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Light-microscopy methods in C. elegans research

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Abstract

Ever since *Caenorhabditis elegans* was introduced as a model system it has been tightly linked to microscopy, which has led to significant advances in understanding biology over the last decades. Developing new technologies therefore is an essential part in the endeavor to gain further mechanistic insights into developmental biology. This review will discuss state-of-the-art developments in quantitative light microscopy in the context of *C. elegans* research as well as the impact these technologies have on the field. We will highlight future developments that currently promise to revolutionize biological research by combining sequencing-based single-cell technologies with high-resolution quantitative imaging.

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Introduction

Its small size, transparency and an easily accessible genetic system make *Caenorhabditis elegans* a great model organism for imaging, a role that has been shaped by several key properties. Apart from very few exceptions its cell lineage is invariant [1] and all individual cells can be followed optically in wild-type and mutant animals using differential interference contrast (DIC) microscopy. Therefore, the complete cell lineage from zygote to adult was described even before the advent of fluorescent proteins [1,2]. *C. elegans* allows to perform experiments involving large numbers of isogenic animals ensuring statistical robustness and making it a powerful model organism for genetic high-throughput screens. Additionally, it is one of the few model organisms that can be imaged in its entirety using electron microscopy (see *Hall D.* et al. [3] for a comprehensive review), and it was the first animal used to express Green Fluorescent Protein (GFP) [4]. Since these early days, light microscopy has made remarkable advances. This review will discuss the current state-of-the-art of light microscopy in *C. elegans* research published over the last 10–15 years, touch on exciting studies these technologies enabled, and discuss future opportunities arising from current developments in sequencing-based single-cell technologies.

Fluorescent proteins

Fluorescent microscopy is a dynamically evolving field and a key element of *C. elegans* research. New fluorophores are continuously developed and adapted for *C. elegans* [5], while new genetic tools ease endogenous tagging of proteins [6]. Fluorescent protein (FP) tags are used for a wide variety of studies. This includes localization and dynamics of proteins, organelles and anatomical structures [7,8], targeted degradation of tagged proteins [9,10] and monitoring of physiological parameters such as concentration of calcium ions or voltage in muscles and neurons [11], that are often combined with quantitative image analysis [12,13].

FPs are either expressed using extrachromosomal arrays [14] or are stably integrated into the genome. Stable integration can now be mediated using CRISPR [6,15], which, however, can still be challenging. Therefore, random or site-specific integration of constructs using bombardment [16] or the mosSCI system [17,18] continue to be commonplace. Notably, constructs limited to only the promoter region can lack regulatory elements, therefore the use of fosmids that include larger regions around a gene can be advantageous [19,20].

Continuously improved GFP variants [21] represent the most prominent FP tags used by the *C. elegans* community, however, non-GFP-derived FPs such as mScarlet [6] or photoconvertible fluorophores for super-resolution imaging [22] are also available [23,24]. Importantly, FPs that show good optical properties *in vitro* or in other model organisms, do not necessarily behave similar in *C. elegans*. Brightness, protein stability, or photostability can be influenced by cellular pH, expression level regulation, maturation times, or silencing of constructs. FP performance can be

controlled by a number of factors including the type of expression construct, codon optimization, tissue-specificity and microscopy set-up [25,26]. A collection of FPs commonly used in *C. elegans* is described in Table 1. However, it is important to note that many new developments in fluorescence technology such as HALO [27] or MS2-based transcription imaging [28] yet need to be adapted to *C. elegans*.

FPs are also the basis for more advanced fluorescent microscopy techniques. Fluorescence recovery after photobleaching (FRAP) can detect protein dynamics in living cells [29,30] (Figure 1a–c) and is for example used to study dynamics of signaling molecules [31] or to measure tissue- or cell-specific protein synthesis rates [32]. Near-TIRF (total internal reflection fluorescence) microscopy enables tracking of individual proteins [33], and Förster Resonance Energy Transfer (FRET) measures distances between FPs of different wavelength with nanometer precision. FRET-distances can be read out directly or through changes in fluorescent lifetime (FLIM-FRET), which was for example used to study chromatin compaction [34].

