**Introduction to Proteomics**
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**Lecture - 30**
**Lab session - Sample Preparation: Tissue Sample Preservation Technology**

So I would like to highlight one area which is usually overlooked when it comes to sort of proteomic experiment, a lot of focus is generally placed on the mass spectrometer because it is very expensive and that is where the results are generated, but it is important to remember that your results are never going to be better than your sample if your sample are low quality regardless of the amount of money you spend on the mass spectrometer the results are still going to be low quality.

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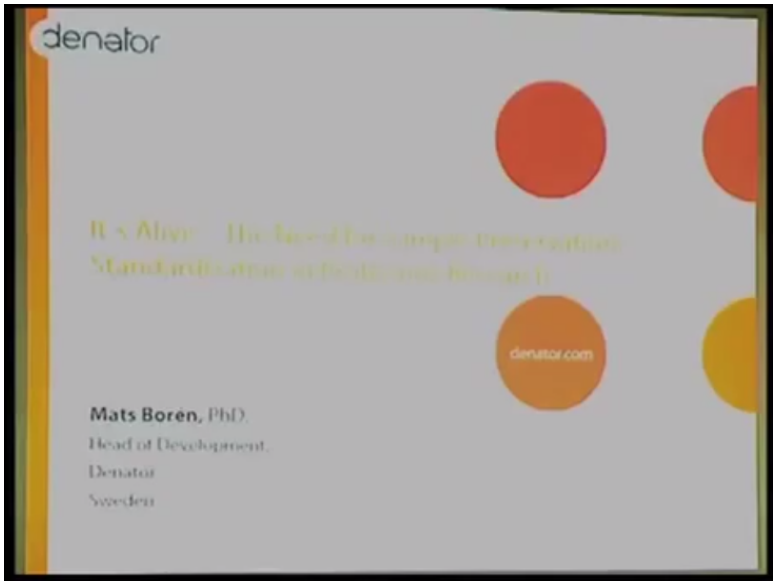


And I represent a company called Denator and we manufacture yes the instrument is stabilizer for stabilizing the molecular composition in the sample.

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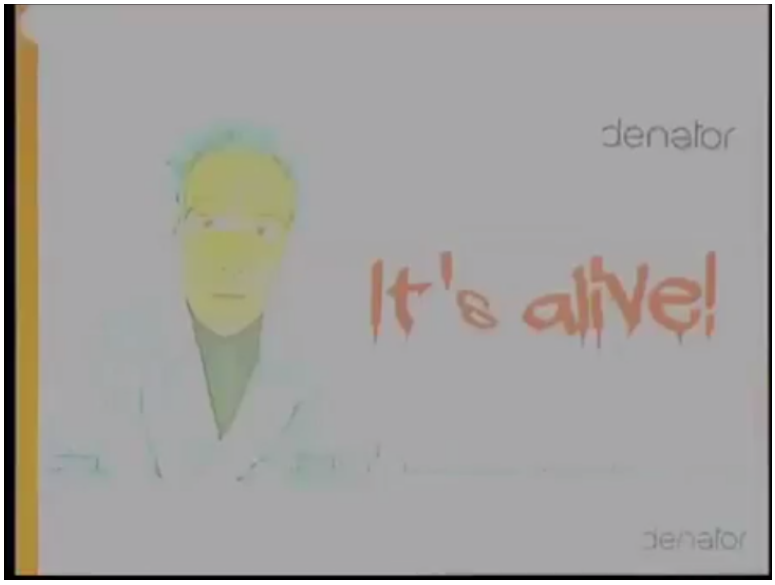


So it is alive this is sort of a central theme of what we do and it is in this case is of course the sample.

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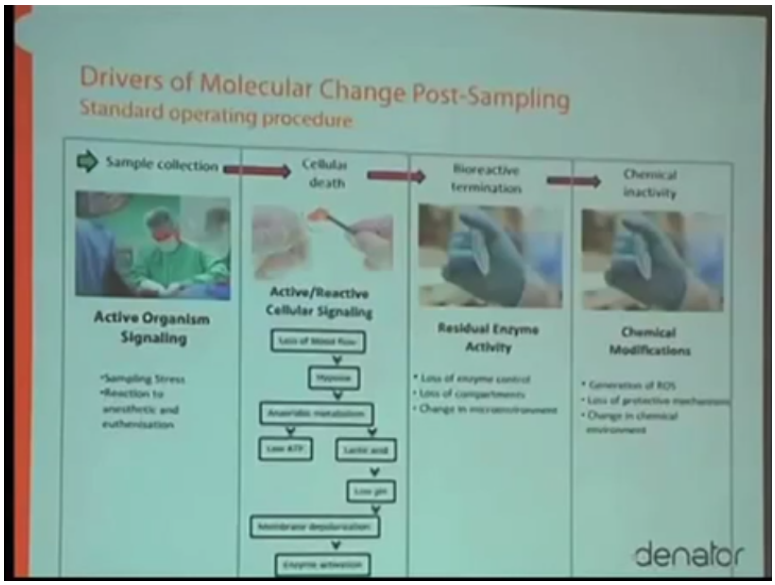


It is very important to remember that once you take the sample, the cells in the sample are still very much alive and they do not know that they have been removed and they are doomed, they are trying to survive because what happens when you remove a sample, primarily speaking about a solid sample like a tumor sample or biopsy sample or something like that.

So first of all, you have a loss of blood flow and it is the blood that transports oxygen and nutrients onto the tissue, so very quickly you will have loss of oxygen, hypoxic situation that will cause the cell to switch to an anaerobic metabolism so instead of using oxygen as the sort

of end product in generating ATP and energy, they will start to sort of generate lactic acid instead.

And lactic acid accumulation will lead to lower pH and this is also leads to so this is a much poorer using oxygen and doing glycolysis in the whole lactic and respiratory cycle is much more efficient when it comes to generate the energy and ATP. So very quickly we have a very low ATP environment in the cells.

So all of these are powerful signals that the cell will sends and try to adopt too and it does so by changing phosphorylations digesting proteins and so on and of course in the longer scale it will also change levels of transcription and translation and so on, but in the very first phases it will start by the primarily phosphorylations and also lipids so sort of degrade them. So this is one area from the moment the sample is taken.

Or really from the moment that if it is an animal the moment the animal dies that is when the blood flow stops that is really when the clock starts ticking and these changes starts to occur. So that is one sort of danger zone until you either freeze the sample or heat stabilized as we are doing. The second sort of danger zone is when you start to extract your sample. Somewhere down the line you generally homogenize your sample and extract it.

And even with inhibitors, even with 8 molar urea, even with high levels of organic solvents, there are enzymatic activity and they will change the sample composition. There is some examples of that later and so that is another area where you can have changes.

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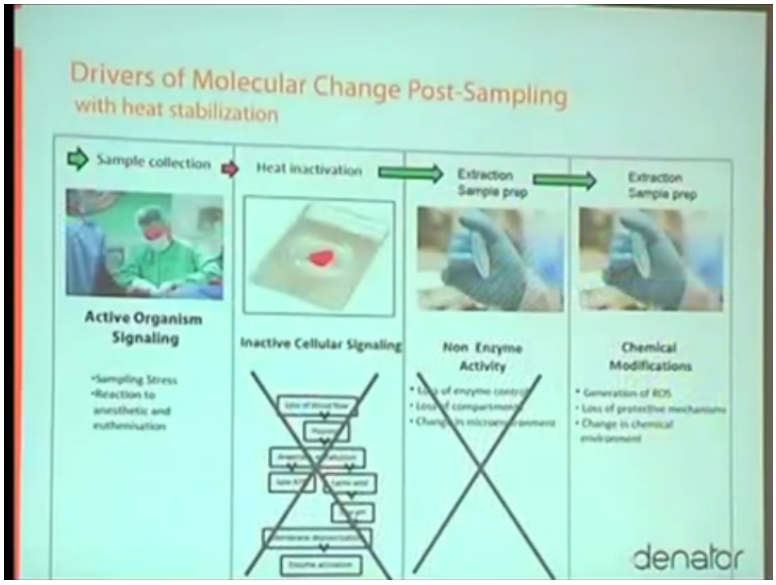


And so we are going to do we want to abolish, remove enzymatic activity because the majority of these changes are due to enzymatic activity. So by removing enzymatic activity preferably as soon as possible after sample collection we stop this cascade or at least the changes brought on by this cascade and you also stop these changes that happen during or after homogenization and extraction.

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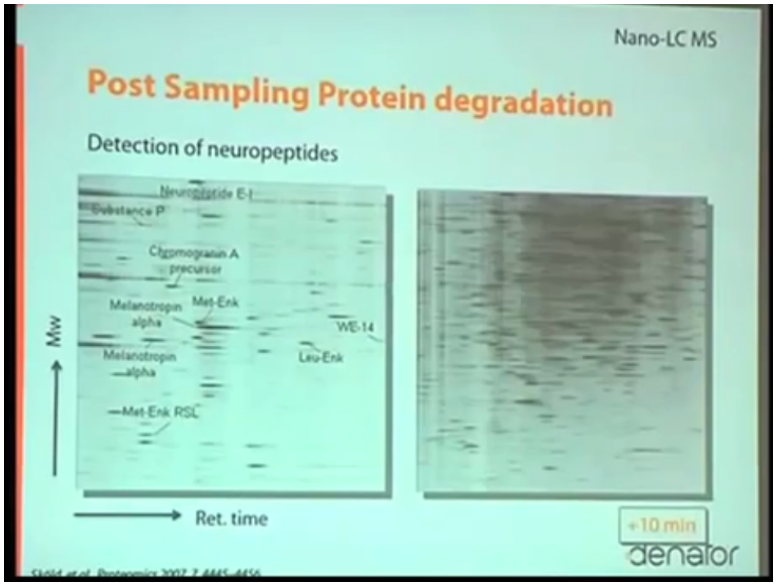


So first there are some examples or you know how does it look? How do I detect post sampling changes in the sample? And then I want to present the stabilizer as such and then there is number of examples where we do comparison between using the stabilizer and using state-of-the-art inhibitors and in fact with freezing. So what we see here is mass spectrometry, is an LC on this axis.

And there is a mass spectrometry, now it is on business so LC-MS analysis and we are detecting peptides. So this is a mice brain extract, we used a molecular weight cut off filter and that will remove everything which is larger than 10 kilodaltons and we are looking at small peptides and the idea here is that that is where we find in this case neuropeptides. So left image has been down from heat stabilized samples.

