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COMMENTARY

# Of what use is connectomics? A personal perspective on the *Drosophila* connectome

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## ABSTRACT

The brain is a network of neurons and its biological output is behaviour. This is an exciting age, with a growing acknowledgement that the comprehensive compilation of synaptic circuits densely reconstructed in the brains of model species is now both technologically feasible and a scientifically enabling possibility in neurobiology, much as 30 years ago genomics was in molecular biology and genetics. Implemented by huge advances in electron microscope technology, especially focused ion beam-scanning electron microscope (FIB-SEM) milling (see Glossary), image capture and alignment, and computer-aided reconstruction of neuron morphologies, enormous progress has been made in the last decade in the detailed knowledge of the actual synaptic circuits formed by real neurons, in various brain regions of the fly *Drosophila*. It is useful to distinguish synaptic pathways that are major, with 100 or more presynaptic contacts, from those that are minor, with fewer than about 10; most neurites are both presynaptic and postsynaptic, and all synaptic sites have multiple postsynaptic dendrites. Work on *Drosophila* has spearheaded these advances because cell numbers are manageable, and neuron classes are morphologically discrete and genetically identifiable, many confirmed by reporters. Recent advances are destined within the next few years to reveal the complete connectome in an adult fly, paralleling advances in the larval brain that offer the same prospect possibly within an even shorter time frame. The final amendment and validation of segmented bodies by human proof-readers remains the most time-consuming step, however. The value of a complete connectome in *Drosophila* is that, by targeting to specific neurons transgenes that either silence or activate morphologically identified circuits, and then identifying the resulting behavioural outcome, we can determine the causal mechanism for behaviour from its loss or gain. More importantly, the connectome reveals hitherto unsuspected pathways, leading us to seek novel behaviours for these. Circuit information will eventually be required to understand how differences between brains underlie differences in behaviour, and especially to herald yet more advanced connectomic strategies for the vertebrate brain, with an eventual prospect of understanding cognitive disorders having a connectomic basis. Connectomes also help us to identify common synaptic circuits in different species and thus to reveal an evolutionary progression in candidate pathways.


**KEY WORDS: Focused ion beam milling, FIB-SEM, Proof-reading, Segmentation**

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## Introduction

With the advent of our capacity to derive the synaptic wiring diagram of a brain, or connectome, complete at the electron microscopic (EM) level (Lichtman and Sanes, 2008), we have entered a newly minted, long-awaited age of brain science.

At the outset of this Commentary it is perhaps worth recalling the words attributed to Benjamin Franklin, subsequently taken up by Michael Faraday (Cohen, 1987), uttered in response to a question from an audience member who queried the value of their new science, either of balloons or electromagnetism, with a reply as follows: 'Madam, will you tell me the use of a newborn child?' The value of the new science of connectomics lies, similarly, not in its existence, nor even its accomplishment, in deriving the synaptic wiring diagram of a brain or connectome, complete at the EM level (Lichtman and Sanes, 2008), but in its prospects: what it can do for us in the future. It offers, or it is claimed (Lichtman and Sanes, 2008) will have, the same sort of enabling technology to the solution of problems of how networks of neurons function as genomics has offered functional studies in molecular biology. The various abstractions of synaptic circuits – generalized mnemonics, models and cartoons (for examples, see Shepherd, 1998) – that until recently were the only alternatives possible, are to be distinguished from the detailed circuits now revealed by a connectome, the exact network of real synaptic connections (Lichtman and Sanes, 2008).

The last 25 years have seen a gradual awakening in neuroanatomy, since Crick and Jones (1993) could rightfully lament its backwardness. That backwardness, which had placed neuroanatomy firmly in the third world of neuroscience, enabled by few effective tools and illuminated by few stars, is now decidedly reversing (Rockland and DeFelipe, 2016), with the field now enormously enriched especially by new genetic (Luo et al., 2008) and imaging (e.g. Knott et al., 2008) methods, and overtaken by a glorious new world of connectomics initiated little more than a decade ago (Lichtman and Sanes, 2008; Bohland et al., 2009).

Connectomics is of course no new idea, merely one now implemented by new sectioning, imaging and reconstruction technologies, and enabled by a powerful combination of computers, imaging technologies, solid state physics and major resources (e.g. Hayworth et al., 2015; Xu et al., 2017) and complemented by powerful genetic technologies (Luo et al., 2008). Together, these overcome the brain's numerical and dimensional challenges (Lichtman and Denk, 2011), and support a rate of progress that is accelerating at a breathtaking and formerly unthinkable pace. No matter that the overall task may still be daunting, the prospects are particularly exciting. The compelling genetic tools of the fruit fly *Drosophila melanogaster* and the small physical dimensions of its brain that favour electron imaging combine to promote the suitability of this tiny brain for the first establishment of a complete connectome of a species capable of complex behaviour (Schlegel et al., 2017). This is just the beginning, of course, to which must be added functional changes

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**Glossary**

**Arbour**
Tree of branching dendrites.
**Bodian method**
The staining of nervous tissue by silver proteinate solutions.
**Calibre**
The diameter of a neurite.
**Cartridge**
A unit column of lamina neuropile.
**Dendrite**
A neurite that is not an axon and is usually branched.
**Golgi impregnation**
The random impregnation of individual neurons by salts of silver chromate, by successive immersion of brain tissue in potassium dichromate and silver nitrate solutions,
**Hub**
A brain region with long-range tracts but lacking local interneurons.
**Local processing unit**
A population of local interneurons with mathematically defined spatial features and branches entangled with each other.
**Milling**
The process of etching a microgroove from the surface of an object, such as an embedded block of brain tissue, in aligned rows, so as to image the block face at successive depths.
**Neurite**
The process or extension of a neuron, referring to an axon (usually single and projecting to a different region) or dendrite (usually branched).
**Neuropile**
The collective thicket of intermingled neurites and their synapses.
**Projectome**
An areal brain map of axon projections between connected hubs.
**Proof-reading**
The adjudication of automated segmentation decisions made to identify neuron profiles.
**Split-GAL4**
An intersectional genetic technique that expresses the yeast transgene Gal4 in two lines A and B, in which line A expresses half the GAL4 protein, which is inactive alone, and line B expresses the other half of GAL4, also inactive alone. Only those cells that are in both lines make both halves, which then assemble into GAL4 and activate a reporter gene.
**T-bar**
The presynaptic organelle for transmitter release at dipteran synapses, comprising a platform surmounting a pedestal.
**Tract**
A cable of multiple axon fascicles.

