MIC Microscopy Workshop 2022
ImageJ Computer lab

Please wear a mask, your neighbor may have a medical condition!

35 sqft = 6 ft away from anybody else

Feel free to ask questions

FIJI SETUP

- Windows: copy “fiji-win64.zip” to the desktop, open and extract “Fiji.app” to the desktop. Run “ImageJ64.exe”, Help > Update ImageJ

- Mac: copy “fiji-macosx.dmg” to the desktop, open it, drag the FIJI icon (left) to the desktop. Click on FIJI, Help > Update ImageJ

- Help > about ImageJ: v. 1.52a

- Copy folder “Images” from fash drives

- Install Image Stabilizer plugin:

Copy “Image_Stabilizer.class” and “Image_Stabilizer_Log_Applier.class” from flash drive

Plugins > Install Plugins and navigate to folder

Restart Fiji

Plugins > Image Stabilizer: “There are no open images”

MIC WORKSHOP, Spring 2022

Vincent Schram, Ph.D.

- **LIGHT MICROSCOPY 1: TRANSMISSION AND FLUORESCENCE**

  Monday May 9, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 2: CONFOCAL, 2P AND LIGHT SHEET**

  Tuesday May 10, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 3: SUPER-RESOLUTION IMAGING**

  Wednesday May 11, B35 / GG607, 11 am - 1:30 pm

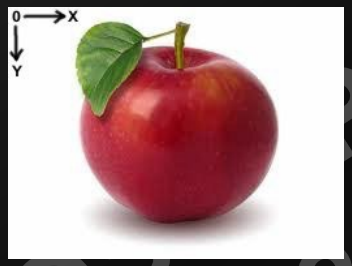
- **IMAGE ANALYSIS WORKSHOP: IMAGEJ**

  Thursday May 12, B35 / GG607, 9:30 am - 12:30 pm / 1 pm - 4 pm

- **CONFOCAL MICROSCOPY HANDS-ON**

  Friday May 20, B35 / GD922, 9:30 am - 12:30 pm / 1 pm - 4 pm

Digital Image

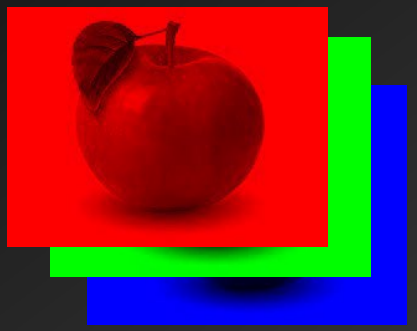


- **Digital image:**
  - Two-dimensional array of intensities
  - Origin = upper left corner

- **Pixel depth = number of bits (0-1) in each cell**

- **Grayscale image:**

1-bit (binary)
8-bit (0 – 255),
16-bit (0 - 65,535)
Look-Up Table (LUT): black to _color_ instead of B&W



- **Color image: Composite of red, green and blue layers**
  - 24-bit = 3 x 8-bits image (R, G, B)

- **Clipping: "washed-out" appearance (CCD)**
  - → re-scale min and max intensity values

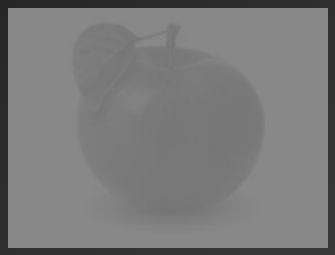
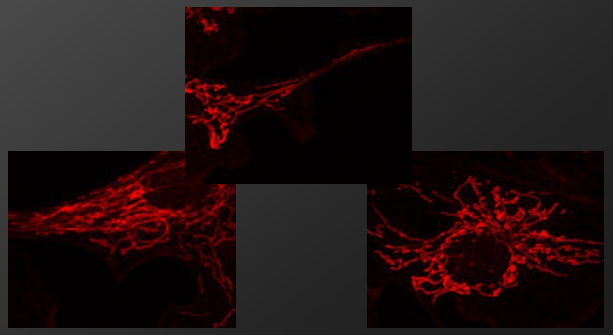
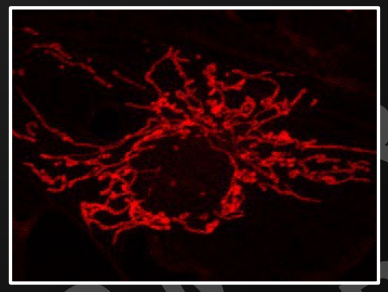


Image Dimensionality

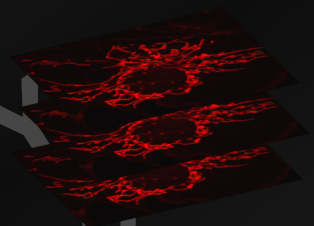
Locations

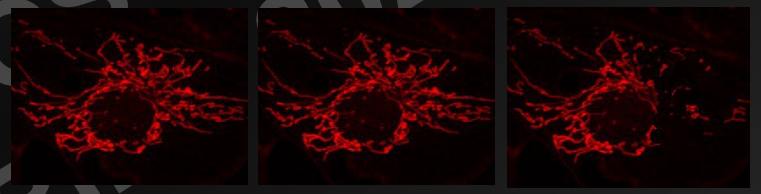


"Flat"



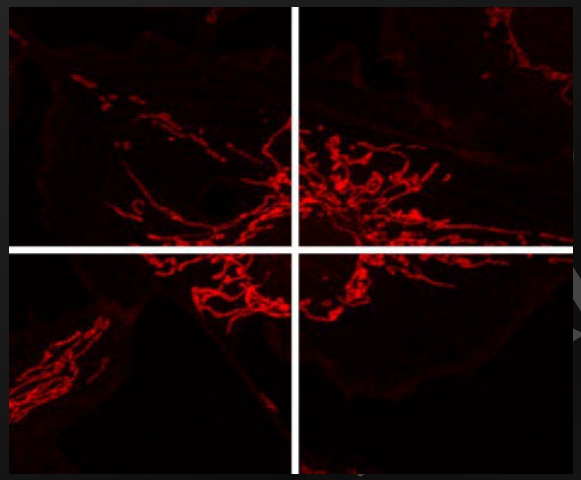
z

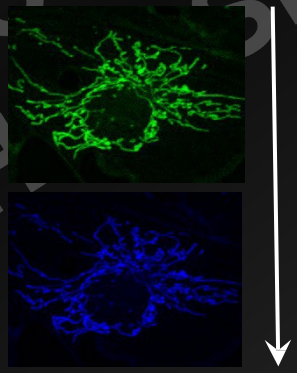




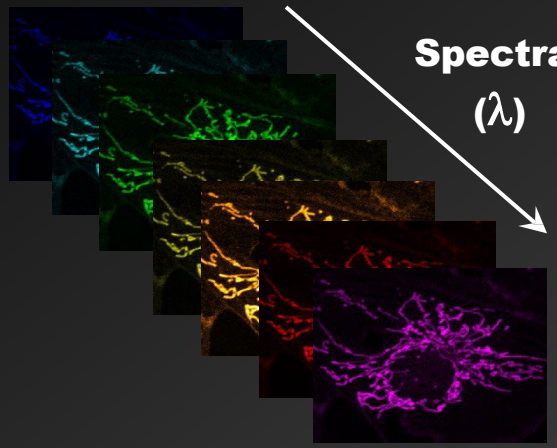
Time

Tile





Color
(Channels)



