Advanced fluorescence microscopy methods for the real-time study of transcription and chromatin dynamics

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In this contribution we provide an overview of the recent advances allowed by the use of fluorescence microscopy methods in the study of transcriptional processes and their interplay with the chromatin architecture in living cells. Although the use of fluorophores to label nucleic acids dates back at least to about half a century ago,[1] two recent breakthroughs have effectively opened the way to use fluorescence routinely for specific and quantitative probing of chromatin organization and transcriptional activity in living cells: namely, the possibility of labeling (i)the chromatin loci and (ii) the mRNA synthesized from a gene using fluorescent proteins. In this contribution we focus on methods that can probe rapid dynamic processes by analyzing fast fluorescence fluctuations.

Introduction: Imaging Chromatin and Transcription in Living Cells Using Fluorescence

The importance of studying the highly dynamic processes taking place in the cell nucleus, such as chromatin dynamics, protein diffusion, transcriptional activity and nucleo-cytoplasmic transport, to name only a few, could not be overstated. Fluorescence microscopy provides an ideal tool to address relevant biological questions in these domains in vivo, at a high temporal resolution and non-invasively.

Robinett et al.[2] originally demonstrated the possibility of labeling a specific chromatin locus with a GFP-like protein exploiting the Lac operator-repressor system. In this first breakthrough publication the authors were able to use a fluorescence microscope to follow the position of a DNA locus over time in cells undergoing mitosis. In a second breakthrough, Bertrand et al.[3] showed two years later that mRNA in a yeast cell could be fluorescently labeled immediately after transcription using

an endogenous probe, and tracked within the cell, exploiting the high binding affinity of the bacterial coat protein MS2 (fused to a GFP) for a specific mRNA hairpin sequence. The authors used the system to observe, for the first time in vivo, the displacement of mRNA containing the ASH1 untranslated region sequence to the yeast bud tip, as it moves along the actin filaments.

Following the pioneering work of Robinett et al., chromatin loci were tracked using fluorescence microscopy, observing constrained diffusional motion of interphase chromosomes.[4] Furthermore transcription downstream of a fluorescently labeled and inducible gene was monitored over several hours using a fluorescent protein reporter; this allowed measuring, for example, morphological changes in chromatin structure upon gene activation.[5]

The two methods were ultimately combined by Janicki et al.,[6] who correlated the amount of fluorescent mRNA, being synthesized from a fluorescently labeled gene array, with its transformation from a heterochromatic state into a transcriptionally active state upon induction.

These works have provided a huge incentive for a quantitative application of fluorescence microscopy in this field of research. In parallel to this, a growing number of nuclear proteins interacting with chromatin or mRNA have also been fluorescently labeled over the years (histones, transcription factors), providing a remarkable palette for the study of transcriptional processes and their interplay with the complex and dynamic chromatin organization within the nucleus of a living cell.

Normally the molecule of interest (e.g. mRNA, chromatin locus, polymerase) is directly labeled with either an endogenous fluorescent protein or a synthetic probe. In a few cases, however, powerful insights are provided by the use of an inert fluorescent reporter, diffusing within the nucleus.

Within the domain of fluorescent methods applied to the study of transcription and chromatin organization, we discuss in this manuscript four areas of recent research: (i) application of fluorescence microscopy to the study of the kinetics of mRNA synthesis during transcription; (ii) advances in fluorescence methods allowing measuring of the displacement both of an actively transcribing gene and of its mRNA within the nucleus; (iii) novel approaches to study chromatin binding kinetics of fluorescently labeled proteins; and (iv) the development of methods to

A Imaging modes

Widefield imaging
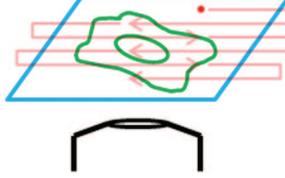
B Point scanning (raster 2D)
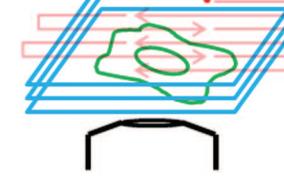
C Point scanning (raster 3D)

D FRAP

D, k_i

Fluorescence

time

promoter   initiation   elongation

termination

promoter binding   abortion
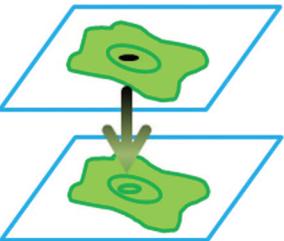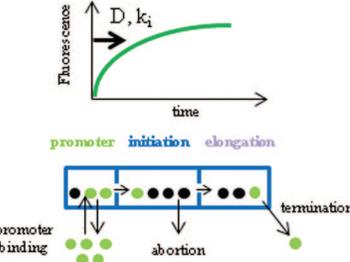
FRAP: provides diffusion coefficient D and kinetics parameters k_i by fitting a model to a fluorescence recovery curve.

