TECHNOLOGY FEATURE

MEASURE FOR MEASURE

Cutting-edge tools that can identify the characteristics of cells are helping researchers to develop more-effective vaccines.

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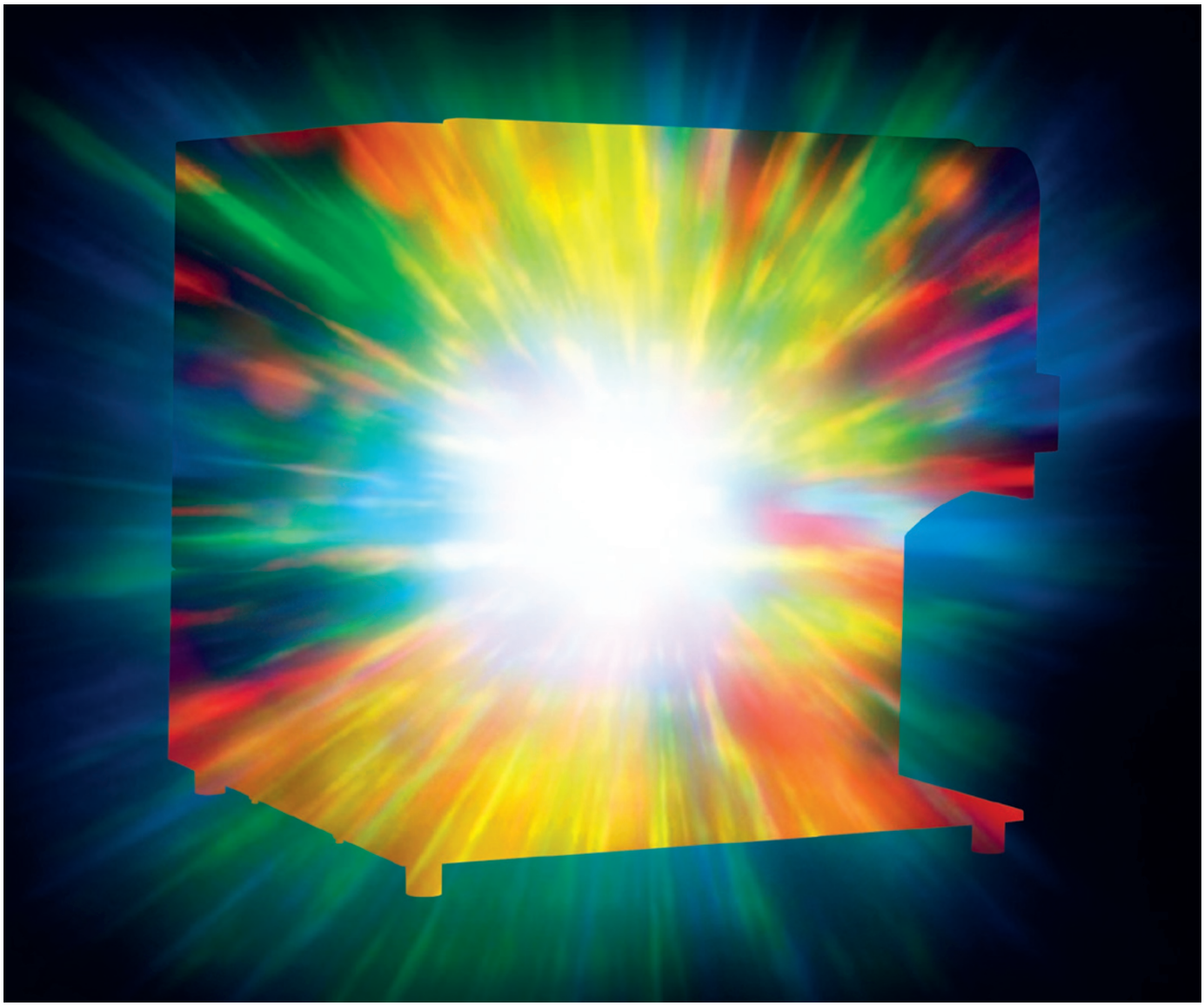


