Introduction to FIJI - workflows v. 1.9

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Contents

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1 working with single channel images

1.1 opening the image and basic info

1. file > open samples > HeLa (IJ:26.2)
2. image > show info (ctrl-i) (IJ:28.3)
3. analyze > tools > scale bar (IJ:30.14.6)

1.2 opening the image and basic drawing

1. **target:** “convert” [geometry-source.tif] into [geometry-target.tif]
2. file > open : [geometry-source.tif] (IJ:26.2)
3. use following tools to convert source into target:
   (a) [palete > straight line] (IJ:19.12.1)
   - try shift

(b) [palete > color picker] (IJ:19.11)
- e.g. choose black as foreground
- try Draw (d) (IJ:27.9-10)

(c) [palete > flood fill tool] (IJ:19.16)
- use again: color or picker to choose right color for filling

(d) [palete > wand tracking tool] (IJ:19.7)
- use to select shapes
- apply Copy (ctrl-c), Paste (ctrl-v), Cut (ctrl-x)
- alternatively select, move selection, choose color and try Fill (f), Draw (d), Clear tools (IJ:27.2 IJ:27.9-10)

(e) [palete > rectangular selection] (IJ:19.1)
i. select a rectangular shape around blue rectangle
ii. apply Copy (ctrl-c), Paste (ctrl-v)

1.3 image inspection

1. open the “single_channel.tif” image (drag & drop, or File > Open)
2. Image > Duplicate (IJ:28.9)
   - shortcut: D (shift-d)
3. Analyze > Histogram (IJ:30.10)
   - Shortcut: h
4. click “live”
5. Image > Adjust > Brightness/Contrast
   (a) play with sliders and Auto and Set and Reset
   (b) observe histogram window–what is changing, what is not?
   (c) click Apply
   (d) what changed?

1.4 adjust brightness/contrast of all open images

1. open three single-channel images, in folder “for_exercise_1.4” (drag & drop, or File > Open)
2. for all images: Analyze > Histogram (IJ:30.10) (Shortcut: h)
   - click “live”
3. for one image: Image > Adjust > Brightness/Contrast

4. adjust contrast with sliders or Auto
5. click Set
6. check “Propagate to all other open images”-box
7. click OK
8. what happened to the histograms?
9. what happened to the images?
10. did pixel values change?
11. pick another image and repeat steps 4–7
12. what happened, what is different, is it better/worse?

1.5 copy pasting from image to image

1. **target:** create a figure illustrating the difference in C. elegans phenotype (see elegance-fig.tif) based on elegans.tif example
2. file > new > image (IJ:26.1)
   - Type: 8bit
   - Slices: 1
   - Fill with: black
3. file > save as > tif : my-elegance-fig.tif (IJ:26.10.1)
4. [palete > color picker] (IJ:19.11)
   - choose white as foreground and black as background
5. file > open : [elegance.tif] (IJ:26.2)
6. find two different phenotypes of a worm; for each worm
   - select worm with [palete > rectangular selection] (IJ:19.1)
   - edit > selection > add to manager (ctrl-t) (IJ:27.12.22)
   - analyze > tools > ROI manager
7. prepare the template as in elegance example (see elegance-fig.tif)
   - [palete > straight line] (ctrl-click to select **straight** mode) (IJ:19.2)
     - use line selection to create two horizontal lines (use shift while drawing)
     - create line; Draw (d); move line down; Draw (d); add to selection for future

- [palete > text tool] (IJ:19.8)
  - add text for “GFP” and “Brightfield”
  - confirm with ctrl-d (or edit > draw)
- use ROI manager to put ROIs into right places (analogous to elegance-fig.tif); for each ROI from original image:
  - paste ROI into new image
  - move it to desired location
  - add it to the ROI manager

8. copy paste selected worms to the new images (two channel per worm)
   - use ROI to find a worm position
   - choose channel: GFP or Brightfield
   - copy selection
   - choose my-elegance-fig.tif window
   - use ROI to select desired paste location
   - paste
   - repeat procedure for all four images
9. file > save (IJ:26.10.1)

1.6 file handling and non-invasive editing

1. file > open samples > blobs (shift-b) (IJ:26.4)
2. analyze > tools > scale bar (IJ:30.14.6)
   - set color, background, position
   - check: overlay, bold
3. file > save as > tiff : blobs1.tif (IJ:26.10.1)
   - saving the image as tiff keeps the scale bar as an overlay, so pixel-values below it are kept

1.7 file handling and invasive editing

1. file > open : [blobs1.tif] (IJ:26.2)
   - from the previous exercise
2. image > info (i) (IJ:28.3)
3. image > overlay > remove overlay (IJ:28.14.7)
4. analyze > set scale (IJ:30.8)

- set scale such as image is 100 um long in each dimension

5. analyze > tools > scale bar (IJ:30.14.6)
   - set color, background, position
   - check: NO overlay
6. file > save as > tiff : blobs2.tif (IJ:26.10.1)
   - load again to check the difference (e.g. check info)

1.8 LUT and calibration bar

1. **target:** verrify that images “looking” alike might be different
2. file > open samples > blobs (shift-b) (IJ:26.4)
3. inspect pixel values (IJ:p.28:Toolbar)
4. image > lookup table > invert LUT (IJ:28.15.1)
5. change color lookup table
   - Image > Look up table > Fire
6. analyze > tools > calibration bar (IJ:30.14.7)
7. file > open > [blobs16bit.tif] (IJ:26.2)
8. change color lookup table
   - Image > Look up table > Fire
9. analyze > tools > calibration bar (IJ:30.14.7)

1.9 8-bit and 16-bit pseudocolor images

1. file > open samples > blobs (shift-b) (IJ:26.4)
2. inspect pixel values (IJ:p.28:Toolbar)
3. image > lookup table > invert LUT (IJ:28.15.1)
4. analyze > tools > calibration bar (IJ:30.14.7)
5. file > open > [blobs16bit.tif] (IJ:26.2)
6. inspect pixel values (IJ:p.28:Toolbar)
   - alternatively use Pixel Inspector (IJ:19.20)
7. analyze > tools > calibration bar (IJ:30.14.7)

