Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 07
Principles of 2D correlation Spectroscopy COSY

Welcome back to the course. In the last class, we were looking at 2D NMR Spectroscopy we looked briefly at how the data is acquired, how the magnetization is transferred from one nucleus to another nucleus. So, we will continue with that today.

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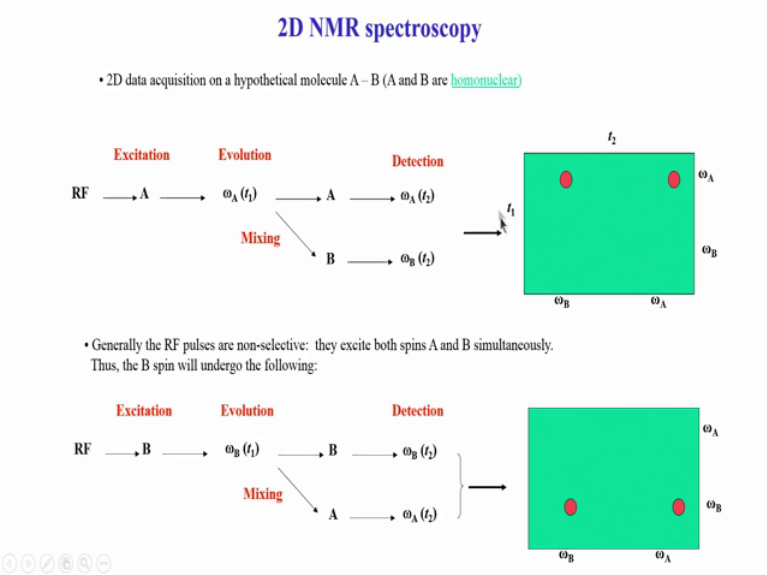

So, this is the slide which was shown last time which showed the schematic of how a 2D NMR experiment works. So, as I said we start from applying first an RF pulse to the nucleus any given nucleus. So, let us say this is one hydrogen proton that excites this particular proton. So, it brings from z-axis to x-y plane. By now you must be now familiar with the concept of going from x-z axis to x-y plane when we apply a 90 degree pulse. So, we can do basically recollect that part recall that part that when you apply a 90 degree RF pulse you excite the magnetization which goes from z axis to x-y axis or x or y-axis.

Now, once it that happens we remove the RF pulse and the spin simply rotates or precesses in the x-y plane. During this process it is also recovering to z-axis and also it is de-phasing in the x and y plane. So, this is a relaxation part which is going on here. And

during that process we transfer the magnetization from A to B, so that is what we do through some kind of a interaction, that means, two hydrogens, two protons A and B should have interaction between them some kind of an interaction.

And typically there are two types of interaction in NMR which we use for transferring from one spin to another, one is called as J-coupling through bond and other is dipolar coupling through space. So, both of this we will use in this course, we will see in this course how we can be it can be used. For now let us assume that there is only J-coupling between A and B. So, these are two hydrogens in a given molecule they are let us say separated by three bonds or even two bonds. So, it could be geminal coupling or a vicinal coupling. So, during this evolution, so we use the word evolution is the time when the spin A is free to rotate in the x-y plane or precess in the x-y plane.

So, this is during that time the spin A precesses with a frequency of A because that is the precessional frequency. And that time period is labeled as t 1, this is one time period. So, this is an FID. So, it should be we as we see in the later slides we will write it as cosine omega A t 1. So, now, this during this period when it is evolving or when it is precessing at the same time simultaneously transfer from A to B is happens. Now, how does that happen as I said that is through the mechanism of J-coupling in this case. So, this is called mixing. We are actually basically transferring, but we have not excited the B pulse. So, it is not mixing in that sense it is essentially just transferring.

So, once the transfer has happened, we then apply another RF pulse like similar to this which acts on both A and B. So, it is not an RF pulse which works only on A, it will also act on A and B. So, that is that mixing pulse it mixes the magnetization which has come from A and now that gets transferred to B. So, now the B nucleus gets excited similar to what happened here and therefore that spin B now starts rotating or precessing after this pulse mixing pulse is applied or after the second RF pulse is applied it starts precessing with frequency of its own which is omega B.

Now, one thing which I mentioned in the last class is this transfer is not 100 percent usually it is a very small fraction of magnetization from A which is transferred to B. Majority of the magnetization of A remains on A itself it is not transferred to B, even though the J-coupling maybe strong enough there the coupling in proton to proton in case of proton to proton it is not sufficient. So, therefore, your magnetization remains a

bulk of it major of majority of it remains on A. So, there are two magnetizations now, when you finally detect, both of them are detected. And these are independent they are different they are separated, but they come together.

