HUGE STEPS ON A TINY BRAIN: Unraveling the Fruit Fly Connectome and Approaches for Comparative Connectomics

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Abstract—A map of the brain's circuits, known as the connectome, is essential for understanding behaviour. Obtaining a high-resolution connectome, where individual neurons and synapses are annotated, has long been a challenge, with most complete connectomes containing just a few hundred neurons and synapses. However, a groundbreaking achievement has been the recent elucidation of the connectome of the fruit fly, *Drosophila melanogaster*, which includes thousands of neurons and millions of annotated synapses. These comprehensive connectomes are invaluable as they enable the comparison of neural connections between different brains, even across species, shedding light on how changes in brain connectivity can impact brain function and behaviour. This review explores the path that led to the creation of the most extensive and detailed connectome to date. It also provides an overview of the strategies for approaching comparative connectomics. The development of these extensive connectomes has been driven by advances in electron microscopy systems and computational tools, which have streamlined the automatic annotation of neurons and synapses. While traditional methods for comparing connectomes have primarily relied on morphological neuron comparisons, there is a growing demand for approaches rooted in the connectivity of neurons. Graph matching emerges as a pivotal technique to determine neuron correspondences across different brains, yet challenges persist in finding an accurate and scalable solution, particularly for large brains.

Keywords— Connectome, *Drosophila melanogaster*, Electron Microscopy, Volume Reconstruction, Comparative Connectomics, Graph Matching

1. INTRODUCTION

Connectomics aims to comprehensively map and understand the nervous system in the most detailed way possible, mostly focusing on the brain. In the literature, connectomes have been defined in several ways. While structural connectomes map the physical connections between various brain structures, functional connectomes illustrate how information flows between these structures. Functional connectomes have proven especially valuable for studying large brains, such as the human brain, given the current infeasibility of obtaining their structural

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connectomes at fine detail [1].

Indeed, the resolution of a connectome depends on the complexity and size of the brain being studied. In human connectome studies, the focus is on connections between large anatomical regions of the brain, typically achieved through techniques like Magnetic Resonance Imaging (MRI) [1]. However, in smaller brains, such as those found in insects, the emphasis shifts to connections between individual neurons [2]. This neuron-to-neuron connectivity is considered the most complete representation of brain circuitry and is referred to as the connectome at synaptic resolution. This fine detail leads us to consider it a connectome in its own right, without the need to distinguish between functional or structural connectomes, as reflected in the literature. Notably, this level of detail has recently been achieved for two specimens, the larva and adult of *Drosophila melanogaster*, the fruit fly, using Electron Microscopy (EM) [3, 4]. EM has long been employed for this purpose, offering optimal nanoscale resolution. In 1986, White et al. used EM to succesfully reconstruct the simpler connectome of *Caenorhabditis elegans* [5]."

The connectome is often referred to as the wiring diagram, and it can be effectively represented as a graph [6]. In this graph representation, nodes represent neurons and edges represent the synaptic connections between them are depicted as the edges. As the number of nodes and edges increases, representing and extracting valuable information from the brain network becomes more challenging. This is where computational algorithms come into play, aiding us in uncovering patterns within the structure that may be linked to specific functions. Comparative connectomics extends this idea further by contrasting connectomes between different individuals, species, or under various conditions [7]. It seeks to understand how variations in neural connectivity relate to differences in function and behaviour across diverse contexts.

The **first part** of this review will explore the efforts and advances in a chronological way that have led to the elucidation of the whole-brain connectome of the fruit fly, *Drosophila melanogaster*. We will start from the reconstruction of the connectome of *C. elegans* (302 neurons) [5] until the very recent publication of the fruit fly connectomes for the larva (3,013 neurons) [3] and the adult (123,978 neurons) [4].

The **second part** of this review will focus on the usage of full connectomes to understand differences or similarities between organisms, the goal of comparative connectomics. We will review methods that utilize morphology for comparisons, as well as computational tools that primarily focus on comparing neurons with the same connectivity pattern.

2. TOWARDS THE COMPLETE CONNECTOME OF THE FRUIT FLY

2.1. Before the fruit fly connectome: mapping hundreds of neurons

1986 was a key year for connectomics at synaptic resolution: White et al. reconstructed for the first time the whole wiring diagram of the *C. elegans* nervous system [5]. They mapped the synaptic connections between 302 neurons. With its remarkable simplicity, it represented a significant breakthrough after about 15 years of dedicated research using Transmission EM (TEM) to study the structure of tiny neural circuits [8, 9]. In all these studies, including the final mapping, the procedure was to slice the brain specimen and to image the slices with TEM. From these, they manually drew and annotated neurons and synapses. Of note, these studies drew inspiration from the foundational work of Santiago Ramón y Cajal in the late 19th century, who described the structure of the nervous system [10].

Decades later, in 2016, researchers reconstructed approximately half of the neurons in the larval *Ciona intestinalis* connectome [11] (177 neurons). They focused exclusively on the Central Nervous System (CNS) due to its distinct separation from the Peripheral Nervous System (PNS), unlike the non-distinguishable systems in *C. elegans*. They prepared the brain specimen similarly to White's method, slicing it for TEM imaging. Advanced software aided in automating tasks for mapping individual neurons. However, the process for synapse detection remained consistent with the manual annotation of vesicle clusters at presynaptic membranes, aligning with the initial connectome's

methodology [5].

Even though these two invertebrate models have a relatively simple nervous system, at least in terms of the number of neurons, they still show some interesting behaviours. *C. elegans* organisms have memory, they learn and they respond to a series of external stimuli [12]. On the other hand, *C. intestinalis* larvae exhibit different swimming strategies to survive [13]. Besides the small size of the brain, invertebrates are attractive models because of the great stereotypy among individuals [2].

2.2. Why the fruit fly?

Connectomics faces the significant challenge of linking neural structure with function. Consequently, researchers often study animals that manifest more intricate functions than the ones described previously. In spite of their relatively minute brain sizes compared to larger vertebrates, miniature insects exhibit a wide spectrum of complex behaviours and structures [2]. Notably, the olfactory system of *D. melanogaster* is of particular interest, as it shares resemblances with the olfactory systems of larger insects and even draws parallels with the olfactory system of vertebrates, including humans [14]. These similarities likely stem from convergent evolution. Besides learning, memory capacities and complex locomotion, the fruit fly is also interesting because of the social interactions that happen during courtship [15].

The genetic tractability of *D. melanogaster* allows for precise manipulation and control of genes, facilitating targeted studies of neural circuits. The fruit fly's genome has been meticulously sequenced, providing researchers with a well-annotated genetic blueprint to explore neural connectivity [16]. Furthermore, its transparent larval stage allows for non-invasive imaging of developing neural circuits, making it an ideal candidate for studying the maturation and establishment of connections [17]. All of these factors have motivated the use of the fruit fly as an intriguing model in neuroscience.

