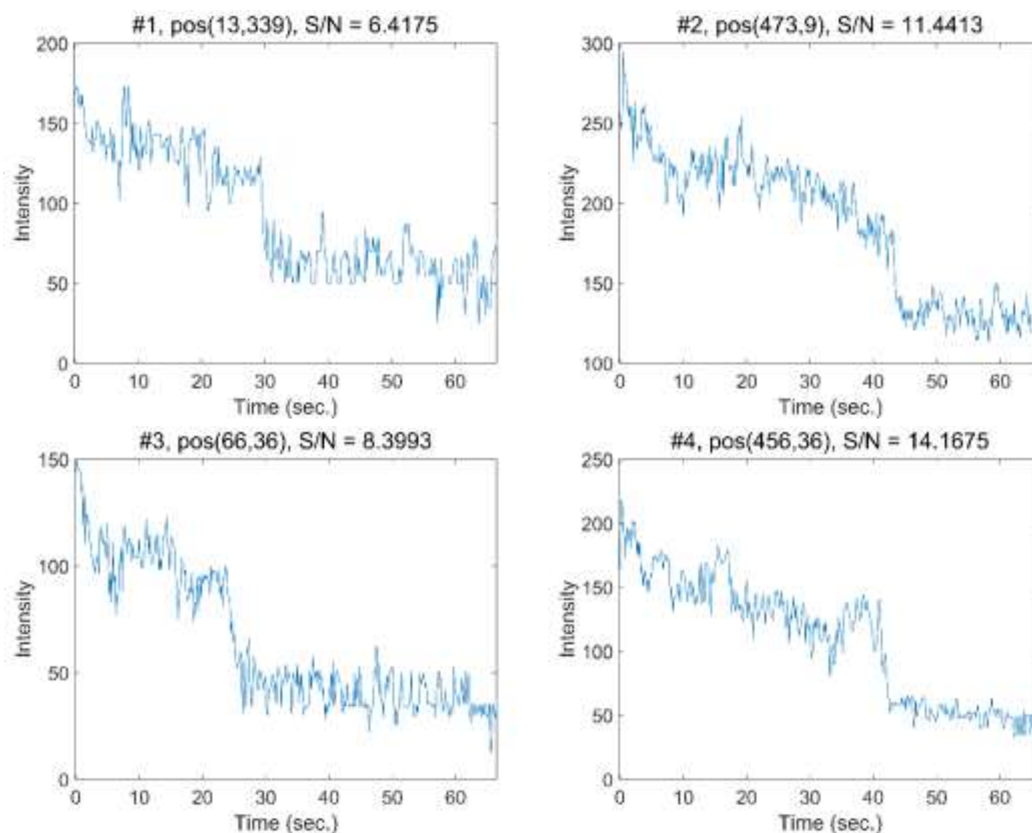
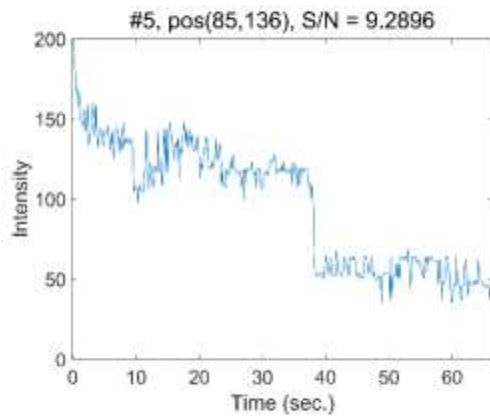
Supplementary Materials