So, now when I do a Fourier transform, I will see a pattern like this. So, here is plotted a t 1 axis here and t 2. Actually when you do Fourier transform, we end up with frequencies. So, this is just t 1, t 2 is just a schematic to show what happens during that period, but actually what you will see in a real spectrum is the frequency scale. So, you can see here for spin A, omega A, it was during t 1 omega A and this combination of omega A and t 2. So, if you look at these two combinations, it we can see that they are both omega as, so that happens here you can see this is this particular peak, here it has omega A during the t 1 in the vertical axis and it is also having omega A during t 2 which is the horizontal axis.

So, therefore, that is a peak, which has just a no particular information it just has a correlation of A with itself. So, we do not have any useful information in this p, but the other combination where you go from A to B, so you see here if I take this combination where it is evolving or it is frequency evolution is omega A during t 1, it then revolves with omega B during t 2 this combination basically will tell us at omega A is during t 1 at is this t 1 axis coordinate and during t 2 it is omega B if I draw a vertical line here. So, this peak is now a very useful peak for us, because it is telling us that there is an interaction or correlation or connection between two spins A and B.

So, this information would not have been possible to obtain in one from 1D NMR; a 1 D NMR would have given A peak separately, B peak separately, but no such cross peak. So, we use the term cross peak in 2D NMR. A cross peak is basically a peak between two different spins which are correlated or connected or interacting via J-coupling or through dipolar coupling. So, now, this is as far as A spin is concerned. But remember I mentioned in this just before sometime that you are spins the RF pulse is applicable to both A and B, so which means if I can excite A in the beginning here that should also excite B the same RF pulse.

Same RF pulse here and here should a work on both A and B. So, now, the B gets excited also at the same time as A. So, this and this are happening simultaneously. So, B you all similar to what happened to A here and B has a frequency omega B. During t 1 again the

same process happens where B is coupled A is coupled to B, B also will be coupled to A. So, this mixing or transfer of magnetization is a same process as here, but only thing is it is A to B here and here it is B to A so, it is a reverse.

Now, after the transfer has happened, we apply another RF pulse that is a mixing pulse which will now excite the one which was not transferred. So, for example, here B was excited it remains excited, it further goes away I mean this is a magnetization which remains on B that as I said majority of it remains on the nucleus, it does not get transferred. Whereas, the one which got transferred to A gets excited the A nucleus is excited by the second RF pulse and that then evolves during the detection time, this is where you physically detect the signal.

Here this here this evolution period there is not detection ok. So, this is something one should keep in mind that we have two t 1 values two t time values t 1 and t 2, but the actual detection physical the detective hardware detects signal only during this period t 2. This is indirect detection this is not actually detected, but then because we capture this combination in a spectrum we can call this as a indirect dimension.

So, this combination now we let us see how it comes in the spectrum it will be similar to this except it is now coming at the B frequency. So, you can see during t 1 this frequency which is B and this is also B. So, this peak is similar to this here A to A and why this is B to B this is this combination omega B t 2 with omega B t 1. So, therefore, this is peak, this peak has no useful information. It has not it is not telling me to what it is connected or correlated. Whereas, this peak the second one here this tells me that omega B and omega A are connected.

Now, this is similar to this peak here also the information was omega A is connected to B. So, you see we get the same information in two times. So, now, these two have to be combined together because as I said both this processes are happening simultaneously so, it not that this happens separately from here.

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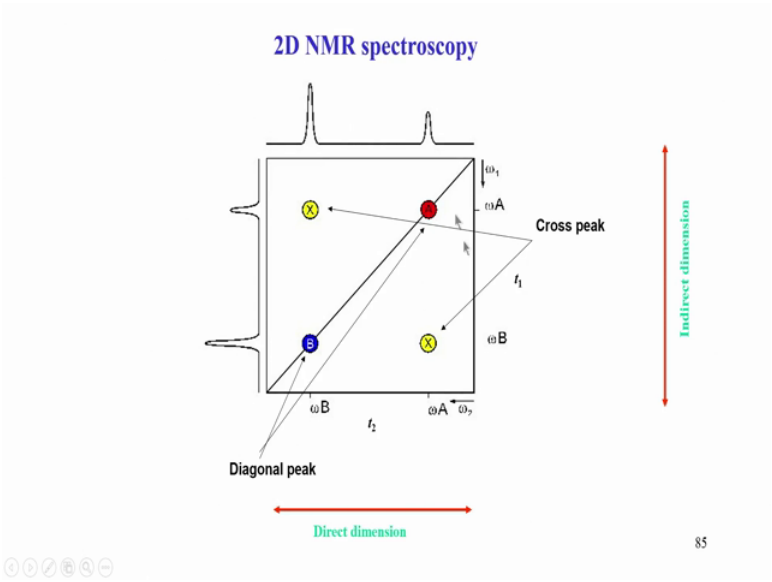

So, when both are together the spectrum also will be a combination of these two. So, when you combine the two spectra or two patterns, essentially what you get is a spectrum like this. So, this is the 2D NMR spectrum. So, you can see here omega A to omega A and B to B and that lies neatly on a what is call as diagonal peak of a square so, this is like a square. Why is it a square, because you have the same frequencies here the same scale and the same frequency in scale here.

