# RGB Linear Unmixing
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The RGB Linear Unmixing module allows the extraction and separation of up to three, significantly different, colours from an ordinary RGB colour image (bmp, tiff, jpeg).

The result will be a new RGB colour image where a map of reference 1 occupies the red channel, reference 2 occupies the green channel and reference 3 occupies the blue channel (this can be separated using the extract colour planes function). In absorption mode maps of each reference in the correct colour for the stain/dye can be created.

Two examples of the panel that control the functions are shown below:
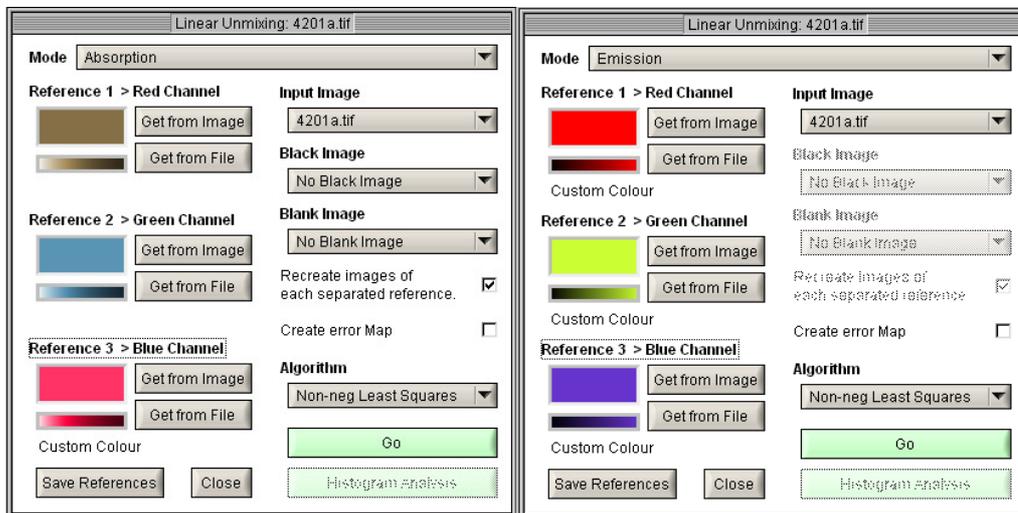
Mode:

The algorithm needs to know whether the colours arise from absorptive dyes or from fluorescence. If your image is from bright-field microscopy (e.g. histology) please select "Absorption". If your sample contained emissive dyes used in fluorescence microscopy, please select "Emission".

Reference 1, 2 and 3:

The 3 colours shown are the references to which to image will be unmixed. The bar below each colour shows how this colour looks at a range of intensities. The references can be changed in one of three ways:

Manual colour selection: Left click on the coloured box and select a new colour.
Get colour from image: Left click on this button and then left click on a pixel in the image.

Get colour from file: Left click this button to select a *.spec file. This can be a previously saved reference from this module or a full spectrum from the spectral imager. If a full spectrum is provided, a 3 point, RGB, reference colour will be estimated from it for use with this module.

If you have only two elements (stains/dyes) in your image, the third refernce should be set to WHITE manually.

Black and Blank images:

In "Absorption" mode the image must be corrected for the black level of the camera and for the microscope illumination.

Corrected image = (input image – black image) / (blank image – black image)

This can be done at capture time (using a program such as the visilog program on the histology microscope, or the image correction function of this program) or using this RGB Unmixing module. You will get better unmixing results if correct using this module by supplying black and blank images. You must do this by opening the black and blank images using the menu option File->Open Image, and then specifying them in the correct boxes of the Linear Unmixing panel.

The black and blank are not needed for "Emission" mode. You will get better results if the images have previously been corrected for the black level, but there is no advantage in doing that here and the option is not provided. (You could use the arithmetic function if this program to perform input image – black image).

Recreate images of each separated reference:

In absorption mode maps of each reference in the correct colour for the stain/dye can be created. This is simply the stain intensity map for each reference but with a colour table (applied colour map) which represents how the stain looks under the microscope.

Create Error Map:

An error map of how well the unmixing was can also be created. This is a greyscale image where zero indicates that the pixel was unmixed extremely well, >0 less so and -1 indicates that the unmixing totally failed at that pixel. The value is actually the integrated absolute error between the pixel value and the result of unmixing with the supplied references. High errors indicate that there may be other elements (stains/dyes) in the image (other than those represented by the three references) or that the references supplied badly represent the elements of the image.

Algorithm:

Non-negative least squares is the more general purpose and robust. Colour Deconvolution may be faster but requires that there are exactly 3 elements (stains/dyes) to the image and the you have good references for all of them. Non-negative least squares can handle just two references if the third is set to white.

Go:

Starts the unmixing. The new images produced will appear in the workspace when complete.

Histogram Analysis:

This calls up a new panel (below) and creates a new image that shows the results of image thresholding.
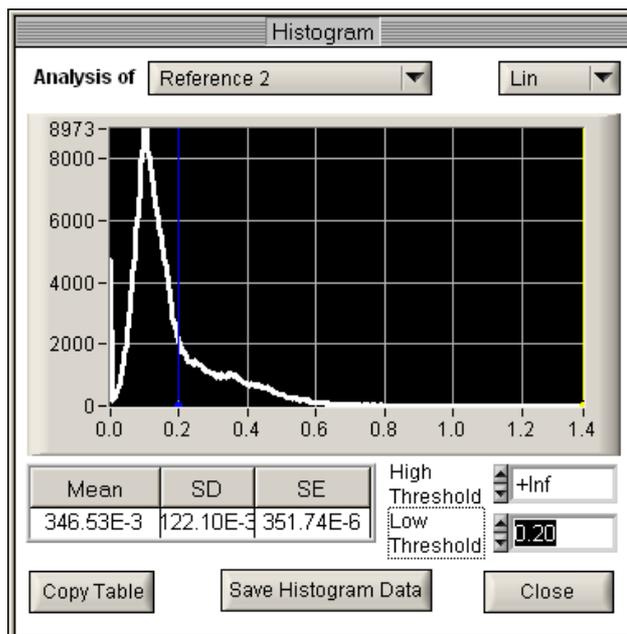
Use "Analysis of:" to choose which reference map to analyse.
The histogram and the image can be threshold to exclude low background staining by typing in a "Low Threshold" or by moving the blue cursor with the mouse. Similarly a high threshold can be set, or you can be certain of including all high values by typing "+inf" in the box. The mean, standard deviation and standard error are reported in the table which can be copied to the windows clipboard (for pasting in MS Excel™ for example) by pressing "Copy table". "Save histogram data" saves the full histogram curve in a text file that can be loaded into MS Excel™, Origin and JMP.