

Reference: Depth-Variant Deconvolution Applied to Widefield Microscopy for Rapid Large-Volume Tissue Imaging

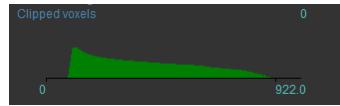
## Supplementary Protocol

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## Acquisition reminders

1. Z-step < 2 \* [the Nyquist limit](#) (e.g., ~1.2  $\mu\text{m}$ , NA 1.0). Z-stack should be arranged so that the start and end include ~50  $\mu\text{m}$  of out of focus information beyond the edge of the tissue.
2. Signal should be acquired to utilize the full dynamic range of the camera without overexposure.

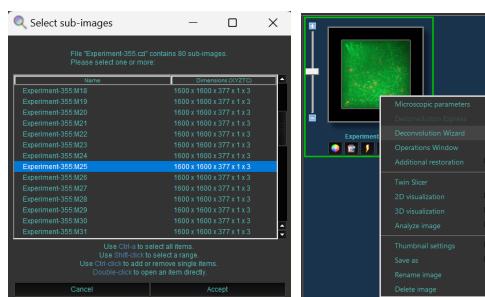


## Creating Deconvolution Template (START here)

For single tile or multi-tile images, start with one tile and create a deconvolution template

### [Loading single tile](#)

1. Drag the image file into the Huygens workspace.
2. For multi-tiled images, select 1 tile (i.e. “sub-image”) to optimize the deconvolution.
3. Once the image loads into workspace, right click and select microscope parameters.

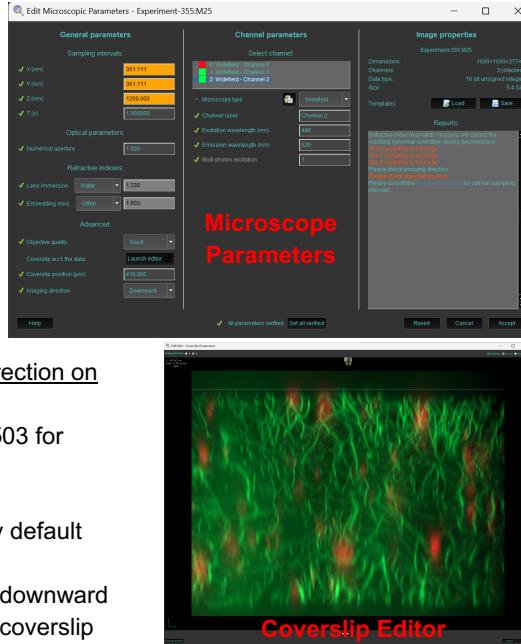


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## Setting microscope parameters

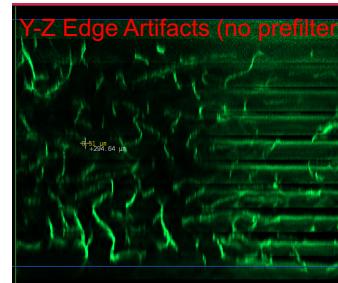
Huygens automatically loads most metadata.

1. Confirm the Z-step size (i.e. 1.2  $\mu\text{m}$ ) and numerical aperture of lens (i.e. 1.0).
2. Refractive Indexes
  - a. Lens Immersion: usually air (1.000) or water (1.330) or spherical aberration correction on lens (1.413)
  - b. Embedding media to 1.503 for ADAPT:RI
3. Advanced
  - a. Keep microscope quality default (good).
  - b. Set imaging direction to downward
  - c. Launch editor to set the coverslip distance, approximate start of true signal.
4. Make sure the microscope type is set to widefield for each channel.
5. Click 'Set all verified' to set all microscope parameters as verified.
6. Save template in new folder.
7. Click 'Accept' to exit the window.



