Interactomics: Basics and Applications

Prof. Sanjeeva Srivastava
Prof. Pratip Saha
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

Lecture – 46
Mass Spectrometry Coupled Interactions- II

In today's lecture, we are going to talk about basics of Mass Spectrometry which could be used for characterizing, identifying the proteins of interest after performing the immunoprecipitation base experiment. So, in the last lecture if you recall we talked about if you want to identify the potential interactors of a protein of interest. You can do immunoprecipitation experiment and then identify the potential interactors using mass spectrometry.

So, today we are going to have an application scientist who is going to talk to you about how to perform these experiments using mass spectrometers and then identify the possible proteins of interest using the software. So, let us have this lecture.

Hi, good morning everyone. So, I am Pratip from Thermo Fisher Scientific, I am the application specialist for mass spectrometry for proteomics and biopharma. So, today till now you have I have done the IP experiments. So, immunoprecipitation you do the antibody precipitation.

So, next from those proteins which have been precipitated, how you identify the protein. Mainly, we will focus on the mass spectrometry part. So, my topic is mass spectrometry analysis from IP experiment to protein identification. So, already you have done the these experiment. So, you have precipitated the protein and you have the protein.

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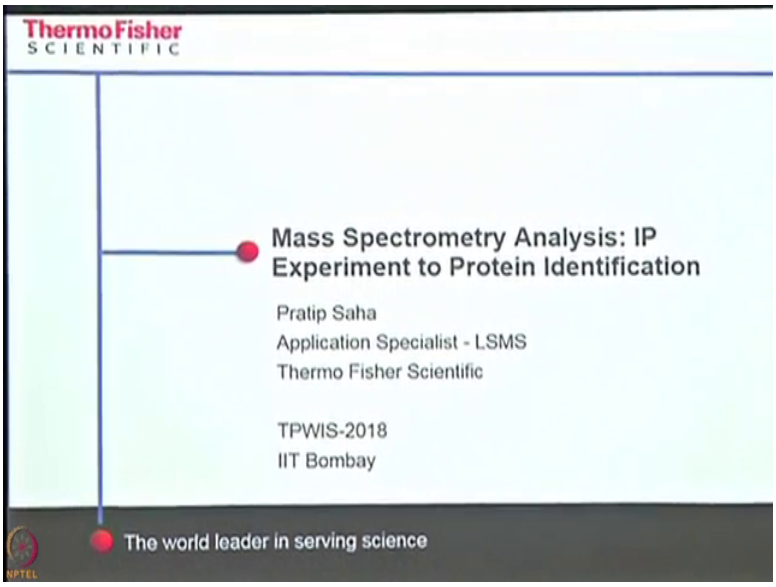

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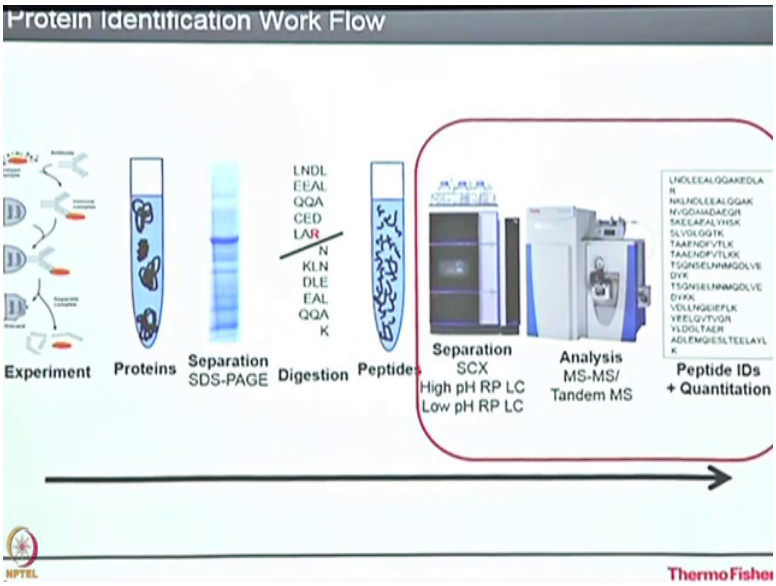

So, what is the next step? Next step; either you start with this protein, you can run a SDS page or in a solution you can digest the protein with some protease which for example, trypsin which cuts after arginine and lysine then it makes a peptides mixture.

So, those peptide mixture is fractionated through LC and introduced to the mass spectrometry where you get the masses of those peptides. And now you search again some database using some search tools you identify those peptides ok. So, today we will discuss mostly on the mass spectrometry part. How you can analyse the data, how you can introduce the peptides into the mass and how can you analyse the data ok.

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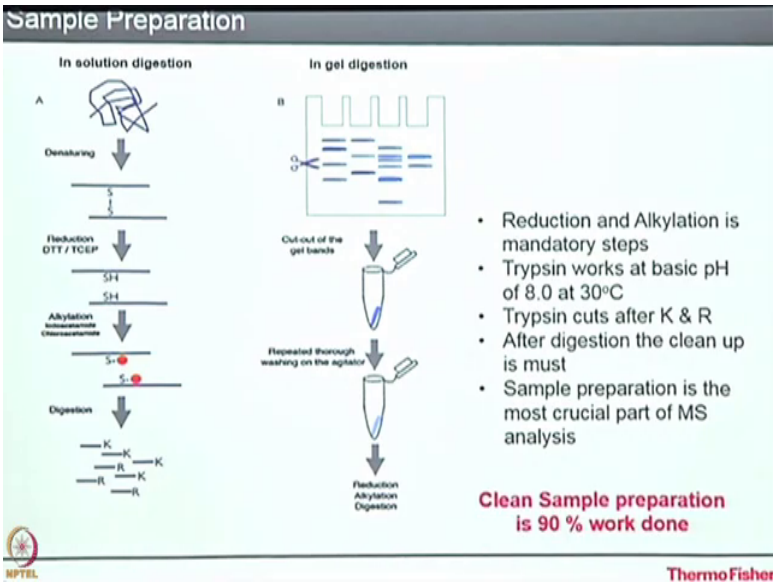

So, before going to that for I am just giving you a brief of the sample preparation. So, you have the IP proteins, proteins you have. Either you can run a SDS page normal SDS page or you can make a solution in solution digestion. So, in both cases you in case of in gel digestion you run the in SDS page, then you cut the band of interest of your protein of interest and cut the band then you digest that band extract. So, when you keep with the trypsin. So, protein gets digested to peptides.

So, once it will be in a peptide form it will come out from the gel bands and you extract those peptides and then further you proceed. So, in case of in solution digestion already there is protein in the solution. So, you have to do the reduction and alkylation, it is very important part. Otherwise, what will happen? There are disulphide linkage. Due to this disulphide linkage protein will not been open up all the parts are is not open up.

So, you have to go through these reduction and alkylation DTT, we are using is very common for a reduction it deduce the disulphides bond and because of reversible reaction you have to use some alkylation part. So, block that sulphide residues.

So, you put iodoacetamide and those disulphides will be blocked by iodoacetamide and then you digest with some proteases. So, most common protease is trypsin which cuts after arginine and lysine. So, that masses basically important part is reduction and alkylation is very important otherwise you will not get 100 percent digestion.

Next, trypsin works in the basic pH. So, when you do the digestion procedure that has to be in the basic pH more than 8 pH ok. And it has to be kept at 30 degree. So, these are the two criteria where trypsin works very well. And then trypsin, we all know trypsin cuts after arginine and lysine then after digestion the clean up procedure.

So, before injection to the in injected to the mass spectrometry we have to clean up your samples. For in gel digestion it is fine the samples are comes pure, but in solution digestion. So, samples may contain some impurities some salts or some other impurities.

So, that will block your MS analysis. That is why the cleanup procedure is very important. So, sample preparation that is why sample preparation is the most crucial part and if you done a proper sample preparation your 90 percent work is done. The next is the basically MS is basically a software driven. So, whatever you are set in the software it will do, but the sample preparation is the most crucial part for yours ok.

Now, comes to the mass spectrometry. So, I am keeping a basics of the mass spectrometry, I am not going in very details on all the parts. So, what is mass spectrometry? Mass spectrometry basically is a production of ions that subsequently filtered or separated by m by z ok.

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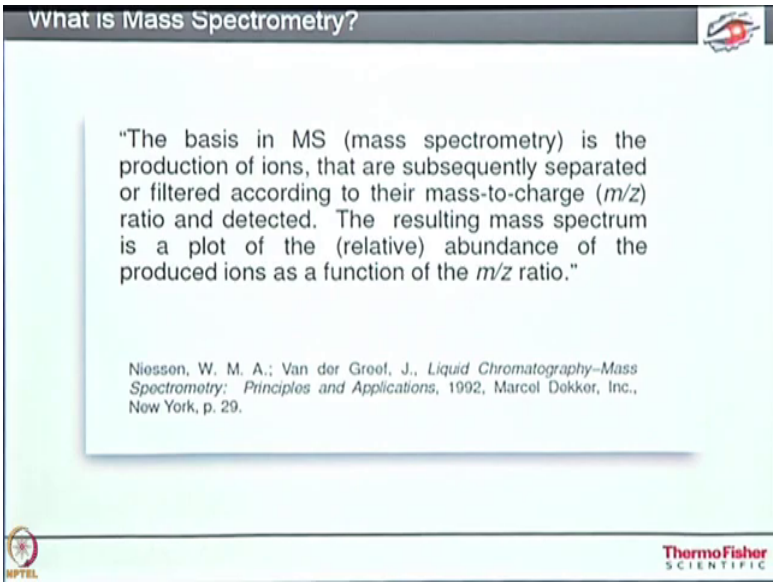

So, where and we are getting a resulting mass spectrum of abundance of produced ion as a function of m by z. So, any mass spectra, if you look into the mass spectra. So, x axis will be the m by z value mass by charge ratio and y axis will be the abundance either relative abundance or the absolute abundance so.

So, now sometimes if you run any samples in MALDI ok. So, MALDI is MALDI TOF away is very common. So, that time you will sometimes you are saying a your mass is coming x axis it is comes as a mass m. So, whatever the m by z value is the m because in MALDI you are getting the plus 1 charge. So, always the m by z value will be equal to your mass, but in case of ESI Electrospray Ionization.

So, that time you will get multiple charges that time m by z value will not be the exact mass. So, do not be confused with this terminology ok.

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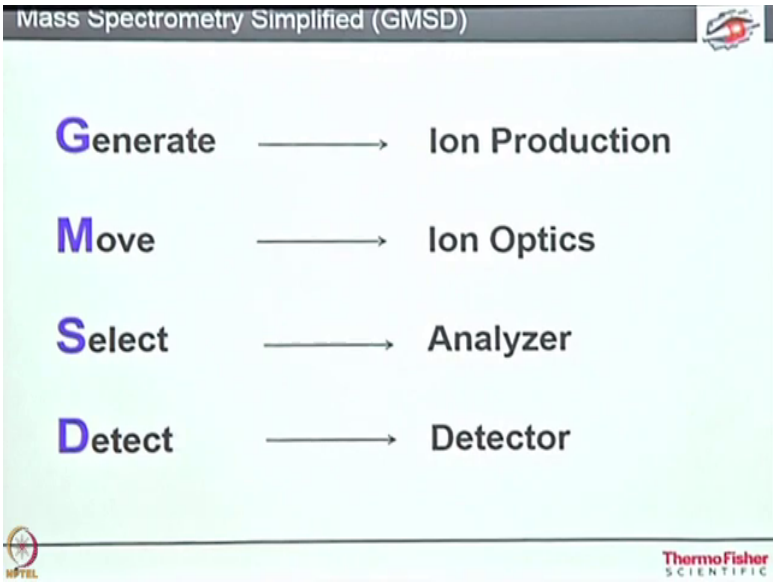

So, now we can simply explain the mass spectrometry in four letters GMSD. So, it has first is a generate. So, you have to be ionize the molecule. Once any molecule can be ionized it can be detected by mass spec. So, that is a simple way to identify. So, it has to be ionized properly the ionization method may be from solid to gaseous, may be liquid to gaseous, then it has to be move properly. So, after ionization till the detector; so, that path should be the proper otherwise what will happen if any molecule touch in the wall that get discharged and it will not be detected in the mass spec.

