

Tutorial for cell nucleus intensity clustering on histochemically stained tissue microarrays.

<http://www.nexus.ethz.ch/> -> Software -> TMARKER

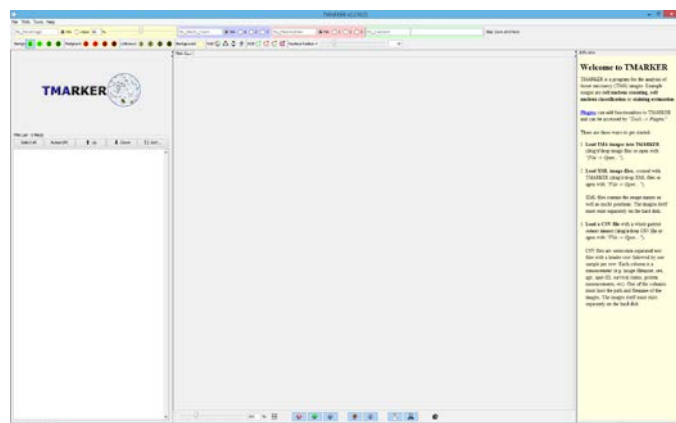
The plugin „Intensity Clustering“ starts on step 7.
Steps 1-6 are only needed to detect nuclei from scratch.

PREREQUISITES:

- Java 1.7 (Runtime Environment JRE).
- Tissue images of IHC stained tissue (see DemonstrationData.zip)

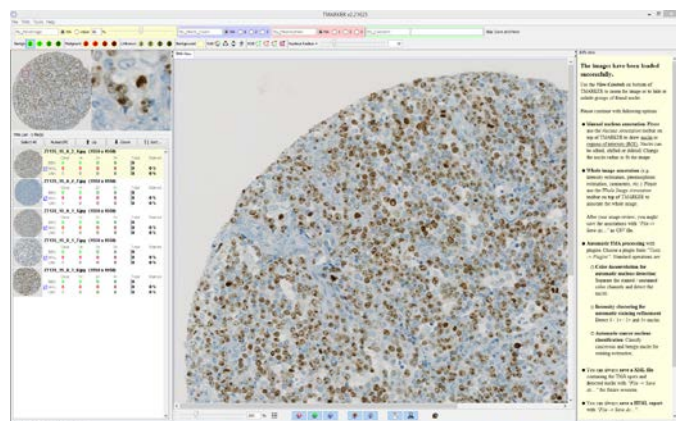
WORKFLOW

1. Open TMARKER.

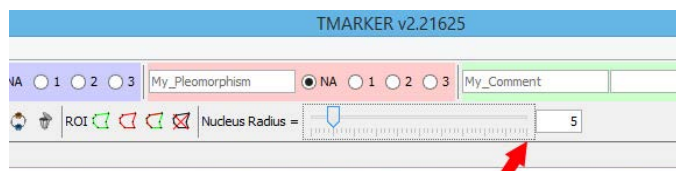


2. Drag and Drop the images (one image per TMA spot) from the demonstration dataset (Folder “RCC”) into TMARKER.

(Or alternatively click “File -> Open...” and select the images).



3. Set the nucleus radius to 5.



4. Select all images that you want to process with nucleus detection.

TMA List - 5 file(s)

Select All Human/PC Up Down Sort...

	Clear	1+	2+	3+	Total	Stained
ZT135_15_A_2_1.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	0	0	0	0	0	0 %
ZT135_15_A_4_7.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	0	0	0	0	0	0 %
ZT135_15_A_1_1.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	0	0	0	0	0	0 %
ZT135_15_A_1_7.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	0	0	0	0	0	0 %
ZT135_15_A_1_8.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	0	0	0	0	0	0 %

5. Open the “Color Deconvolution” plugin in the tools and use following parameters in the upcoming window:

Staining Protocol = H DAB.

