Introduction to image analysis using ImageJ/Fiji

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Get the course materials

https://hms-iac.github.io/fiji-workshop

One-stop resource for everything we'll cover today

Problem: Seeing is Believing

Mask rotates continuously in the same direction
(nose passing right to left on screen)

Can you see the inside of the mask?

https://en.wikipedia.org/wiki/Hollow-Face_illusion

Learning objectives

1. **Motivate** the use of algorithms in image analysis
2. **Introduce** some image-analysis nomenclature
3. **Learn** to use Fiji effectively and reproducibly
4. **Touch** on deep learning

Reasons for Learning About Image Processing

- Make pretty pictures (processing)
  - publications, talks, websites, ...
- Get numbers out of pictures (analysis)
  - cell sizes, vessel lengths, GPF expression level, ...
- Make experiment possible (automation)
  - whole-genome screen: millions of images
- Objectivity and Reproducibility
  - in science, just do it!

Reasons for <u>Not</u> Learning About Image Processing

- …

none

Acknowledgements

- Szymon Stoma, ETH Zurich, Switzerland
  - slides and workflows
- Peter Bankhead, Edinburgh University, Scotland
  - slides and graphics

I.A.C. (Image Analysis Collaboratory)
@ HMS since Sep ‘22

- **Support** and **collaboration:** for image and data analysis projects
- **Teaching:** at HMS and internationally
- **IT:** access to relevant solutions, open- and closed-source
- **R&D:** centered on methods and tools
- **Locations**: LHRRB 105 & Armenise 531D
- **Consults**: Contact Simon
  - simon@hms.harvard.edu

https://iac.hms.harvard.edu/

Image
Analysis
Collaboratory

Who *we* are, currently

Antoine A. Ruzette

Associate

Ranit Karmakar

Specialist Postdoc

Maria Theiss

Specialist Postdoc

Federico Gasparoli

Research Associate

Simon F. Nørrelykke

Director, Lecturer

Backgrounds in

Physics
Biology
Microscopy
Bioengineering
Computer Engineering

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How we operate

Consultations

"Image Clinics"

Teaching and Training

Courses in image analysis

Postdoc training program

Collaborations

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Self-introductions

1. My **name** is *Donald Duck*
2. My **position** is as a *10th year postdoc*
3. My **lab** is *Mad City Labs*
4. My model **system** is *blue whales*
5. I have **data** from *confocal microscopy*
6. A **fun fact** about me is *I pipette at the speed of light*

Optical Illusions

—

Why should you analyze images with computers

Color perception and pattern recognition is individual – science less so

https://www.moillusions.com/perfect-circles-optical-illusion/

Concentric circles !?

https://www.moillusions.com/perfect-circles-optical-illusion/

Identical central discs?

Our size estimate is strongly influenced by the local neighbourhood

http://www.brainbashers.com

Identical central discs?

Yes, the discs are identical

http://www.brainbashers.com

Is the dot half-way up?

Our sense of distance depends on neighbourhood

http://www.brainbashers.com

Is the dot half-way up?

Yes it is!

http://www.brainbashers.com

Which car is bigger?

http://www.brainbashers.com

Which car is bigger?

http://www.brainbashers.com

Which car is bigger?

All cars are same size

(Erroneous, application of subjective/perceptual constancy algorithm by your brain)

http://www.brainbashers.com

Are A and B equally grey?

Intensity perception depends strongly on neighbourhood

http://www.brainbashers.com

Are A and B equally grey?

Yes they are!

http://www.brainbashers.com

Spot the animal

—

Why you should still use your brain
(a pre-trained neural network)

Pattern Recognition

Yes, that is a cow!

http://www.brainbashers.com

Pattern Recognition

Yes, that is a cow!

http://www.brainbashers.com

Using Prior Knowledge

Keyword: Dalmatian dog!

http://www.brainbashers.com

Using Prior Knowledge

Keyword: Dalmatian dog!

http://www.brainbashers.com

A brief history of image analysis

—

ImageJ centric

37 years ago…

NIH IMAGE

What is ImageJ?

ImageJ is an open source image processing and image analysis software for multi-dimensional image data with a focus on scientific imaging.

1987 - NIH Image: written in Pascal for the Macintosh by Wayne Rasband

1997 - ImageJ 1.x: written in Java and also compatible with Windows PC

Since then, ImageJ has been maintained and upgraded (e.g. ImageJ2, Fiji, …)

ImageJ GitHub | Fiji GitHub

Schneider CA, Rasband WS, Eliceiri KW (2012). "NIH Image to ImageJ: 25 years of image analysis". *Nat Methods*. **9** (7): 671–675. doi:10.1038/nmeth.2089. PMC 5554542. PMID 22930834.

What is Fiji?

