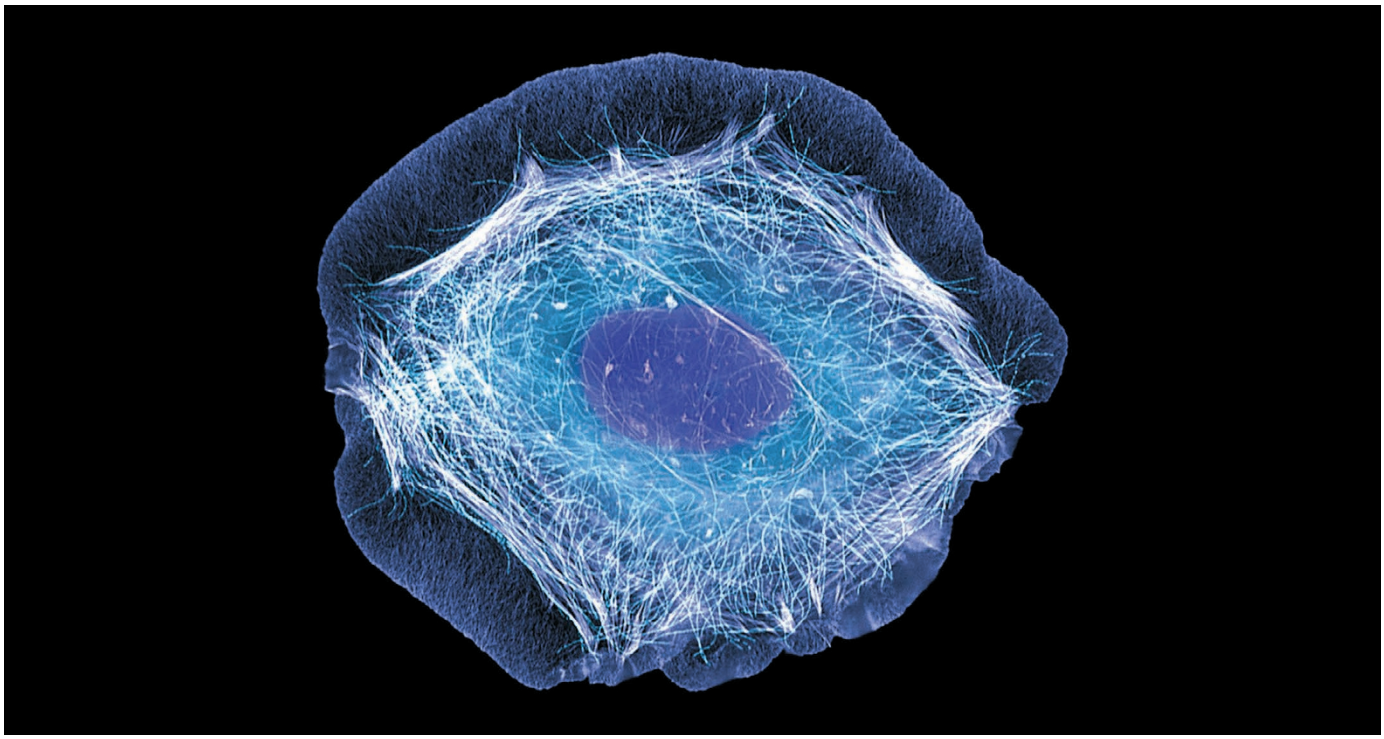
TECHNOLOGY FEATURE

METABOLOMICS: SMALL MOLECULES, SINGLE CELLS

Sensitive mass spectrometry and innovative cell-sampling techniques allow researchers to profile metabolites in single cells, but the field is still in its infancy.

TORSTEN WITTMANN/SPL



Researchers are developing innovative methods to understand the inner workings of individual cells.

