Video Article

A Microfluidic Platform for Longitudinal Imaging in *Caenorhabditis elegans*

Kyung Suk Lee[1,2], Erel Levine[2,3]

[1]Department of Physics Education, Kongju National University

[2]Department of Physics, Harvard University

[3]FAS Center for Systems Biology, Harvard University

Correspondence to: Erel Levine at elevine@fas.harvard.edu

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Abstract

In the last decade, microfluidic techniques have been applied to study small animals, including the nematode *Caenorhabditis elegans*, and have proved useful as a convenient live imaging platform providing capabilities for precise control of experimental conditions in real time. In this article, we demonstrate live imaging of individual worms employing WormSpa, a previously-published custom microfluidic device. In the device, multiple worms are individually confined to separate chambers, allowing multiplexed longitudinal surveillance of various biological processes. To illustrate the capability, we performed proof-of-principle experiments in which worms were infected in the device with pathogenic bacteria, and the dynamics of expression of immune response genes and egg laying were monitored continuously in individual animals. The simple design and operation of this device make it suitable for users with no previous experience with microfluidic-based experiments. We propose that this approach will be useful for many researchers interested in longitudinal observations of biological processes under well-defined conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57348/

Introduction

Changes in environmental conditions may lead to activation of genetic programs accompanied by induction and repression of the expression of specific genes[1,2]. These kinetic changes may be variable among tissues in the same animals and between different animals. Studies of such genetic programs therefore call for methods that allow longitudinal imaging of individual animals and provide precise dynamical control of environmental conditions.

In recent years, microfabricated fluidic devices have been used to study many aspects of response and behavior in small animals, including worms, flies, water bears and more[3,4,5,6,7]. Applications include, for example, deep phenotyping, optogenetic recording of neuronal activity in response to chemical stimuli, and tracking of motor behaviors such as locomotion and pumping[8,9,10,11].

Microfluidic-based approaches hold many properties that could benefit long-term longitudinal imaging of response to environmental cues, including precise dynamical control of the local microenvironment, flexible design that allows maintenance of individual animals in separate quarters, and favorable attributes for imaging. However, maintaining animals in a microfluidic chamber for a long time with minimal adverse impact on their well-beings is a challenge, which requires particular care in the design of the microfluidic device as well as in the execution of the experiment.

Here we demonstrate the use of WormSpa, a microfluidic device for longitudinal imaging of *Caenorhabditis elegans*.[5] Individual worms are confined in chambers. A constant low flow of liquid and bacterial suspension guarantees that worms are well-fed and sufficiently active to maintain good health and alleviate stress, and the structure of the chambers allows worms to lay eggs. The simplicity of the design and operation of WormSpa should allow researchers with no previous experience in microfluidics to incorporate this device into their own research plans.

Protocol

The protocol below uses WormSpa[5], a previously-described microfluidic device for longitudinal imaging of worms. Fabrication of WormSpa (starting with CAD files that can be obtained from the authors upon request) is straightforward but requires some expertise. In most cases, fabrication can readily be done by a core facility or by a commercial company that provides such services. When fabricating the device, make sure to specify that the height of the features is 50 μm.

1. Experimental Setup

1. Mount the microfluidic device on an inverted fluorescence microscope with a motorized stage.
2. Place an orbital shaker close to the microscope, but make sure the vibration from the shaker does not directly affect the microscope. For example, put the shaker on a shelf fixed on a wall that makes no contact with the optical table.
3. Place a syringe pump on the orbital shaker. Tape the pump to the shaker so that it does not jiggle while shaking.

2. Preparation of the Microfluidic Environment

NOTE: During the experiment, four syringes are connected to the microfluidic device via syringe tubes, as outlined in **Figure 1**. This step describes the preparation of these syringes. Unless mentioned otherwise, keep the syringe tubes as short as possible without making them taut.

