Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 34
3D NOESY HSQC

So, in the last class we looked at how distances are briefly extracted from 2D NOESY and there know a best idea that, NMR structure determination by NMR is basically a model building approach.

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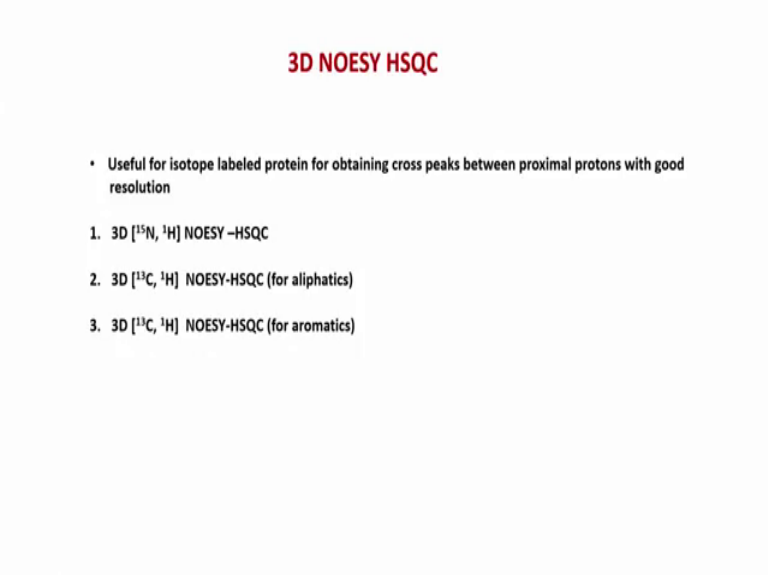

So, now let us look at this 3D NMR experiment, called 3D NOESY - HSQC which is basically very important experiment when it comes to proteins. So, if you recall in a protein we do isotope labeling. So, if you have an isotope labeled protein, then we have to obtain distances based on these experiments in the 3D NOESY that means, you need an N 15 labeled protein, and a C 13 labeled protein. So, there are three types of this 3D experiment. We will see the pulse sequence shortly. There are three types of 3D NOESY experiments which are routinely used in protein NMR.

So, one is called N 15 NOESY – HSQC, C 13 NOESY – HSQC. And now in C 13 we have two types; one is for the aliphatics pin systems, that is for the side chains of hydrophobic hydrophylic amino acids. And you have another C 13 NOESY which is for aromatics pin system that is in the case of phenyl alanine tyrosine cryptofan and so on.

The reason these two are different because, the chemical shifts of aromatics and aliphatics are widely different both in terms of proton and in carbon. In carbon aromatic comes around 140 to 130 ppm whereas, aliphatic comes between 0 to 60, 70 ppm. So therefore, it is difficult to do both aliphatic and aromatic HSQC NOESY or NOESY HSQC in a single experiment. Therefore, these 3D is now split it into two parts, one is exclusively dedicated for aliphatics and one is dedicated to aromatics.

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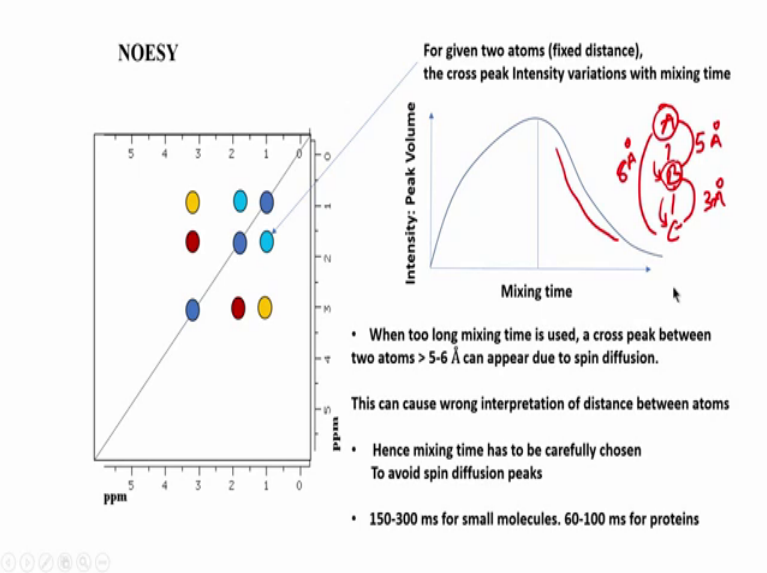

