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Use of *in situ* Hybridization to Localize *Wolbachia* during Embryogenesis in *Brugia malayi*

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Abstract

Wolbachia are intracellular bacteria that are required for development and reproduction in most filarial nematodes. New approaches are needed to improve understanding of the role of *Wolbachia* in filarial biology. Recent studies using fluorescent immunohistology revealed asymmetric segregation of *Wolbachia* in early embryos of *Brugia malayi*, and this helps to explain the restricted distribution of *Wolbachia* in adult worms. However, the distribution of *Wolbachia* in late stages of embryo development or in infective larvae (L3) is largely unknown. In this study we have explored the use of *in situ* hybridization (ISH) to localize *Wolbachia* and *Wolbachia* gene expression during embryogenesis and morphogenesis in *B. malayi*.

ISH with a 16S rRNA probe revealed *Wolbachia* in the lateral cords, oocytes, and embryos of *B. malayi* female worms. This was consistent with prior studies that detected *Wolbachia* by immunohistology. ISH with probes for *Wolbachia* surface protein gene (*wsp*) and *Wolbachia* heme biosynthetic pathway gene (*hemE*) showed that these genes were strongly expressed in early embryos but not in later stage embryos ("comma", "pretzel", or stretched intrauterine microfilariae). A detailed study of the distribution of *Wolbachia* in *B. malayi* embryos using ISH with the 16S rRNA probe documented the asymmetric segregation of *Wolbachia* in early embryos (morulae) and showed that *Wolbachia* were only present in hypodermal cord precursor cells in later stage embryos ("comma" stage or later). Progressively restricted localization of *Wolbachia* during morphogenesis may explain why *Wolbachia* are absent from the genital primordium in L3.

Wolbachia distribution and gene expression vary dramatically during filarial embryo development and across the worm's life cycle. Additional research is needed to understand the tissue-specific population dynamics of *Wolbachia* and to explore how signals from the nematode host might influence the growth, distribution, and gene expression in these bacteria.

Keywords: *Brugia malayi*; *Wolbachia*; Embryogenesis; *in situ* hybridization; Gene expression

Introduction

Wolbachia are α-proteobacteria that are maternally transmitted and widely distributed (as an intracellular symbiont) in insects and in filarial nematodes. It is estimated that up to 65% of insect species harbor *Wolbachia* [1], and most filarial nematode species are infected with these bacteria [2]. *Wolbachia* infection is associated with several phenotypes in arthropod hosts such as cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing [3,4]. In contrast, many filarial nematodes require *Wolbachia* for development and reproduction [2].

These include *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus*, which cause the disabling tropical diseases of lymphatic filariasis and onchocerciasis, respectively. These parasites depend on *Wolbachia* endosymbionts for their development, viability and fertility [2]. This dependence makes *Wolbachia* an interesting target for development of new drugs for lymphatic filariasis and onchocerciasis [5,6]. Animal studies and clinical trials have shown that doxycycline and rifampicin therapies clear *Wolbachia* from filarial worms and inhibit fertility and larval development [7,8]. However, since doxycycline treatment is not practical for widespread use in tropical populations, researchers are searching for other drugs that can more efficiently kill *Wolbachia* in filarial worms [6]. A more comprehensive understanding of interactions between *Wolbachia* and their nematode hosts could lead to identification of new drug targets.

Wolbachia are present in all life stages in *Wolbachia*-dependent filarial species. However, the quantity and distribution of *Wolbachia* vary dramatically during the parasite life cycle [9,10]. The distribution of *Wolbachia* in adult filarial worms is well-documented, with the bacteria being concentrated in the lateral cords and the female reproductive system but not in the male reproductive system [11-13]. *Wolbachia* are asymmetrically separated into different cell lines during early embryogenesis, and this may explain the localization of the bacteria in adult worms [14].

This study employed *in situ* hybridization (ISH) to detect *Wolbachia* and to assess the expression of *Wolbachia* genes within the filarial host. The results include new information on the distribution of *Wolbachia* and changes in *Wolbachia* gene expression during embryo development.

