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Live-cell imaging of the complete life cycle of the bacterial predator, Bdellovibrio bacteriovorus, using time-lapse fluorescence microscopy

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Corresponding Author:	Lukasz Makowski, Ph.D University of Wroclaw Wroclaw, Lower Silesia POLAND
Corresponding Author's Institution:	University of Wroclaw
Corresponding Author E-Mail:	lukasz.makowski@uwr.edu.pl
Order of Authors:	Łukasz Makowski, Ph.D
	Damian Trojanowski
	Jolanta Zakrzewska-Czerwińska
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Lukasz Makowski

University of Wroclaw, Poland
e-mail: lukasz.makowski@uwr.edu.pl

Wrocław, 22.01.2020

Dear Dr. Steindel,

Thank you very much for your e-mail from January 02nd, 2020 regarding the manuscript entitled "Live-cell imaging of the complete life cycle of the bacterial predator, Bdellovibrio bacteriovorus, using time-lapse fluorescence microscopy" (JoVE61105). The revisions of our manuscript have been made (see the Revised manuscript and the answers to the reviewer comments). We hope that our manuscript is acceptable for publication now.

I am looking forward to your kind answer.

Yours sincerely,

Lukasz Makowski

TITLE:

Live-Cell Imaging of the Life Cycle of Bacterial Predator *Bdellovibrio bacteriovorus* using Time-Lapse Fluorescence Microscopy

AUTHORS AND AFFILIATIONS:

Łukasz Makowski, Damian Trojanowski, Jolanta Zakrzewska-Czerwińska

Department of Molecular Microbiology, Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland

Corresponding Author:

Łukasz Makowski (lukasz.makowski@uwr.edu.pl)

Email Addresses of Co-Authors:

Damian Trojanowski (damian.trojanowski@uwr.edu.pl)

Jolanta Zakrzewska-Czerwińska (jolanta.zakrzewska@uni.wroc.pl)

KEYWORDS:

predatory bacteria, *Bdellovibrio bacteriovorus*, prey, *Escherichia coli*, time-lapse fluorescence microscopy, DNA replication, bdelloplast, live-cell imaging, bacterial cell cycle

SUMMARY:

Presented here is a protocol that describes monitoring of the complete life cycle of predatory bacterium *Bdellovibrio bacteriovorus* using time-lapse fluorescence microscopy in combination with an agarose pad and cell-imaging dishes.

ABSTRACT:

Bdellovibrio bacteriovorus is a small gram-negative, obligate predatory bacterium that kills other gram-negative bacteria, including harmful pathogens. Therefore, it is considered a living antibiotic. To apply *B. bacteriovorus* as a living antibiotic, it is first necessary to understand the major stages of its complex life cycle, particularly its proliferation inside prey. So far, it has been challenging to monitor successive stages of the predatory life cycle in real-time. Presented here is a comprehensive protocol for real-time imaging of the complete life cycle of *B. bacteriovorus*, especially during its growth inside the host. For this purpose, a system consisting of an agarose pad is used in combination with cell-imaging dishes, in which the predatory cells can move freely beneath the agarose pad while immobilized prey cells are able to form bdelloplasts. The application of a strain producing a fluorescently tagged β-subunit of DNA polymerase III further allows chromosome replication to be monitored during the reproduction phase of the *B. bacteriovorus* life cycle.

INTRODUCTION:

Bdellovibrio bacteriovorus is a small (0.3–0.5 µm by 0.5–1.4 µm) gram-negative bacterium that preys on other gram-negative bacteria, including harmful pathogens such as *Klebsiella*

pneumoniae, Pseudomonas aeruginosa, and *Shigella flexneri*[1–3]. Since *B. bacteriovorus* kills pathogens, it is considered a potential living antibiotic that can be applied to combat bacterial infections, particularly those caused by multidrug-resistant strains.

B. bacteriovorus exhibits a peculiar life cycle consisting of two phases: a free-living non-replicative attack phase and an intracellular reproductive phase (**Figure 1**). In the free-living phase, this highly motile bacterium, which moves at speeds of up to 160 µm/s, searches for its prey. After attaching to the prey's outer membrane, it enters the periplasm[4,5]. During the interperiplasmic reproductive phase, *B. bacteriovorus* uses a plethora of hydrolytic enzymes to degrade the host's macromolecules and reuse them for its own growth. Soon after invading the periplasm, the host cell dies and bloats into a spherical structure called a bdelloplast, inside which the predatory cell elongates and replicates its chromosomes. The replication process starts at the replication origin (*oriC*)[6] and proceeds until several copies of the chromosome have been completely synthesized[7]. Interestingly, replication of each chromosome is not followed by cell division. Instead, the predator elongates to form a long, multinucleoid and filamentous cell. Upon nutrient depletion, the filament undergoes synchronous septation and progeny cells are released from the bdelloplast[8].

Before *B. bacteriovorus* can be used as a living antibiotic against bacterial infections, it is crucial to understand the major stages of its life cycle, particularly those related to its proliferation inside the prey. Live-cell imaging of *B. bacteriovorus* has been challenging, due to the various morphological forms of the predator and its prey during the complex life cycle. So far, the interactions between *B. bacteriovorus* and its host cell have been mainly studied by electron microscopy and snap-shot analysis[2,9,10], both of which have limitations, especially when they are used to monitor successive stages of the predatory life cycle. These methods provide high-resolution images of *B. bacteriovorus* cells and enable observation of a small predator during the attack or growth phase. However, they do not allow tracking of single *B. bacteriovorus* celsl throughout both life cycle phases.