And we see that the majority of the peaks here have been identified is being sort of no neuropeptides as we expect to find there. A non-stabilized sample with only 10 minutes' postmortem and this 10 minutes here does not really mean much but the majority of what we see here is loads and loads of peptides, but when we go and identify this, the majority of these are degradation fragments of albumin and other sort of housekeeping large high abundance proteins.

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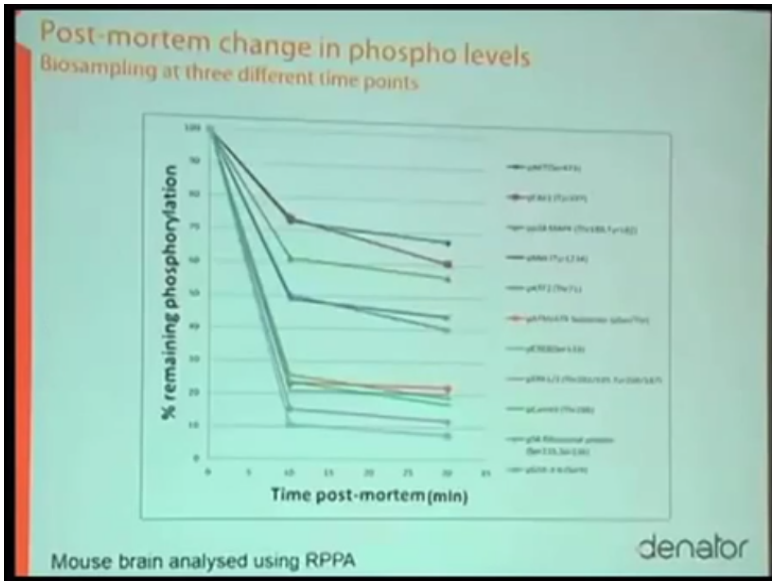


That was for peptides so peptides are one of the class of molecules that we see change rapidly post sampling. The other one is protein phosphorylations. So what we have here is a reverse phase protein array experiment same as your Western Blot experiment. It is a time course experiment so we have mice brain. We remove them as fast as possible and then freeze them rapidly.

We killed the mice, we wait 10 minutes, then we removed the brain or we killed the mice, wait 30 minutes at room temperature then collect the brain and freeze it and then we do this and analyze, extract them in that was in 6 molar urea buffer and we look at the how the levels

of these 11 common phosphorylation change and all of these some of them decrease quite rapidly, some not so much.

The majority of decrease seems to happen during the first time period compared to the second time period. This is probably to do with the disappearance of the ATP that happens quite rapidly in brain tissue.

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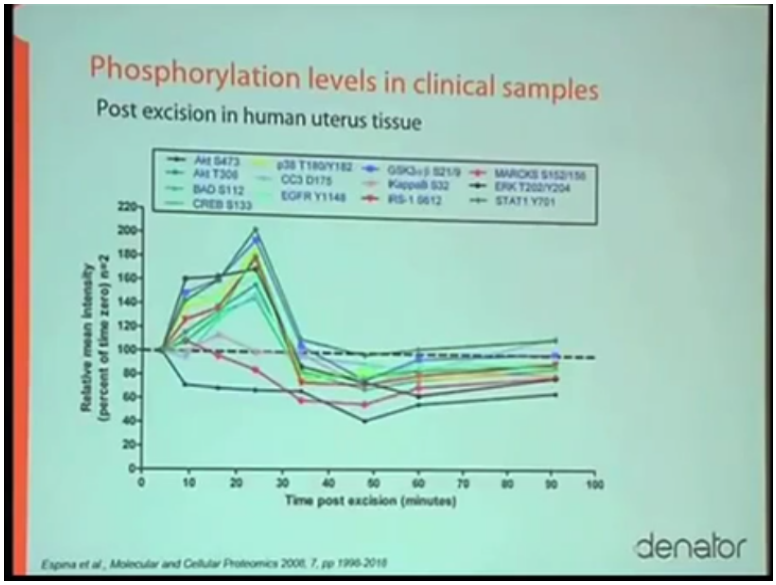


However, it is interesting to know this is the study that we did. This is another study which is published that we have nothing to do with. They are using a different tissue so this is uterus tissue. Some of the phosphorylations are similar. It is a slightly different time scale, slightly longer, there zero is not quite at zero.

There is actually little longer here, but anyway what is interesting here is in this system, the majority of the phosphorylation is actually increase in the initial period and then they decrease. So we do not really know why that is and this is completely different tissue and so it is important to realize that some phosphorylations will go up, some phosphorylations will go down post sampling.

And this may change depending on tissue and also there will be a lot of phosphorylations that we will see later on are stable, they are not they does not change at all, but quite a few will. So again unless you know how your phosphorylations that you are interested in study, how they change, it is important to do time-course studies to understand or my phosphorylation that I am interested in affected by post sampling changes.

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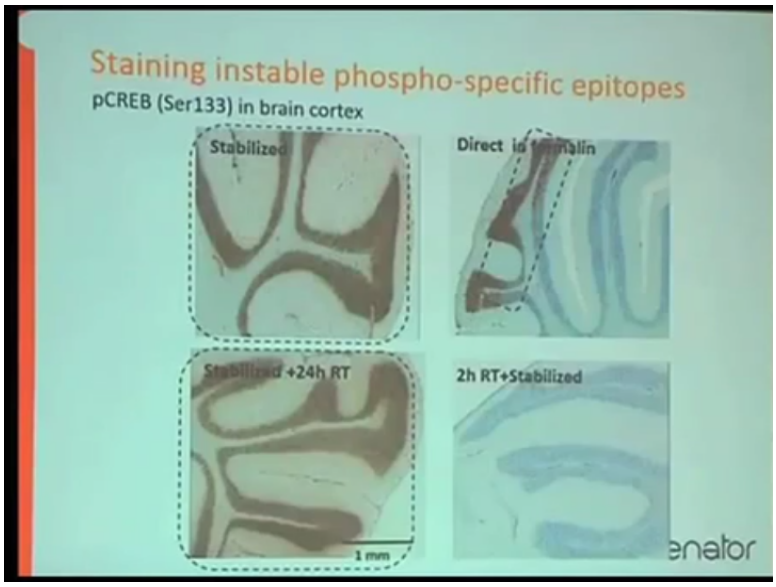


This is another example this is where more specific we are looking in this and this is brain tissue, protein called CREB, specific phosphorylation, so this is immunohistochemistry. A whole brain is either stabilized in the instrument or and then placed into formalin or directly placed into formalin and then we are visualizing this phosphorylation on CREB and this is the one that goes and then we cut the sections.

So that we have the full gradient, this is the coronal section that is sort of goes into the, this is cerebellum, so the hind brain and we see here a clear gradient is at the outside. So the formalin penetrates from here and end this way and we see that where the formalin penetrates rapidly in the outer layer we have a good signal; however, as it goes in we start to have a fainter signal and in here there is basically no signal at all.

Because formalin penetration with depending on tissue is roughly between 1 and 2 millimeters per hour, so these generally takes quite a long time to stabilize and that what we see here. During that time, there is still enzymatic activity, which can change the phosphorylations. So this is where it has been stabilized with the enzymes activities removed. We see this phosphorylation sort of all the way from the surface and inwards.

And if we stabilize it and leave it at room temperature 24 hours and then put it into formalin, will still see very similar staining; however, if we have an ordinary brain only 2 hours at room temperature and then stabilize it, we do not see anything. There is just a tiny, tiny bit on

the very edge here where oxygen penetrates from the atmosphere. So another group of molecules that change rapidly are free fatty acids.

So liquids are generally stored as triglycerols. Each triglycerols has 3 fatty acids and in an effort to recover some little energy these fatty acids are released in the post sampling case. So in this case again time series experiment in this case is to homogenate and this is mainly to illustrate that in the homogenate, there is an active enzymatic process ongoing that releases these free fatty acids.

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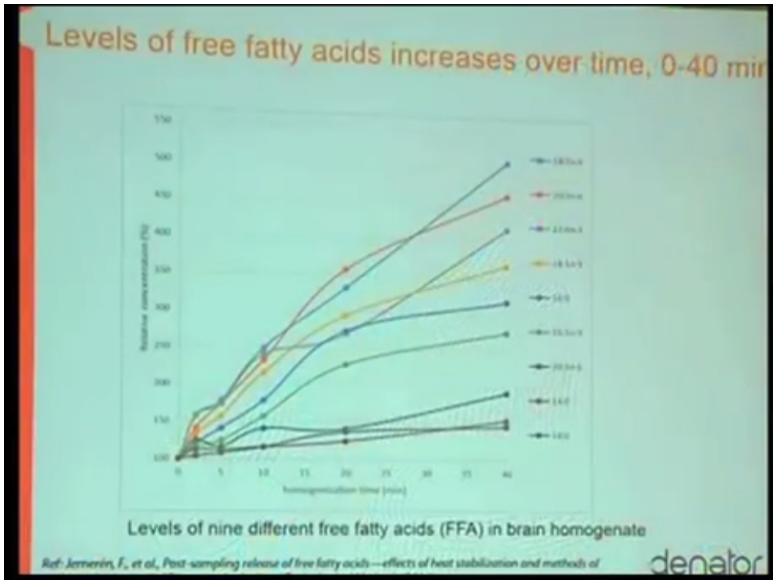


And again we see some are not specifically affected just a very modest increase where some increase very rapidly again emphasizes unless you know how your specific lipids or molecules are changing you do not know they may be changing a little or they may be changing by several 100%. So it is important to investigate these parameters. So that is the few examples of sort of what you can see if you do time course experiments.

