Heidelberg University

BioQuant, IPMB

Biomedical Computer Vision Group

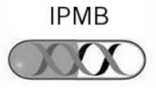
ColocQuant and *ColocJ*:

Software for quantification and visualization of particle colocalization in fluorescence microscopy images

User guide

by B.Sc. Roman Thielemann,

M.Sc. Christian Ritter, PD Dr. Karl Rohr



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Introduction

This guide explains and illustrates how to use the software tools *ColocQuant* and *ColocJ*, developed by the Biomedical Computer Vision Group (head: PD Dr. Karl Rohr). *ColocQuant* is a software tool that performs detection and colocalization analysis of fluorescently labelled viral particles in live cell microscopy images. It allows the user to adjust important detection and colocalization parameters to adapt the image analysis methods to the image data. Results of the analysis are saved in a chosen directory and can be visualized using the software tool *ColocJ*. With *ColocJ*, an ImageJ macro, the user can visualize important information of the performed colocalization analysis. Its goal is to ease understanding and interpretation of the colocalization results in order to obtain insights of subcellular processes.

ColocQuant

ColocQuant is a software tool for the detection and colocalization analysis of colocalized particles in fluorescence microscopy images. It comes with a graphical user interface (GUI, see Figure 1) to make the application more user-friendly. Using the GUI, the user can select the images for performing particle detection and colocalization. Also, the user can choose the directory where the results are saved and select if *ColocQuant* is supposed to perform a double or triple colocalization or both. Furthermore, the GUI allows changing important parameters for the detection, like *Filter size* and *Detection Threshold* for each channel, and the maximal particle distances (*Dmax*) for a colocalization.

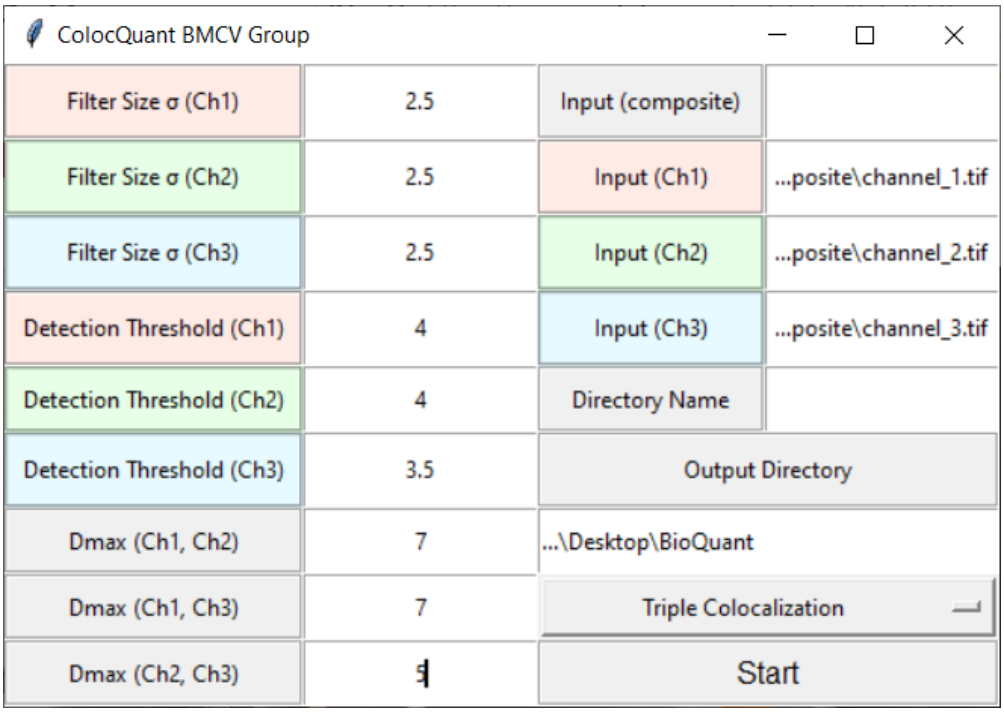


Figure 1: Graphical user interface (GUI) of ColocQuant.

Getting started

1. Open *ColocQuant* by typing `python` on your computer command prompt followed by the location of the *ColocQuant* file on your computer:

   ```
   python C:\Users\BMCV\Documents\ColocQuant\pipeline.py
   ```

   Hint: On Windows, the command prompt can be opened by pressing [Windows + R], typing `cmd` and pressing `Enter`.
2. The GUI will appear on your screen. Here, you can change the parameters for detection and colocalization by clicking on the fields containing the values and changing them manually. Changing the directory name is done analogously.

