



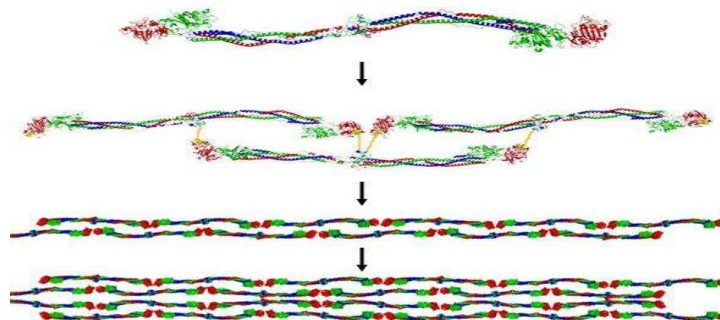
## Combined Optical/AFM Manipulation of Fibrin

In this experiment we will be investigating the mechanical properties of single fibrin strands. In our setup, fibrin fibers are suspended over channels in a “structured surface” that we fabricate here in the lab. We use the AFM-Optical Microscope set-up to observe the fibers as well as the AFM tip as it manipulates the fiber. The optical data depicting the stretching of the fiber can be captured as a movie, while the AFM simultaneously records forces. Both the strain data (pictures/movies showing the stretched length) and the force data are quantitative, and provide independent mechanical information about the fibrin. Together, the two data sets provide the ability to construct a stress-strain relation from which mechanical parameters such as Young’s Modulus, yield strength, tensile strength, and toughness can be derived.

### Preliminary information about Fibrin:

Blood clots form in the event of injury or damage to blood vessels to prevent the loss of blood. Unfortunately, blood clots often form in undesired locations, such as in blood vessels around the heart or brain, resulting in heart attacks or strokes. Fibrin fibers, the skeleton of a blood clot, perform the mechanical task of creating a blockage that stems blood flow.

Fibrinogen is a 340 KDa protein that is made up of two identical halves. In it, there are three peptide chains that are held together by disulfide bonds in trinodular structure with dimensions of about 45nm in length and 4.5 nm in diameter[1-4]. An activator, thrombin, proteolytically cleaves the fibrinogen leaving binding sites open at the end domains. These fibrin monomers assemble into two stranded protofibrils that are thought to aggregate laterally to form fibers. These, along with platelets and other components, make up a blood clot. We have recently discovered that individual fibrin fibers can be stretched to extraordinarily high strain (up to 5 fold extension before breaking) [5]. How the fibrin fibers accommodate such high strain is not completely understood. The experiments outlined here mirror our own studies that are aimed at teasing out the molecular origins of fibrin’s extraordinary extensibility.





## Procedure

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### **Sample Prep A: Structured Surfaces**

Our sample prep procedure is designed to best facilitate clean stretching experiments. We remove the effect of fibrin/surface friction and adhesion effects by suspending the fibers across micron sized channels. Microcontact printing is used to pattern ridges and channels on a glass cover slip. Details on how these “structured surfaces” are created can be found in **Appendix B**.

### **Sample Prep B: Fibrin prep and Labeling**

In this experiment we will be using fibers prepared from human plasma fibrinogen. We are able to produce samples with individual fibers suspended across channels by using fibrinogen concentrations well below physiological levels. Once clots are formed directly on the structured surfaces, the sample is rinsed with buffer and treated with fluorescent beads. Specific details of the fibrin polymerization and labeling procedures can be best answered by Tim O’Brien or Nathan Hudson.

