Pore Extractor 2D Installation Instructions

Sample images and tutorials for Pore Extractor 2D are available on the site:
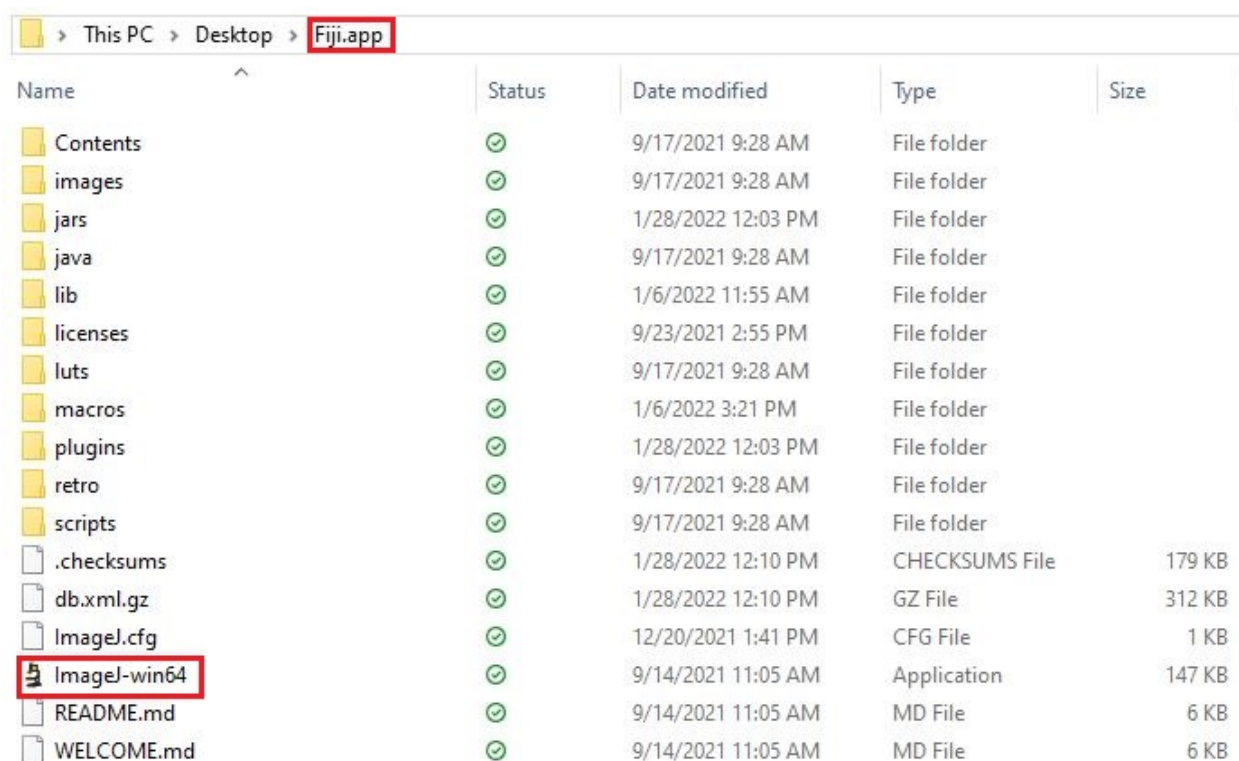https://github.com/MaryECole/PoreExtractor2D

Pore Extractor 2D requires ImageJ FIJI version **1.53g** or later distribution.
Downloads and system requirements can be found at: https://imagej.net/Fiji/Downloads

Unzip the download and extract the Fiji.app folder. If installing on Windows, the folder should be stored somewhere on the user space (e.g. Desktop, Documents), rather than in Program Files, to facilitate write permissions.

ImageJ can be opened by clicking on the application within the Fiji.app folder.

Name	Status	Date modified	Type	Size
Contents	⊘	9/17/2021 9:28 AM	File folder	
images	⊘	9/17/2021 9:28 AM	File folder	
jars	⊘	1/28/2022 12:03 PM	File folder	
java	⊘	9/17/2021 9:28 AM	File folder	
lib	⊘	1/6/2022 11:55 AM	File folder	
licenses	⊘	9/23/2021 2:55 PM	File folder	
luts	⊘	9/17/2021 9:28 AM	File folder	
macros	⊘	1/6/2022 3:21 PM	File folder	
plugins	⊘	1/28/2022 12:03 PM	File folder	
retro	⊘	9/17/2021 9:28 AM	File folder	
scripts	⊘	9/17/2021 9:28 AM	File folder	
.checksums	⊘	1/28/2022 12:10 PM	CHECKSUMS File	179 KB
db.xml.gz	⊘	1/28/2022 12:10 PM	GZ File	312 KB
ImageJ.cfg	⊘	12/20/2021 1:41 PM	CFG File	1 KB
ImageJ-win64	⊘	9/14/2021 11:05 AM	Application	147 KB
README.md	⊘	9/14/2021 11:05 AM	MD File	6 KB
WELCOME.md	⊘	9/14/2021 11:05 AM	MD File	6 KB

