Interactomics: Basics & Applications

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Lecture – 24
Applications of Protein Microarrays in Deciphering PTMs and Biological Networks

As you know, a screening for TAAA or Tumor Associated Auto Antibodies is a novel concept, where the aim is to detect the auto antibodies or antibodies produced in the body much ahead of time. And they have lot of clinical utility especially for the early detection of cancer and other diseases.

In today's lecture, MS Nikita will discuss about a few more Applications of Protein Microarrays using different case studies to provide you a broad understanding of potential of protein microarray based technology. You must understand and appreciate that there are many applications which are possible on different type of protein microarray based technology platforms.

This lecture will also provide you understanding for novel applications for doing various protein interaction studies, post transition modification, tiny substrate screening etcetera using high throughput microarray based platforms. So, let us welcome Nikita for today's lecture.

A very good morning to all of you. In the previous lecture, you have seen how protein microarray can be used to detect the presence of auto antibodies in the bio fluids of cancer patients. In this lecture; we will further look into the applications of protein micro arrays that can be used to understand the signaling network and to understand the time bound post translational modification happening at the cellular level.

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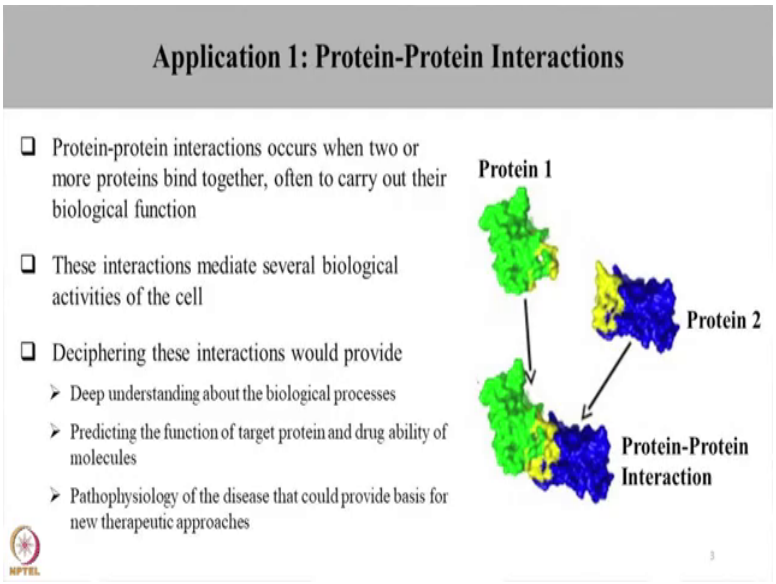

Protein interaction occurs when two or more proteins interact with each other to carry out a biological function. These interactions mediate several cellular processes and understanding these interactions would help in understanding the function of proteins and to identify the disease pathobiology. So, once we dissect these interactions we can end up finding few druggable targets and new therapeutic approaches that can be used to treat the disease.

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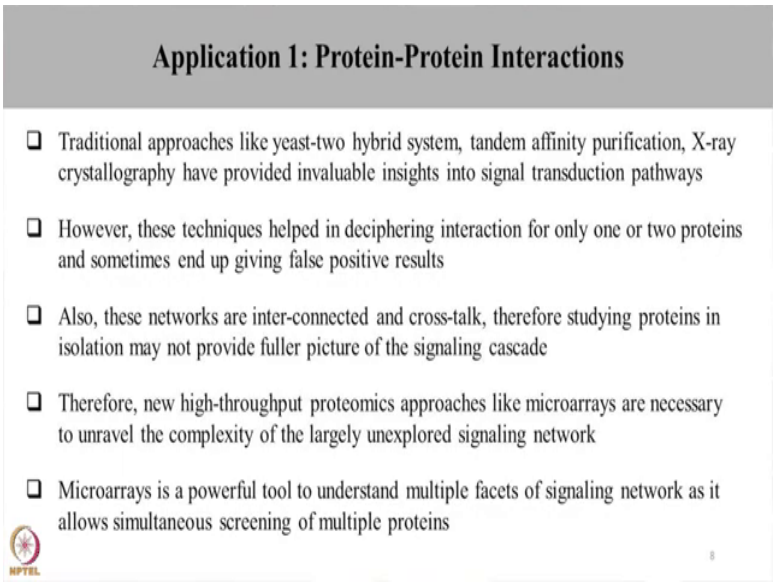

Traditional approaches like yeast-two hybrid system tandem affinity chromatography etcetera have provided invaluable insight into the protein interaction. However, these techniques just look at one or two proteins study these proteins and isolation and sometimes even end up giving false positive results.

These networks the signaling pathways are dynamic therefore; studying up protein in isolation might not provide a fuller picture of the interacting pathway. Therefore, high throughput platforms like protein microarrays can hold immense value to screen multiple proteins together and hence can be used to decipher the protein interactions.

