Mini Review

Improved Methods of Microinjection in *Caenorhabditis elegans*: Automation and Microfluidic Systems

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Abstract

C. *elegans* has become a versatile model organism because of various advantages, such as being a small-sized non-parasitic worm with a transparent body and a short life cycle, along with the capacity to conduct *in vivo* live worm imaging, genetic amenability and cost-effective laboratory maintenance. It has also gained popularity because of the mapping of the developmental fate of every single cell and full genome sequencing of the worm. Further, among eukaryotic multi-cellular model organisms, C. *elegans* has the ability to generate a transgenic line in the shortest possible time compared to most other model organisms, which are being used to perform *in vivo* studies. Two methods for the transformation of C. *elegans* are widely used, namely, the microinjection of the transgene into the gonads and the bombardment of the transgene on the worm. The microinjection of C. *elegans* for the purpose of creating transgenic lines to study the effect of various genetic backgrounds is being carried out in almost every worm-based laboratory throughout the world. However, this technique of microinjection into the worm demands high technical expertise and expensive instruments, while the technique itself is low throughput. Until up to three years ago, the microinjection technique remained more or less same. This review focuses on recent advancements in relation to the microinjection technique, which involves the addition of automation via a computer-assisted system and the involvement of biomicrofluidic systems in order to increase the efficiency and rate of microinjection, thereby reducing fatigue experienced by the researcher when developing transgenic lines of C. *elegans*.

ABBREVIATIONS

C. elegans: *Caenorhabditis elegans*; CAMI: Computer-Assisted Microinjection; DIC: Differential Interference Contrast; CS: Cross Section

INTRODUCTION

A nematode worm known as *C. elegans* has become a very versatile animal model for biological research from the time it was first used by Sydney Brenner during the second half of the 20th century to study genetics, animal behavior and development [1,2]. Furthermore, the worm has become a popular model to carry out various genetic, developmental, pathological, neurological, toxicological and aging research studies with its full genome sequenced, developmental cell fate mapped, and several libraries and mutants available [3]. Genetic modification of a model organism is a popular methodology to understand various aspects of biology and disease in a particular genotype by generating either a knock-out mutant or knock-in transgenic lines. One of the techniques used to generate transgenic lines and to alter the genome of an organism is needle microinjection [4-8]. From the time that needle microinjection was introduced by Félix Dujardin and M.A. Barber, the techniques of microinjection have gained popularity in numerous field of biology from basic to clinical research [9-11].

Bombardment is yet another technique for the generation of transgenic lines, in which worms are shot at with genetic material bound to gold particles from a gene gun (biolistic bombardment machine). Unlike needle microinjection, bombardment can often produce integrated transgenic lines with a low copy number. However, bombardment demands more labor intensive preparation of worms, along with relatively more expensive instruments and materials than needle microinjection [8,12-14]. More comparison between the two techniques can be found in [15].

The conventional method of microinjection of *C. elegans* only differs slightly from that of cultured cells or oocytes. Several

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optical, analytical and digital instruments are required to carry out a successful microinjection procedure. An ample amount of literature is available to understand the basic microinjection procedure and the basic instruments required for this procedure, which can be found in [8,11]. Briefly, the worm is mounted onto a 2% agarose pad (the absorption of moisture content in the worm by the dry agarose pad develops an adhesive force, which immobilizes the worm on the agarose pad; as the agarose pad continuously desiccates the worm, the procedure has to be completed within few minutes of the immobilization of the worm [8]) by immersing in a drop of oil (this prevents the fast dehydration of the worm's moisture to the surrounding air medium by evaporation), after which the worm is rotated to clearly visualize the gonads. Next, the worm is taken to the stage of an inverted microscope, aligned in an appropriate position against the needle. Using a micromanipulator, the needle is brought to the focal plane of the worm and the injection of several picoliters of reagent is performed using the injection system, followed by the recovery of the worm in an appropriate buffer [8]. This procedure demands a great deal of expertise to accurately actuate the needle such that the injection causes the least possible amount of damage to the worm. The current rate of microinjection in the traditional way is ~3-4 min per worm [3,16], which causes the researcher severe fatigue inorder to microinject a sufficient number of worms or multiple strains per day to obtain a transgenic lines. An experienced technician can produce an average of ~>3 independent transgenic lines upon microinjection of 10-50 gonads [5,8]. However, the efficiency and the success rate depend on several factors, such as the expertise level of the technician, the number of gonads being injected, the type of co-injection markers used, the method of screening the transgenic lines and toxicity of injection materials. Further, gaining a proper understanding of the microinjection technique and obtaining the expertise to carry out the technique may take several months for beginners.

