# *Acto3D Instruction*

# Compatible image file formats

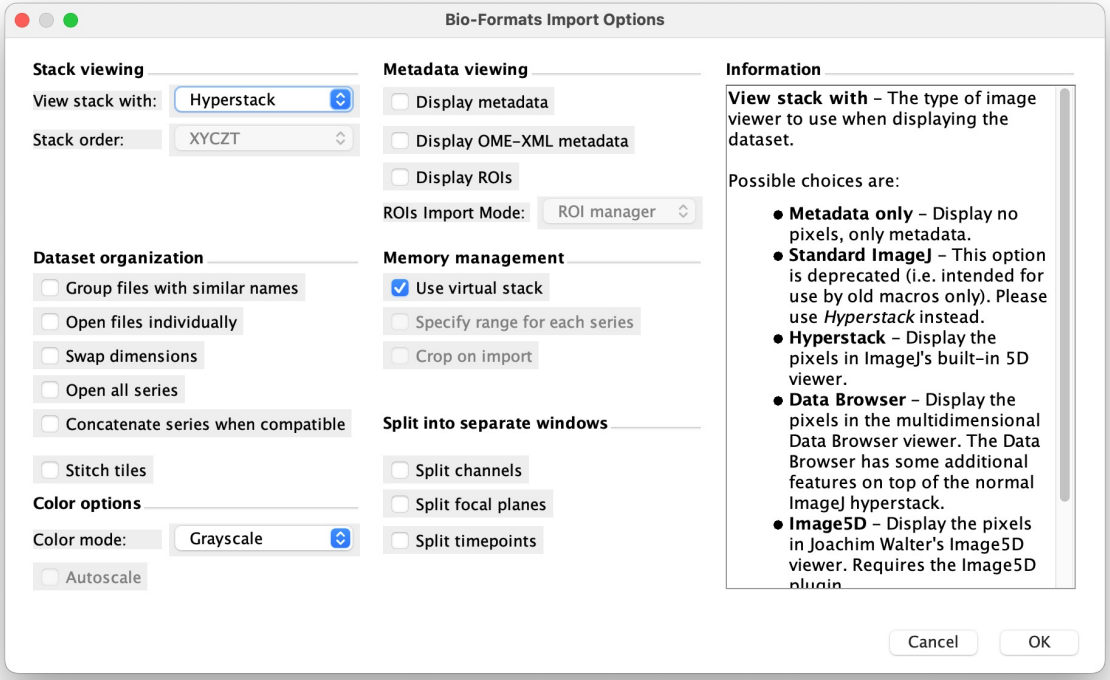
| | 16 bits / channel | 8 bits / channel |
|---|---|---|
| Multipage TIFF converted by Fiji | 1 - 4 channels | 1 - 4 channels |
| TIFF stacks | 16 bits Grayscale image | 32 bits RGBA images (*)<br>24 bits RGB images<br>8 bits Gray scale images |
| PNG stacks | | 32 bits RGBA images (*)<br>24 bits RGB images<br>8 bits Gray scale images |
| JPG stacks | | 32 bits RGBA images (*)<br>24 bits RGB images<br>8 bits Gray scale images |

(*): Normally, the "A" in a 32-bit image refers to the opacity channel, but in Acto3D, the "A" can be used as the data for the fourth channel by storing the data of each fluorescent channel in R, G, B, and A.

**We strongly recommend using FIJI to convert from the microscope manufacturer format to multipage TIFF.**
Please follow the instructions on the next page.

# Convert images to TIFF format

Firstly, you need to convert the manufacture's format to TIFF using Fiji.