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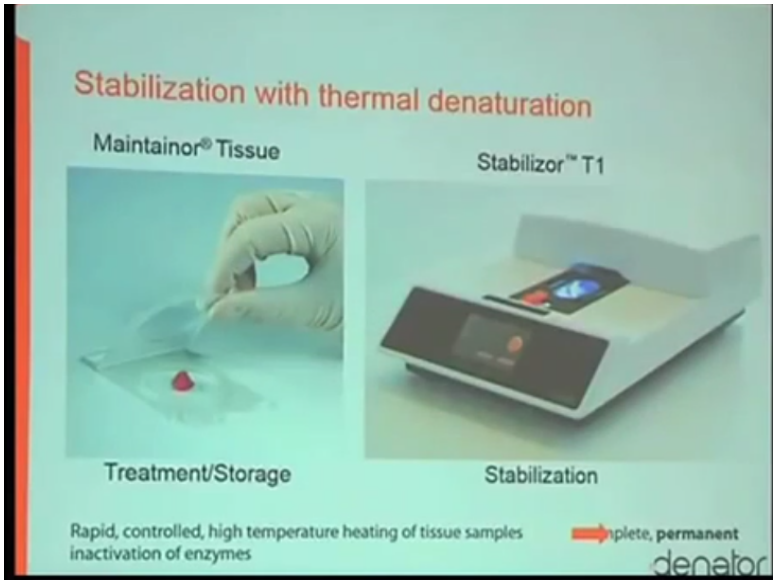


So this is the problem that we are addressing and to do this we stabilize through thermal denaturation. So we use heat, a heat based technology and in the next slide I will show you exactly how it is done and what happens on a molecular level. So we have the instrument itself, there is a cartridge, this is the cartridge for solid tissue, there is a cartridge for liquid samples. There is also a cartridge for DBS, dried blood spots.

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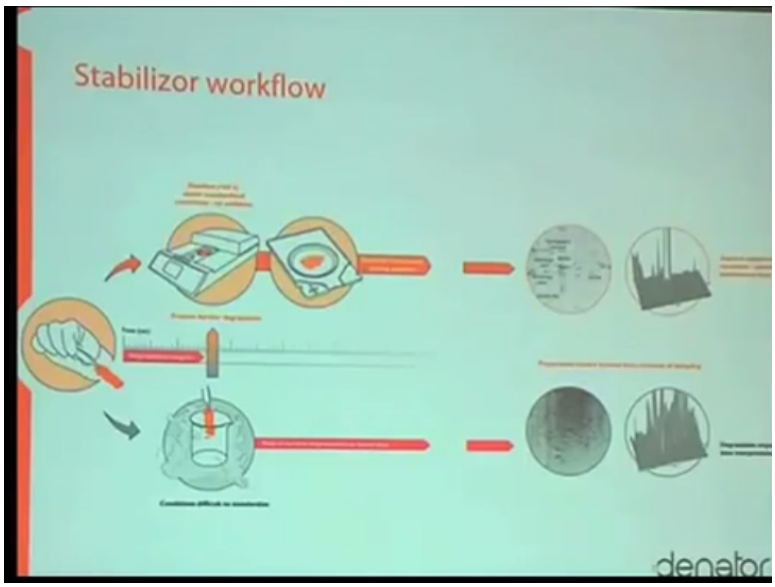


So this system then either be used very closely to the sample source and having the instrument sort of introducing directly into the instrument. It is also possible to use with frozen samples so that you freeze the sample rapidly after excision, transfer it frozen, and when you have it sort of with instrument it is brought frozen into the instrument and directly from a frozen state heated.

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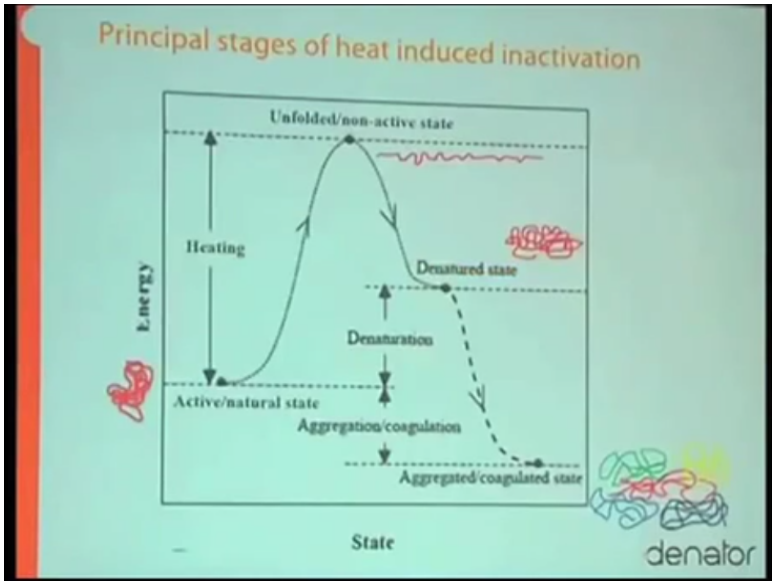


So what happens in the tissue itself or in the sample itself is that so enzymes are proteins and they have a 3-D fold and this 3-D fold is very detrimental, it is very important for the activity. So what we do here, we take the sample, we apply heat. So we add energy, so we heat the sample to roughly 90 degrees centigrade and that causes to proteins and the enzymes to unfold into a linear non-active state.

And then the sample is taken out of the instrument, it cools down and instead of hydrophobic collapse we will get another 3-D fold, which is generally denatured non-active state and this will further as this is done sort of in the tissue, there is lots and lots of proteins in there, we will get an aggregated coagulate state of the proteins locking in this inactive new form of the that is how it works.

So it is a very basic works for more or less all proteins. The one exception that we know of are RNases. RNA degrading enzymes are very short and they are held together by 2 I think it is 2 disulfide bonds. So they denature by the disulfide bonds, stay intact and when they cool down, it will fold back into a native configuration. That is the one sort of class of exception that we have found so far.

But the majority of protein affecting enzymes seems to find sort of a new denatured stable state after they have been heated.

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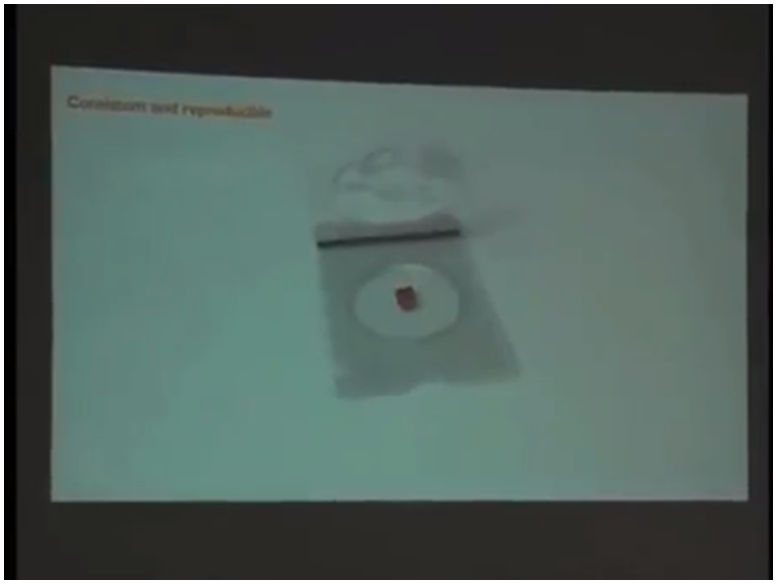

So this is say shortly the movie of how a sample is stabilized in the instrument so that is the card and sample goes in the card, it is sealed.

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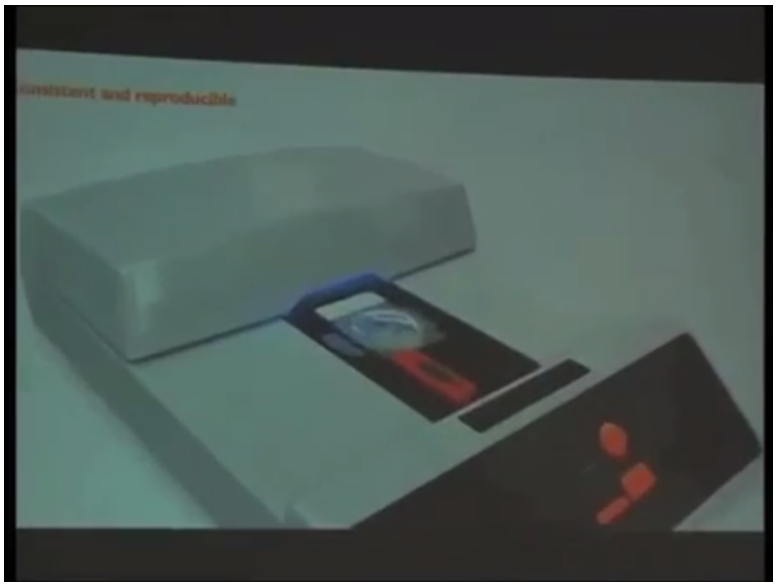

And this you see the upper part here is sort of a dome shaped so the first thing that happens is in the instrument is that air is evacuated as to make sure that the sample is the highest point on the card.

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As it goes in, the height is measured and based on the height and whether or not the sample is fresh or frozen, the time it takes to stabilize will be calculated. The sample is heated it returns out and it is done.

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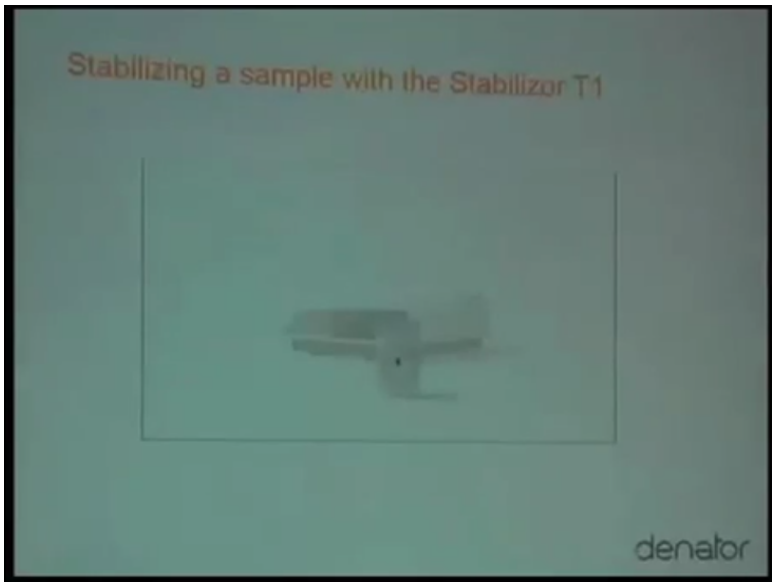


And then the sample is ready for either if you want to extract it right away or if you want to store it for a few hours can be fine at room temperature. If you want to store it for longer time, we recommend that you freeze it preferably with -80. So for a small like a 2, 3 millimeter sample like a biopsy, like a small piece of sample, it is roughly 20 to 25 seconds. If you have a full size mice brain, 6 something like that millimeter, it is roughly 1 minute.

