NEURAL CIRCUITS — OPINION

Structural neurobiology: missing link to a mechanistic understanding of neural computation

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Abstract | High-resolution, comprehensive structural information is often the final arbiter between competing mechanistic models of biological processes, and can serve as inspiration for new hypotheses. In molecular biology, definitive structural data at atomic resolution are available for many macromolecules; however, information about the structure of the brain is much less complete, both in scope and resolution. Several technical developments over the past decade, such as serial block-face electron microscopy and trans-synaptic viral tracing, have made the structural biology of neural circuits conceivable: we may be able to obtain the structural information needed to reconstruct the network of cellular connections for large parts of, or even an entire, mouse brain within a decade or so. Given that the brain's algorithms are ultimately encoded by this network, knowing where all of these connections are should, at the very least, provide the data needed to distinguish between models of neural computation.

Why haven't we yet figured out how the computations that the brain performs really work? We now possess a lot of information about the brain's molecular and cellular building blocks. We know how neurons use action potentials to transmit information over long distances, how they communicate with each other through synapses and which nonlinear chemical or electrical processes they use when performing computations. Exquisite methods exist to record electrical activity and calcium dynamics in individual cells[1–3] and in small populations of cells[4–6]. Powerful modelling tools[7,8] allow the simulation of cellular and network behaviour, given a set of cellular parameters and the network connectivity. However, although we know what most neuronal cell types 'look' like and, to a lesser extent, what their biophysical properties are, information about circuit structure is lacking in most cases.

Structural analysis of the nervous system began in earnest at the end of the nineteenth century with the cellular-level work of Golgi[9] and Cajal[10,11] and with the lesion studies by Broca and others[12,13]. These studies yielded the insights that information flows along neurite 'wires' (mostly in one direction) and that cognitive function is regionalized. The next big step in structural analysis was provided by the electron microscope, which revealed the structure of synapses[14–17] and eventually enabled the reconstruction of the almost complete synaptic wiring diagram of the 302 neurons of the roundworm *Caenorhabditis elegans*[18–20]. However, the simultaneous requirements for wide spatial scope and high resolution, as well as the need to accumulate and analyse a huge amount of data, have for a long time limited electron microscopy analysis to very small neural circuits. Recently, new volume electron microscopy methods such as serial block-face imaging[21–23] and automatic section collection[24], as well as associated analysis methods[25–27], have been breathing new life into complete (connectomic) circuit-diagram reconstruction efforts. Modern molecular tools now allow combinatorial colouring[28] and positive synapse identification[29], thereby expanding the ability to carry out circuit analysis at the light-microscopy level.

We believe that a relative paucity of structural information has held back our understanding of computation in the brain and that, for any complex system, structural information may fundamentally have more power to provide definitive answers to mechanistic questions than functional measurements. To make this case, we will first consider how much activity information we would need to understand the complete computational functionality of any 'device'. We will then explain why structural information is able to distinguish between different functional models or theories of neural computation, and describe several new tools and technologies that are boosting our capacity to acquire detailed structural information on neural circuits. Finally, we will describe how the usefulness of this structural information depends on its resolution, molecular contrast (the ability to distinguish molecular constituents in the image) and spatial scope.

The importance of structural data

Activity versus structure. If our goal is to understand how the brain works, should we put most of our efforts towards a better understanding of its anatomy or its activity? "Show us the activity" seems to have been the refrain through most of the recent decades; and much activity has been mapped in this time. But we believe that we should first ask how much activity or structural information we need to 'reverse engineer' a computational device such as the brain.

To understand this question, let's take as an example a black-box digital electronic circuit with 32 inputs and one output (FIG. 1 illustrates a case with only 16 inputs, as the input–output table for a 32-input device would require more than 500 full pages to display). The responses of this circuit can in theory be completely characterized by looking at activity. This would require us to construct a truth (or look-up) table — a list with one entry for each of the 2^{32} (four billion) possible input patterns, each entry giving the output produced when that input pattern is applied. To describe this circuit

would require us to obtain approximately 4 gigabytes of data. As we start to probe the circuit's responses, a pattern might emerge that suggests that the circuit is simple and can be described using less information than this. However, to be sure that we have a complete description of the circuit, we would still need to apply each of the possible input patterns and record the output that results.

Now take the structural case. The worst case is again a descriptional complexity of 2^{32} if, for example, the device generates its output using a quasi-random (non-compressible) truth table with one entry for each possible input configuration. However, in most cases, if the device response is governed by a moderately complex network of simple circuit elements with functional responses that are understood, such as those of well-characterized neurons or of binary logic gates (elements of the circuit that perform Boolean operations such as 'AND', 'OR' and 'NOT'), we will have gained a complete description of the circuit's behaviour as soon as we have deciphered the structure of that network.