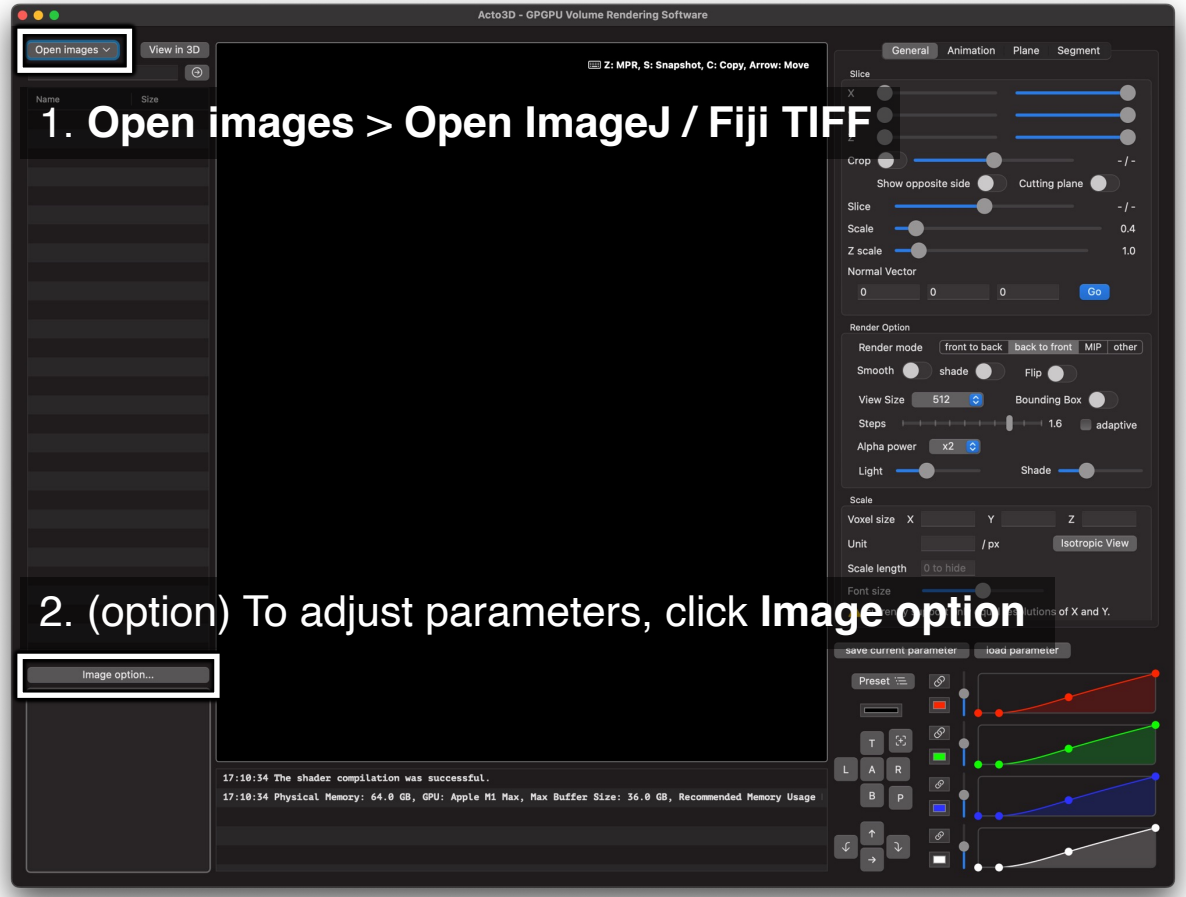



Fiji
(version 2.3.0 / 1.53s)

Set "Color mode" to Grayscale, and turn "Split channels" OFF.
Adjust "Use virtual stack" as needed depending on your memory capacity.

Click **File** > **Save As** > **Tiff...**.

# Load images in Acto3D




```
Physical Memory: 64.0 GB, GPU: Apple M1 Max, Max Buffer Size: 36.0 GB,
```

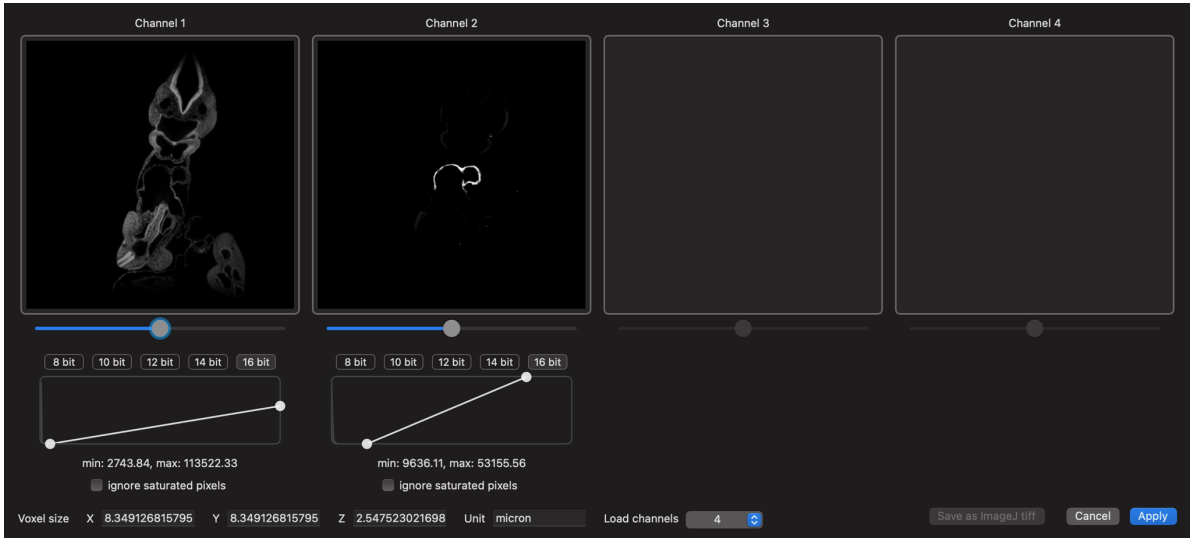
The **Max Buffer Size** represents the maximum amount of memory that can be allocated on the GPU at one time. Images must be accommodated within this capacity.

To calculate the memory required for 3D visualization of the image, use the following formula:

Max Buffer Size = [width] × [height] × [depth] × [channel] / (1024^3)

Note: This formula is applicable irrespective of whether the image is stored in 8-bit or 16-bit.