**Box 1. Electron imaging the fly's entire brain.**

Methods, now partly superseded, originally generated transmission EM images of neuropile regions from ultrathin sections cut from a fly's brain fixed and embedded in Epon. Ultrathin sections are cut at a thickness of about 50 nm on a diamond knife in long series, and collected as ribbons, all as previously reported (Meinertzhagen, 1996), undertaken and reported in outline for the *Drosophila* brain (Zheng et al., 2017 preprint). More recently, developed methods use FIB-SEM imaging, which offers superior *z*-axis resolution that can be matched to that in the *x*- and *y*-axis, to yield isotropic voxels of a brain dissected from the fly's head and prepared for EM using high-pressure freezing or progressive lowering of temperature methods (Z. Lu, K. J. Hayworth, S. Xu, P. K. Rivlin and I. A. Meinertzhagen, unpublished), and embedded in epoxy plastic, Epon. Epon is thermoplastic and cuts at 20 µm on a heated diamond knife to yield consecutive slices (Hayworth et al., 2015). These slices are floated on oil and collected in sequence, and then stored for subsequent FIB imaging. A typical voxel size of 8 nm is selected to yield isotropic resolution in *x*, *y*, *z* and is obtained by imaging and combining four thinner image slices each at a depth of 2 nm. The isotropic volume can be re-sectioned without loss of resolution in orthogonal *x*–*z* and *y*–*z* planes, compared with which the resolution in the *z* plane from a corresponding ssEM image stack is limited by section thickness, typically around 50 nm.

associated with circuit plasticity and neuromodulation, to be considered below.

## The numerical scale of connectomes

The objectives being pursued at the EM level in *Drosophila*, initiated using serial sectioning methods (ssEM) a decade ago (Takemura et al., 2008) and continued comprehensively 5 years later (e.g. Takemura et al., 2013) but now using focused ion beam-scanning electron microscopy (FIB-SEM) (Takemura et al., 2015, 2017a,b) (see Box 1), are also powerfully aided by variously defined wiring networks derived from light microscopy (LM). These identify a total of about 16,000 classes of neurons and their sites of juxtaposition (Chiang et al., 2011; Shih et al., 2015), even if the latter provide only probabilistic rules of connection between neurons and are thus not a true substitute for a synaptic connectome. Some idea of the magnitude of the task ahead can be gained from the numbers of processing centres in the fly's brain. A total of 41 neuropiles and 6 hubs (see Glossary) have been identified with about 58 tracts (see Glossary) connecting these, within which fasciculate the 16,000 classes of neurons, together comprising 43 local processing units (see Glossary) (Shih et al., 2015).

These are large numbers, but still manageable for the human brains that must marshal them. Corresponding data for mammals compare different anatomical features and different species, but some idea of the numerical power of their brains can be gained from the roughly 500 identified regions in the rat's brain between which possible long-range projections had already been identified by Crick and Jones (1993) and the connectivity matrix generated for these 500 (Bohland et al., 2009). Compared with these, more recent comprehensive maps of the mouse brain at the LM level for 295 of 863 identified grey-matter structures, especially for cortico-thalamic pathways (Oh et al., 2014), are to be distinguished from the connectionist literature generated from mammalian brain imaging studies (e.g. Sporns et al., 2005), all of which identify projectomes (see Glossary) (Kasthuri and Lichtman, 2007) rather than connectomes, however. More than that difference between connectome and projectome, i.e. between cells for which actual synaptic contact has been demonstrated and those for which it seems merely likely from the trajectories of their neurites (see Glossary), we remain as deeply ignorant of the exact ways in which brains with more or fewer neurons and synapses actually differ as we did when Bullock (1993) first brought this to our attention. Thus, even though a *Drosophila* brain may have 1/1000th the number of cells of a mouse brain, nobody would assert it is simpler, or that its neurons branch in less complex ways than those of a mouse, as first memorably pointed out by Ramón y Cajal (1923), nor that the behaviour of a fly (e.g. Greenspan, 2004; Greenspan and Ferveur, 2000; Devineni and Heberlein, 2013; Silies et al., 2014) lacks complexity. The tadpole larva of *Ciona* (Ryan et al., 2016) has 1/500th the number of neurons, and these support a behavioural repertoire that is possibly simpler but yet to be extensively explored.

Collectively, these efforts endeavour to fulfil historical objectives (van den Heuvel and de Reus, 2014) that in invertebrates are the logical outcome of approaches from a century ago (Fortuyn, 1920) – approaches that are thus not new, just enabled by new technologies. All have one aim in common: to generate a map of the brain's synaptic networks.

## EM plays an essential role

At the EM level, few precedents exist for true connectomes: most are restricted to the visual system of *Drosophila* and the vertebrate retina (e.g. Helmstaedter et al., 2013; Ding et al., 2016), which share a common circuit design (Borst and Helmstaedter, 2015), and the numerically simple synaptic circuits of certain invertebrates (Bargmann and Marder, 2013). Arthropod visual systems have played a major role, because their composition inherited from the overlying compound eye is modular, with older accounts from the optic lamina in various species: the water flea *Daphnia magna* (Macagno et al., 1973), the horseshoe crab *Limulus polyphemus* (Fahrenbach, 1985) and the fruit fly *Drosophila melanogaster* (Meinertzhagen and O'Neil, 1991) (Fig. 1), all of which depend on a repeating retinotopic composition of cartridges (see Glossary). Connectivity matrices have now been reported more recently for other parts of the *Drosophila* brain, in both the adult (Takemura et al., 2013, 2015, 2017a,b) and larva (e.g. Schneider-Mizell et al., 2016; Gerhard et al., 2017).