This may be considered an unfair comparison because in most cases of reverse engineering a computational device (be it a nervous system or a computer chip), we already know that the descriptional complexity (the minimum amount of information needed to describe the device behaviour completely) is much less than the total number of possible input patterns, for example, because the number of synapses (or transistors) in the circuit is much smaller than that number. Consider the case (FIG. 1) in which the responses to most input patterns follow a simple rule but for a small but functionally important fraction of input configurations the responses deviate from this rule. An example of such a circuit might be one that includes cells that respond vigorously to one particular input pattern but not at all (or only weakly) to most others, such as cells that respond specifically to the presence of certain individuals[30]. This might occur as a result of the addition of a small number of circuit elements (FIG. 1) that increase neither the device's volume nor its descriptional complexity by much and thus add relatively little to the effort needed to determine the structure of the circuit. However, to generate a complete description of the device based on activity measurements, it would still be necessary to run the full gamut of 'stimuli'. An everyday example would be the picking of a lock by, in one case, trying out all the possible keys or, in the second case, by mapping the lock's three-dimensional structure (which is simple even for lock types for which a large number of possible keys exist, only one of which will actually open the lock). Although we have used a feedforward circuit as an example here, the argument that knowledge of the structure (in conjunction with the properties of the components of the circuit) permits the prediction of functional responses remains true for recurrent circuits.

How does this picture change if we move from purely black-box experiments (which can be likened to behavioural measurements) to physiological recordings, which allow us to open up the black box and perform functional measurements from points inside the circuit? It is important to distinguish between 'blind' recordings (those made from random points in the circuit) and recordings that are guided by or put in the context of structural information. In the blind case, we gain some insight into the intermediate results that are used in the computation. This can provide important clues as to how the computation might work, and can be the first step towards building a model, as the example of visual processing in the cortex shows, where the discovery of complex cells[31], such as those sensitive to stimulus orientation, triggered a modelling effort that is still going on today. However, how useful this approach is will depend on what subset of possible input configurations

