Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 03
Observing the NMR Signal

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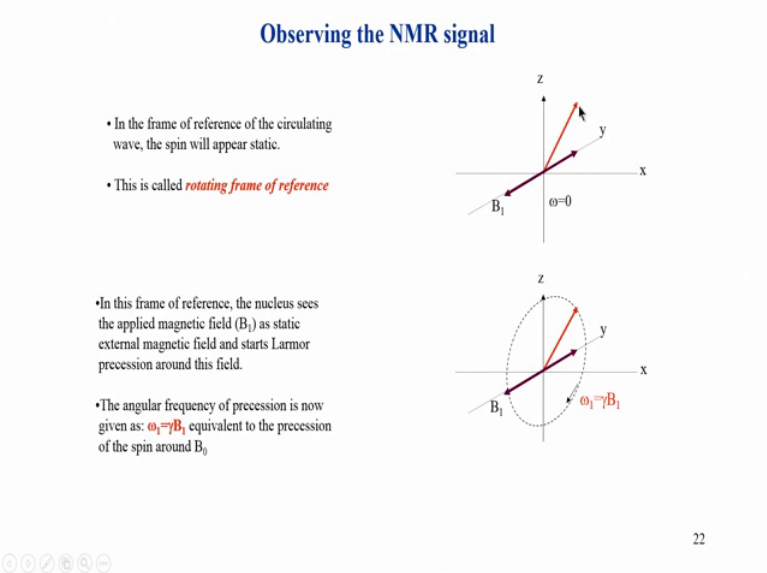

Welcome back to the course. In the last class, we were looking at how do we observe the NMR signal in the rotating frame. So, we introduce the rotating frame of concept which is shown here. You can see when we talk about rotating frame what essentially we mean is that the RF frequency, the RF radiation which is applied is actually also rotating in the x-y plane and that is synchronized with the precession Larmor precession of the spin. So, when that happens we use word resonance. So, in the rotating frame, we are trying to reach a resonance condition such that the frequency or the rotational angular frequency of the B 1 that is a magnetic field which is applied is in synchrony with the spin.

So, under such conditions in the rotating frame, so when you go into a virtual frame, a new frame of reference the spin is now static, because it is now having the same speed or same angular frequency as the magnetic field which is rotating in the x-y plane. So, in such conditions, the spin now starts precessing around B 1. And this is again a Larmor frequency Larmor precession which is happening now around B 1, whereas in the previous case, it was around B 0 which was in the z direction. But we have removed that

affect we are removed the effect of the rotation around the z-axis by going to a rotating frame where omega then become 0. And therefore, the only precession now which is noticeable in the rotating frame is this precession around B 1. And this frequency is now given by omega 1 equal to gamma B 1. So, this is for a given spin.

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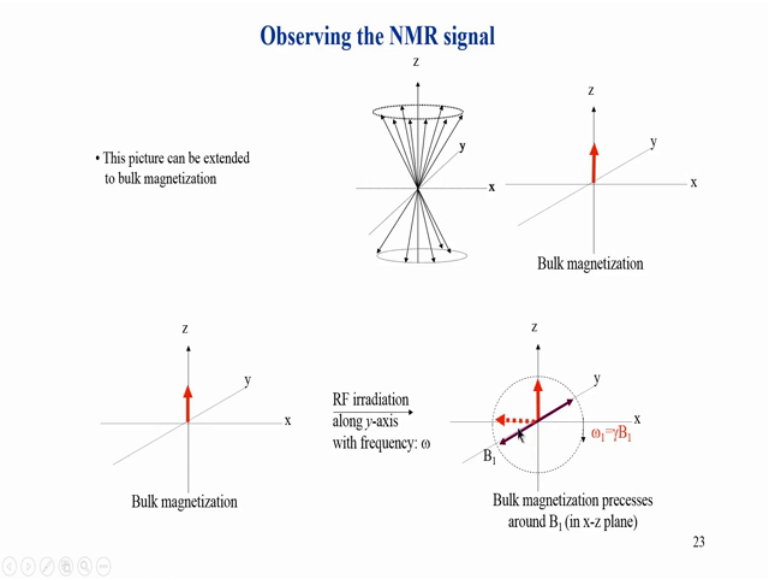

But if you remember, we looked at collection of spins, we never look at a single spin, and if you look at a collection of spins, we represent this collection of spins by a net magnetization or we call it as a bulk magnetization vector, so that is pointing in the positive z-axis. So, now, if you look at this spin how it behaves or this particular magnetization vector, how it behaves under an application of a magnetic field, we will see how it that happens. So, in the similar situation, we apply an RF radiation along the y-axis along this axis and now the spin start precessing along the around the y-axis. So, it is precessing or rotating in the z-x plane. So, you see it is going from plus z it goes to x goes to minus z minus x and so on. So, this is how it keeps rotating in the rotating frame around the applied magnetic field which is B 1.

