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Parallel high-throughput single molecule kinetic assay for site specific DNA cleavage

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TITLE:

Parallel High Throughput Single Molecule Kinetic Assay for Site-Specific DNA Cleavage

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KEYWORDS:

DNA tethering**,** single molecule, DNA cleavage, protein-DNA interactions, restriction endonuclease, site-specific DNA binding

SUMMARY:

A highly parallel method for measuring the site-specific cleavage of DNA at the single molecule level is described. This protocol demonstrates the technique using the restriction endonuclease NdeI. The method can easily be modified to study any process that results in site-specific DNA cleavage.

ABSTRACT:

Site-specific DNA cleavage (SSDC) is a key step in many cellular processes, and it is crucial to gene editing. This work describes a kinetic assay capable of measuring SSDC in many single DNA molecules simultaneously. Bead-tethered substrate DNAs, each containing a single copy of the target sequence, are prepared in a microfluidic flow channel. An external magnet applies a weak force to the paramagnetic beads. The integrity of up to 1,000 individual DNAs can be monitored by visualizing the microbeads under darkfield imaging using a wide-field, low magnification objective. Injecting of a restriction endonuclease, NdeI, initiates the cleavage reaction. Video microscopy is used to record the exact moment of each DNA cleavage by observing the frame in which the associated bead moves up and out of the focal plane of the objective. Frame-by-frame bead counting quantifies the reaction, and an exponential fit determines the reaction rate. This method allows collection of quantitative and statistically significant data on single molecule SSDC reactions in a single experiment.

INTRODUCTION:

Site-specific DNA cleavage (SSDC) is a key step in many genomic transactions. For example, bacterial restriction-modification (RM)[1] and CRISPR[2] systems protect cells from attack by

phages and plasmids by recognizing and cleaving foreign DNA at specific sequences. In type II RM, restriction endonucleases (REs) recognize short 4–8 base pair (bp) sequences via protein-nucleic acid interactions[3]. CRISPR-associated endonucleases, such as Cas9, bind to sites via hybridization of the target site with crRNAs bound to the endonucleases[4]. The creation of site-specific double stranded breaks (DSBs) are also the first step in many DNA recombination events[5]. For example, the diversity of antigen binding regions created by V(D)J recombination requires the recognition and cleavage of specific target sites[6]. Some transposons are known to target specific DNA sequences, as well[7]. Not surprisingly, many site-specific nucleases involved in these processes, such as Cas9, are a key component of gene editing technologies[8]. In addition, novel site-specific endonucleases (i.e., zinc finger nucleases[9] and TALENS[10]) have also been engineered to edit genomes.

Many methods have been employed to measure the kinetics of site-specific cleavage of nucleic acids. These include gel analysis, fluorescence[11,12], and sequencing based methods[13]. A major advancement was achieved with the tethering of microbeads, which allows DSBs in single molecules of DNA to be detected by the motion of a bead after strand separation. In these methods, different types of forces are employed to ensure strand separation and motion of the bead post-cleavage. In one case, optical traps have been used to measure cleavage of DNA by EcoRV[14]. In these experiments, target search is the objective of the investigation, with conditions optimized so that site-specific binding is the rate limiting step. One drawback of optical traps is that only a single DNA can be observed at a time. In addition, a periodic large pulling force has to be applied to test for the strand separation.

Another technique uses a combination of flow and weak magnetic forces to pull on the bead in a continuous manner[15]. In this way, diffusion limited cleavage by NdeI is measured. The method employed allows for the simultaneous measurement of several hundred DNAs at once, allowing for statistical significance to be attained in a single experiment. Experiments based on magnetic tweezers have also been used. In one such study, a retroviral integrase was studied by including a DSB in the insertion oligonucleotide[16]. Successful integration resulted in the incorporation of DSB in the tethered DNA and loss of the attached bead. In a similar study of the ATP-dependent type III restriction endonuclease EcoPI, tens of DNAs were observed in a single experiment[17]. Magnetic tweezers hold the advantage that tension, as well as DNA looping, can be controlled and monitored during the reaction.

Presented here is a highly parallel single molecule method for measuring SSDC kinetics, which takes advantage of recent improvements in large-scale tethering of DNAs. This method is an improvement and extension of previous methods used to measure DNA replication[18], contour length of DNA[19], and cleavage by REs[15]. In this technique, linear DNAs containing a single copy of the recognition sequence are prepared with biotin at one end and digoxigenin at the other. The biotin binds streptavidin, which is covalently attached to a paramagnetic microbead. The DNA-bead complexes are injected into a microfluidic channel that has been functionalized with anti-digoxigenin FAB fragments. The DNA then tethers to the surface attachment points via binding of the digoxigenin to the adsorbed FAB fragments. Weak magnetic forces applied with a permanent magnet keep the bead from sticking non-specifically to the surface. Samples can be

injected rapidly (<30 s) into the flow channel to activate the cleavage reaction. Flow is turned off during data collection. As each DNA is cleaved, the exact time of cleavage can be determined by recording the frame in which the bead moves up and out of the focal plan of the objective, thus disappearing from the video record. A frame-by-frame count of remaining beads can be used to quantify the reaction progress.

Presented below is the complete protocol as well as example data collected using NdeI. As an example of how the technique can be applied, cleavage rates for a range of protein concentrations are measured at two different concentrations of magnesium, an essential metal cofactor. Although this application of the protocol uses NdeI, the method can be adapted for use with any site-specific nuclease by varying the DNA substrate design.

PROTOCOL:

1. Making the flow cell

1.1 Washing the coverslips

1.1.1 Place coverslips in staining jars and sonicate with ethanol (EtOH), then with 1 M KOH (for 30 min each). To avoid KOH precipitation in EtOH, rinse thoroughly with ddH_2O between washes.

1.1.2 Repeat both EtOH and the KOH washing steps 1x for a total number of four washes (two EtOH and two KOH). Store cleaned coverslips in ddH_2O in staining jars.

1.2 Cut loading and exit tubes (8 cm long) using a clean razor and insert into holes in a clean glass slide. Use PE-20 for inlet and PE-60 for outlet. Epoxy for 5 min to secure tubing and trim off any excess tubing.

NOTE: Glass slides measure 2'' x 3'' x 1 mm. Holes are drilled in pairs 15 mm apart. Each pair forms either end of a single channel. The tubing with the smaller ID is used for the inlet, as this reduces the upstream dead volume and thus the mixing time required (**Figure 1**).

1.3 Line up and apply precut double-sided tape with channel pattern cut out over holes on the glass slide. Smooth out with plastic forceps to achieve a good seal.

NOTE: The double-sided tape used in this experiment is 120 µm thick, and the channels are 2 mm wide and 15 mm long. Channels are cut using a knife printer (**Table of Materials**). It is possible to fit up to four channels on a single coverslip. See **Figure 1** for an image of the flow cell.

1.4 After peeling off the backing, apply a clean coverslip (dried with compressed air) over the tape and smooth out again with plastic forceps for a good seal.

1.5 Epoxy the edge off the coverslip to seal the flow cell and let it cure.

2. Preparation of labeled DNA for tethering

2.1 In a PCR tube, prepare 50 µL of PCR reaction mix containing 0.02 U/µL high fidelity DNA polymerase, 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, and 250 ng of M13mp18 vector DNA.

NOTE: Here, the forward primer (biotin-CCAACTTAATCGCCTTGC) and reverse primer (digoxigenin-TGACCATTAGATACATTTCGC) were chosen to amplify a region approximately 1,000 bp long, spanning from positions 6338 to 107 in the circular genome of the M13mp18 DNA. There is a single NdeI site in the middle of the amplified region. The forward primer is 5′ labeled with digoxigenin, which binds the anti-digoxigenin on the coverslip. The reverse primer is 5′ labeled with biotin, which binds the streptavidin-coated beads.

2.2 Insert the PCR tube in thermocycler and follow the cycle as shown in **Table 1**.

2.3 Purify the PCR product with a PCR clean-up kit following the manufacturer's protocol.

NOTE: Using the kit specified in the **Table of Materials**, the typical DNA yield is ~2 µg.

3. Tethering of DNA and beads

3.1 Prepare 10 mL of buffer A (1 M Tris-HCl [pH = 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 1 mg/mL β-Casein, 1 mg/mL Pluronic F-127). Degas in a vacuum desiccator for at least 1 h.

3.2 To functionalize flow cell, inject 25 µL of anti-digoxigenin FAB fragments (20 µg/mL) in PBS into the flow channel. Use gel loading tips to fit into the PE-60 tubing. Incubate at room temperature (RT) for 30 min.

3.3 After incubation, flush the channel by pulling 0.5 mL of buffer A through the channel using a syringe. Take care not to introduce air into the channel.

3.4 After functionalization, mount the flow cell on an inverted microscope. Hook up the outlet tube to a syringe pump and put the inlet tube into a microcentrifuge tube containing buffer A.

3.5 Manually pull at least 0.5 mL of buffer A to flush the system and prime the pump. Let the pump run at 10 µL/min for at least 5 min to equilibrate the system.

3.6 To prepare the beads (**Table of Materials**), vortex the stock bottle of beads and pipette 1.6 µL of the 10 mg/mL stock beads into 50 µL of buffer A, then vortex.

3.7 Using a magnetic separator, pipette out the buffer and resuspend in 50 µL of buffer A, then vortex.

3.8 Repeat step 3.7 2x for a total of three washes. For the last wash, resuspend in 100 µL of buffer A and vortex to achieve a final concentration of 160 µg/mL.

3.9 To complex the DNA and beads, first prepare 480 µL of 0.5 pM labeled DNA substrate in buffer A. Then, pipette in 20 µL of 160 µg/mL bead suspension, making sure to vortex the beads before pipetting. Place on a rotator for 3 min.

