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6 Optogenetic actuation, inhibition, modulation and readout for neuronal networks generating behavior in the nematode *Caenorhabditis elegans*

6.1 Introduction – the nematode as a genetic model in systems neurosciencesystems neuroscience

Elucidating the mechanisms by which nervous systems process information and generate behavior is among the fundamental problems of biology. The complexity of our brain and plasticity of our behaviors make it challenging to understand even simple human actions in terms of molecular mechanisms and neural activity. However the molecular machines and operational features of our neural circuits are often found in invertebrates, so that studying flies and worms provides an effective way to gain insights into our nervous system.

Caenorhabditis elegans offers special opportunities to study behavior. Each of the 302 neurons in its nervous system can be identified and imaged in live animals [1, 2], and manipulated transgenically using specific promoters or promoter combinations [3, 4, 5, 6]. The chemical synapses and gap junctions made by every neuron are known from electron micrograph reconstruction [1]. Importantly, forward genetics can be used to identify molecules that modulate *C. elegans*' behavior. Forward genetic dissection of behavior is powerful because it requires no prior knowledge. It allows molecules to be identified regardless of *in vivo* concentration, and focuses attention on genes that are functionally important. The identity and expression patterns of these molecules then provide entry points to study the molecular mechanisms and neural circuits controlling the behavior.

Genetics does not provide the temporal resolution required to study neural circuit function directly. However, neural activity can be monitored using genetically encoded sensors for Ca^{2+} (*e.g.*, GCaMP and cameleon) [7, 8, 9, 10] and voltage (*e.g.*, mermaid, arclight or VSFP-Butterfly) [11, 12, 13]. In *C. elegans*, imaging studies have focused largely on single neurons in immobilized animals [14]. However, it is now becoming possible to image the activity of single neurons in freely moving animals, and of multiple neurons in three dimensions. Additionally, increasingly sophisticated hardware allows precise spatial control of neural activity in freely moving *C. elegans*, using light activated channels and pumps (see Section 6.2). From a reductionist perspective, the worm model is very exciting because it has the potential to reveal how neural circuits work in enormous detail. This potential has fostered collaborations between physicists, engineers, and neuroscientists. Here we try to convey some of the excitement in this fast moving field.

6.2 Imaging of neural activity in the nematode

6.2.1 Genetically encoded Ca^{2+} indicators (GECIs)

C. elegans has played a key role in the development of optogenetic methods for monitoring the activities of neurons and other excitable cells *in vivo*. The primary tools for optogenetic recordings, in worms and in other organisms, are the genetically-encoded calcium indicators (GECIs). GECIs are engineered derivatives of fluorescent proteins with emission properties dependent on intracellular calcium levels. The feasibility of using GECIs to image calcium in transgenic animals was first demonstrated in *C. elegans*, with the FRET-based ratiometric indicator yellow cameleon [8] used to detect calcium transients associated with pharyngeal muscle activity as well as depolarization-evoked calcium influx in single neurons [15]. *C. elegans* is extremely well-suited to GECI-based optical recordings because of its small and transparent body, through which single-photon illumination can easily penetrate, and its small and well-characterized nervous system, which allows straightforward targeting of GECI transgenes to single or groups of identified neurons. GECIs have been used to record activity from a wide range of *C. elegans* neurons, including sensory receptors, interneurons and motor neurons [16, 17, 18, 19].

The development of GECIs with improved speed and sensitivity has increased the utility of these indicators for neuroimaging in *C. elegans*. While there has been significant improvement in ratiometric indicators, optimization has been particularly notable for non-ratiometric indicators such as GCaMP [9]. GCaMPs, which have been widely used in *C. elegans* [20], are circularly-permuted fluorescent proteins that undergo a large increase in emission intensity upon calcium binding, due to conformational changes that reduce the access of quenching solvents to the chromophore [21]. Successive rounds of mutagenesis, informed by structural knowledge, have led to orders of magnitude of improvement in signal-to-noise ratio and off-kinetics [22, 23]. Despite these significant advantages, a potential disadvantage of non-ratiometric indicators is their vulnerability to motion artifacts that may occur in behaving animals. While the reciprocal change in the emission intensities of FRET donor and recipient fluorophores provides an intrinsic control for such artifacts, non-ratiometric indicators must often be used together with, or in parallel to, a non-calcium sensitive fluorescent protein control [24].

