Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 06
Basic concepts in 2D NMR spectroscopy

We now move on to the second part of the course from 1D NMR to 2D NMR spectroscopy you looked at how briefly how 1D NMR is data is recorded, how the pulses are applied. Now let us look at how 2D NMR is conducted or perform, 2D NMR experiments are very crucial for biomolecular structure determination. In fact, they found the backbone of all the experiments and then multidimensional experiments from there we can go to understand how 3D is build 4D and so on.

So, let us start from a very basic idea why do we need 2D first of all. So, what are the limitations of 1D NMR which give rise to the idea of 2D NMR to begin with.

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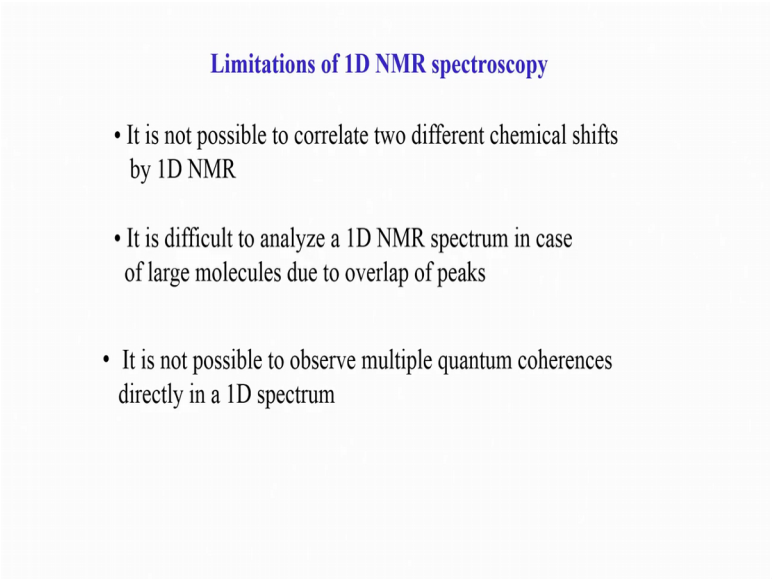

So, one thing is in 1D NMR if you have noticed we cannot correlate two different chemical shift. So, what do you mean by correlation? Correlation basically means let us say I am seeing two different peaks from the same molecule. I will never come to know those two peaks are actually belonging to the same molecule unless I do some special

technique that is in 1D or if I do 2D I can directly get the connection that these two peaks which are from the same molecules are actually correlated or connected to each other.

So, this is called chemical shift correlation between two or more different peaks belonging to a given molecule. So, that correlation is not possible to achieve by in a simple 1D NMR experiment. What 1D NMR gives us, if you have a single molecule it gives us a spectrum, that if I have a mixture of molecules then I do not know which peaks belong to a individual molecules. So, in a mixture typically in protein biomolecules we have amino acids with the proteins, polymers, linear polymers, chains of amino acids and each amino acid which a molecule. We can think of it has a molecule and if I have 100 amino acids I have 100 molecules in one protein chain and each of them will give different set of peaks.

So, how do I know which peak belongs to one particular molecule or amino acid and which set of peaks belong to another amino acid. So, that kind of a correlation is not possible to do you get it in 1D NMR. And the second is that 1D NMR becomes very crowded or become starts overlapping in the case of large molecules. So, when we go to proteins we will see the spectrum is really very complex, meaning the peaks are largely overlap. In the case of methanol or ethanol or very small organic molecules you basically get half a dozen peaks or 7 or 8 peaks, but when you come to large polymers like protein nucleic acids the number of the peaks increases enormously.

So therefore, it is not possible to simply take a 1D spectrum and analyze of a protein; you need to go for 2D NMR and also 3D NMR. So, a third reason which is a very important is that when you look at NMR and 1D NMR we are exciting a nucleus from alpha to beta state, but that is a single quantum transition. We cannot generate multiple we cannot observed, if I do a multiple quantum transition I cannot observed directly NMR. Because, NMR spectroscopy by principle you can only look at single quantum transition or single quantum coherences.

So, this is a slightly technical word. We will not going to detail in this course on multiple quantum NMR. This we can ignore this because, in biomolucules multiple quantum experiments are less sensitive. So, will essentially the two main reasons why we need 2D NMR or 3D NMR for biomolecules is these two that is to correlate different chemical shift two different or more chemical shift and to resolve overlap or get more resolution.

So, let us look at some schematic examples of how these two objectives that can be achieved.

