Interactomics Basics and Applications
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Lecture – 13
NAPPA and its applications in study of antibody immune response in disease and in drug screening-III

As you have seen Nucleic Acid Programmable Protein Arrays or NAPPA is very robust technology where many applications can be performed. You have seen Dr. Joshua LaBaer discussing about the advent of this technology and various type of applications which could be performed on these arrays. However, the nature of microarray experiments are such that you have to perform series of a steps before you can make any meaningful signal or the sense out of the data.

It is like a western broad whether starting from blocking your arrays to doing the incubation with the patient samples or primary antibody, followed by again washing his steps, then secondary antibody incubation and then do the signal detection. So, this whole procedure which is a daylong procedure involves series of washing a steps, drying and again adding the next set of reagents. Imagine that you know you have printed some features on the arrays; some substrate could be glass or nitrocellulose. And, as the day progresses then you are performing next set of experiments on these chips.

Then, if the reagents are not very tightly bound to the substrate, they may slightly wash off or if your binding is not very tight then probably you will see the loss of the signal. To overcome these technology barriers, there is need to come up with better alternatives and the technology provision has to happen. So, that we have more robust microarray platforms available. In this slide Mr. Joshua LaBaer is today going to introduce you to a newer method which is HaloTag based NAPPA technology which they have very recently developed.

It has shown much more promise and very strong signals to do the NAPPA arrays with much more efficiently. So, let us welcome Dr. Joshua LaBaer for today's lecture on advent of NAPPA technologies using HaloTag methods.

So, what I thought we would do today because, you are all such advanced scientists in the area of NAPPA is fast forward a little bit. So, we have talked a lot about the development of the technology, we have talked about the methods for making it. We have talked about some of the applications that we have done with it. What I thought we would talk about today are some of the newer methods that we have been developing the last year or two. So, these are very current in fact, several of them are papers that were published just this last year.

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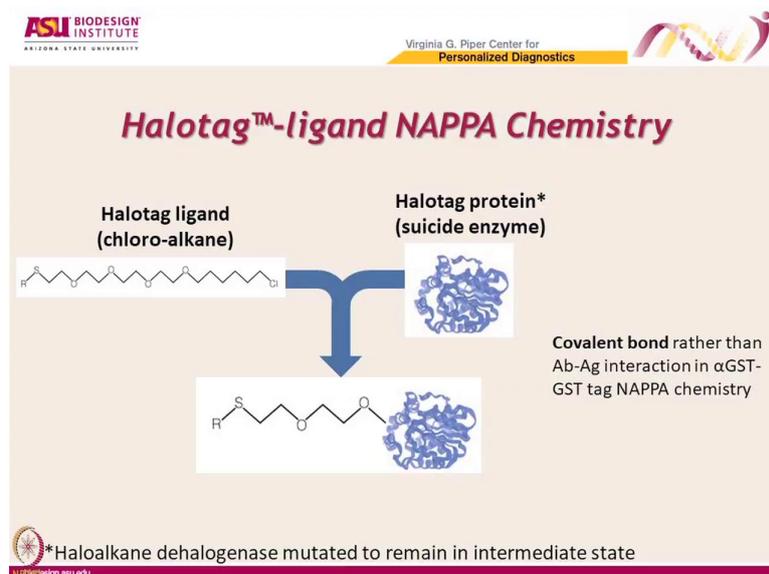
So, one of the things I mentioned the other day was the HaloTag and this is what the HaloTag looks like. This is a chloro alkane. So, it is a aliphatic chain with a chloride at the end.

You can also do the same thing with bromo alkanes, but chloro alkane is a I think one of the preferred substrates and this the HaloTag enzyme is a suicide enzyme. So, it binds to the chloride, it forms a covalent attachment to the chloride and then it gets stuck. And so now, you have this protein that is covalently attached to this chloro alkane. And, this R group here can be any functional group that you want. And so, you can use that to attach the chloro alkane to any surface, you can attach it to beads, you can attach it to a DNA barcode.

And, and that means, that that if you add the halo, if you add this part to your protein; just the way you would add the GST protein. Now, your protein will stick to any of those places covalently, you will capture it in a one directional of method that is permanent, this is this could be potentially very useful. So, one application is that I think I have emphasized several times there that the NAPPA technology primarily displays proteins that are functional and folded.
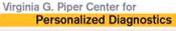
And, and for the most part we believe that is the advantage of the technology. But, there may be circumstances in which you want to measure binding to denatured protein. Perhaps, the epitope you want to find is actually a linear epitope and it is buried inside the protein. So, if you were to try to denature standard NAPPA, what would happen? So, how does NAPPA hold the protein on the array.

Student: GST.

GST and?

Student: Anti GST (Refer Time: 04:55).

And, antigen antibody; so, what happens when you denature antigen antibody?

Student: (Refer Time: 05:00).

They fall apart right. So, if you take the standard NAPPA and put it under denaturing conditions, the proteins on standard NAPPA will all fall off; because they are being held there by a strong, but nonetheless non-covalent interaction. So, imagine now if you could attach the protein to the array in a in a covalent attachment. Now, you could treat it with, you could you could denature it and the proteins would still stay attached right.

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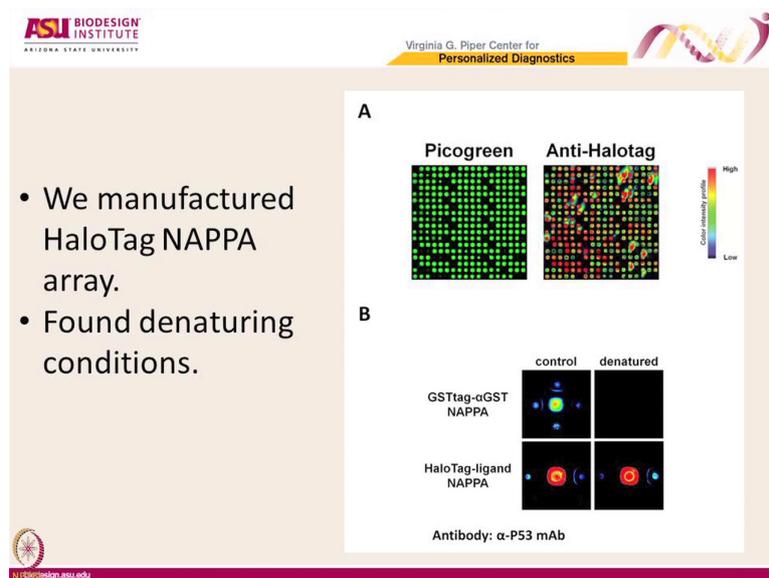
And so, that is what is shown on the slide. So, we made an array and these are with proteins that all have the HaloTag on them. So, this is just like the kind of NAPPA that you have seen before, but the difference is that in this case instead of using the GST tag, we have a HaloTag

and its binding to the ligand. And now you can see for example, this is a P-53 antibody and the GST tag.

