Credit: Chan Zuckerberg Initiative

A singular view of COVID-19

Single-cell analysis sheds light on immune response to COVID-19 infection, enables the rapid discovery of antibody leads, and points to ways to get ahead of future pandemics.

Michael Eisenstein

In late January, Xiaoliang Sunney Xie was in Davos, Switzerland, attending the World Economic Forum — but his mind was miles away, contemplating the coronavirus outbreak raging in his homeland. "Wuhan was locked down," says Xie, Director of the Beijing Advanced Innovation Center for Genomics at Peking University, "and at that time, the situation in China was very serious." Shortly after he returned on 25 January, he and his colleague were already contemplating a clinical research program to isolate potent neutralizing antibodies against SARS-CoV-2, and by 2 February, his team had coordinated with Beijing Youan Hospital to arrange access to patients who had beaten COVID-19.

Xie is neither an immunologist nor a virologist, but he saw an opportunity to focus his group's expertise against the emerging pandemic. "Single-cell genomics is what we do for a living," he says. "And we were fortunate to realize that single-cell genomics is the way to go for this specific problem." He was not alone: as the pandemic unfolded in early 2020, many other researchers recognized opportunities to untangle the complex pathology of this enigmatic virus using single-cell techniques. "I thought it was a unique opportunity to go in with an unbiased single-cell approach … to begin to dissect out what a good immune response to SARS-CoV-2 looks like versus what a bad immune response looks

like," says Stanford University researcher Catherine Blish. "We closed our tuberculosis lab, killed all the TB cultures — much to my horror — and reopened a week later to do SARS-CoV-2 work and began growing our first virus stocks," says Blish.

This mobilization has been remarkably fruitful. High-throughput profiling of patient-derived B cells has propelled antibody drug candidates into clinical trials, while other studies are employing single-cell transcriptomics, proteomics and immune repertoire analysis to chart the process of viral infection and understand how subsequent immunological events determine which patients rebound and which ones rapidly decline.

Ready for action

There's no such thing as good timing for a pandemic, but the research community was undeniably well-positioned in early 2020 to grapple with this crisis. Commercial platforms for profiling the transcriptomic activity of large numbers of individual cells, such as the Chromium system from 10x Genomics, have become increasingly commonplace. Ben Hindson, cofounder and CSO of 10x, notes that his company has counted more than 1,000 papers using the company's technology to perform transcriptomic profiling at ever-growing throughput. "With our current products, you can do about 80,000 cells per run," says Hindson, "and we've released some datasets at the million-cell scale."

These technologies have already proven transformative for immunology. "Previously, we were limited to the use of flow cytometry, and could only measure at most six to eight different parameters," says Shuye Zhang of Fudan University in Shanghai. "With single-cell RNA-seq, you can measure tens of thousands of markers in thousands of cells, which gives very high resolution of the immune landscape." And although there are relatively few demonstrations of these technologies in infectious disease research, a handful of researchers had begun using them to hunt for genomic footprints of viruses in tissue specimens. "We've been working for several years to try to understand what cells are actually infected by a virus in vivo versus being a bystander," says Ido Amit of the Weizmann Institute of Science in Rehovot, Israel, whose team recently demonstrated the feasibility of using single-cell RNA-seq to perform such profiling with viruses like influenza.

Initiatives like the Human Cell Atlas have also created a foundation of technical expertise that could be repurposed for COVID-19 research. "My lab has developed different experimental frameworks to analyze quite a large range of tissues, including the brain, lung, the entire GI tract, liver, kidney and muscles," says Alexandra-Chloe Villani at Massachusetts General Hospital in Boston, who is one of the coordinators of the immune cell component of the Human Cell Atlas. Their workflows are sufficiently sensitive to capture rare cell types representing as little as 0.1% of a sample, and such sensitivity is often essential if one aims to home in on specific cell subsets that drive disease pathology. One of Villani's postdocs called attention to SARS-CoV-2 in early winter, and by February she and her collaborators had already begun collecting specimens from patients with COVID-19.