These *Drosophila* reconstructions in turn were all anticipated by the landmark reconstruction of the entire central nervous system of the nematode *Caenorhabditis elegans* (White et al., 1986; Durbin, 1987). The latter stood largely alone for 30 years, with only a second connectome reported recently (Jarrell et al., 2012). Functional analyses, especially electrophysiological analyses, were initially limited in nematodes, offset only by the advent of calcium imaging, and were confounded by the influence on behaviour of the mechanical properties of the body, and by local computations undertaken by individual neurons (Plaza et al., 2014). The sole connectome reported for any other species is from the entire, asymmetrical brain in the tadpole larva of the ascidian *Ciona intestinalis* (Ryan et al., 2016), for which functional analyses are appropriate but largely lacking. These matrices, which typically report the connections, presynaptic and postsynaptic, for 150–300 neurons (e.g. Fig. 2), obviously still fall far short of the total numbers, an estimated 100,000 (Simpson, 2009; Chiang et al., 2011) or 135,000 (Kaiser, 2015) for the adult fly, of which 10% may be glia, or 12,000 neurons for the first-instar larva (A. Cardona, personal communication).

A single idea is invariably on the lips of many observers. This is the belief, held by not a few, that we can infer the presence of synapses from other evidence, using LM, without the need for specialised EM methods that, while exhaustive, are also exhausting and can only be limited to tiny volumes. This might be possible for sites of juxtaposition seen in LM, and by no means denies the validity of attempts to use such means. Thus, reporting both presynaptic and postsynaptic sites of contact between partner neurons by use of the GRASP (GFP Reconstitution Across Synaptic Partners) method in *C. elegans* (Feinberg et al., 2008) and *Drosophila* (Gordon and Scott, 2009), along with other, newer methods (Wickersham and Feinberg, 2012), most recently the *trans*-TANGO technique (Talay et al., 2017), which demonstrates connections that are functional, do indeed offer that hope. Recent approaches using expansion microscopy (Chen et al., 2015; Chozinski et al., 2016) do likewise. In the worst case, however, this is just wishful thinking: the literature is culpably penetrated by suggestions, more or less explicit, that when two differently labelled neurons viewed by confocal microscopy approach or overlap each other, they are actually connected by synapses. Experience in the fruit fly *D. melanogaster* already shows that even if such data are confirmed by EM, the latter invariably reveals that there are many synaptic sites and nearby synaptic partners within a volume that might not be recognised by molecular markers, while at present EM alone has the resolution to reveal actual membrane contiguity and synaptic organelles. In the fly, synaptic organelles comprise a cluster of presynaptic vesicles surrounding a release site, indicated at most synapses by a dense body, ribbon or T-bar (see Glossary) (Trujillo-Cenóz, 1965; Boschek, 1971; Meinertzhagen, 1996), opposite postsynaptic dendrites (see Glossary), which in fly photoreceptor synapses constitute a tetrad (Burkhardt and Braitenberg, 1976; Fröhlich, 1985).

Below, I will explore the major requirements that face those who seek to compile a connectome, especially in *Drosophila*, and what scientific outcomes are likely to accrue from doing so. Different brains and brain regions differ widely in the costs they impose to compile a connectome and the benefits these confer when complete, in balance sheets we will now consider. Particular significance is attached to how complete the resulting connectome is, how accurate and how permanent.

## Simplicity and determinacy are both key

Brains with few component neurons that are fixed in their numerical and structural composition have a particular role to play in generating

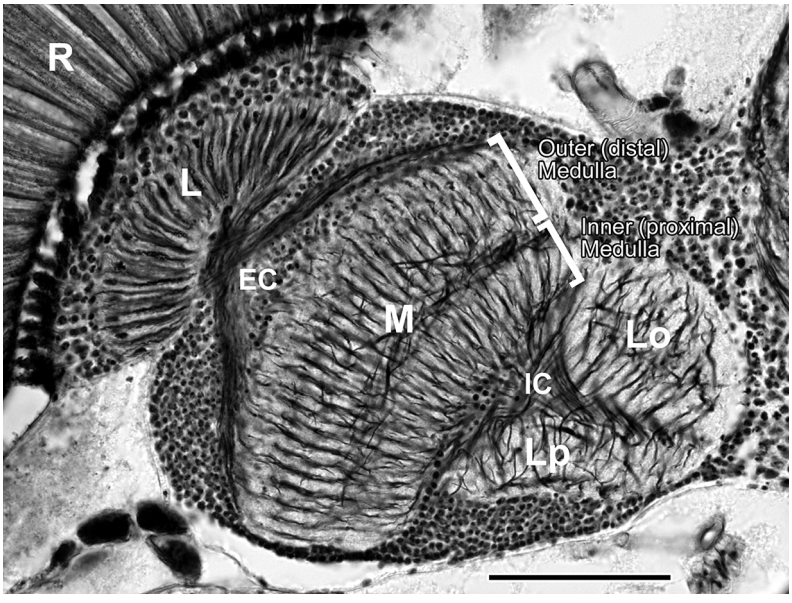


**Fig. 1. The *Drosophila* visual system.** Horizontal section stained by the Bodian method (see Glossary). Horizontal rows of cartridges populate the lamina neuropile (L) and project upon horizontal rows of medulla columns (M) via the external chiasma (EC). The medulla is subdivided into outer (distal) and inner (proximal) strata, and these innervate lobula (Lo) and lobula plate (Lp) neuropiles via the inner chiasma (IC). Each neuropile comprises an array of repeating modules. Bodian stain; scale bar: 50 µm. (From Takemura et al., 2008.)