Presented here is a comprehensive protocol for using time-lapse fluorescence microscopy (TLFM) to monitor the complete life cycle of *B. bacteriovorus*. A system consisting of an agarose pad is used in combination with a cell-imaging dish, in which the predatory cells can move freely beneath the agarose pad while the immobilized prey cells are able to form bdelloplasts (**Figure 2**). This set-up is prepared based on specific strains of both *E. coli* and *B. bacteriovorus*, but the protocol may be easily altered to fit a user's individual strains (e.g., carrying different selection markers, proteins fused with different fluorophores, etc.).

In this case, to visualize *B. bacteriovorus* during the attack phase, a specific strain (HD100 DnaN-mNeonGreen/PilZ-mCherry) was constructed that expresses a fluorescently tagged version of the cytoplasmic protein, PilZ (available in our laboratory upon request)[7]. This strain additionally produces DnaN (the β-sliding clamp), a subunit of DNA polymerase III holoenzyme, fused with a fluorescent protein. This enables ongoing DNA replication to be monitored inside the predatory cells as they grow within bdelloplasts.

Although the described protocol and software used for image acquisition refer to an inverted microscope provided by a specific manufacturer (see **Table of Materials**), this technique may be adjusted for any inverted microscope equipped with an environmental chamber or other external heating holder and capable of time-lapse imaging. For data analysis, users may choose any available software compatible with the individual output formats.

PROTOCOL:

1. Preparation of *B. bacteriovorus* lysate for microscopic analysis

1.1. In a 250 mL flask, set up the coculture by combining 1 mL of fresh 24 h *B. bacteriovorus* culture (e.g., HD100 DnaN-mNeonGreen/PilZ-mCherry) and 3 mL of overnight prey culture (e.g., *E. coli* S17-1 pZMR100) with 50 mL of Ca-HEPES buffer (25 mM HEPES, 2 mM $CaCl_2$ [pH = 7.6]) supplemented with antibiotics when needed (here, 50 µg/mL kanamycin).

1.2. Incubate the coculture for 24 h at 30 °C with shaking at 200 rpm.

1.3. Check that the prey cells are fully lysed by liquid mounted phase-contrast microscopy. At this point, numerous motile attack phase *B. bacteriovorus* cells should be present, and no *E. coli* cell or bdelloplast should be visible.

1.4. Filter the *B. bacteriovorus* culture through a 0.45 µm pore size filter to remove any remaining host cells. Predatory cells (0.3–0.5 µm x 0.5–1.4 µm) freely penetrates 0.45 µm pores, whereas host cells are retained on the filter surface.

1.5. Spin down the filtrate for 20 min at 2469 x *g* and 30°C in a 50 mL conical tube to collect predatory cells. Resuspend the *B. bacteriovorus* pellet in 3 mL of Ca-HEPES buffer (to a final OD_{600} of approximately 0.2) and incubate at 30 °C and 200 rpm for 30 min.

2. Preparation of host cells for *B. bacteriovorus* invasion

2.1. Use a single colony of the host strain (*E. coli* S17-1 pZMR100 is recommended due to various cell sizes resulting in the formation of unequally sized bdelloplasts) to inoculate 10 mL of YT medium (0.8% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl [pH = 7.5]) that has been supplemented with antibiotics, if needed (here, 50 µg/mL kanamycin). Culture cells overnight at 37 °C and 180 rpm.

2.2. Transfer 2 mL of overnight *E. coli* culture (OD_{600} of ~3.0) to a 2 mL test tube and centrifuge at room temperature (RT) and 2469 x *g* for 5 min. Resuspend the pellet in 200 µL of Ca-HEPES buffer.

3. Set-up of the microscope (see Table of Materials)

3.1. Pre-warm a microscopic chamber with a 100x oil immersion objective to 30 °C for at least 1 h before use. This step is required to prevent errors in maintaining focus during the experiment.

3.2. Turn on both the microscope and microscopy automation. Initialize the control software and select the appropriate filters to acquire brightfield/DIC/phase-contrast and fluorescence images, as needed (here: BF images, GFP/mCherry polychromic filter, and emission/excitation for mCherry and GFP).

4. Assembly of layouts for time-lapse fluorescence microscopy

4.1. Mix 200 mg of low fluorescent molecular grade agarose with 20 mL of Ca-HEPES buffer (1% final agarose concentration) and dissolve the agarose in a microwave. The batch can be reused several times after it solidifies, so simply repeat the heating step.

4.2. Pour 3 mL of melted agarose into a 35 mm glass-bottom dish (**Table of Materials**). Allow the agarose to solidify.

4.3. Using a laboratory micro-spatula, carefully remove the agarose pad from the 35 mm dish without disturbing the center pole of the agarose pad. Flip the pad bottom side facing up and place it in a Petri dish cover (**Figure 2**).

4.4. Put 5 µL of concentrated *E. coli* suspension on top of the flipped pad and spread cells in the center pole using an inoculation loop. Add 5 µL of concentrated *B. bacteriovorus* suspension (OD_{600} of ~0.2) onto the *E. coli*-coated surface. Do not spread the cells.

4.5. Quickly return the agarose gel to the 35 mm glass-bottom dish (top side facing down), then cover with a lid. If necessary, remove any air bubbles by gently pressing down the agar against the plate.

5. Conduction of time-lapse fluorescence microscopy

5.1. Place the 35 mm dish in the Petri dish holder such that it will not move during the course of the experiment. Place one drop of immersion oil (1.518 refractive index) onto the objective as well as the bottom of the 35 mm dish.

5.2. Mount the holder onto the stage of the inverted microscope in the microscope chamber.

5.3. Set up the options in the microscope control software to collect multiple images at multiple stage positions over time.

5.3.1. Set the magnification (100x) and polychromic lens (GFP/mCherry). Using an eye piece and brightfield (BF), find the focal plane and open the point list manager.

