A small excitation window allows long-duration imaging of OCR-2-associated vesicles in *C. elegans* sensory neurons

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Abstract

Single-particle imaging using laser-illuminated widefield epi-fluorescence microscopy is a powerful tool to investigate molecular processes *in vivo*. Performing high-quality single-molecule imaging in such biological systems, however, remains a challenge due to difficulties in controlling the number of fluorescing molecules, photobleaching, and the autofluorescence background. Here, we show that by exciting only a small, 5-15 µm wide region in chemosensory neurons in live *C. elegans*, we can significantly improve the duration and quality of single-molecule imaging. Small-window illumination microscopy (SWIM) allows long-duration single-particle imaging since fluorescently labelled proteins are only excited upon entering the small excited area, limiting their photobleaching. Remarkably, we also find that using a small excitation window significantly improves the signal-to-background ratio of individual particles, due to reduced background, mostly caused by out-of-focus autofluorescence. We demonstrate the potential of this approach by studying the dendritic transport of a ciliary calcium channel protein, OCR-2, in the chemosensory neurons of *C. elegans*. We reveal that OCR-2-associated vesicles are continuously transported back and forth along the length of the dendrite and can switch between directed and diffusive states. Furthermore, we perform single-particle tracking of OCR-2-associated vesicles to quantitatively characterize the transport dynamics. SWIM can be readily applied to other *in vivo* systems where intracellular transport or cytoskeletal dynamics occur in elongated protrusions, such as axons, dendrites, cilia, microvilli and extensions of fibroblasts.

Introduction

Living organisms and cells are complex and heterogeneous systems where myriads of processes take place at the same time. The same protein can have different dynamics and functionality, depending on its subcellular localization, conformational state, chemical modification, and presence of interacting partners. Fluorescence microscopy is a great tool for investigating protein dynamics in real-time in their native context [1, 2]. Dynamics of clearly defined structures, such as microtubule bundles, mitotic chromosomes and nuclei can be revealed by means of fluorescently labelling the whole pool of a protein of interest and imaging the bulk fluorescence signal. This generally provides an averaged image of all of these protein molecules, which might keep the stochastic behaviour of individual protein molecules hidden, limiting important insights in the molecular basis of processes of interest. Single-molecule fluorescence microscopy and analysis techniques allow overcoming these limitations and provide spatial information with sub-diffraction limit precision [3-6].

The quality of single-molecule information depends on the number and photostability of emitting fluorophores, signal-to-background (SBR) and signal-to-noise (SNR) ratios, and rate of image

acquisition. All these parameters are intertwined and require optimization for each particular experiment. A sparse density of fluorescing proteins, which is required for tracking and analysing them individually, can be achieved by labelling the proteins with photoactivatable or photoconvertible fluorescent markers and activating only a small fraction of them [3, 7-9]. Alternatively, one can start with many fluorescently-labelled proteins and photobleach them until only a few of them remain fluorescent in the region of interest [10]. The SBR ratio can be enhanced by using bright and photostable fluorescent proteins and by reducing the background that is caused by out-of-focus fluorescence and autofluorescence. Autofluorescence is due to other fluorescing molecules in the sample than the label and is inherent to most biological samples. The autofluorescence spectrum depends on the particular type of cell or tissue and its metabolic state. Generally, it is excited by wavelengths in the ultraviolet and blue and emitted over a wide range of wavelengths in the visible spectrum [11-13]. Imaging conditions have to be optimized in order to collect the maximum fluorescence signal of the labels while minimizing autofluorescence. Out-of-focus fluorescence can be eliminated by exciting only a thin layer of the sample, close to the focal plane of the objective – a principle underlying techniques such as Total Internal Reflection Fluorescence (TIRF) microscopy, Highly Inclined and Laminated Optical sheet (HILO) microscopy, Light Sheet Fluorescence Microscopy (LSFM), which combine video-rate acquisition speeds, with improved SBR [14-18].

In earlier studies, we have shown that it is possible to image and track single molecules within cilia and dendrites of chemosensory neurons in the multicellular organism *C. elegans* (illustrated in Figure 1A) [19-23]. This could be done using laser-illuminated widefield epi-fluorescence microscopy (which is very sensitive to out-of-focus background fluorescence), since the transparent worms have a limited number of ciliated neurons. Furthermore, the dendrites and cilia are thin (100-500 nm) and contain a specific subset of proteins not expressed in other cells. To image ciliary proteins we endogenously label proteins with fluorescent proteins such as eGFP and mCherry. To reach the single-molecule level we use photobleaching to reduce the number of fluorescing proteins, such that only a few molecules are visible in the imaged area at any given time. This strategy allows imaging single-molecule dynamics of proteins involved in intraflagellar transport (IFT), a transport mechanism specific to cilia (eg. Kinesin-2, cytoplasmic dynein-2, etc. [19, 21, 23, 24]) as well as IFT-cargo proteins such as the Ca^{2+}-channel OCR-2, which plays a key role in signalling [22]. We also obtained first insights in how the dendritic transport machinery transports vesicles containing OCR-2 from cell body, along dendrite, towards the base of the cilium. Although our approach has proven successful and insightful for several labeled proteins, we noticed that for some proteins the time during which single-molecule imaging in cilia or dendrite is possible is only brief, limited by the photobleaching of all molecules, severely reducing the amount of data that can be taken from a single sample.

We reasoned that we could prolong the time window in which single-molecule imaging is possible by only exciting a smaller region of the sensory neuron to allow continuous replenishment of 'fresh', unbleached proteins into the imaged region. Here we show that by restricting the excitation area in the x-y plane (Figure 1B) – an approach we call Small-Window Illumination Microscopy (SWIM) – the quality of laser-illuminated widefield epifluorescence imaging can be considerably improved. In the first place, it allows for substantially longer-duration single-molecule imaging *in vivo* in systems where proteins continuously move in and out of the excited area. Furthermore, remarkably, imaging using SWIM minimizes autofluorescence and out-of-focus fluorescence, substantially improving SBR. Finally, we utilize SWIM to reveal the dendritic transport dynamics of OCR-2-associated vesicles to and from the neuron cell-body (soma) to the cilia (Figure 1A).

Results and Discussion

The typical diameter of the excitation area in our set-up, as we have used in previous studies [21], is 50 µm (FWHM, Gaussian; Figure 1B left and Supplementary Figure 1B), covering the field of view of the EMCCD camera (41 x 41 um). To implement SWIM, we placed an iris diaphragm in the excitation path, which allows us to change the size of the excitation beam (Figure 1B). Closing the aperture of the diaphragm to the minimal of 0.8 mm yields an excited area with a diameter of 5-10 µm (Figure 1B, right). Ideally, the diaphragm should be placed at a plane conjugate to the back focal plane of the objective, to obtain the sharpest beam profile. When the aperture width is significantly smaller (< 5x) than the excitation beam, such that only the central part of the Gaussian beam passes through, the cropped beam has a Gaussian blurred rectangular profile (Figure 1B right and Supplementary Figure 1C). Upon imaging reference fluorescent beads immobilized on glass, with the aperture open or closed (excitation window width 7 µm), we find that the signal intensity is not changed for beads near the center of the illuminated window (Bead 1 and 2 in Supplementary Figure 1A, D), while the signal intensity near the edge of the window is changed substantially (Bead 3 and 4 in Supplementary Figure 1A, D). Thus, placing an iris diaphragm in the excitation path allows to readily crop the excitation beam in order to excite a small region of interest in a sample.

