Lecture - 09

NAPPA and its applications in study of antibody immune response in disease and drug screening-III

Welcome to MU codes, on applications of Interactomics, using genomics and proteomics technologies. As you have seen, nucleic acid programmable protein arrays, or NAPPA, is very robust technology, where

many applications can be performed. We have seen, Dr. Josh 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 steps, before you can make any meaningful, signal or the sense out of the data. It's like a Western blot. When you're 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 the day-long procedure, involves, series of washing 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 and you are, performing next set of experiments, on these chips, then if the Regents 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, micro platforms available. In this light, Mr. Josh LaBaer, is today going to introduce you, to a newer method, which is, Hello tech waste, 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's welcome Dr. Josh LaBaer, for today's lecture on, Advent of NAPPA technologies, using hello tag methods. Okay. So, um, what I thought we would do today, because you're you are all, such Advanced, scientists in the area of NAPPA, is fast forward a little bit. So we've talked a lot about the, development of the technology. We've talked about the methods for making it. We've talked about, some of the applications that we've done. What I thought we would talk about today, are some of the newer methods, that we've been developing the last year or two. So these are very current effects. Several of them are papers that were published just this last year.

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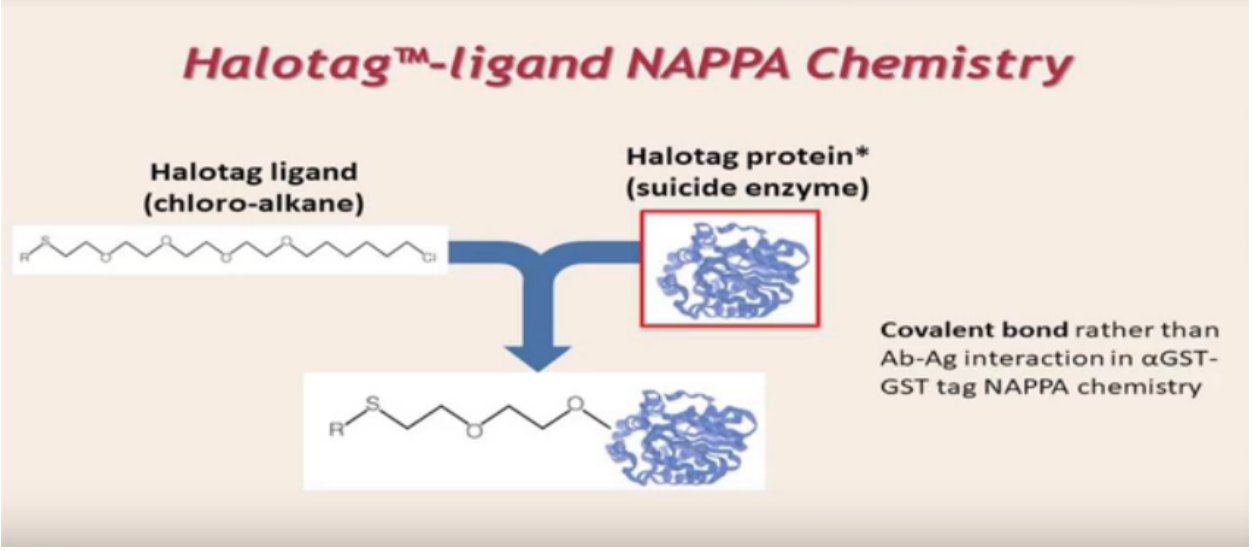

So one of the things I mentioned the other day, was, the halo tag. And this is what the halo tag looks like. This is a chloro alkane, so it's a, it's a, it's a it's a aliphatic chain, with a chloride, at the end. You can also do the same thing with bromo alkanes, but chloro alkane is a, I think of one of the preferred substrates. And this, the halo tag 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'll capture it, in a, in a one directional method, that is permanent. This is, this could be potentially very useful. So one application, is that, I think I've emphasized several times, that,

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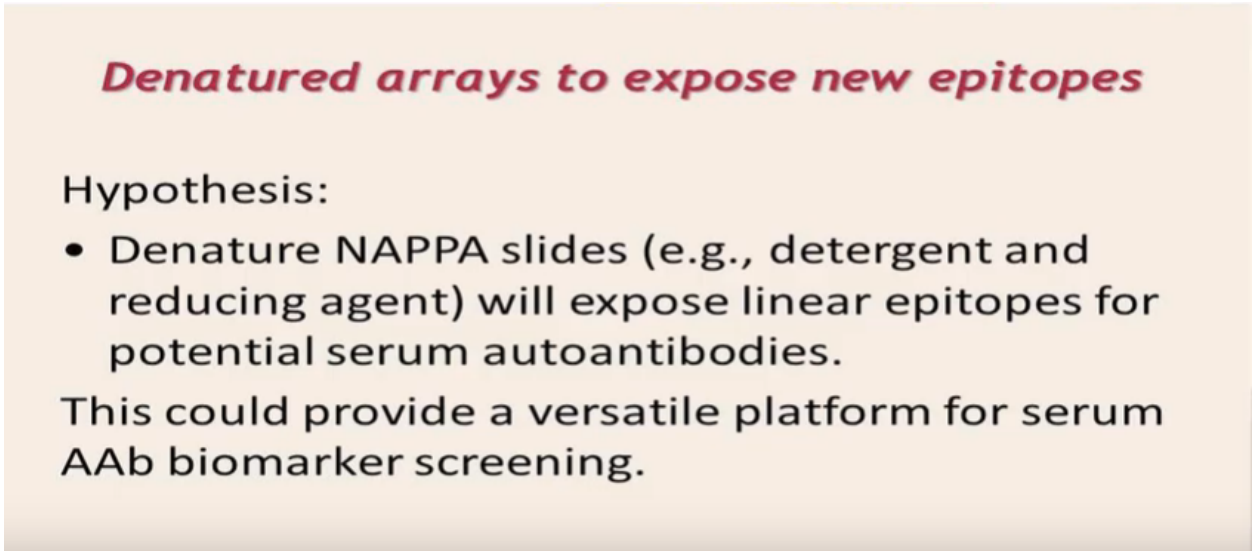

that the NAPPA technology, primarily displays proteins that are functional and folded. And, and, for the most part, we believe that, that is the advantage of the technology. But there may be circumstances in which you want to measure, binding to denatured protein. Perhaps, the Epitope you want to find is actually a linear epitope and it's 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? GST and, and Antigen Antibody. So what happens when you denature antigen antibody? They, they fall apart. Right? So if you take standard NAPPA and put it under, denaturing conditions, the proteins on standard NAPPA, will all fall off. Because they're 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, with, you could, you could denature it and the proteins would still stay attached. Right?

