High-Throughput Imaging of *Bacillus subtilis*

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Abstract

Bacillus subtilis is a widely used model bacterium to study cellular processes and development. The availability of an arrayed mutant library gave us the opportunity to cytologically analyze every mutant and screen for new genes involved in cell shape determination, cell division, and chromosome segregation. Here we describe a high-throughput method to image arrayed *B. subtilis* mutant libraries using wide-field fluorescence microscopy. We provide a detailed description of growing the arrayed strain collection, preparing slides containing agarose pedestals, setting up the microscopy procedure, acquiring images, and analyzing the images.

Key words *Bacillus subtilis*, High-throughput imaging, Automated imaging, Arrayed mutant library, Fluorescence microscopy

1 Introduction

Bacillus subtilis is widely used as a model organism to study processes important for bacteria growth and development. This bacterium is extremely genetically tractable due to its natural competence, which is a physiological state that allows bacteria to uptake extracellular DNA and recombine to their own genome [1]. Natural competence makes it easy to obtain genetically modified strains, and enables high-throughput transformations to add desired traits to existing arrayed mutant collections [2].

While genetic screens such as Tn-Seq [3] are powerful tools to identify essential genes in certain strain backgrounds or growth conditions, they rely on strong growth defects. Cytological screens like the one described here make it possible to discover new factors that are important for cellular processes that may be missed in other screens because of the lack of strong growth defects. Additionally, cytological screens can be used to determine the spatial localization of fluorescent protein fusions in single cells [4]. Furthermore, cytological screening of arrayed mutant libraries can be streamlined by systematic quantification of fluorescence images. Thanks to the
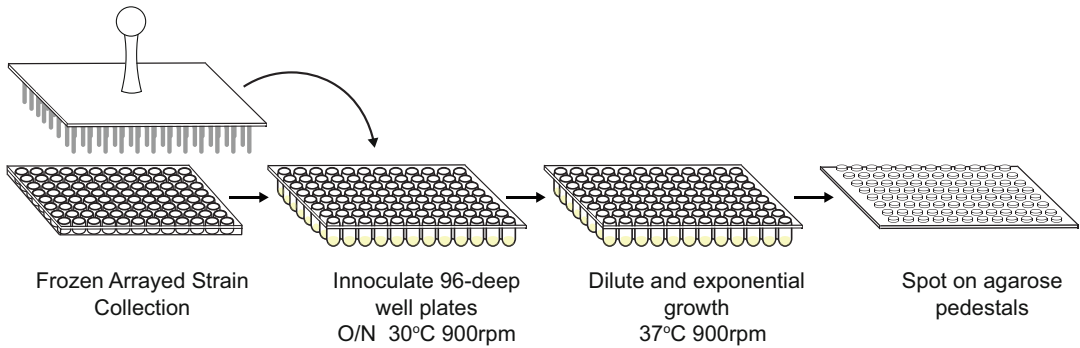
Fig. 1 Overview of the experimental procedure

inherent quantitative nature of fluorescence images, the cytological characteristics of each mutant can be determined by quantifying different aspects of the cells, including cell length, width and area, nucleoid length, width and area, and number of nucleoids per cell. Thus, these quantifications enable the characterization of every mutant and their classification according to the phenotype they display [5–7]. For example, when disrupted, genes involved in cell division generate long cells and mini-cells [8]. When a gene of unknown function displays such phenotypes, this gene would most likely be involved in some aspect of cell division. Therefore, systematic analysis and classification are key in assigning a function to an uncharacterized gene or inferring the pathway the gene is involved in. In this chapter, we describe a detailed method to perform high-throughput imaging and analyses of *B. subtilis* from an arrayed know-out strain collection in which the parental strain expresses HbsU-mGFPmut3 [9] fusion protein and cytoplasmic mCherry [10] to visualize and quantify chromosome and cell dimensions, respectively (Fig. 1).

2 Materials

The arrayed strain collection imaged here is derived from the *B. subtilis* knockout strain collection constructed by Koo et al. [2]. We first extracted the genomic DNA from this strain collection using Promega Wizard SV 96 DNA purification system (Cat# A2370). Then we performed high-throughput transformation of the genomic DNA into a strain expressing HbsU-mGFPmut3 fusion protein and cytoplasmic mCherry. This chapter describes the procedure to image this new arrayed mutant library. We note that this chapter builds on our previous chapter in which we describe tools and protocols to grow and image *B. subtilis* [11]. Here we include quantitative high-throughput tools to characterize *B. subtilis* mutants.

Media components are prepared in Milli-Q water (ddH$_2$O) unless otherwise stated. Where indicated, liquid media are sterilized by autoclaving for 30 minutes (min) in liquid cycle, or by filtering through a 0.22 μm syringe-driven filter or a bottle-top filter. Glassware is sterilized by autoclaving for 30 min in a dry cycle. Sterile media components and glassware are stored at room temperature unless otherwise specified.

2.1 Media and Equipment for the Growth of B. Subtilis

1. Defined rich casein hydrolysate medium (CH medium) [12] component CHI+II: 10 mg/mL casein hydrolysate (Neogen #7229A), 4.7 mg/mL L-glutamate sodium salt monohydrate, 3.2 mg/mL L-asparagine monohydrate, 2.5 mg/mL L-alanine, 2.72 mg/mL potassium phosphate monobasic anhydrous (KH$_2$PO4), 2.68 mg/mL ammonium chloride (NH$_4$Cl), 1.1 mg/mL sodium sulfate (Na$_2$SO$_4$), 1 mg/mL ammonium nitrate (NH$_4$NO$_3$), and 0.01 mg/mL ferric chloride 6-hydrate (FeCl$_3$·6H$_2$O); autoclaved.