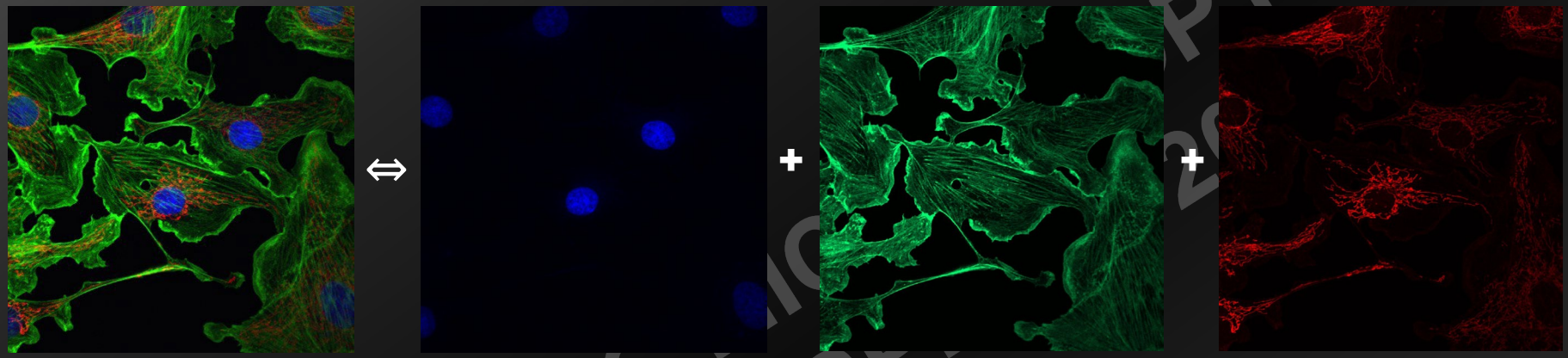
Spectral
(λ)

Any combination + images saved sequentially in single file

→ ***Formatting information critical...!***

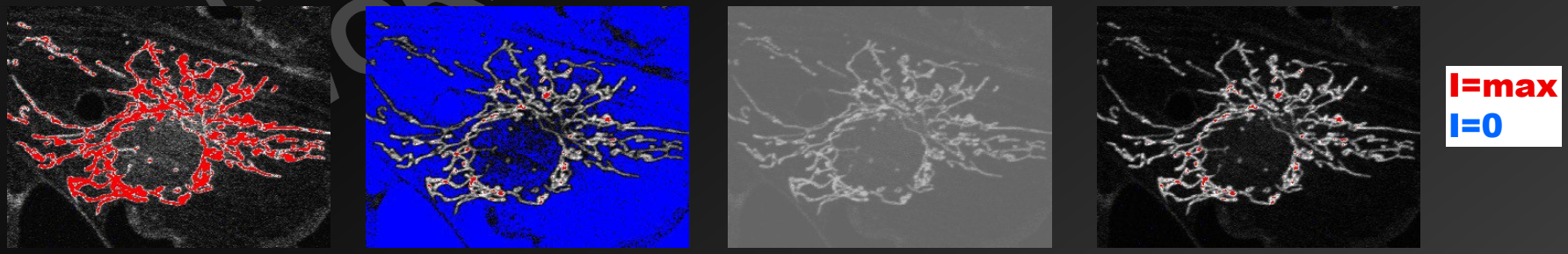
Fluorescence image

- Fluorescence image: bright signal on black background



→ RGB image: red, green and blue color for clean channel separation
→ Tiff: > 3 non-primary layers (yellow, cyan, purple, pink…) possible

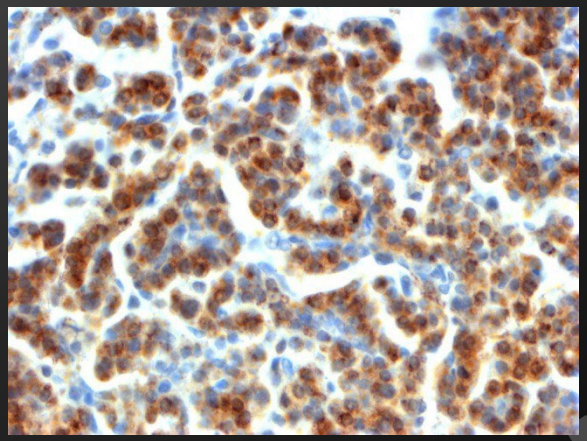
- White and black values: use full intensity scale (some exceptions)



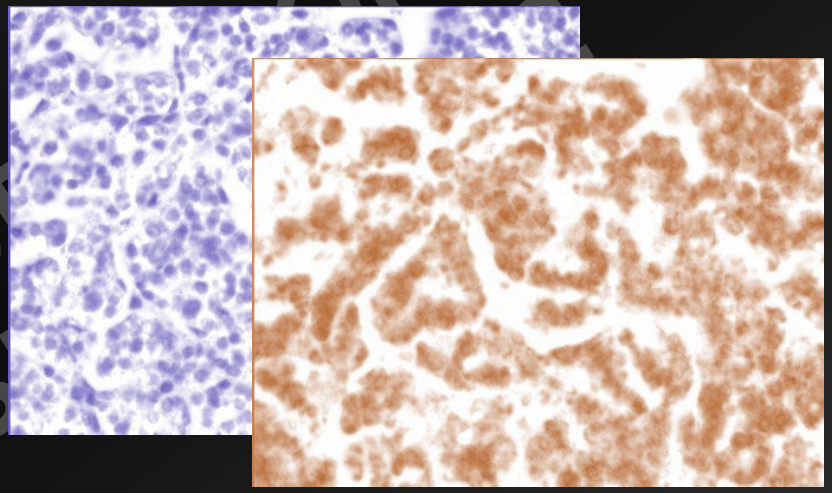
Reduce gain | Adjust offset | Adjust gain / offset | Optimal

