# Single-cell metabolomics hits its stride

An array of new techniques allow researchers to catalog the chemical contents of a single cell, or even a single organelle.

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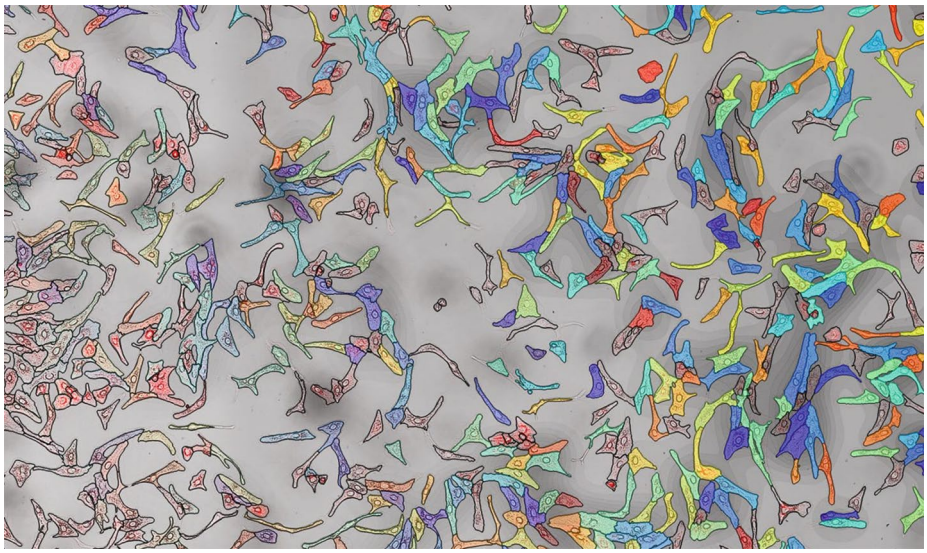
Studying genomics reveals what a cell is capable of; transcriptomics gives a view of what the cell is planning to do. To find out what the cell is actually doing, however, requires proteomics and metabolomics.

"Metabolomics is very important," says computational biologist Theodore Alexandrov of the European Molecular Biology Laboratory (EMBL). "It is the youngest of the omics, but it provides the readout which is closest to the phenotype."

Taking a census of metabolites gives the best view of what chemical processes are taking place in a given cell. Two genetically identical cells can have widely different chemical metabolomes. The cell and the extracellular environment interact dynamically via a variety of molecules, including metabolites. The food we eat can change the cell's metabolome. Cancer cells are well known to fill the tumor microenvironment with chemicals that befuddle the immune system, spur the growth of new blood vessels and launch metastases. In the brain, cells send signals using neurotransmitters, which can then alter the activity of nearby cells.

"Single-cell metabolomics is remarkably helpful," says Anne Le, who studies cancer metabolism at Johns Hopkins University. "Within the tumor of a single patient, you can see subpopulations and you can determine which cells do what."

To advance single-cell metabolomics, researchers have needed to address several key challenges. The metabolome changes rapidly, meaning that even collecting a sample to analyze can alter the metabolome from its native state. Metabolites can vary wildly in their abundances in a single cell, and the tiny quantities present require high sensitivity to detect. Unlike the genome and transcriptome, which consist of just a few different nucleotide bases, metabolites come in all shapes and sizes. When dealing with large biomolecules, it can be tricky to distinguish between isomers, even with the aid of software. "If you look at very complex secondary metabolites which have many, many molecules, the number of possible structural configurations that can have the same atomic mass becomes huge," explains Alisdair Fernie, who studies metabolism in plant cells at the Max Planck Institute of Molecular Plant Physiology. "There's a lack of absolute knowledge on that, even with very precise weighing."



