Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 08
Principles of 2D Total correlation spectroscopy (TOCSY)

Let us continue our discussion on 2D NMR. Now, we looked at the simplest 2D NMR the experiment in the last class that was 2D COSY, where we correlate to hydrogen chemical shifts which are J-couple to each other, but only two bonds or three bonds. It is very difficult as I said to connect hydrogens which are three; four or five bonds away, because there is no direct J-coupling between them. To overcome that problem there is a new experiment or subsequent experiments was develop known as 2D TOCSY, which stands for total correlation spectroscopy as shown here.

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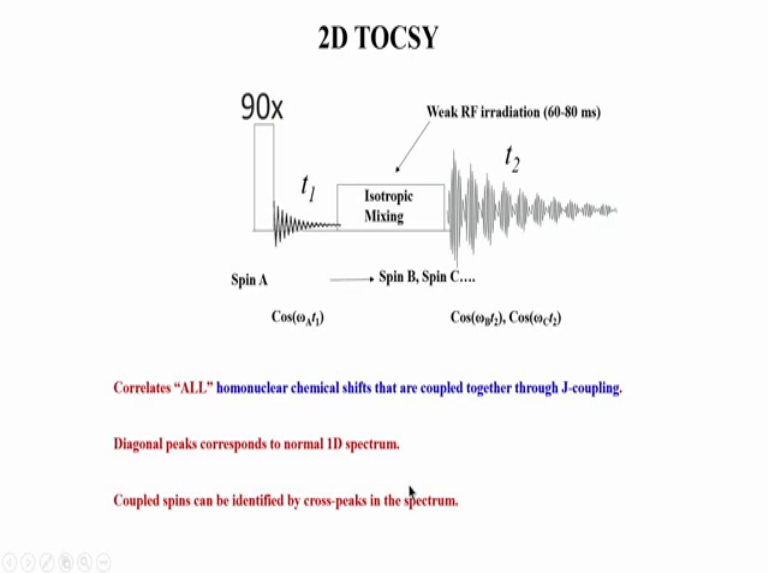

So, this is an abbreviation for this word total correlation spectroscopy. So, let us see how it this works, and what is the additional information we can get over a simple 2D COSY? So, this is the pulse sequence of the 2D TOCSY. Typically, this is an NMR, this how we analyze NMR experiments. What we do is we first look at the pulse sequence how is the sequence of the pulses applied in a given a NMR experiments. And once you practice understanding this type of pictures, you will get used to basically immediately understanding how the magnetization is flowing in this pulse sequence.

So, we call into flow of magnetization. And this is something as an experienced NMR person can immediately look at this diagram, and figure out what is happening. At this stage I would not expect you to obviously, understand fully how this work plus sequence, but the idea basic ideas one should have a feel of that is one is they apply an RF pulse the first pulse is always an excitation pulse in an NMR experiment, and then you evolve the magnetization.

Now, during this revolution period, again similar to what we saw there is a transfer we consider in the COSY experiment. But in TOCSY we do not look at it as that what we do is we assume there is no coupling; let us not worry about any transfer here let us say that there is only chemical shift evolution. So, chemical evolution happens during this period. And as soon after sometime again do not take this FID is the literally the way it is shown here. We do not wait till it is over, it is as I said in a 2D NMR experiment, we always acquire one point and the entire experiment is repeated.

So, we allow it to go for some duration here, and after that this block is applied. Now, what is this block, this block actually is a collection of RF pulses. This consists of 180 degree pulses typically. We will not go into detail of what is inside this box right. Now, you can think of it as a black box. The box basically what is doing it is doing is called isotropic mixing meaning whatever magnetization come here, it is now mixed with all other magnetization. And the output is of this here is the magnetization from A actually lands up all the way in a faraway proton.

So, if you recall in 2D COSY, we said that magnetization does not go beyond three bonds. So, we are looking only at three bond couplings three bond transfer; whereas, here we can actually go up to five bond, six bonds, seven bonds away depending on the of course, the coupling strength and how long we apply this block. But that magnetization actually goes from all the way from one proton to a faraway proton because of something which we do in this block.