We utilized SWIM to image the transport dynamics of vesicles carrying the ciliary channel protein OCR-2 labelled with eGFP (OCR-2::eGFP), in the dendrites of the PHA/PHB neurons in *C. elegans* (Figure 1A). OCR-2, which is produced in the cell bodies, primarily localizes in cilia, which implies that it has to be transported across the ~50 µm long dendrite that connects the soma to the cilium. Using a cropped 491 nm excitation beam, we imaged OCR-2::eGFP in a small region of a dendrite (excitation window width 7 µm) and observed rich dynamics of OCR-2-associated vesicles (Figure 2A and Supplementary Movie 1). Directed transport of vesicles both in the anterograde (soma to cilium) and retrograde (cilium to soma) direction, as well as purely diffusive vesicles, were observed. Although fluorescent proteins bleach in the illuminated region, due to the relatively high laser intensity, it is possible to image continuously for long durations (15 minutes of continuous imaging in Figure 2A), since new, unbleached proteins continuously move into the illuminated region. In contrast, illuminating with the full beam (aperture open) results in bleaching of fluorescing molecules over a much larger region, allowing observation of single OCR-2::eGFP for only a short period of time. Indeed, in the imaged dendrite shown in Figure 2A (Supplementary Movie 1), after opening the aperture following 15 mins of SWIM, to illuminate a much larger section of the worm (beam diameter ~50 µm), OCR-2-associated vesicles could only be observed sparsely for 1-2 mins. In conclusion, SWIM allows imaging of the transport dynamics of OCR-2-associated vesicles in dendrites of *C. elegans* sensory neurons for considerably longer than is possible using standard widefield illumination.

To our great surprise, the BG intensity appeared to be significantly lower when a smaller area was excited using SWIM (Figure 2A and Supplementary Movie 1). We sought to quantitatively characterize the effect of the excitation window width on the BG intensity. Since vesicles contain a varying amount of OCR-2, their intensities vary considerably, which makes quantitative comparison of SBR difficult. To overcome this, we imaged the relatively constant pool of KAP-1::eGFP (the non-motor sub-unit of kinesin-II) in the cilia of PHA/PHB neurons (Figure 2B, Supplementary Figure 2A) [21, 23]. First, we imaged the bulk fluorescence intensity of KAP-1 in the cilia, performing time-lapse imaging (acquisition every 2 s) with low-intensity 491 nm excitation, switching the aperture between open and closed states every 40 s (Figure 2B). We measured the signal and BG fluorescence intensity and observed that

while both signal and BG intensity decrease upon closing the aperture, the BG-corrected signal intensity is relatively independent of the aperture state (Figure 2C upper panel). This results in a substantial increase of the SBR from 3 to 4 upon closing the aperture (Figure 2C lower panel). We performed a similar experiment on single-molecules of KAP-1, using a high-intensity 491 nm excitation laser beam (Supplementary Figure 2A), switching the aperture between open and closed state every 15 s. Again, we observed that signal and BG intensities decrease upon closing the aperture, with SBR increasing by a factor of 1.5 (Supplementary Figure 2B). Thus, exciting only a small region in a sample considerably improves the quality of single-molecule imaging by increasing the SBR of individual molecules. Next, we explored how the SBR depends on the width of the excited area, by imaging the bulk fluorescence intensity of KAP-1 at different aperture widths (Supplementary Figure 2C, Figure 2D). We discovered that the SBR decreases non-linearly with increasing window size, changing rather sharply for small window widths (5 – 15 µm). In principle, it should be possible to increase the SBR further by using even smaller excitation windows. Exciting larger areas (>20 µm) results in the SBR saturating to the value obtained with the full beam. Taken together, it appears that while the BG-corrected signal intensity remains constant, the BG intensity decreases with the reduction in size of the excited region, resulting in the significantly higher SBR.

Since the BG intensity crucially determines the quality of single-molecule imaging we wanted to better understand its source. To this end, we imaged in the tail region of a worm not expressing any fluorescent proteins (and thus only producing BG light upon excitation) using low-intensity 491 nm laser excitation, while periodically opening and closing the aperture. We observed an approximately 70% reduction of the measured light intensity in the central region of the sample with the aperture closed (Figure 2E, left). We repeated these measurements (on the same area of the same worm) after prolonged high-intensity 491 nm illumination with the aperture closed (Figure 2E, middle) and observed that the light intensity nearly dropped to zero with the aperture closed, while it was still substantial with the aperture open. Subsequently, we illuminated the sample with high-intensity 491 nm laser light with the aperture open and repeated the measurement cycle (Figure 2E, right). We now detected only a very small light intensity, both with the aperture closed and open. From these measurements we can draw two conclusions regarding BG light signals produced when exciting live *C. elegans* with 491 nm laser light. (I) The BG signal is mostly due to autofluorescence in the body of the worm, since it nearly disappears after prolonged, high-intensity excitation. The small residual BG after photobleaching might be due to elastic or inelastic (Raman) scattering of the excitation light [25, 26], or incomplete photobleaching. Furthermore, the BG signal is much lower when exciting the worm with 561 nm laser light (Figure 2F), as expected for autofluorescence, which is considered to be more efficiently excited with light on the blue side of the spectrum [12, 13]. (II) A substantial part of the BG signal is due to autofluorescence excited in the sample outside of the (detection) focal plane. This is most evident from the large BG observed upon excitation with the aperture open after photobleaching with the aperture closed (Figure 2E middle). Autofluorescence created outside the focal plane creates a hazy, unstructured BG. These experiments on wild-type worms clearly indicate that the intensity of this out-of-focus autofluorescence decreases substantially when limiting the excitation area in epifluorescence microscopy, which is in line with experiments on fluorescent slides (Supplementary Figure 1B).

In summary, the key to performing single-molecule imaging in neurons of living *C. elegans* is to limit the number of fluorescent molecules of interest and to minimize BG autofluorescence. Both can be achieved with the relatively high excitation laser intensities used for single-molecule imaging. We have

shown here that the BG (mostly due to out-of-focus autofluorescence) can be substantially decreased when only a small region is excited using SWIM. In addition, this allows for longer duration single-molecule imaging, because fresh, unbleached fluorescent molecules of interest can continuously move into the illuminated region of interest. Longer duration imaging not only improves data throughput, it also helps to further reduce the BG thanks to photobleaching by the excitation light.

Having established that reducing the excitation area can substantially improve single-molecule imaging *in vivo*, we utilized SWIM to image the dynamics of OCR-2-associated vesicles in the dendrites of phasmid neurons in the tail of *C. elegans* (illustrated in Figure 3A). As seen in the example movie (Supplementary Movie 2; image acquisition rate 6.67 fps), it was possible to visualize OCR-2::eGFP in a section of both the PHA and PHB dendrites (denoted dendrite 1 and 2 in Figure 3B and Supplementary Movie 2, since we cannot assign them to either PHA or PHB), since both dendrites were in one Z-plane. We observed that OCR-2-associated vesicles are continuously being transported, in a directed manner, across the imaged region of the tens of µm long dendrites, from the soma to the ciliary base (anterograde) and back (retrograde). OCR-2, which is a subunit of a TRPV channel associated with sensory cilia [27], is expressed in the neuron's soma and associates with vesicles formed by the trans-Golgi network [22], that likely constitute the anterograde moving vesicles. The retrograde-directed movement of OCR-2 from the cilia towards the soma was slightly less anticipated. Since OCR-2 primarily localizes at the membrane of the cilia and the periciliary membrane compartment (PCMC), at the transition between dendrite and cilium, we hypothesize that retrograde-moving OCR-2 molecules are associated with endocytic vesicles formed at the PCMC [28], cycling old OCR-2 proteins back to the soma. Further, we observed that a significant portion of the vesicles was purely diffusive, typically photobleaching within the first few seconds of imaging (Figure 2A and Supplementary Figure 4A).