Conventional *C. elegans* microinjection has not changed much since its introduction and the rate of microinjection has remained more or less the same. Therefore, it is necessary to develop automation processes in order to ease technical fatigue and increase the efficiency of the experiment. One event worth mentioning is the collaboration between material science, microfluidics and biology to develop biomicrofluidic devices, which have accelerated various aspect of biological research in the last decade. Microfluidic devices can regulate the fluid properties in micro-or nano-environments using simple mechanical or computer-controlled units, thereby providing greater control over the micro-environments used for any type of scientific analysis. Information on designing and fabricating biomicrofluidic devices, which are cost effective, can be found in [17-19].

This review article explains the latest automation strategies and microfluidic devices, which have been developed for the microinjection of *C. elegans,* thereby making this technique a high throughput technique.

Automation of microinjection for *C. elegans*

In order to increase the rate of microinjection of the worm to create transgenes and to reduce the fatigue felt by technicians,

Gilleland *et al.* (2015), recently developed a CAMI system [3]. This study replaced the traditional use of an agarose pad and oil to prevent the mobilization and fast desiccation of the worm, respectively, along with temperature-sensitive Pluronic F-127 hydrogel (25%) and sodium azide (10 mM) to immobilize the worm on a glass-bottomed multi-well plate. A change in the temperature of Pluronic F-127 hydrogel (from 15℃ to 25℃) will solidify the gel, resulting in complete immobilization of the worm, while the use of sodium azide anesthesia will provide a straightened worm. Thus, the prepared worm is mounted onto a microscope stage, whose temperature is regulated, while a computer-assisted imaging system is used to locate the position of the worm and its gonads, with microinjection performed using a micromanipulator and piezoelectric vibrator. (Figures 1,2) offer a pictorial representation of the automation methodology and successful microinjection of *C. elegans*. CAMI can work at a rate of ~25 s per target gonad. Further, the assessment of the success rate of CAMI showed improved results when compared to the conventional microinjection method, along with reduced fatigue and reduced experimentation time. Thus, Gilleland *et al.* (2015), improved the traditional microinjection system in worms with the introduction of an automated system, thereby increasing the rate of microinjection and the rate of success (Figure 1,2).

Microfluidic devices for microinjection

Zhao *et al.* (2013), developed a semi-open microfluidic device for the microinjection of various chemicals and proteins into a single intestinal cell of *C. elegans* in order to study cell-
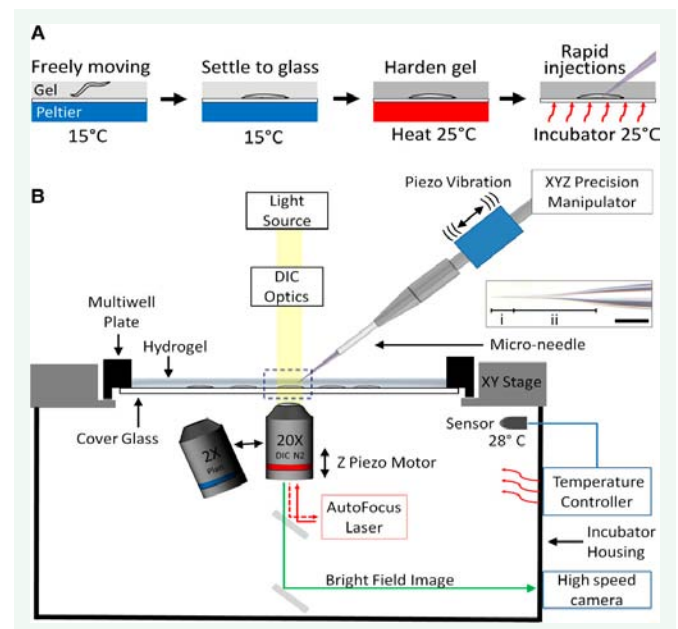

