Introduction to Mechanobiology
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Week – 02
Lecture – 09
Atomic Force Microscopy (AFM)

Hello and welcome to our ninth lecture of the NPTEL course; introduction to mechanobiology. So, in the last lecture we had concluded our discussion on rheological behaviour of ECM networks taking the example of collagen and we discussed that one of the main properties of these networks is this property of strength stiffening or make or the network becoming stiff if you exert force on it. And I argued that this property ensures that tissues do not fail or networks do not fracture when exposed to forces. And then I said that similar behaviour also exists at the level of a single protein and in that regard we started our discussion on this protein called fibronectin right. So, fibronectin is a dimmer and it binds to multi, it is a ECM protein it binds to other ECM proteins including collagen proteoglycans.

And it plays very important roles in cell adhesion wound healing, inflammation, so on and so forth. So, what we want to know is to, how the structure of fibronectin is necessary for its function and particularly how forces regulate functioning of fibronectin through unfolding of its individual domains. So, let me once again introduce the structure of fibronectin. So, you have three modules.

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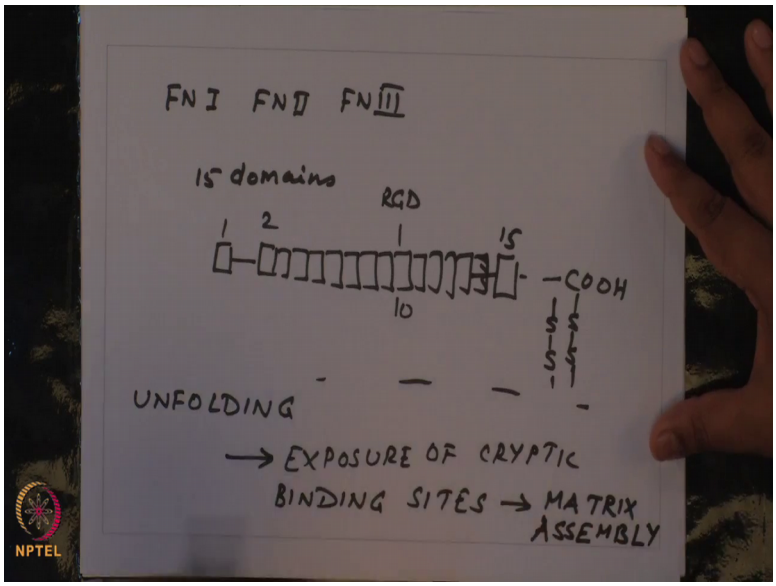

FN 1, FN 2 and FN 3 and these structures are these modules are repeated. So, for example, one intact fibronectin molecule it probably have 28 repeats of FN 3.

So, FN 3 has 15 domains. So, FN 3 is the structure of FN 3 is roughly like this, you have 1 and 2; first and second domain are connected by a linker and then you have these 4, 5, 6, 7, 8, 9, 10. So, in 10 you have the RGD sequence which is important for mediating cell addition and RGD binds to integrins. So, you have these 15 domains actually 14 and 15 are also connected by a linker. So, this is my 15 and towards the end you have your COOH and the disulfide bridges. So, this is the how fibronectin is.

Now it is hypothesized that mechanical stretch actually induces unfolding of these modules or individual domains of it. And exposure of cryptic binding sites by unfolding triggers binding of other molecules which, drives not only matrix assembly as well as regulates cell signally. So, you have unfolding, leading to exposure of cryptic binding sites and then this regulates matrix assembly. So, typically calorimetry has been used for testing the sensitivities of these individual domains, to increasing temperature and these studies have revealed, for example.

