Basics of Quantitative Image Analysis

What you need to know about Microscopy Image Processing … … but never knew to ask

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Before you start writing...

See these slides at:  https://ifn.mpi-cbg.de
under: Teaching

Also available on the Fiji Wiki
✓ Fiji is just ImageJ – batteries included http://fiji.sc
✓ Fiji tutorials
✓ DetectInfoLoss, Colocalization Analysis and more...
✓ Practicals etc. are included in online version...
Topics:

- Images = “Information” (Digital Images)
- What is a pixel?
- Info “about” the image = Meta Data
- Different ways to visualise / display image info
- Quantitative imaging workflow
Quantitative Imaging?  
...what does that mean?

Art or Science?  Photography or Spectroscopy?

Scientific method = measure something

✓ Numerical Results
✓ Statistics!
✓ Computers become useful...
What is Image Analysis / Quantification?

Object: Stick man
Body: 1
Head: 1
Legs: 2 (1 lifted)
Arms: 2 (2 lifted)
Walking left to right…

Minimum: 50
Maximum: 255
Mean: 94.5
Std.Dev.: 93.2
Area: 10x14
Pixels: 140
Pix <255: 42

= Image Analysis/Measurement
= Interpretation of Analysis Result
What is a (Digital) Image anyway..?

✓ it’s a digital “representation” of reality!
✓ it’s an artifact that contains less info than the object!
✓ it’s just numbers! NOT analogue art!

The Image of a point is NOT a point!!!
(Point Spread Function – PSF)

A digital image of ???

Image Analysis
(Brain or Computer)

A stick man?
How do I know?
How can computer know -
algorithm?
Image = Information

Image = “Information”

✓ Quantify / Measure / Analyse
✓ Meta data (what, where, when, how)
✓ Noise / Background

Manipulate Image = Changed Info!!!

Lost Info is lost forever

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Slice | Count | Total Area | Average Size | Area Fraction |
blobs.gif | 46 | 17686.000000 | 384.478261 | 27.2 |
Image Data? What is it?

Intensity – Dye concentration??
Comparison of 2 colours / dyes / proteins??
Noisy Images?
Averaging?
Pixel Time?
Shapes, Movement, Structure?

Internal controls!!

A digital image with 2 channels / colours
What can you see here?
Getting to know “Fiji” better – Fiji is just ImageJ
http://fiji.sc

File - Open Samples – fluorescent cells

Fiji Tutorial –
http://fiji.sc/Detect_Information_Loss

✓ Write a list of things that are wrong with this image.

✓ Why is this image unsuitable for quantitative analysis?
Photographer or Spectroscopist?

We can show you how to take pretty pictures (Art)

or

We can teach you how to get useful information (Science)

You choose!!

This

Is simply a way to “Visualise”

This

0 0 1 0 0
0 1 0 1 0
0 0 1 0 0
0 0 1 0 0
1 1 1 1 1
0 0 1 0 0
0 1 0 1 0
1 0 0 0 1
Photographer or Spectroscopist?

Science vs. Art

Objectivity vs. Subjectivity

What I “think” I see vs. What is actually there

Morphology can also be quantified!
Photographer or Spectroscopist?

Science vs. Art

Objectivity vs. Subjectivity

What I “think” I see vs. What is actually there
“Colour Merge” images could ruin your life

You see: Yellow and Green Circles?

Actually, both circles are the same color!

Moral of the story: You can't measure colour by eye!

Evolution made you this way! Why?
Which colours can you see???
“Colour Merge” images could ruin your life

You see: spirals, of pink, orange, green and blue?

Actually, the green and blue... are the same color!

Moral of the story: Don’t Trust Your Eyes!

Spirals macro in Fiji
Color Inspector 3D
Colocalisation/Correlation

The past: “I see yellow - therefore there is colocalization”

It is NOT possible to objectively decide about colocalization by eye in a red-green merge image!