Besides FP expression, immunofluorescent (IF) labelling is a straight-forward method that can be easily combined with other assays since it does not require genetic engineering [35]. However, it does rely on the availability of specific antibodies for the target protein. Furthermore, it is often limited to fixed tissues, with the notable exception of newly engineered modification-specific intracellular antibodies (mintbodies) [36] (Figure 1d). IF labelling gained additional importance as it can be used as basis for super resolution techniques [22,37].

Nucleic acid imaging

DNA can be visualized directly using stains like DAPI (Figure 1e and f) or indirectly through tagged, DNA-associated proteins like histones (Figure 1a). The repressor and binding site system lacO/lacI allows to follow specific DNA sequences *in vivo*, which, for example, led to the discovery of sequences involved in genome organization [38,39]. 3D genome architecture can be visualized using DNA-FISH (fluorescent in-situ hybridization) based approaches on the level of chromosomal sites up to entire chromosomes [40–42].

The quantification of spatio-temporal gene expression patterns is essential for studying gene function in development. In addition to FPs and IF, transcription can be more directly measured using RNA (fluorescent) in-situ hybridization [43]. This powerful technique is for example used to study translational regulation of mRNAs [44] or localization of miRNAs [45]. Like IF, in-situ approaches do not require genomic engineering but rely on probes complementary to the DNA/RNA of interest. Detecting individual RNA molecules by single-molecule RNA-FISH (smFISH) enables a truly quantitative understanding of transcription in single cells [46–49] (Figure 1e), which can be combined with IF to simultaneously detect RNA and protein [50]. Separate probes for intronic and exonic sequences enables detection of nascent transcription to quantify transcription dynamics [51], an aspect that is still understudied in *C. elegans*. Additionally, the possibility to combine a large number of IF and/or FISH measurements on the basis of the fixed lineage holds exciting potential for *in-toto* studies of gene function.

Lineage tracing and lightsheet microscopy

In order to capitalize on the fixed *C. elegans* lineage, approaches aiming for automated lineage tracing and staging using widefield and confocal microscopy have been developed [13,52–54], thereby facilitating the creation of open resources [55,56]. However, variable nuclei positions and division times, dense nuclei packaging, and fast movements during late embryogenesis and larval stages make robust nuclei assignment a challenging problem that has not yet been solved in its entirety and requires manual correction or annotation.

C. elegans images are traditionally acquired using confocal or widefield microscopy with samples mounted on coverslips. These images provide very high spatial resolution in the lateral (xy) dimension, while showing reduced axial (z) resolution and relatively high bleaching rates (Figure 1g). Consequential sample compression additionally creates physical forces that counteract regular cellular forces [57], thereby modifying cell lineage parameters in live acquisitions [58] and activating compensatory mechanisms [7]. Compression can be circumvented using beads enforcing a minimum distance between coverslip and slide, with the additional benefit of immobilizing larvae and adults for imaging [59,60], albeit at the cost of increased bleaching and reduced imaging speed due to the larger sample volume in z.