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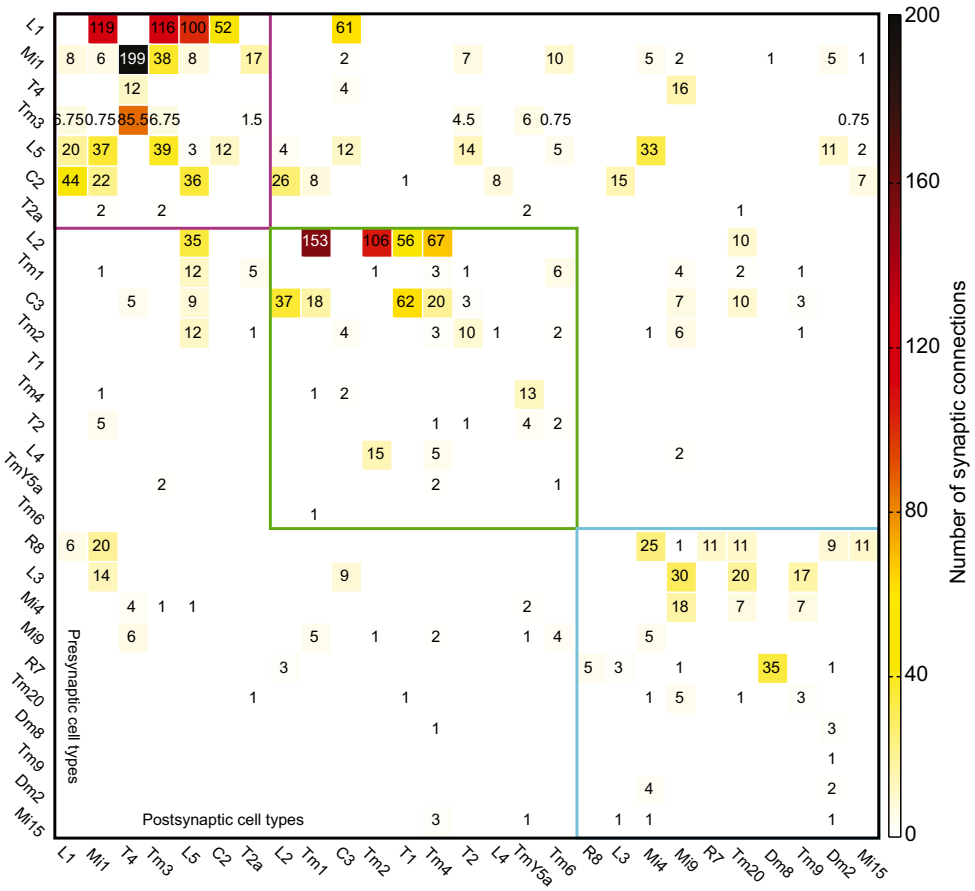


**Fig. 2. Matrix of synaptic connections for a medulla column.** Data assembled from serial sectioning scanning electron microscopy (ssEM) of 2495 synapses formed by 27 modular neurons, presented in the columns. Three pathways, identified via the Louvain clustering analysis, are enclosed in coloured boxes, named for their primary input pathway(s): L1 (magenta), L2 (green) and L3/R7/R8 (cyan). Each pathways is ordered by the total number of its connections within a pathway, and the cell types within each pathway are ordered by the sum of their presynaptic and postsynaptic connections, both in descending order. Synapse numbers range from 1 to >100 between partner neurons (see colour key, right). (From Takemura et al., 2013.)

knowledge about synaptic circuits (Bargmann and Marder, 2013), which argues powerfully for their utility as models for connectomic studies. Small numbers of neurons mean circuits with fewer elements, and usually smaller volumes for reconstruction. There is no shortage of candidate species (Meinertzhagen, 2017), but few satisfy all needs, and none does better than *Drosophila*. All examples are instructing us in the steps required to generate connectomes, but quite reasonably some workers may feel that the marathon is worth joining only when those running it are much closer to the finishing line. By contrast, other attempts start with yet more complex brain regions, such as the neocortex (Kasthuri et al., 2015), for which the methods developed for connectome analysis in *Drosophila* may help to provide a test bed.

Should a first reconstruction in *Drosophila* be made on the brain of a male or a female? Studies on the visual system typically exploit the circuits of the female eye (Meinertzhagen and O'Neil, 1991; Takemura et al., 2008), while sex circuits have greater divergence in male flies, with 19 dimorphisms in males that are highly concentrated in male-enlarged higher brain centres, and only 7 dimorphic lineages with female-specific arbours (see Glossary) (Cachero et al., 2010). These and other uncertainties qualify but in no way invalidate current attempts to compile the complete connectome for a single fly, for which half a female brain is a current goal for the FlyEM team at the Janelia Campus of HHMI, with the greater eventual aim of completing a second connectome for the male.

## Sparse versus dense reconstruction

Two methods, each offering its own advantages, use either sparse or dense tracing methods. The former traces the neurites of particular neurons, cell by cell, and is more rapid (Helmstaedter, 2013), but this method is also liable to inaccuracies because transferring the profile of a neurite from one section to the next may introduce inaccuracies that are not detected. Sparse tracing is therefore best suited to axon tracts or single unbranched neurites especially in ssEM only when there is access to well-aligned images from sections lacking folds and other artifacts. Branching neurites by contrast require dense reconstruction, in which all neurites are traced within a volume, a method that is inherently more accurate because an error made while tracing one neurite soon becomes apparent because all the fibres have to be accounted for (Meinertzhagen, 2016a). In the case of dense reconstruction, no cell can hide or its neurites go undetected in the jungle of others, providing a powerful argument for the comprehensive accounting of all cells.

Collecting image data and aligning them accurately has improved dramatically in the last decade. In this way, it is possible to collect massive datasets of the image profiles derived from neurons. But then the challenge of data analysis intervenes. Images have to be digitised, and their profiles detected and then segmented (reconstructed in 3D). Given the many profiles and image planes incorporated within this process, even a low frequency of errors at each step leads to unacceptable error rates for entire reconstructed neurons. For this reason, each reconstruction has to be checked at each step by proof-readers, a process that for early pre-digital LM studies using entirely manual methods was likened to sewing mail bags (Horridge and Meinertzhagen, 1970), and that even now massively lags behind the time required for preceding steps in the pipeline (Helmstaedter, 2013).