Now, B 1 is actually an electromagnetic radiation, but it has electromagnetic radiation has electric component and the magnetic field component. So, we are now looking at only the magnetic field component and electric field component is ignored because we are looking at nuclear magnetic phenomena. So, the only the magnetic field part of the electromagnetic radiation actually interacts with the spin. So, under this resonance

condition, when this the omega naught, when the gamma equal to omega naught, gamma equal to omega equal to gamma B 0, the resonance condition happens and the spin start precessing.

So, this precession actually happens in we can say this angle is called as 90 degrees this all of us know this a perpendicular. And if you go further it is now 180 degrees. So, we can allow the spin to go by any angle we wish, and we can then stop the rotation. How do we stop the rotation? We stop the rotation by removing this B 1. So, when we remove this B 1, the spin is then frozen in the direction or in the angle to which it has come. So, we can go further, it will be 270 degrees. And if you go further, it becomes 360 degrees.

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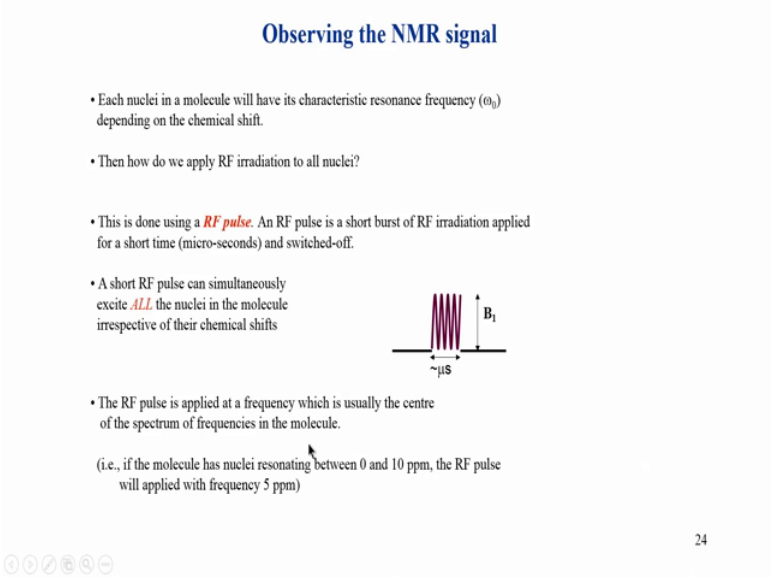

So, this is how basically NMR signal is observed each nuclei in a molecule will have its characteristic frequency depending on the chemical shift. So, we will look at the chemical shift concept shortly. But what basically it means is suppose we have a molecule which has three hydrogens, three different types of hydrogens, each of the hydrogens will have its own omega value that is own Larmor precession frequency, because each of them have a different rotational frequency. Now, therefore, we have to now excite all of them means we have to have resonance for all of them at the same time.

How do we achieve that? We achieve that by applying a pulse. So, this is where the pulse NMR concept comes into picture. So, how do you apply an RF radiation which is on resonance with all nucleis. So, remember on resonance can happen only for a particular

value, but still we can apply a high frequency or a high power frequency such that all the spins are excited or in resonance.

So, this is done using an RF pulse an RF pulse is basically a short burst of RF frequency. So, you can see its depicted here is an electromagnetic radiation of a very short duration. So, typically it is in the microsecond. And the amplitude the height of this is the B 1 which is what we discussed. B 1 is the RF magnetic field applied in the x-y plane. So, this is applied in x or y direction and that is now a very short duration. And after sometime, it is switched off. So, as soon as you switch off the RF field the radiation the spin has rotated by some angle which we saw in the last slide and that is angle at which the spin stops and that we call it as a flip angle that is how much it has flipped. So, the RF frequency is applied at the centre of the spectrum.

So, this is something which was discussed in the previous course, how the in the practical aspects how do we apply an RF pulse. We will not go into detail in this course that the main take home is the RF pulse that is this pulse we shown here has four components associated with it. One is the width of the pulse the duration which is already shown here, which is typically in microseconds. Then you have the height or the amplitude of the pulse which is the strength of the pulse, how strong is the pulse that is represented by this notation by B 1.

Then you have two more parameters one is the phase; the phase meaning it is applied along x-axis or y-axis that is not shown here, but that is important, so, that is it is a third parameter. And the fourth parameter is the frequency of this. So, we can see this actually is having a frequency. So, what is the frequency at which this is applied and that frequency is basically the Larmor that is basically the resonance frequency, so that is in megahertz.