Single-cell metabolomics combined with spatial analysis can help uncover how diseases progress. This image combines light microscopy images of hepatocytes and single-cell intensities of a fatty acid, detected using SpaceM, shown in the jet color map. Using SpaceM, the team observed changes in fatty acid metabolism associated with non-alcoholic fatty liver disease. Credit: Theodore Alexandrov, Luca Rappez

Additionally, to understand how heterogeneous populations of cells interact, it's important to analyze cells in their native context, accurately matching up metabolome data with the physical characteristics and neighborhood of the cell it came from.

## An immense and moving target

All of these challenges make characterizing and quantifying the cell's chemical contents seem impossibly complex. For one thing, the metabolome is far more dynamic than the genome or even the transcriptome. "If you check someone's genes, for example, you'd find that person is one-quarter French and three-quarters Vietnamese," explains Le. "The way you take the sample won't change that result. If you leave the sample at room temperature and come back in five days, the genome will still be the same. But the metabolome won't just sit there waiting for you."

Sample preparation is key to getting an accurate snapshot of the metabolome. Different mass spectrometry setups are compatible with different methods for quenching metabolism, Le says. Snap-freezing with liquid nitrogen is a common method, as is the addition of organic solvents. Using a solvent can also help with metabolite extraction. For example, she points out, acetonitrile is used for both quenching and extraction for matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS).

Hua Tian of Pennsylvania State University, who developed a method called high-energy gas cluster ion beam–secondary ion mass spectrometry (GCIB–SIMS)[1], prepares her samples using frozen hydration. "Frozen hydration is really important," she says. "It freezes the biological system in space and time and provides a snapshot of the near-nature state of the biological system." Freeze-drying or chemical fixation

allow some metabolites to diffuse away, she says, losing a bit of biological information.

Another key difference between genomics and metabolomics is complexity. Although a few tricky regions of the genome remained difficult to sequence, nearly all of it can be sequenced and annotated. Not so for the metabolome: metabolites come in all different shapes and sizes, and they number in the millions. Of these, typically about 5% can be definitively identified from mass spectrometry data.

"Metabolite identification is one of the foundational problems in metabolomics," says Alexandrov. To help tease concrete information out of the jumble of peaks in the spectra, Alexandrov's team created METASPACE, an algorithm for metabolite identification and an open-platform knowledge base for people to share their imaging mass spectrometry data[2].

"This was transformational for us, and now it's also used by about 100 labs around the world," Alexandrov says. Instead of spending years becoming proficient in interpreting mass spectra, he says, a biologist can learn to use METASPACE in a much shorter time, allowing them to make sense of the spectra they collect. "The software is pretty user friendly, and within 2 months they are producing good molecular data, focusing on improving and interpreting the molecular content of the data without the need to manually browse through myriads of spectral peaks corresponding to unknown molecules," Alexandrov says.

Additionally, "there's a lack of linearity between the number of substrates and number of products," explains Fernie. Transcriptomics and proteomics methods might reveal that a cell produces a particular enzyme, and that generates a reasonable expectation that the cell also contains the molecules acted on by the enzyme. But those molecules may form intermediates or get sucked into side reactions, generating unpredictable new products. "There's also a very small minority of non-enzyme-catalyzed reactions, spontaneous reactions, and these are difficult to predict," Fernie says.

Though metabolomics may fall short of measuring the full and complete chemical contents of the cell, new techniques that increase throughput and sensitivity are yielding a wealth of new biological information.

## Demystifying differentiation

The great miracle of vertebrate life is how a single cell, the zygote, contains all the information needed to generate dozens of different specialized cell types. Though the genome is often considered "life's instruction book," a zygote's DNA doesn't change as it reproduces and differentiates. Throughout the stages of development, the genome stays essentially the same, and the changes are driven by the metabolome.

Peter Nemes and his team at the University of Maryland, College Park, have honed a method to analyze metabolites from a single cell of a frog embryo. During embryonic development, the cells divide rapidly, going from one very large cell to two smaller ones and so on, doubling in number and halving in size roughly every quarter of an hour. "Studying single-cell metabolomics in such spatially and temporally developing samples is a real methodological challenge," Nemes says.

