Biomedical Imaging Research Unit
School of Medical Sciences
Faculty of Medical and Health Sciences
The University of Auckland
Private Bag 92019
Auckland, NZ
Ph: 373 7599 ext. 87438
http://www.health.auckland.ac.nz/biru/
jacqui.ross@auckland.ac.nz

Colour Analysis Tools in ImageJ

11 July 2007

Jacqui Ross

Digital Images

= arrays of pixels (picture elements). Each has a value and is made up of bits (8bit, 12bit, 16bit)

Colour Modes/Colour Spaces (3D)

http://en.wikipedia.org/wiki/Color_space

RGB

= **R**ed **G**reen **B**lue

Starts with black and as colours are added, the amount of emitted light is increased (i.e. **additive** colour) = **R**ed, **G**reen and **B**lue « **channels** » with pixel values for every pixel in each channel

= colour digital cameras, computer display, etc.

For example, for a 24bit colour image, **white** = R255, G255, B255; **black** = R0, G0, B0.

Pure **red** = **R255**, **G**0, **B**0, **green** = **R**0, **G255**, **B**0, blue = **R**0, **G**0, **B255**.

All other colours are displayed by varying these pixel values.

HSB/HSV

= **H**ue **S**aturation **B**rightness/Value

Each colour shade (**hue**) has a value.

Saturation is equivalent to intensity, **Brightness/Value** relate to lightness.

Work in combination. Relates to RGB values.

CIE Lab

= Luminance (L) and two colour components (a, b) which work in an opposing way.

Attempts to approximate human vision, therefore has a larger colour gamut.

L* = 0 = black

L* =100 = white

YUV

= Luma or brightness (Y) + colour signal (U, V combined)

Used for analog TV for creating the picture, e.g. NTSC, PAL, SECAM formats

CMY(K)

= Cyan **M**agenta **Y**ellow

Starts with white and as colours are added, the amount of absorbed light is increased (i.e. **subtractive** colour).

= for pigments, printing, etc.

M + Y = red

C + Y = green

M + C = blue

K (black) is added to improve black since C + M + Y is not true black.

Colour Segmentation

RGB Channel Separation

Fluorescence/confocal images

Split into individual channels.

Image – Color – RGB Split. This will give you 3 separate grayscale images which you can then work on independently.

Histological stains (e.g. DAB, AEC, etc.)

First try splitting channels as for fluorescence images since this is the simplest way to work with the images (*Image – Color – RGB split).* Usually doesn't work because stains are not "pure" colours.

Specialised Plugins and Tools

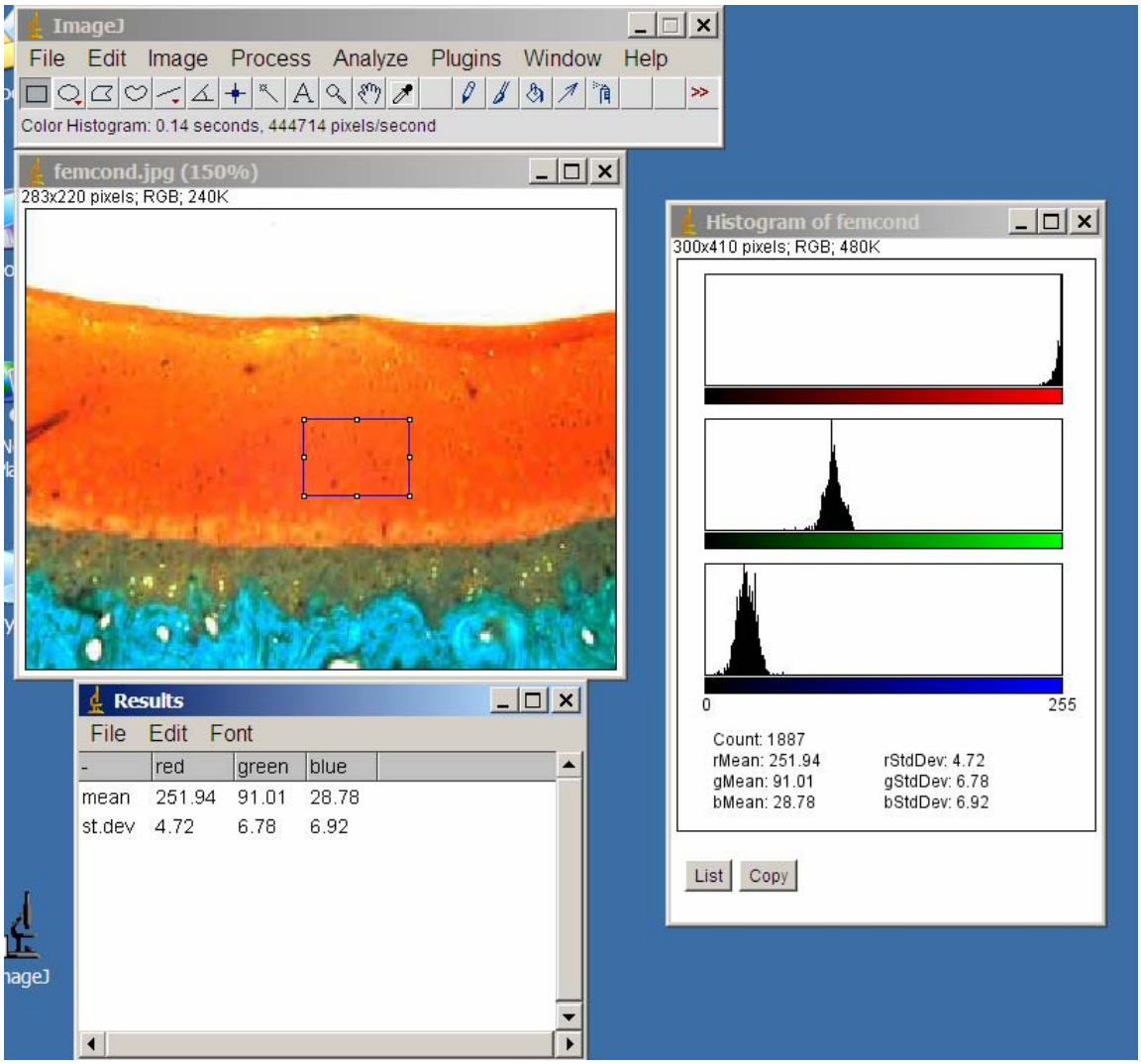
- Color Histogram
- RGB Recolor
- RGB Measure
- RGB Measure Plus
- Color Profiler
- Colour Space Converter
- Convert RGB to Luv, Lab or HSV
- Threshold Colour

- Colour Deconvolution
- 3D Color Inspector

Colour Histogram

http://rsb.info.nih.gov/ij/plugins/color-histogram.html

Gives you histograms of R, G, B values and mean pixel values in a Results table. Works on the whole image and ROIs.



RGB Recolor

http://rsb.info.nih.gov/ij/plugins/recolor.html

Allows linear alteration of the colors in R, G and B channels of RGB images, where:

New color = Old Color*Scaling Factor + Constant

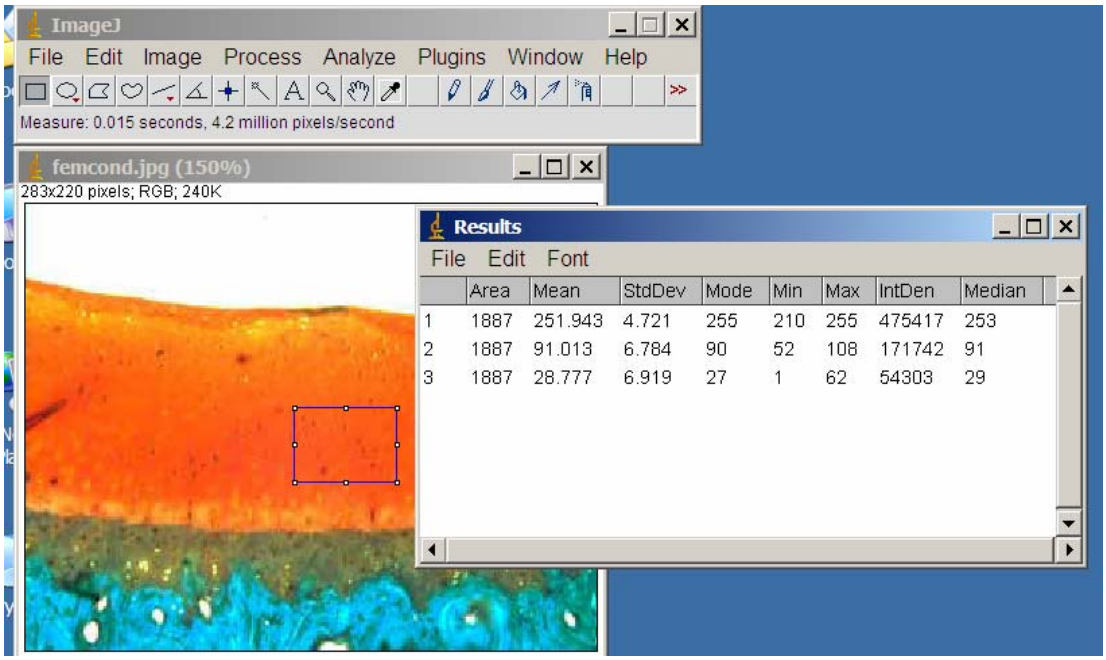
If the new color is greater than 255 it is set to 255. If it is less than 0 it is set to 0.

RGB Measure

http://rsb.info.nih.gov/ij/plugins/rgb-measure.html

Gives you measurements of R, G, B values. Also gives you any other measurements (in R, G, B channels) as selected in *Analyze – Set Measurements*, e.g. area, median, min, max, etc. Works on the whole image and ROIs.