Fiji stands for Fiji Is Just ImageJ

It is a "batteries included" distribution of ImageJ2

Easy to install ("portable" application)

Include a collection of Plugins

Easy to update (https://imagej.net/list-of-update-sites/)

Documentation

12 years ago…

- ***AlexNet*** is published (2012)
  - Wins the *ImageNet Large Scale Visual Recognition Challenge*
  - Error of 15.3%, more than 10.8 percentage points better than #2
  - Possible due to GPUs
- ***CellProfiler*** has been around for 6 years (2006)
- ***Fiji*** (Fiji is just ImageJ) has been around for 5 years (2007)
- ***scikit-image*** released 3 years ago (2009)
- Bitcoin grows from $5 to $13 (remember Silk Road?) (2012)
- U-Net, GANs, and ***Jupyter*** will appear in 2-3 years (2014/15)
- ***AlphaGo*** will beat Lee Sodol in 4 years (2016)
- ***QuPath*** is still 4 years in the future (2016)

4 years ago…

- CellPose is out (2020)
  - “Cellpose: a generalist algorithm for cellular segmentation”
  - Trained on highly varied images of cells,over 70,000 segmented objects
  - Cells don’t have to be star-shaped
  - Web-platform and Jupyter notebooks
  - https://www.nature.com/articles/s41592-020-01018-x
- ZeroCostDL4Mic available
  - Paper still a year in the future
  - Implementation of common DL technologies to microscopy imaging
  - Relies on GPUs and other infrastructure provided by Google Colab.
  - https://github.com/HenriquesLab/ZeroCostDL4Mic/wiki

- *Vision Transformers* (2020)
  - “*An Image is Worth 16x16 Words: Transformers for Image Recognition at Scale”*, Dosovitskiy et al.
  - https://arxiv.org/abs/2010.11929

Today…

Image Analysis Software

forum.image.sc

History of Segmentation

https://x.com/BoWang87/status/1773132444717252718

English as the next programming language

—

LLMs, chatbots and foundation models for bio image analysis

GPT-3.5 GPT-4

ChatGPT

Create a workout plan
for resistance training

Design a database schema
for an online merch store

Explain why popcorn pops
to a kid who loves watching it in the microwave

Write an email
to request a quote from local plumbers

Please write an ImageJ macro that uses StarDist to segment an image from fluorescence microscopy

Free Research Preview. ChatGPT may produce inaccurate information about people, places, or facts. ChatGPT September 25 Version

https://chat.openai.com/

Using ChatGPT for Image Analysis

SI **You**

please segment the nuclei and show me the relevant code you used

Using ChatGPT for Image Analysis

GPT-4, May 2024

SI **You**

please segment the nuclei and show me the relevant code you used

GPT-4o, Oct. 2024

please segment the nuclei and show me the relevant code you used

Using ChatGPT for Image Analysis

GPT-4, May 2024

ChatGPT

The nuclei in the image have been segmented using the following steps:

- The image was converted to grayscale to simplify the analysis.
- Gaussian Blur was applied to reduce noise and detail in the image, which aids in thresholding and contour detection.
- Otsu's thresholding was performed to convert the grayscale image into a binary image, which helps in distinguishing the nuclei from the background.
- Contours were found in the binary image, which correspond to the boundaries of the nuclei.
- These contours were then drawn onto the original image to visually segment the nuclei.

GPT-4o, Oct. 2024

Using ChatGPT for Image Analysis

GPT-4, May 2024

python Copy code

```python
import cv2
import numpy as np
from matplotlib import pyplot as plt

# Load the image
image_path = '/mnt/data/hela-cells.jpg'
image = cv2.imread(image_path)

# Convert the image to gray scale
gray = cv2.cvtColor(image, cv2.COLOR_BGR2GRAY)

# Apply GaussianBlur, which is good for edge detection
blurred = cv2.GaussianBlur(gray, (5, 5), 0)

# Perform Otsu's thresholding
_, thresh = cv2.threshold(blurred, 0, 255, cv2.THRESH_BINARY_INV + cv2.

# Find contours which will give us the nuclei boundaries
contours, _ = cv2.findContours(thresh, cv2.RETR_TREE, cv2.CHAIN_APPROX_

# Draw the contours on the original image
contoured_image = image.copy()
cv2.drawContours(contoured_image, contours, -1, (0, 255, 0), 3)

# Save the output image with segmented nuclei
output_path = '/mnt/data/hela-cells-segmented.jpg'
cv2.imwrite(output_path, contoured_image)
```

GPT-4o, Oct. 2024

python Copy code

```python
from skimage import io, filters, measure, morphology, color
import matplotlib.pyplot as plt
import numpy as np

# Load the image
image_path = '/mnt/data/hela-cells.png'
image = io.imread(image_path)

# Convert to grayscale
gray_image = color.rgb2gray(image)

# Apply Gaussian filter to smooth the image
smoothed = filters.gaussian(gray_image, sigma=1)

# Use Otsu's threshold to create a binary mask of the nuclei
threshold_value = filters.threshold_otsu(smoothed)
binary_mask = smoothed > threshold_value

# Remove small objects and fill holes to improve segmentation
cleaned_mask = morphology.remove_small_objects(binary_mask, min_size=500)
cleaned_mask = morphology.remove_small_holes(cleaned_mask, area_threshold=500)

# Label the segmented regions
labeled_nuclei = measure.label(cleaned_mask)
```

Using ChatGPT for Image Analysis

GPT-4, May 2024

You

please use the blue channel for segmenting the nuclei

ChatGPT

The nuclei segmentation has been performed using the blue channel of the image with the following steps:

- The blue channel was extracted from the original image since the nuclei appear to be stained blue.
- Gaussian Blur was applied to the blue channel to smooth the image, which helps in reducing noise for thresholding.
- Otsu's thresholding was then used on the blurred blue channel to create a binary image where the nuclei are distinguished from the background.

