Interactomics: Basics and Applications

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Lecture – 45
Mass Spectrometry Coupled Interactomics-I

After doing a discovery based proteomic experiment, let us assume we have identified a protein of interest for which you do not know, what is the possible role of that protein? You want to characterize that protein in much more detail. So, possible way of doing that experiment could be, you can do immunoprecipitation and identify that this protein of interest interacts with each other proteins. So, protein interaction networks could at least tell you their possible role that what their protein may have. In this slide immunoprecipitation 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 scholar who will show you how to perform immunoprecipitation mass spectrometry based experiment.

Raise in or a 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 our complex zoom study or an interact home study using mass spectrometry. Though immunoprecipitation predominantly also the co immunoprecipitation is used in even in western blots, where you can use one protein and see whether it is interacting with other the protein and you can use an cross antibody and check it. So, this also is a is basically how IP is conventionally used.

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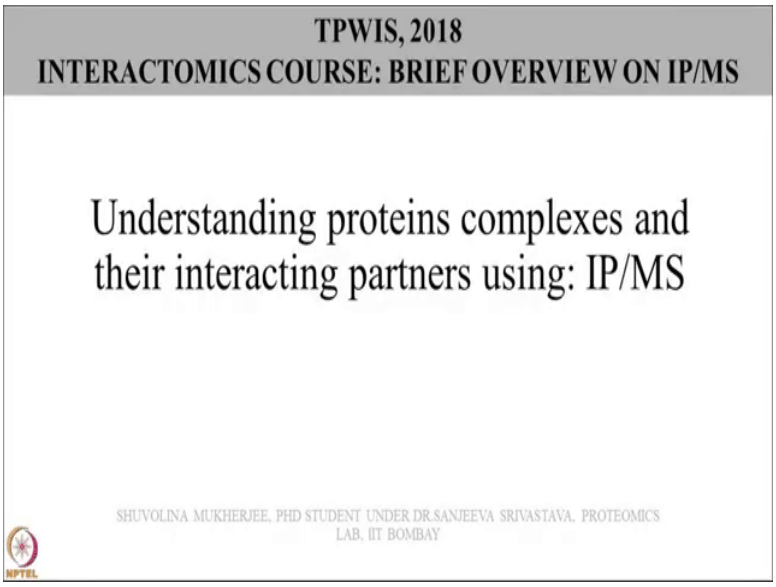

But, here we will be doing it how we will study it using like we in western blot you can identify maybe one interacting partner. But using mass spectrometry you can identify a plethora of proteins from your complex. So, thereby I will be talking about understanding protein complexes and their interacting partners using IPMS.

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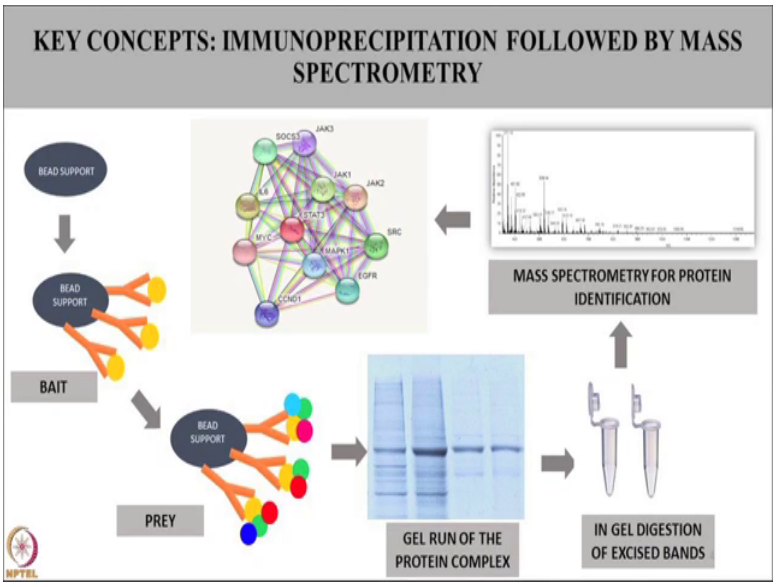

So, basically this is the workflow. So, we will have a beat support majorly people use a Sephora beads people use a agros beads also with protein a or proteins g immobilize because protein a or g will bind to the fc region of the antibody. So, you will conjugate your antibody and then you will incubate this. So, the 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 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, in we will take a case study today that is STAT 3. So, STAT 3 as you know is a part of the JAK STAT pathway and what is if you were interested to see that how which proteins are there potential interactions of STAT 3. So, we will take an anti stat antibody and we will conjugate it with the bead and then STAT 3 will uh, let us assume that this yellow dot

is STAT 3. So, start to 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 STAT 3. 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 this case we will have the whole complex home like this and then we can run on simple SDS space we can excise the bands and do an internalization and give it to the 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, you can see at your identified proteins on the mass spec and you can know how relevant are your hits. Whether at all they are interacting partners of STAT 3, but at least rather than doing a global proteomics like many of you are familiar with wherein, you are 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 in you can use this complex and use a buffer like something that has high pH like a glycine buffer what it will do this interaction will break. The bead and the antibody interaction will break off and then, you can do an in solution digestion and that you can subject to mass spectrometry analysis. So, overall this is the simple workflow of IPMS.