We performed single-particle tracking to obtain more detailed information on the transport dynamics (Figure 3C). Both retrograde and anterograde trajectories appeared, in some cases, entirely directed (e.g. Figure 3D and 3F) and in others, directed stretches where interspersed with diffusive phases (e.g. Figure 3E and 3G). Diffusive vesicles (e.g. Figure 3H) were mostly observed only briefly, mostly near the edges of the excitation area, getting photobleached quickly or moving out of the excited area. We analysed the local velocities of individual trajectories (referred to as point-to-point velocity) by calculating the local slope from the distance-time data (including 3 consecutive frames). For entirely directed anterograde tracks, the velocity is ~2-2.5 µm/s (Figure 3I), while for less purely directed trajectories, the velocity is lower in the more diffusive sections (~1-2 µm/s; Figure 3J). Similarly, for entirely directed retrograde trajectories the velocity is ~1-1.2 µm/s (Figure 3K; negative value indicates retrograde motion), while for less purely directed tracks, velocities range between ~0.5-1 µm/s (Figure 3L). For diffusive trajectories, we calculated the diffusion coefficient from the mean squared displacement (MSD) to be 0.11 $\mu m^2/s$ (for the trajectory of Figure 3H; Figure 3M). To obtain a quantitative measure for the directedness of the motion, we used an MSD-based approach [22, 23, 29] to extract the anomalous exponent (α) from $MSD(\tau) = 2\Gamma\tau^{\alpha}$ (where Γ is the generalized transport coefficient and τ is the time lag) along the track, in the direction of motion. α is a measure of the directedness of the motion, α = 2 for purely directed motion, α = 1 for purely diffusive motion and α < 1 for subdiffusion or pausing [30]. We found that α for apparently entirely directed trajectories (both anterograde and retrograde) is 2 (Figure 3N and 3P), while it ranges between 1.5 and 2 for less directed tracks (Figure 3O and 3Q), and is close to 1 for diffusive trajectories (Figure 3R).

Since directed trajectories displayed some variability in motion, we filtered directed regions of individual trajectories based on the α values, to obtain a better estimate of the velocity of the molecular motors driving the motion. For anterograde trajectories, the average point-to-point velocity from 144 tracks (N = 2865) in 7 dendrites (5 worms) was 1.77 ± 0.05 µm/s (Figure 4A; average value and error estimated using bootstrapping; see methods). Using a threshold of α = 1.95, we find that less directed stretches of trajectories (α < 1.95) show an average velocity of 1.58 ± 0.07 µm/s (N = 1694) and highly directed stretches of trajectories (α > 1.95) show an average velocity of 1.99 ± 0.06 µm/s (N = 1171; Figure 4A). Binning the time points in 0.05 α-value bins, shows that the velocity increases from 1 µm/s (α: 1.5-1.55) to 2.1 µm/s (α: 2-2.05; Supplementary Figure 3A). For retrograde tracks, the average point-to-point velocity from 184 tracks (N = 6375) in 8 dendrites (5 worms) is 0.82 ± 0.01 µm/s (Figure 4B). Furthermore, we find that less directed stretches of trajectories (α < 1.95) show an average velocity of 0.75 ± 0.02 µm/s (N = 4662) and highly directed stretches (α > 1.95) show an average velocity of 0.95 ± 0.02 µm/s (N = 1805; Figure 4B). Binning the velocity data using α values, we find that the velocity increases from 0.55 µm/s (α: 1.5-1.55) to 0.95 µm/s (α: 2-2.05; Supplementary Figure 3B). 40% of the tracked datapoints for anterograde tracks and 28% of the tracked datapoints for retrograde tracks have α > 1.95, indicating that the OCR-2-associated vesicles in the anterograde direction are less diffusive. Since the microtubule bundles in the dendrites of *C. elegans* neurons are minus-end out [31-33], anterograde vesicles are most likely driven by cytoplasmic dynein-1 [34] and retrograde vesicles are driven by kinesin-1 (UNC-116) and/or kinesin-3 (UNC-104) [35, 36]. Thus, using filtering based on α values, we find that in dendrites of sensory neurons in *C. elegans*, cytoplasmic dynein drives OCR-2 vesicles at 2.1 µm/s and kinesin drives retrograde OCR-2 vesicles at 0.95 µm/s.

As described above, some vesicles appear entirely directed, others switch between directed and less directed (having a diffusive component) phases, leading to a substantial variability in speeds. In rare cases, reversal of motion from anterograde to retrograde, or *vice versa*, was also observed (Supplementary Figure 4C), suggesting that OCR-2-associated vesicles can associate with retrograde and anterograde directed motors simultaneously. It is possible that directionality and directedness are tuned by the number and ratio of activated dynein and kinesin motors cooperating to drive a vesicle [37]. Trajectories of vesicles driven by a large number of motors with the same directionality would show highly directed motion. Less directed motion might be observed when a trajectory is due to a vesicle driven by only a few motors of the same directionality. Because of the finite run length of the motors, a vesicle might regularly switch to a transient diffusive state whenever none of the motors connects it to the microtubule lattice. Another cause of less directed trajectories might be vesicles containing opposite directionality motors (i.e. both dynein and kinesin), resulting in transient directed runs, interspersed by periods of tug-of-war or reversals of direction [37, 38].

Next, we explored the nature of the diffusive trajectories. To obtain diffusion coefficients of the diffusive vesicles we tracked 78 events in 7 dendrites (Figure 4C). The distribution of diffusion coefficients for individual tracks, is broad with an average diffusion coefficient of 0.12 ± 0.03 $\mu m^2/s$ (Figure 4D). Remarkably, the diffusivity of single OCR-2 molecules along the ciliary membrane is much lower (~0.03 $\mu m^2/s$ [22]). For these trajectories, the average α along the length of the dendrite is 0.79 ± 0.03 and the average α perpendicular to the dendrite long axis is 0.38 ± 0.03 (Figure 4E), indicating that the motion of the vesicles is slightly sub-diffusive, perhaps due to transient interactions with the microtubule cytoskeleton. We also observed that anterograde and retrograde vesicles occasionally switched to a diffusive state (Supplementary Figure 4B), the mechanism of which is unknown but could

be linked with the disassociation or deactivation of the driving motors. We did not observe diffusive vesicles switching to directed motion. This might reflect, however, a limitation of our imaging approach, in which most diffusive vesicles photobleach quickly and can only be observed for a short period of time.

Finally, SWIM makes it possible to estimate the rate at which OCR-2 proteins move back and forth along an imaged dendritic section. We determined that the distribution of time intervals between individual anterograde (N = 498 in 14 dendrites; Figure 4F), as well as retrograde (N = 468 in the same 14 dendrites; Figure 4G) OCR-2-associated vesicles is exponentially distributed. The characteristic time interval between two anterograde vesicles was 19.8 ± 3.2 s and between two retrograde vesicles was 20.6 ± 4 s (Figure 4H), suggesting that anterograde and retrograde vesicles have similar frequencies. Furthermore, we quantified the intensity of the vesicles and observed a broad distribution of intensities, indicating that the amount of OCR-2 associated to individual vesicles is highly variable (Figure 4I). We also observed that the intensity of the retrograde vesicles (3399 ± 670 a.u.) was on average slightly lower than the intensity of anterograde vesicles (3771 ± 451 a.u.; Figure 4I). This may imply that slightly more OCR-2 proteins move towards the cilium than away from it (to the soma). It is plausible that only part of the OCR-2 returns back to the soma, while the rest is either degraded or extruded out from the cilia, as part of extracellular vesicles [39]. In summary, employing SWIM and single-particle analysis, we provide a detailed, quantitative picture of the dynamics of vesicles transporting OCR-2 proteins along the dendrites of *C. elegans* sensory neurons.

Conclusion

In this work, we have developed a simple technique, referred to as small-window illumination microscopy (SWIM), to perform single-molecule fluorescence imaging for long durations (at least 15 minutes) in sensory neurons of living *C. elegans* worms. In SWIM we remarkably improve the quality of single-molecule imaging by restricting the size of the excitation area in the x-y plane in order to reduce the autofluorescence BG. Potentially, one could further improve the quality of imaging by using SWIM in conjugation with techniques such as TIRF or HILO, to further remove out-of-focus fluorescence in the z-direction. Further, SWIM can be readily extended to perform multi-colour and 3D single-molecule imaging in *in vivo* systems.

We demonstrated the potential of SWIM by providing a detailed view on the nature and dynamics of OCR-2-associated vesicles in dendrites of sensory neurons in *C. elegans*. In our view, SWIM is ideally suited for longer-duration single-molecule imaging in *in vivo* systems such as axons, dendrites, cilia, etc., where fluorescently labelled proteins continuously move in and out of a given area. The dynamics of proteins in such systems are often highly heterogeneous, involving directed and diffusive transport, pausing, directional reversals, velocity variability, switching between modes of transport and tug-of-war behaviour [31, 38, 40-42]. SWIM can help to address open biophysical questions in such *in vivo* systems.

