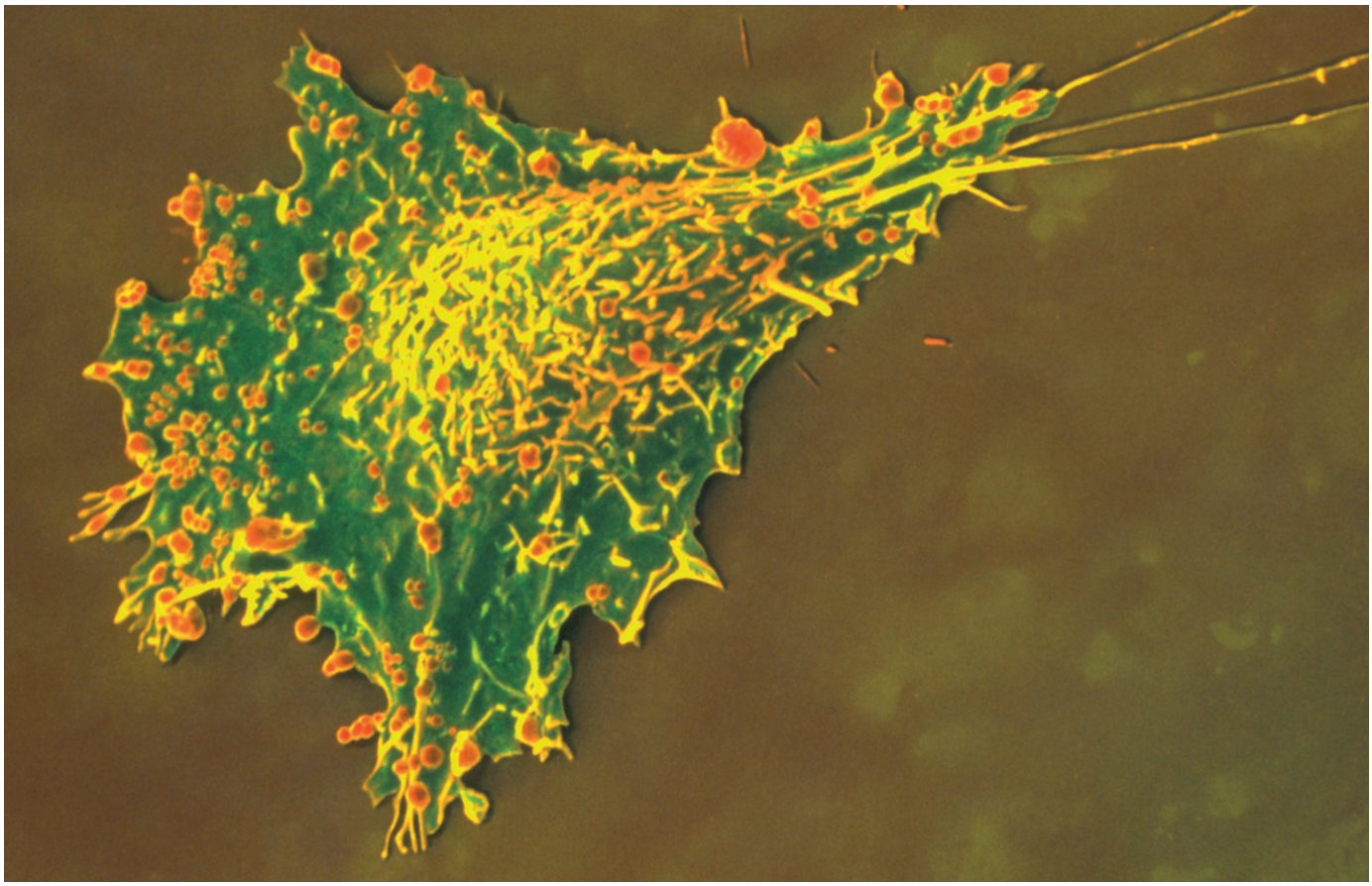
TECHNOLOGY FEATURE

THE DEEPEST DIFFERENCES

To understand biological heterogeneity, researchers are learning how to profile the molecular contents of individual cells.