### **Experiment A: Strain to breaking**

1. After fibrin sample has been inspected to determine quality, place AFM head on the sample. Since the AFM cantilever must be aligned with the objective, you should wait until after you have accomplished the AFM/objective alignment before choosing your fiber.
2. Once fiber is selected use the nM Direct Step (DS) to position the tip off to the side of the fiber.
3. Using DS set the z position to maximum (~12,000 nm). Then bring down in steps of 1000nm until it appears that the end of the tip is at a similar height as the fiber.
4. Using DS, move in x (or y depending on the fiber orientation) across the fiber in steps of 100-200 nm to determine if the end of the tip is low enough to contact the fiber.
5. If the fiber is contacted, move back in x (or y) and raise the tip in z by 200nm. If the fiber is not contacted, lower the tip by 200nm. Repeat. When you are reasonably sure you are contacting the fiber within 200-300nm of the end of the tip, you are ready to stretch. **Important:** when you have found the correct height for the tip, record the x (or y) position of the tip at contact (the x or y position at the very initiation of stretching). This will be essential for accurate analysis later.
6. Place the tip slightly off to the side of the fiber (~500nm).
7. Set the x step (or y) to 25-50nm per step. Select “keep stepping”. Note that for any direction you select now, the steps will be repeated until you de-select direct step.
8. Press the x (or y) arrow and the tip will move slowly toward the fiber, contact it and begin stretching
9. Let the tip stretch the fiber until: a. the fiber breaks b. the fiber slips off the tip and relaxes elastically or c. other (peeling from the side, inelastic deformation).





10. If in step 9, “a” occurs, you are done. Save movie of stretch and evaluate strain at breaking. See analysis section.
11. If in step “b” occurs, repeat the stretch. If “c” occurs, raise the tip, and select a new fiber.

### ***Experiment B: Local strain***

1. Make a standard fibrin sample on the structured surface and label at very low fluorescent bead concentration.
2. Inspect the sample and select a fiber that has prominent (bright) isolated labeled areas on the fiber at several places along its length.
3. Take movies of standard stretching experiment as described in Experiment A.
4. Bring movie up in Spot Tracker software and record trajectories of at least two spots along any straight segment of the fiber.
5. Analyze trajectories to determine local strain between points along the fiber. See Analysis section for instructions on local strain analysis.

### ***Experiment C: in situ methanol buffer exchange***

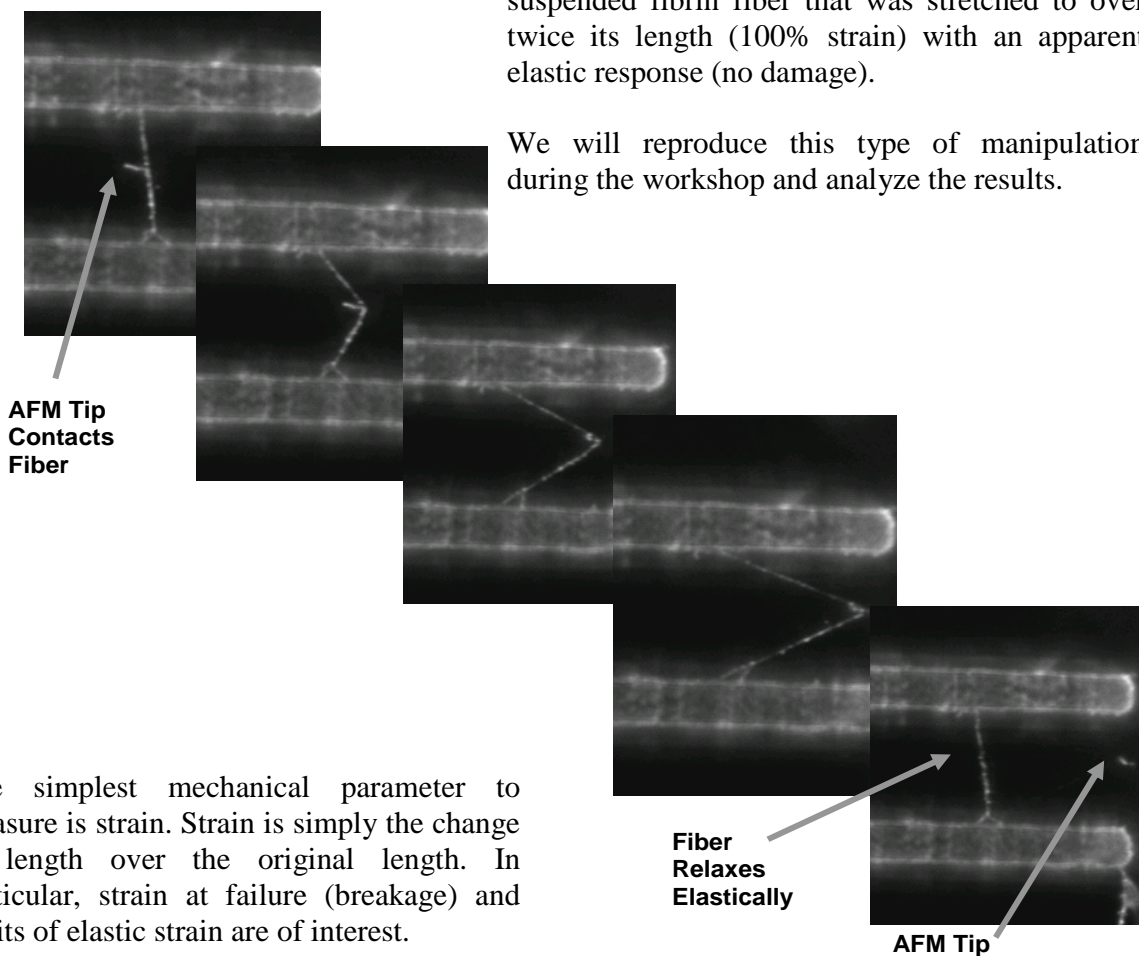
1. Make a standard fibrin sample on a structured surface and identify a region with several target fibers within a relatively small area (such that you can move between them without losing your place. You will want to be able to revisit each fiber you stretch)
2. For each of the targeted fibers, stretch to roughly 100% strain. Ideally you will be stretching the fiber reversibly. If so (if the fiber does not break and you do not see signs of damage), repeat several times. Record the force vs. extension curves for each of the fibers. You should stretch far enough to achieve good signal to noise on your force trace.
3. Carefully remove the AFM from the optical microscope stage. You must be careful not to disturb the position of the stage so that you will be able to maintain alignment of the AFM, objective and sample.
4. Add exchange the normal experimental buffer with methanol by wicking of the buffer and adding 20 $\mu$ L of methanol.
5. Carefully place AFM back on the optical microscope and verify alignment. You will want to stretch the same fibers as in step 2.
6. Repeat stretching experiment performed in step 2. Record force vs. extension curves and for each fiber, and compare to the pre-methanol data.