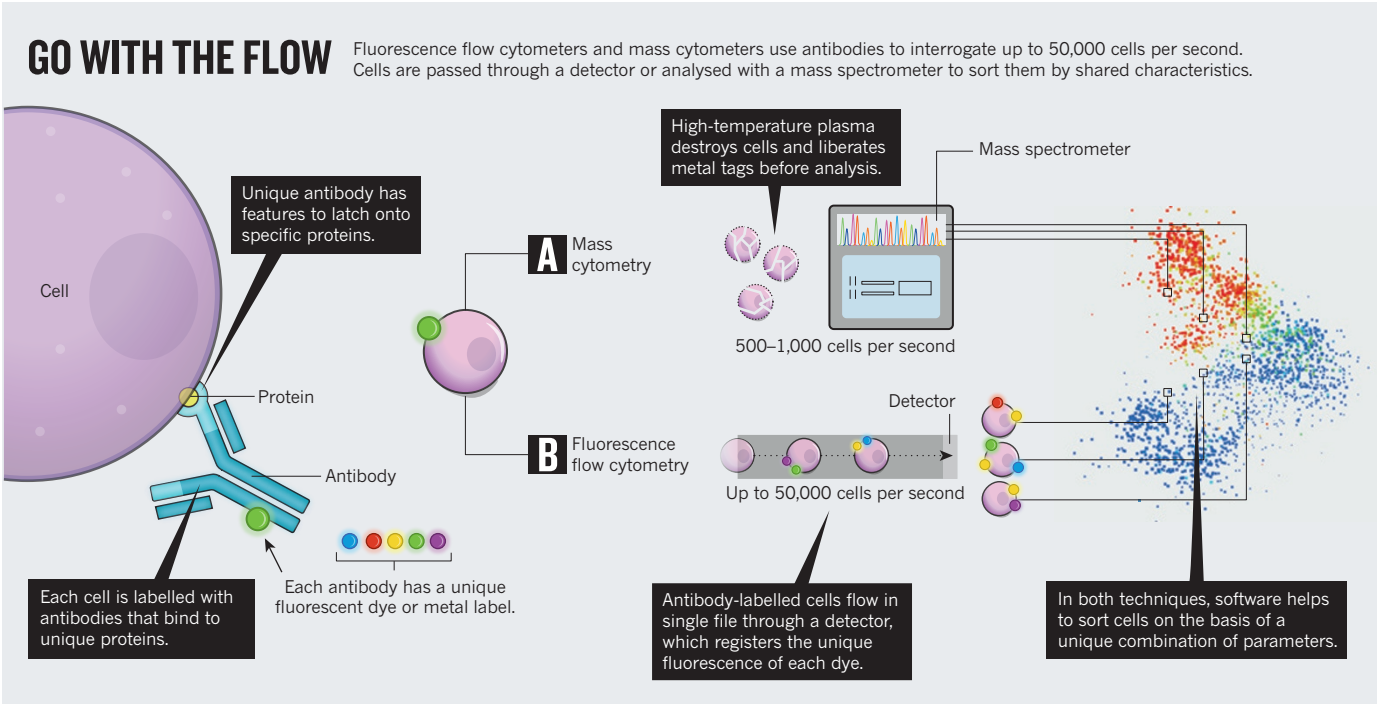
BY JIM KLING

Vaccines are a triumph of science over infection. They have defeated smallpox, which the World Health Organization declared eradicated in 1980, and dramatically lowered the toll of many other infectious diseases.

But not all. The search for vaccines against conditions such as HIV/AIDS and malaria has been hindered by researchers' incomplete understanding of the human immune system, says Mario Roederer, an immunologist at the US National Institutes of Health's Vaccine Research Center in Bethesda, Maryland.

In general, says Roederer, researchers know that a successful vaccine jump-starts antibody production and other defences. But they do not know which of the immune system's thousand or more functional cell types direct the response against individual pathogens. If researchers could identify those cell types, he says, then they could devise vaccines that maximize production of these cells.

