NATIONAL PROGRAMME ON TECHNOLOGY ENHANCED LEARNING (NPTEL)

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Applications of Interactomics using Genomics and Proteomics technologies

Course Introduction by
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MOOC-NPTEL

Applications of Interactomics using Genomics and Proteomics Technologies

Lecture-24
Mass Spectrometry coupled Interactomics-I

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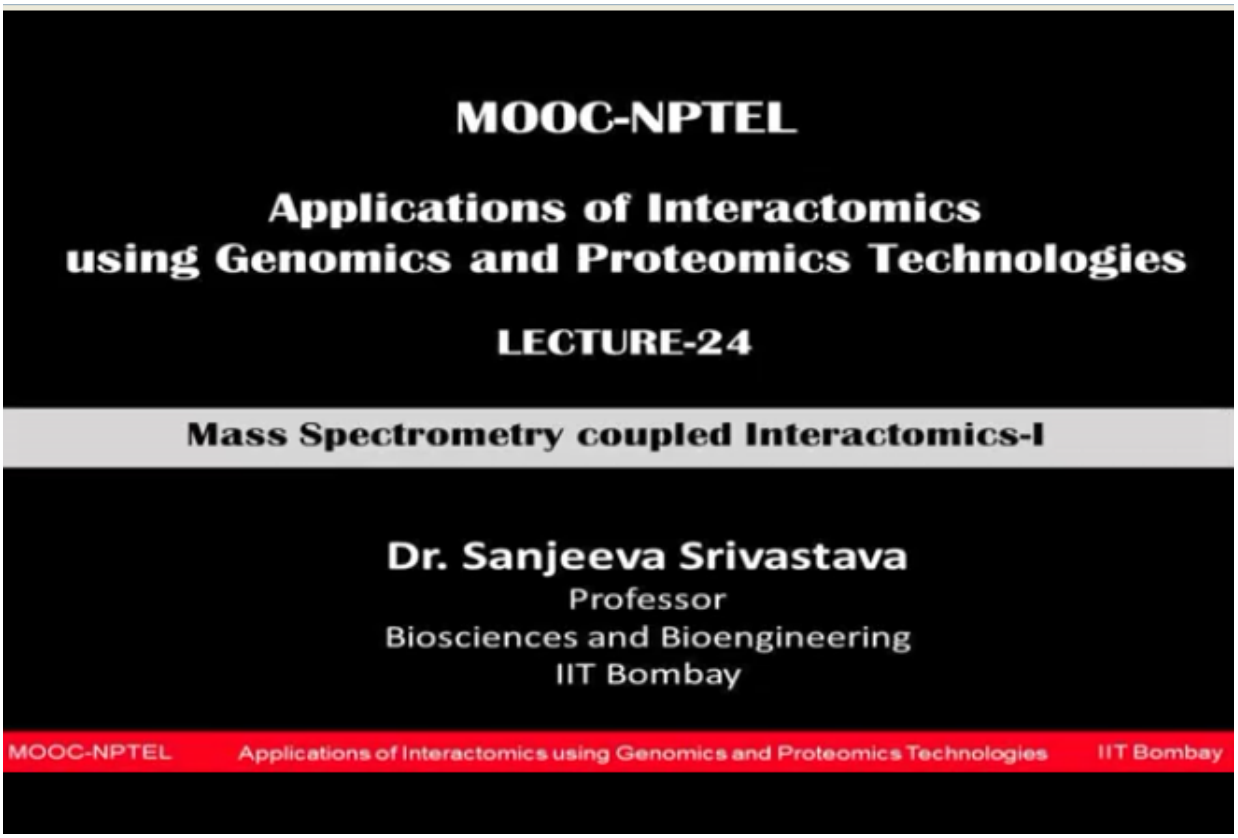

Dr. Sanjeeva Srivastava:- Welcome to MOOC course on Applications of Interactomics using Genomics and Proteomics Technologies. After doing a discovery based proteomic experiment let us assume you have identified a protein of interest for which you do not know what is the possible role of that protein, you have to characterize that protein in much more detail.

So possible way of doing that experiment could be you can do amino precipitation and identify that this protein of interest interacts with other proteins. So protein interaction networks could at least tell you that possible role that what their protein may have. In this slide amino precipitation followed by mass spectrometry have become a very powerful technique to do the protein interaction analysis.

In today's lecture we will have a demonstration by a research is called who will show you how to perform amino precipitation mass spectrometry based experiment.

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Shuvolina Mukherjee: Our bead support and we will conjugate our antibodies with it, and those antibodies are definitely depends on your experiment but they will be specific to a particular protein, right, and that protein you can trap on your antibody and then that protein can act as a bait and on it many of its interacting partners can get bound to, right, so what we will do is we will separate this whole complex, so we will be doing a complexome study or interactome study using mass spectrometry, though amino precipitation predominantly also the co-amino precipitation is used in even in western blots, where you can use one protein and see whether it is interacting with other the protein, you can use in cross identity body and check it, so this also is basically how IP is conventionally used,

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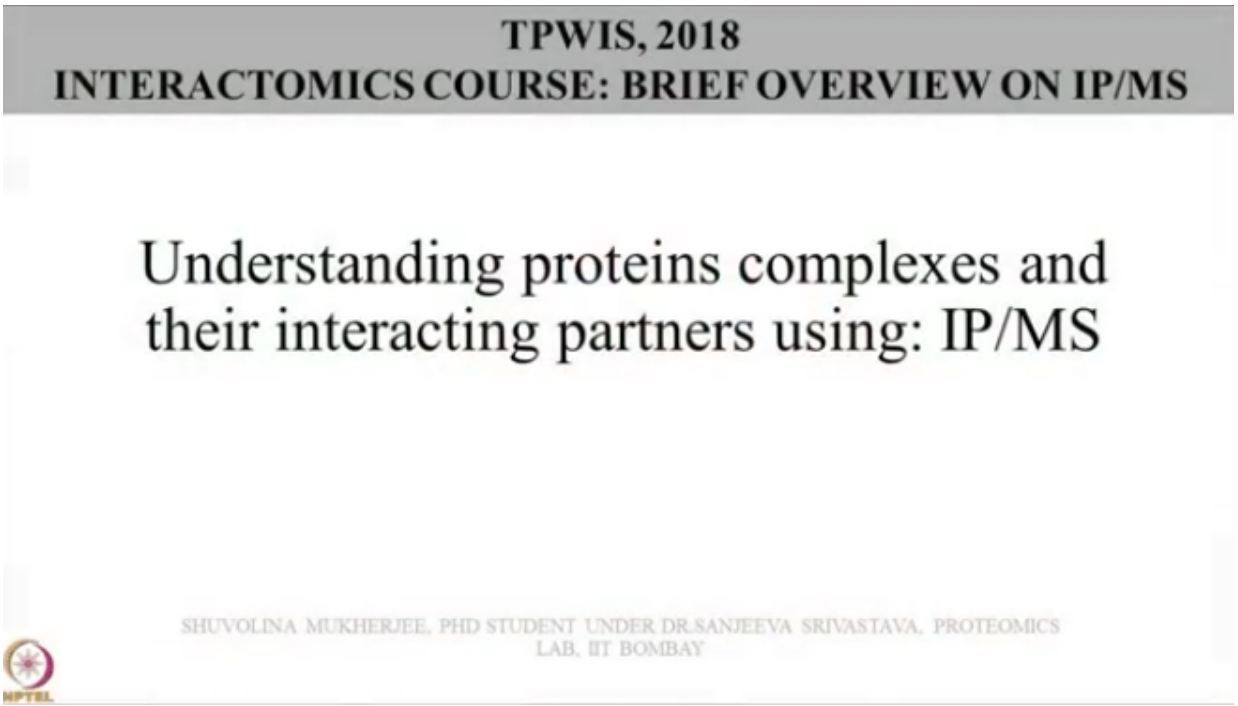

but here we will be doing it how we will study it using like in western blot you can identify maybe one interacting partner, but using mass spectrometry you can identify a plethora of proteins from your complex.

