

Research Article

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Optimization of culture and analysis methods for enhancing long-term *Brugia malayi* survival, molting and motility *in vitro*

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Abstract

Lymphatic filariasis is a neglected tropical disease caused by roundworm parasites such as *Brugia malayi* that spread via a mosquito vector. *In vitro* culture of these parasites provides controlled conditions to understand parasite biology and provides a cheaper way to screen potential micro- and macrofilaricides. Published studies have used a wide array of approaches and metrics regarding *in vitro* cultures of *B. malayi*; as a result, drawing comparisons and identifying the reasons why inability to reproduce outcomes are difficult. This study sought to determine conditions that ensure reproducible outcomes and used evaluation metrics that are easily measured and can be automated to ensure objectivity. We found culturing *B. malayi* third-stage larvae (L3) in endothelial basal media supplemented with 20% fetal bovine serum and 75 μM ascorbic acid in a temperature- and humidity-controlled incubator produced better survival and molting rates as well as longer and more motile parasites than previously reported. The benefit of ascorbic acid seemed to be unique to L3 parasites, as the addition of ascorbic acid to adult parasites had no significant impact on survival or motility. The methods reported in this study will help in designing experiments for both parasite behaviour studies and drug screening applications for disease eradication.

Introduction

Lymphatic filariasis is a neglected tropical disease that has debilitating effects on one-third of the approximately 120 million people currently infected worldwide (World Health Organization, 2011). Lymphatic filariasis spreads via parasitic nematodes that utilize a mosquito as a transmission vector and then migrate to and live in human lymphatics. Infective lymphatic filarial parasites (third-stage larvae, L3) are deposited onto human skin when the mosquito carrying them takes a blood meal. After the parasites mature and molt into fourth-stage larvae (L4) and then into sexually mature adults, they produce sheathed microfilariae that circulate in the lymph and blood stream. While infected individuals can be clinically asymptomatic, their lymphatics are dilated and have altered function (Ottesen, 1994; Norões *et al.* 1996; Dreyer *et al.* 2000). Those with lymphatic filariasis are at risk of developing lymphoedema or elephantiasis, morbid diseases that significantly reduce one's quality of life (Melrose, 2002).

To minimize disease morbidity, the Global Alliance to Eliminate Lymphatic Filariasis (GAELF) aims to eliminate the epidemic by interrupting disease transmission via mass drug administration (MDA) (World Health Organization, 2011). The standard single- and double-drug MDA have been effective at clearing microfilariae temporarily, while adult parasites appear to continue to live albeit undergo a decrease in microfilariae production (Melrose, 2002; Katiyar and Singh, 2011; Ballesteros *et al.* 2016a). To reduce the need to re-dose, studies explored the sterilization of adult parasites by targeting *Wolbachia*, bacteria with an endosymbiotic relation with lymphatic filarial parasites (Rao, 2005; Johnston *et al.* 2010; Lustigman *et al.* 2014; Taylor *et al.* 2014). Recently, a clinical trial demonstrated that a single-dose triple-drug regimen led to microfilariae-free individuals two years after dosing (Thomsen *et al.* 2016). Other macrofilaricides such as auranofin have also been identified through an *in vitro* screening process (Bulman *et al.* 2015). Adult parasites and microfilariae are thought to be the central cause of pathogenic progression of lymphatic filariasis (Dreyer *et al.* 2000; Chakraborty *et al.* 2013). However, very little is known regarding the underlying biology that drives L3 migration towards lymphatic vessels. This gap is due in part to the lack of experimental model systems (*in vitro* and *in vivo*) that allow researchers to study L3 behaviour in detail. Recent advances in organ-on-a-chip approaches have allowed researchers to study physiologic systems with selected *in vivo* complexities with the precision and high-resolution

imaging capabilities that are afforded by *in vitro* studies. To this end, we recently reported on a multicellular platform that allowed L3s to be co-cultured in migratory chambers in the presence of lymphatic endothelial cells and dermal fibroblasts (Kassis *et al.* 2014). While the study showed differential motility behaviour in the presence of cells, it was limited to short-term experiments due to the challenges associated with maintaining a high viability of L3s past a week or so in culture. An improved method to study these parasites in an *in vitro* culture environment will provide the opportunity to gain a better biological understanding of the L3 life-cycle stage and migratory behaviour which could facilitate the development of new therapies to target transmission (by eliminating the establishment of the lymphatic niche) and/or treating lymphatic filariasis-associated morbidities.

In vivo drug assays have the advantage of allowing the investigation of drug effects in their intended physiological contexts, but are low-throughput and costly (Saeung and Choochote, 2013). Such an approach is more suited for the testing of a specific compound of interest (Lakshmi *et al.* 2010; Wenkert *et al.* 2010; Ceballos *et al.* 2014). On the other hand, *in vitro* drug assays can quickly screen for efficacy in a multitude of compounds. Unfortunately, this approach requires large quantities of parasites, which are a limited resource in the adult stage due to current methods requiring cultivation in an animal model. Several studies have explored how to successfully culture *Brugia malayi* from L3 to sexually mature adulthood *in vitro* (Riberu *et al.* 1990; Smillie *et al.* 1994; Falcone *et al.* 1995). Additionally, many attempts have been made to develop an *in vitro* cultivation system that provides a suitable platform for the life cycle of filarial parasites, which would allow for the effective screening of new drugs (Taylor, 1960; Mak *et al.* 1983; Saeung and Choochote, 2013).

Despite several established *in vitro* culturing protocols, a consensus of necessary conditions for successful filarial parasite culture is absent. A variety of methods has been proposed in the literature, all with varying results and definitions of success (Table 1). Mammalian hosts are used to prime filarial parasites for a successful molt and continue to be the platform used to provide *B. malayi* at all life-cycle stages for research purposes (Chen and Howells, 1979; Wisniewski and Weinstein, 1993). Meanwhile, reliable, animal model systems are costly and complex (Saeung and Choochote, 2013). The extensive and current use of animal models indicates a deficiency in the cultivation of *B. malayi in vitro* (Michalski *et al.* 2011). Unfortunately, it is difficult to draw clear comparisons across studies due to a wide variety of approaches and conditions (Table 1). It is hypothesized that the success of *in vitro* culture systems is dependent on the serum source and lot. It may be possible that serum supplementation could provide some factors essential to *in vitro* filarial development and survival, but identification of these factors is complex. For a serum-free method, fatty acids, which can be found in fetal bovine serum (FBS), with the presence of specific species of yeast have been identified to aid in the molting process of this filarial parasite (Smith *et al.* 2000). Another group found that ascorbic acid is required for *B. malayi* to molt from an L3 to an L4; they hypothesize, although it was not actually tested, that ascorbic acid results in the stabilization of the collagen triple helix of the parasite, but *B. malayi* development and survival post-transition were not reported (Rajan *et al.* 2003).

Because a wide variety of non-standardized metrics has been used to quantify parasite viability, the measurements taken are inconsistent. Therefore, evaluating optimal conditions is challenging. Parasite length and width data are likely to be the most consistent metric for determining parasite growth but alone are not enough to characterize filarial health. Survival rate is a useful measurement to gauge parasite viability in culture but is seldom reported, likely due to the low yield of parasites that make it to

adulthood. A measure often used for *in vitro* culture success is molting rate; however, the methods used for determining a molt vary, from counting the number of full cuticles to morphologic analyses of different regions of interest. Variation of parasite length and therefore shed cuticle length could lead to bias and person-to-person variation of the number of molts counted. Motility of the filarial parasite has been assessed, although the methodology is vague and varies with the person performing the assessment. Thus, the goal of this study was to identify key culture components and develop an *in vitro* system that provides reproducible nematode maturation quantified by easily gathered metrics, as an easy and repeatable system is more likely to lead to consistent *in vitro* culturing of *B. malayi* L3.

In this study, various chemical conditions were explored to identify key aspects necessary for successful L3 and adult *B. malayi* culture, while quantitatively tracking various metrics of parasite viability and maturation over one month of culture. The impact of cell culture media, serum levels, co-cultures with cells derived from the dermal layer and the lymphatics, and use of ascorbic acid were all explored. Metrics of *B. malayi* health were defined by (1) survival percentage, (2) molting rates from an L3 to an L4, (3) length and (4) a motility coefficient. These proposed measurements captured various aspects of a healthy nematode. This *in vitro* system allows not only for the successful cultivation of L3 and adult *B. malayi* but also has the potential to provide the foundation for the discovery of more efficient drugs to combat lymphatic filariasis.