One of the best ways to identify those cells is flow cytometry, a technique that analyses and sorts cells according to their distinguishing characteristics — usually proteins on the outer surface — and reveals much about a cell's function and position in the immune ▶



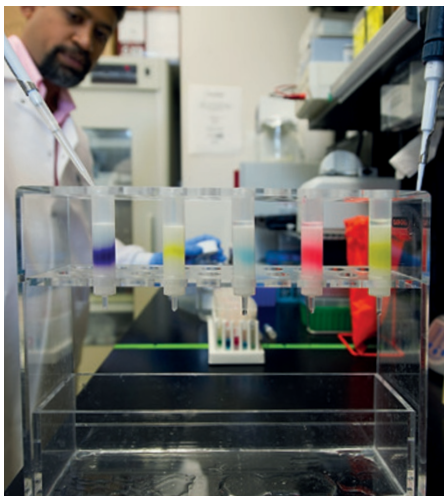
J. KLING; CLAIRE WELSH/*NATURE*

system. But current-generation flow cytometry is detailed enough to place cells only in broad categories — like identifying something simply as a fish instead of as a great white shark. Researchers know that there is a specific predator that they want, says Roederer. "We're just trying to figure out how to find it."

The ability to identify specific cells may also improve researchers' understanding of diseases such as multiple sclerosis, in which the immune system attacks the host's own tissues, and metastatic cancer, in which rogue cells from the original tumour migrate into other tissues.

Such possibilities have motivated researchers to develop two new approaches, each of which promises to double or even triple the limit of conventional techniques by 2016 (see 'Colour bursts').

One approach is a variant of standard flow cytometry that uses a new type of very intense fluorescent dye and can identify 27 proteins. The other, known as mass cytometry, can record 50 parameters such as cell-surface proteins or parts of proteins, says immunologist Garry Nolan at Stanford University in California. It labels the proteins with metal atoms, then records the weight of the atoms within each cell with a mass spectrometer to provide a signature for each cell type. The labels can also help in microscope imaging of tissues. For instance, they show where cell-surface proteins are located in a slice of excised tumour. That adds layers of information about the cell types present in the tumour and hints at their function within the cancerous growth.



By using antibodies labelled with fluorescent dyes, researchers identify the proteins present on a cell.

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WIDEN THE NET

Like the immune system, flow cytometry uses antibodies to seek out proteins (see 'Go with the flow'). First, researchers create an antibody for each protein they want to study, then they label the antibody with a dye molecule that can absorb light and be made to fluoresce in a specific colour.

The sample to be studied is then bathed in the labelled antibodies, which stick to the cells that bear the matching proteins. The antibody-decorated cells are then directed one at a time through a narrow channel. As they pass through, a light pulse triggers the dyes to flash, revealing which proteins are present on the cells. The light from each dye fans out across different wavelengths to produce a readout that looks like a broad mountain peak.

At the moment, flow cytometers can process no more than 18 fluorescent dyes at once because when more than this number fluoresce simultaneously, the shoulders of some spectra will overlap with the crests of others, making the crests impossible to discern.

"I've always been lucky to be surrounded by the necessary technologies."

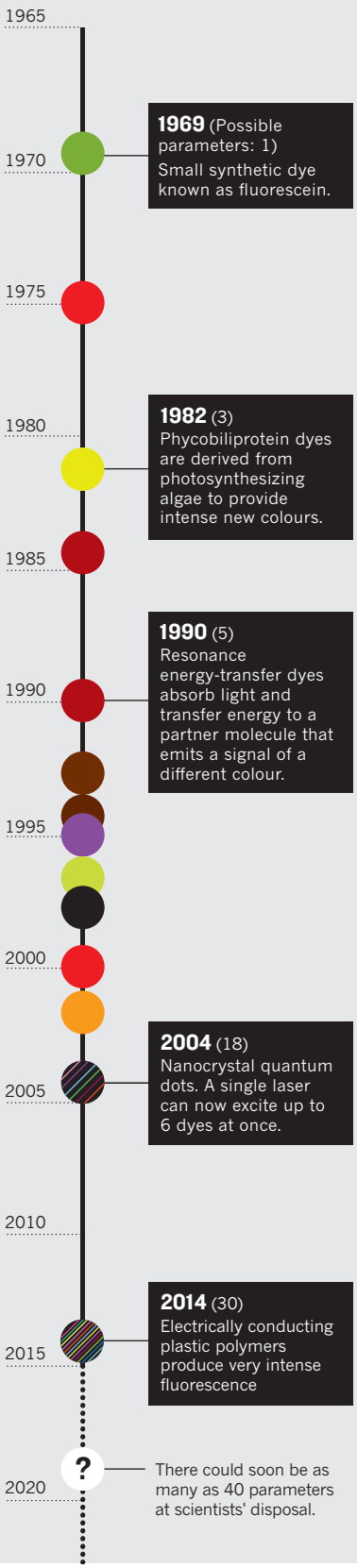
A complicating factor is that the abundance of each protein can vary greatly from cell to cell. A cell that has thousands of a specific protein on its surface will attract many identically labelled antibodies, which combine to produce a bright flash of fluorescence. A rare protein will produce a weaker signal that may get overwhelmed by those from more abundant proteins.