And this block actually if you see it is a weak RF radiation and is applied for 60 to 80 milliseconds. So, this is a mixing time. So, this is thing which you should keep in mind a typical TOCSY mixing time, it is a time we apply this RF pulses or isotropic mixing block is about 60 to 80 milliseconds. One should not apply this for a very long time,

because that will start heating the sample and the sample may actually get destroyed or degraded. So, this is this is important practical aspect which you have to keep in mind.

And once this magnetization has been transferred from A, we will see that also in a diagrammatically shortly, has been transfer from A all the we have to for away proton, all these protons now start evolving during t 2. And therefore, what is happened is we have correlated we have basically by transfer magnetization from one proton to another proton far away we have correlated their chemical shifts.

So, let us look at it little bit more in detail. So, this is shown as spin A. So, spin A is exited by a 90 degree pulse. It evolves by chemical shift omega A t 1. As I said we ignore the J-coupling during this part which we did not in the 2D COSY. In 2D COSY in fact, we needed that J-coupling, but here we will ignore that because for us we already have this which will do the 2D transfer or isotropic transfer.

So, now we apply, so what happens is this cosine this spin which is magnetization on A is transferred to B, C, D and so on as far as possible by the isotropic mixing. But what is the condition, how long can it be transferred it depends on now the coupling between A to B, B to C, C to D and so on. So, what we are trying to do in TOCSY experiment is we trying to exploit that there are coupling between A to B, and then from B to C there is coupling, and from C to D there is coupling, coupling meaning j or dipolar coupling. And because of that coupling we are trying to transfer A to all the way to far away spin.

So, if there is a break, that means, suppose C and D are not coupled, then we cannot go from A to D because that break will stop at C. So, our magnetization will be not be we not be able to transfer all the way up to D or E or F whatever number of spins here because we encounter a break. So, we will see that in say a few example that A to long chain can be transfer, but if there is break it does not work. So, as long as A to B is coupled B to C is coupled and so on it can be transferred. So, once the spins the magnetization from A is transferred to B, C, D and so on this isotropic mixing block is over and then you start detecting whatever has been transfer, so that transfer is now detected. So, B will evolve with this frequency; C will evolve with this frequency D will evolve with this frequency and so on.

But each time each of this evolutions are actually connected to this now, because they are coming from that only, they are big they have come from A only, so that means, we are

correlating A to B because of this connection, and we also correlate A to C, we correlate A to D, and A to e and so on. So, you see form a chemical shift correlation between A and D and C and so on. I just by using this TOCSY concern. So, this is very useful experiment, because it gives us a long range transfer information which you will not get from a simple COSY.

So, correlates all homunculus shifts that are coupled through J-coupling. So, this is important point here in a TOCSY that we correlate all homonuclear chemical shifts, they are couple together meaning there should not be any break A, B, C, D, E are five hydrogen. A should be couple to B, B to C, C to D, and D to E. It cannot be that if A to B is coupled, but there is no coupling B to C, then I cannot go from A to E because of the break.

And now before we look at the actual spectrum, the diagonal peaks which we see in TOCSY is similar to a COSY that is the diagonal peak is just a 1 D spectrum, there is no difference between the diagonal peak of a COSY and a TOCSY. So, diagonal peaks are same. Whereas, a cross peaks are look completely different types. Of course, the two three bond here suppose A to B is three bond that interaction also will be there in COSY. So, as per as near neighbors are concerned meaning two hydrogens which are coupled to each other, there only three bonds away, they will be the similar they will have the similar correlation as in a COSY. But those which are far away that correlation is not there in COSY, but will appear in the 2D TOCSY spectrum.

