



SOFTWARE TOOL ARTICLE

**REVISED** Automated Wormscan [version 3; referees: 2 approved, 1 approved with reservations]

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**Abstract**

There has been a recent surge of interest in computer-aided rapid data acquisition to increase the potential throughput and reduce the labour costs of large scale *Caenorhabditis elegans* studies. We present Automated WormScan, a low-cost, high-throughput automated system using commercial photo scanners, which is extremely easy to implement and use, capable of scoring tens of thousands of organisms per hour with minimal operator input, and is scalable. The method does not rely on software training for image recognition, but uses the generation of difference images from sequential scans to identify moving objects. This approach results in robust identification of worms with little computational demand. We demonstrate the utility of the system by conducting toxicity, growth and fecundity assays, which demonstrate the consistency of our automated system, the quality of the data relative to manual scoring methods and congruity with previously published results.

**Keywords**

WormScan, *Caenorhabditis elegans*, toxicology, software, phosphine

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This version corrects oversights in the Grant information and Acknowledgements sections only. The "ARC Discovery project DP140103513" has been added to these sections, as this was missed out in the previous versions.

See referee reports

## Introduction

Several techniques exist for digitisation and computational analysis of *Caenorhabditis elegans*, with the highest throughput techniques being those that utilise photo-grade flatbed scanners for image acquisition. The first of these, WormScan<sup>1</sup>, lacks post-acquisition automation of data processing, whereas the second, The Lifespan Machine<sup>2</sup>, is more complicated to implement and is computationally intensive. We present a very simple to implement, low-cost system based on automating elements of WormScan, which can score *C. elegans* on agar plates for mortality, size, and fecundity at a rate of tens of thousands of worms per hour with very little operator input.

*C. elegans* is the premier model organism for ageing and toxicological research. They are among the most intensively studied organisms of the last 50 years, which has resulted in the development of powerful genetic and molecular research tools. These tools include an extensive collection of readily available mutant strains<sup>3</sup>, comprehensive and commercially available RNAi libraries<sup>4</sup>, and a large collection of strains that each contain a GFP tagged gene. These strains provide multiple avenues by which genes of toxicological importance can be efficiently investigated, either individually or in combination. Additionally, the size, simplicity, and fecundity of the organism make it a popular choice for toxicological studies<sup>5-7</sup>. Furthermore, *C. elegans* have a very short natural lifespan, making them ideal for studies into the mechanisms of lifespan determination<sup>8-11</sup>. The ability to assay the worms in large numbers makes it possible to address the immense combinatorial challenge of testing large chemical libraries to identify bioactive compounds against a large numbers of strains.

There has been a recent surge of interest in rapid data acquisition to increase the potential throughput and reduce the labour costs of large scale *C. elegans* studies. In 2012, Mathew *et al.* developed WormScan, a low cost analysis tool that uses high resolution flatbed scanners and computer analysis to collect mortality data from *C. elegans* on petri dishes<sup>1</sup>. This system replaces microscopy with commercially available transmission flatbed scanners, and replaces the mechanical stimulus of a platinum wire probe with the aversive stimulus of the bright light from the scanner, which was demonstrated to induce comparable responses. However, while WormScan employed computer image processing of scanned images to replicate human counting, the system was not automated. The 'Lifespan Machine', a more sophisticated, higher throughput system was then developed, which, like WormScan, relies on machine learning to identify worm-like objects<sup>2</sup>. In its current implementation, this system requires sophisticated IT support.

Here we present an automated software system, Automated WormScan. It is extremely easy to implement and use. It employs

low-cost hardware that can be set up by a person with average computer skills in under an hour, and can be used by any researcher with typical computer expertise. Any number of scanners can be run simultaneously from a single standard desktop PC. Three scanners permit an operator to count over ten thousand individual organisms per hour, with only approximately ten minutes of actual operator labour. By comparison, traditional microscope-based counting methods often count fewer than 500 individuals per hour of uninterrupted labour.

The automated system does not depend on machine learning for image recognition, but rather relies on an alternative worm counting method of WormScan, the generation of a difference image from sequential scans, with moving objects scored as live worms. This approach results in robust identification of worms with little computational demand. We demonstrate the utility of the system by conducting toxicity, growth and fecundity assays that demonstrate the consistency of our automated system, the quality of the data relative to manual scoring methods and congruity with previously published results. For this analysis, we use fumigant phosphine as a toxic stress as our lab has studied the toxicology of this compound quite extensively<sup>6,7,12-16</sup>. We do, however, routinely use the system to study the toxic or protective effects of a wide range of other gases and dissolved compounds as well.

## Methods

### Scanning

As with both WormScan and the recent Lifespan Machine system, an Epson v700 (or v800) photo scanner is used to take transmission scans of *C. elegans* grown on a thin lawn of *Escherichia coli* on agar growth medium in 6cm petri dishes<sup>1,2</sup>. In order to minimise the possibility that worms will be in physical contact at the time of scanning and accidentally counted as a single worm, up to 100 worms are scanned per plate. Scans are initially acquired as .tiff images in 16 bit greyscale at 2400 dpi, but are then saved as condensed jpeg images. This ensures sufficient pixel count to be able to distinguish small differences between sequential images, while minimising file size. Up to 12 populated petri dishes can be scanned at a time in each scanner, and all are scanned simultaneously. This is followed immediately by a second scan, each scan taking approximately ten minutes. While all experiments in this study used three scanners simultaneously, there is no absolute limit to the number of scanners that could be utilised in parallel.

### Image analysis and data concatenation

The software for automated analysis of the images exists as two components; a plugin for the FIJI image processing application<sup>17</sup>, and a macro for Microsoft Excel. The FIJI plugin automates a sequence of image manipulations that generates a reliable count of the number of light-responsive individual organisms on each agar plate. The Excel macro then automates the extraction, labelling and concatenation of these counts into a spreadsheet.

### Worm strains and maintenance

Worm maintenance was carried out according to the standard procedures in Wormbook<sup>18</sup>. Briefly, worms were grown on solid nematode growth medium seeded with *E. coli* OP50, and maintained at 20 degrees. Populations of nematodes were synchronised by dissolving gravid adult worms in 1.5% hypochlorite bleach and 0.75M NaOH to release their eggs. Eggs were immediately rinsed

in M9 buffer and maintained overnight at room temperature in M9 buffer with gentle shaking to allow the eggs to hatch. The resulting L1 larvae were then transferred to NGM agar plates seeded with OP50 to initiate growth<sup>18</sup>.

### Phosphine generation and treatment

Phosphine was generated as described in Cheng *et al.*, 2006<sup>7</sup> by dissolving commercially available ammonium phosphate tablets ('Quickphos'; UPL Ltd.) in 5% sulphuric acid in a glass Valmas Chamber. The resultant phosphine was then quantified using a phosphine gas monitor (Canary 'SmarTox-O' PLZZ) and injected through a septum into a sealed glass desiccator, to achieve the concentration required by the parameters of each experiment.

