Week 3

Lecture 3

NAPPA Technology and Protein Arrays-II

Applications of Interactomics Using Genomics and Proteomics Technologies

Welcome to MOOC's course, on applications of Interactomics using, genomics and Proteomics technologies. in last lecture Dr. Joshua LeBaer, give you an overview of proteomics field and to perform high-throughput, proteomics based experiment, they need to generate the Clone repositories and to achieve the high throughput biology and performing proteome experiment what are the key considerations you need to pay attention for generating those cloned repositories once you have obtained large number of Gene clones, now you are ready to perform many experiments and one such experiment, was developed in his lab, which is a novel protein microarray technology, which is Nappa, or nucleic acid, programmable protein arrays. to perform Nappa, if you have these clone repositories available, you have large number of cDNA clones available, you can print them on the chip and imagine that what goes on in our body, to do the central dogma, the transcription and transition process, from the genes, to RNA and the proteins. the same cascade of event could we try to reproduce, could we try to replicate on the chip itself and that was a concept of Nappa, from the DC DNA sequences printed on the glass Slides, could we add the machinery, which is required for in vitro transcription, and translation and use those to synthesize the protein, on the chip itself. Looks, science-fiction, but it was the reality. Dr. Joshua LeBaer and one of his senior most docs, Dr. Niroshan Ramachandran, they made this technology, for performing high-throughput protein microarrays using Nappa. Today dr. Leber, will talk to you about the development of the Nappa technology and how to use this for various microarray, based applications. So let us welcome dr. Leber, for a lecture on Nappa technologies.

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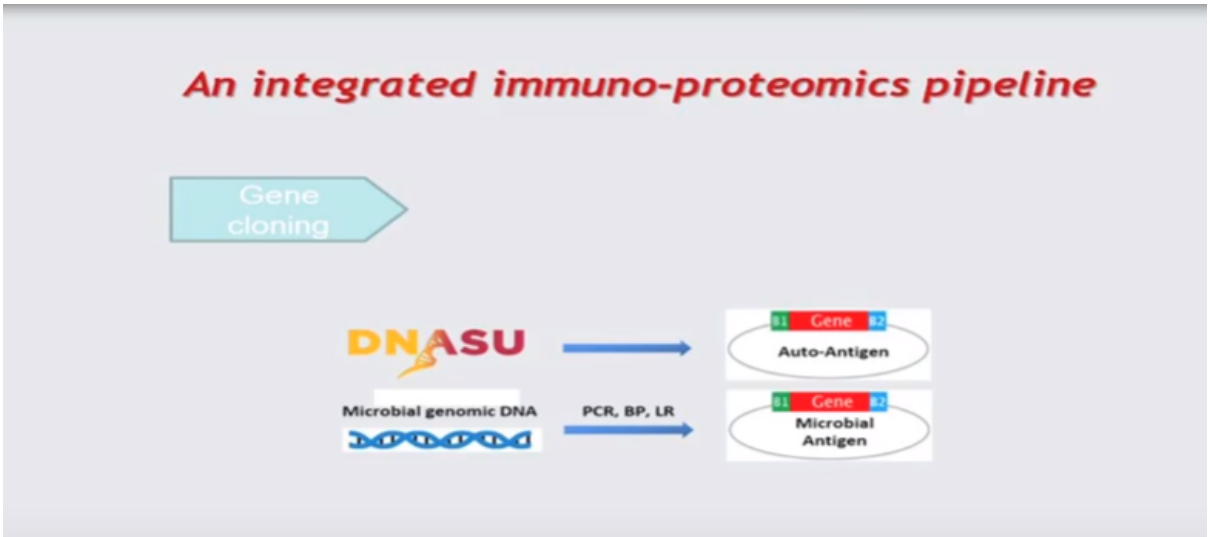

All right, so we're going to, we're going to start now, by talking a little bit about, then the Nappa method, right? And I already I already spent some time talking to you about the gene cloning part, right? So that's how do you make the clones for the genes that you're going to put on your protein arrays.

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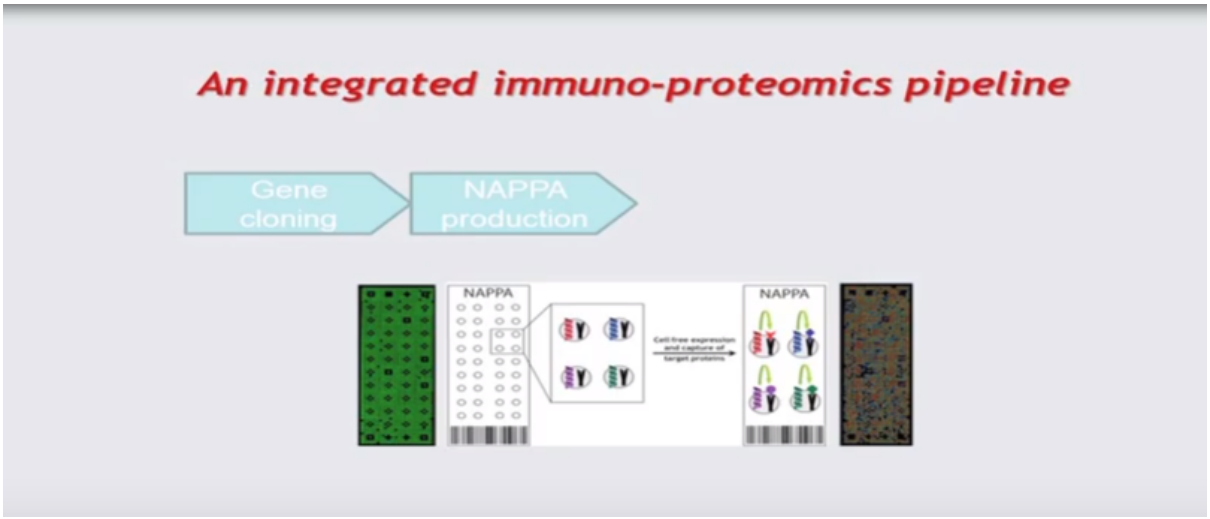

We'll talk a little bit today about Nappa production and I'm going to do it a little bit from a historical perspective. So, how did we come about this method and how does it generally work?

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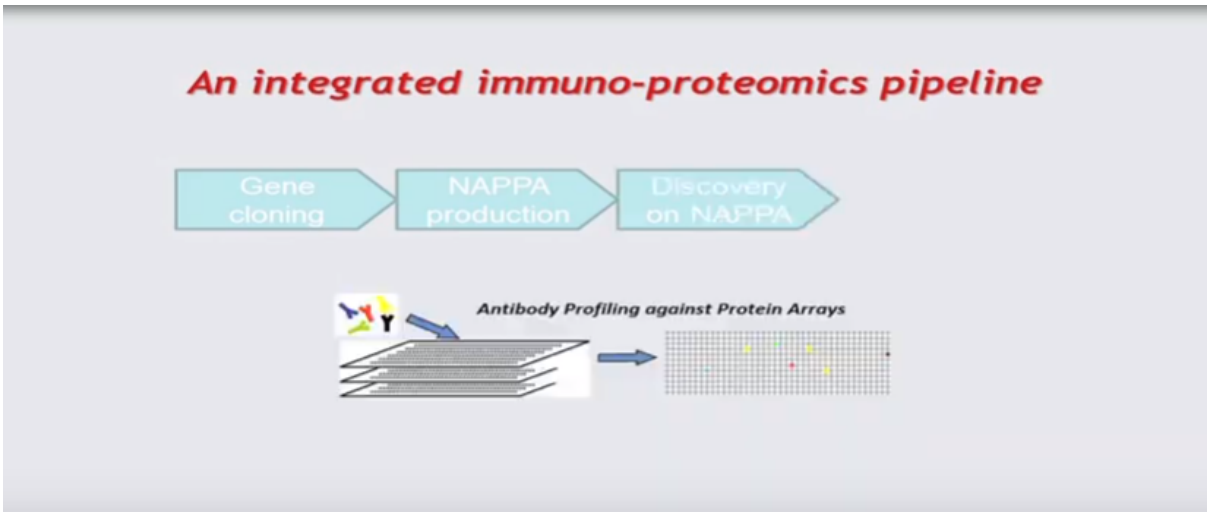

then we'll talk a little bit about,
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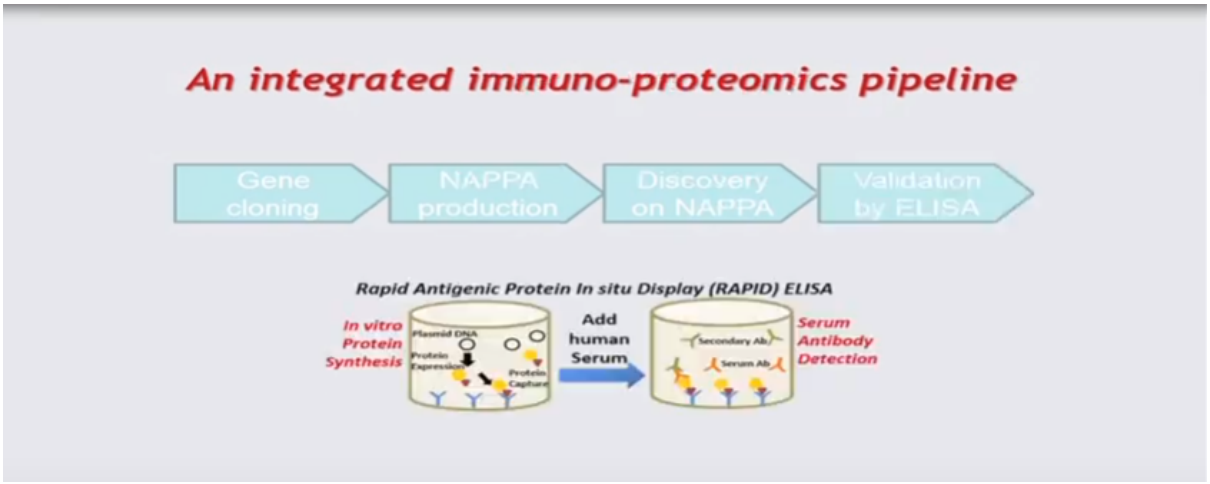

How to do discovery on the platform, and then finally nothing you do works if you don't go back and validate it.

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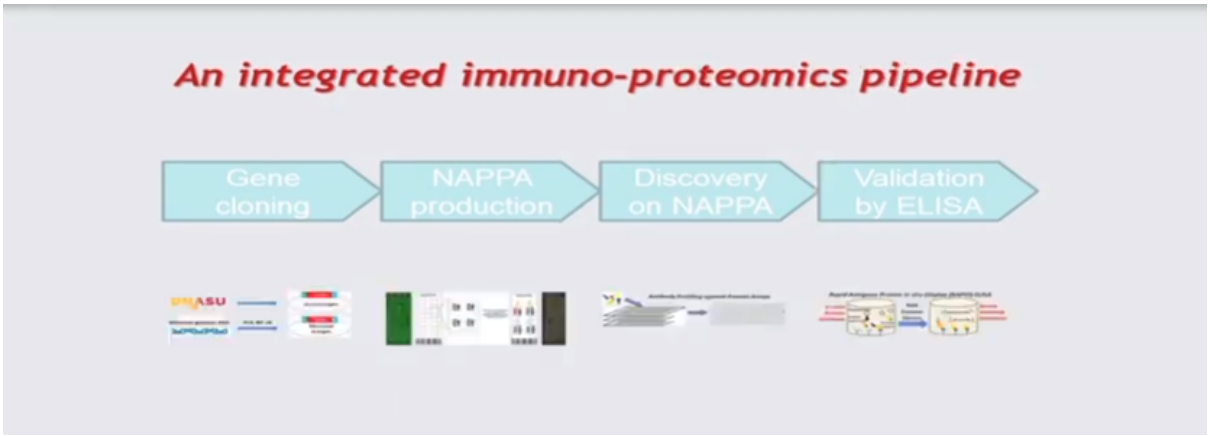

So that's kind of like the whole, end-to-end process.

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So, so let's, let's talk a little bit about protein microarrays, right? What are they and why do we want to do them? And I'm going to point out that, these are a number of the people that were most responsible for doing this work. Niro Ramachandran was really the leader in our group that really pioneered this methodology. Eugenie Hainsworth was the engineer, who did a lot of the work and then some of these folks also contributed, to some of the early methodology. So we talked earlier about, how having a library of expression clones, would allow you to do, all kinds of different studies. Well here's one that we got very interested in, which is Biochemistry. If you want to study the Biochemistry of a protein,

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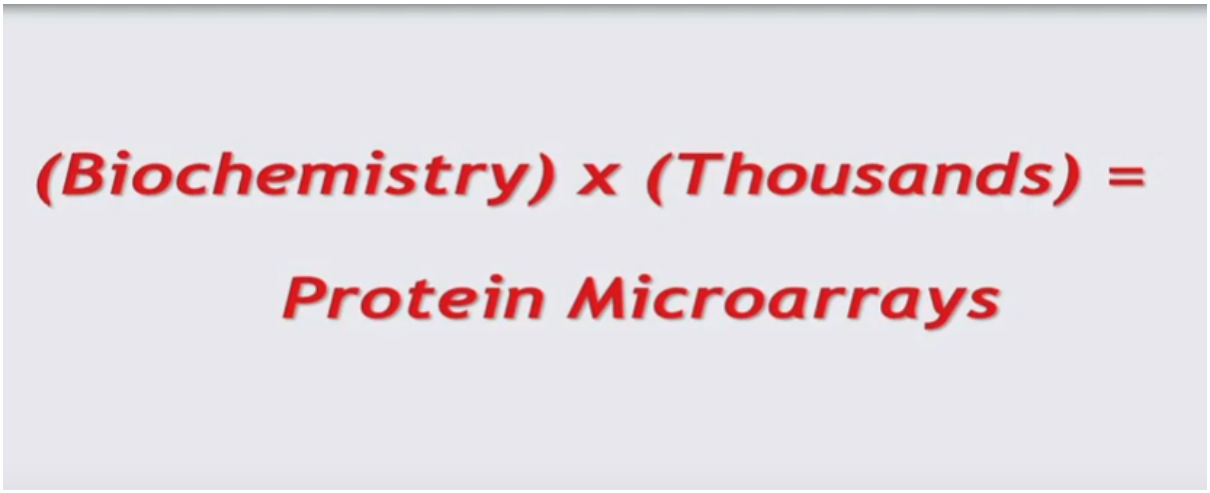

you need to, be able to make that protein and do experiments, and, and if you want to be able to do it times thousands, then that's what led us to the idea of protein microarrays and just to give you some, perspective, when we first began this work, we actually started by trying to do, high throughput protein purification and so we were developing methods, to make proteins and bacteria, lysis bacteria, capture the proteins on, on columns, loot the proteins and then study them in high-throughput. And believe it or not, we still do some of that work, for other reasons, but we realized very quickly, that it's

hard to do high-throughput protein purification. That if you, if you try to isolate lots and lots of proteins, first of all, a lot of the proteins don't purify well, secondly, the ones most of the proteins don't give you very high yield and then third, you don't really know, if the proteins that you're purifying, are going to be, of high quality folded, you know and active. So that's what led us to this idea of protein microarrays. Now there are two kinds of protein arrays.

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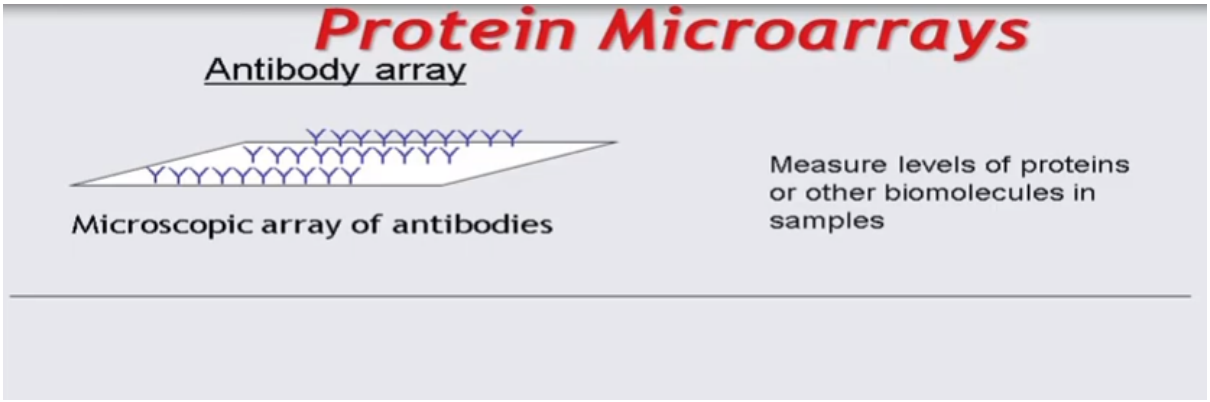

The first kind, which I'm not going to spend a lot of time on, are these, antibody arrays, where you, you print an array, on a microscope slide, you put antibodies down, that recognize different proteins on them and then you use those arrays to probe a sample, to capture, whatever proteins are in that sample, as a way of measuring the levels of those proteins, in the sample.