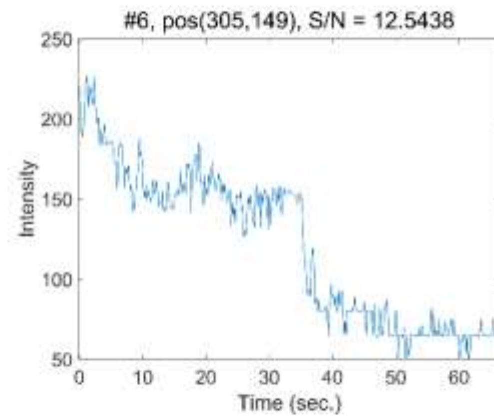
Signal-to-Noise ratio

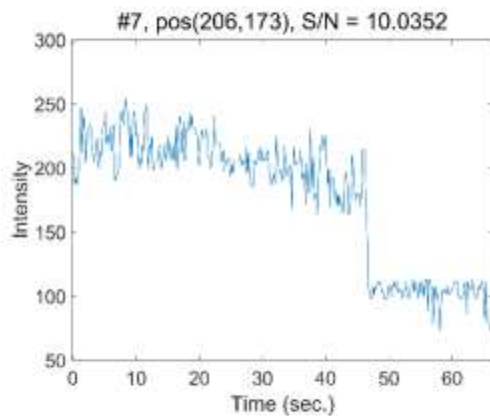
To experimentally test the Signal-to-Noise(S/N) ratio for Cy3 and Atto647N fluorescence dyes, we performed a photobleaching experiment. The exposure time we used is 100ms. Sampling frequency is 10MHz. In Fig. S1 and S2, 20 spots for both Cy3 and Atto647N were randomly selected within same Field-Of-View (FOV), with size of 54.6 μm X 54.6 μm. The S/N ratio for each spot was calculated by definition, 'signal' equaled to the difference of the average fluorescence intensity before and after quenching; 'noise' was calculated from the standard deviation of the fluorescence intensity after quenching. This noise measured the background fluctuation when fluorophores have been quenching.

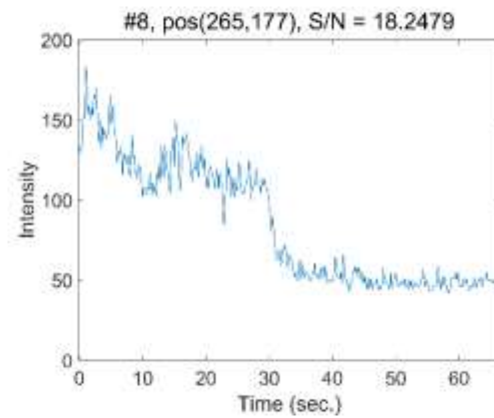
Results were shown in Fig. S1-S3. Fig. S1 show 20 spots traces for Cy3. Fig. 2 show 20 spots traces for Atto647N. Fig. S3 show the average S/N ratio and standard error for both Cy3 and Atto647N. As shown in Fig. S3, the average S/N ratio for Cy3 fluorescent is 15.9000, standard error is 1.7322. The average S/N ratio for Atto647N fluorescent is 34.4155, standard error is 2.5528.

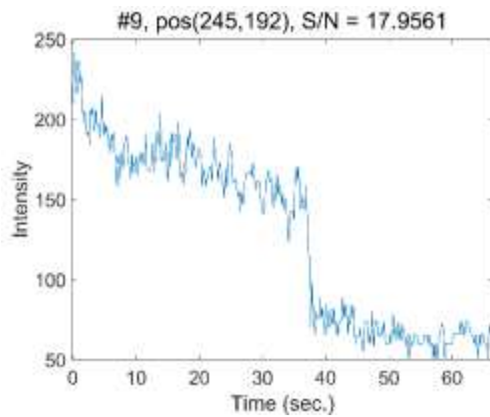


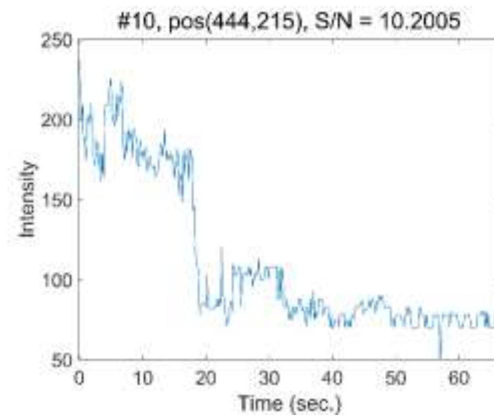

#5, pos(85,136), S/N = 9.2896
Intensity
Time (sec.)

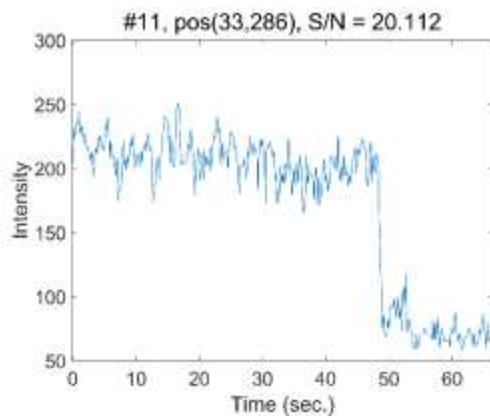

#6, pos(305,149), S/N = 12.5438
Intensity
Time (sec.)

