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MAKING CONNECTIONS

By turning neurons technicolour, Jeff Lichtman exposed the brain's wiring. **Jonah Lehrer** meets the 'unapologetic cell biologist' with ambitions to map every connection in the human brain.

C. SENTER/AP

At first glance, Jeff Lichtman seems to be hanging long strips of sticky tape from the walls of his Harvard lab. The tape flutters in the breeze from the air-conditioner. But closer inspection reveals that this is not tape: it is the brain of a mouse, rendered into one long, delicate strip of tissue and fixed onto a plastic film. When the film is tilted to the light, the tissue becomes visible, like the smear of a greasy fingerprint.

These smudges are the creation of a new brain-slicing machine invented by Lichtman, a molecular and cellular biologist at Harvard University, along with Kenneth Hayworth, a graduate student at the University of Southern California, Los Angeles. Called the automatic tape-collecting lathe ultramicrotome (ATLUM), the machine resembles an old-fashioned film projector with two large reels. At its centre is a fixed diamond blade that cuts continuously into a rotating mouse brain, much like an apple parer. The end result is a seamless sliver of tissue, less than 10 nanometres thick and around 5 metres long, that is deposited on the plastic film spinning around the spools.

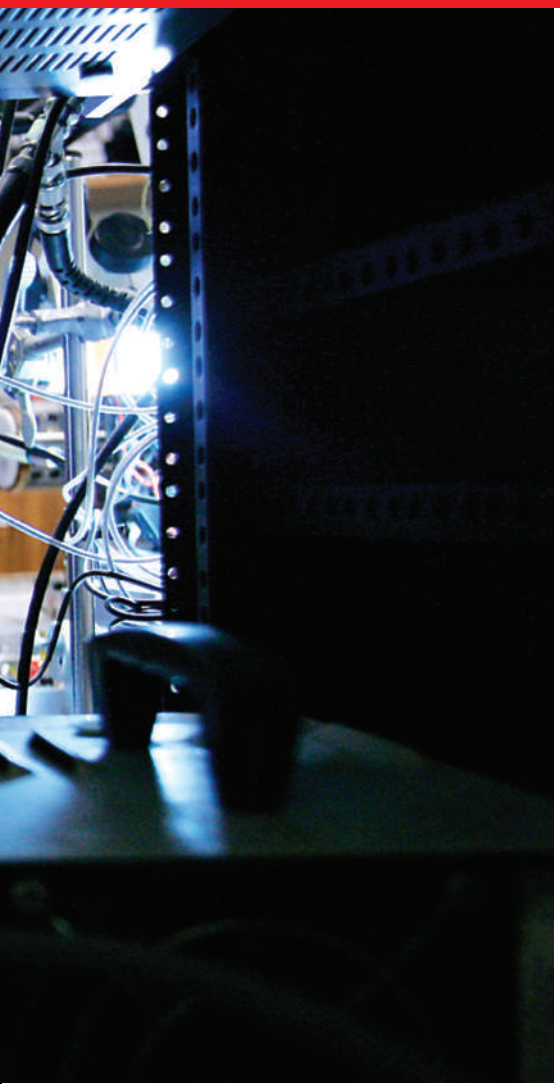
> **"You can learn a tremendous amount, and generate some interesting theories, just by staring at pictures of the brain."**
> — Jeff Lichtman

Although Lichtman appreciates the technical precision of the ATLUM — "That's a real diamond!" he says — he is most excited about its scientific potential. Researchers in his lab are starting to put these slices under an electron microscope to visualize the intricate web of connections between neighbouring neurons. Lichtman eventually hopes to have a 'farm' of several dozen such microscopes scanning tissue around the clock. Even then it would take months, if not years, to capture all the connections in the strip from a single brain. "When you cut the brain this thin, there's just such a massive amount to see," he says. "It does require us to think about imaging on a different scale."