   Hint: How each parameter influences your detection and colocalization results is described in the Section “Changing parameters”.
3. Select the colocalization mode (triple, double, triple and double) or select “detection” to perform only detection without colocalization by clicking on the *Triple Colocalization* drop-down menu.
4. To change the input images, click on the label *Input (Ch*)* for each channel or select a multi-channel image by clicking on the *Input (composite)* label. A dialog window will appear, asking to choose an image. Selecting the target directory works analogously: just click on the *Output directory* button and select a directory.

   Hint: The input images need to be .tif files and should all contain the same number of frames.
5. Start the program by pressing the *Start* button. It will then automatically perform detection and colocalization and save the results.

Changing parameters

With the GUI, the user can change three parameters: *Filter size* and *Detection Threshold* influence the detection results, while the maximal particle distance (*Dmax*) influences the colocalization results.

Filter size: Particles need to have a minimum size to be detected. The size of the smallest detectable particle depends on the *Filter size*. To detect smaller particles, a smaller *Filter size* should be chosen. For the detection of larger particles, increasing the *Filter size* is recommended. However, the *Filter size* does not directly correspond to the size of a particle, i.e. *Filter size* = 3.5 does **not** mean that only particles with a size larger than 3.5 pixels are detected.

Since detection is performed for each image channel separately (Ch1, Ch2, and Ch3), the *Filter size* and *Detection Threshold* parameters can also be chosen separately for each channel, depending on the signal-to-noise ratio.

Detection Threshold:

With the *Detection Threshold* parameter, particles are filtered out based on their intensity. A low *Detection Threshold* allows the detection of particles with lower intensity. If the detection results contain too many particles that are barely visible, i.e., with low intensity, increasing the *Detection Threshold* discards these detections.

Dmax:

The maximal particle distance (*Dmax)* defines how close two particles need to be in order to be considered colocalized. For a triple colocalization, the software allows the adjustment of the distances between all channels separately (see Figure 2). Here, the chosen values correspond to the pixel distances between the particles. Note, the center of the detected particles is used to calculate the distance between two particles.

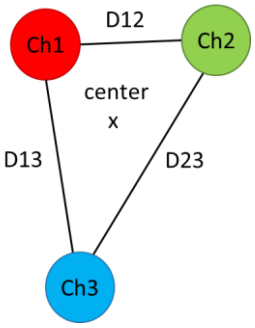


Figure 2: Maximal particle distances (Dmax) for a triple colocalization.

Result directory

The detection results created by *ColocQuant* are saved in a directory whose name and path can be chosen using the GUI. Results within the directory are organized as follows:

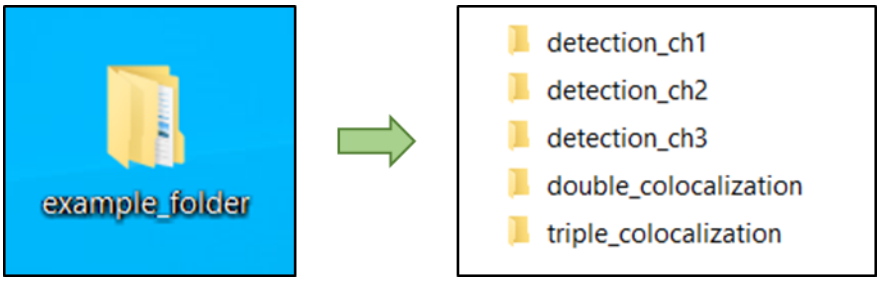


Figure 3: Directory structure created by ColocQuant.

In Figure 3, a three-channel microscopy image was used as input to create both double and triple colocalizations. Detection results are saved separately from the colocalization results in subdirectories named “detection”, one for each channel. Each detection subdirectory contains a “tracks.mdf” file, which can be opened with the ImageJ Plugin MTrackJ to visualize the detection results for this channel. The “tracks.mdf” file, e.g., for channel 1 can be found under `detection_ch1` -> `results` -> `tracks.mdf`.

