Bio-Physical Chemistry
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Lecture - 32
Protein Folding Kinetics: Rapid mixing and Relaxation Techniques (Contd.)

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So, we were discussing stopped flow right. So, this is what we looked at last class it is a part of a Rapid Mixing Technique.

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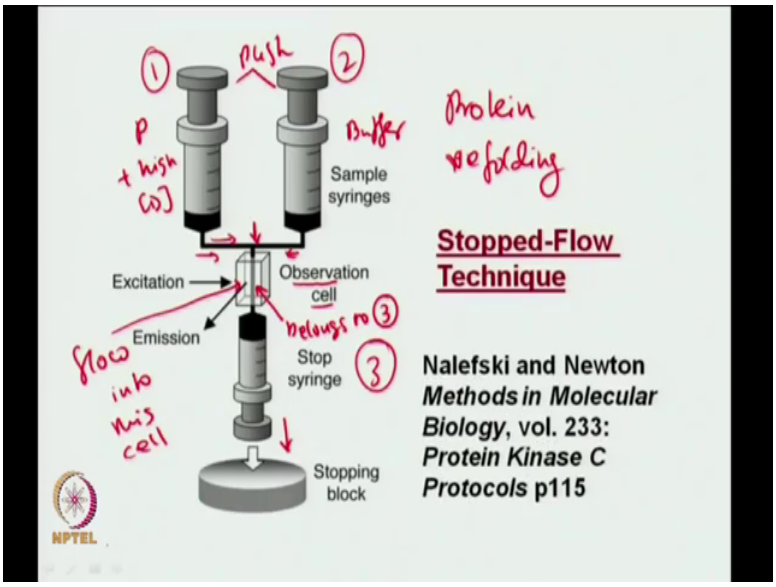

So, this is what the stopped flow is all about, you have two syringes one with protein and high concentration denaturant and the other one is buffer. And there is a third syringe which is known as a stop syringe which stops the flow. So, after the flow is stopped, you can see this is your observation cell right, this is the observation cell and you can see what happens is, this is where you make all your measurements or your data collection.

So, what happens is, whether you have you know whatever technique you are using, say in this case we are using a fluorometer, then what would happen is, the excitation light of the fluorometer that is of the lamp would come like this and then you collect fluorescence at right angles. But essentially that is your observation cell. So, you have stopped the flow and then you are watching the protein to fold back to its native state at whatever final denaturant concentration you have.

So, just hold on with the technique, I will come to the technique later technique means the spectroscopic tool which is fluorescence; but just focus on the technique right now as such right the stopped flow ok. So, essentially I will stop the flow and then you are watching the protein to fold. So, again to put things very simply what you are doing is, once the mixing is complete right you have completed the mixing, then the stop flow syringe gives a stop signal. The protein has already seen the low concentration of denaturant right because you have mixed it. But the protein cannot respond just like that, it takes time to come back to its native state or whatever state under these refolding conditions. So, that is the time you are monitoring.

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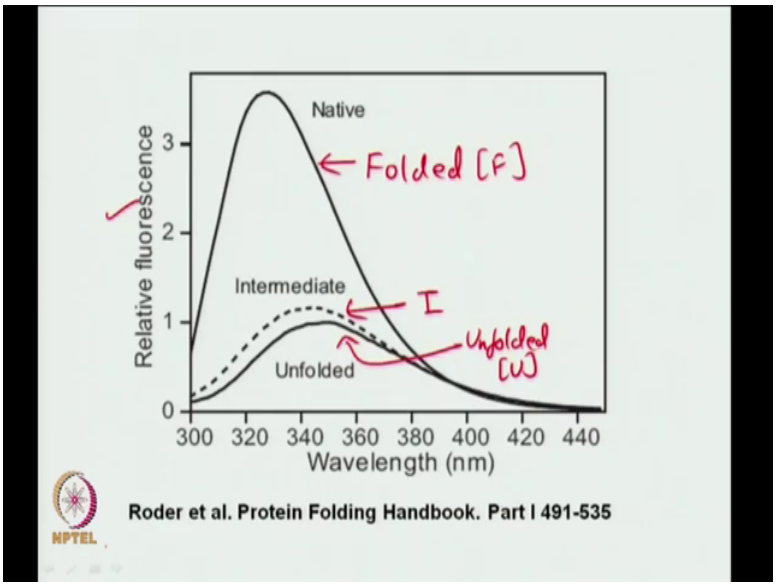

Now, one thing an error I made last class was I will tell you what I was what I am referring to right now ok. So, but before going there this is a change you are looking at. So, in this case you are looking at a change in fluorescence. So, the native or the folded is typically an

emission spectrum of tryptophan, if the tryptophan fluorescence would decrease in this case you can see, when you go from the folded state to the unfolded state the fluorescence is decreasing.

So, if you are doing a refolding; that means, you are going from unfolded to folded what would happen to tryptophan signal? It would increase. So, you would see an increase in the tryptophan signal right.

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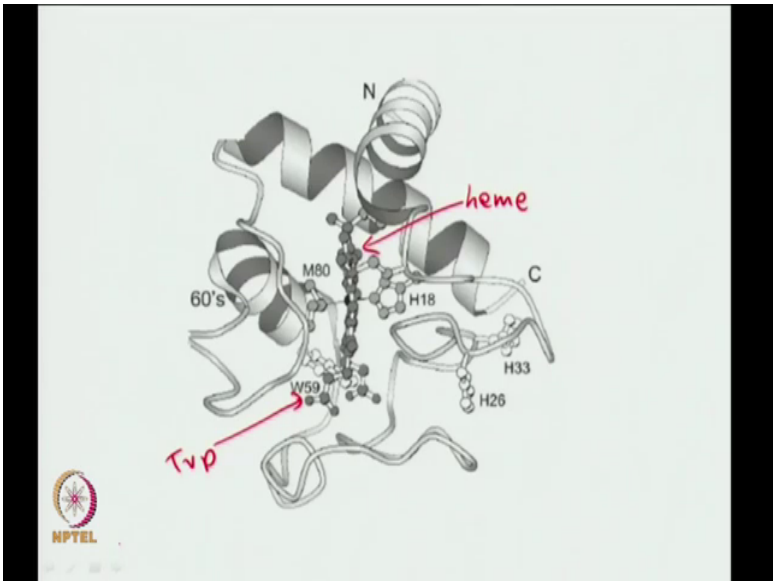

So, on the contrary, I was taking a case which was cytochrome c and where I said just the reverse happens; because in this case the tryptophan there is only one tryptophan which is Trp 59 at the 59th position. In the native state Trp and heme are very close to each other. Trp transfers almost all its all of its energy to the heme.

So, this folded state has the lowest intensity for Trp and the unfolded state has the highest.

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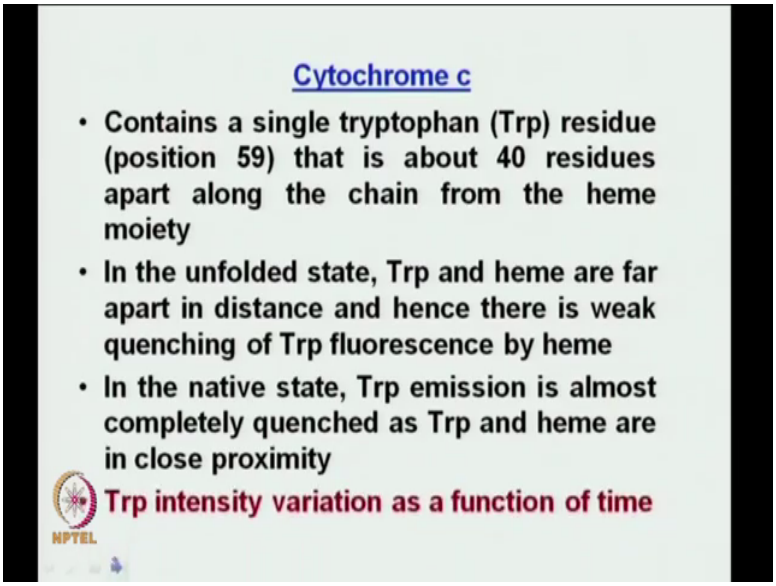

So, then this is how it looks. So, this was about cytochrome c. Let us look at the spectrum. So, this is how it looks.