So, this is the protein array under standard conditions of the protein array and denatured conditions. When I say denatured, I mean we treated it at 55 degree Celsius with SDS. So, that is the pretty harsh treatment and you can see that the GST antibody only binds to folded NAPPA; it does not bind to the nature NAPPA. But, this particular anti P 53 antibody could bind both formats because; we knew this antibody by this antibody here binds to a linear epitope.

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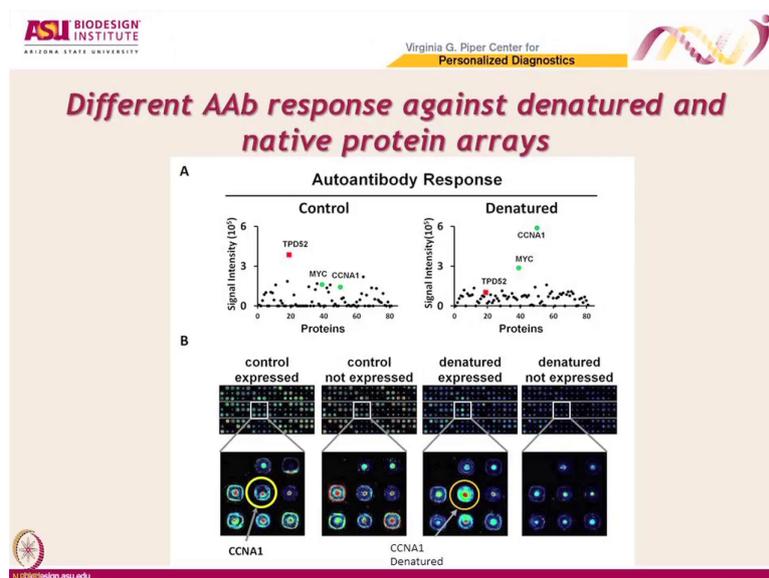
And so, one of the ideas that Ryan had, the student who did this work was is it possible that if you were to take a protein array and display it against serum either in the native format or the denatured format, would you get different immune responses? Could you pick up responses?

For example, under nature conditions and that is what he actually found here. Here you see the denature a protein and then here you see the under standard conditions.

And, here you can see that this antibody here specifically detects yeah under standard conditions and whereas, these two antibodies only detect CCNA1 and MYC under denatured conditions. So, that patient's serum response was different depending on whether they were folded or whether they were not folded proteins. I think you can see that response here, then its binding; this is this response here binding the denatured protein whereas, you do not see it so, strong in this guy here ok. Any questions on that part? Ok. So, then.

Student: Sir, antibody works even in denatured conditions?

No, no the array was denatured, then all the then it was washed and then treated with physiological. So, the proteins were all stretched out on the array surface, you denature the array. Then you rinse off the SDS you rinse and you bring it back to room temperature and now you add it fold in antibody, because if you denatured the antibody, it would not work where most antibodies do not work alright.
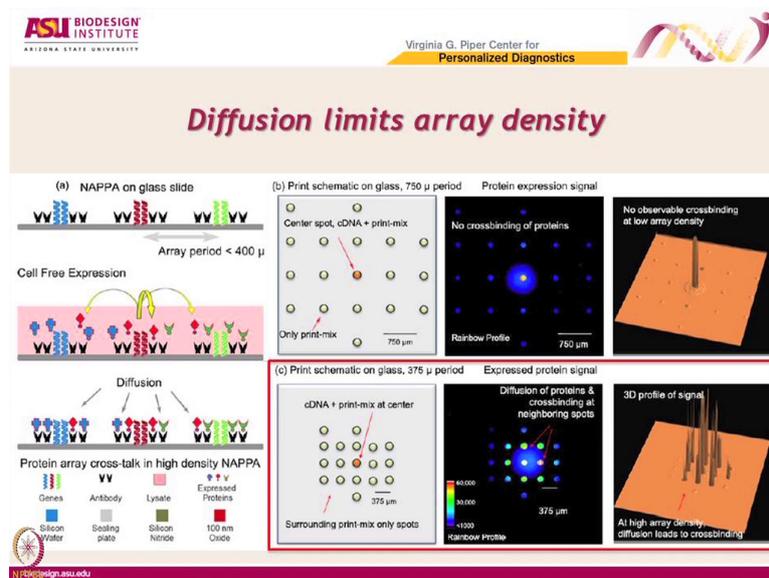
So, we talked about this a little bit earlier in the course, this issue of some a couple people asked the question about protein diffusion. And so, what do I mean by protein diffusion? Well, here are three spots on the array kind of in a in a cartoon and here you see the DNA for each of these genes. And, one of the concerns would be that if you produce the red protein that the red protein could float over and bind in the spot where the blue protein is supposed to be, same could be true over here on the green side.

So, you get mostly red bind to red, but maybe red binds here, maybe blue binds over here. And so, you end up with a circumstance where you have a little bit of mixture at each of these spots and so, that would be that is the concern that a lot of people have. So, we actually went to see if how much of a problem that actually was. So, this is the state this is a configuration of our standard NAPPA, the spacing here between these spots is what we currently print at.

And, what we did in this experiment was we printed the gene here at this spot and in these spots here we printed everything, but the gene. So, the antibody is still there to capture the GST, but there is no gene in that local position. So, what that means, is that if there is any diffusion from here to here, it will get captured by those sides and you will see that as signal ok. And, and what you see is that in fact, there is a little bit of spread around the spot, but it does not really reach over to these neighboring spots and this is a three-dimensional plot of that intensity.