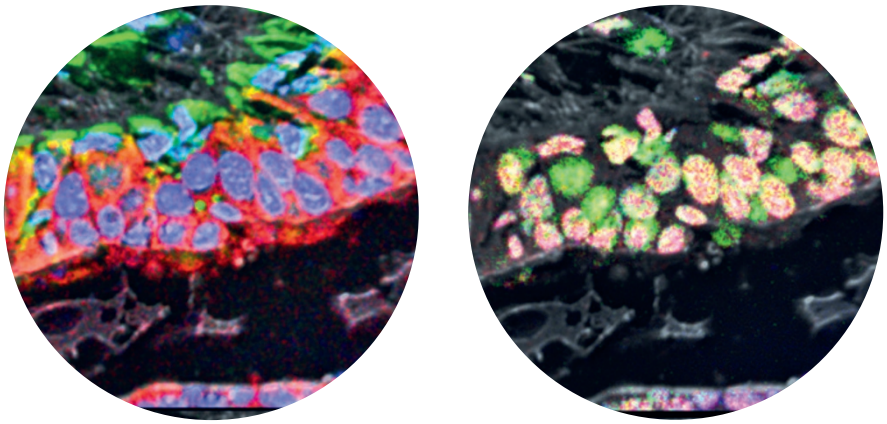
Researchers can compensate by using brighter dyes to label antibodies targeted at the less common proteins, says Roederer. But they often do not know the relative abundances of proteins in advance, he says, so they may have to spend weeks relying on trial and error to work out which dyes to use on which antibodies.

That could well change soon, because researchers have developed a new class of dye[1]. Made from electrically conducting plastic polymers, these dyes act as miniature antennas that absorb energy from the light pulse at multiple points that all fluoresce together, producing a more intense signal. When used in flow cytometry, says Roederer, the intense signal overwhelms any overlap from other dyes, even for low-abundance proteins, and means that more dyes can be used simultaneously. "You have so much more flexibility," says Roederer, whose group has used the dyes to survey 30 proteins commonly found on the

COLOUR BURSTS

Every decade, the discovery of new classes of fluorescent dye (coloured dots) roughly doubles the number of simultaneous measurements (parameters) possible per cell.





MICHAEL ANGELO, STANFORD UNIV.

surfaces of immune cells, although it has yet to publish the results.

Other research teams are using these dyes, which are available from two Californian firms — BD Biosciences in San Jose (where they are marketed as BD Horizon Brilliant dyes), and BioLegend of San Diego (marketed as Brilliant Violet dyes) — but Roederer knows of no one else who has managed to measure more than 18 parameters simultaneously. Measuring more than that requires specialized instruments, software and chemical expertise. "It's not trivial" to do, he says. "I've always been lucky to be surrounded by the necessary technologies."

Roederer now plans to use the dyes to study vaccine candidates against several diseases, including Ebola, malaria, tuberculosis and HIV/AIDS. An Ebola vaccine currently being tested in human volunteers is a priority. Roederer joined a team led by immunologist Nancy Sullivan, also at the Vaccine Research Center, that narrowed down the cells that protect monkeys from Ebola — not quite to shark level, but to about that of a predatory fish[2]. With more dyes, Roederer expects to find his shark and pinpoint the cell populations that give the monkeys immunity. Then Sullivan and Roederer hope to tune the vaccine doses and schedules to get the human immune system to produce and support those cells.

METALS, NOT COLOURS

As Roederer forges ahead with the new dyes, DVS Sciences of Sunnyvale, California, is taking flow cytometry in a different direction. The company, now called Fluidigm, introduced the first commercial mass cytometer in 2009 and debuted its next-generation machine, the CyTOF 2, in 2013.

