SINGLE CELLS: SHUT YOUR EYES AND SEE

Increasingly, researchers are turning to single cells to explore basic biology. Jeffrey Perkel looks at the technology making single-cell experiments a reality.

The Sanford Underground Research Facility in South Dakota, where microbiologists study the deep subsurface microbiome, one cell at a time. Credit: R. Stepanauskas.

In late 2015, Brazilian authorities and the World Health Organization began reporting sharp increases in the incidence of microcephaly in newborn infants. It didn't take long to identify the culprit: Zika virus was a leading suspect, and within months, researchers had effectively proven its link to those birth defects.

Less clear was how a virus whose manifestation in adults is so mild that many are unaware they have it could lead to such catastrophic neurological malformations in newborns. The answer would come, in part, from an unlikely approach now finding increasing use across the life sciences: single-cell biology.

Understanding Zika

About the same time the case was closing on Zika in January of this year, Arnold Kriegstein, Director of the Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research at the University of California, San Francisco, was in the second year of a 3-year \$4.8 million project funded by the Obama administration's BRAIN Initiative to catalog different cells in the human brain. His strategy to map various cells would rely critically on the rapidly expanding field of single-cell biology. And importantly, his findings would prove crucial in understanding Zika.

Studying gene expression in the developing brain is not a new concept; multiple groups have already done so—but on a bulk tissue level. These studies have identified differences in gene expression between regions and over different developmental stages. What they cannot provide, however, are cell-specific fingerprints. “What are the genes expressed by an individual cell type? That's really what we need to know, especially if we're going to try to decompose the brain into its component parts,” Kriegstein says.

To create gene expression fingerprints, Kriegstein's team, working with the Palo Alto-based company Fluidigm, is using an automated microfluidic platform, the Fluidigm C1 Single-Cell Auto Prep system. The C1 captures up to 800 individual cells in parallel

and then isolates, reverse-transcribes, and amplifies their cellular RNA, outputting single-cell cDNAs. Applying that tool to brain tissue, the team sequenced libraries to low depth on an Illumina sequencer and used unsupervised hierarchical clustering to bin profiles into classes, each representing one particular cell type. In an initial demonstration, the team amassed profiles for more than 600 cells from the human brain and differentiated stem-cell cultures, which they were able to separate into four broad groups.

These data turned out to be particularly useful in deconstructing Zika virus pathogenesis. Other researchers had homed in on a handful of potential viral entry factors for Zika in skin cells. But which one, specifically, was the virus using in the brain? And, which cells did it target?

Alex Pollen, a postdoctoral fellow working on the single-cell gene-profiling project, noticed that one candidate Zika receptor, AXL, was highly enriched in radial glial cells, a kind of neural stem cell. “These are exactly the cells that you would want to target if you were trying to produce microcephaly,” Kriegstein says. “If you were trying to produce a smaller brain, it's the neural stem cells that you'd want to somehow damage or destroy.”

Other brain cells also express AXL, including neurological immune cells called microglia, as well as astrocytes, endothelial cells, and certain cells in the eye. Neurons largely lack AXL expression, suggesting they are not viral targets. “It was a very interesting pattern,” Kriegstein says, one that might never have been discovered had his team used bulk analysis techniques. “You really have to look at the gene expression patterns on a cell-by-cell basis,” he says. “And luckily, that's what we'd been doing.”

Unexpected aneuploidy

Other groups also are discovering unique biological insights as they explore single cells. Indeed, publication of single-cell studies has risen sharply, from 46 in 2010 to 332 in 2015. Yet, while

the field is hot, it isn't actually new. Whether via optical or electron microscopy or electrophysiology, researchers have been interrogating individual cells for decades, according to Jonathan Sweedler, Professor of Chemistry at the University of Illinois at Urbana-Champaign. "Most of what we know about a cell"—its morphology, its organellar composition, the nuances of protein trafficking, and more—"was [learned] from single-cell measurements," he says.

What has changed are the questions researchers are asking and the information content of the answers. Once limited to cellular anatomy and physiology, researchers today are armed with next-generation DNA sequencers and mass spectrometers that can interrogate an individual cell's molecular contents.

Sweedler has used microscopy-guided MALDI and capillary electrophoresis-coupled mass spectrometry to probe cell-to-cell signaling molecules in organisms from mollusks to mammals. He has matched the neuropeptide content of individual neurons with the behaviors those cells control in the sea slug, *Aplysia californica*, and probed the peptide signatures of mammalian islets. "The heterogeneity in the suite of molecular players used by the cells in the nervous system, even in adjacent cells, continues to surprise me," he says.

Yet accessing that heterogeneity isn't easy. There isn't enough DNA or RNA in a single cell for direct sequencing, so those macromolecules must first be amplified, leading to inevitable concerns about amplification bias. Proteomics and metabolomics studies don't have that option, so those measurements inevitably push instrument boundaries. And of course, when dealing with individual cells, differentiating biology from stochastic noise takes on added complexity. "I'm sort of a masochist by nature," jokes Michael McConnell, Assistant Professor of Biochemistry and Molecular Genetics at the University of Virginia School of Medicine, who uses single-cell techniques to study genetic variation in the brain.

