Automated smFRET microscope for the quantification of label-free DNA oligos

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Abstract: Single-molecule FRET (smFRET) spectroscopy is a powerful tool for studying inhomogeneous dynamics in biological systems. However, because of the intrinsic variations that accompany the sample sizes, massive data sets are essential to extract statistically reliable information. In this aspect, a simple motorized stage and autofocusing modification can save time without the expense of a high-end automated microscope. In this report, we describe a simple and economical modification of a commercial inverted microscope with a manual stage to automate the data acquisition and measurement process. We collected 8000 images with a 100 ms exposure time in 1000 fields of view in approximately 13 min, where it would take more than 8 h by manual collection. We demonstrated the method with a DNA oligo quantification experiment. In this experiment, the measurement platform is a FRET signal from a dye-labeled DNA duplex containing unmatched base pairs. The target DNA replaces one of the strands because of the formation of a perfect duplex. This thermodynamically driven exchange reaction causes FRET to disappear, which correlated with the DNA concentration. The data are batch processed with the freeware ImageJ. These modifications are feasible and economical for general smFRET experiments.

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1. Introduction

Single-molecule Forster resonance energy transfer (smFRET) spectroscopy measures the fluorescence intensities of two energy-tangled dye molecules at close spatial locations. The reconstruction of signals from individual biomolecules provides inhomogeneous population information and rare event dynamics that cannot be revealed with ensemble methods [1]. However, because one molecule is measured at a time, acquiring and analyzing the data requires intensive labor. Automation of data acquisition is essential in many cases. On the other hand, commercially available motorized stages and perfect focusing systems are expensive. We modified a manual Nikon stage with inexpensive step motors and built an autofocusing system with a quadrant photodetector paired with a PID feedback controller, taking advantage of the back-reflected TIRF laser spot.

To demonstrate our automation system, we quantified a DNA oligo using the smFRET signal as the measurement platform. Nucleic acids play an important role in normal and diseased cell functions [2]. For example, microRNAs (miRNAs) are 20-25 nucleotide noncoding RNAs that suppress the translation of their mRNA targets [3]. miRNAs are dysregulated in cancers, correlating with the dysregulated protein expression in these cells. Therefore, miRNAs are promising cancer biomarkers [4]. However, miRNA profiling is not ready for clinical diagnosis, because of the inconsistency of miRNA quantification via different methods [5]. Most of the current methods, such as microarrays [6], RT-qPCR [7] and NGS [8], require RNA extraction, ligation, cDNA conversion, extensive amplification and even preamplification [7]. Because of cDNA conversion and PCR sequence biases [9,10], different commercial platforms generate diverse quantification results [11,12]. Many methods have been developed to address the inconsistency, but a perfect method has not been found [13]. Alternative methods that can bypass some or all of these steps are possible solutions to avoid the inconsistency [14]. In this report, smFRET signals were generated between two
DNA strands of surface-bound duplexes, which were dissociated by the target oligo (microRNA let-7g's DNA analog) due to the energy levels. This dissociation decreased the FRET pair numbers, which were proportional to the concentration of the microRNA analog. Because the sensitivity of smFRET is high, the DNA molecules are not amplified or labeled. However, to obtain statistically reliable results, large sample sizes are necessary. While manually changing the microscope stage to collect tens of videos is possible, it becomes a tedious and prolonged procedure to collect thousands of videos. The main time-consuming step is moving the stage and refocusing. Although a time as short as 30 s is enough to change the field of view for a skillful researcher, this step amounts to more than 8 h if 1000 videos are needed. To solve this problem, we implement an automotive stage-scanning setup. For capturing data in 1000 fields of view (100 ms exposure time and 8 frames per field), the acquisition time decreased from more than 8 h to 13 min. If more data are necessary, the time difference will be even larger. Accordingly, the massive amount of data was batch processed with macros developed in the ImageJ program. Our method provides an alternative assay to directly quantify nucleic acids.