Lichtman likes to think on a different scale. In recent years, he has become a leading proponent of a new field that is working to create a connectome, a complete map of neural wiring in the mammalian brain. Currently, such a map exists only for the nematode *Caenorhabditis elegans*, which has 302 neurons. The adult human brain, in contrast, contains 100 billion neurons and several trillion synaptic connections. "I know the goal sounds daunting," Lichtman says. He insists that such a wiring diagram is an essential undertaking, because it will allow scientists to see, for the first time, the path that information takes as it is shuttled from cell to cell, and how all these cells and the information they transmit weave together to create a conscious brain.

All in the wiring

As Francis Crick and Edward James wrote in a *Nature* Commentary[1] in 1993, "It is intolerable that we do not have [a connection map of] the human brain. Without it there is little hope of understanding how our brains work except in the crudest way." Thomas Insel, the director of the National Institute of Mental Health in Bethesda, Maryland, notes that many of the most common mental illnesses, from autism

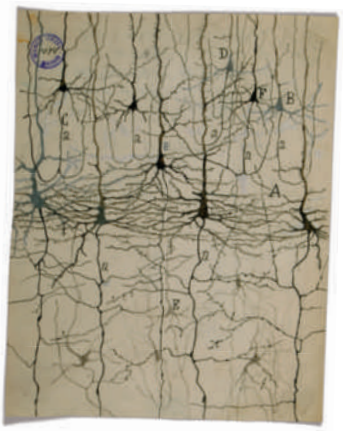


to schizophrenia, seem to be diseases of "faulty wiring", in which the brain has a set of aberrant connections. "The brain needs a connectome, just as modern genetics needed a genome," says Insel. "That's the only way we're going to understand how the brain works at a detailed level, and also what happens when something goes wrong."

As yet, Lichtman, Insel and others have not proposed a formal connectome project — and Lichtman declines to even give a rough estimate of such a project's cost, saying only that it would be a "scary number". But a debate is under way about whether such an undertaking would be worthwhile. Studies have shown that a majority of synaptic connections — some estimates run as high as 80% — are extremely weak and transmit few electrical signals. If that's the case, then a map of structural connections might actually misrepresent the brain's functional organization. "Only a very small proportion of connections seem to drive network activity," says John Isaac, who studies synaptic plasticity at the National Institutes of Health (NIH) in Bethesda, Maryland. "How do you know which connections are important? A wiring diagram won't tell you that." It is also unclear if the connectomes of different individuals could be readily compared in the same way that their genomes can be. Whereas bioinformatics can easily identify two similar genes in different genetic sequences, it is not yet clear what comparable tools will serve for identifying functionally equivalent neurons in two brains — if they even exist — or in a diseased brain versus a healthy one.

Leading connectomics scientists are not deterred by these problems, saying that they will be solved only once the research is under way. "The point is that you don't even know what's important until you see the system in its entirety," says Winfried Denk, director of biomedical optics at the Max Planck Institute for Medical Research in Heidelberg, Germany, and a pioneer of advanced microscopy. "There is a tremendous virtue in completeness."

Lichtman says the criticisms of the connectome are similar to those put forward at the start of the Human Genome Project — and he expects them to die down once the data start coming through. Indeed, Lichtman is so convinced of the connectome's value that he hopes it could transform the way that neuroscientists study the brain. He says that the typical experimental process, in which a scientist sets out to test a specific hypothesis, is simply incapable of deciphering something as complex as the human mind. "History has shown that it's rather tough to come up with good hypotheses about how the brain works," he says. He thinks that scientists should rely more on inductive reasoning — the staple of nineteenth-century scientists — in which hypotheses are generated only after careful observation. "We need to rediscover the power of looking," he says. "You can learn a tremendous amount, and generate some interesting theories, just by staring at pictures of the brain."



Santiago Ramón y Cajal drew the microscopic structure of neurons.

CAJAL INSTITUTE, CSIC, MADRID, SPAIN

Lichtman is perhaps best known for pictures generated by Brainbow, a technique unveiled in November 2007, with which his team engineered individual neurons to emit more than 90 different shades of fluorescent light, from cerulean blue to heather grey, rendering mouse brains as Impressionist landscapes[2]. Lichtman is proud of the images — "People always ask me for screensavers and stuff" — but he says that the beauty of the Brainbow is actually a side effect of the biology. "The prettiness is just the structure of the brain," he says. "All I did was make the structure visible."