So, before we go into this, we should recap one thing in NOESY, which i had mentioned briefly yesterday that you have to now look at this intensity versus mixing time. So, what is the intensity concept here. So, this is a schematic of a NOESY spectrum. This all of us know this is a 2D NOESY where this is called a diagonal peak, and these are the cross peaks between the different hydrogen atoms. So, now if I what happens to this intensity? There is a volume remember when I say intensity it means peak volume as shown here. So, now, as you vary the mixing time in a NOESY experiment, the peak volume will keep increasing. But after some time the peak volume starts decreasing.

Now this is very interesting why does it have to decrease, increasing the mixing time? The reason is, that when in a long mixing time is used what happens is, a cross peak between two atoms starts appearing, even though the two atoms are not close in space. Means they are greater than 5 to 6 Angstrom, but still you will start seeing a peak. Why do you do have, why does that happen? That happens because of a phenomenon known

as pin diffusion. So, let me illustrate with a picture. Suppose i have an atom A and B and C. Suppose the distance between this is 5 Angstrom distance between this is 3. So, the total distance between this is 8.

So obviously, between A to C i do not expect any cross peak. But from A to B I am getting a cross peak, and from B to C I am getting a cross peak. So, what will happen is, when I excite A although C is far away it can first transfer to B and if I use a longer mixing time, slowly B will get transferred to C ok. So, this is why the B intensity starts decreasing like this. Because it is now whatever had come from A is getting passed on to C whatever means the population, the polarization. Whatever had come because of the close distance is getting further transfer to C. So, the intensity of B or the cross peak between A and B stars decreasing. But cross peak between A and C starts increasing. Because now the a magnetization is going to C through B. But A and C should not actually directly transfer, because they are far away.

So, this intermediate spin is now helping to transfer from A to C. But then, the cross peak between A and C will start appearing in a spectrum, and that will tell us, we will get that will be misleading. Because we will interpret that this cross peak means it is less than 5 Angstrom, and that is not correct because that could be because of spin diffusion. So, how do we know that a peak is because of spin diffusion or a correct peak? The only way is, that you mix vary the mixing time and see if the peak intensity is decreasing or increasing. Ok. So, if the peak intensity in the initial mixing time is increasing and later it starts decreasing, then the peak is basically going to spend diffusion peak ok.

So, this is a unfortunate problem this happens very many times. So, typically this 5 what we do is we try to restrict the mixing time to smaller values. Typically for proteins you do not want to go beyond 100, 80 typically is the average used, for small medium sized proteins. 60 millisecond mixing time is used for a larger protein. But not more than 100, because then more than 100, the peaks which you start seeing could be because of this effect, and that will be misinterpretation of data. For small molecules, that is organic molecules which we are not looking at in this course. But there you will have to use a little longer mixing time, and typically that is in the range of 150 to 300.

So, therefore, as mentioned here mixing time has to be carefully chosen to avoid spin diffusion peaks because this can cause wrong interpretation of distance between atoms.

Because you may think that this is somewhere 5 Angstrom. Because they are there is a peak between A and C. But actually it is not it is a longer distance. So, your structure will become wrong. So, this point has to be carefully with experience it comes, and mixing time has to be appropriately chosen.

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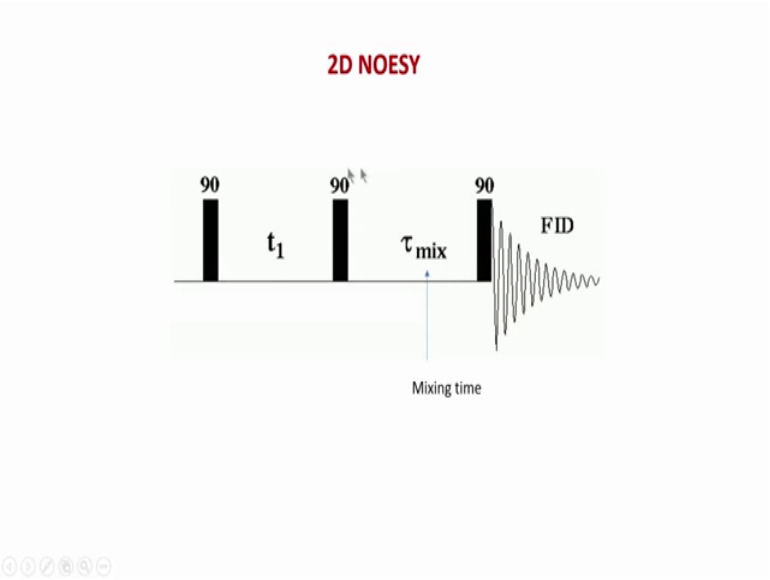