Methods

Parasite material and *in situ* hybridization

B. malayi adult worms and L3 larvae were provided by the Filariasis Research Reagent Resource Center (FR3). Details regarding

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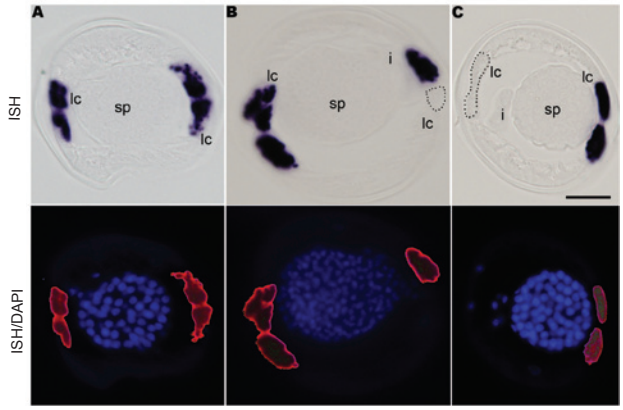

Figure 1: *Wolbachia* are exclusively and unevenly distributed in lateral cords of adult male *Brugia malayi*. Sections A, B and C are cross sections. The top panel shows ISH results obtained with a 16S rRNA probe and the bottom panel shows ISH merged with DAPI staining. The color of ISH staining has been replaced by red to increase contrast before merging with DAPI staining. (A) *Wolbachia* are present in lateral cords on both sides of the worm; (B) no *Wolbachia* were seen in half of the lateral cord on one side; (C) *Wolbachia* are visible in one lateral cord. Abbreviations: i: intestine; lc: lateral cord; sp: developing sperm; dotted circles outline segments of lateral cords without *Wolbachia*; scale bar: 20 µm.

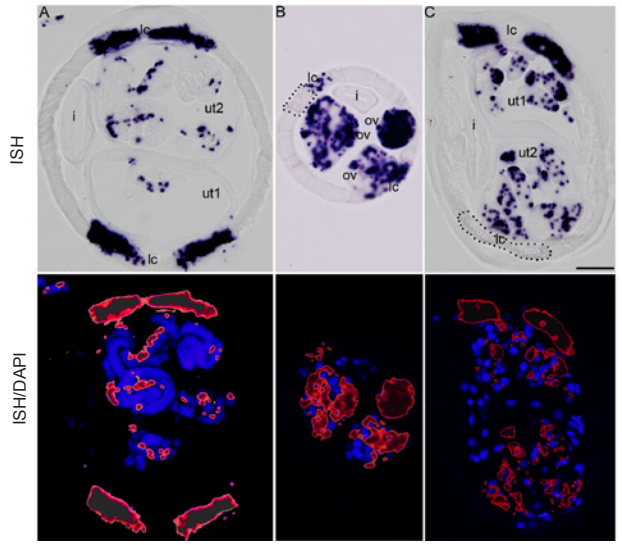

Figure 2: *Wolbachia* are present in oocytes and embryos and unevenly distributed in lateral cords of adult female *Brugia malayi*. Sections A, B and C are cross sections. The top panel shows ISH results obtained with a 16S rRNA probe, and the bottom panel shows ISH merged with DAPI staining. The color of ISH staining has been replaced by red to increase contrast before merging with DAPI staining. (A) *Wolbachia* are present in lateral cords on both sides of the worm and in pretzel stage embryos; (B) developing oocytes in ovaries and half of the lateral cord on one side contain *Wolbachia*; (C) *Wolbachia* are present in this section in morula stage embryos, but they are absent in the lateral cord on one side. Abbreviations: i: intestine; lc: lateral cord; ov: ovary; ut: uterus; dotted circles outline segments of lateral cords without *Wolbachia*; scale bar: 20 µm.