8. file > open samples > blobs (shift-b) (IJ:26.4)
9. add arrow in overlay (IJ:19.13)
    - play with the look of the arrow (i.e. colors, thickness)
    - indicate a blob which you like
    - confirm by ctrl-b (IJ:19.8)
10. add arrow in draw (IJ:19.13)
    - confirm by ctrl-d (IJ:19.8)
11. mark a part of image with overlay brush (IJ:19.18)
    - play with transparency
    - cover blobs marked by arrows with red paint

1.10 pseudocolor image to RGB conversion

1. file > open samples > blobs (shift-b) (IJ:26.4)
2. image > duplicate (IJ:28.9)
3. rename images: left and right
    - image > rename (IJ:28.10)
4. change color lookup table for left and right
    - Image > Look up table > Fire | green
5. duplicate both images (IJ:28.9)
    - name them left-1 and right-1
6. image > stack > tools > combine (IJ:28.6.15.1)
    - select left and right
7. select left-1
    - image > type > Color RGB (IJ:7)
    - check with picker what are the values in pixels (IJ:p.28:Toolbar)
8. select left-2
    - image > type > Color RGB (IJ:7)
9. image > stack > tools > combine (IJ:28.6.15.1)
    - select left-1 and right-1

1.11 color spaces

1. file > open : [blob-combined.tif] (IJ:26.2)
2. plugins > Color Inspector 3D
3. switch “Display mode” to histogram
4. play with color: rotation | saturation | brightness
    - compare RGB space with e.g. LAB

1.12 line-tool operations

goal: familiarize you with manual histogram adjustment and some built in rutines making this task easier

1. file > open samples > blobs (shift-b) (IJ:26.4)
2. image > lookup tables > invert LUT (IJ:28.15.1)
3. analyze > histogram (IJ:30.10)
    - mark live
4. select line tool (IJ:19.2) (IJ:p.28:Interface)
5. analyze > plot profile (IJ:30.11)
    - mark Live
    - move line
    - double click on line icon
    - change line thickness (move line)
6. edit > selection > select none
7. image > adjust > brightness/contrast (IJ:28.2.1)
    - play with settings to achieve white blobs and black background (actually you almost thresholded image)
    - apply when finished (note: pixel values are altered)

1.13 histogram based intensity transformations

1. file > open samples > M51 galaxy (IJ:26.4)
2. (2x) image > duplicate (IJ:28.9)
3. (3x) analyze > histogram (IJ:30.10)

- mark live
- check log button

4. on each image
   - process > enhance contrast (IJ:29.5)
     - normalize checked
   - process > enhance contrast (IJ:29.5)
     - equalize checked
   - process > enhance local contrast (CLAHE)
     - `http://fiji.sc/wiki/index.php/Enhance_Local_Contrast_(CLAHE)`
     - try different setting (block size ~30, histogram bins ~200, max slope: ~30)
     - what settings brings the result close to the (global) histogram equalization?
     - what happens at small block sizes and large max slopes? why?
5. compare the results

1.14 histogram adjustments

1. **target:** familiarize with manual histogram adjustment and HiLo LUT
2. file > open > [dudes.jpg] (IJ:26.2)
3. analyze > histogram (IJ:30.10)
   - mark live
4. image > lookup tables > invert LUT (IJ:28.15.1)
5. image > adjust > brightness/contrast (IJ:28.2.1)
   - play with settings to increase contrast in the image (actually you almost thresholded image)
   - apply when finished (note: pixel values are altered; see histogram)
6. process > enhance contrast (IJ:29.5)
   - equalize un-checked
   - normalize un-checked
7. What is happening when saturated pixels is equal to 5%?

1.15 using 16 bit images to increase precision

1. file > open samples > M51 galaxy (IJ:26.4)
2. image > lookup tables > fire (IJ:28.15)
3. image > duplicate (IJ:28.9)
4. (2x) analyze > histogram (IJ:30.10)
    - mark live
    - check log button
5. on first image:
    - image > type > 8 bit
    - process > enhance contrast (IJ:29.5)
        - equalize checked
6. on second image:
    - process > enhance contrast (IJ:29.5)
        - equalize checked
7. for both img.: image > lookup tables > fire (IJ:28.15)
8. select center of the galaxy with [palete > rectangular selection] (IJ:19.1)
9. edit > selection > add to manager (ctrl-t) (IJ:27.12.22)
    - analyze > tools > ROI manager
    - switch window to second image
    - click ROI you just added that it appears in the second image
10. analyze > set measurements (IJ:30.7)
    - check: mean gray value / standard deviation
11. for both img.: analyze > measure (m) (IJ:29.12.1)
12. for both img.: image > lookup tables > glasbey (IJ:28.15)
13. compare the results

2 working with multichannel images

2.1 create new image

1. **target:** create a sketch of a cell as in [cell.tif]
2. file > new > image (IJ:26.1)
    - Type: 8bit
    - Slices: 2
    - Fill with: black
3. [palete > brush] (IJ:19.4)
    - right click on the [palete > brush]
        - uncheck: “Paint in overlay”
4. [palete > color picker] (IJ:19.11)
    - choose red as foreground
5. Draw an outline of a cell ;)
6. image > color > channel tool (ctrl-z) (IJ:28.5.3)
    - make composite (IJ:28.5.5)
7. Switch a slice with a slider
8. [palete > color picker] (IJ:19.11)
    - choose blue as foreground
9. Draw cell nuclei
10. [palete > brush] (IJ:19.4)
    - right click on the [palete > brush]
        - check: “Paint in overlay”
11. [palete > color picker] (IJ:19.11)
    - choose blue as foreground
12. Draw cell vesicles
13. Inspect pixel values (IJ:p.28:Toolbar)
    - alternatively use Pixel Inspector (IJ:19.20)
    - switch a slice with a slider
    - move inspector between: outline | nuclei | vesicles

14. image > color > channel tool (ctrl-z) (IJ:28.5.3)
    - switch between composite | color | grey
15. file > save as > tif : my-cell.tif (IJ:26.10.1)

2.2 composite images - splitting and merging

1. file > open samples > fluorescent cells (IJ:26.4)
2. image > color > arrange channels
    - click on New 1, and select magenta
3. image > color > split channels (IJ:28.5.1)
4. merge channels to composite
    - image > color > merge channels (IJ:28.5.2)
    - check “create composite” box (IJ:28.5.2)
5. LUT
    - change LUT of the channels (LUT in the “startup tools”)
6. color blindness
    - image > color > simulate color blindness
    - image > color > dichromacy

