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Unraveling Cellular Biochemistry, One Cell at a Time

New technologies help researchers profile the contents of individual living cells | By Bennett Daviss

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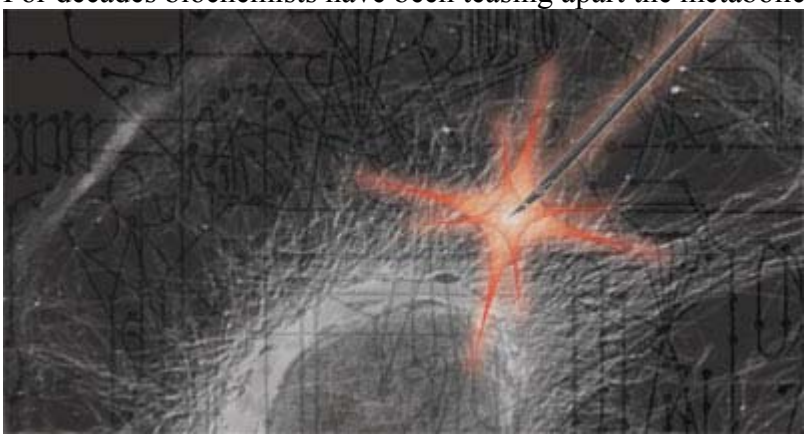


For decades biochemists have been teasing apart the metabolic circuits that power eukaryotic cells. Their descriptions of how enzymes work and play together have illuminated everything from carbohydrate metabolism to DNA replication. Yet technical limitations have forced these scientists to record the behavior not of individual proteins in single cells, but of populations of proteins in millions of cells. The result is an average, idealized description of intracellular behavior, which is the functional equivalent of trying to understand what a group of people is thinking by listening to the roar of a crowd.

Recognizing that shortcoming, a new breed of biochemist is now embracing the single-cell approach. Armed with new electrophoretic and fiber-optic methods, they are asking questions at the cellular level that were previously impossible, and discovering new insights into the behavior of cells along

the way.

CAPILLARY ELECTROPHORESIS Norman Dovichi, professor of chemistry at the University of Washington, Seattle, has been profiling the contents of individual cells for nearly a decade. In 2004 his team used a form of two-dimensional capillary electrophoresis to fractionate the proteins in individual mouse bone precursor cells and in cultured breast cancer cells. The team was able to detect differences in the cellular "protein landscapes" before and after transfection of a new gene, and before and after the onset of apoptosis.[1]

The whole process takes place in a capillary, like the kind used in modern DNA sequencers. Dovichi draws a cell into the capillary, cracks it open, labels the proteins with a fluorescent dye, and separates them by mass. "That gives us a bunch of bands or peaks with many different proteins present in each," he explains. Then he transfers each band in succession to an adjoining capillary, where the proteins are parsed by hydrophobicity using a technique called micellar electrokinetic capillary chromatography. "Combining the two allows us to characterize a sample much better than using either method alone," says Dovichi.

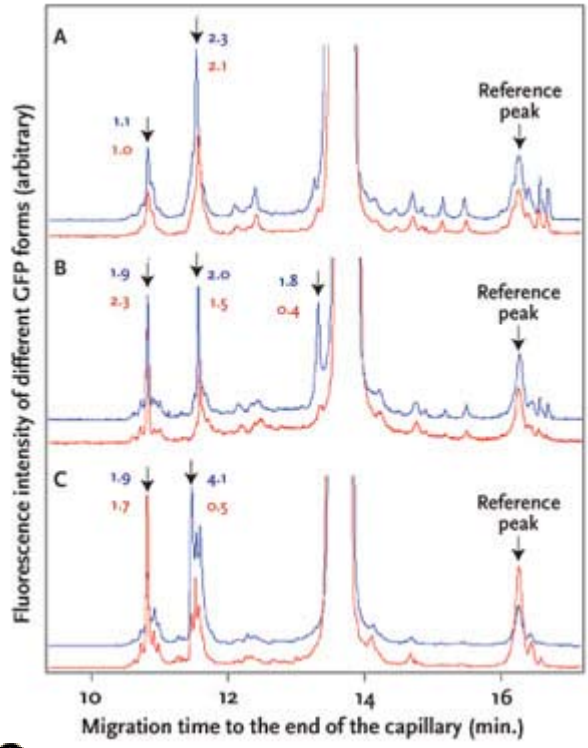
The National Institute of General Medical Sciences awarded Dovichi $680,000 over four years to develop an instrument that can analyze 1,000 cells per day and identify at least 200 distinct proteins in each. The work is on track, he says. "Two years ago, it took us eight hours to analyze a single cell; today it takes us one hour. By this time next year, we'll be doing five an hour .... How we progress from here is based on multiplexing the instrument," he says, but he expects to beat his four-year deadline.

