### Correlative Light and Electron Microscopy in an Intact Larval Zebrafish

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Correlative light and electron microscopy in an intact larval zebrafish

A dissertation presented
by
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to
The Committee on Higher Degrees in Biophysics

in partial fulfillment of the requirements
for the degree of
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Correlative light and electron microscopy in an intact larval zebrafish

Abstract

How does the structure of a living system relate to its function? In neuroscience, an outstanding issue is what can be learned about the brain’s function by knowing how the individual neurons are wired together. We use high resolution serial-section electron microscopy (EM) to map the structure of an entire vertebrate’s nervous system at the synaptic scale. The dataset is collected from an intact larval zebrafish at 4nm x 4nm x 30nm resolution and the imaged volume size is 76 million cubic microns. It contains 170,000 neurons and glia, and offers comprehensive synaptic connectivity information from both the central and peripheral nervous systems. Additionally, we combine complementary information from light and electron microscopy in the same animal. Specifically, we pair electron microscopy with confocal microscopy of the same brain, where the major excitatory and inhibitory neurons are fluorescently labeled. To achieve single-cell precision in the matching of the two datasets, we solve a multi-modal image registration challenge, which handles non-uniform deformations introduced during tissue processing between the two imaging modalities. This correlative electron microscopy data is essential to test computational models for circuit function in larval zebrafish. Most generally, these experiments advance larval zebrafish as a vertebrate model-system in which vastly disparate scales can be bridged – brain-wide synaptic connectivity, brain-wide neuronal activity, and animal behavior.
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TO MOM, WHO HELPED

AND

TO DAD, WHO TOLD JOKES
Acknowledgments

This all started with Florian rollerblading into the first year Biophysics Program seminar room, and showing light-sheet microscopy images of blinking neurons in a zebrafish brain. I was seeing a “thinking” brain for the first time, and I was instantly hooked. As I learned more, I was puzzled - “If brains are connected neurons which compute together, why is it that we do not know more about how brains are wired?” As it turns out figuring out the wiring of a brain is an exceptional challenge that requires bridging spatial scales over several orders of magnitude. Then I got lucky - a few years into my time as a graduate student, Jeff changed the rules of the “wiring” game and it became possible to truly try and get a wiring diagram of a larval zebrafish brain: the central goal of this dissertation.

Florian and Jeff have completely transformed my scientific trajectory. Now we are at the brink of linking brain structure and behavior, and I am deeply grateful to be working on this important and difficult problem with their support and guidance. The sentiment “simplicity is the ultimate sophistication” by Da Vinci rings especially true in science, and I have been continuously inspired by Florian and Jeff’s ability to distill simple insights from this complex problem.

This work would not have been possible without the help and dedication of an excellent team of scientists. Richard Schalek has been there for me from the very first day in the Lichtman lab, through protocol developments, single and multi-beam electron microscope imaging, and most importantly: the 5-day zebrafish cutting marathon, which brought this dissertation into existence. Jonathan Boulanger-Weill procured the transgenic fish lines, and helped with imaging and ground truth tracing. Yuelong Wu developed a fish-specific version of his retake manager software for multi-beam imaging, and also generated the incredibly useful overview 3D...
volume of the fish. Adi Peleg took on the challenge of reconstructing a cohesive 3D full resolution zebrafish volume, and generated 3D image datasets to train and test machine learning algorithms. Kat Boit, Michaela Benedict, and Pallavi Goel, an impressive team of undergraduate students, produced ground truth volumes, which were then used by Michal Januszewski at Google to train machine learning algorithms. Zachary Miller, an undergraduate student as well, did a spectacular job of manually registering light and electron microscopy data. Daniel Berger and Alexander Shapson-Coe helped with navigating the tools for visualizing and processing these large image data - VAST and Neuroglancer.

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Cell and tissue, shell and bone, leaf and flower, are so many portions of matter, and it is in obedience to the laws of physics that their particles have been moved, moulded and conformed... Their problems of form are in first instance mathematical problems, their problems of growth are essentially physical problems, and the morphologist is ipso facto, a student of physical science.

D’Arcy Thompson, On Growth and Form, 1942

1

Introduction

1.1 Biological structure and function

How structure defines function is a question that permeates all of biology. Some of the answers have produced the largest frame shifts in our understanding of nature. Perhaps the greatest success story is Darwin’s insight that structural differences in organisms are proxies for functional diversity, which affect the organism’s ability to survive and reproduce (Fig. 1.1A). He was able to reason out that species are “designed” by means of the natural processes of chance and selection, and thus biology is explainable by the laws of nature, and does not require the acts of a divine creator (see historical review).  

To formulate the principle of natural selection, Darwin recognized that the beaks of Galapagos finches are matched to the available food source on each island. In other words, it was essential that he understood how structure defines function, in order to work out a natural law. Much of modern biology, which is concerned with phenomena beyond the limits of sight, is preoccupied with discovering the means to visualize relevant structures and catalogue their function. Every now and again an organizing principle emerges out of these
Indeed, once it was possible to visualize the structure of macromolecules with X-ray crystallography, Watson and Crick could reason out that DNA is double-helical and that this architecture is the key to a universal biological mechanism for heritable information storage (see Fig. 1.1A):

It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.

Watson and Crick, 1953

This incredible understatement marks the birth of the molecular genetics revolution of the 20th century. It has reshaped how we understand our evolution and ancestry, and how we approach the treatment of disease.

At the turn of the 20th century, Santiago Ramón y Cajal perfected a silver staining technique, originally developed by Camillo Golgi, to sparsely label cells in brain slices and visualize these under the microscope. He reproduced his observations in thousands of meticulous drawings, which are exquisite both in their detail and
their insights into how the structure of the nervous system generates function. By the careful and systematic study of the avian retina and developing brain, Cajal demonstrated that the nervous system is made up of discrete cells. By considering the arrangements of neurons in the brain, he reasoned out that the nerve impulse travels from the dendrites through the cell body and to the axon, famously indicating the direction of travel with arrows (Fig. 1.1C). These findings, presented by Cajal as the neuron doctrine and law of dynamic polarization, form the foundations of modern neuroscience and are nearly as universal as the genetic code.

1.2 Structure and function of neural circuits

In modern terms, Cajal’s diagram from Fig. 1.1C would be called a neural circuit - a collection of neurons which are connected by synapses and together perform a specific function. What an animal perceives of the world and what actions it takes is set by the collection of neurons comprising that animal’s nervous system. There is an astonishing diversity of nervous systems - some have only a hundred neurons, while others are billions of neurons large. Yet, all nervous systems share a common function, which is to produce adaptive behavior that optimizes an animal’s ability to interact with its environment. Finding out the rules by which neural circuits generate adaptive behavior is the core challenge to modern neuroscience.

The nervous system is unique among all multicellular organ systems, because it bridges multiple spatial scales (from microns to meters) and temporal scales (from milliseconds to lifetime) in order to produce actions appropriate to the animal’s circumstances. Neurons are singular among cell types, because their morphology allows them to interact with cells which are not their direct neighbors and are sometimes vast distances away. Combined with electrical signaling, this design achieves information transmission over several spatial scales in tens to hundreds of milliseconds, which is essential for coordinating the animal’s actions with sensory input from its environment. Neurons persist over a lifetime, and their function is shaped by both the animal’s developmental and experience history. Specifically, while the physical connections between neurons constrain the possible neural activity patterns, the activity itself reshapes the structure of the network via morphological changes of the synaptic contacts. This ability of the nervous system to re-wire its connections makes it possible for an animal to learn and adapt to its environment.