2.3 composite images - individual channel corrections

goal: create an image with brightfield / red / cyan channels which shows locations with strongest expression of these fluorophores

1. file > open samples > neuron (IJ:26.4)
2. image > color > channel tool (IJ:28.7.5)
3. for each channel
    - adjust contrast such as final image “conveys the message” (IJ:28.2.1)
    - Make sure to select the same channel in the channel tool and with the slider in the image window otherwise you may be surprised that adjusting has no visible effect
4. Save as jpg or Image > Type RGB color to remove other channels

2.4 microscopy stacks handling

goal: make a video and a picture for publication showing progression of mitosis in time (see: mitosis-montage.tif)

1. open : [mitosis-mixedStack.tif] (IJ:26.2)
2. image > hyperstacks > reorder hyperstacks
    - swap z with t (it is to overcome issues with make montages)
3. image > stacks > tools > make substack (IJ:28.6.15.7)
    - choose only one z slice e.g. 3rd one
4. image > duplicate (IJ:28.9)
    - check duplicate hyperstack
5. image > color > channels tool (shift-z) (IJ:28.7.5)
    - split channels (IJ:28.5.1)
6. for both C1 and C2 images
    - choose LUT Fire (IJ:19.7)
    - create channel label (IJ:19.8)
    - image > type > color RGB (IJ:7)
7. for original two channel image
    - image > type > color RGB (IJ:7)
8. image > stacks > tools > combine (IJ:28.6.15.1)
    - choose left: 2 channel image
    - choose right: C1
9. image > stacks > tools > combine (IJ:28.6.15.1)
    - choose left: merged image
    - choose right: C2
10. image > stacks > series labeler
    - select time and other options; use preview to peek
11. file > save as > avi (IJ:26.10.1)
    - e.g. 10 frames per sec.
12. image > stacks > make montage (IJ:28.6.8)

- play with settings:
  - columns 1
  - rows 5
  - increments 12

13. file > save as > tiff (IJ:26.10.1)

3 image processing: thresholding & filters

3.1 basic concept of thresholding

target: Threshold blob.gif image to create a mask enabling segmentation of blob-like structures (i.e. create an image where the values of all pixels not belonging to blob-like structures are set to 0, and all other pixel values are equal to 255).

1. file > open samples > blobs (IJ:26.4)
2. image > lookup table > invert LUT (IJ:28.15.1)
3. image > duplicate (IJ:28.9)
4. image > adjust > threshold (IJ:28.2.4)
   - set up sliders and Dark background checkbox and threshold the image:
   - use 126 value as threshold
5. note: pixel values are altered
6. (optional) image > adjust > auto threshold

3.2 basic concept of filtering: binary filters

goal: see different binary operations in action

1. open : [blobs-thr.tif] (IJ:26.2)
2. image > duplicate (IJ:28.9)
3. process > binary > watershed (IJ:29.8.12)
4. process > noise > remove outliers (IJ:29.6.5)
   - check preview
   - list bright
5. (process > find maxima) (IJ:29.4)

6. (2x) process > binary > erode (IJ:29.8.3)
7. process > find edges (IJ:29.3)
8. process > binary > fill holes (IJ:29.8.8)

3.3 basic concept of filters: sharpen

1. file > open samples > hela cells (IJ:26.4)
2. process > filters > unsharp mask (IJ:29.11.8)
    - check how it behaves when image is composite / color (use channel tool for this purpose) (IJ:28.7.5)

3.4 basics mathematical operations on single image

1. open : [f2.tif] (IJ:26.2)
2. process > math > add (IJ:29.9.1)
    - play with different functions
    - check what happens when image is 32 bit

3.5 beyond the limits of bits

1. **target:** Perform consecutive addition and subtraction of the same value from an image. Compare the results with original image.
2. open : [spooked_16bit.tif] (IJ:29.9.1)
3. image > duplicate (IJ:28.9)
    - work on the copy
4. process > math > add (IJ:29.9.1)
    - add value: 600
5. process > math > subtract (IJ:29.9.2)
    - subtract value: 600
6. comapare the original and the copy: are they the same? What has happened?

3.6 basics mathematical operations on two images

1. open : [f2.tif] and [f1.tif] (IJ:26.2)
2. process > calculator (IJ:29.13)
    - start with add
    - check what happens when image is 32 bit
    - play with different functions

3.7 seeing JPEG artifacts

target: Save the same image in TIFF and JPEG formats. Compare the differences

1. file > open : [tulip.tif] (IJ:26.2)
2. image > duplicate (IJ:28.9)
    - work on the copy
3. file > save as > jpg (IJ:26.10.3)
    - tulip.jpg
4. [palete > pencil] (IJ:19.19)
    - modify value of only one pixel
5. file > save as > jpg (IJ:26.10.3)
    - tulip-1px.jpg
6. close all jpeg files and reopen them
7. process > calculator (IJ:29.13)
    - image1: tulip.tif
    - operation: subtract
    - image2: tulip.jpg
    - checked: create new window
    - checked: 32 bit result
8. process > calculator (IJ:29.13)
    - image1: tulip-1px.jpg
    - operation: subtract
    - image2: tulip.jpg
    - checked: create new window

- checked: 32 bit result

9. **questions:**
    - What are the differences between images tif and jpg images? Why?
    - How many pixels are affected by changing only 1 pixel in jpg image? Why?

4 background elimination

4.1 dividing by background image

1. **target:** Estimate the local ratio of increase by dividing the image by background. Check the impact of 32-bit image conversion on the quatlity of the result.
2. file > open : [xxx.tif] (IJ:26.2)
3. file > open : [xxx_background.tif] (IJ:26.2)
4. process > calculator (IJ:29.13)
    - image1: xxx.tif
    - operation: subtract
    - image2: xxx_background.tif
    - checked: create new window
    - checked: 32 bit result
5. process > calculator (IJ:29.13)
    - image1: xxx.tif
    - operation: subtract
    - image2: xxx_background.tif
    - checked: create new window
    - unchecked: 32 bit result
6. **question:** what is the reason of posterization ?

