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Using Atomic Force Microscopy to Study the Real Time Dynamics of DNA Unwinding by Mitochondrial Twinkle Helicase

Parminder Kaur [1, 2, *], Hai Pan[1], Matthew J. Longley[4], William C. Copeland[4] and Hong Wang[1, 2, 3, *]

[1]Physics Department, North Carolina State University, Raleigh, North Carolina, USA; [2]Center for Human Health and the Environment, North Carolina State University, Raleigh, North Carolina, USA; [3]Toxicology Program, North Carolina State University, Raleigh, North Carolina, USA; [4]Genome Integrity and Structural Biology Laboratory, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, USA

*For correspondence: pkaur3@ncsu.edu; hwang18@ncsu.edu

[Abstract] Understanding the structure and dynamics of DNA-protein interactions during DNA replication is crucial for elucidating the origins of disorders arising from its dysfunction. In this study, we employed Atomic Force Microscopy as a single-molecule imaging tool to examine the mitochondrial DNA helicase Twinkle and its interactions with DNA. We used imaging in air and time-lapse imaging in liquids to observe the DNA binding and unwinding activities of Twinkle hexamers at the single-molecule level. These procedures helped us visualize Twinkle loading onto and unloading from the DNA in the open-ring conformation. Using traditional methods, it has been shown that Twinkle is capable of unwinding dsDNA up to 20-55 bps. We found that the addition of mitochondrial single-stranded DNA binding protein (mtSSB) facilitates a 5-fold increase in the DNA unwinding rate for the Twinkle helicase. The protocols developed in this study provide new platforms to examine DNA replication and to explore the mechanism driving DNA deletion and human diseases.

Graphic abstract:

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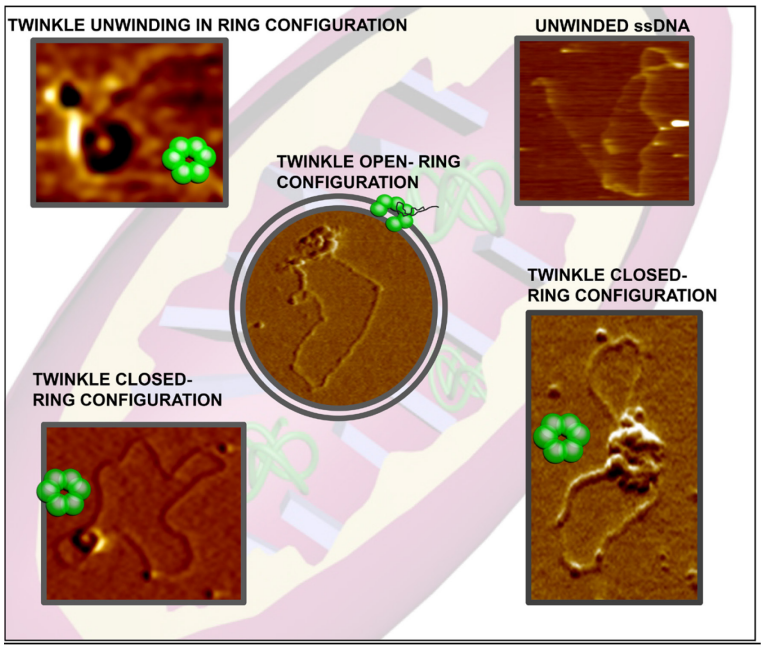

Uncovering mitochondrial Twinkle helicase structural and functional dynamics using Atomic Force Microscopy.

Mitochondrial Twinkle Helicase Dynamics

Keywords: Atomic Force Microscope, Twinkle helicase, Mitochondria, Liquid AFM imaging, Mitochondrial replication, Single molecule imaging

[Background] Human mitochondria contain circular double-stranded DNA (16,569 bp) replicated by DNA polymerase Pol γ in concert with Twinkle helicase and mitochondrial single-stranded DNA binding protein (mtSSB) (Korhonen *et al.*, 2004). Mutations in the genes encoding the core mitochondrial DNA (mtDNA) replication machinery have been associated with human diseases, including progressive external ophthalmoplegia, ataxia neuropathy syndromes, and other neurodegenerative diseases collectively known as “mitochondrial diseases” (Tyynismaa *et al.*, 2005; Goffart *et al.*, 2009; Ylikallio and Suomalainen, 2012; Bhatti *et al.*, 2017; Suomalainen and Battersby 2018). The relationship between dysfunction in mtDNA replication and mitochondrial diseases makes it crucial to understand the molecular mechanism governing mtDNA replication. Although core mitochondrial proteins at the DNA replication fork have been identified (Bogenhagen *et al.*, 2008), the structure and dynamics of mtDNA replication remain elusive. Most of the previous mitochondrial replication studies have been based on ensemble averaging methods using short DNA as template. The transient nature of the protein-DNA interactions presents significant challenges towards elucidating the underlying mechanisms using traditional methods. Using traditional methods, it has been shown that Twinkle is capable of unwinding dsDNA up to 20–55 bps both with and without mtSSB (Korhonen *et al.*, 2004; Sen *et al.*, 2012). To elucidate the mechanism of Twinkle helicase binding and unwinding DNA, we sought to uncover the intricate transient states, which may be obscured by ensemble averaging effects. To fill this knowledge gap, we developed protocols using Atomic Force Microscopy (AFM) imaging in air as well as in liquids

