High Content Microscopy

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Single-cell microscopy is a powerful tool for studying gene functions using strain libraries, but suffers from throughput limitations. Here we describe the Strain Library Imaging Protocol (SLIP), a high-throughput, automated microscopy workflow for large strain collections with minimal user involvement. SLIP involves transferring arrayed bacterial cultures from multi-well plates onto large agar pads using inexpensive replicator pins and automatically imaging the resulting single cells. The acquired images are subsequently reviewed and analyzed by custom MATLAB scripts that segment single-cell contours and extract quantitative metrics. SLIP yields rich datasets on cell morphology and gene expression, which illustrate the function of certain genes and the connections among strains in a library. For a library arrayed on 96-well plates, image acquisition can be completed within min per plate.

Keywords:
- single-cell microscopy
- high-throughput
- multi-well format
- automated microscopy

Reagents

- Granulated agar (BD Diagnostic Systems, BD cat. no. 214530)
- 1.5 large glass cover slips (NEXTERION® Coverslip custom, #1.5; Glass: D263; Size (mm): 110 x 74)
- Immersion oil (for Nikon Ti-E: Nikon immersion oil type NF, cat. no. MXA22024)
- Growth medium (specific to species and condition of interest)
- Singer PlusPlates (Singer Instrument Company, cat. no. PLU-001)
- Singer RePads replicator pins (Singer Instrument Company, cat. no. R P-001 for 96-well plates, cat. no. R P-003 for 384-well plates)
- 500 mL Corning glass bottle (Corning Inc., cat. no. 1395-500)
- Bacteria for imaging (e.g. E. coli MG1655 cells, CGSC #6300)

Additional Products and Services:
- Mouse Monoclonal Antibody
- Rat Monoclonal Antibody
- Rabbit Monoclonal Antibody
- Human Monoclonal Antibody
- Polyclonal Antibody
- Antibody Sequencing
- Hybridoma Sequencing
- CAR T-cells
- Lentivirus production
- Cancer Stem Cells
- Specialty Cell Culture Media
- T-cell Expansion beads

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**EQUIPMENT**

- Computer (for controlling microscope acquisition, installed with software necessary for controlling microscope stage, focus, shutters, and cameras)
- Inverted fluorescence microscope and objective (e.g., ikonclipse Ti with 100X A1.0 oil-immersion objective ikon Instruments)
- Motorized xy stage controller (Applied Scientific Instrumentation, cat. no. MS-2000, firmware 9.2h, with ARRA M L included)
- Camera (e.g., Neo sCMOS camera, cat. no. 5.5 or Zyla sCMOS camera, cat. no. LA-5.5, Andor Technology) with B cables
- \( \mu \)Manager 1.0 (SF, www.micro-manager.org)
- Antivibration table (Thorlabs, cat. no. B3636F)
- Compressed air pump
- Dry heat block (Fisher Scientific, cat. no. 11-718)
- MATLAB R201b (MathWorks)
- (Optional) TwinCam dual-emission imaging system (Cairn Research, TwinCam LS)
- (Optional) Fluorescence light source (e.g., ikon Intensilight mercury lamp, cat. no. MBF 2665, or Xcite 120L, Lumen Dynamics, cat. no. P010-0015)
- (Optional) BNC T-shape adaptor (Thorlabs, cat. no. T325)

**REAGENT SETUP**

**Preparation of agar pads**

To 200 mL of liquid growth medium in a 500 mL Corning glass bottle, add 1.5% (w/v) granulated agar (3 g) and dissolve by microwaving at 50% power. While microwaving, check the solution frequently to avoid overheating or boiling. If the experiment requires the addition of heat-sensitive chemicals, keep the melted agar on a 65°C heat block until thermal equilibrium is achieved and then add the chemicals.

**CRITICAL:** Make the agar medium fresh each time and keep bottles capped to avoid evaporation and contamination.
Remove the lid of a Singer PlusPlate and place the bottom (which is usually used for holding agar) upside-down onto a flat surface such as a benchtop. Pour melted agar onto the flat bottom surface of the plate (Fig. 1a, Supplementary Figure 1a), gently tilt and shake the plate to spread the agar evenly over the entire plate bottom, and use a flame to remove any bubbles if necessary. The surface tension of the hot agar will create a flat surface. One plate requires ~25 mL of melted agar. Leave the plates at room temperature (~22 °C) for 20–30 min to solidify the agar (Fig. 1b, Supplementary Figure 1b).

