

Cutting all the way around	976
Molecular guideposts	978
Box 1: Putting it all back together again	978

## Following the wires

Scientists may have finally developed the techniques to reconstruct complete wiring diagrams for the neuronal circuitry of the human brain. Nathan Blow reports.

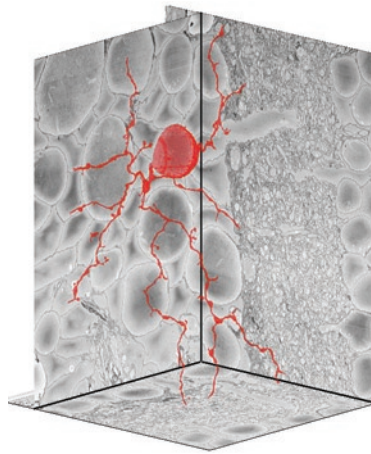
In the 1880s Santiago Ramón y Cajal began examining the structure of the human brain at the cellular level. More than 100 years later our understanding of the structure and cellular connectivity of the human brain is still rudimentary at best. “The amount of structure that we don’t know in the brain is actually the vast majority of it,” remarks Stephen Smith of Stanford University.

The problem with studying the neural connectivity of the brain is one of scope: scientists need to look at a tremendous number of structures in exquisite detail. But the use of electron microscopy might provide the answer to imaging neuronal connections in a rapid-enough fashion and at high-enough resolution to decipher complicated wiring diagrams. “The reason for using electron microscopy is that the smallest wires, or processes, in the brain have diameters that are below the resolution of light microscopy,” says Winfried Denk of the Max Planck Institute for Medical Research, Heidelberg, Germany.

Still many problems have to be solved before a full electron microscopy reconstruction of the neural connections in the human brain becomes a reality. But pieces are starting to fall into place with the advent of new techniques such as serial block-face imaging and array tomography that are being complemented with instrumentation advances such as the automatic tape-collecting lathe ultra-microtome (ATLUM)—making the imaging of these connections only a matter of time.

### Face value

Electron microscopy applied to serial sections of a sample has been used in the past to map the neuronal connections in very simple organisms. In the mid-1980s, John White and colleagues reconstructed a circuit diagram of *Caenorhabditis elegans* by ‘photographing’ each serial section, and then reconstructing



A  $50 \times 50 \times 60 \mu\text{m}$  data cube of the rabbit retina acquired using serial block-face SEM. (Courtesy of Kevin Briggman, University of California, San Diego).

all the neurons and connections by hand<sup>1</sup>. This amazing feat required over ten years to complete for the worm, which has only a few hundred neurons.

One of the major problems with reconstructing neuronal connections using this traditional approach is that each section to be imaged must be first cut from a block of embedded tissue using a microtome, then collected and imaged. “The real problem comes from the fact that you have to handle and photograph these sections, and they are fragile and prone to distortion,” says Denk. Although this fragility is not overly problematic for some electron microscopy applications, when attempting to reconstruct complete neuron wiring diagrams, the distortion or breakage of a single section may cause the loss of crucial wiring information. This led Denk to adapt for electron microscopy a technique used in light microscopy to produce what is now called ‘serial block-face electron microscopy’. The approach is to ‘photograph’ the

surface of the embedded tissue block instead of a pre-cut section, then cut off a section, discard it and photograph the surface again. “The advantage is each section is perfectly aligned with the next section and undistorted,” says Denk.

Serial block-face electron microscopy, however, restricts the form of electron microscopy imaging that can be used. “You cannot use transmission electron microscopy because that does not give you an image of the surface, so you use a reflection (scanning) electron microscope, which looks at the electrons that bounce back from the sample,” says Denk. The use of scanning electron microscopy or SEM does have a few disadvantages as it is slower than transmission electron microscopy. Another issue when using the block-face method with SEM is resolution. Electrons tend to scatter and lose energy as they penetrate into a block of tissue, so to avoid losing too much resolution Denk and colleagues try and force the electrons to remain near the surface of the block. “What we have to do is use low voltage, unlike regular transmission electron microscopy, where you use voltages of 80 or 300 keV, here we use around 3 keV, which is still a lot of energy but low enough so that electrons do not penetrate very far into the sample,” comments Denk. He also notes that this lower voltage limits lateral dispersion of electrons as well.