Plugins – RGB Measure



RGB Measure Plus

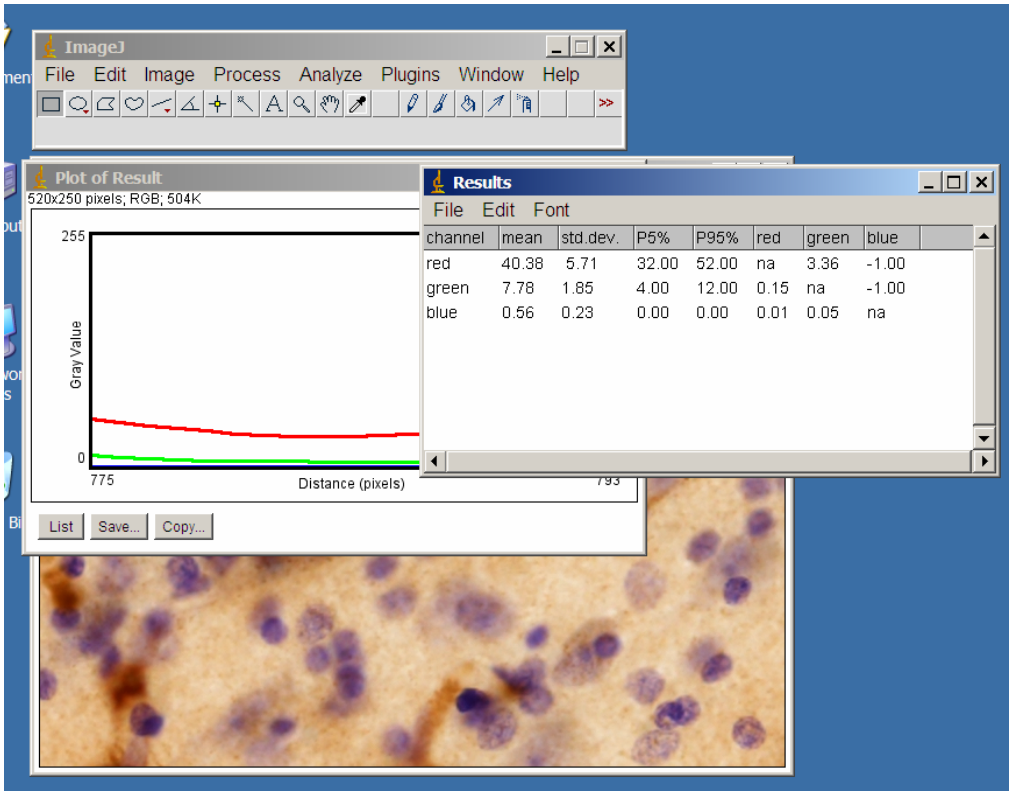
http://rsb.info.nih.gov/ij/plugins/rgb-measure-plus.html

Same as RGB Measure except that you can define thresholds.

Color Profiler

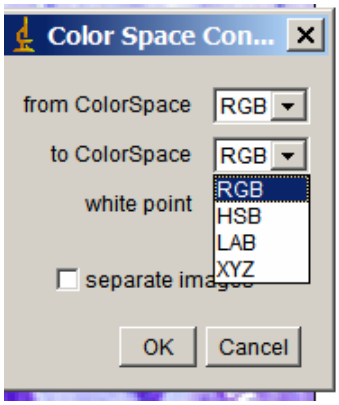
http://rsb.info.nih.gov/ij/plugins/color-profiler.html

A useful tool to determine proportions of R, G, B in the image. Provides a plot of intensity for each channel as well as tabulated results.



Color Space Converter

http://rsb.info.nih.gov/ij/plugins/color-space-converter.html



Convert RGB to Luv, Lab or HSV

http://rsb.info.nih.gov/ij/plugins/color-converters.html

Threshold Colour

http://imagejdocu.tudor.lu/imagej-documentation-wiki/plugins/threshold_colour

This plugin is designed as a filter to either **pass** or **stop** parts of the image being displayed depending on the settings you have selected.

You can choose to see the original image or alternatively select **threshold** to see your selection in black on white.

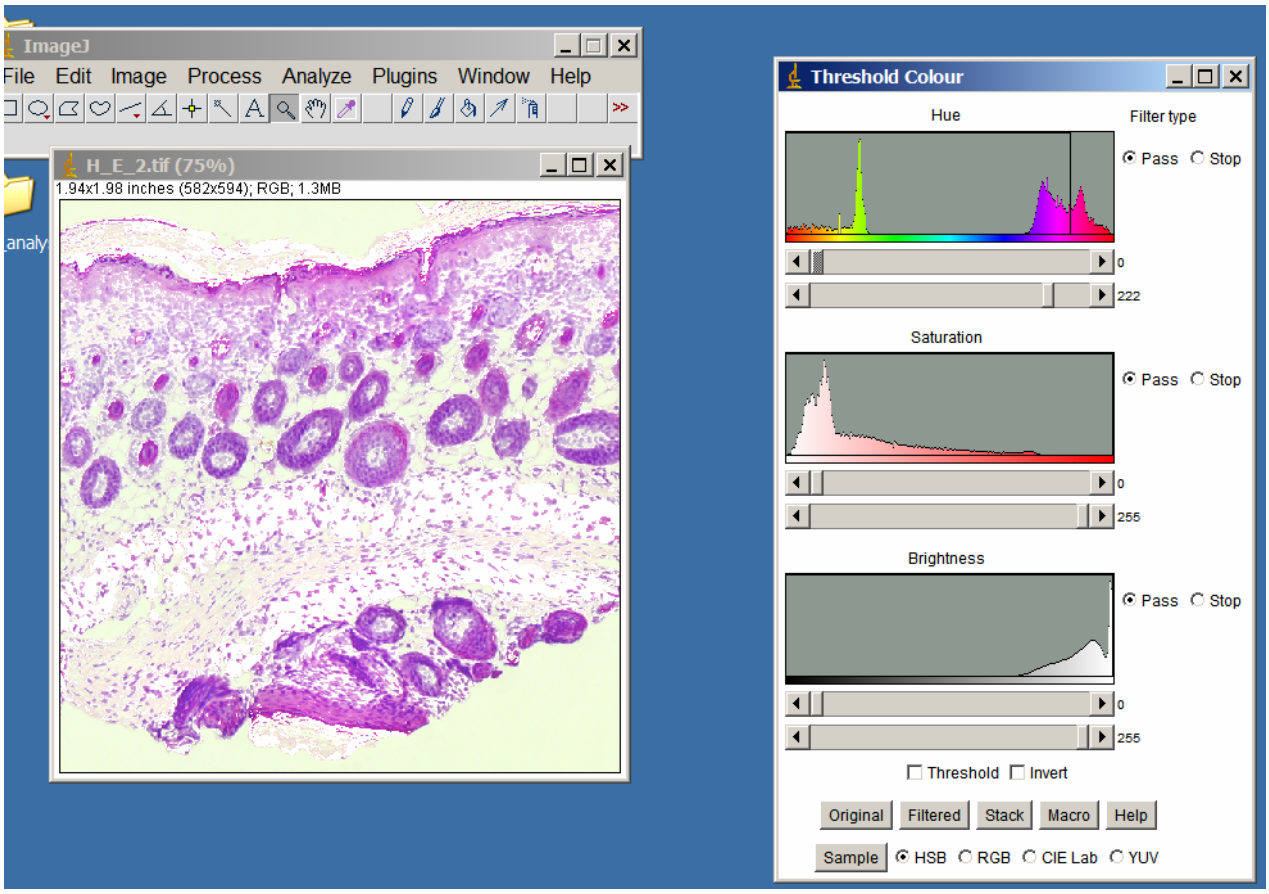
There are 4 colour modes available; HSB, RGB, CIE Lab and YUV.

If you find it useful, you can convert your image using additional plugins such as the

