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# Imaging transcription factors dynamics with advanced fluorescence microscopy methods

Paula Veneri<sup>1,2</sup>, Juan José Romero<sup>1,2</sup>, María Cecilia De Rossi<sup>1,2</sup>, Yanina Alvarez<sup>1,2</sup>,  
Camila Oses<sup>1,2</sup>, Alejandra Guberman<sup>1,2,3\*</sup> and Valeria Levi<sup>1,2\*</sup>

*1 - Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

*2 - CONICET—Universidad de Buenos Aires, Instituto de Química Biológica (IQUIBICEN), Buenos Aires, Argentina*

*3-Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

\*To whom correspondence should be addressed. E-mail: vlevi12@gmail.com, algub@qb.fcen.uba.ar. Phone and fax: 0054-114-786-3426

## ABSTRACT

Pluripotent stem cells (PSCs) are capable of self-renewing and producing all cell types derived from the three germ layers in response to developmental cues, constituting an important promise for regenerative medicine. Pluripotency depends on specific transcription factors (TFs) that induce genes required to preserve the undifferentiated state and repress other genes related to differentiation. The transcription machinery and regulatory components such as TFs are recruited dynamically on their target genes making it essential exploring their dynamics in living cells to understand the transcriptional output. Non-invasive and very sensitive fluorescence microscopy methods are making it possible visualizing the dynamics of TFs in living specimens, complementing the information extracted from studies in fixed specimens and bulk assays. In this work, we briefly describe the basis of these microscopy methods and review how they contributed to our knowledge of the function of TFs relevant to embryo development and cell differentiation in a variety of systems ranging from single cells to whole organisms.

## **1. Transcription factors control stem cells fate**

Pluripotent stem cells (PSCs) are characterized by two basic properties: self-renewal, the ability to proliferate while preserving the same characteristics, and pluripotency, the capability of producing all cell types derived from the three germ layers in response to developmental cues (Hirai et al., 2011). These properties make them an important promise for their high potential both in regenerative medicine and to generate models for multiple diseases for research and drug screening (Trounson and DeWitt, 2016). In 2006, Takahashi and Yamanaka obtained induced PSCs from terminally differentiated cells, the so called induced PSCs or iPSCs (Takahashi and Yamanaka, 2006) further extending the potential applications of PSCs (Takahashi and Yamanaka, 2016). For these reasons it is crucial the comprehension of the molecular mechanisms involved in self-renewal, pluripotency and differentiation of PSCs.

Transcription factors (TFs) work together controlling the complex network of gene transcription activation and repression. They bind to specific DNA regions and recruit other biomolecules, including chromatin modifiers and non-coding RNAs that locally alter DNA accessibility to polymerases. The transcription factors Oct4, Sox2 and Nanog compose the PSCs core and act coordinately to preserve pluripotency (Loh et al., 2006).

Traditional approaches used in studies of TFs function focused either on measuring TF mRNA and protein levels using techniques such as RT-qPCR or Western blot and immunofluorescence, respectively, or analyzing TF-DNA interactions by electrophoretic mobility shift assay (EMSA) or chromatin immunoprecipitation (ChIP). Although these methods provide valuable information on TF function, their output mainly reports average properties of a large cell population missing the intra- and intercellular variability. Moreover, most of these methodologies only provide static snapshots of TFs since they require cell fixation and/or have poor temporal resolution.

In contrast, live-cell imaging allows observing TFs in single living cells with high temporal and spatial resolution. These novel studies revealed a highly dynamic scene in opposition to the traditional view of very stable TFs-chromatin complexes (Perlmann et al., 1990).

## **2. Fluorescence microscopy methods: from a static to a dynamical view of cellular processes**

The fluorescence microscope has become one of the key tools in Cell Biology labs. Microscopes have traditionally provided images of biological specimens with ~200 nm resolution however modern superresolution techniques are pushing this limit farther (Sahl et al., 2017). The groundbreaking innovations in fluorescence microscopy included the development of methodologies that allowed observing molecules as they move in living cells (Liu and Tjian, 2018). These advances, briefly summarized below, are making possible the observation of biological processes in their real context with high spatial and temporal resolution.

*Fluorescence Recovery after Photobleaching (FRAP)* involves the fast and irreversible photobleaching of fluorescent molecules within a region-of-interest (ROI) of the specimen followed by the observation of the recovery of fluorescence attained when photobleached molecules exchange with fluorescent molecules (Axelrod et al., 1976).