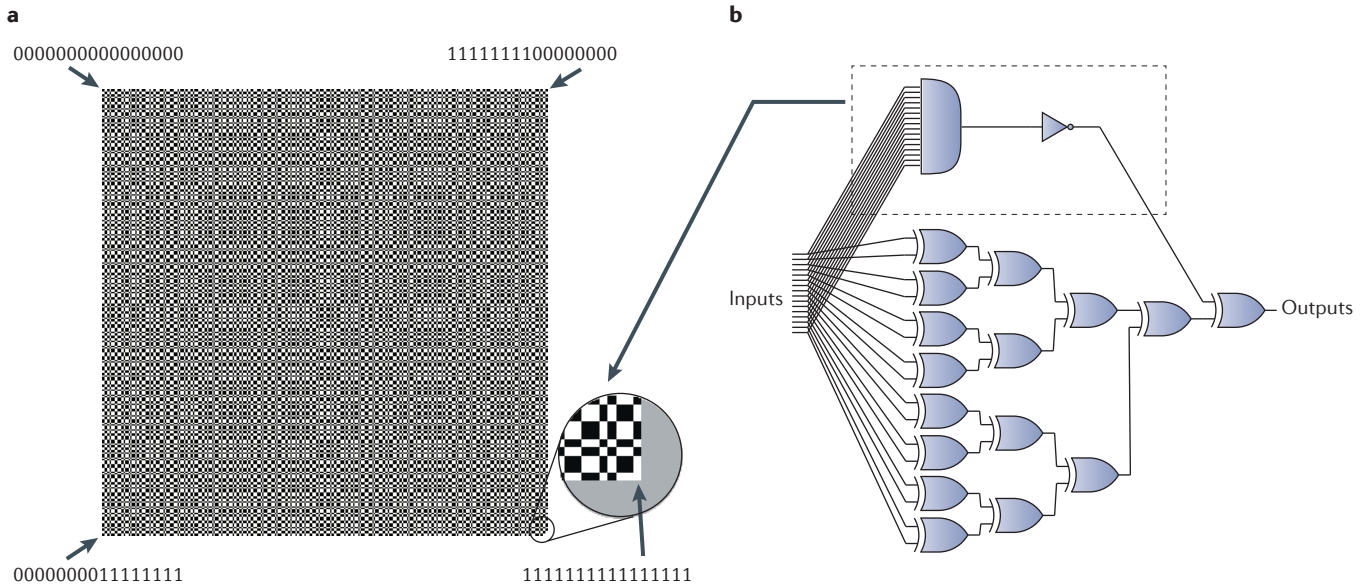

Figure 1 | **Dissecting a computational device.** Two alternative methods of describing a 16-input computational circuit are shown. **a** | A truth table (central square) such as this would result from exhaustive input–output mapping (white and black correspond to the output being active and inactive, respectively). The input configurations that correspond to the four corners of the truth table are shown next to the arrows. The magnified lower right corner shows how the pattern persisting through the rest of the truth table is broken: the output is active rather than inactive for a single input pattern. **b** | A circuit diagram as it might be obtained by structural analysis. The lower branch of the circuit consists of 'exclusive-OR' gates for which the output is true (active) if the inputs are different. This branch is responsible for 99.999% of the truth table and accounts for the regular patterns seen in part **a**. The upper branch is active only for a single input pattern and consists of an 'AND' gate followed by a 'NOT' gate, which gives an output that is true except for when all the inputs are true. When all the inputs are true, the output is inverted (lower right corner of the truth table in part **a**). This illustrates how structural analysis of the circuit can be more efficient than the exhaustive mapping of the truth table for reverse engineering.

(stimuli) are used to probe the circuit, with the possibility that entire classes of salient inputs may be missed. In the 'non-blind' case, structural knowledge can be used to a widely varying degree to target functional recordings or to interpret them post-hoc. This includes the use of gross anatomical information to determine which brain area to record from 'blindly', the use of fluorescent labelling to target recordings to cells of a certain type[32,33] or the post-hoc reconstruction, using 3D electron microscopy, of a tissue region from which functional recordings have previously been made[23,34].

It thus seems to be the case that — somewhat counter-intuitively — for complex systems with many elements, such as circuits made up of many neurons, structural analysis is more efficient than functional analysis in providing comprehensive insight into the functional properties of the circuit. This is because the number of possible functional states of a circuit grows roughly exponentially as a function of the number of circuit elements. However, to actually predict circuit behaviour (for example, by simulating it), both the network of connections between circuit elements and their functional (biophysical) properties need to be very well understood. This is rarely the case for real neurons and was certainly not the case for *C. elegans*, which may be why we are still awaiting the *in silico* 'mind of a worm'. However, even if there is insufficient information about the circuit elements to enable forward modelling, complete structural data can still be very powerful.

Structural and theoretical neurobiology. Ultimately, the value of any experimental approach depends on how efficiently it decimates hypothesis space (eliminates the wrong hypotheses). If there is no danger of being proven wrong, theorizing and model-building become futile. It can be argued that computational and theoretical neuroscience, in a similar way to some areas of physics, suffers from a falsification gap: its practitioners out-think, out-compute and out-simulate what is actually measured. Brain-scale simulations involving millions of neurons[35] are attempted, using what we believe often amount to little more than wild guesses about actual neuronal connectivity.

Theoretical neuroscience has other disadvantages compared with theoretical physics, in which a single very precisely measured quantity can provide strong support for — or falsification of — a theory. By contrast, resolving biological questions often relies on the elimination of the wrong hypothesis by many experiments of limited precision: this is because high precision is usually hard to achieve and, more fundamentally, because much of the structure of a biological object reflects evolutionary history, rather than the following of fundamental principles. For example, the ribosome's functional mysteries were finally revealed only after the determination of the coordinates of a quarter of a million atoms; however, its function might not be affected by changing many of its constitutive amino acids or RNA bases. The ribosome is only one in a long series of cases in which structural biology (that is, the determination of the spatial arrangement of a biological macromolecule's constitutive atoms) has been the arbiter of mechanistic controversies[36].

The mechanistic description inherent in any model must have a corresponding structural substrate. For example, if a model postulates that "the inputs are filtered in different ways and split into parallel channels", there must be neurons representing those channels, and the connections observed must be consistent with parallel channels. Thus, structural information can be used to support or falsify a model. At this point, it is therefore worth considering why there are no well-known examples of structural information disproving a theory. We believe that this may be because theories that are appealing in other ways but are in contradiction to existing structural data are never put forth, and that not enough high-quality structural data have been available to provide falsification for any pre-existing theories.