The maximum size, height is 7 millimeters. So it is a small sample you cannot put a whole human in there or anything but it is a fair amount of sample.

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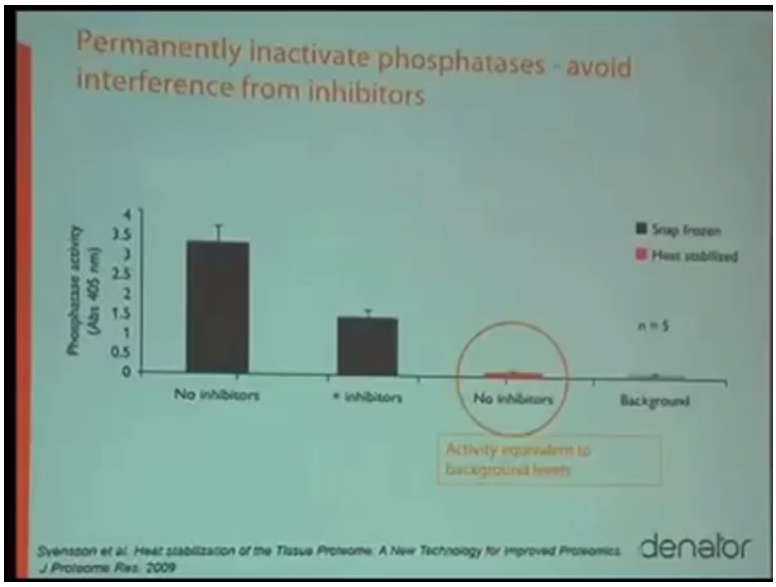


So what happens to enzymatic activity? We have here is an experiment to visualize phosphatase activity. So phosphatases removes phosphorylations and we are using a substrate called P and PP that when the P phosphatase removed, it turns yellow, so what you do is you do homogenate of your sample in this case is mice brain in PBS, you add the substrate, you incubate for roughly half an hour at 37 degrees and then you measure the yellow color.

And that is what we see here, so without inhibitors, we see lots of yellow color, lots of phosphatase activity. This is what we expect to find in PBS in a sample. If we add inhibitors, this is the (()) (18:18) inhibitors from Ross. We see there is a market reduction, but nonetheless roughly 50% of the activity remains in the sample.

If you compare that to the activity level that we detect in the heat stabilized samples, we see that that level is really on the background level. So very low to background level activity detected after heat stabilized seen on these samples.

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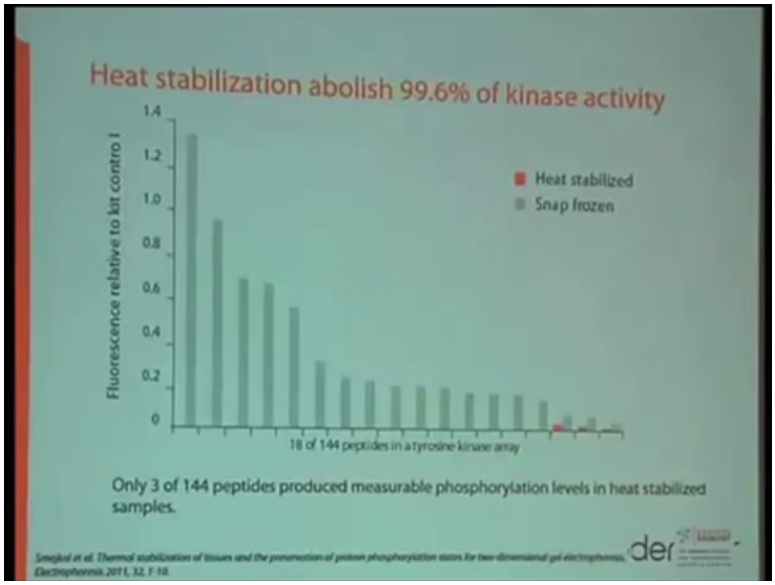


So the other part of the equation is the kinases. The kinases adds phosphorylations to proteins and so this is an array experiment, you have an array with 144 peptides, each peptide contains a phosphorylation site, a tyrosine phosphorylation site. You incubate your extracted samples with this array and if there are kinases in this mixture, they will phosphorylate these motifs.

And then you use tyrosine sensitive specific antibody to detect the level of phosphorylations on these peptides. So here are the 18 most abundant most phosphorylated motifs and again we see that in the snap frozen, we see quite high levels and in the heat stabilized, there is just 3 that shows any type of activity and there is another 120 or so peptides and if all these are summed together.

And we do a percentage comparison we find that 99.6% of the kinase activity has been abolished in the heat stabilized samples.

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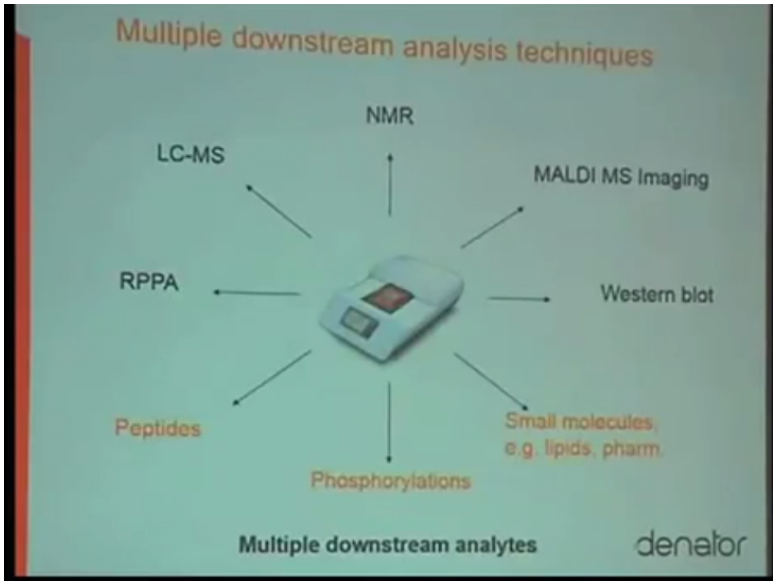


So those were the examples of what happens to the enzymatic activity that will so now let us get over more into the technology and the comparison between using heat stabilization and using standard work flows. So it is an upstream technology and that means that it comes very early close to the sample source.

So in afterwards you can do most ordinary proteomic techniques from Western blot 2-D gels over through LC-MS based technologies, phospho-shotgun and so on. We primarily focus on peptides and proteins phosphorylation that is what we see that those are the class of molecules that were the changes most abundant following post sampling.

There are examples of proteins, which are also degraded, but generally proteins are not degraded as rapidly as phosphorylation and peptides. We have also started to move into their some example that into the lipids, small molecules like pharmaceuticals and so on.

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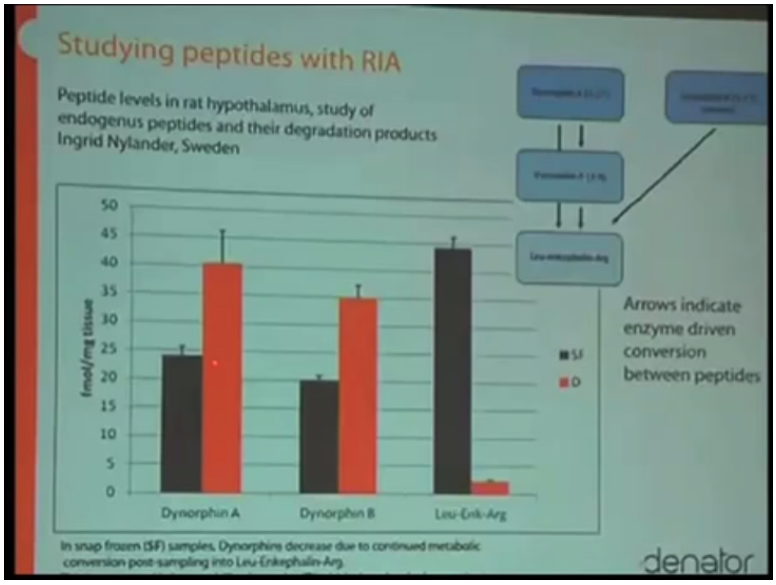


Here is like sample of a 2 peptides Dynorphin A and Dynorphin B and they are enzymatically converted in the body to this short reversion Leu-Enkephalin-Arginine and when we measure these using a RIA radioimmunoassay system. We find that the 2 sort of parent peptide, the Dynorphin A and B we see higher levels in the heat stabilized as the D compared to the snap frozen.

However, when we look at the sort of cleavage product, we see much higher levels in the snap frozen sample. It is important to illustrate these samples, they are taken, they are rapidly frozen from a frozen state, they are dropped into boiling 1 molar acetic acid, boiled for 5 minutes and then they extracted and then the extract is again heated to 95 degrees for 1 minute.

That is the sort of snap frozen as a standard workflow that they use for the samples. The only difference with the heat stabilized are that directly after sampling, they are introduced into the stabilizer snap frozen and then they are extracted and boiled and so on the same way as the other so despite these efforts boiling in this 1 molar acetic acid, very acidic solution, we still have this sort of continued degradation.

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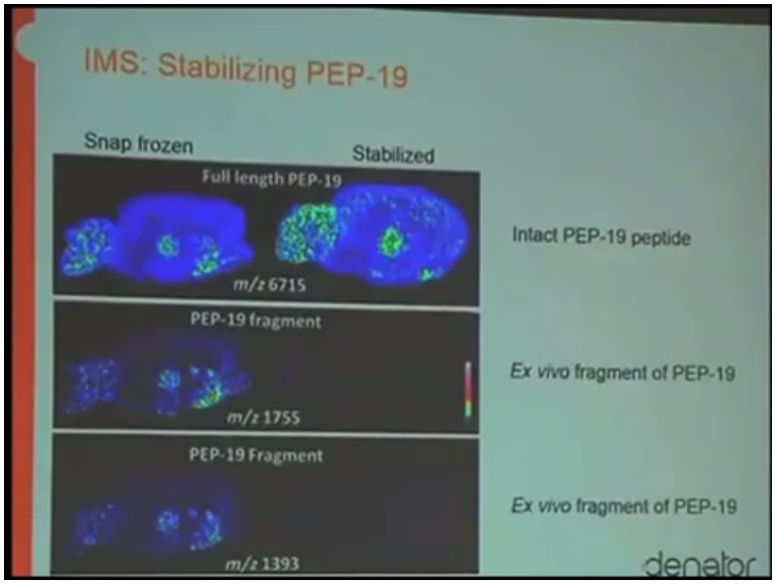


Another peptide example this time, this is the technology called MALDI imaging. You have a thin section of a mice brain in this case so we have here that is the cerebellum, the hind brain, you have the cortex appear and we are looking at the peptide called PEP-19. It is rather large 6715 is the mass.

