COOLLOCALIZER

Image analysis software for confocal microscopy

Walk-through manual

Version 1.0

About CoolLocalizer

Quick description

CoolLocalizer performs images processing and co-localization analyses on images acquired by fluorescence microscopy.

Why CoolLocalizer?

The application was developed by a team of scientists exclusively for other scientist and for their own needs. The other software used was often confusing, unstable (constantly crashing), too complex or loaded with wrong functions, or lacking functions needed. The goal was to develop an application that was

Powerful in doing what it is designed to do
Easy to use
Quick to learn
Equipped with algorithms capable of performing correct quantitations
Keeping the user in total control over the process
Capable of opening a range of formats including Biorad .PIC and Leica TIFF
Capable of saving screenshots and montages as standard image formats including JPEG, GIF, BMP, and others suitable for documentation, image handling and presentations
Possible to run under standard operative systems including Mac OS X and Windows XP, but also Windows 95, 98, 2000, Me, NT, and Mac OS from version 8.6.

We hope that we have succeeded in our goals.
Welcome to CoolLocalizer!

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About this Walk-through

The walk-through is not a complete users manual. As a microscopy user you have probably a vast experience in computer use, file handling, image processing and the application is designed with the intention to be very easy to use and learn. A **Feedback area** on the screen will provide information that sometimes might be useful for how to go about.

You might find it useful to start by using the demo images that came with the software.

> Notes displayed like this indicate more specialized information of sometimes-technical nature. These notes might or might not contain understandable or useful information. Bugs and other known issues are mentioned to save the beta tester from reporting already known flaws.

About supported computer platforms

CoolLocalizer was written in three versions that run under Windows, Mac OS X, and Mac Classic, respectively. The Windows version runs under Windows 95, 98, 2000, Me, NT, and is enhanced for XP compatibility. The Mac OS X version is fully carbonized and runs also in the Classic environment. However, for optimal stability under Mac OS 9 and earlier it is preferred to run the version specifically written for Classic Mac OS. All screen-shots in this manual are taken from the Mac OS X version of the software. Users running Windows or Mac Classic will find that layout details differ somewhat but not significantly from the figures.

How to report feedback

Report feedback by email to the address provided at the end of this Walk-through.

Launching the application

Double-click the application icon. The application opens up.

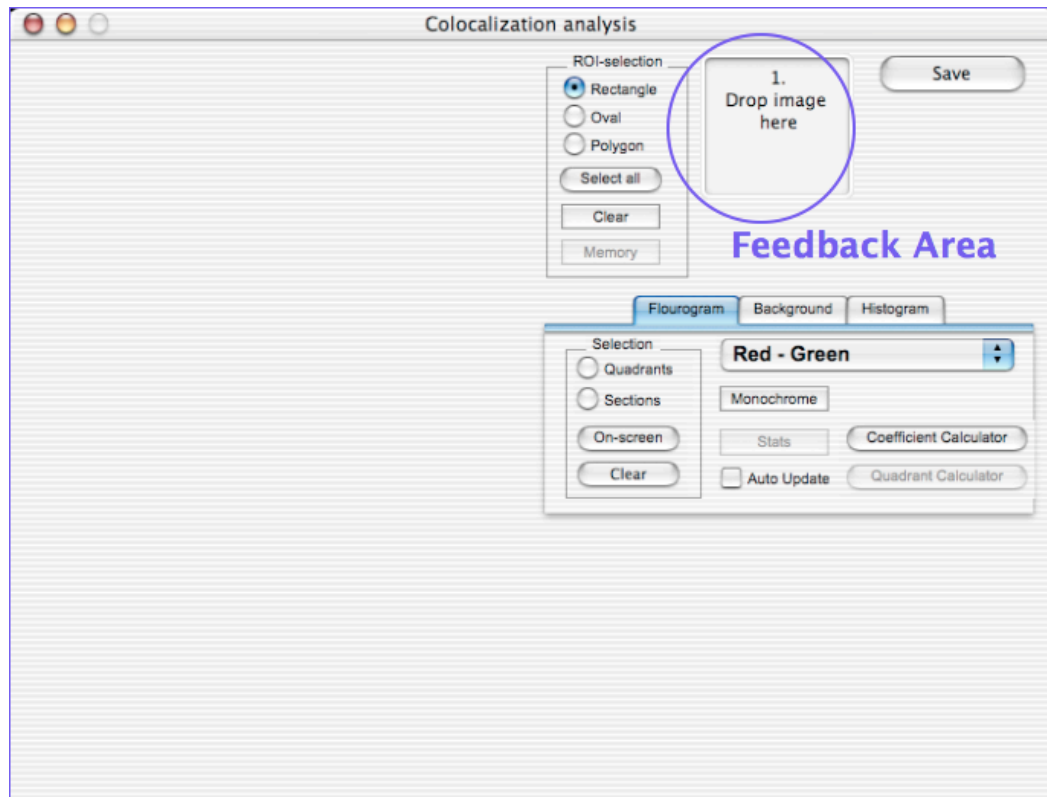**Figure 1: Import images by dragging a file icon into the Feedback area**

Importing images

Importing files

There are two ways images can be imported:

1. Drag-drop method

This is the easiest way to import image files. Drag a file from any location into the Feedback area (Figure 1).

