Colocalisation Analysis

P. Barber

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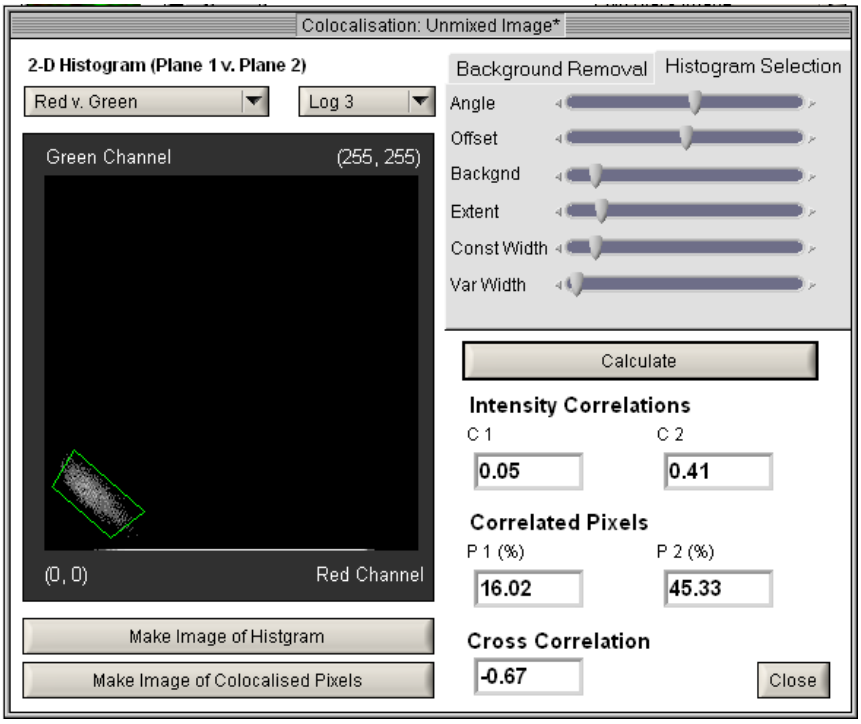
This module allows the determination of how well two stains or dyes are colocalised (how well they overlap) in an image by producing and analysing 2-d histograms.

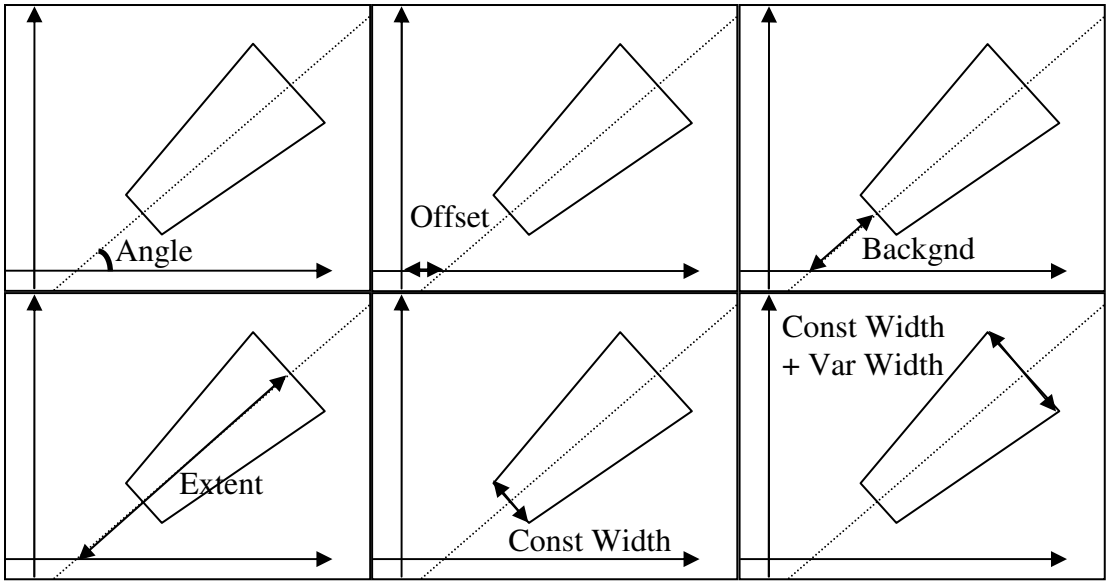
The module works on RGB colour images and works on the intensities in the red, green and blue channels. These may represent staining intensities of three stains on a sample.