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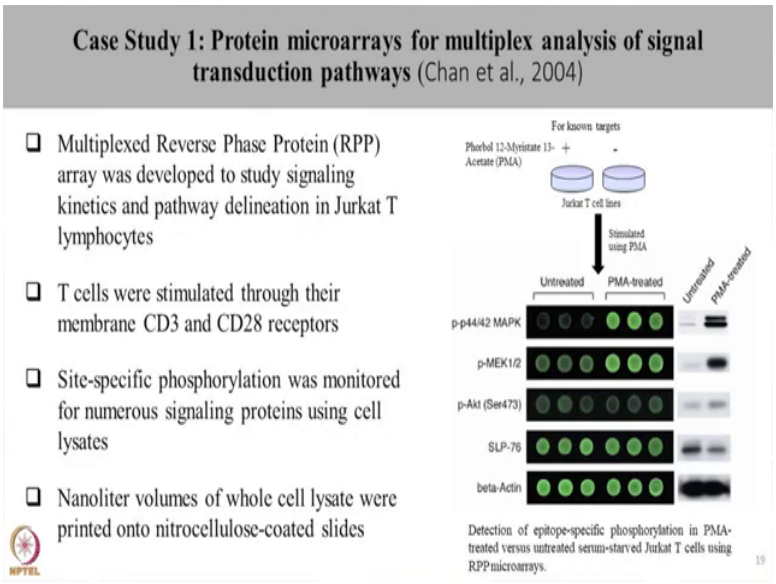

So, let us start with one of the case studies where Chan et al have used protein microarrays to understand the pathway of T lymphocytes upon activation with CD 3 and CD 28 antibodies. Chan et al have made multiplexed reverse phase protein microarray and these protein microarrays were used to study the pathways in T cells which were activated upon a stimulation with CD 3 and CD 28 molecules.

In this current study they monitored the site specific phosphorylation of numerous signaling molecules and they perform a time bound experiment to look into the pathways that are activated upon stimulation with the cell receptors.

So, to check whether this reverse phase protein microarray is working. They first took these Jurkat T cell lines and activated it with PMA. PMA is phorbol 12 myristate acetate, this PMA activates protein kinase C; this protein kinase C once activated leads to phosphorylation of

MAP K and MEK proteins. And hence the phosphorylation was studied, the cell lysate were taken and imprinted in triplicates for the untreated as well as PMA treated cell lines the phosphorylation of these proteins were studied using phosphor antibodies.

In this diagram you can see that map k showed a very good phosphorylation upon PMA treatment. Whereas, the untreated cells did not show any phosphorylation MEK also showed a differential phosphorylation upon PMA treatment. Whereas, Akt which is not a target of protein kinase C showed no change in the phosphorylation levels. SLP 76 and beta actin where users control and no changes were seen in the treated as well as the untreated cells.

The same was verified using western blot which is shown in this image. Once they were sure that this experiment is working they have taken the T Jurkat cell lines.

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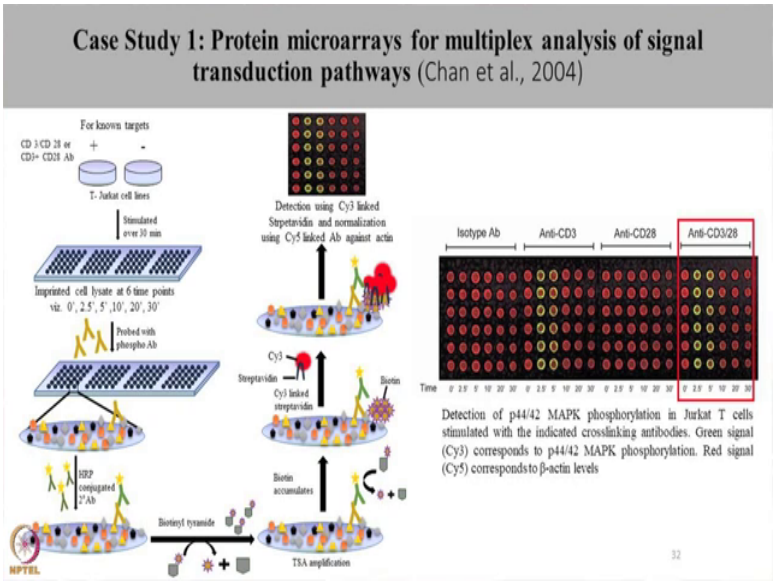

And they have treated it with CD 3 antibody CD 28 antibody or CD 3 and CD 28 antibody in combination. The cell lines were stimulated over the period of 30 minutes and the cell lysates were imprinted at 6 different time points. The time points were 0 minutes, 2.5 minutes, 5 minutes, 10 minutes, 20 minutes and 30 minutes. These cell lysates were then probed with phospho antibodies to look for the phosphorylation status further these slides were incubated with HRP conjugated secondary antibodies. Tyramide amplification was further performed in which HRP catalyzes accumulation of biotinyl tyramide.

This biotinyl tyramide was further detected using streptavidin which was labeled with cy three simultaneously these arrays were also probed with cy 5 length antibodies to detect the level of atom in the cell lysate. Here in the array picture you can see the red spot shows the atom level whereas, the green spots show the phosphorylation status of the cells. This is one of the summary where the phosphorylation of MAPK was studied, the cells were treated with isotype antibodies which acted as control. The cells were treated with CD 3 antibody CD 28 antibody and CD 3 and CD 28 antibody in combination.

