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Metabolomics: Sifting Through Complex Samples

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Look between the covers of any biochemistry textbook and you'll likely find an illustration outlining the major biochemical pathways of the cell and how they fit together. There, in the center, are glycolysis and the citric acid cycle. There's lipid metabolism and the urea cycle, nucleic acid metabolism and amino acid synthesis.

For students of biochemistry, trying to keep those pathways straight can be a frustrating exercise in rote memorization – succinate, fumarate, malate, oxaloacetate! But for basic biochemists, and increasingly for those in the clinical arena, metabolites are where it's at. After all, DNA encodes protein, but proteins often work their magic on small molecules – and very often, those molecules are completely unknown to the scientific community.

"If you measure as many small molecules as you can in a drop of blood, something like 60% of the peaks detected don't match any metabolite anywhere," says Gary Patti, Assistant Professor of Chemistry, Genetics, and Medicine at Washington University School of Medicine. "So there's an incredible wealth of information that is ripe for discovery."

As a postdoc at the Scripps Research Institute in La Jolla, Calif., Patti discovered evidence implicating a metabolite called N,N-dimethylsphingosine in a rat model of chronic pain, identifying in the process a potential therapeutic target for the condition. [1]

To do that, Patti and his research advisor, Gary Siuzdak tapped into a relatively young branch on the 'omics tree, metabolomics. Like genomics and proteomics, metabolomics seeks to catalog and quantify all the metabolites in a living organism, a daunting task. There are probably between 2,500 and 5,000 small molecules in the body, according to Mike Milburn, Chief Scientific Officer at Metabolon, a North Carolina-based service metabolomics provider and discovery firm, and metabolomics researchers typically probe those with either mass spectrometry or nuclear magnetic resonance (NMR).

Siuzdak and Patti used mass spectrometry to survey all the small molecules present in and around the site of injury in

their model rats, as well as the blood. By computationally sifting through all those spectra, they identified compounds whose abundance tracked with the phenotype – in this case, pain.

If you've been contemplating adding metabolite profiling to your own research, read on. We'll identify the new tools, technologies, and trends in the metabolomics space.

Mass spectrometry

Of the two key workhorse technologies of metabolomics research, mass spectrometry and NMR, the former is far more popular, says Oliver Fiehn, Director of the West Coast Metabolomics Center at the University of California, Davis, Genome Center, largely because of sample complexity.

"Mass spectrometry is much easier to couple with chromatography," Fiehn says. "NMR usually is plagued by no separation before it, so you end up with more complex spectra." NMR is also less sensitive than mass spec, though also more quantitative.

Between the instruments in his own lab and a core facility next door, Fiehn says his team has access to a dozen mass spectrometers, including six gas chromatography-coupled instruments (three LECO GC-time-of-flights (TOFs); a GC-quadrupole-TOF (qTOF) from Agilent Technologies; and two Agilent GC-quadrupoles) and six liquid-chromatography systems (two Agilent LC-qTOFs; one Thermo Scientific ion trap; a Thermo LC-linear ion trap-FT-ICR; an LC-QTRAP from AB SCIEX; and one LC-high-resolution-TOF from LECO).

Each of these has different strengths and weaknesses, Fiehn says. For instance, the GC-TOFs are sensitive and fast, but lack accurate-mass capabilities, which makes it difficult to identify and characterize novel metabolites, especially in discovery mode (or unbiased, as opposed to targeted) experiments.

"In unbiased metabolomics, you see a lot of signals you cannot identify that might be important in certain diseases," Fiehn explains. "Then you have to have an accurate mass instrument, so we use the LC- or GC-qTOFs from Agilent, which do give accurate mass."

Siuzdak's lab also uses LC-qTOF instruments from Agilent, as well as AB SCIEX. "The reason why we've chosen that platform is because it gives you the fast scan speed of a TOF but also high accuracy and tandem mass spectrometry capabilities. And the dynamic range is good."

New mass spectrometers have been released over the past few years to support the metabolomics workflow.

Waters Corp.'s Synapt G2-S is a UPLC- or MALDI-enabled quadrupole-TOF featuring ion mobility separation (IMS) capabilities. IMS is an in-instrument gas phase separation strategy that resolves molecules, even chemically identical (but conformationally distinct) ones, by their "collisional cross-section" – that is, by shape. "IMS is a very, very powerful thing," says James Langridge, Waters' Director of Pharmaceutical Discovery. "If you think of trying to identify an unknown metabolite, collisional cross-section provides greater specificity."

According to Langridge, there are three possible kinds of metabolomics studies using mass spectrometry: untargeted (or discovery) metabolomics, which scans in an unbiased way for metabolites whose abundance is changing in response to some event; targeted metabolomics, in which researchers quantify specific known metabolites in, for instance, a given pathway; and "spatial metabolomics," which studies the spatial distribution of small molecules in tissue sections.

Spatial metabolomics is enabled by an application called “mass spec imaging,” in which spectra are collected point-by-point by raster scanning across a sample, for instance, to define spatially where particular metabolites are localized in tissues or even within cells. MS imaging is commonly accomplished using MALDI mass spectrometers, and according to Langridge, the MALDI Synapt G2-S can handle this application, enabled by a proprietary piece of imaging software called MALDI-HDI (high-definition imaging).

Waters also offers the Xevo G2-S QTOF, an atmospheric-pressure electrospray ionization-coupled TOF system, and the Xevo TQ-S triple-quadrupole. Triple-quads excel at targeted metabolomics applications, as they can be programmed to look for and quantify specific ions.

Thermo Fisher Scientific also offers mass specs for metabolomics researchers. The company’s Q Exactive quadrupole-Orbitrap features fast scan speeds (12 Hz), high resolution (up to 140,000 FWHM), and high mass accuracy (1–3 ppm) for compound identification, says Yingying Huang, the company’s Strategic Marketing Manager for Metabolomics.