So, now this two peaks are the cross peaks. They are the useful ones, because they give us a information that A and B spins are correlated. So, you can see that we are getting actually redundant or information twice. So, in principle even say why do we need two time, so that is the design of the experiment is like this. And sometimes it helps because a peak here will be overlap with some other peak, whereas its partner which is exactly the same may not be overlapped.

So, if you cannot identify the cross peak here, you may be able to identify the cross peak here or vice a versa. So, it is useful to have duplicates twice the same frequency same equal information in case of overlaps. So, this is what now we shown here is that we call this dimension that is the indirect dimension that is the t 1 axis, we are not detecting the signal physically here. There is an FID here because anything evolves in NMR by a frequency that is an FID. But that FID is not captured or digitized or detected by the hardware or RF coil, it modulates the intensity of the direct dimension this is the

dimension where actually we see detect physically the signal the signal here whatever is coming out here is detected and Fourier transform and similarly it is modulated by this here.

So, the Fourier transform here as well because here also we have an FID, but it is not an induction decay in the sense it is not inducing the signal in the coil directly. It is only indirectly affecting the signal here. So, these are their basic concepts one should keep in mind when analyzing or understanding 2D NMR that is diagonal peak and cross peak. As we go later a one slight complication will come is that in a heteronuclear NMR in there we will see that later, but you can in heteronuclear NMR what will happen is this axis will be a heteronuclear means carbon or nitrogen, whereas this axis will be proton.

So, therefore, they will not be a same axis in scale. So, you would not get a diagonal peak in heteronuclear, because you never detect carbon-carbon or proton-proton correlation when you are recording a carbon-proton experiment. So, we will see that point later where it is important keep in mind this diagonal peak business or cross peak concept arises mainly in homonuclear NMR where both axis are either proton and proton or both are carbon and carbon.

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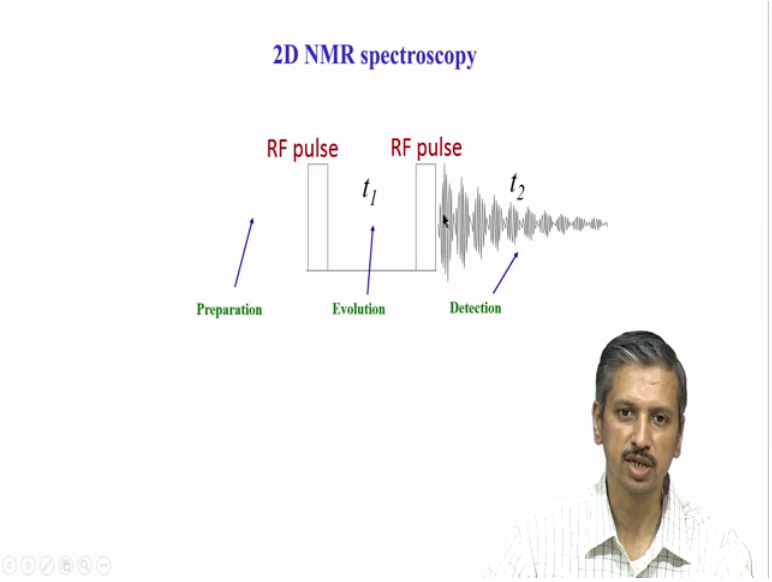

So, let us now go little bit into mathematical detail of how a 2D NMR experiment is made, because unless we go a little bit into a mathematics of this, it will be difficult to capture or gather information on 3D when we go little bit more complicated experiments.

So, let me little bit give a little more detail insight now. So, this period before this pulse is applied. So, this is the first RF pulse if you recall the previous slide we have said we applied RF pulse on spin i, this pulse actually is a pulse before which we have to prepare the system in equilibrium and that is the similar to 1D pulse sequence I showed you in the previous to previous class when we did 1D part and there we had this call as relaxation delay and that depends on the t 1 of your sample.

So, similarly here this preparation period depends on the t 1 relaxation value of the sample or the molecule. Then you apply this RF pulse and during this period is what we saw it is a evolution. And once you evolve there is this omega into t the term will come. And during second RF period, during this period from here to here that is during evolution the magnetization is actually transferred from spin one to spin two, spin A to spin B and that results in another pulse which will caused to excite the transferred nuclei. And that will (Refer Time: 13:58) evolve with its respective frequency that is the detection part which I showed you in the previous slide.

