

Open-source automated imaging systems for  
spatiotemporal gene expression analysis during

*Escherichia coli* colony biofilm development

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時空間的遺伝子発現解析のためのオープンソース  
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## **General introduction**

### ***E. coli* as a model organism**

*Escherichia coli* (*E. coli*) is a facultative anaerobic, Gram-negative rod-shaped bacterium belonging to the family Enterobacteriaceae. Since its discovery by Theodor Escherich in 1885, *E. coli* has been extensively used as a central model organism in life sciences, due to its ease of cultivation, well-characterized genetic background, and short generation time [1,2].

The completion of the whole-genome sequence of the K-12 MG1655 strain in 1997 marked a major transition from single-gene analyses to genome-wide investigations. Since the functions of many of the approximately 4,000 genes remained unknown, comprehensive genetic resources became essential. In response, large-scale collections such as ASKA clone (plasmid clones of whole genes from *E. coli*), Keio collection (systematic single-gene disruptant collection), *E. coli* promoter–GFP transcriptional fusion library and a Venus–YFP fusion-protein library were developed to systematically analyze open reading frames, promoter activities, and protein expression [3-5]. These resources have enabled not only individual gene characterization but also integrative studies of gene regulatory networks, stress responses, and metabolic control [6,7]. As captured in Jacques Monod’s famous statement, “What is true for *E. coli* is true for the elephant,” *E. coli* has served as a model for uncovering universal biological principles. Indeed, seminal studies using *E. coli* have elucidated fundamental mechanisms of transcriptional regulation [8], metabolic flux control [9], gene expression control by cAMP [10,11], and stress responses [12-15], many of which are conserved across multicellular organisms. More recently, *E. coli* has also been employed as a model to study higher-order phenomena such as coordinated behaviors, spatial structuring, and phenotypic differentiation in microbial populations, including biofilms [16-19].

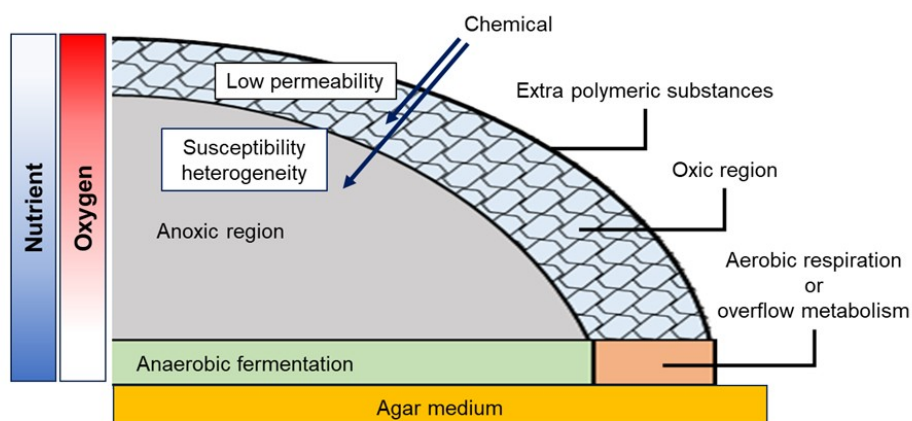
## **Biofilms, a collective mode of growth: biofilm heterogeneity and its spatiotemporal dynamics**

Collective behavior phenomena have been reported in microbial populations, including information sharing through quorum sensing, a cell–cell communication mechanism enabling coordinated gene expression in response to population density, and other cooperative intercellular behaviors [20].

Biofilms are defined as multicellular assemblies in which microorganisms attach to solid surfaces and are embedded in self-produced Extracellular Polymeric Substances (EPS), including curli fibers and cellulose [21,22]. Within biofilms, the three-dimensional architecture restricts molecular diffusion, resulting in spatially heterogeneous distributions of nutrients and oxygen. Consequently, heterogeneity in pH, metabolite concentrations, and redox states emerges [23-26]. In *Escherichia coli* biofilms formed on agar, as examined in this study, such environmental gradients are thought to drive spatial differentiation of metabolic states [16,17,27,28] (Fig. 1). Therefore, metabolic and growth processes targeted by antimicrobial agents become non-uniform within biofilms, giving rise to heterogeneity in drug susceptibility and persistence. In addition, the EPS matrix composed of curli and cellulose physically limits the penetration of antimicrobial agents and disinfectants, thereby protecting cells from external stresses [22,29]. Through the combined effects of metabolic heterogeneity and restricted drug penetration, cells in biofilms exhibit enhanced tolerance compared with planktonic cells, leading to persistent infections and surface contamination of medical devices, catheters, and industrial piping systems. Therefore, understanding how heterogeneity within biofilms is generated and maintained is essential for developing effective strategies to control biofilm-associated problems [30,31].

To investigate heterogeneity within biofilms or other collectives, approaches combining spatiotemporal sampling using Fluorescence-Activated Cell Sorting (FACS) or automated sampling systems with omics analyses have been employed, providing evidence for differences between cells located in the interior and exterior of biofilms [17,32,33]. However, due to destructive and labor-

intensive sample preparation, temporal and spatial resolution are limited. Although reporter-based time-lapse approaches lack comprehensive coverage, they enable higher temporal and spatial resolution by imaging biofilms formed in microfluidic devices or on agar surfaces [34-37]. Microfluidic platforms offer high controllability, allowing precise control of flow rates, medium composition, and the timing of substance addition. However, their implementation and long-term operation entail substantial costs. In contrast, macroscale agar-based biofilm growth culture in Petri dishes or well plates, while lacking perfusion control, can enable simple, low-cost experiments. However, time-lapse imaging of such large biofilms requires continuous use of adapted live-imaging systems, which remain expensive in their commercial form and are not broadly accessible.



**Fig. 1. Model for metabolic heterogeneity and chemical resistance in *E. coli* colony biofilms.**

This model represents a sagittal plane view through the center of a mature biofilm growing on the surface of nutrient agar plate. Oxygen availability is highest at the outer periphery of the biofilm and surface, whereas nutrient accessibility decreases toward the upper regions due to increased distance from the nutrients in agar, resulting in a population of metabolically less active cells. In the lower outer layer, nutrient access is maximum and cells exhibit aerobic respiration or overflow metabolism. In contrast, cells located in the biofilm interior experience reduced oxygen availability due to consumption and physical barrier by the outer layers but retain access to nutrients, leading to predominantly anaerobic fermentative metabolism. The model was reproduced with modifications based on Díaz-Pascual et al. eLife 2021;10:e70794 [17] under Creative Commons Attribution 4.0.

## **Significance and challenges of laboratory automation**

Laboratory automation to improve throughput, efficiency and reproducibility in scientific studies has gained increasing attention. These technologies have been successfully applied to high-throughput experiments [38-40]. However, in many research laboratories, particularly in academic settings, the adoption of automation remains limited, largely due to economic constraints [41]. Moreover, experimental requirements vary widely among laboratories, and existing commercial systems often lack the flexibility to accommodate project-specific customization. In addition, maintaining automated systems usually requires specialized technical expertise, which makes their operation highly dependent on specific personnel. As a result, the widespread adoption of laboratory-scale automation remains difficult.

Under these circumstances, future laboratory automation is expected to shift away from the conventional model of relying on expensive proprietary systems toward an environment in which researchers can design, build, and operate experimental devices according to their own needs. The feasibility of this shift has increased markedly with advances in digital fabrication technologies. In particular, device development using 3D printers and single-board computers has emerged as a promising approach for researcher-driven automation [42]. Because 3D printers allow the fabrication of customized structures from a wide range of materials, researchers can flexibly design mechanisms and components that are not available as commercial products [43]. In parallel, single-board computers such as Raspberry Pi and Arduino are equipped with General Purpose Input/Output (GPIO) pins and camera interfaces, thereby facilitating automated control of motors, LED illumination, and imaging systems. Moreover, electronic components for laboratory automation are available at low cost [44]. Together, these technological advances have been shifting laboratory automation from reliance on commercial equipment toward a model in which individual researchers can freely design and implement their own systems. Nevertheless, comprehensive evaluations of device performance and

detailed reports on practical use cases remain limited, indicating that systematic sharing and accumulation of such knowledge are necessary to support bottom-up progress in scientific research.

Based on this background, the aim of this study was to investigate the spatiotemporal heterogeneity of gene expression profiles in *Escherichia coli* biofilms through the development of low-cost, open-source automated live-imaging systems and their application to biofilm analysis.

In Chapter 1, biofilm formation conditions of *E. coli* colony biofilms on agar were examined and characterized using Congo Red staining. Characteristic wrinkle formation and dye-binding properties associated with curli production were observed in biofilms formed by the parental strains of different *E. coli* collections. Observations using fluorescent reporter strains further suggested spatial heterogeneity in fluorescence intensity; however, single-mode fluorescence imaging systems were insufficient for distinguishing structural features only from fluorescence signals.

Building on these findings, in Chapter 2, we developed an open-source automated imaging system, LOTUS-MP (LOW-cost, Time-lapse, aUtomated, imaging System for Multiple Plate), which integrates red transmitted-light imaging for quantitative measurement of colony area with green fluorescence imaging for time-series analysis of multiple samples. Performance evaluations demonstrated that LOTUS-MP provides sufficient spatial resolution, positional accuracy, imaging-interval precision, and illumination stability for observing colony biofilms. In addition, fluorescence intensity and colony area measurements obtained using LOTUS-MP showed strong correlations with those acquired using commercial imaging systems.

In Chapter 3, to enable more flexible multimodal analysis while ensuring comparability across samples, a more advanced LOTUS system was designed and developed. LOTUS-12 (for 12 Petri dishes) automatically switches between epi and transmitted illumination as well as green and red fluorescence imaging. The number after the hyphen, here 12, refers to the number of wells that the

sample tray can hold. The system was shown to maintain stable positional accuracy, temporal precision, illumination uniformity, and reproducibility during long-term time-lapse imaging. Furthermore, by using technical and biological replicates, LOTUS-12 was shown to enable reliable spatiotemporal tracking of fluorescence intensity dynamics.

In Chapter 4, spatiotemporal promoter activity dynamics within *E. coli* colony biofilms were analyzed using LOTUS-MP and LOTUS-12. This analysis revealed the presence of distinct promoter clusters based on their spatiotemporal expression in the presence or absence of glucose. Analyses showed that stress response promoters were activated at the biofilm center and subsequently spread toward the periphery during biofilm development. Glucose supplementation suppressed or delayed this phenomenon, indicating that nutrient availability modulates the spatial expansion of stress responses within biofilms.

These developments and findings are discussed below.

Unless otherwise stated, it should be noted that all experiments, data acquisition, and analyses presented in this thesis were independently performed by the author.

## **Chapter 1: Characterization of *E. coli* colony biofilm development**

### **Introduction**

In biofilms, the production of EPS, such as curli fibers and cellulose, is a major determinant of physical strength, permeability, and surface properties that lead to the formation of clinically problematic biofilms. At the same time, in the laboratory the extent of EPS production serves as an indicator of biofilm-forming capacity [16,45]. *E. coli* strains commonly used as models in laboratory research may exhibit variable biofilm-forming ability depending on genetic background and environmental conditions. Therefore, it is essential to first clearly characterize strains under biofilm-forming conditions before further analysis.

## Results

### 1. Examination of biofilm formation conditions

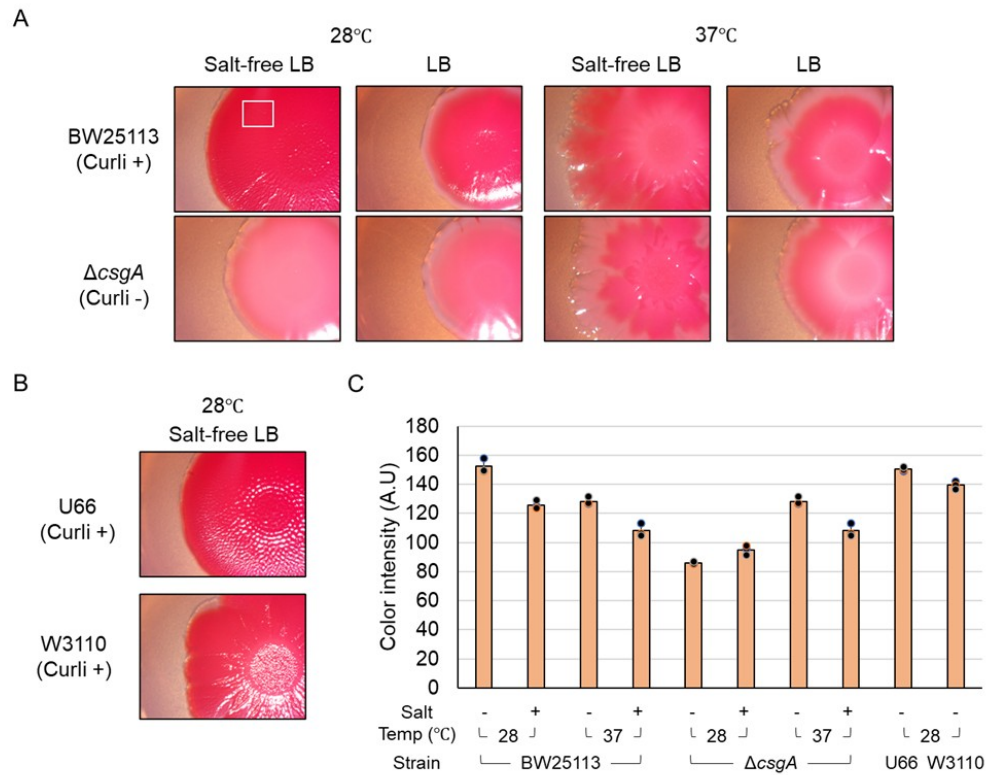
Previous studies have shown that culture conditions, including medium composition and temperature, strongly influence biofilm formation in *Escherichia coli*. Low-temperature conditions (around 28 °C) and low-osmolarity media such as salt-free LB are known to promote the production of EPS such as curli protein and cellulose in *E. coli* [46,47].

In this study, we used biofilms growing at the agar-air interface as previously described by Serra et al. (2013) but using alternative strains. Congo Red can be readily used to assess EPS production because it binds the abundant EPS components curli fibers and cellulose [22]. Based on this, we examined whether biofilm formation is promoted under previously reported conditions (salt-free LB medium and 28°C) in several laboratory *E. coli* strains, including the MG1655 strain carrying the pUA66 plasmid (U66 strain), a widely used K-12 strain used as the host for the promoter reporter collection [5] hereafter referred to as AZ collection, BW25113, the host strain of the Keio collection [4], and the W3110 strain, another commonly used K-12 strain [48]. While cellulose is a common EPS component, all strains used in this study are expected to produce only curli fibers due to a mutation in the cellulose biosynthesis gene *bcsQ* [22].

First, BW25113 and a  $\Delta csgA$  strain (curli major subunit deletion mutant) were cultured on Congo Red-containing agar plates to assess curli production (Fig. 2). When the BW25113 strain was cultured at 28°C in salt-free LB medium, colonies developed pronounced surface wrinkles and exhibited strong Congo Red staining. In contrast, at 28°C in LB medium, Congo Red staining was reduced relative to the salt-free LB condition, and wrinkled structures were rarely observed. Cultivation at 37°C further decreased Congo Red staining in salt-free LB medium, and staining was even weaker in LB medium.

In the  $\Delta csgA$  strain, cultivation at 28°C in salt-free LB medium resulted in a marked reduction in Congo Red staining compared with the wild-type strain, and no surface wrinkling was observed. Similar results were obtained in LB medium at the same temperature. At 37°C in salt-free LB medium, Congo Red staining was observed to spread outward from the center of the biofilm; however, staining was largely restricted to the central region. When cultured at 37°C in LB medium, the  $\Delta csgA$  strain exhibited low Congo Red staining and a simple colony morphology comparable to that of the BW25113 strain under the same conditions.

These results indicate that cultivation at 28°C on salt-free LB medium induces curli-associated biofilm formation in Keio collection host strains. Furthermore, biofilms formed by the U66 and W3110 strains displayed Congo Red staining intensity and surface roughness comparable to those observed in BW25113. Based on these findings, these specific culture conditions were adopted for subsequent experiments.

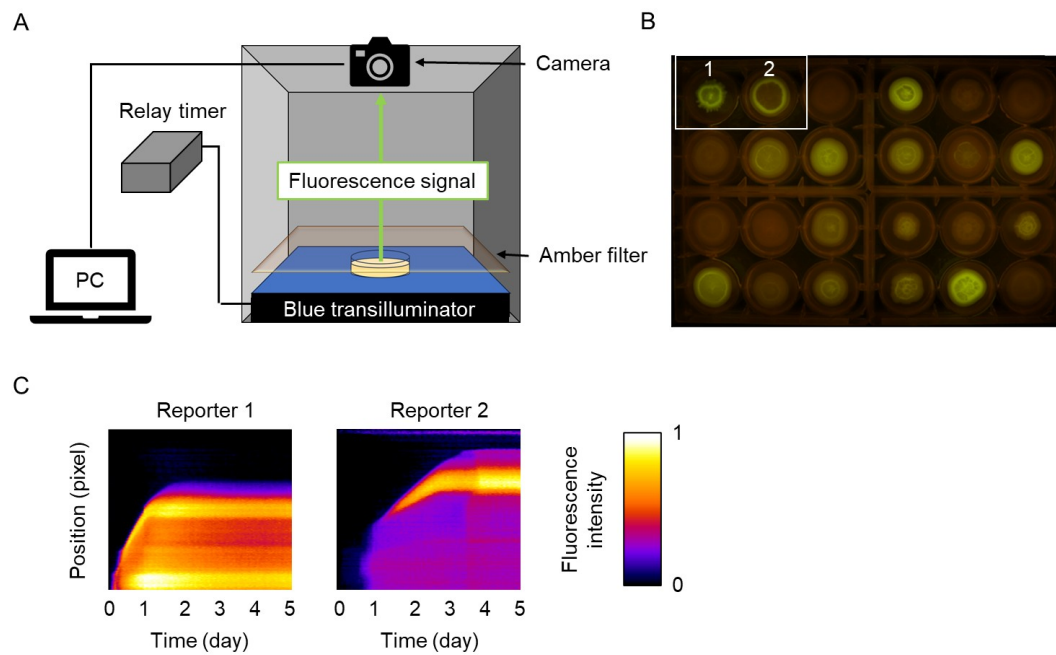


**Fig. 2. Assessment of biofilm formation using Congo Red staining.**

Biofilms were cultured for 8 days at 28°C or 37°C on Congo Red agar plates (1.5%) containing the indicated medium. (A) Stereomicroscopic images of *E. coli* biofilms formed under various culture conditions. (B) Representative images of biofilms formed by MG1655 (U66), the host strain of AZ collection and W3110, used for the YFP-fusion protein collection. (C) Quantification of biofilm red color intensity for each strain and culture condition. The region of interest (ROI) was defined as the middle region of the biofilms (enclosed by white rectangle). Bars show the mean and whiskers represent SD (n=3, technical replicates). Dots indicate data points.

## **2. Limitations of single mode imaging for spatiotemporal analysis of biofilms**

To characterize functional differentiation and heterogeneity within biofilms, we analyzed spatiotemporal changes in promoter activity during the development of biofilms under the conditions described above (salt-free LB at 28 °C). Time-lapse imaging of a subset of the AZ reporter collection was performed using a blue transilluminator and amber filter for detecting GFP fluorescence (Fig. 3). Although distinct fluorescence intensity patterns were observed among different reporter strains, the system was limited to fluorescence detection, which imposed significant constraints. For example, the possibility that the observed increases in fluorescence signal reflect local biofilm thickening due to cell accumulation, rather than true spatiotemporal changes in promoter activity, could not be excluded.



**Fig. 3. Single-mode imaging system prototype and its application to spatiotemporal analysis of fluorescence reporter signal.**

(A) Diagram of the first imaging system prototype. A blue LED transilluminator with a timer-controlled power-supply is used for periodic illumination and synchronized with the shutter activation of an SLR camera controlled with a computer. Green light fluorescence is selected with an amber filter. (B) Fluorescence images of reporter strains in *E. coli* biofilms. Images were acquired 5 days after the start of cultivation. (C) Spatiotemporal changes in reporter fluorescence intensity within biofilms. Fluorescence intensity temporal profiles (600-pixel line) were obtained using Line Selection tool in Fiji.