Like flow cytometry, mass cytometry involves soaking cells in labelled antibodies then squeezing them into a narrow flow to be screened one by one. But that is where the similarity between the two techniques ends. In place of fluorescent dyes, mass cytometry uses rare earth metals, which are absent from living systems, to label the proteins. And rather than illuminating the cells to reveal the presence of proteins, the mass cytometer uses high-temperature plasma to split the cells into their component atoms. These atoms, which now include the rare-earth labels, then get fed into a mass spectrometer to measure the mass and abundance of each metal, and thus the identity and abundance of each matched protein. There are therefore no problems with signal overlap as there are in fluorescent dyes.

A group led by Nolan used the technique to look for specific immune-cell populations in patients recovering from hip-replacement surgery — which, like most traumas, prompts a complex response from the immune system to orchestrate healing. Nolan reasoned that he could identify the immune cells that promote faster recovery in some patients. His team collected blood from each patient before their surgery and at various times afterward, and tracked 31 proteins using mass cytometry. In patients who recovered quickly, the researchers found unique types of immune cells known as monocytes[3]. "We were able to see signatures that were changing and that lined up really well with surgical recovery," says Sean Bendall, a pathologist at Stanford, and a co-author of the study.

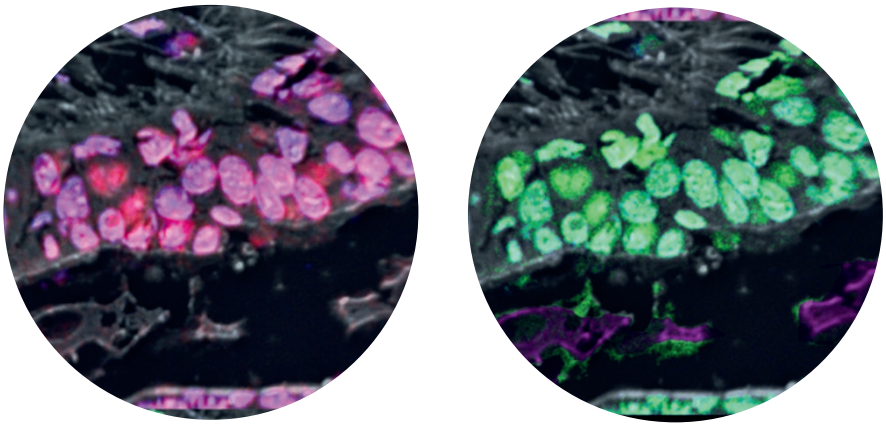
Researchers are now investigating whether it is possible to use these cells to predict which patients are likely to have delays in recovery, and provide interventions that could improve their recovery time, says Bendall.

Mass cytometry and fluorescence flow cytometry compete for some of the same research applications, and researchers tend to have a preferred technique.

Roederer points out that mass cytometry destroys cells, whereas fluorescence flow cytometry can preserve them, and can even isolate and sort them at the same time.

Bendall does not share that concern. He enthusiastically backs mass cytometry, but says that he often hears objections about the destruction of cells. "I feel like that's the 'gotcha' question," he says. Many people will dismiss the technique simply because of that, he explains, but cell destruction "has never been a roadblock to anything we wanted to do". If a mass-cytometry experiment identifies an interesting cell type, he explains, then researchers can

SOURCE: MARIO ROEDERER. DESIGN: CLAIRE WELSH/*NATURE*



Multiplexed ion-beam imaging can reveal different DNA-binding proteins in human breast tissue. Each image highlights four proteins.

always use that information to isolate living counterparts with a traditional flow cytometer.

Fluorescence-based flow cytometry does, however, have the advantage of being faster: it can analyse up to 50,000 cells per second, whereas mass cytometry manages no more than 1,000 per second because the cloud of atoms produced by the plasma takes time to clear before the instrument can accommodate the next cell.

The intense fluorescent dyes are not without their drawbacks. They are so new that little road-testing has been done using high numbers simultaneously — and few papers have been published on them.

That leaves mass-cyotometry advocate Nolan a little sceptical of the technique. "I've yet to see them implemented in a way that would have me jump ship and go back to fluorescence," he says.

PICTURES PERFECT

Nolan, Bendall and Roederer all agree on one thing: the most promising application of metal tags is their potential to improve images of intact slices of tissue — and that cannot be done with either fluorescence flow cytometry or mass cytometry because both techniques require cells to be dispersed into a flowing stream.

One such application, called multiplexed ion-beam imaging, applies metal tags to tissue slices, then showers them with oxygen ions. The oxygen ions react with the metal tags, dislodging them from their accompanying antibodies. A mass spectrometer then measures the metal atoms as they ricochet away from the tissue.