The time evolution of the intensity recovery is then calculated and fitted with either empirical equations (e.g. Angiolini et al., 2017) or, when possible, with equations accounting for the dynamical processes causing the replenishment of the ROI fluorescence (Mueller et al., 2010). Whereas the first approach provides characteristic recovery times that depend, for example, of the size and shape of the photobleached region (Mueller et al., 2010), the second analysis provides quantitative information on the dynamics of the molecules such as diffusion coefficients and binding/unbinding kinetic constants. In many cases, the intensity is not fully recovered evidencing a population of molecules with very low mobility within the temporal window of the experiment.

FRAP and related methods (Lippincott-Schwartz et al., 2003) provide dynamical information with relatively low spatial and temporal resolutions and require a relatively high concentration of fluorescent molecules. In addition, FRAP involves the use of high laser powers that may cause cell photodamage.

*Single Molecule Tracking (SMT)* techniques are exquisite methods that allow observing and localizing molecules with nanometer-precision. SMT makes it possible to follow individual molecules as they move in the intracellular environment with minimal perturbation of the specimen. The development of small, genetically encoded sequences such as Halo and SNAP tags that binds to bright and photostable organic fluorophores

allowed observing proteins labeled with single fluorophores in living cells for longer periods of time (revised in Liu et al., 2015).

The most widely used SMT methods require microscopes that allow illumination of an optically defined z-section of the sample such as TIRF (total internal reflection fluorescence microscopy; Schneckenburger, 2005) and SPIM (single plane illumination microscopy; Huisken et al., 2004). Single TFs are observed as diffraction-limited spots provided that only a small population of TFs is labeled with bright and photostable probes (Presman et al., 2017). The center of these diffraction patterns corresponding to the molecule position is localized with ~2 nm error (Yildiz et al., 2003). In living specimens, the precision decreases to 20-40 nm (Mortensen et al., 2010).

These procedures are repeated in every frame of a time-lapse movie providing trajectories of the molecules that are quantitatively analyzed to obtain motion parameters such as diffusion coefficients and flow velocities (Levi and Gratton, 2007).

In the particular case of TFs, the trajectories are frequently analyzed extracting those regions where the molecules present confined motion that are assigned to chromatin-binding events (e.g. Paakinaho et al., 2017). The analysis of the temporal duration of these events provides information on the lifetime of the TF-chromatin complex.

*Fluorescence fluctuation based methods* analyze spontaneous intensity fluctuations produced within the small, femtoliter-sized observation volume defined in confocal and multiphoton microscopes (Berland et al., 1995; Elson, 2013; Rigler et al., 1993).

In the simplest, single-point fluorescence correlation spectroscopy (FCS) experiment, the laser is focused in a diffraction-limited region of the sample and the intensity is collected as a function of time. Every time a molecule spontaneously enters in and exits out the observation volume causes a fluctuation in the intensity trace; slow-moving molecules produce long-lasting fluctuations while fast molecules introduce fast fluctuations. A careful, statistical analysis of these fluctuations provides quantitative information on the dynamics.

Generalizing these concepts, FCS allows studying any process causing fluctuations in the intensity trace including dynamical processes such as diffusion as well as binding interactions, chemical reactions and photophysical phenomena.

The intensity data is normally analyzed calculating the temporal autocorrelation function that quantifies the self-similarity of the intensity trace at time  $t$  and after a lag time  $\tau$ :

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} \quad (1)$$

where  $I(t)$  represents the fluorescence intensity at time  $t$ , the brackets indicate average values over the time-course of the experiment, and  $\delta I(t) = I(t) - \langle I(t) \rangle$  represents the fluorescence fluctuation at a given time.

This data can be fitted with functional forms of  $G(\tau)$  derived from the theoretical analysis of the molecular mechanism assumed to be responsible for the intensity fluctuations (Elson, 2011; Elson, 2013; Krichevsky and Bonnet, 2002).

As an example, Figure 1 shows representative FCS data obtained in PSCs expressing the TF Oct4 fused to the green fluorescent protein (GFP); the autocorrelation function is shifted to higher  $\tau$  values in comparison to free GFP illustrating the slower motion of the TF due to the interactions with chromatin. In this particular case, the data was analyzed using a model that considers the diffusion of the TFs in the nucleoplasm and their interactions with two populations of fixed, binding sites (Stortz et al., 2017; White et al., 2016).