Given the fruit fly's complex life cycle (i.e., from larval to adult stage) and their sexual dimorphism, a single model cannot comprehensively represent its neural intricacies [18]. For example, a connectome for the larval stage holds great importance in understanding how the nervous system evolves during the fly's development [19]. Additionally, since behavioural differences between males and females are likely supported by differences in their connectomes, models for both genders are essential [20]. In the year 2023, after years of dedicated effort, the full connectomes for the larval stage and female adult have been released [3, 4].

2.3. Connectome of the fruit fly brain: mapping thousand of neurons

Before releasing the complete connectomes of *D. melanogaster*, efforts were directed toward releasing small portions of the fly's brain in a modular fashion. Over the years, various experiments have benefited from advances in both the employed technology and the automation-assisting software for connectome mapping. This part of the review will delve into this modular discovery of the brain, while also highlighting the most pioneering advancements. In doing so, this section will also contribute to a better understanding of the overall structure and anatomy of the fruit fly's brain.

The brain of the adult *D. melanogaster* served as the reference for establishing the insect brain atlas by the Insect Brain Name Working Group in 2014 [21]. This consortium of neurobiologists proposed a consensus framework and nomenclature to describe the structural organization of the insect brain. Figure 1 shows the nomenclature and anatomy of this atlas. For the next subsections of the review, we will adopt this nomenclature. Within the fly's CNS, they identified 43 neuropil units, which are specialized compartments rich in terminal neurite branching, forming dense synaptic regions where signal processing occurs [22]. The majority of these 43 units are located within 12 neuropil blocks, which constitute approximately 90% of the brain. Among the most studied regions, we highlight the mushroom body (MB), the antennal lobe (AL), the optic lobe (OL), and the lateral horn (LH). The remaining 10% of the

brain is composed of fiber bundles that fill the spaces between neuropils and serve as connections. These fiber bundles were named 'landmark fiber bundles' since they correspond to the boundaries of the neuropils.

In addition to the brain, *Drosophila* also has a Ventral Nerve Cord (VNC). A second nomenclature effort expanded the system to include the sub-gnathal regions of the brain [23], which still belong to the CNS but are found adjacent to the lower part of the fly's brain. The VNC is subdivided into the thoracic region and the abdominal region.

2.1. Larval brain (3,013 neurons): the first and "easier" to map

The first large studies of the fruit fly connectome were initiated with larvae examples. They possess smaller brains, making them more manageable for imaging at a nanometer scale, requiring less time and effort. Importantly, the brain structures of the larval stage are homologous to those of the adult fruit fly [21]. While the larval stage does not encompass the full repertoire of adult behaviours, it does exhibit several intriguing adaptive behaviours including sensory-driven responses [24], learning [25], and simple ways of locomotion [26].

Following the success of the mapping of the *C. elegans* connectome [5], researchers attempted to investigate small sections of the *D. melanogaster* larval brain [27, 28, 29]. But they either imaged tiny volumes or reconstructed neurons in a sparse manner, focusing on limited sets and individual neural processes. The process of imaging volumes using serial section TEM (ssTEM) involved meticulously sectioning the specimen at a nanometric scale. Subsequently, manual alignment of these sections was necessary to recreate a 3D volume. With that volume, they needed to reconstruct and trace the profiles of individual neurons and their synaptic connections. These studies were highly demanding in terms of both time and effort. A comprehensive dense reconstruction of the entire larval brain, like what was achieved for *C. elegans*, appeared unfeasible using this approach alone.

Initial advances, the software. Advances in computer-assisted image processing revolutionized the field. A 'Computational Framework for Ultrastructural Mapping of Neural Circuitry' was published in 2009 aiming to image larger volumes with ssTEM [30]. Notably, large sections (i.e., in the x-y plane) were imaged not as a whole, but the microscope acquired images of different regions of the sections individually, known as image tiles (see Fig. 2). With this computational framework, they automated the process of montaging these tiles into a mosaic that represented the whole section, significantly reducing the time required for volume acquisition. Additionally, they proposed an efficient method for registering and browsing such vast datasets, easily scaling up to gigabytes.

TrakEM2 was also introduced with a similar approach [32]. TrakEM2 was utilized for the analysis of the microstructure of a *Drosophila* larval brain hemisphere and a segment of the VNC. Like the previous framework, TrakEM2 incorporated an automatic montage of tiles within the section, and included automatic alignment of sections to create a 3D structural representation (see Fig. 2). Furthermore, they introduced the concept of dense reconstruction after obtaining the large TEM volume. The imaged portion of the hemisphere, excluding the cortex, measured approximately 30x30x30 µm. Serial sections had a thickness of about 50 nm, and they were imaged at a resolution of approximately 3-4 nm per pixel. The researchers divided the entire volume into smaller 'microvolumes,' each as large as 5×5×5 µm. For five of these microvolumes, they performed dense reconstruction, reconstructing all the neurons and connections. By linking microstructural TEM images with macrostructural light microscopy images, they argued that one could more easily establish the position, input, and output relationships of that volume. These five reconstructed microvolumes were located in the calyx and spur of the MB, dorsolateral protocerebrum, and dorsolateral domain of the VNC.

Reconstructing neurons and their synaptic partners within the microvolumes in the latter was done with CATMAID [33], a collaborative annotation toolkit that has been consistently

A)

Synapse-rich neuropils					
optic lobe	*OL*	*central complex*	*CX*	*antennal lobe*	*AL*
lamina	LA	central body	CB	AL glomeruli	GL
lamina dorsal rim area	LADRA	fan-shaped body	FB	AL hub	ALH
plexiform lamina	PLLA			*ventromedial neuropils*	*VMNP*
accessory lamina (*not in flies*)	ALA	ellipsoid Body	EB	ventral complex	VX
medulla	ME			vest	VES
medulla dorsal rim area	MEDRA	protocerebral bridge	PB	epaulette	EPA
plexiform medulla	PLME	noduli	NO	gorget	GOR
accessory medulla	AME	*lateral complex*	*LX*	posterior slope	PS
lobula complex	LOX	bulb	BU	superior posterior slope	SPS
lobula	LO	lateral accessory lobe	LAL	inferior posterior slope	IPS
lobula plate	LOP	*ventrolateral neuropils*	*VLNP*	*periesophageal neuropils*	*PENP*
mushroom body	*MB*	anterior optic tubercle	AOTU	saddle	SAD
calyx	CA	ventrolateral protocerebrum	VLP	antennal mechanosensory and motor center	AMMC
accessory calyx	ACA	anterior VLP	AVLP	flange	FLA
pedunculus	PED	posterior VLP	PVLP	cantle	CAN
pedunculus neck	PEDN	posteriorlateral protocerebrum	PLP	prow	PRW
pedunculus divide	PEDD	wedge	WED	dorsal pharyngeal sensory center	DPS
spur	SPU			*gnathal ganglia*	*GNG*
vertical lobe	VL	*lateral horn*	*LH*	ventral pharyngeal sensory center	VPS
α′ lobe	α′L	*superior neuropils*	*SNP*	anterior maxillary sensory center	AMS
α lobe	αL	superior lateral protocerebrum	SLP	posterior maxillary sensory center	PMS
α_p lobe	α_pL	superior intermediate protocerebrum	SIP	labellar sensory center	LBS
		superior medial protocerebrum	SMP		
medial lobe	ML	*inferior neuropils*	*INP*		
γ lobe	γL	crepine	CRE		
β′ lobe	β′L	clamp	CL		
β lobe	βL	superior clamp	SCL		
β_p lobe	β_pL	inferior clamp	ICL		
		inferior bridge	IB		
		antler	ATL		

