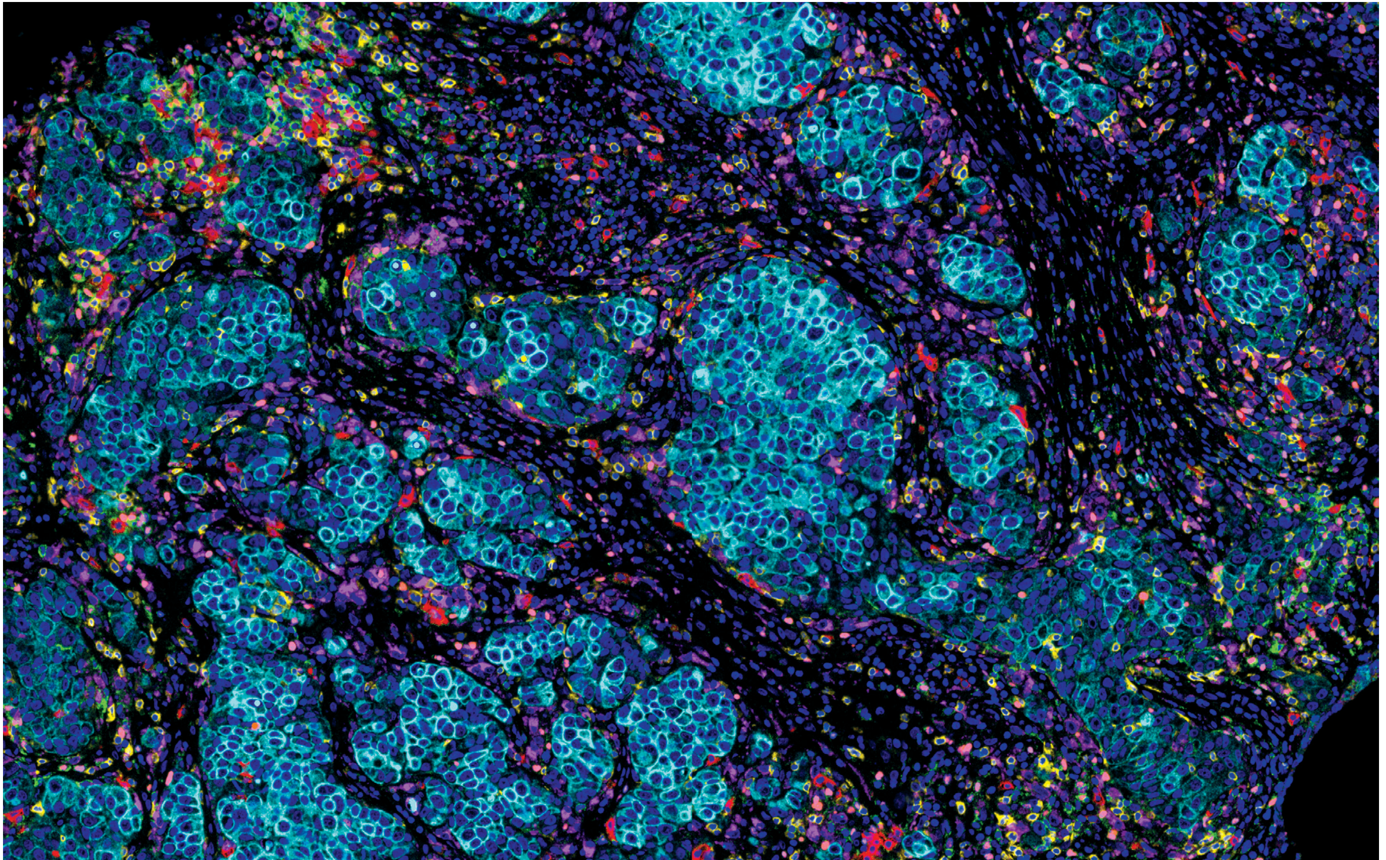
TECHNOLOGY FEATURE

CELLULAR CENSUSES TO GUIDE CANCER CARE

In the age of immunotherapy, cancer biologists are relying on a new generation of tools to learn how the interplay between tumours and immune cells shapes the course of disease.