## Analysis

### Global Strain Measurement



This sequence depicts a manipulation of a suspended fibrin fiber that was stretched to over twice its length (100% strain) with an apparent elastic response (no damage).

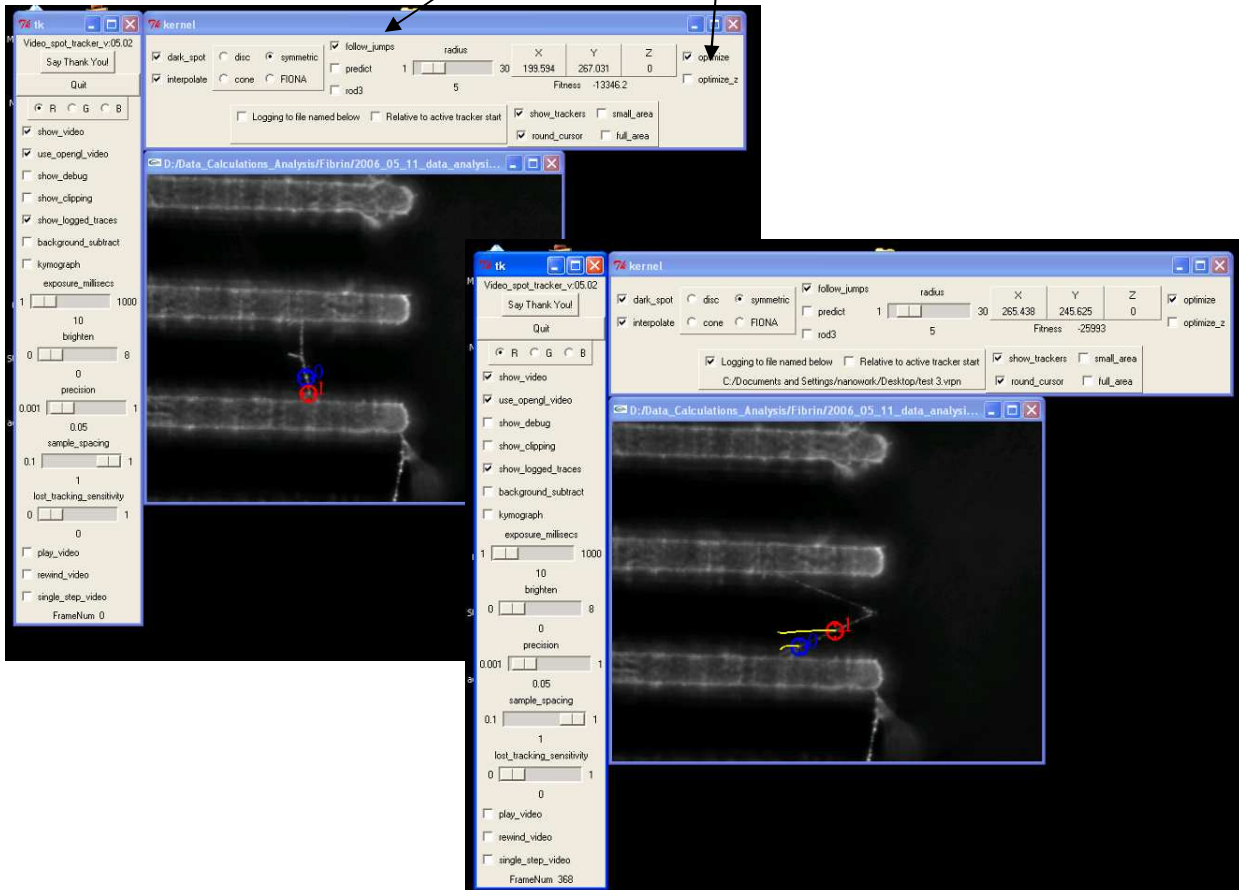
We will reproduce this type of manipulation during the workshop and analyze the results.