3.10 After 3 min, immediately load into channel at a flow rate of 10 µL/min for ~15 min or until sufficient bead tethering is observed.

NOTE: Beads should not be so densely packed that they interact with each other on the surface (see discussion section).

3.11 To wash the channel of all free beads, switch the inlet tube to a fresh tube of buffer A and flow in at 50 µL/min for at least 10 min or until no loose beads are observed.

4. Data collection and analysis

4.1 To prepare for data collection, place inlet tube into a microcentrifuge tube containing at least 100 µL of NdeI (0.25–4.00 U/mL) in buffer A. Lower the permanent magnet over the flow channel, and position the light source off axis for darkfield imaging.

NOTE: Two annular rare earth magnets, epoxied together, are held 8 mm above the active surface of the flow channel using a cantilevered optical post during data collection. A goose neck lamp is used for the off-axis light course.

4.2 Use a commercial microscope, video camera, and data collection software (**Table of Materials).** In the software, click the "**Exposure**" tab, and set "**Exposure Time**" to 10 ms. Click "**Timelapse**" tab and set "**Image Count**" to 600, "**Duration**" to 20 min, and "**Interval**" to 2 s. Click "**Run**" to start data collection.

4.3 On a syringe pump, set the flow rate to 150 µL/min and injection volume to 80 µL. Press "**Run**" at 1 min into data collection. After injection, turn off the pump and close the valve to prevent flow during data collection.

4.4 Once data is collected, open image analysis software (**Table of Materials**). Under the "**File**" tab, choose "**Import**" **|** "**Image Sequence**". Locate the image files in the pop-up menu and click "Open".

4.5 Set the threshold by choosing "**Adjust Theshold**" under the "**Image**" pull-down menu. Use the slider bar to set the threshold value to identify bright spots corresponding to beads in the image.

4.6 Count the bright spots in each frame by clicking "**Analyze Particles**" in the "**Analyze**" pull-down menu. Click "**OK**", then choose "**Yes**" to process all images. Save the results file.

NOTE: This will save a data file that contains the number of beads in each recorded video frame.

4.7 Open data analysis software (**Table of Materials**) and import the results file by clicking "**Import from Test File**" in the "**File**" pull-down menu. Plot the bead count data vs. time.

4.8 Fit the number of beads vs. time by clicking "**Curve Fit**" in the "**Analyze**" pull-down menu. Choose the "**Natural Exponent**" equation and click "**Try Fit**" **|** "**OK**".

NOTE: Only the data recorded after the injection of sample should be included in the fitting region. The fit parameter in the exponent of the fitting function will be the cleavage rate.

REPRESENTATIVE RESULTS:

Using this technique, the SSDC rates of NdeI were measured for a range of protein concentrations (0.25–4.00 U/mL) at two different concentrations of magnesium (2 mM and 4 mM). Each of these conditions was replicated at least twice, with a few hundred to 1,000 tethered DNAs per experiment. **Figure 2** describes the experimental design. **Figure 3** shows examples of data collection and analysis details. **Figure 4** illustrates how the rate depends on protein concentration at the two concentrations of magnesium. It can be observed that at sufficiently low protein concentrations, the rate is proportional to protein and independent of magnesium. For sufficiently high protein concentrations, the rate is dependent on magnesium but independent of protein concentration.

FIGURE AND TABLE LEGENDS:

Table 1: PCR parameters. Shown are temperatures and durations of the thermocycler program steps used in step 2.2 of the protocol. The melt, anneal, and extend steps (steps 2, 3, and 4) are repeated 30x.

Figure 1: Microfluidic flow cell construction. The top glass slide (2'' x 3'', 1 mm thick) is pre-drilled with holes matching the channel pattern. The inlet and outlet tubes are inserted into the holes and fixed with epoxy prior to attaching tape and cover glass. The double-sided tape is pre-cut with channel pattern. The bottom slide (#1 or #1.5 cover glass) is previously cleaned using the protocol described in the main text. Once assembled, the edge of the cover glass is sealed with epoxy.

Figure 2: Experimental design. (**A**) Method of DNA tethering. Double-stranded DNA (1 kbp) labeled with digoxigenin on the 5’ end is attached to the surface of the flow cell via antidigoxigenin-digoxigenin interaction. The 3’ end of the DNA, labeled with biotin, is attached to a microbead via streptavidin-biotin interaction. The NdeI cleavage site is located at the

center of the DNA. (**B**) Experimental setup during data collection showing magnet and objective position. The permanent magnet maintains a weak upward force on the bead during the cleavage reaction.

Figure 3: Example of data collection and analysis. (**A**) Image of region of beads taken before cleavage reaction is initiated. (**B**) Image of same region after reaction is completed. (**C**) Plot of number of beads vs. time (black curve) as determined from each frame of the video record. The shaded green area marks the period of injection of enzyme and is not included in the fit. The data fits a single exponential curve (dashed green curve), with a decay constant equal to the reaction rate.

Figure 4: NdeI cleavage is dependent on concentrations of protein and magnesium. Plot of the measured SSDC rate of NdeI for a range of protein concentrations at two different concentrations of magnesium: 2 mM (blue circles) and 4 mM (green squares). Error bars represent SEM. The dashed curves are trend lines and do not represent fits to theory.

DISCUSSION:

The protocol can be used to measure the kinetics of any SSDC system, provided that the strand separation is observed during the experiment. The detection of cleavage is affected by observing the detachment of the tethered bead and therefore marks the instant of strand separation. All preceding steps occur before the detection of the cleavage; thus, only the total transit time is recorded.

The flow cell coverslip is functionalized via non-specific adsorption of antibody protein to the clean glass. Insufficiently cleaned glass may affect binding of the antibody. In tethering, bead density should be low enough so that beads do not interact. The surface density of attachment points can be controlled by the concentration of antibody during functionalization. The total number of beads depends on the size of the field of view. In this case, a few hundred to 1,000 tethered beads were sufficient for good statistics and avoided bead-bead interactions. During bead injection, surface tethering was monitored via live video. Bead injection was stopped when estimated bead count was between 500 and 1,000 beads.

The fastest cleavage rates that can be accurately measured are limited by the mixing time of the flow cell. The mixing time in laminar flow cells is influenced by several factors. Diffusion to the surface is a key step; therefore, the mixing time depends on the diffusion coefficient of the reactant. The significant shear that occurs in the entrance tubing, which transports the sample to the flow channel from the sample reservoir, can increase the time needed to ensure adequate mixing at the reaction surface in the channel. It was found that the mixing time could be reduced by reducing the upstream dead volume and increasing the flow rate. With an inner diameter of 380 µm and maximum length of 8 cm for the inlet tubing (and flow rate of 150 µL/min), it was found that the injection time could be reduced to ~20 s without affecting the measured cleavage rate. Since the mixing time depends on the diffusion coefficient of the reactant, it should be determined separately for each enzyme or cleavage activator studied.

The tethering method allows for non-specific tether rupture, presumably due to either dissociation of the digoxigenin-antibody complex or release of the antibody from the surface. This results in a reproducible background bead loss rate present before the injection of enzyme of ~3 x 10^{-4} s^{-1}. This systematic effect can be corrected for either by subtracting the background rate from the measured cleavage rate, or by modeling the background in the fitting equation. However, cleavage rates lower than this lower bound will be less reliably measured.

Imperfect surface passivation can lead to improper tethering. This leads either to increased fractions of "stuck" beads, which do not go away during the the experiment, or to beads, which dissociate from the surface very slowly. This creates a higher and possibly sloping baseline in the processed data. It was found that with properly cleaned cover slips and freshly made β-casein stock solution, these effects were minimal for most data sets. For the occasional data sets that show this, modifying the fitting function (to include a sloping baseline) can correct for this effect.

The current protocol can be extended in several ways. Further isolation of mechanistic steps after target site binding can be performed using a pre-binding format, in which protein is injected in the absence of essential cofactors. This idea is tested by injecting NdeI in the absence of magnesium. Under these conditions, the protein binds to its cognate site but does not cleave the DNA. Injecting magnesium after this binding step activated the cleavage resulting in rapid bead loss. The experimental setup also allows for the control of DNA conformation and tension by varying the magnet configuration or adding flow. Under the low forces in these experiments, the DNA becomes partially coiled. Changing the forces slightly can have a dramatic effect on the conformation of the DNA. For example, under buffer conditions in which target search is rate limiting, varying the conformation of the DNA can test for the effect of jumping on the target search. Under buffer conditions in which the hydrolysis step is rate-limiting, varying the force can probe the effect of the DNA tension on phosphodiester bond hydrolysis. It should be noted that under the low magnification used, these conformational changes cannot be observed. The resulting small motions in bead position have to be tracked under higher magnification to verify that DNA conformation can be controlled.

Data analysis can be extended in a variety of ways. This work applies a simple bead counting method followed by curve fitting of a single exponential function. Methods based on residence time analysis can be used, as well[20]. Distributions of residence times of individual DNAs can then be analyzed via curve fitting or by using more sophisticated techniques, such as the generalized method of moments[21].