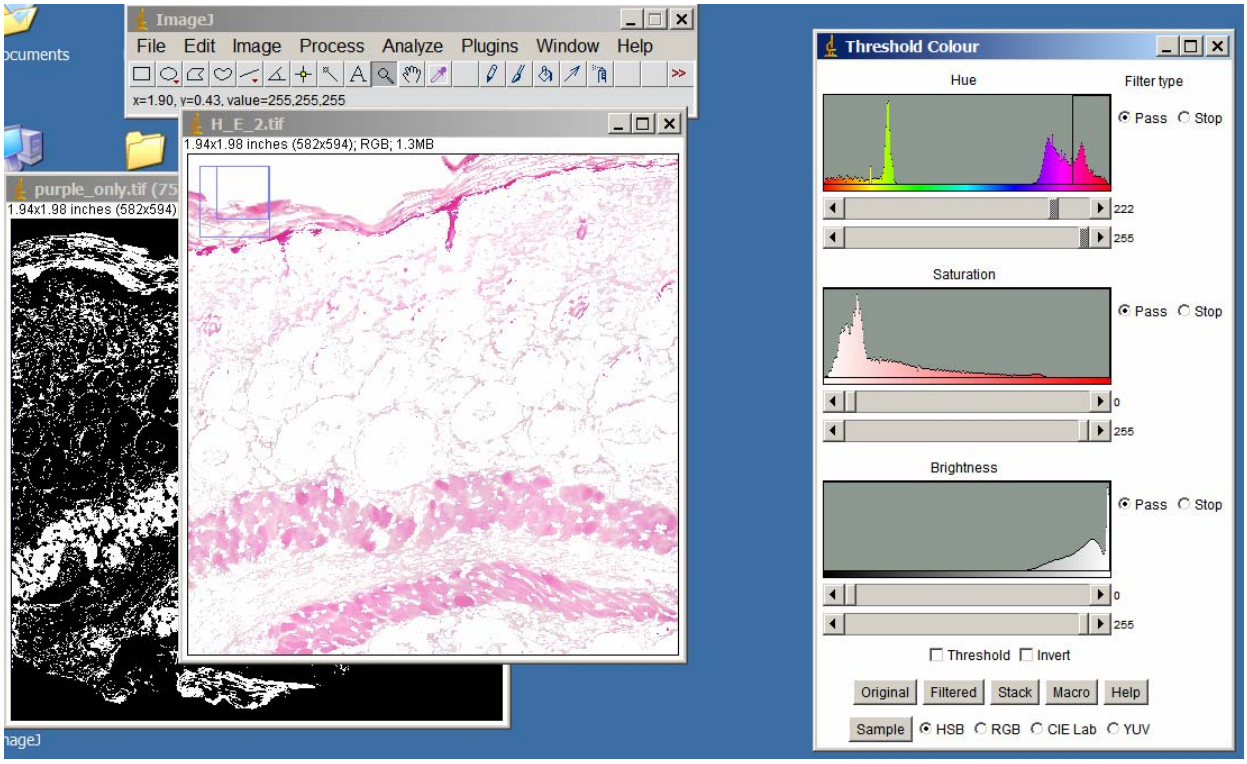
Color Space Converter

HSB

Example 1: Haematoxylin & Eosin stained skin section

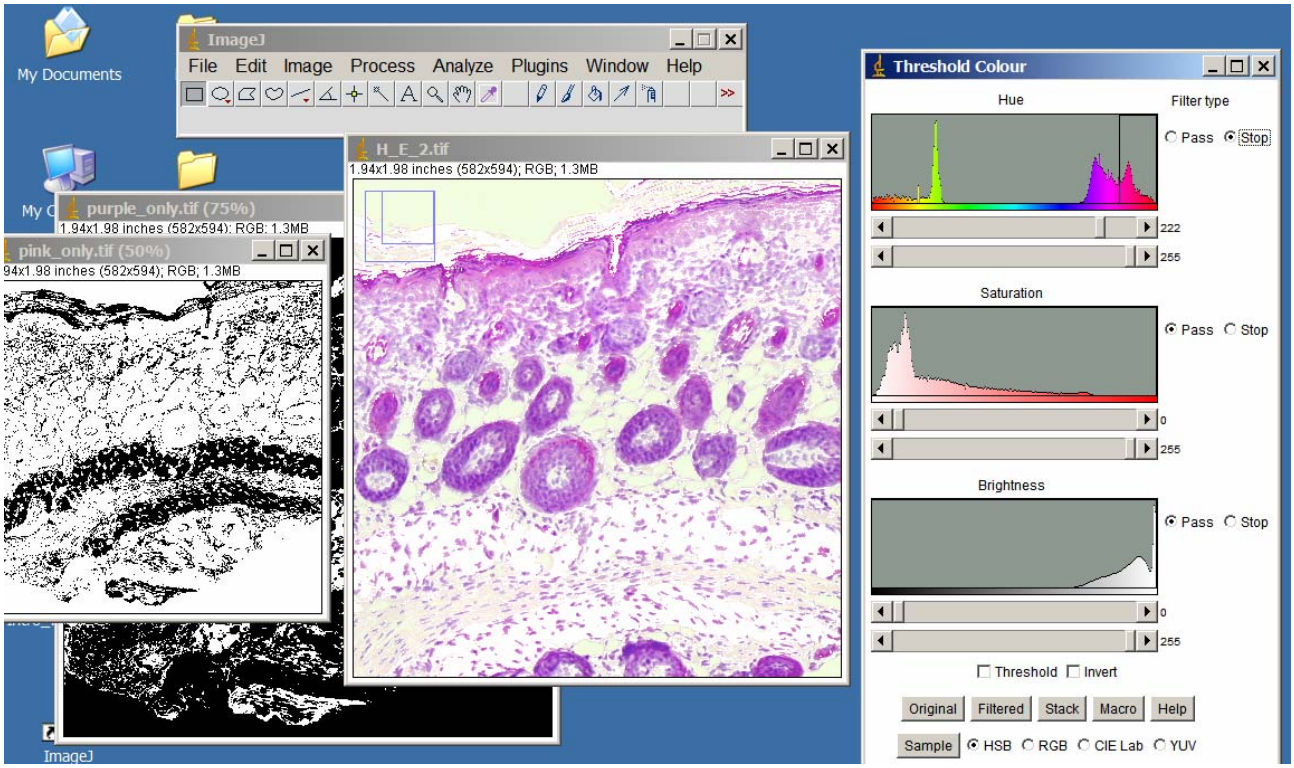


You can see a number of peaks present in the Hue histogram. The ones to the right represent the purple/pink colours present in the image. The green one represents the background which is not totally white.



Change **Pass** to **Stop** to see the other colour (purple). This allows you to check the segmentation.

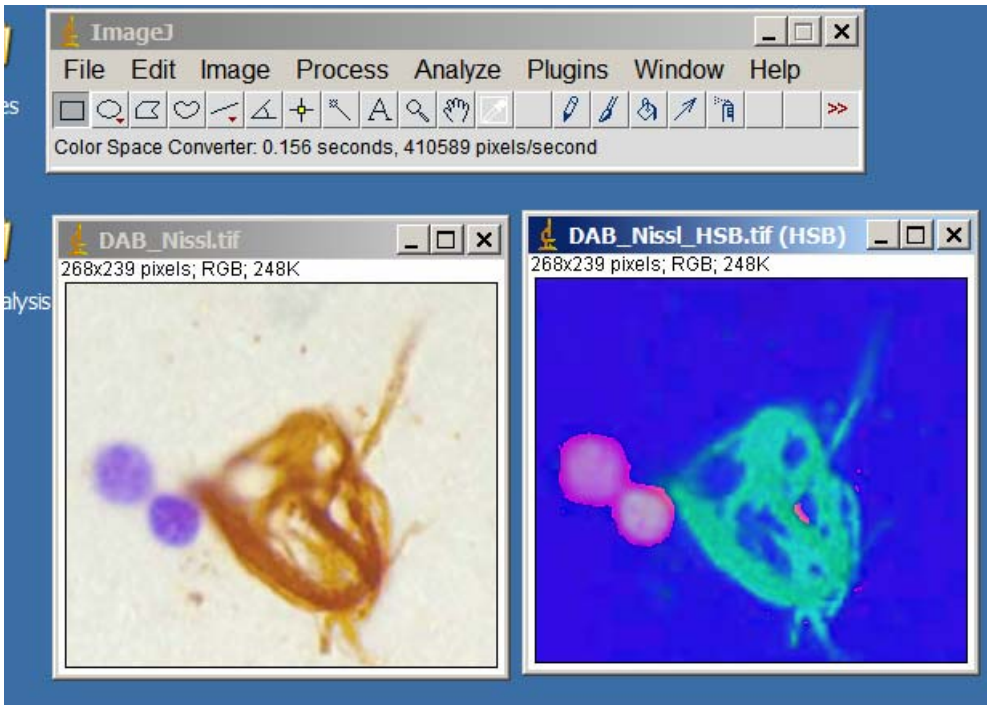
You can also change between **Original** and **Filtered**.



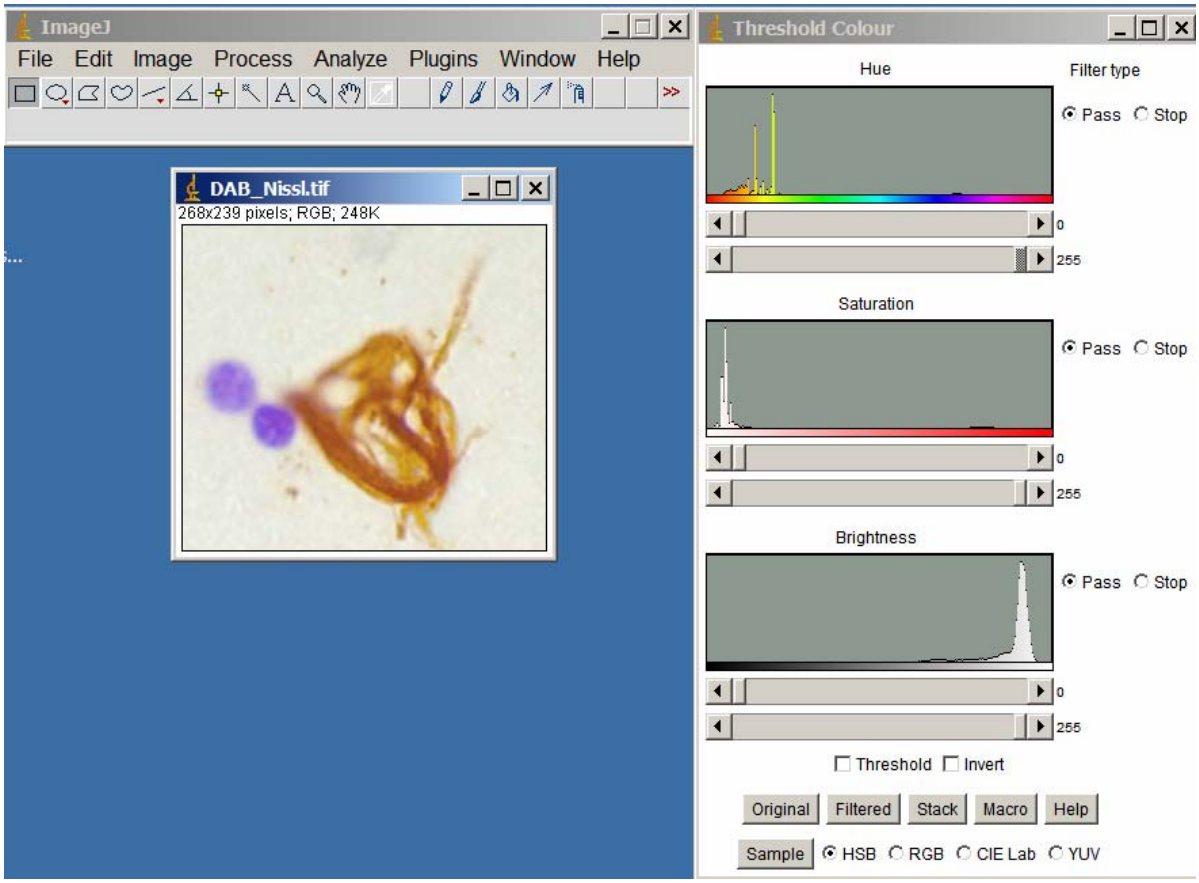
Example 2: DAB/Nissl stained brain section

HSB is a good colour mode to use for DAB because it is often quite dark (saturated) in comparison with the counterstain used (e.g. Nissl, Haematoxylin) so it's easier to segment out. In addition, although DAB has a strong blue component, the hue is quite different from the counterstain.

You can use the **Colour Space Converter** first to see whether HSB shows the components you want to separate as distinct.