1. Prepare an outlet syringe and a syringe tube. This syringe (S1 in **Figure 1**) will later be connected to the outlet of the device through the syringe tube, and used for temporary storage of fluid coming out of the device.
    1. Make an adapter needle by removing plastic parts of a 20 G x 1/2 inch syringe tip, keeping only the needle (the metal part). Do so by holding the plastic and metal parts with pliers and pulling them apart. Remaining plastic residue on the needle can be burnt off with a Bunsen burner.
    2. Bend the needle according to the geometry of the experimental setup while holding its two ends with pliers. In the design described here, bending the needle in its middle to an angle of ~110° is most convenient.
    3. Plug the adapter needle half way into the tube. The other half will later be plugged into the device. The syringe tube should be compatible with a 20 G syringe tip without a leak (for example, tubing with inner diameter of 0.86 mm and outer diameter of 1.32 mm). The recommended tube length is ~50 cm.
    4. Connect a 10 mL luer-lock syringe to the other (open) end of the syringe tube via a 20 G x 1/2 inch syringe tip.

2. Prepare buffer-inlet syringes and syringe tubes. One of these syringes (S2 in **Figure 1**) is used as a reservoir for the fluid that goes into the device and to control the injection flow. The other syringe (S3 in **Figure 1**) is required for buffer exchange, as explained in Step 3.2.
    1. Connect a 10 mL luer-lock syringe (S2) directly to a 3-way valve and connect another syringe (S3) to the same valve via a syringe tube (**Figure 1**): connect S3 to the tube via a syringe tip, and connect the tube to the valve via another syringe tip.
    NOTE: The order in which these materials are connected does not matter.
    2. Connect a syringe tube to the remaining port of the 3-way valve. Prepare another adapter needle as in Step 2.1.2, and plug the needle half-way into the other (open) end of the syringe tube.
    3. Set the valve such that the syringe tube in Step 2.2.2 and S2 are connected while S3 is closed (**Figure 1**).

3. Prepare a worm-inlet syringe and a syringe tube. This syringe (S4 in **Figure 1**) is used for loading worms into the device.
    1. Connect a long syringe tube to a 10 mL luer-lock syringe (S4). Determine the length of this tube from the volume of the fluid that will be used for loading; for example, 100 cm of tubing supports over 2 mL of liquid (also, see step 4.2).
    2. Prepare another adapter needle as in Step 2.1.2, and plug one half of the needle to the other (open) end of the syringe tube.

4. Rinse all the syringes and tubing with S-medium[12] twice. Fill the syringes and the tubes with S-medium, taking care to remove all air bubbles.
5. Connect the outlet syringe tube to the device outlet.
    1. Plug in the adapter needle at the end of the syringe tube to the outlet port.
    2. Inject a small volume of S-medium through the outlet to fill up the device, until a little S-medium comes out of both the buffer inlet and the worm inlet.

6. Connect the buffer-inlet syringe tube to the buffer-inlet port of the device, while plugging the worm-inlet with a pin (stainless steel dowel pin with a 1/32-inch diameter).
7. Inject a constant flow of buffer into the device. Load the buffer-inlet syringe S2 onto a syringe pump[13], and set the pump such that the medium in S2 is continuously injected into the device at a flow rate of 3 µL/min.

3. Loading Bacteria into the Microfluidic Device

1. Prepare *Escherichia coli* OP50 suspended in S-medium[12].
    1. Following a standard protocol, inoculate a 250 mL flask with 50 mL of LB and a single colony, and incubate overnight at 37 °C.
    2. Centrifuge the overnight culture at 4000 rpm for 10 minutes and resuspend the pellet in 30 mL of S-medium.
    3. Filter the suspension through a 5 µm syringe filter. Measure the bacterial concentration by its optical density at 600 nm (OD_{600}), and adjust the concentration to an OD_{600} of 3. This high density is required to keep the worms well-fed.

2. Exchange the buffer in the device with the OP50 suspension.
    1. Prepare a clean syringe (S5) filled with the OP50 suspension. Hold the syringe vertically and tap it several times to collect all the air bubbles at the top.
    2. Turn the 3-way valve of the buffer-inlet syringes to close the connection to the device, and connect the two syringes, S2 and S3. Disengage S2 from the syringe pump.
    3. Disconnect S2 from the valve and connect S5 to the valve. Push out all the air collected at the top of S5 to S3 through the valve until a little bit of the OP50 buffer goes into S3. In doing so, make sure that no air bubble remains in S5 or the valve.
    4. Turn the 3-way valve back to the original position to close the connection between S3 and S5 and connect S5 to the device. Load S5 onto the syringe pump. Turn on the orbital shaker that holds the pump. Set the shaking speed to approximately 200 rpm.
    5. Set the flow rate to be 100 µL/min. The time it takes for the new buffer to arrive to the worms in the device depends on the length of the buffer-inlet syringe tube (around 5 minutes with the tubing described above). A higher flow rate can be employed if a quicker buffer exchange is required.