## Pre-filtering decision point

1. If single Z-stack tile, filtering is optional because edge artifacts can be cropped.
2. If multi-tile image, filtering is almost always necessary.
  - a. If unsure, select an individual tile from the edge of the image, proceed [to the Deconvolution Wizard](#).



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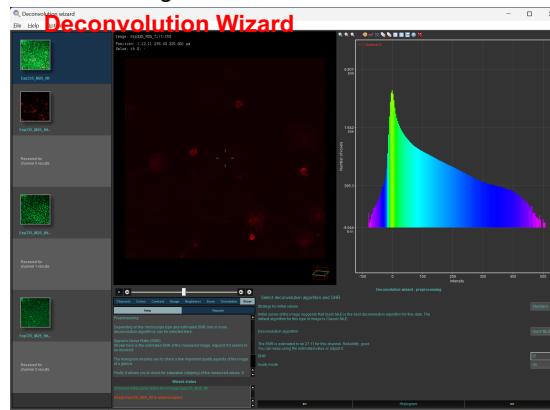
### Pre-Filtering Single Tile

1. Paste following code into Huygens TCL shell (bottom right of workspace).
  - a. Change text in Blue highlight to **name of your image** in the workspace
 

```
Exp335_M25 convert -type int
Exp335_M25 qgauss -> gFilt -sigma {3 3 3} -maxWidth {25} -edge trunc
gFilt minFilter -> min_gFilt -width {9 9 21}
Exp335_M25 - min_gFilt -> Filtered_Tile
```
2. Produces 3 images, Filtered\_Tile is the final product

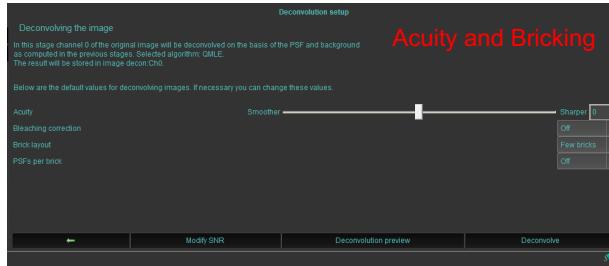
### Deconvolution Wizard

1. Right click on image and select deconvolution wizard.  
Note: If filtering was done, use 'Filtered\_Tile' as the input.
2. Click through the first three windows with the right arrow.
  - a. Window 1: Confirm pixels were not overexposed. Click "Enter Wizard"
  - b. Window 2: No PSF selection. Click right arrow
  - c. Window 3: Cropping optional. Right arrow
  - d. Preprocessing, select the first channel to deconvolve.
3. Deconvolve each channel individually (take notes)
  - a. Select QMLE for most widefield cases, CMLE has use for smoother objects like nuclei
  - b. KEY: Set SNR correctly
  - c. SNR automatically estimated by Huygens but may need to be changed Expect > 40 for good raw images, expect < 25 for filtered images.
  - d. A SNR set inappropriately low will lead to partial deconvolution with low iterations. Too high of an SNR can create noise in the result by considering background as true signal.
  - e. Background subtraction
    - i. For raw images select automatic estimation [Widefield, 0.7 micron] (take note of value).



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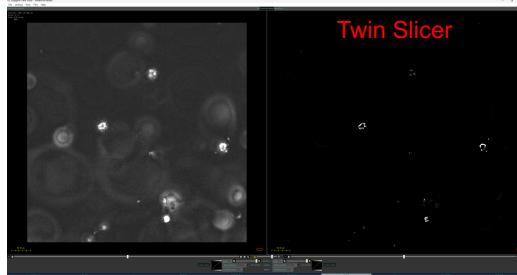
- ii. For filtered images select manual and set background to 0.
- f. Setup: for first attempt keep acuity set to 0 and select few-bricks option.



