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Non-microfluidic methods for imaging live *C. elegans*

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Abstract

There are many challenges to live *C. elegans* imaging including the high motility of the animals and sustaining their viability for extended periods of time. Commonly used anesthetics to immobilize the *C. elegans* for imaging purpose prevents feeding of the animals and can cause cellular physiologic changes. Here we present three adapted or novel methodologies to image live *C. elegans* over different imaging microscopy equipment to allow for visualization of animals by DIC and fluorescence without the use of microfluidic technologies. The methods present here use common microscopy consumables and equipment found in many imaging core facilities and can be easily adapted to fit on multiple microscopy systems.

Keywords

Caenorhabditis elegans; microscopy; live-cell imaging; fluorescence; confocal; widefield

1. Introduction

C. elegans is a soil dwelling androdioecious nematode that has proven to be a useful laboratory model organism since it was first introduced in the early 1970's [1]. The mainly hermaphroditic nature of the adult *C. elegans* has made it invaluable for genetic studies and for assigning gene function. Despite being wildly different morphologically, the genome of *C. elegans* is remarkably similar to humans, having greater than 60% genetic conservation (reviewed in [2]). The advantages of utilizing *C. elegans* as a model organism for studying human biology are many fold, including short generation time, high fecundity and a complete mapping of all cell fates. In addition, *C. elegans* is small (~1 mm long and 50 μ m wide and depth) and transparent, making it ideal for microscopic examination. However, its high motility has made imaging of live *C. elegans* challenging. Traditional *C. elegans* imaging has utilized anesthetics such as sodium azide and levamisole to immobilize the animals [3]. However, the use of these compounds prevents the ability of the animals to feed

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and can also disturb physiological function. For example, the use of sodium azide inhibits the mitochondrial electron transport chain and perturbs cellular activity. Recently, researchers have exploited microfluidic technologies for longer term live *C. elegans* imaging (reviewed in [4]). Microfabricated microfluidic devices use a soft silicone-based polymer material, polydimethylsiloxane, which is ideally suited for both imaging and *C. elegans* viability. The polydimethylsiloxane contains micrometer channels in which the animals are immobilized using pressure, cooling or CO₂ (reviewed in [4]). However these technologies often require a dedicated microscope set up that may not be possible when using core facilities and high-end microfluidic technologies may not be available to all researchers. Thus methodologies that employ simple consumables could make longer term imaging possible on a variety of microscopic equipment.

Long-term live *C. elegans* imaging is dependent on both the culture of *C. elegans* and the microscope technology. *C. elegans* has a high motility rate and is traditionally grown on agar plates, with *E. coli* as a food source at temperatures between 15-25°C [5]. This presents several complications in terms of imaging. *E. coli* and agar do not make good optical mediums and microscopes with light sources producing a lot of heat, such as halogen bulbs, can interfere with *C. elegans* survivability over longer time periods. Additionally, the high motility of *C. elegans* means that extremely short acquisition times are required. Here we describe several methods for longer term imaging of live *C. elegans* that can circumvent these issues.

2. Materials and methods

2.1 Growth and culture of *C. elegans*

All animals were grown by standard procedures as previously described [5]. Briefly, transgenic *C. elegans* were grown on standard NGM media seeded with the *E. coli* strain OP50 at 22°C. For all imaging experiments, late L4 stage animals were picked onto fresh plates 16 hours prior to microscopic examination.

2.2 Generation of mKate2 expression plasmid

The mKate2 cDNA was amplified from the plasmid pmKate2-N (Evrogen, cat # FP182) by PCR using the primers 5'-GCATGCGCTAGCATGGCGAGCGAGCTGATTAAGG-3' and 5'-GGATCCGAGCTCTCATCTGTGCCCCAGTTTGC-3' and cloned into vector pKS2236 [6] which contains the NHX-2 promoter [7] using NheI and SacI restriction sites (underlined). The resultant P_{nhx-2}mKate2 plasmid was then sequenced prior to injection

2.3 Generation of transgenic *C. elegans*

Plasmid microinjection was performed as previously described [8]. Briefly 20 ng/μl of P_{nhx-2}mKate2 mixed with 20 ng/μl of the P_{myo-2}GFP co-injection marker and 80 ng/μl of pBluescript SK- plasmid (Agilent technologies) was injected into the distal arm of the gonads of N2 Bristol wild-type *C. elegans*. The resultant transgenic progeny were then integrated by gamma irradiation as described [9] to generate the strain VK2289 (N2;vkIs2289 [P_{nhx-2}mKate2; P_{myo-2}GFP])

2.4 Preparation of concentrated OP50

OP50 was grown as previously described [5]. After overnight growth the culture is then centrifuged at 5000×g at 4°C for 30 minutes. The clarified supernatant is then removed and the OP50 pellet is resuspended in 1/10th the volume of the original culture with sterile M9 media. This culture is then stored at 4°C for up to 3 weeks.