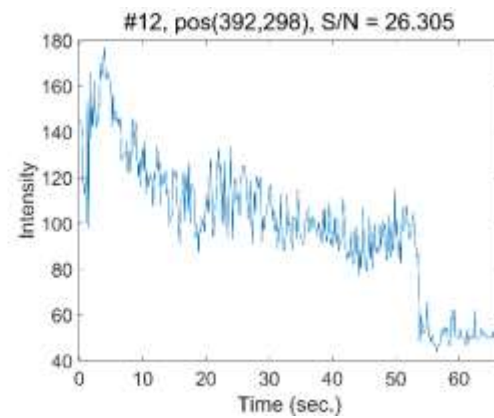

#7, pos(206,173), S/N = 10.0352
Intensity
Time (sec.)


#8, pos(265,177), S/N = 18.2479
Intensity
Time (sec.)


#9, pos(245,192), S/N = 17.9561
Intensity
Time (sec.)


#10, pos(444,215), S/N = 10.2005
Intensity
Time (sec.)


#11, pos(33,286), S/N = 20.112
Intensity
Time (sec.)


#12, pos(392,298), S/N = 26.305
Intensity
Time (sec.)

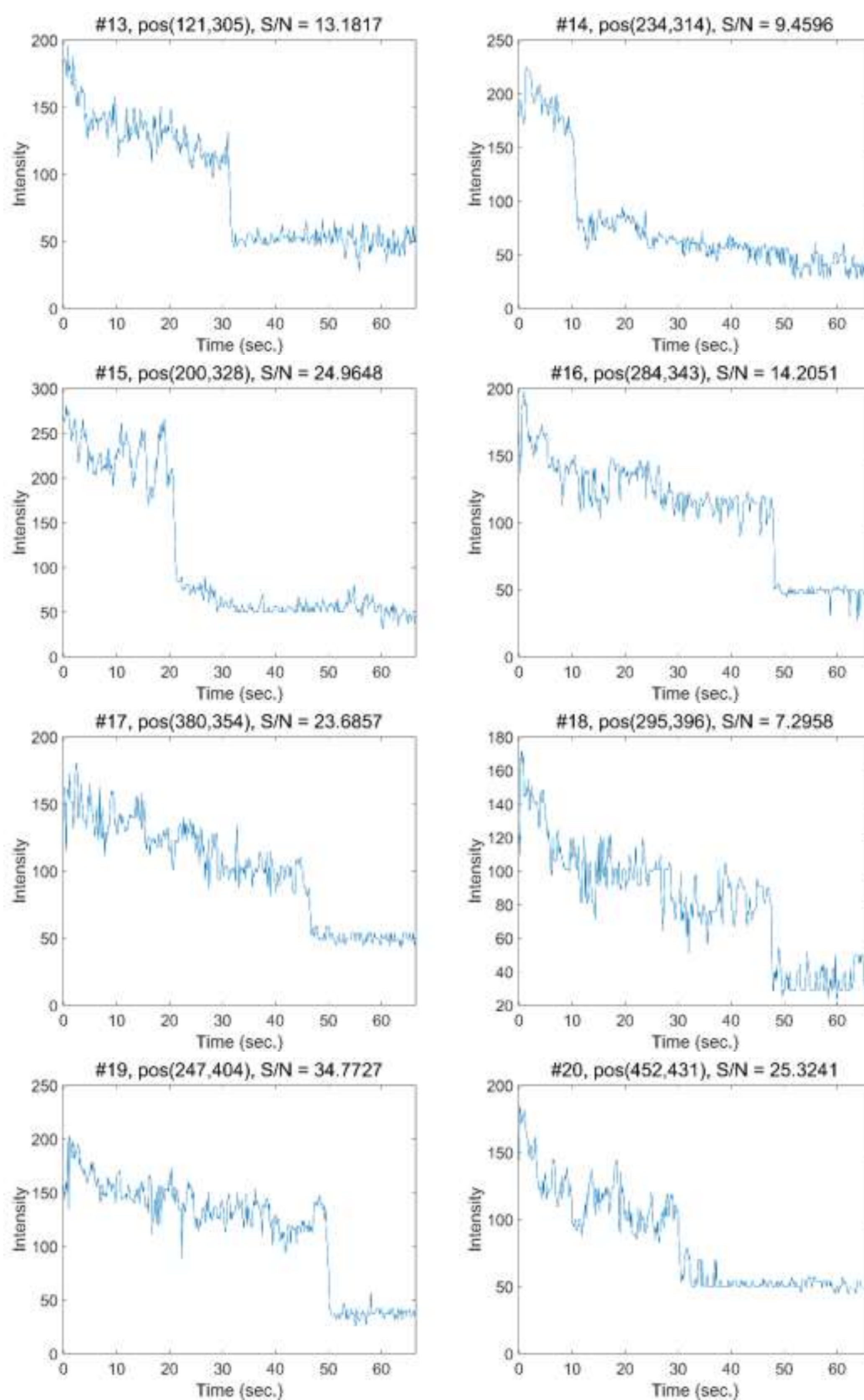
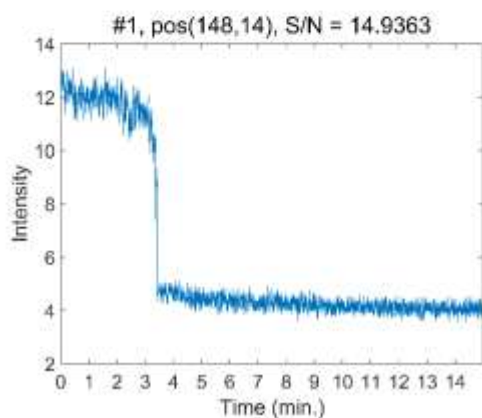
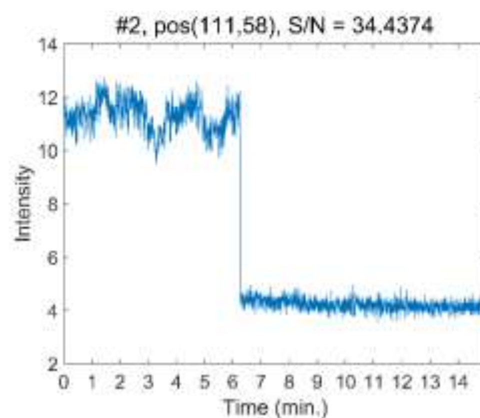
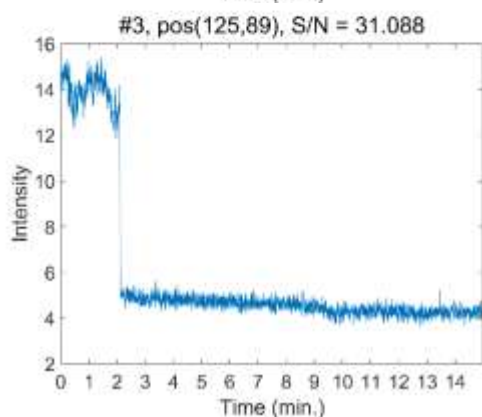


