Video Article

Recording Multicellular Behavior in *Myxococcus xanthus* Biofilms using Time-lapse Microcinematography

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Abstract

A swarm of the δ-proteobacterium *Myxococcus xanthus* contains millions of cells that act as a collective, coordinating movement through a series of signals to create complex, dynamic patterns as a response to environmental cues. These patterns are self-organizing and emergent; they cannot be predicted by observing the behavior of the individual cells. Using a time-lapse microcinematography tracking assay, we identified a distinct emergent pattern in *M. xanthus* called chemotaxis, defined as the directed movement of a swarm up a nutrient gradient toward its source [1].

In order to efficiently characterize chemotaxis via time-lapse microcinematography, we developed a highly modifiable plate complex (Figure 1) and constructed a cluster of 8 microscopes (Figure 2), each capable of capturing time-lapse videos. The assay is rigorous enough to allow consistent replication of quantifiable data, and the resulting videos allow us to observe and track subtle changes in swarm behavior. Once captured, the videos are transferred to an analysis/storage computer with enough memory to process and store thousands of videos. The flexibility of this setup has proven useful to several members of the *M. xanthus* community.

Video Link

The video component of this article can be found at https://www.jove.com/video/2038/

Protocol

Supplies needed:

- Klett meter
- Pipette and tips
- 2.5 ml microcentrifuge tubes
- Microcentrifuge
- CTTYE media:
  - 1.0% Casitone (Difco Laboratories), 0.5% yeast extract (Difco Laboratories),
  - 10.0 mM Tris-HCl (pH 8.0), 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$
- TPM media:
  - 10.0 mM Tris-HCl (pH 8.0), 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$
- Agarose
- Water bath set to 55°C
- Glass microscope slides
- Large glass coverslips
- 2 x 2 cm, 0.5-mm-thick silicone rubber gasket (Grace Bio-Lab Inc.)
- Parafilm (or labeling tape)
- Forceps
- Small binder clips
- Micro-sampling pipette (Fisher)
- 100 µl glass disposable tip (Fisher)
- Kimwipes

Part 1: Cell Preparation

Start by creating a sterile environment.

- Clean workspace, don gloves, and light burner.

1. Start an overnight culture of cells by inoculating into a flask containing CTTYE broth and incubated in the dark at 32°C with vigorous swirling.
2. Once the culture reached a density of 5 x 10^8 cells/ml, pipette 1 ml cells into a 2.5 ml microcentrifuge tube.
3. Pellet cells by centrifugation for 2 min at 16,000 x g (or max speed).
4. Decant and discard supernatant.
5. Wash cell pellet with 1 ml TPM (salt-balanced, nutrient-free media).
    - re-suspend and vortex
6. Re-pellet cells by spinning for 2 min at 16,000 x g (or max speed).
7. Decant and discard supernatant.
   CRITICAL STEP
8. Completely re-suspend pellet in 100 µl TPM media using a combination of pipetting and vortexing.
   IMPORTANT: This step ensures that there are no clumps of cells left in the tube. This may take a while.
9. Set the cells aside at room temperature while preparing the tracking assay plates.

Part 2: Agar Preparation

1. Prepare 50 ml of both TPM (assay media) and CTTYE (nutritive disk) media.
2. Add 0.5 g agarose to each (agarose is less diffractive than agar).
3. Autoclave to sterilize.
4. After sterilization, maintain the media at 55°C in an incubator (or water bath) while constructing the tracking assay plate complexes.

Part 3: Nutritive Disk Construction

1. Place a sterile 0.5-mm-thick silicone rubber gasket on top of a flame-sterilized glass microscope slide, forming a small well.
2. Pipette ~300 µl of the 55°C CTTYE media/agarose into the well created by the gasket on the slide and containing the nutritive disk. The CTTYE media/agarose should mound up.
3. Place a flame sterilized slide (with no gasket) on top of the CTTYE media/agarose.
   IMPORTANT: This slide must be set down at an angle to prevent bubbles from forming.
4. Once the slide is in place, clamp the complex together with mini binder clips - one on each side.
5. Place the clipped complex at 4°C and allow the CTTYE media/agarose to set. This usually takes about 5 min.