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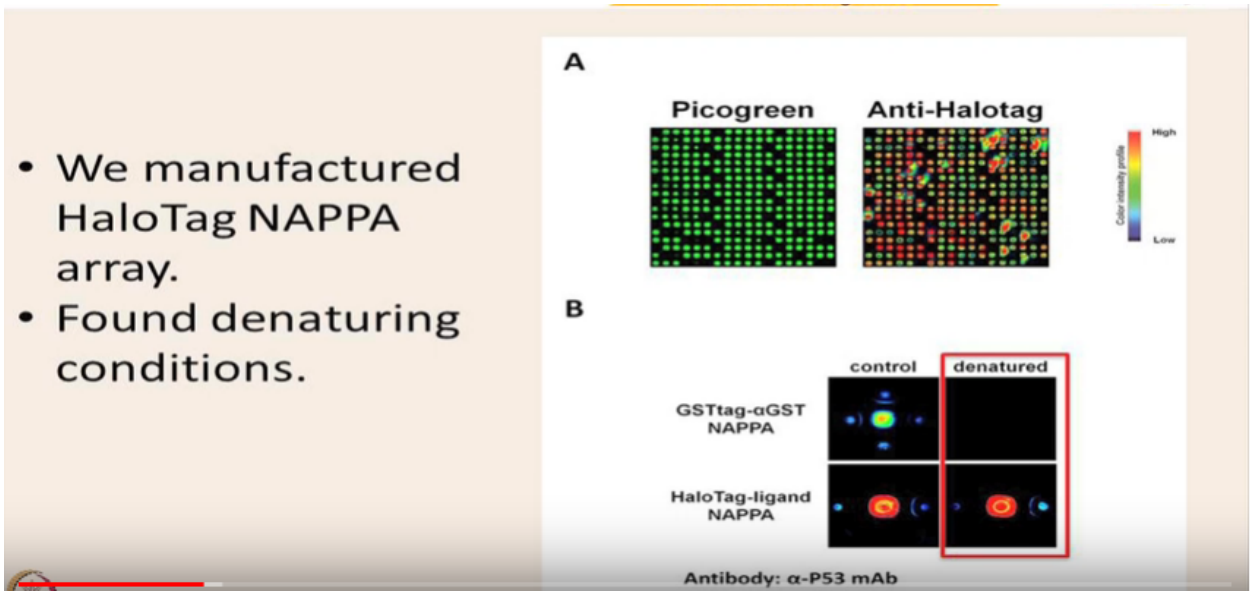

And so that's what's shown on this Slide. So we, we, we made an array and these are with proteins, that all have the halo tag on them. So this is just like the kind of NAPPA that you've seen before. But the difference is that, in this case. Instead of using the GST tag, we have a halo tag and it's binding to the, Legend. And now you can see, for example, this is a p53 antibody and, the GST tag. So this is, the protein array, under, standard conditions of the protein around, denatured conditions. When I say denatured, I mean, we treated it at, 55 degrees Celsius, with SDS. So's a 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 p53 antibody, could bind, both formats. Because we knew this antibody, by this antibody here, binds to a, linear epitope.

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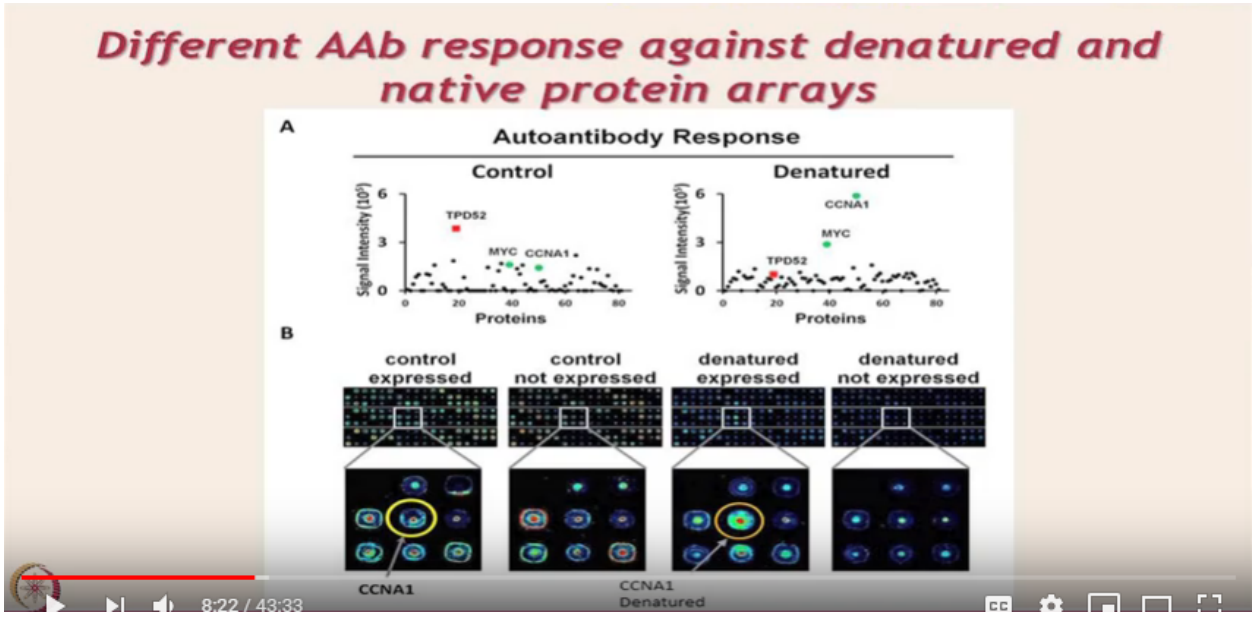

And so one of the ideas that, Ryan had, the student who did this work, was is it possible, that if you were to take a protein array and display it against serum, either, in the native format or the denatured format, would you get different immune responses? Could you pick up responses, for example, under nature conditions? And that's 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, this antibody here, specifically detects, yeah, under standard conditions and whereas these two antibodies, only detect, Cyclin A and Mik, 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. That it's binding, this is, this response here, binding to the denatured protein. Whereas you don't see it so strong, in this guy, here. Okay? Any questions on that part? Okay. So then. No, no, no, the array was denatured, then, 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 and fold an antibody. Because if you denature the antibody it wouldn't work, where most antibodies don't work. Okay. All right.