Because each neuron is labelled with a different hue, Lichtman and others could start to untangle the knot of neural connections in the mouse brain. In this sense, the Brainbow represents an important milestone, as it promises to advance a scientific goal that was first outlined in the 1880s, when the Spanish physiologist Santiago Ramón y Cajal set out to trace the microscopic structure of the nervous system. He used a technique developed by Camillo Golgi that darkly stained a few cells in brain tissue with silver chromate salt. Although Ramón y Cajal was able to decipher the layout of individual cells — he compared its shape to the branches of a tree — the monochromatic pictures made it difficult to parse the connections between two neurons that were identically labelled. Where did one cell end and the other begin?

Context is everything

Lichtman argues that the difficulty of this parsing problem has led neuroscientists to neglect neuronal connections and circuits ever since, and instead fixate on the electrophysiological activity of individual cells and the biochemistry of synapses. "We've developed all these powerful tools that let us see what one neuron is doing," Lichtman says. But in his view, a brain cell by itself can't do anything; it is defined by its web. This is why, he says, it is so essential to develop tools that allow scientists to visualize neural cells and their connections on a massive scale.

In 1970, neuroscientist Paul Redfern at the University of Liverpool, UK, described a peculiar aspect of the nervous system[3]. He measured the electrical signals that stimulate muscles in a newborn rat, and watched how they changed in the first 2–3 weeks of its life. Based on these data, he proposed that when a rat is newborn, each muscle fibre makes connections with a few dozen neurons that grow out from the spinal cord. Then a period of competition ensues until, within a few weeks, most of these connections disappear, leaving only one victorious neuron synapsed to each fibre. Redfern's idea challenged the widespread assumption that the wiring of the nervous system is predetermined and precisely choreographed. And Lichtman, who came across the paper a few years later as an MD–PhD student already interested in the brain, at Washington University in St Louis, was captivated by the question of how the nervous system figured out which neuron would pair with which muscle fibre. "I remember

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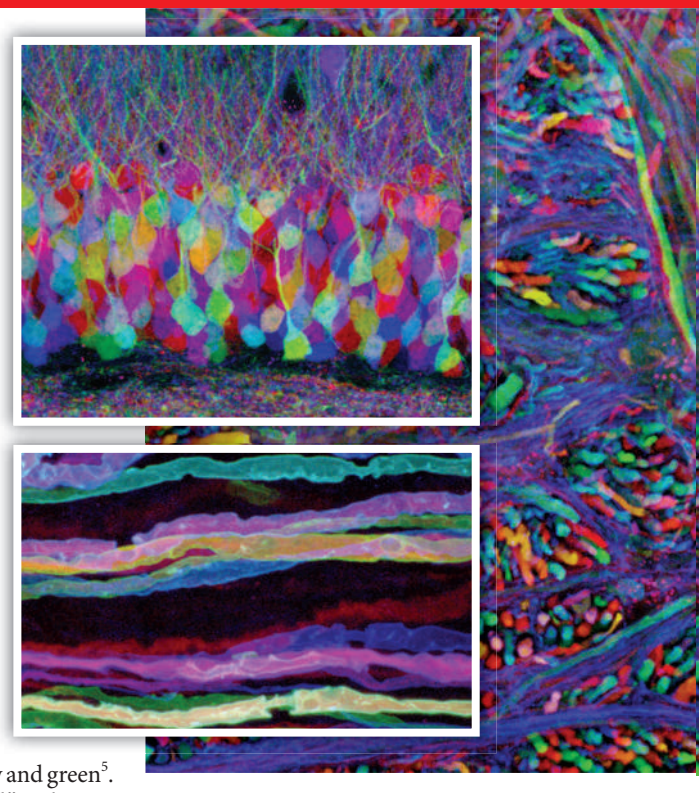
reading that Redfern paper and thinking that that was just the most interesting problem," he says. "I wanted to understand the rules of the game."