Transmission image

- Dark signal on bright background, no RGB color separation



Color
Deconvolution
H&E DAB



- Extended depth of field

→ No easy way to render volume

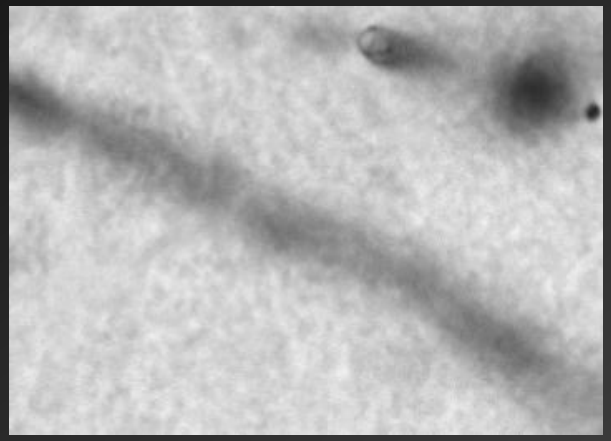
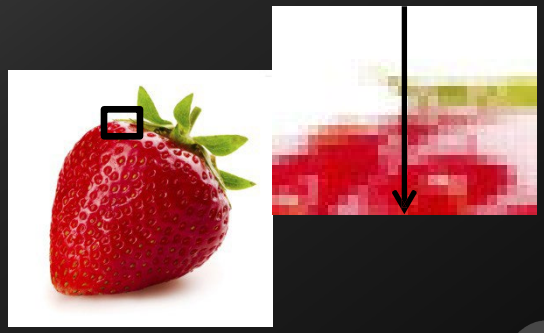
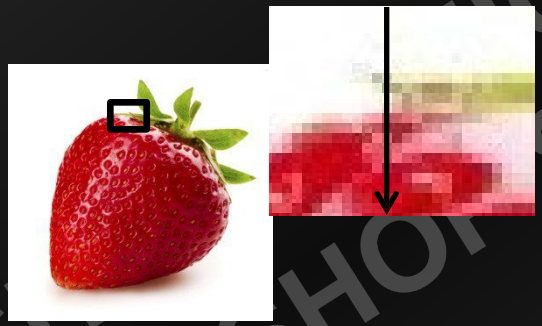


