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Quantifying transcription factor binding dynamics at the single-molecule level in live cells

Diego M. Presman [a,1], David A. Ball [a,1], Ville Paakinaho [a,1], Jonathan B. Grimm [b], Luke D. Lavis [b], Tatiana S. Karpova [a], Gordon L. Hager [a,*]

[a] Laboratory of Receptor Biology and Gene Expression, Building 41, 41 Library Drive, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[b] Janelia Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA

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ABSTRACT

Progressive, technological achievements in the quantitative fluorescence microscopy field are allowing researches from many different areas to start unraveling the dynamic intricacies of biological processes inside living cells. From super-resolution microscopy techniques to tracking of individual proteins, fluorescence microscopy is changing our perspective on how the cell works. Fortunately, a growing number of research groups are exploring single-molecule studies in living cells. However, no clear consensus exists on several key aspects of the technique such as image acquisition conditions, or analysis of the obtained data. Here, we describe a detailed approach to perform single-molecule tracking (SMT) of transcription factors in living cells to obtain key binding characteristics, namely their residence time and bound fractions. We discuss different types of fluorophores, labeling density, microscope, cameras, data acquisition, and data analysis. Using the glucocorticoid receptor as a model transcription factor, we compared alternate tags (GFP, mEOS, HaloTag, SNAP-tag, CLIP-tag) for potential multicolor applications. We also examine different methods to extract the dissociation rates and compare them with simulated data. Finally, we discuss several challenges that this exciting technique still faces.

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1. Introduction

One of the most fundamental tasks that occur inside the nucleus of eukaryotic cells is the process of transcription. The first step in transcription initiation involves the binding of transcription factors (TFs) to specific recognition sequences located at enhancers and/or promoters, which ultimately leads to the assembly of the pre-initiation complex [1]. Built upon biochemical and population-averaging studies, the classical view is that the subunits of the transcription machinery arrive in sequence to form a stable, functional end product. However, live-cell imaging studies have proven that the transcriptional regulatory complex is far more dynamic than originally anticipated, with subunits that quickly assemble and likely not always in a pre-defined order [1–4].

Groundbreaking developments in live-cell microscopy, fluorescence correlation spectroscopy (FCS), and fluorescent labeling have begun to open unique opportunities to study the dynamics of biological systems with high spatial and temporal resolution [5]. In particular, single-molecule tracking (SMT) approaches allow one to follow individual protein molecules in single live cells (Fig. 1A). These technological advances now provide the means for the visualization and the measurement of the *in vivo* behavior of TF-binding events at chromatin targets such as enhancers and core promoters [6]. Several studies have now measured TF-binding events in both prokaryotes [7,8] and eukaryotes [7,9–21]. These experiments have shown that eukaryotic TFs spend most of their time freely diffusing, while only a small portion is specifically bound to chromatin at any given time [9–11,13,20]. In striking contrast, prokaryotic TFs appear to spend most of their time associated with DNA [7]. In all cases, binding events appear to be very fast, on the order of seconds at the most.

Abbreviations: CDF, cumulative distribution function; EMCCD, electron-multiplying charge-coupled device; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; Dex, dexamethasone; GR, glucocorticoid receptor; HILO, highly inclined and laminated optical sheet; JF, Janelia Fluor; sCMOS, scientific complementary metal oxide semiconductor; PDF, probability density function; PSF, point spread function; ROI, Region of Interest; SMT, single-molecule tracking; SNR, signal-to-noise ratio; TF, transcription factor; TIRF, total internal reflection fluorescence; TMR, tetramethylrhodamine.

* Corresponding author.
E-mail address: hagerg@exchange.nih.gov (G.L. Hager).
[1] These authors contributed equally to this work.