AKOYA BIOSYSTEMS



The microenvironment surrounding a breast-cancer tumour (blue) contains different types of immune cells, such as T cells and B cells.

BY MICHAEL EISENSTEIN

Although he is trained as a pathologist, Sean Bendall has become something of a map-maker of late, using cutting-edge protein-mapping technology to chart the treacherous landscape of tumour tissues.

Created with Michael Angelo, Bendall's colleague at Stanford University in California, this technology is providing rich molecular profiles of both cancer cells and their immediate neighbours — most notably, the immune cells in and around the tumour. Bendall is already seeing evidence that these maps could help clinicians to decide on the appropriate treatment for some people. In a 2018 study[1], Bendall and Angelo applied their technique, called multiplexed ion beam imaging (MIBI), to tumour specimens from people undergoing standard chemotherapy for triple-negative breast cancer, which is a particularly aggressive type of tumour. "We identified an immune type that was highly predictive of long-term disease-free survival," Bendall says.

It's easy to think of tumours in stark, binary terms, composed of cells that are either abnormal or healthy. But the reality is messier: cancer cells interact extensively with immune cells, blood vessels and supporting connective tissues. This tumour 'microenvironment' can profoundly affect both the characteristics of the disease and a person's response to treatment, particularly immunotherapy — a type of treatment that helps the immune system to fight cancer. The microenvironment, for example, can determine whether nearby immune cells are switched 'on' or 'off', or even whether they can access the tumour at all. "The tumour is not just a homogenous bag of cells," says Dana Pe'er, a computational biologist at the Memorial Sloan-Kettering Cancer Center in New York. "It's actually an organ. It's just a badly malformed organ."

Armed with tumour-mapping technologies, such as Bendall's, as well as other approaches that can generate detailed censuses of vast numbers of individual cells based on gene expression or protein content, researchers ▸

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are now dissecting the structure and function of the tumour microenvironment. These tools are so new that many researchers are still getting the hang of how best to use them. But the resulting insights could help to usher in a new era of tumour profiling — one that takes a 'big picture' view of cellular ecosystems, such as the number and type of immune cells present, rather than focusing exclusively on individual genetic variants.

"These are features that are really going to impact treatment," says Miriam Merad, a medical oncologist at the Icahn School of Medicine at Mount Sinai in New York City.

A CELLULAR CENSUS

The idea that interplay between the tumour and the immune system might shape a person's disease is not new — immunologist Wolf Fridman has been studying such interactions for 50 years, based on early observations of the response of immune cells called T cells in leukaemia. "I rapidly got the conviction that the location and organization of the immune reaction is very important," says Fridman, who is now an emeritus professor at the Medical School Paris Descartes. However, the broader impact of these interactions didn't become a major focus for oncologists until about ten years ago, with the emergence of powerful immunotherapy strategies.

Pathologists can get some indication of the immune-cell composition of a tumour with conventional techniques such as haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). IHC, which uses enzyme-tagged antibodies that recognize specific molecular features in a tissue specimen, has proved particularly informative in the context of the various drugs known as checkpoint inhibitors. This class of immunotherapy agent blocks specific signalling proteins that can otherwise prevent local immune cells from mounting an effective attack on the tumour, and IHC can reveal the presence of these proteins. "If you don't have a certain level of checkpoint-protein expression in your lung tumour, you basically don't get a response," says Bendall. However, many people that do express these proteins still fail to respond to checkpoint therapy, and researchers are hunting for other immune features that might more clearly predict whether the treatment will be effective. But IHC is not an optimal strategy for finding these, because it can only profile a handful of molecular markers at a time.

