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Super-Resolution Force Spectroscopy Reveals Ribosomal Motion at Sub-Nucleotide Step

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Probing biomolecular motion beyond a single nucleotide is technically challenging but fundamentally significant. We have developed super-resolution force spectroscopy (SURFS) with 0.5 pN force resolution, and revealed that the ribosome moves by half a nucleotide upon the formation of the pre-translocation complex, which is beyond the resolution of other techniques.

Nucleic acids play important roles in a wide range of biological functions.[1] For example, the translocation of mRNA through the ribosome is a critical step for protein synthesis, in which every three nucleotides (nt) form one codon that decodes for one amino acid.[2,3] Investigating the translocation mechanism, or many other processes involving nucleic acids, would prefer directly monitoring the molecular motion with beyond single-nt precision. This is equivalent to ~0.2 nm spatial resolution because the backbone length between adjacent riboses is about 0.4 nm.[1] Structural techniques, mainly x-ray crystallography and cryo electron microscopy, typically have the resolution of a few angstroms, which limits the observation of sub-nt differences.[4,5] Both sequence- and fluorescence-based modalities have not been able to provide single-nt precision.[6-8] Consequently, single-nt displacement is generally considered as the minimum step for ribosome translocation and other translocations in general.[9]

In the field of force spectroscopy, several different methods have been developed to provide single-nt resolution and applied to study molecular motors. Examples include atomic force microscopy and optical tweezers.[10,11] Recently we have reported using force-induced remnant magnetization spectroscopy (FIRMS) to measure the ribosome translocation at three consecutive codons.[12] With force resolution of 2-4 pN, we have revealed single-nt resolution in ribosomal motion. However, no force-based methods have reached resolution sufficient for revealing sub-nt differences, including two previous work using ultrasound.[13,14]

Here, we show the super-resolution force spectroscopy (SURFS) technique with 0.5 pN resolution, by combining accurate ultrasound radiation and an automated atomic magnetometer. It distinguishes nucleic acid duplexes with less than one basepair (bp) difference, thus offering sub-nt resolution. We have observed sub-nt mRNA translocation in the ribosome and further studied the related mechanism.

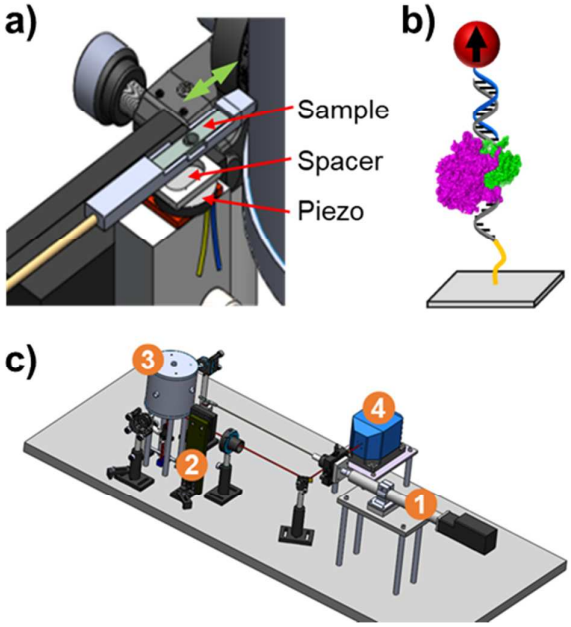

Fig. 1. Principle of SURFS. **a)** Generation of precise acoustic radiation force. **b)** The molecular system. The ribosome complex (purple and green) was immobilized through its mRNA; the probing DNA conjugated with a magnetic particle forms a duplex with the exposed mRNA. **c)** Overall apparatus. 1, motor; 2, ultrasound setup; 3, magnetic shield housing the atomic magnetometer; 4, laser for the atomic magnetometer.

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Figure 1 shows the designs of force generation, molecular scheme, and overall SURFS instrument. A piezo disk was used to generate acoustic radiation force to dissociate molecular pairs in the sample placed above. The driving frequency of the piezo was 1.0 MHz (Supplementary Information). A layer of water was placed between the sample and the piezo as the spacer. The sample was mounted on a linear motor, which shuffled the sample between the piezo location and atomic magnetometer (Fig. 1a).[15] A typical molecular system consisted of an immobilized nucleic acid on the surface and a complementary nucleic acid labelled with a magnetic particle. In the case of the ribosome experiments, the mRNA of the ribosome complex was used for immobilization (Fig. 1b). The magnetic signal of the sample was measured by an atomic magnetometer after application of each force. Dissociation of the nucleic acid duplex was indicated by a decrease in the magnetic signal, due to the randomization of the magnetic dipoles of the dissociated particles.[12,13] The main components of the automated apparatus were shown in Fig. 1c.