E SPT

SPT: provides trajectories of the motion of isolated particles. Can be used to extract diffusion coefficients and to lock-in and follow fluorescence fluctuations around a specific particle

F FCS and PCH

Autocorrelation

delay

Intensity

time

Intensity

frequency
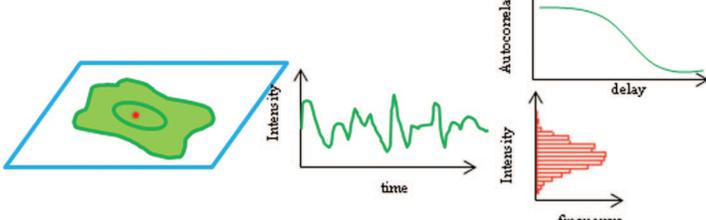
FCS: Diffusion and binding information by fitting a model to a fluorescence autocorrelation curve

PCH: molecular brightness and oligomerization state by fitting a model to the histogram of the photon counts

G pCF

Cross Correlation

delay
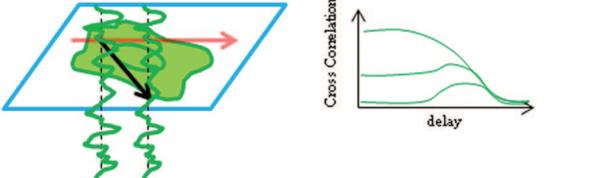
pCF: provides information about molecular flows and obstacles to diffusion by cross-correlating over time the fluorescence fluctuations from two different locations

H RICS
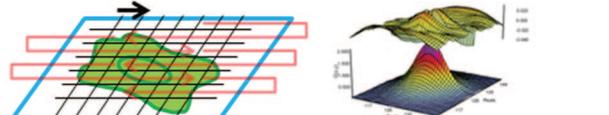
RICS: provides information on diffusion and binding kinetics by fitting a model to a two dimensional spatio-temporal autocorrelation function

I tICS

Autocorrelation

delay
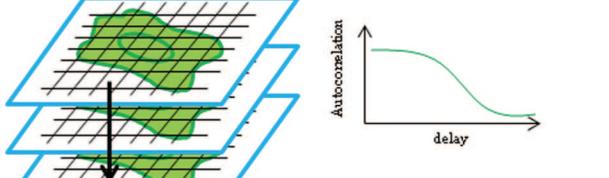
tICS: provides a spatial map of diffusion and binding kinetics autocorrelating in time each pixel of an image sequence . (>10-100 ms timescale)

Figure 1 (opposite). A graphical summary of the principal fluorescence microscopy methods discussed in this manuscript and their application in chromatin and transcription research. (**A**-**C**) Different imaging modes. In widefield imaging a large region of the sample is homogeneously illuminated and imaging is performed using a camera. In Point Scanning, a focused laser spot is typically displaced on the sample in a raster pattern. Multiple planes can be imaged to obtain a 3D reconstruction of the sample. (**D**) In FRAP, a region of the sample is photobleached by intense excitation light, and the recovery of fluorescence is observed over time. (**E**) SPT allows tracking of an individual particle over time. The reconstructed trajectory can be used to obtain information about the diffusion coefficient, regions confining the motion or interaction with other particles or organelles in a cell. (**F**) FCS and PCH are complementary approaches that allow extracting diffusion, brightness and concentration information from the sequences of fluctuations in photon numbers detected from a small excitation volume. Autocorrelation of the time sequence provides the fluorescence AutoCorrelation Function (ACF). Analysis of the distribution of the photon counts provides brightness information to determine aggregation state of the molecules. (**G**) pCF can be performed on any microscope able to do fast line (or circular) scans; it measures as a function of the time delay the cross correlations between the fluctuations at two different spatial positions. It can be used to measure diffusion and flow in the presence of obstacles (**H**) RICS provides a spatiotemporal correlation map of raster scan images. The 2D autocorrelation function can be fit to a model to extract diffusion coefficient and binding rates (**I**) tICS performs temporal autocorrelation on each pixel of a sequence of images. It can provide a spatial map of slow (> 10 ms temporal resolution) diffusion and binding processes.

quantitatively measure by means of fluorescence fluctuations the rate of production and the amount of transcribed mRNA.