So, now let us look at the pulse sequence of a 3D NOESY. Before we look at 3D NOESY, we will again look at 2D NOESY first, because all 3D experiments remember are based on 2D. So, 3D experiments are essentially an extension, of 2D NMR experiments in general. So now, let us see 2D NMR, 2D NOESY experiment. Here we saw that you have 390 degree pulses. The first pulse brings a magnetization to the x y plane and then the magnet spin starts evolving. During that period they we gather the chemical shift, of the each nucleus, hydrogen atom, that is, cosine omega t 1 or sign omega t 1. Then this pulse inverse of magnetization and it goes to z axis or minus z axis. All the protons, this is something which am repeating or recap from the previous part of the course.

So, am not writing it here, am just mentioning it that you invert the protons and then when they go back towards z axis, during this mixing time that is tau mix, they start transferring the polarization to each other. The transfer of polarization is called the Noe effect. Now that transfer from A to B or B to A or A to C and so on. Is then whatever has transferred is further excited, with another 90 degree pulse because remember during this

period it is a long z axis. So, we cannot detect a signal unless we bring it to the x y plane and that is brought by this 90 degree pulse. Then we detect the signal and that is a final detected signal. So, what is important is, the polarization transfer which happened during this period, is captured in the cross speak between t 1 and t 2.

So, this was a basic idea of NOESY, 2D NOESY which have done a very brief mentioned, you can go back to the first part of the course or read the several standard books which gives you a very good detailed explanation of this pulse sequence. So now, let us see from here, how can I extend this pulse sequence to a 3D NOESY experiment in proteins.

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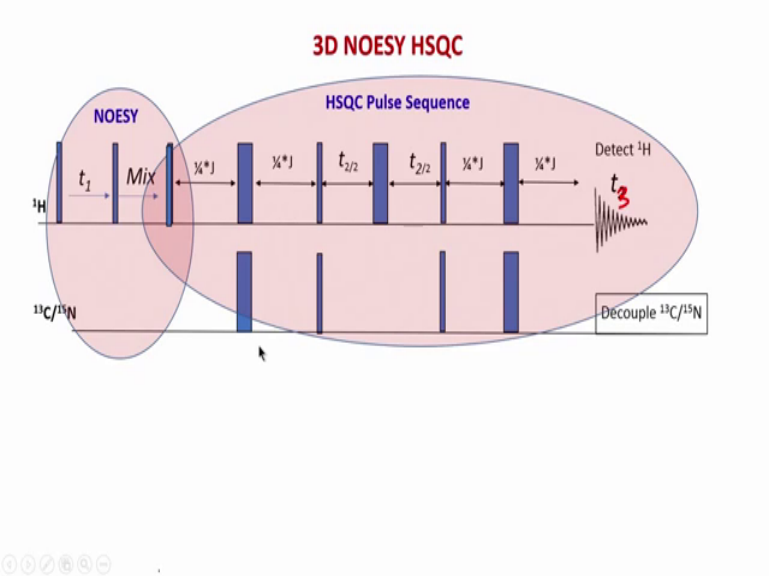

So, what we do is the following; we start from the same experiment first. So, this is the same repetition of the last slide 390 degree pulses, it is a t 1 evolution and then a mixing time. Then what I do is, after I bring this to z x y plane. This last pulse remember bringing some magnetization to the x y plane. This is a NOESY part. we then continue with HSQC part. So, this entire part here is an HSQC pulse sequence. So, if you recall the HSQC experiment, we discussed again in the last part of this course, second first part of the course, we saw that HSQC is basically through inept. First you have the inept part, then you have the evolution part of the hetero nucleus. Now this hetero nucleus can be either carbon or nitrogen. It can be anything, does not matter if there is carbon nitrogen.