GPT-4o, Oct. 2024

Segmented Nuclei (Blue Channel)

Using ChatGPT-4o for Image Analysis

GPT-4o, Oct. 2024 - prompted to close larger holes

make sure to close larger holes, and increase smoothing sigma to 3

Using ChatGPT-4o for Image Analysis

GPT-4o, Oct. 2024 - it helps to know what to ask

compute the convex hull for shapes with a low circularity, to approximate the characteristic shape of a nuclei

Original Image

Convex Hull for Low Circularity Nuclei

BioImage Chatbot

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https://arxiv.org/abs/2310.18351

https://aicell.io/post/bioimageio-chatbot/

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napari-omega

Omega – Harnessing
for Bioimage Analysi

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10.5281/zenodo.10828225

Supplementary Figure 3. Omega's System Architecture. Diagram illustrating the Omega system architecture, encompassing both the user interface and backend components. The user interface is displayed at the top, consisting of the napari viewer, chat window, and AI-enhanced code editor. Key backend elements include the Omega ReAct agent, web server, AI tools within the editor, and the Python code repair module. The architecture employs a cascaded design, with a central dialog loop orchestrated by a primary language model. This model coordinates a suite of specialized tools, each powered by a secondary tool-specific language model that can access online resources, interact with the napari viewer, and integrate functionalities from third-party libraries such as Cellpose and StarDist.

Supplementary Video 1. Omega can segment nuclei with StarDist and perform follow-up analysis. The video showcases Omega's ability to segment cell nuclei in a 2D image using Stardist. Omega successfully segments the nuclei and adds a label layer to the napari viewer. With further instructions, Omega can count the segmented nuclei and create a CSV file on the desktop folder of the machine. This file contains coordinates and areas of all segments, sorted by decreasing area, with one segment per row. Omega also opens the file using the system's default CSV viewer. The video has been sped up by a factor of 2.

Supplementary Video 2. Omega can segment nuclei in a 3D image. This video shows how Omega segments the nuclei in a 3D image displayed in the napari viewer. Omega uses a specialized tool for cell and nuclei segmentation and employs a 'classic' approach that combines single thresholding, specifically Otsu, with watershed splitting to prevent under-segmentation. After segmentation, Omega adds a labels layer to the viewer, and we inquire about the number of segments detected. The response is 27. The video has been sped up by a factor of 2.

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Human red blood cells
DIC microscopy

"BBBC009v1 from the Broad Bioimage Benchmark Collection [Ljosa et al., *Nature Methods*, 2012]."

https://segment-anything.com/

https://segment-anything.com/

Segment Anything in ImageJ/Fiji

Released around March 14th, 2024
Graphical User Interphase
No coding required

Example of point annotations

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https://github.com/segment-anything-models-java/SAMJ-IJ

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Image Analysis Workflow

- There are typically *five* steps in an image analysis
- Often a good idea to structure work along these lines before starting

Think of this even ***before*** you acquire the images!

otherwise image analysis may become only a *post-mortem* on your experiment

Pre-processing → Processing → Post-processing → Measuring → Analysing & Visualising

- **Data-wrangling in general**
  - Image format conversion, incl. lossless compression
  - Renaming
- **Restoration and Reconstruction**
  - Tomographic reconstruction
  - Registration (3D stacks)
  - Stitching (tiled images)
  - Illumination correction
  - Intensity normalisation
  - Deconvolution
  - Filtering (smoothing, sharpening, etc)
  - Grayscale morphological operations
- **Color deconvolution**
- **Channel alignment (chromatic aberrations)**
- …

Pre-processing → **Processing** → Post-processing → Measuring → Analysing & Visualising

- **Image Segmentation**
  - Thresholding
  - Pixel Clustering
  - Edge detection
  - Region growing
  - Level set methods
  - Watershed
  - Model based
  - Multi-scale
  - Trainable pixel-classification
- **Spot detection**
- **Tracking**
- …

Pre-processing → Processing → **Post-processing** → Measuring → Analysing & Visualising

- **Filtering** detected objects based on
  - Size, shape, intensity
  - Texture, location, neighbours
- **Binary morphological operations**
  - Open/close
  - Skeletonise
  - Hit-or-miss transform
- …

Pre-processing → Processing → Post-processing → **Measuring** → Analysing & Visualising

- **Object measures**
  - Size, shape, intensity
  - Texture, positions
  - Local neighbourhood
- **Image measures**
  - Object count
  - Area covered
  - Total intensity
- **Co-localisation**
- …