4.2 background elimination - flat field correction

1. file > open > cell colony (IJ:26.4)
2. use selection to draw a horizontal line across the image (IJ:19.2)
3. analyze > plot profile

- check live

4. process > subtract background
    - click: preview
    - click: create background
    - vary: rolling ball radius
    - try: sliding paraboloid

4.3 background elimination - flat field correction using Image calculator

1. file > open samples > cell colony (IJ:26.4)
2. image > duplicate (IJ:28.9)
3. process > filters > gaussian blur % sigma ~= 30 (IJ:29.11.2)
4. measure mean of blurred image (select it, “a”, “m”) (IJ:29.12.1)
5. process > calculator plus > divide (i1 = image, i2 = blurred image, k1 = mean, k2 = 0)

5 manual measurements and working with rois

5.1 measuring fluorescence within a selection

1. task : measure average flu. in Red channel in neuron
2. file > open samples > neuron (IJ:26.4)
3. image > color > channel tool (IJ:28.7.5)
    - split channels (IJ:28.5.1)
4. close all but green and red
5. work on green image
    - image > duplicate (IJ:28.9)
    - process > filters > gaussian blur (IJ:29.11.2)
        - use preview to set parameters
    - image > adjust > threshold (IJ:28.2.4)
        - threshold to create neuron mask (avoid false negatives)
    - use wand tool to select main part of the neuron (IJ:19.7)
    - analyze > tools > roi manager (IJ:30.14.5)

    - roi manager > add (t) (IJ:27.12.22)

6. choose second copy of green
    - image > adjust > threshold (IJ:28.2.4)
        - threshold to create neuron mask (avoid false negatives)
    - edit > selection > create selection (IJ:27.12.11)
    - roi manager > add (t) (IJ:27.12.22)
    - edit > selection > select none (ctrl-shift-a) (IJ:27.12.2)
    - process > noise > remove outliers (IJ:29.6.5)
        - use preview; remove some of the outliers outside of neuron
    - edit > selection > create selection (IJ:27.12.11)
    - roi manager > add (t) (IJ:27.12.22)
7. analyze > set measurements (IJ:30.7)
    - check: area / area fraction / mean gray value
8. work on red image
    - choose multi point tool (IJ:19.5)
        - select some points in the neuron
        - edit > selection > enlarge (IJ:27.12.14)
        - roi manager > add (t) (IJ:27.12.22)
9. for each selection
    - analyze > measure (m) (IJ:29.12.1)
10. roi manager > more > save selection (IJ:30.14.5)

5.2 measuring geometrical properties in the image

1. task : measure average flu. in Red channel in neuron
2. file > open samples > neuron (IJ:26.4)
3. use polygon selection tool to measure cell body area (IJ:19.1.6)
    - use measure to get the read out after creating polygon (IJ:29.12.1)
        - roi manager > add (t) (IJ:27.12.22)
4. use segmented line tool (IJ:19.2.2) to measure length of few dendrites
    - test shift and alt while adding points (with mouse over a point)
    - use measure to get the read out after creating a line (IJ:29.12.1)

- roi manager > add (t) (IJ:27.12.22)

5. use angle tool (IJ:19.2.2) to measure length of few dendrits
   - use measure to get the read out after creating an angle (IJ:29.12.1)
   - roi manager > add (t) (IJ:27.12.22)
6. roi manager > more > save selection (IJ:30.14.5)

6 automatic measurements

6.1 identifying and measuring objects - basics

1. open : [blobs-thr.tif] (IJ:26.2)
2. image > duplicate (IJ:28.9)
3. process > binary > watershed (IJ:29.8.12)
4. process > noise > remove outliers (IJ:29.6.5)
   - check preview
5. analyze > set measurements (IJ:30.7)
   - check: area / area fraction / mean gray value
6. analyze > analyze particles (IJ:30.2)
   - test different options

6.2 identifying and measuring objects - cells #1

1. **target:** measure distribution of RFP signal inside nucleus across cell population
2. open : [hela1.tif] (IJ:26.2)
3. image > adjust > threshold (IJ:28.2.4)
   - test different option to isolate cells
4. **question:** What are the difficulties?

6.3 identifying and measuring objects - cells #2

1. **target:** measure distribution of RFP signal inside nucleus across cell population
2. open : [hela2.tif] (IJ:26.2)
3. image > color > split channel (IJ:28.5.1)
4. work on blue channel (DAPI)
    - image > adjust > threshold (IJ:28.2.4)
    - process > binary > watershed (IJ:29.8.12)
    - process > noise > remove outliers (IJ:29.6.5)
        - check preview
5. analyze > set measurements (IJ:30.7)
    - check: area / area fraction / mean gray value
    - redirect to: RFP
6. analyze > analyze particles (IJ:30.2)
7. analyze > distribution (IJ:30.4)
    - choose: gray value

7 Segmentation

7.1 DAPI segmentation with thresholding

1. open `hela-1.tif` (single-channel image)
2. change LUT to Grays
3. `Image > Adjust > Threshold`
4. understand the function of the `Dark Background` checkbox (inspect pixel values)
5. try setting sliders manually
6. try different algorithms
    - `Image > Adjust > Auto Threshold`, if you want to see all at the same time
7. try different display options (`Red`, `B&W`, `Over/Under`)—do you understand what they show

8. when happy with result, click `Apply`
9. save the resulting binary image (`File > Save As > Tiff...`) with name “myThresholdHeLa.tif”
10. proceed with `Analyze > Analyze Particles` etc
11. bonus: use morphological operations to clean up results, *before* analyzing particles
12. bonus: smooth, e.g. with Gaussian or median filter, the image *before* thresholding

7.2 DAPI segmentation with Weka

1. open `hela-1.tif` (single-channel image)
2. change LUT to Grays
3. `Plugins > Segmentation > Trainable Weka Segmentation`
4. draw a line outside a nucleus
5. click `Add to class 1`
6. draw a line inside a nucleus
7. click `Add to class 2`
8. click `Train classifier`
9. repeat the last four steps until happy with result
10. click `Get probability`
11. inspect the probability maps, do you understand the meaning of the values of the pixels in the different channels?
12. click `Create result`
13. inspect results, do you understand the meaning of the values of the pixels in the different channels?
    (a) you now have a binary image, but not the kind Fiji likes
    (b) to measure, proceed by thresholding (“set” both threshold values to 1), then Analyze Particles, etc
    (c) alternatively, multiply all values in the Weka output image by 255, then apply Binarize, etc
14. save the resulting image (`File > Save As > Tiff...`) with name “myWekaHeLa1.tif”
15. Bonus round: play with Settings