Part 4: Tracking Assay Preparation - Set Up Plate Complexes

Assemble the components, prepare the slide complexes, place the nutritive disk, pour the TPM media/agarose, separate and dry plate complexes, plate cells, and assemble tracking assay plate complexes.

Assemble plate complex components

1. For each complex, flame sterilized a glass microscope slide
2. Place an autoclaved gasket on top of the slide and set aside (sterilized side up). This will form the bottom of the assay.
3. Flame sterilize a glass cover slip and place it on top of a second glass microscope slide wrapped with labeling tape (or Parafilm) and set aside (sterilized side up). This is used as a support slide to keep the coverslip from cracking. This will form the top of the assay.
4. Flame sterilize a third glass microscope slide and set aside (sterilized side up). This will be used to flatten the agarose.
   IMPORTANT: Make sure the gaskets form a seal with the glass by pressing it down with forceps, otherwise the media/agarose may dry out.

Place nutritive disk

IMPORTANT: Steps 5 through 10 must be done to one slide complex at a time; otherwise the media/agarose could start to solidify resulting in poor movie quality.

5. Remove the nutritive disk complex form 4°C (step 5 from part 3) and pry apart the slides exposing the now solidified CTTYE/agarose.
6. Remove a 1 mm diameter 'nutritive disk' from the CTTYE media/agarose well described above using a micro-sampling pipette with a 100 µl glass disposable tip and place it in the well created by the gasket on the cover slip (step 3 above).

Pour plates

CRITICAL STEP

7. Pipette ~300 µl of the 55°C TPM media/agarose into the well created by the gasket on the cover slip and containing the nutritive disk. The TPM media/agarose should mound up.
   CRITICAL STEP
8. Place the flame sterilized slide with no gasket (from step 3) on top of the TPM media/agarose.
   IMPORTANT: This slide must be set down at an angle to prevent bubbles from forming.

9. Once the slide (from step 5) is in place, clamp the complex together with mini binder clips - one on each side.
10. Place the clipped complex at 4°C and allow the media/agarose to set. This usually takes about 5 min.

Separate and dry plates

IMPORTANT: To prevent the media/agarose from drying out, steps 11 through 20 should only be performed on one slide complex at a time.
IMPORTANT: For best results, steps 11 through 13 should be performed at 4°C.

11. Once the media/agarose has set, remove the binder clips and squeeze the end of the complex to loosen the tape-wrapped slide.
12. Remove the tape-wrapped slide and place it on the bench for further use.
    CRITICAL STEP
13. Using forceps as a wedge, separate the cover slip/gasket/media/agarose complex from the support slide (with no gasket) and discard support slide.
    IMPORTANT: Do not use a prying motion to separate the cover slip/gasket complex. This could result in the cover slip breaking and/or the media/agarose sticking to the support slide.
14. Place the cover slip/gasket/media/agarose complex on the tape-wrapped slide (gasket side up) and remove from 4°C. Place this complex next to a burner to allow all visible moisture evaporate from the newly exposed media/agarose surface - no more than 1 min.
    IMPORTANT: Do not let the media/agarose dry for too long as this could impact the *M. xanthus* swarm behavior.

Plate cells

CRITICAL STEP

1. Once the excess moisture has evaporated, pipette 0.5 µl of the concentrated cells (step 8 from part 1) onto the media/agarose approximately 1 mm away from the nutrient disk.
   IMPORTANT: It is extremely important to make sure that the cells are deposited by moving the pipette straight down and then straight up. This ensures that the swarm will be circular.
   IMPORTANT: It is extremely important not to touch the pipette tip to the media/agarose. This will make a depression on the surface and change the behavior of the *M. xanthus* swarm.
   TIP: Depress the pipette tip before approaching the media/agarose. This will allow a drop of cells to appear on the bottom of the pipette tip and make it easier to deposit the cells.
2. Once the cells are deposited, place the complex next to the burner to allow the cell spot to dry - no more than 20 sec.