# Adjust parameters



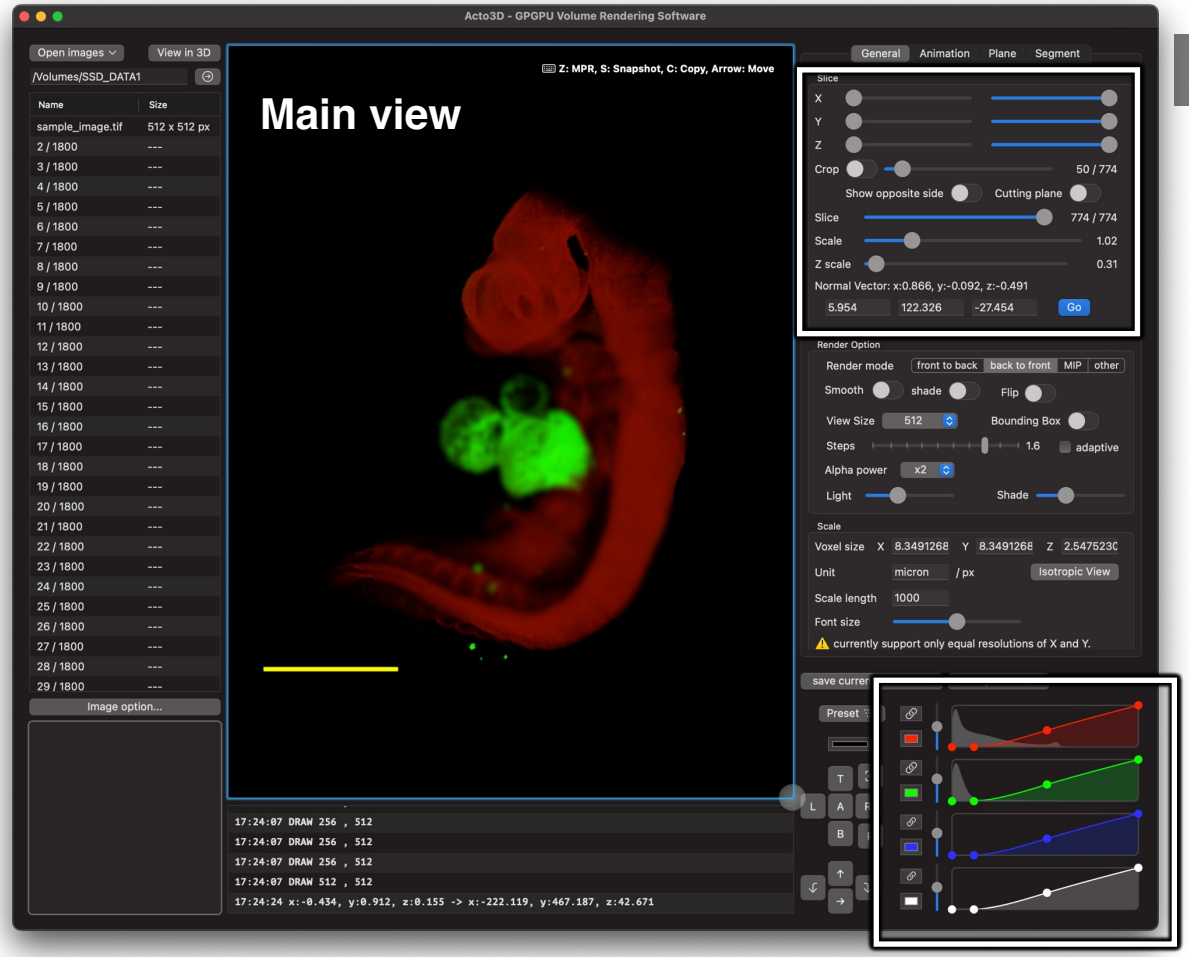

- Adjust display ranges (if adjustments have been made by Fiji, Acto3D will utilize those values)

- Configuring the Voxel Size
  - When loading the microscope manufacturer's format in Fiji, it is automatically configured within the TIFF metadata. In case it’s not present, you will need to ascertain it based on the imaging parameters.

- Selecting the Number of Channels
  - If sufficient memory is available, select 4. If memory conservation is necessary, select 1 for a single-channel image, 2 for a two-channel image, and 4 for three or four-channel images .

- Ignore saturated pixels
  - If there's a potent non-specific signal and you wish to exclude regions with excessively high pixel values, you can set the corresponding pixel areas to 0 by turning this ON.

# Screen Layout

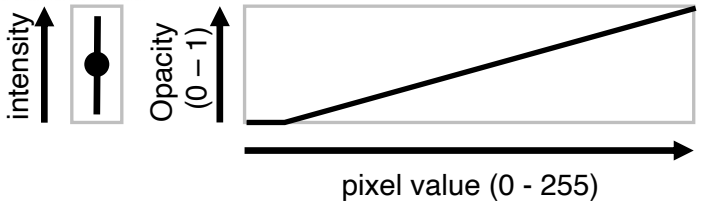



## Slice setting

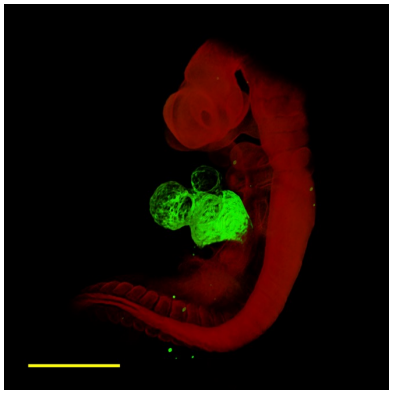
Set the Z scale by dividing the Z voxel size by the XY voxel size. For instance, if XY equals 2 $\mu$m/pixel and Z equals 5$\mu$m/pixel, set it at 2.5. If this has already been configured on page 4, the accurate value will be automatically applied.

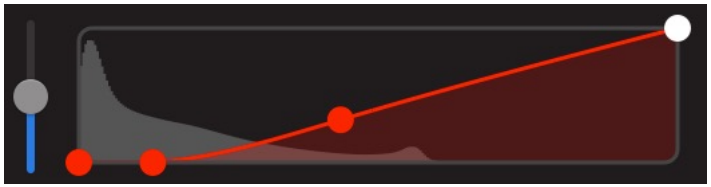
## Color tone and transfer function

In this section, you define the opacity corresponding to each pixel value. Clicking enables you to freely increase the control points. Right-clicking on a control point offers the option to remove it. Right-clicking elsewhere will display additional options.