Worms were grown on NGM agar plates seeded with OP50 at 20°C for 48 hours, by which time they had reached the early L4 stage of development. Prior to exposure to phosphine, the number of live worms on each plate was determined by the Automated Wormscan procedure described in this paper. These plates were then sealed inside the desiccators prior to phosphine injection and were exposed to phosphine for 24 hours. Worms were then removed from the chambers and permitted to recover for 48 hours, at which time the number of surviving worms on each plate was determined by Automated Wormscan.

## Results

### Automated Wormscan implementation and operation

The required software for Automated WormScan can be downloaded as an easy to install complete software package available at doi, [10.5256/f1000research.10767.d152697](https://doi.org/10.5256/f1000research.10767.d152697)<sup>19</sup>. Installation and running of the software are fairly intuitive, but detailed instructions can be found in the comprehensive user guide ([Supplementary File 1](#)). The software is based on the FIJI image analysis software package, version 1.49m. If FIJI is already installed, the plugins and macros can be added to the current installation, as described in the user guide ([Supplementary File 1](#)). A macro designed to work with Microsoft Excel is also provided to assist with data compilation and formatting. The software has been developed to run on a computer running Windows 7 or higher. The minimum system requirements are low, with the program confirmed to run on a desktop PC with an Intel Core 2 processor and 4GB ram.

#### Dataset 1. Automated Wormscan source code

<http://dx.doi.org/10.5256/f1000research.10767.d152697>

A set of twelve 6 cm plates are scanned as described in Methods and in the user guide ([Supplementary File 1](#)). Computational analysis of the images begins with the flipping of the images around the vertical axis to make the plate locations correspond intuitively to their locations on the scanner. This image is then split into 12 individual images corresponding to each individual plate. The second scanned image of the same 12 plates is likewise split into 12 individual images. Each of the 12 individual images from the first scan is paired with the corresponding images from a second scan. These paired images are then precisely aligned using image stabilization (Kang Li. 2008. The image stabilizer plugin for

ImageJ. Available: [http://www.cs.cmu.edu/~kangli/code/Image\\_Stabilizer.html](http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html)) so that each pixel can be compared for a difference in intensity between the two images. The outcome is a greyscale difference image, in which the whiteness intensity of each pixel corresponds to the degree to which that pixel differed in intensity between the first and second scan. This is then converted to true black-and-white with the use of an edge detection function (Boudier T, Meys J. 2015. Edge Detection. Available: [http://imagej-docu.tudor.lu/doku.php?id=plugin:filter:edge\\_detection:start](http://imagej-docu.tudor.lu/doku.php?id=plugin:filter:edge_detection:start)). Particle analysis of this image is then performed, which generates a count of areas on the image that differ between the two scans, as well as circumference measurements for each of these. Size parameters are pre-set to exclude identified areas that are too large or too small to be L4 nematodes (>6mm, <0.4mm in diameter), such as refractive areas of the petri dish or dust particles. It is possible to alter the pre-set parameters to identify and count worms of other developmental stages.

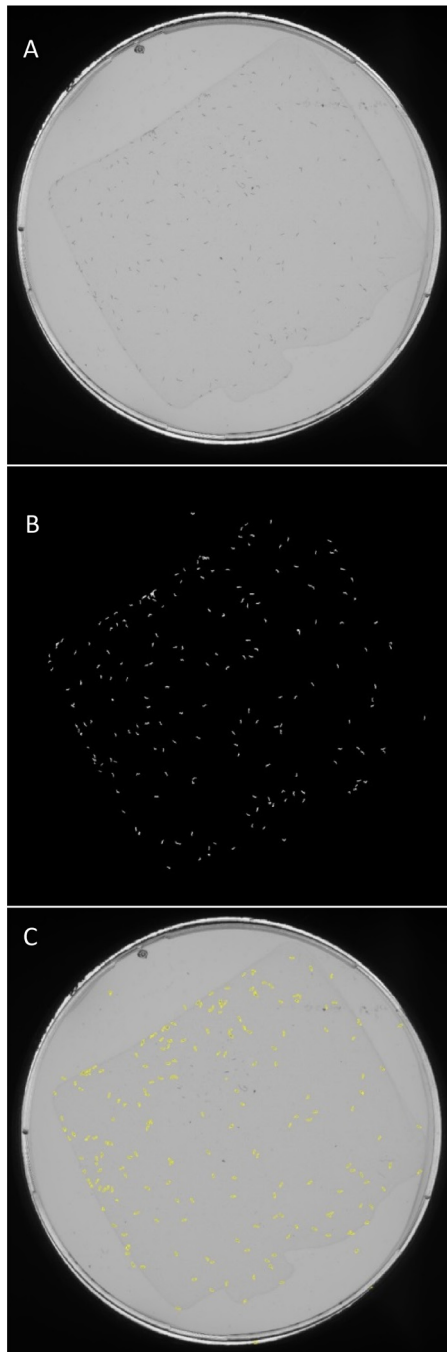
The worm identification, measurement and counting functions are fully automated, so will proceed until an entire folder of scanned images has been processed. A difference image with worm-like features highlighted is created for each plate and is also archived to allow visual confirmation of results and as a permanent record of the experiment ([Figure 1](#)). The processing of a large number of scans (>30) requires several hours. However, a human operator is not required during this entire period, so it is convenient to run the process overnight.

The end product of the computational analysis is a spreadsheet for each individual plate, containing a series of measures of each area of difference (and hence, of each worm). These measurements include circumference, area and circularity, which are potentially useful for comparing worm size or developmental stage. An Excel macro has also been developed, which imports the data from the FIJI plugin into an Excel spreadsheet template ([Supplementary File 2](#)). This results in a table of raw data suitable for statistical analysis and graphing ([Figure 2](#)).

### Assays demonstrating the utility of the system

**Accuracy and consistency of Automated WormScan relative to counting by humans.** The reliance of Automated WormScan on comparing the worm count prior to an experiment to the worm count after an experiment, simplifies the computational burden relative to employing machine learning to identify and assess mortality of worms from a single pair of post-experiment scans. While our procedure makes worm identification extremely robust, errors associated with both the pre- and post-experiment counts contribute to the final error of the analysis. This differs from human counting, as well as from the machine learning procedure originally described in WormScan and utilised by the Lifespan Machine, both of which count live and dead worms from a single set of scans.