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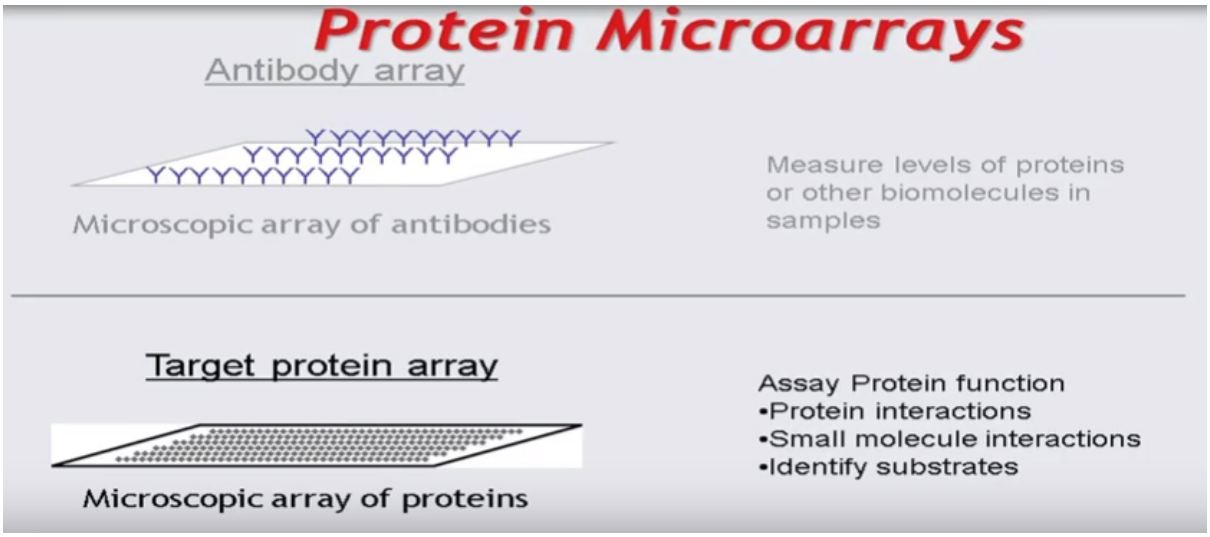

So the goal of this array is to measure the levels of proteins. The protein arrays that, I'm going to talk about, are called target protein arrays and the goal for these protein, these arrays, are to look at the proteins themselves. What do they do? Who do they interact with? How do they fold? What is their function? So, the idea on these slides is that you have a slide and each of these different spots, represents a different protein, on the array.

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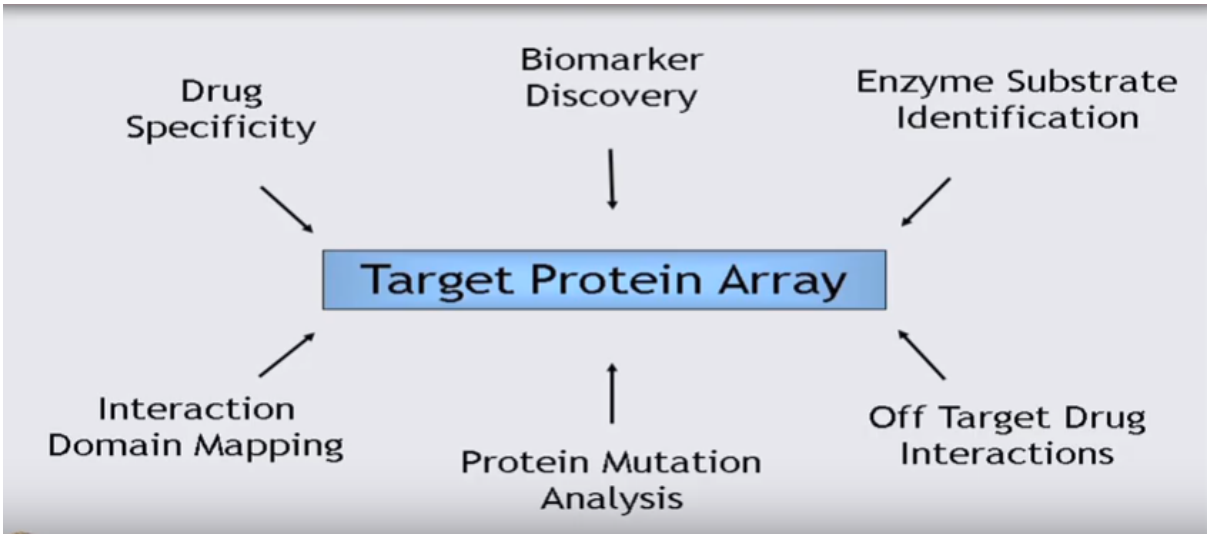

So lots of things that you can do, with a target protein array, You can look at drug specificity, also you can show an example of that later. You can do biomarker discovery, you can do enzyme substrate, sub identification, you can do interaction domain mapping, you can do analysis of, how protein mutate, of gene mutations affect the function of the proteins. You can look at off target protein interactions.

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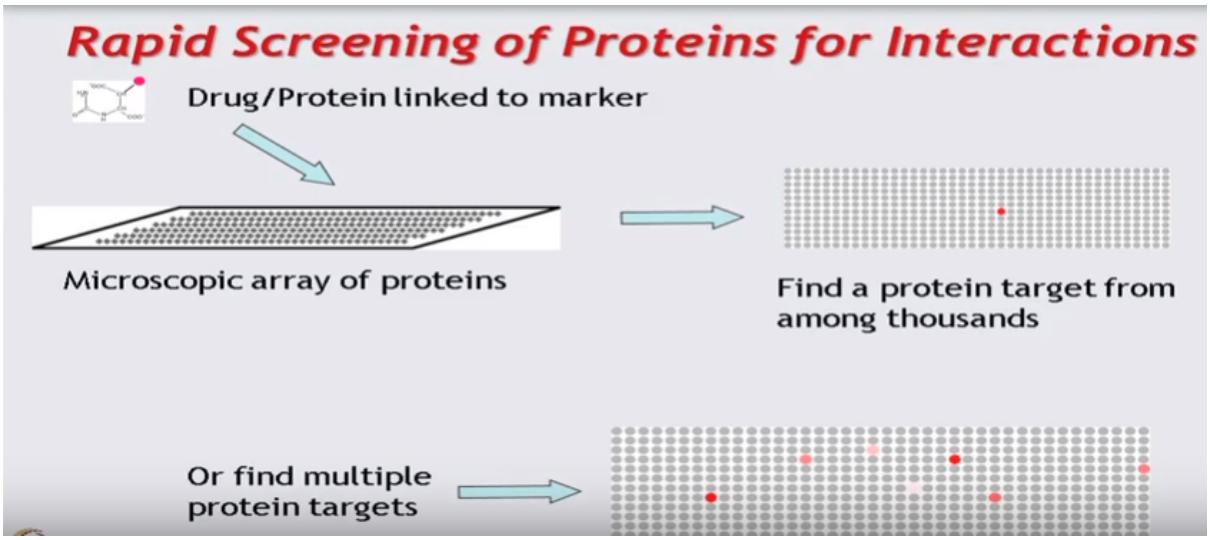

So here's an example of what that might look like. Imagine if you had a fluorescently Tagged, molecule and you probe the array, you can see which protein that, that, that molecule targets. Or, if it turns out that it binds to multiple proteins, you might get it binding to multiple proteins and you might see the differences in the binding and that would give you some sense of its specificity.

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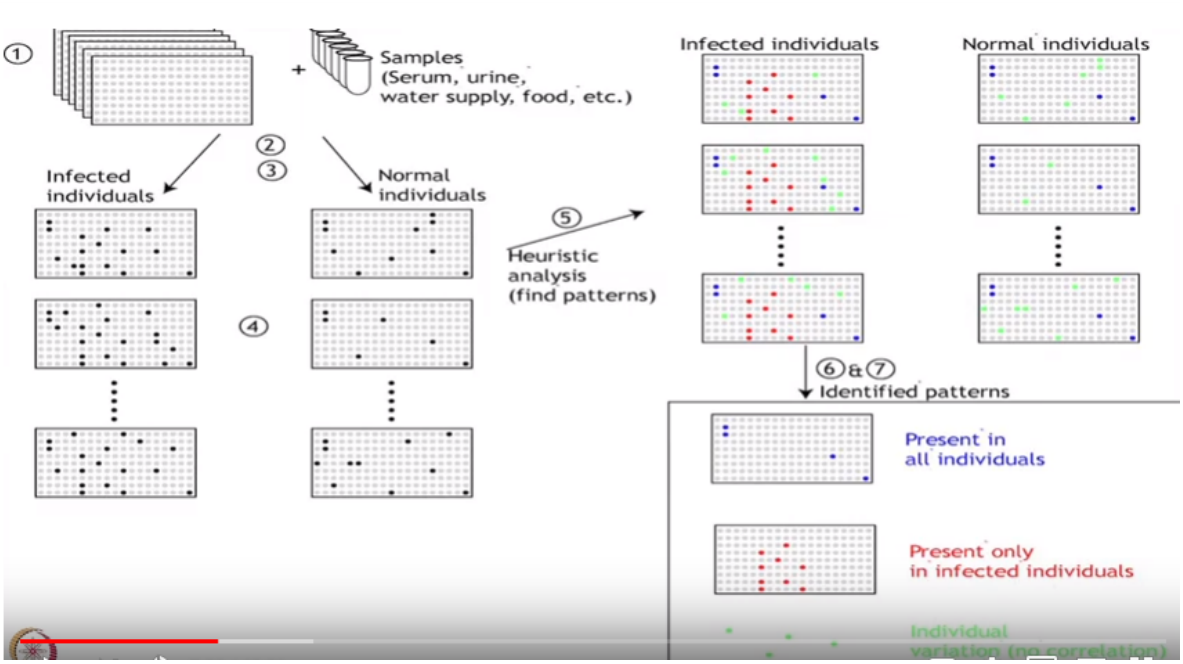

This is the area that, that I've spent a lot of my career on and that is looking for patterns of binding that indicate the presence of disease. I told you yesterday, that, we were looking to do, look for markers for breast cancer. Right? And we did that by looking at the immune response those markers. So imagine you take this serum and you apply it against, in the affected individuals or the normal individuals. Now I've shown that a number of spots light up and I did that for a reason, it turns out, that, even normal people, develop antibodies against some proteins. The problem is that the word normal's in quotes, it's in quotes, 'Because, all of us have medical histories'. We may not have cancer, but we have had other things in our lifetime. And those things can affect your immune system and those things can get responses. So the key here is to know, which responses correlate to cancer and which responses are not related to Cancer. Right?

And so, you have to accept the fact, that there will be these other responses and then the idea is you do, variety of Informatics processing, to stick these, compare these, to these and look for patterns, like this one here, that's present in everybody. Look for these guys, that occur only in the affected individuals and then there will be some that are just, random variation that occur from, from person to person. So that's, so that's another approach that we're doing.

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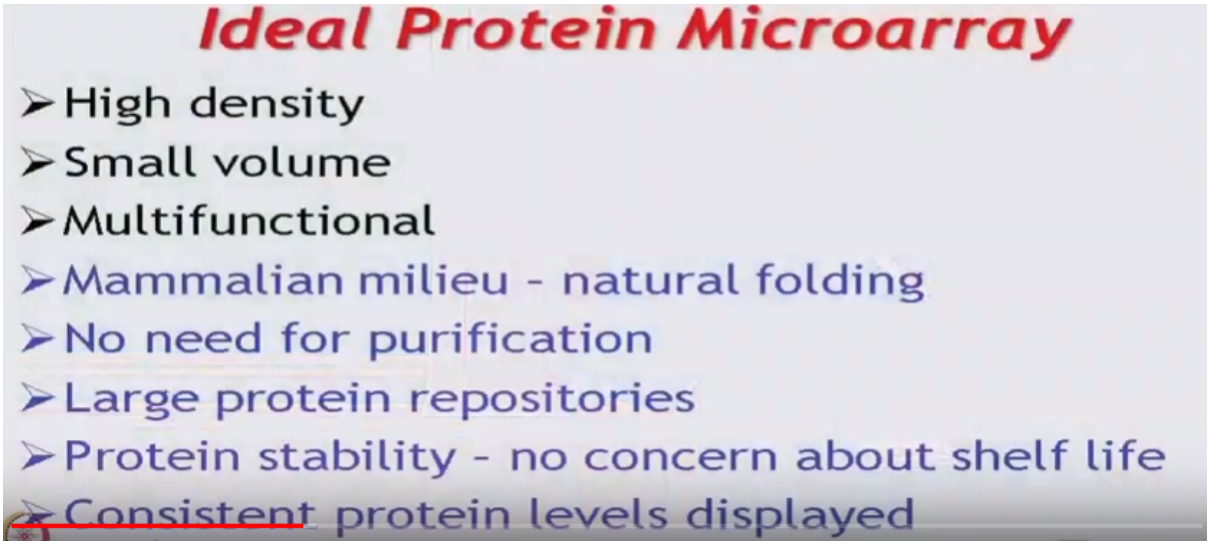

So let's start here, by asking the question, what are the ideal qualities that we want in a protein array? What would make an array, a really good array? So I would argue, the first thing has to be high density. The whole point of a protein array, is to, get lots of different proteins, in a very small space, so you can study it. Of course, you want to be able to work with small volumes, the advantage of a protein array is, that you can take only a couple hundred microliters or a few microliters of serum and test, thousands of proteins, with that information, with that amount. Of course, it needs to be multifunctional; you'd be able to be able to test it, with lots of activities. But then, you, you also want it to have natural folding; you want the proteins, on the array to look like they do in normal circumstances. And ideally, they would be made, in a milieu, similar to the one in which they normally occur. You don't want to have to purify the proteins, because if you have to purify the proteins, then you're going to end up with, all the things that can occur during protein purification. Proteins could lose their folding, they could get low yield, they may not be, in the proper conformation.

You obviously want to be able to test, as many different repositories, as possible, so you want to be able to test any proteins that you'd like. And you'd like, the to not worry about the shelf-life of the protein. Once you print the protein on a chip, there's always this worry that, the clock is starting and the longer it sits there, the more likely it is to stop being active or being well folded. Alright. So and of course you want the levels of protein, from one to the other, to be very consistent, those are the things that you'd really like. Alright, so, so this is the array type, that we're going to talk about today, which is, called, ‘Nappa’, for nucleic acid programmable protein array. And the idea for Nappa, is that we print

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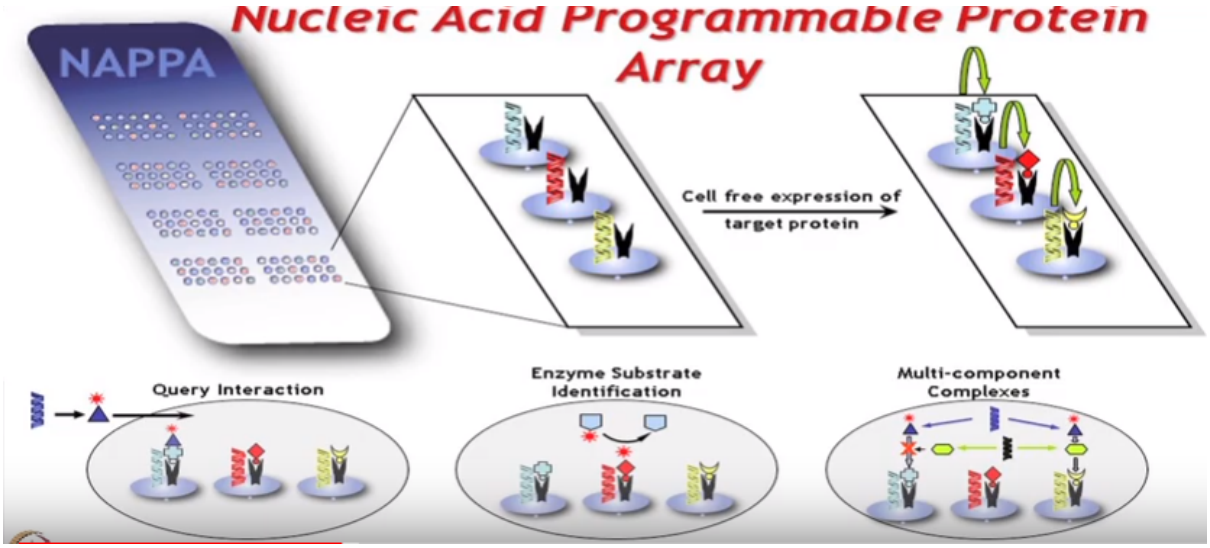

the gene for the protein on the chip and we store the chip, as a DNA chip. So the gene is there, to clone, the ones that we talked about in the first half of today, is on the chip, along with an agent, that's going to capture the protein, when it's made, but the protein hasn't been made yet. Then, on the day of the experiment, we had a cell-free extract, make the protein and capture it and then we; we display the protein, at that time, fresh. And so the idea of Nappa is that, we can do all kinds of studies on it. We can do interactions with specific protein queries, we can do enzyme substrate modification, we can even build multi multi protein complexes. So let me begin at the beginning, by showing you, the, the idea behind Nappa, before it was actually on a protein array.

