Chapter 10

Quantitative Measurement and Analysis in a Synthetic Pattern Formation Multicellular System

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Abstract

Pattern formation has been studied for more than a century in biology. In recent years there are increasing interests in studying it using bacteria and synthetic biology tools to program intercellular communication and cellular response to environment. Quantitative measurement is critical to dissect the interplay between the synthetic gene circuits with underline cellular processes and verify the mechanism determining the pattern formation. Here, we describe simple optical setups for quantitative measurements of the cell density and growth and spatial–temporal dynamic characterization of *E. coli* pattern formation in soft agar plates.

Key words Pattern formation, Synthetic biology, Quantitative biology, Quorum sensing, Time-lapse imaging, Optical density

1 Introduction

One of the fundamental questions in biology is how the cells coordinate their movement and fate to form well-organized spatial–temporal pattern [1–5]. It has been studied in the content of bacterial ecosystems, animal embryo development, as well as stem cell-based tissue engineering. Many molecular components and interactions have been well studied. However the underlying general principles are surprisingly difficult to be identified due to the overwhelming complex physiological context. For instance, the famous Turing model of lateral inhibition for pattern formation, one of the well-received theories by experimental biologists, has been published 60 years ago [6]. However no direct evidence can be found until recently when a group quantitatively measured the key differential diffusivity of Nodal and Lefty to confirm this model in zebra fish embryogenesis [7]. This highlights the importance and difficulty of quantitative biology measurement.

We took an alternative approach using synthetic biology tools [8] to specify and engineer the factors that control patterns formed by populations of *E. coli* cells. Specifically, we engineered the

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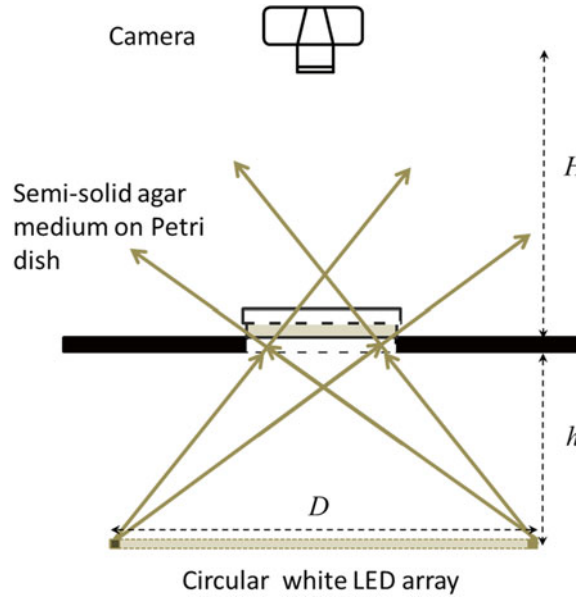

Fig. 1 A schematic view of the pattern formation imaging system. A circular white LED light belt illuminates the agar plate slantwise. Light was blocked except for right beneath the plate. A digital camera is positioned right over the plate so that LED light cannot directly reach it. The dimensions *D*, *h*, and *H* are chosen to enhance the contrast of the cell density variance

quorum sensing systems (LuxI) from *Vibrio fischeri* to *E. coli* cells so that the local cell density is represented by local concentration of signalling molecule *N*-acyl homoserine lactones (AHL). The same cells then sense the local cell density via LuxR-based gene regulation circuit to downregulate expression of CheZ gene and suppress cell motility. This control resulted in a sequential and periodic stripe formation. Careful quantitative measurement and modelling revealed the principle behind controlled individual cell behavior and pattern formation at the macroscale, which is then confirmed by experimental verification of model predicted pattern tuning method [9]. In this chapter we describe the optical methods to measure the spatial–temporal dynamics of the pattern and quantitative measurement of cell density profiles and growth rate in Petri dish that can be adapted for similar studies.