Within these domains, which are obviously not exhaustive of the entire research activity in the field, multiple methods are compared and the most promising and novel approaches to capture fast dynamic processes are critically discussed.

Keeping the focus on fast dynamic processes related to the transcriptional activity, we deliberately did not include a review of the growing body of works generating super-resolution images of the chromatin and nuclear structures (see Flors et al. and references therein[7]). Up to date, most of these works have been performed in fixed samples or have been employed to capture relatively slow dynamic processes.

Kinetics of mRNA Synthesis: Capturing Fluctuations at the Transcription Site

Over the last ten years fluorescence microscopy methods have provided significant insights in the study of the kinetics of mRNA synthesis at an actively transcribing gene (or gene array) in living cells (for a recent review, we refer to[8] and references therein).

The availability of a system such as the MS2-GFP fusion protein, which directly labels the mRNA, has allowed obtaining an estimation of the RNA polymerase elongation speed in living cells. However, transcription kinetics is determined by a complex interplay, still poorly understood of transcription factors, enzyme activity and modifications of the chromatin structure.

To quantify by means of fluorescence the amount of newly synthesized mRNA, it is therefore necessary to follow in 3D the

position of the active gene (or gene array) for extended periods of time, at least minutes, and with a fast frequency response. Within this time span, cell motility, the displacement of the nucleus within the cell, and long range motions of the chromatin itself, may all add up to a significant motion (on the micrometer length-scale) of the region of active transcription. The problem of accurately collecting the fluorescence signal arising from the newly synthesized mRNA on the active gene poses then the problem of accurately tracking (within nanometers) the position of this gene in 3D.

Wide-field imaging systems (**Fig. 1A**), that use state of the art Electron Multiplying Charge Coupled Device (EM-CCD) cameras, offer very high frame rates, up to 50 Hz full-frame, and Complementary Metal Oxide Semiconductor (CMOS) sensors can achieve even higher full-frame rates of 100 Hz. Frame rates as high as 50 Hz have been employed to track in 2D mRNA with sufficient signal to noise ratio during nucleocytoplasmic export.[9] However, the frequency response of a wide field system slows down significantly (1–10 Hz range) if the tracking is extended to the axial dimension,[10] not to mention the increased technical complexity of the optical setup.

Most of the commercially available laser scanning systems (**Fig. 1B**) allow good background rejection by means of either confocal detection or 2-Photon excitation, but the scanning of a 3D volume of the size of the cell nucleus at Nyquist resolution and sufficient signal to noise ratio still requires at least a few seconds (**Fig. 1C**).

Actively transcribing genes have been so far imaged using both widefield and confocal techniques. The aforementioned work of Janicki et al.[6] originally performed time-lapse imaging to capture the dynamics of mRNA synthesis from the 200-repeats of an MS2-coding gene. An increase of MS2-mRNA fluorescence was observed in the first few minutes after transcriptional induction, until approximately 15 min post induction, followed by a steady-state condition. Darzacq et al.[11] employed Fluorescence Recovery After Photobleaching (FRAP) of both fluorescently labeled RNA polymerase II and MS2 coat protein to investigate in detail the kinetics of transcription, collecting fluorescence recovery curves on a laser scanning confocal microscope with a temporal resolution as low as three seconds (the principle by which kinetics information can be reconstructed by means of FRAP is schematically illustrated in the diagram of **Fig. 1D**). The authors fit the data to a mechanistic model in order to extract kinetic parameters of RNA polymerase II transcription. Notably, they observed that only one percent of the polymerases interactions lead to a complete mRNA, that fast elongation rates of 72 bases per second occur and that an individual polymerase can pause for hundreds of seconds leading to so-called transcriptional bursts.

More recently,[12,13] 3D time-lapse microscopy was used to track (0.1 Hz) the activity of an individual gene over extended periods of time (tens of minutes). Larson et al. observed in yeast discrete steps in the mRNA fluorescence trajectory, associated to individual mRNA molecules being synthesized on the gene (one step up) or being released (one step down) (**Fig. 2A**). The position of the MS2-coding sequence with respect to the promoter, together with the release rate, determined the duration of each of

these fluorescence traces. The average dwell time (10–100 s) of an mRNA molecule on the gene was measured from fluorescence autocorrelation measurements. This approach allowed determining that there is no transcriptional memory between initiation events, and that elongation rates display significant variability depending on the cell cycle state.