Mass cytometry by time-of-flight, or CyTOF, can profile tumour cells on a much larger scale. Instead of coupling antibodies to dyes or fluorescent labels, as in conventional flow cytometry, CyTOF uses antibodies linked to metal isotopes to label large numbers of dissociated cells from a tumour specimen. These isotope tags are then rapidly profiled with a mass spectrometer, which can detect and quantify dozens of different markers in parallel for each cell.

In 2017, Merad and her colleagues applied CyTOF to lung adenocarcinoma — the most common form of lung cancer — using as many as 40 different tagged antibodies[2]. Their data revealed how newly emerging tumours move quickly to quell a person's immunity by recruiting immune-suppressing regulatory T cells and impeding the migration of 'effector' cells such as tumour-slaying natural killer cells. "Even at this early stage, when they start to become malignant, they have to start getting rid of these effector cells," says Merad. "These results suggest that reversing immunosuppression very early in tumour development could halt progression and, hopefully, metastasis."

TALLYING TRANSCRIPTS

CyTOF requires prior knowledge of cell-type-specific markers so that researchers can select the appropriate antibodies, limiting its utility in discovery-based research. And Pe'er notes that CyTOF offers little direct information on biological function, indicating only the presence or absence of cellular markers.

Transcriptome analysis techniques such as RNA sequencing (RNA-seq) can overcome some of these limitations. RNA-seq employs high-throughput sequencing platforms to characterize and quantify vast numbers of protein-coding messenger RNA transcripts. This offers a direct window onto which genes are turned off and on in a given cell, revealing the biological activities that take place in a tumour. Researchers have been applying such techniques for more than a decade, but much of this has been 'bulk' analysis — profiling every cell in the tumour at once. That approach can offer useful insights, but it also glosses over any variation between cells, notes Aviv Regev, a computational biologist at the Broad Institute in Cambridge, Massachusetts.

> ***"We get very comprehensive profiles, both the variety of cells, and the variety of molecules inside the cells."***

Regev's lab helped to develop a method called Drop-seq, in which individual cells are encapsulated and prepared for sequencing in individual lipid droplets. During this process, each cell's RNA is assigned a distinct genetic barcode, making it straightforward to determine which transcripts originate from the same cell. "We get very comprehensive profiles — both the variety of cells, and the variety of molecules inside the cells," says Regev. Crucially, Drop-seq and other single-cell RNA-seq methods require no prior knowledge of the genes of interest, and the gene-expression profiles can even be used to reconstruct interactions between cells, as Regev demonstrated last year for melanoma tumours[3]. "We found that malignant cells actually can assume a specific state where they form what are called 'cold niches', where you don't have T cells," Regev says. Such tumour regions could prove more resistant to immunotherapy.

Single-cell RNA-seq is relatively straightforward to implement, with several commercial instruments available. But the technique still poses some important challenges. Perhaps the most significant is that tumour specimens must be shuttled directly from the operating theatre to the laboratory, with only a brief layover in the pathologist's lab. "Single-cell approaches are only really informative on fresh cells, because the RNA is very quickly degraded when we freeze and thaw cells," says Merad.

However, the technology can uncover cell types and cell states that researchers might otherwise not have known to look for, and reveal nuances in immune-cell function that transcend the simplistic categories often used to sort cells. "There's much more variation and many more subsets than we imagined," says Pe'er. "We used to like to rank cells as 'good' and 'bad', in terms of pro- or antitumour, but the system is much more complex, with most cells co-expressing both good and bad programs."

LAY OF THE LAND

For all their power, CyTOF and RNA-seq come with a serious trade-off: destruction of the tumour to access individual cells. This erases the spatial details that can be invaluable for understanding interactions between malignant cells and their microenvironment. Methods such as IHC and H&E staining capture such details, but can reveal only limited information in a single experiment, confounding efforts to assemble a comprehensive portrait of the microenvironment. "I've literally watched pathologists in our department putting slides on top of one another to look at multiple features at the same time," says Bendall. A pair of imaging techniques developed in 2014 can deliver this kind of rich spatial data without the need for stacking and squinting.