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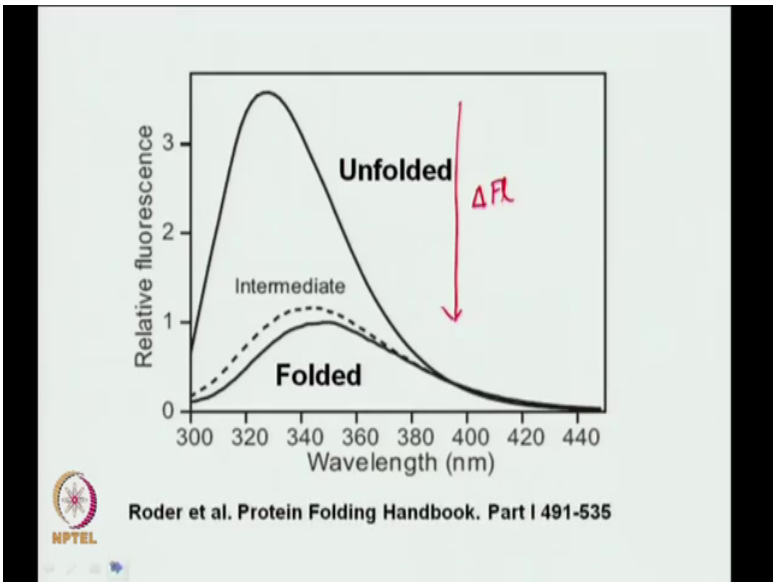

If you go from the unfolded to the folded what will happen is, your Trp intensity would decrease and that is what we are going to monitor as a function of time and this what you are looking at is the change in fluorescence right.

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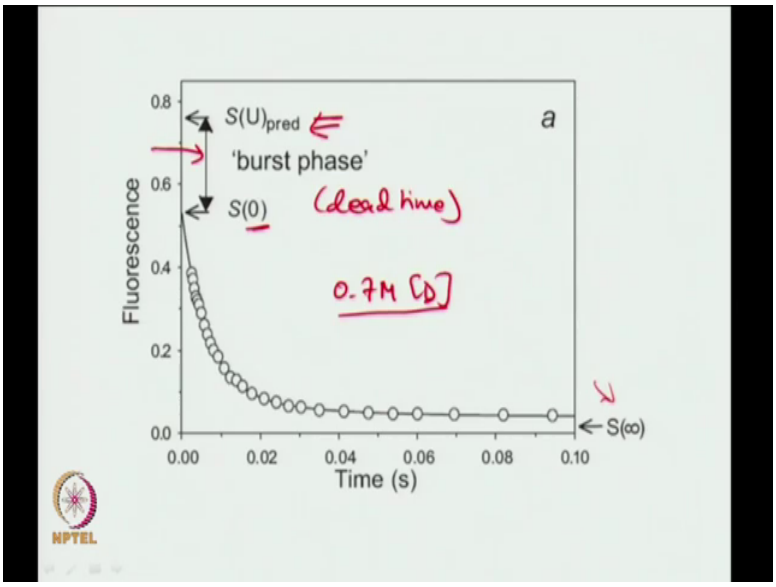

So, then at the very end of the class what we are looking at is something known as a burst phase and now what did we say burst phase was corresponding to? It was corresponding to sometime known as the dead time. So, this is the error this is where I made a made the error. So, when I wrote the expression or definition for dead time what I said was, it is a time lag between when you complete mixing actually that is not true. What I what I meant was, it is a time lag between the time point you start mixing that means, initiate mixing not complete mixing.

So, change complete to initiate. The time lag between you start initiation of mixing that is a first time you start the mixing them and the first time you collect your data point. So, the first time you start mixing the solutions and your first data point this is the time lag between which you do not have any information about the system because you are not being able to collect

any data right. So, that is your lag time and that is your dead time; because that is dead for you cannot see anything or you cannot see anything with regards to the system right.

So, any conformational change that happens during that phase is referred to as a burst phase; because it happens in bursts. Because it happens so, quick for your instrument that you cannot catch it that is why it is called a burst phase right ok. And then what we also said was. So, this typical trace you are looking at this trace what you are looking at is the final concentration is 0.7 molar denaturant say guanidine hydrochloride right. Where they started from was a concentration of 4.5 molar guanidine hydrochloride ok.

So, the initially the protein was taken in 4.5 molar guanidine hydrochloride and then you mix buffer into it rapidly by the stopped flow technique. Such that the final concentration is 0.7 molar of guanidine hydrochloride clear and that is what you are looking at the you know that is the kinetics you are looking at. So, the protein would stop at this signal S infinity and what that the signal at infinity mean? It means that this is the equilibrium signal of the protein at 0.7 molar guanidine hydrochloride; that means, the final denaturant concentration clear.

So, this is the final signal you have right ok. So, this was at S infinity. So, there is another thing called S 0. So, if S infinity as t infinity then S 0 is your t 0. So, t 0 means the time 0. Now this time 0 is not with respect to your initiation, this time 0 is with respect to your first data point you are collecting please keep that in mind. With respect to the system the time 0 is already started a long time back.

Student: Yes sir.

You have not been able to look at it because that was in your dead time. So, time 0 is always with respect to your instrument; that means, how fast or what is the first point you can capture that is your time 0 essentially. 0 time and then your time evolves; that is what the 0 time is. So, then we were discussing one question, the question was what is the significance of this SU predicted? That means, signal U means unfolded state and predicted means what do you

predict; where do you predict it from and this is where you are talking about equilibrium transitions. What did we mean by that?

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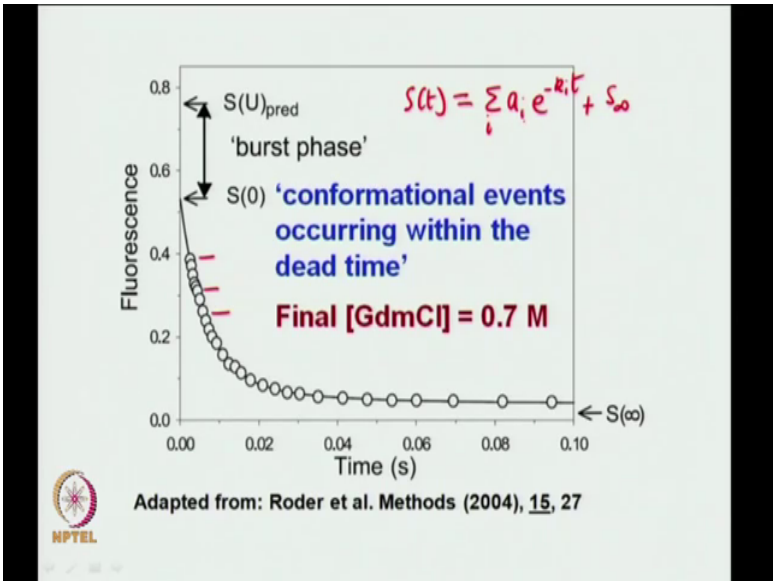

So, let us so, what you can see out here is written for the same graph, the conformation events occurring within the dead time referred to as a burst phase. And here the final denaturant which is guanidine hydrochloride concentration is 0.7 molar. So, well I mean before going further let me tell you what it do is, you look at all these points; you see look at all these points these circles these circles are your data points ok. These circles are the points you collect with the help of your instrument.

