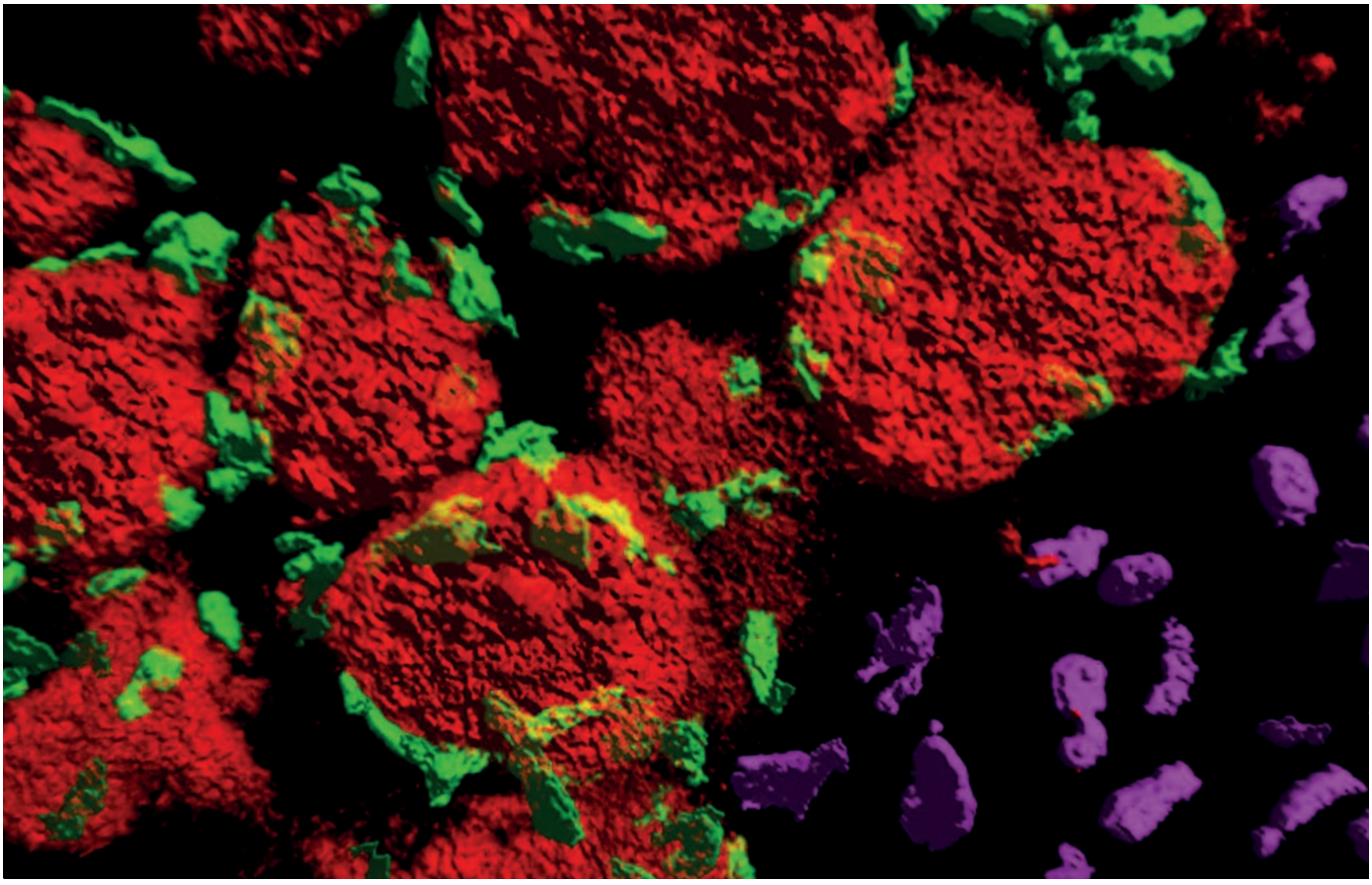

MATTHEW KRUMMEL AND JOHN ENGELHARDT

Two types of immune cells (green and purple) near the border of a tumour (red) are caught in action in this video still.