Pre-processing → Processing → Post-processing → Measuring → **Analysing & Visualising**

- **Exploratory data analysis**
- **Filtering of numerical data based on measured values**
- **Summary statistics**
- **Plotting numerical values**
- **Making movies and montages of images**
- **Record entire work-flow for later reference**
  - Write down what you did and why (each step)
  - Use a version control system
  - Document your code
- …

Processing vs. Analysis

Image Formation
object in → image out

Image Processing
image in → image out

Image Analysis
image in → features out

Obj	Area	Perim
1	324.2	98.5
2	406.7	140.3
3	487.1	159.2
4	226.3	67.8
5	531.8	187.6
6	649.5	203.1
7	582.6	196.4
8	498.0	162.9
9	543.2	195.1

Computer Graphics
numbers in → image out

X	Y	I
-3.54	-2.32	0.50
-2.78	-1.90	0.12
-1.15	0.42	3.09
0.45	1.65	5.89
1.83	2.18	7.72
2.98	3.33	2.07
4.21	3.96	-4.58
5.62	4.54	-11.45
7.16	5.02	-3.63

Computer Vision
image in → interpretation out

The series shows microtubule growth in a live neuron. The average speed of the distal ends is comparable in the cell body, dendrites, axons, and growth cones.

Visualization
image in → representation out

Meijering and van Cappellen. Biological Image Analysis Primer. (2006)

Debugging Fiji and getting help — a workflow

1. **Restart** Fiji
2. **Ask** you right neighbor
3. **Ask** you left neighbor
4. **Ask** an instructor
5. **Search** https://forum.image.sc/

Install and Update Fiji

Download Fiji

https://fiji.sc/

https://imagej.net/Fiji

Fiji is distributed as a portable application,

which means that you do not have to run an installer.

Just download (zip file), unpack and start it.

Update Fiji

(and install/uninstall plugins)

Help > Update…

To update Fiji and/or the plugins.

***Apply Changes*:** Install/Update the listed plugins

***Manage Update Sites*:** opens a list of plugins, you can select which one(s) to install in Fiji

You can have more than one Fiji app!

Graphic User Interface (GUI)

Graphic User Interface (GUI)

Search Bar
(L key shortcut)

**Plugins > Shortcuts > List Shortcuts - list of the default shortcuts*

***Plugins > Utilities > Find Commands - search for Fiji Commands*

Open a file in Fiji

Drag and Drop the file you want to open onto the Status Bar.

“File” menu
“Plugins” menu

https://imagej.net/Bio-Formats

Bio-Format Plugin

import a file - options

Import data from many life sciences file formats (e.g. @NIC .nd2 format)

Bio-Format Plugin

import a file - options

Bio-Format Plugin

import a file

Drag and Drop

If you have a tiff file, "Drag and Drop" does not open Bio-Format Importer.

File > Import > Bio-Formats

or

Plugins > Bio-Formats > Bio-Formats Importer

what is an image?

CITE

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A digital image is a matrix of numbers!

=

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
9	9	16	19	13	13	19	13	26	16	16	13	6	103	179	189	132	33	19	16	16	9	9	6	6	6
13	9	4	13	13	13	16	19	13	23	6	16	23	123	186	192	169	126	26	16	19	13	6	13	16	13
13	13	9	16	9	6	13	19	16	19	6	19	63	199	192	106	29	149	162	113	119	53	9	13	6	13
13	9	16	6	6	19	13	9	23	13	9	6	119	182	149	36	6	39	196	196	176	73	16	9	9	9
6	19	13	9	19	16	13	13	19	9	9	23	142	179	109	13	16	9	39	59	23	19	13	4	9	9
19	13	9	9	16	16	16	9	9	13	6	66	169	172	43	16	9	9	9	13	13	19	16	16	16	9
9	9	6	9	13	9	6	13	4	9	19	116	196	89	9	9	16	16	19	19	9	16	6	16	9	9
13	13	9	23	19	13	9	9	9	6	26	159	219	59	23	9	13	9	6	13	6	19	16	13	16	13
9	23	13	6	6	23	9	19	13	16	66	206	179	13	6	16	13	13	13	16	9	13	9	9	16	13
13	13	23	16	19	19	6	9	19	13	142	255	103	19	13	6	19	9	16	9	16	9	16	13	23	9
6	13	23	9	13	16	13	6	9	53	229	246	39	9	13	13	13	13	9	9	19	13	16	13	13	13
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Pixel = Picture Element

