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Wired For Insight- Recent Advance in *C. elegans* Neural Circuits

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Abstract

The completion of *C. elegans* connectomics four decades ago has long guided mechanistic investigation of neuronal circuits. Recent technological advance in microscopy and computation programs have aided re-examination of this connectomics, expanding our knowledge by both uncovering previously unreported synaptic connections and also generating models for neural network underlying behaviors. Combining information from single cell transcriptome with elegant tools for cell-specific manipulation has greatly enhanced the ability to precisely investigate individual neurons in behaving animals. This mini-review aims to provide an overview of new information on connectomics and progress towards a molecular atlas of *C. elegans* nervous system, and discuss emerging findings on neuronal circuits.

Introduction

The *C. elegans* neural connectome has provided a model for deciphering cellular and molecular basis of animal behaviors for nearly 50 years (WHITE *et al.* 1986). The feat of reconstructing neuronal connections within an entire organism from electron micrographs was attainable largely because adult hermaphrodite animals contain only 302 neurons in stereotypical positions. In past decade, technological advances in electron microscopy and computation algorithms for image acquisition and 3D reconstruction, along with in-depth mechanistic dissection, are expanding our understanding of neuronal circuits in *C. elegans*. In this mini-review, we will first cover principle findings in connectomics and progress towards a molecular and cellular map of the nervous system. We will then highlight examples of how technological advances aided studies of neural circuits, focusing on those related to locomotor circuit. Lastly, we will touch upon emerging findings about gut-brain axis. For comprehensive coverage of many other neural circuits and behaviors, such as sensory perception and transduction, learning and memory, we recommend many excellent reviews (PORTMAN 2017; GOODMAN AND SENGUPTA 2019; KIM AND FLAVELL 2020; LIU AND ZHANG 2020).

Advances in connectomics

One key factor that influenced Sydney Brenner's choice of *C. elegans* for his experimental approach to 'define the unitary steps' in development and in the nervous system (BRENNER 1988) was the beautiful ultrastructure preserved after fixation. This seemingly technical-driven decision enabled the pioneering work to reconstruct the nervous system by White et al (WHITE 2013), modern term 'connectomics'. The first published connectome analysis was on the pharyngeal nervous system that controls feeding behavior (ALBERTSON AND THOMSON 1976), followed by the construction of the ventral nerve cord that controls locomotion (WHITE *et al.* 1976), with the ultimate reconstruction of the entire nervous system published in 1986 (WHITE *et al.* 1986). These precocious connectomes have been an incredible resource for deciphering cellular and molecular details of behavioral circuits.

Technological advance, such as high-pressure freezing sample fixation, automation of tissue sectioning, and digitization of electron microscopy images (ROSTAING *et al.* 2004; MULCAHY *et al.* 2018), played major roles in current connectomics research. Various computational programs on image reconstruction, such as TrakEM2 (CARDONA *et al.* 2012) and Elegance (XU *et al.* 2013), have aided the expansion of connectomic studies (Table 1). For example, using TrakEM2 to align digitized images and Elegance to reconstruct cellular connectivity for all

pharyngeal neurons, Cook et al. re-analyzed the original electron micrographs of pharyngeal nervous system and added anatomical weights for synaptic strength (COOK *et al.* 2020). This re-evaluation of the pharyngeal nervous system connectome not only revealed previously unreported synaptic connections and additional details of the extensive cross-connectivity, but also provided evidence that pharyngeal neurons may have both sensory and motor characteristics.

Sexual dimorphism of the nervous system is a fascinating topic among researchers working with all animal species. *C. elegans* hermaphrodite has 302 neurons, of which 6 neurons are hermaphrodite-specific, and male has 385 neurons, of which 91 neurons are male-specific. The posterior connectomics of *C. elegans* male, completed in 2012, revealed that of the 89 neurons that are shared by both sexes, two-thirds are sexually dimorphic in their wiring (JARRELL *et al.* 2012). Since the posterior region includes most of the male-specific neurons involved in mating behavior, the sexual differences in connectomics may not be surprising. *C. elegans* males also display differences in sexual conditioning of associative learning (Sakai N et al. 2013). Interestingly, two of the male-specific neurons required for this behavior are derived from glia (SAMMUT *et al.* 2015; MOLINA-GARCIA *et al.* 2020). A recent study employed quantitative computation strategies to compare the circuitry of the nerve ring and ganglia in the head for both sexes (COOK *et al.* 2019). Based on the sizes of synapses and input-output connections, they defined 460 nodes in hermaphrodite and 579 nodes in male connectomes. The data show not only how sexual and non-sexual pathways converge on central conserved circuitry, but also reveal synaptic strength differences within the shared central circuitry.

While the early connectome has been an essential resource for determining anatomical details of behavioral circuits, further studies are beginning to uncover variability of connectomics in development. For example, based on new volumetric reconstruction of the legacy electron micrographs of one early adult and one L4 stage (WHITE *et al.* 1983), one study finds that the connectomics is not invariant, rather a precisely wired core circuit is embedded in a background of variable connectivity (BRITTIN *et al.* 2020). Other systematic efforts are being made towards reconstruction of the nervous system from birth to adults (WITVLIET *et al.* 2020). Such studies are deemed to uncover dynamics of synaptic connectivity.

Finer structures and expanded microdomains of neurons revealed by super-resolution microscopy

The small size and compactness of *C. elegans* nervous system are both advantageous for examining neuronal connectivity in intact animals and also impose limitation to decipher microdomains of molecules and cellular structures by conventional light microscopy. Super-resolution microscopy techniques have vied for their usefulness in *C. elegans* (Table 1). Recent efforts have also shown promise of expansion microscopy, which improves resolution of light microscopy by isotropically expanding the tissue sample using a swellable hydrogel polymer complex. Overcoming the permeability issue of *C. elegans* outer cuticle, Yu et al developed a customized protocol called ExCel, with an epitope-preserving ExCel method for antibody staining, and an iterative ExCel method to expand tissue to almost 20x its original size (YU *et al.* 2020). They validated ExCel by mapping synaptic proteins, identifying previously unreported cell junctions, and analyzing expression of mRNAs in multiple individual neurons of the same animal. While the super-resolution microscopy increases detection of structures and molecules, it is limited the depth of imaging within a sample and hardware expense. As an addition to current widefield microscopy, Light sheet microscopy eliminates the effects of illuminating out-of-focus planes by restricting illumination to only the plane of focus, and has been shown that it can be configured for imaging *C. elegans* (VAN KRUGTEN *et al.* 2020; ZHAO *et al.* 2020) (Table 1).

