Interactomics Protein Arrays and Label-Free Biosensors
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Module 06
Lecture 30
Digging Deeper into NAPPA Basic Workflow Surface Chemistry Printing and Assessment

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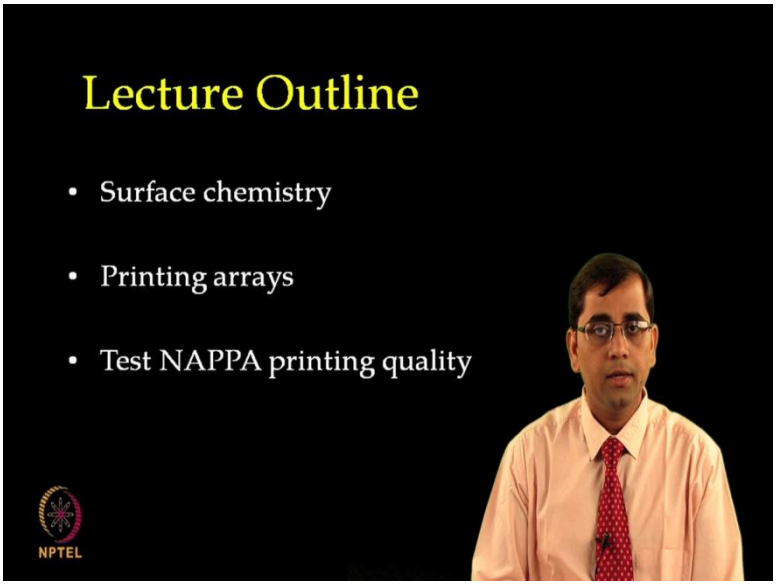

Welcome to MOOC interactomics course. In our previous lecture we discussed about the general work flow of NAPPA focusing on plasma DNA extraction. In today's lecture will speak about the subsequent steps involved in performing NAPPA based experiments including surface chemistry, printing, quality control checks and protein expression. Let us first understand the importance of surface chemistry. The surface chemistry determines the immobilization of proteins and truly defines an essential aspect of protein array fabrication. Orientation of immobilized protein and its binding strength affects downstream interactions in crucial determinants of any study. The carboxyl functional groups on proteins usually bind to the amino surface through electrostatic interactions.

Thiol groups to malamide surface through covalent thioether binding and hydroxyl group to epoxy surfaces through covalent ether binding. Functional groups on protein allow immobilization oxalis surface through covalent or non-covalent bonds based on functional groups presenting them. The nuclear felic residue of proteins are immobilized by reactive surfaces coated with aldehyde, NHS, ester, epoxy side, etc. Cumuligation is an elite based example of side specific immobilization. Non covalent interactions are driven two different

affinity tags like (())(2:26), GST and (())(2:29) to their respective likens or antibodies. After completing the DNA prep you need to now functionalize the glass slide surface for printing purpose.

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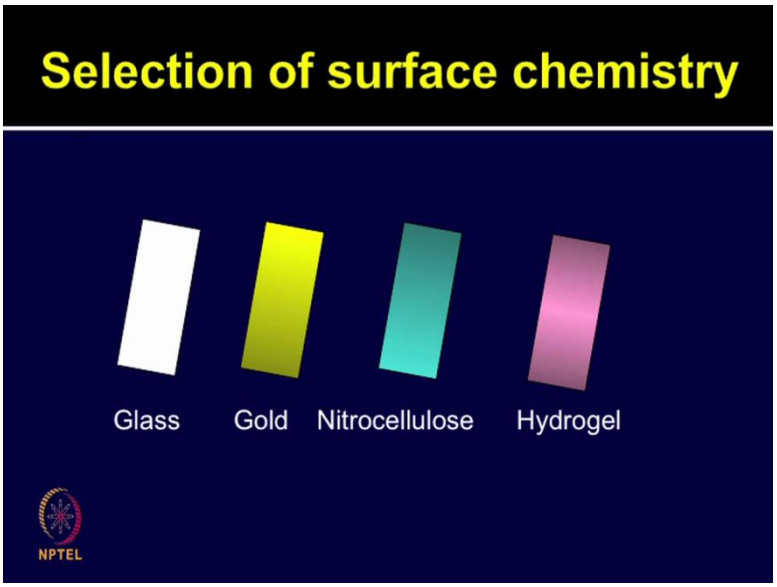

One can use different types of surface for example glass, gold, nitrocellulose, hydrogel, etc. There different properties that one needs to look for selecting the surface; cost is one of the major factor. When you are talking about experiments such as by molecular discovery, in such screening based experiments you need to use 100 of shapes for several 100 of patients and therefore the cost is one of the major fact. If you have glass slide which is cost effective, so it will allow you to perform large number of experiments, in such scenario gold becomes very costly in comparison to nitrocellulose or hydrogels.

The reactivity for the glass is moderate so, is the case with nitrocellulose and hydrogels, gold in comparison has much low reactivity. Absorption to surface is low for gold and glass and is high in case of nitrocellulose and hydrogels. This platform can be made compatible to mass spectrometry using cold surface this depending on the broader goal of the experiments one can choose an appropriate surface. These surfaces can be appropriately fabricated using different types of chemistry including amino groups or thiolistries. Let us first focus on silane based chemistry.

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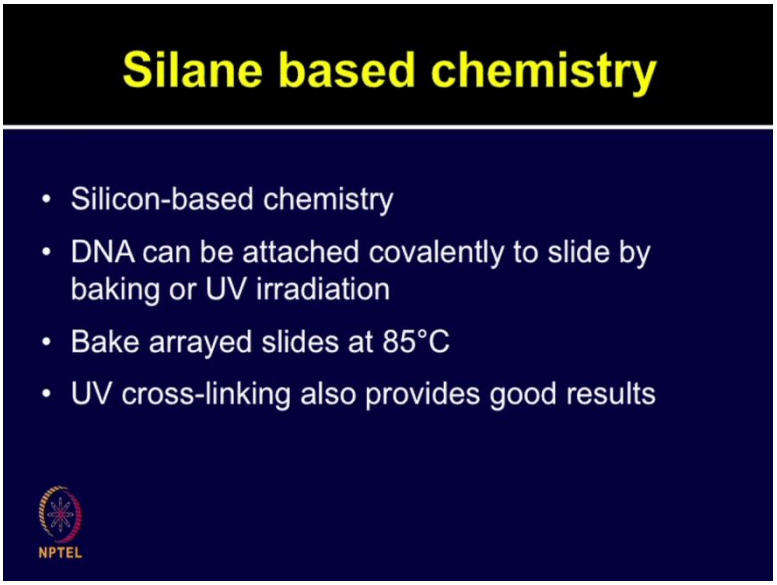

We will talk about printing DNA on the array surface for using cell-free expression based NAPPA microarrays. Silicon based chemicals contain two types of groups such as alkoxy groups like methoxy or ethoxy or organo functional groups such as amino, epoxy , vinyl etc. Thus given our aim DNA can be attached covalently on the glass surface by baking the arrayed surface at 85 degrees. UV cross-linking is also a popular method for DNA immobilization.

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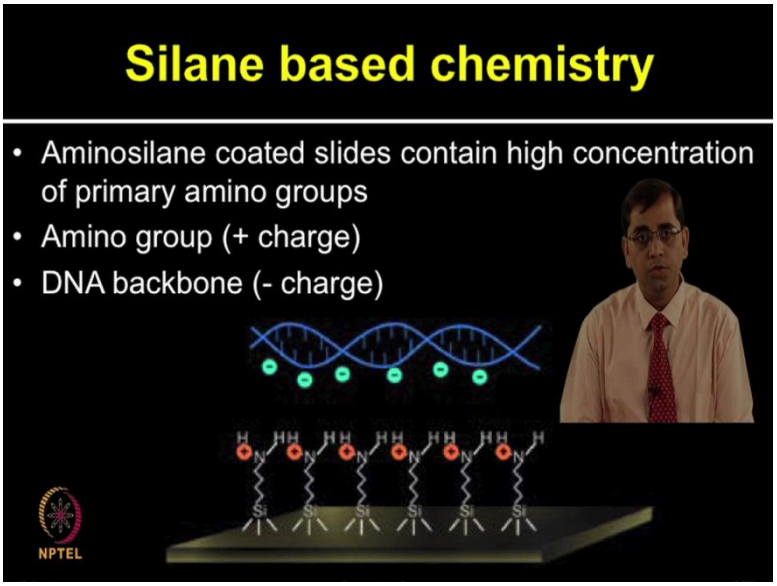

Aminosilane coated slides provide high concentration of primary amino groups on the chip surface. One question that may arises that why should the used this chemistry. Amino groups provide a positive charge when placed in contact with the neutral aqua solution, the groups

becomes positively charge. As you know the DNA backbone possess a negative charge, so DNA backbone forms multiple ionic interactions with positive charge amino group coating as you can see in this glass slide.

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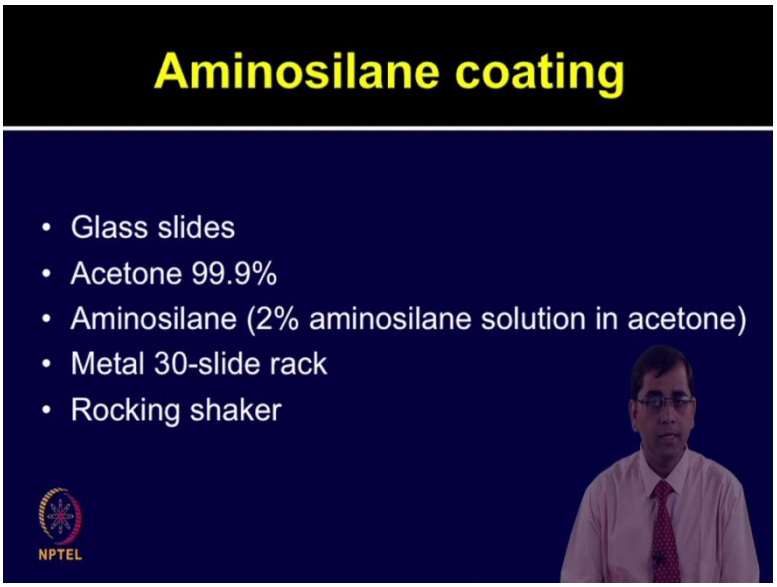

For performing aminosilane coating one need to have glass slides, acetone, aminosilane, using a 2 percent aminosilane solution in acetone works best, then you need a metal slide rack and you need a rocking shaker.

