Advances in Mass Spec Push Single-Cell Proteomics Toward Feasibility

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NEW YORK (GenomeWeb) – While still not so widespread as single-cell genomics or transcriptomics, single-cell protein analyses have gained traction in recent years with companies like Nanostring, Fluidigm, IsoPlexis, and Bio-Techne developing single-cell proteomic offerings.

By and large, though, these systems have relied on immunoassays. And while technologies like Fluidigm's mass cytometry platforms offer relatively high levels of multiplexing for an antibody-based system, they remain limited in terms of the breadth of proteomic profiles they can generate.

In contrast to immunoassay-based approaches, mass spectrometry allows for the broad, unbiased analysis of proteomes of interest. However, mass spec-based proteomics has not been feasible at the single-cell level. This is changing, though, as new approaches and technical advances are making mass spec a suitable tool for single-cell analysis.

"I think it's a very exciting time for [single-cell proteomics]," said Nikolai Slavov, an assistant professor at Northeastern University and the developer of an isobaric tagging-approach to single cell proteomics called Single Cell Proteomics by Mass Spectrometry (SCOPE-MS). "There are huge gains to be made. It's not just about marginally improving over the current state."

Slavov's SCOPE-MS method, which he developed with Bogdan Budnik, director of proteomics at Harvard University's Mass Spectrometry and Proteomics Resource Laboratory, uses tandem-mass-tag labeling to analyze several hundred cells in addition to the single cell that is the actual target of the mass spec run.

This means that while peptides may be present in relatively low abundance in the cell of interest, the are present at higher levels in the overall samples, making them more likely to

be fragmented and detected by the mass spec. This allows researchers to quantify the amount of each TMT-labeled peptide in the single cell of interest while using the total of the peptides pooled across the larger set of cells to identify its sequence.

In the original SCOPE-MS study, published last year, the researchers quantified 538 proteins at the single-cell level in Jurkat and U-937 cells. Currently, Slavov said, his lab can quantify between 1,500 and 2,000 proteins in single cells using the technique. He said they are also able to identify hundreds of protein post-translational modifications.

Tackling throughput

Depth of coverage is important, of course. But equally important is throughput. This is particularly the case in single-cell proteomics, where it is necessary to do many thousands of mass spec runs to sample a reasonably large population of individual cells.

"If we are just able to quantify the proteins of ten cells [at a single-cell level], it's not very useful," Slavov said. "At the single-cell level, we need to be able to quantify thousands of single cells. We need to be able to obtain quantitative information for hundreds of cells per day per instrument."

Key to this is developing an automated sample prep system that can isolate single cells and prepare them for mass spec analysis, said Harvard's Budnik, noting that his lab is in the process of developing such an approach.

He and his colleagues are using fluorescence activated cell sorting (FACS) to place single cells in 96-well plates where they then take them through cell lysis and protein digestion. Their sample prep process avoids use of the mass spec-incompatible reagents used in conventional proteomic workflows, which lets them skip sample cleanup steps and limit their sample loss — an important consideration when working with single cells.

Budnik said work remains to be done on linking the FACS to the liquid handling to the mass spec system, but, he said, once complete, the platform should allow his lab to analyze at least 100 cells per day.

Higher levels of sample multiplexing will also be essential to reaching the throughput required for single-cell mass spec studies. Slavov said he expects that improved isobaric tagging reagents will provide a boost in this regard, noting that he was in communication with several groups who were working on such tools.

He declined to name these groups, but Harvard researchers Craig Braun, Steve Gygi, and Wilhelm Haas published several years ago on an isobaric tagging approach they named Combinatorial isobaric Mass Tags (CMTs) that they said could in theory allow for multiplexing of up to 28 samples at once without any decrease in protein identifications.

The CMT reagents would represent a significant multiplexing boost over existing TMT reagents which currently top out at a 10-plex.

Another important consideration for throughput is the upfront separation used. Budnik noted that in the original SCOPE-MS experiments, the researchers used 180-minute liquid chromatography gradients. They've since brought that down to either 60-minute or 90-minute runs, depending, he said, "on the depth with which we want to look at [a cell]."

Slavov said he was looking into capillary electrophoresis (CE) as a separation technique for single-cell analysis, noting that he believed that approach might be able to bring down separation times to the 10- to 20-minute range.

Bingyun Sun, an assistant professor of chemistry at Simon Fraser University, agreed that CE has potential for single-cell experiments.

"The advantage of that technique is the way it can really focus and achieve the separation with a very small quantity of protein," she said.

However, she added, LC is a more refined technology, at least for proteomic applications. "I would say that LC, because it is more mature than CE, has [higher] capabilities at this point."

Sun noted, though, that LC's maturity as a commercial product makes it more difficult to customize to a newer experimental workflow like single-cell proteomics than an emerging approach like CE.

"I would say that, instrumentation-wise, because LC has been commercialized and matured so that it's simple to use, it's difficult to customize," she said. "In our case we want certain customizations of the [LC] in terms of being able to program the valves and [determining] the size and dimension of the columns."

She added that she believes optimization of LC for single-cell proteomics is perhaps the part of the overall workflow most poised for major advances.

Optimizing ion collection

Last year Sun and colleagues at Simon Fraser published a study in *Journal of Proteome Research* modeling the optimal mass spectrometry settings for single-cell proteomics experiments. In particular, they looked at the ideal precursor ion isolation windows and ion injection times for single-cell work. In the case of these parameters, trade-offs are involved between the volume of ions collected for analysis and the level of interferences and speed of duty cycle.

