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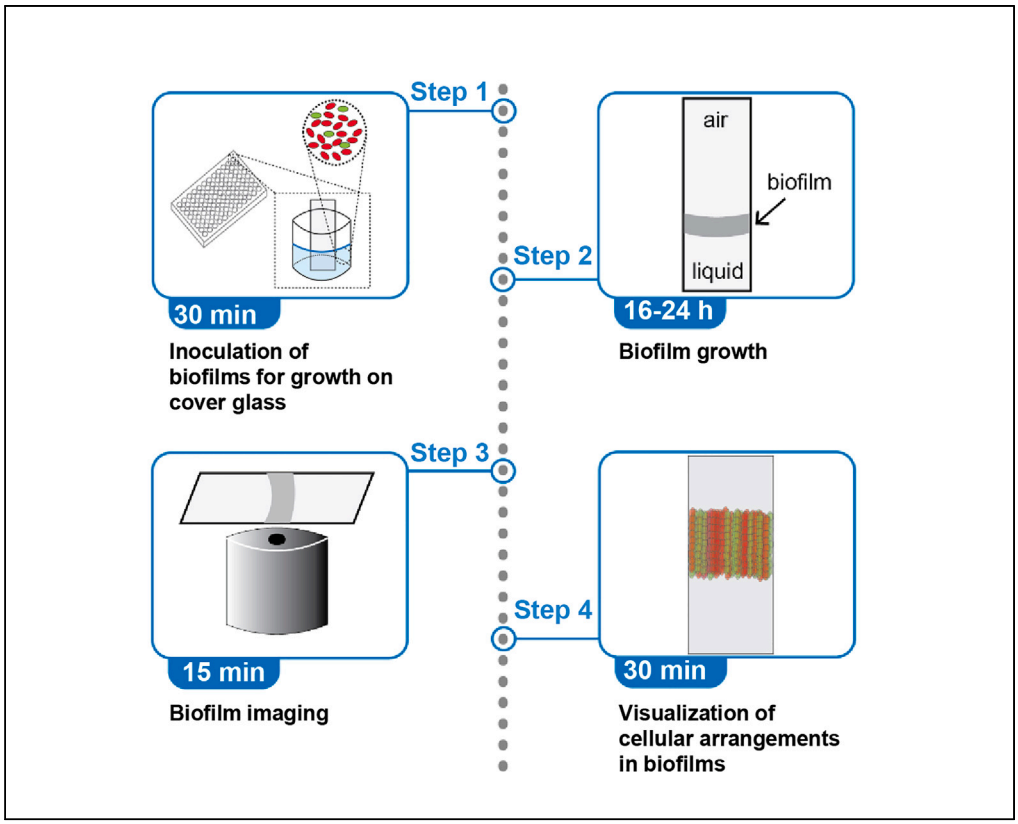
Fluorescence-based protocol for revealing cellular arrangement in biofilms



Standardized assays have greatly advanced the understanding of multicellular bacterial biofilms, but they lack cell-scale detail. Here, we present a fluorescence-based protocol that builds on past assays to reveal the cellular-scale arrangement within biofilms. We describe steps for growing biofilms on cover glass, followed by imaging and visualization of cellular arrangements in biofilms. We have applied this protocol to study *Escherichia coli* biofilms, though it could also be adapted to study biofilm formation in other species.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Cellular-scale visualization of biofilm structure

Fluorescence-based adaptation of widely used standard biofilm assay

Applicable to investigating genetic mechanisms of biofilm formation

Adaptable to validating engineered multicellular bacterial behaviors

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Fluorescence-based protocol for revealing cellular arrangement in biofilms

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SUMMARY

Standardized assays have greatly advanced the understanding of multicellular bacterial biofilms, but they lack cell-scale detail. Here, we present a fluorescence-based protocol that builds on past assays to reveal the cellular-scale arrangement within biofilms. We describe steps for growing biofilms on cover glass, followed by imaging and visualization of cellular arrangements in biofilms. We have applied this protocol to study *Escherichia coli* biofilms, though it could also be adapted to study biofilm formation in other species.