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In this slide various steps involved in performing aminosilane coating are mentioned. On the left side a rocking shaker has glass tray with metal rack containing 30 slides at the inset you

can also see these 30 slides in the metal rack are submerged in the aminosilane solution. On the bottom panel on the right side I have shown that slide can be either centrifuge or dried with the compressed air and those are ready for printing purpose. Aminosilane coating is demonstrated in following animations.

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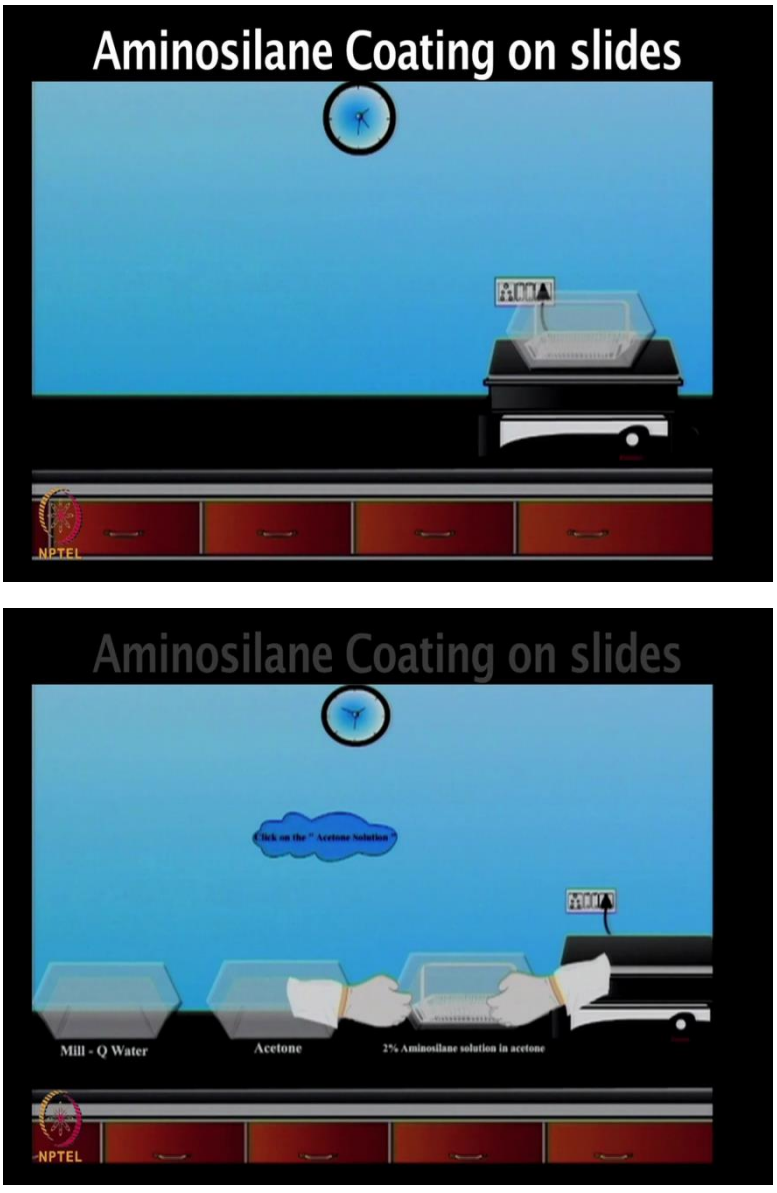

Prepare 300 ml of aminosilane coating solution which is 2 percent of aminosilean reagent acetone; place the slides in metal rack. Treat glass slide in aminosilane coating solution for around 15 minutes in glass box on shaker. Rinse with acetone and followed by brief sensing with MILLI-Q water, after washing steps are done then you can spin dry in the speed rack.

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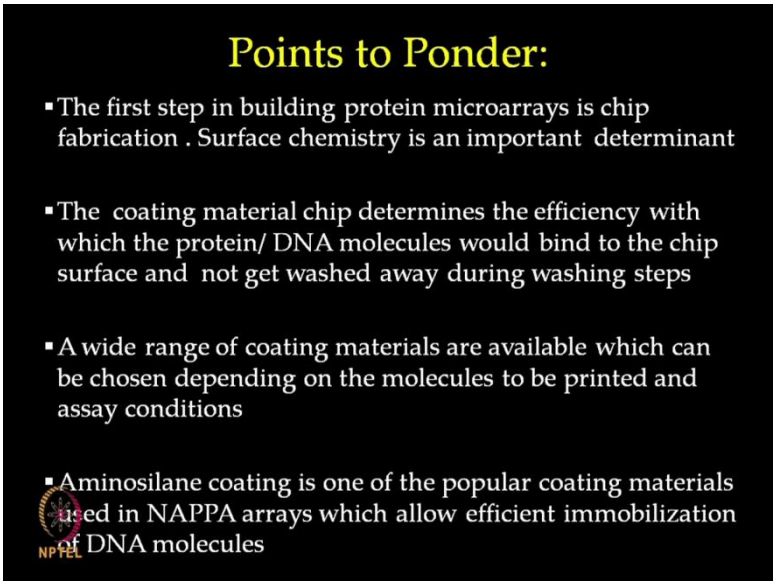

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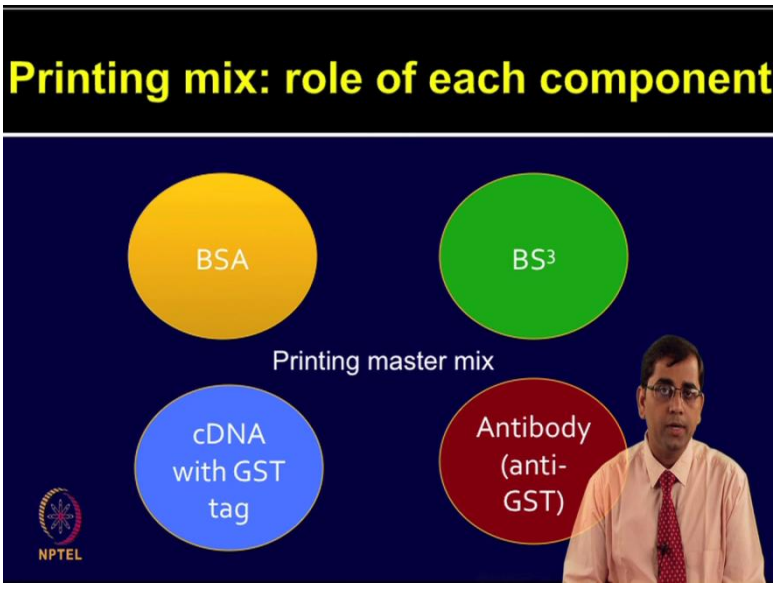

Printing DNA or protein features on the chip surface. First of all we need to make a master mix or printing mix in case of NAPPA as I mentioned earlier you have 4 components BSA, BS3 cross-linker, cDNA containing the GST tag and antibody which is anti-GST body. It will briefly explain the roll of these components. BSA dramatically improves immobilization and efficient expression of the DNA BS3, BSA and capture antibody. They are coupled to the amino coated glass surface with ester terminated homobifunctional cross linker is printed. The cDNA contains GST tag or you can introduced any other tag, then you need to have captured antibody so, that they express proteins can be captured to the antibody to a c-terminal GST tag on each protein.

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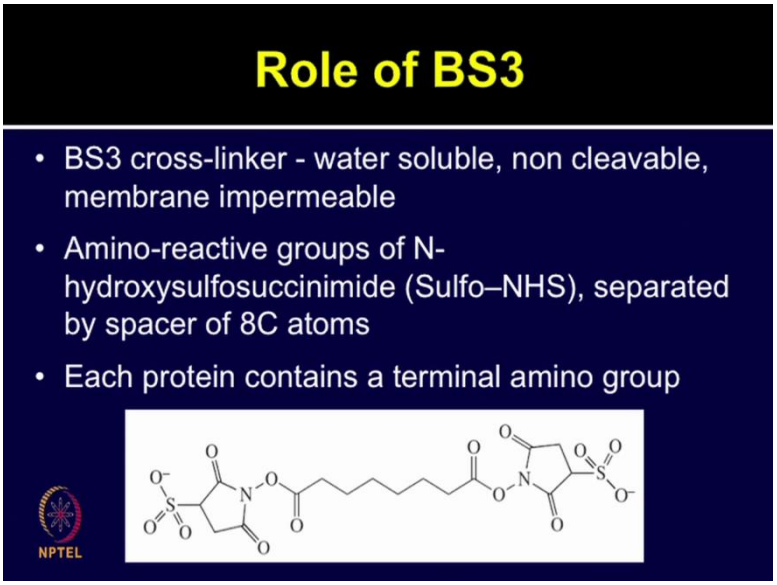

Once we have these 4 components ready, then you are ready to perform the printing. The BS3 Cross-linker is a water soluble, non cleavable, membrane impermeable. The amino-reactive group of Nhydroxysuccinimide (sulfo-NHS) are separated by this spacer of 8 carbon atoms and each proteins contains a terminal amino group. Once we have these components ready, you are ready to perform the printing. You first need to define various parameters on the software.