No colocalization definition + No statistics = No Science

Complementary methods: BioChemical, Optical (FRET, FLIM)
Colour Merge Images?

What are they good for?

Apart from looking pretty... not much.

Scientific conclusions from the image below? - No!

Colours blind people can’t distinguish green and red!

So use Magenta and Green!
Publishing Images
or “how Photoshop can ruin your career”

CCD/PMT sees intensities differently than your eye/brain
LUT? Gamma correction?
Calibrate monitors

Journal Images ≠ Screen Images
Screen = RGB = Visualise
Inks = CMYK = Print

Image = data
Don’t corrupt information!

Compression
Lossless – Yes
Lossy (JPEG) - NO

Always state the exact image processing done!
Publishing Images
or “Don't bother with figure layout, but do make raw data available”

Publishers will kill your images
Suggest layout – they redo it
Provide low res. thumbnail images
Publish link to raw image data

Focus on analysis results
Representative images only:
Not “the nicest one”

Publish processing and analysis method
eg. a macro
Verifiable
Reproducible

Raw Image Data
Must be made available!

JCB Data Viewer
Online image browser
What can you digitise?

Dimensions!

SPACE

INTENSITY

TIME

Wavelength

Colour

\[ \lambda \]
Pixel Size / Spatial Calibration
A pixel is NOT a little square!!!

A pixel is a *point* sample. It exists only at a point.
Digital spatial resolution

Projected pixel “size” at the sample/object is the point sample “**spacing**”

A pixel is not a “little square”

Point sample = Picture Element = PixEl
A pixel is a sample of “intensity” from a POINT in space

A pixel is NOT a little square!!!

Yes!  

A pixel is a sample of “intensity” from a POINT in space

“pixel size” is pixel spacing distance

– not the imaginary pixel edge length!
A pixel is NOT a little square, A pixel is NOT a little square, A pixel is NOT a little square!
(And a voxel is NOT a little cube)

A pixel is a point sample. It exists only at a point.

Maybe it lies on a grid pattern…
…but that's accidental!
Or in our case the PSF (Point spread function = image of a point) of the microscope system!

(a) A 5x4 image.

(b) The footprint of a reconstruction filter. A truncated Gaussian, for example.

(c) Footprint of image under reconstruction.

Dotted line is minimally enclosing rectangle
A pixel is not a little square … So what?

Example – image shrinking
2048 x 2048 pixel electron micrograph – resized to 100 x 100

Wrong
dumb interpolation of square pixels (aliased)

Correct
Gaussian smooth, then down sample

Compare plugins-examples-downsample with Image-scale
What does a point sample from a microscope detector contain?

Image of a point light source = Point Spread Function (PSF)

In the diffraction limited, high resolution case:

The PSF is **bigger** than the pixel / sample Nyquist spacing.
So what does a point sample from a confocal microscope detector contain?

In the low resolution, big pixel case:

The PSF is much smaller than the pixel or sample Nyquist spacing.

We miss spatial information = lower resolution.
Abbe’s diffraction limit / Rayleigh criterion

Limit the resolution of light microscopy

Airy Patterns and the Rayleigh Criterion online tutorial:
http://www.microscopy.fsu.edu/primer/java/imageformation/rayleighdisks/index.html

2 point light sources:

\[ d = \frac{0.61 \times \lambda}{\text{lens N.A.}} \]

\[ d = \frac{0.61 \times 550\text{nm}}{1.4} = 240 \text{ nm} \]
Digital spatial resolution

Projected pixel “size” at the sample/object

The point sample spacing

But what “should” it be?

under sampled  over sampled  good sampling
Pixel Size / Image Resolution

“Correct” image size? 64x64, 512x512, 2048x2048, ...