To establish the error rate of the automated system, an assay was performed to count the number of live worms on agar plates using both traditional human counting and Automated Wormscan. In total, 12 plates of worms were counted in triplicate using a microscope, each time by a different trained human. The same plates were then scanned sequentially three separate times, each



**Figure 1. Movement based identification of *Caenorhabditis elegans* based on pixel intensity difference between two successive scans of an agar petri dish.** (A) A high-resolution scan of a petri dish containing a population of approximately 100 early L4 stage *C. elegans* individuals. (B) A difference image produced from a pixel-by-pixel comparison with a second image taken of the same plate ten minutes later. White regions correspond to a worm that moved from its original location during this time. (C) Regions of interest from the difference image that have worm-like size and shape attributes are identified. Archival images are created with an outline of identified worm-like objects overlaid on the original images.

time in a different scanner, with counts performed using Automated WormScan (Figure 3). We performed a Generalised Linear Mixed-Effects Model<sup>20</sup> in R (version 3.3.2)<sup>21</sup>. Overall, we found that counts produced by human observers *vs* counts produced by Automated Wormscan did not differ significantly ( $P > 0.2$ ), indicating that Automated Wormscan closely replicates human counting.

**Wormscan is able to replicate previously published toxicological data.** We replicated phosphine exposure protocols used by our laboratory to demonstrate that Automated Wormscan generates results consistent with published results derived from manual counting. Depending on the dose of exposure, toxins can impair mobility or alter the shape of the worms, but this does not materially affect the outcome of the analysis. We exposed wild type N2, and a phosphine resistant strain, *dld-1(wr4)*, to a range of phosphine concentrations and obtained survival curves indicating  $LC_{50}$  values of 400ppm for N2 and 1800ppm for *dld-1*. These results, demonstrating a phosphine survival rate for *dld-1(wr4)* 4.5 times greater than wild type, are consistent with a previously published study that used manual phenotype scoring, which found the survival rate of *dld-1(wr4)* to be 4.4 times greater than wild type (Figure 4)<sup>16</sup>.

**Automated wormscan can be used to measure growth rate and fecundity.** While counting living, mobile and stimulus responsive worms is the primary objective of the system, the perimeter of each identified worm-like object is also determined during analysis. We therefore performed an assay to determine how well this data could reproduce published size difference of two different worm strains, N2 and *daf-2*, as *daf-2* is well known to have a slow growth rate and delayed maturation relative to the wild type N2 strain<sup>22,23</sup>. Plates were scanned 48 hours and 90 hours after seeding with synchronised L1 worms. After 48 hours, no progeny were present on any of the plates, and the average perimeter data was recorded for each worm and length was calculated as  $1/2 \times \text{perimeter}$ . Using Automated WormScan, *daf-2* worms were determined to be significantly shorter than wild type worms after 48 hours growth at 20°C, by an average of ~0.25mm (Figure 5). This reflects the well-established size difference of the *daf-2* strain.

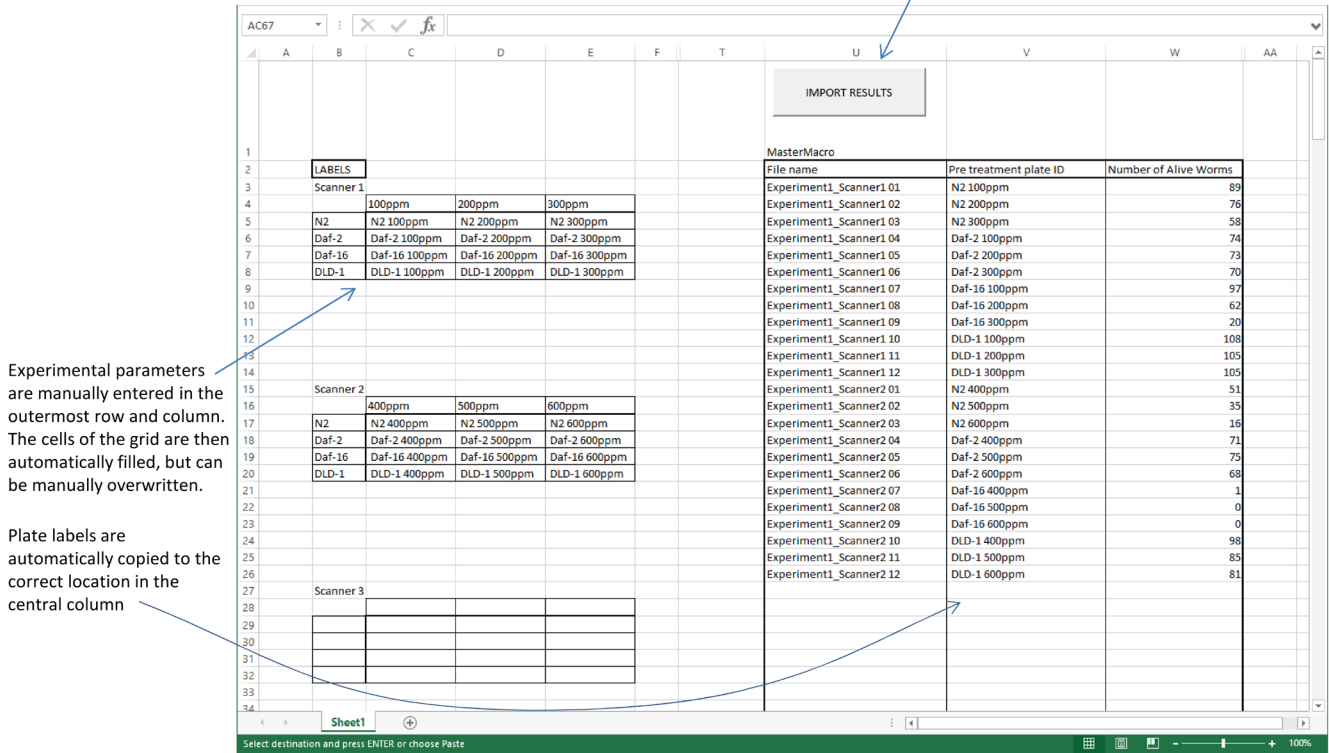
After 90 hours at 20°C, both strains had produced progeny that were easily recognised by their size, with adults being well over 1.5mm in perimeter and juveniles being under 0.5mm. The rate of reproduction of each strain is presented as juveniles present per adult worm (Figure 6). The developmental stage of each individual was confirmed visually on the source image. After 90 hours, wild type worms produce approximately 4 times as many progeny as *daf-2* worms, which is consistent with the well-established decrease in reproduction of the *daf-2* strain.

**Accuracy of automated wormscan using compressed image formats.** We find that it is essential to scan at high resolution to obtain accurately identified worms, but this produces TIFF format image files of individual petri plates of ~60 MB. For the small experiment shown in Figure 4, the 14 data points were generated from 3 experimental replicates, each of which contained 2



## Data spreadsheet

An import macro imports the file name and plate number into the left hand column, and the number of live worms detected on that plate in the right hand column



Experimental parameters are manually entered in the outermost row and column. The cells of the grid are then automatically filled, but can be manually overwritten.