7.3 DAPI double-segmentation with Weka

1. open hela-1.tif (single-channel image)
2. change LUT to Grays
3. `Process > Enhance Contrast > Histogram Equalization`
4. `Plugins > Segmentation > Trainable Weka Segmentation`
5. draw a line outside the cells
6. click `Add to class 1`
7. draw a line inside a nucleus
8. click `Add to class 2`
9. draw a line inside the cytoplams
10. click `Add to class 3`
11. click `Train classifier`
12. repeat the last six steps until happy with result
13. inspect the probability maps, do you understand the meaning of the values of the pixels in the different channels?
14. click `Create result`
15. inspect results, do you understand the meaning of the values of the pixels in the different channels?
    - you now have an image with three values
    - to measure, proceed by thresholding at 0, 1, and 2, to extract each class (use Set and then set both thresholds to 0, 1, or 2)
    - then proceed with Analyze Particles, etc for each of the classes of interest (nuclei and cytoplasm)
16. Bonus round: play with Settings

7.4 DAPI segmentation with StarDist

1. Follow StarDist installation instructions from here
    - `https://imagej.net/plugins/stardist`
2. open `hela-1.tif` (single-channel image)
3. change LUT to Grays

4. `Plugins > StarDist > StarDist 2D`
5. `Model: Versatile (fluorescent nuclei)`
6. click `OK` and wait
7. after result shows
    - select original image
    - select `ROI Manager`
    - check the `Show All` box
8. comment on the result, is it good/bad?

7.5 H&E segmentation with StarDist

1. open `H&E_retina.jpg` (RGB image)
2. `Plugins > StarDist > StarDist 2D`
3. `Model: Versatile (H&E nuclei)`
4. click `OK` and wait
5. after result shows
    - select original image
    - select `ROI Manager`
    - check the `Show All` box
6. comment on the result, is it good/bad?
7. duplicate original image
8. re-scale it by factor of 0.5 in x and y (Image > Scale)
9. apply StarDist again and overlay the ROIs as above
10. is there a difference? why?

8 Spot detection

8.1 manual spot detection with the Multi-point Tool (IJ:19.6)

1. read all the next steps before beginning
2. open `spot_detection/beads_001.tif`
3. right-click (ctrl-click) on the `Point Tool` in the tool bar and select `Multi Tool`

4. double-click on the `Point Tool` in the tool-bar and customize to your liking (test on image, clear points by shift-a)
5. count all beads by clicking on them one by one
6. how long did it take you to click on all the beads (measure with stopwatch the time it took to complete the previous step)?
7. save results to the `ROI Manager` (t) then to file (More » Save . . .)
8. click `measure` in the `ROI Manager` to get number of spots

8.2 algorithmic spot detection with Find Maxima (IJ:29.4)

Forum post about Find Maxima

1. open `spot_detection/beads_001.tif`
2. `Process > Find Maxima ...`
3. check `Preview point selection`
4. try different values for `Prominence` and the three check-boxes
5. try each of the possibilities in the pull-down (remember to click OK to apply your selection)
   - what is the difference and what could the different outputs be used for?
   - tip: shift-u allows you to pin sub-menus of the control panel for quick selection, e.g. the Process menu
6. add you detected maxima (`Point Selection`) to the `ROI Manager` and save to file
7. load saved ROIs from the manual detection exercise and compare results
   - how many beads do you get and how does the number compare to your manual count?

8.3 automatic spot segmentation with thresholding

1. open `spot_detection/beads_001.tif`
2. `Image > Adjust > Threshold ...`
   - select `Otsu`, then apply
3. `Analyze > Set Measurements ...`

```
+ [X] Area
+ [X] Mean gray value
+ [X] Display label
```

4. `Analyze > Analyze Particles ...`

```
+ Show: Overlay Masks
+ [X] Display results
+ [X] Clear Results
+ [X] Summarize
+ [X] Add to Manager
```

5. save ROIs to file
6. Compare results to the previous two approaches
   - Do they differ significantly from each other?
   - Which performed better?

8.4 spot detection with noise

1. Repeat 8.2 with `with_noise_8000.tif`
   - consider smoothing the image first:
     - `Process > Smooth` (this is a 3x3 mean filter)
2. Repeat 8.3 with `with_noise_8000.tif`
   - consider smoothing the image first:
     - `Process > Filters`
     - then pick one
3. Compare and comment on the performance of the two methods
   - Which one is better?
   - Why/how is it better?

8.5 spot detection with variable background

1. Repeat 8.2 with `beads_001_ramp.tif`
2. Repeat 8.3 with `beads_001_ramp.tif`
3. Compare and comment on the performance of the two methods
   - Which one is better?
   - Why/how is it better?
4. What could be done to the image to make thresholding work better?

9 scripting / macros / automation basics

9.1 macro recorder - reproducing edits

1. **target:** save the edit chain for later (to save work and to document parameters used by different filters)
2. plugins > macro > recorder (IJ:31.1.4)
3. file > open samples > mri-stack (IJ:26.4)
4. process > filters > gaussian blur with sigma ~= 2 (IJ:29.11.2)
5. image > adjust > threshold (IJ:28.2.4)
    - select manually threshold such as the head is separated from background
        - ignore small holes
        - uncheck box: calculate threshold for each image
6. process > binary > fill holes (IJ:29.8.8)
7. (optional) use analyze > set scale to calibrate the measurement units
    - 200 pixels is 25 cm
8. **Question:** What are the benefits of storing such a macro?

9.2 macro recorder - repeating actions on stack

1. **target:** calculate the volume of human skull
2. file > open samples > mri-stack (IJ:26.4)
    - use steps below or macro developed in previous excercise
3. process > filters > gaussian blur % sigma ~= 2 (IJ:29.11.2)
4. image > adjust > threshold (IJ:28.2.4)
    - select manually threshold such as the head is separated from background
        - ignore small holes
5. process > binary > fill holes (IJ:29.8.8)
6. (optional) use set scale to calibrate the measurement units
    - 200 pixels is 25 cm
7. plugins > macro > recorder (IJ:31.1.4)

8. analyse > measure (IJ:29.12.1)
9. image > stacks > next slice (IJ:28.6.3)
10. in recorder click `create` button
11. use copy paste to execute the same action many times:

```
run("Next Slice [>]");
run("Measure");
run("Next Slice [>]");
run("Measure");
run("Next Slice [>]");
run("Measure");
run("Next Slice [>]");
run("Measure");
```

12. **Question:** how to compute volume of human skull based on the measurement?