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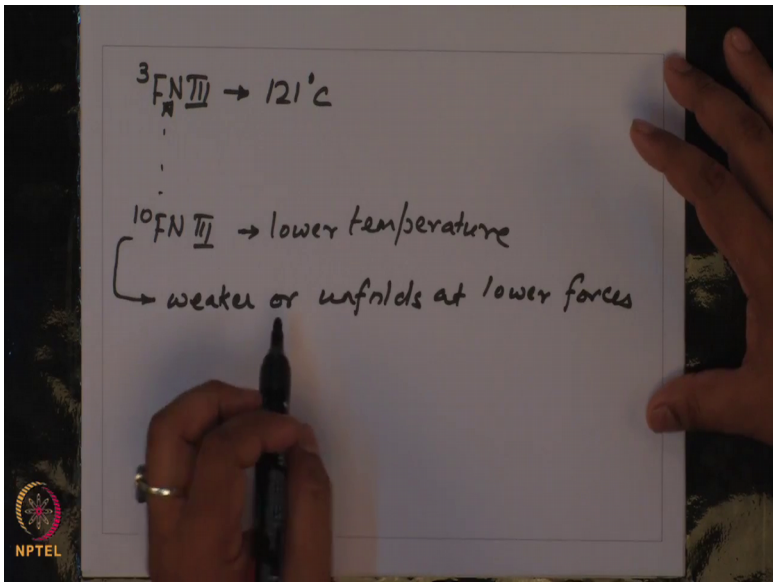

So, third domain of FN 3 unfolds at 120 three degree Celsius so on and so forth.

And tenth domain of FN 3, it unfolds at a lower temperature. So, these would suggest that FN 10 is weaker or unfolds at lower forces. So, because it gets denatured at the lower temperature you can expect it to unfold in vivo at lower forces. So, how would you test this? And this is where the method of AFM which I briefly touched upon yesterday would come in.

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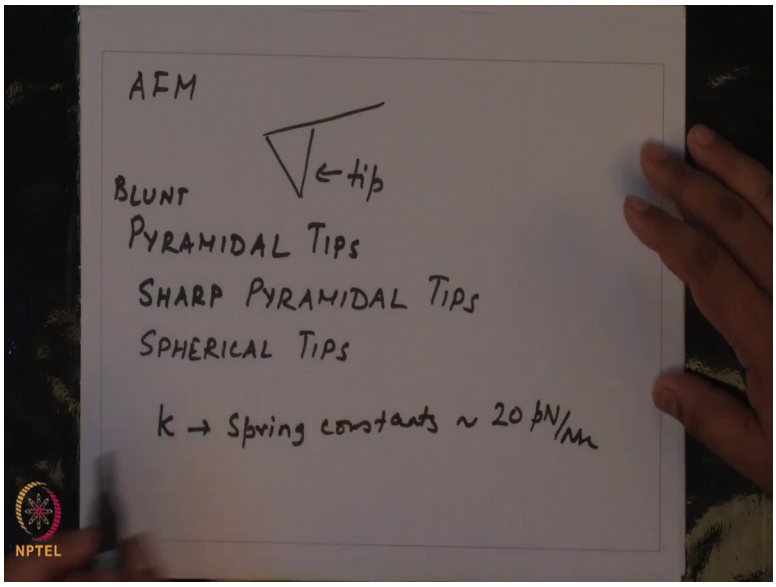

So, in AFM just to summarize, what we had discussed in last class; you have this AFM cantilever. So, this is called the tip you can have various geometries, various tip geometries including more often these blunt pyramidal tips are used. So, you can have pyramidal tips sharp tips. So, or rather, so I will put it as blunt and sharp pyramidal tips as well as spherical tips. So, the stiffness of these tips is dictated by its dimensions. So, for protein unfolding studies you would want to work with tips which have spring constants.

So, spring constants of the tip order 20 Pico Newton per nanometer. So, one more thing I had mentioned in a last lecture was when you want to study protein unfolding. So, you have a substrate.

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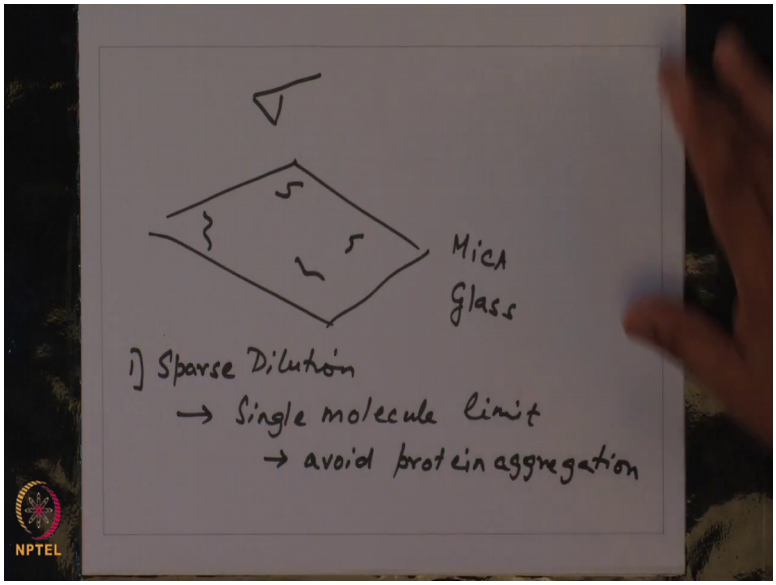