Image Analysis Collaboratory

Images in publications and presentations should be used to ***communicate*** a finding...
not ***be*** the finding

this is your **data**

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
9	9	16	19	13	13	19	13	26	16	16	13	6	103	179	189	132	33	19	16	16	9	9	6	6	6
13	9	4	13	13	13	16	19	13	23	6	16	23	123	186	192	169	126	26	16	19	13	6	13	16	13
13	13	9	16	9	6	13	19	16	19	6	19	63	199	192	106	29	149	162	113	119	53	9	13	6	13
13	9	16	6	6	19	13	9	23	13	9	6	119	182	149	36	6	39	196	196	176	73	16	9	9	9
6	19	13	9	19	16	13	13	19	9	9	23	142	179	109	13	16	9	39	59	23	19	13	4	9	9
19	13	9	9	16	16	16	9	9	13	6	66	169	172	43	16	9	9	9	13	13	19	16	16	16	9
9	9	6	9	13	9	6	13	4	9	19	116	196	89	9	9	16	16	19	19	9	16	6	16	9	9
13	13	9	23	19	13	9	9	9	6	26	159	219	59	23	9	13	9	6	13	6	19	16	13	16	13
9	23	13	6	6	23	9	19	13	16	66	206	179	13	6	16	13	13	13	16	9	13	9	9	16	13
13	13	23	16	19	19	6	9	19	13	142	255	103	19	13	6	19	9	16	9	16	9	16	13	23	9
6	13	23	9	13	16	13	6	9	53	229	246	39	9	13	13	13	13	9	9	19	13	16	13	13	13
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

this is your **result**

this just helps to ***communicate*** the result

Individual Pixel Values in Fiji

CITE

Image Analysis Collaboratory

A digital image is a matrix of numbers!

=

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
9	9	16	19	13	13	19	13	26	16	16	13	6	103	179	189	132	33	19	16	16	9	9	6	6	6
13	9	4	13	13	13	16	19	13	23	6	16	23	123	186	192	169	126	26	16	19	13	6	13	16	13
13	13	9	16	9	6	13	19	16	19	6	19	63	199	192	106	29	149	162	113	119	53	9	13	6	13
13	9	16	6	6	19	13	9	23	13	9	6	119	182	149	36	6	39	196	196	176	73	16	9	9	9
6	19	13	9	19	16	13	13	19	9	9	23	142	179	109	13	16	9	39	59	23	19	13	4	9	9
19	13	9	9	16	16	16	9	9	13	6	66	169	172	43	16	9	9	9	13	13	19	16	16	16	9
9	9	6	9	13	9	6	13	4	9	19	116	196	89	9	9	16	16	19	19	9	16	6	16	9	9
13	13	9	23	19	13	9	9	9	6	26	159	219	59	23	9	13	9	6	13	6	19	16	13	16	13
9	23	13	6	6	23	9	19	13	16	66	206	179	13	6	16	13	13	13	16	9	13	9	9	16	13
13	13	23	16	19	19	6	9	19	13	142	255	103	19	13	6	19	9	16	9	16	9	16	13	23	9
6	13	23	9	13	16	13	6	9	53	229	246	39	9	13	13	13	13	9	9	19	13	16	13	13	13
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Where do these number come from?

Bit depth

Detectors in Fluorescence Microscopy

Image Analysis Collaboratory

The detectors used in fluorescence microscopy are **monochromatic**.

Cameras or PMTs are not able to distinguish between different wavelengths (they just collect photons), you need fluorescence filters to separate your fluorophores.

The detector converts photons in digital numbers (linear relation).

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Each pixel in the digital image has **one digital value** that **depends on** the **intensity** of the signal emitted by the **sample**.

Digital Values = Pixel Intensity Value

The **range** of possible **digital values** is defined by the **bit depth**.

Bit Depth

The **bit depth** defines the range of possible **digital values** that each pixel can have, usually **8**, **12** or **16 bit**.

The **bit depth** is expressed in **grey values**.

bit depth of the **image** = **bit depth** of the **detector**
(Unless you change that during acquisition)

x bit = a range of 2^x grey values

8 bit image = **each pixel** can have 2^8 **grey values** = 256 grey values = **range 0-255**

12 bit image = **each pixel** can have 2^{12} **grey values** = 4096 grey values = **range 0-4095**

16 bit image = **each pixel** can have 2^{16} **grey values** = 65536 grey values = **range 0-65535**

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Digital Value = Pixel Intensity Value = Grey Value

Image Metadata

Image > Show Info…

(cmd) + i

Show the Metadata stored with the file

e.g. bit depth, camera bit depth

display your images

Mapping Image Intensity to Monitor Intensity (LookUp Tables)

Image Analysis Collaboratory

LUT = how the grey values are displayed

LUTs do not change the pixel values

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Image (12 bit)	Displayed color
0	
1	
…	
2000	
…	
4095	

Display a file: Brightness & Contrast

Image > Adjust > Brightness/Contrast

Interactively change the <u>displayed</u> brightness and contrast of the <u>active</u> image.

(cmd) + shift + c

You are NOT changing the pixels values, you are just changing how the image is displayed (unless you click on the “Apply” button).

Histogram

Analyze > Histogram

h

jaehyuk-lee: https://jaehyuk-lee.com/animated-image-histogram/

Histogram

Analyze > Histogram

h

Fiji auto-adjust the range (default option)

Bit depth

Display a file: Brightness & Contrast

Image Analysis Collaboratory

Which image has more fluorescence?