Despite these few drawbacks, the huge appeal of Denk’s approach is in the thinness of slices the researchers are able to cut. As the slices do not need to be collected and manipulated, they can be thinner than for analysis using other approaches. And the thinner the sections that are dissected are, the more information can be imaged and acquired, so none of the wiring data are lost (Box 1). At a routine thickness of 30 nm, Denk notes that they probably would not be able to collect the slices if they wanted to.

### Cutting all the way around

Like Denk, Kenneth Hayworth of the University of Southern California saw a conundrum in the world of neuroscience that he thought he could solve. “It is almost impossible to get the circuit that you are interested [in] mapped,” says Hayworth, “and that seemed to me like a huge disconnect since it can be done

but is not.” He saw this as an automation issue—mapping circuits is a labor-intensive, manual process with the potential for human error. To remedy this Hayworth, along with Jeff Lichtman and Bobby Kasthuri of Harvard University, is developing instrumentation that goes a long way toward automating the process of circuit mapping.

Hayworth, Kasthuri and Lichtman started by redesigning the cutting microtome for electron microscopy. In the original design the tissue goes up and down against a diamond knife, cutting a section that is then floated on water to be collected for imaging. In Hayworth’s new configuration, the tissue block is placed onto a lathe that rotates the tissue around the diamond knife cutting 50-nm or thinner sections with each revolution. The cut section is also floated in the water boat, like in the original design, but Hayworth and Kasthuri added a conveyor belt made of carbon-coated Mylar (a very strong plastic film) that comes up from the water boat and automatically collects each section after cutting. The Mylar film with the sections is then rolled up to save them for imaging at a later time. Alternatively, researchers can directly image these tissue sections sitting on the carbon-coated Mylar, eliminating the need to collect these fragile sections on grids. Hayworth and colleagues coined the term automatic tape-collecting lathe ultra-microtome, or ATLUM, for their device.

Using the ATLUM, Hayworth and his colleagues can now cut and collect hundreds of 40-nm serial sections. But Hayworth wants more from the technology. “This is a work in progress: we obviously want to get to the 10,000 section range, and we think that this technique can actually get to that.” But to collect 10,000 sections, put them on good tape and stain them properly are all bottlenecks that the team is keenly working on.

In addition, Hayworth thinks that the true value of the ATLUM is that the sections are saved and not destroyed. “If you look at the serial block-face technique, it is highly automated and from a reliability point of view better than the ATLUM. They have some resolution limitations that we do not have, but the most important thing that I see is that we collect the section for later imaging, [whereas] they destroy the sections while imaging,” says Hayworth. And collecting the sections has benefits right now because imaging each section is computationally demanding and slows down data acquisition (**Box 1**). Hayworth’s goal is to be able, by collecting all the sections, to create ‘ultrathin-section’ libraries that neuroscientists can use to trace their own circuits of interest. “By collecting these sections and putting them in a library, it allows a level of collaboration that is unheard of in the neuroscience community,” says Hayworth.

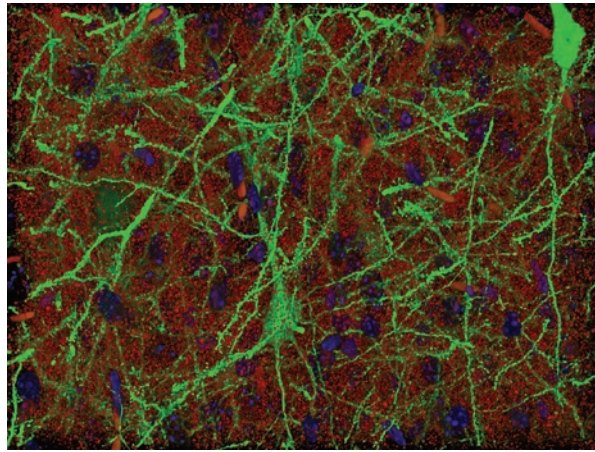
Collecting electron microscopy serial sections as thin as those generated by the

ATLUM also helps overcome resolution issues encountered with the block-face method. “Since we are using a thin section with backscattered electrons, we get scatter from very small spots and increased resolution,” comments Lichtman who points out that the images generated by electron microscopy from ATLUM-cut sections are good enough to clearly resolve synapses without the need for additional staining.