Methods

C. elegans strains

C. elegans strains used in this study are listed in Table S1. Strains expressing eGFP-labelled OCR-2 and KAP-1 were generated earlier in our lab using CRISPR-Cas9 and Mos-1-mediated single-copy insertion,

respectively [43, 44]. Worms were grown according to standard procedures [45], at 20°C, on NGM plates seeded with HB101 *E.coli* bacteria.

Fluorescence microscopy

Images were acquired using a custom-built epi-illuminated widefield fluorescence microscope [10, 21]. In short, the setup was built around an interved microscope body (Nikon Ti E) equipped with a 100x oil immersion objective (Nikon, CFI Apo TIRF 100x, N.A.: 1.49). The system was controlled using MicroManager software (v1.4) [46]. Excitation light was provided by 491 nm and 561 nm DPSS lasers (Cobolt Calypso and Cobolt Jive, 50 mW). Laser power was adjusted using an acousto-optic tuneable filter (AOTF, AA Optoelectronics). For modulating the beam size, an iris diaphragm (Thorlabs, SM1D12, ø 0.8-12 mm) was mounted between the rotating diffuser [21] and the epi lens, at a distance equal to the focal length of the latter. The aperture size of the diaphragm was adjusted manually. Fluorescence was separated from the excitation light using a dichroic mirror (ZT 405/488/561) and emission filters (525/50 and 630/92 for collecting fluorescence exited by 491 and 561 nm, respectively). Images were recorded by an EMCCD camera (Andor, iXon 897).

For imaging live *C. elegans*, young adult hermaphrodite worms were sedated in 5 mM levamisole in M9, sandwiched between an agarose pad (2% agarose in M9) and a coverslip and mounted on a microscope, as described in [10]. The region of interest (either cilia or dendrites) was brought into focus and imaged with an acquisition rate of 6.67-50 fps. Single-particle imaging was performed using high intensity 491 nm excitation ($\sim$1-4·10^3 mW/mm^2 in the centre of the beam), for bulk imaging the excitation intensity was $>$200 times lower. The Perfect Focus System (PFS) of the microscope was used to keep the sample in focus during long-duration experiments.

To obtain intensity profiles of the excitation beam, fluorescent plastic slides (Chroma) were used. For the fluorescence intensity control, we imaged fluorescent microspheres (Tetraspeck, 0.1 um, Invitrogen) diluted in M9 buffer.

Data analysis

Intensity analysis: Initial image analysis was performed in Fiji [47]. Ensemble and single-molecule fluorescence intensities were measured by manually selecting a region containing fluorescent signal and a region next to it within the illuminated area, for the background correction. The size of the excitation beam was determined by manually measuring the region with highest intensity (which corresponds approximately to FWHM).

Tracking of OCR-2 vesicles: OCR-2 vesicles were tracked using a MATLAB-based software, FIESTA (version 1.6.0) [48]. Tracks, corresponding to an event, contain information regarding time (t_i), x and y coordinates (x_i, y_i) and distance moved (d_i), for every tracked time-frame i. The connected tracks were visualized and erroneous or small tracks were manually excluded from further analysis. Erroneous tracking primarily occurred when two or more retrograde, anterograde or diffusive events were too close to one another.

Point-to-point velocity for all tracks: Before calculating the point-to-point velocity, the tracks were smoothened by rolling frame averaging over 10 consecutive time frames, to reduce the contribution due to localization error (typically estimated to be between 10-80 nm, depending on the brightness

of the tracked object). The point-to-point velocity was calculated using the following equation: $v_i = (d_{i+1} - d_{i-1})/(t_{i+1} - t_{i-1})$.

Diffusion coefficient for diffusive tracks: Displacement along the length of the cilia for all diffusive tracks was calculated for discrete time points, and the displacement data were cumulated to calculate the average MSD (Mean Square Displacement) for every discrete time point. The first five points of the MSD thus obtained were then fitted with a linear curve, using the error bars as weights, with the slope of the fit providing the diffusion coefficient for each track.

Calculation of α-value: The x-y coordinates were transformed to dendritic spline coordinates, by defining a spline along the length of the imaged dendrite section and obtaining the distance parallel ($c_{\parallel_i}$) and perpendicular ($c_{\perp_i}$) to the spline, for every tracked datapoint (x_i, y_i). The spline was generated by interpolating a cubic spline curve on a segmented line, drawn manually over all tracks in a given dendritic section (which provides the shape of the dendrite). For each datapoint ($c_{\parallel_i}$, $c_{\perp_i}$), a windowed Mean Square Displacement classifier (wMSDc) approach, described in Danné et al. [29], was used to extract the anomalous exponent value (α) of the time lag (τ) from $MSD = 2\Gamma\tau^{\alpha}$ (where Γ is the generalized transport coefficient), in the direction parallel ($\alpha_{\parallel_i}$) and perpendicular ($\alpha_{\perp_i}$) to the spline. α was calculated analytically, using the following equation: $\alpha = \langle\alpha\rangle = \frac{2}{W-1}\sum_{n=1}^{(W-1)/2}\log\left(\frac{MSD(n\Delta t+\Delta t)}{MSD(n\Delta t)}\right)/\log\left(\frac{n+1}{n}\right)$; keeping a fixed window W = 15 time-frames. Due to the size of the window, all tracks shorter than 15 frames were removed from the analysis. In this paper, $\alpha_{\parallel}$ values are referred to as α, since we primarily discuss the motion along the length of the dendrite and filter velocities based on $\alpha_{\parallel}$ values.

Exponential fit for time-lag distribution: The time-lag between subsequent anterograde (or retrograde) vesicles was extracted by manually selecting events on a kymograph. The characteristic time lag (τ) was obtained by least-squares fitting of the cumulative distribution function (LSF-CDF), where the time lag distribution was used to generate a cumulative probability distribution that was fitted with the CDF: $y = 1 - e^{-x/\tau}$. The average value and error were estimated using bootstrapping (see below).

Vesicle Intensity Analysis: The intensity of a single vesicle was roughly estimated by manually drawing a box on the dendrite, with dimensions sufficient to capture all the light emitted by a single vesicle, positioned right at the edge of the illuminated spot, using Fiji. Because the square was placed at the edge of the illumination spot, we minimized prior excitation and photobleaching which would decrease the measured intensity. The intensity of actively transported vesicles passing through the square was recorded. The background intensity was measured by placing a box of the same dimension outside the dendrite and this was subsequently subtracted from the raw signal to obtain the background corrected value.

Estimating the average value and error for distributions: We utilized a bootstrapping approach to estimate parameters for any given distribution. The distribution (consisting of N measurements) was resampled by randomly picking N measurements from the measured distribution (with replacement) and calculating the median of the resampled distribution. This was repeated 1000 times. The resulting bootstrapping distribution was used to estimate the parameter (mean of the bootstrapping distribution μ) and its error (standard deviation of the bootstrapping distribution σ). All values and errors in this paper use $\mu \pm 3\sigma$.

Information on plots and figures: Kymographs were generated either on FIESTA or using the KymographClear [49] plug-in on Fiji/ImageJ. All the data was analysed and plotted using custom written scripts on MATLAB (The Math Works, Inc., R2021a).