The substrate might be mica or glass and you have let us say these protein molecules are passively adsorbed onto the surface. Now one thing to note is you want to operate at the sparse dilution or rather almost at the single molecule limit, as a single molecule limit so that you can avoid protein aggregation.

So, I also mentioned that on a surface what you do with the tip.

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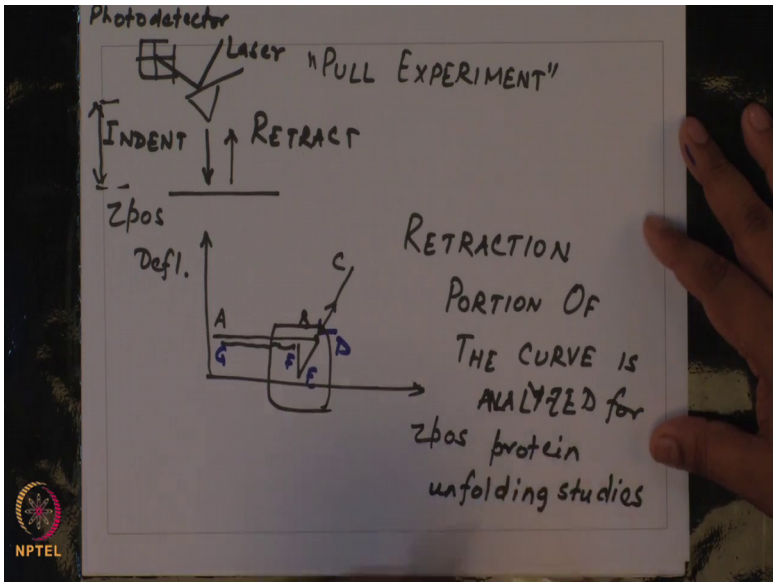

So, if you have a surface whatever be the surface, you do one pull experiment. What is the pull experiment? The pull experiment is essentially, the tip goes down and it stops once it reaches a critical force and then it comes up. So, this is your indent portion and this is your retract. So, your typical force curve on any substrate or let us say on a stiff substrate. So, what you track is the Z position, Z pos in short and the deflection. So, you have a laser which reflects of the back surface of your tip and is collected by a photo detector.

So, this is a photo detector and this is your laser source. So, what you get at the end of it is a curve; Z pos versus deflection. So, your curve will look something like this. So, at point A, point B and C, then D, so A to B and F to G are sections where the tip is actually traveling through the intervening space. It is not in contact with the substrate and between point B to C the tip is actually indenting or deforming the sample. So, for protein unfolding studies we are actually interested in this portion of the curve; the retraction portion of the curve. So, the retraction portion of the curve is analyzed for protein unfolding studies.

So now, again going back to this picture.