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure 1

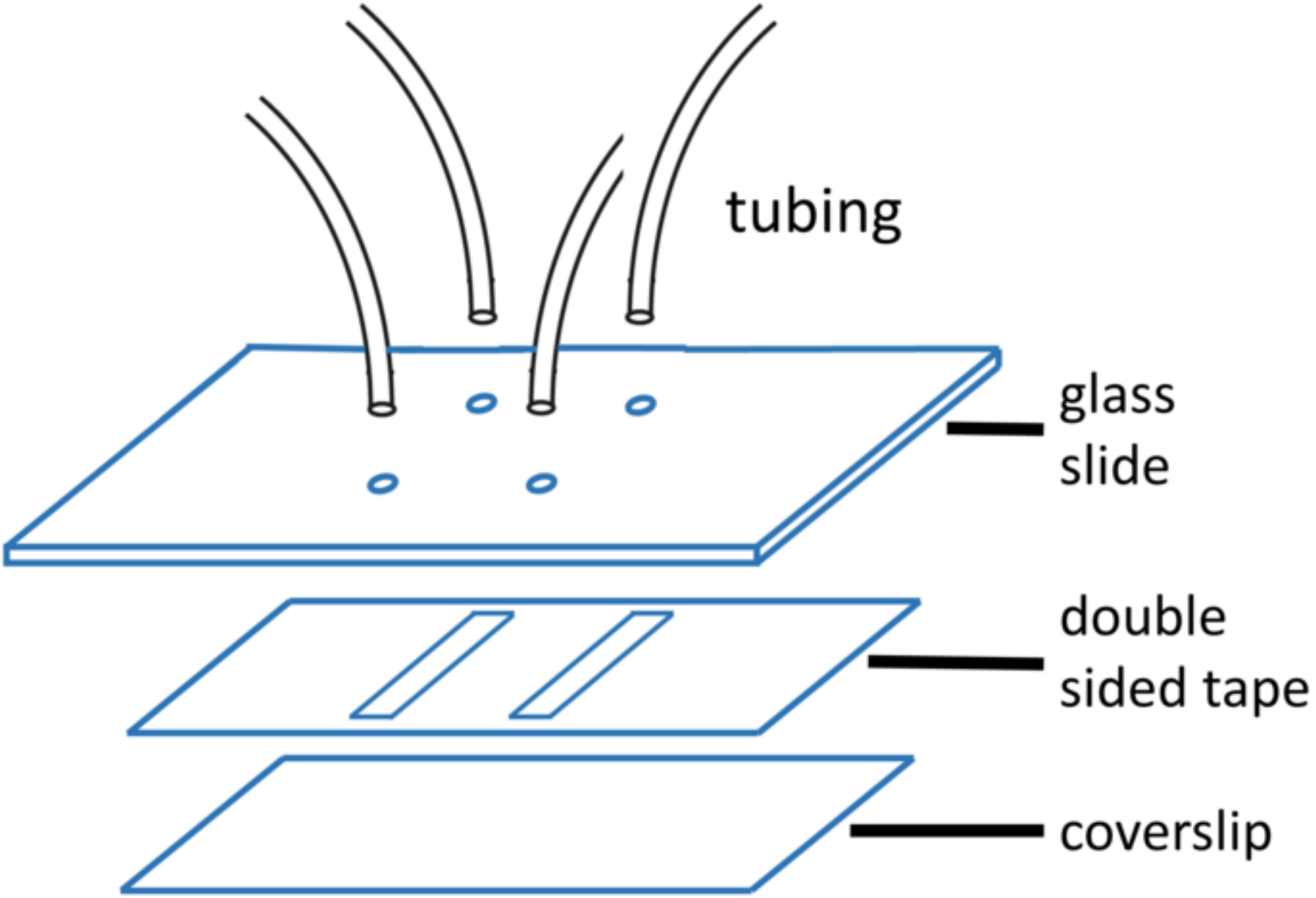
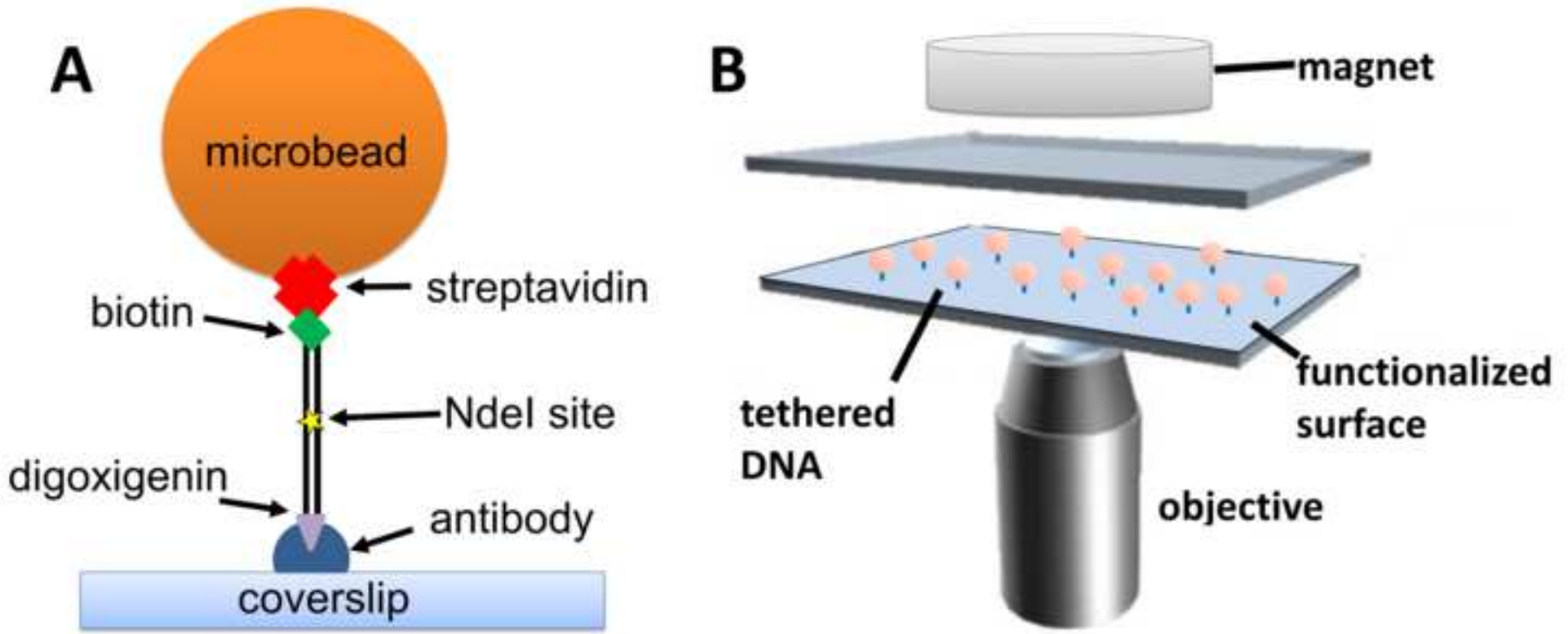