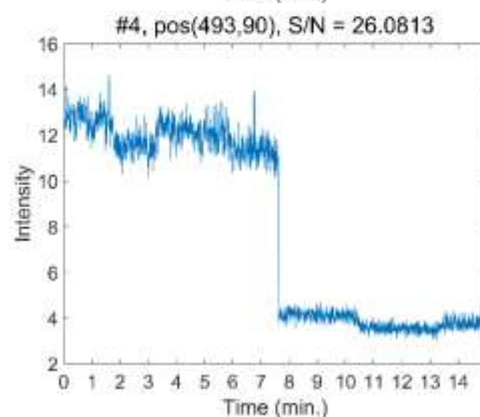
Figure S1. **20 randomly selected spots traces for a photobleaching experiment on Cy3**. The experimental condition was the same as actual sequencing (exposure time, laser intensity, sampling frequency). The x,y coordinate (in pixels) and S/N ratio for each single spot were shown on the title. The entire field-of-view was 54. The average S/N ratio is 15.9, standard error is

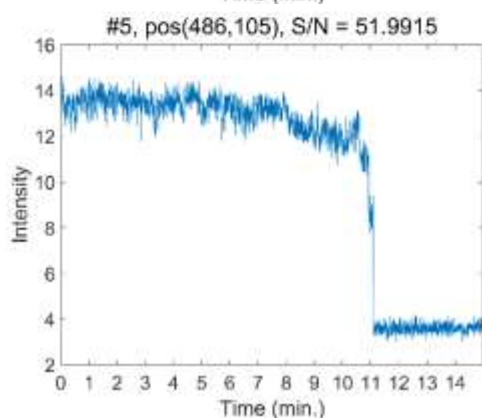
1.7322.