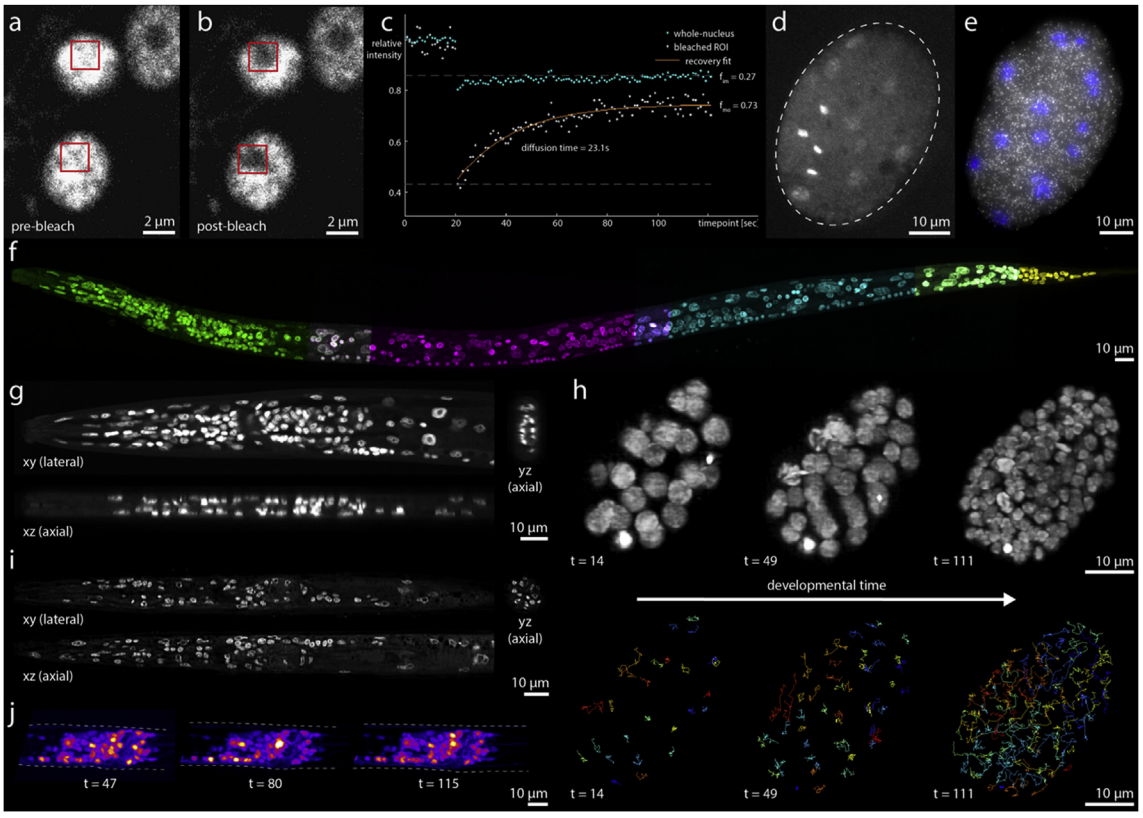
Lightsheet microscopy [61–63] is an emerging technology that enables acquisition with high spatial and temporal resolution while minimizing photobleaching and phototoxicity. Additionally, samples are embedded in agarose or glued to coverslips preserving their normal shape. Significantly increased image quality enabled the development of improved lineage tracing tools for *C. elegans* embryogenesis, often taking advantage of the isotropic spatial resolution [64,65] (Figure 1h). Recent approaches further employ lineage-specific nuclei labelling or photo-conversion [66] in addition to ubiquitously expressed nuclei markers to further ease the nuclei matching problem or to constrain it to subsets of nuclei [12,53,67]. However, to achieve said isotropic resolution (Figure 1i), multiple 3D images of the same

Table 1

Fluorescent proteins commonly used in *C. elegans* research [141–146].

Fluorescent protein	Excitation max. (nm)	Emission max. (nm)	Reference/Source	Comment
mTagBFP2	399	456	Addgene; Sands, et al., 2018 [141]	
mCerulean3	433	474	Sands, et al., 2018 [141]	Used in FRET, paired with Venus or Citrine
CFP	433	475	Addgene; Tursun, et al., 2009 [20]	
mTFP	462	492	Addgene; Kelley, et al., 2015 [142]; Sands, et al., 2018 [141]	Used in FRET, paired with Venus or Citrine
EGFP	488	507	Addgene; Frøkjær-Jensen, et al., 2016 [143]	
Dendra2	490/553	507/573	Addgene; Griffin, et al., 2011 [144]	Photoconvertible (green to red)
mNeonGreen	506	517	Dickinson, et al., 2015 [24]; Heppert, et al., 2016 [5]	
YFP	514	527	Addgene; Tursun, et al., 2009 [20]	
Venus	515	528	Addgene; Tursun, et al., 2009 [20]	Used in FRET, paired with Cerulean
mECitrine	514	529	Sands, et al., 2018 [141]	Used in FRET, paired with Cerulean
mYPet	517	530	Addgene; Dickinson, et al., 2015 [24]; Heppert, et al., 2016 [5]	
mKO2	551	565	Sands, et al., 2018 [141]	
dTomato	554	581	Weinheimer, et al., 2015 [145]	
TagRFP-T	556	585	Addgene; Dickinson, et al., 2015 [24]; Heppert, et al., 2016 [5]	
mScarlet	569	592	Addgene; ElMouridi et al., 2017 [6]; Sands, et al., 2018 [141]	
mRuby2	559	594	Addgene; Heppert, et al., 2016 [5]	
mCherry	586	610	Addgene; Tursun, et al., 2009 [20]; Heppert, et al., 2016 [5]	
mKate2	589	634	Addgene; Dickinson, et al., 2015 [146]; Heppert, et al., 2016 [5]	
mNeptune	599	634	Addgene; Sands, et al., 2018 [141]	

