Bioimage Analysis Course 2022
March 7th, 2022

Cell counting

(as an example of image processing workflow)

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"From images of double-stained cells in different conditions, I need to **calculate the percentage** of those that are single- or double positive."

"I need to **quantify TH-positive neurons** in primary neuronal cultures."

"I need to separately **quantify the amount of single and double positive cells** in different tissue regions from double labeled immuno images."

"How to automatically **count stainings** for PV and PNN positive cells?"

"I want to **calculate the number and area** of myocytes in a transversal muscle section."

"Quantification of **pixel intensity from nuclear envelope vs. cytoplasm** from basic fluorescence microscope"

Cell counting and characterization

Why? (What is your biological question?)

- How many…
  - live / dead cells?
  - cells/droplets/vesicles/bacteria inside a selected area?
- How big...
  - an area/volume?
- How much…
  - protein X is in treated cells vs. control?
  - more protein X is localized in the nuclear envelope relative to cytoplasm?
- How fast…
  - do the cells move/divide/migrate?
- Do the signals colocalize?
  - Colocalization (or co-occurrance) of two different protein clusters

Cell counting and characterization

How? (What are the steps needed to get the result?)

- This lecture: Image processing workflow
- In workshops: How to do it in practice with FIJI ImageJ?

Then what? (What to do with the numbers?)

- Analysis of results, visualizations, statistics
- Conclusions
- Modify protocol, repeat experiment, iterate
- Publish (remember to cite & acknowledge)

Image processing workflow

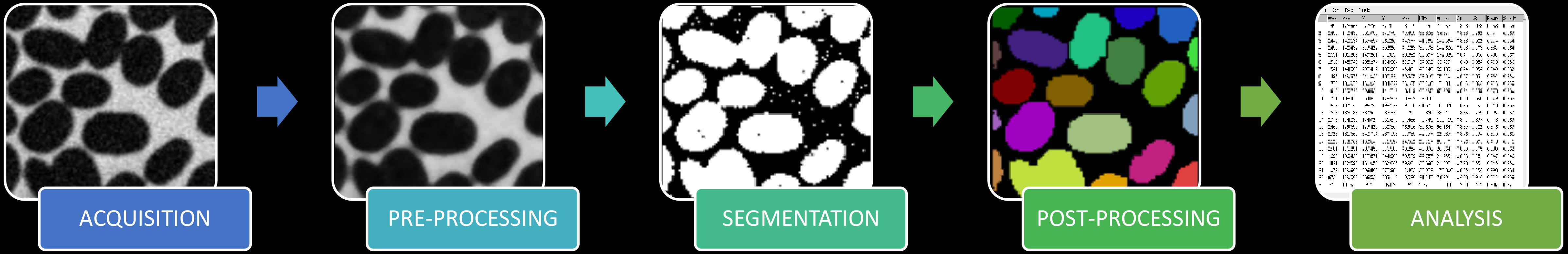


Image processing workflow

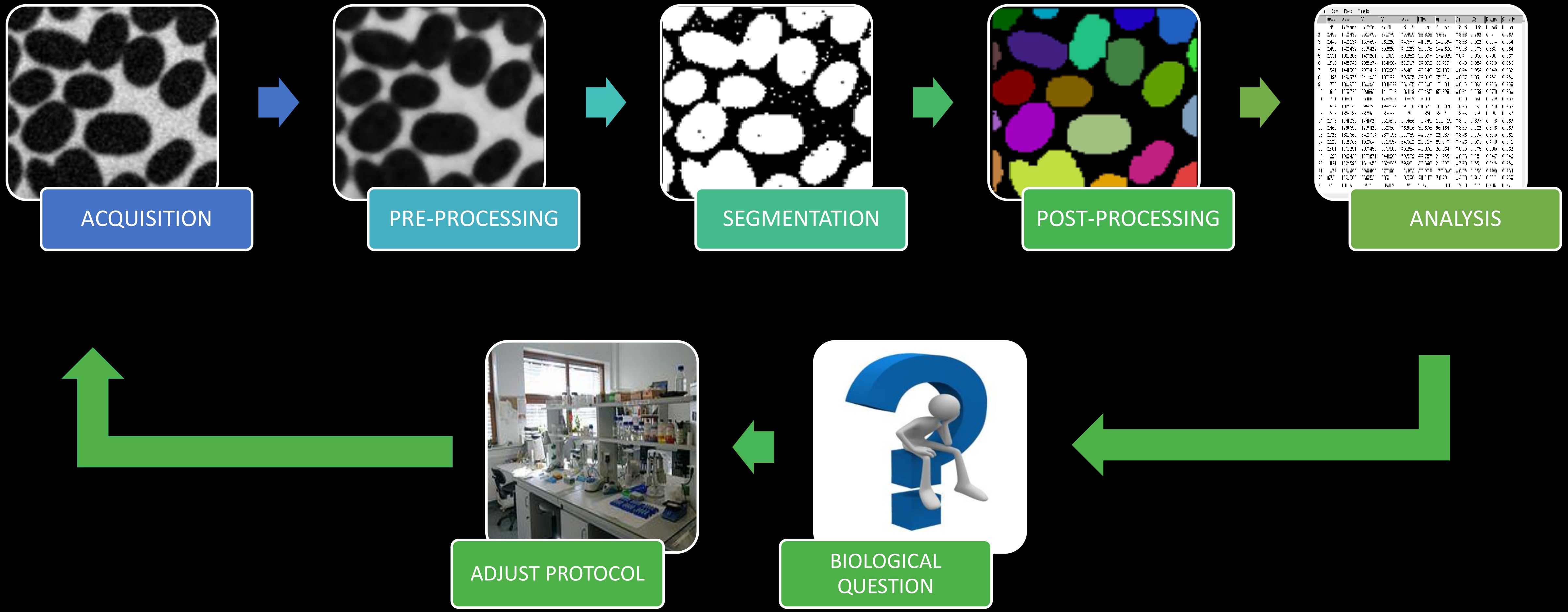
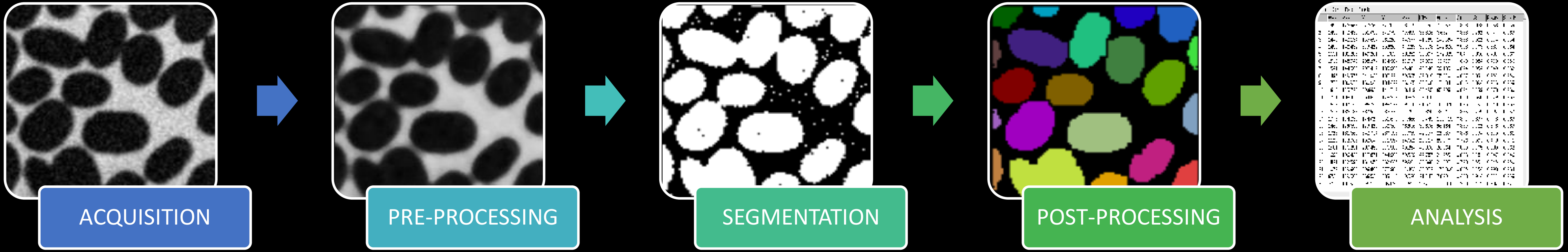


Image processing workflow



ITION

Noise reduction
Background correction
Contrast enhancement
Geometrical correction
Deconvolution etc.

Intensity thresholding
Component labeling
Watershed transform
Contour extraction
Region growing etc.

Regularize object shape
Separate touching objects
Merge separated parts
Fill holes etc.