slide preparation, gene cloning, RNA probe labeling, and the ISH procedure were described in detail previously [15]. In this study, a 424 bp fragment of *16S rRNA* gene (AF051145) specific for *Wolbachia*, a 700 bp *Wolbachia* surface protein gene sequence (*wsp*, AJ252061), and a 415 bp fragment of *Wolbachia* heme biosynthetic pathway gene *hemE* (Q5GTT1) were amplified with these primers: *16S rRNA* forward primer, 5-CAGCTCGTGTCGTGAGATGT-3, *16S rRNA* reverse primer: 5-CCCAGTCACTGATCCCACTT-3, *wsp* forward primer; 5-TTTTTCAGCAACCGCTTTAG-3, *wsp* reverse primer: 5-ATTAAACGCTATTCCAGCTTCTG-3, *hemE* forward primer, 5-TGAAATTCCTGGTGCAAGTG-3, *hemE* reverse primer: 5-TCTCGTTGTTCTTTCGTCCA-3. The amplified fragments were subcloned into PCRII vector (Invitrogen, Carlsbad, CA), and purified plasmids were linearized for RNA probe synthesis. Digoxigenin labeled RNA probes, sense (negative control for ISH) and anti-sense were transcribed in vitro with Sp6 and T7 RNA polymerases (New England Biolabs, Ipswich, MA). No signal was observed with control "sense" probes.

DAPI staining

After ISH, slides were washed in 1 x PBT (PBS plus 0.1% Triton) for 5 min and incubated with DAPI (8 µg/ml in PBT) for 5 min at room temperature (RT) followed by 2 x 5 min washes in PBT. Slides were sealed with ProLong gold anti-fade reagent (Invitrogen) and examined with a fluorescence microscope Olympus BX40 (Olympus, Center Valley, PA).

Cell membrane staining

Cell membranes were stained with wheat germ agglutinin (WGA) conjugated with Alexa fluor 633 (Invitrogen). After ISH, slides were washed in PBS for 5 min and incubated with WGA (1:200 in PBS from 1 mg/ml stock solution) for 10 min at RT, followed by 3 x 5 min washes at RT. Slides were sealed with ProLong gold anti-fade reagent (Invitrogen) and reviewed by fluorescence microscopy.

Photo modification

Acquired photos were merged as layers with Adobe Photoshop CS2 (Adobe, San Jose, CA).

Results

Detection of *Wolbachia* and *Wolbachia* gene expression by *in situ* hybridization

Large amounts of *Wolbachia* were detected in adult *B. malayi* worms by ISH with the *Wolbachia* 16S rRNA probe. *Wolbachia* were detected in the lateral cords in adult male and female worms (Figure 1, Figure 2). However, the bacteria were unevenly distributed along the cords. In some sections, *Wolbachia* were detected throughout both lateral cords (Figure 1A,Figure 2A). In others, *Wolbachia* were only observed in one cord (Figure 1C, Figure 2C) or only in some segments of one cord (Figure 1B,Figure 2B). *Wolbachia* were only observed in lateral cords in males (Figure 1), but the bacteria were also seen in oocytes and all stages of developing embryos in female worms (Figure 2).

As in adult males, *Wolbachia* were only observed in hypodermal cords in third stage infective larvae (L3) which include both sexes (Figure 3).

Wolbachia surface protein is a widely used target for immunohistological detection of *Wolbachia*. The expression pattern of wsp was somewhat different from that of *16S rRNA* gene. ISH signals for wsp were detected in lateral cords in adult male and female worms, and wsp expression was also detected in oocytes and in early embryos (morulae) in female worms. However, in contrast to *16S, wsp* was not expressed in late embryos (pretzel and stretched microfilariae stages) (Figure 4). The heme biosynthetic genes of *Wolbachia* are considered

