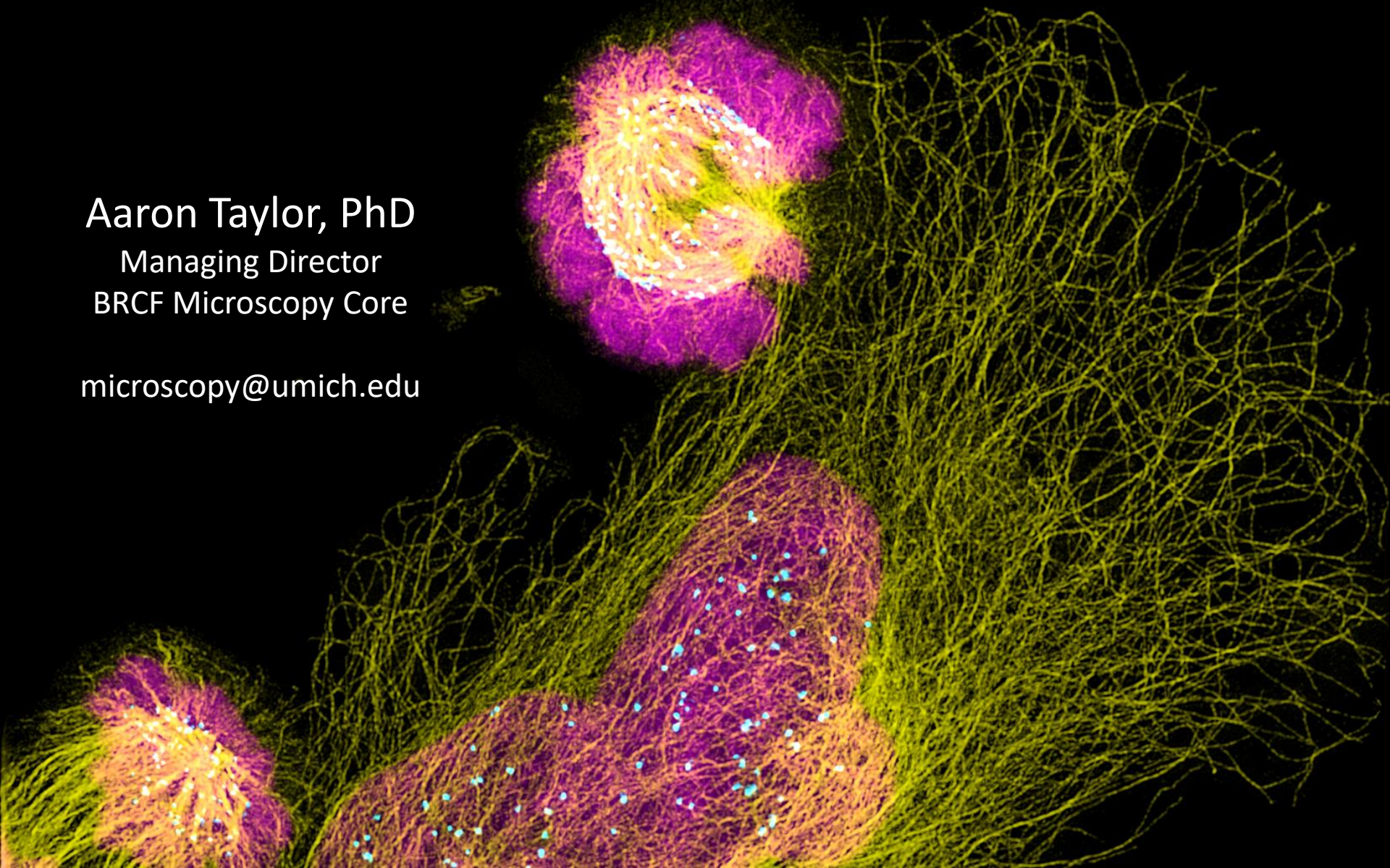
Quick Start Guide to using FIJI and FluoRender for Visualizing Fluorescence Images



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Purpose:

This workshop aims to introduce you to how **FIJI** and **FluoRender** are used to visualize and render fluorescence images. The emphasis is practical - "how to" not "why."

Other workshops will emphasize theory or computation:

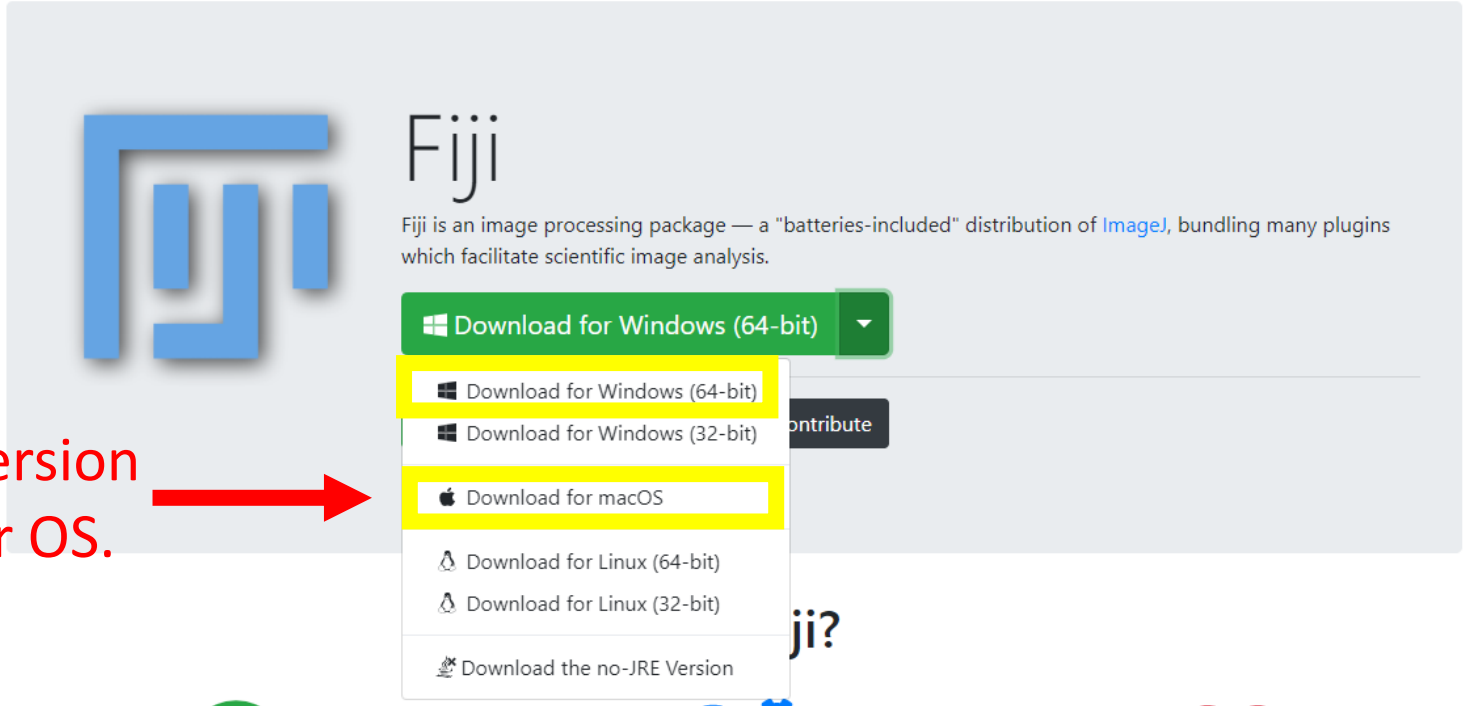
- Key Concepts in Image Processing and Analysis
- Extending FIJI with Plugins and Automation Macros
- Advanced Quantitative Methods in Image Processing

See the Learning and Outreach section of our website for more information:
https://brcf.medicine.umich.edu/cores/microscopy/outreach/

Installing FIJI

Goto: https://fiji.sc/

Select version for your OS.



If your computer is HITS 'Core Imaged', must install FIJI to the Desktop folder

Installing FluoRender

Goto: https://github.com/SCIInstitute/fluorender/releases
Scroll down to find proper executable for your OS…

Latest **Manual and Tutorials** are also available here.

* If you are having problems with installation, contact HITS *

What is ImageJ/FIJI?

ImageJ is an open source and extensible image processing application written in Java in 1997 (runs on any OS).

http://imagej.nih.gov/ij/

ImageJ2 is a re-write of ImageJ according to modern programming conventions released in 2010. The typical user won't see any differences.

FIJI Is Just ImageJ2 with many plugins pre-installed. It is also automatically updated with new plugins when they are available.

https://fiji.sc/

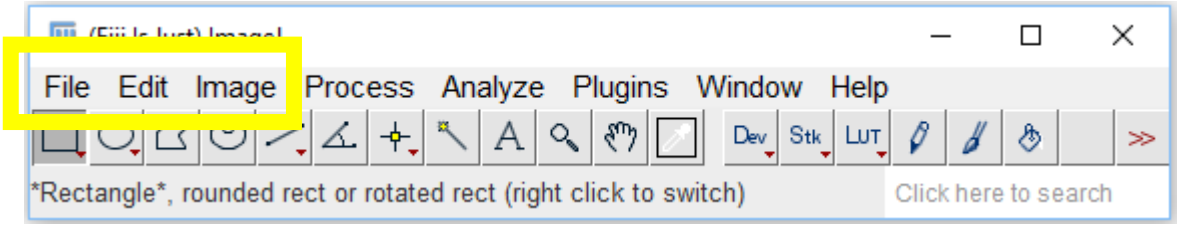
See: Schindelin et al. Fiji: an open-source platform for biological-image analysis. Nature Methods. 2012.

What is ImageJ/FIJI?

FIJI looks small, but it is very powerful *once you know how to use it*.

FIJI is also FREE!

Today, we will focus on the most common and useful commands in the first three menu options.



FIJI does **much more** than what we will talk about today.
Please be curious and explore further on your own!