ColocJ

ColocJ is a software written as an ImageJ macro. It visualizes the colocalization results computed by *ColocQuant* to quantify parameters of subcellular processes. This includes particle size, intensity and color distribution of the colocalized particles, as well as the number of colocalizations per frame. Additionally, *ColocJ* visualizes colocalizations in the fluorescence microscopy images and comes with useful keyboard shortcuts. Visualization can be performed for the entire image or for a selected region of interest (ROI). Also, *ColocJ* is designed modularly and therefore visualization methods can be chosen independently by the user.

Getting started

1. Installing the *ColocJ* macro in ImageJ: Open ImageJ, go to `Plugins` -> `Macros` -> `Install…` and select the *ColocJ*.ijm file. Check if the installation was successful by going to `Plugins` -> `Macros`. *ColocJ* should now be present in the dropdown menu. If not, `Help` -> `Refresh Menus` might help to update the ImageJ menu after installing a new macro. Otherwise, repeat the installation process and make sure to choose the correct file. If necessary, restart ImageJ and repeat the installation process.
2. To visualize colocalizations with *ColocJ*, an image needs to be opened in ImageJ. For full functionality, the TIF-image(s) used for detection and colocalization should be opened as a composite image. This can be done simply by dragging the composite image into the ImageJ GUI.
3. To visualize only data from a ROI, select the region using the **polygon selection tool**. Otherwise, data for the entire image is visualized.
4. Go to `Plugins` -> `Macros` -> *`ColocJ`* to start *ColocJ*.
5. A pop-up window will appear, asking which visualization methods should be used. The user can choose to either visualize double or triple colocalization data, or detection results. Also, additional visualization methods can be chosen here. If *Boxplot* is selected, *ColocJ* will create boxplots for the intensity and size of the particles of computed triple colocalizations, as well as a graph with the number of colocalizations per frame. If *Maxwell/Ribbon* is selected, *ColocJ* will create the Maxwell color triangle or a color ribbon to visualize the color composition of the triple or double colocalizations. The choices *center* and *nodes* determine from which positions the intensity values are taken; this is further explained in the section "Example live cell images" below.
6. Click *OK* after deciding on the visualization methods.
7. *ColocJ* now asks to open a “colocalization directory”, meaning a directory that was created by *ColocQuant*. The data from this directory will then be visualized.

8. *ColocJ* will now visualize the data. A dialog window will appear, displaying the “Total number of colocalizations”. Click *OK* to see the selected visualizations. It is recommended not to use other functions of the computer until the generation of the visualizations is finished, and the dialog window has appeared.

   Hint: *ColocJ* might need some time to visualize data, especially if the option “Maxwell/Ribbon” was checked.

Visualization of Colocalizations

ColocJ can visualize colocalizations in different ways. Most visualization methods are available for both double and triple colocalization data, although the style of visualization might differ. The different types of visualization are introduced below.

Results table

The first type of visualization is the results table, it contains the x- and y-coordinates of all triple colocalizations and the x- and y-coordinates, the intensity and the size of each detected particle involved in a triple colocalization. The table can be saved as an Excel .csv file by closing it.

Markings in the input image

All computed colocalizations are marked in the input image with a circle around them and the number of the colocalization is displayed in the top right of the circle. This number makes it easy to look up quantitative information for a colocalization of interest in the results table. To hide the number, simply use the “hide number” shortcut (see the Section ”Keyboard shortcuts”). For triple colocalizations, all three detected points of the colocalized particles are connected by lines, forming a triangle. Each point of the triangle corresponds to a detected particle.

Boxplots

When the *Boxplot* box is checked in the starting dialogue window of the software, two boxplots are created to display the intensity and size of the colocalized particles. Intensity and size are displayed for each channel separately. The boxplots capture the median value, the lower and upper quartile (in form of boxes) and the 2.5th and 97.5th percentile in the form of whiskers (see Figure 4).

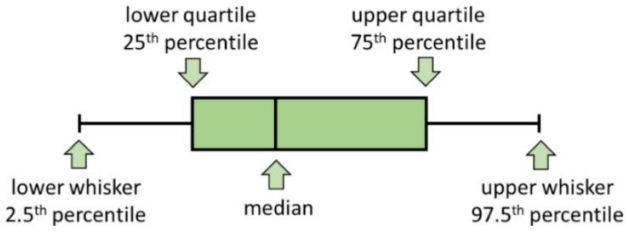


Figure 4: Information of the boxplot visualization.

Colocalizations per frame

In addition to the boxplots, a bar chart visualizing the number of colocalizations per frame is created when the *Boxplot* box is checked in the starting dialogue window.