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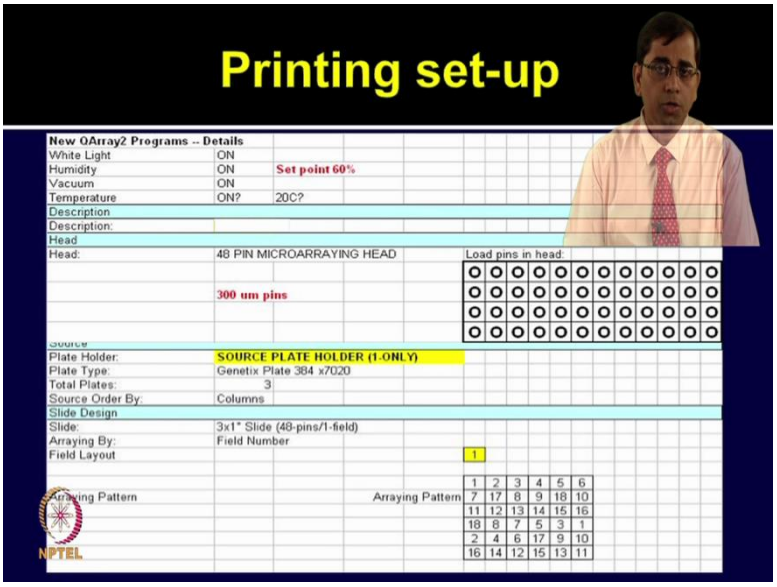

You need to define the types of pins you are going to use. For example, one can use 48 pins for printing several features simultaneously or even go as low as 1 or 4 pins. This could be of

different microns ranging usually between 150 to 300 microns depending on what kind of density you want achieve on the chip.

You want to ensure maintenance of humidity throughout the experiment, 60 percent humidity is ideally maintained. You need to define the source pit which is being used for printing. We must take if we have 1 or multiple plates type of arrays pattern refers. These things have to be programmed before placing 384 well plates containing the masterbits, which would be used for the printing purpose.

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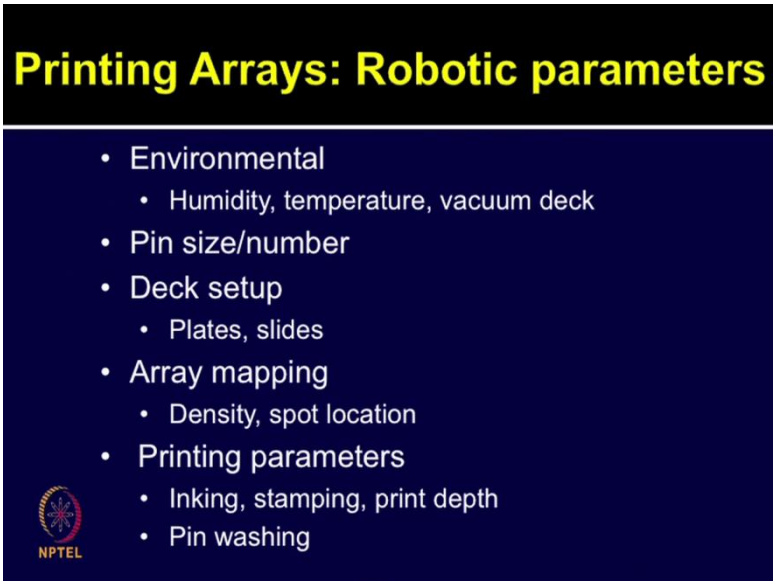

One need to ensure the different type of robotic parameters are in place. Let me show you the animation to explain you the procedure for master mix preparation and the steps involved in printing.

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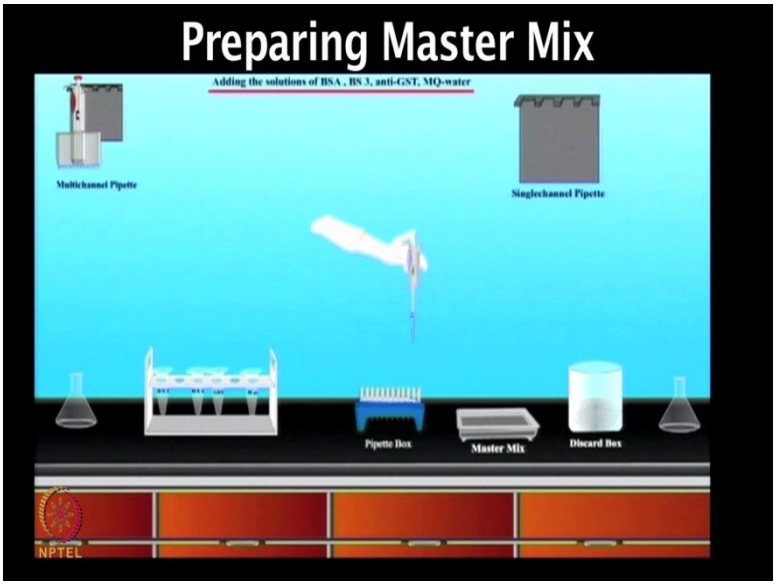

So, prepare enough master mix so, that you can do printing for large number of arrays. The master mix contains DNA, polyclonal GST antibody, BSA and BS3 linker. For detail recipe of each of this reagent you can follow the publication manuscript by Ramachandran Etal 2008 published in mixer methods.

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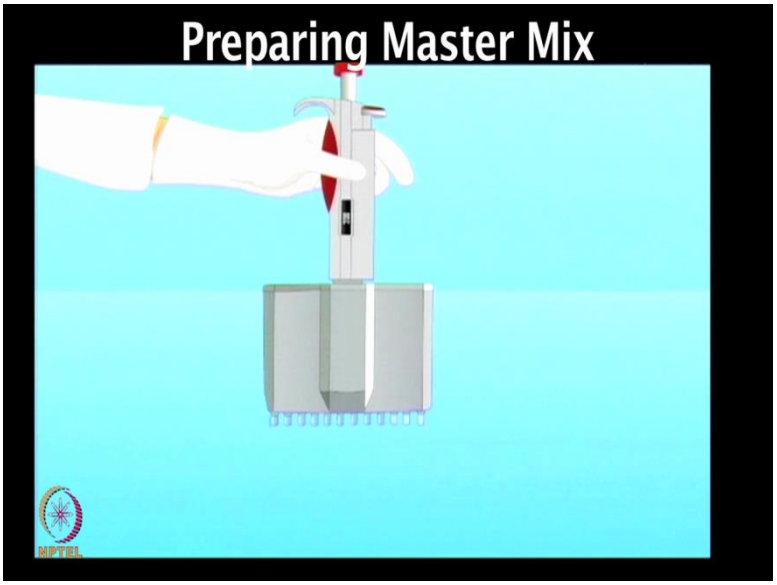

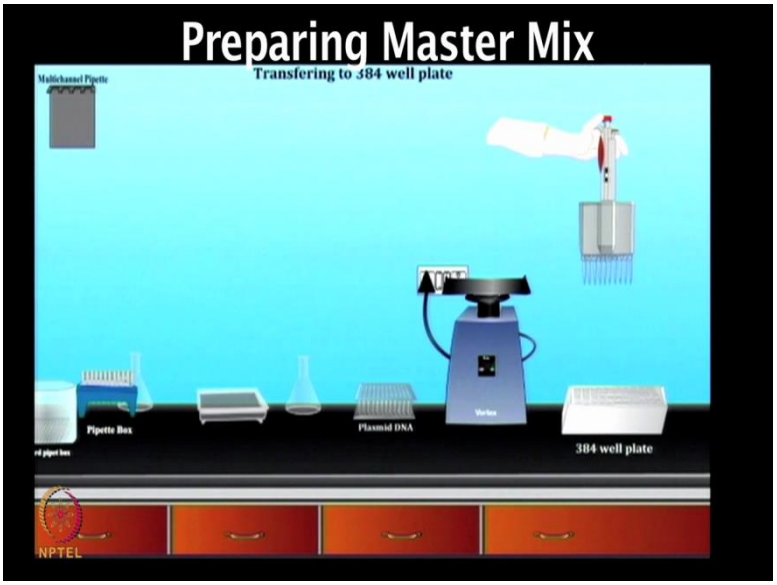

Once master mix is prepared, you can mix it well and then transfer 20 microliter of mater mix to the block containing plasma DNA. Once the master mix will added to all the wells in the block, then it will shake plate for 15 minutes. For printing you will require 384 well plates so now you need to transfer your master mix containing plasma DNA from 96 well plate to the 384 well plate. Again this step can be performed either using multichannel pipettes or by using liquid handler systems. Most master mix is prepared in transfer into the 384 well plates. We can perform the printing step.