red: *level 1, supercategories*
black: **level 2, neuropils**
blue: level 3, subregions

B)

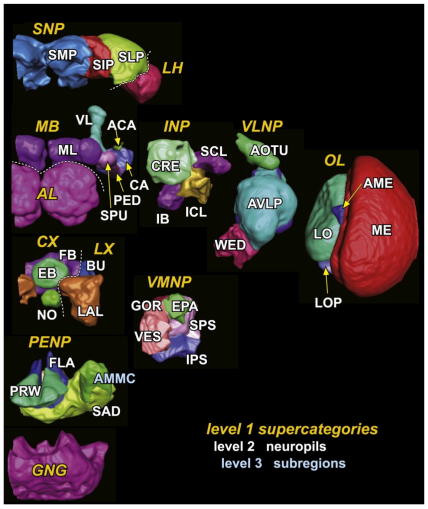

Fig. 1: Nomenclature of the brain of *Drosophila melanogaster* (adult) brain. Adapted from [21]
(A) List of the neuropil nomenclature and hierarchy. These are divided into 3 categories: supercategories or neuropil blocks, neuropil or neuropil units, and subregions.
(B) 3D reconstruction of the right hemisphere of the brain with neuropil annotations, using the same abbreviations as in A. Brain regions are depicted as independent and far from each other for visualization purposes.

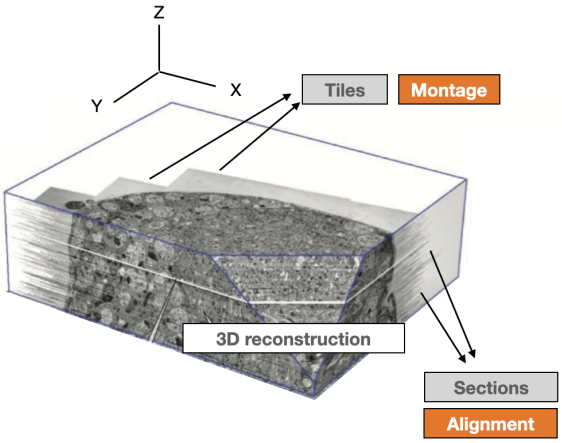

Fig. 2: 3D reconstruction from serial section Transmission Electron Microscopy images to illustrate the difference between tiles (x-y plane) and sections (z axis). Two techniques are needed to obtain the 3D reconstruction: montage of tiles and alignment of sections. Adapted from [31]

utilized in the subsequent studies discussed here. CATMAID was integrated into TrackEM2 and provides extensive support for annotating massive amounts of image data. It enables the navigation of large image stacks, as well as the tracing of neurons and the annotation of synapses. Thanks to this collaborative tool, the reconstruction of the whole connectome has been possible by merging the efforts of different studies into annotating regions of the same imaged brain.

The whole brain data: ssTEM images. In 2015, a team at Janelia Research Campus in Virginia, USA, conducted ssTEM imaging of the entire CNS in a first instar larva and segments of the abdominal VNC [26]. This work can be seen as an enhanced and expanded version of the images generated using TrakEM2 in a previous study [32]. Notably, Ohyama et al. imaged both left and right hemispheres, providing a comprehensive view of the larval brain, and extended their coverage to include a larger abdominal area encompassing segments A1, A2, and A3. While the imaging technology and resolution (about 4 nm per pixel with 50 nm serial sections) were similar, they introduced a novel technique for assembling image tiles into sections and aligning these sections into 3D volumes [31]. This technique effectively corrected distortions that could occur during specimen sectioning or due to the specimen's shape with an elastic constraint. These advancements made Ohyama's image dataset a cornerstone for subsequent larval studies, although the full connectome reconstruction was not provided.

The subsequent reconstructions. In the original study [26], they examined the rolling circuit, the larva's rapid escape response to severe threats, by

reconstructing neurons in the A1 and A3 abdominal segments. These neurons included mechanosensory chordotonal neurons, nociceptive multidendritic-IV neurons, Goro and Basin neurons. Basin neurons acted as interneurons, connecting sensory neurons to Goro neurons, which are involved in locomotion. They also reconstructed second-order interneurons, some of which ascended to the brain for sensory processing, without confinement to specific neuropils. In total, over 300 neurons were reconstructed. Subsequently, two other groups focused on forward and backward locomotion circuits, reconstructing numerous premotor and motor neurons in the VNC [34, 35].

Additional sensory circuits have been explored using the same larval data, contributing to the comprehensive larval connectome. The olfactory circuit was studied by examining both the left and right ALs, the primary olfactory neuropils [36]. This investigation involved tracing 160 neuronal arbors, from olfactory receptor neurons to various types of local and projection neurons. Notably, projection neurons established connections between the AL and the LH as well as the MB calyx. Subsequently, it was discovered that the LH conveyed olfactory information to the premotor circuit through the reconstruction of descending neurons [37]. Additionally, leveraging the MB projections, Eichler et al. successfully reconstructed the entire MB calyx in a later study [25].

The MB is central for learning and memory formation. In the last study, they found that its intrinsic neurons, the Kenyon Cells (KCs), were not only connected to projection neurons connected to olfactory neurons [36] but also to thermal, gustatory, and visual neurons. To further comprehend the learning circuit of the larva brain, this group also reconstructed pre- and postsynaptic partners of modulatory neurons and output neurons within the MB, respectively [38, 39].