For complete details on the use and execution of this protocol, please refer to Puri et al. (2023).[1]

BEFORE YOU BEGIN

Standard bulk-scale assays serve as robust methods for biofilm growth and high-throughput screening of their genetics,[2,3] however information on cellular arrangement in the resulting communities is lacking. To address this, we describe below a step-by-step protocol to efficiently grow biofilms on microscope cover glass and discern their cell-scale structure through high-magnification, multi-fluorescence imaging. This protocol was used to demonstrate the arrangement of clonal multicellular chains in biofilms of multiple *Escherichia coli* strains, including a urinary tract infection isolate, and to identify the roles of adhesins therein.[1] However, this approach can be adapted to study the microscopic biofilm structure in different bacterial species, disease causing clinical isolates, and other microorganisms. The outlined methods will be beneficial to study multicellular community structuring in general, and for the design and testing of engineered biofilms.[4]

1. Prepare appropriate culture media for bacterial cell growth.

   Note: For this protocol, cells were cultured in Luria Broth (LB, Miller; Difco™). Other appropriate media could be used as well. To make LB, 25 mL of LB powder was dissolved in 1 L of deionized water and autoclaved at 121°C for at least 15 min.

   Note: *E. coli* strains MG1655, BW25113, and a urinary tract infection isolate were used.[1] These methods could also be applied to other bacterial species and cell types.

2. Transform cells with constitutively expressed fluorescent reporters.

   Note: For imaging biofilms in multi-fluorescence, a mixture of isogenic cells with at least two different fluorescent reporters should be used to inoculate the biofilm. To facilitate imaging,

we transformed cells separately with plasmids pUA66-p*ompC*::*gfp* (green)[5] and pEB2-mScarlet-I (red).[6] Supplement media with antibiotics to maintain plasmids where necessary. We used 50 μg/mL of the antibiotic kanamycin (kanamycin monosulfate; Goldbio) to maintain these plasmids.

Note: Having cells with at least two different fluorescent reporters would make it feasible to discern any clonally organized cellular structure of biofilms.

Note: It is important to determine that the fluorescent reporters used do not have spectral overlap.

3. A day prior to setting up the experiment, culture strains overnight (16 h) in appropriate media.

Note: We cultured cells in LB media (1 mL LB; supplemented with 50 μg/mL kanamycin), incubated in light-isolated shakers at 37°C and 300 rpm, in 14 mL Round Bottom Falcon Test Tubes with aerating caps (VWR).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli MG1655 (*E. coli* K-12 F- , λ-, *ilvG, rfb-50, rph-1*)	American Type Culture Collection (ATCC)	ATCC 700926
E. coli BW25113 (*E. coli* K-12 *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3)*, λ-, *rph-1, Δ(rhaD-rhaB)568, hsdR514*)	Yale stock collection[7]	CGSC#: 7636
E. coli MG1655 (Δ*flu*::kan(kanR) ; Δ*flu* (cured))	Tranduced from KEIO Collection[7]	N/A
E. coli MG1655 (Δ*fimH*::kan(kanR) ; Δ*fimH* (cured))	Tranduced from KEIO Collection[7]	N/A
E. coli MG1655 (Δ*csgA*::kan(kanR) ; Δ*csgA* (cured))	Tranduced from KEIO Collection[7]	N/A
E. coli MG1655 (Δ*pgaB*::kan(kanR) ; Δ*pgaB* (cured))	Tranduced from KEIO Collection[7]	N/A
E. coli Urinary Tract Infection isolate	This study (Clinical isolate received from Emory Investigational Clinical Microbiology Core)	N/A
Recombinant DNA		
pUA66 *pompC::gfp* (pUA66 derivative containing *gfpmut2* gene under control of *ompC* promoter)	Zaslaver et al.[5]	N/A
pEB2-mScarlet-I (mScarlet)	Addgene[6]	Plasmid#: 104007
Software and algorithms		
LAS X software	Leica Microsystems	N/A
Other		
14 mL round bottom test tubes	VWR	N/A
Suspension culture, 96-well, flat bottom plates	Olympus™ Plastics	N/A
VWR Micro Cover Glass (18 × 18 mm; 22 × 50 mm)	VWR	N/A
Luria Broth (LB, Miller)	Difco™	N/A
Kanamycin monosulfate	GoldBio	N/A
Phosphate buffered saline	Fisher Bioreagents	N/A
Agarose	Fisher Bioreagents	N/A
DMi8 microscope	Leica	N/A

STEP-BY-STEP METHOD DETAILS

Biofilm growth on cover glass using modified hydrostatic assay

Timing: 16–24 h

The following steps outline methods for growing biofilms in hydrostatic cultures,[2] along with the modifications we made to image their cellular structure. In standard assays, cells are inoculated in