S. GSCHMEISSNER/SPL



Studies are uncovering the molecular biology of individual tumour cells, such as this breast-cancer cell, seen in a coloured scanning electron micrograph.

BY CHARLOTTE SCHUBERT

James Eberwine, a neuroscientist with a penchant for invention, helped to pioneer a technique that is now routine. In the early 1990s, he sucked the contents out of a single cell with a pipette, and examined the expression of a handful of genes using molecular techniques that amplify RNA. His data verified a long-held assumption — that electrical activity in a neuron simultaneously changes the abundance of multiple RNAs inside it[1]. But other researchers were sceptical. At the time, just about the only way to detect RNA in a single cell was by labelling molecules using fluorescence *in situ* hybridization. "People were used to using microscopy to look at RNA; they wanted to see it," says Eberwine, who works at the University of Pennsylvania in Philadelphia.

Things have changed since then. Gene-expression analyses leaped forward in the mid-1990s with the invention of the microarray. And the rise of high-throughput RNA sequencing, or RNA-seq, which spits out the sequences of thousands of cellular RNAs at once, has enabled researchers to reveal the collection of active genes in a cell in a single readout.

Eberwine and others are using sequencing, and techniques such as microfluidics and flow cytometry, to profile single cells — cataloguing RNA molecules, sequencing DNA and even profiling metabolites and peptides.

Studies indicate how strongly cells can show their individuality. Brain cells may express as few as 65% of the same genes as their neighbours, according to an unpublished analysis by Eberwine. In the immune system, cells placed in the same category on the basis of surface markers can express different sets of genes, and have different responses to vaccines[2]. And as tumour cells evolve, their genomes quickly ▶

become twisted in unusual ways.

Single-cell techniques let researchers track and catalogue this heterogeneity. They may be the only way to get at some fundamental questions, such as what makes individual cells different biochemically and functionally. How much is each cell influenced by its microenvironment, and what is the role of stochasticity — random 'noise' in the behaviour of cellular molecules?

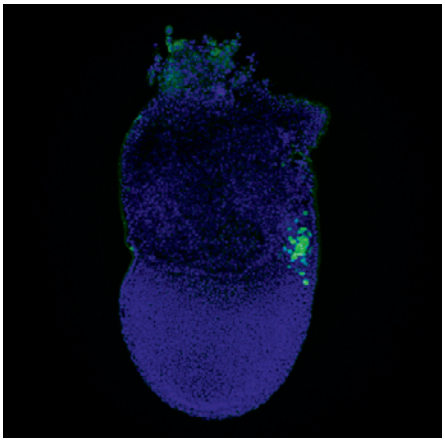
These questions are getting more attention (see page 139). In 2009, Eberwine co-organized a meeting on single-cell analysis at Cold Spring Harbor Laboratory in New York, along with Sunney Xie, a single-cell biochemist at Harvard University in Cambridge, Massachusetts. The meeting drew 47 attendees. This July, 120 people went to the second such meeting. And the US National Institutes of Health (NIH) has launched an initiative to support single-cell techniques (see 'The NIH gets singular').

But single-cell analysis is still an emerging field. Many researchers say that protocols from academic labs are often superior to commercial kits. "With any nascent field, there are lots of different approaches," says Eberwine. "People are trying lots of things to see if they can make the techniques more sensitive, more representative of the state of a cell, easier and cheaper."

PROFILING HETEROGENEITY

The classic biochemical approach is limited, say single-cell researchers. Grinding up and analysing the contents of large pools of cells — a procedure undertaken in thousands of labs every day — averages out the results, says Timm Schroeder, director of the Institute of Stem Cell Research at the German Center for Environmental Health in Munich. But, says Schroeder, "it's the individual cell that makes a decision" such as whether to fire an electrical impulse, migrate or differentiate into a new cell type.

Looking at single cells to uncover the impetus for such decisions means doing fussy experiments on a very small object: a cell might span about 10 micrometres and contain less than 1



S. KIM/M. A. SURANI

Studies can probe single cells, such as those in the mouse embryo that give rise to gametes (green).

picolitre (1×10^{-12} litres) of cytoplasm. And some key regulatory molecules are scarce — just a few, hard-to-detect RNAs can exert a big effect on a cell.