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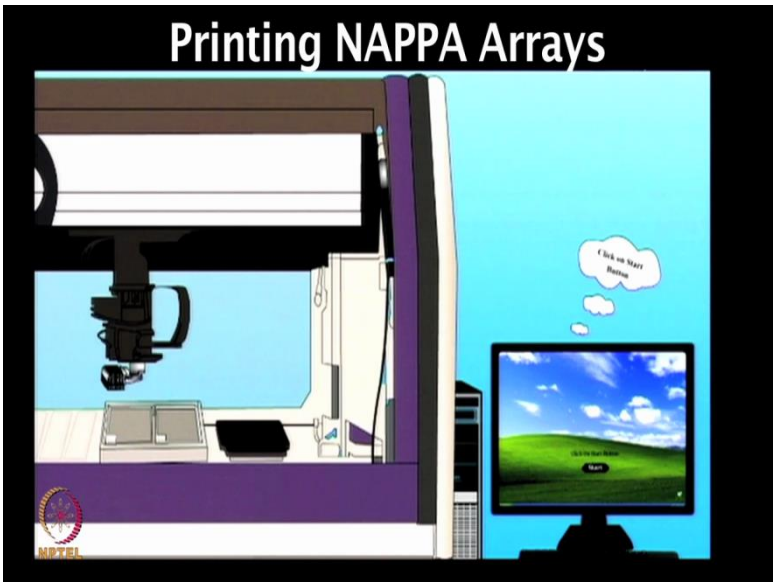

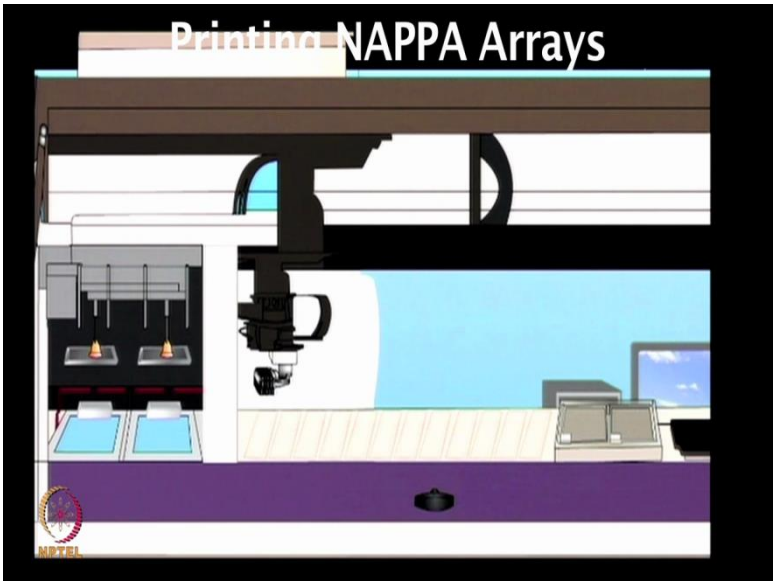

So, we can use these microarrays to print the DNA and master mix on aminosilane coated glass slides. First you need to ensure that pins are washed thoroughly. You can wash with ethanol and water and then entire printing procedure we need to ensure that humidity is maintained at 45 to 60 degrees. One can use different types of pin heads and different type of microarrays for printing the chip, now this master mix can be printed on the chip.

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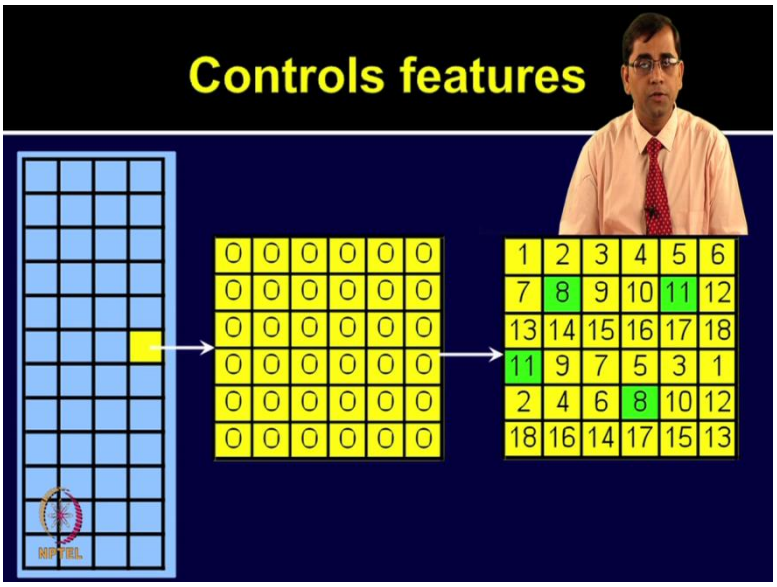

Control features, what are the different types of control features you need to put on the array, Let us briefly discussed that? As mentioned earlier you need to ensure the different types of control features are spread across the array surface and this could be mouse IGG, no DNA, non-spot, EBNA, human immunoglobulins, etc, depending on the diseases contacts the kind of problem you working on you can define good positive and negative controls.

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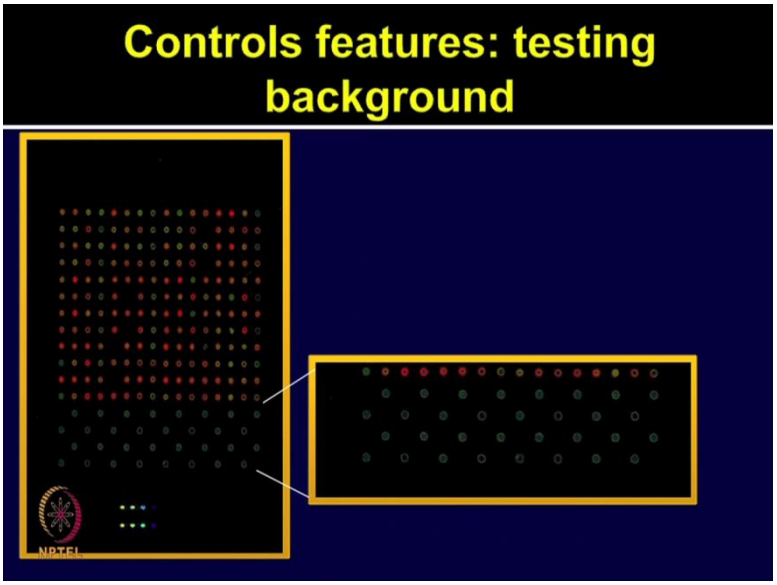

Now to test the background you need to have certain control features. Since we are talking about the NAPPA arrays, we are talking about close containing GST tags. It will be good idea to have different spots with GST alone, so you can have the purified GST in the dilution series. So, you can use the dilution series of increasing concentration of purified GST protein for normalization and doing the calculation for the protein expression later on.

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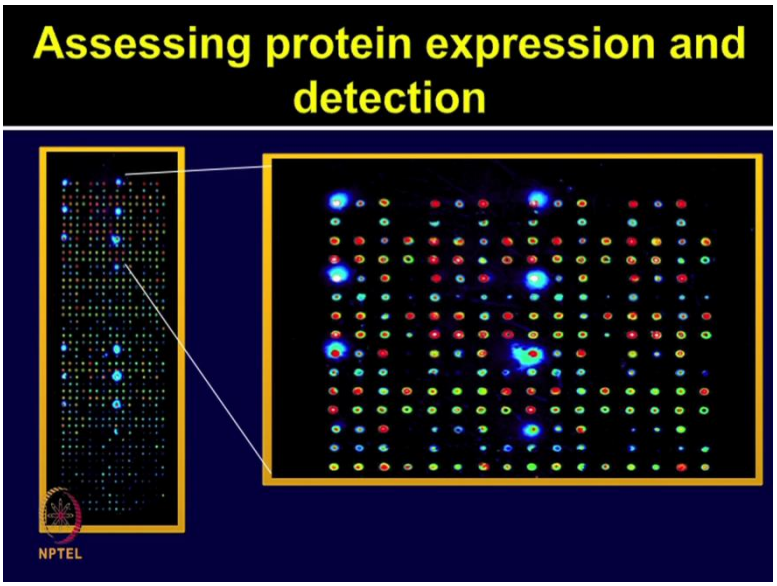

If you are looking for different type of immunological response in by biomarker discovery, etc, you need to ensure that immunoglobulin igG is printed at the varying concentration. It can also be used for statistical analysis that are run by the (())(16:11), so that different types of protein expression can be normalized.

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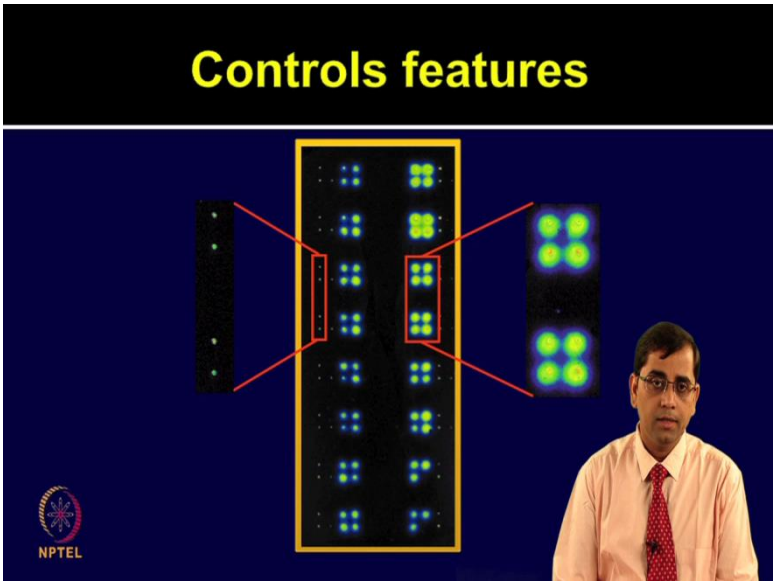

Control features and QC checks are shown here in this slide. The same G has been printed 4 times in the blocks repeated and then negative controls are used to estimate the background noise. What are the different quality check points to be kept in mind for good protein arrays?

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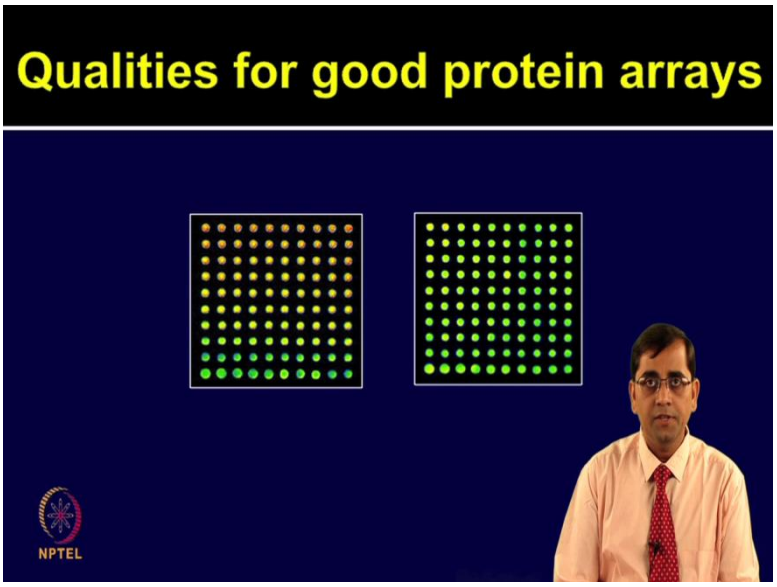

Spot to spot consistency is one of the key features positional consistency has to be there for a large number of slides. Sample loading, sample integrity as well as different types of controls replicates and positional flexibility are needed to maintain the quality of protein arrays.