- g. Observe status, # of bricks, and iterations in reports tab (lower left)

#### 4. Visualization before improvement

- a. Right click original first channel in left hand column. Select 'Twin slicer.'
- b. From the drop down on the right side select "decon"
- c. Select contrast in bottom tab to make equal.
- d. Increase contrast if needed to check that all features are retained even if dimmer relative to brightest signal.
- e. Alternative view: right click on image in left column and select ortho slicer
  - i. Provides adjustable max intensity projection view



- f. Note: Considerations for next attempt
  - i. If deconvolved image is blurry, point spreading remains, or some features remain very dim, more iterations may be required (increase acuity for QMLE)
  - ii. If true signal looks good but there is lots of diffuse noise, the SNR may be too high.

#### 5. Repeat deconvolution with altered parameters (if needed). Hit restart channel

- a. First, SNR can be adjusted and deconvolution repeated
  - i. Use the twin slicer to compare to the first deconvolution
- b. Second, Acuity can be increased on QMLE deconvolution to increase the number of iterations run (start with 25, over iteration can create noise)
- c. Alternatively, CMLE can be tested instead. On the final window start with 80 iterations, turn off bleaching correction, and set to few bricks.

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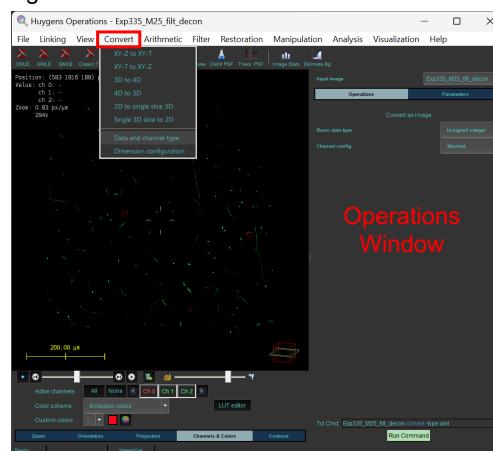
- d. Take notes for each attempt as each attempt will be stored as :T0, :T1, etc. and the preferred attempt must be selected at the very end.
- 6. Repeat the deconvolution process for each channel of the image.
- 7. Then select 'All done.'
- 8. Then select the best deconvolution attempts of each channel to combine into the final image and that will be used for the deconvolution template.



- 9. Hit 'Next,' save the deconvolution template, and close.

### Saving Single Tile Image

1. Right click on image in the workspace, save as an ICS2 file.
  - a. Will save as a 32-bit float
2. Or first convert to 16-bit unsigned integer
  - a. Right click on image to open operations window.
  - b. Click 'Convert' and select data type.
  - c. Then select 'Unsigned integer' for data type and hit 'Run Command'
  - d. May require scaling of intensities, but reduces file size by half.
3. The operations window can also be used as needed to rotate or flip the image orientation (manipulations tab).



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## Automated pre-Filtering of Multi-Tile Images

1. Edit the python script provided at <https://github.com/wowdanlee/3D-Widefield-with-ADAPT-3D-and-Huygens>

```
## USER ENTRY REQUIRED for following 4 lines
myPath = "D:/user/folder/myImageFile.czi"           # paste file path (forward slash required)
destinationPath = "D:/user/project/filteredImages"    # paste the file path of destination folder
Tiles = 9                                              # enter the number of tiles in the image
ImageRoot = "myImageFile" + ":M"                      # enter file name. Defines subImage root for Zeiss file
```

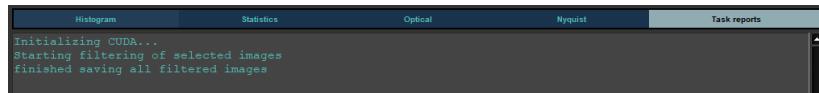
- a. Insert the file path of your multi-tile image file.
- b. Enter the file path of the folder you would like to save your image.
- c. Specify the number of tiles in the image.
- d. Enter image file name (ie. change “myImageFile” to “Experiment-335”).