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ACQUISITION
PRE-PROCESSING
SEGMENTATION
POST-PROCESSING
ANALYSIS

Acquisition

- Very important for good result, but you heard this already…
- Pick the best microscope & acquire optimal images

- ”Best” ≠ Newest
- ”Best” ≠ Most expensive
- ”Best” ≠ Confocal microscope
- ”Best” ≠ The one everyone else in the lab is using

ACQUISITION → PRE-PROCESSING → SEGMENTATION → POST-PROCESSING → ANALYSIS

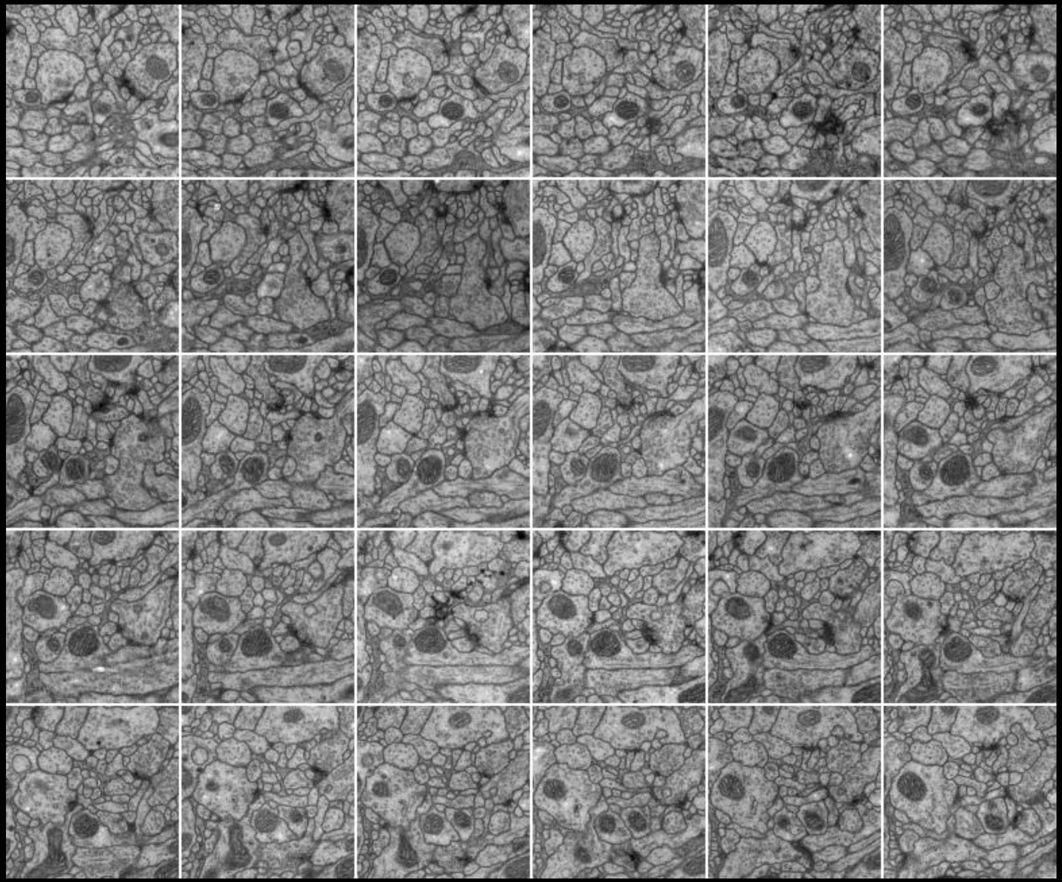
Pre-processing

- Image data format conversion to ensure precise calculations (32 or 64 bit)
- Modify the image using filters to make later segmentation more effective
- Which filter(s) to use is highly dependent on your data, but some commonly useful filters include:
  - Normalization
  - Gaussian blur
  - Subtract background
  - Find edges
  - Deconvolution

ACQUISITION → **PRE-PROCESSING** → SEGMENTATION → POST-PROCESSING → ANALYSIS

Pre-processing – Normalization

- Correction of intensity fluctuations during a time series or a z-stack
- In Fiji
  - Plugins → Integral Image Filters → Normalize Local Contrast



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Pre-processing – Normalization

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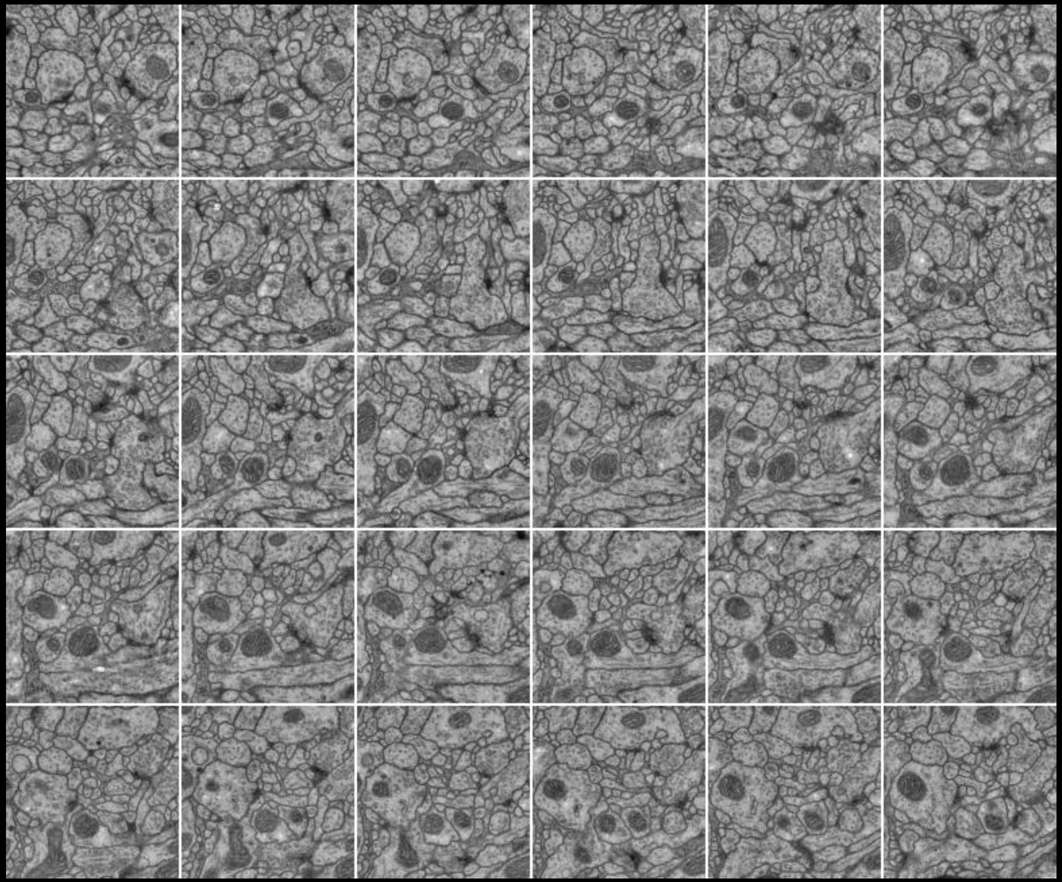