# Adjust transfer function (adjust opacity) (1 / 2)



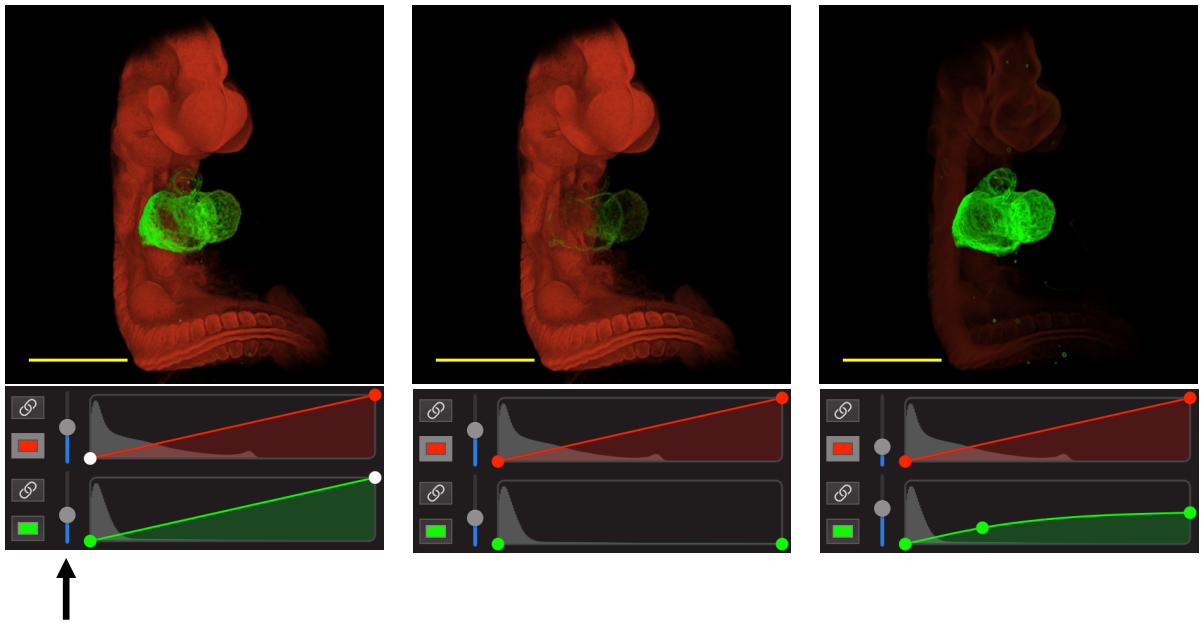


This configuration renders portions with low pixel values transparent, proving effective in scenarios where noise appears significantly dimmer than the original signal, such as non-specific signals.

This adjustment renders areas with pixel values nearing zero as transparent, yet maintains visibility of regions with faint brightness. Portions with existing pixel values turn opaque, enhancing the overall object's observability.

This configuration enables regions with low pixel values to become transparent. By assigning a specific opacity to areas of pronounced brightness, which are regarded as signals, it facilitates the visualization of the interior extending beyond the surface.

# Adjust transfer function (adjust opacity) (2 / 2)

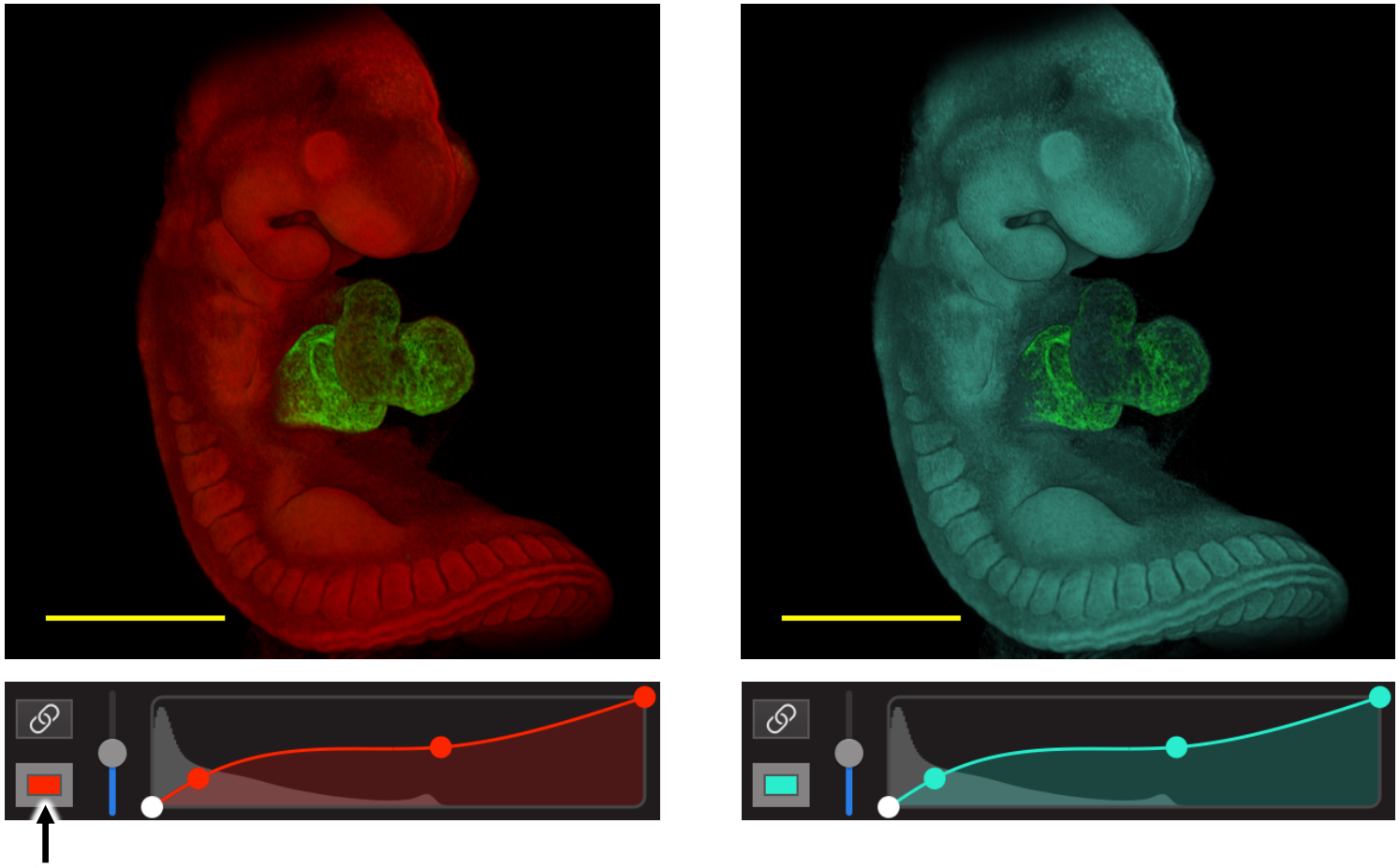


The slider to adjust the pixel intensity for the channel.

Use these settings to make fine adjustments to create your ideal image.