The discovery of multi-dendritic sensory neurons a decade ago has forever changed the stereotypic reputation of *C. elegans* neurons as being simple in morphology (INBERG *et al.* 2019; SUNDARARAJAN *et al.* 2019). Aided by super-resolution microscopy, a recent study presents definitive evidence that *C. elegans* neurons have functional dendritic spines, validating the early notation of spine by White at al (WHITE *et al.* 1986). Using airyscan live imaging of LifeAct::GFP, Cuentas-Condori et al report actin-rich microdomains in the dendritic processes of the ventral cord GABAergic motor neurons (CUENTAS-CONDORI *et al.* 2019). Such microdomains exhibit dynamics that are reminiscent of dendritic spines in mammalian central nervous system neurons. Additional evidence from fluorescence microscopy and EM reconstruction show that the actin-rich spines directly oppose the presynaptic release sites of upstream cholinergic motor neurons. The dendrites also contain cisternae SER-like structures, as well as ribosomes in the spines, consistent with an early report using a split-GFP based ribosome reporter (NOMA *et al.* 2017). Using GCaMP imaging, they detected Ca^{2+} transients within the spines, dependent on activation by cholinergic motor neurons. Further mechanistic studies reveal that the spine density is dependent on actin regulators Arp2/3 complex, the F-BAR protein TOCA-1, and Wave

Regulatory Complex, and is enhanced by cholinergic input during development. Along with recent connectomics studies that have revealed complex morphologies of the ciliary ends of chemosensory sensory neurons (DOROQUEZ *et al.* 2014), these findings raise interest questions, such as how the morphological diversity is regulated, how the microdomains are formed, and how the structural complexity contribute to the function of neuronal circuits.

Towards a molecular and cellular connectomics:

New technology for neuronal identification

It is often stated that a unique advantage using *C. elegans* is the ability to precisely identify cells. This is largely true for the neurons located in the periphery and in the ventral nerve cord. However, determining the identity of neurons in densely packed ganglia, especially around the nerve ring, can be time-consuming, and especially daunting for beginners. In fact, positions of neurons in the ganglia of adult animals are quite variable. For example, a comprehensive analysis of 311 adults using 35 fluorescence reporters for neurons in the head reported large positional variations, posing caveats for position-based cell annotation (TOYOSHIMA *et al.* 2020). Now, a new approach called Neuronal Polychromatic Atlas of Landmarks (NeuroPAL) enables simultaneous identification of neurons in the entire nervous system (YEMINI *et al.* 2021) (Table 1). Key to NeuroPAL is a transgene made of multiple fluorescence proteins expressed under some 40 plus neuronal-type promoters, resulting in not only illumination of all neurons but also marking each neuron with a unique combination of fluorophore pattern. The authors also developed a software package for semi-automated determination of all neuronal identities based on color and positional information. To demonstrate the utility of NeuroPAL they mapped expression patterns of all metabotropic receptors for acetylcholine, GABA, and glutamate, and identified cell fate changes in select mutants. Additionally, by combining NeuroPAL with GCaMP imaging, they recorded whole-brain activity in response to attractive and repulsive chemosensory cues. A parallel study used NeuroPAL to delineate homeobox (hox) genes with neuronal identity (REILLY *et al.* 2020). The *C. elegans* genome encodes 102 hox genes, 70 of which have conserved homologues in vertebrates. By building an expression atlas of 101 hox genes, combined with NeuroPAL, the authors generated a comprehensive analysis of homeodomain protein expression patterns in all 302 neurons. They find that neurons expressing the same homeodomain protein are not usually related by lineage or neurotransmitter expression. Each neuron class expresses a unique combination of homeodomain proteins. This "combinatorial code" consists of an average of four homeodomain proteins. While there are 118

anatomically defined neuron classes, they found 155 distinct homeobox codes, indicating that the homeobox codes may further define neuron subclass identities.

Another potentially powerful technology used a ribosome-tethered GCaMP reporter to link neuronal activity with cell identification (CHEN *et al.* 2020). The authors found that co-expression of a GFP nanobody with other GFP fusion proteins is able to localize GFP fusion proteins to the cell soma and also enhance fluorescence. By tethering GCaMP to a ribosome protein, the reporter has the advantage that the neurons can be identified more easily in whole-brain imaging, with improved signal and temporal fidelity. As a proof of principle, the authors imaged calcium dynamics in *C. elegans* AFD sensory neurons. Further development of this technology may consider taking advantage of many knock-in reporters generated by the *C. elegans* researchers.

Building single-cell molecular expression atlas

In the era of single-cell deep-sequencing, consorted efforts have been made towards a complete molecular atlas for all cell types in an organism. The anatomically well-defined cell types in *C. elegans,* along with technical improvement in isolating single cells, are particularly suited for this kind of approach. Building on early transcriptome analyses of 1-16 cell embryos and L2 larvae (TINTORI *et al.* 2016; CAO *et al.* 2017), a recent large-scale effort has profiled transcriptomes of 86,024 individual cells from *C. elegans* embryos, representing different developmental times from gastrulation to terminal differentiation (PACKER *et al.* 2019) (Table 1). Integrating computation methods with extensive gene expression dataset curated from literature, they mapped each single-cell transcriptome to the known embryonic cell lineage and reconstructed a near-complete molecular atlas of *C. elegans* embryogenesis. This database, 'VisCello', will provide a valuable resource for mechanistic dissection of early events in neural circuit formation.

In the nervous system, efforts to profile neuron-type transcriptomes began nearly two decades ago (ZHANG *et al.* 2002), and have recently cumulated to the CeNGen initiative (HAMMARLUND *et al.* 2018), which aims to establish a complete gene expression atlas for each neuron from larvae to adults. While conventional bulk RNAseq using dissociated and sorted neurons is a proven success (TAYLOR *et al.* 2019), recent implementation of TRAP-seq (Translating Ribosome Affinity Purification) has uncovered neuronal-type mRNA splicing events (KOTERNIAK *et al.* 2020). In combination with forward genetic screens using elegantly designed reporters that

detect alternative splicing *in vivo* (THOMPSON et al. 2019), there is a greater anticipation to advance mechanistic understanding.