These are your points at different time intervals right or different times these are your kinetic data points. Now obviously, once you get a kinetic data point what would you do? You would try to fit it to a model. Now this is an exponential model you fit it to assuming that it is kind of

a two state process right though it is not, you are looking at activation barriers exponentiality comes from that, but what you fit it to is this. Say if this is your S that is your signal t if this is your S that is signal t as a function of time and this is what you fit it to. You fit it to a summation of ai e to the power minus ki times t; plus S infinity.

So, what do we mean by this? So, S as a function of time, you can realize it is your signal as the function of time right for some signal. Then what is S infinity? S infinity means.

Student: (Refer Time: 08:13).

Everything is done this is the final state you have equilibrium; that means, your signal at infinite time, but look at the this one a e to the power minus kt what does it mean? i is. So, what you can realize is it is an exponential fit right because e to the power minus kt. So, what is i mean? i means that you might be having more than one exponent coming in.

See you might be having say i is equal to 1, 2, 3 depending upon how many exponentials you need to fit it. See it can be mono exponential, if it is mono exponential then i would be equal to 1, if it is the by exponential then i would be equal to 2, if it is the triple exponential i would be equal to 3 and so on. So, then if I refers to that exponential you know you know time constant tau or k is in this case it k the rate constant what does a refer to?

A refers to the amplitude; that means, in the total signal. In the total signal say if you have say in the total signal you have just one component what is the amplitude? There is no big deal the amplitude is 1 because you just have one component. Now suppose you have two components i is equal to 1 and i is equal to 2, it might be that both the components contribute 50 percent; that means, equally to that decay.

See in that case a 1 and a 2 would both be equal to 0.5, it might be that one contributes 20 percent the other contributes 80 percent. So, in that in that case a for the 20 percent a 1 say would be 0.2, for the other one it would be 0.8 and so, on. So, this is what amplitude means; that means, it is the contribution of that respective ki component to the exponential decay is it clear right? So, this is how you get your kinetic information; that means, your kinetic

parameters in this case either the rate constant ki or the inverse of that which is the time that is what you get from here ok.

And that is what you finally, use remember where do you use it? You use it and with the help of something else you construct your chevron plot that is how you do it, but this is how you get your k from here.

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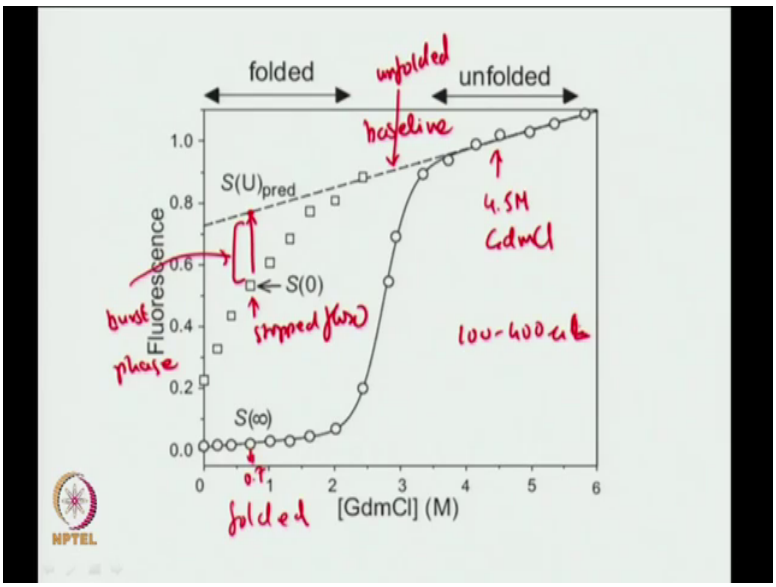

Now, this is what I meant by S U predicted. So, look at this, this is not a time curve what you are doing is you are doing a chemical denaturation of the same protein right. You are going from the folded state which is at the left, you can see this is your folded right.

This is your folded state because you have low denaturant and this is your unfolded state right its written fold; obviously, it is written on the top this is your unfolded state. Now see how it

matches? This is the fluorescence in the folded state the fluorescence is very low is not it? So, that is why it has the value is low. It is also S infinity why is it S infinity? Because whatever you are doing you are doing it at equilibrium and this S infinity is the same S infinity you are getting in your kinetic tress.

Do you realize that? Think of this; say what was the final denaturant concentration in the previous case? It was 0.7 molar. So, say this is 1 this is 0.5, say this can correspond to 0.7 right. So, what S infinity means is, whatever equilibrium value you have at this point; that means, for 0.7 molar guanidine hydrochloride whatever equilibrium value you have from your thermal denaturation of fluorescence should match with the one you get from your kinetic tress because you are finally, talking about equilibrium time.

S infinity is essential equilibrium one point; obviously, I can ah realize that you will not be able to kind of make out is how to you know match these two things. There units might be different here units might be different.

So, generally then what people do is they plot ratios ok. So, that whatever we do? The final thing comes out to be independent of what are you started with right they plot a ratio of singles, but anyway guys do not worry about that. So, this is how it is done this is how you match it between the equilibrium value on the kinetic I mean kinetic equilibrium S infinity.

So, just before leaving this slide you look at S U predicted. Now this is important you look at this dotted line, can you tell me what is this dotted line referring to? First of all what where is this dotted line coming from? It is coming from your unfolded state.

Student: Yes.

If you recall when we were fitting when we were actually going to this fits thermodynamic fits what did we refer to this as? It was certain sort of baseline do you remember we have always had a straight line and then we are fitting it? It was this is. So, this is essentially your what unfolded baseline is not it?

Student: Yes.

This is essentially unfolded baseline. So, what you do is unfolded baseline means what? Unfolded baseline means these are your points and these are your points where they it is essentially unfolded to the right of the curve. Now you extend it, you extend it and if you have done it correctly then every point on that curve would give you the signal for the corresponding unfolded state at that denaturant concentration ok.

So, you can see here. So, this one would correspond to two molar and all this case all these things. So, what you what do you mean by S U predicted? What do you mean by S U predicted is this, before you started mixing before you started mixing; that means, even before the protein at 4.5 molar guanidine hydrochloride was mixed; what was the equilibrium prediction? What was the equilibrium value? The equilibrium value was here.

So, it was 4.5 molar. So, this is 4.5 molar. So, this is 4.5 molar. This is before you started mixing this is before you started mixing. Now think about this, after you have mixed; after you have mixed coming along the same line after you have mixed what was your final concentration 0.7 ok? If it the final concentration is 0.7, it should be somewhere here, this is your S U predicted 0.7 because it is along that unfolded line.

But, look at this, if you remember the curve; if you remember the curve if you go back right here it is. What is the value here? It is about 0.5 something right. Now what is the value here? It is much more than 0.5 is not it? It is what? It is kind of say 0.75 or so. So, whatever you are predicting based on equilibrium value is 0.75; whatever you are getting as the first time point from your stopped flow is which one its not 0.75 what are you getting? You are getting this one from stopped flow.

So, what does it mean? It means this difference this difference, this difference is essentially what? This difference essentially is your burst phase; because this you can never pick up, that is your predicted based on thermodynamic concentrations based on equilibrium, but what you

get from kinetics? Because if your instrument limitation is from S 0 which is not from 0.75, but from 0.5.

So, that portion you miss actually ok. So, this is what your burst phase refers to. So, if you would be doing this correctly if you would be doing the kinetics correctly based on whatever limitations. You have you can always match these two things start an issue, but you have to know what you are looking for or what you are going after. So, good. So, the drawback of this one obviously, you can realize the dead time is close to your 1 millisecond or so, why I said was you can go even down to 500 600 microseconds possibly, if you are really good at the instrumentation right.