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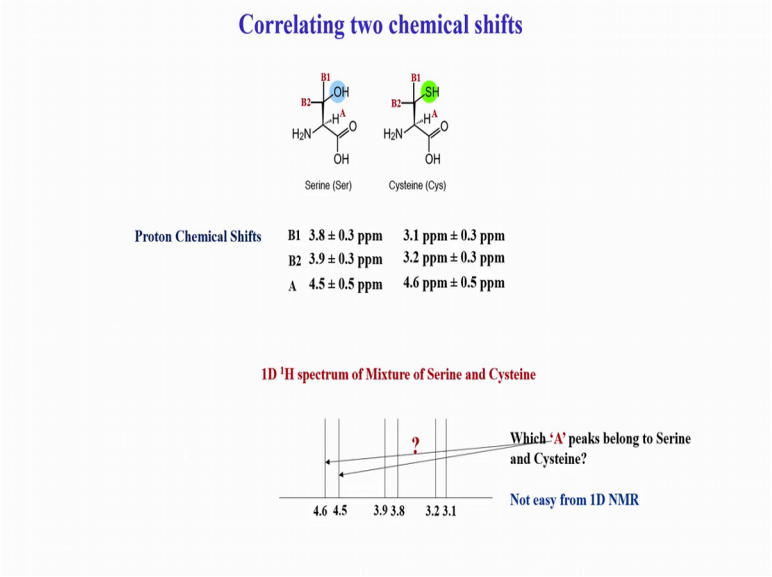

So, let us take an example of 2 amino acids: serine and cysteine. So, this is a very two popular amino acid, they are present in almost all proteins. So, let us say we have say now, if you look at the structures of these two molecules they are pretty similar accept instead of this sulfur here there is an oxygen here. So, as for as structure is concerned and chemical environment of each hydrogen is concerned you expect them to be quite similar, only the difference will be the electro negativity effect of the oxygen versus sulfur. So, these are the values which statistically we have been observed in proteins when you have serine and cysteine.

So, you can see for example, for these two proton there are little bit no downfield shifted in the case of serine because, of the oxygen. They come at 3.8, 3.9 again there is a spread roughly and there is also a chemical shift here there is a spread here, but they are little bit of field and they come around 3.1 and 3.2. I am talking about this B 1 and B 2 and remember these two are not chemically equivalent; these are two different hydrogens. Now if you look at the H alpha we use the word H alpha for hydrogen attach to C alpha here, that H A now comes for these two are almost similar.

You cannot distinguish the 2 molecules based on your H alpha values, but B beta values can probably we use, even H beta value sometimes can come very close. So, this is an

example of a just stick drawing here showing the 3 that the peaks from the serine and cysteine here is shown the beta 1 beta 2 of cysteine, here is a beta 1 beta 2 of serine and here is alpha 1's alpha proton. So now, the question is suppose you record a spectrum of a mixture of serine and cysteine why mixture because, remember other side in a protein you always have serines and cysteines in the same molecule. So, you can think of it as having a serine molecule and cysteine mix together in the same protein molecule.

And therefore, when I record a spectrum of a protein it the serine and cysteine and all other amino acids all will come in the same spectrum. So, let us say have a mixture of just two of these serine and cysteine; how do I identify that this A peaks corresponding to this A here which one corresponds to serine and which one belongs to cysteine. How do I find out? This range given to me here is quite similar, you can see within the standard deviation that is error they almost overlap. So, there is no way we from 1D NMR I can say that this is serine or this is serine. So, this is not easy from 1D NMR. So, this is where 2D NMR is helpful. So, how does 2D NMR help us in this particular case?

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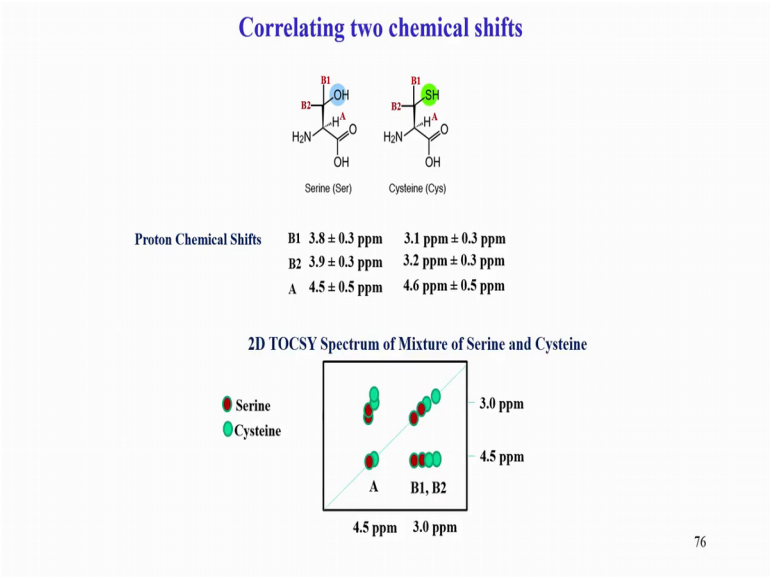

This is using any special experiment called TOCSY or COSY, we will go through that as we go along. Right now, I am just directly giving you an example where 2D NMR helps us to resolve the peaks. So, which you can see by co relating to chemical shifts now what is happening is this 'A' which was shown here they are very close and they are very close here, but in a 2D NMR see they gets separated out.