So, that path should be very proper. Then selection; obviously, you have to separate the molecule because you have a mixture of masses. So, you have to filter which mass you want

to identify you can control that one. And lastly there is a detector ok. So, you have to detect the molecule. So, let us see what are the components are there in the different sectors. So, first ion source, ionization where the ionization happens.

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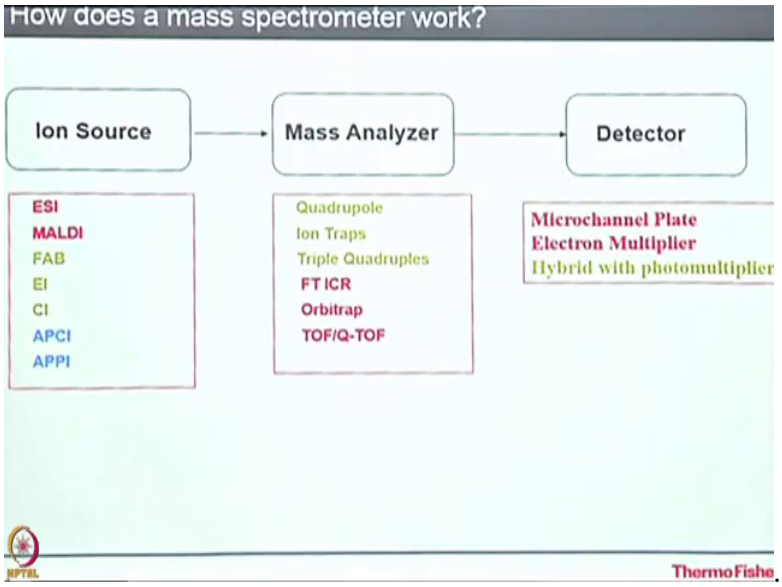

So, there are very different methods of ionization. It comes with the most popular is a ESI and MALDI you already heard about this electrospray ionization which is liquid to gaseous. So, you inject the sample in the solution format where from where you put the high voltage and that high voltage it will makes the ionization. Next is a MALDI, Matrix Assisted Laser Desorption Ionization. So, it is a solid form to gaseous form. What happened in MALDI? You mix the sample with some matrix molecule spot on a plate may keep it for dry and then you should laser that laser gives the energy to ionization.

So, from solid to gaseous space. These are the two most soft ionization where your biomolecules will not degrade in during the ionization. Whatever the other mode of ionization FAB, electron ionization chemical ionization, these are very hard ionization. Where already when you put your biomolecule it will fragment in the source. So, you will not get the exact mass of the molecule.

So, it is applicable only in case of small molecules like chemical compounds. And APCI and APPI, Atmospheric Pressure Chemical Ionization. These two are for nonpolar molecules ok, exclusively for nonpolar molecules. Now come for the mass analyzer means; where you separate your molecules. Most common, everybody knows a quadrupole TOF instrument time of flight time of flight of quadrupole means it quadrupole is not a analyzer I can say it is a filter. It can precisely filter your molecule which molecule you want ion traps.

So, you can put your molecules in the you can trap your molecule inside of vessel and you can make detect that molecule. So, quadrupole ion traps triple quads. These are very low resolution means; here you cannot separate nearby masses. But on the other case FTICR Orbitrap TOF or Q TOF these are high resolution instrument where you can separate the nearby masses ok. So, in IIT, Bombay; we have Orbitrap Technology. It is nothing but a trapping of electron in a orbital motion ok.

So, we put a high voltage and the ions are rotating around the molecule ok. So, we will come to those part and last is the detector. So, you have you require some detector. The better part for Orbitrap is Orbitrap it is itself acts as a analyzer as well as detector. So, you do not require a extra detector for Orbitrap ok. Now we see how the mass spec works what we are doing in the mass spec after injection of sample.

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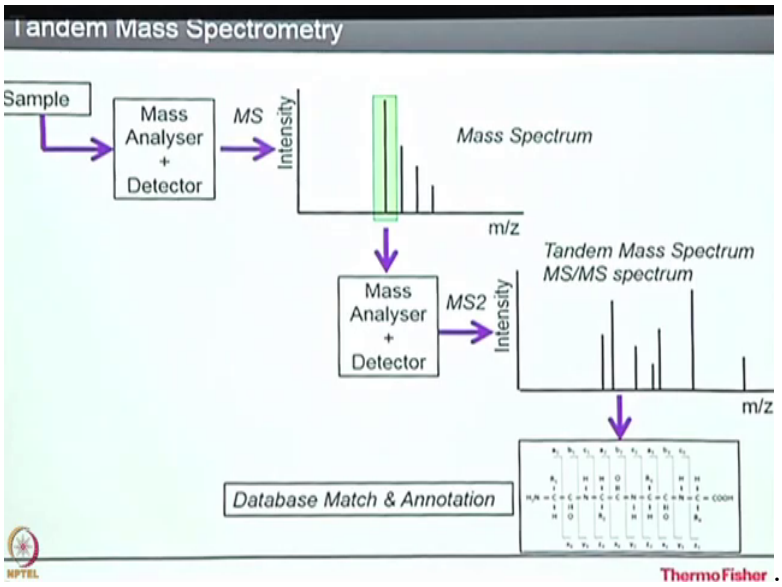

So, we inject a sample to mass spec through the mass analyzer and the detector we get the MS 1 MS spectra, we will get this MS spectra. From these MS spectra; we fragment we will select one of the peak and again we will do the fragmentation, MS 2 and we get this fragmentation. Now this fragmentation will search against the database and we will find out what are the ions are there b and y ions and we get the database match we will get the peptides. From the peptides we can say these protein is present in your sample.

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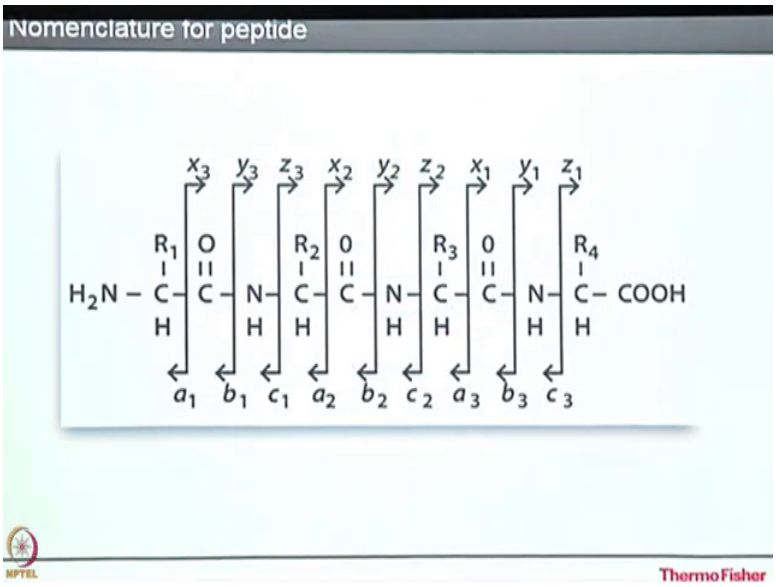

So, now come to the nomenclature of identifying the ions, is a basically nomenclature how you can assign your peptides. So, this is example of a peptide of 4 amino acid R 1, R 2, R 3, R 4 ok. So now, when you applied voltage to fragment the molecule in MS 2. So, this C alpha C, C N and N C alpha, these three bond can be broken. So, this any one of the broken ok.

So, if it is C alpha C bond is broken then the N terminal side. We will called a ions the C terminal side we will call the x ion. Same way C N, this is a peptide bond basically b and y ions and if it is a NC alpha bond then it will be called the c z ions.

So, now there are different kind of fragmentation, different way of fragmentation CID, ACD ETD. So, different different fragmentation will give you different kind of ions. So, in case of CID, you will get only b and y ions. So, in case of ETD you will get c and z ions ok.

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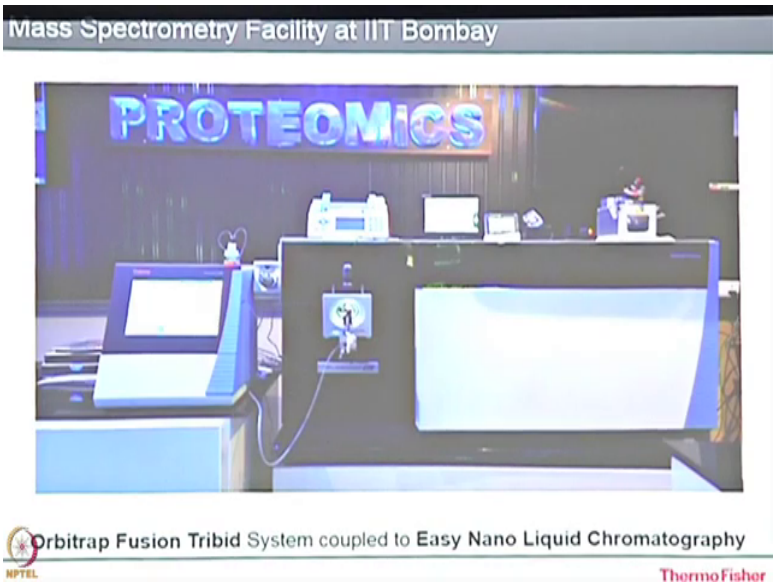

Now, come to the facility here in the IIT, Bombay. So, it has a tribrid instrument Orbitrap fusion with coupled with nano LC. So, why we require nano LC? Because we are doing the proteomics, in proteomics sample is very small amount and it is very precious.

So, we cannot run the normal HPLC where the flow rate is very high. We want to run in the nano LC in a nanoliter flow rate. Now come to the schematic diagram of Orbitrap Fusion. So, Orbitrap Fusion as I told it is a tribrid instrument, it has quadrupole, it has ion trap and it has Orbitrap ok.

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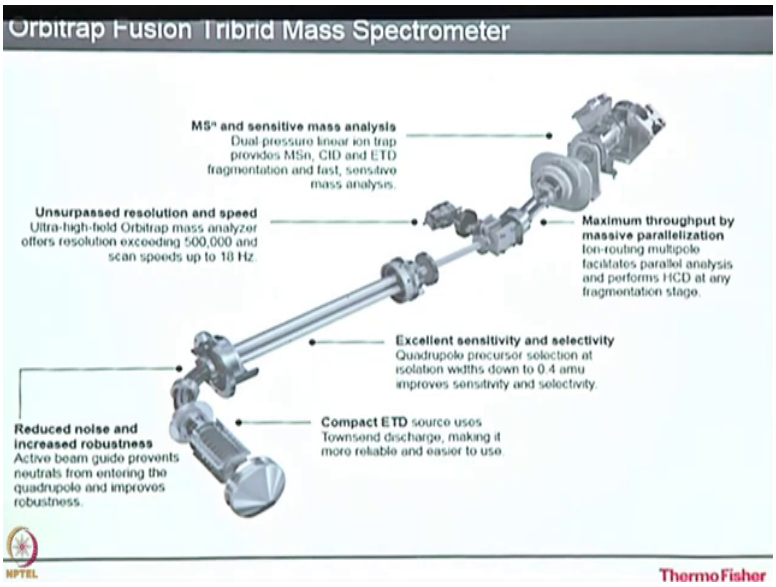

So, ions are inserted from here it gets filtered in the quadrupole first, then it goes to it comes to the ion routing multiple where the ion will store and it will tells you where to go because you have two detector ion trap and the Orbitrap.

So, from here either it will go to the ion trap or it will go to the Orbitrap. So, then Orbitrap it is Orbitrap basically it is a high resolution it will gives you 500 k maximum resolution and lastly the ion trap which is basically a dual cell ion trap, you can store your ion and you can do the detection of our ions and also you have a ETD option.