And so in the full length peptide we see that the distribution pattern in this tissue is relatively equivalent, slightly high in the stabilized tissue, but nonetheless more or less very similar. What is interesting is when we go down and look at the 2 other masses, which have been identified as being degradation fragments from this full length peptide, we see that they are only found in the snap frozen where we have enzymatic activity.

And they have roughly equivalent distribution as you would expect from the degradation fragment.

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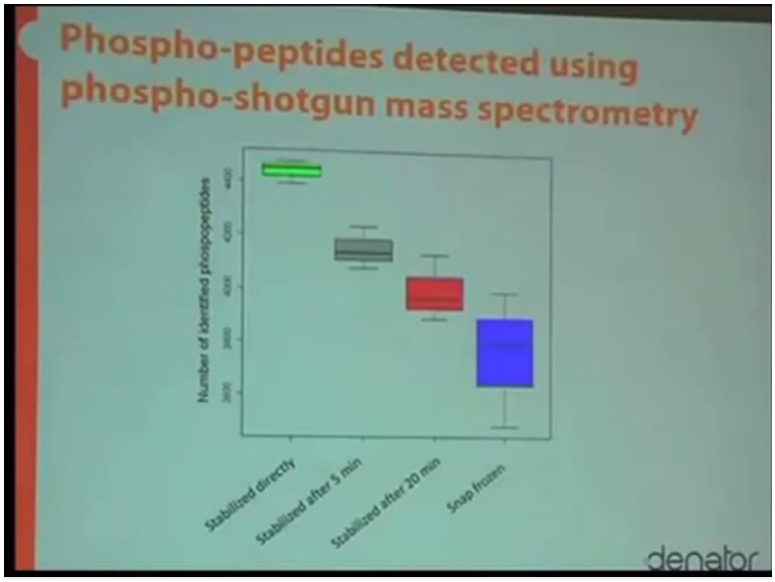


So moving on from the peptides to protein phosphorylations. This is a phopho-shotgun so bottom-up approach, the sample is digested with trypsin, extracted 8 molar urea, digested with trypsin and then titanium dioxide is used to enrich phosphorylated peptides. In the heat stabilized samples that have been into the stabilizer, we find roughly 4400 so this is 3 replicates.

If we kill the animal, wait 5 minutes then heat stabilized, we see a bit of a drop 4200 roughly. If you wait 20 minutes and then heat stabilized, we see further drop to roughly 4000 and we compare that to the snap frozen. So the snap frozen in this case, the brain is removed, put into liquid nitrogen, homogenized in liquid nitrogen and then 8 molar urea with inhibitors is added to the sample.

And then you do the extraction and so on and we see despite the measures 8 molar urea, keeping it cool during extraction and so on, we still see a further reduction to roughly 3700. So this again since it is sort of impossible to know how many phosphorylations are there in the tissue originally, a tool that we generally use is this time-course studies.

By following what happens in the time course post sampling to get sort of an idea how does my molecules of interest change. So on average the majority seems to be that there is a loss of phosphorylations. So anything which is less is probably with time it sort of changes from it is (()) (26:33) and this change is a removal of phosphorylations.

So this sort of tells us that this sample would fewer phosphorylation is longer from the original in vivo situation compared to what we have over here when we see that it sort of the original maybe one could have taken 5000 phosphorylations for example, that is impossible to know.

So this is a pre-study, this was unfortunately not published. He then went on to use the heat stabilizer together with other techniques to study phosphorylations in roughly 14 different rat organs and discovered roughly 36,000 phosphorylations in these 14 tissues.

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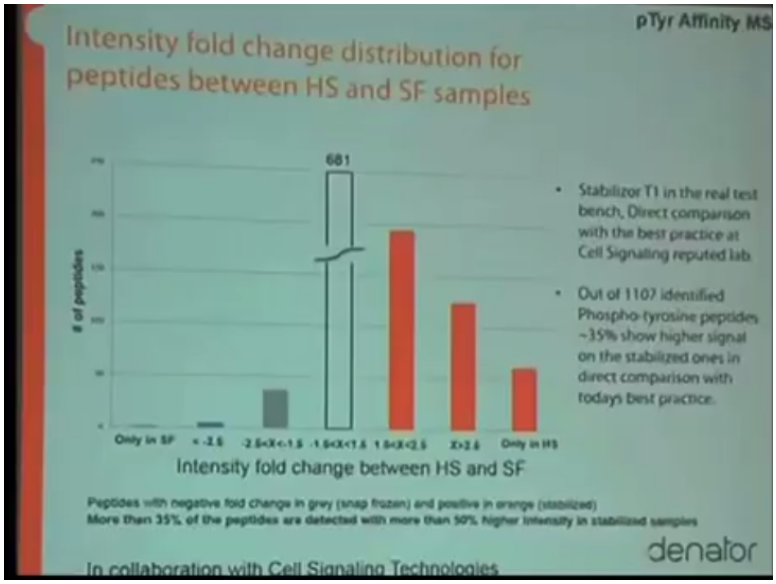


That data is published. This is data from other experiment that we did in collaboration with the company in the US called Cell Signaling Technologies. They primarily produce antibodies and what we see here on this axis is the ratio between the intensity for a peptide in the heat stabilized sample divided by the intensity in the snap frozen sample.

Again snap frozen rapidly after excision homogenized frozen, extract in 8 molar urea with inhibitors that is the standard (()) (28:02) that were comparing with, so anything here the orange bars will have at least 50% higher intensity in the heat stabilized samples compared to the snap frozen samples and there is also a few that decreases. So in this case that would have had higher level of phosphorylations in these snap frozen samples.

So these sort of added phosphorylations during the extraction and postmortem. These would have lost phosphorylations during the extraction. So roughly one-third of the phosphorylations, the tyrosine phosphorylations that we detected in this study have changes

at least 50% from what we think it compared between these 2 experiments so it is also important to note that roughly two-thirds did not change, so lot of phosphorylations does not change.

So if you are lucky and you are looking at 1 or 2 phosphorylations, you may be looking at the stable phosphorylations and that is good for you, but you do not know until you done the experiment. The time-course experiment will tell you whether or not your phosphorylation is stable or not.

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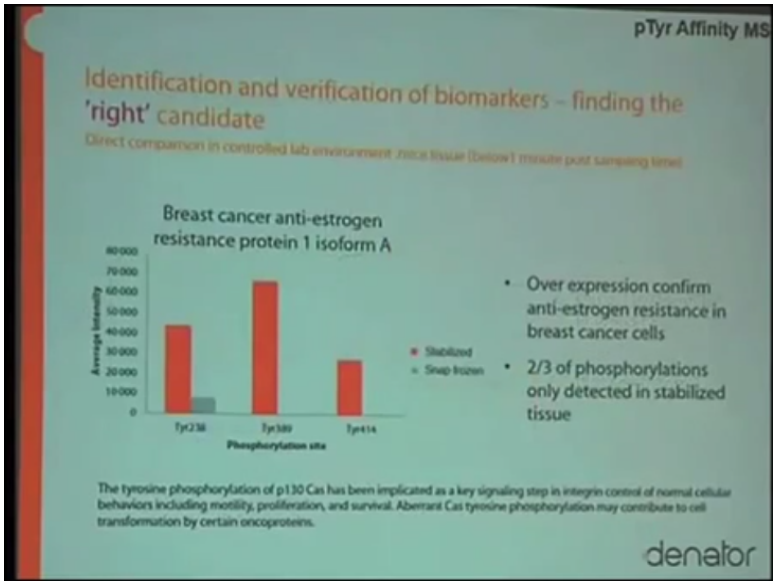


So if we dive down to some specific proteins, we see here this is the breast cancer anti-estrogen resistance protein. It has some sort of connection to breast cancer. We find 3 phosphorylations in the heat stabilized samples where is only 1 and all only very little in the snap frozen sample.

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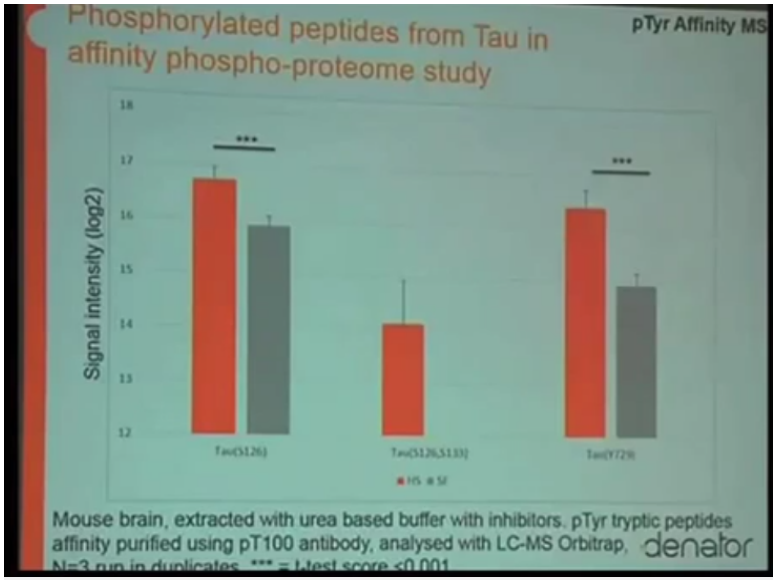


Another sample is tau, again happens to be 3 phosphorylations in the heat stabilized samples, 2 phosphorylations in the snap frozen sample and again we see here that the levels are highly statistically significant. That was examples for phosphorylations.

