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Quantitative PCR Analysis of DNA, RNAs, and Proteins in the Same Single Cell.
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Guest:
Dr. Anders Ståhlberg is Principal Investigator at Sahlgrenska Cancer Center, at the University of Gothenburg in Sweden.

Bob Barrett: This is the podcast from *Clinical Chemistry.* I am Bob Barrett.

Single cells are the basic unit of all organisms, but most scientific investigations use a large number of cells, perhaps of different populations. Understanding cell dynamics and heterogeneity requires single cell analysis.

In the December 2012 issue of *Clinical Chemistry*, a Swedish and US team described a way to perform quantitative PCR analysis of DNA, RNAs, and proteins, all in the same single cell.

Dr. Anders Ståhlberg is a Principal Investigator at Sahlgrenska Cancer Center, University of Gothenburg, and lead author of that paper. He joins us today in this podcast.

Doctor, one type of analyte is usually rather challenging to measure at single cell level. In this study, you show that multiple analytes can be analyzed. What are the major challenges to analyze DNA, RNAs, and proteins all in the same cell?

Dr. Anders Ståhlberg: The overall problem with single cell analysis is that you are actually working with very, very few molecule numbers: very few DNA molecules, very few RNA molecules, and very few protein molecules. Therefore, you cannot really afford to lose any molecules in your analysis, and this is really challenging to do for one type of analyte, but here we actually did it for three different types of analytes.

For example, if you start with 10 RNA molecules and you go on and process your sample. If you lose 5 molecules out of these 10, then you will have quite much of a problem to quantify these reliable. However, if you actually maintain all 10 molecules, you will have a quite robust read-out in the end.

So, therefore, different platforms have emerged over time. So we have platforms that are very good to measure DNA and other platforms that are very good for RNA, and the third type of platforms that are very good for proteins, even if proteins analytes have been a bit more challenging to perform at a single cell level if you don't measure fluorescence signal.

So what we needed to do, was to measure DNA, RNAs, and proteins in the same cell, but we wanted to keep the sensitivity of respective analytic platform and make them compatible to each other. The way we solved this was that we had a unique and newly developed lysis buffer that worked equally good for DNA, RNA, and protein. It was important to balance the efficiency of the lysis buffer without getting any inhibition in downstream reactions.

Furthermore, what we have to do, was to take the PLA-qPCR to a new level, so we actually can analyze very few protein molecules. This had never been done before, but we were able to take this method one step further and actually do reliable quantification of the number of proteins using a qPCR approach in our cells.

Bob Barrett: Doctor, protein quantification usually has some sort of background signal. What's the source of this background and how do you control for it?

Dr. Anders Ståhlberg: There are mainly two sources of error. One is that the PLA probes can bind non-specific to other proteins and the other reason is that they have self-ligation to themselves, and what we did was that we checked what kind of background we had here because if we have a non-specific binding to other proteins, it's much more complicated, but we could show that our problem with background was only due to background binding between the two different PLA probes that we have to use in combination.

Another thing that we never put in our publication was that we actually had another probe set for the same target protein that worked equally efficient, so that means that we were not just lucky of selecting

one very good set of PLA probes, we could actually do exactly the same analysis, but with another set up of PLA probes.

Bob Barrett: But Doctor, in your work, you use a fluorescence-activated cell sorter that collects single cell. Could other methods be used?

Dr. Anders Ståhlberg: Yes, indeed, you could in principle use any method you want. The most common ones in addition to FACS are microaspiration and microdissection, and our setup is fully compatible with these setups. However, I should make a point here that when you do microdissection, I mean that you can do it on living cells. If you have fixed tissue, you have more problem because you need to do something with the fixed material. Working with fixed material you need to develop the lysis conditions in addition to solve the problems that we introduced by using fixation.

The reason why we used FACS in our experiments was that we could make use of one of the benefits of FACS, and that is that you actually can measure the fluorescence, and by doing that, you can actually correlate the fluorescence of the protein we actually quantify with PLA-qPCR measurement. So that was like a verification we could use for our setup, and we saw that we have a very nice correlation between these two approaches.

Bob Barrett: Can this single cell approach be applied on any type of cell?

Dr. Anders Ståhlberg: Yes, more or less all mammalian cells will be fine to use this approach. If you have other cells with a more rigid cell wall like bacteria, you may end up with little bit more problems, because you need to lyse the cell wall and that's a classical problem working with bacteria.

