**Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules**

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**Lecture – 15**
**2D HSQC-TOCSY**

We will look at 2D HSQC-TOCSY experiment today. We in the last class we looked at protein structure analysis using HSQC, but there is very next important experiment which use often in NMR of proteins is 2D HSQC and 3D HSQC-TOCSY. So, let us have a look at how this is designed and developed, in fact this will be very useful for us to understand 3D NMR in the next part when we start.

So, 2D HSQC spectroscopy basically is the same as combining an HSQC and TOCSY in a same pulse sequence. So, let me now take you through a very simple approach how these two experiments HSQC is one experiment, TOCSY is another, how these two can be actually combined and gene[rate]- we can generate 2D HSQC-TOCSY which will give you a very good rich information compared to HSQC or TOCSY separately.

So, this is what is shown here that the essential idea is we are trying to combine simple HSQC experiment which we have already looked at in detail with a proton-proton TOCSY experiment which also we have looked in the homonuclear part. So, now, we are combining a heteronuclear NMR experiment with a homonuclear NMR experiment. And both were 2D experiments as we saw in the previous lectures, but now we are combining them to create a new 2D experiments. So, this is also going to be a 2D experiment, which will be called as HSQC-TOCSY. So, this as I said we will form basis for going into 3D NMR which we will see how 3D NMR are built. So, it will be similar to what we I show you here the 3D NMR experiments are built.

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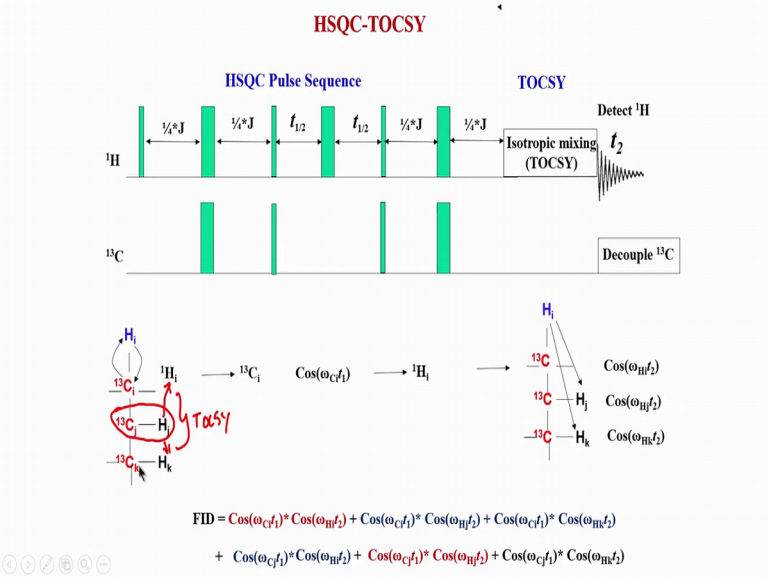


So, let us start again from a basics or recap of HSQC pulse sequence. So, this is what we saw in HSQC part, you start from a proton excitation, you excite the proton pulse. Then there is what is, this called as inept experiment inept module. So, in a inept module, you transfer the magnetization from proton to carbon, or it can be proton to nitrogen as well in the case of nitrogen HSQC. And this is done through this series of these two 180s here, and they are exactly in the centre of delays two delays. So, there is this long delay which is 1 by 4 j, the another delay 1 by 4 j, on either side. So, this j is according to what we saw is depending on the coupling value. So, j is 140 hertz typically the value we get is 1.8 milliseconds here, and 1.8 here.

Now, after this pulse excitation is over, we take it back to z-axis the proton pulse and carbon is excited now to bring it to the x-y plane. And during this period the carbon is in the x-y plane. And it evolves according to its chemical shift value during this period and that is during this t 1 half t 1 half. But if you combine these 2, it becomes t 1. So, again repeating why have you split this t 1 into two parts because we want in the middle, we want to have a 180 degree pulse on proton, and this helps to decoupled the carbon from proton. So, any 180 at the centre of evolution not on the nucleus on another nucleus helps to decouple that nucleus from this evolving nuclei. So, this is the principle behind this idea that if carbon is evolving. And I put a 180 degree in the centre; it decouples my carbon from proton.