So thereby I'll be talking about understanding protein complexes and their interacting partners using IP-MS,
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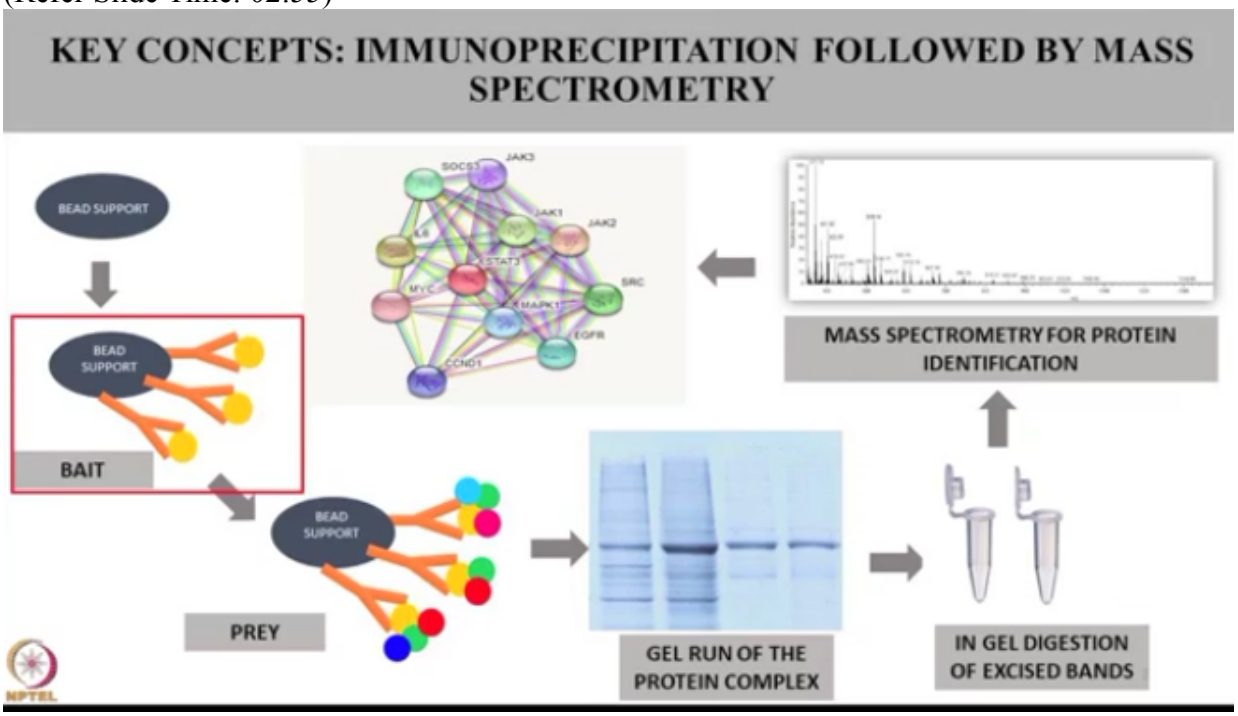

so basically this is the workflow, so we'll have a bead support, majorly people use sepharose beads, people use agarose beads also with protein A or protein G immobilize, because protein A or G will bind to the FC region of the antibody, so you will conjugate your antibody and then you will incubate this, so in the protocol you will incubate your bead antibody conjugate for overnight, so that from your lysate you can trap the protein of interest, so we will call this protein of interest which is bound to your antibody as the bait, and then this bait will actually help you trap all the proteins, the interacting partners of this protein, so we will take a case study today that is STAT3, so STAT3 as you know is part of the jak STAT pathway and what is if you were interested to see that how, which proteins are there potential interactors of STAT3, so we will take an anti-STAT antibody and we will conjugate it with the bead and then STAT3 will, let's assume that this yellow dot is STAT 3, so STAT 3 will bind to the antibody and then all the interacting partners of STAT 3 will, we are expecting that it will come and bind to STAT3.

And then what we can do? So this is only one of the ways that you can do IP, there are many ways to do it, so people also directly use the antibody on the lysate and then they trap the complex, so that can also be done, so there are modifications of it, but in case we will have the whole complexome like this and then we can run in some simple SGS space, we can exercise the bands and do an in gel digestion and give it to mass spectrometry analysis, so what will happen? You can exactly know which molecular regions you are looking at, and you can go back to the database or you can see at your identified protein, so in the mass spec and you can know how relevant are your hits, whether at all they're interacting partners of STAT3, but at least rather than doing a global proteomics like many of you're familiar with, wherein you're identifying everything at least now you have just the way you can enrich your PTM, you can enrich your sample to look at a particular complex, right, so this is the overall methodology.

There is an alternate way to do it and you can use this complex and use a buffered like something that has high PH like a glycine buffered what it will do? This interaction will break, the bead and the antibody interaction will break off, and then you can do an insulation digestion and that you can subject to mass spectrometry analysis, so overall this is the simple workflow of IP-MS.

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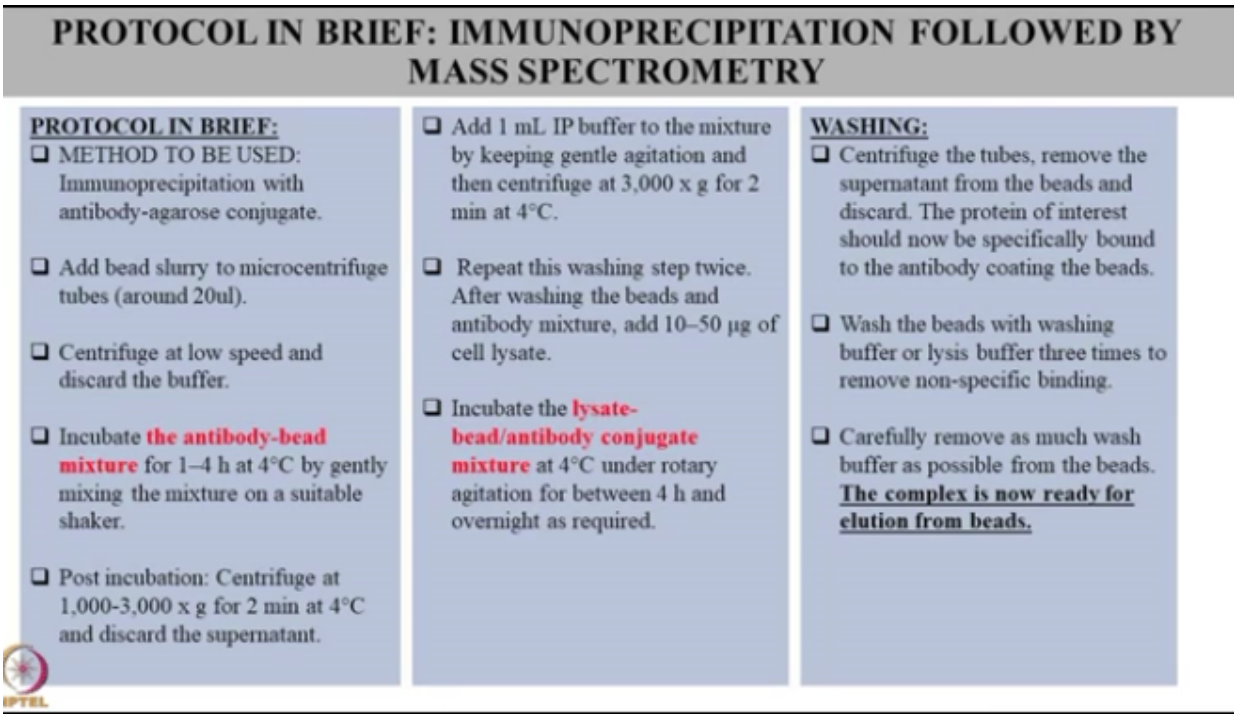

So protocol in brief, what we do is? First of all we will use the bead slurry and we will decant of the buffer, so then the antibody mixture is incubated at 4 degrees for 1 to 4 hours, so this is to ensure that the antibodies properly bound to the bead, because that's very important, that is what will pull, so your sort of doing a pull down SA, we can also say that, right.

And then you will centrifuge to get rid of any like things that are not bound, so unbound things will be, we will get rid of it and then you will wash the mixture using IP wash buffer, so basically it's trace NACL and all this buffers, that will keep your protein intact.

And then but at any point you must remember that for this to be successful you should not disturb the interaction, so all your steps you need to be very gentle, you need not to, you should not use something like very harsh chemical like urea or anything that can denies your protein or even disruptive interaction, so that's something that you should keep in mind, and then this lysate bead antibody conjugate this mixture you can keep for, with a rotary gentle agitation at 4 degrees for overnight binding, so now again it depends on your experiment you can plan it according, if you know like if you are already verified the response using a western blot you will know how much of it, of the protein of interest is in your sample, so accordingly you can design, if you think that this is too abundant maybe you might not go for an overnight incubation, so these things can be optimized.

So further the next day so basically IP takes like a consecutive 3 days, so you can use like a couple of days to get this done, so then in the next day you will again wash it, so again you are washing so as to not have those things that are just weekly bound or maybe as a carry over, you just want to be sure that you're identifying only the complex of your interest, and then you will remove it, you will do an illusion, so again this step is optional you can directly load the whole thing in the gel, so whatever it is it will come up and also you can use the whole complex and do any insulation digestion after the illusion step.