The simplest mechanical parameter to measure is strain. Strain is simply the change in length over the original length. In particular, strain at failure (breakage) and limits of elastic strain are of interest.



## Local Strain measurement

1. Open up Spot Tracker Software
2. Load movie of fibrin manipulation
3. Within kernel panel, select “follow jumps” and “optimize”.



4. Click on 2 or more spots on suspended fiber.
5. Select “logging to file named below” and name the file into which the tracking data will be saved.
6. X&Y tracking data for each spot will be saved into a excel spread sheet.





## Appendices

### Appendix A: AFM Force Calibration for Veeco Explorer / nM

The raw force data collected during AFM force measurements comes from the quadrant photodiode (QPD) and is in units unrelated to force (nA or V depending on the microscope). These raw signals are the “top minus bottom signal” or (T-B) (also called the “internal sensor” signal in the Topometrix and nanoManipulator software) which is proportional to the bending deflection of the cantilever and the “left minus right signal” or (L-R) (also known as the “lateral force” signal) which is proportional to the twisting deflection of the cantilever. Calibration of AFM force data requires determination of the cantilever spring constant ( $k$ ) and the optical lever sensitivity ( $S$ ) of the system. Normal force (cantilever bending) and lateral force (twisting) have separate calibrations with different spring constants and optical lever sensitivities.

The AFM cantilever can be thought of as a spring with a bending and twisting mode. Both modes have a characteristic spring constant (they can be thought of as independent springs). In order to determine forces using AFM, we simply use Hooke’s Law which relates the deflection of a spring from its equilibrium position to the applied force, using a proportionality coefficient called the spring constant.

$$(1) \quad F_N = k_N \Delta z \quad (2) \quad F_L = k_L \Delta x$$

Where  $F_N$  is the normal force or bending force,  $k_N$  is the normal or bending spring constant,  $\Delta z$  is the vertical or bending deflection of the cantilever tip,  $F_L$  is the lateral or twisting force,  $k_L$  is the lateral or twisting spring constant and  $\Delta x$  is the lateral or twisting deflection of the end of the AFM cantilever tip (See Figure 1). Instrumentally,  $\Delta z$  and  $\Delta x$  are not measured directly but are determined by calibrating the raw (T-B) and (L-R) signals with their respective optical lever sensitivity  $S$  (eqns. (3a) and (4a)). Substituting eqns (3a) and (4a) into eqns. (1) and (2), we get the normal and lateral AFM forces in terms of the raw QPD signals and the cantilever force constant (eqns. (3b) & (4b)).

$$(3a) \quad \Delta z = \frac{\Delta(T - B)}{S_{T-B}} \quad (3b) \quad F_N = k_N \frac{\Delta(T - B)}{S_{T-B}}$$