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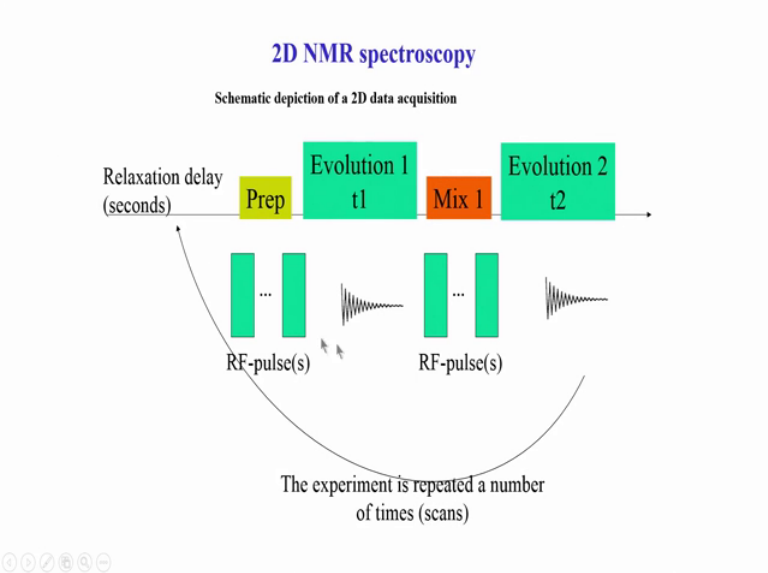

So, this is a schematic of a 2D NMR experiment. So, you start with the preparation period, you have an evolution period, you have a mixing period and then again you have an evolution period. So, one has to keep in mind that preparation period is not just relaxation delay you are also preparing the magnetization to ready to be transferred or

evolve. So, therefore, it also consists of those initial pulses RF pulses the 90 degree which we apply.

During the evolution period, one has to keep in mind there is no pulse applied. So, during this evolution period typically we are letting the spin free to just simply evolve and relax. So, there is a relaxation also happening in this time, but normally we do not consider a relaxation when you analyze the correlation. So, we are looking at 2D NMR spectral correlation, a relaxation is not so important to be worried about or concerned about. So, we assume that we are not allowing it to relax fully, we are not allowing to go up to the end typically the evolution part is stopped here. Somewhere after it reaches about 20 to 30 percent of a signal and then it the second pulse the new set of pulse that is called mixing pulse is applied.

So, mixing pulse is basically now doing the job of transferring actually the polarization or exciting the second set of nucleus from where to which the magnetization has been transferred. And the final detection is again a simple FID which is which decays with time. And this is allowed to complete we do not stop this in between we allow this to complete fully. So, your question may arise in you mind that why is that here we stop somewhere in between, but why is it that we do not do that here. And this is a very important point in NMR this is related to what is called as a measurement time.

So, what happens is suppose let us say I have here a 100 points or let us say this whole set up FIDs if I digitize meaning if I take collect points let us say it corresponds to 1000 points and let us say this also corresponds to 1000 points. So, what happens is for every point here I will have to record 1000 points here. So, how the experiment is conducted is basically you record one point you evolve this magnetization during t 1 for a single point.

And then after that point, you apply the mixing pulse, then you detect the full signal. Then you apply then you go back to the preparation period here, wait for the equilibration, apply the pulse then go for second point; that means, you are evolved it for two points that initially was point 0, now it is 0 and 1. And then again you apply the mixing pulse, so and apply take this volt. So, if you have 1000 points here and 1000 points here, here is 1000 points you will take probably 100 milliseconds, but for every point here you have to record the entire FID here.

So, therefore, if I have to do each point here, so let us say the whole cycle this whole cycle, let us say it takes about 2 to 3 seconds. Then if I have to record 1000 points, I have to wait for 1000 into 3 seconds, because each point I do here will take the entire cycle 5 seconds, I have to come back increase this point, go back record full again come back and so on. So, the measurement time goes a very fast because in 1000 times 5 seconds or 3 seconds I have to record this if I have to record the full FID. Therefore, we normally cut it down, we cut it down to about 200 or 300 let us see here one-third of width and that saves a time, because now I have to do 200 times 5 seconds or 3 seconds depending on how long is this entire sequence.

So, therefore, in indirect dimension in NMR 2D, 3D and 4D, in fact, in 3D and 4D, you will have two more dimensions like this in 3D. So, there we cut it even more. So, we can see as we go to higher dimensions, the signal time becomes very large because of this constraint that every point you acquire here, you have to repeat the whole cycle again, again increment it with another point and repeat the again the entire cycle. You cannot finish this fully and immediately go to this. You have to do this step by step, but this can be done continuously. So, this is the technical problem. And therefore, it is cut down and then some other techniques are used to recover this whatever is the cut down signal.