The current version of Pore Extractor 2D should be installed from its ImageJ update site. This update site also automatically installs the Pore Analyzer tool dependencies BoneJ (Doube et al., 2010) and BioVoxxel Toolbox (Brocher, 2015).

The user will be prompted to close and restart ImageJ several times during this installation process.

1. Open the ImageJ application and navigate to **Help** → **Update.** If the ImageJ installation is new, several default updates may install, the user will be prompted to **Apply Changes**, close ImageJ, re-open ImageJ, and call Help → Update again.
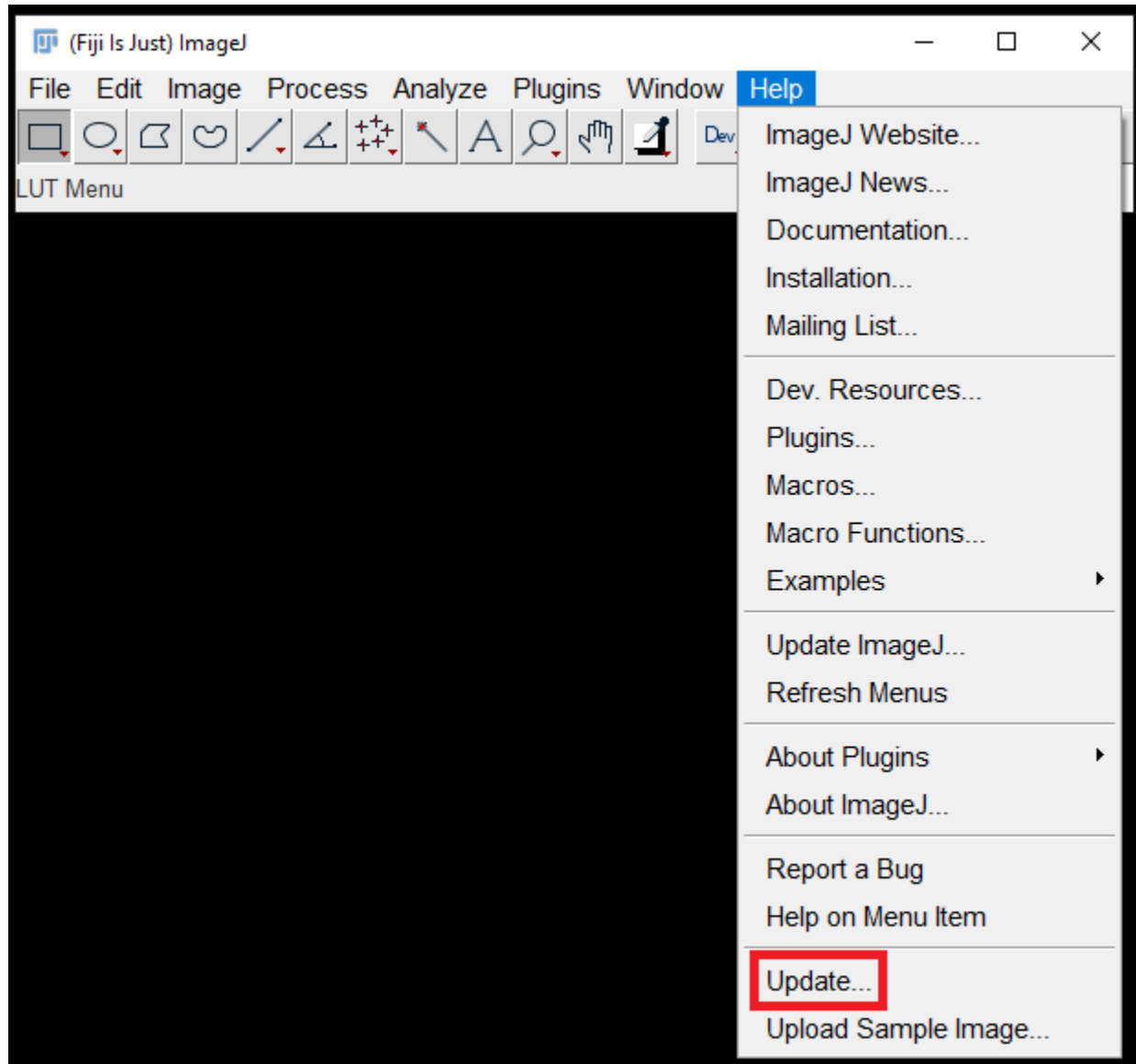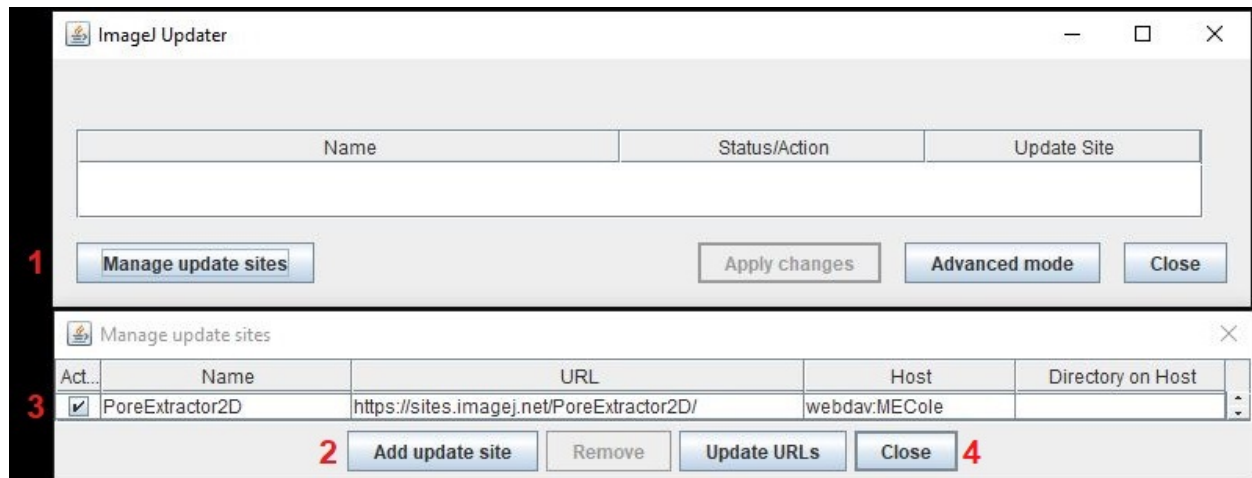
2. Select **Manage update sites,** then **Add update site**, and input the following information:
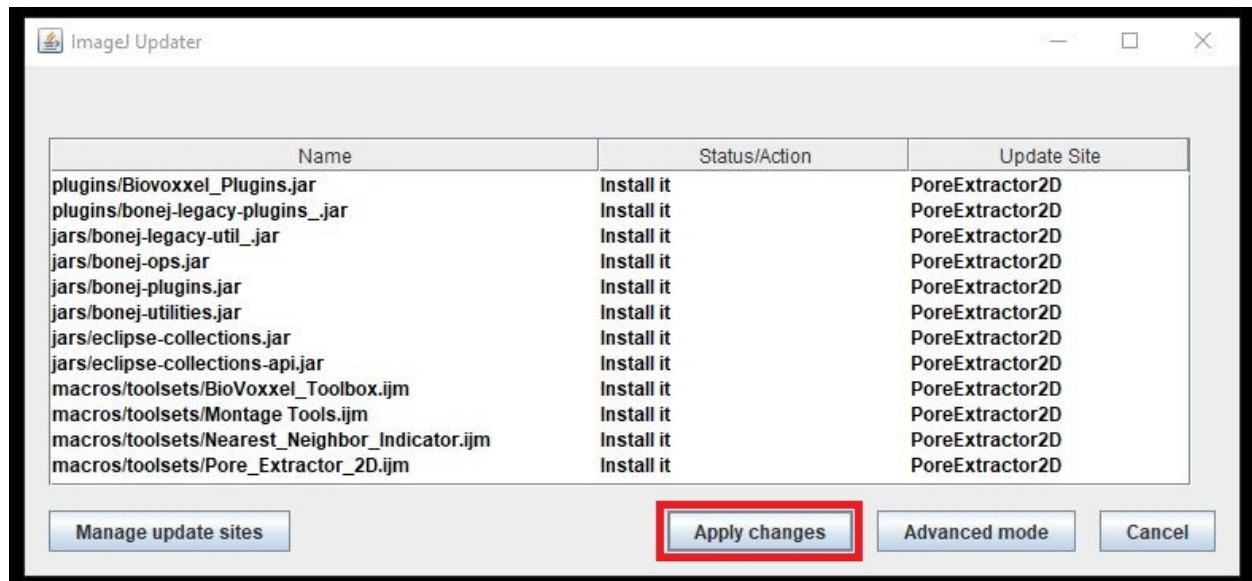
Name: PoreExtractor2D
URL: https://sites.imagej.net/PoreExtractor2D/
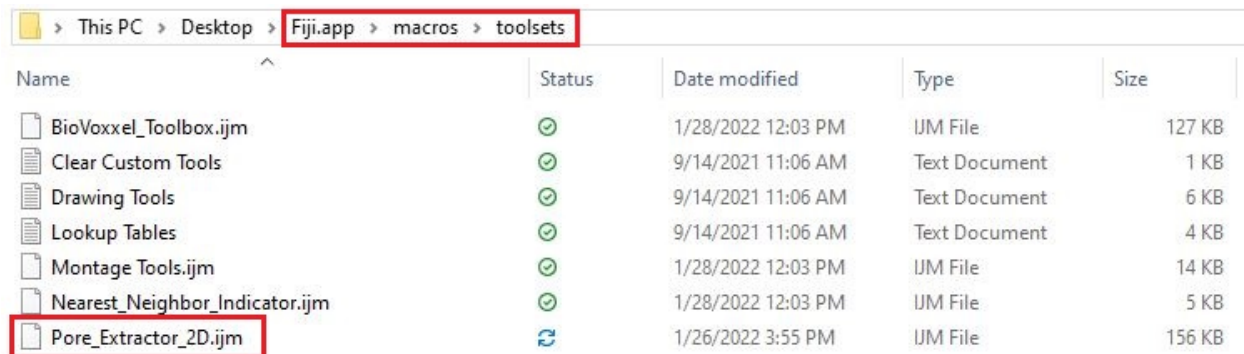**Host**: webdav:MECole