Nyquist – Shannon sampling theory: Proper spatial sampling
2.3 – 3 times smaller than optical resolution (x, y, AND z)

Adjust zoom, binning, and image size (no of pixels)

under sampled  over sampled  correct sampling

1 Airy unit
Harry Nyquist, 1889 - 1976

- Swedish – American
- Engineer in telecommunications
- Worked at Bell labs
- 138 US patents
Nyquist sampling criterion

**Aliasing**: Moiré patterns / loss of information
Nyquist sampling criterion

**Aliasing:** Moiré patterns / loss of information
Nyquist sampling criterion

**Aliasing**: Moiré patterns / loss of information
Nyquist sampling criterion

**General form**
Digital sampling frequency > analogue frequency x 2

**Spatial representation**
Image pixel size x 2.3 = smallest resolvable distance

**Microscopy**
Image pixel size x 2.3 = optical resolution (d)

**Aliasing**
Moiré interference patterns = loss of information
Nyquist sampling criterion

Different “objects” in different places…
but the digital images are identical!
More aliasing problems...

Pixel size relative to projected image

Image of object varies, depending on where it falls on detector

Especially for small objects close to pixel size
Spatial Sampling is fun with Food Items!

Squared paper = CCD Camera

Object size vs. Pixel Spacing?

Image of object varies, depending on where it falls on detector

- Especially for small objects close to pixel size

Pixel size relative to projected image
Nyquist sampling criterion

Resolution - pixel size calculations:

Resolution, \( d = \frac{\text{lambda}}{2 \times \text{NA}} \)

Required Pixel Spacing = \( \frac{d}{3} \)
Nyquist sampling criterion

Optomistic pixel size calculations:
550 nm light ; $d=\lambda/2\text{NA}$ ; $\text{pix}=d/3$:

<table>
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<th>Objective (N.A.)</th>
<th>Optical Resolution limit (nm)</th>
<th>Projected size on CCD (um)</th>
<th>Required CCD pixel spacing (um)</th>
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<td>1400</td>
<td>5.5</td>
<td>2</td>
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<tr>
<td>10x (0.4)</td>
<td>690</td>
<td>7</td>
<td>2</td>
</tr>
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<td>40x (0.75)</td>
<td>366</td>
<td>14.5</td>
<td>5</td>
</tr>
<tr>
<td>40x (1.3)</td>
<td>210</td>
<td>8.5</td>
<td>3</td>
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<td>63x (1.4)</td>
<td>200</td>
<td>12.5</td>
<td>4</td>
</tr>
<tr>
<td>100x (1.4)</td>
<td>200</td>
<td>20</td>
<td>6.5</td>
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</table>

Think about your digital spatial resolution carefully!
Remember !!!
Nyquist told us how to do digital sampling:
\(~1/3 \times \text{smallest feature}.\)
Pixel size / Spatial Calibration

Pixel size is determined by the microscope system!
- CCD photodiode “pixel” size - Magnification X
- Point scanner settings – zoom and image size
- Field of View Size - No. of Samples or “pixels”

It might be changed / lost during processing

It is stored in the “Meta Data”

So .. a dataset for image processing
= Image data + Meta Data!
Practical Session 1a

Getting to know “Fiji” better – Fiji is just ImageJ
http://fiji.sc

File - Open Samples - Embryos or Bridge

Spatial Scaling:
Can you measure the length and area of objects?
→ See Fiji Tutorial - SpatialCalibration (search Wiki)

✓ Analyze - Set Scale, Analyze-Tools-Scale Bar
✓ Line and ROI selection - ctrl M (cmd M)
✓ Rectangle, Oval, Polygon, Freehand, Angle, Point, Wand.
✓ Analyze - Set Measurements (Results – Edit - summarize)
What can you digitise?

Dimensions!