Various computational tools are in use to detect and segment the consecutive profiles of an aligned image stack, and from these to

reconstruct neurons with complex arbours. Progress in undertaking these steps has improved considerably, most recently by advanced algorithms developed at Google using flood-filling algorithms (Januszewski et al., 2016 preprint), which can segment even large neurons with wide-field arbours. These provide greatly improved accuracy and completeness, the two major challenges in connectome reconstructions (Seung and Sümbül, 2014). Regardless of the segmentation tools, many reconstructed neurons require proof-reading (see Glossary). For the latter, unusual or biologically improbable shapes, obtuse branch points, neurites that have a stouter calibre (see Glossary) than their parent neurite, and so on, all suggest a reconstructed tree that is suspect. So, too, do synaptic partnerships that are not congruent for neurites of the same neuron class but are so for those that belong to nearby neurons of a different class. Confirmation can come from 3D views using virtual reality tools to help arbitrate difficult decisions by disambiguating improbable neurites. These are all occasional but still difficult problems in reconstruction, the solutions to which enable operators to detect and interactively adjudicate false merges and disjunctions and arbitrate decisions especially on the splitting of incorrectly conjoined neurites belonging to different cells.

Most such proof-reading operations bring a benefit, although not necessarily in reducing the time taken for reconstructions that can now be made at higher speeds. Often they just enable the proof-reading of yet larger volumes, even if many orphaned neurites may still remain. The common denominator seems to be what can be accomplished within a reasonable time within a career, for example the unit time period spent in postdoctoral training. Current practice at the Janelia Campus is to proof-read 3D reconstructions using the applications Neutu and Neu3 (https://github.com/janelia-flyem/NeuTu), and identify synapses automatically (Huang and Plaza, 2014 preprint). The methods are in constant evolution, however, and subject to future updates. A continual if stimulating tension exists between progress in improving software development tools and the biologists who use these, the latter wishing the former would stop changing things – no sooner is progress obtained using an earlier software version than that version is updated or changed.

## The value of permanence

Like all anatomy, a connectome should be permanent, standing the test of time and repaying the cost and extensive investment in time used to collect it. Structural determinacy and limited variation between individuals mean that just a single connectome can reveal the complete network of a nervous system for all time, so that, accurately reported, the anatomy of any brain need never be repeated if the animal or its sample does not change. How far is this true? Assertion of permanence rests on ignoring or not acknowledging a number of presumptions: that individuals may differ; that the brain may change over time, with ageing and metamorphosis from larval forms; and that it may change as a result of plasticity, maturational, experiential or circadian. For all that fly brains are largely similar, however, we already know that none of these requirements is in fact met by *Drosophila*, and some presumptions are definitely false, especially two forms of plasticity already identified that will be considered further below.

This simple belief in permanence may be thought to underlie much of what has so far been accomplished for the *Drosophila* connectome, despite the inconvenient truth that the fly's brain does in fact exhibit various forms of plasticity (Meinertzhagen, 2001). The extent of this plasticity is only partially known, and only for certain circuits, chiefly in the lamina, because of their tractable features. Thus, flies exhibit synaptic plasticity, depending on the visual experience received during a critical period in the first 4 days of adult life (Kral and Meinertzhagen, 1989), and circadian rhythms in both neurite calibre (Pyza and Meinertzhagen, 1997) and synapse number (Pyza and Meinertzhagen, 1993), as well as in the extent of neurite arbours (Fernández et al., 2008) and no doubt other structural features. We can only imagine the extent of the latter two factors, in particular their consequence for any connectome, either one already elucidated or any of the other brain regions which mostly are not yet explored.

## Different brain regions pose different problems

Most progress in the analysis of the adult fly connectome has been accomplished in neuropiles that have a distinct substructure of repeating elements, so-called glomerular neuropiles (Hanström, 1928), such as are provided by the parallel circuits of sensory pathways: the optic lobe (Takemura et al., 2017a), central body complex or output pathways of the mushroom body (Takemura et al., 2015), for example.

Not all brain regions are modular, however, and some lack a clear substructure visible in LM, but may nevertheless prove to be regularly organised at the circuit level when analysed at the EM level. Nevertheless, such organisation has not been detected in the higher-order olfactory neuropile of the lateral horn, for example (Yasuyama et al., 2003). Yet other systems, for example the accessory medulla (aMe) of the fly's circadian clock (Nitabach and Taghert, 2008), are distributed systems that provide a different challenge. Experience gained from reconstructing modular circuits in the optic lobe and elsewhere will be required to reconstruct such cosmopolitan circuits.

The circuits of the aMe pose a special problem for the fly connectome. There are 100–500 such neurons in insects, most of which express clock genes that interact to generate cell-autonomous, self-sustained circadian rhythms (Hardin, 2005). Given their widespread trajectories, the neurites of aMe neurons project into the circuits of a number of previously uncharted regions of the fly's brain, and will help to identify these. Centred in the aMe (Helfrich-Förster et al., 2007), the output neurons are better known for their peptide content and their cyclical release. Most attention has been accorded to pigment dispersing factor (PDF) (Nässel et al., 1991), one of at least three different neuropeptides reported in aMe neurons (Johard et al., 2009). Some of these neurons also form synaptic contacts that are little acknowledged. For example, in addition to releasing their peptide neuromodulator PDF, neurites, as output pathways from the fly's clock (Meinertzhagen and Pyza, 1996), also provide synaptic input to brain regions such as the pars lateralis and its synaptic circuits (Hamanaka et al., 2005), inputs that await new functional interpretations. Thus, the diffuse neurites of aMe neurons pioneer new and unexplored territories of the fly's brain, many known only in name (Ito et al., 2014), and so we can anticipate entry points to the circuits in those territories, as well as other modulator pathways.

Neuromodulators may also mediate changes in the behavioural state of synaptic circuits, as for example octopamine alters the temporal tuning of directionally selective neurons in the ON motion pathway of the medulla's motion circuits, and their primary input neurons (Strother et al., 2017a).

