NATIONAL PROGRAMME ON TECHNOLOGY ENHANCED LEARNING (NPTEL)

Applications of Interactomics using Genomics and Proteomics technologies

Course Introduction by Prof. Sanjeeva Srivastava

MOOC-NPTEL

Applications of Interactomics using Genomics and Proteomics Technologies

Lecture-15
Applications of Protein microarrays in Cancer Research-11

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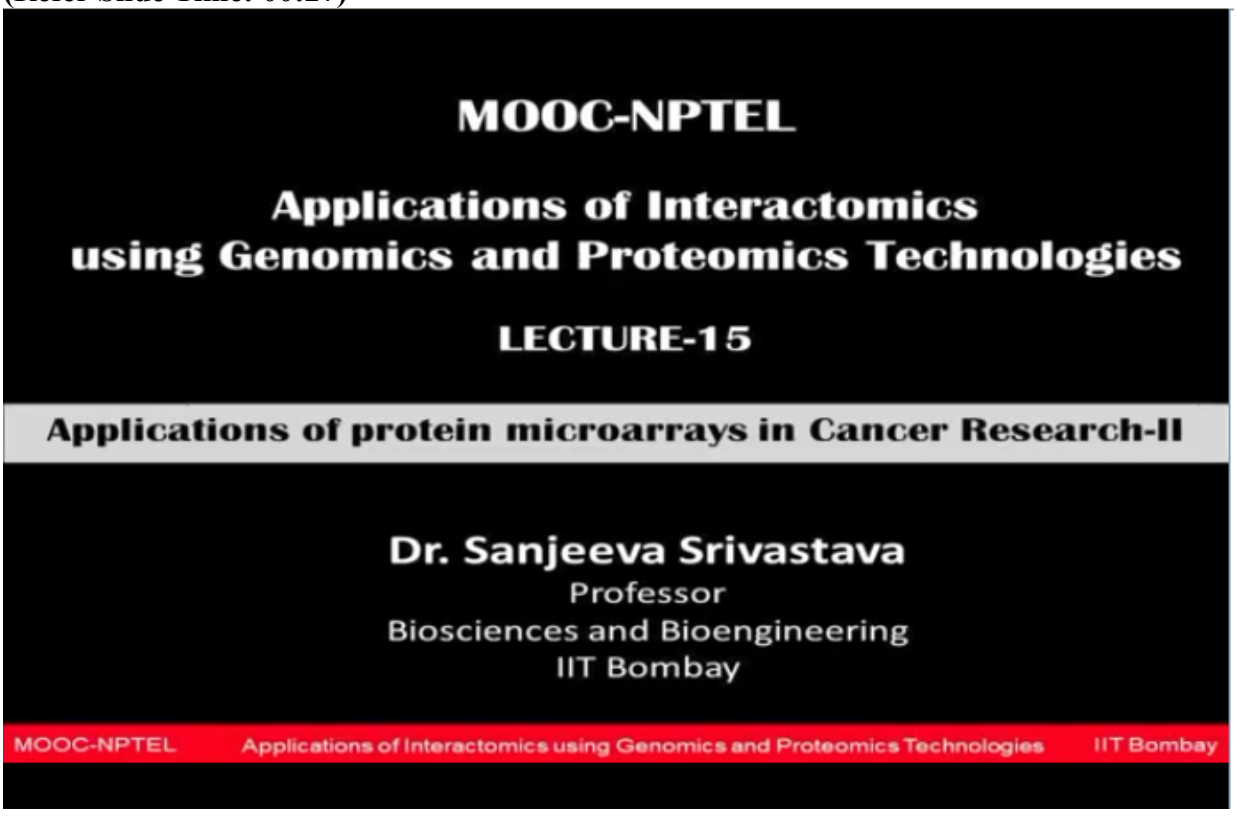

Welcome to MOOC course on applications of interactomics using genomics and proteomics technologies. In the last lecture you are exposed to one of the applications of protein microarrays and the protocol used to screen the presence of tumor associated autoantibodies.

As you know screen for TA is or tumor associated autoantibodies is a novel concept with the aim is to detect the autoantibodies or empty bodies produce in the body much ahead of time, and they have lot of clinical territory 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 the remaining applications which are possible on different type of protein microarray based technology platforms, this lecture this also provide you understanding for novel applications for doing various protein interaction studies, post translation modification kind a substrate screening etcetera using high throughput microarray based platforms. So let's welcome Nikita for today's lecture.

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Ms. Nikita Gahoi: 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 autoantibodies in the biofluids of cancer patients.

In this lecture we will further look into the applications of protein microarrays that can be used to understand the signaling network and to understand the time on post translational modification happening at the cellular level.
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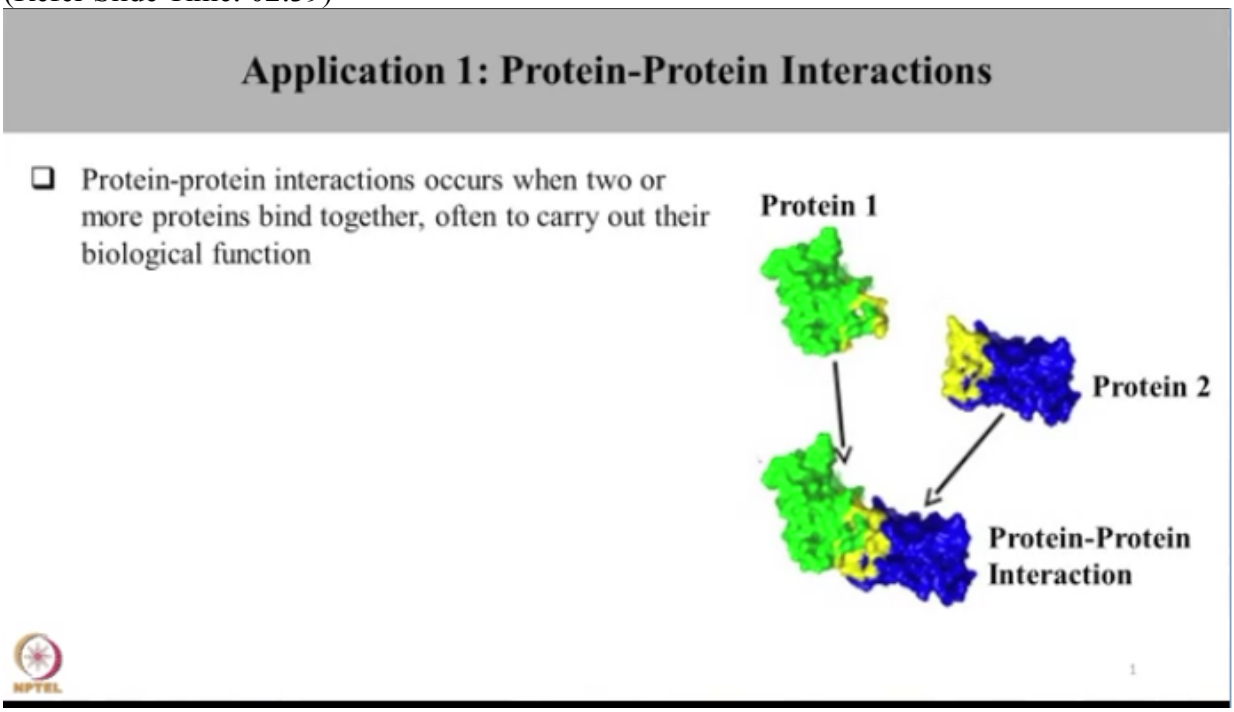

Protein-protein interaction occurs when two or more proteins interact with each other to carry out a biological function, these interactions mediate several cellular processes and understanding these interactions would help in understanding the function of proteins and to identify the disease pathobiology,
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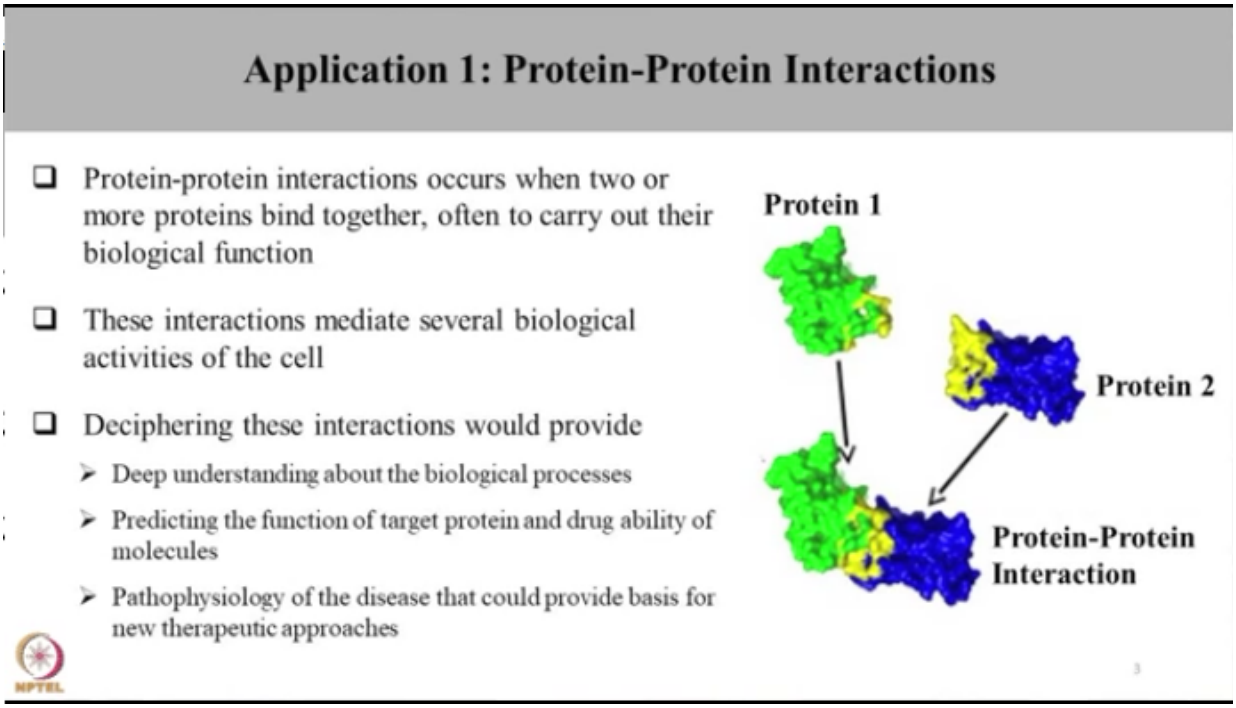

so once we dissect these interactions we can end up findings few druggable targets and new therapeutic approaches that can be used to treat the disease.

Traditional approaches like yeast-two hybrid system,
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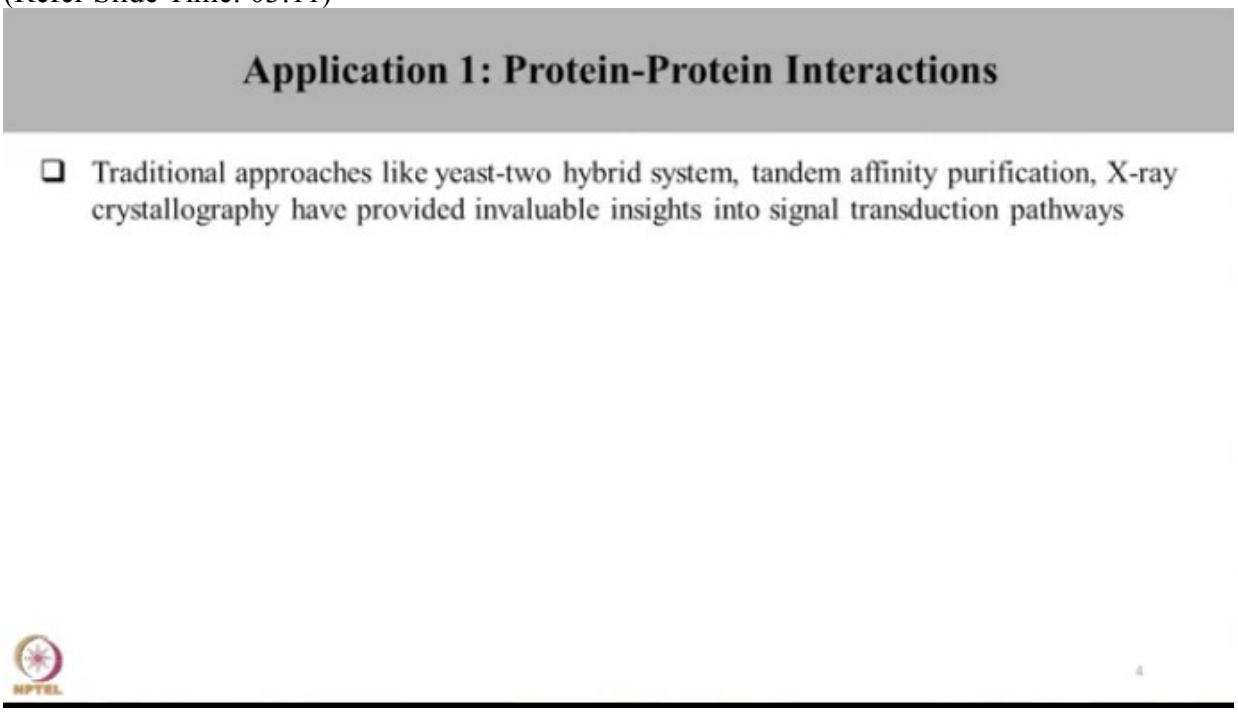

tandem affinity chromatography etcetera have provided invaluable insight into the protein-protein interaction, however these techniques just look at one or two proteins, study these proteins in isolation and sometimes even end up giving false positive results.