In our experiments, the patterns are formed in 0.15–0.35 % and 2 mm thick semisolid agar in Petri dish. The cell density has a typical optical density range of 0.1–3. So it is mostly transparent and very hard to view the patterns of subtle cell density variations. We have developed two easy methods to make instrumentations to measure the patterns. The first one is based on a light scattering principle that enhances the density contrast (Fig. 1). It enables fast two-dimensional pattern acquisition and high spatial resolution but does not provide quantitative density measurement. The second is

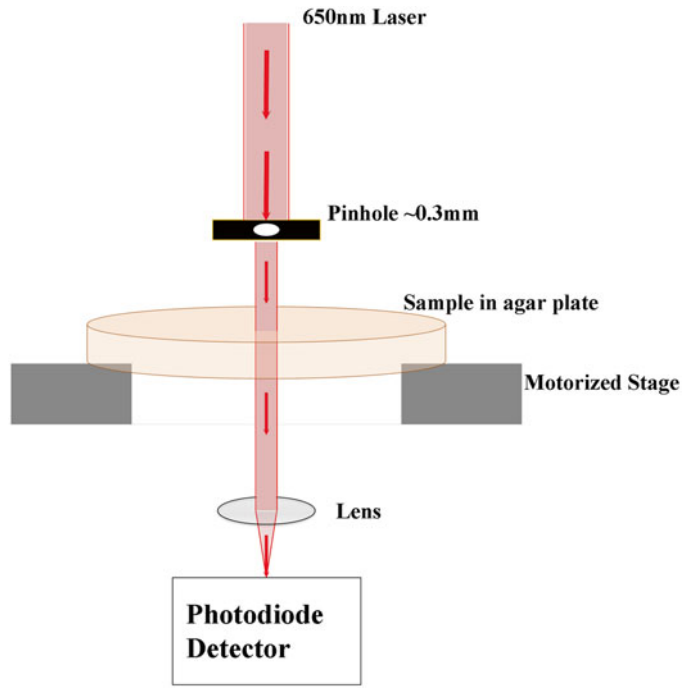

Fig. 2 A schematic view of the customized optical setup for the real-time measurement of the spatiotemporal cell density profile in semisolid agar dishes. A parallel 650-nm laser beam is guided through a 300-μm pinhole, passed through the sample in semisolid agar on the Petri dish at right angle, and collected via a convex lens to a photodiode detector. The light intensity is digitized with a DAQ device and stored on a personal computer. The spatiotemporal scanning is realized with a motorized stage controlled with the PC. The whole apparatus was placed in a warm room (37 °C) throughout the experiment

a direct spatial–temporal optical density measurement setup applied to Petri dish that provides quantitative and spatial–temporal cell density measurement (Fig. 2). Normally bacteria optical density measurement is carried out with run of the mill spectrophotometer and standard plastic cuvette with precise 1 cm optical path length. We utilize similar principle but with extensive calibrations to control for sources of systematic errors.

2 Materials

2.1 Reagent and General Facility

1. Engineered *E. coli* K12 MG1655 strain or other strains to be studied.
2. Semisolid agar plates: 10 ml of Luria–Bertani (LB) medium buffered by 0.1 M HEPES (pH 8.0) containing 0.25 % agar was poured into a 10-cm *Petri* dish and allowed to harden at room temperature for 90 min.

3. Paper template for marking the outline and center of 10 cm Petri dish for consistent seeding of *E. coli* at the center of each semisolid agar plate.
4. A room with constant temperature of 37 °C (*see* **Note 1**).
5. Blackout cloth to insulate ambient light.
6. MATLAB software for data analysis (Mathworks, Natick, MA, USA).

2.2 Bacteria Pattern Imaging

1. Camera and lens: Canon 450D digital single lens reflex camera and EF 50 mm f/1.8 II lens (*see* **Note 2**).
2. Tripod: Benro C-2691 carbon fiber tripod with B1 Ballhead.
3. Camera controller: Canon timer remote controller TC-80N3 for time-lapse image capturing.
4. Illumination and controller: Off-the-shelf LED light belt with 30 0.15 W white light-emitting diodes (LED) per meter, and 12 V regulated power supply of your trust brand.
5. Spirit level: Any brand from hardware stores.
6. Sheet materials for illumination box construction: 5 mm thick transparent polyacrylic sheet from local home improvement stores, professionally cut to four 14 cm-by-40 cm side panels and two 41 cm-by-41 cm top and bottom panels.
7. Light diffuser for LED light belt: Semiopaque plastic tubing with diameter of 5 mm.