Mean:	**4803**	**4803**
Display range:	188-**16828**	188-**45514**

Mean:	**4803**	**4803**
Display range:	188-**16828**	188-**16828**

Display a file: Brightness & Contrast

Image Analysis Collaboratory

Which image has more fluorescence?

Mean:	**4803**	**2074**
Display range:	188-**19540**	112-**7768**

Mean:	**4803**	**2074**
Display range:	188-1**9540**	188-**19540**

Do NOT trust your eyes, rely on numbers!

Display a file: Brightness & Contrast

"Set" button - visually compare images

Use the same acquisition parameters
(per channel) if you want to compare images!!!
(e.g. same exposure time, Illumination intensity,…)

Example	*Exposure time Condition1*	*Exposure time Condition2*
Channel 1	*100 ms*	*100 ms*
Channel 2	*200 ms*	*200 ms*

For a meaningful comparison, you have to extract numbers from your images (analysis). Use the images to support your results.

If you save the images as jpeg/png/tiff, they will maintain the display range you set.

Segmentation with pixel based classifier—exercises

1.3 image inspection

1.4 adjust brightness/contrast of all open images

Images and Colors

Lookup Tables (LUTs)

LUT = how the grey values are displayed

LUTs do not change the pixel values

Image (8 bit)	Displayed color
0	
1	
…	
100	
…	
255	

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Image (8 bit)	Displayed color
0	
1	
…	
100	
…	
255	

=

136	106	152	179
109	209	236	179
103	179	189	132
123	186	192	169

Images and Colors

Lookup Tables (LUTs)

Image > Lookup Tables

or

"LUT" menu in Startup Tools Bar

Examples of LUTs

Image > Color > Display LUTs - to display default LUTs available

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Image Analysis Collaboratory

Images and Colors in Fiji

Choose the right LUT

Which is brighter?

The human eye evaluates intensity best in grayscale

If you are imaging for example a blue fluorophore, you are NOT FORCED to display it in blue!

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Images and Colors in Fiji

Choose the right LUT

Color blind people don't distinguish some colors

POINTS OF VIEW

Color blindness

NATURE METHODS | VOL.8 NO.6 | JUNE 2011 | 441

"If a submitted manuscript happens to go to three male reviewers of Northern European descent, the chance that at least one will be color blind is 22 percent"

Images and Colors in Fiji

Choose the right LUT

Color blind people don't distinguish some colors

Protanope (no red) **Tritanope (no blue)**

Image > Color > Dichromacy *or* ***Image > Color > Simulate Color Blindness***

DAPI
Phalloidin (Alexa 488)
MitoTracker Red

Talley Lambert

RGB Images (still matrix of numbers)

LUTs cannot be applied to RGB Images

RGB image

=

255 0 234	0 255 0	0 186 28	255 255 1
0 0 255	1 199 255	255 0 0	0 255 111
255 0 255	255 255 1	255 255 255	0 0 0
254 105 0	117 117 117	141 137 0	118 0 143

RGB Color image (e.g. jpeg, png) = **Red + Green + Blue**

RGB Color image = 8 bit Red, 8 bit Green, 8 bit Blue = R (0-255), G (0-255), B (0-255)

Image > Type > RGB Color or **Save As…png, jpeg**

Images and Colors in Fiji

The Color menu

Image > Color > Split Channels

Image > Color > Merge Channels

***Split* or *Merge* the channels of the active image**

Images and Colors in Fiji

The Color menu

Image > Color > Channels tools

(cmd) + shift + z

Interactive visualization/handling of each image channel.

Choose how to display your multicolour image: “Color” mode (single channel) vs “Composite” mode (overlay)

Segmentation with pixel based classifier—exercises

2.2 composite images - splitting and merging

Pixel Size

Scale Bar and Pixel Size

Image Analysis Collaboratory

Pixel Size and Scale Bar

If you want to make some physical measurements of your sample (length, size, …), you need to know the pixel size of your image.

w/ Metadata

Image > Show Info…

(cmd) + i

Show the Metadata stored with the file.

Pixel width (Pixel x) = 0.0515 µm
Pixel height (Pixel y) = 0.0515 µm
Voxel depth (Pixel z) = 0.0515 µm

1 µm = 19.4201 pixel (xy)
(1 µm / 0.0515 µm)

Pixel Size and Scale Bar

If you want to make some physical measurements of your sample (length, size, …), you need to know the pixel size of your image.

Image > Properties…

(cmd) + shift + p

Open the image properties windows containing pixel size properties (and multi-dimensional properties)

You can set/read the image pixel size in xyz.

Scale Bar and Pixel Size

What if the pixel size is not stored in the metadata?

If you know the magnification and the camera you used for the acquisition, you can estimate the image pixel size.

image pixel size = camera pixel size/magnification

Example

Magnification = 100x Objective

Camera = Hamamatsu Orca Flash 4

Product number	C13440-20CU
Imaging device	sCMOS
Cell (pixel) Size (μm²)	6.5×6.5
Pixel Array (horizontal by vertical)	2048×2048
Effective Area (horizontal by vertical in mm)	13.312×13.312

pixel width and height:
6.5 μm / 100X = 0.065 μm

Image > properties

If a z-series was acquired, “Voxel depth” is the z step size choose for the acquisition (cannot estimate).