So, very strong intensity at the main feature, almost no signal at the neighboring area; so, in standard NAPPA this is really not a big problem. But if you start to make NAPPA much smaller; so, if you take the 750 microns spacing here and make it 375 spacing so, almost cut it in half yeah. So now, these spots are really close by, now you start to see a little bit of signal bleed over; you see that little green signal.

So, this is the intensity of the spot itself and then these neighboring spots have picked up a little bit of the protein and you can sort of see that in a 3D rendering. So, that tells us is that for the most part under our current conditions we are ok, but if we ever wanted to make our arrays much more dense. So, shift from 2300 proteins, let us say to 10000 proteins we could run into trouble where there would be neighboring spot intensity.
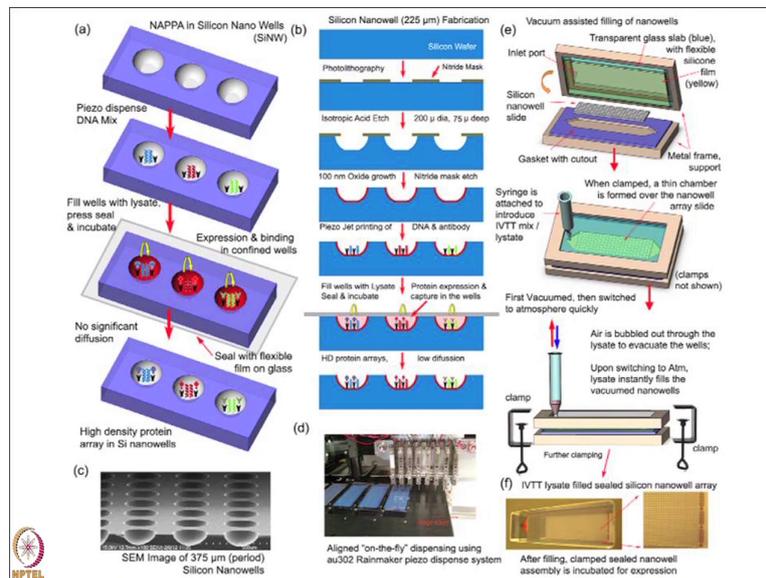
So, we have been thinking about ways to get around that and this is the method that we have developed. What we do is we take silicon, the same material that you use to make computer chips. And, we use the same technical approach that they use what is called photolithography, where by shining light on the surface you create a mask. And, then you etch it with chemical compounds that etch away the surface and you essentially can wear away the surface of the silicon. And, you end up what we do is we create these little what we call them nano wells nano, nano because they are nanometers in size. In fact, in terms of fluid volume they hold picolitres of liquid.

So, they are very very small and we so we edge away those wells. So, here this is the process, use photolithography to kind of create a mask, you use the acid etching to create these wells. And, then they there is a couple of chemical treatments you have and then we print the NAPPA mix into the wells. So, it its well I describe it like it was easy, this is actually quite an

involved process to get this to work. It took a lot of different mapping methods on the on the photolithography side to create wells that had this sort of bowl shape at the bottom.

Because, typically for (Refer Time: 12:43) the photo lithography wants to make a straight wall and a flat bottom and it turns out that the signal intensity was not as good in that format. The other thing it is not tricky that that is not easy is printing the DNA into the well. So, in standard NAPPA, we just have a solid pin printer that just runs along and just makes spots, but here we had to get the liquid right into a much smaller target. And so, we ended up having to use a piezoelectric printer that has a camera in line with the print head and by using the camera to align where the spots are, we can aim this it spits the liquid into the tiny wells.

And, it does so quite accurately, but it is a little bit tedious, but anyway that that is what we do so, you end up printing the DNA and the well. Then once you do that you add cell free lysate across the entire surface of the array and that liquid in is intended to get into the wells. And, then you cover it with a cover slip and you can see that cover slip right there. That also turns out to be non-trivial, non-trivial because when you have small wells like that there is a tendency for the liquid, because its hydrophilic to not want to go into the wells. Because, air gets captured, it basically it there is air in the wells and it gets captured.

So, you have to fiddle a little bit with vacuum pressure and surface pressure to get the air out and get an even distribution of the expression lysate throughout the wells. And, actually the solution that the engineer came up with is quite clever; I will show you that in a moment. I just want to mention this is what this is a scanning electron micrograph of these nano wells in silicon. And, you can see that they have this very nice bowl shape and it turns out that that shape is important. So, what Peter did to seal these wells, he developed a system where he had to plastic laminate surfaces, that flat plastic services that are like this.

And, sandwiched in the middle he has a form of oil, liquid a liquid oil, a clear liquid oil. And so, then what he does and the liquid oil that whole system is connected up to a pressurized system and so, the minute we finished putting in the expression lysate, he adds pressure to the oil. The oil then takes the plastic and does that, it kind of forces it apart and essentially forces the plastic to seal the nano wells. And it does so, instantly and at the same time there is they

apply a little bit of vacuum to the liquid on the surface of the array that pulls out any excess expression lysate.

And, you end up with a sealed surface where you I do not know if you can quite see that, but you end up sealing the silicon well, the nano wells with that with that plastic. So, this is what the apparatus looks like, it there is its evolved and I need to get a better picture of that. This is a little bit of an old slide, but the system works pretty well. This is the piezoelectric printer by the way that is doing the printing and these are these piezo these special piezoelectric nozzles that are very accurate at delivering fixed volumes to each well.

One added benefit for those of you who are NAPPA aficionados is that with these nano wells, we have figured out a way to print the print mix separate from the DNA mix. So, one of the things that you may not appreciate when we normal NAPPA, when we print it has a cross linking agent in it. And, the crossing agent is meant to capture the DNA and the and the protein BSA to the surface of the slides so it stays put. The problem with the cross linking agent is that its, there is a time function attached to it. The minute you activate the cross looking agent, it is a free chemistry that starts to act on your sample. If you let it go too long, everything gets over cross linked and it is no longer functional.

So, the minute you add it to your print mix, the clock starts and you have a certain amount of time to print it before everything gets ruined. Anything that does not get printed that day, whatever is left in your tube its gone forever. So, if you made a lot of DNA to print your arrays, use a little bit of it to print your arrays; all the rest of your DNA is lost. And, remember I mentioned the other day that even though it is not expensive to make DNA, whenever you have to make 10,000 of anything its expensive.