In parallel to gene expression profiling, consorted efforts are evident towards a systematic determination of the cellular expression patterns of gene families. This is largely aided by genome-editing mediated knock-in of fluorescent reporters and fosmid-based reporters, the latter offering the benefit of higher expression levels with more complete genomic regulatory elements (Table 1). For example, the nerve bundles are ensheathed by basement membranes (BM). Using knock-in fluorescence reporters, a comprehensive analysis revealed tissue distribution of 29 BM components (KEELEY *et al.* 2020). Contrary to the presumption that BM simply provides static support, they uncovered dynamic movements of BM proteins that suggest active roles in sculpting microdomains. Another study characterized the expression of 25 innexin genes, which form gap junctions (BHATTACHARYA *et al.* 2019). Over half of the innexins are expressed in the nervous system, and most neuron classes express multiple innexins. While expression of most innexins is stable throughout development, a subset shows dynamic expression over developmental stages, with widespread changes in dauer, an enduring state under harsh conditions. One example includes AIB interneurons, which express two innexin genes, INX-6 and CHE-7, to make dauer-specific electrical synapses with each other and with BAG sensory neurons. Formation of these dauer-specific electrical synapses appears to regulate locomotory and chemotaxis behaviors only in dauer-stage animals. Dauer-specific expression of INX-6 in AIB neurons is dependent on the function of the DAF-16 FOXO transcription factor, a downstream component of insulin signaling. Interestingly, this provides a mechanism that links sensation of environmental changes to plasticity in electrical synapse formation. A similar study examined the expression of 26 metabotropic receptors and dissected their roles in the egg-laying circuit (FERNANDEZ *et al.* 2020). They report the consistent theme that individual neurons express multiple receptors and that many receptors function redundantly. Additionally, some receptors are often positioned to receive extrasynaptic signals, suggesting modulation of neuronal circuits beyond anatomical connections. Such cellular maps will undoubtedly guide functional perturbation to dissect neural circuits.

Advances in neural circuits

Mystery resolved- roles of AS motor neurons in locomotor circuit

C. elegans moves by propagating sinusoidal waves of dorsal-ventral bends, due to coordinated contraction and relaxation of body muscle controlled by the ventral cord motor neurons. The

connectivity of the ventral nerve cord was reported in 1976 (WHITE *et al.* 1976), concurrent with the ground breaking work on postembryonic cell lineage (SULSTON AND HORVITZ 1977). Eight morphologically defined classes of motor neurons (MNs) are arranged in repetitive unites from head to tail (Figure 1A). The VC-MNs innervate specialized muscles for egg-laying. Six classes of MNs form three pairs, designated as VA/DA, VB/DA, VD/DD, based on their synaptic output to ventral (hence first letter V) and dorsal (hence first letter D) body wall muscles. The A/B-MNs form dyadic synapses with muscles and the dendrites of D-MNs as postsynaptic partners. Incorporating the information from electrophysiology studies on Ascaris, which diverged from *C. elegans* about 500 million years ago (VANFLETEREN *et al.* 1994) but shares conserved structure of the nervous system with huge size of neurons for electrophysiological recordings (NANDA AND STRETTON 2010), White et al rightly deduced that A/B-MNs are cholinergic and excite muscles, and D-MNs use GABA to inhibit contraction of muscles on the opposite side. Using laser ablation of neurons, Chalfie et al then delineated the roles of four command interneurons in the head and tail ganglia, now known as pre-motor interneurons, in touch-induced movement (CHALFIE *et al.* 1985) (Figure 1B i-ii). In recent decades, advances in optogenetics and calcium imaging have enabled delineation of neuronal activity patterns in moving animals (ZHEN AND SAMUEL 2015; DILORETO *et al.* 2019). These studies reveal calcium oscillatory pattern in the cholinergic B-MNs, a shared feature for central pattern generators, CPGs (GAO *et al.* 2018). The core pre-motor interneurons, along with local sensory and interneurons, relay sensory information by setting up up- or down-states of the cholinergic excitatory A/B-MNs (Figure 1B) (KAWANO *et al.* 2011; XU *et al.* 2018).

A long-standing unaddressed question arose from counting synaptic innervation number to body wall muscles—the total number of cholinergic innervation from A/B-MNs to dorsal muscles is about half of that to the ventral muscles (WHITE *et al.* 1976). It was speculated that the seventh class, AS-MNs, would fill this gap, because they are cholinergic, receives inputs from pre-motor interneurons and form synapses to the ventral inhibitory VD neurons and dorsal muscles (Figure 1C). However, despite extensive genetic screens and gene expression studies, there was a dearth of reagents specifically labeling AS-MNs until very recently. By designing an intersectional transgene scheme, Tolstenkov et al succeeded to tackle the roles of AS-MNs (TOLSTENKOV *et al.* 2018). They found that activating AS neurons using Chrimson leads to contraction of the dorsal body muscles, with animals displaying a distorted locomotion wave. Conversely, ablation of AS neurons causes animals to move slowly with increased bending angles. While chronic inhibition of these neurons eliminates calcium influx to dorsal muscles,

acute inhibition induces contraction of ventral muscles through disinhibition. Moreover, AS neurons exhibit calcium oscillation that depends on gap junctions with the pre-motor interneurons. The authors conclude that while AS neuron excitation does not appear to play an instructive role in generating wave, they integrate signaling from pre-motor interneurons in both forward and backward movement (Figure 1C).