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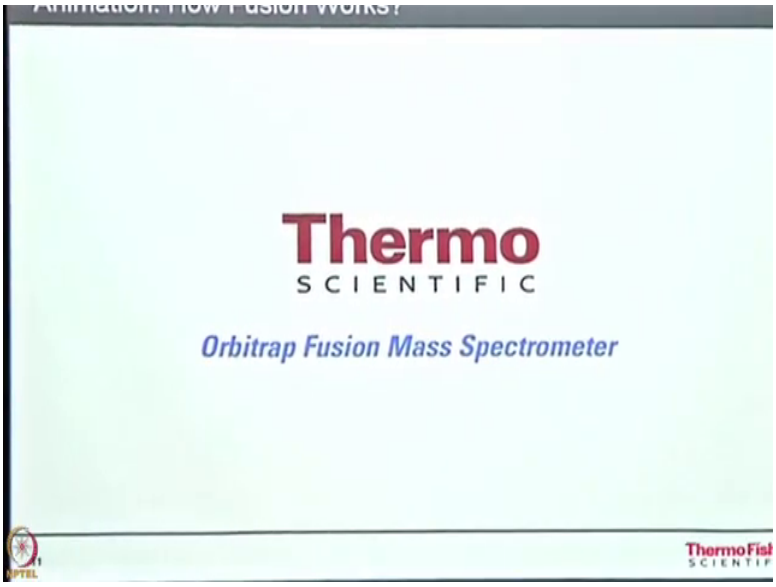

So, in the next slide it will be clear for you we have a video how it works. Now, we are doing first the full MS, MS 1 ok.

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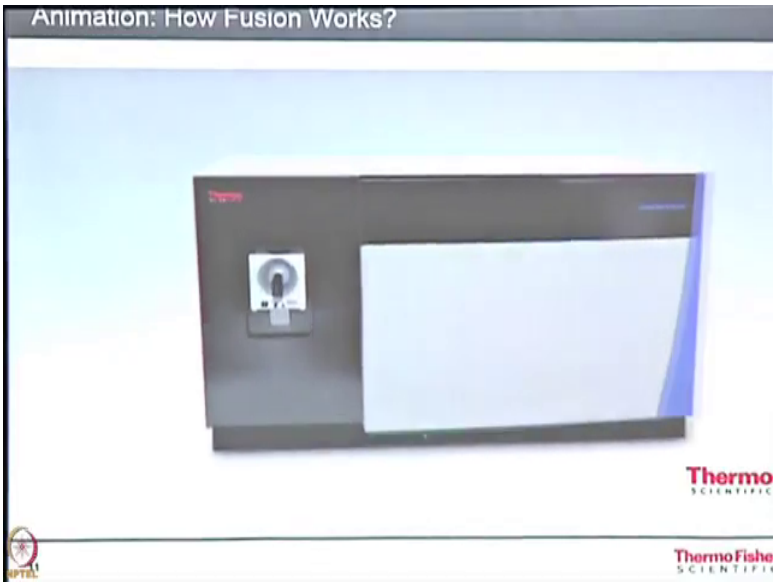

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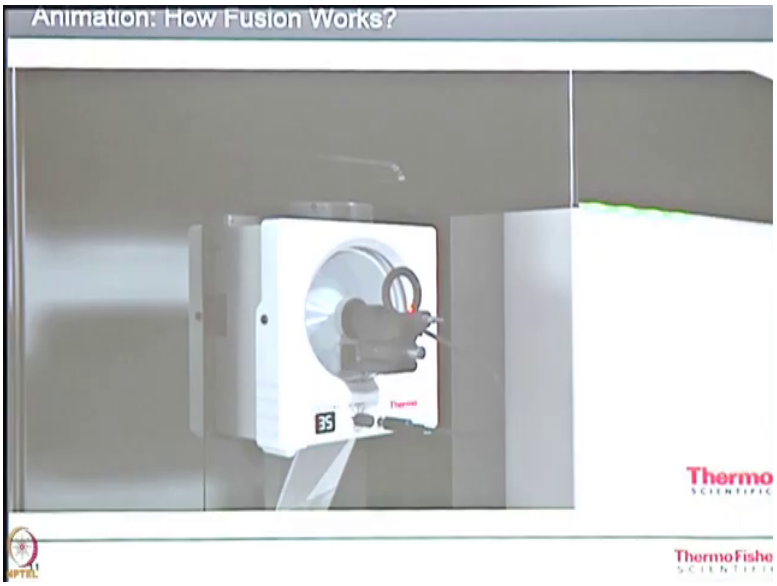
Animation: How Fusion Works?
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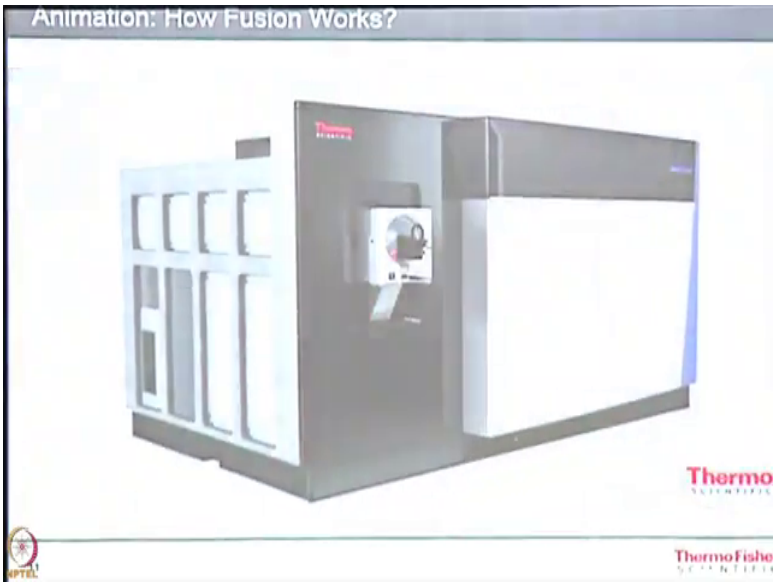

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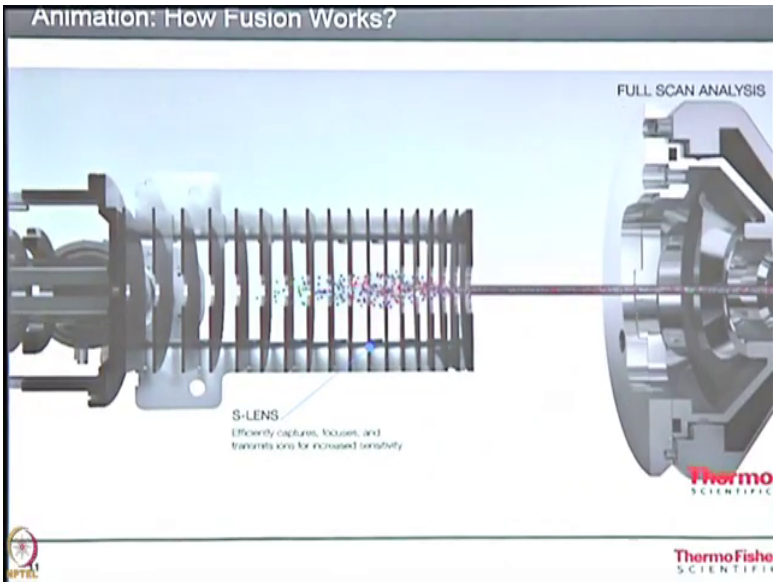

So, now ions will generate here, it will passes through a ion transfer capillary inserting through that. So, after that here what happened ions trying to spread out because all the ions are very highly charged. So, it tries to spreading. So, you need a focusing.

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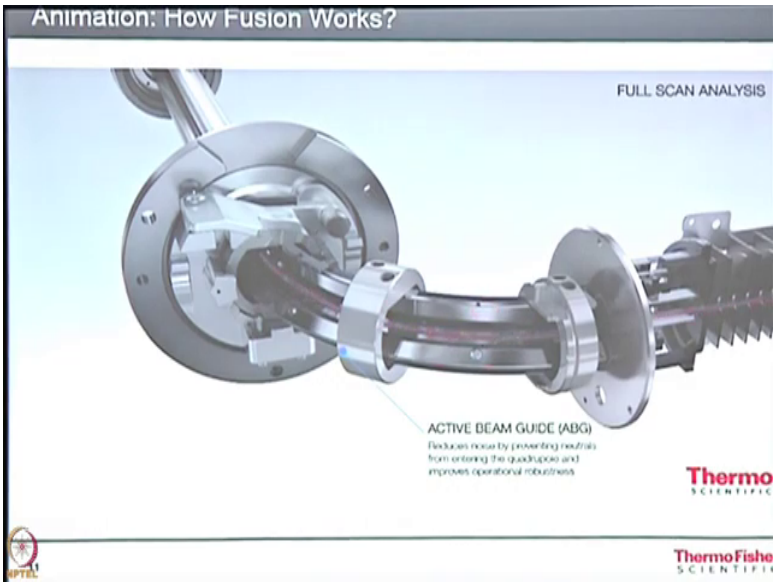

So, it will focus. Then it is called the bend flutter pole bending, where the neutrals are removed. Because in when the ions are inserting to the instrument, there are some neutrals also. So, you with the high voltage difference the charge molecule can bend, but the neutrals cannot bend.

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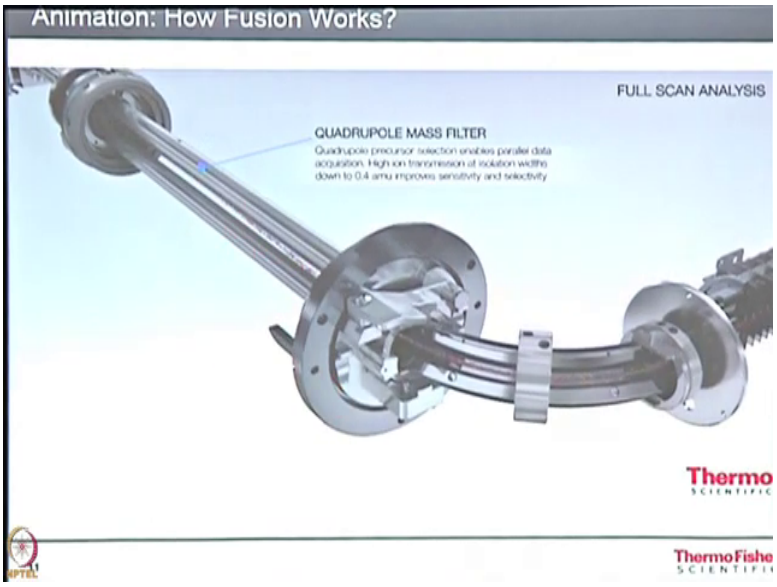

So, after that in the quadrupole, you can select the ions which one you ion, then it first comes to this ion routing multiple where it will first store here.