2.3 Bacteria Cell Density Measurement in Agar Plates

1. One simple optical table with the size of 60 cm by 60 cm or bigger.
2. Motorized stage: Stand-alone LabVIEW programmable motorized one-dimensional stage with at least 75 mm travel.
3. Red laser: 650 nm/2 mW laser diode, DA650-2-3 (Huanic Co., Xi'an, China) (*see* **Note 2**).
4. Voltage-regulated power supply for red laser diode: Any 5 V unit.
5. Pinhole: A thin metal sheet with one 300 μm diameter pinhole drilled/laser burnt through.
6. Red laser intensity detector: Amplified Si photodetector, PDA36A (Thorlabs, Newton, NJ, USA) (*see* **Note 2**).
7. Lens: Plano-convex lens, f=75 mm, LA1208 (Thorlabs, Newton, NJ, USA).
8. Various optomechanical components and mirrors necessary for laser, pinhole, and photodetector fasten and light path arrangement (Thorlabs, Newton, NJ, USA).
9. Adjustable fasten mechanism to lock Petri dish onto the motorized stage.

10. Data acquisition: USB-based DAQ device capable of digitizing analog signal at 12 bit/10 kHz, model USB-6009 (National Instruments, Austin, TX, USA).
11. Personal computer, with LabVIEW programmable software (National Instruments, Austin, TX, USA).

3 Methods

3.1 Construction and Usage of a Pattern Formation Imaging System

A circular white LED array illumination of the Petri dish from below (Fig. 1). The light will not be directly collected by the camera. Instead, *E. coli* scatters the light. The scattered light, which is positively correlated with cell density, is captured by the camera. The procedure for construction and using of this system is described as follows:

1. Level the bottom acrylic sheet panel (41 cm by 41 cm) (*see* **Note 3**) on the floor at a low-traffic region of the warm room.
2. Cut a 1.1 m LED light belt piece (*see* **Note 3**), cover it with a semitransparent plastic diffuser tubing of the same length, connect the two electrode with 2 m insulated metal wire, and connect the other ends of the wire to a 12-V regulated power supply with correct polarity.
3. Fix the LED light belt with diffuser tubing to the bottom panel in a circular loop with about 35 cm diameter, and align its center with the center of the bottom acrylic panel (*see* **Note 4**).
4. Glue the top panel and four side panels of the acrylic sheet together to form a box cover. Remove the inner layer of the non-transparent protective paper of the top panel. Cut and remove a centered circle with diameter of 8.5 cm from the outer/upper paper protective layer to allow the Petri dish to be illuminated. The rest of the paper protective layers are left untouched to block the unnecessary light leaking.
5. Put the box cover exactly on top of the bottom panel with circular LED light belt. Level the top panel. It effectively becomes a special light box. Keep the power supply outside the light box.
6. Put a testing agar plate with faint pattern on top of the circular light-exposed area of top panel switch on the 12-V power supply (*see* **Note 5**). From the top of the agar plate, there should be little LED light directly observable, and the *E. coli* density variations (patterns) should be easily observable as only the light scattered by *E. coli* reaches the eyes (*see* **Note 6**). If not, adjust the diameter of the circular LED light belt and the height of the light box.

7. Position the digital camera right on top of the Petri dish with distance H approximately 50 cm using a tripod and flexible Ballhead. The back of the camera needs to be leveled using a spirit level. Secure the camera using the corresponding fasten knobs on the tripod and Ballhead.
8. Carefully adjust the focus through back LCD panel using the "live view" mode and 10× zoom option. Secure the focus ring of the lens using a piece of post tape (*see* **Note 7**).
9. Set the exposure to ensure best imaging quality. Set the white balance to match the color temperature of the LED light belt. A typical setup is ISO200, f/5.6, and exposure 0.6 s. It needs to be determined using in-camera meters.
10. Connect the camera remote controller to the camera, and set it to take pictures every 10 min. Ensure enough battery charge and storage space on the memory card before experiment.
11. Carefully position a newly seeded semisolid agar Petri dish to the exposed center of top panel of the light box (*see* **Notes 8–10**). Lay a ruler by the plate to provide scale in the acquired images.
12. A large piece of blackout cloth is used to cover the entire system, except for the camera remote controller and power supply, to prevent ambient light from interfering with image acquisition. Record the time of seeding, and start the time-lapse acquisition loops using the remote controller.
13. After experiment, stop the camera remote controller, and take out the memory card. Transport the images to a computer, and use MATLAB program to exam the images. Find one of the three channels (RGB) representing the highest dynamic range without saturation, and use it to generate grayscale imaging for further analysis. Determine the converting factor between pixel and millimeter manually using the image of the ruler (sample images in Fig. 3).