Flexibility of hardwired motor circuit- roles of local interneurons

Behavioral flexibility allows animals to explore possible actions to choose the best route to escape danger or the best reward in a dynamic environment. For *C. elegans*, gentle touch to anterior body activates touch receptor neurons (TRN, Figure 1B), which activates pre-motor interneurons of the backward motor module to generate an initial stereotypic backward movement, which is followed by a turn and reorientation to move forward in a different direction. Early studies have identified a set of sensory neurons, interneurons, and motor neurons required for navigating locomotory escape (CHALFIE *et al.* 1985; GRAY *et al.* 2005), however specific mechanisms for the variation in motor sequence remained unclear. A recent study explored this touch-induced behavioral flexibility quantitatively using optogenetically or thermally activating anterior TRN (WANG *et al.* 2020). By estimating the probability of each motor response measured over time, they determined transition rates from either backwards to forwards movement or backwards to turning movement. Combining calcium imaging and optogenetic stimulation in several local interneurons in the head ganglia, they defined three sub-circuits: backward, forward and turning modules and showed that feedforward excitation between backwards and turning modules promotes turning behavior, likely through electrical synapses between two interneurons AIB and RIV. However, the timing of the turn is likely delayed by feedforward inhibition involving inhibitory glutamatergic signaling from AIB to RIB interneurons. Since the animals continue backwards longer when the turning module is inhibited, there may also be feedback inhibition from the turning module to the backing module to stop backing and begin turning, although the mechanism for feedback inhibition remains speculative. The authors propose that the flexible sequence of locomotion is the result of the combination of feedforward excitation promoting the turn, feedforward inhibition delaying the turn, and feedback inhibition to stop backing, thus integrating classic synaptic chain and mutual inhibition models for generating complex flexible behaviors.

Food modulation of locomotion- roles of dopamine and neuropeptides

In the presence of food, *C. elegans* exhibits a behavior called dwelling- slow forward locomotion with frequent reversals. A study has recently reported a mechanism mediated by the PDE dopaminergic neurons to regulate food-induced dwelling (ORANTH *et al.* 2018). PDE neurons form extensive connections with gas sensing neuron AVK and stretch sensing interneuron DVA. By transcriptomic profiling of AVK neurons, the authors identified the dopamine receptor DOP-3 and showed its activation silenced AVK. AVK releases neuropeptide FLP-1. By screening genetic mutants that impaired FLP-1 neuropeptide signaling, they then identified two GPCRs, NPR-6 and FRPR-7, as FLP-1 receptors that inhibit the downstream motor neurons SMB and another interneuron DVC. On the other hand, PDE neurons activate DVA neurons through the DOP-1 receptor. DVA neurons release both acetylcholine and neuropeptide NLP-12 to activate SMB and the ventral cord motor neurons. Thus, PDE neurons modulate dwelling through both chemosensation via AVK and mechanosensation via DVA by balancing two antagonistic neural pathways.

Circuit regulation beyond neurons: neuro-gut axis

C. elegans encounters many species of bacteria in their habitats, some pathogenic and others non-pathogenic. Recent studies have begun to uncover how hardwired neural circuits intersect with signals in the gut, an emerging topic in neuroscience. While animals are initially attracted to pathogenic bacteria, such as *Pseudomonas aeruginosa*, they later avoid it via aversive olfactory learning through a switch between two neural circuits (ZHANG *et al.* 2005; HA *et al.* 2010). Exposure to *P. aeruginosa* also stimulates an innate immune response that promotes pathogen-avoidance and activates microbial-killing pathways to enhance survival (MEISEL AND KIM 2014). However, specific mechanisms connecting the bacteria in the gut to the aversive learning and pathogen avoidance behaviors remain unclear. Recent studies suggest that intestinal bloating caused by intestinal colonization of bacteria triggers the innate immune response, and the bloating-induced pathogen avoidance requires NPR-1 neuropeptide signaling (Figure 2 red path) (SINGH AND ABALLAY 2019b). There are many emerging examples of bacterial toxins and metabolites that can signal to the host (YANG AND CHIU 2017). Intriguingly, the bacterial GacA virulence gene is required for production of phenazine metabolites, gut colonization of the bacteria, and pathogen avoidance (MEISEL *et al.* 2014; SINGH AND ABALLAY 2019b). Moreover, phenazines induce expression of DAF-7/TGFβ in ASJ chemosensory neurons (MEISEL *et al.* 2014). However, chemosensation of phenazines appears to be insufficient to elicit the pathogen avoidance behavior(SINGH AND ABALLAY 2019a).

An intriguing new mode of signaling from gut bacteria to *C. elegans* has emerged from studies of how different non-pathogenic bacterial food sources affect worm behavior (O'DONNELL *et al.* 2020). Worms fed certain Providencia strains display decreased avoidance of the volatile repellant 1-octanol. Strains that modulate octanol avoidance produce neuromodulatory tyramine that can bypass host tyramine biosynthesis. Since *tbh-1*, a gene encoding the tyramine beta-hydroxylase enzyme that converts tyramine to octopamine and *octr-1*, encoding an octopamine receptor, are necessary for full octanol modulation, the bacterially produced tyramine is likely converted to octopamine in the host and signals through the OCTR-1 receptor expressed on nociceptive ASH neurons to modulate the aversive olfactory response (Figure 2 green path). Additionally, worms colonized by Provincia prefer these bacteria in food choice assays in a manner dependent on the bacterially produced tyramine and host octopamine signaling. Therefore, the Provincia gut bacteria are able to synthesize a neurotransmitter that alters host behavior to promote fitness of both the bacteria and its host (O'DONNELL *et al.* 2020).

Much work has elucidated neuronal control of feeding and intestinal fat storage, revealing input from gustatory, oxygen-sensing, and pheromone-sensing neurons for fat storage in intestinal cells (GREER *et al.* 2008; CUNNINGHAM *et al.* 2012; LEMIEUX AND ASHRAFI 2015; WITHAM *et al.* 2016; HUSSEY *et al.* 2017). A recent study has uncovered a pathway linking olfaction with fat storage without detectable changes in food intake or energy expenditure. Loss of function mutations in *daf-11*, which encodes a conserved transmembrane guanylyl cyclase, result in increased intestinal fat storage and total whole animal triglyceride levels. Expression of *daf-11* is specifically required in AWC olfactory neurons for rescue of the fat storage phenotype. Conversely, optogenetic activation of the *str-2*-expressing AWCon neuron can decrease fat levels, suggesting that sensation of a specific odorant and activation of a single olfactory neuron can affect fat storage. Results of fat storage phenotypes lead to a model whereby the odorant 2-butanone is sensed by and inhibits the *str-2*-expressing AWCon olfactory neuron, which inhibits AIY interneuron from releasing FLP-1/FMRFamide neuropeptides that can signal to the adipose tissue through the NPR-4/NPY receptor, SGK-1/SGK1 kinase, and DAF-16/FOXO transcription factor to control lipid homeostasis (Figure 2 purple path) (MUTLU *et al.* 2020).