6.2.2 Imaging populations of neurons in immobilized animals

Ultimately, understanding how the nervous system works requires us to simultaneously monitor neural activity in populations of neurons. More than half the 302 neurons of an adult *C. elegans* hermaphrodite have their cell bodies located in the head, in a volume of ~80 (x) × 60 (y) × 30 (z) microns [1]. With a 63× objective, all

these head neurons can be imaged in the same field of view, albeit at different focal planes. Optogenetic imaging requires that the target neuron or neurons be kept in the microscopic field of view. The standard solution to this problem is to immobilize the subject animal, typically by gluing it to an agarose pad in a recording chamber. This also makes it possible to apply a precisely defined stimulus to the worm (either a natural stimulus to sensory neurons, like mechanical, chemical, or thermal stimuli [17, 25–31], or by optogenetic actuators – see Sections 6.3 and 6.4); however, immobilization can interfere with the execution of behavior or even the properties of sensory circuits. One way this problem has been surmounted is using microfluidic devices, which immobilize animals more gently than glues and in some cases allow enough freedom of movement for behavior to be assayed [32, 33]. Because *C. elegans* is transparent and thin, imaging across the entire depth of the animal is possible, and optical sectioning methods (*e.g.*, using a confocal microscope) allow fluorescence emitted from different neuronal layers to be collected separately. The high capture rates possible with multi-beam scanning confocal microscopes, such as the spinning disc confocal, can achieve the required high frame rate capture. These approaches are limited by the light budget coming from the specimen, the camera speed, and fluorophore bleaching, rather than the confocal hardware. The efficiency of the latest generation of Ca^{2+} sensors make it feasible to use spinning disc confocal imaging to record the activity of populations of *C. elegans* neurons in immobilized animals. For some neuron groups, confocal sectioning does not provide sufficient resolution to separate closely apposed neurons. Use of Ca^{2+} indicators with different spectral properties (*e.g.*, combining R-GECO [34] or RCaMP [35] and GCaMP) may provide a way around this difficulty. Ultimately, the goal must be to image neural populations in freely moving animals. Although daunting, this is becoming possible in *C. elegans*.

6.2.3 Imaging neural activity in freely moving animals

The transparency of *C. elegans*, combined with improvements in automated tracking microscopy affords the possibility, at least in principle, of monitoring neural activity in behaving animals. Monitoring neural activity in freely behaving animals permits neurons and neural circuits to be linked to behavior. Unlike experiments carried out with immobilized or sedated animals, studying freely moving animals allows temporal relationships between neural activities and behavioral outputs to be established; it enables feedback from motor activity to be identified; and it permits the succession of neural activities that underlie behavioral sequences to be elucidated.

Imaging neural activity in freely moving *C. elegans* presents challenges. Unrestrained animals can move quickly, and enough light has to be collected in a brief time interval to obtain reliable signals. High magnification objectives with high numerical aperture (NA) are optimal for light collection and resolving power. However, they

provide a small field of view and have a shallow depth of field. Their use requires ways to efficiently re-center the moving animal to keep it in the field of view. Several methods have been designed that use feedback loops to rapidly re-center XY motorized stages [19, 36–39]. An alternative solution is to reduce the animal's locomotory activity; for example, by putting it on partially dry, sticky substrates. Shallow microfluidic devices make it possible to minimize movements in the Z axis, by slightly squeezing the animal onto its substrate. An alternative solution to the focus problem involves imaging in 3D, capturing different focal planes by moving either the objective or the stage [40].

Imaging neural activity at low magnification (10× or lower) reduces these difficulties. Low NA lenses have large depth of field. The trade-off is poorer light collection and resolving power. The large dynamic range of the latest generation of Ca^{2+} sensors (*e.g.*, YC3.60 and GCaMP3 and higher) [7, 41] provides sufficiently large signals, such that they can be used with low magnification lenses in favorable circumstances; that is when it is possible to drive strong expression of the sensor without perturbing neural properties and when the neural response is strong.