Then select **Close** to be returned to the **Manage update sites** window.

3. Pore Extractor 2D dependencies will populate in the **Manage update sites** window. Note that only "Pore_Extractor_2D.ijm" will populate if the user previously installed BoneJ and Biovoxxel dependencies. Select **Apply changes**. The user will be prompted to restart ImageJ.

After download, the "Pore_Extractor_2D.ijm" file will appear in the Fiji.app subfolder **macros →
toolsets.**

If the user encounters difficulties downloading from the update site, the
"Pore_Extractor_2D.ijm" file attached with this publication's supplement can be manually
copied into the toolset subfolder. In this case, the user would also need to install BoneJ and
BioVoxxel Toolbox individually from the ImageJ Update menu.

4. Navigate to **Help → Update ImageJ** and update to the latest version (**1.53g or later**).
    ImageJ will close automatically after this update and will need to be restarted.
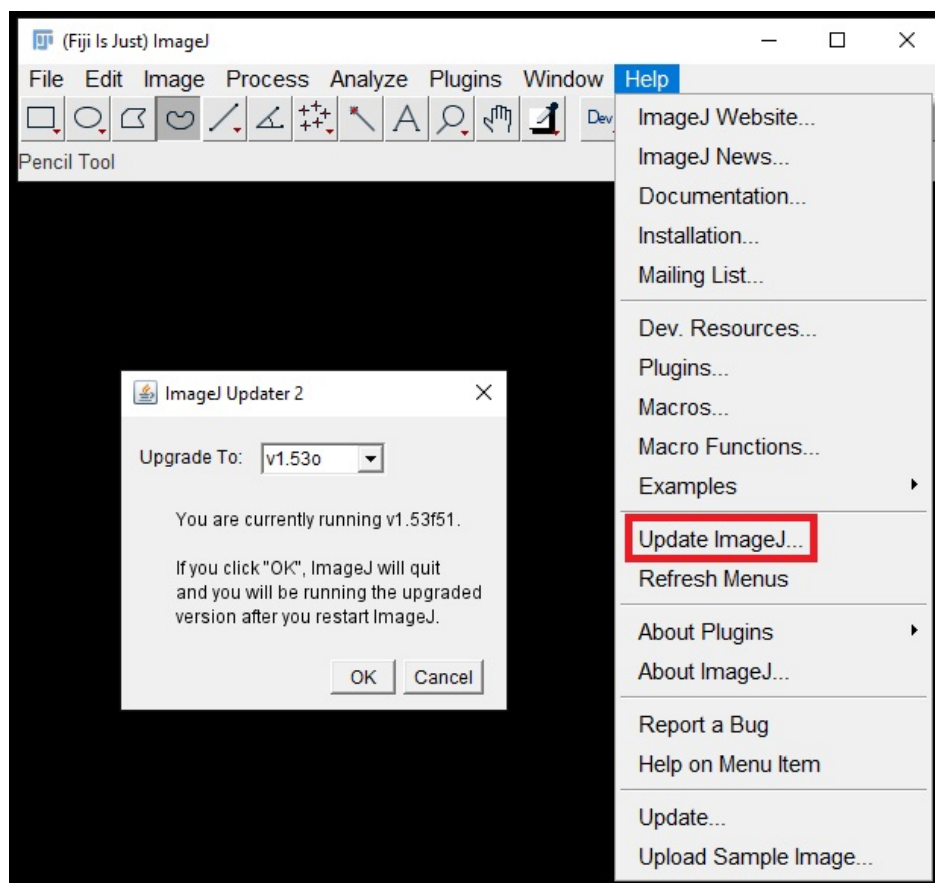
Pore Extractor 2D Session Startup Instructions