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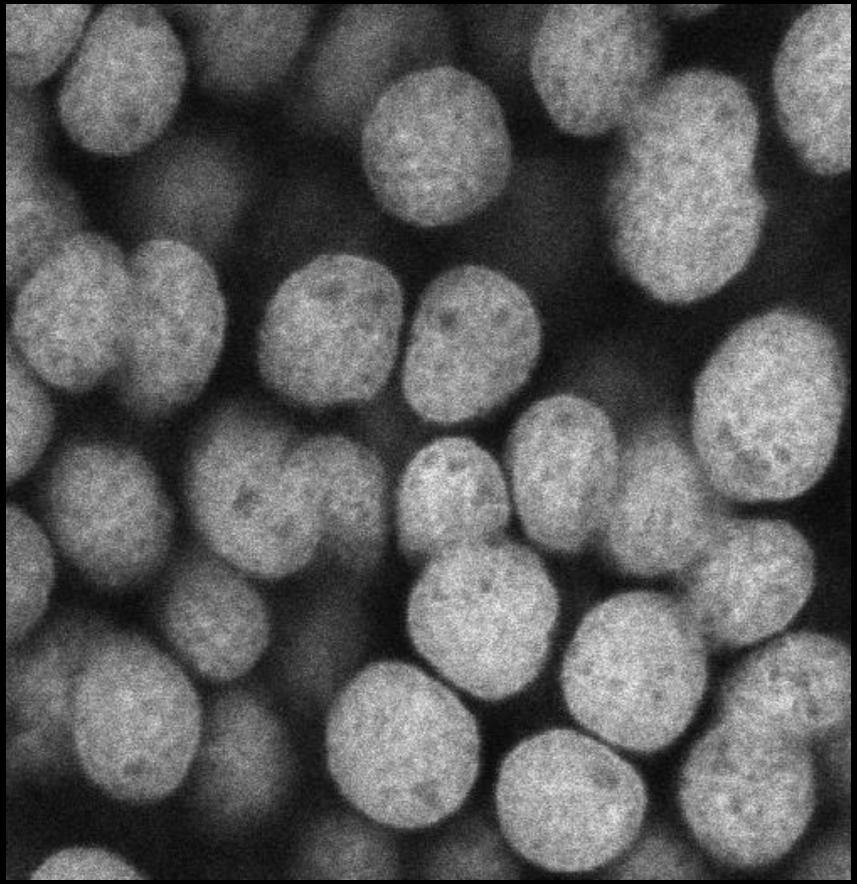
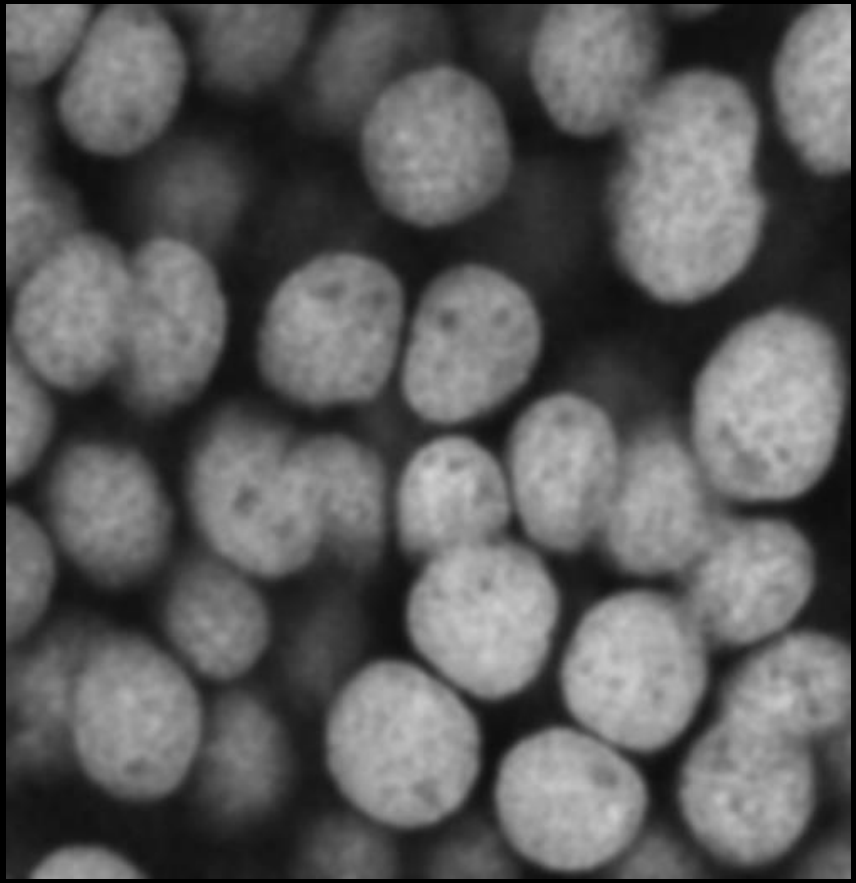
Pre-processing – Gaussian blur





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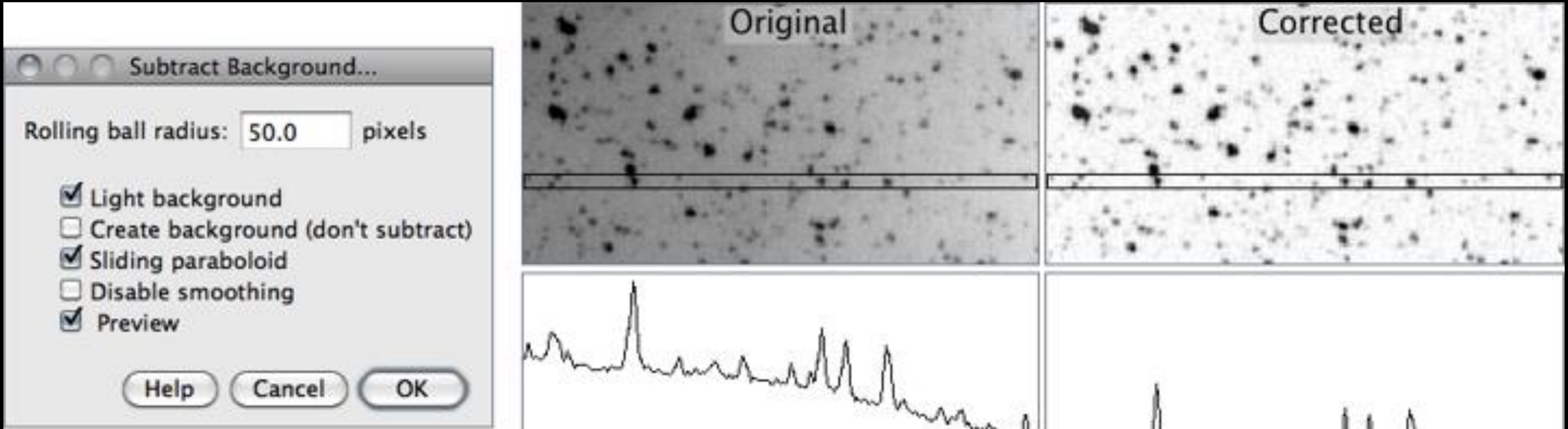
Pre-processing – Subtract background

