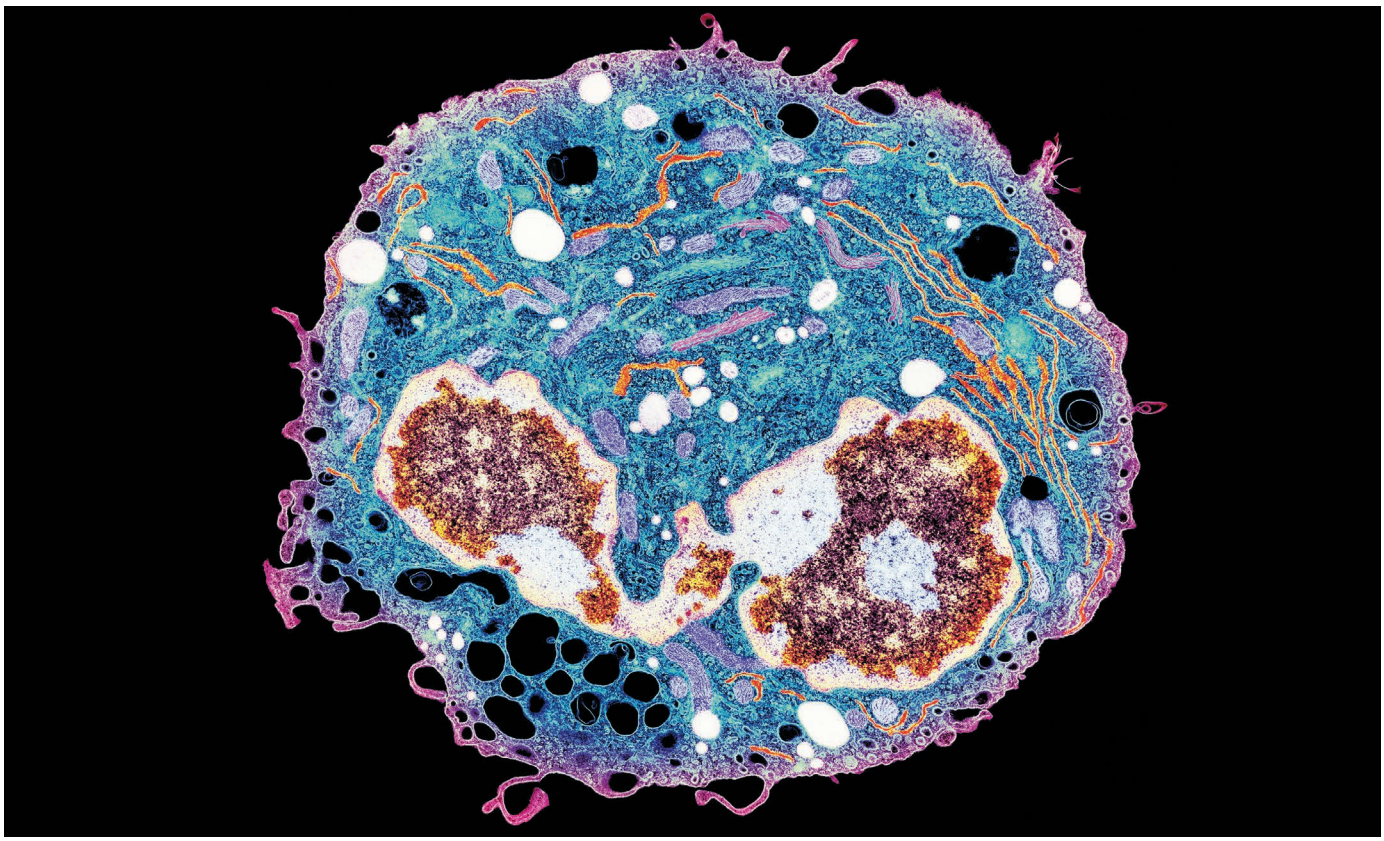
TECHNOLOGY FEATURE

# SINGLE-CELL APPROACHES TO IMMUNE PROFILING

*Protein- and sequencing-based technologies are helping researchers to profile immune cells ever more deeply.*



THOMAS DEERINCK, NCMIR/SPL

**Analysing individual white blood cells can help scientists to identify their specific role in fighting disease.**

BY ESTHER LANDHUIS

The human immune system is a vast, decentralized army. With billions of specialized cells in constant motion, it's incredibly difficult to work out which ones are doing what in any given place or situation. But advances in proteomics and genomics technologies are helping researchers to catalogue the various parts. It is a massive undertaking that is likely to produce 100 billion times more data than the Human Genome Project.

For decades, immunologists have relied heavily on flow cytometry, a technique that involves labelling cells with different fluorescent markers. Over the past eight to nine years, mass cytometry, which uses metal ions to label the cells, has offered more-detailed glimpses into immune responses to diseases such as cancer, tuberculosis and malaria.