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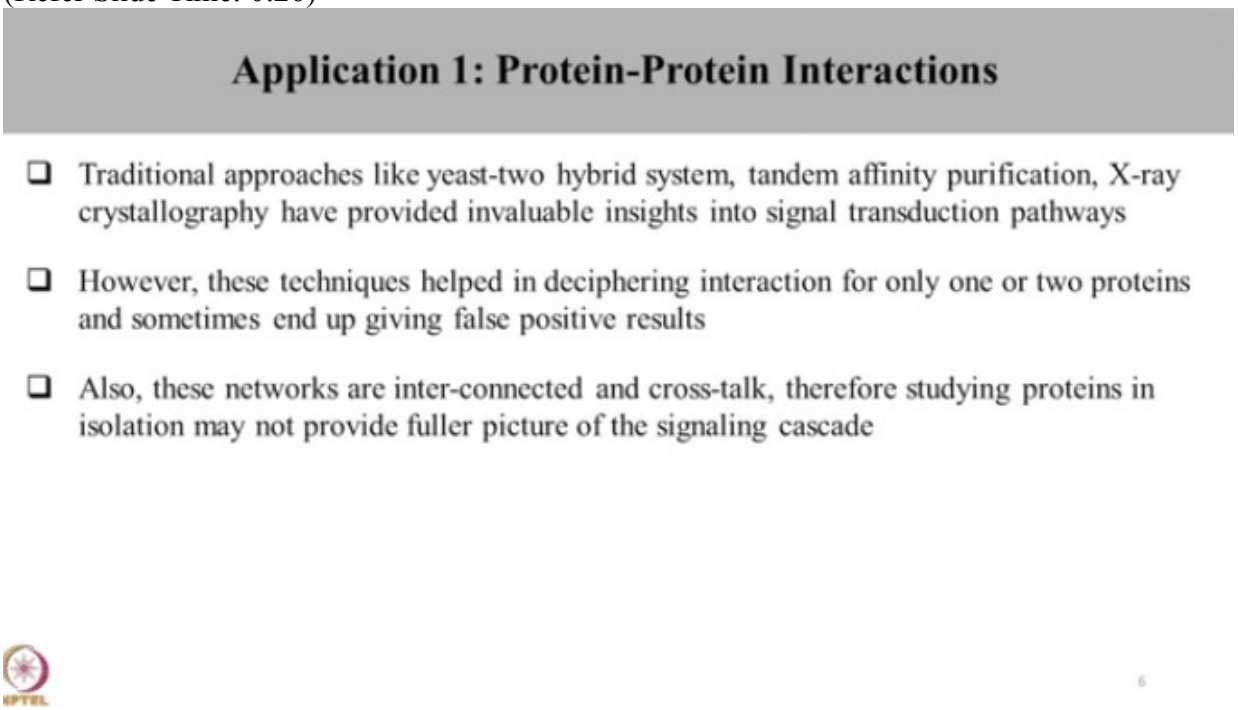

These networks, the signaling pathways are dynamic therefore studying of protein in isolation might not provide a fuller picture of the interacting pathway,
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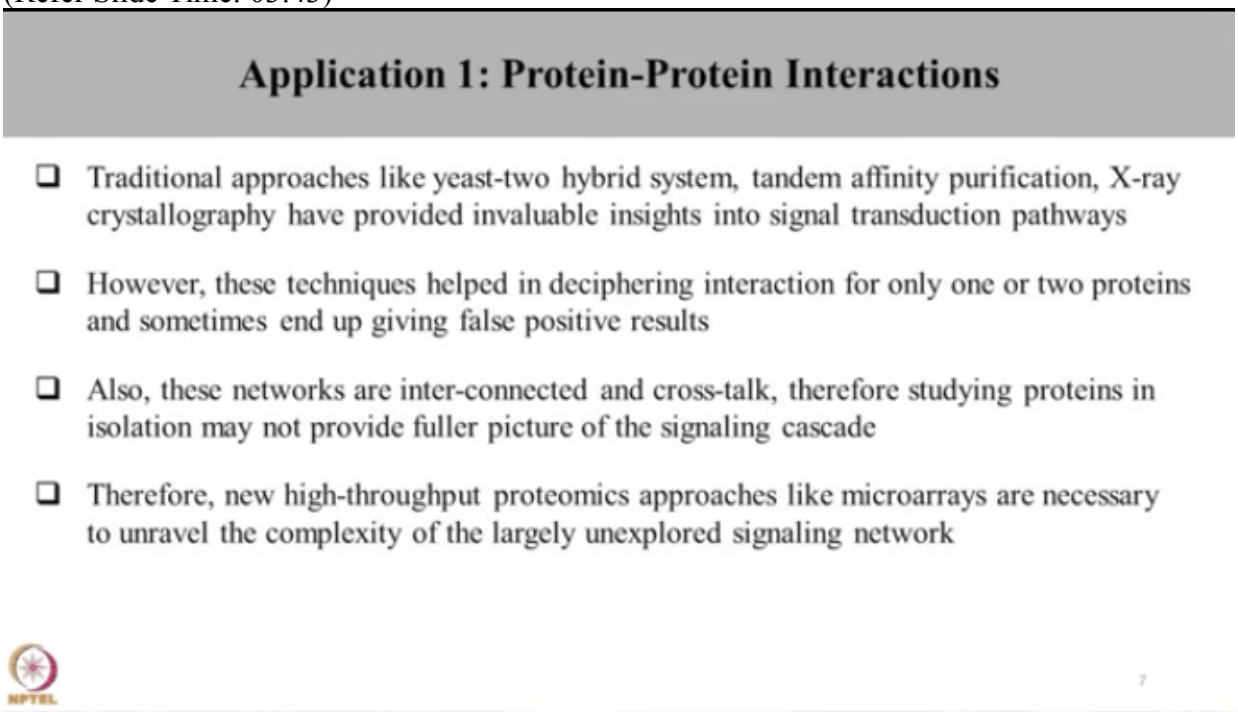

therefore high throughput platforms like protein microarray can hold immense value to screen multiple proteins together,
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Application 1: Protein-Protein Interactions

- Traditional approaches like yeast-two hybrid system, tandem affinity purification, X-ray crystallography have provided invaluable insights into signal transduction pathways
- However, these techniques helped in deciphering interaction for only one or two proteins and sometimes end up giving false positive results
- Also, these networks are inter-connected and cross-talk, therefore studying proteins in isolation may not provide fuller picture of the signaling cascade
- Therefore, new high-throughput proteomics approaches like microarrays are necessary to unravel the complexity of the largely unexplored signaling network
- Microarrays is a powerful tool to understand multiple facets of signaling network as it allows simultaneous screening of multiple proteins

and hence can be used to decipher the protein-protein interactions.

So let's start with one of the case studies where Chan Et Al have used protein microarrays to understand the pathway of T emphasize upon activation with CD3 and CD28 antibodies. Chan Et Al have made multiplexed reverse phase protein microarray and these protein microarrays were used to study the pathways enthesis which were activated upon a stimulation with CD3 and CD28 molecules.

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Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)

- Multiplexed Reverse Phase Protein (RPP) array was developed to study signaling kinetics and pathway delineation in Jurkat T lymphocytes
- T cells were stimulated through their membrane CD3 and CD28 receptors
- Site-specific phosphorylation was monitored for numerous signaling proteins using cell lysates

In this current study the monitor the site-specific phosphorylation of numerous signaling molecules and they performed a time bomb 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 T cell lines and activated it with PMA.
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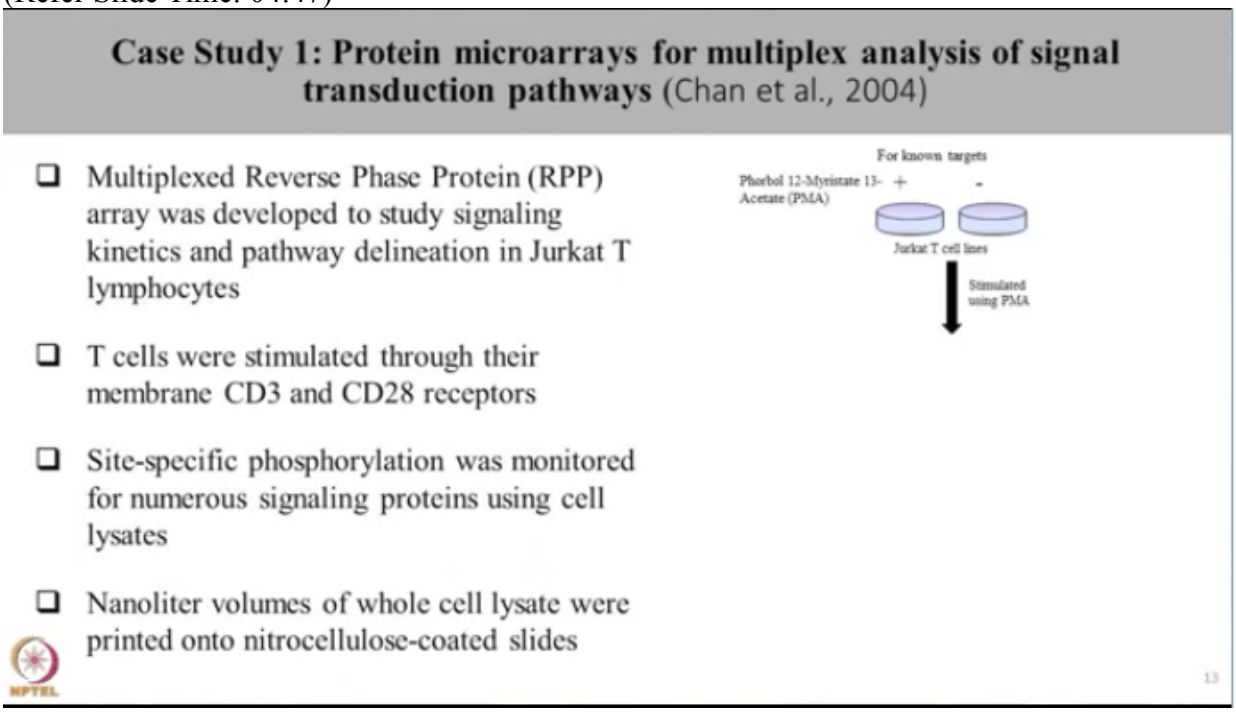

PMA is for Phorbol 12, Myristate, Acetate, this PMA activates protein kinase C, this protein kinase C once activated it leads to phosphorylation of MAPK and MEK proteins, and hence this phosphorylation was studied, the cell lysates were taken and printed in triplicates for the un-treated as well as PMA treated cell lines.
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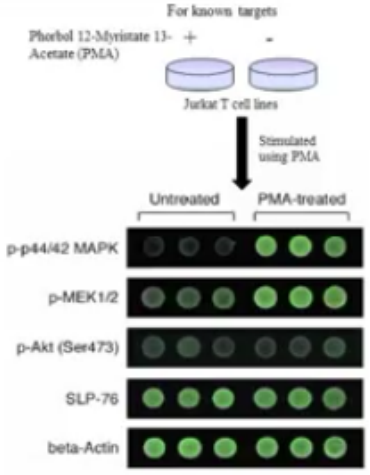
Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)

- Multiplexed Reverse Phase Protein (RPP) array was developed to study signaling kinetics and pathway delineation in Jurkat T lymphocytes
- T cells were stimulated through their membrane CD3 and CD28 receptors
- Site-specific phosphorylation was monitored for numerous signaling proteins using cell lysates
- Nanoliter volumes of whole cell lysate were printed onto nitrocellulose-coated slides



Detection of epitope-specific phosphorylation in PMA-treated versus untreated serum-starved Jurkat T cells using RPP microarrays.