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that reveal a multi-faceted, dynamic structural description of DNA binding and unwinding by Twinkle-mtSSB at the single-molecule level. Studying unwinding reactions using AFM unveils unprecedented dynamic as well structural information of the unwinding reaction, helping us to provide a functional step model of Twinkle unwinding of the DNA. These protocols elucidated the real-time dynamics of Twinkle helicase loading onto DNA in an open ring configuration, switching to a closed ring state for DNA unwinding, and unloading from DNA as an open ring structure. These changes in the Twinkle helicase conformation dynamics are stochastic in nature and would have been lost in ensemble averaging traditional methods. Results from this study using this protocol showed that Twinkle helicase exists in various oligomeric states. Furthermore, time-lapse AFM imaging in liquids showed that Twinkle hexamers are formed through sequential recruitment of individual subunits, and these hexamers can exist and switch between both open and closed ring conformations. These imaging results support a model in which Twinkle helicase loads onto DNA in open-ring conformation and searches along DNA in a closed ring conformation. Also, the Twinkle helicase unwinds the DNA in open ring conformation and unloads from DNA upon reopening of the ring after performing DNA unwinding. Our AFM studies with a model circular dsDNA substrate containing 37-nt ssDNA gaps revealed that Twinkle is capable of unwinding thousands of base pairs when facilitated by mtSSB (~240 bp/min without mtSSB to ~1,265 bp/min with mtSSB) (Kaur *et al.*, 2020). Here, we outline detailed protocols that can be adapted to study other DNA replication proteins *in vitro* using AFM imaging in air and time-lapse buffer imaging. While air imaging elucidates the spatial interaction, time-lapse buffer imaging provides dynamic information on protein-DNA interactions.

Materials and Reagents

1. 100 mM ATP stock solution in deionized (DI) water buffered to pH 7.5 (aliquoted and stored at -80°C) (Sigma-Aldrich, catalog number: A7699)
2. Mica (SPI Chem Mica Grade V-1 50 × 25 mm × 0.15 mm, catalog number: 12001-26-2) (Figure 1)

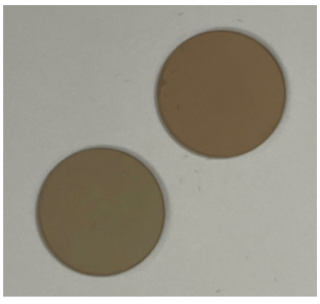

Figure 1. SPI Chem Mica used for Atomic Force Microscopy (AFM) experiments

3. Aminopropyl silatrane (APS) (Shlyakhtenko *et al.*, 2013)
4. HEPES (Fisher Bioreagent, catalog number: 7365-45-9)
5. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 7647-14-5)

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6. Magnesium chloride ($MgCl_2$) (Fisher Chemical, catalog number: 7791-18-6)
7. Glycerol (EMD Millipore, catalog number: 356350)
8. Nonidet P-40 (NP-40) (ThermoFisher Scientific, catalog number: 85124)
9. Nt.BstNBI (NEB, catalog number: R0607S)
10. Amicon Ultrafiltration (Millipore, catalog number: C82301, MW 100K)
11. ScaI restriction enzyme (NEB, catalog number: R3122S)
12. pscwo1 plasmid (Addgene, catalog number: 72300)
13. Helicase Imaging Buffer (see Recipes)
14. APS working solution (see Recipes)
15. Buffer 3.1: 1× Buffer components: NEB (see Recipes)

Equipment

1. NanoDrop 2000C (Thermo Scientific, catalog number: ND-2000C)
2. AFM tips for air imaging – Pointprobe PPP-FMR probes (Nanosensors, spring constant k = 2.8 N/m, Resonant frequency f_r = 70 kHz).
   AFM tips for Buffer imaging – BL-AC40TS (Biolever Mini) probe (Asylum Research, spring constant k = 0.09 N/m, Resonant Frequency f_r = 110 kHz)
3. Centrifuge (to fit Eppendorf 1.5 ml tubes)
4. Cylinder of Nitrogen gas with a pressure regulator and nozzle.
5. Eppendorf tubes (1.5 ml) (Eppendorf, catalog number: 022431081)
6. Scotch Tape
7. MFP-3D – Bio AFM (Figures 2-4) (Asylum Research, Oxford Instruments)