6.2.4 Other genetically encoded indicators of neuronal function

Although GECIs have been the most widely used optogenetic indicators, genetically encoded sensors to monitor other aspects of neuronal function are also desirable. Sensors of presynaptic activity, such as synaptophluorin [42], have been successfully used in *C. elegans*, but their relatively low sensitivity has made them more suitable for measuring neural activity over time than for dynamic neuronal recording [43]. Recently, a genetically-encoded glutamate sensor has been described that can detect synaptic glutamate signaling in intact worms [44]. Similar sensors for other neurotransmitters would clearly be very useful. Finally, the holy grail of optogenetic indicators would be a voltage sensor that could directly measure membrane potential, with sensitivity comparable to the present generation of GECIs. A number of promising candidate molecules have recently been generated [11, 12, 45], though thus far none has been successfully used in *C. elegans*.

6.3 Optogenetic tools established in the nematode

6.3.1 Channelrhodopsin (ChR2) and ChR variants with different functional properties for photodepolarization

C. elegans was the first animal in which Channelrhodopsin-2 (ChR2) and Halorhodopsin (NpHR) were expressed and utilized as optogenetic tools [46, 47]. Muscle contractions or relaxations were the first proof of light-evoked de- or hyper-polarization in

this system, which strictly depended on the presence of the obligate chromophore all-*trans* retinal (ATR). This was confirmed by directly measuring photocurrents in *C. elegans* muscle. In addition, several neuron types were probed using these tools, namely mechanosensory neurons, photoactivation of which evoked an escape reflex, as well as cholinergic motorneurons, in which photoactivation or inhibition evoked downstream muscle contraction or relaxation. The two proteins could also be concomitantly used for independent control of two cell types [47].

Several ChR variants with different functional properties and thus, applicability, could be established in the meantime for use in nematodes. These include C128X mutants, also called "step-function opsins", with a slowed-down photocycle, which allow for photostimulation at low light intensities and for prolonged activation in the minute (or, with repetitive stimuli, hour) time range [48]. The ChR2 variant H134R; T159C exhibits higher expression levels and largely increased photocurrents, thus evoking strong effects at low light intensities, and was combined with the spectrally red-shifted chimeric variant C1V1-ET/ET (a fusion of ChR1s from *Chlamydomonas* and *Volvox*), to achieve independent two-color photostimulation of two different cell types in the same animal [49].

6.3.2 Halorhodopsin and light-triggered proton pumps for photohyperpolarization

Halorhodopsin is a yellow-light-driven Cl$^-$ pump that mediates photoinhibition in *C. elegans* [47]. However, NpHR shows poor membrane insertion in this system, so most of the synthesized protein never reaches the plasma membrane and for successful application, "strong" promoters are needed. Trafficking signals used in mammalian cells to improve this were, unfortunately, not recognized in *C. elegans* [50, 51]. Thus, as an alternative, outward directed H$^+$ pumps from *Leptosphaeria maculans* ("Mac") and Archaerhodopsin3 ("Arch") from *Halorubrum sodomense* were also introduced as hyperpolarizing optogenetic tools [51, 52]. These work very efficiently and respond to blue-green wavelengths, thus allowing inhibition either independent of, or concomitant with, ChR2 mediated activation.

6.3.3 Photoactivated Adenylyl Cyclase (PAC) for phototriggered cAMP-dependent effects that facilitate neuronal transmission

A qualitatively and quantitatively different type of neuronal stimulation can be achieved with photoactivated adenylyl cyclase (PACα) from *Euglena* [53]. This is a protein containing a BLUF domain (blue light sensor using flavin) and an adenylyl cyclase domain. The protein generates cAMP from ATP in a light-dependent fashion, and thus permits it to trigger processes that depend on this second messenger, most prominently to stimulate protein kinase A (PKA) activity. In neurons, PKA activity

appears to increase the rate of priming of synaptic vesicles (SVs), thus more SVs can be released upon neuronal activation. Accordingly, PAC activity accentuates intrinsic activity patterns, but does not override them, like the strong stimulation achieved via ChR2. In *C. elegans* cholinergic neurons, PACα activation led to exaggerated locomotion and an increase in the rate of miniature post-synaptic currents (mPSCs) [54]. However, as PKA has a multitude of downstream targets, it is not clear which other effects may be evoked by PACα photoactivation. PACα from *Euglena* has a comparably high dark activity, thus expression levels need to be carefully adjusted to avoid basal cAMP elevation that leads to compensatory effects. As an alternative, PAC from *Beggiatoa* has been introduced [55, 56], which has very low dark activity and is highly light sensitive due to a slower photocycle, and several labs are using this now in *C. elegans*.