Figure 1



Examples of current state-of-the art imaging approaches in *C. elegans*. (a,b) Example of FRAP analysis on H2B-mCherry signal from *C. elegans* L1 larvae tail nuclei acquired on a Leica SP8 confocal microscope. Red squares indicate areas used for laser inactivation of fluorophores by bleaching and temporal recording to detect protein dynamics. (c) Example analysis of a FRAP experiment. The mobile and immobile fractions are calculated by fitting of the bleaching-corrected fluorescent signal recovery curve. (d) A single image plane through a live embryo expressing a H4K20me1-mintbody fused to GFP is shown ([36], the mintbody line was a kind gift from Akatsuki Kimura). H4K20me1 is detected at mitotic chromosomes in early *C. elegans* development as published in Ref. [139]. (e) Maximum intensity projection of a young *C. elegans* embryo stained with DAPI (blue) and smFISH probes against the W04G3.5 mRNA (grayscale). (f) Maximum intensity projection of a DAPI-stained dauer larva acquired on a scanning laser confocal microscope. Different colors indicate independently acquired, overlapping 3d image tiles, stitched together as described in Ref. [71]. (g) Magnification of a DAPI-stained dauer larva similar to (f), highlighting the physical compaction in z and extension in xy when imaging larva mounted under a coverslip. Single image slices are shown. (h) Maximum intensity projections of lightsheet microscopy images of *C. elegans* embryo development with a spacing of approximately 30s between each acquisition. Shown are three timepoints 14, 49, and 111, with a relative temporal distance of 17,5 and 31 min, respectively. Nuclei are marked by H2B-mCherry and the cell lineage shown on the bottom was manually extracted using MaMuT [65]. Here, each nucleus is assigned a random color and 10 timepoints (5 min) each are shown to illustrate their trajectories. (i) Multiview lightsheet acquisition of a DAPI-stained *C. elegans* dauer larva. Note the natural, round shape in lateral and axial direction compared to (g). The data was stitched, multiview reconstructed and deconvolved as described in Ref [72]. Single image slices are shown. (j) Maximum intensity projections of three-dimensional neuronal activity recordings of a dauer larvae expressing the nuclear GCaMP6s reporter spanning the larva's head region. A dauer larva anaesthetized with levamisole was imaged under a coverslip on a 10% agarose pad on a spinning-disk confocal microscope for 8 min. The larva's head region was imaged in 3D at 1 Hz with a z-step size of 400 nm. Timesteps (t) correspond to 1s.

sample taken from different orientations (*views*) need to be aligned and multiview deconvolved, for which open-source software packages have been developed [68–70]. Additionally, image stitching is required to reconstruct larvae and adults that do not fit into the field of view of confocal, widefield, or lightsheet microscopes [71] (Figure 1f), which, in combination with multiview reconstruction, yields isotropic images of entire *C. elegans* larvae at single-cell resolution [72] (Figure 1i).