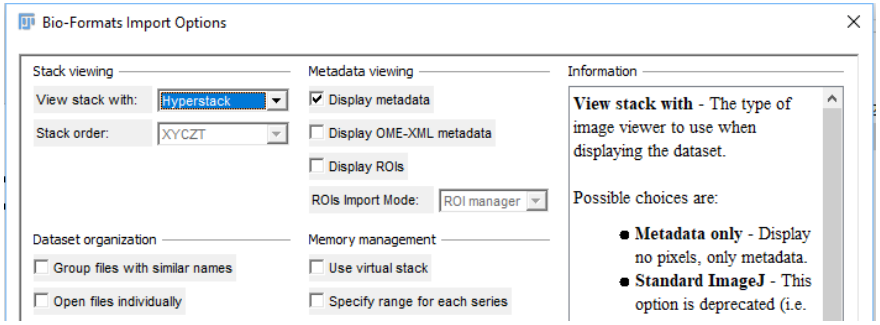
Display of 2D Images

Several Ways to Open Files in FIJI…

1. Drag and Drop the file onto the FIJI menu bar.

   TRY IT WITH Tissue.tif

2. For some proprietary file types, you might first see an options window…



TRY IT WITH Neuron_Vesicles.nd2

   There are useful options here for advanced users, but today we will just press OK.

3. If the image is comprised of many indexed files, use the File -> Import -> Image Sequence command. Use file filtering options as needed.

   TRY IT WITH images in folder Neuron_Slices

Common Image File Types

File types are just conventions for how to organize pixel data and metadata (other information such as acquisition parameters). A given software may be designed to only understand certain file types, but thankfully FIJI will open almost anything!

A few (of many) image file types that FIJI will open:

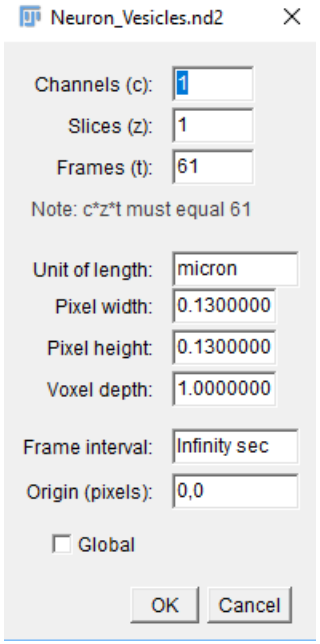
File Types	Purpose
TIF	"Tag Image Format". Very flexible and understood by many softwares
LIF	Leica format; stores meta data so users can 'reuse' settings
CZI	Zeiss format; stores meta data so users can 'reuse' settings
ND2	Nikon format; stores meta data so users can 'reuse' settings
AVI	A (often compressed) movie format
JPG	A compression format for natural images. Generally not for science images.

Getting Information About a File

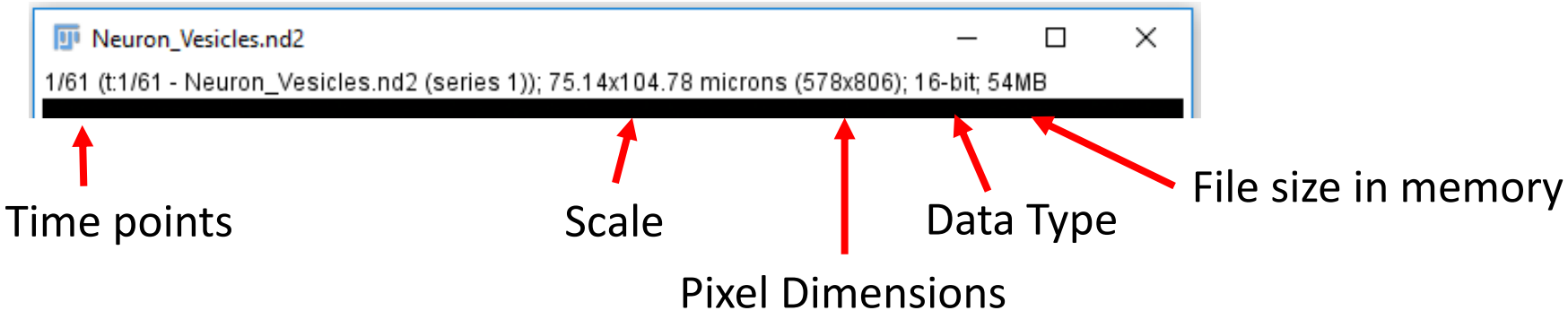
Besides the pixels, images contain other descriptive information called ‘meta-data’.

TRY IT WITH Neuron_Vesicles.nd2

To display this information, use Image -> Properties… and Image -> Show Info

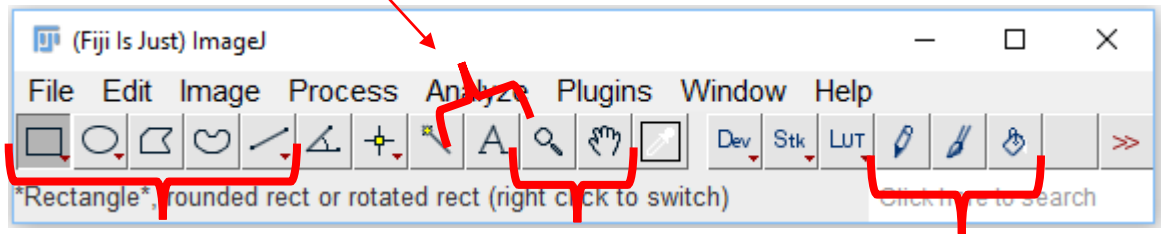


Some of this info is also displayed under the image’s title bar:



Handy Tools…

Drawing text on the image



Zooming/Panning the image

Drawing shapes on the image

Free drawing on the image

Right-click on the buttons to get more options.

TRY IT:

To get a new image: File -> New -> Image (Select Type = RGB)

Use: Edit -> Fill and Edit -> Draw to paint the shapes and text

Image Data Types

A data type is a low-level description of how image pixel data is stored (not how it is organized, which is the file type). Most file types can contain many different data types. Conversely, a given data type can be contained in many different file types.

Data types are important because they limit how an image can/should be manipulated and displayed.

Common Scientific Image Data Types:

- Channel based: 8-bit, 16-bit, or 32-bit per channel
- Color based: 24-bit RGB, HSB, LAB, Indexed Color

Since this workshop is about visualization of fluorescence images, we will care mainly about 8-bit, multi-channel images. (Since a computer monitor can display only about 8-bits per channel).

Channel-based vs RGB Color Data Types

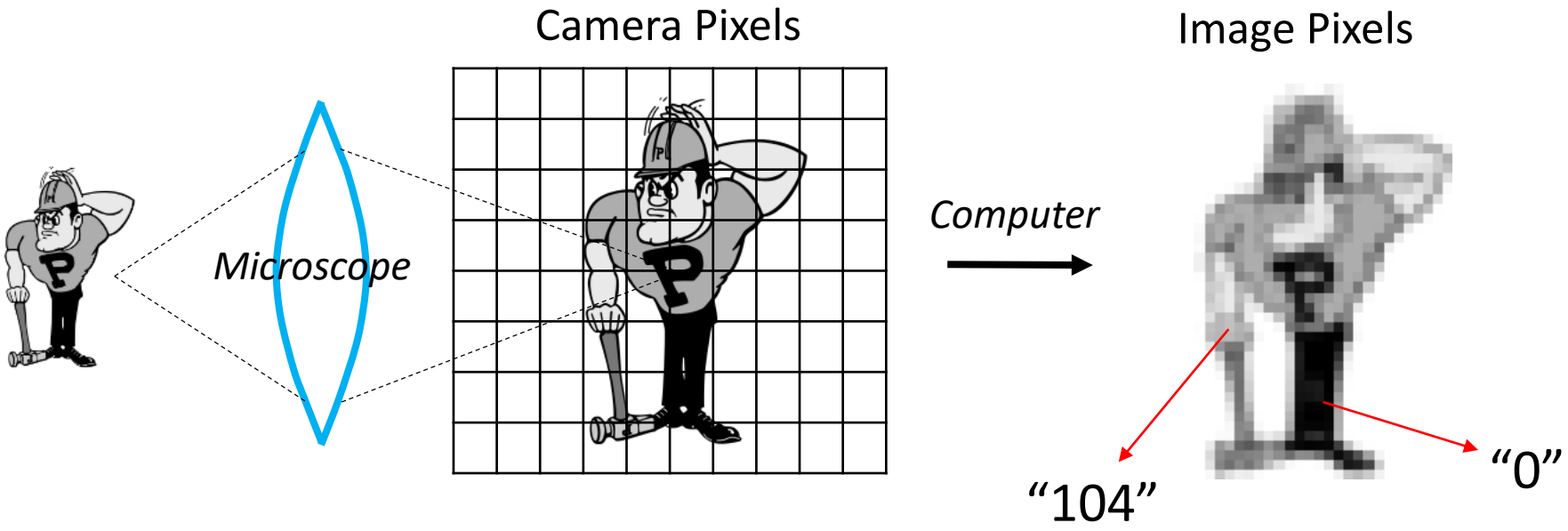
A multi-channel image and RGB color image are COMPLETELY DIFFERENT! They store data differently and should never be used interchangeably.

	Multi-Channel Image	**RGB Color Image**
Purpose:	Storing collections of monochrome images	Storage of a natural color (white light) image.
Channels:	Any number [c1],[c2],[c3],… Channels are independent and can be view separately.	Exactly 3 [c1,c2,c3]. Channels are dependent and always viewed together.
Bit depth:	Any	8-bit only (“24-bit color”)
Pseudo color LUT Support:	Yes	No, the data IS the color
Issues:	Can only be opened using dedicated image processing software	Opens with any image viewer since RGB Color is used by most consumer electronics

Caution: Fluorescence images should NEVER be stored as RGB Color since the original channel information will most likely be lost. (Only exception is if you are making an image purely for visual display in a figure).

Monochrome Images

A monochrome image is a matrix of numbers where the number at each location in the matrix is proportional to the amount of light present at a corresponding location in the sample.



The value of each pixel is encoded as a whole number ("intensity") .

Bit-Depth Describes Number of Intensity Levels

Though we typically count in whole numbers (0,1,2,3…9), computers represent numbers in base-2 (binary) symbols called bits (0,1). Bits are organized into combinatorial blocks of 8. Thus, bit-depth describes the range of intensity levels that can stored at each pixel.