This same idea is applied to both, and then we have the reverse Inept, and we detect the signal. Finally, so this is actually t 3. So, let me correct this here, this is t 3 because, there are three dimensions in this experiment. The very first is t 1, that hetero nucleus evolution here is t 2 and then proton evolution again because you are detecting protons is t 3. So, let me explain now how things go in this figure how there is a magnetization is taken further or taken let us start from this picture here.

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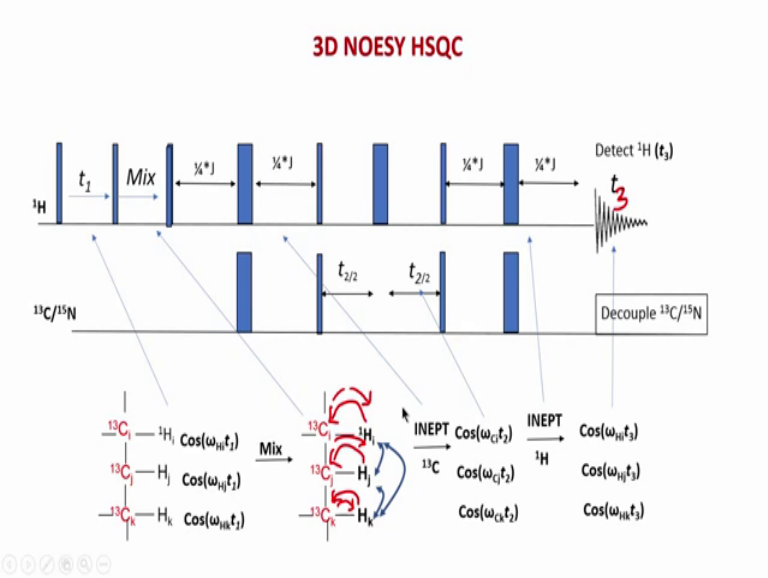

Now, let us go back to this. So, when we excite the proton, by 90 degree pulse let us say this is our molecule, this is a schematic drawing of a molecule. So, this is hydrogen i, j and k. So, when I excite the protons first by this pulse. This first pulse here all the hydrogens are excited. Because this is not a selective pulse, it is a pulse which is applied to all the protons. Say all of them are excited, and they start evolving during this period in with their chemical shift.

So, it is called cosine omega, it can be sign also it does not matter. But am just writing it as cosine; so, cosine i H I, H j, H k, t 1 each of this is during t 1. Now during the mixing part this magnetizations are now exchanged between the different hydrogen atom. So, H j will give some 5 percent of its magnetization to H I. H i will give 5 percent to here, 5 or 6 or 1 percent.

All depends on the distance between them. It depends on the strength efficiency of transfer between them and so on. Smillarly H i and H k will also transfer to each other.

So, they will exchange some few percentage of magnetization to each other. But remember 90 percent is kept with themselves, 90 to 95 percent of magnetization is actually never given to anybody. Only 5 percent exchange goes on, even less than 5 sometimes.

So, this is this tiny tiny transfer between magnetization is what we try to detect. Now, after the mixing is over, then we have come to the HSQC part. In the HSQC part you have this INEPT what is shown here where we transfer the magnetization to this own carbon. So, let me write down here we are transferring to directly attached carbon ok. Now once we do that, we can then excite this carbon the here and during this period t 2, we evolve the carbon now; so, i j k because all three carbons are now coming from this respect to j j k.

So, all three will evolve during this period. Then during this last part that is reverse INEPT, we transport it back to proton. Again there is a slight correction here, it should be t 3. So, we transfer it back during this Inept to proton and what you detect during t 3 is again back to this proton so, here what we have done is we have gone back to this proton. We have gone back to the proton. So, we had only come to carbon to get the chemical shift value.

Once that is done we go back to proton, the same protons from where it came; so, i j and k. Now, in the next slide I will show you how we can get the correlations? Now what kind of information and correlation we get in a 3D NOESY spectrum. But we have to know carefully look at this slide understand, how the magnetization has gone from one nucleus or one spin to another nucleus or a spin ok.