## When are we complete?

A characteristic of insect nervous systems that favours connectomic analyses is that each neuron belongs to a distinct class of so-called identifiable neurons (Hoyle, 1977; Bullock, 2000; Comer and Robertson, 2001) that can be individually named, and distinguished

from other classes by the specific pattern of its synaptic connections, the connections of each class differing from those of other classes (Bullock, 2000). The work of providing detail for each and every connection obviously increases as the number of cells and circuits increases, especially as the number of cell types increases, and as we begin to saturate the number of synapses between specific partners.

In common with those of other insects, *Drosophila*'s neurons are highly determinate in their morphological features, and each class makes a specific class of connections. Cell types reconstructed from FIB-SEM are identified from the locations of their somata, and by comparing the reconstructed shapes of their arbours relative to axon tracts with the 3D shapes of neurons from LM of a large number of split-GAL4 (see Glossary) genetic driver lines (Luan et al., 2006) for a large number of individual aMe cell types. Tools, such as NBLAST (Costa et al., 2016) help match LM of single-cell flip-out images to FIB-SEM segmentations. Congruence between arbours from LM and EM provides important, sensitive 3D validation for EM reconstructions, confirming that human-assisted reconstructions from FIB-SEM capture the same cells as split-GAL4 reveals about the fly's genetic decisions, and enables input and output synaptic sites to be plotted over the reconstructed neurons. In addition to confirming the cell types and their connections, a connectome also reveals where their synaptic contacts are distributed, providing additional functional relevance.

Neuron classes are numerous, and the best-known case is probably from the optic lobe's medulla (Fig. 3), with at least 84 types (S.-Y. Takemura, personal communication), including 57 previously reported from Golgi impregnation (see Glossary) (Fischbach and Dittrich, 1989). Most cells are allocated to over ~760 columns (Ready et al., 1976), corresponding to lamina cartridges from the 27,000 cell bodies in the medulla cortex. If the latter were evenly distributed they would average 36 cells per column (see Meinertzhagen and Sorra, 2001), but in fact only 9 lamina input terminals and at least 13 non-lamina cells, the so-called modular neurons (Figs 2 and 3), are represented in each and every column (Takemura et al., 2015). Other cells are less frequent, having less than one representative cell of each type per column; some most likely have only a few representatives in the entire medulla. The unwelcome burden of such numbers for future connectomicists is to suggest that the morphological characterisation of many cell types will require larger and larger volumes to be densely reconstructed than at present, and yet yield fewer cells, so as to constitute diminishing returns.

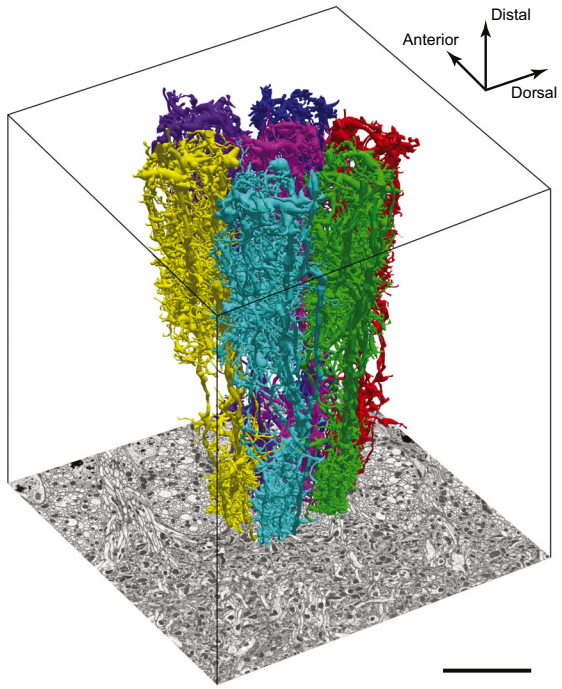


**Fig. 3. 3D reconstruction of four subclasses of modular medulla cells.** Those from seven neighbouring reconstructed columns are shown, revealing the density of reconstructed neurites. Cells are shown relative to a single EM image from which they are reconstructed from the corresponding distal series. Scale bar: 10 µm. (From Takemura et al., 2015.)

## Major and minor pathways, networks and motifs

For synapses, the task of constructing the matrices of connections between densely reconstructed neurons typically reveals pathways with 100 or more contacts and those with fewer than, say, 10, thus a 10-fold range. The former are few and outnumbered by the latter. It is tempting to regard these in functional terms as major and minor, respectively, but of course pathway strength depends not only on synapse number but also on the unit conductance changes arising at each postsynaptic site, information that in general we entirely lack. Moreover, the outcome of large synapse numbers bears equally on the signal to noise ratio of transmission for each pathway. Even so, for pathways constituted by many synapses between the same partners, the existence of each synapse helps to confirm the validity of others, whereas synaptic minorities pose a different problem: is their detection correct, or does it result from any of a number of problems of human provenance, or simply from noise in cell adhesion steps that lead to synaptogenesis? It is customary to refer these pathways to what is called ground truth, a term borrowed from various forms of mapping. In my view, the concept of ground truth is misleading, because there is in fact no practical means to detect a synapse categorically, except by reference to what an experienced proof-reader adjudicates, who can of course also be wrong. Errors of human provenance in synaptic detection are better adjudicated democratically, from the consensus provided by multiple proof-readers, but this naturally multiplies the work of an already huge task and is still not ground truth. Do we then need to know every synaptic contact in order to claim a connection? In a previous careful analysis, repeated proof-reading of the same circuits led to the conclusion that a connection could be confirmed with >95% probability for pathways with at least five synaptic contacts (Takemura et al., 2013), and this probably provides a good yardstick.

The significance of such synaptic minorities is unclear. Recorded in neighbouring columns of the medulla, the number of contacts made by corresponding neuron partners of the same type indicates that <1% of contacts are not part of a consensus circuit (Takemura et al., 2015), providing a measure of the accuracy of identified pathways and of the morphogenetic events that assemble these during synaptogenesis. Various interpretations can be placed on pathways having so few contacts: they may represent silent synapses (Newman et al., 2017), pathways that are plastic and could be strengthened or that were previously stronger, under different functional conditions (Meinertzhagen, 2001). They may also represent pathways that were stronger in ancestral forms, and either down-regulated during the course of natural selection (Shaw and Meinertzhagen, 1986) or available for up-regulation in future generations.