Tolerance = 5

Blur = 2

T_hema = 55

T_dab = 110

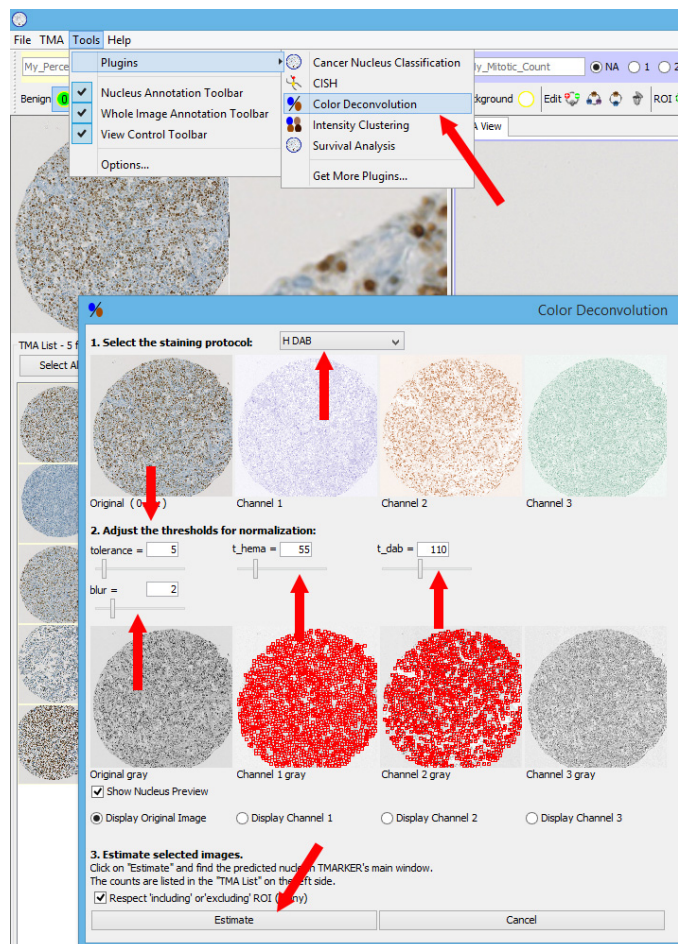
Click on „Estimate“.

This is the **Color-Deconvolution** method. The two color channel images Hema and DAB are separated. They can be seen by selecting “Display Channel 1” or “2” in the plugin after click on “Estimate”.

How are nuclei detected?

The two channels are blurred and local intensity minima are detected in the image. A local minimum is accepted if it is below the threshold and if it has a minimum difference to the neighboring local minimum of *tolerance*.

Accepted nuclei are filtered not to overlap to each other according to their radius.



6. The nuclei are displayed in the main window as “**Unknown**” nuclei (since they do not have a known cancer/benign state – they are just detected).

As the nuclei arise from the *Hema* (unstained) channel and from the *DAB* (stained) channel, they appear in the “**Clear**” and “**3+**” column of the TMA List.

Example here: 2.2k blue nuclei (unstained) and 2.6k brown nuclei (stained) have been found (54% stained).

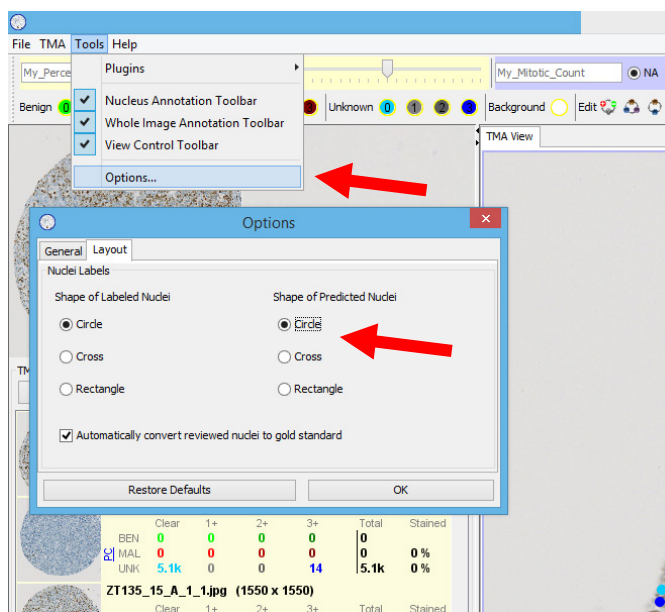
TMA List - 5 file(s)

Select All Human/PC Up Down Sort...