MEDICAL IMAGING

Removing the blindfold

Using a variety of creative imaging techniques, researchers are tracking the dynamic interactions of immune and cancer cells. Their results will guide drug development.

BY KATHERINE BOURZAC

Mark Headley's computer screen displays the cellular landscape of a living mouse lung, software-corrected to ensure the images do not blur with each rapid breath. Headley, a post-doc in immunology at the University of California, San Francisco (UCSF), points to certain features: the spherical black areas are alveoli, air pockets absent of cells; the blue threads are fluorescently labelled structural proteins within cells; and the reddish tubes are groups of labelled platelets flowing through blood vessels. So far, so good.

Then, like something out of a Hollywood B movie, a neon green blob enters the scene: a cancer cell. The blob stretches out. "It looks like it's trying to escape the blood vessel," says immunologist Matthew Krummel, Headley's boss. Green fragments break off the blob. Krummel doesn't yet know what is happening on screen. The cancer cell may be dying, it may be sending out signals to the immune system, or it may be doing something else entirely.

Krummel and other immunologists are making such videos to get a better grasp of the immune system's response to cancer. Trying to understand immunity with conventional static images is like trying to figure out the game of football by looking at a photograph of players on the field, says Thorsten Mempel, a doctor and immunologist at the Massachusetts General Hospital in Boston. By imaging at the cellular and molecular levels, researchers are starting to learn the plays. They can see not just how much a tumour grew or shrank, but the details of what happened and why.

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Cancer immunotherapy comes of age:
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Initially, treatments that recruit a patient's own immune cells to combat cancer were developed without the kinds of studies Krummel and others can now perform. Researchers couldn't see what the cells actually did in the body, so they were unable to spot problems, says Christopher Contag, an immunologist at Stanford University in Palo Alto, California. "We're seeing the failure of a lot of immunotherapies because we went in blindly," he says.

By watching immune reactions to cancer in detail, and turning a lens on what happens during experimental immunotherapies, researchers hope to take the blindfold off.

CELLULAR GPS

Over the past ten years or so, scientists have been watching fluorescently labelled immune and tumour cells in living animals using sophisticated microscopy technology. They have learned that *in-vitro* experiments, which are often the first step in the development of immunotherapies, can be very misleading.

Many of the things that cells do in culture, they don't do in the body. "Every time we set up a new disease model with imaging we find something unexpected," says Contag.

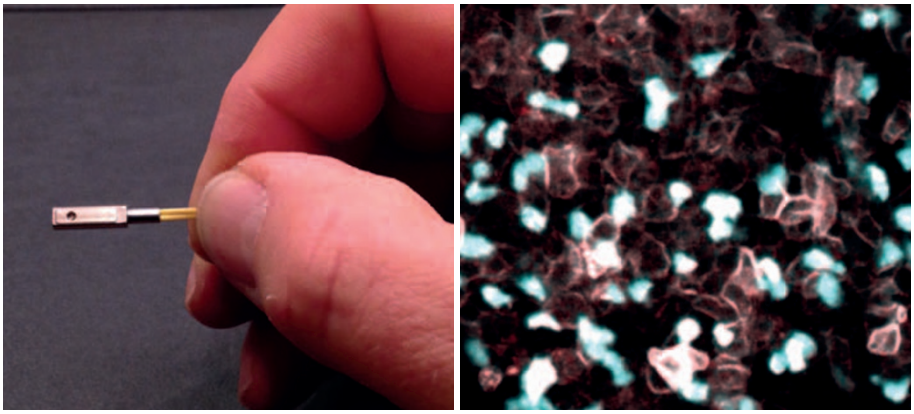
One early finding is that immune cells inside the body take their time. Biologists are using fluorescent labels to track cells and figure out where they are going, how long it takes them, and what other cells they interact with. Immune cells inside the body interact with one another and with cancer cells for much longer than they do outside the body. In cell culture, for example, an immune cell called a cytotoxic T cell will kill a cancer cell in a matter of minutes. In a mouse, however, it's a different story. According to work by Philippe Bousso, an immunologist at the Pasteur Institute in Paris, in the body it takes an average of six hours for a T cell to kill a single cancer cell[1].