Perspectives

The few examples mentioned above represents the tip of the iceberg in *C. elegans* neurobiology research. Although the number of neurons is fixed and their connectivity is well defined, many studies have uncovered multi-functionality of individual neurons and flexibility of anatomically

hardwired circuits. *C. elegans* remains a favorite model to test the feasibility and scalability of new technologies. For example, a recently reported methodology uses a membrane-targeted ascorbate peroxidase Apex2 with polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) to assemble conductive and insulating polymers around specific cells in vivo (LIU *et al.* 2020) (Table 1). When tested in *C. elegans,* it was observed that animals with PANI polymerization on inhibitory or excitatory motor neurons exhibited behavior patterns resembling optogenetic inhibition. Another methodology developed split photoactivatable forms of botulinum neurotoxin serotype B light chain protease, 'PA-BoNT', for rapid and local disruption of synaptic transmission (LIU *et al.* 2019) (Table 1). When each two-fragment pair sPA-BoNT and vPA-BoNT was expressed in *C. elegans* neurons*,* under blue light illumination, animals showed significantly reduced thrashing rates that were recovered after 24 hours in the dark, demonstrating that the PA-BonNT can both conditionally and reversibly alter behavior in vivo. These proof-of-concept results will surely stimulate the efforts for further improvement in their broad applications. With its long-standing reputation for *in vivo* functional dissection, along with rapid infusion of cutting-edge technologies, insights from studying *C. elegans* will continue to expand our understanding of neuronal circuits from microstructures to inter-tissue interactions.

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Table 1: Summary of recently developed analytical and experimental technologies

Techniques	Examples*	References#
Electron Microscopy		
High pressure freezing fixation	Standard since 2004	(ROSTAING *et al.* 2004)
Image reconstruction and analytical methods	TrakEM Elegance Volume EM	(CARDONA *et al.* 2012) (XU *et al.* 2013) (MULCAHY *et al.* 2019)
CLEM (Correlated Light EM)	Phagocytosis	(CHERRA *et al.* 2020)
Light Microscopy		
Expansion Microscopy (ExCel)	Synaptic proteins, mRNAs	(YU *et al.* 2020)
Light Sheet Microscopy	Neurons and muscle	(J *et al.* 2021) (ZHAO *et al.* 2020)
Live imaging super resolution:		
Airyscan imaging	Dendritic spines	(CUENTAS-CONDORI *et al.* 2019)
SIM imaging (Elyra)	Actin-Spectrin membrane cytoskeleton	(HE *et al.* 2016)
Single-molecule localization microscopy (PALM, STORM, STED)	Active zone proteins Clathrin adaptor proteins	(KURSHAN *et al.* 2018) (LI *et al.* 2016)
Genomics		
sc-RNA-seq	Neuroblasts in embryos Mature neurons (CeNgen)	(PACKER *et al.* 2019) (HAMMARLUND *et al.* 2018)
TRAP-seq	Cell- and Tissue-type alternative splicing	(KOTERNIAK *et al.* 2020)
Neuron ID **Neuronal protein expression**		
NeuroPAL	Pan-Neural imaging Neuronal 'homeobox code'	(YEMINI *et al.* 2021) (REILLY *et al.* 2020)
Ribosome tethered GCaMP	AFD	(CHEN *et al.* 2020)
Fluorescent tagging of neuronal proteins (Crispr-Cas9-Knock-in or Fosmid reporters)	Many applications	(DICKINSON AND GOLDSTEIN 2016) (SAROV *et al.* 2006)
Chemo- and Opto-genetics		
Membrane Assembly of Electroactive Polymers	motor neurons and pharyngeal muscle	(LIU *et al.* 2020)
Split Photoactivatable Botulinum Neurotoxin	Synaptogyrin Motor neurons	(LIU *et al.* 2019)
HisCl1-histamine Silencing	Many applications	(POKALA *et al.* 2014)
Neuronal ablation (Reconstituted caspase, miniSOG)	Many applications	(CHELUR AND CHALFIE 2007) (QI *et al.* 2012)

* Examples are abbreviated, please refer to main text or original publications.
# We apologize for not able including all references.

Figure legends

Figure 1. *C. elegans* nervous system and circuit mechanisms for locomotion

A. Graphic representation of the entire nervous system. Dots illustrate neuronal soma, lines nerve processes. Head ganglia consists of majority sensory neurons and interneurons (IN), including premotor command neurons. Tail ganglia consists of sensory neurons and INs. Ventral nerve cord consists of eight classes of motor neurons (MNs) organized in repetitive units from head to tail, and connects to dorsal nerve cord through circumferential commissures (verticle lines). Touch receptor neurons (TRN) sense and transduce mechanic stimuli to pre-motor command neurons to initiate forward or backward movement. Drawings are modified from the art work provided by Erik M. Jorgensen, with permission.

B. Schematics of the *C. elegans* motor circuit components and connectivity in (i) wild type animals and (ii, iii) upon ablation of respective neuronal populations (as faded color), with a snap shot of representative body posture exhibited by adult worm shown below. Hexagons and circles represent pre-motor interneurons and ventral cord MNs, respectively. Orange and blue denote components of the forward and reversal motor circuit, respectively. Taupe denotes neurons that participate both forward and backward locomotion. Reproduced from Figure 1A in Gao et al (2018), with permission.

C. Ventral cord AS MNs asymmetrically regulate dorso-ventral bending during forward and backward locomotion, modified from Figure 7 in Tolstenkov et al (2018) with permission. Left illustration shows functional connections between pre-motor interneuron AVA and AVB with AS MNs, which synapse onto ventral GABAergic VD MNs to inhibit body muscles. Right illustration shows interconnections and functional roles of AS MNs and other VNC MNs during the propagation of the undulatory wave along the body. Cholinergic (orange) and GABAergic (blue) cell types are indicated.

Figure 2. Summary of recent findings within the *C. elegans* neuro-gut axis

Animals ingest bacteria (yellow) that sometimes colonize the gut. Microbial colonization of pathogenic bacteria may induce intestinal bloating, which induces NPR-1 neuropeptide signaling to promote aversion of the pathogenic bacteria (red signaling pathway), based on Singh and Aballay (2019a). Non-pathogenic colonizing Providencia strains produce tyramine (L-Tyr) that may be converted to octopamine within the worm and signal through the octopamine receptor (OCTR-1) expressed by ASH sensory neurons to modulate avoidance of octanol and

influence food choice (green signaling pathway), based on O'Donnell et al (2020). Sensation of the odor 2-butanone inhibits the AWC_{on} chemosensory neuron, which inhibits AIY interneuron from releasing FLP-1/FMRFamide neuropeptides that can signal to the intestine through the NPR-4/NPY receptor, SGK-1/SGK1 kinase, and DAF-16/FOXO transcription factor to control lipid homeostasis (purple pathway), based on Mutlu et al (2020).