Image compression

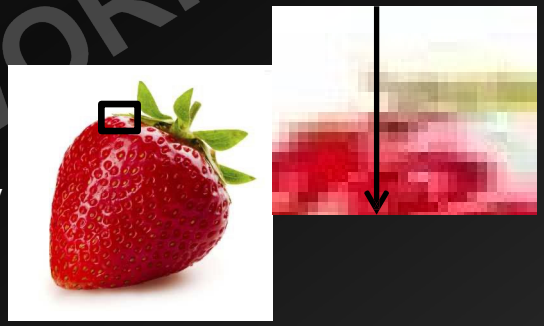
TIF
no compression
485 kB

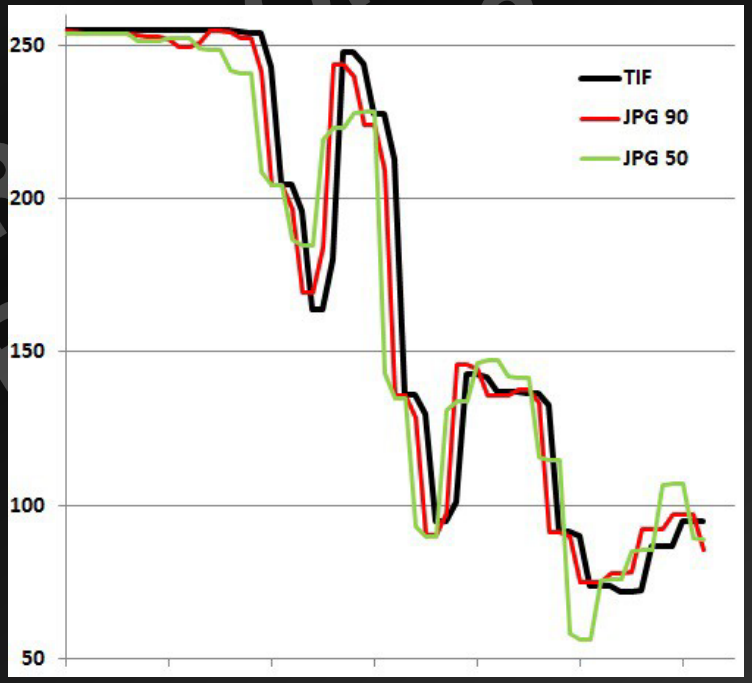


JPG
90% quality
62 kB



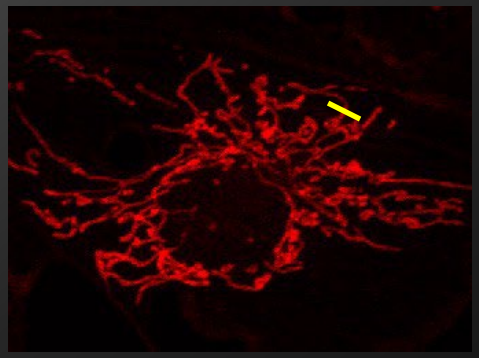
JPG
50% quality
15 kB

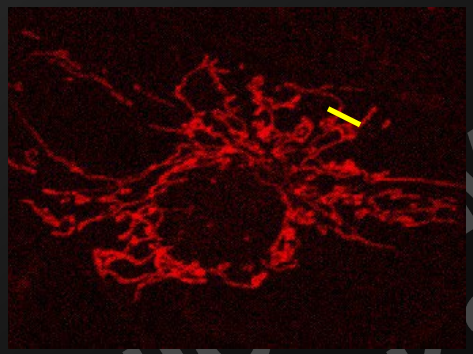


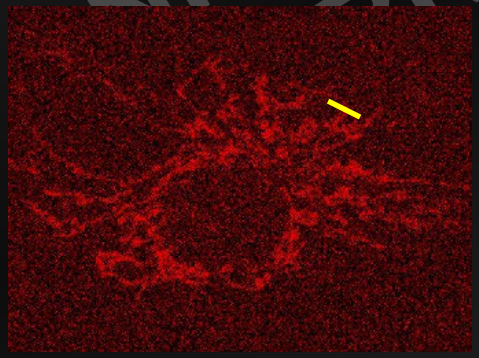


→ ***Do not use compression!***

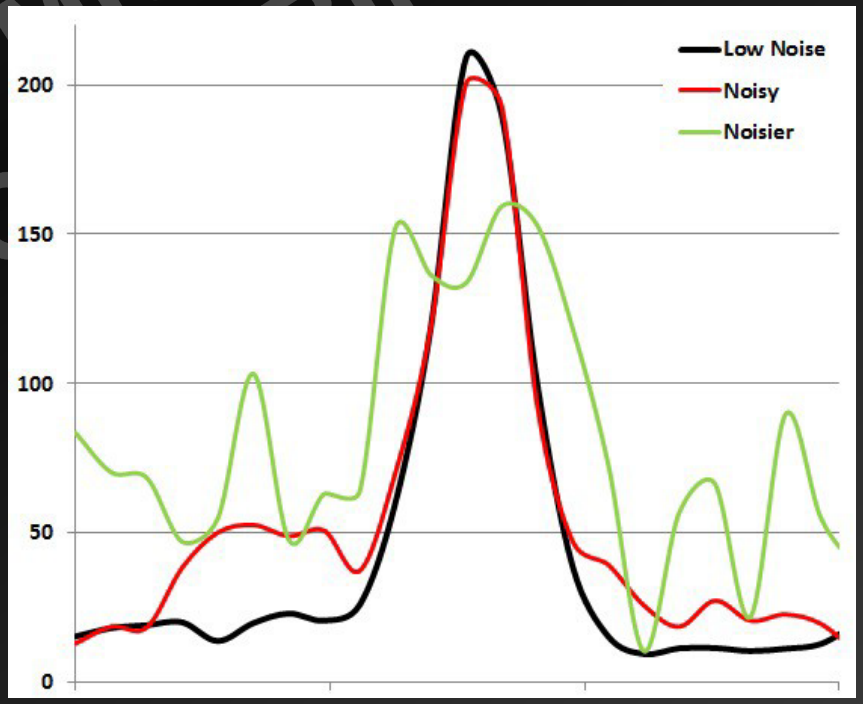
Image noise



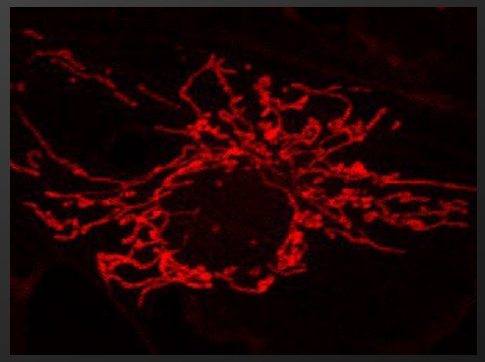


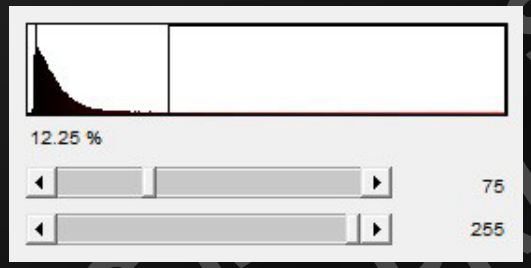


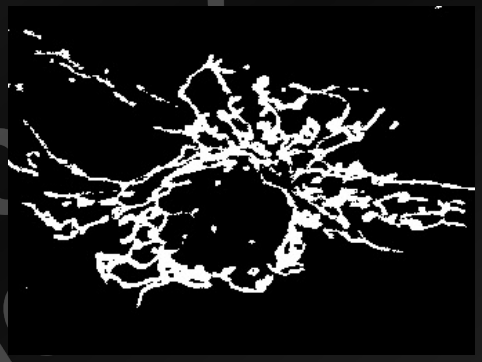
- Noisy image = low signal (camera) or high gain (confocal PMT)
- Noise reduced by image averaging *(last resort...!)*

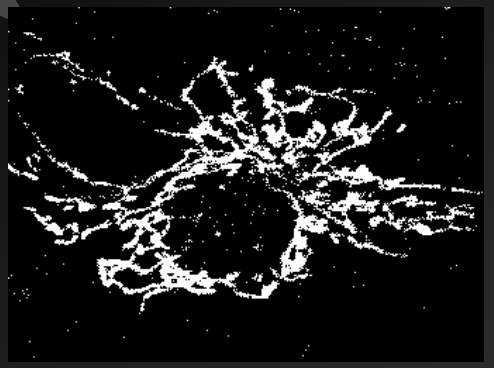


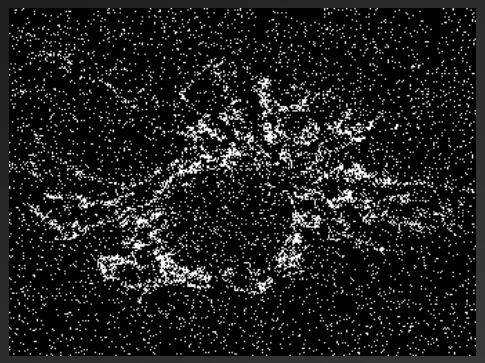
Thresholding











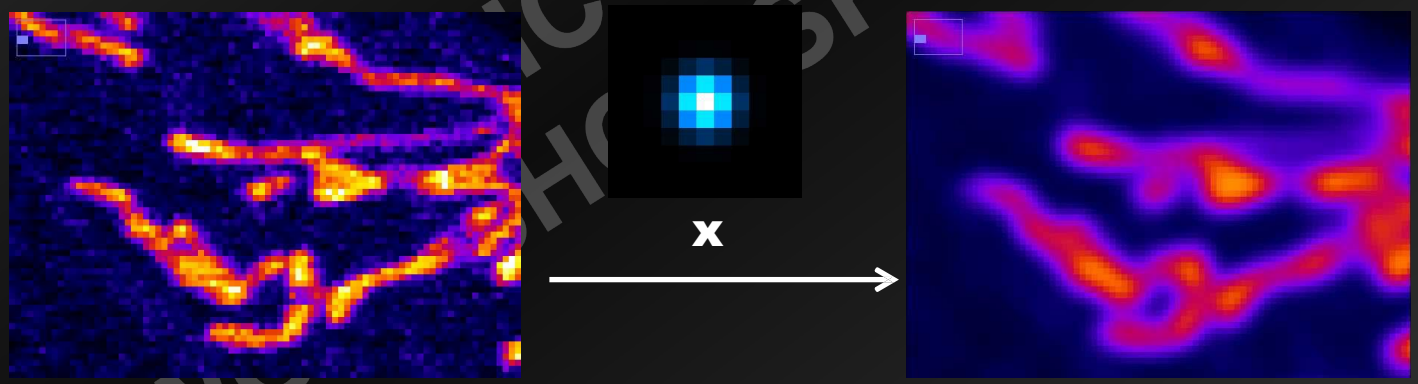
- Bin pixels above / below intensity value(s) (segmentation)
- Primary method to automatically isolate features and create masks (binary images)
- Severely impacted by noise, uneven illumination, uneven background

Filtering

- Filter = structuring element
  → Intensity transformation based on neighbor pixels

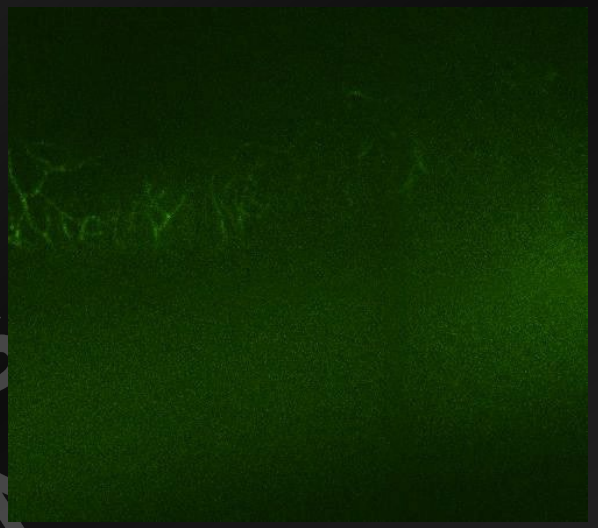
0 0 0	1 1 1	-1 -1 -1	1 2 1
0 1 0	1 1 1	-1 6 -1	2 4 2
0 0 0	1 1 1	-1 -1 -1	1 2 1
null	median	Find edges	Blur