3. Imaging of live *C. elegans*

3.1 Low resolution imaging *C. elegans* in a microtiter plate

The use of a 384- or 1536-well plates means that with low-resolution objectives, an entire well can be taken in a single image. However, multi channel fluorescence imaging may not be possible without extremely fast shutters and filter wheels or newer LED light source technologies. One major advantage of microtiter plates is that multiple worms under different conditions can be imaged, however, too many well positions can impede the speed of image acquisition and reduce the number of timepoints that can be taken. Here we describe a simple method for low-resolution live multi-animal *C. elegans* imaging in a 384-well microtiter plate.

3.1.1 Microscope—The multiwell plate imaging platform ArrayScanV^{TI} (ThermoScientific) which consists of a Zeiss Axiovert 200 inverted microscope with integrated LED light engine, a motorized XY stage and Hamamatsu ORCA-ER CCD camera. The wells containing the worms were imaged using a Carl Zeiss 2.5x Plan NeoFluar objective (N.A. 0.075) with a 0.63x coupler with excitation wavelength of 485 nm and 549 nm every 15 minutes for 24 hours. Acquisition and analysis parameters were controlled using the SpotDetector^{TM} algorithm within the BioApplications modules of the ArrayScan software as described [10]. The autofocus channel was the 485 nm channel.

3.1.2 Protocol for imaging *C. elegans* in a 384 well microtitre plate (adapted from [10])

1. Culture transgenic *C. elegans* as described in section 2 until young adult stage.
2. Pick transgenic animals on to unseeded NGM plates for 10-15 minutes before imaging to remove unwanted bacteria. (Note: Plates are monitored carefully to make sure transgene expression is not affected due to lack of food)
3. Add 50 µl of PBS + 1 µl of concentrated OP50 to the wells of a 384 well plate.
4. Pick approximately 20-25 worms into individual wells of a 384-well optical bottom plate (e.g. Thermo Scientific^{TM} Nunc^{TM} cat #3164730) and allow to settle for 10 minutes before imaging.
5. Seal the plate with with a Breathe Easier sealing membrane (Sigma Aldrich Cat # Z763624)
6. Load the plate into the ArrayScan V^{TI}

3.1.3 Results and discussion—Using the LED light source and single filter sets, the image acquisition speed was fast enough to capture both the fluorescent heads of the

transgenic *C. elegans* as well as the mKate2 fluorescence in the cytoplasm of the intestinal cells (figure 1). Analysis of the mKate2 fluorescent over time was defined as the total fluorescent intensity of the 549 nm channel divided by the threshold of the area computed from 485 nm fluorescence channel. Figure 1A shows the average fluorescence of the mKate2 fluorophore /total number of worms over 9 wells of the 384 well plate over time.

The increase in mKate2 fluorescence is attributed to the hatching and development of the progeny from the adult animals after 16 hours of total imaging time (Fig. 1 E inset). This may be potentially useful for automation of brood size assays. If however, an investigator wishes to study total animal total fluorescence over time without interference from progeny fluorescence, this can be achieved in several ways.

1. Using younger aged animals that will not reach egg laying age by the end of the assay. This however may be problematic in that the total fluorescence intensity will probably increase with development.
2. The use of FUDR to prevent egg development, although this may produce spurious physiological changes not part of this study.
3. The use of SpotDetector algorithm to ignore fluorescence below a certain size and/or intensity to exclude levels from progeny

Since transgenic animals are picked directly into wells, the major advantages of this imaging technique are the ease of use, testing multiple conditions and relatively little manipulation. However, the use of a single low-resolution 2.5x objective with a 0.63x coupler to capture the entire well means that this imaging assay is restricted to fluorescent reporters (e.g. Figure 2) and would be difficult to track protein localization changes. Since the *C. elegans* in this system are highly motile and there is no restriction of movement, animals can also move in X, Y and Z which can cause issues with the autofocus parameters and result in blurry images. To determine if, the use of sodium azide to restrict this movement results in physiological changes and death the experiment was repeated as outlined in 3.1.2 except that 5 mM sodium azide and Sytox Green® (Invitrogen, cat # S7020) was added at a final concentration of 5 μ M to the PBS and OP50 as a measure of worm death [10] (Figure 2). Sytox Green® had no adverse effect on animal viability in the absence of sodium azide (data not shown). The algorithm was adjusted to count the amount of Sytox Green® fluorescence present in the entire *C. elegans* body. Since the pharyngeal GFP fluorescence could no longer be discriminated from the Sytox Green®, we used the initial head count at time 0 as the count of number of animals present in the well. Figure 2A shows the relative mKate2 expression in the intestine sharply decreases after ~2 hours of *C. elegans* exposure to 5 mM sodium azide indicating that the physiology of the animals is being affected. The LT₅₀ of 5 mM sodium azide is ~7 hours. These results suggest that the overall viability is affected by sodium azide and thus is not appropriate for longer-term imaging.

