NATIONAL PROGRAMME ON TECHNOLOGY ENHANCED LEARNING (NPTEL)

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Applications of Interactomics using Genomics and Proteomics technologies

Course Introduction by
Prof. Sanjeeva Srivastava

MOOC-NPTEL

Applications of Interactomics using Genomics and Proteomics Technologies

Lecture-25
Mass Spectrometry coupled Interactomics-II

Dr. Sanjeeva Srivastava
Professor
Biosciences and Bioengineering
IIT Bombay

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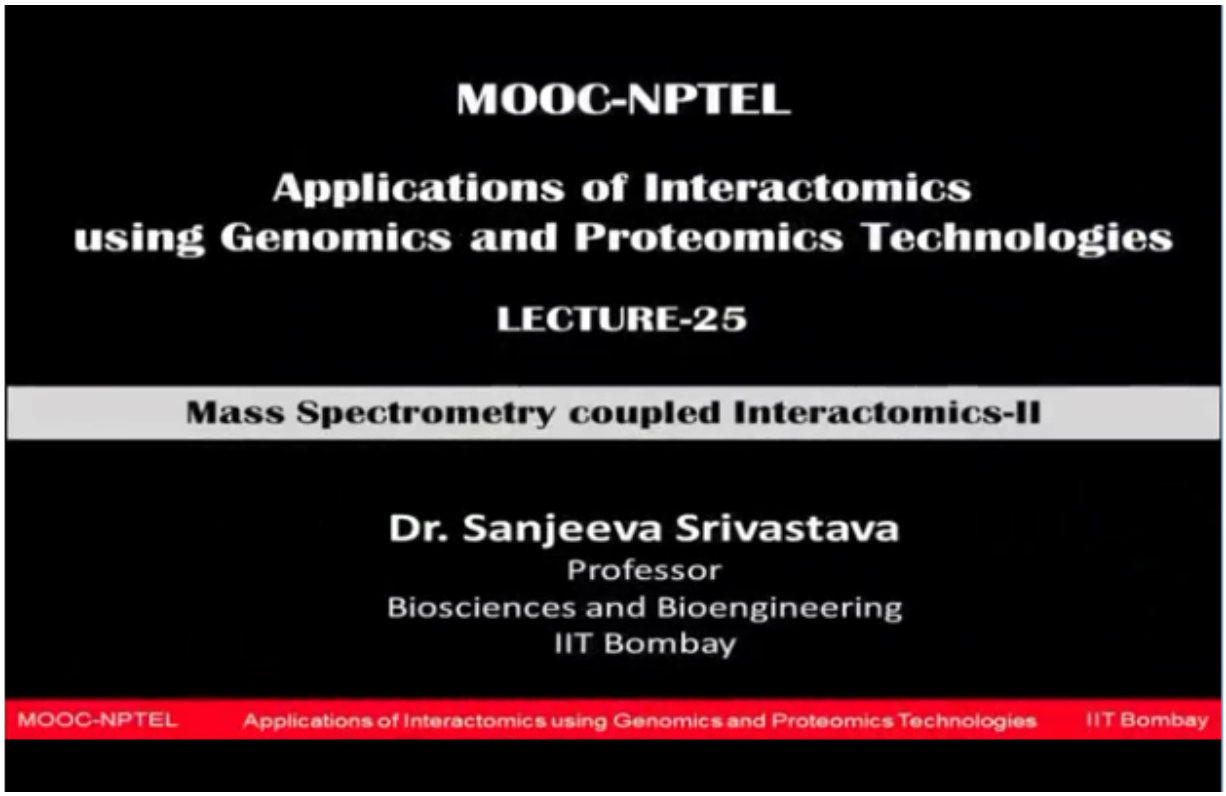

Dr. Sanjeeva Srivastava:- Welcome to MOOC course on Applications of Interactomics using Genomics and Proteomics Technologies. In today's lecture we are going to talk about basic of mass spectrometry which could be used for characterizing, identifying the proteins of interest after performing the immunoprecipitation 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's going to talk to you about how to perform these experiments using mass spectrometers and then identify the possible proteins of interest using a software, so let's have this lecture.

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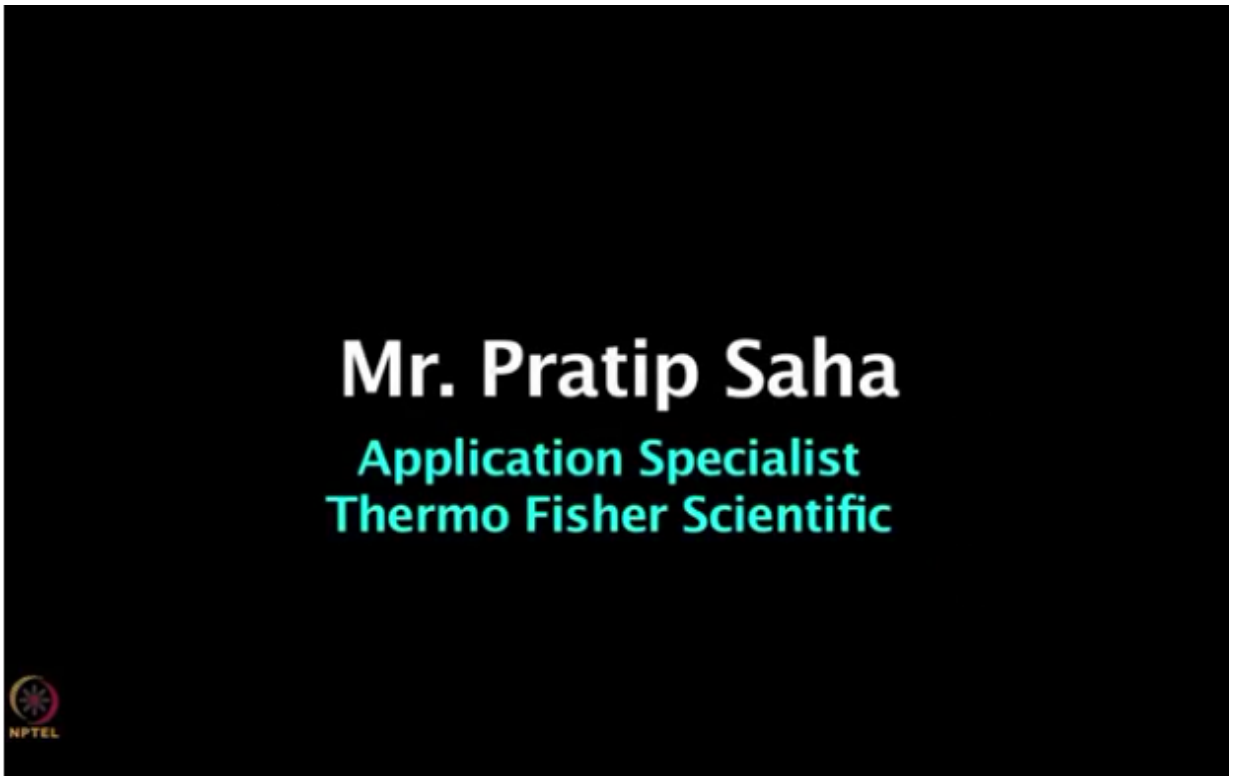

Mr. Pratip Saha:- Hi, good morning everyone, so I'm Pratip from Thermo Fisher Scientific, I'm the application specialist for mass spectrometry for proteomics and bio-pharma, so today till now you have done the IP experiments,

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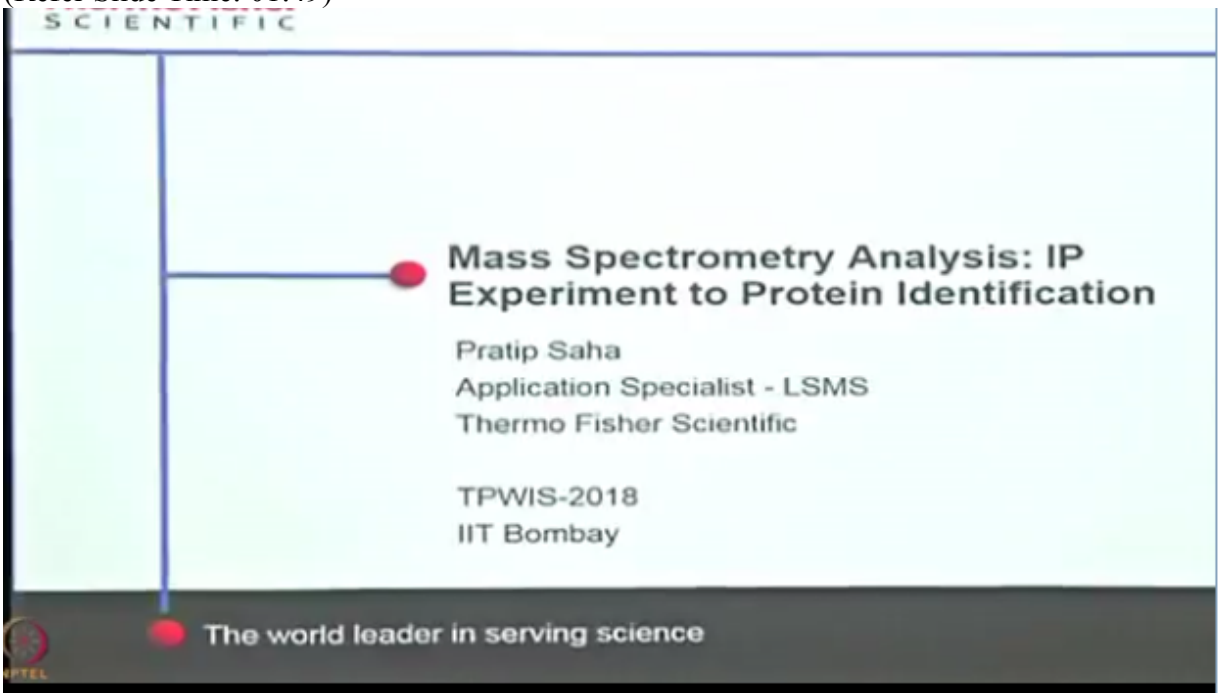

so immunoprecipitation you do the antibody precipitation, so next from those proteins which have been precipitated how you identify the protein.

Mainly we'll focus on the mass spectrometry part, so my topic is mass spectrometry analysis from IP experiment to protein identification, so already you have done these experiment, so you have precipitate the protein and you have the protein,

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so what is the next step?

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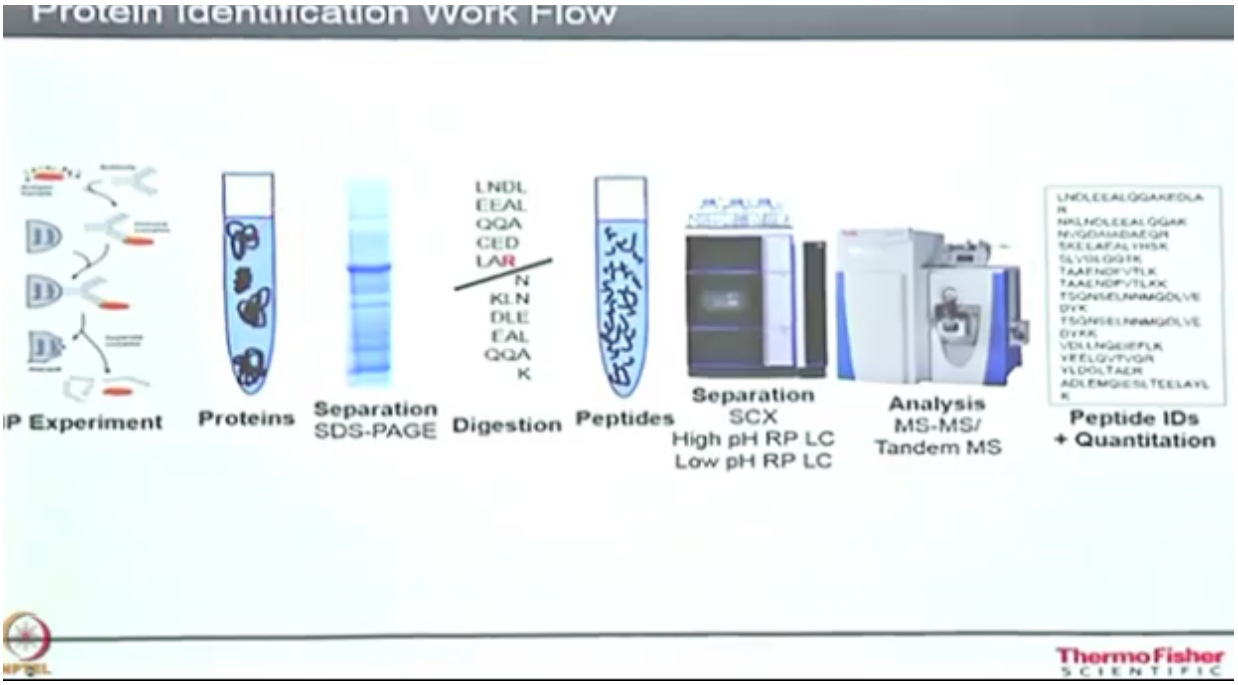

Next step either you start with this protein, you can run a SDS page or in a solution you can digest the protein with some protease which for example trypsin which carts after arginine and lysine, then it makes out peptides mixture, so those peptide mixture is fractionally through LC and introduce to 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, okay, so today we'll discuss mostly on the mass spectrometry part, how you can analyze the data, how you can introduce the peptide into the mass and how can you analyze the data, okay.

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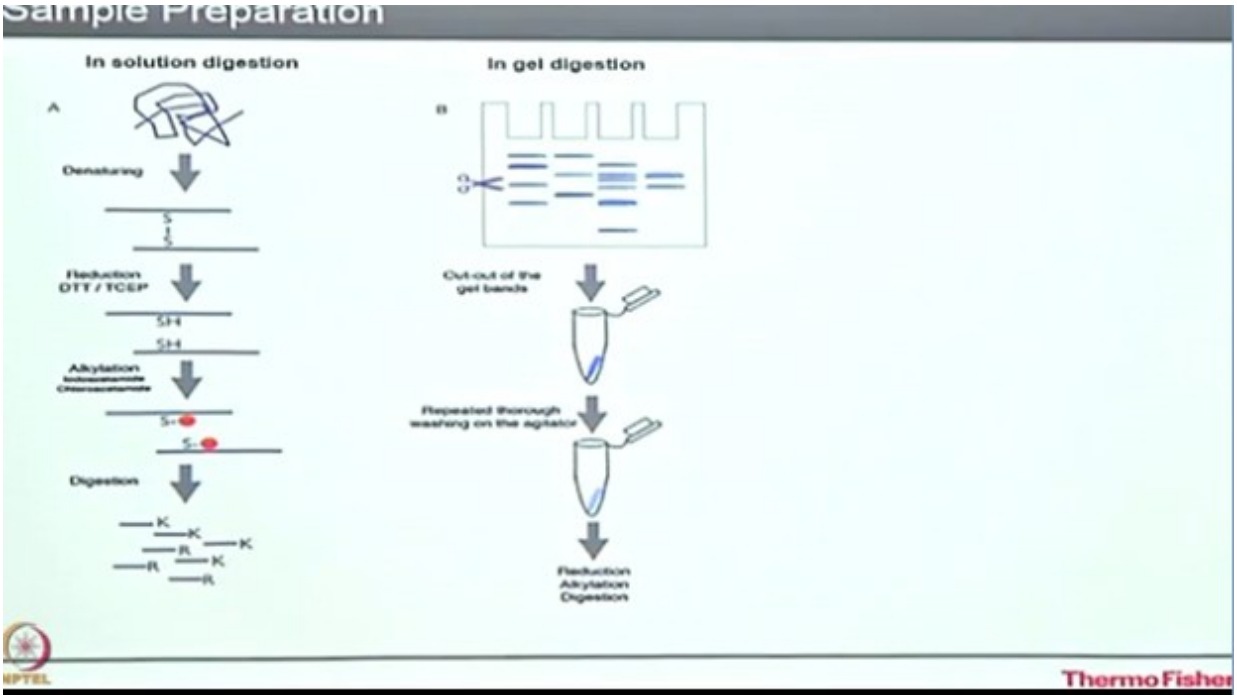

So before going to that for I'm just giving you brief on the sample preparation, so you have the IP proteins, proteins you have either you can run a SDS page, normal SDS page or you can make a solution insulation digestion, so in both cases you, in case of in gel digestion you run the gel in SDS page, then you cart the band of your protein of interest, and cart the band then you digest that band extra, so when you keep with trypsin so protein get 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 cross it.

So in case of insulation digestion already thus protein in the solution, so you have to do the reduction and alkylation, it's very important but otherwise what will happen, there are disulfide linkage, due to this disulfide linkage protein will not be open up, all the parts is not open up, so you have to go through these reduction and alkylation.

