From cellular vulnerability to altered circuit activity

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CHAPTER 2

Assessing motor-related phenotypes of *Caenorhabditis elegans* with the wide field-of-view nematode tracking platform

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Abstract

Caenorhabditis elegans is a valuable model organism in biomedical research that has led to major discoveries in the fields of neurodegeneration, cancer and aging. Because movement phenotypes are commonly used and represent strong indicators of *C. elegans* fitness, there is an increasing need to replace manual assessments of worm motility with automated measurements to increase throughput and minimize observer biases. Here, we provide a protocol for the implementation of the improved wide field-of-view nematode tracking platform (WF-NTP), which enables the simultaneous analysis of hundreds of worms with respect to multiple behavioral parameters. The protocol takes only a few hours to complete, excluding the time spent culturing *C. elegans*, and includes (i) experimental design and preparation of samples, (ii) data recording, (iii) software management with appropriate parameter choices and (iv) post-experimental data analysis. We compare the WF-NTP with other existing worm trackers, including those having high spatial resolution. The main benefits of WF-NTP relate to the high number of worms that can be assessed at the same time on a whole-plate basis and the number of phenotypes that can be screened for simultaneously.

Introduction

Caenorhabditis elegans is a powerful tool in biomedical research because of its relative simplicity, amenability to genetic manipulation, invariant development and short lifespan[1-3]. Moreover, the high degree of similarity of its genetics and cellular complexity to those of its human counterpart has led to major discoveries in the fields of neurodegeneration, cancer, metabolic diseases and aging[4-11]. Over the years, numerous phenotypic assays have been developed to assess various aspects of *C. elegans* fitness. In particular, behavioral and visible phenotypes such as thrashing, crawling and paralysis (**Figure 1**) have been instrumental in discoveries related to the function and development of the muscular and nervous systems[12-15]. In addition, these phenotypes have also been shown to be useful in studies concerning the pathology of muscles and neurons, as is the case in conditions such as aging and neurodegeneration[16-20]. In this context, the two-dimensional sinusoidal wave-like movement (crawling; **Figure 1B**) or the frequency of lateral bends (i.e., thrashing; **Figure 1A**) in liquid are commonly assessed phenotypes in *C. elegans*[11,16,18,20-25]. Various mutations and transgenes are known to disrupt these movement behaviors (**Table 1**)[26,27]. Moreover, as with humans, a decline in muscle function is a common characteristic in aging *C. elegans*, as manifested by a decrease in movement capacity[20,28]. However, not all movement phenotypes appear to correlate strongly with aging; crawling (i.e., maximal crawling velocity) showing the strongest correlation[20]. Yet the fitness of *C. elegans* is most often still assessed by the number of body bends per minute (BPM) in liquid media (thrashing) (**Table 1**). In fact, owing to thrashing's strong correlation with toxicity (**Table 1**), thrashing assays are often the first choice when assessing healthspan in *C. elegans*. Because of differences in both kinematics and the patterns of muscle activity, thrashing and crawling provide distinct but complementary information about *C. elegans* fitness[20,29-32]. This observation suggests that assessing multiple movement phenotypes simultaneously could enhance the characterization of a worm strain[12,33]. Nevertheless, evaluating movement phenotypes manually has long been hindered by difficulties in acquiring

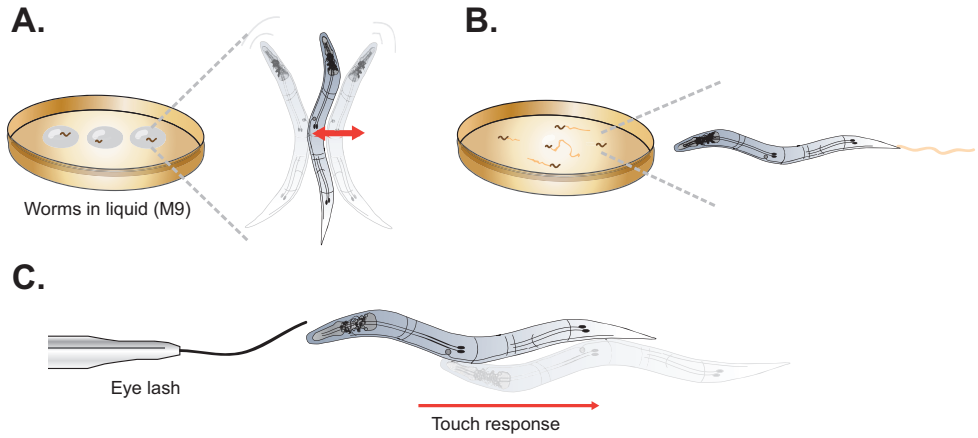


Figure 1: Different assays for assessing movement capacity in *C. elegans*. A) Thrashing behavior is typically assessed by counting the number of c-shaped bends per 30 s or per minute (BPM). For this assay, worms should be in liquid. **B)** Crawling behavior can be assessed by letting worms crawl for a user-defined time and by looking either at the distance they covered or at the maximal speed at which they crawled. **C)** Paralysis of worms can be assessed by scoring worms that are still alive (pharyngeal pumping present), do not move voluntarily anymore and do not respond to a touch with an eyelash (touch-response). These worms are considered to be paralyzed.

Table 1: *C. elegans* disease models and their thrashing capacity as compared to that of control worms

Human disease	Human gene	*C. elegans* gene	Notes	References
Huntington's disease	*HTT* (Huntingtin)	-	Reduced trashing	16, 17
Parkinson's disease	*SCNA* (a-synuclein)	-	Reduced trashing	16
Alzheimer's disease	Amyloid-B (Ab)	-	Reduced trashing	11, 19
Spinal muscular atrophy	*SMN*	*smn-1*	Reduced trashing	21, 22
Duchenne muscular dystrophy	*DMD* (dystrophin)	*dys-1*	Reduced trashing	23
Mitochondrial DNA depletion syndrome	*POLG*	*polg-1*	Reduced trashing	25
Amyotrophic lateral sclerosis	*TARDBP* (TDP43) *SOD1* (G85R & WT)	*tdp-1* *sod-1*	Reduced trashing Reduced thrashing (stronger in G85R)	18 16, 24

WT, wild-type

data—assays are labor intensive, throughput is low, and observer biases cannot be fully excluded[34,35]. In addition, owing to small sample sizes, subtle changes in behavior (e.g., effect size) are hard to identify.

The desire to tackle these difficulties has led to the development of automated worm tracking platforms. Although some of them focus on highly detailed aspects of the movement of single worms[12,36–38], most of them focus on analyzing thrashing and/or crawling behavior of larger worm populations[34,39–42]. We recently developed the WF-NTP as a high-throughput tool[35]. This platform enables the simultaneous analysis of large numbers of worms, as well as multiple behavioral parameters (maximal and average speed, BPM, size, paralysis). The use of a wide field of view and flatfield illumination enabled the experimental throughput to be increased substantially, and the platform allows large population sizes (up to hundreds of worms) to be monitored at the same time[35]. This feature is especially helpful when considering the high intrinsic variability of worm behavior in combination with the aim of detecting more subtle phenotypic changes (i.e., low effect sizes) in a quantitative manner.

Here, we describe in detail how the WF-NTP can be used to study several movement phenotypes in *C. elegans*. In particular, we present a subset of new filters that we included within the software to decrease the number of faulty estimations as compared to manually counted data. Moreover, to make biological interpretations and to validate comparisons of results to those in the existing literature, automated platforms should relate to manual methods; thus we provide the field with optimized software whose output closely resembles manually counted data. Finally, we also provide detailed information about how parameters can be optimized for lab-specific settings and how they can be adjusted and manipulated for different applications.

Technical background of the WF-NTP

The WF-NTP enables researchers to acquire high-quality movies and to perform simultaneous analysis of high numbers of worms and multiple behavioral parameters. By recording worms either in liquid (to assess their thrashing frequency) or on seeded or non-seeded nematode growth medium (NGM) plates (to track their crawling capacity), numerous parameters can be extracted by the WF-NTP software. The system uses a simple optical path in a transillumination geometry for bright-field microscopy data acquisition, along with custom software written in Python to perform

the wormtracking[35]. The system does not rely on live feeds but instead analyzes previously acquired movies, which makes it possible to separate data acquisition and analysis in time and space.

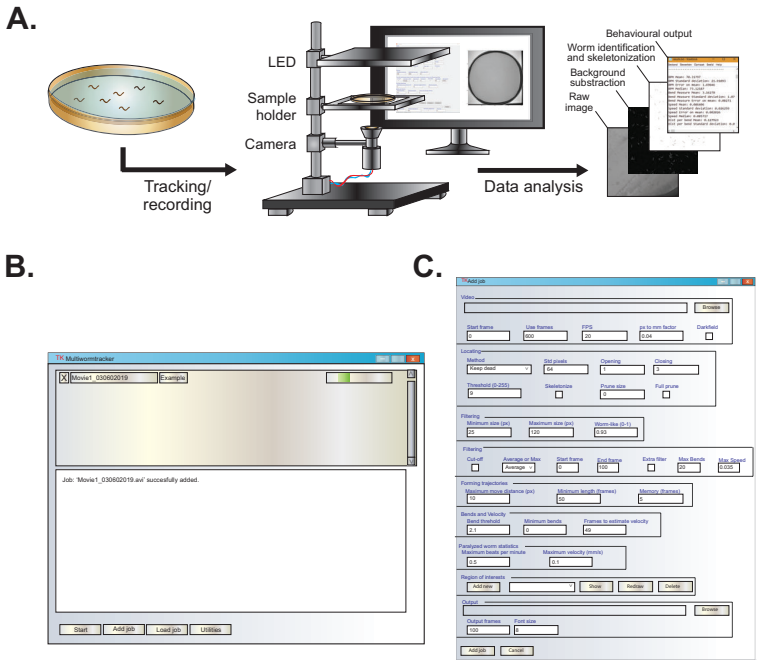


Figure 2: WF-NTP platform and software. **A)** Schematic of the platform used for making recordings. Plates to be analyzed can be placed on the sample holder and recorded for a specific time interval. Afterward, the videos can be analyzed with the WF-NTP software. **B)** Graphical user interface (GUI) of the WF-NTP. If the user clicks on 'Add job', the screen in c will appear. **C)** WF-NTP software screen for the input of the values for specific parameters and filters.

The platform itself consists of three important parts: the light source, the plate holder and the detector (camera with lens), which is coupled to a computer (**Figure 2A**). The choices of light source, optical components and detectors are interrelated and are based on several criteria. First, the light source used is an array of LEDs coupled to a diffuser that together provide flat-field illumination over a large area of up to 100 cm^2 with minimal heat generation. The last criterion is especially important in avoiding stress of the worms during data acquisition. Flat-field illumination is critical to ensuring that data acquisition is independent of the position of the animals in space. In contrast to conventional fixed-focus lenses, we use an imaging lens with an adaptable focal length to ensure usability at different working distances, making it possible to image different area sizes and magnifications (6- and 9-cm plates, multiwell format). Finally, the detector (i.e., the camera) should have a high (~6-megapixel) resolution to allow enough spatial information to be acquired for motility phenotypes to be recorded accurately, as well as the ability to record at ~20 frames per s (f.p.s.) to provide sufficient time resolution for movement analysis. Typically, a computer with a USB 3.0 connection is required to achieve sufficient data transfer rates to enable data from this type of camera to be recorded. By combining these components, it is possible to generate high-resolution movies with uniform illumination and adaptable frame rates for different applications. Moreover, by either working in a closed box or in a darkroom, possible interference

of background light can be eliminated so that acquired movies have the same contrast and quality across different locations in space and over time. Technical details of all suggested components can be found in the 'Materials' section, but it is possible to adapt the choices of elements, as long as the generated movies fulfill the criteria required by our software ('Overview of the procedure').

The WF-NTP software is implemented in Python and originally was generated to work on Windows computers, but it now works on other platforms too, including MacOS (**Figure 2B, C**). This software has been designed to parallelize analysis of multiple movies on the basis of the RAM and CPU of the computer, so that a computer with high calculation power will allow the simultaneous analysis of more videos. Moreover, the software was designed to be flexible in terms of functionality and adaptability. Through a graphical user interface (GUI), users can adjust operation parameters, tracking settings and regions to be analyzed (regions of interest (ROIs)) in a straightforward manner. In this way, the software operation can be adjusted for laboratory-specific parameters, including the type of camera, level of noise filtering, nature of ambient light, achieved contrast and type of assay. The software provides users with two different ways of performing a background correction required for identifying individual worms. The first method, z-filtering, follows a conventional approach and uses temporal differencing to differentiate relevant foreground pixels from the constant background. Although this approach works well for non-stationary objects (e.g.,

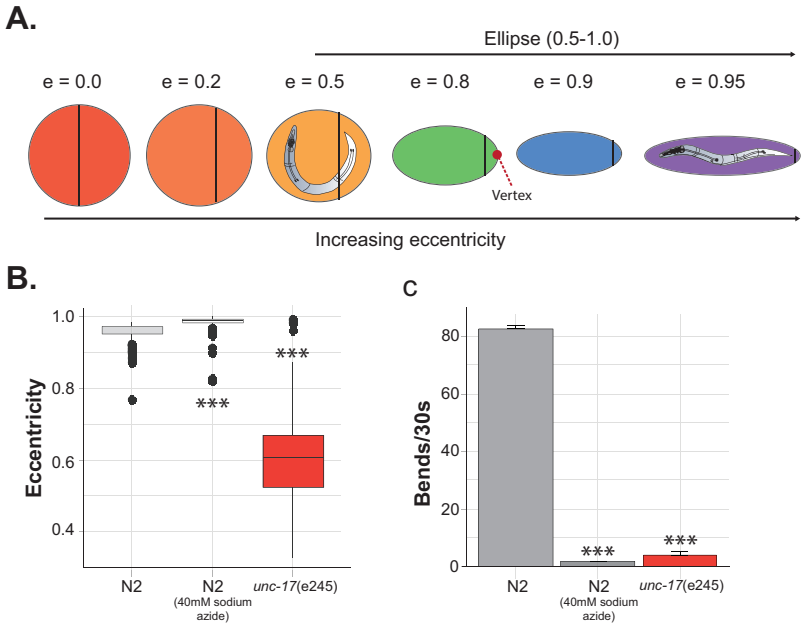


Figure 3: Measurements of the eccentricity of the particles and of the coilers. A) Eccentricity (e) is used as a measure of how nearly circular an ellipse is. Ellipses have an eccentricity between 0.5 and 1.0. Typically, crawling and thrashing worms have an eccentricity close to 0.9 or higher. At the same time, coilers can have an eccentricity close to 0.5. Vertical lines represent the latus rectum, which crosses the focal point at one side of the ellipse. The closer the focal point to the vertex, the higher the eccentricity and the lower the circularity. **B)** Eccentricity of N2 worms, N2 worms treated with sodium azide (which straightens the worms) and coiler worms (*unc-17(e245)*), as measured by the WF-NTP. Kruskal–Wallis test ($P < 0.001$) with post hoc Dunn's test, $n \approx 100–300$ per condition. The dots represent outliers outside the $[Q1 - (1.5) \times IQR$ (interquartile range), $Q3 + (1.5) \times IQR]$ range. **C)** With small adjustments in the software, both paralyzed worms and coiler worms have a low bend rate, which is in line with the bend rate observed by eye. Kruskal–Wallis ($P < 0.001$) with post hoc Dunn's test, $n = 100–300$ per condition. ***$P < 0.001$. Error bars: s.e.m.

worms), stationary particles are often not detected. Consequently, a second method, called 'keep dead', was included that uses adaptive Gaussian thresholding that relies on preselected spatial data. Where the inclusion of immobile worms is critical to a robust analysis in most studies, effects are sometimes only expected in the fraction of mobile worms and then the temporal filtering approach may be more useful. Therefore, the choice between the two background subtraction methods depends on the biological question to be answered. More information on the algorithms underlying the two methods is provided in ref. [43,44].

After thresholding, images are further processed and the morphological noise is removed by two operators from mathematical morphology (opening and closing). The resulting image contains labeled regions with particles of different sizes. By executing object-size filtering, additional background noise is removed in the form of objects too small or too large to be single worms. Finally, an additional option converts worms to single-pixel skeletons, which can then be pruned to remove spurious features. Importantly, this skeleton approach is used for better determination of centroids (i.e. geometric centers) and not to define outlines or to do segmentation of the worms. The degree of noise reduction via mathematical morphology, size exclusion and skeletonizing can all be easily adjusted in the GUI. To ensure that the algorithm is set up in the optimal way (all filtering parameters are adequate) before the start of the analysis, it is possible to create example images for all individual thresholding and filtering steps (**Figure 2A**).

After all these operations have been performed, the remaining labeled regions are identified as individual worms and the coordinates of those regions are stored for each frame. By means of standard tracking algorithms[45], these regions are then linked across the frames for each individual worm. This tracking algorithm allows collisions, for example, worms disappearing and/or overlapping, to take place without directly removing worms afterward. By keeping the coordinates of worms before the collision in memory, tracking is continued when individual worms are detected again. However, this continuation happens only when a collision event takes place for a user-defined number of frames and when worms are within the maximal movement distance per frame. Subsequently, the centroid of each object is used for speed estimations, and changes in eccentricity (i.e., how nearly circular an ellipse is; **Figure 3A**) are used to estimate the extent and frequency of worm bending as a function of time. Here, additional filtering is possible based on worms behaving in a non-anisotropic fashion and lacking ellipsoidal properties (i.e., low eccentricity), or their speed–bend relationship; the number of particles present can be evaluated as well when selected for in the GUI. Potential caveats and outcomes of these and previously mentioned filtering steps will be pointed out in the 'Experimental design' section. A variety of metrics, including BPM, average and maximal speed, paralysis, area per animal, length and average eccentricity are assessed by the WF-NTP software. All metrics are combined in a single text file (averages for whole populations) and a particles.csv file (with all the individual worm values). Moreover, an additional file containing the tracks of individual worms over time is also generated. The WF-NTP software comes with a tool with which to visualize those worm tracks in a color-coded fashion.