The first, imaging mass cytometry, was developed by Bernd Bodenmiller of the University of Zurich, Switzerland, and his colleagues[4]. As with conventional CyTOF, samples are labelled with metal isotope-tagged antibodies, but in Bodenmiller's technique, the labelling is performed on intact tissue rather than on dissociated cells. The specimens are then scanned with a laser that disrupts the tissue and releases the isotope labels, which are analysed with CyTOF. This enables simultaneous measurement of dozens of markers at subcellular resolution across an intact sample with a level of detail that greatly exceeds IHC. "Imaging mass cytometry has an orders-of-magnitude higher dynamic range, and that really brings a much more quantitative aspect to the analysis of immune markers," says Bodenmiller.

The second technique is MIBI, developed by Angelo and Bendall while the two were working in immunologist Garry Nolan's lab at Stanford[5]. MIBI uses isotopically labelled antibodies and a scanning ion beam that subsequently liberates those labels from the specimen, but it uses a different kind of mass

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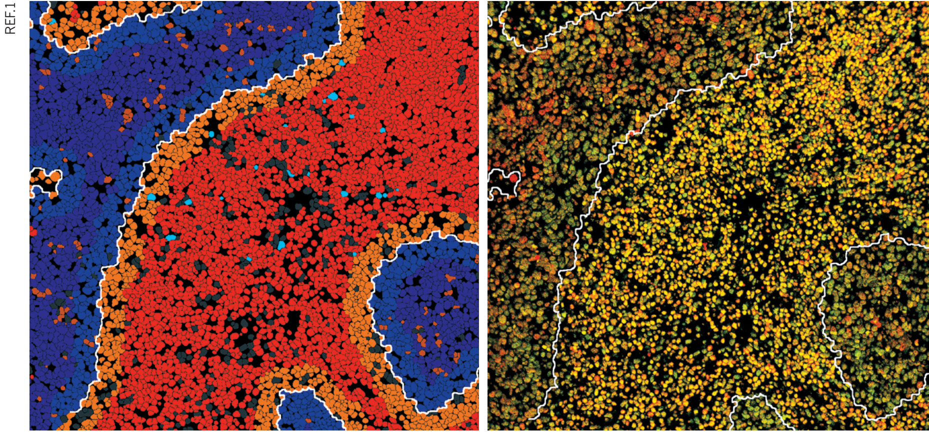

MIBI-generated molecular profiles of tumour and immune cells. Cells are coloured to show either the distance from the tumour border (left) or the expression of genetic markers (right).

spectrometer for isotope analysis. Angelo and Bendall have continued to refine this platform to improve its speed and ease of use, as highlighted in their 2018 work with triple-negative breast cancer[1]. "Instead of taking almost a day to acquire one tiny picture, we ended up acquiring millimetre-square images from an entire cohort of about 40 patients over the course of about a week and a half," says Bendall.

Although they have been used only in a handful of published studies, MIBI and imaging mass cytometry are already highlighting the value of knowing where cells dwell in the tumour microenvironment. "Everyone says that tumours are highly heterogeneous and random systems, but we've found a lot of structure and cell–cell interactions that are not random," says Bodenmiller.

Both imaging mass cytometry and MIBI are now available commercially, but users should prepare for a steep learning curve, Merad warns. "These mass-spec-based systems are quite sensitive to many things," she says. "I think it will require an engineer on site to shield the apparatus from environmental factors like movement and light." And although a growing number of isotopically labelled antibodies are available, developing and optimizing new probes can be a painstaking process. Bodenmiller also notes that these mass-spectrometry-based methods can be less sensitive than fluorescent variants of IHC, which typically use a signal-amplifying strategy that allows the visual detection of even very scarce proteins.