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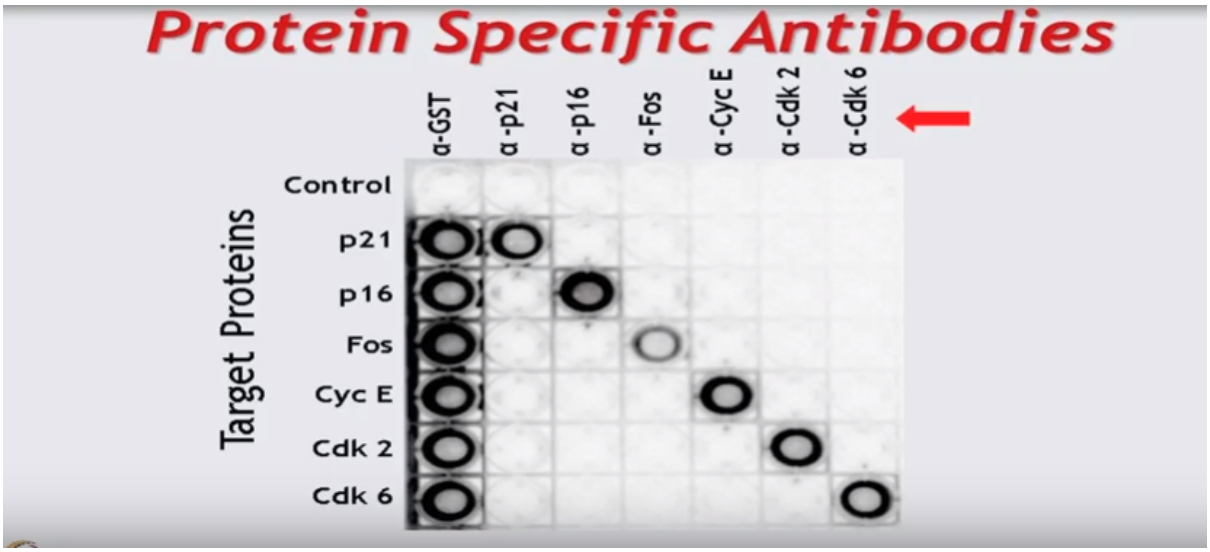

So here what we're looking at is, just making proteins, using cell-free extract, in the wells of, a 96-well dish. Okay? So, the proteins that we make all have a GST tag, at the c-terminus. Remember we talked about; you always have to have a tag. So these guys all have a tag. And so if we, if we, make the protein in these wells and we probe them, with an antibody, that recognizes the tag, they all light up. Right? But, if we probe them with antibodies, that are specific to each individual protein, then only the p21 lights up, at p21, only the 16 lights up, at 16, only the Fos and so on and so forth. So depending on what you use as your antibody, you only get that signal. Now you can use the same approach, to look at, at interaction queries.

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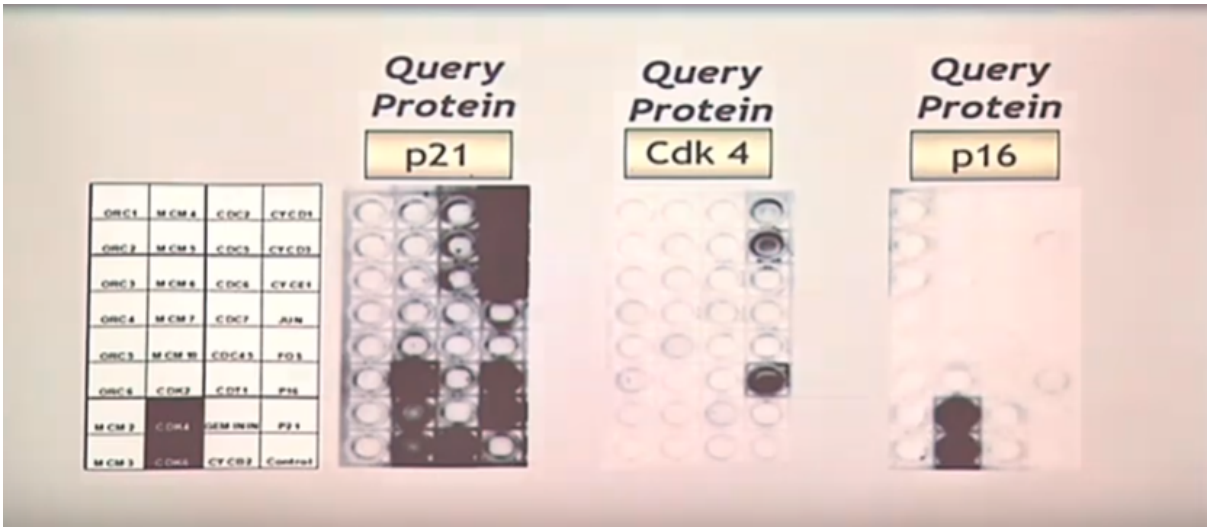

In this case, here's a bunch of different proteins on a chip. Right? And in chip, I mean in quotes, because these are actually the wells of a 96-well dish. If I probe with the protein p21, it binds to all the CDKs, it binds to these Cyclins, it binds, to these guys here and it binds, actually to itself again. So, those are the interactors will be 21. If you probe with a different query, in this case cdk4, it's gonna bind to, the cyclin Ds and it's gonna bind, to p16. And if I probe with p16, it's gonna, it's gonna pick up, these CDKs. So you can see, using different queries, I get different interactors. And again, this is not, a microscopic array, this is a nice as well dish. Well of course, the,

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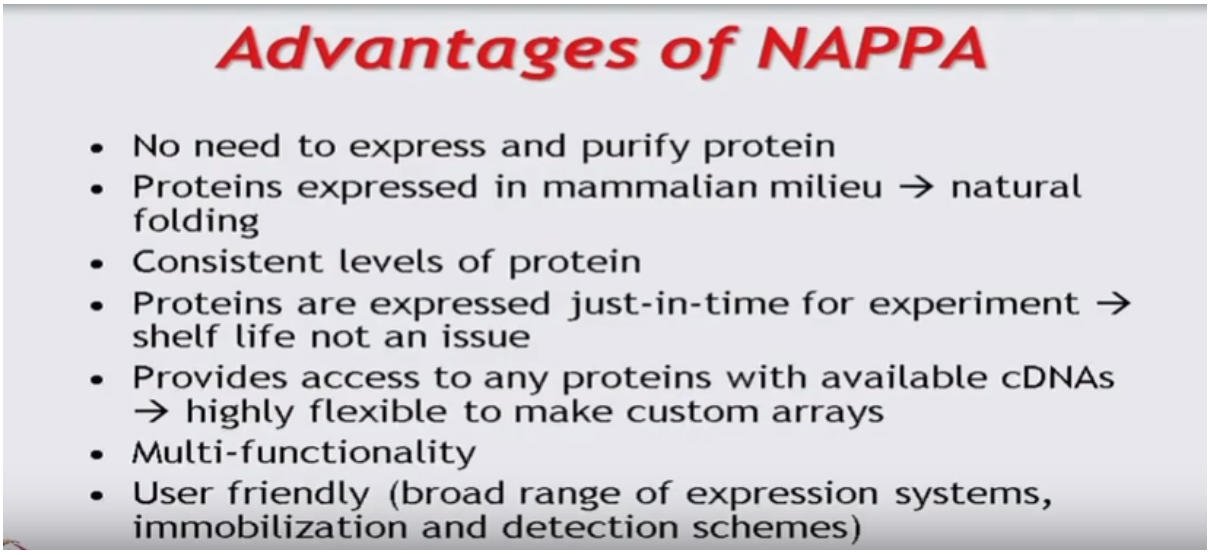

advantage of this is, now, I don't have to express these proteins or purify them. They were made using, mammalian extracts. The levels of proteins were consistent, on these, on this chip. They were made, at the time of the experiment. So I made the proteins, and I tested them, minutes later. And of course, I could do this, general approach, using, any kind of cDNA. If I can clone the gene and make the

cDNA, I can make the protein array. And then, we talked about the multi functionality, in and of course, users can modify this, as they need to. But there's still some challenges.

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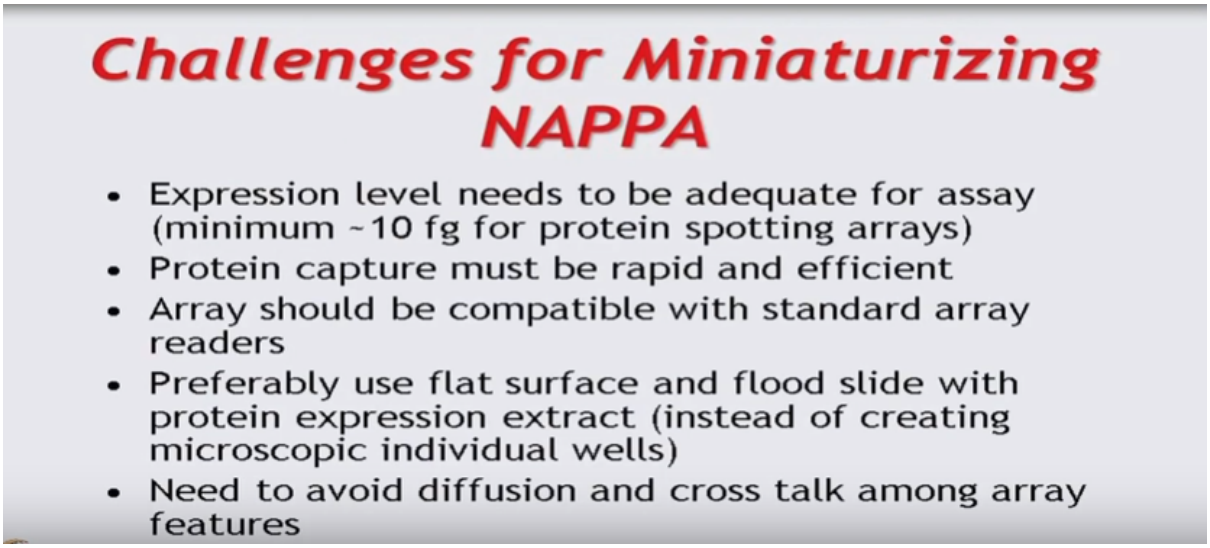

So, we need to make, we need to now, take it from this format, which is on, 96-well dishes and we had to get it onto a microscopic chip. We had to be able to print it on slides, so that we could do, thousands at a time. So we had to be able to deal, with very low amounts of protein. We need to make at least 10 themter grams of protein. We had to get capture that was rapid. We had to find a arrays, that were compatible with standard array readers. At the time, we really wanted to work, on a single slide format that, that, where we can add the extract of the whole slide. We didn't want to have to manufacture these, specialized; methods for expressing in little tiny wells or something like that, I'm gonna come back to that, because we now are moving in that direction. But at the time, we really wanted this to be very simple. And of course, we had to avoid cross talk, from, from, from spot to spot.

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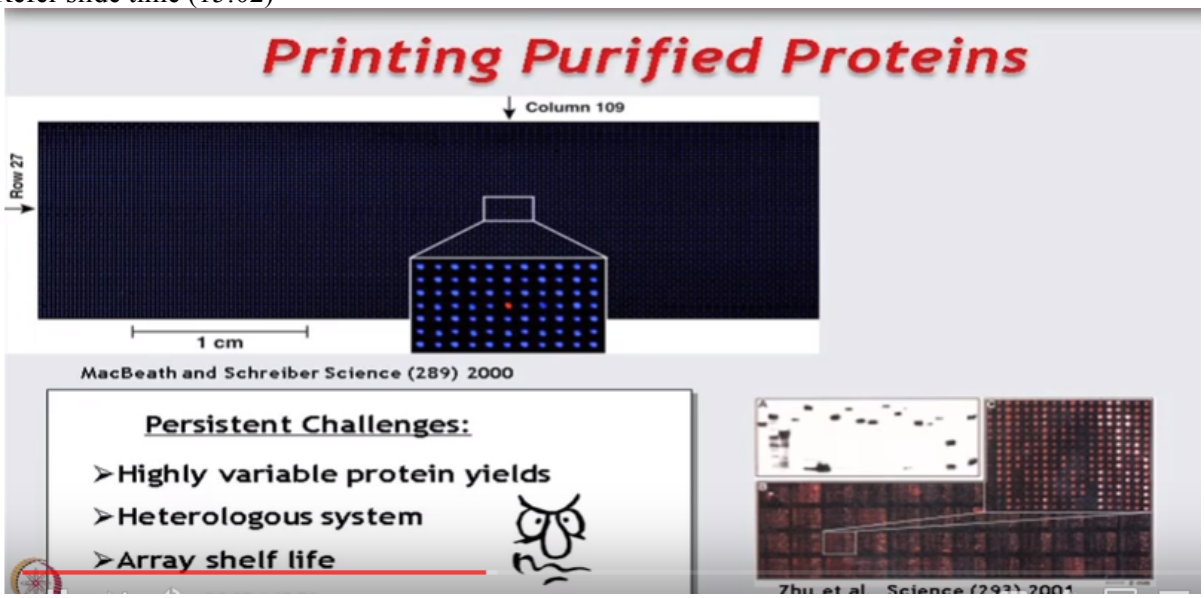

So let me talk about the technology, so most people who built protein arrays, do it by, purifying the proteins, first. They, they, they do, what we started with, which was this high-throughput, purification kind of technology. And they do it in 96 well plates or even 384 well plates. But they have a couple of problems. First of all, you get very highly variable protein yields. So the amount of protein, that you get, from, from one protein to the next, can change over for logarithms. So four, orders of magnitude of difference. They get, they're working typically in heterogonous systems. So they're either purifying in bacteria or they're purifying from yeast or they're purifying from insect cells. And that, in and of itself, introduces some differences. And of course, the biggest concern, I have, is a race shelf-life. So you purify the protein, you store the protein, then you, take the protein out, then you print the protein and then you store the, printed protein. So you have a lot of steps in there. And all those are opportunities for proteins, to lose their shape, lose their folding and not be as functional. And of course, some proteins will stay fine, during all that process, and other proteins will not and you never know, which ones they are. There's no way to tell, which ones, are the good ones and the bad ones. So this is the idea behind Nappa.