6. Once the OP50 buffer spreads throughout the device, set the flow rate back to 3 µL/min.

4. Loading Worms into the Microfluidic Device

1. Prepare a small low binding microcentrifuge tube (650 µL) and fill it with 100 µL of OP50 suspension. Transfer around 20 - 30 age-synchronized young adult worms (46 hours post L1 larval arrest at 25 °C) from an NGM (nematode growth medium) agar plate to the tube by picking them one by one with a worm pick. Age-synchronization can be done following a standard protocol[12].
2. Fill the worm-inlet syringe and syringe tube (S4 in **Figure 1**) with the OP50 suspension. Inject ~500 µL of the fluid into the microcentrifuge tube with worms. Draw the suspension with worms into the worm-inlet syringe tube. Make sure that the syringe tube is long enough (>1 m) and that drawn worms stay in the syringe tube and do not make all the way to the syringe S4.
3. Connect S4 to the worm-inlet. Inject all the worms in the worm-inlet syringe tube into the microfluidic device. Disconnect S4 and the syringe tube. Plug the worm-inlet with a pin.
4. Disengage the buffer-inlet syringe S2 from the mechanical pump (**Figure 1**) and manually control S1 and S2 to push all the worms into separate channels. Pressing S2 will move worms into the channels, while pressing S1 will move them in the reverse direction. Once a channel is loaded, the worm in each channel will block the entry of other worms.
5. Let worms adjust to the new environment for 2 - 3 hours.

5. Setting Up an Imaging Protocol

1. Find all worms that are securely located in the device, and set an imaging position for each of them in the image acquisition software that controls your microscope. Note that in some cases (*e.g.* when higher magnification is desired, or when using cameras with a smaller CCD sensor) multiple imaging positions are required for each channel.
   NOTE: While this can be done manually, some acquisition software packages can load a text file that lists the positions where images should be taken. Since these positions are ordered regularly in WormSpa, a user can write a small script (*e.g.,* in Python or commercial software) that automatically creates such a list. The precise format of this script would depend on the file format expected by the acquisition software (see an example in **Supplementary Material 1**).
2. Set the required frequency of time-lapse imaging. For example, imaging of immune gene response to infection is done at a frequency of 10 minutes per frame. Make sure that all necessary channels (*e.g.* bright-field and the GFP fluorescence) are acquired at every time point.

6. Host Response to Pseudomonas Infection

Note: This step is specific for studying host-pathogen interactions. Alternatively, one can prepare a buffer that contains other environmental cues of interest (biotic and abiotic stressors, drugs, signaling molecules, *etc.*).

1. Prepare *Pseudomonas aeruginosa* PA14 suspended in SK-medium[14].
   1. Inoculate a 250 mL flask with 50 mL of LB and a single colony, and incubate overnight at 37 °C.
   2. Inoculate another 250 mL flask with 50 mL of LB and an aliquot of the culture, and incubate overnight at 37 °C.
   3. Centrifuge the overnight culture at 4000 rpm for 10 minutes and resuspend the pellet in 10 mL of SK-medium. Measure the bacterial concentration and adjust the concentration to OD_{600} = 4.
   4. Transfer the suspension to a clean flask, and incubate at 37 °C for 24 hours and at 25 °C for another 24 hours while shaking. This step mimics the plate-incubation step in the standard killing assay[14].
   5. Filter the suspension through a 5 µm syringe filter. This is to prevent bacterial aggregates from clogging the device.
2. Replace the OP50 suspension in the device with the PA14 suspension, following the same procedure as in Step 3.2. Keep imaging for 10 hours.

Representative Results

Age-synchronized young adult worms (46 hours post L1 larval arrest at 25 °C)[12] were loaded in the device, as described in the protocol. The worms were individually located in separate channels, enabling longitudinal measurement of animals' response to the pathogen. When the experiment is successful, most worms remain in their channels for the duration of the experiment. In this case, images of individual worms are taken simply by placing their channel in the imaging path, avoiding the need to actively locate the animal. **Figure 2A** shows the bright-field images of a single representative worm over the course of 10 hours in the device. While worms are confined within their channels, they can move up- and downstream, and can freely turn their heads. This is critical to guarantee that the device itself does not cause stress or harm the animals.