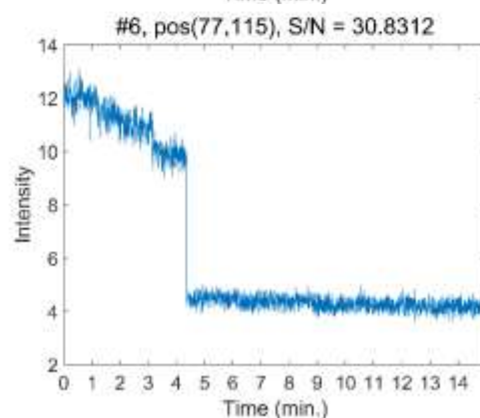


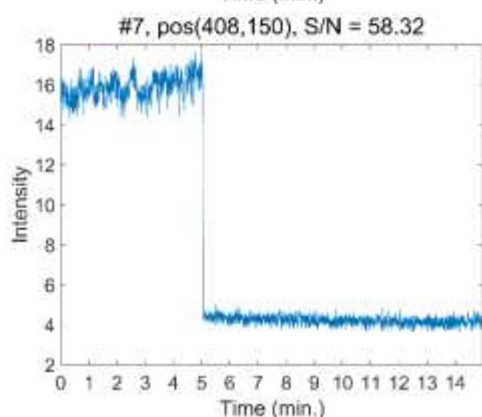


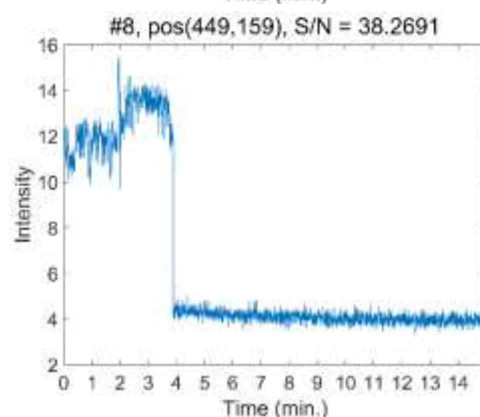


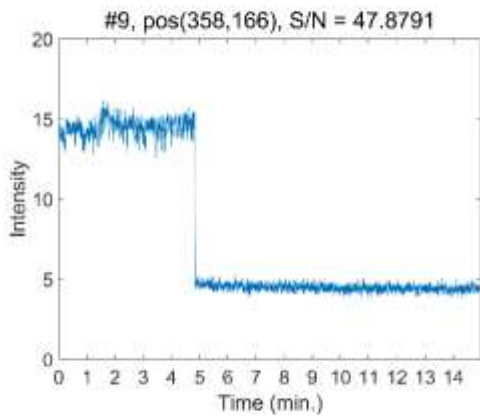


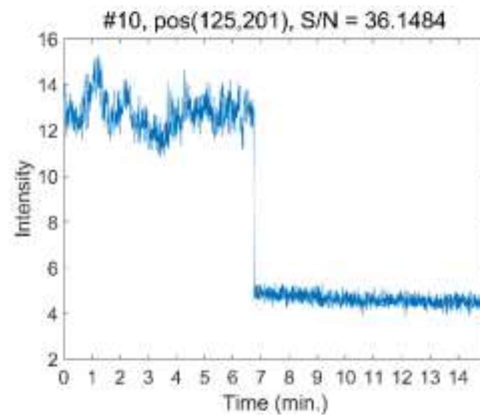


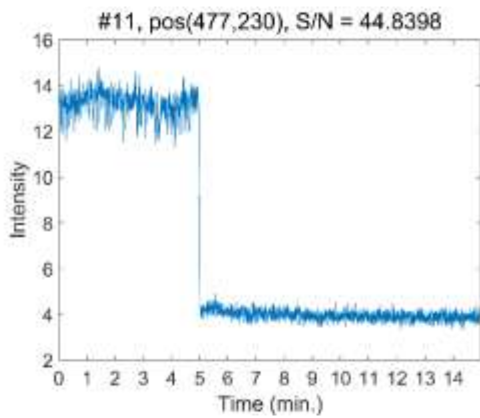


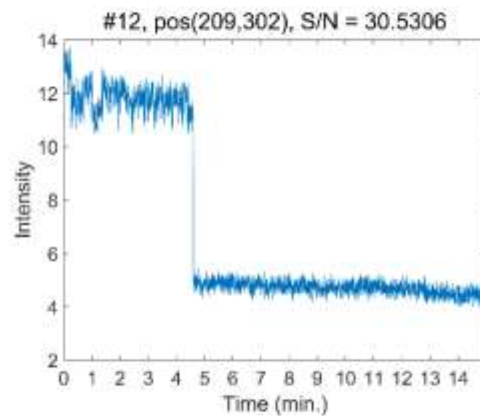


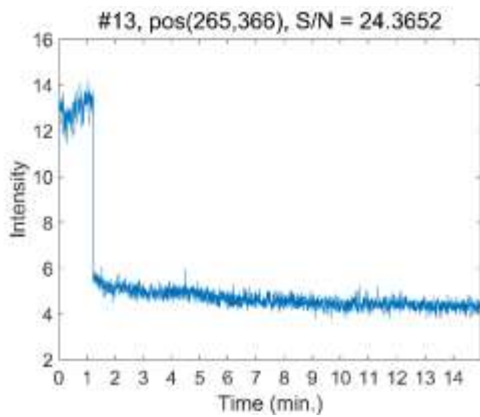


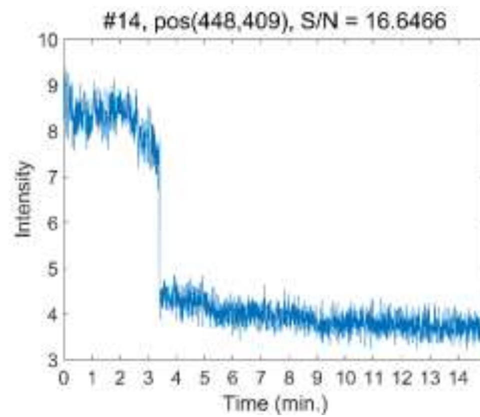

#9, pos(358,166), S/N = 47.8791
Intensity
Time (min.)

