Video Article

Fluorescence Time-lapse Imaging of the Complete S. venezuelae Life Cycle Using a Microfluidic Device

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Abstract

Live-cell imaging of biological processes at the single cell level has been instrumental to our current understanding of the subcellular organization of bacterial cells. However, the application of time-lapse microscopy to study the cell biological processes underpinning development in the sporulating filamentous bacteria Streptomyces has been hampered by technical difficulties.

Here we present a protocol to overcome these limitations by growing the new model species, Streptomyces venezuelae, in a commercially available microfluidic device which is connected to an inverted fluorescence widefield microscope. Unlike the classical model species, Streptomyces coelicolor, S. venezuelae sporulates in liquid, allowing the application of microfluidic growth chambers to cultivate and microscopically monitor the cellular development and differentiation of S. venezuelae over long time periods. In addition to monitoring morphological changes, the spatio-temporal distribution of fluorescently labeled target proteins can also be visualized by time-lapse microscopy. Moreover, the microfluidic platform offers the experimental flexibility to exchange the culture medium, which is used in the detailed protocol to stimulate sporulation of S. venezuelae in the microfluidic chamber. Images of the entire S. venezuelae life cycle are acquired at specific intervals and processed in the open-source software Fiji to produce movies of the recorded time-series.

Video Link

The video component of this article can be found at http://www.jove.com/video/53863/

Introduction

Streptomyces are soil-dwelling bacteria that are characterized by a complex developmental cycle involving morphological differentiation from a multicellular, nutrient-scavenging mycelium to dormant, unigenomic spores.¹,³

Under favorable growth conditions, a typical Streptomyces spore starts to germinate by extruding one or two germ tubes (Figure 1). These tubes elongate by tip extension and grow into a branched hyphal network known as the vegetative mycelium. Polar growth and hyphal branching is directed by the essential protein DivIVA. This coiled-coil protein is part of a large cytoplasmic complex called the polarisome, which is crucial for the insertion of new cell envelope material at the extending tip.⁴,⁷ During vegetative growth, the hyphal filaments become compartmentalized by the infrequent formation of so-called cross-walls.⁸ The formation of these cross-walls requires FtsZ, the tubulin-like cytoskeletal protein that is essential for cell division in most bacteria.⁵ In Streptomyces, however, these vegetative cross-walls do not lead to constriction and cell-cell separation and therefore the mycelial mass remains as a network of inter-connected syncytial compartments. In response to nutrient limitation and other signals that are not well understood, specialized aerial hyphae break away from the vegetative mycelium and grow into the air.³ The erection of these structures initiates the reproductive phase of development, during which the long multi-genomic aerial hyphae become divided into dozens of equally sized unigenomic prespore compartments. This massive cell division event is driven by the synchronous constriction of multiple FtsZ rings within single sporogenic hyphae.²,¹⁰ Morphological differentiation is completed by the release of dormant, thick-walled, pigmented spores.
Figure 1: The *Streptomyces* life-cycle on solid media. This is a model of the life cycle based on classical studies of *S. coelicolor* growing on agar plates. The cellular development of a spore begins with the formation of one or two germ tubes, which grow by tip extension to form a network of branching hyphae. Polar growth and branching of the vegetative hyphae is directed by DivIVA (red). The formation of vegetative cross-walls requires FtsZ (green). In response to nutrient limitations and other signals, aerial hyphae are erected. Arrest of aerial growth is tightly coordinated with the assembly of a ladder of FtsZ-rings, which give rise to the sporulation septa that compartmentalize the sporogenic hyphae into box-like prespore compartments. These compartments assemble a thick spore wall and are eventually released as mature pigmented spores.

The key developmental events of the *Streptomyces* life cycle are well characterized\(^1,3\). However, what is still scarce are cell biological studies that employ fluorescence time-lapse microscopy to provide insight into the subcellular processes underpinning differentiation, such as protein localization dynamics, chromosome movement and developmentally controlled cell division. Live-cell imaging of *Streptomyces* development has been challenging because of the complexity of the life cycle and the physiological characteristics of the organism. Previous studies on vegetative growth and the initial stages of sporulation septation have employed oxygen-permeable imaging chambers, or the agarose-supported growth of *Streptomyces coelicolor* on a microscope stage\(^11-15\). These methods, however, are limited by a number of factors. Some systems only allow short-term imaging of cellular growth and fluorescent proteins before cells suffer from insufficient oxygen supply or grow out of the focal plane due to the three-dimensional pattern of hyphal development. In cases where long-term imaging is possible, cultivating cells on agarose pads limits experimental flexibility because the cells cannot be exposed to alternative growth or stress conditions, and the background fluorescence from the medium in the agarose pads severely limits the ability to monitor weaker fluorescent signals.