So applications of IP-MS, so IP-MS is very widely you know, is being used now because conventional IP methods have also led to understanding of interactions, protein-protein interactions, so integrating with mass spectrometry just takes it to another level, because now you can do functional assays using mass spec as well, and you were people, there are many people who do targeted validation of proteins, peptides, using targeted proteomics for them as well their traditional biologist often raises a question that have you monitored the, you know have you done a western blot, have you done an Elisa, so for them to if you have someone who wants to address both these crowds you can definitely use this technique, so one of the preliminary studies that have come up is this by Mathias Uhlen group,

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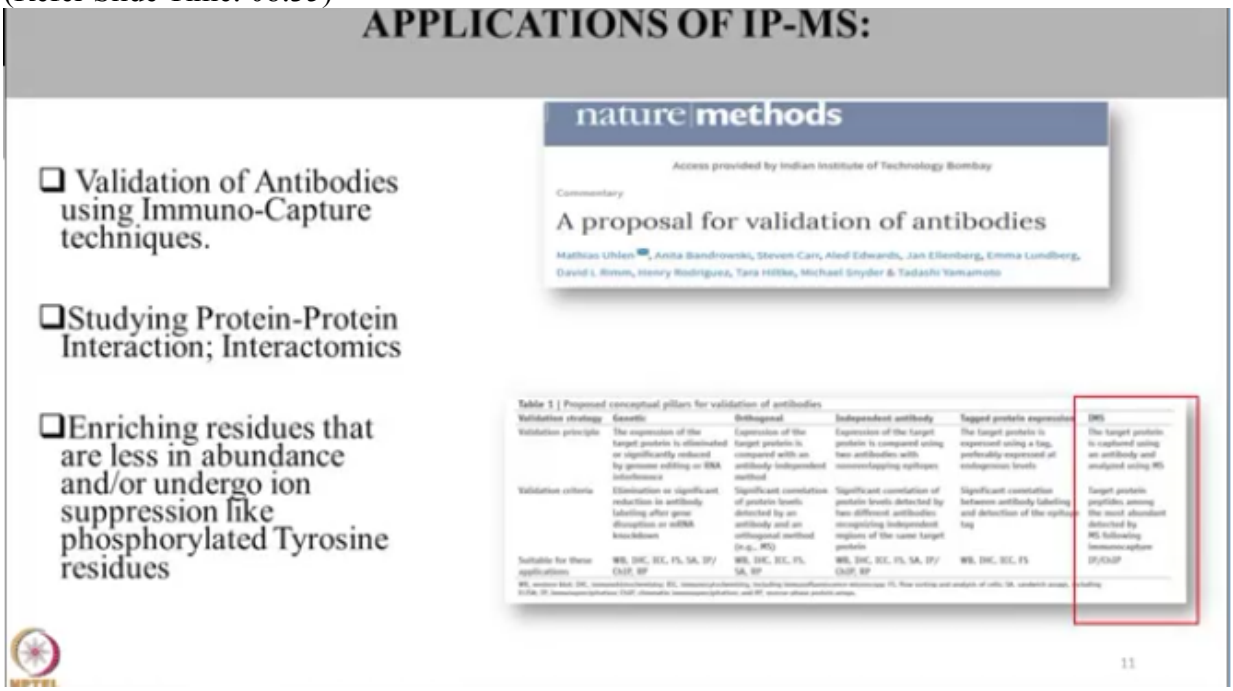

so they have given a proposal by which you can validate antibodies, so hereby now onwards I will be talking about 3 major applications of IP-MS, so there are much many, but in this scope of lecture, so application, one of the applications I'll be talking about is validation of antibodies using immunocapture technique, so immunocapture is again IP-MS, because you were capturing the antibody of your interest followed by a mass spectrometry.

Secondly we will be talking briefly about an identifying protein-protein interaction, that is interactomics, this is what the, this is the demo session that you will be going for, and the third thing is enriching residues that are less in abundance and undergo ion suppression like phosphorylation or even ubiquitinylation and many other PTM's, so because of that it is often very difficult to, you know even if the post translation modification is very complex like a ubiquitinylation, it's not easy to identify, using a global proteomics approach, so we can also use IP-MS for enriching those residues like we can enrich the residues in, we can ubiquitinylate the proteins and see which are the residues getting ubiquitinylated, we can use the tyrosine specific antibody which can bind to the tyrosine residues, tyrosine phosphorylated residues and then we can do mass spectrometry, so thereby there are many things that you can do with this technique.

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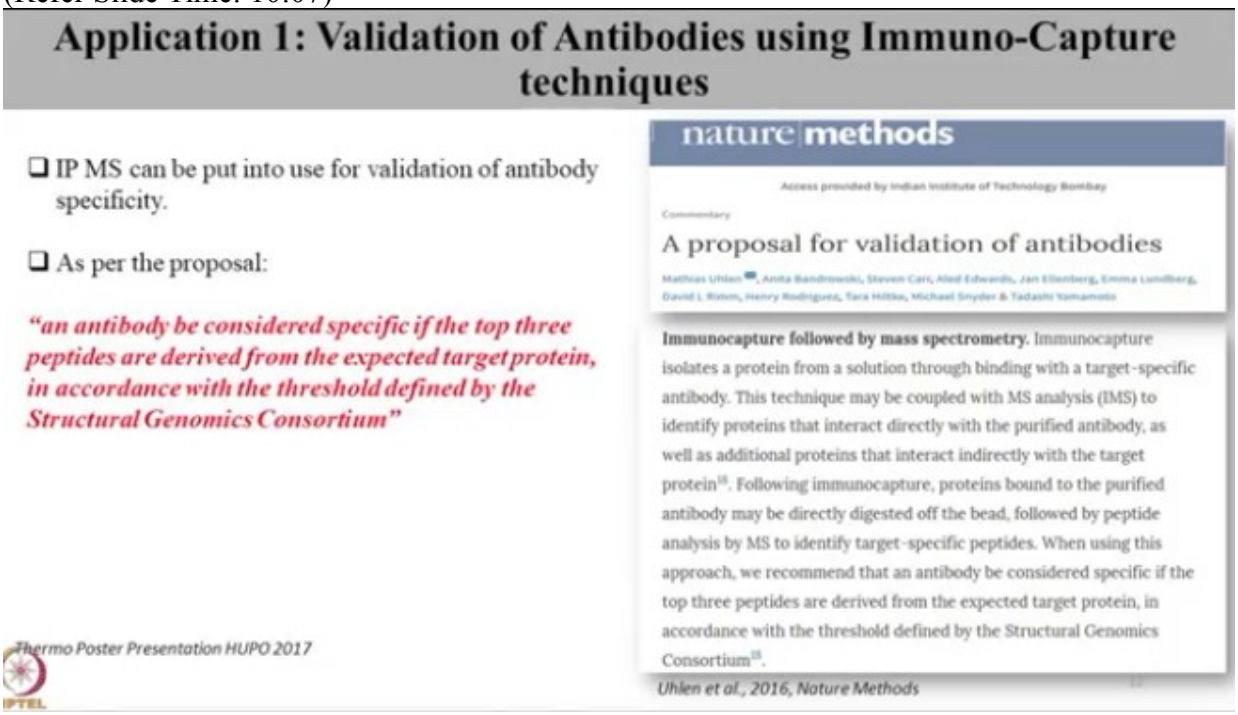

Coming to the first applications, so in this proposal for validation of antibodies where Mathias Uhlen group what they have proposed is that immunocapture followed by mass spectrometry is a very nice way to know whether your antibodies specific or not, so what they are aiming for is, if your antibodies very specific to your target, so if you bind it to a bead conjugate and then you do a immunocapture it should bind only to the protein of your interest, if you do the washing, if you follow the whole IP protocol what should you get is only your protein of interest, and maybe a few interacting partners, right, but so they have a device this statement that is an antibody can be consider specific if the top 3 peptides are derived from the inspected target protein, so if you are looking at an antibody, so you have 3 antibodies, say from vendor ace abcam CST, sigma, so you want to know which one works the best for your experiment, so before probably you are going to do something very, you might use something very expensive or you have some sample which is very precious, so before you proceed you might want to check how's specific in your antibodies, so you can do in immunocapture, and you can just see whether the top 3 peptides that are coming up after mass spectrometry are of the protein of your interest, in this case STAT 3, so if that is coming you can be confident that whichever antibodies most specific will give higher number of peptides or the top hits would be from the protein of interest, so in this way they have proposed that for validating an antibody they have proposed the panel of many studies and one of the studies they have proposed is immunocapture.

