

Introduction to data analysis in R

Quantifying protein co-localization in fluorescence images using the colocr package

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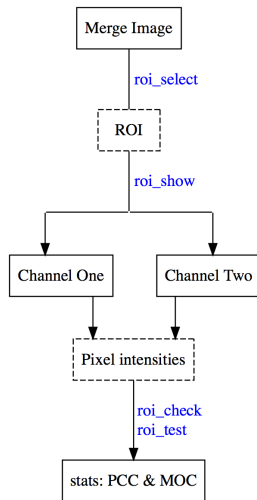
Fluorescence image analysis

A few R packages are available for conducting image analysis, which is a very wide topic.

The colocr package provides a simple straight forward workflow for

1. Loading images
2. Choosing regions of interest
3. Calculating co-localization statistics.

Included in the package, is a web app that can be invoked locally to interactively select the regions of interest in a semi-automatic way.

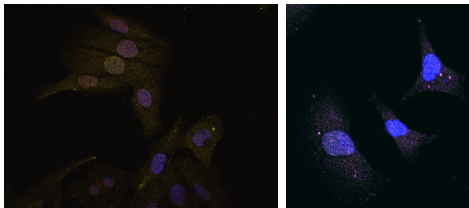


The dataset

The images we will be using come from the DU145 prostate cancer cell line. In this experiment, the cell line was stained with probes for two proteins RKIP and LC3.

The aim of this experiment is to determine, how much of the two proteins are co-localized or co-distributed in the particular cell line.

The files that contain these images are from are named 'image_1.png' and 'image_2.png'.



Loading images in R

We will first learn how to load these images in R and look inside the object to see how R represents images in numbers and pixel coordinates.

```
# load colocr library  
library(colocr)  
  
# load image  
img <- image_load('image_1.png')  
  
# print img object  
img
```

Image. Width: 800 pix Height: 600 pix Depth: 1 Colour channels: 3

This is a four dimensions object of class cimg that contains the image data.

Image representation as numbers

To get a deeper understanding of this object, we can transform it into a data.frame and have a look at it.

```
# transform the object into a data.frame and call str  
str(as.data.frame(img))
```

```
'data.frame':  1440000 obs. of  4 variables:  
 $ x      : int  1 2 3 4 5 6 7 8 9 10 ...  
 $ y      : int  1 1 1 1 1 1 1 1 1 1 ...  
 $ cc     : int  1 1 1 1 1 1 1 1 1 1 ...  
 $ value: num  0.0392 0.0392 0.0392 0.0353 0.0353 ...
```

Here is a breakdown of the dimensions:

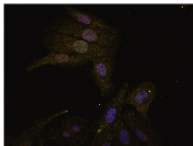
- x and y are the spatial coordinates of the pixels
- cc is the color (channel)
- value is the intensity

Visualizing images

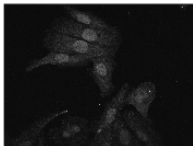
Channels 1, 2 and 3 refer to the red (RKIP), green (LC3) and blue colors (DAPI).

```
# show channels  
library(imager) # to all channel  
plot(img, axes = FALSE, main = 'Merged')  
plot(channel(img, 1), axes = FALSE, main = 'RKIP')  
plot(channel(img, 2), axes = FALSE, main = 'LC3')
```

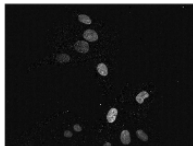
Merged



RKIP



LC3



Selecting regions of interest

To select a region of interest (ROI), we use `roi_select`. By default, the largest contiguous region of the image is selected.

Thresholding means all values below that number are set to 0, and above it to 1.

```
# select the region of interest  
img_rois <- roi_select(img, threshold = 90)  
str(img_rois)
```

```
'cimg' num [1:800, 1:600, 1, 1:3] 0.0392 0.0392 0.0392 0.0353 0.0353 ...  
- attr(*, "label")= num [1:480000] 0 0 0 0 0 0 0 0 0 0 ...
```

The returned `cimg` object contains the original input image and an added attribute called `label` which indicates the 0 (not-selected) or 1 (selected) for each pixel.

Visualizing selected regions

To make sure the selection is appropriately encompassing the ROI, call `roi_show`, which displays

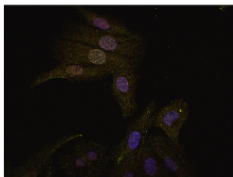
- The original merge image
- A low resolution picture of the ROI
- Highlighted ROIs in different channels

```
# select ROI and show the results  
roi_show(img_rois, ind = c(1, 2))
```

Output in the next slide >>

Visualizing selected regions

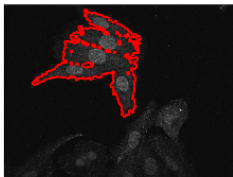
Merge



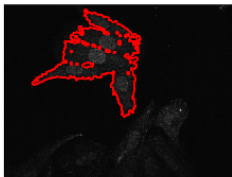
Pixel Set



Channel One



Channel Two



<< Code in the previous slide

The interactive web app

The package provides a simple web app to interactively determine the selection parameters and apply the rest of the workflow. To launch the app, run the following

```
# run the shiny app  
colocr_app()
```

Or visit this page.

colorcr

Main

GitHub

Contact us

Input Panel

Get started by uploading the merge image/s. Then, adjust the parameters to select the regions of interest. Finally, assign a name to the probe used in this image/s to be used in the output.