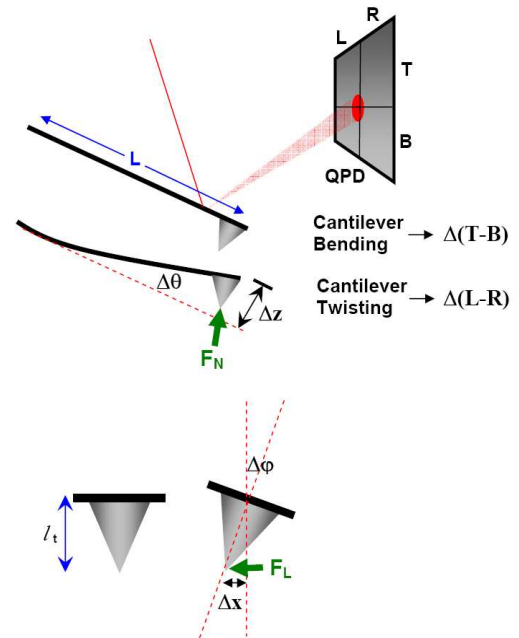


Figure 1.



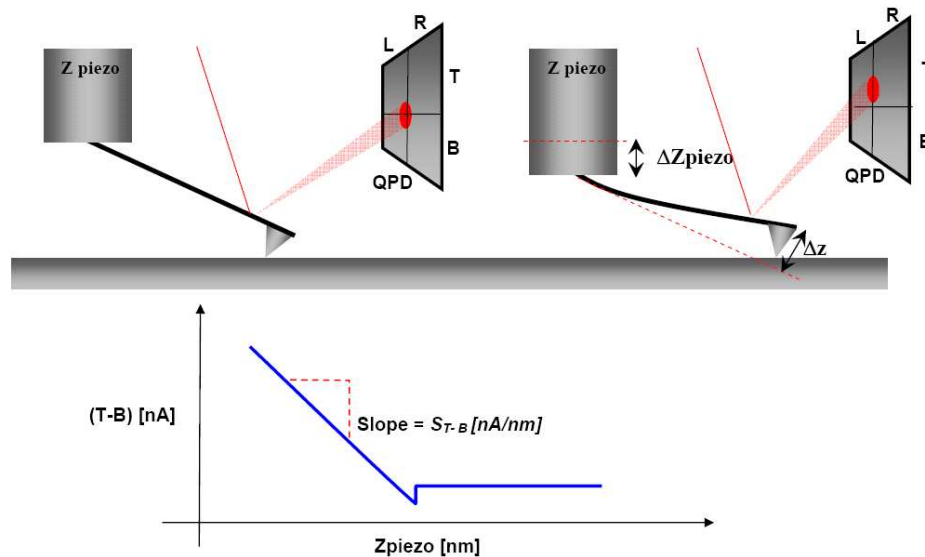


$$(4a) \quad \Delta x = \frac{\Delta(L-R)}{S_{L-R}} \quad (4b) \quad F_L = k_t \frac{\Delta(L-R)}{S_{L-R}}$$

### **Determination of Normal Force Optical Lever Sensitivity**

$S_{T-B}$  is determined readily by taking a force vs. distance curve or “force curve”. The AFM tip is brought in contact with a hard surface. We make the assumption in determining the sensitivity that the surface is “infinitely hard”. This is a reasonable assumption if the effective spring constant of the surface is much higher than the spring constant of the cantilever. For most substrates (glass, silicon, mica) this assumption is valid even for high spring constant cantilevers. This assumption is necessary because we are going to relate the movement of the the piezo (which we know) to the movement of AFM tip end (which we do not know). With the tip in contact with the surface, the z-piezo is actuated pushing the tip down onto the sample and causing a deflection of the cantilever ( $\Delta z$ ). We monitor the (T-B) signal and plot it against the movement of the z-piezo. The slope of this “force curve” is the optical lever sensitivity for the bending mode,  $S_{T-B}$ , in units of  $[nA/nm]$ . The optical lever sensitivity is dependent on the position of the laser on the back of the cantilever as well as the amount reflected laser light. Therefore, the sensitivity needs to be determined for every experiment and re-determined for any change in the laser alignment (including a change in sum signal) during an experiment. Ideally for a given set of measurements, you want to try to avoid changing laser alignment.