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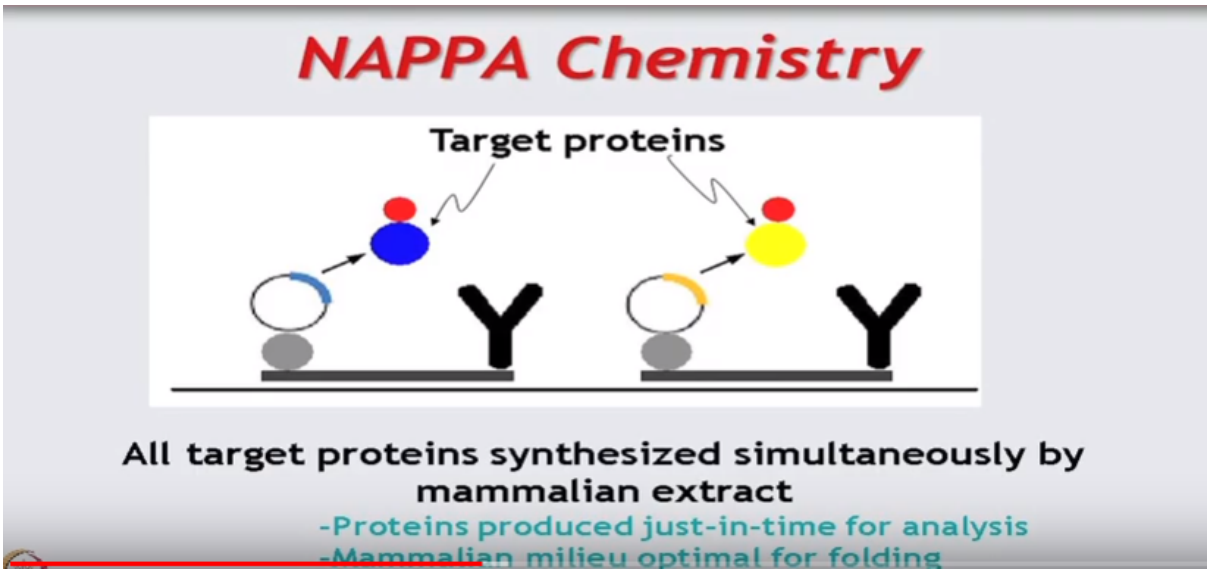

And in the case of Nappa, what we do is, we print the gene, on a plasmid. We add cell-free extract, that makes the protein and this is meant to show that, we have the protein in blue and then a GST tag in red. And then here's a different protein in yellow and a GST tag in red. So we make this at the time of the experiment and then, what's gonna happen is, yeah I just told you that,

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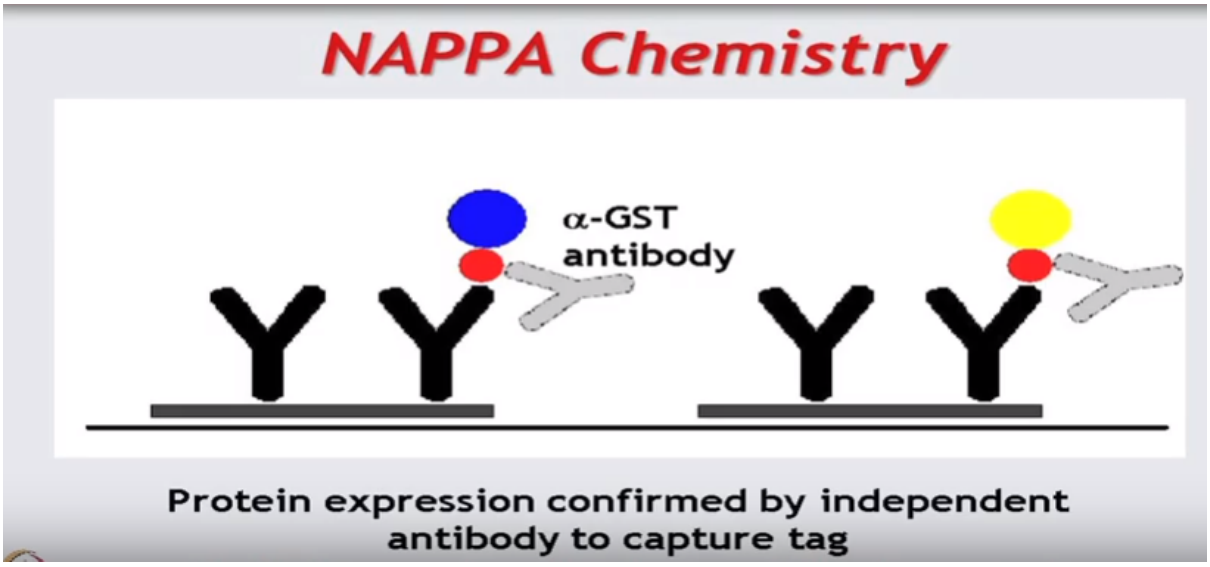

is that, the GST is gonna get captured by the anti GST antibody and now you're displaying the protein on the surface. So it flips upside down, so that the protein part is what's facing up. Makes sense?

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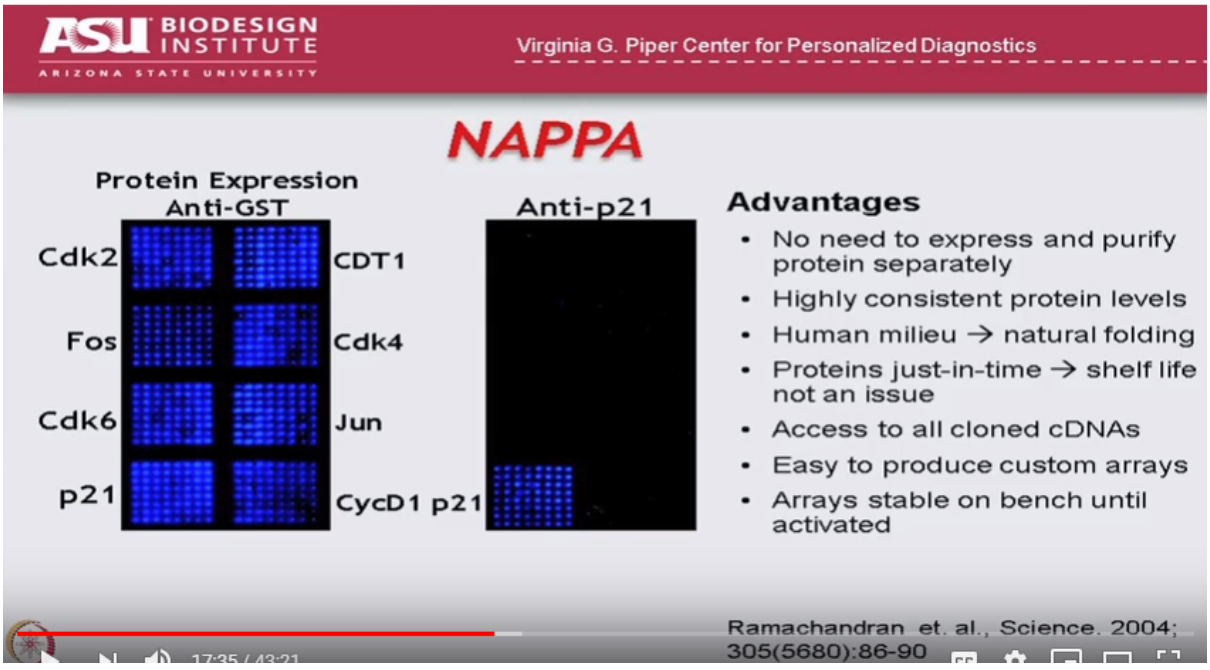

And then this is what it looks like. Here's an early array, printed eight different proteins, 64 times each. And here, we probe with anti GST, that's, that's a way of measuring, how much total protein we have on the array and so all of them light up. And then if we probe it with, anti p21, just remember the little 96-well plate, same idea, now we only get the p21, lighting up. Alright? And I already kind of, covered these, these, these advantages, pretty much the same, ones here, so I won't go over them again.

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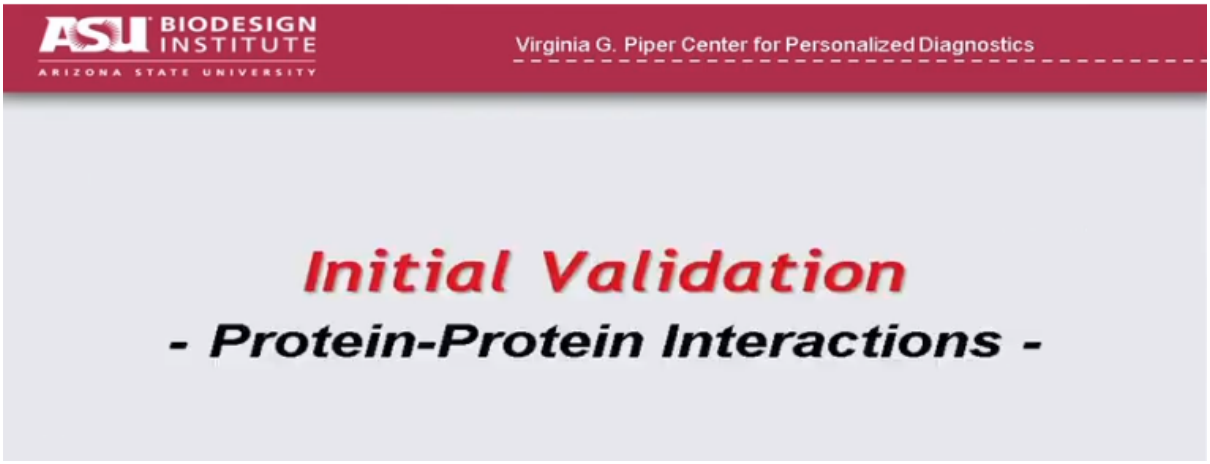

Alright? So, so, here's how we first tested this. We said, decided to do protein, protein interactions.

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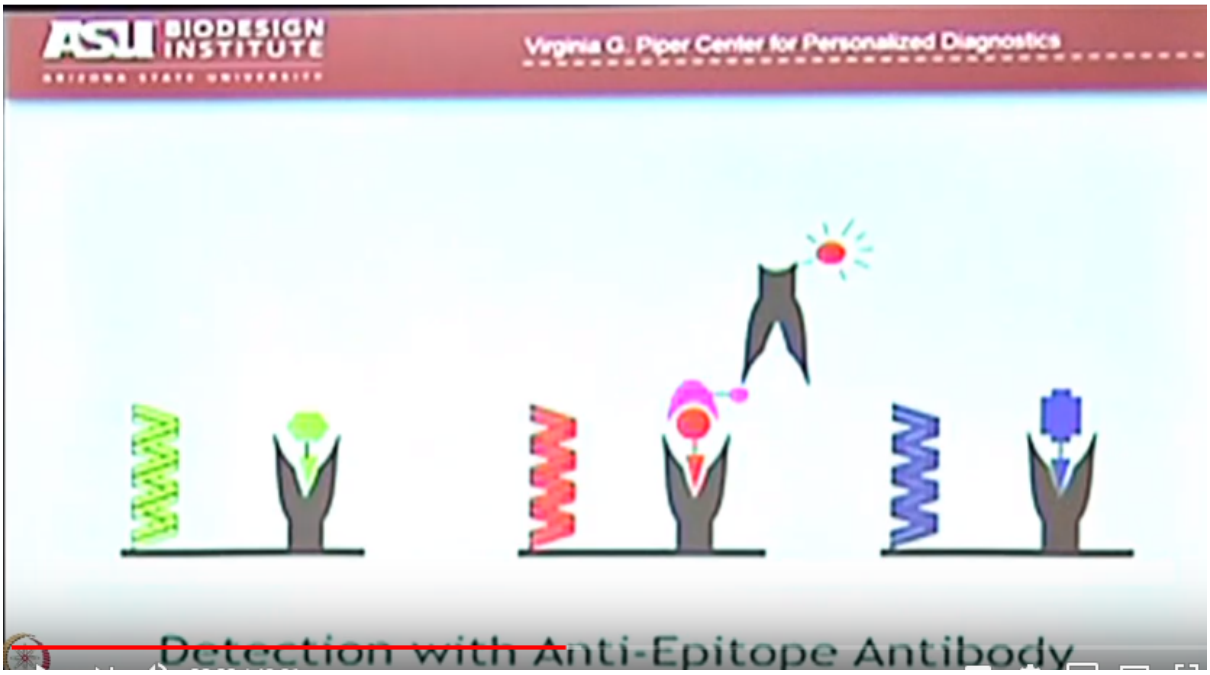

So imagine, that you have three different spots on your array, in fact we had, we had many more than that. But let's just talk about three. This one makes the yellow protein, the red protein and the blue protein. And these are the genes, these are DNA. And then here's the antibody, that's going to capture the GST. Now we want to, we want to ask, do any of these three proteins, interact with the pink protein. Alright? And the, and in the case, in this case, what we're doing is, we're going to add the gene for the pink protein, in the solution, along with these guys, which are attached to the surface. So these guys are, bound to the spot, this, this is free to go, anywhere it wants. We then express the protein. Right? And these three proteins are going to get captured, to the surface of the array, because they have the GST tag, that's going to lock them down to their spot. But this does not have a GST tag, so it's going to float, everywhere on the, with, across the array. And then over time, if you give it time, the query protein, will bind, to the target proteins, if it recognizes them. In this case, it binds to this guy, but not these guys. Right? And so now I can wash away, anything that's not bound and I'm left now, with this guy bound, to this spot. Now how do I know, where it binds? Well I know the identity

of every spot, on the array. I know whatever position it is, which gene it is. And so I know that if this spot lights up, that pink binds to red. Right? And I can detect, that interaction, with an antibody, that has a fluorescent marker on it, that will recognize, either a tag on the pink protein or it can recognize the pink protein, itself. There's all kinds of variations, you can do here. You could use click chemistry, to look at interactions and we've done that. You can, have you know, other molecules that interact with this guy, Avidin and biotin, lots of different strategies. But the bottom line is, as long as you can recognize the query protein, you can determine, where the binding occurred.

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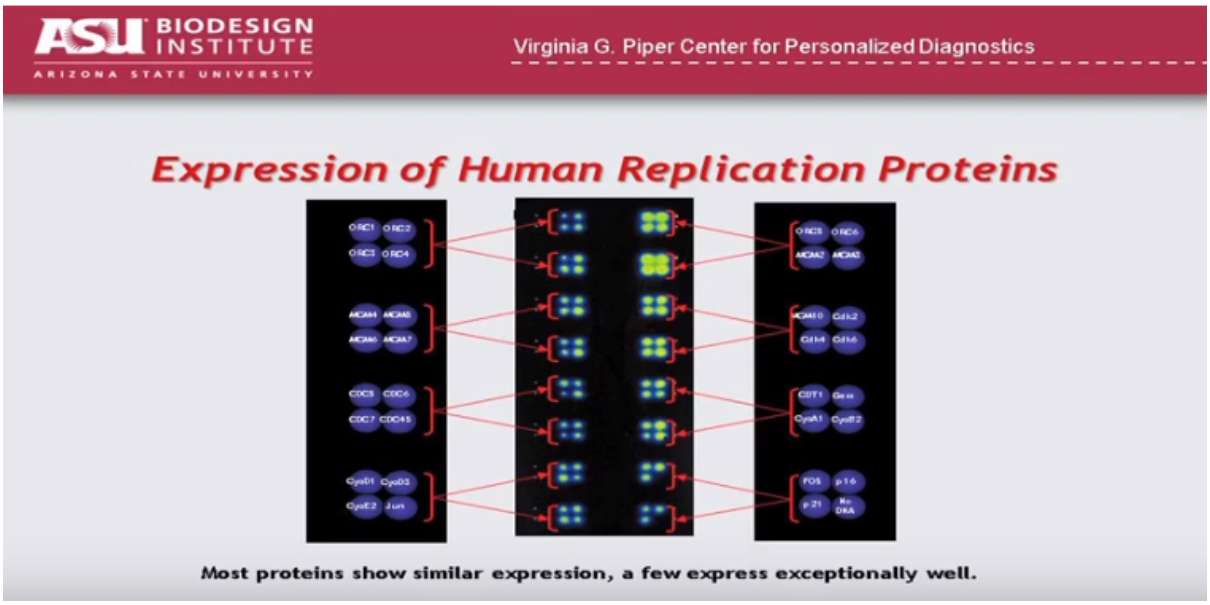

So our first experiment was this guy. We took all the proteins in the human DNA replication complex. These are, this, this is, that collection there, cloned all those genes and then we, printed them and express them. And this is measuring with GST, just to show that they all got made. Okay? And everybody was done in duplicate, so they were all there, in two spots.

