Technologies for microscopic image analysis
显微图像分析技术

第4课

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Overview of this course

第一章　光学显微术　Optical microscopy

1. History of microscopy
2. Principles of optics
3. Bright field microscopy
4. Fluorescence microscopy

第二章　图像数据处理与分析　Image data processing and analysis

1. Processing of digital image data
2. Quantitative image analysis
3. **Application of ImageJ/Fiji software**
4. **Image processing and analysis with the Python coding environment**
5. Bioimage informatics

第三章　先进的显微术　Advanced microscopy

1. Advanced imaging technology: from single molecules to living animals
2. Super-resolution microscopy
3. Other bioimaging techniques

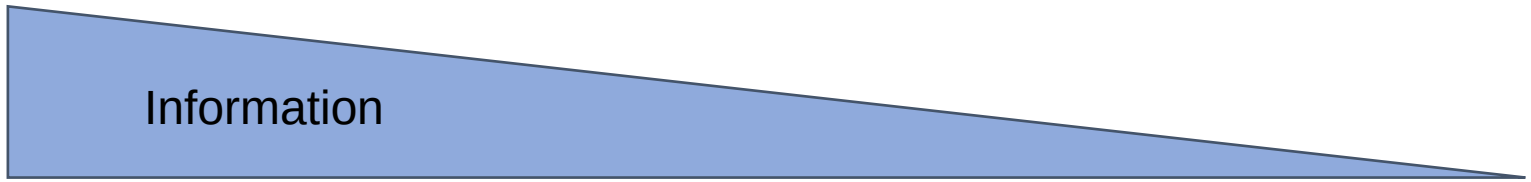
第四章　人工智能的应用　Application of AI

1. Overview of AI technology
2. AI for bioimaging

Image file formats
图像文件格式

For backup | For analysis | For publication/presentation

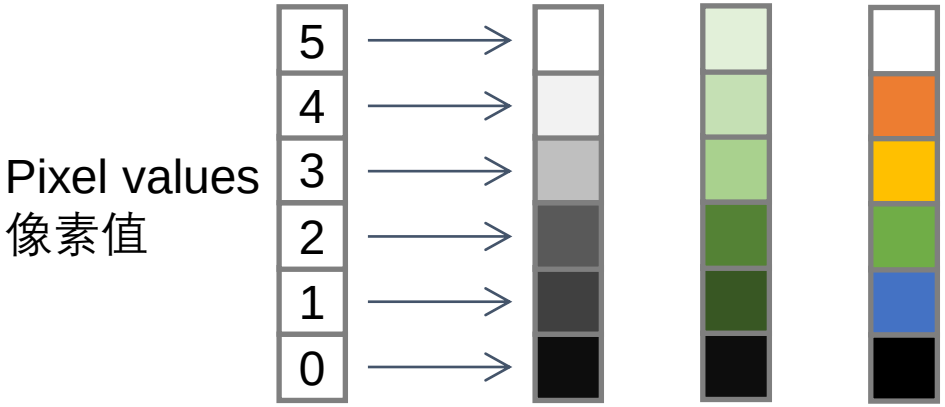
Format	RAW (.lif, .czi, etc)	TIFF	BMP, PNG	JPEG
Bit depth (tone) 色调,灰阶	8/12/16-bit	8/12/16-bit (32-bit)	8-bit only	8-bit only
Additional information (metadata) 元数据	Full (laser power, gain, pixel size, etc)	Full or partial (e.g., pixel size, 1 px = 0.5 µm)	None	None
Compression 压缩	No	No or reversible compression	No or reversible compression	Irreversible compression
Stack (multi-channel, time series) 堆栈	Yes	Yes	No	No
File size	Large	Large	Small	Small



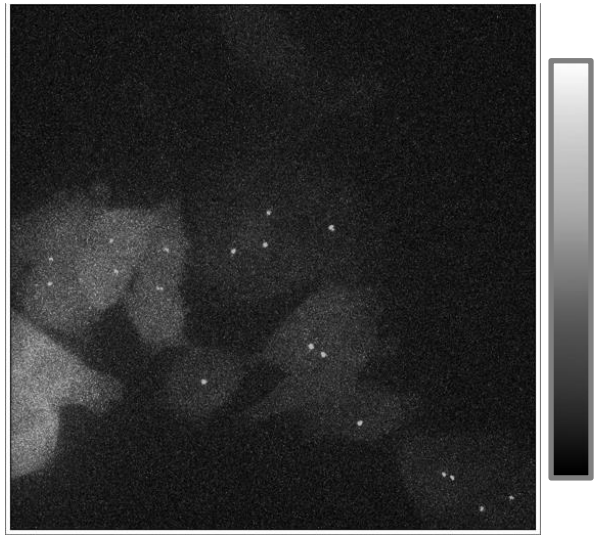
Lookup table
查找表

Lookup table (LUT):
Color code for pixel values

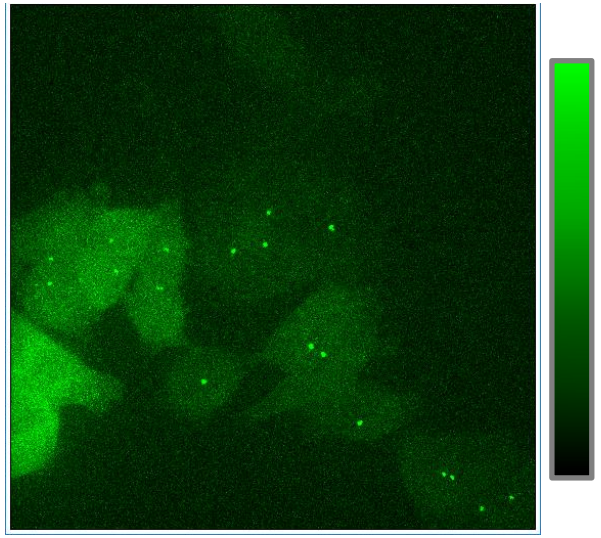
Any color can be assigned for each pixel value
可以为每个像素值指定任何颜色



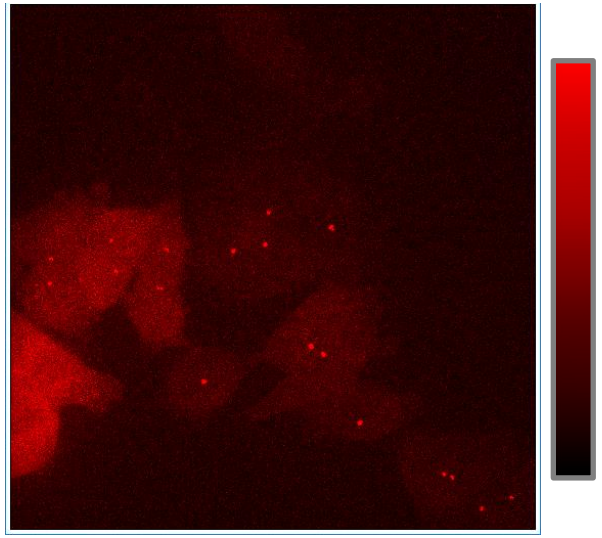
Grayscale



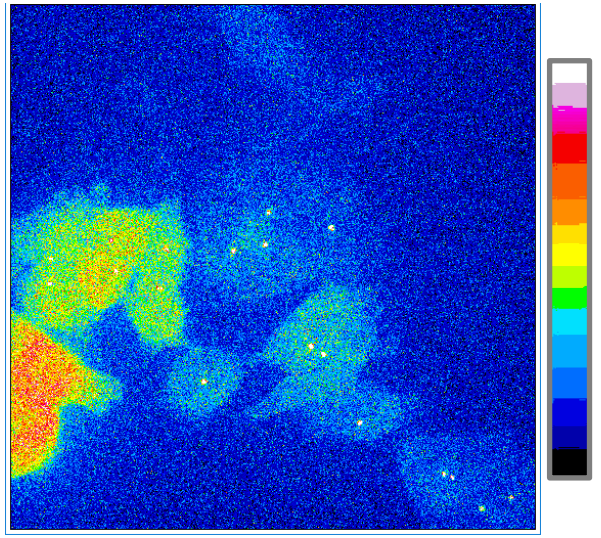
Green



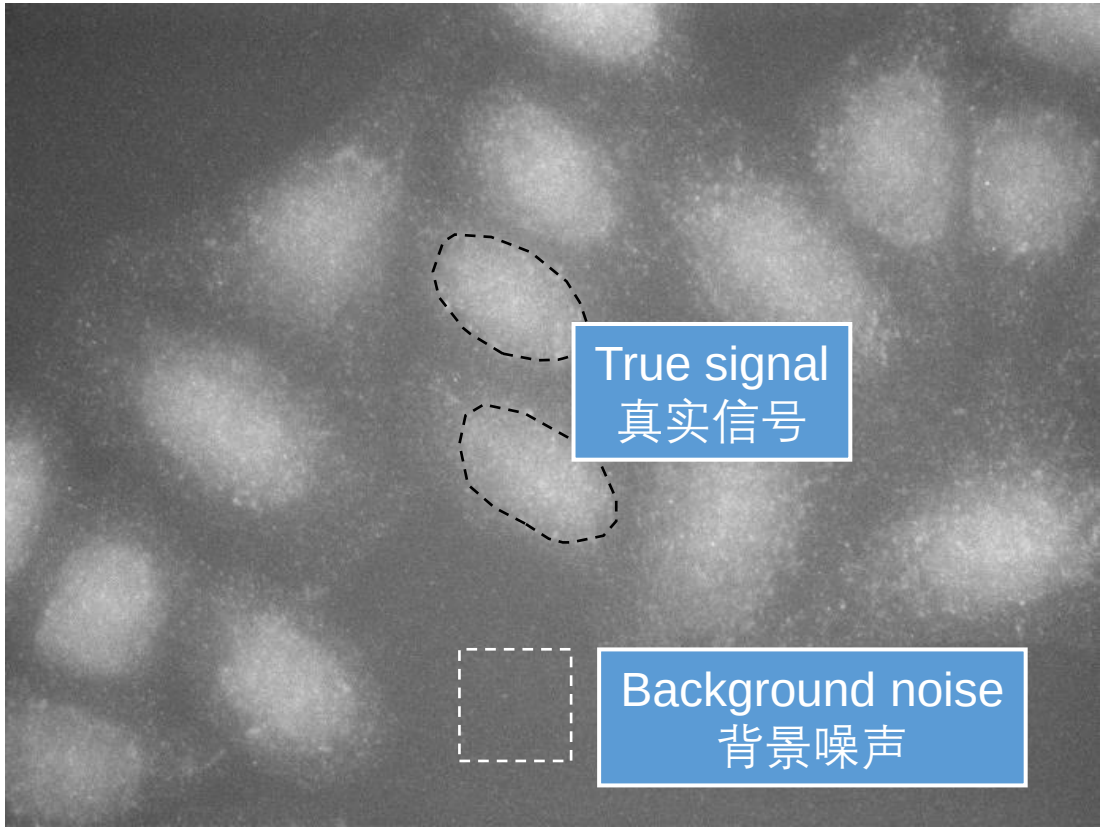
Red



16-colors

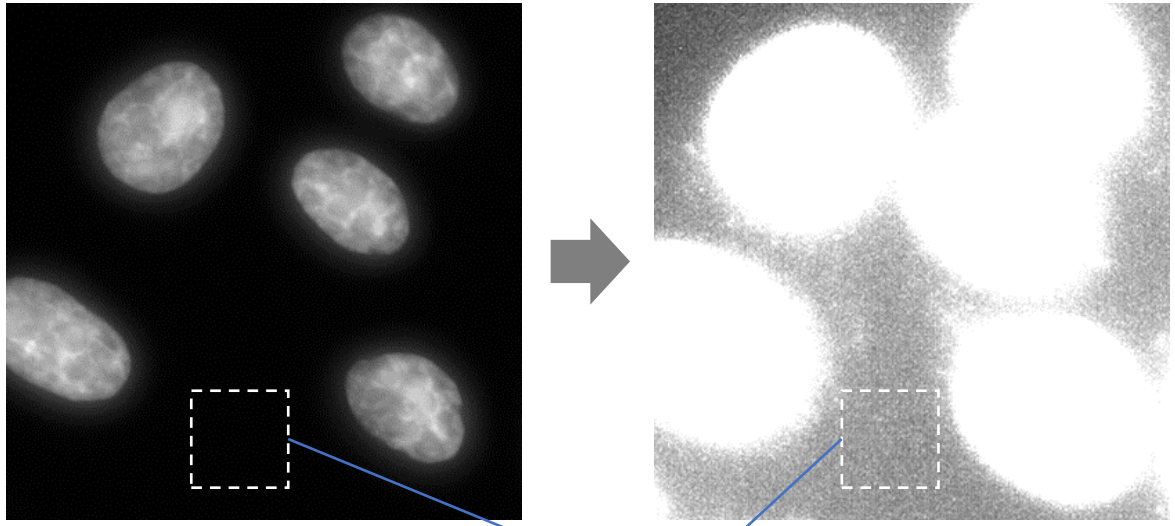


Background noise
背景噪声



No background noise?

High contrast

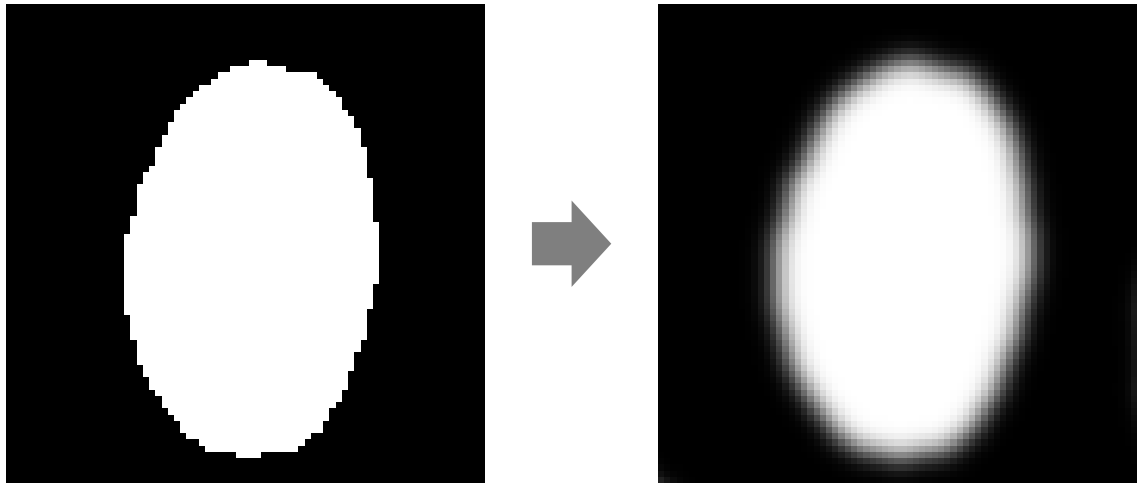


Mean pixel value = 270.16

Every image contains background noise!
每个图像都包含背景噪声!

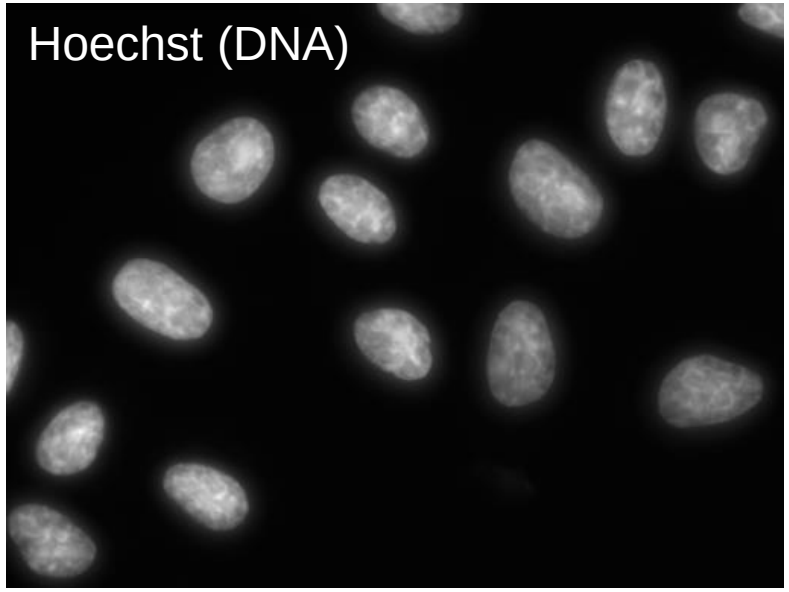
Edge smoothing
边缘平滑

Gaussian blur
高斯模糊

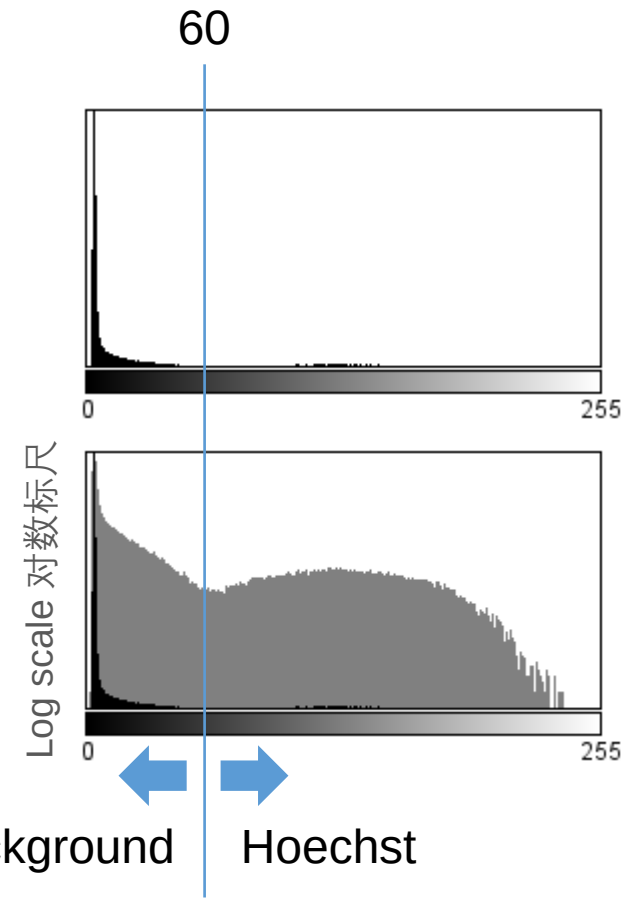


Blurring can also be used to smoothen edges
模糊处理也可用于平滑边缘

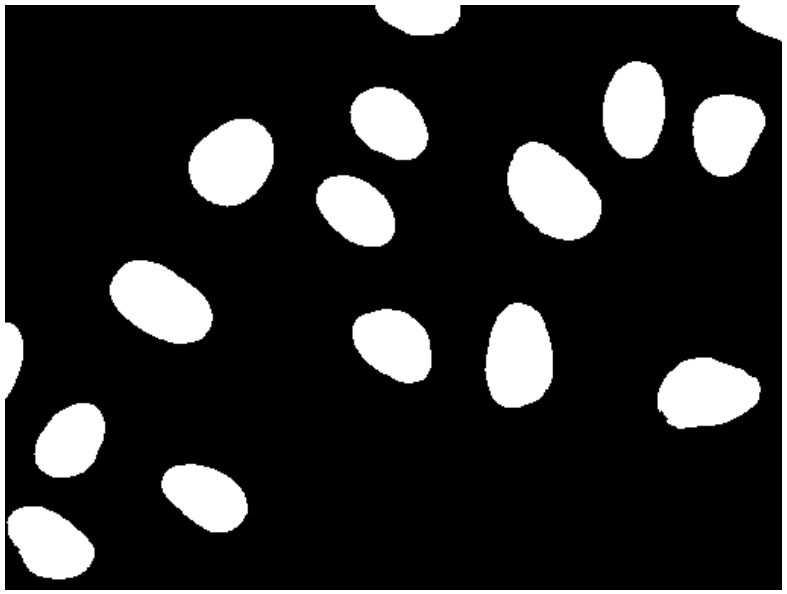
Binarization
二值化



Mean intensity ~100–200
平均强度



Binary image
二值化图像



Binarization/Thresholding
二值化/阈值化

Pixel value ≥ 60 → 255

Pixel value < 60 → 0

Binarization/Thresholding can extract objects to make masks
二值化/阈值化可以提取对象以制作遮罩

ImageJ and **Fiji**: Standard software in bioimaging

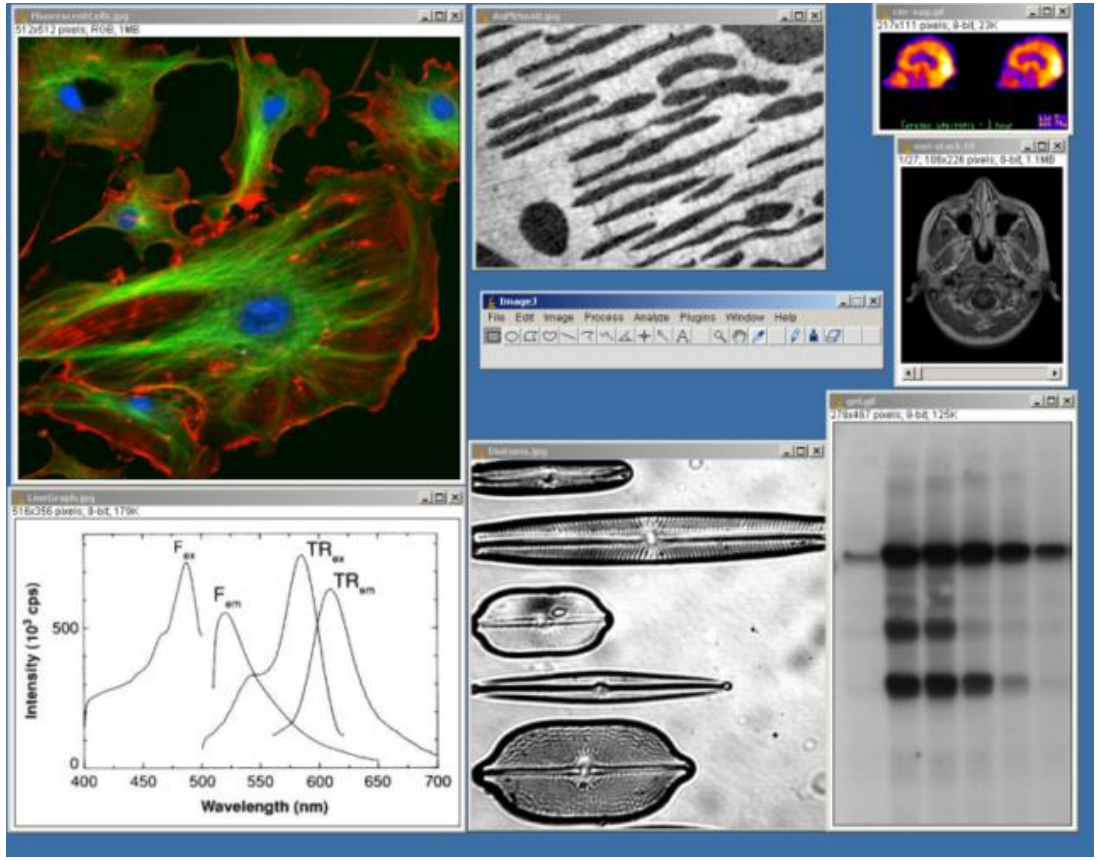
- Java-based image processing/analysis program
- Developed at the National Institutes of Health (NIH) and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin)
- Free and open architecture
- Can be customized with plugins and macros
- Many plugins and macros for bioimage processing and analysis are available
- Fiji is basically identical to ImageJ but with convenient plugins pre-installed

If you are not sure which to install, try **Fiji** first

ImageJ
Image Processing and Analysis in Java

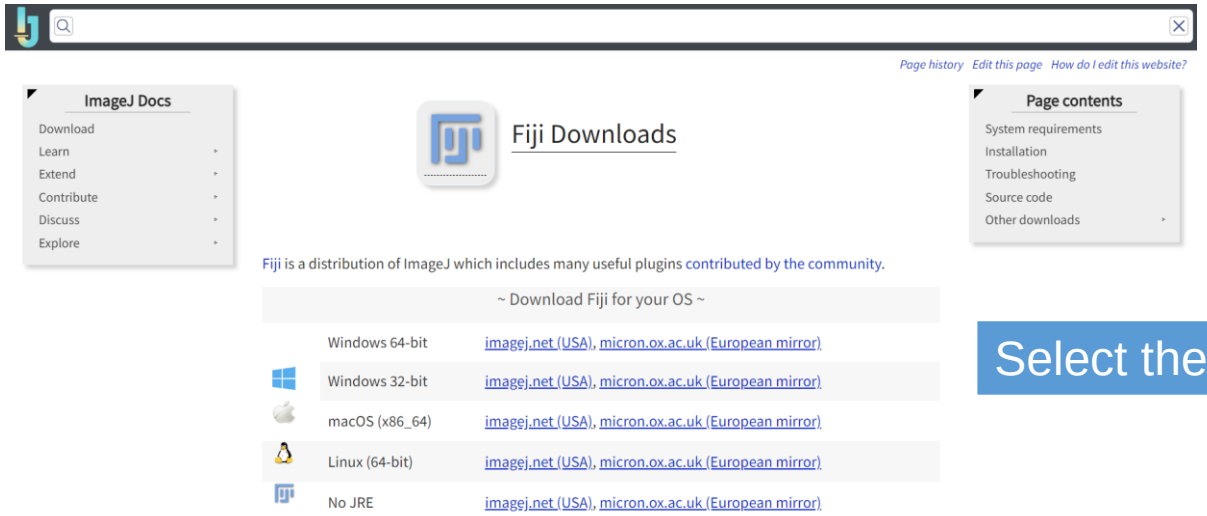
https://imagej.nih.gov/ij/index.html

https://imagej.net/software/fiji/



Installation of Fiji

Download Fiji: https://imagej.net/software/fiji/downloads



Select the OS and download Fiji

Installation

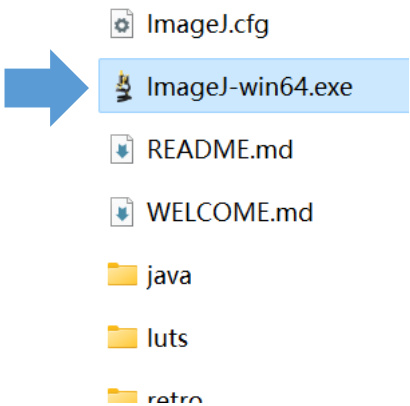
Caution for Windows users

Caution: “Program Files” not recommended!

If you are installing ImageJ2 on Windows, we strongly recommend that you store your ImageJ2.app directory somewhere in your user space (e.g., `C:\Users\[your name]\ImageJ2.app`) rather than in `C:\Program Files` or other system-wide directory. If you move ImageJ2.app to such a directory, modern versions of Windows will deny ImageJ2 write permission to its own directory structure, preventing it from being able to update. See also imagej/imagej#72.

Fiji is distributed as a portable application. That means that you do not have to run an installer; just download, unpack and start it.