**Figure 2.**

Note: angle of cantilever with respect to surface is exaggerated (typically  $<5$  degrees). The approximation that  $\Delta Z_{\text{piezo}}$  equals  $\Delta z$  is accurate to within 1%.

### **Determination of Lateral Force Optical Lever Sensitivity**

Unfortunately, there is no straightforward method of *directly* determining the lateral force optical lever sensitivity,  $S_{T-B}$ . We therefore use a geometrical argument to obtain  $S_{L-R}$  by relating it to the readily determined  $S_{T-B}$ .

### **Angular Optical Lever Sensitivity (AOLS)**

Strictly speaking, the change in the QPD signal is due to a change in the local slope or angle of the cantilever at the point where the laser reflects. This angle local slope or angle,  $\theta$ , is a measure of the bending of the cantilever and is linearly related to the deflection of the end of the tip (in nm) (see figure 1). So, a change in **(T-B)** is a measure of  $\Delta\theta$ :

$$(5) \quad \Delta(T - B) = C_{T-B} \Delta\theta$$

Where  $C_{T-B}$  is proportionality constant relating the (T-B) signal to the bend angle in units of [nA/rad]. The twisting of the cantilever ( $\phi$ ) is measured by a change in the “Left minus Right” signal **(L-R)**.

$$(6) \quad \Delta(L - R) = C_{L-R} \Delta\phi$$

Where  $C_{L-R}$  is proportionality constant relating the (L-R) signal to the twist angle in units of [nA/rad].





We'll call  $C_{T-B}$ ,  $C_{L-R}$  the “*Angular optical lever sensitivity*” for the bending and twisting modes respectively.

## Optical Lever Sensitivity

Since we are interested not so much in the angular deflection of the cantilever but rather the deflection of the tip end in linear units (nanometers), we need to relate the Angular Optical Lever Sensitivity to the Linear Optical Lever Sensitivity (*LOLS*). From here on out, we will when using the term Optical Lever Sensitivity (*OLS*) we will mean *LOLS*. The *OLS* will be in units of [nA/nm]. We will give *OLS* the symbol  $S$  for our calculations.

We want sensitivity values  $S_{T-B}$  and  $S_{L-R}$  that relate our QPD signal to our actual deflection in nm.

$$(7) \quad \Delta(T - B) = S_{T-B} \Delta z \quad (8) \quad \Delta(L - R) = S_{L-R} \Delta x$$

When a beam clamped at one end is deflected a distance  $\Delta z$  in bending or a distance  $\Delta x$  in twisting by a point force as depicted in Figure 1, the geometrical relationships between the deflection angle (at the end of the cantilever) and deflection distances are as follows

$$(9) \quad \Delta \theta (L) = \frac{3}{2} \frac{\Delta z}{L} \quad (10) \quad \Delta \phi = \frac{\Delta x}{l_t}$$

We now substitute the angular terms with linear terms and can now express the

$$(11) \quad \Delta(T - B) = \left(\frac{3}{2L} C_{T-B}\right) \Delta z. \quad (12) \quad \Delta(L - R) = \left(\frac{C_{L-R}}{l_t}\right) \Delta x$$

where

$$(13) \quad S_{T-B} = \frac{3}{2L} C_{T-B} \quad (14) \quad S_{L-R} = \frac{C_{L-R}}{l_t}$$

The reason we have gone through all of this trouble is to relate the lateral force optical lever sensitivity which we can not directly measure (easily) to the readily obtainable normal force optical lever sensitivity which we can find simply by taking a force curve as described above. We're not there yet. With equations (9) and (10) we have 2 equations and 3 unknowns. We know the dimensions of the cantilever  $L$  and  $l_t$ , and the normal force optical lever sensitivity  $S_{T-B}$ . We do not know either angular optical lever sensitivity,  $C_{T-B}$  or  $C_{L-R}$  (which we cannot measure directly), and we're trying to find  $S_{L-R}$ . Our final assumption relates  $C_{T-B}$  and  $C_{L-R}$ . If our QPD is symmetric, that is all four quadrants are equal in size and shape and have similar sensitivities, then we expect that the change in signal for a given laser spot motion along one axis of the diode will create same change in signal as motion along the perpendicular axis. We then can assume that the change of signal ( $\Delta(T-B)$ ) for a change in  $\theta$  should be equal to the change in signal ( $\Delta(L-R)$ ) for the same angular change  $\phi$ . This means the angular sensitivities are the same.