Here we describe a protocol for live-cell imaging of the complete *Streptomyces* life cycle with excellent precision and sensitivity. By growing *Streptomyces* in a microfluidic device connected to a fluorescence widefield microscope (Figure 2), we are now able to monitor germination, vegetative growth and sporulation septation over a time period of up to 30 hr. This is greatly facilitated by the use of the new model organism *Streptomyces venezuelae* because it sporulates to near completion in submerged culture and thereby overcomes the limitation of the classical model species *S. coelicolor*, which sporulates only on solid media\(^16-20\). To help visualize vegetative growth and sporulation, we co-express fluorescently tagged versions of the cell polarity marker DivIVA and the key cell division protein FtsZ.

We are using a commercially available microfluidic device that has been successfully employed for mycobacteria, *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis* and yeast\(^21-25\). The system traps cells in a single focal plane and allows the control of continuous perfusion of culture medium from different reservoirs. In the detailed protocol we take advantage of this feature to expose *S. venezuelae* vegetative mycelium to a nutritional downshift to promote sporulation.

The protocol described is for live-cell imaging of the entire *Streptomyces* life cycle, but alternative media conditions or microscope settings can be chosen if specific developmental stages are of particular interest.
Figure 2: Schematic depicting the experimental work-flow. The three main steps described in the protocol are shown. First, spores and spent medium are prepared from a stationary-phase culture. Second, the fresh spores are loaded into a microfluidic system and S. venezuelae is imaged throughout its developmental life cycle using a fully automated inverted microscope with an incubation chamber to maintain an optimal growth temperature. Third, the time-lapse series obtained is analyzed and processed using the open-source software Fiji.

Protocol

1. Isolation of Fresh S. venezuelae Spores and Preparation of Spent Culture Medium

1. Inoculate 30 ml Maltose-Yeast Extract-Malt Extract (MYM) medium, supplemented with 60 µl R2 trace element solution (TE), with 10 µl spores of strain SV60 [attBϕBT1::pSS209 (PdivIVA-divIVA-mcherry PftsZ-ftsZ-ypet)]. For consistent growth and sporulation of the cells, use a baffled flask or a flask that contains a spring to allow for sufficient aeration.
2. Culture cells for 35-40 hr at 30 °C and 250 rpm.
3. Examine the cells by liquid mounted phase-contrast microscopy. At this point mycelial fragments and spores will be visible.
4. Centrifuge 1 ml cells in a table-top centrifuge at 400 x g for 1 min to pellet the mycelium and larger cell fragments.
5. Transfer approximately 300 µl supernatant containing a suspension of spores to a new 1.5 ml tube and keep on ice. Keep the remaining culture medium for step 1.7.
6. Dilute spores 1:20 in MYM-TE (final concentration: 0.5-5 x 10^7 spores/ml) and keep on ice until needed (see step 2.3).
7. Filter-sterilize 10 ml of the remaining culture medium to obtain spent MYM-TE that is free of spores and mycelial fragments. Use a sterile 0.22 µm syringe filter and transfer spent MYM-TE to a sterile 15 ml tube.
8. Keep the prepared spent MYM-TE for a few days at 4 °C if additional experiments using similar growth conditions are to be conducted.

2. Preparation of the Microfluidic Device

Note: Each microfluidic plate enables up to four independent experiments to be carried out. To avoid contamination of the unused flow chambers, use sterile solutions and working conditions when setting up an experiment.

1. Remove shipping solution from the microfluidic plate and rinse the wells with sterile MYM-TE.
2. Add 300 µl MYM-TE to inlet well 1 and 300 µl spent MYM-TE to wells 2 to 6.
3. Load 40 µl diluted spores from step 1.6 in cell loading well 8 of lane “A” and seal the manifold to the plate according to manufactures instructions.
4. Launch the microfluidics control software, select the appropriate plate type and set up a flow program to prime the flow channel and culture chamber by flowing medium from inlet wells 1 to 5 at 6 psi for 2 min per well.
4. Generation of Time-lapse Movies Using Fiji Software

Note: We note that different commercial and free software packages are available to process time-lapse microscopy images including ZenBlue, Metamorph, ICY, ImagePro or ImageJ. Here we focus on Fiji, which is an open-source image processing program based on ImageJ, and which already provides a number of useful pre-installed plugins.