6.3.4 Other optogenetic approaches

Other optogenetic approaches, *e.g.*, optochemical genetics, where endogenous ion channels or receptors are rendered light sensitive by modifying them with covalently tethered, photo-isomerizable ligands [57, 58], have not been described yet for *C. elegans*. Yet, this approach provides an additional route to triggering neuronal activity, and importantly, allows stimulating individual receptors at the post-synapse. This differs from ChR2-mediated pre-synaptic stimulation, which also spreads to other synapses innervated by the same neuron and may have further effects. This methodology requires carefully designed photoswitchable ligands and strategically positioned cysteines in the respective ion channel, to which the ligands are covalently linked.

An approach to trigger GPCR pathways using optogenetics has been described, in which visual rhodopsin is modified in its intracellular loops that bind and activate heterotrimeric G-proteins. When the respective loops from α- or β-adrenergic receptors were transferred, they mediated coupling to G_s or G_q proteins [59]. This has not been used for *C. elegans* yet, however, along these lines, mammalian rhodopsin and melanopsin were expressed in *C. elegans* motor neurons and coupled to $G_{i/o}$ or G_q pathways, respectively, affecting locomotion behavior [60].

6.3.5 Stimulation of single neurons by optogenetics in freely behaving *C. elegans*

To specifically influence neurons by optogenetics, one can generate animals that express the optogenetic tool in just the neuron of interest, and thus use straightforward whole-field illumination, as only the transgenic neuron will be activated. This can be achieved by expressing ChR2 or other optogenetic tools via conditional expression. Using two promoters whose expression patterns overlap in just the cell of inter-

est, a recombinase is expressed from one promoter to activate an otherwise silent construct for expression of ChR2 from the other promoter [4, 5, 6].

Often, however, single-cell expression is difficult to achieve, such that one ends up working with transgenic animals that express the respective tool not only in the neuron of interest. In such cases, one can restrict the light delivery to just the cell of interest, sparing other cells. Microscope systems have been developed that allow selective illumination of distinct neurons (or rather, body regions) in freely behaving animals. One such system uses an LCD video beamer to project a segmented binary image of the animal onto the body region of the animal that contains the respective neuron(s) of interest [61, 62]. The animal is tracked using a computer-controlled x-y translational stage, and the system is updated at a maximum frequency of 25 Hz, thus ensuring that the respective neuron is faithfully kept in the light. The system can transmit light of different colors and intensity via its three independent light paths, and it was used to analyze circuits for nociception [51, 63]. A second system with basically similar properties was developed (the software is faster; it updates at a maximum frequency of 50 Hz), and uses a digital mirror device (DMD) to reflect light from an expanded laser beam onto the specimen, to generate the light geometries needed for cell specific illumination [64]. This system was used to analyze the influence of proprioception during locomotion [18]. A third system described for selective illumination uses a spatial light modulator (liquid crystal on silicon chip), however this system does not track the animal automatically and thus the animal's movement was restricted in a microfluidic arena. This system was used to analyze the function of O_2 sensing neurons [65].

A recent, remarkable approach, that further developed the tracking and selective illumination systems described above, realized the generation of a virtual environment by using dynamic optogenetic feedback, depending on the automatically detected behavior of the animal [39]. First, the authors observed by Ca^{2+} imaging the activity of AIY interneurons, which act downstream of chemosensory neurons, in animals navigating in an odorant gradient. Then they used animals expressing ChR2 in AIY neurons to mimic the inferred activity of AIY in a virtual odorant gradient, while the animals crawled on a plate without any actual odorants. The tracking system registered the movement of the nose of the animal within the artificial gradient and increased or decreased the light intensity accordingly, when the animal turned its nose towards or away from the virtual chemoattractant source. An earlier attempt towards the goal of generating virtual environments using optogenetics was to "simulate" a region of aversive chemicals, *e.g.*, high osmolar solution. This was done by photoactivating the aversive chemosensory neuron ASH whenever the animals' nose entered the area of the sham aversive chemical [37].