2. Open dialog

Under **File** in the Menu bar, select **Open**. Select the desired image file.

Importing Biorad .PIC files

PIC files require a separate import step. Under **File** in the Menu bar, **select Import Biorad PIC.** The Import window opens up (Figure 2). If you know the order the channels were acquired you might select the sequence using the **RGB Popup menu**, choose RGB for red, then green, then blue etc.

Note: Images with only one channel will be useless for co-localization studies. However, CoolLocalizer can be used to automatically process all Biorad files. One-channel pictures will be displayed as grayscale images but can be converted to color based on the first letter in the RGB Popup menu choice (for green select GRB or GBR etc).

Drag a Biorad .PIC file into the upper area of the window. The image will be automatically processed and presented in the window.
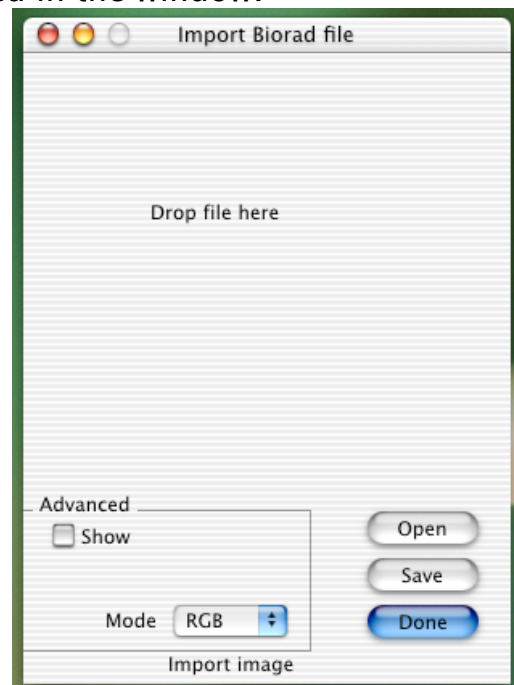
Figure 2: Drag to import Biorad files.

To change the representation of the color channels, modify the sequence using the **RGB Popup menu**.
When the image is displayed correctly, press **Done**. The image will be transferred to the main window. The import window is kept in the background and can be closed at this point or kept open for further imports.

Note: CoolLocalizer can be used as a conversion tool of Biorad images. After importing files images can be exported by dragging the displayed image to the computer desktop (Mac only) or by pressing the **Save** button (all platforms)
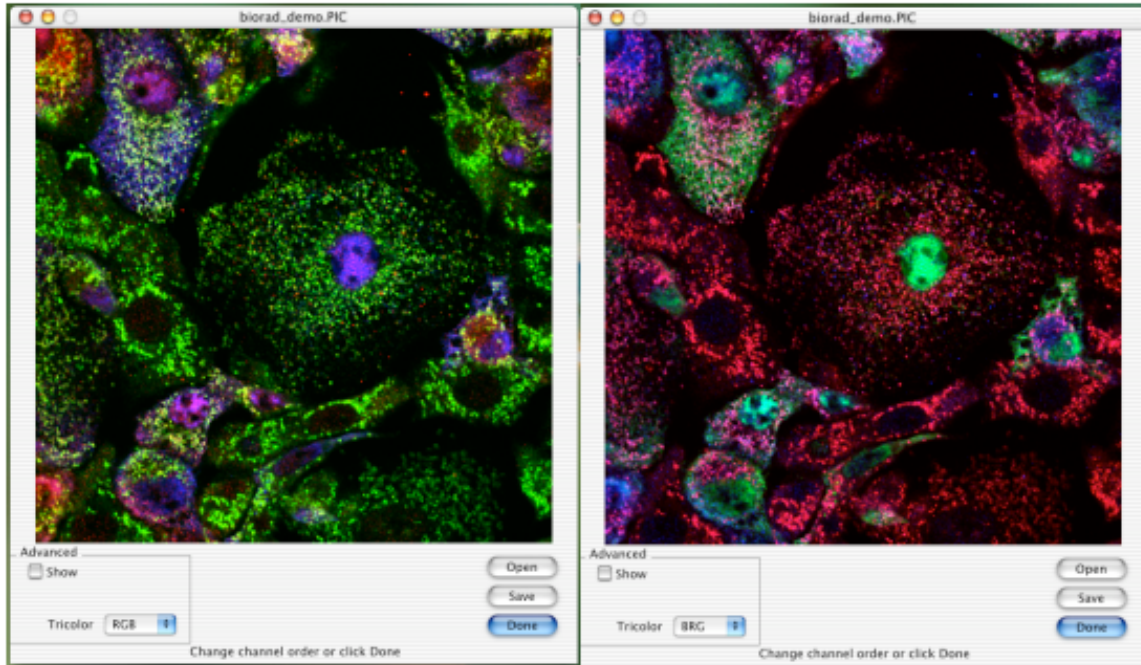
Figure 3: Importing Biorad files. Change the tricolor channel sequence by using the RGB Popup menu