*Neurons are highly specialized and do not divide and make copies of themselves. The turnover rate of neurons in the human brain is practically zero, with the exceptions of two brain regions where stem cells produce neurons at a rate of 1% per year. This is tenfold slower than the rate of skeletal cell turnover.*
The workings of any other organ system are explainable in terms of the functions of the relatively few cell types comprising the system. On the other hand, it is impossible to even describe the function of a neuron without referring to its wiring partners within a neural circuit. Such context-dependence results in an explosive proliferation of neuronal functions. While this feature might be well-suited for generating adaptive behavior, it challenges the notion that a simple description from neurons to behavior is possible.

1.3 From behavior to neurons in three levels

The problem of linking brain and behavior is a problem of scales, which require different levels of abstraction. A central framework in neuroscience for bridging the scales is Marr’s tri-level hypothesis, which views brains as information-processing machines. According to Marr, it is essential to first identify the computational problem that the animal faces - what is being computed and why? The solution can then be formulated in algorithmic terms - how are the inputs and outputs represented and what are the transformations between them. With this prior, we can then make informed guesses about neural computational units that physically realize the proposed algorithms. The computational problem, its algorithmic representation, and its physical realization are “the three levels at which any machine carrying out an information-processing task must be understood”.

The best example of a behavior which has been understood at all three levels is an electro-sensory behavior in weakly electric fish. These fish map their surroundings by discharging a weak, nearly sinusoidal (in time) electric field, and sense disturbances in this self-generated field that are caused by nearby objects. However, if two fish meet and discharge at similar frequencies, this is detrimental to their electrolocation ability - they jam each other’s signal. To avoid this, fish have developed a jamming avoidance response: they change their own signal frequency such as to increase the difference between the two frequencies, see Fig. 1.2A. The computational problem for the fish is to determine if its signal is being jammed, and if so, whether it should increase or decrease its signal frequency. By virtue of anatomy the fish can measure, or represent, the amplitude and phase (time points of zero crossing) of the summed signal and its own electrical signal, but it is blind to the jamming signal. Given these constraints, the algorithmic solution to the problem is to distinguish among the four possible states shown in Fig. 1.2B, which determine if the self-generated signal is higher or lower in frequency than the jamming signal. The physical implementation of this algorithm has been traced from the sensory input to the motor output, and the neural motifs which perform the individual computations are shown in Fig. 1.2C.
Figure 1.2: Jamming avoidance response examined at Marr’s three levels of analysis. A. Computational problem. If two weakly electric fish meet while discharging electric fields at similar frequencies, they change their discharge frequency such that the animal with the lower frequency lowers it further, while the animal with the higher one increases it. Otherwise, if the signals remain close (within 4 – 6 Hz), the summed signal results in a beat pattern like that of a screeching microphone, which is detrimental to the fish’s electrolocation ability. All vertical axes show the electric field, and the horizontal axes - time. B. Algorithmic representation. Given the biological constraints of the fish’s sensors and their placement on its body, the animal can measure the phase (zero crossings) and amplitude of its own signal and of the summed signal. Two variables - the change of the summed signal amplitude over time, and the time delay of zero-crossings of the summed and own signals - define four possible inputs of the circuit which determine if the fish’s own frequency is larger or smaller than that of the jamming signal. C. Physical implementation. Two types of electoreceptors convey phase and amplitude information to cells in the electrosensory lateral line lobe (ELL). These project to the torus semicircularis (TS), where neural substrates for the four input configurations are computed. Next, these are combined in the nucleus electrosensorius (nE) to determine if the discharge frequency should be increased or decreased. The nE drives prepacemaker nucleus (PPn), and subsequently the pacemaker nucleus (Pn) and the electric organ, to change the discharge frequency.
Once behaviors are explained at all three Marr’s levels, it becomes straightforward to make comparisons across animal species. For example, nocturnal birds and mammals face the same computational problem, which is to localize the sound source from the difference in sound arrival times between the two ears. However, they have implemented different neural architectures: birds use a spatial map, where individual neurons report sound location⁴¹, while mammals encode location through the summed activity of a whole neuronal population⁵¹. These differences make sense when considering that the eardrum has evolved separately in mammals and birds¹⁶, and show that evolution can arrive at multiple solutions to the same computational problem. Understanding how the brain works then reduces to cataloguing computational problems and their solutions across organisms in the hope of finding shared principles.

1.4 What are maps of the brain good for?

Practically every Marr’s level three model for the neural implementation of behavior evokes neuronal wiring. Computation in the nervous system is carried out via physical connections among neurons; yet, nearly all existing models have been conceived from measurements of neuronal activity, without visualizing neuronal connectivity. Producing a map of neuronal connections - a *connectome* - in an animal’s nervous system is difficult, because it is a problem of bridging vastly different imaging scales. Specifically, we need to visualize nervous systems across five or six orders of spatial magnitude - from the nanometer resolution required to identify synapses, to millimeter or centimeter-scale imaging necessary to reconstruct whole circuits in model animal systems⁵².

At present, electron microscopy is the only technique which is up to this challenge. It was used to obtain the first complete map of connections in a whole nervous system - the 302 neurons of the worm, an effort which started in the 1970s and took 15 years to complete⁹³. Today, advances in imaging and computing have increased the scale of connectome sizes by two orders of magnitude: all the connections in the brain of an adult fly (100,000 neurons) are currently being mapped in two different animals¹⁰²,⁹⁵. These mapping studies share a common goal to understand how much the wiring of a brain can tell us about how it works. The answers are yet unclear, with opinions greatly outnumbering results, and expressions of hope and skepticism appearing in equal measure⁶².

One point of concern about connectomics is that the overwhelming amount of data and detail in a connectome might preclude insight. It is a feat of mapmaking, which brings to mind Borges’ one-paragraph story *On
**Exactitude in Science.** The story tells of an empire where the art of cartography becomes so precise, that only a map of the same scale as the empire is deemed satisfactory. This map proves to be so useless, that the next generations offer it up to the elements, and forego maps entirely - “in all the Land there is no other Relic of the Disciplines of Geography”

Consider AlphaZero, which is an artificial neural network that taught itself the intricacies of three games: chess, Go and shogi, and outperformed state-of-the-art programs which have specialized in these games. AlphaZero broke new ground by adapting the same artificial neural network architecture to the strategies of three different games, much like a nervous system adapts to different environments. To describe its unprecedented performance, the community found itself squarely in the realm of anthropomorphic language, discussing a computer’s creativity. But even though we have access to all details of AlphaZero’s architecture, it is completely opaque to interpretations. In the words of Steven Strogatz: “AlphaZero gives every appearance of having discovered some important principles about chess, but it can’t share that understanding with us. Not yet, at least. As human beings, we want more than answers. We want insight.”

On the other extreme, there is a worry that the connectome is incomplete, missing important features such as extrasynaptic neuromodulation or neurotransmitter identity, and thus it is likely to fail to capture the dynamics of a living brain. Evidence for this deficit comes from research on the crustacean stomatogastric nervous system, which contains a pair of neural circuit that produce rhythmic behavior in response to food. In this system, the same unchanging set of connections can switch circuit dynamics in the presence of neuromodulators. Specifically, modulators can change the pattern of rhythmic behavior produced by a single circuit, but also they can switch elements from one circuit to the other by changing their activity patterns.