2. TIRF microscope optics

An objective-based TIRF (total internal reflection fluorescence) microscope was built on a Nikon Eclipse Ti inverted microscope (Fig. 1). Two solid-state lasers (532 nm – 75 mW, 640 nm – 20 mW, Crystalaser, Reno, NV) were used. The original laser spots (approximately Ø 0.5 mm) were expanded with a pinhole spatial filter (KT110, Thorlabs) to a size of Ø 1.5 cm and then directed to the edge of a 60 x 1.49 NA oil immersion objective (Nikon Instruments). The light was focused at the back focal plane of the objective and then hit the coverslip of the flow cell at a glancing angle to be totally reflected at the glass-liquid interface. Therefore, only surface-bound molecules can be excited by the 100-nm-thin evanescent wave radiation. The emitted fluorescent light was collected via the same objective, passed through dichroic mirrors, and after separation by a dual view apparatus (Optical Insights) formed mirrored images on a back-thinned CCD camera (532B Cascade II; Photometrics). The sample cell was made of a cover slip (ThermoFisher rectangular cover slips, 24 x 40 mm, thickness #1 (0.17 μm)) and a glass slide (Fisherbrand, 25x75 mm) via double-faced tape. Six channels (3x13 mm) were made in one glass with two holes in each channel. One hole was connected a pipette tips for solution delivery, and the other hole was connected to a syringe or syringe pump for suction (Fig. 1). A few studies have described details on building a TIRF microscope for smFRET experiments [15–17]. The physical setup from our lab is shown in Fig. 8 in the Appendix.
3. Stage motorization and stepping size calibration

To gain statistical significance, it is essential to automate the data acquisition. For the purpose of automating a random scanning of the fields of view of the sample, it is not necessary to implement high-precision expensive automated stages. Thus, two economical SilverPak17C step motors (Lin Engineering) were coupled to the 2D-manual stage actuators. The stepping size of the motor was calibrated with a patterned grid as shown in Fig. 2. Three different motor stepping sizes were measured: P50, P100 and P150 (these are the motor parameters for stepping). For example, at the P50 setting, 7 images were taken at 7 consecutive steps of the stage movement. The images can be aligned with their succeeding image via the same grid in the different images because every grid line has a unique thickness. Therefore, the stage movement step can be measured in pixels by comparing the pixel differences of the same grid in consecutive images. To quantify the displacement more precisely, the grid pattern in each image was obtained via the “vertical profile plot” tool in ImageJ (plots (a-c) in Fig. 2). Each colored profile represents one grid pattern at one stage position. Six offsets with the best overlay of the consecutive profiles were obtained and averaged. This was done similarly for P100 and P150. By visual inspection of Fig. 2, the step size increased from P50 to P150. In Fig. 2(d), the stepping sizes are plotted against the P-parameter, and a linear relationship is obvious. The slope is approximately 2.7 pixels/P. Therefore, the P300 setting moves the stage 800 pixels, which is 58% more than the 512 pixels of the camera area. On the other hand, the standard deviations in Fig. 2(d) indicate that random errors occurred during the stepping. However, because returning back to the exact same field of view is not required in most smFRET experiments, the small uncertainty in the stage movement is not a concern for these experiments, as long as the new field of view does not overlap with the previous field of view. The P300 setting achieved this goal without moving the stage at an excessively large distance. Consequently, even though a more precise estimate of the error can be obtained by hundreds of repeated measurements, it is trivial in the current study. Instead, 6 movements and measurements at each P-setting were sufficient for calibration purposes.
Although the calibration was conducted for vertical movement (Y-axis), the motor moved the stage the same distance along the X-axis. Figure 3(b) shows the detailed stage movements in microns. Each channel is 3x13 mm in size (Fig. 3(a)), and one round of stage movement contains (20 steps down)-(one step right)-(20 steps up). Based on the step calibration, the P300 setting generated a step size of 800 pixels, which corresponded to 216 μm (0.27 μm per pixel on the 512B cascade II camera via a 60x objective). Therefore, the total traveling area was 0.216x4.3 mm², which was 2% of the total channel area. A larger area can be scanned by tuning the motor step size and adding more steps, as well as repeating the 40-step protocol. The latter is a better choice for keeping the data size manageable. The smaller traveling distance that we chose is advantageous for keeping the focus drift small and avoiding large background variations in the surface in one set of 40 fields of view.

Fig. 2. The motor stepping size calibration. For three settings, P50, 100 and 150, consecutive images were taken with a grid on the stage. Because of the distinguishable grid thickness, the stage movements could be deduced by aligning the same grid in different images. To avoid a one-pixel calibration, the alignment was performed by aligning the vertical profile of all the grids (a, b, c). For 7 images, 6 alignments were obtained and averaged to generate the linear line of (d).