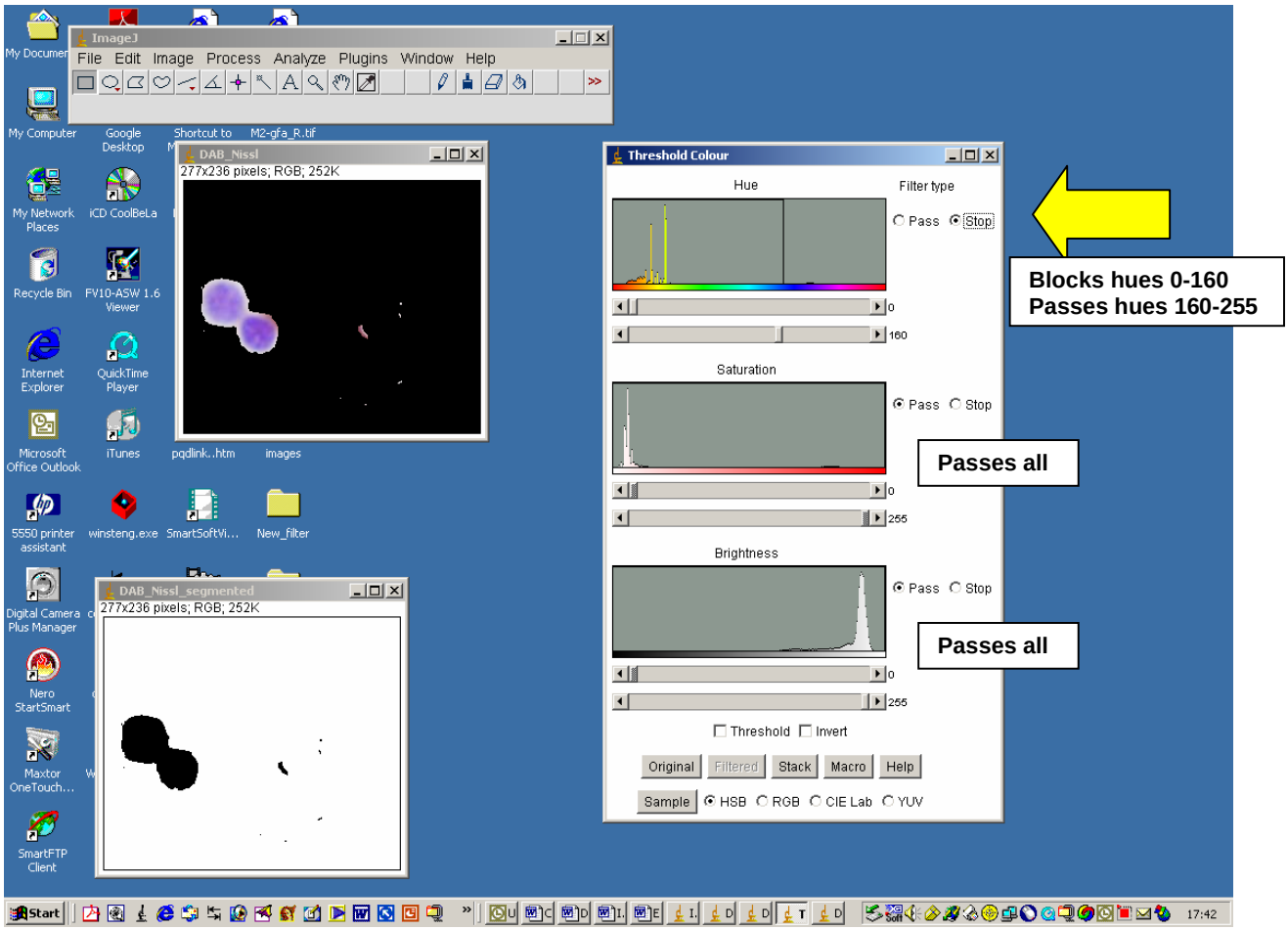


You can then choose to use the original image for segmentation or the newly converted image.

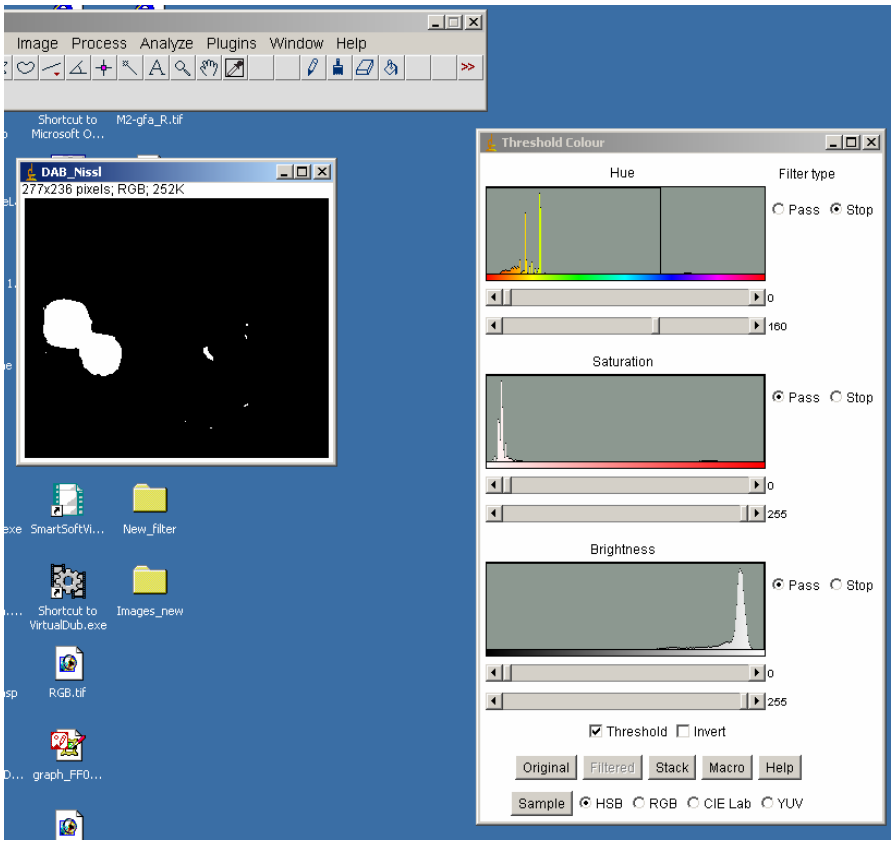


Look at the histograms. If there are a number of peaks present in the hue histogram, it will be easier to segment the image.

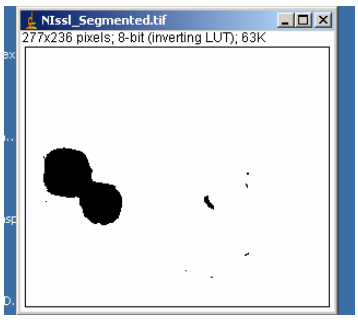
Segmentation of Nissl staining



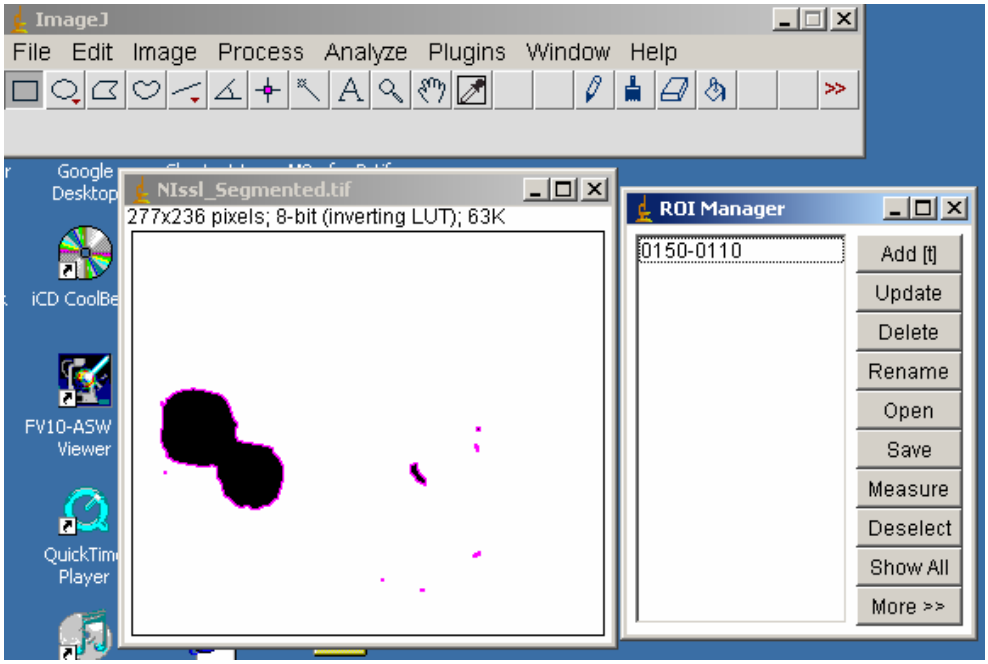
Click on **Threshold** to get a black/white representation. This is still an RGB image. If you want to keep it for analysis, RHS mouse click and *Duplicate*, *Rename* and save. This is the segmented Nissl image.



You can binarise the segmented image by going to *Image – Process – Make Binary*.