What both sides of the connectomics debate agree upon, is that checking our intuition against the reality of a brain’s wiring diagram is invaluable for testing neural circuit models and for generating new hypotheses. In this role, connectomics is making a difference across model systems: in the Drosophila fly and larva - for models of memory, vision, feeding, olfaction, action selection, muscle coordination, developmental rewiring; in the nematode C. elegans - for touch response, decision making, sex-dependent rewiring, comparative evolution; in mouse - for visual processing, muscle coordination; in zebrafish Danio rerio - for odor representation, oculomotor integration, muscle coordination; singing in zebra finch; visual navigation in annelid Platynereis.
1.5  Connectomics in an intact larval zebrafish: testing level three

In this dissertation, we take a connectomics approach to examine how behavior is implemented by neural circuits in a small vertebrate - the larval zebrafish. The larval zebrafish is an excellent model system for linking brain and behavior at all three Marr’s levels. The animals develop rapidly outside the mother and by 7 days post-fertilization (dpf) they have a stable and stereotyped set of behaviors, which have been well documented. These behaviors are amenable to quantitative analysis at the first two Marr’s levels: identifying the computational problem and how it is implemented as an algorithm. The animals are translucent, and we can optically measure neural activity while the animal is behaving; notably, larval zebrafish is the only vertebrate model system in which it is possible to record whole-brain neural activity with cellular resolution. Combined, these features make larval zebrafish research particularly useful for producing neural circuit models of behavior.

The larval zebrafish is small enough to attempt a connectomic analysis, by which we can validate or falsify the growing body of zebrafish neural circuit models. Here, we use serial section electron microscopy (SSEM) to obtain the first connectivity-level map of the nervous system in an intact larval zebrafish - from sensory inputs to motor outputs. To obtain this map, we build on pilot electron microscopy experiments in whole larval zebrafish, and we exploit significant advances in speeding up electron microscopy imaging and subsequent image analysis. In principle, we can test any circuit model in zebrafish, because the imaged volume contains most of the central nervous system together with its sensory inputs and motor outputs. Importantly, in the same animal, we complement this structural data by genetically labeling the major excitatory and inhibitory neurons, and visualizing these with confocal light microscopy (LM). This dissertation focuses on the methods to produce and combine these datasets into a tool for testing neural circuits models in larval zebrafish (Chapters 2, 3, and 4).

†The animal is physically restrained in agarose during these recordings, and is not swimming freely. Instead, the fish experiences a virtual environment - it is presented with stimuli, which are designed to mimic its sensory experience during natural conditions.
Linking neural activity with behavior in larval zebrafish is limited to those behaviors, which can be reproduced while the animal is restrained under a microscope (fictive preparation). When such experimental paradigms cannot be constructed, connectomics may well be the only means by which we gain insight into the neuronal organization of these behaviors.

We consider one such elusive case: rheotaxis - a behavior by which fish orient against an oncoming water current. When the water flow speed is high enough to drag the fish downstream, the fish see the surroundings moving away, and they use this visual cue to orient themselves. Remarkably, fish can perform rheotaxis even in the absence of visual cues, such as when swimming in darkness. To achieve this, they rely on their mechanosensory lateral line which consists of discrete water flow sensory organs called neuromasts (see Fig. 1.3A). Each neuromast holds a collection of hair cells that report two opposing directions of water flow. How these neuromasts are distributed over the fish’s body determines what features of the surrounding water flow the animal can use to orient itself.

Rheotaxis has been quantitatively explored at the first two Marr levels of analysis using the zebrafish as a model system. Larval zebrafish were placed individually in a tube with controlled laminar flow and the lights were turned off - the animals could only use their mechanosensory lateral line to orient themselves. Therefore the computational problem that each fish had to solve was to use local water flow measurements to determine the direction of the stream. By recording the fish’s choices and positions in the tube, Oteiza et al arrived at a simple algorithm: larval zebrafish measure the rotation of the water flow around their bodies and then compare subsequent measurements to make a decision about the direction in which to turn. If the value of the rotation increases, fish make a large angle turn in the direction of the rotation, otherwise they swim straight.

The proposed algorithm makes a strong prediction about the circuitry - there is a neural network for measuring the local water flow rotation. Mathematically the local rotation of a flow field is quantified by the curl, which in turn is related via Stokes’ theorem to the integral of the flow along a contour around that location. Perhaps not coincidentally, the flow sensors of the mechanosensory lateral line are placed in a contour around the animal’s body. To determine if there is rotation and the direction of such rotation, the fish distinguishes among four possible configurations shown in Fig. 1.3B. It follows that the neural network, which we will call the curl detector, sums neural activity from water flow sensors of opposing polarities across the body of the fish. We
Figure 1.3: Zebrafish rheotaxis at the third Marr level. A. Mechanosensory organs called neuromasts are distributed in a line along the circumference of a larval zebrafish. Shown is the organization of the posterior lateral line system which is necessary for rheotaxis\(^\text{19}\). Black dots along the length of the animal mark the locations of neuromasts. Each neuromast contains hair cells which report two opposing directions of water flow: head-to-tail (black) and tail-to-head (red) B. Proposed circuit implementation for curl detection. Two neuronal populations report clockwise and anticlockwise curl. A salient feature of this model is that information from hair cells of opposing polarities is combined across the body of the fish.
propose that this is achieved by two neural populations which report clockwise and counterclockwise direction of rotation, and show a possible circuit implementation in Fig.1.3B.

To look for neural activity correlates of the curl detector, we need to reproduce the effect of a rotating water flow field around the fish while the animal is embedded under the microscope. This translates to stimulating the neuromasts along the fish’s tail such that they bend in opposite directions across the animal’s body (as in Fig.1.3B). It is possible to achieve the effect by either precisely controlled water jet streams in opposite directions, or direct mechanical stimulation of neuromasts on both sides of the fish. Both of these approaches are difficult and yet unsolved engineering problems. It is also possible to exploit gene expression differences between hair cells of opposing polarities to optogenetically stimulate hair cells of a specific polarity, but these tools are yet to be developed and tested.

When neuromasts are visualized with electron microscopy, the function of all cells comprising these sensory organs is apparent from their structure. In particular, how the tufts of hair cell stereocilia are oriented determines the hair cell polarities. In turn, sensory neurons contact hair cells of only one polarity and it is straightforward to keep track of their directional sensitivity all the way to the brain. Thus, by following the neural wiring diagram from neuromasts to the brain, we will be able to test the hypothesis that sensory information from opposing polarities is integrated across the animal’s body.
Whole-animal serial-section electron microscopy in a larval zebrafish

We use high resolution serial-section electron microscopy to map the structure of an intact larval zebrafish at the synaptic scale. We adapt a standard approach of aldehyde specimen fixation followed by heavy metal contrast staining for electron microscopy, which has been used previously to obtain images of a whole zebrafish brain\textsuperscript{17}. Here, we modify the aldehyde fixation protocol to preserve extracellular space in the brain, and we increase the heavy metal staining in order to facilitate imaging and image segmentation. This protocol takes 10 days to prepare a resin embedded fish, which is ready to be cut in sections and imaged with electron microscopy. We combine this protocol with a semi-automated pipeline for serial section electron microscopy to map an intact 7 days old larval zebrafish.
2.1 Dissection and aldehyde fixation

At 7 days post-fertilization (dpf) a larval zebrafish is immobilized in 1.8% LMP agarose in water, and a slab of agarose over the animal’s head is removed with a scalpel to expose the dissection site. The water is replaced with aCSF (Table A.2) and 0.02% (w/v) tricaine for the dissection. A sharpened tungsten needle is used to carefully break the skin over the hindbrain ventricle, with minimal damage to the brain’s surface. The tail is cut just before the caudal fin; if the tail is not cut, access to the notochord is limited, which causes problems with resin infiltration and serial sectioning (see Fig. 2.1 A). To help with downstream handling, the animal is left encased in agarose, with the dissection sites kept free of agarose to allow fixative diffusion. The dissection is completed in less than a minute and the animal is immediately transferred into 10ml of cold fixative solution (6% mannitol, 2% Glutaraldehyde) in a glass scintillation vial, which is kept on ice.