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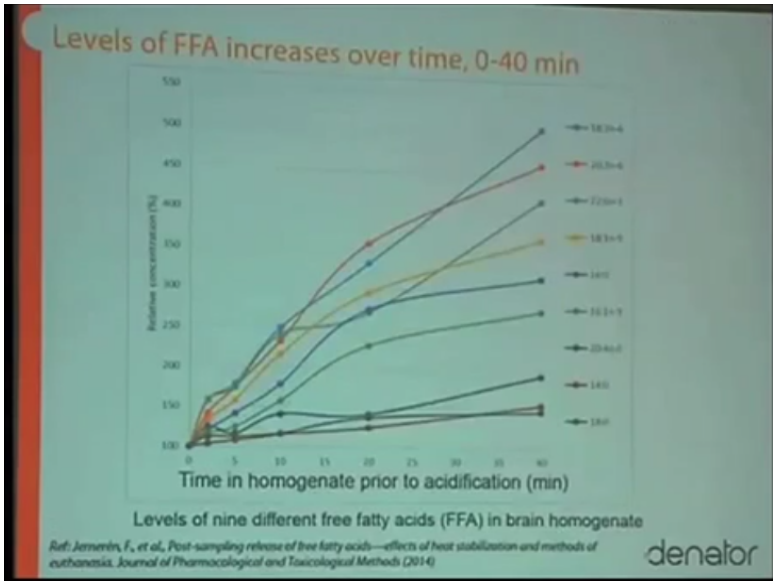


The other sample showed initially the free fatty acids. So just to time course experiment in the homogenate some increase rapidly, some not so much.

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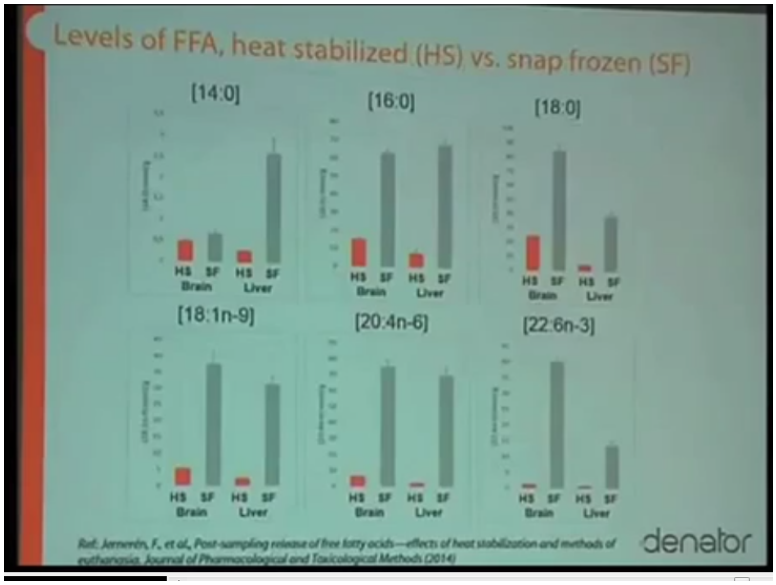


When we do the direct comparison between the heat stabilized sample and the snap frozen sample, so these samples are extracted in acidified methanol. So 100% or 99.9% methanol, 0.1% TFA, so acetic methanol very organic and very denaturing solution. Nonetheless, we see large differences. In some tissue again a difference in tissues so the 14 here we see almost no difference in the brain, large difference in the liver.

I do not know why, but that is just the wait is, in some tissue behave differently than others. So the majority of the free fatty acids show much higher levels in the snap frozen and we were pretty positive that this is due to this ongoing enzymatic change in the sample.

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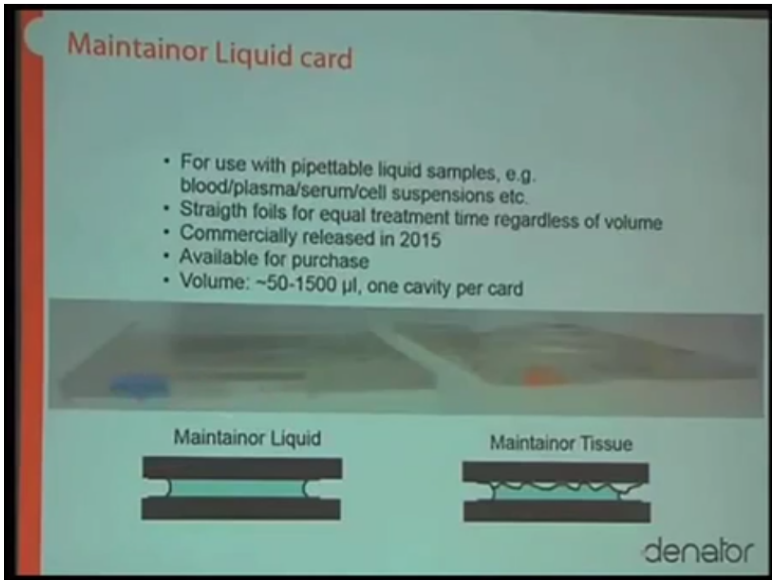


So these were samples from solid samples, is also possible to do more liquid samples like cell cultures, plasma, blood, CSF something like that so we have a card, we call it maintainor

liquid and so the tissue had a dome-shaped to be able to sort of fit around a physical sample. The liquid card consists of 2 membranes and when you add a drop of liquid here, the liquid will be between what sort of stand between the plastic to the Teflon membranes.

And regardless of volume from roughly 50 microliters up to 1.5 milliliters it will have the same height and the height is really what determines the time it takes to heat something.

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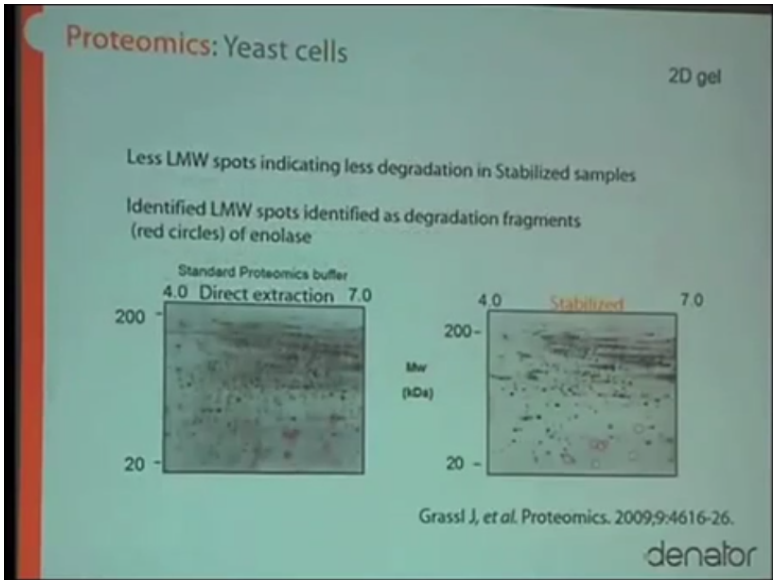


So I also have one example, we just recently released these, but we have 1 example where we will use what we sort of stabilized liquid sample. In this case, it is a yeast live yeast cells, so they are grown, they are harvested and washed and then they are suspended in PBS and then the heat stabilized and then we did the extraction in 8 molar urea and separated them using 2-D gels.

And in this case we focus on these, there is 7 very prominent spots in the standard proteomics buffer extraction which is a direct extraction that are pretty absent in the heat stabilized samples and when these were identified, it turned out all 7 spots were from a protein called enolase. Enolase is presumed to be the theoretical sequence. It is pretty large proteins or pretty high up in the gel that is another indicative.

If you start identifying proteins down here which have a theoretical molar weight up here, then you pretty much know that what you identified is a fragment. So it is also noteworthy that so the background there is sort of hazy background to this gel that we do not find here

because all proteins does not degrade as nicely as this one, but maybe say that 1 to 5% is degraded that does not change the pattern very much.

But it does show up in a sort of background smear that we do not really see here, which is sort of gives you cleaner.

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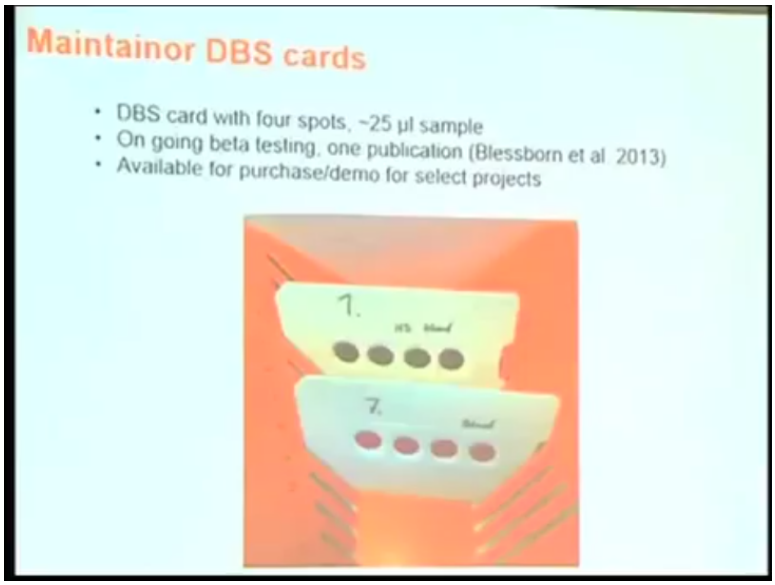


That was the example for the liquids card. We also have another liquids sort of card, dried blood spots.

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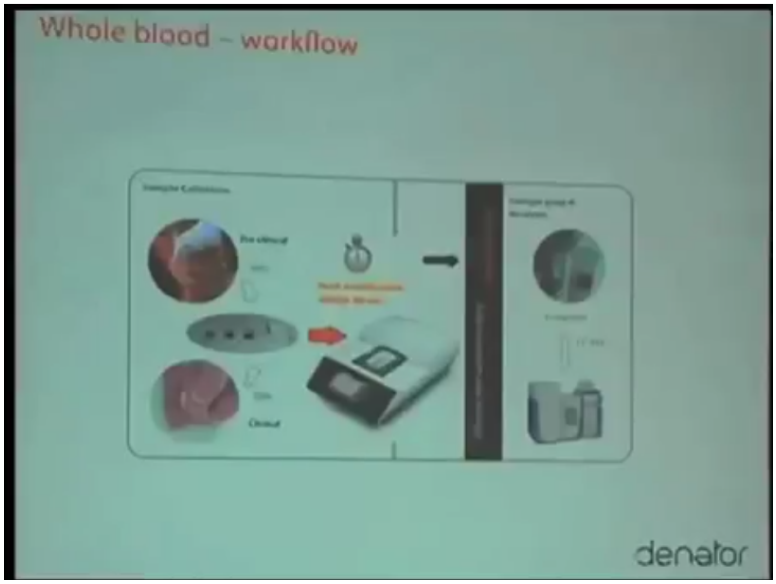


And the dried blood spots, it is a technology where you put drops of blood on a filter paper and then you let this filter paper dry and once it is dry, it is stable and you can send it through ordinary post and you can store it at room temperature and it is a generally very good system

for collecting samples like in rural areas and if you have to have a lots of sample and do not want to invest lot of money in freezers and so on which can be very, very expensive.