Complementing Dovichi's effort is Sergey Krylov. A former researcher in Dovichi's lab, Krylov is now an associate professor of chemistry at Toronto's York University. Working with Dovichi, Krylov realized that studying single cells was the key to many of life's best-kept secrets.

"Early embryogenesis, where you have only a few cells, stem cells which divide asymmetrically – if you want to study these things, you have to deal with single cells," Krylov says. In turn, "understanding the molecular mechanisms of many fundamental biological processes requires simultaneous analysis of a large number of the chemical species within a single cell."

To do that, he and his research group have refined chemical cytometry, the identification of chemical components in individual cells. "Classical cytometry allows simultaneous assay of only a limited number of species, determined by the number of optical channels available, which is rarely more than five," he says. Krylov's team is creating cytometry gear that both expands the number of compounds identified and increases the sensitivity.

Courtesy of Sergey Krylov



SISTERS, BUT NOT IDENTICAL: Differences in GFP expression patterns between sister 4T1 cancer cells stably transfected with a GFP-expressing construct. Panels A-C show pairs of sister cells with varying levels of differences in the GFP expression patterns between the sisters. Numbers indicate relative intensities of adjacent peaks (marked with an arrow) of corresponding colors. The intensities were normalized to those of the reference peak.

But Krylov's chief breakthrough has been the development of what he calls "two-channel chemical cytometry." Two cells are injected into parallel capillaries and opened simultaneously. Their contents are then sorted by the same power source and analyzed by the same laser light to eliminate potential sources of variation. "Our concern was to make sure that what we observe is not a difference in our instrumentation but between cells themselves," he explains.

Such precision is critical because the team uses the instrumentation to address some fairly subtle questions. One example: whether the daughters of a dividing cell really are identical. By comparing expression of green fluorescent protein in a stably transfected cell line, Krylov's team was able to report in 2004 that in fact they are not identical.[2]

"This is a controversy in the cancer paradigm," Krylov says in an interview. "Many people treat all cancer cells as clones because they develop by dividing. What we have shown is that, when cancer cells divide, you see individual chemical signatures from cell to cell almost immediately after division."

Similarly when stem cells divide, one remains pluripotent while another develops into something more specialized. "Some molecular mechanism controls this asymmetry and we know nothing about it because, until now, we couldn't analyze those chemical signatures of individuals."

Now Krylov is working on a high-throughput version of his system that can compare many cells at

once. But first he will have to get a new assistant in his lab, as the graduate student who devoted himself to developing the two-channel instrumentation has left research. Says Krylov, "He said that he would go into something that doesn't require you to work for five years to get a result and then be happy for three days."

CHECKING SUBCELLULAR LOCALIZATION At the University of Minnesota Edgar Arriaga, an associate professor of chemistry, uses capillary electrophoresis to fractionate subcellular compartments instead of proteins. "We wondered if, instead of just dissolving away a cell in order to analyze it, we could gently peel off the cell membrane and release everything inside intact," he says. "From a single cell, we could get one separation pattern that tells us about the mitochondria, another that tells us about the acidic organelles, and so on."

Arriaga sees two immediate and practical applications. One is monitoring the intracellular distribution of a drug. The second application involves aging. As the body grows older, its energy-producing mitochondria, which have their own DNA, stockpile debilitating mutations that pass to their offspring. "If we can begin to look at the accumulation of damaged DNA in individual mitochondria," he says, "we could perhaps start to get an idea of why and how this happens."