Fig. 3. (a) The scheme of the sample cell ensemble. A physical picture is shown in Fig. 1. Holes were drilled on the glass slide, which is shown as the larger rectangle (25x75 mm). The smaller rectangle that circumvents the channels is the cover slip. These two glass surfaces were glued together via double-faced tape. (b) Illustration of the 2x20 rectangular stage movement pattern.
4. Autofocusing setup

An autofocusing system was installed to keep the images in focus during stage scanning. To do this, the back reflection of the laser spot was directed to a quadrant photodiode (PDP90A, Thorlabs). The X and Y displacements of the laser spot from the center were transferred to the PID module (TPA101, Thorlabs) as electronic signals, which were output as either a high or low voltage. The X output (or Y output) voltage was coupled to a third SilverPak17C step motor that was clamped to the fine-focus knob of the microscope. Before acquisition, the focus was manually set, and then the photodiode was moved to the position with “0” displacement. During acquisition, if the focus drifts, the output voltage will drive the motor to crank the focus knob in the opposite direction via motor commands; therefore, a stable focus is achieved. Compared to a commercial “perfect focus” system, no additional light source and optical components were needed, therefore dropping the cost significantly. An illustration of the autofocusing setup and sample motor command is provided in the Appendix (Fig. 9).

5. Synchronization and alternating laser illumination

The motors exhibited excellent time consistency. The dwell time for each field of view can be set by the motor program or controlled by a user-developed Labview interface. For continuing video recording without a pause, the number of frames for every field of view was perfectly consistent. Therefore, synchronization between the stage and camera was not necessary, as long as the first field-changing frame was identified. In addition, a few frames at the beginning and end for each field of view were removed to eliminate the images captured during stage transitions.

On the other hand, the stage movement, camera shutter, and laser shutters can be synchronized with an external function generator (Jameco electronics). This is necessary in alternating laser illumination experiments. For example, to acquire movies with a 100 ms exposure time with alternating green/red laser illumination, the master signal was a 10 Hz square wave (Fig. 4(a)). The signal was halved in frequency by an NTE889M flip-flop (NTE electronics) to control the green laser shutter (Fig. 4(b)). At the same time, this signal was inverted via a CD4013BE op-amp (Texas Instruments) to control the red laser shutter (Fig. 4(c)). The outcome was an alternating image recording of fluorescence emission under green and red laser illumination (arrows in Fig. 4(a)). The same master signal also controlled the time to move the stage using the “Halt” command. For example, command “H04H14” will move the stage every 2 images (“0” and “1” represent L and H voltage; “4” represents the motor I/O input pin).

![Fig. 4. Illustration of electronic signals to synchronize the stage, camera, and the laser shutter.](image-url)
6. smFRET platform

All of the DNA oligos were purchased from IDTdna. The duplex formed between S1 and S2 was prepared by mixing 10 μM S1 and S2 in an exchange buffer (10 mM Tris (pH 8.0), 1 M NaCl, 1 mM EDTA), heated at 95 °C for 5 min and then cooled to room temperature very slowly. The duplex was then stored at −20 °C in aliquots. Before loading into the imaging chamber, the duplex was diluted 100,000 times to a final concentration of 0.1 nM. FRET images of the imaging chamber were first collected as a reference; then, S3 solutions with various concentrations were injected into the sample chamber and incubated at 37 °C for 30 min under water vapor conditions to prevent drying. Afterwards, the sample chamber was washed with buffer extensively to remove free floating DNA strands, and FRET images were taken again. The imaging solution was the same as the exchange buffer with the addition of an oxygen scavenger cocktail containing (3 mg/mL glucose, 100 mg/mL glucose oxidase, 48 mg/mL catalase, and 2 mM trolox). The sequences of S1-3 are tabulated in Table 1.