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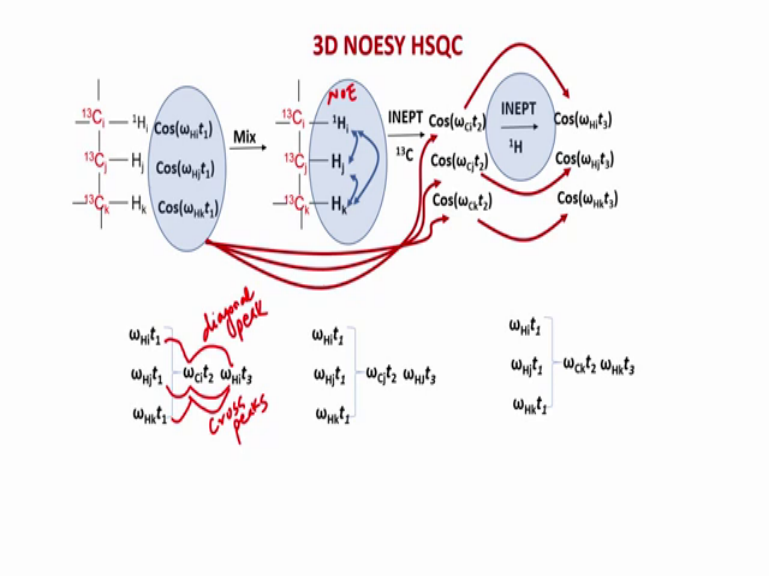

So, this is again the last part that is this part what I showed in down, bottom is repeated here ok. But one thing if you recall, in a NOESY our goal is not to transfer magnetization within amino acids, for example, if this is one amino acid, we are not interested so much in within the atom like this, within the amino acid. And why is that because, this can also be done with a TOCSY. If you recall TOCSY achieves magnetization transfer within a network of coupled spins, that was a definition of TOCSY, and that is this is a network of couple spins means a family and each amino acid we saw is like a family of protons.

So, we do not need this transferred through NOESY, TOCSY can do that. What we are actually interested is basically a transfer to a long (Refer Time: 15:20). So, let us first understand how this transfer has happened. So, you can see that this is the three hydrogen atoms i j and k within the amino acid ok. That has now transferred to this. But because of this inter atomic transfer here, what has happened is whatever was on the H has come to I, whatever was on the j has also come to I, this is because of the NOESY.

So, let me write down here this is because of the NOE effect. So, because of the NOE effect all of these have exchanged their magnetization. Therefore, all these three terms, are transferred to H I. Because H i is of course, the same as H i. So, it is the same as I said 90 percent is with H i, but whatever came from j this would have come from j. This part because this modulation is transferred to j, from j to I; similarly this modulation from k to i so that means, this entire three, are actually transferred to I so, therefore,

when i is evolving a C I. These three now are connected, or correlated with this C I. Then similarly this three will be also correlated to this C j. Why?

Because again if you see here H i is transferring to H j, H k is transferring to H j and H j is retaining 90 percent with its own so, again all the three are transferred are kept on H j and when H j evolves, this is omega C H j term will be connected to these three terms, and same thing for the H k or C k. Because again H k retains 90 percent on its own that is the diagonal peak, but the cross peak are coming from i to k, j to k. So, these modulations that is this terms, are connected to these term also. So, all three with all three. Then each of this gets connected with this that is through Inept. So, that is just C i to h I, C j to H j, C k to H k. Here there is no cross terms, cross meaning there is no term between C i and h i or between C j and H i because this is Inept is only direct transfer ok.

So, you see this is how what happens that all the three hydrogen atoms here, the modulations are connected to each of these three. And each of these three are connected to their respective protons. So, if you look at this in this manner, we can say that we have co-related. So, you remember this word correlation. So, we have co-related the chemical shift of i, j and k, with C i and H i. So, this is the first arrow here, if you follow this arrow this first arrow this here and this here. Then we have also correlated again the same three nuclei with C j and H j, which is the second arrow here. And we have also correlated again the same three atoms with C k and H k. So, you see we are correlated all the three with all the three each other. Ok.

So, this is basically the idea that you have correlating with each other. So, you are getting all possible combinations of connections ok. So, these correlations so, this can be thought of as correlation omega, omega, omega i, omega j. Correlations will be seen in the spectrum as a peek. So, when we say a peek in a NMR spectrum, a peak can be diagonal peak or it can be a cross peak. So, what is a diagonal peak? Diagonal peak is a peak, where you have the same repeated two times or three times you can see here H i, H i is repeated. So, this correlation, that is this correlation becomes a diagonal peak. And these correlations, and this correlation they are cross peaks. This is very important to keep in mind, what is a diagonal peak and what is the cross peak?