The circuits related to gustatory stimuli (i.e., food-intake circuits) started to be studied by first reconstructing those neurons expressing the peptide Hugin within the gnathal ganglia [40]. There were only 20 neurons, including interneurons and efferent neurons, the ones leaving the CNS. While interneurons projected both to the protocerebrum and the VNC, efferent neurons projected to the ring gland and pharynx, two endocrine organs. This circuit was then expanded with the reconstruction of other sensory and motor neurons from three pharyngeal nerves that innervate the gnathal ganglia [41]. Here, they studied how these neurons connected to the gustatory projection neurons previously found in the MB calyx. Finally, a more recent study continued the work about neurons that projected to the ring gland, by reconstructing all the projection neurons that targeted it, including non-Hugin neurons [42]. They merged the reconstruction of sensory neurons from many previous studies [26, 36, 40, 41] to comprehend neuroendocrine outputs as a response to some stimuli.

The larval visual system was also reconstructed for the *Drosophila* larva [43]. Unlike adults, larvae present a much simpler visual organ, the Bolwig organ. But they still present photoreceptors, which are connected to the larval OL. They traced all the neurons innervating the OL and found similarities with the olfactory wiring diagram, the reconstruction of the AL. They both relay information to higher-order neuropils like the MB for associative memory purposes.

It is worth mentioning that all these subsequent publications have reconstructed the neurons manually, like in the original publication of the data [26]. However, all of them benefited from a novel reconstruction tool on CATMAID [44]. It facilitates the reconstruction by informing the user with several measures based on the anatomy of the neuron and the connectivity. The reconstruction is iterative, so independent reconstructors would work on the results of the others, instead of working from scratch and obtaining a consensus map. This made proofreading faster. But still, both reconstruction and proofreading tasks were highly time-consuming. For example, Berck et al. took 763 hours to reconstruct 160 olfactory neurons, and 431 hours to proofread them [36].

The final reconstruction. The very recent completion of the whole-brain connectome of the larva brain merged all these previous reconstructions and added the rest of the circuitry [3] (see Fig. 3). A total of 3,013

neurons and approximately 544,000 synapses are mapped, including the brain inputs (477 neurons), interneurons (2,118 neurons), and brain outputs (418). About half of these neurons had been already described and traced [26, 25, 36, 37, 40, 41, 34, 35, 38, 39, 42]. They reconstructed the remaining 1,507 brain neurons and analyzed connection types, neuron types, hubs, circuit motifs, and brain-VNC interactions. During this intensive reconstruction, validation of the connections was also done with the quantitative measures of CATMAID [44]. But they also introduced the review of neuron pairs based on left-right homology that offers the *Drosophila* brain, which they concluded to be about 93%. They performed this validation based on graph matching.

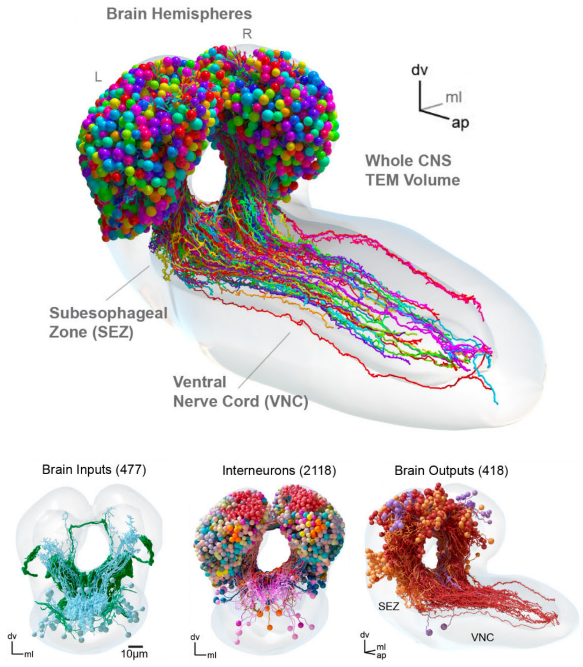

Fig. 3: Connectome reconstruction of the larva brain from the Central Nervous System (CNS) volume obtained with Transmission Electron Microscope (TEM). It shows the general picture for the two hemispheres (R: right, L: left), as well as the division into inputs, interneurons and outputs. Subesophageal zone (SEZ) corresponds the Gnathal Ganglia from Fig. 1. Adapted from [3].

2.2. Adult brain (127,978 neurons): the most complex to map

The adult fruit fly nervous system is far larger and complex than the larval one. In contrast to the larva, adult flies navigate over long distances and can create long-term memories [45]. This is of course besides the basic behaviours that are (mostly) present during the larval stage, although much simpler. They process visual, olfactory, and auditory cues. And they show a more complex locomotion: walking and flying.

Similar to the first efforts on the larval connectome, before the imaging of large volumes of the adult brain, several studies focused on small circuits. These covered small brain regions like a few columns of the Optic Lobe (OL) medulla [46, 47], the α lobe of the MB [48], and a part of the AL [49]. These involved the reconstruction of hundreds of neurons, although the images were specifically obtained for each study independently.

Even though these studies were contemporaneous, or even younger, to the studies done for the whole-brain connectome (2013-2019, while the whole larval CNS was imaged in 2015), the methodology used to image and reconstruct the connections was different. Mainly, because of handling bigger specimens for these experiments on the adult fly. During these years, researchers examined different imaging techniques, including the already mentioned ssTEM and the novel Focused Ion Beam Scanning Electron Microscopy (FIB-SEM). In contrast the studies on the larva connectome, researchers have implemented several versions of automated tools for neuron reconstruction and synapse annotation along the way.

Since the process for the adult brain reconstruction was not as uniform as for the larva case, we will narrow this part of the review. We will focus on two big volume EM datasets: the full adult fly brain (FAFB) [50] and the hemibrain [51] datasets. These two datasets were published with numerous advances.

The whole-brain data: ssTEM images. In 2019, another group at Janelia Research Campus released the complete EM volume of a female adult brain

(full adult fly brain or FAFB) [50]. Similar to the larva brain volume [26], Zheng et al. imaged the brain with ssTEM at a similar resolution (4 nm per pixel, with z-sections of 40 nm). However, they upgraded this imaging technology with a high-speed camera array (TEMCA) and a robot system for exchanging the sections to be imaged. For transforming 2D tiles into a 3D volume, they used a customized version of the elastic montage and alignment used in the larva volume. In total, it took approximately 16 months to obtain all the data.

With the publication of FAFB, they also reconstructed KCs from the MB, as they had been previously studied in studies like [48], and served as a test of the consistency of reconstructions with these data. This reconstruction was done manually on CATMAID with its quantitative anatomical and connectivity measures [44], as done for the larva connectome. Of note, older studies of the adult brain had already made use of semi-automated reconstruction of neural circuits, facilitating neuron segmentation and/or synapse prediction [46, 47, 48, 49]. However, they argued that the quality of the FAFB large image alignments was not sufficient at that time to apply such automatic algorithms.