Assemble assay

1. Once the cell spot has dried, align the slide/gasket complex (from step 1) with the gasket on the cover slip/gasket/media/agarose/cell complex (from step 13) and gently press together to form a seal.
   CRITICAL STEP
2. Clean the surfaces of the slide and cover slip with a Kimwipe to remove the residue left by the Parafilm. The completed assay should resemble Figure 1.
3. Place the completed slide complex on the heated stage maintained at 32°C (slide down, cover slip up) immediately after wipe down (step 15) to prevent condensation form forming. If condensation has formed, let slide complex sit on the heated stage for several seconds before starting the image acquisition software.
4. Choose the appropriate objective (2X, 4X, or 10X).

Part 5: Movie Preparation

CRITICAL STEP

1. Turn on the camera and microscope, and check the light levels (make sure the light is not too intense). Start up the computer and open the image acquisition program.
2. Click the live image window and focus the microscope. The image should appear similar to the one seen in Figure 3. Notice the uniform agar surface.
3. Start acquiring images.
   CRITICAL STEP
4. Check the focus regularly during the first hour, as the media/agar tends to settle causing the focus to drift.
5. Clean work area.
6. Once image acquisition is complete, transfer acquired images for storage and break down the slide complex by soaking in 90% ethanol overnight.
7. Autoclave gaskets for reuse.

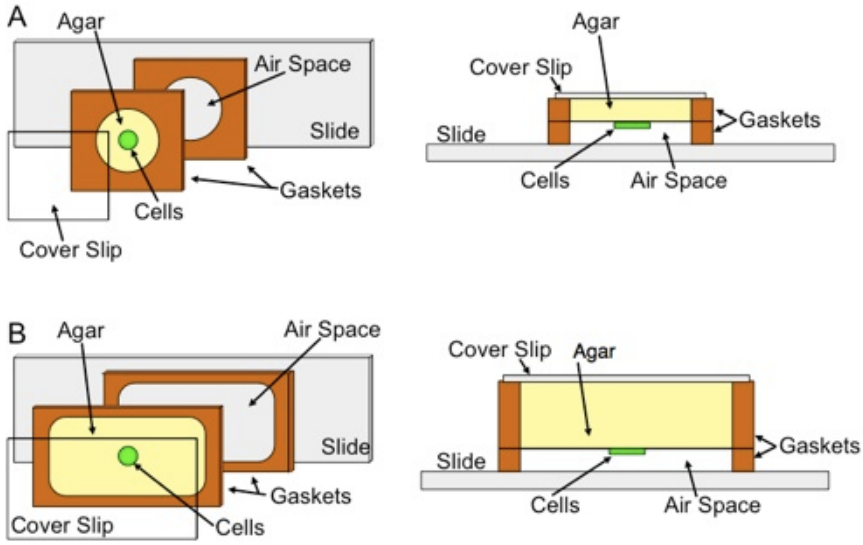

Figure 1. Cartoon illustration of the TM plate complex. (A) shows the basic TM plate complex in exploded view and cross section. (B) shows the use of larger gaskets.