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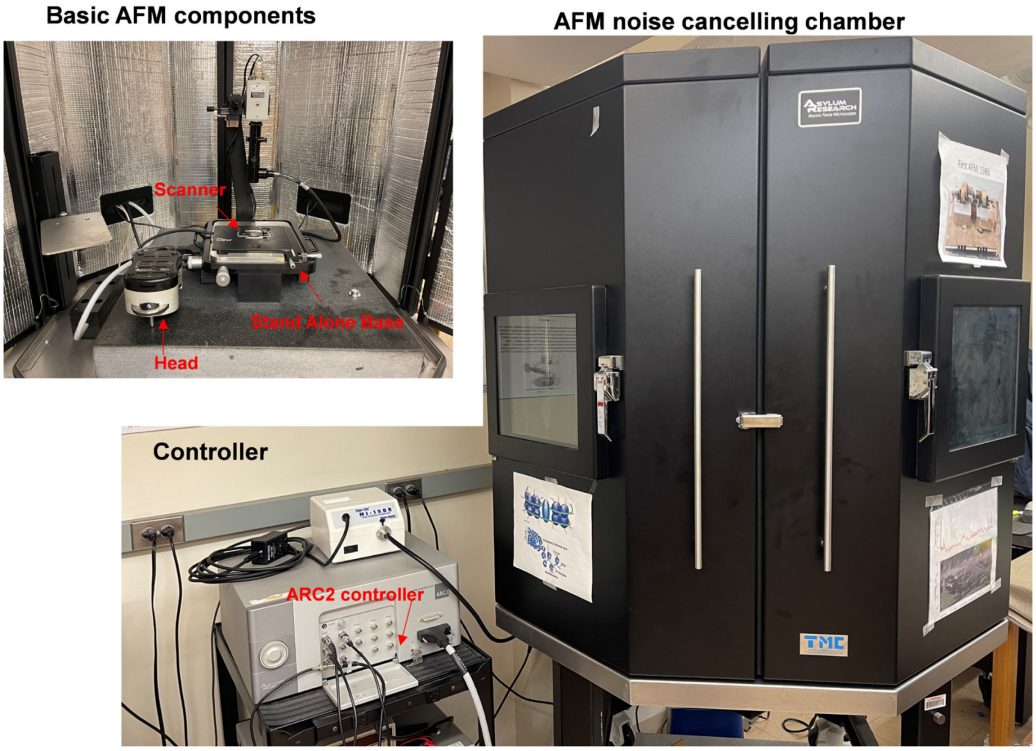

Figure 2. AFM instrument details showing basic AFM components inside the AFM noise canceling chamber with the AC2 controller

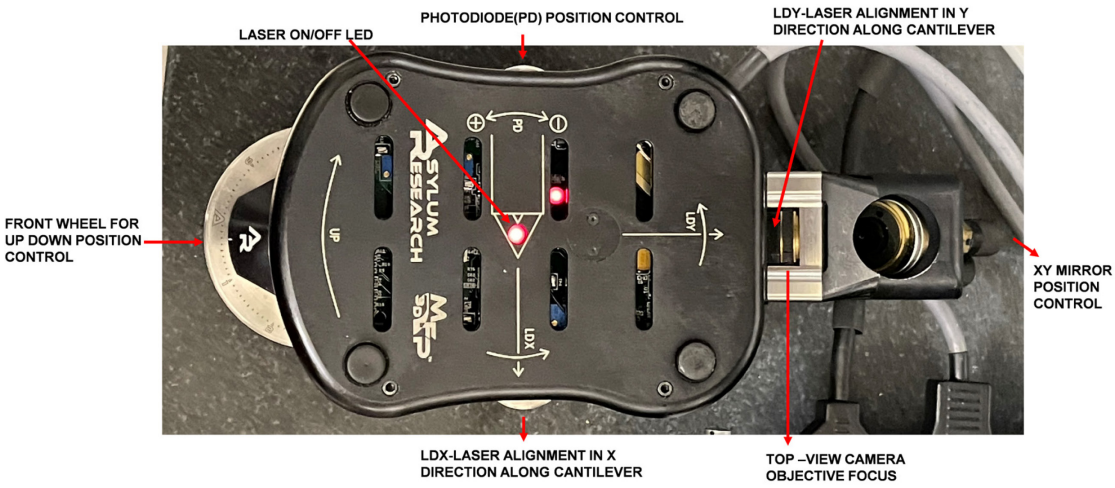

Figure 3. AFM head with labeled controls

Software

1. IGOR Pro 6 (https://www.wavemetrics.com/software/igor-pro-802, WaveMetrics, Portland, OR, USA)
2. MFP3D AFM imaging software or Gwydiion software (http://gwyddion.net/) (Nečas and Klapetek, 2012)

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Procedures

A. Proteins used for unwinding assays

Recombinant human Twinkle helicase with a C-terminal His6-affinity tag was overexpressed in *E. coli* BL21 (DE3) Codon Plus-RIPL (Stratagene). The Twinkle helicase was purified to >95% purity using successive nickel-affinity, anion exchange, and heparin-affinity chromatography (Longley *et al.*, 2010; Kaur *et al.*, 2020). Twinkle helicase protein concentration was evaluated using Coomassie-stained SDS-PAGE gels with BSA standards (Krieg *et al.*, 2005; He, 2011). For a detailed purification protocol, see Longley *et al.* (2010). Human mtSSB protein was overexpressed in *E. coli* and purified as described previously (Longley *et al.*, 2009).