So, now you have essentially wasted all of your 10,000 DNAs, one of the advantages of this platform is that we can print the DNA separate from the print mix which means that we do not add the cross linking agent to the DNA when we print it; which means that whatever DNA is left over you can freeze it and use it another day. And so, you do not have to waste everything that you have used so, that that turns out to be an advantage to us.

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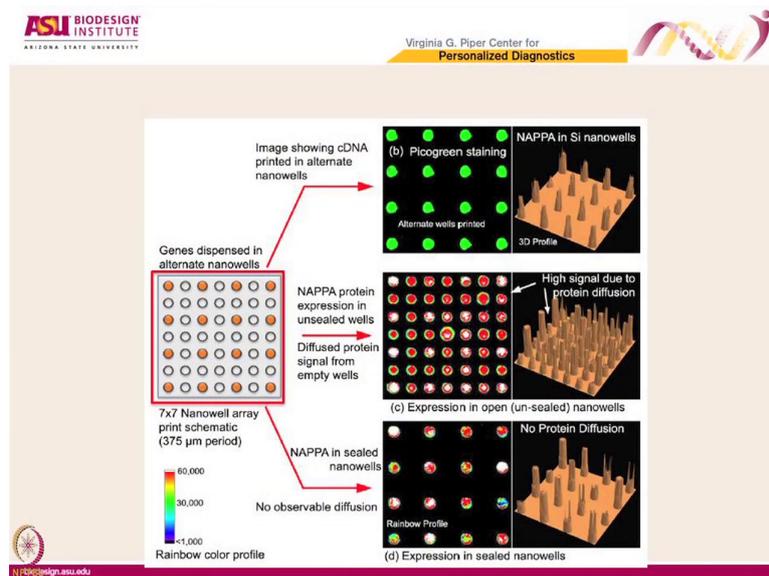
So, this is what this looks like, here we have dispensed genes into nano wells. The genes are in this pattern where they are separated by wells that do not have any expression and you can see how accurately it expresses. You get very clean expression at each spot and despite the fact that these are very close together, you are seeing no intervening spots right. This is if you were to just express it without sealing the well.

So, if you just remember I said we sealed the wells of the plastic, if you left the plastic unpressurized. Now, every now you see how much spreading there is. So, this is the tendency to spread and this is how well the sealing apparatus prevents the spread. So, essentially blocks out completely.
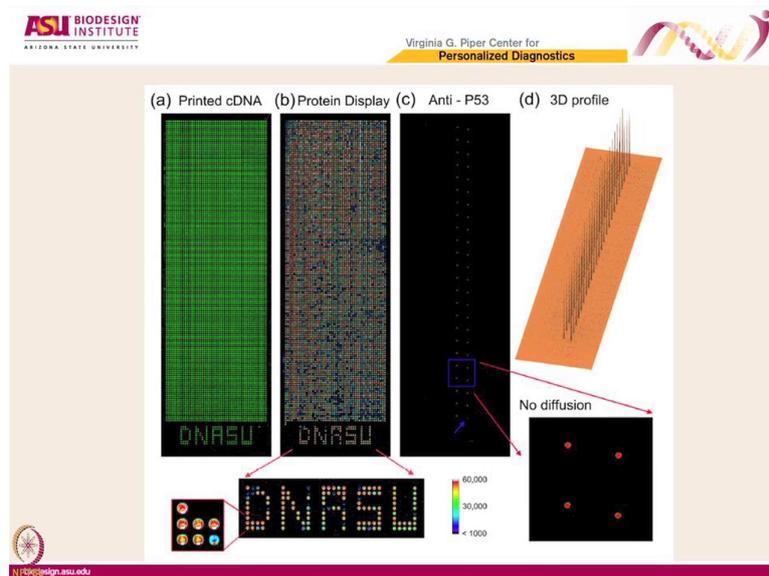
And so, that is how we got this image here. So, what you are looking at here is now an app array that has 10,000 features on it, all expressed in nano-wells. This is the DNA print, this is the protein print and then we stained it with an antibody to one specific protein that we repeated on the array. And, you can see how sharp that is, single spot single spot no diffusion to any of the neighboring spots. And, if you plot that in a 3D image you can see it is just exactly where you want the signal to be.

One of the added benefits of this approach that we did not appreciate when we first developed it is that it turns out be more sensitive than standard NAPPA.
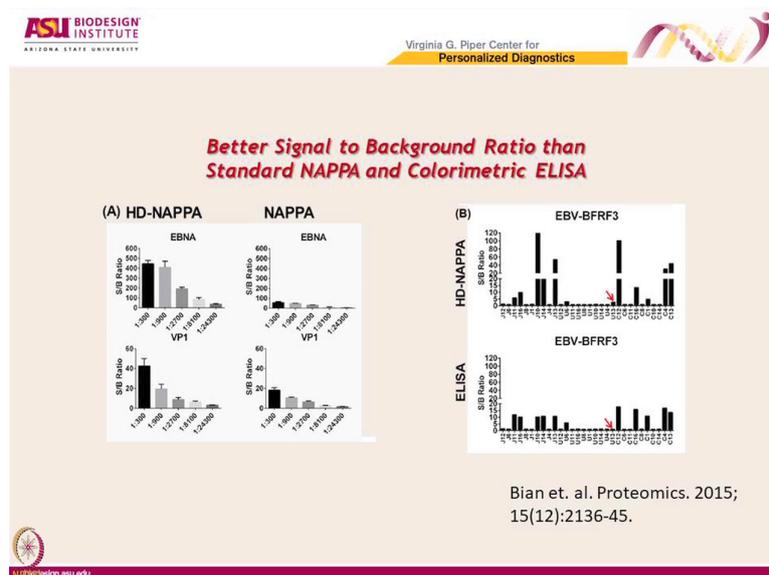
So, we did some comparisons and we looked at this antigen VP 1 and we are doing different dilutions of antibody to ask what is the detection limit on the array platform. This is standard NAPPA and then this is HD NAPPA, we call that High Density NAPPA. And, you can see that you know at every pretty much every dilution we are getting much better detection here then we are here. In fact, you know it kind of plateaus here at you know maybe 50 or 60 at a 1 to 300 dilution. At 1 to 300 on this platform its 450 so, the signal intensity is much stronger on these high density NAPPA arrays yeah.