6.4 Examples for optogenetic applications in *C. elegans*

6.4.1 Optical control of synaptic transmission at the neuromuscular junction and between neurons

Chemical synaptic transmission in *C. elegans* is generally believed to be graded. Thus, it can be conveniently studied using optogenetic methods, as one can stimulate neurons precisely, and cell-type specifically, with a light stimulus (in contrast to electrical stimulation), and the extent of neurotransmitter release can be finely tuned by the light intensity used. As readout, one can quantify evoked behavioral effects, *e.g.*, body contraction following stimulation of cholinergic motor neurons, or record photoevoked postsynaptic currents. Stimulation can be repeatedly done, allowing one to study plasticity. To analyze postsynaptic transmitter receptors, optogenetics allows stimulating transmitter release at synaptic sites only and in native amounts, in contrast to the frequently used pipette-application.

The first optogenetic studies of synaptic transmission were concerned with the neuromuscular junction (NMJ). At this synapse, muscle cells are innervated by cholinergic and GABAergic motor neurons. Liewald *et al.* [66] and Liu *et al.* [67] analyzed transmission from cholinergic and from GABAergic neurons using ChR2-mediated photostimulation. Graded transmission could thus be confirmed for these neurons [67, 68]. A number of pre- and postsynaptic mutants were analyzed, and phenotypes observed by optogenetic stimulation could be compared to those evoked electrically [66]. Postsynaptic ACh receptors at the NMJ were also investigated using optogenetics [67, 69], as well as a GABA$_B$ receptor [68]. Furthermore, PACα was used to photoevoke increased synaptic transmission; however, not by depolarizing the neurons, rather, by increasing the rate at which transmitter vesicles would fuse with the plasma membrane in response to depolarization, as well as increasing the amplitude of postsynaptic currents per release event, indicating higher transmitter content [54]. Additionally, two papers probed the role of gap junctions in the neuromuscular system [70, 71].

Interneuronal synaptic transmission has also been analyzed by optogenetics in *C. elegans*. Previously, such analyses were restricted to neuron-neuron synapses involving sensory neurons, to which a natural stimulus was presented, and the postsynaptic response was recorded. Photo-electrophysiology can be used to analyze interneuronal transmission without a natural stimulus, thus making "central" synapses accessible. Thus far, two different synapses were analyzed by optogenetics, namely between the thermosensory AFD neuron and the interneuron, AIY [72], as well as between the polymodal nocisensory neuron ASH and the premotor-interneuron AVA [73]. In both synapses it was found that transmission was graded, *i.e.*, transmitter release increased proportionally with increasing light intensity. This emphasizes that results obtained from synaptic transmission experiments using optogenetics will depend also on the amount of ChR2 expressed at the respective synapse, and that alterations in ChR2's peak current during prolonged or repeated stimulation have to be taken into account [66, 67].

6.4.2 Optical control of neural network activity in the generation of behavior

In addition to work on the chemotaxis circuit [39], described in Section 6.4, several other neuronal networks and their roles in behavior have been analyzed by optogenetics in *C. elegans*. The first neurons photostimulated were the touch receptor neurons (TRNs) [46]. Photostimulation caused escape behavior, and behavioral habituation was observed upon repeated optical stimulation, just as in mechanically evoked behavior. This could be achieved independently of the MEC-4/MEC-10 mechanoreceptor channel, emphasizing that habituation is not merely due to desensitization of this channel. The TRNs were subject to more detailed work using optogenetics, in which single TRN contribution to behavior was analyzed using patterned illumination [62, 64].