Here you can see that when the cells were treated with CD 3 there was a quick phosphorylation observed at 2.5 minutes which reduced at 5 minutes. But there was no change in the phosphorylation status of MAP K when the cells were treated with CD 28 antibodies. When the cells were treated with the combination of CD 3 and CD 28 as a state phosphorylated MAP K was observed and the signal intensity even at 5 minutes was prominent. Further to study the signal transduction kinetics the authors study the phosphorylation level of phospholipase C gamma protein.

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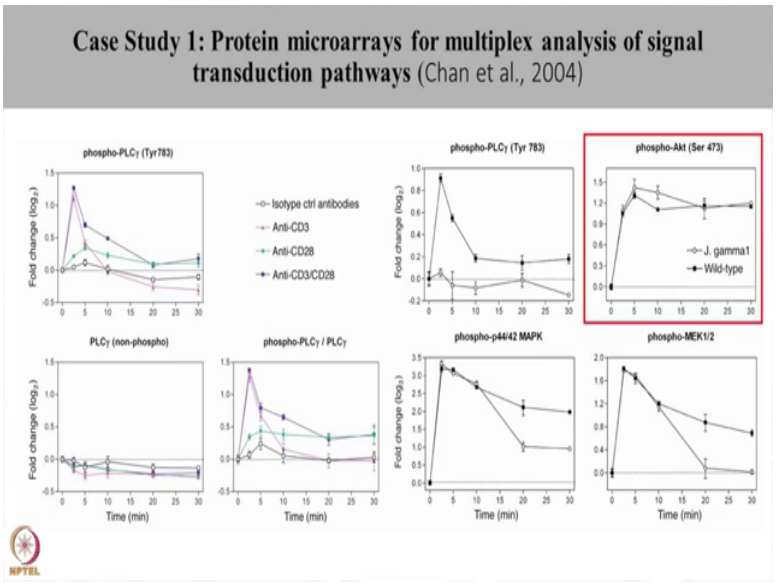

In this, again the cell line toward treated at different time points and cells were treated with isotype antibodies as control with CD 3 antibody CD 28 antibody and a combination of CD 3 and CD 28 antibodies. Also the protein microarray was probed with non phospho antibodies to study the overall concentration of phospholipase c in the cell lysate.

The graph shown here shows the adjusted level of phosphorylated PLC gamma if the total phospholipase c present in the cell. So, in this graph you can see the phosphorylation kinetics did not change when the cells were treated with isotype control antibodies. However, when the cells were treated with anti CD 3 antibodies, a quick phosphorylation was seen at around 2.5 minutes which then dropped at 10 minutes and reached the baseline level.

The green line shows the phosphorylation level of PLC upon treatment with CD 28. Here you can see that although the phosphorylation was less, but then it was sustained till 30 minutes

when the cell lines were treated with both CD 3 and CD 28 antibodies a quick increase in the phosphorylation status of PLC was seen at 2.5 minutes similar to CD 3.

However this phosphorylation sustain and reached the level which was similar to the level that was obtained upon activation with CD 28 to further delineate the signaling pathways or cell line that is j gamma 1 which is a mutant cell line of Jurkat T cells which do not have the phospholipase C was used and wild type T Jurkat cells were seen to study the phosphorylation kinetics of the downstream signaling components.

In this case J gamma 1 cell lines and the wild type cell lines were treated with CD 3 and CD 28 antibody in combination, different time points were studied. In the first graph you can see that the levels of phospho PLC showed an increased phosphorylation at around 5 minutes.

Now, which gradually decreased; however, there was no change in phosphorylation of status seen in the J gamma 1 cell lines. Confirming that these cell lines are deficient in PLC. Further the phosphorylation status of MAPK and MEK were studied and here we can see that in the wild type cell lines, the phosphorylation of MAPK and MEK sustained over 30 minutes; however, in case of mutant cell lines the phosphorylation dropped drastically upon 20 minutes. Stating that phosphorylation of status of MAPK and MEK is dependent on the presence of phospholipase c gamma protein.

Whereas when the phosphorylation status of AKT was seen no change in the phosphorylation status was seen in the wild type as well as in the mutants cell lines. This infers that the presence of phospholipase c does not affect the phosphorylation kinetics of AKT protein. To further understand the signaling events in the T cells Chan et al activated T Jurkat cell lines with CD 3 antibody and with CD 3 and CD 28 antibody in combination.