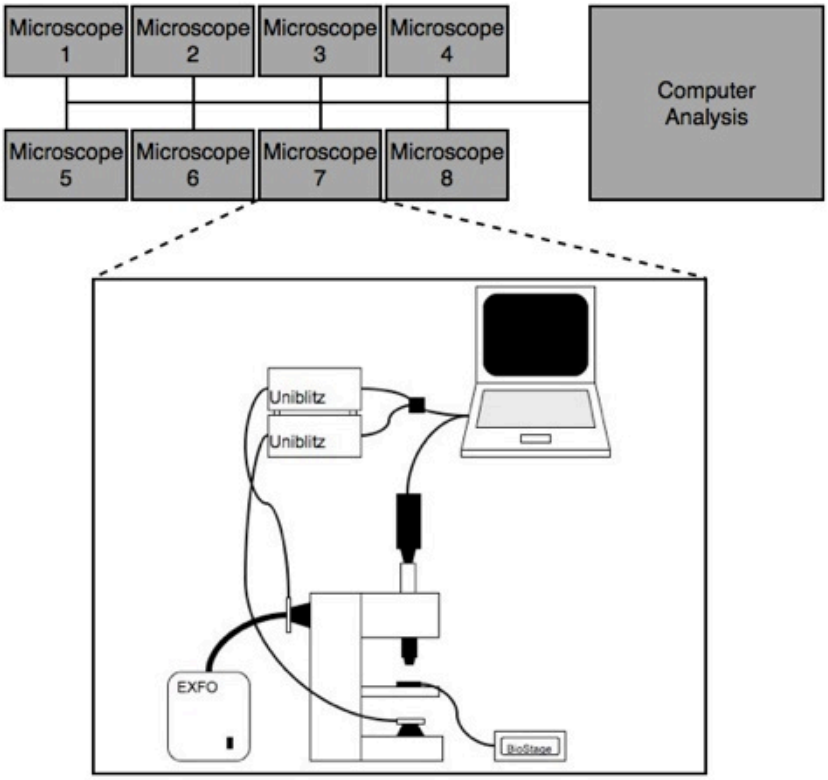

Figure 2. Microscope cluster. Each microscope node (inset) consists of a Nikon E400 microscope, objectives, a heated stage, an Insight camera, and a notebook computer. Each node is networked together and linked to a master controller computer. Two of the nodes are set up with fluorescence capabilities that consist of the EXFO light source and two Uniblitz shutters.

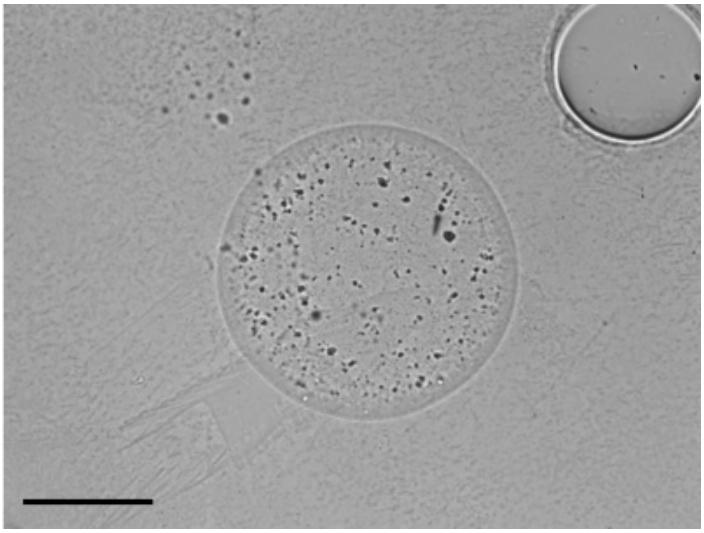

Figure 3. A 20X image of tracking assay apparatus at time = 0. Scale bar, 1 mm.

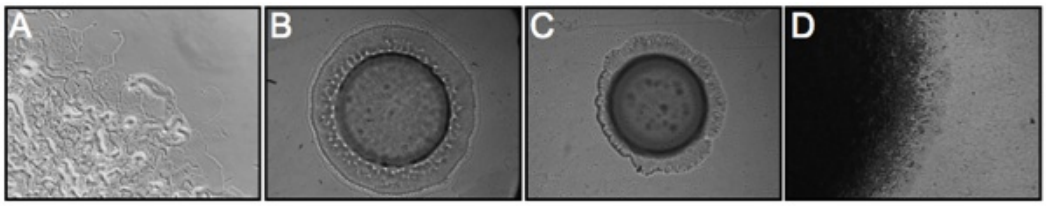

Figure 4. Adaptability of the TM plate complex. (A) a 100X image of *M. xanthus* gliding motility on CTTYE in 1.0% agar. (B) a 20X image of *P. aeruginosa* twitching motility. (C) a 20X image *S. marcescens* swarming motility. Both (B) and (C) were assayed on LB in 1.0% agar. (D) a 40X image of *M. smegmatis* sliding motility on LB in 0.5% agar. This image was captured using the alternative assay configuration seen in Fig 1B.