Maxwell triangle and color ribbon

To visualize the color composition of colocalized particles, a *Maxwell color triangle* is created for triple colocalizations and a *color ribbon* for double colocalizations. Both techniques visualize the color contribution (ratio) of colocalized particles in different channels.

For triple colocalizations, the *Maxwell color triangle* (see Figure 5) represents the intensity of a certain color compared to the intensities of the other two colors. Double colocalizations can be visualized by a *color ribbon* (color gradient; Figure 6). Each computed colocalization is depicted as a black dot in these visualizations.

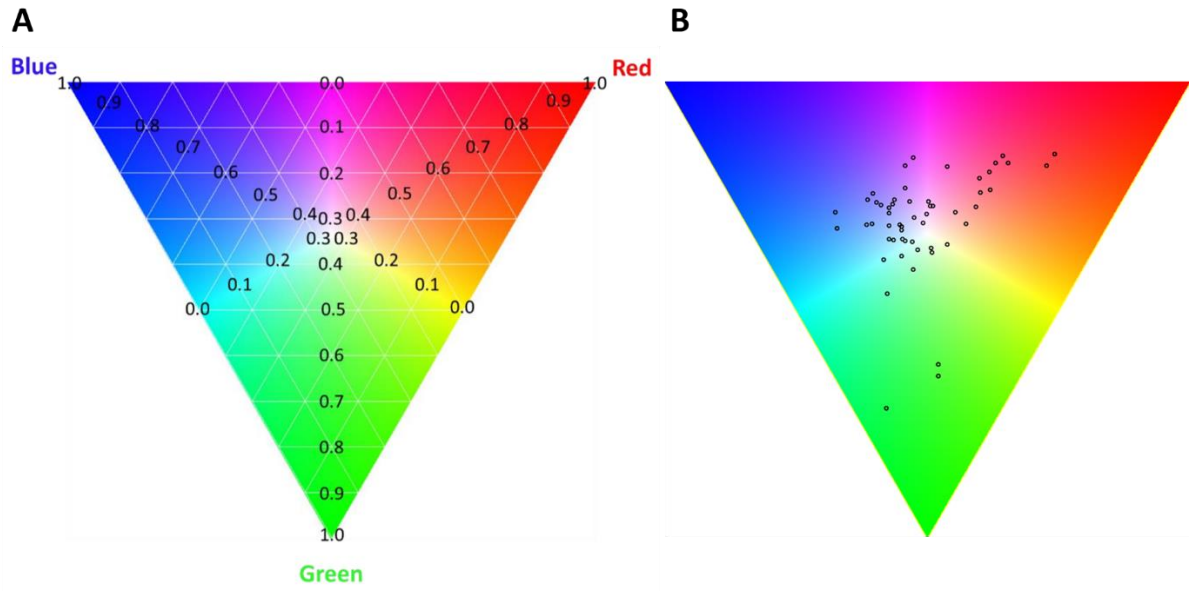


Figure 5: Maxwell color triangles with the three colors blue, red, and green. A) Three coordinate axes ranging from 0 to 1 are drawn to indicate the proportions of the primary colors at different points in the triangle. B) Example Maxwell color triangle with multiple triple colocalizations placed in the triangle based on the intensity values in the different channels. Each black dot represents a triple colocalization.

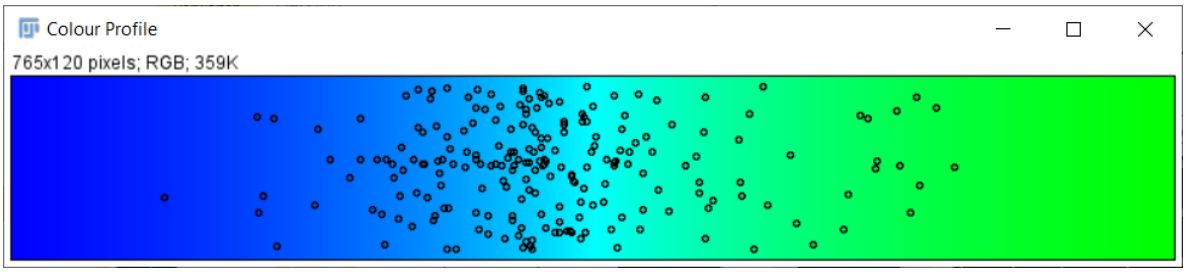


Figure 6: Example color ribbon with a color gradient showing the color distribution of double colocalizations of channel 2 (green) and channel 3 (blue). Each black dot represents a detected double colocalization.

