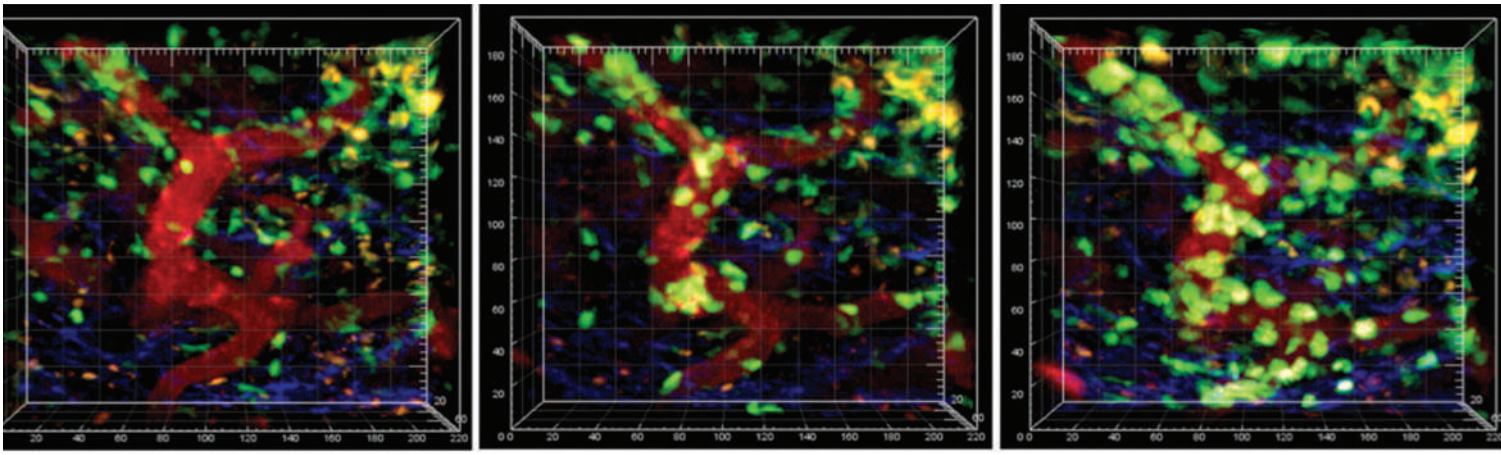


Lights, camera, infection

Multiphoton microscopy is allowing immunologists to watch infections as they happen. **Jeanne Erdmann** pulls up a seat.

Behind the heavy black curtains of his microscopy room, Mark Miller is shooting an action movie. He gives the settings on the multiphoton microscope a once-over while his senior scientist Vjollca Konjufca checks the sedated mouse on the warmed stage. Then Miller flicks a switch on the scanner and red, blue and green images flicker on the computer monitor. He points to what he's looking for. "Right there," he says. Blue-labelled *Salmonella* bacteria gather near the top of a red villus, a finger-like projection from the wall of the mouse's small intestine. The bacteria look like helicopters buzzing around a mountain. It's early afternoon, and we are settling in to watch these bacteria for the next hour.

Miller's experiments, and others like it, are not just gripping the audience behind the curtain — they are gripping a much broader audience of immunologists. Miller, at Washington University School of Medicine in St Louis, Missouri, runs one of the leading labs using multiphoton microscopy to watch infections in living animals in real time.

Less than a decade ago, Miller and other immunologists mostly studied the process of infection *in vitro*, mixing pathogens and the cells they interact with in a culture dish. These simulations had their limits because the cells, much like animals in a zoo, were removed from the environment that influences their behaviour. The advent of multiphoton microscopy allowed researchers to view deep inside living tissues and watch cell biology live and 'in the wild'. This is particularly valuable for the immune system, in which the component cells roam throughout the body's landscapes interacting with pathogens and surrounding cells.

Miller likens multiphoton imaging to a "naturalist studying a herd of gazelles. You infer their function from the behaviour you observe," he says. "The beauty of multiphoton is that you don't have to know what it is you're looking for ahead of time. It just shows itself. We often find things we didn't expect." One thing that researchers didn't expect was the dramatic changes that take place in the first few minutes or hours of an infection, long before symptoms occur. That drama is what's keeping them glued to the screen. "Reality has been eye-opening," says Ronald Germain, an immunologist at the National Institutes of Health in Bethesda, Maryland, who works with the technique.