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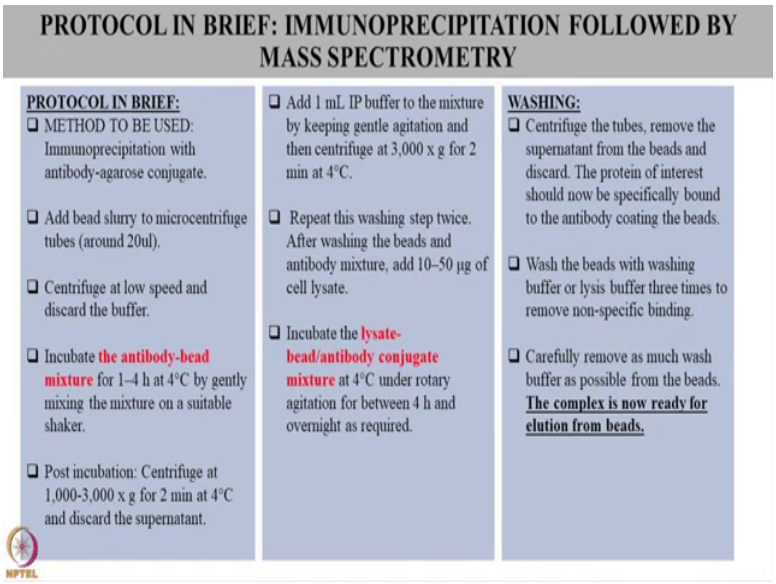

So, protocol in brief, what we do is first of all we will use the bead slurry and we will decant of the buffer. So, then the antibody mixture is incubated at 4 degrees for 1 to 4 hours. So, this is to ensure that the antibodies properly bound to the bead because that is very important that is what we will pull. So, you are sort of doing a pull down assay, 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 its trace NACL and all those buffers, they will keep your protein intact. And then, but at any point you must remember that for this to be successful, you should not disrupt the interaction. So, all your steps you need to be very gentle, you need not to you should not use something like a very harsh chemical like urea or anything that can demixure your protein or even disrupt your interaction. So, that that is something that you should keep in mind and

then this lysate bead antibody, this mixture you can keep for a with the 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 if you think that it is too abundant and 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 consequent of 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 your washing so, so, as to not have those things that are just weakly bound or maybe as a carryover, you just want to be sure that you are identifying only the complex of your interest. And then you will remove it you will do an elution. 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 an indulgence in solution dilation after the elution step.

So, applications of IPMS. So, IPMS is very widely uh, 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 biologists often raises a question that have you monitored the you know have you done a western laws, have you done an Eliza.

So, for them to if you have someone, we who wants to address both these crowds you can definitely use this technique.

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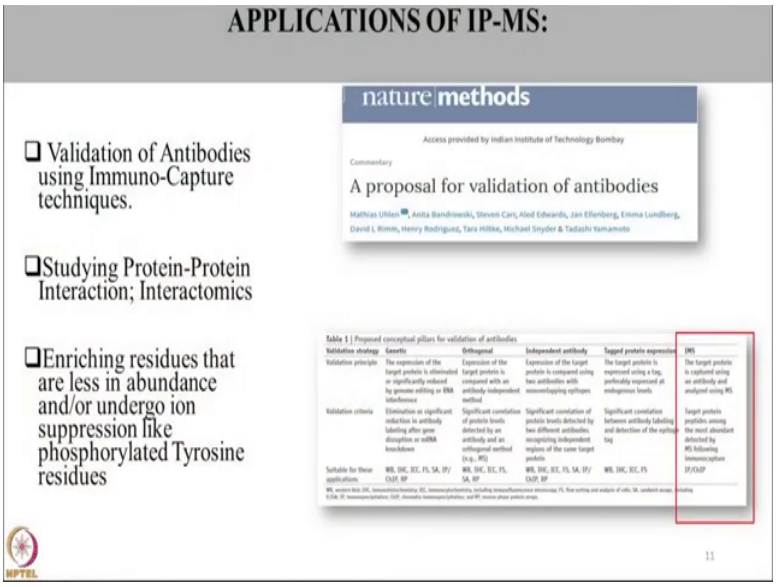
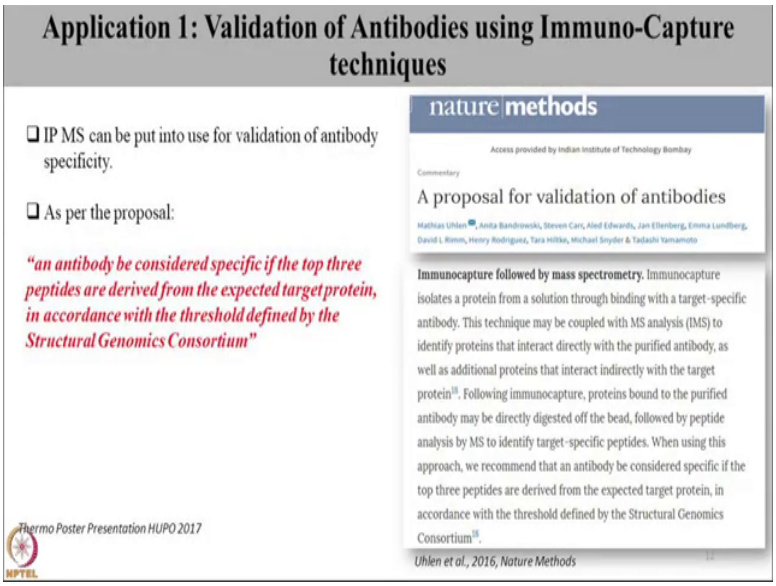