Here, you need to work with stronger lysis conditions, and if you do that you need to, more or less, redo our optimization and see that this new lysis condition is fully compatible with downstream reaction condition, but if you solve these issues you can use the same experimental setup. But again, most mammalian cells will be excellent to use and measure with our approach.

Bob Barrett: Doctor, you found large variability in analyte concentrations among individual cells. How can this be explained?

Dr. Anders Ståhlberg: The variability of RNA and protein levels was quite expected. So our data is very much in agreement with other reports and also other methods, showing that the analyte variability can vary with several magnitudes in seemingly homogeneous cell populations. It's known and has been shown for some time ago that both transcription and translation may occur in bursts, and this variability is partly stochastic. So this variability we see was not really unexpected and it can be explained by these stochastic processes happening in the cells. One interesting thing was that we saw that we had up to four orders of magnitude difference in intracellular levels of the plasmid. This may be a little bit surprising, it's quite well-known that when you do transient transfections of plasmid you get aggregates, but that we had this large variability, was a little bit of surprise at least for me.

Bob Barrett: Correlation studies at single cell level seem to be very informative, were you surprised by the relatively low correlation values between plasmid numbers, transcribed RNA, and translated protein?

Dr. Anders Ståhlberg: Both, yes and no. It has been proved and also shown in many experimental systems that RNA and protein levels correlate quite poorly at cell population level, but this is very much dependant on the system you actually are studying.

In our study we observed significant but moderate or even low correlation between plasmid and the transcribed RNA and this weak correlation can be explained that most plasmid are actually not available for transcription, they may end up as aggregate in the cytoplasm.

So this is one thing that has to do with experimental system we are studying by doing transient transaction.

However, when we look on the correlation between RNA and protein and that was also moderate in our system. It actually tells something about the regulatory effect that we have in our cells, and one thing that is important to remember is that we measure the total amount of RNA and total amount of protein and we do not see the novoproduction of these respective analytes. So if we have a strong or weak correlation, this is very much up to what we actually are studying.

So I was not surprised that we didn't see that large

correlation, anyhow we saw correlation and that I think is what we would expect when we actually introduce the plasmid into the system. I believe that our approach to analyze multiple analytes in the same single cell open up new possibilities to study processes that actually regulate RNA and protein levels and how they are dependent on each other.

Bob Barrett: Another interesting observation was the loss of correlation for a fused in sarcoma or FUS mRNA with some other analytes when you over-expressed FUS, was this expected?

Dr. Anders Ståhlberg: Yes, our hypothesis was that we could see a difference, because there are many different factors that can affect if we have a synchronized or unsynchronized expression level between analytes.
We found that three RNAs in our cells were correlated. It was one mRNA, one non-coding mRNA, and one microRNA, and they were highly correlated compared to the other data that we talked about before, and we thought that we have one common factor that actually regulated these correlations.

We don't know what it is, but it was something that actually made them synchronized in expression. So our hypothesis was that if we over-expressed one of these factors but with another promoter region, in this case CMV, we will get a disturbed synchronization.

And indeed, when we over-express FUS in our cells, we lost the correlation between FUS and these two other RNA molecules, and one of the reason why this happened is of course when that the CMV promoter is different from the endogenous promoter and this addition of a new promoter that dominates expression will actually destroy the synchronization that we spoke about in the beginning.

Bob Barrett: Well, finally, Doctor, how many DNA, RNAs, and proteins can you measure in the same cell?

Dr. Anders Ståhlberg: The answer to this question is related to the first question you asked. It's all about the number of molecules you have in your cell. If you work with highly-expressed analytes, you can more or less analyze any combination.

However, if you work with lowly-expressed analytes, they are much harder to quantify in a robust manner, especially if you're working with proteins and with our experimental setup.

Today I would say, that we can measure one to five proteins in combination with up to 100 different RNA, DNA molecules. But in principle we can scale it up a little bit more, but something that I learned working with single cell analytes for a long time is that it's more important that you have really, really good assays that you actually can trust and that you have a good biomarker.

So it is easy to go for high throughput, but the question is, if you need to do it, or if you actually should do it, I often believe that it's better to have a few good markers that you can actually trust when you analyze them instead of going for many markers and you don't know when you have a reliable readout or not.

Bob Barrett: Dr. Anders Ståhlberg is a Principal Investigator at Sahlgrenska Cancer Center, at the University of Gothenburg in Sweden. He has been our guest in this podcast from *Clinical Chemistry.*

I am Bob Barrett, thanks for listening!