3.2 Construction and Usage of a Spatial–Temporal Optical Density Measurement Setup

The imaging system described above can only qualitatively record the cell density of the pattern. We constructed another optical setup to quantify the cell density profiles. The spatial–temporal optical density measurement setup is designed to measure the attenuation of a 650 nm laser beam after passing through an agar plate with the right angle (Fig. 2). The optical density (OD, absorbance) is proportional to the product of cell density and optical path length (agar thickness). Standard spectrophotometer uses standard cuvette to ensure 1 cm optical path length, so that cell density can be determined from OD readout directly. The semisolid agar in Petri dish (*see* **Note 8**) usually forms an inverse tapered shape with the lowest thickness at the center. To avoid such systematic error on the optical path length, we measured the location-dependent transmittances for known standard cell densities.

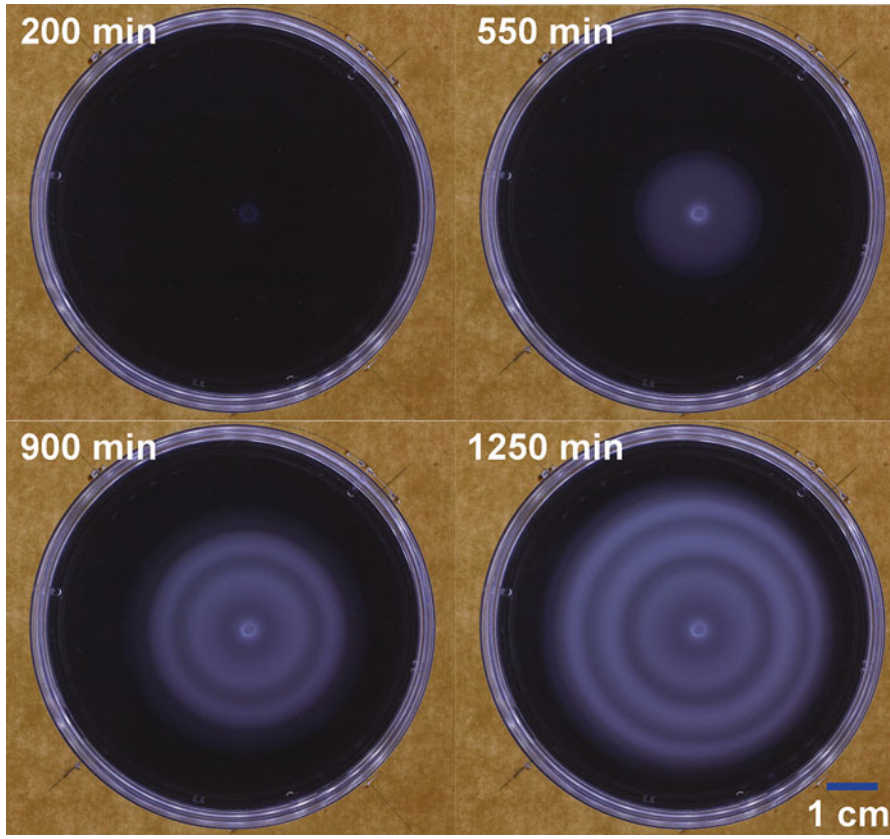

Fig. 3 Time-lapse pattern images of stripe-forming engineered *E. coli* using pattern formation imaging system. A series of images indicate the dynamics and geometry of the pattern formation

Hence we established a series of standard curves for any given radial positions. The step-by-step instruction is given below:

1. Position the optical table at the low-traffic area of the warm room.
2. Secure the motorized stage on the optical table, and level it using the spirit level (*see* **Note 11**).
3. Install a fasten mechanism for gently clamping the semisolid agar plate in place to the motorized stage, and clamp an empty and uncovered Petri dish for optical path adjustment (*see* **Notes 10** and **12**).
4. Fasten the 650 nm red laser diode to a kinematic mount, and position them right on top of the plate.
5. Wear proper laser safety goggle, and switch on the laser diode by connecting it to a 5-V regulated power supply.
6. Adjust the angle of the laser diode by adjusting the kinematic mount so that the reflection laser beam hits right back into the

front lens of laser diode. This ensures the right angle between the laser beam and agar plate.