So, this diagonal will see later is like a 1D. So, this diagonal peak what your diagonal line you are saying here is nothing, but this 1D spectrum here. So, you take this 1D and tilted by 45 degrees, you will end up with this line. So, but so, in the 1D as I said we cannot figure out what is which is which, what if I do a 2D experiment I can separate the two and I can say yes this looks like a field shifted.

So, it is belongs to cysteine, this green colour peaks and this peaks which is red colour corresponds to serine because, that is more downfield. So, how did I know that, because I correlated means, you this remember this word. I connected the chemical shift of the A proton with it is respect you B proton. So, by establishing this correlation I am able to separate or identify serine separately from cysteine otherwise, with just 1D where there was no such correlations to a just peaks alone I could not do that; I would not have been able to do that.

So, this correlation in 2D NMR from 2D NMR or higher dimensional NMR is therefore, very useful for resolving the peaks and also for identifying which amino acid we are looking at. This pattern as we go along with see the pattern becomes unique also for a given type of molecule.

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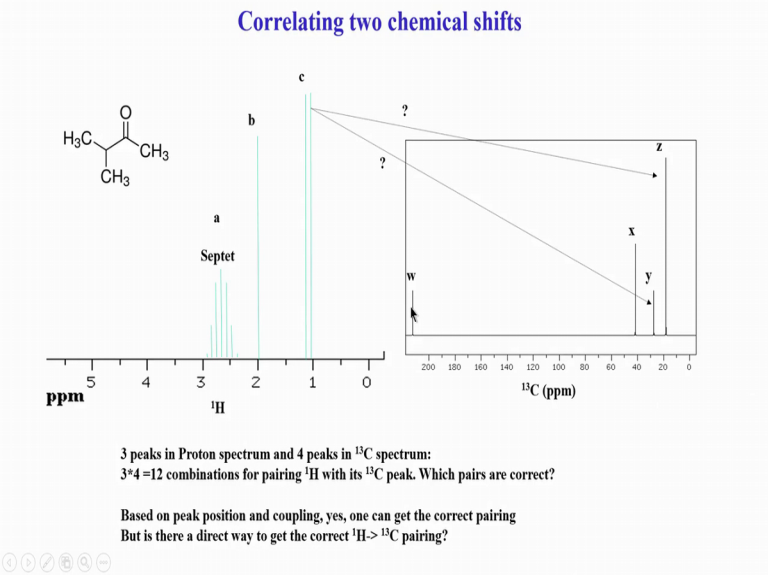

So, this is where 2D NMR helps. This is another example of correlating chemical shifts. So now, let us see this molecule. So, let us see I have recorded a 1D spectrum of this molecule, I will get a very simple spectrum. This is proton spectrum. So, from a proton

spectrum very straight away you can identify which peak is which, this example was taken up in the previous course. So, we will not going to the interpretation of the spectrum. We already will assume that we know which peak is which because, this is a very simple case. Now, for the same molecule if I record a carbon 13 spectrum I will get these peaks. Here you can see there are 3 4 carbons, there is a carbonyl, these methyl these two are equivalent. So, essentially you have 4 carbon peaks which is seen here.

Now my next problem is, how do I correlate which methyl proton here there are 2 methyls: this is one methyl and there is also this methyl. So, how do I know this methyl, these are this methyl is coming because, of this doublet here this CH 3. This methyl carbon does it proton sorry, this is a proton whether this methyl proton does it connect or correlate with this carbon 13 methyl peak or this carbon 13. That information is not there in the separate spectrum which have recorded. Of course, one may say that with experience now, you can always connect which is which because you will know the based on the peak position and coupling yes, you can get the correct pairing because, these two this is very simple example.

But, in generally if you look at in a principal in how do I know which proton and which carbon attach carbon. So, we are looking at this proton separately in a 1D, we are look at this carbon separately in a 1D. How do I connect that 2 chemical shift and that is where 2D NMR plays a important role we use a what hetro nuclear NMR in this case.