1. Transfer imaging data from your time-lapse experiment to a computer that has Fiji installed.
2. Launch program and import the imaging file using "File>Import>Bio-Formats" or simply by dragging the imaging file into Fiji. In the "Bio-Formats Import Options", tick the box "split channels" to obtain a separate image stack for each illumination channel (DIC, RFP, YFP).
3. Optional: To merge separate time-series resulting from a break in the in the image acquisition due to refocusing (step 3.9), open respective image stacks and run "Image>Stacks>Tools>Concatenate" for each channel (DIC, RFP, YFP).
4. Assess the quality of the time-lapse data by scrolling through the image stacks. Look for hyphae that stayed in focus over time, show mycelial growth and eventually form spores (DIC stack). Examine the expected subcellular localization for DivIVA-mCherry at hyphal tips (RFP stack) and FtsZ-YPet forming single rings or multiple, ladder-like structures in vegetative or sporogenic hyphae (YFP stack), respectively.
5. Isolate a region of interest for downstream processing of the images. Time-lapse microscopy often produces big files which may slow down processing by Fiji. It is therefore recommended to identify and isolate a region of interest (ROI) and to perform further image processing steps on this smaller version of the image stack.
1. Select the "Rectangular Selection" tool in the menu and draw a rectangular ROI in the DIC image stack in such a way that the developing hyphae are enclosed by the ROI throughout the image series.

2. Duplicate ROI-stack with "Ctrl"+"Shift"+"D".

3. Click on the name bar of the YFP image stack and select "Edit>Selection>Restore Selection" in the menu to restore the previous rectangular selection from the original DIC stack to the same position in the YFP stack and duplicate the ROI with "Ctrl"+"Shift"+"D".

4. Repeat this process for the RFP stack.

6. Align the images in the three image stacks to remove stage drifts in the xy plane over time. Scroll to the last frame in the DIC stack and select "Plugin>Registration>StackReg> RigidBody" from the menu. Repeat this step for the RFP and the YFP image stack. If necessary, crop the ROI by repeating step 4.5.1-4.5.3.

7. Optional: Adjust brightness and contrast manually for each image stack with the "Adjust Brightness and Contrast Tool" ("Ctrl"+"Shift"+"C") or select "Process>Enhance Contrast" from the menu and apply the "Normalize" command to all images in the stack. It should be noted that the latter command will alter pixel values and therefore may affect downstream image analysis.

8. Optional: Combine individual stacks (converted to the RGB file format, see 4.11) horizontally or vertically using the plugin "Image>Stacks>Tools>Combine".

9. Add a scale bar ("Analyze>Tools>Scale Bar") and a time stamper ("Image>Stacks>Time Stamper").

10. Save modified image stacks as an image sequence in the "tiff" format. To produce a movie, save as "avi". Alternatively, export image series in the QuickTime format using the "Bio-Formats>Bio-Formats Exporter" plugin and select the "mov" file type.

11. To obtain pictures from single frames, select the frame of interest and duplicate the image "Ctrl"+"Shift"+"D". Change the type of the resulting image to "RGB" or "8-bit" under "Image>Type>RGB color or 8-bit" and save the image as "tiff".

Note: Fiji offers a number of additional functions to further annotate or process time-lapse series. Go to Fiji online support for detailed information (http://fiji.sc/Fiji).

Representative Results

The successful live-cell imaging of the entire S. venezuelae life cycle yields a continuous time-series including the key developmental stages of germination, vegetative growth and sporulation (Figure 3, Movie 1). Visualization of the progression through the life cycle is further enhanced by the distinct subcellular localization of DivIVA-mCherry and FtsZ-YPet. During germination and vegetative growth, DivIVA-mCherry exclusively accumulates at the growing hyphal tips or marks newly forming branch points (Figure 4A). These results are in line with the previously reported subcellular positioning of DivIVA. In contrast, FtsZ-YPet forms single ring-like structures at irregular intervals in the growing mycelium (Figure 4B). These structures provide the scaffold for the synthesis of non-constrictive vegetative cross-walls, leading to the formation of interconnected hyphal compartments. The cellular differentiation of growing hyphae into sporogenic hyphae becomes visible by the disappearance of polar DivIVA-mCherry foci, the arrest of polar growth and the concomitant increase of FtsZ-YPet fluorescence (Figure 4C). In sporulating hyphae, the localization pattern of FtsZ-YPet changes dramatically; first helical FtsZ-YPet filaments tumble along the hypha and then, in a sudden, almost synchronous event, these helices coalesce into a ladder of regularly spaced FtsZ-YPet rings. Under the experimental conditions described here, these evenly distributed FtsZ-YPet ladders persist for approximately 2 hr. Finally, sporulation septa become discernible in the differential interference contrast (DIC) images (Figure 4D) and eventually new spores are released.