But the good thing is this, guys the good thing is you can do it with a very small amount of sample and I said it can be anywhere between 100 to 400 microlitres of sample, you can easily use for this one. So, now, we need improvement this is stopped flow, what happens if we do not stop the flow ok?

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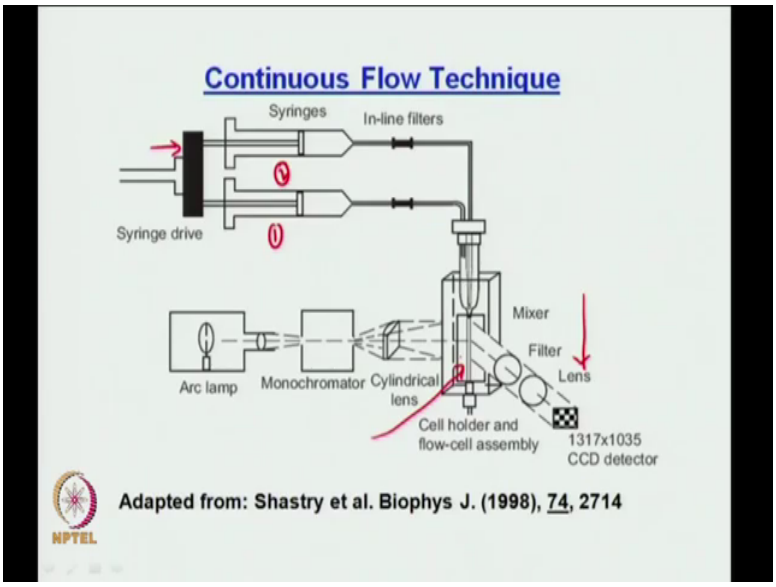

So, then the next one is referred to as a continuous flow technique. So, look how this is different or look how this is similar actually. The stopped flow had two syringes right syringe 1, and syringe 2. Now this one also has two syringes; say syringe 1 and syringe 2, but anywhere in the rest of the apparatus do you see a third syringe? You do not see a third syringe right.

There is no other third syringe you look at it no matter how minutely you look at it there is no other third syringe. So; that means, that syringe which had the function of stopping the flow is not stopping the flow anymore. So, essentially what is happening? What is happening is the same thing in one in syringe 1 you take protein and denaturant in syringe 2 you take buffer then you push them together with the syringe drive, this is your syringe drive you push them together and then they will mix.

So, this is your mixture. So, this is your mixer they will mix here I will show you a blown up portion of the mixer later ok. Why is it called continuous flow? The it is called continuous flow because once you mix these two systems right you are not stopping the mixing; that means, you are not stopping the flow, you mix these and you allow them to flow.

So; that means, you allow them to flow along this line, you allow them to flow along this line and this is your observation cell right. That means, this tubing you are looking at this is where the solution is flowing and you are observing for detectable fluorescence changes. I repeat you have mixed these two solutions in the mixture I show you that to you in the next slide or the next to next slide, after you have mixed them you have let the protein or the mixed solution be as it is you have not stopped the flow. So, what has happened?

So, think about this, the difference is in stopped flow the syringe stopped its flow, but the protein was not flowing anymore right because it has stopped its flow. Now the protein was responding to whatever change it had to. In this case what is happening is you have mixed it and after mixing the solution is going on flowing that is why its called a continuous flow. Now tell me how is the protein responding? The protein is responding in the same way, but because say because this is the tube where it is flowing through right you have not stopped it flow.

Now, think what will happen? At the very initial portion of the tube suppose I have done the mixing, the protein would still be more or less in its unfolded state. As I move down what is happening? The protein is slowly moving towards where? It is folded state so; that means, the distance it travels along that flow tube can be transferred to what? Its time provided you know the flow rate and all these things.

So, again you are not stopping the flow, you are allowing the solution to flow through and the amount of it and the amount or the distance it flows can easily be converted to time. And that time is actually the time which has elapsed since you have started mixing. And at these time points you take fluorescent intensity signals, you record those and you again you get the kinetics. Is it clear or not? This continuous flow clear is the difference between continuous

flow and stopped flow clear right that is why its called continuous flow you are not stopping the flow ok.

You all just letting it flow continuously ok. Sakshi are you sleepy? No. Anyway she is really looking at me its like a face I do not know what face is that, but sorry for boring you. Now, because this is your observation cell, because this is your observation cell like the stopped flow you would need something to observe.

So, you can see here, you have the lamp here you have a monochromator do not worry about the monochromator. So, the one of monochromator ensures this its a mono chromator right mono means single, chromator means colour. So, it helps you figure out only one colour out of a mixture of colours right and then you have a lens then you have a cylindrical lens.

You can see what happens is the lens kind of broadens your beam and then it focuses on the full cell that is a continuous flow cell through, which the protein is flowing ok. Now how do you do the detector collection? If the detector is here its called a CCD you know I will not tell you what a CCD is, it is essentially Charge Coupled Device.

If you guys have digital cameras all these digital cameras are equipped with this, but anyway then you collect the signal based on time points I will show that to you.

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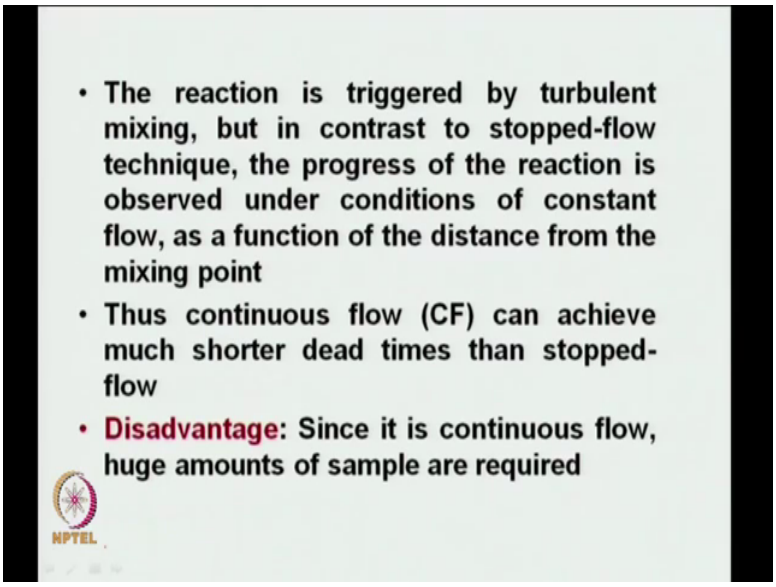

Just go forward. So, the reaction in this case is still triggered by turbulent mixing, but in contrast to stopped flow technique, the progress of the reaction is observed under conditions of constant flow as a function of the distance from the mixing point ok.

Now, try to realize the advantage of this. The advantage of this was or this is as compared to stopped flow is even after the mix even after in stopped flow that observation cell had to be full. The syringe would come back and then it would get an upward thrust that will stop and it would stop it. But see what it means is from the moment that solution comes into the observation cell and the syringe gives a signal to stop it that time is still missing in continuous flow what you are doing? You just mixing and letting it go.

So, that is an advantage you are gaining in terms of dead time right because you have you know stopping the flow; that means, you are you can look at it from a faster time that is one, but tell me what is one major disadvantage?

Student: Require more amount.

You require more amount excellent you require more amount good second. The second is this remember, when we were talking about a flow when we are talking about a flow being converted flow rate right. Going through this distance and then distance been converted into time, what we are assuming is throughout the time we are monitoring it its flow is still the same.

But think about this, the moment you mix it depending upon how you mix it only over a certain distance where its flow be the same after that what would happen? The flow would not be the same; its like reverse flowing through right and source; obviously, is coming down at the source it would be pretty fast but then its coming down on slope. But even then if you are pumping some water right if you are pumping some water what would happen is, very close to the pump what would happen is it would be very fast you move far out out out what would happen the.

