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Single plane illumination microscopy as a tool for studying nucleome dynamics

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ABSTRACT

Single plane illumination microscopy (SPIM) is a new optical method that has become extremely important in recent years. It is based on the formation of a "light slice" in the specimen in which fluorescently tagged molecules are observed. The spatial resolution is close to that of confocal optics, but without the disadvantages inherent to scanning or high laser irradiation doses. A recent development is light sheet fluctuation microscopy, which exploits the dynamic information contained in the fluorescence intensity fluctuations of each image pixel.

Here we review the principles of this method and show some recent applications to the dynamics of transcription factors and chromatin. We show that the dimerization of Fos and Jun proteins is directly linked to their binding to DNA; that nuclear receptor activation changes their intranuclear dynamics; and that the viscoelastic behavior of interphase chromatin strongly depends on the presence of lamin A.

1. Introduction

The transport properties of biological macromolecules that need to access defined sites in the nucleome or to be exported from the nucleus, as well as the dynamics of the chromatin network itself, depend on the properties of the densely crowded network formed by chromatin and other nuclear constituents. Thus, the viscoelastic properties of the cell nucleus and their connection with gene function have become a recent focus of interest.

The packing topology of the chromatin fiber is critical for genome function. Gene activity depends on DNA accessibility, which is determined both by the local folding of DNA around the histone core and by the higher-order chromatin structure. Interphase chromosomes are not randomly intermingled polymer chains, but occupy distinct "territories" in the nucleus. To access and process the genetic information, macromolecules must be transported to specific DNA sites: This process depends fundamentally on the higher-order folding of chromatin. In turn, analyzing macromolecular transport in the cell nucleus can help us understand genome architecture.

Classically, transport dynamics of macromolecules inside the cell nucleus have been determined by light microscopy-related methods, such as fluorescence recovery after photobleaching (FRAP) (for review, see [1,2]) and fluctuation-based techniques such as fluorescence correlation spectroscopy (FCS) [3,4,5,6,7,8,9,10,11,12]. All these methods rely on the observation of fluorescent probes, labelled either by chemical attachment of synthetic fluorophores or through genetic methods such as autofluorescent proteins (GFP and related tags). In FRAP, a region of interest in the biological sample is bleached by a rapid pulse of high laser intensity, and the recovery of fluorescence observed by imaging using a CCD or CMOS camera, or by observing a fixed spot at the position of interest. FCS makes use of the fact that the small confocal volume of a laser scanning microscope (1f l) contains only a few molecules; fluorophores entering and exiting the observation spot will cause significant fluctuations of the fluorescence, which can be analyzed to extract the diffusion coefficient, concentrations and other parameters.

On length scales of the size of the cell, biomolecules are mostly transported by random diffusion. A small particle in homogeneous solution will show normal diffusion, where the mean squared displacement (MSD) $\langle r^2(t)\rangle$ is proportional to the time t. In the presence of obstacles such as the chromatin network in the nucleus, the diffusion can be anomalous depending on the length and time scale of observation. Diffusion is called anomalous if $\langle r^2(t)\rangle \propto t^{\alpha}$, with an anomaly parameter $\alpha \neq 1$. For obstacles, is typically <1; this case is called subdiffusion [13].

FCS was used to show subdiffusion in cell nuclei [11] and chromatin-induced obstruction of the diffusive motion of larger molecules [14,15], whereas smaller molecules diffused normally and no correlation between their mobility and the density of chromatin was found [7]. However, the accessibility of a polymer

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network (such as chromatin in the nucleus) to diffusing molecules does not only depend on the size of the molecules and the global density of the polymer matrix, but also on the spatial arrangement of the polymer chains and their dynamics. In a homogeneous immobile polymer network, molecules are retained if they are larger than the characteristic mesh size. At the same global density, a network with a very inhomogeneous mesh size distribution, i.e., with a tendency of the network fibers to lump together, can leave space for channels that allow large molecules to travel greater distances. To assess the importance of such effects in cell nuclear transport, we need to know the occupation of the nuclear space by chromatin and other macromolecules. Using a combination of fluorescence correlation spectroscopy and confocal imaging, we determined earlier that chromatin occupies about 10% of the nuclear space [16]. However, the overall occupation of cellular compartments by macromolecules is between 5% and 40% [17], i.e., the volume of the cell nucleus contains up to 30% macromolecules other than chromatin. Many studies have looked at molecular crowding as the origin of anomalous diffusion (for a review, e.g., see [18]). In the cell nucleus, however, the contributions of different molecular species to diffusional obstruction and therefore to genome function in general are not clearly understood.

We therefore need methods that allow us to map the position-dependent random motion of proteins and other macromolecules in the cell nucleus, on time scales that are comparable to the time it takes for a molecule to move on a μm length scale. FCS offers such sub-millisecond time resolution, but so far was mostly limited to single-point measurements in a laser focus. Spatially resolved point-by-point FCS in live cells has been pioneered by Wachsmuth et al. [11]; later Dross et al. collected protein mobility maps by point-to-point FCS [7]. However, such measurements are extremely time-consuming and impractical for live cell measurements.