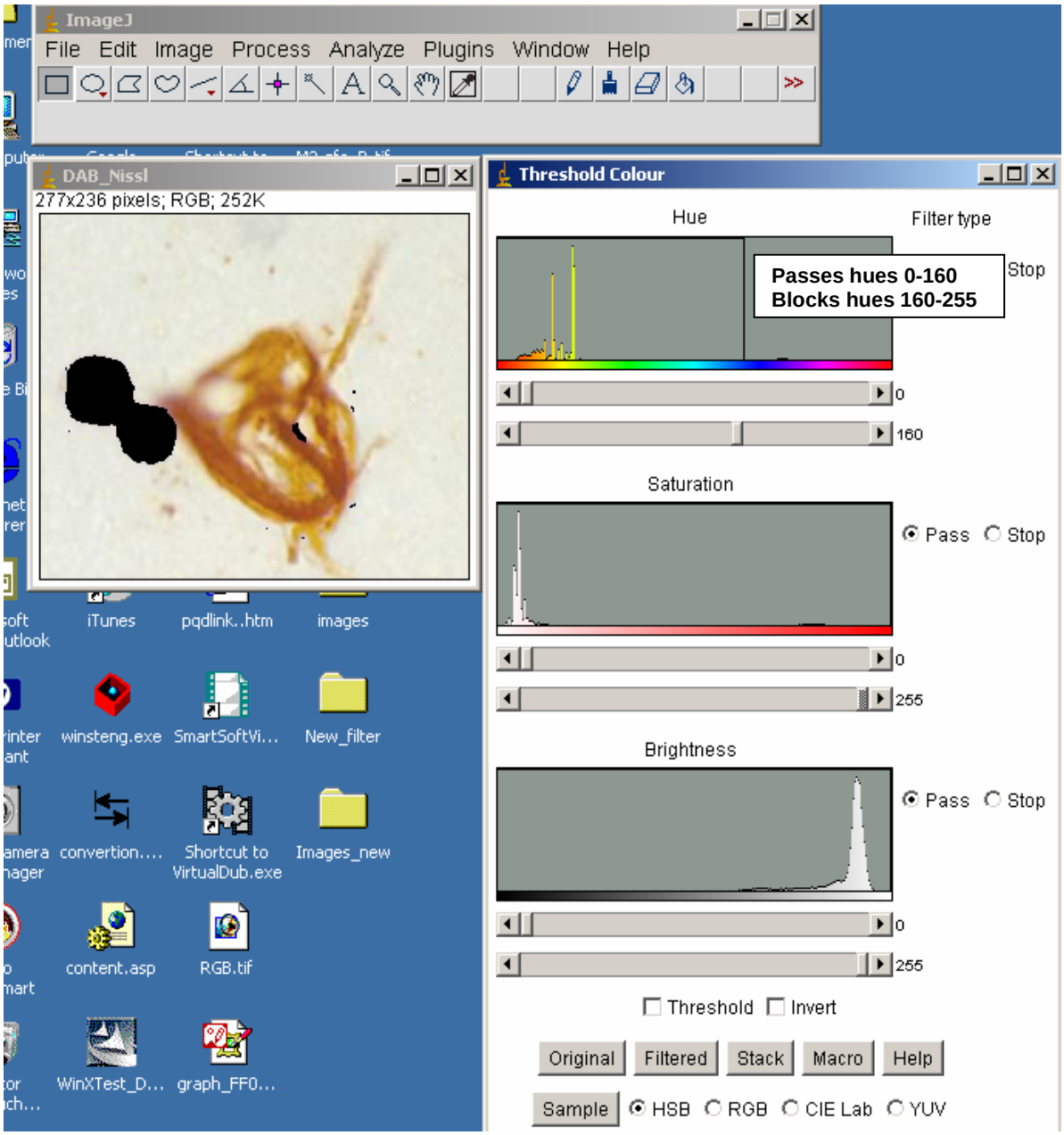
You can then create masks/selections for the **ROI Manager** by going to the Edit menu, e.g. *Edit – Select – Create Selection* and then *Edit – Selection – Add to Manager*. The ROI Manager will then open and add the selection as a ROI.



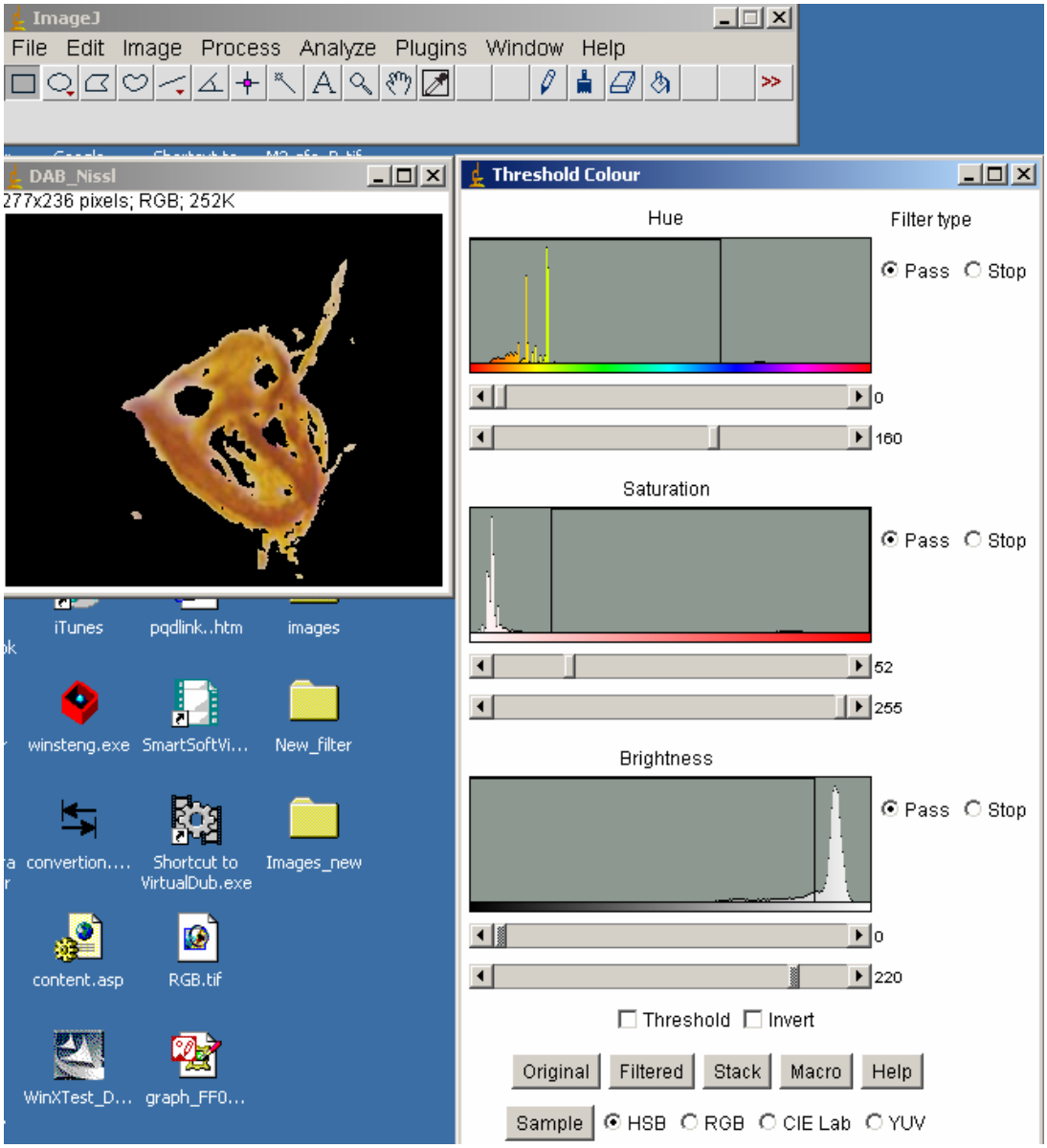
Click on **Original** and turn off **Threshold** to begin segmentation of the DAB staining.

Segmentation of DAB staining

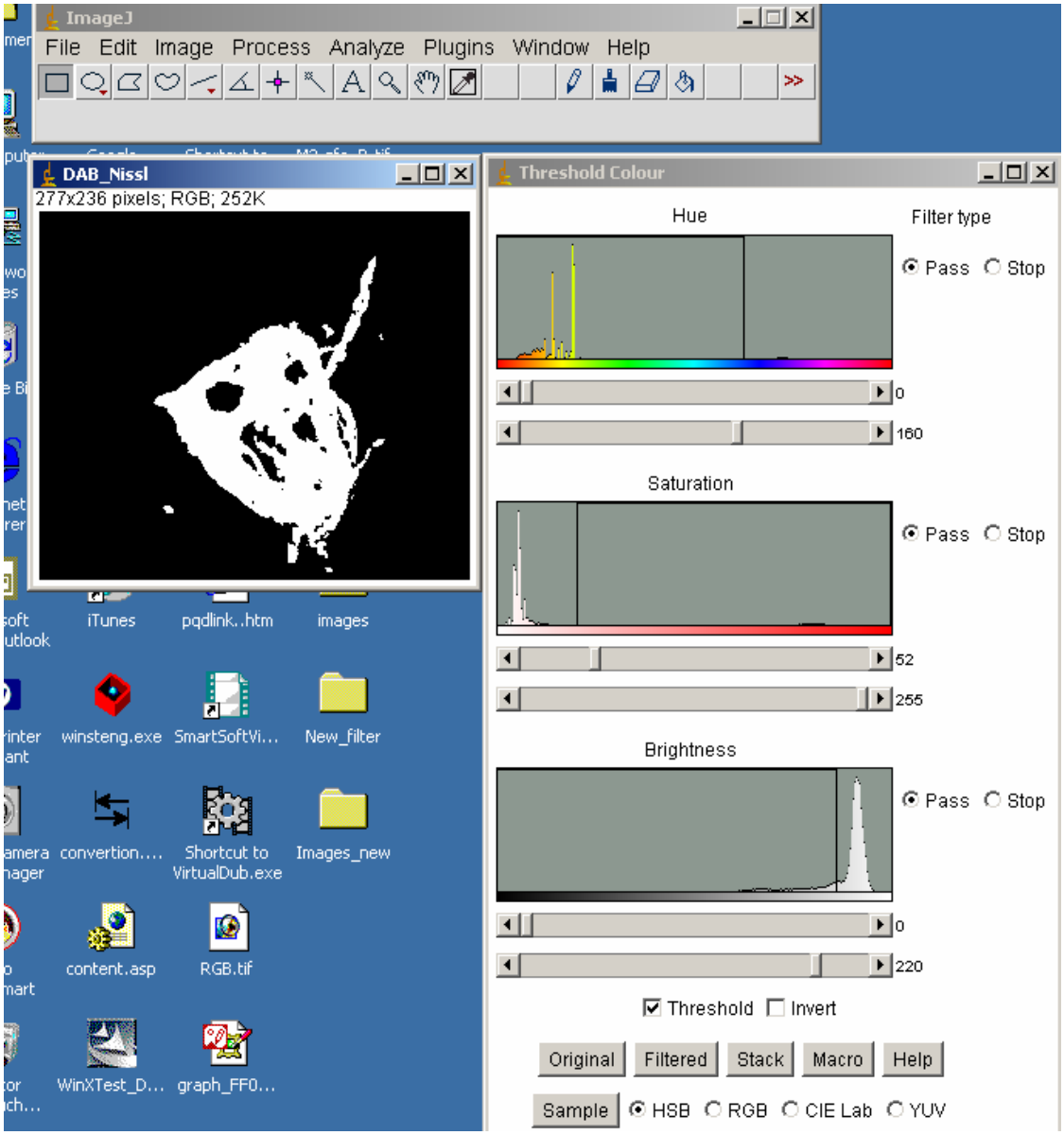
You could start by changing **Pass** to **Stop** (Hue histogram). This will stop the Nissl staining coming through. All other hues will pass which means we now see the DAB staining.