Don’t need to install, just download, unzip, and run the .exe file



Contents of data

Unzip “Data_tutorial_2023.zip” (191 MB; will be 322 MB when unzipped)

HeLa_Hoechst_GM130_CENP-F — Contains 20 TIFF image files; will be also used in the Python tutorial

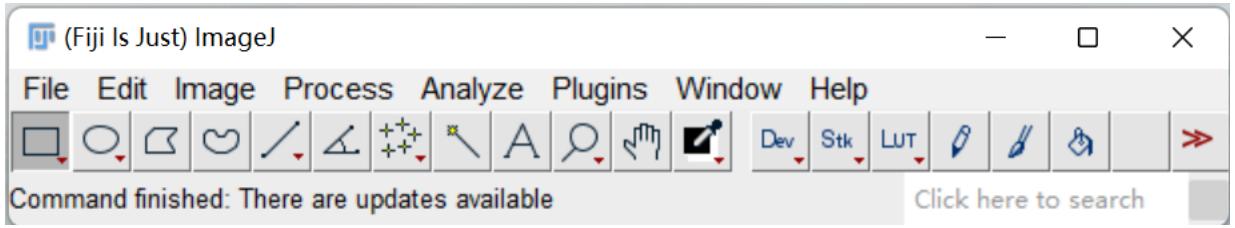
(1) E4.0 Gata6-Tsix-Nanog-2(male).lsm

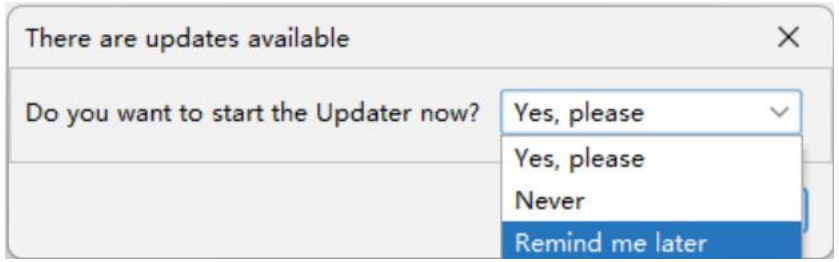
3D4_AcTub_Actin_DAPI.czi

Raw image files (z-stacks of confocal images)

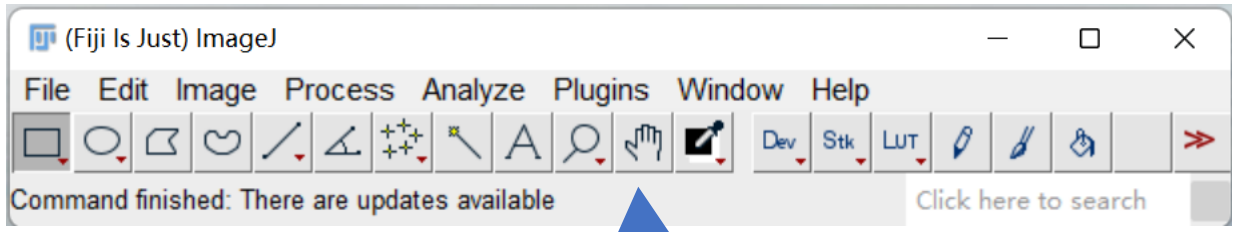
Hoechst_mean.csv — Will be used in the Python tutorial

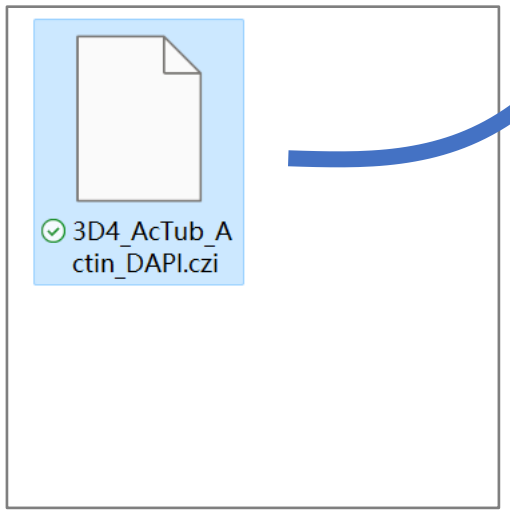
Run Fiji

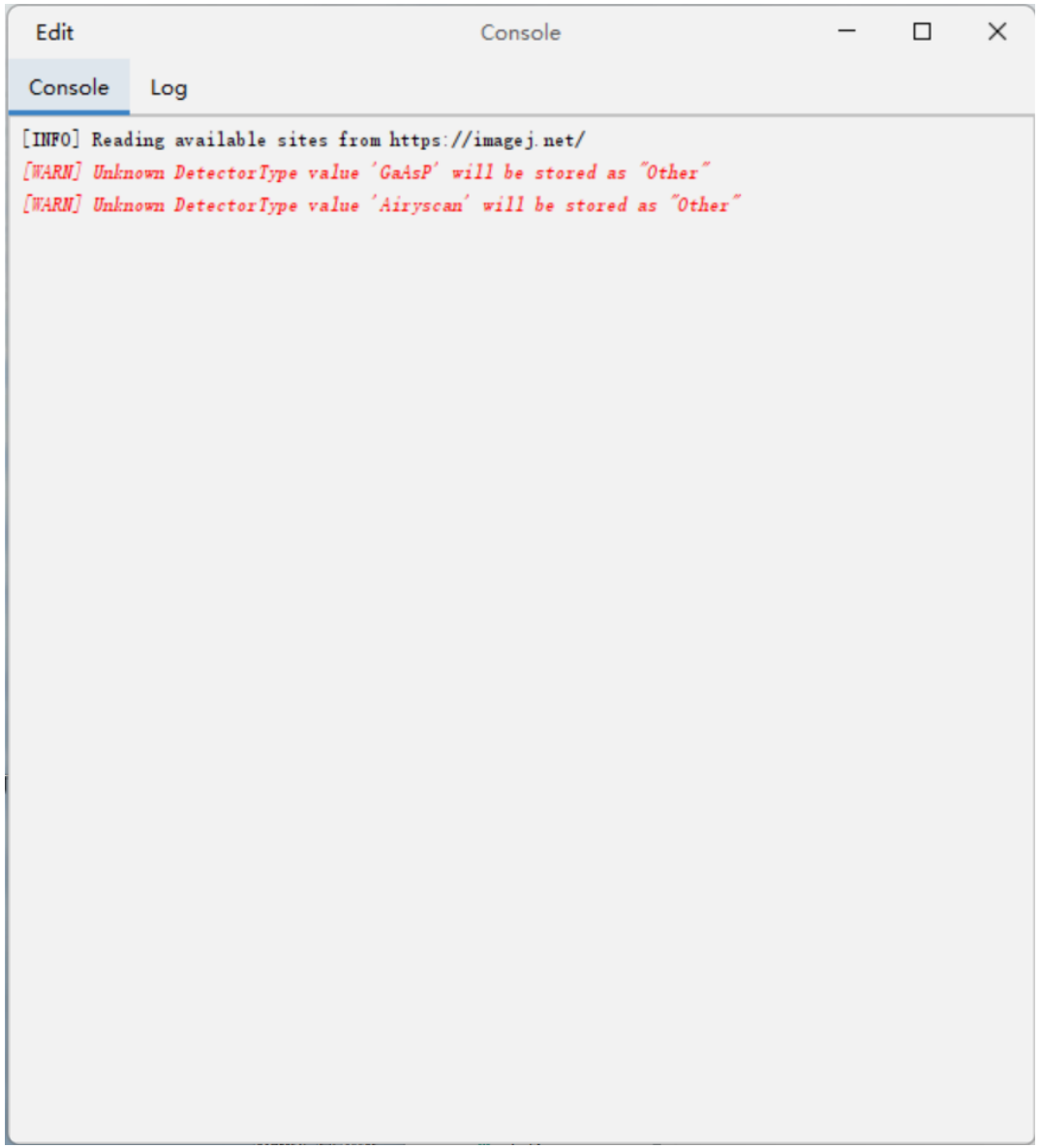

(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
Dev Stk LUT
Command finished: There are updates available
Click here to search


There are updates available
Do you want to start the Updater now?
Yes, please
Yes, please
Never
Remind me later

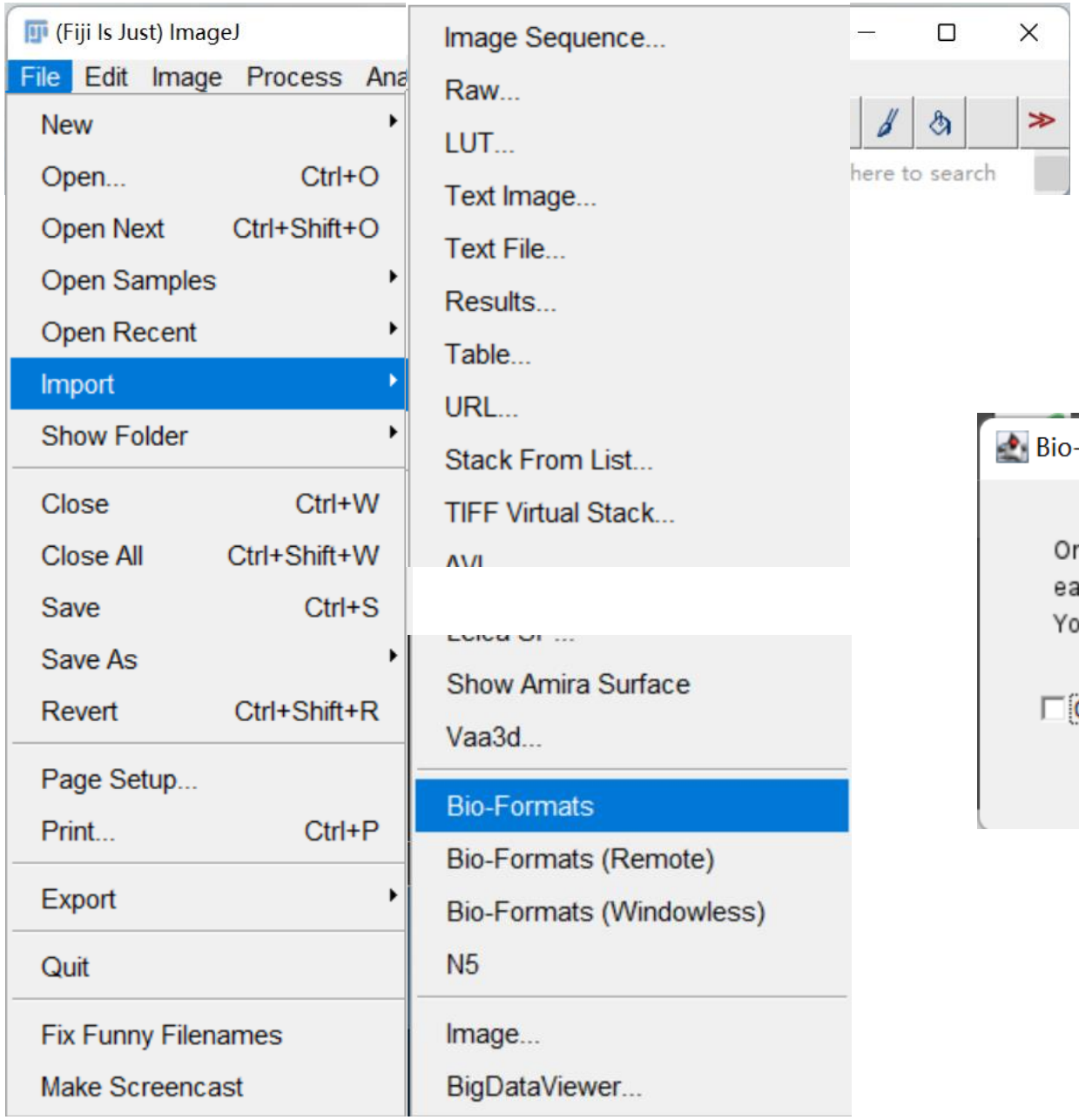
Open a raw file (.czi)

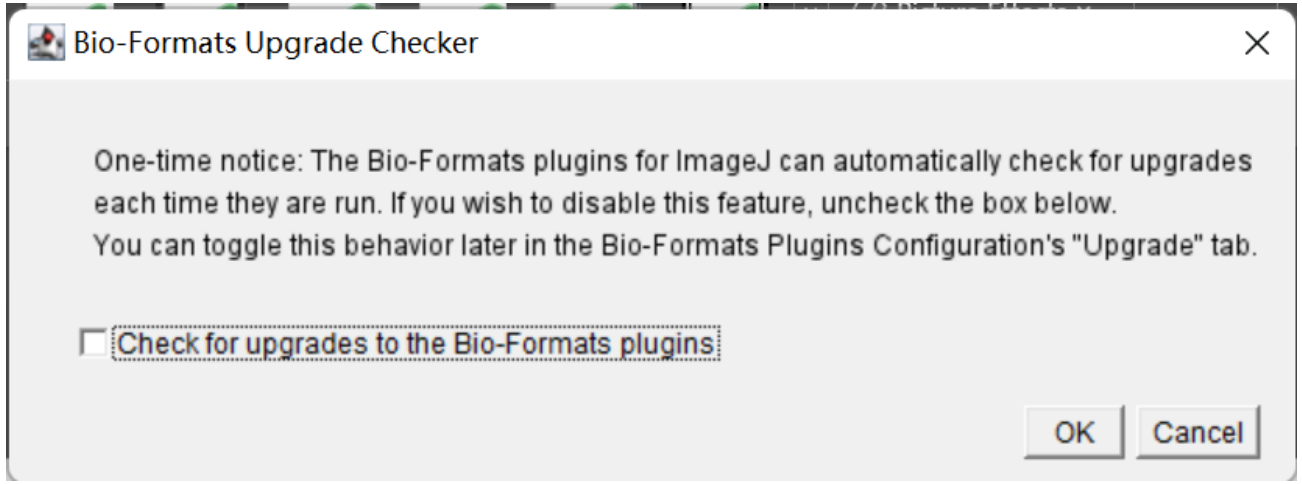




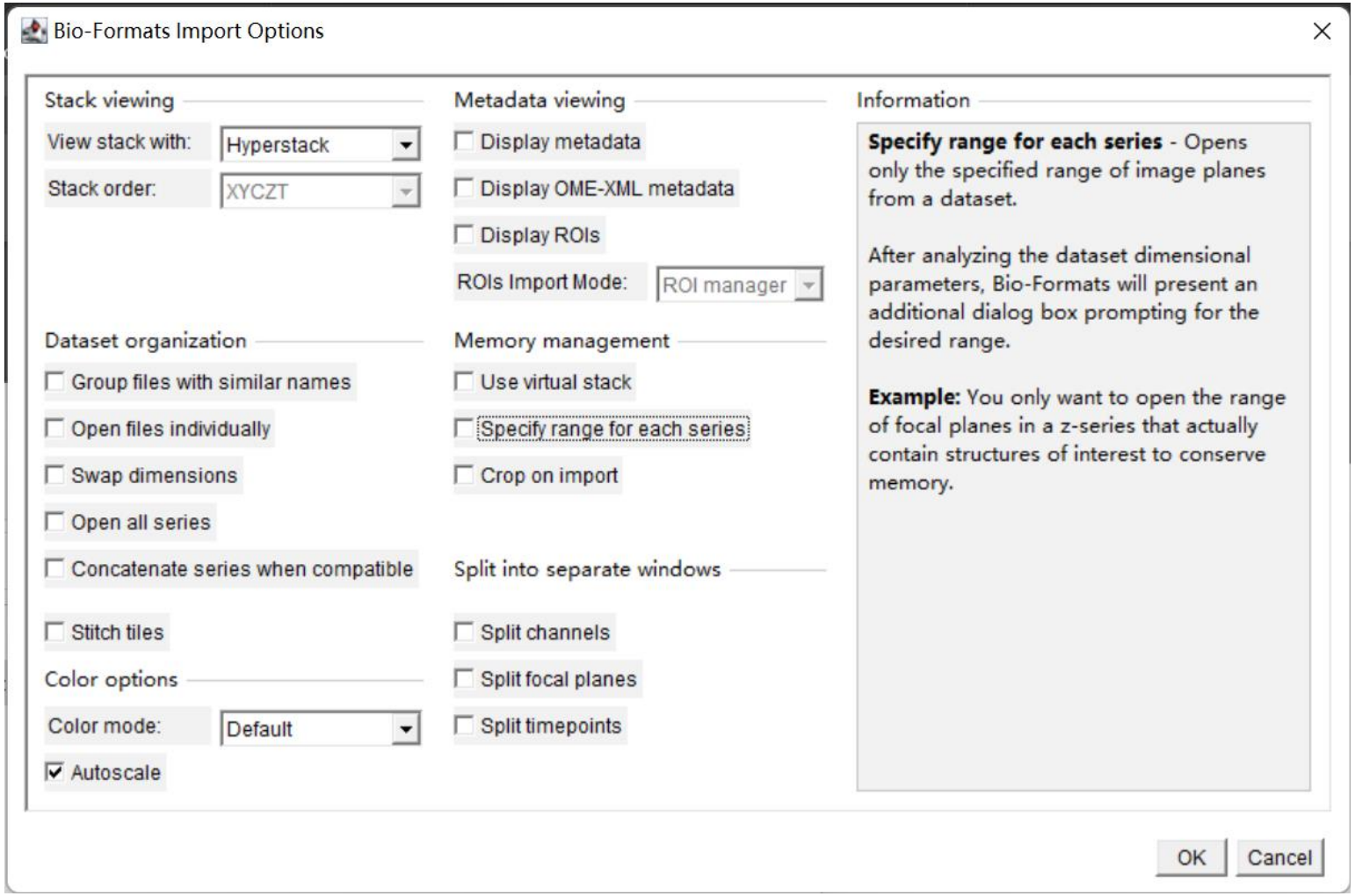


Open a raw file (.czi)

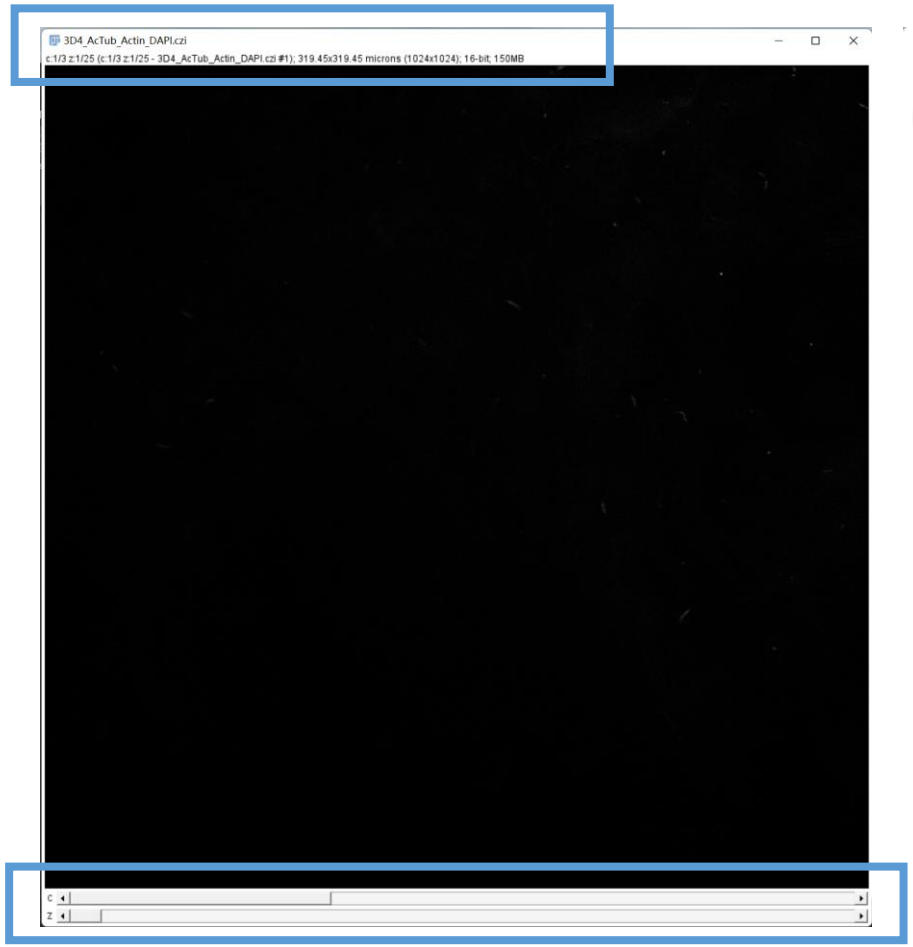




Open a raw file (.czi)



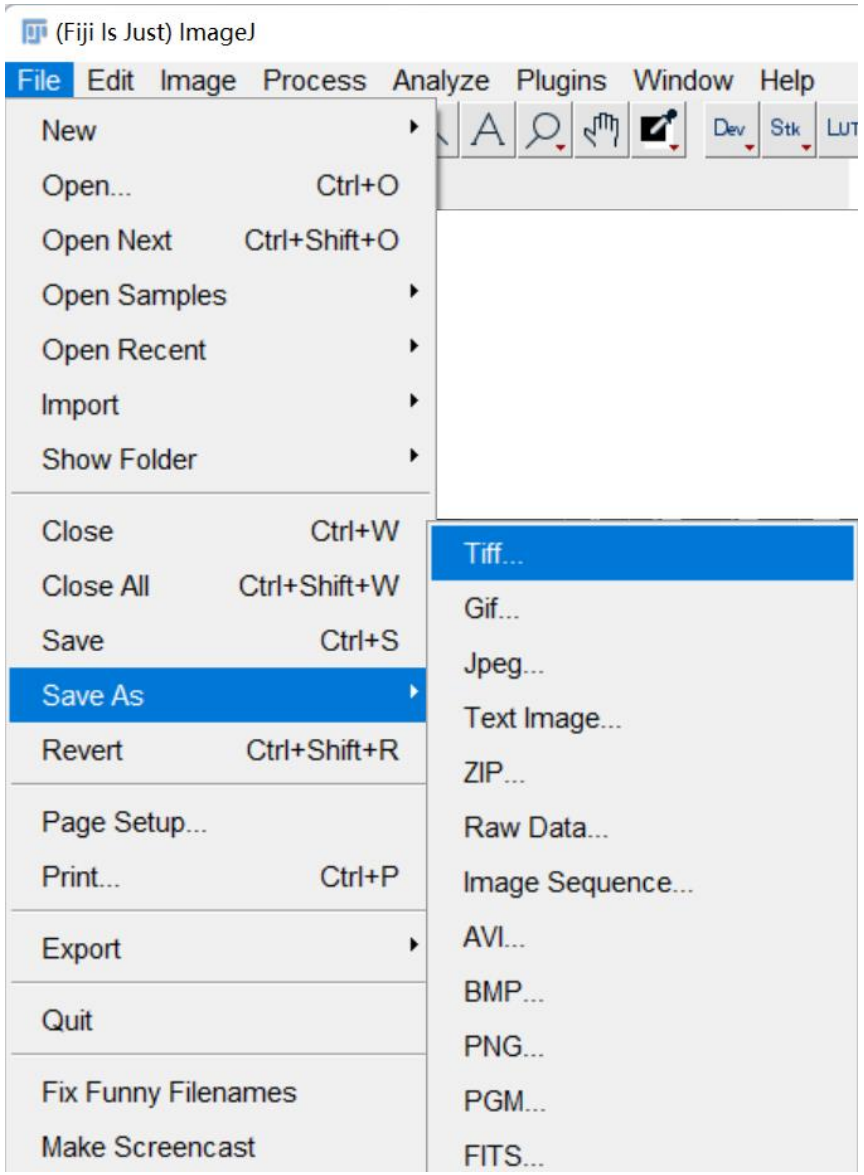
Open a raw file (.czi)



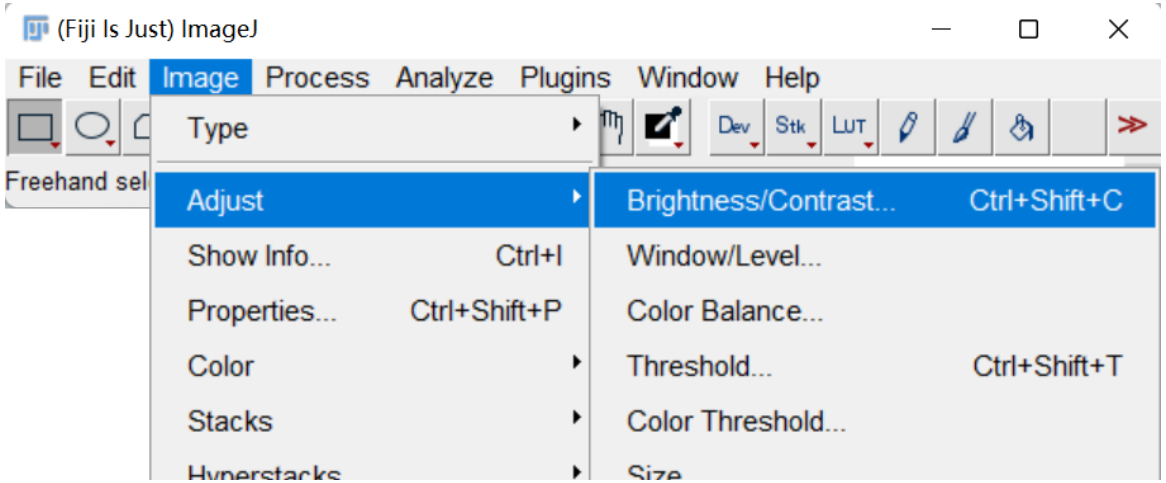
3D4_AcTub_Actin_DAPI.czi

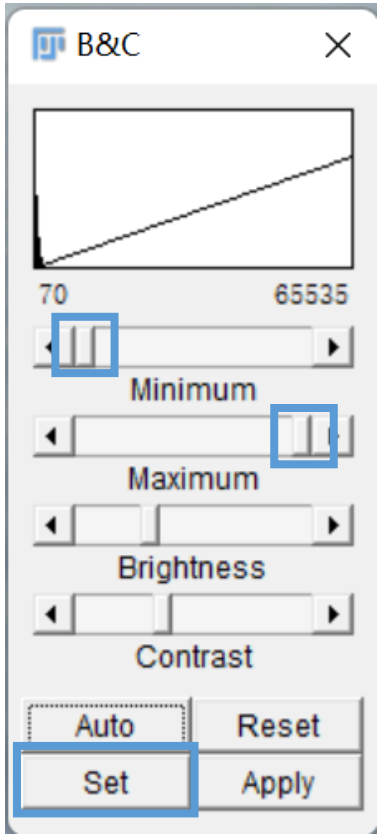
c:1/3 z:1/25 (c:1/3 z:1/25 - 3D4_AcTub_Actin_DAPI.czi #1); 319.45x319.45 microns (1024x1024); 16-bit; 150MB

Save image as a TIFF file



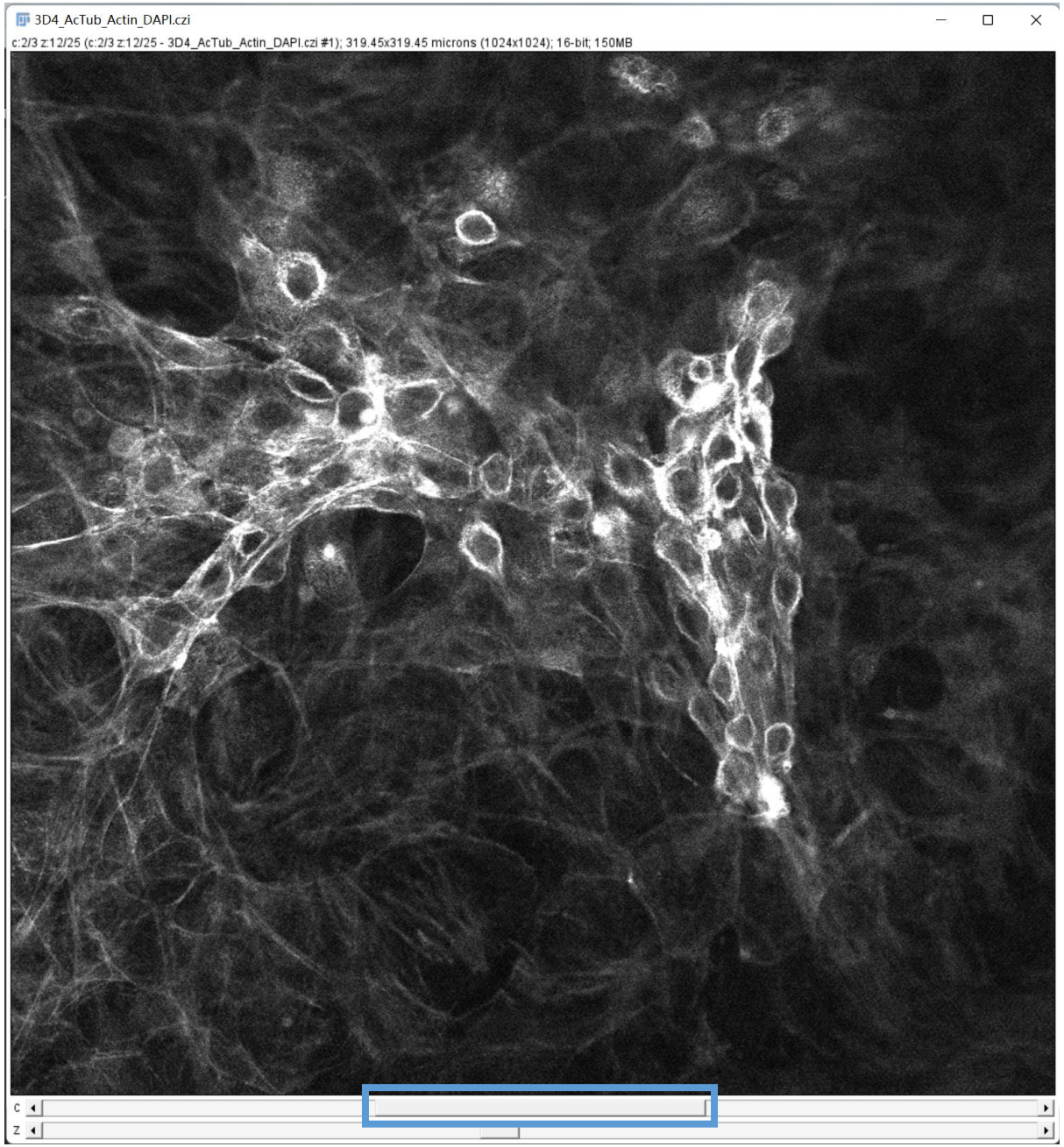
Adjust contrast

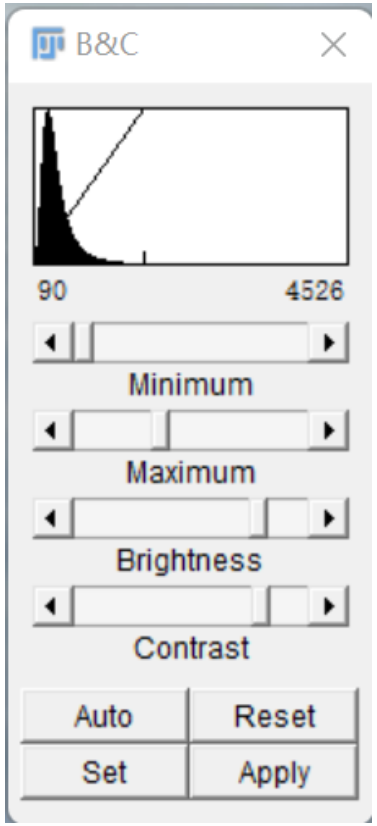




Adjust contrast by sliding “Minimum” and “Maximum” or numbers can be directly input by “Set”