Materials and methods

Brugia malayi receiving, cleaning and preparation for experimentation

Live *B. malayi* L3 were shipped overnight at ambient temperatures from the National Institutes of Health Filariasis Research Reagent Resource Center (FR3) at the University of Georgia (UGA; Athens, GA, USA) in Roswell Park Memorial Institute (RPMI) 1640 (Michalski *et al.* 2011). When received, the parasites were washed three times with RPMI 1640 (Lonza, NY, USA) supplemented with 10% FBS (Lots: L1106, D13057, Atlanta Biologicals, Atlanta, GA, USA) and 1% penicillin–streptomycin–amphotericin (P/S/A) (Gibco, NY, USA). Experiments began on the day *B. malayi* were received, and the parasites were cultured in a 37 °C incubator with 5% CO₂.

Effect of media and serum concentrations on L3 survival

To determine optimal medium and FBS concentrations, *B. malayi* were cultured in a transwell system (Corning, NY, USA) on 12-well plates in order to simplify media changes. More specifically, the parasites were cultured in the transwell-permeable supports that had a 0.4 µm polyester membrane. Each well for the medium tests had an average of 22 parasites with a range of 6–40 parasites and for the serum tests, each well had 26 parasites with a range of 13–42 in 2 mL of total media, which was achieved by transferring 20 µL of L3s suspended in media at a concentration of 700 L3 mL⁻¹ into the transwell. This volume distribution approached was taken as opposed to manually counting parasites per well to minimize L3 contamination but resulted in varying number of L3s per well. Each experimental group had a *n* = 12 performed in four independent experiments. Media changes occurred three times per week (every Monday, Wednesday and Friday) by transferring the transwell of parasites onto a new 12-well plate with 2 mL of fresh warmed media per well.

Three types of media were tested: (1) the standard culture media for *B. malayi* RPMI 1640 supplemented with 10% FBS

Table 1. Overview of culturing methods of lymphatic filariasis parasites

Author	Species	Start stage	End stage	Media	Serum	Cells	Molt percentage to L4 (time) <i>in vitro</i>	Molt percentage to adult (time) <i>in vitro</i>	Maximum survival <i>in vitro</i> from start stage
(Taylor, 1960)	<i>Wuchereria bancrofti</i>	mf*	mf	3% glucose in Ringers solution; Parker 199	Horse	No	N/A	N/A	8 days
(Mak et al. 1983)	<i>Brugia malayi</i>	L3	Adult	RPMI 1640	10% human AB	Yes	17.7% (14 days)	20% (7 weeks)	7 weeks+
(Franke et al. 1987)	<i>W. bancrofti</i>	L3	L4	Franke's NI NCTC 135/Isocove's modified Dulbecco's	10% human plasma	No	65–100% (35 days)	N/A	35 days
(Riberu et al. 1990)	<i>B. malayi</i>	L3	Adult	Franke's NI medium	10% human	No	N/A	85% (60 days)	100 days
(Wisniewski and Weinstein, 1993)	<i>Brugia pahangi</i>	L3	L4	Franke's NI medium	5–20% FBS, FeCS, CS, ABS	Yes	95% (13 days) with FBS and FeCS	N/A	60 days
(Smillie et al. 1994)	<i>B. malayi</i>	L3	Adult	Franke's NI medium	10–15% BAF, CHS, FBS, PHS, SHS	No	0, 0, 0, 8, 10, 24%	0, 0, 0, 1–3%	28, 19, 34, 35, 55 days
(Falcone et al. 1995)	<i>B. malayi</i>	L3	Adult	RPMI 1640	10% pooled human	Yes	100% (14 days)	17% (37 days)	37 days
(Smith et al. 2000)	<i>B. malayi</i>	L3	L4	RPMI 1640	N/A	Yes	32.4%	N/A	13 days (ended experiment)
(Rajan et al. 2003)	<i>B. malayi</i>	L3	L4	RPMI 1640	10% FBS	No	72.2%	N/A	10 days
(Saeung and Choochote, 2013)	<i>B. malayi</i>	L3	L4	HBSS for injection	N/A	No	N/A; only <i>in vivo</i>	N/A; only <i>in vivo</i>	N/A

*mf, microfilariae; FBS, fetal bovine serum; FeCS, non-supplemented calf serum; CS, calf serum; ABS, adult bovine serum; BAF, bovine albumin fraction-V; CHS, commercially obtained human serum; PHS, pooled human serum; SHS, single human serum.

(Atlanta Biologicals) and 1% P/S/A (Gibco); (2) Dulbecco's Modified Eagle Medium (DMEM) (Lonza, NY, USA) supplemented with 10% FBS (Atlanta Biologicals) and 1% P/S/A (Gibco); and (3) endothelial basal medium (EBM) (Lonza) supplemented with 10% FBS (Atlanta Biologicals) and 1% P/S/A (Gibco). Additionally, FBS levels of 0, 10 and 20% were tested to find optimal *B. malayi* L3 survival. All experiments were terminated on day 13 to gather baseline survival after the L3 to L4 transition for selecting the best baseline media and serum conditions that would be used for further optimization (see Table 2). To determine survival, the number of live parasites and total parasites per well were counted and recorded. If no motion was detected for 10 s, then the parasite was scored as dead. This procedure was performed at various time points to generate the survival curve. Occasionally due to media changes, a parasite would be lost, but this loss was accounted for in the statistical analysis.

Based on these results, the new standard medium was the EBM supplemented with 20% FBS (Atlanta Biologicals, Atlanta, GA, USA), 1% penicillin–streptomycin (P/S) (Gibco, NY, USA), 1% Glutamax, 50 μM DBcAMP (Sigma Aldrich, St. Louis, MO, USA) and 1 $\mu\text{g mL}^{-1}$ hydrocortisone acetate (Sigma Aldrich, St. Louis, MO, USA). These additional supplements have previously been shown to support lymphatic endothelia cell (LEC) culture, and these were used in experiments with co-culture as described in the next section (Podgrabinska *et al.* 2002). Experiments were also performed to show that these additional supplements did not affect the survival of *B. malayi* L3 (figures not included).

Effect of co-culture with cells on L3 survival

Human dermal fibroblasts (HDFs) and LECs were used to see whether a co-culture of cells was critical for *B. malayi* survival. These cells were expanded in DMEM supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA, USA) and 1% P/S/A (Gibco, NY, USA). LECs were isolated from human neonatal foreskin as previously described (Podgrabinska *et al.* 2002). They were expanded in EBM supplemented with 20% FBS (Atlanta Biologicals, Atlanta, GA, USA), 1% P/S/A (Gibco, NY, USA), 1% Glutamax, 50 μM DBcAMP (Sigma Aldrich, St. Louis, MO, USA) and 1 $\mu\text{g mL}^{-1}$ hydrocortisone acetate (Sigma Aldrich, St. Louis, MO, USA).

Each cell type was expanded in a T-75 flask coated with type I rat tail collagen solution composed of 50 $\mu\text{g mL}^{-1}$ (BD Biosciences, San Jose, CA, USA) in 0.1% acetic acid (Sigma, St. Louis, MO, USA). Prior to reaching confluence in the T-75, cells were split and seeded at 100 000 cells per well in a 12-well plate for experiments. These cells were seeded on the bottom surface of the well (Fig. 3A). After a day of culture, the media used for all experimental groups was the fully supplemented EBM with 20% FBS levels, and the parasites were added by inserting them in the transwell (refer to Fig. 3A). We tested L3 co-culture with LECs, HDFs and no cells, and each experimental group had a $n = 12$ performed in four independent experiments. Each well averaged 24 parasites with a range of 13–44 parasites. The seeding of cells in each well was performed the day before the experiments started and the day before each media change. Media changes were performed every Monday, Wednesday and Friday by transferring the transwell from the old plate to a new one with freshly warmed media and the newly seeded cells at the bottom of the well, in order to ensure that the cells co-cultured with the parasites had not differentiated or died. LECs were used at passage 8–9 and HDFs at passage 13–15.