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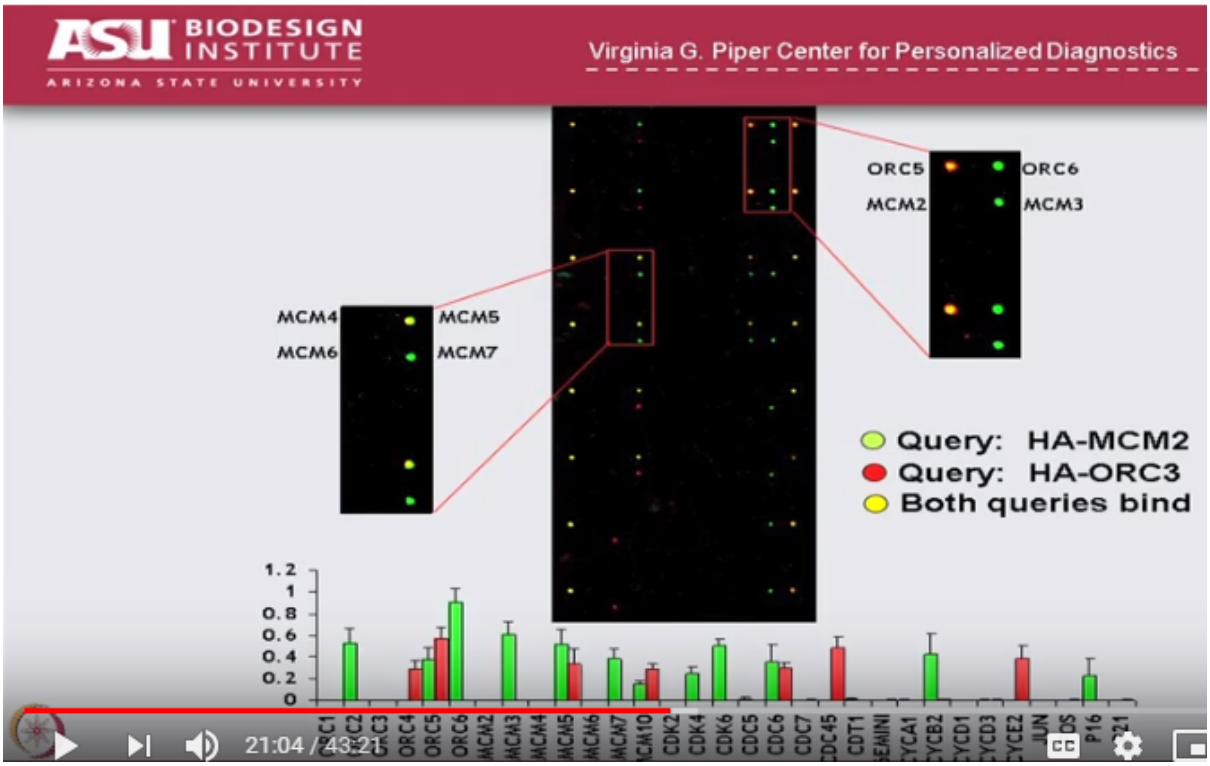

And then we would, we would, query the array, using an antibody, using this protein here, one of the proteins in the set, MCM2. And, and then you can see that, MCM2 binds to ORC5 or ORC6 and MCM3. And here it is binding to these guys over here. And of course, it does it, in duplicates. So we know, it's we're confident of the result. You can do the same thing, with a different protein, this is ORC 3 and again it's binding to certain proteins, but not other proteins. So you have, every other protein on the array, is sort of a negative control. Right? And you can, merge the two images, if you wanted to and even build, use that kind of thing, to build an interaction map,

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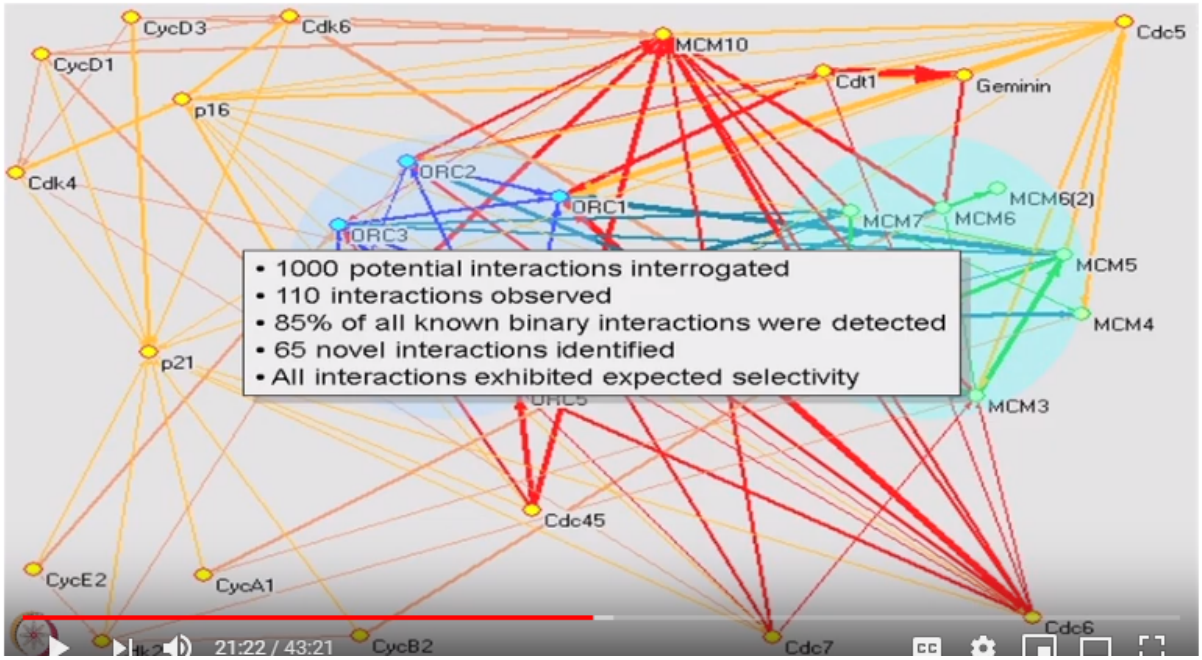

for all the proteins in the complex, with all the other proteins. And that's, effectively what we did. We identified, we queried over a thousand possible interactions, we identified 110 of them, including many new ones, using this general approach. So you can use this to kind of look at, protein, protein interactions. And of course,

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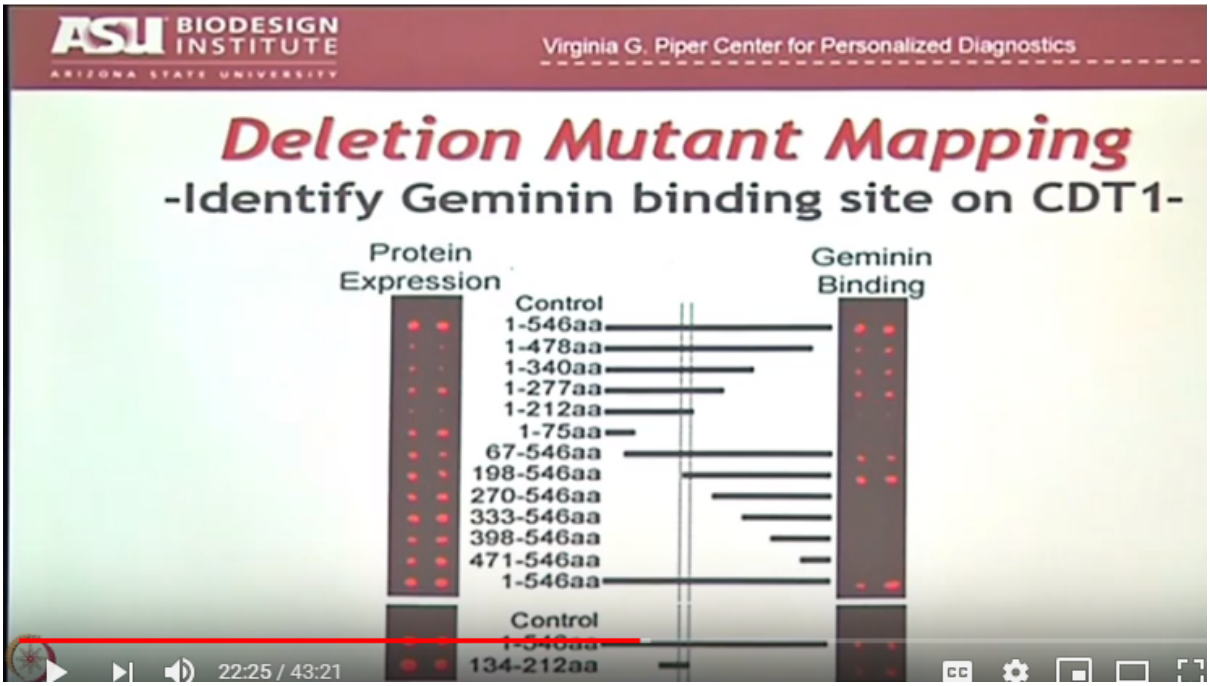

You're not restricted, to looking at, full-length proteins. If you want to map the binding domain, of specific parts of proteins, you can do that. So in this case, we were looking at, where this protein geminin binds, to this protein CDT 1. So we took CDT 1 and we made a series of different deletions. Right? And we showed that, all of them were expressed on the array and then we probed them with

geminin, which interacts with them. And you can see that gemenin binds to some of them, but it doesn't bind to others. So that gives you some sense of where, the binding site is. Right? This line here, if that, if this part of the protein was present, then it always bound. So that map quickly, where two proteins may talk to each other. And then Niro went back and made a very small version of this guy and showed that, it was sufficient for binding.

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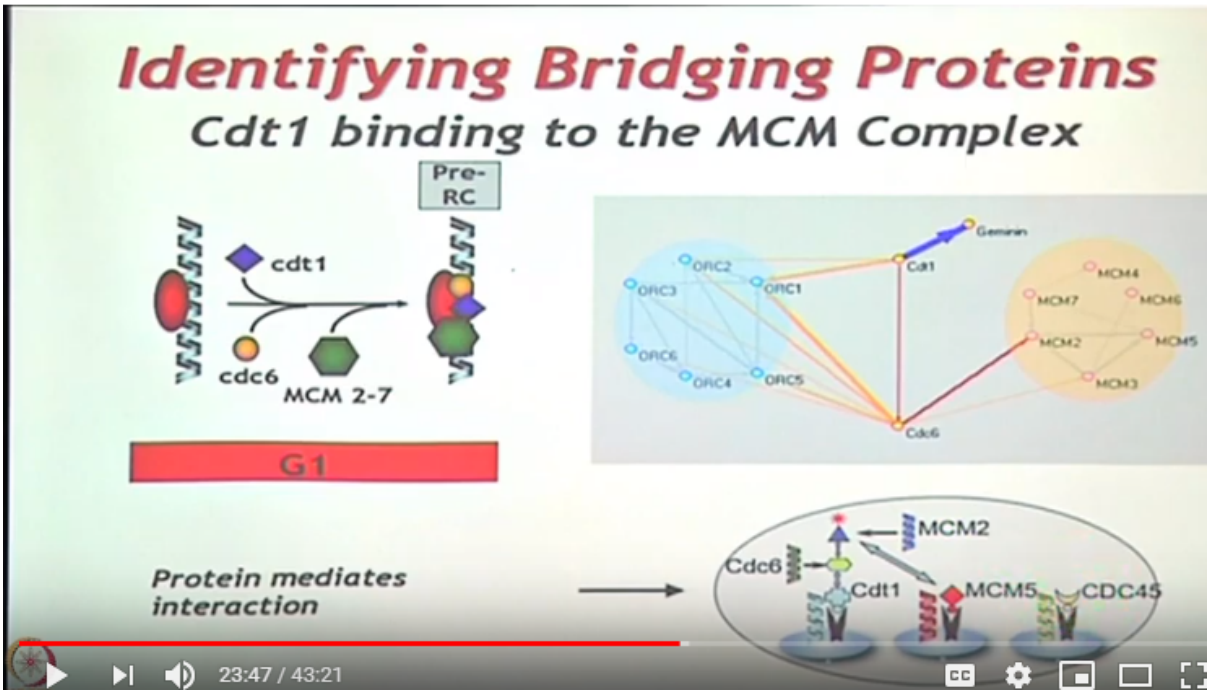

The other thing that you can do, if you want to play with these arrays, is you can actually, do that, you can look at, the possibility of multiple proteins, interacting together. So we knew that, CDT1, bound to the MCM complex. We could tell that by looking, because of biochemical studies that have been done, before we got involved. But we did not see CDT1 directly, interacting with any of the proteins, over here. This is a map that came from that big map, I showed you. And what we did what, we figured out was that, although CDT, did not bind to any of these proteins, it did bind to this protein and this protein bound to that protein. So maybe this protein here, is acting like a bridge protein, its holding, CDT 1, in connection with that complex. Right? So the question was, could we test that on the arrays. Right? And we did that, by, by doing, a couple of things. We, we could probe, MCM2, we knew was in this complex, so we probed MCM 2, against CDT 1, either with, CDC 6 or without it. And we as the control had, MCM 5 and we also had a negative control CDC 45.

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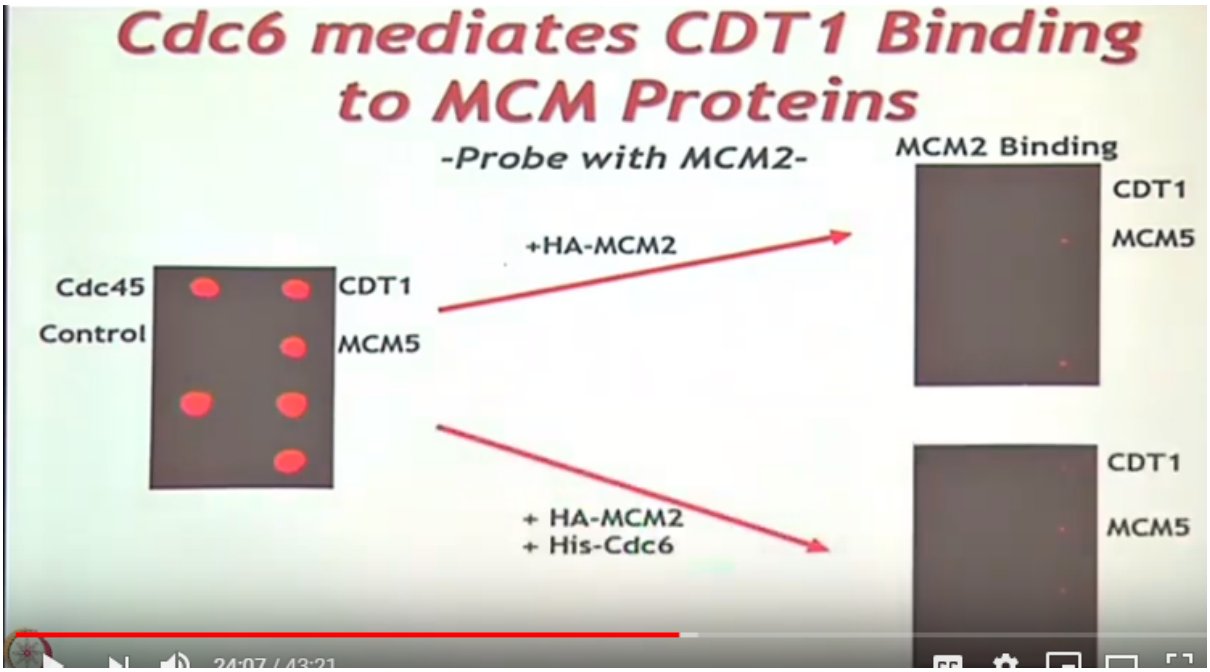

And, and this is just to show you what that looks like. This just shows you, that all the proteins were made on the array. If we add MCM 2, without CDC 6, you don't see any binding here. But you do see, the positive control MCM 5, indicated. If you add MCM 2 + CDC 6, now, now you can see the CDT 1 binding. It's pretty faint, I'm not sure, you can see it where you are, but we definitely observed it. Okay.

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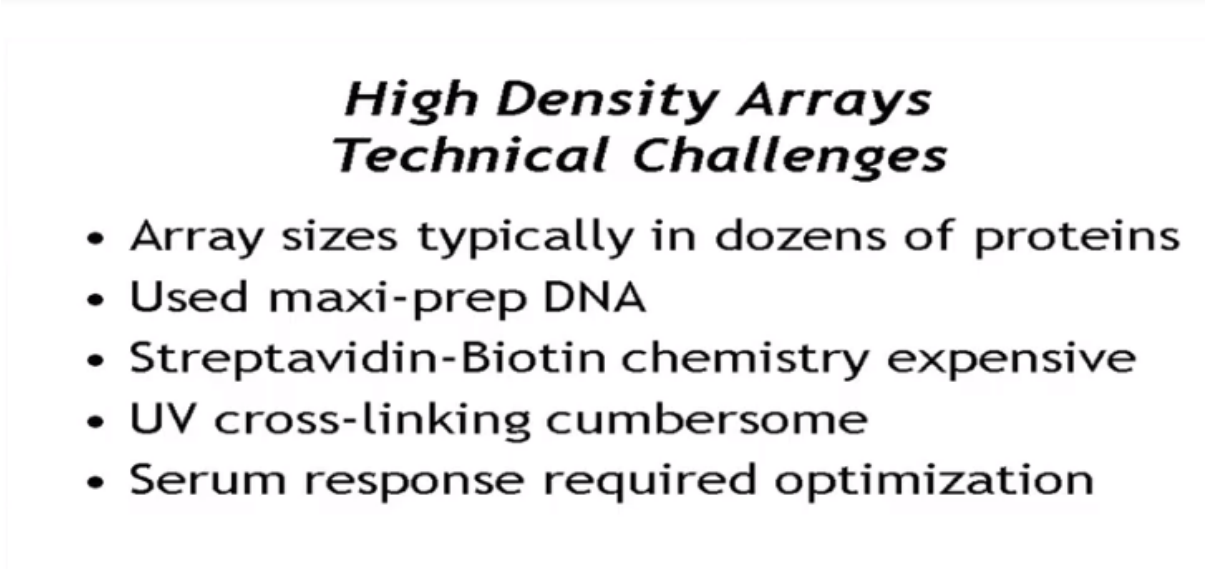

So, well we're going to spend more time later in the course, talking about the high density arrays, but I want to give you a flavour, of, of what we had to do to, get now, from these arrays. Which I showed you, showed; at around you know 50 proteins on them. The goal of course I told you, from the beginning was to get to thousands. So how do we adapt the platform, to get to thousands? So, yeah, so we were working in sort of dozens range, we need, we were, we at those days we were using maxi

prep DNA, if you're going to do thousands of proteins and remember that, what we need to make is, DNA, not protein. And that's advantageous, because it's easier to make DNA than protein, easier to purify it and much easier to quantify, how much you've made. But still, as easy as it is, to make DNA, if you want to do an array of 10,000 proteins, then you have to prepare, 10,000 DNA's. So you need to, you, you can't do that by maxi prep. You have to be working, at small scale, that's reproducible and reliable. Yeah? We were using this Streptavidin biotin chemistry, to do our arrays at the beginning and that clearly, was not going to work. Because it's expensive and it involved having to stick them in the UV box, for a while, it was, really kind of a pain in the butt. Yeah, the UV cross-linking part, as cumbersome. And we needed to do some optimization and we needed to increase our content. So, I'm going to fast forward a little bit. I'm not going to go through all of the, optimization experiments, that we're done. You're gonna learn, how to make these arrays, using our current approach.