DTT we are using, is very common for reduction it reduce the disulfide bond and because of reversible reaction you have to use some alkylation part, so block that sulfide residues, so you put iodoacetamide and those sulfide will be blocked by iodoacetamide and then you digest with some proteases, so most common protease is trypsin which carts after arginine and lysine, so that message basically,
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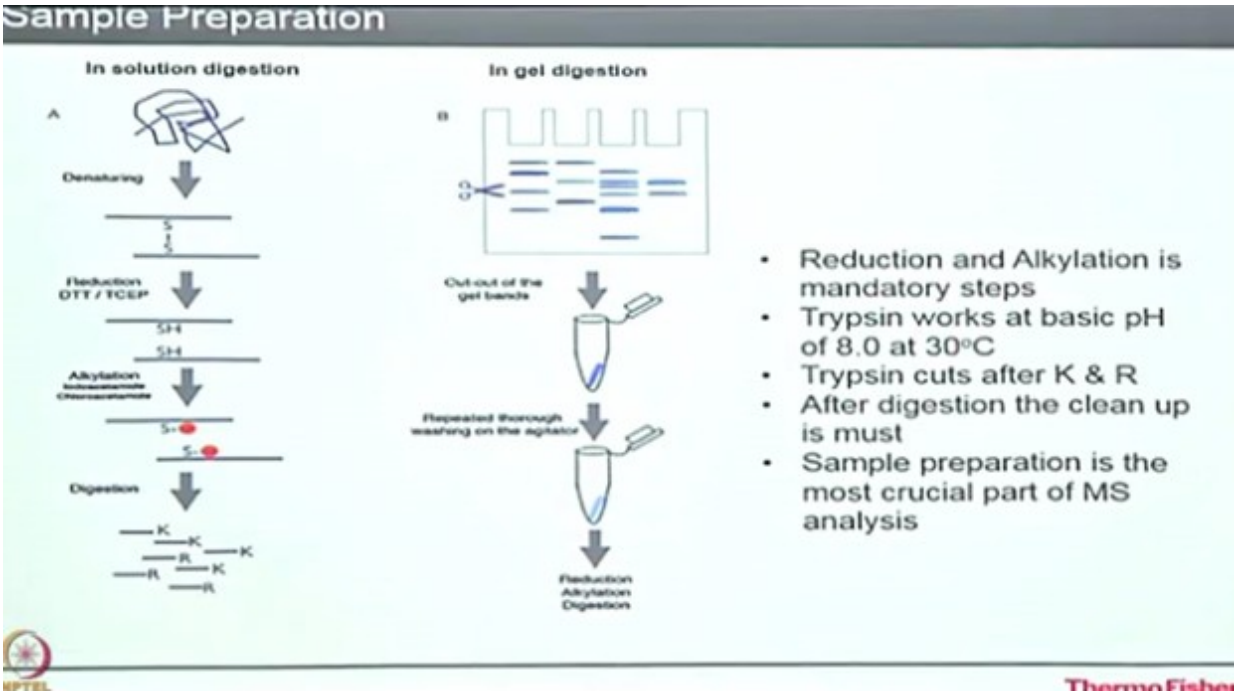

important part is reduction and alkylation is very important otherwise you will not get 100% digestion.

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, okay, 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 carts after arginine and lysine, then after digestion the clean procedure, so before injection do the, injected do the mass spectrometry we have to clean up your samples, for in gel digestion its fine, the samples are comes pure, but in solution digestion so samples may contain some impurity, some salts or some other impurities, so that will block your MS analysis, that's why the cleanup procedure is very important, so sample preparation that's why sample preparation is a most crucial part and if you have done a proper sample preparation your 90% work is done.

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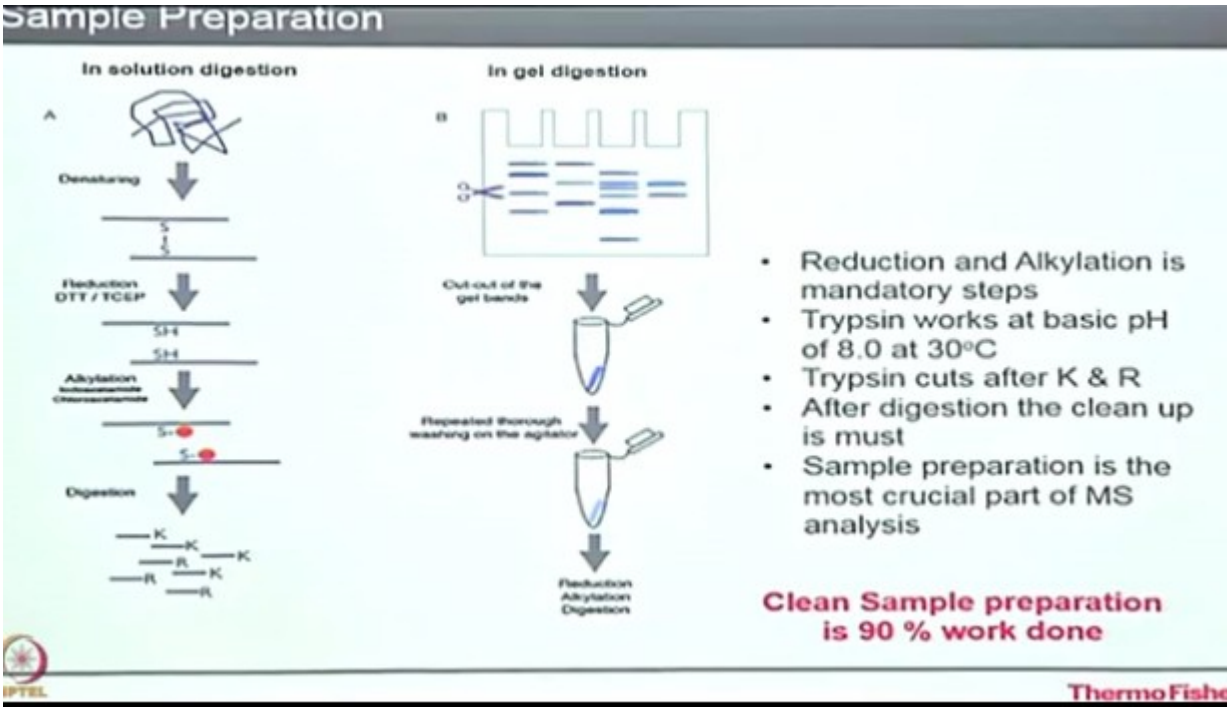

Next is the basically, MS is basically a software driven, so whatever you've said in the software it will do, but the sample preparation is the most crucial part for yours, okay.

Now comes to the mass spectrometry, so I'm keeping a basics of the mass spectrometry, I'm not going in very details on all the parts, so what is mass spectrometry? Mass spectrometry basically is a production of ions, thus subsequently filtered or separated by M/Z, okay so where and we are getting a resulting mass spectrum of abundance of produced ion as a function of M/Z,

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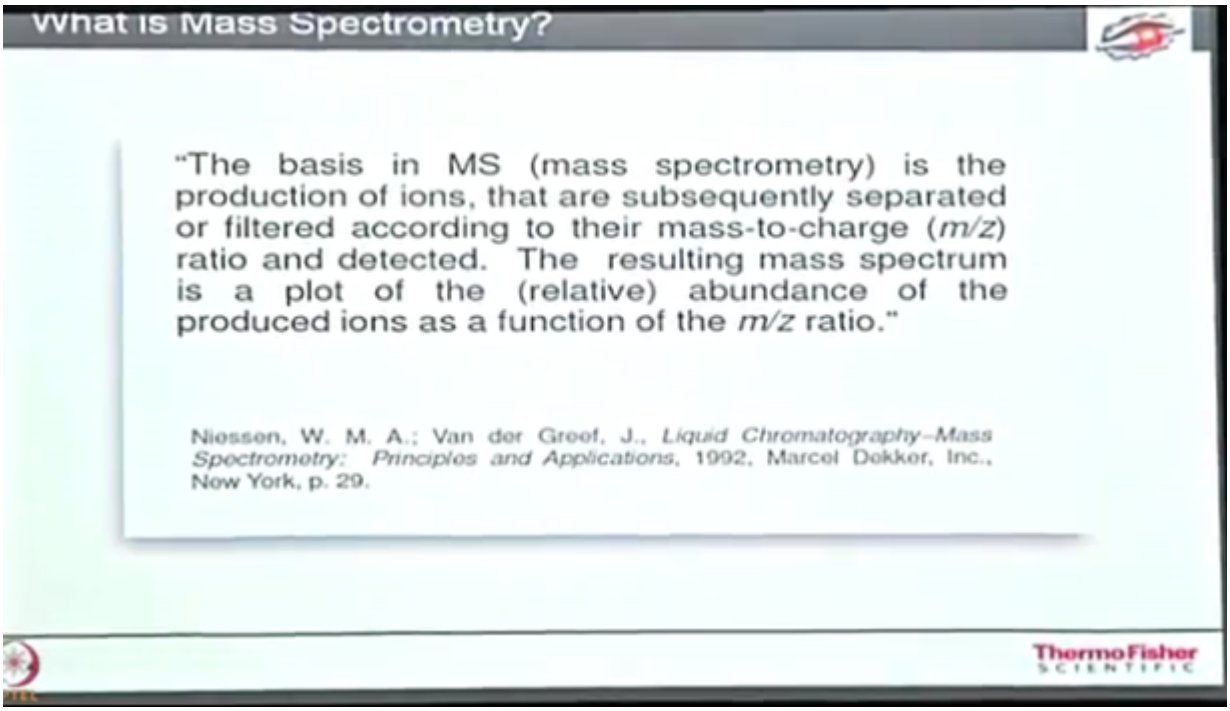

so any mass spectra if you look into the mass spectra so X axis will be the M/Z value, mass/charge ratio and Y axis will be the abundance either relative abundance or the absolute abundance, okay, so now sometimes if you run any samples in MALDI, okay, so some MALDI is, MALDI Tof is very common, so that time you will, sometimes you were saying a your mass is coming, X axis is comes as a mass, M, so whatever the M/Z value is the M, because in MALDI you are getting the class 1 charge, so always the M/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/Z value will not be the exact mass, so don't be confused with this terminology, okay.

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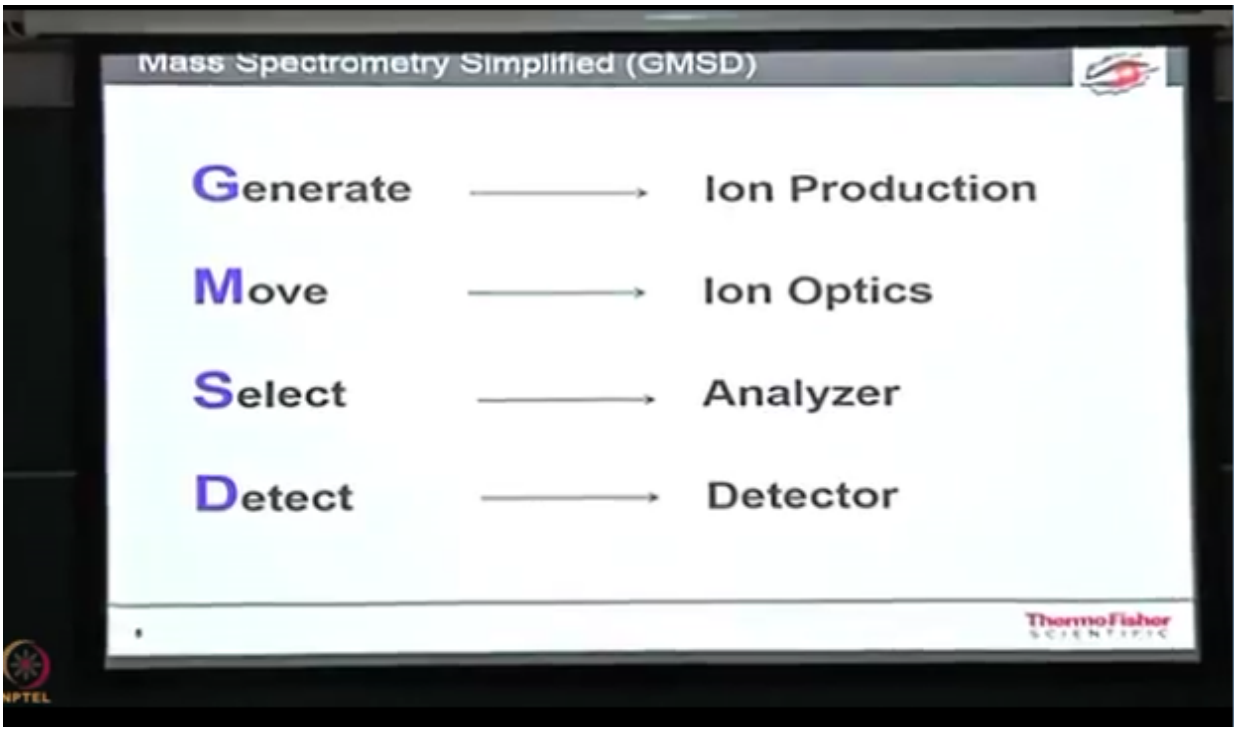

Now we can simply explain the mass spectrometry in 4 letters, GMSD, so it has, first is the generate so you have to be ionize the molecule, once any molecule can be ionized it can be detected by mass spec, so that is a simple way to identify it, so it has to be ionized properly, the ionization method maybe from solid to gaseous, maybe liquid to gaseous, then it has to be moved properly, so after ionization till the detector, so that path should be the proper, otherwise what will happen if any molecule touch in the valve 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, okay, so you have to detect the molecule.

So let's see what are the components are there in the different sectors, so first ion source, ionization, where the ionization happens? So there are very different methods of ionization, it comes with the most popular is the ESI and MALDI,
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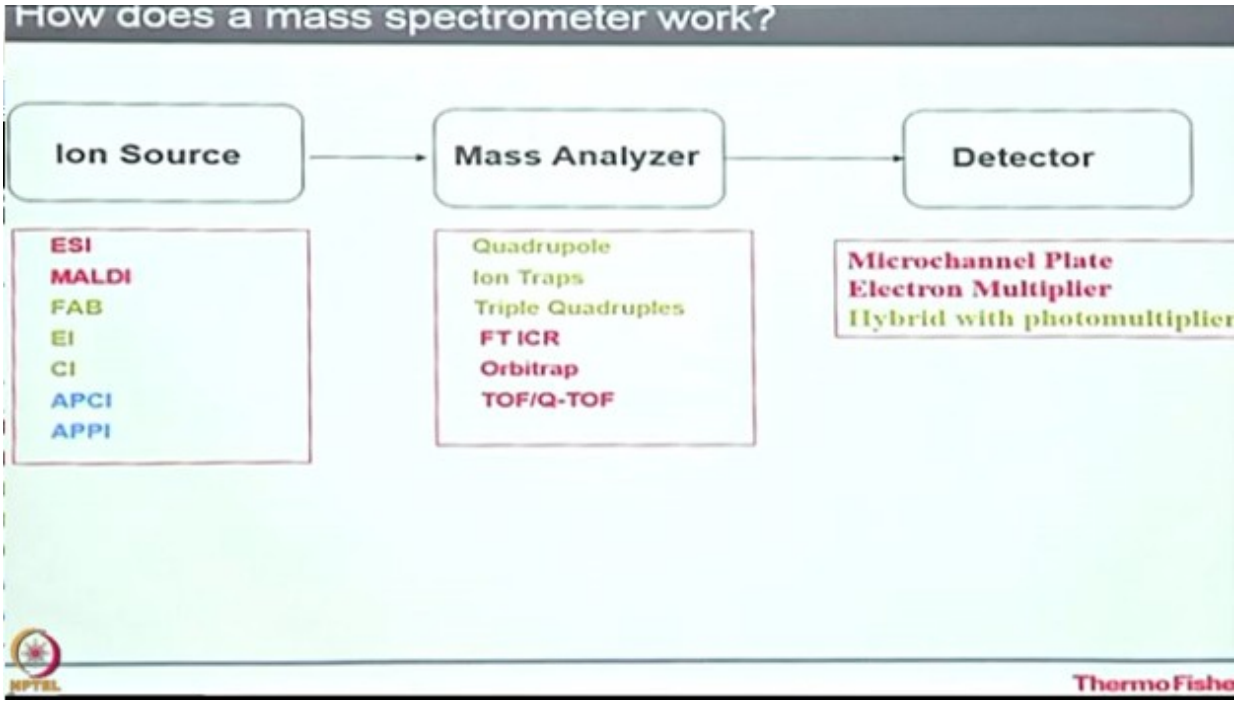

you already heard about this electrospray ionization which is liquid to gaseous, so you inject the sample in the solution from a, from where you put the high voltage and that high voltage it will mix a ionization.

Next is the MALDI, Matrix Assisted Laser Desorption and Ionization, so it is a solid form to gaseous form, what happen in MALDI? You mix the sample with some matrix molecule spot on a plate, 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, electro ionization, chemical ionization, these are the 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, okay, exclusively for nonpolar molecules.

Now come for the mass analyzer, means where you separate your molecules, most common everybody knows quadrupole, TOF instrument, time of light, quadrupole means, quadrupole is not a analyzer I can say it's a filter, it can precisely filter your molecule, which molecule you want, and you can put your molecules in the, you can trap your molecule inside the vessel and you can make detect that molecule, so quadrupole, ion, 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, okay, so in IIT Bombay we have orbitrap technology, it's nothing but a trapping of electron in a orbital motion, okay, so we put a high voltage and the ions are rotating around the molecule, okay, so we'll come to those part.

And last is the detector, so you have, you required some detector, the better part for Orbitrap is Orbitrap itself access the analyzer as well as detector, so you don't require a extra detector for Orbitrap, okay.