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Figures

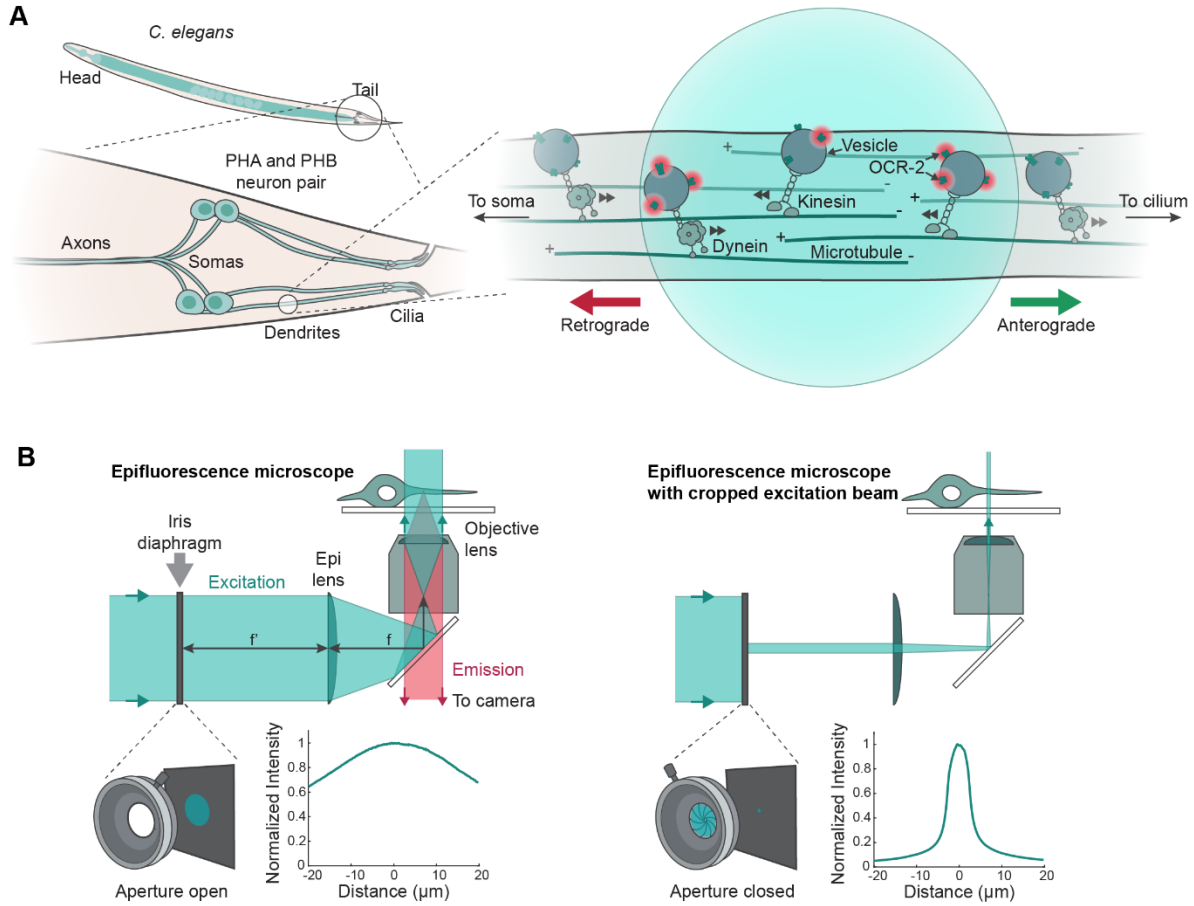


Figure 1: Illustration of small-window illumination microscopy (SWIM) and the imaged biological system. (A) Cartoon illustration of *C. elegans*, highlighting the ciliated chemosensory neurons, PHA and PHB, located in the tail of *C. elegans*. These neurons have dendrites which are tens of µm long, connecting the soma (cell body) to the cilia. Vesicles containing the transmembrane Ca^{2+} channel OCR-2 are transported in the anterograde (from the soma to the cilium) and retrograde (from the cilium to the soma) direction along microtubule bundles in the dendrites, driven by microtubule-based motors, dynein (anterograde) and kinesin (retrograde). The blue circle (illustration to the right) represents the region in the dendrite illuminated using SWIM, with fluorescently-labelled OCR-2 molecules being excited and fluorescing (indicated by red circles) upon entering the excitation region. **(B)** Schematic illustration of SWIM. The sample is imaged using laser-illuminated widefield epi-fluorescence microscopy, where the excitation beam size can be changed using an iris diaphragm at a focal plane conjugate to the image plane of the objective (at the back-focal plane of the epi lens, f). When the aperture of the diaphragm is open (left), the field of view is illuminated with a large parallel beam (beam width ~50-100 µm; see inset left plot). Closing the aperture (right) results in a cropped small-diameter excitation beam (window width ~5-10 µm; see inset right plot).