The Q Exactive’s 12-Hz scan speed means researchers can collect multiple spectra across even the narrow peaks coming off UHPLC separations, says Huang. The system also enables fast polarity switching between positive and negative ion modes. “In one run you can capture information on both polarities, which is very popular because some molecules only ionize in one polarity mode, and that [feature] offers complete [metabolite] coverage.”

Thermo also recently launched the Exactive-Plus, which is like the Q Exactive but without the upfront quadrupole, for use in metabolite profiling experiments. (The instrument can later be upgraded to a Q Exactive if desired, Huang notes.)

According to Huang, the Q Exactive is capable of handling all three phases of metabolomics research – biomarker discovery, compound identification, and biomarker validation. But researchers can also buy dedicated instruments. For instance, the company’s hybrid Orbitrap Velos Pro and Orbitrap Elite instruments excel at compound identification, she says, thanks to their multiple fragmentation strategies and tandem capabilities. The TSQ Vantage triple-quad, with its superior limits of detection and quantitation, is recommended for validation experiments, she says.

Data analysis

By all accounts, instrumentation has advanced to the point that when it comes to metabolomics, the difficulty is not data generation, but data analysis.

“To my mind the biggest challenge in metabolomics, similar to a lot of other ‘omics disciplines, is really the informatics angle – being able to interpret the data,” says Langridge.

The metabolome numbers in the thousands, and each compound may be represented by several peaks. Separating the wheat from the chaff, naturally, requires sophisticated analytical tools.

According to Milburn, Metabolon runs some 300 samples per day in its facilities, picking out and identifying between 500 and 1,000 metabolites per sample. The company has developed software that can automatically run a sample on three different mass spec platforms, pull out the peaks, decide which ones are most interesting, and identify the molecules they represent.

“The heart of Metabolon is a software and technology company,” Milburn says.

Many mass spec vendors have metabolomics platforms of their own, as well.

“It is the software offerings that turn a general purpose analytical instrument into a metabolomics solution,” says Steven Fischer, Agilent’s Marketing Manager for Metabolomics and Proteomics. Agilent offers several software tools for metabolomics, including the Fiehn GC/MS RTL library for metabolomics, a proprietary form of the METLIN database, and Mass Profiler Professional with Pathway Architect.

Thermo’s SIEVE application is a differential analysis tool that identifies the most significantly changing molecules in a metabolomics experiment, says Huang. But one of its most useful features, says Huang, is that SIEVE allows users to correct for solvent background – things like plasticizers that can routinely leech into a sample during processing and can overwhelm more biologically relevant signals. For instance, Huang notes that in one experiment, metabolomics analysis of a rat plasma sample produced some 20,000 ion signals. By removing the solvent background, the system reduced that number to 7,000 peaks, which it then reduced further to just 540 when grouping adducts and isotopomers. “That helps us reduce the false positive rate substantially,” she says.

Huang says a new release of SIEVE (version 2.1) is planned for this year’s ASMS meeting, which will include among other things a pathway visualization tool.

Waters’ metabolomics package, developed with Nonlinear Dynamics, is called TransOmics™. Designed to integrate proteomic and metabolomic datasets, TransOmics can process large sample sets, automatically detect features, perform a quantitative comparison and statistical analyses to differentiate those features that are changing, and finally identify those features from the mass spec data. “It’s our next-generation product, designed from the floor-up with some really nice features,” says Langridge. “We’re really excited about it.”

If you prefer open-source software, Siuzdak and Patti have developed an online data analysis platform called XCMS-Online (described in 2012 in *Analytical Chemistry* [2]; see also this related 2012 *Nature Biotechnology* article [3] explaining an “accelerated workflow for untargeted metabolomics” that relies on XCMS-Online and the lab’s freely available METLIN metabolite database). Anyone can create an account and upload their LC-MS data to the XCMS-Online servers for analysis and visualization. (Even if you don’t have your own data, the team has a few sample datasets you can play around with, including Coke vs. Pepsi.)

One new (and very slick) tool on XCMS-Online is Cloud Plot interactive, which compares two datasets and graphically illustrates, on the background of a plot of chromatographic retention time versus mass-to-charge ratio, features that are up or down regulated, their fold-change, and the statistical significance of those changes. [4] Links on the graph tie the plot to Siuzdak’s METLIN database of over 75,000 metabolite compounds (11,000 of which include mass spec data, meticulously collected by hand.)

According to Patti, his lab has developed other automated tools for rapid compound identification, which he hopes to integrate with XCMS-Online soon. “We want people who are less familiar with metabolomics and informatics approaches to be able to do these experiments,” he says. “That really is our goal.”

With the slate of metabolomics tools now available, that’s a goal that more and more researchers can attain.

Reference

[1] G.J. Patti et al., “Metabolomics implicates altered sphingolipids in chronic pain of neuropathic origin,” Nature Chemical Biology, DOI: 10.1038/nchembio, 2012.

[2] Tautenhahn, R, et al., “XCMS Online: A Web-Based Platform to Process Untargeted Metabolomic Data,” Analytical Chemistry, 84 (11), pp 5035–5039, DOI: 10.1021/ac300698c, 2012.

[3] Tautenhahn, R, et al., “An accelerated workflow for untargeted metabolomics using the METLIN database,” Nature Biotechnology 30,826–828, doi:10.1038/nbt.2348, 2012.

[4] Patti, G, J., et al., “A View from Above: Cloud Plots to Visualize Global Metabolomic Data,” Analytical Chemistry, 85 (2), pp 798–804, DOI: 10.1021/ac3029745, 2013.