B. DNA substrates for the unwinding assays

DNA substrate – Gapped circular DNA substrate (4,060 bp) – was prepared by introducing two clusters of four closely spaced nicks in one strand of pscwo1 plasmid (Addgene: plasmid # 72300) using Nt.BstNBI. These circular substrates were incubated with complementary oligos (Integrated DNA Technologies, IDT) (oligo: plasmid – 10:1 ratio) at 68°C for 30 min, followed by slow cooling to room temperature. Amicon Ultrafiltration was used to remove excess oligonucleotides and short duplex DNA. Diagnostic restriction digestion was used to evaluate the gapping efficiency (85-95%). Linear gapped DNA substrate for AFM studies was generated by digesting the circular gapped DNA with ScaI restriction enzyme using Buffer 3.1 (Countryman *et al.*, 2018; Kaur *et al.*, 2018; Kaur *et al.*, 2020).

C. AFM imaging in air to study DNA unwinding by Twinkle helicase

Using AFM imaging in air, protein-DNA samples are incubated and deposited on a sample surface. These samples are then imaged in air, and the scanning AFM tip captures a snapshot of the protein-DNA static structure-function information at the incubated time. Furthermore, time titration of the protein-DNA sample can provide information on how the protein-DNA interaction changes with respect to incubated time.

1. Prepare freshly cleaved mica surfaces by peeling a layer of mica off using scotch tape.
2. Measure the concentration of the purified gapped DNA using a NanoDrop spectrophotometer. DNA concentration is estimated by measuring the absorbance at a wavelength of 260 nm (A_{260}) using a spectrophotometer. The absorbance of double-stranded DNA at a concentration of 50 µg/ml is 1 and follows a linear relationship with concentration. Using this relationship, we can formulate the unknown DNA concentration as:

   DNA concentration (to be measured in µg/ml) = 50 µg/ml × A_{260}

3. Incubate Twinkle helicase (71.2 nM hexamers) with circular gapped DNA (3.6 nM) in Helicase Imaging Buffer for 1 min at room temperature. Increasing the Twinkle helicase: circular gapped

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DNA ratio can lead to an increased molar ratio of Twinkle per DNA molecule, causing more Twinkle helicase hexamers to unwind the same DNA molecule. Based on the extent of unwinding required and the number of unwinding sites, the ratio and incubation time can be adjusted.

4. Dilute the incubated sample 10-fold in Helicase Imaging Buffer (final volume 20 µl) and deposit the sample directly onto the freshly cleaved mica surface. There is no limitation on the volume of the sample, but there is a limitation on the concentration of the sample as you are performing single molecule studies. Overcrowded samples cannot provide clear information as it is difficult to observe single molecules trajectories. Therefore, the concentration of the sample on the surface should be adjusted accordingly, so that single molecules are clearly identifiable.
5. Wash the deposited samples three times with Helicase Imaging Buffer (300-500 µl) followed by three times with DI water (300-500 µl) and dry the sample with streams of N_2 gas before AFM imaging.
6. Open the MFP3D software and select the standard tab > AC air topography mode from the mode master tab (Figure 3).
7. Load the AFM probe (Pointprobe PPP-FMR probes) on the cantilever holder (Figure 4) and align the position of the laser plot to get maximum SUM signal (laser intensity) and zero deflection. While mounting the AFM tip on the cantilever holder, do not push too far back inside as this can result in misalignment of the AFM tip.

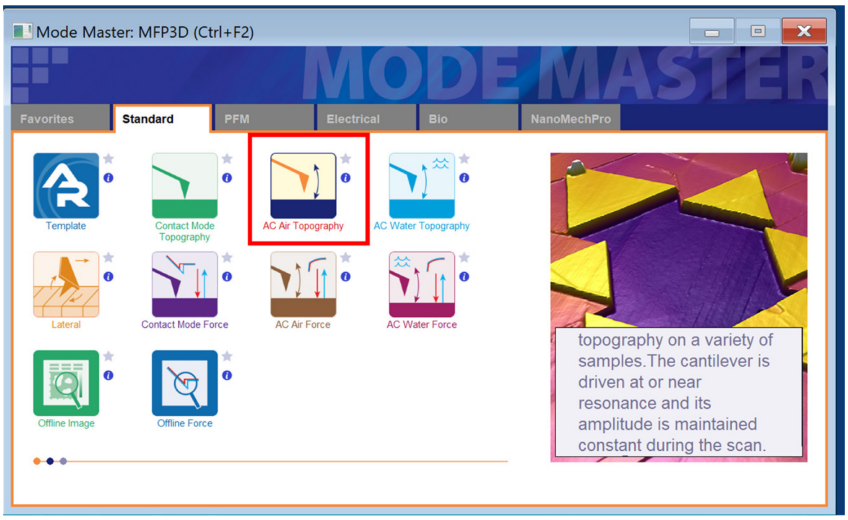

Figure 4. Mode Master tab with AC air topography mode selected (red square)

8. Autotune the mounted probe to the resonance frequency (frequency with greatest oscillation amplitude) with a target amplitude of a 1 V free air amplitude voltage for operation in AAC mode. Adjust the Feedback filter on the Master Panel > Advanced Scan Parms to 1,500 Hz (Figures 3, 5, 6, and 7).