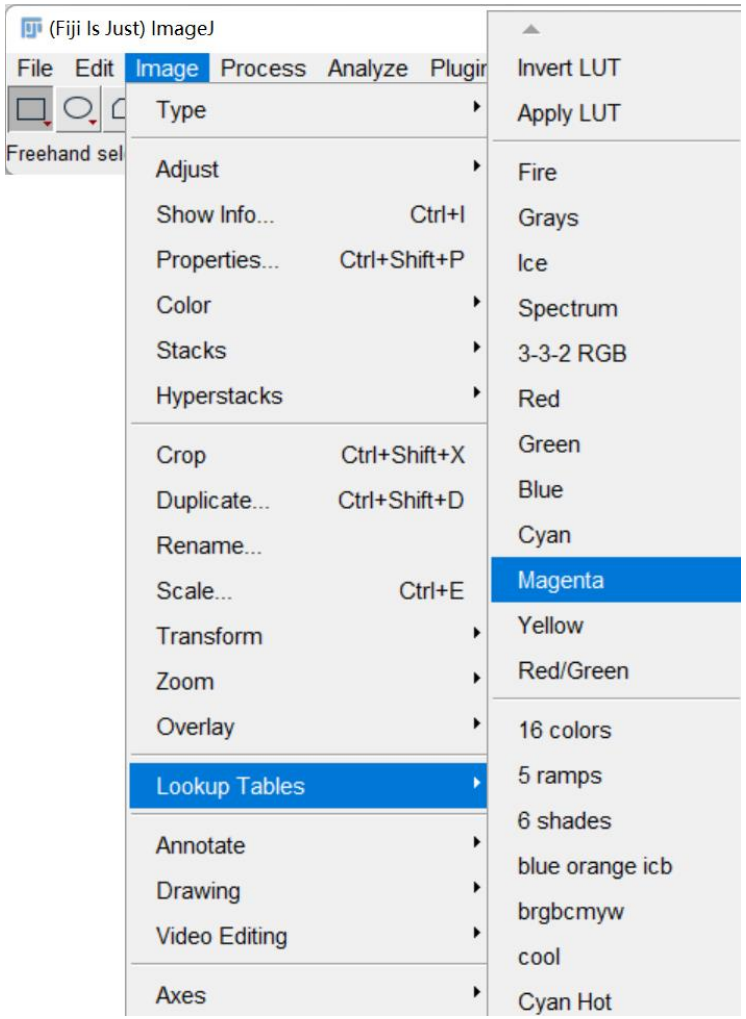
Adjust contrast

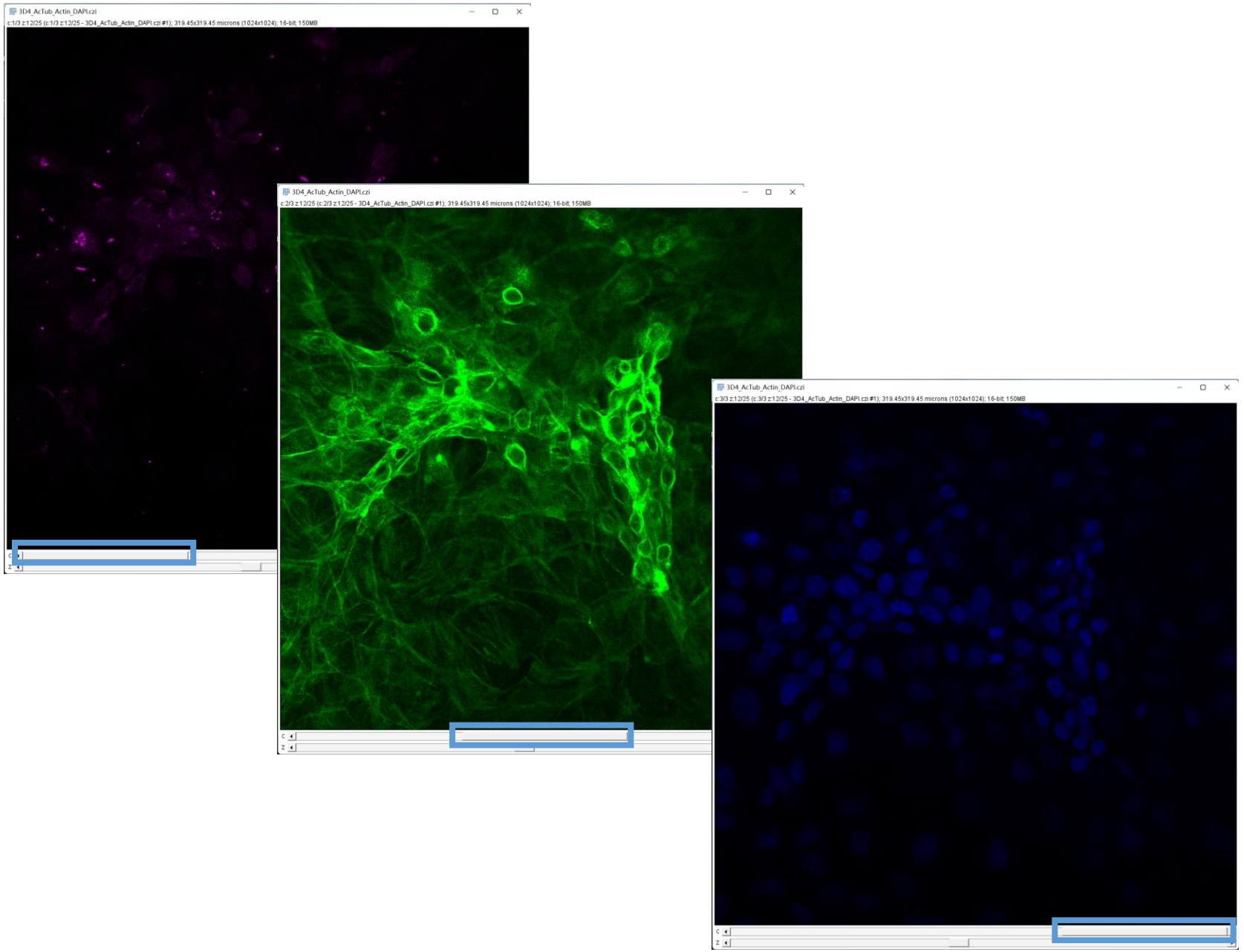




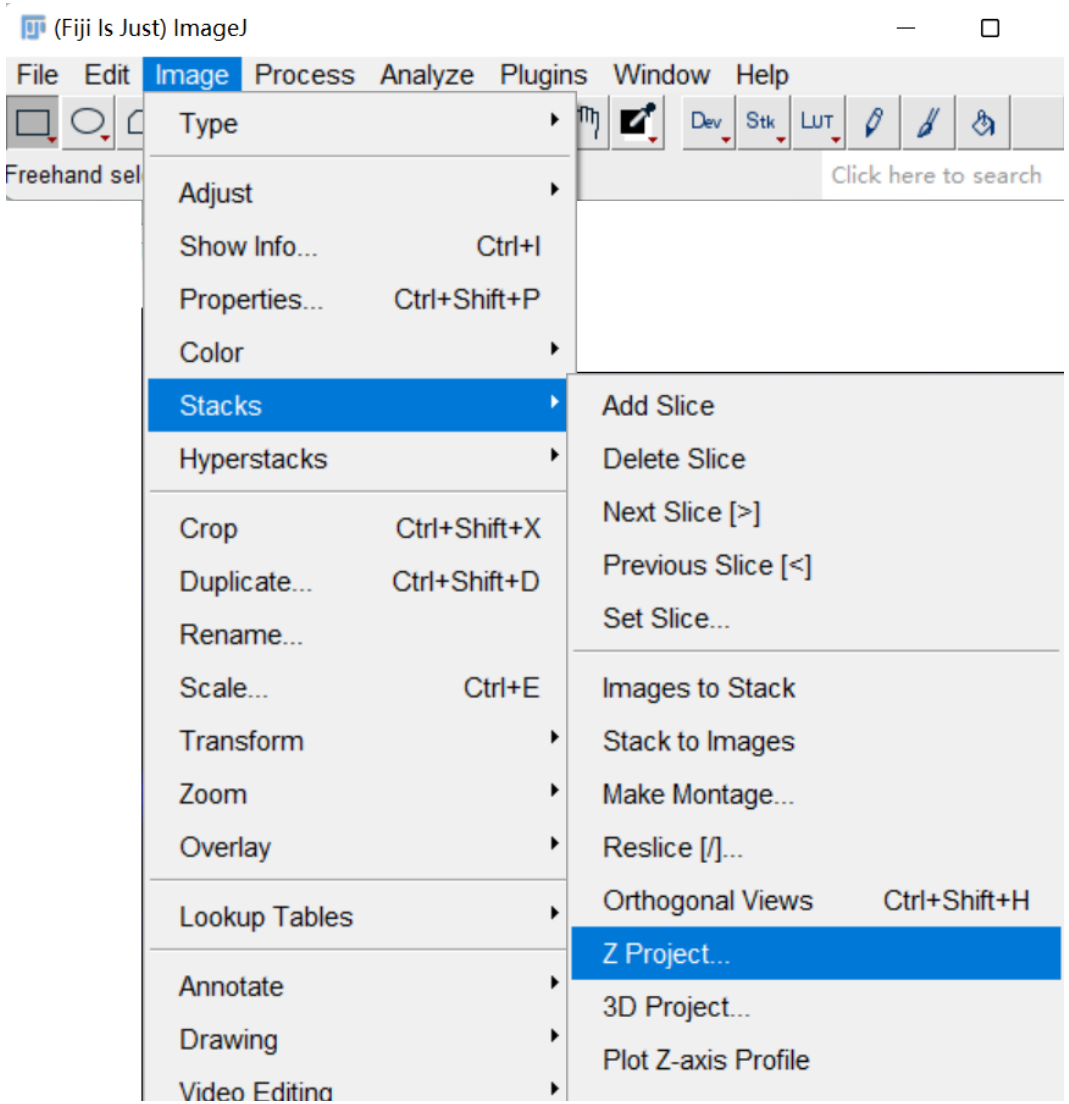
Contrast can be adjusted separately for each channel

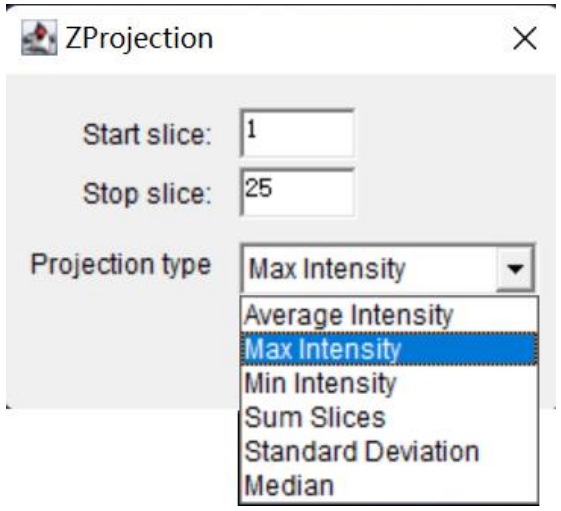
Change lookup tables (LUT)



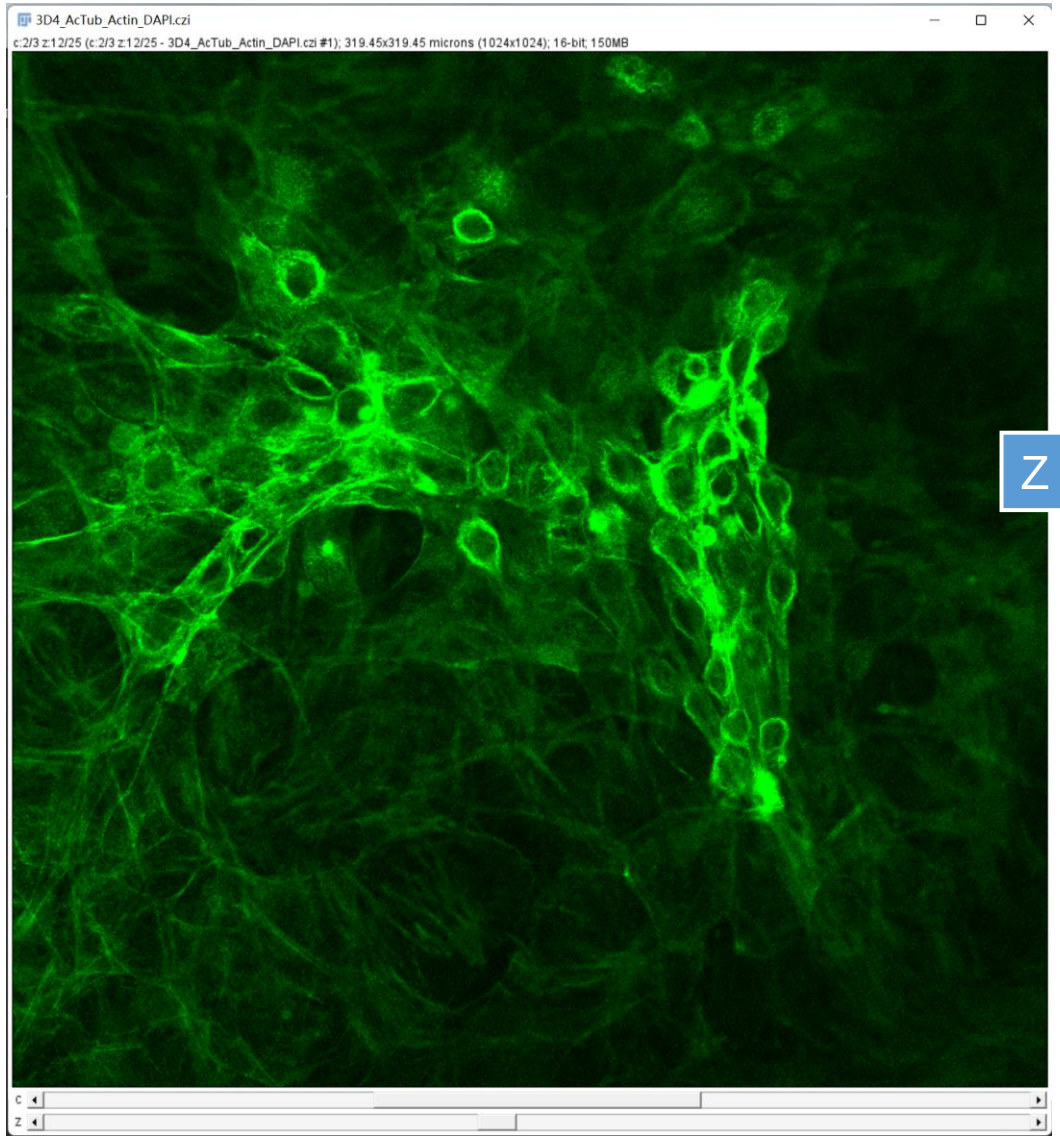


Project 3D image to 2D (max intensity projection; MIP)

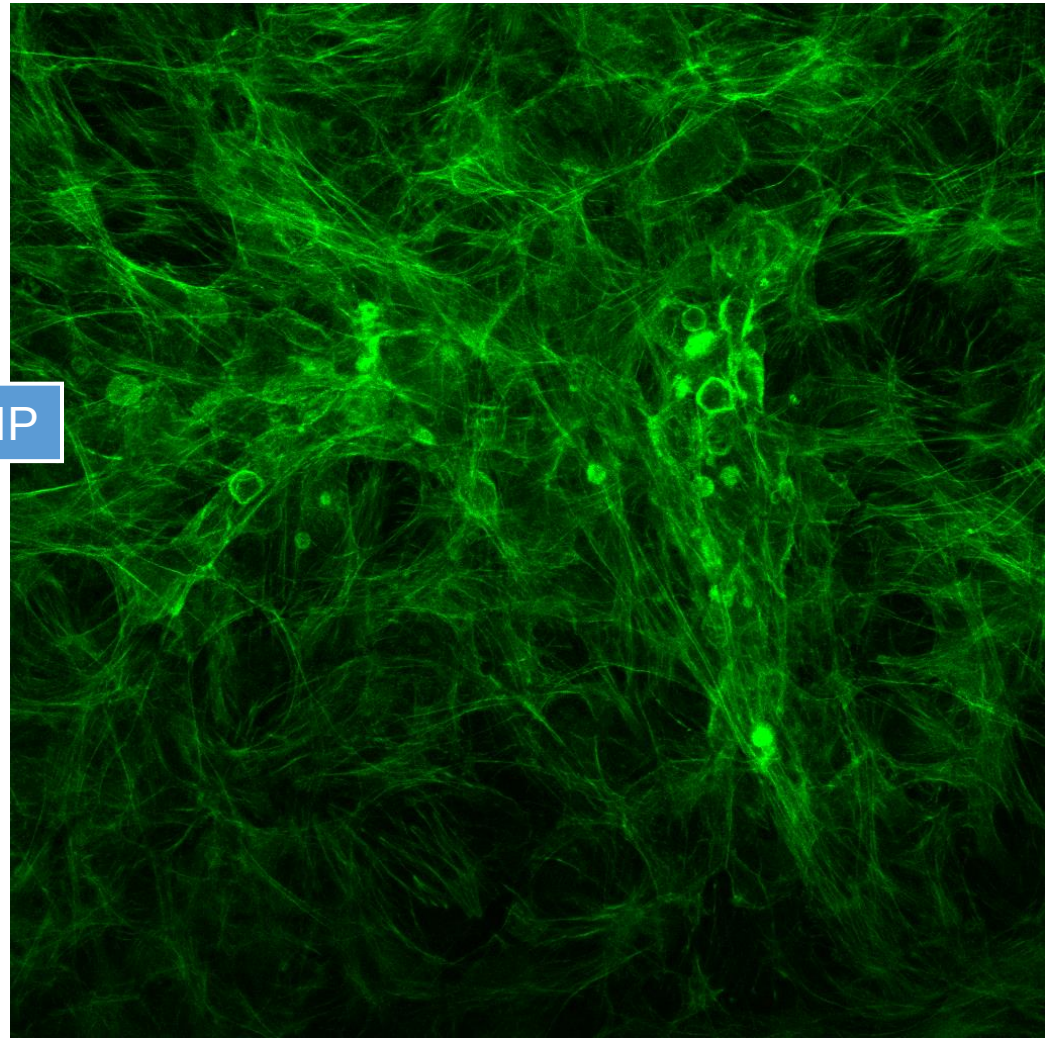




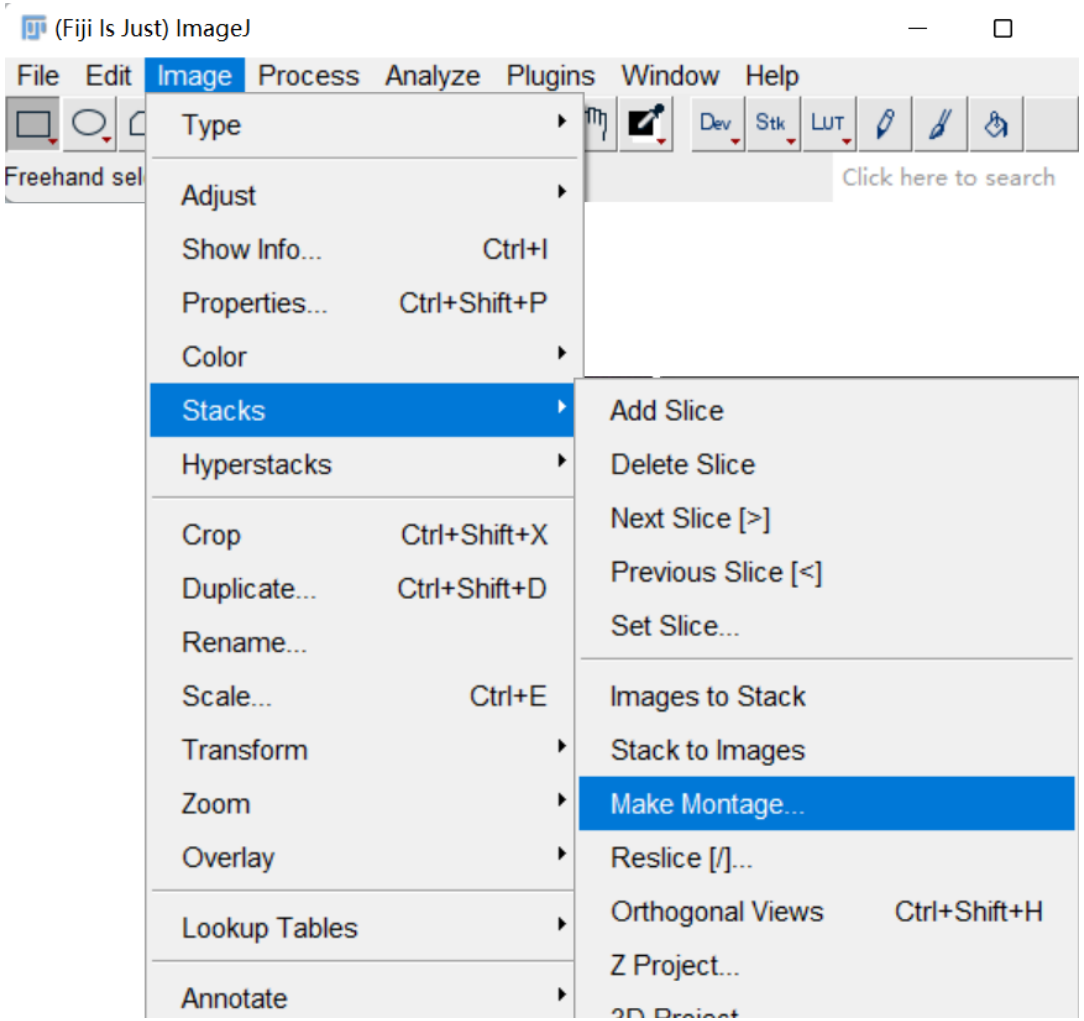
Project 3D image to 2D (max intensity projection; MIP)

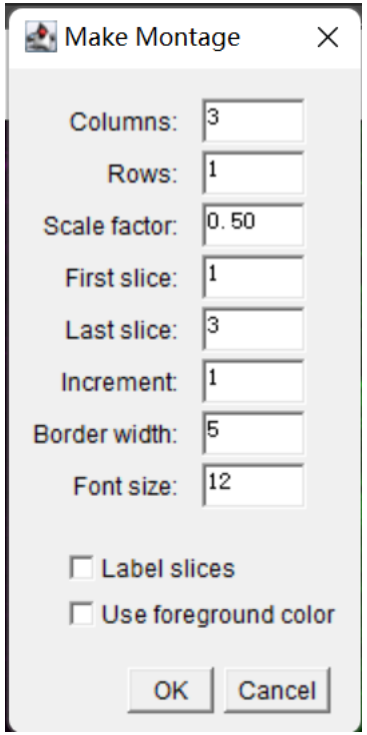


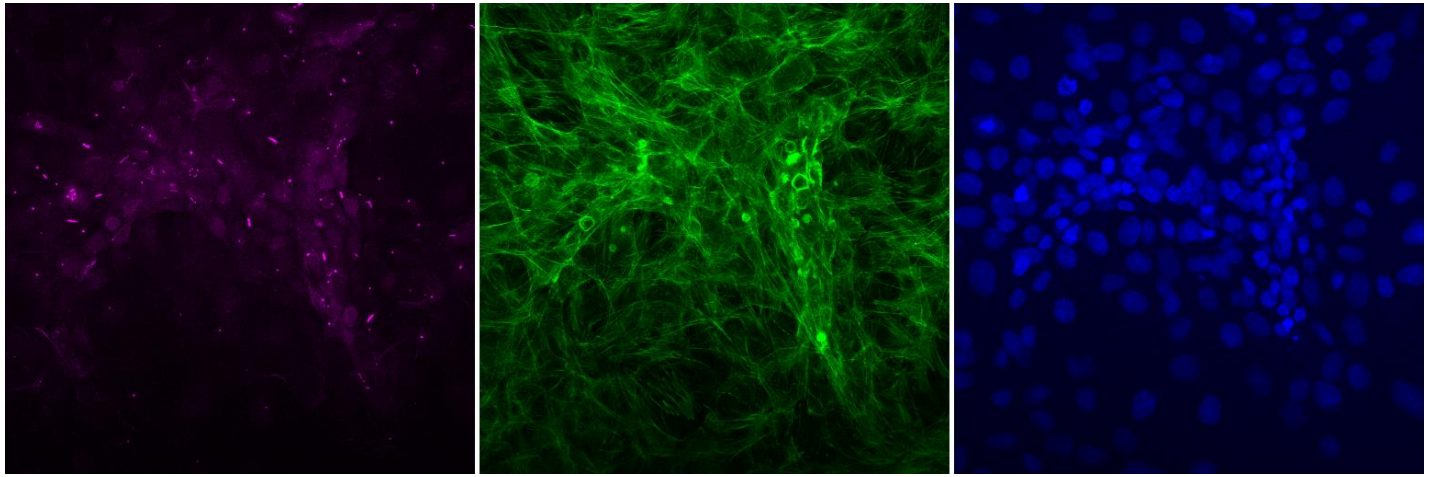
Z stack to MIP



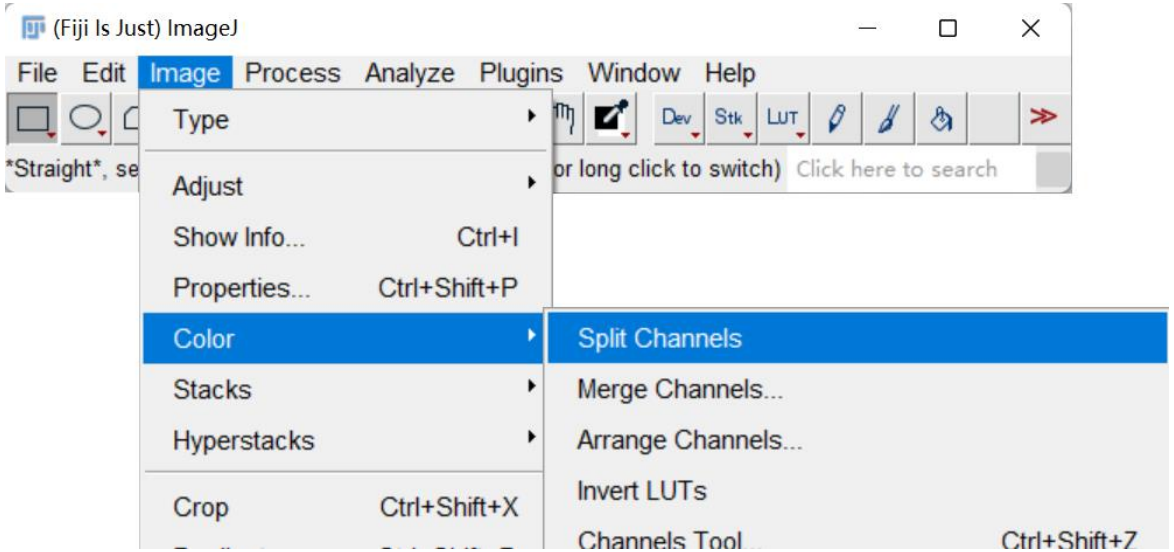
Make montage (arrange images in a grid)