Biorad files with phase-contrast

Phase-contrast images cannot be used for co-localization analysis. However, CoolLocalizer can handle and process such image files as long as the total number of channels do not exceed 3. The phase-contrast image will be handles as a regular channel that is not used by the other colors. To import such images, first select **Import Biorad PIC** under **File**. Check the **Show Advanced box.** Press the **Phase** listbox and select the channel (**R**=red, **G**=green, **B**=blue) that contains the phase-contrast image.
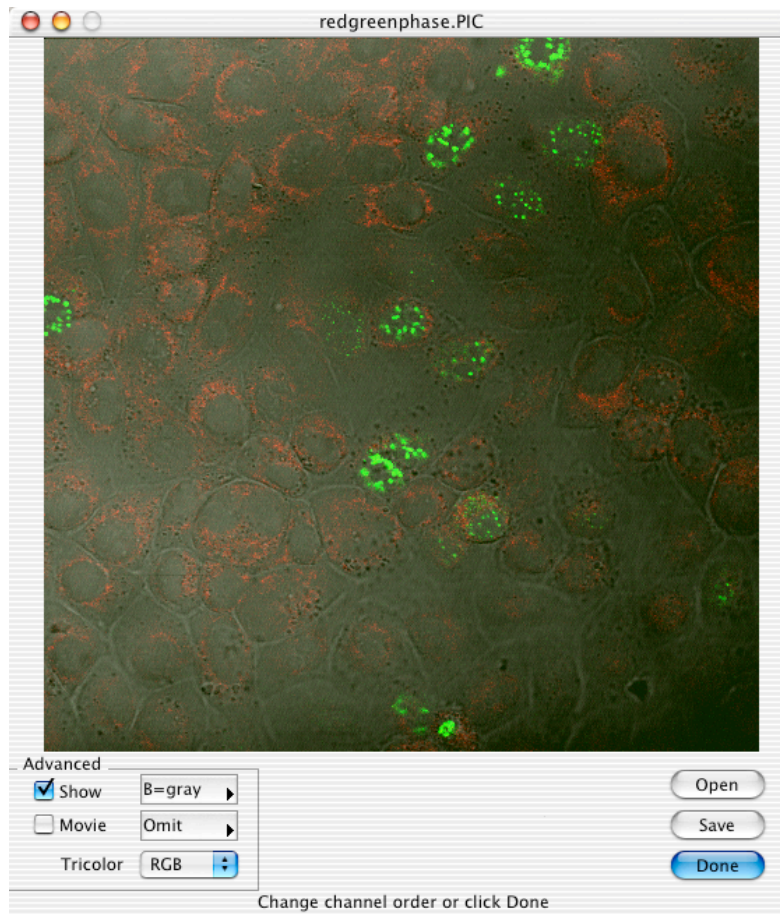
Figure 4: Phase–contrast biorad images can be imported. This image contains red and green channels and a phase–contrast picture. Select "B=gray" to display the non–green/non–red channel as phase–contrast.

Importing sequentially acquired files

Select **Import Biorad PIC** under the **File** menu. Locate the two or three files containing the sequentially acquired channels. Select the file's icons and drag them all together into the **Import Biorad file** window (see Figure 5). If necessary, specify the channel order using the **Tricolor pop-up menu.**