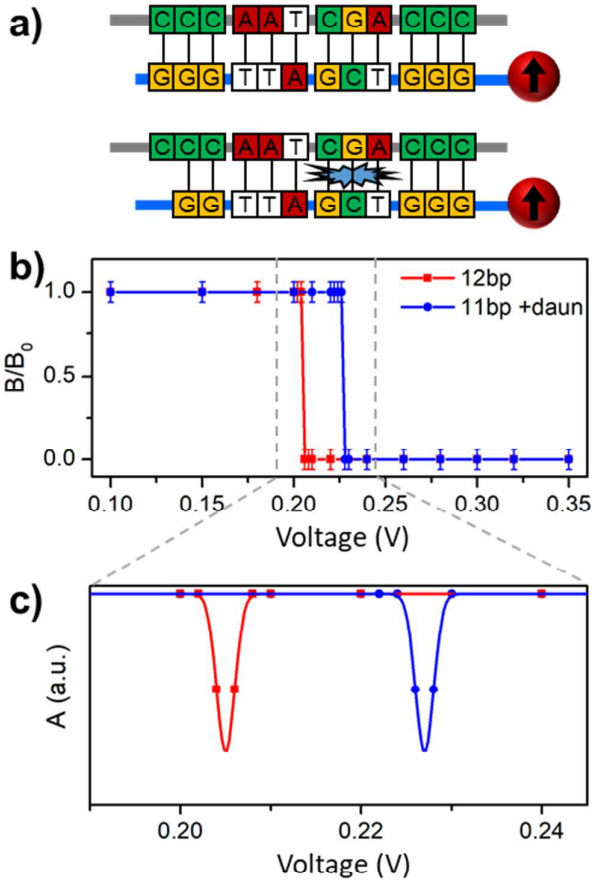

Fig. 2. Resolving two DNA duplexes using SURFS. **a)** Sequences of the 12-bp DNA (top) and 11-bp DNA intercalated with daunomycin (bottom). The star shape represents daunomycin. **b)** Magnetic field profiles for the two duplexes. Daun: daunomycin. **c)** Force spectra obtained from b, shown in an expanded scale.

The initial demonstration of force resolution was carried out for two systems designed to have close dissociation forces. One was a 12-bp DNA duplex. The other was a 11-bp DNA, which had one less bp at the end of the duplex compared to the 12-bp DNA but contained an intercalated daunomycin that specifically recognizes the CGA segment (Fig. 2a).[16,17] The design was based on our previous studies that have shown that adding one additional bp or an intercalated drug increased the dissociation force similarly.[17,18] The results of magnetic signal versus ultrasound amplitude are shown in Fig. 2b. The dissociation voltage was 0.205 V for the 12-bp DNA and 0.227 V for the 11-bp DNA with daunomycin. The results were repeated four times each to confirm the voltage difference (Supplementary Fig. S1). The accuracy of the ultrasound amplitude was ±0.002 V, much better than our previous work.[13] Therefore the two systems were well resolved. As a comparison, they were not clearly resolved by centrifugal forces (Supplementary Fig. S2). The 12-bp and 11-bp with daunomycin exhibited 45±3 pN and 48±3 pN respectively, indicating the force difference of 3 pN was similar to the 3 pN uncertainty. Derivatives of the magnetic profiles in Fig. 2b yielded two negative peaks (Fig. 2c). The peak separation was 0.022 V, and the peak half-width was given by the voltage uncertainty. So the force resolution can be estimated to be 6*(±0.002/0.022) ≈ ±0.5 pN.

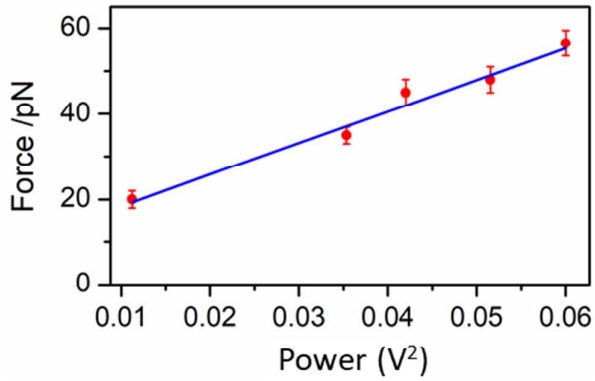

Fig. 3. Voltage-force correlation between the new SURFS technique and centrifugal forces.