SPACE

INTENSITY

TIME

Wavelength

Colour

λ
“Intensity” Digitisation

Remember: Bit Depth

Measured intensity by detector

“Bucket” holds 0-9 electrons

5 electrons counted

Corresponding level in image

Bit depth: 10 (0 to 9) levels

Level 5 selected for RAW data “image”
"Intensity" Digitisation

Bit Depth

“digital” intensity resolution: 10

“real” analogue intensities

“digital” intensity resolution: 20

9 → 19

0 → 0
## “Intensity” Digitisation

### Bit Depth

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<td>2&lt;sup&gt;16&lt;/sup&gt;</td>
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- **Segmentation**: 
- ~ limit of human eye, displays...
- Intensity-related measurements
“Intensity” Digitisation

Bit Depth
for intensity-related measurements

**8 bit**
- Dynamic range: 180

**12 bit**
- Dynamic range: 2800

4095

255

0
“Intensity” Digitisation

Bit Depth for segmentation

8 bit greyscale

1 bit Binary image
Remember:
Intensity / Exposure / Saturation

Do NOT over expose / saturate your image!!!

Why not? → Lost Information!
Use “Look Up Tables (LUT) / palettes to check the saturation

Bye Bye Data!

in range

clipped
overexposed
saturated

255
pixel intensity

0
x
Image Intensity Histograms - Use them!

In Histograms:
easily see problems for image quantification!

OK!  

Lost Info! Clipped!

30 = 0 ???!!!
Intensity Histogram

Fluorescence Microscopy

![Intensity Histogram Images]

- **OK**
  - Count: 524288
  - Mean: 18.561
  - StdDev: 26.465

- **not OK - why?**
  - Count: 524288
  - Mean: 82.504
  - StdDev: 93.452
  - Mode: 4 (101652)
Intensity Histogram

Brightfield Microscopy

Count: 262144  Min: 0
Mean: 191.793  Max: 255
StdDev: 50.337  Mode: 214 (10291)
Intensity Histogram

- **Fluorescence**
  - Count: 524288
  - Mean: 18.561
  - StdDev: 26.465
  - Min: 0
  - Max: 235
  - Mode: 0 (174427)

- **Brightfield**
  - Count: 262144
  - Mean: 191.793
  - StdDev: 50.337
  - Min: 0
  - Max: 255
  - Mode: 214 (10291)
Intensity clipping/ saturation and offset:

- **Bit Depth** – change from 16 to 8. *What happens to the numbers?*

- **Brightness/Contrast**: *Image-Adjust-Brightness/Contrast.*
  *Realize: you can lose data using “Apply”!*

- **Intensity Histograms**: *log scale for fluorescence*
What can you digitise?

Dimensions!

SPACE

INTENSITY

TIME

Wavelength

Colour

\lambda
RGB Color Space

Why RGB? ... because we have red, green and blue sensitive photo receptors in our eyes!

Each “colour” is really just single greyscale numbers!
Each “colour” is really just single greyscale numbers!

So we can represent that information however we like!
better see and also compare different intensity levels

Grayscale - linear

Rainbow lookup table

“original” - linear blue

altered brightness/contrast

data changed/lost!
Line Profile
Line Profile
Line Profile

for measurements

FWHM = “Full Width at Half Maximum”

0.9 µm

50% of max. intensity

Distance (microns)

Gray Value
Line Profile

correct ?
correct !
Intensity Histogram

Count: 524288  Min: 0
Mean: 18.561   Max: 235
StdDev: 26.465 Mode: 0 (174427)

Count: 524288  Min: 2
Mean: 82.504   Max: 255
StdDev: 93.452 Mode: 4 (101652)
2D Histogram

= 

Scatterplot or cytofluorogram
Find a way to visualise what you actually want to see:
Here, we don’t care WHERE the beads are;
We care if they are in the same place or not!
Imaging Experiment Planning:

✓ What **BIOLOGY** am I trying to measure?
  - *Hypothesis?!!?*

✓ Do I need 3D, 4D, xD information
  - *Resolution?*
  - *Sampling: Space, Time, Intensity*

✓ Choose appropriate microscope
  - Don't always use Confocal LSM

✓ Optimise microscope system
  - *get best data from your sample*

✓ Do the right controls!!!

✓ Measure Something
  - *Statistics to test hypothesis*
  - *How many data points/images/cells?*
Imaging Experiment Work Flow

EXPERIMENT HYPOTHESIS
  - What is my experimental hypothesis?
  - How can I test my hypothesis?