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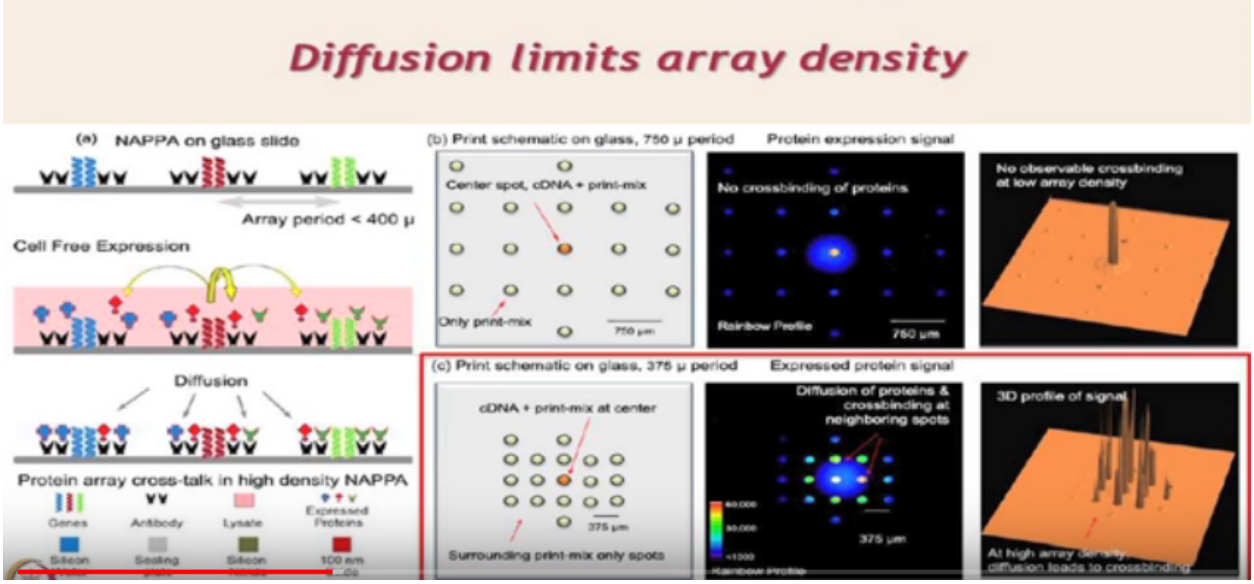

So, we talked about this a little bit earlier in the course. This issue of, some a couple people asked the question about, protein diffusion. And, and, so what what, what do, I mean by protein diffusion? Well here are three spots on the array, kind of in a car, in a cartoon and here you see the DNA for 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 proteins, 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's the concern that, a lot of people have. So we ,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's no gene, in that local position. So what that means is that, if there's any diffusion from here to here, it'll get captured by those sites and you'll see that as signal. Okay? And, and what you see is that, in fact, there's a little bit of spread around the spot, but it doesn't 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. Okay? But, if you start to make NAPPA much smaller? So if you take the 750 micron 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 that, in that 3d rendering. So, so, that tells us is, that, for the most part, under our current conditions, we're okay. But if we ever wanted to make our arrays, much, much, more dense, so shift from 2300 proteins, let's say to 10000 proteins. We could run into trouble, where there would be neighboring spot intensity.

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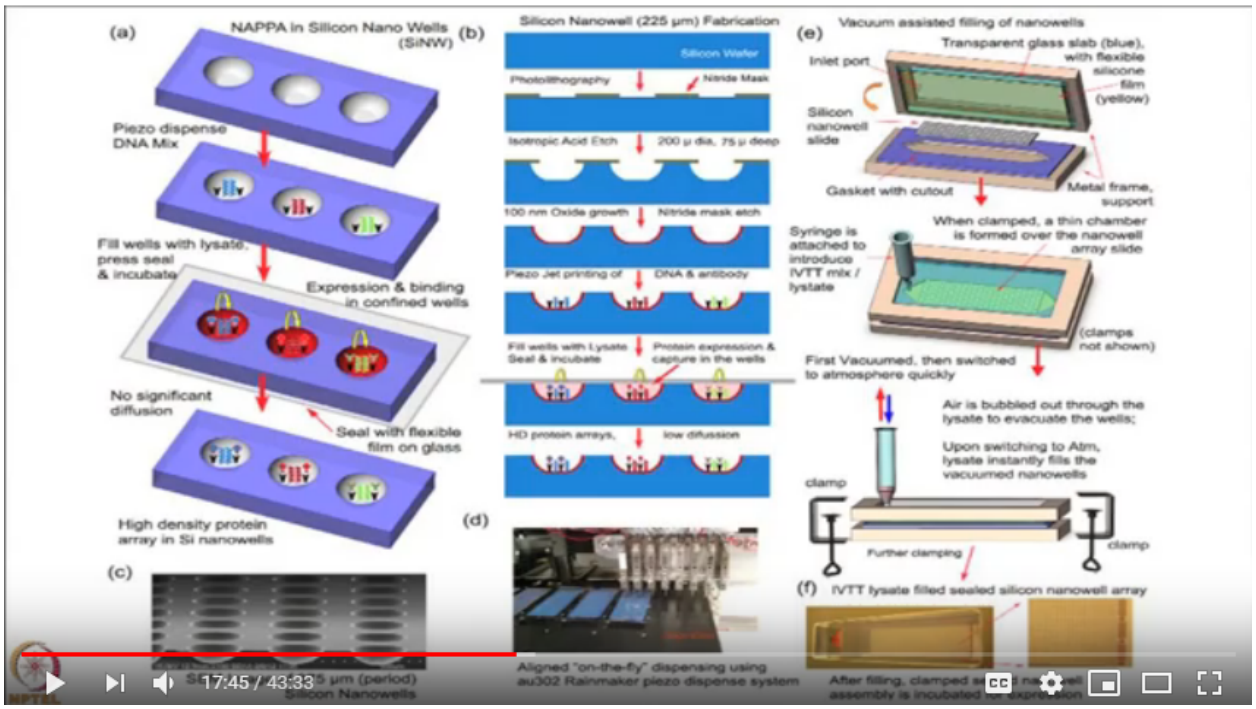

So we've been thinking about ways to get around that. And, and this is the method, that we've developed. What we do is, we take, silicon, the same material, that you used to make computer chips and we use the same, technical approach that they use, what's called, 'Photolithography'. Where by, shining 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're nanometers in size. In fact, in terms of fluid volume, they Hold, Pico liters, of liquid, so they're, very, very, small. And, we so we, etch 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 there's a couple of chemical treatments you have and then we print,

the NAPPA mix, into the wells. So it, it's, 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 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 that's not tricky, that, that's 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's what we do, so you end up printing the DNA in 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's a tendency for the liquid, because it's hydrophilic, to not want to go into the wells. Because air, gets captured, it basically it there's air in the wells and it gets captured. So you have to fiddle a little bit, with, 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'll 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. Okay. So, what, 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, liquid oil, 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, 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 the Nano Wells. And it does so, instantly and at the same time, there's, they apply a little bit of vacuum, to the liquid, on the, the surface of the array, that pulls out any excess expression lysate and you end up with a sealed surface, where you, I don't know if you can quite see that? But you end up sealing the silicon well, the Nano wells, with that, with that plastic over.