So, the frequency of an RF radiation is an megahertz regime that frequency is this shown here. But the strength is depending on how strong you want to excite the nucleus, how much or how long is the I mean how much angle flip angle you want to achieve. So, this is what as I said we apply in the centre of the spectrum.

So, let us say your molecule has nuclei or a spins which are between 0 to 10 ppm. And this is a chemical shift scales which we will discuss again as I said shortly. And then you take the RF pulse is apply, you keep the frequency of the RF pulse somewhere around 5

ppm. So, what happens is it excites or causes resonance for nucleus on either side of 5 ppm. So, it will go on this side 5 ppm and this side 5 ppm. So, the center of the spectrum is what is usually chosen for applying the pulse.

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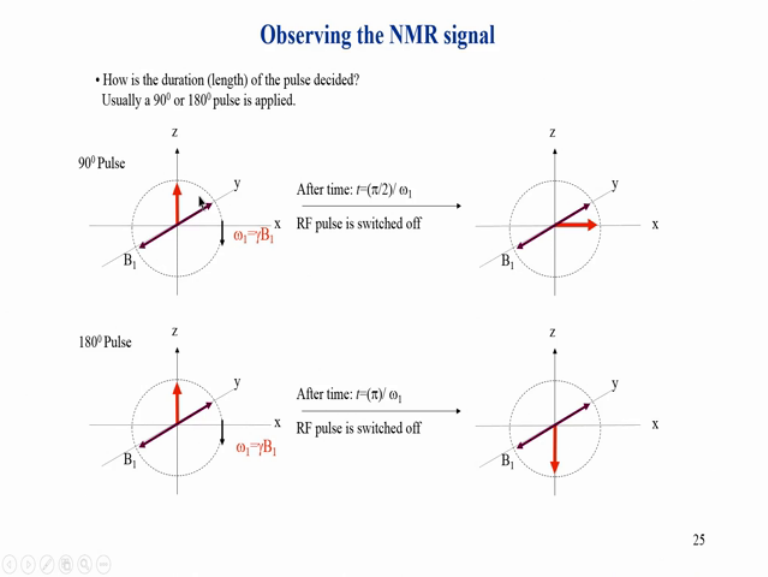

Now, let us look at this concept of pi by 2 and 180 degree pulse which is very important when we go to 2D NMR. In a 1D NMR, 1D NMR is simply applying a magnetic field and you wait for sometime and after that time it has rotated by an angle by 90 degrees. At that moment we switch it off, the RF pulse is switched off. So, this is typically what is done in a 1D NMR, but sometimes you may have to go in a 2D and 3D NMR, we will have to go beyond 90 degrees. So, what is done is you apply the RF field that is B 1 perpendicular to in either y-axis or you can apply it along x and start with the magnetization which is along z-axis. The magnetization flips by 90 degree.

And you continue to apply, do not stop the RF, let it be on. When you keep it on, it goes further and it keeps going it can come up to 180. So, you can switch off at this stage. So, if you switch off at this stage, the signal is now or the magnetization is now located at minus or say 180 degrees from the z-axis, so minus z-axis. So, that if you stop at that point, we say that we have applied a 180 degree pulse. This is a 90 degree pulse. So, we have applied the B 1 as long as the magnetization has gone from z-axis to x-axis. Remember it follows a right hand rule so, it will go on the x-z plane. And if you stop at

180 degrees, it is then called as a 180 degree pulse. So, this is a two basic pulses which are very important.

You can also look at it from here this is called an inversion pulse, because it is inverting you can see it is here and it is just simply inverting a magnetization. I can also invert the magnetization from this direction what you are seeing here, all the way to this direction minus x. So, when you go from a x-axis to minus x, we use the word refocusing pulse. So, both are 180 degrees. So, refocusing pulse is also 180 degrees, inversion is also 180 degrees, but inversion is basically bringing magnetization from z to minus z, whereas refocusing is bringing from x to minus x or y to minus y. We see this in more detail, when we go to 2D NMR.