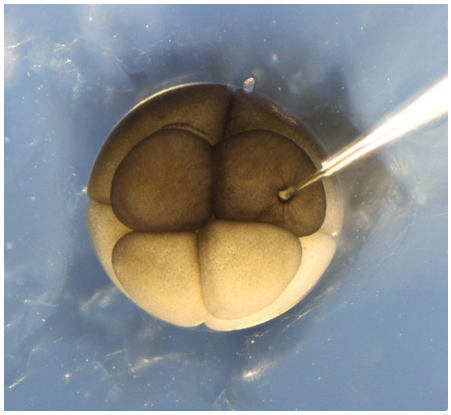
First, while the embryo is in the four-cell stage, Nemes and his team use light microscopy to identify the left-dorsal and left-ventral cells. Using precision microcapillaries that they fabricate in-house, they aspirate a sample of the contents of a single cell. Capillary electrophoresis separates the metabolites, and is followed by electrospray ionization and high-resolution mass spectrometry to identify and quantify the molecules. Importantly, this procedure, called sampling, doesn't harm the embryo's ability to develop into a normal tadpole[3].

"We can remove the capillary and within a few minutes the cell heals its membrane," Nemes says. "After the first sampling, we can come back for a second sampling or even a third."

Because the capillary extraction does less damage than traditional dissection methods, the cell is less stressed. "This is cool because you're much closer to measuring the real metabolome of the cell than in a cell that has undergone oxidative stress," Nemes explains. The ability to re-sample the same cell also allows the researchers to combine metabolomics with proteomics within a live, developing embryo. Any changes in anatomy or behavior as the tadpole develops can then be correlated with changes in the proteome and metabolome.

Achieving sufficient sensitivity posed another challenge. "Unlike single-cell transcriptomics, where you can amplify the entire transcriptome, you cannot do that at the level of the metabolome," Nemes says. "There's no technology available where we can amplify millionfold copy numbers of all the metabolites in the cell. The entire field of single-cell metabolomics comes down to sensitivity."

Nemes's method heightens sensitivity in a few ways. First, the capillary electrophoresis efficiently separates the sample molecules, producing crisp, sharply defined peaks. Then, the team perfected the ionization step. "We build these ion sources ourselves, because we know how to find the proper



Using a specially microfabricated capillary, the Nemes lab can aspirate a sample from a single cell of the eight-cell *Xenopus laevis* embryo. Additional samples can be taken for proteomic analysis, and the embryo continues developing normally. Credit: Peter Nemes

operational conditions to most efficiently convert the molecules to ions," Nemes says. "And we developed custom-built software to extract the very small peak intensities."

Commercial software, he explains, assumes that the metabolites have been separated using chromatography, which produces wider peaks than capillary electrophoresis. "We got these large amounts of data, but we couldn't analyze them," Nemes says. "If we used popular, commercially available software packages, we'd get thousands of false positives. It was a huge dataset that was just noise."

The team collaborated with Chen Zeng at the George Washington University to create their own analysis software, which they called Trace. "We trained an artificial neural network that could recognize the trace sensitive signals in our dataset," Nemes says. "This worked extremely well. 95% of the signals that we find with the Trace software are true signals."

Using their method, Nemes's lab documented the differences in metabolite composition among the cells in the eight-cell frog embryo. They have shown that metabolites drive the differentiation of the stem cells into organ-specific lineages, and that altering the metabolites in a cell can change that cell's fate[4].

"We've discovered novel molecules that are able to alter this stem cell trajectory," Nemes says. "We can actually reprogram an epidermally fated cell to become neural tissue and vice versa by doing single-cell metabolomics."

## Spatial relations

Detecting the metabolomes of individual cells while preserving the spatial

relationships between them adds yet another layer of information. Because a cell's native environment can have a strong impact on its biochemistry, researchers are working on methods to compare single-cell metabolomics data among members of a population.