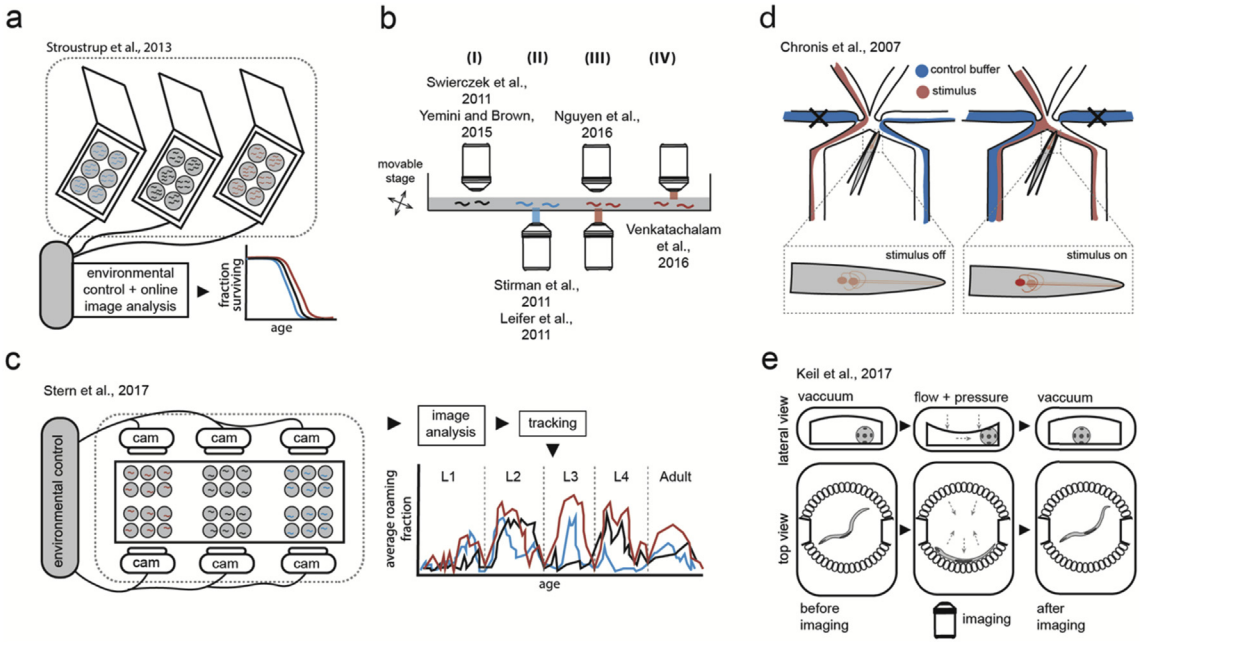
The invariant cell lineage enabled exciting studies. It allowed to identify pathways and the role of chromatin regulators important for lineage commitment by detecting cell lineage variations [73–75]. Cell type-specific FPs enabled screening for mutants affecting temporal or spatial expression patterns [8,76–78]. The stereotypic polarity of cell divisions makes them interesting targets to study spindle orientation, cell–cell interaction and tissue formation [79–81], which are specifically powerful when combined with automated tools [82], physical modelling [83], or optical manipulation [81,84,85]. Moreover, mapping expression patterns onto the cell lineage identified expression profiles that correlate with cell fate changes [86–89] and

lineage tracing enables ablation studies of mechanisms compensating cell loss after induced cell death [59,90,91]. However, since lineaging is not yet fully automatic, these approaches are often only accessible to technology-savvy laboratories. We expect that with the advent of deep learning, fully automated algorithms available through user-friendly open-source software packages such as Fiji [92] will make new and improved, powerful tools more accessible for to the entire *C. elegans* community.