To accelerate the aldehyde fixation, the fish and fixative are transferred from the glass scintillation vial into a small plastic petri dish, and placed in a microwave equipped with a power controller and steady-temperature water recirculator and cold spot. A previously optimized microwave program is executed: Power setting at 100W for 1min ON, 1min OFF (3 repeats), power setting at 300W for 30s ON, 30s OFF (9 repeats). Following the microwave step, the fish is transferred to a cold scintillation vial with fresh fixative, and incubated at 4°C on a nutator for 15 minutes. The microwave protocol and incubation are repeated twice with 10ml 6% mannitol, 2% Glutaraldehyde solution, and then twice with 10ml 6% mannitol, 2% Glutaraldehyde, 0.1M Sodium Cacodylate buffer, pH 7.4 with 2mM CaCl₂. The fish is kept overnight in fresh 6% mannitol, 2% Glutaraldehyde, 0.1M Sodium Cacodylate buffer, pH 7.4 with 2mM CaCl₂ on a nutator at 4°C. For the optimization procedure we used to arrive at this fixative formulation see Appendix A.1.

2.2 Heavy metal staining

On the next day, the fish is washed with buffer - 0.15M Sodium Cacodylate buffer, pH 7.4 with 3mM CaCl₂ - 3 exchanges, 30min each, at room temperature (RT). At this point we also begin processing support tissue for resin embedding alongside the fish sample. For support tissue we use a rat brain which has been kept in a glutaraldehyde fixative. The brain is cut up into ≈ 1.5mm × 1.5mm × 3mm blocks, and a 0.75μm wide hole is punched in each block to house the fish during resin embedding and, later on, section cutting.
We begin the staining process with a chemical reduction, which has been shown to increase in the reactivity of membranes to osmium, thereby enhancing membrane-cytosol contrast. The fish is fixed with 0.8% (w/v) Sodium Hydrosulfite in 60% (v/v) 0.1M Sodium Bicarbonate 40% (v/v) 0.1M Sodium Carbonate buffer with 3mM CaCl₂ (20min, RT). The sodium carbonate and bicarbonate are made separately, filtered and mixed dropwise together in a vial containing the sodium hydrosulfite powder just prior to use. Following a buffer wash (3 exchanges, 10 min each, RT), the agarose encasing the fish is removed carefully with fine tweezers before osmication. The tissue is brittle after osmium impregnation, and it becomes nearly impossible to remove the agarose without damaging the specimen.

To achieve homogeneous and high-contrast staining throughout the whole animal, we use a modified ROTO (reduced osmium OTO) staining protocol. The classical OTO protocol involves two osmium reactions with an intermediate thiocarbohydrazide enhancement step, and achieves homogeneous, but low-contrast tissue staining. On the other hand, a reduced osmium OTO protocol achieves high-contrast staining but inhomogeneous penetration, resulting in a staining gradient across the tissue. Hua et al. obtain high-contrast uniform staining in large tissue blocks with an osmium reaction - to achieve uniform osmium penetration - followed up by a reduction step, which enhances osmium-membrane association, and thereby contrast.

In addition to separating the osmication and reduction steps, we apply a strategy of long incubation times which yields consistent and homogeneous staining in every trial. Specifically, we stain both fish and support tissue with 2% OsO₄ buffered with 0.15M Sodium Cacodylate, pH 7.4 with 3mM CaCl₂ for 4 hours at room temperature, and are then moved to a fridge for overnight incubation on a nutator. On the next day, immediately prior to reduction both fish and support tissue are quickly rinsed with buffer. The reduction step is carried out with 2.5% (w/v) Potassium Ferrocyanide in 0.15M Sodium Cacodylate, pH 7.4 with 3mM CaCl₂ (4hrs, RT). The sample is moved to a fridge for overnight incubation on a nutator.

On the next day, the fish and support tissue are washed in ddH₂O for 30 minutes at room temperature (3 exchanges). During this time, 1% (w/v) Thiocarbohydrazide in ddH₂O (TCH) is completely dissolved in 60°C oven for ≈ 1hr 30min with occasional vortexing. The solution is then filtered with a 0.22μm filter and the samples are incubated for 60 min at room temperature. This is followed up with a long ddH₂O wash (3 exchanges, 30 min each, RT). Next, the second osmication step is carried out with 2% OsO₄ in ddH₂O (4hrs, RT) and followed up with a long ddH₂O wash (3 exchanges, 30 min each, RT).

Finally, the sample is stained with uranyl acetate, a membrane contrasting reagent, which has also been impli-
Figure 2.1: Whole-animal sample preparation for serial electron microscopy. A. Dissection for whole animal fixation. Larval zebrafish rendering by K. Herrera. B. By the end of the heavy metal staining, the tissue is completely black. C. The trimmed resin block with zebrafish sample is ready for serial sectioning. The zebrafish is mounted inside a rat brain block of support tissue (black) and lies in a horizontal orientation with respect to cutting. The length of the coffin-like resin block is 4.2 mm, and the width is 1.6 mm, the obtuse and sharp angles measure $\approx 110^\circ$ and $\approx 60^\circ$ respectively. D. Automated Tape-collecting Ultra microtome (ATUM) cuts the fish into 30nm slices using a diamond knife (blue). The slices are directly picked up by a tape (yellow) moving on a reel. The reel is moving at a speed of 0.3 mm/s, and, on average, 3 sections are collected every minute.

cated in labeling postsynaptic density proteins\(^9\). We completely dissolve 1\% (w/v) Uranyl Acetate in ddH\(_2\)O by sonicating the solution for $\approx$ 1hr 30min. The solution is then filtered with 0.22μm filter and the fish and support tissue incubated overnight at room temperature in glass vials wrapped with aluminum foil. By the end of the heavy metal staining procedure, the fish and support tissue become black and non-transparent (see Fig.2.1B).

2.3 Resin infiltration and embedding

The fish and support tissue are dehydrated and infiltrated with resin, which is then cured to produce a hard block, suited for serial sectioning. Specifically, the sample is washed with ddH\(_2\)O (3 exchanges, 30 min each, RT), and then dehydrated first with serial Ethanol dilutions - 25\%, 50\%, 75\%, 90\%, 100\% (10min each step at RT), followed by 100\% Propylene oxide (PPO, 2 exchanges, 30 min each, RT). Infiltration begins with a series dilution of LX-112 resin (Table A.3) and PPO: 25\% resin: 75\% PPO (> 6hr, < 12hr, RT), 50\% resin: 50\% PPO (> 6hr, < 12hr, RT), 75\% resin: 25\% PPO (> 6hr, < 12hr, RT), 100\% resin (overnight, RT).
Notably, the caps of the vials holding the fish and support tissue are taken off during this step to allow for the evaporation of PPO. Otherwise, there is a risk of leftover solvent which results in a soft resin.

Finally, after a fresh 100% resin exchange (> 6hr, < 12hr, RT) the sample is embedded in 100% LX-112 resin. The sample is assembled directly in a standard coffin mold by carefully guiding the fish inside the support tissue with an eyelash tool. The block is cured in a 60°C oven for 72hrs. The cured sample block is trimmed to a coffin-like shape (Fig. 2.1C) using a Diatome Trim 90 diamond knife mounted on a Leica EM Trim system.

2.4 Serial sectioning, wafer preparation and imaging

The Lichtman lab has developed a mostly automated pipeline for the acquisition of large EM-data volumes. Briefly, the pipeline begins with an Automated Tape-collecting Ultra-Microtome (ATUM), which cuts the resin-embedded sample into thin sections, down to 30nm (see Fig. 2.1D). Specifically, the sections are cut with a diamond knife and continuously collected onto a tape reel. Manual intervention is periodically required to move the knife to a fresh cutting site after cutting between 50 and 100 microns, or to replace the knife altogether after two or three moves. Once cutting is complete, the tape is manually cut into strips and mounted onto square silicon wafers for electron microscopy imaging. An optical image of each wafer is taken with a reflected light microscope (Axio Imager, Zeiss) to create a map of section locations for automated electron microscopy imaging. Each section is then imaged with a 61-beam MultiSEM, Zeiss microscope and its quality is automatically examined in real time with a custom-made software tool.