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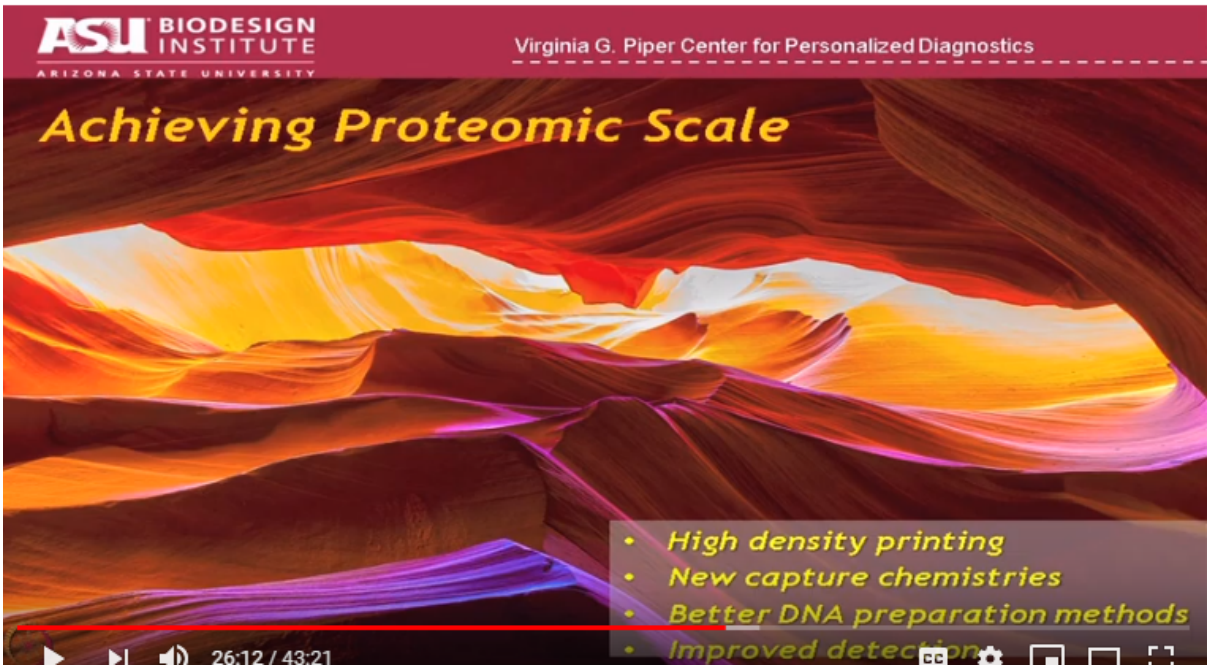

But, we spent a lot of time working on the high density printing, developing a better capture chemistry, figuring out how to make DNA and high throughput. And, and figuring out, how to detect the interactions, in a, in a more ready and easy way.

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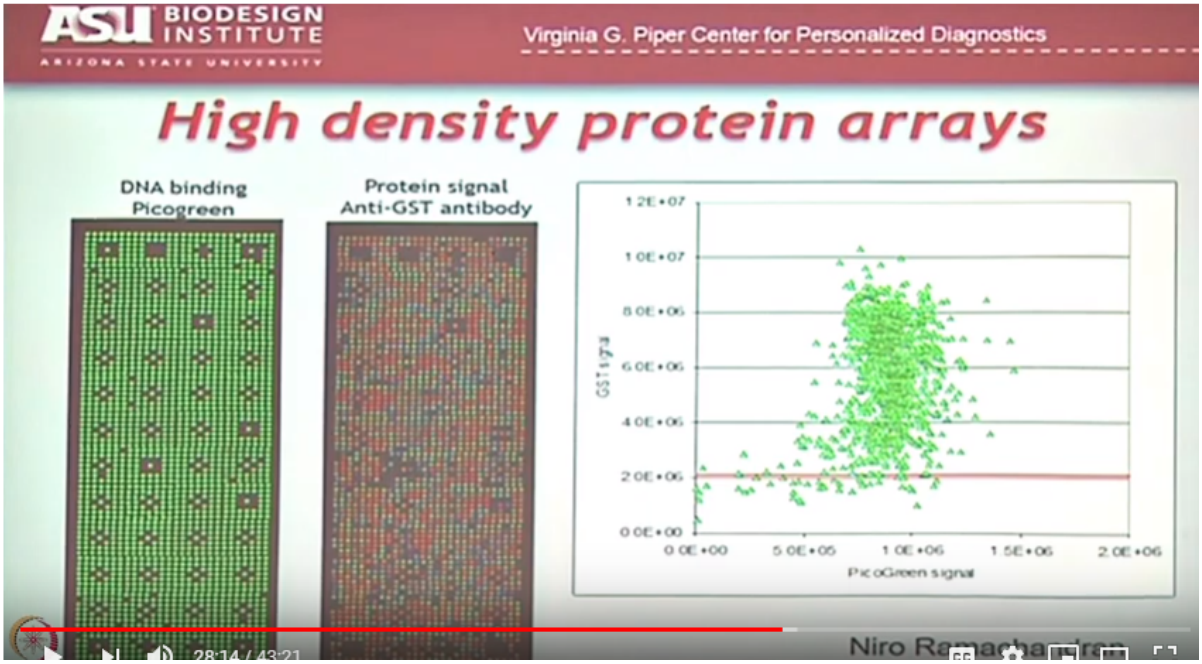

And this is sort of the result, of all that work, what you're looking at, is kind of our current version, of what; we do most of the time for NAPA now. On the left, is one of our typical arrays, around 2300, genes or proteins, printed on the array. The, the signal you're looking at here is the DNA signal. So we stain the chip, every time we make one, for DNA, and that tells us, that our printing was good. Right? Because remember what we're printing is DNA. So if we stain for a Pico up, with Pico green and we see even staining, that means, that we did a good job, of printing, even amounts of DNA. Then we convert it to protein and we measure it with anti GST. And that, tells us how much protein, we have, on the array. And that tells us, that not only to be print well, but we can express and capture the protein as well. Alright? And that's what this plot shows you, here. In the x-axis, is DNA signal, that's the Pico green, in the y-axis, is GST signal, that's the amount of protein made, a couple of things that you can observe here. First of all, in terms of the x-axis, the vast majority of our spots, line up, very evenly, around this area, here.

That means that we do a pretty good job, of printing the same amount of DNA, for every spot. And that's encouraging, means our printing is good. You'll see that there's a few down here, that did not print well, and so, it's good to know that. Secondly, if you look at, that from the top of the expression, to the bottom of the expression, the entire range is within one order of magnitude. So instead of those protein arrays, where you would have, the lowest amount of protein, to the highest amount of protein, being over four orders of magnitude. Now, all of our protein is in a single order of magnitude. In fact, 93%, of these spots, are within two fold, of the mean. Which means, that we're getting very even levels of protein on the chip? And that's exactly, remember, that one of the things we wanted, from an ideal array, was to have very consistent, even levels of protein.

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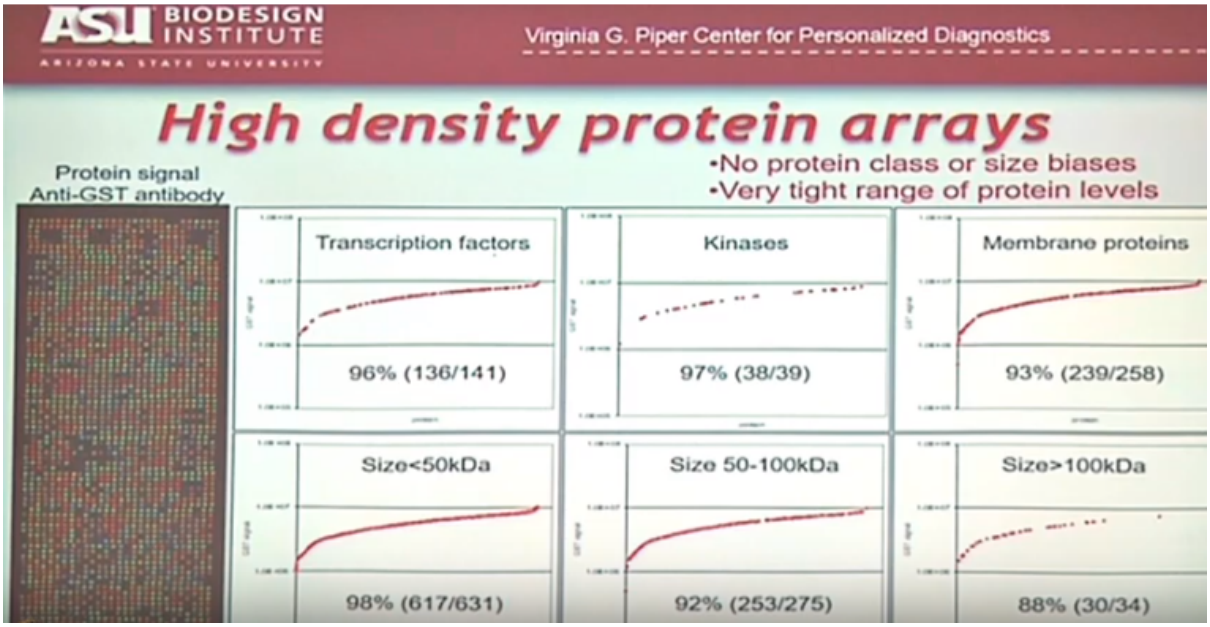

And just to give you, some better sense of that, here's quantification, based on different types of proteins. Going from the weakest is strongest and then, this line here represents the lower the lower end of detection and this is the, higher energy detection. No, no, that's not true here. I'm sorry. The it's right here. I don't have that on this graph, this is just to, signal intensity. But you can see that, we get by and large, 96% of transcription factors, were detectable, 97% of kinases, membrane proteins, are very detectable and then small, medium and large proteins, are all detectable. So, roughly speaking about, 97% of, whatever we print, we can get good expression of. Occasionally, we'll run into proteins, that have, unusual amino acid sequences that make it difficult, to get high yield. But that is by far the exception. So this Is, this is, the method that, we use to purify the DNA or I should say, the method that we used to use. Those of you who know, Sanjeeva, this is, when he was in the lab, this is how he did it.

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This is an automated platform, we had worked out, using a robot, how to do DNA mini preps, using robots and that allowed us to do, if you were really working hard, about 600 a day, in a sort of a team approach, That, that was not easy, but you could do it. Just to give you some frame of reference. When I did DNA mini preps, in my day, if you did 50 in a day, you are working your butt off. But with robots, you could get up to around 600. Okay. Since then, in Arizona now, we have this technology.

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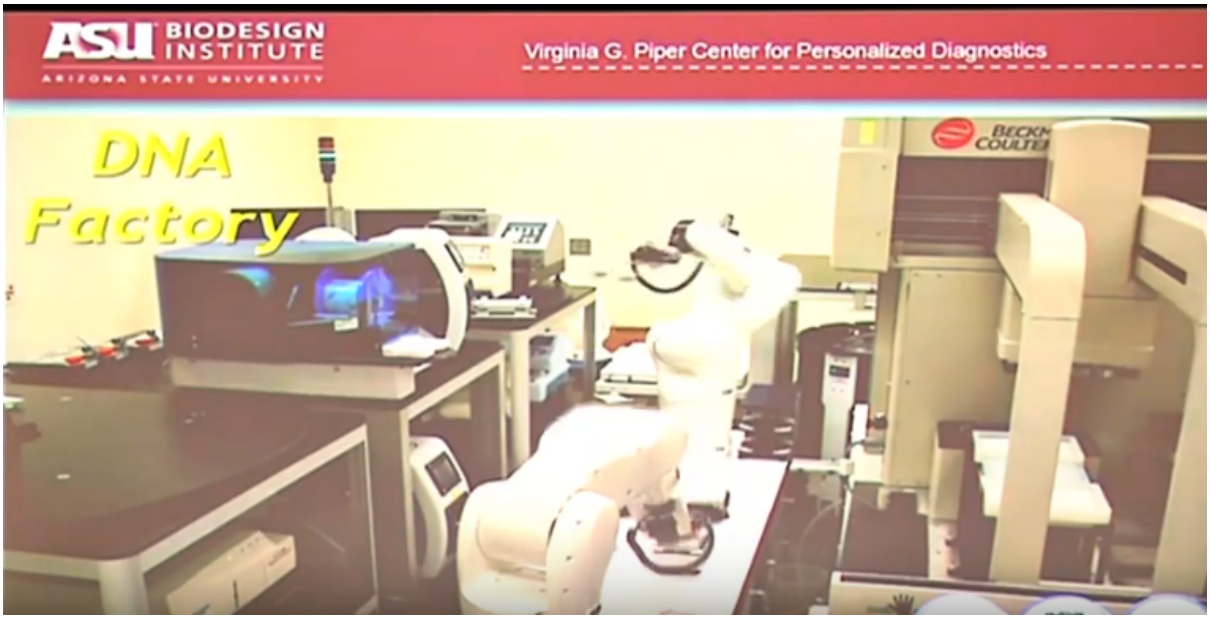

So we've taken that robot technology, which was, we used to call it, ‘Sneaker Net’, which is, you connect, one robot, to the other, by a graduate student, who runs from, one to the other, to an automated platform. And let me see, if I can make this, go. Did that, did that go in? Yeah, so this is what we have, in Arizona now, in the basement, where my lab is located. This is a fully automatic, automated platform, for growing DNA and purifying it. The camera is sitting on an incubator shaker that grows the bacteria, with the DNA in it. This particular shaker, has a specialized door, in the back, so that, these robot arms here, can reach in and take out, each one, at a time, as it needs to and they can put, they pass it back and forth, on that platform there. So hey, this guy's handing it off, to this guy. So he's gonna pick it up now. But they can pass it back, to the centrifuge, which is right there. There’s also a freezer, which is right over here that stores the pellets, after they've been grown. And then, this liquid robot over here will purify the DNA, from the bacterial pellets. You can see, this guy's mixing, by turning it upside down, just the way you would invert, a plate. Right? So, the robots can do that for you, if you want them to. And of course, we have sealers and peelers, to seal the plate, so they don't spill, barcode reader, there's a barcode reader, right down here, which you can't see, that checks the barcode of every plate, to make sure that it's what's supposed to be. And then that, this, this device over here, will read, the, the, optical density of the DNA, after you've made the DNA, the OD 260. So we can actually measure, how much DNA we're making and we the roll can will automatically adjust, the concentration, to make them, what you want them to be. So all in all, we went from doing 50 mini preps a day, to 600 mini preps a day, to now, 4,600 mini preps, in 70 hours, start to finish, growing bacteria, to getting purified DNA. So it really accelerates, what you do and it also gives you a little bit more, certainty, that you were, you're doing is working.

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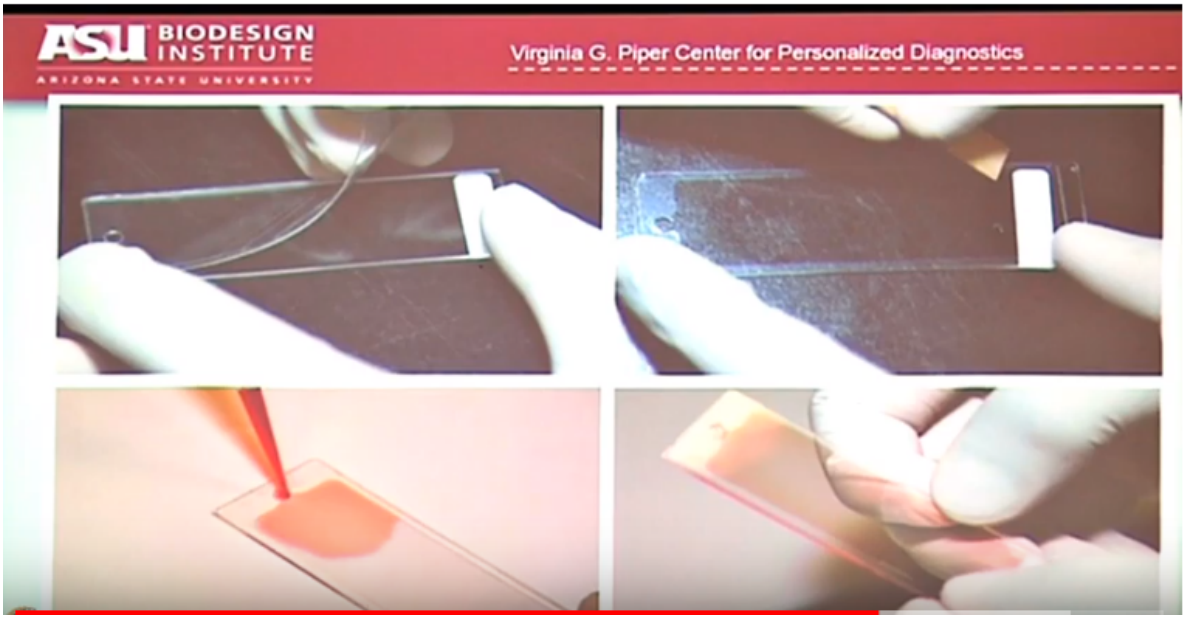

So this is how we, actually make the chips. And I think you guys have examples of this, at your desks? You have this coverslip here that you put on the chip. You, there's a little hole here and the little hole here. You inject your lysate, in here, you fill it all up. Make sure you don't get any bubble, that's always the trick. One of the things you have to learn, how to do. And then you can make the proteins, on the chip, using this approach.