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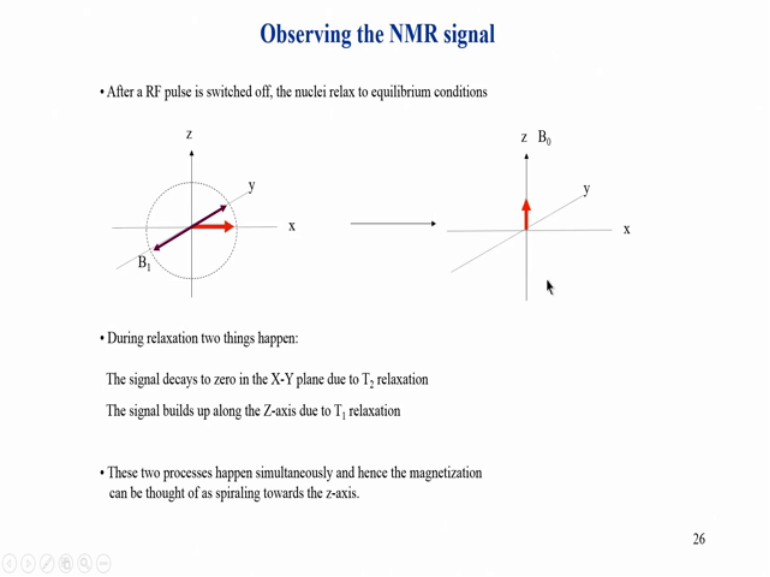

So, this is now what happens after the pulse is stopped. So, this as what I shown here. So, what I said is we first apply a pulse which uptakes the magnetization let us say by 90 degrees. So, let this is a 90 degree pulse. Now, moment you remove the pulse, thus magnetization has to go back to equilibrium, this is the equilibrium picture scenario. So, it has to go from this direction back to this. So, how does it go, it goes via two pathways and those two pathways are called relaxation pathways. So, one is called T 2 relaxation which we will see now shortly and other one is T 1.

So, what is T 2 and T 1, T 2 relaxation is basically the signal is this is signal has to come. Now, what happens when you apply a pulse the signals are all in phase, because you can

see the magnetization have all come to the x-y plane and they are all bunched along x-axis. And now when they go back towards z-axis, they have to again de-phase in the x-y plane because that is a picture we saw in the classical picture classical way that has to happen and that de-phasing of the signal in the x-y plane is called as T 2 relaxation. And T 1 relaxation is happening which happens at the same time as T 2, but it is its different path way in which the signal builds up along the z-axis.

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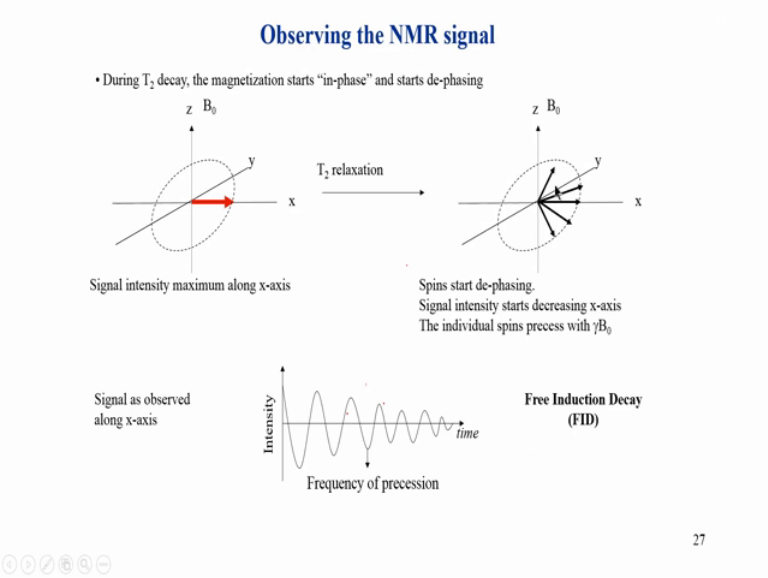

So, let us see little bit more in detail, this two relaxation pathways. So, this is shown here. So, we have brought the signal to the x-y plane or x-axis the signals are all bunched together. Now, during after removing the pulse, the signal is now free to go back to z-axis. It has to go back to z-axis because it has to reach the equilibrium situation. So, this is what happen. The spins starts now de-phasing. This is basically they start spreading out in the x-y plane and then they slowly they spread out. And after sometime they completely de-phased, completely meaning the spin vectors are all spread over this x-y plane and that basically happens slowly and that process is T 2 relaxation. So, T 2 relaxation is nothing but this in decreasing of the signal.

Now, what happens is now let us say we observe this we keep a detector here. So, now we come to the detection part of this NMR signal. So, if I keep a detector along x-axis, I will observe a maximum signal at this point when the signal has come to x-axis that is

here shown in this plot in this picture in this point that is at T equal to 0, time when I start observing the signal as soon as the B 1 is switched off, I will get a maximum signal.

But as soon as the relaxation begins the signals are de-phasing. So, if you see now, they are not all of them along the x-axis they are slowly going away. So, if you take a combination of all of this along the x-axis, because remember detector is only here it can only detect what is along the x-axis. So, if you take a combination of this along x-axis, it has now come down, it is not as good or as strong as the initial time.