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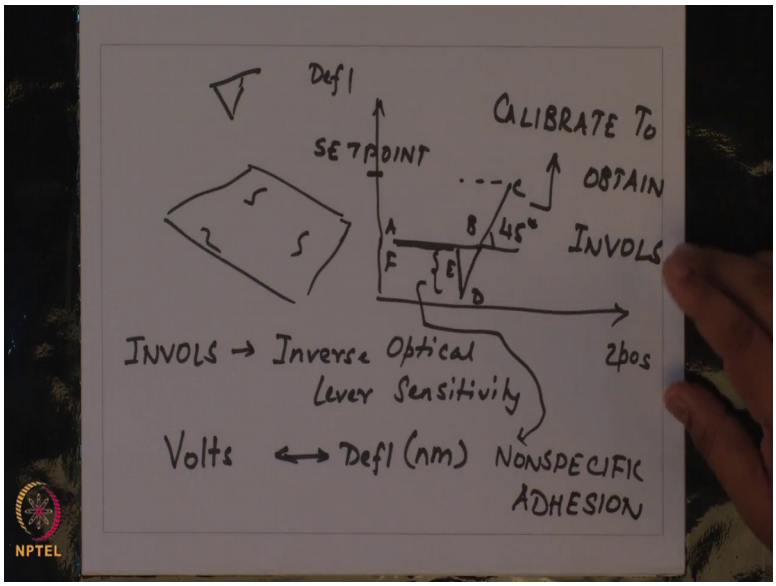

You have these proteins which are passively adsorbed onto the surface. Now you cannot visualize these proteins under the AFM, unless you do an imaging of the surface. So, proteins are below the optical detective element. So, you cannot see the proteins on the surface, that you can really position the tip on top of a protein. So, what you are doing is like a blind man, you are just tapping the surface at multiple different positions with the hope that something will come up. So, how do you know that you have actually touched the protein in one per such pulling cycle? So, let me again draw the force curve, for the case where the tip does not touch the protein you will have a curve something like this; A, B, C, D, E, F.

So, you will have a sharp peak here. So, the sharp peak here is. So, what happens when the tip is traveling through air? So, you have you are traveling to this point between A and B then it touches the surface. So, because the surface is stiffer than the tip, so when you try to go any further it is the tip itself which is getting bend. So, you have a straight line and this line should ideally have a slope of 45 degrees; why? Because the distance by which you move further is the same distance by which the tip gets deformed. So, your deflection and this is how you calibrate to obtain involves. So, involves as I said is called inverse optical lever sensitivity. So, inverse optical lever sensitivity; what is it? It essentially because the instrument is recording everything in terms of volts; what you are interested in is in deflection which is in nanometers.

So, you want to find this calibration, the conversion from volts to nanometers. And that is how it is done? Using this portion of the curve; now when after you have reached the maximum point C and as I mentioned again last class that if you keep going down and down at one point the tip will just break that you do not want to do. So, you want to constrain the maximum displacement, our maximum deflection of the tip and this is typically called the set point or the deflection set point that once the tip reaches this deflection the tip should not go down anymore rather it should just come out so that you can reuse the tip.

So, after, so it reaches this maximum point of deflection C and then it retracts right. So, the tip is now coming up. Now while it is coming up you get this huge peak in force curve between which is point D to E. What is this? This is basically when you have actually indent in the surface you have formed some nonspecific bonds. Now as the tip is trying to come off those nonspecific bonds have to break and this is that. So, this force peak or rather this peak is what is that nonspecific adhesion. So, this is what accounts for nonspecific adhesion, now imagine. So, now, imagine you get a force curve like this.

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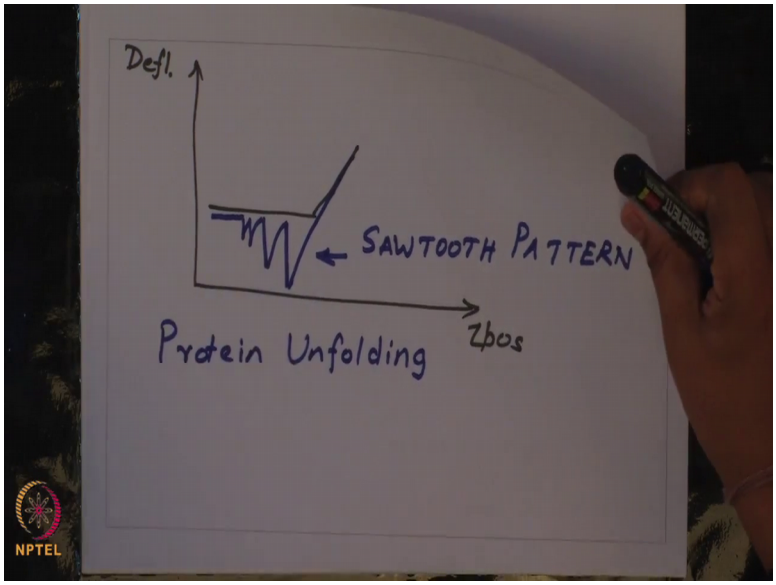

So, I will draw the return with a different colour. So, what you see on the retraction is the formation of these multiple bumps. So, this pattern is called a Sawtooth pattern and if you get this then you are convinced but this is associated with protein unfolding.