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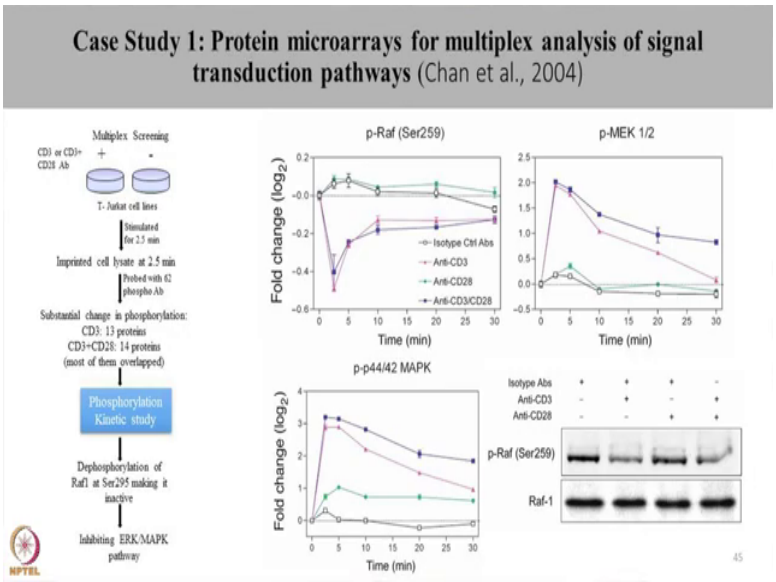

These cell lysates were stimulated for 2.5 minutes and were imprinted in 6 replicates onto the nitrocellulose membrane coded slides. Further the phosphorylation status for the 62 proteins were studied using phosphor antibodies upon activation with CD 3 13 proteins showed a substantial change and phosphorylation when the cells were stimulated using CD 3 and CD 28 14 proteins showed change in phosphorylation of which most of these proteins showed an overlap.

In this study they identify that the Raf 1 protein should de phosphorylation upon stimulation with the antibodies. Further to study the phosphorylation kinetics of Raf protein and its downstream signaling pathway, the cells were stimulated using different combinations of antibodies upon 2.5 minutes steep de phosphorylation of Raf protein was seen in cell lines treated with CD 3 antibody and CD 3 antibody in combination with CD 28 antibodies.

There was no chain seen in the phosphorylation level of Raf protein when the cells were treated with CD 28 antibody. Further, they studied the phosphorylation patterns of MEK and MAPK proteins this phosphorylation pattern matched well with the de phosphorylation patterns of Raf protein. And as the Raf protein de phosphorylated increase phosphorylation of MEK and MAP K seen at 2.5 minutes which substantially degraded over the period of 30 minutes in the cells treated with CD 3 antibodies and the cells treated with CD 3 and CD 28 antibodies in combination.

This de phosphorylation of Raf was further cross checked using western blot to conclude Chan et al studied time dependent phosphorylation kinetics of several downstream signaling molecules in a time dependent manner.

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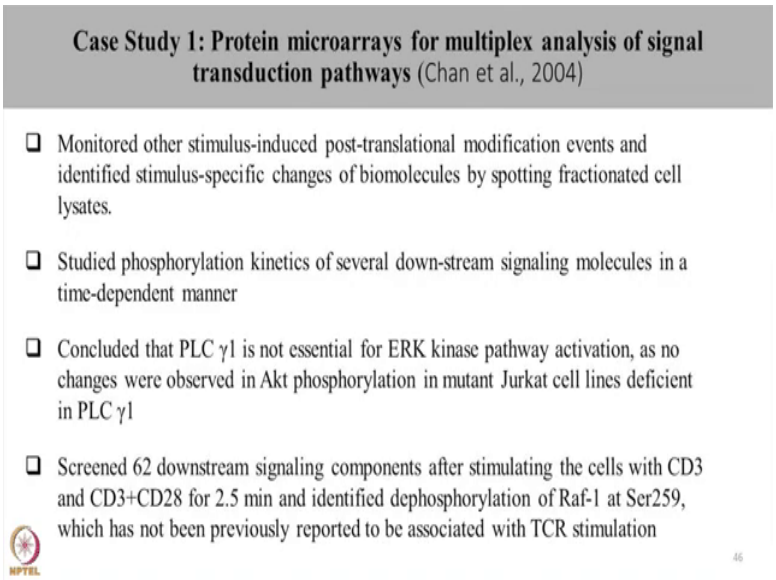

They concluded that PLC gamma 1 is not essential for the ERK kinase pathway as there was no chain seen in the phosphorylation of Akt in the cells that were deficient in phospholipase C protein. Also they screen the phosphorylation level of 62 downstream signaling proteins and identified an novel instance where Raf 1 showed de phosphorylation upon T cell receptor stimulation.

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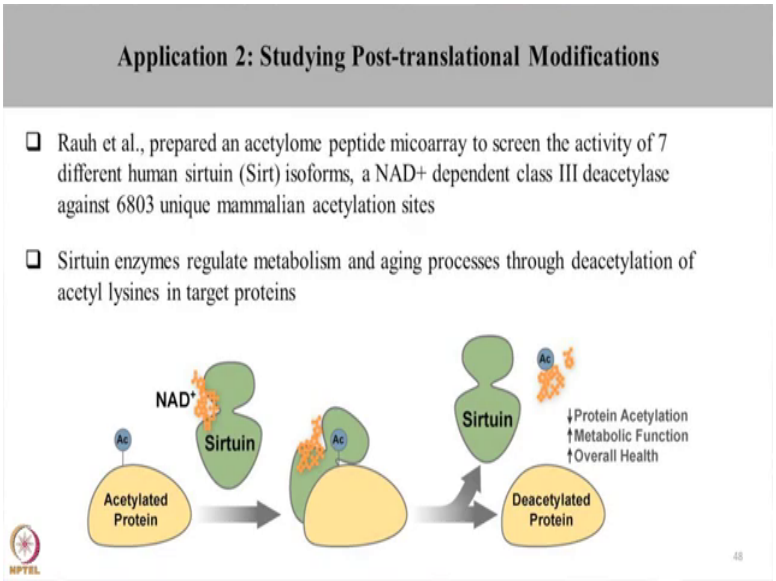