7. Fine-tune the plate and laser beam so that the laser beam could scan right across the dead center of the plate with the motorized stage, and cover most of the radial space of the plate.
8. Position a thin sheet metal with 300 mm pinhole between the laser diode and the plate to generate a thin laser beam and improve the spatial resolution.
9. Position a collection convex lens below the plate to collect the transmission light.
10. Position an amplified photodiode detector so that all the collected transmission light hits the light-sensitive area of photodiode detector.
11. Connect the output of photodiode detector through a DAQ digitization device (*see* **Note 13**) to a computer (*see* **Note 5**), and visualize the light intensity using the accompanying Nation Instrument software for the DAQ. Set the digitization as 12 bit and 10 kHz.
12. Replace the empty plate with a blank semisolid agar plate (*see* **Note 14**).
13. Adjust the gain on the amplified photodiode detector so that, when scanning the empty semisolid agar, the signal collected by the detector and digitized by DAQ is as close to the maximum as possible without saturation. This ensures the maximum dynamic range of the system.
14. Connect the motorized stage through stage controller to the same computer. Write customized LabVIEW program to coordinate the stage movement, data acquisition through DAQ, and data storage. A diagram of the program is shown in Fig. 4.
15. Set the step size to 0.2 mm and total travel of 75 mm for the motorized stage to generate 376 radial positions.
16. At each position, the transmission light was sampled at 10 kHz and 12 bit using the DAQ device. The average of 1,000 data points was used to reduce the noise to the measurement at each position.
17. Set the repeat frequency for this scanning at once every 10 min.
18. Ensure the constant input laser intensity throughout the experiments with a regulated 5 V power supply, verified using the photodetector.
19. Cover the entire setup with blackout cloth using a metal frame to insulate the ambient light and prevent its interference with scanning.

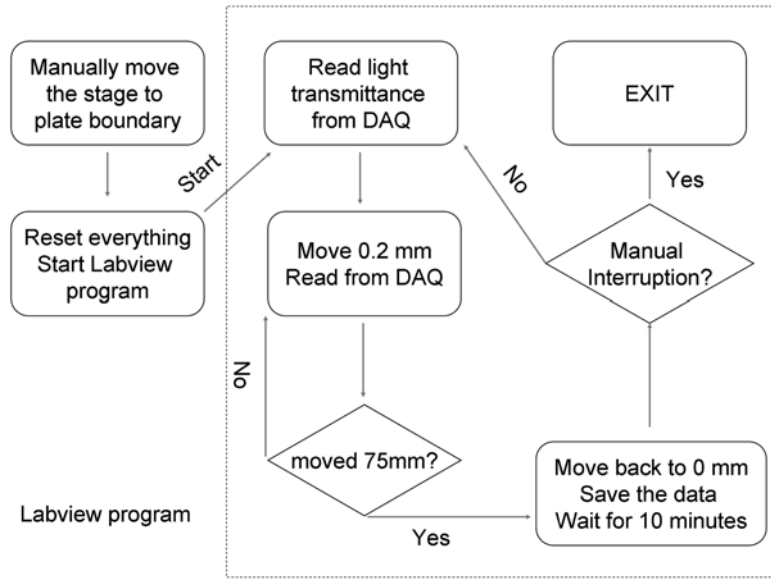

Fig. 4 A diagram of the automatic LabVIEW program performing periodic radial scanning of cell density profiles. The program is highlighted in the *dashed box*

20. The following **steps 21–29** are to obtain standard curves for each position for various known cell densities to calibrate this agar plate optical density scanner.
21. Grow a large amount of *E. coli* cells to mid-exponential phase (OD600 = 0.1–0.2).
22. Stop the cell growth by washing twice with nutrient-depleted LB and concentrate to 9.6×10^9 cells/ml.
23. Subsequently, serially dilute the cells with nutrient-depleted LB. For each cell density, vigorously mix 15 ml of cells with an equal volume of pre-warmed nutrient-depleted medium containing 0.5 % agar and pour into three Petri dishes with 10 ml each.
24. Allow all plates to harden at room temperature for 90 min. The final cell densities ranged from 0.03 to 4.8×10^9 cells/ml.
25. Move the plates into a warm room and place on the motorized stage one by one.
26. Scan the light transmittance radially for each plate, and load all the data using MATLAB program.
27. At each position along the radius, plot the ratio of the output intensities to the input intensities (measured using a blank agar plate), namely, the transmittances T, against the known cell densities for 21 different seeding densities (an example curve in Fig. 5a).
28. Generate totally 376 position-dependent density–transmittance standard curves (using a step size of 0.2 mm for a scanning