The panel is shown below:



The black box on the left of the panel is the 2-d histogram. The intensity in the Red Channel is plotted versus the intensity in the Green channel. The data appears in white and the way it is displayed can be changed using the “Linear, Log, Log 2, Log 3, B/W” control. For an explanation of the 2-d histogram please see the appendix.

The green outline on the histogram shows the currently selected region. This can be modified using the controls at the top right of the panel. “Background removal” simply removes the lower values of the 2 channels. “Histogram selection” allows you to specify a trapezium using the 6 controls which are based on what we expect the distribution of collocated pixels to be (along the diagonal from (0, 0) to (255, 255)).



"Calculate" updates the correlation values, these are:

The Intensity Correlation Coefficients (C_u and C_v) are calculated according to:

$$C_u = \frac{\sum PixelIntensity_{u,coloc}}{\sum PixelIntensity_{u,total}} = \frac{\sum i_{u,coloc}}{\sum i_{u,total}}$$

and similarly for v. (this is the same as Biorad Lasersharp software Cred/Cgreen, and Bitplane Colocalisation ColInt1 and 2)

The % of Pixels Correlated (P_u and P_v) are calculated according to:

$$P_u = \frac{\sum NumberOfNonZeroPixels_{u,coloc}}{\sum NumberOfNonZeroPixels_{u,total}} = \frac{\sum n_{u,coloc}}{\sum n_{u,total}}$$

and similarly for v. (this is the same as Bitplane Colocalisation ColNo1 and 2)

The overall Cross Correlation (CC) is calculated using:

$$CC = \left(\frac{\sum \left(i_{u,coloc} - \bar{i}_{u,coloc}\right)\left(i_{v,coloc} - \bar{i}_{v,coloc}\right)}{\sqrt{\sum \left(i_{u,coloc} - \bar{i}_{u,coloc}\right)^2 \sum \left(i_{v,coloc} - \bar{i}_{v,coloc}\right)^2}} \right)$$

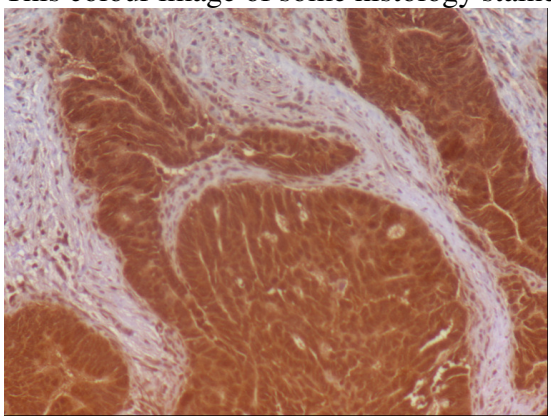
where $\bar{i}$ indicates the mean intensity.

(N.B. this is similar to the Bitplane Colocalisation rho or R1/2 which is abs(CC)*100; information is lost in taking the absolute value).

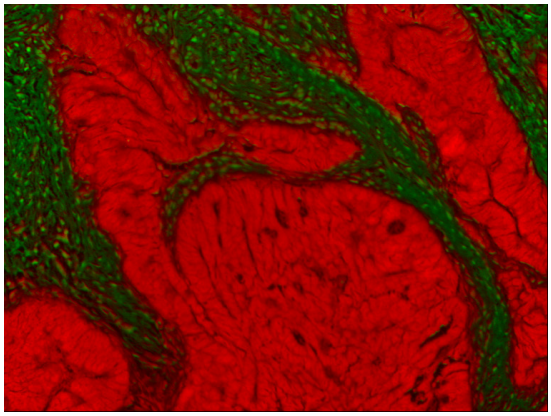
Where "i" indicates pixel intensities and "n" pixel numbers. Subscript "coloc" indicates the pixels within the region (green box), "total" indicates all the pixels.