So, the resolution basically is affected by this problem. So, we are since remember in NMR if you allow the signal to completely go to 0 and if you get this full FID, your resolution is very good you get the best resolution. But here because I am now truncating, so we use this word call truncation. If I am truncating the signal to only a few one-tenth or one or one-third of the points my signal resolution also gets down by one-third. So, the line becomes broader, so that is the drawback in multidimensional NMR spectroscopy that we get broad signals and the broad signals is coming because we have the limitation of not recording the data fully, we have we can only record data as to one-third or one-fourth depending on the time.

So, there are varieties is one of the research of area of research in NMR is how to actually improve the resolution if even if I have not completely getting the data. So, let us say I stop here at 20 percent, so remaining 80 percent is not recorded. So, can I still do something to improve my resolution because my resolution will be very much badly affected if I do not record this fully FID. So, this is area of research, we not go into detail

in this particular course. So, this is the schematic of a 2D NMR this is a result relaxation delay RF pulse, these are again set of RF pulse and so on.

So, this is what I said we have to repeat this n number of times and that depends on how many scans. So, this is so there are two delays here, one delay in the sense, there are two constraints here. One is the relaxation delay which is of the order of seconds and other the constraint is how many times you have to repeat the experiment to get a good signal to noise so, we use about scans. So, more the scans better is a signal to noise, but more the scans longer is experiment going to take. So, this essentially your sensitivity very much depends on these factors, how much time you record, the data how much is distance number of scans and so on.

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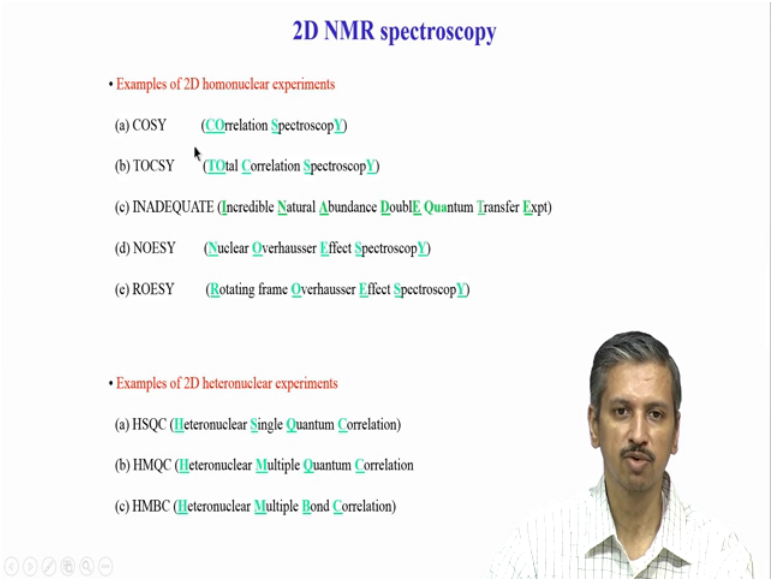

So, these are the set of NMR experiments 2D which we will go through this in this course we will not be able to go through all of them though this for example, COSY-TOCSY something which will see inadequate is something not normally done in the biological samples. So, we will not go through that that was actually covered in the previous NMR course.

NOESY and ROESY are very important for us, because entire structure determination of proteins depends on this experiment NOESY. And ROESY is not so much done for proteins is mainly for peptides, but we will still go through it and get the feel of how this experiments work to we will also look at this is a homo nuclear experiment, wherein

both the axis or hydrogen and hydrogen and whereas in this case we will see heteronuclear experiments where one of the axis is hydrogen, the other axis is carbon or nitrogen.

So, let us start from the very first experiment this is 2D correlation spectroscopy. This is one of the very first NMR experiment also which was developed and slowly it evolved into many more variants. So, the current experiment which we 2D COSY which we use is no longer the one which was originally published there were subsequently many other published versions. But we will go through the most earliest version, because we just want to get an idea of how this works this experiment actually the principles of it.

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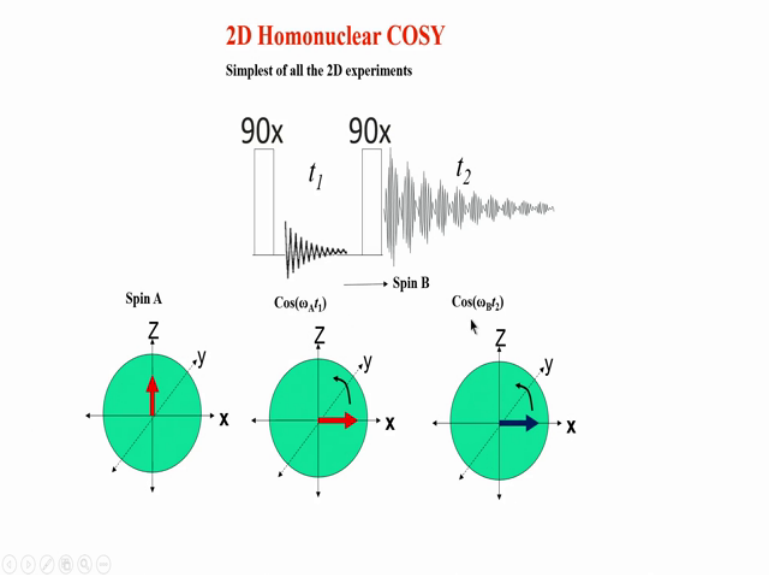