2. CH medium component CHIII: 0.66 mg/mL calcium chloride dihydrate. (CaCl$_2$·2H$_2$O), and 1.21 mg/mL magnesium sulfate anhydrous (MgSO$_4$); autoclaved.

3. CH medium component CHIV: 1.67 mg/mL manganese sulfate monohydrate (MnSO$_4$·H$_2$O); autoclaved.

4. CH medium component CHV: 2 mg/mL L-Tryptophan; filtered and distributed in 10 mL aliquots in 15 mL conical tubes. Store at 4 °C.

5. Round-bottom 96-deep-well plates (Fisher 40002-014).

6. 96-pin replicator tool.

7. Handheld Vacuum Aspirator with 8-Channel Adapter (Argos Technologies HandE-Vac 13050-32).

8. Temperature-controlled plate shaker (Multitron Infors HT).

9. Breathable cover for 96-deep-well plates (Denville Scientific AeraSeal B1212-7S).

10. Sparkle cleaner (VWR 500032-904).

2.2 Components for Imaging

1. Custom-sized glass slides (127 × 85 mm, 1 mm thickness; ordered from Hausser Scientific).

2. Custom-sized cover glass (115 × 75 mm, 0.17 mm thickness No. 1.5; ordered from VWR and prepared by Thermo custom order 115 × 75–1.5-VWR cover glass).

3. Two of custom-made stainless steel molds for 96 pedestals (see Fig. 2 for dimensions).

4. Molecular grade agarose (VWR 12001-870).

5. Microwave oven.

6. Kimwipes.
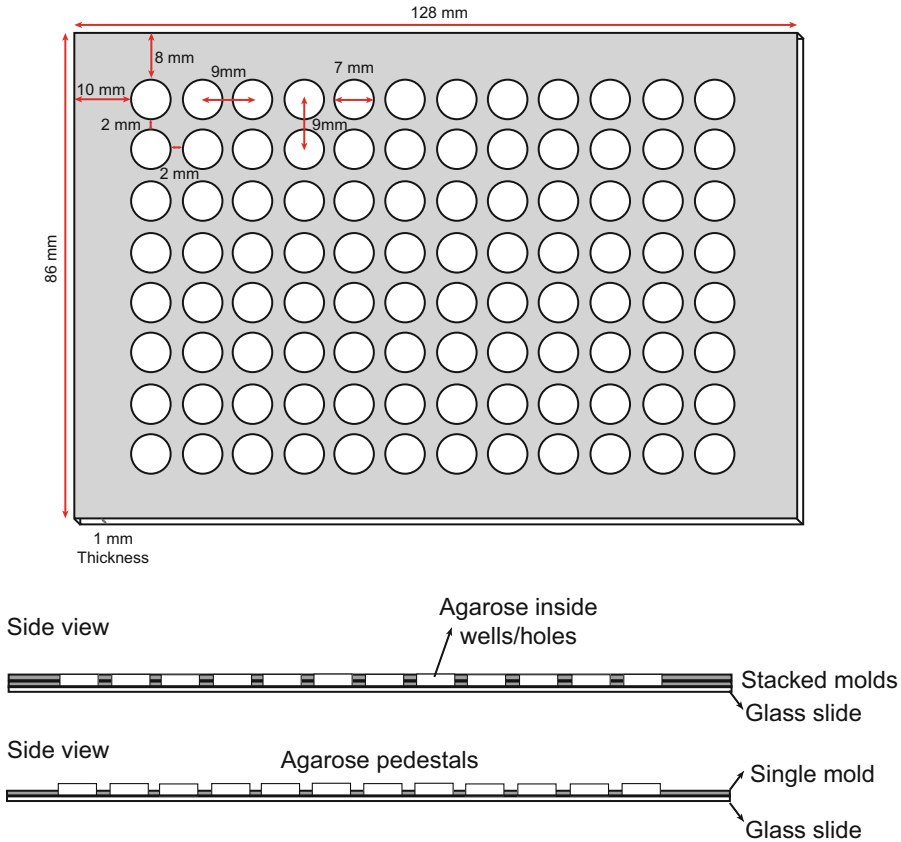
Fig. 2 Dimensions of the custom-made stainless steel molds. Top, dimensions of the molds built in a machine shop. These numbers are needed for the JOBS macro to define the custom plate. Bottom, schematics of the side view of the slide/mold/agarose assembly before (upper panel) and after (lower panel) exposing the pedestals

7. 70% Ethanol in a squirt bottle.

