INTRODUCTION TO FIJI / IMAGEJ – HANDS-ON TRAINING

Applied Microscopy Course – July 2019
Adapted for GloBIAS and CNR Naples – 2025 - Fluorescence image analysis course
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PREREQUISITES:

Download:* Fiji + EMBL Plugin *(or* www.fiji.sc*)

Plugins:

- ***CMCI-EMBL***
- ***STARDIST***
- ***CSBDEEP***
- ***TENSORFLOW***
- ***WEKA***
- ***CLIJ***
- ***ILASTIK***

1. LOADING AND CHARACTERIZING AN IMAGE

Goals: Learn the basics — load an image, get its properties and intensity histogram, convert to 8-bit, and save it.

1.1 Open the image: EMBL → Samples → 1703.tif

Note: EMBL is a plugin that is not included with default Fiji installation.

1.2 Use Fiji to find image properties: type, resolution, width, and height.
→ Image → Show Info

1.3 Show the distribution of pixels as a function of intensity:
→ Analyze → Histogram

1.4 Press the "Live" button to see histogram changes as the image changes.

1.5 Convert the image type from 16-bit grayscale to 8-bit grayscale:
→ Image → Type → 8-bit

1.6 Save the converted image:
→ File → Save As → Tiff

2. UNDERSTANDING BIT-DEPTH AND SCALING

Goals: Understand what happens during 16-bit to 8-bit conversion and how to do it correctly. Use intensity profiles to visualize scaling effects.

2.1 Open: EMBL → Samples → m51.tif

2.2 Use the line tool to draw a line (line ROI) through the bright spot in the middle.
→ Analyze → Plot Profile

2.3 Ensure "Scale When Converting" is checked:
→ Edit → Options → Conversion

2.4 Convert image to 8-bit:
→ Image → Type → 8-bit
→ Re-plot profile and compare it to the 16-bit one.

2.5 Save the ROI using "t" or "Add" in ROI Manager:
→ Analyze → Tools → ROI Manager

2.6 Now uncheck "Scale When Converting":
→ Edit → Options → Conversion

2.7 Reopen the original 16-bit image and reconvert it. Observe what changes.

2.8 Reapply the saved ROI and re-plot the profile. Note the differences.

3. INTENSITY AND CONTRAST ENHANCEMENT

Goals: Compare under-/overexposed images and properly adjust contrast.

3.1 Open gel_inv.tif from:
→ EMBL → Samples

Adjust image contrast. The original image remains unchanged unless "Apply" is used.

Question: What's the problem with setting minimum=80 and maximum=200?

Note: Avoid scaling when quantifying intensity.

4. COLOR DISPLAY

4.1 Look-Up Tables (LUTs)

Goals: Understand LUTs and calibration bars.

4.1.1 Open: EMBL → Samples → cells_ActinDNA.tif

4.1.2 Change LUT:
→ Image → Lookup Tables

4.1.3 Display calibration bar:
→ Analyze → Tools → Calibration Bar

4.1.4 Save image:
→ File → Save As → Tiff

4.2 Multi-Channel Images

Goals: Learn to split and merge channels, use the Composite view and Channels Tool.

4.2.1 Open: EMBL → Samples → RGB_Cell.tif
→ Image → Color → Split Channels

4.2.2 Merge channels back:
→ Image → Color → Merge Channels

- Uncheck "Create Composite"
- Check "Keep Source Images"
- Try changing color assignments.

4.2.3 Merge again but check "Create Composite."

- View channels in Composite / Color / Grayscale mode:
  → Image → Color → Channels Tool
- Adjust intensities of individual channels.

4.3 Color Images

Goals: Work with RGB/HSB, color deconvolution for histological stains.

4.3.1 Open Emphysema_H&E.tif (from samples)

4.3.2 Try splitting channels:
→ Image → Color → Split Channels

4.3.3 Extract Brightness:
→ Image → Type → HSB Stack
→ Duplicate the third channel only.

4.3.4 Use Color Deconvolution:
→ Image → Color → Color Deconvolution
→ Select "H&E"

Note: Do not use this plugin to quantify DAB. QuPath is better suited for this task.

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5. GEOMETRICAL MEASUREMENTS AND MANUAL CELL COUNTS

Goals: Use different ROI tools (line, angle, polygon, ellipse), measure geometric properties, and count cells.