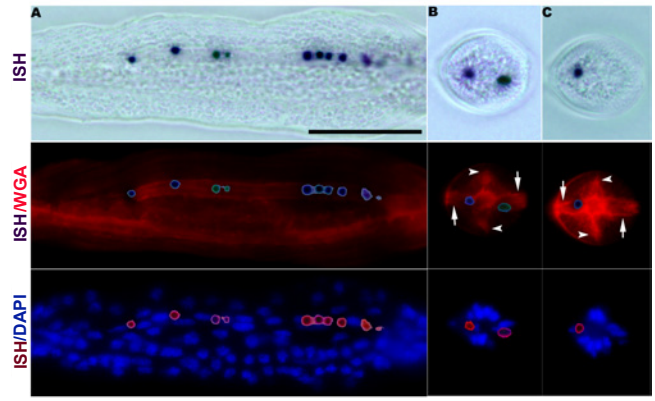

Figure 3: *Wolbachia* are located in hypodermal cords in infective third stage larvae (L3) of *Brugia malayi* by ISH. The top panel shows ISH results obtained with a 16S rRNA probe. The middle panel shows ISH labeling merged with cell membrane staining using wheat germ agglutinin (Alexa fluor 633 conjugated); the bottom panel shows ISH results merged with DAPI staining. The color of ISH staining has been replaced by red to increase contrast before merging with other layers. (A) Longitudinal section to show *Wolbachia* localization along the lateral cord; (B) cross section to show *Wolbachia* localized in two lateral cords; (C) cross section showing *Wolbachia* only present in one lateral cord. Arrows point to lateral cords, arrow heads point to dorsal and ventral cords; scale bar: 25 µm.

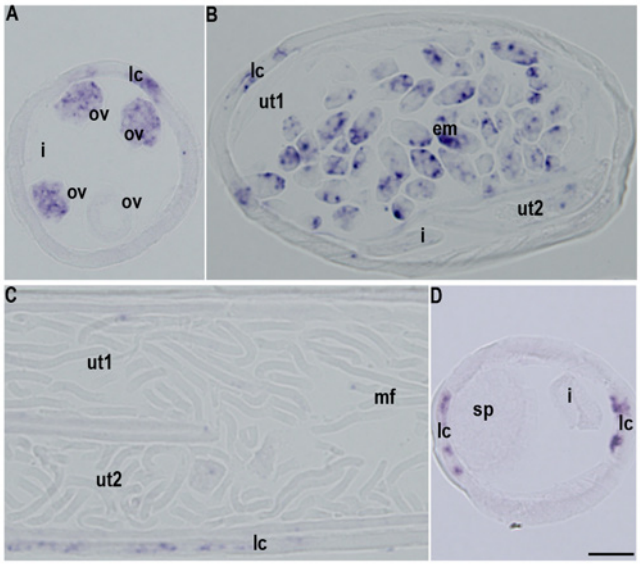

Figure 4: ISH results for expression of *Wolbachia* surface protein gene (*wsp*) in adult *Brugia malayi* worms. ISH signals were detected in both adult female (A-C) and adult male (D) worms. (A) *wsp* expression in lateral cord and some but not all developing oocytes in ovaries; (B) *wsp* expression in lateral cord and early stage embryos (morula stage); (C) lateral cords are labeled, but late stage embryos (stretched microfilariae) are not labeled; (D) only lateral cords are labeled in adult males. Abbreviations: i: intestine; lc: lateral cord; ov: ovary; ut: uterus; mf: stretched microfilariae; sp: developing sperm; em: embryo; scale bar: 20 µm.

to be essential for survival of the filarial host and present potential drug targets [16]. Expression analysis by ISH of any of the heme genes indicates a transcriptionally active pathway and could be of importance. ISH with a probe specific for *Wolbachia* hemE revealed the same expression pattern as wsp (Figure 5). Thus, *Wolbachia* modulate expression of wsp and *hemE* during embryo development.