That said, single-cell studies can be incredibly valuable. That's because even a seemingly uniform cell population differs from cell to cell on multiple levels. The cells may be at different stages of the cell cycle, for instance, or experiencing subtle differences in external

Rusty Lansford is applying single-cell transcriptomics and dynamic cellular imaging techniques to quail embryogenesis. Here, 10 time points of fluorescent protein-labeled primordial germ cells are superimposed to reveal the complexity of cellular motion. Credit: D. Huss, B. Benazeraf, and R. Lansford.

stimulation, all of which can influence gene expression programs. But, at the most basic level of DNA sequence—the genome—cells of the body were traditionally not thought to vary.

As it turns out, that's not strictly true. In 2001, McConnell was a graduate student in the lab of Jerold Chun, then at the University of California, San Diego, when he and his colleagues, including Stevens Rehen and Dhruv Kaushal, reported a remarkable discovery: Using fluorescence microscopy and chromosomal painting techniques, they determined that cells in the mammalian brain were not genomically identical, as researchers and textbooks had thought. Rather, about a third of dividing cells were either missing large chunks of genetic material, or had a surfeit of it, producing a genetic mosaic. "That was the

first description of this type of phenomenon occurring in the brain, to my knowledge," says Chun. A subsequent study in 2003 suggested that this mosaicism could alter gene expression.

In a sense, those findings were not surprising. In the early 1990s, Chun discovered that neurons in the brain express some (though not all) of the same enzymes required for immunoglobulin and T-cell receptor rearrangement in lymphocytes. Yet, which DNA regions (if any) might be their targets in the brain was anybody's guess. In 2001, the first human genome had just been sequenced, so figuring out precisely which DNA sequences were added or missing in these cells was out of the question. That didn't stop McConnell from pondering the possibilities. "I remember saying, somewhat absurdly, facetiously, 'well, when we figure out how to sequence a single cell's genome, then we can really get at this problem!'"

Fifteen years later, he and others are now doing that. In 2013, McConnell, working with his former postdoctoral advisor Fred Gage from the Salk Institute, used sequencing and SNP analysis

of 110 individual cells to delve deeper into the phenomenon of "somatic mosaicism," discovering that 13%-41% of cells in the brain contain megabase-sized copy number variations (CNVs)—duplications and deletions smaller than the aneuploidies described in 2001, yet still likely to impact cellular phenotype. "You could be altering the ion channel composition," he says, "and that can directly affect physiology."

Arnold Kriegstein with a Fluidigm C1 Single Cell Auto Prep station. Credit: S. Bromberger.

Sweedler's single-cell proteomics workflow combines MALDI and capillary electrophoresis-based mass spectrometry in a two-tiered process. Image and data from the Sweedler laboratory at the University of Illinois at Urbana-Champaign, prepared by Troy Comi.

More recently, Gage and his team determined that at least part of the documented somatic mosaicism could be caused by ubiquitous “junk DNA” called LINE-1 elements. LINE elements are retrotransposons that make up some 17% of the human genome. Most of the sequences are dead, but some are still ticking, and they can hop from place to place. In December 2015, Gage reported that outward-directed promoters in those elements can introduce neuropeptide diversity as they move about the genome. And in a recent study in *Nature Neuroscience*, Gage and his team, led by geneticist Jennifer Erwin and computational biologist Apua Paquola, found that even those that don't hop can participate in recombination-based mutagenesis, resulting in relatively large deletions and insertions—a discovery they made using a new single-cell targeted sequencing approach called SLAV-seq (somatic LINE-associated variant sequencing). “The LINES themselves are contributing to this variation, but it's not through jumping in, but through their structure, which allows these cuts to occur,” Gage says.

Among other things, such variation could potentially explain the behavioral differences among otherwise identical inbred animals, Gage says—not to mention sporadic neuropsychiatric disorders in humans, such as schizophrenia, as well as more subtle neurodiversity variants.

Indeed, an analysis of 320 single brain cells using a battery of methods including qPCR, FISH, and CNV detection led Chun's team to discover an apparent link between copy-number mosaicism of the amyloid precursor protein (APP) locus in the prefrontal cortex and sporadic (as opposed to familial) Alzheimer's disease—a finding that highlights the functional consequences of genetic mosaicism.

If nothing else, says McConnell, these results could potentially explain the difficulty researchers have faced in nailing down strong genetic links to certain neurological diseases via genome-wide association studies (GWAS) and genome sequencing. Generally speaking, those studies are performed using blood samples, not brain tissue, based on the idea that the genome in one tissue is identical to other tissues—an assumption now known to be incorrect.

Mice with wings

James Eberwine, Co-director of the Single Cell Biology Program at the University of Pennsylvania Perelman School of Medicine, is one of the pioneers of modern single-cell biology. He has been developing and improving methods for analyzing gene expression at the cellular level since the late 1980s—what he calls “the end of the Jurassic, beginning of the Cretaceous period in terms of molecular biology.”

