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ISBN
9781557529541

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Publication Date
2015

DOI
10.1364/ntm.2015.nm3c.1

3D orbital tracking for super-resolving the dynamics of gene expression

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Abstract: Optical super resolution of fast dynamic processes can be achieved using the 3D orbital tracking technique and fluctuation spectroscopy analysis. Here we show application of the method to the dynamics of loci of gene expression.
OCIS codes: 180.2520; 170.2520]

A critical barrier to progress in the description of sequences of chemical reactions occurring in cells is both in methods applicable to the relevant spatiotemporal scale and the availability of probes that can report on multiples parameters that we believe are important players such as the presence of certain proteins and the local topology of the site of gene expression. Here we describe the development of 3D orbital tracking method that can complement the top-down approach achieved by FISH based techniques. We emphasize that the 3D orbital tracking method is a radical departure from conventional single particle tracking and it better classifies as "nanoimaging".

Multi-cell biochemical assays and single cell fluorescence measurements have revealed that the elongation rate of Polymerase II (PolII) in eukaryotes varies largely across different cell types and genes [1]. Cell-to-cell differences were recently quantified in the amount of transcript of identical genes [2] and stochastic gene expression from an isogenic cell line containing a single fluorescent reporter gene was measured to depend upon the genomic site of the insertion[3]. However, there is not yet consensus whether intrinsic factors such as the position, local mobility or an active molecular mechanism at a genetic locus could be the determinants of the observed heterogeneity [3-6]. We show that the high-speed 3D orbital tracking techniques can resolve at the single cell level multiple, distinct regions of mRNA synthesis within a labeled transgene array. We also show that using fluctuation spectroscopy while tracking we can extract the local PolII elongation rate at each site.

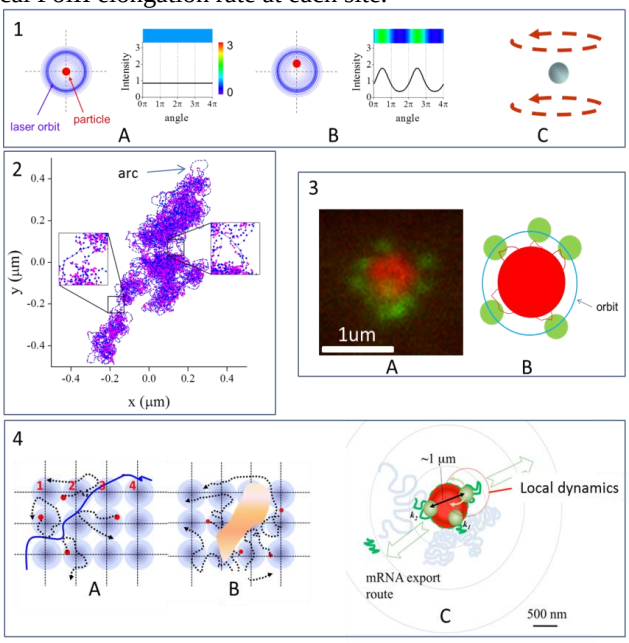

Figure 1. Panel 1. 3D orbital tracking: a laser beam performs an orbit around a fluorescent particle every ms. A) The intensity along the orbit is constant if the particle is at the center. B) If the particle moves, the intensity changes along the orbit at a specific angle and amplitude indicative of the new particle position. The intensity difference between two orbits up and down defines the z-position. A rapid feedback mechanism re-centers the orbit on the particle every ms in 3D. **Panel 2**. Trajectory of a single gene labeled with the Lac repressor-FP. Each point of the trajectory is determined with an error of 6nm. The trajectory of the gene shows regions confined motion and arcs that could be associated with period of gene expression. Each point in this trajectory is averaged for 32 ms. **Panel 3**. A) Image of a gene array (red) and sites of active gene expression (green). B) Schematic of the 3D orbital tracking method monitoring several sites of gene expression. (see Fig 2 for more details). **Panel4**. The pair correlation approach. A) The presence of impenetrable barriers results in lack of cross-correlation among points in the opposite part of the barrier (blue line). B) An obstacle delays the time to move a given distance. C) Demonstration of the pCF (cross-correlation pair correlation function) principle showing the location of barriers to diffusion.