So, you can see this is shown here, it starts coming down. And then after sometime the signal is also rotating remember because of the Larmor precession. So, it comes back again in this position and that is shown here it comes back. But it does not come back fully it has reduced little bit because the de-phasing is re-phasing that is this is called de-phasing and re-phasing is not complete it has decreased a little bit then again it de-phases and so on. So, this de-phasing is going on all the time, but at the same time there is an oscillation this is a frequency happening it is having a frequency at which it is oscillating. And this is nothing but the Larmor precession frequency ok.

So, if the detector basically detects the signal going up and down with a particular frequency and that frequency is nothing but the precessional frequency, so that this signal which is detected is called a free induction decay. The reason we call it as a free induction is because the signal is free, now we are removed the pulse. So, the signal is free to go back to equilibrium. And the reason it is called induction because the signal is detected by the magnets this tiny magnets which are rotating they induced emf in the coil which is kept along the x-axis and that causes a signal to be generated and that is amplified and detected that goes in a oscillating manner. So, this is induction.

So, we are actually detecting the signal by induction not directly, but indirectly through its induction in a coil that is called and the decay basically because the decay the signal you can see is slowly going down and down. It does not come back fully, it goes down and that is because of T 2 relaxation. So, the signal in FID decays purely essentially mainly component main component is T 2 relaxation.

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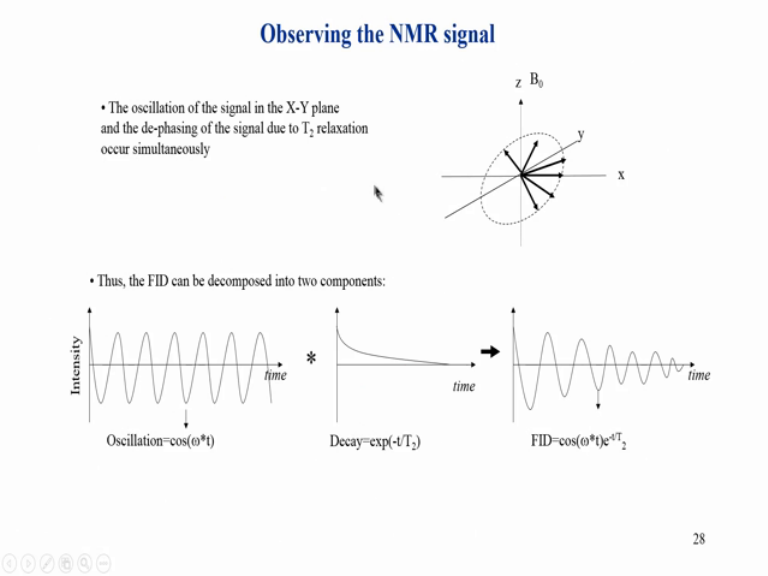

So, this is how the signal is observed in NMR and you can see that here the oscillation in the x-y plane as I said is T 2. Now, how if you look at it, the FID which I showed in the previous slide this one, this picture here you can actually construct the same FID like this. You can think of a pure oscillation because a signal is oscillating because of Larmor precession and that you multiply with a decaying part. This is T 2 relaxation. So, if I multiply the T 2 part relaxation part, which is an exponential decay into a simple oscillation which is cosine I get the FID that is what I showed in the previous slide.

So, the FID is nothing but it consists of two components as oscillation or a frequency component which is cosine or it can be sine multiplied with an exponential decay term. And this is the total actually the FID which is detected and there has to be a multiplicative constant here which we will see later and that constant is basically this height which is a time t equal to 0. And that depends on the concentration or number of protons and so on, so that factor also comes second third factor in this equation.

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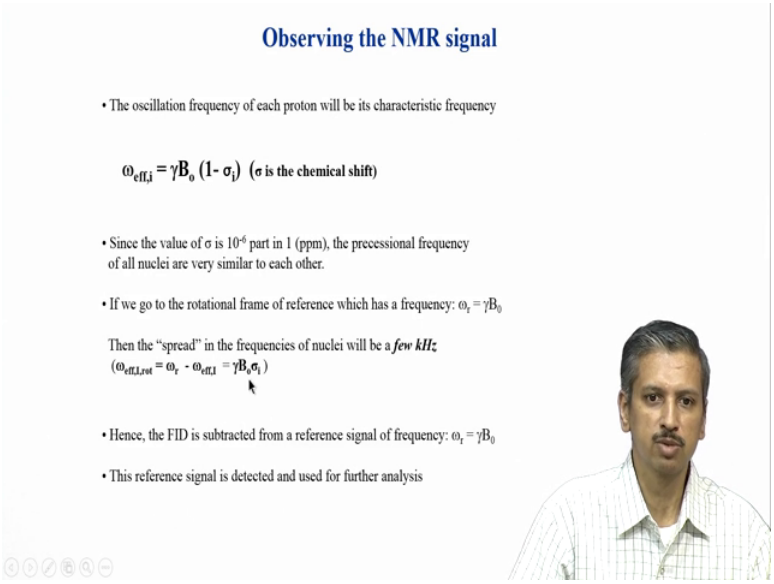