The subsequent 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 3: Snapshot images from a representative time-lapse fluorescence microscopy series of *S. venezuelae* producing fluorescently labeled FtsZ (green) and DivIVA (Red). Shown are selected frames of merged images (top panel: RFP-YFP-DIC, bottom panel: DIC) visualizing the Streptomyces life cycle including germination, vegetative growth and sporulation. Images were taken from Movie 1. Time is in hr:min. Scale bars = 5 µm. Please click here to view a larger version of this figure.
Figure 4: Representative results for a successful time-lapse series of the *Streptomyces* life cycle. (A) Localization of DivIVA-mCherry. Shown are snapshots of two subsequent time-points from Movie 1 with merged images of the RFP and DIC channels. DivIVA-mCherry marks new hyphal branch sites (filled arrow head) and localizes at the hyphal tip to direct polar growth (open arrow head). Scale bar = 10 µm. (B) FtsZ-YPet localization during vegetative growth (arrow head). FtsZ-YPet forms single ring-like structures (upper panel) that do not constrict (DIC, lower panel). (C) FtsZ-YPet (green) localization during sporulation septation. DivIVA-mCherry foci are shown in red, with elapsed time depicted below (hr:min). Scale bar: 5 µm. (D) Corresponding DIC images of the sporulating hyphae from (C) showing the formation of prespore compartments with visible sporulation septa (left image), which eventually mature into a chain of spores (right image). Time is in hr:min. Scale bar = 5 µm. Please click here to view a larger version of this figure.
Movie 1: Time-lapse fluorescence microscopy series of the *Streptomyces venezuelae* life cycle. The movie consists of merged RFP-YFP images (left) and the corresponding differential interference contrast (DIC) images (right). DivIVA-mCherry is shown in red and FtsZ-YPet in green. The time interval between single frames is 40 min. Scale bar = 10 µm. Please click here to view this movie.

**Discussion**

Time-lapse microscopy of the *Streptomyces* life cycle has been technically challenging in the past. Here we present a robust protocol to perform live-cell imaging of the complete life cycle using fluorescent protein fusions to the cell polarity marker DivIVA and the cell division protein FtsZ to help visualize and trace progression through the developmental program (Figure 2).

Central to this method is the cultivation of *S. venezuelae* in a microfluidic device that allows constant medium perfusion and the exchange of normal growth medium (MYM-TE) with spent medium (spent MYM-TE). The change of culture condition is important to the described protocol because the nutritional downshift as wells as any yet unidentified extracellular signals (e.g. quorum sensing signals) in the spent culture medium, promote sporulation, whereas a continuous supply of nutrient-rich medium stimulates vegetative growth with hardly any spore formation (data not shown). Thus, culturing cells in this microfluidic system is superior to culturing cells on agarose because it offers more experimental flexibility and allows long-term monitoring of changes in bacterial growth in response to changing culture conditions. Although the microfluidic plates are designed for single use only, flow channels that have not been inoculated with cells can be used in subsequent experiments. We recommend using all channels of a microfluidic plate within a week, as we have experienced problems sealing the manifold to plates that have been opened for longer times.

When setting up an experiment, we found that freshly prepared spores germinated within two hr of being loaded into the flow chamber, whereas spores derived from a frozen glycerol stock required at least 6 hr before germ tubes emerged (data not shown). This delay in germination extends the length of the experiment and can interfere with equipment availability and experimental conditions. It is also important to start the experiment with perfusion of MYM-TE for at least 3 hr to provide sufficient nutrients for the development and outgrowth of germ tubes. Perfusion with MYM-TE can be extended beyond the initial 3 hr if the experiment is designed to study vegetative growth. While spores provide the preferred choice of starting material, short hyphal fragments can also be loaded into the microfluidic plate. However, the loading efficiency when using sheared mycelium is significantly lower and often requires several loading runs which may present a limitation when examining non-sporulating mutants. Regardless of the type of inoculum used, it is important not to overload the culture chamber with spores or hyphal fragments as this will lead to rapid overcrowding that can interfere with media diffusion and complicate image analysis.

It is important to plan the construction of reporter proteins carefully for use in fluorescence time-lapse imaging in *Streptomyces*. Linker length between the target protein and the fluorescent reporter and the choice of N- or C-terminal fusions can be critical. Moreover, optimal experimental conditions, including imaging frequency and exposure time, need to be determined in advance for each fluorescently-tagged protein. *S. venezuelae* hyphae exhibit auto-fluorescence in the green/yellow channel that may become problematic when imaging a fluorescently labeled protein that is expressed at low levels. In addition, it should be noted that, during germination and the initial vegetative growth phase, *S. venezuelae* is particularly sensitive to short-wavelength light (e.g. when using protein fusions to CFP).

Despite the continuous advances in imaging techniques and fluorescent reporter systems for live-cell imaging of bacteria, most of the software packages (e.g. MicrobeTracker, Schnitzelcells, CellProfiler) for the subsequent automated processing of these kinds of data sets do not support the analysis of images derived from filamentous bacteria with a multicellular life style. Thus, there is a need to develop a suitable algorithm for the quantitative high-throughput analysis of imaging data from *Streptomyces* and other filamentous bacteria.

In summary, the work described here demonstrates the immense potential of *S. venezuelae* as a new model developmental system for the genus, because of its ability to sporulate in liquid. The microfluidic culturing device is simple to use even for inexperienced users. It provides
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Disclosures

The authors have nothing to disclose.

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