Behavioral phenotyping

C. elegans allows to integrate quantitative fluorescent readouts with behavioral experiments. Importantly, the quantitative description of behavioral phenotypes is crucial in order to determine underlying genetic networks that define the molecular basis of behavior. Lifespan measurements in ageing research are a striking example of behavioral imaging that rely on significantly high numbers of measured individuals to infer phenotype–genotype correlations. Although robust and technically simple, manual lifespan measurements suffer from low throughput and lack the ability of recording quantitative image features. Recent improvements enable quantitative, fully-automated analyses that allow extraction of geometric features, e.g. intensities of fluorescent marker genes. By combining automated microscopy with image analysis, the automated "Lifespan machine" is for example able to image and annotate 30.000 animals simultaneously to infer individual death events and provides survival curves for a population under controlled conditions [93] (Figure 2a). While global quantification is powerful, it lacks the ability to study more detailed behavioral phenotypes of individuals. Therefore, custom microscopy solutions to follow individual worms (worm trackers) have been developed [94–96] (Figure 2b). The need to interpret these high-resolution behavioral datasets additionally led to the development of new dimensionality reduction algorithms as well as publicly available software packages [97–99].

Figure 2



Examples of automated imaging set-ups for *C. elegans*. (a) The *C. elegans* lifespan machine. An array of scanners acquires images of living worms on petri dishes over multiple weeks. The image data is processed online and survival curves, specific for different conditions or genetic backgrounds (here represented by different colors) can be extracted [93]. (b) Overview schemes recapitulating microscopy set-ups capable of monitoring freely moving *C. elegans*. (I) Worm tracking devices, recording behavior of individual worms [95,96] (II) Example of two set-ups capable of optogenetically controlling neural circuits within freely moving individuals [130,131]. (III) and (IV) depict two set-ups developed to acquire volumetric neuronal activity data in freely moving worms. (c) Set-up for characterizing behavior of isolated worms across development. An array of cameras captures movies of individuals from different genetic backgrounds (represented by color) over a period of 60 hours within a controlled environment. After acquisition, worm trajectories can be extracted, staged and comparisons between different lines can be drawn [104]. (d) Microfluidic chip for characterizing neuronal activity in response to osmotic stimuli. A single worm can be rapidly exposed to two different stimuli under controlled conditions and at a constant flow rate. By comparing neuronal activity (in either all, or only a subset of neurons) before and after stimulus exposure, neuronal activity can be quantitatively described [140]. (e) Microfluidic chip for transient immobilization of *C. elegans* larvae of any stage enabling high-resolution imaging over time. Automated on-chip valves immobilize individual worms only for the period of imaging, allowing investigation of post-embryonic development [111]. All schemes were adapted from the respective publications and are not to scale.

Whereas worm trackers are usually designed to follow individuals over the course of minutes to hours, following individual animals throughout their entire development is also of interest. For instance, solutions that restrain single larvae within compartments (e.g. microwells) allow following individual developmental trajectories over the entire course of development [100–103]. By comparing behavioral phenotypes of isogenic animals at high spatio-temporal resolution, Stern et al., could for example describe trajectories of spontaneous foraging behaviours at all developmental stages and quantify consistent, non-genetic behavioral biases at the single animal level [104] (Figure 2c).

Microfluidics

While live imaging and tracking of *C. elegans* in their natural laboratory environment is a powerful approach, it complicates to create controlled environments while sample movement limits high-resolution acquisitions. Although anaesthetics [105], glue [106] or polystyrene nanoparticles [59] are commonly used to immobilize worms for the relatively short period of imaging, microfluidic chips represent a powerful alternative. Their flexible layout enables designs specifically adapted to the experiment in terms of worm positioning and controlled supply of stimuli (Figure 2d). Earlier designs were focused on adult worms [107], but more recent adaptations allow imaging of larvae [108,109] and embryos [110]. While most chip designs rely on static physical confinement, Keil et al. recently proposed a design that only periodically immobilizes worms making use of an on-chip pressure trap, which can for example be used to quantify cell divisions during vulval development [111] (Figure 2e). Since more and more laboratories gain access to 3D printing and microfabrication facilities, we are expecting an increase in the number of specific designs adapted not only for specific stages of *C. elegans*, but also more complex experimental frameworks. Ideally, sharing such designs and related results in between labs would be highly beneficial for generating common standards in terms of fabrication and precision, but also for accessing potential biological artifacts caused by immobilization [112].