So, now let us see what is the concept of chemical shift? Because the frequency which I showed in the previous slide each frequency each proton in a molecule has a different frequency so which means it has to now depend on what is called the chemical shift. Why is a the frequency is different because of this concept. So, what is a chemical shift let us look at this like this. So, this is the equation the standard NMR equation without the sigma. So, just concentrate on this part that is omega effect is gamma B 0.

Now, what happens is each nucleus which is written as i here does not actually experience only B 0, it experiences a slightly reduced magnetic field or slightly increased depending on the sign of this term. So, therefore, what we do is we are now writing this term which represents an effective magnet or effective frequency of the nucleus. For example, let us say sigma is 0, then it becomes same as a standard nuclei. But if a sigma is not 0, then this is decreased this is 1 minus this is less than 1 and therefore, this effective reduced frequency is present.

So, this is essentially the concept of chemical shift that chemical shift is essentially reducing the effect of the total magnetic field on the nucleus and therefore, that there is a results in the change in this angular frequency. And each nuclei that is i will have different angular frequencies depending on the value of the sigma i. And that is basically very small value. The sigma i corresponds to roughly 10 to the power minus 6 and therefore it is a very very small number. So, it is just in a ppm scale this number. So, you

can see it is not that it is changing this magnetic field by a lot that is actually changing by very small amount. And that therefore, with the word ppm comes into picture.

So, now, how do we calculate the ppm values and that is what is the calculation which we will go into now. It says that when a frequency, the reference frequency that is say we go into a reference frame, a rotational frame of reference that is what we saw rotating frame, there I can consider the rotational frame frequency as the standard frequency. So, what I have to do basically now subtract this from this, because in my actual rotating frame the frequency experienced by the nucleus will be the rotating frame minus its frequency.

So, remember again if sigma is 0, then these two will cancel because omega r will become exactly equal to omega effective. In such a scenario omega effective in the rotating frame will be 0, but not otherwise in the sense if sigma is not 0, then this value is not equal to 0 and it results in this number which is gamma B 0 into sigma i.

Hence the FID subtracted from the reference signal. So, this is what is standard is typically done for detection. We do not directly detect the signal as such that is FID as such. The FID is subtracted before digitization from the reference frequency and reference frequency is nothing but the frequency that is a megahertz rotating frame of reference. And the reference signal is detected and used for further analysis, so that is basically how practically NMR signals are detected.

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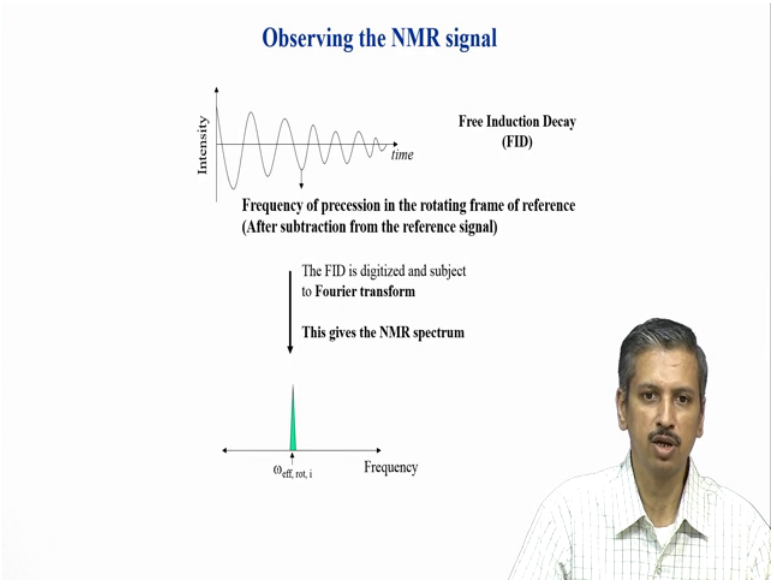

And then we will see now look at example of how we can get multiple signals. So, this is again this picture of a free induction decay. So, you can see here the frequency of precession in rotating frame is now removed, because now this frequency what you are seeing is nothing but the difference between the reference signal and the effective signal frequency of the signal. And therefore, that is 0 this becomes zero if it they match exactly, but they may not match always because they have a chemical shift value in a such situation you will have these oscillation.