Figure 2



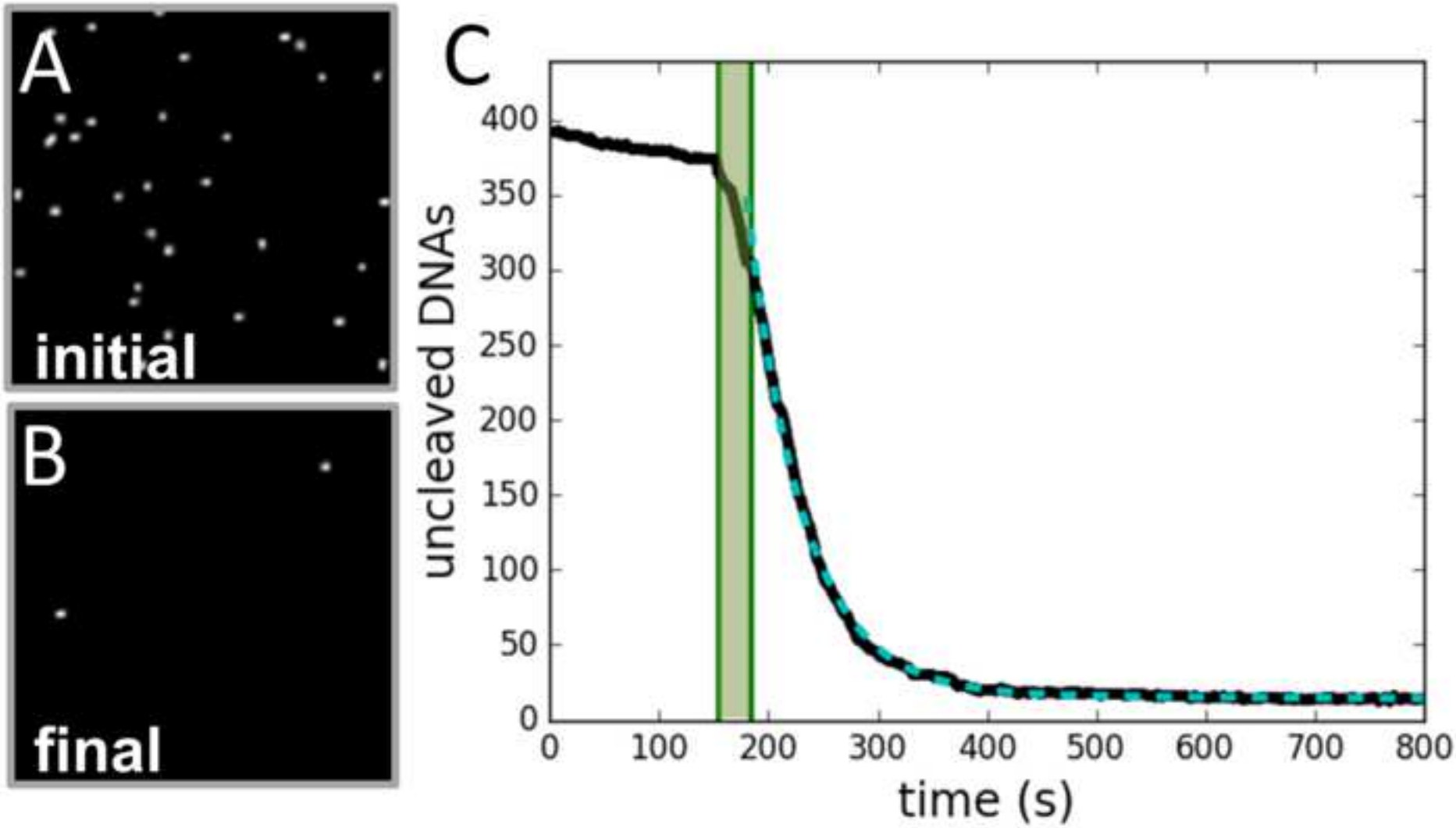


Figure 4

Click here to access/download;Figure;Figure 4.tif

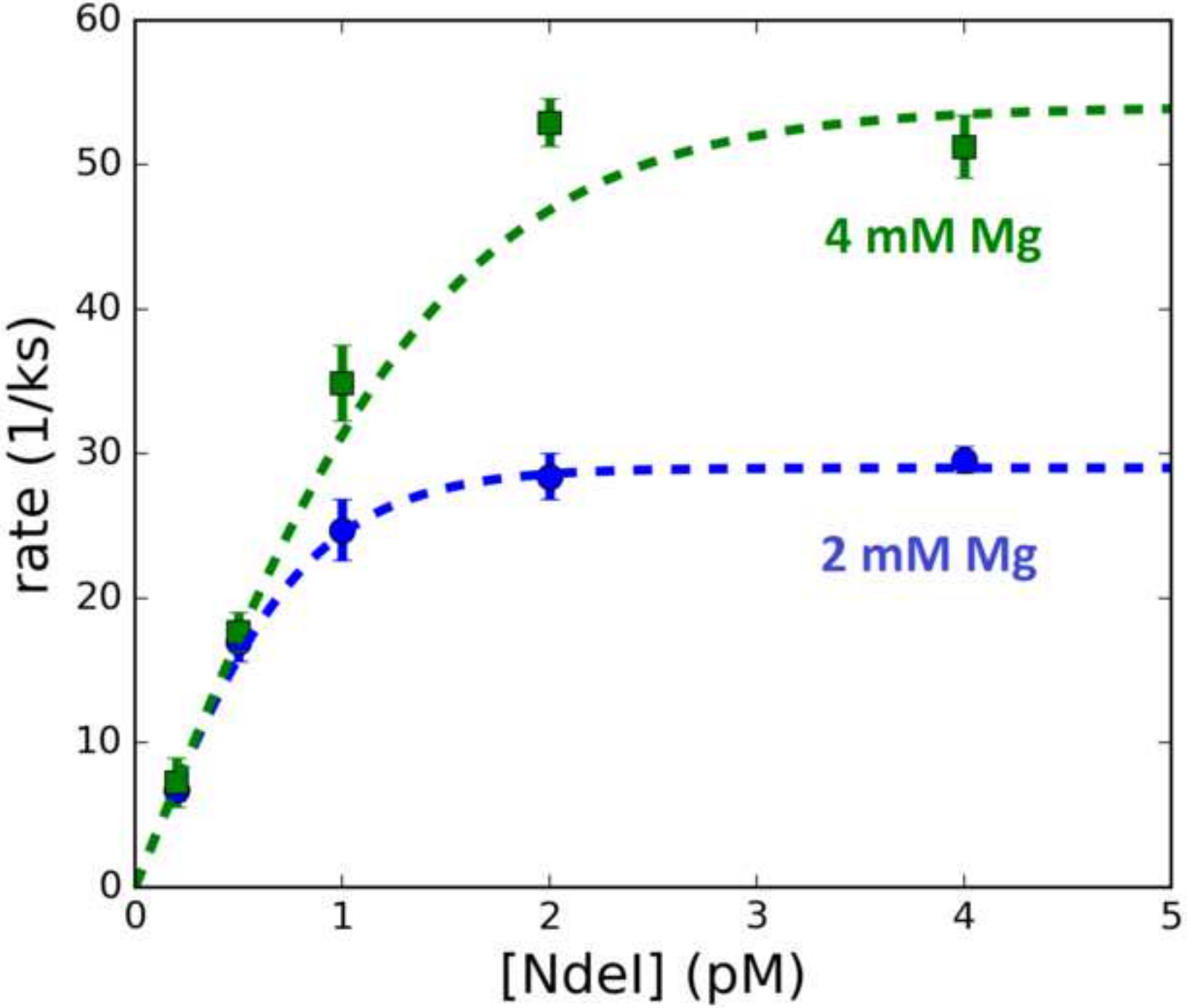


Table 1

Step	Description	Temperature (°C)	Time (s)
1	Denaturation	98	30
2	Melt	98	10
3	Anneal	60	30
4	Extend	72	30
5	Final Extend	72	120

Name of Material/ Equipment	Company/Source	Catalog Number
5 minute Epoxy	Devcon	14250
anti-digoxigenin FAB fragments	Roche Diagnostics	11214667001
camera and software	Jenoptik	GRYPHAX SUBRA
data analysis software	Vernier Inc.	LP
double sided tape	Grace Biolabs	SA-S-1L
Dulbeccos Phosphate Buffered Saline	Corning	21-031-CV
ethanol 95%	VWR	89370-082
forward primer: digoxigenin-CCAACTTAATCGCCTTGC	Integrated DNA Technologies	n/a
image analysis software	National Institutes of Health	ImageJ
inverted microscope	Nikon	TE2000
knife printer	Silhouette	
M13mp18 DNA	New England Biolabs	N4040S
MyOne streptavidin beads	Thermo Fisher Scientific	65601
NdeI enzyme	New England Biolabs	R0111S
PCR cleanup kit	Qiagen	28104
pluronic F-127	Anatrace	P305
polyethylene tubing PE-20	BD Intramedic	427406
polyethylene tubing PE-60	BD Intramedic	427416
Q5 Mastermix	New England Biolabs	M0492S
rare earth magnet 0.5" OD 0.25" ID	National Imports	NSN0814
rare earth magnet 0.75" OD 0.5" ID	National Imports	NSN0615
reverse primer: biotin-TGACCATTAGATACATTTCGC	Integrated DNA Technologies	n/a
syringe pump	Kent Scientific	Genie Plus
β-Casein from bovine Milk	Sigma-Aldrich	C6905

Dear Editor,

We thank you and the reviewers for the thorough and thoughtful reading of the manuscript. In most cases, we have complied, as well as we understood, with the suggested revisions. This has strengthened and focused the manuscript. Note that most of the editorial comments appeared to be standard suggestions, which we have complied with, but we do not list them below. We only list and respond to the editorial comments which appeared specific to our manuscript. For the peer reviewers, we have responded to all of their comments. Our responses are in blue.

Sincerely,
Allen Price

Editorial comments:
Changes to be made by the Author(s):

12. 2: Significance of using M13mp18 vector? Please expand the PCR details. Please include concentrations. What is the region being amplified? Size of the PCR product? Amount of PCR product being purified?

We have modified step 2.1 to include concentrations and added a Note to that section including the information requested. We also added a Note giving typical yield.

13. 3.9: How much DNA was used?

This is now included in step 2.1.

14. 4: Please include how is this done. Please include all the button clicks in the software, knob turns etc.

In section 4 we have included all the software button clicks as well as pump settings. We have listed the name of the software packages and syringe pump in the Table of Materials. In addition, we have added some clarifying Notes to this section.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This article by Matozel describes a high-throughput single molecule assay for looking at site-specific DNA cleavage. The application of this method is illustrated by using NdeI restriction enzyme. The linear DNA molecules are tethered to the coverslip with their free ends attached to paramagnetic beads. The reaction is conducted by monitoring the beads by microscopy and

recording the frame in which they become untethered and float away. Several hundreds to a thousand individual molecules can be observed light microscopy at 10X magnification. From the bead counting, the reaction kinetics can be characterized by using appropriate exponential fit. This article is well written, and the methodologies are described in sufficient detail for other researchers to use this system to monitor site-specific DNA cleavage. The method is attractive in that there is wide interest in site-specific DNA cleavages by restriction enzymes, CRISPR-Cas9 and other DNA cleaving agents. The important of site-specific DNA cleavage in targeted genome editing adds to the significance of the work.

Major Concerns:
None

Minor Concerns:
I have only a few minor comments.
1. The title would seem a bit exaggerated in the use of 'Massively parallel'. Perhaps replacing massively parallel to 'A parallel high-throughput single molecule kinetic assay-----' would be more appropriate.

We have accepted this suggestion and retitled the manuscript "A parallel high-throughput single molecule kinetic assay for site specific DNA cleavage"

2. In the introduction, in referring to site-specific DNA cleavage systems, the authors may wish to add zinc finger nucleases and TALENs.

I have added the following references to these systems in the first paragraph:

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D., "Genome editing with engineered zinc finger nucleases," Nature Reviews Genetics 11 (9), 636-646 (2010).

Joung, J.K. and Sander, J.D., "TALENs: a widely applicable technology for targeted genome editing," Nature reviews Molecular cell biology 14 (1), 49-55 (2013).

3. One had to look at the Table of materials to recognize that the paramagnetic beads used were Myone Dynabeads. Perhaps the brand name could be included in parentheses when first mentioning the beads.

JOVE's policy is not to include company names in the main text, so we have left that section as is. The brand name is listed in the Table of Materials. We have added a reference in section 3.6 directing the reader to the Table.