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Annotation of papers:
* of special interest
** of significant interest

***Bhattacharya, A., U. Aghayeva, E. G. Berghoff and O. Hobert, 2019 Plasticity of the Electrical Connectome of C. elegans. Cell 176: 1174-1189 e1116.**
Innexin genes encode proteins that form gap junctions. The authors reported the expression patterns of 25 innexin genes using engineered fosmid-reporter transgenes. They uncovered dynamic changes of innexins in dauer larvae and characterized how such changes contribute to distinct movement of dauers.

***Chen, Y., H. Jang, P. W. E. Spratt, S. Kosar, D. E. Taylor *et al.*, 2020 Soma-Targeted Imaging of Neural Circuits by Ribosome Tethering. Neuron 107: 454-469 e456**

The authors first found that co-expression of GFP nanobody with GFP-fusion proteins can trap GFP in soma and enhance fluorescence intensity. They then made a ribosomal-tethered GCaMP reporter and showed that in both mouse and *C. elegans* ribo-GCaMP enables simultaneous imagining for neuronal activities and identification of neuronal types.

****Cuentas-Condori, A., B. Mulcahy, S. He, S. Palumbos, M. Zhen *et al.*, 2019 C. elegans neurons have functional dendritic spines. Elife**

This study employed Airyscan super-resolution live imaging with ultrastructual reconstruction to show that actin-rich microdomains are present in the dendrites of *C. elegans* GABAergic motor neurons. They presented a comprehensive set of imaging and functional evidence supporting that such actin-rich microdomains have properties that resemble those described for dendritic spines of vertebrate neurons. Their data provide definitive evidence for the spine annotation in White et al (1986).

***Fernandez, R. W., K. Wei, E. Y. Wang, D. Mikalauskaite, A. Olson *et al.*, 2020 Cellular Expression and Functional Roles of All 26 Neurotransmitter GPCRs in the C. elegans Egg-Laying Circuit. J Neurosci 40: 7475-7488.**

The authors characterized cell-type and subcellular expression of 26 metabotropic receptors. They dissected their roles in the egg-laying circuit using genetic loss of function mutants and transgenic overexpression. Their data suggest functional redundancy of some metabotropic receptors and likely extrasynaptic signaling in modulating neuronal circuits.

***Gao, S., S. A. Guan, A. D. Fouad, J. Meng, T. Kawano *et al.*, 2018 Excitatory motor neurons are local oscillators for backward locomotion. Elife 7.**

This study employed extensive technologies, such as electrophysiology, cell ablation and calcium imaging, to characterize oscillatory network in *C. elegans* motor circuit. They showed that the ventral cord B-type excitatory motor neurons display rhythms, reminiscent of central pattern generators.

***Keeley, D. P., E. Hastie, R. Jayadev, L. C. Kelley, Q. Chi *et al.*, 2020 Comprehensive Endogenous Tagging of Basement Membrane Components Reveals Dynamic Movement within the Matrix Scaffolding. Dev Cell 54: 60-74 e67.**

This study characterized endogenous expression pattern for 29 basement membrane proteins using genome-editing mediated knock-in fluorescent reporters. They further examined dynamics of these proteins using FRAP and found that contrary to the presumption, basement membrane proteins are surprisingly dynamic and may help to sculp domains for tisssue repair and expansion.

***Koterniak, B., P. P. Pilaka, X. Gracida, L. M. Schneider, I. Pritisanac *et al.*, 2020 Global regulatory features of alternative splicing across tissues and within the nervous system of C. elegans. Genome Res 30: 1766-1780.**

The authors demonstrated success implementation of TRAP-seq for cell-type specific expression profiling in *C. elegans.* They identified neuron-type biased alternative splicing events, and uncovered microexons that are prone for alternative splicing. They further defined regulatory motifs for the RNA splicing factor UNC-75/CELF.

***Mutlu, A. S., S. M. Gao, H. Zhang and M. C. Wang, 2020 Olfactory specificity regulates lipid metabolism through neuroendocrine signaling in Caenorhabditis elegans. Nat Commun 11: 1450.**

This study examined how odorant cues regulates fat metabolism. Using genetic and optogenetic approaches, they defined a mechanism by which AWC olfactory neurons regulates the release

of FLP-1 neuropeptide, which acts on NPR-4/neuropeptide receptor in the intestine to modulate SGK-1 add DAF-16 signaling for fat storage.

****O'Donnell, M. P., B. W. Fox, P. H. Chao, F. C. Schroeder and P. Sengupta, 2020 A neurotransmitter produced by gut bacteria modulates host sensory behaviour. Nature 583: 415-420.**

This study started with an obserevation that worms fed certain Providencia strains display decreased avoidance of the volatile repellant 1-octanol. They then showed that some strains produce neuromodulatory tyramine, and further identified the OCTR-1 receptor expressed on nociceptive ASH neurons to modulate the aversive olfactory response. It is a comprehensive study revealing how gut bacteria alters host behavior to promote fitness of both the bacteria and its host.

***Oranth, A., C. Schultheis, O. Tolstenkov, K. Erbguth, J. Nagpal *et al.*, 2018 Food Sensation Modulates Locomotion by Dopamine and Neuropeptide Signaling in a Distributed Neuronal Network. Neuron 100: 1414-1428 e1410.**

This study dissected how food affects patterns of *C. elegans* locomotion. They identified the dopaminergic PDE neurons that can sense food through both chemosensation and mechanosensation. Combining neuron-type transcriptome with testing candidate genes, they delineate a mechanism that dopamine released from PDE acts on two downstream neurons, which then exert antagonistic effects on locomotor circuits.

