

Introduction to Bioimage Analysis

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Part 1. An Overview of BioImage Analysis





What is trivial for a human can be very difficult for a computer:



SKIP



Goal:

Obtaining quantifiable information from microscopy images of biological sample



In the treated group, cell looks 'rounder"...

But how much?



Goal:

Obtaining quantifiable information from microscopy image of biological sample

































How do we get from images to graphs...?







General Bioimage Analysis Workflows





BioImage Informatics Index by Networks of European Bio-image Analysts (NEUBIAS): <u>https://biii.eu/</u>

Biolmage		Q SEARCH Advanced search (under construction)		Category		
O o Index				lector	Туре	Pages
Recent					Training Material	79
					Dataset	25
empanada-napari Component	Image Data Explorer		BaSiC	ClearMag	Software	1,362
Most viewed	er)		DIRmage		As of	March 2023
CellProfiler Collection	3D Slicer m Collection	PhenoRipper Collection	DIPImage Collection	Microtubule tracking in Drosophila Workflow	e end Oocyte	

Reviews

Eliceiri et al., Nat Methods, 2012 Wiesmann et al., J Microsc, 2015 Baroux et al., Methods Mol Biol, 2018

Software name	Primary function
ImageJ	Image analysis
Fiji	Image analysis
BiolmageXD	Image analysis
Icy	Image analysis
CellProfiler	Image analysis
Vaa3D	Visualization and image
	analysis
FarSight	Visualization
VTK	Bioimaging library
ITK	Bioimaging library
OpenCV	Bioimaging library
WND-CHARM	Machine learning
PSLID	Machine learning
Ilastik	Machine learning
CellProfiler Analyst	Machine learning and data analysis
PatternUnmixer	Machine learning
CellOrganizer	Machine learning, modeling and visualization
KNIME	Workflow system

Source: Eliceiri 2012



Image Processing Software @ BOMP









Acquisition





- Acquire with sufficient **sampling rate**
- Use appropriate resolution
- Decide on a **quantitative** or **qualitative** approach
- Use proper **bit depth**
- Avoid saturation
- Use whole **dynamic range**



Sampling Rate



Acquire images with a sufficient sampling rate



- When the sampling rate is too low this is called undersampling and results in a pixelated image
- Particularly important depending on you desired quantificaiton
- Things to consider to avoid undersampling:
 - **Object size.** Determine expected size of the object from literature or experimentally
 - For example: if you wish to image lysosomes 300nm in diameter a pixel size 500 nm would lead to undersampling
 - Increasing the sampling rate allows use to image the shape



While we want sufficient resolution we need to avoid oversampling

- **Time**. Oversampling takes longer to acquire (and costs more!)
- File size. Larger file size means more storage required, slower data handling, longer processing time
- **Object size.** Use a sampling rate appropriate for your sample / quantification
 - > Example of Oversampling:
 - Aim of imaging was to count neurons
 - Acquisition settings used:

	Oversampling	Recommended	
Pixel Size	40 nm	200 nm	
Frame Size	1651 x 1651	512 x 512	
Time	9 min	2 min	
File Size	487 MB	25 MB	
No. neurons	4	4	

Nyquist-Shannon sampling theorem

- A microscope image results from the digital conversion of an analogue signal.
- Fundamental rule in analogue to digital conversion is the Nyquist-Shannon sampling theorem:
 - Sampling frequency must be greater than 2.3 times the bandwidth of the input signal for optimal reconstruction (or use pixels 1/3 the size of the smallest object)

Undersampled (information loss)

Ideally-sampled (Nyquist Sampling)

Oversampled (impractical)

Optical Resolution

Appropriate an **resolution** for our object of interest

Is the aim of your imaging project is more about quantity or quality of images?

	Quantitative	Qualitative	
No. images	A lot	Selected samples	
Image quality / resolution	Sufficient quality	Best possible (oversampling)	
Applications	Large number of samplesStatistical demands	Proof of conceptComplex structuresVisualisation	
Notes	Test settings (& processing)Data management	 Is it scalable? 	

