**Background Subtraction:**

Background subtraction removes uneven illumination of the background, which aids in segmentation.

1. Prepare a dummy coverslip to be imaged for background subtraction. This is a coverslip that has been treated similar to an experimental coverslip, but which has no foreground objects (in this case, cell nuclei).
2. The background image is specific to the objective, optovar, fluorescence intensity, exposure length, camera gain, and excitation wavelength. If any of these parameters are altered while acquiring images of the experimental sample, a new background image must be obtained.
3. Find the focal plane of the dummy coverslip using a confocal microscope and acquire an image stack spanning the average height of an infection mound, by specifying the same number of z slices as would be used to image such a mound. Maintain the same interval between z slices as is used to image an infection mound.
4. Acquire 10-100 such stacks at multiple positions.
5. Create a maximum intensity projection of the stack at each position and generate a median projection across all positions.
6. Remove extremely bright pixels by passing a median filter through the image.

**Flatfield Correction:**

Flatfield correction corrects for uneven illumination of objects in the foreground, such as nuclei at the center of the field of view appearing brighter than nuclei at the corners, to improve accuracy of segmentation.

1. To correct for dapi-stained nuclei, first prepare a concentrated dye slide by adding 5μL of 50mg/mL coumarin dye stock solution to a Micro90-cleaned slide. Cover with a clean coverslip and seal using nail polish. Appropriate dyes for excitation wavelengths other than 405 nm include sodium fluorescein for 488 nm, rose bengal for 561 nm, and acid blue for 642 nm light.
2. Find the focal plane of the concentrated dye slide using a confocal microscope with the Perfect Focus feature or with the aid of bubbles in the concentrated dye slide. Acquire an image stack by imaging both above and below this focal plane and image sufficient z slices such that the fluorescence light emitted by dye is no longer visible at the most extreme z slices.
3. Obtain similar image stacks at various positions on the concentrated dye slide.
4. Acquire a dark image to account for noise in the camera as well as dust along the optical path. To do so, divert light to the eyepiece or close the camera shutter. Then, acquire 100 images in a time series using the same intensity and exposure settings that was used to obtain the flatfield images.
5. Perform a median projection of the dark images.
6. Pass each slice of the flatfield images through a median filter to remove very bright pixels.
7. Subtract the dark image from each slice of the flatfield images.
8. Perform a maximum intensity projection of each dark image-subtracted flatfield image stack.
9. Generate a median projection across all positions.