Reviewer #2:

Manuscript Summary:

The authors present an exciting method for measuring rates of DNA cleavage using a relatively straight-forward method to make a micro-fluidics chamber that can tether DNA through binding of FAB fragments. A number of useful applications are discussed, including extending the technique to change DNA conformation. In addition, a number of suitable points are discussed regarding trouble-shooting of the technique.

Minor Concerns:

In general, this article is well done, and will greatly benefit from a video describing the method in visual form. The title is well-suited, and overall the method is relatively easy to understand. A general comment on the abstract and introduction is that the abstract contains some specific details about the method (i.e. tags used to bind beads to surface and DNA) that are not in the final paragraph of the introduction. Reversing this, with more general details in the abstract and specifics in the introduction will help along the reader in understanding how the assay works overall.

We thank the reviewer for this suggestion. We have re-written the abstract and last paragraph of the introduction as suggested and both read much better now.

Regarding the methods, again these are well written, but could use some clarification in a few spots, as follows:

1. For Section 2. Preparation of Labeled DNAs for Tethering, please provide the specific PCR clean-up kit used. While most commercially available kits are practically identical, there could be slight variations in quality of the final DNA product that may affect the experiment.

We agree with this comment, but cannot list the name of the kit in the main text due to JOVE editorial policy. We have added a Note to section 2 explaining the typical yield we get and a reference to the Table of Materials for the kit name.

2. For Section 3. Tethering DNA and beads, it is not entirely clear how the FAB fragments are binding to the flow cell. Perhaps a sentence or two in the introduction to section 3 could explain for the non-expert. This will be useful if low functionalization of the flow cell is observed.

The FAB fragments bind via non-specific adsorption to the glass cover slip. This technique is commonly used, and we have rewritten the paragraph the reviewer refers to in order to include an explanation. Furthermore, we have moved this paragraph to the discussion where it fits in better, and in response to a request from the editor to move such material out of the protocol and into the Discussion (see lines 291-292).

3. In Section 3.10, the method suggests to load a channel "until sufficient bead tethering is observed". Is there an approximate percentage of the field of view filled, or some way to make a general estimate so that those testing it know when enough DNA is bound to obtain reasonable results?

This is an important point, and we agree that we have not adequately addressed it in the submitted manuscript. In general, one wants as many beads as possible without having them interact with one another. This depends on the size of the FOV of course. We tether until we estimate we have several hundred to one thousand beads, which is the maximum we can achieve before we notice beads interacting with each other. I have added a Note about this in step 3 (lines 192-3) with a reference to the Discussion section (lines 292-8) where I describe it in more depth.

With respect to the Figures, they clearly show the experimental set-up, however an additional figure of a stationary photo of what the microscope shows during the experiment would be beneficial to the reader, to understand the level of resolution. For example, in the discussion it is mentioned how changing the magnetic field can change the DNA conformation. Is this visible in the microscope? If not, how does one know how the DNA conformation is changed? Alternately, having an image and labelling bound vs unbound DNA would also be useful.

A close up of part of an image from data collection is shown in Figure 3A and 3B. The entire FOV would have the image of beads too small to show up well in print or online. To show them more clearly, we modified Figure 3A & B by zooming in more to show the beads better.

Regarding the DNA conformation, we use low magnification to get the benefit of a large FOV for good statistics. The draw back is that we lose the ability to detect small bead movements that result from changes in DNA conformation. Therefore, we are NOT able to detect these DURING DATA COLLECTION. However, one could use higher magnification to observe changes in vertical motion or in-plane fluctuations of the bead as the DNA extends or coils to demonstrate that one can control the conformation using the magnetic or fluid forces. Once that is done, one could go back and collect data under lower magnification wide field. We have not done this, but mention it as an extension of the technique. We have added some clarifying text on this to the Discussion (lines 340-2).

Unfortunately, we were not sure what the reviewer means by "an image and labelling bound vs unbound DNA."

Some minor points regarding Figures 3 and 4. In Figure 3, for continuity, please define what the blue line is in the figure legend, but also, the black dotted line is extremely difficult to see contrasted with the dark blue. A slight change in color of either (perhaps gray?) will help

visually. For Figure 4, a curve of best fit should be plotted to the points to better see the 2-step mechanism described in the discussion, rather than individual data points.

We have altered Figure 3 to use a black curve for the data and a dashed light green curve for the fit. We think this improves the visibility greatly.

In Figure 4, we have added trend curves to better indicate the behavior of the data. Note, these curves are not fits to theory, but merely to make the trends in data more clear. Note that we have removed all text in the Representative Results or Discussion sections refering too interpretations of the data (the 2-step mechanism) in response the Reviewer 3's comments and our decision that such discussion is not appropriate in a methods paper.

The discussion contains some excellent applications of the technique, as well as important methodological points to consider when using this method. Overall, with some minor changes, this method is an excellent tool for single-molecule analysis with many potential applications that have been addressed well by the authors.

Reviewer #3:

Manuscript Summary:

In their appropriately titled manuscript, Matozel and colleagues present their protocol for massively parallel detection of site-specific DNA cleavage at the level of single molecules. The method described permits the quantification of DNA cleavage using widely available materials, software and labeling techniques at low cost. The authors use NdeI as a test case and demonstrate high quality kinetic data acquisition with a clear explanation of sources of background and analytical remedies. The broad utility, do-it-yourself assembly, and extent of imaging makes the manuscript a prime candidate for publication in JOVE.

Major Concerns:

1) The overview of cleavage measurements in the introduction is fairly superficial and uneven. I don't think the authors truly need to elaborate on other approaches, but I would recommend communicating the information as a table instead as text. The technologies listed are 1) gel electrophoresis of DNA fragments, 2) optical traps, 3) magnetic separation (the focus of the manuscript), and 4) magnetic tweezers. These could be organized as 4 rows with "assay," "N", "equipment", "time resolution", "advantages", "disadvantages" and "references" as columns (or to your liking). I would also recommend including DNA sequencing as a 5th option (see Fu 2014 in Nucleic Acids Research and Becker 2019 in Molecular Cell).

We agree with the reviewer that the Introduction needs revision. In order to make it less superficial, we have included discussion of additional techniques in the first two paragraphs

(lines 60, 61, and 64), as well as re-written the final paragraph (lines 84-103) to explain our technique in more depth. We have added three references (including one of the reviewer's suggestions---unfortunately we could not find Fu 2014). The additional references are:

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D., "Genome editing with engineered zinc finger nucleases," Nature Reviews Genetics 11 (9), 636-646 (2010).

Joung, J.K. and Sander, J.D., "TALENs: a widely applicable technology for targeted genome editing," Nature reviews Molecular cell biology 14 (1), 49-55 (2013).

Becker, W.R., Ober-Reynolds, B., Jouravleva, K., Jolly, S.M., Zamore, P.D., and Greenleaf, W.J., "High-throughput analysis reveals rules for target RNA binding and cleavage by AGO2," Molecular cell 75 (4), 741-755. e711 (2019).

The suggestion of a table, although we agree would be very informative, we feel is not appropriate in this context, as we are not attempting a thorough review of the field. The detailed analysis of the techniques that the reviewer requests is beyond what we are trying to accomplish in this methods publication.

2) Some aspects of the assembly process are quite vague: how the double-sided tape is "pre-cut" (what length and width?), where/how to apply the magnet (is it sitting on top of the slide?). The magnets and tape should be included in the table of materials as well. I think the magnet and objective should be included in figure 1 in some schematized form.

This critique is well founded and we have made several revisions to address these shortcomings.

(1) We added Notes to section 1 (lines 122-5, 130-2) in the protocol describing the preparation of the glass slide and the tape, as well as to section 4 (lines 204-6) describing the magnets and how they are supported. The knifer printer, magnets and tape are all in the Table of Materials, and a reference has been added to the protocol directing the reader to the table.

(2) We have added labels to Figure 1 to clarify the construction of the flow cell.

(3) In order to better show the magnet, we have created a new figure which we have added to Figure 2, creating a two panel figure. Figure 2B shows the configuration during data collection, with microscope objective and magnet.

3) The discussion of the representative results is sometimes overaggressively simplified. Cleavage always depends on magnesium and protein concentration (i.e., the concentration of either could at any point be lowered to a level that would abolish cleavage). It should be clearer

that the data shows that magnesium is a required cofactor, that target search is rate-limiting for cleavage at high magnesium:protein ratios, but double-strand break formation is rate-limiting at low magnesium:protein ratios. The results shown also don't address the residence time - magnesium could also slow dissociation in addition to accomplishing cleavage - so the authors should be careful not to speak narrowly about the role of magnesium. Lines 220-229 should be rewritten. Line 276 also gives the impression that cleavage will always happen appreciably faster with more magnesium when the reality is that target-search is rate-limiting.

These are thoughtful and serious criticisms of our interpretation of the data. Making a stronger case for our interpretation would take us beyond what we are trying to accomplish in this manuscript. After hard thought, we chose to remove the sections (the line numbers referred to above) in the Representative Data section as well as in the Discussion that deal with this interpretation. The interpretations of the data are not an essential part of the work, they can be removed without affecting the rest of the article, and cannot be supported adequately (nor should they, we believe) in this format of article. We hope the reviewer will agree that this both improves and better focuses the manuscript. The interpretation of our data is best placed in a paper describing our scientific results, not one reporting the technique.

4) I think the discussion of background in lines 292-304 could be improved. I think it is a strength that the background can be modeled in the fitting equation. It is likely that other systems will have more complicated background processes, and all of these things could be included in the fitting equation (rather than subtracted later).

We agree with the reviewer and have modified the text in the discussion to include his suggestion (lines 315-7).

Minor Concerns:
1) Line 65: Whether gel assays are "the most common" depends on how cleavage events are counted and what information the investigator needs. Many studies require single molecule resolution and can't quite be directly compared. I would characterize gel assays as "common" without the superlative.