So this is what the apparatus looks like. It, there's, it's, it's 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’s doing the printing and these are these, piezo these, special piezoelectric nozzles, that are very accurate, at delivering, fixed volumes, to each well. One, 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 slide, so it stays put. The problem with the cat, cross linking agent is, that it's, it's, there's a time function, attached with. The minute you activate the cross-linking agent, it, it's a free chemistry, that starts to act on your sample. If you let it go too long, everything gets over cross-linked and it's 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 doesn't get printed that day, whatever's left in your tube, it's gone forever. So if you made a lot of DNA, to print your arrays, use a little bit of it to print arrays, all the rest of your DNA, is lost. Remember, I mentioned the other day, that, even though it's not expensive to make DNA, whenever you have to make

ten thousand of anything, it's expensive. So now you've essentially, wasted all of your ten thousand DNA's. One of the advantages, of this platform, is that, we can print, the DNA, separate from the print mix. Which means that, we don't 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 don't have to waste, everything that you've used. So that, that turns out to be an, advantage to us.

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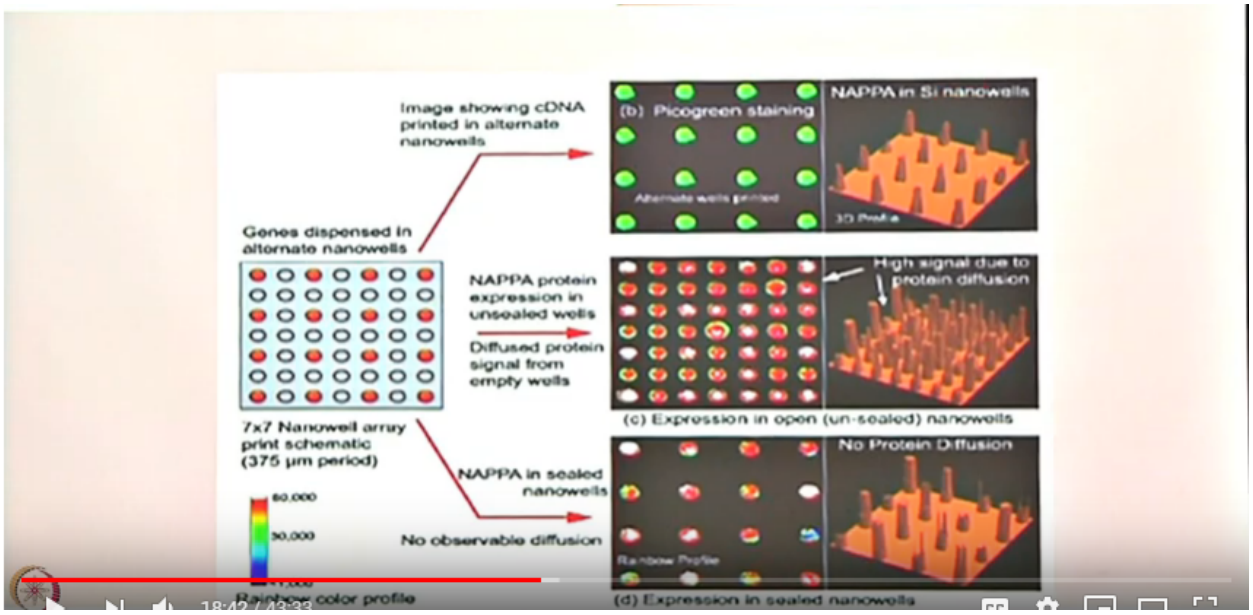

Okay, so this is what, this looks like, here we've dispensed, genes into Nano Wells, the genes are in this pattern, where they're separated by, wells, that don't have any expression and you can see how accurately, it expresses. You get very clean expression, at each spot and despite the fact that these are very close together; you're 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 it essentially blocks out completely.

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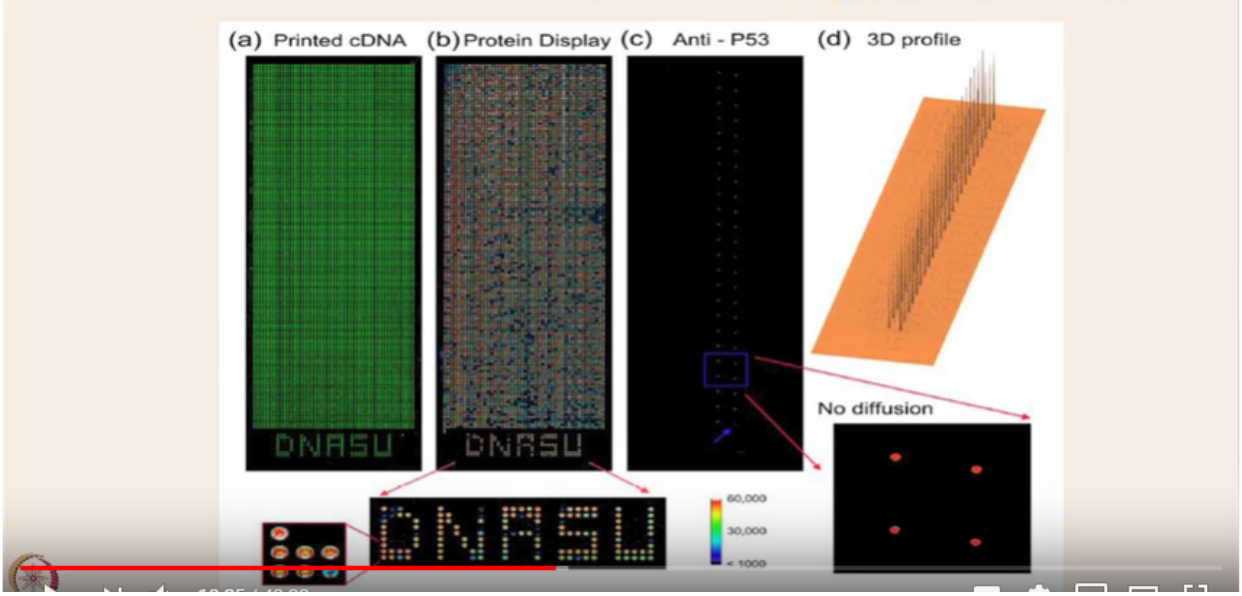

And so that's how we got this image here. So what you're looking at here, is now, a NAPPA array, that has, 10,000 features on it, all expressed in nano Wells. This is the DNA print, this is the protein print. And then we stained it with an antibody, to one specific protein, that we repeated on the array 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's just exactly, where you want the signal to be. Okay, one of the added benefits of this Approach, that we did not appreciate, when we first developed it, is that it turns out to be, more sensitive, than standard NAPPA.