Now, going ahead to the another study where Rauh et al prepared an acetylone peptide microarray to screen the activity of 7 different isoforms of sirtuin against 6800 unique mammalian acetylation sites. Sirtuins are the enzymes which de acetylate the lysine residues in the presence of NAD. In this study database search was performed to look for all the conserved acetylation sites in the mammalian system.

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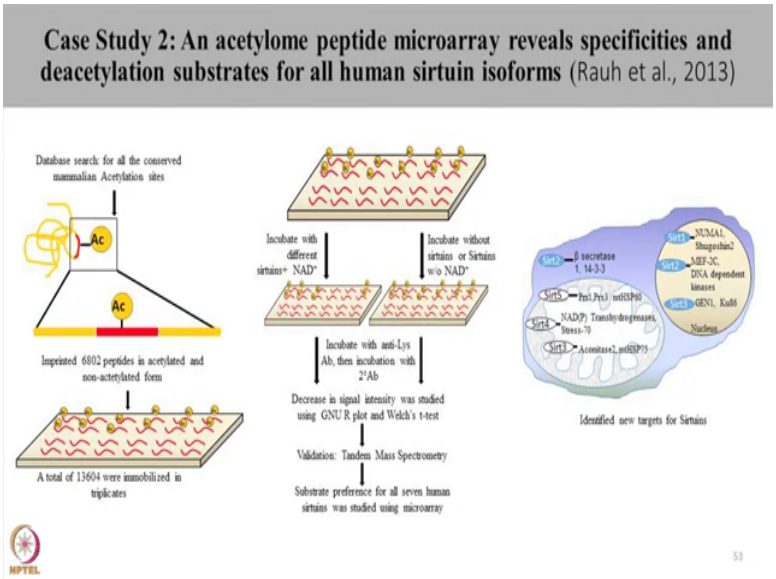

A total of 6802 peptides in its acetylated and non acetylated forms were imprinted. The peptides that were used here had lysine at the 7 position which was flanked by 6 amino acids at the upstream as well as at the downstream. So, therefore, a 13 more peptides were imprinted onto the arrays and a total of 13604 peptides were immobilized in triplicates. This peptide arrays were further incubated with different isoforms of sirtuins and without sirtuins and they were also incubated with and without NAD to check for the activity of sirtuins.

These peptide arrays were further probed with primary and secondary antibody to look for the change in the level of acetylation upon incubation with sirtuins. The decrease in the signal intensity of acetylation was further calculated using Welch's t test, these acetylation patterns were also validated using mass spectrometry and this study resulted in identification of

substrate preferences for different sirtuins isoforms. Further, this a study ended up in identification of new targets for sirtuins.

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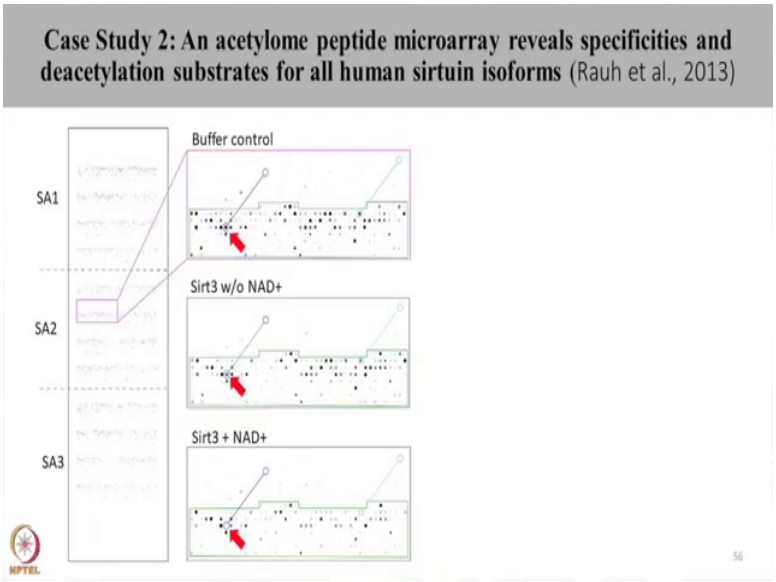

So, first in this study, what they have done is they have printed an array SA represents sub array. These sub arrays were treated with buffer control where no sirtuins were used. These arrays were also treated with sertuins with and without NAD. Since sirtuins need NAD for their activity, there shall be no de acetylation seen when there is no NAD present.