$$(15) C_{L-R} = C_{T-B}$$

Substituting (11) into (9) and (10) and rearranging, we now have the lateral force optical lever sensitivity  $S_{L-R}$  in terms of the normal force optical lever sensitivity  $S_{T-B}$ . With our approximations, they are related simply through cantilever geometrical parameters  $L$  and  $l_t$ .

$$(16) S_{L-R} = S_{T-B} \frac{2L}{3l_t}$$

**Spring constants.**

For an isotropic, homogeneous material, the following beam bending equations relate the bending and twisting spring constants to the geometry and moduli of the cantilever.

$$(17) k_L = \frac{Gwt^3}{3Ll_t^2}$$

$$(18) k_N = \frac{Ewt^3}{4L^3}$$

$G$  and  $E$  are the shear modulus and Young's modulus of the cantilever respectively. The geometrical parameters shown are the width of the cantilever,  $w$ , the thickness of the cantilever,  $t$ , the length of the cantilever,  $L$ , and the length of the tip,  $l_t$ .

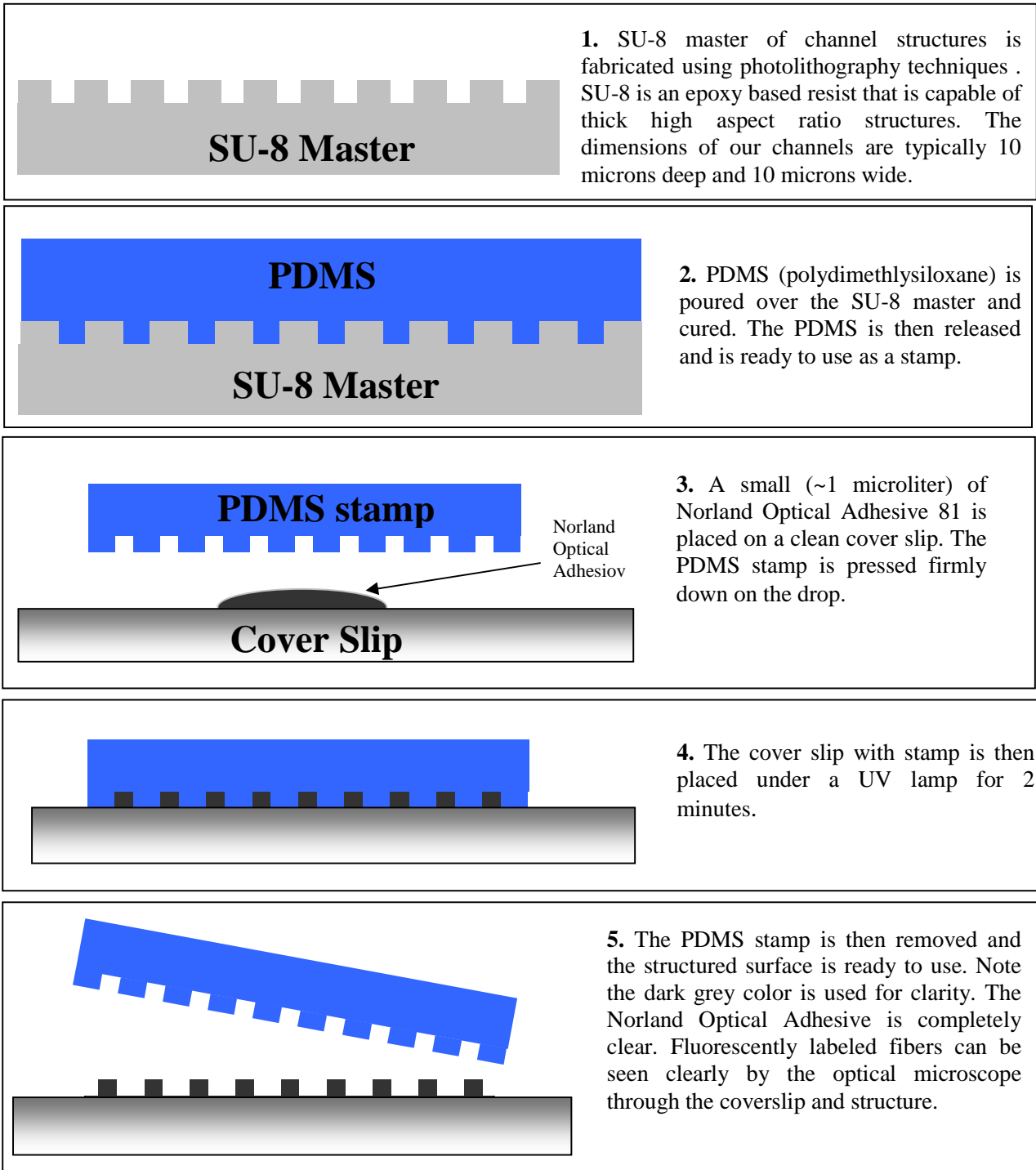
The calibrated force for both normal force and lateral force data can now be obtained using equations 3b and 4b, with the raw data ( $T-B$ ) & ( $L-R$ ) modified by the obtained optical lever sensitivity and spring constant.