Improvements on the original WF-NTP

The initial WF-NTP, introduced in 2018, enabled the detection of small changes in worm behavior, including those occurring upon drug treatment[35]. We discuss here a series of improvements that enable the resulting metrics to be compared with manually acquired data and that allow completely paralyzed worms to be analyzed faithfully. Moreover, we discuss improvements that allow coiling

worms to be analyzed (i.e., if the body of a worm assumes a coil shape when it attempts to move, which is associated with defects in the cholinergic system), to prevent them from being excluded on the basis of their low eccentricity. Because some treatments, genetically or compound related, may induce coiling behavior or paralyze worms completely, we have updated the software to fully take into account these behaviors.

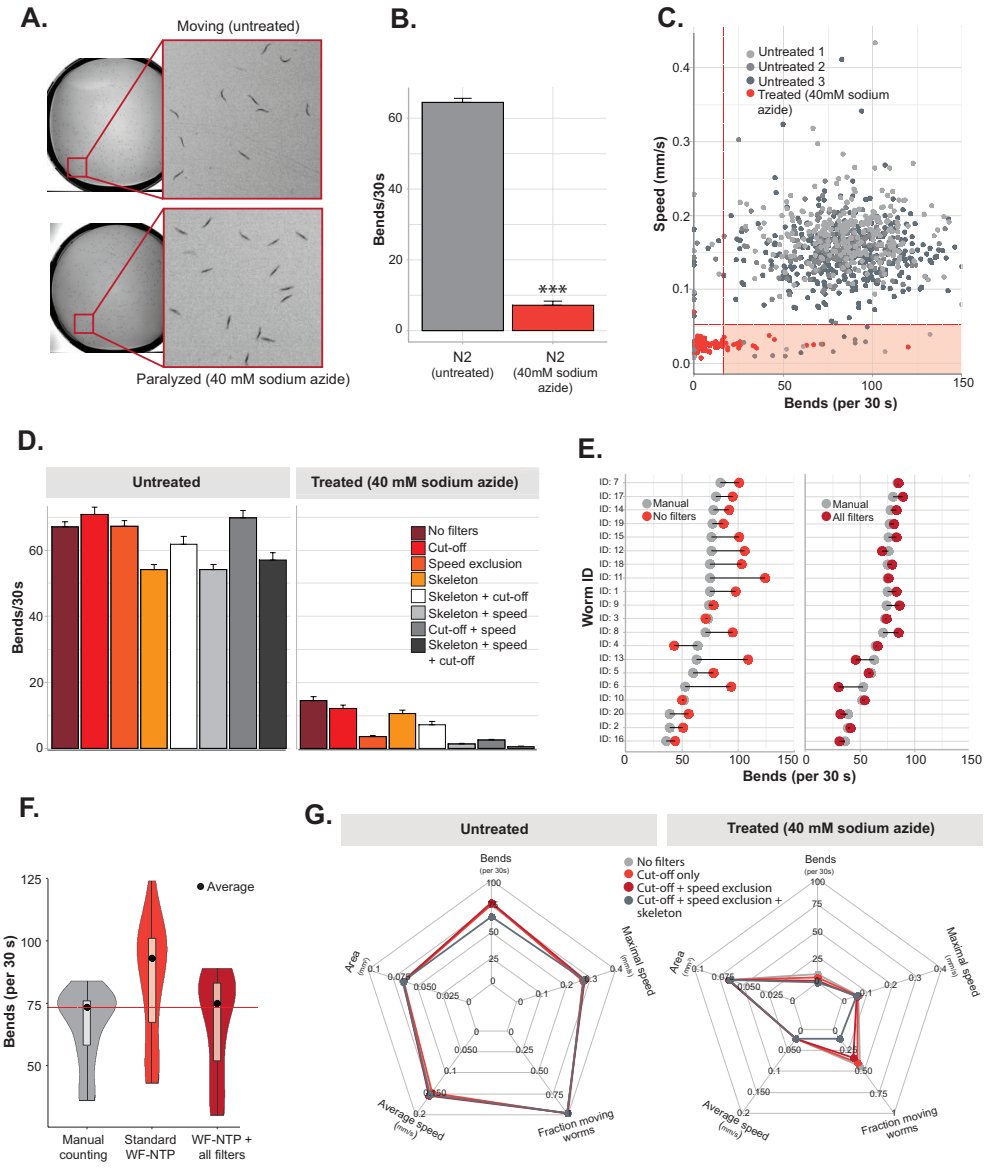
Coilers

The WF-NTP uses the typical eccentricity (**Figure 3A**) of the worms to exclude particles that behave in a non-anisotropic fashion and lack clear ellipsoidal properties. This function was based on the observation that the thrashing frequency of worms that coil is highly overestimated. The underlying algorithm for bend estimation is sensitive to low eccentricity and interprets it as continuous bends. This worm-like filter solved the problem but also created a new one. When a worm has an eccentricity below the user-defined worm-like value ('Experimental outline') in a specific subset of frames, the particle is removed from those frames as if it were not there (but only for bend estimation, not for the other metrics). The other frames, in which it did surpass the worm-like value, are used for analysis of worm bend metrics. As a consequence, BPMs are extrapolated (e.g., inferring the BPM as if the worm were present the whole time) from a set of frames in which actual bends appeared. This procedure also results in BPM overestimates, because the frames in which the worms are not bending (i.e., acting similar to coilers) are excluded. To correct for this problem, the software now substitutes eccentricity values with dummy variables when the actual eccentricity does not surpass the worm-like value. These dummy variables ensure that the bending threshold is not exceeded, resulting in no registered bends in these specific frames. However, these dummy frames, without bending events, are now used to estimate BPM (or bends per 30 s), yielding consistent and biological relevant results (**Figure 3B**,**C**).

Lower-boundary adjustment

We adjusted the WF-NTP for accurate tracking of paralyzed animals. For this purpose, we paralyzed worms with 40 mM sodium azide and recorded their thrashing behavior. When analyzing this kind of movie, one expects to find a thrashing rate (bends per 30 s) of ~0, because the worms cannot move at all (**Figure 4A**). This is, however, not always the case, as illustrated in (**Figure 4B**), where paralyzed objects did have a velocity and a number of bends per 30 s that was >0. Consequently,

Figure 4: Speed–bend and cut-off filters improve the accuracy of the WF-NTP analysis. A) An example of a plate with moving and paralyzed worms. Worms treated with sodium azide have a stick-like appearance. **B)** With the current WF-NTP software, differences between paralyzed and moving worms can be detected by comparing the bend rate (Mann–Whitney U test, $P < 0.001$), $n \approx 200$ per condition. However, sodium azide–treated worms still have an average bend rate that deviates from zero. One representative experiment is shown, repeated three times. **C)** Worms treated with sodium azide have a lower speed than moving worms. However, they appear to bend according to the WF-NTP software. The red dashed lines represent the cut-off values for speed and bend rate that we use for speed–bend exclusion. Particles to the right and below the dashed red lines are excluded (red box). $n \approx 100$ per condition. **D)** Effects of the three filters used separately or together when analyzing paralyzed or moving worms. Clearly, the skeleton filter has the largest effect on the bend rate of moving worms, whereas the speed–bend exclusion filter (speed exclusion) affects paralyzed worms the most, $n \approx 200$. All filters together give the best results, see panel e. **E)** Comparison of manual and WF-NTP counting. Using the new set of filters increases the congruency between manually observed bends and those detected by the WF-NTP. **F)** Population statistics also improve with the new filter methods, mimicking the group statistics of manually counted worms better. Black dots represent the averages, the white boxplots are in the [Q1 − (1.5) × IQR, Q3 + (1.5) × IQR] range, and the red dashed line represents the average of the manually counted data. **G)** All other metrics are unaffected; only the bend rates are reduced by the new set of filters. ***$P < 0.001$. Error bars: s.e.m.



we plotted the number of bends versus the average speed of both untreated and treated (40 mM sodium azide) worms (**Figure 4C**). From this figure it becomes clear that many paralyzed worms have an average speed that exceeds zero but is also lower than that of the non-stationary objects. From this perspective, we generated a speed–bend exclusion filter that removes particles that have a very low speed (comparable to completely paralyzed worms) but still show bends (**Figure 4D-G**), which reduces noise in the bend rate (bends per 30 s) to a great extent without affecting the other metrics (**Figure 4G**). Thus, paralyzed worms are analyzed in a more accurate way, and bending worms now represent the manually counted data more precisely (**Figure 4E,F**). Consequently, we

now provide the field with optimized software that produces data matching manually counted data on a nearly 1-to-1 basis.

We also included a second filter, called 'cutoff', which does not have clear effects on worm metrics (**Figure 4D,G**). This filter investigates the maximal or average number of worms present simultaneously in a movie within a user-defined set of frames. This number is used as an upper limit for the number of worms that are annotated; that is, when a worm moves too far to be recognized as the same worm, it will not be given a new number and will not be tracked anymore. If tracking is accurate and the WF-NTP is set up properly, this filter excludes few particles, because worms are not lost (see **Supplementary Tables 1 and 2**). Therefore, it provides an intrinsic control of the accuracy of tracking that can be used to assess optimization. Moreover, by following only a specified set of particles, it is possible to work with non-weighted averages (see the 'Post-experimental data analysis' section) and judge each single particle as an independent worm.

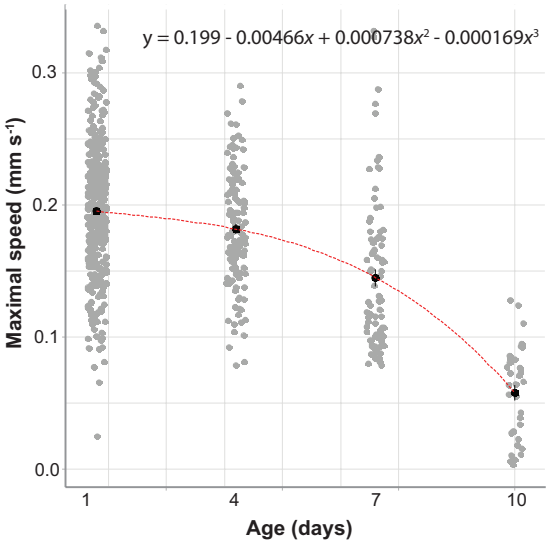


Figure 5: Maximal velocity can be analyzed with the WF-NTP. As shown by ref. 20, maximal velocity declines with age. Here, we show that we can also detect this decline in maximal velocity with the WF-NTP. We made 30-s movies of worms crawling at specific time points (same population of worms), n ≈ 50–150 per time point.

New worm metrics

In addition to the updated algorithms and new filters, we also include new worm metrics. It is thus now possible to estimate maximal speed, eccentricity (as a readout for coilers) and round ratio. Because of potential tracking inaccuracy, the 90th percentile of the speed distribution is used as a representation of maximal speed (**Figure 5**). The round ratio provides a value that represents the fraction of the frames in which the worm eccentricity surpasses the worm-like value. This value gives insight into how long worms behave like coilers. Finally, the eccentricity value represents the average shape of the worm. High eccentricity may represent quickly bending worms (high BPM) or paralyzed worms (low BPM), whereas lower eccentricity may represent slow-bending worms (low BPM, low round ratio) or coilers (low BPM, high round ratio). Indeed, the interpretation of multiple parameters yields relevant information about the behavior of the worms ('Anticipated results'). For more information on these parameters, see also the 'Post-experimental data analysis' section.

Comparison with other worm tracking platforms

Over the years, several trackers have been developed to assess to worm behavior[12,34,36,39–42,46,47]. In **Table 2**, we compare the WF-NTP to those other platforms. Although many of the underlying algorithms and applications are based on similar ideas, making them conceptually similar, critical

Table 2: Comparisons of the WF-NTP with other existing tracking platforms

Name	# of worms	Detection of worms	GUI	Required hardware	Required software	Parameters/ behavior	Required resol.
Worm tracker 2.0 (Shafer lab)	Single	Skeleton and outline	Yes	X-Y stage, camera, windows XP or Vista	Java, ffdshow, MATLAB or MCR	Area, length, width, thickness, transparency, brightness over head and tail	1280x1024
Nemo (Tavernarakis lab)[36]	Single	Skeleton and outline	Yes	Camera	MATLAB (R13) + image Processing Toolbox	Speed, waveform, angles between segments, thickness, distance between head and tail	800x600
Parallel Worm Tracker (Goodman lab)[39]	<50	Centroid	Yes	Camera	MATLAB (R13) + image acquisition and image Processing Toolbox	Size, shape, Speed, tracks, paralysis, turning events	640x480
Multimodal illumination and tracking system (Lu lab)[41]	Single	Skeleton and outline	Yes	Microscope, X-Y stage, camera, projector, filters	LabVIEW (+Vision)	Speed, body shape.	320x240
CoLBeRT (Samuel lab)[42]	Single	Skeleton and outline	Yes	Microscope, X-Y stage, laser, DMD array, frame grabber camera	MindControl (custom, C), MATLAB R2010a	Bending dynamics	1280x1024
Multi worm Tracker (Kerr lab)[34]	<120	Skeleton and outline	No	Camera, frame grabber, background light	LabVIEW (+ vision), C++ (custom), Java	Spontaneous movement, swimming, chemotaxis, response to tapping	4 MP; 2352x1728
Track –a-worm[40]	Single	Centroid and spine	Yes	Microscope, camera, X-Y stage	MATLAB (R2012b)	Locomotion, bending, speed, body shape	640x480
Tierpsy tracker[12]	Multiple	Skeleton, outline, segmen-tation	Yes	Camera, light source (see Worm tracker 2.0)	Python	Postural data, velocity, morphology	1280x1024
CeleST[46]	At least 1-5	*Central body line (skeleton)*	Yes	Camera	MATLAB (2011) with statistics toolbox	Locomotion, speed, curling, reverse swimming, stretch, asymmetry, wave initiation rate	696 x 520 pixel; image resolution : 0.02 mm / pixel (1 mm ~ 50 pixel)
3D-worm tracker[47]	Single	Skeleton	No	Two cameras, a FASTCAM SA1.1 (Photron) with 1024 × 1024 pixel resolution and a PCO.1600 (PCO) with 1600 × 1200 pixel resolution, coupled with two identical objective lenses	MATLAB	Bending vector, turning, backward crawling	550x550

Nematode Tracking Platform (WF-NTP)[35]	<5000	Skeleton and centroid	Yes	Camera, light source	Python	Trashing, locomotion, speed, maximal speed, paralysis, area/length	At least 6 MP (lower/ higher is possible)

differences in implementation, performance and application space exist. Specifically, existing trackers, including the WF-NTP, can be classified on the basis of the type of information that is obtained from the individual video frames: the centroid position or the so-called 'central skeleton'. Most trackers that use a centroid approach (**Table 2**) do not require high-resolution videos, because only a few connecting pixels are sufficient to determine the position of a centroid. The WF-NTP falls in this first category. On the other hand, skeleton-based trackers generally require a high magnification and are sometimes based on the use of a microscope.

Apart from the detection modality, differences between the WF-NTP and other tracking platforms appear in relation to the number of worms that can be followed simultaneously, the type of information that is collected, the different assays that can be performed, the presence of a GUI and the adjustability of the software and platform (**Table 2**). Most of the existing platforms are built to follow single worms over time. By using a high magnification and skeleton-based tracking, large amounts of postural data can be acquired at once (e.g., the direction of movement, angles of movement and ventral or dorsal bends). Some of these trackers, such as WormTracker 2.0, even allow individual worms to be followed in space with the aid of motorized x–y stages. This feature typically comes at the cost of throughput (with the Multi-Worm Tracker being an exception[34]). The centroid-based methodology enables, in general, a higher throughput but does not yield such extensive and detailed postural data as skeleton-based trackers do. Even though the throughput of centroid-based trackers is generally higher, the number of modalities (<50) that can be followed over time is still limited. By contrast, the WF-NTP can follow up to hundreds of worms simultaneously when large plates (9-cmor 14-cm) are used.

Next, tracking platforms with a GUI are generally more user friendly, because prior knowledge of the underlying code, typically either Python or MATLAB based, is not required. However, with software being mainly open source, knowledge of the underlying programming language is an advantage because the software can then be adjusted to specific applications and requirements. It is important to understand how parameters such as bending frequency (e.g., thrashing) are calculated, especially when comparing tracker-generated data to manually counted data and explaining possible differences. Therefore, insights into the underlying concepts are helpful. A few of the skeleton-based trackers apply segmentation to the binarized worm particles; that is, worms are divided into predefined sections such as tail, head and midsection[12]. In this way, angles between two segments, for example, head and tail, can be used to compute an angle evolution over time as a measure for body bends. Other systems, especially those using a centroid-based approach, use changes in eccentricity as a measure for thrashing frequency; the WF-NTP is one of these. The accuracy of these measurements is highly dependent on the resolution of the movies, the efficiency of background subtraction methods and precise morphology. Therefore, it should not be surprising that single-worm platforms using a skeleton/segmental-approach are generally more accurate in estimating bending frequency.