The trick to keeping cell components intact is to make the inside of the capillary as much like the interior of a cell as possible. "It has been very tricky," he says. "We copy the pH, the ionic strengths and balance, the salts; we make buffers that emulate the cell contents. That allows us to do the analysis inside the capillary without losing the information that subcellular components carry individually."

So far, Arriaga's methods can isolate the inner components not of one cell, but of 100 or more at a time. But his instruments, which are modifications of those that Dovichi and Krylov use, "have the sensitivity to analyze the properties of an individual mitochondrion, acidic organelle, or nucleus," he says. Recently, Arriaga's team used the technique to monitor the sub-cellular distribution of aminoacyl tRNA synthetases.[3]

THINKING OUTSIDE THE CAPILLARY Not everyone thinks capillaries offer the best approach to single-cell biochemistry. Tuan Vo-Dinh, director of the Center for Advanced Biomedical Photonics at Oak Ridge National Laboratory in Tennessee, compares capillary electrophoresis to trying to understand how a clock works by smashing it with a hammer and looking at the pieces. "The cells are no longer alive," he says, "and if you work with more than one cell, the data you get is averaged over all of the cells in the sample."

For the past five years, his research group has been perfecting ways to insert fiber-optic probes into living cells and watching cellular processes unfold. His team mounts biological probes, such as antibody molecules, on the tip of fibers perhaps 30 to 40 nm in diameter, and then pushes the fibers

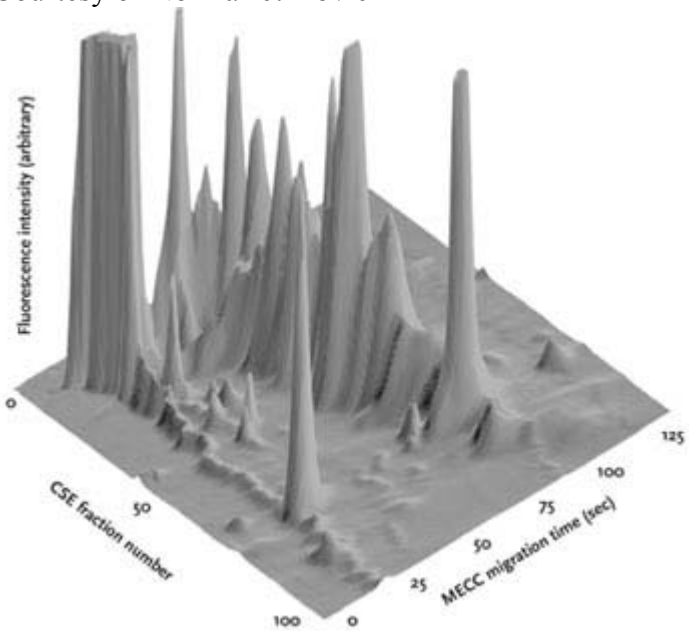
through a cell's outer membrane. When the probe encounters its target, an evanescent wave induced by laser light traveling through the fiber triggers a fluorescence signal, which is picked up by a detector at the base of the probe.

The technology, he says, "let[s] us look for the answers to questions about how cells grow and the specific cellular effects of drugs." In 2002 Vo-Dinh used a bioprobe specific for the carcinogenic pollutant benzo(a)pyrene to detect DNA adducts within individual cells, a biomarker that can indicate DNA damage years before cancer occurs.[4] More recently, his team became the first to witness the onset of apoptosis in real time, using a probe loaded with a fluoregenic peptide substrate for the early apoptotic marker, caspase-9.[5]

Some researchers have asked whether thrusting a foreign object into a cell crowds cellular components and forces reactions that wouldn't occur naturally, and also whether the cells sustain lasting damage. Vo-Dinh respects the concerns but doesn't share them.