Plate labels are automatically copied to the correct location in the central column

**Figure 2. Example data-processing spreadsheet.** A count of live worms from every scanned petri plate is extracted from the output of the FIJI plugin and is associated with its scan number and plate position. Researchers then manually enter experimental parameters to automatically fill the 3x4 grids with presumed plate labels by an auto-completion function. If the design of an experiment does not fit this default model, the labels can be inserted manually. The end result is a single table of raw data from the entire collection of images that can easily be tracked back to the archived images.

technical replicates. As each plate is scanned a total of 4 times, a total of 336 images were generated. Given a TIFF image size of 60 MB, the total storage requirements if using high resolution images would be 20 GB. High resolution images also increase the computational processing demands.

Therefore, we carried out an experiment to see if the performance of our system was compromised by use of compressed image formats. A series of plates of N2 worms in the early L4 stage of development were analysed by Automated WormScan in two different ways. In both cases, the worms were initially scanned as 16 bit greyscale images. In the first case, the images were saved in the lossless TIFF format (~60 MB) by the scanning software. In the second case, the images were saved as compressed high quality jpg images (~1.5 MB) by the scanning software. These images were then processed by Automated WormScan to

compare the number of worms counted per plate in each image format. We found that analysing compressed jpg images had no effect on the number of worms that could be identified by the program (Supplementary File 3).

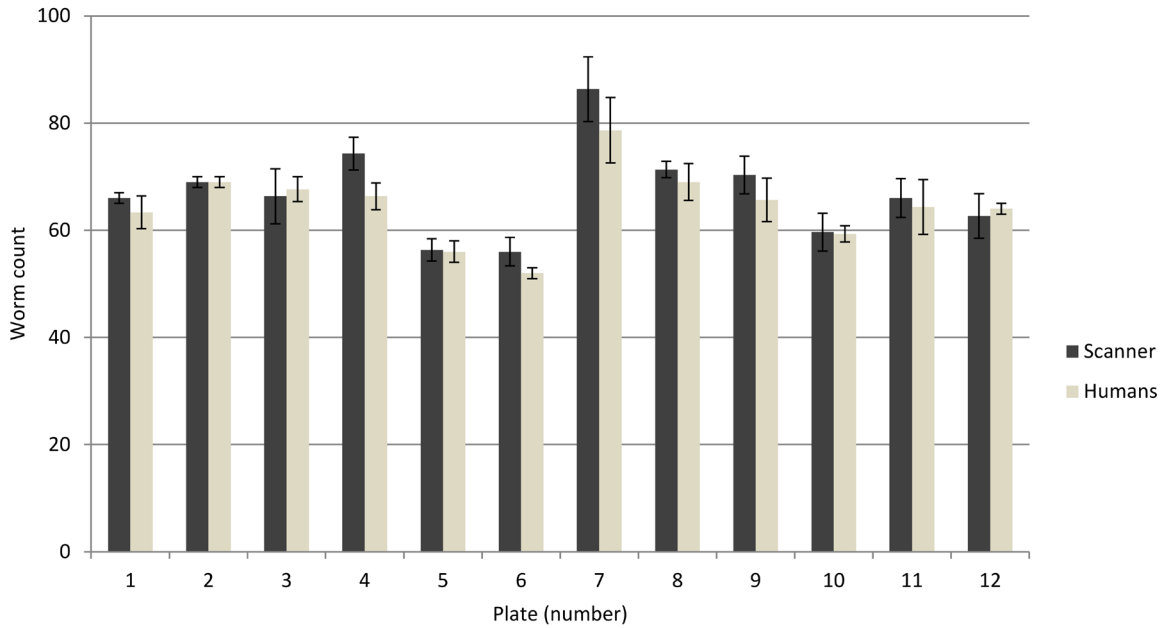
**Dataset 2. Raw worm counts for Figure 3 - comparison of worm counts produced by three different human observers vs three independent scans**

<http://dx.doi.org/10.5256/f1000research.10767.d152698>

**Dataset 3. Raw worm counts for Figure 4 - phosphine mortality curves of wild type and resistant strains of *Caenorhabditis elegans***

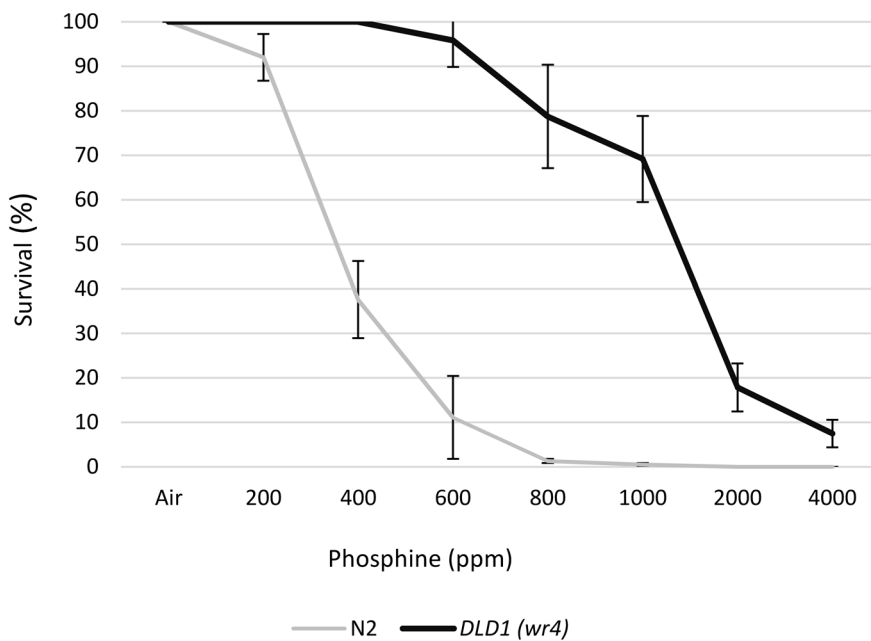
<http://dx.doi.org/10.5256/f1000research.10767.d152699>