Adjust the **Saturation** and **Brightness** sliders until only the DAB staining shows through.

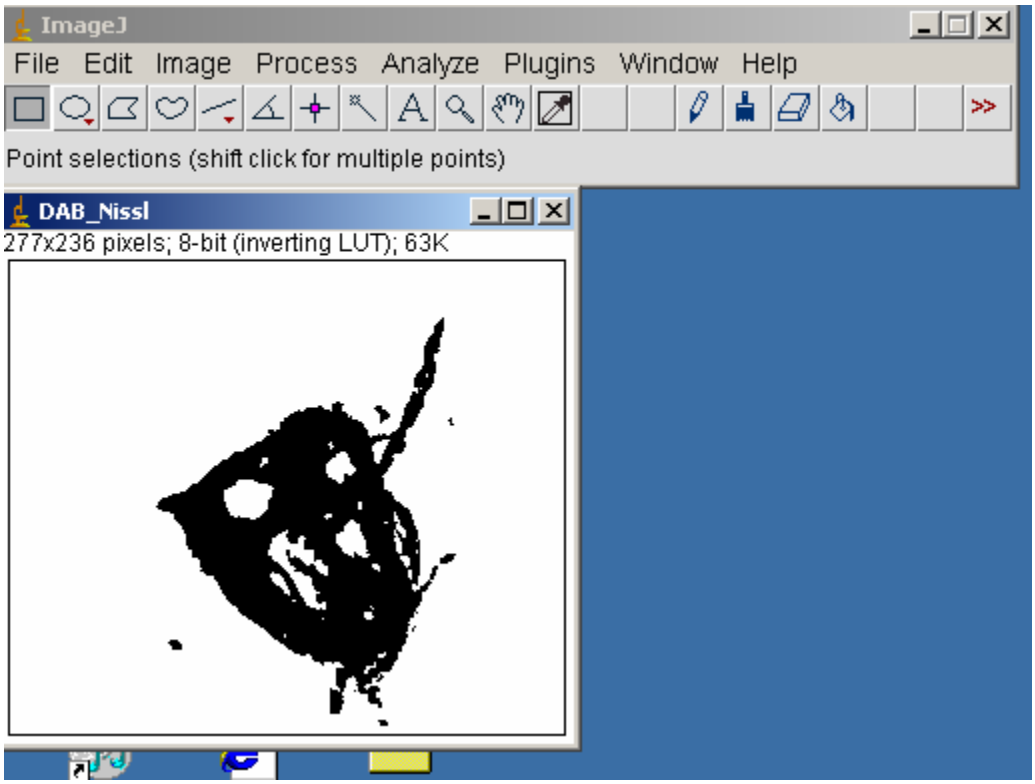


Click on **Threshold**.



RHS mouse click, **Duplicate**, **Rename**, Binarise (*Process – Make Binary*) and save.

Note that for measuring areas/particles, etc. of binarised images, it is the black area (on white) that is measured as the object of interest.


ImageJ
File Edit Image Process Analyze Plugins Window Help
Point selections (shift click for multiple points)
DAB_Nissl
277x236 pixels; 8-bit (inverting LUT); 63K

Colour Deconvolution

http://imagejdocu.tudor.lu/imagej-documentation-wiki/plugins/colour_deconvolution

http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html

Theory: http://home.planet.nl/~ber03728/4N6site/improc/decoplugin/decoexpl/p01.htm

Histological stains are “light absorbing dyes” so can be considered as being subtractive colour. Requires neutral background to work properly. Vectors need to be worked out from single-stained control slides or from ROIs where you can be confident that only one stain is present. The vectors are determined from the optical density of these areas and then normalised.

Vectors have been developed for the following stain combinations:

- Haematoxylin and Eosin (H&E)
- Haematoxylin and DAB (H DAB)
- Fast Red, Fast Blue and DAB
- Methyl green and DAB
- Haematoxylin, Eosin and DAB (H&E DAB)
- Haematoxylin and AEC (H AEC)
- Azan-Mallory
- Alcian blue & Haematoxylin
- Haematoxylin and Periodic Acid of Schiff (PAS)
- RGB subtractive
- CMY subtractive
- User values entered by hand
- Values interactively determined from rectangular ROIs

Example Haematoxylin & Eosin

Analyze – Tools – Color Histogram

First check that the background is neutral. This is best done using a Region of Interest in an unstained area.

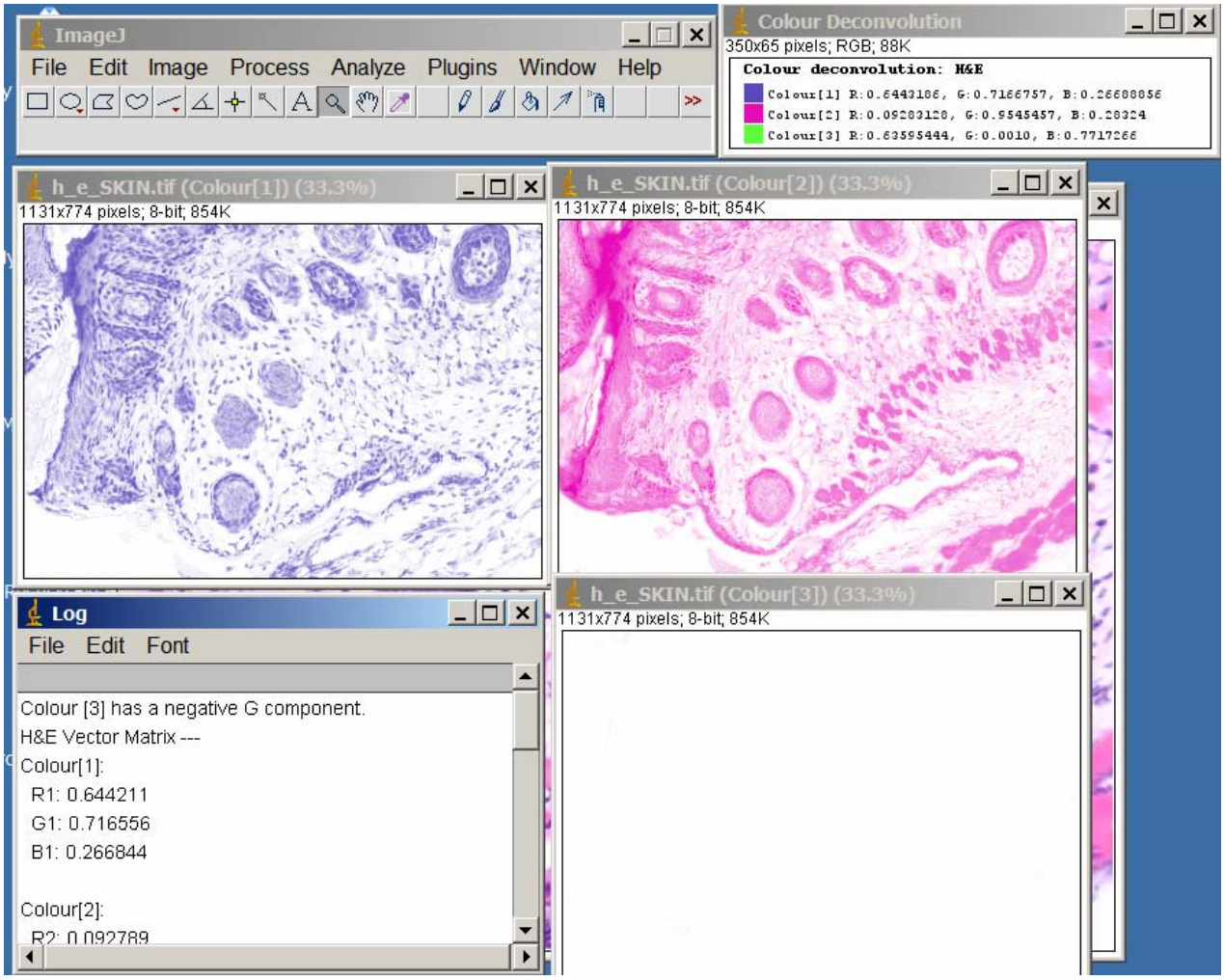
Draw a ROI in a “white” space. Then go to Analyze – Tools – Color Histogram. The R, G, B values should be similar. If they aren’t, then you need to adjust the image using background subtraction (***Process –subtract background***), ***Image – Adjust*** processes, or by subtracting a background image using ***Process – Image Calculator***.

Go to ***Plugins – Colour_analysis-Color Deconvolution*** (or wherever you have loaded the plugin).

Using built-in vectors

Select the vector you want which most closely resembles your image (e.g. H & E), show matrices and click OK.

If there are only 2 colours present that you want to separate, (as in this case Haematoxylin from Eosin), then the result should be one image of each corresponding to the individual stain and another one which is white. You also get a log file and the matrix data. The values have been determined experimentally by the lab group of the person who has developed this plugin.



Using the ROI to define your own vectors

If you have a different stain from those available in the plugin, you can determine your own matrix if you have some clear areas where only one stain is present (using ROI). Where two stains coexist, it may be difficult to separate them out and this “mixed” region may show up in the 3rd image rather than it being white. In this case, you can develop your own vectors experimentally.

Developing your own vectors experimentally

Create one control slide for each individual stain. You then determine the vectors for each stain using the ROI. You can then combine the vectors to get the matrix to apply to the dual/triple stained slides.