Hayworth acknowledges that there is an enormous number of neuroscience researchers waiting for this technology. “I have talked to researchers who have spent 20 years researching a particular circuit in a particular animal, and they have no idea how it is connected.” And although the ATLUM is a ‘brute-force’ approach to obtaining the circuit diagram, Hayworth is confident it will prove very effective.

### Molecular guideposts

“The automated collection of electron microscopy serial sections is coming along beautifully, but the part that is still the big challenge is reconstructing the image,” says Smith. Several laboratories are busy addressing the challenge with a battery of computational



Array tomography rendering of multiple synapses in the brain. (Courtesy of S. Smith.)

tools (**Box 1**). At the same time, Smith hopes that array tomography might be the perfect complement to electron microscopy techniques such as serial block-face imaging or ATLUM for determining neural connectivity<sup>2</sup>. Array tomography can include electron microscopy, but until now Smith and members of his lab have been using the technique for mostly immunological fluorescence applications on thin slices.

“With electron microscopy identifying a structure like a synapse requires a trained eye, and nobody has a good algorithm for that yet, [but] the magic of antibody specificity does the heavy lifting for you in array tomography with immunofluorescence,” explains Smith.

Smith started his research career exploring the landscape of functional neuroscience, but in recent years he has shifted his focus to studying the structure of the brain.

## BOX 1 PUTTING IT ALL BACK TOGETHER AGAIN

Automated serial sectioning for electron microscopy is progressing quite rapidly with technologies such as serial block-face electron microscopy and instrumentation such as the ATLUM. But the problem researchers are now facing is how to use computers to trace the wires and reconstruct networks.

“The reconstruction of wiring diagrams needs to be automated, but it is very hard to get computers to do this as well as people,” says Dmitri Chklovskii from Janelia Farms. Indeed, things that appear very easy to the human eye and brain, such as pattern recognition, are pretty hard for computers.

Chklovskii’s group along with other groups including those of Denk and of Sebastian Seung of the Massachusetts Institute of Technology have been working on the problem of automating the analysis of electron microscopy serial section data. “Rather than performing traditional image processing where we look for edges and stuff like that, we say that we do not know the best strategy to look for these cells but what we do is that we can look at them by hand using students with a training set,” says Denk. These training sets can then be used to train computers using machine learning methods such as neural networks to trace wiring diagrams within electron micrographs.

Although Chklovskii’s group is also using machine learning and training sets for tracing the wiring of the brain, he cautions that even this does not work as well as a person. “The other aspect is that even humans are not perfect, so if you got two humans to look at the same data set they would produce slightly different results,” says Chklovskii.

The other computational issue that will need to be addressed is the resolution of the images used in the reconstruction. “The approach that we are taking is to use the full high-resolution [electron microscopy] images for the reconstruction, which may have much more detail than we need, but possess the details we really want,” says Chklovskii. However, this approach has created a depth problem for Chklovskii and others.

Owing to the details generated by high-resolution electron microscopy researchers cannot always resolve processes or axons. This occurs because, in most electron microscopes, the resolution in *x-y* plane is much better than in the *z* dimension. The *x-y* resolution can be as good as 2–5 nm but the resolution in the *z* dimension is determined by how thick the sections are cut, which is typically 40 nm, or up to 10 times worse than the resolution in the *x-y* plane. This means that objects that run parallel to the *x-y* plane are not well resolved. “You can lose an object if it running parallel to the section, much easier than if it is running through it,” confirms Lichtman, who also says that this can be solved experimentally if you can cut sufficiently thin sections so that the smallest processes are sampled more than once. Chklovskii thinks that in the end the solution to this problem will require both experimental and computational advancement. But he optimistically acknowledges, “it could turn out that one development on one side can resolve everything.”