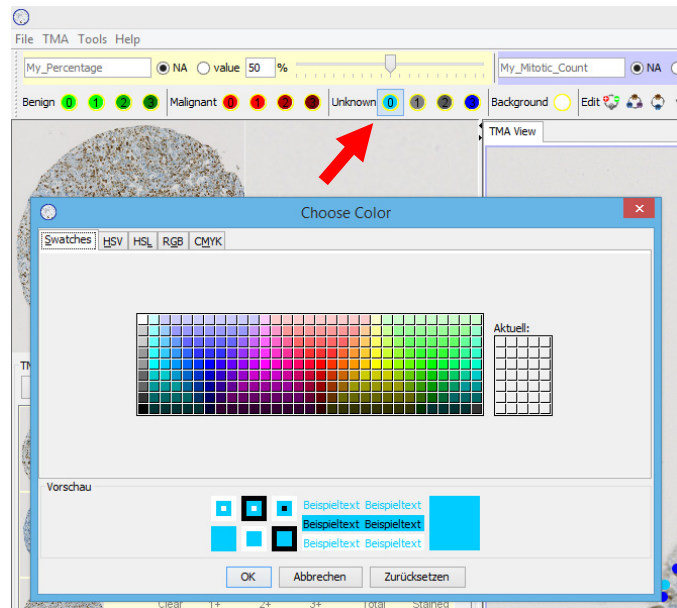
	Clear	1+	2+	3+	Total	Stained
ZT135_15_A_2_1.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	2.2k	0	0	2.6k	4.8k	54 %
ZT135_15_A_4_7.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	5.1k	0	0	14	5.1k	0 %
ZT135_15_A_1_1.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	2.6k	0	0	2.0k	4.7k	43 %
ZT135_15_A_1_7.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	3.7k	0	0	510	4.2k	12 %
UNK	1.7k	0	0	2.8k	4.5k	62 %
ZT135_15_A_1_8.jpg (1550 x 1550)						
BEN	0	0	0	0	0	0 %
MAL	0	0	0	0	0	0 %
UNK	1.7k	0	0	2.8k	4.5k	62 %

TIP: You might want to adjust the shape and the color of the nuclei for better visualization:

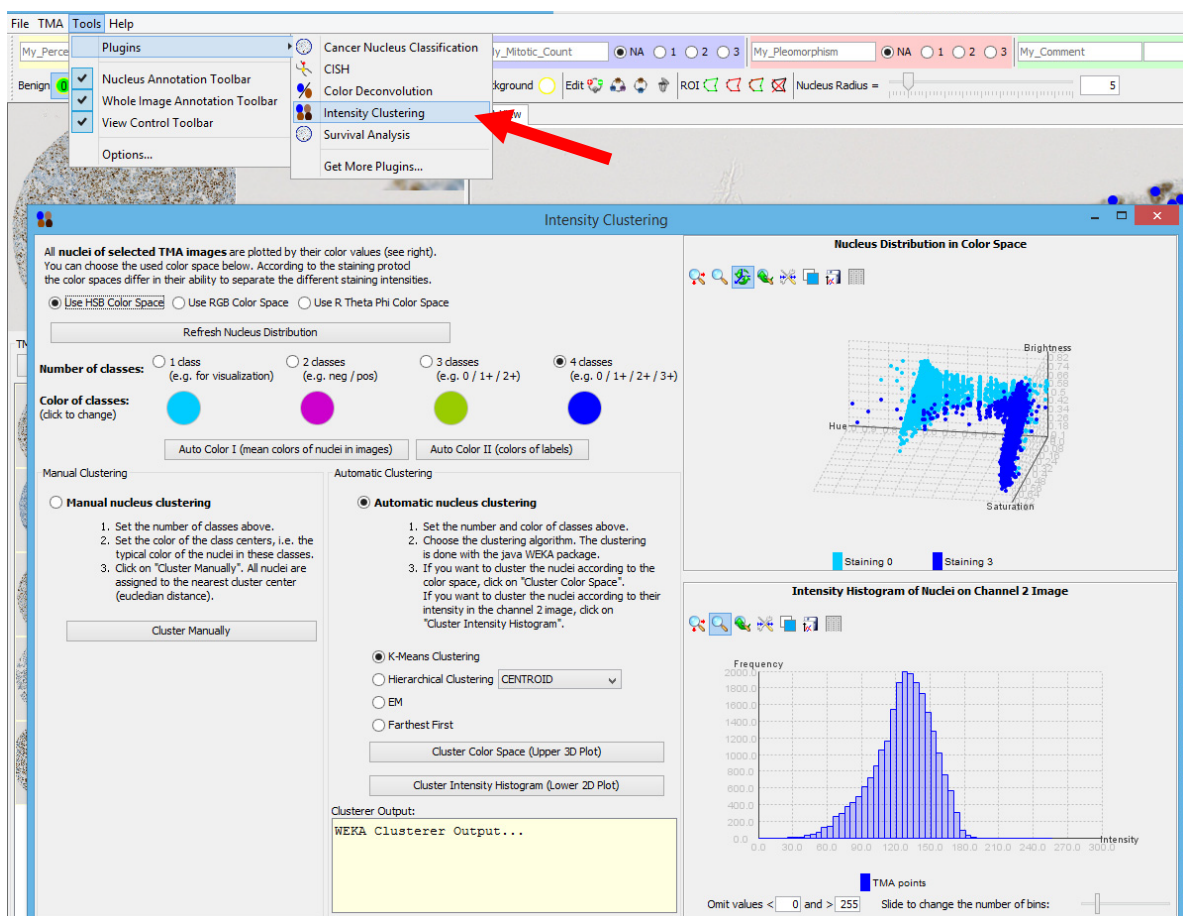
Shape: Click on Tools -> Options -> Lay-out and select “circles”.