- ”Rolling ball” algorithm removes smooth continuous backgrounds

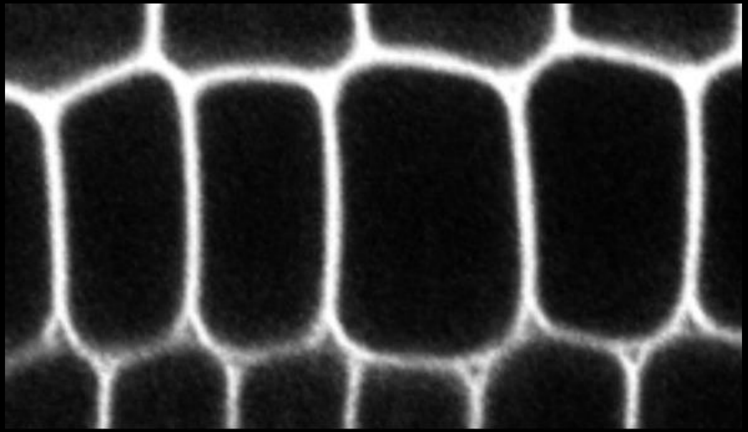


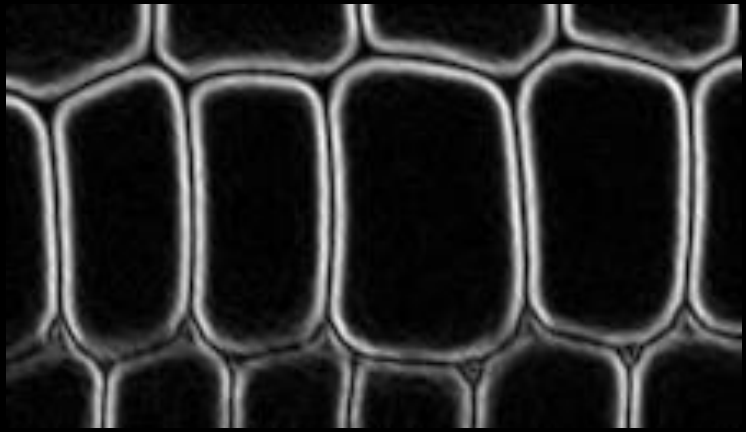
http://imagej.net/Segmentation

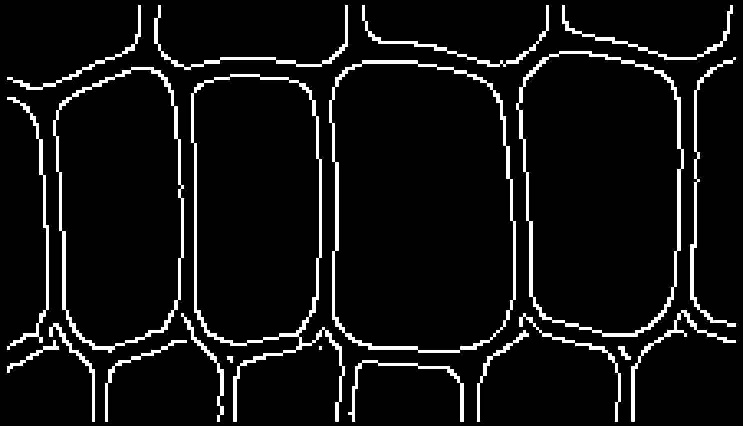
- In high-content analysis, background could also be calculated as an average of large number of (non-confluent) images

Pre-processing – Find edges

- Highlight sharp changes in intensity (by generating vertical and horizontal derivatives of the image)


Original image


Gradient image


Segmented image

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Pre-processing – Deconvolution

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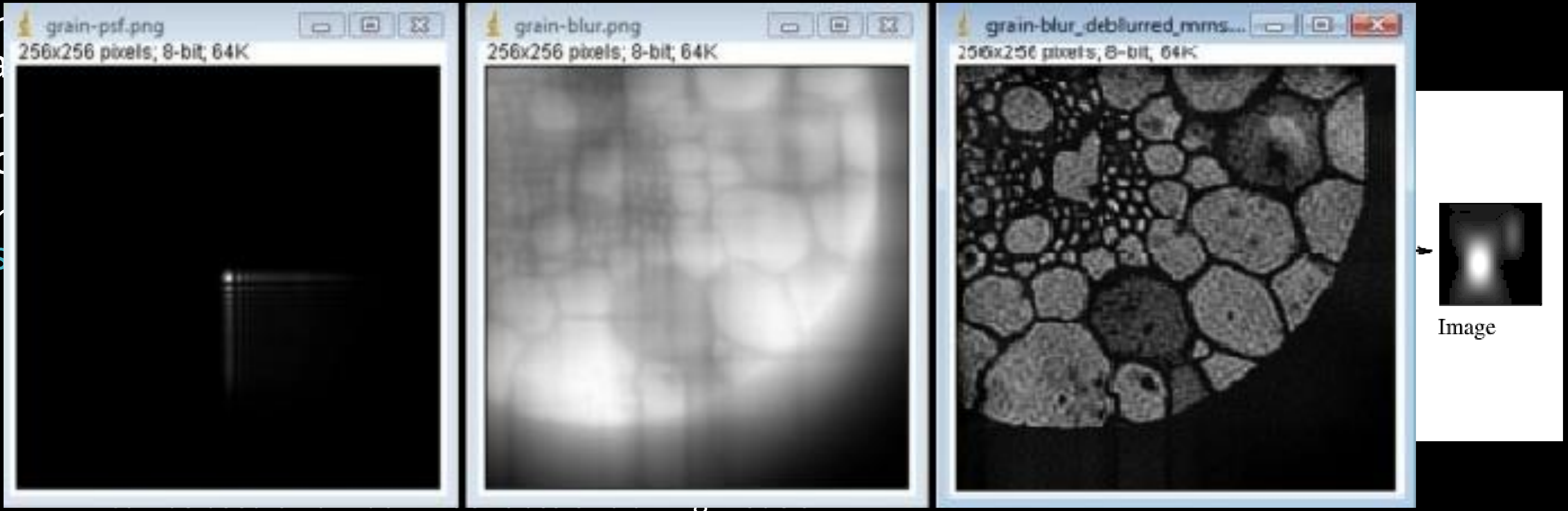

  - Image restoration preserves quantitative relationships in image data
- Consider deconvolution especially if you plan to analyze colocalization

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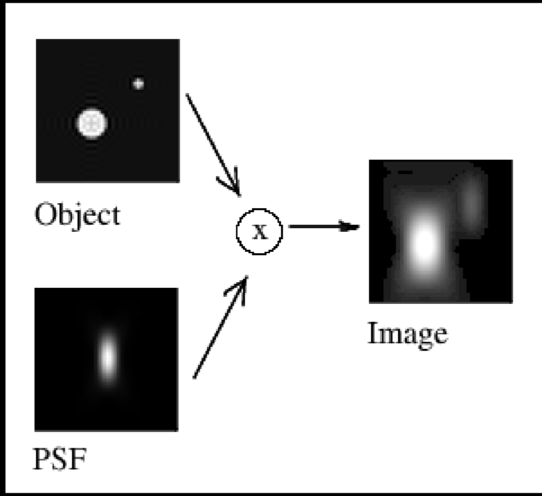
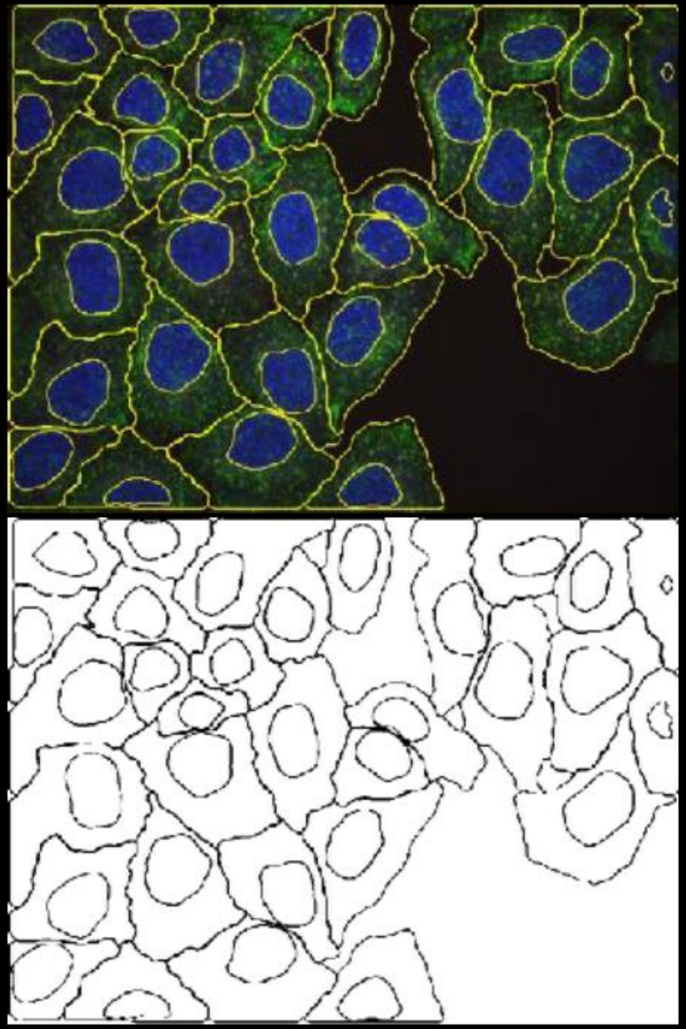
Pre-processing – Deconvolution