Now we see how the mass spec works, what we are doing in the mass spec after injection of sample, so we inject the sample to mass spec through the mass analysis and detector we get the MS1, okay, MS spectra will get this MS spectra, from these MS spectra we fragment,
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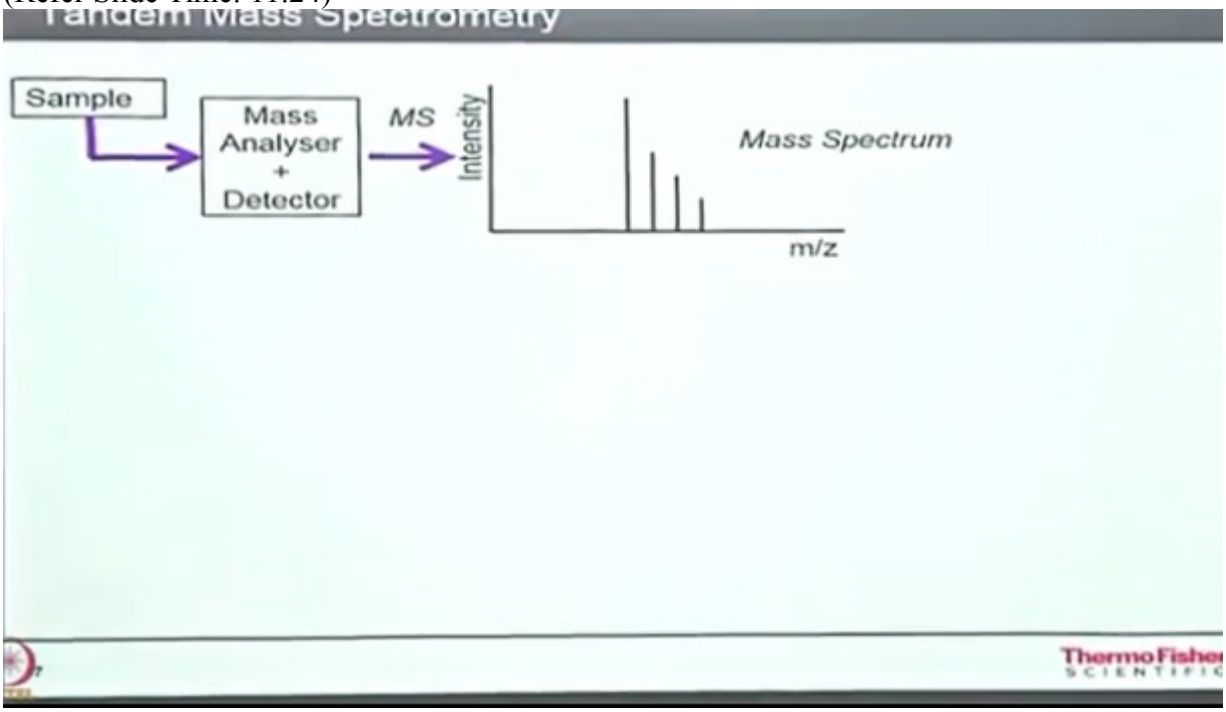

we'll select one of the peak and again we'll do the fragmentation, MS2 and we get this fragmentation,
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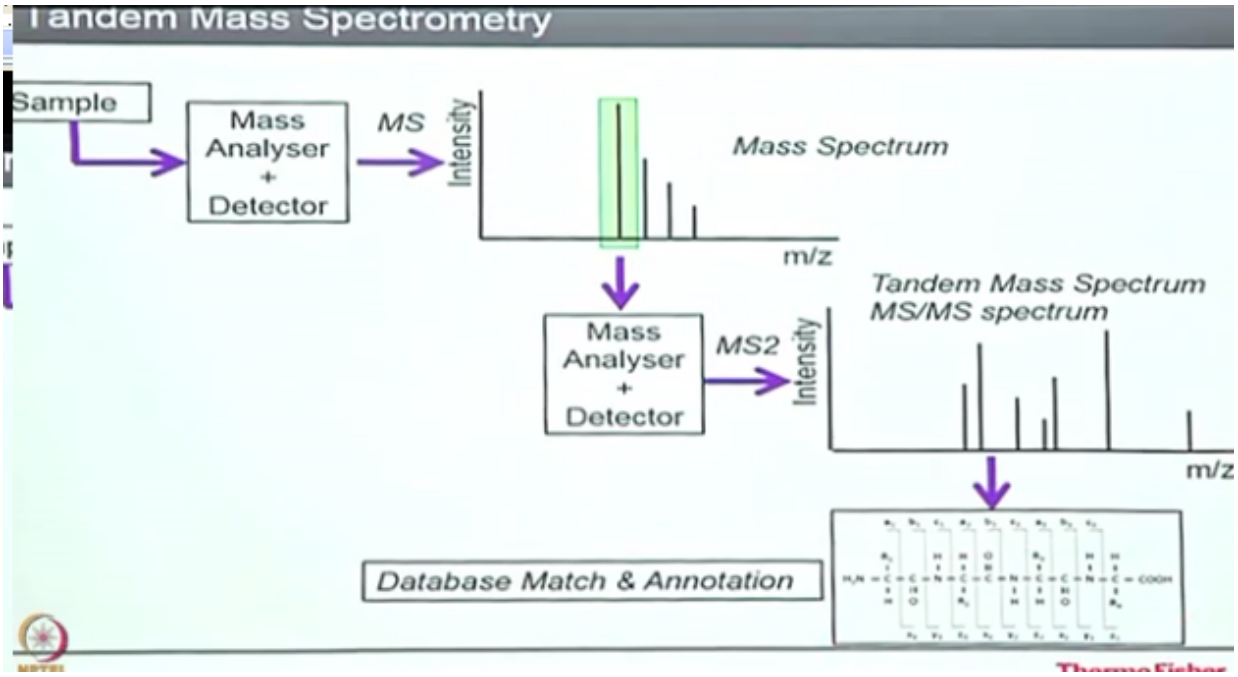

now this fragmentation will search against the database and we'll find out what are the ions are there B and Y ions, and we get the database match, we'll get the peptides, from the peptides we can say this protein is present in your sample, okay.

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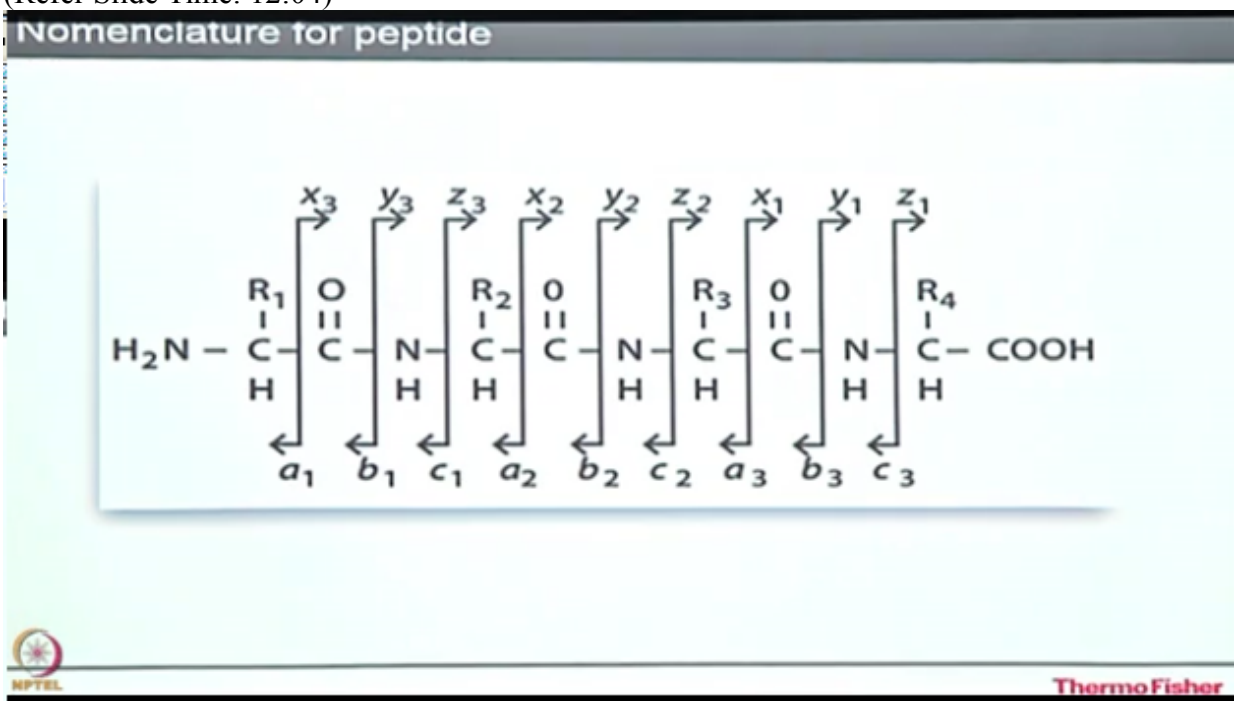

So now come to the nomenclature of identifying the ions, it's a basically nomenclature how you can assign your peptides, so this is example of a peptide of 4 amino acid R1, R2, R3, R4, okay, so now when you applied voltage to fragment the molecule in MS2, so this C alpha C, CN and NC alpha, these three bond can be broken, so this any one of the broken, okay, so if it is C alpha C bond is broken then the intaminous side will call the A ions, while C terminal side will call

the X ion. Same way CN, this is the peptide bond basically, D and Y ions and if it is NC alpha bond then it will call the CZ ions, okay.

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, okay.

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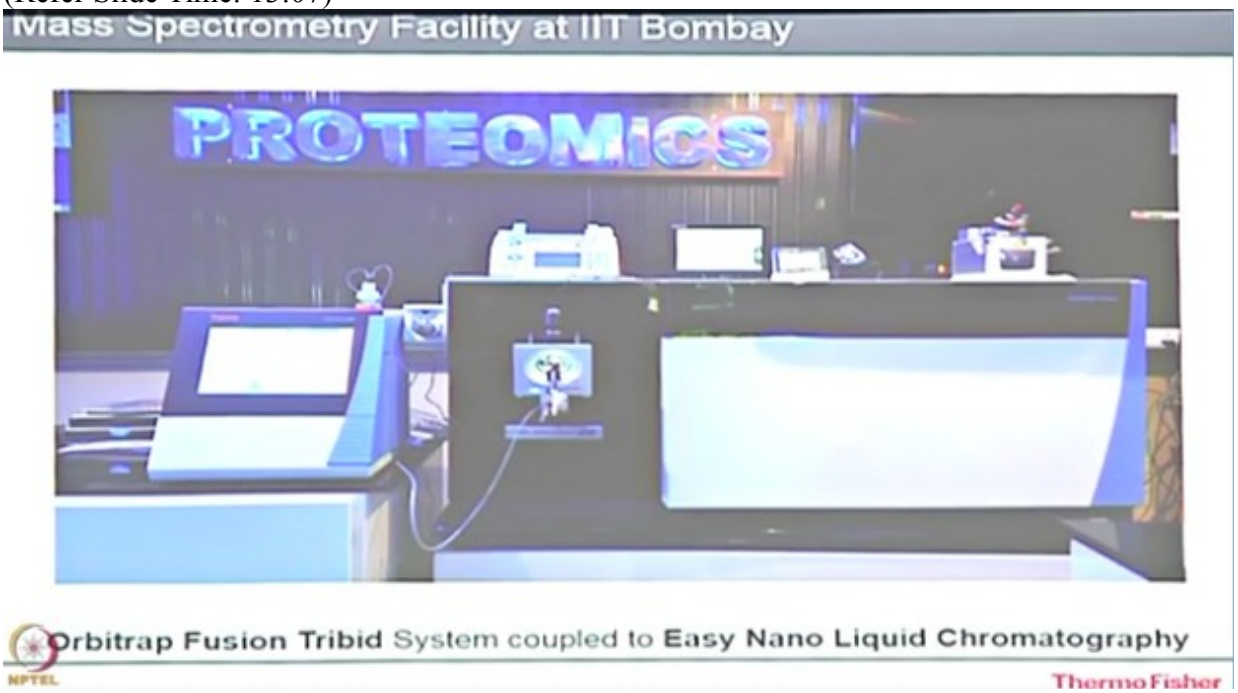

Now come to the facility here IIT Bombay, so it has a tribid instrument, orbitrap fusion with couple with nano LC, so why we require nano LC? Because we are doing the proteomics, in proteomics sample is very small amount and it is very precious so we cannot run the normal HPLC where the flow rate is very high, we've to run in nano LC in a nano litre flow rate, okay.

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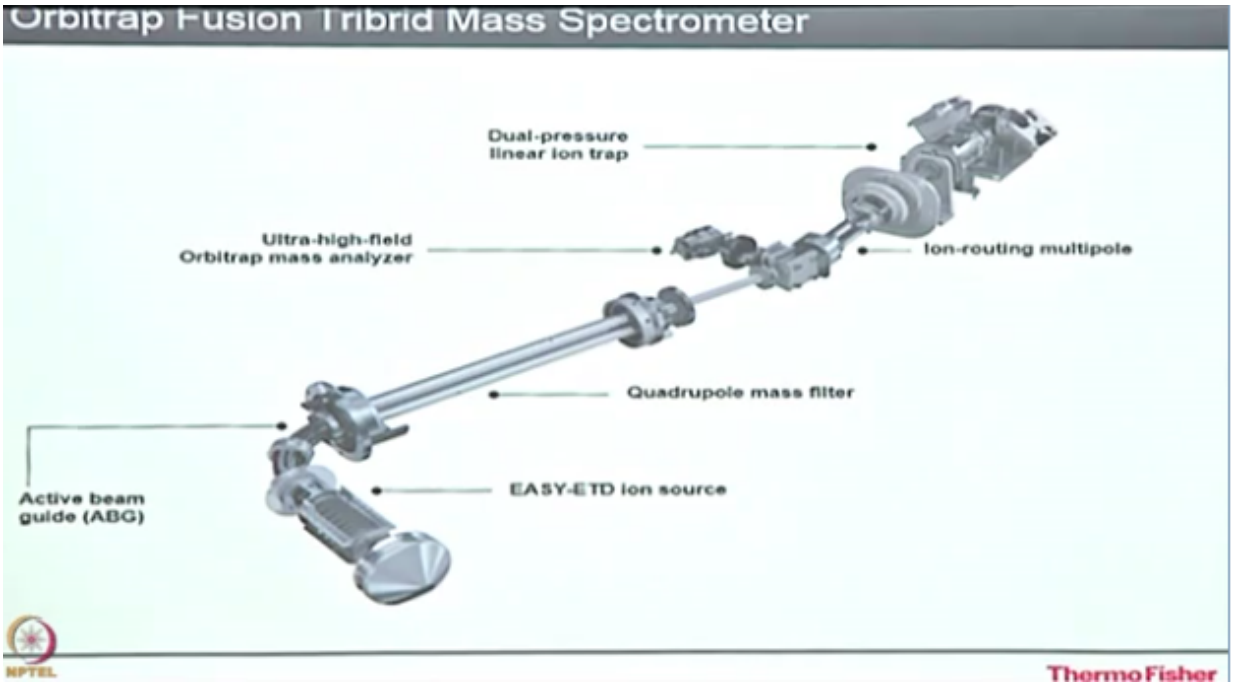

Now come to the sematic diagram of orbitrap fusion, so orbitrap fusion as I told is a tribid instrument, it has quadrupole, it has ion trap and it has orbitrap, okay, so ions are inserted from here, it gets filtered in the quadrupole first, then it goes to, it comes to the ion routing multiple where the ion will store and it will tells you where to go, because you have 2 detector,

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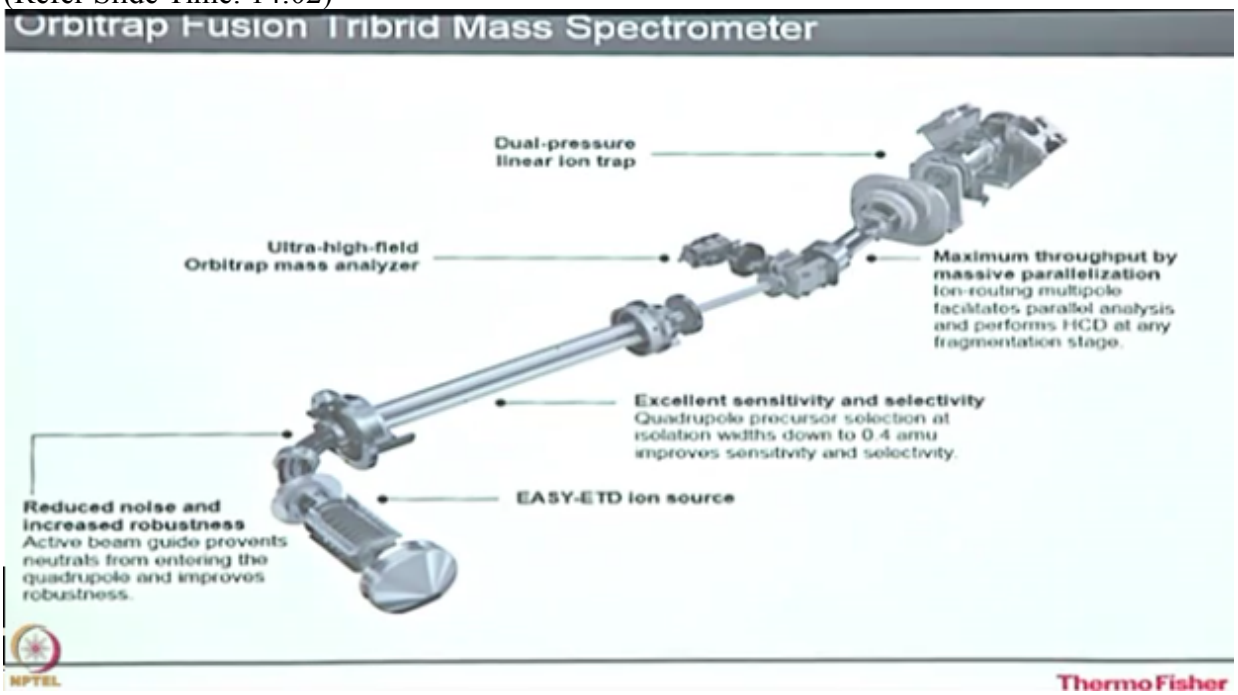

ion trap and the orbitrap, so from here either it will go to the ion trap or it will go to the orbitrap.