The essence of computation, neural and otherwise, is the flow of information along 'wires' and its interaction in 'nodes', which is why knowledge of the network of nodes and their connections is so useful. As pointed out above, the wiring diagram alone is typically insufficient to completely predict the functional dynamics of a circuit. This may be because the properties of the circuit elements (neurons) are not known well enough or are modulated to an unknown degree by various neuromodulators. The lobster stomatogastric ganglion (STG), which is under the modulatory control of many neuroactive substances, is a case in point[37–39]. From a circuit-mapping point of view, the challenge then is to gain a better understanding of the circuit that controls the release of those modulators. Another occasionally overlooked fact is that many physiological recordings are a form of connection mapping. This approach is feasible for circuits made up of a small number of stereotypical neurons, such as the STG, but is bound to fail for even medium-sized circuits with a few hundreds of neurons, simply because the number of cell pairs that would need to be tested becomes too large.

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What information do we need?

How much structural information about a network is needed, and what is the minimum that can be useful? It is hard to see how whole-scale simulations of concrete (rather than abstract) circuits can be truly meaningful without a complete map of all connections, together with the biophysical properties of all synapses, dendrites and axons. Nevertheless, even establishing a fraction of all connections can be useful to test models. The secure knowledge, for example, that no connection exists between two cells or brain regions can be very valuable as it shows that no information can flow directly between those cells or regions, respectively. The absence of a synaptic connection can always be inferred with certainty if no physical contact exists between two cells (even for paracrine signalling, spatial proximity determines effectiveness) or if there are no neural 'wires' connecting two regions.

How much use would a cellular connectome (a complete list of all connections between all neurons) be for just a single brain? An equivalent question might be: how much less do we learn about the brain from reading a single neuroscience textbook compared to reading (and averaging the information contained in) many such books? We may not know whether a pattern of connections or pathways that we find in a single brain is typical or significant. However, if a theory trying to explain a computational ability present in all brains postulates a pattern of information paths, then this pattern has to be present in all normal brains; if it is not, that theory must be wrong. That said, having multiple connectomes could enable us to recognize invariant aspects of the connection pattern, which in turn could inspire the formulation of novel hypotheses and the construction of new theories. Finally, multiple connectomes could help us to link

variations in connectivity to variations in cognitive ability, just as genomes from multiple individuals of the same species link genomic and phenotypic variations (including pathological states).

Tools for structural analysis

Successful imaging experiments depend on the generation of the appropriate contrast, image acquisition as well as image analysis and interpretation. Light microscopy, in particular, is benefitting from the availability of many new cellular stains as a result of molecular engineering. Automation of electron microscopy and light microscopy acquisition has made much larger data sets available, and the automation of analysis is making it easier to access and use the information they contain.

Microscopy technology. Most microscopic imaging in biology is performed using either electrons or light. The light microscope requires a high numerical aperture to resolve subcellular structures, because they are as small as or smaller than the wavelength of light (hundreds of nanometres). Electron microscopy provides better resolution at much lower numerical apertures because electrons have a very small wavelength (39 pm and 3.7 pm for the electron energies of 1 keV and 100 keV that are typically used in scanning electron microscopes (SEMs) and transmission electron microscopes (TEMs), respectively).

Optical sectioning (the confinement of image information to the vicinity of the focal plane) is used by light microscopes, such as the confocal microscope, to resolve structures perpendicular to the image plane. However, this approach is ineffective at the low numerical apertures used for electron microscopy. Instead, one has to either physically cut the sample and/or take projection images (where sample information is averaged along the axis of observation) under different angles of observation. The use of multiple images, each projected in a different direction, is the principle behind stereoscopic depth perception and tomographic reconstruction. Another approach for obtaining depth information in electron microscopy is block-face imaging, which relies on the limited penetration of low-energy electrons into solid materials and thus prevents underlying structures from contributing to the image.

For 3D molecular resolution, the method of choice is electron tomography, whereby a whole series of images of vitrified samples (frozen quickly to prevent the crystallization of water) is taken at different angles[40]; however, to image large volumes of tissue, some form of physical cutting is necessary because even high-energy electrons have only a limited penetration range. Plastic-embedded tissue can be cut into a series of sections (each several tens of nanometres thick) and imaged using a TEM or, after the collection of sections on a solid support, imaged using a SEM[24,41]. Alternatively, the block surface that is left behind after the section is cut off can be imaged using a SEM[21,42]. A variant of this method uses a focused ion beam (FIB) rather than a diamond knife to remove the material from the surface[22]. These approaches have different advantages and drawbacks (TABLE 1). Block-face imaging avoids section distortion and loss because section integrity need not be preserved, furthermore, it allows thinner cutting and thus provides better resolution in the third (z) dimension. The best z-resolution is currently provided by the FIB approach, which suffers, however, from a limited field of view because ablation becomes irregular as the beam propagates along the sample surface for more than a few tens of micrometres. Imaging the sections rather than the block face offers better lateral resolution, the ability to re-examine the sections if needed, and the ability to first cut (which is fast) and then distribute the sections to image them in parallel on multiple microscopes.