8. Fully motorized Nikon Ti inverted microscope equipped with a Perfect Focus System (PFS), a Plan Apo 100×/1.45 N.A. Ph3 oil-immersion objective (or a Plan Apo 60×/1.4 Ph3 objective lens), an Andor Zyla 4.2 plus sCMOS camera, Nikon Ti-S-ER motorized stage with a multi-well stage insert (TI-SH-W; Nikon), filter cubes for GFP (Chroma 49002), Texas Red (Chroma 49008), and a Lumencor Spectra X light engine. The microscope is operated using Nikon Elements software equipped with a JOBS module. Similar microscopes and software can be used.
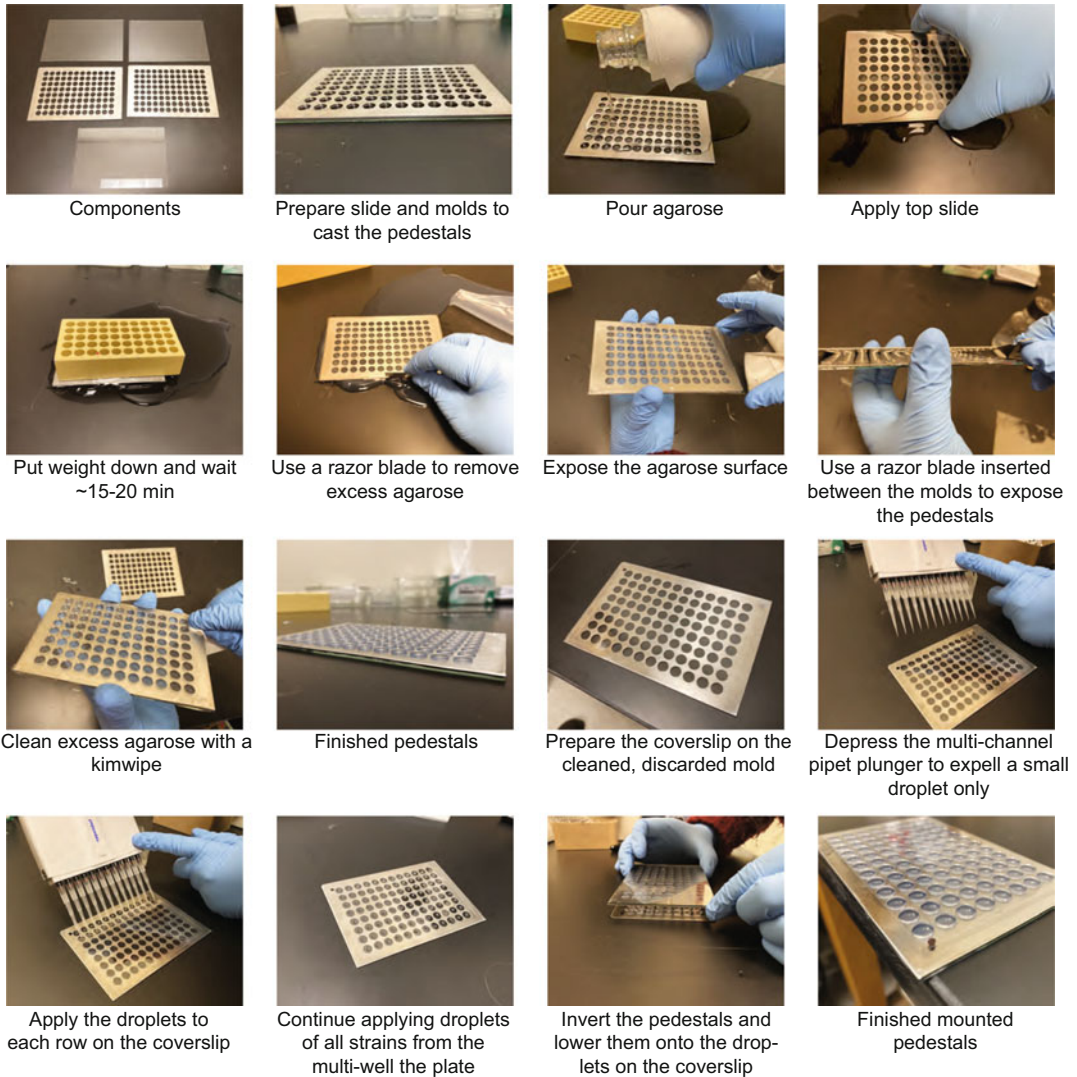
3   Methods

3.1   Exponential Growth of Cells

1. From frozen *B. subtilis* strains stored in 96-well format, inoculate directly into 96-deep-well plates containing 300 µL of CH (*see* **Note 1**). Wipe the foil that covers the frozen stock with 70% ethanol before carefully peeling it from one side. Sterilize the pin tool by submerging the pins in a shallow container containing 100% ethanol and flaming them (*see* **Note 2**). Once the pins are cooled down, firmly press the pinning tool onto the frozen 96-well stock and immediately inoculate into the 96-deep-well plates containing 300 µL growth media (Fig. 1; *see* **Note 3**). Seal the plate with a breathable cover.

2. Incubate the cultures overnight at 30 °C, shaking at 900 rpm.

3. In the next morning dilute the cultures in 300 µL of fresh CH growth medium in 96-deep-well plates to an ~OD_{600} of 0.02. Seal the plate with a breathable cover.