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The phosphorylation of these proteins were studied using phospho antibodies, in this diagram you can see that MAPK 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 were use this 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. (Refer Slide Time: 05:42)

Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)

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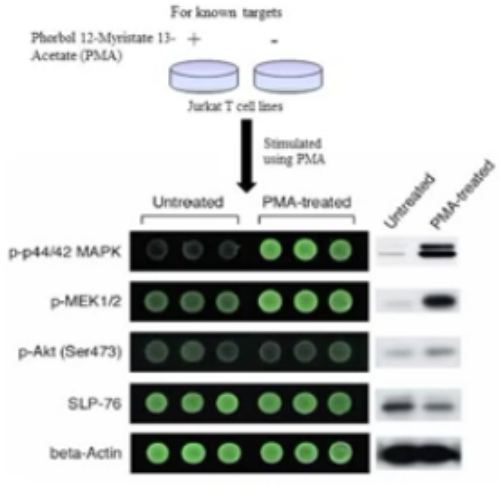

Detection of epitope-specific phosphorylation in PMA-treated versus untreated serum-starved Jurkat T cells using RPP microarrays.

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Once they were sure that this experiment is working they have taken the T Jurkat cell lines and they have treated with CD 3 antibody, CD28 antibody or CD3 and CD28 antibody in combination. The cell lines were stimulated over the period of 30 minutes and this cell lysates were imprinted at 6 different time points.

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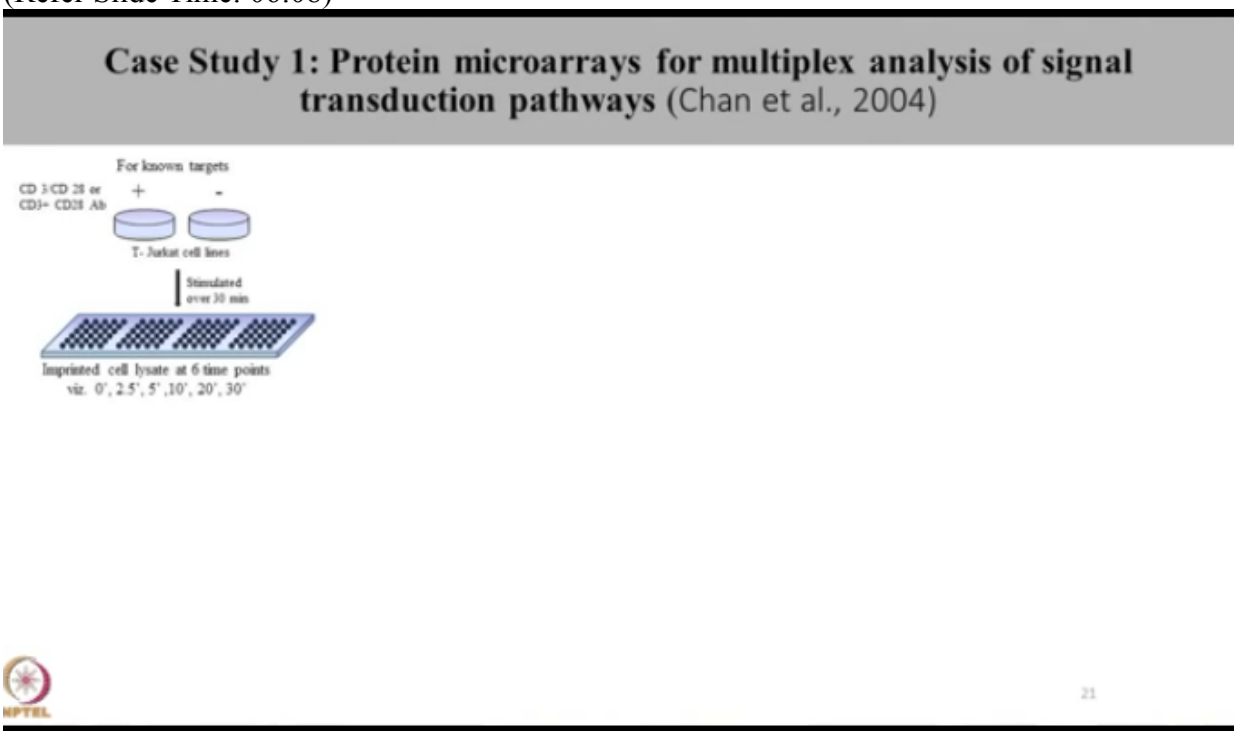

The time points were 0 minutes, 2.5 minutes, 5 minutes, 10 minutes, 20 minutes and 30 minutes, these cell lysates were then probed with phospho antibodies to look for the phosphorylation status.

Further these slides were incubated with HRP conjugated secondary antibodies. Tyramide amplification were further performed in which HRP catalyzes accumulation of biotinylated tyramide, this biotinylated tyramide was further detected using streptavidin which was labeled with Cy3.

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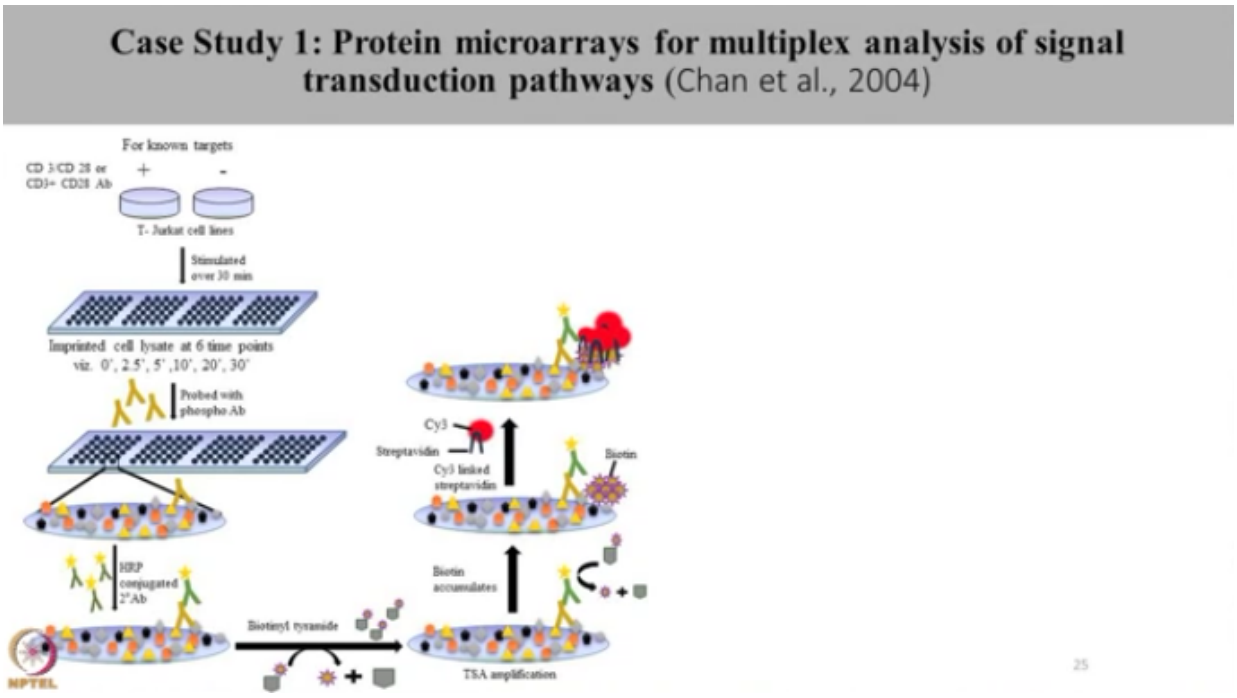

Simultaneously these arrays were also probed with Cy5 link antibodies to detect the level of atom in the cell lysates. Here in the array picture you can see the red spot shows the Et Al level
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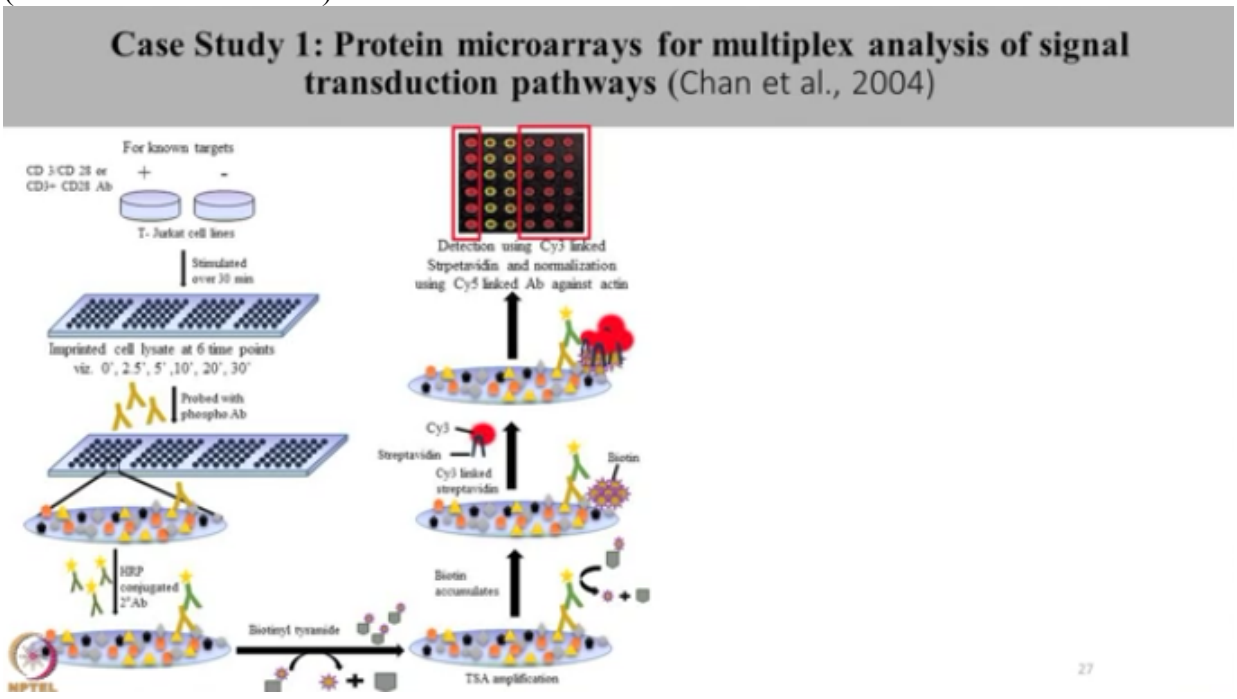