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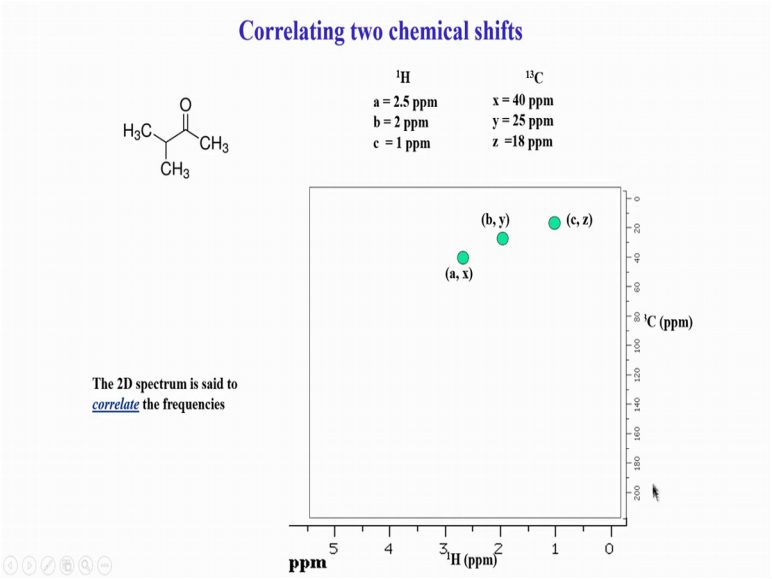

So, this is a hetronuclear NMR spectrum. So, this is a 2D spectrum is set to correlate these 2 frequencies. So, this is again and example we are jumping ahead I am just showing you some 2D NMR spectra, but we will see the principles of how this is generated, how does it work and so on slowly. So, this is a 2D NMR spectrum we get chemical shift correlation, which means I am connecting the chemical shift of one proton with the corresponding carbon to which it is directly attach. So c, z here is one carbon proton pair that is one of this carbon proton pair, now similarly the other carbon proton pair will show a another point in a 2D plain.

So, this is like a simple graph and in a graph I am putting dots. So, one dot has x axis value, and another also perpendicular to it is a y axis. So, this 2 chemical shift are now connected to each other by a correlation. Similarly, here you can see between b and y I am getting a peak and then between a and x I will get a peak. So, you see 2D NMR therefore, helps in connecting the proton chemical shifts with their corresponding carbon partners. So, remember that is important point. This could not have been possible, if I have recorded a proton spectrum separately and a carbon spectrum separately. The connection between the partners is only possible because of the 2D NMR experiment.

So, here you see this axis now is a carbon 13 axis, carbon 13 chemical shift and this axis is hydrogen proton. So, this therefore, we call it as a hetro nuclear means two different nucleus, whereas the previous example which I showed was a homo nuclear experiment is both hydrogen and here also it is hydrogen. So, let us look at some more examples later.

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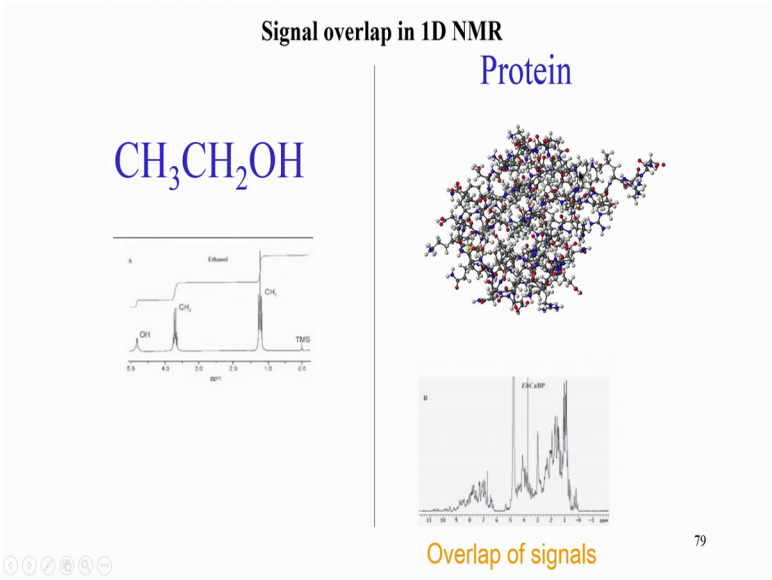

So, this is a case. In case of proteins what happens is there is a lot of signal overlap and that is why as I said we need 2D NMR or in fact, 3D and 4D also comes into picture when you look at very complex biomolecules. So, this is an spectrum of ethanol which all of you know about this a simple 3 line spectrum. But, now if I will show you a spectrum of protein you see this is really complex and you can see here the signals are highly overlap. This is like complete bunch of peaks here. I can hardly even count the number of peaks and why is it so complex, because this is a hydrogen NMR proton NMR spectrum and every peak here corresponds to an every atom in this drawing.

So, this is a ball and stick model of a protein structure 3-dimensional protein structure. So, we can see all this grey coloured atoms belong there hydrogens, these are all hydrogen. So, each hydrogen will give rise to one peak in the spectrum in this spectrum. So, if I have thousand hydrogens in this protein I will end up with thousand peaks here. And why a each peak different because now, each hydrogen has a unique chemical environment. There some of they may be equivalent, some are not equivalent and then there only all different environments, none of the two are in the same environment. So therefore, they have different different values, but when you combine all of them they become a big overlap of peaks.