The Alexandrov lab is developing methods to collect and interpret data from commercially available instrumentation. MALDI-imaging mass spectrometry is one of the most popular techniques for single-cell metabolic analysis. For MALDI, the samples to be analyzed are mixed with a matrix, then irradiated by a UV laser beam. The beam desorbs and ionizes the analytes, sending them into the mass spectrometer. With its minimal sample preparation and high throughput, MALDI–MS is well suited to analyzing large populations of cells, and has been used successfully to reveal heterogeneity among clonal populations of single-celled organisms and to discover rare cell subtypes in tissue samples.

MALDI-imaging mass spectrometry measures mass spectra at different points on a tissue section or glass slide containing plated cells, allowing researchers to create a spatial map of metabolites. However, assigning metabolite intensities to single cells is challenging. "The spatial resolution of MALDI imaging is pretty close, but not as good as microscopy," Alexandrov says. "Our pixel size is close to one cell."

To address this, Alexandrov and graduate student Luca Rappez developed SpaceM, a method that integrates MALDI imaging with light microscopy and digital image processing to precisely match up the mass spectrometry data with the cells they came from[5].

The MALDI laser creates visible burn marks in the matrix, and this is the key to SpaceM. Before performing MALDI imaging, the researchers use microscopy to capture the cells' relative positions, their fluorescence phenotype and any other pertinent information. After metabolite data is collected using MALDI imaging, a second microscope image is taken that shows the positions of the burn marks. These marks provide a visual cue indicating which cells the metabolite data was collected from.

The tricky part is lining up the before and after images with subcellular precision. "To do this, Luca used fiducials, or some features that you can see in both images," Alexandrov says. They create these fiducials by making marks with a black Sharpie pen around the edge of the slide. "The key to achieving subcellular precision was to automatically recognize these fiducials, thousands of them."

SpaceM also provides high throughput, as thousands of cells can be imaged in a single slide. In collaboration with Mathias Heikenwälder's team at the German Cancer Research Center, experts in inflammation, the researchers used SpaceM to analyze the lipid composition of human cultured liver cells, an in vitro model of two lipid metabolic disorders, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). SpaceM revealed that a large subpopulation of NAFLD cells, around a quarter of them, showed accumulation of lipid droplets and neutral lipids, consistent with the metabolic state associated with the diseases. Then, after they induced NASH with an inflammatory cytokine, around 93% of the cells adopted this metabolic profile.

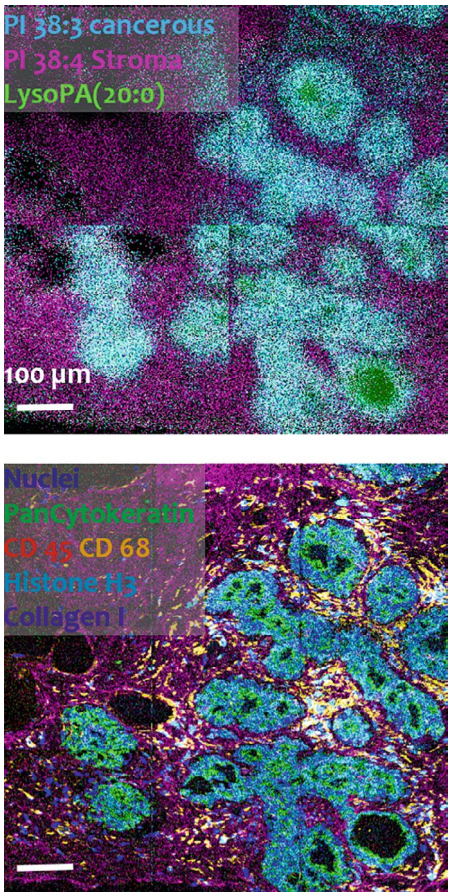
This ability to detect the metabolic heterogeneity of individual cells in a population could also have applications for cancer, Alexandrov points out. "The tumor microenvironment has very special chemical and physical properties," he says. "You have a gradient in oxygen, you have a gradient in metabolic waste like lactate." These chemicals have an effect on the different cells populating the microenvironment, including the vast array of immune cells swarming the cancer. "One of the key applications of single-cell metabolomics will be immunometabolism," Alexandrov says. "If we know the metabolism in every cell, if we know how cancer cells adapt their metabolism, then we can potentially help the immune cells to adapt their metabolism to be more functional in this environment."