The larval zebrafish sample was cut from the dorsal to ventral direction into into 30 nm sections. A total of ≈ 17, 500 sections were cut using 5 Diatome diamond knives and collected onto ≈ 110 meters of tape moving at a speed of 0.3 mm/s over ≈ 5 days. The sections containing the whole brain (≈ 12, 400) were mounted on 89 wafers. To increase contrast, the sections on the wafers were post-stained with uranyl acetate and lead citrate. A total of 47 wafers containing ≈ 6330 sections were imaged at 4nm/pixel resolution with a dwell time of 200ns per pixel. For summaries see Fig. 2.2 and Fig. 2.3.
Figure 2.2: Serial sectioning and electron microscopy imaging. The larval zebrafish is cut in \( \approx 17 \), 500 sections in the horizontal orientation. Of these, \( \approx 12 \), 400 sections contain brain regions and are mounted onto 89 square wafers. We imaged the central 47 wafers, which contain \( \approx 80\% \) of the zebrafish brain. Surface rendering of the imaged zebrafish volume is shown in the top left. The tissue size per section varies continuously in the ventral to dorsal direction (wafer \#1 to \#89). The imaging data (Tb) and time (hrs) per wafer are plotted. Acquiring this amount of data requires 950 hours of imaging time and 350Tb of storage. The wafers containing the ventral-most (wafer \#1 to \#18) and dorsal-most regions (wafer \#66 to \#89) are estimated to require 90 hours and 800 hours of imaging time, respectively. While both sets of wafers contain \( \approx 10\% \) of the brain each, the ventral-most wafers contain only brain tissue, while the dorsal-most regions contain mostly tissue from the body of the fish (see also Fig. 2.3).
**Figure 2.3**: Physical dimensions of the zebrafish EM-volume. For every 16-th section (≈ 0.480 um z-step), we measure the area occupied by the fish (black points) and the area occupied by the brain (red points). Shown are three sections which mark the start, middle and end of the imaged volume. The total imaged fish volume is 76 million cubic microns, out of which 29 million cubic microns correspond to the brain.
Whole-brain correlative light and electron microscopy

We combine complementary structural and functional information from light and electron microscopy in the same animal. Specifically, we pair whole-brain electron microscopy with confocal microscopy of the same brain, where the major excitatory and inhibitory neurons are fluorescently labeled. In mammalian brains, but not in insect brains, it is possible to differentiate inhibitory and excitatory neurons from structural differences in electron microscopy data. The larval zebrafish is a vertebrate, and we suspect, but do not yet know, if any morphological distinction exists. By assigning neurotransmitter identity from the light microscopy images to the cells in the electron microscopy data, we can test for structural differences. The challenge is to solve a multimodal image registration task, which handles non-uniform deformations introduced during tissue processing between the two imaging modalities.
3.1 Confocal microscopy and segmentation at cellular resolution

We use transgenic larval zebrafish in order to distinguish the major excitatory and inhibitory neurons, and to label the blood vessels, which serve as control for the registration of light and electron microscopy datasets. In particular, we cross two transgenic larval zebrafish lines: Tg(flk1:mCherryCAAX; vglut2a:loxP-DsRed-loxP-GFP), which labels vasculature and excitatory neurons, and Tg(gad1b:GFP), which labels inhibitory neurons. The differentially labeled lines are in Nacre (transparent) background. The individual excitatory and inhibitory lines, Tg(vglut2a:loxP-DsRed-loxP-GFP) and Tg((gad1b:GFP), were previously generated by BAC transgenesis. The vasculature line, Tg(flk1:mCherryCAAX), was previously generated using classical transgenesis by cloning of the endothelial-specific flk1 promoter.

At 7dpf a larval zebrafish is immobilized in 1.8% agarose in a glass bottom dish and imaged with a ZEISS LSM 880 confocal microscope (HCBI). The confocal data consists of three 16-bit channels: (1) vasculature and excitatory neurons (flk1:mCherryCAAX; vglut2a:DsRed) excited with a DPSS 561-nm laser, (2) inhibitory neurons (Gad1b:GFP) excited with an 488-nm argon line, (3) transmitted light. The imaged volume encompasses the whole brain and part of the spinal cord; it is comprised of two overlapping boxes each with dimensions 607 μm × 607 μm × 317 μm, and voxel size of 0.506 μm × 0.506 μm × 0.652 μm.

Excitatory and inhibitory neurons are segmented from the image stacks in a series of processing steps. Firstly, pixels corresponding to cells are classified, in 3D, with a machine learning segmentation toolkit (Ilastik). This classification is carried out separately for each image channel. The classification output - a binary mask over the cells - is manually corrected using a Volume Annotation and Segmentation Tool (VAST). The corrected binary masks are segmented into individual labeled objects using distance and watershed transforms in Matlab. For each object, we record the centroid location and then manually verify that it corresponds to a cell body (see Fig 3.1 A-C). Notably, because both the excitatory and inhibitory reporters are expressed in the cytoplasm, separating individual cells is sometimes ambiguous. We discard these ambiguous cases, trading completeness for a high-fidelity segmentation of 35,749 cells (see Fig 3.1 D-E).
3.2 Electron microscopy and segmentation at cellular resolution

An overview image for each zebrafish section is generated by down-sampling the full resolution data to 512nm × 512nm pixel resolution. The overview images for all 6, 300 imaged EM fish sections are automatically aligned with elas-
Figure 3.2: Cell segmentation from electron microscopy images. A. Snippet from a down-sampled EM overview image. Cell nuclei are flat light gray objects surrounded by darkly stained membrane. Scale bar 10μm. B. Binary nuclear mask (red) overlaid onto the EM image. C. Volumetric nuclear segmentation. D. Centroids of all 168, 219 segmented individual cells. E. Nuclear volume distribution of the segmented cells.

tic transformations by a custom algorithm written in Matlab. Specifically, the algorithm uses a sparse set of key sections and aligns them with a heavily regularized method. Subsequently, the rest of the sections are morphed to the key set. This procedure prevents a potential slow drift along the z direction, which would occur if the images were simply aligned sequentially. Finally, the overview images are down-sampled in z to generate a nearly isotropic volume (0.512μm × 0.512μm × 0.480μm). The anterior part containing the head and initial spinal cord segments are cropped out of the EM overview volume and used for registration with the confocal volume.
To segment cells from the EM overviews, we first perform a simple binarization by filtering (in Matlab) the images with lines of various orientations to pick out circular objects such as cell nuclei. In the next step, the simple binary mask is manually corrected using a Volume Annotation and Segmentation Tool (VAST\textsuperscript{8}). The high image contrast and the well-defined object boundaries significantly facilitate the manual mask correction. The corrected binary masks are segmented into individual labeled objects using distance and watershed transforms in Matlab. Objects much smaller than a cell volume are discarded, and the remaining objects are sorted by volume and split into 4 separate groups, which are inspected separately in VAST (the software handles up to $2^{16}$ objects at a time). Nuclei from the three groups with largest objects are nearly perfectly segmented, and manual intervention consisted only in painting in missing pixels for some nuclear masks. The group with the smallest objects contained all falsely split nuclei ($\approx 5,000$), which were easily fused using the Collect tool in VAST. In sum, we are able to achieve a reliable volumetric nuclear segmentation throughout the whole brain and extract 168,219 individual cells (see Fig. 3.2).