Figure 1 Pictorial representation of automated microinjection developed by Gilleland *et al.* (2015). A) The process of immobilizing the worm by the solidification of Pluronic F-127 and the subsequent microinjection of *C. elegans*. B) A schematic representation of the instrumentation set-up of the CAMI system. The dashed box shows the worm being microinjected, while the inlet of the microinjection needle shows the microinjecting region (i) and the tapering region of the microneedle (ii). The figure is reprinted from Gilleland *et al.* (2015), with permission from the Genetic Society of America.
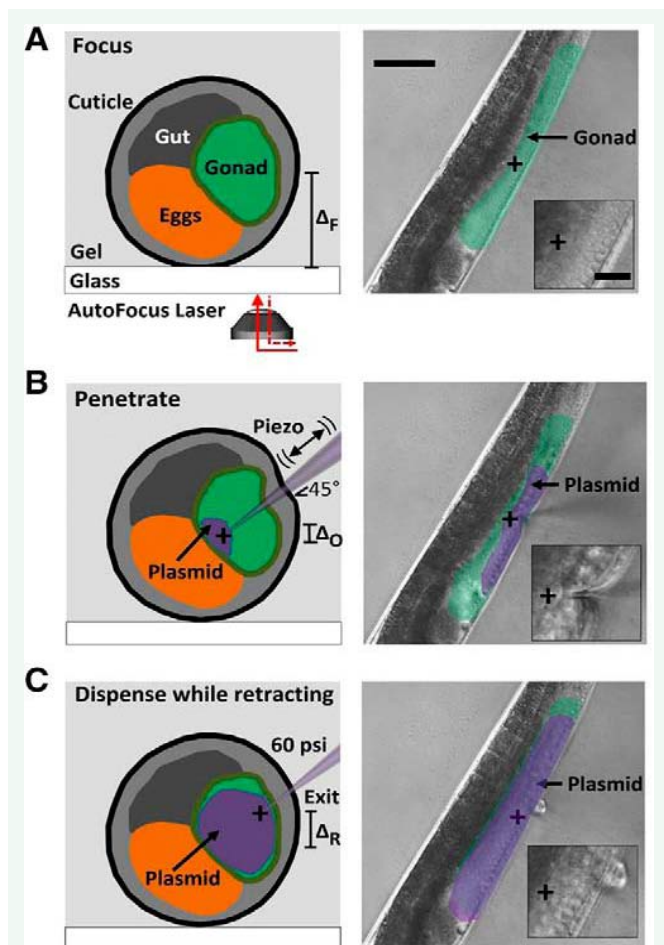
Figure 2 Steps involved in the microinjection of C. elegans gonads using the CAMI system developed by Gilleland *et al.* (2015). The left side offers a graphical representation and the right side shows bright-field DIC image using 20X objective lens. A) Apictorial representation of the CS of worm with different inner organs, in which ΔF represents the distance between the gonads and the glass slide (left); the DIC image of the target region is indicated by a cross, with the gonads marked with a green color (right), while the inlet shows a magnified region with a typical gonadal phenotype. B) The injection of the gonad at a 45° angle using piezo vibration with 5 μm (ΔO) of penetration depth; the injection solution is represented by a purple color. C) The successful microinjection of worm gonads with appropriate pressure, in which the needle retraction distance is 5 μm (ΔR), and the minor damage caused by microinjection. The figure is reprinted from Gilleland *et al.* (2015), with permission from the Genetic Society of America.

cell communication (Figure 3) [20]. This device has several channels and an open chamber. The inlet channel allows the worm to travel near to the suction channels, whose flow is regulated by a buffer channel, and become immobilized by the suction. The open channel provides a space for the microinjection needle's movement in multiple directions, thereby enabling the microinjection of target molecules into the particular region of the worm. The worm can be imaged at the same time, either immediately after microinjection or the worm is recovered in the outlet channel (Figure 4). Although this device can be used for the microinjection of various chemicals into a single cell and imaging, this device was not examined for its efficiency concerning the

generation of transgenic lines by the microinjection of the gonads of *C. elegans*. Based on the design of the biomicrofluidic system, this device will probably provide successful transgenic lines upon the microinjection of appropriate genetic material into the gonads of the immobilized worm. Further, the method for the immobilization of the worm used in Zhao *et al.*(2013), should reduce the damage caused to the worm, compared to the conventional method of microinjection, thereby providing efficient worm handling (Figure 3,4).