Here you can see that there was no change in signal in buffer control as well as when the array was treated with sirtuins without NAD. Whereas, a loss and signal intensity was seen when the arrays were treated with sirtuin in presence of NAD. Concluding that sirtuins need NAD for de acetylation and also confirming that de acetylation is happening on the array.

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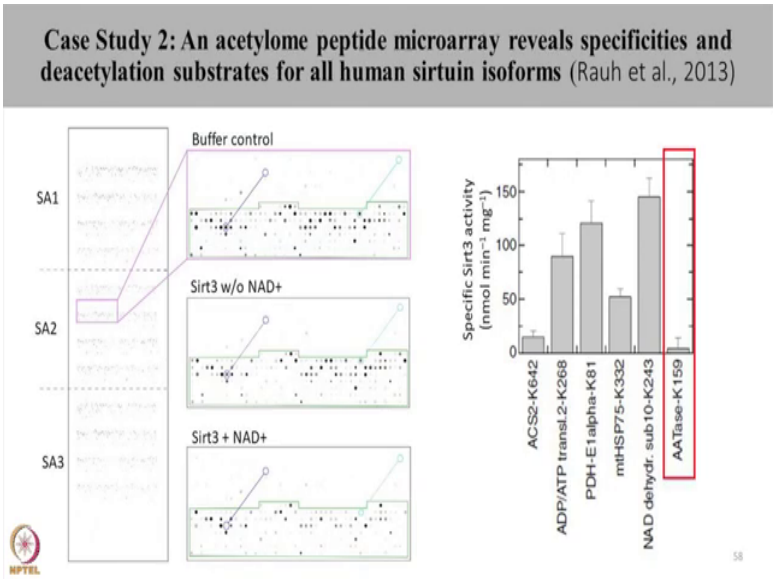

This graph represents the specific activity of sirtuins 3, how the de acetylation is happening for different peptides. The last protein that is AA Tase K was used as a negative control and has shown no change in the acetylation pattern.

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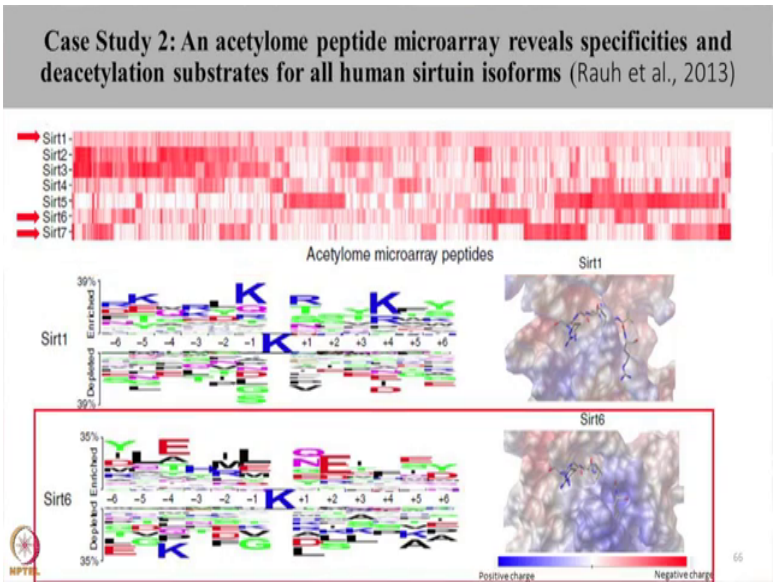

This heat map shows the de acetylation activity of all the 7 isoforms of sirtuins across 6800 peptides sirtuin 1, section 7 locates at the nucleus. However when you look at the target peptides there is a specific pattern of preference of these peptides for sirtuins 6, 7 and 1. If you look properly at sirtuin 6 and sirtuin 7 a specific de acetylation pattern is seen which signifies that these 2 isoform has their specific targets whereas, sirtuin 1 shows a nominal activity across a wide range of substrate in the nucleus.

This logo here shows the peptide that were used the upper panel of the logo that is the enrich section shows the preferred amino acids for each sirtuin, whereas the lower bottom shows depleted amino acid sequence that is the sequence that do not favor de acetylation.

Also the right panel shows the peptide binding groups of these sirtuin molecules whereas, the blue region signifies the positively charged amino acids and the red region shows the

negatively charged amino acids. The peptide preference for sirtuin one is majorly positively charged amino acids that is argenine or lysines as the core has negative charge; therefore, sirtuin 1 prefers the peptides that have positive charge specifically at position minus 5, minus 1, 1 and 4.

Whereas the peptide binding group of sirtuin 6 is majorly hydrophobic and hence the peptide sequence that are specific to sirtuin 6, majorly have hydrophobic residue specifically at minus 1, minus 2, plus 3 and plus 4 sites. However, at plus 2 and minus 4, there is a negatively charged residue. Coming to the another isoforms of sirtuins that is 3 4 and 5 which locates at the mitochondria.