So, one of the preliminary studies that have come up is this by Mathias Uhlen group. So, they have given a proposal by which you can validate antibodies. So, here by on now onwards I will be talking about 3 major applications of IPMS. So, there are much many, but in the scope of lecture. So, application one of the applications I will be talking about is validation of antibodies using immuno capture technique.

So, we know captures again IPMS because, you are capturing the antibody of your interest followed by a mass spectrometry. Secondly, we will be talking briefly about 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 ions suppression like phosphorylation or even ubiquitinylation and many other PTMS.

So, because of that it is often very difficult to you know, even if the if the post translation modification is very complex like a ubiquitinylation, it is 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 ubiquitinated. We can use a tyrosine specific antibody which can bind to the tyrosine residues tyrosine phosphorolated residues and then we can do in mass spectrometry. So, thereby there are many things that you can do with this technique. (Refer Slide Time: 09:48)



Coming to the first application so, in this proposals of validation of antibodies, where Mathias Uhlen group what they have proposed this that immuno capture followed by mass spectrometry is a very nice way to know whether your antibody is specific or not. So, what here they are aiming for is if your antibody is very specific to your target. So, if you bind it to a beat conjugate and then you do an immuno capture 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 s, they have a

devised this statement that is an antibody can be considered specific, if the top three peptides are derived from the expected target protein.

So, if you are looking at an antibody. So, you have three antibodies, say from vendor is or app cam 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 or 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 specific your antibody is. So, you can do an immuno capture and you can just see, whether the top three peptides that are coming up after mass spectrometry are off the protein of your interest in this case STAT 3.

So, if that is coming you can be confident that whichever antibody is more 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 a panel of many studies and one of the studies they have proposed is immune capture.

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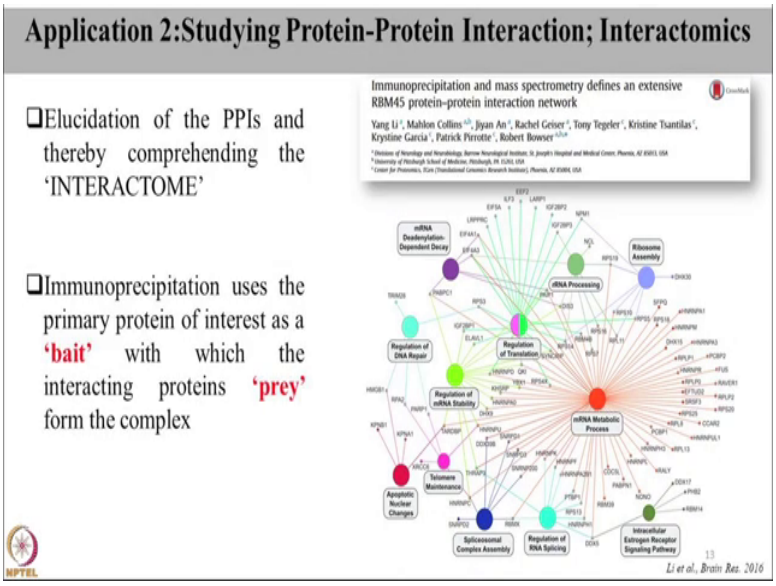

So, then second application is studying protein-protein interaction or the typical interactomics. So, as you can see for any physiological process that you are studying bead in plants, humans, cancer or infectious disease. So, you often find out your list of targets does not involve one protein. You always have something else along with it maybe a transcriptional factor, maybe a protein or another the family of proteins. So, it is never in isolation. So, exactly this is what we can achieve by doing an interactomic study in it. So, we will get to see the PPIs 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 s, you can identify the direct interactor. So, its protein a is interacting with protein b. So, STAT 3 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 are not only identifying the direct interactors, but you are also identifying the co you know in direct 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 weakly bound or the washing might not be efficient. So, you need to verify whatever interactors, you are getting through alternative techniques, but this can definitely give you all a big list of proteins which are potential interactors. So, the application 3 I spoke about is how to study lower abundant residues using IP MS.