So, typically for protein unfolding studies you do not plot the data right this, but rather. So, because you are only interested in this portion of the curve, the curve is plotted in the following way.

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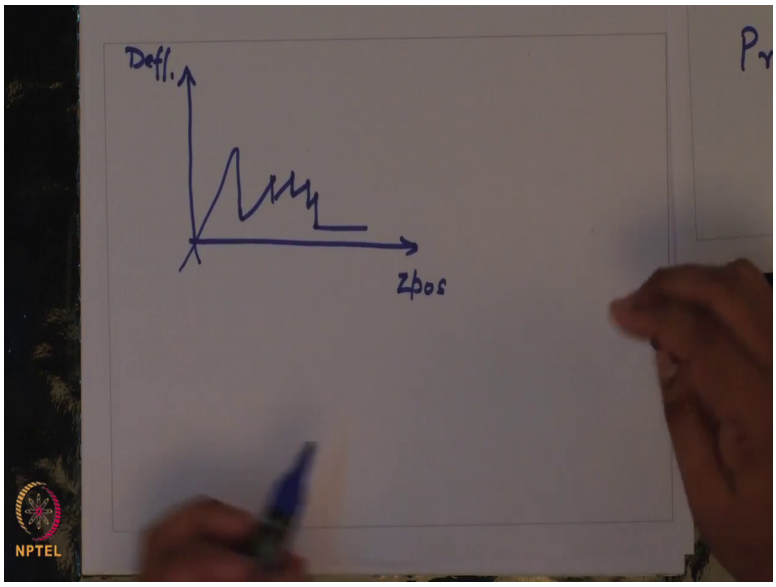

Something along these lines, so you are only. So, what you do is from this curve, you cleave this portion of the curve and you flip it upside down and you flip it. So, you do two mirror images; mirror reflections. So, if you reflect it about this axis, it will come up and then you reflect it about that axis and translate it then you will get this.

So, this is the curve that you are interested in for getting insight into protein folding. So now, let us think as to what is happening, why does it generate this particular structure? And why do we say that this is a signature associated with protein folding? So, let me approximate the protein.

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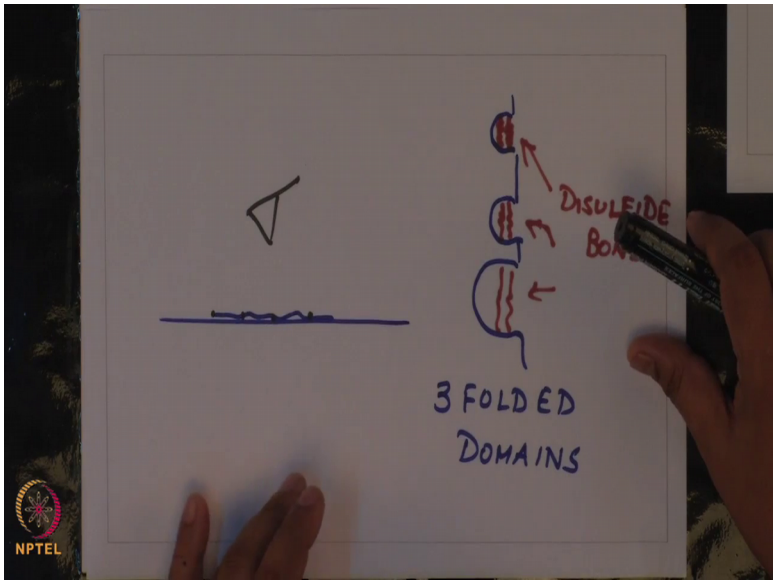

So, on the surface, there is your protein which is lying down and this is your tip. So, if I zoom in let us assume that the protein has some structure like this and these. So, these are disulfide bonds. So, what I have drawn is the imaginary protein which has three folded domains. So, this particular protein that I have drawn has three folded domains.