5.3.2. Select at least 10 positions of interest by moving the stage and storing the coordinates for each position in the microscope software by clicking the **Mark point** button. Avoid positions located close to each other to prevent photobleaching and phototoxicity. Turn the camera valve from the eyepiece to the monitor and calibrate each point by setting the focal plane and clicking the **Replace point** button in the point list manager.

5.3.3. Open the experiment designer and set all experimental parameters in each tab as follows:

5.3.3.1. Unmark the **Z-stacking** box.

5.3.3.2. Choose fluorescent channels and set the optimal illumination settings. Here, the following settings were used: for the mCherry channel, mCherry filter sets (EX575/25; EM625/45), 50% intensity, and 200 ms exposure time; for the GFP channel, GFP filter set (EX475/28; EM525/48), 50% intensity, and 80 ms; and for the BF images, POL channel, 5% intensity, and 50 ms.

5.3.3.3. Select intervals between acquiring images and the total time of the experiment. Here, 5 min intervals were performed over the 10 h period of the experiment.

5.3.3.4. Enable focus maintenance for the chosen positions (for the system used here, by marking the **Maintain focus with UltimateFocus** check box).

5.3.3.5. Select the data folder in which the image files should be automatically saved.

5.3.3.6. Recheck all settings in the microscope software control, then start the time-lapse experiment.

5.4. After the first hour of the experiment, check that all stage positions are still in focus. Adjust the focus during time intervals between image acquisition, if needed.

5.5. When the time-lapse experiment is finished, remove the Petri dish and utilize the 35 mm dish with an agarose pad according to the biosafety protocol.

NOTE: All substeps after step 5.2 are specific for DeltaVision Elite users. Researchers using other systems need to adjust the settings according to individual systems.

6. Processing of time-lapse images and generation of movies using Fiji software

6.1. Transfer experimental data to a computer loaded with the Fiji software. Launch the Fiji program and open an image using **File** | **Open** or by simply dragging and dropping the image file into Fiji.

6.2. In **Bio-Formats Import Options**, choose **View stack with Hyperstack** in the stack viewing options, Click on **Composite color mode** in the color options, and mark **Autoscale**.

6.3. By scrolling through the image stacks, assess whether the cells and bdelloplasts remained in focus over the course of the experiment. Check whether green fluorescent spots from DnaN-mNeonGreen are present within *B. bacteriovorus* cells inside bdelloplasts throughout the growth phase, and ensure that all stages of the predatory life cycle can be visualized.

6.4. To analyze a particular *B. bacteriovorus* cell/bdelloplast, mark it using the selection tools in the Fiji menu (**Rectangle**, **Oval**, **Polygon,** or **Freehand**). Duplicate the chosen region using **Image | Duplicate** or by using **Ctrl** + **Shift** + **D**.

6.5. Adjust the brightness and contrast for each fluorescence channel as follows:

6.5.1. To choose a single channel, open **Channel tools** by clicking **Image | Color | Channels tools** or **Ctrl** + **Shift** + **Z,** and select the channel of interest (**Channel 1**: mCherry, **Channel 2**: mNeonGreen, **Channel 3**: brightfield).

6.5.2. Adjust the brightness and contrast of images in the fluorescence channel by opening the **B&C** menu in **Image | Adjust | Brightness/Contrast** or by clicking **Ctrl** + **Shift** + **C**.

6.6. Add scale bar using **Analyze | Tools | Scale bar**. Add the time stamper to the images by clicking **Images | Stacks | Time Stamper**.

6.7. To save modified images, go to **File | Save As** and choose **TIFF** to save as an image sequence, **AVI** to produce a movie, or **PNG** to save a single image of the current frame.

REPRESENTATIVE RESULTS:

The described TLFM-based system allows individual cells of B*. bacteriovorus* to be tracked in time (**Figure 3**, **Movie 1**) and provides valuable information about each stage of the complex predatory life cycle. The PilZ-mCherry fusion enables the entire predatory cell to be labeled in the attack phase as well as early stage of the growth phase (**Figure 3**). The transition from the attack to replicative phase was visualized not only by the host bdelloplast formation but also by the appearance of the replisome (replication machinery), which was marked by DnaN-mNeonGreen fluorescent foci (**Figure 3**).

As previously demonstrated, the replisome was assembled at the invading cell pole (pili-pole)[7], and more than one replisome was observed within a growing *B. bacteriovorus* filament as the reproduction phase progressed (**Figure 3**). After several copies of the chromosome were synthesized, the replication was terminated (visualized as the disappearance of DnaN-mNeonGreen foci). Finally, the multinucleoid filament underwent septation, and the progeny cells were released from the bdelloplast (**Figure 3**).

The presented protocol describing the image processing using the Fiji software provides a step-by-step explanation of how to produce a movie for publication from the acquired time-lapse series (**Movie 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: *B. bacteriovorus* life cycle in *E. coli* as a host cell. During the attack phase, a free-swimming *B. bacteriovorus* cell searches for and attaches to a host *E. coli* cell. After the invasion, the predatory cell becomes localized in the prey's periplasm, changing the host cell's shape and forming a bdelloplast. The reproductive phase starts with bdelloplast formation. The predatory cell digests the prey cell and reuses simple compounds to build its own structures. *B. bacteriovorus* grows as a long single filament inside the host's periplasm. When the prey cell's resources are exhausted, the *B. bacteriovorus* filament synchronously septates and forms progeny cells. After the progeny cells develop their flagella, they lyse the bdelloplast.