### 3. Unraveling the dynamics of transcription factors in the cell nucleus

From the very beginning, FRAP, SMT and FCS methods constituted attractive tools to study nuclear processes at different levels of complexity ranging from capturing the diffusion of DNA repair factors along the DNA chain *in vitro* (Gorman et al., 2010) to exploring the whole spatial and temporal organization of transcription-related processes in living cells as detailed below.

In a keystone contribution, Mc Nally and Hager (McNally et al., 2000) revealed using FRAP that the interactions between the glucocorticoid receptor (i.e. a ligand-dependent TF) and its DNA targets were fast and transient, arguing against the traditionally view of a stable transcriptional complex. Phair et al. (Phair et al., 2004) also demonstrated by FRAP that a wide variety of chromatin-associated proteins present a fast turnover on chromatin. These studies suggested that the dynamical properties of TF-chromatin interactions play a relevant role in the modulation of gene expression (Normanno et al.,

2012). In this direction, Karpova et al. (Karpova et al., 2008) showed simultaneous fast and slow TF cycling on an endogenous yeast promoter and suggested that the latter represents oscillations in the fraction of accessible promoters rather than the recruitment and release of stably bound transcriptional activators. SMT techniques were first applied in simple organisms such as *E. coli* to quantify the binding/unbinding of the lac repressor to the chromosomal lac operator and its response to metabolic signals (Elf et al., 2007).

SMT and FCS showed that TF-DNA interactions in eukaryotic cells span a wide temporal window from milliseconds to seconds (for example; Brazda et al., 2011; Michelman-Ribeiro et al., 2009; Mikuni et al., 2007; Stortz et al., 2017). The long-lived interactions are frequently assigned to specific binding (Chen et al., 2014) despite nonspecific and specific interactions may overlap temporally (Normanno et al., 2015) suggesting a more complex scenario. In addition, TFs do not distribute homogeneously in the cell nucleus but are frequently concentrated in clusters or foci (Misteli, 2007). FCS also allowed mapping the dynamical distribution of TFs revealing that these proteins partition among nuclear compartments and chromatin-binding sites (Stortz et al., 2017) ultimately regulating their interactions with more specific sites and thus, the final transcription output (Mueller et al., 2013).

SMT and FCS were key techniques to explore in embryonic stem cells (ESC) the dynamics of transcription factors that control pluripotency such as Sox2 and Oct4 (Chen et al., 2014; Kaur et al., 2013). SMT also provided mechanistic insights on the sequential interactions of these TFs with DNA targets and revealed that formation of enhanceosomes involves an initial interaction of Sox2 followed by Oct4 engagement (Chen et al., 2014). Oct4 and Sox2 also contribute to a local chromatin remodeling making it more accessible to other TFs and cofactors (Xie et al., 2017). These results contribute to reveal the highly dynamic and coordinated mechanisms involved in the regulation of transcription programs relevant to pluripotency.

Recent studies in ESC show that Sox2 and Oct4 remain bound to chromatin during mitosis suggesting that certain transcriptional programs are transferred through this mechanism to daughter cells (Deluz et al., 2016; Teves et al., 2016). The quantitatively different Oct4 and Sox2 interactions with DNA during mitosis revealed by SMT and photobleaching-based methods (Deluz et al., 2016) could also contribute to delineate the transcriptional program of daughter cells.

These methodologies are starting to be applied in whole, multicellular organisms to understand the dynamics of transcription factors relevant to development.

For example, FRAP provided estimations of the diffusion and nuclear-cytoplasmic shuttling of Bicoid (BCD) in early *Drosophila melanogaster* embryos revealing new aspects of the mechanisms involved in morphogen gradients formation during development of multicellular organisms (Gregor et al., 2007). Recently, Mir et al. (Mir et al., 2017) used SMT to quantify BCD-DNA interactions along the anteroposterior axis in *Drosophila* embryos. Notably, they found transient hubs, dependent on the factor Zelda and presenting a locally high BCD concentration that facilitate the binding of this TF to specific targets on the DNA. This mechanism plays a key role especially on the posterior region of the embryo where the Bicoid concentration is very low.

FCS also allowed getting insights into the intercellular movement of the TF SHR, that control root patterning and cell fate specification during *Arabidopsis* development (Clark et al., 2016). This study provided quantitative information on SHR mobility, its oligomeric state and the stoichiometry of the SHR-SCR complex involved in cell specification. The authors incorporated these data in mathematical models contributing to understand how these dynamical interactions impact on *Arabidopsis* development.