Using its high-throughput single-cell screening platform, AbCellera went from 5.5 million cells to clinical testing of SARS-CoV-2 antibodies in 90 days. Credit: AbCellera

And as luck would have it, the US Defense Advanced Research Projects Agency (DARPA) recently funded a series of rapid countermeasure development projects through its Pandemic Prevention Platform (P3) initiative, several of which relied on single-cell screening. "The goal was to go from patient to 20,000 doses of countermeasure in 60 days," says Carl Hansen, CEO of Vancouver-based AbCellera, one of the companies involved with P3. "When they first launched … it was considered complete lunacy." But using their proprietary microfluidic platform for the functional characterization of individual B cells, AbCellera was able to home in on neutralizing antibodies for H1N1 influenza within 55 days. "When COVID-19 finally came to North America … we were ready for that and able to turn the platform directly onto that problem," says Hansen.

Other analytical techniques have also emerged as a valuable complement to transcriptomic data. Several COVID-19 studies are employing repertoire analysis, for example — using targeted sequencing of T cell receptors (TCRs) and immunoglobulins to analyze how the adaptive immune system responds to infection and identify receptors that might offer therapeutic value. Before the pandemic, Adaptive Biotechnologies was using its repertoire analysis tools in collaboration with Microsoft to identify useful immunological signatures for detection and diagnosis of disease. In late February, the two companies launched the immuneRACE study to apply the same analytical strategy to COVID-19, hunting for patterns in the TCR repertoire that may prove predictive of patient outcomes.

Proteomic analysis can also fill in critical gaps concerning cell type and function. Villani and colleagues are using a technique called CITE-seq, commercialized by BioLegend as TotalSeq, which employs DNA-barcoded antibodies to detect hundreds of surface proteins from individual cells in parallel with the transcriptomic data gathered by RNA-seq. "There's a lot of key effector molecules that are really not well captured transcriptionally," says Villani. These can be readily detected with a well-chosen antibody, producing richer molecular profiles for categorizing individual cells in a highly heterogeneous mixture. A Seattle-based research consortium headed by the Swedish Medical Center, the Institute for Systems Biology and Merck is also applying a host of multi-omic analytical techniques to blood specimens collected from patients with COVID-19, including a platform developed by IsoPlexis that employs microchip-based immunoassays to profile secreted cytokines and other signaling molecules from individual immune cells. "It basically allows you to perform 35-plex analysis of cytokine secretion from viable single cells," says James Heath, president of the Institute for Systems Biology and cofounder of IsoPlexis.

Material matters

Despite the technological head start, building a clinical research program against a still-unfolding pandemic entails a myriad of challenges, starting with sample selection. For antibody hunters, the choice

of starting material is straightforward: immune cells collected from convalescent patients who have successfully overcome COVID-19 and are therefore likely to have mounted a robust immune response. But one must balance considerations of speed and quality. "You want the immune response to go on for a long time, because the longer it goes, the more likely you are to get nicely affinity-matured antibodies," says Hansen. "At the same time, this is a race to beat the virus and no one can wait the additional three or four weeks to get this in place." James Crowe, Jr., director of the Vanderbilt Vaccine Center, who oversaw the development of an accelerated single-cell-based antibody discovery platform for P3, was able to get hold of some of the first blood specimens from North American patients with COVID-19 back in January. But this proved a dead end rather than an asset. "We were not able to identify good antibodies from those people," says Crowe. "What ultimately worked was obtaining specimens from several individuals who had been infected in Wuhan in December. By the time we got the sample in March, they were now 50-something days out and their immune response had matured."

When the goal is to profile COVID-19 pathology and immune response, specimen selection becomes a thornier challenge. Peripheral blood is easy to collect and can give an informative window on immunological activity — albeit one that may not fully reflect the ground truth of infected tissue. However, blood from infected patients could still prove problematic to process, according to Villani. "These patients were lymphopenic," she says, "so you would get very little blood, with very little cells … and the cells had very poor viability compared to healthy patients." Her group ultimately spent over two months perfecting a blood preparation protocol for their multi-omic analysis pipeline.