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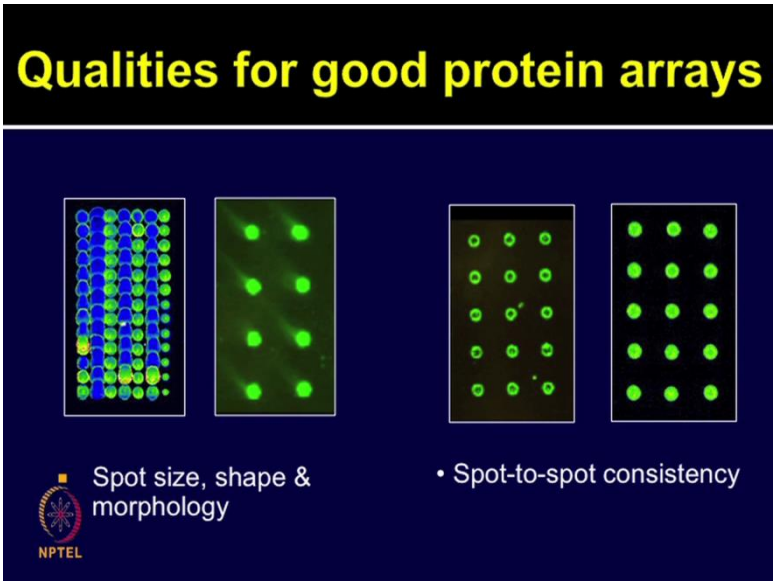

As shown another slide one can encounter different type of problems by printing these arrays. Shown on the left side is spot shape, size and morphology, shown on the right side is spot to spot consistency. Many times this printing issues occur in high throughput type of experiments, so one need to ensure that all these parameters are properly quality control checked. Looking at this spot morphology and the uniformity of this spot, one can think of various type of parameters that could be useful such as spotting solutions, spot capability, maintaining the temperature, maintaining humidity conditions keeping just free environment are important considerations. For printing quality arrays you need to have precise liquid handling system.

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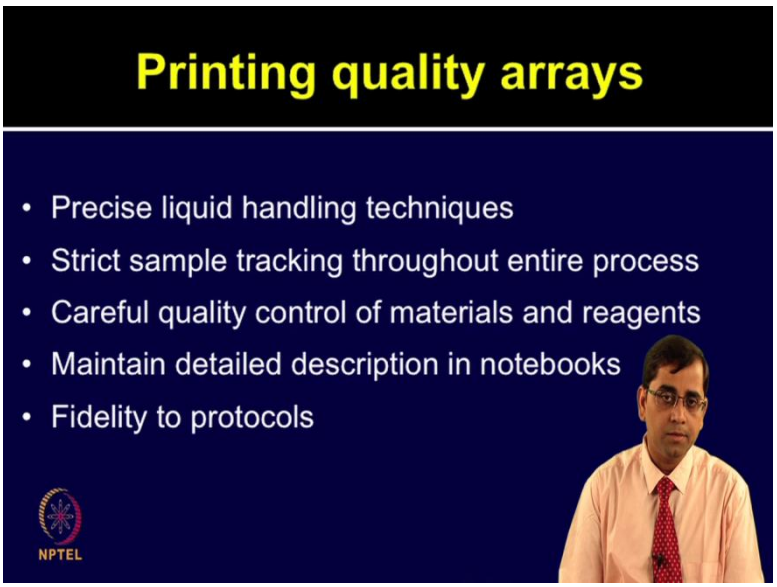

As I have mentioned each step can be automated, you need to keep track of the entire process so you can troubleshoot if anything goes wrong at any step. It is good idea to build the detail log history for each step from cloning, DNA preparation, printing, etc. Now, if you have done step by step optimization and evaluation, there is less chance of making any errors because in high throughput approaches, when we are dealing with thousands of features it becomes important to track everything throughout the experiment and make note of the details.

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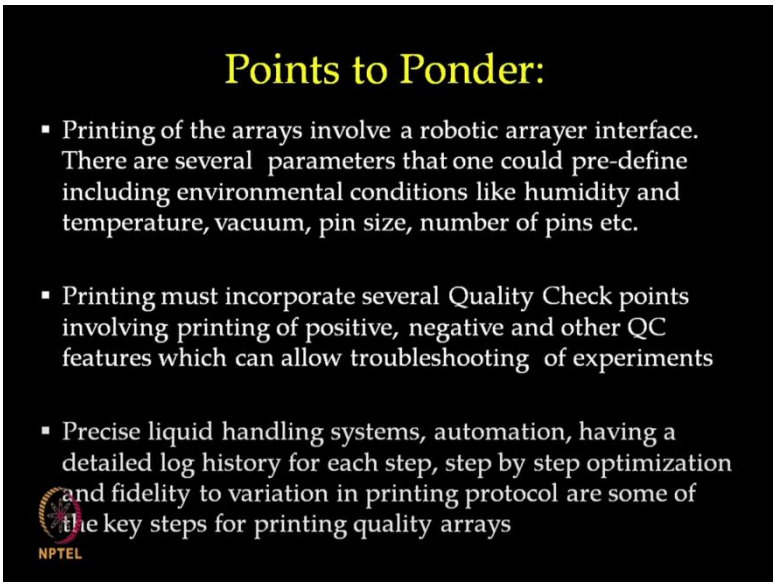

Now, we have done the printing and have shown that printing of its good quality by considering various parameters. However, can we now use this NAPPA arrays for doing further balanceable experiments? So, first of all we have printed DNA so, we to have ensure that DNA printing is good to ensure that one can use pico-green staining. Once the DNA quality is good, one need to ensure that the proteins can express form the DNA using in vitro transcription and translation expression system, so the protein expression needs to be quality checked.

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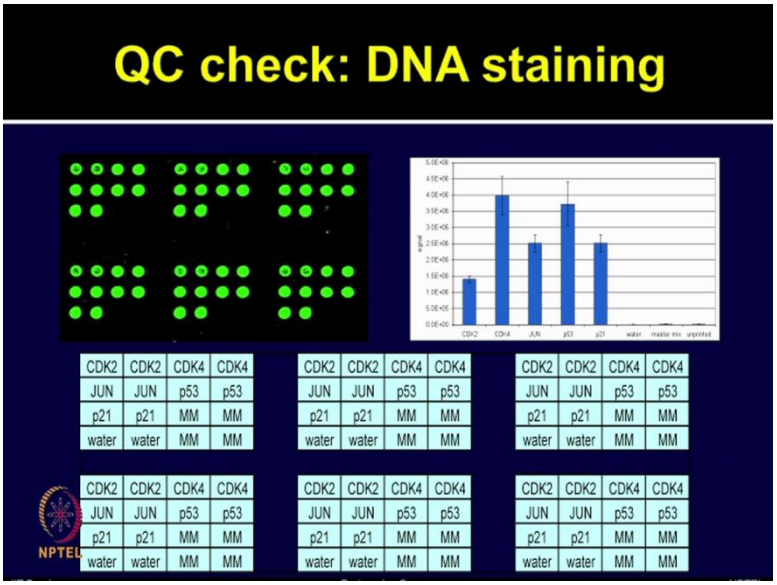

Shown in the slide is small test array, which are used to teach the student from (())(20:07) Harvard laboratories in new York. This actually made by this students Proteomics course. In the bottom you have a map, where only 5 genes are printed CDK4, CDK5, JUN, p53 and p21, each one of those genes are printed in duplicates, then you add master mix and then water. This is a very simple proof of concept test array, these are only 5 features 5 genes, which we have DNA. So ideally you must observe the pico-green staining to check the DNA printing quality. You can see on the upper panel those green spots lighting up indicate that the DNA has been printed properly. One can use the statistical tools for analyzing how good the DNA is printed on the chip surface.

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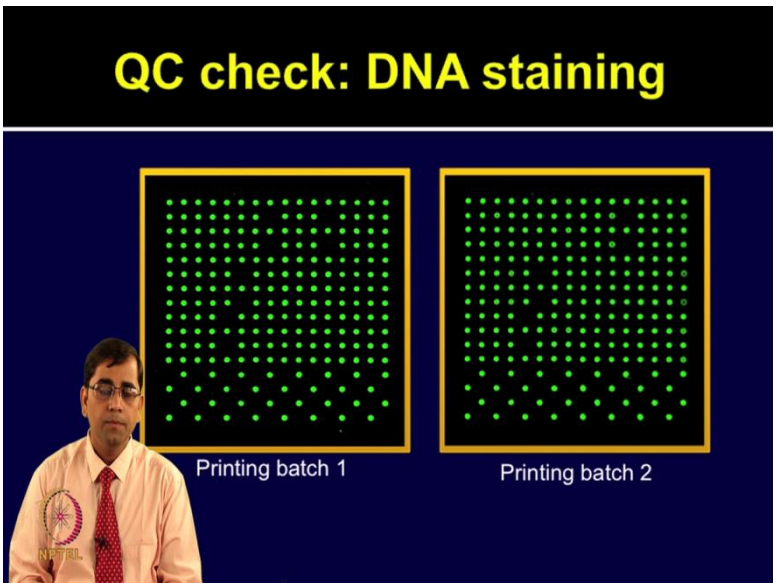

If you are printing 200 slides, 100 NM batch, 110 batches then you need to ensure that both the batches can be used for same experiment and those details comparable. So, batch to batch variability in printing must be minimized for both DNA staining as well as the protein expression. Once the DNA is quality control checked then you need to move on to the protein expression testing.