whereas the green spots showed the phosphorylation status of the cells, this is one of the sub-array where the phosphorylation of MAPK was studied, the cells were treated with isotype antibodies which acted as control,
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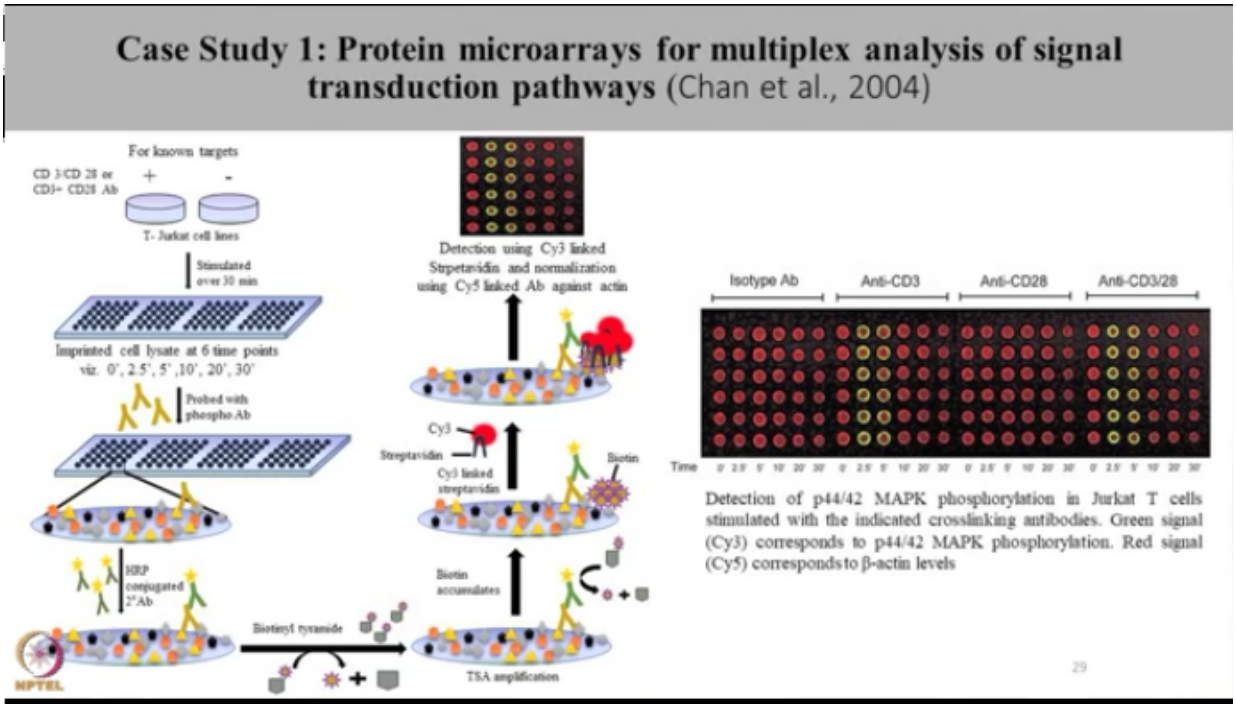

the cells were treated with CD3 antibody, CD28 antibody and CD3 and CD28 antibody in combination.

Here you can see that when the cells were treated with CD3 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 MAPK when the cells were treated with CD28 antibodies.

When the cells were treated with the combination of CD3 and CD28, as a stain phosphorylated MAPK was observed, and the signal intensity even at 5 minutes was prominent. Further to study this signal transduction kinetics of the authors study the phosphorylation level of phospholipase C gamma protein,
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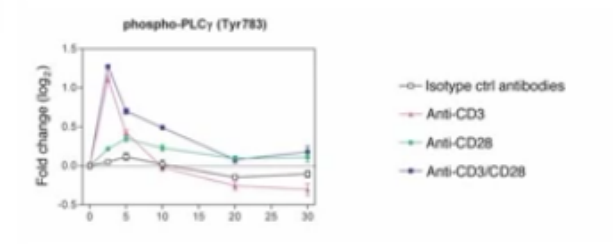
Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)



in this again the cell line were treated at different time points and cells were treated with isotype antibodies as control with CD3 antibody, CD28 antibody and a combination of CD3 and CD28 antibodies also the protein microarray was probed with non-phospho antibodies to study the overall concentration of phospholipase C in the cell lysate.

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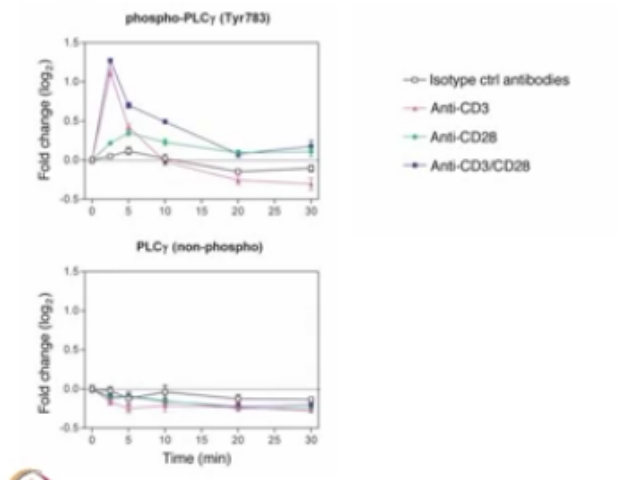
Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)



The graph shown here shows the adjusted level of phosphorylated PLC gamma, so the total phospholipase C present in the cell,

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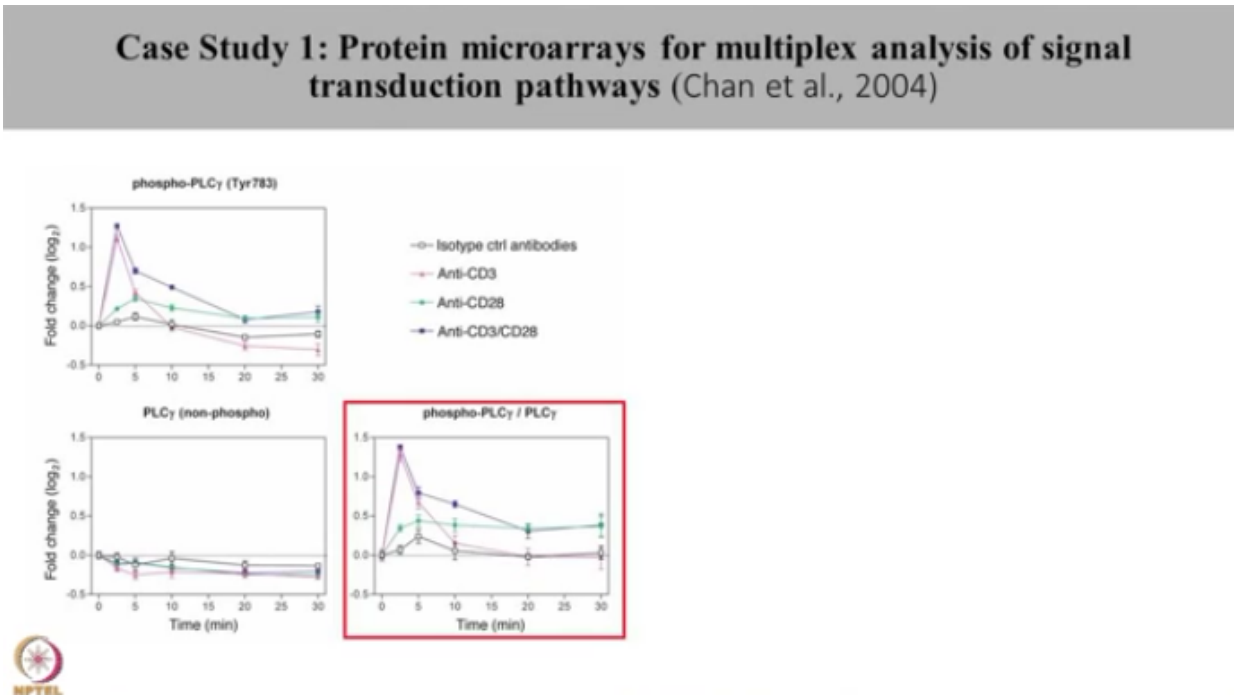

so in this graph you can see the phosphorylation kinetics did not change when the cells were treated with isotype control antibodies, however when the cells were treated with anti-CD3 antibodies, a quick phosphorylation was seen at around 2.5 minutes which then dropped at 10 minutes and reach the baseline level.

The green line shows the phosphorylation level of PLC upon treatment with CD28, 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 CD3 and CD28 antibodies a quick increase in the phosphorylation status of PLC were seen at 2.5 minutes, similar to CD3, however this phosphorylation sustained and reach the level which was similar to the level that was obtained upon activation with CD28, to further daily need the signaling pathways a 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 while type T Jurkat cells were seen to study the phosphorylation kinetics of the downstream signaling components.
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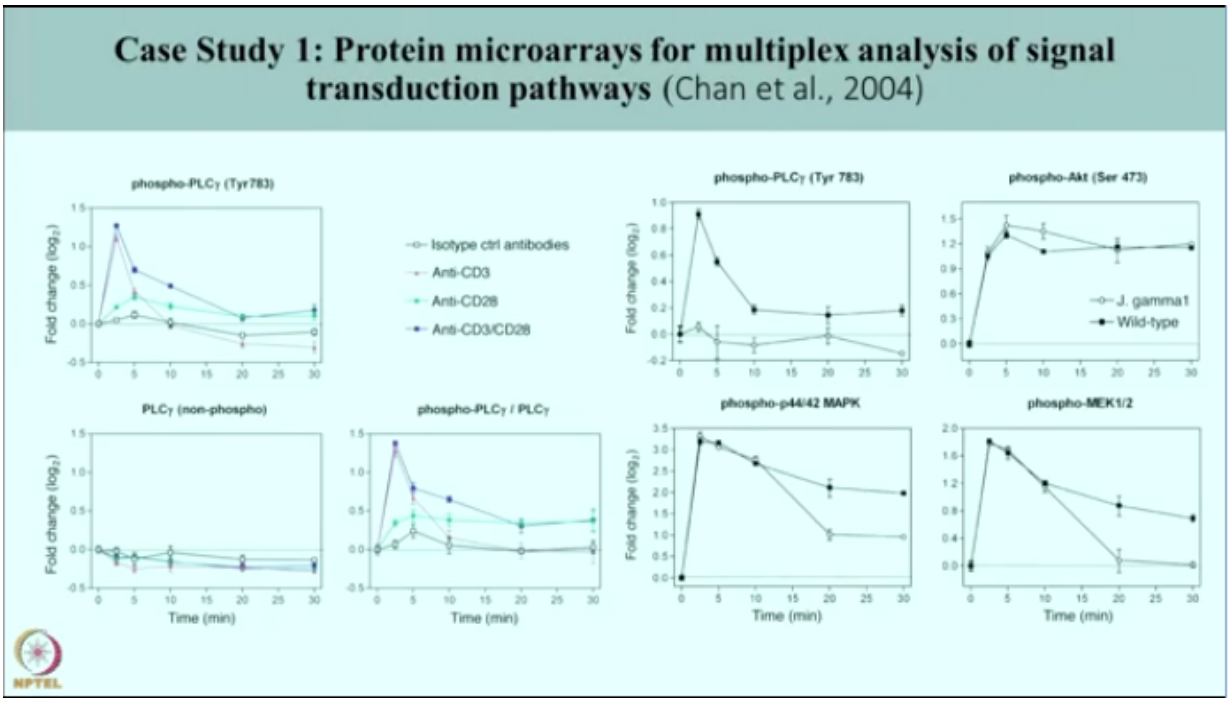

In this case J gamma 1 cell lines and the wild type cell lines were treated with CD3 and CD28 antibody in combination. Different time points were studied in the first graph you can see that the levels of phospho PLC should had increase phosphorylation at around 5 minutes, now which you gradually decreased, however there was no change in phosphorylation 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 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 mutant cell lines. These enforced that the presence of phospholipase C does not affect the phosphorylation kinetics of AKT protein.