3.2 Imaging *C. elegans* at ultra high-speed using widefield microscopy

The recent advance in camera technologies, in particular the sCMOS (Scientific complementary metal–oxide–semiconductor) series of cameras, have allowed researchers to acquire images faster than ever. Thus animals can be imaged without immobilization. The

following method allows for higher magnification and resolution than the microtitre plate. While there are several methodologies for complete immobilization, certain phenotypes may require movement (or thrashing) to study. However, due to microscope limitations, the *C. elegans* still requires a certain degree of impediment from moving from a certain spot. Thus we have developed 2 protocols to study real time *C. elegans* by fluorescent and non-fluorescent means with movement restriction rather than complete immobilization.

3.2.1 Microscope—An inverted microscope with an encoded motorized XY stage, a Lumencor Spectra Light engine and a Hamamatsu ORCA-Flash 4.0 scientific CMOS camera. Animals were visualized using a 10x PlanApo objective (NA 0.45) and taken in both the 470 nm and 555 nm excitation wavelengths. The images were acquired at ~50 fps, with an exposure time of 10 ms and rendered using NIS Elements Ar software (v4.2). Post acquisition image enhancement was performed using Volocity (v6.2.1; PerkinElmer)

3.2.2 *C. elegans* movement restriction protocol 1 - PBS and coverglass method

1. Culture transgenic *C. elegans* as described in section 2 until young adult stage.
2. Into an uncoated 14mm microwell 1.5 cover glass bottom culture dish (e.g. MatTek P35G-1.5-14-C) put 6 µl of PBS and 0.5 µl of concentrated OP50 (Fig 3A, step i)
3. Into the droplet, pick using platinum wire, 10-20 transgenic animals (Fig 2A, step ii).
4. Place a 12mm 1.5 coverglass circles (e.g. Fisherbrand 12-545-81) on top of the droplet (Fig 3A, step iii).
5. Onto the cover glass place a 22 × 22 mm square coverglass (e.g. VWR 48366-227) to prevent evaporation (Fig 3A, step iv).
6. Optional: Seal the square coverglass with a 1:1 mixture of paraffin:petroleum jelly.
7. Prewet a lint-free tissue paper with deionized water and roll into a cylinder of ~0.25 cm width. (Fig 3A, step v)
8. Wrap the inside of the 35 mm dish keeping the tissue away from the viewing area (Fig 3A, step vi).
9. Optional: Place the lid on the petri dish and mount onto the microscope.

3.2.3 *C. elegans* movement restriction protocol 2 – CyGEL™ movement

restriction—CyGEL™ (Biostatus, Ltd) is thermo-reversible hydrogel originally designed to use for non-adherent cells in high-content screening assays. In our hands, CyGEL™ does not completely immobilize *C. elegans* but does restrict movement and can be used for high-speed *C. elegans* imaging.

1. Culture transgenic *C. elegans* as described in section 2 until young adult stage.
2. Prechill an aluminum heating block in ice for at least 30 minutes prior to imaging

3. On the ice cold aluminum block, place an uncoated 14mm microwell 1.5 cover glass bottom culture dish (e.g. MatTek P35G-1.5-14-C) and chill for 10 minutes
4. Onto the coverglass, put 10 μ l of CyGEL, 0.4 μ l of 40x PBS and 0.5 μ l of concentrated OP50 (Fig 3A, step i)
5. Transfer 10-20 transgenic animals into the droplet (Fig 3A, step ii).
6. Place a 12mm 1.5 coverglass circles (e.g. Fisherbrand 12-545-81) on top of the droplet (Fig 3A, step iii).
7. Onto the cover glass place a 22 $\times$ 22 mm square coverglass (e.g. VWR 48366-227) to prevent evaporation (Fig 3A, step iv).
8. Optional: Seal the square coverglass with a 1:1 mixture of paraffin:petroleum jelly.
9. Prewet a lint-free tissue paper with deionized water and roll into a cylinder of ~0.25 cm width. (Fig 3A, step v)
10. Wrap the inside of the 35 mm dish keeping the tissue away from the viewing area (Fig 3A, step vi).
11. Optional: Place the lid on the petri dish and mount onto the microscope.