Yet these techniques barely scratch the surface in determining the precise work of T cells and B cells. These cells are adorned with unique protein molecules — known as T-cell receptors and B-cell receptors — that are exquisitely designed to "recognize specific targets and respond to them in an evolutionary way", says Jennifer Sims, a molecular biologist at the Memorial Sloan Kettering Cancer Center in New York City. Immune cells are almost as varied as people are, she says.

These surface receptors recognize specific molecular features, or antigens, on pathogenic organisms or tumours. When a receptor detects a harmful antigen, it triggers the immune cell to multiply and mobilize for attack. Every receptor is distinct, encoded by combinations of gene segments that are shuffled and recombined as the immune cell develops. Last month, at the annual meeting of the American Association for Cancer Research in Chicago, Illinois, Sims chaired a session on methods for analysing the T-cell repertoire — a set of disease-fighting cells that churn out up to $10^{20}$ unique receptors over a lifetime (see 'A focus on immune receptors').

By studying these receptors — and their B-cell analogues — researchers hope to learn how the immune system responds and evolves during disease. Whereas flow cytometry can reveal the coarse details of how ▶

different cell populations rise and fall, the immune-receptor profiling methods can pinpoint the B-cell and T-cell 'clones' involved. To study these specialized receptors and further expand the power of single-cell analyses, a wave of sequencing-based methods is rippling through the field, says Alex Shalek, a chemical physicist at the Massachusetts Institute of Technology (MIT) in Cambridge, whose lab builds tools to study how cells interact in healthy and diseased states. Just as DNA's four 'letters' can encode any gene in the human body, using unique DNA snippets to tag antibodies paves the way for experiments surveying a theoretically infinite number of proteins, Shalek says.

Plus, such technologies enrich immune analyses by allowing researchers to also measure the transcriptome — the complete set of expressed genes — in the same cells. Although it is less-established than mass cytometry, sequencing-based immune profiling can be done in any lab, hospital or clinic with a DNA sequencer, potentially expanding the technology's reach. And researchers can now choose from at least half a dozen commercial systems and do-it-yourself methods for converting RNA and proteins from individual cells into strings of letters that can be read on a standard DNA sequencer.

Collectively, these new technologies are fine-tuning scientists' understanding of how the various different immune cells act in the microenvironment during disease states, says Sims.

### FLUORESCENCE TO METALS

Flow cytometry is an optical technique that classifies cells on the basis of properties such as size, granularity and the presence of signature proteins labelled with fluorescent antibodies. 'Flow' refers to the mechanics of the technique: cells flow in single-file past a series of lasers and detectors, which read them as they pass.

The technique has been a staple of immunology for decades, but it has shortcomings. The visible-light spectrum limits most experiments to no more than a dozen or so protein markers — too few for analyses that involve small numbers of cells or complex phenotypes.

In 2009, chemist Scott Tanner, then at the University of Toronto in Canada, came up with a solution[1]: mass cytometry (often called CyTOF) blends flow cytometry with mass spectrometry, using metal-conjugated antibodies to boost the number of detectable markers to 50 or so.

The technique gained popularity after geneticist Garry Nolan and his colleagues at California's Stanford University used it to measure 34 parameters at once in human bone-marrow cells — helping them to track simultaneously how a wide variety of immune cells were responding to different drugs[2]. It "lets us dive into really complicated systems with single-cell heterogeneity and rare [cell] populations, and I don't need to know ahead of time where the action is happening", says Sean Bendall, a Stanford biochemist who worked with Nolan on the profiling. CyTOF was originally commercialized by DVS Sciences, which was acquired by Fluidigm in South San Francisco, California, in 2014.

*"It's a very fast-evolving field with a lot of innovation."*

Researchers doing flow cytometry will typically choose a small set of protein markers to stain each kind of immune cell — for example, they may use CD19 to identify B cells, and CD4 or CD8 for T-cell subsets. However, this provides only a coarse level of resolution because the excitation and emission spectra of the fluorescent dyes overlap and muddy the picture. So when Asya Rolls, a neuroimmunologist at the Technion Israel Institute of Technology in Haifa, wanted a comprehensive look at all the immune-cell populations in the mouse brain, she decided to use CyTOF instead[3].

She surveyed 44 antibodies simultaneously, and the approach yielded a surprise: a T-cell subpopulation with unusually high expression of the surface protein CD86 (ref. 4). CD86 isn't usually found on T cells in the rest of the body — it typically decorates the surface of other immune cells and acts to regulate T-cell activation — so the build-up in the brain was intriguing. Mass cytometry is a good tool for discovery, Rolls says.