8 bits can store (2^8) 256 intensity levels, from 00000000 -> 11111111

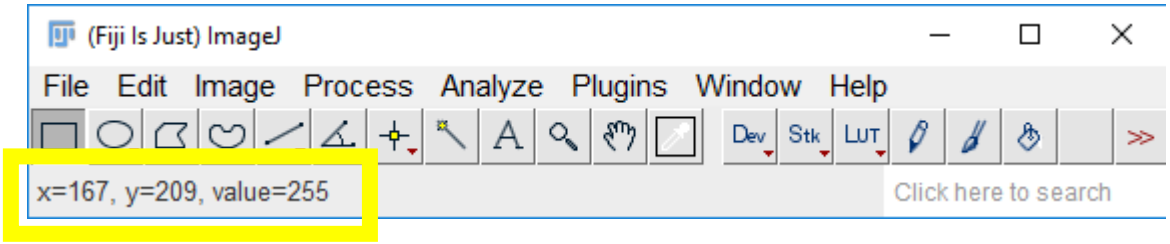
16 bits can store (2^16) 65,536 intensity levels, from 0000000000000000 -> 1111111111111111

Whole Number	Binary Number	Data Type
0	00000000	8-bit
107	1101011	8-bit
255	1111111	8-bit
10,345	10100001101001	16-bit
65,534	1111111111111110	16-bit

Working with Monochrome Images

OPEN Neuron_Vesicles.nd2

Hover over the image and look at the Tool Bar's information panel to see the pixel intensity at each location.



Use: Analyze -> Histogram to get the distribution of pixel intensities for the entire image.

Displaying Monochrome Images

How brightly each pixel's intensity value is displayed on the computer screen is determined by a 'look up table' (LUT). A lookup table is a table or equation that maps pixel intensities onto screen brightness values.

Table:

Pixel Intensity	Screen Brightness
0	0
32	32
64	64
96	96
128	128
160	160
192	192
224	224
255	255

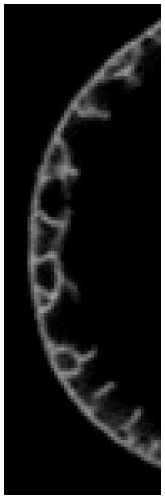
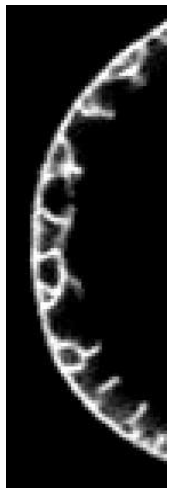


Table:

Pixel Intensity	Screen Brightness
0	0
32	64
64	128
96	192
128	255
160	255
192	255
224	255
255	255



Display saturated.
DO NOT DO THIS

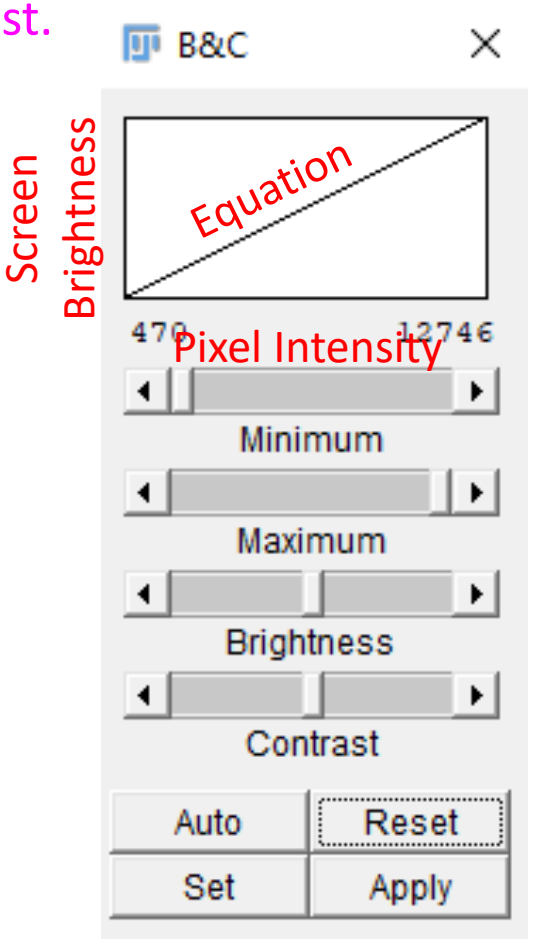
Corresponding Equation: $V = 1*D + 0$ $V = 2*D + 0$

D = (m)*S + b, where m (slope) represents 'contrast' and b (intercept) represents 'brightness'.

Displaying Monochrome Images

OPEN Neuron_Vesicles.nd2

Adjust the LUT using Image -> Adjust -> Brightness/Contrast.



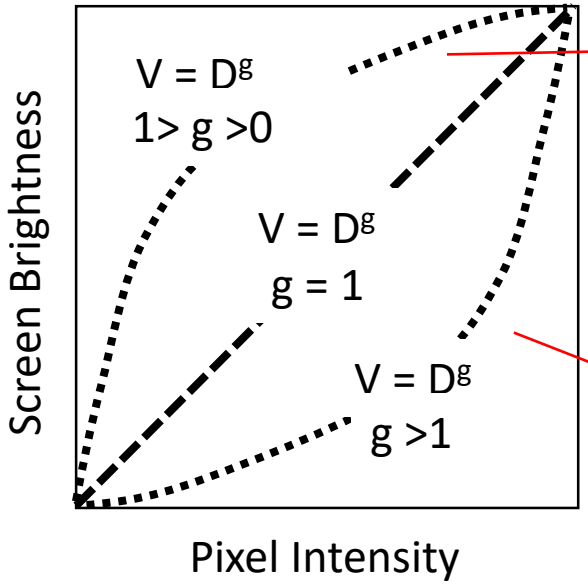
The LUT is illustrated as a line graph, where x-axis is pixel intensity and y-axis is monitor brightness.

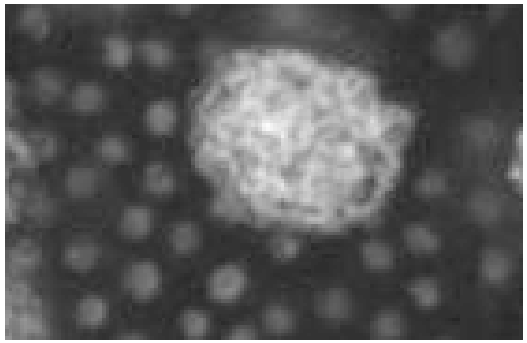
- The Min and Max sliders set the range of pixel intensity values that will be displayed.
- Auto will guess a good range.
- Set allow you to type in exact values.
- Apply applies the LUT to the image.

  Caution: "Apply" changes the raw intensities to the current screen brightness values. Do NOT do this unless you are simply making a figure for visual inspection.

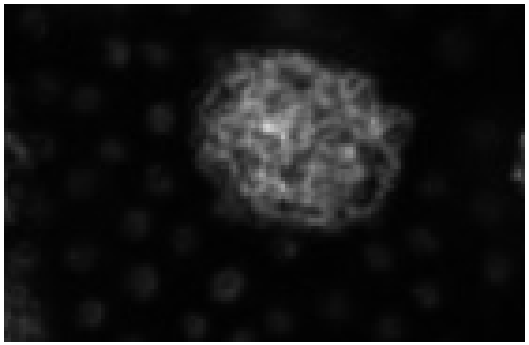
Displaying Monochrome Images

Nonlinear LUTs can be used to specify the pixel intensity / screen brightness relationship (called ‘’). For engineering reasons, a power law relationship is common and the adjustable parameter is an exponent called ‘gamma’.





Gamma < 1 increases relative brightness of low intensity pixels.



Gamma > 1 reduces relative brightness of low intensity pixels.

Caution: Using gamma means the image as displayed no longer accurately represents the relation between the intensities in the raw image.

TRY IT WITH Neuron_Vesicles.nd2

Displaying Monochrome Images

Color LUTs can be used that assign a color to each pixel intensity in the image.

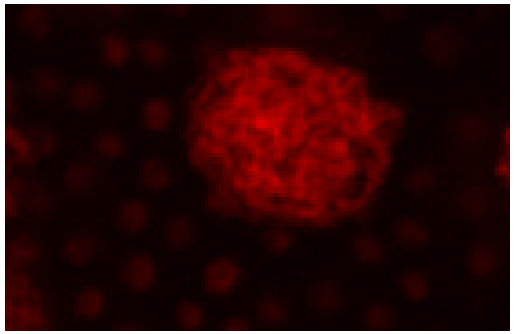
Caution: Our eyes don't see red or blue very well. As possible, use colors from the middle of the spectrum that are closer to green.

TRY IT WITH Neuron_Vesicles.nd2

To apply a color LUT: Image -> Lookup Tables
To see the LUT: Image -> Color -> Show LUT
To create a custom LUT: Image -> Color -> Edit LUT

Red Color LUT:

Pixel Intensity	Screen Red	Screen Green	Screen Blue
0	0	0	0
32	32	0	0
64	64	0	0
96	96	0	0
128	128	0	0
160	160	0	0
192	192	0	0
224	224	0	0
255	255	0	0



Working with Multi-Channel Images

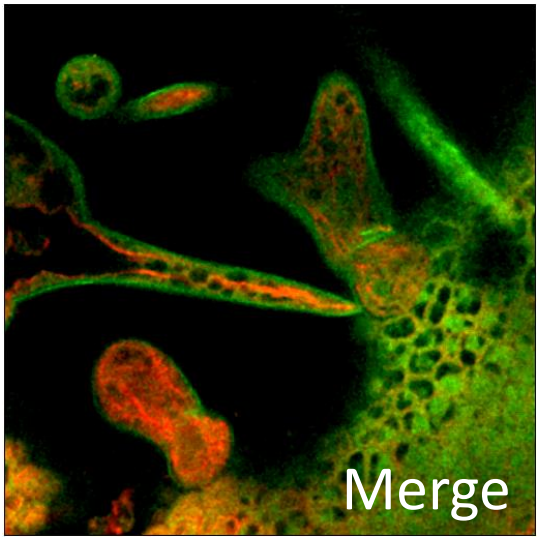
Working with multiple channels is exactly the same as for one channel, only now there are multiple channels, each of which can be adjusted separately.