Neuronal activity imaging

C. elegans is an ideal model to study neuronal activity due to its small, stereotyped, yet relatively complex nervous system, which is mapped for adults in hermaphrodites [113] and, more recently, males [114]. To link neuronal activity to behavior, live imaging of increasingly optimized versions of GCaMP [115], a synthetic GFP variant that increases its fluorescence upon calcium concentration increase, are used to capture neuronal activity patterns (Figure 1j). More recently, fluorescent voltage reporters capable of capturing postsynaptic responses have also been developed [116]. Imaging the fluorescent GCaMP signal over time allows insights into four-dimensional activity patterns and the underlying neuronal circuitry. Similar to embryo lineaging, robust assignment of individual cellular identities over time in living animals has not yet been solved in its entirety [53,54].

While GCaMP expression in a limited set of neurons is an elegant solution, nervous system-wide imaging enables studies without *a priori* knowledge, but requires fast acquisition of image volumes for which spinning disk confocal [117], two-photon microscopy [118] and light field deconvolution microscopy [119] were proposed. They currently enable identification of up to 67% of all head neurons over imaging intervals of up to 18 min [117]. In combination with dimensionality reduction techniques, unknown mechanisms underlying sleep-like and exploratory behaviour [120,121] as well as locomotion [117] could be uncovered. However, since these techniques use physical constriction during imaging, they are often correlated with observations from freely moving animals.

Complementary efforts that approach imaging neuronal activity in freely moving animals additionally require tracking of individual worms. Focussing on subsets of neurons, studies uncovered the role of specific neurons in locomotion [122] as well as mechanisms underlying chemosensory [123] and olfactory response [124]. Recent advances image the entire nervous system of the freely behaving worms [125,126], assigning up to 156 neurons over 8 min of imaging [127].

Moreover, the stereotypic, mapped nervous system of *C. elegans* enables targeted (in-)activation of specific neurons. Laser ablation can be used for irreversible inactivation [90] and behavioural manipulation using optogenetics was first performed in *C. elegans* [128]. Optogenetic activation of individual neurons allows *in vivo* characterization of neural circuitry [129], an approach that has also been extended to freely behaving worms [130,131]. A recently presented method for randomized expression of opsins now also enables hypothesis-free approaches for neuronal network analysis [132].

The field of neuronal activity imaging is currently limited by a lack of tools for robust assignment of all cells over time, a problem that modern microscopy (e.g. lightsheet) in combination with more powerful image analysis could solve. New quantitative assays that automatically correlate light-induced activation of neurons with behavior could then provide a crucial tool to mechanistically characterize neuronal wiring in *C. elegans* and provide significant improvements in our understanding *C. elegans* neuronal circuitry.

Discussion & outlook

Current imaging techniques are of exceptional importance to study dynamic cellular processes in space and time, but they are limited by the total number of biological components (e.g. proteins or mRNAs) that can be recorded in each experiment. This constraint mainly arises from the limited size of the wavelength spectrum, available fluorophores, and capabilities to distinguish multiple colors (typically up to 5 different fluorophores can be resolved). Moreover, since the fluorescent probes have to be chosen prior to any experiment, observed factors are determined *a priori*, which limits the possibility for identification of new targets significantly. Although efforts in the field of RNA imaging illustrate that by multiplexing it is possible to extend smFISH to detect several thousands of mRNAs in one experiment [133,134], they remain technically challenging and were not applied to *C. elegans*, yet. Therefore, current developments in sequencing-based single-cell technologies [135,136] are gaining momentum and challenge microscopy as state-of-the-art method for individually probing all cells in a sample. But although single-cell sequencing technologies are applicable to many species and are able to capture information on a genome-wide level, temporal resolution is limited to static timepoints and there are only first efforts for reconstructing spatial information [137,138]. However, the combination of both technologies potentially enables in-toto, spatio-temporally resolved measurements at cellular level providing an extremely powerful tool for exploratory as well as targeted studies, thus promising to be one of the key technologies in the future. *C. elegans* as a model should contribute to these exciting new developments by focusing more on its unique properties such as its fully-mapped nervous system, opportunity for whole-organism imaging throughout its entire lifespan at single-cell resolution, or the possibilities of computational analysis that its fixed lineage offers.

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