We monitored response of worms to bacterial infection by *P. aeruginosa*, one of the best-studied bacterial pathogens of *C. elegans*. It is also an opportunistic human pathogen which causes infection in cystic fibrosis and immunocompromised patients. Feeding worms with particular strains of *P. aeruginosa*, such as the clinical isolate PA14, leads to lethal intestinal infection, and the virulence factors involved in this process are also implicated in the infection of mammalian hosts.[14,15]

To examine the pattern of changes in gene expression during bacterial infection, fluorescent reporters can be employed. Among many genes that display dynamic response to infection, we monitored the transcriptional response of *irg-1* (infection response gene 1), by imaging GFP fluorescence from transgenic worms expressing *irg-1::GFP* (AU0133 [*agIs17(pirg-1::GFP; pmyo-2::mCherry)*], obtained from the Ausubel lab). *irg-1* is known to be strongly induced in the intestine of infected animals at the early stage of infection by *P. aeruginosa* PA14.[16,17] **Figure 2B** shows time lapse images of a representative worm (the same animal as in **Figure 2A**). Each fluorescence image was taken immediately after the corresponding bright-field image. In the first 5 hours post infection, GFP fluorescence increases only mildly in the mid body and the tail. Then, a substantial increase in fluorescence is observed, starting from the mid body. The total fluorescence in each worm is plotted in **Figure 2C** as a function of time post infection. These data illustrate not only the previously-described induction of *irg-1* upon PA14 infection, but also the worm-to-worm variability in the timing and extent of induction.

The design of the WormSpa microfluidic device includes filters which collect the eggs laid by each individual worm[5]. Once eggs hatch, the L1 larvae are washed through the filter and into the outlet tube. This allowed us to manually monitor the egg-laying kinetics of individual worms during bacterial infection. The time lapse images of the eggs laid by the same worm as in **Figure 2** are shown in **Figure 3A**. These images were taken right after the corresponding bright-field images in **Figure 2A**. The cumulative number of eggs laid by each worm is shown as a function of time post infection in **Figure 3B**, and the average and standard deviation over the entire population are shown in **Figure 3C**. These data capture the animal-to-animal variability as well as the previously-described decline in egg-laying as infection progresses. In contrast, in uninfected animals egg-laying rate does not decline until it peaks at ~54 hours post L1 at 25 °C[5,18].

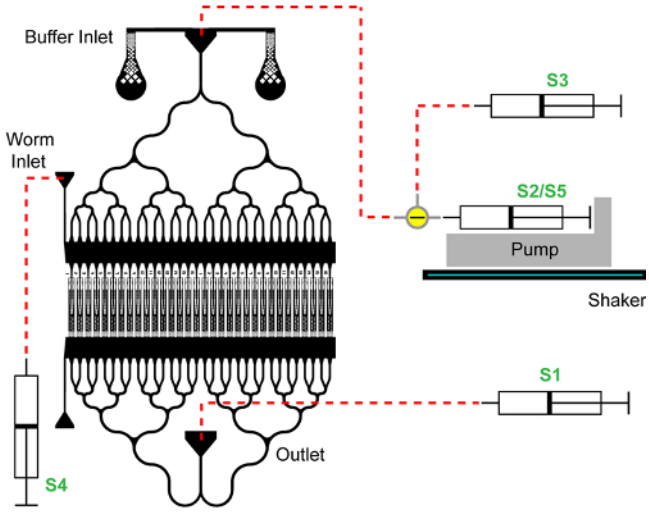


Figure 1: **Experimental scheme of live imaging of worms in a microfluidic device.** Worms are loaded into the device via the *worm inlet*, and are subsequently pushed into separate channels. Bacterial suspension is injected into the device from the *buffer inlet* by a mechanical pump mounted on an orbital shaker. The buffer exits through the *outlet port* of the device. Four syringes (S1, S2/S5, S3, and S4) are used to operate the device. The red dashed lines are syringe tubes connecting syringes and the device. Please click here to view a larger version of this figure.