So, this is EBNA up here, this is VP 1 down here. So, these are two different antigens and you can see that the signal intensity by dilution is much better for the HD than this. We even compared the HD NAPPA to ELISA. So, you would think that ELISA being a full scale chemical method in us in a 96 well tube should be much better in expression. But in fact, we were able to detect signals here on the HD NAPPA that you could not detect it all on the

ELISA. And, then overall the signal intensity by ELISA compared to signal intensity by HD NAPPA, this was nowhere near as strong as that was. So, it in our lab right now this is probably the most sensitive platform we have for detecting interactions.

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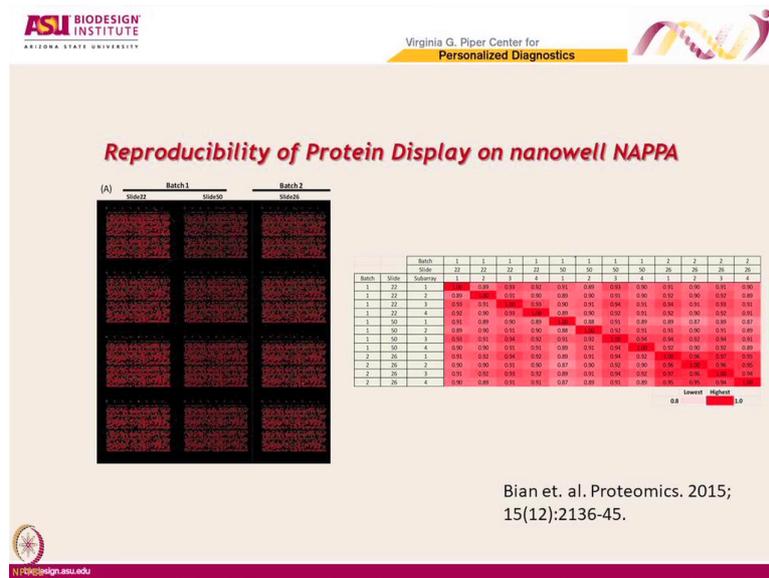
This is just to kind of I will show you that we can print these arrays very reproducibly because, that is one of the things that you want to be able to do. So, I do not know you can see this in this slide. But, this is a single slide array that has 4 sub arrays on it, each of these sub arrays contains 4,000 different spots. So, we have 4,000; so, you a total of 16,000 spots on this slide.

And, and we have repeated it in one batch or in a separate batch and then what we have done is we have done an interaction map, the other kind of correlation coefficients that I have been showing you throughout the course. Every day versus every other day and again as you can

see everything here is in the; you know close to 1.0 and in the certainly above 95 percent in terms of its reproducibility. So, its every bit as reproducible this platform as the standard NAPPA was.

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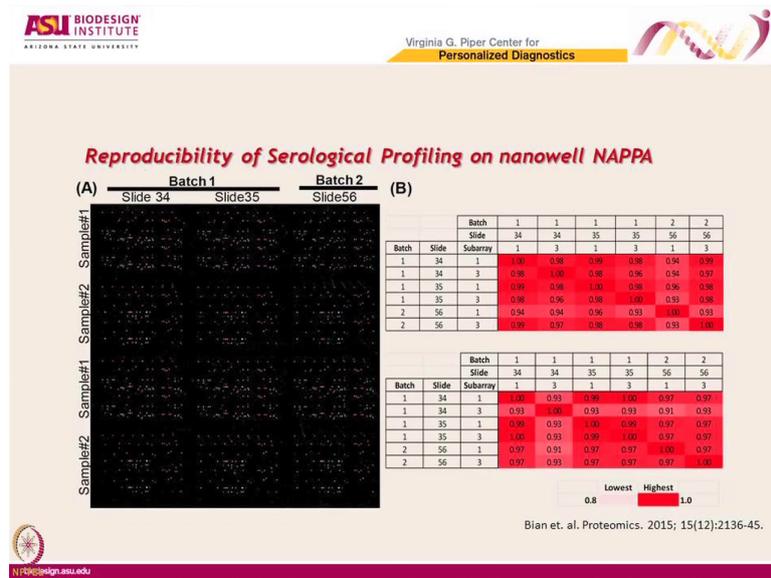
And, then this is probably more relevant to you all is if you actually that was for protein expression. This is now asking if I screen the array with antibodies or serum will the answer I get from array to array from batch to batch be the same. And, again I do not think you can you see the spots there it is a little too dark I think. But, this is the correlation graph and again you can see that nearly everything is in the high 90 percent if not 1.0. So, this is the results you get a line very nicely.

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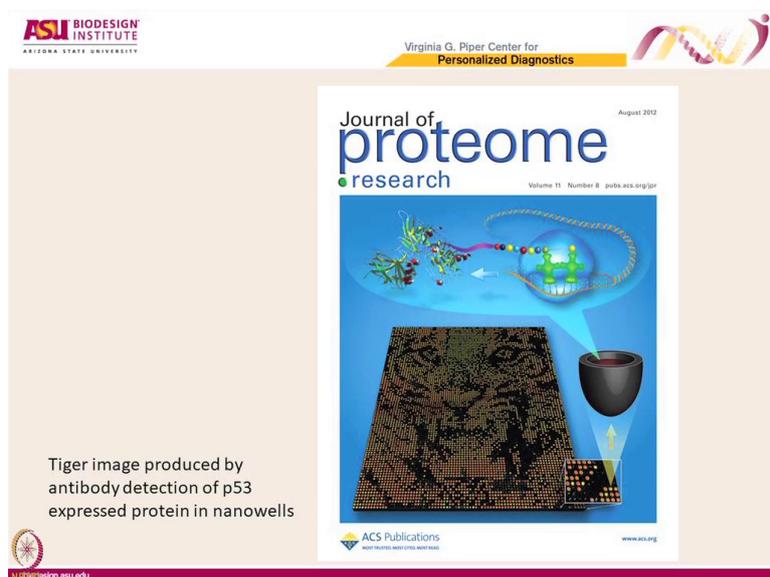
And, so that led us to this picture here. So, this is that we made the cover of Journal of proteome research that month. And, what I will show you this image again what you are looking at here, let me see if I can go here.