## Discussion

In this chapter, we first examined the conditions for biofilm formation in each parental *Escherichia coli* strain. Strong Congo Red staining and wrinkle formation in the parental strains were consistent with expectations based on previous studies [49-51]. In contrast, strong attenuation of Congo Red staining in the  $\Delta csgA$  strain was expected but residual staining may result from binding to uncharacterized components, as also observed at 37°C (Fig. 2A). In addition, previous reports have suggested that Congo Red staining can vary depending on environmental conditions and may also reflect staining of dead cells. Therefore, the present observation is likely attributable to binding to non-curli factors or dead cells [52].

These preliminary results highlighted the need for an imaging system capable of capturing both colony biofilm morphology and density as well as fluorescence. However, commercial live-imaging systems that support multimode observation are costly, and prolonged use of shared instruments is impractical. In addition, existing systems often lack specific features required for precise experimental purposes. These considerations led us to conclude that developing our own imaging system capable of independently capturing both biofilm morphology and fluorescence signals would be advantageous and necessary to pursue screening in a continuous mode and higher throughput.

The following two chapters describe the development of alternative DIY live-imaging systems specifically designed in response to these limitations and for different purposes.

## **Chapter 2: Development of LOTUS-MP, an automated imaging system for biomass quantification and fluorescence imaging of growing biofilms**

### **Introduction**

As described in the General introduction, recent advances in low-cost electronic components and open-source technologies have led to a growing number of studies in which researchers design and build experimental devices tailored to their specific purposes. This trend also extends to microbiology, where high-throughput experimental systems have been developed to track temporal changes in gene expression, biomass, and morphology in bacterial colonies [6-7,53-55]. For example, several studies have reported experimental setups combining custom-built transilluminator boxes with single-board computers to enable time-lapse fluorescence imaging of *Escherichia coli* colonies [7,53]. However, when imaging multiple samples simultaneously at wide angle, ghost signals caused by reflection of fluorescence signals and from plate lids in these systems can introduce noise into downstream analyses. In parallel, automated imaging systems using scanners or transilluminator boxes have been reported for tracking bacterial colony growth under bright-field conditions [6,53-54]. These approaches are suited for quantifying changes in colony density and area, but they do not allow visualization of gene expression using fluorescent reporters.

Thus, most existing open-source imaging systems designed for Petri dishes or single-well plates are limited to either fluorescence imaging or transmitted-light imaging.

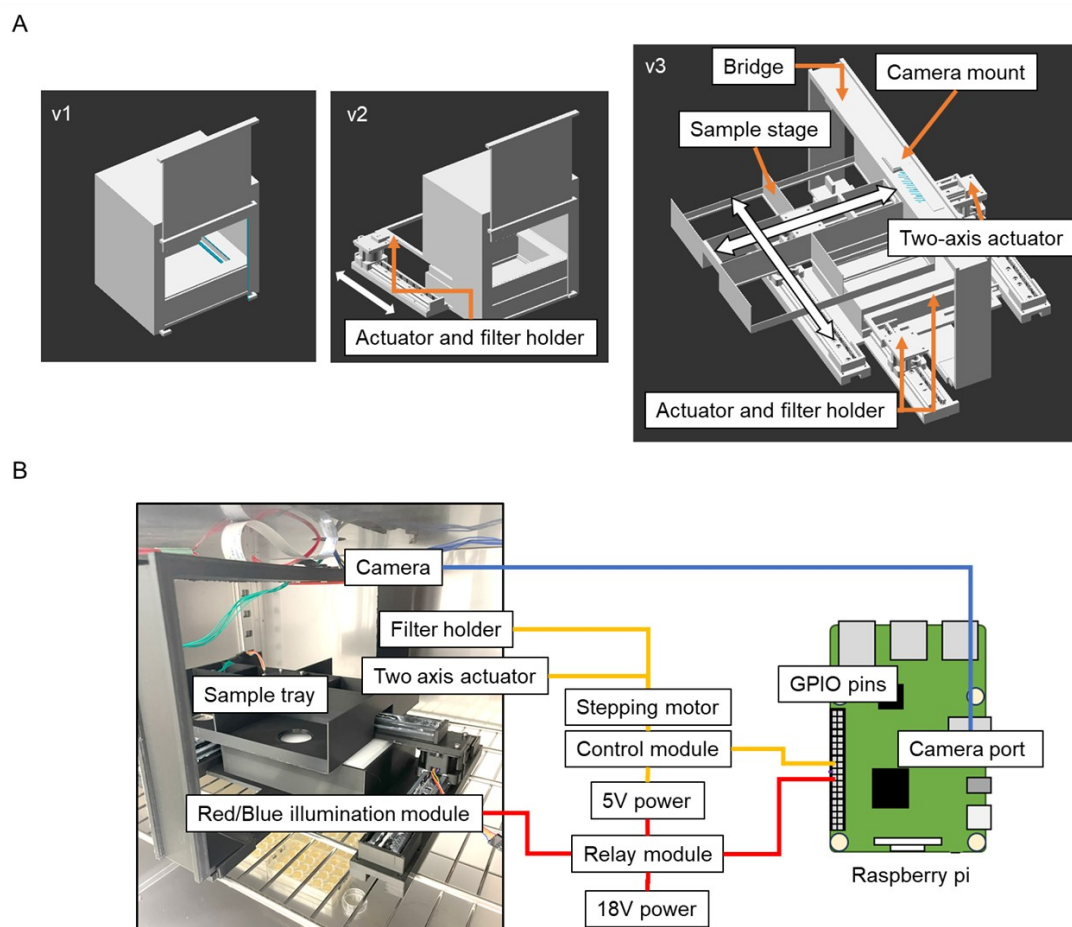
To fill this gap, in this chapter, we developed and validated LOTUS-MP (LOW-cost Time-lapse aUTomated imaging System for Multiple Plates), a multimode imaging system designed to simultaneously capture both biomass and fluorescence signals of colony biofilms.

## Results

### 1. System design

We built an initial version of the imaging system (v1) based on the PFI box [7], but replaced the blue LED illumination module with a dual-color (red and blue) LED module (Fig. 4A), in order to derive biomass estimates from red-light scattering. In the PFI box, a blue filter with a transmission band around 480 nm is used to enhance contrast between background and fluorescence signals. In contrast, our original system was designed without a blue filter to allow for red light transmission. However, because of this, the acquired fluorescence images exhibited low contrast and poor visibility. To address this issue, an improved version (v2) was designed with a robotically movable blue filter. Since a primary goal is to screen multiple samples simultaneously under various conditions, operation of multiple imaging units is required. Parallel operation of multiple units would necessitate individual microcontrollers and illumination modules for each unit. In addition, deploying multiple devices would occupy substantial incubator space and complicate sample handling. To overcome these difficulties, a third version (v3) was designed by introducing a motorized mechanism that allows multiple sample plates to be positioned over a single illumination module.

The position of the sample tray was controlled by stepper motors connected to a two-axis actuator. A red/blue illuminator was controlled using a relay module (Fig. 4B). Image acquisition, as well as relay module and stepper motor operation is managed by a Raspberry Pi computer. With this configuration, the system enables time-lapse, dual-mode fluorescence imaging (green and red) and transmitted-light imaging of four single-well plates.



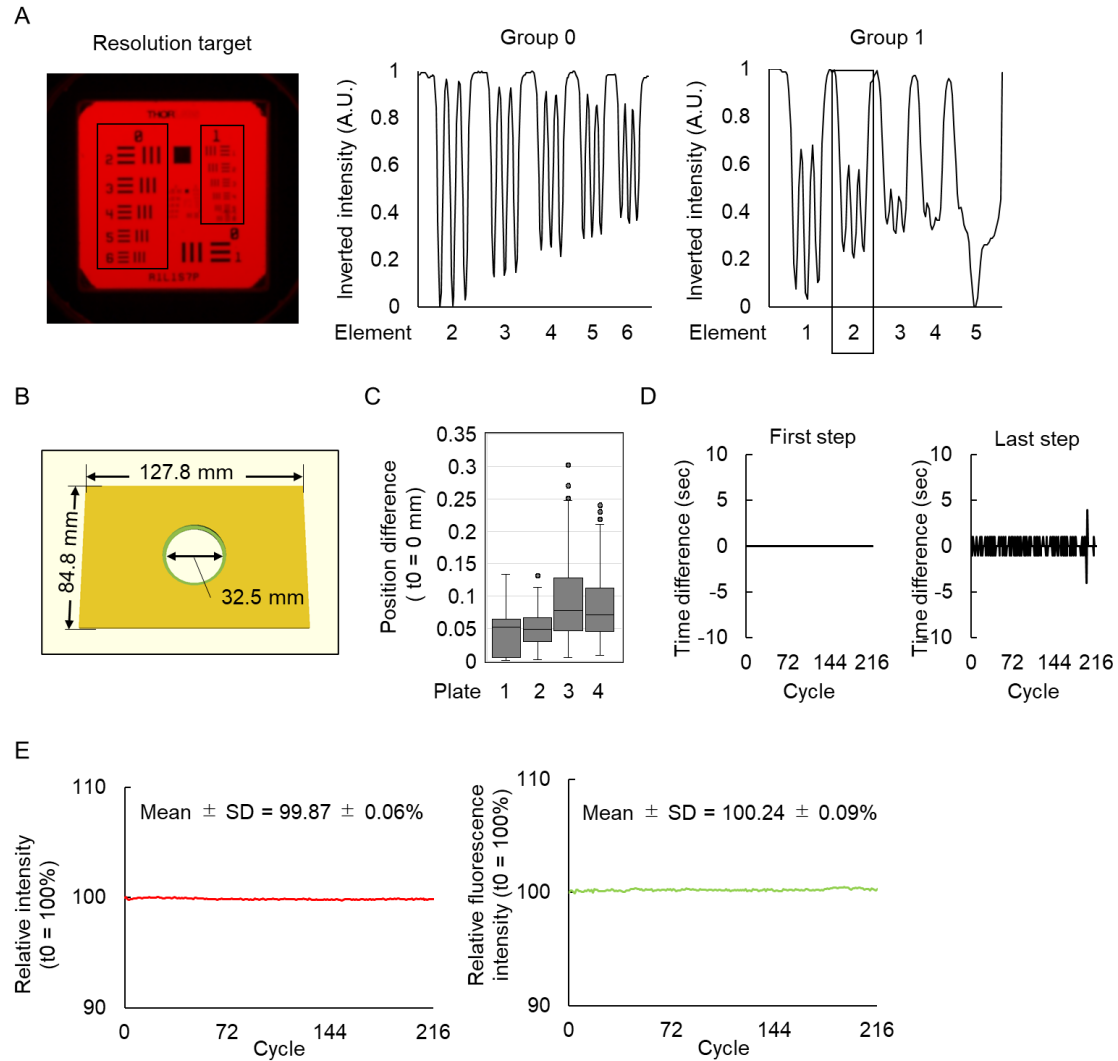
**Fig. 4. Overview of the LOTUS-MP.**

(A) Frame design of versions 1, 2 and 3. The v1 system consists of a blue transilluminator enclosed within a black box. In the v2 system, a movable filter mechanism was added for improved fluorescence image contrast. In the v3 system, a two-axis actuator mechanism was further introduced, enabling the placement and imaging of up to four plates. (B) Connections between Raspberry pi and each component of the LOTUS-MP. The imaging system is centered on a Raspberry Pi 4 Model B, controlling camera, relay modules, and stepping motor controllers. A 16 MP camera (Arducam) is connected to the Raspberry Pi camera port for image acquisition. Relay modules (Wayintop) switch the red and blue illumination modules, which are powered by an 18 V DC power source. Stepping motors (Shanxingan 28BYJ48), driven by control module (ULN2003), actuate a two-axis actuator or a filter holder. A sample tray is mounted on the two-axis actuator. A 5 V DC power source supplies power to the stepping motor control modules and relay modules.

## **2. Benchmark tests: resolution test, accuracy of sample tray alignment and imaging intervals and illumination reproducibility**

To evaluate the spatial resolution of the imaging system, resolution target images were analyzed (Fig. 5A). Because the Michelson contrast (MC) value dropped below 0.1 at group 1, element 2 and above, the effective resolution was determined to be 0.5 mm. Given that the system is intended for imaging microcolonies (>2 mm in diameter), this resolution is sufficient for the intended applications. To assess the accuracy of sample tray alignment and imaging intervals, a plate with an aperture was fabricated, then imaged under red transmitted-light mode for three days (216 cycles) (Fig. 5B-D). Using the initial image as a reference, the maximum positional deviation of the aperture center was approximately 0.25 mm. The difference between actual and ideal timestamps showed no deviation at the start of the imaging process, while a fluctuation of  $\pm 1-4$  s was observed at the end of the imaging process. To evaluate the stability of transmitted-light and fluorescence intensities, a green fluorescent acrylic plate was imaged over time in both red transmitted-light and fluorescence modes (Fig. 5E). The intensity values remained stable throughout the experiment (red:  $99.87 \pm 0.06\%$ ; fluorescence:  $100.24 \pm 0.09\%$ ;  $t_0 = 100\%$ ).

Together, these results demonstrate that the system provides sufficient resolution for colony detection and maintains stable positional alignment, imaging intervals, and illumination intensity during time-lapse imaging.



**Fig. 5. Assessment of imaging intervals, alignment position, illumination intensity and fluorescence intensity stability.**

(A) Determination of resolution. Group 0 and 1 correspond to the left and right bar patterns of the resolution target image, respectively. (B) PLA plate with circular apertures used as a positional reference. (C) Variability in the center position of each aperture across sample holders. The pixel size was calculated using the maximum Feret diameter (0.078 mm/pixel). Dots indicate outliers (216 cycles, each plate). (D) Difference between the actual and ideal imaging intervals at the beginning (left) and end (right) of the imaging sequence. (E) Stability in red light illumination intensity (left) and green fluorescence intensity (right) during temporal imaging.

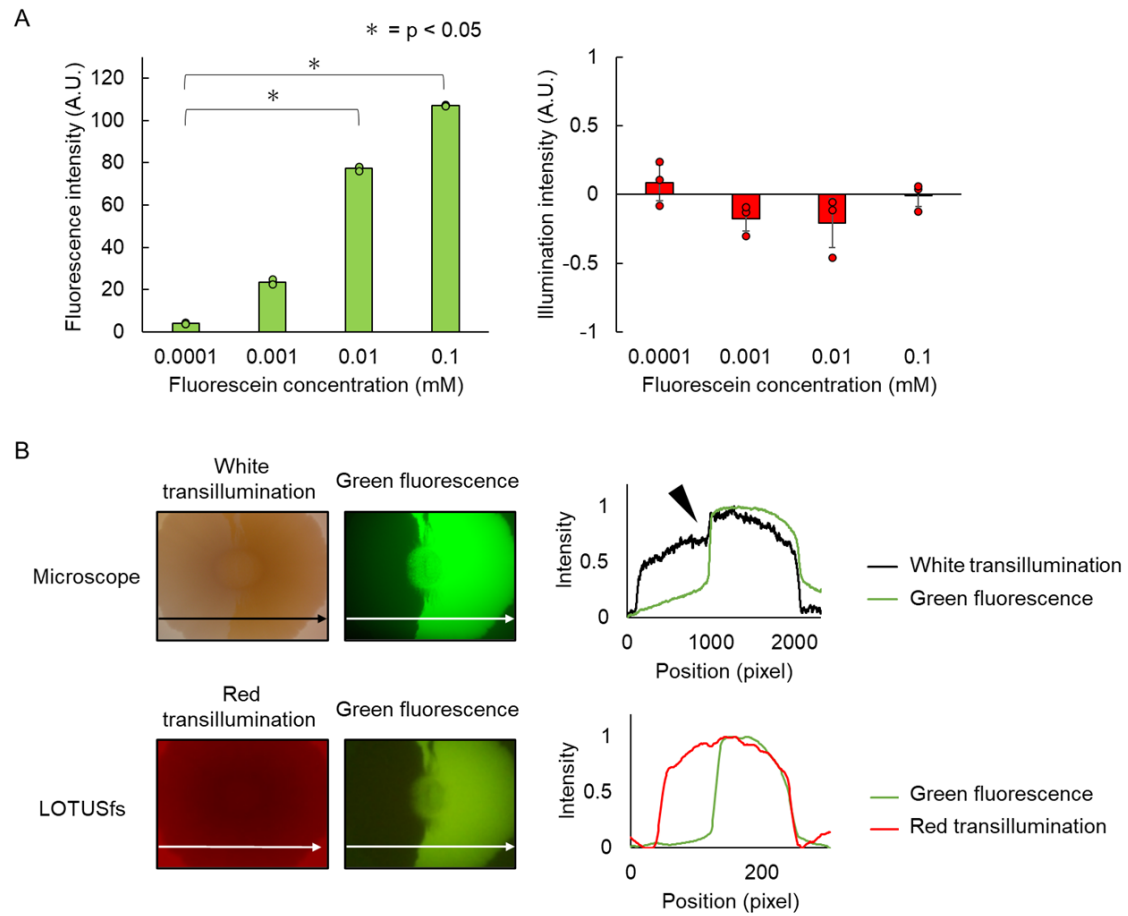
### 3. Capability of red LED illumination to eliminate GFP interference

White LEDs contain blue diode-derived light with a peak wavelength around 450 nm as well as phosphor-derived emission in the range of 550–700 nm [56]. As a result, depending on the excitation spectrum of the fluorescent protein, biomass estimates using white LED illumination can lead to overestimated biomass by absorption in fluorescent protein-expressing biofilms. In addition, widely accepted optical means of measuring bacterial cell density use absorbance measurements in the 600–660 nm range where light scattering by bacterial cells is most specific for cell density. Thus, to both optimize spectral specificity of cell density measurements and minimize green fluorescent protein absorbance a red LED was used as the transmitted light source in this system. However, because the exact emission spectrum of the LED could not be obtained, we experimentally examined whether images acquired with red LED illumination were influenced by GFP or other green fluorescent substances.

Agar plates containing different concentrations of fluorescein were prepared, and both green fluorescence images and red transmitted light images were acquired. While the green fluorescence intensity of the image increased significantly with increasing fluorescein concentration ( $n=3$ ,  $p < 0.05$ , Kruskal–Wallis test followed by Dunn’s test, Fig. 6A), no significant increase was observed in the intensity of red transmitted-light images ( $n=3$ ,  $p > 0.05$ , Kruskal–Wallis test followed by Dunn’s test).

To assess whether GFP affects red transmitted-light imaging, biofilms were formed by co-culturing the U66 strain (promoter-less strain not expressing GFP) and *rsd* reporter strain (strong constitutive expression of GFP) from the AZ collection [5] (Fig. 6B). The resulting biofilms were imaged using both a stereomicroscope (white transmitted-light and green fluorescence modes) and LOTUS-MP (red transmitted-light and green fluorescence modes). In microscope images, fluorescence was detected only in regions containing the *rsd* reporter strain, and a marked increase in transmitted intensity was observed in the white transmitted-light image at the biofilm boundary where the two strains separated

and green fluorescence intensity increased. In contrast, no corresponding change in transmitted intensity was observed in the red transmitted-light images acquired with LOTUS-MP. These results demonstrate that red transmitted-light images obtained with LOTUS-MP are not affected by fluorescein or GFP fluorescence.



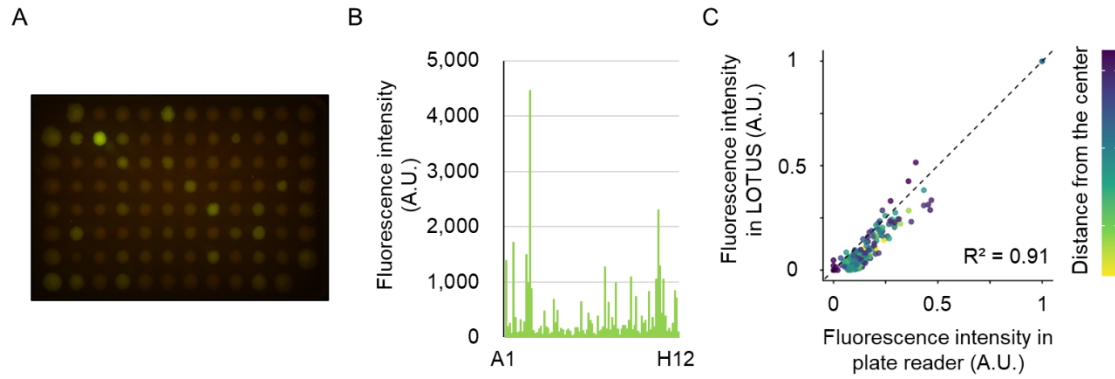
**Fig. 6. Effect of green fluorescence in red transillumination image.**

(A) Green fluorescence and red illumination intensities at each fluorescein concentration ( $n=3$ , technical replicates). (B) Co-cultured biofilm images of microscope and LOTUS-MP with the corresponding red transmitted-light and fluorescence intensity profiles. Biofilms were cultured for 5 days. Arrows indicate the ROI used for profile analysis.