So, now what is the advantage what is it why it is big deal? The big deal here is diagonal peak do not carry any distance information, in a NOESY spectrum. Why? Because, they

are correlating one atom with the same atom. So, there is no question of distance between the same atom to same atom. But cross peak carry information of the distance because the intensity there will now depend on the distance between H i, H j. For this case it will depend on distance between H i, H k. So, you see the cross peaks are important and not the diagonal peak. But we cannot help, we diagonal peak will always be present because the transfer does not take 100 percent, 90 percent to 95 percent is retained on the same atom. Similarly in this combination here you see H j, H j here so, that is a diagonal peak, but these two are the cross peak these two combinations are giving us the cross peak.

In the same manner here H k and H k this combination is the diagonal peak, whereas, these two are the cross peak. So, therefore, one should be able to figure out what is a diagonal peak what is a cross peak and therefore of course, in the spectrum you can very easily find out because a diagonal peak will be a huge peak about 90, 99 percent in intensity compared to a small cross peak which will very weak. So, in principle you do not need mathematically to know, but its good to understand from where diagonally cross peak comes. But in a spectrum it will be very straight forward very easy to identify what is a diagonal peak and what is the cross peak.

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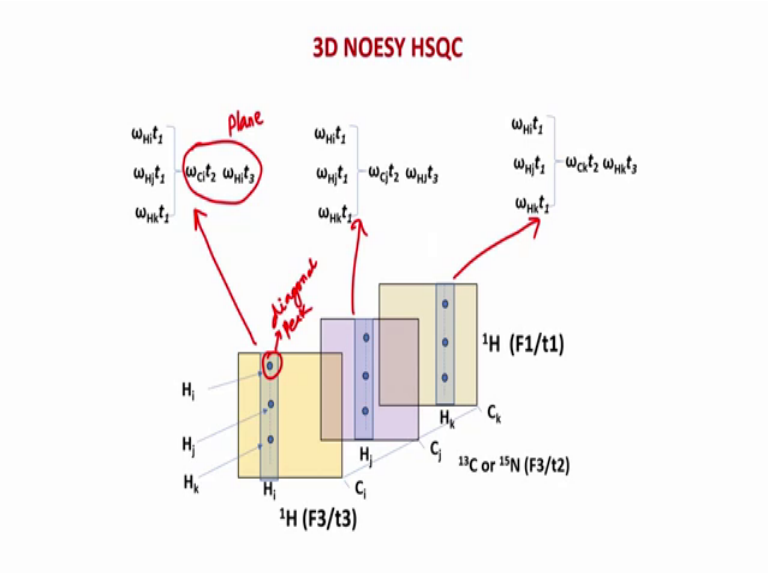

So, this is how the NOESY spectrum will come in a 3D manner this is what I should. As I said we are only illustrating for a single amino acid or within the amino acid I will

show you shortly for a longer distance how it works. I mean for a cross peak to a remote atom or long range atom. Right now let us focus what we get within an amino acid. So, as I said this information can also be obtained from TOCSY but am using this only to illustrate how does the NOESY work, 3D NOESY work. So, you can see the 3D NOESY spectrum will be something like this, where you have three dimensions so H, H 1 will be the 3, 3. You remember F 3 means frequency or time domain is what we saw in the time domain part, but in a spectrum it will be always frequency.

So, this axis is carbon, but remember it can as well be nitrogen. Why? Because if you go back in this program let me go back to this pulse program here it can be i nitrogen or sorry carbon or nitrogen. So, I can do the same thing all the things same for nitrogen also, so everywhere carbon will become nitrogen. So, there is no reason why it should be only carbon or nitrogen the only differences will be in the INEPT part, because if you recall Inept depends on the j coupling. So, j coupling between carbon and hydrogen is different from j coupling between nitrogen and carbon.

So therefore, that is a j coupling difference we have to tune for the difference in carbon and nitrogen. But the mixing time will be the same whether it is carbon NOESY or nitrogen NOESY the proton-proton mixing time will be same. So, this is how the spectrum will look like approximately, and now you can see for each plane this is just for example, for these three atoms. If I am looking at the H i chemical shift am looking at this part here. So, this basically let me show you a correlation. This corresponds to this, this corresponds to this, and this corresponds to this ok. So, for this combination here for a given a H i in a t 3 value look at this here H i in t 3 and C i in t 2, C i in t 2 for that combination that is that is a plane. So, we can say this is like a plane.