Table 1. DNA sequences (S1-S2 forms the platform, and S3 is the analyte), the shaded part formed a stem loop to specifically recognize short DNAs.

| S1  | 5′-CAGCCTTATGGAG CCTG(Cy3)GGAGCTG AACTGTACAAACT ACTACCTCA-3′ |
|-----|-------------------------------------------------------------|
| S2  | 3′-ATGACATGTGTTG TAATGGAGT(Cy5)−5′                          |
| S3  | 3′-TGACATGTGTTG TAATGGAGT−5′                                |

Two smFRET platforms were tested (Figs. 5(b) and 10(b)). In Fig. 5(a), the DNA duplex was tethered to the glass surface via the biotin-streptavidin-biotin interaction via S1 (red strand), which was labeled with biotin at its 3′-end. The S2 (green strand) was complementary to S1 except at its 5′-terminal (an A-A pair), while S3 was completely complementary to S1 (an A-T pair). A set of FRET-paired dyes Cy3-Cy5 were labeled at S1 and S2, respectively, which yielded an average FRET state centered at approximately 0.6 (Fig. 5(c)). Before loading S3, a FRET image is shown in Fig. 5(b) (left panel). In the presence of S3 (canonical let-7g analog), S2 was removed from the duplex and washed away because of the formation of the more stable S1-S3 duplex. This replacement caused dissociation of the FRET-paired dyes and was proportional to the S3 concentration. Consequently, a lower density of Cy5 fluorescence was observed after incubation with S3 (Fig. 5(b), right panel). The number of FRET pairs was counted by the simultaneous emissions from both channels (boxed particles in Fig. 5(b)) and normalized with respect to the Cy3 particles among different fields of view. As reported before, one-step bleaching processes of both Cy3 and Cy5 were observed to verify that the signals were from single dyes only [18]. Figure 5(d) shows the FRET-paired fluorescence emissions from the Cy3 and Cy5 channels superposed with each other. The offset of the alignment between the paired spots is exaggerated for illustration.

In Fig. 10(a), the duplex was the same as in Fig. 5(a), except that the biotin was labeled on . Therefore, after exchange, the Cy5-labeled S2 remained on the surface, while the S1-S3 duplex diffused away in the solution. The exchange efficiency was calculated from the ratio of the Cy5 emission via FRET to the Cy5 emission via direct excitation. Figure 9(b) shows an example of a data set before and after the exchange. The left two images (before exchange) are the Cy5 channel emission (binary data are shown for presentation) via direct excitation and via FRET. The right two images (after exchange) are in the same arrangement. The images were obtained via the abovementioned alternating laser illumination procedure. The advantage of this setting compared to that of Fig. 5 is the removal of nonspecific emission in the Cy3 channel, which is more common than that in the Cy5 channel. Nevertheless, similar results were obtained with either platform.
7. The quantification curve of a DNA oligo

The exchange efficiency calculation is illustrated in Fig. 6. For every glass cell with 6 channels, only buffer as a reference was injected in the first channel. Then, S3 was injected into the other 5 channels at various concentrations. For every channel, approximately 1000 fields of view can be scanned in a reasonable amount of time (one 100 ms/frame movie of 8000 frames takes 13 min). For every field of view, the FRET pair was counted with a FRET fitting program. Then, an ImageJ macro program repeated the counting for the whole movie. The remaining FRET pair percentage (FRET%) was obtained by normalizing the FRET pair value with the Cy3 emission particles (Fig. 5) or the Cy5 direct excitation particles (Fig. 10). Then, FRET% was plotted in a histogram graph. For example, in Fig. 6, the reference average FRET% is 72%, and after exchange with 50 nM S3, the average is 55%. Therefore, the calculated remaining FRET% for this exchange is 55%/72%, which equals 76% (the data in Fig. 7 were calculated similarly). Although the Gaussian FWHM is close to 0.1, the error of
the mean is much smaller because of the $1/\sqrt{n}$ factor (~30). Therefore, automatic scanning to increase the sample size is essential in this method to decrease the error.

As shown in Fig. 7, the exchange was studied in the range of 0-1000 nM S3. Above 80 nM, the exchange reactions were saturated. This is because the binding energy difference for the S1-S2 and S1-S3 duplexes was designed to be very small to potentially distinguish the effect of single nucleotide isomiRs in the future, which are the same miRNAs but with different lengths [19]. In addition, a stem loop was introduced into the S1-S2 platform that provided steric interference, which has been demonstrated to recognize miRNAs specifically in qPCR quantification [7,20]. The linear range for the exchange assay is 5 – 80 nM. Given the typical sample volume of 10 μl, the lower limit amount is 50 femtomol in our method. This range is similar to a single-molecule dual-color detection method, but our procedure is simpler [21]. By changing the signal platform, the sensitivity and specificity can be further optimized, which will be pursued in the future.