WHAT INFO / DATA DO I NEED
  - Dimensions, Resolution, Precision...
  - What controls do I need?

CONTROLS DESIGN
  - How can I get that info?

PLAN INFO ANALYSIS
  - How will the statistical tests work?

EQUIPMENT CHOICE + SETUP
  - What type of equipment is needed
    - System learning
    - Process optimization (Imaging equipment+ sample prep)

DATA ACQUISITION
  - Noise removal, Deconvolution, etc.

IMAGE PROCESSING
  - Intensities / objects

MEASUREMENTS
  - Null Hypothesis rejected? (True/False)

STATISTICAL ANALYSIS
RGB colour space:

- **Colour channels**: Image-Colour-Channels Tool, Split channels etc.
- **LookUp Tables/Palettes**: Image - Lookup tables, or LUT toolbar icon
- **Line Profile**: Analyze – Plot Profile
- **Histogram**: Analyze-Histogram or Plugins-Analyze-2D Histogram
- **Intensity Scale**: Analyze – Tools - Calibration bar
Basics of Quantitative Image Analysis

What you need to know about Image Processing… but never thought to ask

… continued

Session 2
✓ Filtering Images in the spatial, frequency and time domain
✓ Segmentation – finding and measuring objects in images

Session 3
✓ Detect Info Loss, Colocalization Analysis and more
✓ Whatever you find interesting
Image processing in the spatial domain

A) Introduction
   - Neighbourhood
   - Operation on neighbourhood

B) Spatial filters
   - Mean and Median filter
   - Edge detection
A. Introduction

“Transformation or set of transformations where a new image is obtained by neighbourhood operations.”

The Intensity of a pixel in the new image depends on the intensity values of “neighbour pixels”

Neighbourhood (or kernel): pixels that matter
$3 \times 3$
2 x 2 ; shift
Misc.
B: Filtering - the mean filter

Simplest filter: The value of a pixel is replaced by the intensity mean of the neighbourhood pixels.

3x3 example:

\[ a_i^* = \frac{1}{9} (a_1 + a_2 + a_3 + a_4 + a_5 + a_6 + a_7 + a_8 + a_9) \]
The mean filter

Noise removal - typically Gaussian or Poisson noise.

Appears for weak labeling, short exposure time, confocal = few photons detected
The mean filter

The mean filter is a linear filter!

\[ \alpha_{1,1} \quad \alpha_{1,2} \quad \alpha_{1,3} \]
\[ \alpha_{2,1} \quad \alpha_{2,2} \quad \alpha_{2,3} \]
\[ \alpha_{3,1} \quad \alpha_{3,2} \quad \alpha_{3,3} \]

“The new pixel value depends on a linear combination of neighbourhood pixel values”

(The order of several linear filters in sequence does not matter)
The mean filter

Main property: low-pass filter (smooths small objects)
- kernel size influence
- number of successive applications

+ simplest filter – fast
+ it’s a linear filter
+ averages noise, does not eliminate it
+ works against Gaussian and Poisson noise

- blurs images - small details are lost (low pass filter)
- smoothes edges dramatically
- fails for salt & pepper noise
Linear filtering - Properties

✓ Applying a linear filter to an image is the same as: applying it to all parts, then summing the results.

✓ When applying a succession of linear filters: the order filters are applied in does not matter.

✓ Mathematical framework underlying it: Convolution.

✓ We can also reverse the process: Deconvolution.
The Gaussian filter

- smooths Poisson noise
- linear Filter
- makes more mathematical sense than mean filter?
- ...properly spatially sampled image, looks like PSF
- can vary the sigma value: number of pixels
- varying degree of blur.

Gaussian Curve - Bell Shaped function

Gaussian Distribution Function

The full width of the gaussian curve at half the maximum is

$$\Gamma = 2\sqrt{2\ln2}\sigma = 2.355\sigma$$
The median filter

The value of a pixel is replaced by the median of the pixel intensity in neighbour pixels.