4. Put the plates in a plate shaker and grow for 5–7 generations (in our case 30–40 min/generation) at 900 rpm at 37 °C.
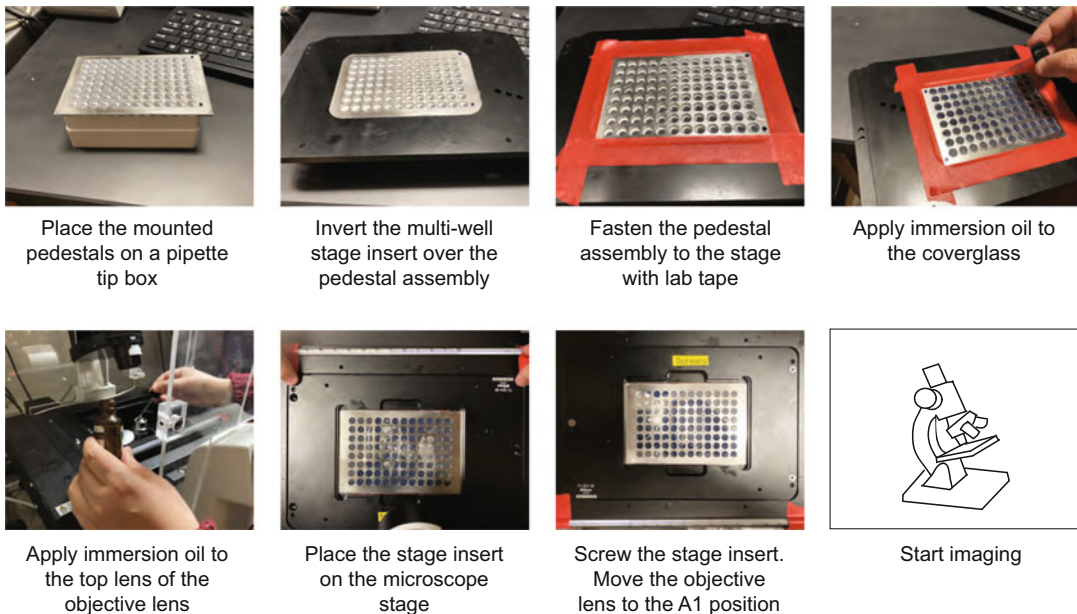
3.2   Prepare 96-Well Pedestals

1. While the cells are growing, prepare 2% agarose solution in growth media to make the pedestals: dissolve 2 g of molecular grade agarose in 100 mL of growth medium. Microwave until homogenous. Keep at 65 °C (*see* **Note 4**).

2. Prepare the agarose pads (Fig. 3, *see* **Note 5**). Clean two custom-sized glass slides per plate by wiping them with a Kimwipe dampened with ethanol from a squirt bottle. Avoid using too much ethanol which will produce strikes on the glass. Next, wipe the slides with a Kimwipe dampened with Sparkle cleaner to remove any water-soluble debris. Layer the glass slides and the stainless steel molds as shown in Fig. 3. Clean an area of the bench with water and then 70% ethanol. Place a glass slide on the bench and the two overlaid stainless steel molds on top. Pour the 2% agarose mixture to the center of the assembly (*see* **Note 6**), slowly moving toward the edges. Immediately after, place a second slide over the agarose-covered assembly at a shallow angle and gently lower it (Fig. 3). Gently place a weight on top to remove excess agarose. Leave on the bench for 15–20 min to allow the agarose to solidify (*see* **Note 7**). Using a razor blade, cut the excess solidified agarose around the assembly and turn it over. The glass slide that was against the bench is now on top (see **Notes 8** and **9**).

3. Carefully remove excess agarose from all 4 sides of the assembly with the razor blade.

4. Expose the surface of the agarose by sliding the top glass slide longitudinally. Avoid pulling, as this will warp and distort the

Components	Prepare slide and molds to cast the pedestals
Pour agarose	Apply top slide
Put weight down and wait ~15-20 min	Use a razor blade to remove excess agarose
Expose the agarose surface	Use a razor blade inserted between the molds to expose the pedestals
Clean excess agarose with a kimwipe	Finished pedestals
Prepare the coverslip on the cleaned, discarded mold	Depress the multi-channel pipet plunger to expell a small droplet only
Apply the droplets to each row on the coverslip	Continue applying droplets of all strains from the multi-well the plate
Invert the pedestals and lower them onto the droplets on the coverslip	Finished mounted pedestals

Fig. 3 Preparation of the 96 agarose pedestal assembly. During imaging, the glass slide is fastened on the stage by lab tape (see Fig. 4). The gap between the cover glass and the mold, which is set by the height of the removed mold, allows the agarose pads to be exposed to air in the humidified temperature-controlled incubator encasing the microscope. For *B. subtilis*, adequate oxygen is important for the health of the cells

agarose. Remove the thin layer of excess agarose from the edges of the mold using a Kimwipe. Slide a razor blade at one of the corners of the assembly, in between the two stainless steel molds. Pry the two stainless steel molds apart by twisting the razor blade. This will expose the agarose pedestals. Wipe the thin layer of agarose from the edges of the mold and around the pedestals (*see* **Note 10**).

| Place the mounted pedestals on a pipette tip box | Invert the multi-well stage insert over the pedestal assembly | Fasten the pedestal assembly to the stage with lab tape | Apply immersion oil to the coverglass |
| Apply immersion oil to the top lens of the objective lens | Place the stage insert on the microscope stage | Screw the stage insert. Move the objective lens to the A1 position | Start imaging |

Fig. 4 Mounting of the agarose pedestal assembly on the microscope for high-throughput imaging. The agarose pedestal assembly is fastened to the stage insert and immersion oil is applied to the surface of the cover glass

5. Clean the discarded slide, mold with soap and water as soon as they are removed, and then rinse them with ddH$_2$O and 70% ethanol. Reuse them for the following steps or for a new batch.

6. Prepare a custom-sized cover glass to mount the strains on. Place a clean and dry stainless steel mold from **step 5** on a clean bench. Place the custom cover glass over the mold, which will guide where to drop the cells on the glass slide (see below). Make a mark at the top left corner of the cover glass with a sharpie to designate where the A1 well/pedestal will be (Fig. 3; *see* **Note 11**).

3.3 Mounting B. subtilis Strains on the Agarose Pedestals

1. Once the agarose pedestals are exposed, spin down the 96-deep-well plate containing exponentially growing *B. subtilis* at 1000 g for 2 min.