5.1 Open blobs.tif

5.2 Set measurement features:
→ Analyze → Set Measurements → Check Area and Perimeter

Tip: Use "Limit to threshold" for thresholded areas only.

5.3 Use Line Tool to measure distances.

5.4 Use Angle Tool for cell angle measurements:
→ Analyze → Measure

5.5 Use Polygon and Elliptical selections to measure area and perimeter.

5.6 Count nuclei manually: use Point Tool with Shift+Click

5.7 Remove a point with Alt+Click

5.8 Get cell count:
→ Analyze → Measure

Tip: Double-click Point Tool to enable Auto-Measure and Add to ROI Manager.

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6. ROI, CALIBRATIONS, AND MORE MEASUREMENTS

6.1 Intensity Measure and ROI

6.1.1 Open fluorescent_Cells.tif
→ Split channels
→ Use blue channel
→ Set Measurements: Area, Mean gray value, Integrated density, Perimeter

6.1.2 Select ROI, zoom if needed, and add to ROI Manager (shortcut: "t")

6.1.3 Measure ROI:
→ Analyze → Measure

6.1.4 Repeat for 2 more ROIs

6.1.5 Enable "Show All" in ROI Manager

6.1.6 Rename ROIs to nuc1, nuc2, nuc3

6.1.7 Highlight all ROIs and use "Measure" to get data for all at once

6.1.8 Switch to red channel. Create oval ROI.

6.1.9 Save selection:
→ File → Save As → Selection (e.g., "ROI red")

6.1.10 Measure 3 areas using this ROI.

6.1.11 Save results:
→ File → Save As from Results window (Excel format)

Tip: *Use Results → Options to include headers and row numbers.*

6.2 Calibration

6.2.1 Open image with 25µm scale bar

6.2.2 Draw line over scale bar →
→ Analyze → Set Scale
→ Distance = 25, Unit = µm

6.2.3 Add scale bar to image:
→ Analyze → Tools → Scale Bar

6.2.4 Save the image

Note: Global calibration overrides Bio-Formats metadata. To remove, click "Click to Remove Scale."

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7. AUTOMATIC SEGMENTATION

Goals: Segment using threshold, apply watershed, count objects, and save results.

7.1 Open RGB_Cell.tif → Duplicate as "Original"

7.2 Convert to grayscale using Green channel (not Image → Type → 8-bit!)

7.3 Apply threshold:
→ Image → Adjust → Threshold
→ Select Otsu/Intermodes → Apply

7.4 Use Watershed:
→ Process → Binary → Watershed

7.5 Count with Analyze Particles:
→ Set size/circularity
→ Check: Display Results, Clear Results, Summarize, Add to Manager
→ Option: Exclude on Edges

7.6 Use Wand Tool to inspect object size manually

7.7 Save Results and Summary:
→ File → Save As

7.8 Overlay results:
→ Use ROI Manager → Flatten → Save

Tip: To measure intensity from original image, use "Redirect To" in Set Measurements.

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8. MASKING

Goals: Analyze only specific image regions using masks.

8.1 Repeat steps from Exercise 7 up to 7.8

8.2 Select ROI to mask an area
→ Edit → Selection → Create Mask

8.3 Return to segmented image →
→ Edit → Selection → Select None

8.4 Use Image Calculator:
→ Process → Image Calculator
→ Image1 = Segmented, Image2 = Mask, Operation = AND

8.5 Analyze Particles on the masked result

Question: What is the difference between masking with image math vs. duplicating a selected area?

Exercise: Measuring Cell Fluorescence

Contributed by Martin Fitzpatrick – University of Birmingham
Data: Mitocheck 2D+t

1. Select the cell using ROI tools (rectangle, circle, etc.)
2. Set measurements:
   → Analyze → Set Measurements
   → Check Area, Integrated Density, Mean Gray Value
3. Measure:
   → Analyze → Measure
4. Select background area (no fluorescence) and measure similarly.

Tip: For accuracy, take 3+ background samples.

5. Copy Results table to spreadsheet.
6. Calculate:
   $$CTCF = Integrated\ Density - (Area \times Mean\ Background)$$
7. Plot data

Note: *Rounded mitotic cells may appear brighter due to compact size — be cautious with raw integrated density.To overcome that issue, you need to:*

NORMALIZE CTCF BY CELL AREA:

$$Fluorescence\ per\ unit\ area = \frac{CTCF}{Area}$$

Source:
https://blog.martinfitzpatrick.com/measuring-cell-fluorescence-using-imagej/
