COMMENTARY

A promising approach to molecular counting problem in superresolution microscopy

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Molecular Counting Problem in Superresolution Microscopy

Cellular processes are often controlled by aggregates of protein complexes. Recent studies have witnessed that the stoichiometry, or the relative composition of protein complexes, can be dynamical (1–3). Quantifying the stoichiometry of these complexes, not only at the mean level but also its full distribution, is therefore a grand challenge of molecular biology. This is exactly the issue that Rollins et al. (4) address using state-of-the-art superresolution imaging data (5–8).

Conventional protein-counting methods include quantitative fluorescence microscopy (9) and fluorescence photobleaching (1, 2). These methods all rely on diffraction-limited methods. The maximum spatial resolution is typically a few hundred nanometers for counting proteins. In contrast, superresolution microscopy can overcome the diffraction limit, not only in vitro but also in living cells. One of the superresolution methods is termed photoactivated localization microscopy (PALM) (5).

PALM is now broadly available to cell biologists. It works by genetically encoding onto the protein subunit a photoswitchable fluorescent protein (FP). The FPs can then be activated one at a time by exposing the cell to low-intensity light. In the cell, the protein subunits containing FPs have assembled into their respective protein complexes. This process can be captured and quantified by the intensities of FPs; the FPs eventually photobleach one at a time. Therefore, the signals originated from multiple FPs of one protein complex within a diffraction-limited spot can be separated in time, but they cannot be separated in space. The spatial location of each stochastically activated FP can then be identified by searching the center of the spatial Gaussian distribution where the photons originate.

Although each fluorescent intensity spike should in principle represent the FP associated with activation of one protein subunit and subsequent corresponding photobleaching, the real situation is more complex. FPs stochastically blink. In other words, they may stochastically turn on and off reversibly before photobleaching. Therefore, registering the number of fluorescent peaks can overcount the number of FPs.

To resolve this issue, one may first characterize the photophysics of the FPs in vitro. On the basis of this information, one can then correct for how many times one expects an FP to blink on average. Although this procedure may seem reasonable at first sight, the issue is not that easily resolved. In fact, the photophysics of the FP depends on its local cellular environment (10).

Rollins et al.'s method inspires the research community by showing that quantitative insight from complex data can be inferred from models with no adjustable parameters.

Whereas the superresolution microscopy has provided the data necessary to resolve protein complexes at length scales smaller than the diffraction limit, quantitatively determining protein complex stoichiometry in a reliable way still remains a grand challenge (11).

A Possible Resolution to Single Molecular Counting Problem

Rollins et al. (4) provide a possible solution to the single-molecule counting problem mentioned above. The authors do so by showing that protein subunits can be counted correctly while simultaneously inferring the FPs photophysics.

To solve the overcounting issue, Rollins et al. (4) apply a maximum-likelihood method in statistics. The authors address the following question: What FP photophysics and FP number are most likely to reproduce the data? The elegant model they provide for the fluorescence of a collection of FPs is motivated by the formalism of aggregated Markov models. These models were first exploited in biophysics in the context of the analysis of ion channel patch-clamp experiments (12–15).

In contrast to thresholding methods (11), Rollins et al. (4) rely on a stochastic approach. This approach treats the photophysics of the FP (i.e., its activation, blinking, and photobleaching) stochastically as it occurs in the cell. Only a stochastic approach can naturally treat inherently stochastic effects. These effects include overlapping peaks, when one FP activates before the previous FP has photobleached, or superposing peaks, where two FPs fluoresce at the same time. The effects mentioned can occur under circumstances that cannot a priori be discounted (such as slow photobleaching/fast blinking, slow photobleaching/fast activation, many FPs, and so on). Rollins et al. (4) also address the problem of missed transitions by an FP (because of a camera's finite acquisition time).

Although this study is focused on PALM data (5), the methodology presented by Rollins et al. (4) should provide a broad framework in which to think about the analysis of other imaging data. This includes, in particular, other superresolution methods, such as stochastic optical reconstruction microscopy (6).

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