****Packer, J. S., Q. Zhu, C. Huynh, P. Sivaramakrishnan, E. Preston *et al.*, 2019 A lineage-resolved molecular atlas of C. elegans embryogenesis at single-cell resolution. Science 365.**

This work represents a milestone in the single-cell transcriptome studies of the embryonic development. They mapped transcriptome from >80,000 single cell that represents different developmental time in embryogeneis with the known cell lineage of *C. elegans.* They were able to reconstruct a nearly-complete molecular atlas for each cell over the full course of embryonic development. Their work provided important insights for how such analysis can be scaled up in complex organisms. The VisCello database generated in this work will provide a valuable source to understand wiring of the nervous system.

***Reilly, M. B., C. Cros, E. Varol, E. Yemini and O. Hobert, 2020 Unique homeobox codes delineate all the neuron classes of C. elegans. Nature 584: 595-601.**

This is a systematic characterization of 101 hox genes in *C. elegans,* using the NeuroPAL system (Yemini et al, 2021). The data identified hox-code for each type of neurons.

***Tolstenkov, O., P. Van der Auwera, W. Steuer Costa, O. Bazhanova, T. M. Gemeinhardt *et al.*, 2018 Functionally asymmetric motor neurons contribute to coordinating locomotion of Caenorhabditis elegans. Elife 7.**

This study tackled the roles of the ventral cord AS-class motor neurons in locomotor circuit. They devised intersectional manipulation strategy to ablate or activate AS neurons. Combined with calcium imaging and tracking of locomotion bending patterns, they provided functional evidence that AS neurons excite the GABAergic VD motor neurons to integrate electric and synaptic inputs from pre-motor interneurons in both forward and backward movement.

***Wang, Y., X. Zhang, Q. Xin, W. Hung, J. Florman *et al.*, 2020 Flexible motor sequence generation during stereotyped escape responses. Elife 9.**

This study investigated the circuit components in touch-induced motor response. They quantitated the sequence of animal movement, following optogenetic activation of touch

receptor neurons. They defined the pre-motor interneuron circuits into backward, forward, and turning modules, and further deduced how the network of feedforward excitation and feedforward inhibition from local interneurons generate flexible motor outputs.

****Yemini, E., A. Lin, A. Nejatbakhsh, E. Varol, R. Sun *et al.*, 2021 NeuroPAL: A Multicolor Atlas for Whole-Brain Neuronal Identification in C. elegans. Cell 184: 272-288 e211.**

This technology began with an engineered multi-color transgene that is made of > 40 plus neuron-type promoters driving a combination of fluorescent proteins, resulting in each of 302 neurons with a unique color code. They demonstrated the utility of NeuroPAL in defining gene expression pattern and changes in expression in genetic mutants. They further combined NeuroPAL labeling with whole-brain imaging in live animals under chemotaxis.

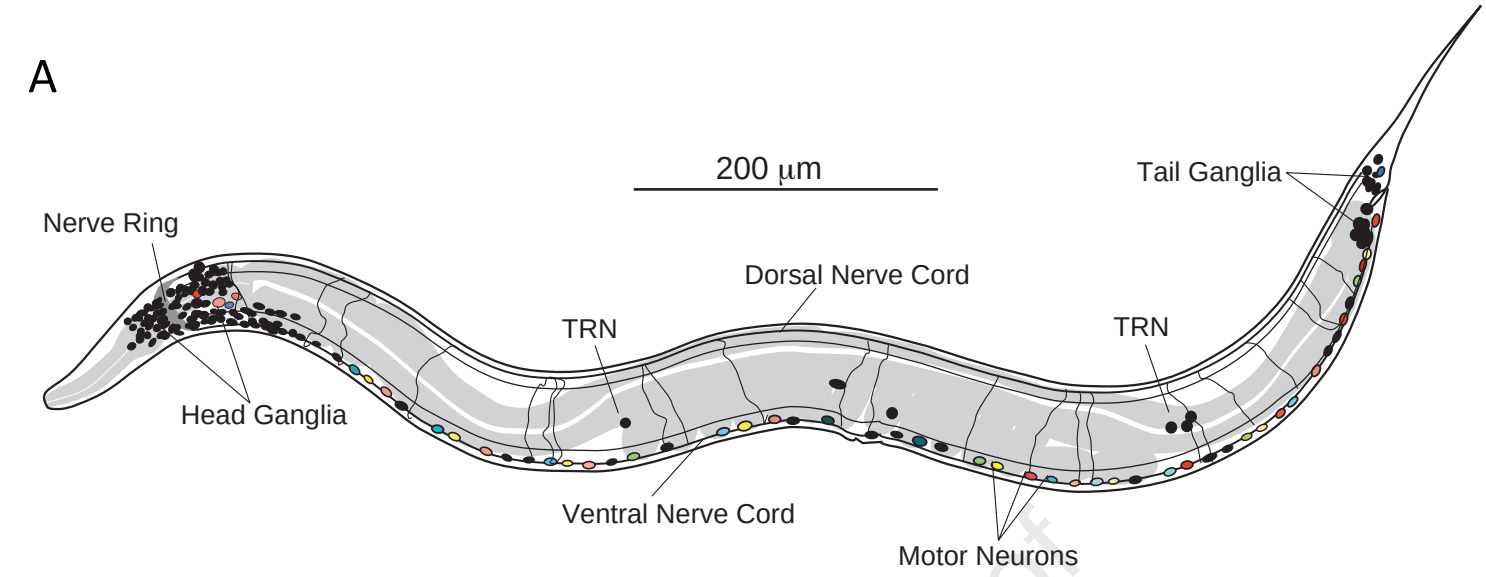
***Yu, C. J., N. C. Barry, A. T. Wassie, A. Sinha, A. Bhattacharya *et al.*, 2020 Expansion microscopy of C. elegans. Elife 9.**