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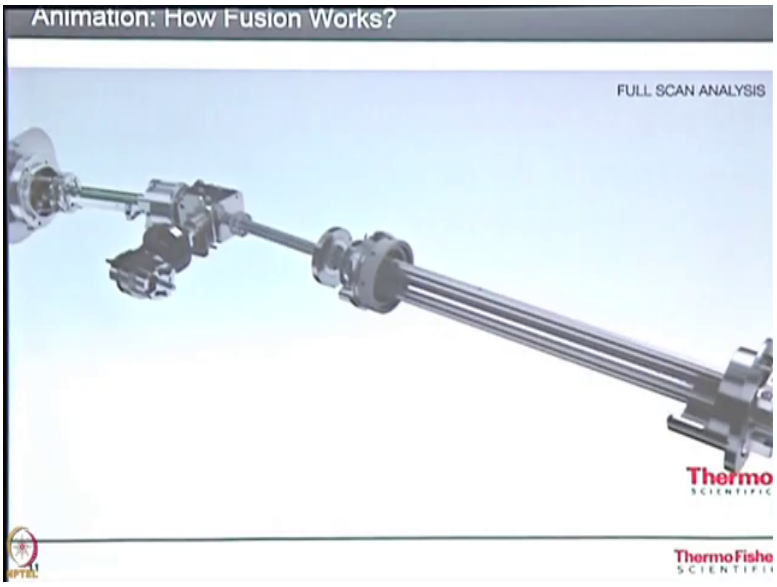

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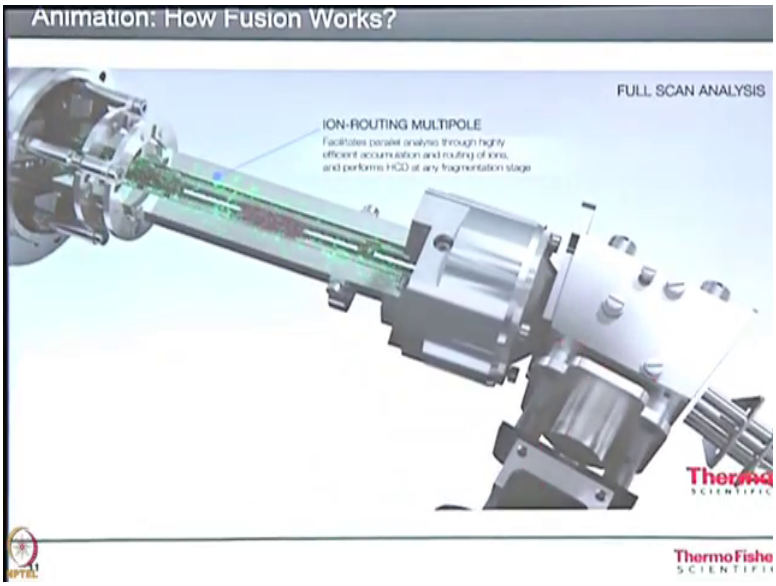

So, here is the first (Refer Time: 15:26) where it will be decided either it will go to the Orbitrap or it will go to the ion trap ok.

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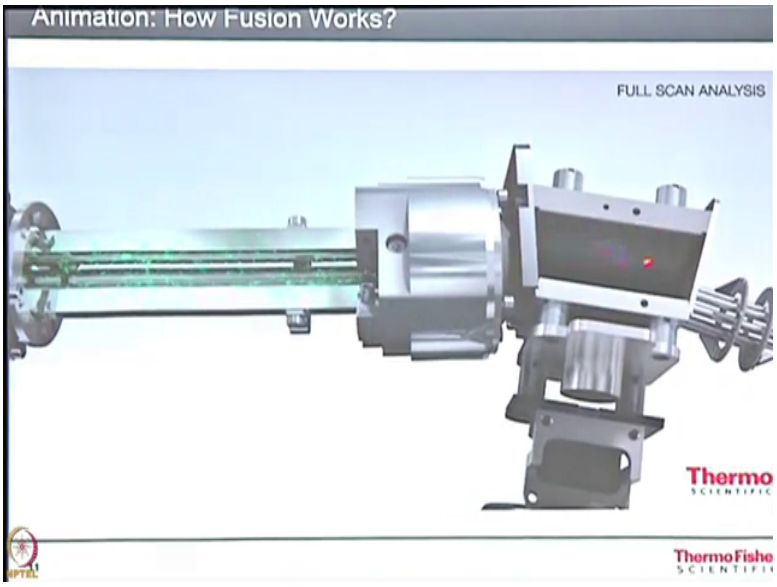

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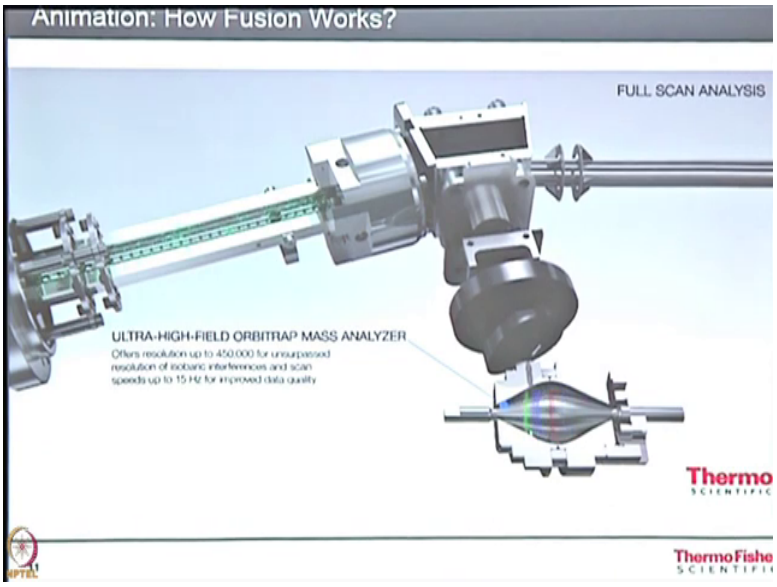

So, now it will come to the Orbitrap, MS and then it is been detected in the Orbitrap ok. So, now, it is a full scan what happened in the MS MS.

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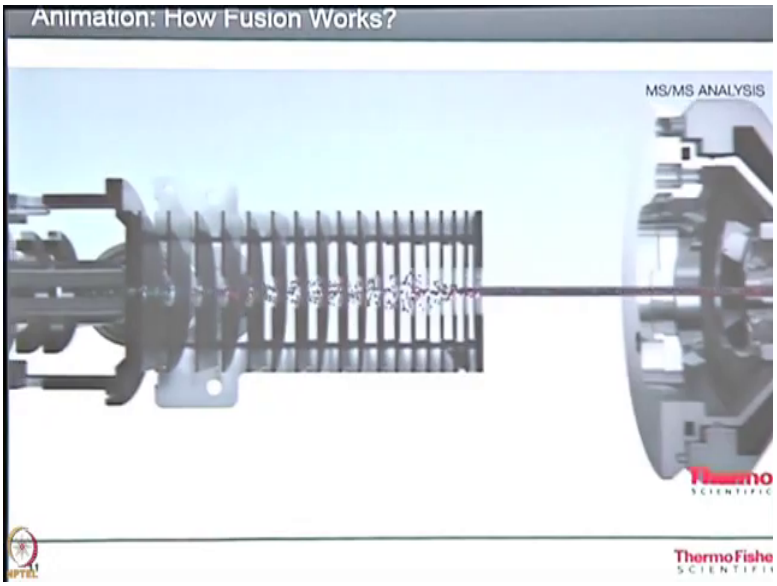

So, in case of MS MS, only one molecule has been filtered from this mass. Only one molecule filtered and a watt gets fragmented and then it will be detected MS 2.

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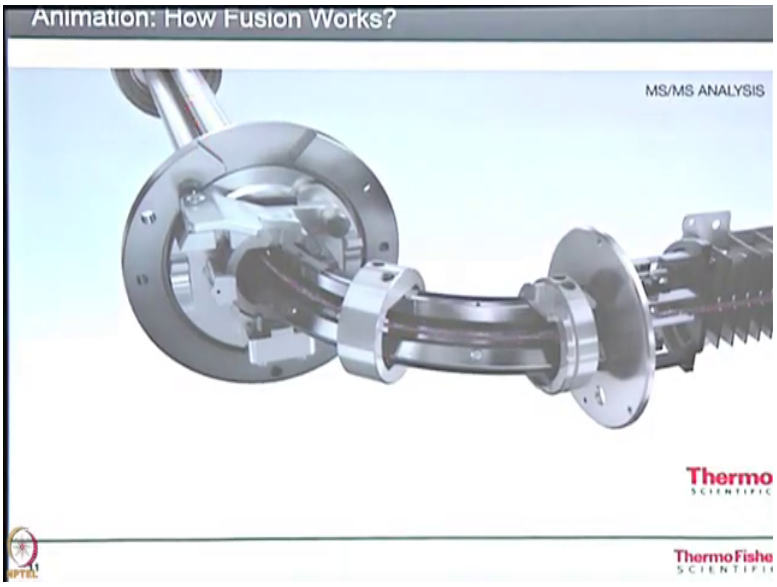

So, same thing happened ions are going. So, inside quadrupole the only one molecule will be there so, other will be filtered out. Now that molecule go to this un routing multiple and get fragmented.

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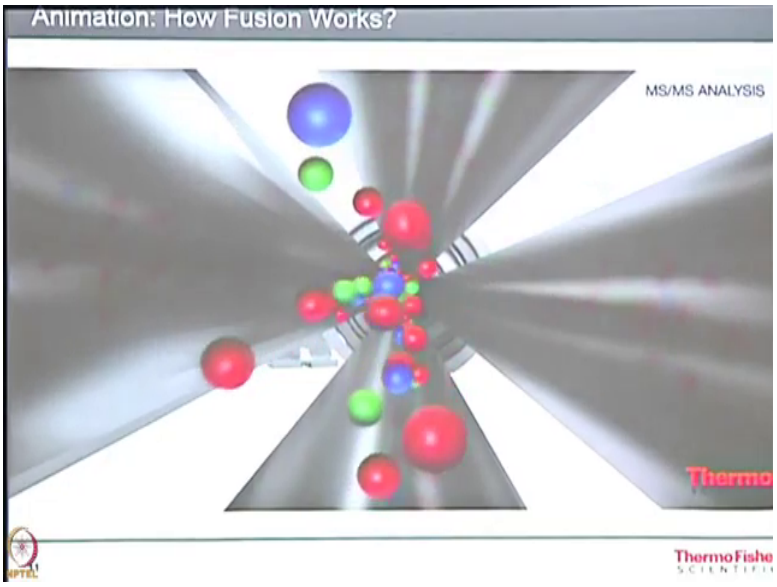

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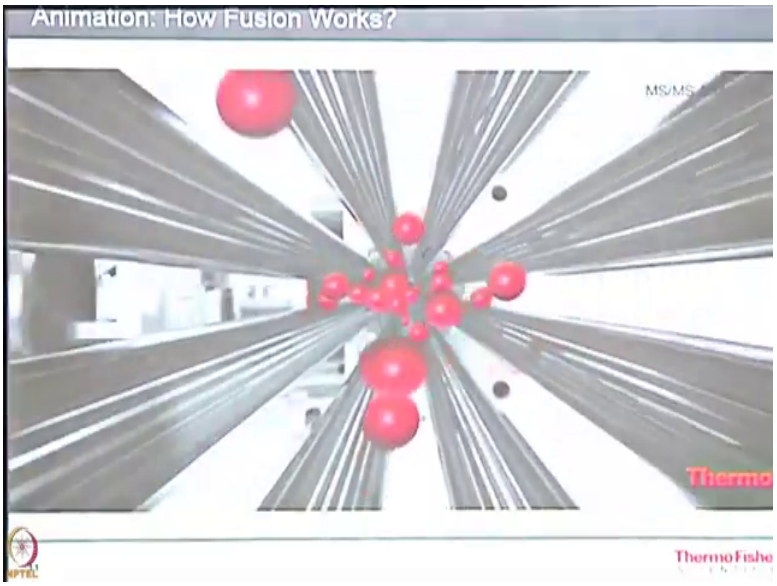
Animation: How Fusion Works?
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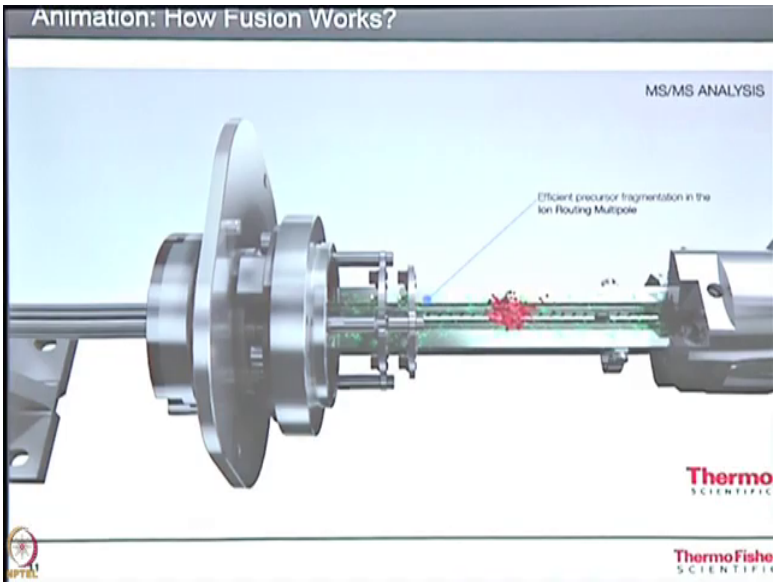

So, here it will fragmented in the ACD mode colli it is nothing, but the collision induced dissociation with high energy.