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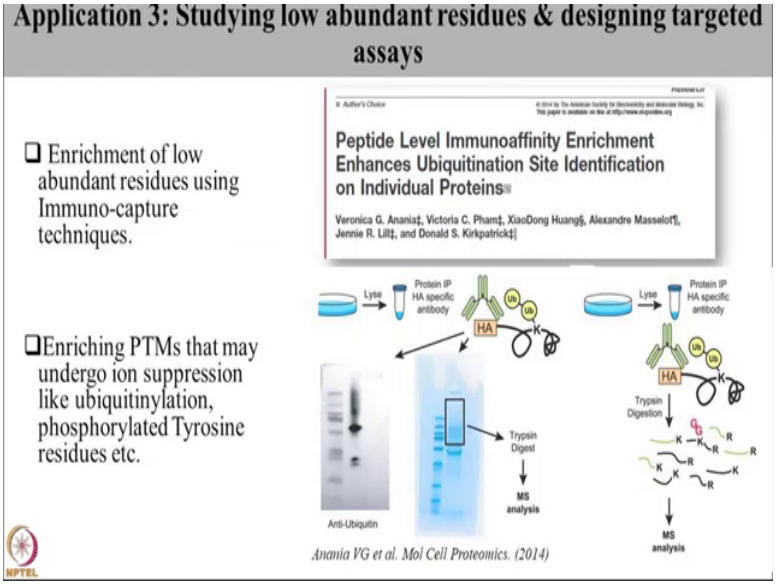

So, again taking one of the case studies. So, in the weekend enrich our samples in the residue that we are interested to look into. So, just the way phosphoproteomic study can be done

using TIA 2, but you would have be aware of the TIA 2 technique it only enriches setting and trionine. You would hardly see any tyrosine residues because tyrosine residues a are very less in the whole proteome and b they are very transient in nature sometimes they are phosphorolated sometimes they are not phosphorolated. So, its hard to get.

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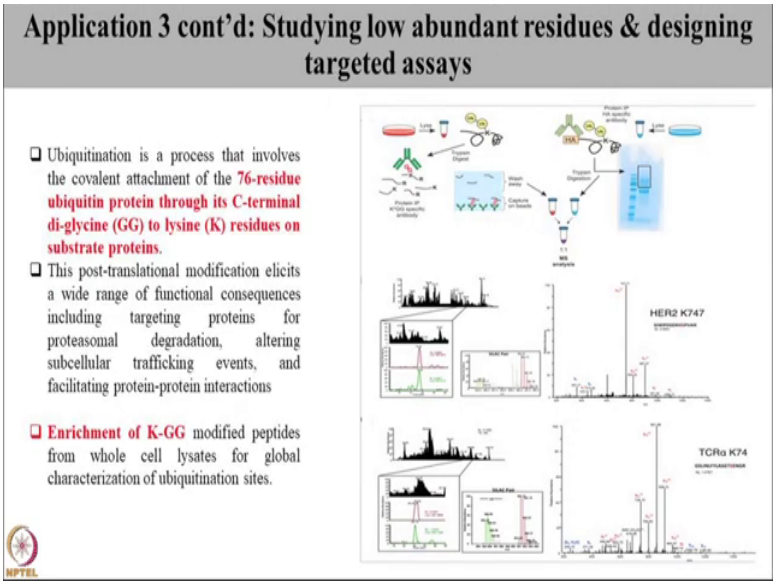

So, what you can do is you can, you can take an antibody. You can pull the tyrosine phosphorolated residues and you can perform an MS to be very confident that you are getting the tyrosine phosphorylated residues. Similarly, in one of the studies what they did is. So, they studied ubiquitinations using IP MS. So, this is the study by Anania et al. So, they have done peptide level immuno affinity enrichment and enhancing the ubiquitination sited identification on individual proteins.

So, as you can see first of all they have monitored the I you know response or the ubiquitinylation using an anti ubiquitin protein. So, one of the steps before you proceed for an 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 subject it 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 substrate 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. And then what they have seen this they have seen in even in the mass spectrometry they can identify the sites in which the ubiquitinylation in 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, where you in you conjugate the bead with the antibody followed by the centrifugation and of course, the lysate and antibody beat conjugation how it happens in the live session.

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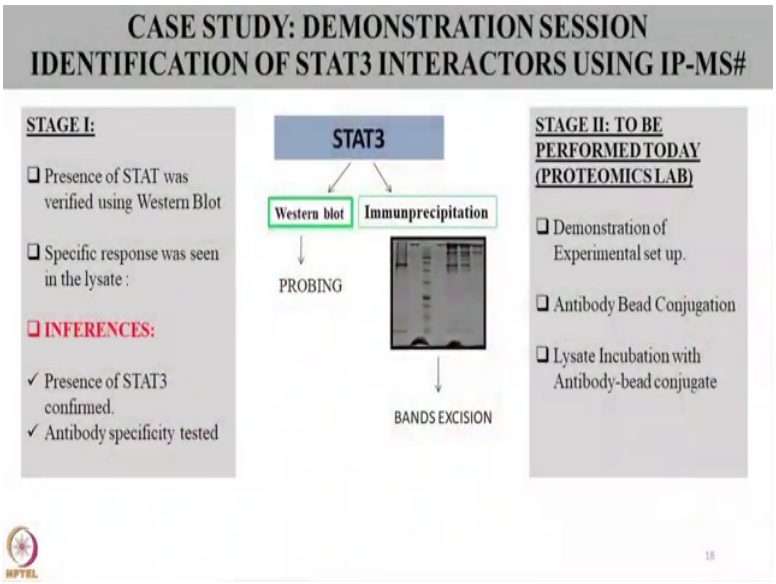

So, what we will do is, we will we have spread this experiment into 3 stages. So, stage 1 is we have verified the STAT 3 is present in the tissue lysate that we are probing with. So, presence of STAT 3 was verified using the western blot.