Figure 2: Schematic depiction of the experimental workflow. The agarose pad is removed from the 35 mm dish and flipped so that the bottom side faces upwards. Fresh suspensions of predatory cells and overnight culture of prey cells are placed on the flipped pad to coat the "bottom" side. The agarose pad is then flipped to its original orientation and placed back in the 35 mm dish, which is mounted onto the inverted microscope stage in the microscope chamber.

Figure 3: *B. bacteriovorus* life cycle followed by time-lapse fluorescence microscopy. (**A**) Free-living predatory (*B. bacteriovorus* DnaN-mNeonGreen/PilZ-mCherry) and host (*E. coli*) cells. (**B**) Attachment of *B. bacteriovorus* to *E. coli*. (**C**) Bdelloplast formation. (**D,E**) Filamentous growth and chromosome replication. (**F**) Termination of chromosome replication. (**G**) Start of synchronous filament septation. (**H**) Bdelloplast lysis and release of progeny cells. Upper panels show brightfield images, lower panels represent merged brightfield and fluorescence channels. Scale bar = 1 µm, time = hh:min.

Movie 1: Time-lapse imaging of *B. bacteriovorus* life cycle in *E. coli* as a host cell. Subcellular localization of DnaN-mNeonGreen (green) in strain HD100 DnaN-mNeonGreen/PilZ-mCherry. Red channel indicates labeling of the predator cells with cytoplasmic PilZ-mCherry. Grey indicates brightfield. Images were collected every 5 min.

DISCUSSION:

Due to the increased interest in using *B. bacteriovorus* as a living antibiotic, new tools for observing the predatory life cycle, particularly predator-pathogen interactions, are needed. The presented protocol is used to track the entire *B. bacteriovorus* life cycle, especially during its growth inside the host, in real-time. Moreover, the application of a strain producing fluorescently tagged beta clamp of DNA polymerase III holoenzyme enabled monitoring of chromosome replication progression throughout the reproduction phase of *B. bacteriovorus*.

A critical step in this method is the proper preparation of layouts in the 35 mm dish. A challenge in observing the life cycle of *B. bacteriovorus* under the microscope is to establish conditions that support predatory growth (involving other gram-negative bacterial cells as host cells) and provide for predatory cell motility. One of the crucial aspects of effective microscopic observations is the need for a uniform distribution of prey on the agarose pad, which is achieved by spreading the cells with an inoculating loop. The host cell density cannot be too high, and the chosen observation points should contain a sufficient number of separate host cells. Meanwhile, the *Bdellovibrio* cells should be motile enough to actively search for their prey. Thus, they should not be spread out or air-dried before the pad is flipped back over and placed into the dish. Sometimes greater volumes of predator suspension are needed to provide sufficient motility in a thin layer of fluid between the agarose and glass surface.

A µ-Dish is used here; however, this is not essential for the protocol. Any glass-bottom dish could be used. An advantage of the µ-Dish is the optimal height and volume of the agarose path, which enables the coculture of predatory and host to be placed on the bottom side of the agarose pad. It is also important to use fresh *B. bacteriovorus* cells in the experiments, as the cells lose their motility during prolonged storage. A limitation of this protocol is that, at the single field of view, users can count approximately 100 predator cells and 100 host cells, but the MOI can be estimated at 50%–60%.

Current knowledge about the *Bdellovibrio* cell cycle is largely based on studies that have employed *E. coli* or *P. aeruginosa.* This system allows monitoring of *B. bacteriovorus* preying on a variety of bacterial pathogens, including *Salmonella* spp., *S. flexneri, Proteus mirabilis,* or *K. pneumoniae*. Moreover, this platform may be useful in experiments involving multidrug-resistant pathogens. Thus, it may help to facilitate improvements in the genetic engineering of *B. bacteriovorus* as a live antibiotic in human and veterinary medicine, particularly to combat multidrug-resistant pathogens.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

