For all its importance, the human brain remains an enigma. It contains and controls everything that makes humans human, yet researchers know little about what truly makes it tick. NIH Director Francis Collins has called the brain “the most complicated biological structure in the known universe. We’ve only just scratched the surface in understanding how it works.” Naturally, researchers are working hard to address that information gap. Some are exploring these mysteries using whole-brain imaging. Others are mapping activity at the cellular level. Still others have taken a reductionist approach, tracing the wiring of the brain’s seemingly limitless circuitry.

The idea, explains Winfried Denk, Director of the Department of Biomedical Optics at the Max Planck Institute for Medical Research in Heidelberg, Germany, is that if you know how something is put together, you can begin to explore how it works and, equally importantly, how it does not. If two regions are connected by neurons, that means those regions communicate. Conversely, a lack of wiring means they do not—information that can help neuroscientists judge models of brain function. “Neurons don’t use cell phones,” Denk quips. Connectivity data, he says, “guides your thinking into how information flow can occur in the brain.”

Researchers can draw limited maps for specific neurons of interest using light microscopy techniques, even in large brains when processed with the CLARITY method. But light microscopes cannot yet reveal all the nanometer-thin wires and synapses that connect neurons to one another—only electron microscopy (EM) can do that. “The wires define the computations that are possible by the circuits,” says Albert Cardona, a group leader at the Howard Hughes Medical Institute’s Janelia Research Campus.

Neuroanatomists have long recognized the potential value in mapping the brain’s wiring diagrams, a collection of data that has been coined a “connectome.” But the technology hasn’t been available to make such efforts practical, or at least scalable. That’s no longer the case, however. Recent studies have used EM to map pieces of the fly eye and mouse retina, data that have helped settle a long-standing debate on how animals sense motion. Buoyed with these results, researchers are now setting their sights on an exponentially bigger challenge: mapping the brain from stem to cortex.

**Neural Cartography**

To get a sense of the challenge of brain mapping, consider what has been accomplished to date. Only one organism’s entire neuronal wiring has been traced, that of the nematode worm, *Caenorhabditis elegans*. Sporting all 302 of neurons linked by some 7600 “synaptic connections,” the nematode connectome was published in 1986 in a mammoth 340-page treatise in the *Philosophical Transactions of the Royal Society* by John White and colleagues in Sydney Brenner’s group in Cambridge, UK (1). The team built their map using serial section electron microscopy by encasing a worm in plastic, then making a series of transverse slices perpendicular to the animal’s long axis. Every third section, each about 50-nm thick, was photographed and printed onto a transparency. From 8000 such images, the authors painstakingly traced every axon and neurite from end to end.
In 1991, Ian Meinertzhagen, now a senior scientist at Janelia Farm and University Research Professor at Dalhousie University in Halifax, Nova Scotia, used essentially the same approach to map a small piece of the *Drosophila* eye (2). The sections are so thin "you can't see them directly with the naked eye; you see them by their interference color, by the light that’s reflected off their face." They’re also incredibly small, because the electron microscope images at such high resolution, and the plastic from which they are cut has a consistency "rather like mozzarella cheese." In trying to cut, position, and image several thousand such slices, mistakes are inevitable. "The biggest frustration is that you can cut a long series and then all of a sudden there will be a break and you'll have an interruption in the series and then you have to make a decision whether to go on."

The *Drosophila* eye is a well-studied system for connectomics researchers. "The whole thing is absolutely crystalline in its arrangement," Meinertzhagen says, with regular columns of neurons underlying each eye facet, or ommatidium. It also has the benefit of having a relatively straightforward structure-function relationship, in which the photoreceptors and underlying neural columns act like the pixels of a CCD camera. "It performs a well-defined computation," says Dmitri Chklovskii, group leader for neuroscience at the Simons Center for Data Analysis, a research division of the Simons Foundation in New York City.

In his original study, Meinertzhagen and his graduate student spent a year imaging a dozen cells and some 600 sections, then devoted another year to tracing all of the fibers. He revisited the problem nearly 20 later, joining forces with Janelia Farm researcher Louis Scheffer and Chklovskii (then also at Janelia) to map 379 neurons and 8637 synapses in a single column of the fly medulla, a deeper relay station of the eye (3).

To build their new map, the team, led by postdoctoral fellow Shin-ya Takemura, used the same serial EM approach, peeling off 50-nm sections of tissue and imaging them by transmission electron microscopy (TEM). "The huge difference now is that images can be collected digitally," Meinertzhagen says—a fact that also accelerates data analysis, or "segmentation," the process by which the neuronal arbor is fleshed out into a three-dimensional form. In this case, the team used a "semi-automated pipeline" where a computer takes a first crack at following the neurites, after which 10 human proofreaders double-check the work.