"It's really important to understand the metabolic states of the different cells in the content of tissue without dissociation," says Tian. "This is the new frontier of biological analysis. It will help understand why some immune cells can penetrate into the tumor microenvironment and some not."

Tian works with GCIB–SIMS, a method that preserves the spatial relationships between the cells without requiring special preparation. GCIB–SIMS bombards the cell with an energetic beam of ions, dislodging ions that fly off and are collected in a mass spectrometer. The technique combines the chemical specificity of mass spectrometry with imaging resolution approaching 1 micron, small enough to image a single cell. Using gas cluster ion beams causes less damage to biological specimens than using monoatomic ion beams, because the beam's energy is divided among thousands of gas molecules. It also results in less fragmentation and can be used to detect larger-mass biomolecules.

In collaboration with Sadia Sheraz and John Vickerman at the University of Manchester, Tian has developed a new SIMS protocol using water cluster ion beams, which is pushing the sensitivity limits even further. "The water cluster ion beam is probably the most effective desorption source I have ever seen," says Tian. "Particularly running it with our newly constructed ion gun, operating at high voltage to focus the beam. We have a cooling pad through the beam column that will also focus the lens, and we can focus this beam down to 1 micron."

Tian analyzed breast cancer tissue samples with multimodal SIMS imaging, combining the water cluster ion beam with one that uses carbon clusters. The water cluster beam detects the metabolites without damaging the samples, allowing a second pass. The cells are stained with lanthanide-labeled antibodies specific to protein markers identifying the cell types, and these are detected using the carbon cluster beam. The antibody panel targets




The same breast cancer section, imaged first with $(H_2O)_n$-GCIB-SIMS to detect lipids and metabolites and then with $C_{60}$-SIMS (carbon cluster SIMS) to detect the lanthanide-tagged antibodies used as cell type markers. This method allows the researchers to precisely localize different cell types in the tumor microenvironment and compare their metabolomes. Credit: Hua Tian

T cells, B cells, macrophages and tumor epithelial cells, as well as chromatin and extracellular matrix proteins.

"With these two sets of images, we can integrate the lipids, metabolites and proteins in single cells," Tian says. "We see some interesting phenomena that are in some ways consistent with previous bulk analysis, but some are not. Moreover, we provide a more comprehensive visualization of the tumor microenvironment." For instance, tumor epithelial cells had an enhanced antioxidant pathway, whereas rapidly dividing cells had high levels of gangliosides.

This multimodal approach opens the door to much richer investigations into the interactions between cancer cells and immune cells, as well as cell–cell interactions in other systems. Still, a cell isn't just a homogeneous sac of chemicals, evenly distributed throughout. Some researchers are looking even closer, at individual organelles, again using MALDI–MS, nanospray ionization mass spectrometry and GCIB–SIMS to come at the problem in different ways.

## Smaller and smaller

"How do you identify what a cell type is?" asks Jonathan Sweedler, who studies analytical neurochemistry at the University of Illinois. Early microscopists named different brain cells based on their shape, or how they looked under the microscope. Astrocytes, pyramidal cells, chandelier cells—these names are evocative but reveal little about the cells' function. As neuroscientists developed methods for measuring biochemicals in the brain, cells became defined by the chemicals they release: cells could be dopaminergic, serotonergic, GABAergic. But these designations don't entirely overlap with the morphological categories.