- Gaussian filter

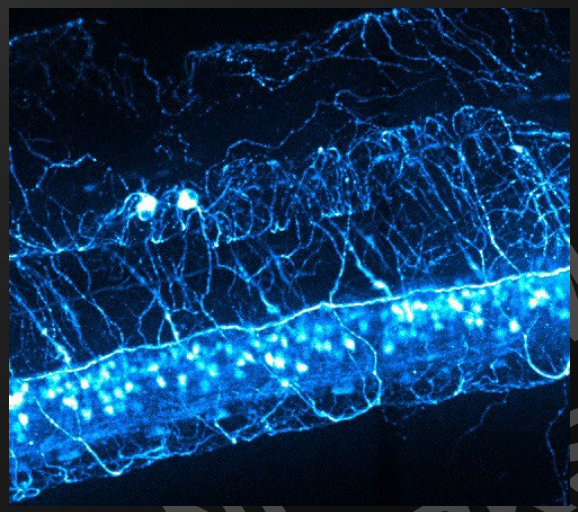


- Other type of filters: Binary (erode, dilate)
  Time-based (frame before / after)
  Frequency-based (Fourrier transform)

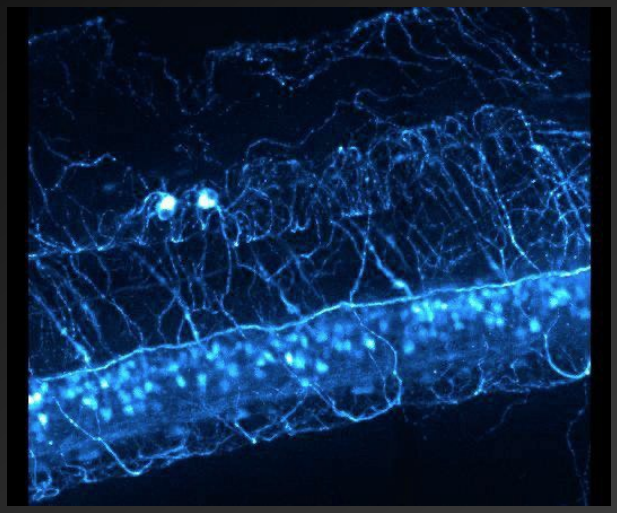
Projection



- Stack = series of images
  - Focal planes → volume
  - Time → kinetic



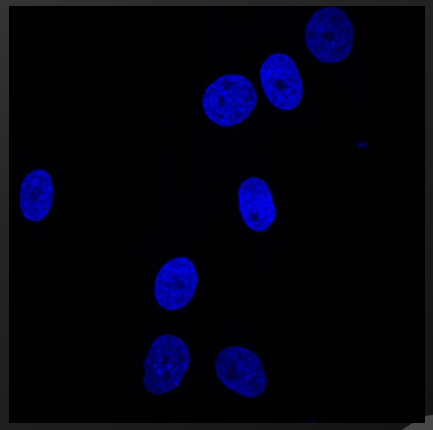
- Maximum Intensity Projection (MIP):
  Retain highest-intensity pixel for each x,y location



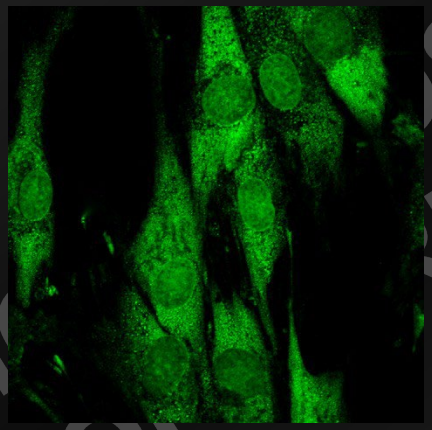
- 3D projection: illusion of volume
- Time series → trajectory (MIP)

Channel cross-talk

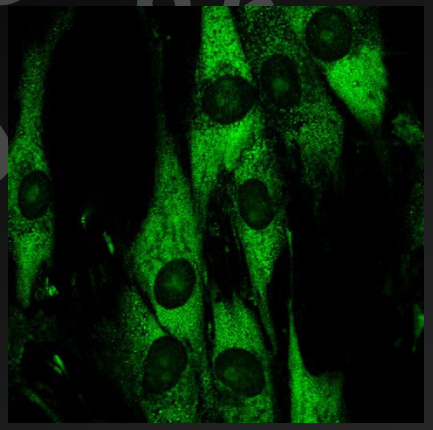
- Crosstalk: signal from one channel spills onto another (up A)
  - → Sequential instead of simultaneous recording



Dapi

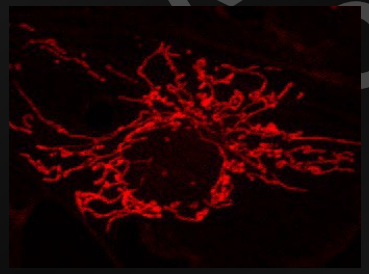


Alexa 488 (simultaneous)

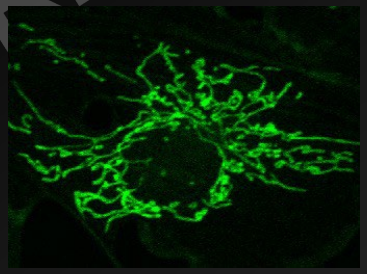


Alexa 488 (sequential)

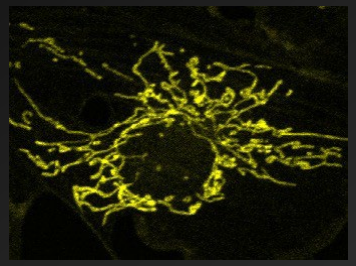
- 100% overlap / colocalization = cross-talk…!