So then orbitrap, its orbitrap basically it's a high resolution it gives you 500K 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 ions, and also you have a ETD option, 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, MS1, okay, so now ions will generate here,

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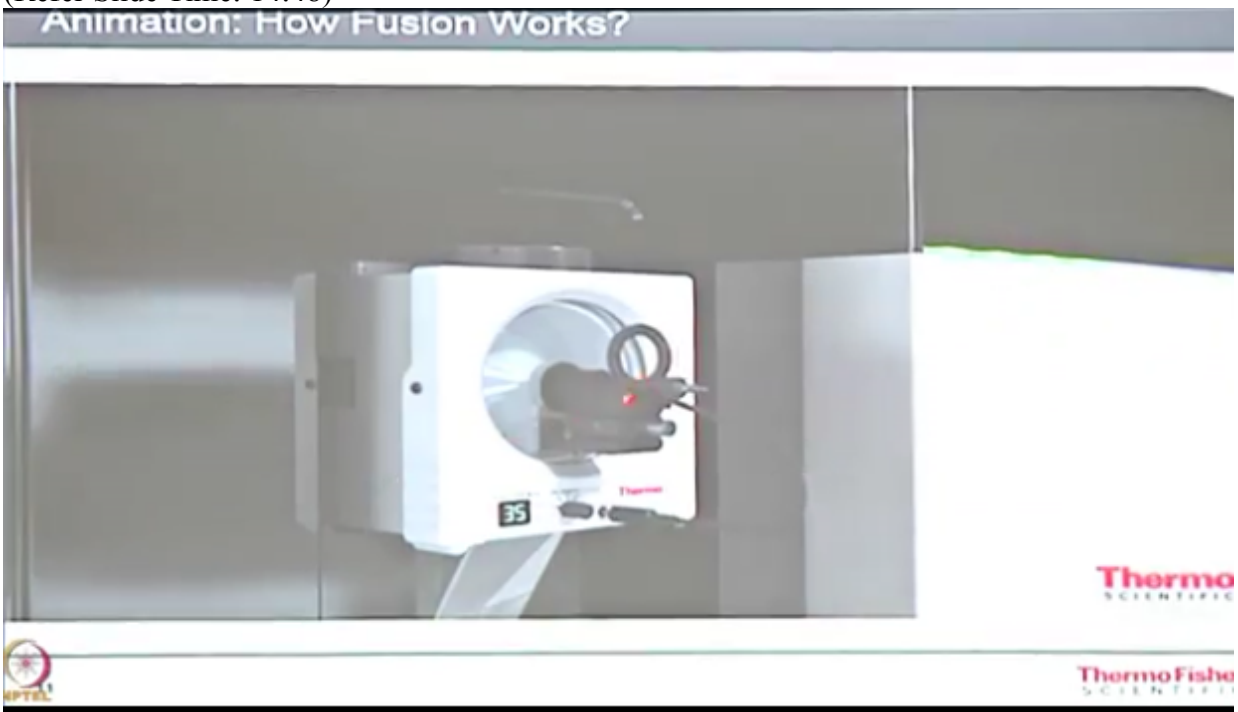

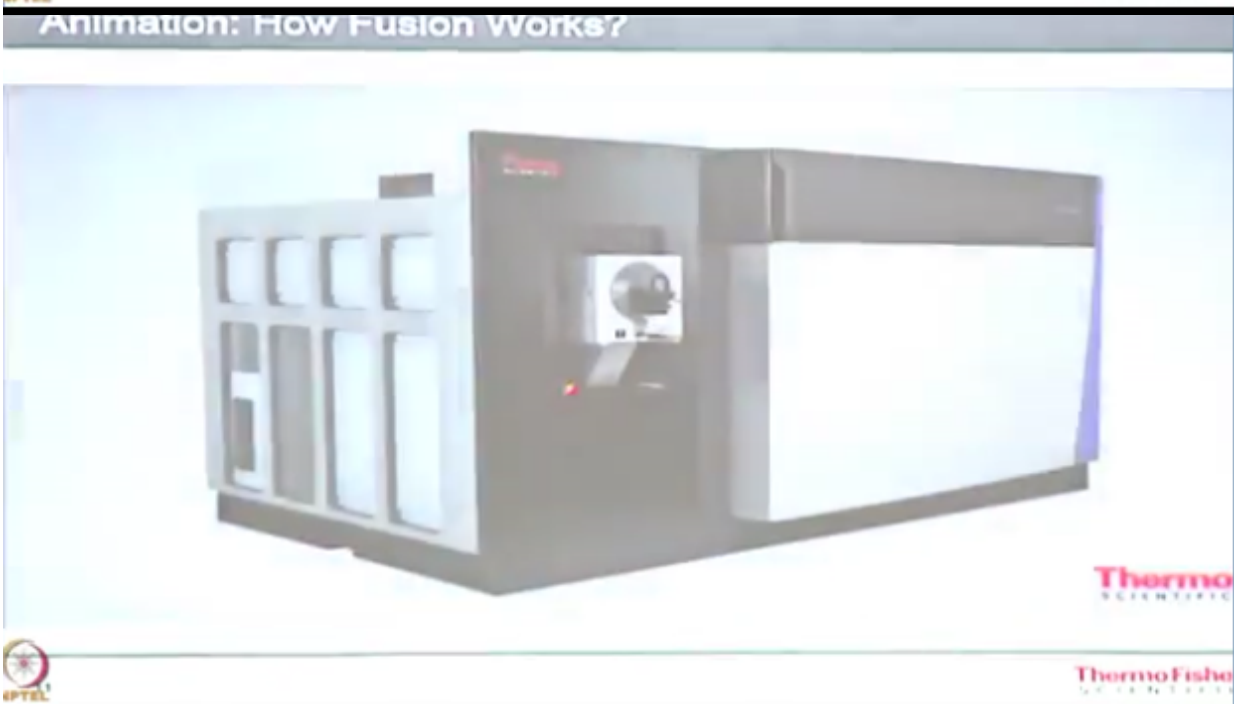

it will passes through a ion transfer capillary inserting through that, so after that here what happen ions trying to spread out, because all the ions are very highly charged, so it tries to spreading, so you need a focusing, so it will focus.

Then it's called the bent flatapole, bending where the neutrals are removed, because when the ions are inserting to the instrument there are some neutrals also, so with the high voltage difference the charge molecule can bend but the neutrals cannot bend,
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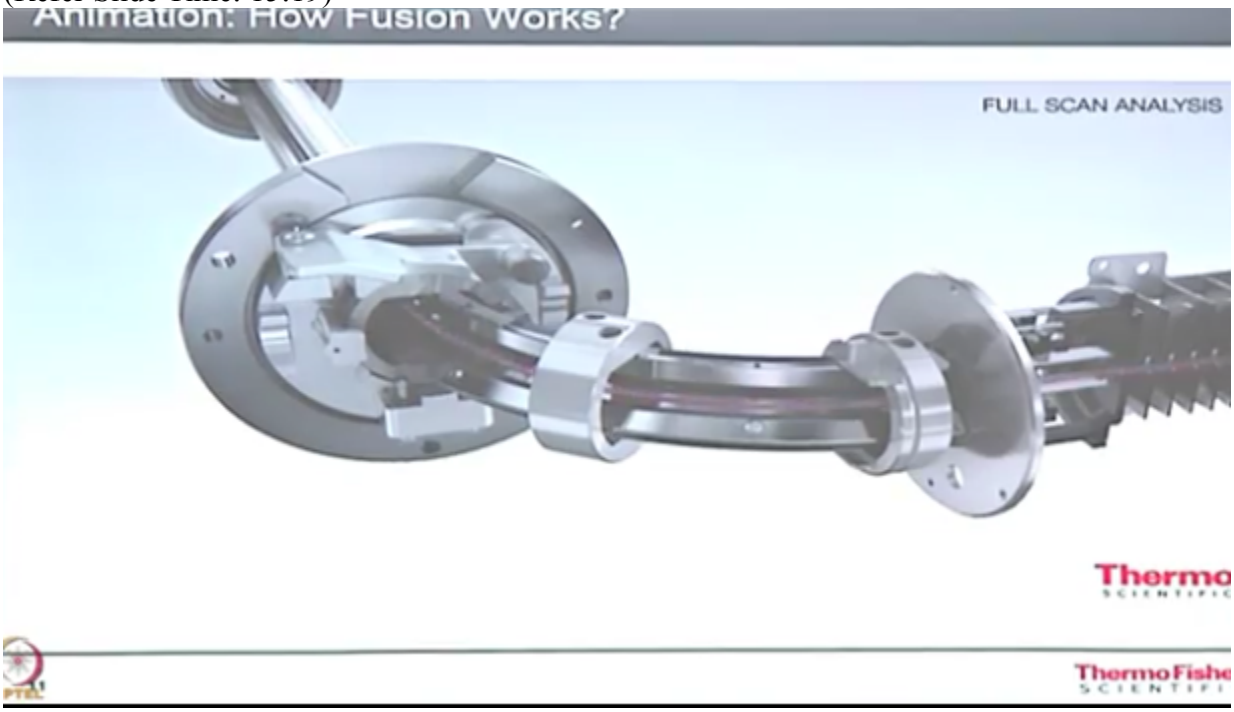

so after that in the quadrupole you can select the ions which one do you want,
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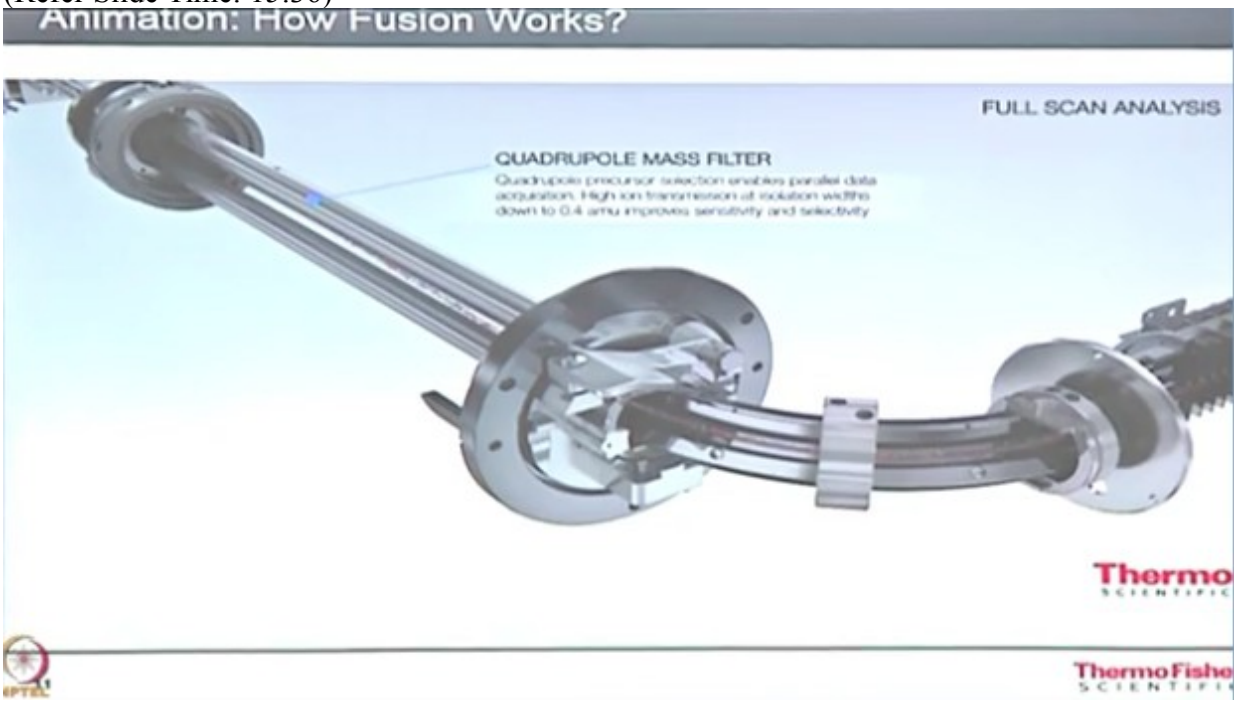

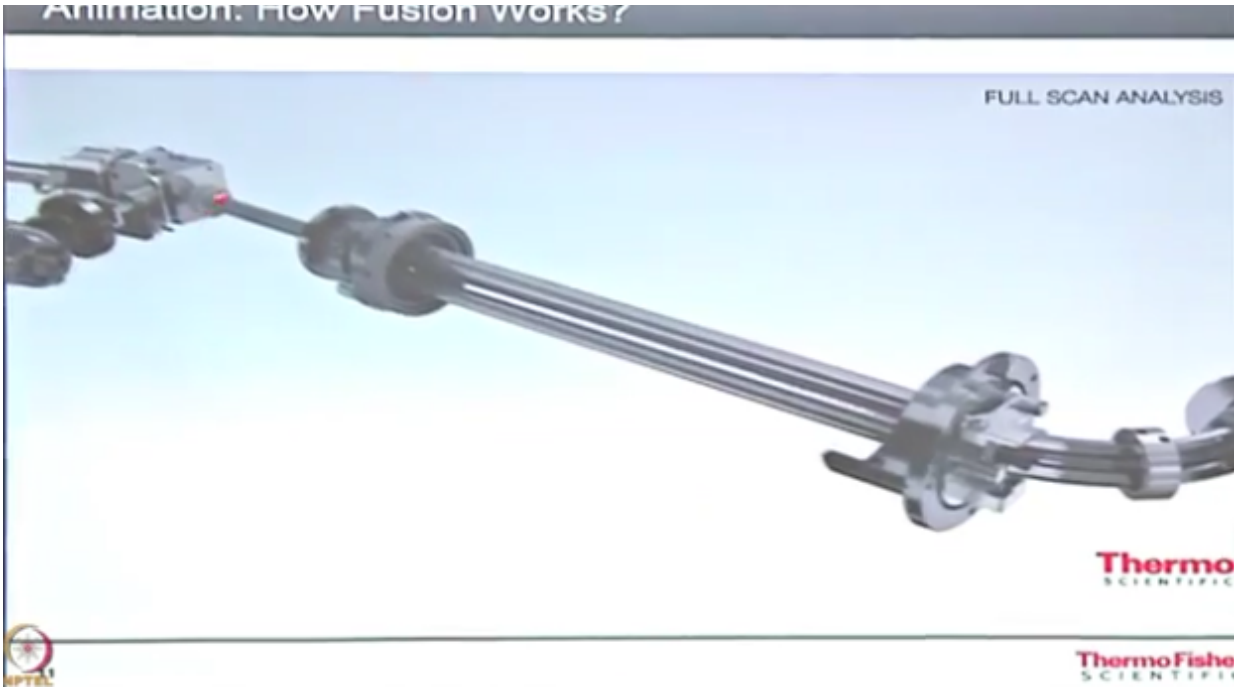

then it first comes to these ion routing multiple where it will first store here, so here is the full stoppage where it will be decided either it will go to the orbitrap or it will go to the ion trap,

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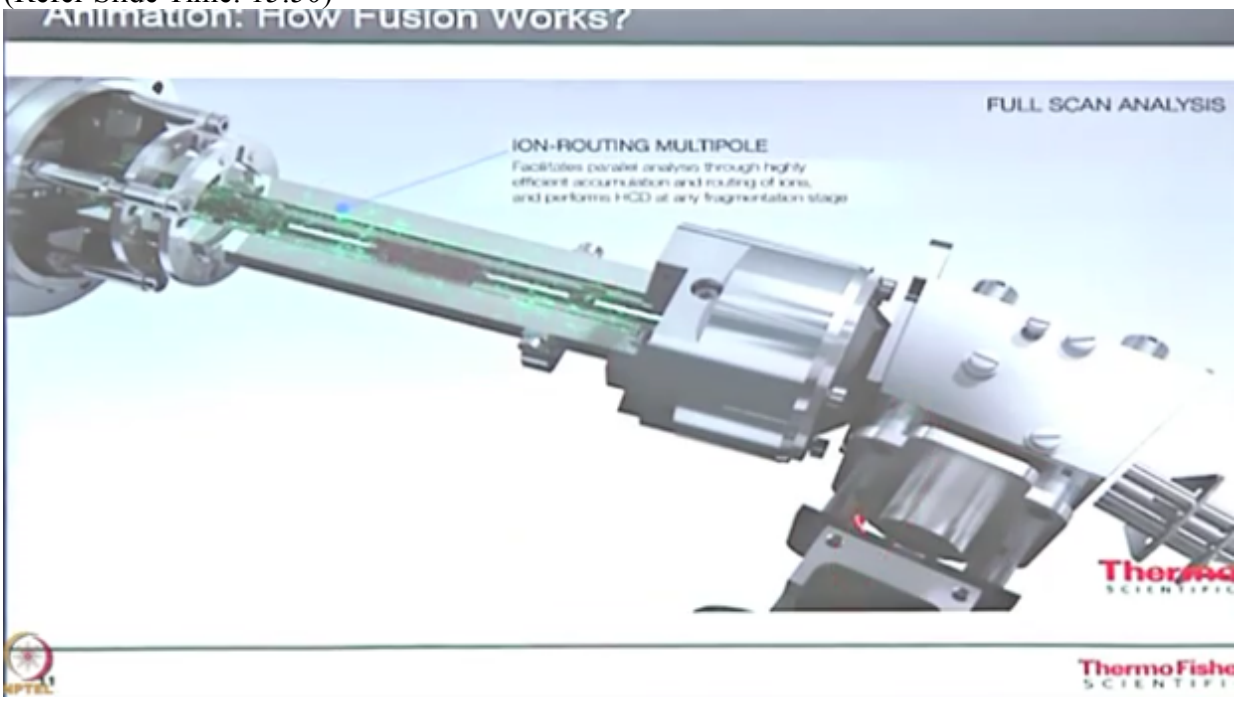

okay. So now it will come to the orbitrap, MS, and then it's been detected in the orbitrap, okay, so now it's a full scan.