3.2.4 Results and Discussion—Using the microscope set up described above frame rates of >50 frames per second were achievable by reducing the size of the image capture over periods of >10 seconds. This means that real time imaging of the *C. elegans* was possible (Supplemental videos 1 and 2). Figure 3 (B-K) shows images captured at 2.5 second intervals in both the green and red channels. The PBS and 2-coverslip method of movement restriction was able to keep the *C. elegans* in the same position but had faster head and tail movement (figure 3 B-F; supplemental video 1) whereas the CyGEL^{TM} movement restriction methodology slows head and tail movement but allowed small positional movement (figure 3 G-K; supplemental video 2). This movement restriction protocol with ultra high-speed imaging is ideally suited to allow for fast protein and physiological dynamic changes to be measured (e.g. calcium fluxes and protein degradation substrates) but could also be adapted to study movement phenotypes using DIC imaging (supplemental movie 3).

3.3 High resolution resonant scanning confocal live *C. elegans* imaging

Protein and organelle movement requires high-resolution 3 dimensional imaging over time. Such imaging requires a higher degree of immobilization than the high-speed widefield imaging in section 3.2. Even with complete immobilization minute movements can cause imaging artifacts, such as blurring, associated with traditional confocal microscope systems. The use of resonance scanning systems greatly enhances acquisition speed and therefore is more advantageous than traditional confocal scanning systems. Here we describe a simple immobilization technique using nanoparticles adapted from previous studies [11, 12] to study fluorescent proteins at high resolution.

3.3.1 Microscope—A Leica SP8 tandem scanning inverted confocal microscope with 2 HyD and 1 PMT detectors, 6 laser lines at 405, 456, 488, 514, 568, 633 nm wavelengths, an

encoded motorized XY stage, a SuperZ™ galvanometer Z-drive and a high speed 12000 Hz resonant scanner. Tiled images (8×8) over 50 Z slices with an A.U. of 1 were collected in 2 wavelengths simultaneously using a PlanApo 40x oil objective (N.A. 1.3) at ~30 fps every 15 minutes for 16 hours. Images were acquired and rendered using Leica LAS AF software (v3.1). Post-imaging processing was performed using Volocity (v6.2.1; PerkinElmer)

3.3.2 Nanoparticle immobilization protocol (adapted from [11, 12])

1. Culture transgenic *C. elegans* as described in protocol 2 until young adult stage.
2. Make 6% agarose in PBS and keep heated.
3. Using a prewarmed Pasteur pipette transfer ~200µl into an uncoated 14mm microwell 1.5 cover glass bottom culture dish (e.g. MatTek P35G-1.5-14-C)
4. Immediately place a 22×22 mm square coverglass (e.g. VWR 48366-227) on top of the agarose and press down on the edges.
5. Allow the agarose to set then using coverglass forceps remove the coverslip. Invariably the agarose will stick to the coverslip square. If not, then using a scalpel score around the circular microwell and using forceps remove the agarose circle and place on the square coverglass. Trim and scrape any excess agarose away using a scalpel.
6. Add 10 µl of 2.5% by volume 0.1 µm polystyrene beads (Polysciences cat #00876-15), 0.4 µl of 40x PBS and 0.5 µl of concentrated OP50 to the center of the microwell. *Note, in previous studies, 0.05 µm beads have been shown to be slightly more effective than 0.1 µm beads* [12].
7. Transfer 10-20 transgenic *C. elegans* to the bead/PBS/OP50 mix
8. Use forceps to transfer the agarose circle to the microwell and place 20 µl of distilled water on top of the agarose pad.
9. Place a square place a 22×22 mm square coverglass (e.g. VWR 48366-227) on top of the agarose and move into position gently with forceps.
10. Seal the square coverglass with a 1:1 mixture of paraffin:petroleum jelly.
11. Prewet a lint-free tissue paper with deionized water and roll into a cylinder of ~0.25 cm width.
12. Wrap the inside of the 35 mm dish keeping the tissue away from the viewing area.
13. Place the lid on the petri dish and mount onto the microscope.

