**Hyphal Escape Assay: image analysis guidelines**

Related to Figure 1 and Figure S1.

Additional guidelines on the image analysis conducted in this study, including image file conversion, generating images used to correct vignetting and CellProfiler image analysis project files. Please note that the image file types may depend on the microscope used for image acquisition.

***Leica Image File conversion***  
  
Before image analysis, leica image files (.lif) were converted to .TIF files using the “bfconvert” command in the “bftools” set of command lines, developed by Bio-Formats (www.openmicroscopy.org/bio-formats/). Download the bftools command line package here:

https://docs.openmicroscopy.org/bio-formats/5.7.1/users/comlinetools/index.html

Using PC, the following steps can be taken to convert .lif files into .TIF files:

1. Download the bftools zip folder. Extract the zip folder contents (bftools folder): select the main PC drive (C, for example) as the folder destination.
2. Open the Command Prompt (type "cmd" in search bar).
3. Next, type: *cd C:\bftools*
4. Press ENTER. Now create a destination folder for your .tif (output) image files (“lif\_TIFF”, for example). Now type: *bfconvert "C:\*directory of your .lif file, i.e. *Documents\LIF files\livecelloutputfile.lif" "C:\*directory of your destination folder, i.e. *Documents\lif\_TIFF\image\_P%%s\_T%%t\_C%%c.tif"*
5. Press ENTER.

This will convert your images. If you receive the error *“JAVA is not recognized as an internal or external command*”, adjustments of the Java directory within your PC’s system variables may be needed.

To create timelapse clips (instead of individual .TIF files for each timepoint), remove the timepoint portion in the bfconvert command as follows:

1. *bfconvert "C:\*directory of your .lif file, i.e. *Documents\LIF files\livecelloutputfile.lif" "C:\*directory of your destination folder, i.e. *Documents\lif\_TIFF\image\_P%%s\_C%%c.tif"*
2. Press ENTER.

***Generating images used to correct vignetting in Cellprofiler***  
  
Four different filter cubes captured the following images: calcofluor white (CFW) hyphae (DAPI), Ttomato- expressing *C. albicans* (TXR), DRAQ7- staining macrophage nuclei (Y5) and CellTracker Green- staining macrophages (GFP, first timepoint only). For each of these images, vignetting (uneven background) was corrected for using the “ImageMath” module in CellProfiler 2.1.1. In order to do this illumination correction images were made using ImageJ 2.0.0-rc-69.

Illumination correction image instructions, using ImageJ 2.0.0-rc-69:

1. For a given channel, select at least four images of media only (no cells) taken with the same illumination settings as for infection condition wells. Open these images in ImageJ.
2. Then select Image, Stacks, Images to stack, and click OK (the name is not important here).
3. Now select Image, Stacks, Z project, project type, select "median”. Now click OK.
4. Select Process, Filters, Gaussian Blur, set the radius to 16. Now click OK.
5. Find the maximum intensity value
6. Select Analyze, Set measurements, check min & max grey value. Click OK.
7. Select Analyze, then Measure. Note maximum intensity value.
8. Now select Process, Math, Divide, enter the maximum value noted down in the previous step. This should give an image with intensity values very close to 1 in the centre (max) and lower across the vignetting profile.

Now save this image:

1. Select File, Save as, Tiff , select an appropriate name (i.e. "IllumCor" plus month/year).

This process has to be repeated for each channel (DAPI, TXR, Y5 and GFP). We recommend A) not using illumination correction images taken on a different microscope than the images being analysed, and B) using up-to-date illumination correction images for analysis.

***CellProfiler image analysis templates and additional information***

CellProfiler 2.1.1 was used for all image analysis in this study. As described in methods, three main image analysis pipelines were used to quantify hyphal escape and macrophage death: 1) CellTracker Green counts, 2) DRAQ7 death counts, 3) Hyphal escape.   
Below is a list of image analysis modules used in each pipeline. For more detail on the image analysis see example project files for each pipeline:

https://github.com/FABOlivier/Hyphal-escape-image-analysis-CellProfiler-pipelines.git

**CellTracker Green counts:**

*ImageMath  
IdentifyPrimaryObjects  
ExportToSpreadsheet*  
  
**DRAQ7 death counts:**  
*ImageMath  
IdentifyPrimaryObjects  
ExportToSpreadsheet*  
  
**Hyphal escape:**  
for dTomato images:  
*ImageMath  
Smooth  
ApplyThreshold*  
  
for CFW images:  
*ImageMath  
Smooth  
EnhanceOrSupporessFeatures  
ApplyThreshold  
Morph*  
  
for both dTomato and CFW images:  
*MeasureImageAreaOccupied  
ExportToSpreadsheet*