So, this is the problem with 1D NMR, in case of biomolecules we do not we cannot deal with 1D NMR, we can only look at the spectrum and say a few things. Because, 1D is

not so useless in protein and biomolecules you can definitely use it for initial testing of the sample. But, beyond that for one complete structure analysis it is not useful. So, now coming back to this; let us say another reason why we need 2D NMR is this one overlap. So, this is something which we did not deal with, we looked at correlations. Now let us look at an case of an overlap. What do you mean by an overlap?

Let us say that two peaks again the same example of serine and cysteine in a protein; let us say those two peaks are exactly in the same position. So, you see this peak actually there are two peaks here, but you do not see it as two. You are looking at it, it looks as one. Why does it have a look as one, because the chemical shift values of H alpha they are supposed to be slightly different. But, because the error range is also high there is a possibility that the overlap or they meet exactly at the same place.

So, now in this spectrum you cannot say which one is you may think there is some missing peak because, is only one here but actually there are two here.

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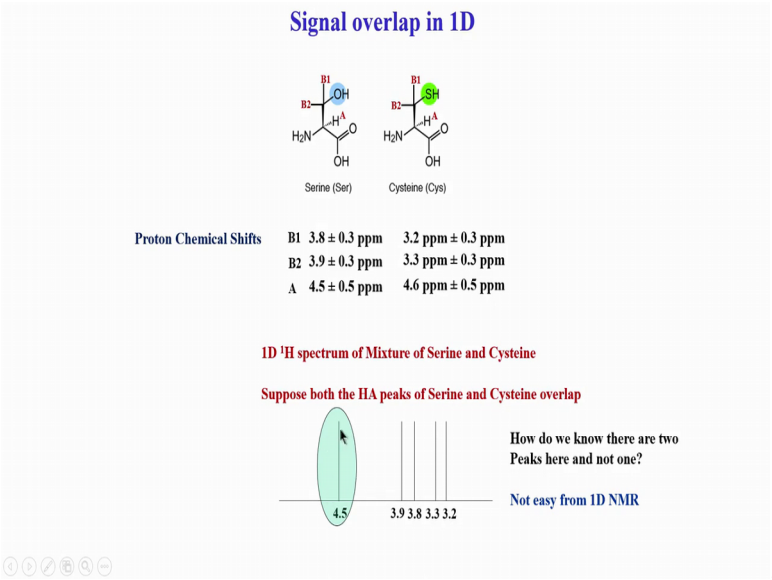

So, how do you identify that we cannot do it from 1D of course, intensity of this peak may become double because it are 2 protons, but that is sometimes not always easy to understand look at. So, we therefore, need a 2D NMR again here to figure out the there actually 2 H alphas here. So, this is what is done again with a 2D NMR possible with 2D NMR. So, the same tocsy spectrum which I showed earlier it is shown here, but I here

what is happening now if you look along this line, we can see that this A they are both serine and cysteine had the same value so, they are overlapped here.

But when I go up again because of the correlation I am able to separate the two because established a correlation between A and B and I am able to see there are two different types of peaks; that means, there are 2 molecules or mixture of there is a one serine here, one cysteine here, because I am able to go along this direction and correlate them with the other chemical shift because these are separated. If B 1 and B 2 if they were overlap then I would have still have the problem because, if A and B overlap I mean a overlap cysteine and serine, if A overlaps and B is also overlap then would not have been possible even with a 2D NMR.

But, fortunately because B 1 B 2 are different and H alpha H A was same by correlating them to the respective B partners I am able to separate them and identify them as 2 different molecules here. So, this is another advantage of 2D NMR how it helps in resolving the peaks. So, let us move on further to 2D NMR, how it is actually performed in a spectrometer.

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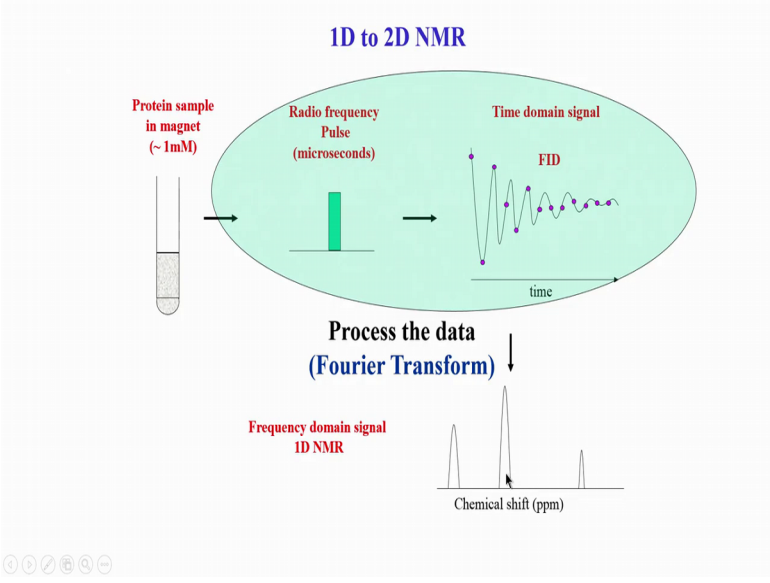