In this article we have focused mainly on electron microscopy techniques because currently only electron microscopy can unambiguously resolve synapses and the finest neurites. However, several super-resolution

Table 1 | **Comparison of different volume electron microscopy methods and two super resolution light-microscopy methods**

	Serial-section electron microscopy		Serial block-face electron microscopy		Super-resolution light microscopy[45]	
	Section collection on TEM grids, TEM imaging	**Section collection on solid support, SEM imaging[24]**	**Diamond-knife cutting, SEM imaging[21]**	**Focused ion beam ablation, SEM imaging[22]**	**Stimulated-emission depletion nanoscopy and variants**	**Photo-activation localization microscopy (also known as STORM)**
Depth (z) resolution	40 nm section thickness, improved by tilts[84]	<30 nm section thickness*	23 nm section thickness[23]	5 nm abrasion thickness‡	~30 nm (strongly dependent on fluorophore properties)	15 nm (strongly dependent on single-fluorophore brightness)
Lateral (xy) resolution	2 nm	< 4nm	< 10 nm	< 10 nm	~30 nm	~20 nm
Advantages	Staining of sections possible after cutting, sections available for re-imaging, parallelized imaging possible		Negligible distortion, no loss of sections, fully automated cutting and acquisition		Multiple colours, optical sectioning possible	
	Highest lateral resolution, fastest imaging rate (TEMCA)[34]	Automatic section collection, large section areas, lower likelihood of section loss	Large areas possible	Highest isotropic resolution	Live cell imaging	Cheap
Drawbacks	Section distortion, folding and loss		En-block staining necessary, sections destroyed during cutting		Limited sample thickness, high fluorophore density needed	
	Section loss and distortion most severe	Tilt series more difficult than in TEM	Cutting debris on block face	Limited field of view	Limited speed (point scanning)	Many images of the same field needed

SEM, scanning electron microscope; STORM, stochastic optical reconstruction microscopy; TEM, transmission electron microscope; TEMCA, transmission electron microscope fitted with a fast camera array. *J. Lichtman, personal communication. ‡H. Hess, personal communication.

(or sub-diffraction) light microscopy (SR-LM) methods have been invented in the past decades[43–45], and their resolution continues to improve. Although not (yet) competitive with electron microscopy in resolving power, SR-LM can be used *in vivo* and, crucially from a structural-imaging perspective, allows multispectral imaging. The ability to image multiple colours can be used to measure the colocalization of molecules, such as different synaptic proteins[46], and could expand the ability of combinatorial fluorophore labelling techniques to follow thin neurites and resolve ever-smaller synaptic contacts. As SR-LM techniques expand their ability to optically section thicker samples, they may allow the creation of 3D data sets, with the need for actual sectioning reduced or completely eliminated. One caveat is that in order to take advantage of the increased resolution, ever-denser labelling will be required, which may be difficult to achieve.

Owing to the still limited spatial scope of 3D electron microscopy techniques, correlating light microscopy and electron microcopy data from the same tissue will be essential. For sizable electron microscopy volume data, 'geometry matching' — registration of the light microscopy and electron microscopy data sets using the shapes of individual neurons and locations of unambiguous landmarks such as blood vessels — is possible[23,34] even without special labels that are visible in both the light and the electron microscope.

Staining techniques. Nothing can be discerned without contrast in the sample. Tissue preparation for volume electron microscopy still relies mainly on variants of traditional fixation and staining protocols[47–50], with staining based on the selective binding of heavy metals to certain cellular structures such as membranes or protein aggregates. 'En-bloc' staining, whereby all staining is completed before the tissue is embedded in plastic, is essential for serial block-face techniques because the application of solutions to the block face after each cut is impractical in the vacuum of the microscope chamber. However, even section-imaging approaches can benefit from the uniformity and freedom from precipitation artefacts that en-bloc staining provides compared to post-cutting contrast amplification. Increasing the contrast by increasing the concentration of heavy-metal atoms[23,51,52] provides a great advantage because the radiation dose needed to obtain a given signal-to-noise ratio (SNR) falls with the inverse square of the specimen contrast. (The difference between signal and background is proportional to the contrast, and the noise is proportional to the square root of the dose. Thus if the contrast is halved, four times the dose will be required to provide the same SNR). If the metal concentration is increased sufficiently[47,49–52], the sample becomes conducting. This further improves the SNR because block-face imaging of conducting samples does not require charge compensation by low-vacuum operation[23].