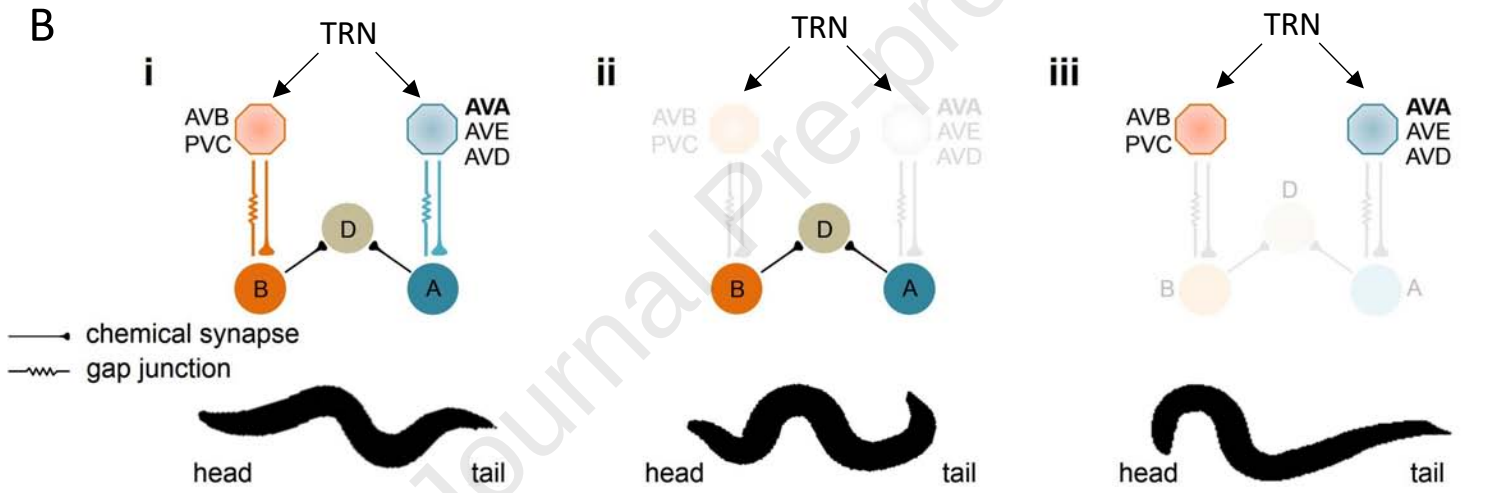
This study developed step-wise protocols to implement expansion microscopy.

Table 1: Summary of recently developed analytical and experimental technologies

Techniques	Examples*	References#
Electron Microscopy		
High pressure freezing fixation	Standard since 2004	(ROSTAING *et al.* 2004)
Image reconstruction and analytical methods	TrakEM Elegance Volume EM	(CARDONA *et al.* 2012) (XU *et al.* 2013) (MULCAHY *et al.* 2019)
CLEM (Correlated Light EM)	Phagocytosis	(CHERRA *et al.* 2020)
Light Microscopy		
Expansion Microscopy (ExCel)	Synaptic proteins, mRNAs	(YU *et al.* 2020)
Light Sheet Microscopy	Neurons and muscle	(J *et al.* 2021) (ZHAO *et al.* 2020)
Live imaging super resolution:		
Airyscan imaging	Dendritic spines	(CUENTAS-CONDORI *et al.* 2019)
SIM imaging (Elyra)	Actin-Spectrin membrane cytoskeleton	(HE *et al.* 2016)
Single-molecule localization microscopy (PALM, STORM, STED)	Active zone proteins Clathrin adaptor proteins	(KURSHAN *et al.* 2018) (LI *et al.* 2016)
Genomics		
sc-RNA-seq	Neuroblasts in embryos Mature neurons (CeNgen)	(PACKER *et al.* 2019) (HAMMARLUND *et al.* 2018)
TRAP-seq	Cell- and Tissue-type alternative splicing	(KOTERNIAK *et al.* 2020)
Neuron ID **Neuronal protein expression**		
NeuroPAL	Pan-Neural imaging Neuronal 'homeobox code'	(YEMINI *et al.* 2021) (REILLY *et al.* 2020)
Ribosome tethered GCaMP	AFD	(CHEN *et al.* 2020)
Fluorescent tagging of neuronal proteins (Crispr-Cas9-Knock-in or Fosmid reporters)	Many applications	(DICKINSON AND GOLDSTEIN 2016) (SAROV *et al.* 2006)
Chemo- and Opto-genetics		
Membrane Assembly of Electroactive Polymers	motor neurons and pharyngeal muscle	(LIU *et al.* 2020)
Split Photoactivatable Botulinum Neurotoxin	Synaptogyrin Motor neurons	(LIU *et al.* 2019)
HisCl1-histamine Silencing	Many applications	(POKALA *et al.* 2014)
Neuronal ablation (Reconstituted caspase, miniSOG)	Many applications	(CHELUR AND CHALFIE 2007) (QI *et al.* 2012)

* Examples are abbreviated, please refer to main text or original publications.
# We apologize for not able including all references.





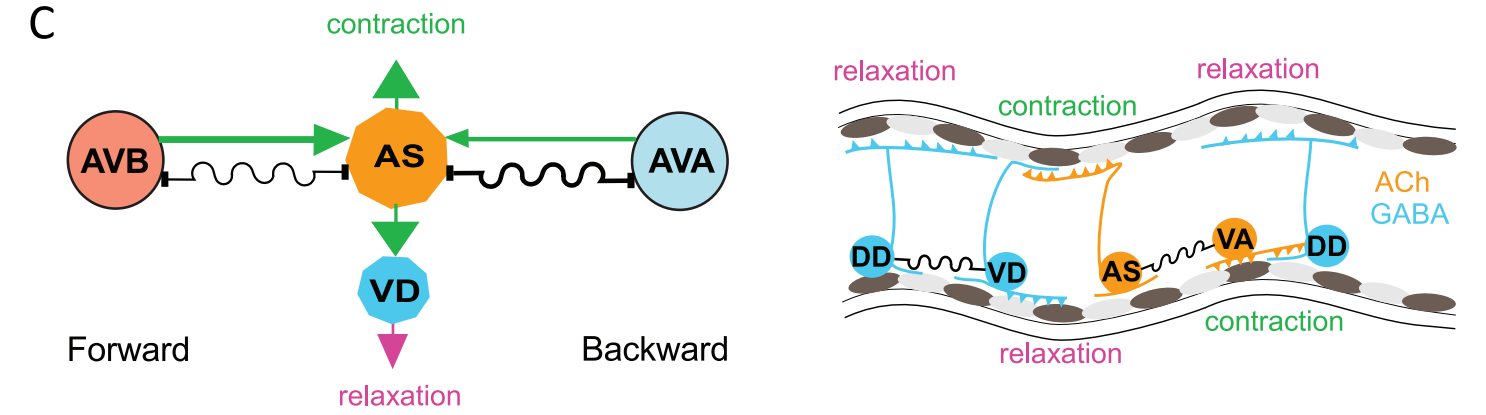


Figure 2. Summary of neuro-gut interactions.