Take neighbourhood (e.g. 3x3)

| 5  | 112 | 86 |
| 235 | 88  | 211 |
| 137 | 233 | 108 |

Sort it

5
86
88
108
112
137
211
233
235

Take median

112
The median filter

<table>
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<th>Median filtered:</th>
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</thead>
</table>
| 5 9 6 6 9 5 9  
9 5 9 7 8 7 9  
8 9 8 6 7 9 9  
9 9 7 9 6 9  
6 5 8 6 9 6 7  
9 7 9 9 8 6 7  
7 9 5 6 7 6 6 | 0 5 6 6 6 6 7 0  
5 8 7 7 7 9 7  
8 9 8 8 7 9 7  
6 8 8 8 7 9 6  
6 8 8 9 8 7 6  
6 7 7 8 6 7 6  
0 7 6 6 6 6 0 |

outlier

noise elimination

The outlier value has been completely removed from the dataset
The median filter - what is it good for?

“Salt & pepper” noise removal

* Typically appears for very weak labeling, high detector gain etc.

Original:

![Original Image]

Median filtered:

![Median Filtered Image]
The median filter

+ Typically good for “Salt & pepper” noise removal
+ Eliminates noise
+ Edge-preserving

- Slower than mean (not such a problem anymore... computers are fast)
- NOT linear
(1) File - Open Samples – bat cochlea volume
(2) File – Import – URL…
http://pacific.mpi-cbg.de/samples/colocsample1bRGB_BG.tif

(1) Convolve a simple binary image
✓ Process – Filters – Convolve (play with different kernels)
✓ Process – Filters – Gaussian Blur (change sigma, in px)

(2) Noisy sample image
✓ Mean and Median Filter (change pixel number, kernel size)
✓ Gaussian Blur … and Gaussian Blur again… and…
Morphological Filters

Binary Images
(plus variants for grayscale images)

Erode
Dilate
Open
Close

... done using spatial filters - kernels
Morphological Filters

Erode: Removes pixels from the edges of objects.

The size and shape of the kernel matters!
Morphological Filters

Dilate: Adds pixels to the edges of objects.

Again, the size and shape of the kernel matters!
Morphological Filters

Open:
Performs an erosion operation, followed by dilation. This smoothes objects and removes isolated pixels.

\[ A \ominus B = (A \ominus B) \oplus B. \]

Again, the size and shape of the kernel matters!
Morphological Filters

Close:

Performs a dilation operation, followed by erosion. Again, this smoothes objects and fills in small holes, but differently.

Again, the size and shape of the kernel matters!
Morphological Filters

In Fiji/ImageJ - Greyscale images:
Use Maximum and Minimum filters for Dilate and Erode respectively.

Minimum...
grayscale erosion: replace each pixel with the min pixel value of pixel's neighborhood.

Maximum...
grayscale dilation: max pixel value of pixel's neighborhood.
Morphological Filters

Options...

Settings for Binary submenu commands

**Iterations**: the number of times performed.

**Count**: the number of adjacent “other” pixels necessary before a pixel is + or - from object edge.

Check **Black background** if the image has white objects on a black background.

If **Pad edges** when eroding is checked, Process>Binary>Erode does not erode from the edges of the image.

Also affects Process>Binary>Close: erodes from the edges unless this checkbox is selected.
Time? Just another dimension

Dealing with multiple images files: time stacks, timelapse movies, 3D stacks, …

- Intensity over time
- Kymographs

L929-RlCgfp - G1 - NZ - ablation 3.5s - 06/11/14 - try11a

total speed of cortex movement: 17.0 μm/mn
Motion blur

Motion blur = average over time
Does this happen in your sample? Frame Rate?
Fourier Image Filtering

- FFT, filter out parts, Inverse FFT: Mess up the image. Can you extract high and low frequency information?

- **Use circle selection and Edit - Fill:** Set foreground colour to black.