And then the protein is present, we can have two incidences. Number 1 that we have stat three in the sample because it is coming up in the western blot and number 2 the antibody is 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 STAT 3 like we have enriched trapped STAT 3 on the antibody and then we have done the whole IP experiment and we have excise the band and it has been subjected to mass spectrometry.

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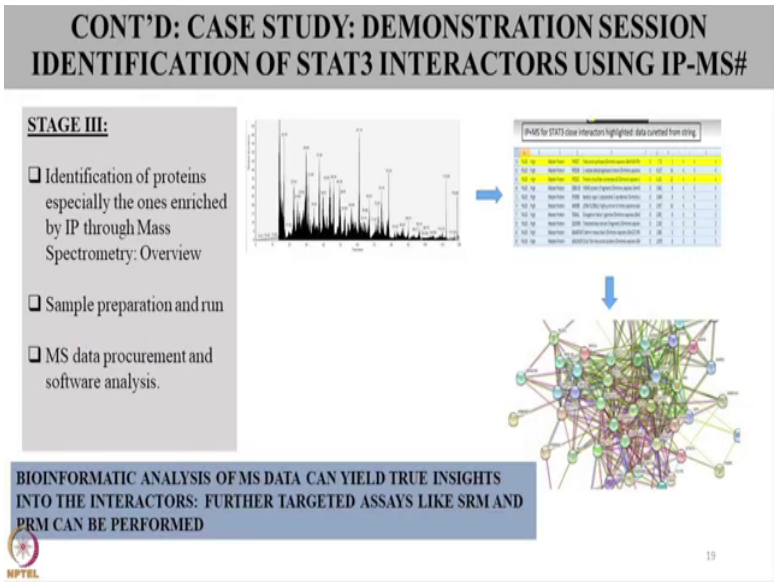

So, the stage 3 of the experiment is identification of the proteins, specifically the ones that has been enriched to IP. So, we are not taking the whole sample we are just taking those bands that have come specific and we are subjecting it to mass spectrometry. So, you will get a list of proteins and you can then do bioinformatic analysis. So, you can do a scoring based on string, you can string dB which is an open access software there are others offers as well.

And you can come up with a 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. We will demonstrate with this technique in detail as well as provide you the brief protocol how to perform these experiments. Let us have this demonstration of experiment.

Hello students. So, in continuation with the lecture, where we learnt the theoretical aspects of IP MS that is immuno affinity and immunoprecipitation base pull down 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 immuno immunoprecipitation based mass spectrometry, we will be doing the pull down of protein of whose interacting partners you are interested to look into. So, for example, or let say a protein s 100 a 10 and. So, you want to learn what are the interacting partners of s 100 a 10. So, for that first of all you need to have an antibody which is specific to s 100 a 10.

So, for that we need the antibody of the protein of interest whose interacting partners you will be finding out.

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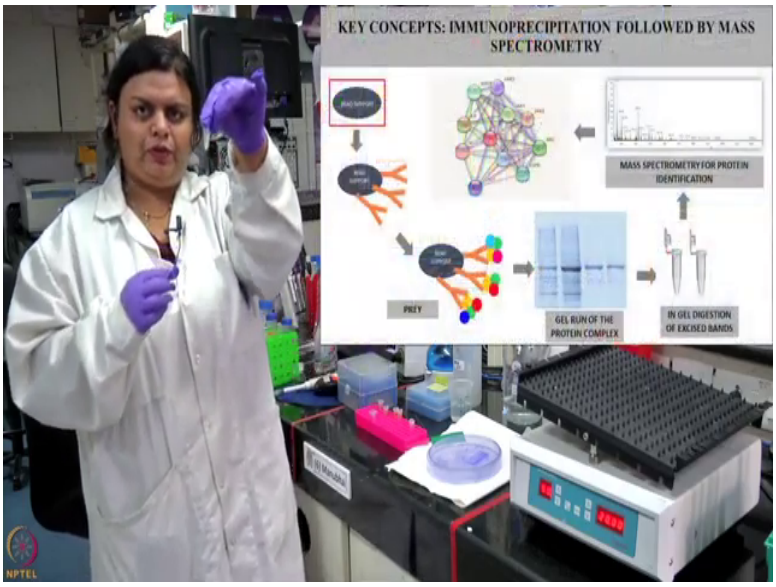

So, here first of all we will take the bead. So, this is protein a or protein g beads you can take. So, basically the protein a beads have the protein a which is derived from bacteria and it is a highly specific and it binds to the fc region of the antibody in that way we will conjugate the antibody of interests 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 specific to the protein of interest, we will try to pull it down that protein from your biological sample.