Merge image

Browse... 2 files

Upload complete

Threshold

1 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85 87 89 91 93 95 97 99

Shrink

1 2 3 4 5 6 7 8 9 10

Grow

1 2 3 4 5 6 7 8 9 10

Fill

1 2 3 4 5 6 7 8 9 10

Clean

1 2 3 4 5 6 7 8 9 10

Tolerance

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 0.99

ROI Num

3

Probe Name

LCS

What are the different tabs for?

Each of the below tabs provide a view of your image, data and analysis output. The different tabs are connected and are updated automatically whenever the input panel is used.

- Select ROI: Choose regions of interest by adjusting the input parameters.
- Pixel Intensities: Check the scatter and density distribution of the pixel intensities from the two channels.
- Tabular Output: View the different colocalization co-efficients in tabular format.
- Graph View: View the co-localization co-efficients in graphical format.

Select ROI

Pixel Intensities

Tabular View

Graph View

Image

Channel One

Image

Channel One

Pixel Set

Channel One

Pixel Set

Channel One

Pixel Set

Image0003.jpg : Average PCC: 0.83 and Average MOC: 0.95 Image0001.jpg : Average PCC: 0.88 and Average MOC: 0.98

Selecting multiple regions

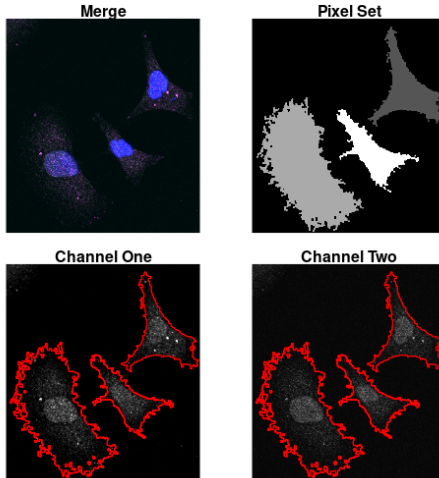
If we want to select the all cells, we modify the different parameters in `roi_select`. The selection parameters are

- threshold parameter before
- shrink fitting pixels in a structure
- clear removing small isolated elements
- n the number of regions to select

```
# load 'image_2.png' as img2  
img2 <- image_load('image_2.png')  
  
# select and show three regions of interest  
img2_roi <- roi_select(img2, threshold = 90,  
  shrink = 10, clean = 10, n = 3)  
roi_show(img2_roi)
```

Output in the next slide >>

Selecting multiple regions



<< Code in the previous slide

Pearson's correlation coefficient

Pearson's correlation coefficient (PCC) is the co-variance of the pixel intensity from two channels. The mean of the intensities is subtracted from each pixel which makes the coefficient independent of the background level.

The PCC is calculated as follows

$$\text{PCC} = \frac{\sum_i (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 \times \sum_i (G_i - \bar{G})^2}}$$

where R_i and the G_i is the intensities of the red and green channels and the \bar{R} and \bar{G} are the average intensities.

PCC value of 1 means positive and -1 negative perfect correlation. 0 means there is no correlation between the pixel intensities.

Manders Overlap Coefficient

Manders Overlap Coefficient (MOC) does not require subtraction of the mean. Therefore, the values are always between 0 and 1. Also, the MOC is independent from signal proportionality.

$$\text{MOC} = \frac{\sum_i (R_i \times G_i)}{\sqrt{\sum_i R_i^2 \times \sum_i G_i^2}}$$

where R_i and the G_i is the intensities of the red and green channels.

Calculating correlation statistics

The `colocr` package implements both PCC and SCC in `roi_test`.

Invoking the test is a one function call on the selected regions of interest.

```
# Calculate the PCC  
tst <- roi_test(img2_rois, type = 'both')  
tst
```

	pcc	moc
1	0.8798341	0.9303114
2	0.8963802	0.8990838
3	0.8882729	0.9386888

`roi_test` returns a data.frame with a column for each of the desired statistics: 'pcc' and 'moc', and a row for each region of interest.

References

1. Ahmed M, Lai TH, Kim DR. colocr: an R package for conducting co-localization analysis on fluorescence microscopy images. PeerJ. 2019;7:e7255. doi:10.7717/peerj.7255.
1. Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol. 2011;300(4):C723-C742. doi:10.1152/ajpcell.00462.2010.

Summary

What you learned

- Fluorescence image analysis
- Understanding images
- Loading images in R
- Selecting regions of interest
- Calculating correlation statistics

What's next

- Practice ([Link](#))
- Homework ([Link](#))
- What to learn next
 - Intermediate R Course ([Data Camp Free Interactive Course](#))
 - R for Data Science ([Book](#))