So, this is the 1D spectrum recap we saw that in 1D NMR experiment you take the NMR sample you apply radio frequency pulse you get an FID which is a free induction decay and the new Fourier transform and you get a spectrum. So, this is as per as 1D is concern, but in 2D we do not stop at this we have to now correlate two different

frequencies. So, that is very important point here with one single FID, I only get a set of frequencies I do not get the connection between this frequency and this frequency or this frequency and this frequency by doing by simply doing one FID.

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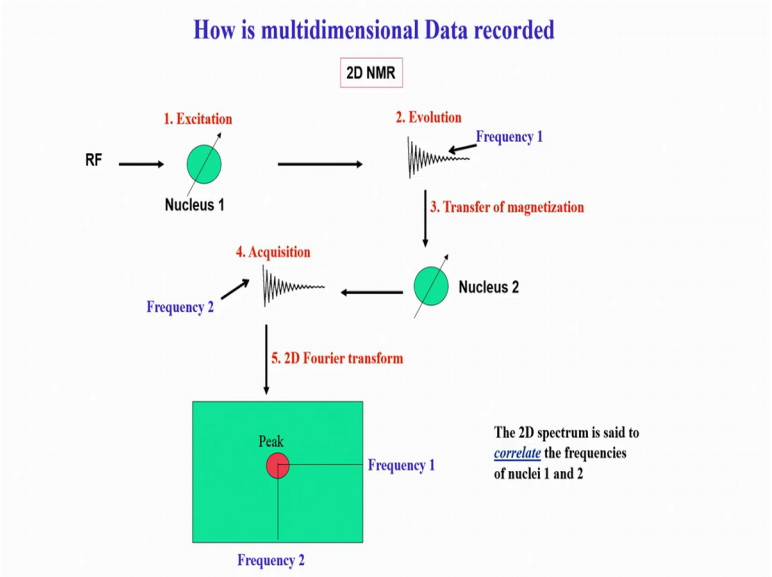

So, how do we do the 2D NMR and this is what is shown here. So, you start from first apply in RF this energy the excitation pulse that is a 90 degree pulse to a given nucleus let us see some hydrogen atom. Now let it after you apply the pulse you remove the pulse and let it evolved with time, evolve meaning let it move in frequency. So, this is a FID of this nucleus when the RF has been removed after the RF has been removed.

So, it is now a free induction decay FID, but during this period before this FID is over what we do is, we transfer the magnetization which is decaying. So, before it decays we have to we cannot wait till decay during this process as it is happening, we transfer the magnetization to a second nucleus. How do we transfer it? There are verities of ways you can transfer this polarization we use about polarization or magnetization: one is using the J coupling suppose this nucleus 1 and 2 are J couple to each other these is a coupling between the 2.

So, therefore, remember J coupling is a very important parameter which is required in NMR to transfer magnetization. So, J coupling is a if it is strong it is easy to transfer, if the J is value is weak it is not easy to transfer. So, let us say that we have a coupling between these 2 nuclei. So, during the evolution or during the frequency FID of the first

nucleus I transfer the energy or magnetization the second nucleus and then after the second nucleus has now got the magnetization I remove the pulse.

Because this excited this transformer may happened because through J coupling the now I exide this nucleus I exide this nucleus because, it has now got the magnetization from here and that magnetization which has received from here. Now, it again it has his own FID, but this FID is coming because it has got the magnetization from here and therefore, it is has now it is own frequency which is the frequency of nucleus 2. So, you see what we have done here is, we first allowed the nucleus 1 to have an FID of it is own which is it is own chemical shift value whatever be the chemical shift of nucleus 1 and during that process we transfer this to another nucleus and we excited that nucleus which now goes which has his own FID because of it is chemical shift.

So, we have now 2 chemical shifts, we took chemical shift number 1 here transferred it and then recorded second chemical shift. So, what we have essentially done we have correlated are connected 2 chemical shifts of 2 hydrogens because of their interaction their possibility of interaction through J coupling. So, this is what is shown here, if I down to a Fourier transform this is not a 1D Fourier transform, this is Fourier transform of this FID and Fourier transform of this FID, but now because this is coming this and these are coming from here.