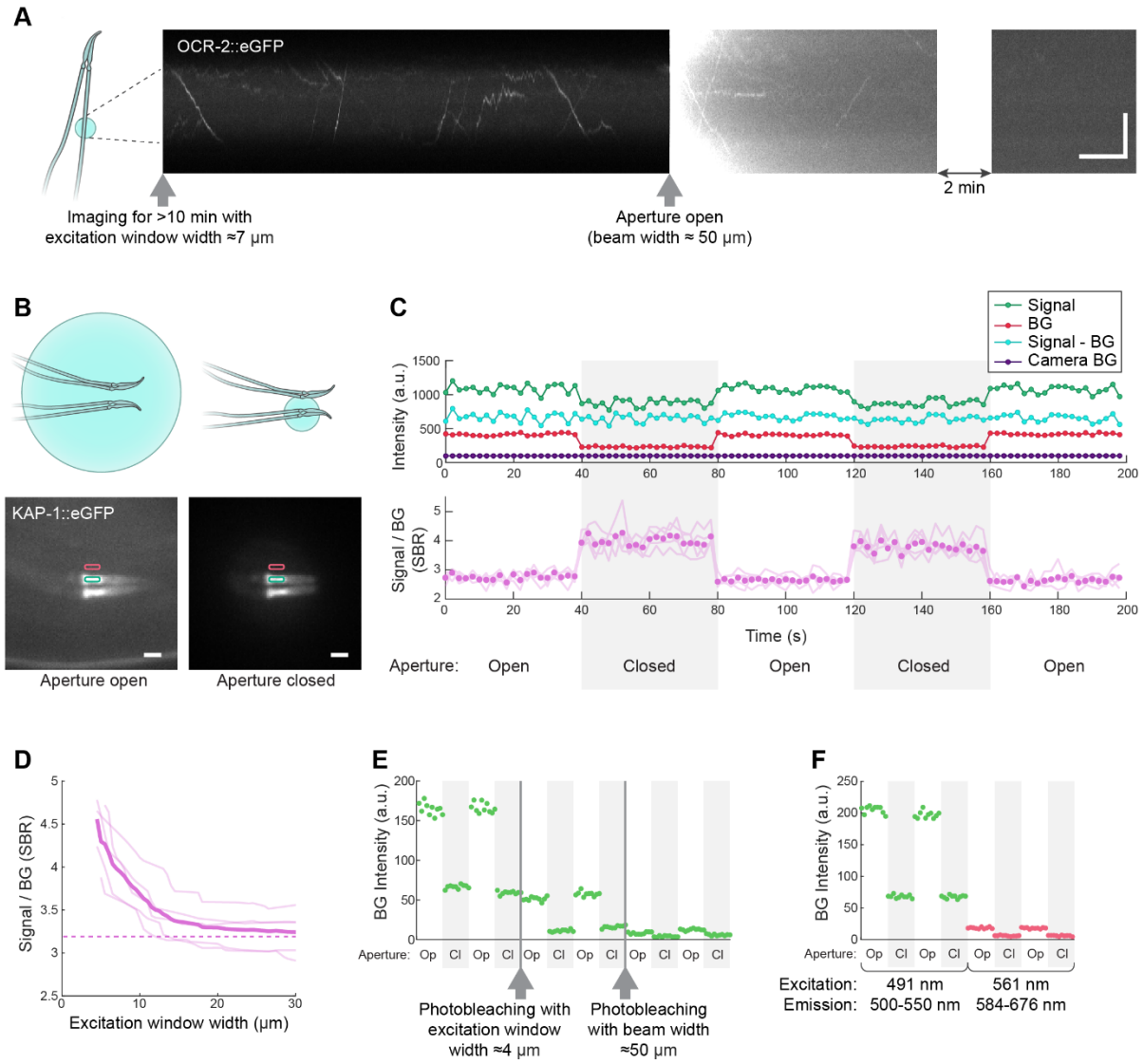


Figure 2: SWIM enables long-duration imaging and improved SBR (signal-to-background ratio). (A) Segments of a kymograph (time-intensity plot; timing of segments indicated) along a dendrite (also see **Supplementary Movie 1**), where OCR-2::eGFP-associated vesicles were imaged using a cropped 491 nm laser beam (excitation window size ~7 µm; 6.67 fps). OCR-2-associated vesicles entering the small excitation window were continuously imaged for ~15 mins. Upon opening the diaphragm at 15 mins, exposure by the entire beam (width ~50 µm), quickly photobleached all OCR-2-associated vesicles within the larger excitation area and no more events were observed. Scale bars: 10 s (horizontal), 5 µm (vertical). Brightness and contrast are the same for all three segments. **(B)** Top: Schematic of imaging location (ciliary base) and approximate excitation window widths when the diaphragm aperture is open (left) and closed (right). Bottom: Time-averaged images of KAP-1::eGFP illuminated with aperture open (left) and closed (right). Regions, where the signal (green) and the background (red) intensity were measured, are shown. Scale bar: 1 µm. **(C)** Top: Signal intensity (Signal), BG, the difference (Signal - BG), and the camera offset (Camera BG), measured over time in the regions indicated in B. Bottom: SBR as a function of time obtained from 5 worms (light magenta lines; mean indicated with circles). The aperture state is indicated below the x-axis. **(D)** Mean SBR obtained from 5 worms imaged at different excitation window widths (individual events plotted in light magenta). The most drastic change in SBR happens at small window widths up to ~10 µm. Dotted line indicates SBR (mean from 5 worms) when the aperture is completely open. **(E)** BG intensity imaged with aperture alternatingly opened (white region) and closed (grey region) every 20 s. Arrowheads indicate the points at which the sample is photobleached (illuminating with 491 nm laser at maximal

power for 2 min, first time with aperture closed (excitation window size ∼4 µm), second time with aperture opened (beam width ∼50 µm). **(F)** BG intensity excited by 491 nm and by 561 nm light, collected with emission filters 525/50, and 630/92, respectively. Aperture is alternatingly opened (white region) and closed (grey region) every 20 s.

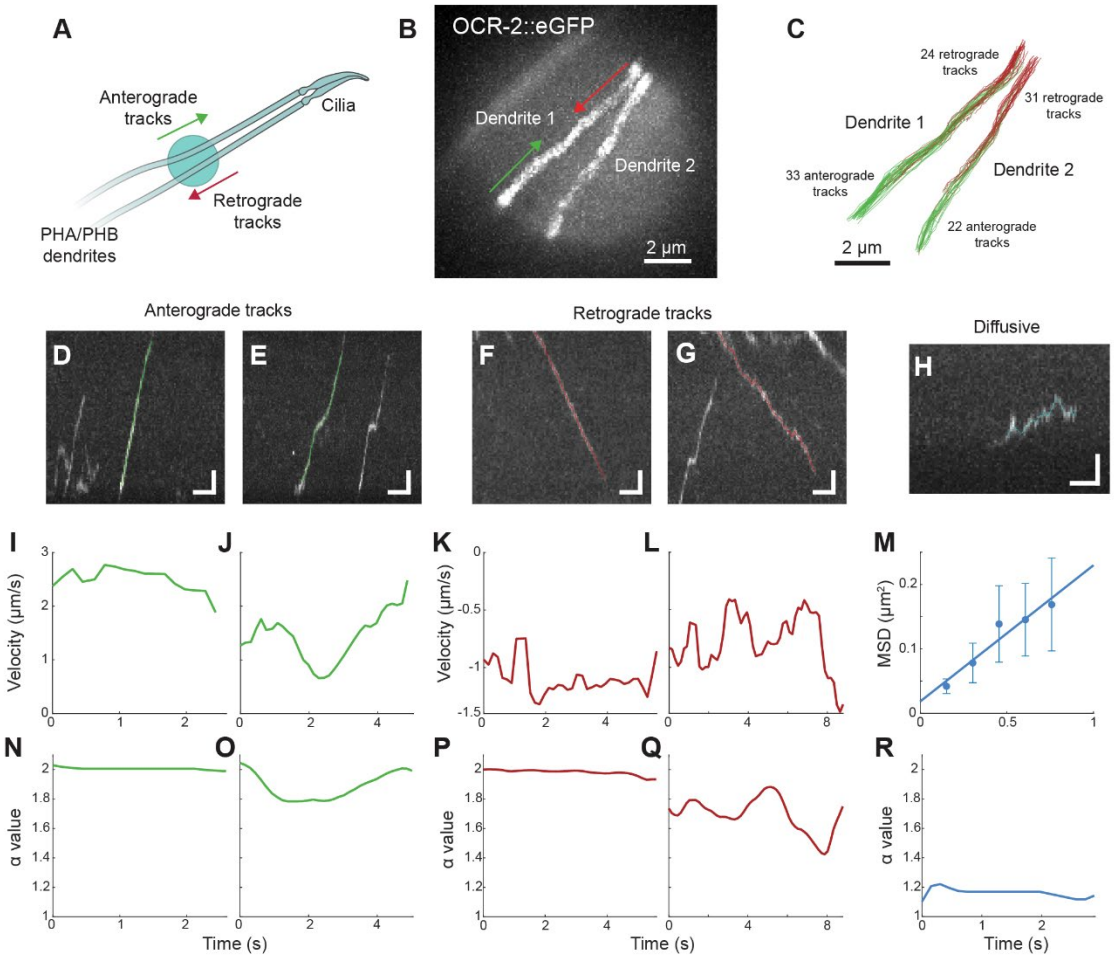


Figure 3: Single-particle tracking of OCR-2-associated vesicles in dendrites, imaged using SWIM. (A) Illustration of SWIM in dendrites of phasmid neurons (left). **(B)** Maximum projection of an example movie (also see **Supplementary Movie 2;** imaged using 491 nm laser at 6.67 fps) shows the section of PHA and PHB dendrites (labelled dendrite 1 and dendrite 2) illuminated by a small excitation window (window width ~11 µm), where the dynamics of OCR-2:eGFP associated with vesicles can be visualized. **(C)** Single-particle tracking in the example movie yields 33 anterograde (soma to cilium) and 24 retrograde (cilium to soma) tracks in dendrite 1, and 22 anterograde and 31 retrograde tracks in dendrite 2. **(D-H)** Kymographs (from dendrite 1) overlayed with track data showing typically observed anterograde (D-E; green tracks), retrograde (F-G; red tracks) and diffusive (H; blue track) events. Horizontal scale bar: 2 s; Vertical scale bar: 1 µm. **(I-L)** Point-to-point velocity plotted over time, for the anterograde (I-J) and retrograde (K-L) tracks calculated for the events shown in D-E and F-G. **(M)** Diffusion coefficient for the diffusive event shown in H calculated using MSD analysis is 0.11 $\mu m^2/s$. **(N-R)** Alpha value, calculated using a windowed mean-square displacements-based approach, plotted over time, for the anterograde (N-O) retrograde (P-Q) and diffusive (R) tracks corresponding to the events shown in D-H. Alpha value is ~2 for purely directed transport and ~1 for purely diffusive transport.