"If you have an astrocyte that's next to neurons using glutamate or GABA as a neurotransmitter, it will have more of the proteins required for taking them up and processing them," Sweedler explains. "If you have an astrocyte in another area of the brain that's near dopamine- or serotonin-releasing neurons, and it has to deal with those molecules, it would have different transporters and enzymes. Do these differences mean it's a different cell type?"

The same cell can change its metabolite lineup if it's moved to a different area, so Sweedler is less interested in cell type designation than cell state. "Transcriptomics gives you the potential of the cell, what building blocks it could make," he says. "Proteomics tells you the building blocks it did make. The state it's in is reflected in its metabolome."

Neural cells package chemical messenger molecules, such as hormones and neurotransmitters, into lipid-lined sacs called dense-core vesicles or electron-lucent vesicles. Sweedler and his colleagues set out to adapt MALDI–MS to analyze these individual vesicles[6].

"We've been doing single-cell peptidomics for 20 years," Sweedler says. "This paper takes it to the next step and demonstrates that we could make measurements on an object smaller than a single cell. A cell contains lots of vesicles that eventually fuse with the membrane and release their contents. Our goal both was to push the technology forward and to ask the question, How heterogeneous [are these vesicles]?"

The researchers scatter thousands of organelles across a microscope slide with enough space between them that the laser will hit only one item at a time. Next, they image the slide and its objects with a microscope. Then, a machine-learning system classifies the images, identifying the coordinates of the objects most likely to be dense-core vesicles. These locations are used to guide the mass spectrometer laser to target only those objects. Automating this process, Sweedler says, enables the method to capture thousands of spectra in a single slide.

"The hardest part, which makes sense, was adapting our single-cell approaches to much smaller organelles. You have to find the objects when they're below a micron in size," Sweedler says. "That took a surprising amount of time."

To develop the technique, Sweedler's lab used cells from the sea slug, *Aplysia californica*, because their organelles are fairly large, around 0.5–2 microns in diameter. They identified three distinct populations of dense-core vesicles that contain distinct, though overlapping, distributions of peptide hormones.

The ability to peek inside a vesicle can improve our understanding of diseases involving cell-to-cell signaling. Hormones and neurotransmitters play a role in physiological functions as diverse as circadian rhythms, maintenance of water–sodium balance and social bonding.

"There are cases where you can have a single prohormone, and you can cleave and package some of the peptides that are produced from one side of a furin site versus the other into different vesicles," Sweedler says. "Does that imply that the same cell could release different peptide hormones depending on how you stimulated it? These types of fundamental questions aren't known."

Changes in the specific hormones released by cells can change behaviors, he points out, so cataloguing the distribution of hormones and other signaling molecules within cells could help identify when things go awry. "You first have to have a good way of measuring something before you know its exact impact on disease," Sweedler says.

## Inside the trash compactor

Although vesicles enable communication between cells, lysosomes could be what keeps our bodies young. Most eukaryotic cells contain lysosomes, membrane-bound organelles full of digestive enzymes to break down cellular proteins or destroy bacteria and viruses. They also have very active metabolism. "Lysosomes are essential for maintaining energy and metabolic homeostasis, signal transduction, and recovery of damaged proteins and organelles," explains Wei Xiong, of the University of Science and Technology of China, in Hefei.

Lysosomes play a role in cellular senescence as well as cancer, and understanding the metabolic processes taking place inside could unlock new information about how to slow down aging or about new drug targets for tumors. But lysosomes are highly heterogeneous: a single cell may contain hundreds, all differing in size, density and enzymatic composition. Although researchers have identified a few different types of lysosomes, "so far, there is no satisfying classification method for lysosomes," Xiong says. "Considering their enormous heterogeneity in physiological and pathological processes, the development of single-lysosome technology is a prerequisite for fully understanding the features of different types of lysosomes."