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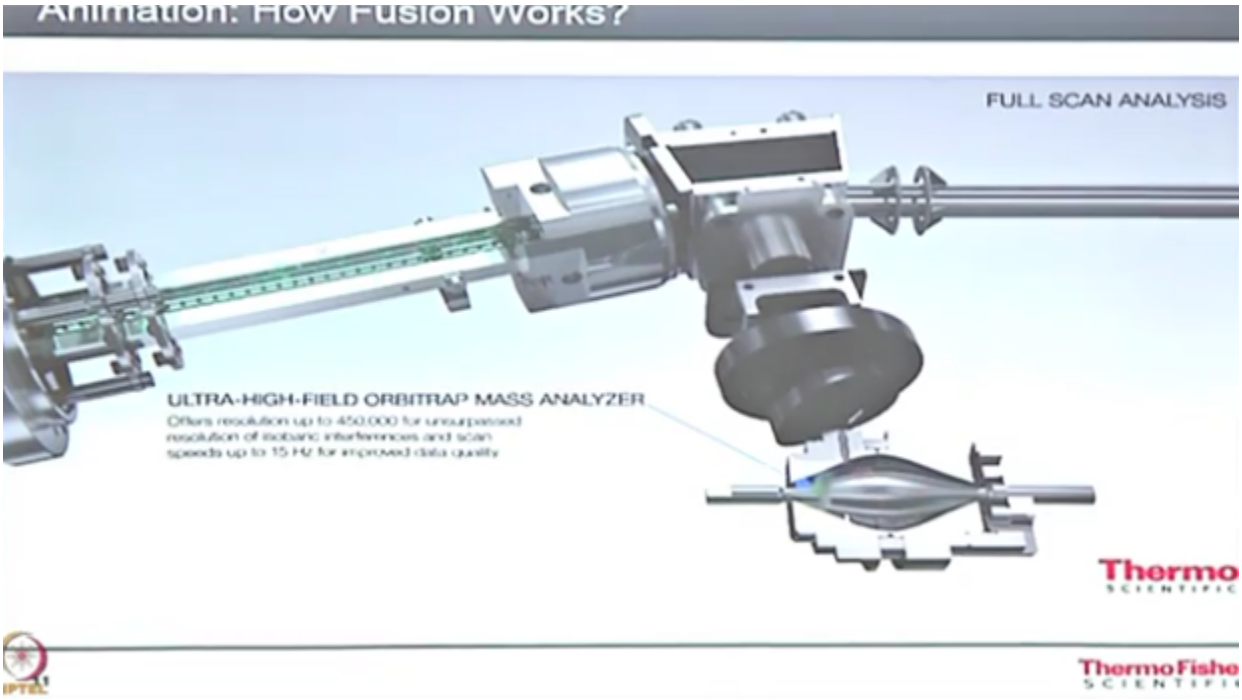

Now what happened in the MS-MS? So in case of MS-MS only one molecule has been filtered from this mass, only one molecule filtered and that gets fragmented and then it will be detected MS2, so same thing happen ions are going, so inside a quadrupole the only one molecule will be there, so other will be filtered out, now that molecule go to this ion routing multiple and get fragmented, so here it will fragmented in the ACD mode, it's nothing but the collision induced decision with high energy,

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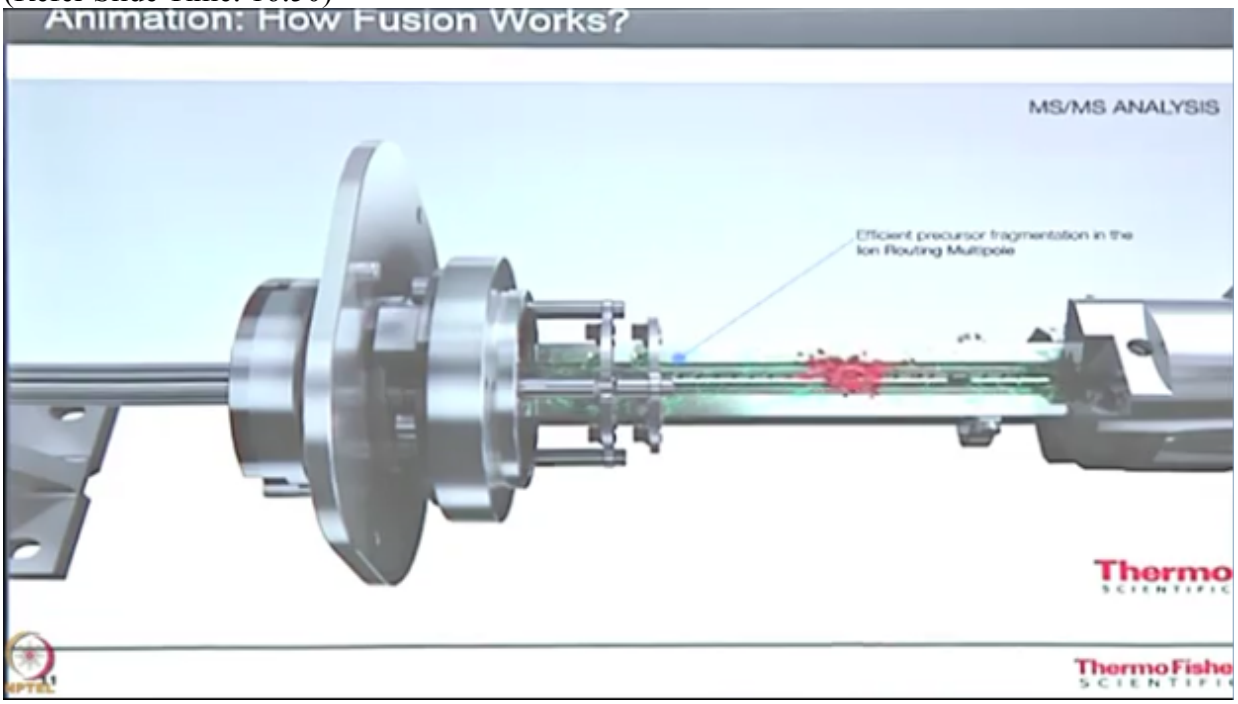

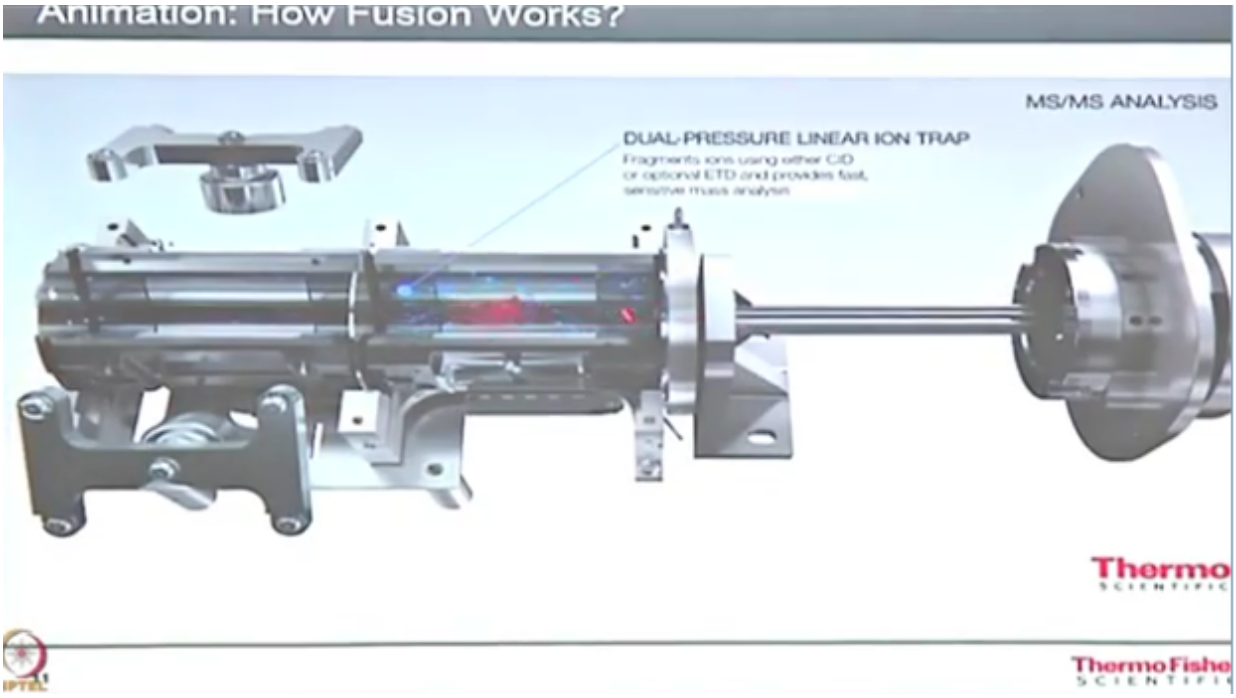

so here after fragmenting here it goes to the ion trap where it is stored first and then trapped here and then is get detected.

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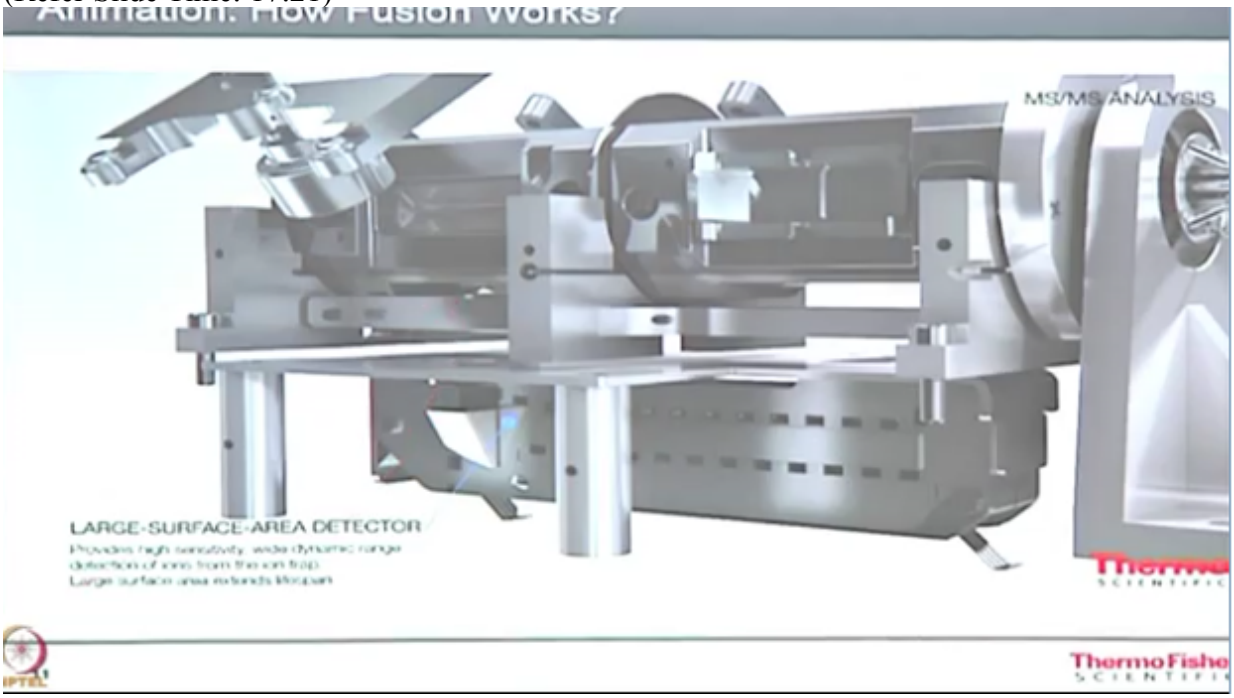

Okay, so it's detected here with the large surface area detector, so it's a very parallel reaction basically, parallelly it is going on, so when the first MS is going on here MS1 at the same time the MS-MS is going on simultaneously, so it's a parallelly both the detector is working, okay so from MS scan it can do 20 MS-MS, it can select 20 peaks from 1MS scan and it can do the MS-MS of 20.

Okay now come to the specification of fusion orbitrap,
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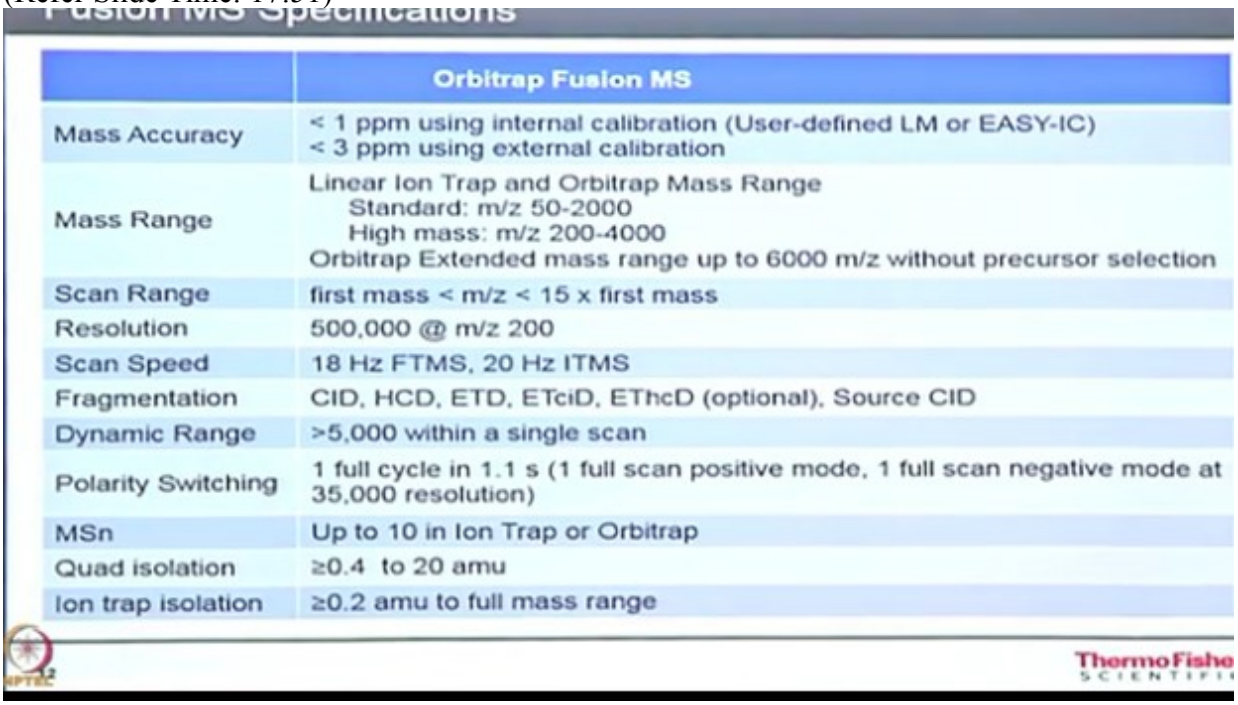

so first is the mass accuracy, mass accuracy which tells that is less than 1 PPM for internal calibration and with less than 3 PPM with external calibration, so what does it mean by external and internal? So every instrument requires a calibration, so you have a standards, you inject the standard so 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 in that time we will get the internal calibration, so that's why when we do the internal calibration the calibration will be less than 1 PPM.

Now the mass range, so you can acquire the mass range from 50 to 6000, M/Z, not the mass, okay, so M/Z, so bigger the molecule the more the charge state and it will come inside this range, but you cannot scan the total mass range 50 to 6000 in one shot, so you can scan the first mass and the highest is the 15x of the first mass, so for example if you start from 100 M/Z you can scan up to 1500 M/Z, so if you want to scan the full mass range then you are going to divide the scan range, so start from 100 to 1500 and 1500 to 6000, then resolution you can get the maximum resolution of 500K at 200 M/Z.

Scan speed 18 hertz per minute, so fragmentation, so you have different, 3 different kind of fragmentation CID, ACD and ETD, and there is a basically mixture, combination of any 2 ETD-ACD and ETD-CID, so in case of CID what? It's basically collision induced decision, so what happen in that collision induced decision? You collide your, you inject some a neutral gases that will collide with your sample molecule and get fragmented, that is CID.