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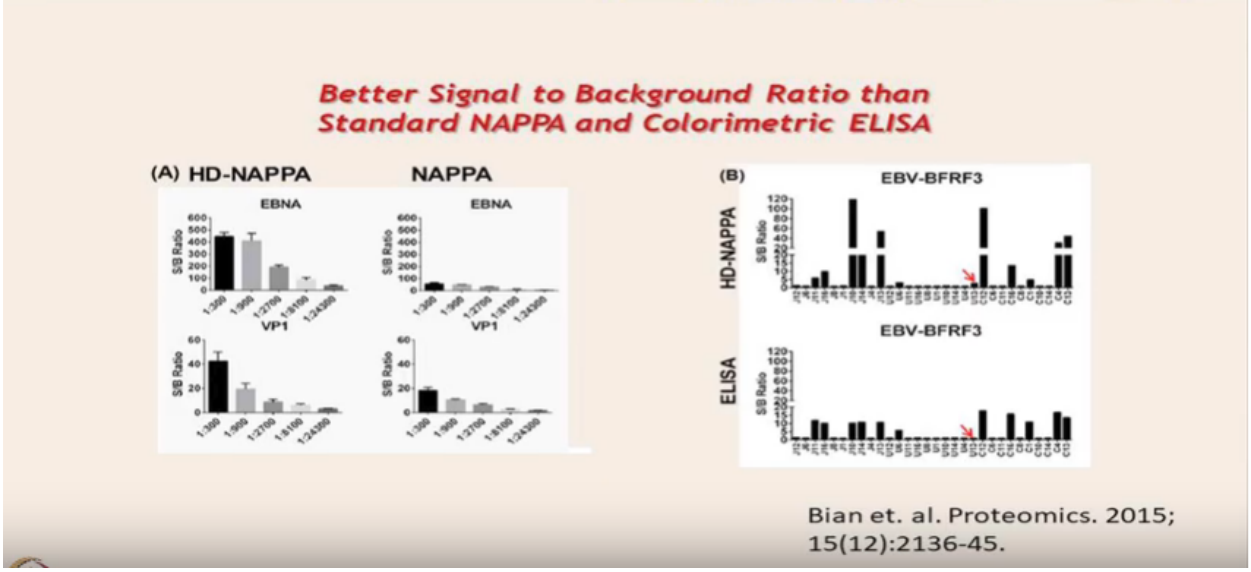

So we did some comparisons and we looked at, this, this antigen VP1 and we're doing different delusions of antibody, to ask, what, what's the detection limit, on the array platform. This is standard NAPPA and

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're getting much better, detection here, that we are here. In fact, you know, it kind of, plateaus here, at, you know maybe, 50 or 60, at one to three hundred Dilution. At one to three hundred on this Platform, it's four hundred and fifty. So the signal intensity is much stronger, on these high density NAPPA arrays. Yeah, so this is Ebna, up here, this is VP one 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 Eliza. So, you would think that Eliza, 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 Eliza, and then overall, the signal intensity by Eliza, compared to signal intensity by, HD Napa, this was, nowhere near as strong as that was. So it, it, in our lab right now, this is probably the most, sensitive, platform; we have, for detecting interactions.

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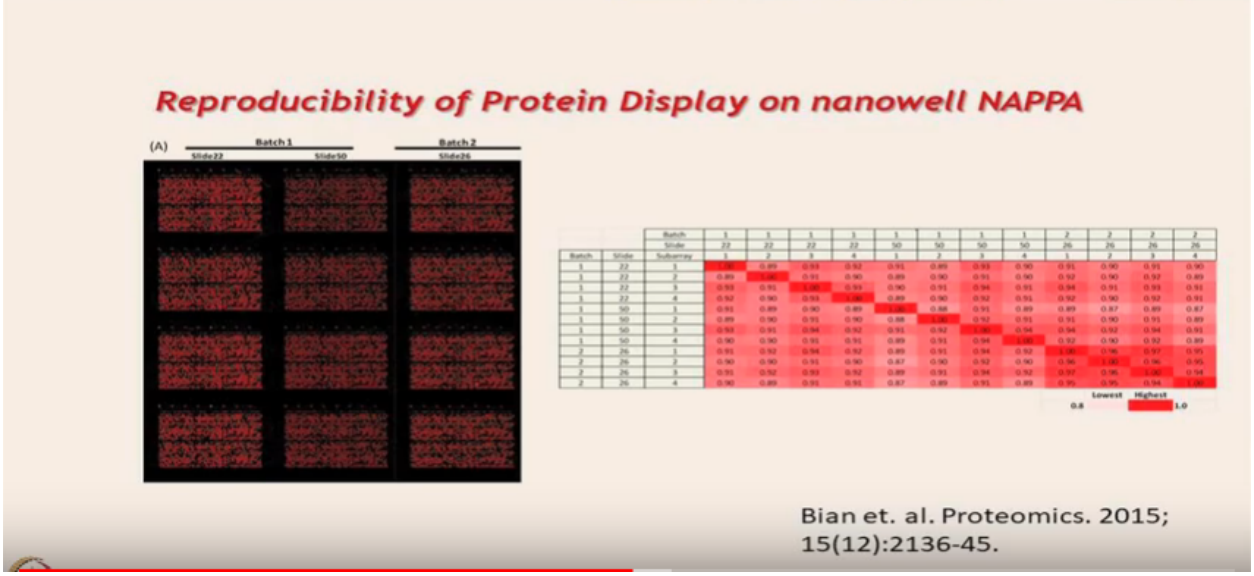

This is just a kind of, assure you, that we can print these arrays, very reproducibly, because that's one of the things, that you want to be able to do. So, I don't know, if you can see this in this light. But this is, a single slide array that has four sub arrays on it. Each of these sub arrays, contains 4,000, different spots. So we have 4,000, 4,000, so you, a total of 16,000 spots on this slide. And, and, we've repeated it, in one batch or in a separate batch. And, and, then what we've done is, we've done an interaction map, the other kind of correlation, coefficients, that I've been showing you, throughout the course. Everyday 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 it's every bit is reproducible, this platform, as the, the standard NAPPA was.