If brains can be deconstructed into constituent circuits, those circuits can be compared without reference to the brain's complex

structure and morphogenesis. It then becomes clear that certain combinations of neurons especially of three- and four-element circuits, those in which a presynaptic neuron has three or four postsynaptic partners, appear more frequently than by chance, and that such circuit motifs are also over-represented elsewhere in nature, in genetic networks and population biology, for example (Milo et al., 2002). Some years ago it likewise became clear that neuronal circuits embody motifs that are shared with those of engineering and other human-made circuits, as so-called small world networks; these have in common that highly connected local sub-networks are linked together by a smaller number of long-range connections (Watts and Strogatz, 1998). This resemblance between brains at different taxonomic levels hints at the possibility of yet deeper ones lurking within the connectomes of all brains.

## A world of numbers

No commentary on the new world of connectomics can quite avoid the topic of numbers. Questions immediately arise of how many, how fast and at what cost can connectomic data be produced? For example, an early study in 2013 required a concussive 20,000 annotator hours to yield raw skeletons, representing 0.64 m of neurites (Helmstaedter et al., 2013), fed by a band of compliant and even eager students with sharp eyes, quick brains and nimble fingers, as well as the requisite motivation and time, and the skill and experience of their organisers. Science on this level is no longer possible in most institutional settings, and it is therefore encouraging that proof-reading rates have now become faster, or distributed (Arganda-Carreras et al., 2015). But of course no sooner do they accelerate than larger volumes are considered for reconstruction and these increases move in to take up the slack. As a result, image files have become huge, typically terabytes, but with petabyte files or more coming into range for the entire brain of *Drosophila*. Moreover, FIB imaging is slow, currently about $10\times10^6$ $\mu m^3$ per year per machine (H. F. Hess, personal communication), about one-quarter the volume of a *Drosophila* brain. This means that imaging time can be rate limiting. The prolonged imaging times using FIB-SEM, even for the tiny volume of the *Drosophila* brain, are being accelerated (e.g. Xu et al., 2017), and can of course be offset by running parallel machines, each independently and with minimal operator intervention. Given these challenges identified above, who then would embark on such a huge task and why? The first answer must be foremost: only those with sufficient resources and scientific motivation. A second reason may be to drive advances in artificial intelligence, deep learning and neural networks theory (Lecun et al., 2015). We will now come to a third answer, one that is biological.

## The point of it all

Finally, how may we link its connectome to the brain's higher-order functions and, especially, to behaviour? Motor behaviour is, after all, the brain's chief biological output, the phenotype that has been selected over many generations so as, ultimately, to generate the structure of the brain (Tosches, 2017).

We may assert that a connectome is required to understand the network functions of any brain, and thereby the causal basis for behaviour, but certainly none would make the claim that knowledge of a brain's connectome is of itself sufficient. In particular, we acknowledge the role played by gap junctions, neuromodulators, dendritic integration, glia and other functions beyond the role of synaptic circuits (Bullock et al., 2005). Neuromodulators, in particular, play a troubling non-connectionist role (Nusbaum et al., 2001, 2017; Bargmann, 2012) that is largely unquantified in *Drosophila*, although not in outputs from the circadian clock (Shafer et al., 2008; Liang et al., 2017). Shafer et al. (2008) monitored output from only a single set of clock neurons signalling with PDF and used imaging that does not distinguish synaptic from non-synaptic signalling. As one example of its utility, EM connectomics in such situations would be most useful to help interpret increasing examples of transmission by small-molecule fast neurotransmitters accompanied by neuropeptide co-transmitters (Nässel, 2018). Despite this qualification, the author asserts the primacy of connectomic knowledge in elucidating the function of any and all brains. These and other limitations have been discussed (e.g. Morgan and Lichtman, 2013).

Knowledge of the fly connectome enables us to interpret functional interactions revealed either by imaging (e.g. Seelig et al., 2010; Akerboom et al., 2012; Simpson and Looger, 2018) or by electrophysiological recording (e.g. Behnia et al., 2014; Mauss and Borst, 2016) of neuronal activity, both challenging pursuits, neither of which can be discussed further here. It also fuels computational analyses and predictions, which are technically easier and sometimes valuable, offering insights from and for readerships in electronic and engineering fields. Connectomes also allow simulations of the interactions in circuits, which, while functional and empirical, in most cases still lack an experimental foundation based on detailed knowledge on signalling and postsynaptic conductance changes. Critical to the latter are the distributions of presynaptic inputs to dendritic arbours, which are specific to the class of input neuron and clearly not random (Fig. 4).

In *Drosophila* we have powerful genetic tools. These can be used to target transgenes in a cell-specific manner that either selectively activates (for review, see Simpson, 2009) or silences (Kitamoto, 2001; Mauss et al., 2017) individual links in a synaptic pathway, enabling us to determine the behavioural outcomes and thus demonstrate a causal link between the activity of single neurons in a circuit and the components of a complex behaviour that these generate. In this way we can establish the causal basis of complex behaviours. Not only is *Drosophila* richly endowed with behaviours of all sorts from memory to alcoholism, as can be assessed by a selection drawn at random from Annual Review of Neuroscience (e.g. Greenspan, 2004; Greenspan and Ferveur, 2000; Devineni and Heberlein, 2013; Silies et al., 2014), but also a wide selection of cell-specific Gal4 lines is available (Pfeiffer et al., 2010; Chiang et al., 2011; Jenett et al., 2012; Shih et al., 2015) that can ensure the targeting specificity of such instrumental manipulations (Venken et al., 2011). Such manipulations have been reported for the visual system in particular, for motion sensitivity (e.g. Rister et al., 2007; Mauss et al., 2017; Strother et al., 2017b), and spectral vision (Gao et al., 2008), but also for many other behaviours. We emphasise that such analyses establish the causal basis of identified behaviours, and not merely the higher description of these.