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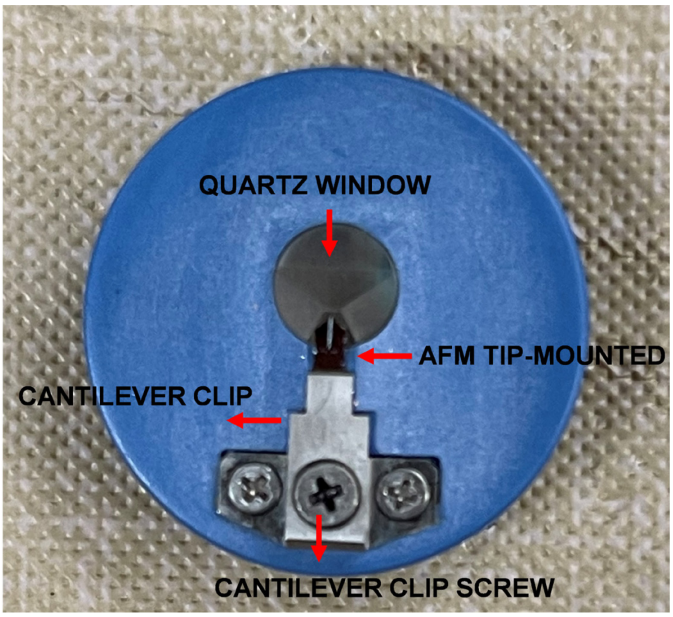

Figure 5. Labeled cantilever holder parts with mounted AFM tip

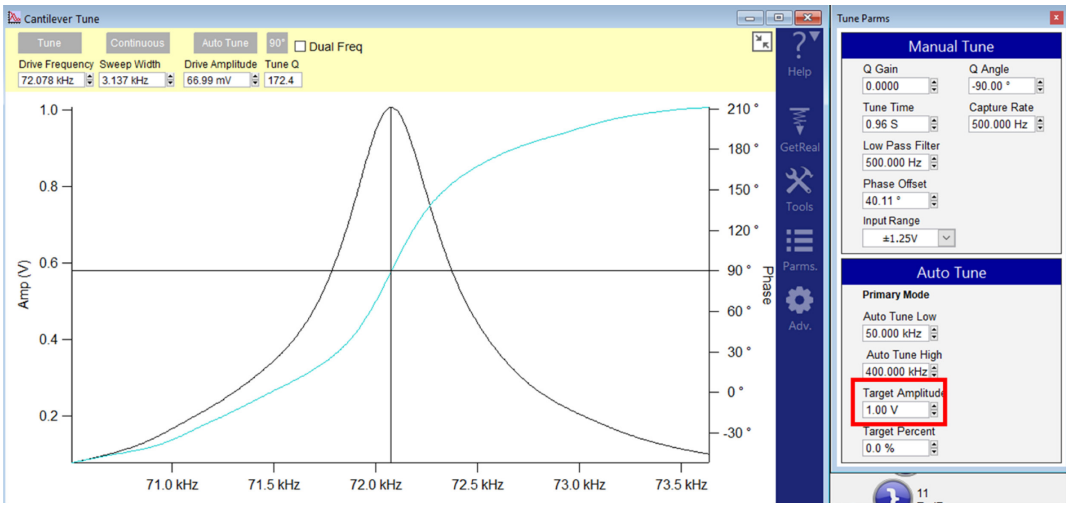

Figure 6. Cantilever tune and Tune Panel tab of the Master Panel showing target amplitude of 1 V selected (red square)

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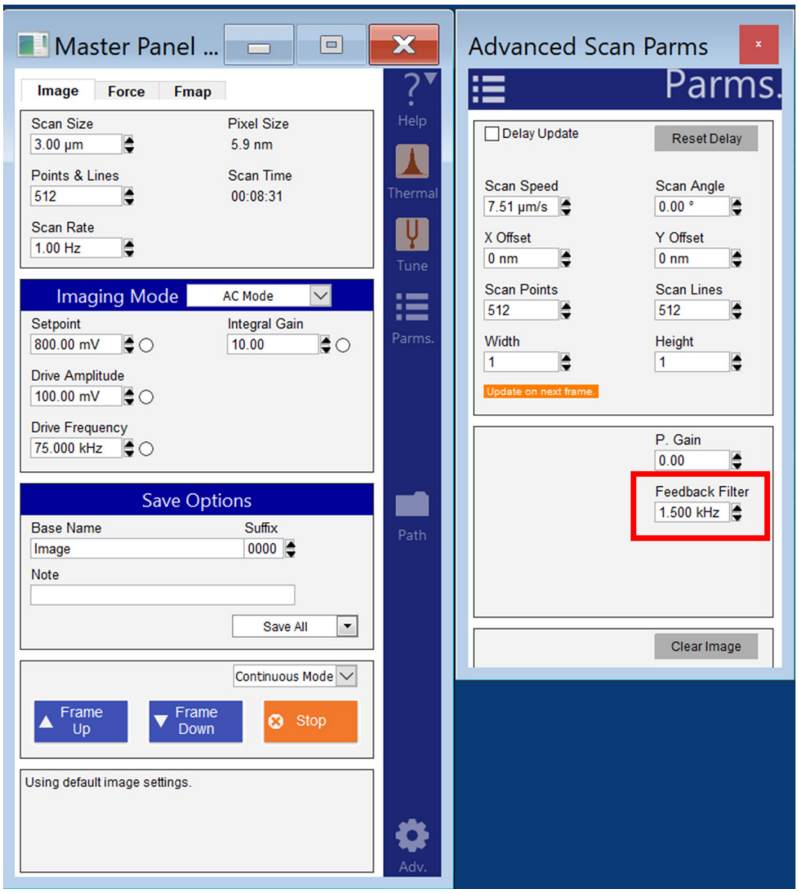

