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Jan.23,2004

Biocomplexity Faculty Search Committee
c/o Prof. Rob de Ruyter van Steveninck
Biocomplexity Institute
Indiana University
Swain Hall West 117
Bloomington, IN 47405-7105

Re: tenure-track faculty application

Dear Prof. R. de Ruyter van Steveninck,

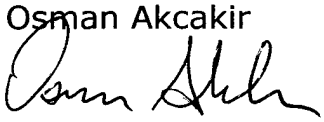
I would like to apply to the faculty position your department has advertised on A.I.P.'s web site. I am particularly interested in applying single-molecule techniques to topics which range from measuring single-molecule protein dynamics during protein folding-unfolding to measuring molecular transport dynamics in living organisms, as patterns are formed.

I believe that my background in single-molecule and fluorescence correlation spectroscopy methods, as well as my recently acquired skills in molecular biology and biochemistry make me an ideal candidate to engage in the kind of interdisciplinary research your institute wishes to foster.

Although I have a collaborator for one of my projects (morphogen gradient quantification), I am also open to collaborating with scientists on your campus on other projects.

Thank you for your consideration, and I look forward to hearing from you in the near future.

Sincerely,
Osman Akcakir



Research Proposal *by* Osman Akcakir

The dynamics of molecules is critical for living things; from the molecular level of complexity which includes protein dynamics (including the dynamics of protein folding, *i.e.* intramolecular dynamics, to the cellular and organism levels of complexity, where the dynamics of signaling molecules within cells and between cells ensures appropriate responses to stimuli (intermolecular dynamics). I am interested in experimentally probing the dynamics of biological matter at these 2 levels of complexity using the methods of single-molecule spectroscopy and fluorescence correlation spectroscopy.

Single-Molecule Protein Folding

Proteins that don't fold properly into their native 3-dimensional structure do not function properly either. Protein misfolding is known to be a primary cause of many diseases including Creutzfeld-Jakob disease (CJD) and Alzheimer's disease. Unfortuantely, the principles governing the folding of proteins into a unique structure are poorly understood. It is often modeled as a chemical reaction between two species, a folded species and an unfolded species. This simplification obscures the true complexity of the reaction however. The first misleading assumption is that of a single unfolded species. In reality there is an ensemble of unfolded conformations. Transitions from different unfolded conformations to the folded conformation may be associated with a distribution of barrier heights, which leads us to the second misleading assumption, that there exists a well defined barrier height to folding. The non-Arrhenius temperature dependence of the folding kinetics points out this shortcoming (simple reactions show an Arrhenius-like temperature dependence). Conventional techniques perform measurement on $>10^{12}$ proteins at one time, causing results to be ensemble averaged. By immobilizing fluorescently labeled proteins, imaging with high numerical aperture optics and confocally located sensitive photon detectors, signals from single proteins may be measured. In this way the heterogeneity of the system may be resolved.

I have choosen to work with a small protein domain *src SH3*, which exists as a protein adaptor module in several hundred different proteins. It is also a good model system for studying protein folding with single molecule techniques. The time scales of folding and unfolding (20ms and 10s) are ideal for single-molecule fluorescence studies. Equilibrium fluctuations resulting in folding and unfolding events may be measured. Had the folding reaction occurred much faster, characterizing it would be limited due to poor photon statistics, and unfolding reactions occurring at much longer time scales would be prohibitive due to photobleaching of the fluorophore. The plasmid DNA for a double cysteine mutant of *src SH3* was kindly gifted by D. Baker (U. of Washington). I have successfully expressed the protein, labeled its cysteine residues (one at each terminal) with Alexafluor 488 maleimide, and purified the doubly labeled fraction with ion-exchange chromatography. This doubly labeled fraction shows a large increase in fluorescence (due to unquenching) upon unfolding with a denaturant, guanidine chloride (as confirmed by ensemble fluorescence measurements). Data from a single molecule shows equilibrium fluctuations (see figure 1). This data is effectively the reaction coordinate for the end to end distance (i.e. fluorescent labels are at each end). Other reaction coordinates may be genetically engineered (i.e. by mutating a particular residue to cysteine, which may be subsequently labeled) and monitored by using FRET (Fluorescence Resonance Energy Transfer) when spectroscopically compatible dyes areused, allowing distance resolution down to the 10A range.