We revised lines 63-4 to remove any characterization of gel methods as "most common."

2) Line 115: I was hung up on the use of "alternation." I see the logic after reading the following step, but it is clearer in 1.1.1 to say "sonicate with EtOH and then with 1 M KOH" and specify the repeats in 1.1.2.

We have reworded that step for clarity, making use of the suggested wording. We also corrected a typo. The total number of wash cycles is 2 not 4.

3) Line 182: The concentration is a little confusing as written. Rewrite to "Prepare 480 uL of 0.5 pM labeled DNA substrate"

We have made this correction.

4) Line 186: It is unclear how "sufficient bead tethering" is assessed. I am assuming the beads should be visible, and the bead density is no longer visibly increasing. Please elaborate.

We have made several revisions to address this point. Please see our response to comment 3 by Reviewer #2.

5) Line 215: Please include the need for excluding data in the period of mixing (approximately 20 seconds). This is very important for data analysis, so it shouldn't be left to the discussion.

This is now explained in a Note after step 4.8 (lines 236-7). We have also highlighted in green the injection period in Figure 3 and inserted relevant text in Figure 3 caption explaining this.

6) Line 235: The authors must mean inches, not feet, for the slides (" instead of ').

Corrected, thank-you.

7) Figure 1: Please add text labels to the components on the figure itself.

We have added labels as requested.

8) Figure 3A-B: I think further zoomed-in panels would help. It is quite difficult to see the spots on printouts.

We have revised the figure as requested.

9) Figure 3C: Please highlight the mixing time segment of the plot.

The injection period is now highlighted in green in the figure and caption modified to explain this.

10) Table 1: Include the 30 cycle repeat somewhere in the table.

We were not sure how best to indicate this in the table. We do mention this in the legend for the table and we have added a column to number the steps, iterating the exact steps which are repeated in the table legend. We hope this makes it more clear.

screen capture 1

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screen capture 2

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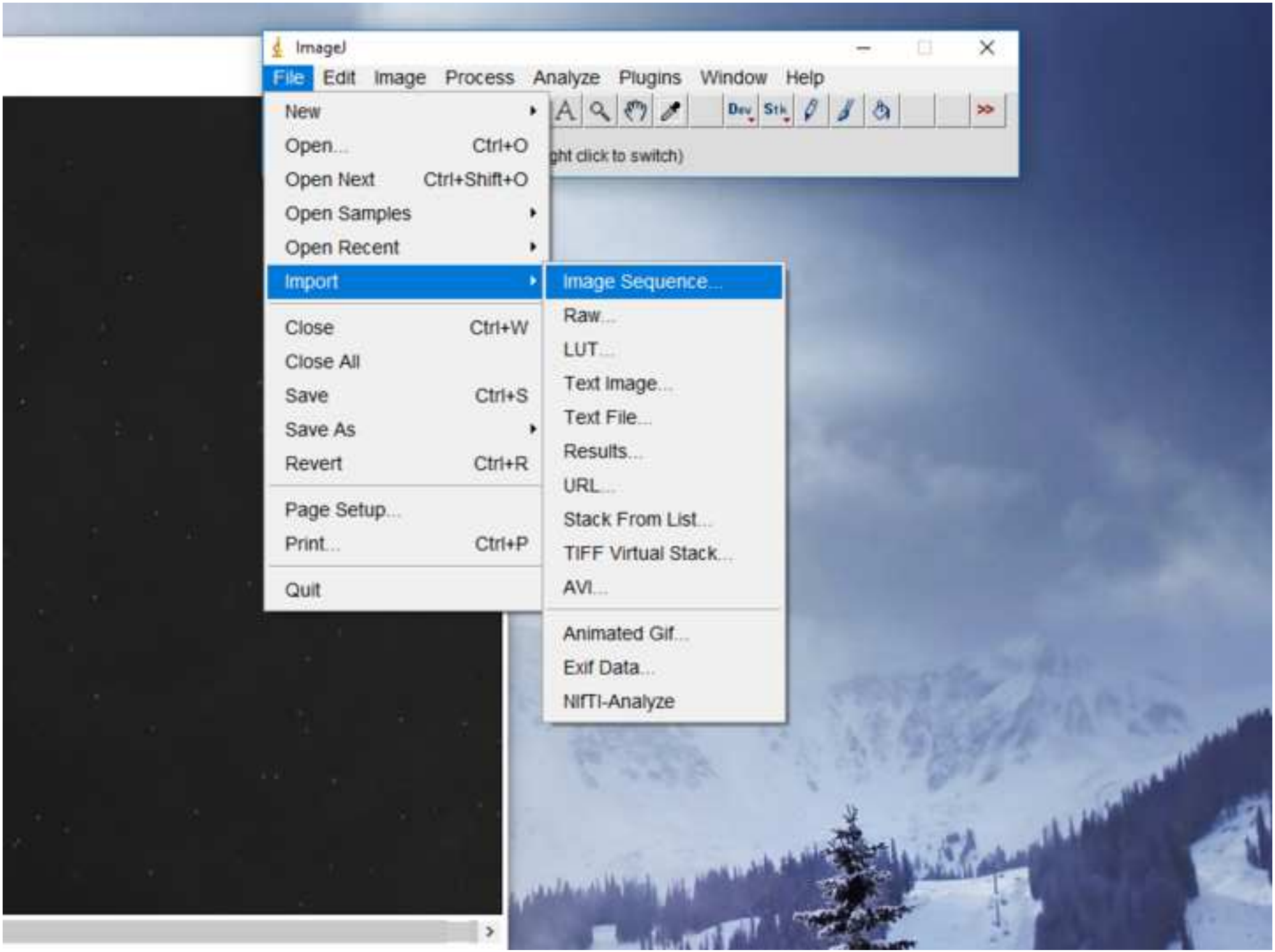

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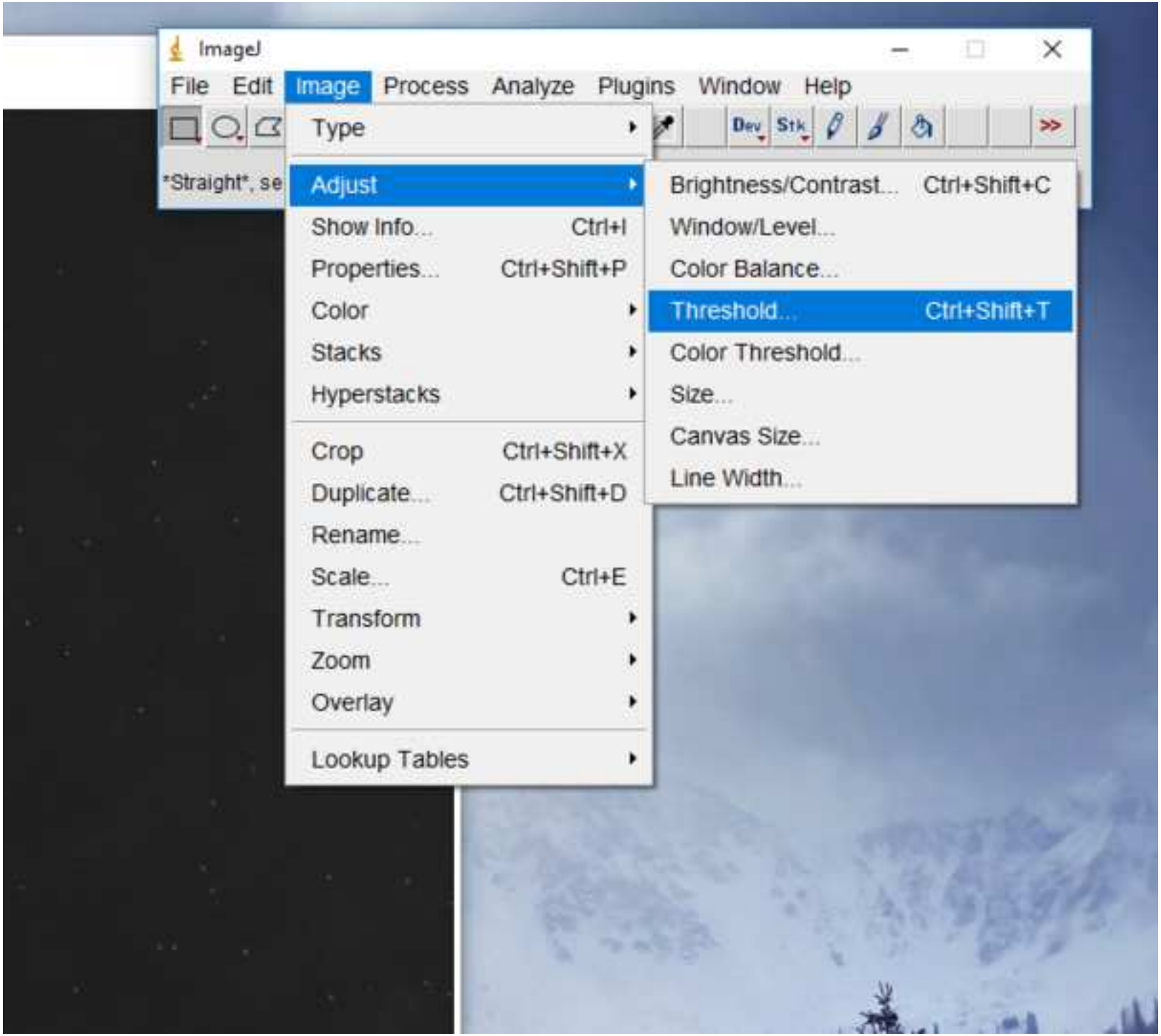