BY MARISSA FESSENDEN

Sitting in his first-floor office, in an industrial lab space that opens onto a field of grazing cattle, analytical chemist Renato Zenobi explains one of the fundamental problems facing today's cell biologists. He traces out a curve representing the average concentration of a molecule in a theoretical cell population — a simple bell-shaped distribution. That distribution, he explains, can obscure complexity. To prove the point, he sketches two curves overlapping either side of the single peak, each representing a distinct phenotype in the population — and also consistent with that bell-shaped curve. "To really figure out if the distribution is multimodal or bimodal, you need to go down to the single-cell level," says Zenobi, at the Swiss Federal Institute of Technology (ETH) in Zurich.

Cell heterogeneity is why some bacteria in a clonal population can develop antibacterial resistance. It also gives rise to the different cell subpopulations in the brain. And it explains tumour relapses. The tools to detect those differences are only just becoming available.

"Recent technological advancements — especially those made just in the past two years — have revealed that individual cells within the same population may differ dramatically," says Ananda Roy, programme leader of the National Institutes of Health (NIH) Common Fund working group for single-cell analysis in Bethesda, Maryland. "And these differences can have important consequences for health and disease." Funders worldwide have queued up to support single-cell research. The NIH has invested in special initiatives to support single-cell profiling, starting with US$2 million in 2014, and almost 60 groups have now received awards under the programme. A collaboration between universities and companies in Japan launched the Society for Single-Cell Surveyor, which awards grants and holds symposia on single-cell analysis and technologies. And in October, experts discussed launching the International Human Cell Atlas Initiative, which aims to chart every type of human cell and its properties — an ambitious task that relies heavily on single-cell analysis.

Much of this work focuses on revealing cell-to-cell differences at the DNA level. Yet profiles of genes and epigenetic modifications merely outline a cell's potential. The rapid, dynamic responses a living cell has to its environment are better reflected in the metabolic transformations — and the resulting patterns of small molecules — that keep the cell powered, cycling and communicating with others.

"The metabolome is most directly related to the phenotype," says Caroline Johnson, an ▶

analytical chemist at Yale School of Public Health in New Haven, Connecticut. It reveals the products of the genome and its protein output, as well as metabolites from diet, drugs and toxic compounds.

Yet its complexity means that metabolomics has lagged behind other 'omics'. Unlike DNA and RNA, metabolites cannot be amplified. Although some metabolites can reach millimolar concentrations, a single cell offers a limited volume for analysis. Exquisitely sensitive methods are needed to detect rarer compounds — and the abundances can change in seconds in a living cell. Metabolites also have a bewildering variety of structures. The Human Metabolome Database contains records for more than 42,000 metabolites, from sugars to peptides to cofactors. But the total may be significantly higher, and single analytical methods often struggle to capture the chemical diversity.

Still, the field is advancing, thanks to leaps in detection capabilities, increasingly sophisticated ways of isolating and handling single cells and developments in bioinformatics. "We are getting close to making single-cell metabolomics robust," says Jonathan Sweedler, an analytical chemist at the University of Illinois at Urbana–Champaign. "I can't see exactly how it is going to work, but I look at the increase in detectability and throughput of mass spectrometry and I say it will happen."

PUSHING SENSITIVITY

Profiling individual yeast cells allowed Zenobi's group to spot two phenotypes lurking in a genetically identical sample — one characterized by low levels of a metabolite called fructose 1,6-bisphosphate, and another with high levels[1]. The difference probably comes down to different glucose-utilization strategies. The insight doesn't have immediate biomedical applications, Zenobi admits, but it does illuminate a fundamental way in which cells work.

To draw such insights, Zenobi's team uses sophisticated techniques to isolate cells and to boost the sensitivity of its analytical approaches. Researchers generally use either mass spectrometry or nuclear magnetic resonance (NMR) to drive metabolomics studies. But because NMR is less sensitive, mass spectrometry has emerged as the method of choice, and there are many variations to improve its detection abilities, throughput or simply make it easier to pull the contents out of a cell. "The whole arsenal of mass spectrometry has been thrown at this problem," Zenobi says.