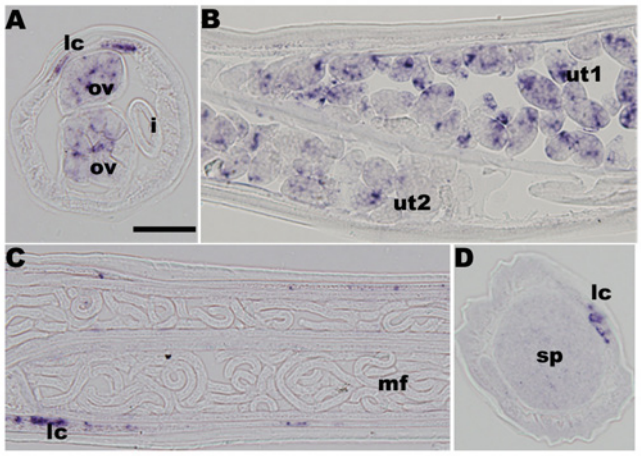

Figure 5: ISH results for expression of *Wolbachia* heme biosynthetic pathway gene (*hemE*) in adult *Brugia malayi* worms. ISH signals were detected in both adult female (A-C) and adult male (D) worms. (A) *hemE* expression in lateral cord and developing oocytes in ovaries; (B) *hemE* expression in early stage embryos (morula stage); (C) lateral cords are labeled, but late stage embryos (stretched microfilariae) are not labeled; (D) only lateral cords are labeled in adult males. Abbreviations: i: intestine; lc: lateral cord; ov: ovary; ut: uterus; mf: stretched microfilariae; sp: developing sperm; scale bar: 20 µm.

Distribution of *Wolbachia* during embryogenesis

We examined the distribution of *Wolbachia* in adult female reproductive organs by ISH with 16S rRNA probe. *Wolbachia* were diffusely present in unfertilized oocytes in ovaries (Figure 6A). In fertilized oocytes, *Wolbachia* segregate to the poles prior to the first cell division (Figure 6B). As early as the first cell division, *Wolbachia* were asymmetrically separated into precursors of the soma and germ line [14]. Early divisions after the first cell division produce early morula stage embryos as the somatic and the germ line precursors further differentiate into various cell lines [14]. In early morulae, the *Wolbachia* were asymmetrically segregated during cell division, and variable numbers of *Wolbachia* were distributed into different daughter cells (Figure 6C). However, some *Wolbachia* were still present in most cells at this time. In late morula embryos more cells lack *Wolbachia*, and the quantity of *Wolbachia* in different embryos appeared to vary (Figure 6D), although this could be an artifact related to sectioning. *Wolbachia* were absent from most cells in comma stage embryos, and the bacteria were mostly confined to cells on the edge of the embryos that probably are precursors of hypodermal cords (Figure 6E). In later pretzel stage embryos, *Wolbachia* were clearly localized in one or two longitudinal dotted lines along the edges of the embryos (Figure 6F). This pattern was also observed in mature embryos (stretched microfilariae inside adult females) (Figure 6G) and in L3 (Figure 3).

Discussion

Prior studies have used methods such as PCR, immunohistology (IH), and electron microscopy (EM) to study *Wolbachia* in filarial worms. Each method has advantages and disadvantages. PCR is simple and very sensitive, but it does not provide information on localization of *Wolbachia* in the worms. IH provides localization information, but it does not provide direct information on gene expression or viability of the bacteria. Also, some of the antibodies used for IH produce significant background staining, because they also label mitochondria (e.g. anti- HSP60, anti-GroEL) [17,18]. EM is useful for visualizing