Also with manual reconstruction of FAFB, Bates et al. released a full inventory of olfactory projection neurons that connected the AL to higher brain centers like the MB calyx and the LH [52]. The main difference with Zheng's reconstruction was the usage of a partial automatic segmentation tool to refine the reconstruction and search for missing arbors as the final step.

The hemibrain data: FIB-SEM images and reconstruction. In parallel to the FAFB efforts, another Janelia's group imaged a portion of the central brain of a female adult fruit fly [51]. Not only the images were released, but also the whole dense reconstruction at the synaptic level. In this case, they used FIB-SEM, which had been already used for the sparse reconstruction of small brain regions. Referred to as the hemibrain data, it contains around 25,000 neurons and 20 million synapses. It covered the majority of the right hemisphere, apart from the OL, periesophageal neuropils, and gnathal ganglia.

FIB-SEM was preferred in this case due to the high-quality requirements when using automated algorithms for the reconstruction. In contrast to ssTEM, FIB-SEM does not require previous nanometric sectioning of the specimen as it can image volumetric specimens. However, some sections were needed to speed up the image acquisition. This allowed parallelizing the process with two FIB-SEM machines. The no-sectioning translates to a higher quality alignment and images at isotropic resolution since z-axis resolution is no longer limited to the section depth. However, all this comes at a cost of time. It took about 2 years to image the hemibrain with two FIB-SEM machines running in parallel, compared to the 16 months of FAFB imaging with ssTEM [50].

The computational reconstruction of this volume implemented several advances. For neuron segmentation, they used machine learning algorithms that included flood-filling and generative adversarial networks [53, 54], which were trained with the manual reconstruction of some parts of the hemibrain. On the other hand, the prediction of synapses was performed with a custom iterative approach that combined model re-training with manual proofreading. After automatic reconstruction, several passes of human proofreaders refined the results. Proofreading was facilitated by a focused proofreading technique with machine suggestions on reconstruction tools like NeuTu [55] and Neu3 [56], similar to CATMAID [33] but optimized for larger datasets.

Some studies have used this partial connectome to study circuits within the central complex [57], the MB [58], and the AL [59].

The final reconstruction. Very recently, the whole-brain connectome of the female adult fly has been published [4] (Fig. 4). The FlyWire consortium [60], a big group of researchers from around the world, has reconstructed the whole FAFB volume. It contains 127,978 neurons and 53 million synapses. In contrast to the hemibrain [51], this includes both hemispheres (central brain), gnathal ganglia, and both OLs (as depicted in Fig. 4). The VCN is not covered within this connectome, but external studies focused on its reconstruction [61, 62]. Computational reconstruction was followed by a great effort of

proofreading.

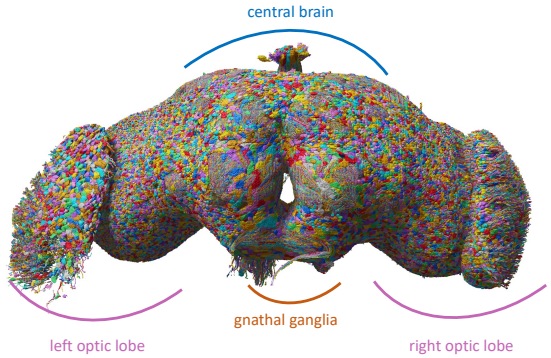

Fig. 4: Connectome reconstruction of the adult brain from the female adult fly brain volume obtained with Transmission Electron Microscope (TEM) [4, 50]. The volume contains both hemispheres that make up the central brain, the two optic lobes, and a part of the gnathal ganglia. Adapted from [4].

Reconstructing automatically all the neurons and their synapses on FAFB was possible with an improved method of section alignment [63], as suggested by Zheng et al. [50]. This pipeline includes key elements like convolutional networks for fine-tuning alignment, vector voting for robustness, and division of the volume into blocks for speedup. All these successfully solved problems like folds or cracks present in the sections. Alignments of such quality allowed the automatic segmentation of the neurons, as described in [64]. Briefly, the segmentation pipeline consists of several steps: (1) detecting of boundaries between neurons, (2) classifying voxels as cell body, dendrite, glia, and blood vessels, and (3) segmenting the resulting boundaries. Convolutional networks were also used for step 1 and 2. Finally, a similar network predicted postsynaptic sites with a vector that also pointed to the presynaptic partner [65].

The collaboration of FlyWire members, including specifically trained proofreaders, permitted them to proofread these automatic reconstructions with the Connectome Annotation Versioning Engine (CAVE) (paper under prep.). In some cases, the neurons to be checked were prioritized with an automatic selection of the neuron nuclei [66]. Thanks to the automation improvements that we mentioned, the proofreading time was reduced from 50 person-years for the hemibrain [51] to 30 person-years for the whole brain.

A comparison between the partial connectome of the hemibrain with that of the whole brain was done in a companion study [67]. They argue that this large connectome at the synaptic level paves the way for further studies of comparative connectomics.

3. Approaches for comparative connectomics

With the initial reconstructions of these connectomes, researchers aimed to assess differences in neurons and their connectivity across various developmental stages [19, 68], and in some cases, even across different species [69]. This research question has been coined as comparative connectomics: the quantification of variations across connectomes, potentially linked to adaptations in behaviour and cognition [7].

Next, we will briefly introduce some studies that focus on connectome comparisons based on morphological similarities between neurons, but our specific focus will be on how two connectomes can be compared based on the connectivity of the neurons.

3.1. Morphology-based comparisons

Thanks to the remarkable neuronal stereotypy observed in invertebrates [2], researchers may identify homologous neurons based on morphology, position, and branching patterns. This stereotypy allowed for the comparison of connectomes across different life stages, revealing principles of brain maturation due to the rewiring of neurons in *C. elegans* [68]. In contrast, *Drosophila* larvae showed conserved circuit connectivity when comparing approximately 173 reconstructed neurons from two different instar larvae [19]. However, they concluded that the development of fruit fly larval brains involved an increase in neuron size and synaptic inputs.

Morphological traits also aided in the identification of 20 homologous neuron pairs

in the pharynx of *C. elegans* and another nematode, *Pristionchus pacificus* [69]. Comparing these neurons across species revealed extensive rewiring of connectivity, likely related to divergent feeding behaviours.

Recently, Schleget et al. aimed to make comparisons between the full connectome (FlyWire [4]) and the partial connectome (hemibrain [51]) of two fruit flies [67]. They achieved this by matching each FlyWire neuron with one of the defined hemibrain neuron types using NBLAST [70], which measures similarity based on neuronal position and geometry. This analysis revealed variable connection weights between the two individuals.

Despite the stereotypy observed, some studies have reported variability in body position and neuron trajectory during these comparisons [67, 68]. Additionally, it is important to note that stereotypy may not hold in the olfactory circuit, leading to high inter-individual variability in terms of morphology [71].