CRITICAL: The plate must remain on a flat surface until the agar solidifies in an undisturbed environment to yield a flat and smooth agar surface for imaging.

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**EQUIPMENT SETUP**

Software installation and setup

SLIP runs on MATLAB and controls a μManager GUI for image acquisition. Install μManager 1.4 (or the current version; UCSF, www.micro-manager.org) and MATLAB (MathWorks), and then open the SLIP source code (Supplementary Source Code, or visit https://bitbucket.org/kchuanglab/slip2 for the current version) in MATLAB to perform the initial setup as detailed in Box 1.

**Box 1**

Initial software setup after installation

On our computer, μManager is installed in the default folder C:/Program Files/Micro-Manager-1. The path is hard-coded in some scripts and may require modification. The steps below are for a computer running Windows.

1. Find all .jar files located in the μManager folder: run MMsetup_javaclasspath.m in MATLAB. This script creates MMjavaclasspath.txt under the MATLAB default directory.
2. Run edit([prefdir '/MMjavaclasspath.txt']) in MATLAB to open the MMjavaclasspath.txt file in the MATLAB editor. Then run edit([prefdir '/javaclasspath.txt']) to copy all contents of MMjavaclasspath.txt to javaclasspath.txt.
3. Save javaclasspath.txt.
   In MATLAB, run edit('librarypath.txt'). At the end of the file, add a line with the μManager installation directory, for example, C:/Program Files/Micro-Manager-1.4. Save librarypath.txt.
5. Restart MATLAB.
6. Right-click computer on the desktop or start menu.
   .lick Properties Advanced system settings, highlight the Advance tab, and click Environment variables.
   .n the System variables list, highlight Path and click dit. o not delete anything. At the end, add ;C:/Program Files/Micro-Manager-1.4 or the appropriate μManager installation directory (do not omit the leading semicolon).
9. Save the changes and restart the computer to make the change of path effective.

**TTL setup**

During image acquisition, SLIP uses TTL signals to trigger between the camera and stage controller, circumventing the external controls between each image acquisition and thus speeding up the experiment. TTL signals have inactive (low voltage) and active (high voltage) states, and hardware can communicate through the switch of states. In this setup, when the camera finishes one exposure, its TTL signal switches from the active to the inactive state. This change at the camera’s arm port sends a TTL signal to the stage controller’s input port, which directs the stage controller to move to the next imaging site. Once the stage movement is done, its TTL state switches and sends a signal through its output port to the camera’s external triggering port, directing the camera to start another exposure. To set up TTL, first use BNC or other compatible cables to connect the camera and the stage controller to each other for TTL signals: connect the camera’s arm port to the stage controller’s input port, and then connect the camera’s external triggering port to the stage controller’s output port. An optional step is to connect the camera’s fire port to the input port of the light shutter, such that the shutter opens only during camera exposure. This step can be important for long-term time-lapse imaging to minimize photo-toxicity.
Since TTL is hardware-dependent, we recommend that users optimize their hardware settings to achieve the fastest possible acquisition. The main factors that determine total imaging time are camera exposure and stage movement. Acquisition time scales linearly with camera exposure time (Supplementary Figure 2a), thus reducing exposure time can speed up acquisition, but potentially at the cost of reducing image quality due to lower signal intensity. For stage movement, the time-consuming part is stage acceleration and deceleration between two imaging sites. We have found that the acceleration speed can impact total imaging time by ~3-fold, with an optimal time for acceleration for our system of 0–50 ms that minimizes total imaging time (Supplementary Figure 2b). We recommend that users test their stage controller to achieve the best results.