Nevertheless, one important point should be considered when selecting the proper platform for an experimental purpose: many tracking platforms were initially developed to track locomotion of *C. elegans* on a solid surface (e.g., agar) and not in liquid media. Therefore, only a few trackers (**Table 2**) focus on thrashing behavior as primary tracking goal[34,35]. Although the

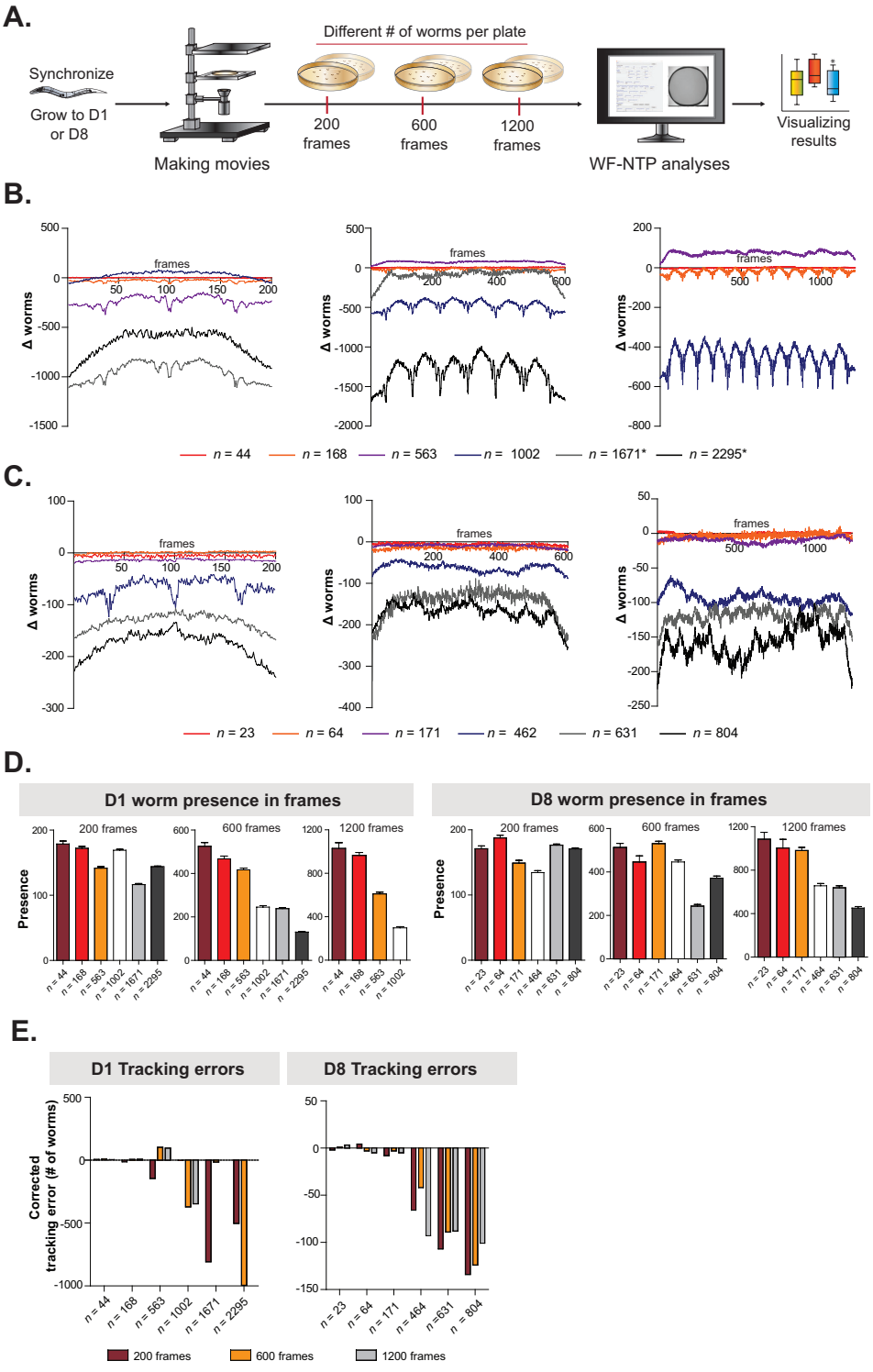
platforms that focus in particular on crawling behavior can be modified and adapted to also analyze thrashing worms, one should be aware that this might not be a standard feature. The same applies to other behavioral assays that some worm trackers offer (**Table 2**). In addition, several integrated applications exist that go beyond the scope of this protocol. For example, integrating optogenetic strategies into some existing platforms makes it possible to stimulate specific neuron populations when assessing specific behaviors[41,42,48].

In summary, there is no shortage of methods to collect and track worm behavior. Given the large set of approaches, one should choose a platform that fits the type of behavior to be analyzed and the biological question to be answered. This choice depends greatly on the amount of detail required, the expected effect size and thus the number of worms needed, the number of conditions to be tested (throughput) and the type of behavior to be assessed.

Advantages and limitations of the WF-NTP

On the basis of comparisons with other tracking platforms (**Table 2**), the WF-NTP offers important benefits for the tracking of a large population of worms at the same time. Its throughput is substantially greater than those of other existing methods and is one of the main advantages of the WF-NTP. Larger sample sizes are often required because of the high intrinsic variability of worm behavior and/or the sometimes-subtle effects of compounds or genetic interventions. In fact, the WF-NTP provides a flexible platform for performing genome-wide screens or compound screens in relation to defects in movement capacity. In particular, the ability to track multiple worms in multiple regions (i.e., in a multiwell plate) and to analyze different movies simultaneously makes the WF-NTP an outstanding application for these types of studies. By offering researchers a way to analyze all worms on an agar plate instead of a zoom-in region, observer and population biases can be avoided. At the same time, the platform offers software that can be used for both crawling and thrashing assays, because data on both types of behavior can be collected. The improvements of the WF-NTP contribute to high-accuracy bend estimations as compared to manually counted data (**Figure 4E,F**) and provide additional postural information (e.g., coiler-like behavior, **Figure 3**) that makes the platform even more flexible in terms of usage.

Nevertheless, the WF-NTP also has some limitations in regard to accuracy, the type of information that can be collected and the effort that one should put into optimization. In the 'Comparison with other worm tracking platforms' section, we pointed out that the detection modality of the trackers influences the number of parameters that can be derived from the videos. Particularly when segmentation is included, postural data can be collected from single worms[12]. The WF-NTP uses a centroid-based approach and does not collect segmental data about postures, angles or speed. This may be a clear disadvantage when considering its use in the field of phenomics, which is the acquisition of high-dimensional phenotypic data on an organism-wide scale. An important assumption within this field is that the right phenotype to look for can be difficult to determine *a priori*. In fact, the phenotypic effects of perturbations—for example, genetic manipulations—are difficult to predict when taking aspects such as pleiotropy into account. Therefore, performing phenotyping as extensively as possible may yield valuable information about the effects of interventions on phenotypes[12,33,38,49]. For example, the Tierpsy Tracker, which uses the phenomics approach, offers the user a platform with adequate resolution and a segmental approach that makes it possible to extract morphological and behavioral information at the same time[12]. Nevertheless, one should always take throughput into account, because there is a clear


A.
Synchronize
Grow to D1 or D8
Making movies
Different # of worms per plate
200 frames
600 frames
1200 frames
WF-NTP analyses
Visualizing results
B.
Δ worms
frames
n = 44
n = 168
n = 563
n = 1002
n = 1671*
n = 2295*
C.
Δ worms
frames
n = 23
n = 64
n = 171
n = 462
n = 631
n = 804
D.
D1 worm presence in frames
D8 worm presence in frames
Presence
200 frames
600 frames
1200 frames
E.
D1 Tracking errors
D8 Tracking errors
Corrected tracking error (# of worms)
200 frames
600 frames
1200 frames

◂ **Fig. 6 | Tracking accuracy at different worm densities and time intervals in 9-cm plates. A)** The experimental pipeline: worms were aged until adulthood D1 or D8 before movies were generated. Worms were pipetted in different densities onto tracker plates, counted and then recorded for 200, 600 and 1,200 frames. Subsequently, all the movies were analyzed with the WF-NTP software. Tracking accuracy is visualized in b–e and described in Supplementary Tables 1 and 2. **B)** The 'Δ worms' (e.g., the difference between the actual number of worms present per frame and the number of worms detected by the WF-NTP with a preselected cutoff filter) per frame at different worm densities and time intervals at adult D1. Negative values imply that fewer particles were detected by the WF-NTP than were actually present; this might be due to collisions and overlap, which will result in particles being excluded because of their size. A positive number means the opposite; this might be due to background being recognized as particles (e.g., residuals of the plate edges). Clearly, from >500 particles, the 'Δ worms' value increases exponentially, making the tracking results less reliable. *In the conditions n = 1,671 and n = 2,295, the 1,200-frames data are missing because of memory errors; the linking is too complex with so many worms at such a time interval at a standard computer. **C)**, The Δ worm per frame values (e.g., the difference between the actual number of worms present per frame and the number of worms detected by the WF-NTP with a preselected cut-off filter) at different worm densities and time intervals at adult D8; see b. Clearly, the deviation from the actual number of worms present is smaller when worms are older (and bigger) than when they are younger. This implies that tracking is more accurate when worms are bigger/older. **D)** Average worm presence at different worm densities and time intervals at adult D1 and D8. At >500 worms, the worm presence declines steeply, which becomes especially clear at longer time intervals. The chance of collisions increases in these cases, with the consequence that particles are lost and filtered on the basis of their combined size. **E)** Tracking errors as represented by the maximal number of worms present at the same time measured by the WF-NTF, as compared to manually counted numbers. Going higher than 400 worms reduces the number of worms picked up by the WF-NTP. In other words, increasing worm numbers does not yield more information per se, because, for example, worms are filtered out due to collisions. Tracking errors are lower when using larger worms (compare D1 with D8), which are more easily detected. Error bars: s.e.m.

trade-off between the extent of postural data that can be collected and the number of worms that can be followed simultaneously.

Tracking large number of worms simultaneously also entails clear challenges. In fact, when particle populations are large, one should definitely consider the potential collisions as a perturbing and limiting factor. Even though the tracking algorithm takes collisions into account, the accuracy of tracking is definitely influenced by such events. In fact, the software does not guarantee that two particles that collide will be recognized as the same particles after the collision; that is, objects may be swapped. This aspect may create unwanted noise and affect the tracking accuracy. Therefore, most trackers discard worms during collision events or allow users to manually select individual worms before and after collision events in order to connect tracks. By using wide-surface screening approaches—meaning large surfaces that are recorded with relatively few worms—and by allowing only very short collision events to take place, the WF-NTP software deals relatively well with this recurrent issue.

However, even though high numbers of worms can be tracked at the same time by the WF-NTP, the tracking accuracy decreases with increasing sample size (**Figure 6, Supplementary Tables 1 and 2**). When using 9-cm plates for recording purposes, tracking errors—as expressed by several parameters in **Figure6B-E**—become more evident at a sample size >500 worms. In these specific situations, collision events and background subtraction start to interfere with the software's ability to localize individual worms. As a consequence, particles are lost during the tracking procedure and subsequently quantitative data per worm are reduced. Also the length of recording should be taken into account: short recordings reduce the chance of additional collisions and worms being lost (**Figure 6B,E**). Although the number of worms per plate should be limited to ensure high-quality data, one should be aware that acquiring recordings for 400–500 worms at the same time still takes only 30–60 s. Moreover, parallel processing of movies with the WF-NTP software is possible. Therefore, the throughput of the WF-NTP remains high.

Another limitation may derive from the algorithm that is responsible for the estimation of bending frequency (thrashing). It has been previously stated that conventional methods used to extract bending frequency from videos—by making use of peaks of angles or extrema of eccentricity against time—can be confounded by the presence of random fluctuations. Moreover, setting the threshold to distinguish real bends from noise has proven to be difficult at times[50]. Indeed, optimization can be challenging when using the WF-NTP for the first time. Before the analysis, one should decide upon the values of multiple parameters, all of which have effects on the outcome of the tracking data. To avoid this kind of optimization, Buckingham et al.[50] developed a method in 2008 to automatically measure bending frequency without the use of morphometry and segmentation. Instead, a principal component analysis (PCA) that results in a covariance matrix is used to find the interval between two significantly similar frames as an estimation of BPM. Although this method bypasses morphological assumptions, which is in contrast to the WF-NTP, it allows only low throughput. Consequently, for this PCA method to work, worms should preferably be spatially restricted. In this way, movies can be split into smaller areas containing individual worms to make reliable covariance matrices[50]. Although individual bending frequencies calculated by the WF-NTP may sometimes slightly differ from manually counted bending frequencies, we show in **Figure 4F** that population statistics appear to be similar for the two methods. This shows that the WF-NTP provides the user with a platform to screen high number of worms simultaneously without loss of accuracy or reliability. Furthermore, by using the optimal parameters and interrelated verification steps shared in this protocol, optimization should be straightforward for any researcher using the WF-NTP for a lab-specific context.

Alternative applications

The WF-NTP provides a flexible platform for performing genome-wide screens and compound screens in relation to defects in movement capacity, that is, thrashing and crawling. Although the platform was originally developed to assess thrashing behavior of *C. elegans*[35], crawling behavior can also be studied. The software is open source and freely available; with some programming knowledge, the underlying code can be adjusted relatively easily. We strongly recommend that researchers do so and share additional features and changes with the community. In this way, alternative applications may arise over time.

In addition, because many assays in the *C. elegans* field depend on changes of speed and/or direction of movement, clever experimental design may directly give rise to other applications (**Figure 7**). For example, the effect of the acetylcholine esterase inhibitor aldicarb is often used to interfere with cholinergic synaptic transmission. In the presence of aldicarb, acetylcholine continues to accumulate, which eventually causes persistent muscle contraction followed by paralysis[51,52]. Generally, the fraction of paralyzed worms at specific time intervals is used as a measure of aldicarb sensitivity and thus of the relative efficiency of cholinergic synaptic transmission[51,52]. In **Figure 7A,B**, we show that analyzing 30-s crawling movies at specified time intervals with the WF-NTP is an efficient way of studying this aldicarb-induced paralysis. For example, by using 12-well plates, one can analyze multiple conditions and/or strains at the same time (using the ROI-selector).

Finally, the WF-NTP can also be used to analyze chemotactic behavior. As illustrated in **Figure 7C**, gradual regions around an attractant or repellent can be selected with the ROI-selector in the WF-NTP software. By either making a 1-s (20-frame) movie as an endpoint measure or making

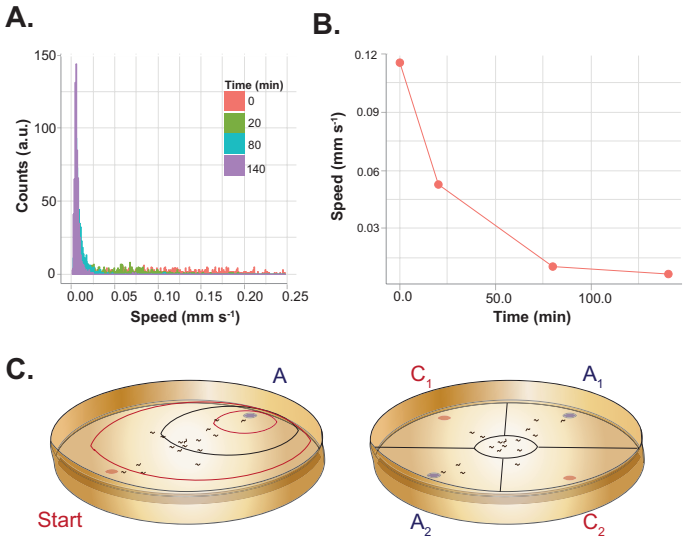


Figure 7: Aldicarb and chemotactic assays can be performed with the WF-NTP. A) Distribution of average crawling speed at specific time points after aldicarb treatment. The larger the time interval, the narrower the speed distribution, n ≈ 200 per time point. **B)** The average speed goes down with time after aldicarb treatment. **C)** Two possible experimental setups to assay chemotaxis. A, attractant; C, control.

multiple 1-s movies at specified time intervals, one can track the number of worms per region as a measure of attraction or repulsion. Optionally, one can follow worms continuously, so their direction of movement can be visualized by the Plot path function. In conclusion, by adding new features to the software and by using a clever experimental design, the WF-NTP software may open new avenues for the automated analysis of behavioral assays beyond those measuring thrashing and crawling.

Overview of the procedure

Performing tracking experiments with the WF-NTP is technically a simple procedure that consists of five main stages: (i) pre-experimental procedures; (ii) collection and preparation of worm samples; (iii) video acquisition; (iv) actual tracking with the WF-NTP software; and (v) post-experimental data analysis. An overview of the experimental procedure is given in **Figure 8**. Optimization of stages ii–v is required to obtain reliable estimations of the tracking parameters, such as thrashing (bend rate) and crawling speed. We strongly recommend taking note of our proposed optimization steps ('Experimental design' section) before continuing on to the Procedure.

Pre-experimental procedures

The pre-experimental procedures should be performed at least 1 d before the actual WF-NTP experiment but will generally start a few days earlier because the worms should be allowed to grow and reach the age of interest.