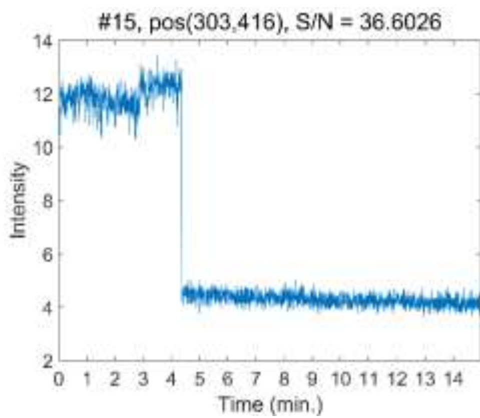

#10, pos(125,201), S/N = 36.1484
Intensity
Time (min.)

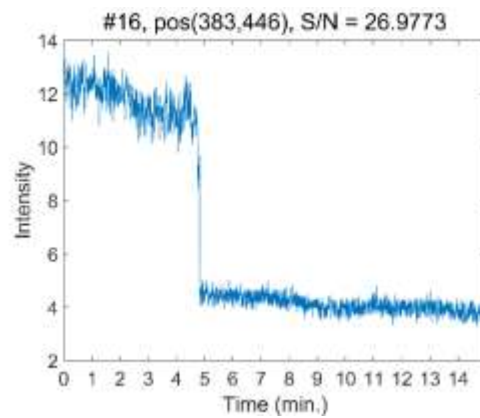

#11, pos(477,230), S/N = 44.8398
Intensity
Time (min.)


#12, pos(209,302), S/N = 30.5306
Intensity
Time (min.)


#13, pos(265,366), S/N = 24.3652
Intensity
Time (min.)


#14, pos(448,409), S/N = 16.6466
Intensity
Time (min.)


#15, pos(303,416), S/N = 36.6026
Intensity
Time (min.)


#16, pos(383,446), S/N = 26.9773
Intensity
Time (min.)