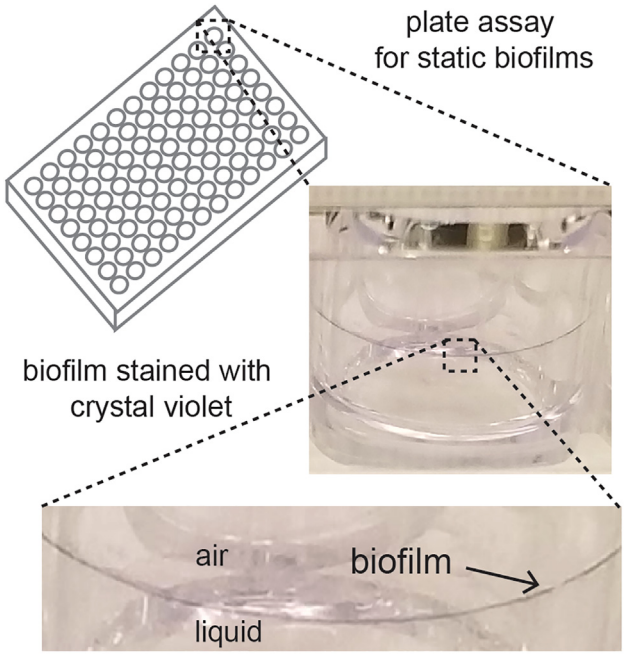

Figure 1. Illustration of standardized hydrostatic assay for biofilm formation in microtitre plates

microtitre plates, and biofilms eventually assemble at the surface-air-liquid interface.[2] Biofilms are stained to measure the extent and robustness of their formation (Figure 1).[2] We adapted these methods to grow biofilms on microscope cover glass from a mixture of cells containing either red or green fluorescence. Our modifications make biofilms formed through these assays amenable for high-magnification, cellular-scale imaging.

1. Grow biofilms using 96-well microtitre plate static assays.[2]
2. Fill wells of microtitre plates with 200 μL of fresh LB containing 1:10 dilution of overnight cultures, such that green and red fluorescent cells are mixed in a 1:10 ratio (Figure 2). This initial inoculum can be diluted as desired.

   Note: 1:10 mixture of fluorescent cells is beneficial as the color added in higher concentration serves as the background to make patterns for clonal cell organization within biofilms evident. Ratio of the different color fluorescent cells could be varied as desired.

   Note: Alternatively, cells without fluorescence can be used as background, and red and green fluorescent cells could be added in lower amounts to visualize clonal cell arrangements (this case is illustrated in Figure 5). When including cells without fluorescent reporters, it is important to not use antibiotic while inoculating biofilms so that the cells without plasmids are not affected. As cells without plasmids may not have antibiotic resistance, antibiotics should not be used when the biofilm inoculum contains non-resistant cells.

   Note: A lower dilution factor can be used for the inoculum for growing biofilms. Since our investigation also utilized mutant strains that relied on aggregation, we used a more concentrated inoculum to increase robustness of mutant biofilms for comparison of their structures to wild type.

3. Carefully break 18 × 18 mm cover glass into pieces big enough to fit inside the wells of a microtitre plate.

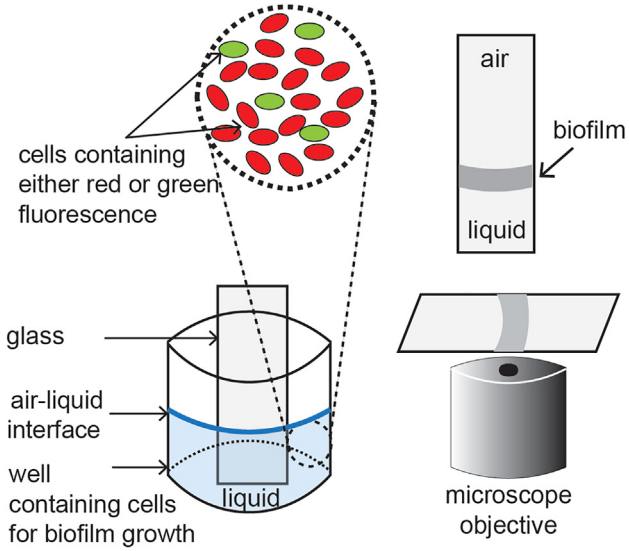

Figure 2. Illustration of modifications to standardized assay to image cellular arrangement in biofilms

Note: In the study, we broke cover glass into pieces by taking 3–4 cover glass squares at a time and using a glass slide as a solid support to break them into smaller, approximately rectangular pieces. Glass cutting tools can also be used if desired. We recommend sterilizing cover glass pieces by autoclaving.