### Human vs. Scanner worm counts

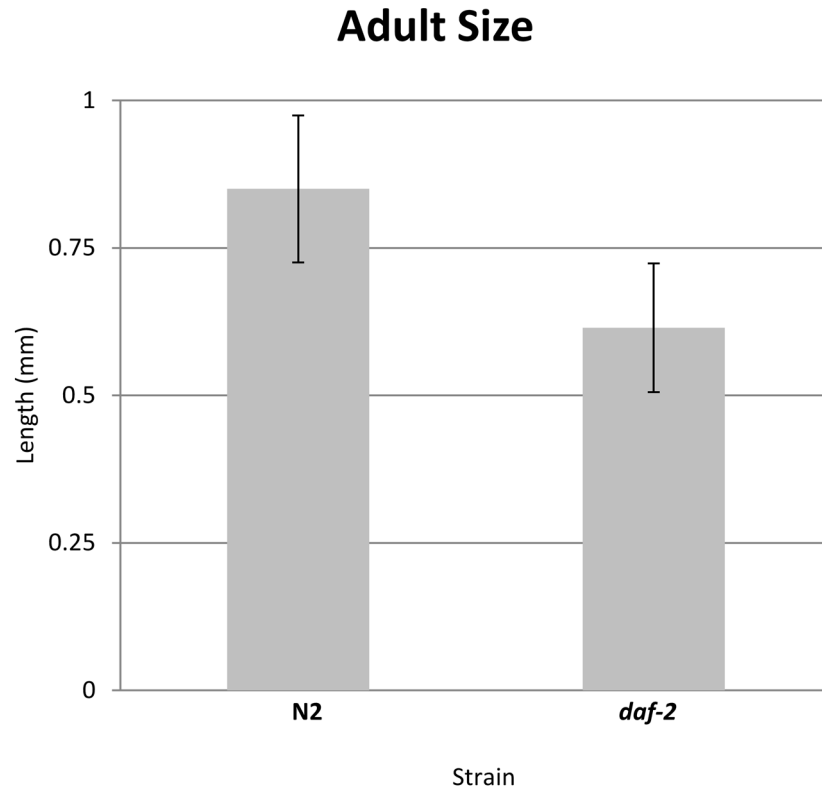


**Figure 3. Comparison of worm counts produced by three different human observers vs three independent scans.** Variability in numbers of individual worms counted by Automated Wormscan on the same plate is not significantly different from the variation in counts between three independent human observers using traditional methods. Error bars indicate standard deviation over three independent counts.

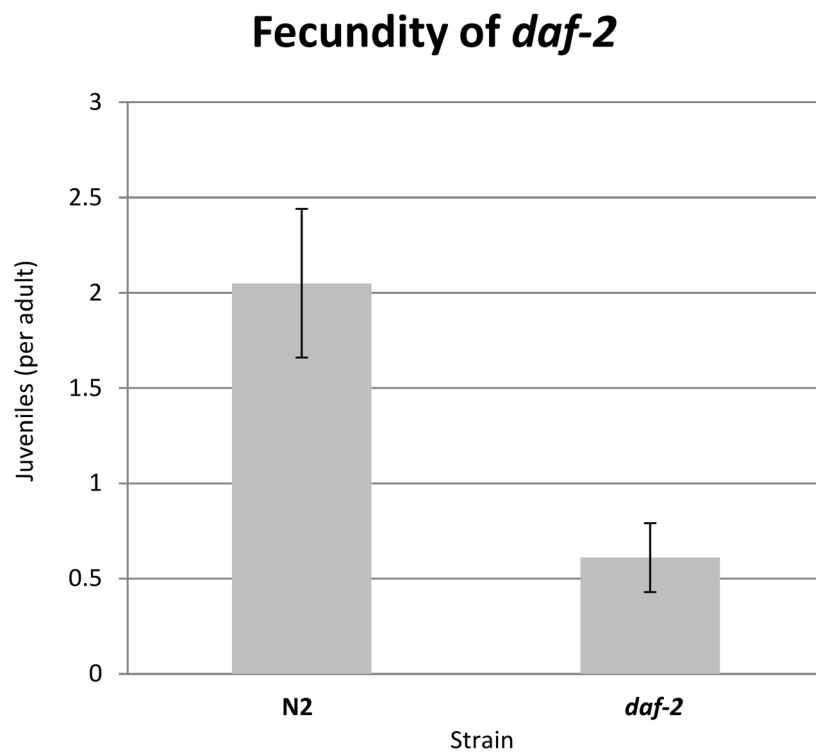
### Survival



**Figure 4. Phosphine mortality curves of wild type and resistant strains of *Caenorhabditis elegans*.** Automated Wormscan accurately duplicates previously published phosphine toxicology results. Wild type worms (N2) display an LC<sub>50</sub> of ~400ppm, while the phosphine resistant mutant *dld-1(wr4)* display an LC<sub>50</sub> of ~1800ppm, congruous with recently published results<sup>9</sup>. Error bars indicate standard deviation over three experimental replicates.



**Figure 5. Growth rate of *daf-2* worms compared with wild type.** Length was calculated as  $\frac{1}{2}$  of the perimeter measurement determined by Automated WormScan. Error bars represent standard deviation over three experimental replicates.



**Figure 6. Reduced fecundity at 90 hours of *daf-2* worms compared to wild type.** Fecundity is expressed as juvenile-sized objects (<0.5mm perimeter) per adult-sized object (>1.5mm perimeter) as counted by WormScan. Error bars represent standard deviation over three experimental replicates (>600 juvenile individuals scored).



**Dataset 4. Raw worm counts for Figure 5 - growth rate of *daf-2* worms compared with wild type**

<http://dx.doi.org/10.5256/f1000research.10767.d152700>

**Dataset 5. Raw worm counts for Figure 6 - reduced fecundity at 90 hours of *daf-2* worms compared to wild type**

<http://dx.doi.org/10.5256/f1000research.10767.d152701>

## Discussion

Automated Wormscan is an extremely low cost system (scanner USD\$500, 3TB internal hard drive \$125, hard drive dock \$25, scanner plate holder \$50, standard PC with Windows operating system) capable of replacing manual counting of worm populations and scoring of survival. We have demonstrated that the system is capable of replicating previously published toxicological results, while also yielding greatly improved speed and throughput. The system is remarkably easy to set up, requiring less than 60 minutes. Once set up, very little operator time is required to carry out the image acquisition and analysis. An archival record is automatically stored as a compressed image file on which identified worms are highlighted. Accuracy of record keeping is enhanced by the ability to collect all image files for an experiment in a single folder and process them all at once, with the output being a single Excel spreadsheet of data with filenames and plate numbers transferred directly from the image files.

*C. elegans* toxicological assays are often limited to only a handful of strains, treatments, time points and/or chemical concentrations, due to the inability of researchers to quickly score very large numbers of individual worms. The use of an automated mortality scoring system removes this significant bottleneck in the throughput of most toxicological assay designs. This greatly improves the speed at which such experiments can be performed and enables complicated experiments to be contemplated, such as the simultaneous determination of the combinatorial effects of many substances at a variety of concentrations. Furthermore, the increased throughput enables the use of larger numbers of technical replicates and extra experimental replicates, which provides greatly improved statistical power that previously would have been impractical due to the labour required.

Automated WormScan is one of several systems recently developed that utilise image capture and computer-based phenotype assessment to improve the throughput or accuracy of such *C. elegans* assays<sup>1,2</sup>. Automated WormScan uses very inexpensive and simple hardware, requiring only a basic desktop PC and at least one Epson v700 (or v800) scanner and a plate holder to align petri plates on the scanner (LabPro Scientific; part LPST12-1). Automated WormScan requires no modifications or alterations to the scanners and the software is also especially easy to install and simple to use. Setup can be accomplished in under an hour by anyone with basic computer skills. In addition, scans are taken while the worms are on an NGM agar plate, which is the typical format for most *C. elegans* assays. This means the

scanning can be incorporated into existing experimental methods without requiring alterations to the experimental procedure.