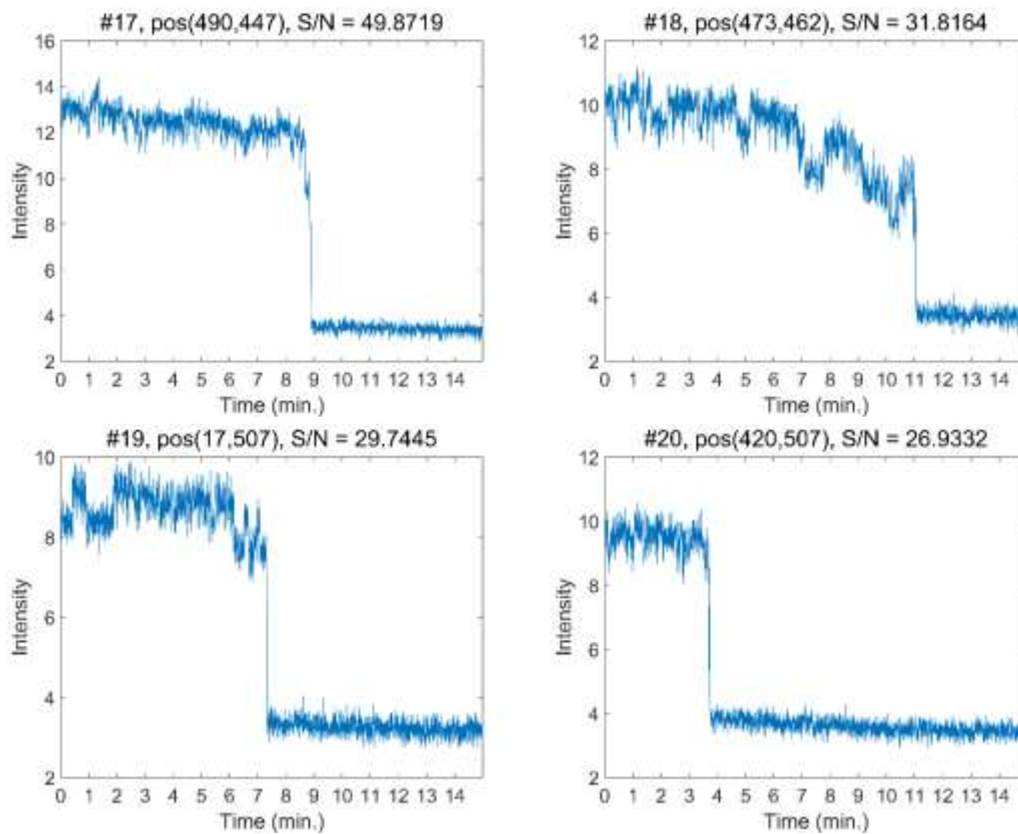


Figure S2. **20 randomly selected spot traces for a photobleaching experiment on Atto 647**. The experimental condition was the same as sequencing(exposure time, laser intensity, sampling frequency). The x,y coordinate (in pixels) and S/N ratio for each single spot were shown on the title. The entire field-of-view is 54.6 μm x 54.6 μm. The average S/N ratio was 34.4155, standard error was 2.5528.

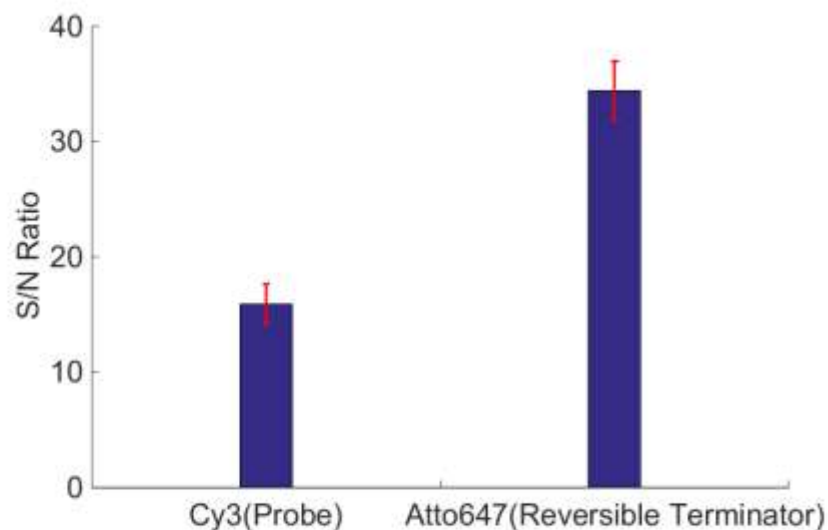


Figure S3. The average S/N ratio and standard error for both Cy3 and Atto647N.