The TRNs evoke backward locomotion by influencing signaling in the premotor interneurons AVA, AVD and AVE, which control A-type motor neurons for backward locomotion, while signaling to the interneurons AVB and PVC mediates forward locomotion through activation of B-type motor neurons [74, 75, 76]. The locomotion premotor interneuron circuits, and other neurons involved in the control of forward versus backward locomotion, were subject of several optogenetic studies. The generally accepted role of the AVA and PVC interneurons to control backward and forward locomotion, respectively, have been confirmed by direct photostimulation or photoinhibition [4, 51, 62, 63]. Other cells through which backward locomotion can be influenced by optogenetics are the RIM interneuron [77], as well as a second circuit acting in parallel to the "classic" AVA B-type motor neuron circuit for backward locomotion, which involves inhibitory signaling from AIB to RIM [38]. How locomotion itself is evoked and influenced by motor neurons and how proprioception leads to propagation of the locomotory wave of contraction and relaxation, and influences the body posture, has been subject to an elegant study using optogenetics, imaging and carefully designed microfluidic devices, to manipulate parts of the animal's body, and of the nervous system [18].

Locomotion is also controlled by other sensory neurons. Aversive mechanical or chemical sensory perception, which can also be interpreted as nociception, leads to rapid escape responses. Here, the polymodal neuron ASH (and modulation of its output), as well as the harsh touch receptors FLP and PVD, were analyzed using optogenetics [37, 63, 77, 78]. For PVD, optogenetics were further used to identify and study genes acting within the nociceptor, downstream of the primary nocisensor molecules, for encoding and/or transmission of these signals to downstream circuits [63].

Further sensory modalities that were addressed by optogenetic manipulation (and other methods) are the sensory circuits for oxygen and CO_2 [65, 79], and for temperature [80], as well as circuits for mechanosensation used during male mating behavior [81].

6.5 Future challenges

6.5.1 Closed-loop optogenetic control and optical feedback from behavior and individual neurons

To fully understand how a neuronal circuit generates behavior, one would need to achieve true optogenetic *control* over a neuronal circuit, rather than optogenetic *perturbation*, as is currently the case. *Control* would require an "optical voltage clamp", which would allow suppressing all intrinsic activity, and instead imposing the activity from outside, using optical signals. This type of manipulation should be coupled with a non-invasive but fast feedback, such that activity patterns and the extent of signals could be monitored, and corrections or new control paradigms be imposed accordingly by the optogenetic manipulation, in a closed-loop. Furthermore, in this way, it would be possible to stimulate or inhibit particular cells and determine the effect on other neurons in the circuitry. What exactly do we mean by "closed loop optogenetics", and how could this be achieved?