Mass spectrometry involves ionizing a sample to lend a charge to its constituent molecules. The charge then means that magnetic plates can nudge the molecules as they fly through a vacuum. Each molecule deflects by a different amount, depending on its mass-to-charge ratio, such that by the time the molecules reach the detector, they have resolved into their component parts. The data appear as a complicated plot of unidentified peaks, each corresponding to a different molecular entity.

The method is straightforward — except when applied to single cells. Attempting to detect just a few molecules in a vanishingly small volume pushes the limits of modern instrumentation.

Zenobi uses a specialized silicon slide to individually deliver hundreds of single cells into the mass spectrometer. To the naked eye, the slides seem to be covered with a fine black mesh. The mesh is a coating of a polymer called polysilazane, which has been laser micro-machined to create hundreds to thousands of reservoirs, each a couple of hundred micrometres in diameter. When the researchers add a dilute solution of cells to the slide, the coating's repellent properties ensure that some liquid, and one or two cells, end up in each reservoir. The researchers then direct the spectrometer's laser to target each well in turn. One configuration of these slides, called microarrays for mass spectrometry (MAMS), is available from MilliporeSigma in St. Louis, Missouri.

Across the hall from Zenobi's office, graduate student Robert Steinhoff demonstrates how the slide fits into a mass spectrometer. The researchers use matrix-assisted laser desorption/ionization (MALDI) coupled to a time-of-flight analyser, which requires them to spot their slides with a chemical matrix to drive ionization. By using a matrix that minimizes interference with the signal given off by small molecules, the team can detect metabolites in the low attomole (10^{-18} moles) range — and do so for about 1,000 cells per chip, which is relatively high throughput in the single-cell world.

"Individual cells behave very interestingly and unexpectedly."

Steinhoff is also working to alter the substrate used in his reservoirs. "Making nanostructures — pillars — within the spot helps us see things more reliably," he explains. The mechanisms behind this effect aren't yet clear, but Steinhoff says that the cells end up clinging to the tops of the silicon columns.

Sweedler has developed a high-throughput approach that uses a computer to guide the laser to individual cells spread across a slide. His team can process about 10,000 cells per slide in this way. But for a more comprehensive look at the metabolome, Sweedler takes it one cell at a time. He uses a modified patch-clamp tool, which typically records cellular electrical signals, to withdraw roughly three picolitres of cytoplasm (about 10–40% of the total volume) from individual brain cells and deliver it into a mass spectrometer.

The limited throughput restricts analysis to a few dozen cells per experiment. Still, Sweedler's group has used it to detect about 60 metabolites from 30 neurons and astrocytes in slices of rat brain[2]. The team focused on neurochemicals such as glutamate, but also detected amino acids and derivatives of ATP, among others. From that, they compiled unique profiles for the different cell types, providing a window into the cell-to-cell heterogeneity that makes the brain so complex, Sweedler says.

DIFFERENT STROKES FOR DIFFERENT SIZES

When dealing with single cells, size matters. Plant cells can be anywhere from 10 to 100 micrometres across. Mammalian cells tend to be smaller, on the scale of 10–20 micrometres. Microbial cells are smaller still, reaching down to the submicrometre range. As cell size varies, so too does the volume and thus the absolute number of metabolites. "It is obvious from an