> "It is thus likely that we will soon have staining techniques that provide good contrast for both the pursuit of wires and the detection of synapses."

To reconstruct wiring diagrams, we need to reliably follow the axons and dendrites over at least hundreds of micrometres and reliably detect synaptic contacts. Our ability to follow neurites in 3D electron microscopy volume data is aided by stains that emphasize the cell surface, such as the precipitate produced by the oxidation of di-aminobenzidine (DAB): a reaction that is catalysed by horseradish peroxidase (HRP) that has been introduced into the living tissue before fixation[23]. This leads to an insoluble polymer with a high affinity for osmium[53]. However, staining protocols that make it easy to follow the wires by de-emphasizing intracellular structures also make it harder to identify synapses on the basis of vesicles and synaptic densities. A way out of this dilemma may be to use synapse-targeted chromophores that — upon illumination — trigger the formation of the DAB polymer. Although photoconversion using chemically synthesized chromophores is a well-established technique[54,55], protein-based and thus genetically targetable chromophores suitable for photoconversion have only recently become available[56]. Another possibility is the genetic targeting of HRP[57] or another oxidizing enzyme. It is thus likely that we will soon have staining techniques that provide good contrast for both the pursuit of wires and the detection of synapses.

Although correlations between a synapse's functional parameters and its shape are likely and have been quantified in some cases[58,59], more work is needed to establish this correspondence more tightly and to extend it from merely estimating synapse strength to other parameters, such as the degree of use-dependent transient potentiation and depression. Additional information may be provided by cell-type data, which should, in most cases, be deducible from the neurite morphology that readily emerges[23,34] from large-scale 3D electron microscopy.

Beyond monochrome. Despite its resolution, it's all greys in electron microscopy. Colour has been and still is the almost exclusive domain of the light microscope. A long history of dye chemistry[60] and, more recently, the molecular engineering of protein-based fluorophores[61,62] has provided us with a range of staining options for fluorescence imaging. However, it is often underappreciated that in order to have, for example, even a single fluorophore molecule per ($10\,nm^3$) voxel a concentration of 1.6 mM is needed, which is already higher than commonly achievable by protein expression. Potentially very useful for structural circuit analysis is the combinatorial generation of cell-unique fluorescent colours (also known as 'brainbow')[28,63,64], which allows the pursuit of wires thinner than the resolution limit, the trans-synaptic complementation of split fluorescent proteins[29] and trans-synaptic viral infection[65–68], the latter two of which enable the identification of synaptic contacts between two cells at the light-microscopy level. Viral tracing, which is synapse-specific but seems to still miss a large fraction of synaptic contacts, is more useful for sparse reconstruction targeted to a specific question[69–71], rather than for dense, connectomic reconstruction.

In plastic-embedded tissue, proteinaceous material can be stained by heavy metals in order to provide contrast for electron microscopy but its molecular identity has to be inferred, in most cases, from its location in relation to recognizable subcellular, mostly membraneous structures. This makes it generally impossible to use the electron microscope to map, for example, the distributions of ion channels, which are the major determinants of the electrical response of a cell, or of the enzymes involved in biochemical signalling cascades. Most current techniques that combine antibody labelling with electron microscopy result in reduced ultrastructural preservation that is insufficient for reliable circuit tracing, mainly because of weak fixation, which is necessary to preserve endogenous antigens and membrane permeabilization treatments (however, see REFS 72,73 for an account of good preservation while allowing access to exogenous antigens). Another possibility would be to exemplarily map, at the light microscopy level, protein