Student: (Refer Time: 23:07).

Tide would slow down the same thing happens here; that means, for the constancy of the flow to remain, you have to put in a turbulent mixing and even if you do that, even if you do that only over a certain time. That means, only over a certain length of the cell would it would the flow remain constant because after that the flow changes.

So, the time interval now changes ok. So, the limitation is in stopped flow you do not have this the limitation is in continuous flow you cannot you cannot monitor it for S infinity. Because you are limited by that length of the flow cell over which it has a constant flow velocity that is the limitation of continuous flow its a major limitation actually. So, thus

continuous flow can achieve much shorter dead times than stopped flow right; we just discussed. The disadvantage is as was pointed out since it is continuous flow, huge amounts of sample are required.

Now, again realize this in stopped flow you are stopping mixing, in continuous flow the syringe is always being pushed; always being pushed, pushed because you have to maintain a continuous flow right. Because if there is no flow there is no signal as simple as that. You push, push, push and you push on until what? Until you do not have any sample you have run out of sample ok.

So, that obviously, is a huge disadvantage especially for expensive proteins or expensive chemicals if you are using those ok.

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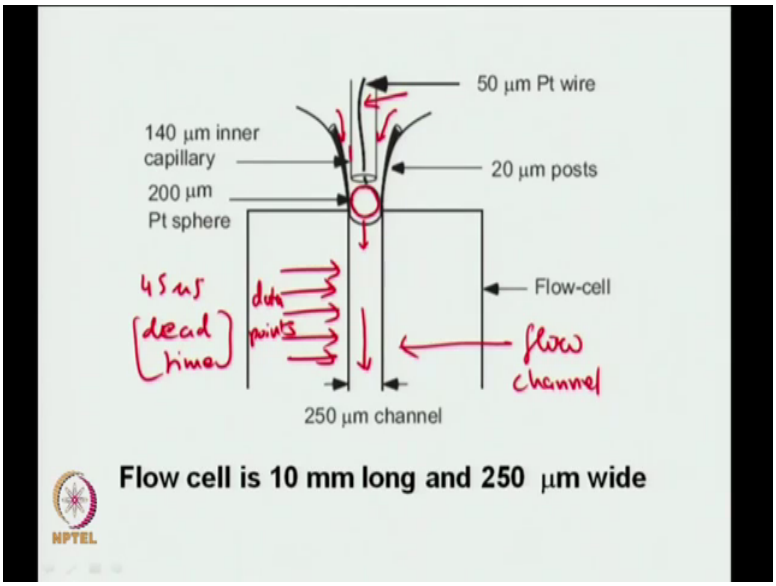

Now, this is you are really looking at the instrument now this is a zoomed in portion of the mixer; now see what they have done. You have to maintain turbulent mixing you have to maintain turbulent mixing to maintain a decent enough flow rate over a certain length of the capillary or the flow tube.

So, first of all what is this 250 micron channel? This is your flow channel ok. So, this is you can see this is your flow cell and this is your flow channel. So, what do you mean by the flow channel? Flow channel means after mixing the protein and buffer is flowing through this ok. So, this is where it is flowing through good.

Now, but before flowing it has to get mixed. So, the way it is done is you see this is a platinum wire, you can see this is a platinum wire out here right. So, this is. So, this is the platinum wire right. Now at the end of the platinum wire you can see this small circle. So, the platinum wire has to be has been drawn out into a circle.

So, what they do is what they do is, you have two solutions right two syringes. So, one syringe solution comes like this and the other syringe comes like this through. So, this is your this is your inner capillary you can see this is your inner capillary. So, whatever space you have whatever space you have the solutions come through this ok.

You are pushing these two. Now what people have found out is if you push it with enough force and you make the solutions slight over sphere the mixing is very turbulent and because the mixing is very turbulent then mixing is very fast. So, that is why they have a drawn out like this. Now this is an experimental method; obviously, people had have spent time behind doing this ok.

But this this was this was discovered some time ago. So, once you do this, then the mixed protein starts flowing out ok. Based on this instrument based on this instrument or the instrument I just showed you based on this instrument the dead time the dead time is 45 microseconds this is your dead time. The dead time is 45 microseconds now tell me what was the dead time for your stopped flow?

Student: 1.

About 1 millisecond right. Can you imagine the improvement you have done just by removing the stop method or just by removing the stop syringe?

It is a huge improvement and whether its more than enough and that is a different issue that will depend upon the system, but it definitely it is coming down from 1 millisecond to 45 microseconds its by no means small ok. Now what will happen is because this is the flow cell you will be getting data points throughout the flow cell ok. So, these are your data points you will be collecting. So, these are your data points because along the flow there means along the length of the flow tube is your time that is what it means, now what do you mean by that ok?

So, the flow cell in this case is 10 millimeter long and 240 250 microns wide 250 micrometer.

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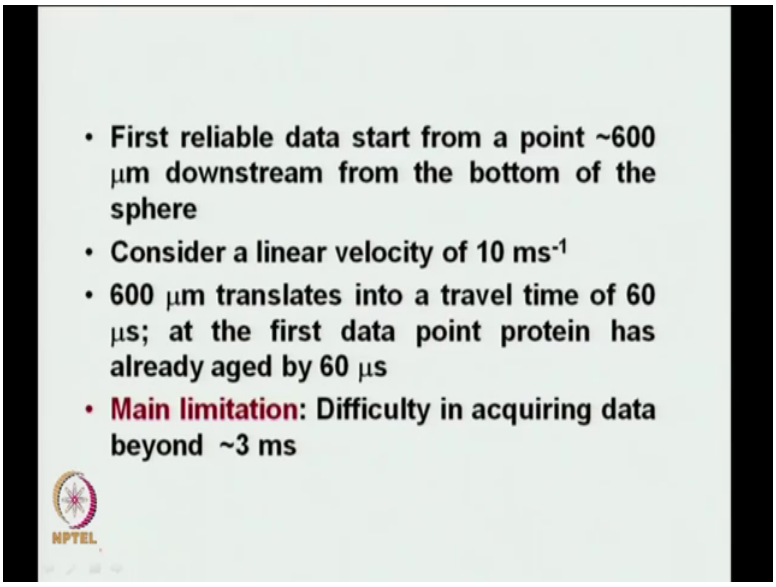

So, here the first reliable data start from a point which is about 600 micron downstream from the bottom of the sphere, you remember the sphere; the sphere on which the mixing occurred right. So, from the bottom of the sphere if you draw take a ruler draw distance 600 micron then the 600 micron is the first point where you start taking reliable measurement good. Now what does this translate to in terms of time? Consider your linear flow velocity of 10 meters per second.

If that is a case when 600 micron travels or translates into travel time of what? 600 microseconds right. Say at the first data point; that means, the protein is already aged by what? 60 microseconds. So, this is how you do it. Now do you understand the meaning of distance being converted to time? So, at whatever point you would take along the flow cell, whatever distance it is from the mixer you know exactly depending upon the flow rate what

time it corresponds to and that time you put on the x axis and you put your fluorescence intensity out there and then you will get the curve again.

Clear or not? Not many of you are nodding your heads. Is it from not understanding or is it from because you are too tired? I have to know the difference now. Anyway again, if you move along the flow cell each point of the flow cell there is a distance you have moved from the bottom of the sphere can be converted to a time point. The same thing you did for the stopped flow, but you did not have to do that the machine itself did it for you right the software rather.

So, what is the main limitation? The difficulty in acquiring data beyond 3 milliseconds now this is what I was referring to; based on 3 milliseconds you can calculate right how much distance I can go beyond that I cannot go any more because my flow now becomes very erratic, I do not have a constant steady flow and hence I cannot take data beyond that point of 3 milliseconds right.