Bousso was one of the first to look at tumour–immune cell interactions in living animals using multiphoton microscopy, which allows researchers to view cells as deep as 400 micrometres beneath the skin. Conventional microscopes, which use single photons of visible light to excite fluorescent particles, can peer only about 50 micrometres deep. The infrared light used for multiphoton microscopy is of a lower energy than visible light, so the fluorescent dyes must absorb multiple photons in order to get excited and shine; but infrared light also travels deeper into tissue without scattering. In living mice, 400 micrometres is deep enough to view breast, prostate and skin-cancer tumours.

This multiphoton microscopy enabled Bousso to take a new look at adoptive-cell therapy (see 'Honing that killer instinct', page S13), a treatment in which killer T cells are taken out of the body, trained to target cancer cells, then multiplied and reinjected. His results suggest that one reason these therapies don't work well for most cancers is because each T cell takes more time to kill a single cancer cell than expected, and solid tumours are made up of a massive number of cells. For these therapies to work, the dose of T cells might need to be much greater than what's been tested to date.

The broader implication, however, is that cancer researchers might be wise to re-evaluate their approach. Cancer immunology studies conducted *in vitro* are not always realistic, and conclusions about cell behaviour should be tested in animal models before being accepted and used as the basis for new therapies. "What's going on *in vivo* is difficult to access," says Bousso. The only way researchers can know for sure is to observe their subjects.

Such studies have found immune cells dilly-dallying inside the body. Krummel, for example, recently published research showing killer T cells located at the periphery of breast cancer tumours in mice, interacting with other immune cells called dendritic cells, but never entering the tumour[2]. It was just one more piece of evidence that, in the battle between the immune system and cancer cells, there are many other factors that must be better understood and considered in designing new immunotherapies.



An implantable microscope (left) is being developed to capture immune-tumour cell action (right).

CHEMICAL EAVESDROPPING

To get a better understanding of these complex interactions, researchers are now moving beyond simply tracking cells' locations. "We're trying to get a more detailed picture, at the subcellular and molecular level," says Wolfgang Weninger, a cell biologist at the University of Sydney in Australia. "We don't understand what T cells do when they're put back into the body."

Capturing these details presents imaging researchers with major technical challenges. There are only so many colours of fluorescent proteins, therefore parsing the signal becomes problematic — a few years ago, imaging studies were limited to just four labels at a time. To see more, Krummel's group at UCSF has built its own microscope with two US$150,000 infrared lasers that fire at intervals of 1/30th of a second, each at a different wavelength, to excite different imaging labels that are activated by different colours of light but emit similar colours. Using this method, the group can image six labels at once.

"We're seeing the failure of a lot of immunotherapies because we went in blindly."

Whereas Krummel's microscope relies on speedy frame rates, other researchers are able to get detailed information by snapping higher-resolution pictures. They're taking advantage of a technique called super-resolution microscopy, which makes it possible to image individual protein molecules *in vitro* both on the surface of and within cells. The technique appears to defy the laws of physics. That is because conventional lenses focus light on a spot with a minimum diameter of half the wavelength of light — a barrier called the diffraction limit; researchers, however, have devised ways to get around this constraint.

One early adopter of super-resolution microscopy in immunology is Daniel Davis, a biophysicist at the University of Manchester in the UK. Davis has built a microscope that uses not one but two lasers. One laser excites fluorescently labelled proteins in his sample, while the second laser creates a doughnut-shaped beam of light around the outer rim of the first, cancelling out the excitation of dyes in this region before they have a chance to shine. Typical confocal microscopy collects fluorescence from as small a field as 200 nanometres. But Davis's technique effectively narrows the focus of the light to a donut hole of just 10–20 nanometres, enabling the detection of single molecules (see 'Light show', page S12).