Force-voltage correlation can be obtained by comparing the SURFS results of a series of DNA duplexes with centrifugal force results. The DNA duplexes were 10-bp, 11-bp, 12-bp, 11-bp with daunomycin, and 12-bp with daunomycin. The sequences and force profiles from both techniques are in the Supplementary Information Fig. S3. The values of centrifugal forces from FIRMS were plotted against the square of the voltages on the piezo (Fig. 3). This is because acoustic radiation force is proportional to the ultrasound power,[19] i, e., the square of voltages for a given piezo element. A linear correlation was obtained, yielding

$$F = 736 * V^2 + 11$$

This linear correlation (R^2 = 0.98) serves as a guideline for force amplitude. The force resolution based on this correlation was approximately 0.6 pN at 42 pN, consistent with the previous estimation. The dissociation forces obtained by SURFS for the 12-bp

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DNA and 11-bp DNA with daunomycin are therefore 41.9 and 48.9 pN respectively, based on this correlation.

The established SURFS technique was then used to determine the positions of the ribosome on the mRNA at two distinct states. The ribosome complexes were synthesized and purified as previously (Supporting Information).[20] As shown in Fig. 4a, one was the post-translocation state (Post) in which the $tRNA^{Lys}$ (MFEK) binds the ribosome at the P-site after the formation of a peptide bond, representing the starting point of decoding the next codon for arginine. The other was the pre-translocation state (Pre) in which the $tRNA^{Arg}$ (MFEKR) binds at the A-site of the ribosome, representing the finishing point of decoding the arginine-codon.[21] The ribosome is ready to move to the next codon. It has never been pursued whether the ribosome would have moved at this state, before the putative translocation step. In our probing scheme, two DNA probes were used, termed as P12 and P11, which would form 12-bp and 11-bp duplexes, respectively, with the uncovered mRNA at the 3′-end.

Surprisingly, the SURFS results indicate, for the first time, that the ribosome indeed has moved towards the 3′-end of the mRNA by approximately half a nt in Pre (Fig. 4b). The results using P12 showed the dissociation voltage was 0.138 V for the Pre and 0.166 V for the Post, indicating a weaker duplex between Pre and P12. The corresponding forces calculated from the force correlation were 25.0 and 31.3 pN, respectively. Because the force difference was substantially larger than the force resolution of 0.5 pN, this represents the different ribosome locations on the mRNA between Pre and Post, i.e., the ribosome has moved a small distance on the mRNA. Each profile was repeated four times to ensure reproducibility (Supplementary Fig. S4).

We then confirmed this movement by using P11 to probe both ribosome complexes. We were supposed to observe the same dissociation voltages for Pre and Post because this shorter probe does not reach the ribosome (Fig. 4a). As expected (Fig. 4c), both cases gave 0.110 V (19.9 pN), indicating the strength of the 11-bp DNA-mRNA duplexes. We also performed an independent FIRMS study for the P11 Post duplex, which gave a dissociation force of 21 pN, further validating the force correlation (Supplementary Fig. S5). The force difference between P11 to P12 for the Post was 11.4 pN, representing one nt; but the force difference between the Pre-P12 and Post-P12 duplexes was only 6.3 pN, nearly half the amount to 11.4 pN. This small force difference can only be revealed conclusively with SURFS. The result suggests that the translocation step is not one process, and the following translocation step is not exactly 3 nt. This observation has important implication to understanding the high fidelity of the ribosome to only translate the canonical reading frame.

The small ribosome displacement during the transition from Post to Pre correlates well with the literature reports that the Post is in a classical state while the Pre is in a hybrid state, in which a small-angle head swivel is possible.[22-24] We hypothesized that the head swivel motion dragged mRNA towards inside of the ribosome by half a nt. To test this hypothesis, we used antibiotics to manipulate the ribosome conformation. Shown in Fig. 4d, we tested the Post with puromycin and Pre with spectinomycin. When the Post was incubated with puromycin, the force profile showed the same result as the Pre. This indicates that puromycin was able to induce the ribosome motion that caused half a nucleotide movement along the mRNA. It has been shown that puromycin removes the peptidyl chain attached to the p-site tRNA, allowing the formation of the hybrid state.[25-27] This result also proved that the A-site tRNA accomodation was not the cause for the mRNA difference between Pre and Post, because the puromycin treatment did not change the vacant A-site conformation in the Post complex.