So, there is no proton carbon coupling which is present during this period. And following this period, we then transfer the polarization back to proton, and this is through in the reverse inept. So, in the reverse inept, we basically go back to the proton magnetization or polarization which is something which we have seen in HSQC. This again the same as a inept here. So, we have 1 by 4 j, 1 by 4 j, and here also we have 1 by 4 j, and 1 by 4 j. So, we can see that this part is same as this part, there is no difference. It is just that this is called as forward inept and this is called as a reverse inept.

Now, we can see here the magnetization at this point will be completely on the hydrogen proton. And then what we did in the HSQC was we started evolving this magnetisation, we started detecting this magnetization. And during that period, we had decoupling on the carbon present. But in HSQC-TOCSY, now we want to combine this HSQC with a TOCSY. So, what I need to do now yeah I need to add a TOCSY part in this here, and then that becomes an HSQC and a TOCSY. So, before we look at the TOCSY part here, this is this look at the magnetization again, how the magnetization is going from proton to carbon.

So, now look at this schematic drawing here of a molecule. So, let us say your molecular structure is something like this. So, what we are looking at here in the first part here is that we started from a proton which is we can label as i and I transfer the polarization to carbon through this inept ok. And then in HSQC that is what is shown here, we transfer to carbon. And we had this carbon chemical shift evolution and then we transfer it back to proton, and this is exactly what this is basically what we did in HSQC.

But now what we will do is we will add an additional part and that is the TOCSY part. So, you can see here, this is the TOCSY mixing now. So, what is the part doing here, it is basically mixing or transferring the magnetization from one proton to another proton. So, if you look at this point here, here, where the arrow is pointing we are basically on the proton magnetization ok. So, we are in the, I that is H i, which is this H i, I have taken this H i as an arbitrary H i. So, now, from here onwards it has now it is in the x-y plane, we have not detecting this proton yet it is in the x-y plane. Now, if I apply a mixing like this for some duration if you recollect the duration time for this should be around 80 to 60 to 80 milliseconds along.

So, during this period, the magnetization from one proton or that i proton is transferred to every other proton in the spin system. So, remember we have to remember that magnetization goes within the spin system only; it does not go out of the spin system. The transfer of magnetization from one proton to the remaining proton takes place only within a given network of coupled spin. So, we are having all j coupling to each other. So, this hydrogen is j coupled to this hydrogen, this hydrogen is j coupled to this hydrogen, but this need not be directly couple to here. So, i is couple to j, j is couple to k, and therefore, i gets couple to k through j and that is that long-range interaction which is through TOCSY mixing happens to TOCSY mixing.

Now, during this period, we use the power level etcetera similar to what we do in a regular TOCSY. So, this is nothing different from a regular 2D TOCSY. But you can see now what I have done here I have taken an HSQC part and simply added a TOCSY mixing. So, this is how we design or we construct multi-dimensional NMR experiments, we take one experiment and we concatenate meaning we add to it or append another experiment, and the combination is done in a way such that the required information is given to is comes out.

So, we will look at the information now. So, now, during this period t 2 the magnetization evolves on that all the hydrogens wherever the magnetization has been transferred. Because now this was in x-y plane at this position here, from here it got transferred to here and here and there it is, there these protons are also now in the x-y plane, and all of them now start getting evolve they getting detected during the t 2 period and during that period we are decoupling as usual. Now, this chemical shifts of each of this hydrogen is just written as H 1, H, H i, H j, H k. So, they will evolve during t 2 and we can say that the revolution is captured as cosine omega h I H j and H k t 2.

So, now if you look at this correlations what we have done with this experiment, now we have correlated or connected the chemical shift of carbon of one carbon i to another to its own proton which is HSQC. So, this combination that is H C i t 1 and H i t 2 is nothing but the HSQC peak which you all of all of us have now seen it in the previous lectures, this is what we got in the HSQC. But what additional information now we have obtained because of the TOCSY is that we have correlating now C i with also H i, H j. So, you see this is not a direct coupling, because that was two bonds away. So, you can see that j is here, this carbon C i is actually two bonds away.

So, I remember I told you in HSQC we do not tune the coupling to two bond, we tune the coupling only to one bond carbon and proton. But here you can see now we have got information of C i chemical shift correlated with H j; not only that we have now got C i chemical shift correlated to H k as well, because all these combinations will come this cosine into this cosine, this cosine into this cosine, and this cosine into this cosine. So, these three combinations all of them will come which I will show you shortly. So, these three have been correlated with this carbon which would not have a happened in a normal HSQC. In a normal HSQC, I could not I would not have seen the interaction or correlation of this chemical shift of this carbon with this proton, and this proton because of this delay which is tuned only to this coupling.