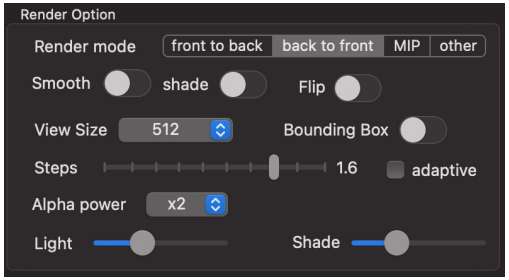
While it's feasible to designate opacity for each channel, by default, the opacity at a specific sampling point is configured to the maximum value among the four channels. This is to avoid unnatural visualizations, like semi-transparent structures in the background appearing when there are opaque objects in the foreground. If you wish to increase the transparency of a particular channel, you can fine-tune its intensity using the slider.

# Change color



You can specify the color tone for each channel.

# Rendering options (1 / 2)




**Render mode** **Press ‘Z’ key** at any time to generate **MPR** images

Choose the algorithm to employ for volume rendering. The default choice is 'back to front', yet 'front to back' necessitates fewer calculations and typically generates a comparable image. MIP (Maximum Intensity Projection) is a method that adopts the pixel with the highest brightness along the line of sight. In an image using MIP, the depth becomes indiscernible.

**Smooth** This configuration utilizes linear interpolation to ensure smooth sampling along the line of sight.

**Shade** When sampling along the line of sight, this option takes into account the gradient with surrounding pixels.

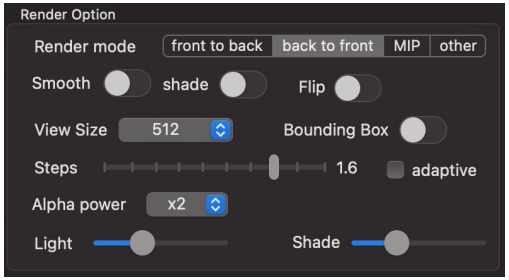
**Flip** This option enables you to toggle between arranging the image stack from back to front or front to back, effectively offering the capability to horizontally flip the image.

**View size** Choose the size of the image to generate.
Irrespective of this value, the original image employed for rendering remains unchanged in size.

**Bounding box** Display the outer frame of the 3D volume.

**Steps** Determine the interval for sampling along the line of sight. Use 1.0 to sample at the same interval as 1 pixel. However, since this can significantly increase computational demands, the default is set to 1.6, with some degree of thinning. Note that when the number of images is small, or the resolution of the image is low, the image might exhibit jagged edges; setting the interval below 1.0 can mitigate this roughness. Please note that in volume rendering, the final image alters depending on the number of times the same transparency level is overlaid.

# Rendering options (2 / 2)



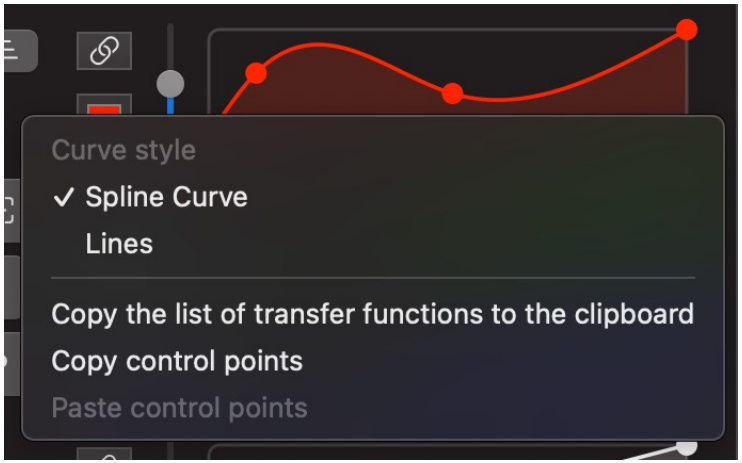

**Adaptive** This setting is associated with the view size and steps mentioned above. When OFF, a 3D image is created from the original image and finally adjusted to the view size. When ON, the image size is adjusted (typically reduced) to match the view size first, and the 3D image is constructed based on that modified image. Both methods maintain the isotopic display, but in many cases, enabling the ON option significantly reduces the number of calculations required.

**Alpha power** In volume rendering, many images are overlaid. Without setting the transparency to a very low value, only the surface is usually visible. Consequently, the alpha value specified in the graph is squared by default (falling within the range of 0.0 to 1.0). If the transparency becomes excessively pronounced, you can bypass this adjustment by selecting an x1 setting. Conversely, if increased transparency is desired, you can elevate it to x3 for a cubed effect.

