New and Notable

Scan and Conquer: A Novel Approach to the Analysis of Interactions by Molecular Brightness Determination

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The investigation of molecular mechanisms in living cells is an important step toward understanding processes in health and diseases and for the development of therapeutic interventions. Viruses are a particularly important problem, since we have, in most cases, very limited remedies and rely mainly on vaccinations if available. A better understanding of the different stages of the viral life cycle on a molecular level would provide new clues of where and how to intervene and block viral infection, reproduction, or shedding. In this issue of *Biophysical Journal*, Smith et al. (1) address a crucial step in viral assembly by investigating how group-specific antigen (Gag) matrix domains of different viruses bind to plasma membranes as a first stage to assembly. For this purpose, they have extended fluorescence brightness analysis methods to the complex environment of living cells.

The quantitative analysis of the functions, actions, and interactions of molecules in cell biology has received considerable attention in the last decades with ever-improving instrumentation and analysis methods. One particular branch of fluorescence techniques relies on the analysis of molecular brightness to determine molecular aggregations and interactions. In essence, one measures the number of photons detected from a particle and then calculates how many fluorophores had to be present within that particle to yield that number of photons. Since its inception (2,3), the analysis of molecular brightness has made tremendous advances from single-point confocal measurements in vitro to highly multiplexed measurements in living cells using camera-based approaches (4,5).

This works well as long as all particles are randomly distributed within the observed volume. However, cells are complex three-dimensional structures where proteins can reside in and exchange between different compartments with different stoichiometries. And because the observation volume of light microscopes, characterized by the point-spread function, is on the order of hundreds of nanometers in the radial direction and micrometers in the axial direction, one will in general observe many different particles with different stoichiometries at very different nonrandom locations, simultaneously. Thus, a distinction among proteins that reside in the cell cytoplasm, on the cell membrane, or in other compartments is difficult, and their brightness analysis poses a particularly challenging problem.

Smith at al. (1) have developed a new approach to this problem, so-called *z*-scan fluorescence fluctuation spectroscopy (z-scan FFS) by combining *z*-scanning with brightness analysis. Axial *z*-scanning has been used in the past for determining laser profiles (6) or for analyzing errors in fluorescence correlation spectroscopy data (7), until Benda et al. (8) inverted the problem and demonstrated that if the laser profile is known one can determine diffusion coefficients in fluorescence correlation spectroscopy more accurately. More recently, it was also used for the correction of molecular brightness analysis (9). Here Smith et al. (1) extend *z*-scan FFS as a tool to distinguish molecules of different brightness in layers, which are smaller than the axial size of the point-spread function of the microscope.

Using H-Ras as a model, they demonstrate that they can distinguish plasma membrane-bound molecules from a cytoplasmic pool and then apply *z*-scan FFS to matrix domains of Gag polyproteins of human T-cell leukemia virus type 1 and human immunodeficiency virus type 1. Gag proteins fulfill a range of different functions in the lifecycle of a virus, but in particular can oligomerize and bind to the plasma membrane for viral assembly. This class of protein is therefore an ideal target for *z*-scan FFS, which can distinguish monomeric and oligomeric complexes in the cytoplasm and on the membrane. Interestingly the authors observe differences in the distribution of the Gag proteins of the different viruses, implying that their mechanism of assembly is different. Extensions of these studies to full-length proteins will provide a more detailed picture of these processes.

The article gives a detailed overview of the different problems faced by molecular brightness analysis in three dimensions. To be able to disentangle the brightness values of molecules in the different layers, the geometry of the layers; the brightness of the molecules in the layers; and the contributions of neighboring layer intensity had to be taken into account. The authors, to our knowledge for the first time, determine the influence of these different effects by simulations and experiments. This renders *z*-scan FFS a powerful tool to determine molecular stoichiometries and interactions in live cells. But future refinements could extend the applicability of the technique.

A question that remains an open issue is whether *z*-scan FFS can be used in systems in which the underlying three-dimensional structure is not known. In this work, the authors assume a layered structure with features much smaller than the resolution limit, which is justified for a membrane/cytoplasm distinction. But this becomes

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more complicated when, for example, the nucleus is included in the model, or even organelles. Future studies will have to show where the limits of *z*-scan FFS lie. And although *z*-scan FFS has been shown here to work with two-photon excitation, a wider spread of the technology would aid in the development of the theory for one-photon excitation, as the authors noted themselves (9) and as has also happened for other brightness analysis methods (10), for which these instruments are more readily available.

Finally, to address environments which do not only have a layered structure but have a general three-dimensional structure, scanning could be extended also in radial directions. After this pioneering work of Smith et al. (1), these other extensions are on the horizon. *Z*-scan FFS is an important step toward quantitative analysis of molecular interactions in a three-dimensional cell environment and will surely find many applications.

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