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Hopefully, you can appreciate that there is a tiger in that image. So, this is of what you are looking at is an actual protein array. We have printed different amounts of DNA encoding the p 53 protein; we then express the p 53 protein in the array in these nano wells.

And, then probe the array with anti p 53 antibody with a fluorescent tag on it. And, what you end up seeing is because of the different amounts of DNA you can get an image of the tiger's face. So, this was the first time we ever did an image using a protein array alright.
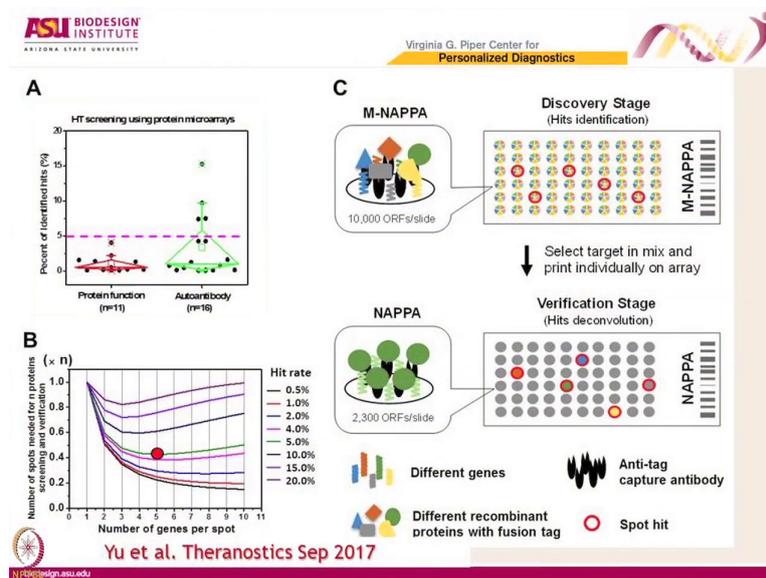
So now, alright so, I want to move on to a slightly different topic then, this is another way about getting a lot of information onto the array. So, imagine you know the whole goal here is we want to we want to test as many proteins as we can, when we when we screen an array. In the current NAPPA format on plain glass slides which is what most people can use, because all the technology I just showed you is kind of fancy and you have to have special instruments to do it.

Most people would rather work on a plain glass slide the way you have, the problem is in our current platform we can only put about 2300 proteins on that slide. But, that limitation is only true if at each spot you only have one protein, but what if you put more than one protein at each spot? So, maybe what do you all think, could that work? What would be the issues? So,

the idea here one of these ideas that came to me in the shower is that we could print multiple genes at each spot. So, what would be the issues? Why would you not want to do that?

Student: Specificity, specificity of the binding.

So, tell me what you mean by the specificity of the binding?

Student: Cross reactivity in each spot (Refer Time: 24:31).

Say, say it again.

Student: Cross reactivity in each spot.

Ok, not that is not that way that was not exactly what I was worried about, what other people think.

Student: Quantitation.

Say it again.

Student: Quantitation.

So, tell me what you mean by quantitation?

Student: We have to eventually quatitate to get the graphs or bolts. So, then we have to take individual values to get their individual quantitation in the end.

Ok. Alright. So, that is the so, so she is saying how are you going to understand in to the individual contributions and I think that that is a fair concern right. So, I have got let us let us say I put three proteins in the spot right. Now, now in the same spot all three proteins will be

there? If I get a signal I will not know which of those three proteins was the target right, I will not be sure yeah.

Student: All the three proteins will have the same tag?

Well all of them have what?

Student: Same tag like.

The same tag yeah, yeah they will be they will be captured by the GST tag that is the idea yeah. One could imagine a much more sophisticated version of this, where you had three sets of proteins with different tags that would be an elaborate method, but potentially one that could work for sure right. So, but nonetheless so, that could be an issue. So, here was my reasoning when the idea occurred to me. So, whenever I do an experiment on NAPPA and I screen an array of thousands of proteins and I get hits.

The first thing I do after I get those results is I repeat them right, I want to make sure that if my array told me that the antibody bound to protein x, that if I really try it again with another protein x it still works right. So, I believe that all scientists are obliged to repeat their experiments to make sure that they are correct. So, it occurred to me that if I did the experiment with a multiplexed spot that had multiple proteins, I was going to repeat it anyway. But, this time instead of repeating them as mixed, I could repeat them as individual proteins.

And so, I would be confirming that they were binding, but at the same time I would be identifying which spot was the one that contributed to the signal. So, I would get sort of two benefits for one in the second round experiment. And, the net effect would be that I could screen many more proteins on a single slide and then in the end do much less work to get the same information. So, so, one of the questions we had now that strategy has limitations to it right.

It one of the assumptions of that strategy is that when you screen the array the first time that the fraction of proteins on the array, that will be detected is small right; because, if the fraction of the proteins on the array is high, then the whole time savings thing goes out the window. Now why is that? What do you think? So, imagine now, I have an array that has will make a simplified array, it has a 100 spots on the array and each of the spots on the array has five proteins in it ok. If I screen the array and I get two spots that light up, how many possible targets do I have?

Student: Ten.

Ten possible targets right. So, my next day when I go to verify I have to I have to do ten different spots and then I and all have done my job right. Now, let us go to the other extreme, let us imagine for that 100 spot array that 95 spots light up. How many potential targets do I have?

Student: 95 times 5.

95 times 5, right. And so, how many spots am I going to have to do the next day? Pretty much, as if I had started with you know five arrays each one with one spot each. So, I am back to doing the same job, I would have done if I had not multiplexed. So, the multiplex idea works when the tart, when the hit rate is low and it does not work so, well when the hit rates high. And so, you can actually mathematically evaluate what is the best or most optimal number of spots to mix based on the likelihood of a hit rate ok.

And so, we actually did that, we did that develop the equations and we actually looked at it; I am not going to go through the math. We first we looked at we looked at the frequency of hit rates for different types of studies that were published in the literature. So, the first question we asked was on average if you are doing a protein interaction study, if you are doing an a you know auto antibody study of all the targets that people study and there is when they do their experiments what fraction of proteins light up?