Since the system focuses on the ability of the animals to move under stimulus, the assay is very robust, with a demonstrated ability to consistently count live worms with the same accuracy as a human. The robustness of the system is achieved largely by avoiding the machine learning steps integrated into the primary component of WormScan, in favour of the simpler difference image analysis method that was originally developed for monitoring survival in lifespan experiments<sup>1</sup>. This design decision increases the amount of handling required compared to the machine learning strategies of the original WormScan and Lifespan Machine systems<sup>1,2</sup> (i.e. two pairs of scans must be taken instead of one). In practice, this requirement is not onerous. Automated Wormscan is not capable of resolving multiple worms in close contact. In practice, this is not usually a problem, as clearly demonstrated in [Figure 1](#). The solution is simply to use  $\leq 100$  worms per plate, in which case the simplicity of the Automated WormScan software does not significantly impinge on the ability of the system to replicate human counting. Automated WormScan will not work with mutants or experimental conditions that induce aggregation of worms, but the same is true of the other counting systems and probably human counting as well.

Automated WormScan is time and labour efficient, requiring ~10 minutes of operator time per 60 plates (5 scanners) and 20 minutes of scanning time (during which an operator need not be present). Once all scans have been completed and saved to a single folder, another 5 minutes of operator time is required to start the analysis software. The software requires about 30 seconds to analyse each pair of images (but this requires no operator involvement). Thus 6,000 individual *C. elegans* (at a density of 100 per plate) can be analysed with 15 minutes of operator time and about 60 minutes of elapsed time. The total capacity of the system is limited only by the number of scanners that are used in parallel to generate the source images, so the system can be scaled to suit the requirements of the user.

User bias in methods that involve manual counting is a known source of data variation<sup>24</sup>, especially between institutions<sup>25</sup>. The use of this automated method eliminates this possibility while simultaneously improving consistency. Furthermore, the use of the light stimulus obviates the need to open the plates and physically touch the animals, which minimises the potential for contamination and removes the possibility of physically damaging the animals.

While the lifespan machine utilises modified scanners to reduce temperature fluctuations, the focus of Automated WormScan for mortality based toxicology assays means that worms do not spend much time in the environment of the scanner and are able to be housed in temperature controlled conditions for the great majority of time, obviating the need to modify the scanners to compensate for temperature. Also, while Lifespan Machine requires modifications to the scanner to adjust the instruments focal plane, the robustness of difference imaging produces accurate counts of worm populations using entirely unmodified scanners.

Since compressed image formats do not hamper the ability of Automated WormScan to accurately quantify *C. elegans* populations, we utilise compressed .jpg files, rather than the much larger TIFF images utilised by other systems<sup>1,2</sup>. This obviates the need for greater than terabyte data storage for most applications, and brings the system specifications required to run previously computer resource intensive image processing phases into the range of a standard modern desktop PC.

### Software and data availability

Dataset 1. Automated Wormscan source code: doi, [10.5256/f1000research.10767.d152697](https://doi.org/10.5256/f1000research.10767.d152697)<sup>19</sup>

License: GNU General Public License

Dataset 2: Raw worm counts for [Figure 3](#) - comparison of worm counts produced by three different human observers vs three independent scans. doi, [10.5256/f1000research.10767.d152698](https://doi.org/10.5256/f1000research.10767.d152698)<sup>26</sup>

Dataset 3: Raw worm counts for [Figure 4](#) - phosphine mortality curves of wild type and resistant strains of *Caenorhabditis elegans*. doi, [10.5256/f1000research.10767.d152699](https://doi.org/10.5256/f1000research.10767.d152699)<sup>27</sup>

Dataset 4: Raw worm counts for [Figure 5](#) - growth rate of *daf-2* worms compared with wild type. doi, [10.5256/f1000research.10767.d152700](https://doi.org/10.5256/f1000research.10767.d152700)<sup>28</sup>

Dataset 5: Raw worm counts for [Figure 6](#) - reduced fecundity at 90 hours of *daf-2* worms compared to wild type. doi, [10.5256/f1000research.10767.d152701](https://doi.org/10.5256/f1000research.10767.d152701)<sup>29</sup>

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### Supplementary material

**Supplementary File 1: Automated Wormscan hardware and software installation and setup guide.** A step-by-step guide to installing and setting up all hardware and software necessary to run Automated Wormscan. Includes troubleshooting information.

[Click here to access the data.](#)

**Supplementary File 2: Automated Wormscan Excel Mastersheet.**

[Click here to access the data.](#)

**Supplementary File 3: TIF scans vs JPG scans.** Data is live worm counts produced by Automated Wormscan from 12 plates scanned first in JPG and then in TIF file format.

[Click here to access the data.](#)

**Supplementary File 4: Template for making a scanner guide.**

[Click here to access the data.](#)

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# Open Peer Review

Current Referee Status:



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## Version 3

Referee Report 18 January 2019

<https://doi.org/10.5256/f1000research.19096.r42550>



**Nathaniel Szewczyk** 

MRCARUK Centre of Excellence for Musculoskeletal Ageing Research, University of Nottingham, Nottingham, UK

According to the summary of revisions only a correction to the funding acknowledgement was added, therefore I do not feel any further review is required as the revisions don't address my previous review.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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## Version 2

Referee Report 15 September 2017

<https://doi.org/10.5256/f1000research.13593.r25727>



**Nathaniel Szewczyk** 

MRCARUK Centre of Excellence for Musculoskeletal Ageing Research, University of Nottingham, Nottingham, UK

According to the summary of revisions only a supplemental material was added, therefore I do not feel any further review is required as the revisions don't address my previous review.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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## Version 1

Referee Report 21 March 2017

<https://doi.org/10.5256/f1000research.11609.r20572>



**Courtney Scerbak** 

Departments of Biology and Chemistry, Earlham College, Richmond, IN, USA

The ability to automatically count live *C. elegans* on agar plates accurately, in a time-efficient manner, and with relatively low cost is of great utility to many *C. elegans* researchers. This manuscript and its supplementary materials provide an open access tool to allow researchers to do just that. I especially appreciate the discussion of the benefits of keeping an archival record (of images and data) in the reproducibility of scientific research. Overall, the authors describe the use and demonstrate the accuracy of this tool well enough to allow other groups to troubleshoot its application to their particular needs.