A time resolution of 10 s is adequate if one is interested in measuring the dwell time of kilobase-sized mRNA molecules on a gene. However, this is generally not sufficient to directly measure the step-wise enzyme activity of an elongating polymerase. Elongation at the reported speeds of up to 70 bp/s implies that a novel MS2-stem loop is synthesized in less than a second. Therefore, the analysis of fluorescence fluctuations arising from the addition of an MS2-EGFP dimer to the mRNA would require a frequency response of the order of at least 1–10 Hz.

The possibility of attaining a higher temporal resolution while tracking an actively transcribing gene in vivo (therefore ensuring that the collected fluorescence intensity is always proportional to the amount of mRNA present on the site) may help shedding further light on debated issues such as RNA polymerase II pausing. In certain instances, it was suggested that polymerases pause on the gene, reflecting an individual property of this enzyme;[11] in others, it was proposed that the heterogeneous dwell times observed for mRNA molecules on a gene rather reflect heterogeneities in the elongation speed that depend upon the cell cycle.[12]

Fluorescence lock-in tracking approaches (**Fig. 1E**), which evolved over the last ten years, may provide an ideal tool for the study of the problem of in vivo transcription at the single molecule level. Ragan et al.[10] demonstrated, for instance, the possibility of performing fast 3D z-stacks, limited in range to a region of interest, that can follow a bright fluorescence object using a software feedback mechanism. Kis Petikova and Gratton[14] and Levi and Gratton[15] proved that circular orbits of a laser scanning microscope combined to a software feedback system can be used to follow at high spatial (10–50 nm) and temporal (50 Hz) resolution for the 3D position of a fluorescent particle.

Levi et al.[16] successfully applied this method to the tracking of a gene array within the chromatin of living cells, fluorescently labeled using the Lac repressor-EGFP system. The high frequency response of this system allowed demonstrating sizable (100 nm range) and fast (0.3–2 s) jumps of the fluorescently labeled region of the chromatin. Besides raising very interesting questions on the possible relationship between the microscopic displacements of regions of the chromatin and their transcriptional activity, these observations point to the requirement of collecting any fluorescence fluctuation of the labeled mRNA on timescales at least one order of magnitude smaller than those investigated so far.[17]

Orbits with a circumference ranging from 0.5 to 1 μm can be performed using galvanometer scanners in as fast as 0.5 ms (2D),[18] and the entire orbit cycle necessary to track the 3D position of the fluorescent gene (and mRNA) can be completed in about 2 ms (**Fig. 2B**). With RNA polymerase II reportedly transcribing in a range between 1320[12] and 4300 bp/minutes,[11] many fluorescence fluctuations of the MS2 system associated to transcriptional activity, that are lost within the 1–10 s timescale of typical time-lapse microscopy (**Fig. 2A**), could be detected using this method[19] (**Fig. 2D**).

Furthermore, the possibility of obtaining high resolution trajectories of the transcribing locus will enable us to investigate the interplay between transcription kinetics and local chromatin structure and mobility[20] (**Fig. 2C**).

mRNA Displacement in the Nucleus and Chromatin Organization

As the newly synthesized mRNA is released from the transcription site, it moves within the nucleoplasm until it reaches the nuclear membrane, where it undergoes export through the nuclear pore complexes.

Over the past 15 y many approaches have been employed in trying to assess the nuclear diffusion coefficient of mRNA, and to determine if its motion within the nucleus is purely diffusive, or it relies on energy dependent processes.

This problem remains of great interest and is still controversial, since many questions regarding the interaction of mRNA particles with the surrounding chromatin remain unanswered. It is well known that chromatin regions can undergo different types of motion, either diffusive-like motion confined to a region of the nucleus,[21] confined motion connected by 100 nm-sized jumps[16] or even long range motion of a few microns from the periphery to the nuclear center upon transcriptional induction.[22] However, the knowledge of higher levels of chromatin structure and dynamics remains incomplete (Belmont et al.).[23]

A comprehensive review of mRNA tracking by means of fluorescence methods was recently published by Park et al.[24] Among the techniques used are FRAP (**Fig. 1D**), Fluorescence Correlation Spectroscopy (FCS) (**Fig. 1F**) and Single Particle Tracking (SPT) (**Fig. 1E**).