Pore Extractor 2D must be installed each time a new session of ImageJ is opened.

1. Select "Pore_Extractor_2D" from the "More Tools" (**>>**) dropdown menu, and the toolkit icon will appear in the ImageJ toolbar.
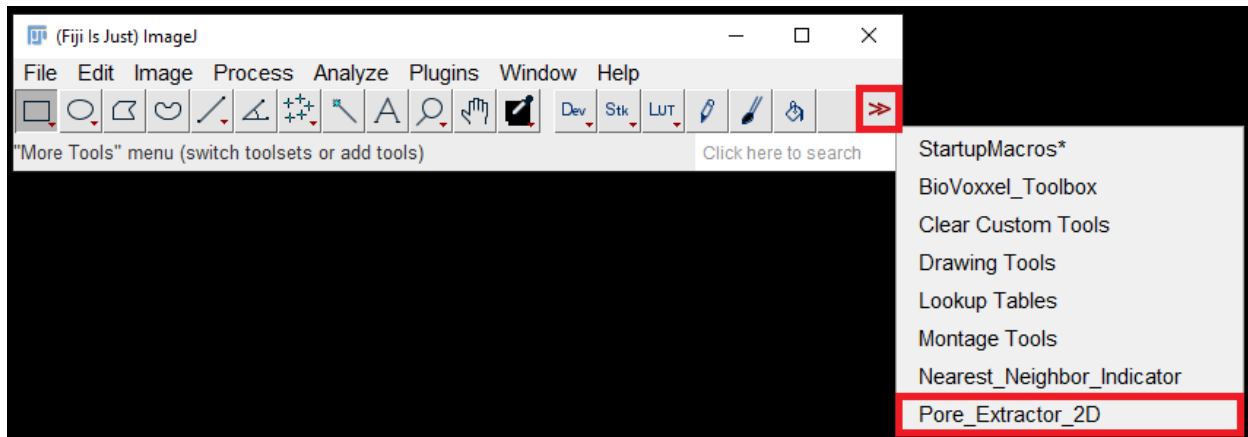
2. Click the Pore Extractor 2D toolkit icon to select a macro from its dropdown menu.