However, the problem here is that the from the moment that you put a drop of blood on the filter paper card until it is dry depending on humidity roughly 1 to 2 hours and during this time, you have a liquid sample at room temperature with enzymatic activity that can degrade your sample. So we do is that put the drops on the port on the instrument, heat and activate the enzymes, take it out, let it dry and then you go downstream analyze.

We have analyzed together with a group in Thailand, a number of pharmaceutical compounds. They want to do DMP case studies you know how quickly there is a molecule of pharmaceutical as you take how quickly it is digested in the body, very many pharmaceutical compounds are differently digested between different people.

And then it is important to know if you are a high digester, you need to have a higher dose in order for it to have the same sort of benefit, same efficacy on the dry because otherwise you will have a very low level of active drug in your system and you may not have and vice versa if you are a very low digester, you may get very high, you get toxic levels when that is not good either.

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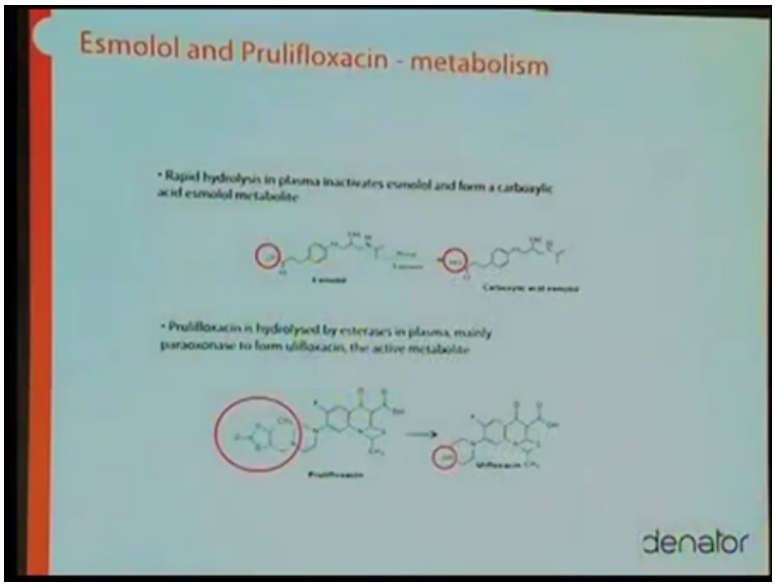


So there is number of examples in the paper, here are some of the examples. So this drug esmolol, it is given as a pro-drug and esterase in the blood converts this into the active form and this is another form, this is given, this is what is converted to.

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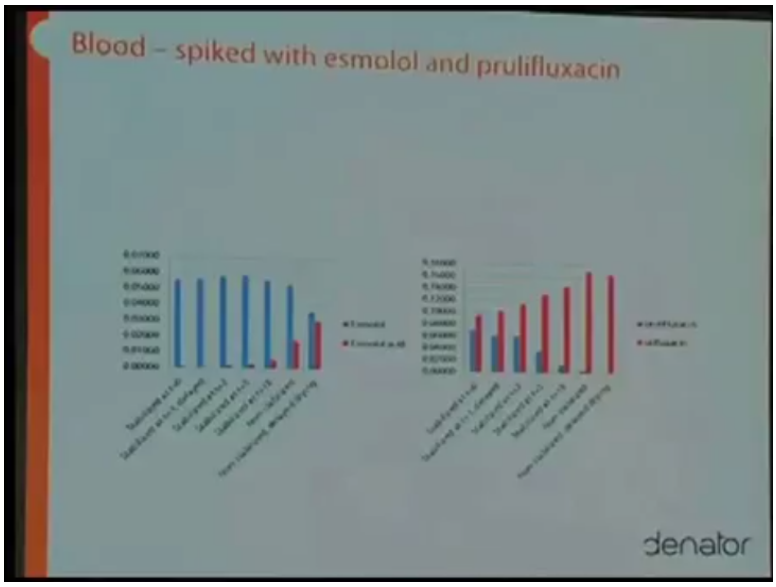


Again a time-course study to see what happens post sampling or in this case what happens after it is placed on the filter paper. So the blood is collected, it is spiked, so we add these 2 compounds as spiking compounds, pure synthesized compounds into the blood and then we either put a drop on, stabilize directly, wait 1 minute, 2 minutes, 5 minutes, 10 minutes and we see here the blue that is the mother the 1 we spiked with.

The levels stays roughly similar the degradation fragment, the active molecule slowly increases with time and in the non-stabilized case where we let it just dry at room temperature the way this is to suppose to work we see quite high levels of the degradation product. If we delay the drying, we put it in the plastic bag for 1 hour before we remove it and let it dry, we see that there is sort of conversion continues.

So the conversion continues as long as it wet and as long as there is enzymatic activity and when we remove the enzymatic activity, we see very low levels of the other degradation product. This example is even more striking, same type of experiment and here it really, really shows if you wait 1 minute, 2 minutes, 5 minutes, 10 minutes, the mother compound is rapidly degraded.

And here already at a time 0 more than half of the compound that we added has been converted to the degradation fragment and when we look at the non-stabilized, the standard way working with this we see at almost all have been converted into this active form.

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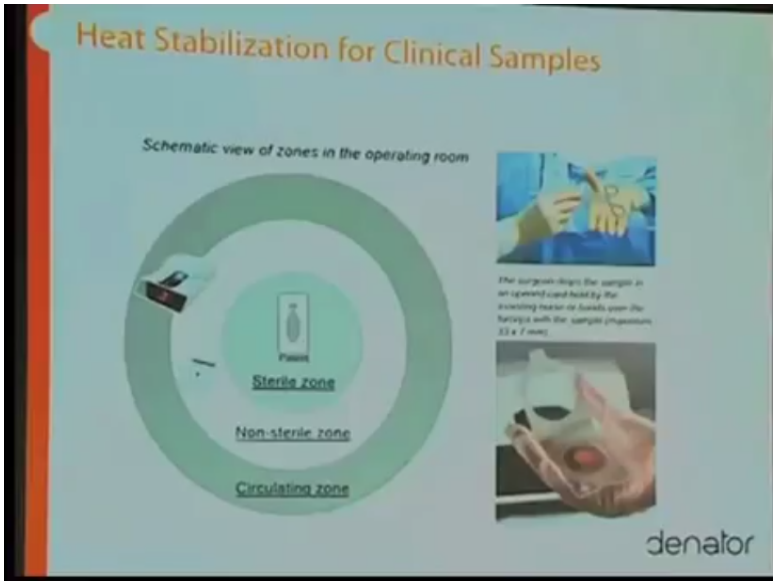


That was the examples, some words abusing the heat stabilized in a clinical setting. The majority of work that has been done with is done in university setting, laboratory animals primarily. It is being used in some hospital setting for clinical research. It is not in routine use but people are interested in changing the way treatment are being done in a few cases primarily involving biopsy sampling.

So in this case, the patient in the operating room is surrounded by a sterile zone and we have the instrument sort of on the border between the non-sterile zone and the card itself sort of goes in and the surgeon either drops the piece of tissue in the card or hands the forceps with the sample and it is placed into the card that is generally how it is used.

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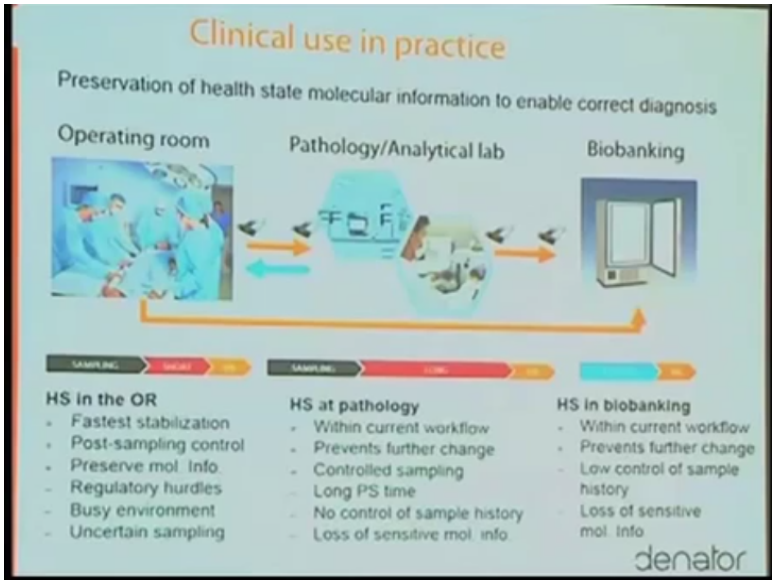

So the stabilized can be integrated sort of in the workflow, the samples generally emanates from some sort of operating room. They generally go through pathology and analysis lab and sometimes part of samples end up in a biobank. So the samples can either they stabilized, they can either be integrated right where the samples are taken. Then sort of stabilize for a short post sampling time as possible.

It is also possible to integrate it at the pathology lab to freeze the samples to have them frozen and then when they arrive at the pathology lab, you can do the stabilization. We have also done studies taking sort of fresh samples that have between 30 and 45 minutes post operation time and even with that time we see an improvement in a number of phosphorylations that we detected.

So it is also possible to have it here or as it arrives at the biobank or also here as the biobank sends out the samples to the customers and there is a number of pluses and negatives depending on having it in these ways.