Once mass cytometry reveals which cells and molecules to focus on, however, a flow cytometer often proves more useful for follow-up analysis. Flow cytometry is faster: it can sort more than 10,000 cells per second, whereas mass cytometry handles only about 1,000. CyTOF experiments also tend to use many more antibodies per sample, so any mistakes are costlier. And if you will still need the cells after the analysis, CyTOF is not an option because it vaporizes them before reading their metal tags.

But in its favour, mass spectrometry can target many more parameters per cell — including chemical compounds, collectively called the epigenome, that attach to DNA and influence how a cell uses its genetic instructions. Targeting the epigenome, a Stanford team found last month that immune cells from older people have much greater epigenetic heterogeneity than do those of younger adults[5]. These data support the long-held assumption that the human immune system deteriorates with age because of cell-to-cell variations in gene expression.

### DNA-BASED PROFILING

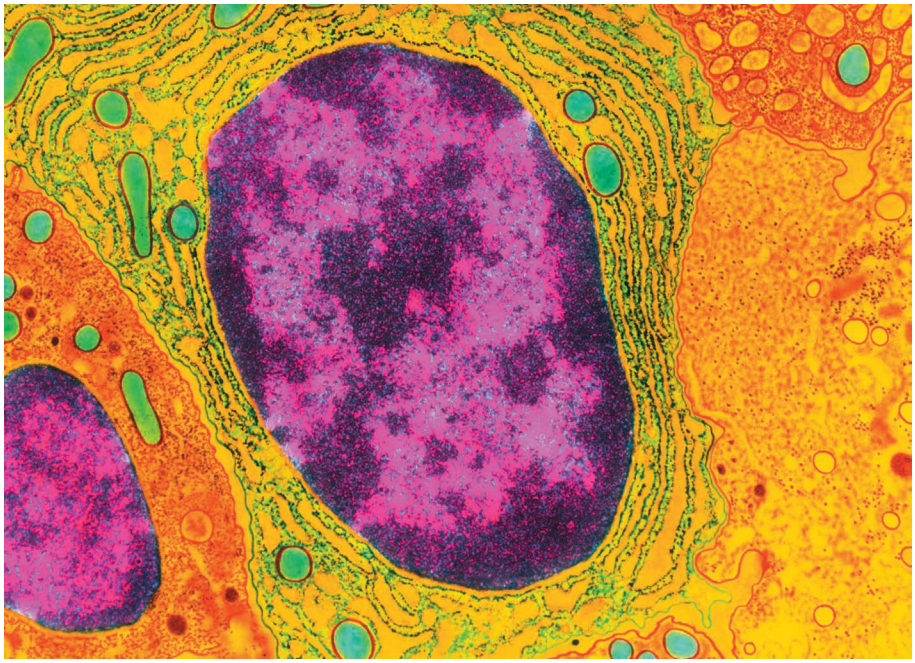
The initial wave of mass-cytometry papers in the early part of this decade caught the attention of Adam Abate. Abate is a physicist at the University of California, San Francisco, who builds tools known as microfluidics devices, which are used to miniaturize the work and form a parallel with biological experiments. "I was reading Garry Nolan's papers," he says. "We had journal clubs on them." Those studies convinced Abate of the value of simultaneously analysing many parameters in the same cell, a process known as multiplexing. The advent of mass cytometry had already boosted the number of protein markers it was possible to analyse in one shot, from 10–15 to perhaps as many as 100. But Abate started asking, "why stop at a hundred? Why not go bigger?" Human cells have around 20,000 protein-coding genes, and more than 100,000 protein variants. "We need more multiplexing," he decided.

## A focus on immune receptors

Trying to pinpoint which of the trillions of T-cell and B-cell receptors in the immune repertoire contribute to a person's disease might seem like an impossible task. But specialized sequencing approaches are enabling researchers to give it a try. About half a dozen companies now offer services or kits for reading out the shuffled gene sequences of T-cell and B-cell receptors from cell samples. By tallying those sequences and watching how they change, researchers can track which clones of cells have key roles in disease.

Adaptive Biotechnologies in Seattle, Washington, offers the whole process. Researchers can send in chunks of frozen tissue and pay for the DNA isolation. They can also submit purified DNA or RNA samples and have the company run the analyses and send back processed data — spreadsheets with lists of nucleotide sequences identified in that run, and their frequencies. Other companies support a more do-it-yourself approach. ArcherDx in Boulder, Colorado; Takara Bio in Mountain View, California; iRepertoire in Huntsville, Alabama; and New England Biolabs in Ipswich, Massachusetts, sell multiplex PCR primers that researchers can use to amplify T- and B-cell receptor gene segments from their own samples.

Each system has pros and cons. Some are great for frozen tissue, others for formalin-fixed, paraffin-embedded samples. Still others are sensitive to interference from tumour-cell DNA. One challenge is shared by all platforms: it can be hard to distinguish real biological changes, such as a small expansion of certain cells during an immune response, from the technical errors that arise from use of the enzymes. E.L.