3.3 Registration between confocal and electron microscopy images

There are three major challenges to the registration between confocal and electron microscopy. Firstly, the images are of two very different modalities - cell objects, for instance, are intensity-based in the confocal microscopy volume and boundary-based in the electron microscopy volume. Secondly, the cells represented in the light microscopy data ($\approx 36,000$) are only a fraction of all cells in the electron microscopy data ($\approx 170,000$). Lastly, the chemical processes for electron microscopy, resin embedding and subsequent cutting of the zebrafish into sections introduce nonuniform stretching or squeezing (i.e. elastic) deformations between the two datasets.

To register the confocal and electron microscopy volumes we use BigWarp\textsuperscript{10} - a tool for landmark-based image registration. BigWarp allows for interactive, on-the-fly registration and manual identification of point-correspondences (landmarks) between two image sets via the BigDataViewer\textsuperscript{68} tool in Fiji\textsuperscript{71}. The tool generates a non-rigid transform that matches the point correspondences exactly and interpolates the rest of the image with thin plate splines (TPS). The resulting deformation is smooth and invertible; that is, points are not allowed to pass through each other. We use the interactive feature of BigWarp to generate an initial matching between the confocal and electron microscopy images\textsuperscript{*} with slightly fewer than 1,000 manually identified landmarks.

\textsuperscript{*}Prior to BigWarp matching, the confocal volume is downsampled to isotropic resolution $0.650\mu m \times 0.650\mu m \times 0.650\mu m$, and the images are flipped in the horizontal and z-directions in order to match the orientation of the EM-volume.
This number of landmarks begins to strain the practical use of the interactive BigWarp, as the time needed to calculate the deformation increases with the number of points used. With this list of landmarks, we achieve a close global matching, and single-cell matching in some sparse regions. However, we fail short of single-cell precision particularly in the densely packed cell regions, due to an overall lack of distinguishing features.

To fine-tune the registration, we match the segmented light microscopy (LM) and electron microscopy (EM) cell point clouds. We develop a Matlab algorithm which uses an Iterative Closest Point (ICP) procedure to match groups of cells in the light microscopy volume to cells from their neighborhood in the electron microscopy data. ICP finds the closest rigid transformation (translation and rotation) between two point clouds, and is a good approximation when small local volumes are considered. Specifically, we consider all groups of 3 – 8 closest LM cells and used ICP to find matches for their configurations among the 20 neighboring EM cells. This procedure generates a large list of putative matches between LM and EM cells, which is then filtered: only matches with an average root mean square error of $1 - 1.8$ pixels, rotation smaller than $0.1$ radians, translation less than $10 - 20$ pixels, and matches which occur at least twice were considered good. These threshold
Figure 3.4: Correlative light and electron microscopy with single cell precision. A. Maximum intensity projections of confocal image stacks with fluorescently labeled major excitatory neurons and vasculature (green, left) and major inhibitory neurons (red, middle) in a larval zebrafish. Maximum intensity projection of the zebrafish outline is shown in gray for both panels. This light microscopy (LM) data is registered to electron microscopy (EM) images of the same animal. Overlay between the LM and EM data (EM in gray) is shown for a single plane within the EM volume (right). B. Detailed view of the matching between imaging modalities. The location of this region is marked by a dashed rectangle in A. The outlines of cells and blood vessels from EM appear white in the overlay image. The cell bodies of excitatory neurons and the outlines of the blood vessels from LM appear in green. The cell bodies of inhibitory neurons from LM appear in red. Both cells and blood vessels are precisely matched between LM and EM.
values are determined empirically by testing for threshold combinations which do not produce ambiguities. Next, the matches are added to a list of landmarks, which is used to generate a new TPS transform of the point clouds, and the matching procedure is repeated. This iterative procedure is carried out automatically until no new matches are found, at which point we can manually add new landmarks in (the few) poorly matched regions and repeat the procedure. Finally, we generate a list of 8,473 landmark points spaced at least 12 points (6μm) apart from each other (see Fig. 3.3), which achieve single-cell matching between LM and EM across nearly the entire zebrafish brain (see Fig. 3.4).

3.4 Assignment of functional identity to cells in electron microscopy

Once the light and electron microscopy volumes are registered to each other we can assign excitatory and inhibitory identity to cells in the electron microscopy images. The accuracy with which we assign identities depends on the registration quality between the datasets.

The natural choice for a quality metric for our matching procedure is the distance from landmark points. Specifically, we consider a cell to be matched well between the light and electron volumes when the distance from its centroid to the centroid of its closest landmark is below a certain threshold: 25 pixels, which is equivalent to 12.5μm, or ≈2 cells away. This is approximately the size of the cell neighborhoods we use for fine-tuning the registration.

How accurately we assign functional identities to the cells in electron microscopy also depends on how well we can extract cell objects from the light microscopy data. As discussed in Section 3.1, the automatic segmentation of light microscopy images undercounts the genetically labeled cells. On the other hand, the segmentation of electron microscopy cells is significantly more complete, precise and volumetric (Section 3.2). We consider three strategies for assigning functional neurotransmitter identities using these features of the data:

1. Centroid distance: use the point cloud segmentation results to assign excitatory or inhibitory identity to the electron microscopy cell, whose centroid is closest to the labeled cell in light microscopy. The first drawback to this strategy is that we know we are undercounting the cells. The second drawback is that

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†For example, a large rotation threshold could lead to two groups of LM cells matching onto the same EM neighborhood. In such cases, when the matching is not one-to-one, the algorithm does not produce a TPS transform. Importantly, the thresholds are kept low, because the initial manual matching with BigWarp takes care of large rotations or nonuniform deformations, and the cells are within a diameter or two of their matches.
assignment can be ambiguous, because the errors from registration and centroid segmentation add up and affect the relative positioning of cells. This is especially concerning in tightly packed areas.

2. Average cell intensity: use the volumetric segmentation of electron microscopy cells to obtain the average intensity per cell from each image channel. We find that in both channels the distributions of average intensities are exponential, and setting a threshold intensity cuts off dim cells, especially in the dorsal regions of the fish.

3. Image overlays: for each electron microscopy cell, record an image snippet of the overlay between electron microscopy and excitatory, and inhibitory channel respectively (see Fig. 3.5A). Manually inspect the snippets and make one of three calls (excitatory, inhibitory, unassigned).

We choose to use the third strategy, because we can leverage the physical shape of the cells in making the calls. Therefore, we also remove any misaligned cells and improve accuracy. The drawback to this method is that it is slower. To speed things up, we use a custom Matlab script to automatically extract and present the snippets to a human annotator, who only has to key in ‘e’, ‘i’, or ‘n’ as an input to the script. Additionally, we leverage the average intensity of the cells (strategy #2), such that the script can make an initial guess about the cell identity, with which the user can agree. Thus, a single person can make \( \approx 10,000 \) calls per day. Using this approach, we find 23,798 excitatory neurons (Fig. 3.5B), and 10,628 inhibitory neurons (Fig. 3.5C).