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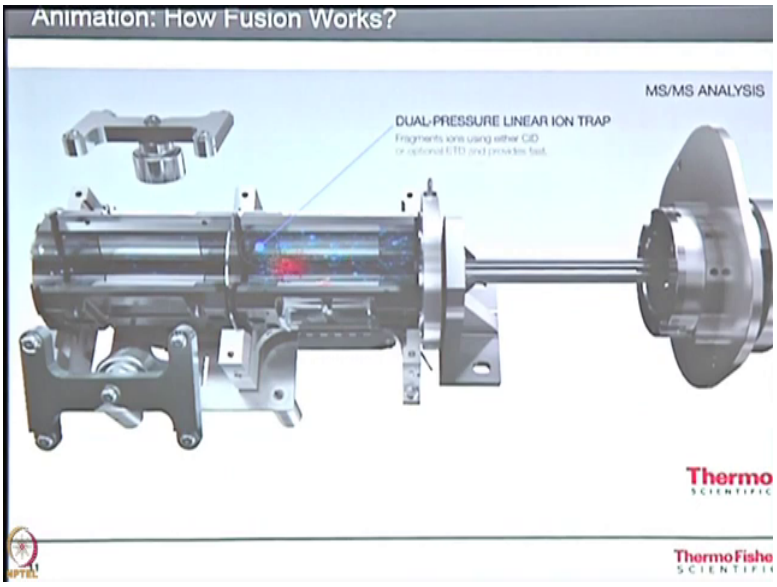

So, here after fragmenting here it goes to the ion trap, where it is stored first and then trapped here and then it is get detected.

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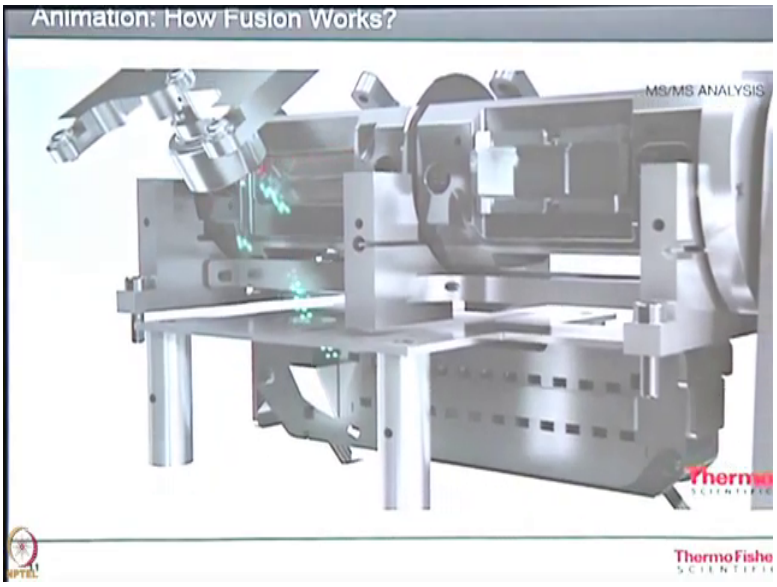

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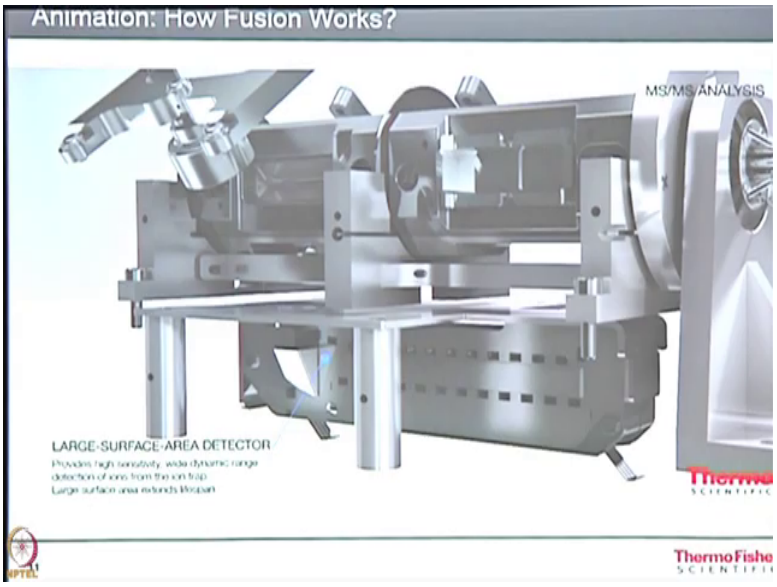

So, it is detected here with the large surface area detector.

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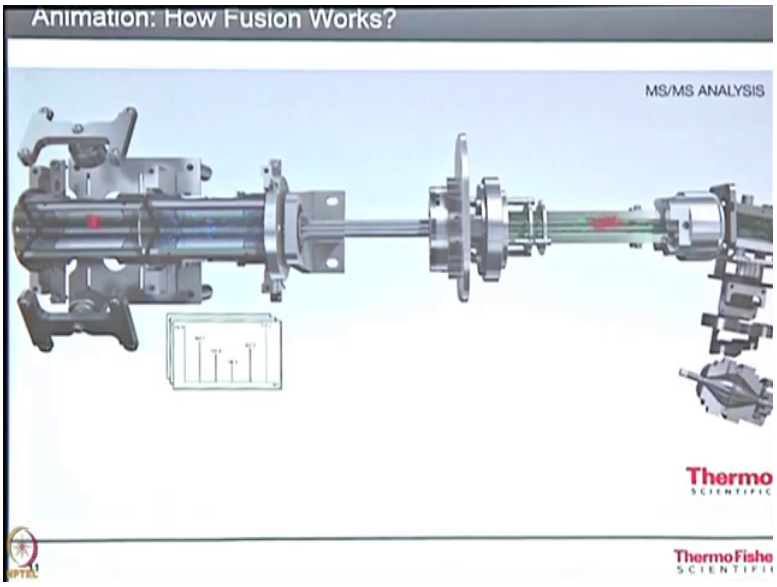

So, it is a very parallel reaction basically parallely it is going on.

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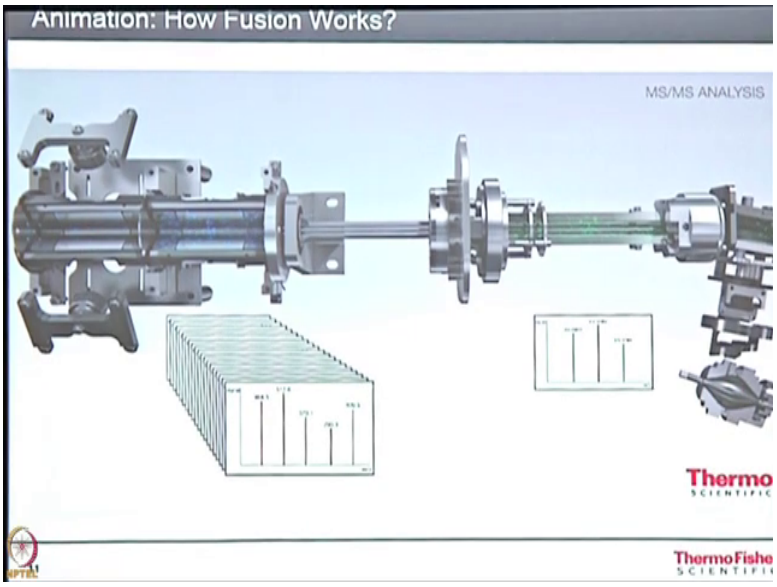

So, when the first MS is going on here MS 1, at the same time the MS MS is going on simultaneously. So, it is a parallelly both the detector is working ok. So, from one MS can it can do 20 MS MS. It can select 20 peaks from one MS can and it can do the MS MS of 20 ok.

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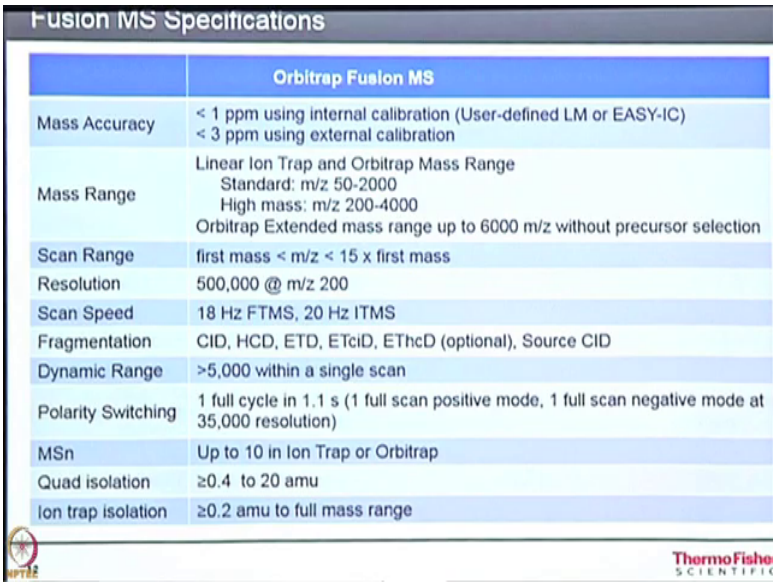

Now, come to the specification of fusion Orbitrap. So, first is a mass accuracy: Mass accuracy is tells that a is less than 1 ppm and for internal calibration and with less than 3 ppm with external calibration. So, what does it mean by external and internal. So, every instrument requires a calibration.

So, you have a standards, you inject the standard. So, you have the mass of the standards and against that masses, exact mass you calibrate your instrument. So, that is the simple external calibration. Now in case of internal calibration we fix one of the masses as a internal standard.

We fix that mass and do the again calibration. So, that in that time, we will get the internal calibration. So, that is why when we do the internal calibration the calibration will be less,

less than 1 ppm. Now the mass range; so, you can acquire the mass range from 50 to 6000 m by z, not the mass ok so, m by z.

So, bigger the molecule, the more the charge state and it will come inside the this range. But you cannot scan the whole total mass range 50 to 6000 in one shot. So, you can scan the first mass and the highest is a 15 x of the first mass. So, for example, if you start from 100 m by z, you can scan up to 1500 m by z.

So, if you want to scan the full mass range, then you going to divide the scan range. So, start from 100 to 5 1500 and 1500 to 6000. Then resolution you can get the maximum resolution of 500 k at 200 m by z. Scan speed 18 Hertz per minute so, fragmentation.

So, you have different 3 different kind of fragmentation CID, HCD and ETD. And there is a basically a mixture combination of any two ETD, HCD and ETD, CID. So, see in case of cid what it is basically collision induced dissociation. So, what happen in that collision induced dissociation? You collide, your you inject some neutral gases that will collide with your sample molecule and get fragmented that is CID. HCD is high energy collision induced dissociation.

So, this is nothing but CID with higher energy. We are putting higher energy to get better fragmentation. And ETD is a electron transfer dissociation. What happened there? You put a reagent molecule which ejects a anion. That anion will collide with the sample molecule and get fragmented, that is called the ETD.

So, according to your sample objective what do you want to do according to you have to select the fragmentation mode. Then, polarity switching means it is not required for the proteins, but in case of small molecules if you do not know in which mode either it is a positive mode or a negative mode it will be ionized.