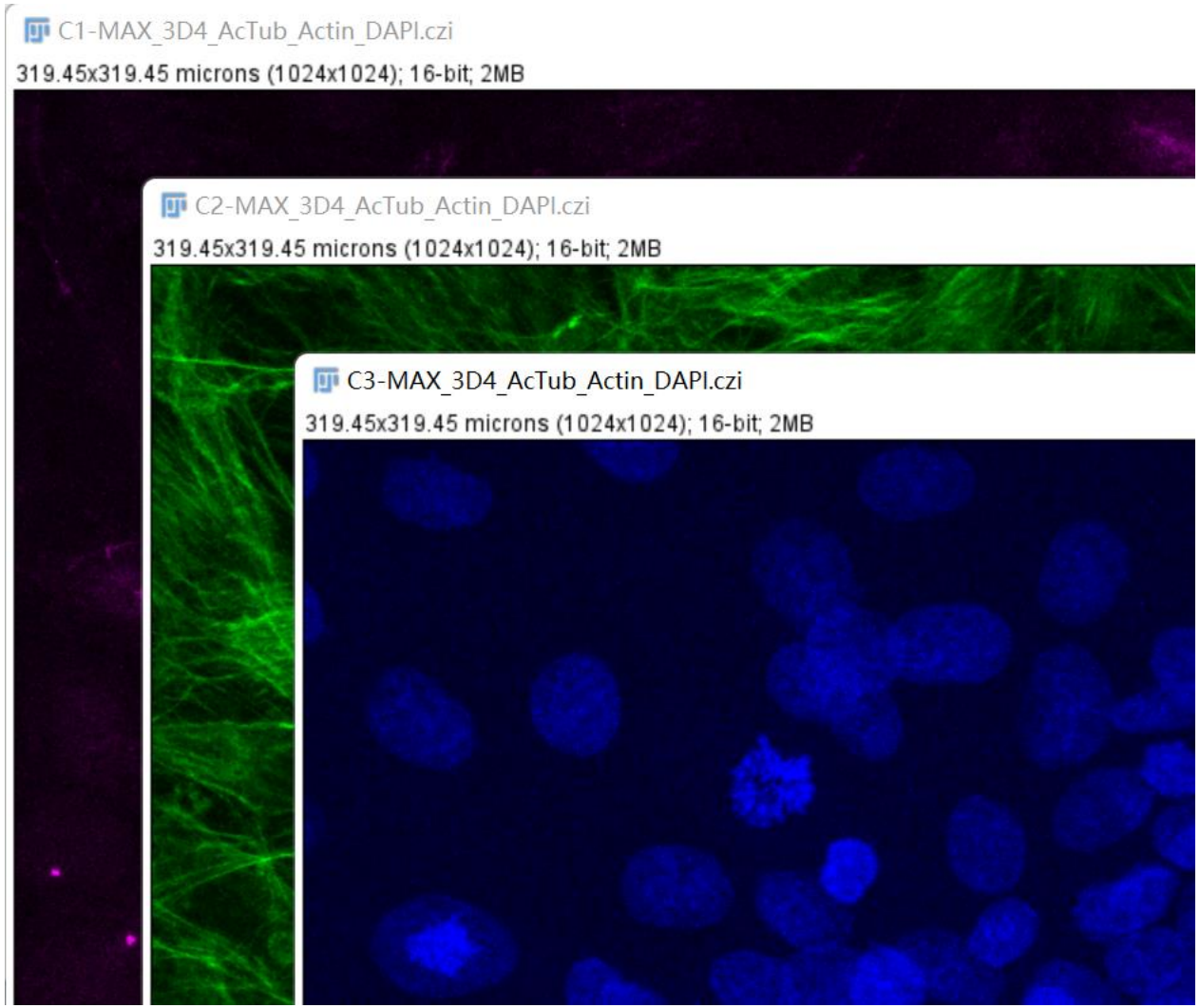




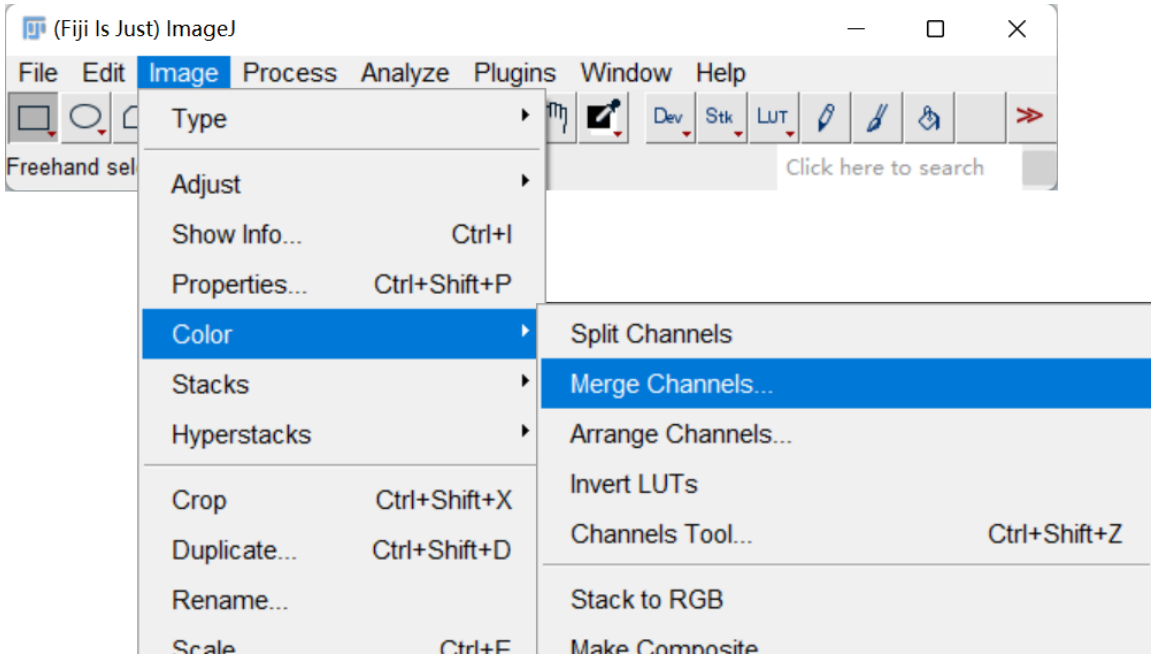


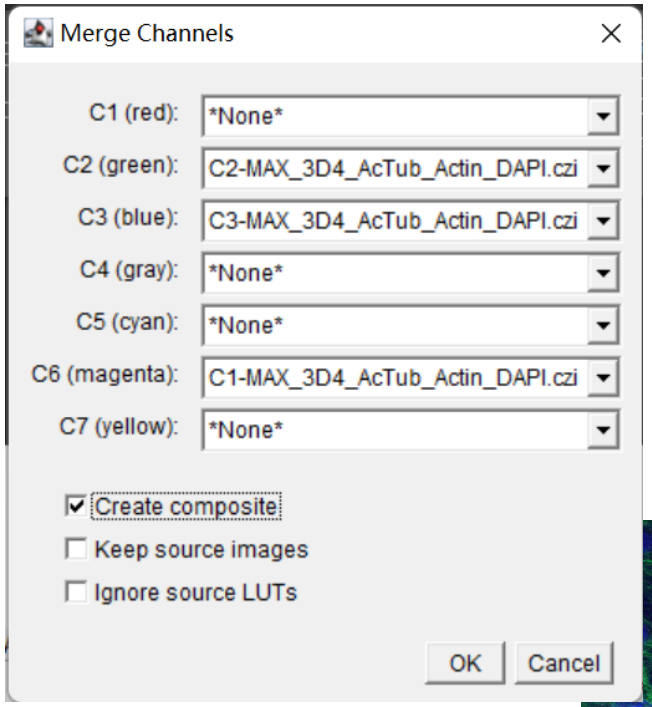
Merge channels

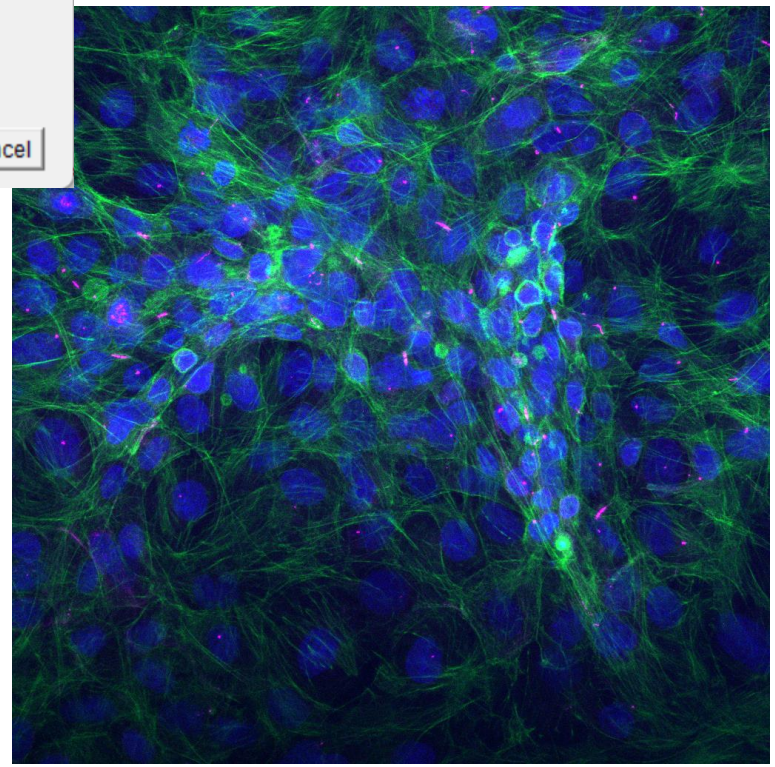




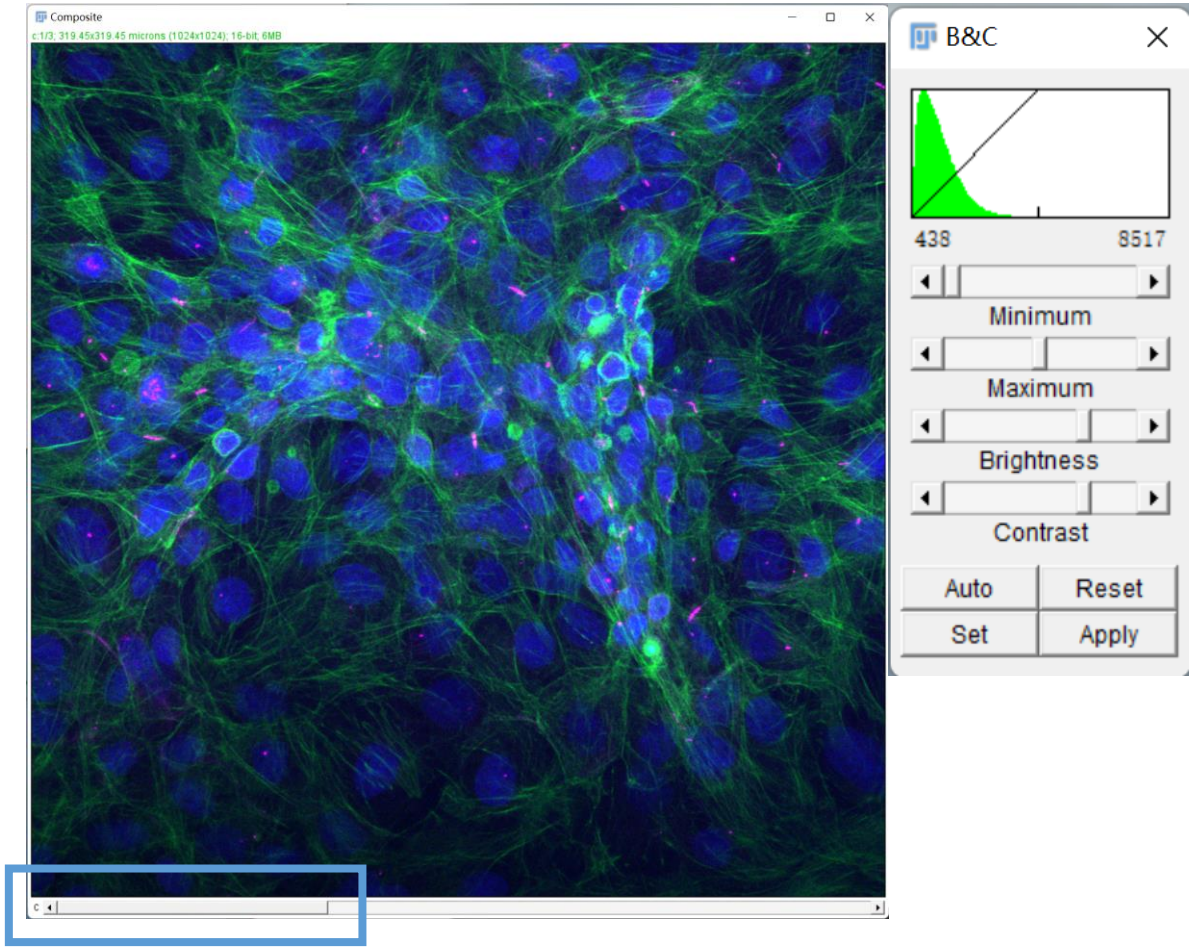
Merge channels

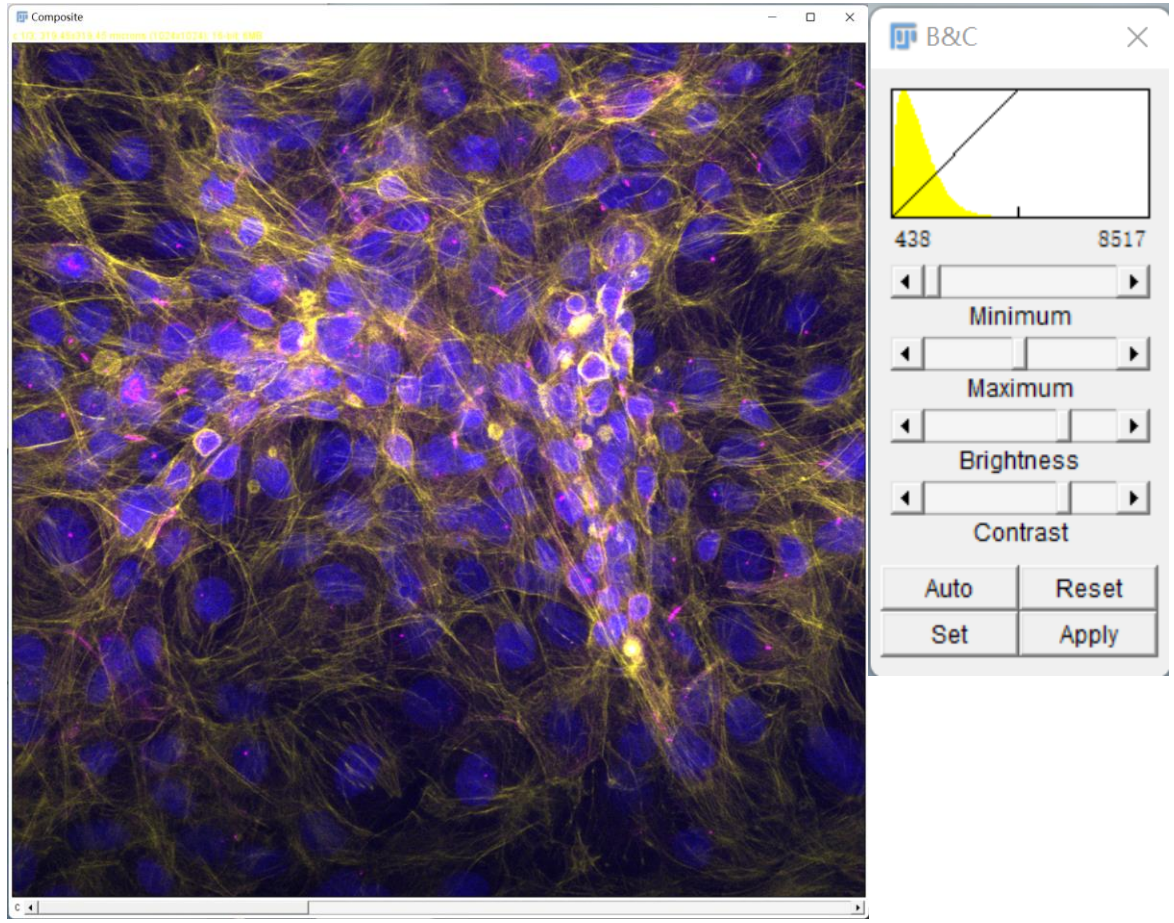






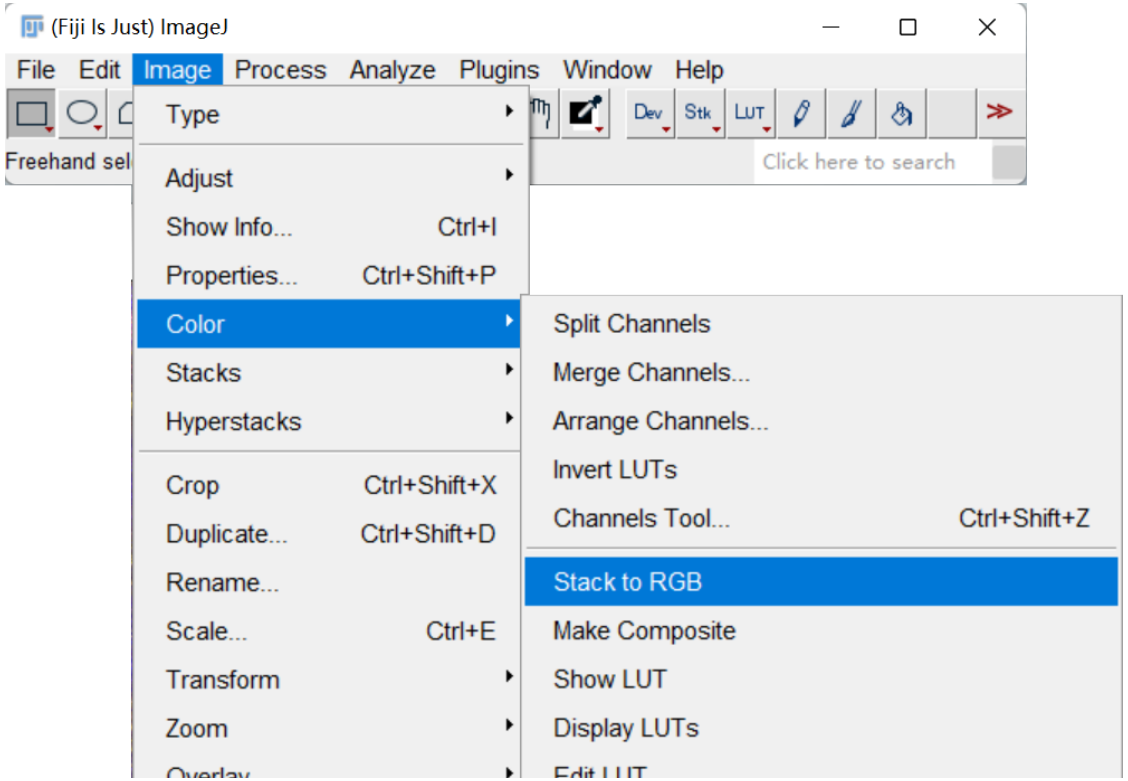
Merge channels

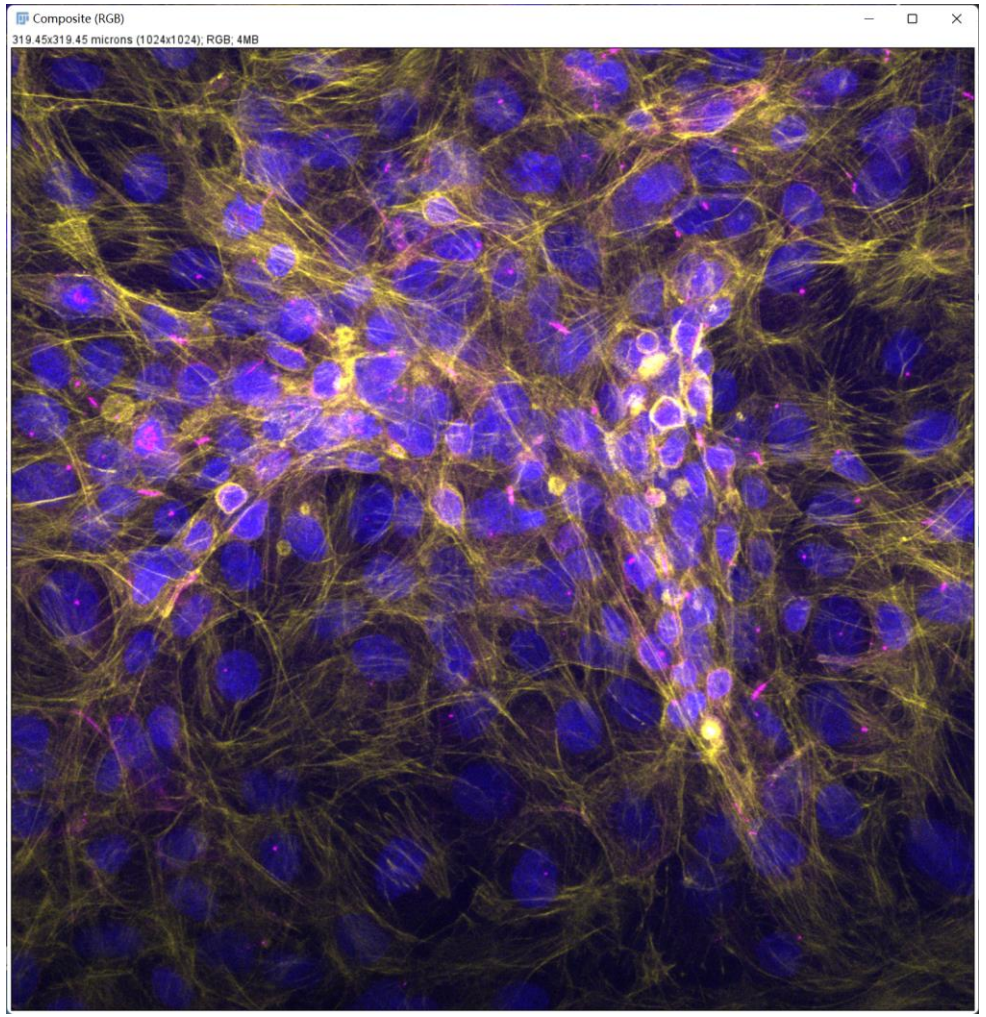




You can still change contrast and color (LUT) in each channel

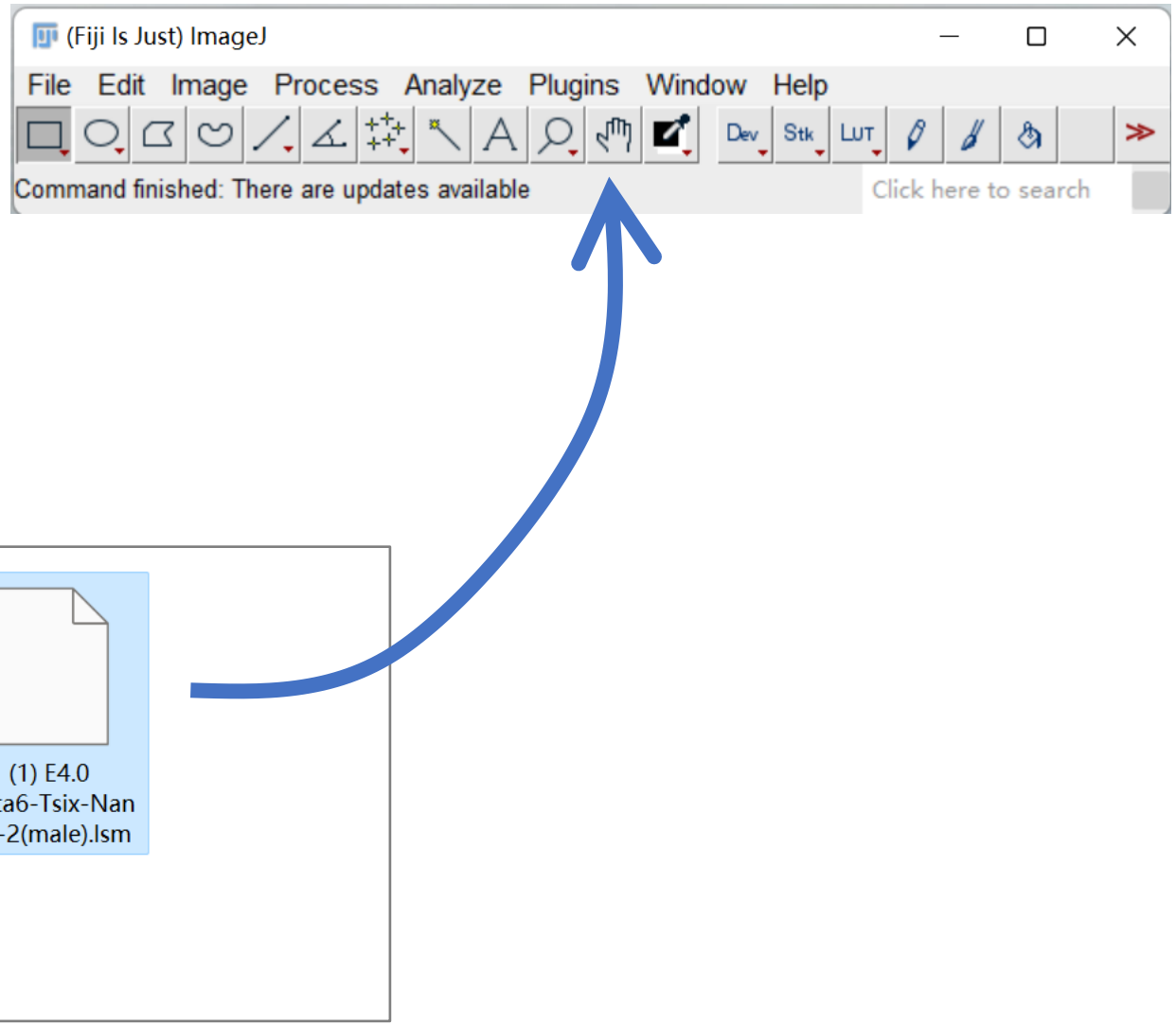
Convert to RGB image

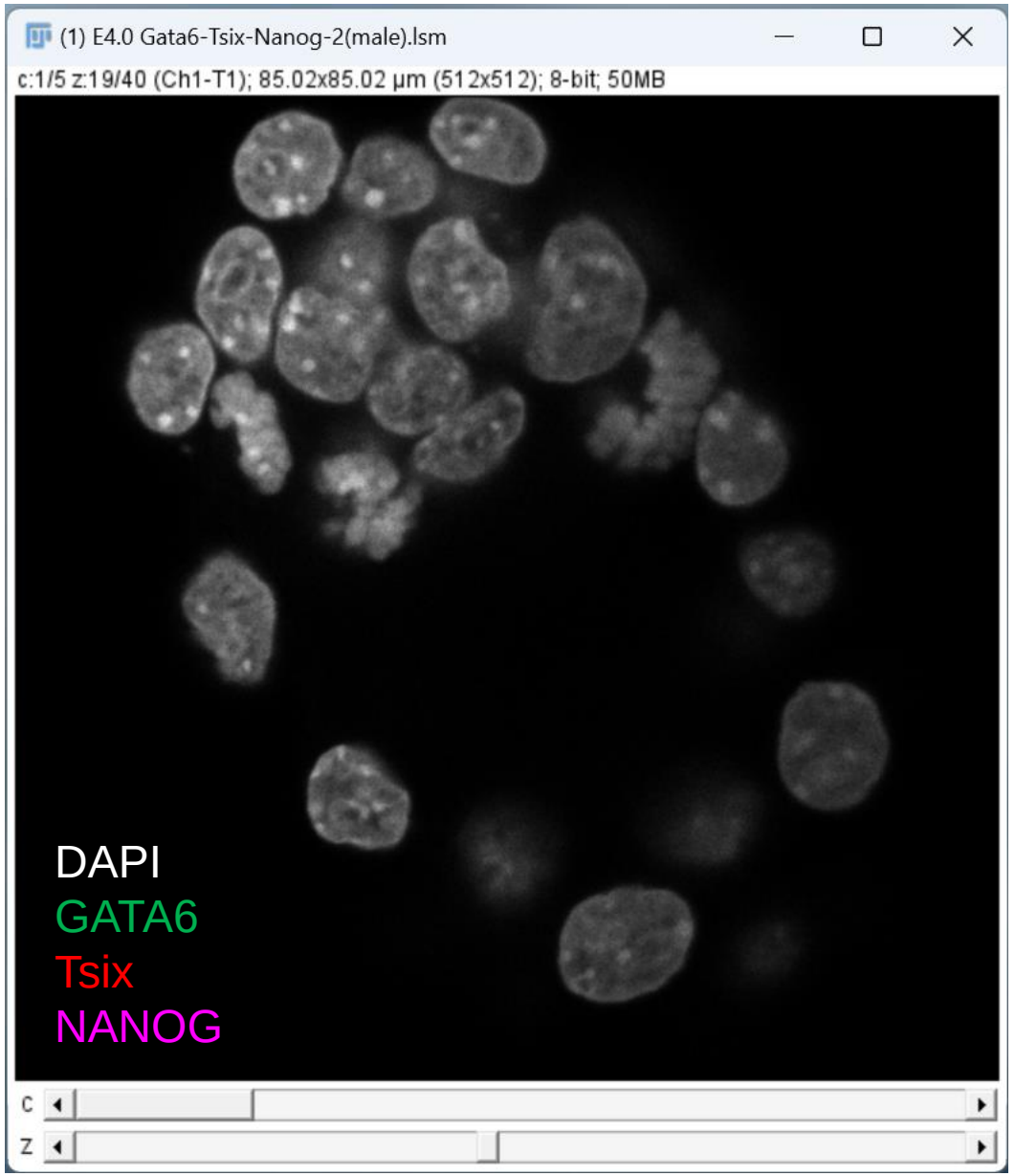




- Merged images still have channels and can be saved as TIFF (supports multi-page image format)
- If saved as BMP or JPEG, only the single channel that is selected will be saved
- Conversion by “Stack to RGB” will enable to save merged image as BMP or JPEG

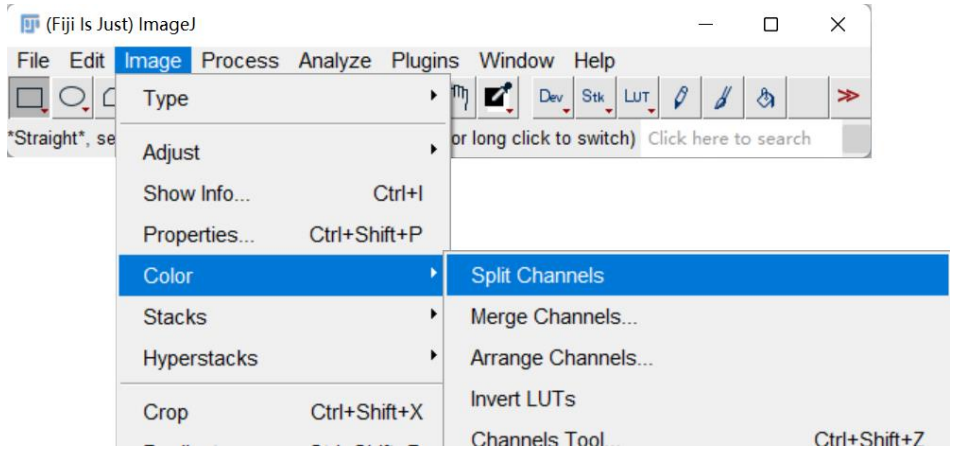
3D projection

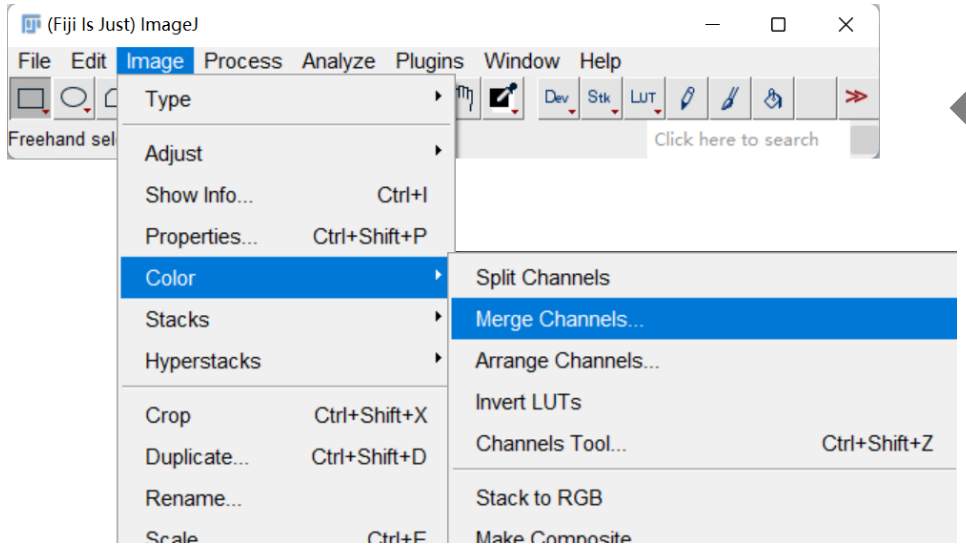


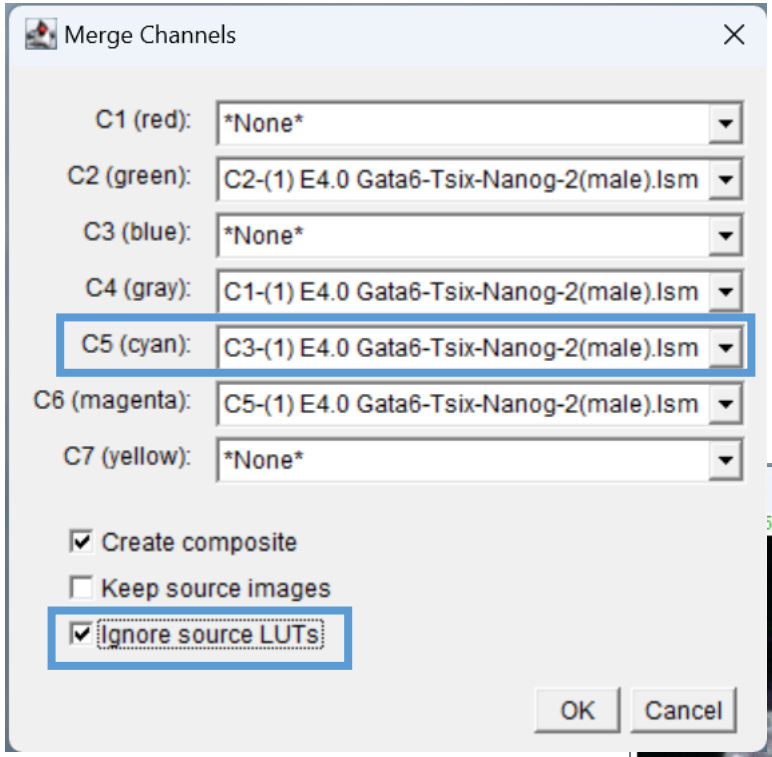


Multi-channel Z-stack images of a mouse embryo at E4.0
Downloaded from a database SSBD
https://ssbd.riken.jp/database/project/114/ (Shiura and Abe, Sci Rep, 2019)