So, what is done next is that you Fourier transform this FID. So, this FID is digitized. Remember this, this concept you have to digitize a FID and subjected to Fourier transform and that gives as a finally the NMR spectrum. So, the technique of Fourier transform is something again we will not be able to go in detail, what Fourier transform is a mathematical approach. What it does is, it takes a signal which is varying with time and it then gives out the frequency components present in that signal.

So, if there is a one particular signal one present, it will give a one peak. If there are many signals, it will give you many peaks that is what we will see now if you have multiple signals.

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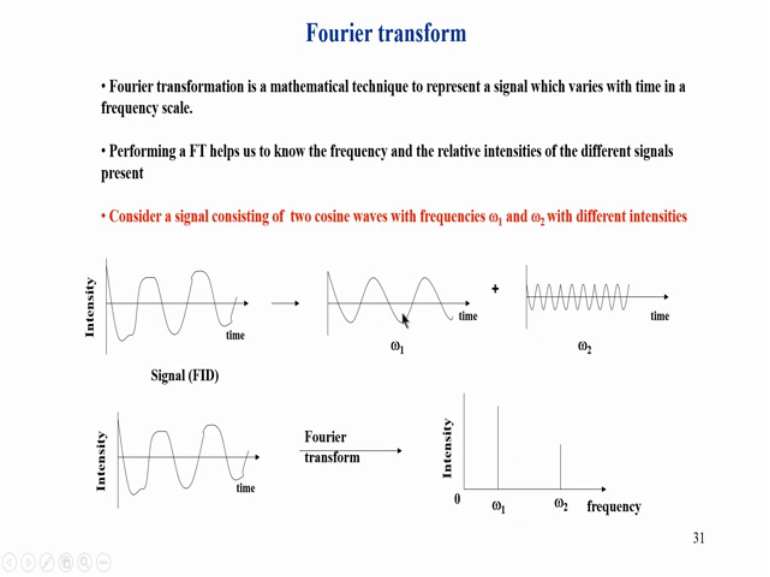

This is what is shown here that Fourier transform basically takes a any signal which varies with time. So, this is a signal let us say an arbitrary signal which is detected by NMR because let us say we have two cosine waves. So, we may have two signals, let us

say two protons in my molecule, in such scenarios for example, let us say methanol if you are looking at a molecule such as methanol you will have two frequencies CH 3 and OH. So, you have two frequencies present.

So, FID will be a combination of those two because remember the detector does not care how many frequencies are present. It simply adds the two FIDs and then that is what looks like this. But this actually may be a component two components present here, one could be a low frequency and one could be a high frequency. Not only that the amplitudes are also may be different for example, in a CH 3 in of methanol that has three times stronger than CH-OH of methanol which is only one proton. So, you see the amplitude also can vary the frequency also will vary. So, this is how we can decompose or we can separate the frequencies into two components.

And now if I do a Fourier transform of this signal, remember I do not know these frequencies I only have this in my hand that is what is detected and stored in the computer and only that can be used for Fourier transform after digitization. Remember this is a digitized signal although, it is shown as a continuous signal, it is not really a continuous signal it is a digital signal means it is discrete values at every point there are equal the number of points here and then the signal is taken at those points. Say if you take a discretized signal and you then subject it to Fourier transform, it will give me two frequencies, because there are two frequencies present as per this picture here.

Now, if you look at this, this is one belongs one comes at the frequency which is the frequency of this particular k signal, and other frequency the peak and other frequency is this signal. And now the height of the peak of this particular peak depends on this amplitude here. If this amplitude is three times this or two times that, it will now depend on that ratio. So, you see the amplitude also I mean the NMR Fourier transform also tells us the amplitudes relative amplitudes of the signals. And this relative amplitude is something which is unique to NMR spectroscopy because it gives us a way to quantify the signals and this is that one of the property unique properties NMR which we listed in the previous classes, where you can see that is what is used in several of the applications.

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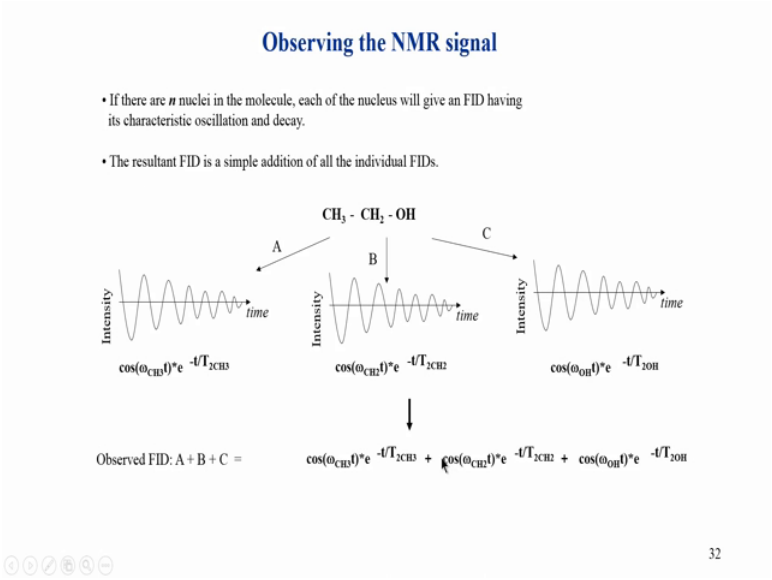