So, this COSY experiment is a simplest 2D NMR experiment you can think of. It essentially the same similar to what we just discussed about the 2D NMR. So, you start from applying a 90 degree pulse which can be along x-axis or it can be along y-axis. You can take assume it is x-axis then you apply this pulse once you apply you are spin A which is along Z axis, because before this pulse this is in equilibrium during this preparation period, it now comes into the x y plane or x-axis. In this case because x pulse is apply it should be y-axis, but does not matter right now let us assume that it is now in the x or the y-axis.

Now, during this period t 1, it precesses. So, since this arrow is shown as anticlockwise you can think of it as clockwise or anticlockwise, it precesses or goes around the Z-axis.

So, Z is now vertical. So, you have to please keep in mind that this is a vertical axis. So, x and y plane is horizontal. So, this green circle we shown here is actually is a plane x y plane. So, now, it tries to go in the x y plane and that precession mathematically is written like this cosine omega into t 1. So, this is a simple rotation of this spins, this the red color in the x y plane.

Now, during that period when it is precessing we transfer or we transfer the magnetization to B through this j-coupling concept, if there is a J-coupling between A and B, so that means, the minimum requirement is that there should be a coupling between A and B two spins. Now, once you do this transfer to B, now the B has to be excited because B is still along Z axis we are although I said we excite both A and B here, but the magnetization which has come from A to B is still along Z, B also will be x y plane, but again it transfers to A and the transferred part is along Z. So, we have to keep in mind when I say that B has to be excited it means it is a spin magnetization which has come from A that part has to be excited, it is not the spin B has to be excited in the beginning A and B both are excited by this pulse ok.

So, this something which you have to go back to the previous few slides ago, where we showed that A and B are equal and opposite. So, whatever happens to A happens to B, but with the transferred portion from here to here when we transfer the magnetization in the spin B part which is transferred is still along Z and that has to be brought into x y plane that is done by applying another pulse. And this is what i said we call it as a mixing pulse.

And once you do that the spin is on x y plane now and A is also in the x y. Because remember A does not transfer all of its magnetization to B only part of is this transfer the remaining part is still on A. So, A also remains like this is this rotating B also. So, both of them are rotating now in this during this period t 2 ok. So, that is basically what is the this mechanism. So, this is basically A and B both are correlated only because A and B both are connected to each other by J-coupling.

Now, if you look at it from the B point of view, you will have this concept, you will have the idea you will have it atom call cosine omega B into t 1, because A and B are equal and same. And during this t 2 you will have cosine B into t 1 t 2 cosine in a into t 2

omega A into t 2. So, A and B will be reversed during t 1 and t 2. And this, so these two are the cross peaks which we saw in the previous slides.

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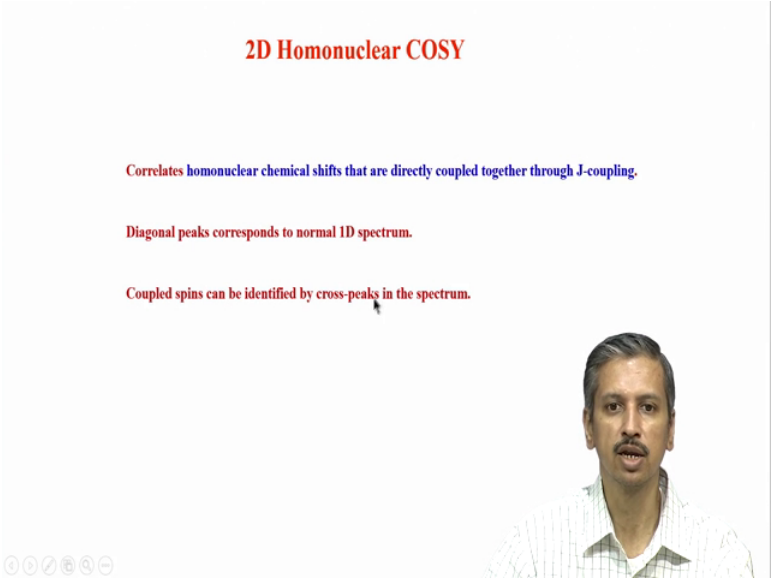

So, what is the advantage of this experiment homonuclear COSY? It correlates to proton chemicals shifts or carbon chemical shifts that are directly coupled through J-coupling. So, as I said this J-coupling is a very important requirement without this you cannot transfer the coupling transfer the magnetization from one screen to another. Diagonal spin corresponds to the 1D spectrum and this is something which I mentioned last class also. Diagonal speaks peaks actually in 2D NMR homonuclear NMR, do not carry any information, they are actually redundant information, they are only telling us A is connected to A which anyway we know.