> **"Multiphoton imaging is like a naturalist studying a herd of gazelles."**
> — Mark Miller

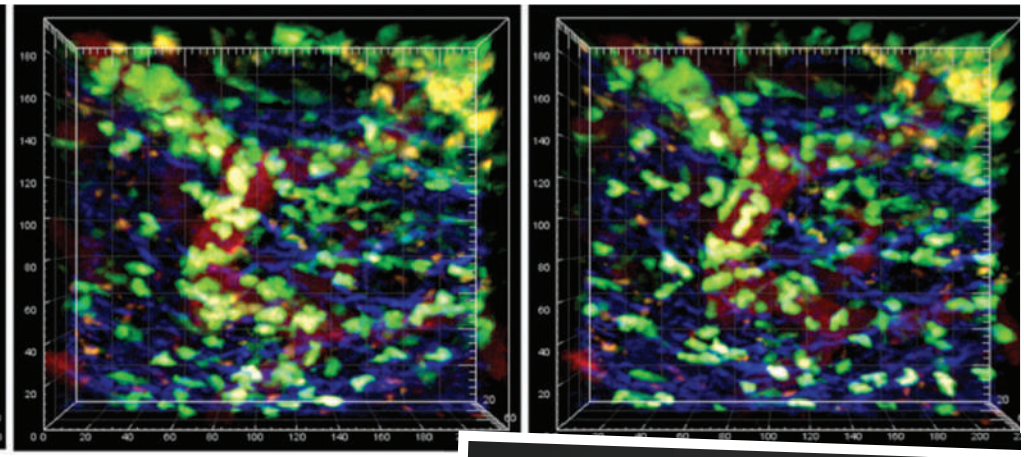
Set dressing

Konjufca started her experiment at 11:00 a.m. She took a mouse that had been genetically engineered so that several types of cell in the immune system — including neutrophils, lymphocytes, dendritic cells and macrophages — emit light in the fluorescent microscope. Then she sucked into a syringe some 10,000 *Salmonella*, also labelled with a fluorescent tag, and injected them into the mouse's intestine. She also injected a fluorescent label that will be taken up by epithelial cells lining the villi, painting them red.

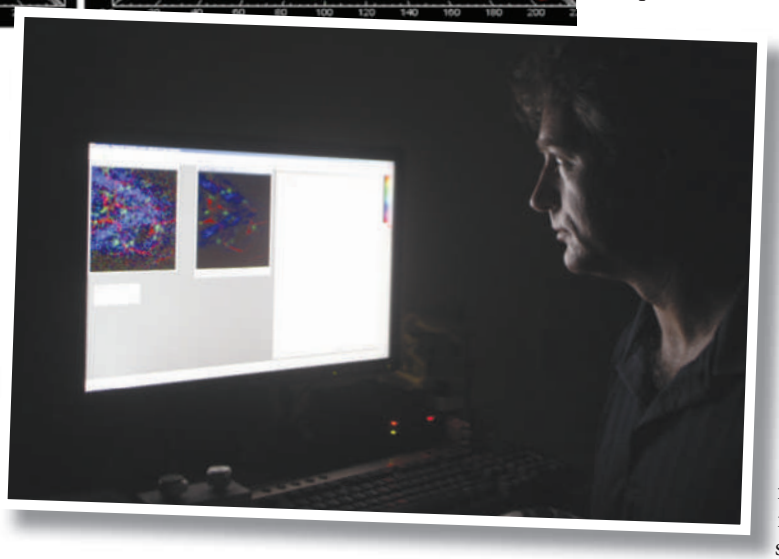
Konjufca does about two experiments like this every week as part of a project to study how *Salmonella* invades the body to cause food poisoning, work she hopes might help produce an oral animal vaccine that protects the food supply. She wants to work out the exact sequence of events during an infection: how the bacteria breach the gut wall and various immune cells arrive on the scene; how dendritic cells in the gut villi engulf and process some of the *Salmonella*; and then which cells transport the broken-down *Salmonella*, in the form of antigens, to the lymph nodes and spleen. "We don't necessarily know which cells go where and at which time point," says Konjufca.

For her experiments she uses both wild-type bacterial strains and ones with different virulence genes mutated. Mutations introduced into *Salmonella* to make a harmless vaccine strain often make the bacteria less able to invade the intestine and provoke the immune system, says Konjufca. The trick is to make a harmless *Salmonella* that still induces a strong and lasting immune response. Konjufca is trying to understand this by watching the mouse's response to these differing strains.

None of the work has been published. For now, she is still hammering out the technical details, such as how many bacteria to inject. Too few, and she may not recruit enough of the white blood cells such as neutrophils, the first responders to infection. Too many, and she will



V. KONJUFCA/T. GRAF



T. GANNAM/AP PHOTO

Over about an hour, neutrophils (green) pour out of blood vessels (red) to fight *Salmonella* in a mouse gut. Right: Mark Miller looks on.

no longer be mimicking a physiological response. She also needs to figure out how long to wait after injecting the bacteria. If she waited, say, three to five hours, they may have already exited the small intestine and she would miss what happens there. Today she plans to wait around two hours. Ironing out these technical details is more than half the battle; otherwise there will be nothing to see.