So, let us say that if we have three signals, so this is in this particular case I have taken example of ethanol. So, in ethanol you have OH, CH 2 and CH 3, this is again I am just going giving another example of how Fourier transform works, how NMR signals come out. So, you can see there are three signals. So, this is one particular frequency corresponding to CH 3, second frequency corresponding to CH 2 and third corresponding to OH. And all of these signals are coming together from the sample. So, the detector cannot distinguish between this individual signals. And all of them come together and each of them now has this form, each of them is a FID individual FIDs, but the FIDs are added together to give the signal.

So, this one has if T 2 relaxation of its own, this will have a different T 2 this will have different T 2. And when we do a Fourier, when you combine the signals, we will get three different I mean terms which are added together. So, this is a combined FID or observed FID which is simple linear addition of A plus B plus C. So, one thing again which is missed out here and you should keep in mind is a amplitude. So, you see here this is CH 3. So, it will be three times long stronger. So, it will be three times higher in amplitude compared to this is two times and this is one. So, you this will be three times this term will have a factor three here, this term will have a factor two here, this term will have a factor one here.

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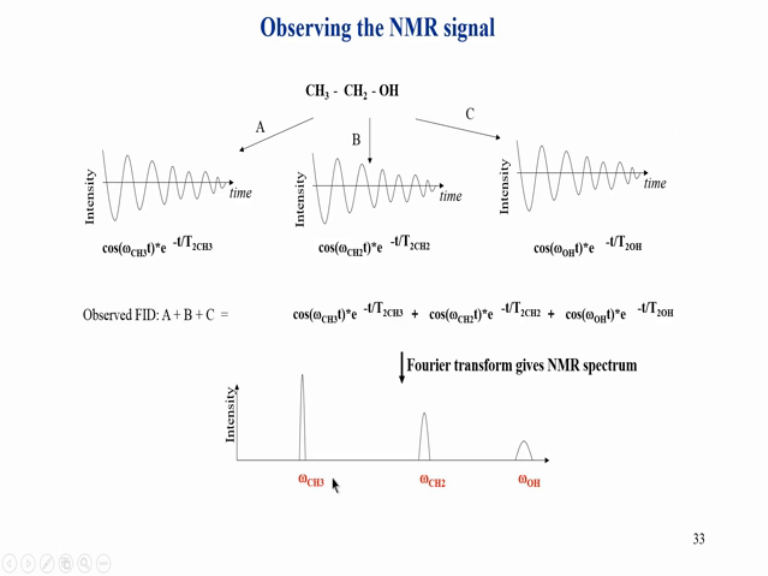

So, that is how if you do a Fourier transform of this signal which is the combined FID which is detected at the detector, we will get end up getting three signals. And these three signals now corresponds to the each of these. And one of them as I said omega CH 3 is shown three times long stronger compared to CH 2 and compare to OH. So, one thing you will notice in this spectrum, so this is a NMR spectrum which is the display which is shown here and is what is displayed in NMR spectrometers, the peak is not anymore has single line which was shown in the previous slide. It has a width you can see there is a width to this line this has also a width.

So, what we do is we do not simply take the height of the peak when we calculate the number of protons, we take the area. So, the area of the peak is actually considered for calculation and the area of the peak relatively depends on the number of protons. So, if you take the area of this peak, it will be three times the area of this peak and this peak will be two times area of this peak. So, if we take the relative areas it is 3 is to 2 is to 1.

So, again remember NMR gives us the relative number of protons and relative number of relative quantification, if you need absolute if you know want to know the exact concentration of ethanol in your sample, that would not be possible by simply taking a spectrum like this. For that you will have to use a reference compound of known concentration. And that known concentration area which you will calculate will then

decide will tell you how much is a concentration of the molecule of your interest that is how we calculate the absolute concentrations.

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So, this is how basically NMR signals are detected. In the next class, we will look at one-dimensional specrtrum and look at more parameters such as chemical shifts and spin-spin coupling.