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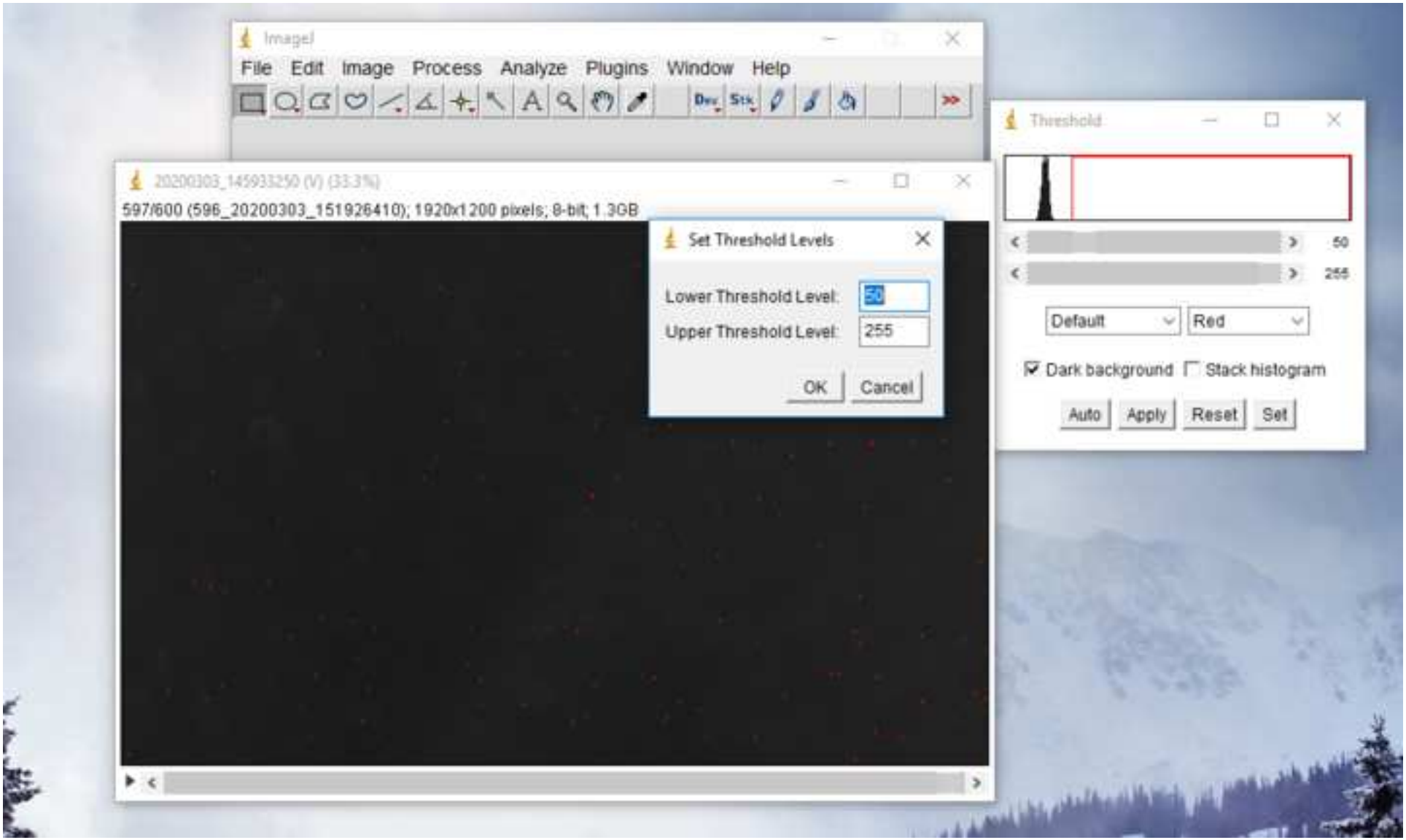

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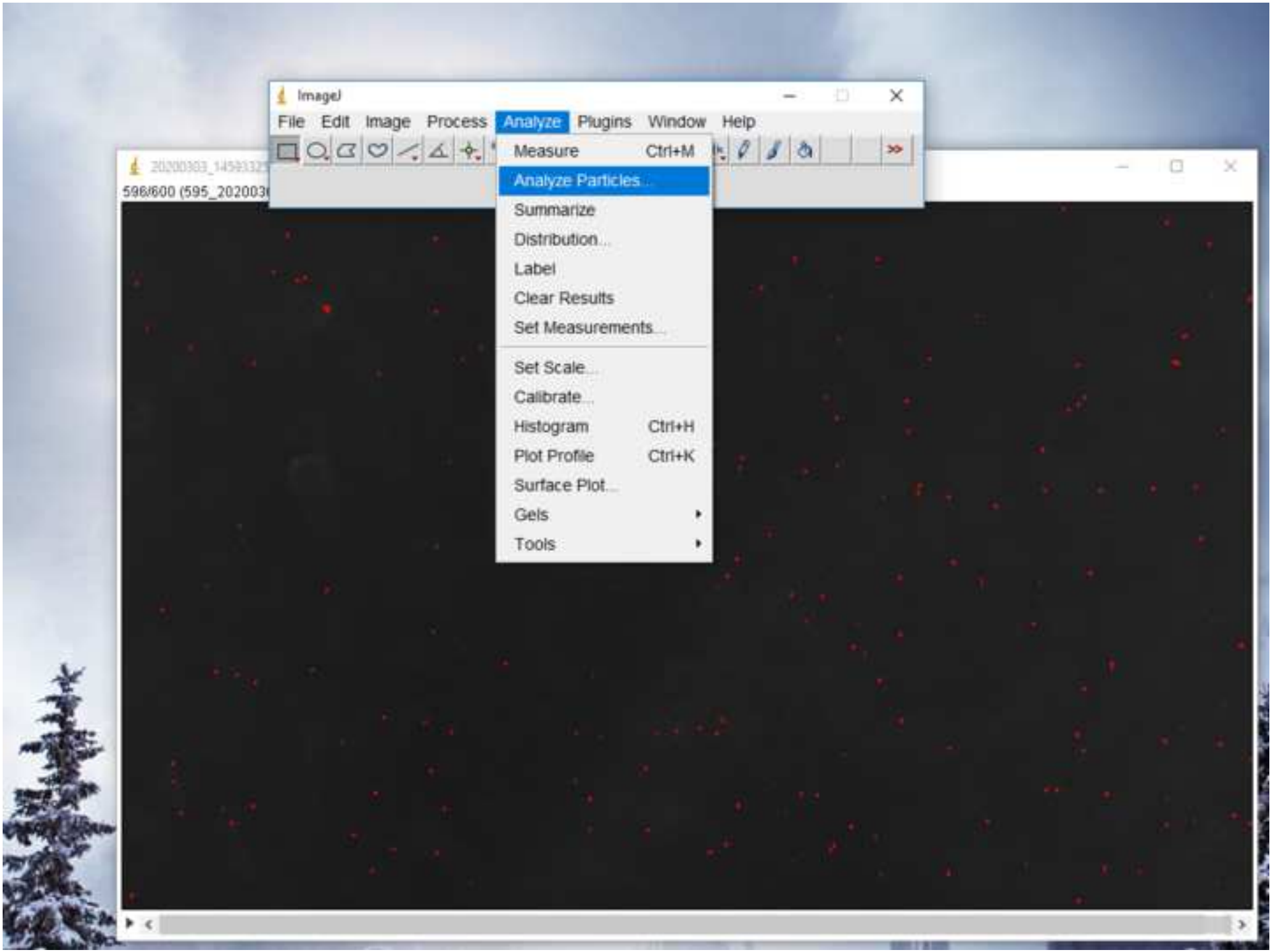

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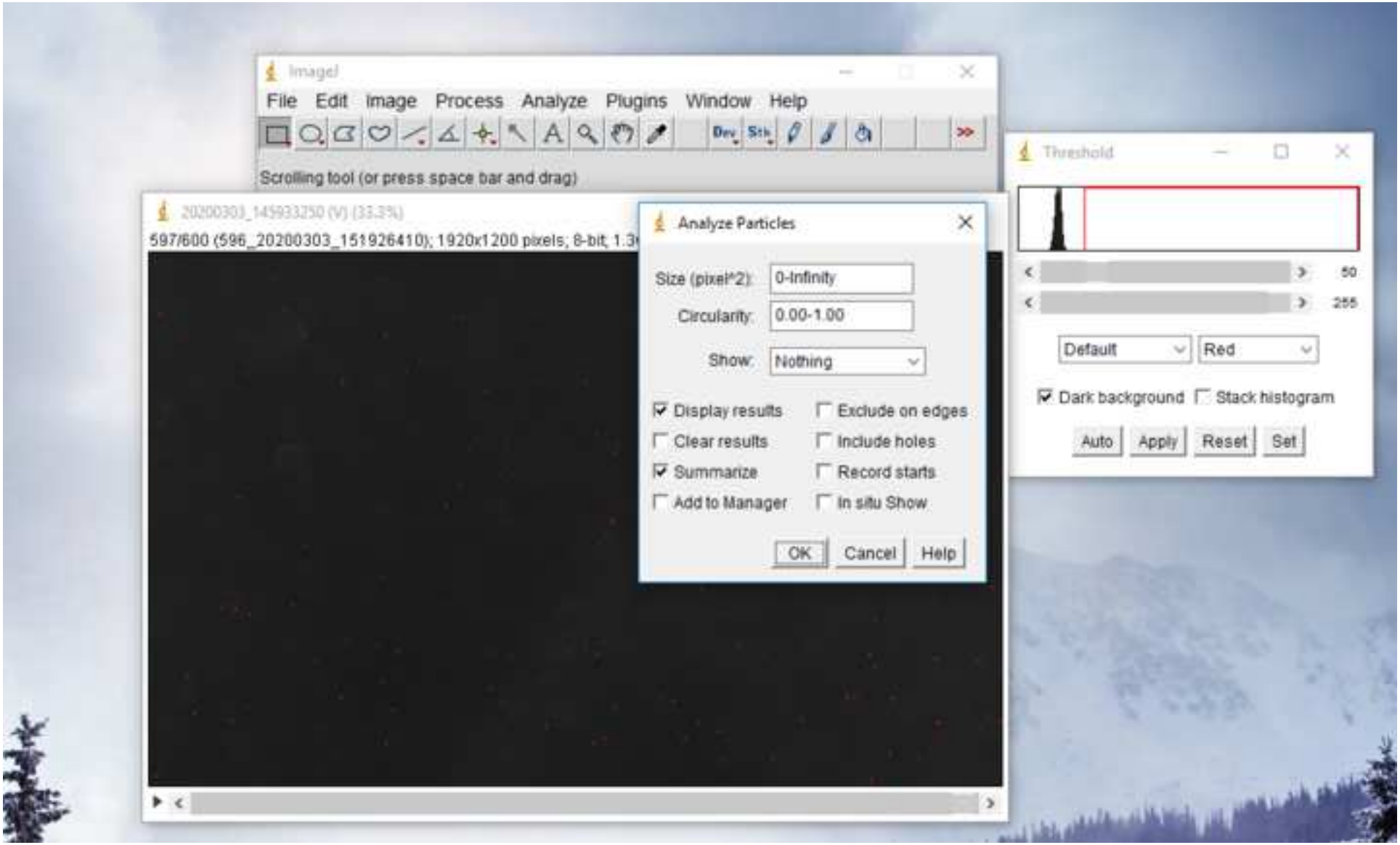