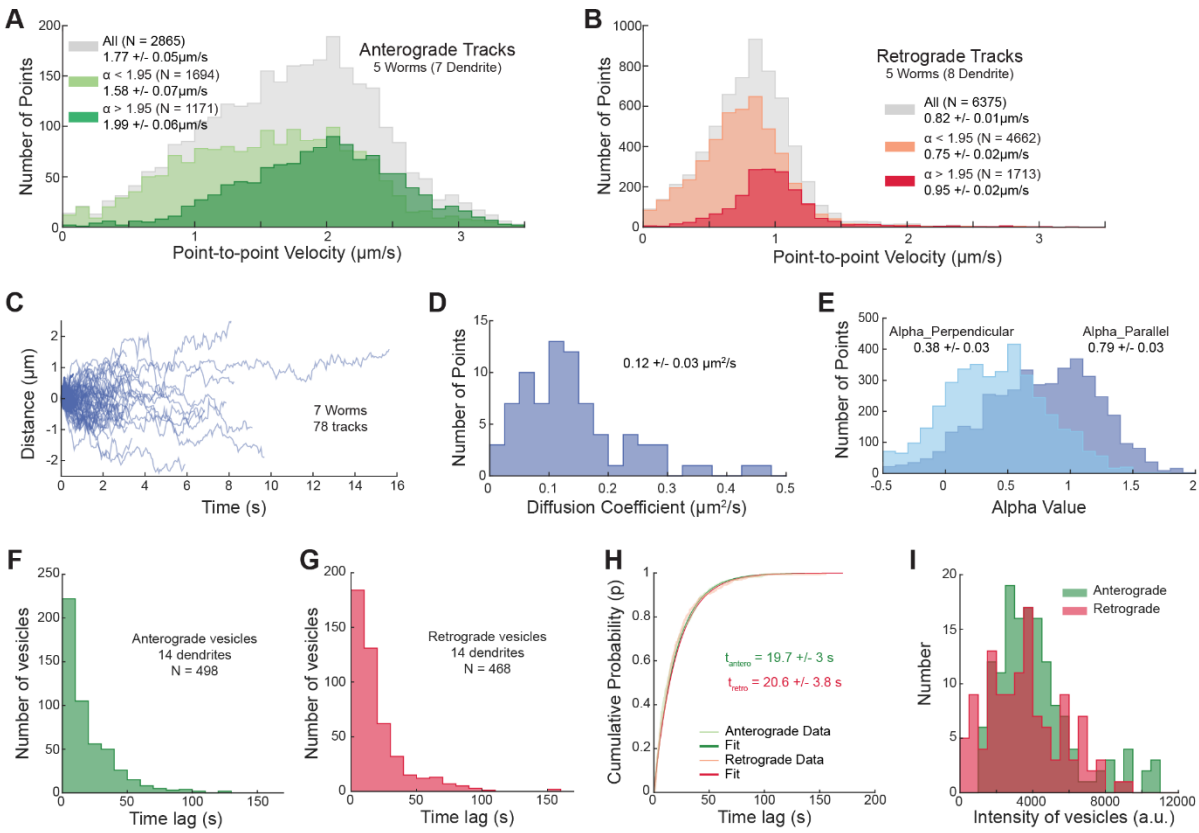
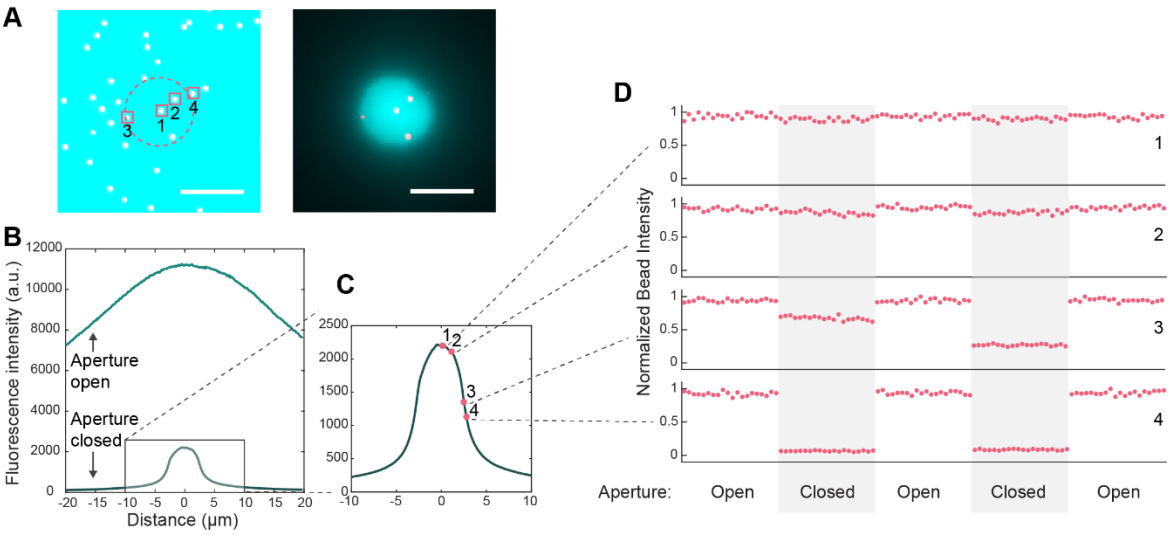
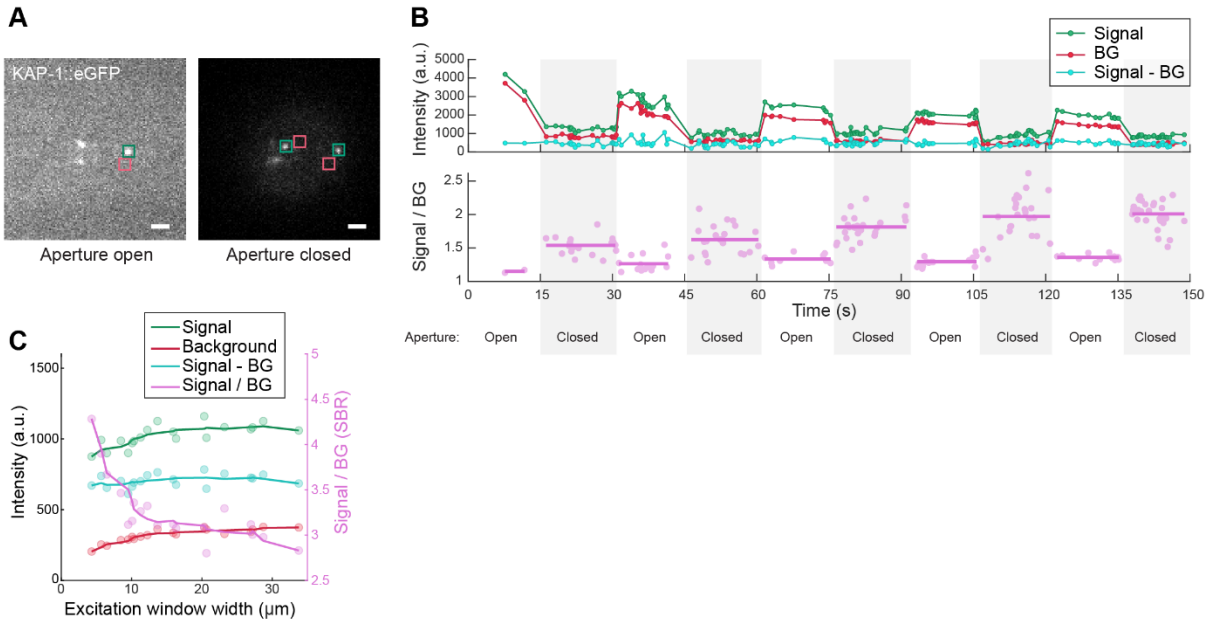