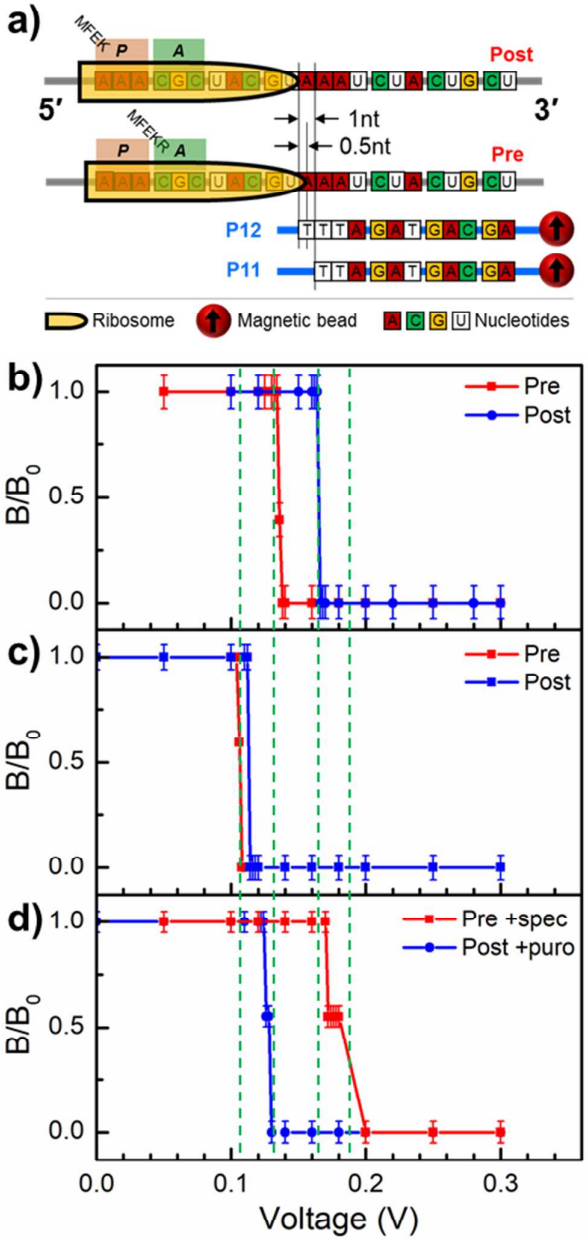

Fig. 4. Probing ribosomal motion. **a)** The probing scheme. Two different ribosome complexes, Post and Pre, were studied by two DNA probes with different lengths, P12 and P11. **b)** The

different magnetic field profiles obtained by SURFS using P12. **c)** The overlapping magnetic field profiles obtained using SURFS using P11. **d)** Manipulation of the ribosomal motion using antibiotics. Puro: puromycin; spec: spectinomycin. The dashed lines indicate steps of approximately half a nucleotide.

When the Pre was incubated with spectinomycin, two distinct features were observed. The first one was at 0.170 V and the second one at 0.185 V. The two features were well resolved. The former was nearly identical as the result in Post, indicating that this binding mode of spectinomycin resets the head swivel of the 30S back to its classical position. This is consistent with structural studies in that spectinomycin binds on the second hinge of the 30S neck, thereby prohibiting the head swivel.[28,29] There are two possibilities for the stronger force at 0.185 V (36.2 pN): one due to a possible unidentified ribosome motion in the literature, and the other due to the direct binding between mRNA and the probing DNA. In other words, for the latter case spectinomycin acts as a drug binding with the DNA-mRNA duplex. We have measured the dissociation voltages for the duplex without the ribosome, in the absence and presence of spectinomycin (Supplementary Fig. S6). No difference was observed in between the two cases. Therefore, the feature at 36.2 pN probably indicates a second binding mode of spectinomycin onto the ribosome, causing the mRNA to bind with the probing DNA stronger by nearly half a nucleotide, or on the order of a single hydrogen bond.

In conclusion, we developed the SURFS technique with 0.5 pN resolution, which was sufficient to reveal sub-nt resolution in biomolecular motion. We revealed that from the Post state to the subsequent Pre state, the ribosome moves spontanesouly by nearly half a nt, which has not been observed using other techniques. This motion correlates well with structural studies. In addition, a new binding mode for spectinomycin was identified. Our technique is complementary to x-ray crystallography for mechanistic studies of the ribosome, because we use robust and unbiased probes to provide precise local conformational changes. More intermediate states may be identified in ribosomal translation, which are currently challenging or controversial for other techniques.[30-33] In addition, other biological motors, such as RNA and DNA polymerases, can also be investigated. The sub-nt resolution will be valuable for revealing transcription fidelity of RNA polymerase, for which only single-nt stepping and pausing have been reported.[34]

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Conflicts of interest

There are no conflicts to declare.

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