Example:

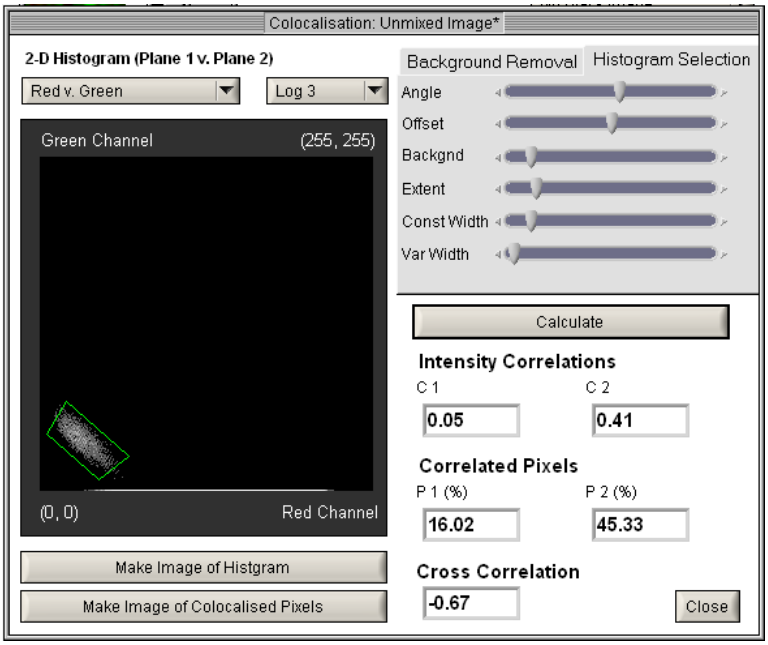
This colour image of some histology stained with DAB and haematoxylin (Hx),



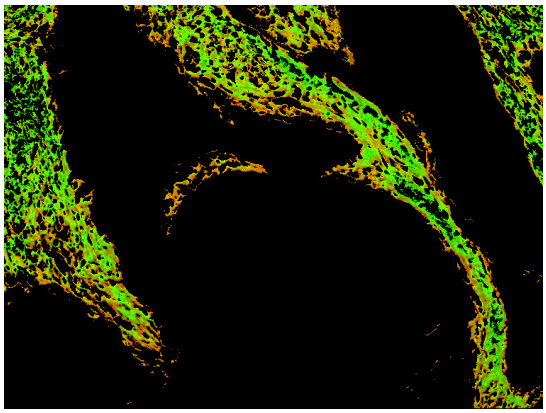
Can be separated (unmixed) into the image below where red represents DAB stain intensity and green the Hx stain intensity.



With the Colocalisation module we can create and segment the 2-d histogram:

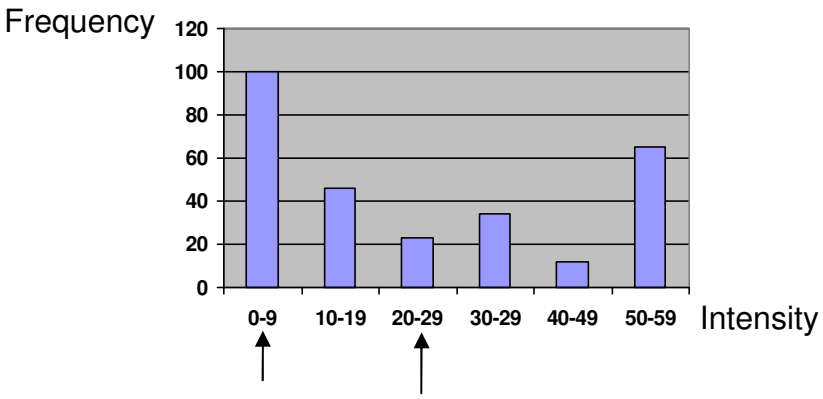


We can see that some pixels are vaguely colocalised but the cross correlation is –ve indicating that the red and green are inversely correlated, red is strongest where green is not and vice versa.
If we then "Make image of colocalised pixels" we get the image below which shows where the DAB and Hx overlap. We would expect the Hx to stain all the tissue but the DAB staining is too strong for RGB unmixing to 'see' the Hx underneath. We should really use the spectral imager to separate these stains rather than use the ordinary colour image.