#### **4. Image uniformity across colony position**

To assess whether images acquired with LOTUS-MP are comparable to data obtained using commercial instruments, reporter strains were cultured and the green fluorescence intensity of individual colonies was measured using both a plate reader and LOTUS-MP. Comparison of fluorescence intensities between the two systems revealed a strong correlation in green fluorescence measurements ( $n=3$ ,  $R^2 > 0.73$ , Fig. 7). In addition, images of plates containing the U66 strain were acquired using a scanner and LOTUS-MP in red transmitted light mode. Area measurements showed strong correlation ( $n=3$ ,  $R^2 > 0.73$ , Fig. 8) between the two systems, whereas mean inverted pixel intensity values (biomass proxy) showed a weak correlation ( $R^2 < 0.5$ , Fig. 8).

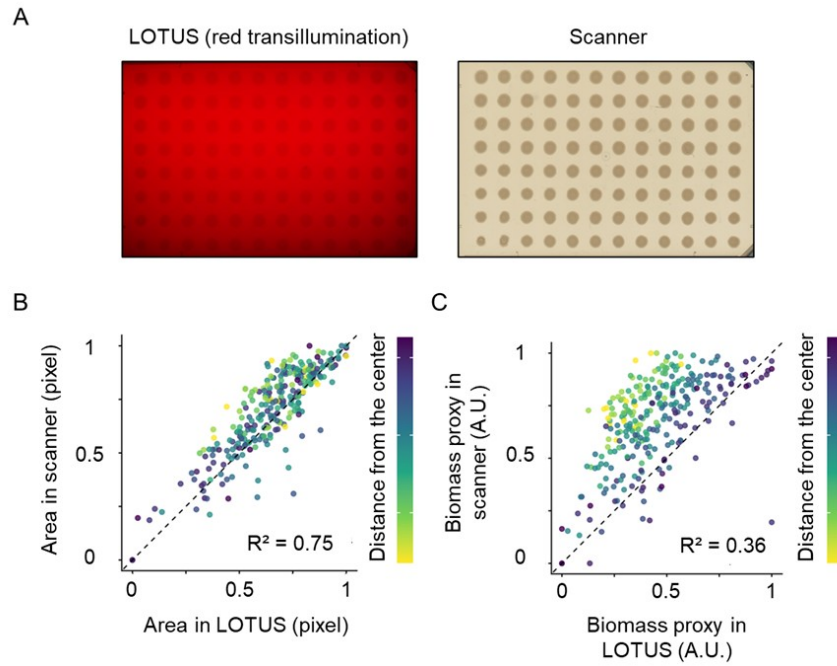
These results indicate that LOTUS-MP can quantify colony fluorescence intensity and colony area in a manner comparable to a plate reader and scanner, respectively, whereas optical density-related measurements of biomass derived from inverted intensity are not directly comparable to those obtained using a scanner.



**Fig. 7. Comparison of fluorescence intensity with plate reader.**

(A) Green fluorescence image of reporter collection on the imaging system. Biofilms were cultured for 3 days. (B) Green fluorescence intensity of colonies measured by plate reader. (C) Scatter plot of fluorescence intensities measured by the LOTUS-MP plotted against those obtained from the plate reader ( $n=3$ , technical replicates). Data points are colored by the distance of each well from the center of the 96-well plate.

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**Fig. 8. Comparison of area and mean inverted pixel intensity with scanner.**

(A) Red transillumination image (left) and scanner image (right). Biofilms were cultured for 3 days. (B) Scatter plot of areas measured by LOTUS-MP plotted against those obtained from the scanner ( $n=3$ , technical replicates). (C) Scatter plot of mean intensities ( $n=3$ , technical replicates). Data points are colored by the distance of each well from the center of the 96-well plate. Data from well H12 were excluded from the analysis because the measurements were affected by shadowing from the sample tray.

## Discussion

In this chapter, we developed LOTUS-MP, a time-lapse imaging system for colony biofilms, and conducted benchmark tests. The spatial resolution of LOTUS-MP is lower than that of commercial microscopes or scanners; however, it is sufficient for detecting bacterial colonies ( $\geq 3$  mm in diameter), which are the focus of this study. The positional drift during time-lapse imaging was approximately 0.25 mm, about half of the system's resolution, but at the scale used here (approximately 120 pixels per colony), this level of drift is unlikely to substantially affect kymograph visibility, analysis, or quantification. Likewise, deviations in imaging intervals are not expected to have a major impact, given the acquisition intervals of 10–20 min. The weak correlation observed for inverted intensity measurements compared with a scanner is likely due to attenuation of colony inverted intensity toward the center caused by illumination intensity gradients, as suggested by the Euclidean distance encoded in the data point color distribution in the scatter plots. This positional effect on intensity could be corrected by flat-field normalization in future work. Considering the number of plates that can be imaged simultaneously, the fabrication cost of LOTUS-MP is approximately one quarter the cost of existing systems [6,7]. The use of red transmitted-light images allows the semi-quantitative analysis of biofilm biomass without interference from green fluorescent proteins. However, this benefit does not apply when red fluorescent proteins are expressed, and it will be necessary to examine whether red fluorescence signals interfere with red transmitted-light measurements. Nonetheless, since the system employs a long-pass filter, it is capable of detecting red fluorescence.

Overall, LOTUS-MP enables time-lapse imaging of multiple plates in two modes and allows semi-quantitative analysis of colony area and fluorescence suitable for quick screening of multiple samples. However, its ability to capture biofilm morphology remains limited, biomass normalization can require additional steps due to non-uniform illumination and the small size of biofilms limits the spatial details that can be captured. Therefore, in Chapter 3, we describe the development of an improved imaging

system designed to overcome the limitations of the basic LOTUS-MP platform, enable independent imaging of individual samples, and minimize positional effects as much as possible.

## **Chapter 3: Development of LOTUS-12, a flexible, multimodal automated imaging system**

### **Introduction**

The imaging system developed in the previous chapter enables quantification of fluorescence intensity and colony area; however, because inverted intensity values derived from red transmitted-light imaging vary across different sample positions, transmitted-light images cannot be used reliably to normalize fluorescence signals. In addition, the large distance between the camera and samples with respect to biofilm diameter limits spatial resolution. The excitation light source is also bulky, time-consuming to assemble, spectrally limited to two colors, and not easily customizable. To overcome these limitations, we examined existing open-source imaging systems with the aim of enabling multimode imaging of multiple samples while maintaining comparability across samples. Among these, 3D-printed microscopes have attracted attention as low-cost and reproducible devices [57]. While these microscopes can acquire bright-field or fluorescence images of samples, simultaneous observation of multiple samples requires multiple units, leading to increased cost and inefficient use of laboratory space. To address this issue, systems employing multiple cameras to image multiple samples in parallel have been proposed [58]. However, these systems are limited to white-light illumination and lack fluorescence imaging capability. More versatile systems have also been reported [59,60]. Although these platforms enable time-lapse imaging of multiple samples, their use of RGB LED illumination results in poor excitation wavelength selectivity for fluorescence imaging, and they lack mechanisms for automated optical filter switching. Consequently, they are not well suited for live-imaging with multiple fluorescent reporters. Thus, while existing open-source imaging systems incorporate many useful elements, few integrate the functionalities required to simultaneously monitor biofilm morphology and reporter dynamics.

To address this gap, we developed a live-imaging system that integrates advantageous features from these existing platforms as described in our previous publication [61]. In this chapter, we describe the design concept of this system and present the results of its performance evaluation.

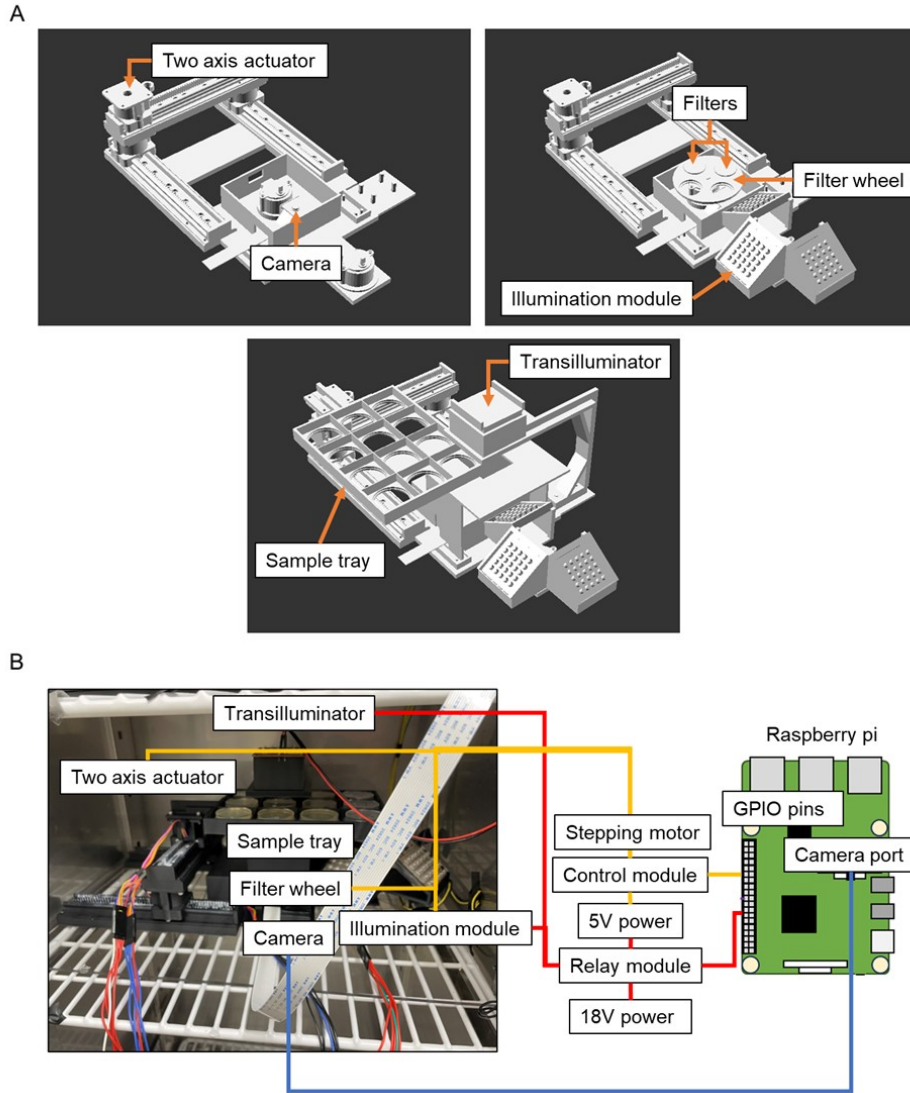
## Results

### 1. System design

The imaging system required for this study must meet four key criteria: (1) epi-illumination to visualize surface structures such as wrinkles and ring patterns; (2) trans-illumination to quantify biomass; (3) excitation illumination modules and optical filters for fluorescent reporters such as GFP and RFP; and (4) an alignment mechanism that automatically positions each sample for imaging. To satisfy these requirements, we developed LOTUS-12 (the number indicates the maximum number of samples that can be imaged simultaneously) (Fig. 9).

In LOTUS-12, a sample tray is moved above a fixed camera by a two-axis actuator, allowing sequential imaging of twelve 36.7-mm-diameter Petri dishes (Fig. 9A). The illumination modules are positioned to provide appropriate light for samples located above the camera, while an optical filter wheel is placed directly above the camera to ensure proper filtering. A transilluminator is fixed above the camera to enable transillumination mode.

The positions of the sample tray, illumination modules, and filter wheel are controlled by stepper motors connected to the two-axis actuator (Fig. 9B). Illumination is switched on and off using a relay module. Image acquisition, as well as control of the relay module and stepper motors, is controlled by a Raspberry Pi.



**Fig. 9. Overview of the system design of LOTUS-12.**

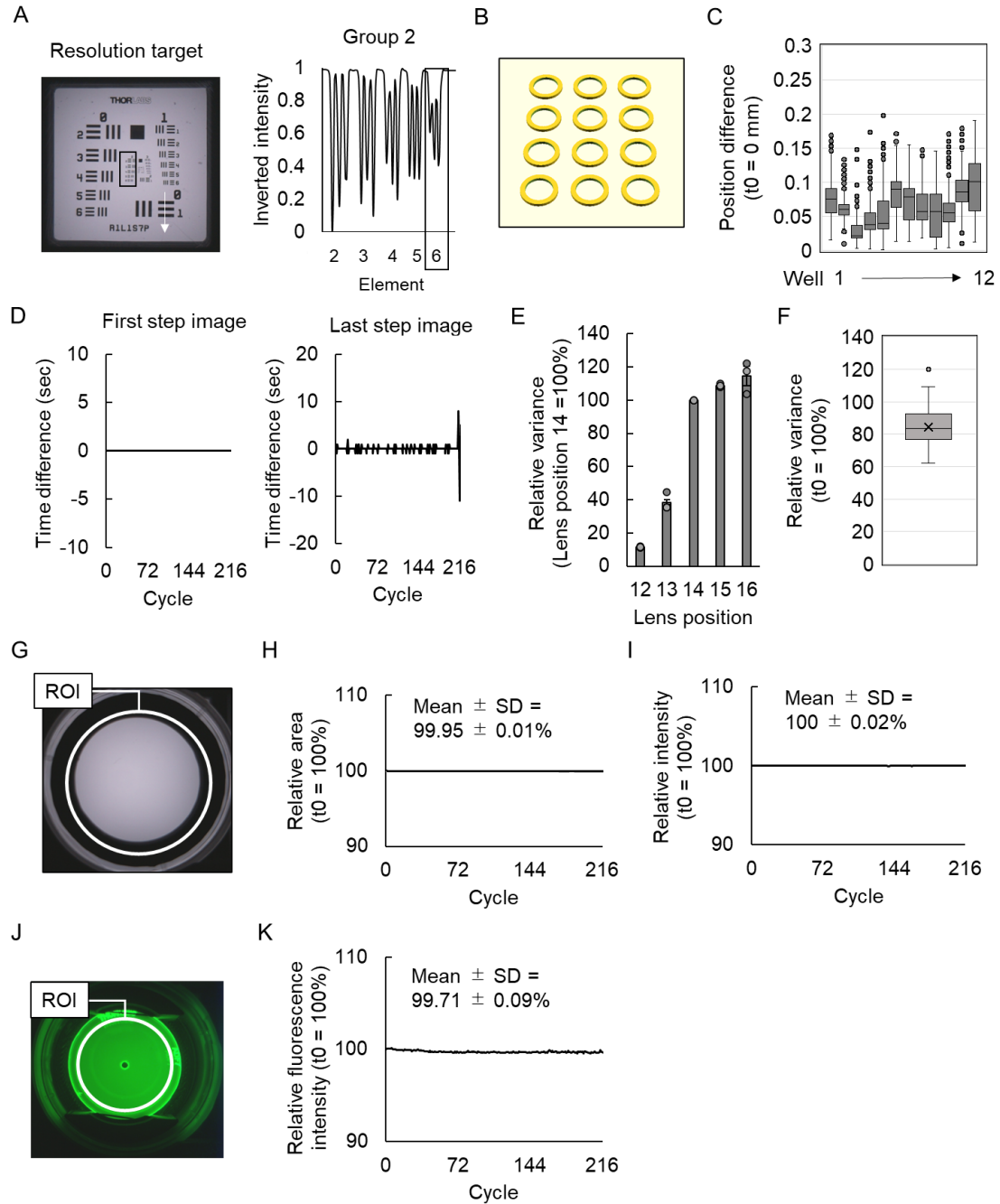
(A) Frame design of LOTUS-12. (B) Connections between Raspberry pi and each component of the LOTUS-12. The imaging system is built around a Raspberry Pi 4 Model B, which controls camera, relay modules, and stepping motor controllers. A 64 MP camera (Arducam) is connected to the Raspberry Pi camera port for image acquisition. Relay modules (Wayintop) switch the illumination modules for each LEDs, which are powered by an 18 V DC power source. Stepping motors (Shanxingan 28BYJ48), driven by ULN2003 control modules, actuate a two-axis actuator, filter wheel and illumination module. A sample tray mounted on the two-axis actuator. A 5 V DC power source supplies power to the stepping motor control modules and relay modules.

## **2. Benchmark test: resolution assessment, alignment accuracy, imaging-interval precision, focus drift, and the stability of quantitative metrics including intensity and area.**

To evaluate the spatial resolution of the LOTUS-12, images of a resolution target were analyzed. Because the MC value dropped below 0.1 from group 2, element 6 onward, the minimum spatial resolution was estimated to be at least 0.14 mm (Fig. 10A).

Next, to assess the temporal reproducibility of LOTUS-12, we evaluated alignment accuracy, imaging-interval precision, focus drift, and the stability of quantitative metrics including intensity and area. To examine alignment error and imaging-interval accuracy, PLA disks were imaged for three days (216 cycles), and images were acquired in four modes: white transillumination, white epillumination, green fluorescence, and red fluorescence for each sample position (Fig. 10B). Tracking the center coordinates of the apertures in transmitted-light images revealed a maximum positional deviation of approximately 0.2 mm across all sample positions (Fig. 10C). Analysis of timestamps indicated no deviation at the beginning of the acquisition script, while a maximum deviation of ~10 s was observed at the final acquisition (Fig. 10D). Overall, the imaging intervals remained stable throughout the experiment. To assess potential focus drift, resolution targets were imaged while varying the camera focus position, and the Laplacian variance of the ladder (Group 0, Element 1) was quantified (Fig. 10E). When the target was imaged over time and Laplacian variance was measured in the same manner, the temporal fluctuations remained stable and did not exceed values observed under intentional focus changes (Fig. 10F). As these results alone could not fully exclude focus drift, we further evaluated the stability of area and intensity values measured within ROIs under white transmitted-light and green fluorescence modes. In both modes, temporal fluctuations in area and intensity were within 1% (Fig. 10G–K).

Together, these results demonstrate good temporal reproducibility of images acquired with LOTUS-12 during time-lapse imaging.