Finally, we can also use these results to estimate how many cells are missed by the automatic confocal segmentation. We segmented 35,749 cells in light microscopy, 3697 of which fall outside the imaged electron microscopy volume, leaving 32,052 inside the volume. Of these cells, 26,632 are close to landmarks and satisfy the registration quality criteria. These cells are split into 8,381 inhibitory, and 18,251 excitatory cells. The counts are 79\% and 77\%, respectively, of the manually assigned calls. Thus, the automatic confocal segmentation misses at least 21\% of the cells in the volume.
Figure 3.5: Assigning functional identity to cells in electron microscopy A. LM/EM overlay snippets for three cells from electron microscopy. The cell under consideration is marked by a blue dot. The overlays with the excitatory and inhibitory channels for the same cell are stacked vertically. The intensities within each panel have been enhanced with a CLAHE filter. B–C, 3D renderings of the excitatory and inhibitory cells next to the cell size distributions. Both distributions have the same mean, but the excitatory neurons have larger variation.
Automatic methods for whole-brain connectomic reconstruction from electron microscopy images

Automatic image processing methods are indispensable for reconstructing anatomy from serial section electron microscopy images. To achieve this goal, these methods address three major challenges. First, the 2D electron microscopy images are aligned - that is, computationally stacked into a coherent volume to recover the 3D information. Second, the aligned images are segmented, which means that image pixels are assigned to neuronal or glial objects. Third, to build the neural wiring diagram, image pixels belonging to the pre- and post-synaptic partner at synapses are labeled and then used to assign connectivity to the neuronal objects.
4.1 Full resolution electron microscopy image alignment

Reconstructing the coherent full-resolution (4nm × 4nm × 30nm) zebrafish volume from images of serial sections is accomplished in two steps. The MultiSEM microscope images each section in a series of overlapping tiles, and these are first stitched together to form a coherent 2D image of the section. With a single 24-core machine + GPU, the stitching transformations for the entire set of images (350Tb) are computed in less than 2 weeks. The 3D alignment process requires significantly more computational time and resources, as well as parameter optimization, manual proofreading and intervention to correct alignment error.

The alignment of the dataset is still ongoing a year after the imaging has been completed. The majority of this time has been devoted to understanding the new challenges that come with aligning images from an entire animal. Specifically, automatic alignment has been optimized for brain tissue alone, which is relatively homogeneous and tightly packed with neurites. The latter is advantageous for the alignment procedure, which relies on finding many correspondences between the features in one section and the next. The larval zebrafish, on the other hand, is inhomogeneous with relatively featureless regions dispersed throughout the volume: such as the ears, muscles and vast swaths of tightly packed cell bodies. This challenge is additionally exacerbated by problems inherent to serial sectioning electron microscopy, such as partial or thin sections, wrinkles and post-staining artefacts.

4.2 Ground truth for training machine learning segmentation algorithms

In parallel to the full-resolution alignment, we are generating ground truth for machine learning automatic segmentation algorithms. We align four sub-volumes from different regions in the fish: wafers 19&20, 28&29, 32&33, and 41&42 (See Fig.2.2), corresponding to sections 1-276, 1220-1505, 1781-2065, and 3006-3247 (See Fig.2.3). From these we choose 6 training boxes for manual annotation (see Fig. 4.1). The training boxes are chosen from neuropil regions with different image statistics, such as different extracellular space fraction, or global neurite orientation (transverse or longitudinal).

Tiles are referred to as mFOVs (multiple fields of view) and they contain 61 single-beam FOVs (fields of view) arranged in a hexagonal pattern.
Figure 4.1: Dense volumetric ground truth segmentation. To train machine learning image segmentation algorithms, we manually label all neurites in six $4\mu m \times 4\mu m \times 8\mu m$ subvolumes. The volumes are chosen from neuropil regions with different image statistics, representative of the data heterogeneity. Each volume requires 150-200 hours to complete and proofread.
To carry out manual segmentation we use VAST, where objects are labeled by painting their area in each section to reconstruct their 3D shape. Each training box is segmented by either one or multiple annotators, and all annotated volumes are proofread using two different methods by a single expert. In particular, each segmented object is proofread in VAST by following it through all sections in the training box, and then inspecting its 3D shape using Autodesk 3ds Max. The proofread volumes are shared with the Connectomics team at Google Research who then train a flood-filling network for automated segmentation of neurons and glia, and test it on unlabeled images (see Fig. 4.2). The performance of the automatic segmentation is found to be comparable to what has been observed with other datasets. Thus the zebrafish dataset is analyzable with the available automatic segmentation tools.

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1VAST does not support simultaneous collaborative annotation. Instead, each annotator works independently on the same dataset and the segmentation results (VSS files) are combined in VAST using a merge function.

2VAST has its own 3D viewer, but it requires re-loading every time a new object is visualized. However, VAST also exports all objects as .obj files and these can be loaded in Autodesk 3ds Max, which significantly speeds up proofreading.
Figure 4.2: Examples from automatic image segmentation. The ground truth volumes are used to train a flood filling network algorithm which is tested on unlabeled images. A. Automatic segmentation of a glial cell (pink) and spiny dendrite (green) in the zebrafish cerebellum. B. Automatic segmentation of a spiny dendrite (green) and an axon (blue), which makes multiple synapses with the dendrite. The Insets show raw EM images with labeled objects corresponding to the 3D structures. The size of the 3D objects is limited by the image stack depth (8μm), not the segmentation algorithm.
4.3 Ground truth for training machine learning algorithms for synapse identification

An electron microscopy connectome is a map of the connections formed by chemical synapses, and it generally does not include electrical synapses. Chemical synapses are identifiable in electron microscopy images by several features. In mammals, the synaptic cleft shows up as an electron-dense site of apposition between the membranes of two neurons. Synaptic vesicles from the presynaptic neuron appear as circular objects near the cleft. Typically, when the synapse is excitatory, the cytoplasm of the postsynaptic neuron shows an electron dense postsynaptic density (PSD). The appearance, specifically the relative contrast, of the synaptic cleft and PSD depends on the synapse type, staining and imaging. The larval zebrafish is a vertebrate and its chemical synapses resemble those in mammals. The synapses with spiny dendrites in the zebrafish cerebellum are particularly evocative.

Here, we survey the chemical synapses across the brain volume with the goal of providing ground truth for machine learning algorithms. We focus on the three synapse-containing training boxes: from the cerebellum, the dorsal hindbrain and the tectum. The boxes from commissure regions contain bundles of neurites which do not make synapses with each other. In each box, we identify the synapses in 3D, and label separately the pre- and post-synaptic partners. In all volumes, the presence of a putative presynaptic site is unambiguous, because the synaptic vesicles are clearly visible structures. It is also straightforward to identify putative post-synaptic sites which are in contact with a putative pre-synaptic site. The appearance of the synaptic cleft, however, is variable. It is generally more difficult to identify the synaptic cleft in regions with tightly packed neurites, such as in the optic tectum. A single annotator has densely labeled two training boxes (cerebellum and hindbrain), counting 397 and 395 chemical synapses in each, respectively. Annotation by multiple experts is still necessary to estimate the error rate in synapse identification.

We can make an estimate for the task ahead. There are 170,000 cells in the dataset, split approximately equally between neurons and glia. Half of the 30 million cubic micron brain volume is taken up by cell bodies, and the other half by neuropil. If the density of connections is 3 synapses per cubic micron, we expect 90 million synapses, which amounts to an average of 1000 synapses per neuron.

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1 Unless there is sufficient extracellular space to force a connection apart from a contact.
2 From these numbers we estimate the average density of synapses in a neuropil region to be 3 synapses per cubic micron.
Figure 4.3: Dense ground truth segmentation for synapse detection. A. Cerebellum region. Raw and labeled image snippet containing synapses. Cyan and purple colors mark pre- and post-synaptic neurons. The synapse is reconstructed in 3D by labeling all image sections containing that synapse. The reconstructions of all synapses within the training box are shown on the right. B. Hindbrain region. Raw and labeled image snippet containing synapses. C. Tectum region. Raw and labeled image snippet containing synapses.
Conclusion

We acquire the first comprehensive electron microscopy images of neurons and their connections in the nervous system of an intact vertebrate, the larval zebrafish. We complement this data with information about a neuron’s excitatory or inhibitory function, which we obtain from confocal microscopy images of genetically labeled cells in the same animal. The combined dataset is a valuable tool for testing current circuit models for behavior, and for generating new circuit hypotheses.