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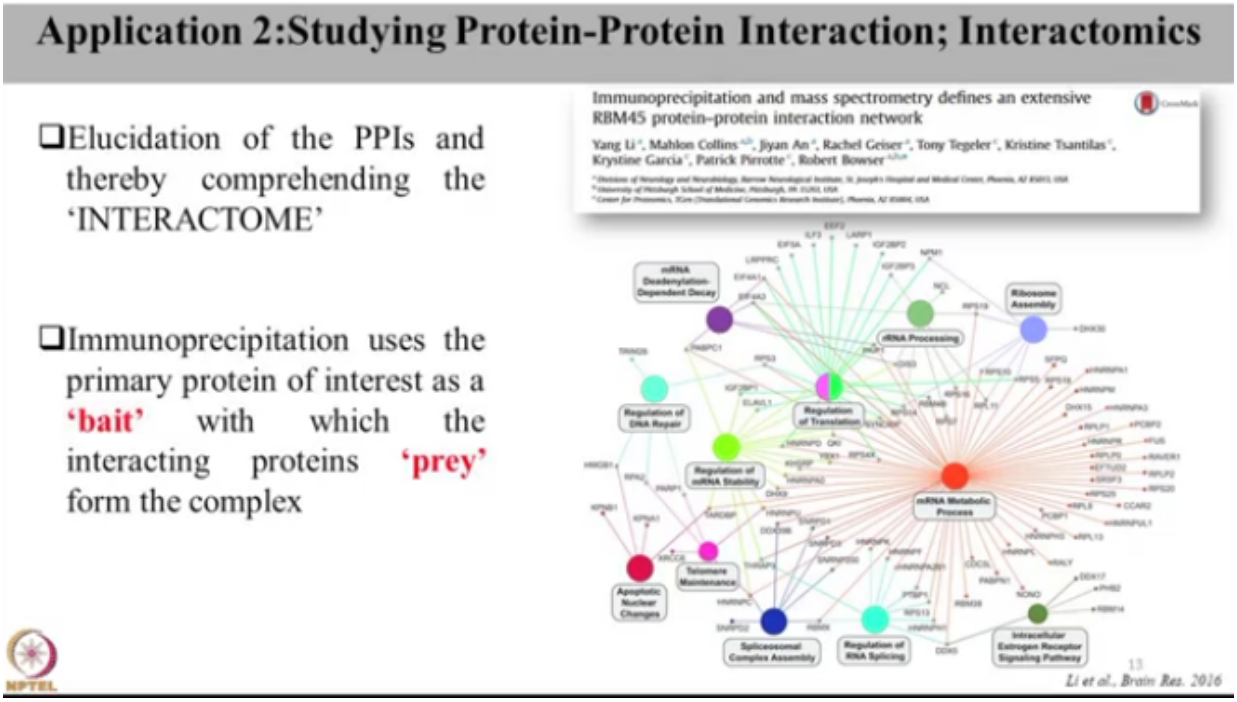

So the second application is studying protein-protein interaction, or the typical interactomics, so as you can see for any physiological processes that you are studying bait in class, humans, cancer, or infectious disease, so you often find out your list of targets does not involve one protein, you always have something else along with it, maybe a transcriptional factor, maybe a protein another, the family of protein, so it's never an isolation, so exactly this is what we can achieve by doing an interactomic study in it, so we will get to see the PPI's or protein-protein interactors, so in this way we can map the whole interactome using IP-MS.

So in the first slide I had mentioned that the protein of interest can be used as a bait, in which the various interactors will come and bind to the bait, and so you can identify the direct interactors, so is protein A is interacting with protein B, so STAT3 is direct interactor say June or whatever, binds to STAT.

So now even you can identify proteins that are bound to be or June or whatever it is, so you're not only identifying the direct interactors, but you also identifying the co you know indirect interactors, thereby you can map it and you can form a map of the interactome, of course this alone might not be the ultimate experiment, because there are hundreds of other things that they can be some molecules that are weekly bound or the washing might not be efficient, so you need to verify whatever interactors you're getting through alternative techniques, but this can definitely give you all big list of proteins which are potential interactors, so the application three I spoke about is how to study low abundant residues using IP-MS,
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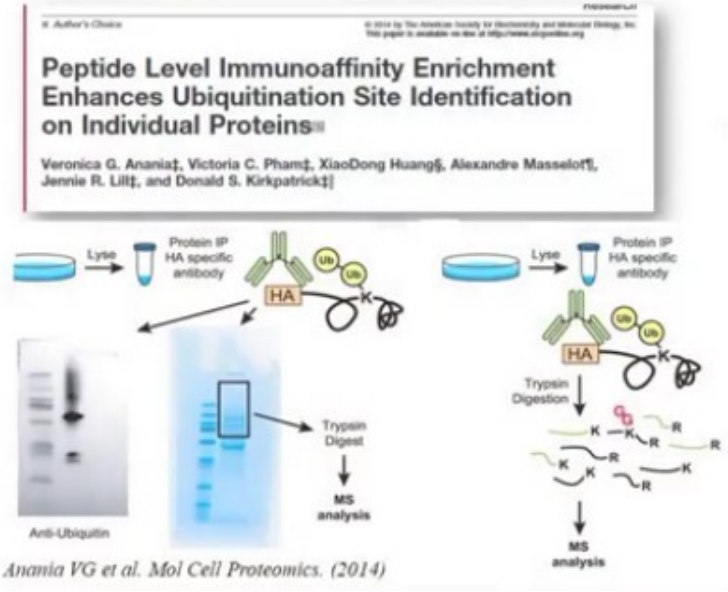
Application 3: Studying low abundant residues & designing targeted assays

- Enrichment of low abundant residues using Immuno-capture techniques.
- Enriching PTMs that may undergo ion suppression like ubiquitinylation, phosphorylated Tyrosine residues etc.



Anania VG et al. Mol Cell Proteomics. (2014)

so again taking one of the case studies, so we can enrich our samples in the residue that we are interested to look into, so just the way phosphoproteomics study can be done using TIO2, but you would be aware of the TIA2 technique, it only enriches serine and _13:43_ you would hardly see any tyrosine residues, because tyrosine residues A are very less in the whole proteome, and B they're very transient in nature, sometimes they are phosphorylated, sometimes they are not phosphorylated so it's hard to get.

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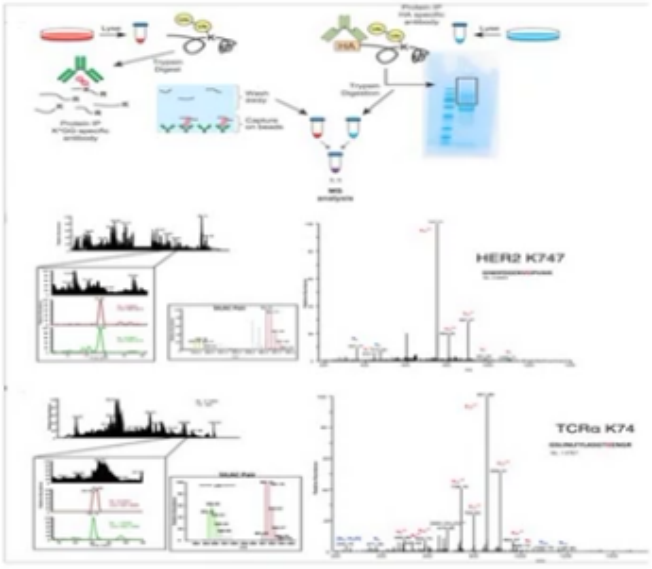
Application 3 cont'd: Studying low abundant residues & designing targeted assays

- Ubiquitination is a process that involves the covalent attachment of the **76-residue ubiquitin protein through its C-terminal di-glycine (GG) to lysine (K) residues on substrate proteins**.
- This post-translational modification elicits a wide range of functional consequences including targeting proteins for proteasomal degradation, altering subcellular trafficking events, and facilitating protein-protein interactions
- **Enrichment of K-GG** modified peptides from whole cell lysates for global characterization of ubiquitination sites.



So what you can do is you can take an antibody, you can pull the tyrosine phosphorylated residues and you can perform an MS to be very confident at you are getting the tyrosine phosphorylated residues.

