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Review Article

RNA folding dynamics by single-molecule fluorescence resonance energy transfer

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ABSTRACT

Single-molecule fluorescence resonance energy transfer (smFRET) microscopy has become an increasingly popular tool to study the structural dynamics of RNA molecules. It reveals, in real time, the structural dynamics of these molecules that would be otherwise hidden in ensemble-averaged measurements. Here we present a detailed protocol for performing smFRET using total internal reflection fluorescence microscopy, including RNA preparation, optical setup, separation of the dual color channels, sample immobilization and data acquisition and analysis.

1. Introduction

Over the past two decades, single-molecule experiments have yielded a wealth of answers to important biological problems. For instance, they have been used to investigate mechanisms of molecular motors such as ATP synthase [1–3], myosin and kinesin [4,5], the DNA packaging engine of bacteriophage phi-29 [6,7] and the ribosome [8,9]. The most attractive aspect of single-molecule experiments is that they directly reveal the structural dynamics of individual molecules, which would otherwise be hidden in ensemble-averaged experiments [10–15]. There are many approaches that have been used in single-molecule microscopy, such as fluorescence correlation spectroscopy (FCS) [16], optical and magnetic tweezers [17,18], atomic force microscopy [19], confocal microscopy [20] and total internal reflection fluorescence microscopy (TIRFM) [21,22]. Each approach presents advantages and disadvantages that have been exploited to study a wide variety of biologically relevant questions. Here, we focus our attention on single-molecule spectroscopy using TIRFM excitation and FRET detection to study the folding kinetics of RNA molecules.

Non-coding RNA molecules play numerous roles in cellular process such as protein synthesis, regulation of gene expression, RNA editing and viral replication [23–25]. According to the RNA world hypothesis, RNA may have initiated life on Earth [26]. In order to become biologically active, non-coding RNAs must first fold into their correct secondary and tertiary structures. However, RNA folding potential energy surfaces can be very rugged and replete with kinetic traps [27], which can result in the formation of inactive intermediate structures. To fully understand the structure–function relationship in RNAs, it is important to map their folding potential energy surfaces. Over the past decade, numerous smFRET studies have shed new light onto this process [12,28–30]. Here, we review a detailed protocol for using single-molecule FRET microscopy to study the folding dynamics and kinetics of RNA. First, we describe the necessary instrumentation for the total internal reflection microscope and single-molecule FRET detection. Then, we describe in detail the sample preparation. Lastly, we explain how to analyze the resulting data to obtain relevant thermodynamic and kinetic information. Other reviews have previously described the principle of smFRET [21,22], here we provide more detailed protocols.

2. Description of method

2.1. Total internal reflection fluorescence microscopy

To achieve single-molecule fluorescence detection, it is essential to decrease all sources of background fluorescence. TIRFM helps achieve this goal by reducing the excitation volume to a thin sheet at the interface between the microscope slide and the sample solution [31,32]. Using an inverted microscope (IX-71, Olympus, Center Valley, PA) the sample solution is located between a quartz microscope slide (Finkenbeiner, Waltham, MA) and a coverslip (VWR, West Chester, PA). In TIRFM, the laser excitation beam (532 nm, 3 mW, CrystaLaser GCL-532-L, Reno, NV) reaches the solution at an incidence angle larger than the critical angle (θ_c) at which the beam is totally reflected and does not penetrate in

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solution (Fig. 1). This critical angle can be easily determined using Snell's law

$$\theta_c = \sin^{-1}\left(\frac{n_{sol}}{n_q}\right), \quad (1)$$

where n_q and n_{sol} are the index of refraction of the quartz slide and the buffered solution, respectively (Fig. 1a, note that $n_q > n_{sol}$ in order to obtain a critical angle). Above θ_c, an evanescent wave is formed at the slide–solution interface, which penetrates only a few hundred nanometers in solution, typically 100–150 nm. Therefore, only those fluorescent molecules that are within this distance from the interface are excited by the evanescent wave. Any other source of background fluorescence in solution remains unexcited. There are two common approaches used to generate TIRF: prism-based and objective-based TIRF (Figs. 1 and 2).

(i) Prism-based TIRF: In this approach, a quartz Pellin-Broca prism (CVI Melles-Griot, Albuquerque, NM) is used to bring the laser beam to the quartz slide. To avoid optical gaps between the prism and the slide, an index-of-refraction-matched immersion oil (Cargille, Cedar Grove, NJ) is used to ensure contact. Fig. 1a shows a schematic diagram of the setup. The laser beam is reflected toward the prism by a mirror (M, Newport, Irvine, CA) at a distance (d) from the prism. The beam is focused by a BK7 lens (L, effective focal length f_1 = 100 mm, Newport, Irvine, CA). To achieve TIRF, the angle of incidence θ_3 must be larger than θ_c, which can be easily controlled by adjusting the height (h) of the mirror (M). The maximum height h_{max} can be calculated as

$$h_{max} = d\sqrt{\frac{n_q^2 - n_{sol}^2}{n_{air}^2 - (n_q^2 - n_{sol}^2)}}, \quad (2)$$

where n_{air} is the index of refraction of air. Typically, d and f_1 are fixed distances and only h is varied. To assist with beam alignment, the lens (L) should be installed on a three-dimensional micrometer translation stage (Newport, Irvine, CA). To pre-align the laser beam, a low magnification air objective (20×, Olympus, Center Valley, PA) can be used to position the TIRF spot near the center of the field-of-view by observing the scattered light through the microscope's binoculars. The TIRF spot can then be precisely aligned on the center of the single-molecule objective (60×, water immersion, Numerical Aperture = 1.2, Olympus, Center Valley, PA) by adjusting the lens position on the micrometer translation stage.