Special effects

What is routine for Konjufca today was ground-breaking in 1990, when multiphoton microscopy made its debut[1]. In standard confocal microscopes, a single photon of light excites an electron in a fluorescent tag called a fluorophore, and light is emitted as the electron drops back to its ground state. But the high energy of light needed to excite the fluorophore quickly breaks it down and is damaging to cells. The innovation of Winfried Denk, now working at the Max Planck Institute for Medical Research in Heidelberg, Germany, was to excite the fluorophore using the simultaneous arrival of two or more photons of longer-wavelength light. These wavelengths are less damaging and, most importantly to immunologists, they typically penetrate at least 200 micrometres into tissue.

Neurobiologists, desperate to see deeper into the living brain, seized on the technique. Immunologists were not far behind. Miller began using the technique in the early 2000s, while working as a postdoc in Michael Cahalan's lab at the University of California, Irvine, and in collaboration with neurobiologist Ian Parker, also at Irvine. He viewed the compartment of the lymph nodes where naive T cells, a type of lymphocyte, recognize antigen and become activated, a necessary step in readying the cells to respond to future infections. When Miller tried out Parker's multiphoton system on a lymph node snipped out of a mouse, his first reaction was surprise. He thought the T cells would be moving in unison along a gradient of chemicals called cytokines. Instead, he saw them weaving around very fast. "I couldn't believe how quickly and randomly T cells moved," he says. "They looked so purposeful and excited."

Miller used these images to calculate the velocity of the cells, showing that they can reach speeds greater than 25 micrometres a minute. And he suggested that the random movement has a purpose, by helping a T cell range across a broad territory and find the 'antigen-presenting cell' carrying the precise antigen it recognizes. In 2002, the work was published in *Science*[2] as part of a trio of advanced microscopy papers exploring the dynamics of T cells[3,4].

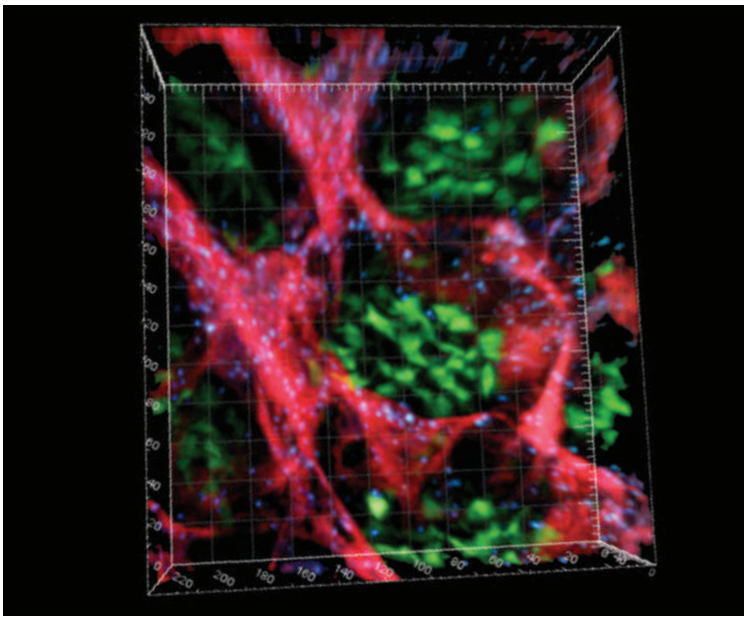
At that time, Miller estimates that only a handful of immunology labs were using multiphoton microscopy. Researchers were excited to just observe the cells and get a picture of how these dynamics build up the host's defences. "It revolutionized the ideas that immunologists had about how immune responses come about," says Ulrich von Andrian, an immunologist at Harvard Medical School in Boston, Massachusetts.

Live action

These days, Miller says, it seems as though every major immunology department has set up a multiphoton system or wants to. "I get requests for advice all of the time." And much of the work has gone from studying the immune cells on their own to the infection as a whole. This makes for a more complex experiment. The tissue must be kept still, insulated from the pumping of the heart or the contractions of the gut, and the pathogen must be introduced without disturbing the surrounding tissue.

When microbiologist Agneta Richter-Dahlfors, of the Karolinska Institute in Stockholm, mastered the technique to watch the first few hours of a kidney infection, she worked with surgeons to inject bacteria into single rat kidney nephrons the size of a human eyelash. The images, some of the first showing an infection in real time, revealed that just two to three bacteria are enough to attach to the mucosal membrane and set up a colony[5]. Within three hours, the oxygen tension in the nephron drops to zero and blood flow to the area halts. "These analyses cannot be performed in any setting other than a live animal," says Richter-Dahlfors. Researchers have now observed a range of bacterial infections, including *Listeria monocytogenes* in the mouse's footpad, a model system that Miller developed with his postdoc Bernd Zinselmeyer to show how immune cells are recruited in the early stages of infection[6].