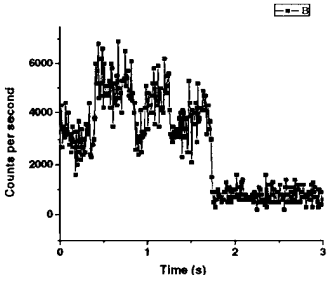


Figure 1: Single-molecule trace of doubly labeled src SH3, immobilized in agarose gel.

I wish to apply single-molecule fluorescence techniques to immobilized proteins to study folding and unfolding events one protein at a time. In this way ensemble averaging is removed, and the unfolded state ensemble and the kinetic barriers of each member of the ensemble may be mapped out. In addition I would like to probe, at the single molecule level, to test the hypothesis that the non-Arrhenius temperature dependence of folding rates is due to a distribution of unfolded states and barriers to the folded state.

2-photon FCS microscopy

At the level of the organism, I wish to probe the transport dynamics of signaling molecules in the developing fruit fly (Drosophila). I propose to do this with a 2-photon FCS (Fluorescence Correlation Spectroscopy) microscope, which is ideally suited for quantifying very low concentrations (e.g. sub-nM) of fluorescent molecules in living organisms. Non-linear excitation confines absorbtion to the focal plane, minimizing photobleaching and phototoxicity, an important advantage. Using genetic engineering, it is possible to fuse a fluorescent protein (Green Fluorescent Protein) to a protein of interest, for tracking purposes. By defining a microscopic probe volume with a high-numerical aperture objective, single molecules of GFP-fusion protein fluctuating in and out of this volume element may be detected. An autocorrelation of this signal yields both absolute concentration information and mobility information. In contrast, traditional fluorescence microscopy including confocal microscopy gives the researcher a static spatial map of the structure, and is blind to the fluctuating processes occurring within each pixel. FCS can not only quantify the absolute concentration of labeled proteins in a given region, but it can also characterize the physical origin of fluctuation process (e.g. passive diffusion or active transport) by the nature of the autocorrelation function decay.

Measuring morphogen gradients *in vivo*

One of the most amazing features of the 'genetic program' for development is its ability to generate patterns in space and time. How can a 3-D map be expressed in the linear sequence of DNA? How do the cells know where to go and what to differentiate into? The development of these patterns is principally in response to intercellular signaling. Looked at as a complex system, we may consider an embryonic organism as a collection of cells, each a node which may both generate signals and process signals (i.e. via signaling molecules/proteins and membrane receptors), based on a set of rules. To completely define such a system, the physical processes governing, 1. signal generation, 2. signal transport and 3. signal transduction must be quantified. Theoretical models based on coupled reaction-diffusion mechanisms as well as gene network dynamics have been proposed to explain pattern formation in various model organisms. While many models make qualitatively accurate predictions, quantitative validation has been missing due to the absence of experimental data. My research plans involve measuring the absolute concentration and transport properties of an essential class of signaling proteins called morphogens, directly in living organisms, during development.

Morphogens are generated in a localized area, and activate specialized membrane receptors on surrounding cells, causing a signal which turns on a transcription factor, thus altering the expression of that cell. It is believed that cells respond to the morphogen gradient in a concentration dependent way. Different morphogen detection thresholds elicit different responses; for example, a cell may differentiate into a particular type of cell, as opposed on another type depending on the concentration of morphogen detected. So, positional information is encoded in this gradient, with cells far away from the source of the morphogen detecting a low morphogen concentration, and responding differently to a cell close to the morphogen source, which detects a high morphogen concentration. A deeper understanding of morphogenesis (development of pattern formation) is likely to lie in the detailed spatio-temporal

features of this gradient and the underlying physical processes governing its generation and maintenance. For example, in Drosophila (fruit fly), the morphogen Dpp (Decapentaplegic) is involved in many different stages of morphogenesis, including wing formation in the imaginal wing disc of the developing embryo. In the wing disk, Dpp is secreted by a column of cells lying along its anterior-posterior axis (see figure 2). I propose to measure: 1. the absolute concentration of Dpp-GFP in both the anterior and posterior domains as a function of distance from the anterior-posterior axis, 2. the transport properties of Dpp-GFP, as measured by the decay profile of the autocorrelation function. By analyzing the decay profile of the measured autocorrelation function, I wish to determine the physical process underlying Dpp gradient formation; is it passive diffusion, active transport or some other process?