Many established techniques are only now being applied to single cells. Fluorescent tagging and microscopy can be used to analyse molecules that have already been characterized. To profile previously unexamined molecules, there is transcriptome analysis — cataloguing the set of RNAs expressed in a cell — as well as high-throughput methods based on microfluidics or flow cytometry. But getting such techniques to work on single cells is not easy, says Schroeder, whose research involves long-term imaging of individual bone-marrow cells. Single-cell applications are, he says, "at least one level more demanding and complex than the conventional approaches".

And the unexplored biological terrain is vast. "We don't even know what we are getting into in terms of heterogeneity," says Sherman Weissman, a geneticist at Yale University in New Haven, Connecticut.

Whatever the study, the first step is generally getting hold of the cells. When Eberwine first struggled with studying gene expression in a neuron 20 years ago, it was difficult even to get intact RNA out of a single cell. Eberwine solved the problem by capturing the material in the same pipette used to measure electrical activity. Now, researchers can use a variety of techniques to pick out single cells, from enzymatic digestion, which releases cells from tissues, to laser-capture microdissection. But it is still tricky, says Eberwine. "A major technical issue is how do you do that initial capture."

Eberwine has used his pipette-capture system to study individual warm-sensitive neurons[3], which regulate core body temperature and underlie fever. Together with Tamas Bartfai, a neuroscientist at the Scripps Research Institute in La Jolla, California, and his colleagues, Eberwine examined the cells' transcriptomes. The researchers identified transcripts for G-protein-coupled receptors — potential drug targets — that went undetected in screens of pooled cells.

As techniques improve, they are letting researchers explore the heterogeneity within a cell. By cutting branches, or dendrites, off neurons, Eberwine and his colleagues have discovered that RNA in dendrites can retain nucleotide sequences that target the RNA to that location[4]. They could not have found such information by analysing the RNA of an entire neuron.

"It's the individual cell that makes a decision."

Developing technology will produce even more fresh data. Most biologists will need to work closely with computational biologists to evaluate the huge data sets that will result from cataloguing thousands of molecules in numerous single-cell experiments.

EXTRA-LOUD AMPLIFICATION

Perhaps the best-known single-cell profiling technique is transcriptomics. Azim Surani, a developmental biologist at the University of Cambridge, UK, uses this method to examine cells of the early embryo, which are hard to study in large batches because they are so rare. He is tracing how, such cells turn into pluripotent embryonic stem cells in culture.

Surani has adapted a single-cell protocol for the polymerase chain reaction (PCR) to work with RNA-seq. To do this, he has collaborated with technical experts such as Kaiqin Lao, a molecular cell biologist at Applied Biosystems in Foster City, California (a subsidiary of Life Technologies in Carlsbad). In the cells of the early mouse embryo, the team detected the expression of some 12,300 genes — 75% more than were detected by microarray techniques[5]. Lao says he can now get his PCR technique to work with 1 picogram of RNA — one-tenth the amount of RNA in a typical cell.

The published protocol amplifies molecules only if they are no more than 3 kilobases long, so it misses about 40% of transcripts, says Lao. He and his colleagues are using different

The NIH gets singular

The challenges of single-cell analysis have caught the attention of the US National Institutes of Health (NIH). The agency has launched a programme to fund advances in single-cell research, with a budget of around US$90 million over five years from the NIH Common Fund, which backs science that crosses disciplines. Grant applications are due early next year, and the NIH expects to make the first awards by September 2012, says Andrea Beckel-Mitchener, a programme officer at the NIH campus in Bethesda, Maryland. The programme will fund new techniques in areas ranging from microscopy to biochemistry, and foster their commercialization. The NIH also sees a big need for tools to examine cells in their natural environment.

Many of the techniques need an extra push. "It's still really difficult for individual labs to move into that area; the group of researchers who work on this is still highly specialized," says Beckel-Mitchener. "If you want to reach the next level you really have to push the envelope." C.S.

enzymes to increase that; Lao can now amplify 10-kilobase transcripts, corresponding to about 99% of transcription, he says.