SELECTED METABOLOMICS SOFTWARE

Name	Description
Human Metabolome Database	Free database of metabolites in the human body, including details relevant to chemical profiles, molecular biology and clinical chemistry. Links to pathway and structure-viewing tools and to other databases.
KEGG Pathway	Visualization tool linked to a collection of manually drawn pathway maps (KEGG database) with ability to locate and colour specific metabolites and pathways.
LipidBlast	A tandem-mass-spectral database for lipid identification.
MapMan	Tool for converting large data sets, such as those from gene-expression arrays, into metabolic pathway diagrams. Focuses on plant metabolomics.
MetaboAnalyst	Complete workflow for metabolomics data processing and analysis for both mass spectrometry and nuclear magnetic resonance data with common statistical analyses and heat-map visualizations.
Metabolites Biological Role (MBRole)	A server that integrates chemical and biological annotations from a handful of databases to identify potentially important metabolites and other molecules.
MS-Dial	Downloadable program that spots and identifies peaks in mass-spectrometry data, developed for small molecules.
OpenMS	Open-source library for C++-based data analysis and management that includes infrastructure for software development. Mainly for processing raw data.
XCMS Online	Cloud-based platform for metabolite profiling from raw liquid-chromatography and mass-spectrometry data and for data sharing.
Yeast Metabolome Database	Manually curated collection of yeast metabolites and their chemical properties, with links to spectral and chemical databases.

analytical perspective there will be no single method that can deal with all these volumes," says Akos Vertes, a chemist at George Washington University in Washington DC. His lab uses different methods for different cell sizes.

For the largest cells, the team uses a sharpened optical fibre to deliver infrared light directly into the cell. The light excites the oxygen–hydrogen bonds in the water of a cell, causing the cell to explode and eject its contents. The escaping material then meets an aerosolized, ionized liquid called an electrospray, which charges the molecules for mass spectrometry. The advantage of this technique is that single cells can be profiled while still embedded in tissue. But it also can be very slow, because each cell generally needs to be poked with the fibre by "a very patient graduate student", Vertes says. He recently automated the process, using a computer to manoeuvre the sample stage.

The smallest cells are deposited on a nanopillar-covered surface, also made of silicon although fabricated differently from Steinhoff's. Imaging the entire surface reveals where the instrument's ion beam needs to aim to target single cells. With this method, the team can routinely detect metabolites at femtomole (10^{-15} moles) levels[3]. But the investigators estimate their lower limit of detection at about 800 zeptomoles (one zeptomole is 10^{-21} of a mole), or about 482,000 molecules.

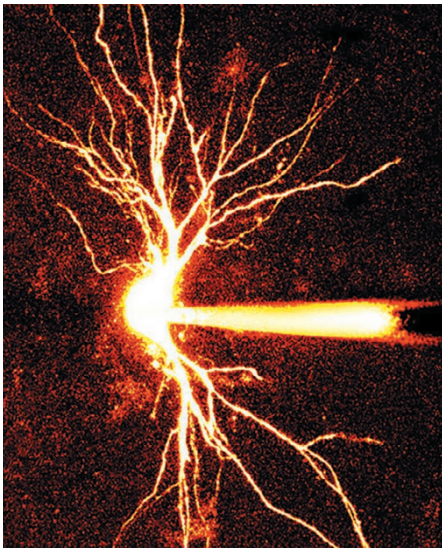
It is possible to go even smaller. Bioanalytical chemist Andrew Ewing at the University of Gothenburg in Sweden examines the small-molecule content of neural vesicles. These vesicles are key to delivering and releasing chemicals into the space between cells to facilitate cell-to-cell communication. "It's tricky because you get very little signal with so few molecules in there," he says.

Ewing uses an approach called nanoscale secondary ion mass spectrometry (NanoSIMS), which bombards a sample surface with a high-energy beam of caesium ions. This assault ejects charged particles from the surface, and they can then be analysed in a mass spectrometer to determine the material's composition. Ewing's group uses the method to assess distribution of the neurotransmitter dopamine. And by correlating the NanoSIMS data with transmission electron microscopy to observe a single vesicle as it loads and unloads dopamine, the researchers determined that a vesicle's inner shape might regulate how quickly this process happens[4].

SEEING IS BELIEVING

At the RIKEN Quantitative Biology Center in Osaka, Japan, chemist Tsutomu Masujima uses a video feed to target single cells for mass spectrometry.

"Individual cells behave very interestingly and unexpectedly, so I like to see [them] as much as possible," Masujima says. His approach involves inserting a nanospray needle directly into a cell under video observation, sucking out the contents and then using the same needle to inject the contents into the mass spectrometer. Adding the visual component allows his group to complete delicate manoeuvres, such as capturing and analysing the amino acid and lipid contents of single white blood cells and tumour cells circulating in the blood[5].