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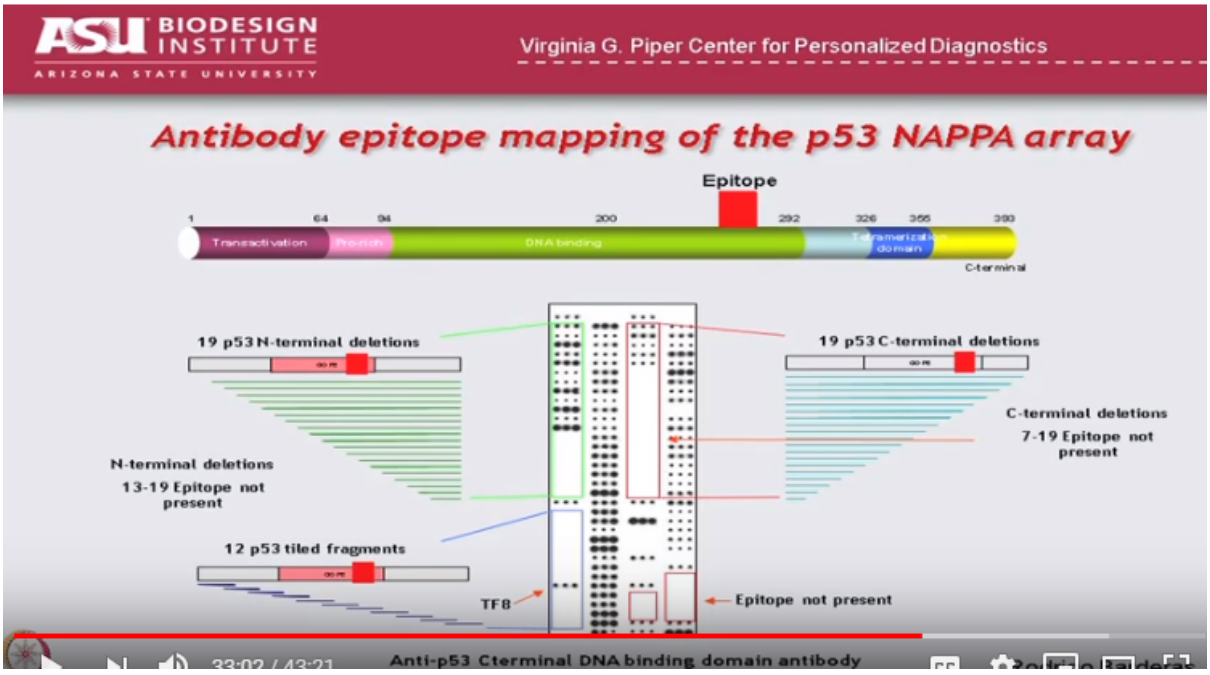

This just indicates that you can, map, a more high debt, a higher throughput version, of mapping, where binding occurs. Here's an example of an antibody that was binding to the p53 protein. We did a series of n-terminal deletions and you can see it binds, to all of them, until it gets to here. We did C

terminal deletions and again, bind to here then, you don't see it. And we did fragments that walk across the protein and of course it binds to just, that one there.

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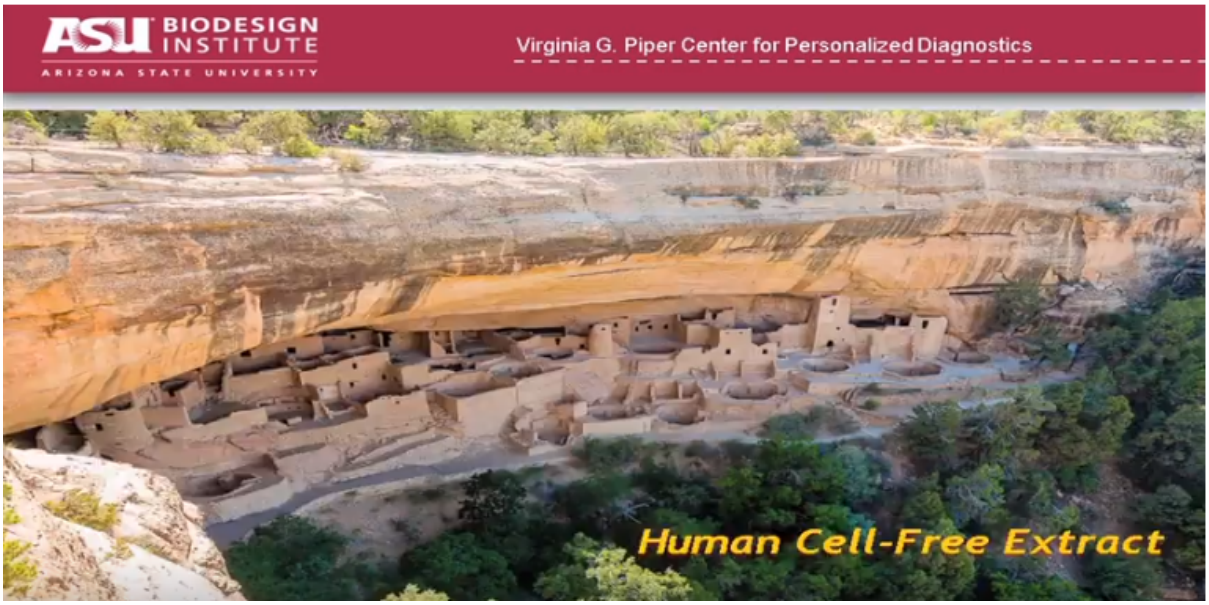

So there's been a couple of, advances, that that have occurred, in the last, I would say several years. When we first started this work, we were using, reticulocyte lysate, from, from rabbits, to make proteins.

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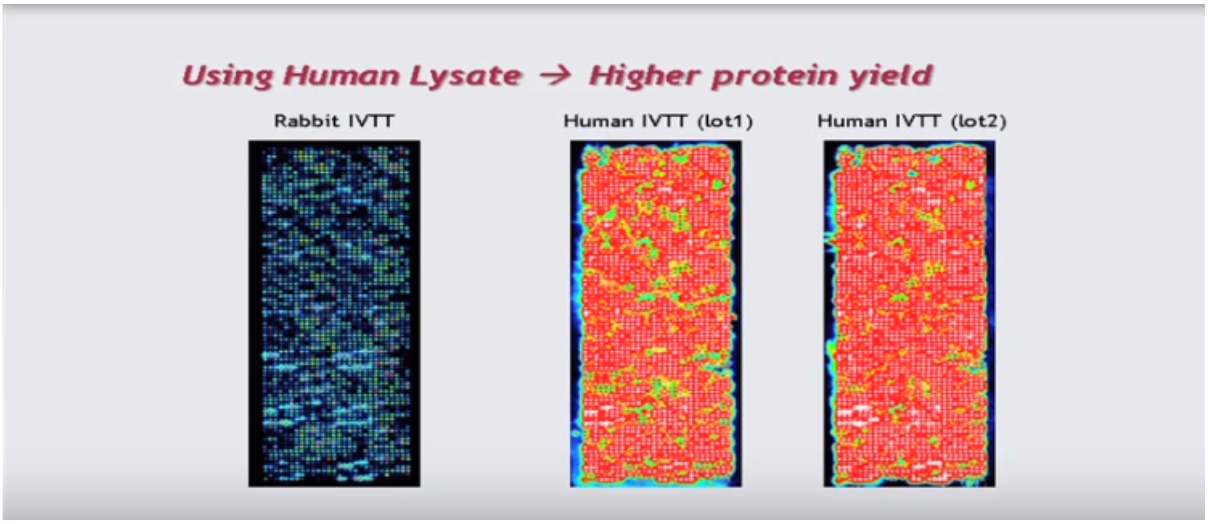

That was the expression lysate that we used to make proteins. And I will say, that still works, ok. This is what, that looks like. So you've been seeing, arrays that look like this, now, most of the day. Blue is okay expression, Green is better expression, Orange is really good expression, Red is like amazing expression. Okay? So that gives you some flavour of the, thing. And we were quite happy, with this. But, then the patent, ended on that and new companies came out. And a new version of lysate, came out, that was made from human cells. The advantage of the human lysate, was that it, it came from, a

purified cell line. So, you didn't have to get, so much variation, from animal to animal. And this is what the signal looked like, just unbelievable signal, about 15 times stronger, than we could get from the rabbit lysate. This is, whole human extract that includes, human ribosomes and human chaperone proteins, which means, that there are proteins in the lysate that held these proteins, fold, in their natural, in their natural shape.

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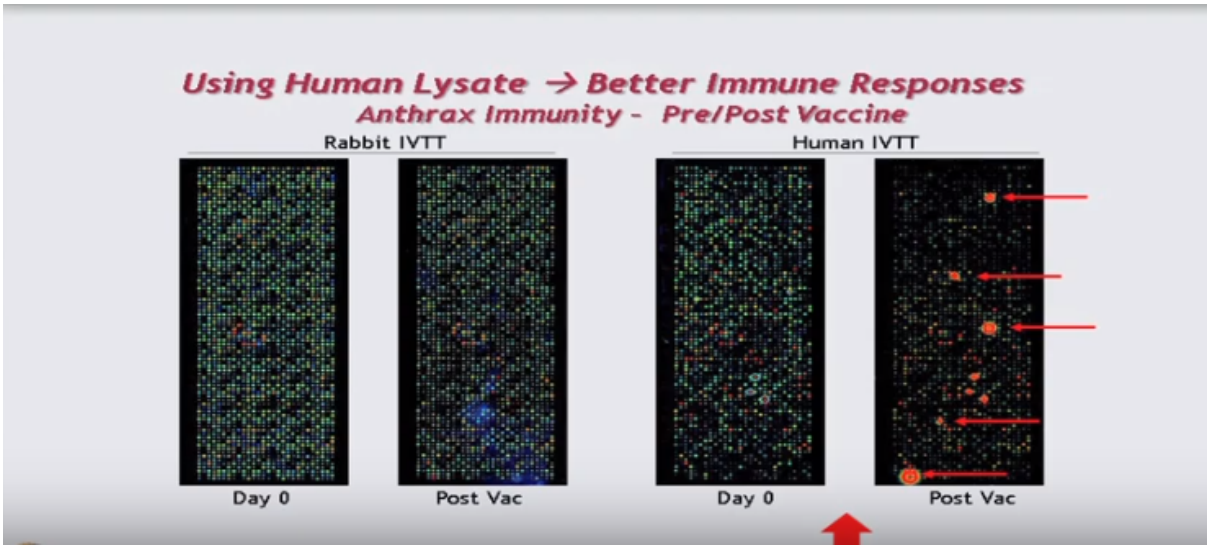

The other advantage of the human lysate is that, it's less likely, to inhibit immune reactions. So one of the problems that we used to see with rabbit reticulocyte, was, every once in a while, when we were looking at, this is a person who was vaccinated, with an antibody, to these to this anthrax. That even after vaccine, we saw no response, on the chips. If we made the same chip, with human lysate, we saw very good responses. Probably what's happening is, in the human lysate, I mean, in the rabbit lysate, there are, because it's from blood, there may be, inhibitors of immune response there and those were blocking it, on the chips. But the human lysate comes from a purified cell line, there's no immune system around and so you don't get that problem.

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So I'm going to, show you just a couple of, applications of this approach and then, maybe even just one.

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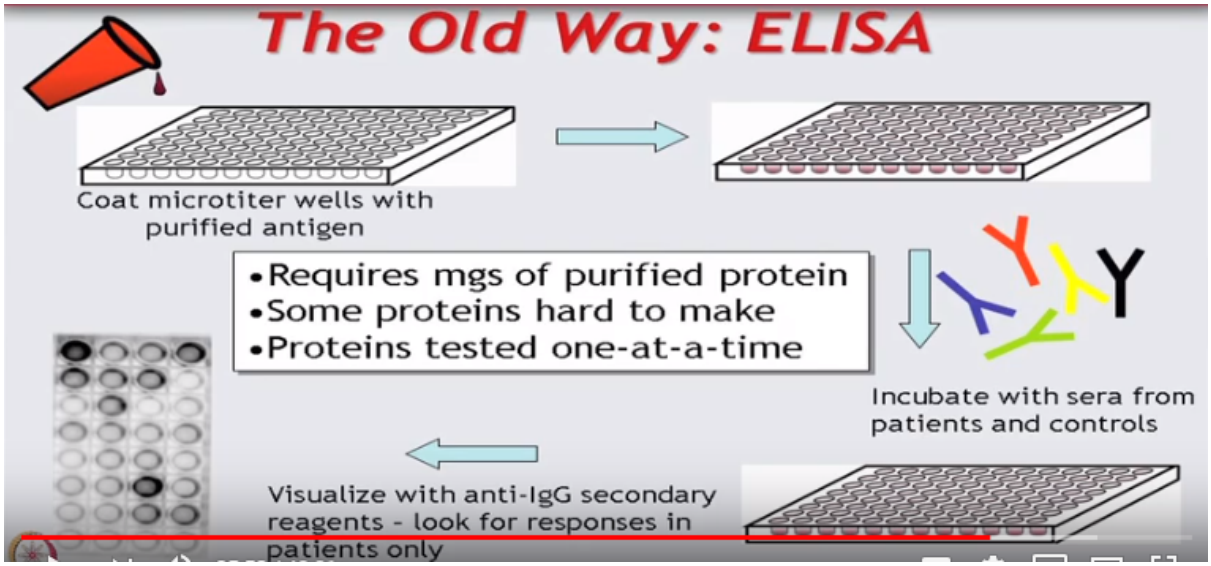

So, we've talked about immune responses, the classic immune responses, by Eliza, where you, you coat, the well the 96-well dish, with your protein, you add serum, to the, the, the wells. And then, here's a patient who had a strong response and here's a patient who had no response. Of course, if you do Eliza's, you're doing one protein, at a time. It typically, requires a lot of protein to do that. And, and, and some proteins are very hard to make to begin with.

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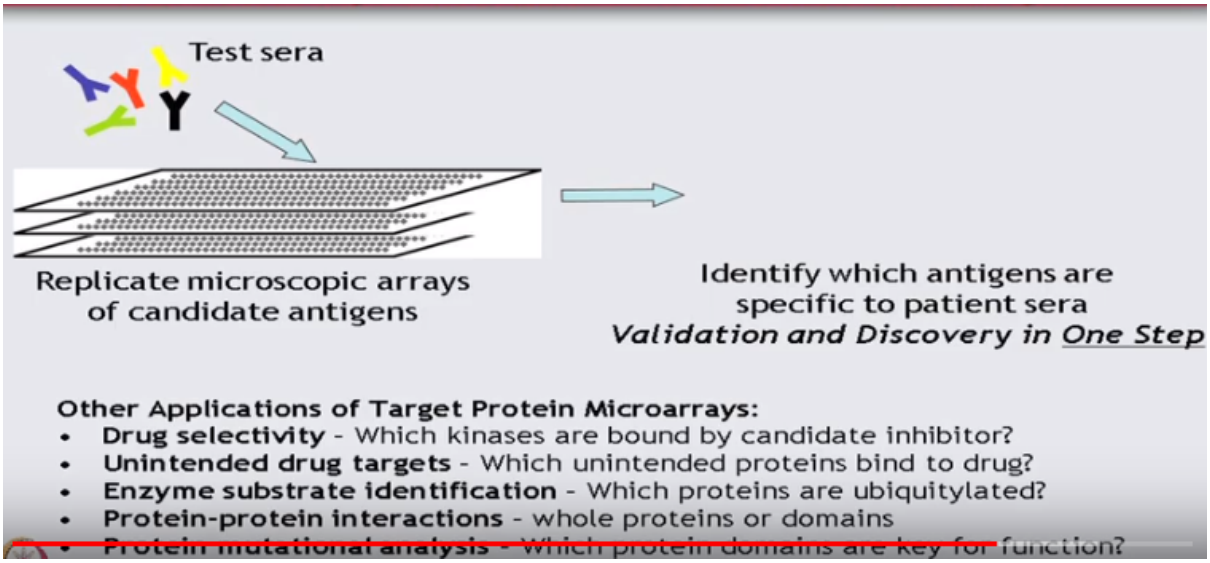

So, of course, what we'd like to do is this. Take a chip that displays thousands of proteins, that. Okay, so the idea is, you probe a chip, with serum and then various spots on the chip light up. And we've talked about all these, different types, of assays, so I won't believe with that.