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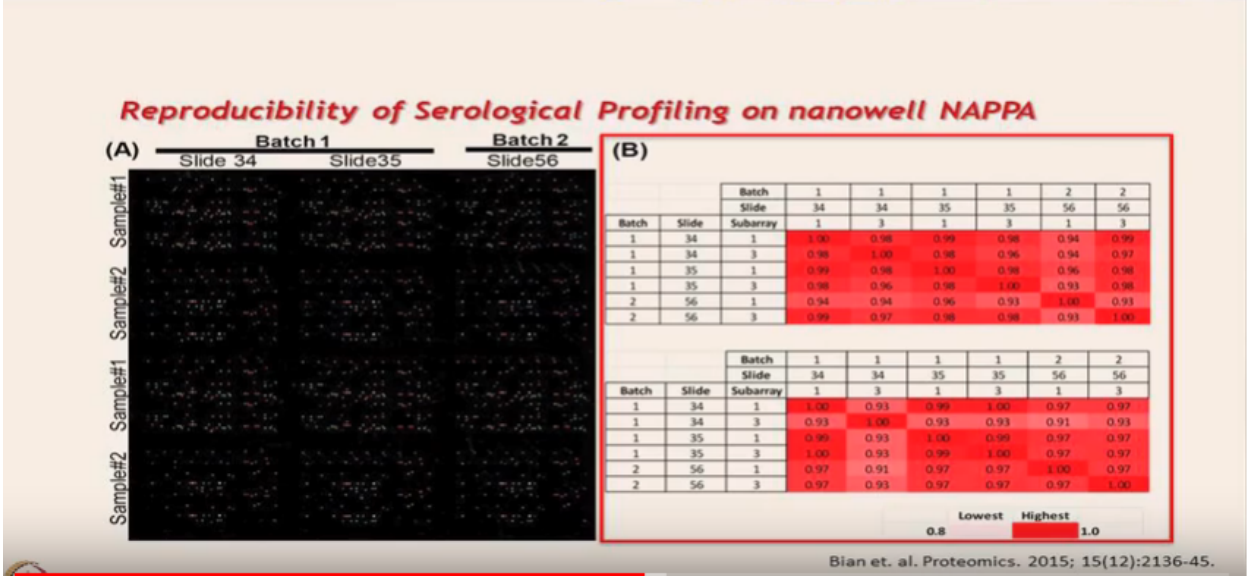

And then this is, probably, more relevant to you all, is if you actually, that was for protein expression, this is now asking, if I screen the array, with antibodies or serum, will the answer I get, from array to array, from batch to batch, be the same? And, again, I don't think, you can, can you see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

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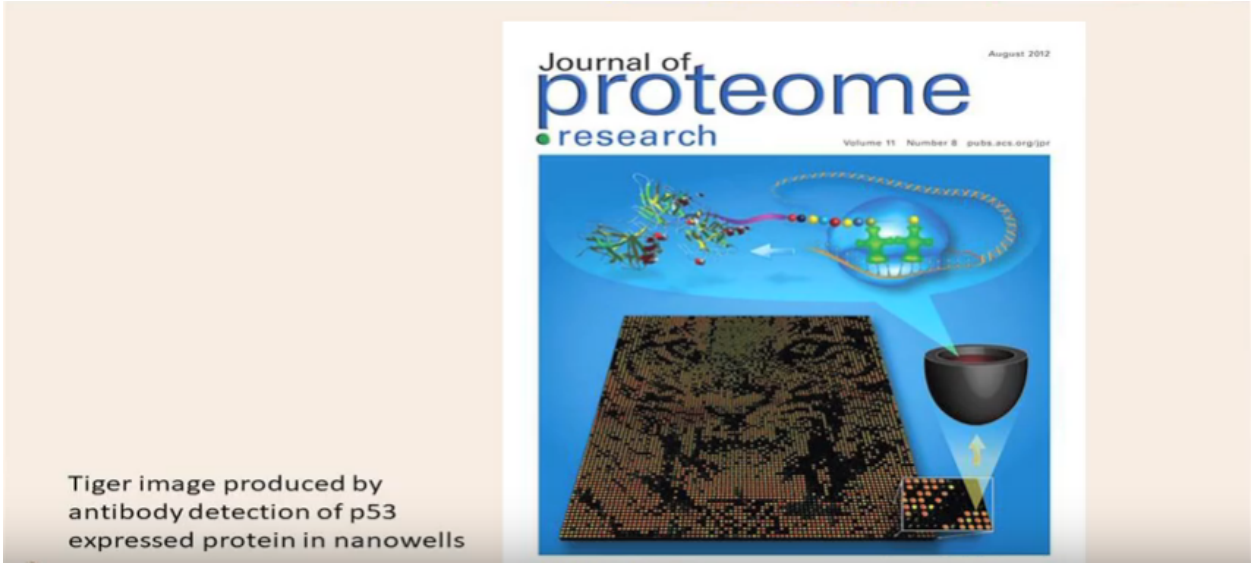

And so that led us to this picture here. So this is that we made the cover of Journal of proteome research, that month. And what, I'll show you this image again. What you're looking at here. Let me see if I can go here.

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Hopefully you can appreciate, that there's a tiger, in that image. So, this is of a what you're looking at is an actual protein array. We've printed different amounts of DNA, encoding the p53 protein. We then, express the p53 protein in the Array, in these nano WELLs. And then probe the array, with anti p53 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, Tigers face. So this was the first time we ever did an image, using a protein array. Alright, so now. Alright.

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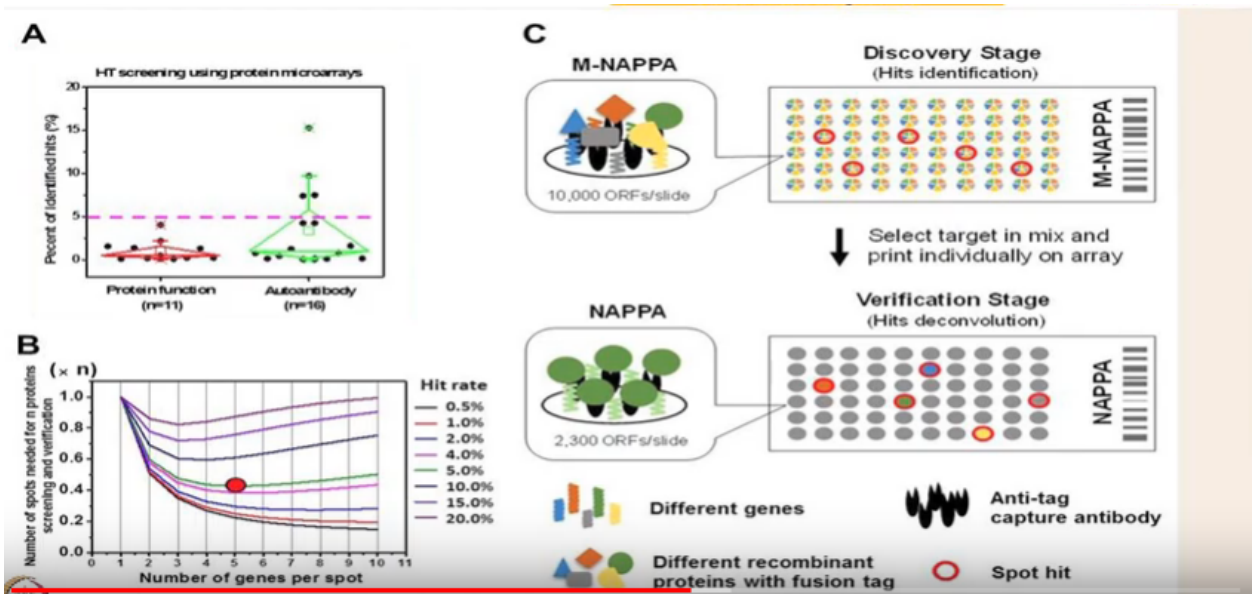