**Fig. 10. Resolution test, assessment of alignment position, imaging intervals stability, focus drift, illumination intensity and fluorescence intensity fluctuations.**

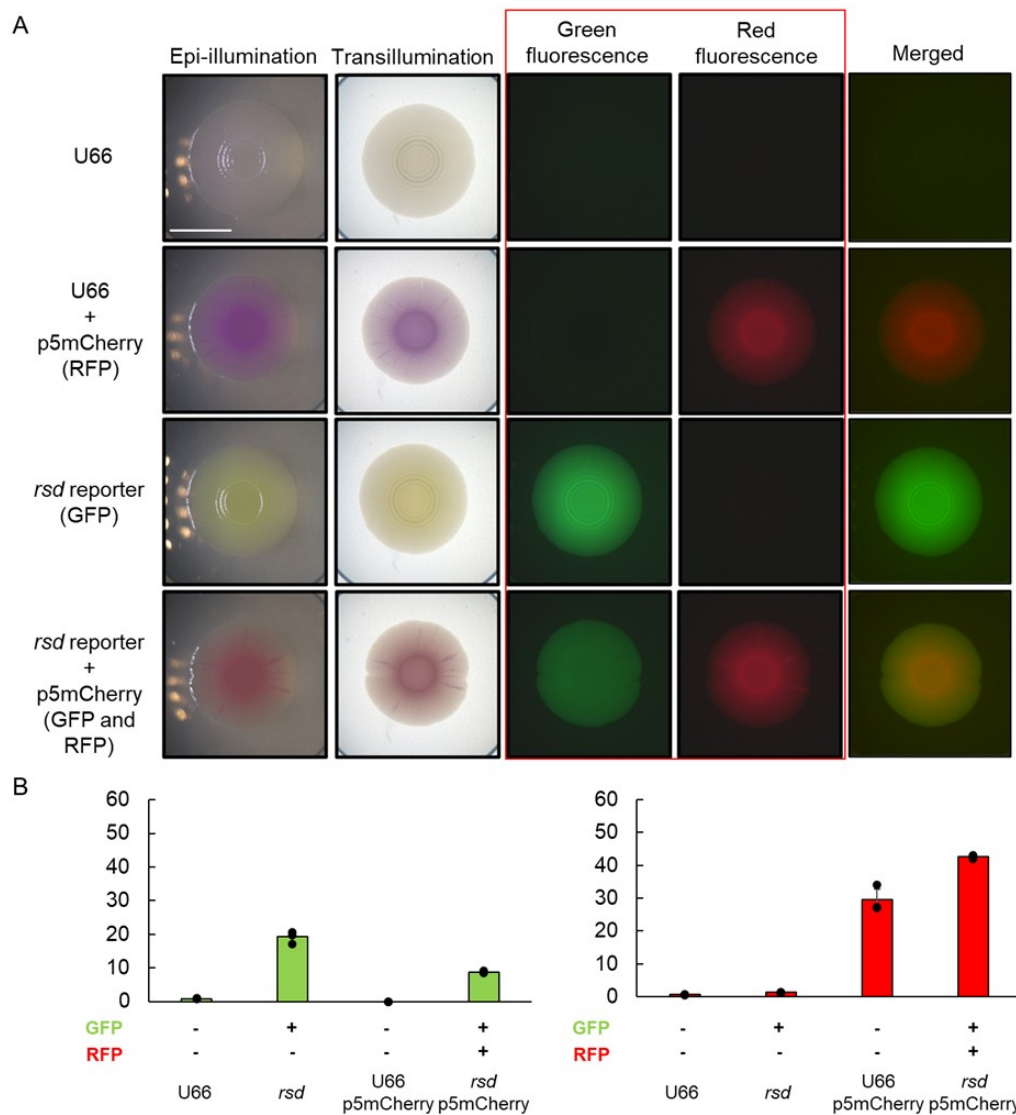
(A) Resolution target image (left) and plot profile of group 2, corresponding to the bar patterns enclosed by the black square in the resolution target image (right). (B) PLA disks with circular hole (25 mm in diameter) used as imaging targets. (C) Variability in the center position of each aperture across different sample holders. The pixel size was calculated based on the maximum Feret diameter

(0.078 mm pixel<sup>-1</sup>). Box plots indicate the median and interquartile range (IQR); whiskers extend to 1.5×IQR, and dots represent outliers (216 cycles, each dish). (D) Difference between the actual and ideal imaging intervals at the beginning (left) and end (right) of the imaging sequence. (E) Laplacian variance measured at each lens position. ROI (white arrow) was shown in (A) (n=3, technical replicates). (F) Temporal variability of Laplacian variance during time-lapse imaging (216 cycles). (G) White transillumination image of a PLA disk. (H and I) Temporal stability of the measured area (H) and illumination intensity (I) within the ROI under white transillumination mode. (J) Green fluorescence image of a fluorescent disk. (K) Temporal stability of green fluorescence intensity within the ROI. Figures reproduced with modifications from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

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### 3. Quality control: LEDs and optical filters

We next evaluated its utility for biological applications by monitoring biofilm development with transcriptional reporter strains expressing fluorescent proteins. For this, we used U66 strain and the *rsd* reporter strain. To generate dual-reporter strains, the U66 strain and *rsd* reporter strains were transformed with the p5mCherry plasmid which constitutively expresses mCherry [62]. The strains were cultured on salt-free LB agar medium for 5 days. Biofilms were imaged in four modes (Fig. 11A), revealing structure (epi-illumination), biomass (transillumination) and distinct fluorescent protein expression patterns. Fluorescence intensity was also quantified in each biofilm (Fig. 11B). This analysis revealed minor crosstalk in which some GFP signal was detected in the red channel of *rsd* reporter strain biofilms. GFP emission overlaps with the 665 nm long-pass filter window and may explain this phenomenon. Both fluorescent proteins were detected in the *rsd*+p5mCherry biofilms, however, GFP signal intensity was reduced compared to the *rsd* single-reporter strain, possibly due to metabolic burden of dual-plasmid maintenance or optical cross-excitation effects. Together, these results demonstrate that the selected LED/filter combinations are appropriate for dual-fluorescence detection with commonly used fluorescent proteins, though users should remain aware of minor cross-talk effects.



**Fig. 11. Multimode imaging of biofilms with LOTUS-12.**

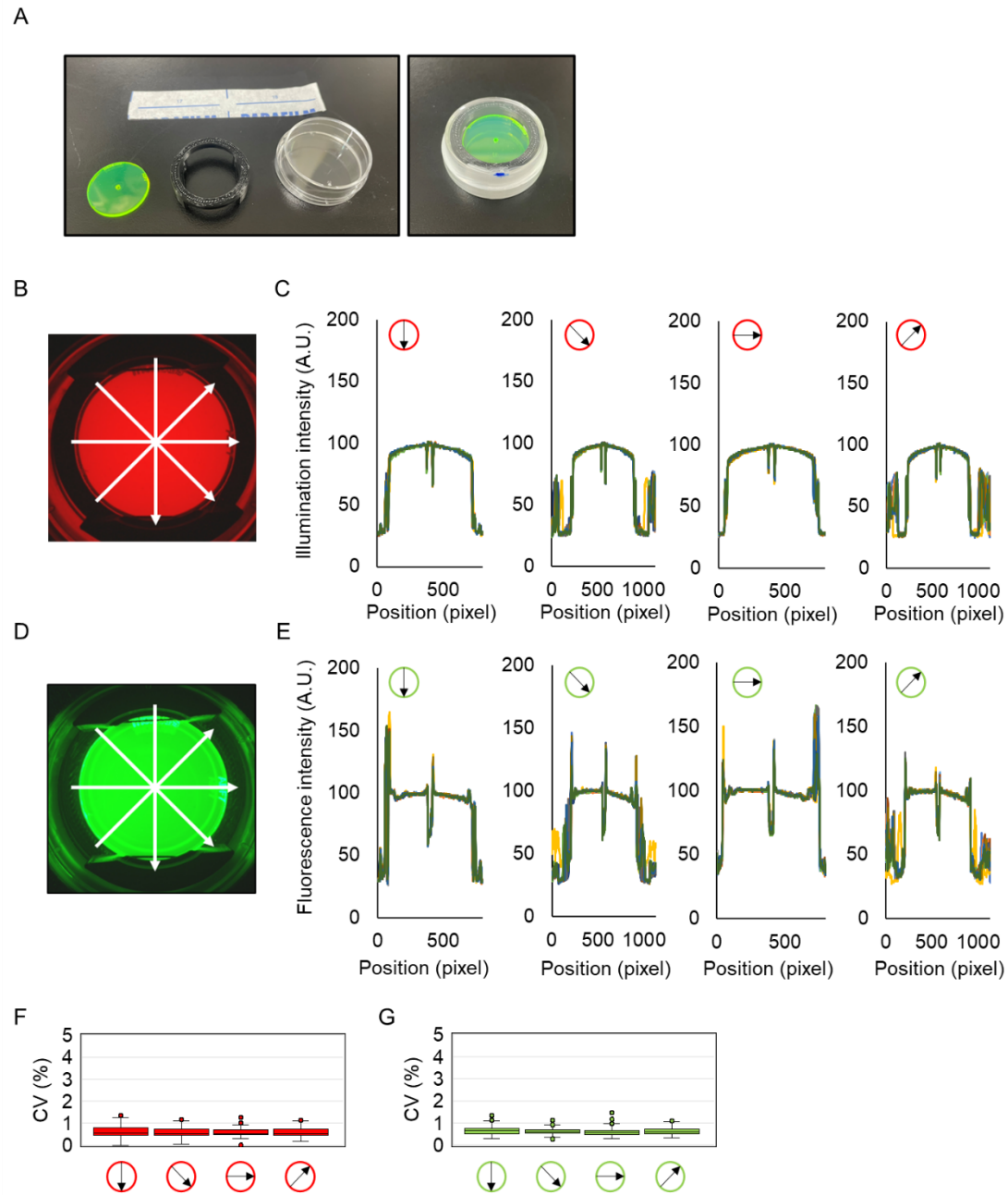
(A) Bright field and fluorescence images of biofilms containing one or two reporters were captured at biofilm growth experiment endpoint. Displayed samples are: U66 (promoter-less), U66 + p5mCherry, *rsd*, and *rsd* + p5mCherry strain. Green fluorescence images were obtained using a green band-pass filter and blue LED. Red fluorescence images were obtained using a long-pass filter and green LED. Merged images were created by overlaying green and red channel fluorescence images. The scale bar indicates 1 cm. (B) Green and red fluorescence intensities detected in the biofilms (n=3, technical replicates). Figures reproduced with modifications from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

#### **4. Quality control: Illumination uniformity across wells**

LOTUS-12 is operated by sequentially positioning sample wells on top of the camera through movement of the sample tray, and deviation in the well positioning can lead to differences in the imaging conditions. We therefore checked whether the detected fluorescence signals could be compared across wells. Alignment disks emitting green and red fluorescence (ADF-7, Thorlabs) were placed in a Petri dish together with a scaffold made by a 3D printer and the lid immobilized with Parafilm (Fig. 12A). The dish was placed in the sample tray, positioned above the camera, and both images acquired under red transillumination and green fluorescence images were captured for all twelve sample positions (Fig. 12B and D). The light intensity of each pixel was analyzed along the four directions (Fig. 12C and E).

The fluorescence intensity patterns detected in all wells overlapped for all image analysis directions with large peaks corresponding to the disk edges and central hole. In addition, spatial variation in light and fluorescence signal across the disk center ( $\pm 250$ -pixel region away from the central hole) was low (median CV < 1%, n=12) in all directions (Fig. 12F and G).

These results show that illumination is relatively uniform across well position and sample area.



**Fig. 12. Evaluation of fluorescence intensity flatfield uniformity using alignment disks.** (A) Alignment disk, 3D-printed adapter and 36.7 mm dish, are shown separately and assembled. (B and D) Red transillumination and fluorescence intensity were measured at each well position using both green alignment disk at each pixel position along the four directions. The resulting intensity profiles are shown for the red transillumination image (C) and green fluorescence image (E). (F and G). Box-and-whisker plots showing directional variation in CV values of pixel intensity over a 250-pixel window that excludes the central hole and disk edges for all well positions. Figures reproduced with

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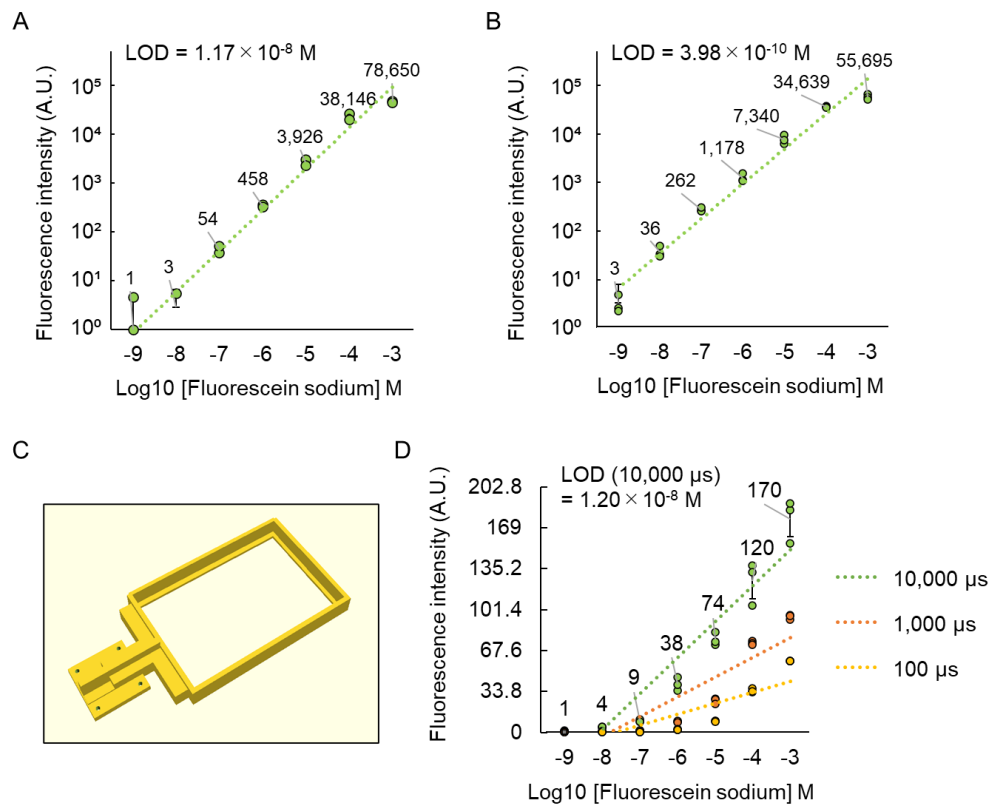
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## **5. Comparison with commercial instruments**

To evaluate the potential of this system, we compared its sensitivity and reproducibility with commercially available instruments, a standard fluorescence plate reader and laser scanner.

### **5.1 Sensitivity analysis using fluorescein**

To compare sensitivity, we used different known concentrations of fluorescein and analyzed their signals on LOTUS-12, Mithras LB940 plate reader and Amersham Typhoon laser scanner. The LOD was  $1.20 \times 10^{-8}$  M for LOTUS-12 modified with a microplate tray,  $1.17 \times 10^{-8}$  M for the plate reader, and  $3.98 \times 10^{-10}$  M for the laser scanner (Fig. 13). The LOTUS-12 LOD for fluorescein is comparable to the plate reader but about 30-fold less sensitive than the laser scanner.



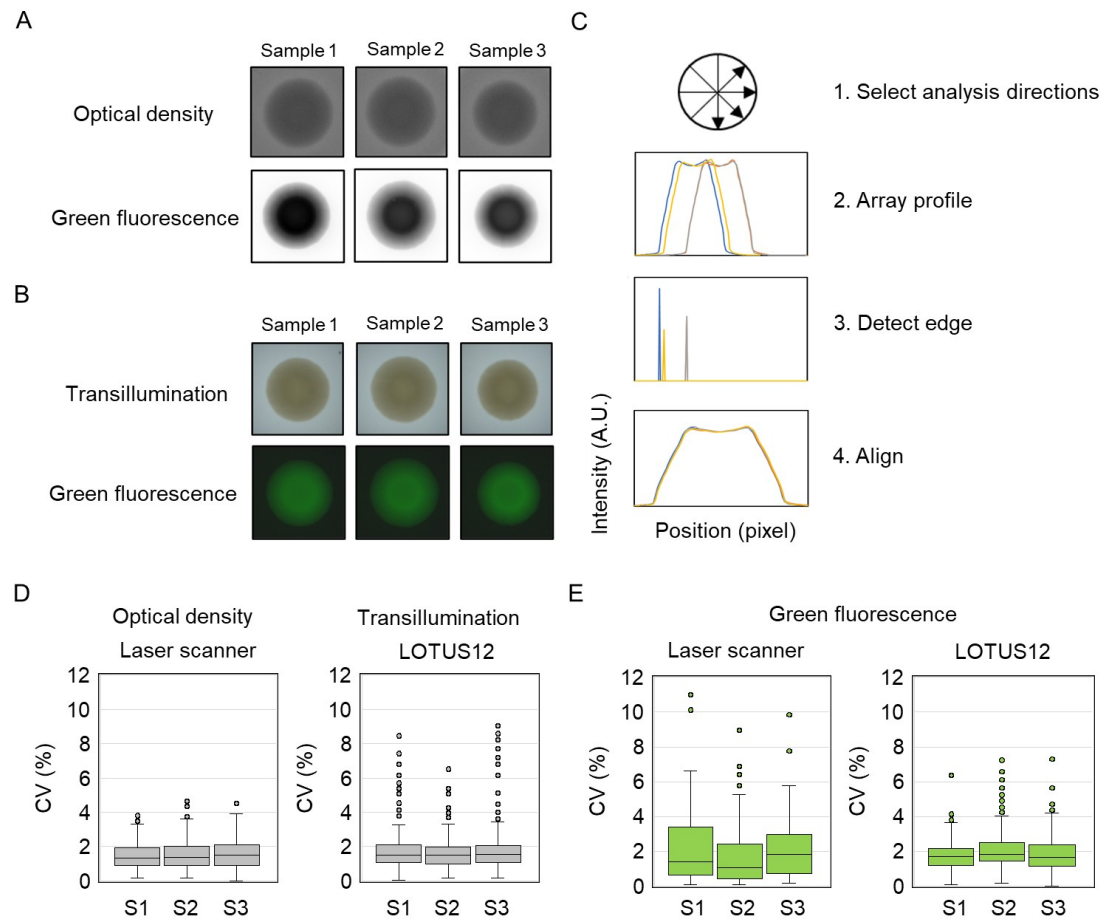
**Fig. 13. Evaluation of fluorescence detection sensitivity using fluorescein and demonstration of expandability to the plate reader.**

Sequential dilutions of a sodium fluorescein solution were poured in triplicates into wells of a 96-well plate to serve as standard samples ( $n=3$ , technical replicates). (A and B) The green fluorescence intensity for each concentration was measured using: (A) a plate reader (Mithras Berthold) and (B) a laser scanner (Amersham Typhoon). (C) To allow for comparisons with LOTUS-12, a plate sample tray was printed and attached to the system. (D) Fluorescence images were captured at different shutter speed and the fluorescence intensity per well was measured. Individual data points are shown and error bars indicate SD. Figures reproduced with modifications from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

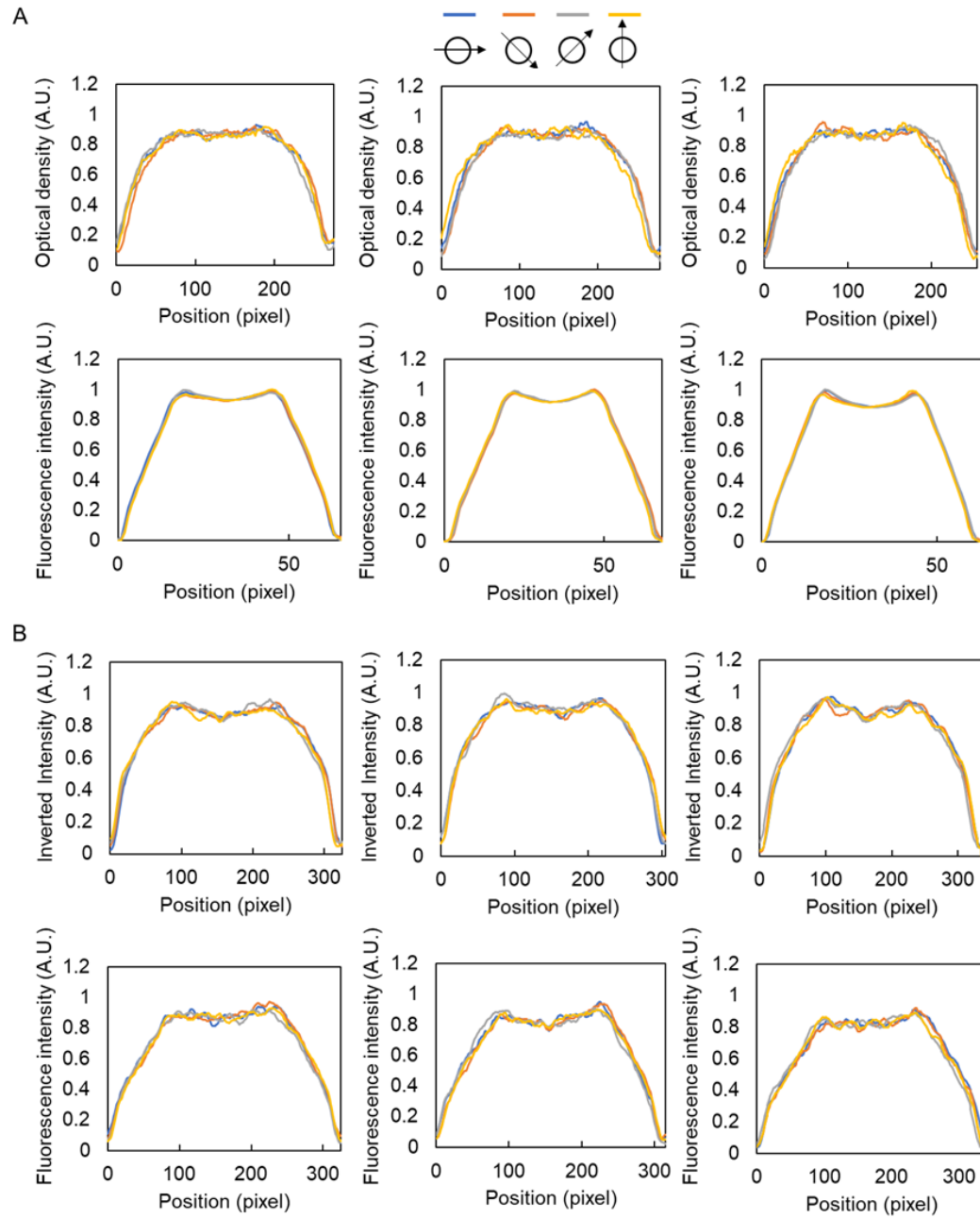
## 5.2 Uniformity and reproducibility within biofilms

Images of *rsd* reporter strain biofilms cultured for three days were acquired using LOTUS-12 and a laser scanner, under transillumination (LOTUS-12) or OD mode (Typhoon), and green fluorescence mode (Fig. 14A-B). To assess radial uniformity, intensity profiles were extracted using Line Selection tool in Fiji through the center of the biofilm from four directions and aligned at the position where the inverted intensity or fluorescence intensity began to increase (Fig. 14C). The aligned profiles were plotted relative to the horizontal analysis direction. Additionally, the spatial CV per pixel was calculated for each direction for all pixels (Fig. 14D-E).