To further understand the signaling events and the T cells Chan Et AL activated T Jurkat cell lines with CD3 antibody and with CD3 and CD28 antibody in combination,
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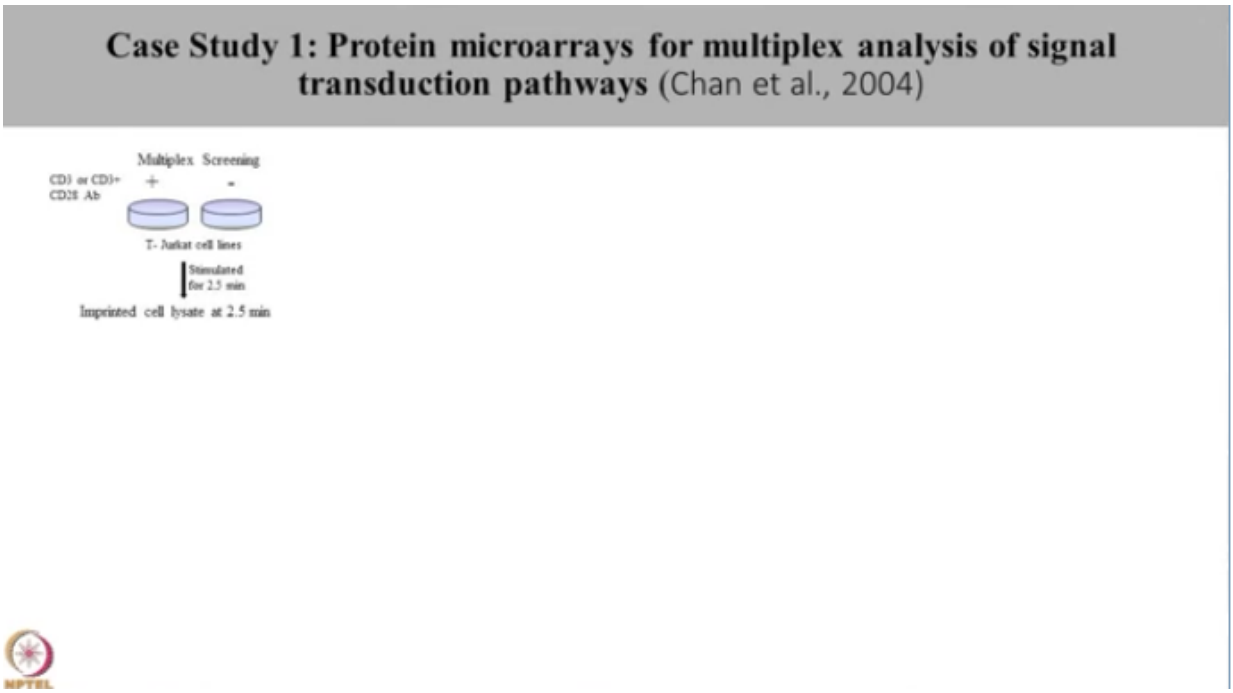

these cell lysates were stimulated for 2.5 minutes and were imprinted in 6 replicates on to the nitrocellulose membrane coated slides, further the phosphorylation status for these 62 proteins were studied using phospho antibodies. Upon activation with CD3, 13 protein showed a substantial change in phosphorylation, when the cells were stimulated using CD3 and CD28, 14 protein 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,
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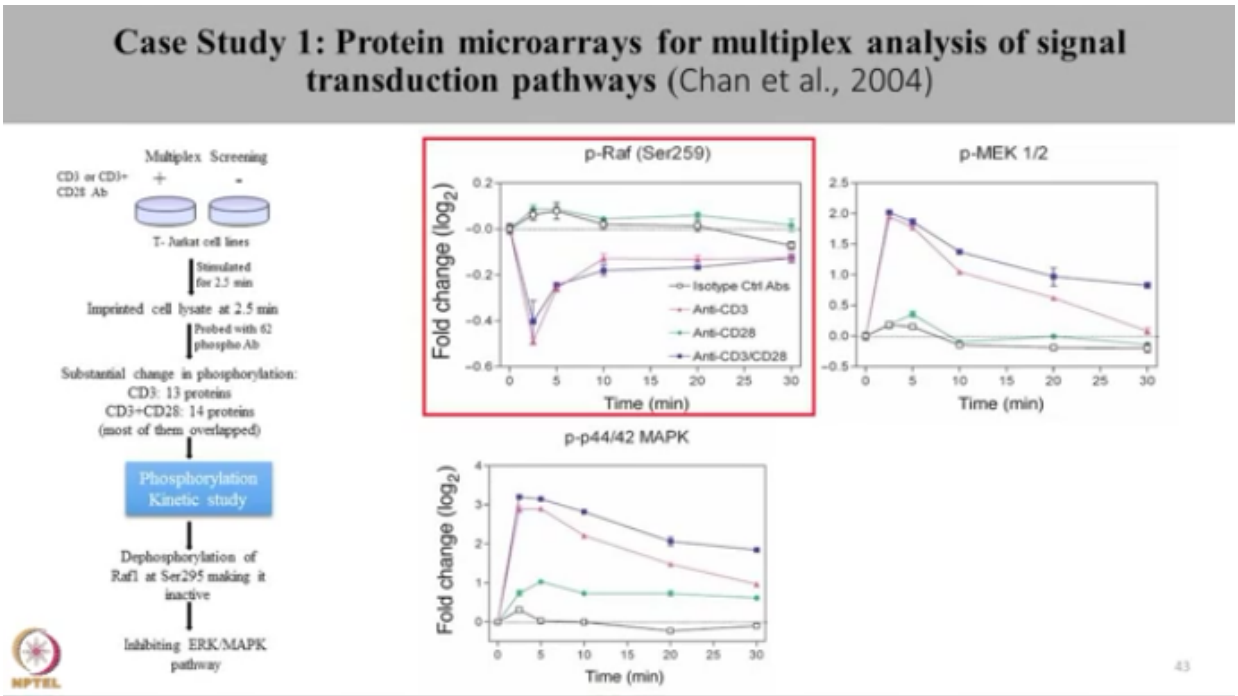

upon 2.5 minutes step de-phosphorylation of Raf protein were seen in cell lines treated with CD3 antibody and CD3 antibody in combination with CD28 antibodies, there was no change seen in the phosphorylation level of Raf protein when the cells were treated with CD28 antibody.

Further they studied the phosphorylation patterns of MEK and MAPK proteins, this phosphorylation pattern matched well with the dephosphorylation patterns of Raf protein and as the Raf protein dephosphorylated increase phosphorylation of MEK and MAPK was seen at 2.5 minutes which substantially degraded over the period of 30 minutes in the cells treated with CD3 antibodies, and the cells treated with CD3 and CD28 antibodies in combination.
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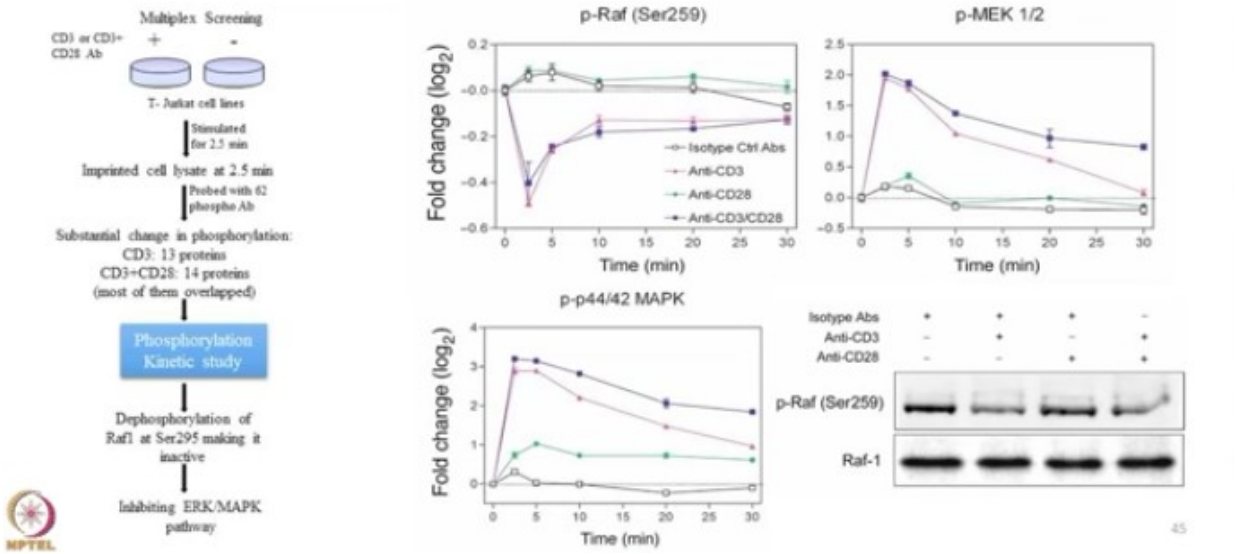
Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)



This dephosphorylation 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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Case Study 1: Protein microarrays for multiplex analysis of signal transduction pathways (Chan et al., 2004)

- Monitored other stimulus-induced post-translational modification events and identified stimulus-specific changes of biomolecules by spotting fractionated cell lysates.
- Studied phosphorylation kinetics of several down-stream signaling molecules in a time-dependent manner
- Concluded that PLC γ1 is not essential for ERK kinase pathway activation, as no changes were observed in Akt phosphorylation in mutant Jurkat cell lines deficient in PLC γ1
- Screened 62 downstream signaling components after stimulating the cells with CD3 and CD3+CD28 for 2.5 min and identified dephosphorylation of Raf-1 at Ser259, which has not been previously reported to be associated with TCR stimulation

they concluded that PLC gamma 1 is not essential for the ERK kinase pathway as there was no change seen in the phosphorylation of AKT in the cells that were deficient in phospholipase C protein also this screen the phosphorylation level of 62 downstream signaling proteins and identified a novel instance where Raf 1 showed dephosphorylation upon T cell receptors stimulation.

Now going ahead to the another study where Rauh Et Al prepared an acetylome peptide microarray to screen the activity of 7 different isoforms of Sirtuins against 6800 unique mammalian acetylation sites.

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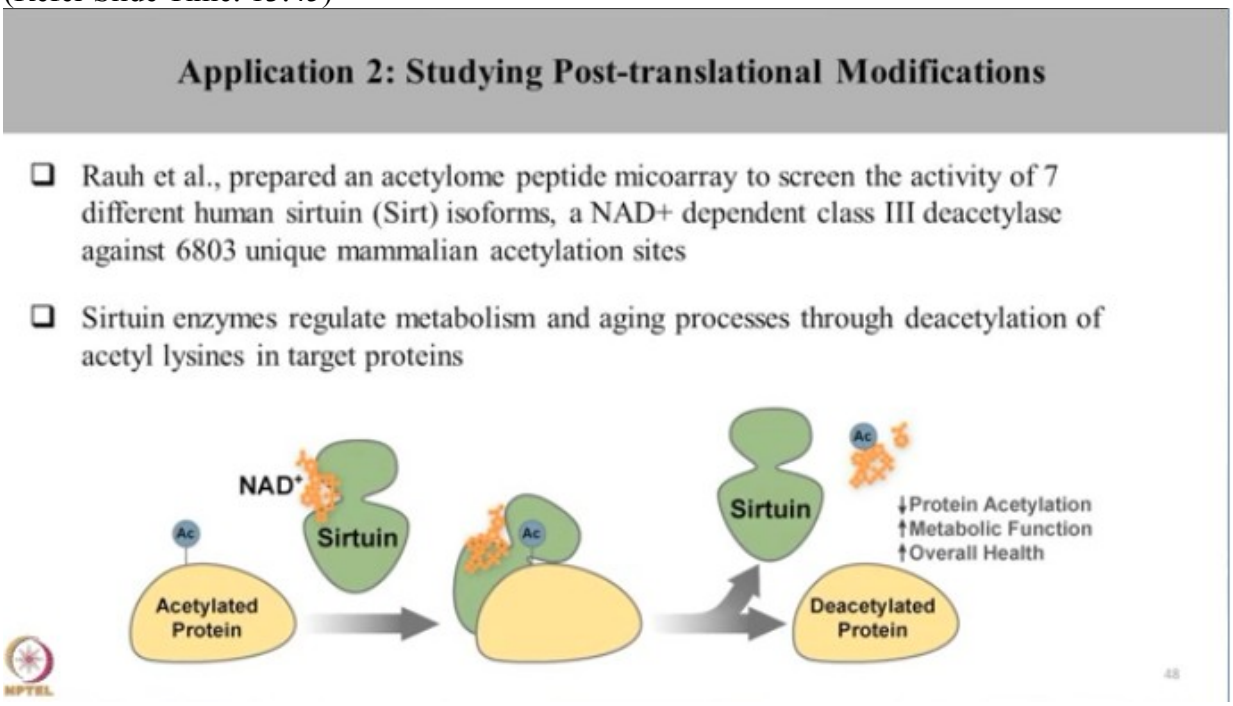

Sirtuin are the enzymes which deacetylate the lysine residues in the presence of NAD, in this study database search was performed to look for all the conserve acetylation sites in the mammalian system. A total of 6802 peptides in its acetylated and non-acetylated forms were imprinted, the peptides that were used here had lysine at the seventh position which was flank by six amino acids at the upstream as well as at the down streams,

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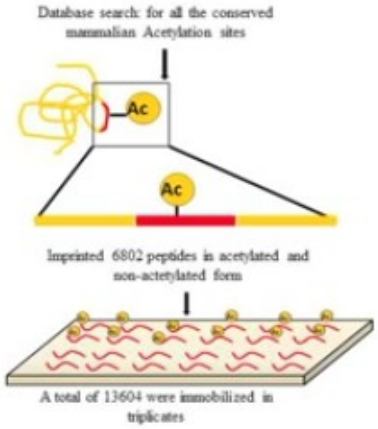
Case Study 2: An acetylome peptide microarray reveals specificities and deacetylation substrates for all human sirtuin isoforms (Rauh et al., 2013)



so therefore a 13 MAD peptides were imprinted on to the arrays and the 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 groved with primary and secondary antibody to look for the change in the level of acetylation upon incubation with sirtuins.