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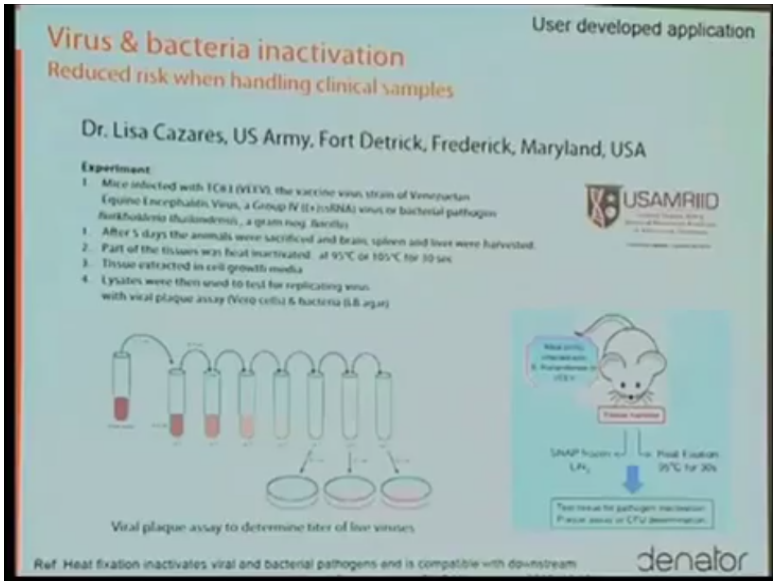


So apart from stabilizing the molecular content of the sample, there is some other potential benefits with using the system. So a couple of years ago, researcher at the US army, Lisa Cazares, she approached us at the trade show and said I think I have a killer application for your instrument, I want to test that and what you wanted to do, wants to use the heat stabilized system to inactivate bacteria.

Because she is working in BSL-3, BSL-4 high containment laboratories with very scary diseases and in order to get samples out from there, she had to put the samples into formalin for 21 days and after that it was practically impossible to do any sort of molecular characterization of these samples because everything was so tightly fixed in the sample. So she has gone on to test the system and the first publication is out.

So in this publication she shows that she infects laboratory animals with either it is a model virus strain or a bacteria. Again, she does a sort of dilution and takes the sample, homogenize and dilutes to see the number of live pathogens that she has in the various tissues.

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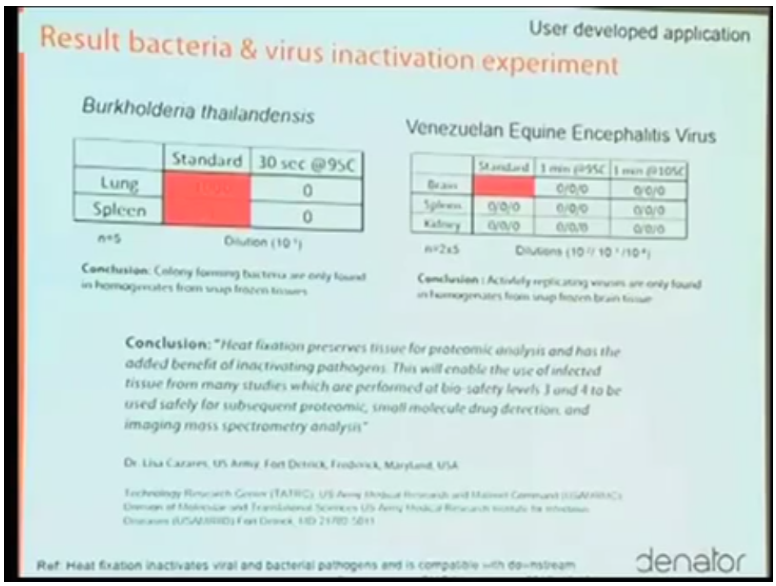


And for the bacterial case, primarily she finds it in the lung and to some extent in the spleen, in the standard non-treated animal in the non-stabilized animal. In the stabilized samples, she cannot find any live bacteria of this species and it is a same thing for this encephalitis, so it is a brain virus and she is fancy in the brain not in the spleen and kidney so that is the way it supposed to be; however, after heat stabilization she is not able to detect any live bacteria.

So she is now sort of expanding this and to see which different type of bacteria and viruses this can useful for. I generally see this as a way to reduce the unknown loads. If you have samples from say a human sample and you do not really know what type of pathogen this patient may or may not have this can be a way in addition to stabilizing the biomolecules, it can also reduce the level of pathogens in the sample.

We do not get into anything with this application, so you sort of have to verify this through your regulatory, but we definitely think this is an interesting side benefit and it is being tested right now with the CDC for tuberculosis samples and so far it looks good. So will see what happens with this application.

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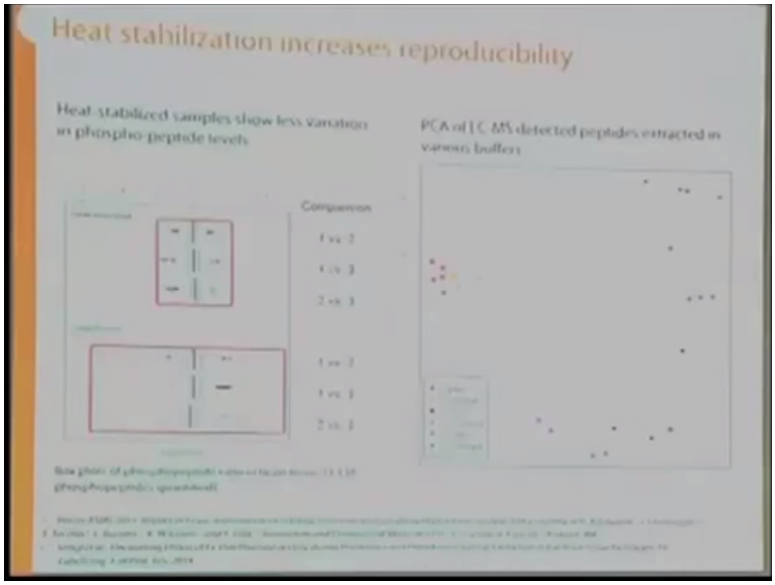


Another sort of side benefit which we see every now and then quite often in many studies is an increase in reproducibility. So what we see here is a comparison of the intensity. This is the shotgun experiment and we are comparing the intensity of sample of the peptide in sample 1 versus sample 2 and if it is more in sample 1, it would end up on this side of the zero.

And if it is more in sample 2, it ends up on this and we see that in for the heat stabilized samples, the samples are much more similar compared to the snap frozen when you see here. A better way of showing this is through using a PCA plot. So principal component analysis where all the very ability in the sample is sort of plotted onto 2 or more, in this case 2 principle components.

So every sample becomes one spot, so in this case with the dots with the same color that is one sample group, they will have the same treatment. When they cluster closely together that means that they are more similar compared to the groups over here which are more spread apart and are more different.

And so these are various extractions on peptides using different extraction buffers, all urea based and after that there are some various incubations at generally 37 degrees. This was experiment we did to show that there are enzymatic activity in 8 molar urea buffers and 2 molar urea buffers and so on but it also shows quite elegantly how the 2 heat stabilized groups cluster very closely together as well as within the groups.

Whereas all the snap frozen the regularly without the heat stabilization, they are both more spread apart the groups and also within the group you have a sort of wider spacing indicating that they are more this similar more different.

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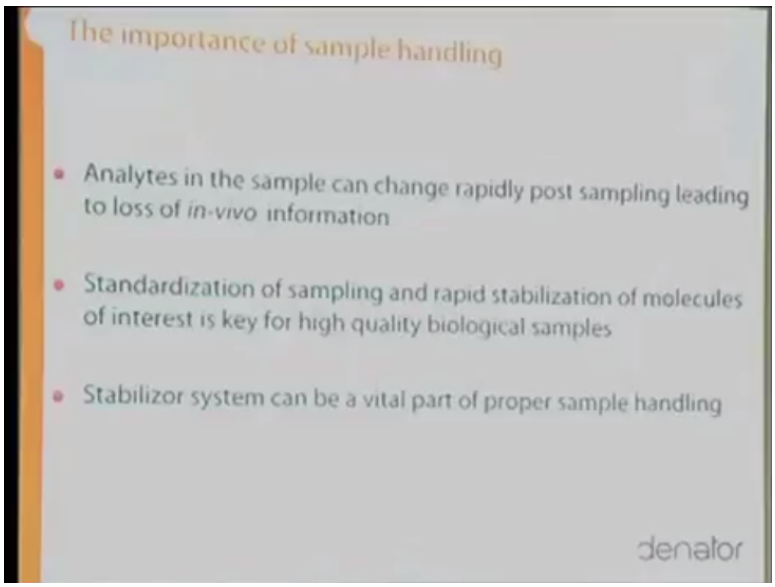


That was the last of the examples and I just want to finish off by really pointing to unless it is unclear to anyone that we think that it is very, very important that rapid changes are happening post sampling and it is key to do high quality biological studies to standardize sampling and stabilize the molecules and we think that the stabilized system can be one component of this standardization.

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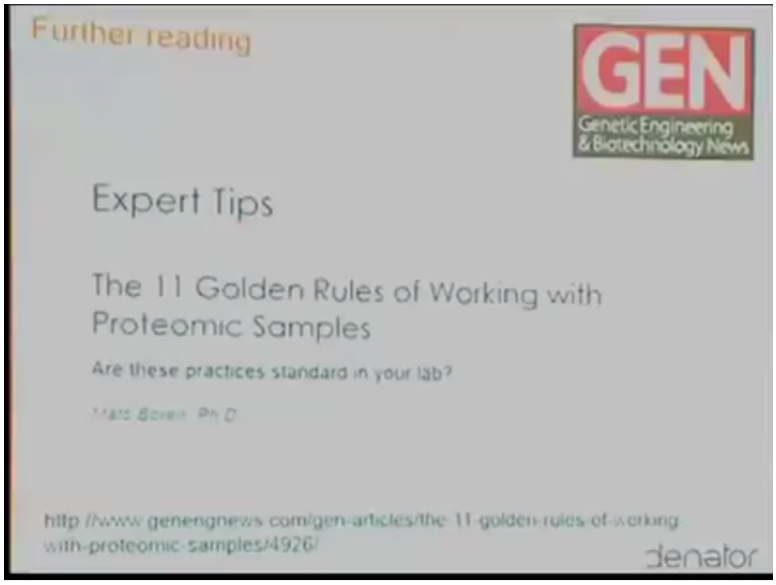


And I just want to add with the little self-promoting. This is a small article I did a few years ago. It is called the 11 golden rules of working with proteomic samples. It is freely available on the web and it is 11 sort of points goes to the sampling all the way from the collection to the inner data analysis and sort of points out what are important to consider to the entire experimental phase, so just Google the golden rules of proteomics and you will find it.