In all analyzed directions, the intensity from the center to the edge of the biofilm was symmetrical and the trend was similar for both instruments (Fig. 15). These results indicated that the inverted intensity and fluorescence intensity distribution of the biofilm showed little difference across analytical directions. Overall, biofilm intensity profiles from LOTUS-12 showed directional uniformity (median CV < 2%) comparable to a commercial laser scanner.



**Fig. 14. Comparison of biofilm image profiles between LOTUS-12 and a laser scanner.** Triplicate biofilms were imaged on a laser scanner (A) or LOTUS-12 (B) in bright-field (top) or fluorescence (bottom) modes. (C) Image profiles were analyzed in four directions and aligned as shown. Directional uniformity of signals are calculated as CV for all samples on both instruments for transillumination (D) and fluorescence (E). Box plots show the median, interquartile range (IQR) and whiskers extend to the most distant data point within 1.5 X IQR. Sample sizes were as follows: Laser scanner (OD mode), 290 pixel-window; green fluorescence mode, 60 pixel-window; and LOTUS, 330 pixel-window. Figures reproduced from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.



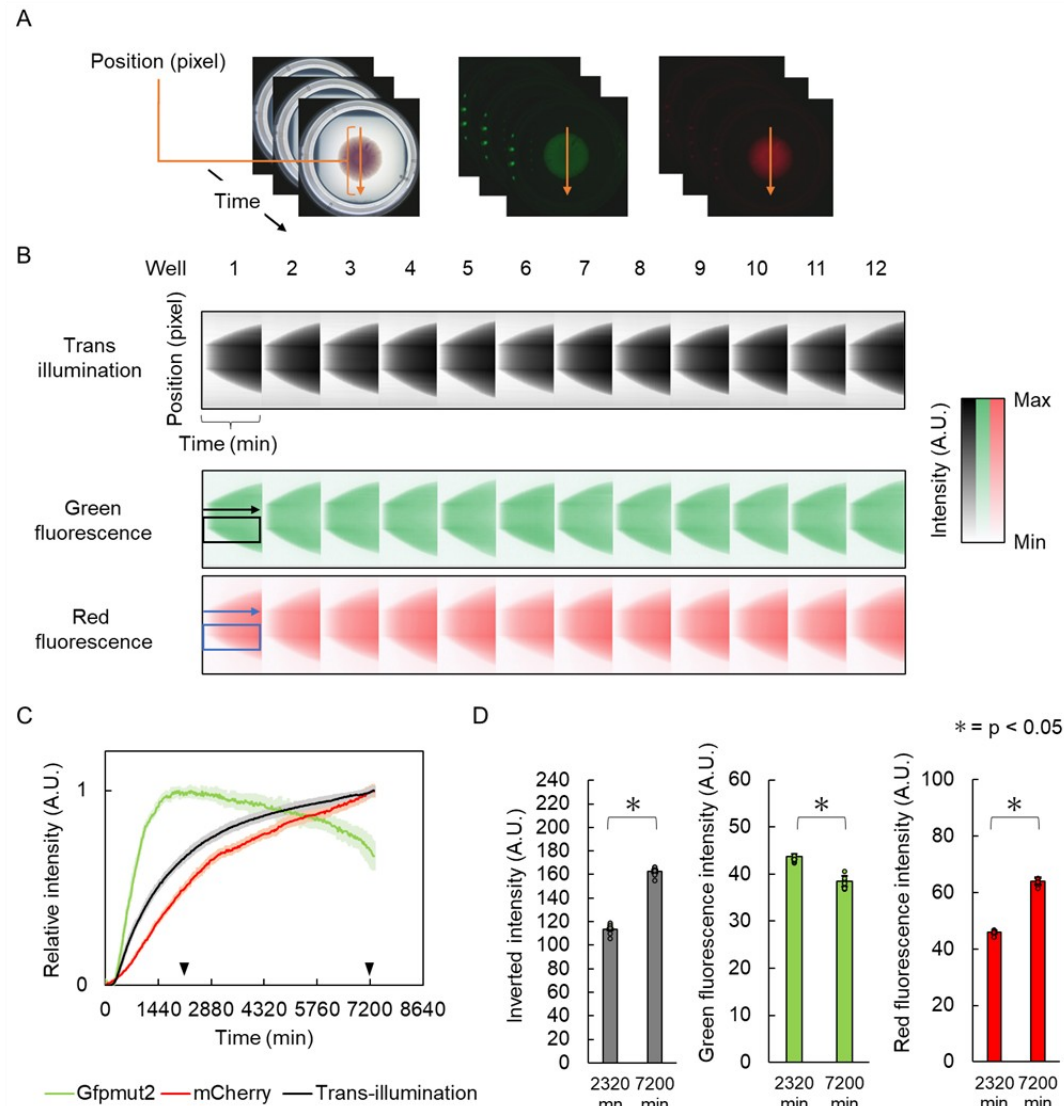
**Fig. 15. Comparison of biofilm spatial biomass distribution and fluorescence profiles on laser scanner and LOTUS-12.** Images of triplicate biofilm samples acquired using a laser scanner (A) and LOTUS-12 (B) were analyzed in four directions to generate intensity profiles for biomass (transillumination, top) and fluorescence of the *rsd* reporter strain biofilms (bottom). In each plot, profiles from all four directions are overlaid. Figures reproduced from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

## 6. Biological demonstration of LOTUS-12 performance

### 6.1 Multimode time-lapse biofilm imaging

To demonstrate LOTUS-12's ability to capture the growth and dynamics of fluorescent protein expression in living biofilms, bright-field epi and transillumination, green and red fluorescence imaging were tested with dual-plasmid reporter strain (*rsd*+p5mCherry).

Spatiotemporal analysis (kymographs) revealed the dynamics of fluorescent protein distributions (Fig. 16A-B). GFP fluorescence intensity initially increased throughout the center of the biofilm, expanding radially before decreasing in the central region after 2320 min (Fig. 16C). On the other hand, mCherry fluorescence increased continuously without subsequent decrease. Quantification showed that biomass proxy (transillumination) and mCherry fluorescence increased significantly between 2320 and 7200 min (peak and endpoint, respectively) while GFP fluorescence decreased ( $n=12$ ,  $p < 0.05$ , Welch's t-test, Fig. 16D). The apparent GFP fluorescence decrease in dual-reporter analysis may reflect actual regulation, but technical artifacts cannot be excluded. As mentioned earlier, minor spectral cross-talk was detected despite the use of a 665 nm long pass filter. Further validation would be required to clarify this. Overall, LOTUS-12 demonstrated reproducible quantitative detection of temporal changes for both fluorescent reporters.



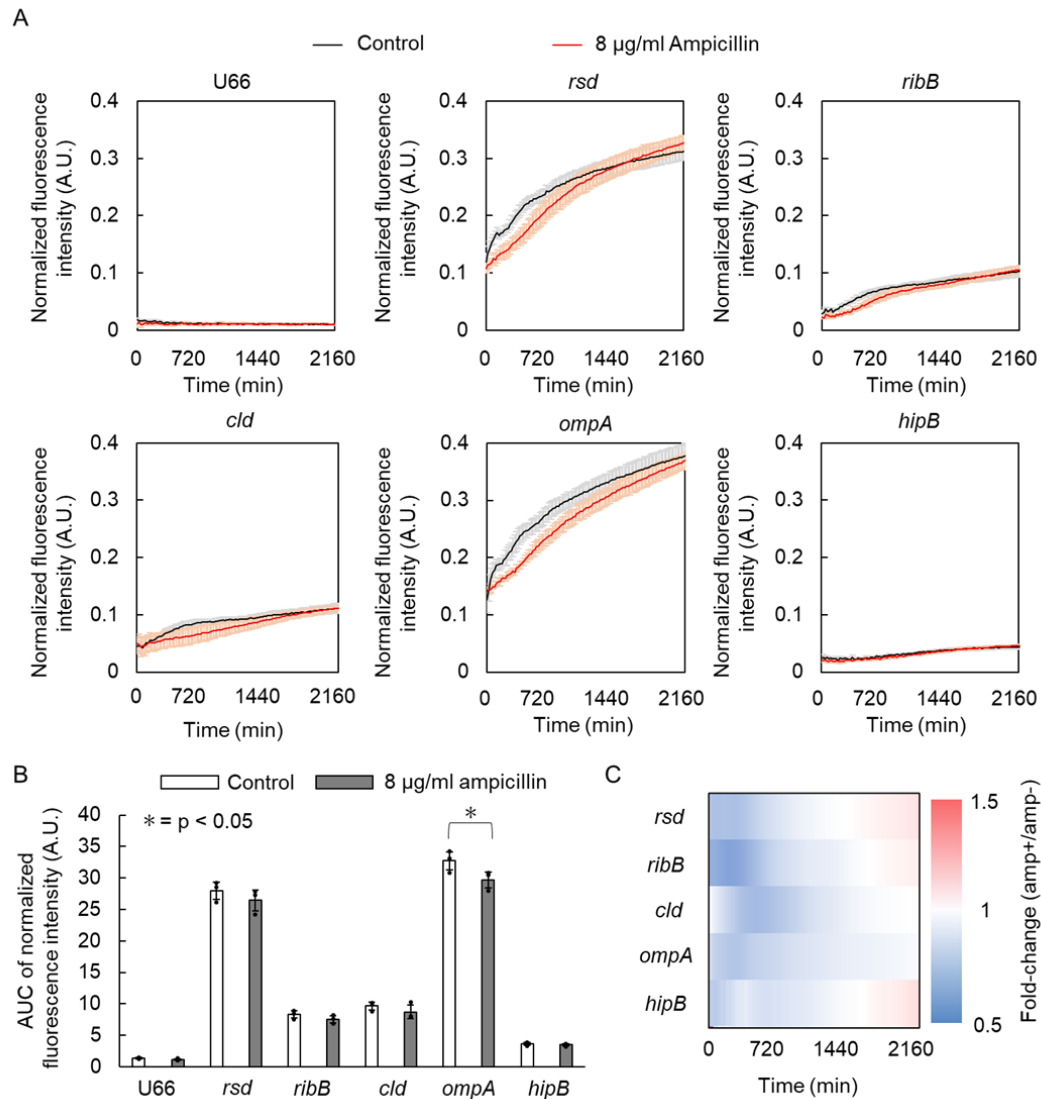
**Fig. 16. Spatiotemporal fluctuations of fluorescent reporters in growing biofilms.**

(A) Time-lapse images collected during the growth of *E. coli* biofilms expressing GFPmut2 and mCherry were analyzed along the orange arrow and the pixel intensity was measured. (B) Kymographs showing pixel intensity along the spatial (horizontal axis) and time (vertical axis) dimensions. Color intensity is displayed as the relative intensity in either bright-field (transillumination), green (GFPmut2) or red (mCherry fluorescence). (C) Transmitted light or fluorescence intensity of *E. coli* biofilm growth. Shading represents the SD. (D) Integrated intensity values for transillumination or fluorescence signals are plotted for the two time points highlighted by arrowheads in (B) (2320 and 7200 min). The error bars show the mean  $\pm$ SD ( $n=12$ , technical replicates). Figures reproduced with modifications from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

## 6.2 Monitoring sub-MIC ampicillin response

To evaluate LOTUS-12's ability to quantitatively measure GFP expression level, we investigated the effect of sub-MIC ampicillin treatment on biofilms. The six reporter strains tested were: U66 (promoter-less), *rsd* (regulator of sigma D, strong constitutive), *ribB* (riboflavin biosynthesis), *cld* (chlorite dismutase), *ompA* (outer membrane protein A), and *hipB* (antitoxin/DNA-binding transcriptional repressor, persister formation). Biofilms for each strain were grown on salt-free LB agar medium with or without the addition of sub-MIC ampicillin (8 µg/ml), for three days at 28°C. To account for delayed growth in the sub-MIC group, growth curves were temporally aligned by setting  $t = 0$  as the onset of detectable growth (defined as the earliest time point where biofilm area began to increase stably following its minimum value). Results showed that normalized fluorescence intensity (intensity/biomass proxy value) increased over time in all promoter strains except the U66 strain (Fig. 17A). U66 and other promoter strains showed trends toward reduced AUC with ampicillin treatment; however, except for *ompA* ( $n=3$ ,  $p < 0.05$ , Welch's t-test, Fig. 17B), these differences were not significant, likely due to limited statistical power. Temporal analysis of fluorescence intensity (fold-change with ampicillin vs without) showed an initial decrease (0-720 min) followed by an increase in signal intensity at later time points ( $t > 1500$  min) for most strains (Fig. 17C).

This biphasic pattern may reflect adaptive responses to cell wall stress. Also, LOTUS-12 demonstrated reproducible quantitative detection of temporal changes for fluorescent reporters among biological replicates.



**Fig. 17. Effect of sub-MIC ampicillin treatment on GFP expression level in selected *E. coli* reporter strains during biofilm growth.** (A) Temporal changes in biomass proxy normalized fluorescence intensity for (U66 promoter-less) and five selected reporter strains. Error bars indicate the standard deviation (n=3, biological replicates). (B) Change in integrated fluorescence intensity over the whole biofilm area. The AUC was calculated from the first time point at which normalized fluorescence intensity was detected until experimental endpoint (n=3, biological replicates). The bar shows the mean and error bars indicate SD. (C) Mean heatmap of fold-change in normalized fluorescence intensity during biofilm development (ampicillin presence vs absence) (n=3, biological replicates). Figures reproduced from Sakai et al. (2026) [61] under Creative Commons Attribution 4.0.

## Discussion

We developed and validated LOTUS-12, a multimode automated time-lapse imaging system, for measuring growth, morphological changes, and gene expression dynamics in bacterial biofilms. LOTUS-12 improved upon our earlier laboratory imaging prototypes by implementing epi-illumination to reduce reflection artifacts and modular LED arrays and filters for flexible wavelength selection.

In the LOTUS-MP system developed in Chapter 2, variation in inverted intensity across colonies depending on sample position represented a limitation. In the LOTUS-12, however, samples are imaged under nearly identical conditions, thereby eliminating this positional dependence.

To evaluate issues commonly encountered during live-imaging, such as image degradation caused by condensation, temperature fluctuations within the incubator during system operation, and potential phototoxic effects, we also assessed variations in the signal-to-noise (S/N) ratio. Details of these evaluations can be found in recent publication [61]. In the published paper, data were acquired using white LED transillumination [61]. However, in Figure 12 of this thesis and in the experiments described in Chapter 4, red LED transillumination was used to remove interference from fluorescent signals.

Well positioning accuracy was good ( $\pm 0.3$  mm) and sufficient for macroscale biofilm imaging but may require corrections for higher magnification applications or smaller samples. When this is of concern, such deviations can be reduced using alignment algorithms during image analysis. Both positioning drift and imaging cycle time could be reduced by using higher-voltage and higher-torque stepper motors and commercially available actuators. The measured resolution of LOTUS-12 ( $< 0.14$  mm) is limited but appropriate for the intended macroscale applications (1-25 mm). Higher magnification is possible using alternative cameras (e.g., Raspberry Pi HQ camera and lens) and appropriate lens adapters. The use of higher-specification single-board computers could enable faster

and higher- resolution imaging with real-time image analysis for automated sample alignment corrections.

We found that the sensitivity of LOTUS-12 in fluorescence mode was comparable to a commercial plate reader but about 30-fold lower than a laser scanner likely due to differences in detectors (CMOS vs PMT) and illumination (LED vs laser). Comparison of fluorescence intensity profiles obtained using a laser scanner and LOTUS-12 revealed that profiles acquired with LOTUS-12 were more rounded in shape (Fig. 15). This difference is likely attributable to halo contributions from fluorescence in neighboring regions and to image curvature effects, which influence the intensity recorded per pixel in LOTUS-12 images.

Several other open-source imaging systems have been developed for time-lapse imaging [7,53,57-60]. While they may excel at specific tasks, they do not integrate the multi-sample, multi-mode imaging features of LOTUS-12 for comprehensive biofilm analysis. Some such as Picroscope [58] allow multi-sample imaging but in a single bright-field illumination mode. Others enable fluorescence but lack bright-field or automated filter exchange [7,53] or can only image single samples [57]. Finally, while openIVIS and others combine multi-mode imaging, these systems use RGB LEDs and lack automated filter selection [59,60]. A feature comparison is shown in table 1.

**Table 1. Comparison of LOTUS-12 with existing open-source imaging systems**

| System         | Multi-sample | Comparability across samples | Multi-color                 | Automatic filter exchange | Reference |
|----------------|--------------|------------------------------|-----------------------------|---------------------------|-----------|
| PFlbox         | ✓            | Limited                      | Limited (only fluorescence) | -                         | [7]       |
| Núñez et al.   | ✓            | Limited                      | Limited (only fluorescence) | -                         | [53]      |
| Flypi          | Limited      | -                            | ✓ (RGB LED)                 | ✓                         | [57]      |
| Picroscope     | ✓            | ✓                            | Limited (only bright field) | -                         | [58]      |
| Gervasi et al. | ✓            | ✓                            | ✓ (RGB LED)                 | -                         | [59]      |
| OpenIVIS       | ✓            | Limited                      | ✓ (RGB LED)                 | -                         | [60]      |
| LOTUS-12       | ✓            | ✓                            | ✓ (Customizable)            | ✓                         | [61]      |

While detailed comparative cost-effectiveness analysis is not presented, LOTUS-12 development costs (< \$600 USD, S1 Table) represent greater than 90% reduction compared to a basic commercial system with similar functionality (e.g., Celloger Nano, clear-field + green fluorescence mode only,

without well alignment functionality, \$8850 USD). Replacement costs for most components are minimal (< \$200 USD) and no obvious degradation was observed over six months of continuous operation except for gradual LED light intensity decrease which does not affect relative measurements.