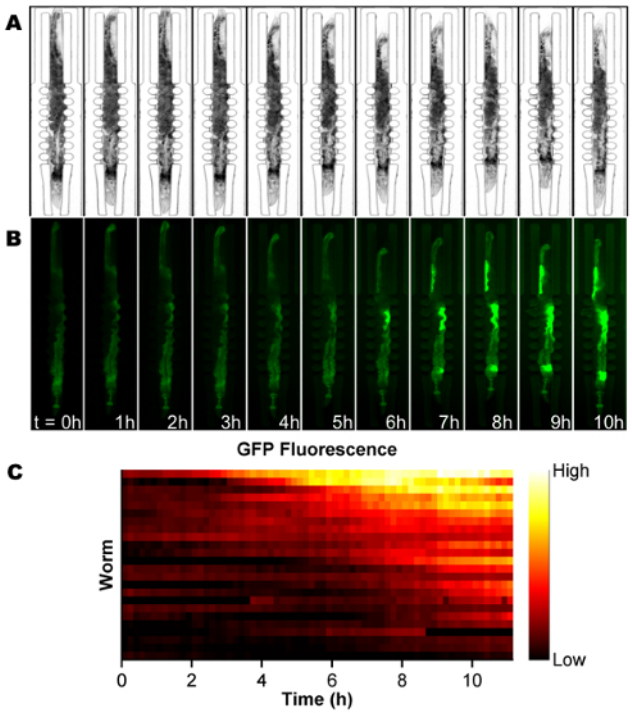


Figure 2: **Representative gene expression kinetics.** Time lapse images of a representative worm taken by **(A)** Bright-field and **(B)** GFP fluorescence microscopy. The leftmost images were taken just before the pathogen was introduced, whereas the rightmost images were taken 10 hours post infection by PA14. The time interval between images is 1 hour. **(C)** The time course of total *irg-1*::GFP fluorescence in individual worms. Each row corresponds to a single animal. Rows were sorted in descending order of mean fluorescence. Fluorescence intensity is color-coded and the color scale is shown on the right. Please click here to view a larger version of this figure.

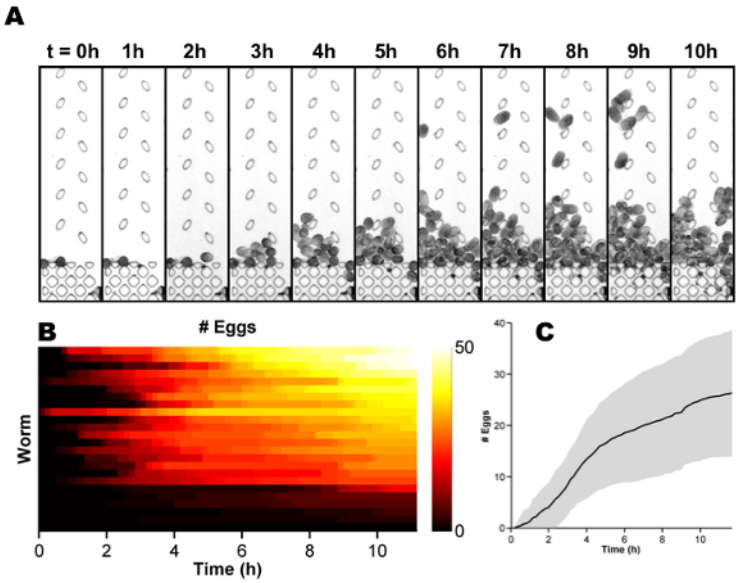
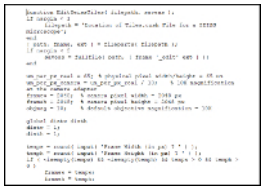


Figure 3: **Representative egg-laying kinetics. (A)** Time lapse images of the number of eggs laid by the same worm shown in **Figure 2A** and **B**. The leftmost images were taken just before the pathogen was introduced, whereas the rightmost images were taken 10 hours post infection by PA14. The time interval between images is 1 hour. **(B)** The time course of cumulative number of eggs in individual worms. Each row corresponds to a single animal. Rows were sorted in descending order of the total number of eggs laid per worm. The number of eggs is color-coded and the color scale is shown on the right. **(C)** The average number of eggs laid per worm as a function of time. The gray area indicates ± one standard deviation. Please click here to view a larger version of this figure.