Lichtman soon discovered why such a basic question remained unanswered: it was virtually impossible to visualize and track individual neurons over time. Although light microscopy had advanced radically since Ramón y Cajal, and scientists had found fluorescent dyes and other new staining techniques, most of these still rendered every neuron the same colour and so were unable to distinguish which cell connected to which. Think of the tangle of cables running from an overloaded socket: if the wires are all black, it's difficult to know which wire connects to which appliance. But if each wire is painted a distinct colour, it's suddenly possible to find the one to disconnect. The insight, which Lichtman began working on as a young assistant professor at Washington University, was that the easiest way to distinguish these cells was with a splash of colour.

His first attempt, published in 1985, involved electrically stimulating axons so that they took up various fluorescent probes[4]. The experiment was successful — each neuron was a unique colour — but painstaking, because the neurons had to be stimulated one at a time. Lichtman knew he would need to take a different tack to study neurons en masse, and the development of green fluorescent protein in the 1990s, followed by its many-coloured spin-offs, gave him the tools he needed.

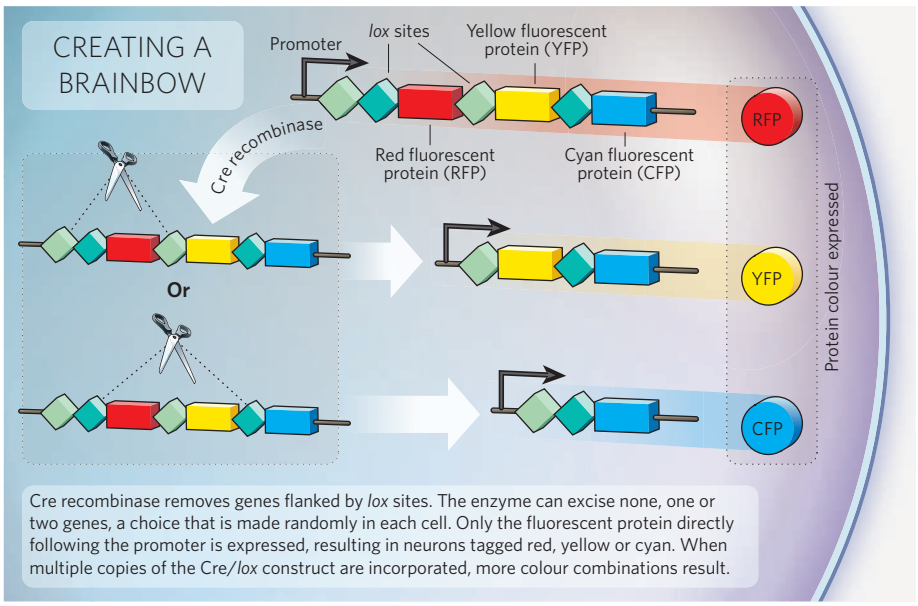
In 2000, Lichtman collaborated with Joshua Sanes, a professor of molecular biology who was then at Washington University but who moved to Harvard University in 2004, the same time as Lichtman. They began generating lines of mice that expressed different fluorescent proteins in some cells of their nervous system, so that some mice had neon-blue neurons whereas others had cells of red, green or yellow. The results were extremely variable, with some mouse strains expressing a fluorescent gene everywhere and others showing fluorescence in only a small percentage of cells. Sanes and Lichtman began crossing these different lines of mice, so that a strain expressing a few blue-labelled cells would be mated with a 'yellow' line with many labelled cells. The end result was mouse brains with neurons expressing three different shades: blue, yellow and green[5]. "That's when I started to get excited," Lichtman remembers. "We still couldn't see that much, but I began to appreciate how powerful these fluorescent cells could be, if only we could engineer more colours."