2. Using a handheld multi-well vacuum tool, aspirate most of the supernatant, leaving ~20–50 µL of the growth medium (*see* **Notes 12** and **33**).

3. Using a 12-well multichannel pipette set to 15 µL, gently pipette up and down to resuspend the cell pellets in each well, avoiding air bubbles. Take 15 µL of each cell suspension from one row of the deep-well plate (e.g., A1–12; *see* **Note 13**). Gently depress the plunger of the multichannel pipette to expose a small droplet of cell suspension in each pipette tip,

without letting them fall (Fig. 3; *see* **Note 14**). Touch the surface of the cover glass to spot each droplet (*see* **Note 15**).

4. Repeat **step 3** until the cell suspension from each well is spotted on the cover glass.

5. Pick the exposed agarose pedestals, invert them so that they are facing down, and carefully lower them onto the cover glass with the arrayed droplets of cells (*see* **Note 16**).

6. Image the arrayed strains immediately (see below; *see* **Note 17**).

3.4 High-Throughput Imaging of B. subtilis *Strains Using Wide-Field Fluorescence Microscopy*

1. Mount the 96-well pedestal assembly onto a multi-well stage insert. To do so, place the glass-slide side of the pedestal assembly on an empty pipette tip box, so that the cover glass side of the assembly is facing up (Fig. 4). Invert the multi-well stage insert and place it over the pedestal assembly. Fasten the pedestal assembly to the stage insert by sticking all four sides with lab tape (*see* **Note 18**).

2. Apply immersion oil to the surface of the cover glass using an eye drop applicator or a small brush (*see* **Note 19**).

3. Apply immersion oil to the top lens of a Plan Apo 100×/1.4 oil objective lens (*see* **Note 20**).

4. Before mounting the stage insert onto the microscope stage, ensure that the nosepiece is in the lowest position (*see* **Note 21**).

5. Attach the stage insert to the microscope stage by fastening the screws with a #2 Allen key. Be careful not to overtighten the screws as they can break.

6. Optimize acquisition settings to produce high signal-to-noise ratio images (*see* **Note 22**). Proceed with semiautomated Subheading 3.5 or automated Subheading 3.6 image acquisition depending on the capability of your acquisition software.

3.5 Option 1: Setting Software for Semiautomated Image Acquisition

1. Position the pedestal assembly so that the Plan Apo 100×/1.4 Ph3 objective lens is below the pedestal at A-1 position (Fig. 4). Slowly bring the nosepiece up until the immersion oil on the lens is in contact with the surface of the cover glass. Turn on the Perfect Focus System (PFS) and continue carefully bringing the nosepiece up using the fine focus knob until the PFS is engaged. Alternatively, look down the eyepiece using transmitted light and carefully focus on the surface of the agarose (*see* **Notes 23–25**).

2. Once the cells are in focus, adjust the optics in the transmitted light path for Koehler illumination to ensure bright and even illumination across the field of view (FOV). This is critical to produce high-contrast and -resolution phase-contrast images.

3. Set up the experiment in Nikon Elements ND acquisition. In the λ tab, enter the optical configurations to be imaged (e.g., GFP, mCherry). In the XY tab, generate a relative XY array of points or FOVs to be imaged. To do so, Check "Relative XY" in the XY tab, click on custom, and in the pop-up window select the well plate tab. Enter the number of FOV to be imaged (rows and columns; e.g., 3×2) and enter the distance between points, which should be greater than the size of the FOV with a particular camera and total magnification (*see* **Note 26**). Enter a distance that would not create overlapping points. Note that the distance is in mm. Click on apply to create the points. The relative positions will appear in the XY tab.

4. Select a stage speed of <5 mm/s to move from pedestal to pedestal without losing focus. To do so go to Devices>Manage devices, highlight Nikon Ti, and click on Configure Devices. In the pop-up window select the appropriate speed.

5. Use the XYZ navigator tab to move from pedestal to pedestal automatically (right click on the workspace and select it under Acquisition controls). Make sure that the distance is set to 9 mm, which is the distance between the centers of adjacent pedestals. Use the arrows to navigate from pedestal to pedestal. Click on the arrow once; the stage will move from the center of one pedestal to the next.

6. Adjust the focus if necessary and press run to automatically acquire multipoint, multicolor fluorescence images (*see* **Note 27**).

7. Continue until images for all 96 pedestals have been acquired (*see* **Note 28**).

3.6 Option 2: Setting Software for Automated Image Acquisition

If the acquisition software is equipped with a JOBS module, a custom macro can be implemented to perform the automated acquisition of all 96 pedestals. This will enhance the high-throughput capability of the system (*see* **Note 29**).

1. Perform **steps 1** and **2** in Subheading **3.5**.

2. Set the stage speed to 1 mm/s.

3. Create a custom JOBS to automatically acquire 6 FOV per agarose pedestal, using the PFS and two-pass software-based autofocusing routines to maintain focus (example in Fig. **5**). The steps are described below.

4. In the Plate Definition window, click "Custom Plate" to create a custom plate with the mold dimensions displayed in Fig. **2**.