- FFT bandpass filter
What is “Image Segmentation”? 

“Greyscale” image

Foreground background
What is “Image Segmentation”? 

“Scalar Intensity” image

“Binary” image
What is “Image Segmentation”?

```
1  65  13  55  2
2   3  34  2  1
4   0  31  1  2
1  33  3  54  3
56  3  2  1  34
```

“Scalar Intensity” image

```
0  1  1  1  1  0
0  0  1  0  0
0  0  1  0  0
0  1  0  1  0
1  0  0  0  1
```

“Binary” image
What is “Image Segmentation”?

“Scalar Intensity” image

Labeled objects
What is “Image Segmentation”?

High Information Content
65536 pixels, 0-255 value

Lower Information Content, but easier to interpret biological meaning…
45 “objects” with properties: size, shape, intensity etc.
“Thresholding”
(Intensity Histogram Split)

Clear difference between foreground and background?
Image not very noisy?

Choose an intermediate grey value = “threshold”
Determines foreground and background.
How to choose the grey level for thresholding?

Look at pixel intensity histogram of whole image...

Is there an obvious place?
“Thresholding”
(Intensity Histogram Split)

Histogram is bimodal, so put threshold in the trough between the peaks!

Note, in this case:
Foreground = “dim” objects
Background = “bright” objects
“Dumb Global Threshold” (Subjective - User Biased)
Computed Global Threshold
Objective - Reproducible

ImageJ - Image - Adjust - Threshold - Auto (=Make Binary):

Initial guess of Threshold, $T$
Compute mean pixel intensity of background and foreground
$T_{\text{new}} = 0.5 \times (\text{mean of foreground} + \text{mean of background})$
Iterate until $T_{\text{new}}$ no longer changes.

Note:
Manual threshold set? Make Binary uses that dumb threshold!
(1) File - Open Samples – Blobs (inverse)
(2) File – Open Samples – Clown

(1) Thresholds

✓ Image – Lookup Tables – Invert LUT
✓ Process - Binary - Make Binary (default method)
✓ Image - Adjust – threshold: Adjust the thresholds, then set them to make binary
✓ Image - Adjust - Auto Threshold and Auto Local Threshold
✓ Many more methods, and “local” method

(2) Statistical Region Merging
How can we find the edges?
Edge Detection

What is an “edge”?

“Hard Edge” - Adjacent black / white pixels

“Soft / Fuzzy Edge” - common in images. Especially for small diffraction limited objects like vesicles/membranes. Noise makes edges look softer
Edge Detection "Image Gradient"

What is a “Gradient Image”?

Rate of change of pixel intensity (1st derivative)

What is a “Gradient Image”?

Y = pixel intensity gradient
Edge Detection “Image Gradient”

What is a “Gradient Image”? 

Rate of change of pixel intensity (1st derivative)

Image

Hard edge

Soft edge

Gradient Image
"Image Gradient" - How?

- find edges with x and y components
- compute total gradient magnitude
- approximates 1st derivative of image

Sobel filter - 3x3 convolution filter pair in x AND y

<table>
<thead>
<tr>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>0</td>
<td>+2</td>
</tr>
<tr>
<td>-1</td>
<td>0</td>
<td>+1</td>
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</tbody>
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<td>0</td>
</tr>
<tr>
<td>-1</td>
<td>-2</td>
<td>-1</td>
</tr>
</tbody>
</table>

| \(| g_x | + | g_y | = | g | \)
Gradient Image - Real Sample:

Real / Biological images:
✓ Sobel filter
✓ many edges
✓ many weak edges from noise
Remove weak edges?