To carry out this project, we harness the significant increase in imaging speed when using multiple electron beams in parallel. The pilot electron microscopy volume of a larval zebrafish was imaged with a single electron beam, and it took 100 days to acquire a $6 \times 10^7 \text{ um}^3$ volume at $20\text{nm} \times 20\text{nm} \times 60\text{nm}$ resolution. Now, we use 61 beams in parallel to acquire a $8 \times 10^7 \text{ um}^3$ volume in 60 days at $4\text{nm} \times 4\text{nm} \times 30\text{nm}$ resolution. The difference in resolution between the two datasets is the difference between following a subset of neural wires, and mapping a full connectome.

Curating the images into a cohesive 3D volume and mapping neurons and synapses is without doubt only
manageable by implementing automatic image processing methods. Consider just the zebrafish brain, which takes up 30 million cubic microns. At full image resolution, it takes about a second for a human to view all objects inside a cubic micron. At this speed, it would take a full year to simply view the entire brain. Annotating all objects inside a cubic micron takes an hour, so a human would spend 3,400 years segmenting the images. Labeling synapses is faster: about 10 minutes per cubic micron, or just 600 years of synapse annotation. Here, we do enough of all of the above to train machine learning algorithms, which will take over the task of annotating the images.
A.1 Aldehyde fixation for extracellular space preservation

The neurons and glia in a living brain are separated from each other by as much as 20% extracellular space (in mammals)\(^8^6\). The presence of extracellular space aids the connectomic reconstruction of neurons and synapse identification, including gap junctions\(^6^6\). However, standard aldehyde fixation protocols for electron microscopy produce tissues in which neurites and glia are tightly packed together. There are two general methods by which extracellular space can be preserved - (i) high pressure freezing and fixation by substitution with osmium\(^8^6\), and (ii) by supplementing the aldehyde fixation with sugars\(^1^8,^1^9\). While the high pressure freezing method yields the best extracellular space preservation, it only does so with small specimens (i.e. C. elegans) - otherwise, in larger samples such as a zebrafish, ice crystals form and cause catastrophic cracks across the tissue. This leaves us with the task of optimizing the sugar-supplemented aldehyde fixation approach for the purpose of zebrafish fixation: see Fig.A.1 and Fig.A.2. An invaluable resource for learning about specimen preparation for electron microscopy is the book “Principles and Techniques of Electron Microscopy” by M. A. Hyatt\(^3^9\).
A.2 Reagents and equipment

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<td>501778</td>
<td>WPI</td>
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<td>Tricaine</td>
<td>A–5040</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Mannitol</td>
<td>16220</td>
<td>EMSs</td>
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<tr>
<td>Glutaraldehyde, 25% solution</td>
<td>15700</td>
<td>EMS</td>
</tr>
<tr>
<td>Osmium tetroxide, 4% solution</td>
<td>19190</td>
<td>EMS</td>
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<td>T–2137</td>
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<td>Potassium ferrocyanide</td>
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<td>Sigma-Aldrich</td>
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<td>Sodium hydrosulfite</td>
<td>71699</td>
<td>Sigma-Aldrich</td>
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<td>Uranyl acetate</td>
<td>22400</td>
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<tr>
<td>Propylene oxide</td>
<td>02524 – AA</td>
<td>SPI</td>
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<td>Ethanol</td>
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<td>EMS</td>
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<tr>
<td>LX-112 kit low-viscosity</td>
<td>21212</td>
<td>Ladd</td>
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</tbody>
</table>

Table A.1: List of reagents and vendors

A.3 Solutions

NaCl | 64 mM
KCl  | 2.9 mM
HEPES| 10 mM
Glucose| 10 mM
Sucrose| 164 mM
MgCl2 | 1.2 mM
CaCl2 | 2.1 mM

adjust to pH 7.5 with NaOH
osmolarity 323.8 mOsm

Table A.2: Artificial cerebrospinal fluid (aCSF) recipe
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tr>
<td>LX-112</td>
<td>31.2g</td>
</tr>
<tr>
<td>NSA</td>
<td>9.4g</td>
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<tr>
<td>NMA</td>
<td>19.4 g</td>
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<td>BDMA</td>
<td>0.6g</td>
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**Table A.3:** LX-112 resin recipe.
Figure A.1: Fixative effects on extracellular space preservation (part #1). The loss of extracellular space (ECS) during aldehyde fixation is due to the influx of sodium chloride and water inside cells, causing the swelling of neurites and glia. This can be overcome by replacing the sodium chloride with a molecule which cannot enter the cells, i.e. a sugar. However, this replacement needs to occur before the aldehyde fixation. For a whole-zebrafish preparation, aldehyde fixation is achieved by means of diffusion though a surgical opening in the skin above the animal’s hindbrain. Extracellular preservation thus depends on the choice of fixative components and their relative diffusion rates. To optimize this selection, we develop an assay for evaluating extracellular space preservation as a function of fixative choice. Specifically, we cut the anterior portion of the animal, removing the eyes and leaving a single wide opening for the fixative to enter (cut site is shown as red dashed line across the zebrafish). The animal is left in fixative overnight on a nutator at 4°C. We only modify the fixative components and keep the rest of the electron microscopy protocol the same across conditions. Finally, to evaluate the extracellular space preservation we screen the tissue by taking serial sections 100μm apart and measuring the extracellular space from images of these sections (cut sites are shown as gray dashed lines across the zebrafish). Extracellular space measurements are performed using ilastik, and errors are evaluated from multiple 4μm² neuropil sub-regions within the same section. A. Zebrafish neuropil with three different fractions of extracellular space (blue). B. It is exceedingly common to use paraformaldehyde (PFA) and glutaraldehyde (GA) fixation because PFA diffuses more quickly into the tissue and speeds up fixation. However, PFA severely limits the preservation of ECS, which is much improved using only GA for the fixation. Furthermore, it is recommended to use 1.5%-2.5% GA for fixation of animal tissues, and indeed this concentration range does not lead to excessive shrinkage of the neurites while preserving the ultrastructure and ECS. C. The choice of vehicle composition (buffer and/or sugar) and its osmolarity also affects extracellular space preservation. For buffer, we choose to use Sodium Cacodylate buffer, supplemented with CaCl₂ (the buffer is abbreviated as NaCac) which at sufficiently high osmolarity has been shown to preserve extracellular space in some tissues. As a sugar, we choose to use mannitol which is smaller than sucrose and thus diffuses quicker, but is still big enough to not cross over the cell membrane. As a rule of thumb, 100mM NaCac has osmolarity of ~200mOsm, and 1% mannitol has osmolarity of ~50mOsm. In the left and right panels, the total vehicle osmolarity is 300mOsm and 400mOsm, respectively. There is a dramatic difference when the total osmolarity of the vehicle comes from only sugar or only buffer. However, an additional factor that needs consideration is that GA has different crosslinking activity at different pH values. Unbuffered GA is acidic, while buffered GA has neutral pH and a higher crosslinking activity. We explore the effects of vehicle composition on extracellular space preservation further in Fig.A.2.
Figure A.2: Fixative effects on extracellular space preservation (part #2). A. We explore the effect of different mannitol concentrations on ECS. Mannitol concentration higher than 6% Mtl does not make a difference, and concentrations smaller than 4% Mtl are insufficient. B. We observe that the effects of sugar and buffer add up nonlinearly. Sugar and buffer, in combination, prevent from extreme shrinking and because the pH is neutral, the crosslinking activity of GA is higher and ultrastructure preservation is improved.
References