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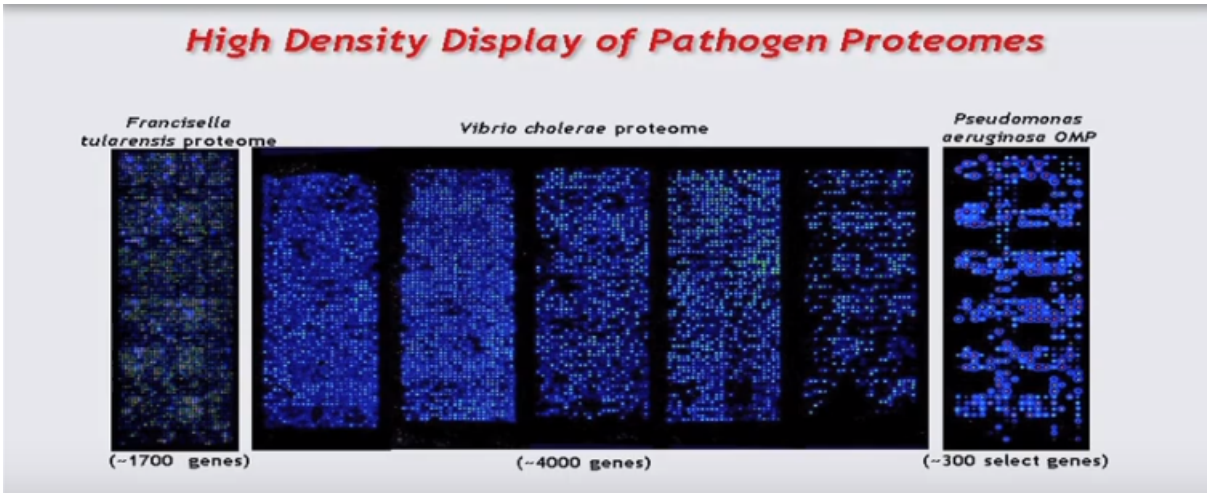

So, so let me give you one example. We'll talk about more, as the course goes on. This is pathogen proteins. Right? And, what you're looking at here, I remember, I mentioned to Alenia, when you ask your question that is the entire proteome, of Francis Satuluna Elisis. In fact it's the entire proteome, and duplicate. So we got them, all into a single array. So, because we in the end, we got that to work. These five chips are cholera, so, Vibrio cholera. And then this chip here, is just outer membrane, proteins, from an organism called,' Pseudomonas aeruginosa', which is the organism, that causes, pseudo pneumonia aeruginosa which is the, organism that causes Pseudonomial pneumonias. It's a even cause of death and patients who have a disease called, 'Cystic fibrosis. I'm gonna spend a little bit more time, on this guy. So there are around 300 proteins on there. We were working with a collaborator, when we were in Boston. Steve Lawrie was interested, in identifying, developing, a vaccine, against Pseudomonas. Because this was the leading cause of death, in patients with, with CF, and. Also a major cause of death, of patients in the hospital, who are, incubated, who are otherwise, immune compromised. This is an organism, by the way, that we've all been exposed to. It's in the

environment, all the time. Most of us if we're healthy don't get infections, but under certain circumstances, you get infections.

So he, his idea was, the proteins on the outer membrane of the bacteria, are the most likely to be, inducing an immune response, and to respond to, a vaccine. So he wanted to look at, which of those proteins was Immuno genic. His idea was, he was going to purify those proteins and then test them. Now if you've ever tried, to purify a membrane protein, you know how difficult that is. It's hard to purify proteins in general, but purifying membrane proteins, is a, is a nightmare. And so he, he boldly went ahead, to clone, to purified 300 of these and I think he got 4. Alright? So we suggested, to try the array, because we knew the arrays can make membrane proteins, pretty well. And in fact, they did.

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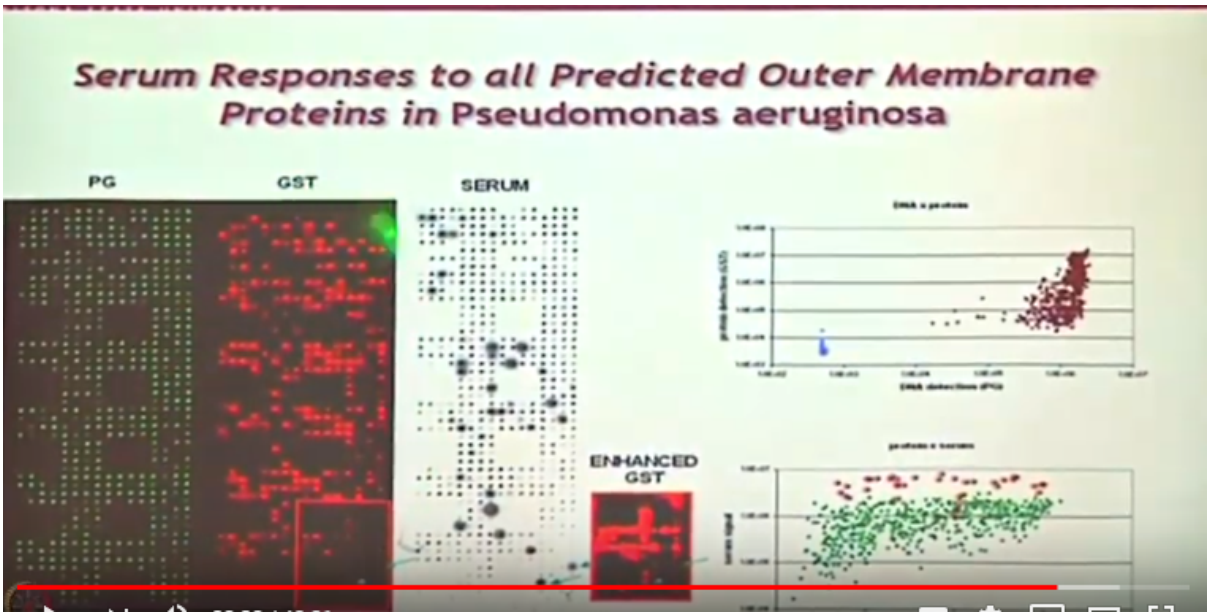

So here's the chip, the DNA stain, here's the chip, the, the protein stain. You notice that, they're all red and they're all expressed. So the membrane proteins were well made on the chip. And then we probed them with patients' serum and you can see that this patient is responding, to certain proteins, on the chip.

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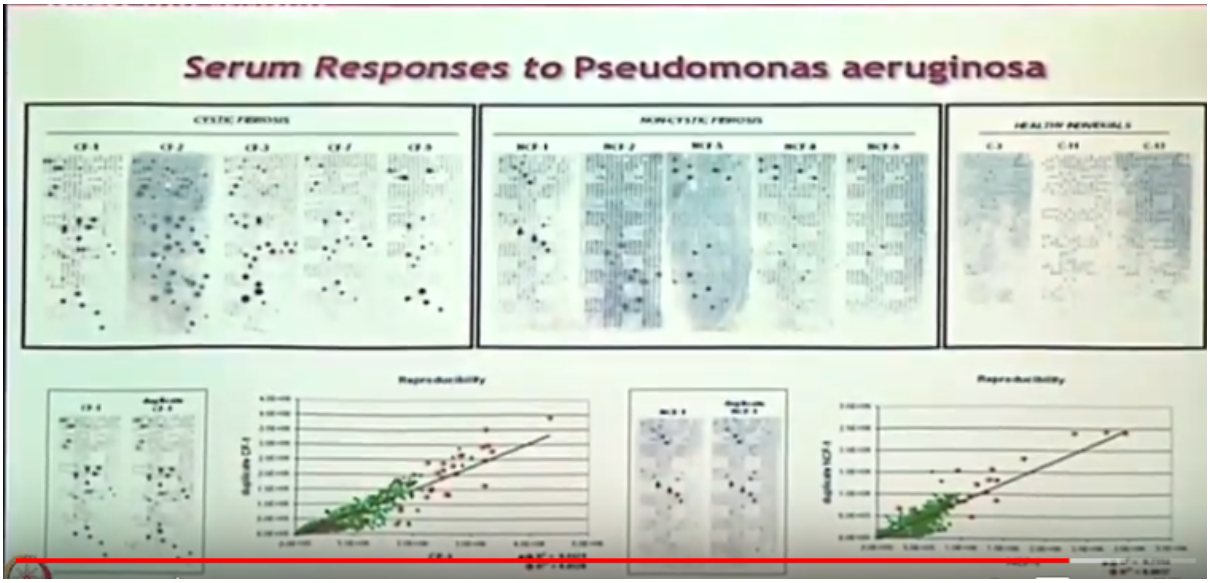

So then you can ask the question, well are there, common responses? Because, if you're going to make a vaccine, you want it to be one, that's common, that works for most people. Yeah? So, here he took a number of patients, with cystic fibrosis, who had documented Pseudomonas infections. Here's a group of, non CF patients, who had, who still had documented infections, but just they didn't have CF and then here are healthy controls. This is just to show you, that the responses were very reliable, from chip to chip. And if you start looking carefully at this, you'll start to see a pattern emerge. Certain features show up over and over again. So that pair there, is there, it's there, it's there, it's there and there and there and there. Same, it's, those two spots are the same protein. Everybody's eye on duplicate, they show up repeatedly. That's a sign, that, that particular protein is what we call, 'Immuno Dominant'.

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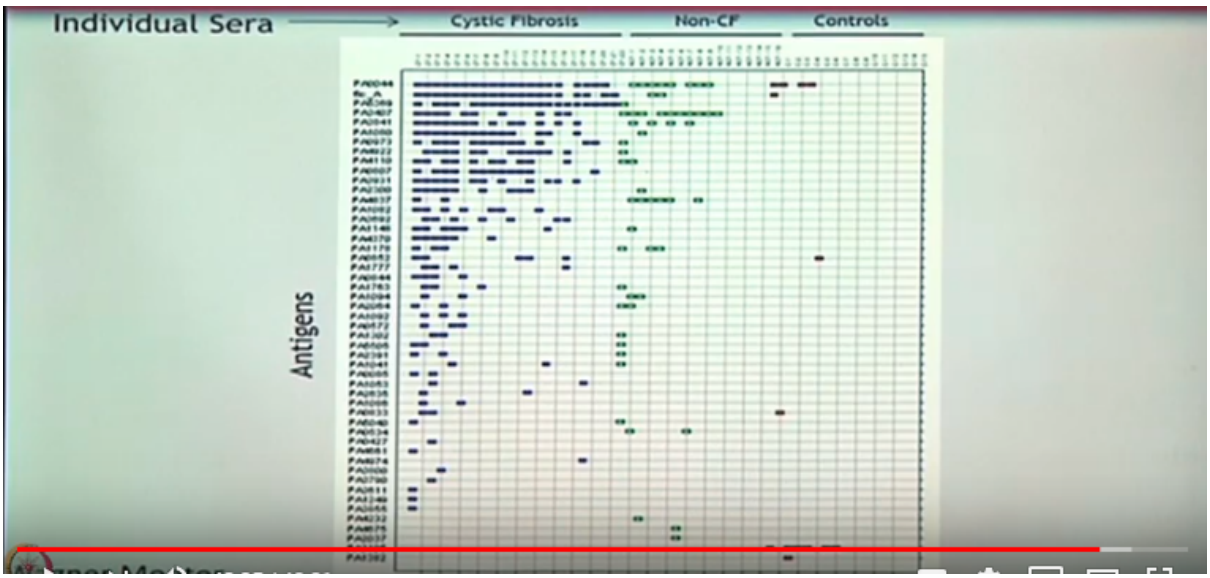

And, if you look at, patients and columns and antigens and rows, you'll see that, these top twelve or so antigens, show up in numerous patients. And so this is the group of proteins that we should be looking at, to think about developing a vaccine. Now there's a couple of things to remember. First of all, you can't make a vaccine until you know that those proteins actually induce a protective response,

so we haven't done that part yet. The second thing is, that, no single protein, worked for everybody. It turned out, that to get everybody; you had to get a mixture of a few proteins. And I think, that's gonna be a common thing, I know that's gonna be a common theme, moving forward, in developing biomarkers, is that, it's gonna be rare, that a single biomarker will work for everybody. Eventually you're going to need, multiple biomarkers, because different people have different responses.

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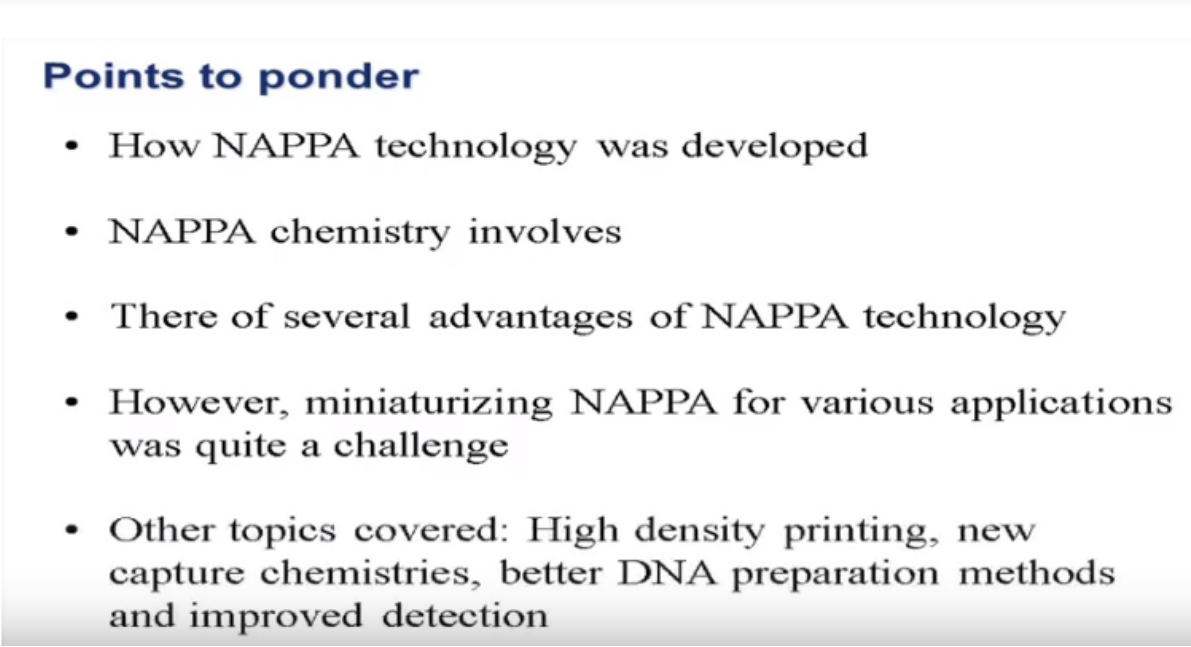

So hope you got, a very good overview of, how this fascinating technology, nucleic acid, programmable protein array, was developed. The kind of thought process, of generating these resources, especially protein, without having the protein, expression and purification, was definitely, one of the revolutionary concepts, in the proteomics field. The Nappa chemistry, was explained by Dr. Dr. Joshua LeBaer in detail and it also now, familiar, with, what are the advantages of using, this technology platform. Of course there are challenges, in naturizing these, assure these features, to do the high-density printing. But those were overcome, with many innovative ways. And Oklahoma has talked to you, about, high-density printing, the new capture chemistries, the different modified ways of DNA preparation and the improve detection technologies, which have really progressed, the initial versions, of Nappa technology, to the very latest, much more easy and reproducible and high-throughput, Nappa based platforms.

I hope now you are, very convinced, that, using cell free expression microarrays, could overcome any limitations of, protein expression and purification. You need not to limit yourself to express and purify each protein of interest and large number of proteins, to be purified, before you can do a protein microarray experiment. Even if you have CDNA, for the genes of interest, you can still do the protein microarray based experiments and Nappa could be one of the very, powerful technologies, to do these kinds of experiments. I hope you got, some understanding, of this novel technology and a basic of, some applications which could be performed, using up arrays.
Thank you very much
Thank you very much.