So, they 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 a patient derived tissue or tumor and you want to isolate the protein of interest. So, for that we will do the we will 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 antibodies 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 our SDS gel and then we will do the gel exertion followed by the mass spectrometer.

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So, as you can see we have the beads here. So, first we have to add wash buffer in this beads. So, usually what we will do is, we will usually use the pre chilled wash buffer. So, this buffer is already chilled. Why this is done is because the beads need to get like activated prior to the

binding with the antibody. So, the once you put up the. So, you just give it a little bit of a tap and once you put the beads and the buffer. So, the beads will swell after some time and then you can perform a centrifugation step followed by which we can put the antibody.

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So, we will just use, we will just place it in a container and we will gently rock it for some time.

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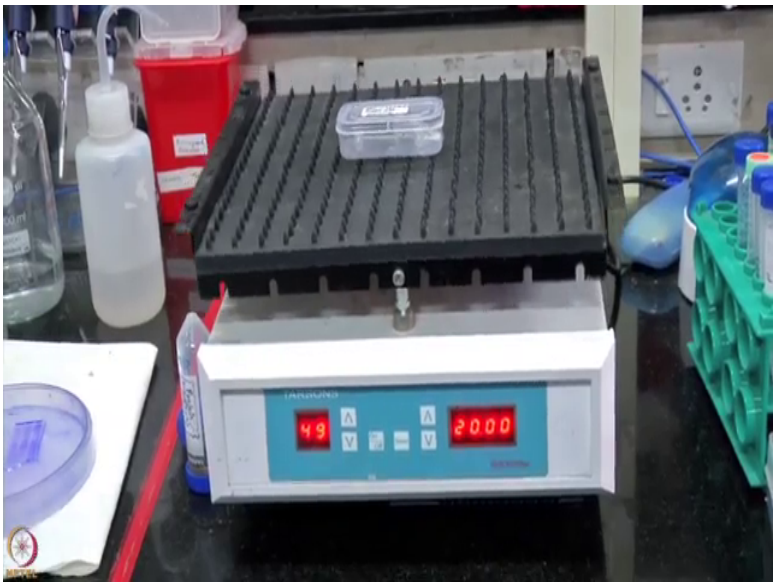

So, that the buffer is uniformly distributed between the beads.

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So, once it is mixed properly, you 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 touch with the buffer and then we can do a centrifugation.

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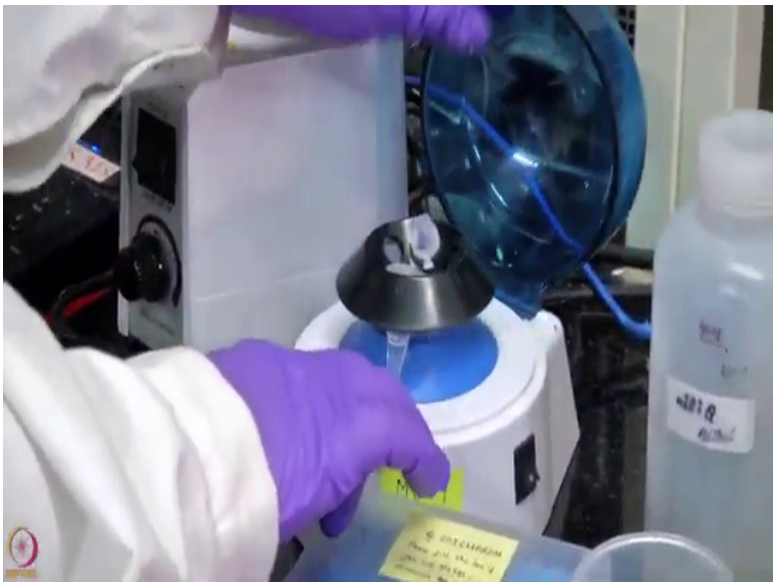

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So, now that your beads are activated, you can get rid of the wash buffer put some fresh wash buffer and in this and in this you can now add the antibody. So, usually 2 to 5 2 to 5 microgram of antibody is usually used, but then it depends on the protein of interest and how specific your antibody is you can use monoclonal antibody as well as polyclonal antibody. So, for example, if you are trying to pull down a protein which has the many of the family members like for example, if you are pulling down something like an axiom. So, there are many an axiom proteins that are present in the human or mammalian tissues and axiom a 1 a 6 a 2.

So, no matter how specific your antibody is these all the members of this protein will definitely cross react. So, according to the protein that you want to select, you need to optimize how much time you want to like keep this binding solution. So, you can go either for

an 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.

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So, now the bead is already activated using the wash solution to which we will add the antibody. So, after adding the antibody it is not advised that you would 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 rocker. 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 it there is already a pre cleared lysate and after this step is done your beads are already containing.