So, if let us assume that your tip. So, your tip hold so, where the tip touches the protein is not in your control. So, the tip might hit the protein from this point, from this point, from this point, from this point. So, you have no control where the tip actually touches the protein . So, imagine that the tip actually came in contact with the protein; at some point of the protein. So, what will happen in our previous curve?

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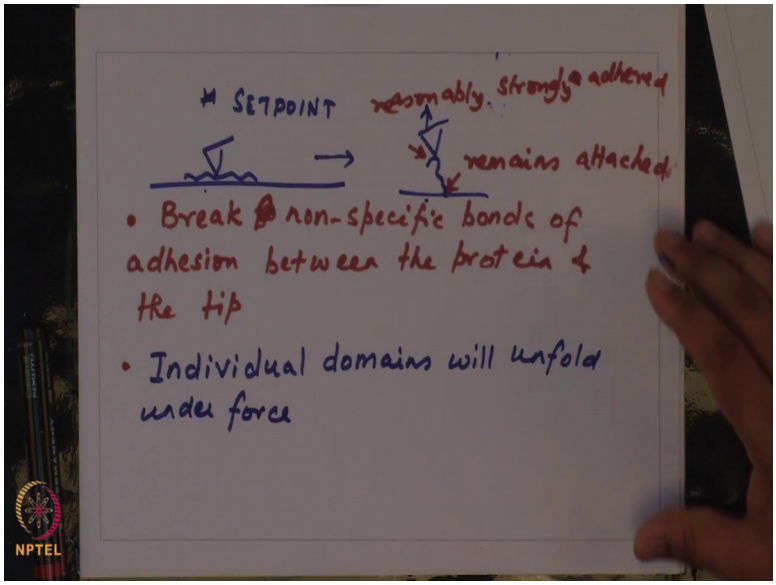

So, you have this structure, the tip has touched the protein. From here let us say so and you have reached the maximum or you have reached the set point. From here the tip has to go up. So, once the tip starts going up, you will have let us say a situation like this.

Now, because the protein was adsorbed onto the surface, for the tip to go from this point to this point you have to break; forces break the nonspecific bonds of adhesion between the protein and the tip. So, that is point one when you retract. So, as you are retracting this is step one. Step two; so when the, with the tip is going up it is actually, so you know this will work if at least this end, this end remains attached. So, if the entire protein detaches from the surface then the entire thing will come off and you will not get any signature.

So, one end of the protein must remain attached to the substrate. So, what will happen? Let us assume if one end of the protein remains attached to the surface. So, you think of a protein as a chain, this as a rubber band. So, when you try and this end is also reasonably strong adhesion, strongly adhered. If both these ends remain attached, so when you try to bring the tip up as you try to bring the tip up, so it will exert forces on your individuals disulfide bonds. So, it is like exerting a force here the tip. So, this end is anchored and your tip is trying to come off, tip has gotten attached somewhere here and the tip is trying to come off.

So, it will transmit. So, if this end is strongly attached and this end is strongly attached then force will get transmitted to each of these bonds and the force required to disulfide to break the disulfide bond will break the once your force switches that magnitude. So, what you have is the case of a, so you will have individual domains will unfold under force. So, this is the second step, so one by one.

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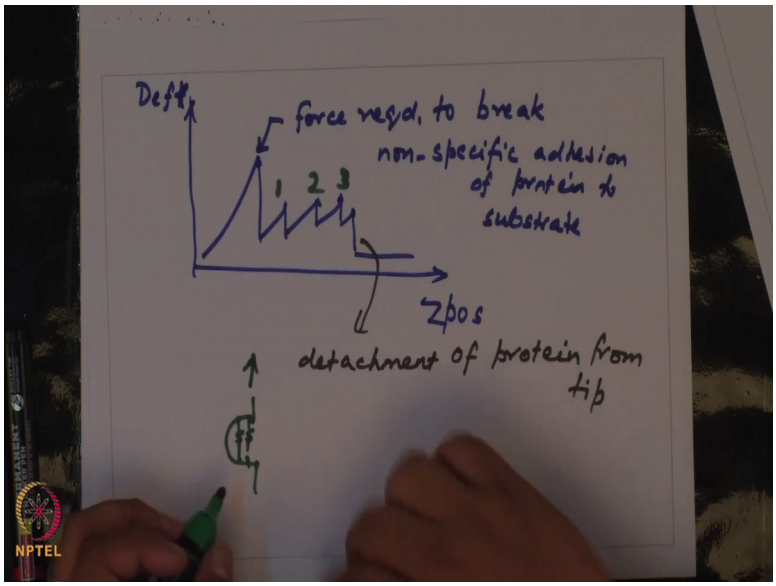