Appendix A: The 2-d histogram

The 1-d histogram is usually made up of a number of bins, into which pixels are placed according to their intensity. The result is a plot of frequency versus intensity.



This peak indicates that there are a lot of pixels with an intensity near zero (black).

There are a less pixels in this intensity range.

In the 2-d histogram each pixel has two values, a red intensity and a green intensity (say), and now bins are formed based on these two intensities, in a 2-d grid:

Red range Green range

Etc.						Etc.			
0-9 50-59					50-59 50-59				
0-9 40-49				40-49 40-49					
0-9 30-39	10-19 30-39	20-29 30-39	30-39 30-39						
0-9 20-29	10-19 20-29	20-29 20-29	30-39 20-29						
0-9 10-19	10-19 10-19	20-29 10-19	30-39 10-19						
0-9 0-9	10-19 0-9	20-29 0-9	30-39 0-9	40-49 0-9	50-59 0-9	Etc.			

When the histogram is formed each bin is given a value according to how many pixels have intensities in the correct range for both channels (red and green).

In bmp or tiff images, the red, green and blue channels can take a value in the range 0-255. So we form an array of bins 256 square, (0, 0) at the bottom left to contain the frequency of pixels with both channels zero, and (255, 255) at the top right to contain the frequency of pixels with both channels at 255, and all the other combinations in between.

When displayed, the values given to the bins are converted into brightness (screen intensity) so that areas of the histogram where there are a lot of pixels appear white. Since the values given to the bins can be over a wide range we need several options for displaying them so that we can see what we want to see, these are:
A linear look-up table, where brightness is proportional to value
Three logarithmic look-up tables, where brightness is proportional to log(value)
A B/W (black and white) display mode where all bins at zero are black, all other bins are fully white.

Appendix B: Bitplane and Biorad Correlation Calculations

Text from Biorad and Bitplane documents:

Biorad:

The theory behind co-localisation analysis

LaserSharp version 3.0 and later contain co-localisation analysis functionality. The program calculates two values which represent

the proportion of colocalising objects in each component of a dual-colour image. These values are called co-localisation coefficients. The calculations are based on Pearson's correlation coefficient which is a well trusted means of describing the degree of overlap between patterns or images. The co-localisation coefficients are calculated according to the following equations:

$$C_{red} = \frac{\sum_i R_{i,coloc}}{\sum_i R_i}$$

Where;

$\sum_i R_{i,coloc}$ = The sum of intensities of all red pixels that also have a green component. (i.e. those within the coloc region of the histogram – PB)

$\sum_i R_i$ = The sum of intensities of all the red pixels in the image.

Bitplane:

ColNo1: The ratio of colocalisation pixels (voxels) in a layer (map) to the number of pixels with a non-zero intensity in channel 1 given in percent.

$$ColNo1 = \frac{NumberOf(colocalisedPixels)}{NumberOf(Pixels_{Channel1,greyValue>0})}.100$$

Cross Correlation: The cross correlation coefficient between the intensities of all
colocalisation pixels of the contributing channels given in percent. The cross correlation coefficient between the intensities of all colocalised pixels of the contributing channels given in percent. The cross correlation coefficient can be used as a measure of similarity between the channels masked with the colocalisation map. The masked images, on which the calculation is based, consist of grey values of the original channels, which are set to zero when the pixel is not colocalised. A value of 100% means identity and a value near 0 means that the colocalised channel information is uncorrelated. The cross correlation coefficient R1/2 is determined according to:

$$R1/2 = ABS\left(\frac{\sum(i_1 - \bar{i}_1)(i_2 - \bar{i}_2)}{\sqrt{\sum(i_1 - \bar{i}_1)\sum(i_2 - \bar{i}_2)}}\right).100$$