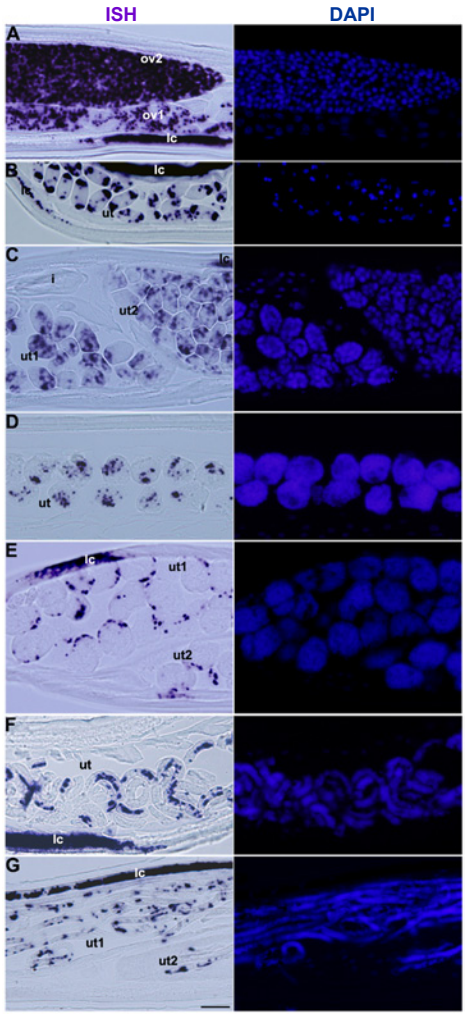

Figure 6: *Wolbachia* are asymmetrically segregated into different cells during early embryogenesis and are eliminated from all cells except hypodermal cord cells during morphogenesis. *Wolbachia* were labeled by ISH using a 16S rRNA probe (left panel), and DNA was stained with DAPI (blue, right panel). Developing oocytes in ov1 are more advanced than in ov2 and embryos in ut1 are more advanced than in ut2. (A) *Wolbachia* are present in most of the developing oocytes in ovaries; (B) *Wolbachia* moved predominantly to the poles after fertilization; (C) in early morula stage, *Wolbachia* are asymmetrically segregated in many blastomeres; (D) in the late morula stage, *Wolbachia* are present in a smaller number of cells; (E) in comma stage embryos, *Wolbachia* are absent in most cells and concentrated in hypodermal cord cells; (F) in pretzel stage embryos, *Wolbachia* were only detected in hypodermal cord cells; (G) in stretched microfilariae, *Wolbachia* were only visible in hypodermal cord cells. Abbreviations: i: intestine; lc: lateral cord; ov: ovary; ut: uterus; scale bar: 20 μm.

Wolbachia, but it is difficult to get an overview of their distribution in different parts of the worms with this technique. The present study used ISH to detect *Wolbachia* in filarial worms. ISH localized the *Wolbachia* in tissues and organs of the worms as well as IH. ISH results with a *16S rRNA* probe showed that *Wolbachia* were present in the lateral cords in adult male and female *B. malayi* worms, and in oocytes and embryos in females. These results showed that *in situ* can be used as an alternative to other methods for localizing *Wolbachia*. Our results confirm prior results obtained by IH and EM [11,13,17,19], and this consistency helped to validate the ISH method. Advantages of *in situ* are that it provides information on bacterial viability and gene expression [20]. *Wolbachia* of filarial parasites cannot be cultured or easily isolated from host tissue. Therefore, expression analysis of *Wolbachia* genes is sometimes difficult because the amount of *Wolbachia* mRNA is often very low relative to mRNA transcribed from the nuclear genes. ISH hybridization offers the opportunity to study *Wolbachia* gene expression within the bacterial cells and in relation to the infected host tissue. The ISH probes used in this study were specific for *Wolbachia*; ISH signals were not observed in *B. malayi t*issues that do not contain *Wolbachia*.

The distribution of *Wolbachia* in adult male somatic tissues (lateral cords) in *B. malayi* worms is different from their distribution in *Drosophila* and other insects. For example, *Wolbachia* are sometimes present in germline stem cells in male *Drosophilia*; they are also present in cells involved in spermatogenesis, and only discarded in elongated sperms [4]. In the wasp *Nasonia vitripennis*, *Wolbachia* are observed throughout the whole testis and present in 28% of developing spermatocytes [21].

Although, we are not able to distinguish future males from females in sections of filarial L3, we did not observe *Wolbachia* outside of the hypodermal cords in sections that sampled at least 1000 L3 larvae. This suggests that *Wolbachia* are not present in germline precursors in L3, and this would be consistent with their later absence in the reproductive system of male worms.