So, you can do the both scan positive scan and the negative scan within a 1 second. So, you can run that one way MSN as it is a ion trap. So, you can do 10 MSN means MS MS 2, MS 3, MS 4 up to 10 theoretically. Isolation in quadrupole you can go minimum 0.4 amu, means;

plus minus 0.2 daltons. So, the, what that means? Means; if you want to identify if you want to identify one molecular m by z of 100 so, you can scan between 99.8 to 100.2. So, that narrow window you can scan so.

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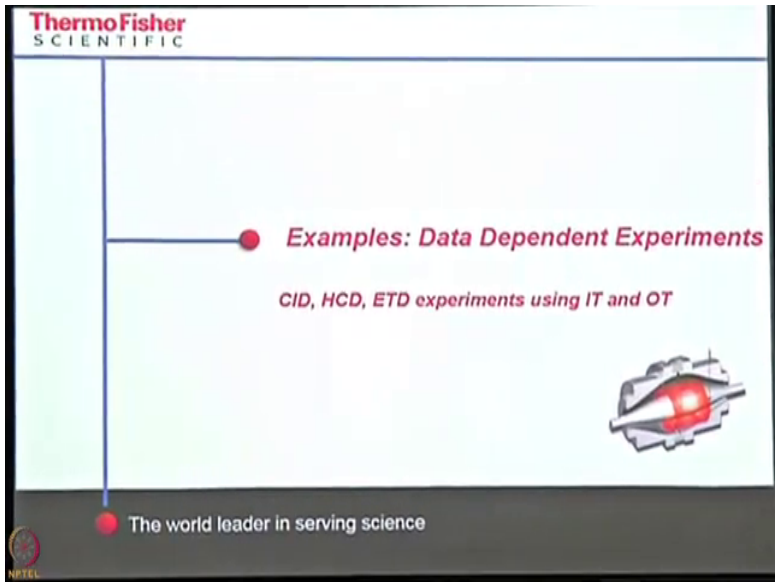

Now comes see some experiments where means how the instrument works in different workflows. Because we cannot show you in the instrument part, so we have some animation where we can see how the instrument do the MS and MS MS.

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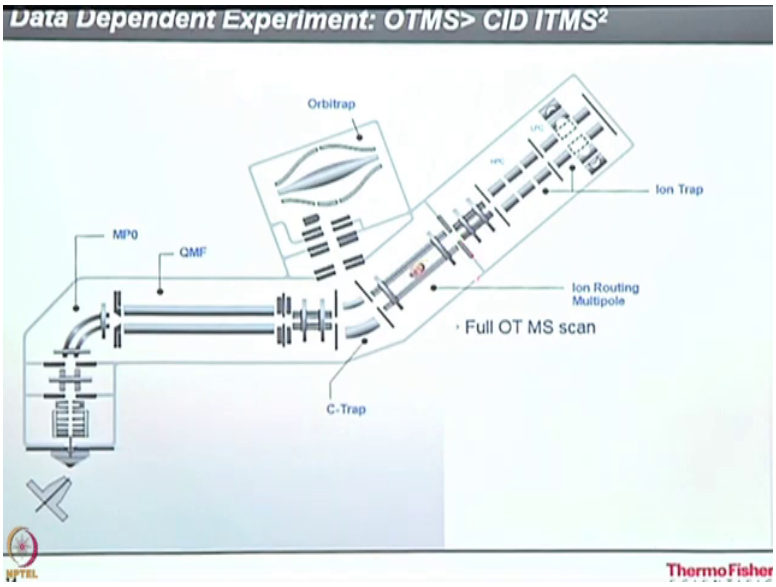

So, first experiment tells k MS in OT Orbitrap and MS MS doing the through the CID it will do in the ion trap. So, CID will be happened here. So, what happened first? The ions will be detected here MS. From that MS scan 1 molecule 1 at a time it will be filtered here and fragmented here ok. So, let us say so, the first full MS will go.

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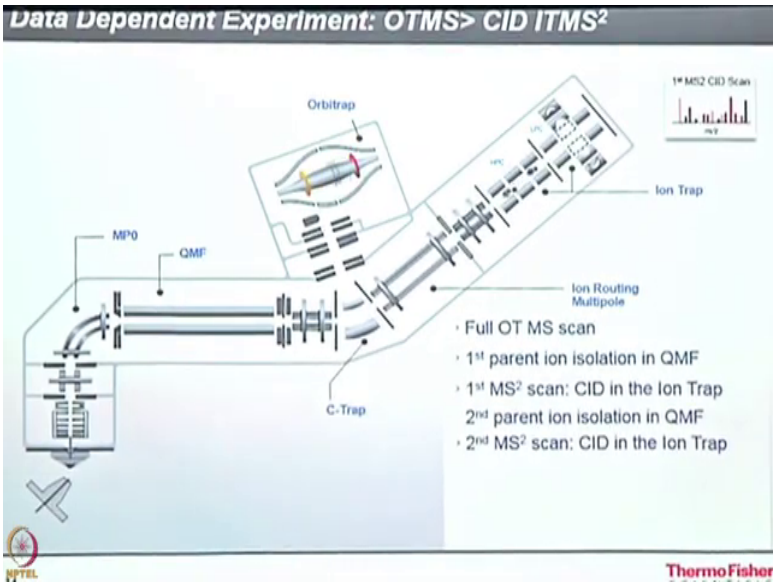

So, the first mass it will first it will stop here, then it will comes to Orbitrap. When the full MS is going on we are doing the parallelly. So, the second, first parent ion has been selected here and it will be stopped there first then it will go for the fragmentation and the HPC cell. When it is going fragmenting the second ion is waiting here. So, you are not losing any molecule as well as the time ok. So, it is a parallel reaction.

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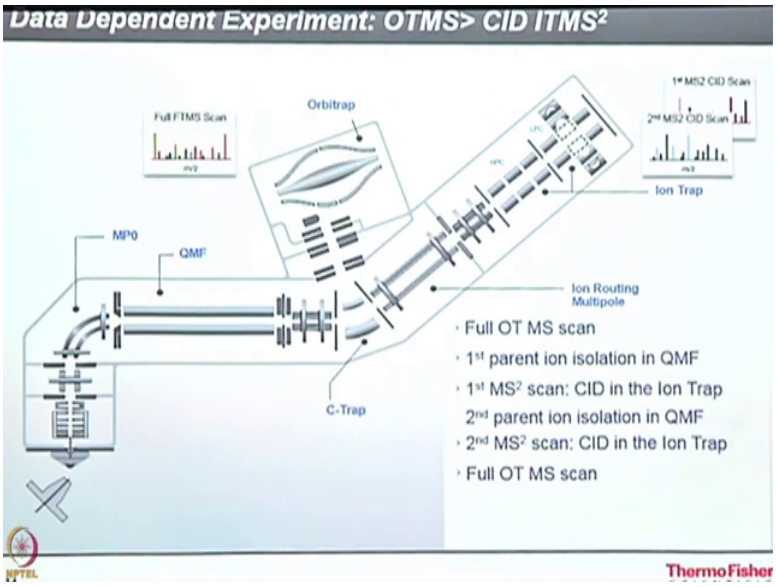

So, when all the MS MS scan is MS 2 scan is over then only you will get the MS scan. So, at the end of the full scan you will get MS scan as well as MS MS scan.

So, you are not losing any time as well as any molecules samples. So, like second experiment is a HCD. On the first experiment we are doing in the CID what happened there in that case the fragmentation happens here. Now in case of CI HCD, the fragmentation will happen here ok.

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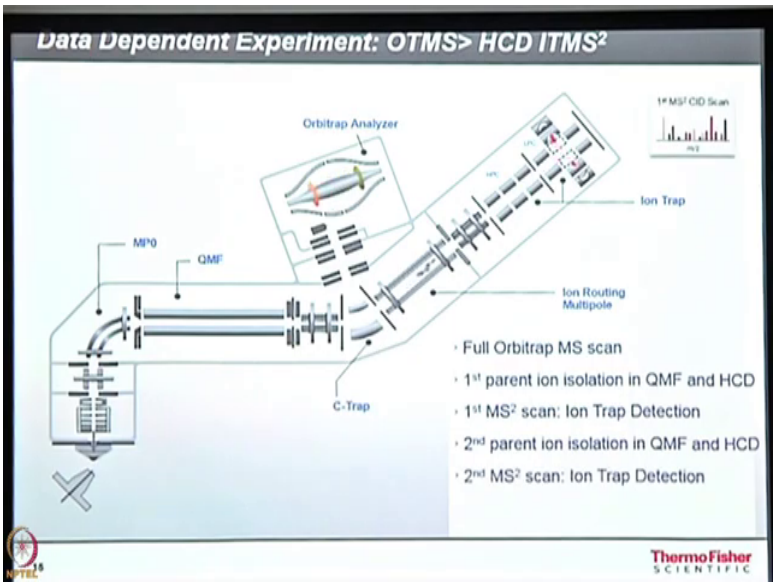

So now, same way the full MS, it will stop here then, it will go to Orbitrap for analyzing. Now, the first mass will comes here and get fragmented. When it will be sending to the ion trap at the say same time the second mass will come on. So, you are not the same way you are doing the parallel reactions.

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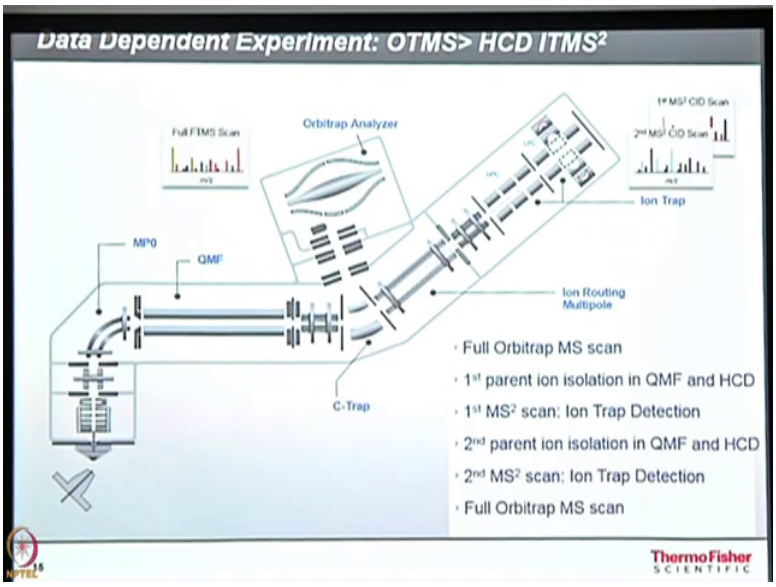

So, after all the MS scan MS 2 scan you will get the MS MS scan.

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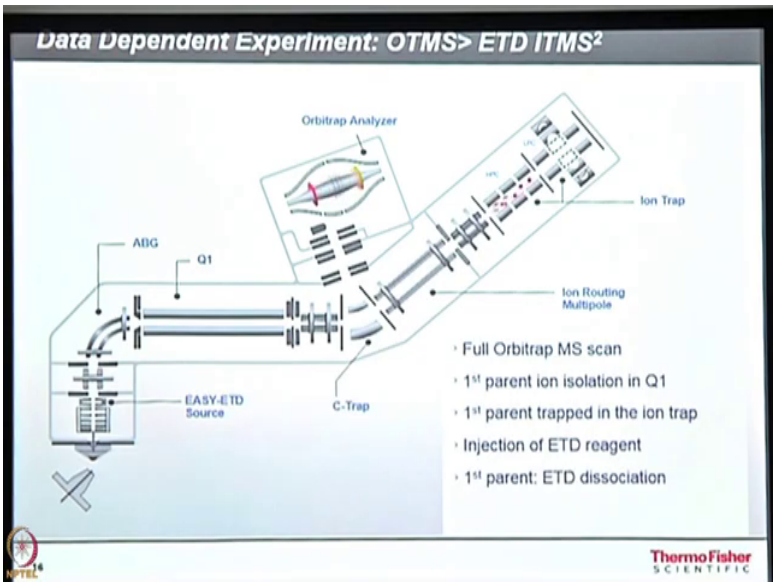

Now, next is the ETD, what happens in case of ETD? So, even the ETD fragmentation will happen in the ion trap also. So, first is the full MS in same way the full MS comes to Orbitrap. Now the first ion has been isolated in the quadrupole and first stop here then it will injected to the ion trap. Now I told k in ETD you are required reagent which gives you the anion so that reagent will comes from here.