As anticipated in the previous section, and schematically illustrated in **Figure 1D**, FRAP can measure the diffusion coefficient of a species by following the recovery over time of the fluorescence signal in a bleached region due to diffusion of unbleached molecules. As illustrated in **Figure 1F**, FCS measures the diffusion of a molecular species using the average duration of fast fluorescence fluctuations arising from the passage of the fluorophores in a small excitation volume. SPT localizes the position of isolated particles (such as single fluorescent particles or particles much brighter than their background) and calculates the trajectories of their motion.

In 1998 Politz et al.[25] investigated by means of FRAP and FCS the diffusion of mRNAs in the nucleus following hybridization with short fluorescent oligonucleotides. Diffusion coefficients ranging from 0.1 to 10 μm²/s were measured by both techniques, the spread in diffusion coefficients being ascribed to the log-normal size distribution of the mRNA of typical culture cell lines, which peaks at about 2.2 kb.[26] Free mRNA in solution diffuses at 10 μm²/s, leading the authors to postulate the possibility of mRNA freely diffusing in the nucleus as it would do in an aqueous solution.
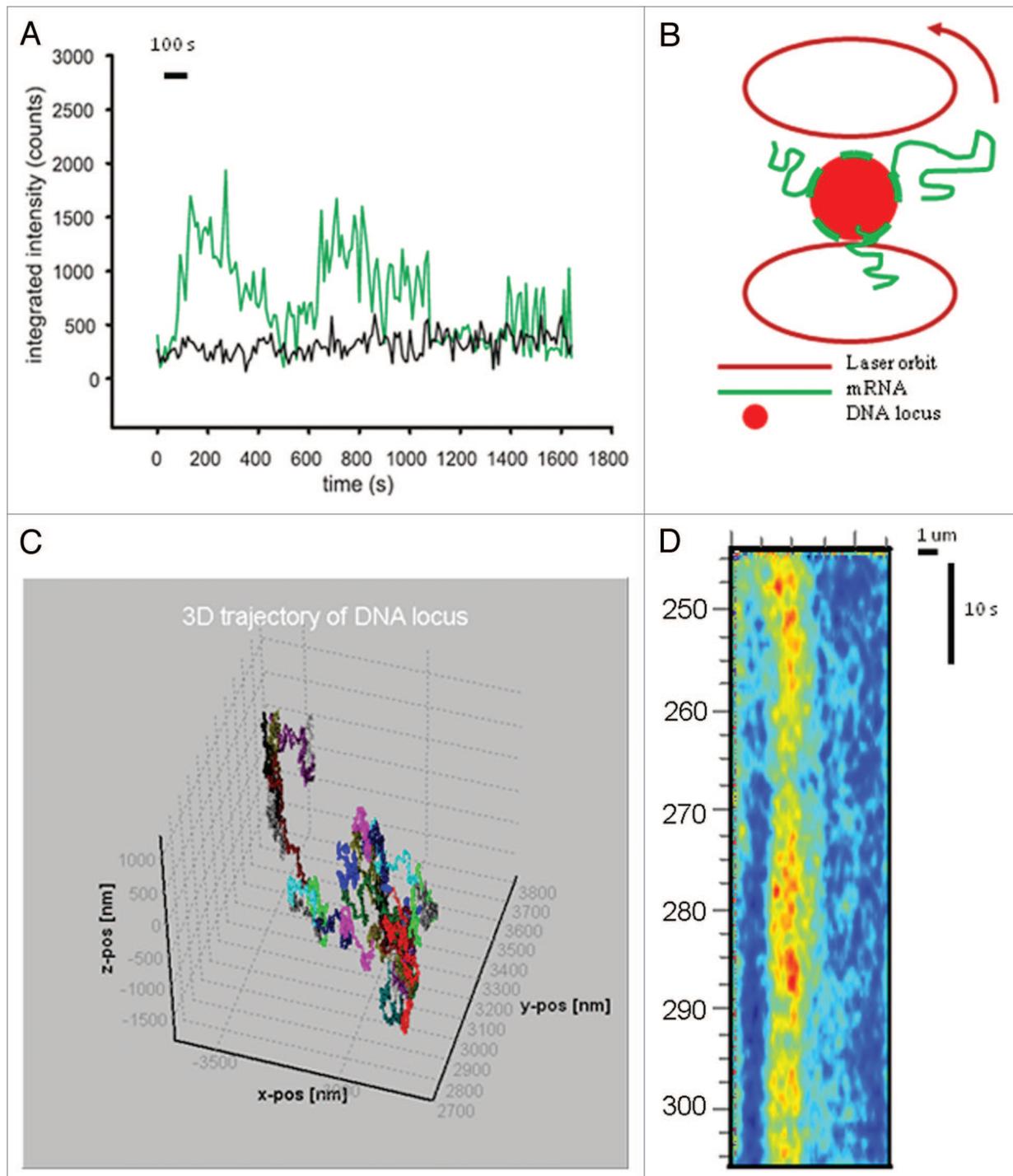
Figure 2. mRNA-MS2 fluorescence intensity collected using the 3D orbital tracking method. (**A**) Fluorescence intensity over time collected using 3D time-lapse imaging in a confocal laser scanning microscope. One point is collected approximately every 20 s (adapted fromLarson et al[12]). (**B**) Configuration to record mRNA labeled MS2-EGFP fluorescence intensity using the orbital tracking method. A circular orbit above, and one below the particle are used to calculare its x,y,z position. The integrated fluorescence intensity along each orbit cycle is represented as a line in the kymograph in (**D**). (**C**) Reconstruction of the 3D trajectory of the lac repressor-mCherry gene array within the nucleus. (**D**) Kymograph (or intensity carpet) of the fluorescence emission from MS2-EGFP labeled mRNA scanned along a circular orbit locked-in to a lac repressor-mCherry gene array. One line is collected every 32 ms.