Effect of ascorbic acid on L3 health

While assessing the role of ascorbic acid on L3 *B. malayi* survival, the transwell system was discontinued due to the inability to

image the parasites while in culture. Parasites were cultured in 12-well plates filled with 2 mL of EBM supplemented with 20% FBS (Atlanta Biologicals, Atlanta, GA, USA), 1% P/S (Gibco, NY, USA), 1% Glutamax, 50 μM DBcAMP (Sigma Aldrich, St. Louis, MO, USA), 1 $\mu\text{g mL}^{-1}$ hydrocortisone acetate (Sigma Aldrich, St. Louis, MO, USA) and the addition of 75 μM ascorbic acid. Each well averaged 16 parasites with a range of 8–24 parasites. Each experimental group had a $n = 12$ performed in two independent experiments. Due to contamination in two wells, the final sample size analysed was $n = 10$. Media exchanges were performed every Monday, Wednesday and Friday by manually exchanging 75% of the medium in the well. All experiments were stopped on day 30, and all the live *B. malayi* for each experimental group were shipped overnight at ambient temperatures in the appropriately supplemented media to the UGA for *B. malayi* molting assessment. For each experimental group, all the live parasites were aggregated into a 1.5 mL centrifuge tube filled with 1 mL of supplemented EBM with 20% FBS. Groups were blinded to UGA prior to molting assessment. Every 4 days, survival of the remaining parasites was manually assessed as described above, and videos were taken for subsequent analysis, as described later in ‘Data acquisition and analysis of L3’. A random sample of three L3s was taken per well for video analysis, as it was difficult to individually image every parasite in the well.

Effect of media types and ascorbic acid on adult female health

Experiments performed on adult female *B. malayi* mirrored those performed on *B. malayi* L3. Females were chosen because they release microfilariae and are the primary target of macrofilaricides. To assess whether media type affected the *in vitro* survival of adult female *B. malayi*, the three media used were RPMI ($n = 16$ in two independent experiments), DMEM ($n = 16$) and EBM ($n = 16$); each were supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA, USA) and 1% P/S (Gibco, NY, USA) to parallel the test described for L3. Later, to determine if ascorbic acid played a role in adult female *B. malayi* survival, the parasites were cultured in EBM supplemented with 10% FBS and 1% P/S ($n = 24$ in two independent experiments) and the same supplemented EBM with the addition of 75 μM ascorbic acid ($n = 24$). Adult female *B. malayi* were cultured in a 24-well plate at a density of one parasite per well; this density allowed for a more automated assessment of survival. Each well contained 2 mL of media. Media changes occurred three times per week (every Monday, Wednesday and Friday) by manually exchanging 75% of the media in the well. Videos were taken every hour for 3 weeks and were later analysed.

A summary of the experimental conditions can be found in Table 2.

Data acquisition and analysis on L3

For this study, 10 s video segments for the *B. malayi* L3 were captured with a Guppy Pro camera (Allied Vision, Exton, PA, USA)

at a resolution of 780×580 pixels and frame rate of 25 frames/s under $25 \times$ total magnification using a Zeiss inverted microscope. After the videos were collected, they were analysed to gather information about *B. malayi* motility and length.

A LabVIEW programme was developed to obtain motility measurements automatically from the captured videos. The programme allows the user to specify the regions of interest (i.e. the well containing the parasites) for which they would like to obtain motility data, then the programme runs the analysis on all videos stored in the video acquisition folder; analysing all the experimental videos with only one required region of interest (ROI) setup. The region selection functionality was purposefully not automated to allow a higher level of flexibility for use in custom high-throughput screening (HTS) plates, microfluidic devices and any type of well plate. Finally, parasite motility is quantified using a Lucas–Kanade optical flow algorithm implemented in LabVIEW (National Instruments, Austin, TX, USA). All the magnitudes of the velocity vectors obtained between two subsequent frames were summed for an entire well and then averaged over the length of the video segment giving an aggregated motility metric for a particular parasite (Kassis *et al.* 2014; Schudel *et al.* 2015). The motility coefficients were then normalized to the average motility coefficient (of all groups) on day 1.

Survival percentages were calculated per well. The length of *B. malayi* was calculated at various time points ($n = 10$ in two independent experiments). Using the videos, a single frame was manually isolated where the parasite is mostly ‘straight’ or uncoiled. ImageJ was used to calculate the length of the parasite.

Molting

To determine the per cent of L3s that molted at day 30, 20% of the total parasites were retrieved and the live parasites were separated and sent to the UGA for morphological analysis. Larvae in micro-fuge tubes were heat-killed by placing them in a heat block at 60 °C for 2 min. Slides for mounting the larvae were prepared by dispensing 200 μL of molten 5% agarose in the centre of a glass microscope slide flanked by spacer slides of slightly greater thickness. A second glass slide was quickly placed over the agar perpendicular to the slide underneath to create a thin agarose pad. Larvae were transferred to the agarose pads in small volumes of culture media (<10 μL) followed by a coverslip.

Larvae were examined at $200 \times$ magnification under an Olympus IX51 inverted microscope and photographs were taken with a Canon Vixia HF M52 attached via a Martin Microscope adapter, model MM99-58 (Martin Microscope, Easley, SC, USA), and a Diagnostic Instruments DBX 1.0X C-mount adapter (SpotImaging, Sterling Heights, MI, USA). To distinguish the larval stage of the parasites, the anatomical features of the anterior and posterior ends were examined, previously detailed by Mak *et al.* (1983). L3 are characterized by a tapering anterior end, whereas the end of the L4 is more globular in shape. Three terminal papillae can be seen on the posterior end

Table 2. Experimental conditions

Experiment	Stage	Media	Serum (%)	Cells	Other supplement	n	Length of experiment
A	L3	RPMI, DMEM, EBM	10	N/A	N/A	12	13 days
B	L3	EBM	0, 10, 20	N/A	N/A	12	13 days
C	L3	EBM	20	HDF, LEC, none	N/A	12	13 days
D	L3	EBM	20	N/A	75 μM ascorbic acid	10	30 days
E	Adult	RPMI, DMEM, EBM	10	N/A	N/A	16	21 days
F	Adult	EBM	20	N/A	75 μM ascorbic acid	24	21 days

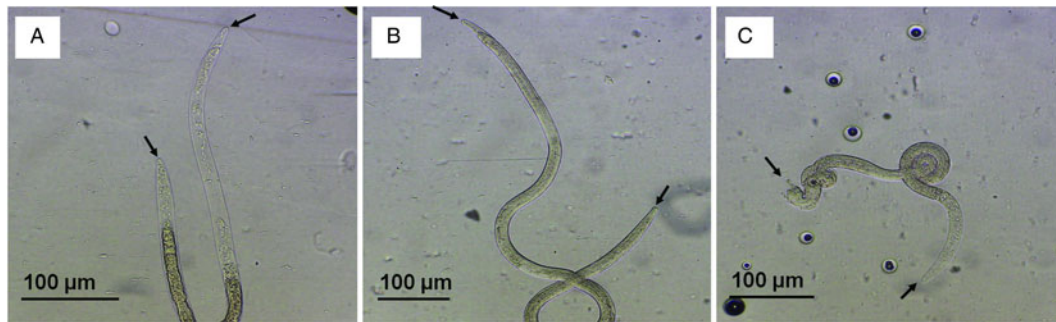


Fig. 1. Representative images of live *Brugia malayi* on day 13 at stages (A) L3, (B) L4 and (C) unknown. Arrows indicate features used to identify larval stage.

of the L3, while these are absent in the L4. Based on these features, larvae were classified as L3, L4 or ‘unknown’, in the case of damaged parasites or parasites of ambiguous molting status. Representative images of stages L3, L4 and unknown are found in Fig. 1A–C, respectively.