With some more clarification in the manuscript, I believe this manuscript will have an even greater practical impact on many *C. elegans* research groups. Below are a list of suggestions and questions:

1. In the Introduction, you should mention or at least refer to more techniques that are currently available for automated *C. elegans* handling and data collection. For example, see the WormBook chapter on Microfluidics: [http://www.wormbook.org/chapters/www\\_microfluidics/microfluidics.html](http://www.wormbook.org/chapters/www_microfluidics/microfluidics.html)
2. When referring to Matthews *et al.* (2012) in the Introduction, it would be worthwhile to describe in more detail their findings that the light of the scanner (a) works to detect mortality and (b) habituates the worms to the same degree as using a platinum pick. This would address any concerns about potential detrimental health effects of the scanner light right in the introduction.
3. Is there a reason you use this particular scanner (Epson v700 or v800)?
4. General Methods questions:
  1. Can this program be adapted to analyze different sized plates?
  2. Is there a particular thickness the agar needs to be or will any depth work?
  3. How does your group actually get an estimated 100 animals onto each plate?
5. Worm Strains and Maintenance should mention which exact strains/genotypes were used and acknowledge the CGC if applicable. The “*daf-2*” strain examined later in the manuscript has no genotype mentioned that I can find.
6. You mention in the results section (pdf page 3) the different measurements that are available in the end product after analysis: circumference, area and circularity. None of these measurements are listed in Figure 2 – do these data appear in a different output spreadsheet? Also, in Figures 5 and 6, you mention using perimeter to calculate the length of worms. Is perimeter another measurement that is included in the automated end product after analysis? If so, please add that item to the list in the results (given that you use it in the data presented in the Figures). If not, please provide more details on how perimeter was calculated from the automated end product dataset.
7. Potential typo – page 4, under “Automated wormscan can be used to measure growth rate and fecundity” – second mention of the *daf-2* strain is missing the “-2.” Also, related to #5 above, it

would be best to name the full strain name and genotype again in this section.

8. The protocol used to determine fecundity in Figure 6 should be described as a section in the methods. Doing this would likely address many of my concerns/questions below in #9.
9. I have many questions and concerns related to Figure 6:
  1. I am not surprised to see that *daf-2* mutants have fewer viable progeny than N2, but how are there only 2 juveniles per adult in the wildtype strain? How was this data collected? Does it align with data collected manually by a trained observer with a microscope? If so, it would be useful to add that comparison to this Figure.
  2. Did you start this experiment with ~100 adults per plate and then end up with ~300 on the N2 plates? Did you remove the parents at some point or are they on these plates as well? Please clarify the number of adults and progeny per plate analyzed by the scanner as limiting the number of animals per plate was an important limitation to this tool.
  3. Did you set the size exclusion to include all animals on these plates at once? Or did you run the Automated Wormscan plug-in two times (once for the objects with >1.5mm perimeter and then again for the objects <0.5mm perimeter)?
10. Are there limitations to using this tool in lifespan studies? Can the scanners pick up the slight movement of the head or tail of older individuals that are no longer mobile?
11. Are there any other mutations or scenarios when you would not recommend using this tool? For example, are there specific mutations prevent the animals from responding to the scanner light?

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 06 March 2017

<https://doi.org/10.5256/f1000research.11609.r20573>



**Nathaniel Szewczyk** 

MRCARUK Centre of Excellence for Musculoskeletal Ageing Research, University of Nottingham, Nottingham, UK

There is little doubt that some *C. elegans* researchers will find the software provided by the authors to be a valuable addition in automating analysis of *C. elegans* grown on standard *C. elegans* laboratory medium. The authors provide good validation data of the principal elements of the analysis for counting and assessing non-moving worms following toxic insult in Figures 3 and 4 which should give others confidence in the ability to employ this software.

However, I found the narrative which I viewed as largely a “sales pitch” for the software was both unneeded for this journal and distracting. Similarly, I was quite disappointed at the lack details of the software, what data is captured, how one might modify the software in obvious ways of interest to *C. elegans* researchers, and what values returned from the software might represent with regards to *C. elegans* biology. While my specific comments below may seem extensive, they are largely and broadly summarized in the preceding two sentences (e.g. make the introduction and discussion more balanced



and make the methods/results/case use more descriptive/detailed).

Specific points that should be addressed in a revision (listed more or less in the order that they came up reading the manuscript in the presented order):

1. This manuscript does not appear to comply with the software tool article template. Specifically, the Methods section is lacking both the implementation and operation sections. Additionally, the authors have opted for a Results section but mostly describe non-novel data and therefore, perhaps should have used a Use Cases section.
2. The Introduction should make some mention of other “digitisation and computational analysis of *Caenorhabditis elegans*.” It is certainly not the case that only the WormScan and The Lifespan Machine exist. Early efforts developed a “wormsorter”, I believe in the 1970s, which has now been commercialized as the COPAS<sup>1</sup> and is fairly standard in many *C. elegans* labs. A quick google search returned loads of other efforts at automation a few examples: drug discovery<sup>2</sup>, gene expression<sup>3</sup>, and locomotion<sup>4</sup>. There are also an entire host of micro-fluidics devices that are continually being published and some reference to this is also probably warranted.
3. “*C. elegans* is the premier model organism for ageing and toxicological research.” Needs to be changed, while I might agree with “is a premier model organism for...” it is certainly not the case that the toxicology field agrees with this statement as explained in a recent review on the worms’ role in toxicity testing<sup>5</sup>. NB. This review also explains, briefly, the current state of play for large scale toxicity screening and explains why axenic medium is preferable for toxicology screening.
4. Related to point (3), If you want to make argument about the utility of this analysis for toxicology, better references for the use of *C. elegans* as a model system for toxicology should be provided, especially as your paper doesn’t really go through toxicology endpoints in much detail. The above review is one example but there are other older excellent reviews as well.
5. Related to point (2), if you want to talk about the “recent surge” of interest in automated and high throughput experimentation and analysis on *C. elegans* you really need to cite other examples, particularly microfluidics which, frankly, is where the surge is.
6. Given that your analysis relies on worm movement you really need to be explicit about the limitation of using this device for ageing studies. Specifically, worms are immobile prior to death and this can last several days. Thus, your device will generate falsely truncated survival curves in lifespan assays.
7. Given that I had a new post-doc count roughly 150,000 worms in 75 min last week I don’t find the number of animals that can be processed with this system vs. a manual system at all convincing. If you want to argue about throughput I would stick to the number of conditions that can be analysed as being where the saving is (we only looked at 5 conditions in that 75 min).
8. “Cheng *et al.*, 2006<sup>7</sup>” should be “2003”
9. A better description of what the plugins and macros “do” and “how they do it” should be included, probably in an implementation section in methods.
10. Related to point (9) there needs to be enough detail such that someone who wants to modify the software can easily do so. For example, what if someone wants to image different size petri dishes

or even 24 well tissues culture?