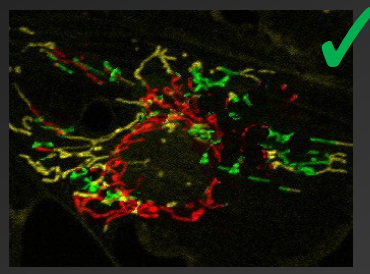


+



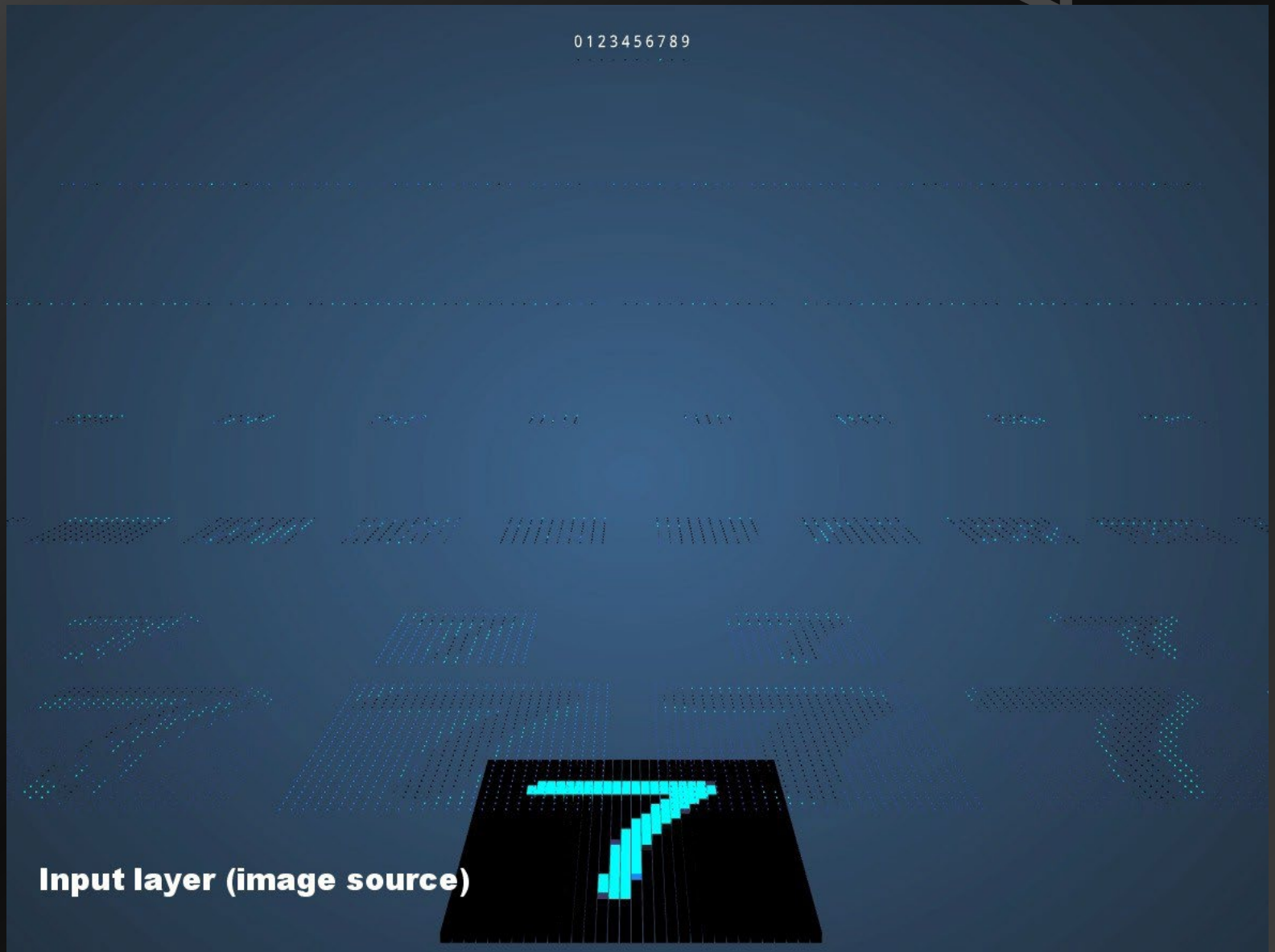
=





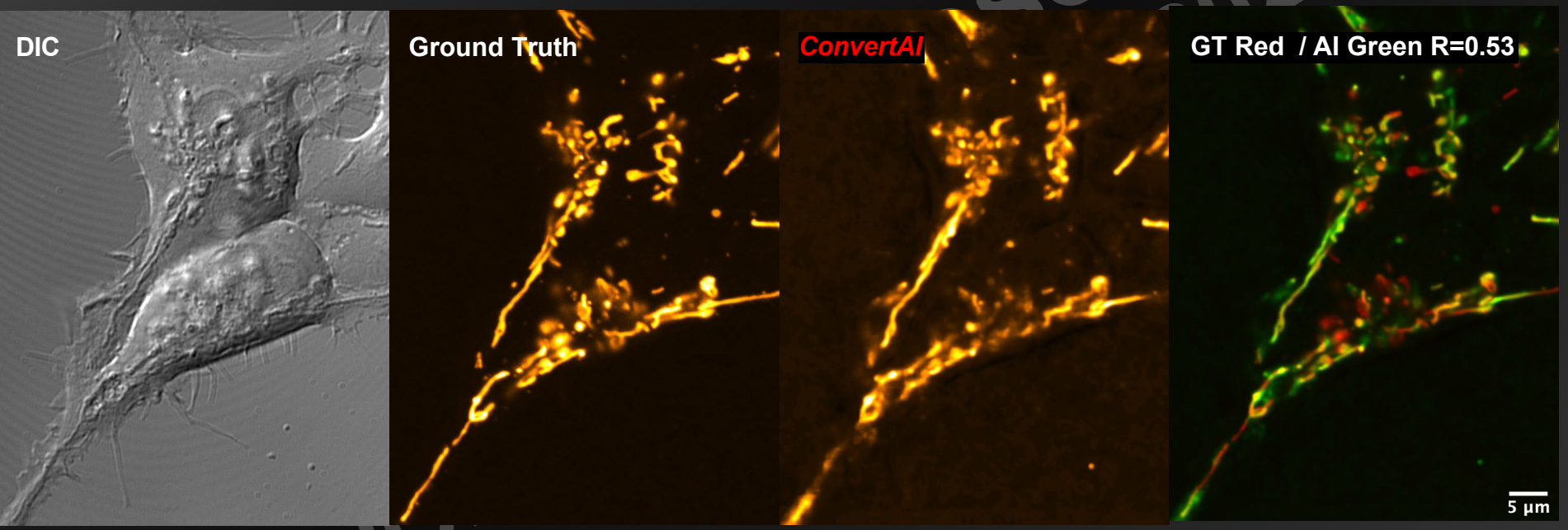
AI Processing

- **Self-optimizing convolutional network:**



AI applications

- Too messy for “hard” (conventional) processing
- Denoising, segmentation, feature extraction, deblurring, resolution enhancement, etc…



→ No explicit model / rationale
→ Training set + computer time
→ “Image forecast”, validation (required, save your CNN)
→ *Use only when hard processing fails*

Bias In Image Processing

- User-selected field of views are already heavily biased
  - → Document selection criteria

- Not an exact science
  - → Carefully document processing workflow
  - → Rationale for user-adjustable parameters (threshold, radius…)
  - → Optimize each step and overall workflow (wet bench)

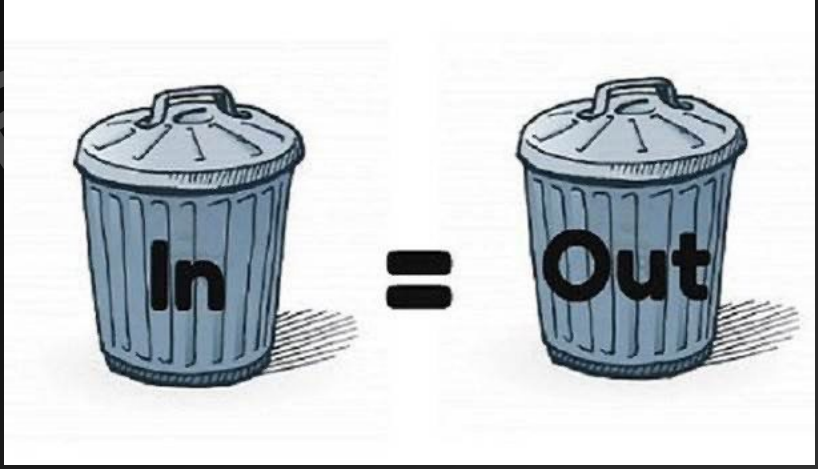
- User Bias = Qualitative data
  - → Relative comparisons only
  - → User bias not specific to microscopy data
  - → Microscopy results used in combination with other data

→ ***Trust your own judgement***

→ ***Trust your eyes: not visible = unlikely to exist***

BEST EFFORT TO RECORD HIGH-QUALITY IMAGES...!