In a first approximation, we can treat neurons in *C. elegans* as simple entities that receive excitatory or inhibitory input, and accordingly release more or less transmitter themselves, at all of their synapses, and/or influence other cells by gap junction electrical coupling. Control over membrane potential can be achieved using light-gated ion channels or pumps, like ChR2 or NpHR, Mac and Arch3. Some of these can be independently controlled by light of different wavelengths; the most spectrally separated are ChR2 and NpHR. If these proteins are co-expressed, and if the mediated photocurrents are strong enough, one could, in principle, very precisely regulate the membrane potential of that neuron using these two optogenetic tools. The neuronal membrane, of course, is not unaffected by such exogenously induced currents – rather, voltage gated Ca^{2+}- and K^+-channels (it is generally accepted that there are no voltage-gated Na^+-channels in *C. elegans*) will respond to this by currents of their own, which will affect the membrane potential. In order to compensate for this, one would have to be able to very quickly measure (at sub-millisecond timescales) the membrane potential, and induce compensatory light-gated currents accordingly. In a behaving animal, non-immobilized and non-dissected, this can only be realized with a fast optical voltage sensor, *e.g.*, genetically encoded, whose excitation and emission spectra would have to be far removed from the ChR2 and NpHR spectra, such that the optical readout of membrane potential does not *per se* interfere with the optogenetic actuators. Above all, the behavior itself also needs to be tracked and interpreted, such that feedback to the optically controlled neuron can be computed and integrated into the stimulus regime. This would require that training of the software needs to occur, such that for a given inferred activity, it can be predicted which behavioral outcome this may have. Similar to the work by Kocabas *et al.* [39], recording membrane voltage from the neuron of interest during spontaneous behavior may first be used to learn which natural activity patterns are associated with which type of behavior. A summary of these points is shown in Figure 6.1.
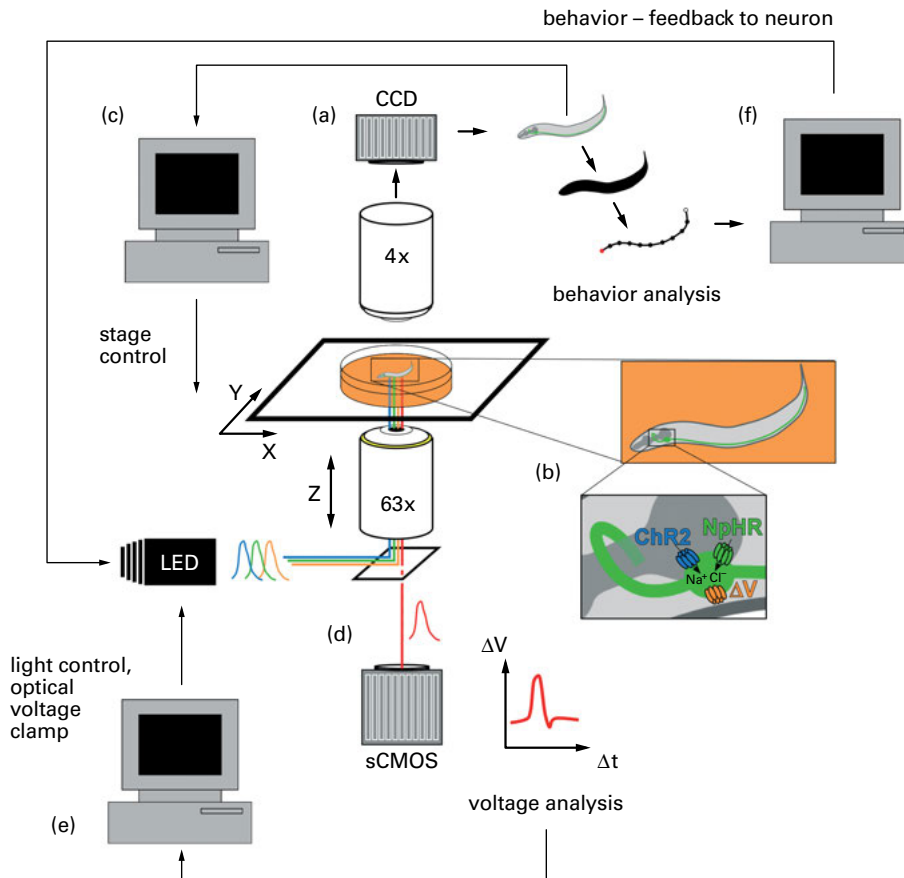
Figure 6.1: How closed-loop optogenetics could work. (a), (b) An animal expressing an optogenetic actuator (ChR2) and inhibitor (NpHR), as well as an optical voltage sensor (ΔV) is allowed to crawl on a culture dish. (c) The position of the animal is monitored by a low magnification objective and used to steer an XY translational stage, to follow the animal. (d) A high NA, high resolution objective is used to image voltage signals, and to guide light to the optogenetic actuators. This objective can be used to quickly scan through different focal planes, to image several neurons. (e) Voltage signals are used to quickly compute a feedback of light signals to the optogenetic actuators, to keep the neuron membrane potential at the desired membrane voltage. (f) Behavior can be analyzed on-line, to provide feedback signals to the actuators that would allow true optogenetic control of the behavior.

To truly recapitulate natural behavior this way, one likely has to incorporate information and control of several neurons simultaneously, a major challenge for both the molecular biology to generate the respective transgenic animal, but also for microscopy hardware, readout and light delivery techniques, tracking and most importantly, for software development that allows fast computing and rapid dynamic feedback to the light actuators in each of the neurons involved.

6.5.2 Requirements for integrated optogenetics in the nematode

What do we need to work out for a closed loop all-optical system for control of animal behavior?