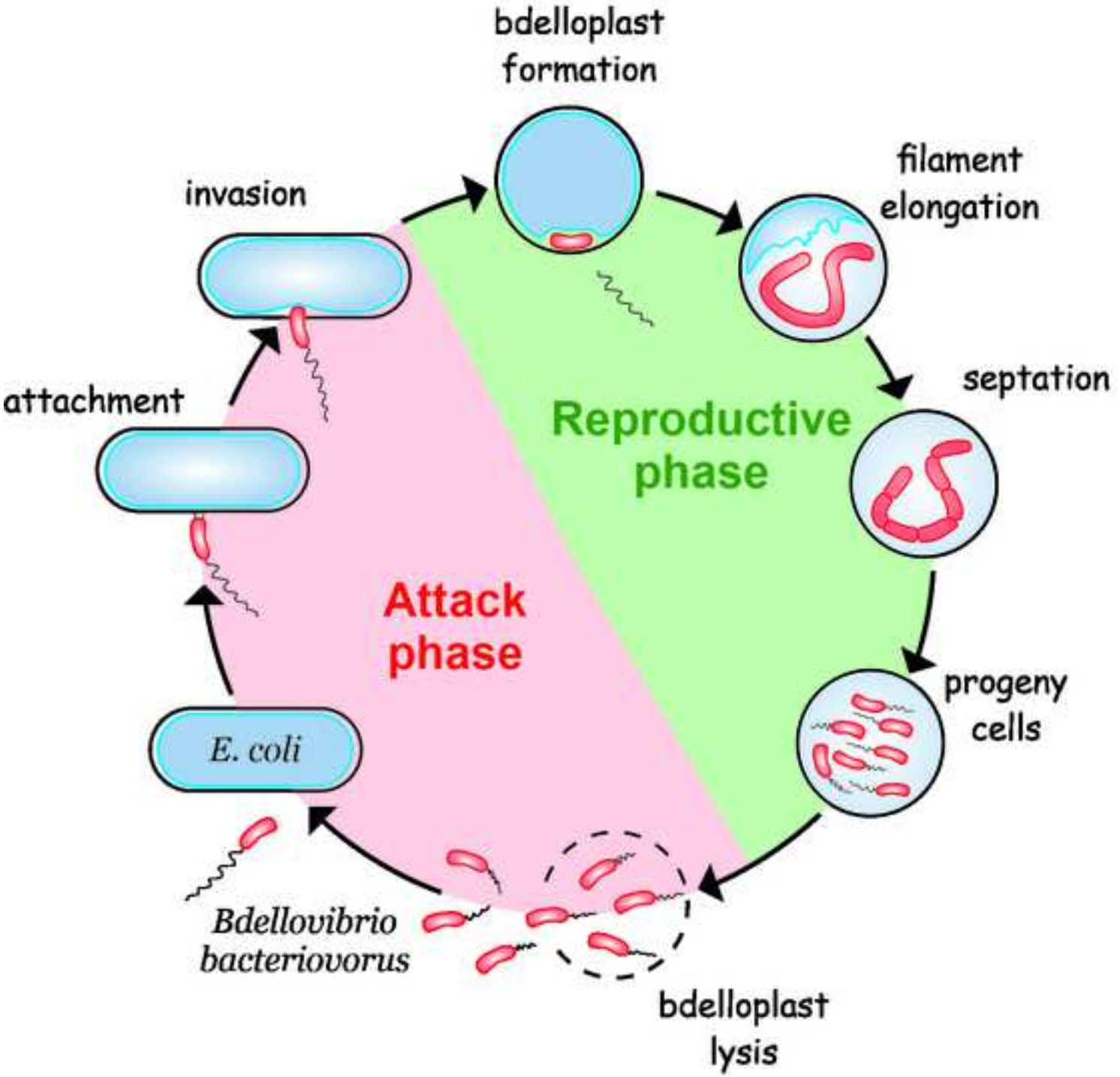


Figure 2

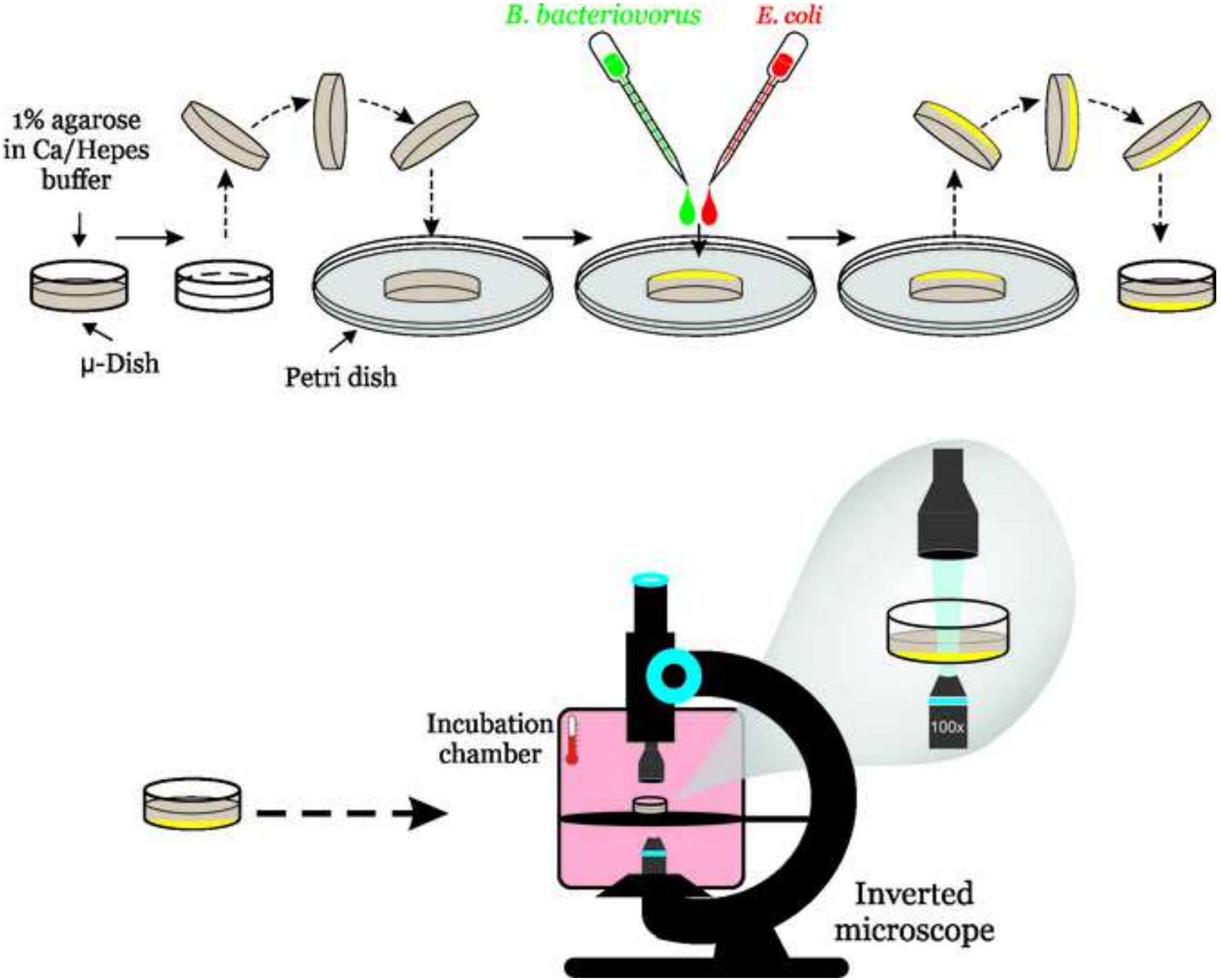
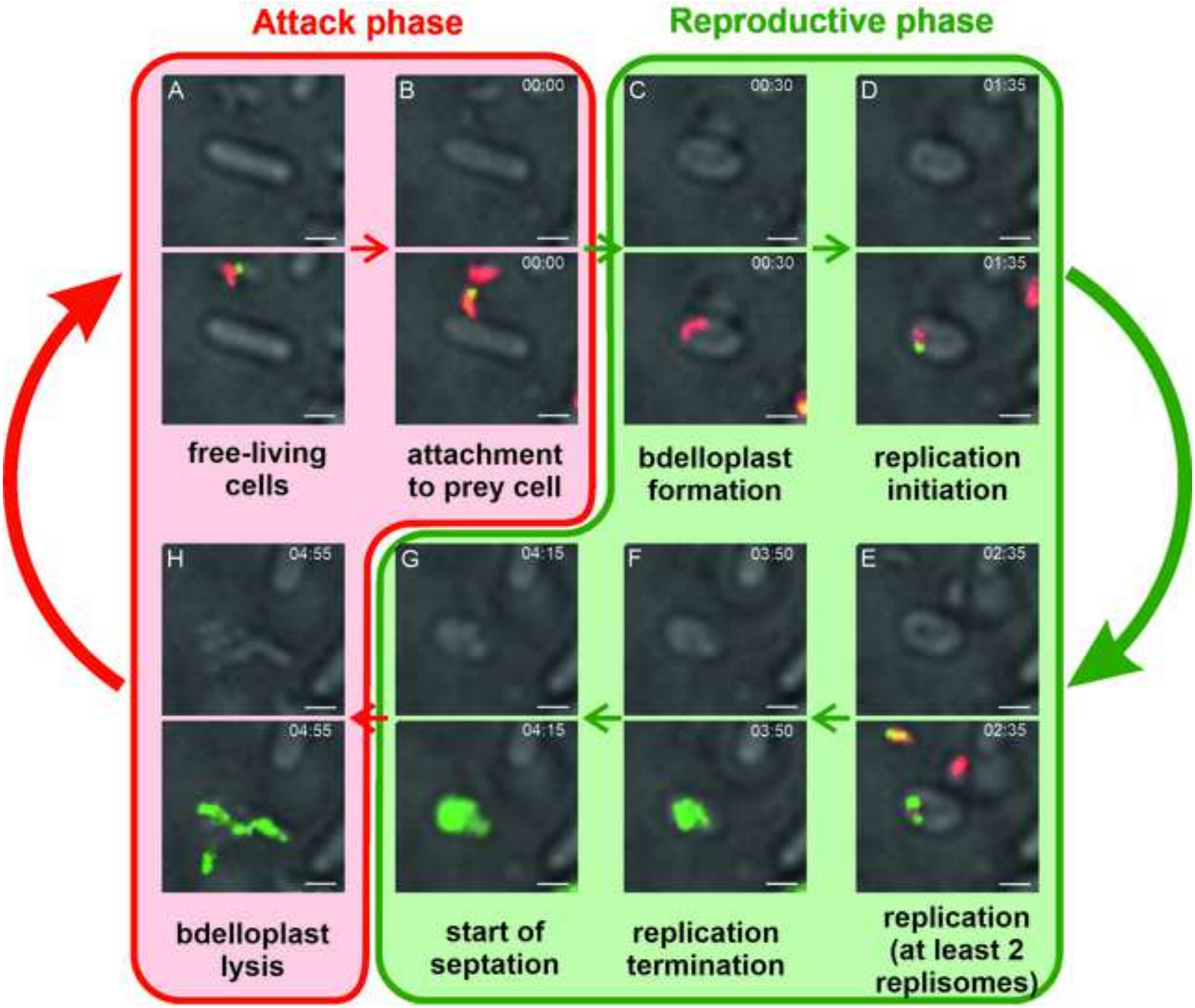


Figure 3



Movie 1

Click here to access/download

Video or Animated Figure

Movie 1 Makowski et al..avi

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Centrifuge	MPW MED. INSTRUMENTS	MPW-260R
CertifiedMolecular Biology Agarose	BIO-RAD	161-3100
Fiji	ImageJ	https://imagej.net/Fiji
Glass Bottom Dish 35 mm	ibidi	81218-200
Microscope	GE	DeltaVision Elite
Minisart Filter 0.45 µm	Sartorius	16555----------K
Start SoftWoRx	GE	

Comments/Description

Rotor ref. 12183

low fluorescence agarose for agarose pad

Open source image processing package

uncoated glass

Microtiter Stage, ultimate focus laser module, DV Elite CoolSnap HQ2 Camera, SSI assembly FP DV, kit obj. Oly 100x oil 1.4 NA, prism Nomarski 100x LWD DIC, ENV ctrl IX71 uTiter opaQ 240 V

Cellulose Acetate, Sterile, Luer Lock Outlet

Manufacturer-supplied imaging software

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was read carefully and all spelling or grammar issues were corrected.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

The manuscript is correctly formatted according to JoVE guidelines.

3. Please define all abbreviations before use, e.g., PFU

Now, all abbreviations are defined before use.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript (and figures) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Eppendorf, µ-Dish, ibidi

All trademark, registered symbols and company names have been removed from the manuscript.

Summary:

1. Please include a separate Summary section (before the abstract) that clearly describes the protocol and its applications in complete sentences between 10 and 50 words, e.g., "Here, we present a protocol to …

The summary section has been added before the abstract.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The Protocol is less than 2.75 pages.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We clearly explain how to perform each step of the protocol.