- Environmental effects and imperfections in the imaging system cause the recorded images to be degraded by blurring and noise
- The information about the blur is usually given in the form of a point spread function (PSF).
- Image deconvolution is the process of reconstructing or estimating the true image from the degraded one
  - Using experimental or theoretical PSF
  - Spectral filtering methods
    - Include many image deblurring techniques, e.g. Wiener filtering
    - Implicit assumption that the blur is spatially invariant
  - Iterative methods
    - Can be used on a much wider class of blurring models
    - Image restoration preserves quantitative relationships in image data
- Consider deconvolution especially if you plan to analyze colocalization



Segmentation

- ”Process of partitioning a digital image into multiple segments”
- Typically used to locate objects and boundaries



http://imagej.net/Segmentation

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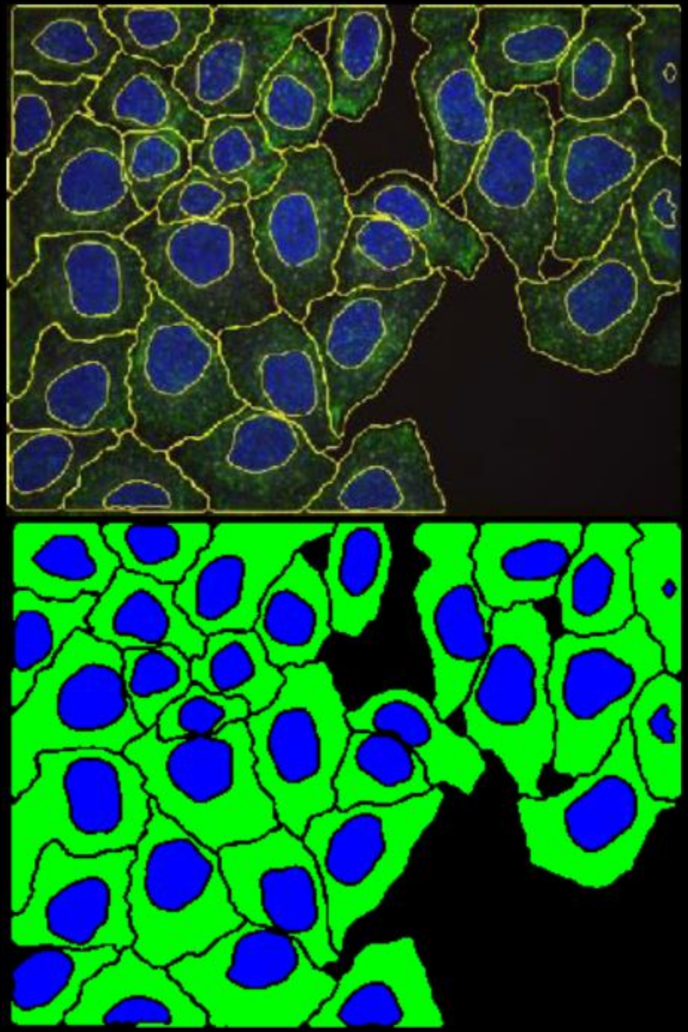
Segmentation

- ”Process of partitioning a digital image into multiple segments”
- Typically used to locate objects and boundaries
- More precisely: process of assigning a label to every pixel in an image such that pixels with the same label share certain visual characteristics (intensity, color, texture…)



http://imagej.net/Segmentation

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Segmentation

Commonly used basic methods

- Intensity thresholding (manual/automatic, global/local)
- Morphological methods (watershed)
- Shape detection (e.g. circular objects, straight lines)

More advanced methods

- Region detection using similarity criteria
- Iterative clustering methods
- Machine learning

Segmentation – Thresholding

- Most frequently used segmentation method is based on histogram analysis

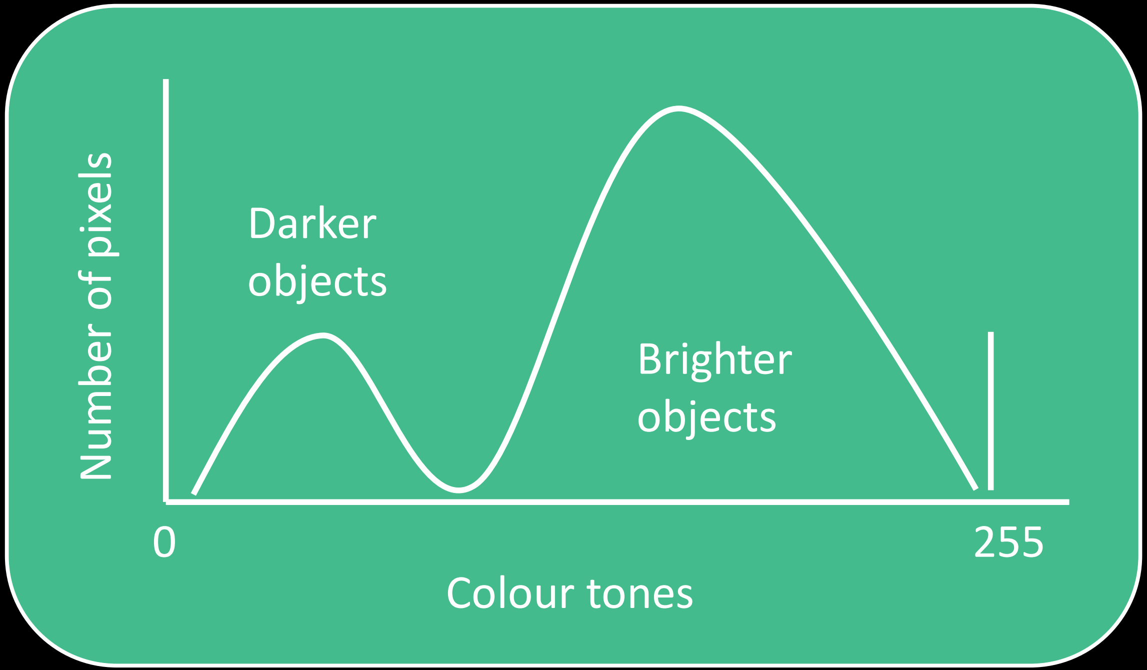
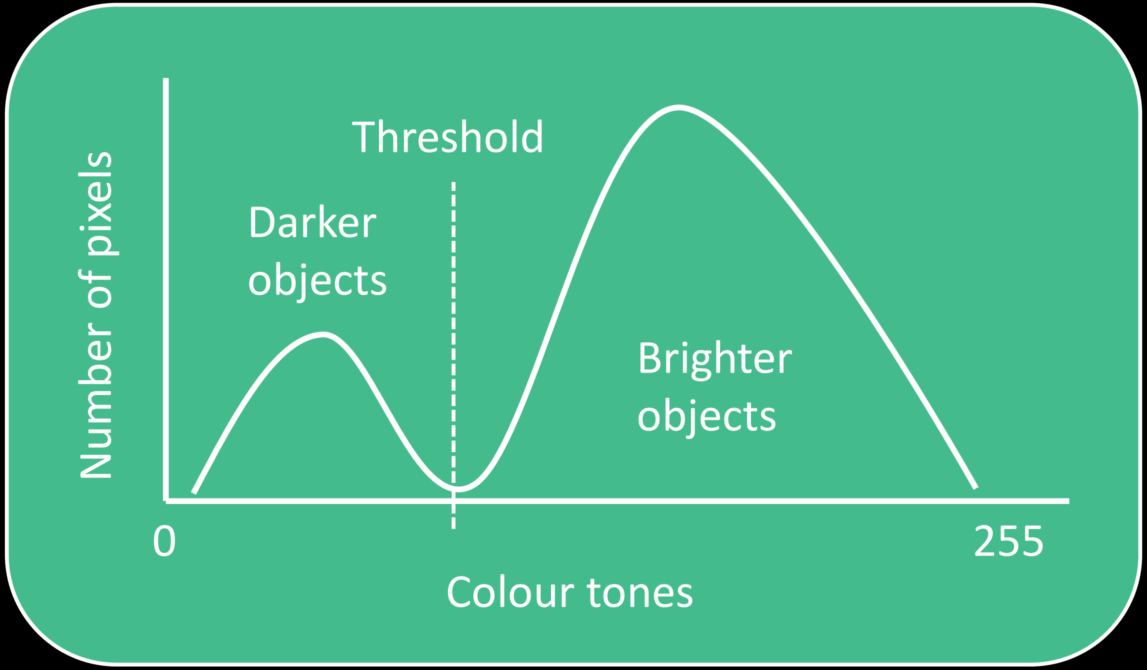