According to Eberwine, who has also taught courses on single-cell methods at Cold Spring Harbor, there's little doubt single-cell methods provide information that would otherwise be obscured at the population level. His lab has used such techniques to document cell-to-cell differences in mRNA abundance, demonstrate cytoplasmic RNA splicing, and capture RNAs in neural dendrites, an area once thought to be largely denuded of transcripts.

But he also stressed that the technology has to be used carefully. “The key, the one thing I'd like for people to take home [is], it's relatively straightforward to do single-cell analysis now,” Eberwine says. “But it's not that straightforward to do good single-cell analysis.” Data interpretation and controls are key.

That's a lesson Rusty Lansford, Associate Professor of Radiology and Developmental Neuroscience at the University of Southern California and Children's Hospital Los Angeles, has taken to heart.

Lansford is a recent convert to single-cell -omics, having teamed with Eberwine to combine the technique with his own approach: long-term live-cell imaging of vertebrate embryogenesis. His model: the Japanese quail, *Coturnix japonica*.

Quail, Lansford explains, are a “goofy” but “logical” choice for such studies. Mice are far more widely used, but imaging embryogenesis in a mammal requires anesthetizing and immobilizing a pregnant female and opening a window into the uterus to watch what happens—treatments that may well influence biology. Chicken eggs are a popular alternative, but chickens are relatively large, require a dedicated facility, and reach sexual maturity slowly. Quail, which are smaller and able to reproduce earlier, occupy the middle ground—“a mouse with wings,” as Lansford puts it.

The Lansford lab has been developing methods to image cellular movement during quail development. In a typical experiment, he expresses a red fluorescent protein under a constitutive promoter in the nuclei of all cells of an embryo, then crosses that animal with another transgenic that expresses yellow fluorescent protein under the control of, say, an endothelial cell-specific promoter. He then images the developing embryos through a hole in the egg using a confocal or two-photon microscope to track where each cell goes.

After a period of development, specific cells are excised, cell types are identified using immunohistochemistry, and then RNAs are sequenced. While the data have not yet been published, the

From left, Apua Paquola, Rusty Gage, and Jennifer Erwin are exploring LINEs in single cells. Credit: J. Belcovson, Salk Institute.

team used their strategy specifically to label primordial germ cells, finding that they can observe a transition in transcriptome composition in individual cells as they differentiate from stem cell-like to more mature germ cells.

These experiments are challenging to perform, but the data analysis is far more difficult. “We are learning to embrace the uncertainty inherent in single-cell experiments,” Lansford says. “We must view certain events with an understanding of the stochastic nature of cellular events.”

Host-pathogen interactions

For Ramunas Stepanauskas, Director of the Bigelow Laboratory Single Cell Genomics Center in East Boothbay, Maine, single-cell biology is not so much about stochasticity as expediency.

Stepanauskas is a microbial ecologist—a tough field of study because the vast majority of microbes cannot be cultured. Over the years, microbiologists have devised multiple strategies to address the unculturable problem, including both 16S ribosomal RNA profiling and metagenomics. Shotgun metagenomic techniques are particularly effective for identifying individual genes that are encoded by a microbial community, Stepanauskas says. But working out how those genes fit together into whole genomes can be difficult. Making the connections between separate DNA molecules in a cell, such as those of the host and an infecting virus or a symbiotic cell, is even more so.

The most recent iteration of culture-free technology is single-cell genomics, which for Stepanauskas means capturing indi-

vidual cells by FACS, lysing them, amplifying their genomes, and sequencing the resulting DNA—all of the DNA. That includes not only the genomes of the microbes themselves, but also any DNA they may have picked up from the environment, including that of the pathogens that have infected them.

“The composition of DNA molecules inside a cell can be quite complex,” Stepanauskas

explains. “By analyzing all of it, we can see how they fit together, to figure out who is eating whom, who is infecting whom, and the symbiotic interactions in the community.”

Applying single-cell sequencing to seawater, Stepanauskas’ team found lytic viral infections in a third of microbial cells sampled in the sunlit ocean, including pathogens targeting Marinimicrobia, Verrucomicrobia, and several groups of Proteobacteria that are key components of the ocean’s microbiome but resist cultivation in the lab. In contrast, the slow-growing Firmicutes, *Desulforudis audaxviator*, which makes its home in one of the world’s deepest gold mines, contained only those viruses that had integrated into the host genome—a slow-burn form of infection. “It’s an interesting adaptation to the extremely low-energy environment in deep subsurface where lytic infections may be hard to sustain,” he says.

There’s no denying single-cell methods have brought unexpected biology to light, and continue to do so. In his talks, Eberwine likes to close with a quote from James Joyce’s *Ulysses*: “Shut your eyes and see.”

“We all have preconceived notions about what biology is telling us,” he says. “But you do experiments to assess whether or not that’s true or not.” Increasingly, those experiments are being done on single cells. Who knows what they will tell us next.

Written by Jeffrey Perkel, Ph.D.

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