becker, 1999; Becker & Weckert, 2004). In brief, we will use nanotechnolgy to try to improve methods for obtaining well-ordered two-dimensional crytals or arrays of proteins. We will collect diffraction data from supported two-dimensional crystals or arrays of membrane proteins in single-shot experiments using ultrashort, ultrabright X-ray pulses of the LCLS, where the diffraction is expected to be faster than much of the damage. Data will

be collected via grazing-incidence diffraction on separate samples in different orientations, or perhaps as a tilt series on separate samples. Data will be scaled, merged, and suitably analyzed, depending on the coherence properties of the diffraction from the samples, to yield electron-density maps, and ultimately, refined structural models.

A key issue in this endeavor is the crystal size. Indeed, experimental results with synchrotrons obtained via grazing-incidence diffraction on supported bacteriorhodopsin monolayers (Verclas *et al*., 1999), and on monolayers of membrane-associated, water-soluble proteins (Weygand *et al*., 1999, 2002), have provided measurements of multiple Bragg rods. However, the samples needed for these studies are large and tend to be rotationally disordered, yielding Ewald cuts through a powder-like diffraction pattern, precluding structure determination of such complex molecules so far. For the LCLS, initial calculations (Weckert, 2001; Becker & Weckert, 2004)) suggest that the ultrabright, ultrashort pulses will allow much smaller samples to be utilized, roughly on the order of about 10-^{S}Bm diameter, which is roughly in the range of the ordered patch size of bacteriorhodopsin (which is perhaps the best-behaved membrane protein for forming two-dimensional crystals by standard techniques), giving reason to believe that this experiment should be feasible. Compared to two-dimensional crystals in EM, the required crystal size for the LCLS may turn out to be rather large. However, compared to supported bilayers that are currently used for diffraction at synchrotrons, which are on the order of many mm^2, this is very small.

Advances in nanotechnology for ordering samples on surfaces will be important for this endeavor. Although EM has some advantages (Henderson, 1995), an appeal of this proposal is that the ability to do grazing incidence-diffraction experiments with X-rays, ie. below the critical angle where the penetration of the incident beam into the supporting material is exponentially reduced, is particularly amendable to trying a wide variety of nanotechnology methods for ordering the samples. While the intriguing proposal of others to use single-particle scattering to determine structures is attractive for overcoming the crystallization problem (Neutze *et al*., 2000), it is possible that physics may place a lower limit on the size of biomolecules that might yield data near atomic resolution with that methodology. In the two-dimensional case, one may overcome the damage-limiting physics by increasing the size of the crystal or array to an optimal size that will allow the experiment to be done. Thus, probably the greatest challenge of the experiments will be to obtain reproducible, two-dimensional crystals that are nearly atomically ordered over the dimensions of the crystals needed to perform the experiments. The recent emphasis on nanotechnology gives cause for hope that this hurdle might be overcome.

How?

Thus, the scientific thrust here will include efforts to improve techniques for preparing samples, as well as efforts to establish methods for collecting and analyzing the data to ultimately yield structures at or near atomic resolution. Practically, the proposal can be separated into 5 tasks. Some of these may overlap with other Letters of Intent.

(1) Sample preparation

Initial work will be done with proteins that have been shown to readily crystallize in two-dimension, such as bacteriorhodopsin (Verclas *et al*., 1999), bacterial surface-layer proteins (Weygand *et al*., 1999, 2002), and other photosynthetic proteins, trying to push the limits of what can be achieved at current synchrotron sources. But some new techniques that will be pursued include:

(i) Shallow wells or pedestals will be generated in materials such as silicon wafers using e-beam lithography (Warren, 1992), along with techniques to adjust hydrophilicity and hydrophobicity of these features and their barriers as desired. Membrane proteins in lipid bilayers will be crystallized on these substrates via dialysis in the presence of external fields to attempt to influence the crystalline order ("Field-Enhanced Self Assembly"). It has been shown that UV-lithography-generated corrals can maintain a fluid environment in membrane bilayers (Groves & Boxer, 2002). The presence of water above and below the lipid bilayer in such environments is important, not only for the fluidity during nucleation and growth of crystals, but also since exposure of proteins to the air:water interface can lead to protein denaturation. The primary fields that will be applied to attempt to improve crystalline order will be magnetic. Proteins are weakly diamagnetically anisotropic, and although the orientational effect of a very high magnetic field on a single protein molecule is small, the effect is additive, and significant orientation effects can be observed on protein assemblies (Torbet, 1987) and on membrane bicelles and biomembrane mimetic liquid crystals (Losonczi & Prestegard, 1998; Firestone *et al*., 2000). It has been reported that three-dimensional crystals of lysozyme grown in magnetic fields are aligned to the field (Astier *et al*., 1998), and that moderate magnetic fields can make small improvements on crystalline order (see Lubbert *et al*., 2004). To increase the magnetic response of proteins in supported membranes for alignment, paramagnetic ligands, such as lanthanides (Veglia & Opella, 2000) may be bound. However, other fields will be tried, such as optomechanical manipulations ("optical tweezers", Ashkin & Dziedic, 1987), and perhaps electric fields (Groves *et al*., 1996; Groves & Boxer, 2002). Laser tweezers have been used for the micromanipulation of monomolecular hexatic phase domains at water surfaces (Wurlitzer *et al*., 2001; Fischer & L^osche, 2004), and further modifications of this technique will be attempted. Also, lasers will be used to generate interference patterns on wells during crystallization, which, in the simplest view, may modify order by generating stripes of gentle heating. Besides achieving crystalline order, part of the challenge is to find the right conditions and geometries to avoid edge effects in the diffraction, and to reproducibly make high-quality samples. After ordering, samples will likely be cryocooled for ease of manipulation.