YOU CANNOT ANALYZE YOUR WAY OUT OF BAD IMAGES...! (even w/ A.I.)



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- Help > about ImageJ: v. 1.52a

- Copy folder “Images” from flash drives

- Install Image Stabilizer plugin:

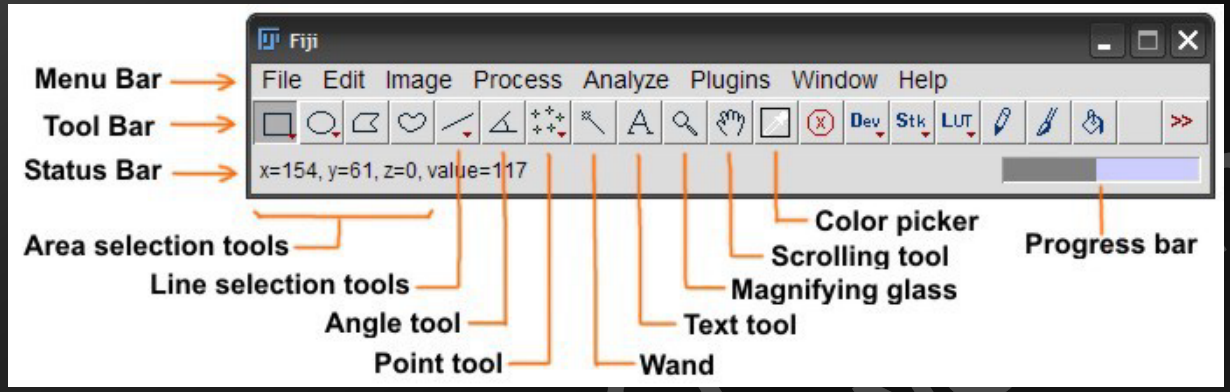
Copy “Image_Stabilizer.class” and “Image_Stabilizer_Log_Applier.class” from flash drive

Plugins > Install Plugins and navigate to folder

Restart Fiji

Plugins > Image Stabilizer: “There are no open images”

FIJI = IMAGEJ



- **Public-domain image analysis, created by Wayne Rasband at NIH.**
- **Written in Java → Cross-platform (Windows / Mac / Linux)**
- **Large number of free plugins, community support**
- ***Micro-manager: acquisition version, good driver support***
- ***Jack of all trades → convoluted, redundant menus***
- **Free and fast alternative to Photoshop: www.Irfanview.com (Windows)**
  → Cropping, annotations, color adjustment, batch processing, etc…

Setup & Introduction

- Memory and Threads: Edit > Options > Memory & Threads, restart
- Open Fluocell.tif, zoom in & out, pan, pixel location and values
- Tool area: right click for options (down arrow = popup menu, broken vs. straight line), double-click for more options (line width).
- Draw an area, click on it and move / resize. Select an area, Edit > Copy, click outside area, Edit > Paste. Click inside ROI and drag. Click outside area. Edit > Undo (1 level only), File > Revert.
- Draw an area, Image > Crop. Edit > Undo. Draw an area, Image > Duplicate. File > Revert.
- Open Fluocell color.tif, Image > Color > Split Channels, LUT > Red, Green and Blue to each, check each Image > Type = 8 bit (w/ palette), Image > Color > Merge Channels, uncheck Create Composite, OK.

File > Close all

Calibration & Overlay

- **Set scale: Open Ruler.tif, draw reference line between landmarks, Analyze > Set scale, enter known distance. Click anywhere, Analyze > Set scale, show 2.49 pixels / micron. File> Close.**

  Open Fluocell.tif, Analyze > Set scale (1 pixel = 0.28 μm), OK, Analyze > Tools > Scale bar (destructive)

- **Text: double-click text icon, select font size, color = red, click on image, type text, Don't click!, Image > Overlay > Add selection (non-destructive, Image > Overlay > Show / Hide). Image > Overlay > Flatten, destructive, image becomes RGB to accommodate red color. File > Close All.**

- **Calibration bar: Open Fluocell.tif, LUT > Fire, Analyze > Tools > Calibration bar (overlay option). File > Close**

- **Manual time stamp: open Live.tif, select color with color picker (default=black). Image > Stack > Time stamper, (know) interval = 4 sec., loc. 200, 10, uncheck '00.00' format, suffix: sec, OK. "\" or wheel. File > Close**

- **B/C: Open Uneven.tif. Image > Adjust > Brightness/Contrast, Auto = normalize. Apply to commit *(change data!)*. Close.**

Measurements

- **Plot profile: open Dapi.tif, draw line across particle, Analyze > Plot profile (Ctrl-k), List, File > Save as. Click Live, move and redraw line.**

- **Measure: draw region, Analyze > Set Measurements, Area, Mean gray value, SD, Min & max, OK. Analyze > Measure (Ctrl-M)**

- **Multi-measure: draw region, Analyze > Tools > ROI manager > Add, draw other regions and add them, Measure (ROI man.). Close ROI manager.**

- **Intensity distribution: draw region, Analyze > Histogram, click Live and move / resize region. Close histogram.**

- **Object counting: right-click on Point Tool > Multi-point tool. Click on multiple objects in image, Ctrl-M = coordinates.**

- **Distances: Analyze > Set Scale 0.28 μm/pix, draw line, Analyze > Tools > ROI manager > Add, draw segmented line, add to ROI manager, Measure.**

Particle Analyzis

- **Binary image: open Dapi.tif, Image > Adjust > Threshold, Click Dark Background, Make objects red, Apply = make binary (1-bit).**

Process > Binary > Make binary equivalent to Apply. File > Revert.

- **Distribution**

Set scale at 0.28 μm/pix, threshold and binarize (Objects may be dark, check intensity). Analyze > Set Measurements, click Area + Center of mass. Analyze > Analyze particles, show outlines (always!), display results + exclude on edge + clear results, OK. Close Measurement, File > Revert.

- **Morphometry:**

Set scale at 0.28 um / pix, Image > Duplicate, Image > Adjust > Threshold, make objects red, Apply (convert to binary), Analyze > Set Measurements, Area + Mean + SD + Center of mass, redirect to Dapi.tif (greyscale).

Click on binary image, Analyze > Analyze particles, set size classifier, OK. File > Close.