Following this original report, a number of other works have reported measurements of the mRNA diffusion coefficient, mostly using fluorescent single particle tracking techniques, although both the size and labeling strategy of the mRNA molecules varied greatly. We have summarized the values reported in the literature for the mRNA diffusion coefficient, to the best of our knowledge, in **Table 1**. We shall briefly note here that the use of fluorescent protein chimaeric constructs to label the

Table 1. Comparison of the diffusion coefficients from selected publications, with the indication of the mRNA molecule size and the technique that was used in the measurements.

Size (kb)	Diffusion coefficient (μm²/s)	Technique
Distribution, mean 2.2	0.1–10	FRAP-FCS[25]
3.4	0.01–0.09	SPT[27]
4.8	0.03	SPT[46]
0.4 and 0.8	0.2	SPT[28]
3.3	1.3–3.5	SPT[9]
3.4	0.35	TIFCA[37]

mRNA requires to consider the additional effect of the labeling constructs adding up to the molecular weight of the mRNA particle (mRNAp), potentially affecting the diffusional properties both by steric hindrance and/or interactions with other proteins and nucleic acids within the nucleus.

The striking variability in the measured values for the diffusion coefficient may partly owe to the size of the particle and to the different techniques employed to measure it, but ultimately it has to be ascribed to the difficulty of establishing the impact of chromatin architecture on the nuclear diffusion of relatively large complexes, such as the mRNA particles. This is further confirmed by the divergent reports about the effect of energy depletion (-ATP) on the diffusion coefficient of the mRNAs. In certain cases,[27] an ATP-dependence of diffusion was observed, whereas in others,[28] it was not possible find evidence of any energy-dependent process driving the diffusion of the mRNA particles.

This problem was recently revisited from a different direction, and by employing novel methodological approaches. Instead of labeling the mRNA and trying to follow the motion of individual molecules in the nucleus, the study of the diffusion of a minimally interacting and relatively inert probe, such as a GFP-like protein with a nuclear targeting sequence, was proven to be a very effective approach to address the impact of chromatin architecture on nuclear diffusion in living cells.[29,30]

FCS performed in multiple points[29] provided a map of diffusion heterogeneity within the nucleus. In addition, no apparent correlation was observed between the diffusion coefficient of EGFP oligomers and the local chromatin density.

Besides single point FCS, the use of pair Correlation Functions (pCF) was demonstrated to be advantageous in the study of diffusion processes taking place within the nucleus.[31] FCS lacks the spatial resolution to resolve diffusion heterogeneities within the PSF of the microscope, and single particle tracking allows following only isolated molecules. Pair Correlation analysis (**Fig. 1G**) is unique in allowing to measure the time that it takes a particle to travel between two spatially distinct points. Furthermore, the method bridges single particle tracking and FCS by providing single molecule sensitivity. Computing the time when the maximum of the cross-correlation between two distinct points occurs, it is possible to calculate not only the diffusion coefficient of a fluorescent species but also the degree of topological connectedness between two distinct regions.

This is information that FRAP does not provide, since it is unknown from where the fluorescent species replenish the bleached volume. On the other hand, single particle tracking provides limited statistics, and is typically confined to 2D.