More invasive procedures are needed to survey the antiviral war being waged in the lung. Amit, Zhang and collaborators collected bronchoalveolar lavage specimens, in which a flexible probe is deployed down the patient's airway to rinse the inner lung with fluid and then collect the cells that are released. For the cohort enrolled in PA-COVID-19, an observational study being run out of Charité-Universitätsmedizin Berlin, researchers also collected cells from the upper airway using a specialized brush. This produced a more complete portrait of the respiratory tract, but required considerably more work. "Samples had to be processed in a very timely manner," says Leif Erik Sander, one of the study's coordinators.
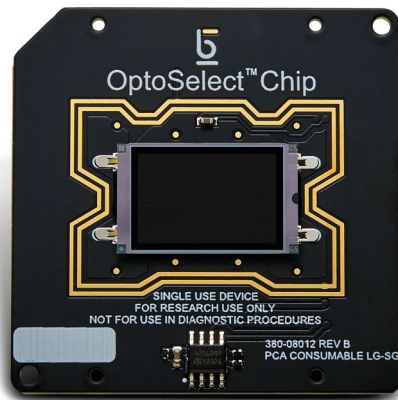
From warp speed to light speed: Berkeley Lights' optofluidic technology uses light to move cells into tiny wells for physical and functional characterizations. Credit: Berkeley Lights

"And obviously these samples have mucus and cellular debris to remove." Villani's group is also collecting autopsied tissues from a variety of organs in patients who died from COVID-19 — a challenging and time-sensitive process, but one that could offer critical insights into fatal disease.

Researchers also had to adapt existing protocols to the rigors of biosafety level 3 (BSL3) conditions. "You cannot have sharps, you have to limit your movements — you kind of have to rethink all of the protocols," says Villani. Blish notes that her team initially used the Seq-Well technique, a relatively low-cost protocol for transcriptome analysis that does not require a specialized instrument, which was developed by her collaborator Alex Shalek at the Broad. Since transitioning to BSL3, however, she has switched to 10x, which offers a more streamlined workflow that requires less effort in a high-risk environment. And above all, these extended sessions of BSL3 work often proved exhausting and emotionally draining. "They always worked long hours — 8, 9, sometimes 11 hours," says Xie. "And it was scary because we didn't know then just how dangerous the virus was."

Hot on the trail
Fortunately, this hard work is paying dividends. In March, researchers led by Markus Hoffmann and Stefan Pöhlmann at the Leibniz Institute for Primate Research in Göttingen, Germany, identified the cellular targets bound by SARS-CoV-2. The spike (S) protein of the virus interacts with a protein called angiotensin-converting enzyme 2 (ACE2), which mediates cellular entry. However, the S protein must also undergo 'priming' before the virus can

enter, which is achieved through cleavage by the protease TMPRSS2.

Single-cell technologies are now enabling researchers to seek out cells where these two genes are coexpressed. Shalek and José Ordovas-Montañes at Boston Children's Hospital recently performed such an analysis with single-cell transcriptomic datasets from various tissues. "The first surprise was actually just how few cells express these genes," says Ordovas-Montañes. "We started wondering if this was real signal or just noise within the data." But after careful analysis, they were able to confidently determine that ACE2 and TMPRSS2 are consistently coexpressed in type 2 pneumocytes, the lung epithelial cells that produce the surfactant that coats the alveoli, and the mucus-generating goblet cells of the nasal epithelium, as well as the enterocytes lining the intestinal wall.

Sander and colleagues also noted the relative rarity of ACE2 and TMPRSS2 coexpression and gained insights into the dynamics of infection. "The viral infection itself actually drives or upregulates the expression of ACE2," says Sander. "We speculate that this might be a 'feed-forward' mechanism for the virus." They linked this to an interferon-response pathway — a signaling network that normally marshals an antiviral immune response, but which may be hijacked by SARS-CoV-2 to accelerate infection. Ordovas-Montañes and Shalek saw a similar pattern in their data, whereby ACE2 expression correlated strongly with upregulation of interferon-stimulated genes, and confirmed this connection in cell culture experiments. "Working with primary human epithelial cells from the nasal cavity, there is a really nice dose–response of more interferon, more ACE2," he says.

Amit's team developed an analytical tool called Viral-Track to capture signatures of viral RNA alongside the cellular transcriptome, revealing actively infected cells in their bronchoalveolar samples. They observed notable differences between severely ill patients who required ventilation and those with more moderate disease. "In the milder patients, within seven to ten days, you have clearance of the virus or it goes to very low titer," says Amit. "But in the severe patients, the viral loads are high … it's a continuous infection." Viral-Track data can also detect other viruses, and Amit reports that such co-infection scenarios are associated with a more dire prognosis. "That is very worrying when we start thinking about the winter and influenza," he says.