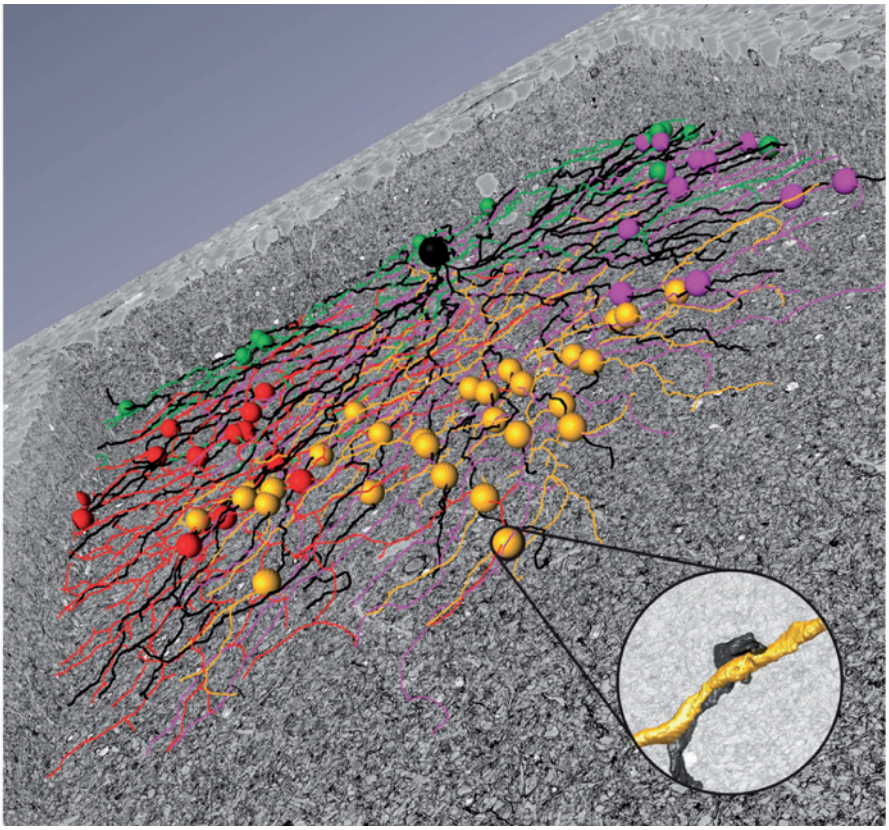

Figure 2 | **Cells in the retina manually reconstructed from serial block-face electron microscopy data.** A cut-away of mouse retina acquired for the study of the direction-selectivity circuit in retina[23]. The image shows a reconstructed starburst amacrine cell in black and the locations of its synapses (inset) onto direction-selective retinal ganglion cells. The synapse locations are colour-coded by the functionally recorded preferred directions of the direction-selective retinal ganglion cells.

distributions in individually labelled cells of a known cell type. This, however, requires the commonly made but unproven assumption that the computationally relevant aspects of, for example, the ion-channel profiles are stereotypical for each cell type.

The 3D distribution of epitopes can be determined without the need for tissue penetration of antibodies by serial sectioning of material embedded in a polymer that preserves antigenicity (array tomography)[74]. Multiple epitopes can be mapped by array tomography in each section by combining multicolour light microscopy and multiple apply–image–strip cycles using different antibodies each time. The section thickness (well below 100 nm) determines the z-resolution, which is much better than that of confocal or two-photon microscopy, and section imaging is well suited for single-fluorophore localization by SR-LM. Array tomography may yield, for example, a catalogue of synapse types[75] or the maps of ion channel distributions, which are much needed for realistic biophysical models of dendritic computation.

Analysis techniques. There is no natural sensory modality that generates 3D volume images. Our imagination is, however, able to represent objects that have a complicated internal structure. Somewhat surprisingly, such a mental representation can be built by viewing successive cross-sectional images. This is particularly effective when the images are shown in quick succession and in registry, as is easily done using computers, which can also be programmed to capture human annotations. Thus, individuals can manually trace neurite connections, and several software packages are now available[25,76–78] for this purpose, each optimized for slightly different types of 3D electron microscopy data.

Although this speeds up human vision-based analysis (to approximately 200 µm of traced neurite length per hour for manual skeleton tracing[25] in 3D electron microscopy data) compared to flipping photographs by hand and marking them with felt-tipped pens, automated computer-based analysis will be needed to completely reconstruct even volumes as small as 1 mm^3. As one mistake in following a neurite will typically lead to the loss (or assignment to the wrong pre- or postsynaptic cell) of hundreds if not thousands of synaptic connections, the reliability of such an analysis method has to be rather high[23,25]. Because no precise mathematical description of the visual features of a cell boundary is available, one has to rely on heuristic algorithms and test different parameter settings against human-generated 'ground-truth' (or 'gold-standard') data. This process can be automated and performed for many parameters (such as the connection weights in an artificial neural network) simultaneously in a process called machine learning, which is generating some promising results[27,79,80].

Highly reliable segmentation (the grouping of voxels into objects) may be the most difficult aspect of automated analysis, but the development of automatic detection of synapses is indispensable as well. One complicating factor, which was already mentioned, is that cell-surface staining methods that facilitate neurite segmentation tend to de-emphasize the intracellular structures that are useful for the identification of synapses. For some synapse types a connection can be inferred from the geometry of the contact, as is the case for the starburst-to-ganglion-cell synapse in the retina[23]. It is an open question whether this will eventually be possible for most or even all synapse types.