In 2002, Jean Livet joined the Lichtman lab as a postdoc and immediately began working on the problem. "Jeff would always say to me, 'More colours! We need more colours!'" says Livet, now at the Vision Institute in Paris. "The idea of a fluorescent brain became like an obsession." Livet's elegant solution, which he devised after several weeks of "doodling in the lab notebook", was to use a DNA-engineering system called Cre/*lox*. Cre is an enzyme that deletes or inverts any section of DNA flanked by *lox* sequences. (The Cre/*lox* system is often used to delete genes from specific tissues and create knockout mouse strains.) Livet realized that if he could build a genetic construct that contained multiple fluorescent proteins flanked by *lox* sites — he chose red, blue and yellow — he could force the Cre enzyme to randomly 'choose' which colours to erase and which to leave (see 'Creating a Brainbow'). "It would be like rolling the dice in each cell," says Livet, "so you never know what colour you will get."



Brainbow-coloured nerve cells in the brainstem (main picture), in the dentate gyrus of the hippocampus (inset, top) and in a peripheral nerve.

J. LIVET

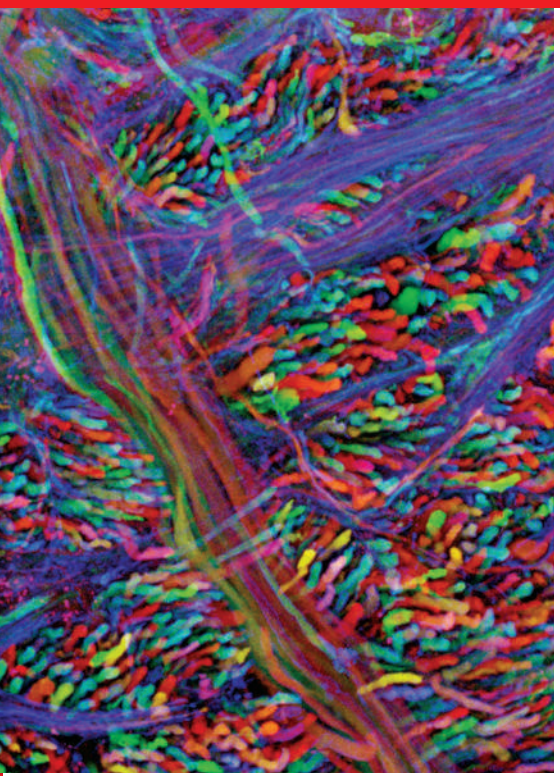


Cre recombinase removes genes flanked by *lox* sites. The enzyme can excise none, one or two genes, a choice that is made randomly in each cell. Only the fluorescent protein directly following the promoter is expressed, resulting in neurons tagged red, yellow or cyan. When multiple copies of the Cre/*lox* construct are incorporated, more colour combinations result.

At the end of the Brainbow

The reality, however, far exceeded their optimistic imagination. Because the mice incorporated multiple copies of the genetic construct carrying red, blue and yellow proteins, the Cre enzyme mixed up a far more extensive colour palette. "We thought we would just get cells expressing a single fluorescent colour, such as blue or yellow," says Lichtman, "but instead we got cells expressing blue-blue-blue-yellow, or blue-yellow-yellow-yellow, and so on. It was a staggering result, to see a brain expressing 80 or 100 distinct colours." Other scientists immediately recognized the potential of the Brainbow (a name that Lichtman devised, and with which he is clearly pleased), and have begun applying the technique to visualize the nervous systems in a variety of lab animals, including zebrafish and fruitflies. "It's an incredibly exciting technique," says Insel. "We've had such a crude map of connections for so long, and this is one of those advances that allows us to really think about how the brain is wired at a cellular level."

With his fluorescently tagged mice, Lichtman finally had a way to solve the problem that had fascinated him since reading Redfern's paper about 25 years earlier. By making maps



of blood vessels and other landmarks visible under the microscope, he could return again and again to precisely the same spot of a neuromuscular junction in a newborn mouse's neck, and know from the neuron's colours exactly which cells remained and which were pruned back[6]. Researchers had mostly assumed that the pruning process followed the classic Hebbian model — "cells that fire together, wire together" — and that the cell with the most synapses on the target muscle, and so transmitting the most electrical impulses, would always gain control. But by observing the process, Lichtman has found that this isn't the case. "Sometimes, the cell with only 20% of the synaptic territory takes over," he says. "The underdog wins."