So, a diagonal peak is actually very difficult thing to get rid off. So, may the again a research problem in NMR has been to how to remove diagonal peak from a two homonuclear experiments, because anyway that does not carry much information. And the problem with the even and second problem is it has very strong nature peak intensity. So, diagonal peak is something which comes is very high intensity and its spoils spectrum several time many a times. So, we like to normally get rid of it, but in something not so easy to do that. So, we have to leave that. And couple spin how do we identify whether two spins A and B are coupled, they can be identified by simply looking

at the cross peaks. So, the cross peak is the main important information carrying peak in the 2D spectrum.

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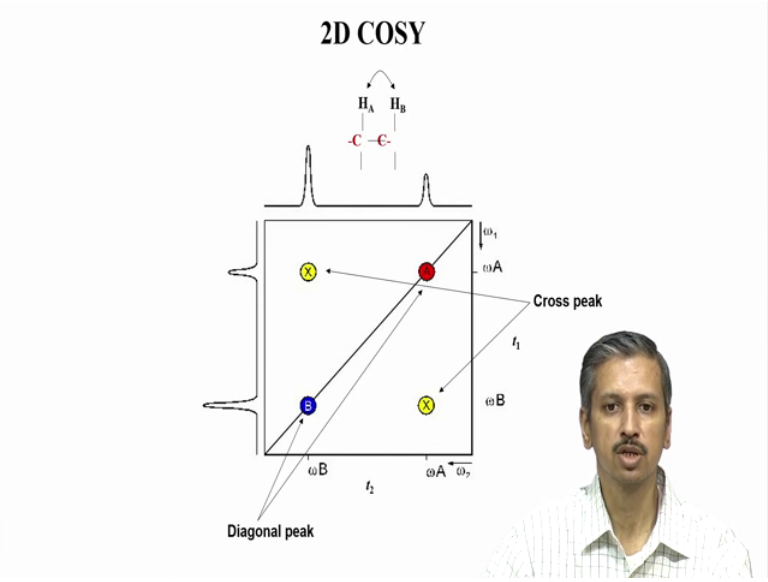

So, this is how 2D COSY spectrum looks this is very simple schematic which is similar to what I showed you in the previous few slides ago that you have a diagonal peak followed by you have a cross peak. So, these two basically are the most important for us. And again they are repeat they are repeated that actually duplicates whatever information is here is same as here. It is as I said it is good to have duplicates because one peak may not give enough information, because it may be crowded you may not be able to extract the value, whereas the second peak maybe useful, say and vice versa.

Whereas, this diagonal peak is something which has no value to us and therefore, so this is basically what is shown here you need basically coupling between A and B. So, here it is shown as a vicinal coupling meaning there is one two and three bond hydrogen to hydrogen, proton to proton coupling or you can have at two bond coupling.

But very rarely you can have a coupling between this proton and very far away. So, COSY actually exploits up to three bond; four bond coupling and five bond is very difficult to detect with a COSY experiment. And therefore, we cannot actually go beyond few atoms carbon atoms. So, if you want to do that let us say you have a molecule which has a long chain which has let us say this four-five carbons. And then if you want a connection or correlation between this hydrogen and an hydrogen located a far away, we

then have to use a TOCSY experiment which we will see next. So, right now let us see what the other things we can see with COSY experiment and this is the cross peak and diagonal peak which we have already referred to previously.

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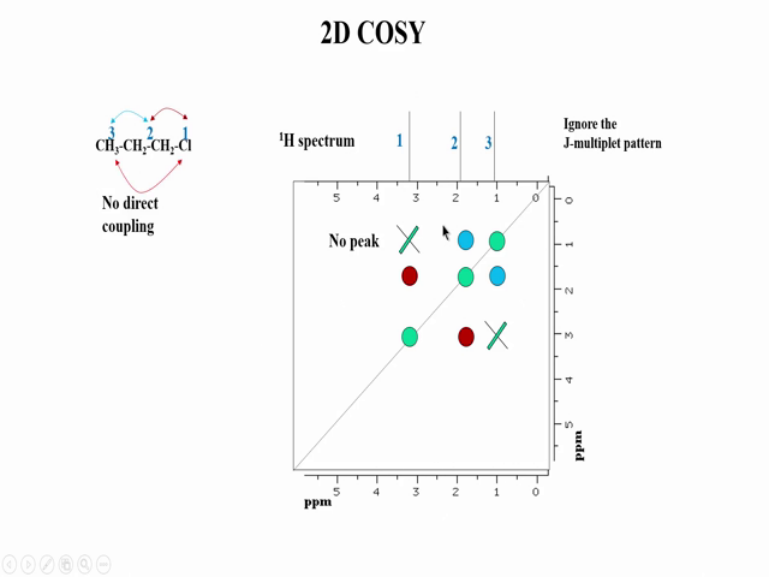