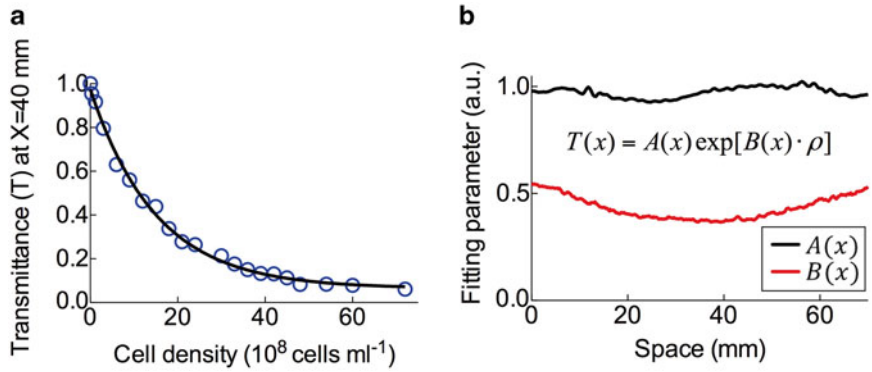

Fig. 5 The calibration curve of light transmittance versus real cell density in semisolid agar plates. (**a**) An example of the fitted curve of the transmittance as the function of cell density at a specific position. (**b**) The spatial distribution across the center of the dish, of the two fitting parameters for the standard curve, $A(x)$ (*black line*) and $B(x)$ (*red line*). This is derived from 376 standard curves (scanning range = 75 mm; step size = 0.2 mm)

range of 75 mm), and fit the results to an exponential function for each position x:

$$T(x) = A(x)\exp\left[B(x)\cdots\rho(x)\right],$$

where $A(x)$ and $B(x)$ are the position-dependent fitting parameters (Fig. 5b).

29. From these experimentally determined standard curves, we could invert them to compute the real cell density profile $\rho(x)$ from the measured transmittance profile $T(x)$.
30. For each experiment, place the semisolid agar plate seeded with engineered *E. coli* cell on the motorized stage, double check the scanning alignment, turn on the computer and power supplies for every component, cover with the blackout cloth, record the seeding time, and start the scanning.
31. After the experiments, convert the recorded light transmittance profiles $T(x)$ to the real cell density profile $\rho(x)$ for each time point.
32. For growth curve in semisolid agar, we uniformly mix the cell in semisolid agar plate, scan the transmittance periodically, and carry out the computation (see an example growth curve in Fig. 6). Growth parameters can be extracted and used in the mathematic models.
33. For measurement of cell density profile and dynamics, seed a small drop of engineered *E. coli* at the center of a semisolid agar plate, load it onto the setup, turn on the computer and power supply for laser diode and photodetector, load the LabVIEW program, cover the entire setup except for the computer, and start the periodic scanning (an example of such measurement in Fig. 7).

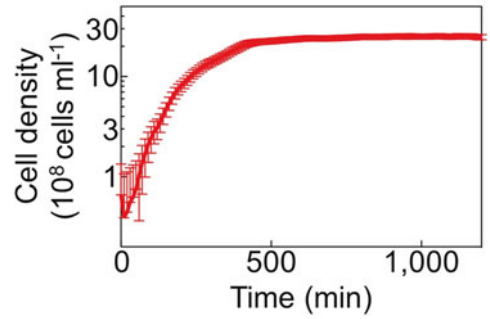

Fig. 6 A growth curve of *E. coli* in a Petri dish containing 10-ml 0.25 % LB agar. After seed culture and preculture growth, cells were diluted 200-fold into the pre-warmed LB media containing 0.25 % agar. Cell–agar mixtures were poured into Petri dishes, allowed to harden at room temperature for 90 min, and then moved back into a 37 °C incubator. At fixed intervals, cell density measurement is performed and computed