So I want to, move on to, a slightly different topic then. This is another way, about getting, a lot of information, onto the array. So, imagine, he, you know, the whole goal here is, we want to we want to test as many proteins, as we can, when, we when we screen an array. In the current NAPPA format, 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, 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, is that, we could print, multiple genes, at each spot. So what would be the issues? Why would you, not want to do that? So tell me what you mean by, 'the specificity of the binding'? Say, say it again? Okay, not, that's, not, that way, that wasn't exactly, what I was worried about. What, what other people think? Say it, say it again? Okay, so tell me, what you mean by Quantization? Okay. Alright. So that's, the so, what, so she's saying, how you gonna understand the individual contributions.

And I think that, that's a fair concern. Right? So I've got. Let's, let's 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 won't know, which of those three proteins, was the target. Right? I won't be sure? Yeah? We'll all of them, have what? The same tag, yeah. Yeah. They'll be, they'll be captured, by the GST tag. That's 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, when the idea occurred to me. So, whenever I do an experiment, on NAPPA and 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're correct. So it occurred to me, that if I did the experiment, with a multiplex the spot, that had multiple proteins, I was gonna 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 hundred spots on the array and each of the spots on the array, has five proteins in it. Okay? If, if I screen the array and I get two spots that light up. How many possible targets do I have? 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 I've done my job. Right, now let's go to the other extreme. Let's imagine, for that hundred spot Array that ninety five spots light up. How many potential targets do I have? Ninety five times, five. Right? And so how many spots am I gonna 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'm back to doing the same job, I would have done, if I had not multiplexed. So the multiplex idea, works when the tart, when they hit rate is low. And, it doesn't work so well, when the hit

Rate is high. And so you can actually mathematically evaluate, what's the best or most optimal number of spots, to mix, based on the, likelihood of a, of a hit rate. Okay?
And so we actually did that, we did that develop the equations and we actually looked at it. I'm not going to go through the math. We first, we looked at; we looked at the frequency, of, 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're doing approaching interaction study, if you're doing a auto antibody study, of all the targets that people study and there's, when they do their experiments, what fraction of proteins light up? And so this, that's what this is, this is the percent of identified hits. And you can see, that this is the five 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're less than five percent. So that's promising. Right? That means that, this strategy could be, a big time saver, if I can make this strategy, work. Right? Okay, so then the question was, what's the optimum, number of spots, we can do? Maybe making a assumption, that the hit rate, is 5%. Even though I think that's probably a little high, for most of them. I think it's a fair assumption. It, it, you Know, it's a more conservative estimate, if we can satisfy that one, we're certainly going to take care of everything, that's 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, five spots, per gene. After which it doesn't 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, five, five genes, per spot. Right? Right there. And this purple line, is the, this green line here, is the 5%. See that 5% there? That's the 5% line. That came out to about five. These guys are also pretty good at, five. When you get up to here, when you get up to ten, fifteen, twenty 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 2,300 spots or twenty five, let's 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 five proteins, per spot and that's what, these different colors, are meant to indicate, five different proteins, per spot. Then you would, you would screen that, and you, let's say you get these, five hits, one, two, three, four, five. You take each of these five hits, that's 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 five proteins was a hit and it, it confirms, that it was a hit. It tells you for sure, yeah, that was real. And so that's and so that's, that would be the strategy. That makes sense? So you're, we call that, 'The Deconvolution step'. It's sort of verification stage and Deconvolution stage. Okay, so. Does it work? So what would be an experiment, to make Sure that it's working? So one of the Questions that come up is. If I put five Spots, five proteins, genes, in a spot, will they all make protein? What if only one makes protein, the other four don't? Now we already know from NAPPA, other, other nap experiments, that every almost, every gene we print, makes protein? So we're confident about that piece. But you can imagine that somehow mixing them on the spot, could be a problem. Right? So how would you test that? You could look at, you could look at that, if you, if, the problem is that, for most protease, we don't have the functionality. What other ideas we got? You could certainly test them, one by one, in the mix, in the mix.
That's, that's how we went about it. Right? So what we did is, we said, well let's make mixtures of proteins, for which we have antibodies. So in this case, we're testing only proteins, that we can come back and test. And then we're gonna 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's, if I have a mixture of proteins, where one might be made in a greater quantity, than another. What, what kinds of

things, would I want to think about? Where could a bias, come in? If I, if I have a protein, that's 15 kilo Daltons, in a protein that's 80 kilo Daltons, will I see a difference? What do you think? Why might I see a difference? Right, so how do, how do proteins get made? Right, they get made by adding one amino Acid, after another, using TRNAs, on the ribosome. Right? So the the amount of, amino acids you have to add, to get to 15 kilo Daltons, is a lot shorter, than the amino acid ,you have to add, to get to 80 kilo Daltons. 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 gonna work. Okay. Alright.

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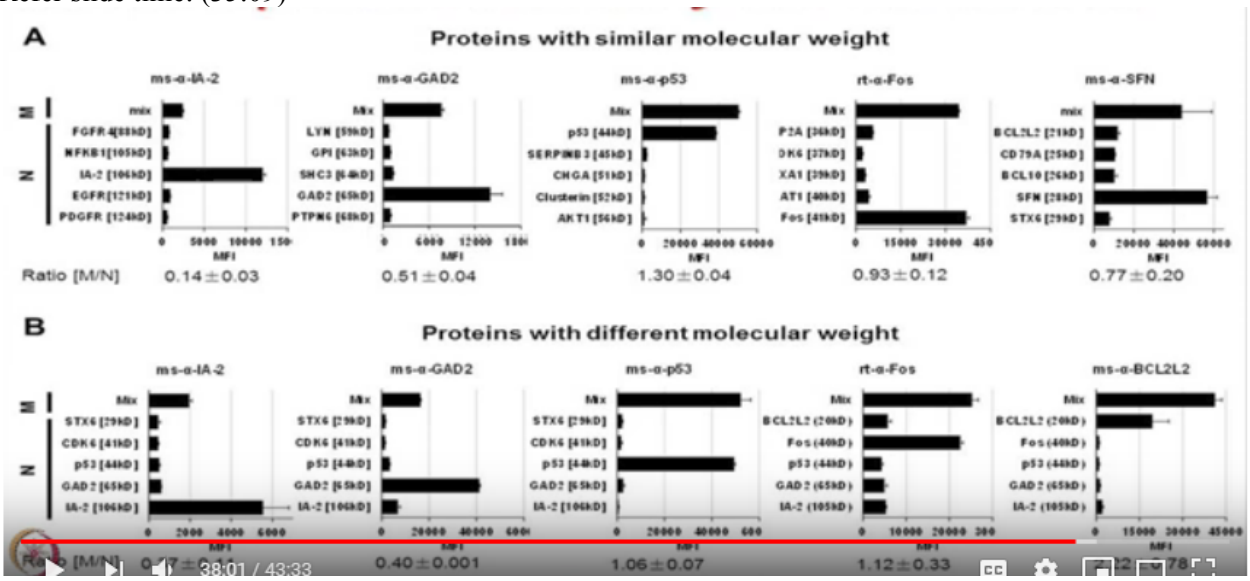