Similarly in one of the studies what they did is, so they studied ubiquitinylation using IP-MS, so this is the study by Anania Et AL,
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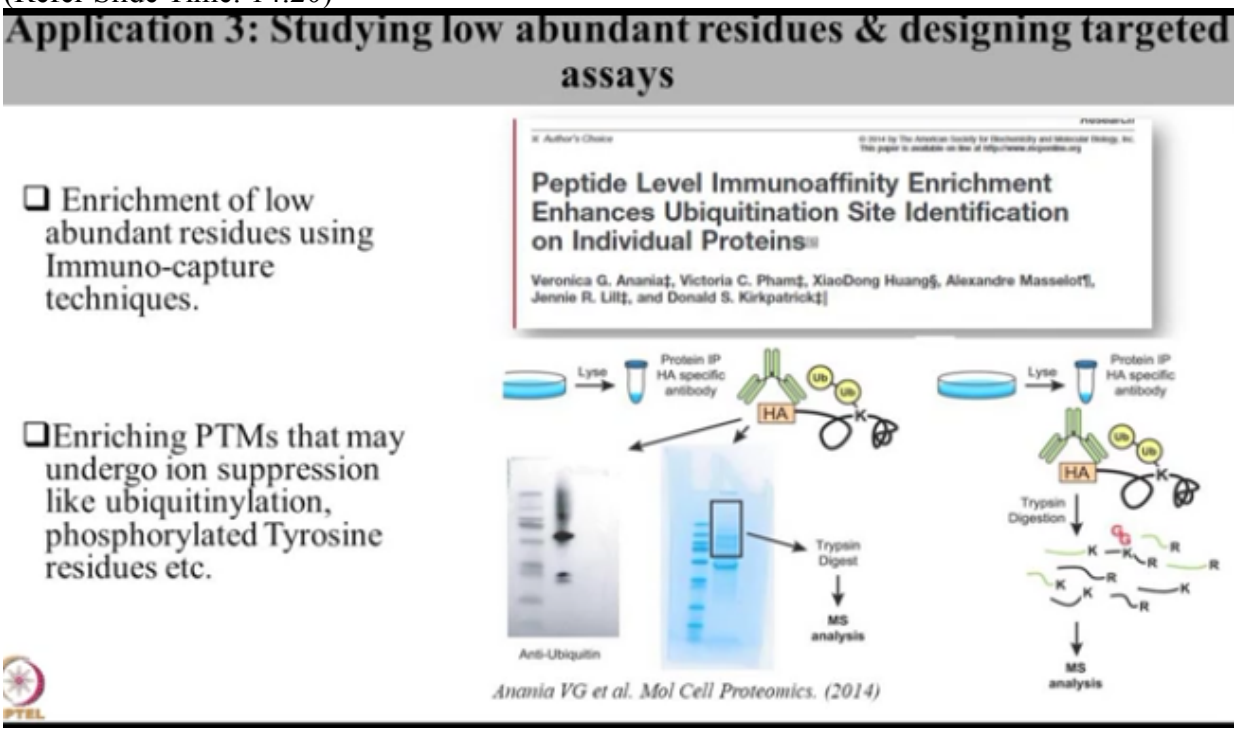

so they have done peptide level amino affinity enrichment and enhancing the ubiquitinations identification on individual proteins, so as you can see first of all they have monitored the you know response, or the ubiquitinylation using an anti-ubiquitin protein.

So one of the steps before you proceed for IP-MS you should check the response or the number of residues present or the protein of interest using a western blot. And secondly you can do a you know you can do your IP experiment followed by so you can use the antibody and then the bait protein and then do an IP and then you can run in the gel and then you can make fractions of it and directly subjected to mass spectrometry, so in this case what they did is so ubiquitinylation involves a glycine to lysine residues, so the ubiquitin attached to the lysine residues of the sub-straight protein, so what they have done is they have enriched the sample using this KGG stretch, so they have actually tried to only enrich samples that have that, yeah, ubiquitinylation,
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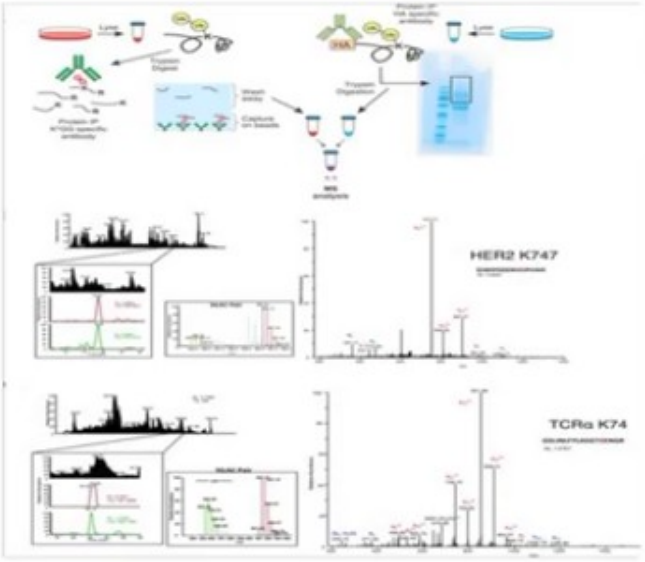
Application 3 cont'd: Studying low abundant residues & designing targeted assays

- Ubiquitination is a process that involves the covalent attachment of the **76-residue ubiquitin protein through its C-terminal di-glycine (GG) to lysine (K) residues on substrate proteins.**
- This post-translational modification elicits a wide range of functional consequences including targeting proteins for proteasomal degradation, altering subcellular trafficking events, and facilitating protein-protein interactions
- **Enrichment of K-GG** modified peptides from whole cell lysates for global characterization of ubiquitination sites.



and then what they have seen is they have seen in even in the mass spectrometry they can identify the sides in which the ubiquitinylation, so they can see the stretches of K and G, thereby they can be confident that these are the, they have identified the proteins that are enriched in ubiquitinylation.

So now today's case study we will be doing a small IP experiment of course the whole stretch of experiments is beyond the scope of this course, but what we will see is we will see that those small steps wherein you conjugate the bead with the antibody followed by the centrifugation

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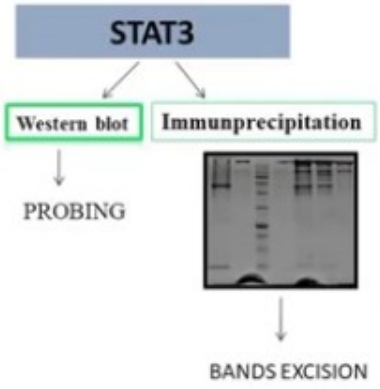
CASE STUDY: DEMONSTRATION SESSION IDENTIFICATION OF STAT3 INTERACTORS USING IP-MS#

STAGE I:

- Presence of STAT was verified using Western Blot
- Specific response was seen in the lysate :
- **INFERENCES:**
  - ✓ Presence of STAT3 confirmed.
  - ✓ Antibody specificity tested



STAGE II: TO BE PERFORMED TODAY (PROTEOMICS LAB)

- Demonstration of Experimental set up.
- Antibody Bead Conjugation
- Lysate Incubation with Antibody-bead conjugate

and of course the lysate and antibody bead conjugation, how it happens in the live session, so what we will do is we will, we have split this experiment into three stages, so stage 1 is we have verified the STAT3's present in the tissue lysate that we are probing with, so presence of STAT3 was verified using the western blot, and then the protein is present we can have two incidences, number 1 that we have STAT3 in the sample, because it is coming up in the western blot, a number 2 the antibodies very specific, because it has not given multiple bands, so then we have moved on to the IP experiment wherein we have done, we have immobilized STAT3 like we have enriched a trap STAT3 on the antibody, and then we have done the whole IP experiment and we have exercised the band and it has been subjected to mass spectrometry, so the stage 3 of the experiment is identification of the proteins,

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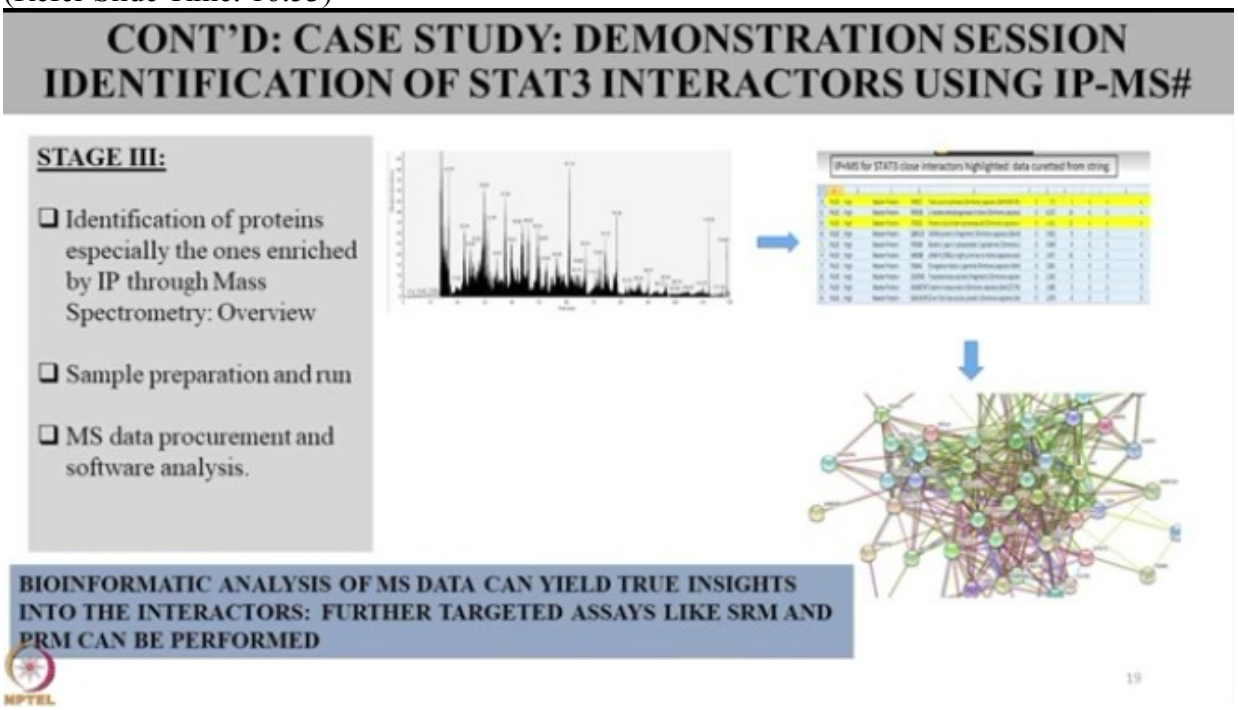