9.3 automatic iteration

1. **target:** avoid copy pasting - use iteration instead
2. continue from last example or open preprocessed file
   - file > open > [mri-stack-binary] (IJ:26.4)
3. make sure that the code looks in the following way:

```
for (currentStep=0; currentStep<100;currentStep++){
  run("Next Slice [>]");
  run("Measure");
}
```

4. **Question:** what is the issue with this approach?

9.4 automatic iteration - introducing function for stop condition

1. **target:** avoid fixed stop condition and exchange it by more accurate mechanism
2. continue from last example or open preprocessed file
   - file > open > [mri-stack-binary] (IJ:26.4)
3. make sure that the code looks in the following way:

```
for (currentStep=1; currentStep<=nSlices();currentStep++){
  setSlice(currentStep);
  run("Measure");
}
```

9.5 adding macro to the menu

1. **target:** add macro to the menu
2. continue from last example or open preprocessed file
   - file > open > [mri-stack-binary] (IJ:26.4)
3. switch to code editor and make sure that the code looks in the following way:

```
macro "measure stack" {
  for (currentStep=1; currentStep<=nSlices();currentStep++){
    setSlice(currentStep);
    run("Measure");
  }
}
```

4. language > ImageJ Macro
5. save > .ijm
6. plugins > macros > install... (IJ:31.1.1)
   - select the macro you just saved
7. (optional) test following FIJI tool
   - image > stacks > plot z axis profile (IJ:28.6.13)

9.6 iterations and variables in the macro: multi-measurement

1. **target:** prepare the evenly distributed selection over the image v.1 This can be used e.g. in FRAP experiment analysis.
2. open : [frap.tif] (IJ:26.2)
3. test code:

```
// initialization
x = 20;
y = 20;

// iterations
```

```
for (nbr_x=0;nbr_x<5;nbr_x++){
  for (nbr_y=0;nbr_y<5;nbr_y++){
    makePoint(x+x*nbr_x, y+y*nbr_y);
    run("Enlarge...", "enlarge=5 pixel");
    roiManager("Add");
  }
}
```

9.7 automatic measurement and saving to a file

1. **target:** prepare the evenly distributed selection over the image v.2
2. test code:

```
// initialization
roiManager("reset");
x = 220;
y = 110;
nbr_sensors = 4;
delta = 12;

// iterations to create selection
for (nbr_x=0;nbr_x<nbr_sensors;nbr_x++){
  for (nbr_y=0;nbr_y<nbr_sensors;nbr_y++){
    makePoint(x+delta*nbr_x, y+delta*nbr_y);
    run("Enlarge...", "enlarge=5 pixel");
    roiManager("Add");
  }
}

// measurement and save results
roiManager("Multi Measure");
fn = getInfo("image.filename");
saveAs("Results", "/Users/sstoma/Desktop/Results-"+ fn +".txt");
// exchange previous line with your path
```

9.8 user input via GUI

1. **target:** prepare the evenly distributed selection over the image v.3
2. test code:

```
// initialization
roiManager("reset");
x = 20;
y = 20;
```

```
nbr_sensors = 4;
delta = 12;

// GUI
Dialog.create("Please specify parameters:");
Dialog.addNumber("Size [px]: ", 5);
Dialog.show();
size = Dialog.getNumber();

// iterations to create selection
for (nbr_x=0;nbr_x<nbr_sensors;nbr_x++){
  for (nbr_y=0;nbr_y<nbr_sensors;nbr_y++){
    makePoint(x+delta*nbr_x, y+delta*nbr_y);
    run("Enlarge...", "enlarge="+ size +" pixel");
    roiManager("Add");
  }
}

// measurement and save results
roiManager("Multi Measure");
fn = getInfo("image.filename");
saveAs("Results", "/Users/sstoma/Desktop/Results-"+ fn +".txt");
// exchange previous line with your path
```

9.9 user input via image window / selection

1. **target:** prepare the evenly distributed selection over the image - add user selected zone of selection and prepare it to work in the batch mode v.4
2. test code:

```
// initialization
roiManager("reset");
x = 20;
y = 20;
nbr_sensors = 4;
delta = 12;

// GUI
Dialog.create("Please specify parameters:");
Dialog.addNumber("Size [px]: ", 5);
Dialog.show();
size = Dialog.getNumber();

// get the input for location from the image
setBatchMode("show");
```

```
waitForUser("Draw ROI, then hit OK");
getBoundingRect(x, y, width, height)

// iterations to create selection
for (nbr_x=0;nbr_x<nbr_sensors;nbr_x++){
  for (nbr_y=0;nbr_y<nbr_sensors;nbr_y++){
    makePoint(x+delta*nbr_x, y+delta*nbr_y);
    run("Enlarge...", "enlarge="+ size +" pixel");
    roiManager("Add");
  }
}

// measurement and save results
roiManager("Multi Measure");
fn = getInfo("image.filename");
saveAs("Results", "/Users/sstoma/Desktop/Results-"+ fn +".txt");
// exchange previous line with your path
```

9.10 batch mode - basics

1. **target:** Create a macro to segment nuclei in a single frame. Your macro should input a row to the Results containing area for each nuclei in the image. Process all files in the hela folder.
2. open : [hela/hela-1.tif] (IJ:26.2)
3. plugins > macro > recorder (IJ:31.1.4)
4. image > adjust > threshold (IJ:28.2.4)
   - select: triangle
   - check: dark background
5. analyze > analyze particles (IJ:30.2)
6. make sure that the code looks in the following way:

```
// processing
run("Gaussian Blur...", "sigma=2 stack");
setAutoThreshold("Triangle dark");
run("Convert to Mask");
run("Set Measurements...", "area mean display redirect=None decimal=9");
run("Analyze Particles...", "show=[Overlay Masks] display exclude");
```

7. process > batch > macro
   - input: select folder containing [hela-1.tif]-[hela-4.tif]
   - output: select folder out in the folder containing [hela-1.tif]-[hela-4.tif]

8. click process
9. modify to save results

```
// initialization
outPath = "/Users/sstoma/Desktop/materials/images/hela/out/";
// exchange previous line with your path

// processing
run("Gaussian Blur...", "sigma=2 stack");
setAutoThreshold("Triangle dark");
run("Convert to Mask");
run("Set Measurements...", "area mean display redirect=None decimal=9");
run("Analyze Particles...", "show=[Overlay Masks] display exclude");

// saving
saveAs("Results", outPath+"Results.txt");
```