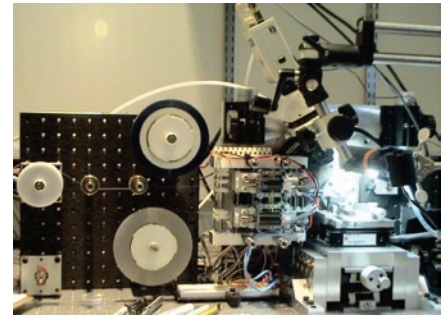
“Structure has a single right answer and that is refreshing,” says Smith. “We invented array tomography to really get at the molecular architecture of the brain.”

“Biologists have this love-hate relationship with immunofluorescence,” says Smith; “it has lots of annoying limitations and pitfalls, ... one of which is that it has never worked well in tissues.” If a piece of tissue is fixed so the biomolecules are well-preserved, the problem is that antibodies can only penetrate a distance of 5–10  $\mu\text{m}$  even after multi-day incubations. And within that 5–10  $\mu\text{m}$  depth there can be a gradient of antibody penetration. Even if imaged with perfect efficiency the results obtained are not quantitative because you do not know how well the antibodies really penetrated. But Smith thinks array tomography might provide an answer to this dilemma.

Array tomography uses a traditional microtome to create sections that are 70–200 nm thick. After the cut, the sections are layered onto a silicon array that has been coated with a slightly hydrated polymer.

“There is a trade-off between finesse and speed,” says Smith. “We use the 70-nm sections when we want the highest possible resolution and the 200-nm sections when we want to chew through a bunch of tissue faster.” The use of thin sections attached to the polymer allows uniform penetration of antibodies and effective staining throughout the section. Because of the uniform penetration, results from array tomography can be quantitative. An additional benefit is that the sections can actually be stripped from the antibody and stained again. Smith says that researchers in his lab have stained sections with up to 40 different antibodies, and they “have not yet found the upper limit.”

Smith thinks this method would even be well suited to studying neuronal circuitry in transgenic mice where individual neurons are labeled. Recently, so-called ‘brainbow’ mice have been engineered with multiple markers for a variety of different neurons in the mouse brain. “My final fantasy is to use the brainbow mouse as a Rosetta stone to create computer algorithms, trained and



ATLUM—a modified microtome for automated cutting and collection of thin sections for electron microscopy. (Image courtesy of K. Hayworth.)

schooled by fluorescence data, for the analysis of the electron microscopy data sets,” says Smith.

1. White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. *Philos. Trans. R. Soc. London Ser. B* **314**, 1–340 (1986).
2. Micheva, K.D. & Smith, S.J. *Neuron* **55**, 25–36 (2007).