2. Save the python script.
3. To run, in the Huygens Python shell, type: `source("C:/user/myScript.py")`.



4. Images will be filtered and saved to your destination folder in the background
  - a. “finished saving all filtered images” will print in the task window when done.



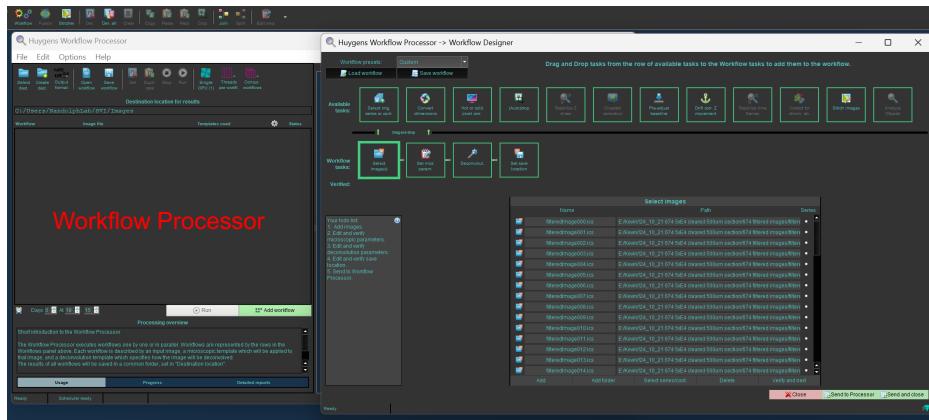
## Batch Processor for Deconvolution of Many Tiles

Once the microscope parameter and deconvolution templates have been produced, a multi-tile image can be automatically deconvolved and stitched using the ‘Stitcher’ window. However, if the stitching is unsatisfactory, rerunning the stitching also requires repeating the deconvolution which is the more time intensive part. Thus, in the case of very large images, it is recommended to batch deconvolve every tile first and then stitch using those deconvolved tiles.

1. Open the workflow processor window.
2. Click ‘Add workflow.’
3. Align workflow tasks in bottom row as Select images, Set micr. param., Deconvolution, and Set save location
4. Hit add folder to identify the filtered images for deconvolution.
5. Load in the micr. param. template, and set to override meta data (verify all).
6. Load in deconvolution template.
7. Select a folder location to save files, and set file type to ICS2.

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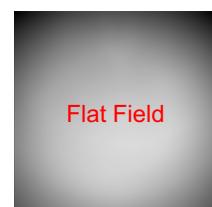
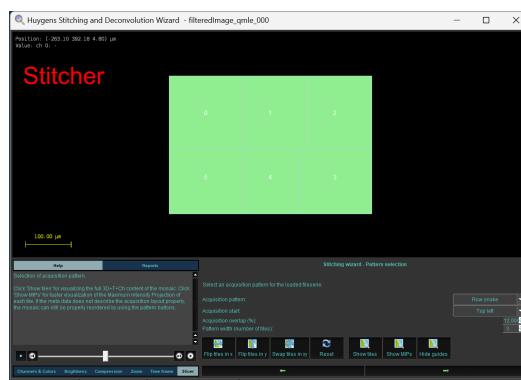
8. Hit send and close (does not start yet).
9. Then in the workflow processor click 'Run.'
10. Each image will be deconvolved and saved automatically.