Another technique to amplify a cell's RNA is antisense RNA (aRNA), an *in vitro* transcription technique from Eberwine and his colleagues[6], in which a cell's RNA is copied into a stable DNA library, with each DNA molecule containing a short sequence recognized by an RNA polymerase. The polymerase uses the DNA library to make multiple copies of the RNA.

"Every neuron is probably different from every other neuron."

Each approach has its advantages, and its problems. Bias can be introduced to PCR when certain sequences dominate during amplification, so approaches based on this technique are less quantitative than aRNA. But aRNA is less efficient than PCR, and can take days, notes Weissman.

Commercially available aRNA kits include TargetAmp from Epicentre Biotechnologies of Madison, Wisconsin (owned by Illumina of San Diego, California), and MessageAmp from Ambion of Austin, Texas, which is owned by Life Technologies. Both can work for single cells, says Eberwine. Companies such as NuGEN in San Carlos, California, and Sigma-Aldrich in St Louis, Missouri (in partnership with Rubicon Genomics of Ann Arbor, Michigan), have products designed for small amounts of RNA, and some say that their systems can work for single cells. It is unclear when Life Technologies might release a product based on Lao's method, but both Eberwine and Lao report that their single-cell techniques are being used successfully in other labs.

THE GENOME GAP

Many researchers want to analyse not just the transcriptome of a cell, but the underlying genome. This would be particularly relevant for cancer cells, with their warped DNA, and Life Technologies is offering US$1 million to the first researchers to sequence the entire genome and RNA content of a single cancer cell using the company's technology.

Nicholas Navin, a geneticist at the MD Anderson Cancer Center in Houston, Texas, is one of only a handful of researchers who have sequenced the genomes of single cells from eukaryotic organisms. This year, in collaboration with Michael Wigler, a geneticist at Cold Spring Harbor, and his colleagues, Navin sequenced the DNA of 100 individual cells from different parts of each of two human breast tumours, tracing how the cancer evolves as it spreads[7]. It took several years and cost about $2,000 per cell; the cost has since fallen to about $200 per cell, he says. In the end, Navin was able to reliably cover about 6% of the genome of a cell — enough to assess some larger copy-number aberrations, but not to look at the accumulation of point mutations during tumour evolution.

The limitation, say Navin and other researchers, is the technique used to amplify the DNA: whole-genome amplification, which

Beyond amplification

Unlike DNA and RNA, some cellular molecules cannot be amplified. Very few labs have tried to profile proteins, metabolites or peptides in single cells. "The analytical challenges are extreme," says Renato Zenobi, a chemist at the Swiss Federal Institute of Technology in Zurich.

To assess populations of proteins, some researchers have used fluorescent tagging. Sunney Xie, a single-cell biochemist at Harvard University in Cambridge, Massachusetts, for instance, has harnessed a microfluidic device to quantify the set of proteins and RNA in the *Escherichia coli* bacterium, using a fluorescent-protein reporter library[12].

Sherman Weissman, a geneticist at Yale University in New Haven, Connecticut, has hopes for ribosome profiling, a molecular technique to monitor protein production. The procedure involves obtaining a sequence of RNAs (about 28 nucleotides long) that have been sequestered by protein-making ribosomes[13,14]. The method works with pools of cells, but it may have potential for single cells, says Weissman.