Another simple microfluidic system has been reported, where the passive immobilization of the worm takes place in a narrow channel, while the microinjection of worm gonads is carried out by a single degree of the freedom of movement of the microinjection needle, which is integrated into the microfluidic system. The rate of microinjection of a single worm in this system is reported to be around ~25 s per worm [21]. One of the greatest advantages of this device is that it does not require a high level
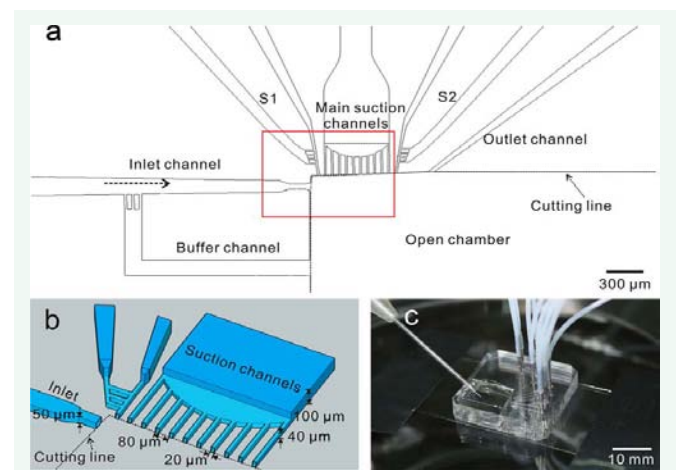

Figure 3 Microfluidic channel designed by Zhao *et al.* (2013). a) A graphical representation of the device designed by Zhao *et al.* (2013) with different regions and channels in the device. b) A zoomed-in depiction of the area contained in the red box in (a), with appropriate dimensions and measurements. c) A photo of a microfluidic chip fabricated by Zhao *et al.* (2013). The figure is reprinted from Zhao *et al.* (2013), with permission from Elsevier.
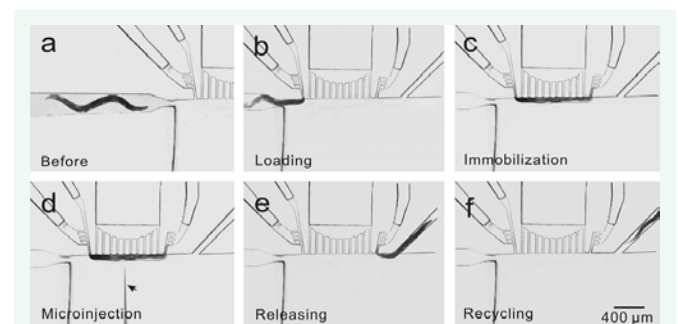

Figure 4 Various stages involved in the microinjection of worms using a microfluidic chip developed by Zhao *et al.* (2013). (a-f) the step-by-step inflow of worms into the chip, and the immobilization, microinjection and recovery of worms. The figure is reprinted from Zhao *et al.* (2013), with permission from Elsevier.
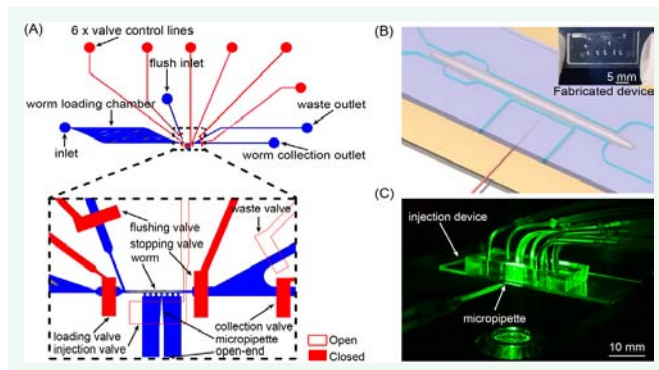
Figure 5 Microfluidic device developed by Song *et al.* (2016). A) Different types of valves in the upper fluid chamber layer (blue) and lower pneumatic valve layer (red); the dotted box shows the channels from the area in which microinjection is performed. B) A pictorial representation of the zoomed-in area of microinjection. C) A photo of the microfluidic chip fabricated by Song *et al.* (2016). The figure is reprinted from Song *et al.* (2016), with permission from AIP Publishing LLC.
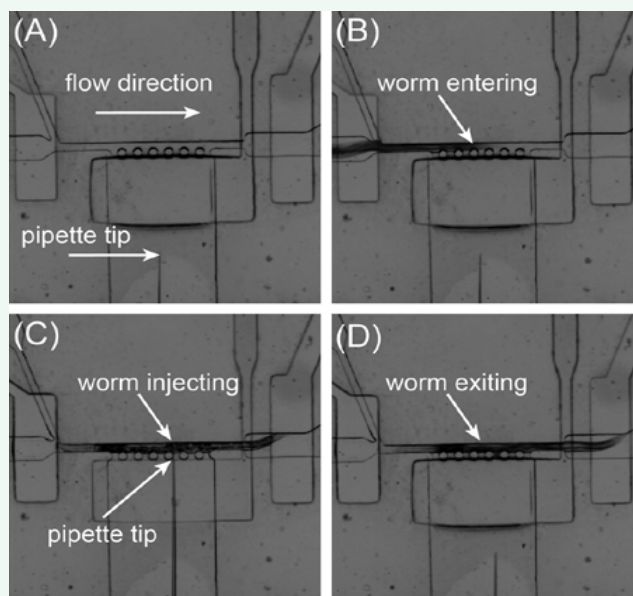