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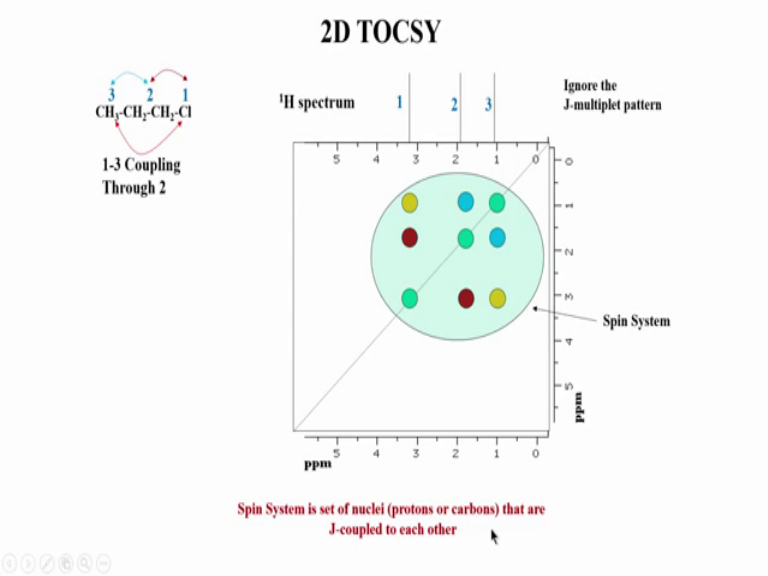

So, let us again look at the same molecule which we saw earlier, so the propyl chloride. You can see here I have this hydrogen which is this label has shifted this hydrogen is 1; this hydro proton is 2; this proton is 3. Now, in a 1 D spectrum again similar to what I said in the previous class we will just see three lines. And again I am telling ignore the J-coupling right now we are only concerned with the chemical shifts. So, we are looking at chemical shift correlation. So, we not worry about the final details of J-coupling, it does not matter for us in the analysis of 2D or even for that matter in 3 d. So, we need J-coupling for transfer no doubt about it. But we do not need J-coupling to appear in the spectrum or we do not need to see them very in a more detail. So, we will ignore that. So, this is a 1D spectrum.

So, now, if I draw 2D spectrum how will I get what kind of pattern will I get in a 2D TOCSY. So, this is a diagonal peak which is similar to a 1D spectrum and similar to what we saw in the COSY. So, there absolutely no difference as far as diagonal peak is concerned between a TOCSY and a COSY. But let us analyze that cross peaks now more carefully. So, you can see here between proton 1 and 2, there is three bond coupling. And therefore, because of these two coupling because of this two three bond 2 protons, you will see cross peak. This is similar to COSY. There is there was no difference between this and a COSY the same thing is drawn here again. And you can see here also it is same to COSY. So, there is actually no difference till here at this point.

But now in a COSY, we could not see a coupling between proton 1 and 3 because that was far away, but now you can see here if there is a intermediate two is present here, so in this molecules. So, 1 is coupled to 2, and 2 is coupled to 3. So, in a TOCSY, I can connect 1 to 3 via or through the coupling to 2, so that is basically the advantage of TOCSY that I am able to get a new cross peak between a proton and another proton which otherwise will not be seen in a COSY spectrum, and that is basically because we are able to use this isotropic mixing concept to transfer magnetization from one spin to a faraway spin. And this is something which very you not be seen in any of these 2D experiment COSY experiment.

So, therefore, TOCSY was a very important development in NMR for assignment of molecules, because you see the entire molecule you can get in a spectrum. So, this is the like, suppose let us see I have a mixture of two molecules, and let us say I have one more molecule present here which have not showing here now. But if they were present that molecule would have again a connection between among its protons, but it will be different from this pattern, because it will be different molecule.

But by looking at this you can see this like a square grid by looking at this grid like thing, I can get the full the details of chemical shift of one molecule which are connected to each other, and I will get this same details for another molecule where also I will get connections between the atoms of that molecule. So, by using TOCSY one of the most important applications in TOCSY is to identify what is called as a spin system. The spin system is a word which very often is used in NMR in biomolecules where spin system basically consists of a system or a set of spins which are coupled to each other by J-coupling.