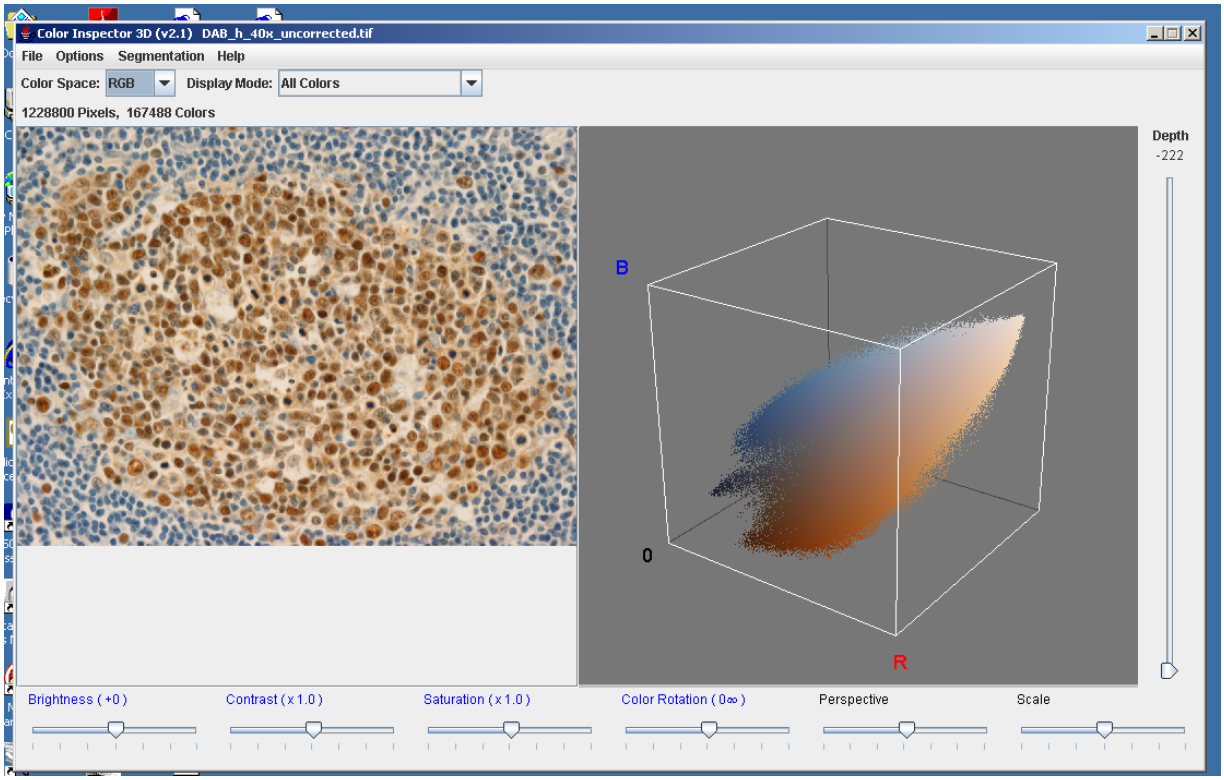
Note that this plugin can be used with stacks.

3D Color Inspector

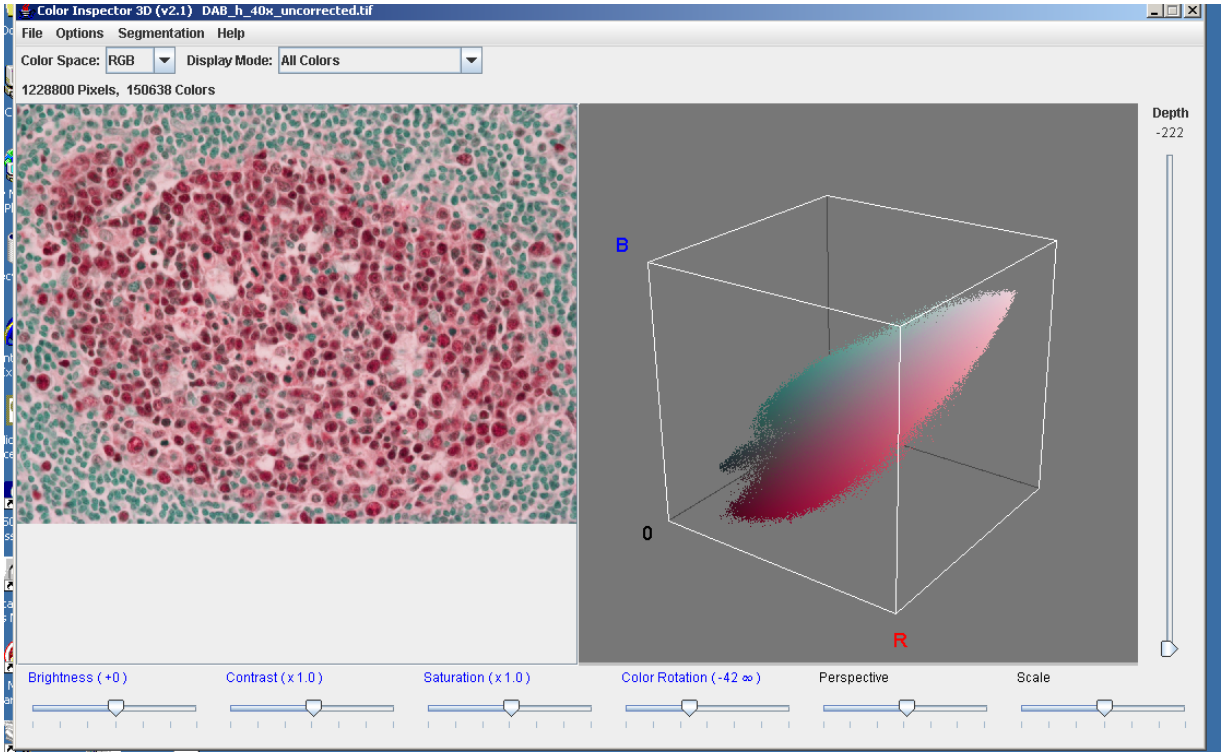
http://rsb.info.nih.gov/ij/plugins/color-inspector.html

http://www.f4.fhtw-berlin.de/~barthel/ImageJ/ColorInspector//help.htm

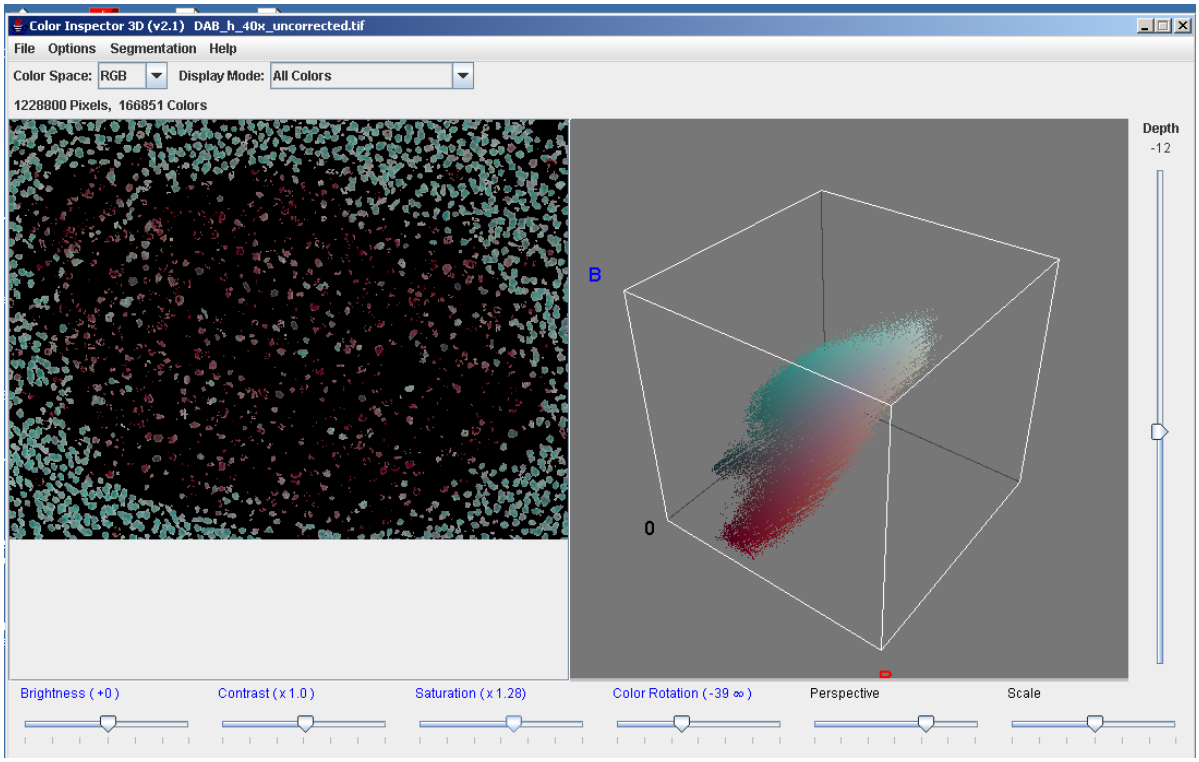
Allows you to change the colour display and save a modified image.



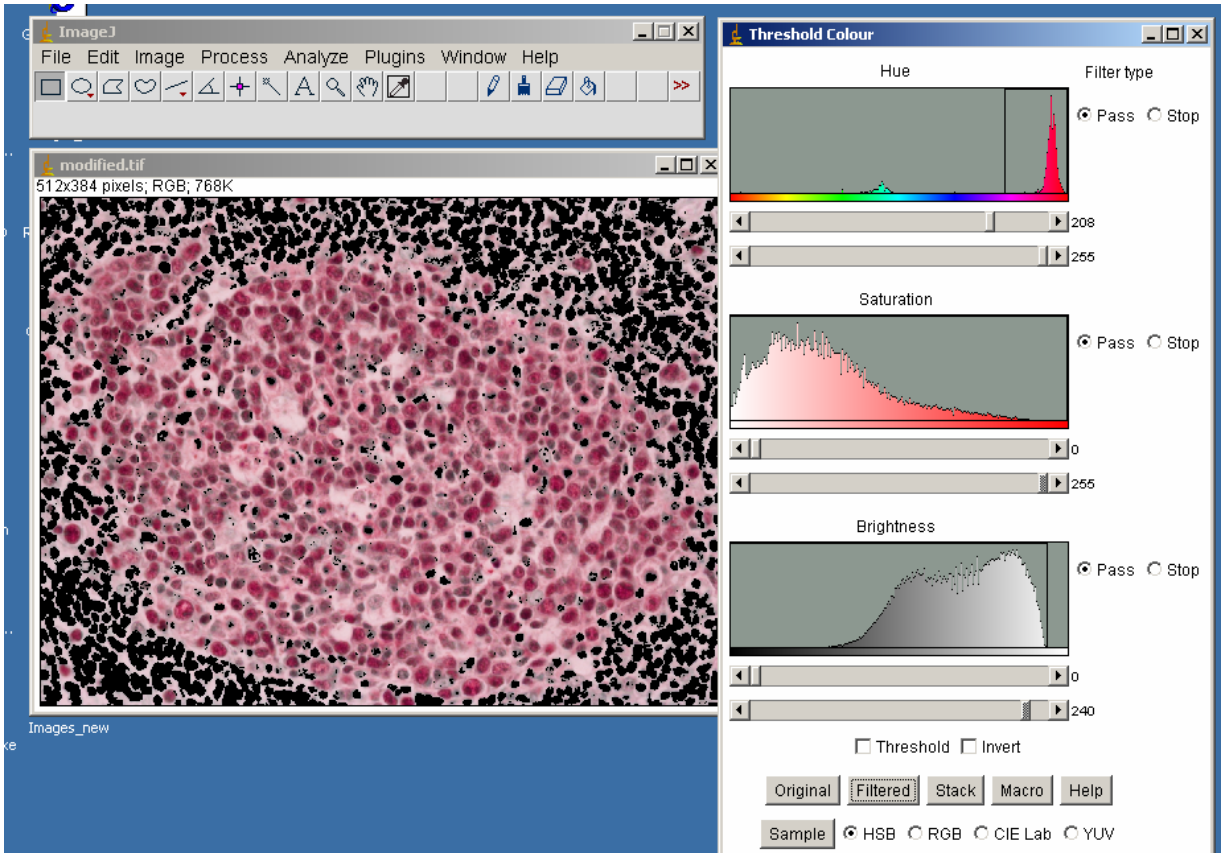
By changing the colour rotation (or other attributes), you can improve contrast which may make segmentation easier.