Note: We recommend preparing 3–5 replicates.

Note: Alternatively, cover glass pieces can also be inserted in wider wells, e.g., 6-well plates. Wells should be filled such that the liquid covers at least three quarters of the height of these wells.

4. Insert cover glass pieces perpendicularly inside the wells for the biofilm to grow on. Use tweezer when handling cover glass pieces (Figure 3).
5. Incubate plates overnight (16–24 h) or for desired duration for biofilm formation without shaking at 37°C.

Note: Though this protocol is optimized for biofilm growth of *E. coli* cells in LB media, different cells and media conditions can be tested through these methods.

⚠ CRITICAL: It is important prevent movement and disturbances during incubation. Unwanted vibrations could disrupt the assembling biofilm. It is best to leave the plate undisturbed in a stationary incubator for biofilm growth.

⚠ CRITICAL: It is essential to prevent evaporation of liquid during biofilm growth. For this, we used custom plastic lids to cover plates while incubating them overnight. The lids were lightly placed to cover but not seal the plates. They were high enough so as to not disturb the cover glass pieces. We also placed a water bath in the incubator to increase hydration.

⚠ CRITICAL: When preparing the cover glass, appropriate precautions should be taken to avoid injury from sharp glass pieces. We recommend using gloves, cut resistant guards for fingers, eye shield, and lab coat while preparing cover glass pieces for biofilm growth. Remaining glass pieces should be disposed with broken glass waste.

Sample preparation for biofilm imaging

Timing: ~30 min (depending on the number of samples)

As expected in static approaches, biofilms assemble at the air-liquid interface on the cover glass pieces inserted in wells of microtitre plate. The following steps are optimized for sample preparation for imaging biofilms formed on the cover glass.

6. Using a tweezer, carefully take out the cover glass piece from a well.

   Note: The submerged part of the cover glass corresponds to the liquid region and the part of the cover glass not in the liquid corresponds to the air region. The biofilm is formed and distinctly visible at the air-liquid interface.

7. Lay this cover glass piece flat on a fresh glass slide. Label the glass slide with any necessary cell names or conditions that are being tested.
8. Cover the glass piece with formed biofilm using a fresh 22 × 50 mm microscope cover glass.

   Note: This step is necessary to cover the glass piece containing the biofilm so that it can be imaged on a microscope.

9. Fix the 22 × 50 mm cover glass on the glass slide by taping its edges (Figure 3).

   ⚠ CRITICAL: In step 8, before placing a fresh cover glass to cover the glass piece with biofilm, we recommend waiting for at least a minute for the excess liquid to dry. Spreading of liquid over the entire cover glass piece could confound biofilm imaging.

   ⚠ CRITICAL: In step 8, we recommend applying the fresh 22 × 50 mm cover glass at ~45° angle, starting from the air to the liquid portion of the cover glass piece with biofilm.

Figure 3. Representative images for experimental set up

(A) Prepared 96-well plate for biofilm growth. Inset depicts cover glass pieces inserted in wells for the biofilm to grow on. Plates were loosely covered with a lid and incubated at 37°C.

(B) Image of cover glass with biofilm prepared for imaging. Cover glass pieces taken out from wells were placed on a glass slide and a 22 × 50 mm cover glass was fixed on top by taping the edges.

This would prevent spreading of unwanted liquid phase cells over the biofilm and air regions, which could confound imaging.

High magnification biofilm imaging

Timing: 1–2 h (depending on the number of samples)

Following steps outline the procedure for high magnification imaging of biofilms formed on the cover glass pieces prepared in the previous section. We used Leica DMi8 microscope with a DIC HCPL APO 63X oil immersion objective, equipped with 1.6× magnification changer, Hamamatsu ORCA-Flash 4.0 camera, and Lumencore Spectra-X light engine. This system allowed for wide-field imaging of biofilms in differential interference contrast (DIC) and fluorescence (red and green channels) imaging modalities. Confocal microscopy could also be applied to resolve the 3D structure of these communities.