We have recently used FCS to quantify the dynamics of TFs in developing mouse early embryos and found variations on DNA-Sox2 interactions among blastomeres of the 4-cell embryo that correlate with the cell fate of the progeny (White et al., 2016).

In summary, these works constitute a firm demonstration of the applicability of these technologies to study TFs dynamics not only in isolated cells but in a wide variety of organisms. Also, the possibility of quantifying interactions between molecules labeled with spectrally different fluorescent probes predicts further applications of these methodologies to explore the complex temporal and spatial network of interactions that define gene expression. In this context, we foresee that these methodologies will constitute fundamental tools to gain comprehension of the mechanisms involved in the regulation of gene expression of developing organisms. Moreover, the examples reviewed here illustrate the bright future of advanced fluorescence microscopy and show how these methods are opening a new era for quantitative biology.

**LEGEND TO FIGURE**

**Figure 1: Exploring the dynamics of Oct4-GFP in PSCs with fluorescence correlation spectroscopy.** (A) Overlay between the transmission and confocal images of a colony of mouse embryonic stem cells showing cells expressing Oct4-GFP (green). (B) The laser was focused in a diffraction-limited volume (yellow point) within a cell nucleus and the intensity was collected as a function of time. Scale bars: 10  $\mu\text{m}$ . (C) Autocorrelation curve obtained for Oct4-GFP (red), the figure also shows data obtained in cells expressing GFP (blue) to illustrate the delayed dynamics of Oct4-GFP partially due to interactions with chromatin.

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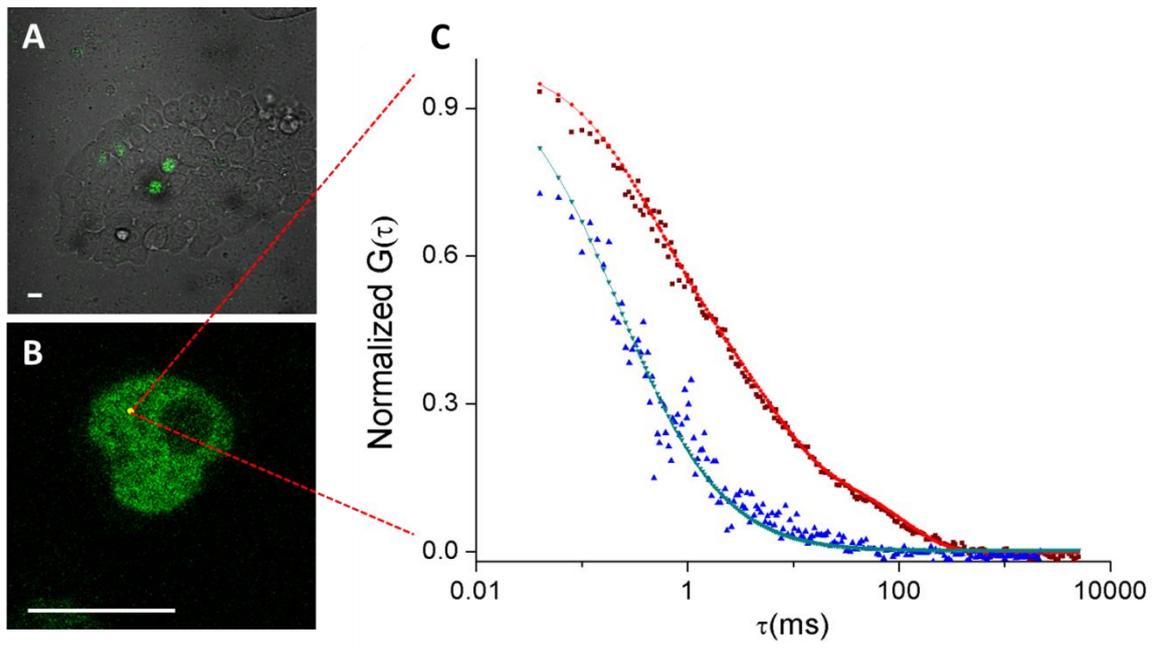
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FIGURE 1



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**Highlights**

Innovations in microscopy allow observing molecules as they move in live cells

FRAP, SMT and FCS are attractive tools to study nuclear processes in single cells

These methods reveal new aspects of gene regulation in developing organisms

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