So, this is mathematically is what is shown here you can see that the FID which I finally, detect here this t 2 is looks like this now we have cosine c 1 into t 1 cosine c 1 t 1 omega which is this into the H 2 H 1 t 2 which is this. So, this is coloured in red, because it is now the same as an HSQC peak. This is no different from HSQC if you go back in slides in the previous lecture, you will notice that we did see this correlation also in HSQC. So, there is no different from that.

But additionally we have got two more inform correlation for this particular molecule if the molecule is longer we would have got even more longer correlations long range correlations. What we are saying now right now in this case in this schematic drawing we have only H j and H k, but C i is getting correlated with the H j and C i is also correlating with H k. So, these are the two special additional chemical shift correlations which we would not have got in a normal HSQC, but by adding this TOCSY part we are able to get this information ok.

So, this is what is shown here additional correlation that is all the same that is now we can see this suppose if I do the detection on this particular proton suppose I do transfer the magnetization from H j to C j. So, suppose I now if I look at H j into C j interactions, this is again direct HSQC peak, but now because of the TOCSY H j will also correlate with H i, and H j will also correlate with H k ok.

So, now when we are looking at these combinations, so let me write this here. So, now, suppose we focus on this complete pair this hydrogen is correlated with this, this hydrogen is correlated with this because of TOCSY. The TOCSY is helping us to

correlate this proton to this proton. Now, this is what is shown in this particular here picture in the bottom line. The bottom here you can see, so we are looking at this pairs, so now, this is an HSQC peak this is what we will normally get in HSQC. But in this HSQC-TOCSY we will also correlate this carbon with this proton because of this transfer; and this carbon also gets correlated to this proton because of this transfer here. So, that is what is shown here, so that is also will possible in TOCSY.

So, TOCSY basically correlate this carbon with these two three protons; this carbon with these three protons; and this carbon with these three protons. So, all carbons get correlated with all protons. So, HSQC-TOCSY is a experiment beautiful experiment which helps us to find all connectivity between all protons and all carbons that something you would not get in a regular TOCSY, neither you will get in a regular no HSQC. You need this combination HSQC-TOCSY to connect all carbons with all protons in a given spin system. So, you have to remember this is only in a given spin system. If there was a break here and the remaining carbons were away from the break, we cannot connect that, we need to connect we can connect only within a family of coupled protons.

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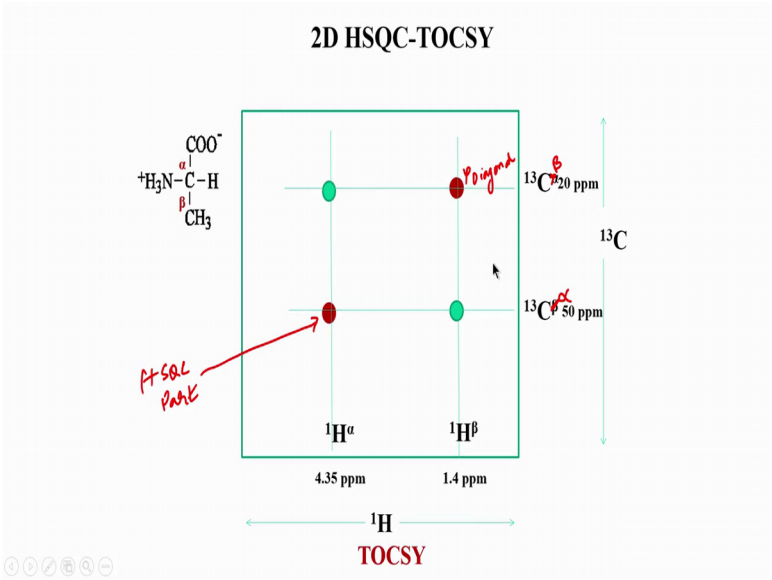


So, let us look at the peak pattern what you would expect in a regular HSQC-TOCSY. And this is a simple amino acid alanine which is given here. So, we can see in alanine we have this proton CH 3, and there is a proton H which is C alpha H alpha. Now, in a

regular HSQC, what you would expect to get, in a regular HSQC you expect to get the correlations of this carbon to this proton these two pairs one pair, and another pair would be this pair. So, you will basically get two peaks.