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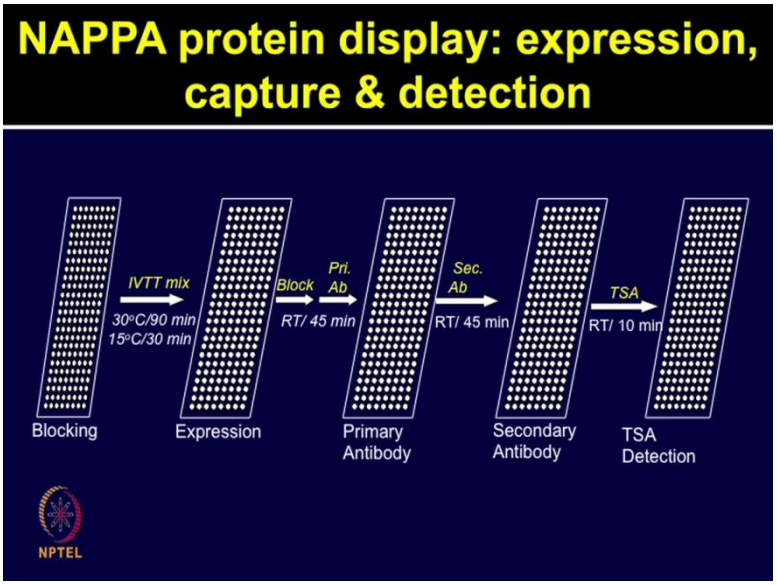

In this slide I have shown schematic of various steps involved in performing NAPPA based protein expression, so once you have slide you need to block the exposed area to avoid non specific binding. Then you can use IVTT system so the proteins are expressed and then you want to detect the signal so you can add the primary antibody. In this case here anti GST antibody followed by the secondary antibody containing either Si 3 or Si 5 levels or one could also use horseradish peroxidase based tyramide signal amplification system TSA based system.

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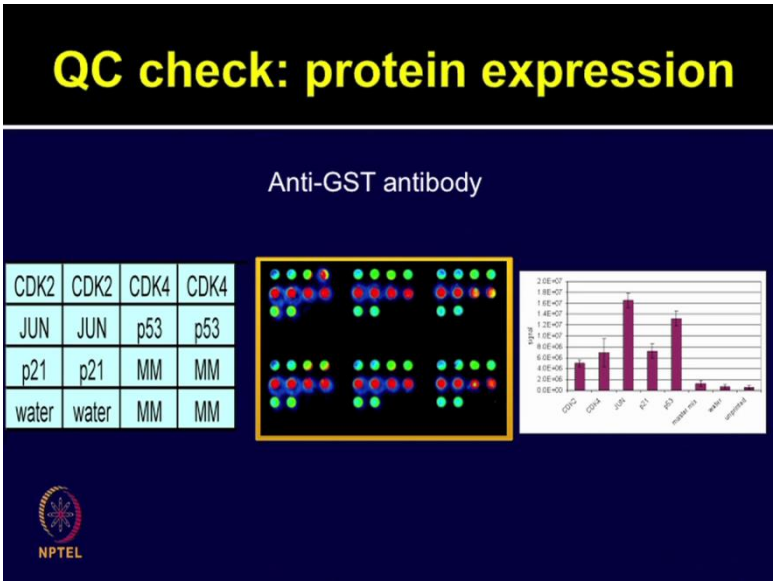

Here the proof of concept small array, these proteins need to be QC here. One could use anti-GST antibody and now you can see that only 5 proteins in duplicates are expressed, where as master mix and water are blank and same can be plotted on the graph which is shown on the right hand side. Now you have tested protein expression, but you want to ensure, how specific is the protein expression?

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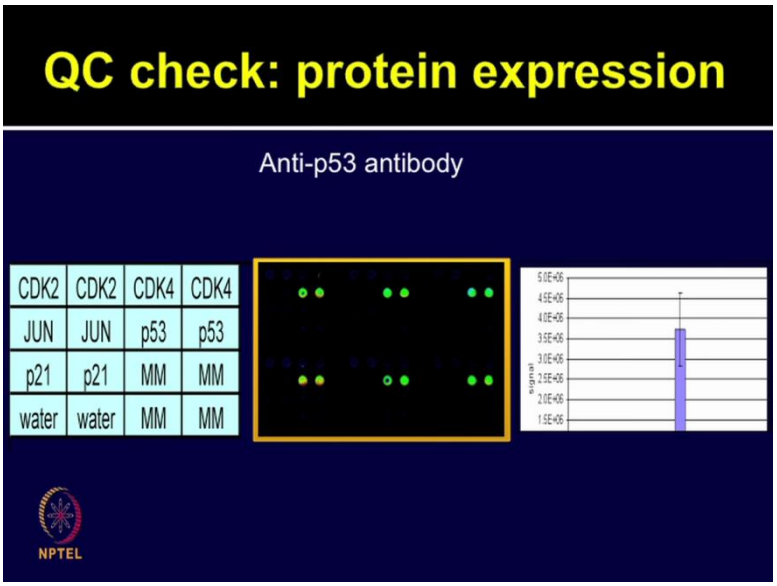

To ensure the specific protein expression one can also use protein specific antibody. In this case we have used Anti-p53 antibody to find out p53 protein printed on the chip surface. As you can see in the middle panel, only one pair of a spot is lightning up which shows that only p53 can be detected. The intensity can be plotted in graph as shown in the right panel.

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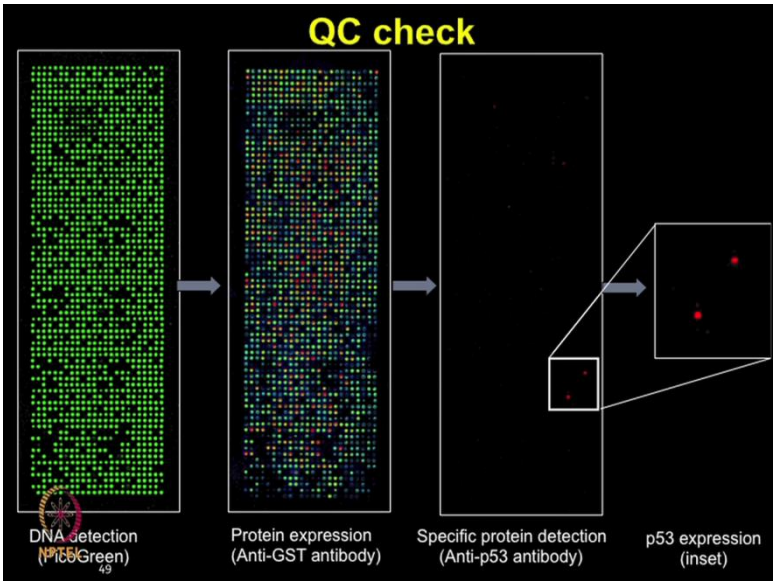

This is an overview of the quality control checks that one has to perform during whole NAPPA procedure. Before you use this printed arrays for testing any biological questions you need to ensure that DNA is properly printed, which is shown in the left side with pico-green stain. Next to that, protein expression by using anti-GST body and protein specific expression by using anti p53 antibody.

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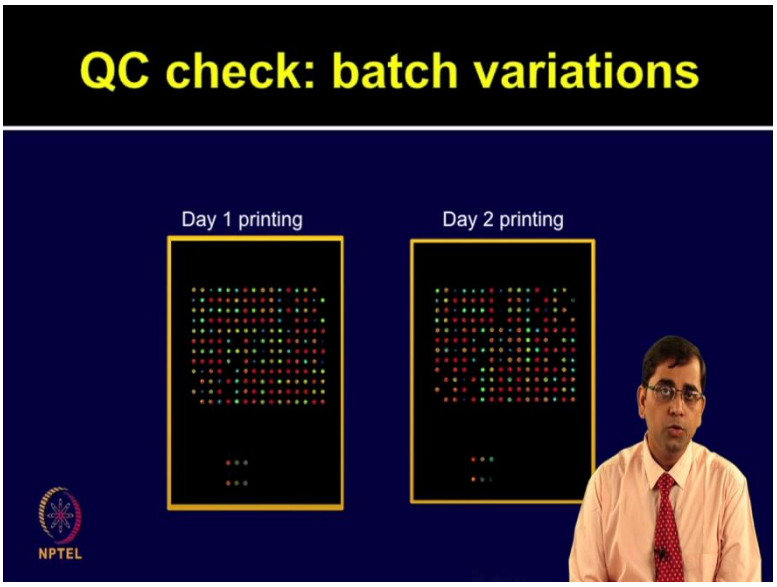

Again, one need to look into the batch to batch variability for the protein expression. If you print GST purified protein in different concentration as shown in the bottom side those can be used to determine how much protein is produced on each feature.

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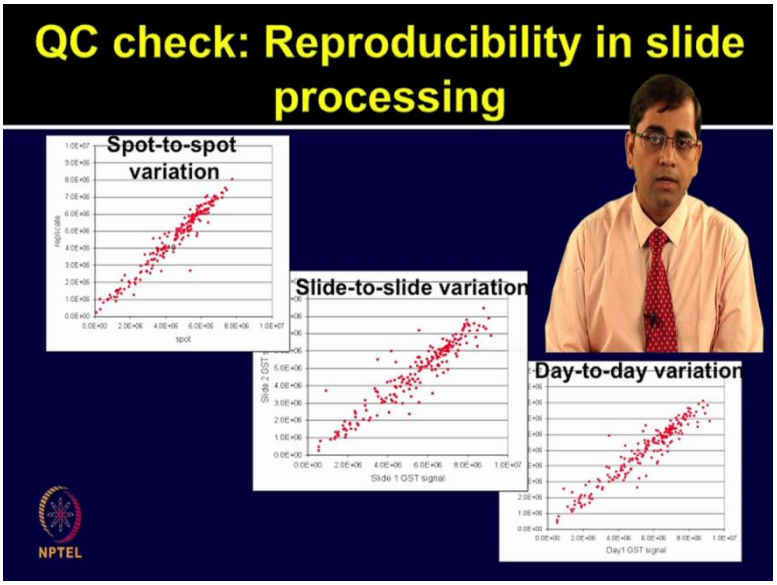

So batch to batch coefficient of variation sample to sample cv as well as replicates cv should be calculated. Now, once you have looked that day to day variations, then also look at different types of other variabilities such as spot to spot, slide to slide and day to day. Again plot it as shown in this graph this shows that NAPPA arrays are quite reproducible. Let me show you this animation for quality control checks and how to detect DNA and protein expression on this chip surface.