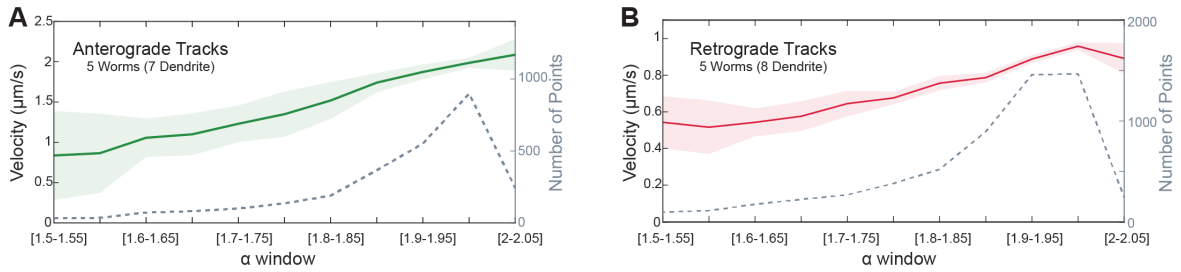
Figure 4: Statistical analysis of the motion of OCR-2-associated vesicles moving in and out of the illuminated region. (A) Histograms of point-to-point velocities obtained from 277 tracks of OCR-2-associated vesicles moving in the anterograde direction, in 7 dendrites (5 worms). Average velocity obtained from all data points is 1.85 ± 0.04 μm/s (N = 4073; grey). For data points with α < 1.95, average velocity is 1.72 ± 0.06 μm/s (N = 2481; light green), while, for data points with α > 1.95, it is 2.02 ± 0.05 μm/s (N = 1592; green). **(B)** Histograms of point-to-point velocities obtained from 228 retrograde tracks, in 8 dendrites (5 worms). Average velocity obtained from all data points is 0.82 ± 0.01 μm/s (N = 7157; grey). For data points with α < 1.95, the average velocity is 0.76 ± 0.02 μm/s (N = 5161; light red), while, for data points with α > 1.95, it is 0.95 ± 0.02 μm/s (N = 1996; red). **(C)** Distance-time plots of 78 tracks of diffusive OCR-2-associated vesicles, in 7 dendrites (7 worms). **(D)** Histogram of diffusion coefficients of the tracks in C, with average diffusion coefficient 0.12 ± 0.03 $\mu m^2/s$. **(E)** Histograms of the α-values in the direction perpendicular (light blue; 0.38 ± 0.03; N = 4028) and parallel (dark blue; 0.79 ± 0.03) to the length of the dendrite. Average value and error are estimated using bootstrapping. **(F)** Histogram of the time lag between subsequent OCR-2-associated vesicles moving in the anterograde direction, in 14 dendrites (N = 498; 9 worms). **(G)** Histogram of the time lag between subsequent OCR-2-associated vesicles moving in the retrograde direction, in the same 14 dendrites (N = 468). **(H)** The time-lag distribution in F and G plotted as a cumulative distribution function, overlayed with the least square fit to the function, in green and red for the anterograde and retrograde vesicles, respectively. The characteristic time between subsequent anterograde OCR-2 vesicles is 19.8 ± 3.2 s and between subsequent retrograde OCR-2 vesicles is 20.6 ± 4 s. **(I)** Histograms of intensities of anterograde (N = 146; 6 dendrites in 4 worms) and retrograde (N = 122; 8 dendrites in 5 worms) vesicles from two dendrites in the same worm. The average intensity of anterograde vesicles was 3771 ± 451 a.u. while the average intensity of retrograde vesicles was 3399 ± 670 a.u. Average value and error are estimated using bootstrapping.



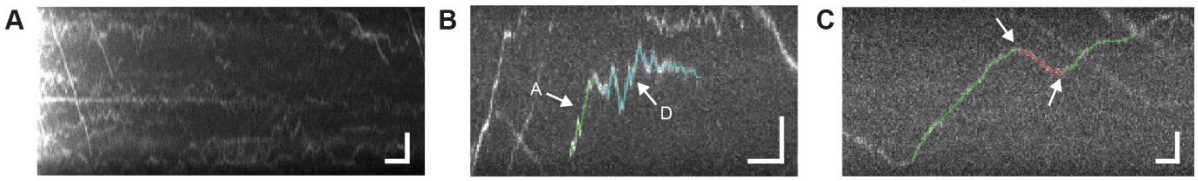
Supplementary Figure 1: Effect of shape and size of the excitation beam on signal intensity. (A) Overlay of fluorescent microspheres (red) and fluorescent slides (cyan) excited with aperture open (left; beam width ~50 µm) and closed (right; excitation window width ~5-10 µm). Scale bar: 5 µm. **(B)** Intensity profile of fluorescent slide excited with 491 nm laser, with aperture open and closed. The fluorescence intensity is ~5.5x lower, when the aperture is closed. **(C)** Zoomed-in intensity profile from B, and the relative positions of fluorescent beads, shown in A. **(D)** Normalized fluorescent bead intensity (BG subtracted) for the four beads indicated in A and C, imaged with aperture open and closed. For Beads 1 and 2 located close to the center of the beam, signal intensity does not depend on the aperture width. Signal intensity of Bead 3 critically depends on the width of the aperture. Note: Slightly different aperture widths results in different intensity during the first and second "Closed" positions. Bead 4, visible when the aperture is open, is not illuminated by the cropped excitation beam.



Supplementary Figure 2: Effect of excitation window width on the signal and background intensities. (A) Example image frames from single-molecule imaging of KAP-1::eGFP with aperture open (left) and closed (right). Regions in which signal and BG intensities were measured are indicated in green and red, respectively. Scale bar: 1 µm. **(B)** Top: Signal, BG, and Signal-BG of single KAP-1::eGFP molecules as a function of time. Bottom: SBR measured for individual frames (circles) and the mean value at different aperture states (horizontal lines). The aperture state is indicated below. **(C)** Signal, BG, Signal - BG (left y-axis), and SBR (right y-axis) measured for KAP-1::eGFP, similar to Figure 2B-C, at different excitation window widths.



Supplementary Figure 3: Velocity of anterograde and retrograde tracks of OCR-2-associated vesicles, filtered based on the local α-values associated with the tracks. (A) For anterograde tracks, average velocity of data points binned over alpha windows with bin width = 0.05, moving from 1.5 to 2.05, show that velocity increases from ~1 µm/s to ~2.1 µm/s. Number of datapoints in each bin is indicated by the dotted grey line (right y-axis). **(B)** For retrograde tracks, average velocity of data points binned over alpha windows with bin width = 0.05, moving from 1.5 to 2.05, show that velocity increases from ~0.55 µm/s to ~0.95 µm/s. Number of data points in each bin is indicated by the dotted grey line (right y-axis). For all distributions, average value and error are estimated using bootstrapping.



Supplementary Figure 4: Diffusive tracks of OCR-2-associated vesicles. (A) Kymograph along the dendrite taken at the start of imaging showing the initial bleaching of many diffusive and stuck OCR-2-associated vesicles. Scale bars: 3 s (horizontal), 2 µm (vertical). **(B)** Kymograph, overlayed with track data, of a vesicle that first demonstrates directed motion in the anterograde direction along the dendrite (green, A), whereafter it switches to a diffusive state (cyan, D). Scale bars: 3 s (horizontal), 2 µm (vertical). **(C)** Kymograph, overlayed with track data, of a vesicle that shows directional switching from anterograde (green) to retrograde (red) and back to anterograde (green) as indicated by the arrows. Scale bars: 1 s (horizontal), 2 µm (vertical).

Supplementary Movie 1: Imaging OCR-2-associated vesicles in the dendrite of a sensory neuron in *C. elegans*, using SWIM and epifluorescence microscopy. OCR-2::eGFP dynamics in the dendrite were imaged with a closed aperture (excitation window width ~7 µm) for the first 15 min. After 15 min, the aperture is opened (beam width ~50 µm), with imaging performed for another 5 min. Four sections of the acquired stream (acquisition rate 6.67 fps) are shown, with time indicated in min:sec (5x sped up). Brightness and contrast of section 3 are different from the other fragments.

Supplementary Movie 2: OCR-2-associated vesicles imaged in the dendrites of sensory neurons in *C. elegans*, using SWIM. OCR-2::eGFP dynamics in a pair of PHA/PHB dendrites (labelled Dendrite 1, Dendrite 2) imaged with the aperture closed (excitation window width ~11 µm) for 15 min at frame rate 6.67 fps. Time is indicated in min:sec (7.5x sped up).

Table S1: *C. elegans* strains used in this study

Strain	Genotype	Short notation	Source
N2	wild type	N2	CGC
EJP501	*vuaSi001 [ocr-2::eGFP] IV*	OCR-2::eGFP	van Krugten et al., 2022 [22]
EJP13	*kap-1 (ok676) III; viaSi1 [BP20; Pkap-1::kap-1::eGFP; cb-unc-119(+)] IV*	KAP-1::eGFP	Prevo et al., 2015 [21]