Imagine the scale of that problem: TEM images have an x-y resolution of about 4 nm. But each section is 50 nm thick. Thus, the data set is like a stacked series of thin planes separated by wide gaps. Nerve fibers can be thinner than those gaps, as small as about 20 nm in some cases, so as they branch and thin out, they can disappear from the data set. They also can pop up seemingly out of nowhere. To make the process slightly easier, Chklovskii’s team used processing software to colorize the images so that each neuron is a different color, imparting to the otherwise grayscale EM images a muted rainbow palette that Meinertzhagen calls “a beautiful jungle.” Still, he says, the reconstruction process is “concussively boring”—you can take a crack at a similar problem at openconnectomeproject.org—and it took some 14,400 person-hours to complete.
Extracting Value

Connectome research can help weigh models of how the brain works, says Moritz Helmstaedter, Director at the Max Planck Institute for Brain Research in Frankfurt, Germany, who in 2013 charted nearly 1000 neurons from a piece of mouse retina measuring 114 \( \mu \text{m} \times 80 \mu \text{m} \) (4). That is especially true in brain regions where structure and function are less obviously connected. “The structural data can help us say, okay, this is possible because this is how the neurons are actually wired up, and this may not be possible, this other model, because that’s not how the neurons wire up.”

Based on their analysis of the neural connectivity of the fly medulla, for instance, Chklovskii and his colleagues predicted that certain cells in the circuit provide key time delays in how the eye senses motion, supporting a model called the Hassenstein-Reichardt elementary motion detector. The idea is that as objects move from right to left across the visual field, they first activate the right-most photoreceptor, then the next one in line. Signals from the two receptors feed into a downstream neuron similar to a logical “AND” gate, which fires when both signals are received telling the brain that the object is moving. To make that work though, the signal from the right-most receptor must be delayed slightly so that the two signals arrive at the same time.

This was the prediction, but how to prove it? A picture of neural tracings can identify neuronal type from position and shape, but not what they do. A connectome, after all, is just an atlas—it contains no functional information. “It’s like a road map, but we do not know the traffic,” explains Hongkui Zeng, Senior Director for Research Science at the Allen Institute for Brain Science.

That’s where electrophysiology comes in. Chklovskii passed his finding on to Rudy Behnia, a postdoctoral fellow at New York University, during a conference at Janelia Farm in March 2013. At the time, Behnia says, she had been studying the fly visual system for some four years and was already recording electrical activity from several key cells in the pathway. But she didn’t know about one particular cell type, called Tm3.

Armed with this new information, Behnia quickly acquired a new fly line with Tm3 cells fluorescently labeled. The results indicated that the final circuit does, indeed, exhibit the expected time delay, just as Chklovskii predicted. “With this new result, we could propose that two pairs of neurons have processing properties that are consistent with them being delayed and non-delayed lines of two correlators, one for moving light edges and the other one for dark edges,” Behnia explains. She published her findings earlier this year in Nature (5). “We could not have written this paper without their data.”

Speed Up, Scale Up

To date, connectome studies have handled small, relatively local circuitry. But even that has been painfully laborious. If researchers hope to scale their work to whole brains, they need to accelerate the process.

One approach is serial blockface EM (SBEM), where a tissue sample is placed inside a scanning electron microscope outfitted with a microtome. The instrument images the blockface by backscattered electron scanning electron microscopy (SEM), removes a thin section, and repeats. According to Helmstaedter, who used SBEM in his retinal reconstruction, the process produces a somewhat more isotropic data set than separating the slicing and imaging steps, as Chklovskii did, which produces voxels measuring about 4 \( \times \) 4 \( \times \) 50 nm. “We gave up a little bit on x-y resolution, going for 15 or 16 nm,” he explains. “In z, however, we had 25 nm,” a thickness that makes it easier to trace fine neurite fibers.
The retinal data set was collected by running the SBEM instrument 24 hours a day, 7 days a week, for 6 weeks, Helmstaedter says. “But that’s nothing compared to the almost 30,000 hours of human labor that went into the analysis.”

To analyze that 250-gigabyte data set, the team employed hundreds of amateur student annotators who combed over the data, each armed with a laptop and filled external hard drive. Among other things, the analysis revealed the presence of a previously unknown bipolar cell type called XBC, which the team identified based on cell shape. But, because Helmstaedter and Denk used a staining method that did not highlight synapses directly, their proofreaders had to infer position by cell-cell contacts, a less accurate approach.