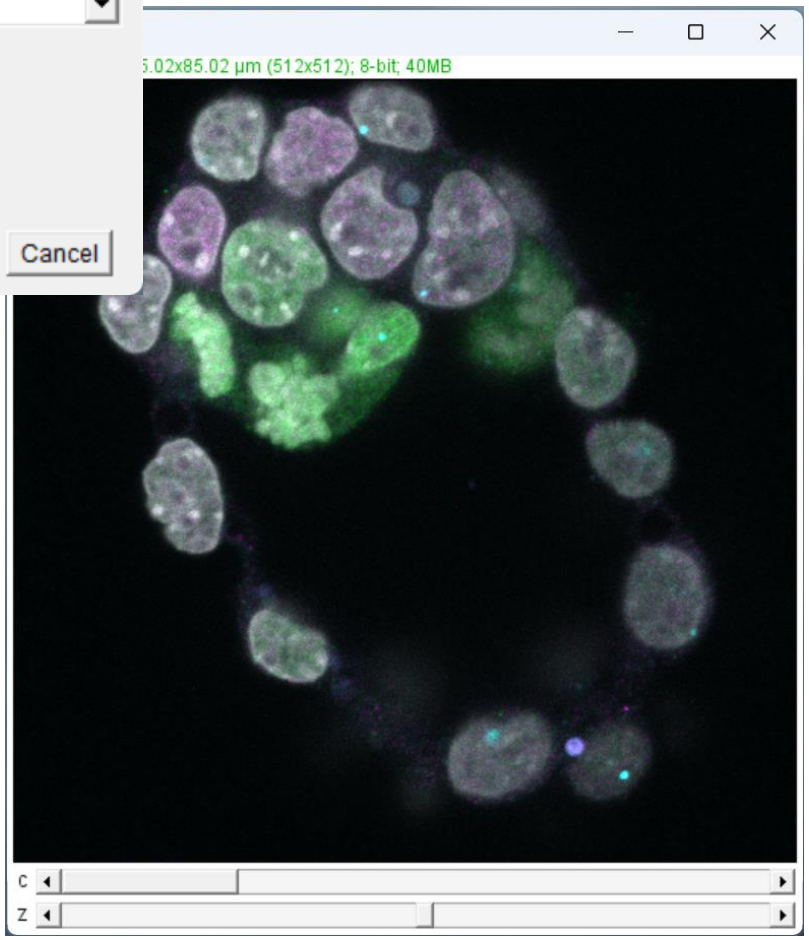
3D projection



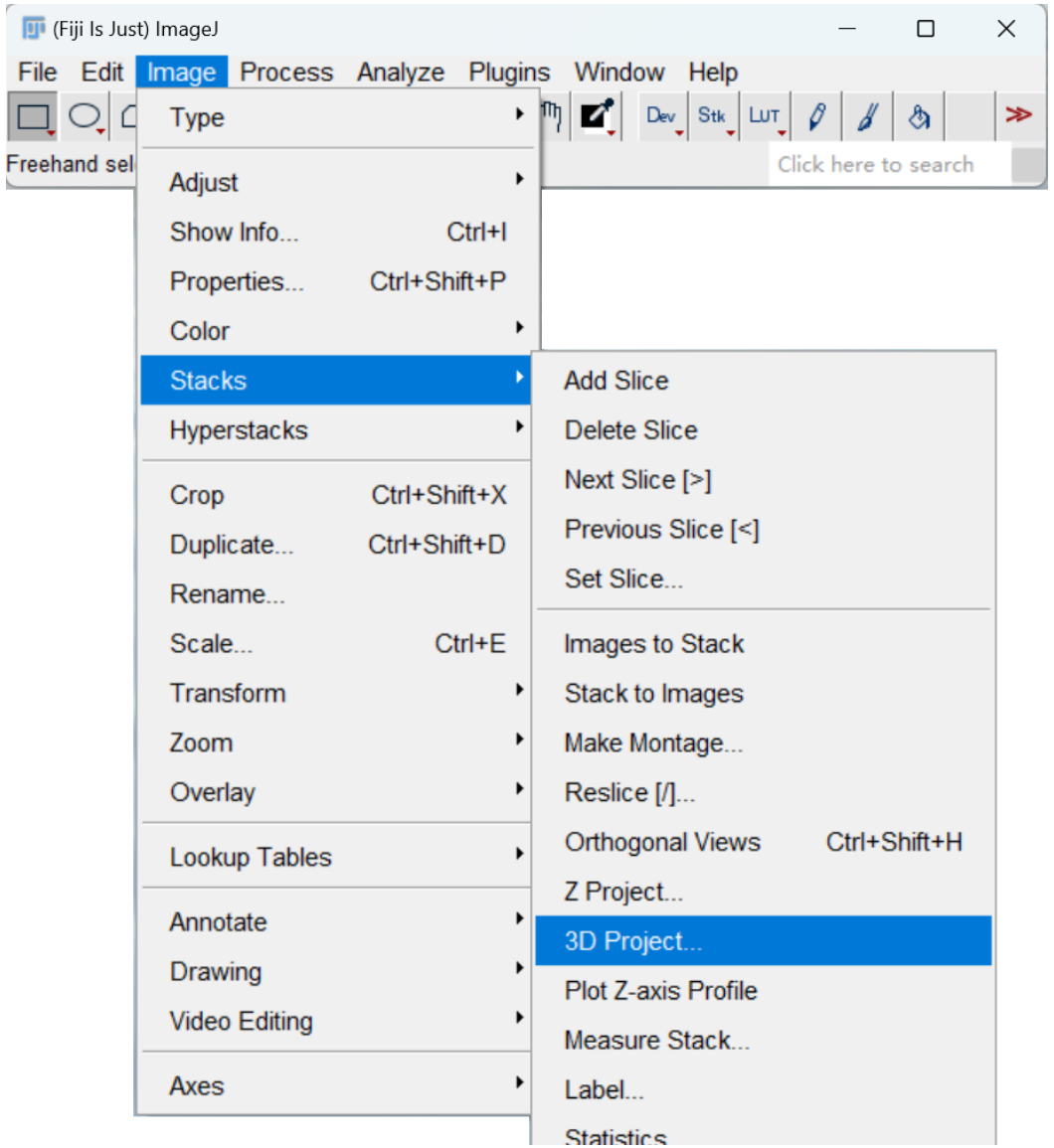


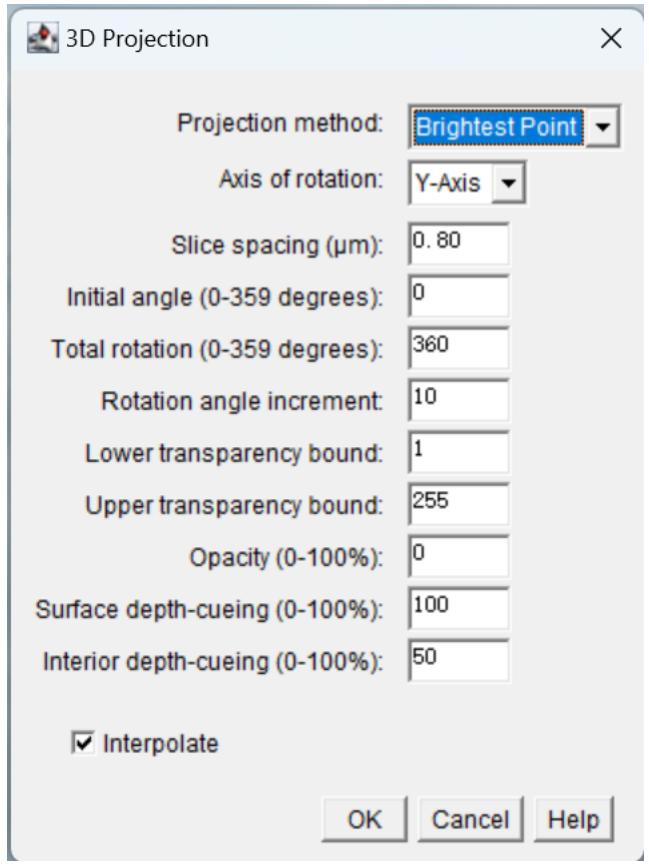


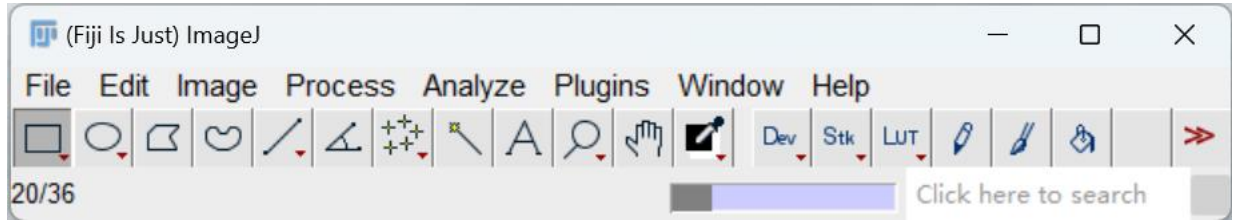
Let's change Red to Cyan



3D projection

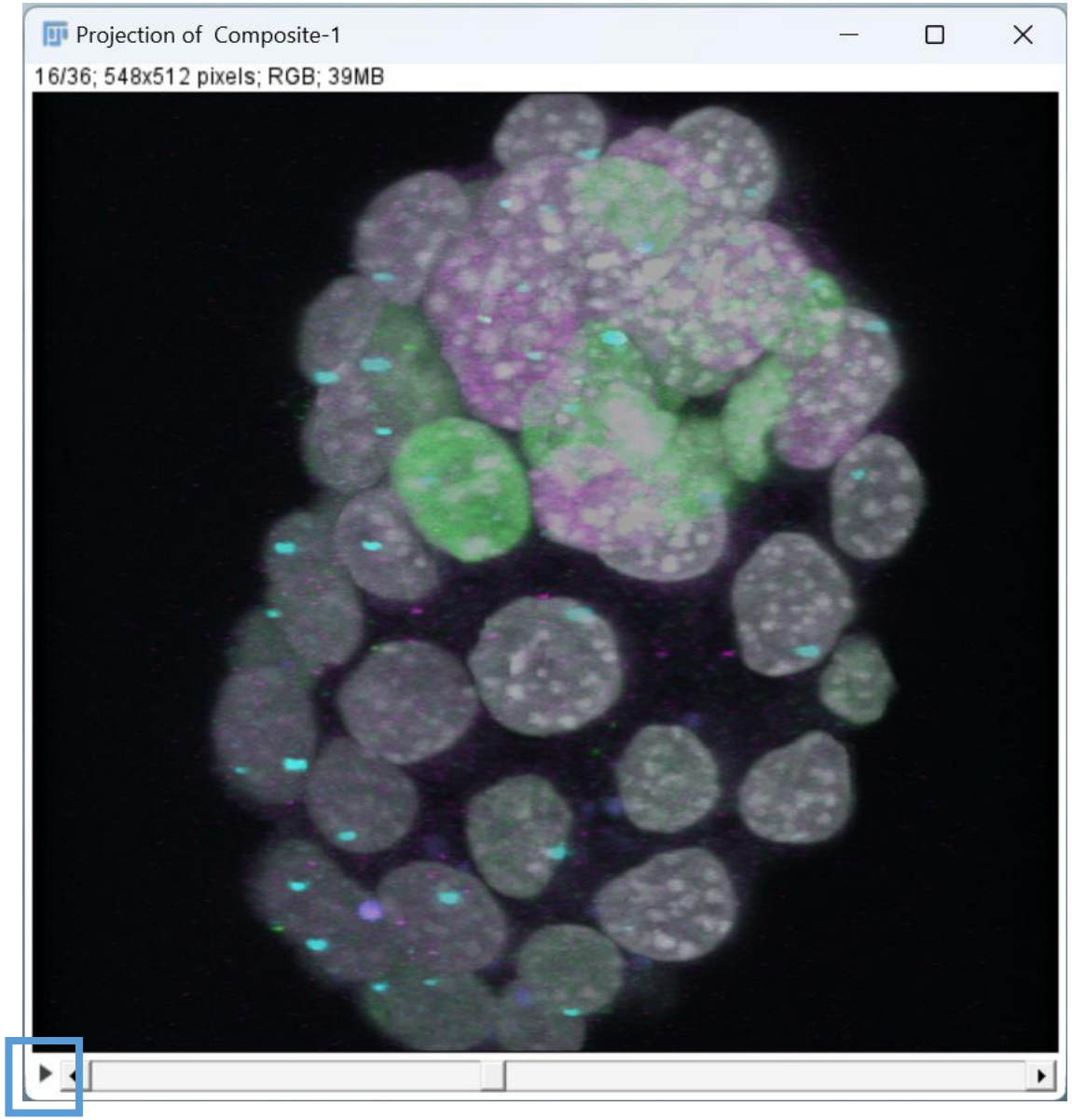






It may take a while

3D projection

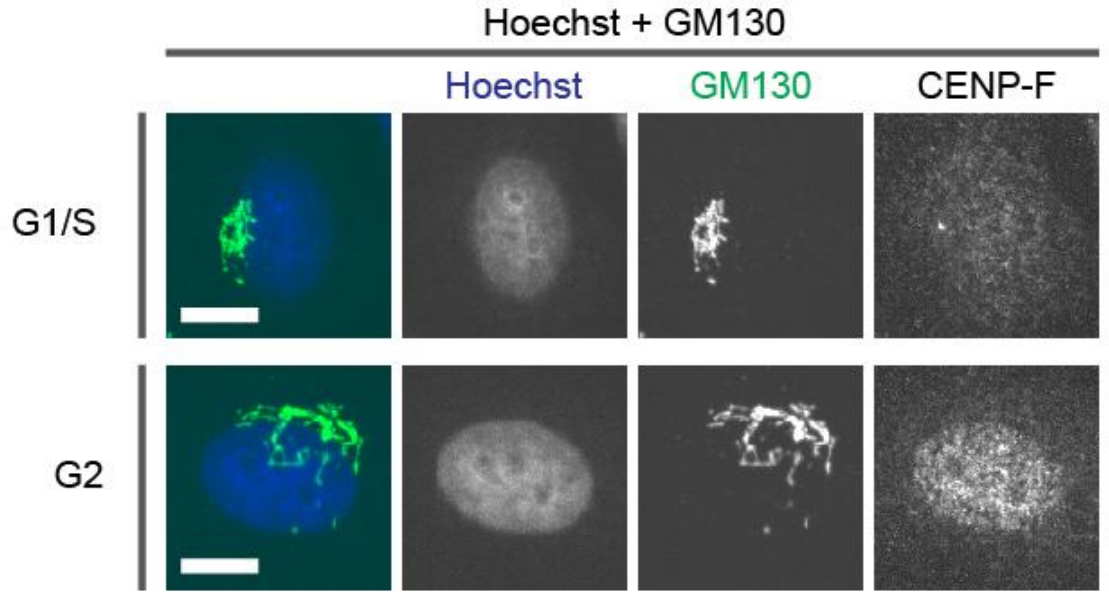


Click here for animation, right-click for config

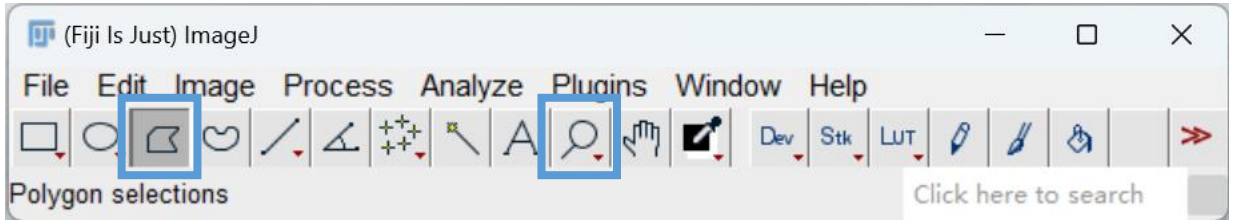
Elongated along Z-axis
(lower resolution compared to XY)

Measurements

- Use image data from Nagao et al., 2020 (“HeLa_Hoechst_GM130_CENP-F”)
- Files are MIP of z-stack confocal images in TIFF format
- These are HeLa cells fixed with PFA and stained for DNA (Hoechst), Golgi (GM130), and CENP-F
- CENP-F is a cell cycle marker:
  CENP-F is localized to the nucleus of cells in G2 phase, but absent in the rest of the cell cycle

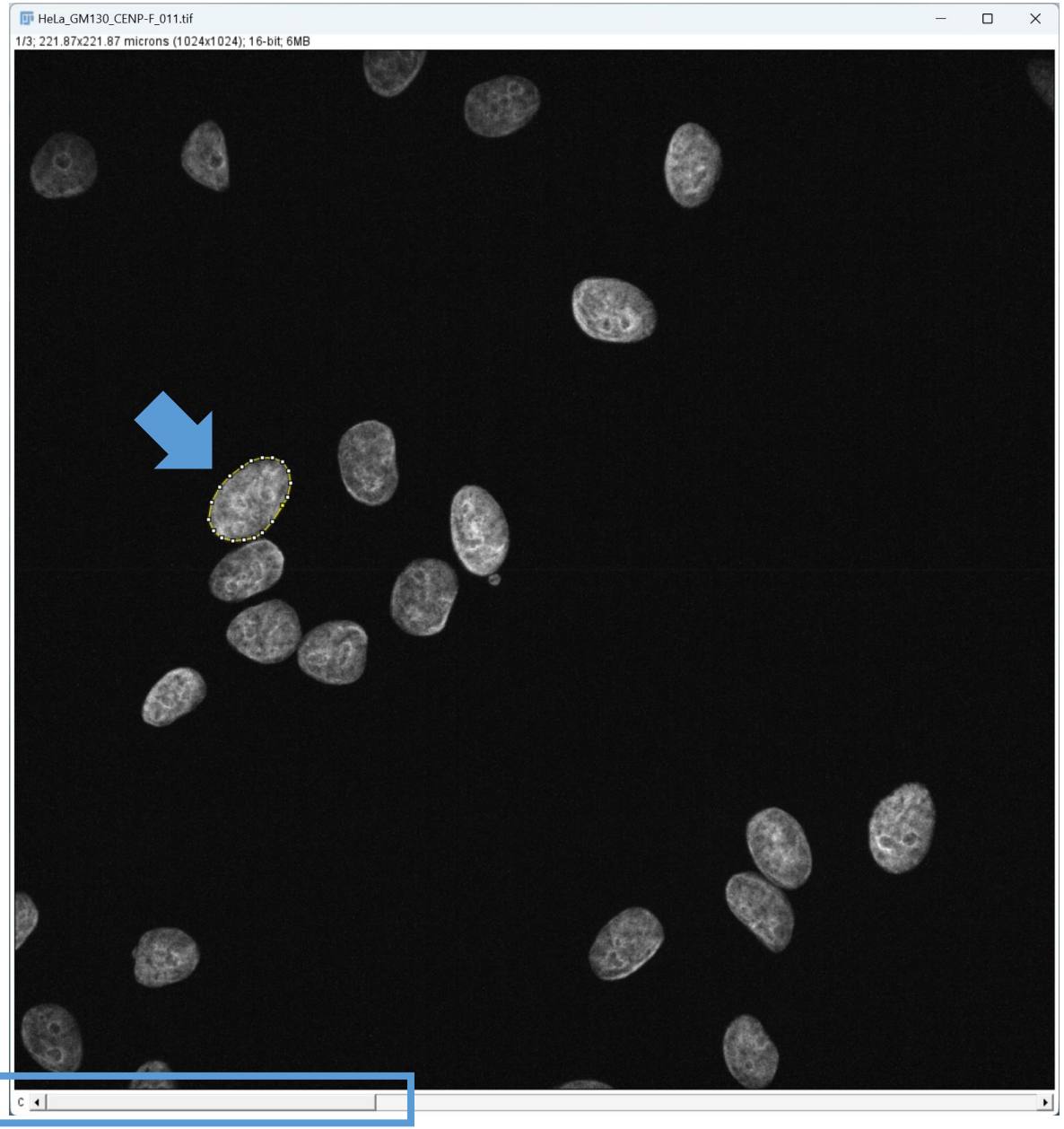


Measurements



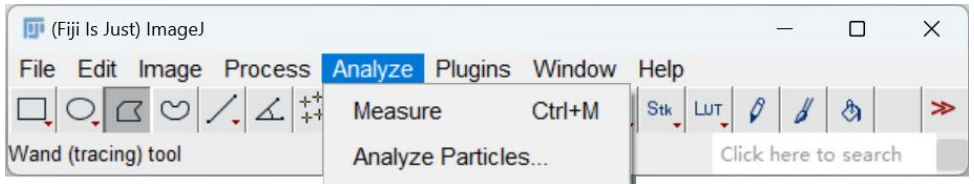
Use “Polygon selections” to select **region of interest (ROI)**