Now, Xiong and colleagues have created a single-lysosome mass spectrometry (SLMS) platform that combines lysosomal patch-clamp recording with induced nanoelectrospray ionization mass spectrometry[7]. This allows the simultaneous detection of the lysosome's electrophysiological properties and its metabolome.

Based on their metabolite content, the lysosomes could be classified into five subtypes. Two of these subtypes corresponded to previously postulated lysosome types, autolysosomes and endolysosomes, and the other three are novel. "We surprisingly found that there was a striking one-to-one correspondence between the five subpopulations of lysosomes from completely different cell types," Xiong says.

The SLMS method also successfully detected a significant drop in amino acids, saccharides and their derivatives, suggesting that as the cell ages, lysosomes

become less efficient at breaking down large biomolecules. Similarly, when they tested cancer cells, the researchers found that certain metabolites increased and some decreased in the different types of lysosomes.

"More and more evidence shows that senescence and cancer are closely related to lysosomes and their internal metabolic processes," Xiong says. By studying metabolic function changes in different types of lysosomes during aging or cancer, he says, it may be possible to develop new anti-aging or anti-cancer drugs.

## Whence biosynthesis

Spatially resolved metabolomics can also provide evidence for how a cell is conducting its chemical business, such as biosynthesis. When cells divide, they need a way to make their own building materials. De novo purine synthesis is the process by which cells make more purine nucleotides from scratch. This method is favored by cancer cells and other fast-proliferating cells, whereas normal cells tend to recycle their purines through a salvage pathway.

Working with Stephen Benkovic, who studies enzyme complex assembly and kinetics at Penn State, Tian used GCIB–SIMS to look for "purinosomes," biosynthetic hotspots in the cell that would prove the hypothesis of enzyme clusters, or the so-called "metabolon." Purine biosynthesis draws on input metabolites from the mitochondria, and the researchers hypothesized that the intermediates and end products of de novo purine biosynthesis would be localized in confined spots near the mitochondria rather than diffused throughout the cell.

Indeed, that is what they found. "We used the GCIB–SIMS to localize the intermediates," Tian says. GCIB-SIMS provided a high-resolution map of the cell, made up of voxels 1 micron by 1 micron in size—small enough to capture a purinosome. They found direct evidence for metabolic channeling within the purine-synthesizing enzyme complex and for the association with mitochondria. They discovered discrete pockets of high concentration of the intermediate metabolite AICAR, as well as other telltale intermediates of the purine biosynthesis process[8]. "This really opens a new opportunity for in situ single-cell metabolomics," Tian says. "It could assist the discovery of therapeutically important metabolic vulnerabilities for cancer treatment."

## Toward the clinic

As techniques for single-cell metabolomics continue to proliferate and improve, it's easy to imagine a metabolomics revolution taking the baton from the earlier omics and finally bringing the potential of personalized medicine to fruition. Metabolomics poses more up-front challenges than genomics or transcriptomics, but as researchers start to overcome those challenges, the benefits of metabolomics become more prominent.

"Metabolomics, which is based on mass spectrometry, is the cheapest and fastest of the omics," says Alexandrov. "Sequencing, by comparison, is very expensive. Single-cell genomics revelations are grand and transformational, they show a lot of really cool stuff, but the associated costs make it very hard to apply them in a clinical setting."

For example, Alexandrov envisions a personalized medicine approach for cancer using metabolomics on organoids grown from patients' tumor samples. Instead of treating the patient with a drug and waiting to see whether the cancer responds, multiple drugs could be tested simultaneously on organoids. Single-cell metabolomics could then reveal how tumor cells responded to each drug. "If we can measure this, then we can select the therapy that restores the healthy state of cells in the best way, and we can recommend it for the treatment," Alexandrov says.

"The real advantage is these techniques are opening up the possibility of looking at what cells actually do in nature and not what their capacity is in an artificial system," says Fernie. "That's the power of the approach." ❐

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Published online: 3 December 2021
https://doi.org/10.1038/s41592-021-01333-x

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