Keyboard shortcuts

With the installation of *ColocJ*, multiple keyboard shortcuts are also installed and can be used in ImageJ. Table 1 shows a list of available shortcuts and their key bindings. These key bindings might overwrite native ImageJ key bindings. For example, the [s] shortcut natively saves the current image as a .tiff, but our *Show overlay* shortcut overwrites this function. To use the native shortcut for saving the current image, simply press [ctrl + s].

Table 1: Keyboard shortcuts that are available after installing ColocJ with their key bindings, names, and description of their function.

Key binding	Name	Description
s	Show overlay	Shows overlay of the currently active image.
h	Hide overlay	Hides overlay of the currently active image.
1	Channel 1	Hides/shows channel 1 of a three-channel image.
2	Channel 2	Hides/shows channel 2 of a three-channel image.
3	Channel 3	Hides/shows channel 3 of a three-channel image.
n	Hide number	Hides/shows the number of the colocalizations marked in the image.
y	Detection Ch1	Hides/shows detection results for channel 1 of the currently selected *ColocQuant* directory.
x	Detection Ch2	Hides/shows detection results for channel 2 of the currently selected *ColocQuant* directory.
c	Detection Ch3	Hides/shows detection results for channel 3 of the currently selected *ColocQuant* directory.
d	Detection count framewise	Counts the number of detections per frame for the entire image or in a selected ROI.
m	Binary movie	Allows the user to choose a threshold for each channel and creates a binary movie. Regions where detected particles in **all** channels overlap are shown in white. Works for multi-channel 16-bit images with one or multiple slices.

Example live cell images

ColocQuant and *ColocJ* were designed for multi-channel fluorescence microscopy images as input. Below, example images (from J.Y. Lee, R. Bartenschlager) with detection and colocalization results are shown.

Example visualizations using *ColocJ* of colocalized particles overlaid with the composite image are shown in Figure 7. In Figure 7A, no ROI is selected, and colocalizations are visualized for the whole image. In Figure 7B, an ROI is selected, and visualization is only performed for the selected area.

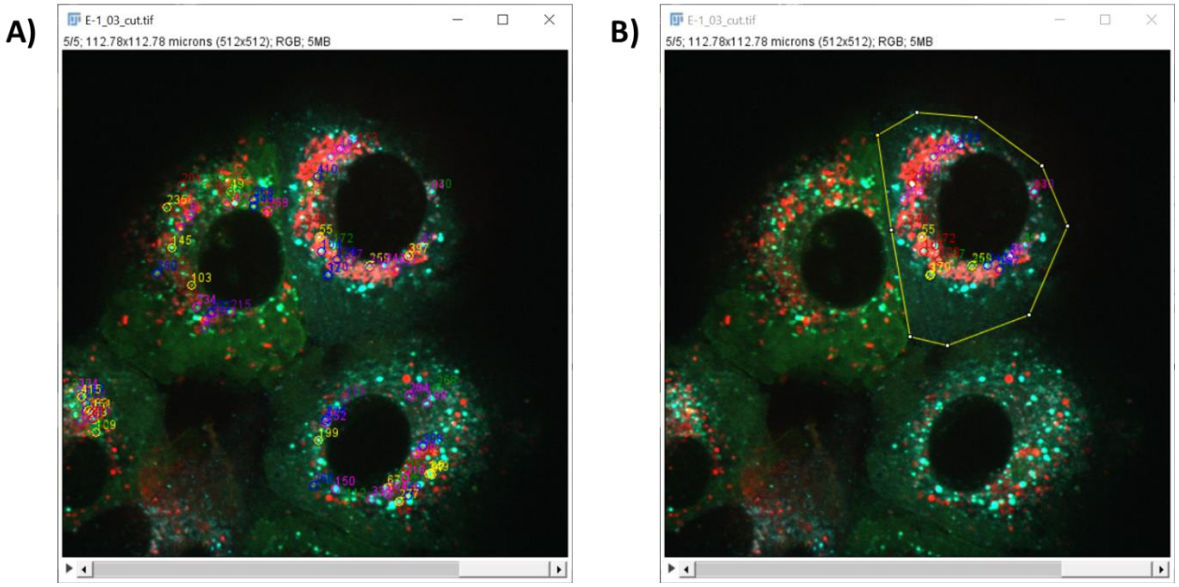


Figure 7: Visualization with ColocJ and selection of an ROI. A) No ROI was selected. The colocalization results for the whole image are visualized. B) An ROI was selected using the polygon selection tool. Only colocalization results in the ROI are visualized.