Age synchronization. Comparing behavior such as thrashing or crawling speed between different worm strains requires strict age synchrony because the quantity of these behaviors clearly changes during development and aging[20]. Hence, it is important to assess the rate of development of the worm strains used to anticipate potential differences. When assessed, worm strains can be age-synchronized (by hypochlorite treatment or egg laying) several days before the experiment[53].

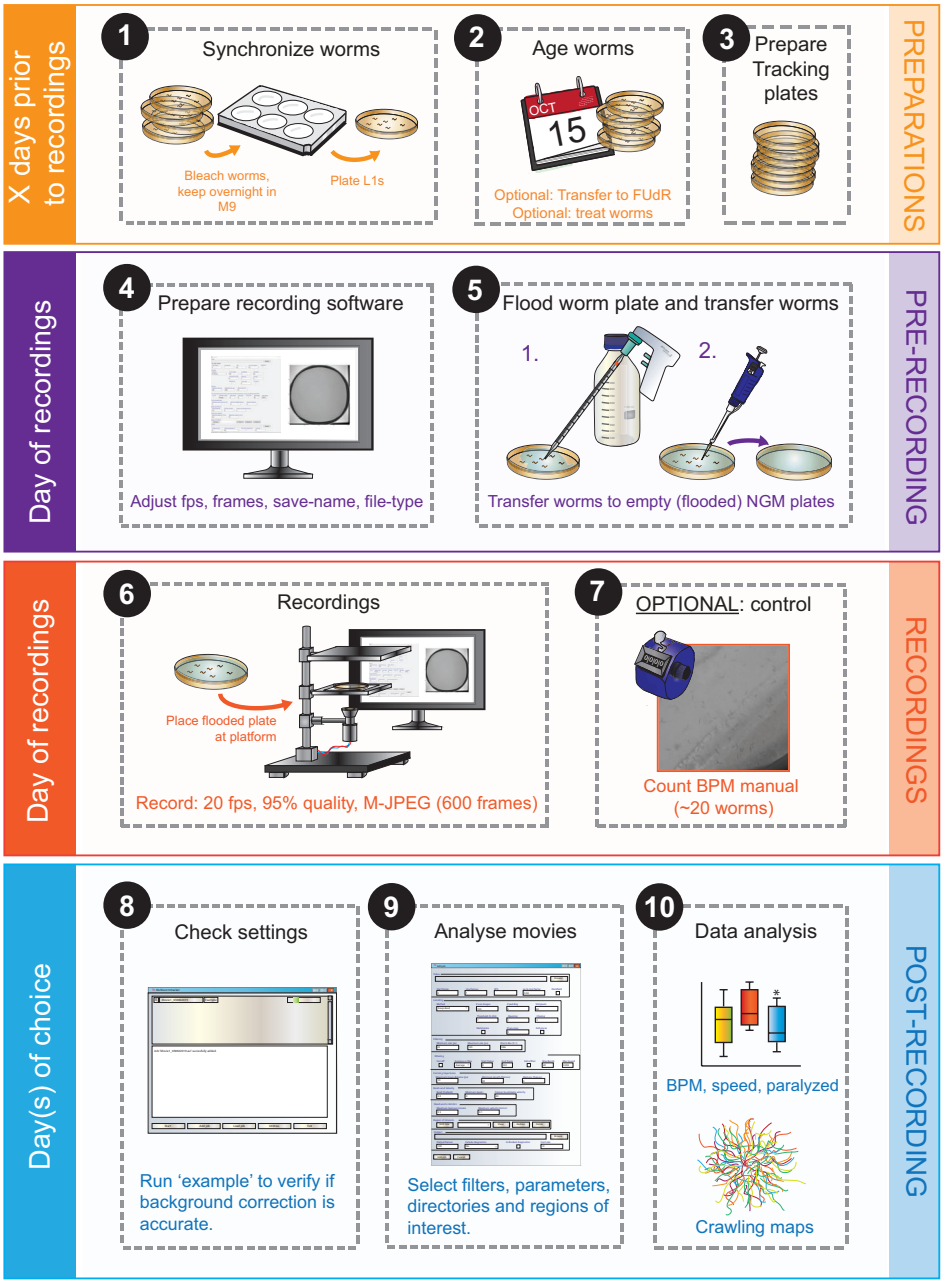


Figure 8: Experimental workflow and outline. Overview of experimental procedures involved in preparing and performing a WF-NTP experiment with C. elegans. The Procedure consists of (1) synchronizing the worms, (2) aging the worms, (3) preparing tracker plates (Steps 18 and 19), (4) preparing the recording software (Steps 20–32), (5) collection and preparation of worm samples (Steps 33–35), (6) recordings (Steps 36–40), (7) optional manual assessment of behavior to verify accuracy of the WF-NTP, (8) preparing and optimizing the WF-NTP software (Steps 41–49), (9) tracking with the WF-NTP software (Step 50), and (10) post-experimental data analysis (Steps 51–53). See main text for a more detailed explanation of all steps involved. Some image components were adapted from ref. 54, Springer Nature America, Inc.

As described previously by Koopman et al.[54], to prevent eggs from hatching or offspring from developing, plates containing 5-fluorodeoxyuridine (FUdR) can be used starting when the worms are young adults. It is important to understand that FUdR can alter biological aspects of the worms, as evidenced by changes in, for example, lifespan and worm size[55–57]. When there are clear reasons to avoid FUdR treatment, other techniques can be used to maintain synchronized worm cultures as described in ref. 58.

Tracking plates. Preparing 'tracking plates' is another important part of the pre-experimental procedures. To make reproducible videos, without adjusting background subtraction and morphological parameters for each movie, tracking plates should be of the same consistency and thickness. In **Table 3**, we provide suggestions for the volume of NGM 'tracking medium' required per well and plate size.

Table 3: Optimized variables for tracking purposes

Plate size	NGM medium (ml)	Volume M9 buffer (ml)[a]	Number of worms[b]	Camera position/distance to plate holder (mm)[c]	Pixel to mm conversion[c]
3 cm	2	1.5	50	130/170	0.034
6 cm	8	2-3	100	130/170	0.034
9 cm	20	5	300	110/190	0.040
6-well	2-2.5	1.5	50	70/230	0.054
12-well	1	0.5	20	70/230	0.054

[a]Flooding with M9 buffer is required only when thrashing is assessed. For crawling behavior, dry NGM plates are used. [b]These are optimal numbers in our experience; higher or lower number of worms are absolutely feasible, but take note of the tracking errors as evidenced by **Figure 6.** [c]The camera position and pixel-to-millimeter conversion ratio are linked; changing the camera position will affect the pixel-to-millimeter conversion.

Table 4: Frame rates and settings for different behavioral readouts

Assay	Suggested format	Frame rate (f.p.s)	No. of worms	Total frames	Total recording time
Thrashing	9-cm plate[a]	20	<500	600-1.200	30-60 s[b]
Crawling (maximal velocity)	9-cm plate[a]	20	<300	600-1.200	30-60 s[b]
Crawling (long-term)[c]	9-cm plate[a]	3	<200	1.800	10 min

[a]Nine-centimeter plates are the preferred format because larger areas lower the chance of collisions. [b]We prefer a recording time of 30 s because this gives a good estimation of healthspan and at the same time lowers storage requirements. [c]Long-term crawling is mainly used to generate crawling maps as visual representations of crawling capacity; long recordings such as these will increase the chance of tracking errors (**Figure 6**).

Collection and preparation of worm samples

Both collection and preparation of worm samples should take place on the same day as the recordings. In fact, recordings should be performed almost directly after collection of the samples. When collecting the nematodes for a WF-NTP experiment, several aspects should be taken into account. First, before removing worms from their plates, it is important to make note of any abnormalities, for example, fungal infections and the amount of food present (starvation). These observations may be critical in the interpretation of the data, because stress (e.g., metabolic stress) influences behavior[59]. When studying thrashing behavior, NGM tracking plates should be flooded first with M9 buffer before collecting the worms; suggested volumes are shown in **Table 3**. Then worms are floated by pipetting a small volume of M9 buffer onto their growth plates. In contrast to several other assays, it is not necessary to wash the worms after collection; a small volume of worm suspension can be directly transferred to the (flooded) tracking plate. In **Table 3**, we give some recommendations on the number of worms that can be used for simultaneous tracking in different

Box 1: Tracking parameters

- *Video*. This input section asks which movie the user would like to analyze.
- *Start frame.* The start frame is automatically set to 0, but if one prefers to skip the first few frames in the analysis, the number can be adjusted.
- *Use frames.* This is the number of frames that are used for analysis; it is automatically updated when a video is uploaded (it recognizes the number of frames of that particular video). If one adjusts the 'Start frame', the number of used frames is added to that number.
- *FPS.* This is the frame rate per second. The number is automatically adjusted when a video is uploaded (it recognizes the frame rate of that video). The number is used to calculate several time-based metrics (velocity, BPM). If the automatically generated number is wrong, adjust accordingly.
- *Px to mm factor.* This number is used for calculations of area, length and speed. On the basis of the camera-to-plate distance, this number needs to be adjusted. Suggested numbers can be found in **Table 3.**
- *Darkfield.* When the worms appear as white instead of black particles in the video (reversed contrast), 'Darkfield' should be selected.
- *Method.* This is the background subtraction method. As described in the 'Technical background of the WF-NTP' section, there are two methods to choose from: 'Z-filtering' or 'Keep dead'. Normally 'Keep dead' is selected in order to also include immobile particles (e.g., paralyzed worms). If there is a valid reason to exclude stationary particles, 'Z-filtering' can be selected.
- *Std pixels.* This parameter affects tracking only when the 'Keep dead' method is selected. It represents the area that is used for Gaussian adaptive thresholding. Normally, the preselected number (i.e., 64 std pixels) is used. When working with very diluted worm populations, it sometimes helps to make the area smaller in order to remove background noise (fewer pixels are used for thresholding). However, we very rarely adjust the preselected number.
- *Threshold (0-255).* The grayness of a pixel that is used as the lower limit for detection purposes. Making the number larger results in a more strict subtraction of background. Values between 7 and 9 are often used (9 is preprogrammed).
- *Opening.* This represents a mathematical morphological function that removes small objects from the foreground. Simply stated, this parameter reduces additional noise. The higher the number, the more strict the noise reduction. Values of 1 or 2 are often used (1 is preprogrammed).
- *Closing.* This represents a mathematical morphological function that removes small holes in the foreground. Simply stated, it connects foreground pixels in close proximity. The higher the number, the more foreground pixels are connected. Values of 3 or 4 are often used (3 is preprogrammed).
- *Skeletonize*. This is used to convert worms into single-pixel skeletons. It is used together with 'Prune size' and 'Full prune' to remove spurious features of objects. This command is optional; without skeletonizing, particles can also be perfectly detected. However, making pruned skeletons increases the accuracy of centroid and eccentricity estimation. The analysis will take a longer time to complete when skeletonizing is included.
- *Prune size versus Full prune.* The amount of spurious features that are removed. We normally use 'Full prune' as an option. But one can also manually select a 'Prune size'. Prune size refers to the number of iterations that are performed to skeletonize the worms.
- *Minimum and maximum size.* By executing object-size filtering, additional background noise is removed; that is, objects too small or too big to be a single worm are excluded. The 'Minimum' and 'Maximum size' determine which particles are removed. Normally values between 25 and 120 are used for 9-cm plates. Smaller worms sometimes require the 'Minimum size' to be lowered.
- *Worm-like (0-1).* Worm eccentricity. All values >0.5 fit with ellipse-shaped particles, which is a requirement for worms. When worms have an eccentricity that is lower than the value of the 'worm-like' factor, their eccentricity is not used for bending frequency estimation. These specific frames are 'ignored' (i.e., dummy variables substitute the actual eccentricity), but speed and coordinates are still estimated. This is essential for the WF-NTP software, because worms with low eccentricity are often overestimated in terms of bending frequency; that is, the software cannot deal with very round particles. This value should be optimized carefully (0.88–0.93), because too-high values will underestimate the number of bends and too-low ones will overestimate bends; see 'Experimental design' section for further details.
- *Cutoff.* Provides the user with an extra filter. Goes together with 'Average or Max' and 'Start and end frame'. The average or maximal number of worms present in a specified frame interval is used to determine the number of worms to be followed. See 'Experimental design' section.

- *Extra filter*. Speed–bend exclusion filter, see 'Improvements on the original WF-NTP' section. If worms have a bending frequency higher than 'Max bends' and a speed lower than 'Speed', particles are excluded from analysis.
- *Max bends and Speed.* See 'Extra filter'. Values for these two parameters should be decided upon specifically for individual lab settings, see 'Experimental design' section.
- *Maximum move distance (px).* The maximal distance a worm can move during two adjacent frames to be considered the same worm. When one uses movies with low frame rates (e.g., 3 f.p.s.) values of 10 are normally used. With a frame rate of 20–30, a value of 5–10 is sufficient. Setting the 'Maximum move distance' to a high value will result in worms being swapped between frames.
- *Minimum length (frames).* The number of frames a worm should be present in to be included for analysis. In this way, only worms with high 'presence' will be used for analysis (this can be useful because BPM is extrapolated (e.g., the metric is inferred from the time the worm was present) when worms were present for only a small amount of time).
- *Memory (frames).* By keeping the coordinates (positions) of worms before the collision in memory, tracking is continued when individual worms are detected again. However, this happens only when the collision event took place for only a user-defined number of frames: 'Memory'.
- *Bend threshold.* When exceeding this threshold, a movement is considered to be a bend. The value is preferentially set to 2.1, and we rarely change this.
- *Minimum bends.* When one prefers to exclude worms on the basis of a minimal amount of bends, this parameter can be adjusted. However, this value is normally set to 0, so stationary particles are also included in analyses. Note that this parameter can actually substitute for 'Z-filtering', because values >0 will result in the exclusion of stationary particles from analysis.
- *Frames to estimate velocity.* The number of frames that are used to estimate the velocity of a worm. Because the speed of a worm changes constantly, setting this value too high will result in an underestimation of maximal velocity, but a very accurate estimation of average speed. Vice versa for too-small values. However, the 'Average speed' per 'Frames to estimate velocity' is averaged eventually over all estimates, and therefore we suggest using low values. Our standard is 50 frames to estimate velocity (2.5 s with a frame rate of 20 f.p.s.). This value should always be at least one frame fewer than 'Minimum length (frames)', because successive frames are used to for velocity calculation.
- *Maximum bends per minute.* Parameter for 'paralyzed worms statistics'. If worms exceed this value, they are considered to be 'moving'. Worms are considered 'paralyzed' when they do not surpass the values of both 'Maximum bends per minute' and 'Maximum velocity'. Note that these are different parameters than 'max speed and bends', which are used for the speed–bend exclusion filter.
- *Maximum velocity (mm/s).* Parameter for 'Paralyzed worms statistics'. If worms exceed this value, they are considered to be 'moving'. Worms are considered 'paralyzed' when they do not surpass the values of both 'Maximum bends per minute' and 'Maximum velocity'. Note that these are different parameters from 'max speed and bends', which are used for the speed–bend exclusion filter.
- *Regions of interest (show, redraw, delete).* By selecting 'Add new', an ROI can be selected by marking a region with the mouse. When the window is closed, a name can be assigned to this specific region. With 'show', the ROI can be visualized; with 'redraw', it can be changed; and with 'delete', it can be removed. By selecting 'Add new' multiple times, multiple ROIs can be selected simultaneously.
- *Output.* This is the output section of the tracking analysis. It asks the user to select a location and name for the output directory to be generated.
- *Output frames*. This value determines how many tracking example frames will be produced (after subtraction, filtering and other commands). So, if one analyzes 600 frames and uses an 'output frame' value of 600, 600 images will be produced. In each frame, the number of bends and the ID of each worm is also annotated. In this way, one can make movies of the example frames with, for example, ImageJ and look at the bend estimations over time. The value is automatically set to 0, because we normally do not use this option. In the optimization pipeline, we tend to select 100–200 output frames in order to decide upon the right parameters.
- *Font size.* This is the font size of the bend numbers as annotated in the frames generated by 'Output frames'. It is standardly set to 8.

sized plates or wells (**Figure 6 and Supplementary Tables 1 and 2**). For further information about the optimization of the number of worms per video, see the 'Experimental design' section.

Video acquisition

After the worms have been collected and prepared, one can immediately start the recordings. This is important, because the time between preparation and recordings should be as short as possible and should be similar for all conditions. Normally, worms are allowed to acclimatize for 30 s in M9 buffer before recordings are started (for thrashing). Acclimatization takes place at the plate holder of the WF-NTP. In this way, motion of the liquid—due to movement of the plate—is eliminated by the time the recording starts. Videos need to fulfill specific requirements when the WF-NTP is used for analysis: videos should be in .avi format (M-JPEG) and should contain >2 frames, and the edges of the plates should be visible in the video frame (no zoom-in). This is mainly important in preventing worms from moving off the screen and being lost, or preventing worms from entering the screen that were not detected at the start of the analysis. In **Table 3**, we give recommendations for the camera–plate distance to use for different plate sizes. The frame rate can be set to any number; we highly recommend using 20 f.p.s. for thrashing assays and 3–20 f.p.s. for crawling (3 f.p.s. for long recordings, e.g., 10 min, and 20 f.p.s. for short ones, e.g., 30 s; **Table 4**). Importantly, the numbers in **Table 4** are mainly based on tracking accuracy, as evidenced by **Figure 6**, and experimental experience in our labs (data not shown). It is important to understand that when worms move quickly, a higher frame rate is required for accurate tracking (because an individual worm can move only a user-defined number of pixels between frames to be recognized as the same worm). However, using a frame rate >30 f.p.s. does not have any additional effect on parameter outcome but will increase the size of the movies (data not shown). We normally prepare the video capture software in such a way that the videos can be immediately started when the worms have been collected (**Figure 8**).