Figure 6 Various stages involved in the microinjection of a worm using the device developed by Song *et al.* (2016). A) The microinjection chamber and the direction of the flow of fluids. B) A worm entering into the immobilization channel. C) The injection of the immobilized worm. D) The removal of the worm after successful microinjection. The figure is reprinted from Song *et al.* (2016), with permission from AIP Publishing LLC.

of expertise with regard to microinjection technique when using this system since the injection microneedle has a restricted (i.e., single) degree of the freedom of movement, making it accessible to beginners.

Automation combined with microfluidic systems

A new microfluidic device system, in combination with an automated computerized algorithm, was developed by Song *et al.* (2016), [16,22], thus predisposing the microinjection technique

from a low throughput to a high throughput technique. The microfluidic device is a two-layer device, where the upper layer (fluid channel layer, blue - see (Figure 5)) is used for loading and mobilizing the worm into immobilization chamber, as well as for microinjection and recovery of the worm, whereas the lower layer (pneumatic valve layer, red - see (Figure 5)) is used to regulate the fluid flow in the upper chamber. The microfluidic device and other auxiliary units required for microinjection were connected to a robotic system in order (i) to regulate the movement and positioning of worms, (ii) to run an imaging algorithm to analyze the worm-loading status and to position the microinjection needle, and (iii) to control the process of injection into the worm. This automated system, combined with a microfluidic chip, attained a rate of ~9.5 s per worm, resulting in a successful high throughput microinjection of *C. elegans*. Further, analysis of microinjected worms for potential damage, caused by the injection procedure using physiological parameters and survival rate, showed no significant different between the worms subjected to the injection procedure and controls (Figure 5,6).

DISCUSSION AND CONCLUSION

It can be observed that the development of automation and microfluidic chips to ease the complex procedure of the microinjection of the nematode worm *C. elegans* has only gained momentum in the past five years. Further, it can be noted that the number of microfluidic devices and automations available for various types of worm studies, such as immobilization and microscopy, behavior analyses, metabolomics studies, microsurgery, drug screening and worm sorting [23,24], is greater compared to the available devices for microinjection. Thus, it can be said that updates to the already existing devices and the development of new devices for the purpose of efficient microinjection will be a primary focus among researchers in the upcoming years. Although the above-mentioned devices have increased the efficiency and rate of injection that can be performed, from a few to several hundreds of worms, yet more efficient methods for the development of transgenic lines in worms are anticipated by worm research communities. This can be achieved by designing complex automation methodologies or by developing new strategies, which involve viral-induced infection and the integration of transgenes into a worm's gonadal oocyte genome, thereby increasing efficiency in the development of transgenic worm lines.

However, it is also disheartening to highlight the fact that the commercial availability of microfluidic devices to enhance worm research is virtually nil [23]. Therefore, an important collaboration between scientists and industry should be encouraged in order to make devices commercially available, which will fulfill the needs of the rapidly growing worm laboratories around the world.