ColocJ marks colocalized particles in the input image with a yellow circle (Figure 8). The center of this yellow circle is the center of the colocalized particles. If triple colocalized particles are visualized, *ColocJ* uses a triangle to visualize the positions and distances between the three colocalized particles. This is illustrated in Figure 8: In the upper row, the three-channel composite image is shown with the marked triangle (left), and a schematic representation explaining the corners of the triangle (right). Single-channel images are shown in the lower row to further visualize how the triangle represents positions and distances between the colocalized particles.

The user can select from which positions the intensity values are taken to create the Maxwell color triangle or the color ribbon in the starting dialog of *ColocJ*. When selecting *Maxwell* or *Ribbon* (***center***), the intensity values for each channel are taken at the center of the colocalization (Figure 9). When selecting *Maxwell or Ribbon* (***nodes***), the intensity values are taken from the node positions of the triangle (Figure 8), which are the positions of the detected particles for each channel.

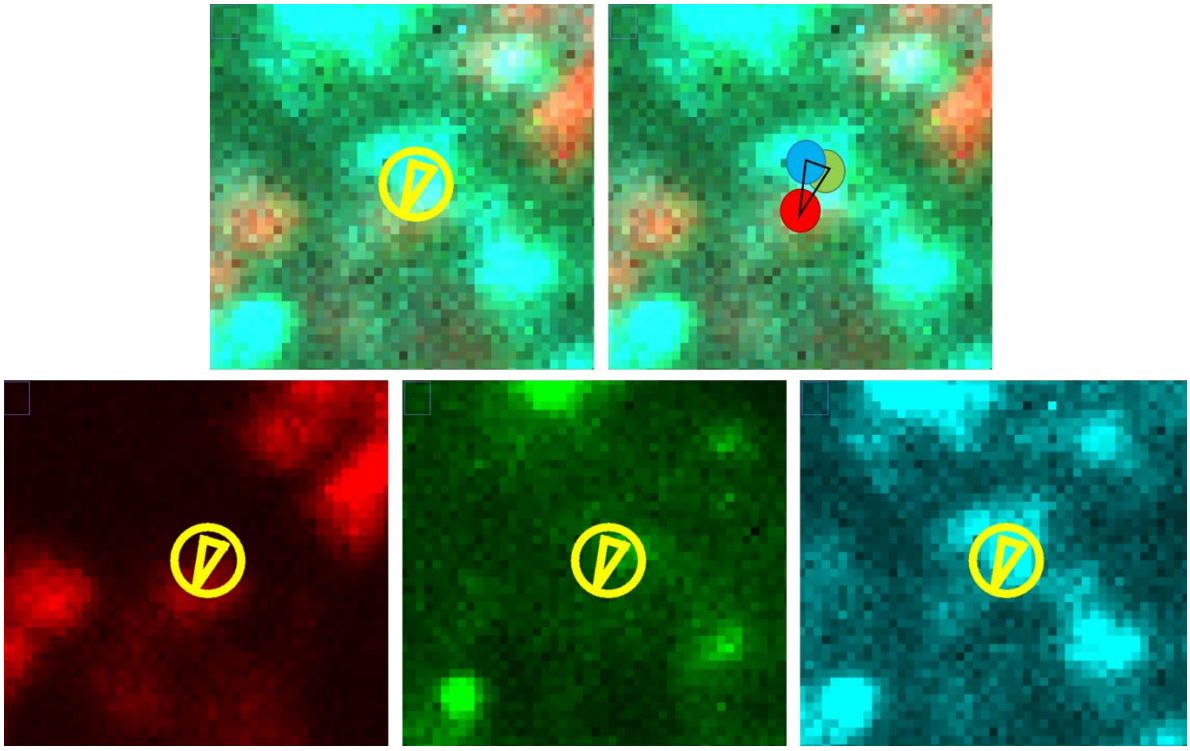
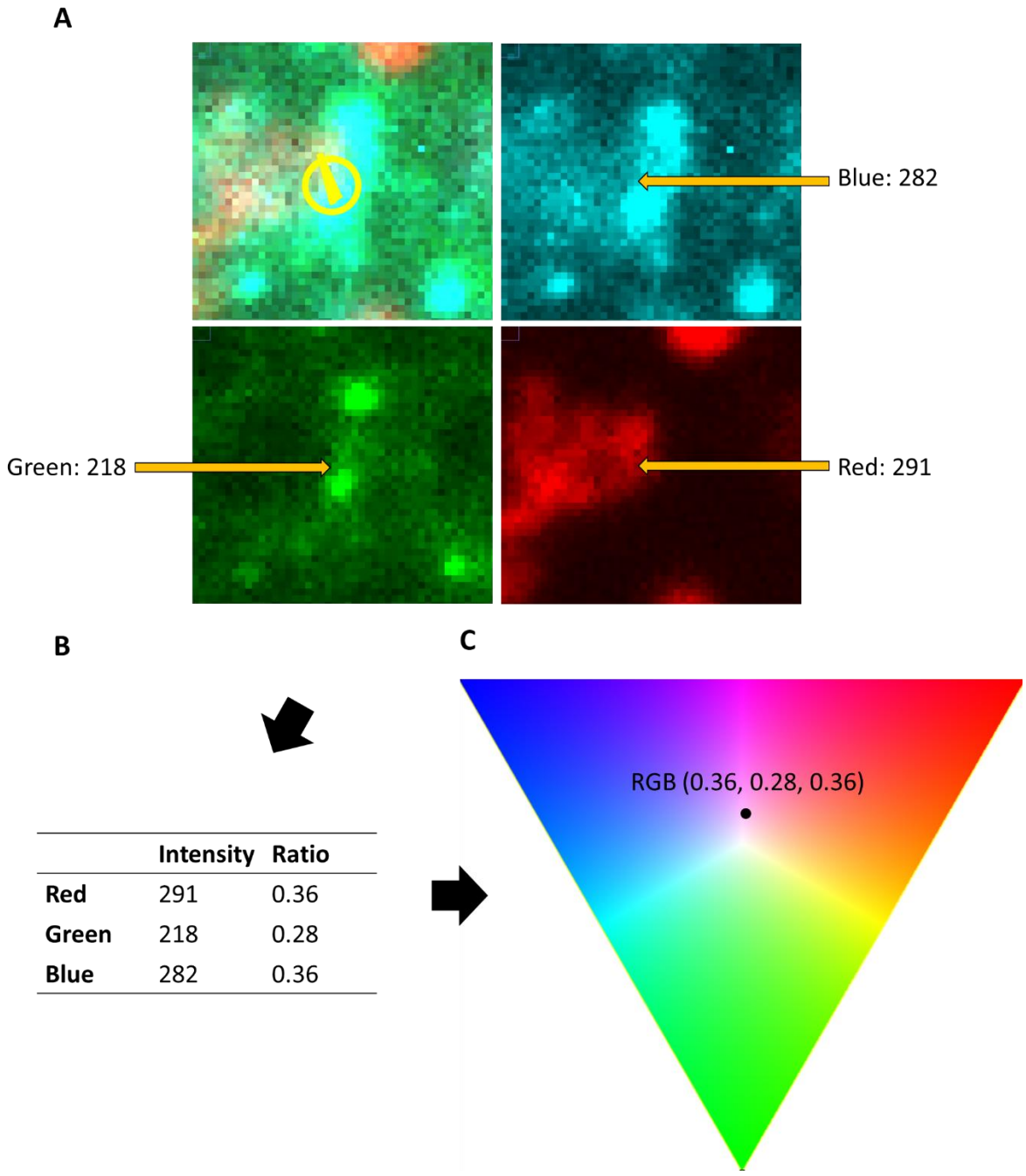


Figure 8: Marked triangle in a three-channel composite (upper left) and in the respective single-channel images (bottom row).

ColocJ can visualize the color composition of triple colocalized particles in a Maxwell color triangle and the color composition of double colocalized particles in a color ribbon. Figure 9 shows an example of how a triple colocalized particle is placed in a Maxwell triangle. Intensity values are taken from the center of the triple colocalized particle for each channel separately, indicated by the orange arrows (Figure 9A). The ratio between each intensity value and the intensity values in the other two channels is calculated (Figure 9B), and the ratios are used to place the triple colocalized particle in the Maxwell triangle (Figure 9C).