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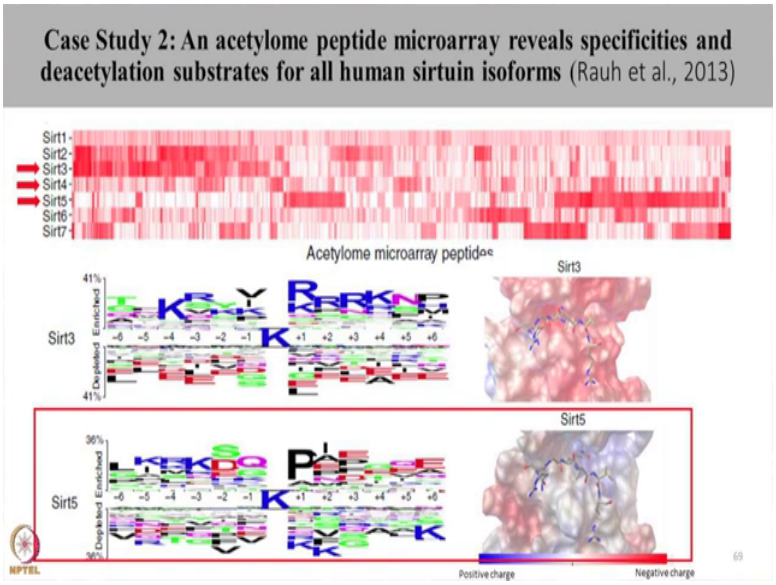

Here also you can see that there is a huge change in the preferences of the peptides that are selected by all the three different isoforms. In this you can see that the peptide binding group

for sirtuin 3 is highly negative owing to that the peptides that are preferred by sirtuin 3 had a lot of positively charged residue specifically argenine at the upstream of the acetylated lysine.

For sirt 5 you can see that at position 1, proline was predominantly present the other upstream amino acids were either positively charged or nonpolar whereas, the downstream amino acids were majorly positively charged.

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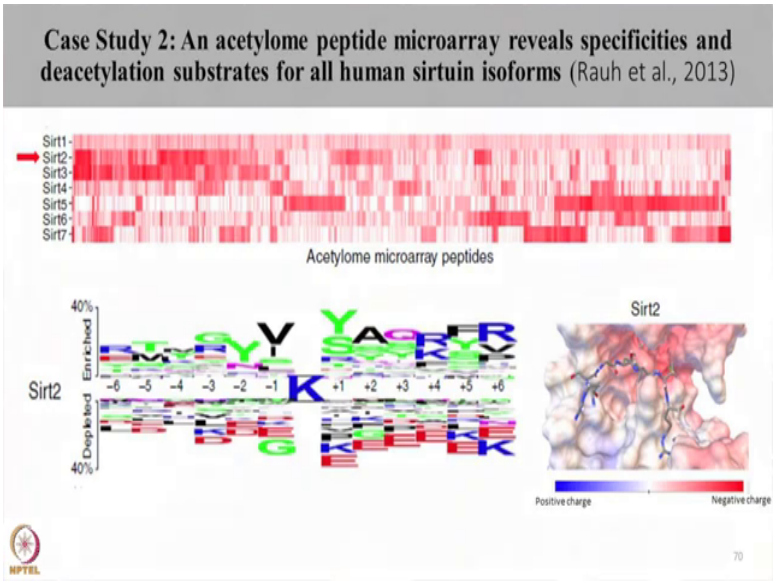

Coming to the another sirtuin that is sirt 2 which is majorly found in the cytosolic region of the cell you can see that the peptide binding group for sirtuin 2 is highly negative and therefore, it does favors the presence of negatively charged amino acids in the sequence preference. Except for sirtuin 4 all other sirtuins have some or the other targets for de acetylation; however, there was no identified substrate for sirtuin 4.

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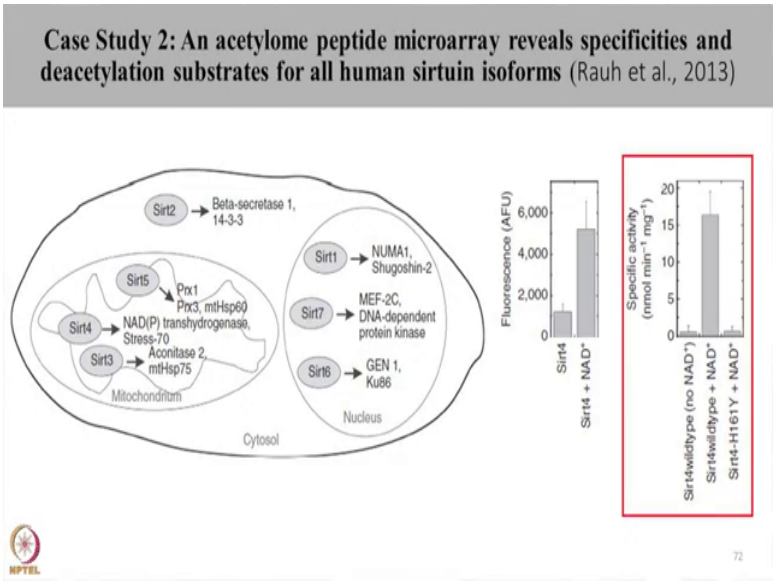

In the fluorescence sirtuin 4 showed a low, but consistent de acetylation activity upon incubation with NAD. From this study, NADP trans hydrogen is a stress 70 protein was found to be one of the substrate a specific for sirtuin 4. To confirm this de acetylation activity of sirtuin 4 the de acetylation of NADP trans hydrogen is stress 70 protein was performed using wild type sirtuin without NAD, wild type sirtuin with NAD and a mutant sirtuin which do not have the de acetylate activity.