For instance, using a larger isolation window will allow more ions into the analyzer for measurement, but it will also increase the amount of precursor ion interference, which can reduce data quality and, ultimately, the number of proteins an experiment is able to identify and quantify. Likewise, allowing for a longer ion injection time will increase the volume of ions in an analysis, which could up identifications. But this comes at the cost of a slower duty cycle, meaning a reduction in the number of MS2 scans and, as a result, a reduction in identifications.

In their *JPR* work, Sun and her co-authors developed a mathematical model identifying the conditions under which single-cell experiments will accurately identify the largest number of proteins.

Slavov said he and his colleagues were exploring other approaches to increasing the volume of ions they are able collect for their single-cell analysis. One strategy is data-independent acquisition mass spec, in which the instrument accumulates multiple ions at the same time.

"Let's say that instead of accumulating one ion at a time as we normally do, we accumulate ten ions at a time," he said. "Then I can afford to accumulate each of them at ten times the accumulation length without decreasing [duty cycle] because they accumulate in parallel."

DIA mass spec is not compatible with the conventional isobaric tagging approach used in the SCOPE-MS method, which quantitates peptides based on reporter ions generated by isobaric tags upon fragmentation. However, Slavov said it could work with an alternative isobaric tagging approach developed by Martin Wühr, assistant professor of molecular biology at Princeton.

The Wühr approach is inefficient when using conventional isobaric tagging reagents, but groups including Wühr's and the lab of Max Planck Institute of Biochemistry researcher Felix Meissner have developed new tags optimized for the approach.

Slavov noted that another potential approach for improving ion accumulation is using a method like trapped ion mobility spectrometry (TIMS) to collect ions that can then be injected into the mass spec. Max Planck Institute of Biochemistry researcher Matthias Mann has developed a workflow based on this idea for use on Bruker's timsTOF Pro mass spectrometer. Called PASEF (Parallel Accumulation - Serial Fragmentation), the method combines collection of ions via TIMS with rapid quadrupole switching on a QTOF instrument to enable the fragmentation of multiple simultaneously eluting precursor ions and has demonstrated sensitivity and speed gains of up to 10-fold compared to other mass spec approaches.

Mann and his colleagues have used these gains to move to smaller sample sizes without sacrificing depth of coverage. In a presentation at last year's annual meeting of the Human Proteome Organization, he showed data from a PASEF experiment in which his lab identified 1,803 proteins in a sample consisting of 15 HeLa cells. Commenting on the experiment last year, Mann said he believes that the technique could work with even smaller sample sizes.

Informatics gaps

Better informatics tools could also improve single-cell analyses, Slavov said, noting that his lab was collaborating with proteomics firm Biognosys to develop software aimed at single-cell experiments.

Lukas Reiter, chief technology officer at Biognosys, said his firm is seeing a trend towards lower sample sizes in its contract research business and added that, "given the success of single-cell transcriptomics, we might see a trend toward single-cell proteomics a few years down the road."

He noted existing antibody-based approaches to single-cell protein analysis, but said that these "allow you to monitor only a limited number of proteins, and you need very good antibodies to do it."

"Classical [mass spec-based] proteomics can really offer something different here, which is why we are interested in it," he said.

Biognosys has traditionally focused on DIA and other targeted mass spec methods but has more recently expanded its offerings to cover traditional mass spec workflows including isobaric labeling experiments. Reiter said that DIA combined with the Wühr lab's alternative isobaric tagging approach could be useful for single-cell analysis, but he said his work with Slavov is currently focused on a more classical isobaric labeling workflow.

"What we can add there is, because typically you don't have a lot of ions to collect MS2 spectra [in single cell experiments], we can use the general approaches that we use in DIA based on libraries to make the [peptide] identification process better," Reiter said. "We have tools like normalized retention times and consensus spectra identification, and we can simply transfer these concepts to a more classical database search and use them to increase the identification rate in single cell proteomics."

And then there is the need for tools to make use of the single-cell data once it's been collected, Sun noted.

"Once we have [data on] thousands of cells, we will have these multidimensional datasets where we will want to extract biological information," she said. She added that her group was looking at existing tools for analysis single-cell transcriptomic data and single-cell protein data generated with technologies like mass cytometry to identify useful approaches for analyzing single-cell mass spec data.

Slavov said single-cell mass spec research is still focused primarily on technology development, but he said his lab is beginning to explore practical applications of the approach. One application his lab is investigating is using single-cell analysis to rationalize methods of directed stem cell differentiation.

"Currently, if we want to make, for instance, beta cells of the pancreas to cure diabetes, or heart cells, we start by taking stem cells and throwing various chemicals at them using some combination of intuition, guessing, and trial and error," he said. "This is very inefficient, very expensive, and very slow, and I think that if we can make [proteomic] measurements in single cells and we can analyze that data appropriately we might derive rational approaches that can substantially enhance the efficiency of directed stem cell differentiation and help develop regenerative therapies for all types of diseases."

The other obvious application, Slavov said, is for research into the origins of cancer.

"We have been looking at mRNA, because that is what we could measure with single-cell methods," he said. "And that has been informative and useful. But the thing I would like to look at is what are the signaling pathways that are dysregulated in cancer. To the extent that we can measure proteins and their post translational modifications in single cells, that can better inform us about the different signaling processes in cancer, and that will serve as a stronger basis for precision medicine cancer therapies."

"I think these are two very broad avenues of applications," he said. "Of course there are other ones, but these are the main ones I see at the moment."

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