A major advance in the analysis of dynamical processes in live cells is brought about by simultaneous acquisition of FCS data across entire lines or regions. Specifically, combining FCS with light sheet microscopy, also called single plane illumination microscopy (SPIM) [19] allows "mobility imaging" of entire two-dimensional cross-sections, providing diffusion coefficients, flow velocities and concentrations of fluorescent proteins. Two-color fluorescence cross-correlation spectroscopy (FCCS) also allows imaging of molecular interactions in live cells [20].

Here, we will review the technique of SPIM-FCS and SPIM-FCCS as applied to mobility imaging inside cell nuclei and present some recent examples from our own work.

2. Methods

2.1. Fluorescence correlation spectroscopy (FCS)

In FCS, the autocorrelation function G(τ) of the detected fluorescence signal *F(t)* is determined either by dedicated autocorrelator hardware or in software. G(τ) is defined as

$$G(\tau) = \frac{\langle F(t) \cdot F(t+\tau) \rangle}{\langle (t) \rangle^2} \tag{1}$$

The fluorescence intensity detected from a fluorophore at position **r** in the focus of a standard confocal microscope is proportional to the molecular detection efficiency ψ (r), which is the product of the illumination and detection point spread functions: ψ(r) = $PSF_{ill}(\mathbf{r})PSF_{det}(\mathbf{r})$. If we approximate both $PSF_{ill}(\mathbf{r})$ and $PSF_{det}(\mathbf{r})$ by three-dimensional Gaussian functions with $1 = e^{2}$ half widths w_0 in the lateral and z_0 in the vertical direction, G(τ) for a molecule with diffusion coefficient D becomes

$$G(\tau) = \frac{1}{N} \cdot \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1} \cdot \left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2} \tag{2}$$

Here N is the average number of molecules in the focal volume $V_{eff} = \pi^{3/2} w_0^2 z_0$. Since $N = c/V_{eff}$ at a molar concentration c, the concentration of particles can be directly determined from the amplitude of the autocorrelation function.

Since in light sheet microscopy the detector is usually a camera with square pixels (see below), the autocorrelation function for particles undergoing normal Brownian motion in a light sheet becomes [20]:

$$G_\gamma(\tau) = \frac{1}{N} \cdot \left\{ \mathrm{erf}\left(\frac{a}{\sqrt{4D\tau + w_\gamma^2}}\right) + \frac{\sqrt{4D\tau + w_\gamma^2}}{a \cdot \sqrt{\pi}} \cdot \left[\exp - \frac{a^2}{4D\tau + w_\gamma^2} - 1\right] \right\}^2 \cdot \left(1 + \frac{4D\tau}{z_\gamma^2}\right)^{-1/2} \tag{3}$$

Here *a* is the size of the pixel image in the observation plane, w_γ the lateral width of the objective PSF, z_γ the thickness of the light sheet, and *N* the mean number of particles in the observation volume.

2.2. Single plane illumination microscopy (SPIM)

In SPIM (Fig. 1) the sample is illuminated with a laser beam focused into a thin sheet by cylindrical optics (sub μm to a few μm). The fluorescence signal in this slice is observed perpendicularly to the direction of illumination. Compared to confocal scanning microscopy, only the observed part of the specimen is illuminated, thus reducing background signal, photodamage and laser-induced stress in a living sample. Furthermore, the two-dimensional image is not produced sequentially by scanning, but simultaneously in the entire observation plane (Fig. 2).

The optical resolution of SPIM is comparable to confocal laser scanning microscopy; typical light sheet thicknesses can be 1–1.5 μm, and the lateral resolution is defined by the numerical aperture of the observation lens, typically less than 0.5 μm. The fluorescence image is taken by a fast CCD or SCMOS camera with typical acquisition speeds of ≈1000 frames/s. Thus, each pixel of the image contains the time-dependent fluorescence intensity in the corresponding observation volume with millisecond time resolution. For images consisting of only one or a few lines of pixels the time resolution can be even an order of magnitude faster [5,21] (Fig. 3).

2.3. SPIM-FCS

The SPIM-FCS device used here has been recently described in detail [20]. In brief, two solid-state lasers at 488 and 594 nm (Cobolt AS, Sweden) were combined co-linearly through a system of mirrors and telescopes, and a vertical light sheet formed by a horizontally oriented cylindrical lens in front of a 10X/NA0.3 illumination objective (Nikon). For live cell measurements, the light sheet was focused into a sample cell filled with cell culture medium, into which the observation objective lens was immersed at the working distance of 2.3 mm from the center of the light sheet. Cells were grown on small pre-cut pieces of cover slips (size: 5…10 mm^2, thickness: 0:28…0:32 mm). The slips were held by self-closing tweezers and introduced from above into the sample chamber. The chamber was filled with FluoroBright DMEM, a clear, non-fluorescent, non-scattering cell culture medium, which sustains the cells over the duration of the measurements (typically 30–90 min per sample). The glass slip was typically positioned under an angle of <45° with respect to the light sheet, to avoid light being reflected into the detection objective, and to optimize the