Imaging system and analysis of adult *B. malayi*

For the adult parasites, an imaging system was designed with readily available components that can be acquired from common suppliers. The resulting setup is modular in nature with each module containing two webcams (Logitech 905c Switzerland, or any compatible USB camera) incorporated within a repurposed storage drawer frame (Walmart, Bentonville, AR, USA). A

Huion 17.7 inch LED light board (Amazon.com, Seattle, WA, USA) was used to illuminate the plates from above and provide contrast of the parasites for the camera. Each single module is capable of holding two cell culture plates, or any custom-made plate or device. The cameras are connected directly to a laptop that sits on top of the cell culture incubator via a USB interface (Fig. 2A–C). The modular nature of the hardware allows the setup to be scalable. In addition to the hardware, a user-friendly LabVIEW-based interface was designed to allow the user to specify the duration of the video to be captured as well as the interval between the videos. The software override the webcam’s built-in autofocus mechanism and allows the user to focus each camera individually to obtain the best image possible for each plate. Once set-up is complete, the user starts acquisition and the programme

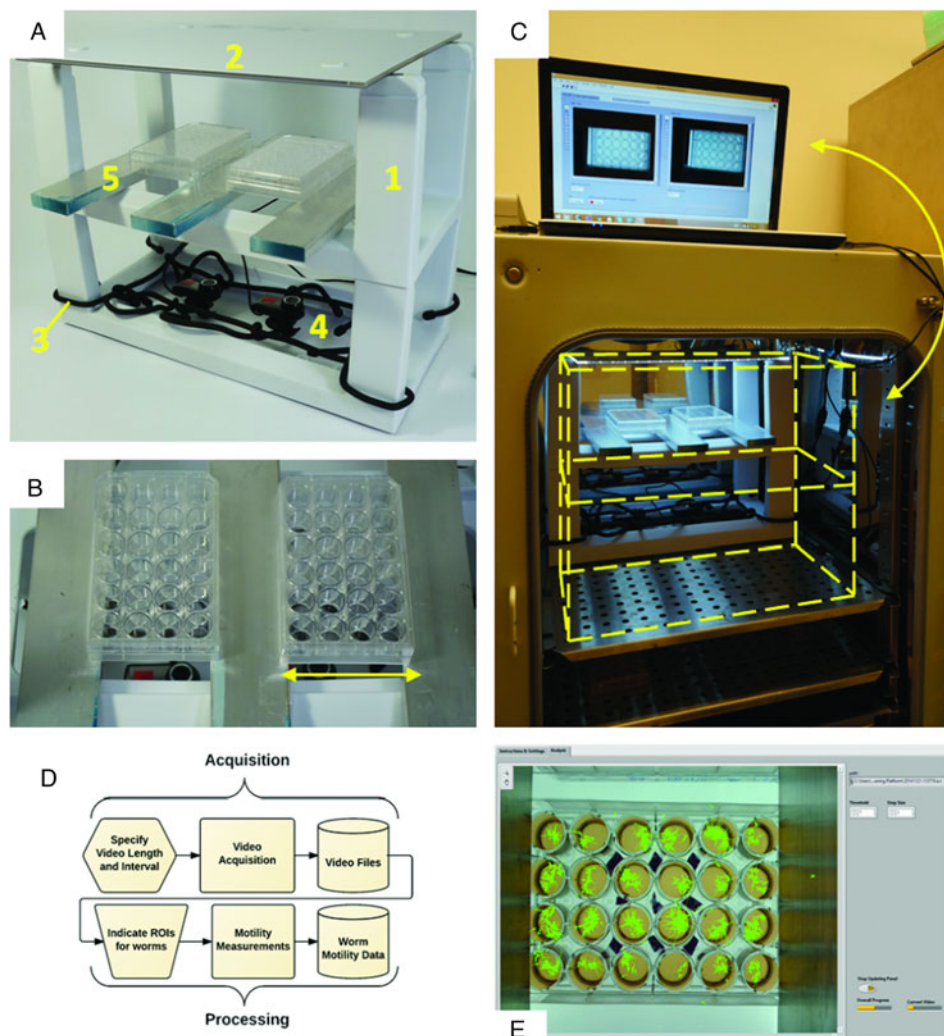


Fig. 2. Cheap, modular imaging system is built from readily accessible parts and can be placed in an incubator creating a low-cost parasite monitoring system capable of video acquisition and quantification. (A) Overview of imaging system and components: (1) Sterilite Narrow Modular Drawer; (2) Huion LED Light Board; (3) Heavy Duty Twist Ties; (4) Logitech Webcam C905; (5) Rectangular aluminium Bar. Total cost is \$97.24. (B) Setup of 24-well plates positioning on aluminium bars directly over cameras. (C) Demonstration of incubator setup showing space for additional imaging unit (dashed box), and real-time imaging setup with laptop equipped with LabVIEW (arrows). (D) Block diagram of acquisition and processing steps of the two LabVIEW-based virtual instruments (VIs). (E) Screenshot of the user interface to analyse the average motility for each parasite for each video segment in a specific region of interest.

automatically acquires the videos and saves them as time/date-stamped compressed AVI files. The videos are acquired in a sequential manner, where only one camera is recording at a time. Such an implementation provides tremendous flexibility in the number of cameras connected and allows the laptop to only need the processing resources to be able to run one webcam at a time, since a typical 5 s, 960 × 720 pixel video requires around 50 megabytes of storage space.

To assess motility for adult female *B. malayi*, the same approach used for the L3s was implemented using a custom LabVIEW virtual instrument (VI) (Fig. 2D and E) with the exception of video post-processing. Due to some lighting and motion artefacts, the videos were post-processed in Adobe After Effects to remove both light banding (due to exposure-LED light mismatch) using a third-party plug-in (Flicker Free) and stabilized using the built-in 'Warp Stabilizer VFX'. Stabilization was necessary in this case due to vibrations present in the incubator resulting from other concurrent experiments in the incubator. All motility coefficients were normalized to the average motility coefficients of the first day of the control group for each experiment. Parasites with a normalized motility coefficient <0.01 were considered dead as that was determined to be within the pixel noise of the system. To determine survival, the number of live parasites and total parasites per well were counted and recorded. All VIs can be found at <https://github.com/TKassis>.

Validation of the imaging system with SNAP

In order to demonstrate the use of the system, the effect of the nitric oxide (NO) donor S-N-acetyl D,L penicillamine (SNAP, Sigma, St. Louis, MO, USA) was quantified on parasite motility in a concentration-dependent manner. This experimental configuration has been used in the laboratory to monitor parasite motility and health over time, as well as evaluate parasite responses to anti-parasitic agents (Kassis et al. 2014; Schudel et al. 2015). Freshly isolated adult female *B. malayi* parasites were obtained from the FR3 (Michalski et al. 2011) and underwent the wash procedure previously described. Individual parasites were subsequently plated in 2.5 mL of complete EBM with 20% FBS in a 24-well culture plate pre-incubated at 37 °C in 5% CO₂ for 1 h prior to the addition of parasites. The NO donor, SNAP, was reconstituted in phosphate-buffered saline (PBS, Sigma, St. Louis, MO, USA), adjusted to the correct pH, and added to each treatment well, with PBS alone being added to the control wells. Following the addition of treatments, the parasites were incubated and 5 s video segments at 10 min intervals were recorded. Video acquisition was carried out continuously for 90 h.

Statistical analysis

The Gehan–Breslow–Wilcoxon test was used for the statistical analysis of the per cent of *B. malayi* survival in various conditions

for both L3 and adult stages. To analyse overall differences in motility for *B. malayi* L3 and adults due to ascorbic acid supplementation, a Mann–Whitney test was used. A two-way analysis of variance followed by Sidak's test to correct for multiple comparisons was performed to compare between the two groups at specific time points among the three media groups for both *B. malayi* L3 and adults. Main group effects and time effects were analysed. All analyses were run in Prism 6 (GraphPad Software Inc, La Jolla, CA, USA) and significance was defined as $P > 0.05$ (not significant – ns), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****). All data are presented as a mean ± s.d.

Results

Endothelial basal media with 20% FBS enhances parasite health

Measuring per cent survival after 13 days of culture allowed us to assess the baseline effects of various media and media supplement conditions on *B. malayi* L3. Specifically, by day 13, *B. malayi* L3 should have molted or attempted to molt (Mak et al. 1983; Smith et al. 2000; Rajan et al. 2003); failure to successfully transition in this time frame would result in immature parasites that then die before reaching adulthood. Parasites were cultured in the medium supplemented with 10% FBS; EBM, DMEM and RPMI resulted in 65, 45 and 16% survival, respectively (Fig. 3B) ($P < 0.0001$). Compared with RPMI, the commonly used media for *B. malayi* (Smith et al. 2000; Rao and Well, 2002; Rajan et al. 2003; Winter et al. 2013), EBM and DMEM enhanced survival ($P < 0.0001$ and $P = 0.0034$, respectively). Next, levels of FBS were assessed in EBM, since this appeared to be the most effective medium of those tested. 20, 10 and 0% FBS in EBM led to 80 ± 12, 65 ± 9 and 11 ± 8% survival by day 13, respectively (Fig. 3C). The highest level of serum (20%) enhanced the survival of *B. malayi* L3 significantly when compared with 10% and 0% FBS levels ($P = 0.0075$ and $P < 0.0001$, respectively). All further experiments with L3s were carried out with 20% FBS in EBM as the baseline media.