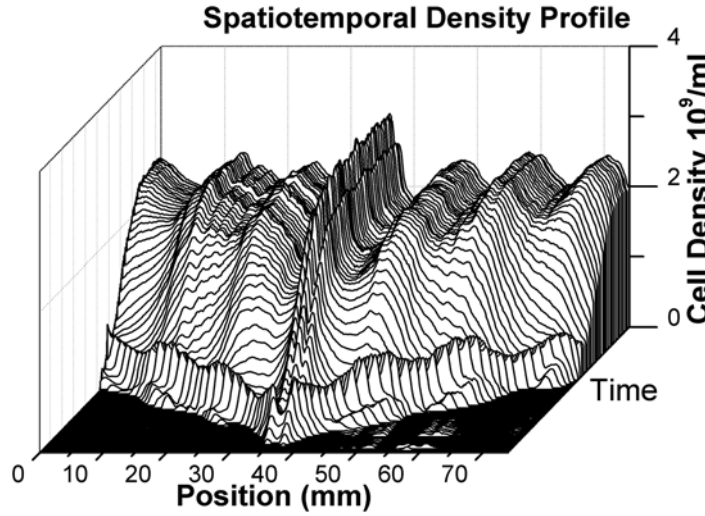

Fig. 7 Spatial–temporal cell density profiles provide quantitative characterization of the pattern-forming *E. coli*. This surface plot represents a typical stripe pattern

4 Notes

1. If the warm room is not available, it is possible to reduce the pattern formation imaging system to fit into a regular incubator. The optical density measurement system is too big to fit into any incubator. Therefore an environment chamber with adjustable temperature control will need to be built around the setup. Alternatively, one can implement this setup using a microscopy with environment chamber and motorized *x*–*y* stage.
2. With the optical setups running at 37 °C constantly, one biggest issue we faced is the reliability of the electrical and optical

components, as most of them are not designed for this temperature. We have narrowed done to the list of components after several rounds of trial and error, such as the laser, photodiode detector, and camera.

3. The dimension of the light box and circular LED light belt can be optimized manually prior to ordering the acrylic panel.
4. The light diffuser tube is not necessary if the LED density on the light belt is high enough. Colored LED should also be OK, but testing is needed.
5. The pattern-forming *E. coli* might be temperature sensitive. For the imaging system, the heat generated from the LED might heat up the light box significantly and alter the temperature of the agar plate. We found that by lowering the voltage of power supply to the LED light belt, this heating effect can be greatly attenuated. Heat-dissipating holes can be drilled to the side panels of the light box to reduce the heating effect. The computer could heat up the area around optical density measurement setup. It can be either moved further away or using a lower power one, such as a laptop computer instead.
6. This scattering imaging is very sensitive to imperfection in the surface of the exposed top acrylic circle as well as the Petri dish. Care needs to be taken to avoid scratching them or accumulate dusts. Alternatively it can be cut away and replaced with a thin glass circle for easy maintenance.
7. Any digital single-reflex camera will work due to its large CCD sensor size and higher sensitivities. Prime lens has better optical quality than zoom lens and also better light collection. Some lens, when pointed down, cannot lock the focus properly. It can be solved by using either tape to stop the drifting or the closest focus so that no further drifting is possible. To increase the dynamic range of the images raw image files instead of jpg files can be stored. But it takes much larger storage space.
8. Special care needs to be taken to make the agar plates for ensuring even cooling down of the agar. The table for pouring the plates needs to be leveled. For every stack of plates, the bottom one has different temperature as it is closest to the table; so it is neglected.
9. Consistent seeding of the *E. coli* at the dead center of the plate helps to obtain consistent good measurements. We use a printout paper sheet to align the seeding for every plate.
10. Semisolid agar plate is very gentle, so it needs to be handled with care, including appropriate leveling, and gentle movement with motorized stage.
11. It is also possible to perform two-dimensional scanning of the optical density of the agar plate if motorized *x*–*y* stage is available.

It will take much longer time if the same step size for one-dimensional scanning. The scanning time should be kept significantly shorter than the cell doubling time and characteristic time scale for pattern formation. If it is not possible, then increasing step size or reducing the scanning area is required.

12. When clamping the agar plate on the motorized stage, it is critical to use minimal force, as any force might deform the plate and interfere with density measurement.
13. Electronic devices, such as DAQ, should be pressed against the stainless steel optical table to alleviate heat building up within the devices.
14. For every experiment, a couple of blank agar plates are needed to obtain the spatial blank reading.

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