10. modify the code (**target:** save each result in separated file):

```
// initilization
fileName = getInfo("image.filename");
outPath = "/Users/sstoma/Desktop/materials/images/hela/out/";
// exchange previous line with your path

// Process all images finishing with .tif
dir = getDirectory("Choose a Directory where the HeLa tif files are");
dirresults = getDirectory("Choose a Directory where to save results (segment
list = getFileList(dir);

for (imagenumber=0;imagenumber<list.length;imagenumber++)
{
  if (endsWith(list[imagenumber],".tif")){
    open(dir+list[imagenumber]);
    run("Clear Results");
    // processing
    run("Gaussian Blur...", "sigma=2 stack");
    setAutoThreshold("Triangle dark");
    run("Convert to Mask");
    run("Set Measurements...", "area mean display redirect=None decimal=9");
    run("Analyze Particles...", "show=[Overlay Masks] display exclude");

    // saving
    saveAs("Results", outPath + "Results-" + fileName + ".txt");
  }
}
```

9.11 batch mode - full control on processing files

1. **target:** Create a macro to segment nuclei in a single frame. Your macro should input a row to the Results containing area for each nuclei in the image. Process all files in the hela folder. Do not use the batch execution in FIJI - iterate on files in the folder instead.
2. open : [hela/hela-1.tif] (IJ:26.2)
3. plugins > macro > recorder (IJ:31.1.4)
4. image > adjust > threshold (IJ:28.2.4)
   - select: triangle
   - check: dark background
5. analyze > analyze particles (IJ:30.2)
6. make sure that the code looks in the following way:

```
// Process all images finishing with .tif
dir = getDirectory("Choose a Directory where the HeLa tif files are");
outPath = getDirectory("Choose a Directory where to save results (segmentati
list = getFileList(dir);

// in list elements are numbered from 0
for (imagenumber=0;imagenumber<list.length;imagenumber++)
{
  if (endsWith(list[imagenumber],".tif")){
    open(dir+list[imagenumber]);
    run("Clear Results");
    // processing
    run("Gaussian Blur...", "sigma=2 stack");
    setAutoThreshold("Triangle dark");
    run("Convert to Mask");
    run("Set Measurements...", "area mean display redirect=None decimal=9");
    run("Analyze Particles...", "show=[Overlay Masks] display exclude");

    // saving
vfileName = getInfo("image.filename");
    saveAs("Results", outPath + "Results-" + fileName + ".txt");
  }
}
```

10 workflow: tracking

10.1 create image with moving dots

1. **target:** create an image with moving objects (dots)
2. make sure that the code looks in the following way:

```
macro "create_image_with_moving_objects"{
  // initial variables
  nbr_frames = 40; // number of frames in the image
  color1 = 150; // color of first obj.
  color2 = 250; // color of sec. obj.
  canvas_size = 200; // size of the image
  step = 4; // progress in x between frames
  delta = 15; // difference in position between two objects in x
  x = 5; // initial position x of object
  y = 5;  // initial position y of object
  width = 10; // baseline size of object in x
  height = 10; // baseline size of object in y

  // empty image with noise
  newImage("Image", "8-bit black", canvas_size, canvas_size, nbr_frames); //
  run("Salt and Pepper", "stack");

  for(i=1;i<=nbr_frames;i++){
    setSlice(i);
    // first dot
    setColor(color1,color1,color1);
    fillOval(x+i*step, y+i*step, width, height);
    // second dot
    setColor(color2,color2,color2);
    fillOval(delta+x+i*step, canvas_size-(y+i*step), width*2, height*2);
  }
  run("glasbey");
}
```

10.2 tracking: process single image

1. **target:** for **current** frame of the stack find the x, y of the center of the dot
2. make sure that the code looks in the following way

```
macro "tracking_process_single_image"{
  run("Median...", "radius=2 slice");
  setThreshold(0, 100);
```

```
  run("Convert to Mask", "method=Otsu background=Light only");
  run("glasbey");
  run("Find Maxima...", "noise=25 output=List light");
}
```

10.3 tracking: process whole stack

1. **target:** for **all** frames of the stack find the x, y of the center of the dot
2. make sure that the code looks in the following way:

```
macro "tracking_process_stack_v1"{
  r = newArray();
  for (i=1;i<=nSlices();i++){
    setSlice(i);
    run("Median...", "radius=2 slice");
    setThreshold(0, 100);
    run("Convert to Mask", "method=Otsu background=Light only");
    run("glasbey");
    run("Find Maxima...", "noise=25 output=List light");
    run("Next Slice [>]");

  // workaround for Find maxima overwriting results at each step
    for (j=0;j<nResults();j++){
      x = getResult("X", j);
      y = getResult("Y", j);
      print(x, y, i);
      temp = newArray(x, y);
      r = Array.concat(r, temp);
    }
  }
  Array.show( r );
}
```

10.4 tracking: process whole stack - enabling linking of the objects

1. **target:** for **all** frames of the stack find the x, y of the center of the dot as well as some object features (i.e. size, mean grey value).
2. make sure that the code looks in the following way:

```
macro "tracking_process_stack_v2"{
  // clearing previous result and preparing image copy
  run("Clear Results");
  rename("Image");
```

```
  run("Duplicate...", "duplicate");
  rename("orig");
  selectWindow("Image");

  // iterating for each slice in the stack
  for (i=1;i<=nSlices();i++){
    setSlice(i);
    run("Median...", "radius=2 slice"); // removing noise
    setThreshold(0, 100); // hardcoded thr. for image
    run("Convert to Mask", "method=Otsu background=Light only");
    run("glasbey"); // changing LUT to get false collors to better distingui
    run("Set Measurements...", "area mean standard center median skewness ar
    run("Analyze Particles...", "display slice");
    run("Next Slice [>]");
  }
}
```

11 workflow: FRET

11.1 preparation part 1 - image import

1. **target:** open .lif image and prepare it for further editing
2. file > open : [FRET_biosensor.lif] (IJ:26.2)
   - configure the importer
   - disable: all series
3. choose 3rd serie
4. image > stacks > z project (IJ:28.6.11)
5. image > color > split channel (IJ:28.5.1)
6. select blue channel (ch0)
   - file > save as > tif : cfp.tif (IJ:26.10.1)
7. select yellow channel (ch1)
   - file > save as > tif : yfp.tif (IJ:26.10.1)