specifically the ones that has been enriched to IP, so we are not taking the whole sample, we are just taking those bands that have come specific and we are subjecting it to mass spectrometry, so you will get a list of proteins and you can then do bio-informatic analysis, so you can do a scoring based on string, you can, string DB which is an open axis of where there are other software as well, and you can come off with the score and you can do other experiments to see that how the list of protein you are getting from mass spectrometer how closely they are the interactors of your interest, and thereby you can form a interactome map.

Dr. Sanjeeva Srivastava: We will demonstrate to this technique in detail as well as provide you the brief protocol how to perform these experiments. So let us have these demonstration sections.

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Shuvolina Mukherjee: Hello students so in continuation with the lecture where we learnt a theoretical aspects of IP-MS, that is immunoaffinity and immunoprecipitation based cool down and followed by mass spectrometry. Today we will see what are the steps in which way we can perform this experiment, and how to do that for your biological problem.

So first of all just to brief you a little bit about the steps, so majorly in the immunoprecipitation based mass spectrometry, we will be doing the cool down of protein of whose interacting partners you are interested to look into, so for example let's say protein say S100 A10, so you want to learn what are the interacting partners of S100 A10, so for that first of all you need to have an antibody which is specific to S100 A10, so for that we need the antibody of the protein of interest, whose interacting partners you will be finding out,
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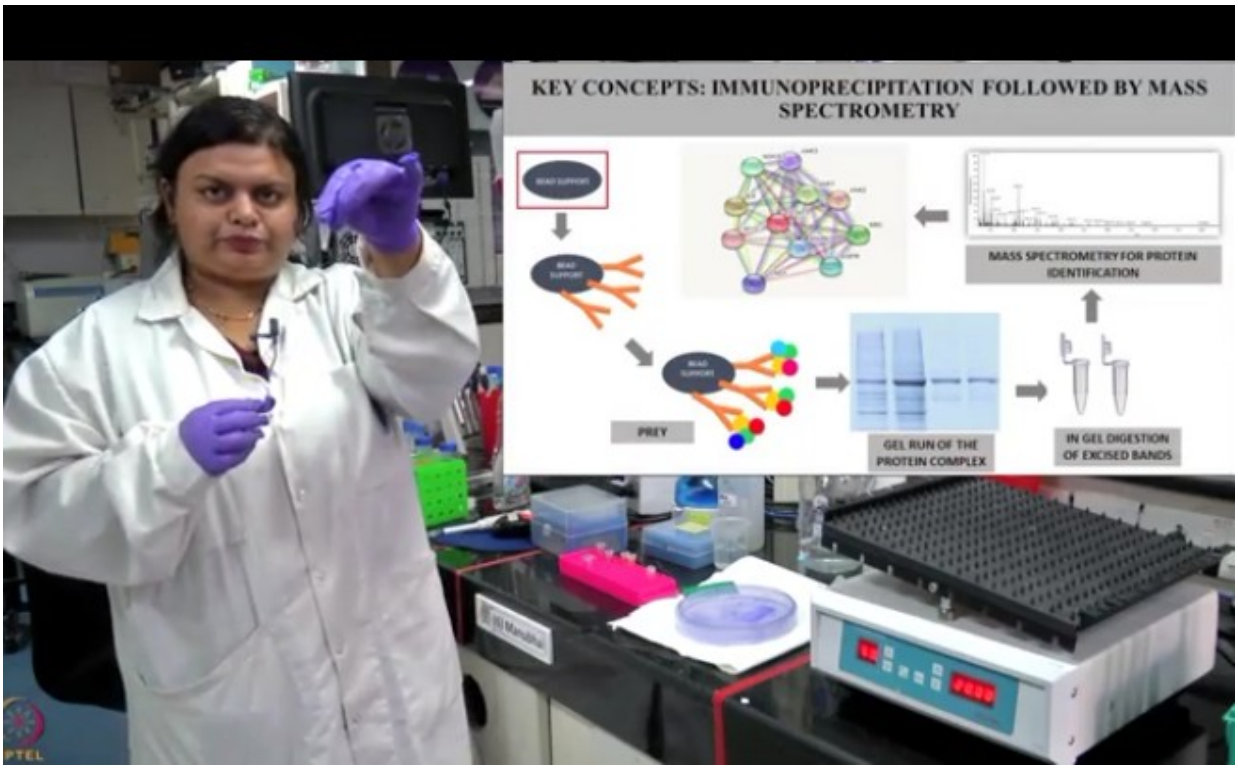

so here first of all we will take the bead, so this is protein A or protein GB you can take, so basically the protein A beads have the protein A which is derived from bacteria, and it is highly pacific and it binds to the FC region of the antibody, in that way we will conjugate the antibody of inflix with the bead that is present in your tube.

So first of all we will prepare an bead antibody conjugate and then because the antibody is pacific to the protein of interest, we will try to pull down that protein from your biological sample, so we will perform the experiments in 3 steps, number 1 we will prepare the antibody bead conjugate, number 2 we will be doing the pre-clearing of the lysate, so for example you want to look into a specific cell line, or patient derived tissue or tumor and you want to isolate the protein of interest, so for that we'll do the,
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we'll first take the lysate of your sample of interest, so this lysate contains thousands of proteins and you do not want to have a very high background.

So first we need to do a step which is called the pre-clearing of lysate, and then the last step is using the antibody and bead the conjugation the mixture where the antibody is already bound to the bead and using this pre-clearing lysate which is the lysate which is clear of all the noise, or all the background proteins that we will incubate overnight, and this is the way we will actually try to get all the interacting partners together in a big complex which we will subsequently run in a SGS gel, and then we will do the gel execution followed by the mass spectrometry.

So as you can see we have the beads here, so first we have to add wash baffle in these beads, so usually what we'll do is we will usually use the pre-chilled wash buffer, so this buffers already chilled, why this is done is because the beads need to get like activated prior to the binding with the antibody, so the ones you put the, so you just give it a little bit of tap and once you put the beads and the buffer, so the beads will swell after sometime and then you can perform a centrifugation step, followed by which we can put the antibody, so we'll just use, we'll just place it in our container,

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and we will gently rock it for some time, so that the buffer is uniformly distributed between the beads.

So once it is mixed properly we can also again do a gentle tap and do an inversion, so that there is nothing like there are no beads that are not in construct the buffer, and then we can do a centrifugation, so now that your beads are activated you can get rid of the wash buffer, put some fresh wash buffer, and in this you can now add the antibody, so usually 2 to 5 microgram of antibody is usually used, but then it depends on the protein of interest and how specific your antibody is, you can use monoclonal antibody as well as polyclonal antibody, so for example if you're trying to pull down a protein which has a many of the family members like for example if you're pulling down something like annexin, so there are many annexin proteins that are present in the human non mammalian tissues, annexin A1, A6, A2, so no matter how specific your antibody is, these all the members of this protein will definitely cross reacts, so according to the protein that you want to select you need to optimize how much time you want to keep this binding solution, so you can go either for a overnight binding at 4 degrees, or you can do a binding for 2 to 4 hours depending on your experiment.

So now what we will do is, we will add the antibody to the activated beads, so now the bead is already activated using the wash solution to which we will add the antibody,
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so after adding the antibody it is not advise that you do a very vigorous shaking or mixing, so you just gently tap it so that everything is properly mixed, and then you again place it in a container and put it on a blocker, so either you can leave it for 2 to 4 hours or you can do the step overnight.