Tracking with the WF-NTP software

In anticipation of tracking analysis with the WF-NTP, the software should be prepared and programmed. Although the exact steps are described in the Procedure, many parameters should be decided on that require clarification. Starting the software begins by launching the 'multiwormtracker_app.py' file, which provides a user-friendly interface for generating an experimental template with all the parameters needed to control the WF-NTP software during the experiment (**Figure 2B**). A tracking procedure is normally started by clicking on 'add job', which causes a screen with multiple commands and settings to appear. In **Box 1**, we provide a brief overview of these parameters.

Although many of the parameters in **Box 1** are preprogrammed, optimization of several values is necessary when one installs the WF-NTP software for the first time in a lab; see 'Experimental design'. When all values have been adjusted accordingly (confirm edits), a tracking analysis can be performed. A movie appears in the GUI with an 'example' button next to it. By running an example image that is the result of all individual thresholding and filtering steps (**Figure 9**), one can determine whether the algorithm is adjusted in an optimal way.

The results of the tracking procedure ('Start') are summarized in a text file and a particles.csv file that can be found in the created directory ('Output'). A variety of metrics, including bend rate (BPM), average and maximal speed, paralysis, area per animal, length and average eccentricity can be found in the text and particles.csv files. In addition to the text and the particles.csv files, images of all the thresholding steps, a track.py file and a settings.json file can be found in the same directory (see our demo data at https://doi.org/10.17863/CAM.46983; these files will help users become familiar with the WF-NTP software and learn about its output). The

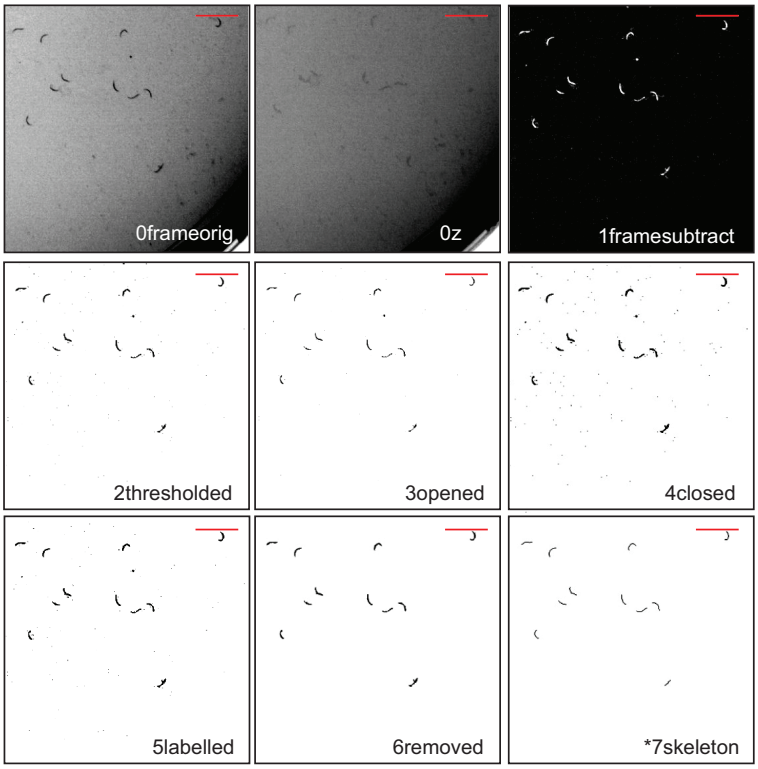


Figure 9: Background subtraction example files. The 9* images that are generated when an example or real analysis is performed with the WF-NTP. The first three images (0framorig, 0z and 1framesubtract) show the original images and the first two filter effects (z-filtering and frame subtraction). These images are normally not used for optimization, because we do not recommend changing parameters related to these images. 2thresholded shows background subtraction; 3opened and 4closed show the results of mathematical morphology; 5labelled shows the images with labeled particles; 6removed shows the particles that remain after size exclusion; and 7skeleton shows the skeletonized worms. *When 'skeleton' is not selected in the setup, the ninth image will not be generated. Scale bars, 0.34 cm.

settings.json file is particularly noteworthy: all used parameters and values are saved in this file, which can be used as a template for subsequent analyses when movies are comparable (e.g., same camera–object distance, same tracking plates and same contrast). By loading the settings.json file ('Load job') and adjusting the 'Video, 'ROI' and 'Output' commands to the new movie, a new analysis can be prepared more quickly. We highly recommend making several 'settings.json' files for different conditions (e.g., plate sizes). Change their names accordingly; the WF-NTP software recognizes files with a .json extension as input files. A batch mode is also available, for example, when loading multiple .json files at the same time.

Post-experimental data analysis

To interpret the data and compare strains and treatments, individual analyses should be combined, because one analysis contains the data of one movie (and often one strain). The data in the text file (population statistics) and the particles.csv file (individual worms) can be easily manipulated in programs such as Excel or R. The text file will contain different parameters, starting with a summary of the filters used and how many particles were excluded on the basis of these settings. This

provides information on the quality of the tracking. When a lot of particles are excluded with the selected filters, specific parameters are not adequately optimized (e.g., the speed-bend exclusion filter is set to strictly) and/or the tracking is of low quality. In this specific scenario, it might be wise to have a look at the 'Experimental design' section. Next, all the population statistics are summarized. The population statistics are helpful for getting a quick feel for the outcome of the analysis. Even statistics on 'moving' versus 'paralyzed' worms are shown. However, although the population statistics may come in handy, one should keep in mind that all averages are 'weighted' averages. So the weight of an individual worm relative to the average depends on the fraction of time that the specific worm was present (**Eq. 1**). The standard error of the mean is then calculated by dividing the standard deviation by the square root of the maximal number of worms that are present at the same time. This can be of help particularly when the cut-off filter is not used. In that case, the raw data will contain more single particles than the maximal number of worms. This is due to particles not being recognized as the same object (because they moved too much or worms enter and leave the ROI) and thus being differently annotated. On the other hand, the raw values (such as velocity and BPM) for each individual worm are not weighted. We tend to use the non-weighted, individual values for analysis (these can be found in the particles.csv file) because the exact sample size can be easily determined. Moreover, if one prepares and optimizes the tracking parameters very strictly and adjusts the 'Minimum length (frames)' parameter to a higher value, most worms will be present for the whole movie.

$$Weighted\ average\ (metric) = \frac{\sum_{worm=1}^{worm=n} Metric(worm) \times presence\ (worm, frames)}{\sum_{worm=1}^{n} presence\ (worm, frames)} \quad (1)$$

in which n = total worms and $frames = \sum_{frame=1}^{frame=end\_frame} worm(frame)$, if the worm is present in frame: 1, else 0

Consequently, extrapolation is barely taking place (because if worms are present during the whole movie, making inferences from the metrics is not needed), and population statistics of the non-weighted (average) values will be similar to the (average) weighted ones. The individual metrics that can be found in the particles.csv file are summarized in **Box 2**. Statistical analysis can be

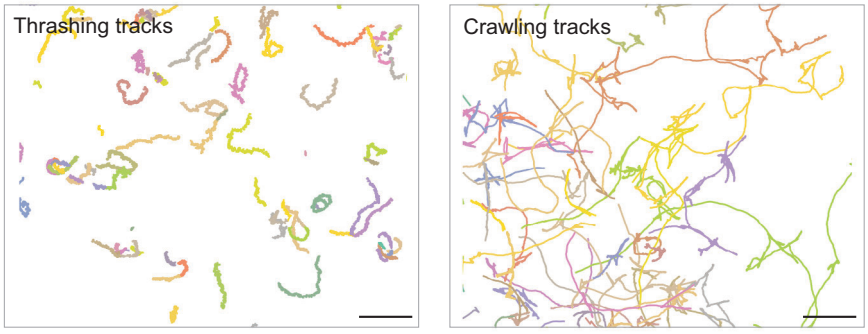


Figure 10: Examples of color-coded tracks made by the WF-NTP. The WF-NTP enables users to plot individual worm coordinates of successive frames in a color-coded fashion. The left image shows the characteristic tracks of thrashing worms: the centroid moves from left to right because of bending, which results in a typical zigzag line. The right image shows tracks of worms that were crawling. Each colored line represents a single worm. Scale bars, 0.4 cm.

Box 2: Individual worm metrics that can be found in the particles.csv file

- *Particle.* The particle ID. This number corresponds to the number annotated in the 'Output frames' images (when selected).
- *BPM.* The number of bends per minute. This is an extrapolated number when a worm does not appear in all frames. However, by making use of a peak to peak' function, this is statistically corrected. This value should be interpreted together with 'round ratio'.
- *Bends/time.* The number of bends per movie. This is an extrapolated number when a worm does not appear in all frames.
- *Speed.* The median velocity of worms in millimeters per second.
- *Max speed (90th).* An estimation of maximal velocity in millimeters per second. Because of tracking inaccuracy, the 90th percentile of speed is used as a representation for maximal speed.
- *Distance per bend.* The distance that a worm moved during 1 bend. This could be used as an indicator for 'force'. It is especially useful when examining 'thrashing'.
- *Area/length.* Estimation of the size of the worms. When using the 'skeleton' filter, this parameter represents length instead of size.
- *Appears in frames.* The presence of a worm in frames.
- *Moving.* 'TRUE' implies that a particle is mobile; 'FALSE' means it is stationary and considered to be 'paralyzed'. These numbers are based on the 'paralyzed worm statistics' and the values appointed to 'maximal velocity' and 'maximal bends'. It is important to realize that we do not know whether worms are actually paralyzed when we use the term as defined in literature. Normally worms are considered paralyzed when they are not dead (pharyngeal pumping is still present), are not moving and do not show a touch response. The first and last criteria cannot be assessed with the WF-NTP, and therefore one should take this observation with a pinch of salt. It may, however, provide a rough estimation of the number of stationary particles, which can be used as an representation for 'paralysis' when, for example, comparing several paralysis-inducing compounds.
- *Region.* The ROI a particle is assigned to. When assessing multiwell plates, regions are helpful for assigning worms to the right condition. Statistics per region are also included in the text file.
- *Round ratio.* The fraction of worm eccentricity that surpasses the 'worm-like' value. When values are 0, all frames are used for bend estimation. A values of 1 implies that worms have a small eccentricity and may act as coilers. Here, all frames are excluded for bend estimation. So BPM is 0. In other words, when worms do not move, it can either be due to worms acting similarly to coilers and being excluded from analysis or worms not surpassing the bend threshold and acting as paralyzed particles.
- *Eccentricity.* The average roundness of a worm. Small values (0.5–0.7) represent worms that have a shape that is close to a circle, whereas larger numbers (>0.9) point toward bending worms or dead ones. Interpret this together with BPM.

performed on all parameters. Comparing different analyses will require specific parameters from different particles.csv or text files to be combined in a statistical program such as R. Interestingly, by using the 'Utilities' option in the GUI of the WF-NTP, one can export the population statistics of multiple text files to .tsv files at once. In addition, the 'utilities' option also provides a way to load track.py files and visualize them with the 'Plot path' function. In this way, an image with color-coded worm tracks can be generated and used to illustrate, for example, differences in average speed between strains or conditions (**Figure 10**).

Experimental design

Overview of parameter optimization

The videos may differ with respect to magnification, contrast and resolution. At the same time specific worm characteristics may influence worm tracking, including the size of the worms, their transparency and their specific types of behavior (e.g., coiling). Therefore, it is essential to optimize some important parameters for both lab-specific settings (only once) and specific types of experiments (depending on the consistency of multiple factors in the lab, this may be required each time). Optimization of three important aspects is discussed further in the following sections: the

optimization of background subtraction and particle recognition, optimization of particle exclusion on the basis of selected features and optimization of worm numbers. By taking into account the parameters and optimization steps, one can adjust the protocol to each individual experiment and lab-specific context. The step-by-step Procedure can easily be used to perform optimization runs when parameters in specific steps are adjusted as described in the following sections. Here, we also provide users with easy but reliable control experiments to verify and optimize specific settings. We highly recommend making settings.json files for different experimental setups (e.g., different assay types and plate sizes) after optimization, as described in the 'Tracking with the WF-NTP software' section, so that one can use these files for future experiments with the WF-NTP.

Optimization of 'primary' background subtraction and particle recognition

Optimizing background subtraction requires interpretation of the thresholding images and comparing them with the original video. As stated previously, whether one performs an 'example' output or a complete tracking analysis, one of the outputs is a sequential series of images that represent all thresholding steps (**Figure 9**). We advise making tracking plates with NGM as specified in **Table 3** and making a simple movie of synchronized control worms (e.g., N2 worms) in liquid (see **Table 3** for worm numbers and amounts of M9 buffer), using the following criteria: 600 frames, 20 f.p.s., M-JPEG compression quality: 95. Start a WF-NTP example run with this movie without changing the preset values. Change only 'Video', 'ROI' and 'Output'. We strongly advise creating an ROI that excludes the edges of the plate, because sometimes residues on the edges are recognized as particles. Add the analysis to the GUI and start an 'example' run. Eight or nine—depending on whether the 'Skeletonize' function is selected—different images will be generated, of which the .jpg files 0framerorig, 2thresholded, 3opened, 4closed, 6removed and 7skeletonized provide clear information about how well the subtraction is performed. In **Figure 11** we show which parameters affect specific images. In addition, we also show how incorrect values assigned to those parameters (too-high or too-low values) affect these images and how one can improve those settings.

Optimization of 'secondary' particle exclusion on the basis of selected features

When background subtraction is optimized, one can proceed to 'secondary' particle exclusion. As stated previously, additional filtering is required to reduce noise and faulty estimations and to approximate manually counted data as much as possible. Again, we advise making three simple movies of the following synchronized worm populations: untreated control worms (the movie from the previous optimization can be used), sodium azide– treated worms (40 mM in M9 buffer) and a strain (e.g., RM908) that shows 'coiling' behavior; all should be placed in M9 buffer and the following criteria should be used: 600 frames, 20 f.p.s., M-JPEG compression quality: 95). Using the previously acquired 'optimal' background subtraction parameters, run a complete analysis without the speedbend exclusion filter ('Extra filter') and skeleton function (optional: the cut-off filter can be used). Plot the number of bends and average speed of the untreated and treated worms in a graph (as in **Figure 4C**) and estimate at which speed and bending frequency inaccurately tracked particles—for example, stationary objects in the movie that appear to thrash and have speed in the analysis—can be excluded (lines in **Figure 4C**; note that sodium azide–treated worms should be completely immobile). Note that speed and thrashing ability decline with age (**Figure 5**), so when analyzing worms of different ages, adjust the speed-bend exclusion to the oldest worms.

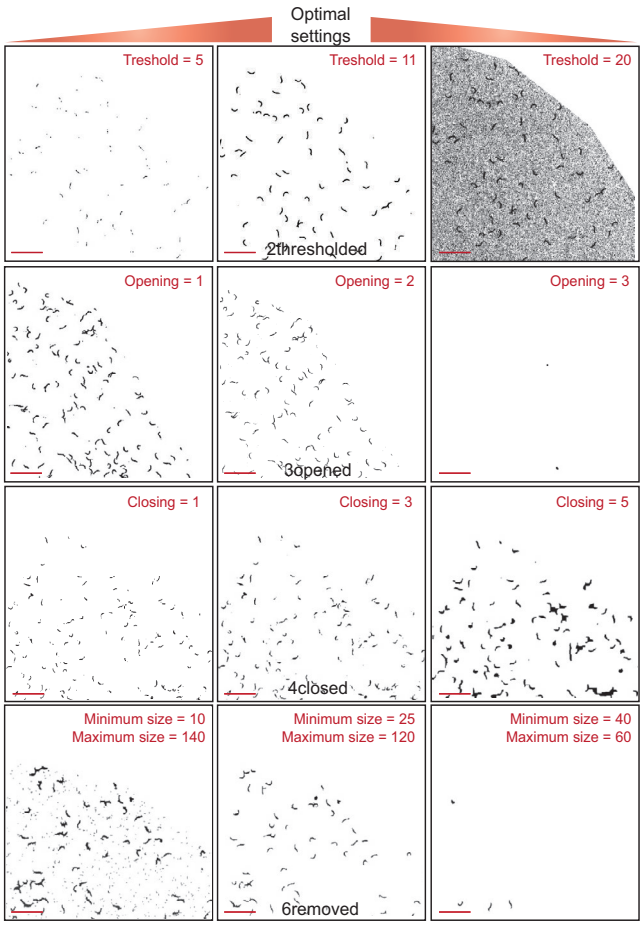


Figure 11: Examples of faulty parameters. The 2thresholded, 3opened, 4closed and 6removed images can be used to optimize specific parameters, as annotated in the example images above. The left column shows specific values for parameters that are not set strictly enough, whereas the right column shows values for the mentioned parameters that are too strict. The middle column shows 'optimal' settings for these specific movies. The images resulting from wrongly assigned values can be used to optimize parameters. Some striking features that should be recognized include too-low values for 'opening' will result in multiple worms being recognized as one individual worm, too-high values will fragment individual worms. Too-low values for 'closing' have severe effects only when 'opening' is set too high, whereas too-high values will result in worm clumps. Note that these worm clumps will be later excluded on the basis of size because wrong values for opening and closing will result in a high degree of particle exclusion on the basis of size. Finally, setting the size-exclusion values too strictly will result in a high loss of worms, whereas less strict values will result in the inclusion of particles that are not worms (e.g., food particles, plate edges). Scale bars, 0.5 cm.