So, it will inject a reagent it is reagent calls a fluoranthene. So, that is a carcinogenic. So, it will gives you some anion it will make the anions and get fragmented that molecules.

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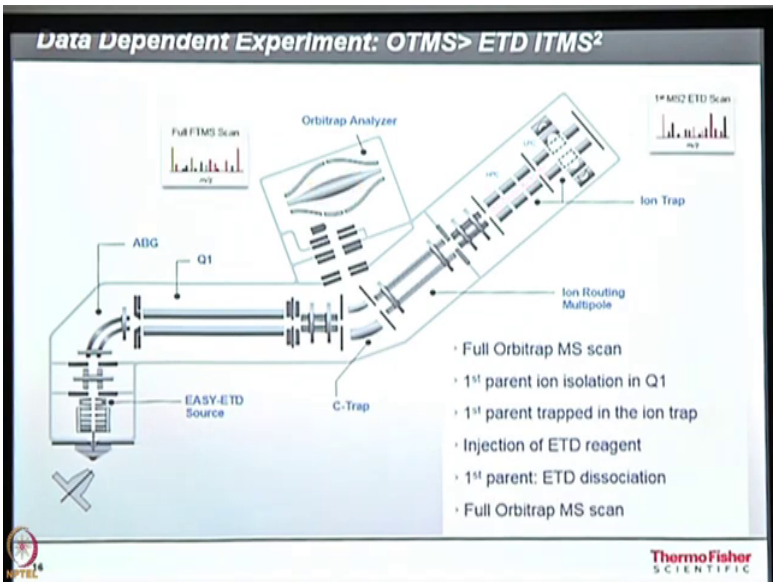

So, once the fragmentation is happened. So, it will give you the MS 2 data as well as MS MS data. So, ETD is a very specialized case only when you are going for the PTM analysis. So, Post Transfer and Modification like phosphorylation or acetylation or glycosylation in those cases you have to use this ETD reaction.

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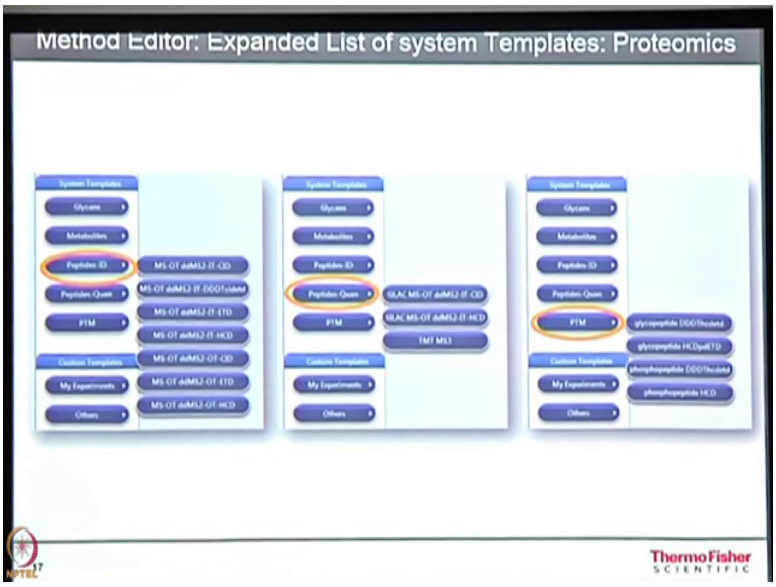

So, in the instrument the better part is that you already there are already the templates for all type of analysis. So, you do not have to start from the scratch. So, you just click on the which method you want to use either it is identification or is a quantification or is a PTM work. So, you have to select that method and run the method ok. So, there are already templates are made.

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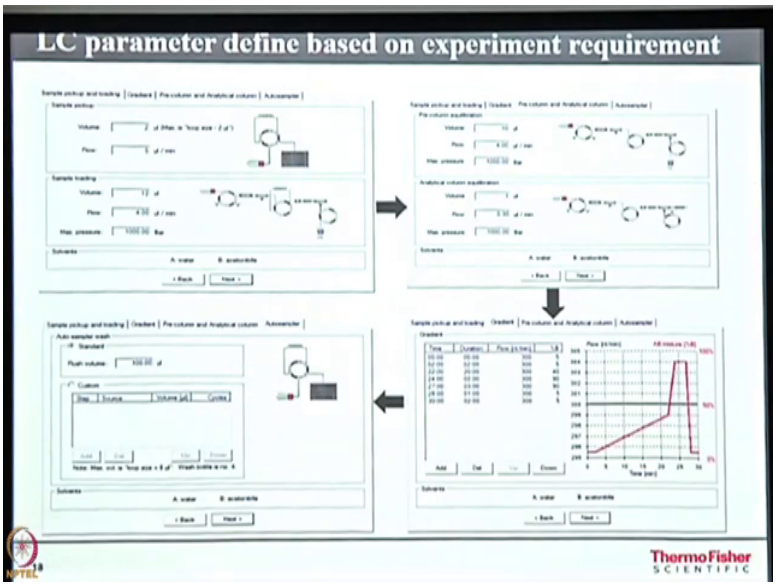

Now, before going to the next step, so, LC is another big part because in the front end we are putting a LC. Why we will require a LC for this kind of analysis? Because the peptide length maybe from 300 to 2000 m by z. So, we did the mass we cannot separate all the m by z s. We can separate the nearby masses by the resolution, but we cannot separate the in the range.

So, we need to separate those peptide. So, that we put a LC be in the front line where either with the basis of hydrophobicity or any other parameters we separate those peptides and elute one by one and injected to the mass spec ok. So, these are the different parameters we are using for setting up the LC run. Here the important part is a gradient. So, you have to choose a proper gradient though that gradient should be on the complexity of our sample.

So, now here I am showing you 30 minutes gradient. So, it is work for a simple one protein like bsa or single protein, but when your complexity when you are working with the

wholesale lies it that time you have to increase the gradient for 2 hours, 3 hours, 4 hours. So, right that like that you have to work on the gradient part not in the MS part ok.

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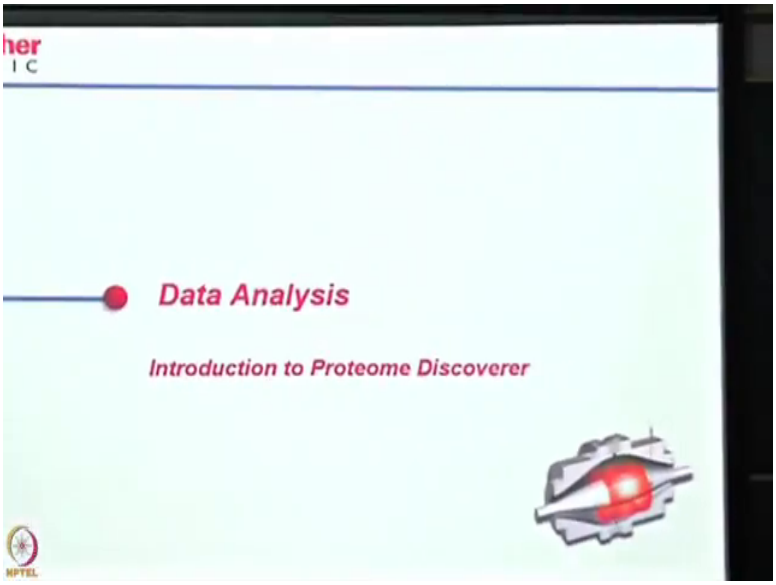

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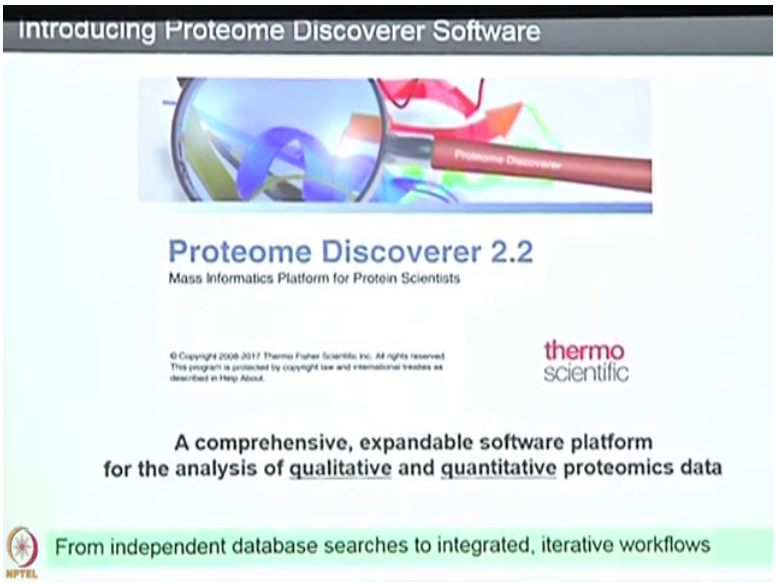

So, now come for the some data analysis part. For data analysis we have protein discoverer 2.2, that is a comprehensive and expandable software for qualitative and quantitative. So, you can identify as well as quantify the proteins. Here the quantification is a relative quantification not a absolute quantification.

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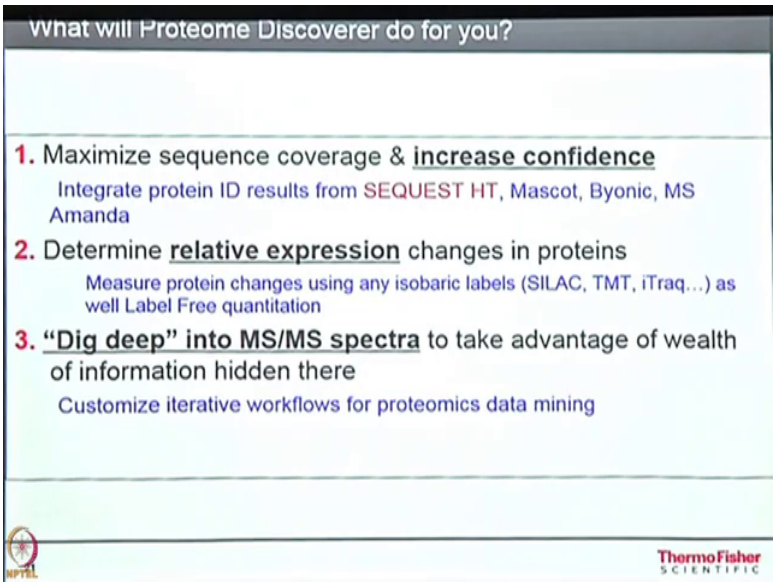

So, what it has? Basically, it will have some different search engines like SEQUEST, Mascot Byonic, Amanda out of the SEQUET is a free available. So, it will comes with the integrated with the protein discoverer other search engines mascot or byonic those has to be purchased with the license.

So, actually what this searching engines works I means, how it to works. So, when you have run a sample human sample you get the data MS MS data. So, now, you tells the software search engine k, you extract the protein you set the database of human.

So, the search engine will digest theoretically digest those protein from the human and make a theoretical mass list and then the theoretical mass list will be compared with the experimental

mass list at the MS label. MS label first MS 1 label when it will much match with the MS label then, it will come for the MS 2 label.

If it is matching the MS 2 label and get those b and y ions to get the sequence then it will tells you that peptide is present, then it will give you this proteopeptide is present means this protein also be present there ok. So, it will measure determined relative quantification. So, you can do relative quantification either it is a label free or a rebel reaction like TMT, SILAC, iTRAQ.