10. Set up glass slide, prepared in steps 6–9, on the microscope for imaging.
11. After focusing, capture a wide-field view of the biofilms using red and green fluorescence channels. Image biofilms at high magnification to resolve their structures at the cellular level.

Note: We overlaid images captured using the red and green fluorescence channels using LASX software to visualize clonal internal structure of the biofilm (Figure 4). We recommend capturing images at 100× resolution. We also used DIC to image biofilms in bright field.

Imaging of clonal communities detached from growing biofilms

Timing: ~1 h (depending on the number of samples)

The following part of the protocol outlines methods to image unattached biofilm cells and/or clonal structures.

12. Using a tweezer, take a piece of cover glass with formed biofilm out of the well of microtitre plate.
13. Hold the cover glass perpendicularly over an agarose pad[8] made of phosphate-buffered saline (PBS).
14. Wash the biofilm by pipetting ~50 μL (or desirable volume depending on size of substrate) of PBS over the cover glass. Let the liquid run-off from washing fall on the agarose pad.
15. Cover the agarose pad with a fresh piece of microscope cover glass. Image using DIC and fluorescence (red and green channels) imaging modalities to analyze the washed biofilm (Figure 5).

Note: Excitation and emission for fluorescence microscopy was performed at 470 nm and 500–550 nm for green fluorescence and 510 nm and 592–668 nm for red fluorescence, respectively. Fluorescent exposures were 30 ms at 30% intensity.

EXPECTED OUTCOMES

The methods outlined in this protocol allow high magnification (63–100×) imaging of biofilms to understand their cellular organization. Using these methods in *E. coli*, we found that biofilms were composed of clonal cellular chains aligned in parallel (Figure 4).[1] We applied these methods to understand the cell-scale role of important biofilm adhesins by imaging biofilms assembled by their mutants.[1] Biofilm washing experiments demonstrated the presence of rosette-configured clonal communities in the liquid-run off, suggesting that *E. coli* biofilms are composed of multicellular communities that attach as clonal units (Figure 5).[1]