So, this is the disadvantage of one of the disadvantages of a continuous flow technique right ok. So, obviously, continuous flow does a good job in giving you a very faster; that means, allowing new a very smaller dead a very small dead time as compared to stopped flow.

But then it has to stop not before your S infinity because the S infinity is not necessarily reached at 3 milliseconds; that means, your protein might not have stopped or might your protein might not be done refolding at 3 milliseconds it might take a longer time to refold then what would you do? What would you do then? You stop your experiment go home sleep.

No what would you do?

Student: Stop flow, (Refer Time: 30:39).

Good. So; that means, you combine the continuous flow with what? Stopped flow. So, you can combine these two techniques you can you can take the advantage of both these techniques combine them and get a really decent time window ok.

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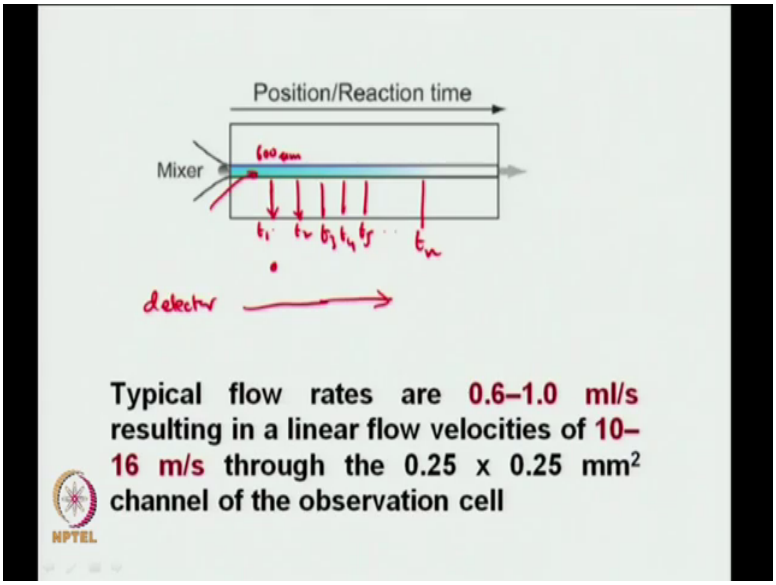

So, here so, this is what I was saying. So, this is your position which is getting converted to reaction time, this is your flow cell you are looking at. So, typical flow rates are 0.6 to 1 millilitre per second, which results in linear flow of velocities of 10 to 16 meters per second right and this was 250 micron by 250 micron cross sectional area right.

So, its 0.25. Now this is the other part. See suppose this is the time point. So, suppose this is your, what was the first time point for data collection in this case, you remember?

Student: 600 micron .

600 micron right. So, you say this is 600 micron this is the first time point right. Now as you move; as you move this is t 1, this is t 2, t 3, t 4, t 5 and so, on right ok. Now first look at that the intensity of the colour of the blue line which is this is. So, this is the blue.

Student: Yes sir.

Blue is the flowing solution. Why do you think the intensity is changing from more intense to a less intense why do you think so?

Student: Mixing (Refer Time: 32:08).

Yeah, we are mixing, but then what is why is intensity changing?

Yes.

Student: (Refer Time: 32:12).

Or why is intensity decreasing?

Student: (Refer Time: 32:15).

Because you are talking about cytochrome c, remember the tryptophan intensity would be decreasing as you go to the folded state right. So, here in the unfolded state you start with a very maximum intensity; that means, very high intensity. As you go forward the protein folds tryptophan intensity decreases and hence this colour shading also goes to the goes in the lighter direction right number 1. Number 2 is here is a problem now try to realize the problem the problem is, if I have to take if I have say one detector and if I have to take fluorescence intensity points at t 1, t 2, t 3, t 4, t 5 and so on; and so, on and say this is tn right.

And if my detector is a point detector; that means, it can only take one data point at one time what will I have to do or what will you have to do? What will you have to do is manually or by some other you know by writing a program you would have to take the detector and slide it isn't it? Along the flow cell do you realize what I said? Say suppose this is your detector and suppose this is a detector and the detector takes only one point, it is a point detector it takes only one point one fluorescence intensity at one time point; that means, at one distance ok.

So, suppose now this one takes a 600 micron, then for the next one you will have to move the detector along the flow cell, but that is a bad way of doing it right now what would you do? Instead what would you do is why cannot I do this? Instead of having one detector I can have what? A series of detectors at different time points so; that means, if I cover almost the whole flow cell right with different detectors, I will have enough information about my different time points.

Now, being really ingenious what you can think about is, why cannot I take one detector and use that one detector to get all the time point simultaneously, it is called an array detector. And an array detector means an array detector means it is like an array detector; that means, if one say if you are talking about one detector is a one point detector, it has one detection element and array detector will be having what? A series of detection elements it is called an array right a number array. So, it is a detection array series of detector elements. And, hence if you put that here each detector element would get the time point would get a different time point along the flow cell and hence you will get the kinetic trace ok.

Now, that is the idea this was the drawback initially because you will have to move your detector along the flow cell along the line.

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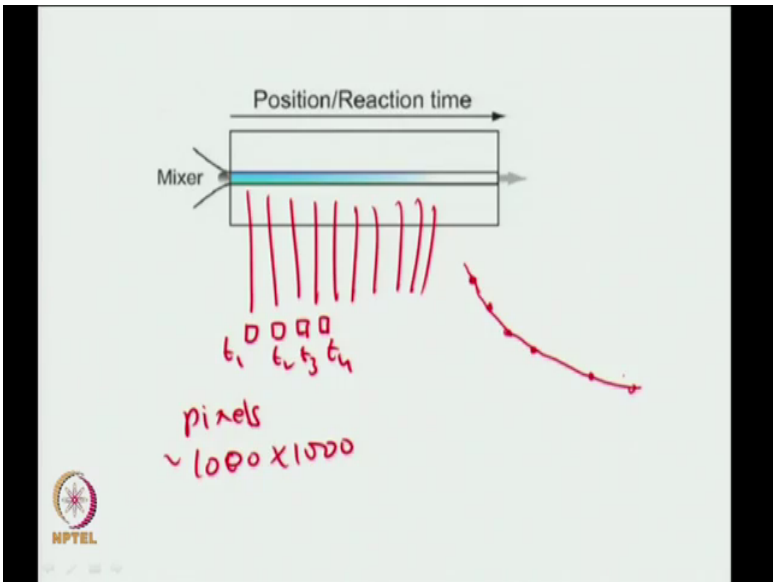

So, what people have come up with now and this is a real good advancement; is something known as a CCD. A CCD is referred to as a Charge Couple Device. So, what it does is this ok. So, you have all these fluorescence intensities coming here. A CCD is broken into pixels you do not have to know this just listen to me. So; that means, it has many pixels say it has say 1000 by 1000 say 1000 by 1000 this is an example.

So; that means, an area has 1000 by 1000 pixels. So, what you can do is now, you can take one you can say one pixel corresponds to this pixel is a small house small house another pixel a small square corresponds to this, another pixel corresponds to this another pixel corresponds to this and so, on ok. So, the CCD can program like that. So, instead so, essentially what you are doing is essentially what you are doing is now, if you go back to this slide.

Now, you can see the CCD detector can you see the small squares out here like a checkerboard like a chessboard? So, these are your pixels. So, the pixels now try to realize this, you can see this you can see this beam of light coming out right this is a one beam of light this one and this is another beam of light right so; that means, you have the full fluorescence beam coming out.