Image histogram

- Plots the number of pixels for each tonal value
- In an 8-bit image zero represents black and 255 represents white pixel value
- Peak at maximum value means signal saturation (overexposure)

Segmentation – Thresholding

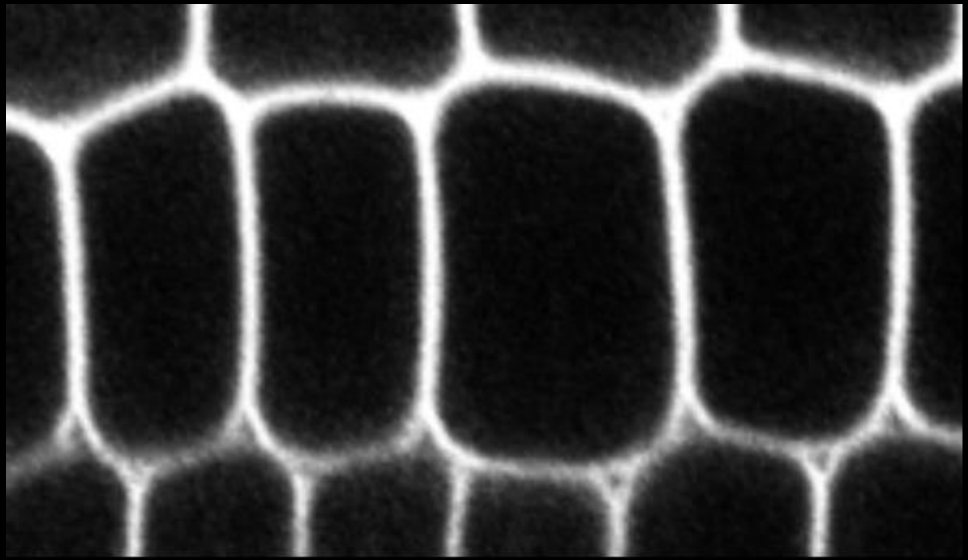
- Set an intensity value which separates the background and foreground



Difficulties

1. Valley may be invisible or so broad that it is difficult to locate a minimum
2. Number of minima due to the type of details in the image (multi-modal histograms)
3. Uneven illumination
4. Noise

Segmentation – Thresholding



Original image

Thresholded image

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Segmentation – Thresholding

Threshold too low

Threshold too high

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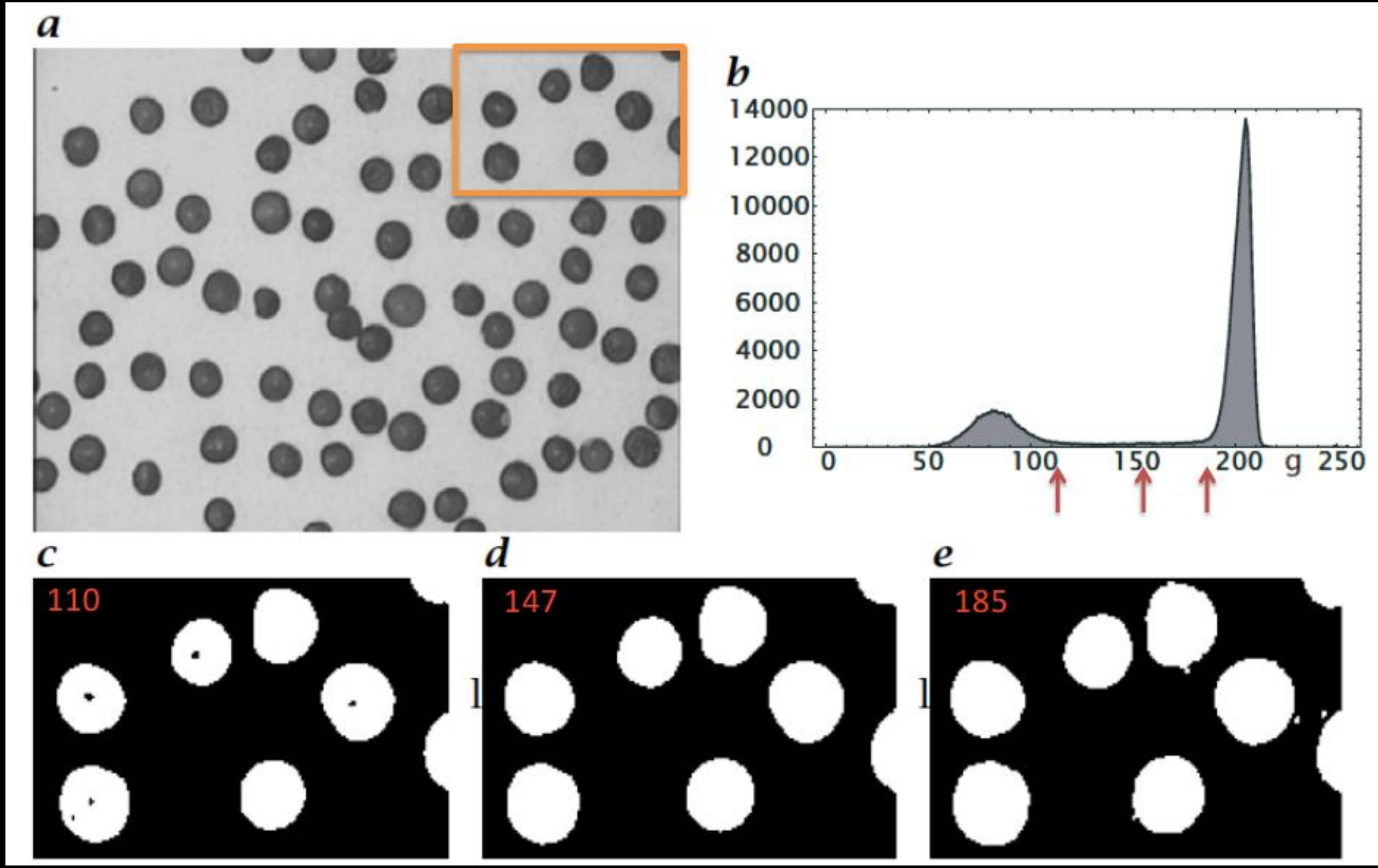
Segmentation – Thresholding



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Segmentation – Global or local threshold

- Image binarization using a global or local intensity threshold?
- One global threshold p for mapping every pixel (i,j) into a binary (black or white) pixel, e.g. in case of an 8-bit image