Co-culture did not enhance L3 health

While it remains unclear why filarial parasites target the lymphatics when establishing a host infection, we decided to test the effects of co-culture with human LECs and HDFs as factors secreted from these cells would come in direct contact with *B. malayi*. L3s were cultured in the presences of either LECs, HDFs or no cells, which resulted in 86, 83 and 80% survival by day 13, respectively (Fig. 3D). Per cent survival was not significantly enhanced by the presence of human LECs or HDFs by day 13 ($P = 0.5680$). Because of the lack of obvious benefit of co-culture, at least over this 13-day period, long-term *B. malayi* L3 survival experiments were conducted without cell co-culture.

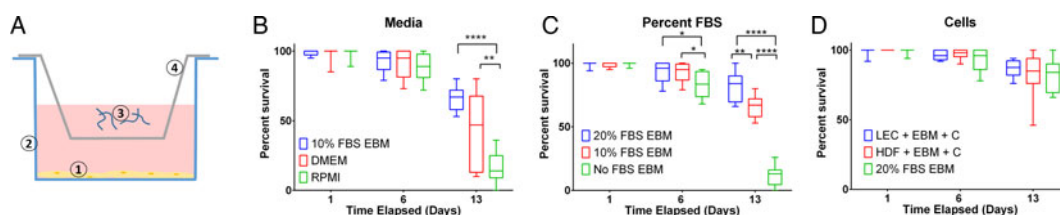


Fig. 3. Per cent survival of *Brugia malayi* in various media, FBS per cent levels and cells presence. EBM with 20% FBS-cultured without cells results in enhanced per cent survival of *B. malayi* L3. Time points were taken at days 1, 6 and 13. (A) Schematic of the Transwell system. The cells (1) are seeded at the bottom of the well (2), while the parasites (3) are seeded in the transwell (4). (B) Per cent survival of *B. malayi* L3 was recorded at various time points for three different media. (C) Per cent survival of *B. malayi* L3 was recorded at various time points for three levels of FBS. (D) Per cent survival of *B. malayi* L3 was recorded at various time points for two cell lines cultured with a collagen layer, denoted as C.

Ascorbic acid promoted L3 health

To assess *B. malayi* health for longer term culture using the optimized media conditions outlined above, multiple metrics (per cent survival, *B. malayi* length, per cent molting and motility coefficient) were tracked over the course of a month. To further enhance survival and maturation, we investigated continuous supplementation of the media with 75 μM ascorbic acid as this concentration is similar to what is found in human serum and has been shown to aid in *B. malayi* L3 molting, presumably through its role in collagen synthesis and cuticle formation (Rajan *et al.* 2003; Schleicher *et al.* 2009). Overall per cent survival was found to be significantly improved between parasites cultured with and without ascorbic acid ($P < 0.0001$). By day 30, per cent survival was 69 and 46% with and without ascorbic acid, respectively (Fig. 4A). On day 30, 75% of the surviving parasites had molted when ascorbic acid was present in the media, while no molts occurred in parasites cultured without ascorbic acid (Fig. 4B). On day 30, average *B. malayi* lengths were 1.9 ± 0.1 and 1.7 ± 0.2 mm with ($n = 10$) and without ($n = 10$) ascorbic acid, respectively (Fig. 4C). This difference was found to be significant with $P = 0.0009$, albeit represents a modest amount of growth. In addition to enhancing molting, the supplementation of ascorbic acid resulted in a significant increase in the motility coefficient and length of *B. malayi* by day 30 ($P = 0.0004$). Overall, the motility coefficients were significantly different between the control and ascorbic acid supplemented groups ($P = 0.0142$). It can be noted that this difference can be seen starting on day 14 ($P = 0.0012$) and persisting through the remaining time of experimentation. By day 30, the average motility coefficients for the control and ascorbic acid supplemented groups

were significantly different at 0.72 and 4.49, respectively (Fig. 4D, $P = 0.0004$). The continuous presence of ascorbic acid led to increased *B. malayi* per cent survival, per cent molting, length and motility of live parasites. However, it did not produce any adult *B. malayi* after 30 days in culture.

Imaging system provided a platform for adult *B. malayi* analysis

Having found a condition to successfully culture *B. malayi* L3, we were interested in whether the same conditions would have beneficial effects on the long-term culture of adult *B. malayi*. Given the larger size of adult parasites, we utilized a low-cost system that could be placed directly in the incubator for continuously monitoring parasite health over the entire experiment. We performed a concentration-response experiment with a NO donor to establish the sensitivity of the system to detect changes in motility. Parasite motility exhibited a concentration-dependent response to SNAP with a significant decrease in motility at 5.3 mM SNAP, which occurred within 6 h. Control parasites cultured with PBS showed no reduction in motility (Fig. 5A). From the motility data obtained, the effect of the concentration on per cent inhibition (defined as the percentage of the baseline motility each parasite exhibited) was calculated. For the two highest concentrations, 2.7 and 5.3 mM, all parasites died within 6 h of treatment (Fig. 5D). The rate of death was highly correlated with concentrations except for an unexplained middle concentration of 0.3 mM, which did not cause any death within the time course of experimentation (two independent experiments). The IC_{50} value was calculated at specific time points from each concentration curve

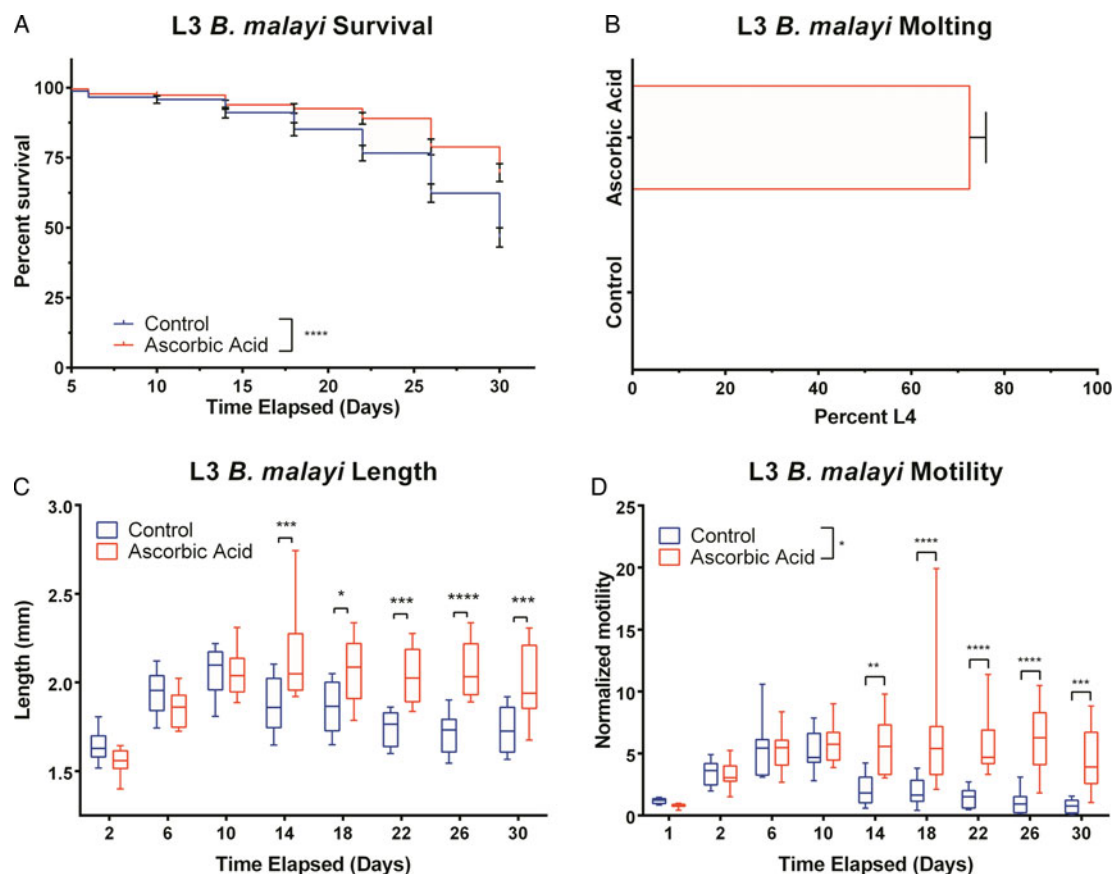


Fig. 4. Continued presence of 75 μM ascorbic acid enhances survival, motility and development of *Brugia malayi* L3 *in vitro*. When 75 μM ascorbic acid is added to EBM over the course of 30 days *in vitro*, *B. malayi* (A) percentage survival was significantly higher with the presence of ascorbic acid. The parasite's (B) percentage molting was 100% and greater with the presence of ascorbic acid. The parasite (C) length was 1.9 ± 0.1 mm with the presence of ascorbic acid when compared with 1.7 ± 0.2 mm when cultured without ascorbic acid. *Brugia malayi* (D) motility is greater with the presence of ascorbic acid.