Then plot the eccentricity of control worms, sodium azide–treated worms and coilers. The eccentricity should be the lowest for the coilers and the highest for the sodium azide–treated worms (**Figure 3B**). Use the eccentricity of the control worms and coilers to determine the appropriate value for the 'worm like' parameter. We tend to look at the lowest eccentricity of untreated worms and the highest of the coiler worms and a pick a value in between (e.g., in **Figure 3B**: ~0.88–0.93). Optimizing the 'worm like' value is less important than the optimizing speed-bend exclusion filter ('Extra filter'). In our experience, values between 0.88 and 0.93 give similar results. Use the 'worm like' factor and the estimated cut-offs for the 'speed-bend exclusion filter' ('Extra filter'), select the 'Skeletonize' function and run the analysis again. The sodium azide–treated worms should now have a thrashing frequency close to 0 (BPM), whereas the untreated worms should be barely affected by the 'speed-bend exclusion filter'. Note that the estimated values for the speed-bend exclusion filter ('Extra filter') for sodium azide–treated worms can also be used for the 'maximal velocity' parameter for the paralyzed worm statistics.

As an extra verification step, we normally set 'output frames' to 600 in these optimization runs. When loading these images into ImageJ and making a combined stack (images to stack), one can follow the bends of individual worms according to analysis by the WF-NTP. Follow the number

of bends over time for 15–20 worms and compare this with the number that is annotated next to particles: do they match? If they differ greatly for all the counted worms (**Figure 4E**), the settings are not optimal yet. When worms are, for example, appearing and disappearing in successive frames, this may be an indication that the background subtraction is not optimal (e.g., particles are removed on the basis of size or there are contrast differences between frames). Also have a look at the results file and see how many particles are excluded by the cut-off filter: if this number is low, tracking is relatively good. If it is high, one probably needs to change 'Maximum move distance' or background subtraction.

Optimization of worm number

In the 'Advantages and limitations of the WF-NTP' section we discussed the presence of collisions as a potential perturbing factor in accurate tracking. Both the software and hardware are equipped with features to correct and deal with these kinds of events. However, when one simply takes into account the chance of a collision as a function of screening surface and worm number, experiments can be designed in a collision-reducing way. **Equation 2** shows the formula for the average number of collisions in a single frame (p) as a function of worm surface (w), worm number (N) and screening area (A)[35]. In other words, using a large recording surface with fewer worms will decrease the chance of collisions taking place. In **Table 3** we give some recommendations on the optimal worm number per recording format. Using other plates for tracking purposes requires some optimization of worm number by either performing experimental trial and error or making use of **Eq. 2.**

$$p = [w] \times \frac{(N)^2}{A} \qquad (2)$$

Controls and replicates

Experimental control groups are helpful for determining whether the experimental setup works and verifying that changes in worm metrics can actually be assessed. Typically, including an established mutant/RNAi condition that increases or decreases the worm metric of interest (e.g., thrashing or crawling speed) helps to determine whether the experimental setup is sensitive enough. Such controls are normally based on manually counted and collected data and, therefore, also represent a way to relate to manual methods, making results easier to validate and interpret biologically. In **Table 1** we show some (disease) genes associated with reduced thrashing, and the use of compounds such as aldicarb will also lower bending frequency over time. On the other hand, knockdowns or knockouts of e.g., *tdo-2*[60] and *snf-1*[61] will result in increased thrashing.

Including sodium azide–treated worms or coilers, as discussed in the 'Experimental design' section, is not necessary for every experiment. However, it may provide an intrinsic control with which to verify that tracking settings are appropriate each and every time. When many strains or conditions are examined at the same time, it may be worth also including sodium azide–treated worms as an internal control. Such a control may also facilitate the relative comparison between different experiments, as the lower boundary of the WF-NTP analysis is determined. We strongly recommend that experimental setups be repeated at least three times, so one does not have to rely on one experiment with high numbers of 'technical' replicates when drawing conclusions. Because worms are hermaphrodites, all their offspring have the same genetic background if they

are homozygous. Although epigenetic differences may arise, worms that are synchronized together and grown and aged on the same plates are very similar: the difference between 'technical' (e.g., measuring the same sample multiple times) and 'biological' replicates (measuring different biological samples) appears to be rather unclear in this kind of situations. Therefore, we highly recommend repeating experiments separated in time.

Materials

▲ **CRITICAL** For equipment and reagents related to standard *C. elegans* maintenance, we refer readers to ref. 62.

Biological materials

- *C. elegans* N2 (*Caenorhabditis* Genetics Center (CGC): *C. elegans* wild isolate)
  ▲ **CRITICAL** N2 strains are used in the described protocol, but the protocol can be used for any other strain or for strains treated with RNAi from the Ahringer or Vidal library[63,64].
- *C. elegans unc-17(e245)* (CGC, cat. no. RM908)
- *C. elegans zgIs15[Punc-54::alpha-synuclein::YFP(xScaI)N2(xPvuII)]* (CGC, cat. no. OW40)
- *C. elegans rmIs126[Punc-54::Q0:YFP]V* (CGC, cat. no. AM134)
- *C. elegans dvIs15[Punc-54 vector + Pmtl-2::GFP]* (CGC, cat. no. CL2122)
- *C. elegans dvIs100[Punc-54::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP]* (CGC, cat. no. GMC101)

Reagents

- Sodium azide (Sigma-Aldrich, cat. no. S2002)
  !CAUTION The electron transport chain (ETC) inhibitor sodium azide can be acutely toxic even at low doses. Personal protective equipment should be worn at all times while handling these reagents; wear gloves and protective clothing. Sodium azide requires extra caution because it changes rapidly into a toxic gas when mixed with water or acids.
- Agar (Invitrogen, cat. no. 30391049)
- Casein digest (BD, cat. no. 211610)
- $CaCl_2$ (Fisher Scientific, cat. no. C/1500/53)
- Cholesterol (Fisher Scientific, cat. no. C/5360/48)
- K_2HPO_4 (Merck, cat. no. 1.05101.1000)
- KH_2PO_4 (Merck, cat. no. 1.04873.1000)
- Na_2HPO_4 (Acros Organics, cat. no. 424380010)
- NaCl (Merck, cat. no. 1.06404.1000)
- $MgSO_4$ (Fisher Chemicals, cat. no. M1000/60)
- dH_2O
- Ethanol

Equipment

- Computer with at least 16 GB RAM; MacOS and Windows 10 have been extensively tested (Windows 7 too, but additional troubleshooting might be required)
- Aluminum breadboard (200 × 200; Thorlabs, cat. no. MB2020/M)

- Post holders with spring-loaded hex-locking thumbscrew (12.7 mm, L = 100 mm (five pack; Thorlabs, cat. no. PH100/M-P5)
- Post holders with spring-loaded hex-locking thumbscrew (12.7 mm, L = 30 mm (five pack; Thorlabs, cat. no. PH30/M-P5)
- Optical post for camera (L = 250 mm; Thorlabs, model no. TR250/M
- Optical post for lamp and sample holder (L = 30 mm; five pack; Thorlabs, cat. no. TR30/M-P5)
- Dovetail optical rail (2; Thorlabs, cat. no. RLA600/M)
- Dovetail rail carrier (3; Thorlabs, cat. no. RC1)
- Dovetail rail spacers (2; five pack; Thorlabs, cat. no. SC1/M)
- Mounting base (Thorlabs, cat. no. BA2)
- Sorbothane feet (4; Thorlabs, cat. no. AV5/M)
- M6 cap screw and hardware kit (Thorlabs, cat. no. HW-kit2/M)
- M4 cap screw and hardware kit (Thorlabs, cat. no. HW-kit1/M)
- Metric balldriver and hex key set (15 pieces; Thorlabs, cat. no. TC3/M)
- Sliding-arm sample holder (Comar Optics, cat. no. 132 BR13)
- Monochrome camera (1-inch; Grasshopper USB 3.0; Edmund Optics, model no. GS3-U3-41C6M-C)
- Universal power supply and GPIO leads for Flea3 cameras (Edmund Optics, cat. no. 86-784)
- USB 3.0. cable (Edmund Optics, cat. no. 86-770)
- Backlight (Edmund Optics, cat. no. 88-508)
- High resolution lens (16-mm focal length; Edmund Optics, cat. no. 86-571)
- Power supply with tinned leads (24 V; Edmund Optics, cat. no. 66-855)
- Glass slide (10 × 10 cm; from a picture frame)
- Tubes (15 ml; Sarstedt, cat. no. 62554502)
- Tubes (1.5 ml; Greiner Bio-One: 616201)
- Serological pipettes (10 and 25 ml; Sarstedt, cat. nos. 86.1254.001, 86.1685.001)
- Pipette boy
- Pipettes (20 and 200 µl)

Software

- WF-NTP software and plugins can be downloaded from https://github.com/impact27/WF_NTP (to ensure the latest updates of the WF-NTP software) or at https://doi.org/10.5281/zenodo.3630199 (to ensure the version described in this paper: V3.3.3. The software runs under the license of Attribution- NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0: https://creativecommons.org/licenses/by-nc-sa/4.0/)
- Demo data, with which to become familiar with the WF-NTP software, can be downloaded from https://doi.org/10.17863/CAM.46983
- Python v.3.8 can be downloaded from https://www.python.org/downloads/
- Camera recording software, FlyCap2 viewer, can be downloaded at https://www.ptgrey.com/support/downloads

Reagent setup

M9 buffer (1x). Combine 3 g KH_2PO_4, 6 g Na_2HPO_4 and 5 g NaCl and add dH_2O to bring the volume to 1 liter. Sterilize by autoclaving and add 1 ml of 1 M $MgSO_4$ after cooling. Store at room temperature (~20–25 °C) for up to several months.

Sodium azide stock (400 mM). Add 26 mg sodium azide per 1 ml dH_2O. Store at room temperature; make up to 24 h before use. 0.4 ml of 400 mM of sodium azide solution is required per 9-cm tracking plate.

(Optional) cholesterol in ethanol. To make 5 mg/ml cholesterol in ethanol, add cholesterol to ethanol according to the following ratio: 5 mg of cholesterol:1 ml of ethanol. The solution can be stored at room temperature for several months.

Tracker plate medium. To make 1 liter of tracker plate medium, mix 17.5 g of agar, 7.5 g of casein digest and 3 g of NaCl together in a 1-liter bottle. Add ~1 liter of dH_2O (fill up to the 1-liter mark) and autoclave (~121 °C for 20 min).

(Optional) When cooled (to ~60 °C), add 1 ml of 1 M $MgSO_4$, 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in ethanol and 25 ml of 1 M phosphate buffer (0.868 M KH_2PO_4, 0.132 M K_2HPO_4, pH 6). Note that it is not necessary to add buffers to NGM plates when they are used only for tracking purposes. However, when you keep worms for a longer period at those plates, you may choose to add those buffers anyway.

Procedure

▲**CRITICAL** For protocols related to standard *C. elegans* maintenance, we refer readers to ref. 62.
▲**CRITICAL** *C. elegans* age synchronization should take place in advance depending on the age of interest. Before starting the experimental procedures on day 2 of the protocol, make sure that worms are of the appropriate age (as described in ref. 53).

Installing Python and camera software ● TIMING 30–60 min (only once)

1| Download the software package from https://github.com/impact27/WF_NTP; this includes the WF-NTP software and additional libraries. Unpack the software. Download Python from: https://www.python.org/downloads/ (v.3.8).
2| Execute the python.exe file and install the program.
▲**CRITICAL STEP** Make sure 'add python to Path' is selected to ensure that the software works properly. (In Windows this option should be specifically selected.)
? TROUBLESHOOTING
3| Launch a terminal (e.g., cmd for Windows, terminal for MacOS). In the terminal, select the location in which the WF-NTP software will be unpacked (see notes following this step on how to select locations in a terminal). Subsequently select the general library name of the WF-NTP software ('WF_NTP-master', when downloaded from GitHub). When the appropriate location is selected (..\WF_NTP-master), type `pip3 install numpy` and press 'Enter', when finished, type `pip3 install .` and press 'Enter'; all the libraries and the software itself will be installed at once. Wait until 'Running setup.py install for multiwormtracker... done' appears before continuing to the next

step. If one would like to test the script with an included 'pytest' in Step 5, make sure to install pytest by typing `pip3 install pytest` after installing all the other libraries.
Step 3 summary:

- Select the appropriate library: `C:\…\WF_NTP-master >`
- Type `pip3 install numpy` and press 'Enter' (`C:\…\WF_NTP-master >pip3 install numpy`).
- Type `pip3 install .` and press 'Enter' (`C:\…\WF_NTP-master >pip3 install .`)
- (Optional) Type `pip3 install pytest` and press 'Enter' (`C:\…\WF_NTP-master >pip3 install pytest`).

▲CRITICAL STEP When opening a terminal, a location will already be visible (e.g., C:\Users\Tracker). When the WF-NTP software is unpacked at a different disk, for example, D, one can use the command: `C:\Users\Tracker>d:`, followed by pressing 'Enter' to switch directly to that disk. Paths can be removed with the command `>cd\`, for example, `C:\Users \Tracker>cd\`, followed by pressing 'Enter'. This will yield 'C:\Users'. Adding paths is managed by typing `>cd X`, for example, `C:\> cd WF_NTP-master`, followed by pressing 'Enter'. This will yield 'C:\WF_NTP-master'.
▲CRITICAL STEP When the software is downloaded from https://doi.org/10.5281/zenodo.3630199, the library name will be different from WF_NTP-master: impact27-WF_NTPe18580a. We suggest renaming it to WF_NTP-master before continuing.
▲CRITICAL STEP Note the dot (.) in `'pip3 install.'` The dot should be included.
? TROUBLESHOOTING
4| Click on the 'multiwormtracker_app.py' ('WF-NTP-master' > 'run_script' > 'multiwormtracker_app.py') (Windows) or launch the executable file (MacOS, right mouse click; open with Terminal) to verify that everything is working: a window as shown in **Figure 2B** should appear. Launching multiwormtracker_app.py for the first time will take a few seconds.
5| (Optional) A test_multiwormtracker_script.py file ('WF-NTP-master' > 'multiwormtracker' > 'test') is included in the multiwormtracker directory. Running this script, by clicking on it, will reveal whether the WF-NTP scripts create the correct files for a given settings.json file. It will say 'passed' when everything works correctly.

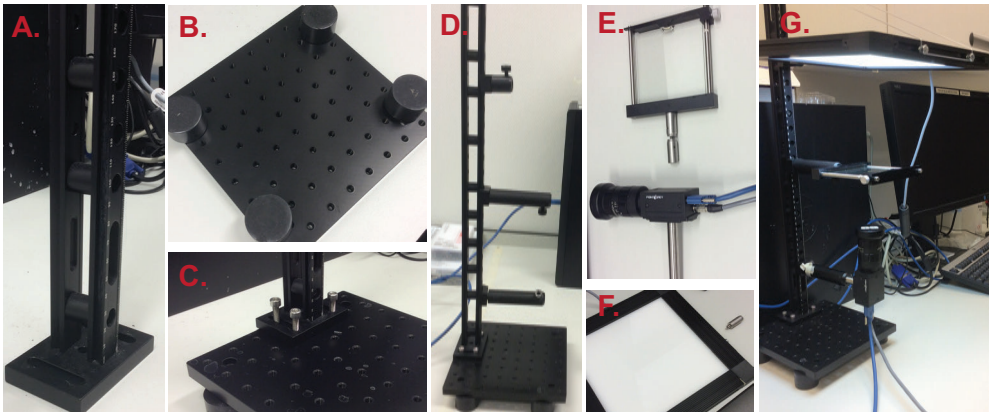


Figure 12: Guidance images for platform assembly. **A-G)** The different steps involved in the assembly of the WF-NTP are illustrated and correspond with the given steps in the Procedure: **A)** Steps 8 and 9, **B)** Step 10, **C)** Step 11, **D)** Step 12, **E)** Steps 13 and 14, **F)** Step 13, **G)** Steps 16 and 17.

? TROUBLESHOOTING

6| Download Point Grey's FlyCap2 software: https://www.ptgrey.com/support/downloads

7| Click below the header 'FlyCapture SDK' at 'Download' (choose the latest FlyCap 2 viewer software).

Installing the platform and camera • TIMING ~60 min (only once)

▲ **CRITICAL** We highly recommend placing the worm tracker in a darkroom or in a box without external light.

8| Connect the two dovetail rails together with the dovetail rail spacers (5–7 spacers are sufficient; **Figure 12A**).

9| Connect the combined dovetail rails to the mounting base (**Figure 12A**).

▲ **CRITICAL STEP** Use the M4 and M6 screws for attachment. Make sure that the dovetail rails are attached properly.

▲ **CRITICAL STEP** Mount the dovetail rails in such a way that the scale starts from 0 at the bottom. This is necessary for attaching the three dovetail rail carriers in Step 12 in the correct manner.

10| Screw the four Sorbothane feet into the aluminum breadboard (all four corners) (**Figure 12B**).

11| Attach the dovetail rails and mounting base to the aluminum breadboard (**Figure 12C**).

12| Attach three dovetail rail carriers to the post holders and attach them to the rails (top = height: 480 mm and post holder L = 30; middle = height: 300 mm and post holder L = 100mm; bottom = height: 110 mm and post holder L = 100) (**Figure 12D**).