5. Align the custom pedestal plate in the Align Plate window, to ensure that the distance between pedestals is correct (*see* **Note 30**).
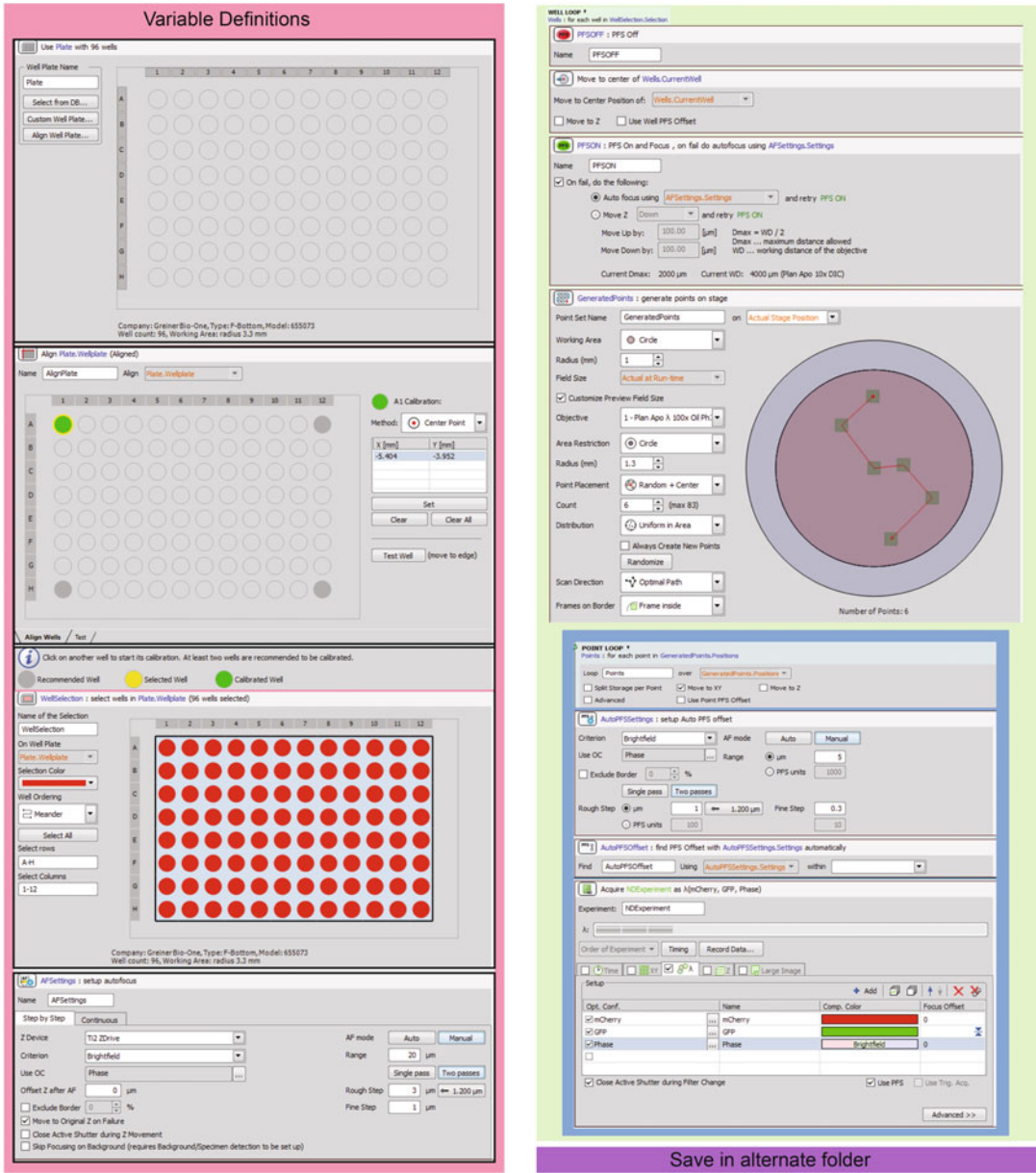
**Fig. 5** Example of custom JOBS macro to fully automate acquisition of the pedestal assembly

6. Select the pedestals to image by highlighting the wells in the custom plate displayed in the WellSelection window.

7. Define the AutoFocus settings by selecting the "Manual" mode with a 20 μm range with "Two passes." Set the first coarse pass to 3 μm and the second fine pass to 1 μm. Use a transmitted optical configuration (Phase) to avoid photobleaching and phototoxicity. In case of AutoFocus error, uncheck "Move to original Z on failure."

8. Add a Well Loop in which the PFS turns off, the stage moves to the center of each pedestal, the PFS is re-engaged, and 6 FOV or positions are generated in a random position within each pedestal to avoid bias.

9. Within the Well Loop, set up a Point Loop in which the macro will perform an autofocusing procedure using the PFS (Manual Mode, 5 μm range, 1 μm coarse step, and 0.3 μm fine step) in each point generated for each pedestal.

10. Define the acquisition parameters that contain the optical configurations for each well, such as the fluorescence and transmitted light channels (e.g., GFP, mCherry, Phase).

11. Select the directory to save the images.

12. Run the custom macro.

3.7 Characterization of Mutant Phenotypes by Quantitative Image Analysis

There are many open-source software available to quantify transmitted light or fluorescence images of bacterial cells [13–19]. We used the Oufti application to analyze the images of our arrayed fluorescent strain collection [14] (www.oufti.org). Oufti allows subpixel detection of cells and nucleoids using either phase contrast or fluorescence signal. Additionally, this software enables batch processing and quantification of the detected cells or structures.

1. Launch the Oufti application to open the main GUI.

2. Click on the "Phase" button to load the mCherry fluorescence images (which will provide information on the cell dimensions; *see* **Note 31**).