GOING MESOSCENE

At the Allen Institute for Brain Science, Hongkui Zeng, Senior Director for Research Science, is pursuing connectomes on a completely different scale. Instead of the so-called “micro-connectomes” of Helmstaedter and Chklovskii, which precisely map local circuitry, Zeng and her team chart connectivity across the entire brain, albeit at lower resolution.

Earlier this year, her team described a “mesoscale connectome of the mouse brain,” which they assembled by injecting GFP-expressing viruses at each of 295 sites in one hemisphere of the mouse brain, then sacrificing the animals, carving their brains into 140 100-micron slices, and tracing the fluorescence through the entire organ (6).

In total, the team imaged more than 1231 brains, each comprising 750 gigabytes of data collected in a single 18.5-hour run. With six instruments running in parallel five days a week, the entire data set was collected in about a year.

To interpret the data, researchers at the Allen Institute undertook “an amazing computational effort,” Zeng says. First they aligned the data sets to one another to create a “computationally averaged brain.” Then, they projected those data onto a standardized anatomic template to create the final navigable online data set (connectivity.brain-map.org). All told, “the project took 18 FTEs 4 years to complete.”

The resulting data set allows researchers to explore and develop hypotheses. “This really provides a way of mapping out a circuit,” she says—for instance, to figure out which brain regions are or are not involved in some biological process. -J.P.
Today, Helmstaedter is moving forward, studying a piece of cortex measuring about half-a-millimeter on a side, involving data sets 100 times larger than the retinal work—a size made necessary by the much larger neurons of the cortex. “The bounding box of each single neuron is definitely at the 0.5-mm if not millimeter and beyond scale on each side,” he explains.

To accommodate the larger size, Helmstaedter says he has accelerated the imaging process by, among other things, switching from a photomosaic imaging strategy to continuous imaging. “We’re about a factor of 30 or 40 faster now than we were in the retina data set in terms of imaging.”

Another approach is implementing a multi-beam EM system to parallelize the imaging process. Carl Zeiss Microscopy has developed one multi-beam SEM (mSEM) system with 61 scanning beams that Denk anticipates will reduce imaging time another 50–60-fold.

At Janelia Farm, group leader Harald Hess is pioneering yet another strategy, focused ion beam SEM (FIB-SEM). FIB-SEM uses a beam of gallium ions to slowly carve away at the sample as it is imaged. For Hess, the approach offers two primary advantages—it produces exceptionally thin sections (about 4-nm thick) and, because the sample doesn’t move, easier reconstruction. “We get about twice as much connectivity to the synapses as you could … with thicker sections,” he says.

But at 4 nm per section, FIB-SEM data accumulate slowly, leading Hess and his team to spend nearly 5 years trying to improve the instrument’s up-time. “We need to run these things continuously for months or even years,” he explains. Initially, the system failed every few days. But over time, the team learned to be preemptive and mean-time-between-failures today is measured in months. “This finally is getting us to a limit where we can now get nice complete neuropil modules from the *Drosophila* brain,” he says.

### Going “Whole Hog”

Others are working to take connectomics to the whole-brain level. Cardona, for instance, is about a quarter of the way through a project mapping all 10,000 neurons in the *Drosophila* larvae, a 7-terabyte data set comprising 5000 sections, which he obtained from the Janelia Fly EM Project Team in a manner similar to Chklovskii’s study. To annotate the data, Cardona enlisted experts from 22 labs...
around the world to trace connections in their brain regions of interest—an approach that is distinct from Helmsstaedter’s paid student annotators, as well as from public annotation via the online game called EyeWire. “[Experts] know already more or less the shape and position and structure of these neurons,” Cardona explains. “So they can go an find them in the EM when they know what to expect. On top of it, they’re very motivated because they need this circuitry as a complement to the scientific manuscripts they are writing and for their theses.”

Cardona and his collaborators are currently developing algorithms to improve annotation efficiency to make it practical to scale up to an adult fly brain.

Meanwhile, Denk has set his sights on mapping the mouse brain to completion. “We are going whole hog,” he says.

It won’t be easy, Denk acknowledges. Sample staining gets progressively more difficult for larger samples, thanks to slower diffusion rates, and the SBEM microtome cannot handle a whole-brain-sized block, so his team is developing a larger one. Even if they can fix that problem, the system is entirely too slow at present; Denk estimates a whole mouse brain could consume 50 to 100 petabytes of data and take tens of years at the current pace. “We are not that patient,” he admits, but the workflow is constantly improving, a trend sure to continue with the anticipated mSEM scanner and advances in neural reconstruction algorithms.

While not everyone in the neuroscience community agrees such efforts are the best use of limited resources, Behnia, at least, sees value. “Knowing where the synapses can be or where they are, is going to give you a lot of information.”

“It guides our experiments,” she says.