	Intensity	Ratio
Red	291	0.36
Green	218	0.28
Blue	282	0.36

*Figure 9: Flowchart showing the process of how a triple colocalized particle is visualized in the Maxwell color triangle based on the intensity values in the different channels. A) Intensities are taken from the **center** (indicated by orange arrow) of the triple colocalization for each channel. B) The ratio of the intensities is calculated. C) Colocalized particle is placed in the Maxwell triangle based on its intensities.*

Figure 10 shows the same procedure for a double colocalized particle being placed in a color ribbon.

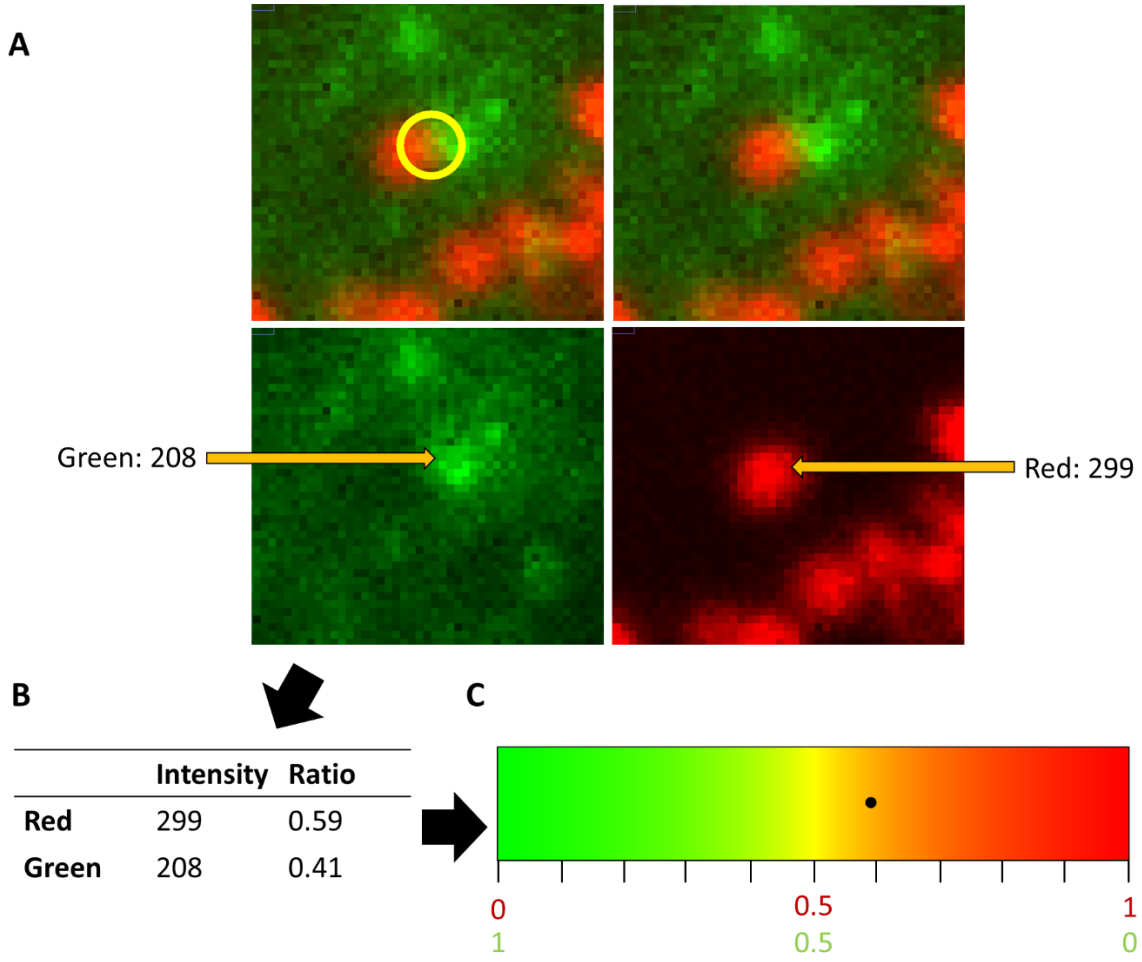


*Figure 10: Flowchart showing the process of how a double colocalized particle is visualized in the color ribbon based on the intensity values in the different channels. A) Intensities are taken from the **center** (indicated by orange arrow) of the double colocalization for each channel. B) The ratio of the intensities is calculated. C) Colocalized particle is placed in the color gradient based on its intensities.*