So, there is no break here. All these three are belonging to one set of spins which are coupled, and we use the word spin system, but we will see one example where this the same molecule, so we have to keep in mind in this case one molecule is one spin system because all the protons are connected. But you can have in one molecule two different spin systems because of breaks. So, we will see that shortly. So, this is what I was leading to we use the word spin system which basically it looks at the entire set of connected spins as a one family, one family meaning one set of spins. So, spin system is a set of nuclei that are j coupled to each other.

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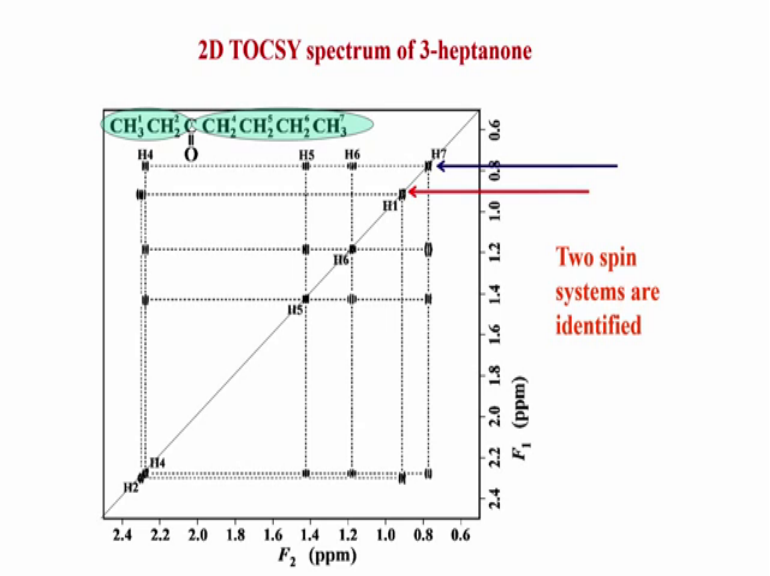

Now, let us look at an example. This is a real spectrum of a molecule 3-heptanone which we saw in for COSY. So, here you can see that here we have 4 protons, but there is a break here. Break meaning there is a carbonyl here which is not allowing the coupling to go from 2-2 and proton 4 to 2 because you see the there is no proton here. So, for example, if I want to do a TOCSY connection between this proton which is labeled as 4 to this proton which is 2, I need to depend on one bond which is this carbon proton, two bond, three bond and another fourth bond to this proton. So, I need four bond J-coupling if I want to correlate a TOCSY see a correlation in TOCSY spectrum between 2 and 4.

So, as I just said that is not possible we are not, there no direct coupling. So, this basically breaks this whole molecule into two parts. In one part, it is one spin system which is these two protons which are connected to each other couple to each other by J-coupling. And these set of four protons which are connected to each other by again J-coupling. How, because 3 is connected to 2 by J-coupling, 2 is couple to this sorry 7 is coupled to 6, 6, 6 couple to 5, 5 is couple to 4. So, by this kind of chain of coupling, this is important term coupling chain network, we are able to connect all this four through in a TOCSY experiment.

So, we can see here will start from if you look at let us say 7 here, so this is a diagonal peak which I said nothing but a 1D spectrum of the molecules. So, you start from 7, I can either go horizontal or I can go vertical we can choose whichever way we want. So, let

us go horizontal because it is labeled here. So, if I start from h 7, I am seeing a correlation to h 6 and this is a cross peak now we can see that some small multiple lines and that is because of the J-coupling multiplied which I said we will ignore we will not bother about it, but in a 2D NMR they will show up, because of the high resolution.