Figure 7. Main tab and Advanced Scan Parms tab of the Master Panel showing Feedback filter at 1.5 kHz (red square) in AAC air imaging mode

9. Image the sample deposited on mica surface using repulsive mode by adjusting the target percent to -5%-10% of the free air amplitude, enforcing the tip to remain in the repulsive regime (phase signal < 90°) while scanning the surface.
10. Capture the images at a scan rate of 1-2 Hz and a resolution of 512 × 512 pixels.

D. AFM Imaging for DNA unwinding by Twinkle helicase facilitated by mtSSB

1. Incubate Twinkle helicase (71.2 nM hexamers) and increasing concentrations of mtSSB tetramer (17.8 nM, 35 nM, 54 nM, and 142 nM) with circular gapped DNA (3.6 nM) in Helicase Imaging Buffer for 1 min at room temperature. Follow this order of addition to the incubated samples: Helicase Imaging Buffer, circular gapped DNA, and Twinkle and mtSSB simultaneously. Higher concentrations of mtSSB are observed to facilitate the unwinding reaction.
2. Dilute the samples 10-fold in Helicase Imaging Buffer (final volume 20 µl) and deposit directly onto the freshly cleaved mica surface.
3. Wash the deposited samples with Helicase Imaging Buffer (300-500 µl) three times followed by DI water (300-500 µl) three times and dry the sample with streams of N_2 gas prior to AFM imaging.

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Image the sample deposited onto a mica surface using AC mode on MFP-3D-Bio AFM with Pointprobe PPP-FMR probes (Nanosensors, k = 2.8 N/m, f_r = 70 kHz) as described in the previous section (Figures 1-3).

4. Capture the images at a scan rate of 1-2 Hz and a resolution of 512 × 512 pixels.

E. AFM imaging in liquids to capture real-time dynamics of Twinkle helicase on circular gapped DNA

To elucidate the dynamics of protein-DNA interactions, time lapse buffer imaging of the protein-DNA is done. In this mode of AFM imaging, the protein and DNA are not bound strongly to the surface and have restricted freedom to move around while imaging of the sample by the AFM tip in a layer of respective buffer (100-200 µl). This helps to visualize the dynamics of the protein-DNA interactions in real time as the tip is scanning across the sample surface in the native state.

1. Prepare a freshly cleaved mica surface by peeling a layer of mica off the scotch tape.
2. Deposit 30 µl of the APS working solution and incubate for 15 min.
3. Wash the APS solution with DI water (300-500 µl) three times and dry with streams of N_2 gas.
4. Check the concentration of the purified gapped DNA using a NanoDrop spectrophotometer.
5. Incubate 0.7 nM circular gapped DNA onto the APS treated mica surface for 15 min. Wash the deposited DNA with Helicase Imaging Buffer (300-500 µl). Add 100-200 µl of Helicase Imaging Buffer to the washed sample.
6. Select a low spring constant AFM imaging probe for imaging in liquids (AFM probes used in this experiment – BL-AC40TS, Asylum Research, spring constant k = 0.09 N/m, Resonant Frequency f_r = 110 kHz).
7. Open the MFP3D software and select standard > Topography > AC water Topography mode (Figure 8).

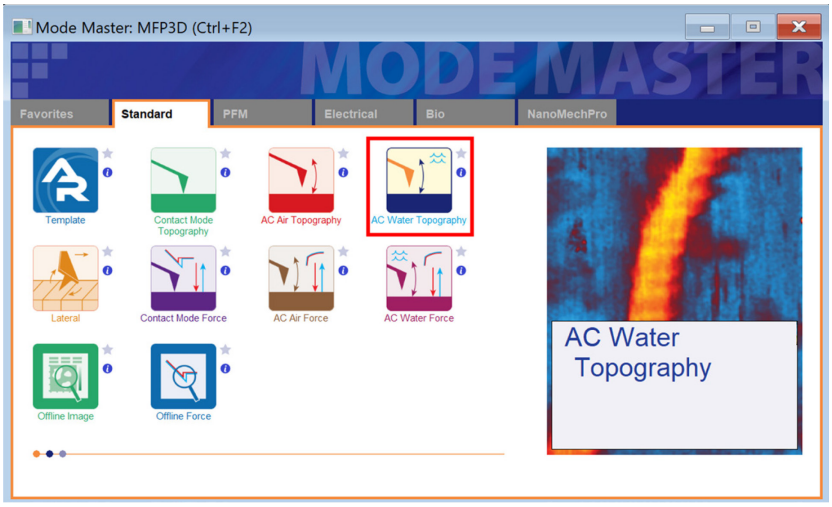

Figure 8. Mode Master tab with AC water Topography mode selected (red square)