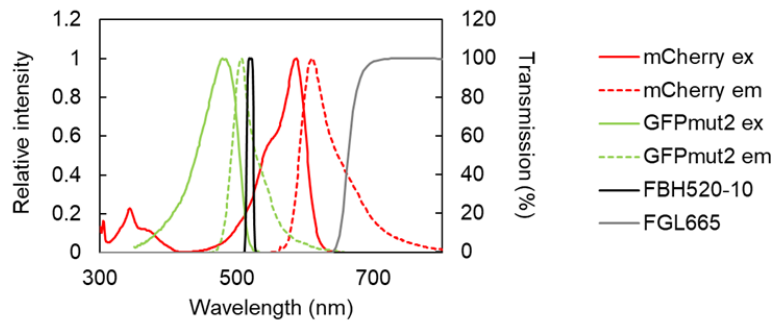
LOTUS-12 successfully captured spatiotemporal patterns of fluorescent protein expression within growing biofilms for up to 5-days. However, we observed reduced GFP intensity in the dual-reporter strain (*rsd*+p5mCherry) compared to the single-reporter strain (*rsd*), particularly near the center of the biofilm (Fig. 11B). This persisted even when illumination was minimized, ruling out photobleaching as the primary cause. One possible explanation is that the plasmid copy number differences (low copy pUA66 (GFP) reporter vs high-copy p5mCherry (RFP)) may result in preferential recruitment of transcriptional machinery components (RNA polymerase and sigma factors) toward high-copy plasmids [5,62]. Alternatively, spectral overlap between mCherry excitation and GFP emission may result in energy transfer processes in which GFP-emitted photons excite mCherry instead of reaching the camera (Fig. 18) [63]. Such findings emphasize the need for careful controls and consideration of possible spectral interference when interpreting dual-reporter data, especially in dense bacterial populations like biofilms. Additionally, discrepancies between biomass proxy (transillumination) and mCherry fluorescence intensity (Fig. 16D) likely reflect extracellular matrix contributions, or visible light absorption by mCherry, leading to overestimated biomass values. LOTUS-12 reliably detected temporal changes in GFP expression levels within biofilms exposed to sub-MIC ampicillin, with good reproducibility across biological replicates (Fig. 17). Sub-MIC ampicillin treatment initially reduced fluorescence intensity across all reporter strains (0-720 min), followed by a recovery near control levels or higher after 2160 min. Since the constitutive *rsd* promoter displayed similar dynamics, fluctuations may reflect cell number changes rather than actual promoter activity responses [7]. Although the reporter strains were selected to represent a diversity of responses to ampicillin based on a previous study using the same strains [7], our results differ quantitatively, likely due to differences

in growth conditions affecting ampicillin stress response [64,65] rather than contradicting previous findings. These include our use of biofilm-inducing conditions (salt-free LB at 28°C) instead of LB at 37°C, large biofilm size vs small colonies, and our longer experiments that ran for 36 hours vs 18 hours. The reproducibility of our results demonstrates the capacity of LOTUS-12 for comparative quantitative analysis of biofilm gene expression dynamics while highlighting their dependence on environmental perturbations. In addition, our observations show that fluorescence changes reflect the interplay between biomass, cell density and reporter expression and LOTUS-12 simultaneous multi-mode imaging can enable deconvolution of these factors and better characterization than single-mode systems.

LOTUS-12's modular design can allow straightforward adaptation to different sample formats such as 96-well plates by modifying the sample tray and adjustment well positions. However, light containment may become an issue with narrow wells to prevent cross-well illumination and phototoxicity, as is commonly implemented in plate readers. Further improvements may include the integration of imaging with sensors for monitoring pH, dissolved oxygen, and temperature, enabling simultaneous acquisition of environmental and imaging data [66]. LOTUS-12 has a compact design that allows it to be placed directly inside a standard incubator. This provides practical flexibility in space-limited laboratory environments and enables parallel operation of multiple units. In addition, the sample tray size and movement range can be easily modified to create configurations tailored to specific experimental needs. The comparison table with a commercial system is shown in table 2.

**Table 2. Comparison of LOTUS with a commercial imaging system**

|                         | <b>LOTUS-12</b>                                      | <b>Commercial System (e.g., Celloger Nano)</b>         |
|-------------------------|------------------------------------------------------|--------------------------------------------------------|
| Cost                    | < \$600 USD                                          | ~\$8,850 USD                                           |
| Imaging modes           | Bright-field + fluorescence (customizable)           | Bright-field + fluorescence (fixed configuration)      |
| Magnification           | Macro-scale standard, but customizable               | Fixed optical configuration; limited user modification |
| Light source and filter | Modular LEDs with customizable wavelength selection  | Pre-installed LED modules                              |
| Multi-sample imaging    | Motorized stage scanning                             | Fixed-stage multi-well imaging                         |
| Sensitivity             | Comparable to plate reader; lower than laser scanner | Higher, optimized commercial optics                    |
| Detector                | CMOS                                                 | CMOS (for commercial)                                  |
| Sensor integration      | Expandable (pH, dissolved oxygen, temperature, etc.) | Rare / optional                                        |
| Software                | Open-source                                          | Proprietary                                            |
| Hardware modifiability  | Fully modular and upgradeable                        | Limited modification                                   |
| Maintenance cost        | Low component replacement cost                       | Higher proprietary servicing cost                      |



**Fig. 18. Emission and excitation spectrum of GFPmut2 and mCherry and transmission**

Properties of optical filters. The green and red lines show the excitation (solid line) and emission (dashed line) spectra of GFPmut2 and mCherry, respectively. The black and grey lines indicate the wavelengths transmitted by each filter. Spectrum data were obtained from FPbase ([FPbase :: The Fluorescent Protein Database](#)). Figures reproduced from Sakai et al. PLOS ONE 21, e0339652 (2026) [61] under Creative Commons Attribution 4.0.

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## Chapter 4: Application to spatiotemporal gene expression analysis

### Introduction

The main original objective of this study was to explore spatiotemporal patterns of gene expression and characterize how division-of-labor emerges in large bacterial communities under variable and dynamic environmental conditions. A previous study in our laboratory demonstrated spatial heterogeneity of protein expression within *Escherichia coli* colony biofilms (Y. Fujino, 2024, unpublished). In that study a cellulose producing *E. coli* K-12 strain (AR3110) was used to produce biofilms that exhibit greater three-dimensional complexity than the strains used here [22]. Biofilms were sampled temporally at 2, 6 and 12 days and separated into two layers, by shaking in PBS, representing the agar-sticking bottom layer and cellulose-matrix enclosed upper layer. In addition, whole 12-day old biofilms were dissected into distinct regions radially (center, middle, and edge). All samples were analyzed by proteomics, including protein extraction, proteolytic digestion followed by peptide purification and LC-MS/MS analysis. Among over 2000 proteins identified, the results revealed a group of 329 proteins that were found to be locally enriched in the center, middle, or edge regions of the biofilms. This study represents one of the first in-depth but low spatiotemporal resolution analyses of large colony biofilms. Guided by these results and to obtain complementary higher-resolution spatiotemporal information of gene expression, we applied the LOTUS imaging systems to spatiotemporal mapping of promoter activity dynamics within biofilms.

Unpublished datasets (Y. Fujino and Y. Hoshiba) were used only for promoter selection and comparative reference, with permission where required, but otherwise all data presented are original and produced by the author of the thesis.

## Results

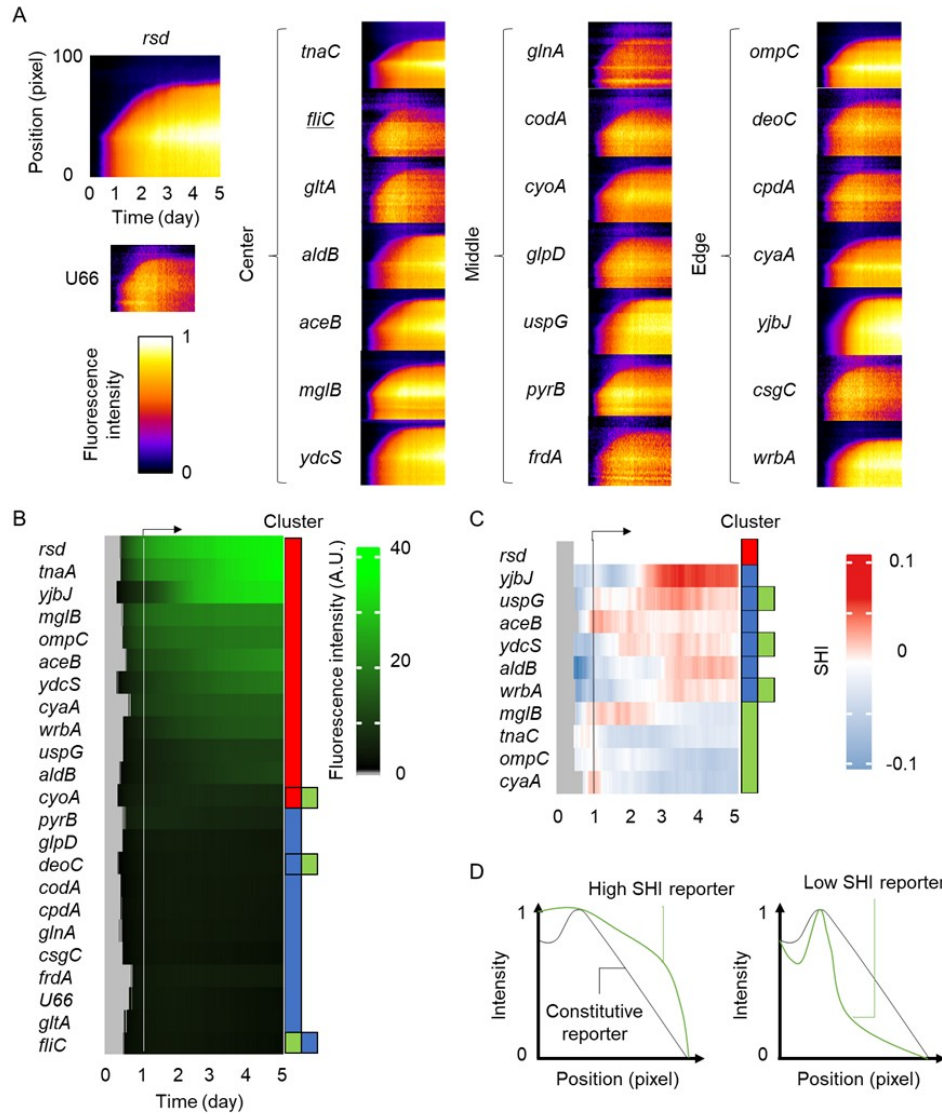
### 1. Identification of promoters with spatiotemporally heterogeneous expression

Based on the result of proteomic analysis, we selected reporter strains from the AZ promoter collection corresponding to proteins that were found to be enriched in the center, middle, or edge regions of biofilms. Seven reporter strains were chosen for each radial region. In addition, U66 (promoter-less) and *rsd* (constitutively high expression) reporter strains were included as negative and positive controls, respectively. Biofilms produced from all these strains were analyzed using the LOTUS-MP system (Fig. 19A). To visualize the spatiotemporal data, kymographs were generated from transillumination and green fluorescence images. This revealed diverse spatiotemporal expression patterns among the reporter strains. Several strains showed initially weak fluorescence that decreased further at later stages (Day 5), similar to U66, suggesting background autofluorescence not specific for these promoters. Other strains showed distinct patterns, including sharp fluorescence profiles localized near the inoculation site (e.g., *tnaC*) and relatively flat fluorescence profiles spreading radially from the biofilm center (e.g., *yjbJ*) as the biofilm expanded. Other reporters showed fluorescence profiles that either reflected the local biomass, like *rsd* (e.g., *mglB*), or exhibited spatial patterns that did not clearly belong to either defined profile type (e.g., *aldB*).

To objectively classify clusters showing increasing or decreasing fluorescence intensity trends over time, in a way that is robust to differences in response timing, Dynamic Time Warping (DTW) clustering was used. This analysis separated the reporters into two groups: fluorescence intensity that stably increased over time and fluorescence intensity that decreased in a manner comparable to the negative control U66 (Fig. 19B). Using the former group, a Spatial Heterogeneity Index (SHI) was calculated at each time point, and fluctuations in SHI were used for further classification by DTW clustering.

This analysis identified two major clusters, with increasing (red) and decreasing (blue) spatial heterogeneity (Fig. 19C). The increasing SHI cluster included *yjbJ* (putative stress response protein),

*uspG* (universal stress protein), *aceB* (malate synthase), *ycdS* (putative ABC transporter / polyhydroxybutyrate synthase), *aldB* (aldehyde dehydrogenase), and *wrbA* (NAD(P)H:quinone oxidoreductase), which exhibit relatively uniform expression with minimal differences between the biofilm center and edge (Fig. 19D). In contrast, the decreasing SHI cluster included *mglB* (D-galactose/methyl-galactoside ABC transporter), *tnaC* (*tnaAB* operon leader peptide), *ompC* (outer membrane porin), and *cyaA* (adenylate cyclase), which appear to show pronounced differential expression between the biofilm center and edge.



**Fig. 19. Spatiotemporal analysis of reporter fluorescence in biofilms using LOTUS-MP.**

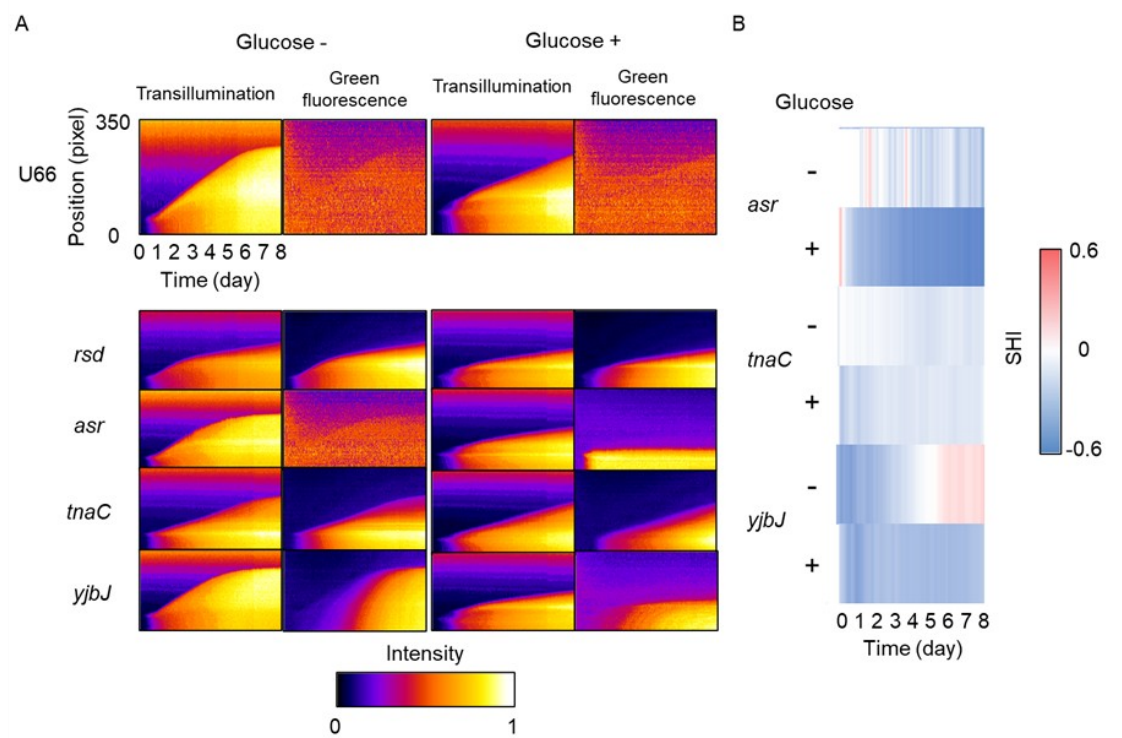
(A) Spatiotemporal changes in reporter fluorescence intensity. Biofilms were cultured for five days. Kymographs were generated using temporal fluorescence intensity data (100-pixel line). Fluorescence intensity is normalized (min-max) for each panel (B) Clustering of temporal changes in fluorescence intensity. Clustering was performed using data collected from day 1 onward, when biofilm fluorescence became detectable in all strains. The heatmap represents mean values (n=3, biological replicates). Clustering was performed using three biological replicates. A single color indicates that all three replicates were assigned to the same cluster, whereas multiple colors indicate replicate-wise variation; in such cases, the cluster most frequently assigned is shown on the left. (C) Clustering of temporal changes in the SHI (n=3, biological replicates). (D) Representative fluorescence profiles illustrating cases in which SHI increases or decreases.

## 2. Spatiotemporal analysis of promoter activity accounting for biofilm morphology and the effect of glucose supplementation on the growing biofilms

Because imaging in the transillumination mode with the LOTUS-MP does not ensure quantitative comparability of biomass (Fig. 8), SHI was calculated using the SMRI (spatial mean relative intensity) across the biofilm radius. However, to confirm that these SHI values truly reflect heterogeneity within the biofilm, normalization with biomass distribution is required. Therefore, imaging was performed using LOTUS-12 to achieve more detailed analysis. In addition, to examine how local metabolic activity influences the formation of heterogeneity within biofilms, glucose was added to induce shifts in carbon source utilization through catabolite repression and to probe possible effects on spatial distributions of promoter fluorescence signals.

The reporter strains used included *yjbJ* and *tnaC*, which showed characteristic SHI dynamics in the previous analysis. In addition, *asr* (low-pH responsive) [67], which has been reported to exhibit strong localized expression upon glucose addition (Y. Hoshiba, 2024, unpublished), was selected as a positive control, in which a change in the spatial heterogeneity index (SHI) was anticipated. In the previous chapter, we confirmed that biomass measured by red transmitted-light imaging in LOTUS-MP was affected due to positional bias; therefore, SMRI was calculated using fluorescence images of the constitutively expressed *rsd* reporter. In contrast, LOTUS-12 images each sample individually, minimizing positional effects in red transmitted-light images. Accordingly, fluorescence signals were normalized to the inverted intensity obtained from red transillumination images at each time point, and the resulting values were used to calculate nSMRI and SHI.

The *yjbJ* reporter strain exhibited a fluorescence signal spreading outward from the biofilm center, whereas *tnaC* showed regions of reduced fluorescence intensity at the biofilm edge (Fig. 20A). Corresponding changes in SHI were also observed (Fig. 20B). In contrast, under glucose-supplemented conditions, the fluorescence signal of *tnaC* reporter, after an initial delay, increased gradually over time, while *asr* and *yjbJ* displayed localized fluorescence at the center.



**Fig. 20. Spatiotemporal analysis of reporter fluorescence in biofilms using LOTUS-12.**

(A) Spatiotemporal changes in reporter biomass density (red transillumination image) and fluorescence intensity. Biofilms were cultured for 8 days in salt-free LB medium with or without glucose. Kymographs were generated using temporal fluorescence intensity and inverted intensity data (350-pixel line) displayed in the transillumination panels. (B) Mean heatmap of temporal SHI fluctuation for each reporter strain (n=3, biological replicates).

## Discussion

In this chapter, long-term live-imaging using LOTUS-MP and LOTUS-12 was employed to evaluate the spatiotemporal dynamics of gene expression within biofilms. Our results demonstrate that spatial heterogeneity of gene expression varies and can be classified into at least two distinct patterns based on their temporal behavior. The cluster in which the spatial heterogeneity index (SHI) increased over time includes genes such as *yjbJ*, *uspG*, *aceB*, *ycdS*, *aldB*, and *wrbA*, which are associated with the stress response [68-72]. These expression patterns are also consistent with existing models suggesting that the proportion of starved or oxygen-limited cells increases in the biofilm core during maturation [17]. In contrast, the cluster in which SHI decreased over time comprised genes such as *mglB*, *tnaC*, *ompC*, and *cyaA*, which are regulated by nutrient accessibility [11,73-75]. The sharp spatial expression profiles of these genes suggest higher expression near the inoculation site. This pattern may arise from enhanced nutrient availability at the periphery of the initial inoculation site during early cultivation, leading to spatially biased gene expression. However, because each cluster also contained reporters with relatively modest spatial changes compared to strongly patterned reporters such as *yjbJ* or *tnaC*, it is possible that some genes exhibit mixed characteristics of both clusters or are constitutively expressed.

We selected promoter candidates that were enriched in the center, middle, or edge regions of the biofilm based on previous proteomic analysis and examined their spatial dynamics using fluorescence imaging. The regional enrichment observed in the proteomic dataset were reflected in the kymograph and SHI results only for *tnaC* (center), *mglB* (center), *yjbJ* (edge), *uspG* (edge) and *wrbA* (edge) (Table 3).

**Table 3. Protein-level enrichment locations and corresponding SHI of individual promoter fluorescence within biofilms**

| Promoter    | Center, middle or edge | Top or bottom | SHI |
|-------------|------------------------|---------------|-----|
| <i>aldB</i> | center                 | top           | +   |
| <i>tnaC</i> | center                 | bottom        | -   |
| <i>aceB</i> | center                 | top           | +   |
| <i>ydcS</i> | center                 | top           | +   |
| <i>mglB</i> | center                 | -             | -   |
| <i>uspG</i> | middle                 | bottom        | +   |
| <i>cyaA</i> | edge                   | top           | -   |
| <i>ompC</i> | edge                   | top           | -   |
| <i>yjbJ</i> | edge                   | top           | +   |
| <i>wrbA</i> | edge                   | top           | +   |

For the other genes, endpoint spatial enrichment did not correspond to their dynamic expression patterns. Several factors may explain this discrepancy. Even assuming similar biofilm development for the cellulose-producing AR3110 strain used for proteomics compared to the MG1655 strain used here, the spatial proteomic analysis was performed only on 12-day-old mature biofilms, whereas our study monitored promoter activity temporally during biofilm development. In addition, proteomics measures directly protein abundance, while our analysis reflects transcriptional activity. Sampling regions in the proteomic study also depended on manual dissection, which may introduce variability. Importantly, none of the genes analyzed here showed sharply localized expression as suggested by the proteomic endpoint data. Thus, interpreting spatiotemporal gene expression solely from endpoint omics data with limited spatial resolution may be misleading.