The dual camera setup enables the simultaneous capture of two images at different wavelengths using two identical cameras (Fig. 2a). The cameras should be aligned before imaging. For TTL setting, one camera (the master camera) communicates with the stage controller to coordinate exposure and stage movement at the same time, it triggers the other camera (the slave camera) for a synchronized exposure in the other channel. To set up the cameras, first connect both cameras to the computer. Open μManager. Click Tool → Device Property Browser, and find the fields Multi camera-Physical camera 1 and Multi camera-Physical camera 2. Set their values to be the two cameras installed. Assign the slave camera to Multi camera-Physical camera 1, and the master camera to Multi camera-Physical camera 2. In addition to the TTL connections for the master camera, also connect the fire port of the master camera to the external triggering port of the slave camera. An optional step is to connect the master camera fire port to the input ports of the light shutters for both phase and fluorescence light sources to control exposure. A B T-shape adaptor may be needed for this optional step.
1. Grow overnight liquid cultures of bacteria from stocks in deep 96-well or 384-well plates. It is recommended to include appropriate internal controls (e.g. wild-type cells) on the plate.

2. The next day, dilute overnight cultures 1:200 (or as desired) into fresh medium. Incubate until cells reach a desired density. Cell density directly affects the number of cells acquired for each strain during imaging, as well as the cellular physiological state. For E. coli and B. subtilis, we recommend incubating 2–3 h after the 1:200 dilution to reach a cell density of OD600~0.1, which typically yields enough cells (usually tens to a hundred) in each field of view. With this density, most cells are well separated from others, permitting easier and more accurate segmentation. We recommend that users perform preliminary tests to determine an optimal cell density for their particular applications. Such tests are facilitated by SLIP, whereby the user can serially dilute an overnight culture in a 96-well plate and rapidly acquire images of populations from different dilutions.

3. Use a replicator pin to transfer the liquid culture from Step 2 onto a solidified agar pad (see REAGENT SETUP). Each transfer carries ~0.1 μL of liquid from each well. If desired, repeat the transfer several times to increase cell densities (Fig. 1c, d, Supplementary Figure 1c, d). It is important that the replicator pin does not directly contact the agar surface; the liquid should drop onto the surface. Direct contact of the pin with the agar may damage the flat agar surface and cause cell clustering and focus drifting, which lead to poor-quality images.
Wait for the agar pad to absorb liquid droplets. Keep the plate on a flat surface to avoid liquid flow and cross-contamination. The agar pad takes ~10 min to absorb all droplets at room temperature, or ~5 min in a 37 °C warm room. Check progress visually by inspecting light reflected from the surface at a tilted angle.

CRITICAL STEP: All droplets should be completely absorbed by the agar pad before the next step. Unabsorbed liquid allows the cells to move above the surface of the pad, causing blurred images and potentially leading to cross-contamination.

5. (Optional) Mark the locations of the droplets at A1 and H12 (or other strain positions that will be used for calibration) on the back of the plate after the droplets have been absorbed. These marks will make it easy to find the strains during calibration.

6. Clean a large glass cover slip (110 x 74 mm) with compressed air and place the cover slip on top of the agar pad (Fig. 1e, f, Supplementary Figure 1e). Touch the pad with one side of the cover slip and slowly put down the other side, avoiding large air bubbles. If large bubbles occur, gently press the cover slip with a gloved finger to push bubbles to the edge of the cover slip, where they will dissipate.

(Optional) If the experiment requires cell growth before imaging, the pad can be incubated at the desired conditions before imaging. We have performed overnight incubation at 37 °C and have not observed significant drying of the pad.

7. (Optional, only for oil objectives) Add immersion oil to the cover glass. Spread the oil evenly across the entire imaging surface (Supplementary Figure 1f).

CRITICAL STEP Due to the large imaging area, it is important to spread the oil over the entire imaging surface such that the objective is always in contact with the oil during imaging, which reduces focus loss. Be careful not to add too much oil, especially on an inverted microscope, as excess oil may drip onto the objective turret.
8. Mount the agar plate onto the microscope stage, with the sample side facing the objective. Manually move the stage to the A1 strain and focus on the cells.

9. Start SLIP by running SLIP.m in MATLAB. This script will open the μManager window. Choose the appropriate μManager configuration file, and the program will initialize (Fig. 2b).

10. Click 'Calibrate' in the SLIP window, and SLIP will display the calibration menu. After specifying the strain locations for calibration, use the Live imaging feature in μManager to locate the center of these strain positions. The positions of all strains on the plate will be calculated based on the calibration.