Using pCF analysis, Hinde et al.[30] have effectively demonstrated that there is a DNA-dependent molecular flow within the mammalian cell nucleus. It was observed, for the first time, that high DNA density regions act as a networked channel, and that molecular flow from high to low DNA density regions is limited.

Chromatin Binding Proteins

The study of the motion of a non-interacting fluorescent probe in the nuclear environment, such as GFP, can be instrumental in understanding the dynamics of chromatin organization and the impact that chromatin has on the release and diffusion of the mRNA away from the active gene. However, a large number of proteins within the nucleus have a functional role that is mediated by their interaction with the chromatin.

Another field in which fluorescence methods have advanced our understanding is in the study of chromatin binding proteins and complexes. As opposed to the study of GFP motion, the study of nuclear binding proteins aims at extracting the residence times of otherwise fast diffusing proteins on the relatively slow chromatin fibers.

Historically, this field was investigated by means of FRAP. FRAP temporal resolution, however, is limited by the acquisition speed of the microscope, and therefore is able to highlight high affinity interactions (long residence times) rather than faster millisecond phenomena. FCS has also been employed for these studies (for a recent review see Erdel et al.[32]); however, the bleaching, the slower chromatin dynamics, and the difficulty of distinguishing slow diffusion and binding from the fit of the autocorrelation function to a model, have proven to be limitations for the widespread use of this approach.

More recently, temporal Image Correlation Spectroscopy (tICS) (**Fig. 1I**) was discussed as a method to measure slow kinetics of chromatin binding proteins in a spatially resolved manner.[33] The field saw a further development with the recent application of spatio-temporal image correlation methods to quantify the interactions of the ISWI family of chromatin binding proteins. Erdel et al.[34] performed FRAP by taking into account the spatio-temporal evolution of the bleaching pattern in a confocal microscope raster-scan image, ultimately being able to resolve transient chromatin binding kinetics below 2 ms for these proteins.

In more general terms, the challenge associated to spatio-temporal image correlation spectroscopy methods is to effectively distinguish diffusion and binding processes[32].

With the use of a well-established image spatio-temporal correlation spectroscopy method, such as RICS[35](**Fig. 1H**), it is possible to distinguish diffusion and binding from the shape of the spatio-temporal autocorrelation function. This can be intuitively understood since diffusing molecules contribute to a broadening of the spatio-temporal image autocorrelation, whereas immobile

Table 2. Experimental observables accessible using advanced fluorescence methods.

Process	Technique	Observables
chromatin motion	Single particle tracking	type of motion,[4,22] jump-size distribution[16]
transcription kinetics	time-lapse imaging, FRAP, single particle tracking	autocorrelation time of transcription fluctuations,[12,13] polymerase kinetic parameters[11]
nuclear mobility of proteins and nucleic acids	FCS, FRAP, SPT, tICS, RICS, pCF	Diffusion coefficient of proteins[29,30,33,34,47,48] and mRNA,[9,24,25,27-29,34,46,48,49] binding/unbinding rates,[32,34,47,48,50] obstacles to diffusion and intranuclear flow[30]
oligomerization state of proteins in the nucleus	PCH	Molecular number and brightness[37,42]

molecules yield a contribution to the spatial autocorrelation that reflects the point spread function of the microscope. Provided the pixel dwell times of a few microseconds commonly employed in this method, RICS was demonstrated to be well suited to detect binding equilibria in the microseconds to 100 ms range. Analysis of simulated data of diffusing and binding molecules shows that binding to either fixed or random locations with binding times of 28 ms and 160 ms, respectively, could be easily detected by fitting the RICS autocorrelation function to the appropriate model, obtaining also a diffusion coefficient in agreement with the simulated data.[35] RICS was recently applied to obtain diffusion maps for HP1α in the cell nucleus[32] and to measure MS2-EGFP-labeled mRNA diffusion.[36]

Quantitative Determination of the Amount of Transcriptional Output

Finally, besides transcription kinetics, nuclear diffusion of mRNA and quantification of chromatin binding protein residence times, a novel and important application of fluorescence fluctuation methods is the ongoing attempt to extract quantitative information on the amount of mRNAs or proteins present in the nucleus (e.g., by investigating how many fluorophores decorate mRNA-MS2 complexes (and analogous systems)[37] or by measuring protein copy numbers (for a review we refer to Li and Xie[38] and references therein).

Most of the work performed in this direction has either exploited single molecule approaches or brightness information contained within the fast fluorescence fluctuations collected in a scanning microscope.