Application to a biological question

Even though the retina is one of the most-studied and best-understood parts of the vertebrate CNS, its remaining mysteries are only slowly being solved. As so much is already known about the retina, insights gained using novel methods can be checked for plausibility. In a recent study (FIG. 2), large-scale volume electron microscopy was used to examine the connectivity of starburst amacrine interneurons and ganglion cells, which project out of the retina[23]. The study found that starburst cell dendrites preferentially connect to direction-selective ganglion cells (DSGCs) with a preferred direction of motion (the direction that elicits the strongest response in a DSGC) that is the opposite to the direction in which the dendrite points. The preferred direction of motion was determined by pre-electron microscopy two-photon calcium imaging. This confirms the notion that DSGCs inherit their direction selectivity from starburst amacrine cell dendrites, which function as independent direction detectors[81].

Although the retinal study used serial block-face electron microscopy[21], another study[34] — also combining functional and structural analysis — acquired their electron

microscopy data using serial sections and imaged them using a transmission electron microscope fitted with a fast camera array (TEMCA). This study investigated the orientation-selective circuitry in the cerebral cortex but found no evidence that inhibitory cells preferentially collect inputs from excitatory cells with one particular orientation tuning.

Conclusions and future perspectives

Today. That neural circuits can be reconstructed in detail by electron microscopy has been known at least since the work on *C. elegans* was carried out[18]. A recent revival of this approach was triggered by several technical developments in microscopy, such as serial block-face imaging. It has the chance to be sustained not only because data acquisition has been improved and automated but because a major bottleneck in the creation of connectomic brain maps has been removed. Computer technology has progressed to a point where storing data for an entire mouse brain at 20 nm isotropic resolution (60 petavoxels) is almost conceivable. Furthermore, because techniques for computerized analysis are rapidly progressing, there is hope that the analysed volumes can be scaled beyond what is possible even with the combined efforts of many human tracers.

The next 5 years. The tools are in place to image, with volume electron microscopy techniques, tissue pieces up to at least 1 mm^3 in size. This should allow the structural analysis of several physiologically well-studied local circuits, including the retina and the cortical 'column'. When needed, functional information can be acquired for the same piece of tissue before electron microscopy imaging, using calcium indicators and two-photon microscopy.

The Achilles heel of structural neurobiology is likely to remain the analysis of the vast amount of data generated. Relief may come from miraculous improvements in staining and imaging technology that make it obvious to even the most basic algorithm where one cell ends and another begins. It is more likely, however, that steady improvements in image processing (probably relying heavily on machine-learning techniques) will reduce error rates and thus the human proofreading effort. This will increase the volume of tissue that can be completely reconstructed or the fraction of cells that can be reconstructed in a given volume.

The next 'n' years. When will we be able to say: "Mission accomplished"? It is still inconceivable that we will be able to acquire and analyse the amount of data needed for a human cellular-level connectome (200 exavoxels), even when using the rosiest predictions of how storage and computing capabilities will grow. Also, although we can easily make the case for why cellular connectomes are useful to decipher the function of local tissue regions with a very high degree of connectivity (for most neurons, a large fraction of the cells they receive inputs from have their somata within 1 mm or so), it is harder to make the same case for long-range connections. The value of atomic-level structural biology is greatest for the understanding of how individual biological macromolecules function. For the understanding of macromolecular complexes, the identity, location and orientation of the constituents is the level of description needed. It is still unclear how the integration of light microscopy information with electron microscopy-level descriptions will ultimately be accomplished. Will it be necessary to use labels that can be seen both in the light and electron microscope or will matching the cellular geometry be the method of choice?

Moving beyond the brain's algorithms, connectomics may ultimately allow us to read memories[82] if, as many neuroscientists believe, memory is stored in the pattern of synaptic connections and strengths. Given that it can survive the shutdown of almost all neural activity (the electroencephalogram is flat during deep anaesthesia[83]), long-term memory must certainly be encoded in the structure of the brain and thus be readable by some form of structural analysis.

In conclusion, new techniques for staining, imaging and analysis have accelerated progress in structural neurobiology (some may prefer to still call it neuroanatomy), thus creating prospects for obtaining complete wiring diagrams for some insect brains and for parts of the mammalian brain, and suggesting that we may be able to answer questions about the overall network connectivity of specific cells.

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Competing interests statement
W.D. declares competing financial interests: see Web version for details.

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