Using the Colour Deconvolution plugin in ImageJ

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Good for separating components of histological stains (e.g. DAB, AEC, H&E, etc.) since they cannot be easily separated by splitting into the red, green, blue channels recorded by colour cameras.

Colour Deconvolution – developed by Gabriel Landini

Download the current version of the plugin from the page below;

http://www.mecourse.com/landinig/software/cdeconv/cdeconv.html

Unzip the files and place them into your Plugins folder. You can put them into a subfolder if you want to do that.

Histological stains are “light absorbing dyes” so can be considered as being subtractive colour. The plugin requires images to have a neutral background to work properly. Vectors should be worked out from single-stained control slides or from ROIs where you can be confident that only one stain is present.

Vectors have been developed for the following stain combinations:

- Haematoxylin and Eosin (H&E)
- Haematoxylin and DAB (H DAB)
- Feulgen Light Green
- Giemsa
- 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-DAB

Pre-processing – ensure a neutral background
First check that the background is neutral. This is best done using a Region of Interest (ROI) in an unstained area.
Draw a ROI in a “white” space using one of the drawing tools from the toolbar. Then go to Analyze – Tools – Color Histogram. The R, G, B values should be similar. If they aren’t, then you may need to adjust the image using background subtraction (Process – subtract background) or Image – Adjust processes.

Go to Plugins – Colour_analysis-Color Deconvolution (or wherever you have loaded the plugin).
Using built-in vectors

The window below will appear;
Click on the drop-down box. You can see a number of different options. Hopefully, one of these will work for your staining.

If there are only 2 colours present that you want to separate, (as in this case Haematoxylin from DAB), 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 if you have selected Show matrices and haven’t selected Hide legend. Note that if you later create a macro, then Show matrices should be turned off and Hide legend turned on. The values for these built-in vectors have been determined experimentally by the lab group of the person who has developed this plugin.

Please note that even if your stains are the ones listed, the vectors may not work perfectly because the formulation of the stain may be slightly different resulting in different colour/absorbance properties.
Select the vector which most closely resembles your image (e.g. in this case, we select H DAB, which is for Haematoxylin-DAB), **show matrices** and click **OK**.
Three image windows will appear. In this case, **Colour 1 = Haematoxylin**, **Colour 2 = DAB** and **Colour 3 = residual** (this should be close to white if the separation is working perfectly).

If Colour 3 has some stain in it as shown below but you are still getting good enough separation to complete your segmentation/analysis e.g. in the case of cell counting/area, etc. then you don’t need to worry. However, if it is incomplete and some structure that you need to include is appearing in this image, then you may need to create your own vectors. This would ideally be done by using images of single stains and then combining the vectors.
Using the ROI to define your own vectors

If you have a different stain from those available in the plugin, or if the inbuilt vectors are not working properly, you can determine your own matrix if you have some clear areas where only one stain is present (using ROI).

To do this, zoom up on your image in an area where both stains are well represented separately using the **Magnifying glass tool** from the toolbar.
Select the **Rectangle tool** from the toolbar.
Then go to the **Colour Deconvolution** plugin as before. Select **From ROI** from the drop-down box.
You should also select **Show matrices** but not **Hide legend**. The legend stores the values that you may need in order to create your own stored vector and/or include in a macro.
Click **OK**.
The message below appears. Click **OK**.

![Message](image)

Draw a region of interest over an area where only one stain is represented. It can be small if necessary although a larger area provides better sampling. Right-click the mouse after you have drawn the ROI.
The window below will appear. Again, click **OK** and then draw another ROI for the other stain and right-click.

The window below will appear. You can draw another ROI in the “white/unstained” area and then right-click or otherwise, just right-click to end.
As shown below, using the ROI has resulted in a much better separation of the two stains. There is very little in the residual image compared to using the inbuilt vector even though it is “correct” for this image.
Comparison of results

<table>
<thead>
<tr>
<th>Built-in H DAB vector</th>
<th>ROI – user-defined values</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image 1" /></td>
<td><img src="image2.png" alt="Image 2" /></td>
</tr>
<tr>
<td><img src="image3.png" alt="Image 3" /></td>
<td><img src="image4.png" alt="Image 4" /></td>
</tr>
</tbody>
</table>

The haematoxylin images look similar other than being a slightly different hue. However, the DAB image from the built-in vector appears to show slightly less staining as well as being a different hue.
Aarea measurements (automatic threshold) confirm this as below (uncalibrated – pixels squared).

**Built-in H DAB vector**

Where two stains coexist and there are very few pixels where only one stain is represented in the image (or if there is a general diffuse background stain), it may be difficult to separate them out since it’s impossible to find an area without the mixed stains. This “mixed” region may show up in the 3rd image rather than it being white. In this case, you should develop your own vectors experimentally from single stains.

**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 and can also be used in a macro for batch processing.