As schematically described in Figure 1 and the associated 4 panels, the 3D orbital tracking method (briefly described in panel 1) has the following capabilities i) can track with nanometer precision in 3D the center of mass of one or more fluorescent particles (panel 2) ii) can determine the molecular dynamics around the particles (panel 3) and iii) can determine obstacles to diffusion around the particle providing unique information about the local chromatin structure (panel 4).

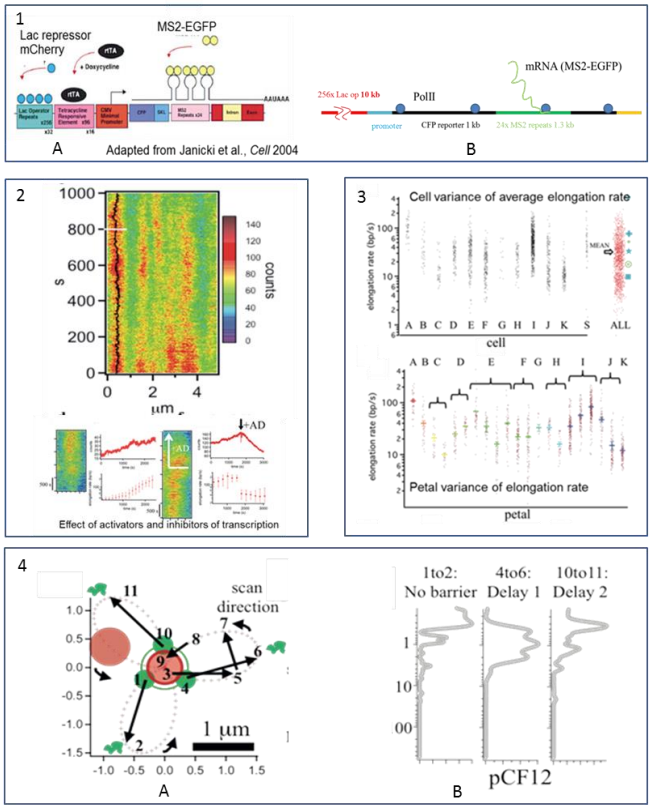

Figure 2 Panel 1. Gene array in U2OS 263 cell line. The Lac repressor is labeled with mCherry and the MS2 protein with EGFP. A maximum of 24 MS2 proteins decorate each mRNA. **Panel 2**. Kymograph of the fluorescence intensity collected along the entire orbit, reflecting the presence of five globular regions or petals. Black line shows the angular trajectory of one of the petals. The center of the petal is localized with a precision < 6 nm. At one petal, Dox induces increase in fluorescence and AD stop the fluorescence. **Panel 3**. Cell and petal variance of elongation rate extracted from the fluctuation analysis at each petal. The average of a given petal is different from the nearby petal for the length of the experiment (1000s). **Panel 4**. Pair cross-correlation analysis of mRNA molecules leaving the petals and flowing into the nucleoplasm. A) The laser performs a trefoil orbit and the lobes of the trefoil reach into the nucleoplasm about 1 μm away from the center of the array. A distance of 12 pixels along the rows of the carpet is used to calculate the pCF kymograph. B) pCF correlation at short times in the 1to2 position indicated diffusion of mRNA molecules away from the petals. The pCF 4to6 highlights a delay for the mRNA to reach the nucleoplasm from this petal. The amount of delay for the mRNA in reaching the nucleoplasm is again different when looking at the last petal (10to11)

As shown in Figure 2 panel 1, we are using a cell line originally described by Janicki et al [7]. Each copy of the 200 gene repeats composing of the transgene array can be visualized by means of a Lac Operator cassette[8] and an MS2 cassette allows nascent mRNA to be decorated by a fusion of EGFP with MS2, a coat protein which recognizes a specific and repeated hairpin sequence in the mRNA molecule[9] . Upon Doxycycline (+Dox) induction the array transitions from a heterochromatic state to an euchromatic condition following histone exchange that removes H3K9 methylation[7]. Images of the cell line (Fig. 1, panel 3) show that mobile mRNA foci or petals (green) surround the denser chromatin array (red). The number of petals varies from cell to cell, from a lower number of one, up to seven distinguishable loci. After ten to fifteen minutes following induction, the fluorescence from the petals reaches a steady state that is maintained over a timescale of hours. Each petal undergoes sizable angular displacements in the reference system centered on the gene array (Fig. 2, panel 2). The fluorescently labeled gene array (Fig. 1 panel 3, red dot) also displays a highly dynamic behavior, owing to overall chromatin motion.