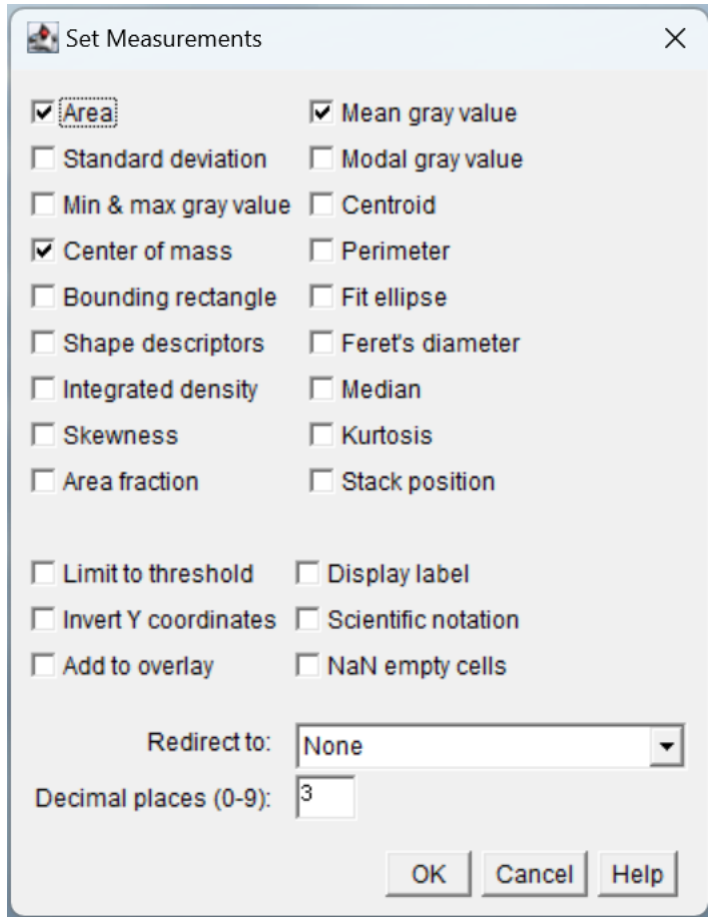
“Magnifying glass” is often helpful

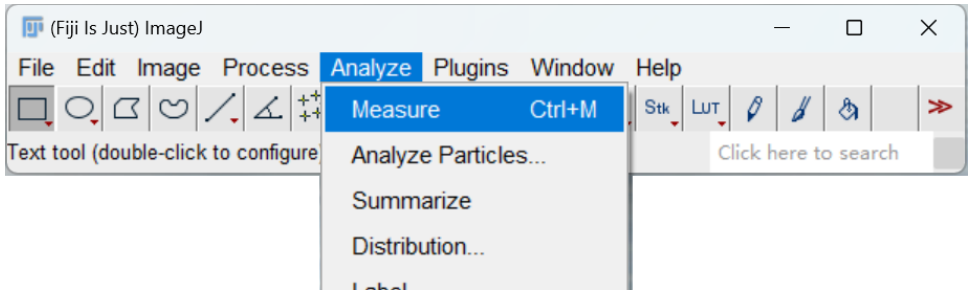


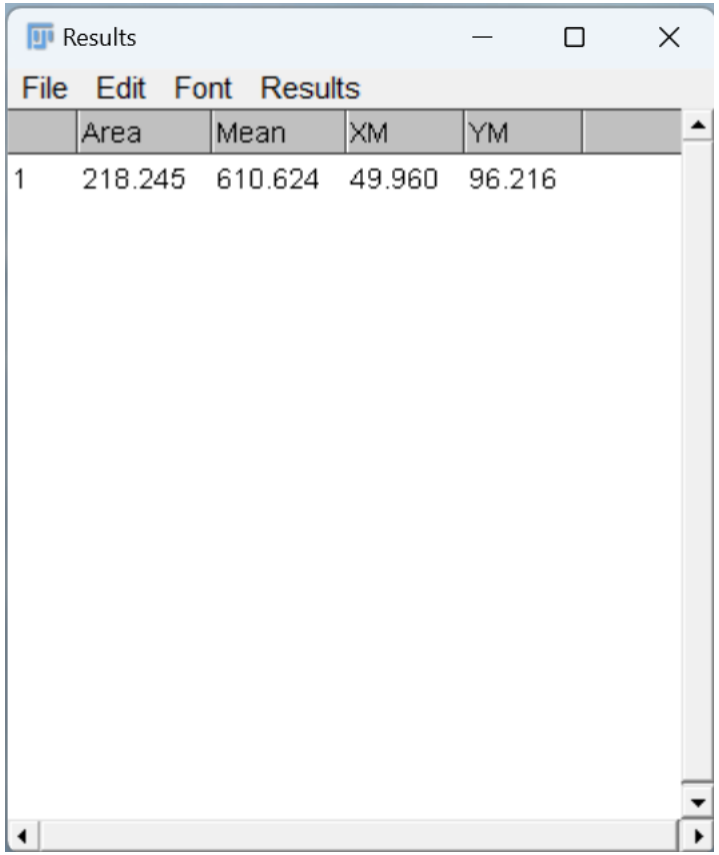
Hoechst (DNA) channel

Measurements



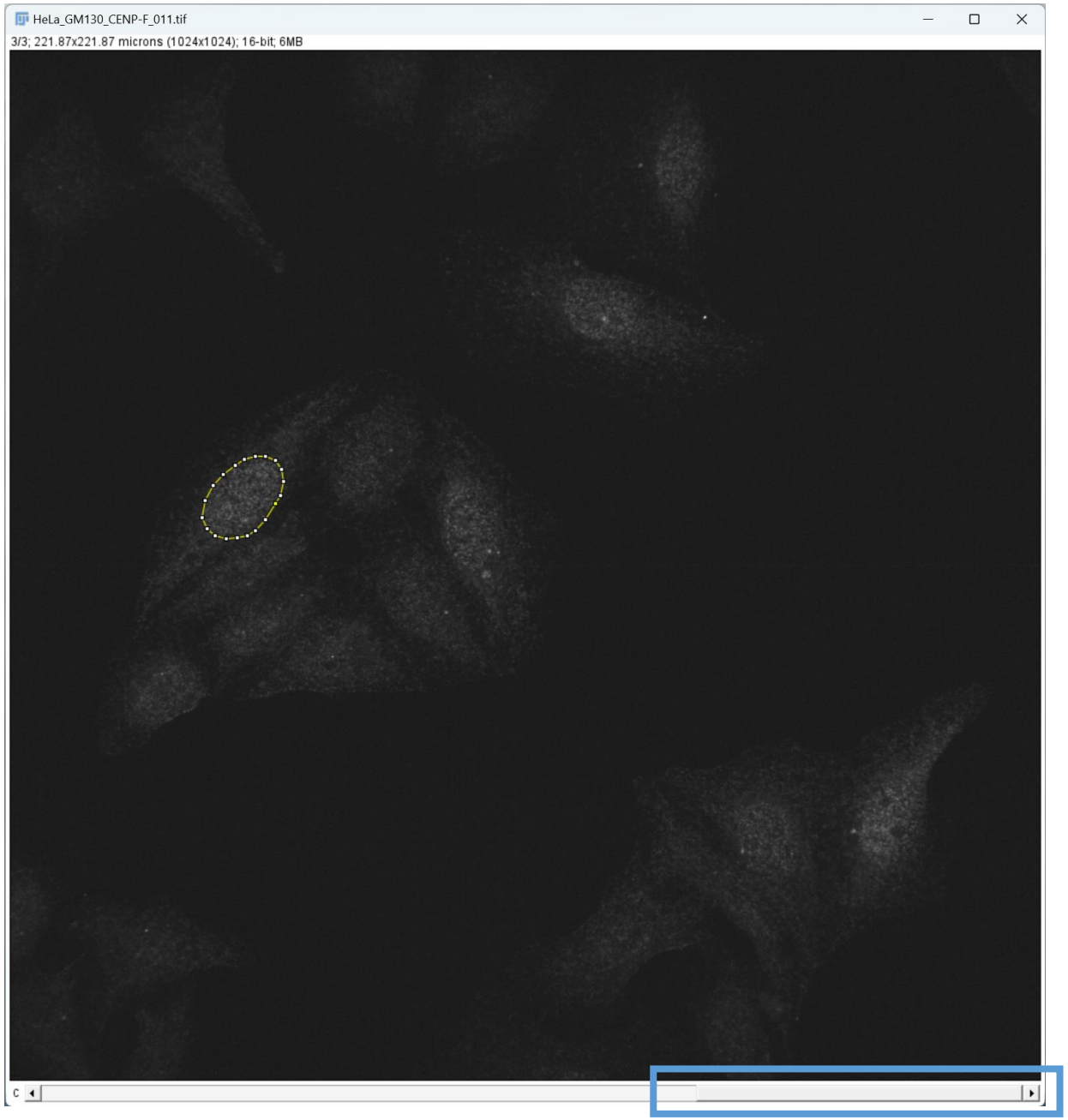






You can choose what to measure

Measurements



Change to CENP-F channel

Results

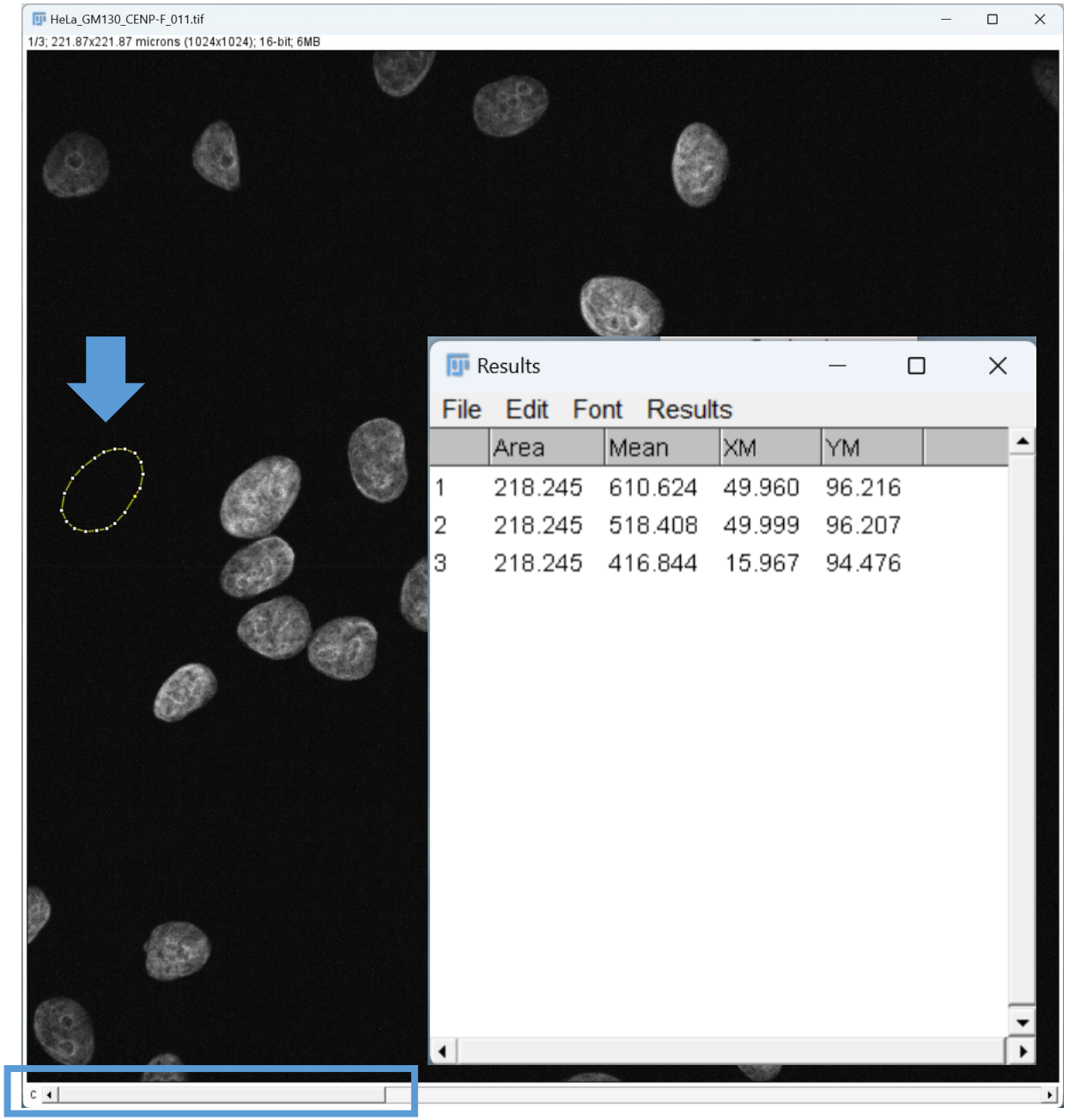
File Edit Font Results

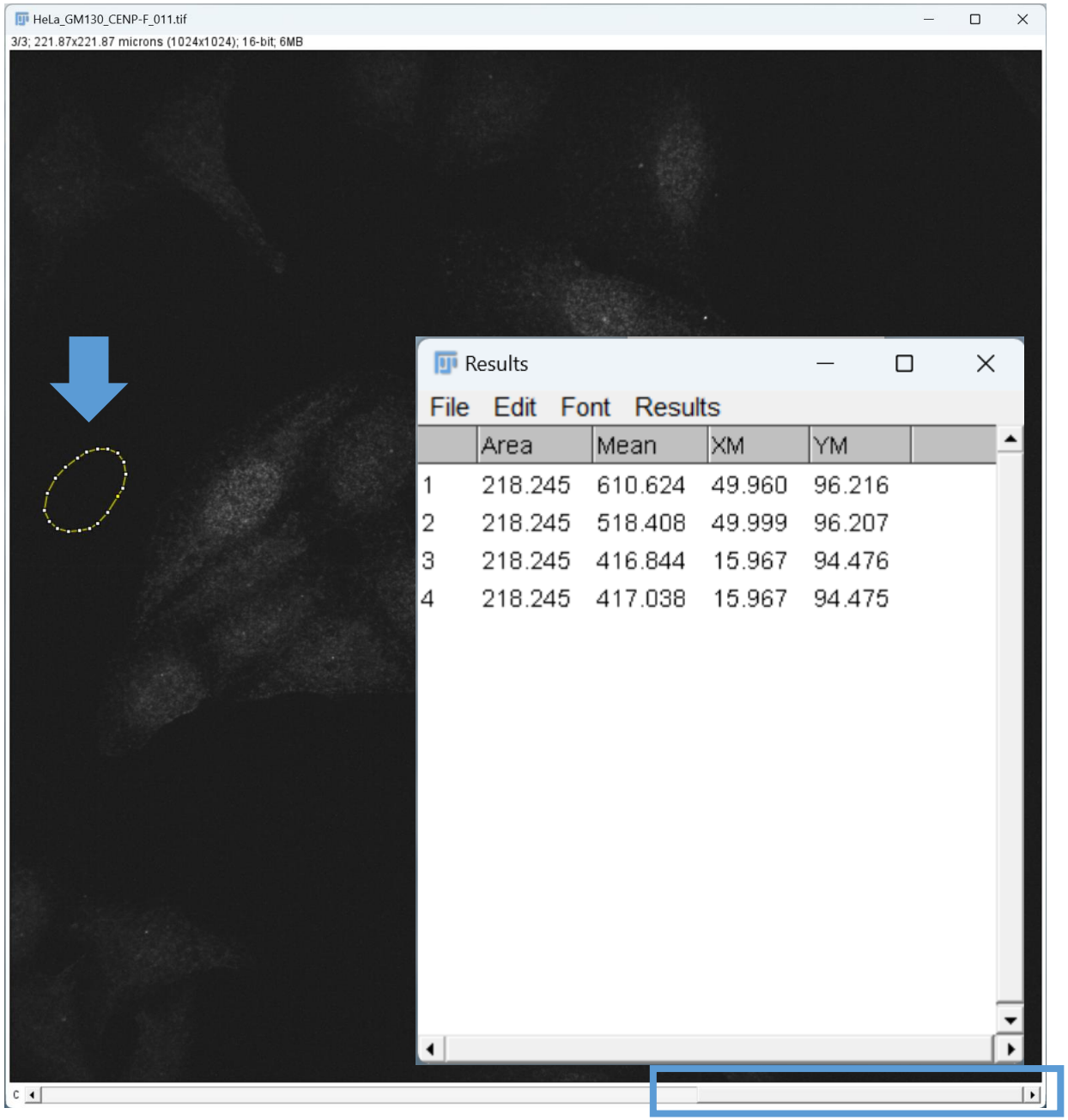
	Area	Mean	XM	YM
1	218.245	610.624	49.960	96.216
2	218.245	518.408	49.999	96.207

Measured values are listed in the “Results” window

Measurements

Measure non-cell background values for each channel





Measurements

	Area	Mean	XM	YM
1	218.245	610.624	49.96	96.216
2	218.245	518.408	49.999	96.207
3	218.245	416.844	15.967	94.476
4	218.245	417.038	15.967	94.475

The results can be exported as a CSV file or just copied and pasted to Excel

Organize data

	Area	XM	YM	Hoechst_Mean	CENP-F_Mean	Hoechst_Background	CENP-F_Background
1	218.245	49.96	96.216	610.624	518.408	416.844	417.038

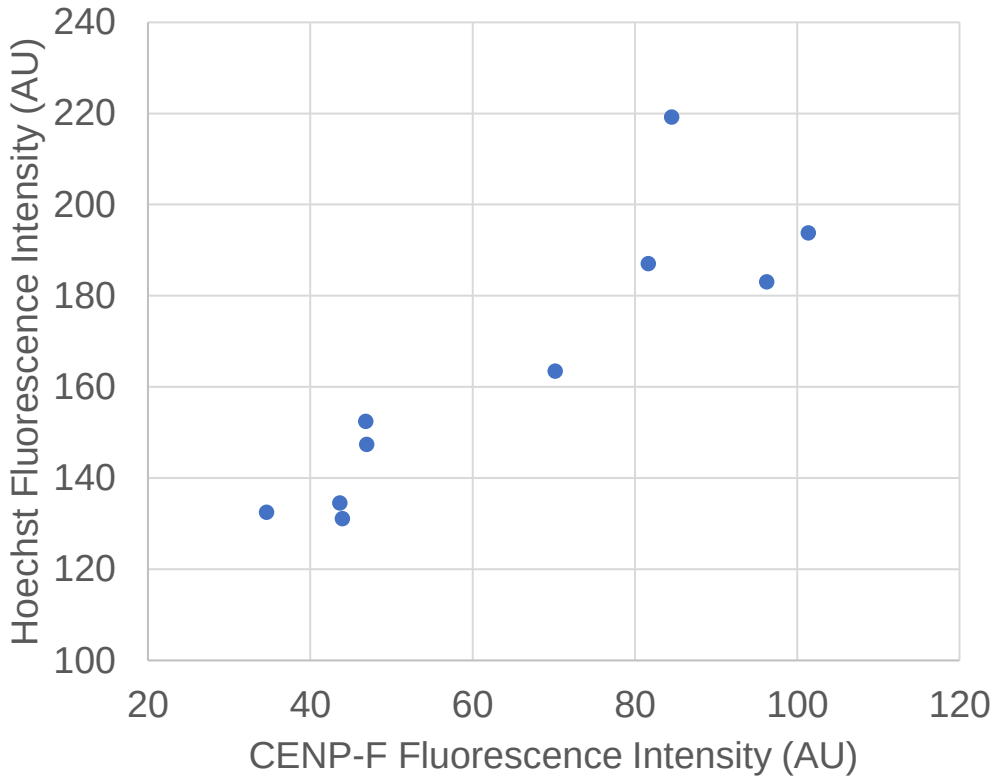
Continue measuring for other cells

Background subtracted

	Area	XM	YM	Hoechst_Mean	CENP-F_Mean	Hoechst_Background	CENP-F_Background	Hoechst_Mean_Subtracted	CENP-F_Mean_Subtracted
1	218.245	49.96	96.216	610.624	518.408	416.844	417.038	193.78	101.37
2	144.918	49.491	111.098	569.259	463.875	416.844	417.038	152.415	46.837
3	186.933	75.385	87.92	547.918	461.008	416.844	417.038	131.074	43.97
4	178.06	98.968	102.581	636.069	501.593	416.844	417.038	219.225	84.555
5	180.079	87.271	116.675	551.35	460.691	416.844	417.038	134.506	43.653
6	200.875	127.647	55.515	603.902	498.703	416.844	417.038	187.058	81.665
7	177.966	163.276	170.249	580.302	487.246	416.844	417.038	163.458	70.208
8	208.856	189.479	166.266	599.898	513.308	416.844	417.038	183.054	96.27
9	158.203	68.134	128.697	564.245	463.991	416.844	417.038	147.401	46.953
10	173.835	130.323	190.729	549.371	451.669	416.844	417.038	132.527	34.631

Same values may be used for a single image

Measurements

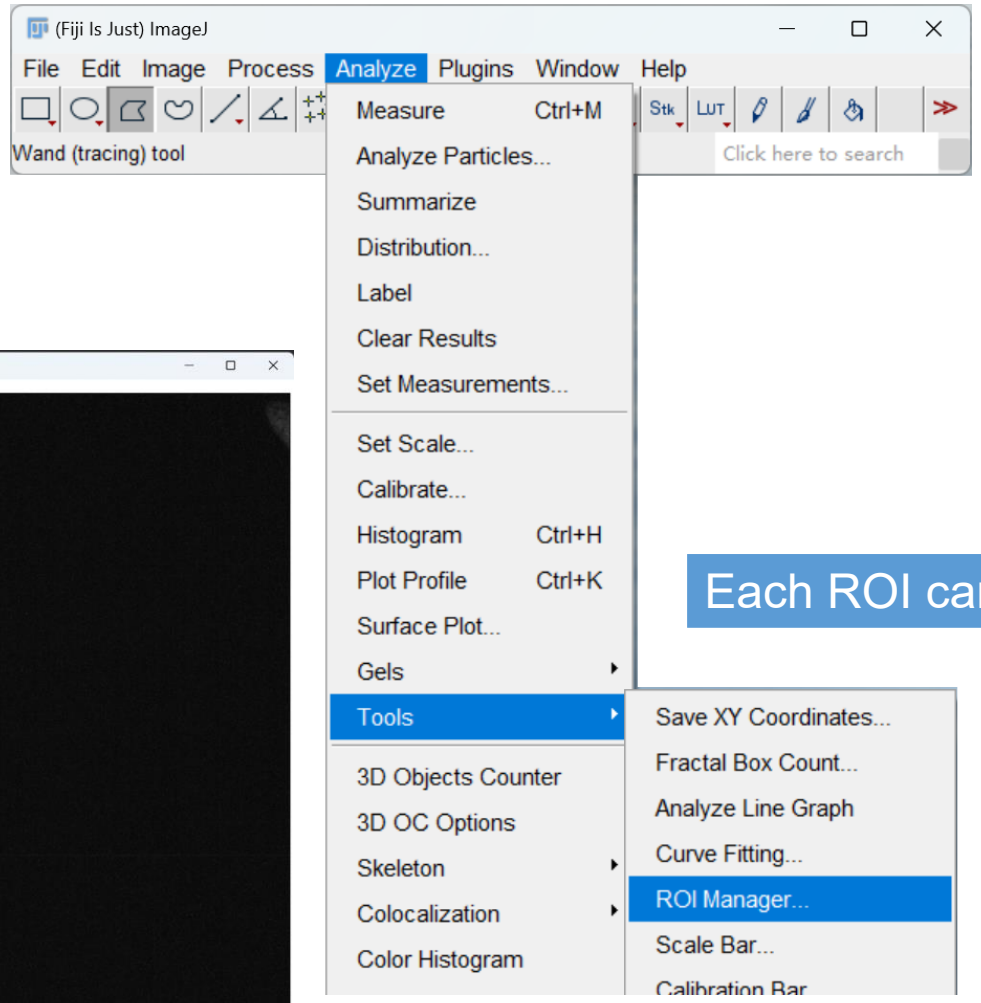


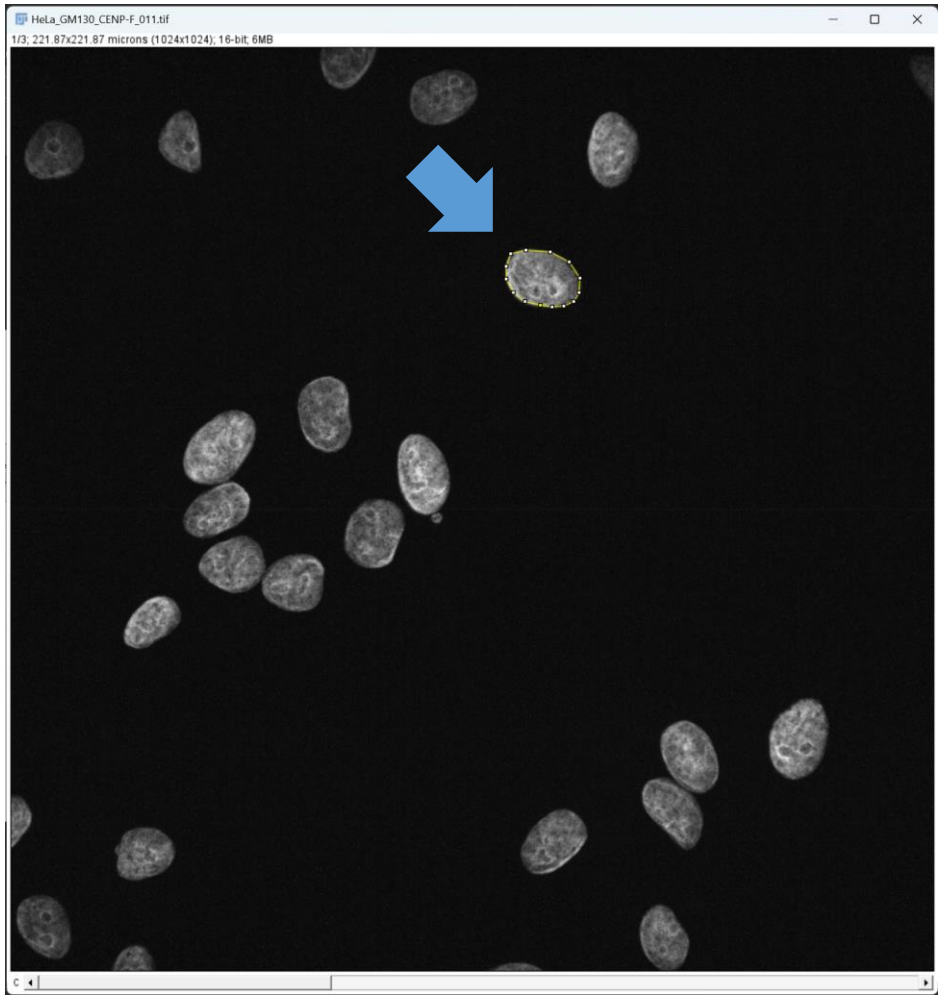
Scatter plot of background-subtracted mean fluorescence intensities

- Plotting data from just 10 cells shows clear correlation
- How will it look when data from more cells (100–200 cells) are plotted?
- You can try: Draw regression line, calculate correlation coefficient, etc
- What does this correlation mean?

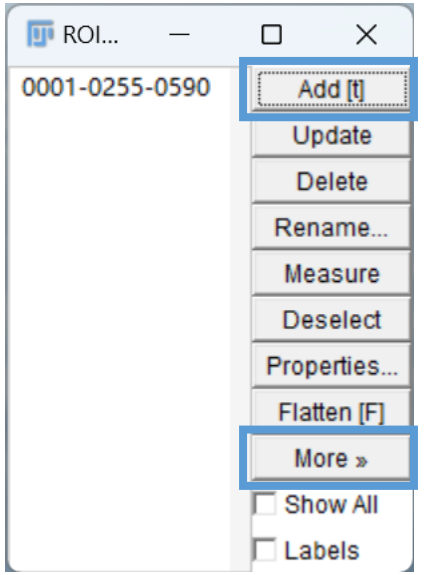
Term paper

Measurements





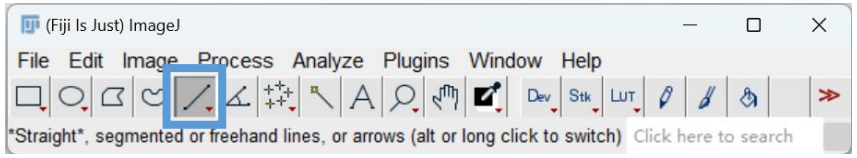
Each ROI can be registered with “ROI Manager”

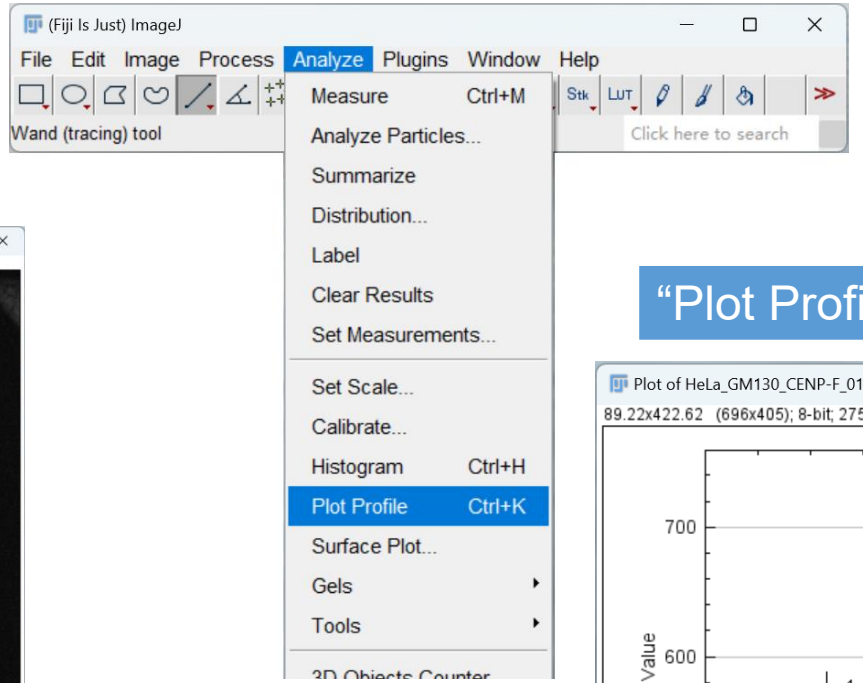


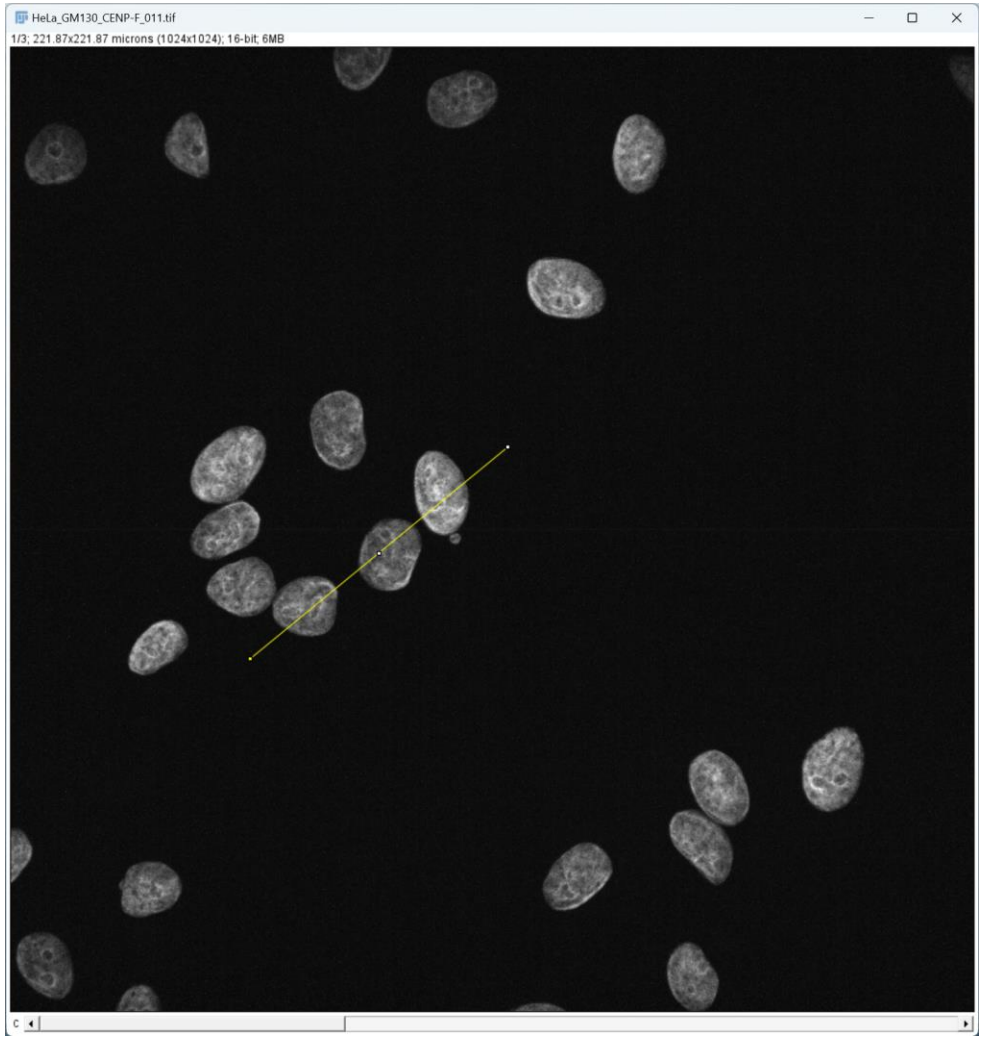
Select ROI and click “Add”