Both the increasing and decreasing SHI clusters included genes enriched in either the upper (top) or lower (bottom) layers of the biofilm. Because our imaging system projects depth information onto a two-dimensional image, vertical differences are compressed and cannot be directly separated in the SHI analysis. However, by comparing SHI dynamics with top–bottom enrichment data, we can infer possible spatial progression patterns. For example, *uspG*, which is associated with anaerobic stress and enriched in the lower layer, showed increasing SHI. This suggests that its expression may begin

in the lower central region and spread relatively quickly toward the edge as oxygen becomes limited. In contrast, *tnaC*, enriched in the center-bottom region, showed decreasing SHI, suggesting more localized expression near the inoculation site with slower outward expansion. Other stress-related genes (*yjbJ*, *wrbA*, *aldB*, *ydcS*) and nutrient-responsive genes (*aceB*, *mglB*, *cyaA*, *ompC*) appear to initiate in the upper central region and gradually extend toward the periphery, consistent with starvation and metabolic stress spreading from the biofilm center.

Imaging with LOTUS-12 revealed that glucose supplementation was associated with changes in the spatiotemporal expression patterns of the *asr*, *yjbJ*, and *tnaC* promoters. At early stages of growth, glucose represses the utilization of other carbon sources and results in organic acid production and local pH reduction, which is likely responsible for the observed induction of *asr* expression [67,76]. Such pH changes may promote the expression of anti-adaptors, thereby attenuating RssB–ClpXP–dependent degradation of RpoS and leading to its accumulation, which in turn may underlie the central localization of *yjbJ* expression [77,78]. In addition, cells located in the outer regions of the biofilm are likely less susceptible to starvation due to higher glucose accessibility. The progressive peripheral expansion of the *tnaC* reporter suggests that indole production increases at the growing edge of the biofilm after structural maturation has been established. Because TnaA-mediated tryptophan degradation generates indole, this spatially restricted activation may locally modulate persister formation and antibiotic resistance [79,80]. Accordingly, TnaA-dependent indole production may contribute to the formation of spatially heterogeneous tolerance zones within the biofilm. The delayed induction of *tnaC* observed upon glucose addition is likely attributable to reduced intracellular cAMP levels caused by catabolite repression [81]. The recapitulation of these trends in the improved LOTUS-12 confirms that the observed spatiotemporal expression patterns reflect intrinsic physiological properties rather than imaging artifacts, demonstrating the robustness of both platforms. GFP chromophore maturation requires molecular oxygen, and reduced fluorescence would be expected in

oxygen-limited regions such as the biofilm core [25]. However, clear fluorescence from reporters expected to be highly expressed in the bottom layer of the biofilm was detected, suggesting that the bottom region is likely microaerobic rather than strictly anaerobic. These findings indicate that, for sufficiently high GFP expression levels, LOTUS-MP can reliably detect fluorescence signals across biofilm depth, although without resolving their vertical position.

## Conclusion

In this study, to analyze spatiotemporal gene expression in colony biofilms, we designed, developed, and validated two different imaging systems, the LOTUS series. LOTUS-MP enables simultaneous semi-quantitative analysis of four multi-well plates in two modes, suitable for preliminary screening of strains or conditions. LOTUS-12 enables more detailed, reproducible, four-mode, quantitative analysis of morphology and fluorescence in up to 12 samples. The LOTUS platforms provide accessible and practical options for multimodal live-imaging under constraints of personnel, time, space, and budget. In addition, the benchmark tests performed during system development serve as useful references for future imaging device design. We further applied the LOTUS systems to investigate spatiotemporal gene expression in *Escherichia coli* colony biofilms. We demonstrated how stress responses spread from the biofilm center toward the periphery and how glucose supplementation spatially and temporally regulates the activity of a subset of promoters. These findings may help identify localized vulnerabilities within biofilms and contribute to a better understanding of persistence and resistance mechanisms in biofilm architecture. As future perspectives, further improvements to the LOTUS platform can be envisioned. Enhancing the optical system would improve spatial resolution and fluorescence sensitivity, and the incorporation of higher-quality lenses and more uniform illumination would enable more precise quantification of fine-scale expression patterns. In addition, expanding automation and implementing a multi-sample stage would increase throughput, making large-scale screening and systematic condition testing more feasible. Finally, extending the system to real-time monitoring of proteome dynamics using fluorescent fusion proteins would allow simultaneous tracking of transcriptional and protein-level changes, thereby providing deeper insight into functional networks within biofilms.

## **General materials and methods**

### **Development of LOTUS series**

#### **System design**

The device framework was designed using OpenSCAD (v2021.01). To account for the dimensional accuracy of the 3D printer, a clearance of 0.5 mm was applied to all contact interfaces between parts. Gears, rails, and the motor box were based on the *Linear mechanism* design (Thingiverse ID: 2707156). The gear rail was extended using FreeCAD (v0.21.2).

#### **3D printing**

The device framework was 3D-printed using black PLA filament (0.75 mm, ELEGOO) on an Anycubic Kobra 2 Max printer. G-code was generated with Anycubic Slicer (v1.3.0). The extruder nozzle and build plate temperatures were set to 220°C and 60°C, respectively, with a layer height of 0.2 mm. To prevent print detachment, adhesive was applied to the build plate. Automatic printing settings were used to minimize the amount of support material for all parts.

#### **Single-board computer and camera settings**

System control was implemented using a Raspberry Pi 4 Model B. The Legacy OS (64-bit, released March 12, 2024) was installed on a microSD card using Raspberry Pi Imager (v1.8.5). The camera module (Arducam IMX519 autofocus 16 MP) was set up according to the official Arducam documentation (<https://docs.arducam.com/Raspberry-Pi-Camera/Native-camera/16MP-IMX519/>). Operational scripts were daemonized using the Linux process management system Supervisor.

#### **Control of relay module and stepping motor**

Illumination control was achieved using an electromagnetic relay module equipped with a TUEJING JQC-3F (T73, 10 A, 250 VAC). A stepping motor (28BYJ-48) was driven by a stepping motor controller based on the ULN2003. One GPIO pin was used as input to the relay module, while

four GPIO pins were used to control the stepping motor driver. All IC logic circuits were powered with a 5 V supply.

### **Design of illumination modules in LOTUS-MP**

The illumination module consisted of a printed circuit board (PCB), LEDs, resistors, and male pin headers. The PCB was designed using KiCad (v8.0) and mounted with 180 two-color flux LEDs (10 × 18 array), arranged as 36 parallel sections, each containing five LEDs connected in series. A 180-Ω resistor was used for each blue LED section, and a 390-Ω resistor was used for each red LED section. Resistor values were calculated using Ohm's law. A 4-pin header was soldered onto the PCB to allow connection to an external power supply. The illumination module was powered by an 18 V, 1 A external supply.

### **Design of illumination module in LOTUS-12**

The illumination module consisted of a universal prototyping board, LEDs, resistors, and male pin headers. Each board was populated with 25 LEDs (5 mm diameter), arranged as five parallel sections, each containing five LEDs connected in series. A 100-Ω resistor was soldered to each LED section. A 2-pin header was soldered onto the board for connection to an external power supply. The illumination module was powered by an 18 V, 1 A external supply. Excess portions of the board were removed after assembly.

### **Measurement of actuator step size**

Sample positioning. Sample positioning was achieved using stepping motors connected to linear actuators along the x and y axis. Actuator step size was measured using a Vernier micrometer. The number of steps was increased from 0 to 640 in increments of 32, and the travel distance after each increment was recorded. Linear regression was used to determine the relationship between motor steps and displacement distance, allowing calculation of steps required for a given displacement.

### **Assembly**

Assembly instructions and setup procedures for LOTUS-12 are available on GitHub ([GitHub - mrobert7/MSB\\_imaging](#): For files for imaging system development).

## Image acquisition: Camera and optics

For LOTUS, images were captured on an Arducam autofocus 64 MP or 16MP camera module connected to a Raspberry Pi camera port via a 1 m ribbon cable.

## Imaging acquisition setting of LOTUS-MP

Green fluorescence was excited using blue LEDs and selectively detected through an amber acrylic filter (Kanase Light, Orange Clear, 3 mm thickness). Blue LED illumination was applied through a blue acrylic filter (Kanase Light, Ultramarine, 3 mm thickness). The imaging interval was set to 20 min to match the optimal growth rate of *Escherichia coli* [82]. The imaging conditions for each mode are described in table 4.

**Table 4. Imaging settings on LOTUS-MP**

| Transillumination red image |                 | Green fluorescence image |                 |
|-----------------------------|-----------------|--------------------------|-----------------|
| Image format                | PNG             | Image format             | PNG             |
| Image resolution            | 2312 × 1736     | Image resolution         | 2312 × 1736     |
| Contrast                    | 1               | Contrast                 | 1               |
| Brightness                  | 0.1             | Brightness               | 0.1             |
| Shutter (μs)                | 700             | Shutter (μs)             | 50000           |
| Denoise                     | off             | Denoise                  | off             |
| Autofocus manual            | Lens position 8 | Autofocus manual         | Lens position 8 |
| Gain                        | 1               | Gain                     | 3               |
| Auto white balance          | 1.5, 2.0        | Auto white balance       | 1.5, 2.0        |

## Imaging acquisition setting of LOTUS-12

No optical filters were used for transmission or white epi-illumination imaging. Green fluorescence was excited using blue LEDs (465 nm; LP-5B4SCHJ12, LED Paradise, Tokyo, Japan) and detected through a band-pass filter (FLH-532–10, Thorlabs, Newton, NJ, USA). Red fluorescence was excited using green LEDs (520 nm; LP-5SG4SCHJ15, LED Paradise) and detected through a long-pass filter (FGL665, Thorlabs). Imaging conditions for each mode are described in table 5.

**Table 5. Imaging settings on LOTUS-12**

| Transillumination image |                  | Green fluorescence image |                  | Red fluorescence image |                  |
|-------------------------|------------------|--------------------------|------------------|------------------------|------------------|
| Image format            | JPEG             | Image format             | JPEG             | Image format           | JPEG             |
| Image resolution        | 1920×1080        | Image resolution         | 1920×1080        | Image resolution       | 1920×1080        |
| Contrast                | 1                | Contrast                 | 1                | Contrast               | 1                |
| Brightness              | 0.1              | Brightness               | 0.1              | Brightness             | 0.1              |
| Shutter (μs)            | 250              | Shutter (μs)             | 3000             | Shutter (μs)           | 30000            |
| Denoise                 | off              | Denoise                  | off              | Denoise                | off              |
| Autofocus manual        | Lens position 14 | Autofocus manual         | Lens position 14 | Autofocus manual       | Lens position 14 |
| Gain                    | 2                | Gain                     | 2                | Gain                   | 2                |
|                         |                  | Auto white balance       | 1.5, 2.0         | Auto white balance     | 1.5, 2.0         |

## Comparative imaging and measurement

### Sensitivity analysis using fluorescein

Fluorescence detection sensitivity was compared for LOTUS-12, a Mithras LB940 multimode microplate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) and a Amersham Typhoon laser scanner (Cytiva, Marlborough, MA, USA) using serial dilutions of fluorescein sodium salt (Nacalai Tesque, Inc., Kyoto, Japan) ( $10^{-3}$  to  $10^{-9}$  M) dispensed into a 96-well plate in triplicates. Green fluorescence intensity was measured in each well on all instruments and values obtained without fluorescein (0 M) (background) were subtracted from all measurement values. To adapt LOTUS-12 for use with microplates, a sample tray for the well plate was designed, printed, and attached to the 2D actuator (Fig 13C). Then, all 96 wells were imaged in sets of  $2 \times 2$  wells while varying the shutter speed. The fluorescence intensity per well was measured using the captured images. Settings for each instrument are described in table 6.

**Table 6. Settings for each instrument**

| Plate reader (Mithras LB940) |               | Laser scanner (Amersham Typhoon) |              |
|------------------------------|---------------|----------------------------------|--------------|
| Plate type                   | 96-well plate | Laser name                       | 488 nm       |
| Counting time [s]            | 0.05          | Filter name                      | Cy2 525BP20  |
| Measurement mode             | by plate      | PMT Type                         | Multi-alkali |
| Excitation filter            | F485          | Voltage                          | 250          |
| Emission filter              | F535          | Laser power mode                 | High         |
| Excitation aperture          | Normal        | Pixel size (μm / pixel)          | 200          |
| Lamp Energy                  | 1000          |                                  |              |
| Counter Position             | Top           |                                  |              |

### **Uniformity and reproducibility within biofilms**

For imaging comparisons and validation, biofilms images were acquired using an Amersham Typhoon laser scanner under the same conditions as those used for imaging fluorescein using FluorescenceFMS.

### **Image uniformity across colony position**

To compare area and inverted intensity, GT-X970 was used for transillumination imaging as a reference device. Images were encoded in tiff format. A Mithras LB940 multimode microplate reader was used for measuring the green fluorescence intensity.

### **Detail imaging in macroscale**

Olympus SZX7 (DF PLAPO 1× objective) stereomicroscope was used for macroscale imaging. The camera was controlled using Swift imaging (ver 3.0) with a 70 ms (epi-white illuminated image) and a gain of 500%.

## Bacterial strains and growth condition

**Table 7. Strain and plasmid used in this study**

| Strain         | Reference | Identifier                                                                                                                      |
|----------------|-----------|---------------------------------------------------------------------------------------------------------------------------------|
| <i>E. coli</i> |           |                                                                                                                                 |
| BW25113        | [4]       |                                                                                                                                 |
| <i>ΔcsgA</i>   | [4]       | <i>E. coli</i> BW25113 <i>csgA::kanR</i>                                                                                        |
| W3110          | [48]      |                                                                                                                                 |
| U66            | [5]       | <i>E. coli</i> MG1655 strain carrying pUA66 plasmid                                                                             |
| <i>aceB</i>    | [5]       | <i>E. coli</i> MG1655 strain carrying pUA66- <i>aceB</i> (Inserted <i>aceB</i> promoter region to multicloning site of plasmid) |
| <i>aldB</i>    | [5]       | MG1655 strain carrying pUA66- <i>aldB</i>                                                                                       |
| <i>cld</i>     | [5]       | MG1655 strain carrying pUA66- <i>cld</i>                                                                                        |
| <i>codA</i>    | [5]       | MG1655 strain carrying pUA66- <i>codA</i>                                                                                       |
| <i>cpdA</i>    | [5]       | MG1655 strain carrying pUA66- <i>cpdA</i>                                                                                       |
| <i>csgC</i>    | [5]       | MG1655 strain carrying pUA66- <i>csgC</i>                                                                                       |
| <i>cyaA</i>    | [5]       | MG1655 strain carrying pUA66- <i>cyaA</i>                                                                                       |
| <i>cyoA</i>    | [5]       | MG1655 strain carrying pUA66- <i>cyoA</i>                                                                                       |
| <i>deoC</i>    | [5]       | MG1655 strain carrying pUA66- <i>deoC</i>                                                                                       |
| <i>fliC</i>    | [5]       | MG1655 strain carrying pUA66- <i>fliC</i>                                                                                       |
| <i>frdA</i>    | [5]       | MG1655 strain carrying pUA66- <i>frdA</i>                                                                                       |
| <i>glnA</i>    | [5]       | MG1655 strain carrying pUA66- <i>glnA</i>                                                                                       |
| <i>glpD</i>    | [5]       | MG1655 strain carrying pUA66- <i>glpD</i>                                                                                       |
| <i>gltA</i>    | [5]       | MG1655 strain carrying pUA66- <i>gltA</i>                                                                                       |
| <i>hipB</i>    | [5]       | MG1655 strain carrying pUA66- <i>hipB</i>                                                                                       |
| <i>mglB</i>    | [5]       | MG1655 strain carrying pUA66- <i>mglB</i>                                                                                       |
| <i>ompA</i>    | [5]       | MG1655 strain carrying pUA66- <i>ompA</i>                                                                                       |
| <i>ompC</i>    | [5]       | MG1655 strain carrying pUA66- <i>ompC</i>                                                                                       |
| <i>pyrB</i>    | [5]       | MG1655 strain carrying pUA66- <i>pyrB</i>                                                                                       |
| <i>ribB</i>    | [5]       | MG1655 strain carrying pUA66- <i>ribB</i>                                                                                       |
| <i>tnaA</i>    | [5]       | MG1655 strain carrying pUA66- <i>tnaA</i>                                                                                       |
| <i>uspG</i>    | [5]       | MG1655 strain carrying pUA66- <i>uspG</i>                                                                                       |
| <i>wrbA</i>    | [5]       | MG1655 strain carrying pUA66- <i>wrbA</i>                                                                                       |
| <i>ydcS</i>    | [5]       | MG1655 strain carrying pUA66- <i>ydcS</i>                                                                                       |
| <i>yjbJ</i>    | [5]       | MG1655 strain carrying pUA66- <i>yjbJ</i>                                                                                       |

|                      |                      |                                                                                       |
|----------------------|----------------------|---------------------------------------------------------------------------------------|
| <i>rsd</i>           | [5]                  | MG1655 strain carrying pUA66- <i>rsd</i>                                              |
| U66p5mCherry         | This study           | <i>E. coli</i> MG1655 strain carrying pUA66 and p5mCherry                             |
| <i>rsdp</i> 5mCherry | This study           | <i>E. coli</i> MG1655 strain carrying pUA66- <i>rsd</i> and p5mCherry                 |
| <i>ptsI-yfp</i>      | H. Mori, unpublished | <i>E. coli</i> W3110 <i>ptsI-yfp</i> (C-terminal in-frame fusion at the native locus) |
| <i>prpC-yfp</i>      | H. Mori, unpublished | W3110 <i>prpC-yfp</i>                                                                 |
| <i>fumA-yfp</i>      | H. Mori, unpublished | W3110 <i>fumA-yfp</i>                                                                 |
| <i>tktA-yfp</i>      | H. Mori, unpublished | W3110 <i>tktA-yfp</i>                                                                 |
| <i>glcB-yfp</i>      | H. Mori, unpublished | W3110 <i>glcB-yfp</i>                                                                 |
| <i>acs-yfp</i>       | H. Mori, unpublished | W3110 <i>acs-yfp</i>                                                                  |
| <i>crp-yfp</i>       | H. Mori, unpublished | W3110 <i>crp-yfp</i>                                                                  |
| <i>gpmA-yfp</i>      | H. Mori, unpublished | W3110 <i>gpmA-yfp</i>                                                                 |
| <i>crr-yfp</i>       | H. Mori, unpublished | W3110 <i>crr-yfp</i>                                                                  |
| <i>mgo-yfp</i>       | H. Mori, unpublished | W3110 <i>mgo-yfp</i>                                                                  |
| <i>gntR-yfp</i>      | H. Mori, unpublished | W3110 <i>gntR-yfp</i>                                                                 |
| <i>galM-yfp</i>      | H. Mori, unpublished | W3110 <i>galM-yfp</i>                                                                 |
| <i>pgk-yfp</i>       | H. Mori, unpublished | W3110 <i>pgk-yfp</i>                                                                  |
| <i>sdhC-yfp</i>      | H. Mori, unpublished | W3110 <i>sdhC-yfp</i>                                                                 |
| <i>mdh-yfp</i>       | H. Mori, unpublished | W3110 <i>mdh-yfp</i>                                                                  |
| <i>gntK-yfp</i>      | H. Mori, unpublished | W3110 <i>gntK-yfp</i>                                                                 |
| <i>aceA-yfp</i>      | H. Mori, unpublished | W3110 <i>aceA-yfp</i>                                                                 |
| <i>agp-yfp</i>       | H. Mori, unpublished | W3110 <i>agp-yfp</i>                                                                  |
| <i>gpmI-yfp</i>      | H. Mori, unpublished | W3110 <i>gpmI-yfp</i>                                                                 |
| <i>sdhD-yfp</i>      | H. Mori, unpublished | W3110 <i>sdhD-yfp</i>                                                                 |
| <i>fumB-yfp</i>      | H. Mori, unpublished | W3110 <i>fumB-yfp</i>                                                                 |
| <i>alsI-yfp</i>      | H. Mori, unpublished | W3110 <i>rpiB=alsI-yfp</i>                                                            |
| <i>poxB-yfp</i>      | H. Mori, unpublished | W3110 <i>poxB-yfp</i>                                                                 |
| <i>frdB-yfp</i>      | H. Mori, unpublished | W3110 <i>frdB-yfp</i>                                                                 |
| <i>arcA-yfp</i>      | H. Mori, unpublished | W3110 <i>arcA-yfp</i>                                                                 |
| <i>ptsG-yfp</i>      | H. Mori, unpublished | W3110 <i>ptsG-yfp</i>                                                                 |
| <i>pfkA-yfp</i>      | H. Mori, unpublished | W3110 <i>pfkA-yfp</i>                                                                 |
| <i>talB-yfp</i>      | H. Mori, unpublished | W3110 <i>talB-yfp</i>                                                                 |
| <i>idnK-yfp</i>      | H. Mori, unpublished | W3110 <i>idnK-yfp</i>                                                                 |
| <i>pykF-yfp</i>      | H. Mori, unpublished | W3110 <i>pykF-yfp</i>                                                                 |
| <i>tpiA-yfp</i>      | H. Mori, unpublished | W3110 <i>tpiA-yfp</i>                                                                 |