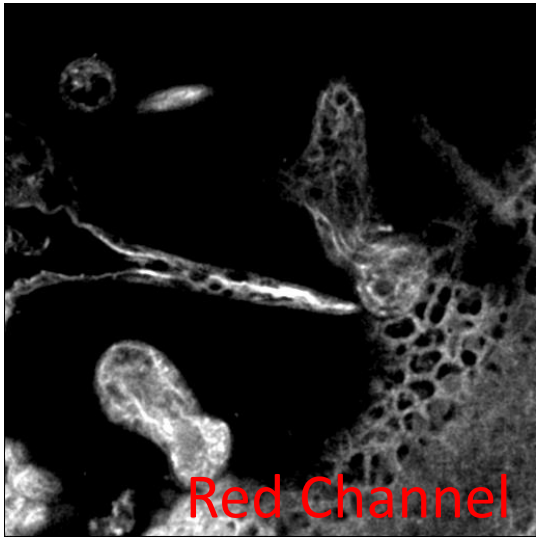
Open Organoid.czi

Use slider a bottom of image window to switch between channels.

Use Image -> Spilt Channels to get the channels as individual images.

Use Image -> Color -> Channels Tool to display selected channels only.





Converting Between Image Data Types

Converting between data types is done using the Image -> Type menu options.

Open Organoid.czi

Adjust LUT and then convert to 8-bits / channel.

Caution: Information is lost during this step, since there are now fewer intensity level values per pixel. For our purposes of visualization, this is not important

Make channels green, magenta, and blue. Convert to RGB color.

Caution: Channels are now no longer separable and in general can never be re-separated. However, non-image processing software such as Photoshop or other graphic software do not support channels and are only designed to work with RGB color, as this is a standard format used by commercial cameras.

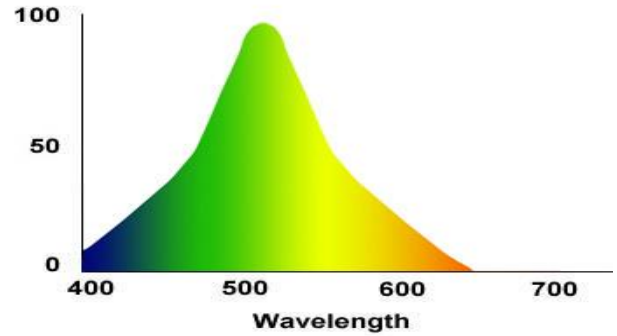
Try converting back to channels

Why does it fail?

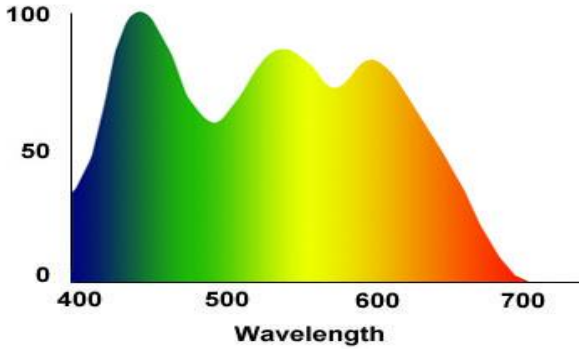
Be careful when displaying in color!

We don't perceive colors equally! Color perception is brightness dependent and brightness perception is color dependent. Color perception is also influence by surrounding colors.

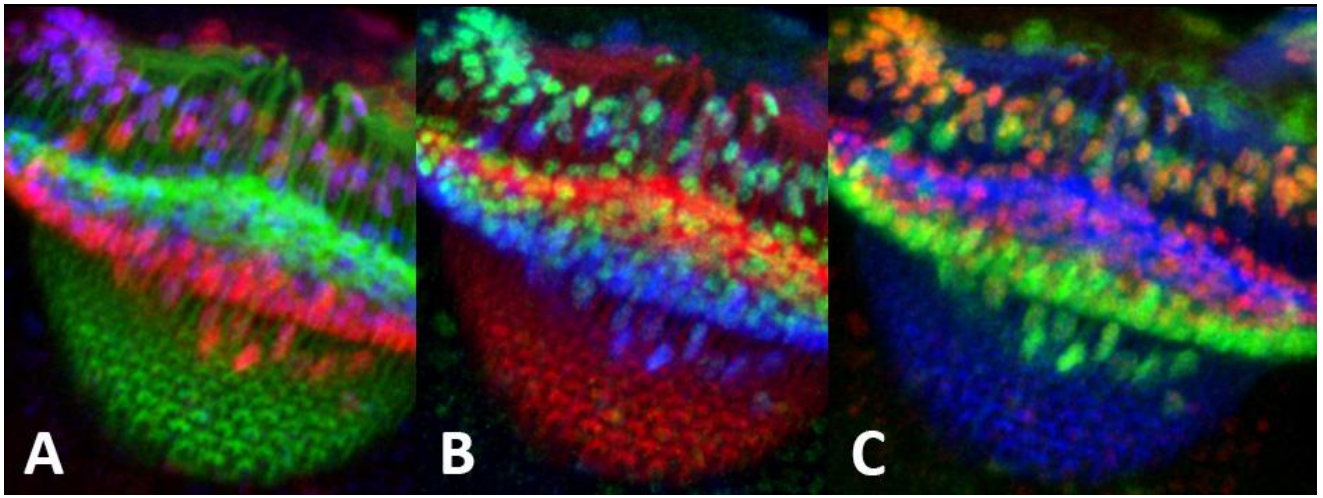
Dark-adapted spectral sensitivity



Light-adapted spectral sensitivity

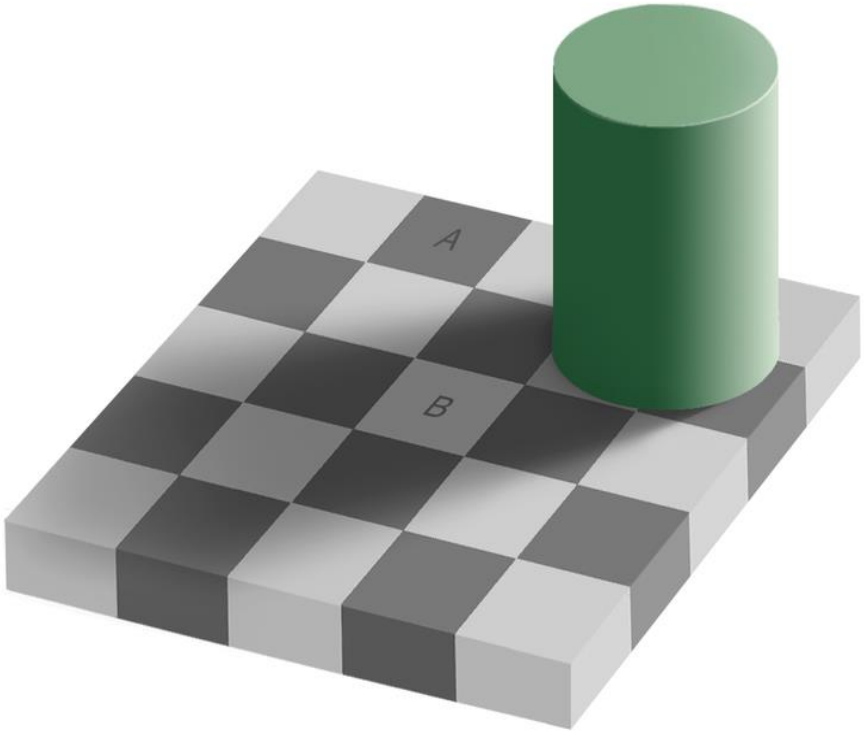


http://starizona.com/acb/ccd/advtheorycolor.aspx



Even be careful when looking at a single monochrome image!

Even intensity perception is scene dependent.



A and B have exactly the same intensity!

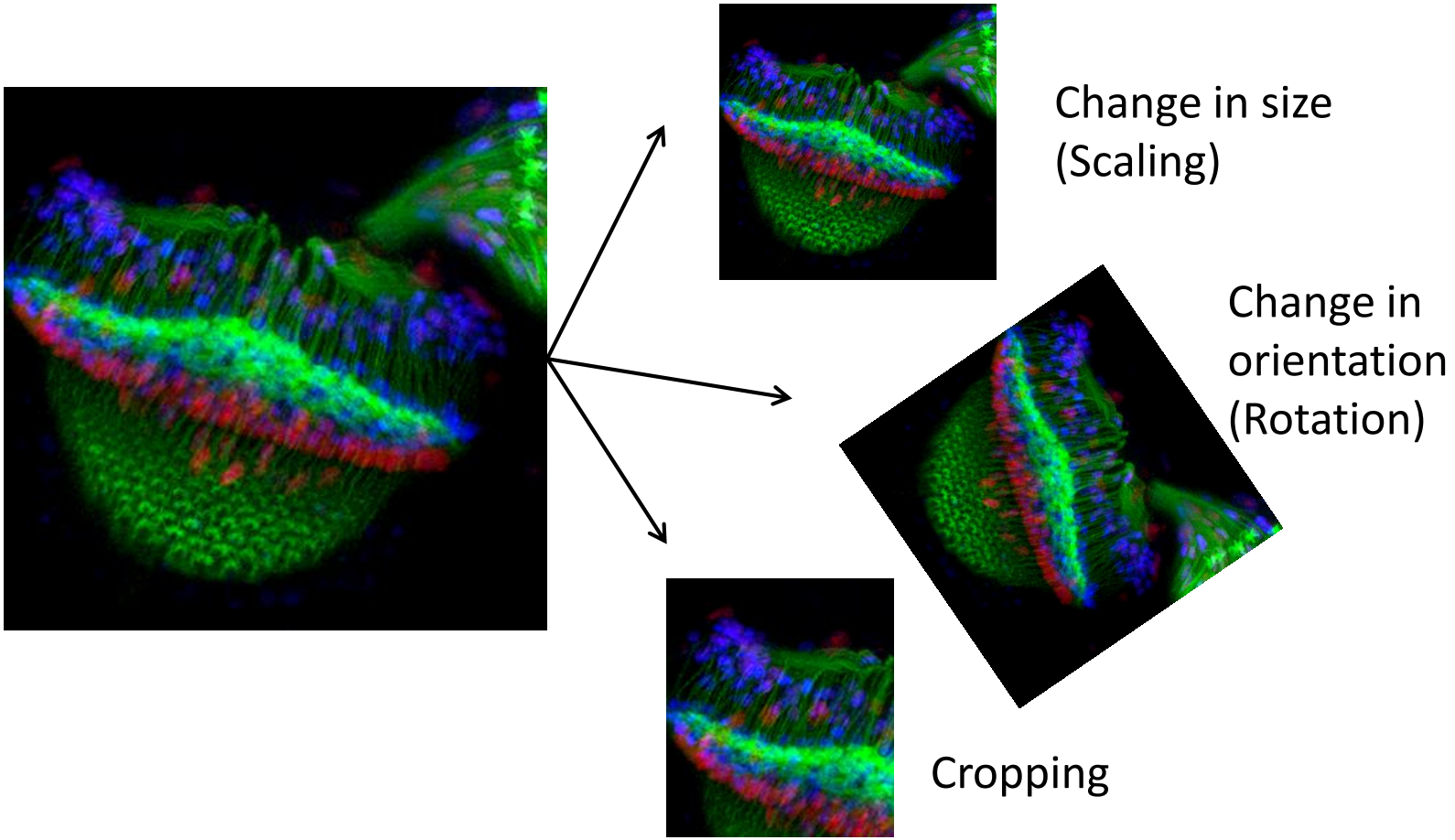
http://starizona.com/acb/ccd/advtheorycolor.aspx

Caution: Though this workshop is about image display, images should NEVER be analyzed by looking at them! Image displays are simply illustrations intended to highlight features established through other quantitative means.