Now, from h 6 I am getting from h 7, I am getting correlation to h 5 as well and that you can see here 7 to 5 will not come if I had in if I had COSY spectrum. This happens only in a TOCSY because of this intermediate coupling to 6. So, 7 to 6 is a pure TOCSY effect, 7 to 5 is a pure TOCSY effect that will not happen in a COSY. Now, you can see we are also getting from 7 to 4, because again because of this intermediate coupling between 7 and 6, 6 and 5, and 5 and 4, I am able to go all the way from correlation from 7 to 4.

And now if you look for each of this others spins, the same similar situation similar situation happens 6 is coupled to 7, this is this peak here; and then 6 is coupled to also 5, 6 is also couple to 4. So, 6 is couple to either side on this side 7, and this side 5 and 4. Similarly, 5 is couple to 6, and 5 is couple to 6 and 7, 5 is also couple to 4. So, all are couple to each other. So, you see it is like a square all with all. So, this is the typical pattern which you get in a TOCSY spectrum.

But you see now we are not able to get any coupling or any cross peak between 7 and 2 here all the way, so that you see where is 2 here this is here if you draw a vertical line here I am not see any peak close to 4 here. Here I am not seeing that because there is a break here of because of this carbonyl. And therefore, I am not able to connect from 7 to 2. But 2 and 3 let us look at 2 and 3, so 2 and 3 is not shown here, but 2 and 3 actually are connected to each, other because they are j couple to each other this is like a simple two spins.

So, this is one spin system consisting of 2 and 3, one you can see this is one family or one set of spins. And this set of spins is another set which is couple to each other in a family. So, this is one spin system, and this is one spin system. So, there are two spin systems in the same molecule. So, therefore, one should be very careful and not assume that spin system is one entire molecule, has spin system basically is a set of spins within a molecule which are coupled. So, in a given molecule, you may have more than one spin system, one hundred more than one different spin system.

But for us unfortunately in biomolecules one each amino acid is one spin system. So, this is the connections which are shown here as I said we have two spin systems in this molecule. So, in a bio molecule, we do not have to worry because in biomolecule there is no such break. So, the one amino acid a given amino acid what about one of the twenty amino acid naturally occurring all of them from one spin, each molecule is one full spin system. So, there is no such multiple spin system.

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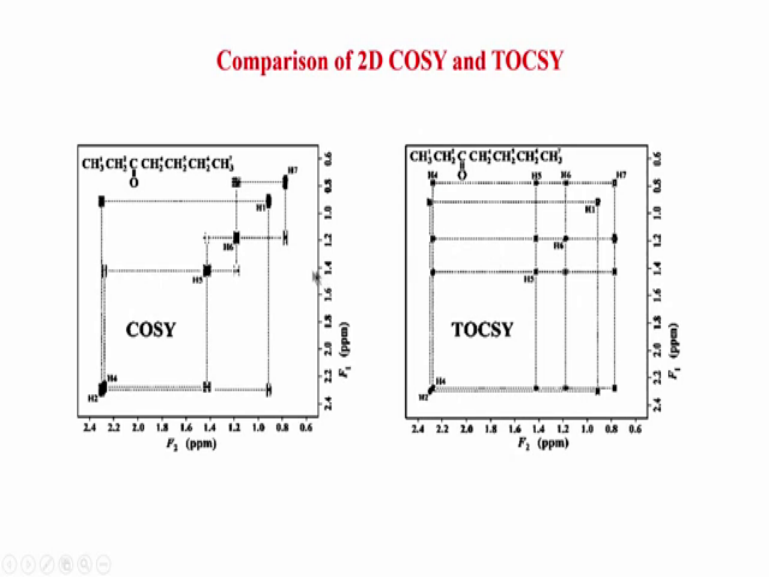

So, this is a comparison now of TOCSY and a COSY for the same molecule. So, you can see there are many additional peaks which are appearing in TOCSY and not there in that COSY. And this is something which we already saw. This is because of the point concept that you have and far range coupling that TOCSY is possible to transfer all the way, whereas, you cannot do that in a COSY in a COSY only are restricted to three bond coupling.