And so, this that is what this is, this is the percent of identified hits and you can see that this is the 5 percent mark right here. And, most of the protein function studies and well I would say all the protein function studies and most of the auto antibody studies are down in the a couple percent range. Certainly, they are less than 5 percent so, that is promising right; that means, that this strategy could be a big time saver if I can make this strategy work right ok. So, then the question was what is the optimum number of spots we can do? Maybe making the assumption that the hit rate is 5 percent, even though I think that is probably a little high for most of them.

I think it is a fair assumption, it you know that it is a more conservative estimate; if we can satisfy that one we are certainly going to take care of everything that is lower than that. And so, we did we did the math and this is the optimum number of genes for spot and you can sort of see that that you get more and more savings up until you get to about 5 spots per gene after which it does not really get better. And, then it gets worse again because of the whole problem of having to do too many duplicates the next day.

And so, the sweet spot here was around 5 genes per spot right, right there and this purple line is the or this green line here is the 5 percent. See that 5 percent there that is the 5 percent line that came out to about 5. These guys are also pretty good at 5. When you get up to here, when you get up to 10, 15, 20 percent response rates you need maybe to question whether the strategy is a good one for you. Does that make sense? And so, the idea here then is you could take on a standard NAPPA now, that has 2300 spots or 25 let us say for the for the sake of argument, we can do 2500 spots.

You could print the entire 10,000 open reading frames on one slide doing 5 proteins per spot. And, that is what these different colors are meant to indicate, five different proteins per spot. Then you would you would screen that and you let us say you get these 5 hits 1, 2, 3, 4, 5; you take each of these 5 hits that is 25 possibilities. You print a second array that has the 25 hits on it and you screen that the next day and that does two things for you. It tells you which of those 5 proteins was a hit and it confirms that it was a hit, it tells you for sure yeah that was real.

And so, that is and so, that is that would be the strategy, does that make sense? So, here we call that the deconvolution step, it is sort of verification stage and deconvolution stage ok. So, does it work? So, what would be an experiment to make sure that its working? So, one of the questions that comes up is if I put 5 spots, 5 protein genes in a spot will they all make protein? What if only one makes protein the other four do not? Now, we already know from NAPPA other NAPPA experiments that every almost every gene we print makes proteins. So, we are confident about that piece, but you could imagine that somehow mixing them on the spot could be a problem right. So, how would you test that?

Student: Checking the functionality of the proteins.

You could look at you could look at that if you if the problem is that for most proteins we do not have the functionality. What other ideas we got?

Student: We can check them one by one.

You could certainly test them one by one in the mix.

Student: In the mix.

In the mix that is that is how we went about it right. So, what we did is we said well let us make mixtures of proteins for which we have antibodies. So, in this case we are testing only proteins that we can come back and test. And, then we are going to ask the question, if I mix a bunch of proteins together, if I test it will I find the protein? Now, is there a are there features that we need to consider where one let us say if I have a mixture of proteins, where one might be made in a greater quantity than another. What kinds of things would I want to think about?.

Where could have bias come in? If I have a protein that is 15 kilo Dalton's in a protein that is 80 kilo Dalton's, will I see a difference? What do you think? Why might I see a difference?

Student: The one which is 50 KDa will produce faster.

Right. So, how do how do proteins get made right? They get made by adding one amino acid after another using tRNA's on the ribosome right. So, the amount of amino acid you have to add to get to 15 kilo Dalton's is a lot shorter than the amino acid you have to add to get to 80 kilo Dalton's right. And so, you could imagine that if you have proteins of different sizes in the same spot, that the small protein could get churned out a lot faster than the big protein and you might have a bias from that. So, we tested that too because we want to make sure that the method was going to work.

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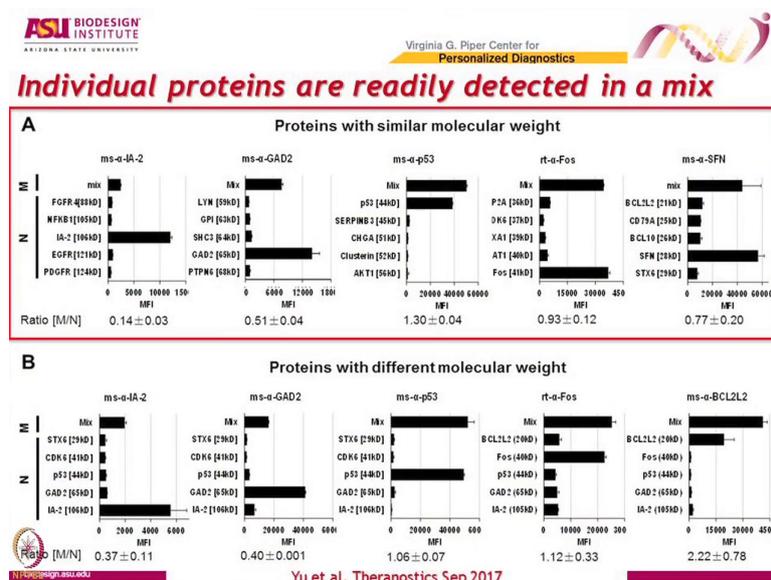
Alright; so, here what you have is so, let me work you through this experiment. On the top versus M that stands for mixed NAPPA or multiplex NAPPA we printed a mixture of 5 genes and then express them. And, then we also on the same slide separately printed each of those

spots individually ok. And, then what we did is we probed, we probed that array with an antibody that recognized one of the proteins in the 5. And, asked even though it was expressed in the mixture did we detect it? And, did we detected as well as we did in the mixture as we did by individual? Ok.

And, you can see for this IA 2 protein we detected it in the mix, we detected it much better as a single spot. So, to some extent this protein did not do as well in this group as it did there, but it was still we could still measure it. So, we would not have missed that in the study. Here is another one GAD 2, we get we can detect in the mix. We can see it as a single protein, here is anti p 53; it turns out that the mix was even better than the single protein was. Here is anti Fos, you can see that the mix was about the same as the in the individual spots. And, here is I cannot read that, s f i SFN and again you can see that there they are comparable, that showed us that the system was basically working now.

