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Biologists Wanted: New Fluorescence Fluctuation Tools for Cell Biology

Katharina Gaus[1,2,*]
[1]European Molecular Biology Laboratory Australia Node in Single Molecule Science, School of Medical Sciences, and [2]Australian Research Council Centre of Excellence in Advanced Molecular Imaging, University of New South Wales, Sydney, Australia

A change in perspective can make the difference between a problem being unsolvable and a new line of investigation opening up. In fluorescence microscopy, we often take snapshots of the distribution of fluorescent molecules and thus only have a bird's eye view of a biological sample. Approaches that are based on analyzing fluorescence fluctuations change our perspective and allow us to view the biological world from the point of the view of the fluorescent molecule itself. In recent issues of the *Biophysical Journal*, extensions of fluorescence fluctuation tools have been reported that provide new opportunities to the cell biologist (1,2).

The key advantage of fluorescence correlation spectroscopy (FCS)-based methods is that they provide a measure of the diffusive behavior of the fluorescent molecules in live cells at low to moderate concentrations. These diffusion signatures are important because they describe the immediate cellular environment as experienced by the molecule of interest. As cellular environment is exceedingly heterogeneous, measuring a large number of diffusing molecules at many places within the cell becomes critical. Here FCS-based methods have an advantage over single-particle tracking; sampling more molecules in shorter periods of time not only makes the life of the experimentalist easier but also the resulting data more statistically reliable.

In its original experimental format, FCS is performed only at selected single spots within the sample. Varying the spot size, for example with stimulated emission depletion, can probe whether membrane proteins diffuse freely, are confined within domains, or experience hindered diffusions through the presence of obstacles such as cortical actin (3,4). While these forms of FCS tell us how molecules diffuse, they do not tell us where they go or how diffusion differs across the cell. Two different approaches that address these types of questions, do so by including a spatial dimension into FCS-based analysis: spatiotemporal image correlation spectroscopy (STICS) and pair correlation function (pCF) where intensity images or fluorescence intensity along a scanned line are recorded over time.

In STICS and related methods (5–7), the spatial cross correlations are calculated for each pair of images that are separated by the time interval, τ. The width of the correlation function indicates the spatial scale and rate of transport. The time-dependent increase in width directly maps the average diffusion while any translation of the correlation peak indicates directed motion. Thus both diffusion and translation can be extracted from STICS measurements. One of the challenges, however, has been the capture of very fast moving molecules (7). A recent publication in the *Biophysical Journal* has now overcome this issue by developing a mathematical framework that accounts for in-frame motion (2). With these improved fitting procedures even the diffusion of GFP in bacteria could be captured at 25 frames/s. A combination of the improved STICS fitting procedure with compartmentalizing the imaging in many small regions of interest (8) could yield maps that display the heterogeneity in molecular diffusion within a single cell.

Previously, pCF (9) or raster image correlation spectroscopy (10) were the fluctuation tools of choice for fast moving molecules. In pCF, the cross-correlation of fluorescence fluctuations is calculated for each pair of points and lag times along the scanned line. As pCF asked whether molecules could diffuse freely along the direction of the line scan, this approach particularly lends itself to probe accessibility into or within large cellular compartments such as the nucleus (11) but can also be multiplexed with other imaging modalities such as fluorescence resonance energy transfer (12). We recently combined pCF with a molecular brightness analysis to map the diffusion of transcription factors as a function of their oligomeric state (13).

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*Correspondence: k.gaus@unsw.edu.au

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Di Rienzo et al. (1) have now extended pCF to a two-dimensional image analysis (2D-pCF), essentially closing the gap between the 2D mapping capabilities of STICS with the directional analysis inherent to pCF. In this approach, a starting point is selected and the cross correlation to all other points in the image calculated so that the resulting 2D-pCF data is a 3D matrix. To make data visualization easier, the authors plotted the data in an image-mean-squared displacement format, where the length of the arrow indicates how far the molecule could go from the starting position in the indicated direction in the time interval τ. At short lag times, the diffusion directions may be isotropic but permanent diffusion barriers become immediately apparent at larger lag times. This is equivalent to short and long trajectories in single-particle tracking but the advantage of 2D-pCF is that one can select any point within the image. An example shown by Di Rienzo et al. (1) is how the diffusion of the membrane-bound kinase H-Ras was affected by its proximity to focal adhesions.

With the new fluctuation tools at hand, cell biologists can now map the heterogeneities in diffusion signatures across a cell with unprecedented temporal resolution. An improvement in spatial resolution can be achieved with structured illumination microscopy, for example (14). There are many applications where these analyses could provide biologically valuable information such as in membrane diffusion (5,7), protein trafficking, nuclear import and export, and the search strategies of transcription factors in the context of the nucleus architecture (12,13). It is now up to the cell biology community to take advantage of these imaging tools. A change in perspective could yield surprising discoveries.

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