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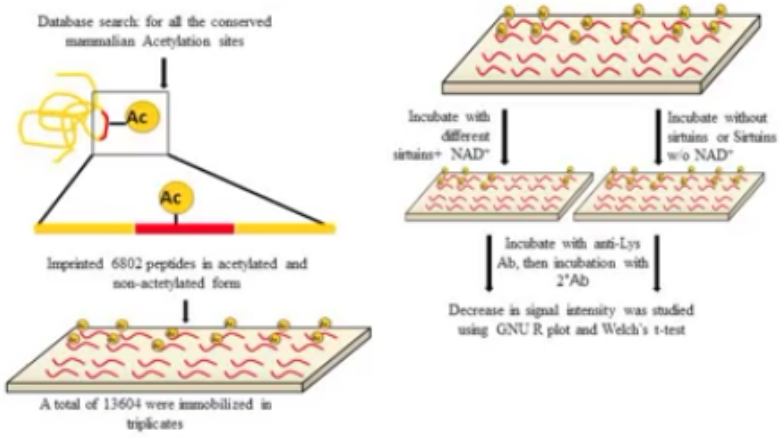
Case Study 2: An acetylome peptide microarray reveals specificities and deacetylation substrates for all human sirtuin isoforms (Rauh et al., 2013)



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The decrease in the signal intensity of acetylation was further calculated using Welch 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 study ended up in identification of new targets for sirtuins.
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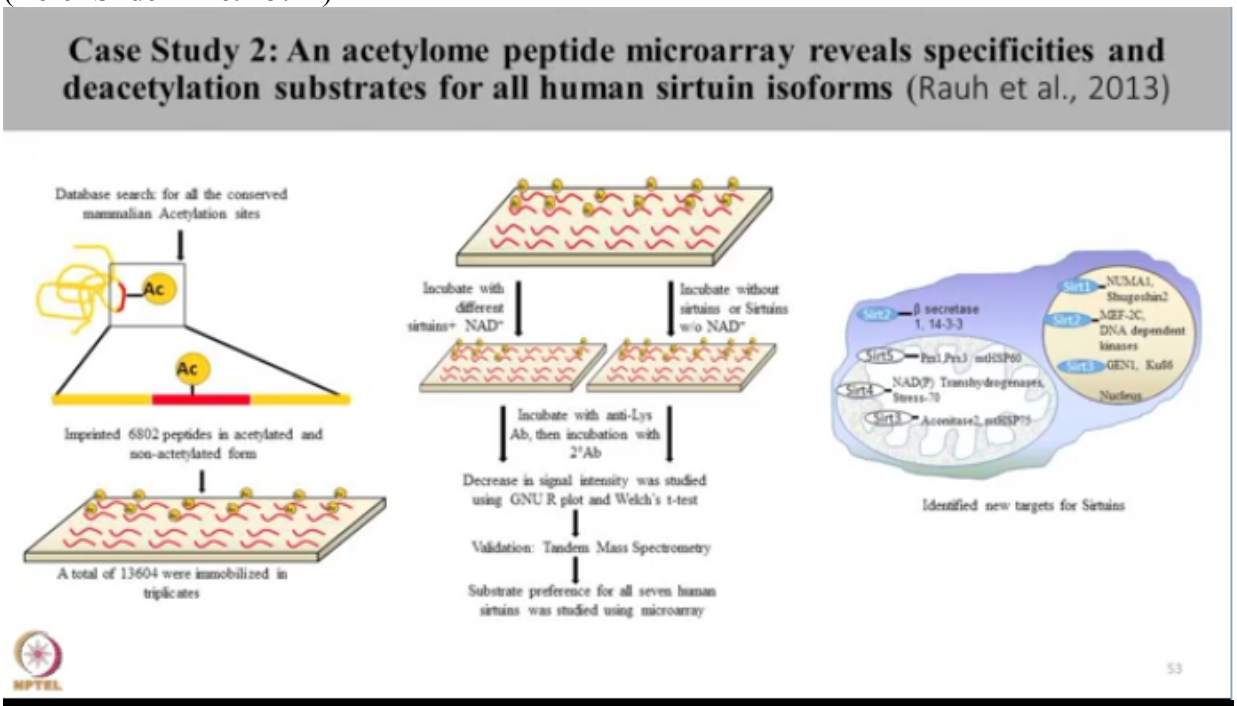

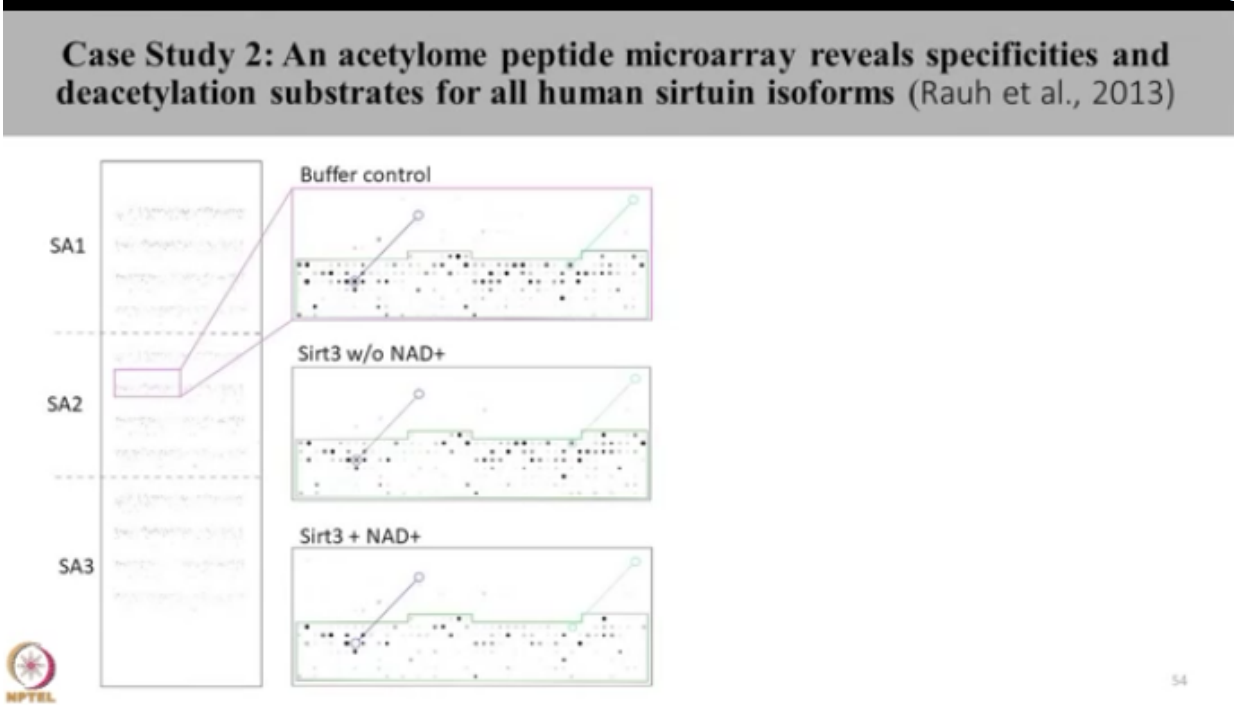

So first in this study what they have done is they have printed an array, SA represents subarray, these subarrays were treated with buffer control were no sirtuins were used, these arrays were also treated with sirtuins with and without NAD. Since sirtuins need NAD for their activity there shall be no deacetylation seen where 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 in signal intensity was seen when the arrays were treated with

sirtuins in presence of NAD, concluding that sirtuins need NAD for deacetylation and also confirming that the deacetylation is happening on the array. This graph represents the specific activity of sirtuin 3, how the deacetylation is happening for different peptides.

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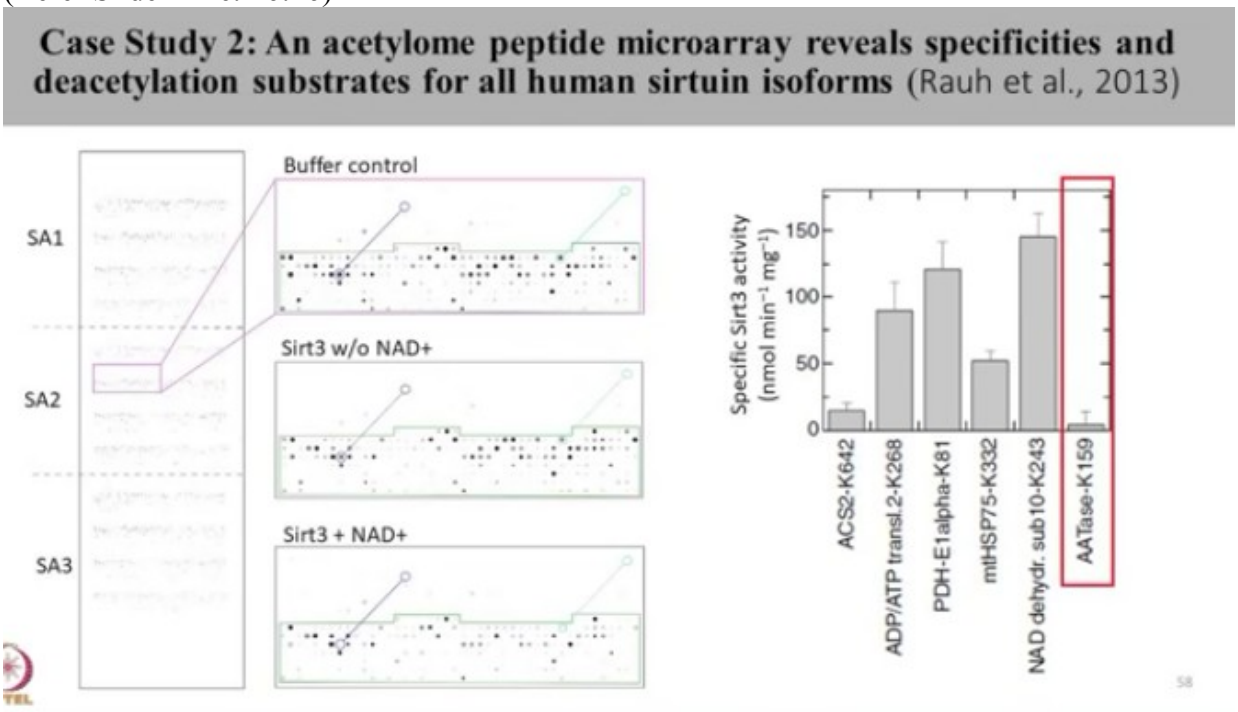

The last protein that is AATase K was used as a negative control and has shown no change in the acetylation pattern,