ACD is high energy collision induced decision, 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 happen there? You put a creation molecule which ejects an ionize, that ion will collide with the sample molecule and get fragmented, that is called the ETD.

So according to your sample objective what you want to do, according to you have to select the fragmentation more. Then polarity switching means it's not required for the proteins but in case of small molecules if you don't know in which mode either it's a positive mode or a negative mode it will ionize, so you can do the both scan positive scan and the negative scan within a one second, so you can run that one way.

MSn as it is a ion trap so you can do 10 MSn means MS-MS, MS2, MS3, MS4 up to 10 theoretically. Isolation in quadrupole you can go minimum 0.4 AMU, means plus minus 0.2 daltons, so what that means? Means if you want to identify, if you want to identify 1 molecular M/Z of 100, so you can scan between 99.8 to 100.2, so that narrow window you can scan, okay so.

Now comes, see some experiments, means how the instrument works in different work flows, because we cannot show you in the instrument part,
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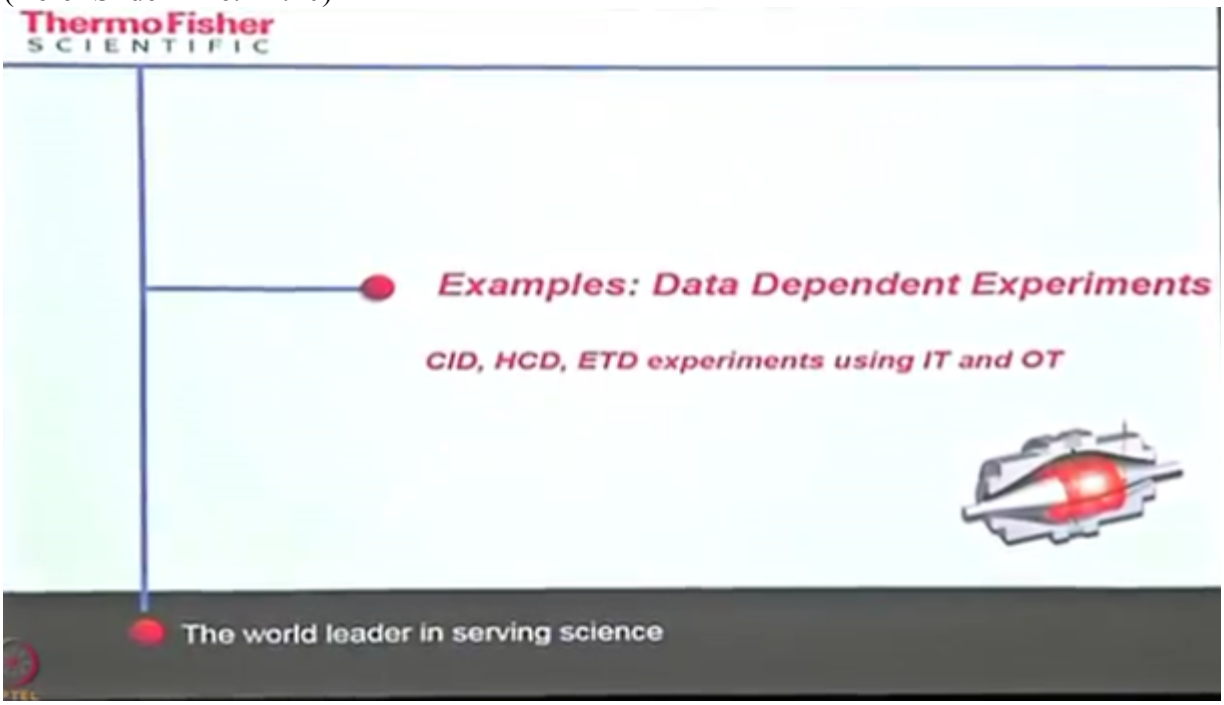

so we have some animation where we can see how the instrument do the MS and MS-MS,
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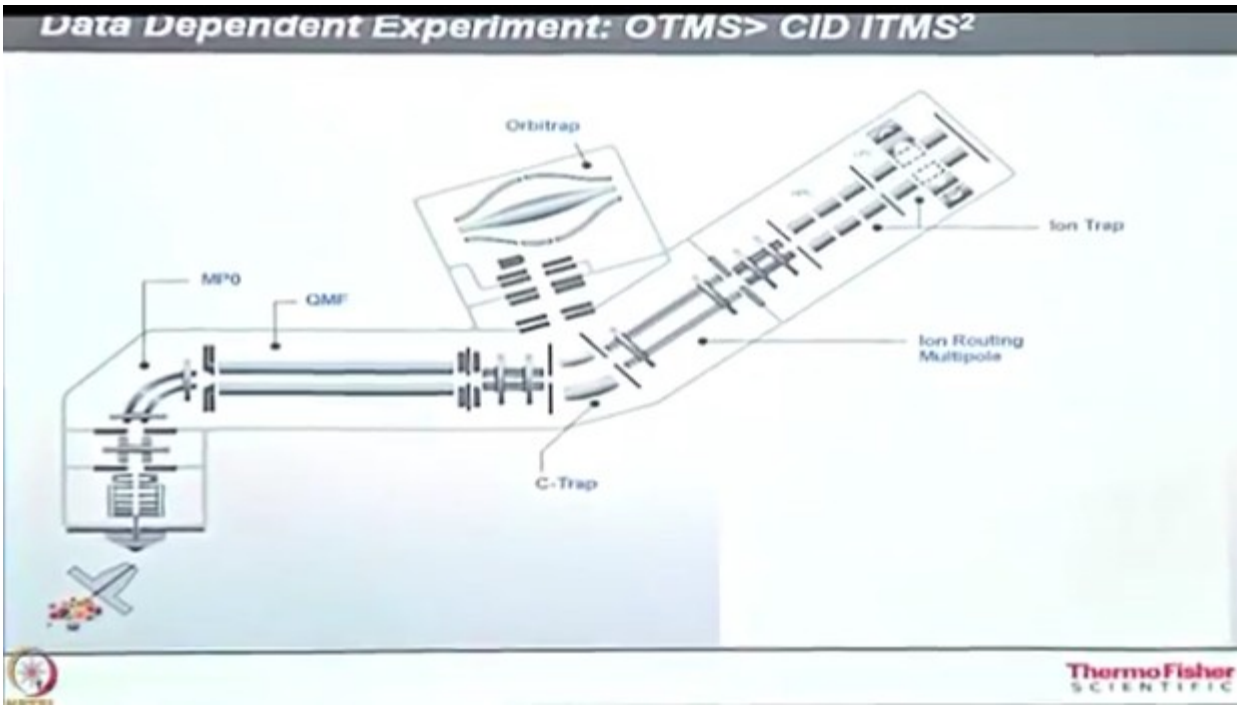

so first experiment tells a MS in OT orbitrap, and MS-MS doing the through the CID it will do in the ion trap, so CID will be happened here, so what happen first? The ions will be detected here MS, from that MS scan one molecule, one at a time it will be filtered here and fragmented here, okay, so let's say, so the first full MS will go, 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're doing the parallel, so the first parent ion has been selected here, okay and it will be stop there first, then it will go for the fragmentation in the HPC cell,

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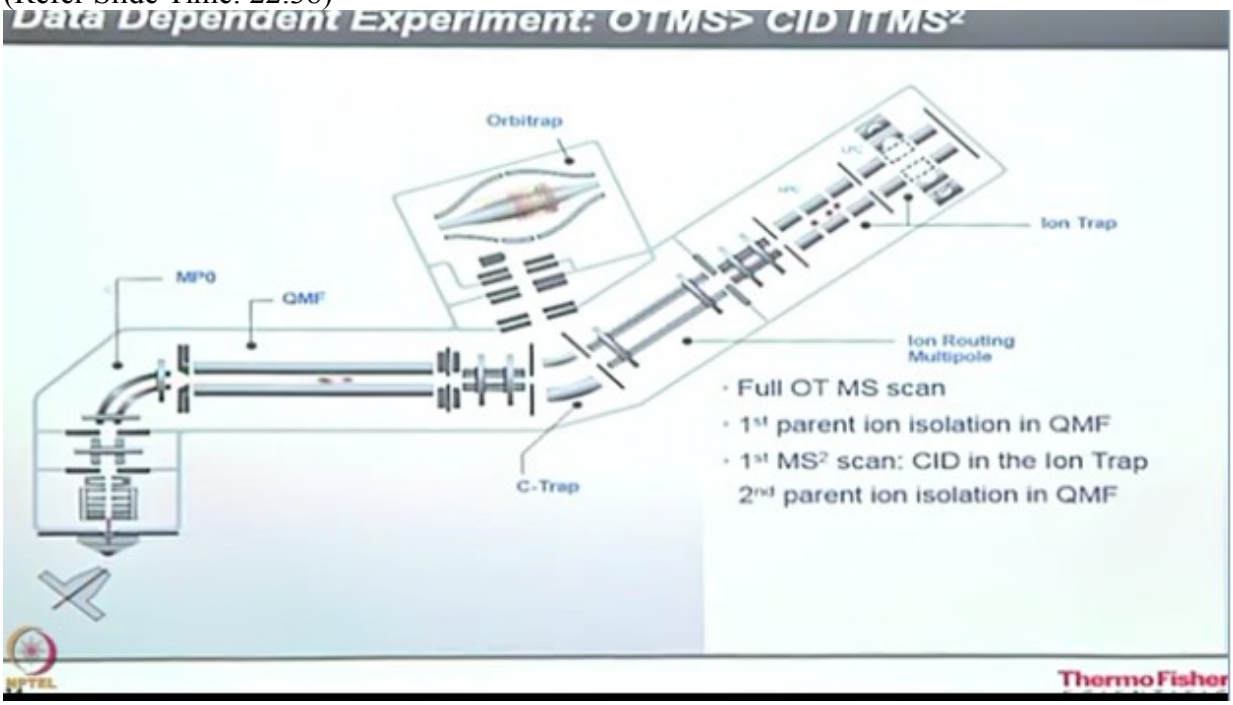

when it is going fragmenting the second ion is waiting here, so you are not losing any molecule as well as the time, okay, so it's a parallel reaction.
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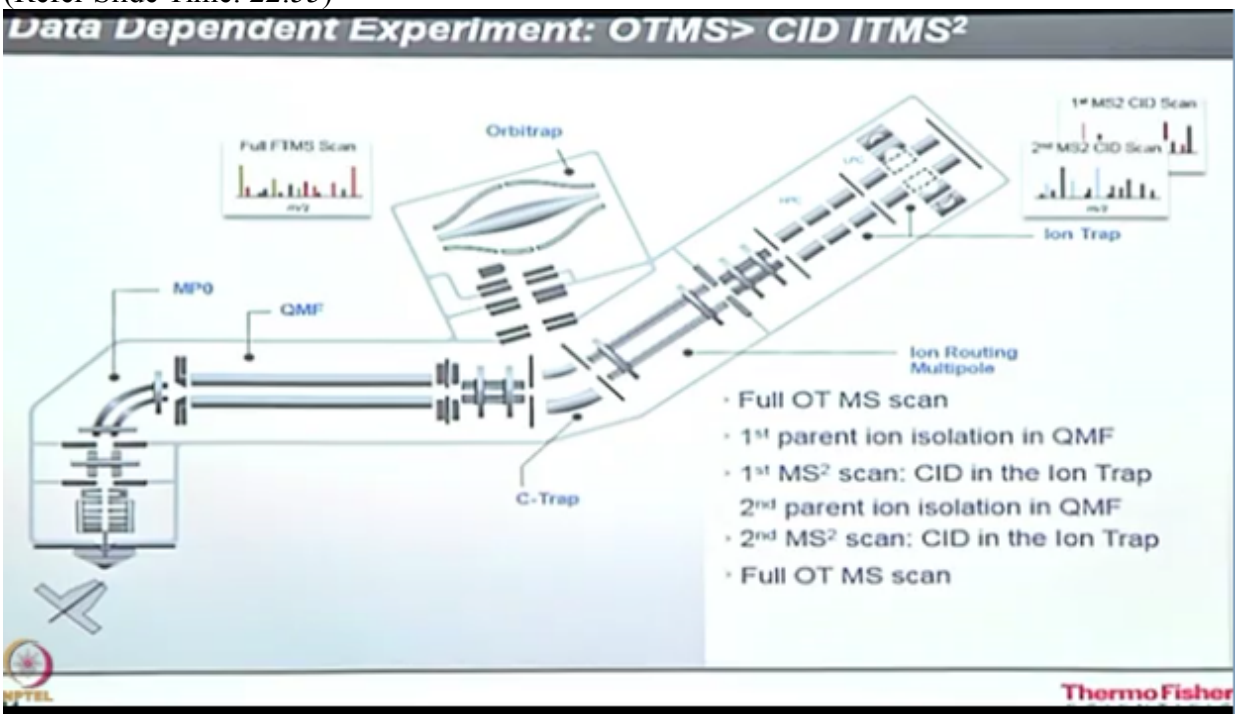

So when all the MS2 scan is over then only you will get the MS scan, so at the end of the full scan you will get MS scan as well as MS-MS scan, so you are not losing any time as well as any molecules, samples.

So like a second experiment is ACD, and the first experiment we are doing in the CID, what happened? That in that case the fragmentation happens here, now in case of ACD the fragmentation will happen here, okay, 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 that is same time the second mass will come up, so you are not the same way, you are doing the parallel reactions.
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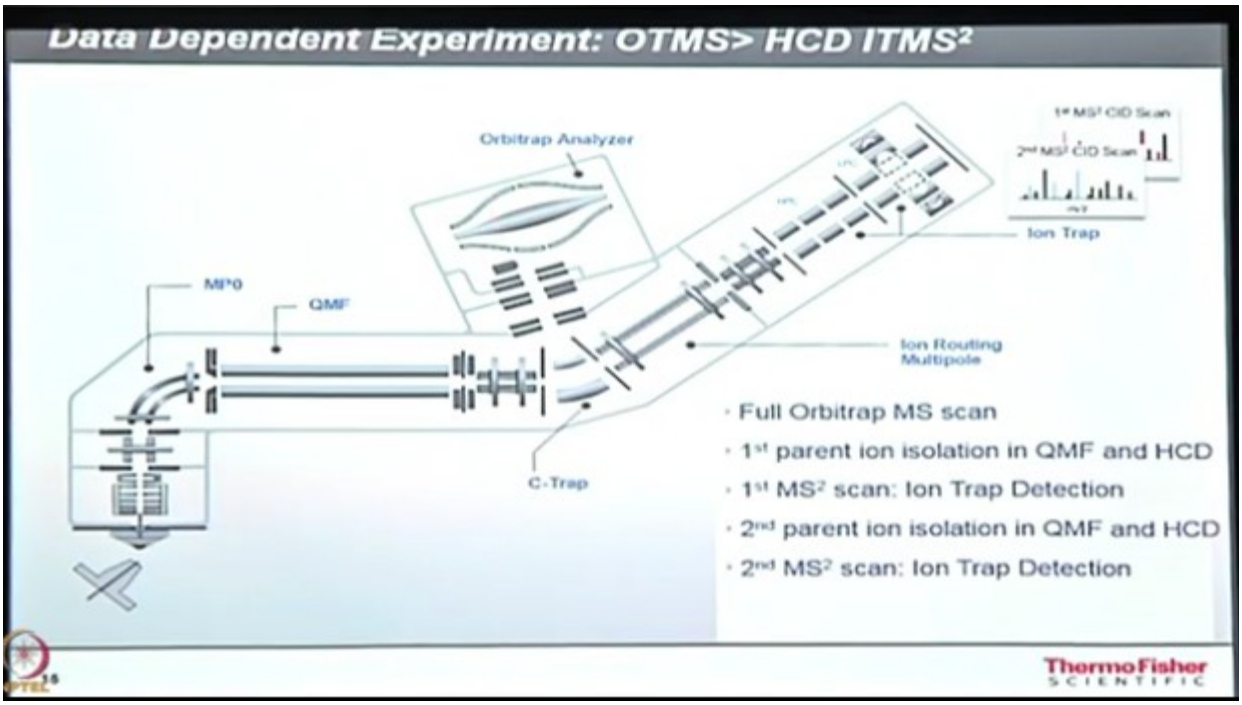

So after all the MS2 scan you will get the MS-MS scan.

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 is been isolated in the quadrupole and first stop here, then it will injected to the ion trap.