Knowledge of the fly's connectome serves to interpret functional data. This is a significant reversal of historical practice, which has mostly sought anatomical connections that confirm functional interactions. Instead, connectomes now enable us to predict function. One particular example provides a case in point. An early serial-section EM reconstruction of medulla neurons identified a class of distal amacrine neuron Dm8, and the major input this receives from UV-signalling R7 terminals (Takemura et al., 2008). Dm8 was identified chiefly because its neurites are stout and were easily reconstructed using ssEM in this early study; many other cells with more slender neurites have since been reconstructed using FIB-SEM methods. The major input from R7 terminals suggested Dm8 should be UV sensitive, as behavioural tests indeed revealed, from

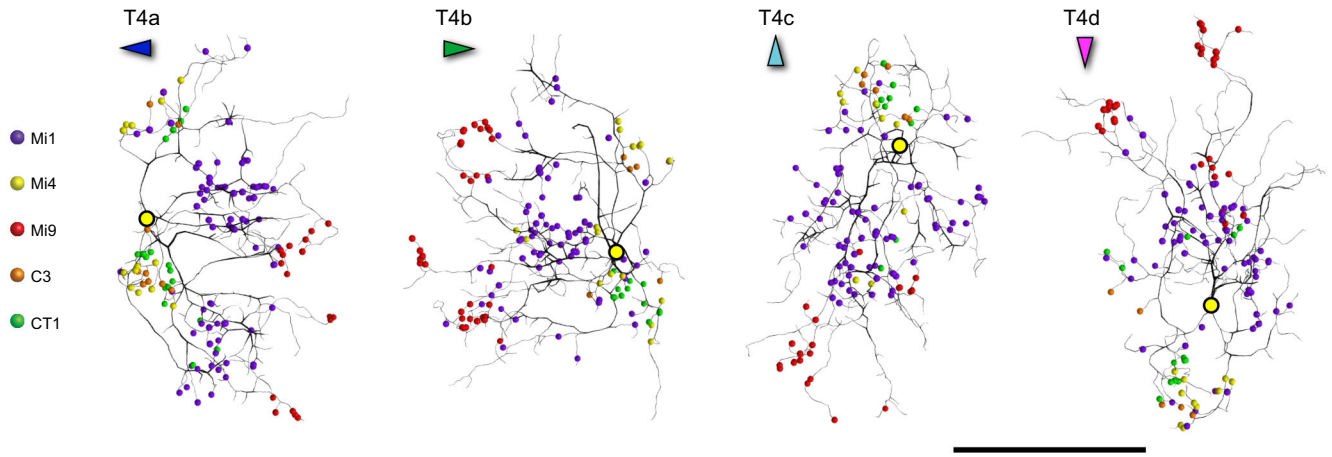


**Fig. 4. Distributions of synaptic inputs onto T4 dendrites.** Reconstruction of the dendritic trees of four subtypes of motion-sensing medulla output neurons (T4a–T4d) and seven other cell types that provide synaptic input to these. Colours of puncta correspond to the input synapses from cell types (see key) and occupy distinct positions out along the length of the dendrite. Dendrites spread in one of four predominant directions relative to the locations of the T4 axon (yellow circles). Scale bar: 10 µm.

the analysis of UV phototaxis when transmission to Dm8 was silenced (Gao et al., 2008). Thus, this early connectomic pathway revealed a rather nondescript neuron with clunky neurites, one that would have been easily overlooked, as the pathway for an elementary visual behaviour mostly not found worthy of prior study. Anatomy thus conscripted behavioural physiology. So, we move from using anatomy to explain functional interactions between identified neurons in a synaptic circuit to predicting those interactions from connectomic anatomy, reversing the more usual sequence of explanation until now, from function to structure, to one of from structure to function. Thus connectomes now enable us to predict function.

An important revelation from all connectomic information derived to date is the synaptic richness with which adjacent neurons are connected morphologically. This far surpasses the extent of interactions revealed by behavioural or electrophysiological evidence. The latter both have large implicit selection criteria: first, that one only finds what one seeks and, second, that the starting point for most investigations has, until now, been behaviour, in particular a suitable range of laboratory behaviours. The richness of synaptic connections reflects not only these behaviours but also a large body of 'unknown unknown' behaviours. A final example endorses this conclusion. In the recently reported connectome of the *Drosophila* mushroom body output lobe (Takemura et al., 2017b), many of the synaptic motifs uncovered were entirely unanticipated, despite more than 30 prior years of extensive study devoted to the role of the insect α-lobe in learning and memory. Their discovery and very existence indicate future behavioural roles yet to be discovered, so the connectome will prove most valuable in revealing the entire envelope of synaptic interactions, and from this the full range of possible behaviours in *Drosophila*.

How may other species contribute to the immediate goal of a connectome? Marine invertebrate larvae, many with essential virtues of small size, suitable for EM and complete transparency, suitable for LM, provide rich opportunities, but currently lack genomic and genetic data, and consequently are poorly qualified for functional studies: few will achieve greatness. As mentioned previously, *Ciona* provides a powerful model system in chordate biology that is widely utilised for comparative genomics and ripe for functional analysis of its larval connectome (Ryan et al., 2016, 2017), and, for the future, it and the tiny brains of other invertebrate larvae (Meinertzhagen, 2016b) provide Nature's untapped bounty for connectomic analyses (Meinertzhagen, 2017).

This brings us to the beginning of our Commentary: of what use is a connectome? A connectome is a map, a highly complex map and possibly even one that can change or that may vary trivially from fly to fly. Its use is mostly to enable us, as travellers, to find a way through the complex network of the brain's circuits – one that is absolutely essential to navigate an unknown synaptic network of enormous complexity, even in the tiny brain of *Drosophila*. Accomplishing this goal would have been unthinkable a decade ago; equally, the lack of access to a complete connectome will be unthinkable a decade hence.

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