**Light** Adjust the overall brightness.

**Shade** If shade is turned on, it increases the effect of the gradient with surrounding pixels.

# Check transfer function

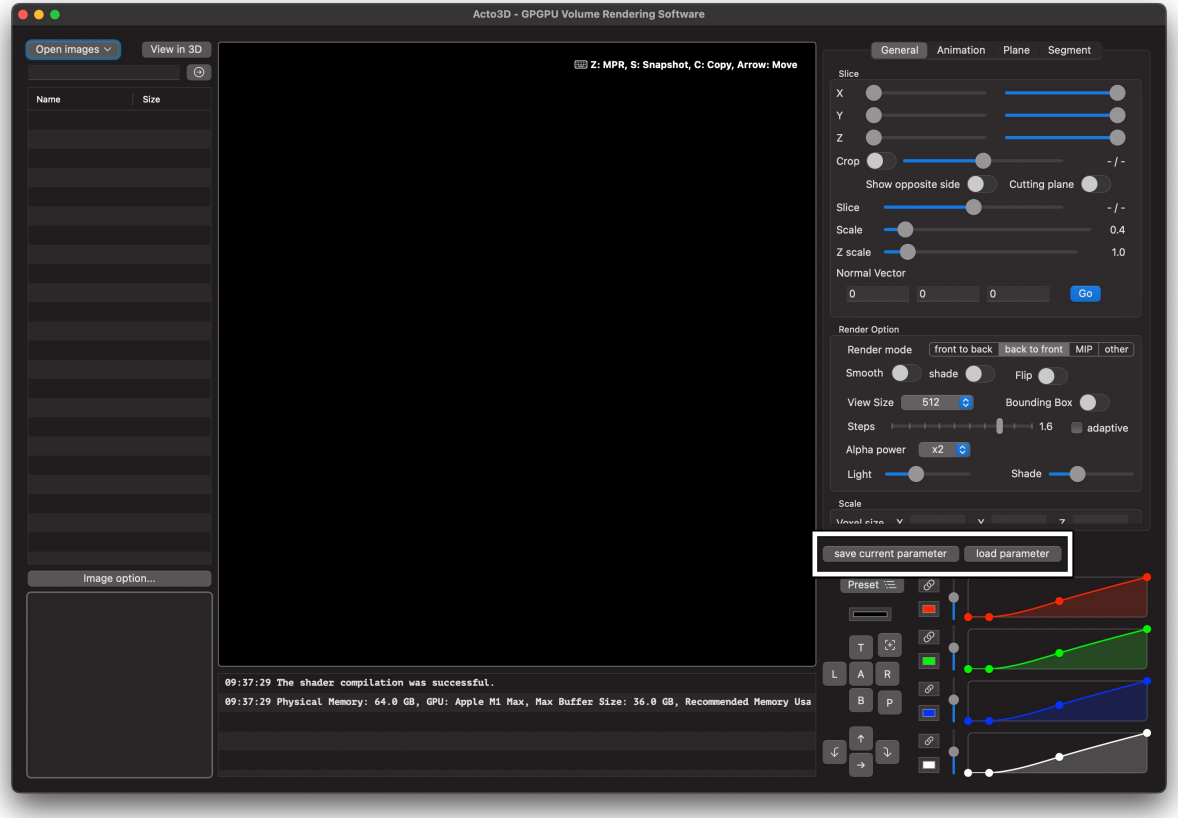



To obtain the the transfer function:
Right click in the graph area
(in places other than control points)

| | A | B |
|---|---|---|
| 1 | Pixel value | Opacity |
| 2 | 0 | 0 |
| 3 | 0.1 | 0.00193923 |
| 4 | 0.2 | 0.00387845 |
| 5 | 0.3 | 0.00581763 |
| 6 | 0.4 | 0.00775676 |
| 7 | 0.5 | 0.00969583 |
| 8 | 0.6 | 0.01163481 |
| 9 | 0.7 | 0.01357369 |
| 10 | 0.8 | 0.01551245 |
| 11 | 0.9 | 0.01745107 |
| 12 | 1 | 0.01938955 |
| 13 | 1.1 | 0.02132785 |
| 14 | 1.2 | 0.02326598 |
| 15 | 1.3 | 0.02520389 |

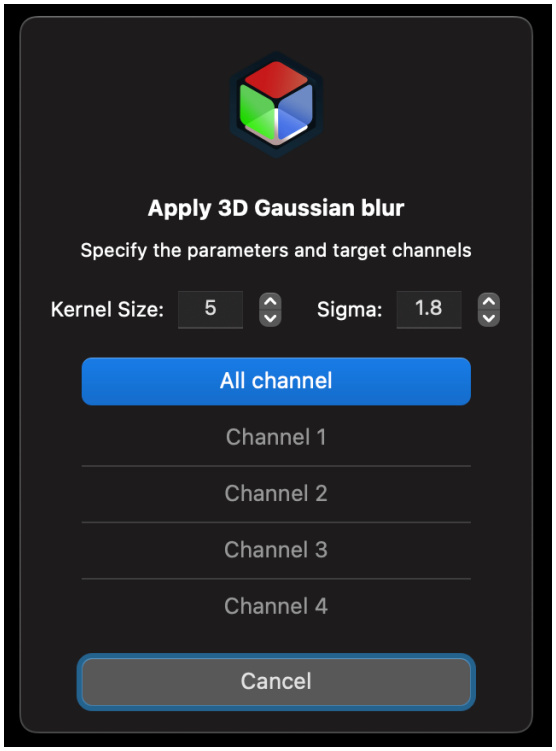
You can paste these values in an Excel file.

# Save and load parameters




Using these buttons, you can save and load the parameters.

# Apply filter (Gaussian 3D filter)




In menu bar, click: **ImageProcess** > **Gaussian 3D**

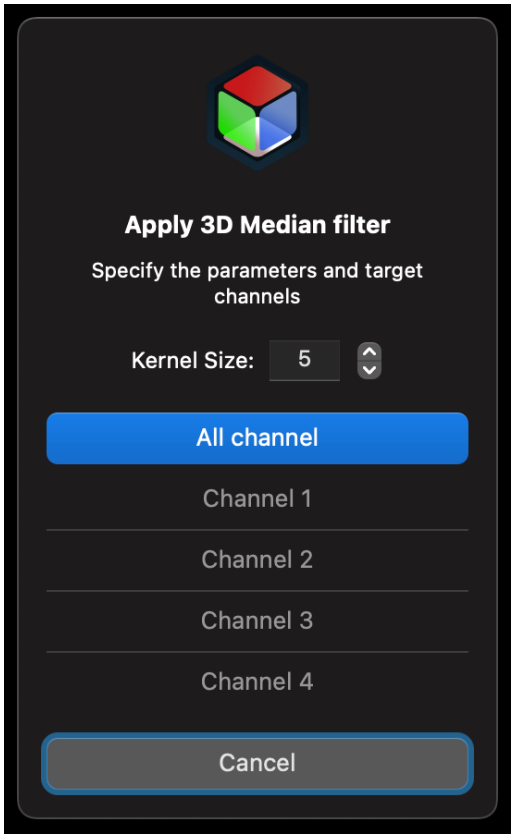
Determine the kernel size, sigma and target channels.
The kernel size **must be an odd integer**.

This process requires additional memory allocation.
When 'All' is selected, the size of the temporary buffer requires 'width x height x depth x 4' bytes.
If a specific channel is selected, the size reduces to 'width x height x depth' bytes.

If there is insufficient memory available, the filter should be applied to each channel individually.

As the kernel size increases, the time required for the process also increases.