So, this FID actually is coming from this signal you see we get a peak corresponding to the 2 frequencies. So now, they are got correlated or connected because a first frequency gave towards transferred and then it the second frequency was evolving on its own and it got the energy from the first nucleus so therefore, they are correlated. So, if you have such pairs in my molecule different pairs of nucleus 1 and nucleus 2 then I will get different peaks in my spectrum. So, each peak in this spectrum we now correspond to each of the pairs of frequencies to which they are correlated. So, this is important point here we are able to now separate we are able to correlate 2 frequencies by transferring magnetization in this manner.

So, the 2D spectrum is therefore, the same in the word being repeated is it is correlates the frequencies of nucleus 1 and 2.

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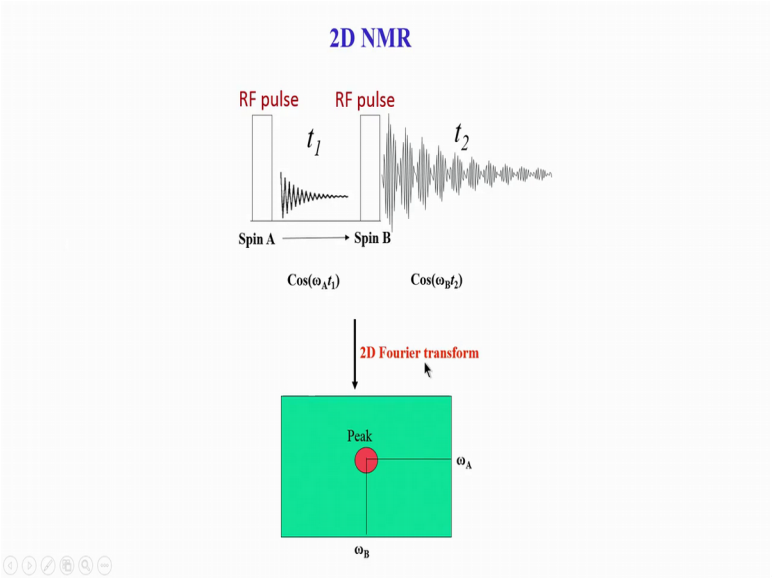

So, this is shown in the pictorial form. So, whatever I we discussed in a qualitative manner here showing here now if you draw it in a diagrammatic manner it is shown in this particular form. So, we see we start from an RF pulse we excited the first nucleus and then there is spin A, one let us say one nucleus A then it started having it is chemical shift value evolution. Now I remember if you remember recall we wrote the formula for FID in the first few classes and the formula for FID was written as an oscillation cosine omega t and A t 2 part.

So, right now we will not focus on the t 2 decay, let us only focus on this oscillation you see here this frequency and this frequency is of the spin A because, we have exited spin A. Now during this process from here to here we transfer the magnetization from A to B this happens while this is going on. So, this is simultaneous we do not wait for this to be over and then transfer because nothing will be left, this happens during this evolution. Now during after this when the spin B has received the magnetization from A, we excites spin B by another expectation pulse and that now starts the excitation after excitation, the spin B starts evolving or processing and that gives rise to another FID which is the spin B's frequency.

So, that is what is can be represented by cosine omega B into t 2. So, we can see here the important point here is we have 2 time axis, why do we have 2 here you can see from this slightly from this picture we have 1 FID which corresponds to one time axis because

this is time we have another time axis which is we call it as t 2. So, we have one time domain signal and we have 2 another time domain signal. Now these 2 are combined and when you do a Fourier transform along this axis and this axis, you will get this peak which says that these 2 are correlated.

So, the correlation is coming of the 2 frequencies because of these two values omega A and omega B. So, if omega A and omega B is if there is another omega A prime omega B prime another pair of atoms you will get another peaks somewhere wherever depending on the chemical shift value. So, each pair of a spins A and B which are connected by J coupling or through another mechanism like dipolar coupling and so on, any interaction between the two can be used to transfer magnetization from one spin to another and that can be used to generate a 2D correlation.

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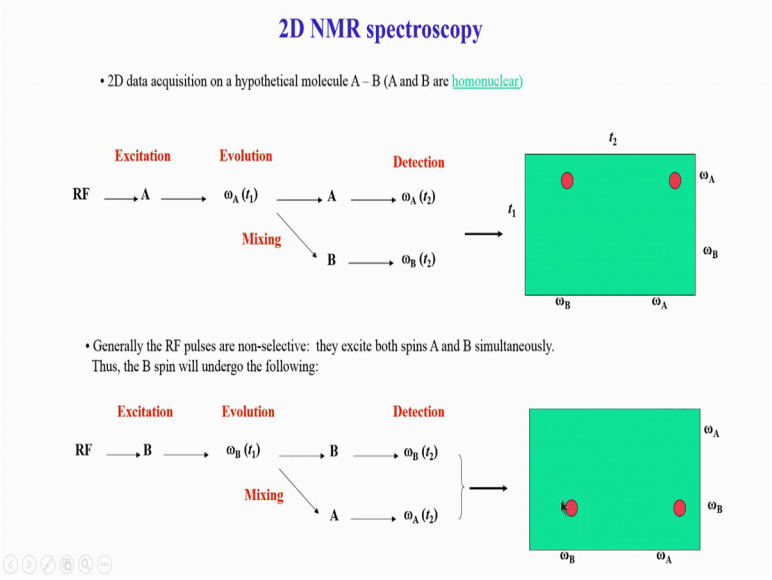