13| Attach optical posts to the camera (L = 250 mm), to the sliding-arm filter holder (2× L = 30 mm: attach them to each other) and to the light source (L = 30 mm) (**Figure 12E,F**).

▲ **CRITICAL STEP** By removing one screw at the side of the light source, an optical post can be attached at the same position. Move this optical post to the middle before tightening the connection.

14| Attach the lens to the camera and plug in the USB 3.0 cable and the universal power supply (**Figure 12E**). The power supply itself should be connected again to the 24-V supply.

15| Connect the camera construction to the lowest post holder (110 mm).

16| Attach the sliding-arm filter holder (with glass) to the middle post holder so that the middle of the glass is positioned exactly above the camera lens (**Figure 12G**).

17| Attach the backlight (attach to power supply) to the top post holder (**Figure 12G**).

▲ **CRITICAL STEP** The light should be manually connected to a power plug. We also connect the power plug to a light switch; in this way the light source can be switched off without unplugging the cable.

■ **PAUSE POINT** At this point, the WF-NTP is ready to use. Experiments can be performed whenever possible.

? TROUBLESHOOTING

Preparation of tracking plates (including autoclaving) • TIMING ~3 h (day 1)

18| Pour the tracking plate medium into 9-, 6-, or 3-cm plates or 6- or 12-well plates; see **Table 3** for the volumes.

▲ **CRITICAL STEP** To speed up worm tracking, we suggest using either a pump or a pipette (serological pipette) to dispense the correct volume of medium per dish. In this way each tracking plate will have the same volume and thickness, and settings do not have to be changed between conditions or recordings (e.g., Step 40 will be easier).

19| Let the plates dry overnight before use.

▲ **CRITICAL STEP** Plates should be made at least 1 d before the actual movies are made. However, one can also make a large batch of tracking plates and store them at 4 °C for a longer time (up to ~2 weeks).

■ **PAUSE POINT** At this point, the Procedure can be paused. Tracking plates can be stored at 4 °C for up to ~2 weeks before use.

? TROUBLESHOOTING

Preparing camera hardware and software ● TIMING ~5 min (day 2)

20| Adjust the tracking platform to the appropriate height for optimal screen resolution purposes. Depending on the size of the plates that one is using, adjust the platform to the numbers described in **Table 3**.

21| Turn on the stage light for the tracker and use this light to assess the clarity of the glass stage.

22| (Optional) If dusty, clean the glass stage with 70% ethanol. Ensure that no clear visible residues are on the plate before starting to record movies.

23| Remove the lens cap of the camera

▲ **CRITICAL STEP** The lens should always be capped to prevent dust from getting on the lens. If there are clear dust particles visible, use an air duster to remove them.

24| Start the FlyCap2 software and click on 'configure selected' in the lower right corner.

25| Adjust the camera settings to the appropriate frame rate depending on the tracking goals ('Auto' should be unchecked for 'FrameRate'). See **Table 4** for the suggested numbers in different conditions.

▲ **CRITICAL STEP** Check the current frame rate in the video window (bottom of the screen); this number should be equal to the just-set frame rate.

? TROUBLESHOOTING

26| Close the window and click on 'OK'.

27| Press 'F9', or click on 'File' > 'Capture Video or Image Sequence'.

28| In the new window, fill in the 'save filename' field by selecting the appropriate directory and adding a clear filename.

29| Fill in the number of frames in the 'Saving options' panel; the optimal number of frames depends highly on the behavior being investigated (crawling or swimming). See **Table 4** for suggested settings.

30| In the 'Recording mode' panel, select 'buffered frames'.

31| Finally, select the 'Video' option and not the 'Image' settings. Set 'Video Recording Type' to 'M-JPEG', 'Frame rate' to the one selected in Step 25, 'AVI split size to '0' and 'JPEG compression quality' (0–100) to '95'.

32| The software is now ready for making movies. Before clicking on 'start recording', prepare the worms.

Preparing worms ● TIMING ~2–5 min per strain (day 2)

33| Take a full growth plate with worms that are synchronized and at the age of interest. Add M9 buffer (2–3 ml) to the worm plate and swirl them to float the worms.

▲ **CRITICAL STEP** Strict age synchrony is required for reliable estimations of the behavior. Make sure that worms are synchronized (by hypochlorite treatment, or egg laying and/or FUdR) and that

they are the age of interest on the day of the recordings. (See he 'Pre-experimental procedures' section for further information.)

34| (Optional; only for thrashing studies) Add the appropriate volume of M9 buffer to a tracker plate (see **Table 3**).

35| Use a P200 pipette to transfer a bit of the worm suspension generated in Step 33 to an empty tracker when assessing crawling or to the tracker plate from Step 34 when assessing thrashing. We normally aim to transfer at least 100 worms for recording purposes. **Table 3** gives an overview of the suggested number of worms per tracker plate format.

▲ **CRITICAL STEP** When assessing crawling, make sure to let the worm suspension dry out on the tracker plate before starting the assay. Otherwise, the worms will still 'thrash' and not crawl.

? TROUBLESHOOTING

Recordings ● TIMING ~1–10 min per strain (day 2)

36| Place the tracker plate (from Step 35) on the glass of the stageholder (do not use the glass plate of the stageholder when working with multiwell recording plates).

37| Make sure the worms are in focus by adjusting the lens.

▲ **CRITICAL STEP** The edges of the tracking-plates should be visible for tracking purposes. Do not zoom in too much.

38| Let the worms 'acclimatize' for 30 s.

▲ **CRITICAL STEP** This step is very important! By means of waiting, liquid movement will be reduced and external forces that move the worms will be eliminated, which increases the accuracy of the tracking software in the following steps.

▲ **CRITICAL STEP** If one is treating worms with sodium azide, make sure to add 1/10th (of the M9 buffer volume) 400 mM sodium azide to the M9 buffer and swirl gently before acclimatization.

▲ **CRITICAL STEP** When crawling is assessed, acclimatization is not necessary because there is no liquid involved in this assay. Make sure that the worms actually crawl before starting the recording.

? TROUBLESHOOTING

39| Click on 'start recording'.

40| When multiple strains and/or conditions are tested, make sure to first repeat Step 28 (changing the saving name) before repeating Steps 36–39.

■ **PAUSE POINT** At this point, the experimental part of the Procedure is complete; the WF-NTP analysis can be performed at any time afterward.

Analyzing movies with WF-NTP ● TIMING ~40–80 min per movie (~8 in parallel)

41| Start the WF-NTP software by clicking on the 'multiwormtracker_app.py' (GUI) file, which can be found in the 'run_script library' ('WF-NTP-master' > 'run_script').

▲ **CRITICAL STEP** A black screen labeled 'python.exe' will appear next to the GUI (Multiwormtracker); this screen can be ignored. All messages about potential errors and tracking will appear in the logger canvas of the GUI.

? TROUBLESHOOTING

42| Click 'Add job' to start a new analysis.

▲ **CRITICAL STEP** 'Load job' can be used if one has already analyzed a movie before by selecting a 'settings file'. In this way, all the parameters that were selected in a previous run can be directly imported. We suggest making 'settings.json' files for different conditions (different plates, heights)

so that tracking can be sped up between experiments. See the Tracking with the WF-NTP software' section.

43| The window that appears requires of the user to set several parameters. We have already explained those parameters and pre-programmed standard values in the WF-NTP that provide a perfect starting point (see 'Experimental design'). Adjust those parameters when necessary. The preprogrammed settings are as follows: 'Threshold', 9; 'Opening', 1; 'Closing', 3; 'Minimum size', 25; 'Maximum size', 120; 'Worm-like', 0.93; 'Maximum move distance', 10; 'Minimum length', 50; 'Memory', 5; 'Bend threshold', 2.1; 'Frames to estimate velocity', 49.

▲**CRITICAL STEP** Use the advice in the 'Experimental design' section to improve and adjust the values of all parameters.

▲**CRITICAL STEP** The 'Frames to estimate velocity' value should always be at least 1 frame less than the 'Minimum length' value. If one uses the 'Cut-off' filter, the frames used to estimate the maximum number of worms should be equal or less than the number of frames in the movie.

? TROUBLESHOOTING

44| Decide which filters to use: we highly recommend using the 'Cut-off' ('Max frames', 0–200), 'Extra filter' (e.g., speed-bend exclusion filter, with 'Max bends', 20; 'Max speed', 0.035; for day 1 (D1) worms; speed can be adjusted up to 0.055) and 'Skeletonize' filters together because they give the best representative results (**Figure 4**). When using the 'Skeletonize' function, select 'Full prune'.

▲**CRITICAL STEP** Use the advice in the 'Experimental design' section improve and adjust the values of all parameters.

45| Select a region of interest (ROI). We strongly advise creating an ROI that excludes the edges of the plate, because sometimes residues of the edges are recognized as particles. This function will also speed up the tracking software when worms are allocated to only a specific space. This function is also highly recommended when analyzing multiple-well formats, because separate wells can be selected and analyzed at the same time.

46| (Optional) Select 'Output frames'. This will yield the trimmed images used for tracking with the worms being numbered. We normally use those images only to check for proper tracking during the optimization pipeline as described in the 'Experimental design' section.

▲**CRITICAL STEP** The number of output images selected should be equal to or less than the number of frames in the movie.

47| When all parameters have been selected and filled in, select an 'Output' directory and fill in a 'Save name'. Then, one can click on 'Add job'.

48| Click on 'example'. In this way, output files are generated in the selected directories, showing all thresholding steps. Have a look at these images: are worms visible throughout all stages? If so, the settings appear to be correct. If not, go back to Step 43 and use the advice in the 'Experimental design' section to figure out which parameters are probably faulty and should be changed.

▲**CRITICAL STEP** By running the 'example' function, a settings.json file is also generated in the selected directory. This file can be uploaded to the WF-NTP software by clicking on 'load job'. We strongly advise first optimizing the parameters for one movie (or an experimental collection) and then running the 'example' function. In this way, parameters can be optimized for all movies at the same time.

49| Click on 'load job' and select the just-optimized settings.json file. Adjust the movie and the export name and click on 'OK'. Repeat as often as there are movies in the experiment.

50| If everything is filled in, use the 'Start' button to start the analyses.

▲ **CRITICAL STEP** In the logger screen (see Step 41) the video shape will appear: for example, 'video shape (2048:2048)', followed by the lines 'Sizes' and 'Example frame outputted'. If everything is OK, the progress of the tracking can be followed by the line 'locating in frame X/600'.

■ **PAUSE POINT** At this point, the WF-NTP analysis part of the Procedure is complete. Postexperimental analysis can be performed at any point afterward.

? TROUBLESHOOTING

Post-experimental analysis • TIMING 1–2 h

51| Open the text files of the analyzed movies and have a look at the population statistics. Do they show values that fit the movies? If not, repeat Steps 41–50 and use the advice in the 'Experimental design´ section to improve the tracking accuracy.

▲ **CRITICAL STEP** For optimization purposes, we tend to count 15–20 random worms manually in the videos and compare those averages with the averages from the WF-NTP. They should be comparable. If 'output frames' is set to 100 frames, one can also have a look at the bend estimations in those frames. If the numbers are deviating widely from the manual data, look at the thresholding steps (Step 43) to make sure background subtraction is optimal and the extra filters (Step 44) are not set too strictly.

52| Open the particles.csv file in Excel, read the raw data (particles.csv) into R, or use any other statistical program to calculate averages and perform statistical testing of the individual worm metrics. The researcher can decide which metrics are of interest and how they should be analyzed.

▲ **CRITICAL STEP** It is also possible to export the population statistics of all the analyzed movies in one experiment to a .tsv file by using the 'utilities' > 'export to .TSV' function in the WF-NTP GUI. These statistics can be used to do quick comparisons. Note that all numbers are weighted averages; see the 'Post-experimental data analysis' section for further details.

▲ **CRITICAL STEP** Which parameters should be of interest greatly depends on the type of assay (e.g., thrashing or crawling). Clearly, bending frequency (BPM) is not of interest when assaying crawling.

53| (Optional) Visualize the color-coded worm tracks by loading a track.py file (in the GUI: 'Utilities' > 'Plot path'). The image will be automatically saved as .pdf and .png files.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

Table 5: Troubleshooting table.

Step	Problem	Possible cause	Solution
2, 4, 41	The GUI.py does not launch	'add python.exe to Path' was not selected during the installation of Python	Reinstall Python and make sure to select 'add python.exe to Path' before continuing
		The libraries were not installed	Install the included libraries by opening a terminal, selecting the library of the WF-NTP software and typing `pip3 install .` or `pip install .` Check the software by running the test_multiwormtracker_test.py file in a terminal
3	Some libraries are not installed	Visual C++ 14.0 is required for mahotas (Windows)	Go to www.visualstudio.microsoft.com/downloads and download Visual Studio 2019 for free. Within the Visual Studio installer, select 'desktop development with C++' and click on 'install'. When

			finished, restart the computer and try typing `pip3 install .` again
		No module named 'numpy'	Type pip3 install numpy into the terminal. When finished try `pip3 install .` again
5	No module named 'pytest'	No module named 'pytest'	Type pip3 install pytest into the terminal. When finished try `'pip3 install .'` again
17	The backlight appears to sag a little or appears unsteady	The weight of the lamp, in combination with only one attachment point, can cause sagging	Attach wires to the front of the backlight and attach those to the dovetail rails; in this way the backlight can be lifted
19	Tracker plates are dried out or are contaminated	Plates were stored too long	Make new tracker plates
		Plates were not stored at 4 °C	Make new tracker plates
25	The frame rate of the camera is too low	The camera is not connected to a USB 3.0. port	Connect the camera to a USB 3.0 port before use
		The USB 3.0 ports are not recognized as being so	Reinstall the USB ports; install new or update drivers
35	Worm suspension is cloudy	Worm plates were contaminated	Preferably exclude the infected conditions. Infections can affect behavior
		There is still a lot of *E. coli* (e.g., OP50) on the plates	When pipetting only a small amount of the worm suspension onto the tracker plates, a bit of cloudiness should not affect the recordings. Otherwise, worms can be washed once with M9 buffer before pipetting onto the tracker plates
38	Worms float to one specific side during the recording	The plate holder is not level	Make sure the plate holder is exactly horizontal and level
		Worms were not allowed to acclimatize for 30 s; there is still liquid movement	Make sure to wait at least 30 s before starting the recordings
41, 43, 50	There is an error in the logger	No particles are detected	Check the thresholding images; do worms appear over there? If not, change the settings accordingly Select a smaller ROI in the WF-NTP software or record a plate with fewer worms to start with
		Too many particles are detected	
		Need more frames than 'frames_ to_estimate_velocity' value	Make sure that the value of 'frames to estimate velocity' is at least one frame fewer than 'Minimum length (frames)', because successive frames are used for velocity calculation
46	The variation between technical replicates (within one run) is large	Linking problem is too complex	Reduce the maximum move distance or memory values to reduce the complexity. Otherwise, choose a smaller ROI
		No track file saved	Reduce the number of frames to be analyzed or choose a smaller ROI

• TIMING

Preparation, age synchronization and aging of worms: ~2–20 d.

Steps 1–7, installing Python and camera software (only once): ~30–60 min.

Steps 8–17, installing the platform and camera (only once): ~60 min.

Steps 18 and 19, preparation of tracking plates (including autoclaving): ~3 h (day 1).

Steps 20–32, preparing camera hardware and software: 5 min (day 2).
Steps 33–35, preparing worms: 2–5 min per strain (day 2).
Steps 36–40, recordings: 1–10 min per strain (day 2).
Steps 41–50, analyzing movies with WF-NTP: 40–80 min per movie (approximately eight in parallel).
Steps 51–53, post-experimental analysis: 1–2 h.