   NOTE that on an inverted microscope, the plate has to be inverted on the stage. Therefore, well A1 will be on the top right, if viewed from the top of the plate holder. Once calibrated, the SLIP tool will also recognize this flipping and save images acquired at the top right corner as position A1.

TROUBLESHOOTING

11. Set the desired imaging parameters (plate layout, strains to image, number of images for each strain, exposure time, etc.) Typically, imaging a 5x5 grid (25 images in total) for each strain yields data from enough cells for the statistical analysis of interest. Variation in mean E. coli cell width and length across fields from the same well is ~3% (Supplementary Figure 3). The number of images should be adjusted based on cell densities. Since image acquisition is only a small fraction of the total time required for SLIP, the imaging grid size can be selected based on the strain(s) with the lowest density without substantially affecting the SLIP time.

12. For time-lapse imaging, set the desired imaging interval and total time points (Fig. 2b(v)). Typically, a full 96-well plate requires at least ~1 min to image one time point, and acquisition time scales linearly with the number of images acquired for each well (Fig. 1h) the defined interval should be longer than the total acquisition time for one time point.
13. In the SLIP window, check that ‘Pause on PFS loss’ (Fig. 2b(vi)) is enabled. With such a large imaging area, PFS loss is likely. Enabling ‘Pause on PFS loss’ allows SLIP to pause image acquisition for the user to manually fix the focus before the next strain. Thus, although SLIP does not require PFS-loss checking, this strategy improves data quality.

14. Select channels for imaging in the selection box (Fig. 2b(vii)). Note that for two or more channels without a dual camera setup (Fig. 2a), image acquisition will take significantly longer.

15. Once all parameters are set, start image acquisition. Fix the focus when necessary.

   If ‘Pause on PFS loss’ is enabled, imaging progress should be monitored so that acquisition can continue after PFS loss. **TROUBLESHOOTING**

16. Segment the cells. Our laboratory has developed a custom MATLAB software, Morphometrics, for segmenting and analyzing phase images of bacteria cells. General steps to use the software are included below. Other software packages, e.g., Oufti and MicrobeJ, can be applied similarly. The segmentation algorithm masks dark objects in a phase-contrast image and thereby identifies the cells. Next, it implements a custom filter to exclude non-cell objects based on their contour shapes. In principle, parameters for cell segmentation depend on the contrast and brightness of images and need to be optimized for each image. However, in our experience, since lighting and exposure conditions are very similar for all images in one experiment, parameters can be set based on a small subset of images (e.g., three images from three strains across the plate) and then used to segment the entire dataset.
17. Compile the data. After segmentation, properties such as cell area, contour curvature, and contour length are readily calculated and stored. We then apply filtering algorithms to remove micro-colonies that were not accurately segmented based on contour curvatures and a pill meshing algorithm to calculate an internal coordinate system and neutral axis for each cell. For rod-shaped cells, cell length and width are extracted from these measurements. The analyzed results for each cell are stored in a MATLAB structure with multiple fields, and the structures for all cells are saved as .mat files. The user can choose fields for further analyses or incorporate custom metrics as new fields into the data structure.

18. Analyze the data. Depending on the biological system and questions of interest, the compiled dataset can be used to extract information about the dimensions and growth (in the case of time-lapse experiments) across strains and/or chemical environments. For instance, the mean and standard deviation of morphological parameters such as width and length can be calculated for each strain in the 96- or 384-well plate (Fig. 3a, b).