So, basically what you happens is, when you record a 2D spectrum you as I said the you transfer the polarization or monetization from one spin to another during that process we actually do not transfer the entire magnetization. So, what happens is during this period when it is evolving a part of it is retain on it is own and a part of it is transfer to the second spin that part which is transfer to the second spin we use a word mixing.

So, mixing is a phenomenon or a process and which the magnetization from one spin is now converted to the magnetization of the second spin, but mixing is only part of the story and about a last fraction of magnetization just does not mix it remains on it is own.

So, what happens to that that actually continues to evolve or continuous to process with the same frequency which happened during this period. So, therefore, these 2 if you look at these 2 condition these to correlation there actually not correlating to different spins. The correlating the chemical shift of A with A which has no information and that becomes a diagonal peak will see that shortly, where as this mixing is very important for us because this is where it correlates omega 2 the frequency of B with A.

Now the same thing is going on during the spin B. So, before that let us that look at this picture here. So, where is what is showing here that because of this process this pathway I am getting one correlation which is omega A with omega A which is this here you see omega A is here omega A is here. So, this is peak which is not correlating actually 2 different spins it is correlating spin A with it is own and this second correlation of second transfer here is correlating omega A with B.

So, if you look at this axis here it is t 1 and during t 1 if you draw a line here during t 1 it is omega A this is what you shown here and during t 2 that is if you draw a vertical line here and here it is A that is this peak and t 2 B which is here ok. So, this is how these 2 peaks are generated in the spectrum, but this is not completely over you have the same process going on from the B spin, the B spin also has the same phenomenon the B spin transfers part of his energy to A, because A is couple to B so, B is also couple to A.

So, any this is a process which is equal and opposite; so, that the spin B also transfers to A during the same time when A is transferring to B. So, this is mutually of equal and opposite, but B also the same problem it will retain a large fraction of magnetization on it is own. And therefore, that will generate now a similar pattern, but it will be happening on the B side.

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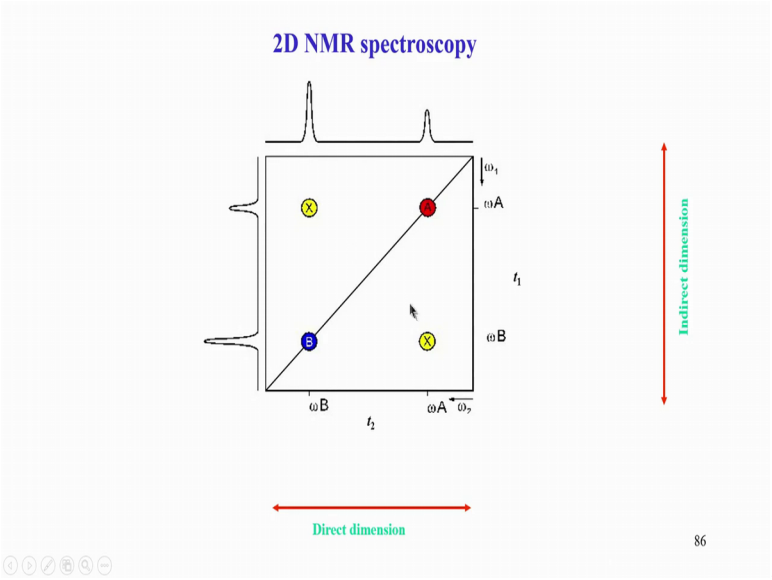

So, the total spectrum what you actually obtained is a combination of those 2 and that becomes a spectrum like this. So, this is nothing, but a combination of this pattern here coming from A and this pattern coming from B and they are equal and opposite because both behave in the similar manner both are hydrogens. So, they cannot be distinguished and therefore, when you record a spectrum complete 2D spectrum it look like this.

So, this peak this here is this set of peaks A with A you can see here is correlating A with A and this is correlating B with B this is called a diagonal peak and it has no information it is not correlating to different chemical shift. But, look at these 2 peaks these are very important peaks which are giving us the information that A and B are connected and that is called a cross peak.

So, a cross peak is essentially the chemical shift correlation between 2 hydrogens and we call this dimension where, the FID is the first dimension with a FID evolved as a indirect dimension and the direct dimension is when actually the data is collected.

We will see more details of this in the next class as you go to the detail acquisition part.