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The antibody of your of your interest, we will now put the pre clear lysate into the antibody of bead conjugate. So, here the beads have your antibody and now, we will add the pre cleared lysate. Again you can give a gentle tap and then put it back in the container and place it back on the locker. 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 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 we will despin it we will give it brief spin, we will store a flow through in a separate tube and now we will perform washing. Why do we have to do washing? Because if you want to reduce the background that is if you; if you consider this mixture it has lots of proteins which are also binding to the

antibody bead conjugate which is also it could be not very strong interactors of your protein. So, the moment we add buffer uh, we will give it a gentle mix. So, what will happen is all this 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 stir and then again do the centrifugation. After giving a gentle spin, we will 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 elution buffer which has your less lesser pH that is you can use trace glycine as a buffer. So, what it will do is it will disrupt the bonds now it will disturb the linkage and it will elute out the complex from the bead. So, we will use the elution buffer and again give it a gentle tap, after that we will again put it in the locker and leave it for few minutes. Make sure this is not too harsh like, you do not do it though do a vigorous mixing or shaking or even in this rotator because the, you do not want to lose the interacting partners that have already bound to the protein of your interest.

So, but this now, we are sure that we have got rid of all the background that 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 illusions that even the bond between the antibody and the proteins of interest starts getting disrupted. So, what we will do is, we will again use a little bit of IP buffer and we will perform a spin. Once this is done you have to take the supernatant and put it in a fresh vial. So, this supernatant now contains your protein of interest as well as all its interacting partners. So, there can be two ways by which you can go for the protein identification. Number 1 you can go for the in solution digestion of that elude.

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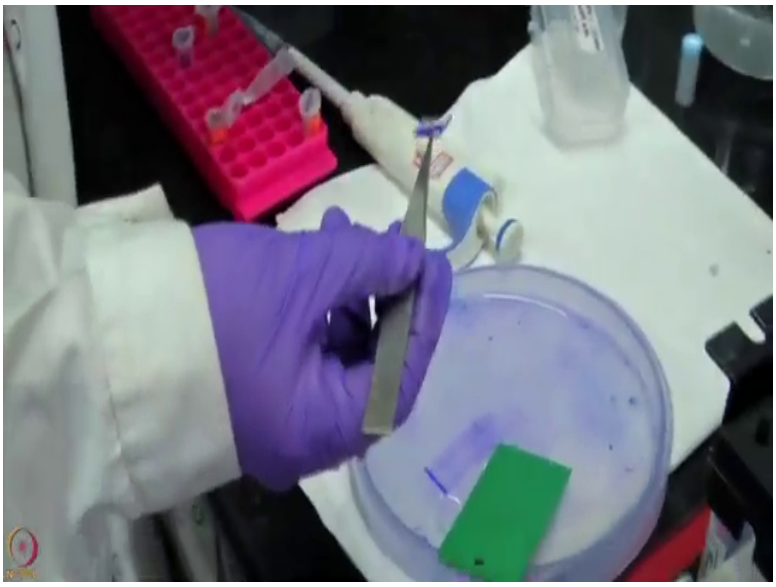

Number 2: you can performance in gel digestion. So, here you can see when you run the protein in a gel you will get different bands and now you do not know what is what. So, what happens is various proteins that are present in the mixture will get separated in the SDS. So, what we will do is we will do though 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 will cut this lane into 4 pieces.

So, this you can see there is a prominent band here, this looks like the protein of interest and probably many of the proteins that have become formed the complex with it. So, this we will cut into for the smaller fractions, we will we will take the band that has been excised 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 elude the peptides out.

So, this is the digestive peptide and now we will inject it for mass spectrometry analysis. To summarize the entire experiment, we have now performed immunoprecipitation of a protein of interest from a mammalian cells. So, we have taken the cell lysate and we have pre cleared it using beads and similarly in the another section of it what we have done is, we have conjugated and 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 its interacting partners also binds to that protein. So, that we can get a big complex or a complex zone or we can get the interactome or that is present in the cell lysate.

So, after that what we did is we ran the sample in the gel and we excise the bands and this bands would now cut into small fractions and were digested after digestion, we have take 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 a coupling a very traditional method like a immunoprecipitation with something advanced like mass spectrometry is that you get best of both worlds. That is while antibody based detection can detect even a very low abundant protein mass spec 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 very promising approach to look into a mechanistic details of how a cell performs, how a signalling cascade performs and hence it is emerging as a very prominent tool in the interactomic 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 it an insulation digestion of the precipitated complex. And, when that is done what we have to do with we have to now submit the samples for mass spectrometry analysis.

