Measuring Nanodistances in Cells

Assessing Stoichiometry from Nanoscale Molecular Separation

Understanding how complex processes such as receptor signal transduction work in cells requires knowledge of the structure-function relationships underlying the composition of protein complexes. Characterization of the oligomerization state of complexes requires the measurement of distances around 10-15 nm, too long for fluorescence energy transfer (FRET), but too small for optical resolution. Here we discuss various approaches that are being taken to measure these distances in cells.

Spatial Resolution Requirements for Cell Biology

Understanding the structure-function relationships of biological macromolecules requires us to determine structure at high resolution. This can range from resolutions around 0.1 nm for detailed protein structure, around 1-10 nm for measurement of inter-molecular distances, and 10-20 nm to measure inter-unit separation and therefore the oligomerization states of protein complexes. Atomic resolution structures can be obtained from x-ray crystallography [1], while electron microscopy provides structural information at the nanometer level [2]. Fluorescence Resonance Energy Transfer (FRET) [3] is sensitive to distances in the range of ~2-8 nm, and is therefore applicable to the study of protein conformation, e.g. distances between domains.

Nanoscale Imaging Methods
Measurement of distances in the 10-20 nm range is more challenging, but is important. For example, there is a need to understand how the oligomerization state of tyrosine kinase receptors in different cellular contexts affects receptor activity. A good example of this is the epidermal growth factor receptor (EGFR), which is expressed or highly expressed in a variety of human tumors of epithelial origin [4]. Structural studies of EGFR indicate that the inter-unit separation in receptor oligomers should be in the region of 10-15 nm [5]. This distance falls in a "resolution gap" between FRET and optical microscopy, which is diffraction-limited around 200 nm at best.

In recent years, a number of so-called "super-resolution" optical methods have been developed, that break the diffraction limit for light microscopy.

These include stimulated emission depletion (STED) microscopy [6], near-field scanning optical microscopy (NSOM) [7], photo-activated localization microscopy (PALM) [8], fluorescence imaging with one-nanometer accuracy (FIONA) [9], single-molecule high resolution imaging with photobleaching (SHRImP) [10], nanometer-localized multiple single-molecule (NALMS) microscopy [11] and single-molecule high resolution co-location (SHREC) [12]. Theoretically, many of these techniques have the potential to measure distances in the required range. However, there are challenges in applying them to the cellular environment. NSOM is not well-suited to the wet conditions required for living or lightly fixed cells. STED and PALM are essentially ensemble imaging techniques that are not easily applied to the measurement of the distance between two or more specific molecules in the crowded cell environment.

SHRImP, SHREC, and NALMS can measure distances between molecules with better than 10 nm resolution. Like PALM, all these methods "beat" the diffraction limit by imaging single molecules, fitting the point spread function (PSF) of the
microscope, and locating its center with nanometer accuracy. NALMS uses single molecule detection to count discrete steps in traces of fluorescence intensity versus time from diffraction limited spots, each step corresponding to the activation or bleaching of a single fluorophore, and measuring the change in the PSF of the spot before and after a step.

In SHRImP, global fitting is carried out on spots before and after bleaching, producing nearly identical results to the sequential NALMS method. SHREC again uses PSF fitting, but two different fluorophores with different spectral characteristics are used, imaged in distinct channels which must be very accurately registered to determine intra-molecular distance. In all these techniques, spatial resolution is ultimately limited by the signal-to-noise ratio of the data, which determines the precision with which the center of the PSF can be determined. For this reason, SHRImP, SHREC, and NALMS have so far been demonstrated only in "clean" samples such as purified, immobilized molecules on glass, and in the presence of antifade reagents.

**Difficulties of Nanoscale Imaging in Cells**

The environment of mammalian cells is not conducive to the collection of high signal-to-noise data. The main source of background noise is intracellular autofluorescence, arising from molecules such as NADPH and flavins [13]. A common approach to reduce this when looking at membrane proteins is to use total internal reflection (TIRF) excitation (fig. 1) [14].

TIRF creates an evanescent field on the coverslip on which the cells are cultured. The field reduces exponentially with depth, and only penetrates approximately 300 nm into the sample. Thus, fluorescence is not excited in the bulk of the cell, reducing autofluorescence. This enables single molecule detection, as shown in figure 2. Single molecules are clearly visible, but there is also background noise. This results from residual autofluorescence, scattered light, and fluorescence from out-of-focus fluorophores.

**Current Developments**
In our laboratory we are developing new methods to improve the accuracy of intramolecular distance measurement in cells and tissues. One of our microscopes is shown in figure 3 [15]. The microscope is supplied with laser illumination through an optical fiber via a TIRF slider accessory. Splitting optics (Cairn Optosplit III) divide the image into three spectrally distinct but spatially identical channels, allowing simultaneous imaging of three different fluorophores. Fluorescence is detected by an EMCCD camera (Andor iXon).

Of particular note is the environmental chamber/incubator that surrounds the microscope stage. This is required to maintain the stage at a constant temperature and is particularly important for nanometer distance measurements, as temperature changes of fractions of a degree result in stage movements greater than the distances measured. We currently achieve sub-10 nm positioning resolution in cells by using new data fitting algorithms that perform well in the noisy environment of cells (manuscript in preparation). Crucially, our techniques give robust estimates of errors, allowing the significance of distance measurements to be properly determined. Figure 4 shows typical single molecule traces and accompanying position measurement from labelled EGFR complexes in fixed HeLa cells.

**Forward Look**

It would also be desirable to apply the same nanoscale distance measurements to tissue sections, such as tumor biopsies. Many anti-cancer therapies target EGFR, and single molecule imaging could classify patients according to receptor behavior, with the aim of choosing the most appropriate therapy for the individual tumor type. The tissue environment is even more challenging than the cellular one, with increased background contributions from autofluorescence and scatter, as well as the problem of achieving TIRF excitation in a sample that has not been grown directly on the coverslip. However, preliminary measurements in our laboratory indicate that it will be possible to achieve similar levels of distance accuracy to that in cultured cells.

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**References**


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