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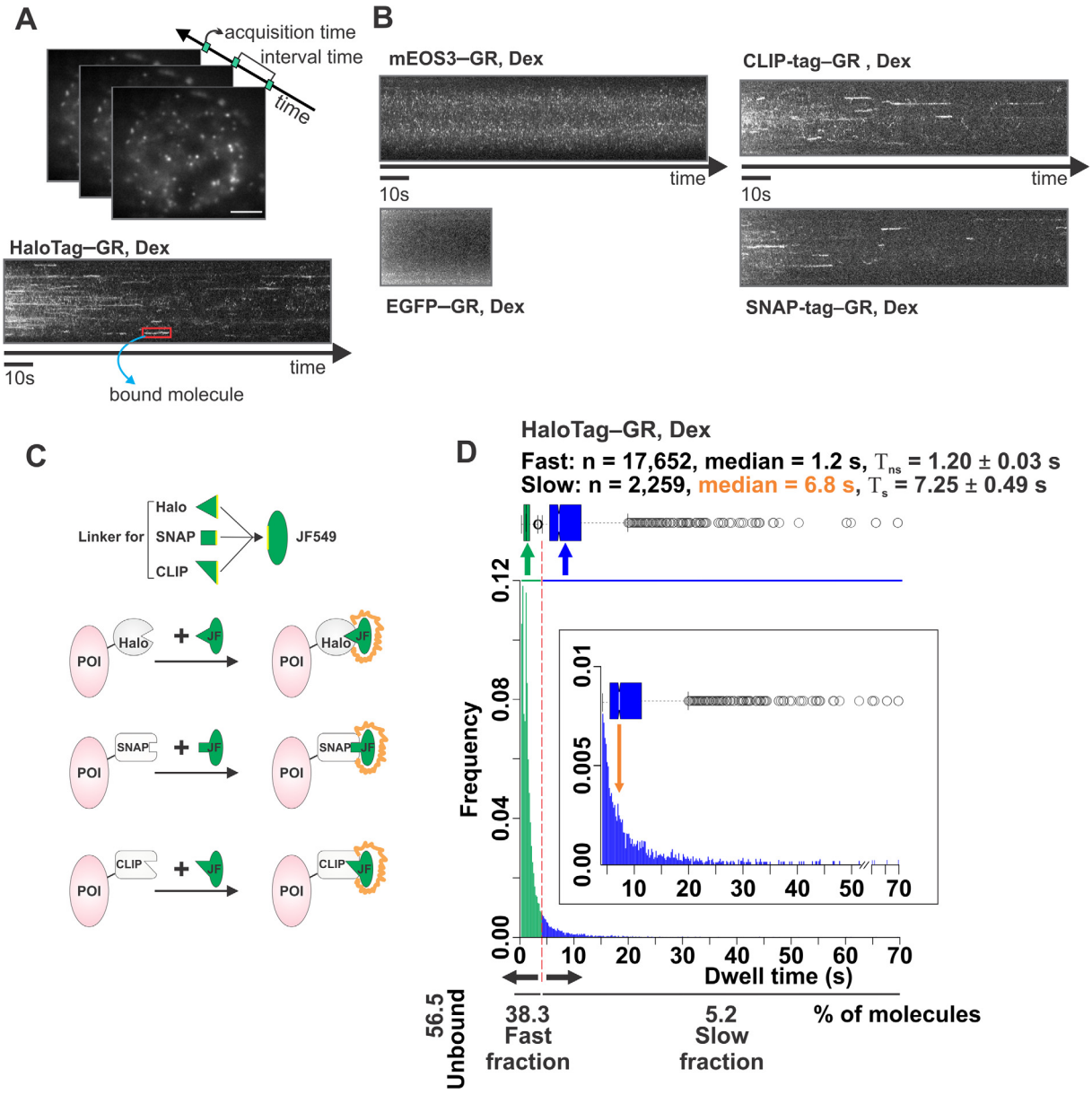

Fig. 1. Single-molecule tracking (SMT) of GR molecules tagged with different labels. (A) The SMT technique visualizes individual molecules as bright diffraction-limited spots and tracks their movement or lack thereof over time. Hence, one can directly identify bound molecules as those that stop moving and remain stationary. HaloTag–GR labeled with Janelia Fluor 549 (JF_{549}) HaloTag ligand can be visualized as such diffraction-limited spots under HILO microscopy. Scale bar 5 μm. A stack of images is taken from a single live cell with 10 ms acquisition time and 200 ms interval time. These parameters are used in order to capture most of the diffusing molecules as well as the stationary ones, while still being able to monitor longer binding events without excessive photobleaching. If molecules remain stationary, the time-projection stack will reveal a continuous signal that represents a bound GR molecule in a dexamethasone (Dex) exposed cell (red box). (B) Time-projection stacks from cells transfected with mEOS3-GR (upper left), EGFP-GR (lower left), CLIP-tag–GR labeled with JF_{549} (upper right), or SNAP-tag–GR labeled with JF_{549}cp (lower right) in Dex treated conditions. (C) Schematic representation of HaloTag, SNAP-tag, and CLIP-tag post-translational fluorescent labeling system. Organic fluorescent dye (green oval), such as JF_{549}, is made specific for different tags by changing the linker region (equilateral triangle, square, and isosceles right triangle). Each tag has different binding pocket specific for certain linker region. Protein-of-interest (POI) will become fluorescent when JF_{549} with right linker binds to the corresponding tag. (D) Distribution of residence times from individual GR (+Dex) stationary tracks, either in a histogram or in a Box-plot. A continuum of bi-exponentially distributed bound molecules is typically observed, based on the fitting of the survival distribution. The fast short-lived (T_{ns}, non-specific) and slow long-lived (T_s, specific) fractions are color-coded (green and blue, respectively). In-set shows only the T_s population where the orange arrow points at the median value. The number (n) of tracks obtained, and the median dwell time in fast short-lived and slow long-lived fraction is shown above the histogram. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

There has been intense interest in understanding how TFs find their way to their targets and several mechanisms have been proposed (reviewed in [6] and [22]). In this work, we will focus on how to performed SMT experiments to extract information regarding the binding characteristics of TFs, *i.e.* the residence time and bound fractions. We will describe key aspects of the technique: fluorophores, label density, microscope set-up, acquisition conditions and tracking analysis. Finally, we will discuss the advantages and disadvantages of this new methodology and perspectives for the future.

2. Single-molecule tracking for extracting binding characteristics

Single-molecule microscopy requires a bright and photostable fluorophore, low labeling density, a sensitive camera (capable of