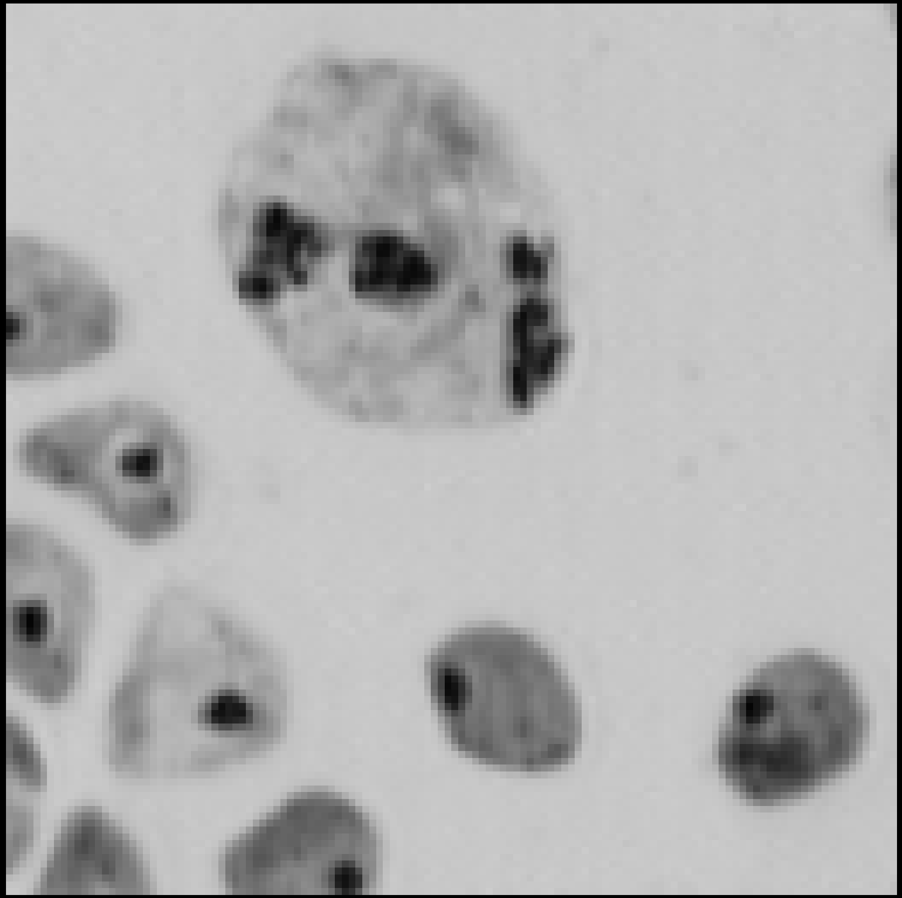
$$O(i,j) = \begin{cases} 0 & if \quad I(i,j) \leq p \\ 255 & if \quad I(i,j) > p \end{cases}$$

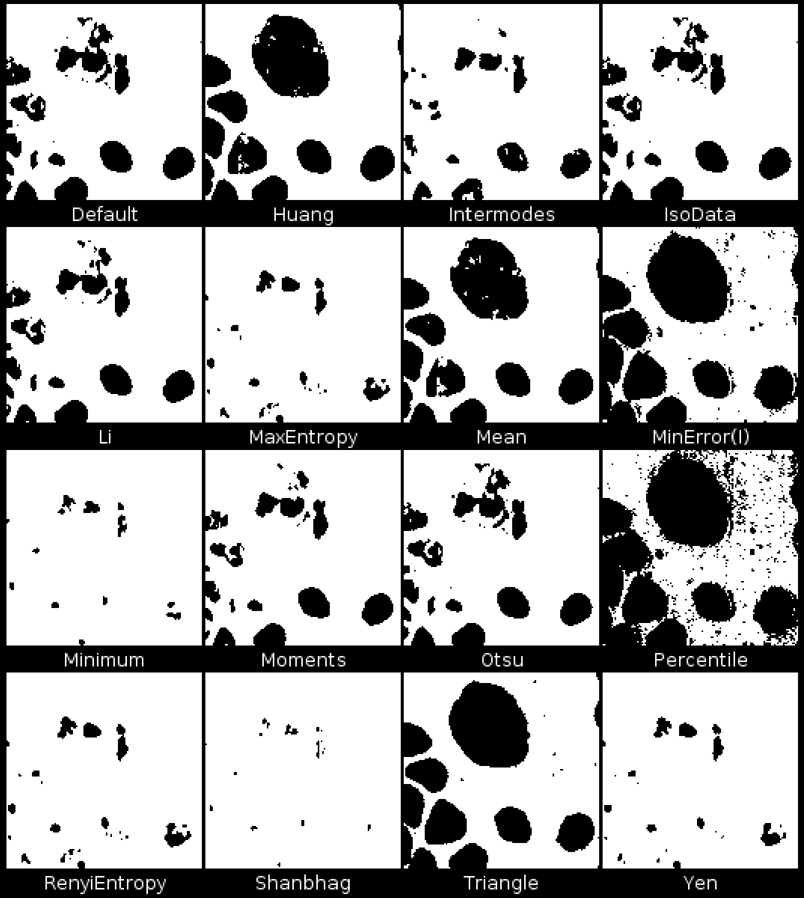
- A global threshold can typically be used when creating large connected regions and reducing the number of small-sized darker regions (artifacts).
- A local threshold adapts the threshold value on each pixel (i,j) to the local image characteristics (neighboring pixels). It can be used for correcting issues like uneven illumination.

Segmentation – Automatic thresholding

- Same manual threshold over a collection of images?
  - NOT recommended due to fluctuations in intensity across images
- Automatic threshold by optimizing some objective criterion that can be:
  - Statistical (e.g. maximization of inter-class variance, entropy…)
  - Probabilistic (e.g. minimization of pixel classification error…)
  - Structural (e.g. circularity of detected objects...)
- How do I know whether my threshold is correct? YOU DON'T!
- How to choose in Fiji? Try them ALL!

Segmentation – Automatic thresholding





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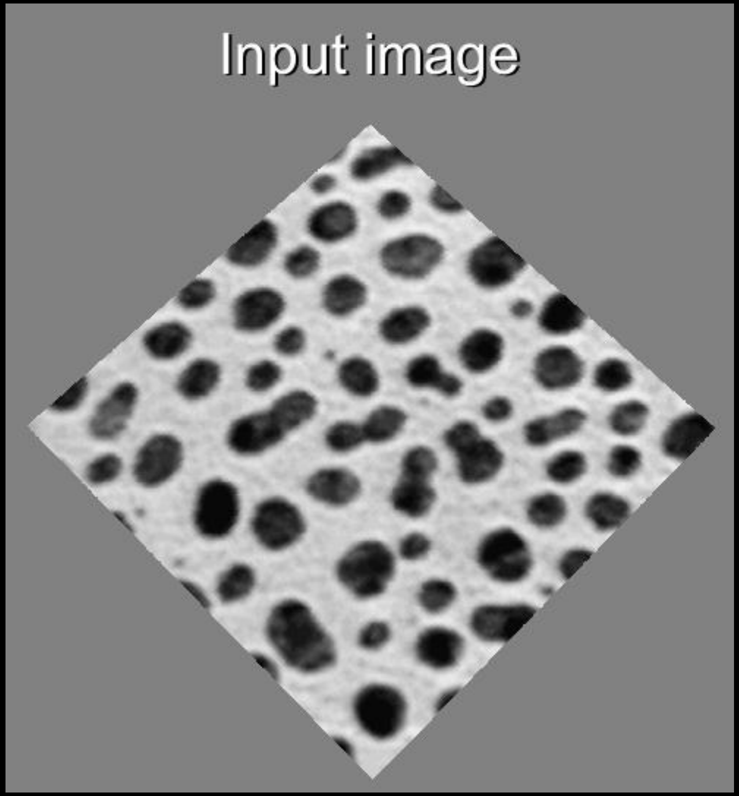
Segmentation – Morphological methods