When the substrate was incubated with wild type sirtuin 4 and with NAD a strong de acetylation activity was seen confirming that NADP trans hydrogen is a stress 70 protein is a substrate for sirtuin 4.

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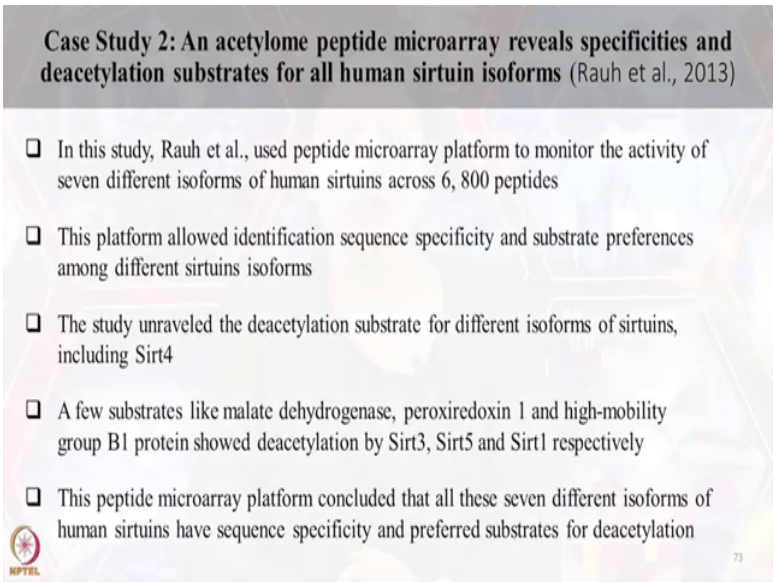

To conclude this study has used microarray platform to parallely screen around 6000 peptides for 7 different isoforms of sirtuin. Using this platform, the authors were able to identify the sequence of specificity for different isoforms of sirtuins. They also identified a substrate for sirtuin 4 which is NADP trans hydrogen is a stress 70 protein, further they confirmed that malate dehydrogenase protein is one of the target of sirtuin 3 and peroxiredoxin 1, peroxiredoxin 3 and mitochondrial protein HSP 60 are targets for sirtuin 5.

This peptide micro array platform concluded that all these different 7 isoforms of human sirtuins have a sequence of specificity and preferred substrate for de acetylation. To conclude protein micro arrays holds immense potential in identifying new targets in delineating the pathway and hence providing a deeper insight into the signaling kinetics at cellular level.

Thank you.

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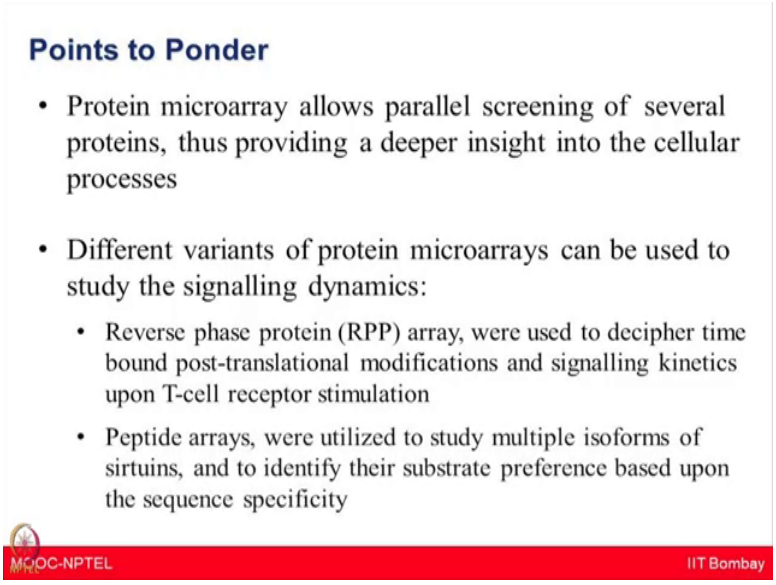

I hope by now, you understood that there are wide applications of protein microarray could be achieved especially from different type of protein microarray based technology platforms in the areas of detection for novel protein interactions, post transition modification in high throughput manner. These examples would have also provided you an insight into the utility of protein micro array based technologies for screening several proteins in parallel providing a holistic understanding of the post transition modification and signaling networks.

In the next lecture; we will talk about how to make arrays and print your own chip using novel printing technologies. So, I will see you next lecture and we will talk to you about the latest

advancements in this area and how you can make your own arrays and recent developments in the areas of printing technologies.

Thank you.