Color: Double click on the Unknown Label and select the color.



7. Once the nuclei are found in the image, we **cluster the nuclei by staining intensity**. For this, select all TMA spots. Then click on *Tools -> Plugins -> "Intensity Clustering"*.





The Intensity Clustering Plugin Window

Top left, the color space can be chosen. Mostly, the HSB (hue, saturation, and brightness) color space works best separating blue and brown nuclei. You can also try RGB (red, green, blue) and R (radius) theta phi color space.

Select HSB for this tutorial.

Then, the number of clusters can be defined. Select the number of expected intensity classes in the images (e.g. 4 for “clear”, “minor”, “moderate” and “strong staining”).

Select 4 for this tutorial.

Thereafter, the colors of the classes are shown. For the manual clustering, these colors define the manually drawn cluster centers (i.e. the typical colors of the nuclei in these classes). For the automatic clustering, these colors are only for visualization in the 3D plot.

Then, select the clustering procedure (manual / automatic).

- For **manual clustering**, the nuclei are clustered according to the indicated colors of the classes, based on Euclidean distance.
- For **automatic clustering**, select the preferred clustering algorithm (default K-Means Clustering) and let the plugin select the best clusters.

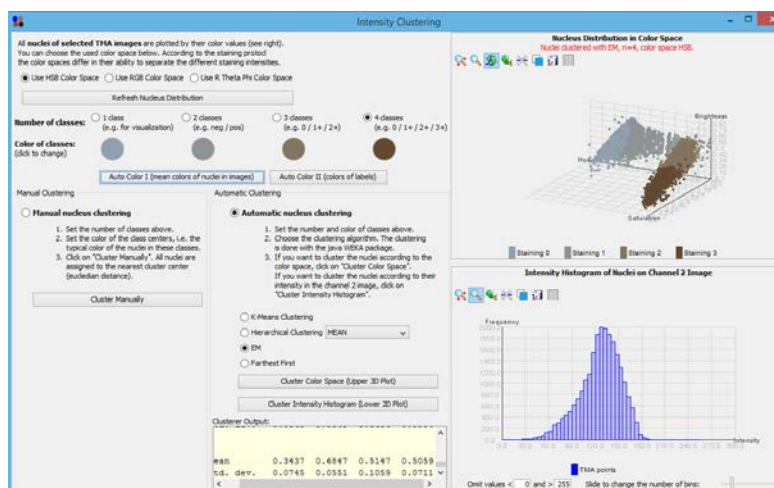
Cluster Color Space: The plugin clusters the nuclei based on their distribution in the 3D plot (top right). If you see clear intensity clusters in the selected color space, this might be the best solution.

Cluster Intensity Histogram: The intensity histogram (bottom right) results from the color-deconvolution channel 2 (DAB-Channel) from the “Color Deconvolution” Plugin. It is only available if the color deconvolution has been performed previously. If you see two or more clear peaks in this histogram, this might be the best solution.

Select Automatic clustering with EM Clustering for this tutorial. Press “Cluster Color Space”.

TIP: Drag the upper right 3D Plot to inspect the found cluster assignments.

TIP: After clustering, click on “Auto Color I” to see the mean colors of the nuclei in the individual clusters.





8. The nuclei in the main TMARKER window are immediately clustered accordingly. The number in the TMA List have been updated.

Note that some of the previously “un-stained” nuclei can now be clustered as “1+”, and vice versa. Therefore, the staining percentage might change after clustering.

If the staining estimation is “correct” depends on your problem and control. You might e.g. change the number of clusters to three classes instead of four influencing the staining percentage.

TMARKER provides the tool to reproduce the nucleus numbers once you have justified reasonable parameters.