The rates of elongation that we have obtained from the intensity fluctuation analysis at each petal and among many cells are in excellent agreement with elongation rates recently measured using biochemical assays (Fig. 2, panel 3). We also confirmed the previously reported large variability in elongation speeds, but this time in the same cell and at loci in close proximity[4]. We observed that different cells display a different degree of variance in the

observed PolII elongation values but surprisingly, a sizable contribution to this variability arises from differences between the elongation rates of PolII on distinct petals within the same cell (Fig. 2, panel 3).

The 3D orbital tracking method allows the determination of the center of mass of the petal with nanometer resolution, similarly in concept to the stochastic optical reconstruction microscopy (STORM) or photo-activated localization microscopy (PALM) principle which is based on center of mass determination. After correction for the changes of the positon of each petal, the remaining fluorescence fluctuations arise only because of kinetic processes, such as increases in fluorescence signal due to the addition of MS2 fluorescent subunits as PolII elongates, or decreases in fluorescence due to the release of fluorescently labeled mRNA molecules as shown in a long time scale by the addition of activators or inhibitors of transcription in Figure 2, panel 2.

It is then possible to reconstruct both the fluorescence and spatial trajectory of each of the petals over an unprecedented duration (up to hours) and temporal resolution (32 ms in this case because several orbits were averaged). Figure 2, panel 3 summarizes the elongation rates obtained for petals in the same cell and rates obtained in a number of single cells analyzed.

The observation of significant elongation variance across multiple, closely spaced, identical transcription sites within the same cell, measured at the same time, allowed for the first time comparing the kinetic variance of the transcriptional process in a context that rules out extrinsic sources of variance, such as cell to cell differences, cell-wide abundance of transcription factors or PolII [2]. Our analysis allows visualization of changes in PolII elongation kinetics within an individual petal over very long time intervals, up to hours, as demonstrated by following the activation of a transcription site or by monitoring the effect of a common transcription inhibitor such as AD on the elongation rate, as displayed in (Fig. 2, panel 2).

What happens of the transcripts after they are synthesized at a specific locus of expression? Here we describe how to extract further information about the dynamics surrounding the site of transcription in the context of the 3D orbital tracking. Figure 2, panel 4 shows the use of the Pair Correlation Analysis to correlate the fluorescence fluctuation in the region surrounding the transcription loci to determine the trajectory of the transcripts after leaving the loci. The pair correlation method is based on spatial cross-correlation in the fluorescence intensity arising from two distinct points at a distance d as explained in the caption of Figure 2, panel 4. A cross correlation signal indicates that molecules that leave one point can reach the other. The delay in such spatial cross-correlation reflects the average physical length of the path taken by a molecule [10, 11]. For this measurement we switched from a circular to a trefoil orbit that allows us measuring diffusion and flow of mRNA molecules across different paths surrounding the transgene array. We focused in particular on the motion from the petals far into the nucleoplasm (point 1 to 2, 4 to 6 and 10 to 11 in Figure 2 panel 4A) which probes the ability of mRNA to leave or re-enter the core of the transgene array. Each of these pairs of locations is spaced 12 pixels along the orbit. Strong spatial cross-correlation is observed between the petals and the distal extremities of the orbit lobes, confirming that most of the mRNA molecules that reach further into the nucleoplasm originate from the transcribing petals (Fig. 2, panel 4B). Barriers to diffusion are instead observed for displacements towards or away from the center of the array, confirming that mRNA synthesis occurs on the surface and that the chromatin density within the array impedes any mRNA diffusion or flow. Finally, mRNA nucleoplasmic diffusion is detected at the distal extremities of the trefoil lobe. Interestingly, a significant difference can be observed in the molecular flow of mRNA molecules that originate from each of the three petals (Fig. 2, panel 4B) which shows that the average delay experienced by mRNA molecules leaving the three positions marked as 1, 4 or 10 in reaching the nucleoplasm is different [12].

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