3. Click on "Load parameters" on the bottom right corner of the GUI window (*see* **Note 32**).

4. Optimize the segmentation parameters to ensure that the application detects the cells correctly (*see* **Note 33**). Once optimized, save the parameter set for batch analysis.

5. Click on "Signal 1" button to load the HbsU-mGFPmut3 fluorescence images. These will provide information on the dimensions of the nucleoids.

6. Select ObjectDetection from the top menu to detect the nucleoids within the cell outlines.

7. Optimize the segmentation parameters and save them for batch analysis.

8. Once the analysis for all strains is obtained, use the built-in tools to obtain statistics on cell dimension (length, width, standard deviation of length, standard deviation of width) and nucleoid characteristics (length, width, standard deviation of length, standard deviation of width, number of nucleoids per cell; see Fig. 6). Classification of these cellular characteristics will facilitate assigning functions to uncharacterized genes [5–7] and generating hypotheses for further validation.
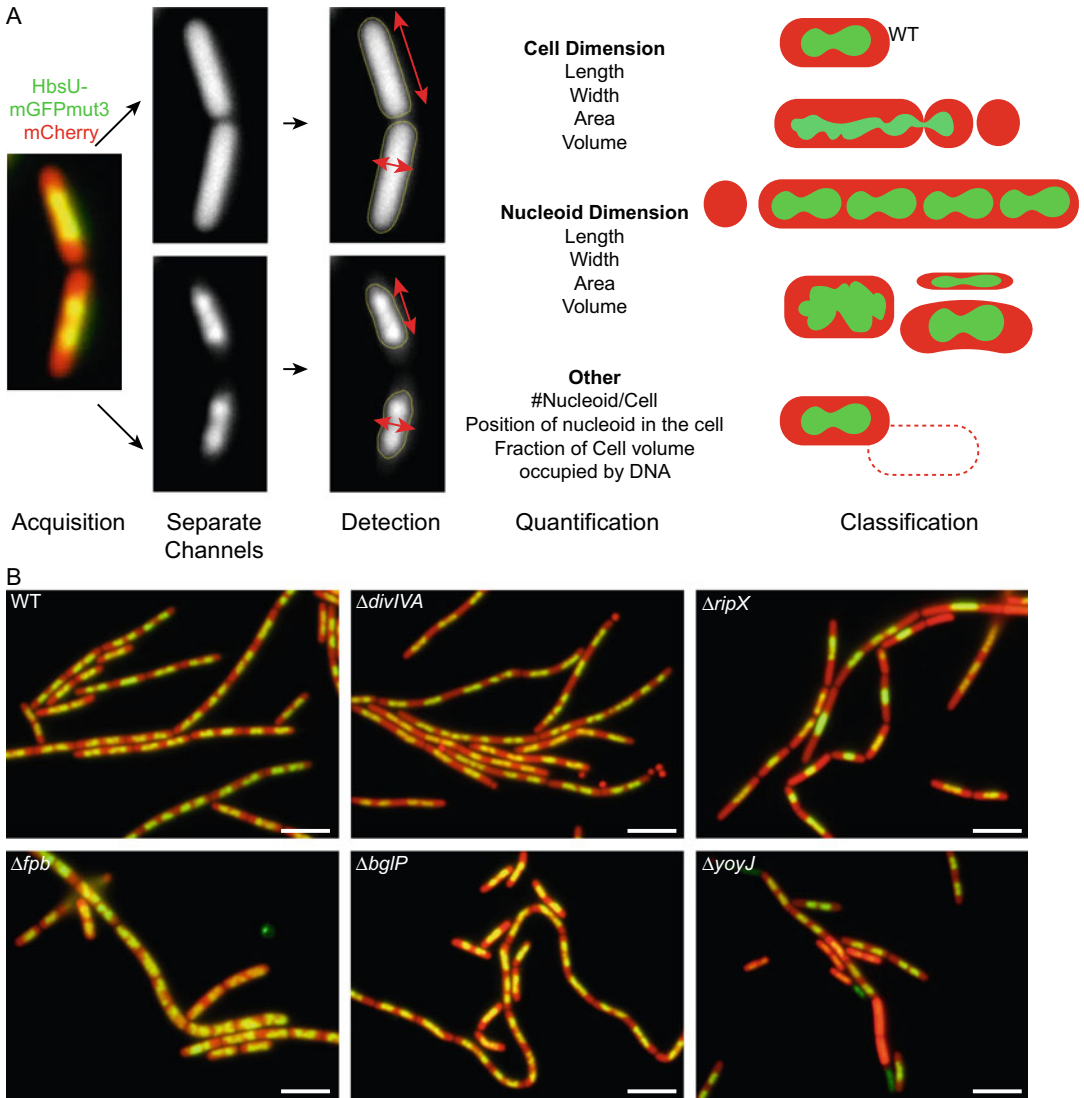
Fig. 6 Analysis pipeline and sample images from the screening. (**a**) Overview of the analysis pipeline to classify each phenotype. (**b**) Fluorescence images of phenotypes displayed by individual mutants. Green, HbsU-mGFPmut3; red, mCherry. Scale bar, 5 μm

4   Notes

1. CH medium has lower background fluorescence compared to LB medium, making it more suitable for wide-field fluorescence imaging.

2. Ensure that the Bunsen burner is away from the ethanol container and that no ethanol is dripping from the pin before flaming to avoid fire hazards.

3. Inoculate cultures at the end of the day to prevent overgrowth in the next morning.

4. To prevent agarose from boiling over, heat the agarose solution in the microwave at 5-s pulses and gently swirl the bottle to mix thoroughly between pulses, until the agarose is fully melted. Use caution to prevent burns.