Anticipated results

Interpretation and considerations

The goal of using the WF-NTP is to quantify different behavioral aspects of *C. elegans* in an automated manner while maintaining the best aspects of manual counts. In this way, it is also possible to relate to manual methods and results already described in literature and to make it easier to validate and interpret biological results. For optimal interpretations of worm metrics, one should sometimes look at combined output metrics. For example, when combining BPMs with round ratios and eccentricities, one can distinguish between paralyzed (non-moving) worms and worms that coil or stay in a specific position. Thus, a high eccentricity points toward quickly bending worms (BPM) or toward paralyzed worms (low BPM), whereas a lower eccentricity may point toward slowly bending worms (low BPM, low round ratio) or coilers (low BPM, high round ratio). As stated in the 'Controls and replicates' section, using specific controls for metrics of interest reveals whether the setup is sensitive enough to detect differences. For example, the thrashing rate should differ between control worms and neurodegenerative strains (**Table 1**). To verify and test the ability

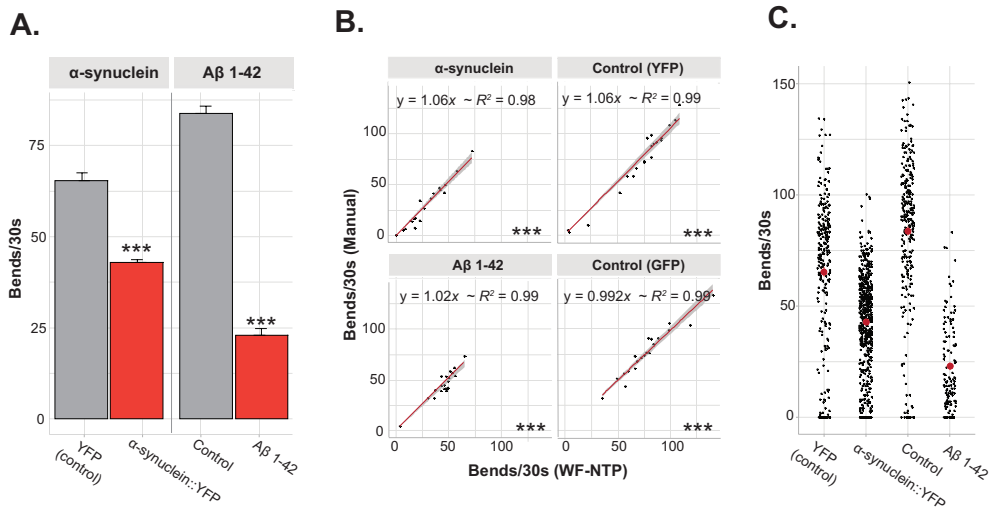


Figure 13: Examples of analyses carried out with the WF-NTP. A) The expression of either α-synuclein in muscles (*Punc-54::α-synuclein::YFP*; OW40) or Aβ in neurons (*Punc-54::Aβ-1–42::unc-54 3'-UTR*; GMC101) results in a decrease in thrashing capacity as compared to those of their controls (*Punc-54::Q0:YFP*; AM134 and *Punc-54 vector + Pmtl-2::GFP*; CL2122, respectively). Mann–Whitney U test $P < 0.001$, n ≈ 100-200 per condition. One representative experiment is shown, repeated three times. **B)** Correlations between manually counted worms and bend rates estimated by the WF-NTP show an R^2 of almost 1. Spearman's r ($P < 0.001$ for all conditions): α-synuclein: 0.84; control (YFP): 0.97, Aβ 1–42: 0.97; control (GFP): 0.94, n ≈ 100–200 per condition. Red lines represent the trend lines of the linear regressions. **C)** The distribution of bend rate is large within a specific worm population, because observer biases are excluded and all the variation of a worm plate is included, n ≈ 100–200 per condition. Red dots represent the means. ***$P < 0.001$. Error bars: s.e.m.

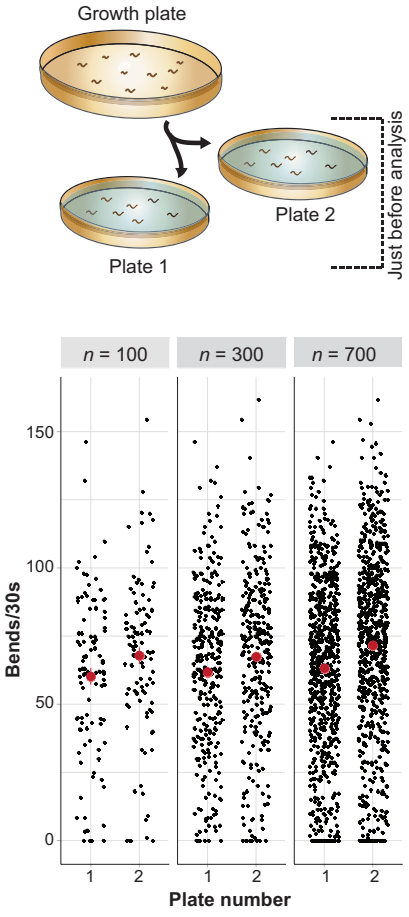


Figure 14: Effect of sample size on statistical significance. Increasing the sample size of worms to be analyzed increases the chance of finding significant differences. N2 worms from one growth plate were divided over two tracking plates and recorded directly after each other. The individual movies were analyzed with the WF-NTP. Mann–Whitney U test: n = 100, P = 0.075; n = 300, P = 0.023; n = 700, P < 0.001. Cohen's d = 0.17 (small). Errorbars: s.e.m.

of the WF-NTP to measure thrashing rate differences, we tested two different neurodegenerative models that express human disease genes in their muscles. Similar to what has been described in the literature (**Table 1**), worms expressing Aβ or α-synuclein in their muscles show a lower thrashing frequency than control worms (**Figure 13A**)[11,16,19]. These results underline the strength of WF-NTP in analyzing worm behavior in an accurate manner. The observation that manually counted particles and WF-NTP-analyzed particles have a correlation (R^2) value of almost 1 (**Figure 13B**) supports this statement.

The variation of, for example, bending frequency is large within a single condition and may differ between assays (**Figure 13C**). By analyzing whole plates instead of 15–20 manually picked worms, observer biases are excluded and whole populations are assessed, including the weak and paralyzed worms (and sometimes a bit of noise too). Larger variation is one of the consequences of automated analysis as compared to manually counted data. Although this variation makes it harder to find differences between treatments or strains, it can be counteracted by increasing the sample size. However, one important consideration should be taken into account when increasing the sample size in relation to significance. Using higher numbers of worms per condition increases the chance of finding significant differences; it increases the power of a study. For those doing power analyses on regular basis, this should not be surprising because studies with small effect sizes require high sample sizes. Indeed, large sample sizes allow researchers to detect subtle differences with small effect sizes. However, small effects are not always clinically relevant, and this should be kept in mind at all times.

To illustrate this statement, we show (**Figure 14**) that increasing the sample sizes of N2 worms may eventually lead to statistical significance (P < 0.001), although the worms that are compared are from the same strain and population batch. They are genotypically similar and their environment has been the same during growth. This statistically significant difference applies to a small effect size. Consequently, the researcher should consider whether this difference is (clinically) relevant. Indeed, continuing to increase samples sizes only to find significance should not be the

underlying reason for using the WF-NTP. Adequate power analyses are highly recommended before performing recordings[65]; in this way the required sample size can be determined correctly.

Interestingly, there are also situations in which differences with small effect sizes may actually be relevant. *C. elegans* has traditionally been considered a poor candidate for drug-related assays because of the worms' relatively inefficient uptake of compounds caused by impermeability of their cuticle to non-water-soluble compounds[66–68]. With this in mind, treatments with small effect sizes could actually be clinically relevant, because the size of the effect may be affected by a poor uptake of the compound. If there are indications of an uptake problem as the underlying reason for a small effect size, *bus* mutants, which have increased cuticle permeability[69], can be used to help explore this possibility. In this case, a larger effect size is expected in the *bus* mutants.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The associated raw data from Figs. 3, 4, 5, 6, 7, 13 and 14 can be accessed via https://doi.org/10.17863/CAM.48480 and demo-data can be accessed via https://doi.org/10.17863/CAM.46983. All other images and movies can be requested via the corresponding authors.

Code availability

The WF-NTP software and plugins can be downloaded from https://github.com/impact27/WF_NTP or https://doi.org/10.5281/zenodo.3630199 (to ensure the version described in this paper: v.3.3.3). We highly recommend downloading the software from GitHub to ensure the latest updates and improvements. The software runs under the license of Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0): https://creativecommons.org/licenses/by-nc-sa/4.0/ The code in this protocol has been peer reviewed.

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Author contributions

M.K. performed most experiments, optimized the final experimental pipeline, adjusted the current WF-NTP software and added new applications and wrote the manuscript together with R.I.S. and E.A.A.N., with contributions from all authors. Q.P. was extensively involved in rewriting, problem solving, editing and optimization of the WF-NTP software. R.I.S. performed experiments on the neurodegenerative strains and helped to verify parameters of the WF-NTP software. M.P., M.V., C.M.D. and T.P.J.K. pioneered the use of the WF-NTP and developed the initial software and

platform. M.K., R.I.S. and E.A.A.N. were extensively involved in discussions and interpretations of results.

Competing interests

The authors declare no competing interests.

Additional information

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Supplements

Supplementary table 1: D1 worms recorded at different worm densities and time-intervals

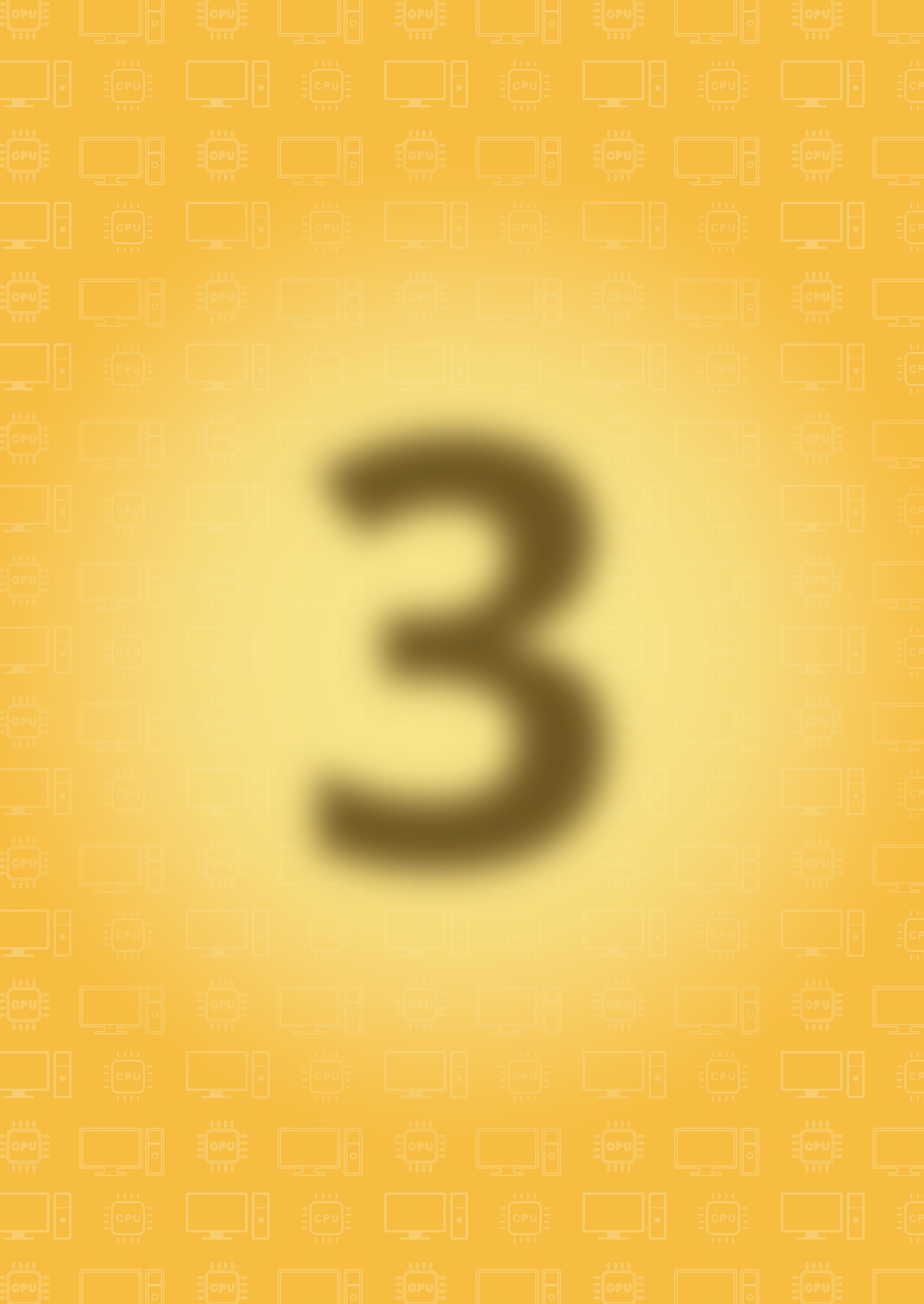
Time recorded (s) / frames	Total worms (manually counted)	Total worms tracker[a]	Tracking errors[b]	Maximal worms at the same time[c]	Tracking errors after correction[d]	Δworm per frame[e] ± SD	Presence (frames)[f] ± SD
10 / 200	44	52	8	51	7	2.74 ± 1.74	178.8 ± 31.90
30 / 600	44	67	23	53	9	4.31 ± 2.074	525.3 ± 123.9
60 / 1200	44	76	32	49	5	1.73 ± 1.31	1029 ± 364.3
10 / 200	168	173	5	155	-13	30.48 ± 9.77	172.1 ± 34.38
30 / 600	168	295	127	173	5	20.26 ± 12.04	467.1 ± 173.9
60 / 1200	168	469	301	175	7	18.22 ± 12.91	964.2 ± 346.4
10 / 200	563	571	8	418	-145	233.7 ± 46.03	128.0 ± 48.25
30 / 600	563	1125	562	665	102	71.79 ± 12.56	416.6 ± 202.1
60 / 1200	563	1705	1142	659	96	74.10 ± 10.97	610 ± 413.6
10 / 200	1002	1312	310	1001	-1	40.68 ± 19.93	169.1 ± 47.97
30 / 600	1002	1729	727	631	-371	475.6 ± 59.11	245.1 ± 179.6
60 / 1200	1002	3373	2371	656	-346	450.2 ± 53.88	297.5 ± 278.8
10 / 200	1671	1319	-352	864	-807	951.0 ± 95.30	116.4 ± 47.63
30 / 600	1671	4951	3280	1657	-14	100.9 ± 78.26	238.4 ± 168.9
60 / 1200	1671	memory error*	memory error*	memory error*	memory error*	memory error*	memory error*
10 / 200	2295	2602	307	1793	-502	664.5 ± 129.9	143.7 ± 52.15
30 / 600	2295	5273	2978	1300	-995	1307 ± 170.1	130.0 ± 98.41
60 / 1200	2295	memory error*	memory error*	memory error*	memory error*	memory error*	memory error*

[a]This is the total number of particles that are detected by the WF-NTP. [b]The tracking error is the difference between the total number of worms that are actually on the plate and the number detected by the WF-NTP. Negative values imply that fewer particles are detected by the WF-NTP than are actually present, this might be due to collisions and overlap, which will result in particles being excluded due to their size. A positive number means the opposite, when worms are getting lost and then tracked again, they will be assessed as a new particle – the number of worms will increase. This might happen after collisions or due to small tracking inaccuracies (e.g. the centroid moving too much between adjacent frames). [c]The maximal worms present at the same time is a value generated by the cut-off filter, which looks for the number of worms per frame. [d]The tracking error after correction is the value generated by subtracting the actual number of worms from the maximal worm number. Clearly, the cut-off filter reduces tracking errors in terms of number of worms. [e]The Δworm number provides the difference between the actual worm number per frame and the number detected by the WF-NTP. In other words, by how many 'worms' does the WF-NTP differ from the real data (absolute values are used)? [f]The presence in frames is the number of frames in which a worm is on average present – value should be compared with the initial frames that are filmed (first column).

Supplementary table 2: D8 worms recorded at different worm densities and time-intervals

Time recorded (s) / frames	Total worms (manually counted)	Total worms tracker[a]	Tracking errors[b]	Maximal worms at the same time[c]	Tracking errors after correction[d]	Δworm per frame[e] ± SD	Presence (frames)[f] ± SD
10 / 200	23	26	3	21	-2	5.66 ± 2.03	170.9 ± 21.05
30 / 600	23	62	39	24	1	5.62 ± 2.65	512.5 ± 90.82
60 / 1200	23	47	24	26	3	1.23 ± 0.96	1084 ± 294.2
10 / 200	64	72	8	68	4	1.725 ± 1.16	187. ± 33.42
30 / 600	64	107	43	61	-3	15.14 ± 3.49	445.7 ± 177.6
60 / 1200	64	194	130	59	-5	4.11 ± 2.96	1003 ± 338.4
10 / 200	171	157	-14	163	-8	14.05 ± 1.68	149.0 ± 47.15
30 / 600	171	207	36	168	-3	9.54 ± 3.38	530.0 ± 149.9
60 / 1200	171	277	106	166	-5	9.57 ± 3.70	980.7 ± 362.1
10 / 200	464	416	-48	398	-66	66.20 ± 16.00	134.4 ± 45.20
30 / 600	464	683	219	422	-42	62.26 ± 9.67	446.1 ± 191.2
60 / 1200	464	955	491	371	-93	93.44 ± 9.85	656.1 ± 420.3
10 / 200	631	604	-27	524	-107	132.6 ± 14.28	176.4 ± 41.80
30 / 600	631	1299	668	542	-89	143.5 ± 30.95	242.7 ± 371.8
60 / 1200	631	1543	912	543	-88	120.2 ± 10.55	637.7 ± 439.2
10 / 200	804	797	-7	670	-134	176.1 ± 24.14	170.7 ± 44.51
30 / 600	804	1436	632	680	-124	168.7 ± 22.48	371.8 ± 194.9
60 / 1200	804	2378	1574	703	-101	158.3 ± 21.19	451.9 ± 372.8

[a]This is the total number of particles that are detected by the WF-NTP. [b]The tracking error is the difference between the total number of worms that are actually on the plate and the number detected by the WF-NTP. Negative values imply that fewer particles are detected by the WF-NTP than are actually present, this might be due to collisions and overlap, which will result in particles being excluded due to their size. A positive number means the opposite, when worms are getting lost and then tracked again, they will be assessed as a new particle – the number of worms will increase. This might happen after collisions or due to small tracking inaccuracies (e.g. the centroid moving too much between adjacent frames). [c]The maximal worms present at the same time is a value generated by the cut-off filter, which looks for the number of worms per frame. [d]The tracking error after correction is the value generated by subtracting the actual number of worms from the maximal worm number. Clearly, the cut-off filter reduces tracking errors in terms of number of worms. [e]The Δworm number provides the difference between the actual worm number per frame and the number detected by the WF-NTP. In other words, by how many 'worms' does the WF-NTP differ from the real data (absolute values are used)? [f]The presence in frames is the number of frames in which a worm is on average present – value should be compared with the initial frames that are filmed (first column).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