"A mammalian cell is three to 10 microns in size while our probes are never bigger than 200 nanometers," he points out. "Also, cell membranes are very soft and fluid. It's not like poking a hole in a hard object" like an eggshell. As test subjects, he chooses cells that haven't yet divided. After probing them, he makes videos of the cells' division and tracks the offspring to check for damage. So far, he hasn't observed any.

Courtesy of Norman J. Dovichi



ANATOMY OF A SINGLE CELL: The protein content of a single MC3T3 cell revealed through two-dimensional capillary electrophoresis. Fluorescently labeled proteins were separated first by capillary sieving electrophoresis (CSE), the capillary equivalent of SDS-PAGE, and then by micellar electrokinetic capillary chromatography (MECC), which separates proteins based on their interaction with micelles. Components were detected by laser-induced fluorescence as they exited the capillary.

Raoul Kopelman, professor of chemistry and physics at the University of Michigan, remains skeptical.

As a chemist, Kopelman is steeped in the commandment that a sensor mustn't interfere with what's being detected. "But biologists aren't always that careful," he says. "Not having had any good tools, they pushed molecular probes or unprotected fluorescent dyes into cells. The trouble with those naked dyes is that proteins may interact with them, which alters the signal that they give, or that the dye may bother [cellular contents], he says. "That mutual interference is what we're avoiding."

In 1997, Kopelman and colleague Martin Philbert realized that tiny bits of harmless polymer could be used to carry sensors inside a living cell and report its findings directly as light. "People often think of photons the way they think of electricity," he says. "They think you need a wire, so they use fibers. But the fiber is cumbersome and you have to work very hard not to hurt the cell with it. Who needs it? Photons travel without fibers, so you can send them directly into the cell and get them directly out."

Kopelman tags his polymer fragments, called PEBBLEs (probes encapsulated by biologically localized embedding), with enzymes, fluorophores, or dyes and typically delivers them into cells using liposomes or a gene gun.

Each PEBBLE is pocked with pores whose sizes reflect the analyte being detected. "The polymer matrix is made so that the analyte will get through, but not the large proteins that would bother the sensing, and so the sensing element can't get out," he explains. When the targeted element links with the sensor, Kopelman reads the resulting fluorescent signal with a microscope. Recently he designed PEBBLEs that could measure dissolved oxygen using paired oxygen-sensitive and oxygen-insensitive fluorescent dyes.[6]

To make things easier, Kopelman uses PEBBLEs that blink when they meet an analyte. The group uses "lock-in" amplifiers that automatically subtract the nonblinking background, making measurements easier. The researchers also can surround "pebbled" cells with a rotating magnetic field, enticing the PEBBLEs to blink at steady rates. With that rate as a marker, Kopelman and his team can measure the physical properties, such as viscosity and drag forces, of the cellular interior.

PEBBLEs have oncology applications, too. PEBBLEs laced with iron oxide can bind to, and contrast starkly with, cancer cells in magnetic resonance imaging scans. And grains designed to bind with tumors can be loaded with pharmaceuticals for precise delivery. In tests, the PEBBLEs have carried photoactive drugs to tumors in rats' brains and killed the tumors following irradiation by a laser beam delivered through a tiny hole drilled into the animals' skulls. "Our PEBBLEs," Kopelman says, "have gone from being sensors to being actuators."

The National Cancer Institute has awarded Kopelman $12.5 million to further develop these applications. Others, like Vo-Dinh, are also pursuing medical opportunities. "In a disease like leukemia, for example, you want to remove the blood and treat it without putting back a single cell that is still diseased," says Vo-Dinh, whose lab is now developing a high-throughput clinical

application.

Given how new the field is, it will likely be a long time before either PEBBLES or optical fibers make their way to the clinic. But on the research front single-cell techniques could ultimately lead to a new class of science: single-cell 'omics. Kopelman envisions a future for his PEBBLEs in metabolomics research. "To follow what's happening in a cell, you need to be able to measure chemicals in real time" and locate them within the cell, he says, "and that's what the PEBBLEs are promising to do." Reflecting on his own technology, Arriaga says, "This will take years to fully develop, but eventually this could become another form of high-throughput analysis similar to proteomics."

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