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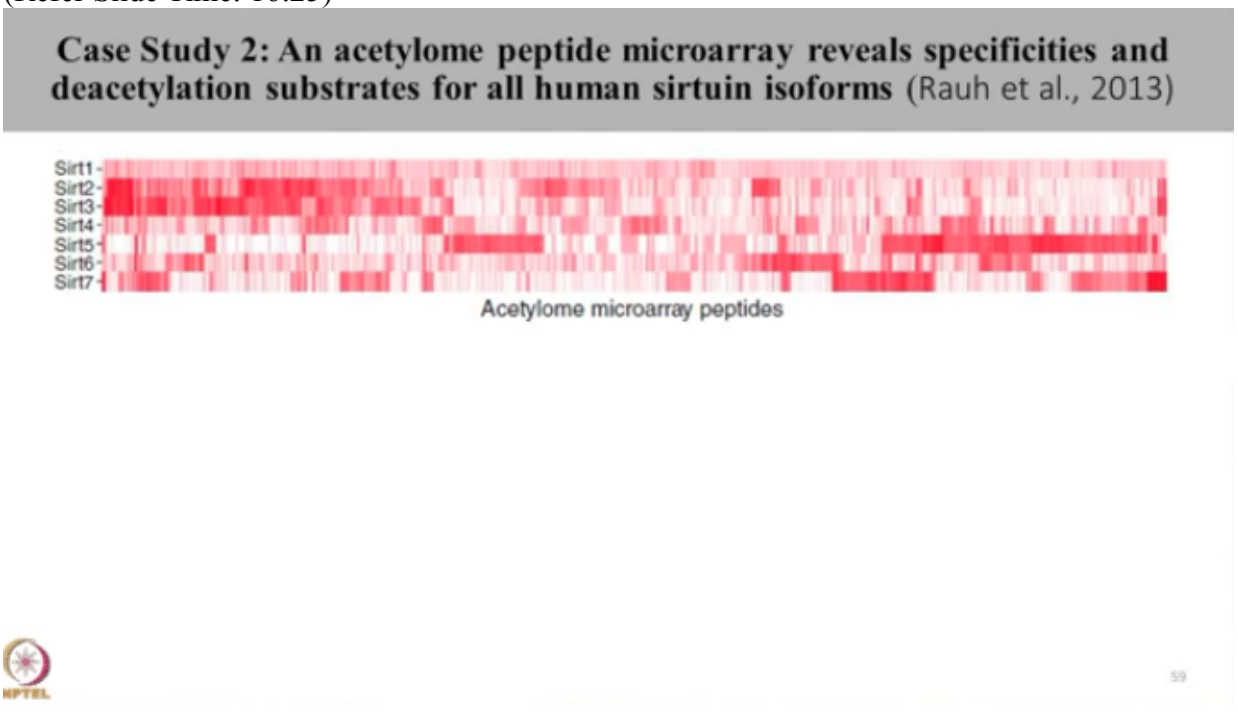

this heat map shows that deacetylation activity of all the seven isoforms of sirtuins across 6800 peptides, sirtuins 1 6 and 7 locates at the nucleus, however when you look at the target peptides, there is a specific pattern of preference of these peptides for sirtuins 6, 7 and 1.

If you look properly at sirtuins 6 and sirtuins 7, a specific deacetylation pattern is seen which signifies that these two isoform has their specific targets whereas sirtuin 1 shows a nominal activity across a wide range of sub straight in the nucleus,
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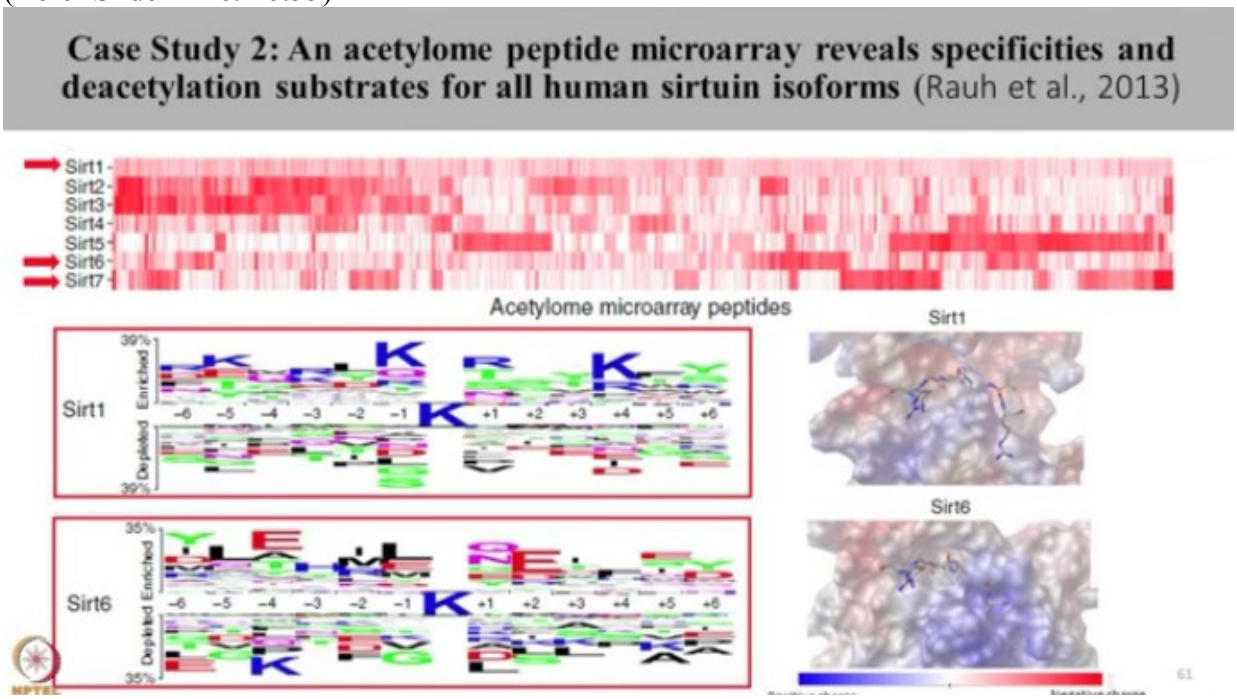

this logo here shows the peptide that were used, the upper panel of the logo that is the enrich section shows the preferred amino acids for each sirtuins, whereas the lower bottom shows depleted amino acids sequence that is the sequence that do not favor the deacetylation. Also the right panel shows the peptide binding grooves of these sirtuins 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 sirtuins 1 is majorly positively charged amino acids that is arginine or lysines as the code has negative chart therefore sirtuin1 prefers the peptides that have positive chart specifically at position -5, -1, 1 and 4, whereas the peptide binding grove of sirtuin6 is majorly hydrophobic and hence the peptide sequence that are specific to sirtuin6 majorly have hydrophobic residue, specifically at -1, -2, +3 and +4 sites, however at +2 and -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, 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 grove for sirtuin 3 is highly negative,
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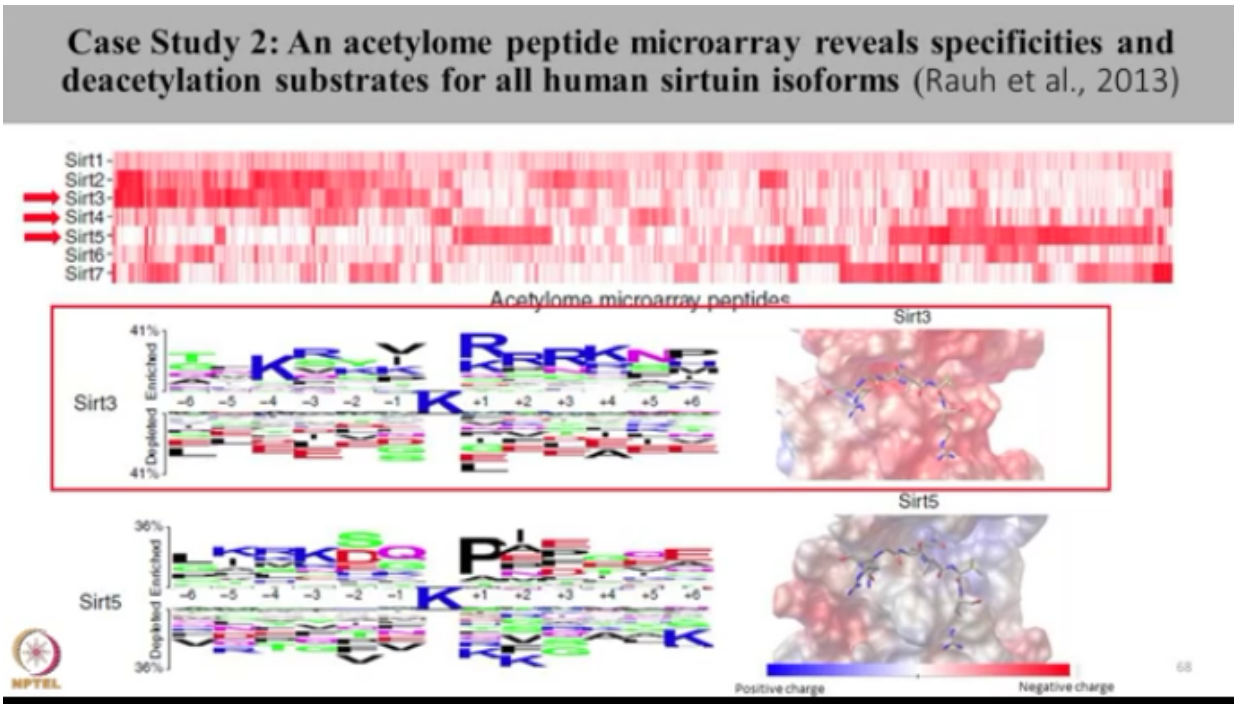

going to that the peptides that are preferred by sirtuin 3 had a lot of positively charged residue specifically arginine at the upstream of acetylated lysine.

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

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 grove for sirtuin 2 is highly negative
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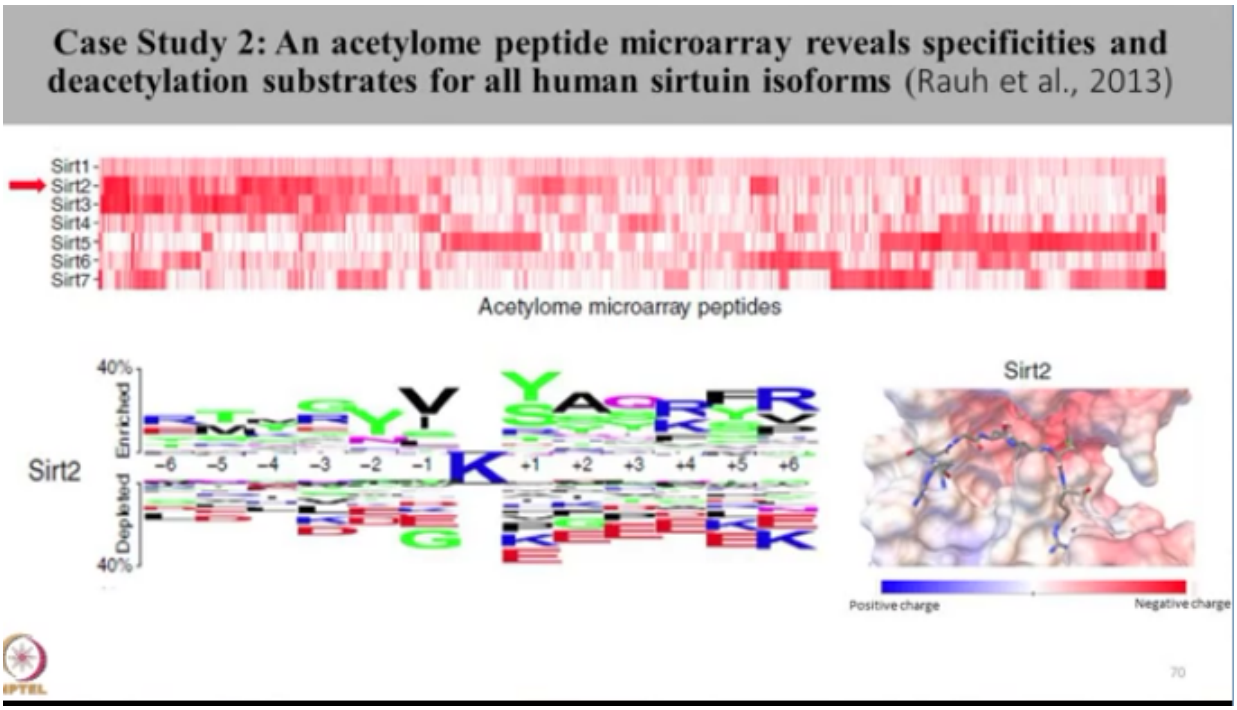

and therefore it disfavors the presence of negatively charged amino acids in the sequence preference, except for sirtuin 4 all other sirtuins have some or the other targets for deacetylation, however there was no identified substrate for sirtuin 4.