11.2 preparation part 2 - averaging

1. **target:** prepare images for further editing
2. file > open : [cfp.tif and yfp.tif] (IJ:26.2)

3. select blue channel (cfp.tif)
    - process > filters > gaussian blur % sigma ~= 2 (IJ:29.11.2)
    - file > save as > tif : cfp-smoothed.tif (IJ:26.10.1)
4. select yellow channel (yfp.tif)
    - process > filters > gaussian blur % sigma ~= 2 (IJ:29.11.2)
    - file > save as > tif : yfp-smoothed.tif (IJ:26.10.1)

11.3 preparation part 3 - masks

target: prepare masks

1. file > open : [yfp-smoothed.tif] (IJ:26.2)
2. image > adjust > threshold (IJ:28.2.4)
    (a) test different option to isolate chromatine
3. file > save as > tif : mask.tif (IJ:26.10.1)

11.4 preparation part 4 - ratios

target: prepare image with ratios

1. file > open : [cfp-smoothed.tif and yfp-smoothed.tif] (IJ:26.2)
2. process > calculator plus > divide (i1 = yfp, i2 = cfp, k1 = 1, k2 = 0)
3. file > save as > tif : ratio.tif (IJ:26.10.1)

11.5 analysis - problem: cell population

target: measure the change of signal in ratio.tif image

1. file > open : [ratio.tif] (IJ:26.2)
2. analyze > set measurements (IJ:30.7)
    - check: mean gray value / standard deviation
3. analyze > measure (m) (IJ:29.12.1)
4. move to next time point; repeat;. . .
5. image > stacks > plot z axis profile (IJ:28.6.13)
    - if does not work: image > hyperstacks > re-order hyperstack
        – swap t with z
6. question: what is the problem with results?

11.6 preparation part 5 - cropping

target: prepare image with ratios

1. file > open : [all previously prepared images] (IJ:26.2)
2. choose a cell in transition to anaphase - make sure the field of view keeps only this cell during all time-points
   - [palette > rectangular selection] (IJ:19.1)
   - edit > selection > add to manager (ctrl-t) (IJ:27.12.22)
   - use roi manager to move selection between images
     - analyze > tools > ROI manager
3. image > crop (IJ:28.8)
4. save all files adding “-crop” postfix

11.7 analysis - problem: chromatin change

target: measure the change of signal in ratio-crop.tif image

1. file > open : [ratio-crop.tif] (IJ:26.2)
2. analyze > set measurements (IJ:30.7)
   - check: mean gray value / standard deviation
3. analyze > measure (m) (IJ:29.12.1)
4. move to next time point; repeat;. . .
5. image > stacks > plot z axis profile (IJ:28.6.13)
   - if does not work: image > hyperstacks > re-order hyperstack
     - swap t with z
6. question: what are the issues with results?

11.8 analysis - problem: manual labor

target: measure the change of signal in ratio-crop.tif image limited to chromatin

1. file > open : [mask-crop.tif] (IJ:26.2)
2. [palette > wand selection] (IJ:19.7)
   - add chromatin from current time-point
   - edit > selection > add to manager (ctrl-t) (IJ:27.12.22)

- move to next time-point
- repeat

3. file > open : [ratio-crop.tif] (IJ:26.2)
4. analyze > set measurements (IJ:30.7)
   - check: mean gray value / standard deviation
5. select the right ROI; analyze > measure (m) (IJ:29.12.1)
6. move to next time point; repeat;. . .
7. question: what are the issues with results?

12 workflow: scratch assay

12.1 part 1

1. **target:** estimate the area occupied by cells in single timepoint
2. file > open : [scratch/control-single-frame.tif] (IJ:26.2)
3. image > duplicate
4. process > filters > gaussiab blur
   (a) sigma: 5
5. threshold
   (a) Method: Moments
   (b) Background: Dark
6. image > invert
7. process > binary > Distance Map
8. threshold
   (a) Method: Moments
   (b) Background: Dark
9. select original image
10. image > type > 8-bit
11. image > color > merge channels
    (a) C1: original image (after conversion to 8-bit)
    (b) C4: mask
    (c) Create composite: True
    (d) Keep source images: True

12.2 part 2

1. **target:** estimate the area occupied by cells in each time point (extend part 1 and identify the main problem)
2. file > open : [scratch/control.tif] (IJ:26.2)
3. repeat steps from part 1 on the stack

12.3 part 3

1. **target:** estimate the area occupied by cells in each time point
2. file > open : [scratch/control.tif] (IJ:26.2)
3. image > duplicate
4. image > process > subtract background
   (a) Radius: 30
   (b) Preview: True
5. process > filters > gaussiab blur
   (a) sigma: 5
6. threshold
   (a) Method: Moments
   (b) Background: Dark
7. image > invert
8. process > binary > Distance Map
9. threshold
   (a) Method: Moments
   (b) Background: Dark
10. select original image
11. image > type > 8-bit
12. image > color > merge channels
    (a) C1: original image (after conversion to 8-bit)
    (b) C4: mask
    (c) Create composite: True
    (d) Keep source images: True

13 various useful tools

13.1 installing plugins

1. download plugin from webpage: `http://bigwww.epfl.ch/algorithms/esnake/`
2. unzip, drag and drop to FIJI
3. create new canvas
4. draw two white discs on black background
5. plugins > ESnake
    - target brightness: bright
6. click OK

13.2 using line selection to make a “straighten” image

1. file > open samples > nile bend (IJ:26.4)
2. use selection tool for freehand selection
    - make the line thickness adjusted to cover whole river
3. edit > selection > straighten (IJ:27.12.17)

13.3 using 3D viewer

1. file > open samples > confocal series (IJ:26.4)
2. image > properties > voxel depth x10 % to get decent aspect ratio
3. plugins > 3D viewer
4. add > from image % the resampling factor is a downsampling factor
5. play with displayed colors
6. view > start/stop animation
7. view > change animation settings
8. view > record 360 degree rotation
9. file > save as > avi % try the different compression options uncompressed, jpg, and png

13.4 preparing images for publications

- FigureJ plugin:
  - `http://imagejdocu.tudor.lu/doku.php?id=plugin:utilities:figurej:start#installation_for_fiji`