You can combine a quantitative and qualitative approach!

Qualitative AND Quantitative

Qualitative (Confocal laser-scanning microscope)

- Multiple z-stack images acquired at 20x, stitched together and used to create a maximum intensity projection.
- Time per image = 5 min. File size = 74 MB
- A ROI was cropped for analysis.

Confocal Imaging (Qualitative)

- Whole fish was imaged with single image at 2X.
- Time per image = 200ms. File size = 4 MB
- A ROI was cropped for analysis.

Widefield Imaging (Quantitative)

Bit-Depth

Use higher **bit depth** for intensity measurements

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Binary + Digit = Bit
1 bit = 0 or 1

Dynamic Range & Saturation

Use whole dynamic range

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- Images to be compared **MUST** be acquired (and processed) using the same settings
- Save the image using the microscopy raw format (CZI, LIF, OIB, ND2... OME-TIFF) to preserve metadata.
- Avoid saving as standard TIFF, JPG, PNG...

More detail on our other microscopy seminar series

https://microscopy.unimelb.edu.au/optical-microscopy/workshops-resources

Optimising Acquisition After Training

- **Optimisation:** Fine tuning your settings is critical for the best results and efficiency
- Adaptation: Adjusting your setting for new samples (avoid copying settings)
- **Troubleshooting:** Identify problems...lookout for large files, slow imaging, difficult data management
- **Reporting:** Always document the acquisition tools used and save templates where possible

If in doubt reach out to BOMP for advice and guidance!

Case Study in Sub-optimal Setup

Question. Simple Yes/No question - is marker (blue) in cells (purple)?

Warnings!

- Raw data size is 61.5 GB
- File opening in Zen took 45 min
- 12 tiles x 45 slices x 5.32 s per frame = 48 min

Suggestions for improvement:

- Tiling, 12 tiles used but only half of 25 % image contains ROI
 - ✓ Take low resolution overview instead
- Z-stack, 60 % of slices are empty ~40 GB
 - ✓ Check first and last slice before starting
 - ✓ Crop after acquiring
- Sampling, objects are ~2 μm in size but pixels are 0.15 μm
 - ✓ Minimum requirement is 0.86 µm (6x smaller)
- Scanning mode was set to unidirectional and doubled acquisition time
 - ✓ Use bidirectional scanning

With correct settings: File = approx 1.6 GB Acquisition = approx. 1.5 min

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Pre-processing

Raw microscopy image

Pre-processing

Fluorescent signal from object

Think of all the lenses, filters and mirrors fluorescent light has to pass through to get to the detector!

- De-noising
- Background correction
- Bleaching correction (in time, in Z)
- Alignment
- Deconvolution

Median Filter Deep learning Gaussian Filter Raw data (preserves edge) (smoothing, blurring) ę

Noise2Void (Krull 2019) https://github.com/juglab/n2v

Image : Celltrackingchallenge dataset Fluo-C2DL-MSC

Background Subtraction

'Rolling Ball' (Castle and Keller 2007)

Shading Correction

BaSiC (Peng 2017)

Flat Field Correction

Suppress stripes (Bandpass Filter)

Bleaching correction

'Bleaching Corrector'

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'Bleach Correction'

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Alignment

Drift correction in x, y, z, t

'Correct 3D Drift' (Parslow 2014) 🛓 🗊

Chromatic shift correction

'TransformJ' (Meijering 2001) 🛓 🏢 'Chromatic Aberration corrector'

Stitching

'BigStitcher' (Preibisch 2009) Π

More details: <u>https://imagej.net/Category:Registration</u>

Deconvolution

Mathematical image restoration method

- Increase resolution in x,y,z
- Increase contrast
- Remove noise

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Thereby improves the quality of data visualisation and analysis