Video 1. A time-lapse video of an swarm subjected to the tracking assay.

Video 2. A time-lapse video of an *M. xanthus* swarm where 1% of the cells are fluorescently labeled. Alternating phase-contrast and fluorescent images were captured and overlaid to elucidate the position of fluorescent cells within the swarm. This video was captured on CTTYE in 1.0% agar.

Video 3. A time-lapse video of *M. xanthus* gliding motility. This video was captured on CTTYE in 1.0% agar.

Video 4. A time-lapse video of *P. aeruginosa* twitching motility. This video was captured on LB in 1.0% agar.

Video 5. A time-lapse video of *S. marcescens* swarming motility. This video was captured on LB in 1.0% agar.

Video 6. A time-lapse video of *M. smegmatis* sliding motility. This video was captured on LB in 0.5% agar using the alternative assay configuration seen in Fig 1B.

Discussion

Time-lapse microcinematography (TM) has become a standard approach to studying prokaryotic motility [2-7]. Traditionally, TM is performed by using filter paper wicks, thin agar pads, or agar slabs as substrates [8-11]. These methods are adequate and cost effective when used to generate image sequences for general illustrations of bacterial movement. However, if image sequences must result in the generation of reproducible and quantitatively rigorous data, these methods are time consuming and somewhat unreliable. For example, variations in these techniques caused by human error could lead to a wide array of inaccuracies, from irregularities in the agar surface that could dramatically affect the behavior of the bacteria being studied to differences in the focal plane from one side of the assay substrate to the other. To solve these problems, we have designed a TM plate complex that is of sufficiently consistent quality to yield reproducible results by employing silicone gaskets that are both inexpensive and reusable (Fig. 1). In addition, the plate complex is highly modifiable and has proven to stay hydrated and sufficiently oxygenated for more than a week over a variety of media types and agar concentrations.

To facilitate the generation of time-lapse movies, 8 Nikon E400 microscopes were outfitted with 2X, 4X, and 10X objectives each. In addition, an Insight camera (Diagnostic Instruments, Inc.), and a heated stage (20/20 Technologies) was acquired for each microscope. Each camera was linked to a notebook computer on which the highly modifiable image acquisition software SPOT (Diagnostic Instruments, Inc.) had been installed. All the various components were assembled into nodes, each consisting of a microscope, three objectives, an Insight camera, a heated stage, and a controlling computer. Each of the eight nodes were networked together into a cluster and linked to a storage computer which is used to compile, analyze, and store the time-lapse videos generated by the cluster (Fig. 2). The storage computer was outfitted with a 1 terabyte RAID system, which is needed to store the vast amount of data generated by the cluster.

Once complete, the plate complex is placed on the heated stage of the microscope and tracking assay is initiated. Images are acquired at preset intervals that are determined based on the motility rate of the cells. For example, individual *M. xanthus* cells move at a rate of approximately one cell length per minute. If images are acquired of an *M. xanthus* swarm at that same rate (one image per minute), the resulting time-lapse video will appear smooth during playback. Once the image acquisition is complete, the images are compiled into a sequential matrix and played back at a sufficient rate that they appear to be moving (Video. 1). This time-lapse video can now be analyzed using a variety software packages.

Variations on Assay. The TM plate complex is highly modifiable and can be used to visualize many different microorganisms under a variety of conditions. Swarm visualization can be performed using phase-contrast microscopy (which records the behavior of the swarm as a single entity), fluorescence microscopy (which records the behavior of individual fluorescent cells that have been diluted in a non-fluorescent population), or a combination of both (which overlays alternating sequential phase-contrast and fluorescent images) (Video 2). The plate complex has been used to successfully generate time-lapse videos of several prokaryotic behaviors including *M. xanthus* gliding motility, *Pseudomonas aeruginosa* twitching motility, *Seratia marcescens* swarming motility, and the novel *Mycobacterium smegmatis* sliding motility (Fig. 4 and Videos 3-6).

Disclosures

No conflicts of interest declared.

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