Scale Bar and Pixel Size

Add pixel information to your images from a reference (same magnification)

Analyze > Set Scale…

Set the pixel size information starting from an object with a known dimension.

****Perform multiple measures and average them to be more precise***

Scale Bar and Pixel Size

Add scale bar to your images

Analyze > Tools > Scale Bar…

- “Overlay" means that the scale bar is not embedded in your image. You can remove it: “*Image >Overlay > Remove Overlay*”.
- If you save the image as tiff, the scale bar will be saved as an overlay.
- If you save as jpeg/png, the image will have the scale bar embedded.

Segmentation with pixel based classifier—exercises

1.6 file handling and non-invasive editing

1.7 file handling and invasive editing

Image Processing

—

Segmentation

- Segmentation is the **division** of an image into **discrete regions**.

Segmentation

Input

Semantic

Background

Instance

Background
Nucleus 1
Nucleus 2
Nucleus 3
…

How do we get segments?

- Thresholding-based
- Interactive tools based on classic machine learning
- Deep-learning based (Stardist, Cellpose)

Thresholding

The easiest way to segment an image is often by applying a *global threshold*.

This identifies pixels that are above or below a fixed threshold value, giving a *binary image as the output.*

https://bioimagebook.github.io/chapters/2-processing/3-thresholding/thresholding.html

Thresholding

Original, 8 bit grayscale
Blobs: Fiji example

Thresholded

Instance Segmented

Instance Segmentation in FIJI**:** keeping white (*connected-*) objects.

Thresholding method

Select only a **range** of **digital values** in the image.

8 bit image (0 - 255)

Image
Analysis
Collaboratory

Thresholding method

Select only a **range** of **digital values** in the image.

in **Fiji**: **Image** > **Adjust** > **Threshold…**

(cmd) + shift + t

8 bit image (0 - 255)

The result of the thresholding process is a **Binary Mask**.

Image Analysis Collaboratory

Thresholding method

Generate a **binary mask**.

8 bit image (0 - 255)

0 **or** 255

0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0
0	0	0	0	0	0	0	0	0	0	255	255	255	0	0	0

Binary because the image has only **two** pixel values,
one for the **selected pixels** and **one for** the "**discarded**" **pixels**.

In Fiji the two pixel values are **0** and **255.**

Image
Analysis
Collaboratory

Segmentation with thresholding—exercises

7.1 DAPI segmentation with thresholding

skip “Analyze Particles”

Thresholding method

What can go wrong?

Usually, if you apply **thresholding** to the **"ORIGINAL" image** (the one you get out of the microscope), you won't be able to precisely **select all/only the pixels** you are interested in.

- **Fluorescence label** (e.g. DAPI)
- **Background** (uneven illumination, out-of-focus light, aberration, …)
- **Noise** (detector read noise, Poisson noise, …)

Filters

Where is Waldo?

Where is Waldo?

Where is Waldo?

Filtering

Neighborhood Transformation

Linear Transformation

Smoothing: Mean, Gaussian

Edge detection: Sobel, Canny

Non-linear Transformation

Smoothing: Median

Cleaning: Morphology

Weights are defined by the kernels

Weighted average = Multiply, add, divide

One neighborhood at a time

Neighborhood defined by the kernel size

Convolution is weighted average in local neighborhood

Terminologies

A **kernel** is a small matrix used in image processing for convolution.

A **filter** is a broader term that refers to any technique that modifies an image.

Convolution operation

Image

px1	px2	px3	px4	px5	px6	px7	px8	px9
px10	px11	px12	px13	px14	px15	px16	px17	px18
px19	px20	px21	px22	px23	px24	px25	px26	px27
px28	px29	px30	px31	px32	px33	px34	px35	px36
px37	px38	px39	px40	px41	px42	px43	px44	px45
px46	px47	px48	px49	px50	px51	px52	px53	px54
px55	px56	px57	px58	px59	px60	px61	px62	px63
px64	px65	px66	px67	px68	px69	px70	px71	px72
px73	px74	px75	px76	px77	px78	px79	px80	px81

convolution ✪

3 x 3 kernel

k1	k2	k3
k4	k5	k6
k7	k8	k9

=

(px1 * k1 / ksum) +

(px2 * k2 / ksum) +

(px3 * k3 / ksum) +

(px10 * k4 / ksum) +

(px11 * k5 / ksum) +

(px12 * k6 / ksum) +

(px19 * k7 / ksum) +

(px20 * k8 / ksum) +

(px21 * k9 / ksum) =

c1

ksum = k1 + k2 + k3 + k4 + k5 + k6 + k7 + k8 + k9

Adapted from Federico M. Gasparoli

Convolution operation

Image

px1	px2	px3	px4	px5	px6	px7	px8	px9
px10	px11	px12	px13	px14	px15	px16	px17	px18
px19	px20	px21	px22	px23	px24	px25	px26	px27
px28	px29	px30	px31	px32	px33	px34	px35	px36
px37	px38	px39	px40	px41	px42	px43	px44	px45
px46	px47	px48	px49	px50	px51	px52	px53	px54
px55	px56	px57	px58	px59	px60	px61	px62	px63
px64	px65	px66	px67	px68	px69	px70	px71	px72
px73	px74	px75	px76	px77	px78	px79	px80	px81