So, let us take an simple example of a molecule this will this propyl chloride a single chain amino acid sorry molecule straight chain. So, we can see this is carbon number 1, carbon 2, carbon 3, and this is the 1D spectrum of the proton for proton 1, proton 2, proton 3. So, proton 1 is closest to chlorine. So, it is most noun field shifted. Proton 2 is slightly away, so the inductive effect is less. And proton 3 is farthest from the chlorine, so proton 3 is having the least inductive effect. So, this is 1D NMR spectrum.

You can see that if I draw a diagonal peak now, so diagonal peak is simply correlation of three with 3 to proton 2 with 2 proton 1 with 1, so that is nothing but the same 1D spectrum drawn in a diagonal way. But now let see the cross peak. So, you see now here this hydrogen 1 and 2 are actually three bonds away if you calculate this is the vicinal hydrogens. So, if they are equivalent I mean let us assume if they are equivalent here then these two protons are three bonds away and therefore, in a COSY spectrum they will give cross peak they are correlated because 1 and 2 are close to each other by three bonds. Similarly, 2 and 3 are also connected to each other because 2 and 3 are while away by three bonds. So, they will also show a cross peak between 2 and 3.

So, this is the pattern which you will expect in a COSY spectrum. I will not see any correlation between one and three because 1 and 3, these two hydrogens are really far away they are five bond separated. So, therefore, it is very difficult to four bond separated. It is very difficult to get information about them by simply doing a COSY, COSY will not your because the coupling between 1 and 3, J-coupling between proton 1 and 3 will be very small. So, this is why we will not see any peak here because of no direct coupling.

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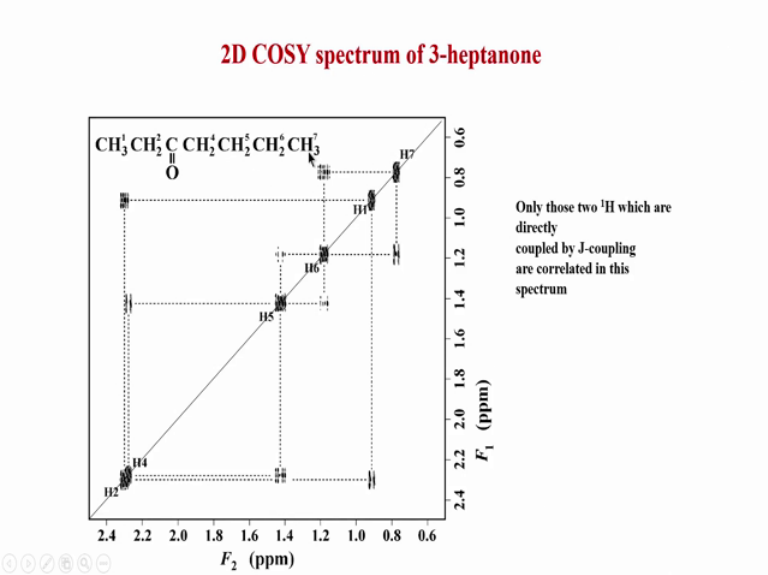

So, this is an example of another real spectrum, this is taken from book and you can see here these are all different atoms. So, these are all different correlations observed between different atoms of the different hydrogens. So, we can see here only those two hydrogens protons which are directly coupled by J-coupling are correlated in this spectrum. So, that means, 7 will be correlated with 6, 6 will be correlated with 7 and 5. 5 with 6, and 5 with 4; 4 with 5, but 4 will not be coupled to 2, because 2 is far away there is a carbonyl which is coming in between. Similarly, here 2 will be coupled to 3 and 3 will be coupled to 2, but within only these two you will not see any coupling the other spins.

So, this brings us to the end of 2D COSY spectrum. In the next class you look at 2D TOCSY experiment which helps us to actually go to the full chain here except of course, there is a break here, but I will be able to see in a TOCSY spectrum correlation from here

this number 2 to 3, all the way this is a number 2 to 7, 4 to 7, so that information will come from TOCSY and we will look at it in the next class.