3.3.3 Results and discussion—Figure 4 shows maximum intensity projections of cropped sections of a transgenic *C. elegans* at 0, 2, 4, 8, 12 and 16-hour time points of either the GFP fluorescence in the pharynx of the animal (Fig 4 A, C, E, G, I, K) and the cytoplasmic mKate2 expression (Fig 4 B, D, F, H, J, L). The movement of the head region indicates that the *C. elegans* is still alive at the end of the imaging time period. The apparent decrease in both GFP and mKate2 levels seen are due to microscope drift over time

(supplemental figure 1). This can be avoided by the use of larger Z-stacks or by the use of automated focusing algorithms and hardware. This imaging system ideal for fluorescent protein or probe tracking in 3 dimensions over time and could also be used for more complicated imaging modalities such as FRAP or FRET.

4. Conclusions

We present here 3 different imaging techniques that can be used to image live *C. elegans* without the use of microfluidic technologies. The technique chosen is dependent on the question being asked. For example, if a researcher is interested in lysosomal tracking then the higher resolution 4 dimensional confocal imaging technique would be more suitable, whereas if a researcher was interested in calcium spikes then high-speed widefield imaging would be of choice. Each immobilization technique presented here is easily adaptable to the microscope set up's available to them which would make core microscopy labs more amenable to the *C. elegans* researcher.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Live low-resolution *C. elegans* imaging in 384-well microtitre plate. Approximately 20-25 transgenic *C. elegans* (VK2289) expressing GFP in the pharynx under the control of the *myo-2* promoter and mKate2 in the intestine under the control of the *nhx-2* promoter [7] were imaged every 15 minutes using the ArrayScanV^{TI} for 24 hours in both the 488 nm and 569 nm wavelengths. (A) The relative mKate2 expression in the intestine was calculated by taking the mKate2 total intensity of the well/GFP fluorescence area of the well (number of worms) for each time point and dividing by the mKate2 total intensity at time 0 for 9 wells. Error bars represent the standard deviation from the mean. (B-F) Images collected for one of the wells at the indicated time points by the SpotDetector^{TM} BioApplication for calculation of the indicated fluorescence. Inset shows a magnified region to indicate the presence of L1 progeny from the adults that results in the increase in relative mKate2 fluorescence after 16 hours.

Fig 2.

Sodium azide immobilization causes physiological changes and subsequent death in *C. elegans*. Approximately, 20-25 transgenic *C. elegans* (VK2289) expressing GFP in the pharynx under the control of the *myo-2* promoter and mKate2 in the intestine under the control of the *nhx-2* promoter [7] were imaged every 15 minutes using the ArrayScanV^{TI} for 24 hours in both the 488 nm and 569 nm wavelengths. (A) The relative mKate2 expression in the intestine was calculated by taking the mKate2 total intensity of the well for each time point/GFP fluorescence area of the well (number of worms) at time 0 and dividing by the mKate2 total intensity at time 0, averaged for 3 wells (red markers). The amount of death was calculated using Sytox® Green fluorescence for each time point /GFP fluorescence area of the well (number of worms) at time 0, averaged for 3 wells. Error bars represent the standard deviation from the mean. (B-D) Overlay images depicting the detection of the GFP pharyngeal marker (green), the mKate 2 (red) and SYTOX® Green dye (magenta) from a

representative well with the SpotDetector BioApplication algorithm at the time points indicated.

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Fig. 3.

High-speed widefield imaging of live *C. elegans* (A) A schematic representation of the *C. elegans* movement restriction protocols outline in sections 3.2.2 and 3.2.3. (B-K) Representative images taken from the continuous high-speed widefield imaging experiments with movement restriction via coverslip (B-F) or CyGEL™ (G-K) methods of VK2289 at the indicated timepoints. Images were acquired and rendered using NIS Elements software (v4.2) in both the 470 nm and 555 nm channels. Movies were created using Volocity software (v6.1; PerkinElmer). Images at the indicated times points are taken from supplemental movies 1 (B-F) and 2 (G-K). The scale bars depict either 100 μm (B-F) or 50 μm (G-K).

Fig. 4.

High resolution 4-dimensional confocal imaging of live *C. elegans*. Maximum intensity projections of transgenic *C. elegans* strain VK2289 of the pharyngeal region (A, C, E, G, I, K) or the intestinal region (B, D, F, H, J, L) expressing GFP or mKate2, respectively, at the indicated time points. Three-dimensional Z-stacks were acquired over approximately 50 planes over an 8×8 tilescan image every 15 minutes for 16 hours using a 12KHz resonant scanner. Images were acquired and rendered using Leica LAS AF software (v3.1) with both the 488 nm and 568 nm laser lines. Scale bars indicate 30 μ m.