In this top experiment we tried to restrict the study to proteins of similar size. So, these are these are all around 100 kilo Dalton's. These are around 60 kilo Dalton's, these are on 50. You can see that they are different, they are roughly different sizes. So, we tried to group the proteins by similar size to avoid that problem I described earlier. But, then (Refer Time: 36:47) who did this work decided what the heck, let us just see what happens if we mix them randomly; you know is it a problem and he did that down here.

So, these are these this is a 100 kilo Dalton's, here is 23 kilo Dalton's and yet we still detected this one even though these other even though these guys are much smaller than that one. So, even though they were smaller they did not seem to inhibit. Same is to here, this guy is 65 kilo Dalton's it is with a much bigger protein than some smaller proteins. And so, in every case we are able to detect the protein either in the mix or by itself.

And so, that gave us a lot of confidence and these are just some of the data, he did much more of it. But, it gives you the idea that you know if you mix the proteins, you can still detect individual proteins in the mix. You still have the issue having to figure out which one is which, but that will come later.
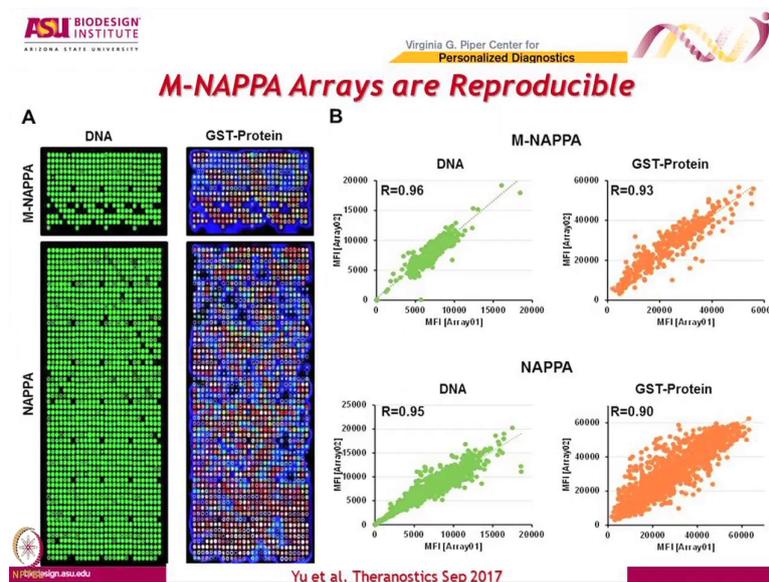
So, we decided to try to print a whole array and this is the array we printed, we wanted to make sure the array was reproducible. So, you guys have seen this plot over and over again. But we did this on every experiment; so, I have to show it to you because I want you to get in them, get used to the idea that part of the job of doing these sorts of studies is doing the quality control; because the experiments only work if you do the quality control alright. So, this is the array printed with DNA, this is the array made with protein and this is doing comparing the DNA from different arrays.

And, then comparing the protein levels from different arrays, just to show that there they are reliable. I am I am just going to tell you briefly this is a group of proteins up here printed as a mixed array. So, that is what we call multiplexed NAPPA. So, each of these spots here contains 5 proteins of piece, the same proteins that are here are down here as individual proteins. So, this was an experiment that we set up so, that we could compare how did the

single individual proteins express compared to the mixed protein expressed right. Because, we are still trying to test the notion that the mixed NAPPA will the multiplexed NAPPA will give us the same result that we were looking for, oh yeah.

Student: I was wondering the input DNA for each individual protein.

Yes.

Student: Is same?

Yeah, we added roughly the same amount of DNA for each one.

Student: (Refer Time: 39:16).

 What?

Student: Can we change them?

 You there is a limit to how much DNA you can print and so, I have think of what we did is we took the normal concentration and cut it by 4 and then mixed that 5 times. So, it was a the overall concentration was about 25 percent higher than normal, but it was roughly the same as what we normal print, but this time it was made up of five different genes.

Student: Do we imprint different moles of DNA for smaller proteins and just (Refer Time: 39:49).

Yeah your yeah. So, technically if you look at this if you look at the you know the molarity of the different DNAs, that there is technically more moles of the smaller genes. It was too complicated to figure that all out and adjust for that and Shabo was not willing to do it, even though I suggested it. So, so, but it seemed to work mixing. So, the idea was you know if and

I do not remember exactly what our final print concentration is these days, I think it is like 2 or 300. But, we think they took the standard concentration and cut it by 1 to one-fourth.

And, then mixed that together with the other guys and then and actually what happens is if you just if you just mix 1 plus 1 plus 1 plus 1 plus 1 right, then each one of those becomes one-fifth of the concentration right. And, then you and the overall concentration is still the same, if they are all at the same concentration. So, that is in fact, how he did it alright, it is a good question though.

Student: Suppose, we have 5 UTR, then almost have short 5 UTRs. So, generally initiated a translation more efficiently will not that effect?

Well that is an interesting question, in normal biology that would make sense; keep in mind that remember here there are no UTRs. All of these genes have been cloned into an expression vector, they all have identical upstream regions; there are there yet it is a t 7 polymerase yeah. So, it is a different circumstance, but that is a good point for the standard biology yep.

(Refer Slide Time: 41:30)

Points to Ponder

- Halo-tag chemistry of protein immobilization and its application in identification of the buried epitopes
- Application of Nano-well technique in printing of high-density NAPPA arrays
- Multiplexed NAPPA arrays and its utility in screening increased number of proteins as compared to conventional NAPPA arrays
- The comparison of m-NAPPA array with conventional arrays and ELISA resulted in better results for m-NAPPA array

In today's lecture you have learnt that how using a very strong covalent bonding chemistry involving HaloTags based NAPPA, you can now perform high density piezo printing. And, the assay quality and reproducibility tremendously improved by incorporating these newer methods. And, that is really a good lesson for all of us to really see that you know a technology can be started but, there is a need to improvise it further.

And, bring in the new creative elements so, the technology can be much more reproducible and can also serve the much sensitive assays on the same surface. In this slide they thought of improvising NAPPA for the high density printing as well as much more strong and robust binding was really accomplished by incorporating these new creative methods. These concepts will be continued and discussed in the next lecture.

Thank you.