Supplementary Material 1: A sample script that creates a text file containing the imaging positions for all channels in WormSpa device. Please click here to download this file.

Discussion

Microfluidic tools provide multiple benefits in studying worms. Imaging in a PDMS device offers higher imaging quality as compared with a standard NGM agar plate. Multiple images can be taken from a single worm, in contrast with traditional methods in which animals are picked from the plate and mounted on a microscope slide for imaging. In addition, the microenvironment in which worms reside can be kept constant or modulated as desired, permitting precise mapping between the composition of the environment and the response of the animals.

Here we demonstrated how to perform a longitudinal measurement of host response to bacterial infection by imaging the response of multiple individual worms to the opportunistic pathogen *P. aeruginosa* PA14 using a custom microfluidic device (WormSpa). Both bright-field and fluorescence images were taken at multiple times without perturbing the ongoing experiment. In particular, the kinetics of egg-laying and the *irg-1* gene expression were probed every 10 minutes, a significant increase in temporal resolution over traditional methods[14,15].

In this device, longitudinal measurements of multiple worms can be performed by revisiting the saved location of each worm at any given time. This was demonstrated in **Figures 2C** and **3B**, where we quantify the *irg-1*::GFP fluorescence and the eggs laid by individual worms. These figures show that worms display broad range of kinetic profiles. As previously shown in single-cell studies[19,20,21], this individuality can be used to understand the design of genetic circuits, as well as the correlation between distinct pathways[19,22].

For successful experiments, it is critical to load worms into the microfluidic device quickly. While worms crawl on agar surface, they tend to thrash in liquid environment, and this behavior induces a genetic program in which AMP activated protein kinases are involved[23,24]. To minimize this undesirable perturbation, it is important to place worms in their separate chambers quickly, as the microstructures in the chamber and the air permeability of PDMS provide worms with an environment that mimics solid surface.[5] Although applying higher pressure helps loading worms faster, pressurizing the device too much could induce mechanical stress on the worms. Worms that are severely stressed do not feed or lay eggs, resulting in a clearly observable bagging phenotype[25]. After loading, worms are fed *E. coli* OP50 for 2-3 hours, allowing the worms to adjust to the microfluidic environment and providing the researcher an opportunity to observe the animals and discard those that were damaged.

The size and spacing of microstructures in each chamber are optimized for the worms grown for 46 hours from L1 arrest at 25 °C. The structures may be scaled up to study older worms or reduced to examine L4 larvae which are smaller in size. However, worms are not able to successfully molt in the chamber, precluding study of post-embryonic development in this device. For such applications, methods such as WorMotel[26] may be considered. Since worms are confined but not immobilized in WormSpa, a successful durable experiment depends on whether the animals stay in the chamber. We find that this can be challenging for early-stage larvae, for male adults, and for some hermaphrodite mutants.

Instead of using a single buffer inlet (at the center of the top, **Figure 1**), the two upside down balloon-shaped inlets can be used simultaneously. Filling the two syringes with different buffers, one can generate temporally structured environments by pressing on different syringes at different time. This, for example, allows study of adaptation to changing environment.[27,28]

As mentioned above, the microfluidic device described here can be used for a broad range of other applications. These include studying responses to other environmental cues. Such responses may include changes in gene expression and egg-laying behaviors, as exemplified here, but also changes in metabolism and fat accumulation (*e.g.* through fat staining by Nile Red), neuronal physiology and signaling (*e.g.* by calcium imaging and optogenetic perturbations), changes in motor behaviors such as pumping, defecation and head movement (via automated quantitative imaging of time-lapse sequences), and more.

Finally, this approach is largely automated and reduces the labor required for long experiments over many hours, even days. Once the locations of individual worms are saved in the data acquisition software, the experiment consists of automatic recording of images while the mechanical pump controller maintains the constant flux of buffer into the device. Importantly, fabrication of a WormSpa device does not require any more than standard soft lithography protocol, and its design and operation are simple enough even to researchers with no previous experience in microfluidics. With the various advantages discussed above and relatively simple preparation steps, this approach can be helpful to those who are considering longitudinal measurement of live worms under precisely controlled conditions.

Disclosures

The authors have nothing to disclose.

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