J. T. AERTS *ET AL./ANAL. CHEM.*

The cytoplasm of a cell can now be extracted and its contents analysed.

But it also allows his team to estimate molecular abundance. Often, such a seemingly trivial calculation is complicated, because researchers aren't sure exactly how much volume they are analysing. So Masujima and his colleagues use 3D microscopy to observe the cell before and after removing a small amount of cytoplasm. By measuring the resulting deformation, they can deduce the volume they removed as well as the location from which it was taken[6]. In one case, they sampled a cytosolic metabolite called methionine sulfoxide, determining that they had captured 5.9 zeptomoles of it.

MAKING SENSE OF THE DATA

Fortunately, says Gary Siuzdak, a chemist who heads the Scripps Center for Metabolomics and Mass Spectrometry in La Jolla, California, bioinformatics tools are available to make sense of such findings.

Siuzdak's centre runs a cloud-based metabololomic analysis platform called XCMS Online (see 'Selected metabolomics software'), whose 12,000-plus users have collectively shared more than 120,000 jobs. Few of those jobs have involved single cells, Siuzdak acknowledges, but that doesn't mean that they are inherently incompatible with the software. "On the bioinformatics side, I don't see a major issue with doing these experiments," he says.

Rather, the main challenge with single-cell metabolomics is one of instrumentation: devices that can analyse enough single cells, and enough metabolites per cell, for the results to be statistically meaningful. "The primary issue with single cells is that the hardware still needs to be improved," Siuzdak says. Studies tend to profile tens or maybe a hundred different molecules. But a single yeast cell can have some 600 metabolites[7]. As a result, even the most sensitive analytical techniques are picking up only the easiest-to-detect, most prevalent molecules in a cell; less-common ones fall below the radar.

A possible solution to this problem, says computational biologist Jianguo Xia of McGill University in Montreal, Canada, may lie in population-based tools and in various 'omics. "The pipelines and tools that have been developed for metabolomics data sets generated from bulk cell populations can be reused," he says. All that may be needed are slight modifications to the data-processing and normalization methods that researchers use. "Single-cell transcriptomics is already established, and we can learn from it to accelerate bioinformatics development for single-cell metabolomics," he adds.

Other researchers are pursuing single-cell strategies that are based on methods other than mass spectrometry — in particular, using living cells. Matthias Heinemann, for instance, who collaborated with Zenobi during his postdoc, now heads a molecular systems-biology group at the University of Groningen in the Netherlands. There, he uses his background in biochemical engineering to "find other ways to zoom into single cells". In one approach, his group uses fluorescent molecular sensors to quantify metabolites, such as ATP. In a forthcoming paper in *Molecular Cell*, his group has used time-lapse microscopy to watch levels of ATP and another metabolite, NADH (which is autofluorescent), oscillate as the cells go through the cell cycle[8].

Key technical challenges have already been solved when it comes to single-cell metabolomics, Heinemann says. "What is needed now is to do tedious development and validation work." The process might not be glamorous, he admits, but it is essential if single-cell metabolomics is ever to make the leap from proof of concept to answering fundamental biological questions.

"We are often so happy to detect unique molecules, but my point is: why?" Masujima says. "What is behind this finding, why does this molecule come up?" Without that insight, techniques run the risk of being mere gimmicks that fail to address questions of biological significance. "I don't want to be a scientist who does that kind of science," he says. ■

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CORRECTION

The Technology Feature 'Metabolomics: small molecules, single cells' (*Nature* **540,** 153–155; 2016) erroneously stated that Matthias Heinemann was a former postdoc in Renato Zenobi's lab. Although he worked with Zenobi, Heinemann was a postdoc in another lab at the time. Also, Heinemann's background was in biochemical engineering, not analytical chemistry.