ISH showed that *Wolbachia* were not evenly distributed in lateral cords of adult worms. This uneven distribution of *Wolbachia* in lateral cords has also been observed by IH staining [14]. It is unknown whether *Wolbachia* can spread between syncytia of the lateral cord, although it has been suggested that *Wolbachia* may use cell fusion to populate the hypodermal cords [14].

In addition to studies with the 16S rRNA probe, we also performed ISH with a probe for the gene *wsp*. ISH results with the *wsp* probe were mostly similar to results obtained by ISH with a 16S rRNA probe and by WSP IH staining [10,17]. All of these methods label *Wolbachia* in lateral cords, oocytes and early embryos. However, unlike the 16S rRNA probe and WSP IH results, the *wsp* rRNA probe did not label late stage embryos (pretzel stage larvae and stretched microfilariae). In late stage embryos, wsp may not be actively transcribed (therefore, not detected by *wsp* ISH), but WSP proteins are still present in the cells (detectable by WSP IH). This suggests that *wsp* gene expression is down-regulated in stretched mf and that *Wolbachia* gene expression varies during embryo development. Thus, besides providing information on localization and the concentration of *Wolbachia* in parasite tissues, ISH can be used to study the expression of individual *Wolbachia* genes during embryo development and in different stages in the life cycle of the parasite.

The population dynamics of *Wolbachia* in *B. malayi* has been investigated by quantitative PCR which demonstrated changes in *Wolbachia* DNA content in different life stages of the parasite [9,10]. A recent study used IH staining to study the distribution of *Wolbachia* in early embryogenesis [14]. The present study used ISH to assess *Wolbachia* during embryogenesis in *B. malayi* worms. We found that *Wolbachia* were present in almost all developing oocytes; this result was similar to that reported for *Drosophila* (transmission rate 97%) [22-24]. ISH showed that *Wolbachia* were concentrated at the poles of fertilized oocytes. Landman et al. [14] reported that subsequent cell divisions establish the somatic blastomere AB and the germ line blastomere P1 which further divided into ABa, Abp and MS, E, C, D, P4

blastomeres, respectively [14]. Our results confirm that *Wolbachia* are asymmetrically distributed into the AB and P1 blastomeres and their descendent blastomeres.

As embryos developed to the comma stage, *Wolbachia* became concentrated on the edge of the embryos and lined up in a curved structure. In later embryos (pretzel and stretched microfilariae), *Wolbachia* were only observed along two longitudinal dotted lines that probably correspond to developing hypodermal lateral cords. *Wolbachia* were not seen in other cells in these stages. *Wolbachia* distribution patterns in pretzel stage embryos and in stretched intrauterine microfilariae were similar to those seen in L3 larvae. Therefore, *Wolbachia* seem to be eliminated from all cell types other than hypodermal cord cells as embryos develop from the comma to the pretzel stage.

We do not know how *Wolbachia* are eliminated from some cell lines. One possible mechanism is dilution during cell proliferation. During early embryogenesis, *Wolbachia* numbers are fairly constant (approximately 70 per embryo) as the number of nematode cells increases from one to more than 100 per embryo [14]. Due to the asymmetric segregation of *Wolbachia*, the bacteria become concentrated in cells of blastomere P1 origin. The mechanism of this segregation is not understood. However, dilution does not explain the absence of *Wolbachia* in germline precursor cells in late stage embryos, L3, and adult males, since germline precursor cells contain many *Wolbachia* during early embryogenesis. More research is needed on this topic.

In conclusion, filarial worms require *Wolbachia* for reproduction and development, and clinical trials have shown that *Wolbachia* represent an "Achilles' heel" for these worms. Further understanding of the *Wolbachia*-worm relationship may help us to better exploit this vulnerability. This study has contributed to this effort by demonstrating that ISH can provide interesting new information on the modulation of *Wolbachia* numbers, distribution, and gene expression in filarial worms during embryo development and across the life cycle.

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