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Cells are motile (and hence difficult to image in a single plane)</td>
<td>Liquid droplets not fully absorbed by pad before placing the cover slip</td>
<td>Wait longer until all droplets are absorbed before Step 6</td>
</tr>
<tr>
<td></td>
<td>Too many/too few cells</td>
<td>Cell density of the liquid culture not optimal</td>
<td>Adjust back-dilution volume and/or incubation time before spotting cells onto agar pad; note that incubation time can affect cell size and/or shape—</td>
</tr>
</tbody>
</table>
### Troubleshooting advice for common problems, with the associated step and possible explanation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>PFS loss for many positions</td>
<td>Agar particles not fully melted</td>
<td>Fully melt agar before pouring pads. Melted agar should be homogenous without visible particles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agar surface not flat during solidification</td>
<td>When preparing agar pads, keep the plate on a flat surface until the agar solidifies in an undisturbed environment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Replicator pin touched agar surface in Step 3 and damaged the agar surface</td>
<td>Avoid direct contact of replicator pin with agar surface; surface tension should be sufficient to transfer droplets to the agar surface</td>
</tr>
<tr>
<td></td>
<td>Insufficient immersion oil on the cover slip</td>
<td>Spread more immersion oil evenly across the imaging region</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PFS loss for some positions on the edge</td>
<td>Agar pad not flat at the edge</td>
<td>Some extent of unevenness at the edge is expected due to surface tension, and can be minimized by using the right volume of melted agar so that the agar surface is at the same height as the plate edge</td>
</tr>
<tr>
<td></td>
<td>No cells are found in certain strain positions</td>
<td>Little or no cell growth in the liquid culture</td>
<td>Check the OD of the liquid culture before transferring onto the pad to make sure cultures are sufficiently dense</td>
</tr>
<tr>
<td></td>
<td>Calculated strain positions differ from the actual positions</td>
<td>Repeat calibration and make sure to set the centers of the calibration droplets</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 1. Schematic of pad preparation and imaging**

(a) Melted agar is poured onto the bottom surface of a Singer PlusPlate. (b) Melted agar is spread evenly by gently tilting and shaking the plate. A flat agar surface is generally achieved when the plate is left on the benchtop and solidifies without disturbance. The agar layer is ~2 mm in thickness. (c, d) Bacteria cultures are transferred onto an agar pad using a 96-well replicator pin. (e, f) After the agar pad absorbs all the liquid (~5–10 min depending on temperature), a large glass cover slip is used to cover the agar surface. (g) Schematic of stage movement during image acquisition for a 96-well plate. Left: to minimize travel distance, the stage moves across the wells in a zigzag manner, first from A1 to A12, then backward from B12 to B1, etc. Middle: for each strain, the stage moves across an imaging grid in a similar zigzag pattern. An example of a 3x3 grid is shown. The grid size is enlarged relative to the droplet size for illustration purposes. Right: a typical image acquired by SLIP. (h) For a 96-well plate, total image acquisition time scales linearly with number of images per strain, with a slope of 200 ms/strain/image or 0.33 min for each additional image across 96 strains.
Figure 2. Experimental setup
(a) Configuration for dual-color cameras. (b) User interface of SLIP software. The key setup parameters are highlighted. (i) Plate layout selection: the user selects 96-, 384-, or 1536-well format. (ii) Illustration of the whole plate and the selected wells (yellow) for image acquisition. (iii) Imaging grid setting: for each well, SLIP generates a grid of non-overlapping neighboring imaging sites and acquires images at these sites. (iv) Exposure time setting. (v) Time-lapse settings. (vi) ‘Pause on PFS’ setting, which allows the user to manually correct any instance of loss of PFS. (vii) Channel setting: the highlighted channels are selected for image acquisition.
Figure 3. Typical results from SLIP

(a) Changes in mean cell width (left) and length (right) during imaging of an E. coli MG1655 culture in exponential growth transferred to all 96 wells of a plate. Twenty-five images (5x5 grid) were acquired at each well to visualize enough cells for accurate quantification of cellular dimensions. Image acquisition required ~12 min. Due to growth on the pad, cell length increased by ~10% from the first well to the 96th, while cell width remained roughly constant. Blue data points are mean ± standard deviation. Red lines are the best linear fit of mean values. Purple data points are cell length corrected for the linear growth effect represented by the red line, and the linear fit to the corrected data is shown in yellow. For clarity, the error bars for purple data points are not shown.

(b) Typical images from a SLIP screen of E. coli MreB-msfGFP mutants. Mutants with altered cell dimensions (larger or smaller) are easily identified.

(c) Cellular dimensions of strains in an MreB-msfGFP mutant library quantified by the MATLAB software package Morphometrics17. Data points are mean ± standard deviation (n > 200 cells per strain).

(d) Typical terminal phenotypes of strains in a B. subtilis CRISPRi essential gene knockdown library, where cells were grown on an agar pad with the CRISPRi inducer xylose overnight to deplete one essential protein in each strain. Wild-type cells showed no shape defect and grew into homogeneous lawns (left), whereas we visually identified a variety of morphological and/or growth defects by depleting essential proteins.
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