Filters

- **Background: Open Uneven.tif. Draw line full diagonal, Ctrl-k. Click on Uneven.tif, Process > Subtract background , uncheck light background, 50 pix rolling ball, OK. Click image, Ctrl-k. Open out-of-plane.tif, repeat.**

- **Noise: open Noisy.tif, Process > Noise > Despeckle, File > Revert, Process > Filters > Median. Close.**

- **Stripes: open Banding.tif. Process > FFT > Bandpass Filter, down to 100, up to 2 pix, horizontal stripes, all options off.**

- **Binary filters: open Noisy.tif, Image > Adjust > Threshold, Apply. Process > Binary > Options, Count = 4**
  Process > Binary > Erode, Process > Binary > Dilate. Close.
  Redo with Process > Filters > Gaussian Blur on greyscale image.

Stacks - z series

- **Dimensions: Open Root Red.tif, browse in z with slider. Close.**

Open Root Dual Color.tif, browse in z and color. Close all.

- **Import / export: Open Live.tif, File > Save as > Image sequence, start and increment number (create new folder!). Close file. File > Import > Image sequence, click on open, check Sort names numerically, OK.**

→ *Build a stack to batch-process multiple images…!*

- **Maximum Intensity Projection:**

Open Root Red.tif. Image > Properties, check / set x, y z at 0.28/0.28/0.39 μm / pix. Image > Stack > Z-projection, select Max intensity, OK. Close.

- **3D projection: Click Root Red.tif, Image > Stack > 3D projection brightest point, y-axis rotation, 180 w/ 5 deg increment, interpolate, OK (Try different conditions). Plugin > 3D viewer for real 3D.**

- **Open Root Red.tif, draw a line, Image > Stack > Reslice (kimograph). Delete line from stack (click), Image > Stack > Reslice (resample volume, orthogonal sections). Close all.**

Stacks - Time series

- **Intensity: open Live.tif, draw region, Analyze > Set Measurements, Mean gray value + SD + Stack position, Analyze > Measure (Ctrl-M) = 1 plane only. Image > Stacks > Plot z-axis profile.**

- **Multi-measure: Analyze > Tools > ROI manager > Add, draw and add other regions, More > Multi-measure, select all slices + one row per slice. Close.**

- **Temporal color code: open Track.tif. Image > Hyperstack > Temporal color code, Lut = Fire, create scalebar. Can be used to depth-code z-stacks. File > Close All.**

- **Enhance contrast: Neurite.tif, Process > Enhance contrast, uncheck all options except Process all slices. Can be used with flat images**

- **Image stabilizer: on first slice of Neurite.tif, Plugins > Image stabilizer, translation, output to new stack. File > Close All.**

EXERCISES

Exercise 1: Motion Tracking

- **Open Track.tif. Establish position of bead versus frame number.**
  ***Hint*: threshold**

Solution:

Image > Adjust > Threshold, Dark background, Apply.
Analyze > Set Measurements, Center of Mass.
Analyze > Analyze Particles

→ ***If problem thresholding: substract background***

Exercise 2: Colocalization

- **Open Mito Division.tif. Analyze colocalization between green and red channels**

***Hint*: separate colors, smooth, Analyze > Coloc. > Coloc. Threshold**

Solution:

Image > Adjust > Brightness / Contrast, Auto (both channels)

Excessive noise: Process > Filter > Gaussian Blur, 1.5, Preview, OK

Image > Colors > Split Channels

Analyze > Colocalization > Colocalization Threshold

Assign channels 1 and 2, no ROI, Red/Green combo, check Show Colocalization Map, Scatter Plot and Set Options. Click OK

Options: Check Show Linear Reg. Sol., Show Thresholds and Pearson's Above Threshold, % Image Volume Colocalized. Click OK

Control:

Green and red images: Image > Type > 8 bit, Image > Color > Merge
Coloc. Pixel Map: Image > Type > 8 bit, LUT > Yellow, Adjust B/C

Compare both images

→ *100% yellow overlay = Crosstalk, not colocalization*

Exercise 3: Dynamic Intensity meas.

- **Open Live.tif. Measure intensity of cell in lower left quadrant**

***Hint*: crop image and isolate cell based on size. Morphometry.**

Solution:

Image > Stacks > Z Project…, Max Intensity
Draw ROI around cell
Click on Live.tif, Edit > Selection > Restore Selection.
Image > Duplicate, check Duplicate Stack.
Process > Filters > Gaussian blur, sigma=1, OK, all slices
Click on Live-1.tif, Image > Duplicate
Image > Adjust > Threshold, Apply
Analyze > Set measurements, check Area, Mean grey value, SD and Stack position. Redirect to Live-1.tif (greyscale), OK.
Click on Live-2.tif (binary)
Analyze > Analyze Particles, size: 150 μm² -inf, Show outlines, Check Display results, Include holes. Uncheck Exclude on edge.
Process all images, OK.

→ ***Show Outlines = critical consistency check***

Exercise 4: RNAscope

- **Open RNAscope.czi, estimate number of dots per cell**
  Hint: *Process > Binary > Voronoi for cell outlines*

Solution:

Image > Color > Split Channels, Image > Adjust > B/C, Auto

1) Dapi image: Image > Duplicate
   Process > Filters > Mean, radius=10
   Image > Type > 8 bit
   Image > adjust > Auto local threshold, Niblak, r=15
   Image > adjust > Threshold, 1-255, Apply
   Analyze > Analyze Particles, 40-inf, show masks, exclude on edges
   Process > Binary > Erode, then Dilate, then Watershed.
   Image > Duplicate → (1). On dup., Process > Binary > Voronoi
   Image > Adjust > Threshold, 1-255 Apply → (2)
   Process > Image Calculator, Add (1) and (2)
   Image > Adjust > B/C, lower Brightness, Set. Image > Type > RGB
2) RNAscope image: Process > Subtract Background, 10
   LUT (Fn key) > Fire, B/C Auto. Image type > RGB, Add to above

→ ***Possible to segment challenging images without AI***

Exercise 5: Batch processing / Scripting

- **Open BRDU image folder, measure nucleus size on each image**
  Scale = 0.8 um / pixel

Solution:

Plugins > Macro > Record

Open BRDU1.tif

Analyze > Set scale, 1 pixel = 0.8 um, OK

Image > Adjust > Threshold, uncheck Black background, Apply.

(clear additional threshold entries from log)

Process > Binary > Erode, then Process > Binary > Dilate

Analyze > Set measurement, Area.

Analyze > Analyze particle, 50-inf., Display Results, Exclude on edge, *uncheck Clear Results* (do not Show Outlines, requires variables...!)

Recorder window: copy all lines except first

(Do not close a single window until that point!)

Process > Batch > Macro, select input folder, paste macro in window

Close all windows, Process.

→ ***Batch processing dangerous, check consistent image quality...!***

FIJI TAKE-HOME MESSAGE

- **Pros:**
  - **Powerful and extensible with plugins, scriptable**
  - **Community support (http://rsbweb.nih.gov/ij/)**
  - ***FREE***

- **Cons:**
  - **Menus not very intuitive**
  - **Plugins not always stable**
  - **Not scalable to large images**

→ ***Hard processing / AI cannot turn crap into gold:***

CRAP IN = CRAP OUT

→ ***No "undisclosed" image manipulation***

→ ***Do not batch-process unless consistent image quality***