Basic Manipulation of 2D Images

Basic Image Manipulations

Besides, changing how pixel intensities are displayed, we may also want to change the image's coordinate system. There are several common ways to do this:



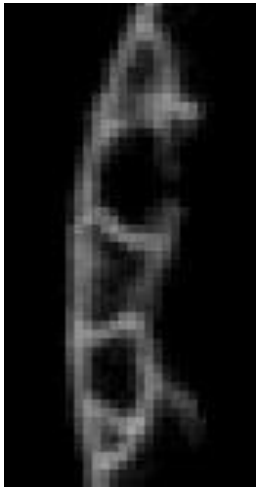
Changing Image Size

Enlargement means creating a finer coordinate system by adding new pixels 'in between' existing pixels. New intensity values must be created for the new pixels.

	0	1	2
0	5	8	..
1	2	4	
2	:		

Double image size…

(Scale = 2x)



	0	0.5	1	1.5	2	2.5
0	5	?	8	..		
0.5	?	?	?			
1	2	?	4			
1.5	:					
2						
2.5						

Open a single slice from Neuron_Slices folder

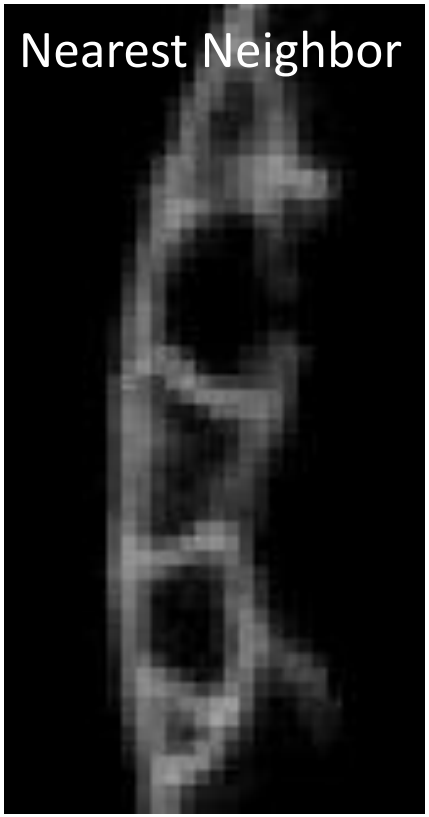
Use: Image -> Scale. Scale values >1 are enlargement.
Scale values <1 are reduction.

Caution: These operations create a new image with more/less pixels. This is NOT 'zooming', which merely enlarges the display of the image on the monitor.

Nearest Neighbor Scaling

‘Nearest neighbor’ method creates the new intensity values by duplicating the nearest measured intensity value (enlargement) or by simply removing pixels (reduction) as needed.

Original



Nearest Neighbor looks pixelated because no new intensity values are created, but it displays only acquired data.

Bicubic Scaling

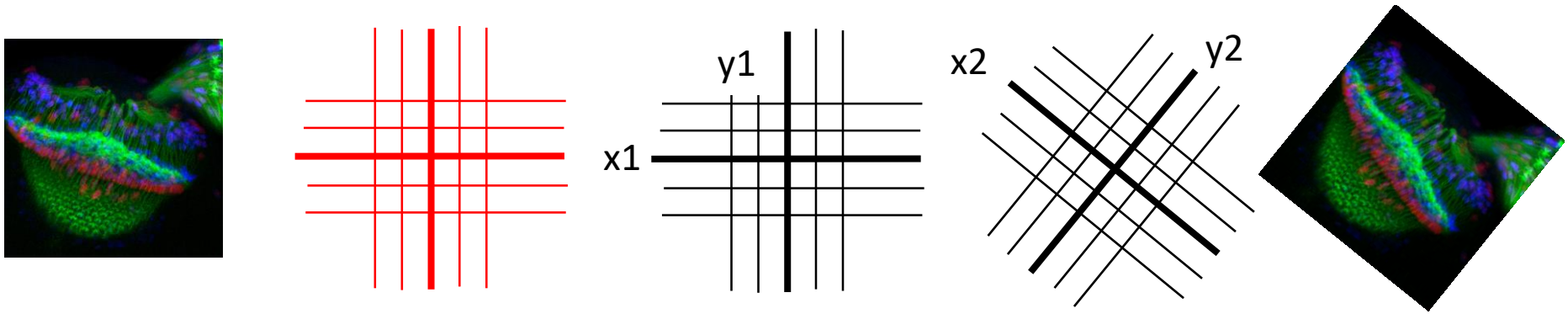
‘Bicubic’ converts fits a curve to a 4x4 neighborhood of known intensity values and then samples from this curve.

Original

Bicubic looks smooth due to the interpolated intensity values, but most of the image consists of interpolated values, not acquired data.

Image Rotation (center point)

An image's coordinate system can be rotated. Unless the rotation is 90 degrees, the rotated coordinate system will not align with the coordinate system of the screen. Again, some scheme is needed to create new intensity values.



Rotation θ : $x2 = \cos\theta\,(x1) - \sin\theta\,(y1)$

$y2 = \sin\theta\,(x1) + \cos\theta\,(y1)$

Use: Image -> Transform -> Rotate TRY IT

Other Coordinate Operations

Use: Image -> Crop

Image -> Transform -> Flip

Image -> Transform -> Translate

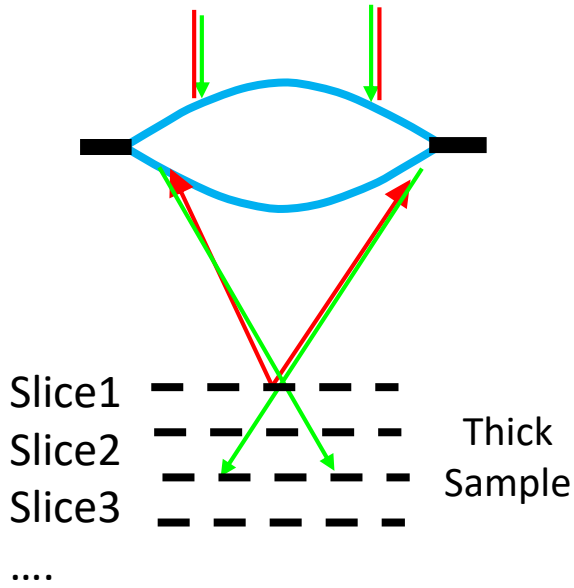
TRY IT

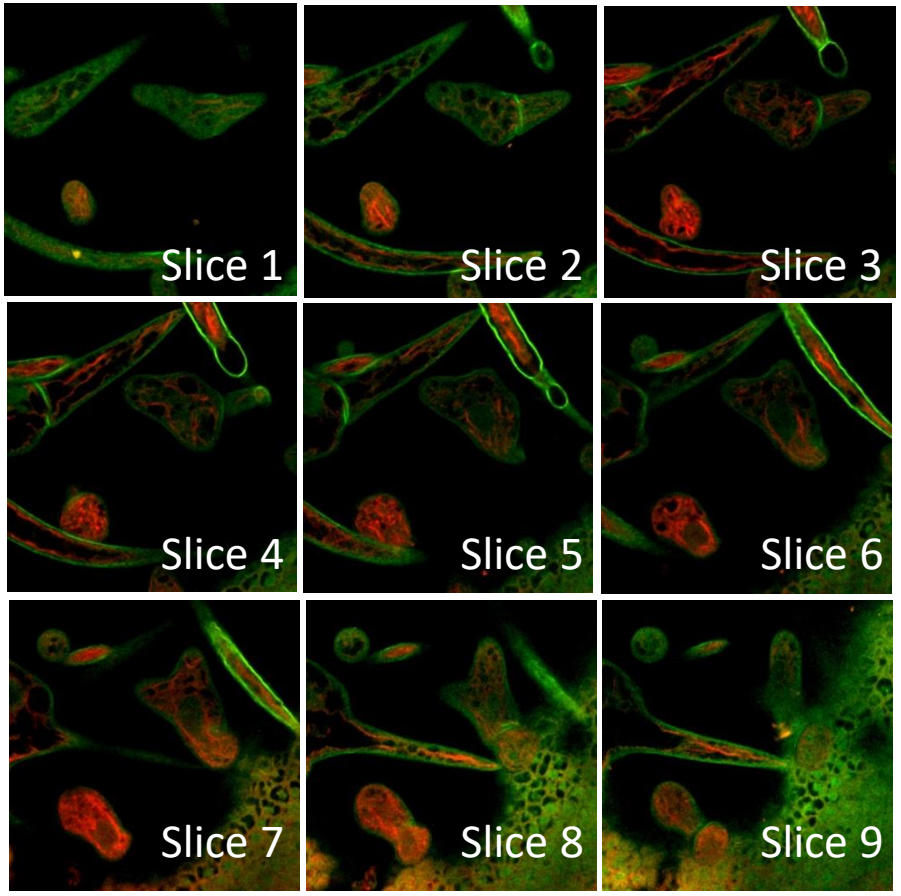
Display of 3D+ Images

How Microscopic 3D Images Are Acquired

In microscopy, 3D images are acquired via collecting a sequence of adjacent 2D images that have limited depth of field. Such a collection of slices is called a ‘z-stack’ and can be represented as a 3-dimensional matrix.