convolution ✪

3 x 3 kernel

k1	k2	k3
k4	k5	k6
k7	k8	k9

=

(px2 * k1 / ksum) +

(px3 * k2 / ksum) +

(px4 * k3 / ksum) +

(px11 * k4 / ksum) +

(px12 * k5 / ksum) +

(px13 * k6 / ksum) +

(px20 * k7 / ksum) +

(px21 * k8 / ksum) +

(px22 * k9 / ksum) =

c2

ksum = k1 + k2 + k3 + k4 + k5 + k6 + k7 + k8 + k9

	c1	?						

Adapted from Federico M. Gasparoli

Convolution operation

Image

px1	px2	px3	px4	px5	px6	px7	px8	px9
px10	px11	px12	px13	px14	px15	px16	px17	px18
px19	px20	px21	px22	px23	px24	px25	px26	px27
px28	px29	px30	px31	px32	px33	px34	px35	px36
px37	px38	px39	px40	px41	px42	px43	px44	px45
px46	px47	px48	px49	px50	px51	px52	px53	px54
px55	px56	px57	px58	px59	px60	px61	px62	px63
px64	px65	px66	px67	px68	px69	px70	px71	px72
px73	px74	px75	px76	px77	px78	px79	px80	px81

convolution ✪

3 x 3 kernel

k1	k2	k3
k4	k5	k6
k7	k8	k9

=

(px3 * k1 / ksum) +

(px4 * k2 / ksum) +

(px5 * k3 / ksum) +

(px12 * k4 / ksum) +

(px13 * k5 / ksum) +

(px14 * k6 / ksum) +

(px21 * k7 / ksum) +

(px22 * k8 / ksum) +

(px23 * k9 / ksum) =

c3

ksum = k1 + k2 + k3 + k4 + k5 + k6 + k7 + k8 + k9

	c1	c2	?					

Adapted from Federico M. Gasparoli

Different Types of Kernel

0	0	0	0	0
0	1	1	1	0
0	1	1	1	0
0	1	1	1	0
0	0	0	0	0

1D Mean Filter

2D Mean Filter

3D Mean Filter

0	0	0	0	0
0	1	2	1	0
0	2	5	2	0
0	1	2	1	0
0	0	0	0	0

1D Gaussian Kernel

2D Gaussian Kernel

3D Gaussian Kernel

0	0	0	0	0
0	-1	0	1	0
0	-1	0	1	0
0	-1	0	1	0
0	0	0	0	0

0	0	0	0	0
0	-1	-1	-1	0
0	0	0	0	0
0	1	1	1	0
0	0	0	0	0

1D Prewitt Filter

2D Prewitt Filter

3D Prewitt Filter

0	0	0	0	0
0	1	-1	1	0
0	-1	2	-1	0
0	1	-1	1	0
0	0	0	0	0

1D Sharpening Filter

2D Sharpening Filter

3D Sharpening Filter

Mean Filter

Gaussian Filter

Effects of Different Gaussian Kernel

Kernel

Blurred image

Edge Filters

Prewitt Operator

Different Filters in Action

0	0	0	0	0
0	1	1	1	0
0	1	1	1	0
0	1	1	1	0
0	0	0	0	0

Mean Filter

0	0	0	0	0
0	1	2	1	0
0	2	5	2	0
0	1	2	1	0
0	0	0	0	0

Gaussian Filter

0	0	0	0	0
0	-1	-1	-1	0
0	0	0	0	0
0	1	1	1	0
0	0	0	0	0

0	0	0	0	0
0	-1	0	1	0
0	-1	0	1	0
0	-1	0	1	0
0	0	0	0	0

Prewitt Filter

0	0	0	0	0
0	1	2	1	0
0	2	5	2	0
0	1	2	1	0
0	0	0	0	0

Sharpening Filter

Filtering — exercises

3.4 edge filters - vertical stripes
3.5 edge filters - horizontal stripes
3.6 edge filters - checkerboard

Filtering — exercises

7.2 DAPI segmentation with filters and thresholding

Interactive tools based on classic machine learning

Standalone: Ilastik

https://www.ilastik.org/

For histopathology

https://qupath.github.io/

As a Fiji plugin

https://imagej.net/plugins/tws/

WEKA: Waikato Environment for Knowledge Analysis: collection of free machine learning and data analysis software developed by the University of Waikato, NZ

Trainable WEKA Segmentation (in ImageJ/Fiji)

Trainable WEKA Segmentation (in ImageJ/Fiji)

Tips

Segmentation with pixel based classifier—exercises

7.2 DAPI segmentation with Weka

Breakpoint at 12: Create results