ROI data can be saved for later use

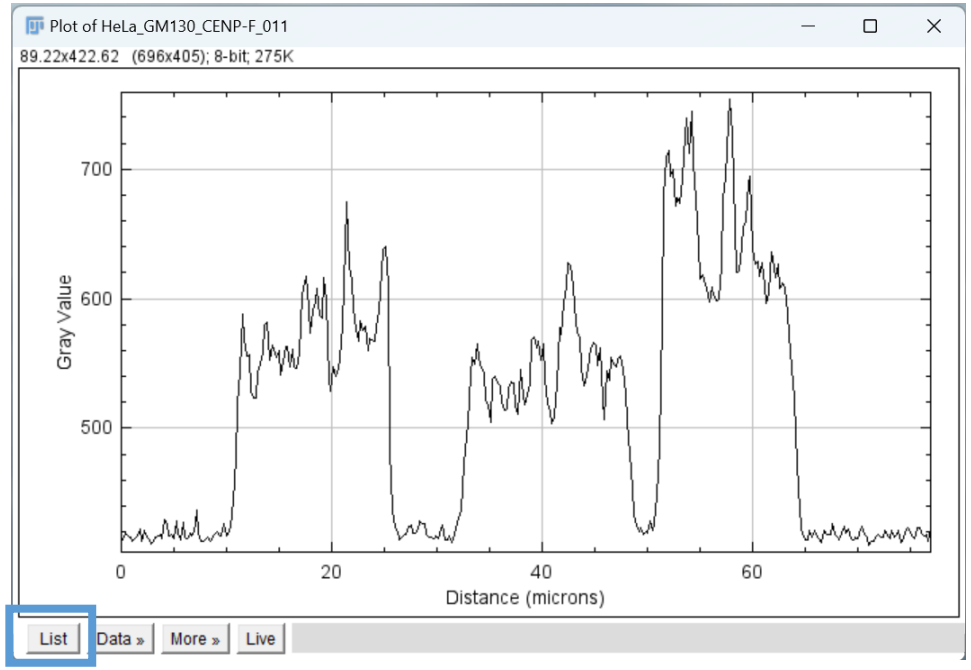
Measurements







"Plot Profile" is another convenient tool



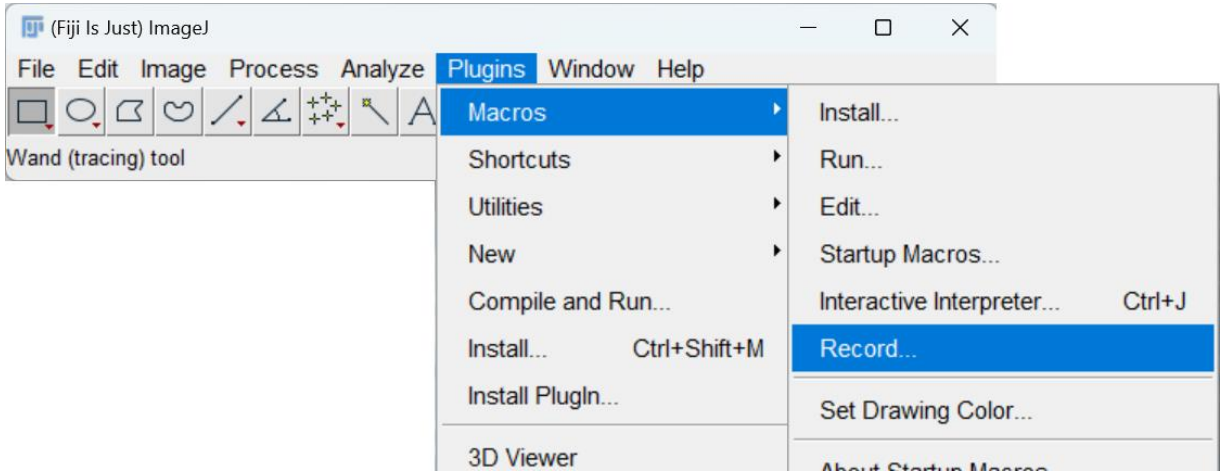
List can be exported

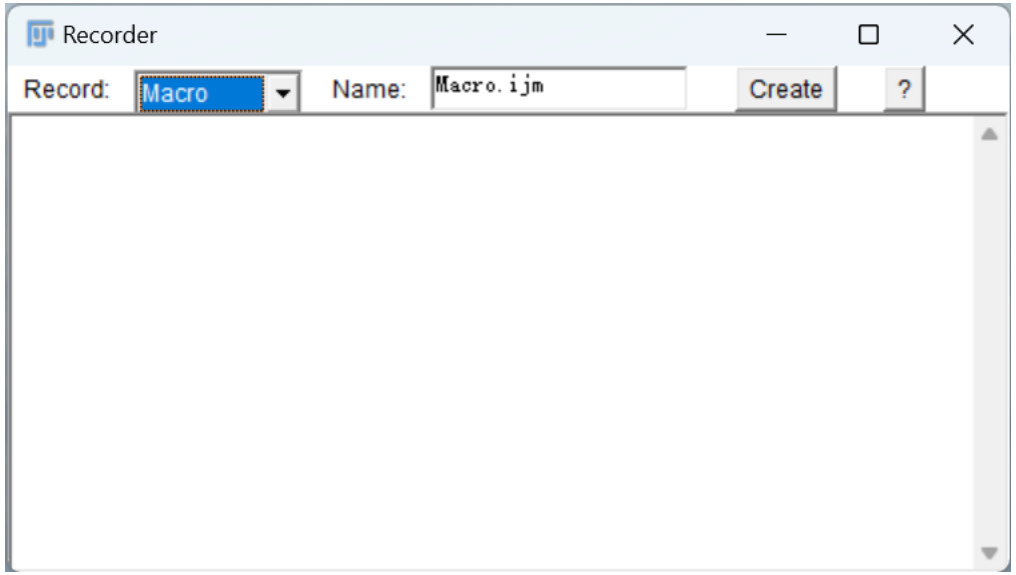
Macro

Processes can be automated using macros (distributed by someone or your own)

Introduction into Macro Programming
https://imagej.net/scripting/macro

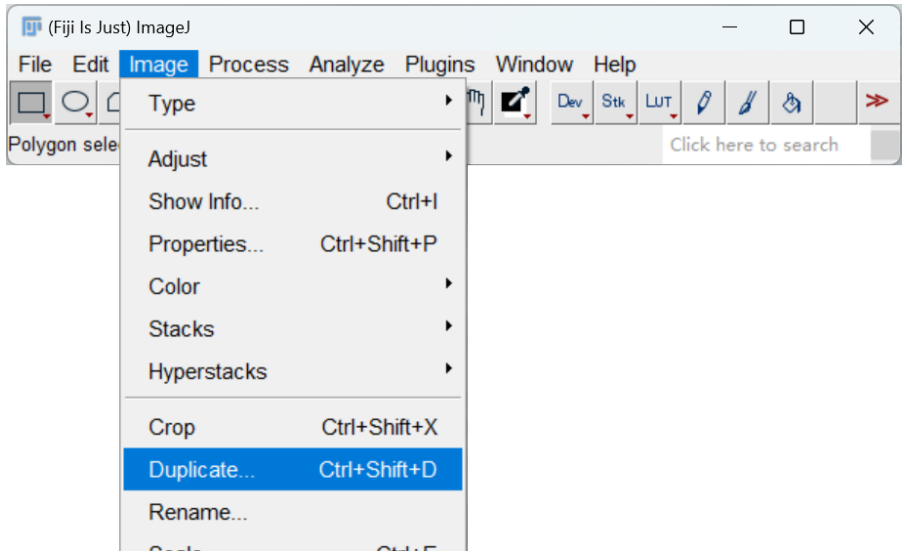
Built-in Macro Functions (References)
https://wsr.imagej.net/developer/macro/functions.html

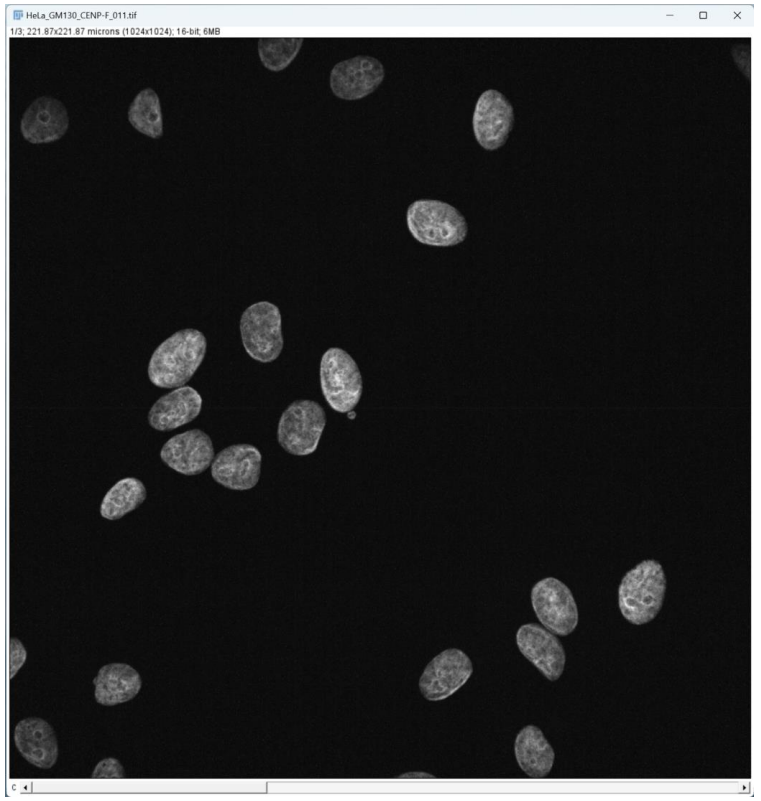


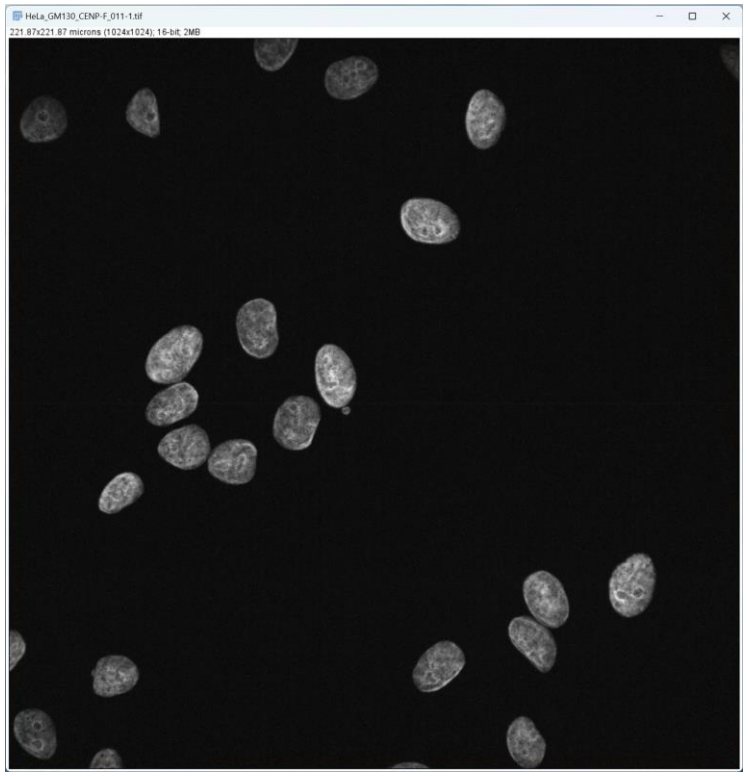


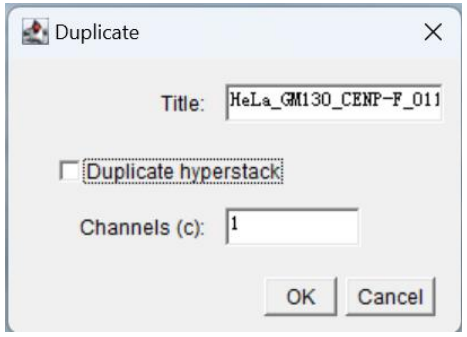
Processes can be recorded to assist writing a macro

Macro: Automatic counting of nuclei

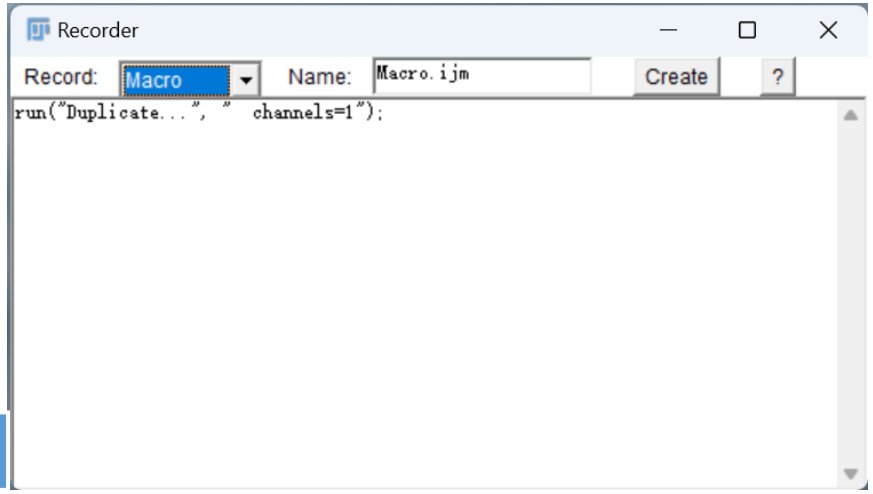






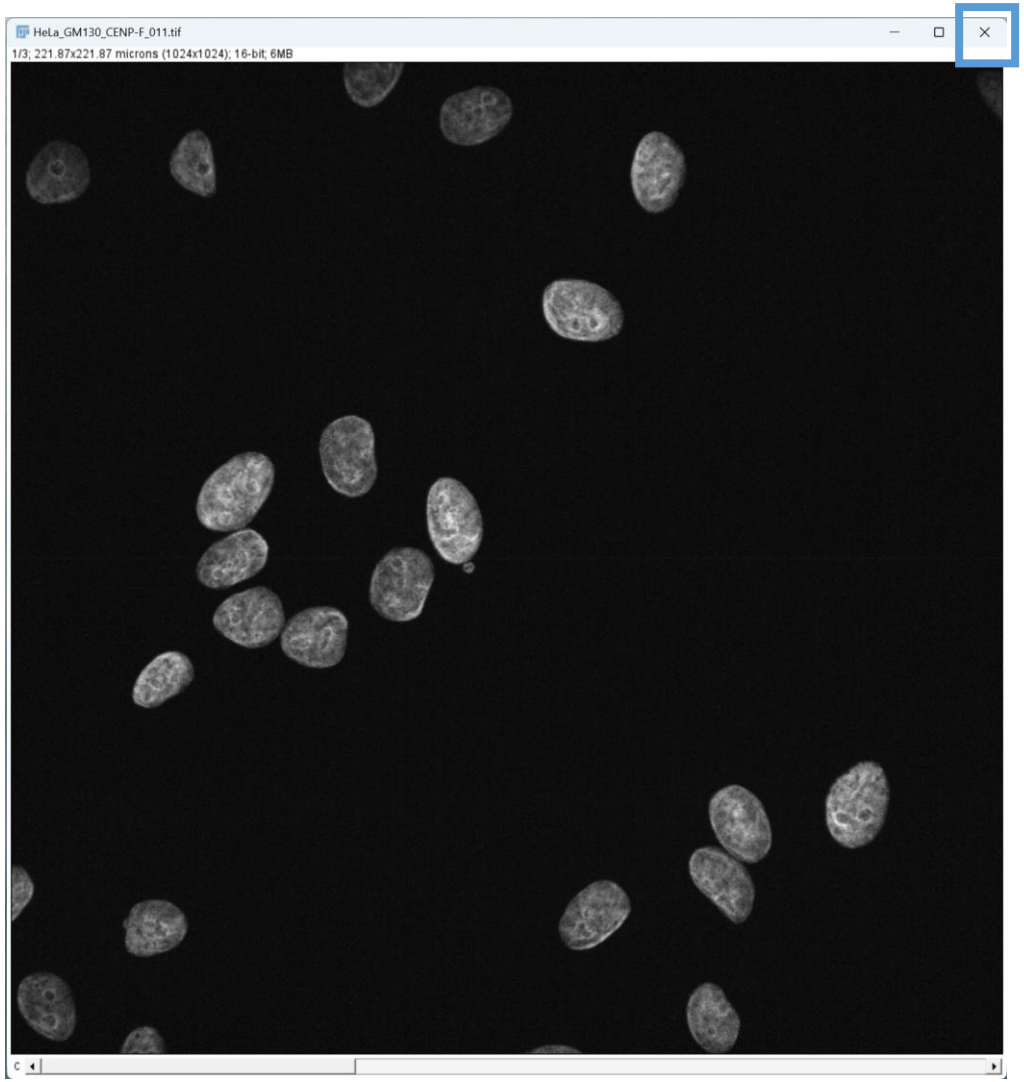


Open “Hela_GM130_CENP-F_011.tif” and extract 1 slice (nucleus channel) from a stack

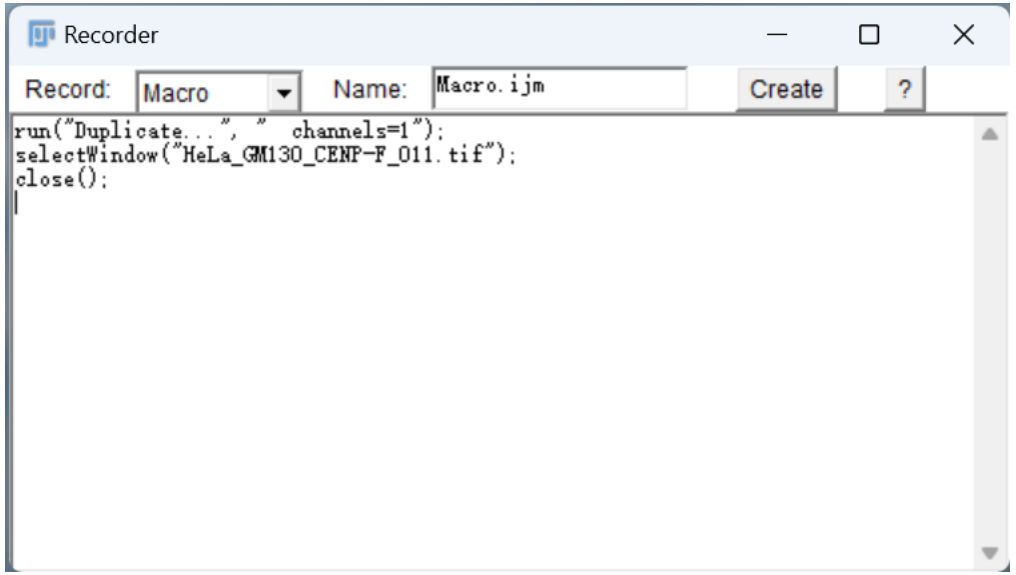


The process is recorded here

Macro: Automatic counting of nuclei

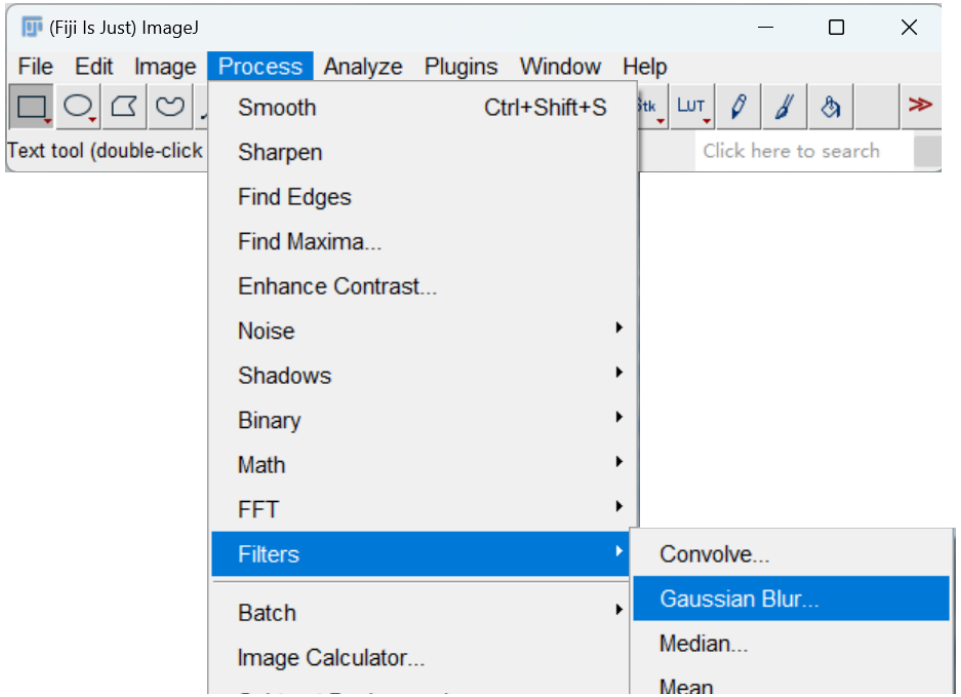


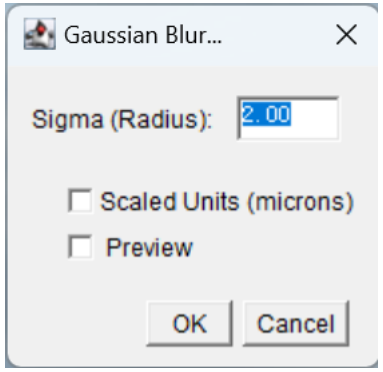
Select the original image and close

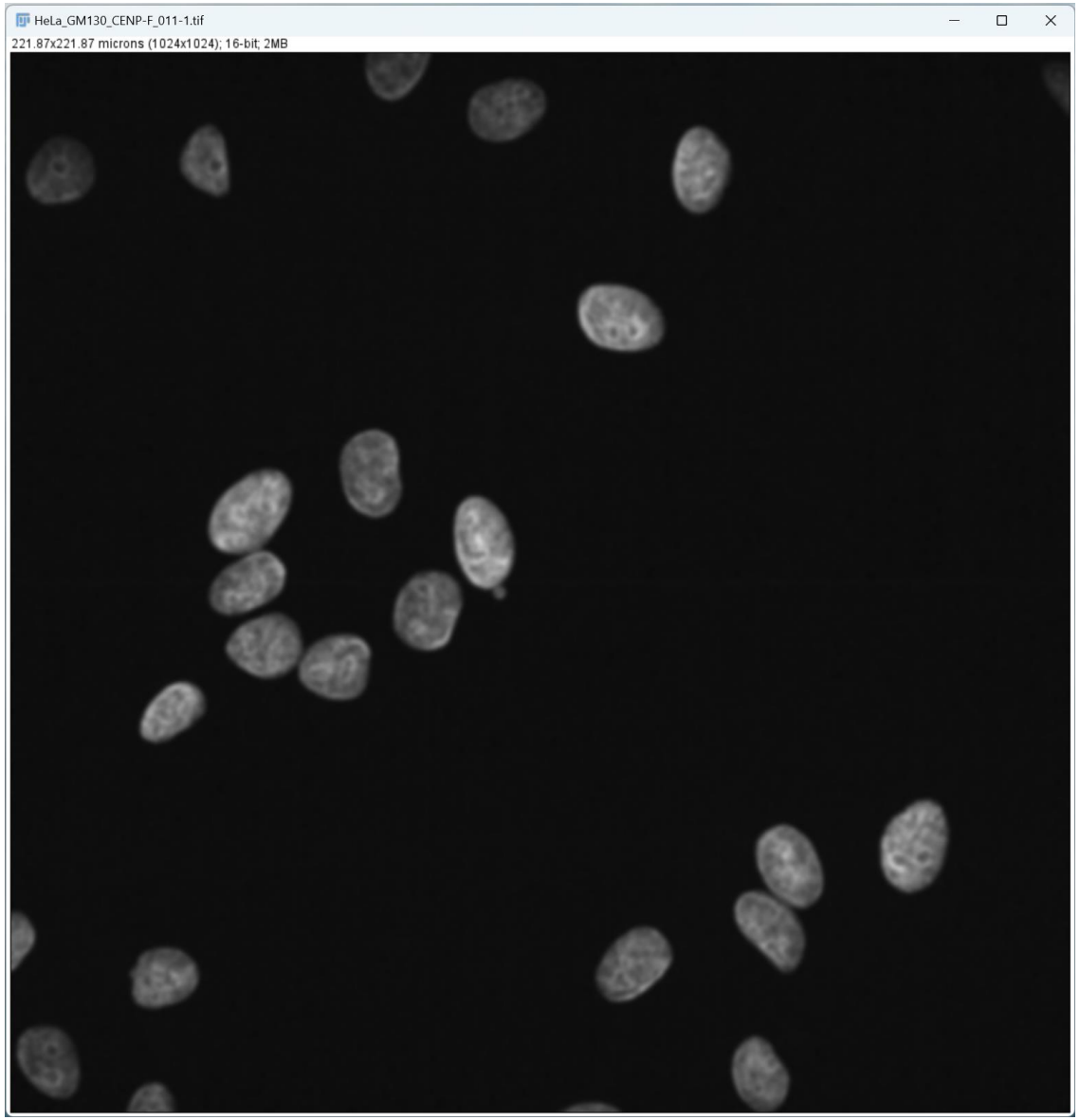


All processes will be recorded here

Macro: Automatic counting of nuclei

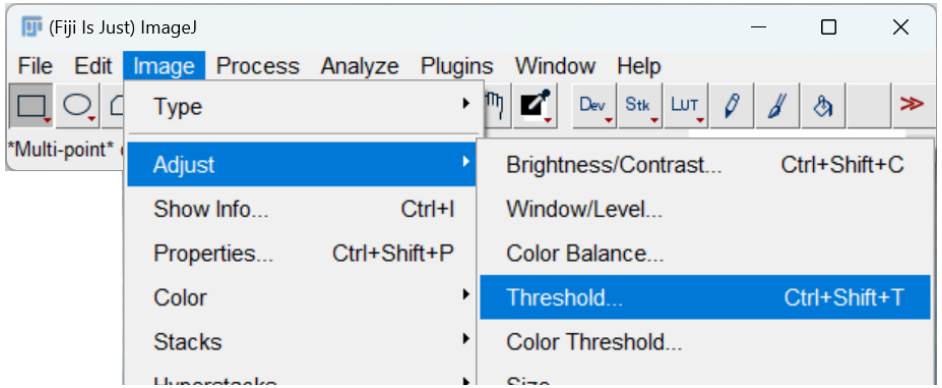


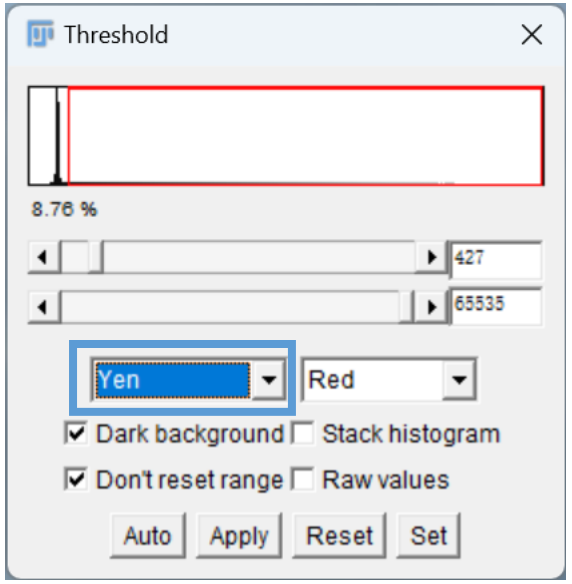


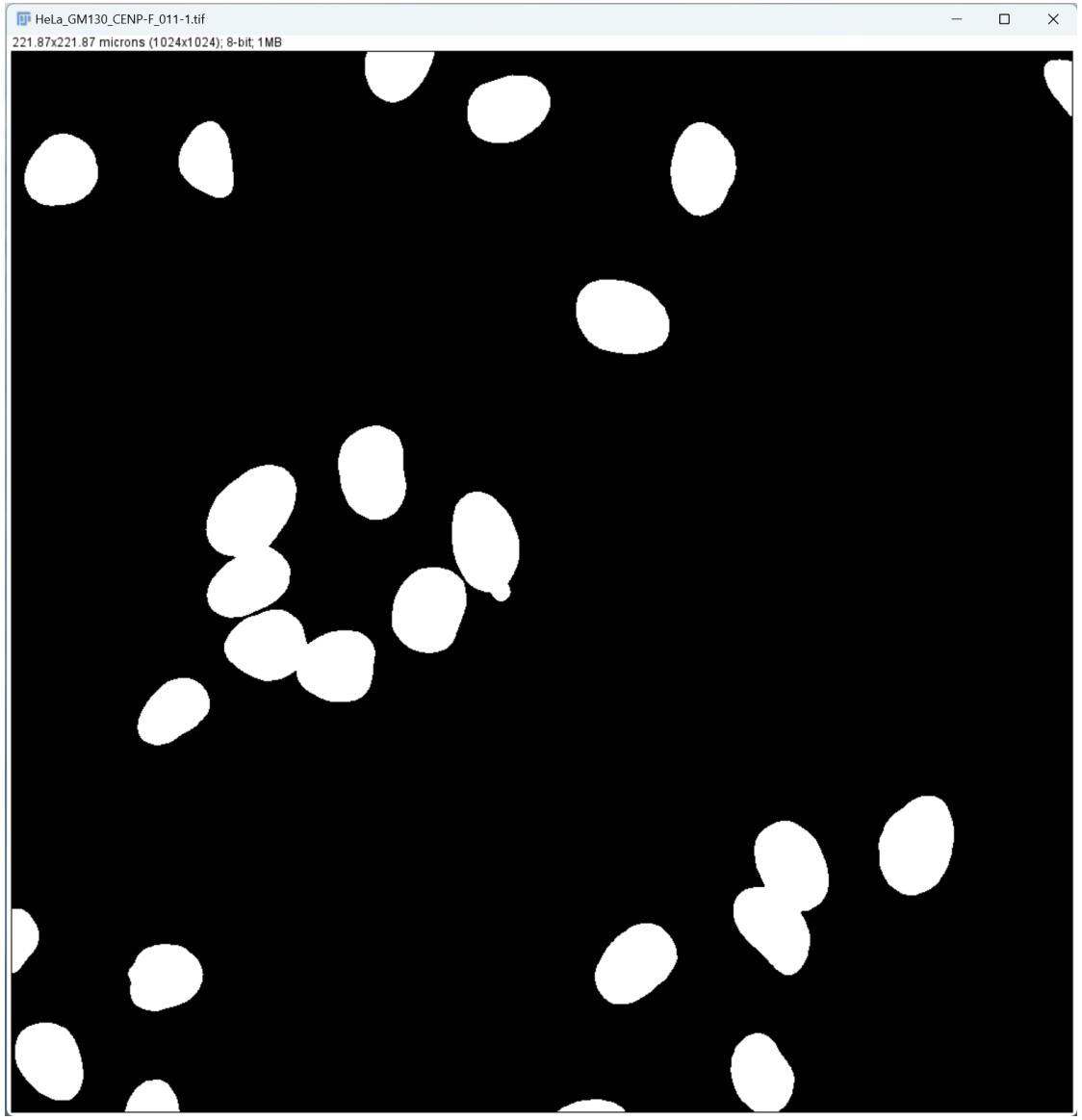


- Use Gaussian blur filter to smoothen the outlines of nuclei
- “Preview” is convenient to determine the radius