# Apply filter (Median 3D filter)




In menu bar, click: **ImageProcess** > **Median 3D**

Determine the kernel size and target channels.
The kernel size **must be an odd integer**.

The median filter is time-consuming to process.

This process requires additional memory allocation.
When 'All' is selected, the size of the temporary buffer rewuires 'width x height x depth x 4' bytes.
If a specific channel is selected, the size reduces to 'width x height x depth' bytes.

If there is insufficient memory available, the filter should be applied to each channel individually.

As the kernel size increases,
the time required for the process also significantly increases.

# Segmentation

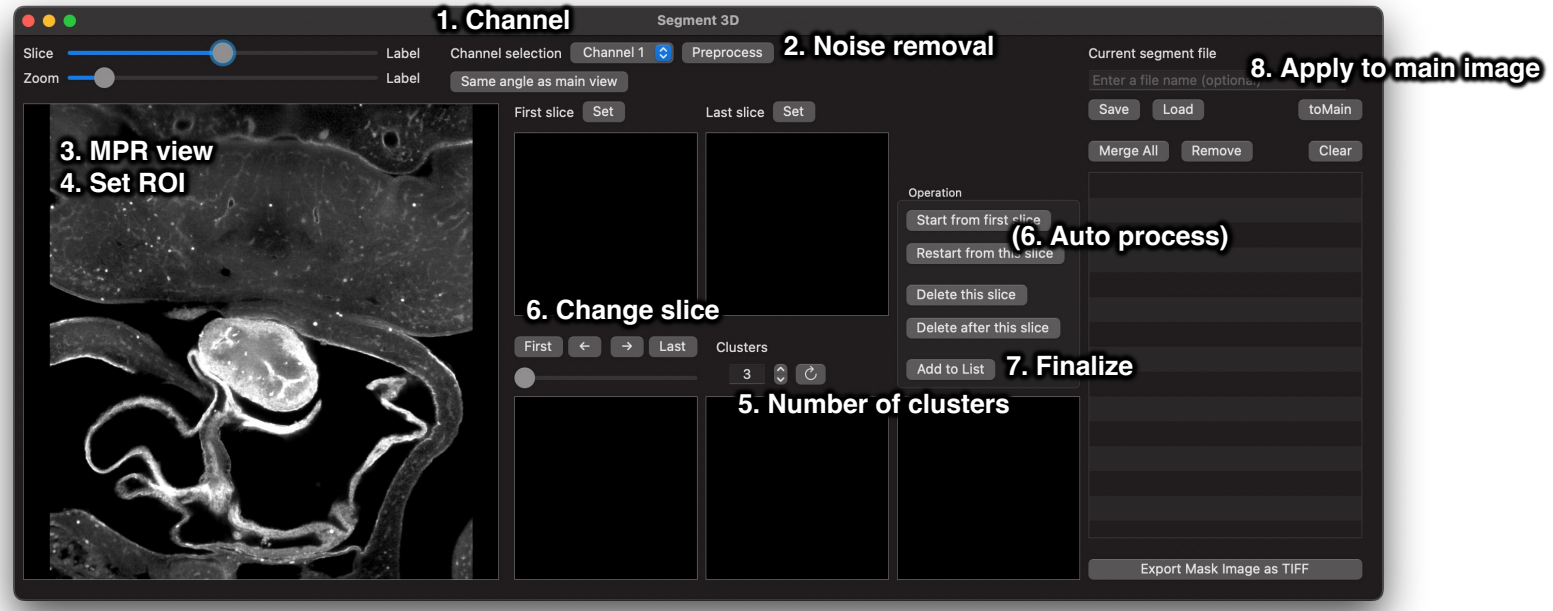
The application operates based on the “iterative” k-means approach as described in the paper.
For details on operation, please refer to Supplementary Video 3.

Load the Image, first
**Navigate to the Segment tab** and select **'3D segment**’.

1. **Channel Selection**: Choose the desired channel you wish to work on.

2. **Noise Removal**:
   Click on the 'Preprocess' button to apply a 3D gaussian blur with
   kernel size 7x7x7 and sigma 1.8. If you wish to utilize different parameters,
   either perform the aforementioned process or use external applications like Fiji for noise removal.

3. **Manipulating MPR View**:
   The MPR view can be adjusted using the mouse. If the orientation in MPR is difficult,
   adjust to the desired angle in the main view and click '**Same angle as main view**' to match the rotation.



# Segmentation

4. **Selecting the Region of Interest**:
   Press and **hold the Command (⌘) button** and use the mouse to highlight the desired region.
   Use the slider to adjust the region, then **set the first** and **last slices**.

5. **Clustering**:
   Set the number of clusters (default is 3). Click the **'Update'** button to execute clustering.
   If the clustering doesn't appear accurate, either repeat the update or adjust the number of clusters.
   Once satisfactory clustering is achieved, click on the desired region within the cluster image.
   This will display a mask image on the right side.

6. **Change slice / Auto-process**:
   Use the ← and → buttons to manually process each slice or click the 'Auto-process' button.
   While in auto-processing mode, if a substantial change is identified in the generated mask image, a confirmation dialog will appear. If everything is satisfactory, press 'Restart' to resume from the previous slice.
   If you intend to adjust the number of clusters partway through, you can delete the clustering for subsequent slices and redo the process

7. **Finalizing the Segmentation**:
   Once satisfied, click 'Add to List'.
   Due to the complex tubular structure of the pharyngeal arch artery, adjust the view angle to get a perpendicular cross-section. Repeat these steps for each segment from various viewpoints.
   Click **'Merge All'** on the right to combine all the segmentation data and generate a mask image.
   This enables a 3D observation of the segmented structure when applied to the main texture.

8. **Apply to Main texture**:
   Click this button to choose the channel of the main texture where you wish to save the mask image. As demonstrated on page 4, an image comprises four channels. This process entails substituting one of these channels with the mask image you've generated. Should you already possess image data for all four channels, you must replace one of them. Furthermore, on page 4, if you opt to utilize just 1 or 2 channels, you are limited to those particular channels.