3.2. Connectivity-based comparisons

In the comparison between the hemibrain and the FlyWire connectome [67], when a neuron could not be paired with a neuron type based on morphology, they opted to link it based on the connectivity pattern of the neurons. Notably, the annotation of *Drosophila* neurons based on their connectivity had been already proposed by Scheffer et al. [51]. This non-morphology-based comparison is somewhat similar to the comparison that Winding et al. did between neurons in the left and right hemispheres of the larva connectome [3]. Here, they treated the two sides of the brain as two independent connectomes. These two were translated into graphs, and they finally used graph matching to pair neurons from both.

Next, we will introduce the concept of graphs for illustrating connectomes, as well as discuss the graph matching problem and some existing approaches that aim to solve it.

The connectome as a graph. The connectome can be represented as a network or graph. A graph is formally defined as:

$$G = \{V, E\} \tag{1}$$

where $V = [N]$ is the set of nodes, or vertices, and $E \subset V \text{x} V$ is the set of edges. For a graph G with N nodes, we have an adjacency matrix A of size NxN. The elements of A describe the edges between each node pair.

For the cases we are discussing (i.e., connectomes at the synaptic level), we will have the following representation: nodes of the graph will correspond to individual neurons, while the edges that connect these nodes will correspond to synaptic connections. Such definition of nodes and edges has been widely used for this kind of connectomes, but further discussion on the nature of nodes or edges is found in [72].

Since different pairs of neurons can be connected at different synaptic strengths, the connectome produces a weighted graph. For *D. melanogaster* it has been proven that the number of synapses between two neurons is correlated with the synaptic strength [73]. Thus, these synaptic counts are used as the edge weights. Finally, the graph is directed because chemical synapses only occur in one direction.

This representation has been used, for example, to analyze the graph properties of the hemibrain connectome of the fruit fly [74]. They concluded that the fly brain is not wired randomly, that fly brain neurons have many inputs and outputs, and that paths in the fly brain are short. Furthermore, the simplification of the connectome into a graph should facilitate the comparison with other connectomes.

The graph matching problem. Before comparing two connectomes, or graphs, one must ensure a clear understanding of which neurons, or nodes, in one connectome correspond to those in the second connectome. Mathematically, this challenge is known as the graph matching or graph alignment problem. Solutions for this problem are interesting and not limited to neuroscience; it finds applications in other disciplines such as molecular biology for protein-protein interactions and social sciences (for a further review and discussion, see [75]).

Graph matching or alignment can be categorized as local or global. In global alignment, the objective is to align and compare entire graphs, whereas in local alignment, the emphasis is on specific subregions within the graphs [75]. Therefore, when comparing complete connectomes, global alignment is the preferred approach. We will now delve into this type of alignment.

The quadratic assignment problem and its fast approximation. Formally, graph matching seeks to find the alignment of the nodes of the two graphs such that the number of edge disagreements is minimized. From two graphs G_1 and G_2 with N nodes (defined as in Eq. (1)), one can obtain their respective adjacency matrices A and B. These matrices are NxN and their elements represent how each pair of nodes are connected within the graph. The graph matching (as described in [76]) can be defined as the following optimization problem:

$$\begin{aligned} &\text{minimize } ||AP - PB||_F^2 \\ &\text{subject to } P \in \mathscr{P} \end{aligned} \tag{2}$$

where $\mathscr{P}$ is the set of nxn permutation matrices, and $||\cdot||_F$ is the matrix Frobenius norm. In turn, this formulation is almost identical to the quadratic assignment problem (QAP), which tries to optimize the assignment of facilities to locations [77]. This is considered an NP-hard problem because no solution has been found that works in polynomial time [78], making it impossible to work with more than 20 nodes. This is because of the size of the set of permutation matrices. For n nodes, there are $n!$ possible matrices. So when n=20, there are $2.43\text{x}10^{-18}$ permutation matrices to be tested.

Since graphs representing connectomes are often quite large, solving the alignment of connectomes using brute force methods becomes impractical. To address this challenge, researchers have developed heuristic and approximation algorithms to find near-optimal solutions without exhaustively exploring the entire set of possible permutations. One such algorithm is FAQ, which stands for Fast Approximation for QAP [76].

Motivated by applications in connectomics, Volgenstein et al. introduced the FAQ algorithm. It approximates the graph matching problem by relaxing the set of permutation matrices (denoted as $\mathscr{P}$) to encompass a broader range of potential solutions (denoted as set D). This relaxation aims to find local optima rather than the global optimum and employs a well-described optimization procedure, as detailed in the paper. Once the local optimum (i.e., the optimal matrix within the relaxed set) is found, it is projected back to the original $\mathscr{P}$. FAQ does not explore all possible solutions but iterates until it reaches a local optimum. This approach significantly reduces computation time, enabling the alignment of larger networks.

Along with the publication of FAQ, they used it to match the C. elegans connectome with a permuted version of itself, both consisting of 279 nodes, demonstrating optimal performance [76].

Seeded graph matching. Let us now shift our focus from synaptic-level connectomes to explore alternative approaches used in the context of human connectomes. In these connectomes, the constituent nodes represent distinct anatomical brain regions, while the edges signify the connections between these regions, distinct from synaptic connections [1]. In such cases, MRI records signals from these regions, which are subsequently mapped to a predefined brain parcellation scheme. However, the conventional method of comparing connectomes solely by matching nodes based on this parcellation is suboptimal due to inherent inter-subject variability [79]. Consequently, the application of graph matching techniques becomes imperative. Nonetheless, the initial parcellation still holds utility, akin to serving as a foundation for the alignment process. Notably, seeded graph matching has been investigated for human connectome matching (with 70 nodes), employing varying numbers of parcellation seeds to facilitate the alignment [80].

Seeded graph matching with FAQ was also tested on the connectome of *C. elegans* [81]. Specifically, they aimed to match the graph representing chemical synapses with the graph illustrating gap junctions between neurons. However, only a few nodes were correctly matched, as confirmed through validation against ground truth. Notably, in this study, they also experimented with matching graphs of varying node numbers, while FAQ

typically assumed matching two graphs of the same size.

In spite of the low performance of this proposed seeded FAQ strategy on real connectomics data [81], this method was used to compare the left and right hemisphere for the larval connectome [3], including about 900 ipsilateral neurons in each graph. As seeds, they used neurons that had been matched based on morphology in previous studies [36, 25]. Matched pairs needed to be manually reviewed. Pedigo et al. have recently suggested including contralateral neurons and treating the graph matching problem as a bisected graph matching with four subgraphs (two for ipsilateral neurons on both sides, and two for contralateral neurons going from left to right, and vice versa) [82]. This formulation improved the performance of automated pairing of bilaterally homologous neurons.