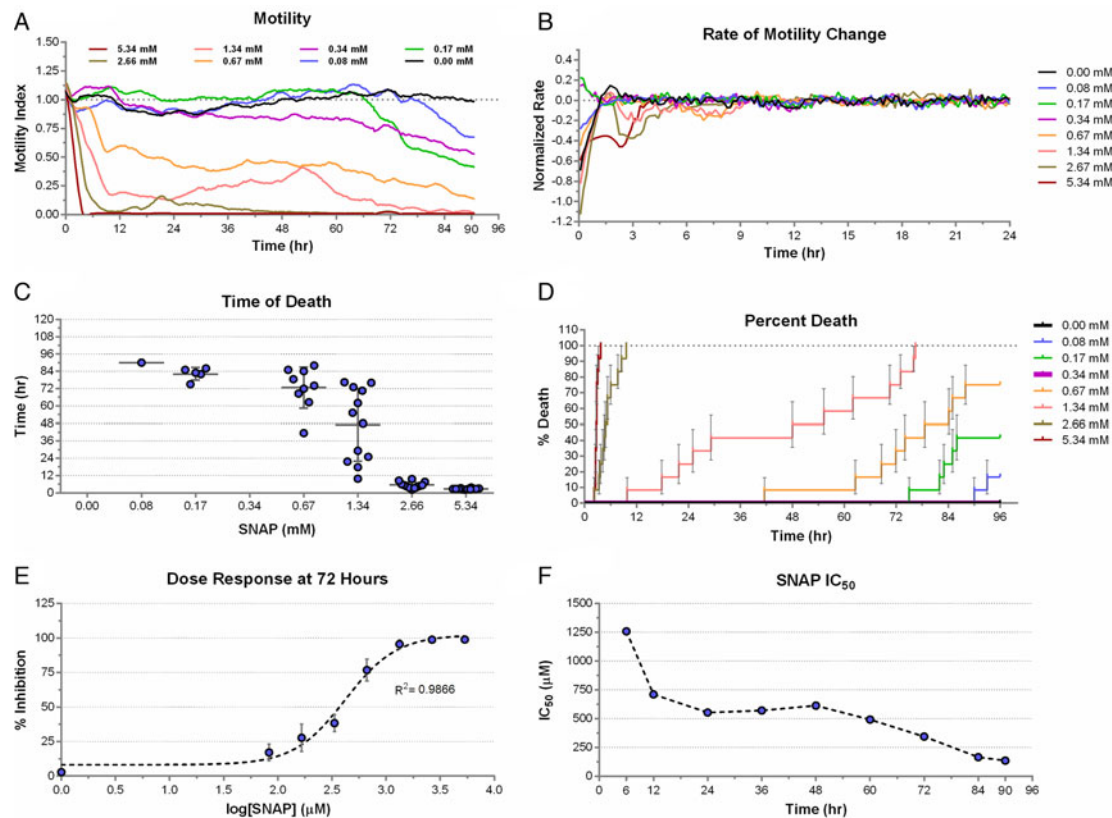


Fig. 5. Response of adult female *Brugia malayi* to nitric oxide. (A) The control parasites show no reduction in motility unlike parasites exposed to S-N-acetyl D,L penicillamine (SNAP) that exhibit a concentration-dependent response. (B) The rate of motility change, plotted for the first 24 h, shows that initially the lower concentrations seem to have a stimulatory effect by slightly increasing motility while the higher concentrations immediately begin to show their efficacy by reducing the motility coefficient. (C) Time of death for each parasite is concentration dependent. A SNAP concentration of 0.34 mM did not cause any parasite death. (D) Survival curve where the y-axis is the percentage of parasites that have died. (E) Concentration response at a single time point, 72 h. (F) IC_{50} values can be obtained from the concentration response curves and plotted as a function of incubation time clearly relating the decreased concentration required for longer incubation times.

(72 h, Fig. 5E) and revealed that longer exposure to the drug reduces the IC_{50} (Fig. 5F).

EBM and RPMI enhanced adult female *B. malayi* health

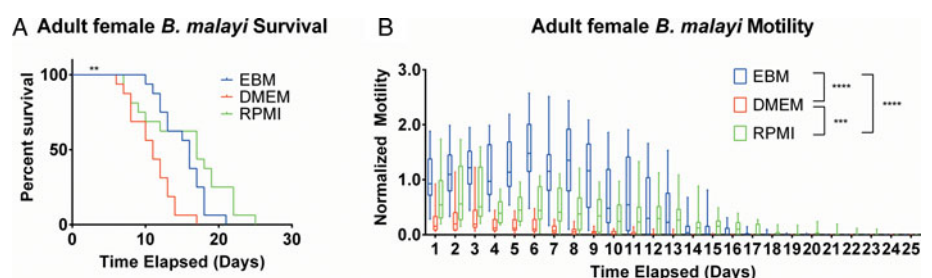
After establishing the utility of the adult parasite imaging analysis system, comparisons of EBM, DMEM and RPMI supplemented with 10% FBS and 1% P/S were carried out to see if adult *B. malayi* responded similarly to L3s. Overall per cent survival differences among the groups were significant with $P = 0.0028$. Culturing the parasites in EBM, DMEM and RPMI resulted in 62.5, 18.75 and 62.5% survival, respectively, by day 13 (Fig. 6A). The last surviving parasite in each condition lasted until days 20, 16 and 24. Meanwhile, the median survivals are days 16, 11 and 17 in EBM, DMEM and RPMI, respectively. While the survival of parasites in DMEM was significantly worse than those in EBM and RPMI, there were no significant differences in survival between EBM and RPMI, which is in stark contrast to the results

obtained with L3 larva. Overall, the motility coefficients in the EBM condition were significantly greater between EBM vs DMEM ($P < 0.0001$) and EBM vs RPMI ($P < 0.0001$). Furthermore, there was a significant difference in motility between DMEM and RPMI ($P = 0.0009$) (Fig. 6B). However, by day 14, there were no significant differences in mean motility among the groups (EBM vs DMEM, $P = 0.5153$; EBM vs RPMI, $P = 0.9534$ and DMEM vs RPMI, $P = 0.2404$), likely because the parasites were in very poor health for all conditions by this time point.

Ascorbic acid does not aid adult female *B. malayi* health

To determine whether ascorbic acid had an effect on the longevity of adult *B. malayi* *in vitro*, parasites were cultured with or without ascorbic acid as described above. No significant differences in survival were observed with ascorbic acid supplementation (Fig. 7A, $P = 0.4599$). The last surviving parasite lived until the end of the experiment at day 19 for the EBM only condition and day 18 for

Fig. 6. Adult female *Brugia malayi* cultured in EBM, DMEM and RPMI result in differences in survival and motility. (A) *Brugia malayi* percentage survival by day 13 is 62.5, 18.75 and 62.5% in EBM, DMEM and RPMI, respectively. The last surviving parasite in each group lived until days 20, 16 and 24, respectively. Among the three groups, there was a significant difference. (B) *Brugia malayi* motility was increased when cultured in EBM, compared with DMEM and RPMI. By day 14, no differences were found in parasite motility among the three groups.