Move objective in z relative to sample





2D Displays of 3D Images

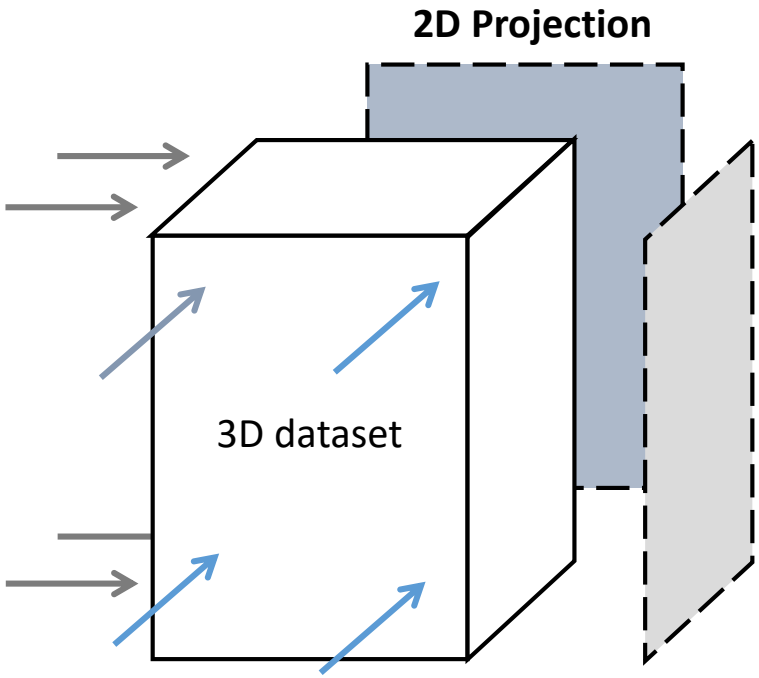
Computer screens are inherently 2D, so the 3rd dimension of information in an image is difficult to visualize. The 3D data must be collapsed into 2D, and information is always lost during this step.

<u>Common display options:</u>

- Project the 3D data onto a 2D plane. This works OK as long as the signal is sparse (not highly overlapping in the z direction).
- Interactively visualize the collection of 2D slices one-at-a-time (scroll through the planes). With practice, this can work well.
- Create a 3D rendering of the data. Always throws away or obscures internal information.

Intensity Projections

Intensity projections are some mathematical operation on the pixel intensities along a set of parallel ray paths through the data.



Open Organoid.czi

Use: Image -> Stacks -> Z Project…

Max Intensity (show brightest pixel) and Sum Slices (sum all pixels) makes sense for fluorescence images where signal is bright and background is dark.

Caution: Max Intensity projection does NOT preserve total intensity and should never be used prior to measuring / displaying intensity information. It is strictly a 'structural' display.

Rotating Intensity Projections

Though a projection is just a 2D image, making a projection from many different angles and playing them back as a movie gives some impression of depth due to parallax.

Use: Image -> Stacks -> 3D Project…

Try it with Organoid.czi

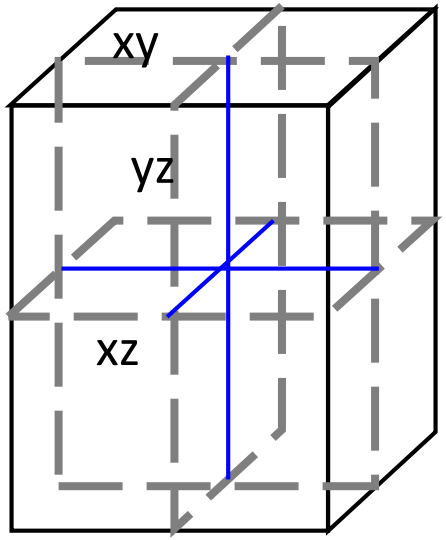
FIJI lacks a good interactive 3D Viewer. We will use FluoRender for this later! But, for a quick interactive 3D view try: Plugins -> 3D Viewer

Caution: 3D Viewer is very limited and often freezes/crashes. It is not recommended for most purposes.

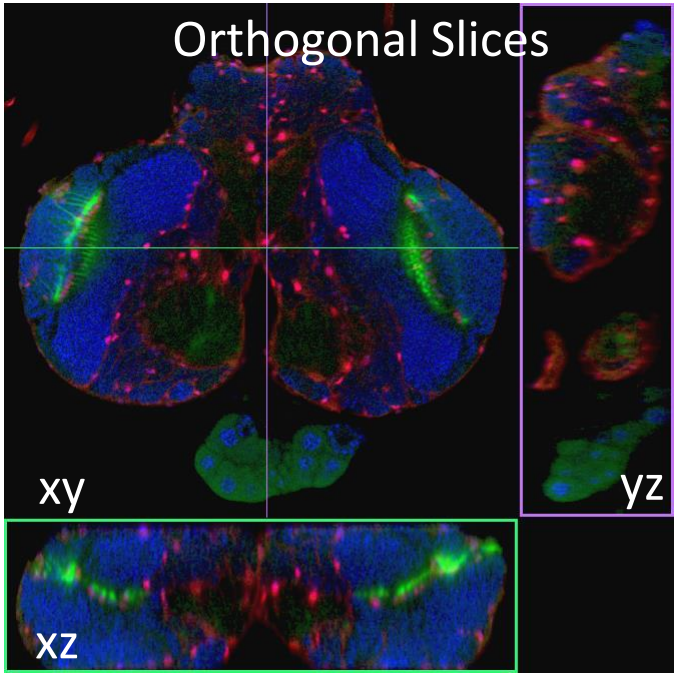
Orthogonal slices

'Orthogonal slices' displays a set of 3, 2D images that are each from mutually perpendicular planes through the 3D volume. The three planes intersect at the location of the cursor. The raw pixel data is shown directly, though only a small fraction of data is seen at any one time.

Orthogonal planes



3D dataset



Use: Image -> Stacks -> Orthogonal Views

Try it with Organoid.czi

Saving Images in FIJI

Image File Type Considerations

After opening, the image must be saved as some file type.

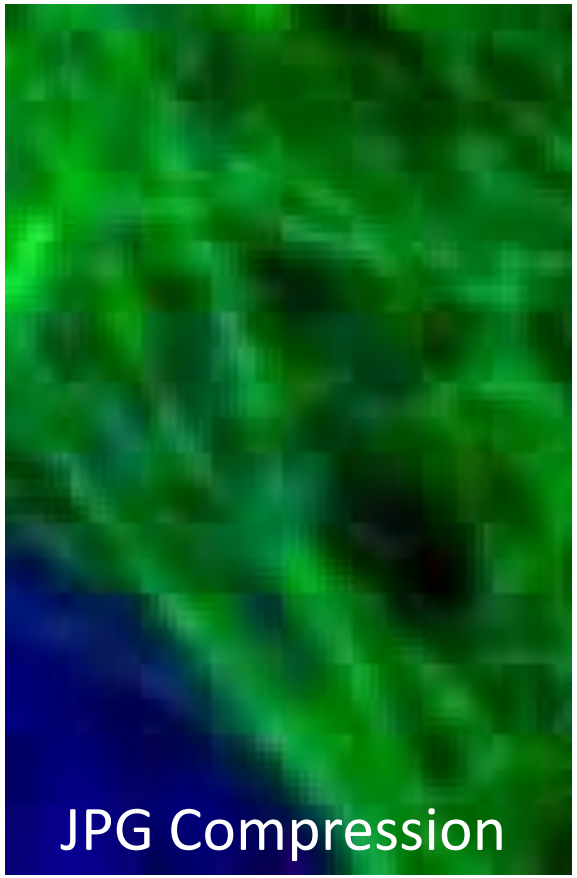
- If you only adjusted LUTs, the data has not been changed so there is no need to re-save.
- If the data type (bit-depth) or raw pixel values have been changed, then re-save as a separate file so as not to lose the raw data.

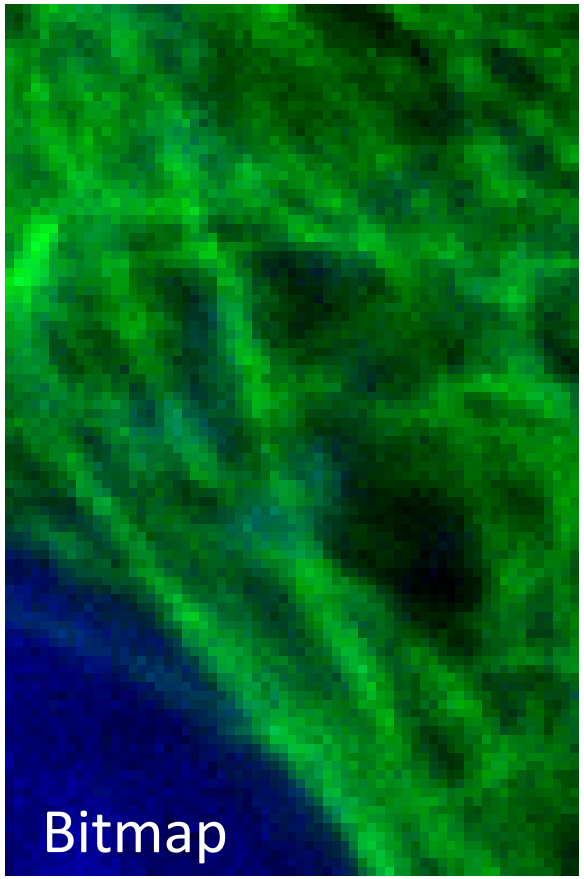
File Type	Purpose
TIF	Precisely stores all pixel values. Compatible with all data types and any number of channels / dimensions. Usually the best option.
JPG	A lossy (data lost) compression format designed for natural (white light) images. Does not support channels (will get whatever channels are displayed). Generally should not be used for fluorescence images. Journals will not accept. Could be used if goal is to create a quick snapshot to email or use in a presentation. Set compression level using Edit -> Options -> Input/Output
AVI	A movie format. Typically uses lossy compression in space and time.

Bitmap vs JPG Compression

Bitmaps (such as TIF) save each pixel value individually. JPG compression splits an image into blocks and then discards certain colors and details within each block to approximate the original image. Approximation may be very poor at high compression levels.

Notice ‘blockiness’. Compression occurs within each block.