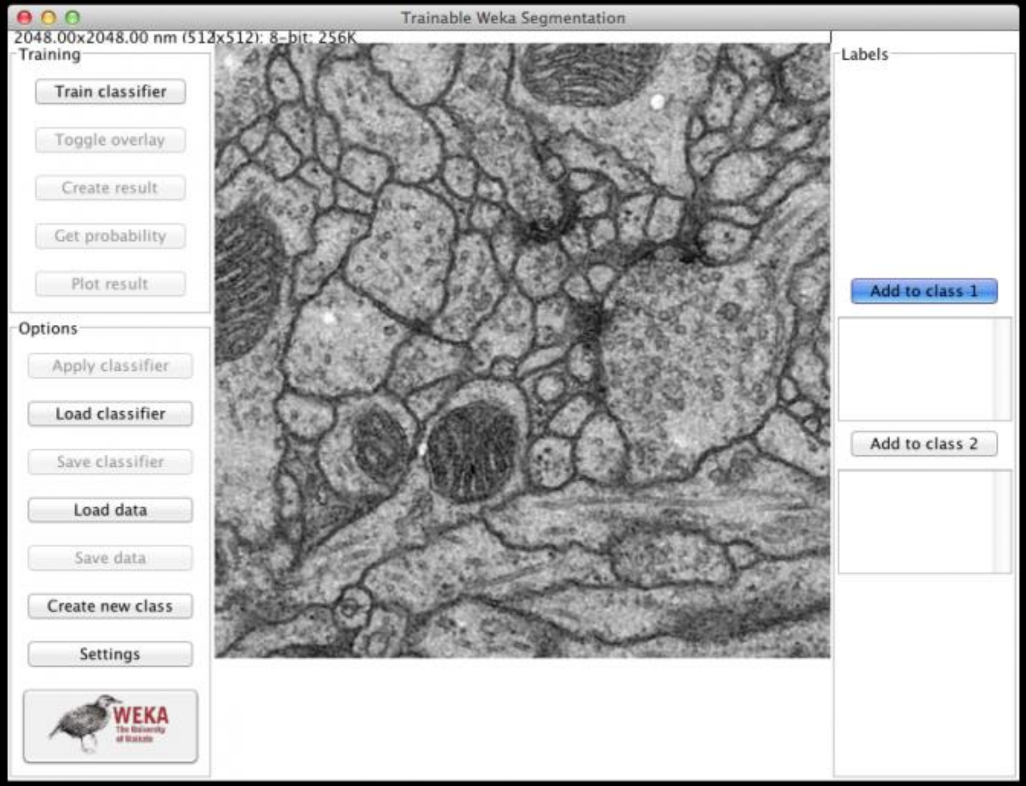
- Watershed segmentation
  - Process → Binary → Watershed
- GUI in Fiji
  - Plugins → MorphoLibJ → Segmentation → Morphological Segmentation
- Algorithm is expecting an image where the boundaries of objects present high intensity values (usually as a result of pre-processing with a gradient or edge detection filter or a distance map).



http://imagej.net/Morphological_Segmentation

Segmentation – Machine learning methods

- Trainable Weka Segmentation
  - Image segmentation based on pixel classification
  - Combines a collection of machine learning algorithms with a set of selected image features to produce pixel-based segmentations
- GUI in Fiji
  - Plugins → Segmentation → Trainable Weka Segmentation



https://imagej.net/Trainable_Weka_Segmentation

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Post-processing

Morphological reconstuction

- Exclude objects on image edges
- Exclude objects based on other attributes
- Fill holes
- Separate touching objects (e.g. watershed, erosion)
- Merge separated parts (e.g. dilation)
- Regularize object shape

Analysis

- Calculations within and between selected regions of interest (ROIs)
- Object properties
  - Area, min/max/mean intensity, standard deviation, roundness etc.
  - In Fiji
    - Analyze → Set Measurements
    - Analyze → Measure
- Colocalization
  - More about that tomorrow

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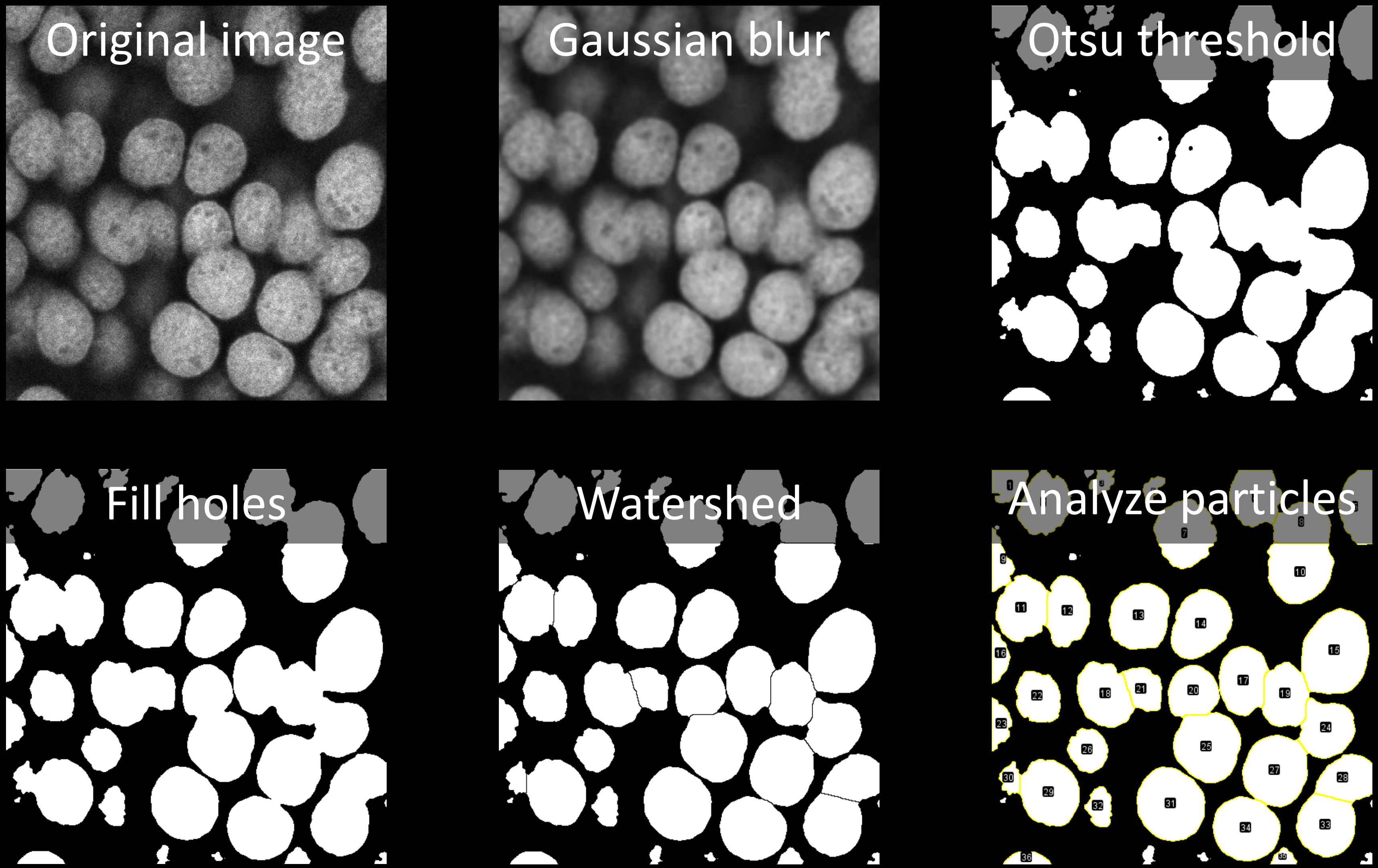

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FIJI hands-on workshops

Group A: Monday 7.3. at 13.30-17.00

Group B: Wednesday 9.3. at 9.00-12.30

- Practical 1: Basics of FIJI/ImageJ (~45 min)
- Practical 2: Cell counting and characterization (~45 min)
- Practical 3: Simple macros (~60 min)

Acknowledgements

- Neubias: http://eubias.org/NEUBIAS/