Similarly for the lysate you can do a same step wherein you incubate instead of the antibody you incubate the lysate with the bead, so here there is already a pre-cleared lysate, and after this step is done your beads are already containing the antibody of your interest,
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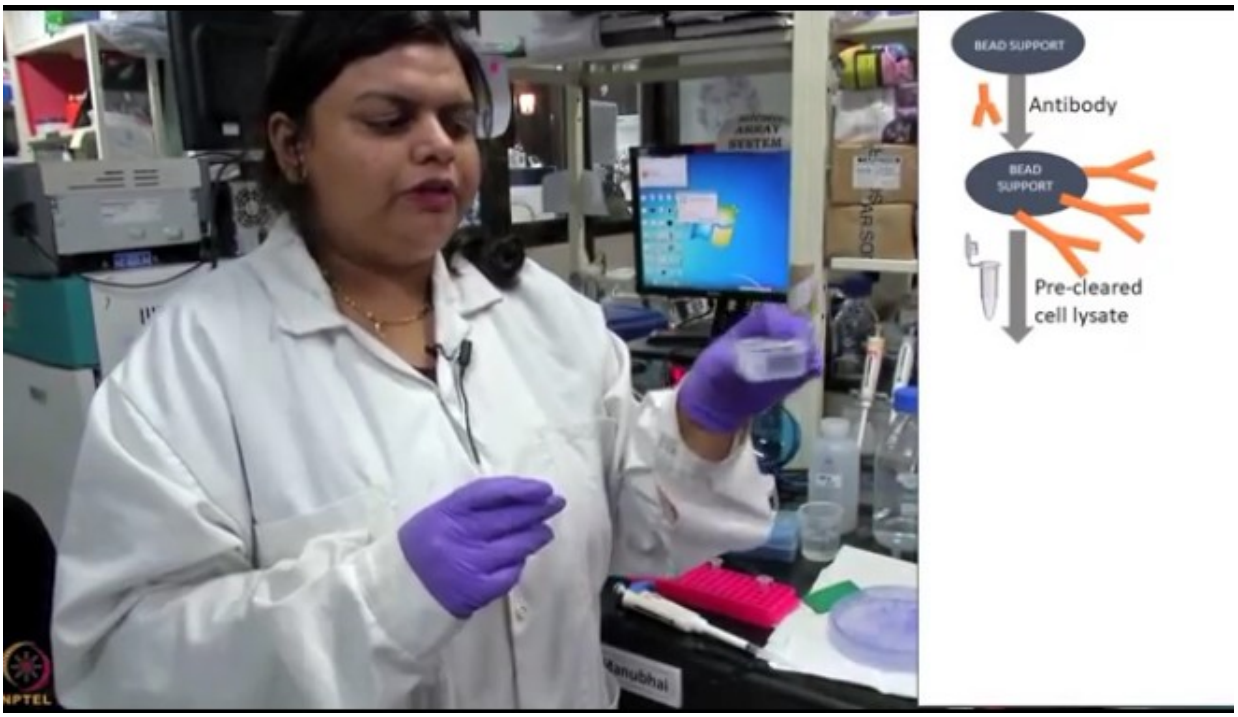

we will now put the pre-clear lysate into the antibody bead conjugate.

So here the beads have your antibody, and now we will add the pre-clear lysate, again you can give a gentle tap and then put it back in the container and place it back on the rocker, after the incubation you can see that now the beads which are conjugated with the antibody will bind to the protein of your interest from the lysate,

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from the pre-cleared lysate, so after this what will happen is you will already have the antibody bound to the protein of your interest and ideally from the lysate all other proteins which are interacting with the protein of your interest will stick to it and form a complex, so what we will do is we will discard the flow through, we will spin with, we will get it a brief spin, we'll store the flow through in a separate tube, and now we will perform washing, then why do we have to do washing? Because if you want to reviews the background that is if you consider this mixture it has lots of proteins which are also binding to the antibody bead conjugate which is also, which could be not very strong interactors of your protein, so the moment we add buffer we will give it a gentle mix, so what will happen is all these loosely bound protein will get washed away, so you will usually, we perform a washing for like 2 to 5 times again depending on the protein of your interest and your specificity of the antibody you can optimize these parameters.

So we will gently mix the buffer with the complex and then we will give it a gentle store and then again do the centrifugation, after giving a gentle spin, we'll again store the flow through in the separate tube, this step will be repeated for 2 to 3 times you can even go up to 5 times depending on the specificity of your antibody and then what we will do is, so now we only need to look at the bead and the conjugated complex, so what we will do is there are two ways to go about it, so either you can use an illusion buffer which has a lesser PH that is you can use neprilysin as a buffer, so what it will do is, it will disrupt the bonds now, it will disrupt the linkage and it will elute out the complex from the bead, so we'll use the elution buffer, and again give it a gentle tap.

After that we will again put it in the rocker, and leave it for few minutes, make sure this is not too harsh like you don't do it, do a vigorous mixing or shaking or even in this rotator, because you don't want to lose the interacting partners that have already bound to the protein of your interest, so with this now we are sure that we have got rid of all the background that might interfere with our study, and now we have only the antibody bound to the protein of interest and the interacting partners.

After the elution step even the bond between the antibody and the proteins of interest starts getting disruptive, so what we will do is we will again use the little bit of IP buffer, and we'll perform a spin, once this is done you have to take the supernatant and put it in the fresh while, so this supernatant now contains your protein of interest as well as all this interacting partners, so there can be two ways by which you can go for the protein identification,
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number 1 you can go for the insulation digestion of this elute, number 2 you can perform an in gel digestion, so here you can see when you run the protein in a gel you will get different bands and now you don't know what is what, so what happens is various proteins that are present in the mixture will get separated in the SDS, so what will do is we will do the in gel digestion, so we will cut the gel and we will make small, small pieces depending on the number of fractions you want to do.

For example I'll cut this lane into 4 pieces,
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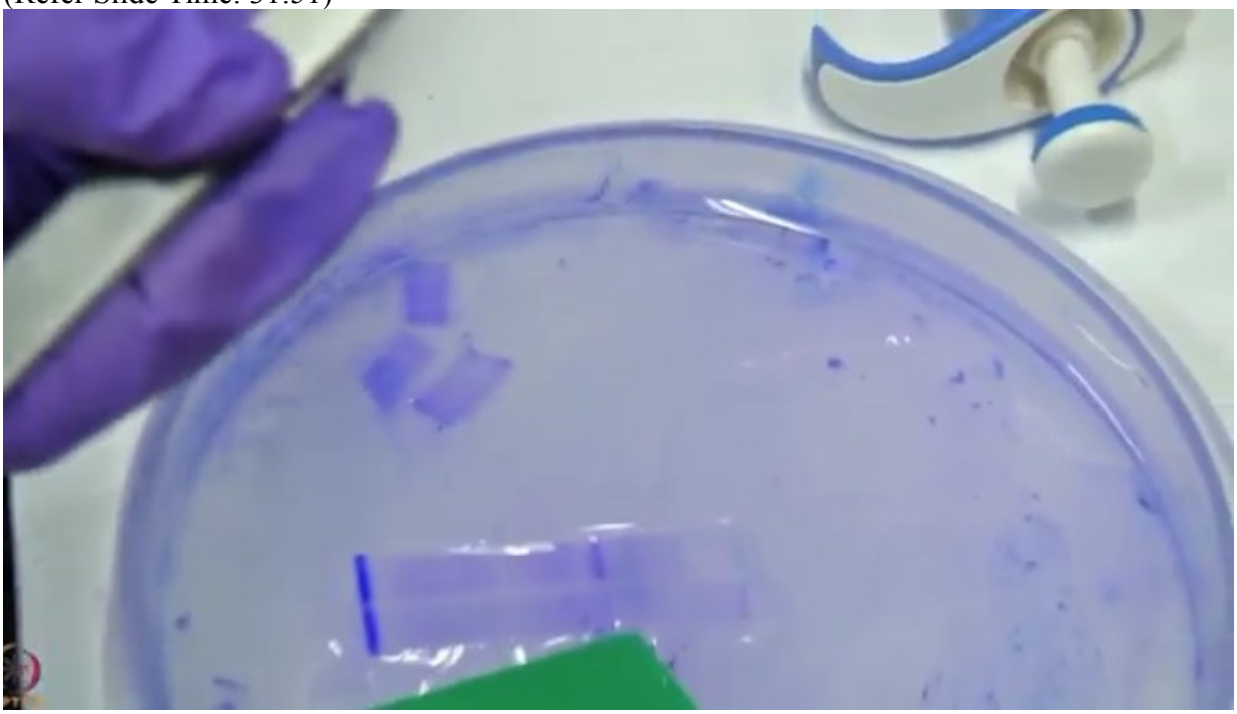

so this you can see there is the prominent band here this looks like the protein of interest and probably many of the proteins that have form the complex with it, so this we will cut into further smaller fractions, we'll take the band that has been exercise from the gel and we will perform a in gel digestion, so here this you can see this small band may have hundreds of proteins which are potentially the interacting partners of the protein of interest against which you had used the antibody, so after this in gel digestion would be done and you will elute the peptides out, so this is the digested peptide and now we will inject it for mass spectrometric analysis.