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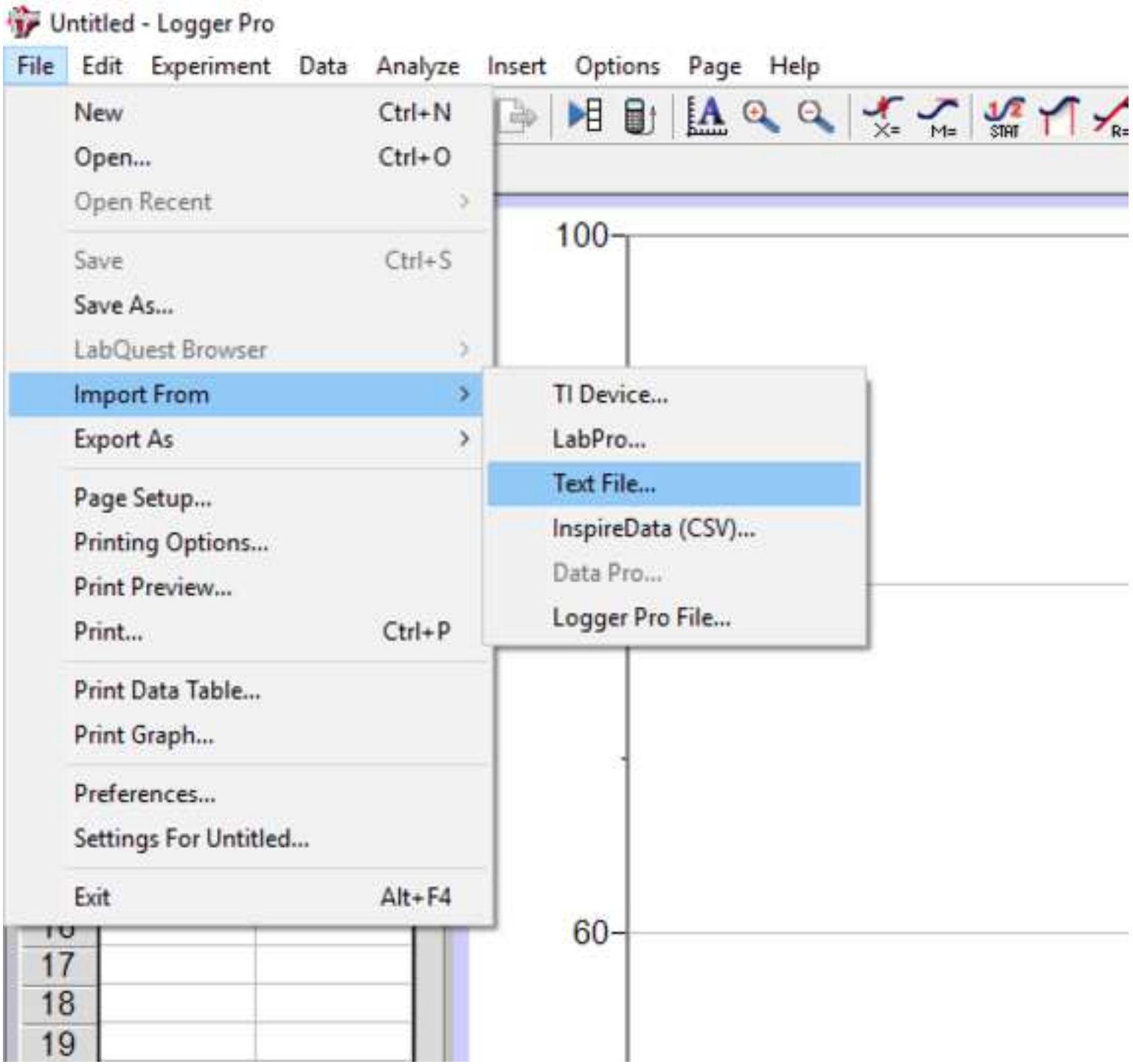

screen capture 8

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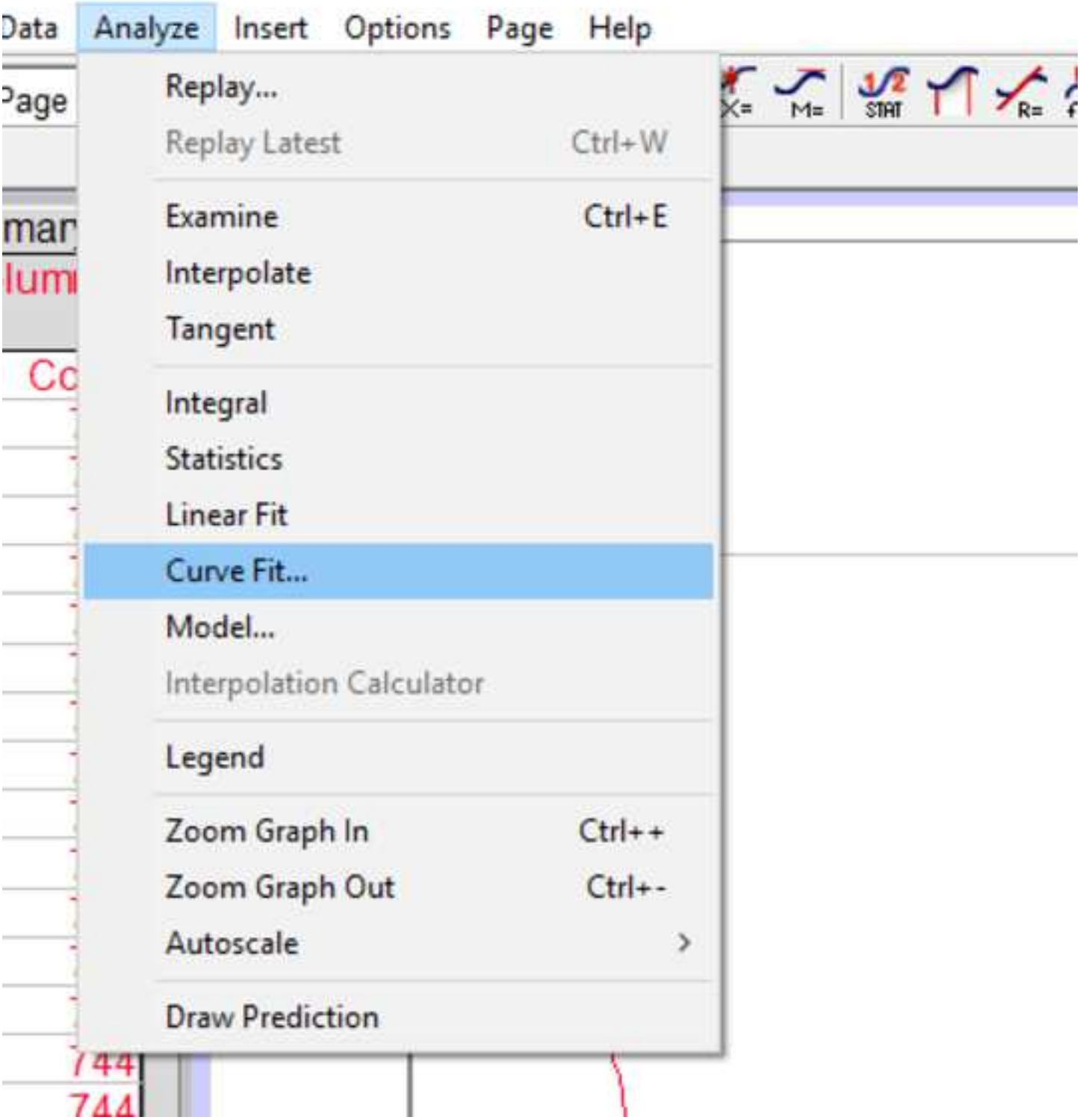

screen capture 9

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