Pore Extractor 2D Recommended Starter Settings

Pore Extractor 2D requires user-selected settings for *Image Pre-Processing* and *Pore Extractor* macros. The following settings are recommended as a starting point for this optimization. Users are encouraged to test and modify these settings to optimize cortical pore identification for their own samples.
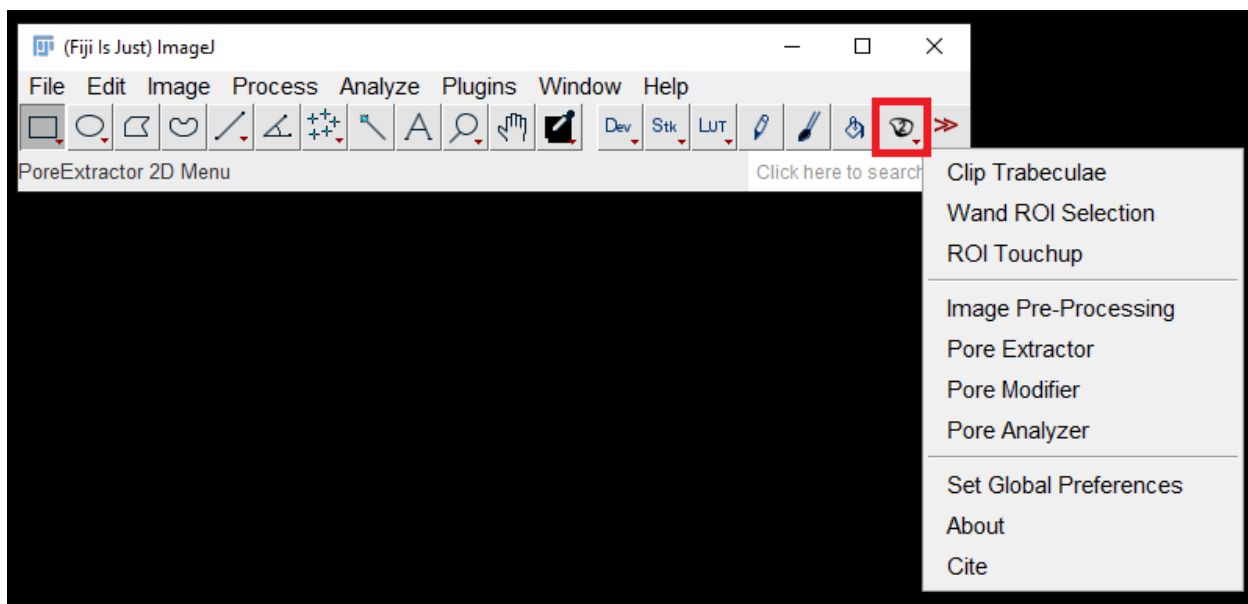
Image Pre-Processing Recommended Settings

1. **Enhance Global Contrast** (Normalize) – 0.3% Saturation (default)
2. **Subtract Background** (Rolling Ball) – 50.0 px (default)
3. **Gaussian Blur** – 2.00 px (default)
    a. Recommended for samples with 100x or greater magnification, as 40x magnification samples already have some inherent blurring of pore borders
    b. Also recommended for "noisy" images with surface debris, tissue discoloration or bleaching, or bubbling and partitioning inside large pore spaces
4. **Save Image Channels** (All)
    a. Recommended for stained samples. Select the grayscale color channel that minimizes the intensity of tissue staining for further processing in *Pore Extractor*
    b. If resulting grayscale images are faint, run Image Pre-Processing without the Subtract Background option.

Pore Extractor Recommended Workflow

Expect Proximity, Not Perfection

Due to the color heterogeneity of histological bone tissue, *Pore Extractor* will not perfectly select all pore spaces. You will always need to run *Pore Modifier* to remove some incorrectly selected tissue, and to add or alter some pore selections.

Choose *Pore Extractor* settings to select **small-to-medium** sized pores, which are the majority of cortical pores in a typical section. These pores tend to have a uniformly colored lumen and complete border.

- Very small pores tend to be removed by the smoothing cycle(s) and minimum pore size filters necessary to exclude tissue noise.
- Large "trabecularized" pores near the endosteum tend to be excluded by the circularity filter necessary to exclude tissue noise, either because they have incomplete borders that cannot be filled, they are sub-segmented by internal bubbling or debris, or they are irregularly shaped.
- It is quicker to add these pores back in *Pore Modifier* than it is to delete a large amount of selected tissue noise. Aim to minimize tissue noise over selecting every pore.

For a good-quality histological section, you should aim to select about **60 - 75%** of all pores with *Pore Extractor*.

Thresholding Recommended Workflow:

1. Follow the load dialog prompts.
   a. Load the cleared and (optionally) preprocessed brightfield image
   b. Set the image scale in pixels/mm

2. The *Thresholding* Method popup dialog will appear. Select Try *All*, which will cycle through each thresholding option in turn.
   a. Keep the default Auto Local Phansalkar radius of 15 pixels.
   b. Click OK.

3. *Pore Lumen Extraction* will prompt user interaction.
   a. Move the **top** slider to the **right** in the popup Threshold window, until light pore **spaces** are selected in **red**.
   b. Minimize speckles of red inside the bone tissue (outside of the pores). Clusters of speckles should not exceed the size of pore spaces. A scattered red "television static" appearance inside the tissue is normal.
   c. If you have moved the top slider all the way to the right (255) and large clusters of red persist inside the tissue, change the dialog option to "Do Not Use Pore Lumen Threshold." This is common if you are using a grayscale image.
   d. **Do not click Apply on the Threshold popup!** Instead, click **OK** in *the Pore Lumen Extraction* popup dialog to continue.