Now, typically C alpha comes around C beta. So, this is slight error here, let me correct this, this should be C alpha and this should be C beta ok. So, this is what we should keep in mind. So, now, a typically what you would expect is a C beta will correlate with h beta ok, so that will come around 20 ppm; and H alpha will correlate with C alpha. So, this is HSQC part ok. So, this is HSQC part. So, I can write it here this is basically the HSQC part ok. So, this is not different, this is nothing which is new we already we have seen this with several examples. We took glutamine; we saw for other molecules.

Now, what is the additional information that we get from TOCSY, the TOCSY now gives you this part because C beta is now correlated with H alpha that is this green peak here ok. So, and C alpha it is correlated with H beta and that is this peak here. Thus you can see the C alpha chemical shift is correlated with H beta; and C beta here is correlated with H alpha ok. So, but please keep in mind this is not a diagonal peak. So, I should write it here clear this is this is not diagonal. This is already a cross peak. So, this is also a cross peak and this is also a cross peak. So, it looks like as if this looks like a diagonal for some people and this looks like cross peak.

But according to this, this, this is already a across peak because remember this is proton axis and this is carbon axis. So, there is there is no question that this is this is a cross peak, this is actually not a diagonal peak. But now additional cross peaks are available now, and that additional cross peak is coming because of the TOCSY mixing which we saw in the previous slide that this hydrogen is now correlated with this carbon, and this proton is correlated with this carbon ok. So, we can see this is a cross talk between two carbon.

So, the entire spin system now is available based on this pattern. So, you see if I look at the square or this is not actually a square a rectangular pattern, so along this line if you look along this line, I am getting from one carbon to all the hydrogens in the molecule in a given spin system. Although you I may say molecule, but the molecule can have two spin system as we saw in the hepta known case. So, if basically within one spin system

which is in amino acids as I mentioned last time, one amino acid is actually one family, one spin system.

So, in a one amino acid you can correlate one carbon with all the hydrogens or protons in that molecule. Similarly, you can see another carbon in the same molecule I correlate with all the hydrogen. So, basically all hydrogens with one carbon, and all hydrogens again with another carbon. So, if I look at them, they will look like this that all hydrogens will come here, and all carbons will come here ok. So, let us see now I say little more examples, another few examples of this system. So, this is a TOCSY part. So, that is dimension what you are seeing in the horizontal axis is proton, but is because we are getting all that protons in this line we are calling it as a TOCSY line or a TOCSY pattern, because all protons are connected to each other.

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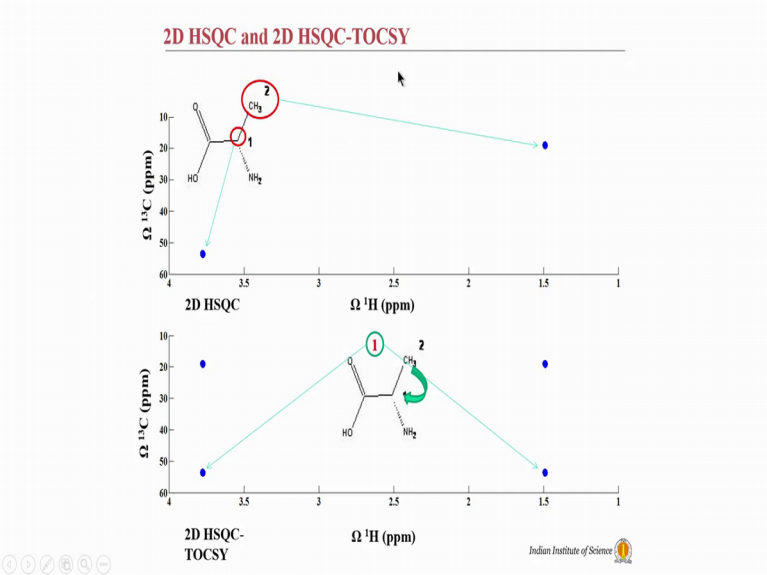


So, let us say that we have these two molecules. So, for example, we have this molecule which is an amino acid here, and this is glutamic acid or aspartic acid. Now, here we can see that there is this HSQC peak here. And what is this peak here this is the beta proton ok. So, this is the beta, these are alanine here. So, you can see for alanine, you can see the same pattern which were I showed you earlier, these two peaks. And then this is this proton because it comes down it will shifted. Remember we mentioned this in the previous part when we look at HSQC that H alpha come somewhere around 50-55 ppm,

and H beta comes around 1.5 ppm. And this is something which we saw here just in the previous slide as well.