So, this is basically about 2D NMR TOCSY, we will move onto 2D Nuclear Overhauser Effect spectroscopy. And this is now a completely different type of transfer for magnetization from one spin to another. And this is very very important experiment in NMR. In fact, this experiment which actually resulted in bimolecular applications of NMR and which subsequently also resulted in the Nobel Prize Prof. (Refer Time: 19:22). So, Nuclear Overhauser Effect or NOE is a important effect in NMR which actually is

used in routinely for not only small molecules in a bio molecules for every particular any other aspect as well.

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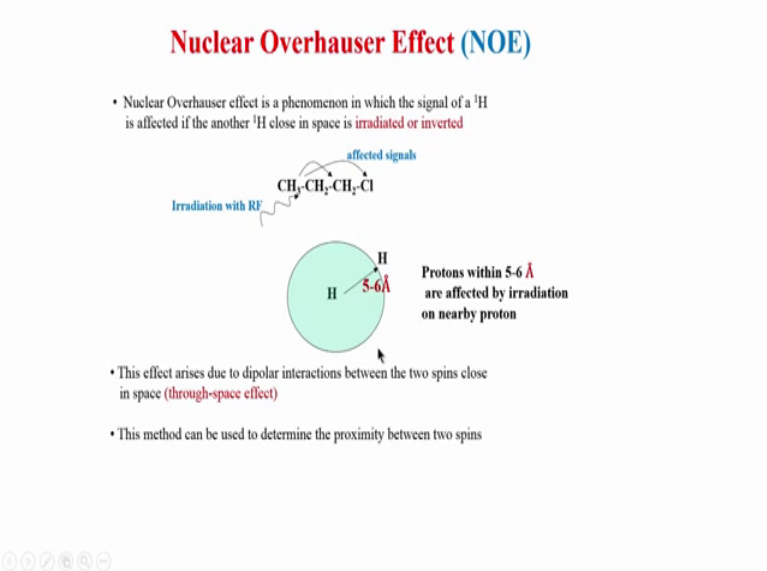

So, before we understand how Nuclear Overhauser Effect works, we have to look at or nuclear NOESY experiment works. Let us look at this concept of what is NOE effect. So, NOE effect basically is an effect which is through-space interaction. So, we this effect happens because of through space interaction basically each proton. So, if you recall the very first slide in the course very first few set of slides we said that each nucleus is actually a dipole magnetic dipole. So, each nucleus has a magnetic dipole moment.

So, if you have two hydrogens in a molecule there also two dipoles. So, any two hydrogen or any two hydrogens you take any hydrogen for that matter any proton is a dipole is a magnet dipole is a tiny magnet. So, therefore, any two protons, now interact with each other. They interact meaning they will they will couple to each other. They will have dipolar coupling between the two. This is just a natural phenomenon which is not in our control, it is just any two dipoles you bring close to each other they will start interactive.

So, now, look at look at this molecule here which we have been looking in the last few slides. So, here these two hydrogens or these two these have to very close to each other in space. So, remember we are looking in space interaction now. So, we are not talking about through bond. So, they may be 3 bond way or 5 bond way, but in space that if you

draw three-dimensional structure of this molecule, in three-dimensional space, they will may come close to each other. So, when we say close what do you how close is considered as close. So, typically in NOE experiment we say 5 to 6 angstroms is the distance which we can think of has close meaning, any, any, any two hydrogen which are within 5 to 6 angstroms or any two protons which are within 5 two angstroms are affected or by each other through by dipolar coupling.

So, therefore, now if I eradiate, that means, I apply a signal only on this proton, this is let us assume that we are able to do that. How we do that we are not going to go into detail, let us say I am able to do that that eradiation because it is now couple to this hydrogen its effects the magnetization of this as well. This is a pure, is simple logic that if I have A and B two spins, and if they talk to each other if I disturb A, B is also disturb. So, any two spins which are coupled to each other or any two atoms, if I disturb one atom and if there is a coupling between this one and two, the second is also affected. So, therefore, this is also affected.