Genomics researcher Joakim Lundeberg of the KTH Royal Institute of Technology and stem-cell biologist Jonas Frisén at the Karolinska Institute, both in Stockholm, have devised a simpler, although lower resolution, alternative. Their spatial transcriptomics approach entails placing tumour specimens directly onto glass slides arrayed with thousands of oligonucleotides, such that each region of the sample corresponds to a distinct sequence barcode. The tissue is then made permeable, allowing its mRNA to diffuse out and be captured by the immobilized oligonucleotides. Once the remaining tissue is eliminated, the RNAs can be sequenced, with the associated barcodes revealing where each transcript was captured.

Spatial transcriptomics lacks single-cell detail, but it captures information that would be difficult to obtain at the protein level, particularly for proteins that are scarce or secreted into the extracellular space. In a paper[6] posted on the bioRxiv preprint server last year, Lundeberg's team used the approach to profile immune-cell activity in breast tumours. "We could actually see within one single biopsy, in one part of the tumour, you will have immune cells infiltrating the tumour," he says, "while on the other side of the same tumour, the immune cells are decorating the tumour without going into it."

A DIFFERENT DIAGNOSIS

Such methods are already demonstrating clinical utility. A 2017 collaboration between Bodenmiller and Pe'er revealed that certain immune profiles can provide prognoses for people with one form of kidney cancer[7]. "We derived an equation from our data that we could use to compute progression-free survival for those patients," says Bodenmiller. Similarly, Merad and Regev have used insights from tumour-microenvironment surveys to identify courses of combination therapy that might overcome drug resistance and immunosuppression in lung, breast and other cancers; clinical trials of those treatment courses are in development.

However, building the experimental and analytical pipelines for comprehensive microenvironment profiling can place a heavy burden on clinical facilities. Merad's department has assembled a closely integrated team of surgeons, pathologists, technologists and cancer biologists, and a multimillion-dollar arsenal of cutting-edge machinery that includes three CyTOF machines, three single-cell RNA-seq instruments and a MIBI platform. "This technology is very expensive and labour-intensive, and we have a lot of things that we are still optimizing," she says. "I've never been as excited, but I've also never been as tired."

Such an investment is out of reach for many cancer centres, but findings from these pioneering facilities should soon trickle down to the broader community, revealing biomarkers and disease profiles that can be routinely detected with less-expensive technology. International efforts such as the Human Cell Atlas are assembling publicly accessible 'field guides' for classifying cells on the basis of genomic, transcriptomic and proteomic data, with an emphasis on tumour microenvironments. And as part of the Pan-Cancer Atlas initiative, researchers, including computational biologist Ilya Shmulevich of the Institute for Systems Biology in Seattle, Washington, have profiled the immune- and tumour-cell composition of more than 10,000 tumour specimens[8]. These data have since been deposited into the Cancer Research Institute iAtlas, a freely available digital resource that clinical researchers might one day use to classify their own samples, says Shmulevich.

Importantly, there seem to be substantial commonalities in the microenvironments that can form, even among diverse tumour types. As part of the Pan-Cancer Atlas effort, Shmulevich and his colleagues were able to sort various tumours, representing 33 different cancer types, into 6 categories on the basis of the immune status of the tumour. These subtypes, in turn, reflect how the person's immune system reacts to the tumour, and might therefore indicate how likely a course of immunotherapy is to succeed. Shmulevich's team, in collaboration with computational biologist Joel Saltz at Stony Brook University in New York, subsequently found that it could use machine learning to computationally identify patterns of immune-cell organization that correlate with these different subtypes based only on conventional H&E stained slides[9]. "You can look at spatial organization and how clustered T cells are, or how close they are to the tumour margin," he says. "That information is now starting to be diagnostic and perhaps prognostic and predictive of response to therapy."

Pe'er is now hoping for something of a slowdown in the breakneck pace of technology development, to allow the science to catch up. "We just got a whole bunch of new toys," she says. "Now we need to work on developing more computational methods and collecting more patient data from more cohorts to understand what these toys can tell us." ■

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