Macro: Automatic counting of nuclei

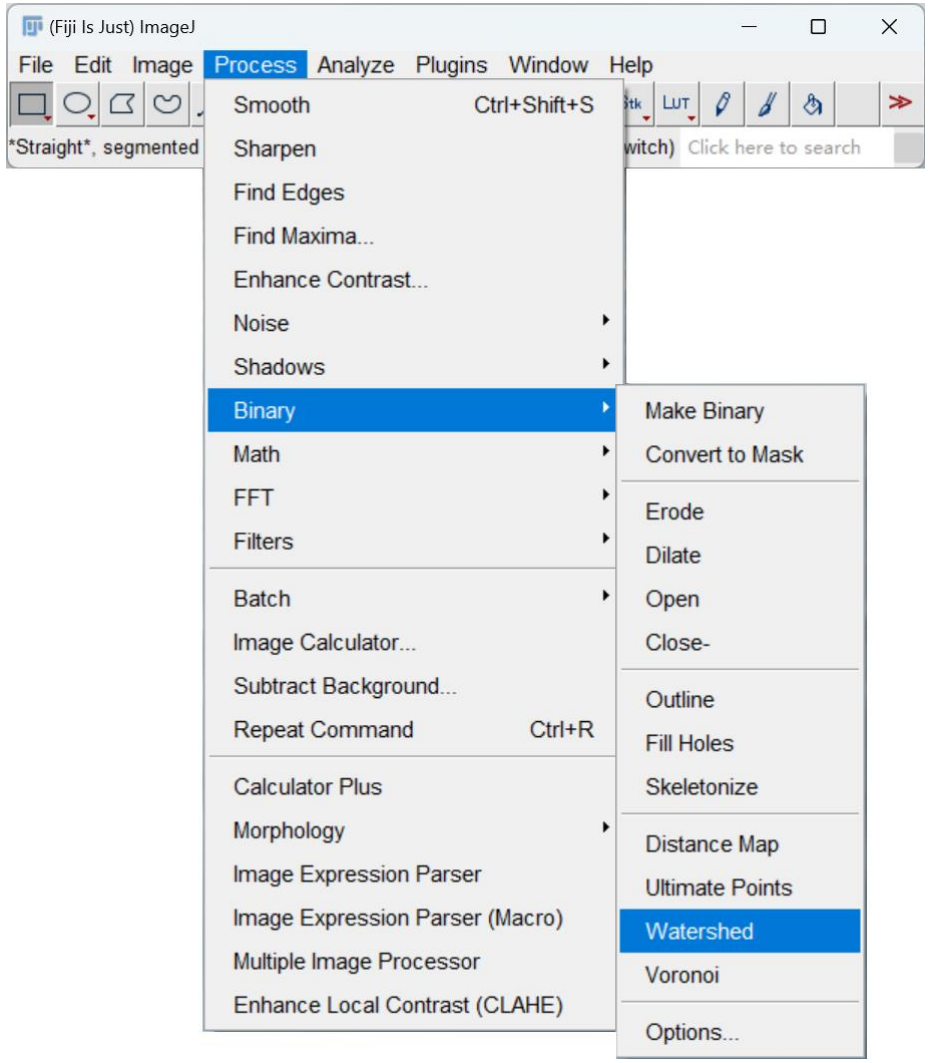


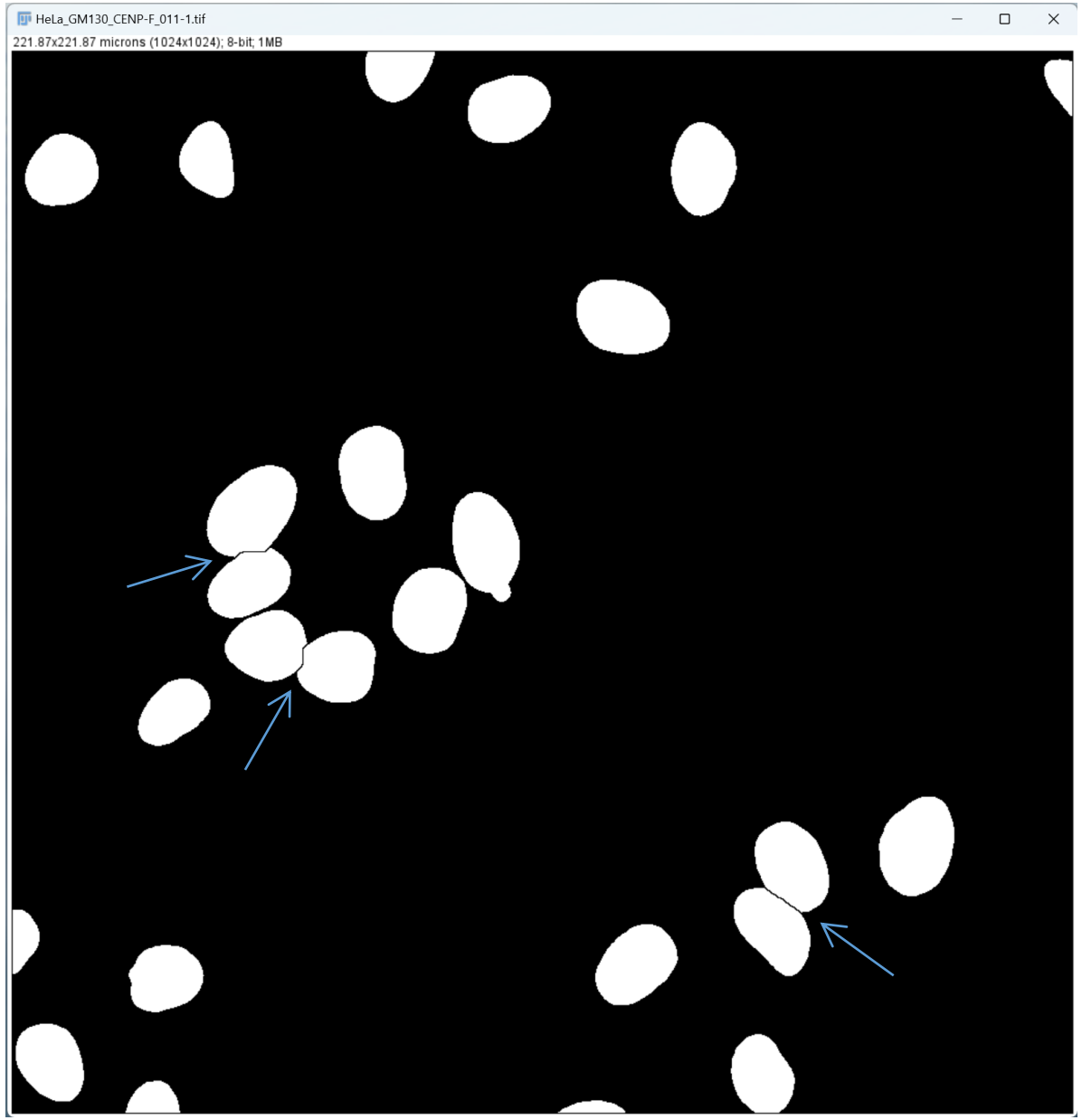




Binarization either manually or using an automation method (“Yen” in this case)
Note: Thresholding value (and thus selection of method) significantly affect the result

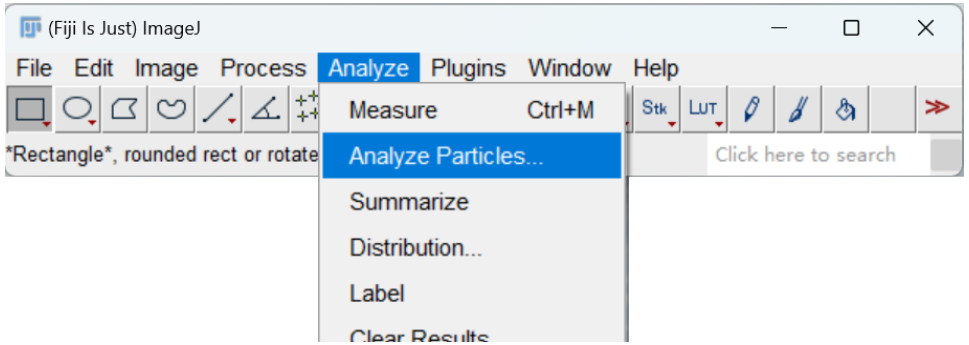
Macro: Automatic counting of nuclei

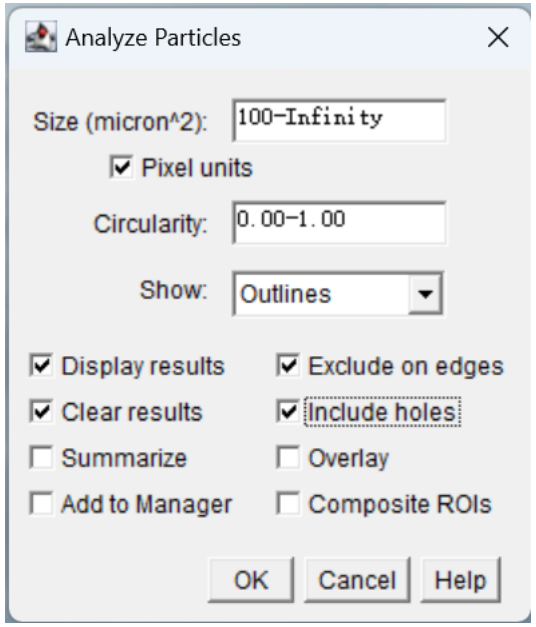


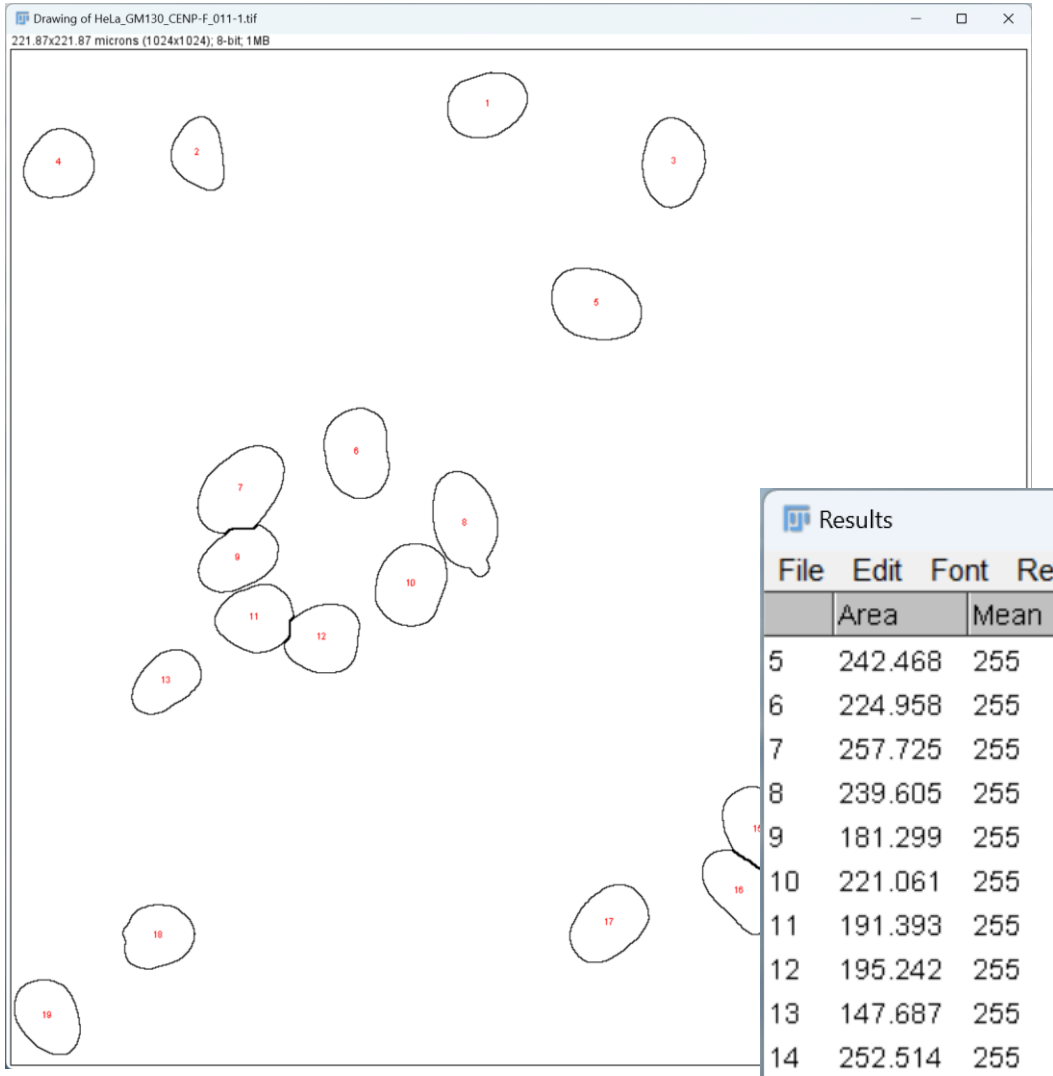


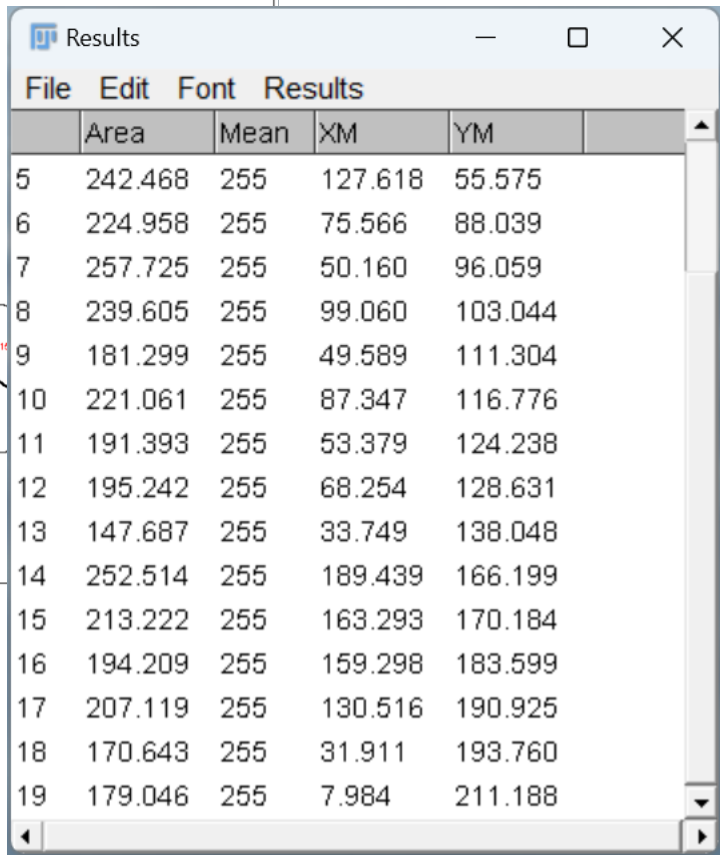
- Segment fused nuclei
- “Watershed” is a popular algorithm

Macro: Automatic counting of nuclei





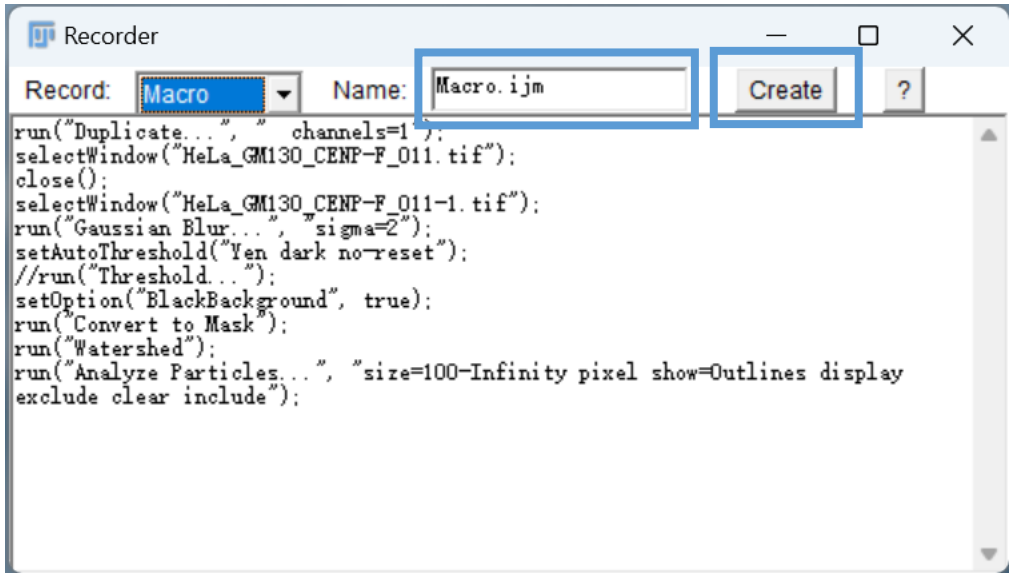




	Area	Mean	XM	YM
5	242.468	255	127.618	55.575
6	224.958	255	75.566	88.039
7	257.725	255	50.160	96.059
8	239.605	255	99.060	103.044
9	181.299	255	49.589	111.304
10	221.061	255	87.347	116.776
11	191.393	255	53.379	124.238
12	195.242	255	68.254	128.631
13	147.687	255	33.749	138.048
14	252.514	255	189.439	166.199
15	213.222	255	163.293	170.184
16	194.209	255	159.298	183.599
17	207.119	255	130.516	190.925
18	170.643	255	31.911	193.760
19	179.046	255	7.984	211.188

- Automatic measurements of segmented nuclei
- Many options to try and see what will happen

Macro: Automatic counting of nuclei

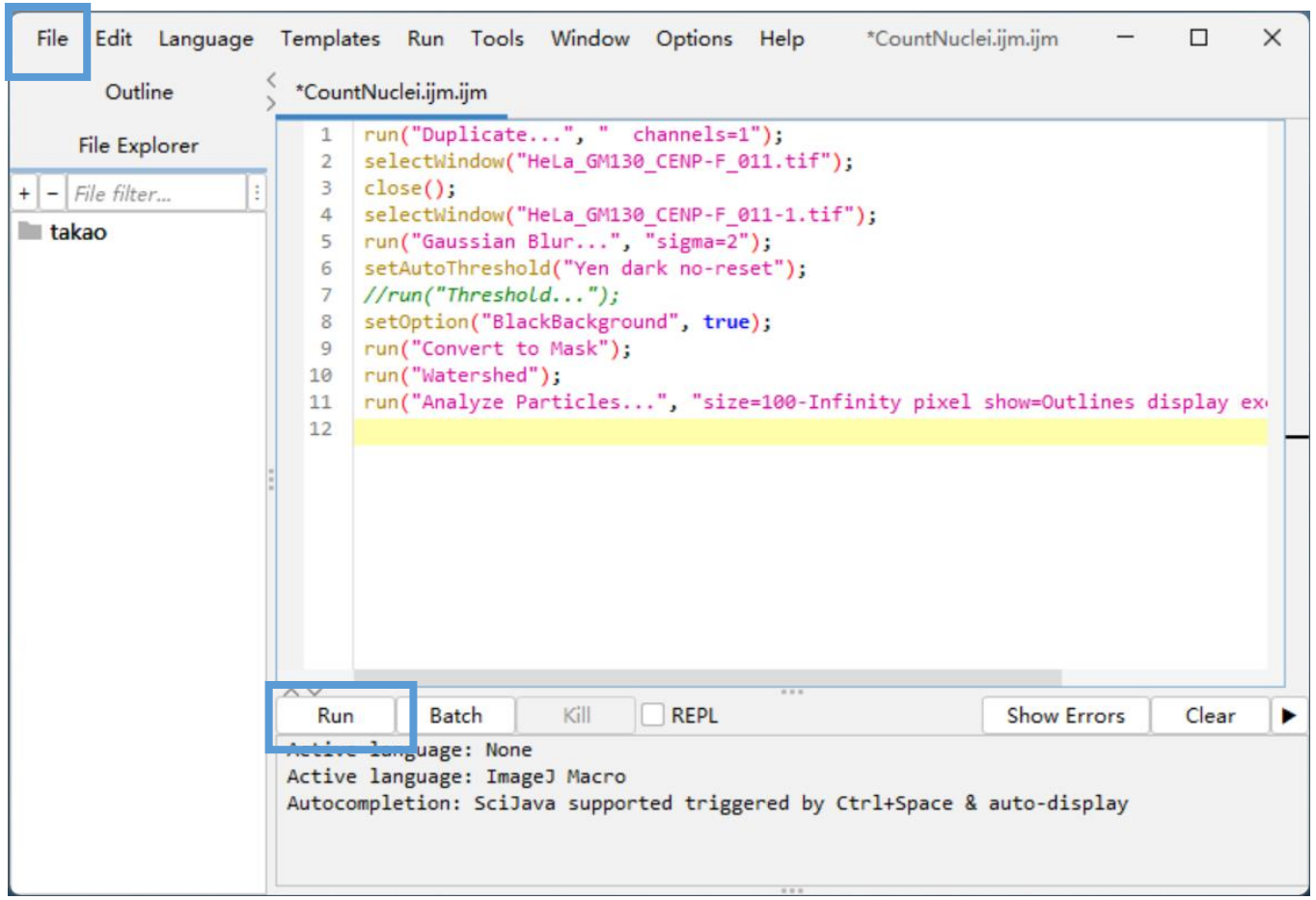


- Recorded processes look like this

- Change the name (optional) and
  click “Create” to convert it to a macro

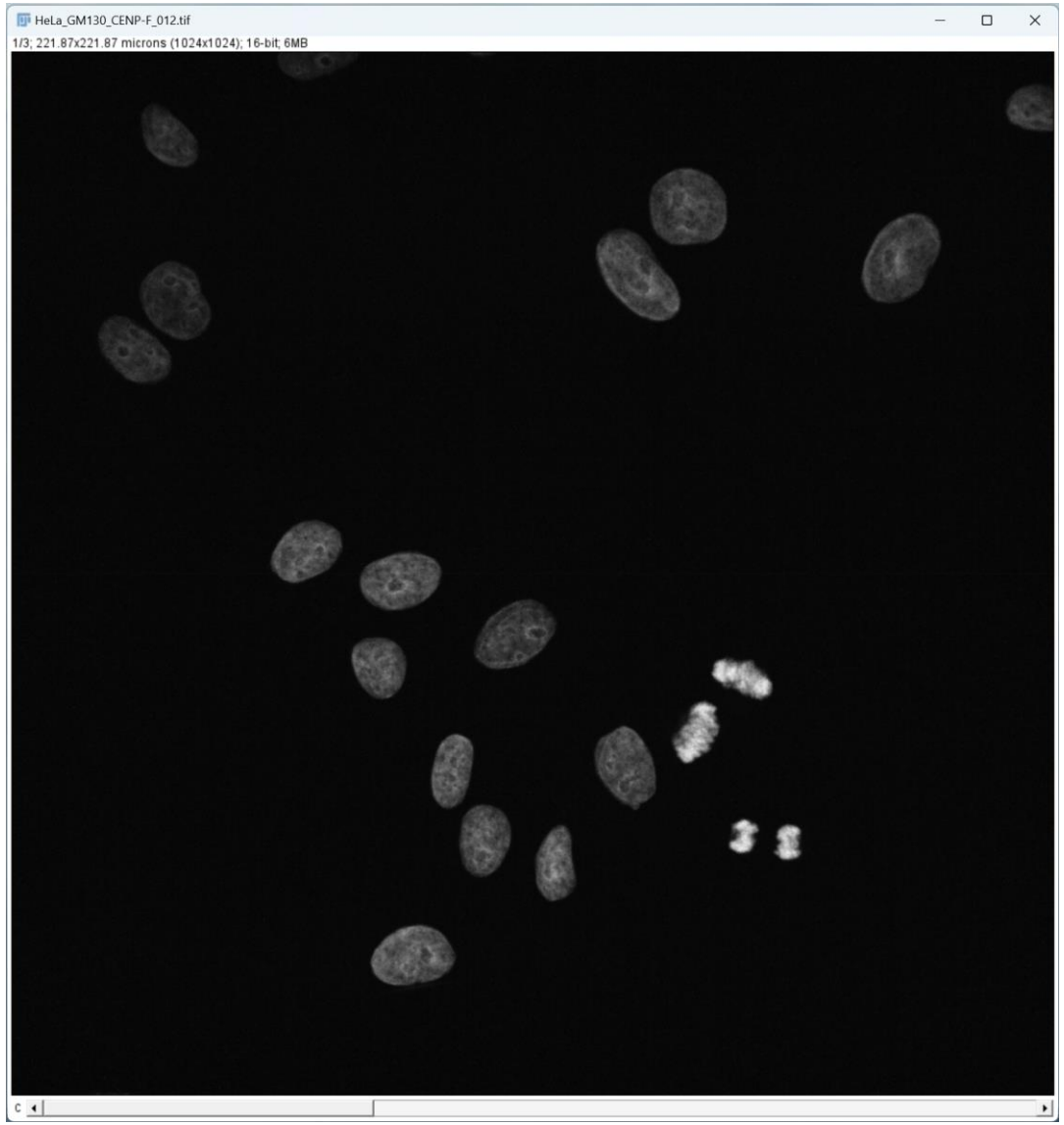
- Created macro can be saved and opened
  like image files

- “.ijm” or “.txt” works with no differences



Open the image file again and click “Run” to see if it will work

Macro: Automatic counting of nuclei



Try with another image

… will result in error because of different file name

```
1  run("Duplicate...", " channels=1");
2  selectWindow("HeLa_GM130_CENP-F_011.tif");
3  close();
4  selectWindow("HeLa_GM130_CENP-F_011-1.tif");
5  run("Gaussian Blur...", "sigma=2");
6  setAutoThreshold("Yen dark no-reset");
```

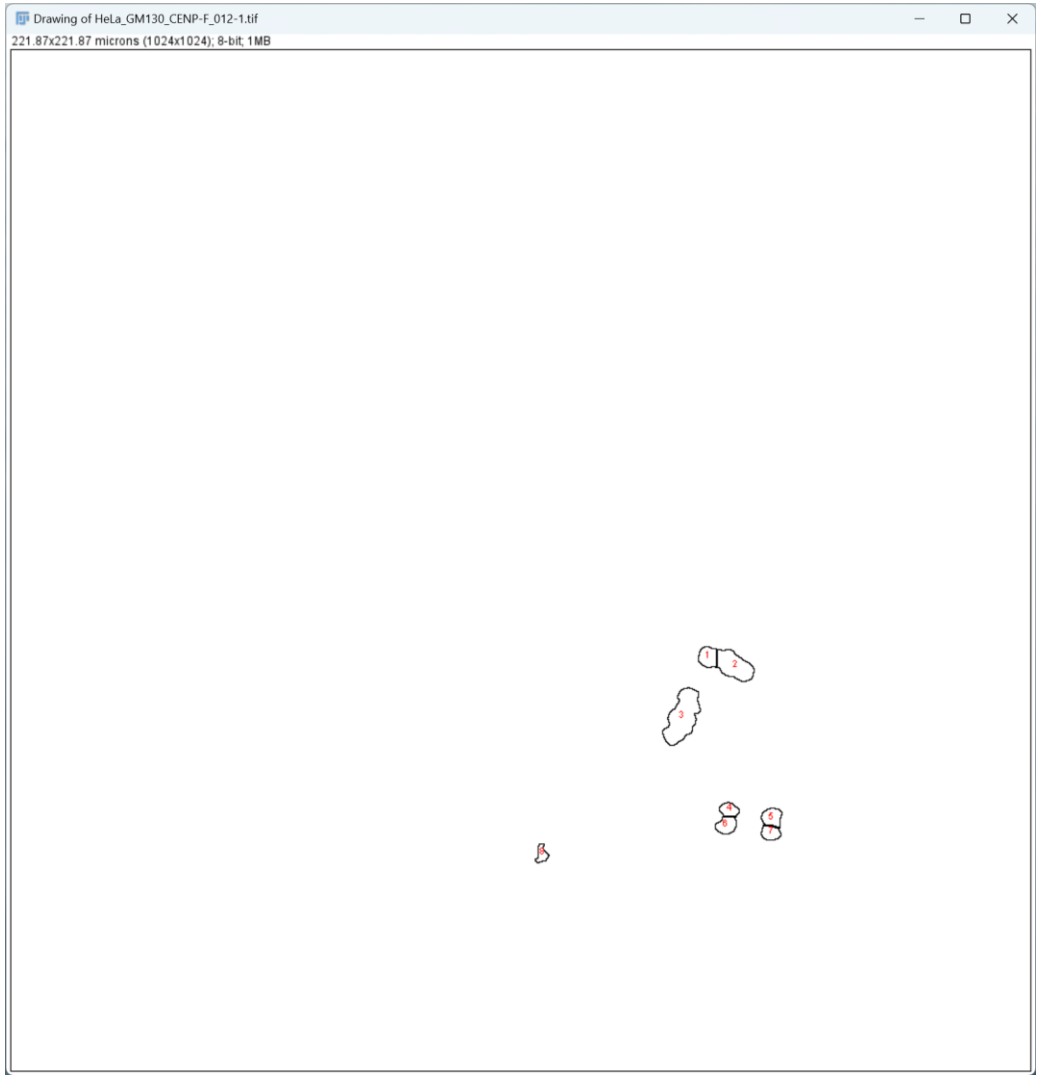
Macro: Automatic counting of nuclei

```
run("Duplicate...", "  channels=1");
selectWindow("HeLa_GM130_CENP-F_011.tif");
close();
selectWindow("HeLa_GM130_CENP-F_011-1.tif");
run("Gaussian Blur...", "sigma=2");
setAutoThreshold("Yen dark no-reset");
//run("Threshold...");
setOption("BlackBackground", true);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=100-Infinity pixel show=Outlines display ex
```

```
image_title1 = getTitle(); // get the image title (file name)
run("Duplicate...", "  channels=1");
image_title2 = getTitle(); // get the title of the duplicated image
selectWindow(image_title1); // select and close the original image
close();
selectWindow(image_title2);
run("Gaussian Blur...", "sigma=2");
setAutoThreshold("Yen dark no-reset");
//run("Threshold...");
setOption("BlackBackground", true);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=100-Infinity pixel show=Outlines display exc
```

Use “getTitle()” function to make it more flexible

Macro: Automatic counting of nuclei



Results

File Edit Font Results

	Area	Mean	XM	YM
1	14.881	255	151.593	131.863
2	36.992	255	157.326	133.732
3	59.666	255	146.032	144.847
4	9.718	255	156.190	165.057
5	13.989	255	165.523	166.675
6	13.051	255	155.615	168.444
7	10.563	255	165.220	170.098
8	6.619	255	115.403	174.719

- Now the macro worked for another image, but bad result…

- Binarization (thresholding) method needs to be optimized

- You will always need such trial-and-error processes