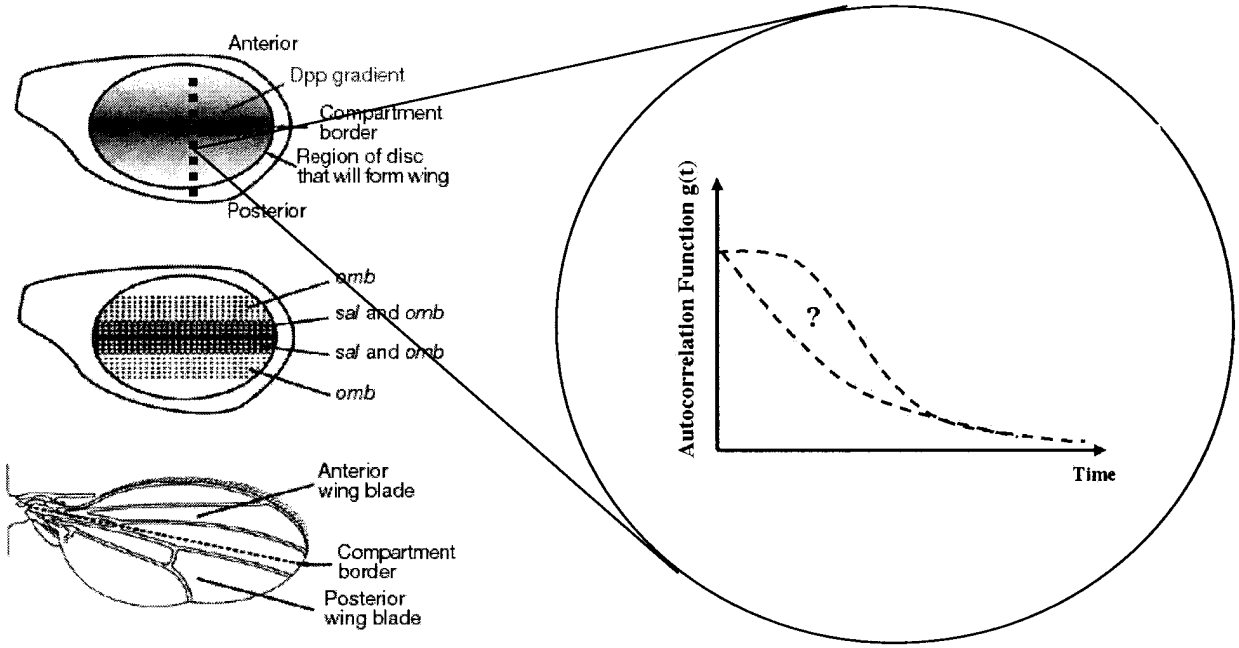


Figure 2: Imaginal wing disk on the left, at successive stages of growth top to bottom. Dpp concentration and dynamics probed by 2-photon FCS and fitting the acquired autocorrelation function(right).

What physical parameters of the morphogen gradient system determines the length scales and time scales of the evolving structures of the organism during development? To what extent are they determining factors of pattern formation during development? It would be interesting to see how modification of these physico-kinetic parameters (e.g. inhibiting active transport, decreasing morphogen production rate etc.) modify the morphogen gradient, and thus the developmental end-product of the organism. How robust is the morphogen gradient to such perturbations? By quantifying these effects I would like to be able to test the extent to which physical processes determine developmental fate.

Prof. S. Cohen (European Molecular Biology Laboratory, Heidelberg, Germany) has agreed to collaborate on this project, and will provide the necessary fly mutants (e.g. Dpp-GFP).

The molecular biology and biochemistry skills I have learned at Michigan will allow me to continue the single-molecule research independently. I believe that my background in single-molecule spectroscopy and 2-photon FCS microscopy makes me an ideal candidate to probe these questions, which are exciting frontiers on the interface of physics and biology.