Defenses in disarray
These studies have also uncovered intriguing patterns in the immune response to

SARS-CoV-2, as well as mechanisms that might explain why the disease spirals out of control. For example, Blish and colleagues observed a considerable shift in the innate immune cell populations that are often the first line of defense against disease. "The absolute depletion of the multiple innate immune cell subsets in the peripheral blood was really striking," she says. "The inflammatory environment is clearly much more induced in the lung than it is in the periphery." Accordingly, the lung exhibits a parallel reconfiguration of innate immune cell populations. Zhang notes a striking disappearance of alveolar macrophages, which normally regulate and restrain the immune response, in the airways of severely ill patients. These are instead replaced with proinflammatory cell types that pump out chemokines — potent molecular signals that attract other immune cells to the lung. Sander's team observed a similar pattern, and he notes that this recruitment does not appear to be beneficial to patients. "The fact that we don't see a strong correlation between viral loads and the resulting immune response sort of indicates that it's not necessarily more efficient in clearing up the virus," he says.

The adaptive immune response, which recognizes and responds to specific pathogen-associated epitopes, also gets derailed in severe disease. Harlan Robins, CSO of Adaptive Biotechnologies, notes that data from immuneRACE suggest that active suppression of the T cell response may be occurring in sicker patients. "Of the people who do really badly, it seems like 20–25% seem to end up with very little T cell response," he says. Amit and colleagues have likewise noted considerable weakening of the cytotoxic T cell response in lung tissue from severely ill patients, and he believes that this may be one of the critical factors contributing to disease progression. "My hunch is that B cells play a relatively minor role," says Amit. "But T cell response seems to be really critical … and outcomes that are worse are labeled by specific subsets of T cells." It remains unclear whether these cells are being actively killed off by an out-of-control inflammatory response or merely suppressed, although preliminary data from the Seattle consortium offer the unexpected finding that inactive 'exhausted' T cells are specifically accumulating in sicker patients. "Curiously, clusters of CD4+ and CD8+ T cells that display exhaustion markers also appear to be the most proliferative," says Heath.

Single-cell analyses also offer some cause for optimism in terms of immunity-modulating clinical interventions. Several studies have highlighted an apparently prominent role for the proinflammatory cytokine interleukin-6 (IL-6) in COVID-19 pathology. "IL-6 is highly elevated," say Sander. "But we're still not sure where all the IL-6 is coming from — it's not necessarily coming from immune cells, but could be from stromal cells." Some studies suggest that drugs such as tocilizumab, which blocks the IL-6 receptor, may be effective in bringing some patients with COVID-19 pneumonia back from the brink of death, although the recently concluded COVACTA trial by Roche failed to demonstrate clinical benefit from this treatment. Careful profiling of IL-6 activity could help guide clinicians to the subset of patients most likely to benefit. Sander also notes that various chemokine-targeting drugs have been developed that could potentially quell excessive recruitment of innate immune cells to the infected lung.

Betting on B cells

It remains unclear how the B cell response influences the course of infection, but there is compelling evidence that therapeutic antibodies could give clinicians an edge against COVID-19. Multiple studies suggest that plasma from convalescent patients may prevent severe disease from turning critical or fatal, and that this effect is mediated by neutralizing antibodies against SARS-CoV-2. Such plasma preparations will inevitably be a mixed bag in terms of antibody efficacy, but single-cell analysis is allowing researchers to rapidly screen for specific antibodies that offer the greatest antiviral stopping power. Crowe considers such antibodies a particularly promising tool in the therapeutic armamentarium. "Vaccines require the host to respond, and not all hosts can respond optimally — for instance, the elderly, at highest risk from SARS-CoV-2," he says. In contrast, an effective antibody drug can potentially confer instant immunity.

Companies including Regeneron and Vir Biotechnology are testing antibodies that were isolated by using flow cytometry to screen patient-derived serum for B cells that recognize S protein. These isolated cells are then subjected to a PCR procedure that amplifies immunoglobulin-coding sequences for subsequent cloning and characterization. This strategy has yielded antibodies that can mitigate and possibly prevent COVID-19 pathology, but is also somewhat slow and inefficient, according to Xie, who opted instead to use a 10x-based approach to rapidly characterize much larger numbers of patient B cells. "In two months, we screened 60 convalescent patients and obtained 8,600 potential antibody sequences," he says. Bioinformatic analysis allowed his group to home in on several hundred candidates with conserved structural features suggesting they might neutralize SARS-CoV-2, and functional characterization of these yielded one particularly potent candidate that reduced viral loads in the lungs of infected mice by 2,500-fold.