Davis recently used super-resolution microscopy to watch what happens when an immune cell called a natural killer (NK) cell attacks its target[3]. NK cells are part of the innate immune system, the first line of defence that targets cancer cells and foreign invaders non-specifically. NK cell's ability to kill abnormal-looking cells may be what prevents some people from getting cancer in the first place. These cells kill by delivering a membrane-bound payload of deadly proteins called a granule. But as the interior of NK cells is crowded with a thick mesh of structural proteins, Davis wondered how the granules passed through the mesh.

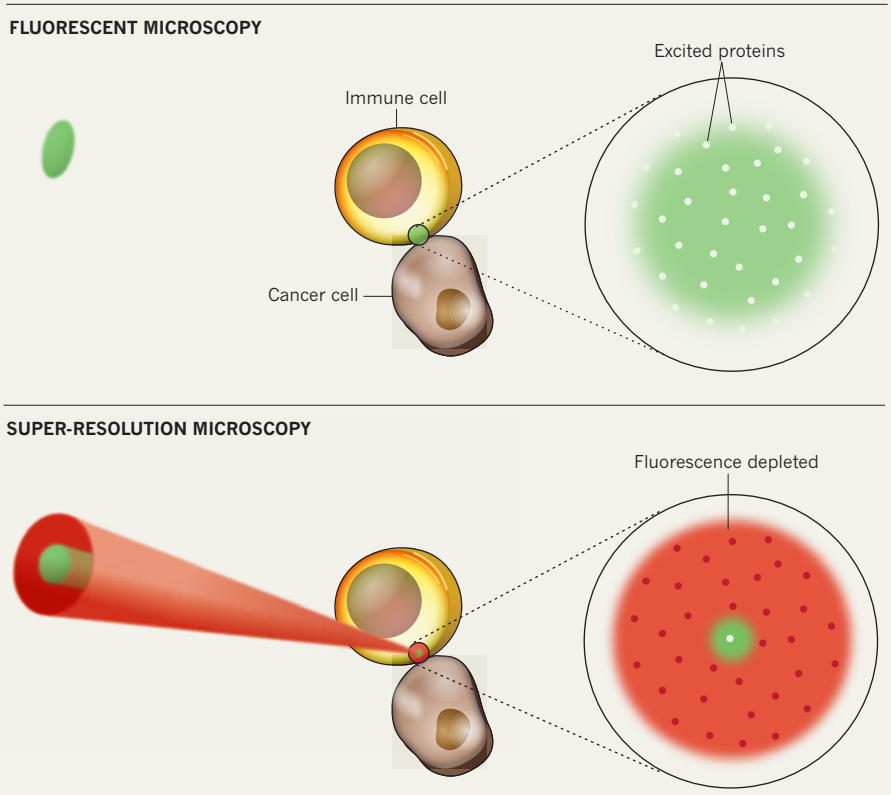
The super-resolution microscope provided the answer as Davis's group watched the process unfold. Directly beneath the cell membrane at the immune synapse, the protein structure that forms a bridge between a killer immune cell and its target, the structural proteins clear a pathway out of the cell for the granule. Although this new insight into cell biology is fairly basic, Davis believes these kinds of studies will lead to new drug targets.

BIG PICTURES

Davis reckons this is just the beginning. Eventually, he says, "we want to see where every single protein is on the surface of these cells." That's key to understanding the cell-level decisions that determine a cancer patient's prognosis. Immune cells receive multiple, often conflicting signals — some activating, some calming — that they must integrate before

LIGHT SHOW

Fluorescent microscopy uses a laser beam (green) to excite labelled proteins inside a cell so they shine, but it illuminates a field containing several molecules. In order to image a single molecule, a second laser beam (red) narrows the imaging field so that single proteins can be detected.



determining whether or not to kill a cell. The subtle details of these cellular actions, Davis says, are at the root of the variation among people both in their tendencies to develop cancer in the first place and in their response to treatment.