Now I told that in ETD you required a region which gives you the anion, so that region will comes from here, so it will inject a region, it's region cause the flow and thing, so that is the cursor eugenic, so it will give you some anion, it will make the anions and get fragmented that molecules, so once the fragmentation is happened so it will give you the MS2 data as well as MS-MS data,

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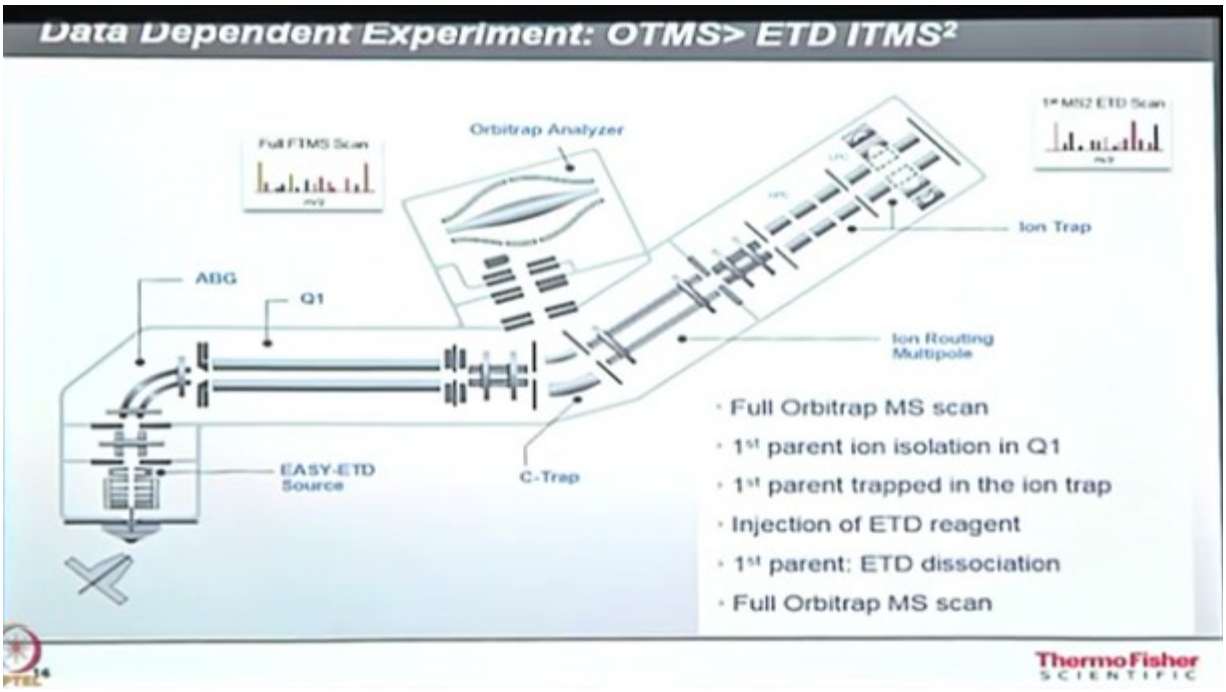

so ETD is a very specialized case only when you are going for the PTM analysis, so post translational modification like phosphorylation or acetylation or glycosylation, in those cases you have to use this ETD reaction.

So in the instrument the better part is that you already, there are already the templates for all type of analysis,

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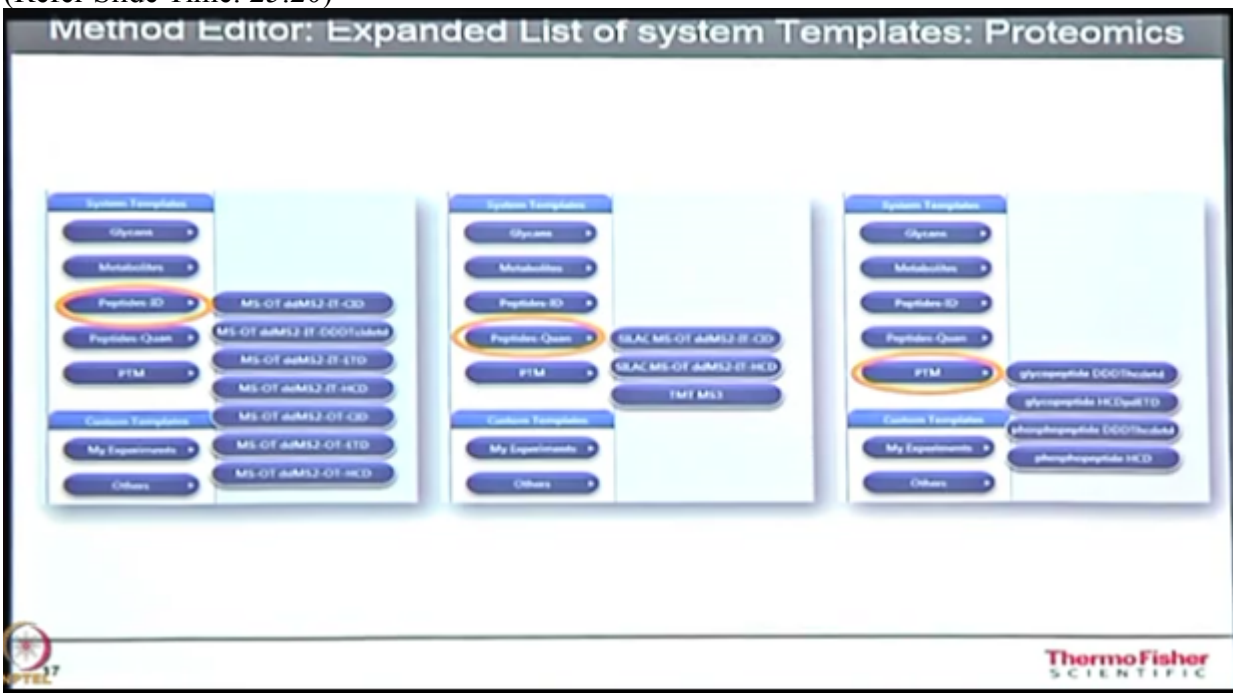

so you don't have to start from the scratch, so you just click on the which method you want to use either its identification or it's a quantification, or is a PTM one, so you have to select that method and run the method, okay.

So there are already templates are made, now before going to the next step,
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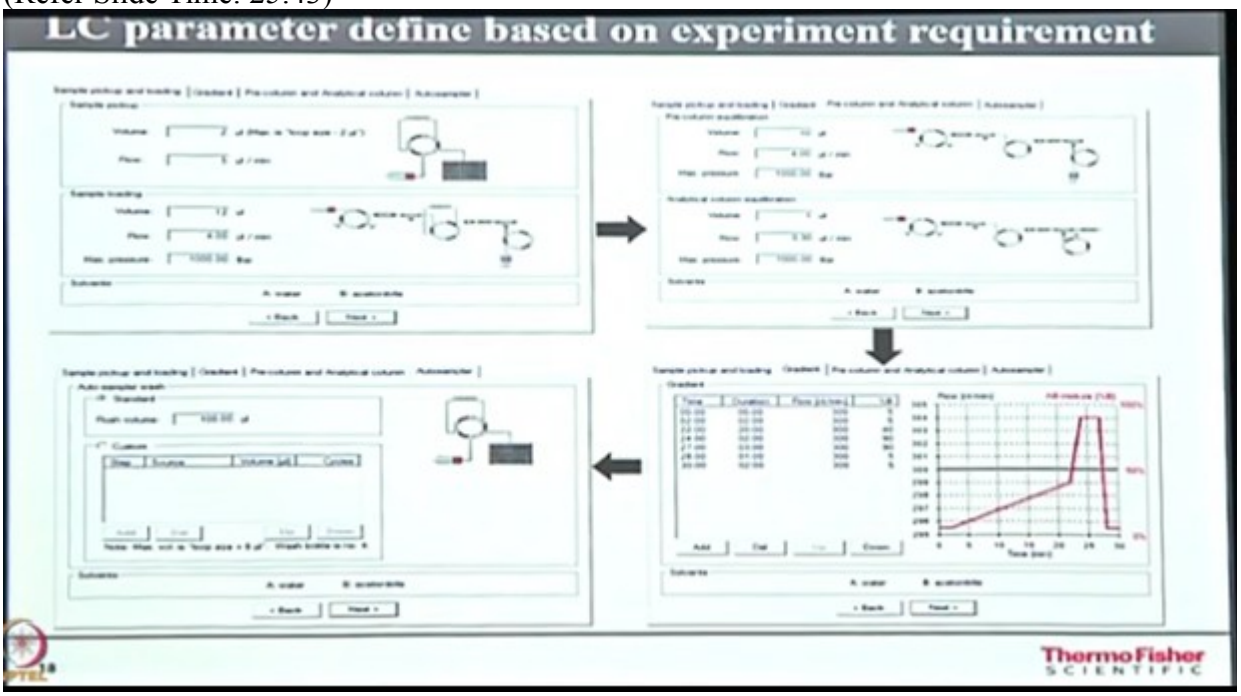

so LC is another big part because in the front end we are putting LC, why we required a LC for this kind of analysis? Because the peptide length curve maybe from 300 to 2000 M/Z, so we did the mass, we cannot separate all the M/Z's, we can separate the nearby masses by the resolution, but we cannot separate that in the range, so we need to separate those peptide, so that we put a LC in the front line where either with the basis of hydrophobicity or any other parameters we separate those peptides and elute 1/1 and inject it to the mass spec, okay, so these are the different parameters we are using for setting of the LC run, here the important part is the gradient, so you have to choose a proper gradient, so that gradient should be on the complexity of the sample, so now here I'm showing you 30 minutes gradient, so its work for the simple one protein like BSA or single protein, but when your complexity, when you're working with the wholesale lysate that time you have to increase the gradient for 2 hours, 3 hours, 4 hours, okay, so like that you have to work on the gradient part, not in the MS part, okay.

So now come for the some data analysis part,
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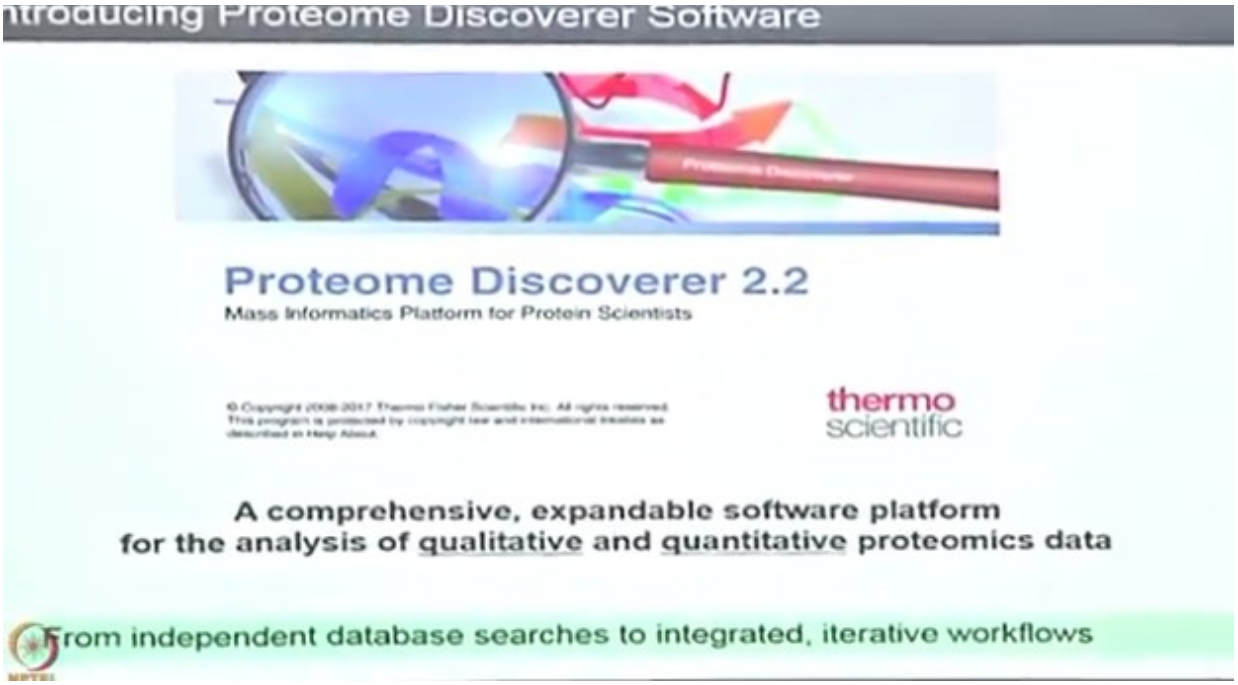

for data analysis we have protein discoverer 2.2 that is a comprehensive and estimable software for qualitative and quantitative, so you can identify as well as quantify the proteins. Here the quantification is a relative quantification not a absolute quantification.

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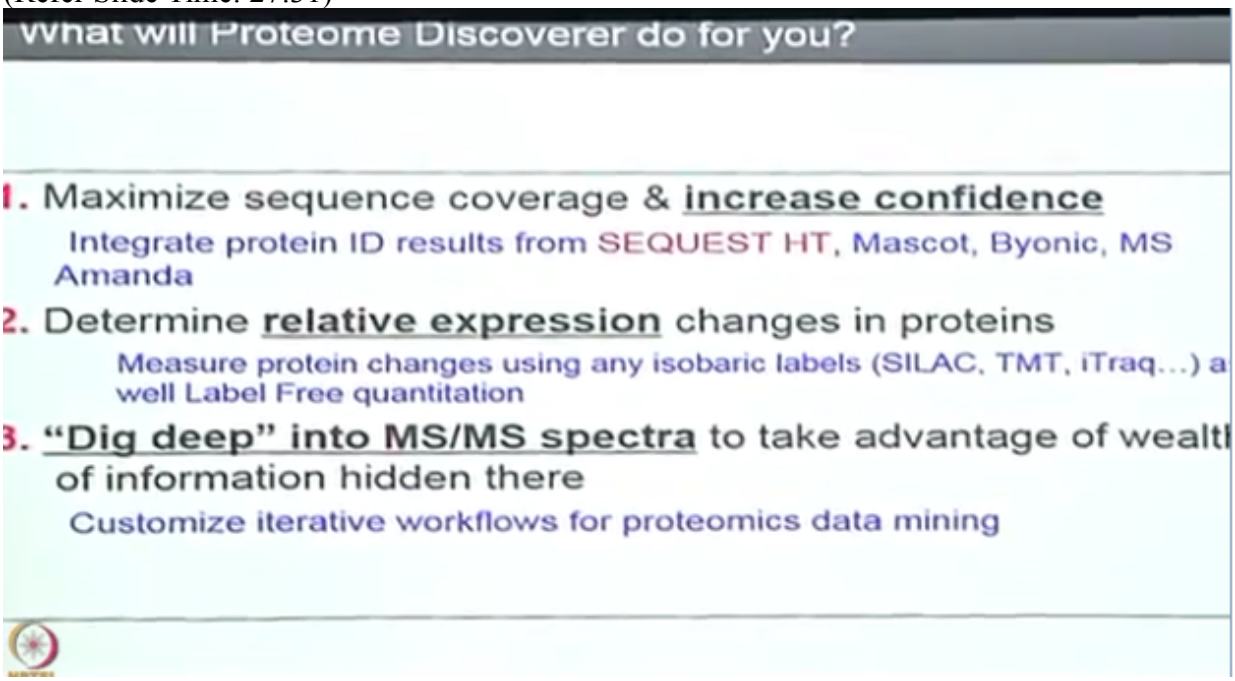

Okay so, what it has basically it will have some different search engines like Sequest, Mascot, or Byonic, Amanda, out of this Sequest is a free available so it will comes with the integral, with the protein discoverer, other search engines Mascot or Byonic those has to be purchased it has licensed, so actually what this search engine works, I means how it 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 level first, MS1 level when it will match with the MS level then it will come for the MS2 level.

If it is match in the MS2 level and get those B and Y ions to get the sequence then it will tells you okay there peptide is present, then it will give you okay, this protein peptide is present means this protein also be present there, okay, so it will measure determine reading quantification so you can do ready quantification either it's a level free or liberal reaction like TMT, silac or,

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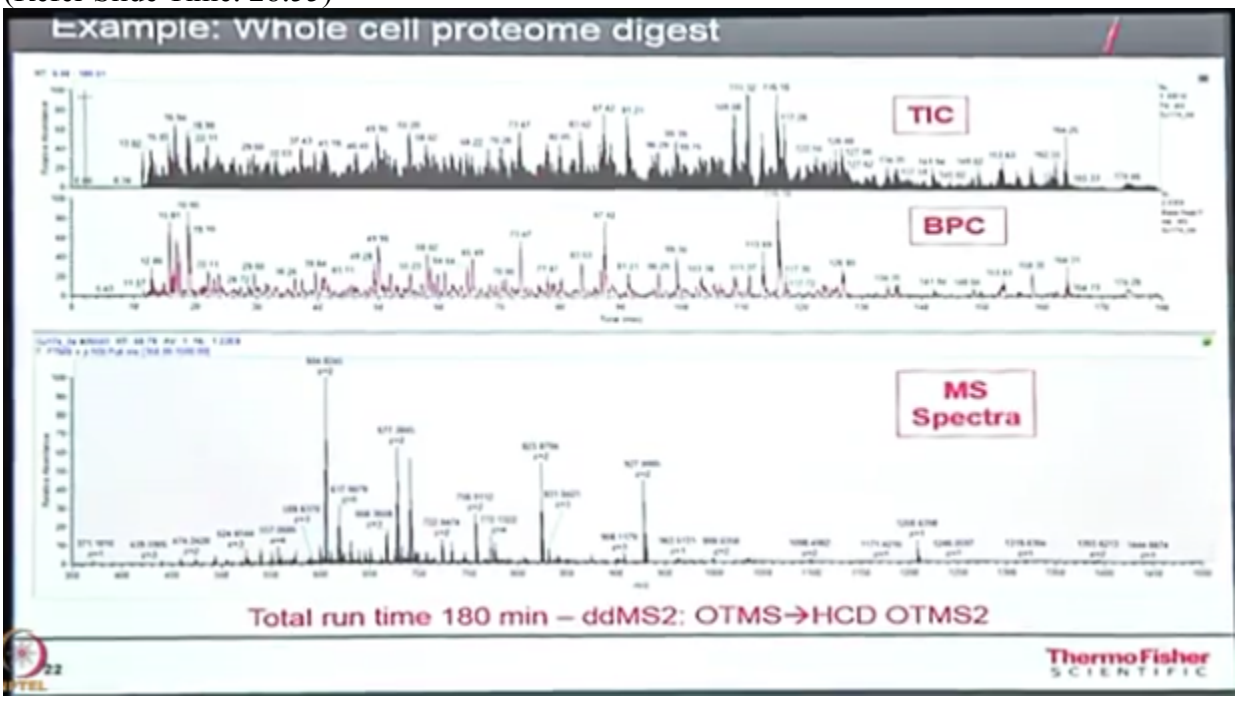

so here is an window where you will see the first of is a wholesale lysate run for 120 minutes, we run a DDMS-2 method, we do OT at MS and ACD also in orbitrap, okay, MS2 also detected in orbitrap, we are not using ion trap here, it's a 120 minutes, 180 minutes run, it's a wholesale lysate.