✓ Threshold the gradient image
✓ Smoothing filter beforehand
“Canny” Edge Detection

Remove weak/noisy edges - keep strong
Gaussian smooth image + hysteresis threshold gradient image

Make edges sharp - 1 pixel wide
Non maximal suppression of gradient image
Watershed Algorithm:

... mountains, lakes and oceans

Height = Image Intensity

View From the Side
Watershed Algorithm:

... mountains, lakes and oceans
Watershed Algorithm:

... mountains, lakes and oceans

2 flooded areas

View from above

Image Intensity
Watershed Algorithm:

… mountains, lakes and oceans

More rain
  =
Increase
“threshold”
Watershed Algorithm:

... mountains, lakes and oceans

One flooded area

A and B merge
Watershed Algorithm:

... mountains, lakes and oceans

Make a “Dam” at the “Watershed line”
Watershed - to find object number

Sometimes there are just too many to count by hand ...
Watershed to separate touching objects

- Euclidian Distance Map
- Ultimate Eroded Points
- Fill with water from UEP until hits edge of object, or dams between objects
Practical Session 2d

Getting to know “Fiji” better –
Fiji is just ImageJ (Batteries included)
http://pacific.mpi-cbg.de

File - Open Samples - Blobs

Watershed Segmentation and Analysis

 ✓ Invert, Make Binary, Watershed, Threshold, Analyze Particles: Separate and measure touching objects

 ✓ Search the Wiki for NucleiWatershedSegmentation tutorials
### Standard Text Book:

*Digital Image Processing 2nd Ed., Gonzalez and Woods, Prentice Hall*

### Fiji and ImageJ:

- **Fiji Wiki and docs**: [http://pacific.mpi-cbg.de](http://pacific.mpi-cbg.de) (also: Installation)
- **MacBioPhotonics plugins collection for microscopy**: [http://www.macbiophotonics.ca/downloads.htm](http://www.macbiophotonics.ca/downloads.htm)

### Image Processing Facility

- **Intranet** - Services and Facilities - Image Processing Facility
- **Wiki** - info for beginners - tips - software documentation:
  

### Imaging Facility Network (IFN):

[https://ifn.mpi-cbg.de](https://ifn.mpi-cbg.de)

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**Email**: ipf(at)mpi-cbg.de
The Fourier transform

The Fourier transform is a way to obtain a new representation of the data (a bit like the 2D histogram from earlier).

It is best suited for data with repetitive patterns, as it highlights those.

And … don’t worry about the maths for now…
The Fourier transform

Bird song

**Detail of the signal:**
Delay between peaks: ~ 0.35 ms

**FFT of this looks like:**
Peak in FFT: ~ 3 kHz

Equivalence: spatial domain vs. Fourier or Freq. domain

\[
\frac{1}{3000} \approx 0.33 \text{ ms}
\]

Peak in FFT gives frequency or periodicity of pattern

Like iTunes frequency spectrum
The Fourier transform – in 2D images

Real image

Central point: non-varying part of the image (mean)

Pattern of points:
always symmetrical!
further = smaller
higher freq = smaller object

FFT (zoomed)

Angle of pattern gives pattern orientation

Diffraction pattern?

FFT (zoomed)
The Fourier transform – in 2D images

Real images… are rarely that clear

S. pombe cells (Tolic lab)

FFT
The inverse Fourier transform

Fourier image and real image contain same information → so it’s possible to reverse the process:

Before: [Image 1]  
After: [Image 2]  
Changed her mind: [Image 3]

Same thing happens physically in a microscope. FT image is in the Back Focal Plane of Objective!
Can use as a filter for detail:

Low frequency pass

High frequency pass
... a filter for periodic noise:

The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.
... a filter for periodic noise:

Laser intensity noise from a bad AOTF... can be removed by frequency filtering in the correct spatial direction.
... during “Deconvolution”:

Take Image and PSF image
+ Do Fourier transforms
+ Image FT / PSF FT
+ Reverse FT of result
=

Deconvolved image with much improved contrast and less out of focus signal.

A metaphase human cell stained for DNA (red), centromeres (blue) and the anaphase promoting complex/cyclosome (green). Recorded by Claire Acquaviva, Pines lab.

Left part: original data
Right part: deconvolved with Huygens Professional.