Lichtman says that neurons that are simultaneously competing to form synapses with several muscle fibres will deploy resources depending on the outcome of all those competitions. A cell that has yet to win a synaptic competition will be able to send more resources to its remaining synapses. (As Lichtman puts it, "being a loser makes you more likely to win somewhere else".) The end result is an exquisitely organized circuit, in which every neuromuscular junction is properly plugged into the nervous system. "The Brainbow allows you to watch this competition in real time," says Lichtman, who hopes to image similar competitions in the brains of mice. "You can see the purple cell win, or watch the turquoise cell retreat. It might seem disorderly, but this is how a brain is built, one competition at a time."

Even as the Brainbow has been illuminating aspects of brain building, Lichtman has been working on refining the method in his pursuit of the connectome. One of his priorities is to visualize the fine-grained branches of the neuropil, the web of dendrites and axons that protrude from either end of a neuron and form synapses with neighbouring cells. The neuropil is too thin to discern with ordinary light microscopes, whose resolution is fundamentally limited by the wavelength of light. This means that objects smaller than 200 nanometres can't be reliably detected. (A typical synaptic cleft, for instance, is between 20 and 40 nanometres, making it all but impossible to see.)

In the past five years, several teams have come up with methods to improve this resolution, allowing fluorescent light microscopy to approach the resolution of electron microscopy. Lichtman is now working to image the neuropil with Xiaowei Zhuang, a professor of chemistry at Harvard University who developed one such high-resolution technique called stochastic optical reconstruction microscopy (STORM). The advantage of this method over electron microscopy is that it can be readily combined with the spectral information of the Brainbow, so that the dendritic arbors of each cell are properly identified. Lichtman refers to this project as "BrainSTORM". The challenge, however, is turning STORM — a labour-intensive method in which each image requires extensive computer processing — into an automated method capable of capturing image after image through the entire brain of a living mouse.

Gathering resources

Lichtman knows that mapping the brain is an epic and expensive undertaking, and it is not one that he can complete alone or with a single method. Some neural connections will be best imaged with the Brainbow, BrainSTORM and other sophisticated microscopy techniques, whereas others — perhaps those buried too deep in the brain to be seen by fluorescence imaging — will be better viewed with ATLUM and electron microscopy. Gathering and processing these images for just one brain will require a new level of standardization and industrialization, something he is working towards with the electron-microscope farm and automated BrainSTORM. "The sheer quantity of connections means that all of these techniques need to be extremely scalable, so that we can construct and analyse massive data sets," he says.

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> — Thomas Insel

Lichtman is starting to collect the resources he will need to work on this scale, and is leading a 'connectome consortium' that includes Zhuang and Sanes. Last November, the group won a US$10-million grant from the Howard Hughes Medical Institute to show that a high-throughput version of BrainSTORM is feasible. The NIH is funding the development of the next generation of Brainbow mice, and Microsoft Research is assisting with some of the complex computational aspects of the project. Given the resources, Lichtman is confident that the connectome will be completed. He cites recent large-scale neuroscientific projects, such as the gene-expression map created for the Allen Brain Atlas, as proof that such an ambitious endeavour is possible.

C. SENTER/AP

Jeff Lichtman's microtome turns mouse brains into a single strip of tissue, fixed to a plastic film.

And in the meantime, Lichtman takes solace in the beauty of his Brainbow images. If other neuroscientists can appreciate their full potential, he thinks, it could change the way they think about the brain. They will see the organ not as a mass of discrete anatomical areas, or as a collection of chemical ingredients, but as a vast loom of connected cells.

"This is what we are," Lichtman says. "Lots and lots of connections." ■

Jonah Lehrer is a freelance writer based in Boston, Massachusetts.

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