4. *Pore Border Extraction* will prompt user interaction.
   a. Move the **bottom** slider to the **left** in the popup Threshold window, until dark pore **borders** are selected in **red**.
   b. Minimize speckles of red inside the bone tissue (outside of the pores). Clusters of speckles should not exceed the size of pore spaces. A scattered red "television static" appearance inside the tissue is normal.
   c. If you have moved the bottom slider all the way to the left (0) and large clusters of red persist inside the tissue, change the dialog option to "Do Not Use Pore Border Threshold." This is common if the tissue has dark discoloration.
   d. **Do not click Apply on the Threshold popup!** Instead, click **OK** in *the Pore Border Extraction* popup dialog to continue.

5. *Auto Local Thresholding* will run automatically.
   a. All image windows will disappear while this function runs in the background. This may take several minutes, depending on image size and computer processing power. A pop-up progress dialog will alert you that the function is still running.

6. All thresholding outputs will appear in tiled windows. Select your choice from the drop-down menu, then click OK.
    a. Manual Pore Lumens and/or Manual Pore Borders will not appear if you selected the option to not use that threshold
    b. For better visibility, you can use the *Zoom* tool (keyboard shortcut [4]) to right-click and zoom in, and the *Scrolling* tool (keyboard shortcut [5] or hold [Space]) to grab and drag the image while zoomed.
    c. Choose the thresholding output that maximizes **filled, isolated pores** while minimizing large clumps of white inside the bone tissue
    d. For tissue with minimal debris or discoloration, *Auto_Local_Phansalkar* or *All_Thresholds_Combined* is typically the best option
    e. For tissue with more surface debris or discoloration, one of the *Manual* outputs is typically the best option.

Morphological Modification Recommended Workflow:

Following thresholding, the *Morphological Modification* popup dialog will appear. You will also see displayed 1) the brightfield cross-section, titled with its image name and 2) the thresholded pore image, titled as *Current_Pore_Modification*.

This is a repeating modification loop that you can return to until you are satisfied with final pore selections. You can select multiple modifications to run at once, by checking multiple boxes. However, it is recommended to test out a single modification, view the results, and accept or reject that change, before testing and adding the next modification.

Before or during modification, you can visualize pore selection from the current image modification by selecting "Preview Selected Workflow Option(s)" and clicking OK.

If, at any point, you want to discard morphological change(s) you have already accepted, select "Revert to Original Thresholded Image" and click OK.

A. Close and Fill Pore Borders

Try the following steps if many pores appear as incomplete, unfilled "rings." **If most small to medium sized pores are already filled in, skip to *B: Smooth Pore Borders.***

1. In the *Morphological Modification* popup dialog, check the box for "Close and Fill Pores (1 pixel)".
    a. Do not check any other boxes.
    b. Select the preview option "Preview Selected Workflow Option(s)."
    c. Click OK and wait for the modification to run.

2. The output of your Close and Fill Pores modification will appear as *Preview_Pores.*
    a. The *Pore Filter Settings* popup dialog will appear.

3. **Did large regions of non-pore tissue merge into white patches?** Reject Close and Fill, then proceed to smoothing.
    a. In the *Pore Filter Settings* popup dialog, select "Skip Pore Display" and click OK.
    b. The *Accept Morphological Changes* popup dialog will appear. Select "Discard Change" and click OK.
    c. You will be returned to the *Morphological Modification* popup dialog.
    d. The *Current_Pore_Modification* image will revert to its previous state.
    e. Continue to ***B: Smooth Pore Borders.***

4. **Are there still many pores that appear as incomplete, unfilled "rings"?** Reject Close and Fill and re-run with a higher pixel value.
    a. In the *Pore Filter Settings* popup dialog, select "Skip Pore Display" and click OK.
    b. The *Accept Morphological Changes* popup dialog will appear. Select "Discard Change" and click OK.
    c. You will be returned to the *Morphological Modification* popup dialog.
    d. The *Current_Pore_Modification* image will revert to its previous state.
    e. Repeat ***A. Close and Fill Pore Borders*** with the "Close and Fill Pores" setting increased to 2 pixels.