Specific Protocol steps:

1. 1.4: How do you measure PFU?

In the revised manuscript, information about PFU was replaced with OD_{600} measurement. PFU is no longer mentioned in the manuscript.

Figures:
1. Please provide a legend for Movie 1.
Legend for Movie 1 has been added.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
All materials and equipment used in the Protocol are indicated in the Table of Materials.

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.
Symbols have been removed from Table of Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Makowski et al. describe a live-cell imaging method to visualize the life cycle of the small Gram-negative bacterial predator, Bdellovibrio bacteriovorus. B. bacteriovorus bacterium has the ability to kill a range of Gram-negative bacterial strains, including several pathogen (Escherichia coli Klebsiella pneumoniae, Pseudomonas aeruginosa and Shigella flexneri…), yet investigating its lifecycle and particularly its proliferation inside the prey remains a technical challenge. This protocol provides a convenient way to monitor interactions between B. bacteriovorus and the prey cells in real-time, and describe the two phases of its proliferation cycle, i.e., free-living non-replicative attack phase and an intracellular reproductive phase. To distinguish between these two phases, the authors use a B. bacteriovorus strain producing a fluorescently tagged PilZ cytoplasmic protein together (attack phase) with a fluorescently tagged β-sliding clamp subunit DnaN, which is the associated with the DNA polymerase III holoenzyme (replicative phase). This technique is mostly based on the preparation of an agarose pad mounted on cell-imaging dishes, which allows observing the cells moving freely in the 2D focal plan of the microscope. This protocol can be adapted to investigate interactions between B. bacteriovorus and a range of prey species. The microscopy sample preparation is simple and well described, as well as the imaging procedure.
Undoubtedly, this protocol with be useful to anyone willing to perform real-time microscopy of B. bacteriovorus life/predatory cycle in interactions with a range of prey species.

Major Concerns:
The method presented in this manuscript claims to allow time-lapse visualization of B. bacteriovorus at the single cell level. However, the microscopy images presented here hardly allow to observe individual B. bacteriovorus cells (movie and figure 3). Indeed, the BF images do not provide enough contrast to see predatory cells within the prey, and the quality of the fluorescence (both red and green) images is rather poor (pixelized)(The fluorescence signal might have been saturated in the movie and in Figure 3.). Consequently, the abstract statement "during its growth inside the host at the single-cell level" appears inappropriate. The authors might want to comment on that point (resolution, cell size, fluorescent signal intensity) and explain more clearly if and when B. bacteriovorus cells can be observed at the single-cell level. This might not be a problem for the scope of this manuscript, but this should be clarified throughout the manuscript.
B. bacteriovorus is a small (0.3-0.5 by 0.5-1.4 µm) bacterium, therefore it is hard to get high-resolution images of this bacterium in the traditional widefield fluorescence microscope using

100x objective lens. The „single-cell" phrase was used to highlight the single prey cell (i.e., to point out that observations of the whole cell cycle arise from the single predator cell attacking the prey at the 'stoichiometry' is 1:1). The pixelization is a result of picture magnification and one has to bear in mind that the diameter of the blelloplast is approximately 2 µm. HD100 DnaN-mNeonGreen/PilZ-mCherry strain expresses a fluorescently tagged version of the cytoplasmic protein, PilZ that enables localization of the predatory cell inside the host cell. We removed the "single-cell level" phrase from the whole manuscript.

Also, this protocol explain sample preparation and image processing (producing the time-lapse file), but no analysis procedure is provided. All reference to image analysis should be removed (l.161).
We agree, we did not provide any procedure for images analysis. References according to image analysis have been removed from the manuscript.

Minor Concerns:
1. Preparing the B. bacteriovorus lysate for microscopic analysis
l.89: It might be useful to indicate the OD600 equivalent to a final concentration of ~9x108 PFU/ml predatory cells.
We removed information about plaque-forming units (PFU) and replaced it with OD_{600} of predatory cells.

2. Preparing host cells for B. bacteriovorus invasion
l.95: It is not clear why the authors recommend to use E. coli S17-1 pZMR100 as a prey.
Any Gram-negative bacteria can be used as a host in the presented protocol. We recommended *E. coli* S17-1 pZMR100 due to its various cell sizes resulting in the formation of unequally sized bdelloplasts. We added that information to the manuscript.

l.101: It would be useful to indicate the final concentration of E. coli cells wanted after resuspension, in OD600 equivalent and PFU/ml (as for B. bacteriovorus in 1.4.)
We agree with the referee. Now, we added OD_{600} value to the manuscript.

4. Assembling the layouts for time-lapse fluorescence microscopy
l.112: Most molecular-grade agarose exhibit some levels of autofluorescence, which might be a problem for subsequent microscopy imaging (fluorescence background). The authors might want to recommend a specific agarose with low levels of autofluorescence.
The specific agarose that we recommend is listed in the Table of Materials.

l.113: The final 1% agarose concentration should be indicated.
The final concentration of agarose was added to the manuscript.

5. Conducting the time-lapse fluorescence microscopy
l.152: The statement "set the correction values (100 nm, 10 interactions)" needs to be explained in more details.
We deleted that statement from the manuscript as it is not crucial for the protocol.

6. Analyzing the time-lapse images and generating movies using the Fiji software
l.161: The title should read "Processing" rather than "Analyzing", as no analysis procedure is explained in this section.
We agree that "processing" is a more appropriate word. Thus, the title has been changed.

l.214: "B. bacteriovorus filament synchronously septates". The word "synchronously" is inappropriate here, as it induces the idea that all division septa form simultaneously. The data presented do not provide enough space- nor time-resolution to support this statement. The authors should rather state that septa are for in a certain time-interval (corresponding to the time-lapse frame rate in Frame/min).
We agree that our data do not provide evidence for the synchronous septation of *B. bacteriovorus* filament but in the text, we refer to the work done by Fenton et al. (2010, J Bac), who showed that *B. bacteriovorus* filament undergoes synchronous nonbinary septation inside the host cell.

l.225: "byrepresentative". Space missing.
Space has been added.