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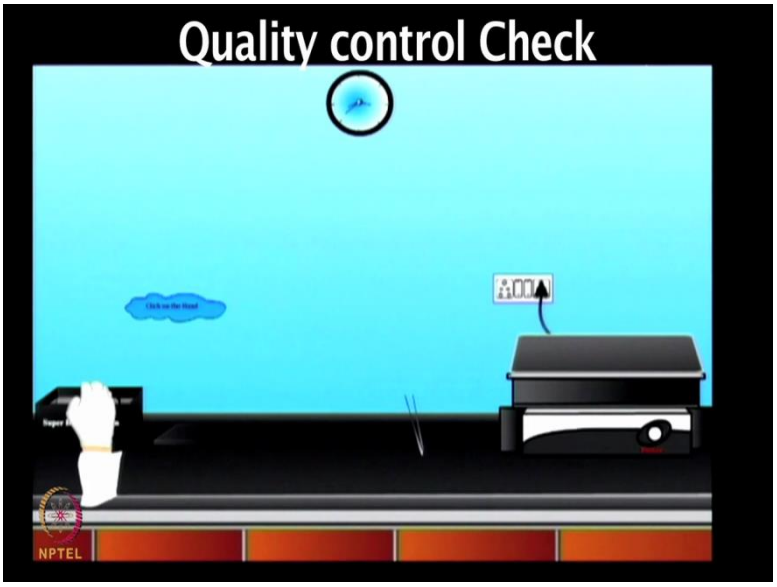

Once array are printed then we need to perform the quality control checks whether DNA printing was appropriate on this chips. This tool for microarrays is merely DNA, so in an activated array all following reaction elapses in the solution and in the real time therefore,

restriction caused by the instability of proteins occur very rarely. To perform the DNA staining first prepare the pico-green stock solutions diluted in the milk or super block.

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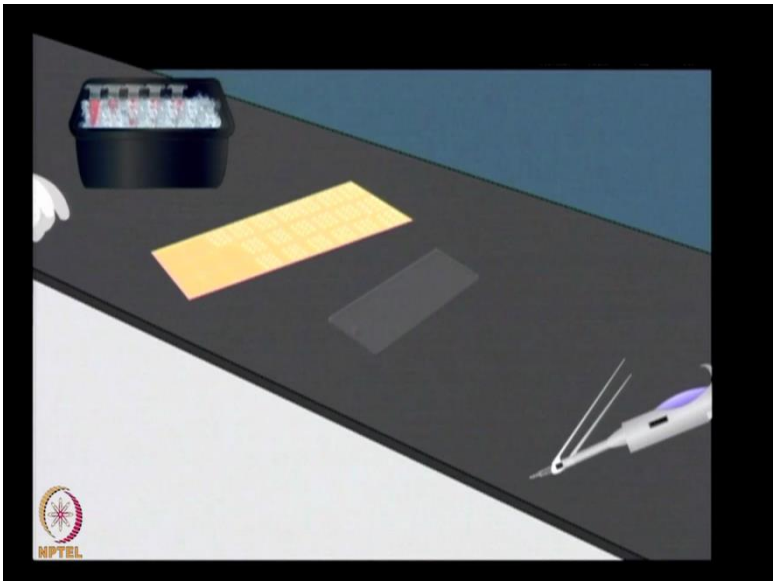
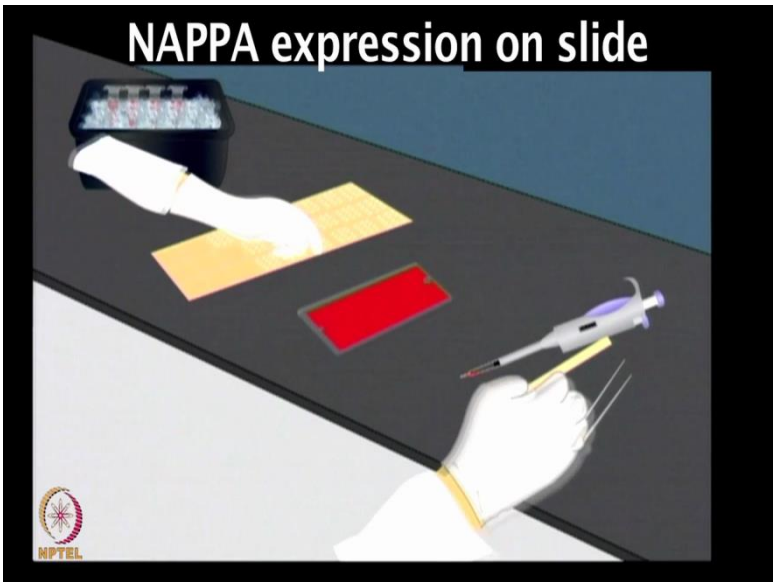
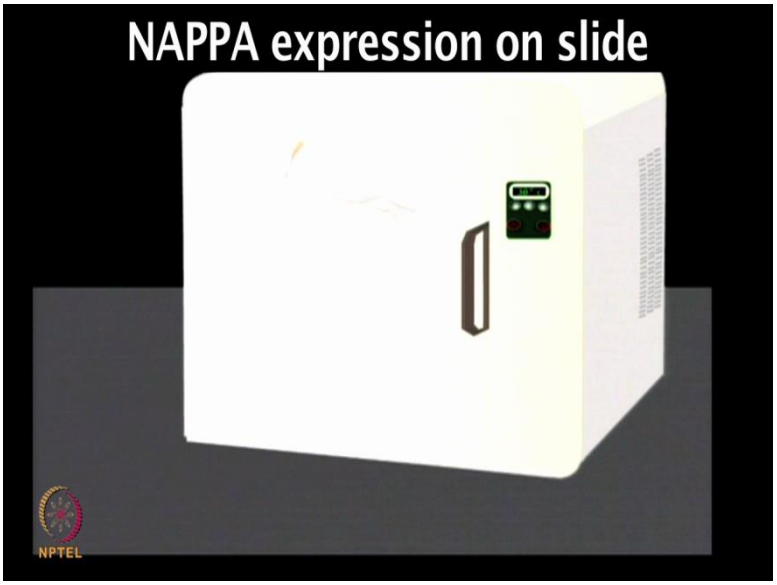
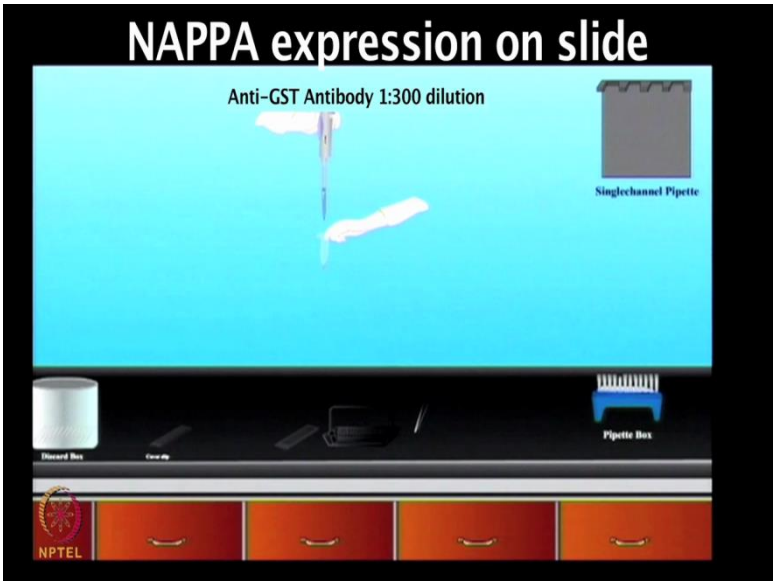






Block the chips to the superblock for an hour an after blocking is done, then we can use the pico-green mix to place on to these printed slides. Let us first look at the NAPPA expression on the slide and then I will describe about scanning for both DNA printing and the protein expression at the same time. So first you need to prepare the in vitro transcription translation mix which contains TNT buffer, T7 polymerase, amino acid, RNAs inhibitor and DEPC water, apply that on the slide which contains the hybridweld gas kept and then seal the pores. Now this printed slide which contain plasma DNA, they have the in vitro transcription translation mix shown in the red colour here and next step will be to incubate slide for the protein expression step.

(Refer Slide Time: 27:52)



So incubate the chips for 1.5 hour at 30 degrees for protein expression followed by 30 minutes at 15 degrees which allows a protein to bind on the anti capture antibody. After the this incubation is done, then we need to wash the slides so remove the hybrid well wash with milk and PBS for 3 times 3 minutes each and block the slide with milk or super block at the room temperature for an hour. After blocking is done, then we need to drain the excess liquid on a paper towel and then we are ready to perform the incubation with the primary antibody. So apply primary antibody which is mouse Anti-GST in this case because we are looking for the protein expression and all the close contain GST tag. So, we need to add the primarily antibody on the chip which is already express proteins are there and after adding primary antibody we need to incubate it for an hour at the room temperature.

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Now place the cover slip so, that liquid is uniformly placed and then we need to perform washing steps after an hour, proper washing is important for all the micro arrays experiments. Now we need to add secondary antibody in this case anti mouse HRP and incubate again for an hour at the room temperature. After secondary body incubation, we need to wash PBS 3 times and then apply tyramide signal amplification system for signal detection. After adding the TSA solution incubation then we needs to wash the arrays in water very quickly so that excess TSA is removed. Now, we need to dry the slide with the compressed air or drying step can also be performed by using centrifugation.