Now, because this is affected this is also affected there is a chain reaction. So, basically NOE initially 1D NOE which started in the very beginning work like this that you eradiate RF you taken RF pulse, you eradiate on a particular selectively on one particular proton, the entire protons spin system, again remember the word spin system all the atoms in this spin system protons which are close to each other they will be effected. So, therefore, their signal in NMR spectrum is also affected. So, this is one of the earlier concept which started in NMR. So, this is sorry this is slightly this arrow has got shifted I am this is not chlorine you are not talking about signal of chlorine, this arrow actually has to point to this is hydrogen. So, this hydrogens to hydrogen, this hydrogen to this hydrogen.

So, the eradiation zone as I mentioned that is how far away should the proton be two proton so that they affect each other is the 5 to 6 angstroms. But remember this proton and this proton is not 5 6 5 or 6 angstroms it is maybe more than that. But because A is connected to B and B is close to C, there is an effect on A to C like a TOCSY. So, there is a long range effect just because there is a intermediate hydrogen which released carries the magnetization from A to B to C. So, this was we use the word spin diffusion. So, in NMR NOESY experiments there is basically a very important concepts that is called spin

diffusion, which talks about this transfer of polarization or magnetization from one hydrogen to another hydrogen because they are very close to each other.

So, NOESY therefore is very information very important experiment which gives information of the proximity between two atoms. So, example let us say here this two hydrogen, we do not know what is a disaster? We do not know the distance. But if I disturb this hydrogen by eradiation, and if it affects is hydrogen in a spectrum immediately I can conclude that these two hydrogens are within 5 to 6 angstroms.

So, this is basically the weight to we look at NOESY, it is very useful to tell telling us the proximity between two hydrogen atoms. So, it immediately tells us, yes, these two hydrogens are closer than 5 to 6 angstroms. And this is an information which we normally do not get from TOCSY and COSY. TOCSY and COSY only telling us the chemical shift correlation between 2 hydrogens in through Bond experiment.

But imagine now a structure a very complicated molecule like a protein or nucleic acids or any other complicated system, where there are not three atoms, but there are hundreds of atoms. So, imagine that for a time for the time being. So, if I have hundreds of hydrogen or thousands of hydrogens, one hydrogen may come close to some hydrogen number 67 or 150 or 360. Now, that interaction will never happen through bond, because 300 bond away proton; obviously, cannot have any J-coupling. But it may come close in space like this. So, this is proton number 1 and let us say this is proton number 300 which is really very far in through bond, but it has come in close through space because of the three dimensional structure property of the molecule.

In that if that happens then that proton number 1 actually affects the proton number 300; if I eradiate proton number 1, proton number 300 is affected. So, you see I get distance information proximity am not getting exact distance value, but at least I know that they are within this distance range. And I can immediately conclude that a proton number 1 and proton number 300 or is within 5 angstroms, but actually if you calculate through bond they may be 300 bonds away or 310 bonds away which means is they are really very far if you consider through bonds, but they are very close if you considered them through space. So, this is a very great advantage of a 2D NOESY experiment or NOESY in general there are different variants of NOESY experiments one D NOESY 2D NOESY a transient NOESY which will see only 2D translate NOESY in this course because that

is the NOESY experiment which is very useful for analyzing the structures of bio molecules.

And again one word before we end this class today is that it is basically the dipolar interaction. So, you have to remember TOCSY and COSY were through bond interactions and NOESY and ROESY which also will come soon these two experiments rely on through space interaction. And together they constitute the very important set of experiments which can be further extended to heteronuclear NMR. So, we can have heteronuclear NOE, heteronuclear COSY, heteronuclear TOCSY which will look in heteronuclear part and all this together are very useful for bimolecular studies.

So, we will take up the full details of how 2D NMR NOESY experiment. We will specifically look at only transient NOESY; we will not look at a steady state NOE in this course, because that is a very old technique which is not used anymore in bio molecules. So, we look at the 2D transit NOESY experiment in the next class.