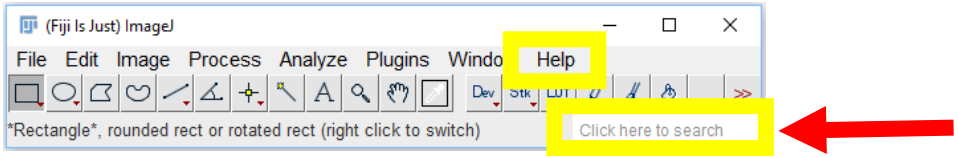




Getting Help with FIJI After Today…

1. The **online manual** is excellent and detailed…
   (but does not cover plugins.. Plugins have their own websites)
   https://imagej.nih.gov/ij/docs/guide/146-30.html

2. Type the name of the menu command into the toolbar…



3. Google it…

4. Attend other workshops that cover more advanced topics

5. microscopy@umich.edu

* If you are having problems with installation, contact HITS *

FluoRender for Interactive 3D Rendering and 3D Movies

What is FluoRender?

A major drawback of FIJI is that there is no great option for 3D rendering of multichannel volume views. FluoRender fills this gap.

FluoRender is an open source (in C) application specifically designed for 3D rendering of multichannel volume views.

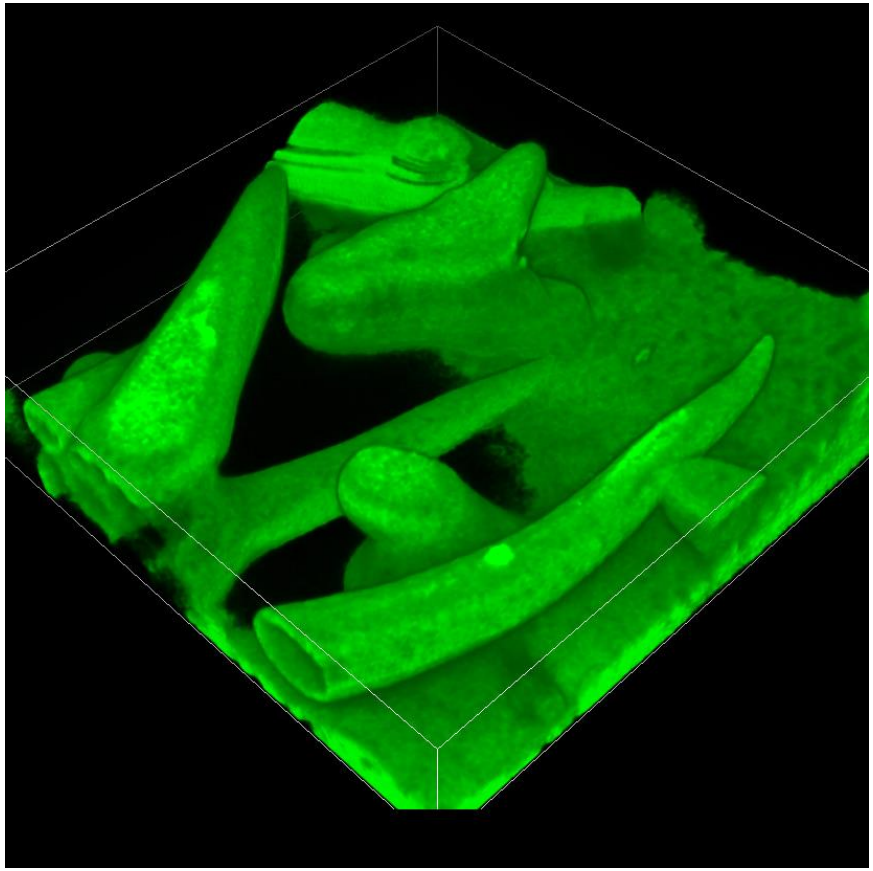
http://www.sci.utah.edu/software/fluorender.html

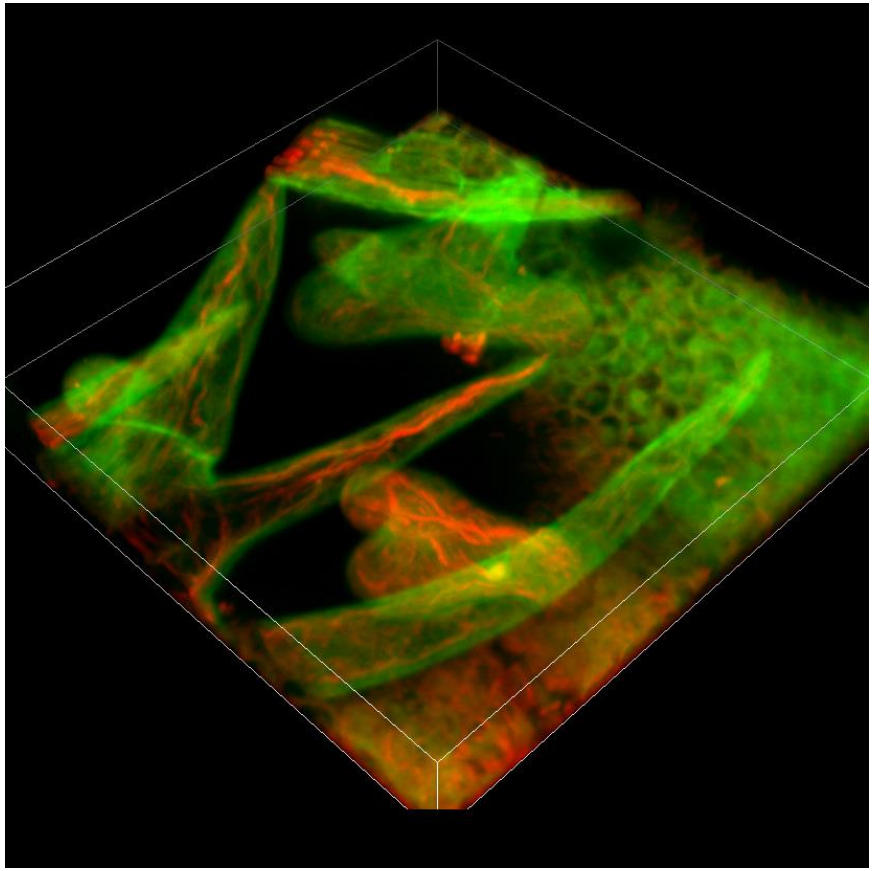
FluoRender does **much more** than what we will talk about today. Please be curious and explore further on your own!

See: Wan et al. FluoRender: joint freehand segmentation and visualization for many-channel fluorescence data analysis. BMC Bioinformatics. 2017.

What is a ‘3D Rendering’?

A 3D rendering is a 2D display created in a way that provides the *illusion* of depth.





Key Features of a 3D Rendering

Features that provide the *illusion* of depth are perspective, shading, and parallax. These are typically user adjustable.

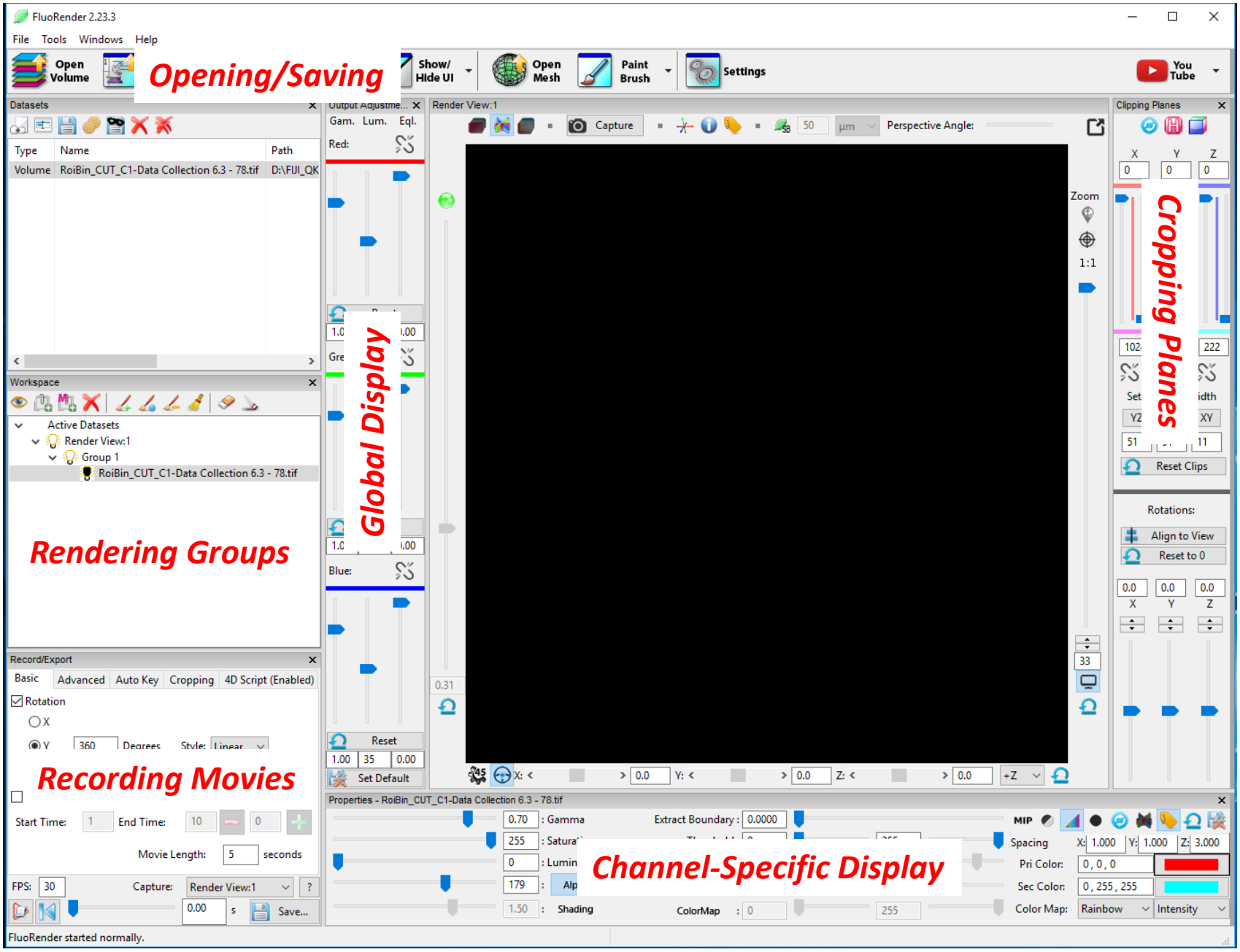
- **Perspective:** Parts of an object more distant from the viewer in the display space are drawn relatively smaller, according to vanishing points. FluoRender uses 3 vanishing points, one for each axis of the data.