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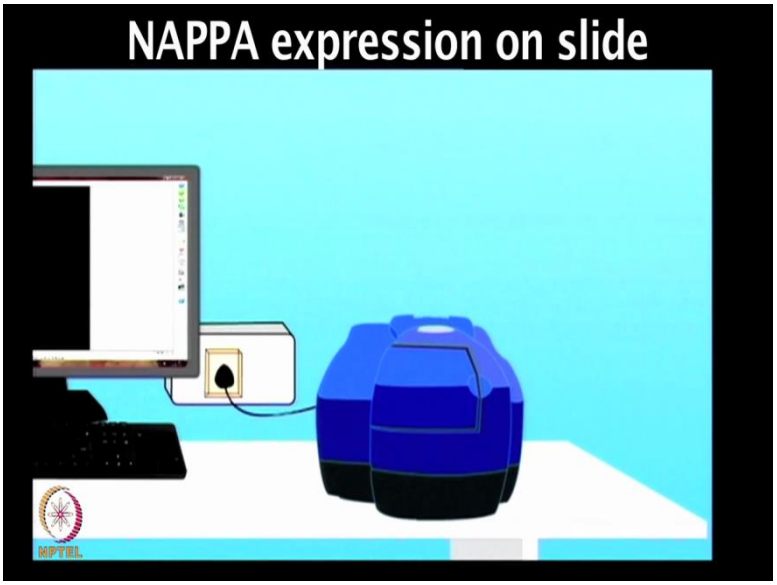
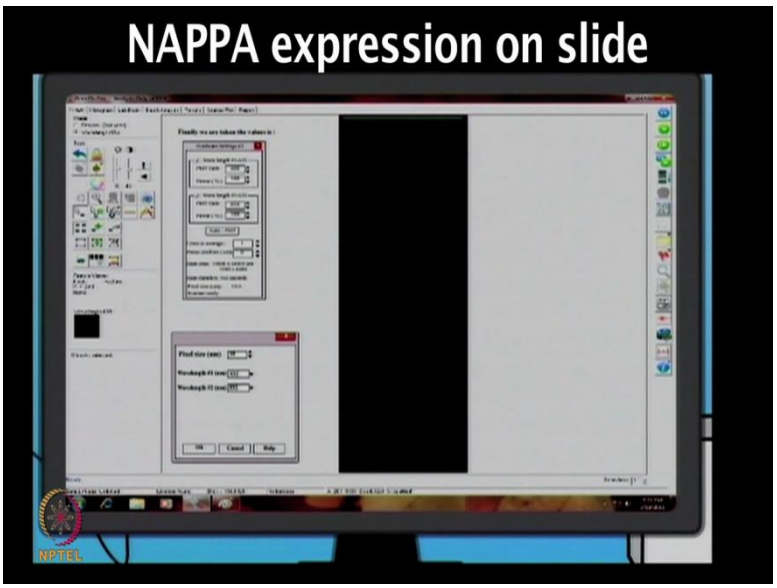
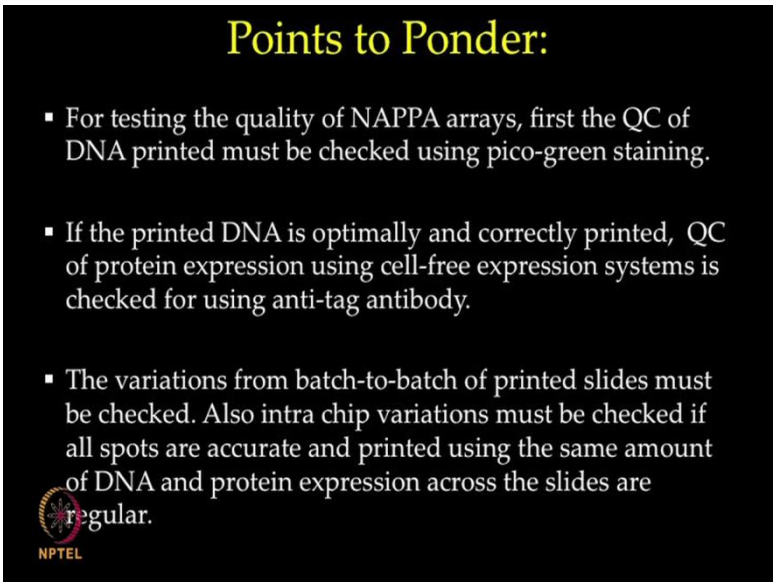




Now we will see animation for the scanning slides whether you done QC experiment of DNA detection or protein expression now you need to scan the slides, the different types of

scanners are available including the those one which are automated and multi slides can be scanned in the automated way. First you can preview the scan and then you can adjust different types of settings to see the signal better, then you can look at each region in the (())(31:25) view and look for the signal.

(Refer Slide Time: 31:43)



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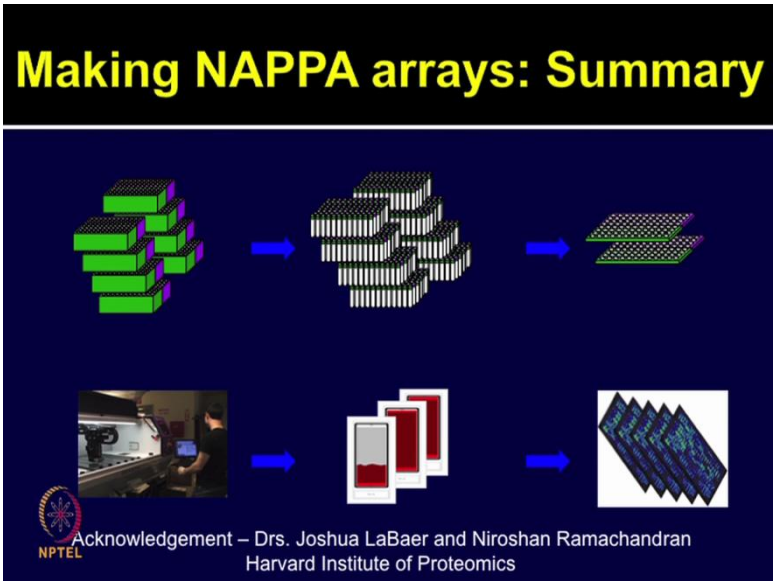
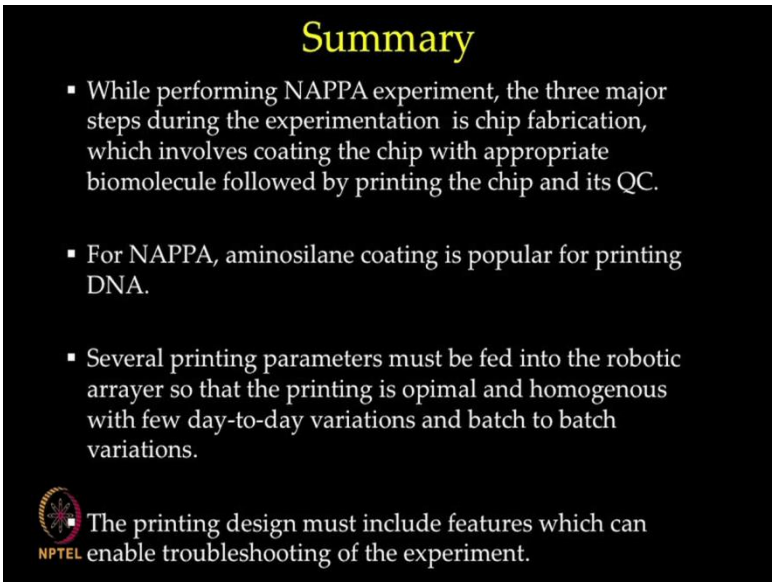
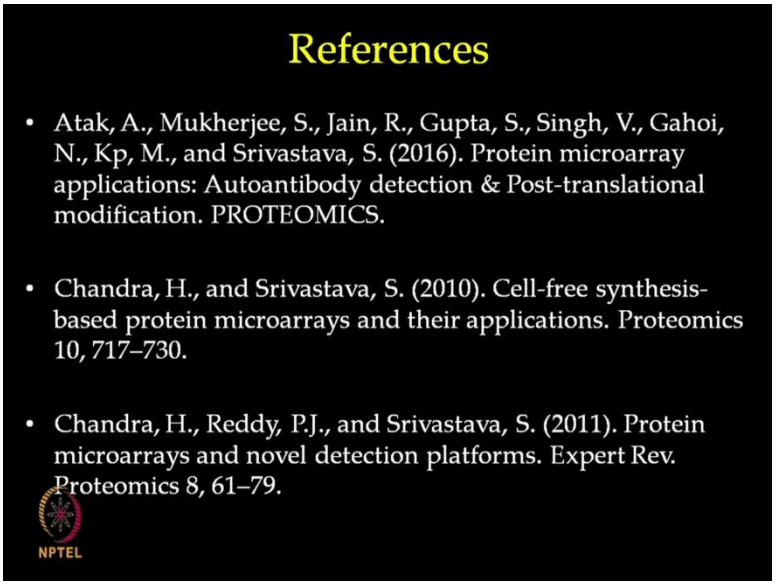


In summary, today we discussed about how to make NAPPA protein arrays. Similar workflow can be followed for other type of cell free expression based microarrays as well. To make the NAPPA arrays having access to the see (()) (32:05) is required. One can perform recombinational cloning or obtain good clones from various repositories and grow them in cultures, the purified plasmids along with master mix can be used for spotting on arrays.

Now these can be used on the micro arrays which can do the printing. Once printed these slides can be stores at the room temperature. Once you have performed all quality control checks, these arrays can be used for different types of biological application. This concludes our discussion on detail workflow of constructing NAPPA proteins arrays. In the next few lectures, we shall talk about data generation, interpretation and detail overview of microarray data analysis Thank you.

(Refer Slide Time: 33:18)





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