So, if I were to draw the Sawtooth profile one by one. So, this force, so I have just drawn three peaks. So, in this particular case for the you know imaginary protein with three domains you have three peaks plus two additional peaks here for one particular situation; deflection. So, this peak corresponds to force required to break nonspecific adhesion of protein to surface. The last peak, this peak corresponds to detachment of protein from tip. So, your real data is really your peaks; 1, 2, 3 is the data that you want. So, what you happen? So, if you focus on one particular peak, what you are doing is you are trying to bring the tip up, up, up. So, if you were to assume that each of these bonds. So, you are exerting this force, the bond is getting stretched, stretched, stretched. So, your force keeps on increasing and at one point once the bond breaks, there is an instantaneous drop in the force. So, that is why this drop is instantaneous.

So, this peak end corresponds to bond stretching, at this point bond breaks and then there is an instantaneous drop in force. So, if you have multiple domains. So, for the case in which you had three domains, let us assume; this is the weakest and this is the strongest.

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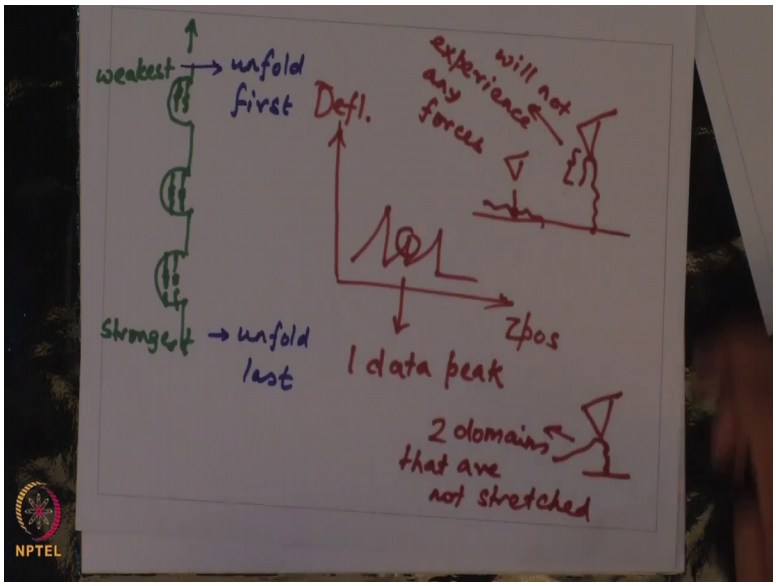

So, this would mean that when you are exerting force since it is, so it is equivalent to a bunch of springs which are connected in series. So, when you are exerting force then the weakest link will unfold first and the strongest link will unfold last.

So, I mentioned that in this force. So, in the force curve that I drew here you see you have a signature where there are three plus two peaks, but for a protein of domain, three domains you might have a situation like this. So, what you see is you are only seeing one data peak, why is that? Because it is very much possible if your protein is like this right, the tip actually gets in contact some point in between. In that case, when the tip is pulling it up your configuration will look something like this.

So, this portion of the protein will not experience any forces. So, in essence if for example if this is your first domain your first domain will not unfold, but second and third will. So, in the case I have drawn it is the case where let us say it is something like this. So, you have two domains that are not stretched. So, with that I end our lecture today.

So, to summarize we have seen how the AFM can be made use of for studying protein unfolding and what is the signature of protein unfolding, that you can say that this means based on this signature I can claim that this is protein unfolding. So, in the next class we will continue with this and see how to analyze our data and what are the design

principles associated with coming up with protein constructs for understanding the protein unfolding of fibronectin. Thank you for your attention.