You can also do simple colour segmentation within 3D Inspector as shown below:



Or segment the modified image using other plugins, e.g. **Threhold Colour**.


ImageJ
File Edit Image Process Analyze Plugins Window Help
modified.tif
512x384 pixels; RGB; 768K
Images_new
Threshold Colour
Hue
Filter type
Pass Stop
208
255
Saturation
Pass Stop
0
255
Brightness
Pass Stop
0
240
Threshold Invert
Original Filtered Stack Macro Help
Sample HSB RGB CIE Lab YUV

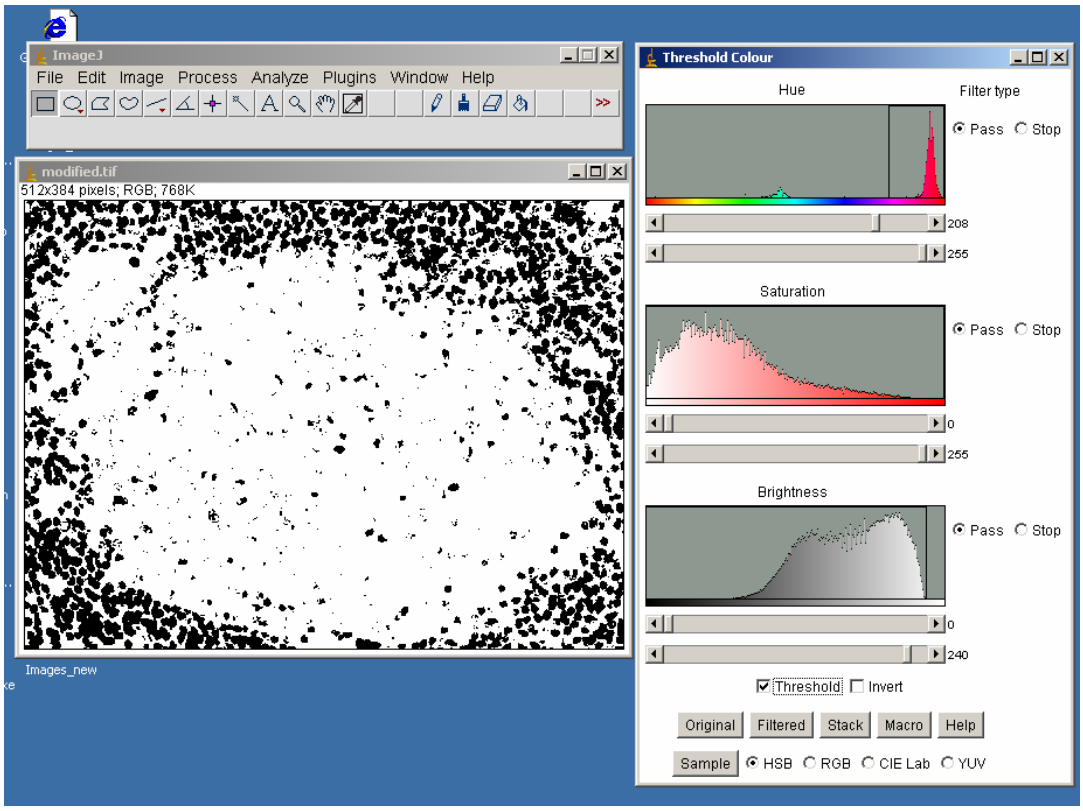

ImageJ
File Edit Image Process Analyze Plugins Window Help
modified.tif
512x384 pixels; RGB; 768K
Images_new
Threshold Colour
Hue
Filter type
Pass Stop
208
255
Saturation
Pass Stop
0
255
Brightness
Pass Stop
0
240
Threshold Invert
Original Filtered Stack Macro Help
Sample HSB RGB CIE Lab YUV

Image Correction Tools

Built in Command: Process – Subtract Background

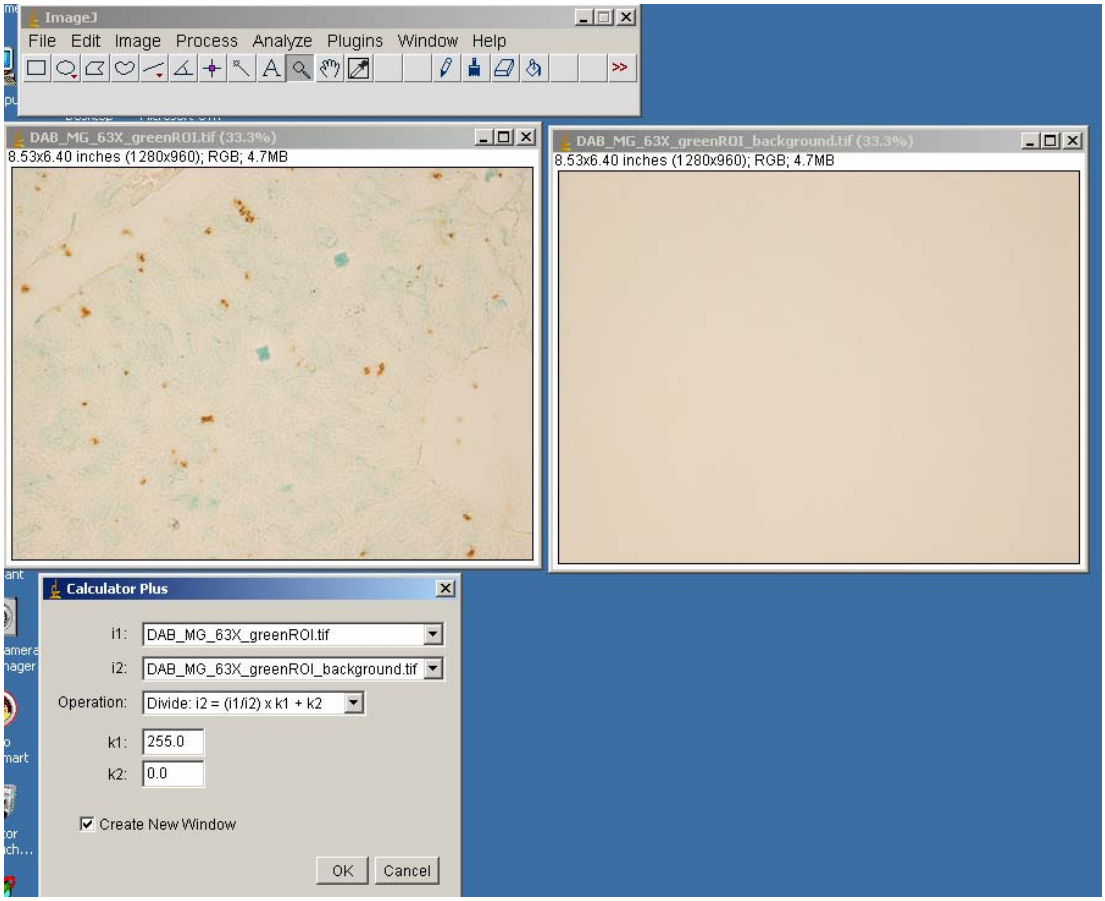
http://rsb.info.nih.gov/ij/docs/menus/process.html

Uses rolling ball algorithm, choose radius according to the size of features, choose light background for brightfield colour images.

Calculator Plus

http://rsb.info.nih.gov/ij/plugins/calculator-plus.html

Uses image and blank image together with a multiplication factor of 255 as shown below:



Shading Corrector

http://rsb.info.nih.gov/ij/plugins/shading-corrector.html

Works only on 8 bit images. Requires a blank image also as for Calculator Plus.

Colour_Correct

Available at: http://www.dentistry.bham.ac.uk/landinig/software/software.html

Does not correct for uneven illumination but compensates for inadequate lamp temperature by allowing the setting of “black” and “white” points.

Background Correction

Grayscale fluorescence 8bit images only at the moment. Corrects for uneven illumination.
http://rsb.info.nih.gov/ij/plugins/background.html

More….

If you want to read more about image correction for brightfield microscopy, go to:
http://imagejdocu.tudor.lu/imagej-documentation-wiki/how-to/how-to-correct-background-illumination-in-brightfield-microscopy

Filters, enhancement, etc.

Don’t forget to use these tools if necessay, e.g. *Process – Enhance contrast* or Median or Unsharp Mask filter, to improve ability to segment the colours.

Acknowledgements

Thanks to the following people for providing images or slides:
Lorraine Rolston
Brent Beaumont
Ursula Byrne
Claire French
Suzanne Reid
Susanne Franke

Thanks also to **Dr. Gabriel Landini** (School of Dentistry, The University of Birmingham, for clarification of the Colour Deconvolution plugin.