**Nathan Blow is the Technology Editor for *Nature* and *Nature Methods* (n.blow@boston.nature.com).**

## SUPPLIERS GUIDE: COMPANIES OFFERING ELECTRON MICROSCOPY AND NEUROBIOLOGY IMAGING INSTRUMENTS AND REAGENTS

Company	Web address
4pi Analysis Inc.	<a href="http://www.4pi.com">http://www.4pi.com</a>
Alpha Omega	<a href="http://www.alphaomega-eng.com">http://www.alphaomega-eng.com</a>
Applied Precision	<a href="http://www.api.com">http://www.api.com</a>
Applied Scientific Instrumentation	<a href="http://www.asiimaging.com">http://www.asiimaging.com</a>
Bioptonics	<a href="http://www.bioptonics.com">http://www.bioptonics.com</a>
Chemicon (Millipore)	<a href="http://www.chemicon.com">http://www.chemicon.com</a>
Chroma Technology	<a href="http://www.chroma.com">http://www.chroma.com</a>
Energy Beam Sciences	<a href="http://www.ebsciences.com">http://www.ebsciences.com</a>
EDAX	<a href="http://www.edax.com">http://www.edax.com</a>
Electron Microscope Sciences	<a href="http://www.emsdiasum.com">http://www.emsdiasum.com</a>
Evident Technologies	<a href="http://www.evidenttech.com">http://www.evidenttech.com</a>
DVC	<a href="http://www.dvcco.com">http://www.dvcco.com</a>
Gatan, Inc.	<a href="http://www.gatan.com">http://www.gatan.com</a>
GE Healthcare	<a href="http://www.gehealthcare.com">http://www.gehealthcare.com</a>
Hitachi High Technologies America	<a href="http://www.hitachi-hta.com">http://www.hitachi-hta.com</a>
Intracellular Imaging	<a href="http://www.intracellular.com">http://www.intracellular.com</a>
Invitrogen/Molecular Probes	<a href="http://invitrogen.com">http://invitrogen.com</a>
ISS Group	<a href="http://www.iss-group.co.uk">http://www.iss-group.co.uk</a>
JPK Instruments	<a href="http://www.jpk.com">http://www.jpk.com</a>
JOEL USA Inc.	<a href="http://www.joelusa.com">http://www.joelusa.com</a>
Kodak	<a href="http://www.kodak.com">http://www.kodak.com</a>
Leica Microsystems	<a href="http://www.leica-microsystems.com">http://www.leica-microsystems.com</a>
LightLab	<a href="http://www.lightlabimaging.com">http://www.lightlabimaging.com</a>
Lighttools	<a href="http://www.lighttools.com">http://www.lighttools.com</a>
Lumafluor Inc.	<a href="http://www.lumafluor.com">http://www.lumafluor.com</a>
Molecular Devices	<a href="http://www.moleculardevices.com">http://www.moleculardevices.com</a>
Nikon	<a href="http://www.nikon.com">http://www.nikon.com</a>
Olympus America	<a href="http://www.olympusamerica.com">http://www.olympusamerica.com</a>
Omega Optical	<a href="http://www.omegafilters.com">http://www.omegafilters.com</a>
Optic Solutions Inc.	<a href="http://www.opticsolutions.com">http://www.opticsolutions.com</a>
Optical Imaging	<a href="http://www.opt-imaging.com">http://www.opt-imaging.com</a>
Optronics	<a href="http://www.optronics.com">http://www.optronics.com</a>
Oxford Instruments	<a href="http://www.oxford-instruments.com">http://www.oxford-instruments.com</a>
Photometrics	<a href="http://www.photomet.com">http://www.photomet.com</a>
PicoQuant	<a href="http://www.picoquant.com">http://www.picoquant.com</a>
Princeton Instruments	<a href="http://www.piacton.com">http://www.piacton.com</a>
Promega	<a href="http://www.promega.com">http://www.promega.com</a>
QI Imaging	<a href="http://www.qiimaging.com">http://www.qiimaging.com</a>
Qioptiq	<a href="http://www.qioptiq.com">http://www.qioptiq.com</a>
Semrock	<a href="http://www.semrock.com">http://www.semrock.com</a>
SemTech Solutions, Inc.	<a href="http://www.semtechsolutions.com">http://www.semtechsolutions.com</a>
Scientific Instruments and Applications	<a href="http://www.sia-cam.com">http://www.sia-cam.com</a>
STS Elionix	<a href="http://www.sts-elionix.com">http://www.sts-elionix.com</a>
Tescan USA	<a href="http://www.tescan-usa.com">http://www.tescan-usa.com</a>
Thermo Fisher Scientific	<a href="http://www.thermofisher.com">http://www.thermofisher.com</a>
VayTek	<a href="http://www.vaytek.com">http://www.vaytek.com</a>
Veeco	<a href="http://www.veeco.com">http://www.veeco.com</a>
Zeiss Nano Technology Systems Division	<a href="http://www.smt.zeiss.com">http://www.smt.zeiss.com</a>