Now, if you look at this particular case now in HSQC-TOCSY, I am able to now get additional peaks. And these additional peaks are because of this interaction. So, this peak as beta is correlated all the way to H alpha and that is a TOCSY pattern this H alpha and H beta. Similarly this H beta and H alpha they are connected to each other and that is a TOCSY, so I am saying this TOCSY peak here these two are repeated here. And one repeat one line corresponds to the H beta C beta here which is 20 ppm and another C a line corresponds to C alpha which is around 55 ppm. And it is something we saw in the previous slide and this is just a repetition of that.

So, how does HSQC-TOCSY help us in assignment. So, remember one of the goal in protein NMR which we will see eventually if we want to assign a protein, so we are trying to assign amino acids. So, suppose I have 100 amino acid in my protein, I have 100 spin systems in the molecule ok. So, therefore, I need to identify each and every spin system for my spectrum. So, this experiment TOCSY helps us to do that.

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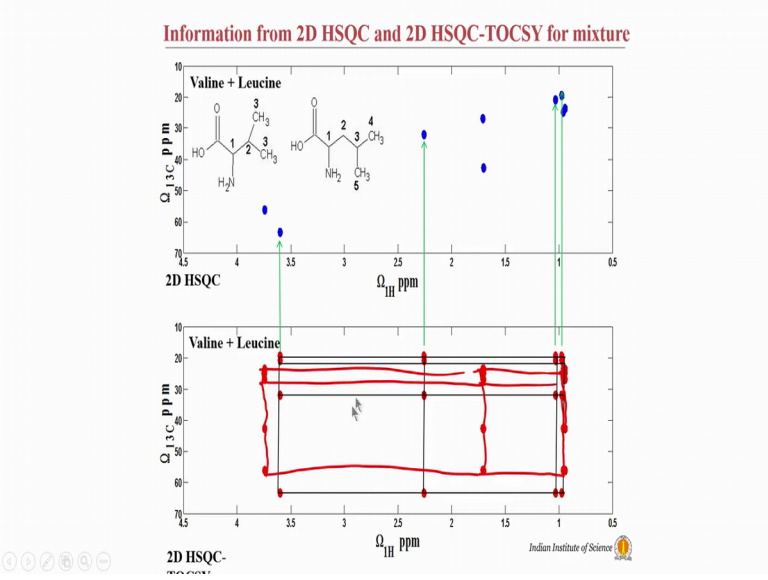


So, let us see how it helps. Suppose I have a mixture of valine and leucine and this is a something which I told you in a protein, we will have many amino acids like this. In fact, we may have many valines, you will have many leucines. So, you will end up with a mixture of many amino acids. So, it is a similar to a mixture. Although they are all join

together by a peptide bond, but each amino acid is a molecule is a is a separate molecule is separate family. So, we can imagine protein chain which consists of many amino acids joined together actually as a mixture of molecules, mixture of amino acids.

So, as far as TOCSY is concerned there all similar to having 10 valine, 10 leucines, 10 alanines and so on. So, let us say that a simple example. Here we have a valine and a leucine mixed together. So, how will the mole spectra look in HSQC-TOCSY and how do you identify molecules it is shown here. So, this is HSQC of the mixture ok. So, now, here you may find it difficult to say which is leucine. You may refer to a database or a text book, but remember in a biomolecules this is never possible to say which is which. Here, yes, we can say these are mrthyls, but methyls can also correspond to methyl from leucine or methyl, so methyl from leucine here or a methyl from valine right.

So, we cannot simply say that this is methyl of valine, this is methyl of leucine etcetera these is not easy to do that. Here to some extent yes we can guess from based on the chemical shift values which peak corresponds to leucine and which peak corresponds to valine. For example, this could be leucine based on the database; and this could be valine. So, this kind of knowledge will only help here that is the knowledge of prior structure, but knowledge of structure will not help us to say anything about this part here, because here all methyles and knowledge will not tell us about here ok.