8. Add 2-3 droplets of Helicase Imaging Buffer on the AFM probe before mounting the AFM head onto the base.

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9. Align the laser spot on the cantilever for maximum SUM signal (laser intensity) and zero deflection. The SUM signal obtained in liquids was observed to be lower than the SUM signal for the same AFM probe in the air.
10. In the Master panel, select capture Thermal Data and Do Thermal. Zoom in on the broad peak and fit it. Right-click on the fit to move the fit to the tune (Move Freq and Phase to the tune) (Figure 9).
    Note: The resonance frequency in liquids is 1/3rd of its value in air.

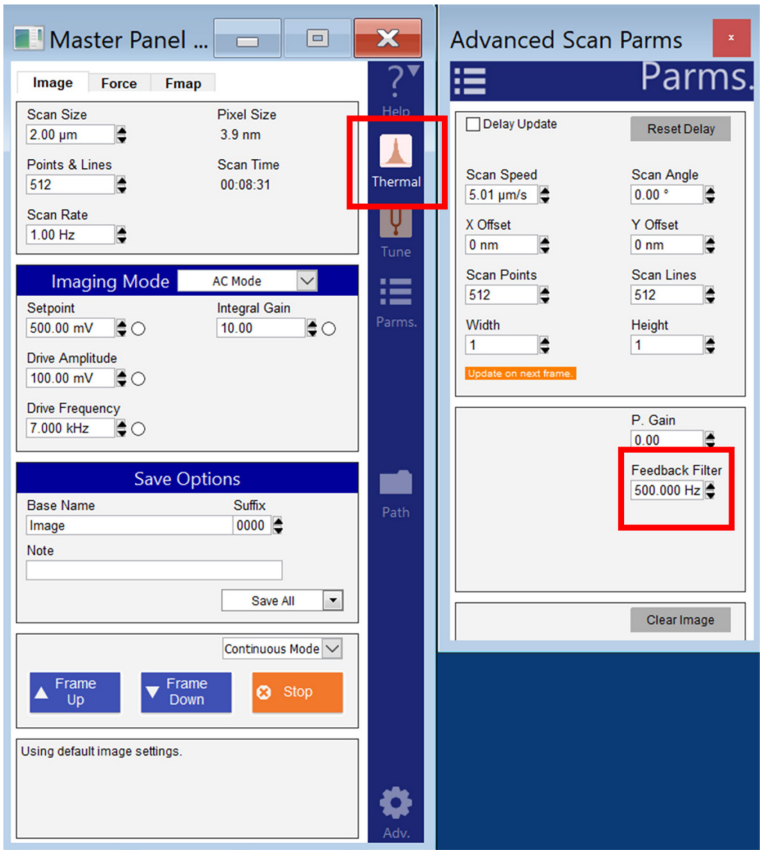

Figure 9. Main tab and Advanced Scan Parms tab of the Master Panel showing Thermal tab and Feedback filter at 0.5 kHz (red square) in AAC water imaging mode

11. Select the Tune tab in the Master panel and turn on 'Append Thermal.' Set the sweep width to 30 kHz. Click on the 'One Tune.' Choose the peak that overlaps the most with the thermal and set it as Drive frequency.
12. Center the phase at resonance to 90° by clicking on the 'Center Phase' button.
    Adjust the Feedback filter on the Master Panel > Advanced Scan Parms to 500 Hz (Figure 5).
13. Increase the Drive Amplitude to get 0.5-0.7 V of the free air oscillation amplitude.
14. For imaging biological samples, typically use 80% of the free amplitude as setpoint. The setpoint was adjusted approximately between 0.4-0.55 V.
15. Images were captured at a scan rate of 2-4 Hz and a resolution of 512 × 512 pixels. A typical

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sample DNA coverage includes 8-10 DNA molecules at a 1 μm × 1 μm scan size.

16. Incubate Twinkle helicase (71.2 nM hexamers, 10 μl) in Helicase Imaging Buffer for 5 min at room temperature.
17. Dilute the Twinkle helicase 10-fold in Twinkle Helicase Buffer and very slowly add the diluted Twinkle helicase (50-70 μl) into the liquids on top of the deposited DNA using a pipette tip while it is being scanned by the AFM tip.