5. **Are pores suitably filled?** Accept Close and Fill, then proceed to smoothing.
    a. In the *Pore Filter Settings* popup dialog, select "Skip Pore Display" and click OK.
    b. The *Accept Morphological Changes* popup will appear. Select "Accept Change" and click OK.
    c. You will be returned to the *Morphological Modification* popup dialog.
    d. The *Current_Pore_Modification* image will now match the closed and filled *Preview_Pores* image.
    e. Continue to ***B: Smooth Pore Borders.***

B. Smooth Pore Borders

In almost all cases, smoothing is required to reduce the pixelated noise of pore borders. Smoothing can also cut off extraneous connections to tissue caused by thresholding or Close and Fill operations. This operation will remove pore border projections that are a smaller diameter than the selected smoothing value (typically 2 – 10 pixels).

1. In the *Morphological Modification* popup dialog, check the box for "Smooth Pore Borders." Do not check any other boxes.
    a. If you did **not** accept a Close and Fill operation: Enter 5 pixels.
    b. If you **did** accept a Close and Fill operation: Enter 7 pixels.
    c. Select the preview option "Preview Selected Workflow Option(s)."
    d. Click OK and wait for the modification to run.

2. The output of your Close and Fill Pores modification will appear as *Preview_Pores.*
   a. The *Pore Filter Settings* popup dialog will appear.

3. In the *Pore Filter Settings* popup dialog, keep all default settings and click OK.
   a. The *Confirm Pore Selection* popup dialog will appear.
   b. Pores that pass the default minimum size (> 300 μm^2) and circularity (> 0.3) thresholds will display as cyan-colored overlays on the brightfield image and on the modified *Preview_Pores* image.

4. **Is it difficult to see the pore selections on the brightfield image?** Change the selection color to better contrast with your brightfield image.
   a. In the *Confirm Pore Selection* popup dialog, select "No, Adjust Pore Filter or Color" and click OK.
   b. The *Pore Filter Settings* popup dialog will appear.
   c. Under "Change Pore Color," select a color that contrasts with your brightfield image. Red or green are good choices for an unstained bone section. Cyan or green is a good contrasting color for a section stained pink with basic fuschin.
   d. You can also switch the pore selection type from "Outline" to "Filled." A filled selection is more visible than an outline, but it can also obscure the true boundaries of the pore.
   e. Click OK to apply these changes to the pore color and selection type.
   f. For better visibility, you can use the *Zoom* tool (keyboard shortcut [4]) to right-click and zoom in, and the *Scrolling* tool (keyboard shortcut [5] or hold [Space]) to grab and drag the image while zoomed.
   g. Repeat this step until you can see the pore selections on the brightfield image. Then proceed to the next questions.

5. **Do pore borders still appear too pixelated, jagged, or connected to surrounding tissue?** Increase the smoothing threshold.
   a. In the *Confirm Pore Selection* popup dialog, select "No, Adjust Morphometry" and click OK.
   b. The *Accept Morphological Changes* popup dialog will appear. Select "Discard Change" and click OK.
   c. You will be returned to the *Morphological Modification* popup dialog. The "Smooth Pore Borders" pixel value will be set to the cycle you just tested.
   d. The *Current_Pore_Modification* image will revert to its previous state.
   e. Repeat **B. Smooth Pore Borders** with the "Smooth Pore Borders" value **increased** by one or more pixels.

6. **Do many regions of bone tissue appear as small, round blobs that are approximately the same size as the pores?** Decrease the smoothing threshold.
    a. In the *Confirm Pore Selection* popup dialog, select "No, Adjust Morphometry" and click OK.
    b. The *Accept Morphological Changes* popup dialog will appear. Select "Discard Change" and click OK.
    c. You will be returned to the *Morphological Modification* popup dialog. The "Smooth Pore Borders" pixel value will be set to the cycle you just tested.
    d. The *Current_Pore_Modification* image will revert to its previous state.
    e. Repeat ***B. Smooth Pore Borders*** with the "Smooth Pore Borders" value **decreased** by one or more pixels.

7. **Are pore borders suitably smoothed, but A) too much tissue is also selected or B) too many small pores are excluded?** Modify pore size and/or circularity thresholds.
    a. In the *Confirm Pore Selection* popup dialog, select "No, Adjust Pore Filter or Color" and click OK.
    b. The *Pore Filter Settings* popup dialog will appear.
    c. **Increase** pore size and/or circularity (range: 0 to 1) to exclude more tissue
    d. Or **Decrease** pore size and/or circularity (range: 0 to 1) to include more pores.
    e. You will likely need to repeat this cycle multiple times to fine-tune your selection.
    f. Once you are satisfied, in the *Confirm Pore Selection* popup dialog, select "Yes, Save ROIs and Exit Macro" and click OK. The macro will export the final pore ROI set.