Now, understand the significance, this is your flow cell isn't it? This is your flow cell through which it is flowing you look at the beam is not the beam covering the whole flow cell through which the protein is flowing? What it means is because you have covered the whole flow cell; that means, no matter where you are at any given time, I just want at one shot you are collecting all the respective intensities with the CC at the same time. Because your instrument is designed in such a way with lenses and all these combinations that the whole cell is being focused at one point and then the detection is also occurring from there from the whole cell at one point clear or no ok.

So, this is how the excitation is, you can look at the excitation this is excitation right see the excitation is not a small line it is a band. What does that band covered? The band covers the whole length of the flow cells through which your protein is flowing [FL] ok. Now once that is done then because the protein is flowing it is also emitting; that means, fluorescing throughout the flow cell. And then you collect that with a combination of filters and lenses on your CCD and then what you get is and then what you get is this.

At each and every time point you have a certain florescent trace. So, let us say this is t 1, this is t 2, this is t 3, this is t 4 now let me this do a qualitative plot; t 1 would be high the highest, t 2 would be little low, t 3 would be lower, t 4 lower and so, on and so, if you join this what would you get? Would not you get another kinetic trace right.

So, this is how you get a kinetic trace from continuous flow. Only in this case you know stopping the flow or you taking time points along the flow cell to get the florescence intensity. Pin drop silence [FL] full reflection [FL] full reflection [FL] problem [FL] full transmission

[FL] problem [FL] (Refer Time: 38:43) [FL]. Now did you understand did you understand the continuous flow technique?

Student: Yes.

Right.

It is really important you understand it why because this is what is done presently this is what quite a few groups have and people are still working on it. And, if you would be doing research depending on which group you land into you might have to work on a development of this technique. That means, you make a faster dead time; that means, you make a smaller dead time; that means, you make a faster continuous flow and people are still trying to bring about improvements on this technique ok.

These are by no means old these are very recent techniques modifications are still being done guys listen from me [FL] ok.

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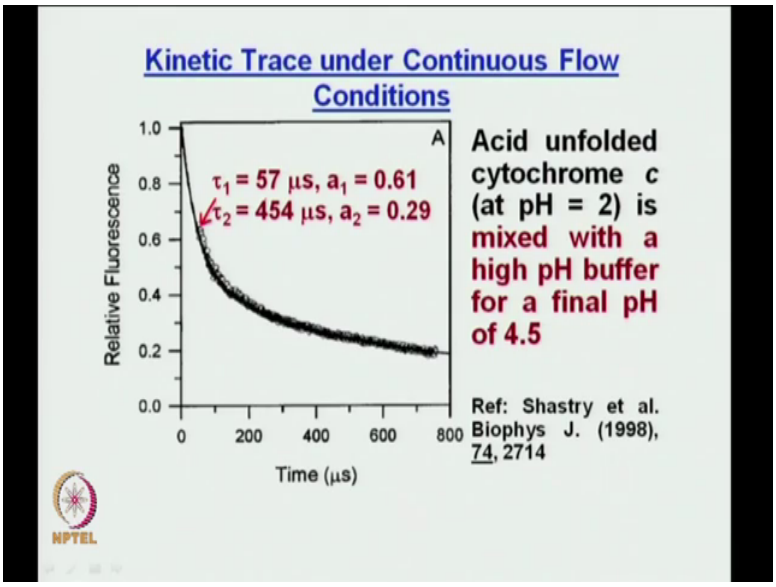

Now, let us look at the kinetic trace under continuous flow conditions. So, this is under continuous flow you can see, this is the first point I started taking data right the small circles, but look at the problem, the problem is I start from here, but where do I end I am not ending close to 0 where am I ending? I am close somewhere at the top right why is it that? It is because I has that limitation right.

I cannot go beyond 2 or 3 milliseconds; that means, anything beyond that I cannot follow using in continuous flow. So, I have to stop beyond before so; that means, I start very early, but because you start early I also stop early essential that is what continuous flow is doing for you right now. So, this is a typical case where you are taking cytochrome c at pH is equal to 2, at pH is equal to 2 it is essentially unfolded low pH you know its a pH denaturation.

Then what you do is you mix it with a high pH buffer and bring it to a final pH of 4.5 and so, it starts folding again at 4.5 and these are the two time constants see these are the two time constants. So, you are fitted using that whatever equation I wrote, one is a 57 microsecond right and the other one is a 454 microsecond right.

Now, this is a time constants these are not your ks these are tau's with the corresponding amplitudes. See one has a amplitude of 60 percent and the other one has amplitude of?

Student: 29.

29 percent. Now you can realize why it does not add to 1. The reason is your decay is not complete because you have to stop at about what? 2 milliseconds or so, your decay is not comeplete how would you know it; because you have farther components which you cannot see right, it is a problem with a method.

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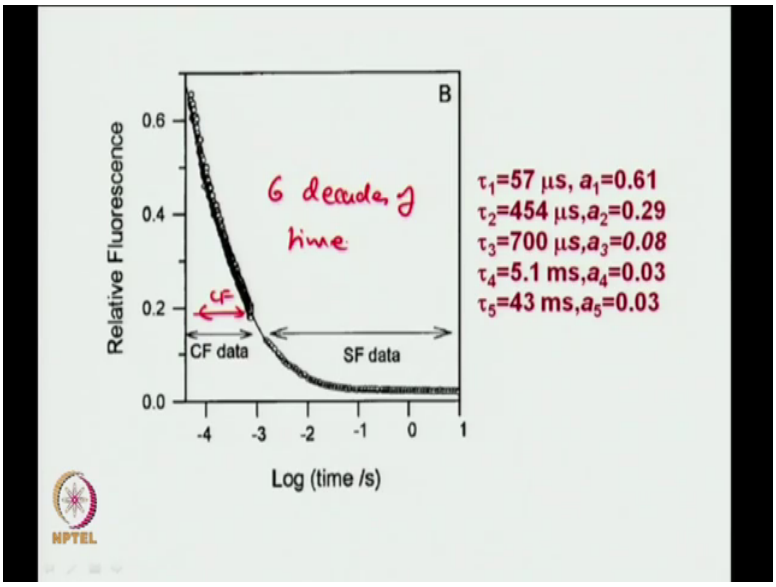

So, now as you guys suggested what do you do is, you combine both of these. So, here from here to here is a continuous flow you can see this is a continuous flow CF, I am writing it on the top again and beyond that here is a stopped flow technique. So; that means, you take two you combine these two to get the full trace right.

Now, look at the x axis, this is your log time tell me what does minus 4 to 1 mean to you in terms of a log time? What does minus 4 to 1 mean to you? Minus 4is how many seconds? If log time is minus 4 its how many seconds?

Student: 10 to the power minus 4 .

10 to the power minus; 4 which is 100 microseconds what was the dead time of our continuous flow?

Student: (Refer Time: 42:19) seconds.

45 microseconds right dead time was 45 microseconds. So, this 100 microsecond is coming from the continuous flow right. Now you move forward the last one is what? 10 isn't it? one is 10 right 10 seconds so 10 seconds cannot come from continuous flow where is it coming from?

Student: Stopped flow.

Stopped flow. So, now, if you go from minus 4 to 1 how many decades of time have you crossed? Minus 4 to minus 2 is 1 decade.

Student: 10.

Right it is a 10 right minus 3 to minus is a another decade how many decades of time have you crossed?

Student: (Refer Time: 42:55).

So, minus 4 to 1 is.

Student: (Refer Time: 42:56).

6 decades right minus 4 to 0 4 rather or how many 1, 2, 3, 4, 5, 6 right 6 decades and one including minus 4; that means, using this combination you have been able to monitor a protein folding from or over 6 decades of time realize the importance of this. This time span is not easily achievable in any other technique ok.

So; that means, you are monitoring over 6 decades of time where do I write? So, let me go forward first then I will see. So, now, so, this is 6 decades of time over which you monitor your protein folding, when you had only continuous flow how many time constants that you have? 2 look at the two 57 or 454.