So first window it tells that TIC, Total Ion Chromatogram, so like HPLC you are getting UV chromatogram, here we'll get on the basis of total ions it will make a chromatogram, so that's why it's called the total ion chromatogram.

Second window is called the BPC, Base Peak Chromatogram on the basis of highest intense speed for the each intension time, okay, 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/Z, Y axis is let be abundance, and it will see different charge states are there +2. +3, +4 because of ESI you will get the different charge states, okay.

Now when we search this data in PD with this two workflow, so in PD you have 2 workflows, one is the processing workflow where we search the data against the database,

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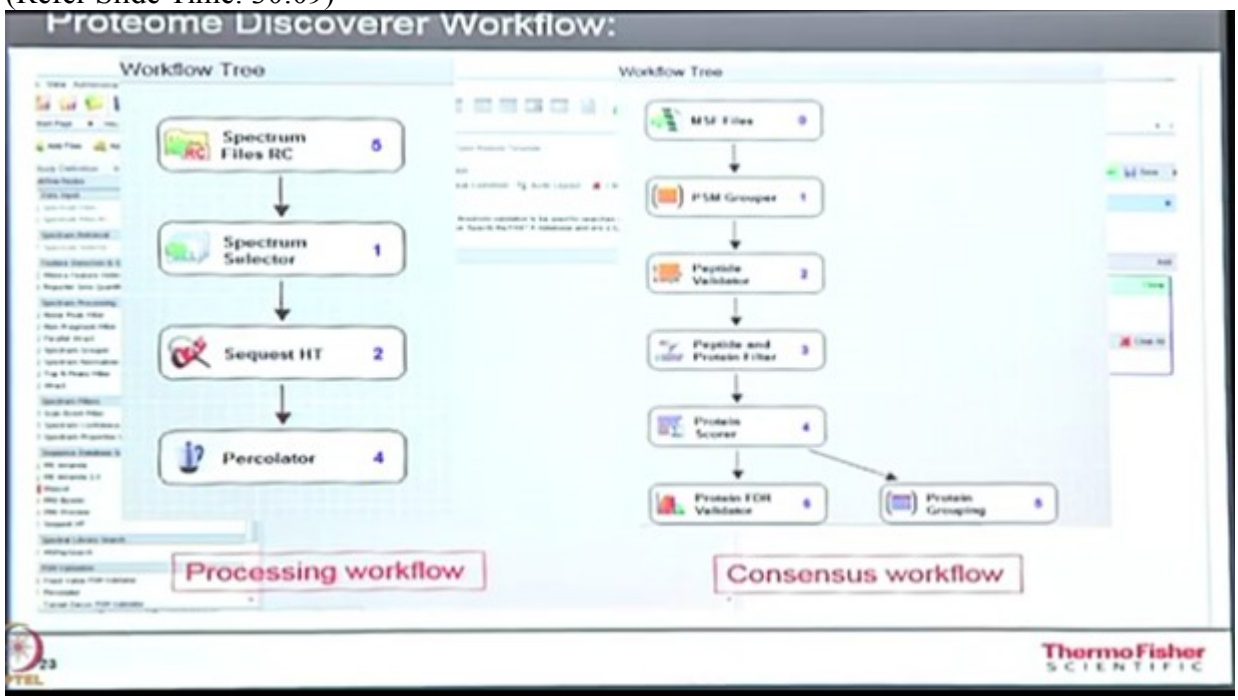

and we are using the sequenced and after getting the peptides, those peptides maybe the false positive or false negative, so you have to validate those peptide, so those has been done in the consensus of step where we do the peptide validation as well as protein validations, and we'll ultimately get the data, so here the string, we are doing the validation at 1% FDA, so what is that mean? If you run the sample 99 times the protein will identify it, maybe in 1 time it not be identified, so in such stringency we are detecting those, validating those peptide and we are telling okay this peptide is present, so then the report is very good for published.

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 that's 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 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, okay, so here we have identified almost 2,255 protein groups, so it comes like from that dataset it have extracted these many mass spectra which has, when we match against the database we are getting this many 23,000 PSMs and out of this PSMs we are getting this many 2255 protein groups, so right now I'm talking about the identification just how to do, so fusion is not for identification, it can do many works,

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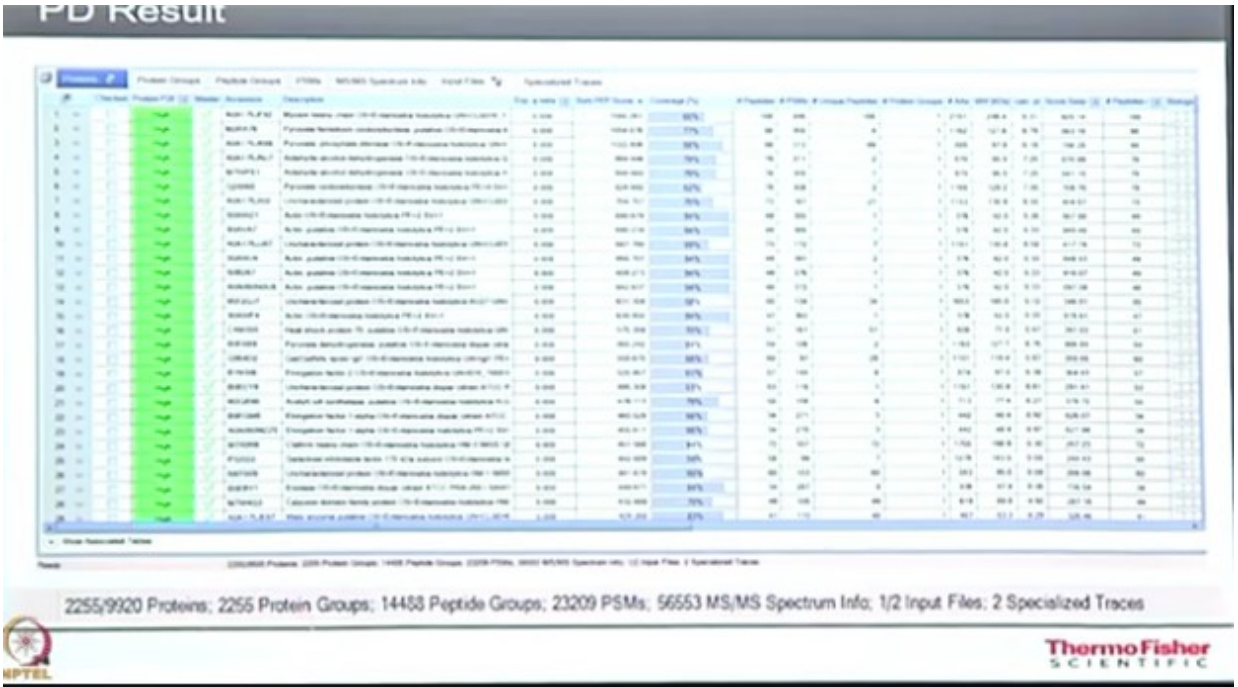

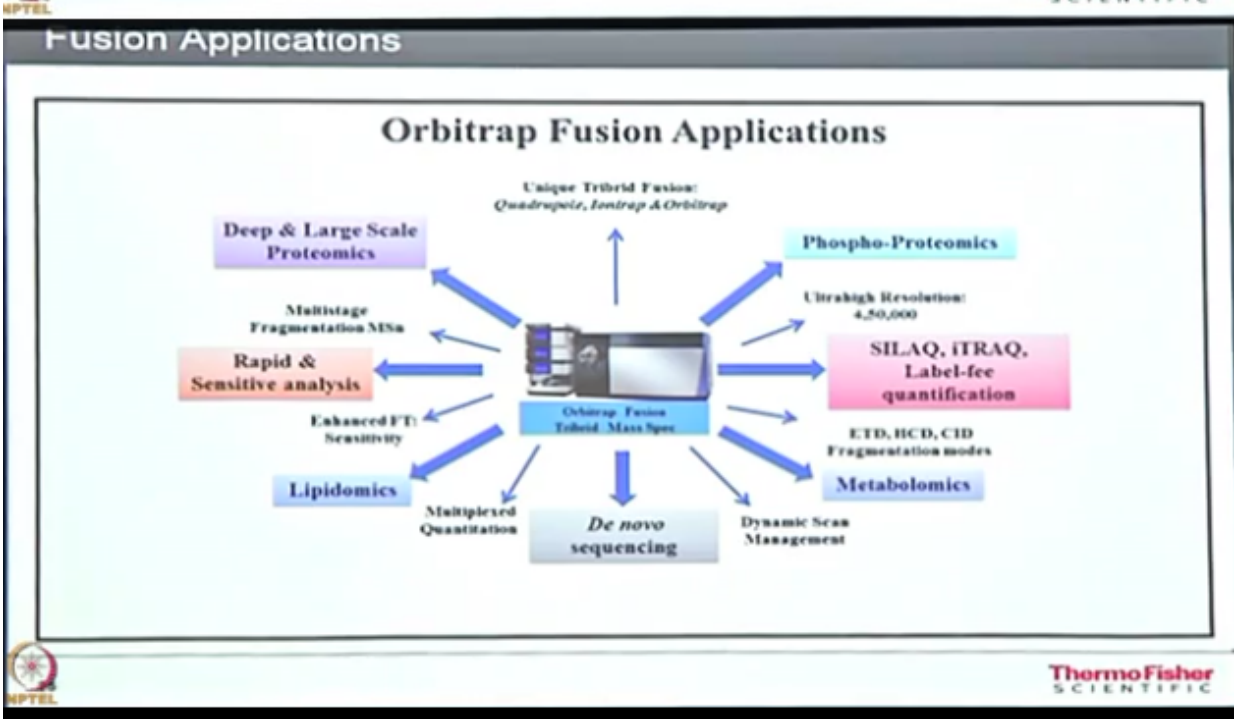

so right now we are doing the deep large analysis and the level free or level reaction to relative quantification, so it can do the phosphoproteomics, it can do the lipidomics, it can do the metabolomics, so you can use as instrument for different kind of analysis.

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Lastly this is a wave side planetorbitrap.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, notes the publish data or methods, so you can registered here and you will get the free updates, okay.

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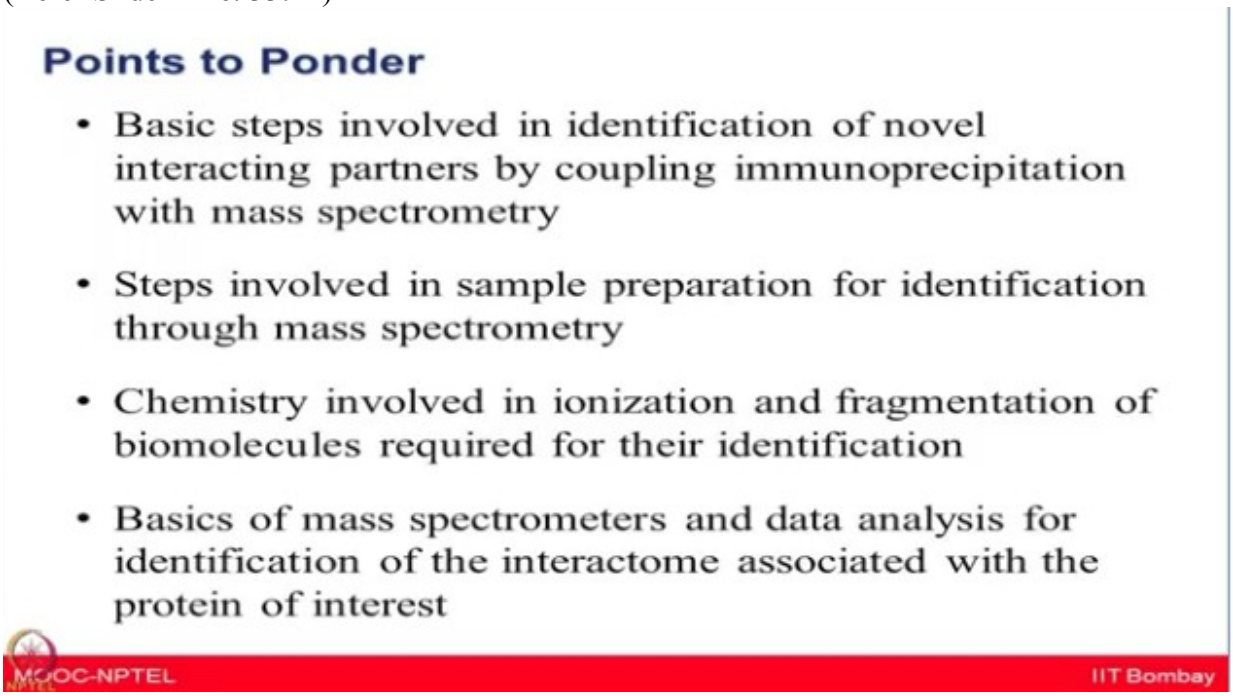

Dr. Sanjeeva Srivastava:-Therefore the last two lectures you are now familiar that if you have a protein of interest which you want to dig deeper you want to further characterize that protein and want to understand the possible role of that protein, and easy way of moving forward could be to do immunoprecipitation experiment followed by protein identification using mass spectrometry.

In today's lecture you have seen how to take the complex which you have illiterate 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 axis software mascot to do the data analysis where you can easily identify the proteins of interest, there are many good softwares available even commercially even which we have shown today is proteome discoverer.

In general I hope these two lectures have given you some basics and understanding about how to study the bio-molecular interactions using immunoprecipitation followed by mass spectrometry base experiment. Thank you.
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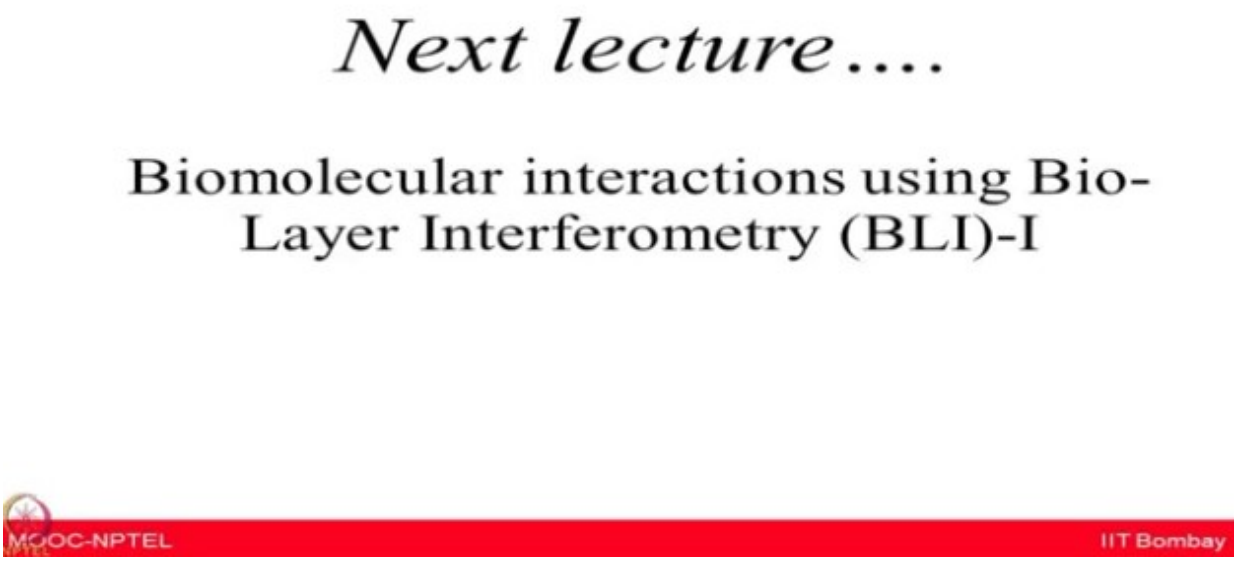

Prof. Sridhar Iyer

NPTEL Principal Investigator
&
Head CDEEP, IIT Bombay

Tushar R. Deshpande
Sr. Project Technical Assistant

Amin B. Shaikh
Sr. Project Technical Assistant

Vijay A. Kedare
Project Technical Assistant

Ravi. D Paswan
Project Attendant

Teaching Assistants

Apoorva Venkatesh **Nikita Gahoi**

Shalini Aggarwal

Bharati Sakpal
Project Manager

Bharati Sarang
Project Research Associate

Nisha Thakur
Sr. Project Technical Assistant

Vinayak Raut
Project Assistant