|                 |                      |                            |
|-----------------|----------------------|----------------------------|
| <i>ybhA-yfp</i> | H. Mori, unpublished | W3110 <i>ybhA-yfp</i>      |
| <i>pfkB-yfp</i> | H. Mori, unpublished | W3110 <i>pfkB-yfp</i>      |
| <i>pgi-yfp</i>  | H. Mori, unpublished | W3110 <i>pgi-yfp</i>       |
| <i>pgl-yfp</i>  | H. Mori, unpublished | W3110 <i>ybhE=pgl-yfp</i>  |
| <i>maeA-yfp</i> | H. Mori, unpublished | W3110 <i>sfcA=maeA-yfp</i> |
| <i>ldhA-yfp</i> | H. Mori, unpublished | W3110 <i>ldhA-yfp</i>      |
| <i>fur-yfp</i>  | H. Mori, unpublished | W3110 <i>fur-yfp</i>       |
| <i>rpoS-yfp</i> | H. Mori, unpublished | W3110 <i>rpoS-yfp</i>      |
| <i>gapA-yfp</i> | H. Mori, unpublished | W3110 <i>gapA-yfp</i>      |
| <i>gpmB-yfp</i> | H. Mori, unpublished | W3110 <i>ytjC=gpmB-yfp</i> |
| <i>sucB-yfp</i> | H. Mori, unpublished | W3110 <i>sucB-yfp</i>      |
| <i>zwf-yfp</i>  | H. Mori, unpublished | W3110 <i>zwf-yfp</i>       |
| <i>adhP-yfp</i> | H. Mori, unpublished | W3110 <i>adhP-yfp</i>      |
| <i>infA-yfp</i> | H. Mori, unpublished | W3110 <i>infA-yfp</i>      |
| <i>gnd-yfp</i>  | H. Mori, unpublished | W3110 <i>gnd-yfp</i>       |
| <i>maeB-yfp</i> | H. Mori, unpublished | W3110 <i>maeB-yfp</i>      |
| <i>ackA-yfp</i> | H. Mori, unpublished | W3110 <i>ackA-yfp</i>      |
| <i>narL-yfp</i> | H. Mori, unpublished | W3110 <i>narL-yfp</i>      |
| <i>rpoN-yfp</i> | H. Mori, unpublished | W3110 <i>rpoN-yfp</i>      |
| <i>sucD-yfp</i> | H. Mori, unpublished | W3110 <i>sucD-yfp</i>      |
| <i>talA-yfp</i> | H. Mori, unpublished | W3110 <i>talA-yfp</i>      |
| <i>fnr-yfp</i>  | H. Mori, unpublished | W3110 <i>fnr-yfp</i>       |
| <i>rpoH-yfp</i> | H. Mori, unpublished | W3110 <i>rpoH-yfp</i>      |
| <i>glk-yfp</i>  | H. Mori, unpublished | W3110 <i>glk-yfp</i>       |
| <i>fecI-yfp</i> | H. Mori, unpublished | W3110 <i>fecI-yfp</i>      |
| <i>fumC-yfp</i> | H. Mori, unpublished | W3110 <i>fumC-yfp</i>      |
| <i>rpiA-yfp</i> | H. Mori, unpublished | W3110 <i>rpiA-yfp</i>      |
| <i>fis-yfp</i>  | H. Mori, unpublished | W3110 <i>fis-yfp</i>       |
| <i>gltA-yfp</i> | H. Mori, unpublished | W3110 <i>gltA-yfp</i>      |
| <i>rpe-yfp</i>  | H. Mori, unpublished | W3110 <i>rpe-yfp</i>       |
| <i>aceB-yfp</i> | H. Mori, unpublished | W3110 <i>aceB-yfp</i>      |
| <i>frdC-yfp</i> | H. Mori, unpublished | W3110 <i>frdC-yfp</i>      |
| <i>dcuR-yfp</i> | H. Mori, unpublished | W3110 <i>dcuR-yfp</i>      |
| <i>sdhA-yfp</i> | H. Mori, unpublished | W3110 <i>sdhA-yfp</i>      |
| <i>frdA-yfp</i> | H. Mori, unpublished | W3110 <i>frdA-yfp</i>      |

|                 |                      |                       |
|-----------------|----------------------|-----------------------|
| <i>fliA-yfp</i> | H. Mori, unpublished | W3110 <i>fliA-yfp</i> |
| <i>gcd-yfp</i>  | H. Mori, unpublished | W3110 <i>gcd-yfp</i>  |
| <i>sucA-yfp</i> | H. Mori, unpublished | W3110 <i>sucA-yfp</i> |
| <i>pps-yfp</i>  | H. Mori, unpublished | W3110 <i>pps-yfp</i>  |
| <i>pykA-yfp</i> | H. Mori, unpublished | W3110 <i>pykA-yfp</i> |
| <i>aceF-yfp</i> | H. Mori, unpublished | W3110 <i>aceF-yfp</i> |
| <i>glpX-yfp</i> | H. Mori, unpublished | W3110 <i>glpX-yfp</i> |
| <i>tdcE-yfp</i> | H. Mori, unpublished | W3110 <i>tdcE-yfp</i> |
| <i>fbp-yfp</i>  | H. Mori, unpublished | W3110 <i>fbp-yfp</i>  |
| <i>fbaA-yfp</i> | H. Mori, unpublished | W3110 <i>fbaA-yfp</i> |
| <i>eno-yfp</i>  | H. Mori, unpublished | W3110 <i>eno-yfp</i>  |
| <i>sdhB-yfp</i> | H. Mori, unpublished | W3110 <i>sdhB-yfp</i> |
| <i>adhE-yfp</i> | H. Mori, unpublished | W3110 <i>adhE-yfp</i> |
| <i>frdD-yfp</i> | H. Mori, unpublished | W3110 <i>frdD-yfp</i> |
| <i>pflB-yfp</i> | H. Mori, unpublished | W3110 <i>pflB-yfp</i> |
| <i>fruR-yfp</i> | H. Mori, unpublished | W3110 <i>fruR-yfp</i> |
| <i>aceE-yfp</i> | H. Mori, unpublished | W3110 <i>aceE-yfp</i> |
| <i>fbaB-yfp</i> | H. Mori, unpublished | W3110 <i>fbaB-yfp</i> |
| <i>pck-yfp</i>  | H. Mori, unpublished | W3110 <i>pck-yfp</i>  |
| <i>pta-yfp</i>  | H. Mori, unpublished | W3110 <i>pta-yfp</i>  |
| <i>lpd-yfp</i>  | H. Mori, unpublished | W3110 <i>lpd-yfp</i>  |
| <i>tktB-yfp</i> | H. Mori, unpublished | W3110 <i>tktB-yfp</i> |
| <i>ppc-yfp</i>  | H. Mori, unpublished | W3110 <i>ppc-yfp</i>  |
| <i>rpoD-yfp</i> | H. Mori, unpublished | W3110 <i>rpoD-yfp</i> |
| <i>sucC-yfp</i> | H. Mori, unpublished | W3110 <i>sucC-yfp</i> |
| <i>acnB-yfp</i> | H. Mori, unpublished | W3110 <i>acnB-yfp</i> |
| <i>acnA-yfp</i> | H. Mori, unpublished | W3110 <i>acnA-yfp</i> |
| <i>icd-yfp</i>  | H. Mori, unpublished | W3110 <i>icd-yfp</i>  |
| Plasmid         |                      |                       |
| p5mCherry       | [62]                 |                       |

p5mCherry was developed by Brian Pflieger and obtained from Addgene (plasmid # 92023).

The E. coli promoter collection [5] was obtained from Horizon Discovery:

<https://horizondiscovery.com/en/non-mammalian-research-tools/products/e-coli-promoter-collection>

All recombinant DNA experiments were reviewed and approved by the Recombinant DNA Experiment Safety Committee of Kyoto University. Applications were submitted through the Kyoto University Application System for Experiments Using Living Modified Organisms (KUELMO), and experiments were performed only after obtaining official approval.

### **Bacterial growth and biofilm formation in isolated condition**

Bacteria from 15% glycerol stocks stored at -80°C were streaked onto Luria-Bertani (LB) agar plates (1% Bacto tryptone (BD Difco, Sparks, MD, USA), 0.5% Bacto yeast extract (BD Difco), 1% sodium chloride (Nacalai Tesque, Inc., Kyoto, Japan) and 1.5% Bacto Agar (BD Difco)), and incubated for 16 h at 37°C. Biofilms were prepared according to Serra et al. [22] with the following modifications. For biofilm preparation, single colonies were inoculated into 2 ml of LB medium and incubated for 16 h at 37°C while shaking at 250 rpm. Overnight cultures were diluted with salt-free LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract) to OD<sub>660</sub> = 0.1. Salt-free LB containing 1.5% Bacto agar was sterilized by autoclaving at 120°C for 20 min, cooled and maintained at 60°C, and 5 ml was poured into 36.7 mm Petri dishes. Dishes were left to solidify and dry with the lid closed for 30 min, and then dried with the lid open for another 30 min. A volume of 5 µl of the diluted overnight culture was then spotted onto the salt-free LB agar plate, and the plates were dried with the lid closed for 30 min. Plates were sealed with parafilm to prevent evaporation and incubated at 28°C. Antibiotics were added when required: 100 µg/ml ampicillin sodium, (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or 25 µg/ml kanamycin sulfate, (Nacalai Tesque, Inc., Kyoto, Japan) or both [5,62]. For sub-MIC experiments, 8 µg/ml ampicillin was used with 1 µl spotting volume to approximate the conditions used by French et al. [7]. For cultures in 12-well plates, the medium volume was set to 1 ml and the spotting volume was adjusted to 3 µL.

### **Bacterial growth and biofilm formation on single-well plates for screening**

Bacteria from glycerol stocks were inoculated into 200 µl LB medium in 96-well plates and

incubated without shaking for 16 h at 37°C. Overnight cultures were diluted with salt-free LB medium and inoculated onto salt free LB agar plates using a 96-pin replicator and incubated for 3 days at 28 °C. Antibiotics were added as required for reporter strain selection (25 µg/mL kanamycin for the AZ collection and 25 µg/mL chloramphenicol for the YFP fusion reporter collection).

### **Preparation of competent cells and transformation**

Competent cells were prepared using the calcium chloride method [83]. A single colony was inoculated into 2 ml of liquid LB medium and incubated for 16 h in a shaker at 37°C and 250 rpm. Then, 20 µl of overnight cultures were inoculated into 2 ml of LB liquid medium and incubated at 37°C for 2 hours at 250 rpm. The cultures were then washed twice with 50 mM calcium chloride solution on ice to prepare chemical competent cells. Plasmid purification was performed using the QIAprep Spin Miniprep Kit (Qiagen). A volume of 5 µl of plasmid solution was added to 25 µl of chemical competent cells and the cells were incubated on ice for 30 min, heat shocked at 42°C for 1 min, and then incubated on ice for another 2 min. A volume of 270 µl of LB medium was added to each transformant and incubated at 37°C for 1 hour with shaking at 250 rpm. Finally, 100 µl of the transformed cells were inoculated onto solid LB medium supplemented with antibiotics (ampicillin 100 µg/ml and kanamycin 25 µg/ml). Plates were incubated overnight (approximately 12-16 hours) at 37°C for colony formation.

### **Quantitative image analysis**

Image analysis was performed using Fiji ImageJ (v1.54g; National Institutes of Health, Bethesda, MD, USA). All images were converted to 8-bit format prior to thresholding and measurement. For bright-field and scanner images, image intensity was inverted.

### **Quantification of Congo Red staining intensity using a stereomicroscope**

A rectangular ROI was applied to the mid-region of the biofilm, and the mean intensity within the

ROI was measured and used as the Congo Red staining intensity.

### **Measurement of spatial pixel intensity**

The distributions of inverted intensity and fluorescence intensity in the resolution target and colonies were measured by applying a linear ROI and using the “*Plot Profile*” function.

### **Generation of kymograph**

For kymograph generation, all images were background-subtracted using the initial image prior to analysis. Linear ROI was drawn across the center of each biofilm to extract intensity values and generate radial profiles. The resulting intensity arrays were chronologically arranged and imported into Fiji as text images. Kymographs were colored using the “*Pseudocolor Image Look-Up Table*” function.

### **Measurement of alignment position and mean intensity**

ROIs were applied to enclose the circular apertures in the fabricated PLA plates and PLA disks, and images were binarized using the “*Auto Threshold*” function (Otsu, dark, no reset). The circular aperture regions were selected using the “*Make Selection*” function, and changes in the measured center coordinates were calculated. Pixel size was determined from the Feret diameter of the circular aperture. When required, the mean intensity within the selected region was measured.

### **Biomass proxy and fluorescence quantification of whole biofilm**

The influence of green fluorescence was removed by extracting the red channel images using the “*Split Channels*” function. To correct for temporal changes or uneven transillumination, the “*Image Calculator*” tool was used to subtract the initial image from all subsequent images. Transmitted-light images were binarized by applying “*Auto Threshold*” within an ROI enclosing each colony. The colony region was selected using the “*Make Selection*” function and defined as the inner colony region, while the surrounding area selected by “*Make Inverse*” was defined as the outer region. Mean intensity and area were measured for both regions. Mean intensity within the colony ( $I_{inside}$ ) was normalized

by subtracting that of the outer region ( $I_{outside}$ ) [6]. We refer to this metric as “biomass proxy” throughout the manuscript, noting that while this measurement correlates with transmitted light attenuation through the biofilm and provides reliable relative comparisons under our experimental conditions, it should not be considered equivalent to spectrophotometric absorbance or optical density measurements.

$$biomass\ proxy = I_{inside} - I_{outside}$$

Due to extracellular matrix contribution, it can only provide an estimate of cell density. However, it provides a reliable biomass proxy for relative comparisons under similar experimental conditions and for samples in which no major quantitative changes in matrix compositions are expected. Under the conditions used here, the values were highly correlated with those obtained from fluorescence values of a constitutively expressed promoter. For fluorescence measurements, the colony outline ROI obtained from red transmitted-light images was applied to the corresponding fluorescence images, and mean fluorescence intensity was measured and normalized in the same manner. The fluorescence intensity of the biofilm was determined by multiplying the difference between the mean fluorescence intensities of the inner and outer regions by the area. Normalized fluorescence was calculated as the total signal intensity divided by the biomass proxy value at the corresponding time point. Normalization of fluorescence intensity using a biomass proxy was performed only for data acquired with LOTUS-12.

### **Resolution determination**

A resolution target (R1L1S7P; Thorlabs) was imaged, and the spatial resolution was determined from the smallest element within each group in which the stripe pattern could be clearly resolved. Stripe patterns with a Michelson contrast (MC) of  $\geq 0.1$  were considered resolvable, according to

established criteria [84,85]. Resolution values were calculated using the line-pair density table provided in the Thorlabs *Resolution Test Targets* product specifications.

[https://www.thorlabs.co.jp/newgrouppage9.cfm?objectgroup\\_id=4338](https://www.thorlabs.co.jp/newgrouppage9.cfm?objectgroup_id=4338)

### Focus drift quantification

A Laplacian filter was applied to image of the resolution target to emphasize edges. The processed image was used to obtain the profile of the ladder (Group 0, Element 1) using the Line Selection tool in Fiji, and the variance of all pixels was calculated overtime.

### Spatial heterogeneity index (SHI)

We used an index to estimate how much fluorescent protein expression levels vary spatially from the expected fluorescence level from biomass contribution (local expression enrichment). Biomass contribution is derived either from a constitutively expressed promoter (*rsd*) fluorescence signal or from the biomass proxy obtained from red transillumination intensity. Variation from the biomass intensity profile for each pixel and time point (kymograph) is defined as the spatial heterogeneity index. For kymograph data obtained using LOTUS, the standard deviation of the intensity profile at 20 min after the start of cultivation was defined as background noise, and pixels exceeding three times this value were defined as signal. Fluorescence intensities corresponding to the detected signal regions ( $I_x$ ) were normalized to the maximum intensity ( $I_{max}$ ) to obtain relative intensity profiles comparable between samples. The sum value of the normalized fluorescence profile divided by number of pixel ( $N$ ) defined the spatial mean relative intensity ( $SMRI$ ).

$$SMRI = \frac{1}{N} \sum_{x=1}^N \frac{I_x}{I_{max}}$$

The  $SMRI$  of the constitutively expressed reporter ( $SMRI_{fluor}$ ) was subtracted from that of the target reporter ( $SMRI_{fluor,const}$ ), and the resulting value was defined as the spatial heterogeneity index

( $SHI_{LOTUS}$ ).

$$SHI_{LOTUS} = SMRI_{fluo} - SMRI_{fluo,const}$$

For data acquired using LOTUS-12, the same procedure was applied to transillumination images, and SMRI was calculated ( $SMRI_{trans}$ ). The fluorescence-mode SMRI was divided by the transillumination-mode SMRI to obtain a morphology-normalized SMRI ( $nSMRI$ ). The difference between this value and the SMRI of the constitutively expressed reporter ( $nSMRI_{const}$ ) was defined as SHI in LOTUS-12 ( $SHI_{LOTUS-12}$ )

$$nSMRI = \frac{SMRI_{Fluo}}{SMRI_{Trans}}$$

$$SHI_{LOTUS-12} = nSMRI - nSMRI_{const}$$

Analyses were performed using kymograph data from the edge of the biofilm (0 pixel) to the center (half of the full biofilm pixel width). To avoid artifacts caused by agar surface damage during inoculation and shadows from the well plate, the outermost 10 pixels on each side were excluded from the analysis.

## Statistical analysis

All validations and experiments were performed in triplicate unless otherwise specified. Data are presented as the mean  $\pm$  standard deviation (SD). Statistical significance was evaluated using Welch's  $t$ -test for comparisons between two independent groups and Kruskal–Wallis test followed by Dunn's test for comparisons among multiple groups. All statistical analyses were performed using Jamovi

version 2.7.13. Reproducibility of measurements was quantified using the coefficient of variation (CV). The temporal CV was calculated from the mean and SD of image profiles across time points. Spatial CV was derived from the mean and SD of pixel intensities at corresponding positions across different sample wells. The limit of detection (LOD) was defined as five times the SD of the mean blank value. Box-and-whisker plots show the median and interquartile range (IQR), with whiskers extending to the largest value within  $1.5 \times \text{IQR}$ . Outliers were identified as data points falling below the first quartile minus 1.5 times the interquartile range (IQR) or above the third quartile plus 1.5 times the IQR. Prior to DTW clustering, each time series was z-score normalized. Pairwise DTW distances were calculated without warping constraints using the R *proxy* package in R [86]. Hierarchical clustering was then applied based on the DTW distance matrix using average linkage, as implemented in the package. The number of clusters was set to three to classify distinct temporal expression patterns. In experiments with biofilms, replicates derived from the same preculture under identical conditions were defined as technical replicates, whereas replicates prepared from independent overnight pre-cultures inoculated separately from different colonies were defined as biological replicates.

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## **Publication list**

The present doctoral thesis was prepared based on the following publication.

LOTUS: A low-cost time-lapse automated imaging system for spatio-temporal analysis of microbial colony or biofilm development

Ryunosuke Sakai, Yifan Zhao and Martin Robert.

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