In addition to the seeded graph matching mentioned earlier, there is another variant known as soft seeded graph matching [83]. In this approach, the FAQ algorithm is initialized with matrices based on prior information, containing the seeds. However, these seeds are not strictly maintained in the final alignment. In a recent study, brain parcelation information was used as the initial matrix to kickstart the alignment of human connectomes with FAQ [84]. This approach demonstrated improved performance compared to other initializations of the initial matrix (including random initialization), resulting in greater connectome similarity.

While the seeds in the studies we have described so far are derived from biological knowledge, some algorithms use pairs of nodes known to be topologically similar (i.e., in terms of connectivity) as seeds. Among these, are GHOST [85]* and WL-Align [86]. Prior to commencing the optimization of the alignment process, they assign a signature to each node that captures the local neighborhood's topology. These signatures, known as spectral signatures, are vectors that describe each node independently. Then, the distance between these vectors is computed on a one-to-one basis. Neuron pairs with smaller distances are finally selected as seeds for the optimization process. WL-Align [86] optimizes the alignment differently than FAQ after this seeding step. It has been tested for aligning human connectomes, both from the same subject or from different subjects, and showed similar efficiency to FAQ but a superior performance in alignment.

An important limitation once again is the scalability of these algorithms. The time required for FAQ, its seeded versions and WL-Align scales cubically with n (i.e. the number of nodes of the graphs being compared). For instance, Volgenstein et al. [76] conducted calculations for a graph with 100,000 nodes, a scale comparable to that of the *Drosophila* adult brain, and determined that the graph matching process would necessitate approximately 20 years of computation on a standard laptop.

The graph matching problem beyond the quadratic assignment problem. In addition to these methods closely related to QAP, there are other solutions in the literature that have formulated the problem differently. They differ in the score they aim to optimize in order to obtain the alignment (i.e., different from the equation for QAP, Eq. (2)), or in the algorithm itself that generates possible solutions for the alignment.

We will present two scores, S^3 and graph edit distance, and the most representative algorithms used to optimize it. Even though these studies were originally focused on aligning networks of protein-protein interactions [87, 88, 89], the algorithms have been tested in the field of comparative connectomics with (human) structural connectomes [90].

- **S^3 or symmetry structure score**

  Briefly, S^3 aims to compute the topological similarity between the two aligned graphs by taking into account the edges that are conserved between the two [87]. It has been shown to be superior to other proposed metrics for computing similarity, as it penalizes both alignments that map denser graph regions to sparser ones and alignments that map sparser graph regions to denser ones. Since the mathematical formulation of this is beyond the scope of the review, we refer the reader to the original paper [87] for a detailed definition.

Among the proposed algorithms to optimize S^3, are the so-called simulated annealing [88] and genetic algorithms [87].

Simulated annealing

Simulated annealing aims to approximate global optimization using a method inspired by metallurgical annealing principles [91]. In the context of graph matching, it starts with an initial alignment, which may be random, and computes a score that assesses the difference between the graphs. In this case, S^3. To minimize this score, the solution iteratively transitions to a neighboring state by altering some mappings between pairs. Initially, these variations occur more freely, but they gradually become more selective due to the decrease in a temperature parameter that limits the probability of accepting a new change.

The algorithm SANA (Simulated Annealing Network Aligner) [88] was mainly created for protein networks (in the order of thousands of nodes), but it has been recently extended and tested for comparing human connectomes (with hundreds of nodes) against the same connectomes but with added noise (i.e., by intentionally changing some connections withing the graph) [92]. Simulated annealing was also employed to compare human connectomes from different subjects without relying on predefined brain parcellation, as demonstrated in [79]. Despite optimizing a simpler score than S^3, this approach revealed a higher degree of similarity in alignment compared to simply comparing connectomes based on anatomical brain parcellation [79].

Genetic algorithms

Genetic algorithms simulate evolution and are guided by the principle of selecting the fittest solutions to optimize a problem [93]. They start with an initial set of solutions and then, they combine these solutions to create descendant solutions. In each generation or iteration, solutions that are more "fit," as determined by the score being optimized, are more likely to generate descendants.

MAGNA (Maximizing Accuracy in Global Network Alignment) utilizes this genetic framework for protein graph matching [87]. This method aims to optimize the S^3 score and has been effective even when starting with a random initial population of alignments for large networks. MAGNA was employed in a benchmark study aiming to align human connectomes with their noisy counterparts [90]*.

- Graph edit distance

  Graph edit distance is a fundamental measure used in inexact graph matching. It quantifies the similarity between pairs of graphs in a tolerant manner, accounting for errors [94]. In essence, it calculates the number of node and edge deletions and insertions needed to transform one graph into another, representing the aligned graphs.

  GEDEVO, short for Evolutionary Graph Edit Distance, is an algorithm designed for graph matching [89]. It employs a genetic algorithm with the objective of minimizing the total cost associated with edge insertions and deletions required for the matching process. GEDEVO was featured in the same benchmark study mentioned previously [90]*.

  While graph edit distance is valuable for solving the graph matching problem, it is worth noting that its computational complexity can be a limiting factor, particularly as it scales cubically with the size of the graph. Additionally, it has found use in quantifying the similarity of brain networks when node correspondence is known [95].

**In a benchmark study [90], Milano et al. evaluated various aligners [85, 87, 89] for their ability to align the original human connectome with noisy versions of those connectomes (i.e., by changing some connections). Notably, MAGNA demonstrated superior performance in this context, compared to GHOST and GEDEVO.*

4. Discussion

Connectomics, the comprehensive mapping and understanding of the nervous system, might offer insights into both structural and functional

connectivity in the brain. This review has explored connectomics with a focus on the fruit fly, *Drosophila melanogaster*, covering the remarkable journey from earlier connectome reconstructions, with just a few neurons, to recent advances in mapping both the larval and adult fly brains [3, 4]. *D. melanogaster* has been regarded as a model for biology, and the publication of the connectome will make it an invaluable reference point for understanding neural connectivity.

A significant amount of work has been undertaken to progress from reconstructing the connectome of *C. elegans* [5], which consists of just a few hundred neurons, to that of the fruit fly, which encompasses thousands of neurons. However, the fundamental approach has remained unchanged: utilizing EM to capture brain sections at a nanometric scale, ultimately resulting in a 3D reconstruction. While ssTEM systems have been predominantly used for imaging the *Drosophila* brain, particularly in the larval stage [26], FIB-SEM has emerged as another suitable technique for synaptic-level brain imaging [51]. For both imaging methods, numerous enhancements have been implemented throughout the process. These advancements include the use of high-speed camera arrays, the automation of sample exchange during the imaging process, and the parallelization of imaging with multiple machines through specimen partitioning [51, 50].