![Reference vs After exchange histogram](image.png)

**Fig. 6.** The FRET% histogram of the reference and exchange channels. The actually exchange efficiency is normalized to the reference.

8. Conclusions

We have developed an automated smFRET scanning method. To demonstrate this method, we measured the DNA analog of let-7g. The FRET platform is specifically designed so that S3 can dissociate the duplex, and the duplex can be measured by decreasing the FRET pair numbers. Tracking on the FRET pair eliminated most background fluorescence noise. The advantages of the method are as follows. 1. The intact miRNA analog was quantified. Therefore, RNA labeling or ligation steps are circumvented. 2. The sensitivity of single-molecule detection eliminated the signal amplification step, which is indispensable and a major source for sequence bias in current methods [11]. 3. High throughput capability. We collected 8000 images in 1000 fields of view in approximately 10 min using automatic scanning with an autofocusing setup. The rate-determining factors are the camera integration time and length of the image sequence, which are both redundant in the current study. Typically, a 0.1 s integration time is sufficient for reliable FRET measurements, and 0.1 million FRET pairs are statistically robust [18]. Three hundred FRET pairs per field of view is a “medium” reading, so scanning 0.1 million/(300 per field) = 300 fields of view is enough
to quantify one analyte. Considering the time for stage movement and stabilization (~0.2 s), one field of view requires 0.1 s x 2 (for two laser illuminations) + 0.2 s (stage movement) = 0.4 s. Therefore, the scanning time for one sample can be shortened to 3 min. It will take approximately 19 h to measure 384 miRNAs (300 fields of view each), which is practical. The economic modification described here is useful for other FRET experiments. For practical applications, such as quantifying short nucleotides in biosamples, the compositions are more complicated than what we have demonstrated here. With a FRET signal, most nonspecific fluorescent signals from biosamples can be filtered. However, similar nucleotide analogs, such as miRNA isoforms, will interfere with the FRET exchange-induced signal because of the partial exchange reactions by the analogs. This is a limitation of our method and other methods that rely on the canonical base pairing of nucleic acids. In the future, we will address this challenge by designing a panel of diverse signal platforms with different relative stabilities for the target and its analogs, and the exchange efficiencies of the platforms for all of these analytes will be obtained. The concentration of the target will be determined by the exchange efficiency pattern in all of the platforms instead of one. Therefore, contributions from analogs can be deconvoluted.

Fig. 7. Quantification curve of analyte S3 from 0 to 1000 nM. The insert shows the exchange linear region. The error bars are adapted from the “σ” values of the Gaussian distribution. The errors of the mean are much smaller because of the large sample sizes.
Appendix

Fig. 8. Physical photo of the TIRF set up. A thin blue line illustrated one of the laser paths. The most important component was the beam expander (manual available with KT110 of thorlabs). The microscope is at the end of the light path.

Fig. 9. (Left) Illustration of the auto-focusing set up. The laser reflected light was detected by the quad photodiode PDP90A. Because the very close distance between the in and out laser spots, the rectangular neutral density filter was used and it was placed at the edge of the reflected laser spot. The signals of X- and Y-displacements were sent to the PID controller TPA101. At the perfect focus point, the X- (or Y-) signals fluctuated around the zero, and were amplified to be high or low voltage by the TPA 101. One of these signals was sent out to the I/O input of the motor, which was clamped to the focusing knob of the microscope via its Z-axis kit. The command to the motor caused it to crank the focus knob to opposite directions with H/L signals. For example, when using the command (/4F0S0P200F1S14P200R), the motor will move the Z-position to reach a “low voltage” signal if the focus is giving a “high voltage” signal; and vice versa. During the back and forth adjustment, the laser spot was kept constant on the PDP90A, which was the perfectly focused position. In this command, the first half (F0S0P200) and second half (F1S14P200) cranked the focus-knob at different direction; and (R) looped the commands infinitely. (right) A physical picture of the optics is shown. The totally reflected laser light was directly by a neutral density filter to a mirror, which relayed the light to the quad photodiode, PDP90A.
Fig. 10. (a) The alternative platform with Cy 5-labeled S2 being tethered on the surface. (b) The images are presented as binary data for display. The FRET pair % were calculated by the ratio of the particles under green laser radiation to those under red laser radiation. Each panel represent half of the camera area of 512x256 pixels.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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