5. Prepare the agarose pedestals ~20 min before harvesting the cells to ensure that they are not overdried.

6. Use caution when pouring the molten agarose over the pedestals, as it can cause burns. An excess of agarose is needed to prevent air bubbles.

7. The agarose makes everything slippery, and the glass and molds will shift. Carefully align the layers so that the holes in the two molds are superimposed. Hold the assembly for ~1 min to stabilize it.

8. Some agarose may get in between the bench and the slide, sticking the assembly to the bench. Hold the assembly by the long sides, avoiding pressing down, and pull toward you.

9. Using this side of the assembly ensures that the pedestals are even and flat.

10. The bottom slide and the bottom mold are not removed. They provide stability to the pedestal array. The molds and glass slides can be reused as long as they are kept clean.

11. Instead of being spotted on the agarose pedestals, the cells will be spotted on the surface of the cover glass. The mold at the bottom of the cover glass serves as a guide of where to spot the cells.

12. Avoid sucking the pellets up and losing the cells. Change the pipet tips to avoid cross contamination between strains. Start a new box of pipet tips and use the same column as the one in the plate when removing the supernatant. This reduces mistakes.

13. Only 1–2 μL of the 15 μL cell suspension will be spotted on the cover glass. Taking a larger volume than needed avoids air bubbles.

14. When using the multichannel pipette, firmly attach the tips to ensure optimal performance. If one of the droplets does not come out, continue anyway. Then use a regular pipet to spot the missing ones.

15. Be careful not to slide the cover glass over the mold when spotting the cell suspensions. This would lose reference of the positions. If this happens, start over with a new cover glass.

16. This prevents air bubbles and overdrying of agarose while spotting the cells. The cover glass will be fastened to the pedestals by surface tension.

17. To image several plates, stagger the timing of the experiments so that the next plate is ready ~1 h after the previous one.

18. Avoid placing the tape over the cover glass, as it will put pressure on the pedestals and squeeze them.

19. Do not apply excess oil as it will drip over the microscope components. Apply the oil on the surface of the cover glass over the pedestals, sufficient so that the top lens will be covered at all times with oil as it moves from each pedestal to the next.

20. Critical! Avoid directly touching the top lens with the applicator so as not to damage the objective lens.

21. This prevents damaging the objective lens.

22. To achieve a good signal-to-noise ratio (SNR) in a fluorescence image, adjust the light intensity and/or the exposure time so that the range of intensities within an image covers the dynamic range of the camera used. This depends on the bit depth (e.g., 12 bit or 16 bit; number of possible intensity levels in an image) and the associated camera gain. The human eye cannot distinguish enough intensities in a 12-bit image, and therefore it is critical to evaluate the histogram of intensities to determine whether the range of intensities is appropriate. Fluorescence background introduces noise and therefore reduces SNR. Finally, ensure that the exposure time and power density of the light source do not photobleach the fluorophore, as this can result in photodamage and irreproducibility.

23. Here we use a fully motorized, inverted Nikon Ti microscope equipped with a Perfect Focus System to maintain focus. The specific microscope is not required for this project, and any that has similar capability would likely work.

24. Caution is needed when focusing to prevent damage to the objective lens.

25. Although a phase-contrast objective lens reduces fluorescence signal, it produces high-contrast images of bacterial cells, improving their detection using transmitted light. Ensure that the appropriate phase-contrast annulus is set up in the condenser turret for the phase-contrast objective lens used (e.g., Nikon Plan Apo $100\times/1.4$ Ph3 requires the Ph3 annulus to be positioned in the light path above the condenser lens).

26. To estimate the size of the FOV, multiply the size of the photodiode array (pixel) in the camera chip (e.g., 2048×2048), by the physical size of the photodiode (e.g., 6.5 μm) and divide by the total magnification (objective magnification and any additional optovar magnification, e.g., 60×1). In the example above, $2048 \times 6.5/60 = 221.9$ μm. The distance between positions in each pedestal should be at least 0.25 μm to prevent imaging overlapping FOVs.

27. Move from left to right, down one row, right to left. This will minimize the acquisition time.

28. The acquisition time in a semiautomated acquisition is ~35–40 min. Make different dilutions in the morning to ensure that the next plate will be ready to mount after the current plate is imaged. 4–6 plates can be imaged a day.

29. Making the pedestals even is key in the success of the automated acquisition. Set the speed to 1 mm/s. Apply enough oil on the surface of the cover glass. Avoid air bubbles between the cover glass and the pedestals. The PFS with longer working distance range performs better at maintaining focus.

30. The alignment routine only needs to be done once per imaging session as the dimensions and the position of the pedestals within the stage insert are constant.

31. Oufti detects objects from images loaded in the "Phase" channel in the main GUI. However, the detection can be done using transmitted light or fluorescence images. Ensure that the Invertimage value is set to 1 in the segmentation parameters.

32. Oufti contains several parameter sets that can be loaded. These are presets for different bacterial cell types. Optimization of the parameters will be required. See the tutorials provided in www.oufti.org for details.

33. Image quality is key in successful segmentation and detection in any analysis software. Images that contain too many cells close to each other will be challenging for segmentation. If cell density needs to be adjusted, modify the volume of the remaining medium at Subheading 3.3, **step 2**. At the analysis step, revision of the cell outlines is important for successful detection of the cells or nucleoids.

Acknowledgments

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