(ii) Objective-based TIRF: While prism-based TIRF is a straightforward approach to easily generate the evanescent wave, it also represents an important disadvantage: the presence of a prism on top of the slide may prevent further manipulation of the sample. To overcome this difficulty, the evanescent wave can be generated through the objective (Fig. 2), however, the beam alignment is more complicated. A detailed list of required optics has been described elsewhere [33]. The laser beam is initially expanded to ~25 mm diameter by a pair of lenses (L1 and L2) with different focal lengths. The focal lengths, f_1 and f_2, respectively, depend on the initial laser beam diameter. For a 0.4 mm diameter beam, the focal lengths are f_1 = 5 mm and f_2 = 400 mm, and the lenses are positioned 405 mm from each other. A third lens (L3) focuses the expanded laser beam onto the back focal plane of the objective (60×, oil immersion, Numerical Aperture = 1.45, Olympus, Center Valley, PA). The difficulty in objective-TIRF is to properly align the excitation beam in the objective to achieve TIRF (Fig. 2b). The green path reaches the center of the objective at an angle larger than θ_c and successfully generates the evanescent wave, while the incidence angle of the red path is smaller than θ_c, and the beam is refracted. The signature of objective-TIRF is a bright laser beam that is totally reflected at the coverslip with no laser beam refraction through the coverslip. For an objective with a 1.45 numerical aperture, there is only a small range of angles to achieve TIRF, therefore, it is crucial to focus the beam accurately on the back focal plane of the objective. Another important issue to achieve objective-based TIRF is to ensure that the excitation beam travels parallel to the vertical axis. The use of irises (Thorlabs, Newton, NJ) can be helpful for this purpose. The beam alignment procedure is as follows:

1. Focus the objective onto the coverslip.
2. Align the laser beam using mirrors M1 and M2 in the absence of lenses L1, L2 and L3 to achieve near-TIRF. M2 should be installed on a two-dimensional (horizontal plane) micrometer translational stage to help accurately align the beam parallel to the vertical axis of the microscope.
3. Install the beam expander (L1 and L2) while ensuring that the beam travels through the center of the lenses and maintains the alignment.
4. Install L3 to ensure that the beam is focused on the back focal plane of the objective. To align the beam accurately, make slight adjustments to the position of L3. Installing L3 on a two-dimensional (vertical plane) micrometer translational stage facilitates this operation.
5. Objective-based TIRF is difficult to achieve and maintain. Occasionally, the focal point drifts during data acquisition, preventing long-time acquisition. However, regular glass slides can be used to reduce the cost of each experiment.

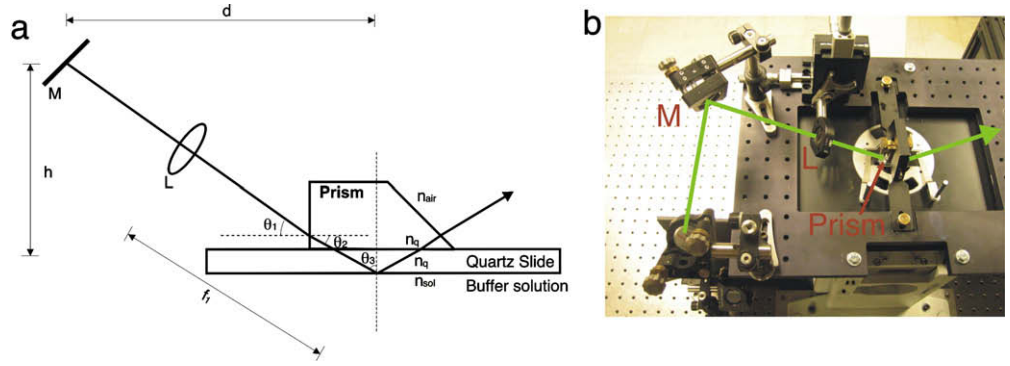

Fig. 1. (a) Schematic diagram of prism-based TIRF. The excitation beam is reflected by a mirror (M) toward the lens (L) to reach the prism at the appropriate angle (θ_1). The beam ultimately reaches the slide–solution interface at the total internal reflection angle ($\theta_3 = \theta_c$). The refraction indices of the prism, quartz slide and aqueous solution are n_q and n_{sol}. The mirror is located at a distance d and height h from the objective, which are adjusted to achieve total internal reflection. The lens is located at a focal length f_1 from the objective. (b) Photograph of our prism-based TIRF setup. The optics are labeled consistently with the diagram.