Two and a half hours after Konjufca injected the *Salmonella*, she reaches into the cage and gently picks up the sedated mouse. She puts it on the microscope stage, and covers it with a velvety, mouse-sized sheet to keep it as comfortable as possible and maintain a stable body temperature. Miller started working with *Salmonella* in the past two years. He likes studying bacterial infections in the gut because it's a common route of infection. It's also home to



V. KONJUFCA/M. NUSSENZWEIG

Dendritic cells (green) and *Salmonella* bacteria (blue) face off in the villi of the mouse small intestine (red).

non-pathogenic bacteria, so he can explore how immune cells tell friend from foe.

Miller built his own multiphoton imaging system at a cost of about half-a-million dollars by customizing a standard fluorescent microscope. It can probe hundreds of micrometres deep into tissue and record images at video rate. Today, he moves the microscope stage around until he finds an area of the small intestine largely empty of undigested food. Along the arc of the villus, the square epithelial cells show up like a necklace of perfect teeth. Some of the bacteria have already crossed the epithelial barrier and found their way to the lamina propria, an area at the bottom of the villi that is rich in dendritic cells and other antigen presenting cells.

> **"Reality has been eye-opening."**
> — Ronald Germain

Towards the talkies

Researchers aren't certain what happens at the epithelium during an infection. One possibility is that the *Salmonella* infect and then kill the epithelial cells, releasing bacteria that dendritic cells engulf. These cells process the bacterial constituents and stick them on their surface as antigens that will stimulate an immune response. Another idea has come from Germain's work suggesting that dendritic cells reach extensions across the lumen and fish bacteria out[7].

Miller collects a stack of images from multiple villi, at intervals of 20–30 seconds, data the computer will compile into three-dimensional time-lapse videos. By 2.30 p.m., the team has watched most of the *Salmonella* reach dendritic cells at the base of the villi. Miller shuts off the microscope, turns on the lights, and pulls back the curtain. The team transfers the data to a computer in an adjoining room.

Microscopy doesn't provide as complete a view of the infection process as researchers might like. "There's a lot of people thinking in molecular terms for their entire careers," says Miller, "and they're resistant to the idea that imaging is providing useful information because they don't feel it's quantitative." But he and other advocates of the technique disagree. They say that the quantification comes from measuring how fast cells move, which direction they move in and whether or how often they stick to other cells. They say that what's missing is a qualitative aspect that gives more information about why cells behave in certain ways. Following cells to an infection site is good, but what happens when they get there? Are the cells signalling, and if so, how?

Immunologist Phillipe Bousso, of the Pasteur Institute in Paris, compares the footage to silent movies: showing how cells move and interact, but not how they communicate biochemically[8]. What would give them 'sound', he says, would be biochemical or genetic assays that could be used in conjunction with microscopy. Jost Enninga, a biochemist also at the Pasteur Institute, is developing and patenting such assays, including one designed to trigger fluorescence in epithelial cells when they encounter pathogens. Another idea is to combine microscopy with real-time monitoring of bacterial gene expression, by dissecting out and analysing tissues at various time points.

Another limitation with microscopy is that the scene is not complete. Any cells not engineered to fluoresce disappear into the black background. "[Researchers] know the cells are there but don't necessarily think it's a disadvantage not to see them," says von Andrian. "If everything revealed itself it would be chaos."

It's a wrap

Later that afternoon, and over the next week, Miller and Konjufca review the videos of the experiment and compare them to earlier ones. They are pleased to see that in the most recent movies the villi are well defined and the bacteria visible on both sides of the epithelium. It shows that they got the set-up right, and that the timing between the injection and the imaging was spot-on. A film of an earlier experiment shows some of the neutrophils in local blood vessels rushing by at several hundred micrometres per second, while other neutrophils respond to inflammatory signals and slow down. The slowed cells then crawl along the inside of the vessel until they reach one spot where they all seem to squeeze through the wall and pour out of the blood vessel like bees swarming through a crack in a fence.

In her next experiments, Konjufca will wait longer before filming, so she can start to study in more detail how the cells cross the epithelium and which cells transport bacteria away from the gut. She'll then follow them to the liver and spleen to study how *Salmonella* interact with T cells and dendritic cells there. She hopes to understand how all this is different in the attenuated vaccine strains. And Miller is working with a computer scientist to develop software to track cells outside the field of view. The microscopic field is small and cells wander in and out of sight. He also wants to analyse their migratory behaviour as it's happening.

Even without these special effects, the surprise and discovery that each movie brings is enough to keep him going, Miller says. "That's the excitement that you get when you're in the lab. That's the motivation," he says. "'What are we going to see today?' We may have some ideas but we may be completely surprised." ■

Jeanne Erdmann is a freelance writer based in Wentzville, Missouri.

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See multiphoton movies at http://tinyurl.com/n35ufp and http://tinyurl.com/mb6l45