So here, here what you have is, so let me walk you through this experiment. On the top, where it says M, that stands for Mixed NAPPA or Multiplex NAPPA. We, we printed a mixture of five genes and then expressed them. And then we also, on the same slide, separately printed, each of those spots, individually. Okay? And then what we did is, we probed; we probed that array, with an antibody, that recognized one of the proteins, in the five. 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. Okay? And you can see for this, IA2 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, we wouldn't have missed that, in the study. Here's another one, GAD 2. We got, we get, we can detect it in the mix. We can see it as a single protein. Here's anti p53, it turns out, that the Mix, was even better, than the single protein was. Here's anti Foss, you can see that the mix was about the same, as the, the Mick, the individual spots and here's, I can't read that, s fi, SFN and again, you can see that, there, they're 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 Daltons, these are around 60 kilo Daltons, these are on 50. You can see that, they're different, they're roughly different sizes. So we tried to, group the proteins, by similar size, to avoid that problem I described earlier. But then, Shaba, who did this work, decided; what the heck, let's 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 hundred kilo Dalton's, here's 23 kill Dalton's and yet, we still

detected this one, even, even though these, other, even though, these guys are much smaller, than that one. So even though they were smaller, they, they didn't seem to inhibit. Same, same is true here. This guy is, 65 kill Dalton's, is with a much bigger protein, than some smaller proteins. And so, in every case, we're 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 not, 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, but, that will come later.

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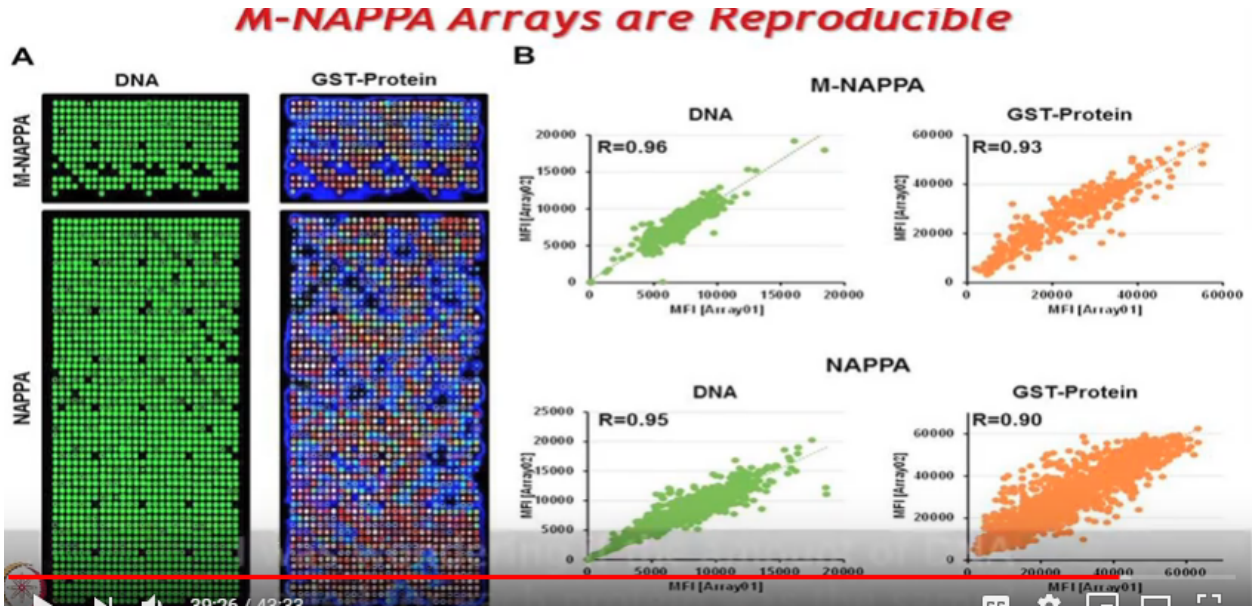

So, we, we decided to try to print a whole array and this is the, the array be printed, we want to make sure, the array was reproducible, so you guys have seen this plot, over and over again. But we do this, on every experiment. So I have to show it to you, because I want you to, get in them, get used, 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, they're, they're Reliable. I'm 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 five proteins, a 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 single individual proteins Express, compared to the mixed protein, expressed. Right? Because we're still trying to test the notion that, the mixed NAPPA, will, the multiplex NAPPA, will give us the same result that we were looking for. Oh yeah? Yes. Yeah. We added roughly the same amount of DNA, for each one. What? Yeah, you, there's a limit to how much DNA you can print and so, I think 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% higher than normal. But it was roughly the same, as what we normal print. But this time, it was made up of, 5 different genes. Yeah, yeah, your, yes, so technically if you, look at this, if you look at the, the, you know, the molarity of the different, DNA's, that, there's technically more moles, of the smaller genes. It was too

complicated, to figure that, all out and adjust for that and Shaba wasn't willing to do it, even though I suggested it. So, so, but it seemed to work ok. Mixing, so the idea was, you know, if and I don't remember exactly, what our final print concentration, is these days. I think it's like two or three hundred. But we think, they took the standard concentration and cut it, by 1 to 1/4 and then mixed, mixed that together, with the other guys. And then, in actually, what happens is, if you just, if you just mix, one plus, one plus, one plus, one plus, one. 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're all at the same concentration. So that's, in fact how he did it. Alright. It's a good question, though. Well that's an interesting question. In, in, in normal biology, that would make sense. Keep in mind that remembers here, there are no UTRS. All of these genes have been cloned into an expression vector. They all have identical upstream regions. There, I, there yet it's a t7 polymerase. Yeah, so it's a different circumstance. But that's a good point. For standard biology. Yep. In today's lecture, you have learnt, that how using a very strong, covalent bonding, chemistry, involving hello tags, Base NAPPA. You can now perform, high density, piezo printing and the assay quality and reproducibility, tremendously improved, by incorporating, these newer methods. And that's 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 versus, on the same surface. In this light, 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.