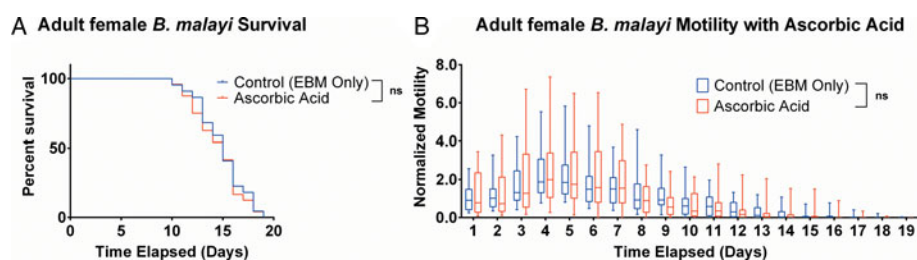


Fig. 7. Adult female *Brugia malayi* cultured with 75 μ M ascorbic acid in EBM results in no difference in survival and motility. (A) *Brugia malayi* percentage survival began to decrease on day 10. The last surviving parasite lived until the end of the experiment for EBM only and day 18 for the EBM with ascorbic acid supplements. (B) The motility coefficient for parasites cultured with ascorbic acid was not significantly different from that of those cultured in only EBM.

the EBM with ascorbic acid supplementation condition; the ones who died had a median survival of day 15 for EBM only and with ascorbic acid. The motility coefficients in the EBM only and EBM supplemented with ascorbic acid were not different ($P = 0.3906$).

Discussion

The aim of this study was to determine *in vitro* culture conditions for *B. malayi* L3 to enhance parasite health. We explored varying types of culture medium, levels of commercially available serum, co-cultures with LECs and HDFs, and the addition of ascorbic acid. In addition to current tools that look at per cent survival and molting rates, we automated the analysis of videos capturing parasite behaviour to output a parasite motility coefficient.

Performing biological studies on *B. malayi in vitro* has been shown to be a valuable biological tool (Ballesteros *et al.* 2016b). When comparing the results of our study with previous studies, certain aspects of the results are significantly improved, while previous methods have achieved better success in other aspects. A recent study has indicated that dysregulated genes appeared in the adult *B. malayi* when cultured *in vitro* indicating stress (Ballesteros *et al.* 2016b). Cultured in 10% FBS supplemented RPMI 1640 indicated adult parasite death by 5 days, similar to what we saw in our RPMI conditions. It is worth noting that studies which have achieved culture for periods >30 days and molting to the adult stage suffer from one of two drawbacks: the rate of total parasite viability is very low (<20%) or the success required patient-specific human serum, thus making the platform inaccessible to other researchers. Mak *et al.* successfully cultured adult *B. malayi* using RPMI with 10% human AB serum and LCC-MK₂ cell co-culture. While 100% of the surviving parasites molted to L4 at 28 days and were longer than the molted parasites from our study, only 51.35% of the original parasites were still alive at this time point indicating a lower survival rate than what was achieved here. Riberu *et al.* (1990) was also able to grow adult *B. malayi* with high viability in cultures that were supplemented with human serum (Franke *et al.* 1987), but this success was highly dependent on the individual that the serum was collected from and has been difficult to reproduce (Smillie *et al.* 1994; Falcone *et al.* 1995; Smith *et al.* 2000). Smillie *et al.* required fresh human serum to see 24% of parasites molt to L4s, with 1% of these parasites molting to a 'young adult'. Falcone *et al.* were able to culture out to L4/young adult stages using RPMI 1640 with 10% human serum (pooled from three individuals) and co-cultured with Jurkat cells and fibroblasts; however, only 15% of the initial larvae were alive at this point. A variety of serum supplements, ranging from fetal bovine to human, has been used, but none has been able to consistently produce *B. malayi* adults with reasonable yields (Wisniewski and Weinstein, 1993; Smillie *et al.* 1994).

Although the formula for many commercial cell culture media is proprietary, EBM is comprised of more amino acids and inorganic salts than the other media tested that may provide the necessary conditions for improved *B. malayi* culture. Serum also provides some vital factor(s) in L3 development. Typical cell culture of LECs uses a maximum of 20% serum supplementation.

Our attempts to culture L3 in 100% serum resulted in 100% death by day 7; thus, we did not explore other serum levels. Studies suggest that cells were a requirement for healthy parasite cultivation *in vitro* (Mak *et al.* 1983; Falcone *et al.* 1995; Smith, 2000). Our study did not show an improvement with LEC and HDF co-cultures, and this discrepancy could be explained by the supplements present in the culture conditions, which may provide the necessary factor(s) typically provided by cell types not studied in this paper. It is possible that the LECs could provide a beneficial survival factor at later stages in the life cycle; however, we did not investigate the benefits of cell co-culture beyond 13 days and thus further study is warranted in order to draw conclusions in this regard. It was advantageous, however, that the *in vitro* system could be simplified by removing the requirement of cells in the parasite culture by providing the appropriate medium and supplements.

Ascorbic acid plays an important role in collagen formation in that it stabilizes the collagen triple helix by regulating enzymes that hydroxylate amino acids, lysine and proline, at body temperature (37 °C) (Gerster, 1987). Since the cuticle of *B. malayi* is composed primarily of collagens, it is critical for the L3-to-L4 transition and may even be necessary for the L4-to-adult transition since *B. malayi* undergoes another molt to reach adult stage. Because adult parasites no longer molt, we hypothesize that the addition of ascorbic acid would not aid in survival and motility due to their lack of need for extensive collagen formation, albeit they need to produce collagen for microfilariae production. While we did see released microfilariae from the adult parasites, it is unclear whether microfilariae development occurs prior to receiving the parasites from the FR3 and we did not attempt to quantify the difference in MF production between ascorbic acid supplemented parasites and controls. We were interested in how a constant source of ascorbic acid could affect parasite health on a longer time scale at 75 μ M ascorbic acid, which is the optimal concentration and is the concentration found in human serum (Rajan *et al.* 2003; Schleicher *et al.* 2009). The combination of ascorbic acid and serum-supplemented EBM leads to reproducible results, and this knowledge is an important step towards determining the necessary *in vitro* conditions for *B. malayi* L4 growth to adulthood. Additionally, ascorbic acid has been found to be unnecessary for *Caenorhabditis elegans* growth due to its cultivation at 15 °C, which is a temperature where collagen stability does not require the hydroxylation of prolines. Thus, it would be interesting to explore the role of ascorbic acid as a supplement in the cultivation of other mammalian filarial parasites *in vitro* (e.g. *Brugia timori*, *Wuchereria bancrofti*, *Onchocerca volvulus*, *Litomosoides sigmodontis* and *Loa loa*) and assess their ability to successfully molt and progress *in vitro* due to a more stable collagen helix (Rajan *et al.* 2003).

The *in vitro* culture conditions described in this paper are limited in that L4 *B. malayi* have not successfully molted and matured into adults. With our approach and experimental setup, we are not able to track individual parasite molting from L3 to L4 and motility data that would allow us to correlate molting and motility. The motility metric used to define a healthy parasite assumes live parasites are motile and therefore maintain

a greater motility coefficient. Additionally, the platform to monitor adult parasites requires constant light to illuminate the plate for data collection. It has been seen that microfilariae of *W. bancrofti* have granules that act as photoreceptors responsible for negative phototaxis (Aoki et al. 2011). Thus, for future works where motion behaviour is studied, the platform setup may need to be altered to ensure that natural motility behaviour is exhibited. Additionally, studies that reported cultivation of *B. malayi* adults all utilized human serum. Thus, in conjunction with an altered setup, future experiments would integrate our findings of a richer medium, higher serum levels and continued presence of ascorbic acid with the use of human serum to continue efforts to culture *B. malayi* to adulthood.

In conclusion, we have demonstrated that there is a reproducible method of culturing (1) *B. malayi* L3 to L4 and (2) adult female *B. malayi* in an *in vitro* environment for well over a week. Using per cent survival, per cent molting, length and motility, the health of *B. malayi* could be quantified. With this knowledge, we systematically determined the combination of medium, serum level and supplements that provide a high rate of molting for surviving L3s and more motile adult female parasites. This platform to culture L3s reproducibly into L4s is the first step to effectively culture *B. malayi* *in vitro* in a manner that provides a sufficient supply of adult parasites for research needs ranging from drug testing to more fundamental understanding of *B. malayi* behaviour.