Advancements in software and computational tools have also played a crucial role in obtaining large connectomes. In the past century, researchers had to manually align the imaged sections and rely on hand-drawn diagrams to trace the skeleton of each neuron and annotate each synapse. Today, the automatic alignment of imaged sections, leading to the creation of a 3D volume and its subsequent visualization, is the standard practice [33, 56, 55].

While manual reconstruction of neurons was sufficient for obtaining the larva connectome, the automatic reconstruction of neurons and the annotation of synapses, often facilitated by machine learning algorithms, became imperative for constructing the adult connectome [4]. These annotation processes are time-consuming, and this is where automatic tools shine, as they make it feasible to map larger brains. It is important to note that the adult brain, with approximately 100,000 neurons, is orders of magnitude larger than the larval brain, which comprises just 3,000 neurons.

We believe that these advancements in imaging processes and automatic reconstruction and annotation will facilitate the acquisition of other connectomes. The same technology could prove invaluable in creating a map of the male adult fly's brain, considering the current one is for the female. Furthermore, it could be extended to study other *Drosophila* species with different behaviours. There might be even potential for application in larger brains, such as those of small vertebrates.

However, we must acknowledge two limitations. Firstly, while chemical synapses and gap junctions were identified in the connectome of *C. elegans*, there is no annotation of gap junctions or electrical synapses for the connectome of *Drosophila*. Gap junctions may indeed play a crucial role in neural circuits, and their absence from this data could lead to important insights being overlooked. It would be intriguing to explore the possibility of obtaining the electric connectome to complement the chemical synaptic connectome. However, it is important to note that annotating gap junctions would be an enormous undertaking, given the high resolution required to visualize them, as discussed in [26, 50].

The second limitation pertains to proofreading. Manual proofreading is essential to correct errors that may arise from the automatic reconstruction of neurons and synapse annotation. Although the time required for proofreading has been significantly reduced, from 50 human-years for the hemibrain dataset to 30 for the entire adult dataset [51, 4], it remains a significant challenge. Nevertheless, we are optimistic that the development of more accurate automatic reconstructors and annotators will expedite this process. Additionally, there are specialized proofreading tools available that prioritize the review of 'unusual' reconstructions (used in [4]), facilitating more efficient error correction.

On the other hand, we have provided an overview of comparative connectomics and the methods used to address it. As mentioned earlier, it is intriguing to explore how differences in brain connections might relate to behavioural variations, such as between

males and females or across different *Drosophila* species.

While research has compared neurons from various datasets, seeking bilateral homologous neurons [3] or making cross-species comparisons [69], these studies primarily rely on matching neurons based on morphological traits like neuron position, trajectory, or synaptic pattern. This approach is motivated by the stereotypy seen in invertebrate nervous systems [2]. However, it is worth mentioning that stereotypy does not always apply universally, and matching neurons this way (by comparing several images or reconstructed neurons) can be highly labor-intensive or incorrect.

To overcome these problems, comparing connectomes based on the connectivity of the neurons is of great interest, as well as annotating or classifying neurons this way. For this, one needs to simplify the connectome to a graph, with neurons as nodes and synapses as edges. In order to compare two graphs, it is crucial to know which nodes of one network are matched with which nodes of a second network. Graph matching or alignment seeks to solve, but it is a really hard mathematical problem.

In this section of the discussion, we have focused on global alignment solutions, particularly algorithms that approach alignment in a manner applicable to solving QAP approximately. Notably, we highlight FAQ [76], which has already been employed to compare the two hemispheres of the larval *Drosophila* connectome. Furthermore, we have explored enhancements to this algorithm using seeds to anchor neuron pairs that have been morphologically confirmed to match [81, 83]. However, the accuracy still needs to be higher and FAQ can not be used for large networks, as in the case of the adult brain of the fly.

In addition to the QAP-based formulation of graph matching, we have sought out alternative algorithms that utilize different methods to achieve optimal alignment between two networks, like simulated annealing [92] or genetic algorithms [87]. Our search has been limited to algorithms employed in connectomics graphs, even though these algorithms have been initially thought for protein-protein interaction networks. To our opinion, these algorithms are currently inadequate for effectively comparing connectomes.

While our primary interest lay in algorithms designed for matching connectomes at the synaptic level, we extended our search to encompass studies involving the human connectome due to the limited literature available in this domain. Human connectomes differ from synaptic-level connectomes as they depict connections between brain regions rather than individual neurons. However, this shift in focus, though unconventional, has shed light on the fact that these two areas of research are not as distinct as they might initially seem. While human connectomes are often compared using brain atlases as a reference, there is a growing need to match them without such predefined templates due to inter-subject variability [79]. Consequently, graph matching techniques are proving to be crucial not only for synaptic-level connectomics but also for higher-level connectomics studies.

Although the task ahead is undeniably impressive, the huge steps that were taken to reconstruct the complete larval connectome instill confidence that upcoming efforts will ultimately succeed in comparing connectomes, whether at the synaptic level or within the realm of human connectomes. These endeavors hold the potential to yield profound insights into the interplay between brain connectivity and its functional implications.

A. Layman summary

Connectomics might sound complex, but it is essentially about creating a map of the brain, much like a geographical map with cities and roads. In this case, cities are neurons, and roads are connections called synapses, allowing neurons to communicate. Mapping the brain in this way is challenging, especially for larger brains. Researchers have spent years working on the connectome of the fruit fly, which, despite its size, is the largest connectome achieved so far.

To visualize neurons and synapses, researchers use electron microscopes with high precision. This technique was initially used to map a tiny worm's hundred neurons in the eighties. Today, it can

handle larger brains like the brain of the fruit fly, thanks to advancements in electron microscopy, enabling larger samples and faster imaging with high-speed cameras and multiple machines.

After obtaining brain slices through microscopy, computers piece them together to create 3D images. While manually tracing neurons and annotating synapses is time-consuming, computational tools now assist with both tasks. This automation has enabled the mapping of the entire adult fruit fly brain, which contains about 100,000 neurons.

These detailed connectomes open doors to comparing brains —for example, male vs. female or larva vs. adult— known as comparative connectomics. It helps us understand how differences in the connectome relate to variations in behaviour. While traditional methods focus on comparing neurons based on their appearance and location, this is not always effective. Instead, we can pair neurons based on their connections to others, which should provide more accurate comparisons and insights into neuron function. However, matching neurons across different brains based on connectivity is a complex mathematical problem known as graph matching. It involves representing the connectome as a network or graph and matching one neuron from the first connectome with another neuron from the second connectome. Although research is ongoing, a definitive solution has not yet been found.

We hope that the technology behind the fruit fly connectome can be applied to map the brains of larger species. Additionally, we see room for improvement in mathematical approaches to better compare connectomes, helping us uncover the relationship between brain connections and behaviour.

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