Crowe's group has pursued a multiplatform approach to antibody generation, running 10x analysis in parallel with screening on the Berkeley Lights Beacon platform. Beacon uses a microfluidic system to capture large numbers of individual B cell clones and enables researchers to directly measure functional information about the trapped cells in a highly parallel fashion. "We could test whether any cells were secreting antibodies that blocked virus binding to receptors — it's a neutralization assay with a single cell," says Crowe. He notes that both this platform and the 10x workflow isolated antibodies of roughly equivalent quality, although the Berkeley Lights system offered a valuable shortcut in terms of assessing antibody properties. Their pipeline operates with breakneck speed, requiring less than a month from the receipt of samples to the identification of a pair of lead candidates that protected nonhuman primates against SARS-CoV-2 infection. Candidates from both platforms are now being clinically tested by AstraZeneca and IDBiologics, a startup founded by Crowe.

AbCellera's ultra-fast antibody generation process also relies on a specialized microfluidic system for the high-throughput characterization of individual B cells. Their platform analyzes multiple credit-card-sized modules that each contain enough microscale chambers to capture 500,000 individual B cells, which can then be rapidly profiled in terms of their binding and neutralization capabilities. "You isolate millions of different cells, scan them using imaging and machine vision, recover only those cells that make antibodies with the property you want, and then move those downstream into single-cell genomics," says Hansen. In partnership with Eli Lilly, the company was able to proceed from serum screening to first-in-human dosing of a lead antibody candidate within 90 days, and their molecule is now in phase 3.

Most neutralizing antibody isolated to date targets a specific section of S protein known as the receptor binding domain (RBD), which mediates binding to ACE2. Accordingly, researchers have generally performed antibody screening using either the full S protein or the isolated RBD to specifically enrich B cells that recognize these antigens and thereby stack

the odds. "If we didn't do this functional selection, we had a 1% success rate," says Xie. "This selection allowed us to increase our yield from 10 to 20%." This makes sense biologically, as Crowe notes that RBD-specific antibodies seem to be a common feature of immune repertoires from recovered patients. However, there may be other, less obvious vulnerabilities waiting to be discovered, and Adaptive Biotechnologies is now working with Amgen to profile patient B cell repertoires and their predicted epitopes in a target-agnostic fashion. "Spike is just one protein out of the viral genome, and there's three other surface proteins," says Robins, "so we're going to let the immune system tell us what it's binding."

In the fast lane

It is sometimes easy to overlook that this has only been an active field of research since December. Given that timeframe, the progress made against this new disease is nothing short of astonishing. This has been facilitated by unprecedented levels of collaboration and data-sharing, with research data being released into public databases and preprint archives almost as quickly as it can be analyzed and curated. The patients themselves have also proven to be enthusiastic volunteers, even if they may not benefit from their contributions. "These patients are so sick and yet they're still willing to give their time and blood, and the family members that just lost a loved one but are willing to say yes to autopsies … I find it incredibly humbling," says Villani.

Much of this work is still waiting to bear fruit, particularly in terms of understanding the timeline of exposure, infection, sickness, and eventual outcome of death or long-term recovery. Accordingly, many of these studies have a longitudinal component, wherein patients contribute multiple specimens over time. "We're sampling roughly around the time they get diagnosed, a week later, and then about a month later … and I'm hoping to reconsent for two years," says Heath, "because if you get this disease with any severity and you recover, you're going to be different — this is not a casual infection." With rich multi-omic datasets collected over extended periods, it might become possible to untangle the molecular and biological processes underlying the heterogeneity of symptoms and outcomes that have confounded clinicians and COVID-19 researchers thus far.

But perhaps the most exciting outcome of this work is the recognition that scientists have the tools at hand to go from complete ignorance of a pathogen to manufacturing potentially effective countermeasures in a few months. As his team's SARS-CoV-2 antibodies move into clinical testing, Crowe is already looking forward with a planned initiative called AHEAD 100, which will deploy his group's single-cell pipeline to pre-emptively generate candidate therapeutics against the 100 pathogens with the highest pandemic potential. "There are so many viruses poised to be the next outbreak," he says. "And that's the promise of these technologies — not just to act fast, but to act efficiently and ahead of time." ❏

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