(ii) Fv or Fab fragments of antibodies, or other molecular affinity ligands (Rodi *et al*., 2001), will be covalently linked to surfaces to organize two-dimensional protein arrays. For example, it has been shown that TiO_2 surfaces can support lipid bilayers (Starr & Thompson, 2000), and that defect sites in such materials can be chemically reactive (Dimitrijevic *et al*., 2003). The challenge will be how to pattern the arrays with linkage sites in ways that are nearly atomically perfect. Scanning methods, mechanical methods, chemical rulers, or laser interference fields are among methods that may be tried; this is a type of question that many in the field of nanotechnology pursue. However, for structure determination, translational disorder may not be as disruptive as rotational disorder. If a flexible covalent linker is used, magnetic tags may be added to improve the rotational order. In another type of hybrid method, gold nanoparticles ligated to antibodies will be used to optomechanically direct the in-plane alignment of two-dimensional protein crystals, as it has been demonstrated that hexatic monolayer phase domains can be manipulated via colloidal particles on which forces are exerted by laser tweezers (Wurlitzer *et al*., 2001; Fischer & L^osche, 2004).

(2) Calculations

Initial calculations indicate that crystals of roughly 10-:m diameter are in the range for structure determination near atomic resolution (Weckert, 2001; Becker & Weckert, 2004). Such calculations will be improved, and will include taking into account background scattering, signal-to-noise on detectors, the number of crystals necessary to determine a

structure, the effects of various types of disorder on the diffraction, and perhaps more sophisticated dynamic models. The aim is to refine estimates of what sample properties must be achieved in order to determine structures at or near atomic resolution. Also, the results may influence how the diffraction data are interpreted.

(3) Programs

The diffraction patterns from these samples may show significant speckle and interference phenomena, which may require direct Fourier inversion techniques akin to those represented in other Letters of Intent for the LCLS (involving I. Robinson, and E. Weckert). However, to a first approximation, it may be desirable to treat the Bragg-rod diffraction somewhat like the kinematic scattering of three-dimensional crystals. That is, for a given still X-ray shot on a sample, cuts through multiple Bragg rods will be collected on an area detector, and for multiple samples, these can be processed, merged, and scaled analogous to reflections from three-dimensional crystals collected by the rotation method. A program will be developed for this purpose.

(4) Phasing

Members of the team have expertise in sophisticated phasing techniques. As above, phasing may be possible via "oversampling" or holography, and these techniques will be investigated in conjunction with other programs. Again, short of perfect optics and samples, it may be desirable to phase samples by methods more akin to traditional methods. For example, adaptations of Single Wavelength Anomalous Dispersion (Dauter, 2002), using sulfur or iodine, may be particularly desirable. Part of this work may include collaborating to investigate edge shifts of elements and compounds in ultrahigh fields. Also, it may be desirable to incorporate phasing information into the supporting material or affinity-reagent linkers. Some examples of two-dimensional sample preparations are listed in Becker & Weckert (2004).

(5) Once electron-density maps are generated, standard techniques for model building and structure refinement will be readily applied.

Thus, by these efforts, we aim to create improved techniques for structure determination of membrane proteins. There can be additional advantages from pursuit of this goal (Becker & Weckert, 2004). First, X-ray diffraction experiments and electron microscopy experiments are complementary. Whereas electron microscopy/diffraction experiments yield a Coulomb-Potential map, X-Ray crystallography experiments yield an electron-density map; these are related by the Poisson equation (Chang *et al.*, 1999). If both experiments are conducted on similar samples to minimize systematic differences, careful comparison of the two may yield detailed "dielectric maps", providing rich experimental information on electrostatics. Electrostatics, which are often calculated but rarely measured, are commonly thought to be the most important forces on protein function. Second, there are advantages for observation of time-resolved, dynamic states in crystals of bio-molecules. Crystallography is very powerful in that it yields detailed, three-dimensional snapshots of molecules in well-defined states. But to understand transitions between states, such as in catalysis, ie. to investigate true intermediate states directly, it is necessary to perform time-resolved experiments. Such time-resolved experiments typically employ a light flash to trigger a reaction, using bio-molecules with intrinsic pigments, such as in photosynthesis, vision, or phototaxis, or using artificial caged compounds as the trigger. For a variety of reasons, these experiments are very difficult; despite these challenges, it is important to pursue such studies. Ultrafast X-ray experiments on two-dimensional crystals might help to alleviate some of the problems. For

example, data could be collected more rapidly, and synchronization of triggering reactions might be relatively improved. Finally, if indeed nanotechnology ultimately yields convenient new ways to form two-dimensional protein arrays, then X-ray crystallography on two-dimensional crystals could potentially become a very important area for water-soluble proteins as well, bypassing the need to grow three-dimensional crystals.

We thank you for your consideration regarding this proposal, and we eagerly look forward to the new science that the LCLS will enable!

Sincerely,

Michael Becker, Ph.D.
Spokesperson for this proposal

Some comments regarding in-hutch instrumentation: for in-hutch instrumentation, we look to partner with scientists at SLAC or elsewhere. Some instrumentation design features that seem desirable may include:

- a rapid, sample-changing robot for two-dimensional "crystals on a chip"
- a goniometer/diffractometer with the ability to rapidly position the samples, and to find grazing incidence and other angles
- the ability to visualize tiny samples
- a rapid area detector, decoupled from the diffractometer/goniometer
- a cryosystem
- a housing chamber in vacuum

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Team members:

Our team consists of scientists with diverse backgrounds and a broad range of expertise, but with a healthy modicum of overlap. With the contacts that we have, including additional scientists to provide a variety of proteins for experiments will not be a problem. In alphabetical order, the team members are:

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