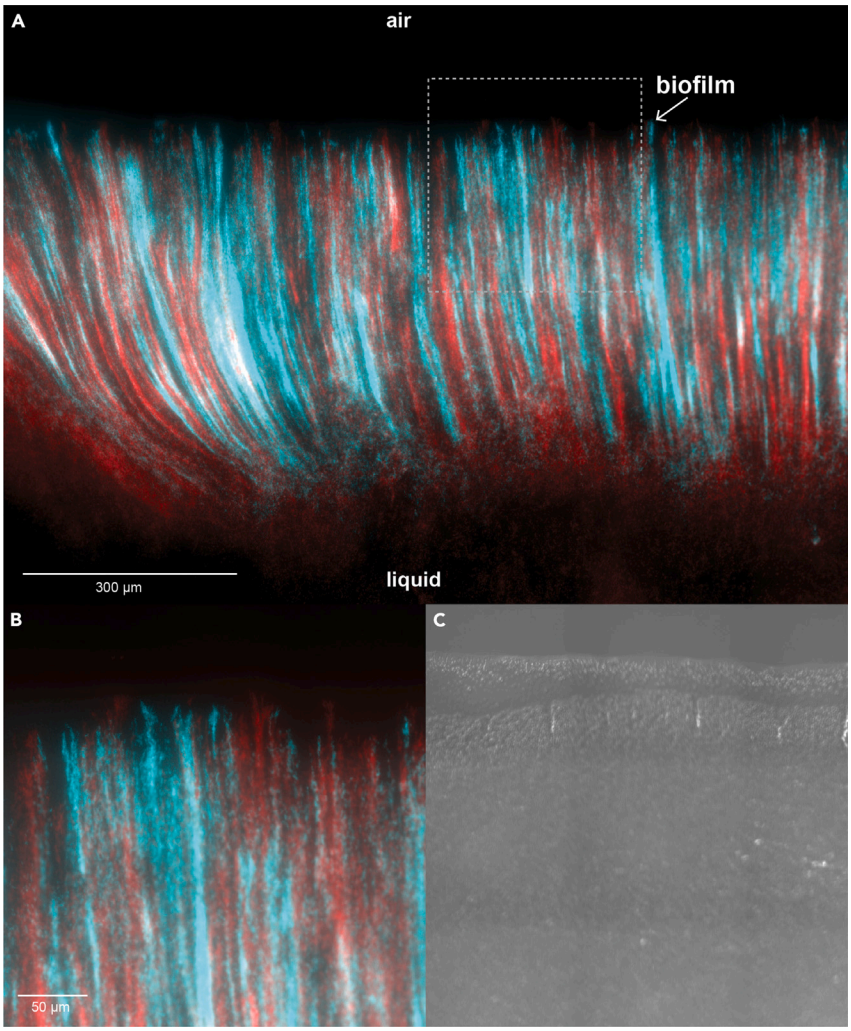

Figure 4. Imaging cellular arrangement in biofilms

(A) Representative image of biofilm imaged through the described procedure. Biofilms were inoculated from a 1:10 mixture of cells with fluorescence (red and green combined in equal amounts; green is represented with cyan in this image) and cells with no fluorescence.

(B and C) Highlighted region is magnified in (B) (multi-fluorescence) and (C) (bright field). The altered cell-mixing ratio for this biofilm illustrates the case described in the notes, where the inoculum contains cells with no fluorescence (in higher amounts) and fluorescence (in lower amounts; containing an equal ration of red and green fluorescent cells). In this case, cells without fluorescence served as background to visualize red and green (depicted in cyan here) cellular chains.

LIMITATIONS

This protocol is not suitable for imaging dynamic assembly of biofilms and only supports single time point imaging. In *E. coli*, imaging sample preparation would disrupt any unattached chains that are in the process of forming the biofilm and hence their behavior is unobserved.

TROUBLESHOOTING

Problem 1

Drying of liquid prior to biofilm formation (step #5).

Potential solution

In order to image biofilms, it is essential to prevent drying of liquid in the wells of the microtitre plates. Placing a lid over the plate would help with preventing evaporation, however care must