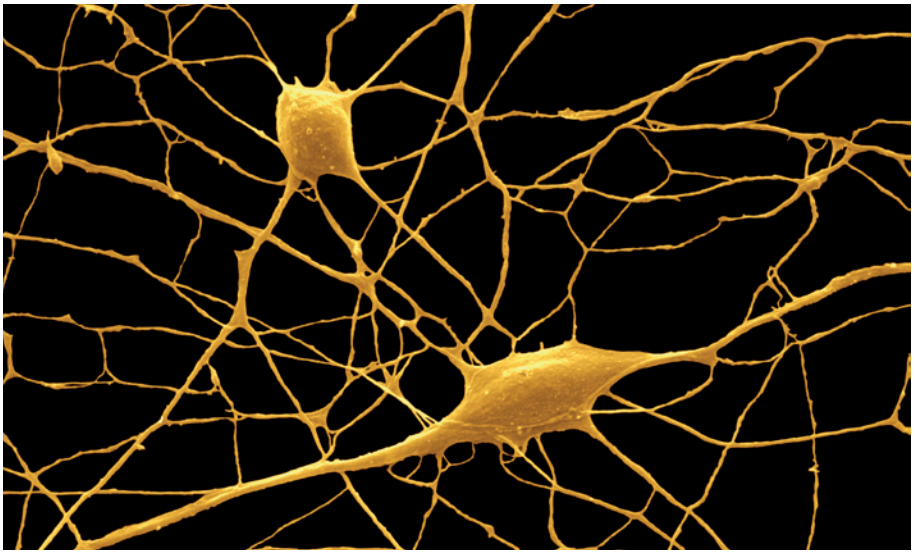
Daniel Chiu, a chemist at the University of Washington in Seattle, has a device that can add reagents to label a cell or organelle and send its contents — one molecule at a time — to an instrument that can count fluorescent molecules[11]. His team has hooked this up to another microfluidic system: a high-throughput device to isolate single cells from a population. With this two-step system, the group can isolate cells and analyse their molecular components. The researchers have adapted the system for the detection and analysis of circulating tumour cells and are launching a company based on the technology: MiCareo, in Taipei, Taiwan.

Researchers working with Garry Nolan, a geneticist at Stanford University in California, and Scott Tanner, a biomedical engineer at the University of Toronto in Canada, are revamping a tried and true high-throughput technique — flow cytometry, which relies on the detection of fluorescent tags, and generally measures six to ten parameters simultaneously. The team has developed 'mass cytometry'. The technique can simultaneously measure 34 parameters in a single cell[15], including protein phosphorylation and cell-surface antigens, and has the potential for some 100. The instrument that they use evaluates up to 1,000 cells per second, and relies on antibody-bound tags consisting of rare-earth elements detected by mass spectrometry. It is sold by DVS Sciences in Sunnyvale, California, and costs US$600,000.

Mass spectrometry is routinely used for profiling proteins and metabolites, but does not have the sensitivity of optical techniques such as fluorescence. Very few proteins are present at more than 100,000 copies per cell, so most are below the limit of detection for commercially available mass spectrometers. But researchers are making progress, mainly with more abundant metabolites and peptides. Researchers at the University of Illinois at Urbana–Champaign, for instance, have used matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to profile the major metabolites and peptide neurotransmitters released in a large neuron of the sea slug *Pleurobranchea californica* in response to hunger[16]. In unpublished work, Zenobi and his colleagues have generated a map of the yeast metabolome using MALDI mass-spectrometry data from single cells. He says that he can detect metabolites in the low attomole range (around 1 million molecules), and can identify more than 200 correlations — for instance, whether increases in one metabolite correlate with decreases in another.

Other researchers use different mass spectrometry methods. Cynthia McMurray, a neuroscientist at Lawrence Berkeley National Laboratory in Berkeley, California, is constructing single-cell metabolic maps from thin sections of brain, comparing healthy, aged and diseased mice using nanostructure-initiator mass spectrometry. She ultimately aims to create a three-dimensional metabolic map of the brain.

Zenobi is negotiating with Sigma Aldrich in St Louis, Missouri, about commercializing a micrometre-scale device[17] that prepares multiple single cells on an array for analysis by MALDI mass spectrometry. The device may be available as early as next year. "Once people realize these types of analyses can be done, they will jump on them," he says. C.S.



D. MCCARTHY/SPL

Researchers are going beyond single cells to profile parts of cells, such as neuronal extensions.

relies on an enzyme that copies some genomic regions but skips others. By tweaking this step, Navin says, he is now exceeding 50% coverage of the genome of a human cell, although his work has not yet been published.