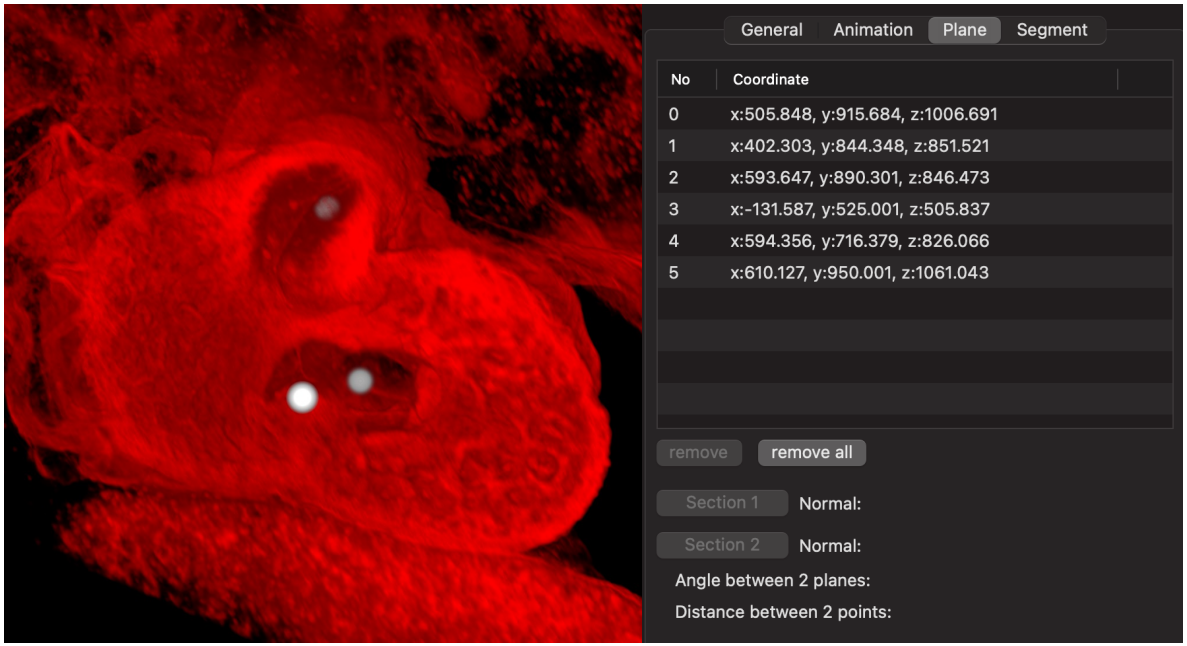
# Animation

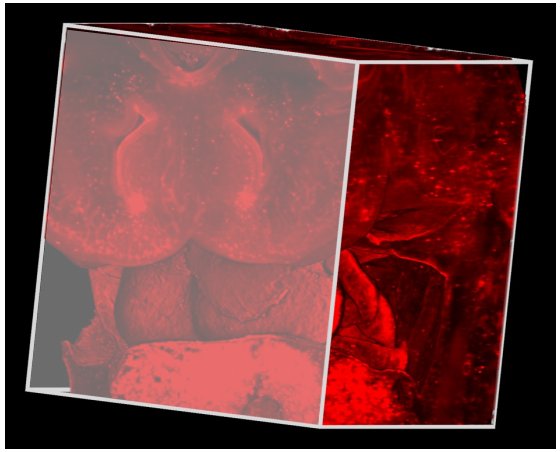

You can create videos in the Animation tab.
To create an animation, save the parameters at the beginning and the end.

By setting the parameters for both the beginning and the end, you can smoothly transition through most of the settings available in Acto3D, such as movement, rotation, and color adjustments.

The video size will match the pixel width specified in the Movie size.

# Point plotting



Even if the object is transparent and you can see through it, it still exists as image data. Therefore, if you don't click on the appropriate slice, the surface will be plotted.

By **left-clicking** within the 3D image, you can plot a specified coordinate.
However, if you click where the entire image is displayed, only the surface of the cube (not the 3D object) will be plotted.

Thus, it's **necessary to adjust to the appropriate slice before clicking**.
Tip: By pressing the Z key and switching to MPR view before clicking, you can plot at the correct location.

You can verify these in the Plane tab, where you can make cuts across planes passing through multiple plots, or calculate distances between points and angles between planes.

# Custom shader

In menu bar, click: **Shader** > **Open Shader Directory**

Place the **.metal** files written in Metal Shading Language (MSL) in this directory.
You can also create subdirectories if needed.
The **.metal** files located in this directory will be compiled
when Acto3D is launched or when the Re-compile button is pressed.

Caution: If there's an error in any of the .metal files,
**Acto3D will disable all custom shaders** and will only use the built-in default shaders.

Please follow the rules provided below.

- The first three lines should be placed at the beginning of the shader file.
  These are necessary for Acto3D to recognize the shaders and display them in the menu.

- To avoid internal errors, please refrain from using special characters.

- The kernel function name must be unique and not duplicate any other shader files.

Sample.metal

```
// Label: built-in Front To Back
// Author: Naoki Takeshita
// Description: Standard front to back rendering

kernel void preset_FTB(device RenderingArguments    &args       [[buffer(0)]],
                       uint2                        position    [[thread_position_in_grid]]){

}
```

These lines are necessary to identify the shader file.

# Custom shader

Sample.metal

```
// Label: built-in Front To Back
// Author: Naoki Takeshita
// Description: Standard front to back rendering

kernel void preset_FTB(device RenderingArguments    &args       [[buffer(0)]],
                       uint2                        position    [[thread_position_in_grid]]){
```

**Geometrical calculation**

**Codes for MPR (Macro)**

```
    for loop{
```

**Bounding Box**

**Crop and Cutting plane**

**Sampling and Compositing process**
**Apply transfer function**
**Apply shade**

```
    }
}
```

**For detailed code and behavior, please refer to the comments provided in the shader file.**