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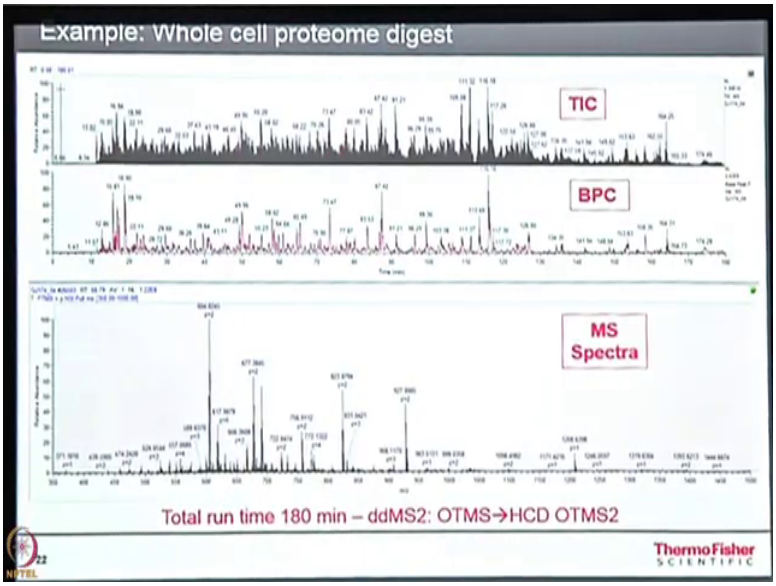

So, here is an window where you will see the first half is the whole cell lysate run for one twenty minutes we run a ddMS2 method. We do OT at MS and HCD also in Orbitrap ok, MS 2 also detected in Orbitrap. We are not using ion trap here it is a 120 minute 180 minutes run it is a whole cell lysate.

So, first window it tells that TIC, Total Ion Chromatogram. So, like in HPLC you are getting UV chromatogram here we will get on the basis of total ions it will make a chromatogram. So, that is why it is called the total ion chromatogram. Second window it is called the BPC, Base Peak Chromatogram. On the basis of highest intense speed for the each retention time ok.

So, that is called the base peak chromatogram. Now if you click anywhere you will get like this of MS spectra this is the real MS spectra where x axis is the m by z y axis is a relative abundance and it will see different charge states are there plus 2, plus 3, plus 4 because of ESI, you will get the different charge states ok.

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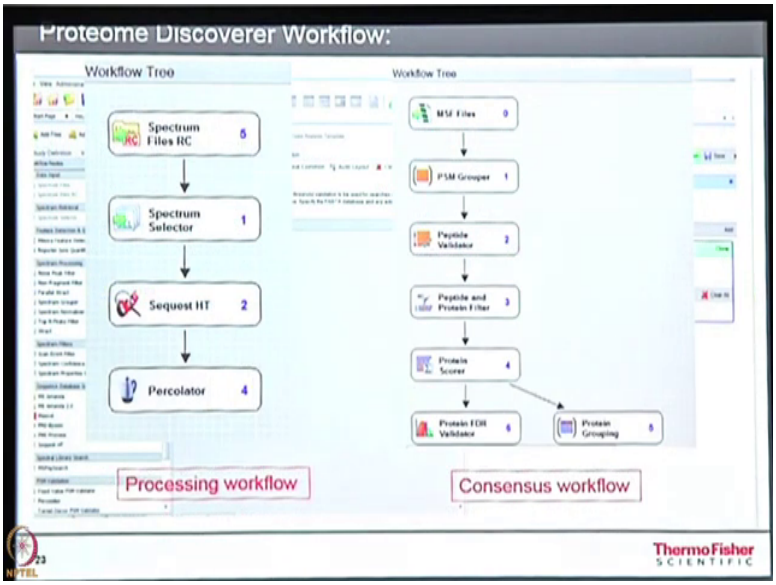

Now, when we search this data in PD with different with this 2 workflow. So, in PD, you have 2 workflows; one is the processing workflow where we search the data against the

database we are using the sequest and after getting the peptides those peptides may be a false positive or false negative. So, you have to validate those peptide.

So, those has been done in the consensus step where we do the peptide valuation as well as protein validations. And we will ultimately get the data. So, here the string we are doing the validation at one percent FDA. So, 1 what is that mean? If you run the sample 99 times, the protein will identified maybe in 1 time it not been identified.

So, in such stringency we are detecting though validating those peptide and we are telling this peptide is present. So, then the report is very very good for publishing.

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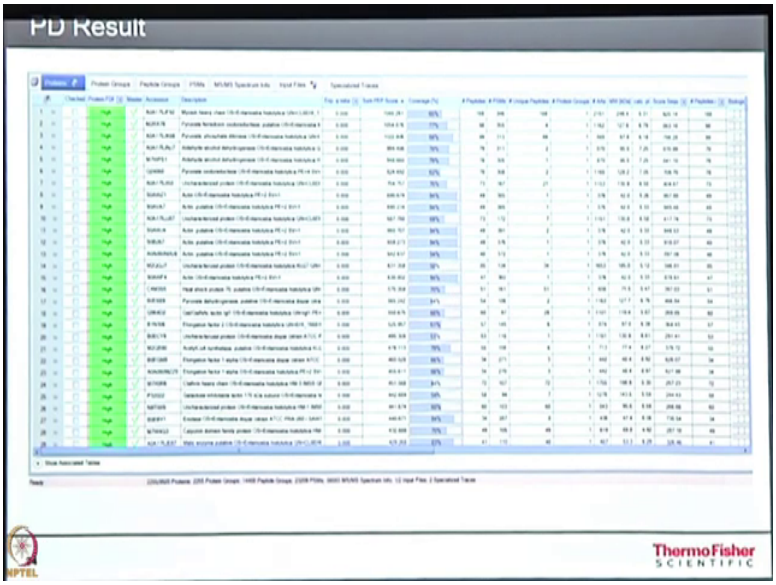

So, now what after search the data what is the report format. So, it comes like this. So, these are giving you the protein information, what are the protein match and the sequence coverage how many peptides has been matched. So, here important thing is the unique peptides.

So, unique we are looking for the unique peptides. How many unique peptides we have identified. So, what does mean by unique peptide unique peptide is those peptides which are present only in those proteins not any other protein in the included in the database. So, where what database you are using in that database that peptide is not present in any other protein. So, we are looking for at least 2 or more unique peptides those kind of data will be the very good data ok.

So, here we have identified almost 2255 protein groups. So, it comes like from that data set it has extracted this many MS MS spectra which has when we match against the database we are getting this many 23000 PSMs and out of this PSMs, we are getting these many 2 2 s 5 5 protein groups.

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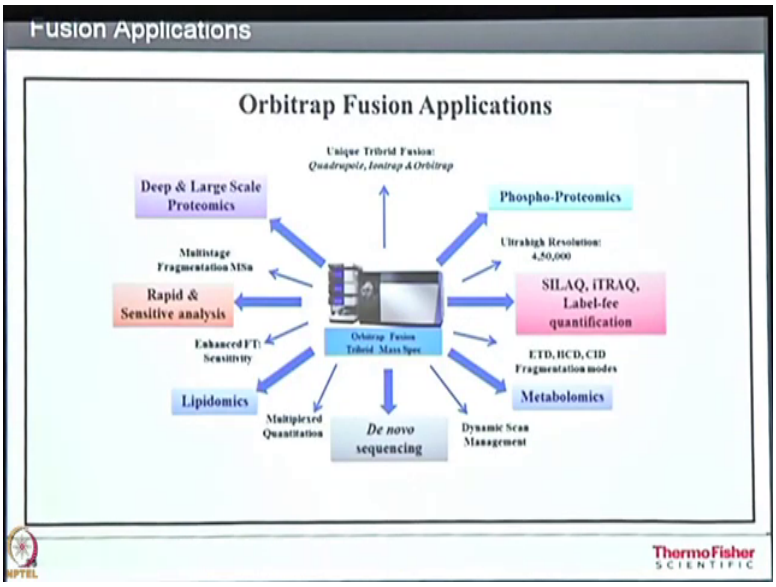

So, right now I am talking about the identification just. How to do? So, fusion is not for identification it can do many works. So, right now we are doing the deep large analysis and the label free or label reaction to relative quantification. So, it can do the phospho proteomics, it can do the lipidomics, it can do the metabolomics. So, you can use as instrument for different kind of analysis.

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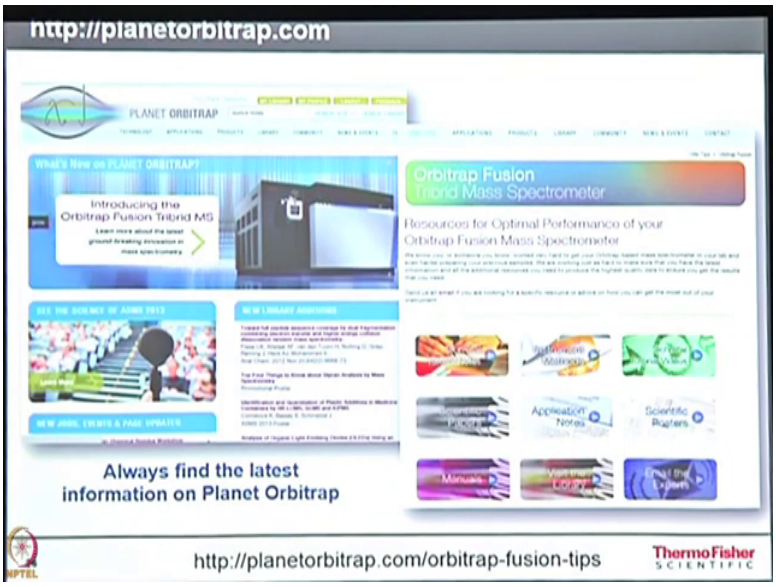

Lastly this is a website planet Orbitrap dot com where you will get all type of information of Orbitrap in regarding application wise if you are working on proteomics. So, you will get all type of application and notes the published data or methods. So, you can register here and you will get the free updates ok.

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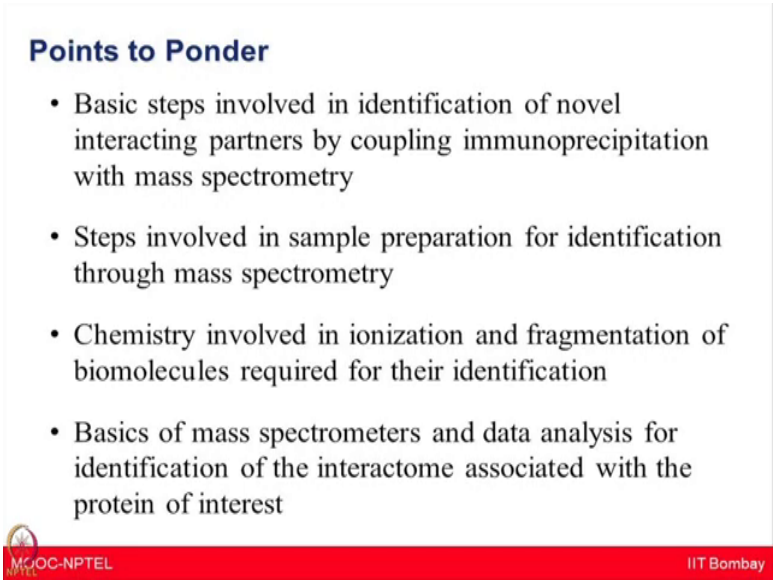

And from the last 2 lectures, you are now familiar that if you have a protein of interest which you want to dig deeper, you want to further characterize their protein and want to understand the possible role of that protein. An easy way of moving forward could be to do immunoprecipitation experiment followed by protein identification using mass spectrometry.

In today's lecture you have seen how to take the complex which you have eluted out from the IP experiment and identify the potential interactors using mass spectrometry. There could be many softwares which could identify the potential proteins of interest. In fact, I would recommend you to use one of the open access software mascot to do the data analysis where you can easily identify the proteins of interest.

There are many good softwares available even commercially one which we have shown today is proteome discoverer. In general I hope these 2 lectures have given you some basics and

understanding about how to study the biomolecular interactions using immunoprecipitation followed by mass spectrometry based experiment.

Thank you.