Michael Angelo, a pathologist at Stanford, says he has pushed the technique to 45 parameters per cell. The method also records the cell's position within, say, a tumour. "I think it's extraordinarily cool. That will never be done by traditional imaging or flow-cytometry-based technologies," Roederer says.

Last year, Nolan's group used the technique in tissue samples from people with breast cancer using ten metal-labelled antibodies. The technique produced a high-definition picture of the tissue that could then be displayed in different colours to reveal the location of individual proteins within the section[4].

In a related approach, immunologist Bernd Bodenmiller at the University of Zurich in Switzerland and chemist Detlef Günther at the Swiss Federal Institute of Technology in Zurich and their colleagues have developed a companion instrument. It records the position of the cells and directs an ultraviolet laser at the tissue to methodically strip away labelled cells and send them to a mass cytometer for analysis[5]. The instrument can analyse up to 40 tags, Bodenmiller says. However, there are now more metal tags available, so researchers will probably soon conduct experiments with even more parameters, says Scott Tanner, Fluidigm's chief technical officer.

Images produced by this technique could illuminate how cells respond to local conditions within a tumour, such as low-oxygen environments. "It gives you yet more information about the nature of that sample," says Tanner.

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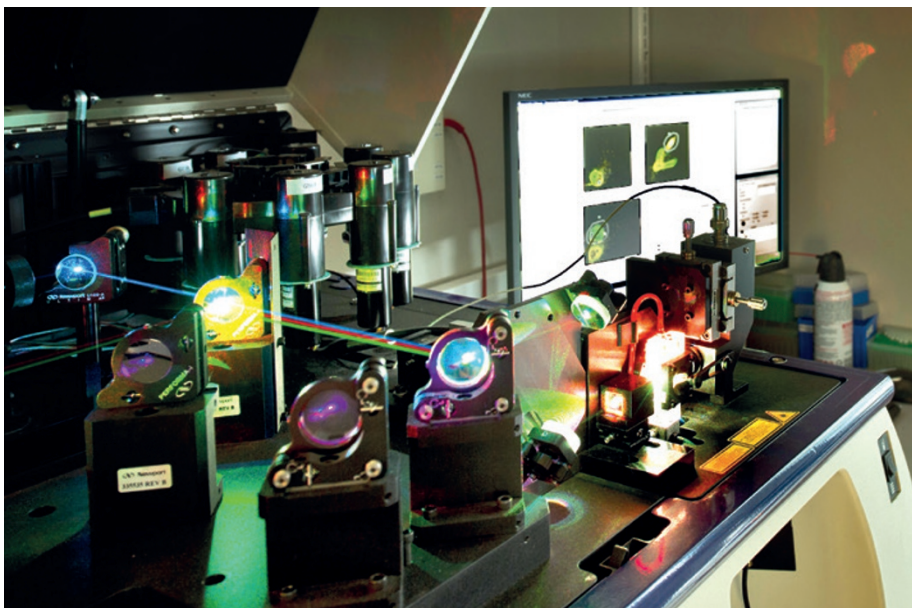
Fluidigm has licensed the laser ablation chamber and given prototypes to several academic research teams. It hopes to begin selling the device this year.

Imaging mass cytometry could be applied beyond cancer, Tanner says. Neurobiologists tell him that they hope to use it to examine how neurons are distributed in the brain or spinal cord. The ability to analyse multiple proteins could help researchers to decipher the functions of neural cells and how they relate to the cell's location within a network of linked neurons.

Roederer and Nolan are constantly pushing the boundaries, but Roederer says that he often encounters scepticism of how useful the improvements are. When he achieved 8 parameters, researchers questioned if that many were really necessary. "When I got to 12, people were saying 'Is that enough? Are you done yet?'," he recalls.

He is not done. He hopes to get to 40 parameters next year, and even more after that. "Every time we reached a new ceiling, we were looking to crack it within a couple of years." ■



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In fluorescence flow cytometry, lasers stimulate dyes to flash a unique colour and the instrument registers those flashes to characterize each cell.

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4. Angelo, M. *et al. Nature Med.* **20,** 436–442 (2014).
5. Giesen, C. *et al. Nature Meth.* **11,** 417–422 (2014).