11. Related to point (9) there needs to be more explanation of what the size parameters are likely to represent for example what likely corresponds to an L1 (can you image an L1?), and L2 (can you image an L2?), an L3, and L4, and adult, etc.
12. Related to point (9) and (11), what are circumference, area, and circularity indicative of? What do different values likely mean, what are the strengths and limitation of each measurement with regards to individual worms?
13. Check your worm count in Figure 1, the legend says approximately 100 early L4 stage worms but a quick manual count (prompted by a quick visual estimate of more than 100) suggests closer to 200. If this is closer to 200 and you are making statements about 100 worms being optimal for maximal reliability either provide a better example image or revise your statements to reflect 200 (in which case additional details of how you get the approximate number that you put down should be provided, for example are you counting worms, visually estimating, automatically putting down counted worms (as the COPAS could do)).
14. Please provide details of what parameters were used to generate the worm count in Figure 3. Are these likely to be L1, L2, L3, L4, Adult, some combination? And did visual conformation match the expectation for animal stage?
15. Similar to (14) please provide details of what parameters were used to generate the survival curve and what stage these are likely to represent and if visual conformation matched expectations. (As written your paper suggests you dosed L4 animals and they remained L4 animals but this does not match your lab's past work so I suspect this is a lack of clarity in this paper).
16. Provide details for what strain of *daf-2* was used and temperature of cultivation.
17. Similar to (14) please provide details of what parameters were used to generate this length data and how this compares with life stage and visual confirmation. In both instances the estimated lengths seem too short for the populations to be entirely adults, this should be reflected in the title (perhaps Size after 48 hr growth from L1 is a better title?). NB. You refer to perimeter in the figure legend and results section but perimeter is not a parameter that is otherwise mentioned in the description of the software.
18. Figure 5 does not provide a growth rate as a single data point is provided. I suggest simply removing "rate" from the title may fix this problem.
19. The well-established size difference of *daf-2* strains is for width (for example Depuydt et al.<sup>6</sup>) yet you are claiming your length data reflects this well established difference. Please provide a reference for how your length data match past published literature for *daf-2* length at a comparable growth point.
20. In the results you state "adults being well over 1.5mm in perimeter and juveniles being under 0.5mm." This does not seem consistent with past published literature where L4 and young adult animals are similar in length with juveniles being in a somewhat linear distribution of smaller sizes down to the size of a newly hatched L1 and adults being in a somewhat linear distribution of larger sizes up until mid-late adulthood. Please clarify/correct this statement. N.B. this relates to point

- (14) and the related points all of which request more clarity of what is being measured and how it precisely relates to visual inspection.
21. Figure 6, Fecundity is typically number of eggs produced. I suggest changing the title to progeny produced or viable progeny produced in two(?) days of egg laying. The figures seem low as your wild-type data are suggesting 2 viable progeny for each adult where >200 are expected. To be honest, from the description you provide it sounds like you have measured a decline in juveniles that results from both a developmental delay in *daf-2* and decreased fecundity. Thus, you really need to be clearer about what you are really detecting and if you want to make clear claims about fecundity you should run a more appropriately controlled example.
  22. If you are going to make claims about the Wormscan improving workflow etc (for example “greatly improves the speed at which such experiments can be performed”, you should discuss what other bottlenecks will occur. For example, in your set up how many plates can you put in a desicator at once? There is a reason, afterall, that high throughput systems go, largely, to liquid culturing. Similarly, do plates need to be poured at a specific depth for optimal use in the scanner, does this add time?
  23. I would disagree that most *C. elegans* assays are done on NGM plates, these days molecular endpoint and/or sub-cellular endpoint are much more common than movement (perhaps excluding toxicology and/or ageing). You might revise the sentence.
  24. It is certainly not true that human counting is precluded in conditions that induce aggregation. One simple disaggregates the worms to count them. This statement should be removed from the discussion.
  25. Since it is clear that mobility defects impair use of the system some guidance as to how little mobility is required would be quite helpful in the discussion as would be discussion of if this can be overcome by changing the scan parameters.

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**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Referee Report 06 March 2017

<https://doi.org/10.5256/f1000research.11609.r20569>



**Elena M. Vayndorf**

Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK, USA

The authors present a system, including easy-to-acquire equipment, software and instructions, for counting live, moving and stimulus responsive *C. elegans*. The authors also demonstrate that human counting vs. Wormscan system counting of animal survival produces similar results that are not significantly different. In addition, the Wormscan system is able to count worm size and live progeny number and these numbers are similar to human scoring.

The Wormscan system can be a useful tool for toxicological studies and teaching laboratory experiments. Overall, the authors have outlined methods for setting up the system, addressed its limitations and performed a set of proof of concept experiments that demonstrate the system's novelty and utility. The Wormscan will make a useful teaching tool for high school and college laboratories or any laboratory looking to setup a simple, high-throughput survival *in vivo* assay.

I have the following questions/suggestions for the authors to consider for revision (in no particular order):

1. In addition to the provided documentation, the authors should consider making a short video tutorial that guides the user step by step in data acquisition and analysis. What may seem «easy» or obvious, can sometimes be less so, especially when setting up the system for the first time.
2. Fig 3 - please clarify the age of the animals and add to figure legend.
3. Fig 4 - over what time period were these data collected over? Add to figure legend.
4. Figure 6 - how many adults were used for the progeny assay?
5. What happens at the very end of life/survival when the animals do not move at all but are still alive? How can the Wormscan system distinguish between living vs. dead animals? Please address this tail end of the curve. Or is this point moot because you are always comparing to the moving control group? If so, please clearly state this in your write-up. Also, in this regard, it would be interesting to determine if the Wormscan can pick up whether an animal is dead even when it is not moving on a petri dish by scanning over a longer period of time.
6. In regard to point in #5 above, beyond toxicological studies, do you envision this system for use in aging lifespan studies? Please address in the discussion.
7. Perhaps I missed this - if the scan takes 10 minutes, are the animals exposed to a bright light for the entire duration of the time? Why does it take so long to make a single scan? Is it due to the high resolution necessary for image acquisition? In this regard, I am glad to see that overheating was

not a problem, but if the time of the scan and overall stress level could be decreased, perhaps this system could be expanded to other types of experiments e.g. motility scoring? This could be an avenue to explore in the future, and perhaps this point could be addressed in the discussion?

8. Is it possible to make this work with a Mac? Or does that depend on scanner compatibility? As it is clear that you have done a lot of testing of the scanner component of the Wormscan system, perhaps in the discussion, you could address the minimum scanner and PC requirements that are needed to set this up with other scanners beyond those that work on the Windows system? \$500 may not seem like a lot for a scanner, but it may still be prohibitive for some researchers and teachers, so a thorough understanding of the requirements of the most expensive piece of the system, the scanner, would be most useful.
9. «The required software for Automated WormScan can be downloaded as an easy to install complete software package available at doi, [10.5256/f1000research.10767.d15269719](https://doi.org/10.5256/f1000research.10767.d15269719) I was not able to access this link (it was circular for me and took me back the main page, I tried it from multiple sources).
10. Please clarify in figure legends and/or methods how many times experiments were repeated and how many biological replicates were completed.
11. Consider expanding the first paragraph of or supplying addition citation to your «Introduction» by doing a more thorough review of the technologies currently available for high-throughput image acquisition and phenotyping of *C. elegans*. For instance, see this review by Kinser and Pincus 2016: <http://www.sciencedirect.com/science/article/pii/S104474311630046X>

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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