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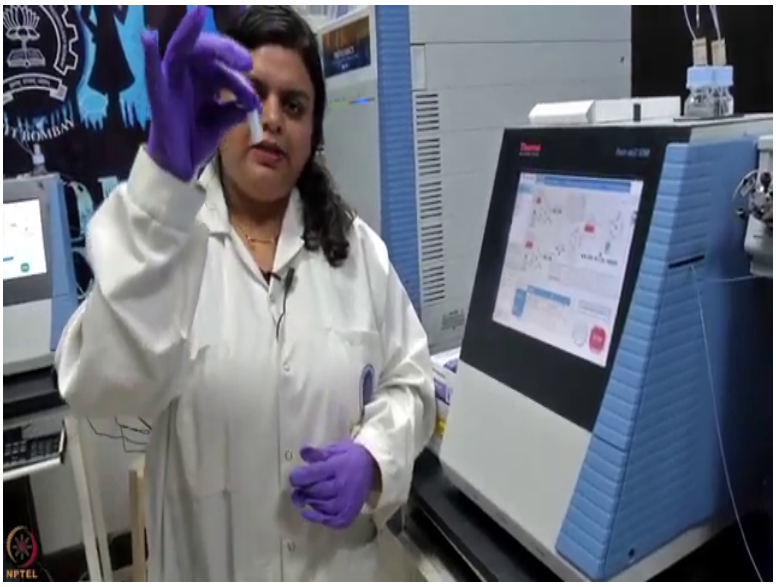

So, now we will be injecting our sample in the mass spectrometer.

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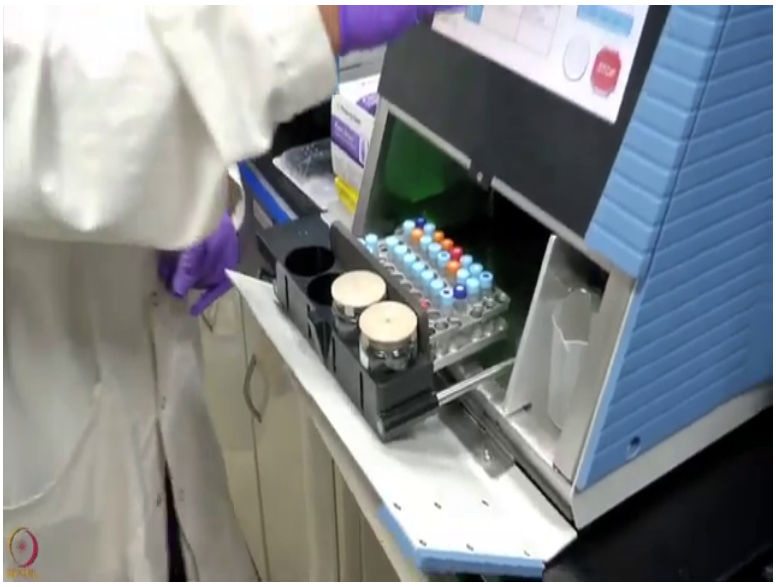

So, now the sample will be done using the LC gradients and the mass spectrometer and we will be possibly able to identify the interacting partners of our protein of interest against which the antibody was used. Therefore, IP MS gives a very nice approach to identify not only just one protein, but also its interacting partners and all not only that one of the advantages it has over traditional immunoprecipitation approaches while immuno immunoprecipitation mostly follow is the followed by a western blot in which another counter antibody is used to see whether you can see the protein of interest binding to other interacting partners.

There are issues related to antibody specificity and cause 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 to the bait and the preys can be characterized very confidently using the mass spectrometry and in the next lecture you will learn in detail how this approach works.

Thank you.

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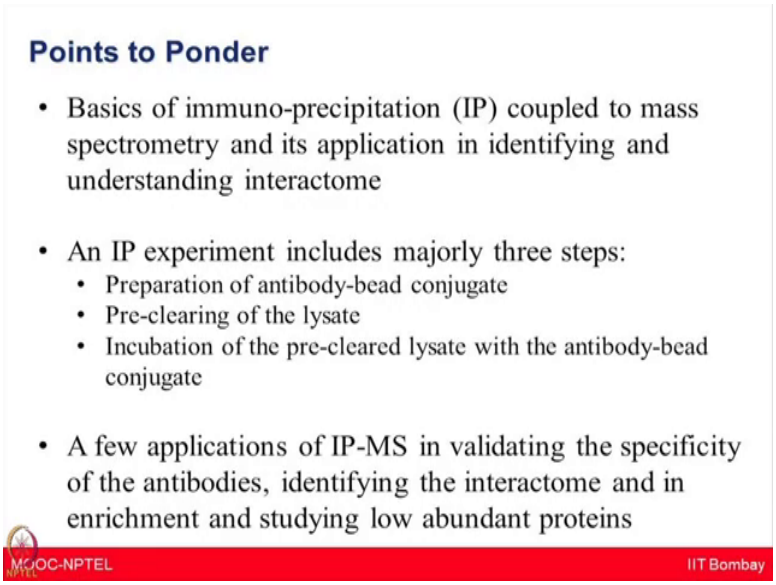

Hope now, you are all familiar with the entire workflow of immunoprecipitation followed by mass spectrometry based experiment. You may have realized that it is a good way to characterize protein complexes and the response to regulatory mechanisms. The IP experiment allows hundreds of proteins to be identified in a single experiment; however, it is also important to keep in mind that the majority of proteins identified in this experiment may also be known as 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 just the sticky proteins who are coming along with the interactors of interest.

This is looking ways to improve the accuracy of identifying the interactors which could be drawn from the IP experiment. However, the way mass spectrometry technologies have now become more 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.