Now, when I have used this stopped flow how many mode you have? You have three mode. So, these three you were missing when you are doing the continuous flow; however, when we are doing this stopped flow, you are missing the first two.

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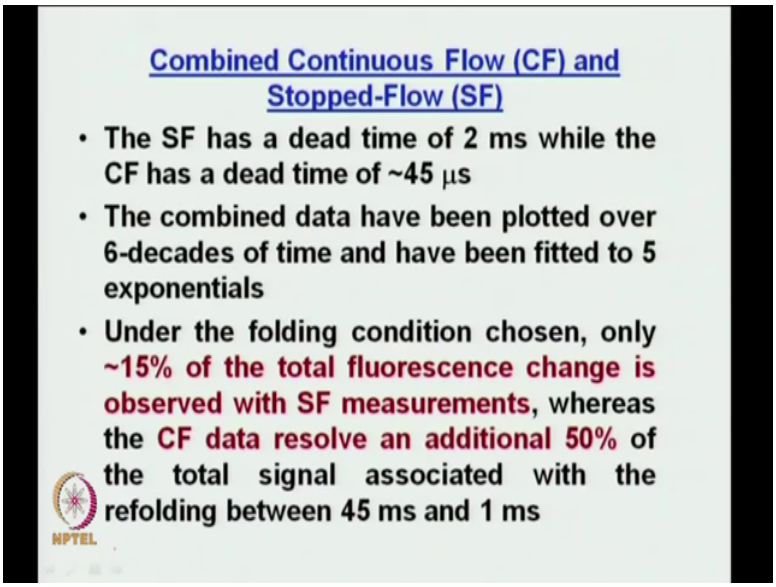

So, then some points about the combined continuous flow which is CF and the stopped flow the SF techniques. The stopped flow has a dead time of about 2 millisecond in this case while the continuous flow has a dead time of 45 microseconds good.

The combined technique a data have been plotted over 6 decades of time right and have been fitted to 5 exponentials. Now this is important right its not going to have for every protein this is cytochrome c we are talking about. Under the folding condition chosen only 15 percent of the total fluorescence change is observed with stopped flow experiments whereas, the CF data which is the continuous flow data resolve an additional 50 percent of the total signal right.

So, do you realize the importance of this? So, now go back and see. From here to here is almost 50 percent of the total signal from here to here its just about 15 to 20 percent. So, if you were taking stopped flow you have to missing out and what? 50 percent of the total signal the initial part. When you are using continuous flow, you are getting that 50 percent then, but then you are mixing the missing the rest which you are getting from stopped flow right.

So, the resolve an additional 50 percent of the total signal associated with the refolding between 45 milliseconds and 1 sorry this should be microsecond 45 microsecond and 1 millisecond.

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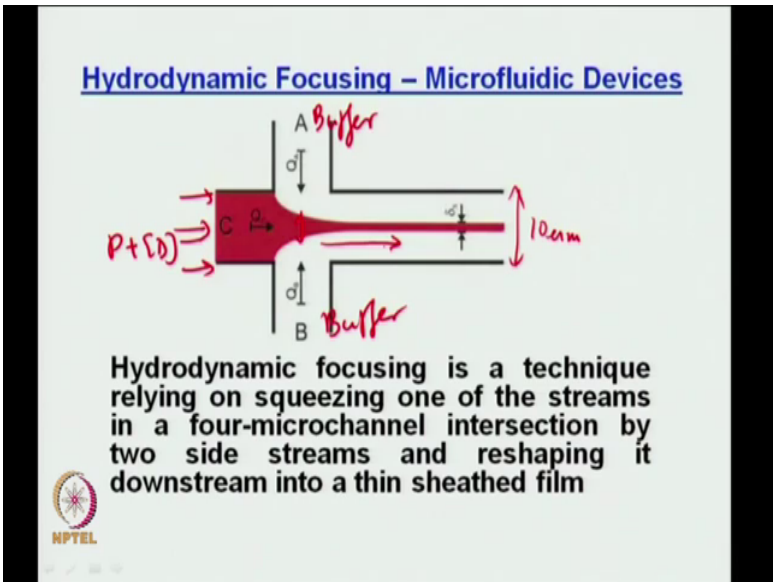

And this is the last point I will quickly introduce before I end the class, you know people are smart the people just do not sit they try to they try on improving these things.

So, what they have done is now, they have given rise or they have come up with a technique known as continuous flow technique, the same continuous flow technique, but it is based on micro fluidics. Have you heard of the word micro fluidics ok? This concept is very simple micro means; obviously, small, fluidic means you are talking about flow of fluids. That means, if you are talking about micro fluidics you will be talking about flow through micron sized channels; that means, channels which are very small see.

So, if I show you what it is, it is based on a technique called hydrodynamic focusing. As it says hydrodynamic focusing is a technique relying on squeezing one of the streams in a four micro

channel intersection by two sides streams and reshaping it downstream into a thin sheathed film ok. Lot of words, but the take home point is very simple this is what it as.

This is your protein plus denaturant ok. Now here there are no syringes right now. Here you can have syringes attached to these channels, we are talking about a channel, this is a micron size channel. The width of the width this width you are talking about this width is typically 10 micron. What was a width for your continuous flow technique? Do you remember?

Student: 250.

250 micron someone was awake 250 micron. So, you can see how small it is right. Now because there is still a continuous flow technique, but your flow cell is small in size; that means, the amount of sample you will be using is also small; because if volume is small right. Now what do you do is because this is the protein plus denaturant, you have to look at refolding what you do is from these two sides you flow in what?

Student: (Refer Time: 47:57).

Buffer. So, see what happens ok. It is like if you are traveling in a crowded train or a bus, you are standing here, initially the bus was pretty empty. Now it is very crowded initially you are spreading your lengths like this, now people come from all sides say two sides and then you just pushed like this right.

So, the space you occupy is small right now the same thing is happening here. Initially these two side channels were not there the flow would be like this ok. Now what are you doing is, this is the protein and denaturant; now what you are doing is you have two channels on these two sides, you are also flowing buffer from these two channels.

So, what will happen? It will press squeeze this small; now remember these are flow techniques, this is a rapid mixing technique right. So, mixing depends upon the distance over which you mix. So, the smaller the distance over which you will mix, the smaller the distance

over which you will mix the better it is for you the faster you will mix it. Now see what happens, initially the flow was like this right this was your flow this was the breadth of the protein flow.

Now, what happens? You see the mixing flow, it mixes if this is whole of 10 micron over what dimension does it mix? It mix say about 2 or 3 microns is it is it not really small? So, the actual mixing is occurring over what? Just a very small area and after that the solution flows like this.

So, this is referred to as hydrodynamic focusing; that means, you are focusing it pretty hard from the sides on a channel which is go like this, which is going like this and you are squeezing it into a thin stream. I will tell you in the class tomorrow can you imagine what the dead time is achieved in this case? Guess.

Student: (Refer Time: 50:02).

What is the dead time any guesses? If you come very close a coffee.

Student: Less than 45 microseconds. .

You give me a coffee now ok. Now you treat me with a coffee that was a bad guess. Everyone knows it is less than a 45 microseconds ok. So, all of you take note of this point yeah, any ideas? Come on come on the faster you tell the faster you go come on.

Student: 1 microsecond.

Student: 1 microsecond.

1?

Student: 5 microseconds.

[FL]. So, you get a coffee and a sandwich it is 5 microseconds. So, it is 5 microseconds. So, that is the fastest you can mix it, the mixing dead time is 5 microseconds and after that you can look at ok. So, this is called microfluidic mixing.

Student: (Refer Time: 50:58).

People are really doing it nowadays.

Student: (Refer Time: 51:00).