To summarize our entire experiment we have now performed immunoprecipitation of a protein of interest from mammalian cells, so we have taken the cell lysate and we has pre-cleared it using beads and similarly in the another section of it, what we have done is we have conjugated an antibody specific to the protein with the bead, so both of these steps were done and then the mixture of the antibody bead conjugate was incubated with the lysate, the protein of interest gets captured by the antibody and all this interacting partners also binds through that protein, so that we can get a big complex, or a complexome or we can get the interactome or that is present in the cell lysate, so after that what we did is we ran this sample in the gel and we exercise the bands and this bands would now cut into small sections and were digested, after digestion we have separated the peptide, and this peptide would now be injected into the mass spectrometry to see what are the proteins, what are the other proteins that have interacted with our protein of interest.

The advantage of coupling a very traditional method like immunoprecipitation with something advance like mass spectrometry is that you get best of the both worlds,
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that is while antibody based detection can detect even a very low abundant protein, mass which can help you identify that protein as well as several other proteins which are potentially the interacting partners of the protein of interest, therefore IP-MS is a very promising approach to

look into mechanistic details of how a cell perform, how a signaling cascade performs and hence it is emerging as the very prominent tool in the interactomics studies, so as you saw I already briefed you about the experimental workflow of IP-MS that is how we perform the immunoprecipitation using the antibody, after the process is done we also saw that we can perform in gel digestion as well as we can go for an insulation digestion of the precipitated complex, and now when that is done what we have to do is we have to now submit the samples for mass spectrometry analysis, so now we will be injecting our sample in the mass spectrometer.

So now the sample will be run using the LC gradients and the mass spectrometer, and we will be possibly able to identify the interacting partners of our protein of interest against which the antibody was used, therefore IP-MS gives a very nice approach who identify not only just one protein, but also its interacting partners, and not only that one of the advantages it has over traditional immunoprecipitation approaches while immunoprecipitation were mostly followed by a western blot in which another counter antibody is used where to see whether you can see the protein of interest binding to other interacting partners, there are issues related to antibodies specificity and cross reactivity, in this case whatever has been pulled down by the antibody and whatever has been bound to the bait or that is the protein of interest, so the bait and the preys can be characterized very confidently using the mass spectrometry, and in the next lecture you will learn in the phase how this approach works. Thank you.
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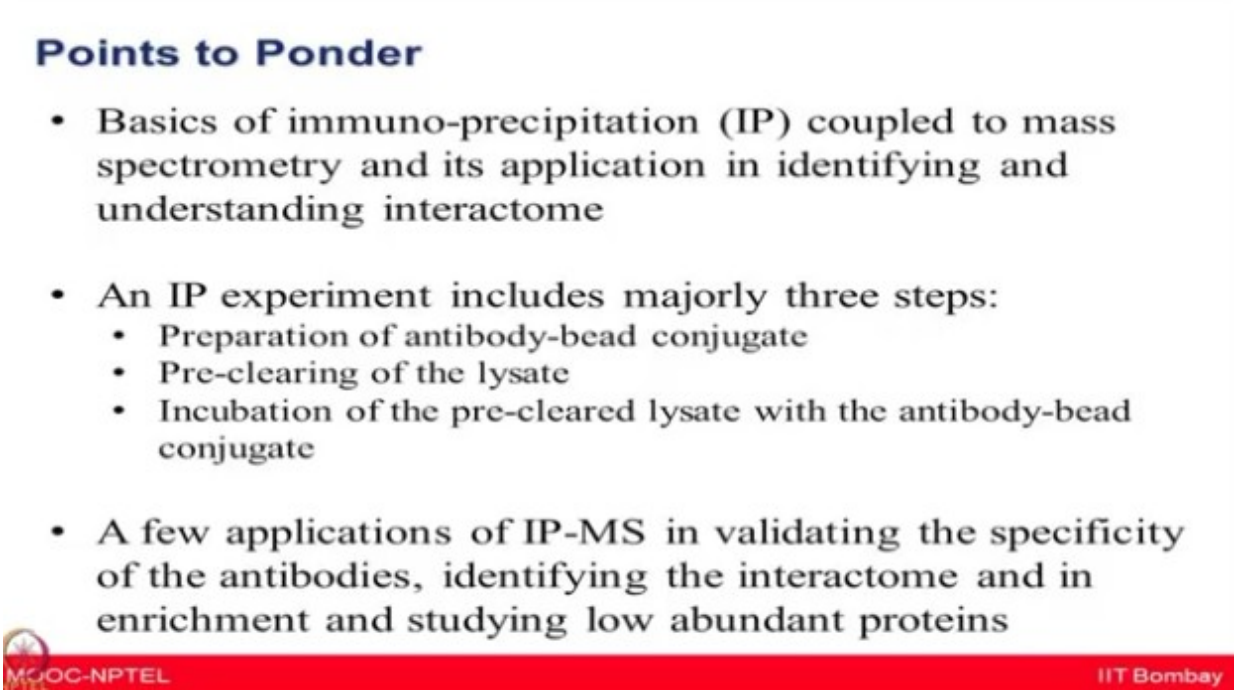

Dr. Sanjeeva Srivastava:- Hope now you all are familiar with entire workflow of immunoprecipitation followed by mass spectrometry based experiment. You may have realized that it is a good rate to characterize protein complexes and their response to regulatory mechanisms. The IP experiment allows hundreds of proteins to be identified in a single experiment, however it is also important to keep in mind that a majority of proteins identified in this experiment may also be non-specific binders, and now you need to do further investigation about from the possible list of interactors how many of those could be the real or direct

interactors, or how many could be your sticky proteins who are coming along with the interactors of interest.

This is now looking based to improve the accuracy of identifying these interactors which could be drawn from the IP experiment, however the way mass spectrometry technology is have now become a robust and easy to operate, it is now not very difficult to perform immunoprecipitation followed by mass spectrometry experiments, we will give you some basics of mass spectrometry and how to perform these experiments in next class. Thank you.
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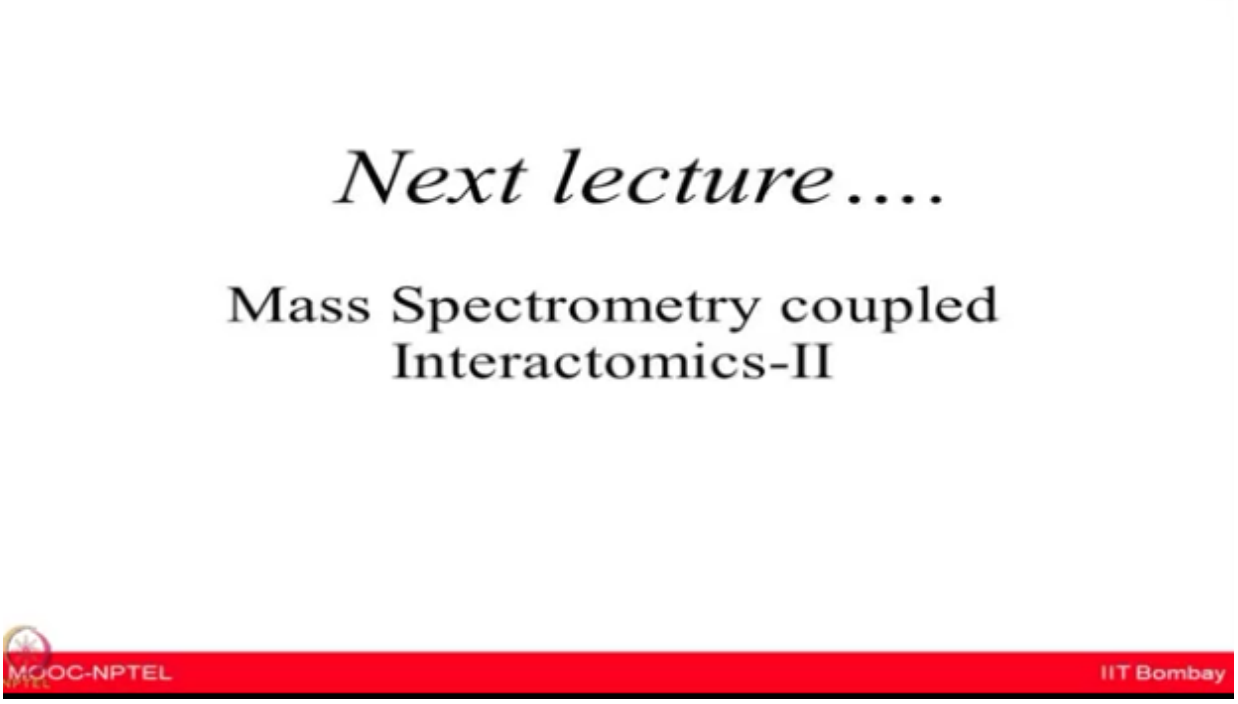

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