Optogenetic actuators and inhibitors for optical voltage clamp and combination with GECIs:
Here, we may already have a good set of tools that allow us to achieve this type of control, particularly ChR2 and NpHR, whose action spectra are nicely distinct. These tools have been fused into a tandem protein [82], which allows for precise control of the relative expression levels, and reduces uncertainties in the extent of effects of illumination with each light wavelength, due to otherwise variable expression levels of either protein. Unfortunately, there is significant overlap in the action spectra of optogenetic modifiers and indicators; thus, recording neural activity with indicators such as GCaMP or cameleon can lead to inappropriate activation of channelrhodopsin in the same animal. To some extent this problem can be overcome by using patterned illumination and sophisticated optics to separately excite the neurons whose activities are modified and monitored [77]. However, in moving animals, or in circuits where the neurons are closely packed, this approach has limitations. Another option is to shift the activation spectrum of either the modifier or activator, typically to a longer wavelength. Some progress has been made on this front, with the development of red-shifted indicators such as RCaMP [35, 63] and R-GECO [34]. However, the signal-to-noise ratio of RCaMP is relatively low, and R-GECO is susceptible to photoconversion, which could lead to artifacts when used together with channelrhodopsin [35]. Thus, improved red-shifted indicators with better spectral separation and better sensitivity are clearly desirable.

Optical measure of membrane voltage:
This is a much more challenging problem, as currently existing genetically encoded voltage sensors provide rather small signals and are slow, and thus far have not been widely used in *C. elegans*. Since they generally do not produce action potentials, *C. elegans* neurons also do not exhibit very large voltage changes, making their resolution by voltage indicators even more challenging. The excitation and emission spectra of the currently available voltage sensors are also not compatible with independently using ChR2 and NpHR in the same cells, as the sensors are based on fluorescent proteins requiring excitation in the same spectral range [12, 83–85]. An exception may be the recently described finding that retinal in proteins like Arch3 or proteorhodopsin can act as a red-fluorescent sensor for membrane voltage [45, 86]. However, the fluorescence of retinal is extremely dim, such that it is not conceivable that this tool will produce sufficient signal in the tiny *C. elegans* neurons. One way to possibly increase the fluorescence, and to shift the spectrum to the far red, could be retinal analogs with altered π electron system; such chromophores can be simply

provided to *C. elegans* with the bacterial diet. They would, however, also be affecting the actuator rhodopsins (ChR2 and NpHR), unless they would be specifically incorporated in Arch and not the other proteins.

Integrated behavioral tracking, voltage imaging and on-line analysis software:
This is not a trivial task either, as the computations required to detect a worm, follow it, determine its outline, segmenting it to guide the light delivery, imaging voltage signals and interpreting them, and the interpretation of a particular behavior are time consuming. At the same time, these computations need to be fast, such that the system can update itself with at least a frequency of 25 Hz, 50 Hz or even faster. The hardware to achieve this may exist already, as several imaging and tracking systems have been developed; they may have to be wed in a fruitful way. Light delivery in at least two color channels is needed, and a third color channel needs to be used for voltage sensor excitation and imaging. Integrating all information will likely require novel and ingenious algorithms, and extremely fast computers.

Optics, microscopes, cameras:
Neurons of *C. elegans* are not found in the same focal plane, at least if they are to be imaged at somewhat high resolution, and high NA objectives are needed for imaging, particularly if the fluorescent signals are small. The field of view of such objectives is too small to image the whole animal and to track behavior at the same time, thus two optical systems are required, one at high and one at low magnification [37]. To image and to deliver light to neurons in different focal planes, fast scanning in the third dimension (z) will be required, generating an additional challenge. Fast cameras with high resolution are available now (sCMOS cameras), that allow high frame rates and can capture larger fields of view, allowing one to image bigger portions of the animal. They produce high volumes of image data, the handling of which requires large memory and powerful computers.

An all-optical imaging, tracking, and actuation system will be further complicated by the biology to be studied; additional, unknown neurons will influence the behaviors controlled via the chosen set of optically targeted neurons. This may involve physical synaptic connections that can be probed, and since they are mapped, the neurons potentially influencing the cells under study are known [1]. However, the "wireless network", *i.e.*, remote signaling by neuromodulators and neuropeptides, is much more difficult to monitor and to control, and thus the systems we laid out here will still be only marginally able to recapitulate signaling and network activity occurring during natural behavior.

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