GOPAL MURTI/SPL

**Adaptive immune cells, such as this mature B cell, can show a staggering diversity of antigen receptors.**

So Abate looked at trying a completely different kind of molecular tag: short DNA sequences. The four-letter genetic alphabet can be used to create an enormous number of unique sequences, known as barcodes, for cells or molecules of interest. Abate stained cells with DNA-labelled antibodies and shuttled them into a machine that broke the cells open and spliced the antibody's barcode to a second barcode that identifies the cell from which it came. He could then analyse those linked barcodes using a DNA sequencer. To get single-cell resolution, he turned to microfluidics. His team developed a method called Abseq, which injects individual antibody-stained cells into 10-micrometre droplets that contain unique DNA barcodes for both the antibody and cell[6]. Abseq works more slowly than flow cytometry, but it can survey a theoretically limitless number of proteins in individual cells. And that could help to pinpoint which cells are driving immune activity in various diseases. This is especially important for diseases such as cancer, in which each tumour has unique features that could engage — or suppress — different immune cells.

However, the real power of Abseq lies in its potential to integrate with other single-cell methods, says Sam Kim, a postdoc in Abate's lab who helped develop Abseq. Because it generates information that is readable by sequencing, it can assign multiple types of 'omics data — proteomes, transcriptomes, even disease-causing genetic mutations — to each cell, Kim says.

Mission Bio, a biotechnology company in South San Francisco, licensed the Abseq technology to build just such a multi-omics system. At a meeting of the American Society of Human Genetics in Florida last October, the company launched a first-generation system for analysing gene mutations, and it is now developing protein- and RNA-profiling components. BD Biosciences in San Jose, California, is developing similar technology: last September, it released its Rhapsody system for single-cell profiling of predefined or custom gene panels. Rather than probe the whole transcriptome, BD's technology focuses on several hundred transcripts, which cuts the number of sequencing reads about tenfold, says Nikhil Rao, global product manager for the company's Rhapsody system. "You save a tonne of money if you don't waste sequencing on genes you're not interested in." The company is scheduled to expand the Rhapsody platform to include protein detection with its own AbSeq assays — developed independently of Abate — later this year.

## DO-IT-YOURSELF APPROACHES

Single-cell transcriptome analyses can also be used to profile the immune system. These methods offer "a tremendous opportunity to look genome-wide at what a cell is trying to express — what it's thinking at a given moment", says Shalek.

Both commercial and homegrown options are available. In 2015, two teams at Harvard Medical School reported on their development of methods, called inDrop[7] and Drop-seq[8], which use microfluidics devices to analyse RNA expression genome-wide from thousands of single-cell droplets in parallel. A paper published in February[9] describes how to build a compact version of Drop-seq from 3D-printed parts.

On the commercial side, the California company 10X Genomics offers a high-end instrument that can convert cell samples into sequencing-ready data in a day, and Blacktrace Holdings in Royston, UK, offers both an all-in-one system and modular components for building your own.

Still, droplet-based methods have drawbacks. They don't work for clinical samples that contain only a small number of cells. And reagents are wasted when loading cells at low densities to ensure only one goes into each droplet.

Shalek and MIT chemical engineer Christopher Love worked through these issues to create a single-cell RNA-sequencing system called Seq-Well[10], which floats cells onto a silicone array containing 86,000 sub-nanolitre wells. "Think of it as a big ice-cube tray," Shalek says. BD's Rhapsody system uses a similar approach, capturing cells in tiny wells.

A key advantage of Seq-Well is its portability, Shalek says. Members of his lab have taken the device to Thailand to help MIT colleagues analyse cells infected by the malaria parasite *Plasmodium vivax*. They have also trained a team at the Africa Health Research Institute in Durban, South Africa, to use Seq-Well to identify infected immune cells in lymph-node samples taken from people with HIV.

Strategies for collecting multiple 'omics data sets are moving forwards, too. Last year, for instance, two teams independently published methods — called REAP-seq[11] and CITE-seq[12] — for analysing proteins and messenger RNA simultaneously in single cells. The New York University Langone Medical Center and the West German Genome Center are gearing up to offer similar services. Researchers can send us samples, "and we will do the single-cell analyses", says Pratip Chattopadhyay, who directs the New York unit. The German centre, a new multi-institution collaboration headquartered in Bonn, is mobilizing a panel of experts for consultation on the technologies.

"It's a very fast-evolving field with a lot of innovation," says Joachim Schultze, a tumour immunologist at the German Center for Neurodegenerative Diseases in Bonn. "That also means nobody has any idea where this will be in ten years — which technologies will make it, or how many we will have." ■

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