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References

- Aoki Y, Fujimaki Y and Tada I (2011) Basic studies on filaria and filariasis. *Tropical Medicine and Health* **39**, 51–55.
- Ballesteros C, Tritten L, O'Neill M, Burkman E, Zaky WI, Xia J, Moorhead A, Williams SA and Geary TG (2016a) The effects of ivermectin on *Brugia malayi* females *in vitro*: a transcriptomic approach. *PLoS Neglected Tropical Diseases* **10**, 1–19.
- Ballesteros C, Tritten L, O'Neill M, Burkman E, Zaky WI, Xia J, Moorhead A, Williams SA and Geary T (2016b) The effect of *in vitro* cultivation on the transcriptome of adult *Brugia malayi*. *PLoS Neglected Tropical Diseases* **10**, 1–20.
- Bulman CA, Bidlow CM, Lustigman S, Cho-Ngwa F, Williams D, Rascon AA Jr, Tricoche N, Samje M, Bell A, Suzuki B, Lim KC, Supakorndej N, Supakorndej P, Wolfe AR, Knudsen GM, Chen S, Wilson C, Ang KH, Arkin M, Gut J, Franklin C, Marcellino C, McKerrow JH, Debnath A and Sakanari JA (2015) Repurposing Auranofin as a lead candidate for treatment of lymphatic filariasis and onchocerciasis. *PLoS Neglected Tropical Diseases* **9**, 1–18.
- Ceballos L, Mackenzie C, Geary T, Alvarez L and Lanusse C (2014) Exploring the potential of flubendazole in filariasis control: evaluation of the systemic exposure for different pharmaceutical preparations. *PLoS Neglected Tropical Diseases* **8**, e2838.
- Chakraborty S, Gurusamy M, Zawieja DC and Muthuchamy M (2013) Lymphatic filariasis: perspectives on lymphatic remodeling and contractile dysfunction in filarial disease pathogenesis. *Microcirculation* **20**, 349–364.
- Chen SN and Howells RE (1979) The *in vitro* cultivation of the infective larvae and the early mammalian stages of the filarial worm, *Brugia pahangi*. *Annals of Tropical Medicine and Parasitology* **73**, 473–485.
- Dreyer G, Noroes J, Figueredo-Silva J and Piessens WF (2000) Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. *Parasitology Today* **16**, 544–548.
- Falcone FH, Zahner H, Schlaak M and Haas H (1995) *In vitro* cultivation of third-stage larvae of *Brugia malayi* to the young adult stage. *Tropical Medicine and Parasitology* **46**, 230–234.
- Franke ED, Riberu W and Wiady I (1987) *In vitro* cultivation of third stage larvae of *Wuchereria bancrofti* to the fourth stage. *American Journal of Tropical Medicine and Hygiene* **37**, 370–375.
- Gerster H (1987) Human vitamin C requirements. *Zeitschrift für Ernährungswissenschaft* **26**, 125–137.
- Johnston KL, Wu B, Guimarães A, Ford L, Slatko BE and Taylor MJ (2010) Lipoprotein biosynthesis as a target for anti-*Wolbachia* treatment of filarial nematodes. *Parasites & Vectors* **3**, 99.
- Kassis T, Skelton HM, Lu IM, Moorhead AR and Dixon JB (2014) An integrated *in vitro* imaging platform for characterizing filarial parasite behavior within a multicellular microenvironment. *PLoS Neglected Tropical Diseases* **8**, e3305.
- Katiyar D and Singh LK (2011) Filariasis: current status, treatment and recent advances in drug development. *Current Medicinal Chemistry* **18**, 2174–2185.
- Lakshmi V, Joseph SK, Srivastava S, Verma SK, Sahoo MK, Dube V, Mishra SK and Murthy PK (2010) Antifilarial activity *in vitro* and *in vivo* of some flavonoids tested against *Brugia malayi*. *Acta Tropica* **116**, 127–133.
- Lustigman S, Melnikow E, Balakrishnan S, Contreras A, Nandi V, Liu J, Bell A, Unnasch TR, Rogers MB and Ghedin E (2014) Potential involvement of *Brugia malayi* cysteine proteases in the maintenance of the endosymbiotic relationship with *Wolbachia*. *International Journal for Parasitology: Drugs and Drug Resistance* **4**, 267–277.
- Mak JW, Lim PKC, Sim BKL and Liew LM (1983) *Brugia malayi* and *B. pahangi*: cultivation *in vitro* of infective larvae to the fourth and fifth stages. *Experimental Parasitology* **55**, 243–248.
- Melrose WD (2002) Lymphatic filariasis: new insights into an old disease. *International Journal for Parasitology* **32**, 947–960.
- Michalski ML, Griffiths KG, Williams SA, Kaplan RM and Moorhead AR (2011) The NIH-NIAID filariasis research reagent resource center. *PLoS Neglected Tropical Diseases* **5**, e1261.
- Norões J, Addiss D, Santos A, Medeiros Z, Coutinho A and Dreyer G (1996) Ultrasonographic evidence of abnormal lymphatic vessels in young men with adult *Wuchereria bancrofti* infection in the scrotal area. *The Journal of Urology* **156**, 409–412.
- Ottesen EA (1994) The human filariases: new understandings, new therapeutic strategies. *Current Opinion in Infectious Diseases* **7**, 550–558.
- Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS and Skobe M (2002) Molecular characterization of lymphatic endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16069–16074.
- Rajan TV, Paciorkowski N, Kalajic I and McGuinness C (2003) Ascorbic acid is a requirement for the morphogenesis of the human filarial parasite *Brugia malayi*. *Journal of Parasitology* **89**, 870–871.
- Rao RU (2005) Endosymbiotic *Wolbachia* of parasitic filarial nematodes as drug targets. *Indian Journal of Medical Research* **122**, 199–204.
- Rao R and Well GJ (2002) *In vitro* effects of antibiotics on *Brugia malayi* worm survival and reproduction. *The Journal of Parasitology* **88**, 605–611.
- Riberu WA, Atmosoedjono S, Purnomo, Tirtokusumo S, Bangs MJ and Baird JK (1990) Cultivation of sexually mature *Brugia malayi* *in vitro*. *American Journal of Tropical Medicine and Hygiene* **43**, 3–5.
- Saeung A and Chochoche W (2013) Development of a facile system for mass production of *Brugia malayi* in a small-space laboratory. *Parasitology Research* **112**, 3259–3265.
- Schleicher R, Carroll M, Ford ES and Lacher DA (2009) Serum vitamin C and the prevalence of vitamin C deficiency in the United States: 2003–2004 national health and nutrition examination survey (NHANES). *The American Journal of Clinical Nutrition* **90**, 1252–1263.
- Schudel A, Kassis T, Dixon JB and Thomas SN (2015) S-nitrosated polypropylene sulfide nanoparticles for thiol-dependent transnitrosation and toxicity against adult female filarial worms. *Advanced Healthcare Materials* **4**, 1484–1423.
- Smillie CL, Vickery AC, Kwa BH, Nayar JK and Rao UR (1994) Evaluation of different medium supplements for *in vitro* cultivation of *Brugia malayi* third-stage larvae. *Journal of Parasitology* **80**, 380–383.
- Smith HL (2000) Investigating development of infective stage larvae of filarial nematodes. *Frontiers in Bioscience* **5**, 95–102.

- Smith HL, Paciorkowski N, Babu S and Rajan TV** (2000) Development of a serum-free system for the *in vitro* cultivation of *Brugia malayi* infective-stage larvae. *Experimental Parasitology* **95**, 253–264.
- Taylor AER** (1960) Maintenance of filarial worms *in vitro*. *Experimental Parasitology* **9**, 113–120.
- Taylor MJ, Hoerauf A, Townson S, Slatko BE and Ward SA** (2014) Anti-*Wolbachia* drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis. *Parasitology* **141**, 119–127.
- Thomsen EK, Sanuku N, Baea M, Satofan S, Maki E, Lombore B, Schmidt MS, Siba PM, Weil GJ, Kazura JW, Fleckenstein LL and King CL** (2016) Efficacy, safety, and pharmacokinetics of coadministered diethylcarbamazine, albendazole, and ivermectin for treatment of bancroftian filariasis. *Clinical Infectious Diseases* **62**, 334–341.
- Wenkert D, Ramirez B, Shen Y and Kron MA** (2010) *In vitro* activity of geldanamycin derivatives against *Schistosoma japonicum* and *Brugia malayi*. *Journal of Parasitology Research* **2010**, 716498.
- Winter AD, McCormack G, Myllyharju J and Page AP** (2013) Prolyl 4-hydroxylase activity is essential for development and cuticle formation in the human infective parasitic nematode *Brugia malayi*. *The Journal of Biological Chemistry* **288**, 1750–1761.
- Wisniewski N and Weinstein P** (1993) Growth and development of *Brugia pahangi* larvae under various *in vitro* conditions. *The Journal of Parasitology* **79**, 390–398.
- World Health Organization** (2011) *Global Programme to Eliminate Lymphatic Filariasis: Progress Report on Mass Drug Administration, 2010*. Geneva, Switzerland.