This is a plane we are looking at, then in that plane we will get three chemical shift along the F 1 direction or t 1 axis which is why because it is t 1. So, all the three chemical shift i, j, k are coming. So, you can say that now here which is a diagonal peak? This is the diagonal peak. Although it does not locate diagonally, but this is a diagonal peak because it is a H i and H i in the same plane. Now if you look at this plane here, this corresponds to this set of correlations. And in this set this now C j, H j plane, so they are in the C j, H j plane. In that plane am seeing along this axis along this H j chemical shift three peaks again coming from i, j, k. Here i, j and k. Similarly for this plane, am getting correlations to i, j and k but I am in the C k, H, k plane am in the C k, H k plane ok.

So, this is how the correlations are near is seen in a NOESY HSQC. Now we can analyze this in a strip manner. So, we can cut out strips like this and put displayed in a computer and also analyze like that. So, this is how 3D NOESY HSQC's are analyzed in a stripped manner.

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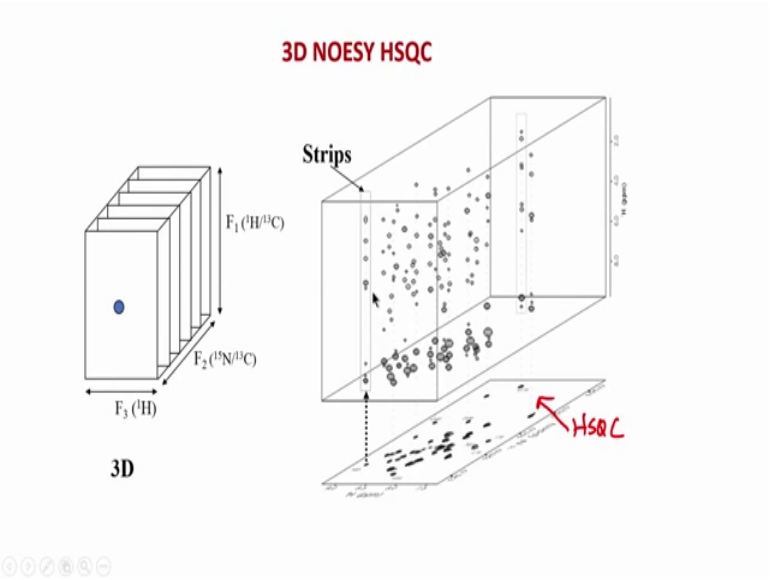

And this is something will we will see like in a 3D spectrum. So, this is how it is. So, this is what I showed you, the 3D plane board and this is some spectrum of a real molecule. So, the NOESY HSQC of some molecule is like this. So, what we do is a base. So, if you look at this here, look at this. F 2, F 3. F 2, F 3 is nothing but an HSQC. That means, if I take the top view, a top view of this projection of the spectrum it is nothing but a HSQC. So, I can write it here it is nothing but, then HSQC. This plane is in HSQC. What kind of HSQC? It can be either carbon HSQC or it can be nitrogen HSQC, and along this dimension, in the third axis I am seeing cross peaks.

So, if you recall in the previous slide we looked at H i, H j, H k etcetera and that is what is H i, H j shown here ok. But one thing if you notice again and go back to this slide as I mentioned we are all looking within the same amino acid, example was shown like that ok. So, I said that this information this spectrum also can be obtained in TOCSY HSQC. So, TOCSY, if I take a TOCSY HSQC 3D which we saw in the 3D part in the previous part of this course; the first part we saw the similar pattern is obtained there also. So, then what is the difference and difference actually is in a NOESY we will see additional

peaks. Not only the peaks from one amino acid, that is the same amino acid we will also get peaks from far away amino acid, because of the noe effect. And that effect is something what we are looking for. We are not interested in getting peaks from the same amino acid.

But we are interested in getting peaks from the neighboring amino acid, from far away amino acids because, those are the ones which carry the distance information. And this is something which we will see in the next class that how far away amino acid use correlations two peaks in the same amino acid spectrum and 3D NOESY spectrum and from there we will see how we can extract distances and calculate the 3D structure of the protein.

We will see that in the next class.