Reviewer #2:
Manuscript Summary:
Makowski and colleagues present a protocol for imaging the life cycle of Bdellovibrio bacteriovorus in a high-resolution fluorescence microscope setup. Crucially, by simple co-incubation of Bdellovibrio and its prey under an agarose patch, the protocol allows to cover all important steps of the Bdellovibrio life cycle, both in the free-living and the intracellular stage.
This may be a useful asset for the community, and the manuscript is largely well-written.

Major Concerns:
1. The protocol careens between descriptions that are very specific for the setup used by the authors (e.g. "50% intensity and 80 ms exposure") and very general descriptions (e.g. "fresh lysate of B. bacteriovorus"). This should be changed to a more consistent style, e.g. by first stating the general intention (such as "use an exposure that will allow to stay in the range of the microscopy sensor throughout the timecourse") and then describing the precise settings used by the group, which might serve as a starting point for other researchers (such as "80 ms exposure with 50% intensity using the xxx/xx filter set on a Deltavision microscope").
We agree that microscope setups are very specific, but we believe that detailed information provides a good start for imaging parameters optimization for other than DeltaVision users.

2. On a related note, for the descriptions that are imprecise (such as the "fresh lysate" or "5 ul of suspension" without OD measurement), it should be stated whether there is a precise value for the measured quantity (e.g. cell density), whether this can vary within a certain range, or whether this does not affect the outcome of the experiment at all. This information is essential for anyone trying to use the protocol.
OD_{600} was added for both *E. coli* and *B. bacteriovorus* suspensions used in the protocol.

3. Intermediate checkpoints for the success of the protocol, a rough estimate of the success rate of the protocol and possible problems should be included.
Critical steps (preparation of the layouts and vitality of the *B. bacteriovorus* cells) are detailed in the discussion section.

4. Possible controls (Bdellovibrio only, prey only, buffer only) should be included.
In our opinion, these controls are not necessary for the manuscript, which focuses on the technical aspects of the protocol. They should be included when the protocol will be used in research studies.

5. The used strain (HD100 DnaN-mNeonGreen/PilZ mCherry) might be a valuable resource for other researchers. Please indicate if and how this strain is available (upon request, Addgene or other repository).
The strain has already been published (Makowski et al., 2019, Appl Environ Microbiol 85:e00730-19). Moreover, we added information "available in our laboratory upon request" in the manuscript.

6. Is the used Ibidi micro-dish essential for the success of the protocol, or are there alternatives? From my understanding of the protocol, any inversible agarose patch should work. Please comment on this point (in addition to lines 242-252, which are excellent).
Ibidi µ-Dish itself is not essential for the presented protocol, but it proved to be the most adequate for the imaging due to its construction: "µ-Dish is not essential for the presented protocol. Any glass-bottom dish could be used. The advantage of µ-Dish is the optimal height and volume of the agarose path, which enables the co-culter of predatory and host to be placed on the bottom side of the agarose pad".

7. In the discussion, the limits of the protocol should be discussed, e.g. how many cell invasions and reproductions can be monitored in one experiment/field of view?
We added the short sentence regarding the protocol limits: "The limitation of this protocol is that, at the single field of view, we can count approximately 100 cells of predator and 100 cells of the host but MOI can be estimated at 50-60 %".

Minor Concerns:
1. Lines 55/56, briefly list advantages and disadvantages of the alternative options mentioned.
We added the short sentence presenting the advantages and disadvantages of alternative options: "These methods provide high-resolution images of *B. bacteriovorus* cells and enabling the observation of a small predator during the attack- or the growth-phase, but they do not allow to track single *B. bacteriovorus* cell throughout both life cycle phases.".

2. Lines 82/91, what is the pH of the Ca-HEPES buffer? In case the pH is not set, please mention this.
pH (7.6) of Ca/HEPES buffer has been added.

3. Line 88, it would be helpful to mention the size of Bdellovibrio and/or the Bdelloplast and the outcome of the filtration at this stage.
We added the sentence: "Predatory cells (0.3-0.5 µm x 0.5-1.4 µm) freely penetrate 0.45 µm pores, whereas host cells are retained on the filter surface".

4. Lines 120/122, how many Bdellovibrio and prey cells (and which resulting MOI) are contained in the used volumes? (If this is not specified, an estimate might already be helpful.)
We did not measure the number of *E. coli* and *B. bacteriovorus* cells in the cell suspension, so we did not mention this in the manuscript. The ratio of predatory cells to prey cell was approximately 2:1.

5. Lines 128-159, this part is extremely specific for the used microscope. Focusing on the more general aspects of the protocol and the intended outcome might be more helpful.

Our intention was to provide detailed setting informations of the microscope we used. We think that detailed informations about filter sets and exposure time is an important part of the presented protocol, at least for the DeltaVision users.

6. Line 129, conducting fluorescence microscopy through a plastic petri dish with two different fluorophores might lead to chromatical aberration. Did the authors test this, or can they comment on this aspect?

Microscopy was conducted through the glass-bottom (0.17 mm thickness) dish and we did not notice any chromatic aberrations.

7. Line 130, shouldn't the refractive index change with the used fluorophores?

The refractive index was assigned by the microscope software. To calculate this, several factors are taken into account, such as temperature of observations, coverslip thickness refractive index of the mounting medium, etc. The refractive index of the immersion oil should not be changed when working with different fluorophores.