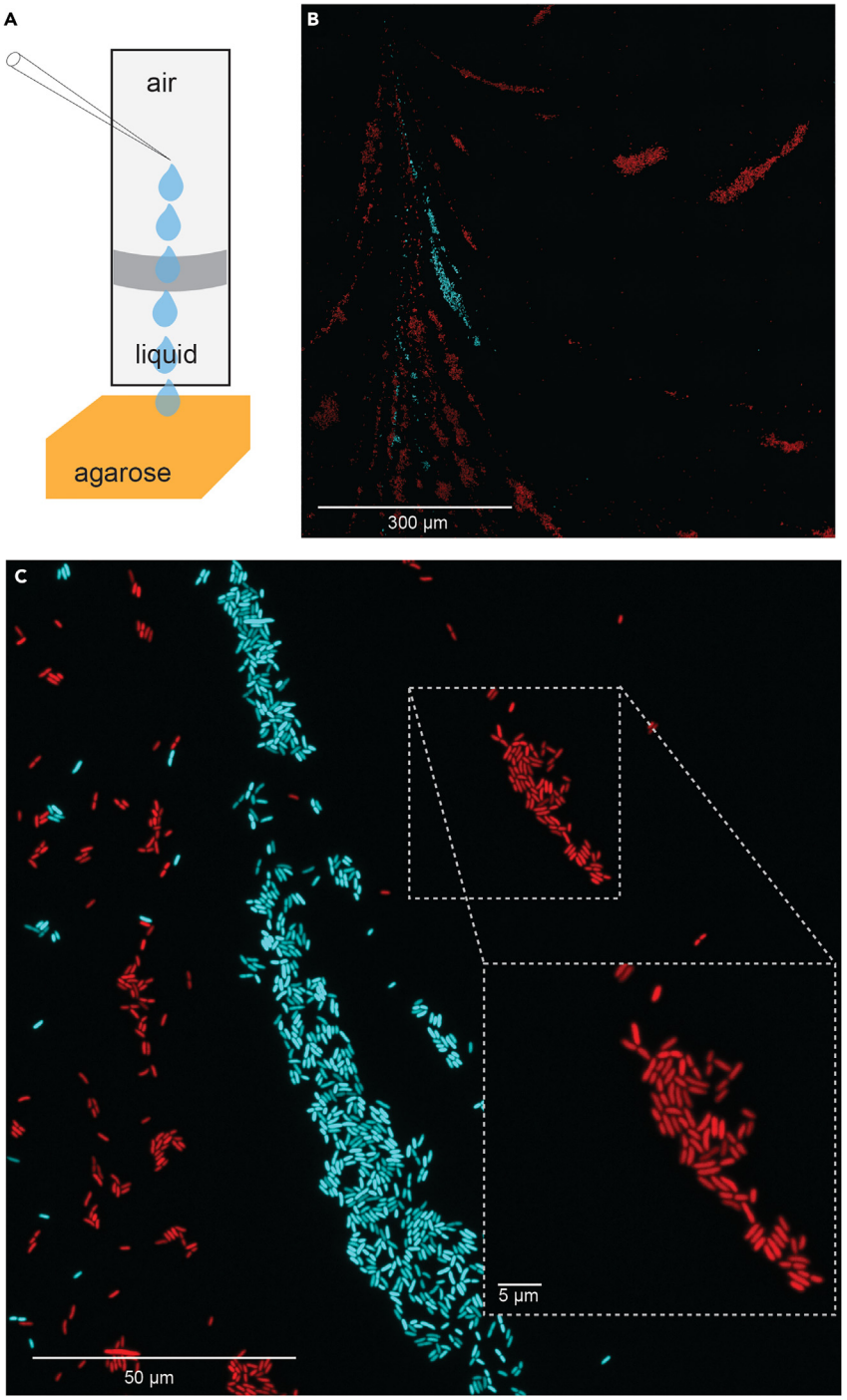

Figure 5. Clonal communities washed from biofilm

(A) Illustration of procedure for washing biofilms.

(B and C) Representative image of clonal communities washed off of a biofilm: (B) wider field of view of the agarose pad and (C) a higher magnification example. Biofilm was grown from a 1: 10 mixture of cells containing green (represented as cyan here) and red fluorescence respectively. Washing procedure is described in steps 12–15.

be taken so that the plate is not sealed so that there is aeration, and that the cover glass pieces are not disturbed. Placing a water bath in the incubator during biofilm growth also helps with increasing hydration.

Problem 2

Spreading of liquid over the cover glass piece with formed biofilm during sample preparation (step #8).

Potential solution

During sample preparation to image cover glass pieces with formed biofilms, it is possible that excess liquid on the portion of the cover glass below the biofilm can spread. This would scatter the liquid phase cells and confound imaging of biofilms. This can be prevented by waiting for about a minute to allow for the liquid to dry before covering it with a fresh microscope cover glass. Additionally, placing the fresh cover glass at an angle, starting from the air region of the biofilm cover glass piece to the liquid region would prevent unwanted spreading of excess liquid.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kyle R. Allison (kyle.r.allison@emory.edu).

Materials availability

This study did not generate new unique materials.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

D.P. performed all experiments and analyses with assistance from X.F. and K.R.A. D.P. and K.R.A. designed experiments, discussed results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Puri, D., Fang, X., and Allison, K.R. (2023). Evidence of a possible multicellular life cycle in *Escherichia coli*. iScience *26*, 105795. https://doi.org/10.1016/j.isci.2022.105795.

2. O'Toole, G.A. (2010). Microtiter dish biofilm formation assay. JoVE, 2437. https://doi.org/10.3791/2437.

3. Pratt, L.A., and Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. *30*, 285–293. https://doi.org/10.1046/j.1365-2958.1998.01061.x.

4. Leech, J., Golub, S., Allan, W., Simmons, M.J.H., and Overton, T.W. (2020). Non-pathogenic *Escherichia coli* biofilms: effects of growth conditions and surface properties on structure and curli gene expression. Arch. Microbiol. *202*, 1517–1527. https://doi.org/10.1007/s00203-020-01864-5.

5. Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M.G., and Alon, U. (2006). A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat. Methods *3*, 623–628. https://doi.org/10.1038/nmeth895.

6. Balleza, E., Kim, J.M., and Cluzel, P. (2018). Systematic characterization of maturation time of fluorescent proteins in living cells. Nat. Methods *15*, 47–51. https://doi.org/10.1038/nmeth.4509.

7. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006.0008. https://doi.org/10.1038/msb4100050.

8. Young, J.W., Locke, J.C.W., Altinok, A., Rosenfeld, N., Bacarian, T., Swain, P.S., Mjolsness, E., and Elowitz, M.B. (2011). Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat. Protoc. *7*, 80–88. https://doi.org/10.1038/nprot.2011.432.