- **Shading:** More distant objects are relatively dimmer. (Which is distinct from shadowing, where objects cast simulated shadows).

- **Parallax:** Upon interaction, objects move relative to one another depending on their relative distance from each other.

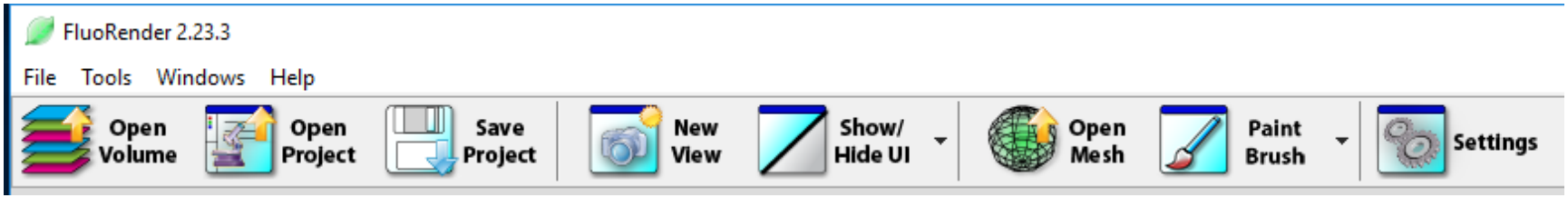
True 3D images require each eye be presented with a slightly different view (called binocular disparity). We will not cover true 3D methods today.

FluoRender GUI Overview



FluoRender GUI Overview

OPEN Tissue.tif

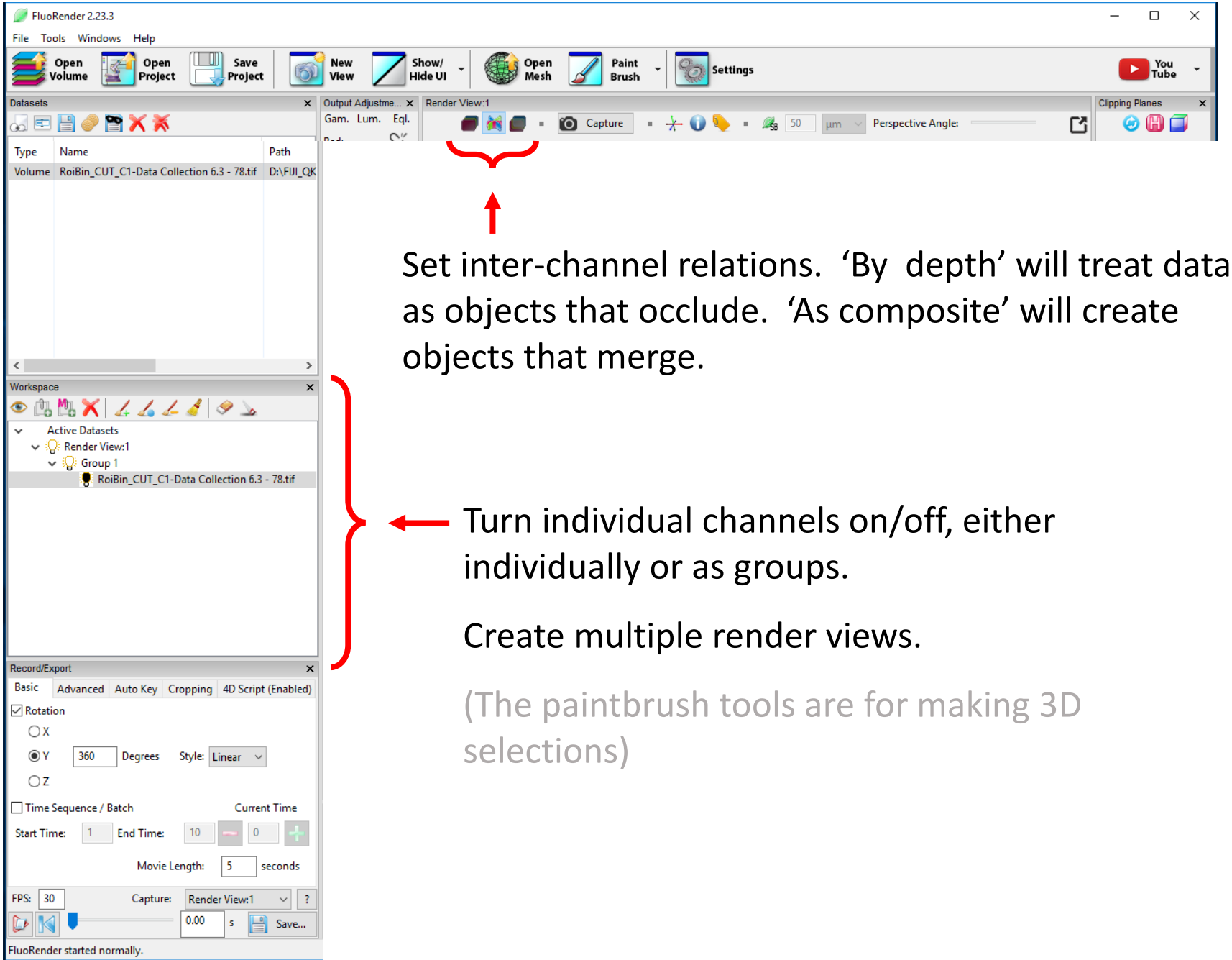


Opening Data Files. Not nearly as flexible as FIJI, so stick with TIF. (Can also bridge to FIJI). Use 8-bit images to make files smaller.

Opening/Save Project. A "project" is a rendering that you have created in FluoRender.

Only option of note for today is ability to set a background gradient.

Working with Channels



Set inter-channel relations. ‘By depth’ will treat data as objects that occlude. ‘As composite’ will create objects that merge.

Turn individual channels on/off, either individually or as groups.

Create multiple render views.

(The paintbrush tools are for making 3D selections)

Adjusting Channel Specific Displays

Gamma: Relative display of dimmer vs brighter intensities.

Saturation: How colorful.

Luminance: How bright.

Alpha: Relative 'transparency' of pixels along the depth of the dataset.

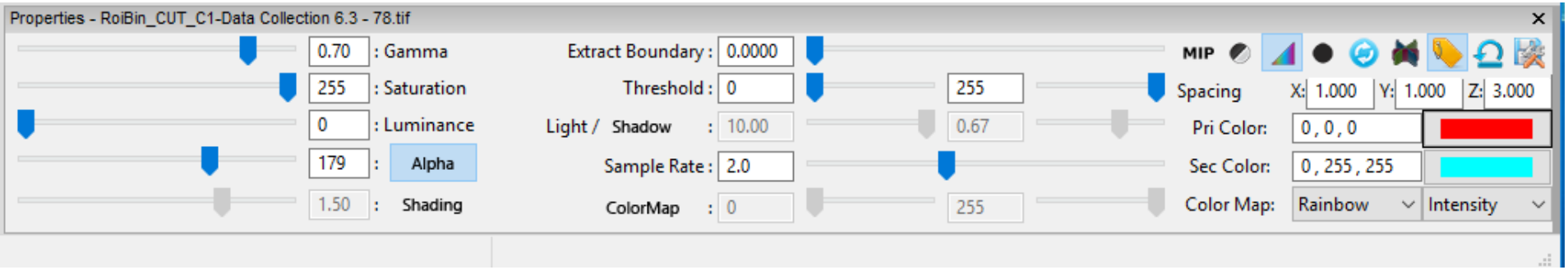
TRY IT

MIP: Show maximum intensity projection.

Sample Rate: Scaling of data before display on screen. Speeds up large files, at loss of data resolution. (Larger values = fewer pixels).

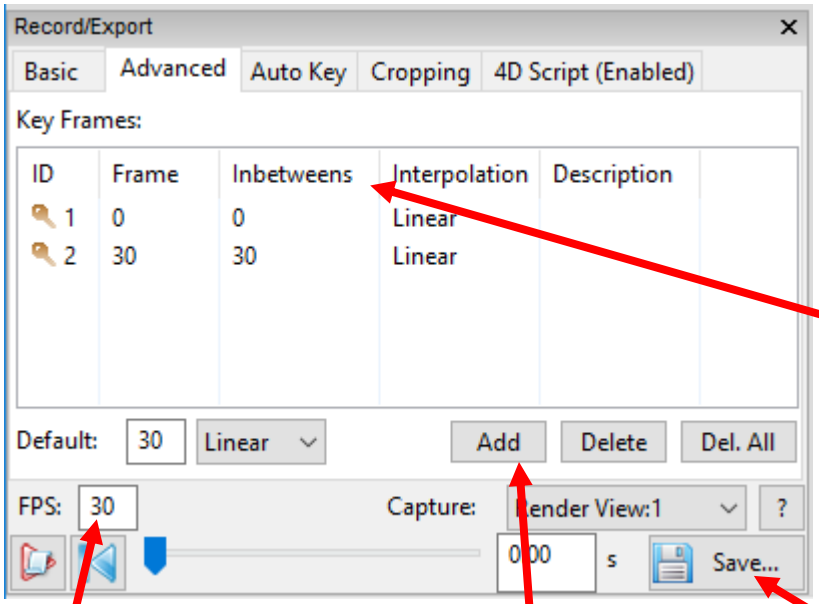
Color Map: Use spectral lookup tables.

Spacing: Spacing of data in screen space. Usually z resolution is worse than xy so z should be larger as appropriate.



Making Movies

Go to Advanced tab…



TRY IT

Inbetweens is the number of frames used to interpolate between views and so also sets timing.

Press Save when you are finished at want to create the movie.

Frames per second that the movie will play at. 10-30 fps recommended.

Press Add to record the current view. What you see on the screen is what you will get.

Getting Help with FluoRender After Today…

1. The FluoRender home page has a complete manual and video tutorials... http://www.sci.utah.edu/software/fluorender.html

2. Read the help file in the menu bar.

3. Google it…

4. Attend other workshops that cover more advanced topics

5. microscopy@umich.edu

Thank you!