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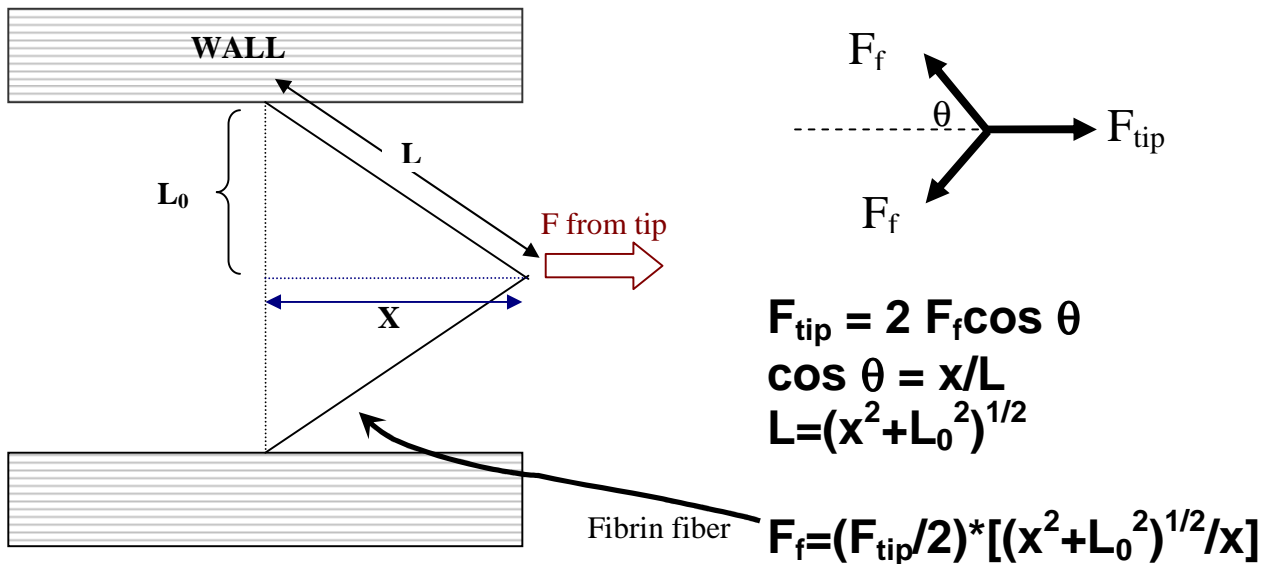
**Appendix B: Fabrication of Structured Surfaces**





Appendix C: Force Analysis

Diagram of Stretched Suspended Fiber



**$L_0$ :** This is the initial (unstrained) half-length of the fibrin fiber as recorded by the optical microscope.

**Extended Length  $L$  :** This is the length of the each half of the fiber during the manipulation.

$$L = (x^2 + L_0^2)^{1/2}$$

**Extension or strain ( $\epsilon$ ):** This is the strain

$$\epsilon = (L_0 - L) / L_0.$$

**Force applied to Fiber  $F_f$ :** The lateral force as measured by the AFM is related to the force being applied to the fiber as indicated in the diagram above. The raw data that is recorded during a manipulation includes the lateral distance the tip travels,  $x$ , as well as the raw uncalibrated lateral force  $F_{tip}$ .





## References

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