So, what we need to do now is to record a TOCSY. So, HSQC we will combine a TOCSY and we will record this HSQC-TOCSY which will give us this spin pattern shown here. So, here you can see that within a given molecule that is within leucine I will form all the connections all the peaks will be connected to each other, whereas within valine all peaks will be connected to each other. So, by looking at the connectivity pattern like this you can see that these two peaks which were here they were actually connected to each other.

So, these two peaks are connected to each other. So, that is why they form a square pattern if you look carefully here. Then these two peaks are connected to this peak here, and this peak here that is this peak here and the peak here. So, you can see that this family or these set of protons are actually connected to each other and they form the spin system ok. So, spin system is basically a network of connections between protons to each other.

Now, what is the advantage another advantage, we have is that we also get the carbon chemical shift on this axis, which earlier was not possible from a regular HSQC from regular TOCSY. A proton-proton TOCSY if you recall in the homonuclear part of this which we dealt in this course, we saw that there is no carbon involve. Similarly, HSQC has no TOCSY involved. But in this kind of a spectrum, I am able to actually correlate carbon with proton not only that I am able to get the complete spin system in the same line of protons. So, by doing this simple joining and matching by spin peak pattern, I can actually identify that all these peaks belong to one family that is one spin system and that could be leucine or valine depending on one information if I have any information.

For example, if I know that this line belongs to leucine, then that means, the entire family now becomes leucine. If I say that this is could be valine based on the data base, then this whole becomes valine. So, although I did not know within these two which are valine and leucine; similarly I did not know which has leucine which has valine. But by connection of the protons with each other, now I am able to separate or distinguish leucine from valine.

Now, what about the remaining spin system this is this green colour lines are basically connecting these peaks to the respective peaks in the HSQC. Now, what about the other molecule, the other molecule is here, this is not shown here. So, let me draw that for you. So, basically we are connecting this line and this will be connected to this sphere, connected to here and this will be connected to this here. So, that will form another spin system which is the valine.

So, you can see that the two spin system are actually very nicely separate from each other and they form a spin system. So, these two spin system, therefore can be now distinguished from each other, and that is how basically the NMR experiments NMR spectroscopy works that we record a TOCSY like this, and we try to identify the pattern like this. And from the pattern like this, we try to see what kind of amino acid that could be, so that is valine, then we assign it to valine.

If it looks like a amino acid leucine or glutamine or any other amino acid, we then assign it to the respective amino acid. Similarly, we may find another spin system which are connected to each other, and they may now correspond to another amino acid. And that could be again depending on what is there in the proteins or protein may consists of

asparagines, serine, threonine, or they may be another valine in the same molecule. So, based on that, we can see this is another one valine or another leucine in the molecule them in the protein.

But then we do not know which leucine is which, it could be number 1, this could be leucine number 20 and so on that information is not coming from here. So, you see there are two problems in protein and NMR. Number 1 is given that a number of amino acids, this is something which we will repeat again in the second part, but I am just jumpingead to tell you what is the problem that we are trying to address in protein NMR, the two challenges are. Number 1 that I have let us say 10 leucines, 4 valine, 3 argenine and so and so forth. There is a multiple copies of a same amino acid. It is not that there is only 1 valine, not 1 argenine, 1 cerine and so on.

So, our for task is to first figure out what are the different amino acids present in the protein. So, that for that you need to know the sequence of amino acids, but let us say even if I know the sequence, I need to identify whether I am really getting 4 leucines according to the sequence in my spectrum or not, so that is one task is to identify the spin system that is what is TOCSY you helpful for use full for, but the next task is that which leucine is which number.

So, if I have 4 leucines in my spectrum which I am seeing like this, which leucine is which number we do not know. So, that correspondence that is leucine number is this one leucine is leucine number 4, other leucine is leucine number 20; third leucine is leucine number 16 that kind of a correspondence is not available from this ok. So, that part of course, comes from another experiment which is three 2D NOESY-TOCSY NOESY-HSQC which we will see later or from 3D NMR experiments though this is what we will see.

So, basically one has to build several experiments to get different type of informations that is basically the heart of NMR. In NMR spectroscopy the whole task is to keep on designing NMR experiments like HSQC, HSQC-TOCSY, 3D NMR and different experiments, so that we get better and better information about the protein. So, in the next class we will basically look at protein NMR, 3D NMR, and we will see how 3D NMR experiments are built what kind of information we can get from 3D NMR. And

then based on that knowledge, we will then start looking at the structures of protein, and then start looking at how NMR can be used to solve the structures.