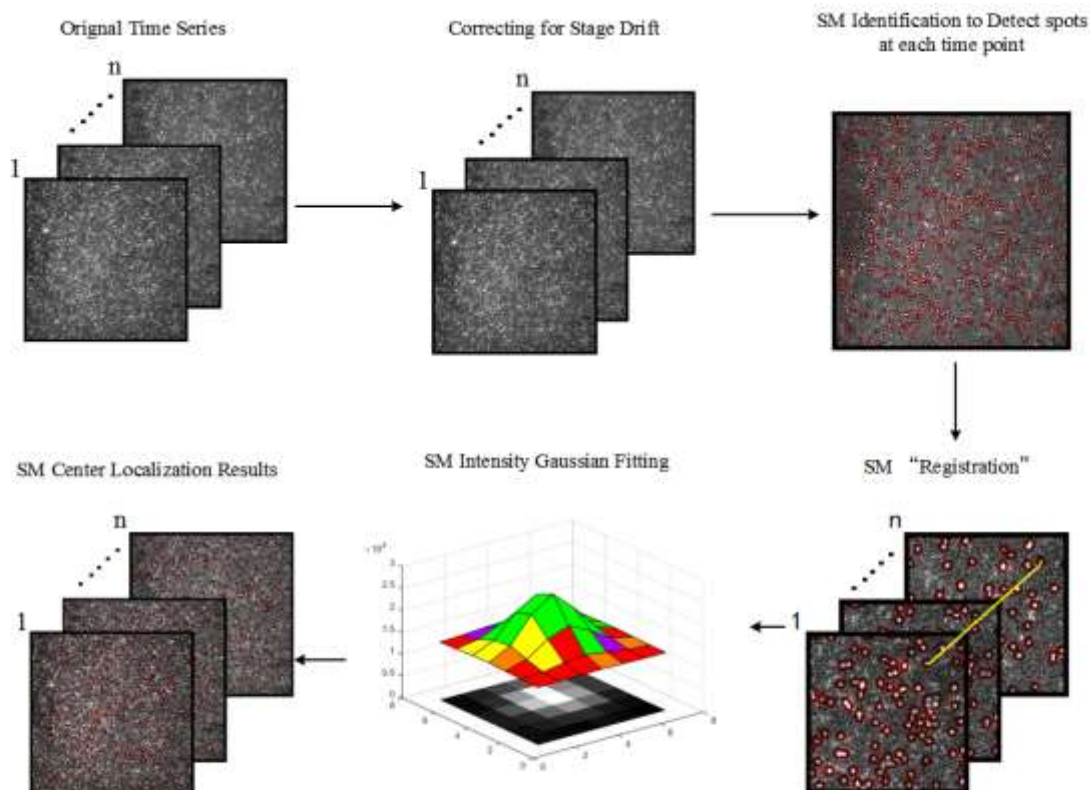


Figure S4. **Schematics for imaging processing**.

Increasing the photostability by imaging buffer

Crucial to the performance of single molecule experiments is the fluorophore and its lifetime. The life time of a fluorophore is the time until photophysical bleaching occurs or until radical oxygen species damage the tethered molecules. Therefore, imaging buffers containing enzymes oxidizing a substrate—so-called oxygen scavengers—are employed to create anaerobic conditions and thus increase the lifetime by preventing oxygen-based reactions. The oxygen scavenger containing 30% acetonitrile and scavenger buffer (130 μL HEPES/NaCl, 8μL 120 mM Trolox in MES, pH6.1, 15μL DABCO in MES, pH6.1, 15μL 1M glucose, 24μL 50 mM NaI, and 8μL glucose oxidase). We test the efficacy of imaging buffer by imaging capture probe with Cy3 label on the 3' prime end immobilized on the coverslips. A on-site video recorded the real time changes of the amount of the spots in one field of view(54.6μm×54.6μm, laser power, 100mW, exposure time, 100ms). The efficiency of the image buffer was compared with water by accounting the total spots on the detected field of views. The imaging buffer can significantly prolong the life time of the fluorophore especially in 1min (Fig. S5). In our experiment of sequencing, a typical exposure time for each adding of oligonucleotide is 400ms(100ms×4). 20 cycles during sequencing lasted 8s during which lost of the spots caused by photobleaching is not significant.

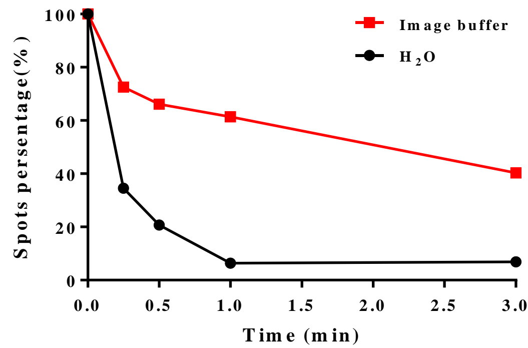


Figure S5. The efficiency of imaging buffer

Sequencing errors

The sequencing error was validated by repeating the sequencing experiments four times. Each single raw sequence was aligned to the reference and the number of positions disagreeing with the reference was recorded. The error rate was calculated separately for each DNA template.

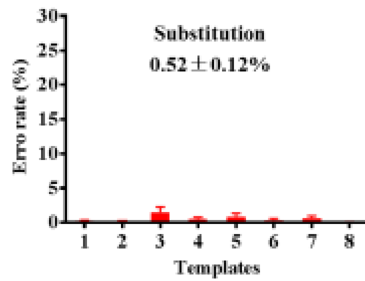


Figure S6. The average error rate in four repeated experiments. In each experiment, 8 DNA templates were mixed and sequenced. The average substitution error rate is 0.52%.