Baroux 2018

www.svi.nl Image created by Dr. Jeff Tucker and Dr. Holly Rutledge from NIEHS, NIH, USA

Object Detection

image

Object Detection

• 1) Pixel segmentation

- Conventional
- Machine learning
- Deep learning
- Template matching

• 2) Binary processing

Image Segmentation Workshop (<u>https://microscopy.unimelb.edu.au/</u>)

- Find threshold using 'negative control' image or from the 'background' area
- To be 'objective', apply same threshold to all images

Conventional Segmentation

But

- Many 'real' images have different background levels
- Background level may change over time in the same sample in live cell imaging

To compensate different background issue,

- Test different Auto-threshold methods
- Apply same auto-threshold algorithm for each image

Machine Learning

- Thresholding doesn't work well with non-fluorescence images e.g. brightfield, phase contrast, colour, or EM images
- Segmentation using few manual annotations
- Interactive, user-friendly
- No machine learning expertise required

Input Data

- Automatically extracts inherent image features or structures
- Models can be trained with ground truth annotations
- Pre-trained models compare input data to a known target
- Some 'pre-trained' models for bioimage analysis are accessible via user friendly software
 Image analysis are accessible via

U-net (Falk 2019)(via DeepImageJ)

StarDist (Schmidt 2018)

Template Matching

- No manual annotation
- Minimal computation computes the probability to find one (or several) template images provided by user into a large image
- Good for finding similar structure in time-lapse or tissue micro-array
- No programming skill required

Watershed

Fill Holes

Skeletons

Dilate

Ultimate eroded points

Measurement / Data Analysis

Raw microscopy image

- Number
- Intensity
- Shape
- Distance (spatial analysis)
- Colocalisation / Co-occurrence
- Tracking

(Yamashita 2015)

Count per region of interest (ROI)

Avoid manual counting!

- Time consuming/ Impossible
- Biased

Number of Cell / Measured Area (volume) Number of Cell / container (ROI)

Treatment

Mean Intensity = sum intensity / area 38 4

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- Treated cells are brighter ٠
- Treated cells shows 2 distinctive ٠ populations

4 8 11 11 12 0 0 1 0 2 10 19 21 21 25 0 1 7 24 39 40 38 42 2 16 45 64 64 57 59 0 5 32 76 97 94 81 79 0 0 1 11 57 112 135 128 105 93 0 1 17 76 139 159 151 121 96 0 1 21 88 154 170 165 128 93 0

- Mean
- Minimum
- Maximum
- Median
- Sum
- StdDev

Measuring Fragmentation

Coefficient of Variation (StdDev/Mean)

Shape/ Morphology

2D: Area 3D: Volume

Shape/ Morphology

Min. bound rectangle

Aspect ₌ Ratio

Orientation (Angle) =

Neuronal Morphology

3D Volume

Branch & Spine

Branch level, length, angle..

Convex Hull 3D

Sholl analysis: Branch number per concentric shell

(Binley2014)

Neighbour relationships ?

Nearest Neighbour Distance (centroid-centroid) Minimum Separation Distance (edge to edge)

Finding touching objects

Result (spots touching cell)

Colocalisation

molecules are on the <u>same location</u> molecules are <u>interacting</u>

- Correlation Is there a relationship between intensities? (Pearson's)
- Co-occurrence Are the fluorophores are generally near each other ?
 - Intensity based : Mander's Coefficient
 - Object based : overlapping area or volume

Quantification is heavily dependent on the resolution of acquisition system so careful planning is required

(North 2006)

Reporting

Raw microscopy image

Performing Bio-image Analysis

- Complex workflows require a detailed reporting
- Reporting also includes being able to track down your data

Reporting Analysis (or Acquisition)

Aim:

- 1. Reproducibility (individually or within a group)
- 2. Knowledge retention
- 3. Materials and Methods

Reporting / recording:

- Take note of main steps and parameters in your analysis workflow (acquisition)
- Add experimental information in files e.g:
 - channel descriptions in Imaris
 - editing Track names in Zen
 - Adding comments in any program if possible

Save templates / settings files

 Programs with exportable settings files are Imaris, Huygens, CellProfiler, Harmony

Imaris templates

Representative example

- Pros: reproducibility, contains more information than you can write down
- Easier troubleshooting

Imaris Example

• FIJI recorder can record all processing steps and save as a text file

🗊 Recor	der -		\times
Record:	Macro 💌 Name: Macro.ijm		Create
makeRect run ("Cro run ("Bri run ("Enh setMinAn run ("Sub run ("Tif	<pre>angle(1285, 1177, 1130, 1130); p"); ghtness/Contrast"); ance Contrast", "saturated=0.35"); dMax(0, 255); tract Background", "rolling=50") f");</pre>	;	^
			~

Automate Analysis

ImageJ Macro writing workshop (<u>https://microscopy.unimelb.edu.au/</u>)

- Workshops run once a year, but workshop resources available online all-year round!
 - https://microscopy.unimelb.edu.au/bomp/capabilities/workshops-resources
- Good source for getting knowledge
- Other good resources:

Forum on Bioimage analysis <u>https://forum.image.sc/</u>

ImageJ learn https://imagej.net/Introduction

Cell Profiler <u>https://cellprofiler.org/</u>

NEUBIAS academy <u>https://neubiasacademy.org/</u>

Thank you

Biological Optical Microscopy Platform

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BOMP Workshop Schedule

Date	Day	Format	Time	Event	Туре
7th March	Tuesday	1hr	11-12pm	Launching Seminar : Stellaris 5	Seminars
14th March	Tuesday	1hr	10-11am	Introduction to: Bio-image Analysis	Seminars
15h March	Wednesday	1hr	10-11am	Launching Seminar : LSM980	Seminars
28th March	Tuesday	1day	10-12pm & 2-4pm	BOMP workshop series: FIJI/ImageJ for Beginners	Workshop
30th March	Thursday	1day	10-12pm & 2-4pm	BOMP workshop series: FIJI/ImageJ for Quantification	Workshop
27th April	Tuesday	1day	9-1pm	BOMP workshop series: Cell Profiler	Workshop
Early May		1hr x 2	10-12pm	Introduction to: Fluorescence Microscopy	Seminars
29th June	Thursday	1day	10-12pm & 2-4pm	BOMP workshop series: Huygens deconvolution	Workshop
25th July	Tuesday	1day	9-12pm & 1-4pm	BOMP workshop series: IMARIS 3D image analysis - Basic	Workshop
27th July	Thursday	1day	9-12pm & 1-4pm	BOMP workshop series: IMARIS 3D image analysis - Advanced	Workshop
15th Aug	Tuesday	1hr	10-11am	Focus on: Lightsheet Microscopy	Seminar & Demo
29th Aug	Tuesday	1hr	10-11am	Focus on: High Content Imaging	Seminar & Demo
5th Sep	Tuesday	0.5day	9-12pm	BOMP workshop series: Zen Intellesis	Workshop
12th Sep	Tuesday	1hr	10-11am	Focus on: Super-Resolution Microscopy	Seminar & Demo
26th Sep	Tuesday	1day	10-12pm & 2-4pm	BOMP workshop series: Colocalisation	Workshop
11th Oct	Tuesday	1day	10-12pm & 2-4pm	BOMP workshop series: QuPath - Fluorescence	Workshop
24th Oct	Tuesday	0.5 day	9-12pm	BOMP workshop series: FIJI/ImageJ Macro writing for biologists - Day1	Workshop
26th Oct	Thursday	0.5 day	9-12pm	BOMP workshop series: FIJI/ImageJ Macro writing for biologists - Day2	Workshop
28th Nov	Tuesday	1day	10-12pm & 2-4pm	BOMP workshop series: Image Segmentation	Workshop