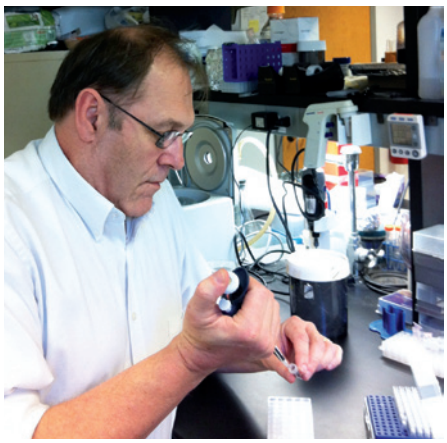
Navin is not the only one tackling this problem. At the Cold Spring Harbor Meeting this year, Xie said that he and his colleagues had been able to sequence 85% of the genome of a mammalian cell. The paper describing it has not yet been published — but researchers who have seen the data are impressed. "Sunney nailed it," says Lao.

This is welcome news to researchers such as Fred Gage, a neuroscientist at the Salk Institute for Biological Sciences in San Diego, California, who wants to sequence individual neurons. He has found that long interspersed elements (LINEs) — DNA sequences that can move around in the genome — form new insertions when neurons are born from neuronal stem cells[8]. Every neuron probably contains unique LINE insertions, with most cells having between 80 and 300. "Every neuron is probably different from every other neuron," says Gage.

ONE DEVICE, LOTS OF INFORMATION

Questions on single cells often lead researchers into difficult experimental terrain. For help in navigating such tricky territory, Gage recommends collaborating with the best technical experts; he is working with Roger Lasken, a leader in sequencing unculturable microbes at the J. Craig Venter Institute in San Diego.

But many researchers venturing into single-cell analysis will be on their own, so techniques will have to become more automated, integrated and kit-like, says Jonathan Sweedler, a chemist at the University of Illinois at Urbana–Champaign. "Researchers will be able to buy a device that has 48 steps incorporated into one platform," he says. Widespread uptake of single-cell analysis will also require high-throughput analyses of dozens to thousands of cells to tease out measurement errors from real heterogeneity.



T. SCHOCHET

James Eberwine's ingenuity in pioneering single-cell protocols has led to dozens of patents.

Several companies are working on these goals, offering miniature devices that integrate multiple steps for the high-throughput analysis of single cells. Fluidigm of South San Francisco, California, markets a microfluidic system that can simultaneously analyse 96 genes in 96 individual cells using quantitative PCR. Fluidigm systems have been deployed to uncover previously unrecognized subsets of immune cells[2], and to examine variability in the response of single cells to cytokine signalling[9]. RainDance Technologies of Lexington, Massachusetts, also sells microscale kits to analyse single cells.

Usability and high throughput are a boon for miniature devices. Integrated steps also help to conserve precious samples, and small volumes can aid the dynamics of biochemical reactions — for instance, they can reduce amplification bias in PCR reactions, notes Stephen Quake, a bioengineer at Stanford University in California, and co-founder of Fluidigm.

"Working with small volumes gives you some real technical advantages," says Quake, whose lab is harnessing microfluidics to develop a technique for single-cell transcriptomics, and has created a device to isolate and sequence single chromosomes[10].

PUTTING IT ALL TOGETHER

But high-throughput techniques will be limited if what they measure is too simple. To grasp how a cell works, "you need to understand not just chemistry, but spatial and temporal information", says Daniel Chiu, a chemist at the University of Washington in Seattle. To integrate these analyses, his lab combines microfluidics, nanomaterials and optics.

Chiu's team has developed a technique for single-cell nanosurgery using a 'vortex trap', an optical method that can manipulate organelles or liquid droplets. The group has isolated single mitochondria from cells and prepared them for analysis on a 'droplet nanolab', which deploys the vortex trap to fuse droplets and change the concentration of reagents[11].

Chiu's lab has also developed microfluidic devices for quantifying fluorescently tagged molecules, and for detecting and analysing cells that are rare in a population, such as tumour cells circulating in the blood (see 'Beyond amplification').

Ultimately, a combination of techniques will be necessary for researchers to attain their goal of measuring multiple parameters in a single, living cell. "The more parameters you can define — the transcriptome, the peptide-ome, how a cell looks, how it responds to drugs — the more information you are going to get out," says Eberwine.

Eberwine is confident that these methods will emerge, even if it takes years. "I think we will be successful," he says, "and if we are not, somebody else will be." ■

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