Although it is still early days for *in vivo* cell-imaging research, it has potential clinical implications. Imaging the immune system in detail can help researchers who are working on therapies to stay on the right track, says Stanford's Christopher Contag. Indeed, over the past decade, with the help of imaging, his group has been developing a novel immunotherapy both *in vitro* and in mice. They've recently applied to start a clinical trial.

Rather than using immune cells that recognize specific antigens associated with a patient's cancer, Contag's group is developing a therapy based on a class of innate immune cells called natural killer T (NKT) cells, which are distinct from the NK cells in Davis's study. The NKT cells are loaded up with tumour-killing viruses and are, says Contag, professional tumour-homing cells. They're experts at finding tumours, but slow at killing them. The viruses, on the other hand, do a poor job of locating the tumour — Contag refers to these viruses as "dumb particles" because they're not very good at finding their way around. But two days after the viruses reach the tumour, they multiply their numbers a million-fold and endow the NKT cells they inhabit with major tumour-killing prowess.

Loading innate immune-cell navigators with viruses might make for a killer combo, but it was only with the advent of better imaging that the possibility was taken seriously. In fact, researchers had dismissed the idea as a non-starter until Contag studied the dynamics of the system under the microscope.

> ***"Every time we set up a new disease model with imaging we find something unexpected."***

This lack of interest in the strategy made sense, given the previous state of knowledge about how NKT cells behave. It takes about 48 hours for the virus to incubate in the cell and emerge. But because mouse circulation is speedy, researchers assumed that once the virus-loaded NKT cells were injected back into the mouse they would reach the tumour in two hours — way too soon to do much good. And during these two days things could go wrong: interactions with other cells in the tumour's proximity, for example, might prevent the NKT cells reaching their target.

Imaging studies, however, dispelled these fears, revealing that viral release in mice is actually ideal. For whatever reason, the virus-infected cells reach peak accumulation within a mouse's tumour 48 hours after injection, not the expected two hours. In Contag's videos, all the tumour cells are infected simultaneously and bloom fluorescent green with labelled virus as it multiplies exponentially, eventually causing the tumour to collapse like a deflating balloon. And over time, the virus elevates the animals' immune response so that they're protected against recurrence.

Contag hopes to see the same response in late-stage ovarian cancer patients in his proposed clinical trial as he has in animals: not just temporary remission followed by a relapse, but long-lasting immune protection against the cancer. He believes that the immune response elevated by the viral infection can lead to the formation of memory immune cells that respond to and fight back against any tumour regrowth. The tumours tend not to grow back in mice; ideally, the same would happen in people.

The Stanford group is now working on an implantable microscope to do cell-level imaging of tumours deep inside the body and their response to immunotherapies. The device, which his group recently began testing in animals, is about the size of a fingernail and sends and receives light through optical fibres that snake in and out of the body. Its resolution, 0.1 micrometres, is half as good as that of a conventional microscope. However, Contag says that it avoids the need to anaesthetize the mice, cut them open and place them on a microscope objective and enables the researchers to take images over much longer periods.

Now that detailed, cell-level imaging has expanded immunologists' view of therapies in animal models, Contag wants to bring that capability to clinical trials, too. He is developing a non-invasive version of his microscope, which looks much like a laser pointer connected to a cable. A clinician could hold it against a patient's skin and use it to count labelled cells flowing past in blood vessels close to the skin. Such a device could provide a non-invasive way to spot circulating tumour cells — which signal that a tumour is coming back, or metastasizing, or both — in patients.

Detailed, dynamic imaging is enabling immunologists to better understand what really happens in that fast-moving game of immune-system football. Being able to watch the players in real time will lead to more insightful research and smarter drug development. "By understanding the rules," says Mempel, "we can change the game in our favour." ■

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2. Engelhardt, J. J. *et al. Cancer Cell* **21**, 402–417 (2012).
3. Pageon, S. V. *et al. Sci. Signal.* **6** ra62 (2013).