Brightness analysis is the study of the amplitude of the photon bursts as opposed to the study of their temporal separation, which in conventional FCS provides the information on the diffusion coefficient of the species under investigation (**Fig.1F**). The exact theory to extract concentrations and the oligomerization state from the photon counts sequence was originally proposed by Chen et al.,[39] following their interpretation and theoretical modeling of the Photon Counting Histogram (PCH). While the temporal behavior of the fluctuations is best described by the autocorrelation function, the amplitude of the fluctuations is described by its probability distribution. The PCH describes the probability of detecting k photons per sampling time in a typical fluctuation spectroscopy experiment, and can be modeled taking

into account the concentration of the fluorescent particles and their oligomerization state.

The original theory of the PCH was further developed by Mueller,[40] ultimately leading to approaches such as Time Integrated Fluorescence Cumulant Analysis (TIFCA),[41] based on the analysis of the fluorescence cumulants, that allows taking into account both brightness and diffusion information. With this method, it is possible to extract the apparent brightness of a fluorescent sample, provided that one has a reference brightness calibration for the fluorescent monomer. Titration of the apparent brightness against the concentration of the fluorophore allows calculating the oligomerization state of the sample.[42]

This method allowed observing that in the widely used MS2 system only 20–30 EGFP fluorophores are present at any time on the mRNA molecule, against an expected number of 48 (the MS2-EGFP construct binds as a dimer to each stem loop).[37] However, for concentrations of free MS2-EGFP above 0.1 uM, the molecular brightness measured in the nucleus was observed to be constant with respect to concentration. Interestingly, this method was also used to measure mRNA diffusion coefficient, yielding a diffusion coefficient of 0.35 um^2/s for the MS2-mRNA complex in the nucleus.[37]

Concluding Remarks

Novel methods that decorate both chromatin loci and mRNA molecules as they are synthesized in the nucleus of living cells have been available for over 15 y thanks to the pioneering work of Belmont and colleagues[2] and Singer and his research group.[3] However, it is only in the last five years that significant advances in the methods and instrumentation for fluorescence microscopy have allowed taking full advantage of these developments. A summary of the experimental observables accessible using these techniques is reported in **Table 2**.

The kinetics of the processes taking place in the nucleus during transcriptional activity span a timescale ranging from milliseconds, such as is the case for the residence time of proteins on the chromatin, to slow fluctuations that are in the range of seconds or minutes, such as the dwell time of an mRNA molecule on a gene during elongation and before release.

The temporal resolution of the fluorescence methods currently available shed significant light on the slower end of this temporal range, but research in the field was characterized until

a few years ago by a search for methods to probe the faster times. Only recently, techniques able to perform accurate and quantitative measurements on millisecond to second timescales have been employed in the field.[9,10,16,29-31,34,37,43]

Fast three dimensional particle tracking based on feedback imaging in a scanning microscope appears as a very promising avenue to investigate transcription kinetics on an active gene as well as to address the issue of the possible interplay between transcriptional activity and chromatin movements.

The dynamics of mRNA in the nucleus, traditionally investigated by means of FRAP, FCS and single particle tracking, may greatly benefit by fluorescence cross correlation methods, particularly suited to address this question, given the complex topological structure of the chromatin and inter-chromatin space.[30]

Imaging correlation spectroscopy methods, traditionally limited to studies of membrane proteins dynamics, are now extended to studies of chromatin binding protein kinetics, allowing to resolve binding times in the milliseconds range (reviewed by Erdel et al.).[32]

Finally, fluorescence brightness analysis provides the first truly quantitative tool to extract number of molecules from fluorescence microscopy experiments employing the MS2-GFP or analog mRNA labeling systems.[37]

Up to now, fast transcription kinetics and protein dynamics could be effectively probed only by means of scanning microscopes, given the intrinsic 3D structure of the nuclear environment and the requirement for off-plane fluorescence rejection.

Nevertheless, recent developments in the field of light sheet microscopy (for a recent review see Weber and Huisken) [44] hold the promise to significantly increase the signal to noise ratio of camera-based measurements of fluorescently labeled chromatin loci or mRNA molecules.

The foremost challenge ahead to further increase our understanding of the field is probably the combination of fluorescence fluctuations methods already established in laser scanning microscopy with camera based (EM-CCD or CMOS) wide-field systems.[45]

Another significant development is expected by the challenging combination of chromatin and mRNA fluorescent labeling methods with super-resolution techniques, such as STED, PALM/STORM and Structured Illumination Microscopy, in order to capture dynamic processes as well as high resolution features.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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