Data analysis

Measure the unwinding DNA length from images collected from dried samples

1. Measure the height of ssDNA (200 ± 104 pm, Gaussian peak) and dsDNA (371 ± 116 pm, Gaussian peak) using cross-sectional analysis in the MFP-3D AFM imaging software to identify dsDNA and ssDNA product (Kaur *et al.*, 2020) (Figure 10).

   Steps for measuring the height of the dsDNA:

   a. Open the Analyze Panel window and select Draw with Mode – Free Hand (refer to Figures 10 and 11).
   b. Trace lines perpendicular to the contour of the imaged DNA.
   c. Adjust one pointer towards the peak of the cross-section of the DNA and the second pointer to the mica surface.
   d. The dZ value indicates the height of the DNA being measured.

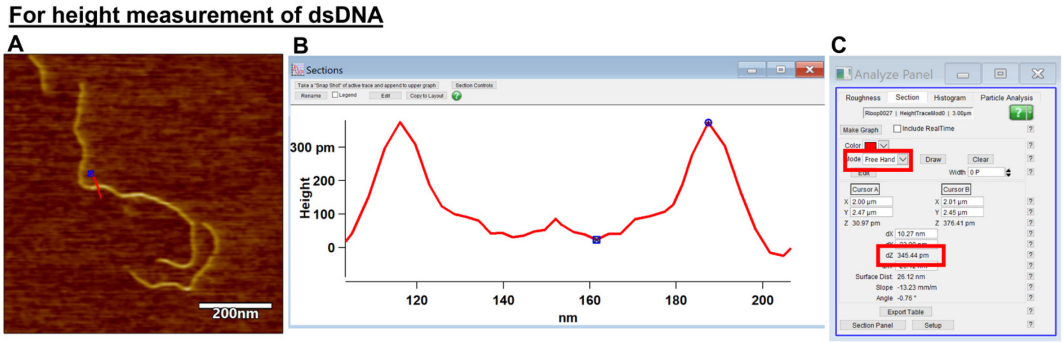

Figure 10. Height measurement steps of a (A) DNA AFM image (B) with its cross-sectional analysis showing a height measured in the (C) analyze panel (dZ) (red square)

2. Measure the contour length of the unwound ssDNA, characterizing it with the height of the ssDNA and make a histogram to plot the average unwinding length. The average unwinding rate is the average DNA unwinding length divided by the sample incubation time before deposition onto the mica surface (Kaur *et al.*, 2020) (Figure 11).

   Steps for measuring the contour length of any DNA:

   a. Open the Analyze Panel window and select Draw with Mode – Free Hand (refer to Figures 10 and 11).

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b. Trace lines overlapping the contour of the imaged DNA.
c. Adjust one pointer to the starting point of the DNA and the second pointer to the end of the DNA in the cross-section.
d. The dXY value gives the contour length of the DNA being measured.

Note: Before measuring the length of the unwinded DNA, measure the height of the DNA to make sure it is ssDNA. The height of ssDNA is lower than the height of dsDNA, as mentioned above.

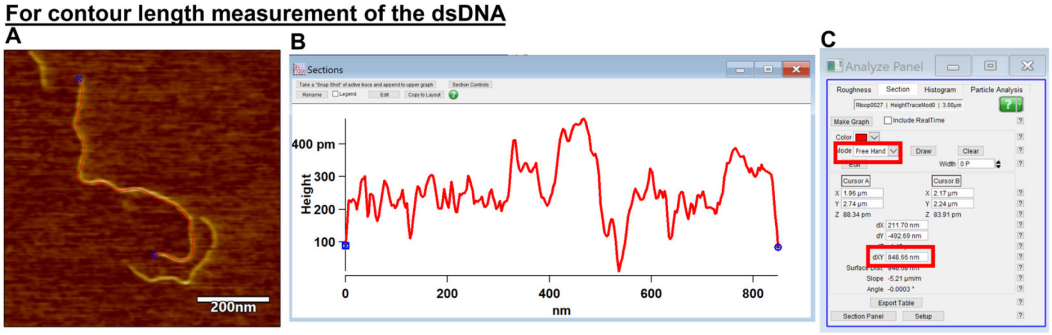

Figure 11. Contour length measurement steps of a (A) traced DNA AFM image (B) with its cross-sectional analysis showing a height measured in the (C) analyze panel (dXY) (red square)

Recipes

1. Helicase Imaging Buffer (Kaur *et al.*, 2020)
   20 mM HEPES (pH 7.5)
   100 mM NaCl
   7.5 mM $MgCl_2$
   7% Glycerol
   0.2% NP-40
   4.5 mM ATP
2. APS working solution (Shlyakhtenko *et al.*, 2013)
   a. Prepare 50 mM APS solution by dissolving 58 mg in 5 ml DI water.
   b. Dilute the stock solution to a ratio of 1:300 to make a working solution.
   c. Seal the stock solution in an Eppendorf tube with parafilm and store at room temperature.
3. Buffer 3.1: 1× Buffer components: NEB
   100 mM NaCl
   50 mM Tris-HCl (pH 7.9)
   10 mM $MgCl_2$
   100 µg/ml BSA

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Competing interests

The authors declare no competing financial interests.

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