REFERENCES

1. Riddle DL, Blumenthal T, Meyer BJ, Priess JR. (1997) Section I: The biological model. In *C. Elegans* II (Second Edition). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

2. Brenner S. The genetics of behaviour. British Medical Bulletin. 1973; 29: 269-271.

3. Gilleland CL, Falls AT, Noraky J, Heiman MG, Yanik, M.F. Computer-assisted transgenesis of *Caenorhabditis elegans* for deep phenotyping.

Genetics. 2015; 201: 39-46.

4. Kimble J, J Hodgkin, T Smith, J Smith. Suppression of an amber mutation by microinjection of suppressor tRNA in *C. elegans*. Nature. 1982; 299: 456-458.

5. Stinchcomb DT, Shaw JE, Carr SH, Hirsh D. Extrachromosomal DNA transformation of *Caenorhabditis elegans*. Mol Cell Biol. 1985; 5: 3484-3496.

6. Fire A. Integrative transformation of *Caenorhabditis elegans*. EMBO J. 1985; 5: 2673-2680.

7. Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 1991; 10: 3959-3970.

8. Evans TC. Transformation and microinjection. Worm Book. The Online review of *C. elegans* Biology. 2006.

9. Barber MA. A technic for the inoculation of bacteria and other substances into living cells. J Infect Dis. 1911; 8: 348-360.

10. Feramisco J, Perona R, Lacal JC. Needle microinjection: A brief history. In J Feramisco, R Perona, JC Lacal (eds.) Microinjection. Springer: Basel. 9-15.

11. Shanmugam MM, Santra TS. Microinjection for single-cell analysis. In TS. Santra, FG. Tseng (eds.) Essentials of Single-cell Analysis (First Edition). Springer-Verlag: Berlin Heidelberg. 2016; 85-130.

12. Wilm T, Demel P, Koop HU, Schnabel H, Schnabel R. Ballistic transformation of *Caenorhabditis elegans*. Gene. 1999; 229: 31-35.

13. Praitis V, Casey E, Collar D, Austin J. Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. Genetics. 2001; 157: 1217-1226.

14. Berezikov E, Bargmann CI, Plasterk RH. Homologous gene targeting in *Caenorhabditis elegans* by biolistic transformation. Nucleic Acids Res.

2004; 32: 40.

15. Rieckher M, Kourtis N, Pasparaki A, Tavernarakis N. Transgenesis in *Caenorhabditis elegans*. Methods Mol Biol. 2009; 561: 21-39.

16. Song P, Dong X, Liu X. A microfluidic device for automated, high-speed microinjection of *Caenorhabditis elegans*. Biomicrofluidics. 2016; 10: 011912.

17. Zhang M, Wu J, Wang L, Xiao K, Wen W. A simple method for fabricating multi-layer PDMS structures for 3D microfluidic chips. Lab Chip. 2010; 10: 1199-1203.

18. Ren K, Zhou J, Wu H. Materials for microfluidic chip fabrication. Acc Chem Res. 2013; 46: 2396-2406.

19. Fujii T. PDMS-based microfluidic devices for biomedical applications. Microelectronic Engineering. 2002; 61-62: 907-914.

20. Zhao X, Xu F, Tang L, Du W, Feng X, Liu BF. Microfluidic chip-based *C. elegans* microinjection system for investigating cell-cell communication in vivo. Biosensors and Bioelectronics. 2013; 50: 28-34.

21. Ghaemi R,Tong J, Ravi Selvaganapathy P, Gupta BP. Microfluidic device for microinjection of *Caenorhabditis elegans*. In 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences. Freiburg, Germany. 2013.

22. Dong X, Song P, Liu X. An automated robotic system for high-speed microinjection of *Caenorhabditis elegans*. In IEEE International conference on robotics and automation (ICRA). Seattle, WA, USA. 2015.

23. Muthaiyan Shanmugam M, Subhra Santra T. Microfluidic devices in advanced *Caenorhabditis elegans* research. Molecules. 2016; 21.

24. Gupta B, Rezai P. Microfluidic approaches for manipulating, imaging, and screening *C. elegans*. Micromachines. 2016; 7: 123.