## Stitching

### [Stitching after batch processing](#)

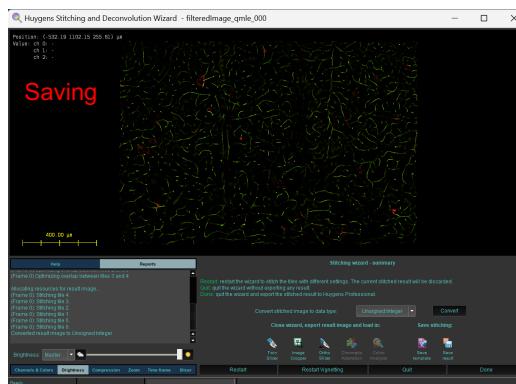
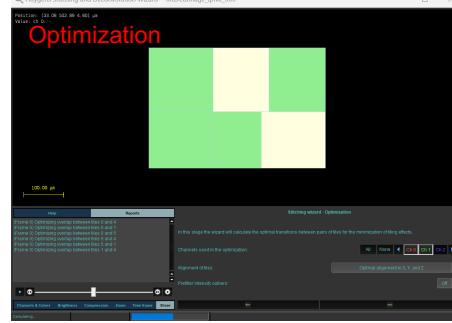
1. Open the stitcher from the menu at the top of the workspace.
2. Load files: Select all deconvolved files and open
  - a. Ensure numerical ordering of tiles matches the order of acquisition (ie. 001).
3. Next, manually input tile pattern metadata to order ->
4. Next, select all the tiles.
5. Vignetting (optional) off or set to manual
  - a. For manual vignetting a flatfield image can be provided ->
  - b. Prepare flatfield by imaging Z-stack of even fluorescent signal and create a median projection.
  - c. Apply a Gaussian filter to smooth the 2D image in Huygens.



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- i. TCL code: `myImage qgauss -> flatfield -signma {9 9 1} -edge trunc`
  - d. More information can be found on [Huygens website](#).
6. Tile position optimization in X, Y, and Z dimensions
- Select the channels with best signal and features throughout the image.
  - Slow optimization due to reading tiles off hard drive, can be redone a second time faster if optimization results bad.
7. Load the micr. parameters template.
8. Do not add a deconvolution template if images were already deconvolved with workflow processor.
9. Hit stitch tiles.
10. Stitched result in slicer view, adjust brightness, compression in lower left.
- If doubling, observed hit restart and change optimization parameters.
11. Convert to unsigned integer (as seen above) and click "save result" as ICS2.
- Can be saved as 32-bit float first, as conversion to 16-bit may require scaling of intensities

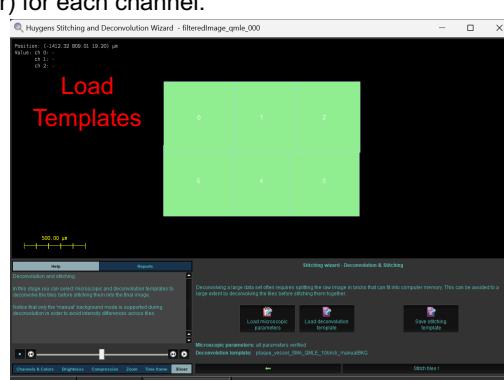
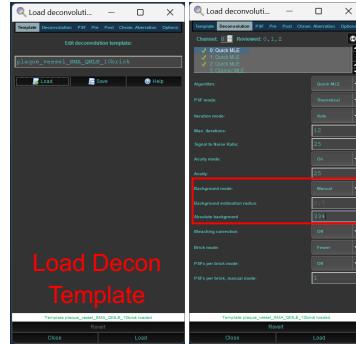
Commented [DL1]: Link



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### For direct stitching and deconvolution (without batch processing)

1. Drag original image into Huygens workspace
2. Ctrl-A to select all tiles, and click 'open.'
3. Select tiles to stitch.
4. Ensure the metadata for tile acquisition pattern and overlap is correct.
5. Vignetting correction is optional, see above.
6. Optimize tile positions in all dimensions, selecting best channels for alignment.
7. Load the microscope parameter template.
8. Load the deconvolution template.
  - a. Change bkg mode to manual, enter abs value (recorded earlier) for each channel.
9. Save the modified template under a new name and reload it.
10. Click stitch tiles.
  - a. Each tile will be deconvolved then stitched together.
  - b. Note: If final stitch unsatisfactory, restarting will require repeating deconvolution of each tile.



For all scenarios, ICS2 files were then loaded into the Imaris file converter and then visualized using Imaris software.