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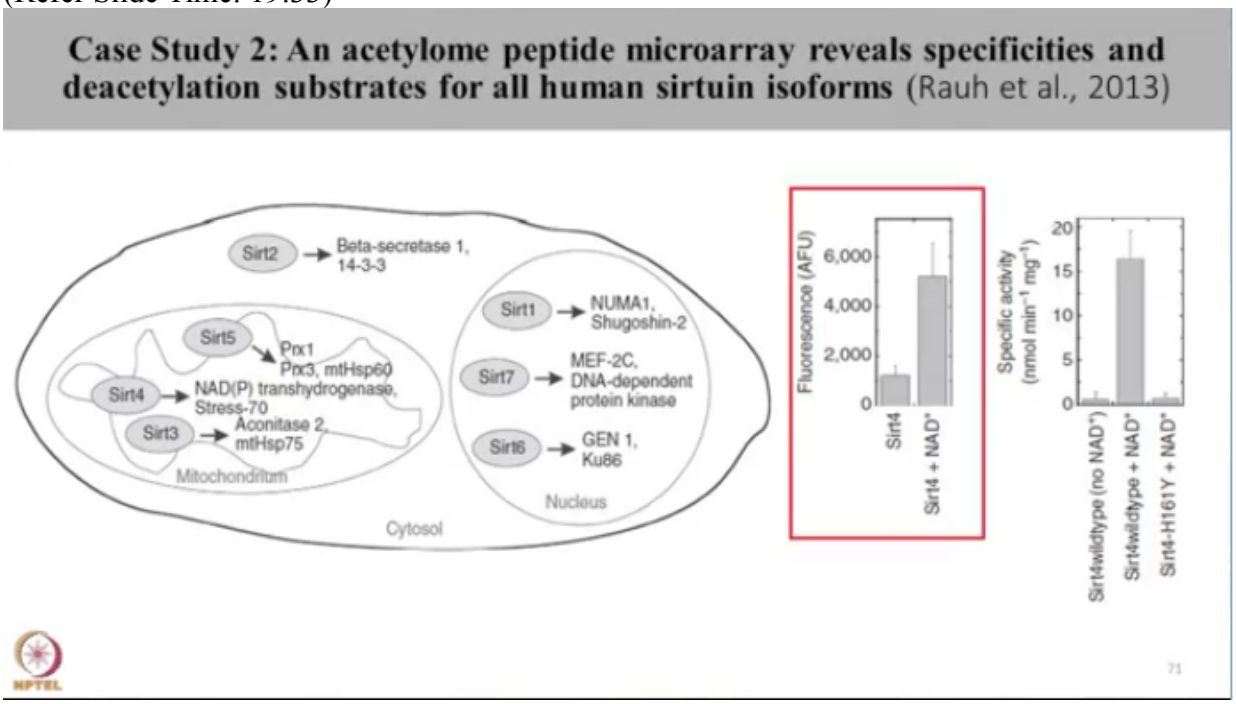

In the fluorescence SA sirtuin 4 showed a low but consistent deacetylation activity upon incubation with NAD, from this study NADP transhydrogenase stress 70 protein was found to be one of the substrate specific for sirtuin 4 to confirm this deacetylation activity of sirtuin 4, the deacetylation of NADP tranhydrogenase stress 70 protein was performed using wild type

sirtuin without NAD wild type sirtuin with NAD and mutant sirtuin which did not have deacetylase activity.

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

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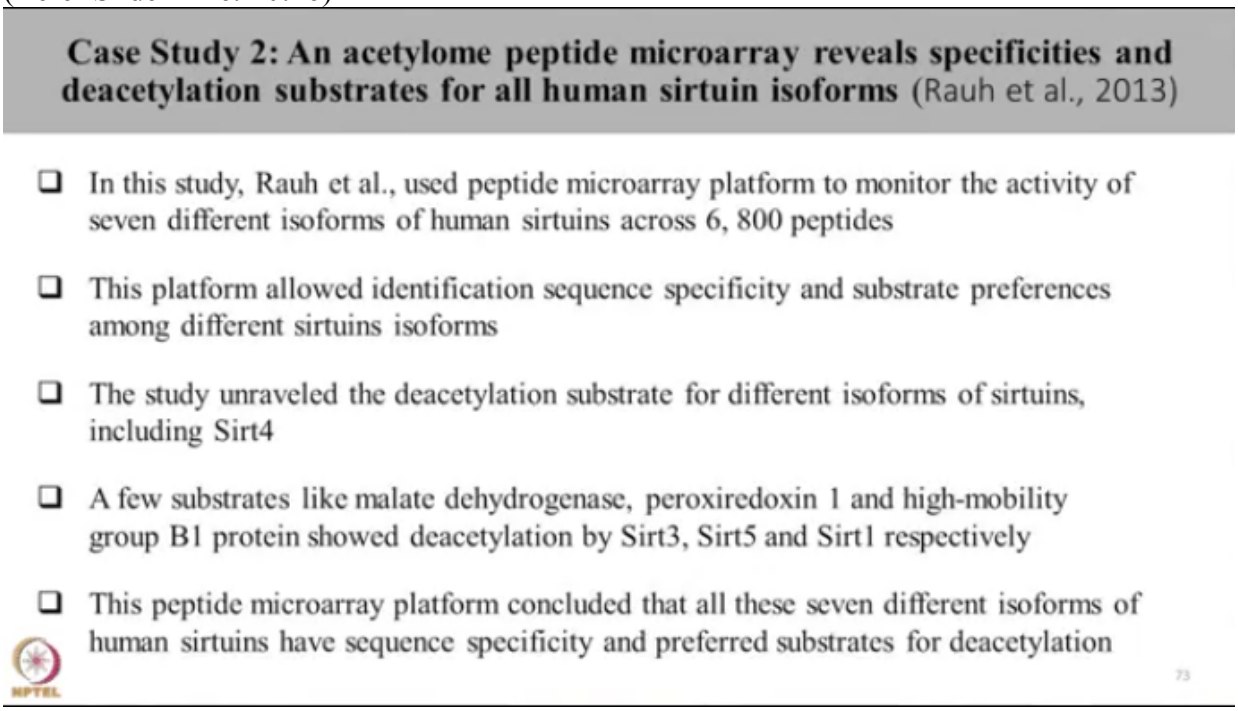

To conclude this study has used microarray platform to parallelly screen around 6000 peptides for 7 different isoforms of sirtuin, using this platform the authors were able to identify the sequence specificity for different isoforms of sirtuins, they also identified a substrate for sirtuin 4 which is NADP transhydrogenase 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 microarray platform concluded that all these different 7 isoforms of human sirtuins have a sequence specificity and preferred substrate for deacetylation. To conclude protein microarrays 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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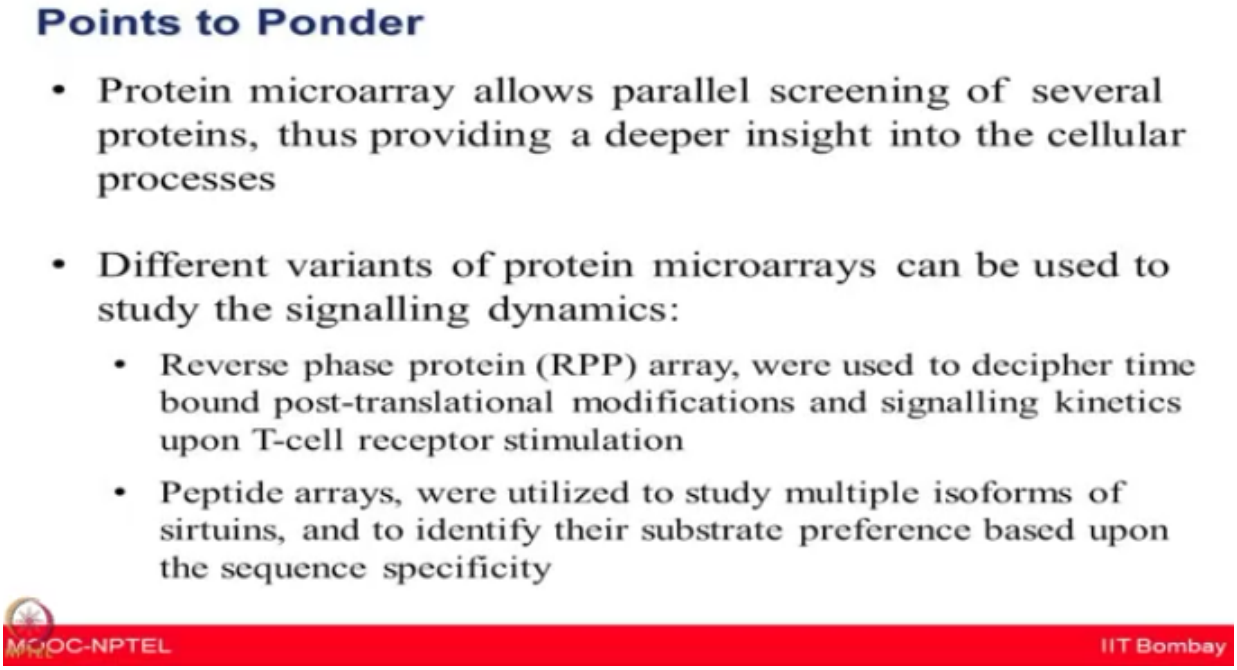

Sanjeeva Srivastava: I hope by now you understood that there are wide applications of protein microarray could be achieved especially from different type of protein microarray based technology platforms in the areas of detection for novel protein interactions, post translational modification in high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

In the next lecture, we'll talk about how to make arrays and print your own chip using novel printing technologies. So I will see you in next lecture, I will talk to you about the latest advancement in this area, and how you can make your own arrays and recent development in the areas of printing technologies. Thank you.
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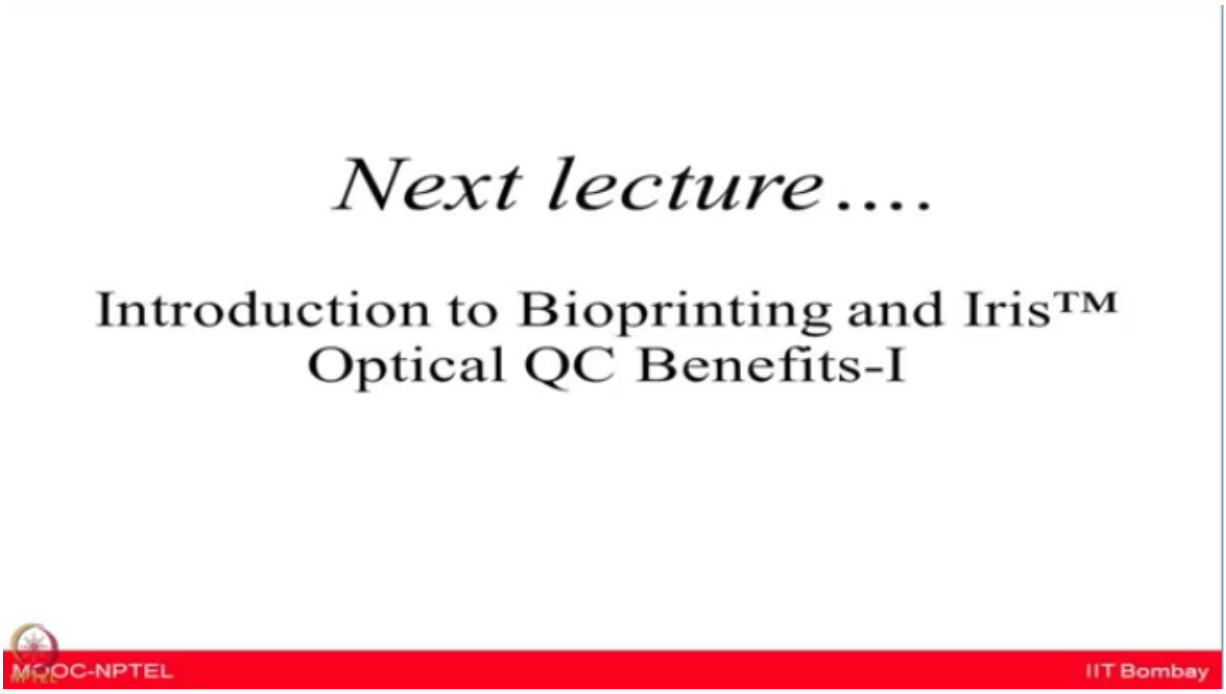

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