Note: For reasons why and an example on the necessity of performing sequential aqcuisition, see the section "Why acquire sequentially?" in Appendix 1 at the end of this manual.
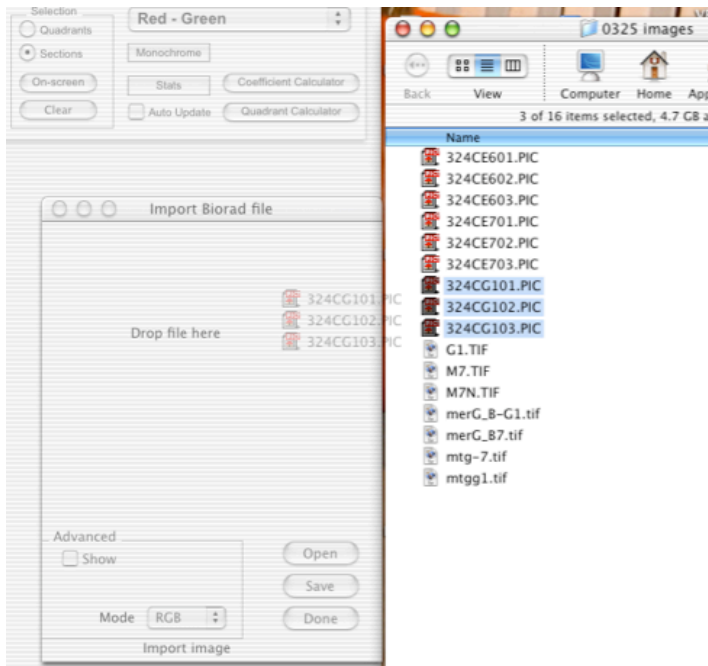
Figure 5: Importing images with sequentially acquired channels.

Importing movies and Z-stacks

Sequential images (movies and Z-stacks) are handled identically by CoolLocalizer.

Define background levels

Background removal is not required for further processing. This step is therefore optional. As will be seen later, background can easily be handled by simply omitting pixels below certain intensities in quantitations and calculations. However, it might be useful to once and for all assign intensity levels below background to zero.

Activate background handling

Point the mouse to the **Tab** called **Background**.
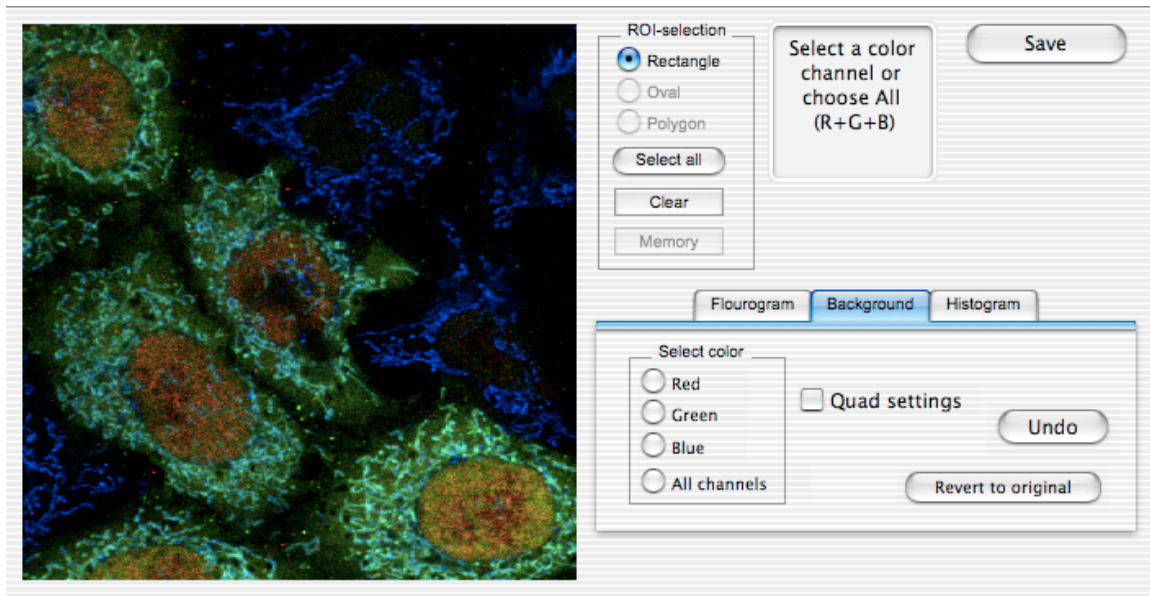
Figure 6: Background handling is activated by selecting the "Advanced" tab.

Process background for all channels

This option is useful when the image contains an area that is supposed to be black.
Press the radio-button labeled **All**. On the imported image, draw a rectangle that only contains pixels of the background level.

> Note: It is common among image processing software to remove background by subtracting with the specified background intensity. This has the effect that color-saturated pixels falsely appear as non-saturated. The background handling in CoolLocalizer, aimed at performing co-localization analyses, therefore instead sets pixels with background intensities to zero.

Process background for specific color

Press the radio-button labeled with the desired color channel. On the imported image, draw a rectangle that only contains pixels of the background level.

The image will be automatically processed for all colors.

Figure 7: Select one or all channels and draw a rectangle containing background only.
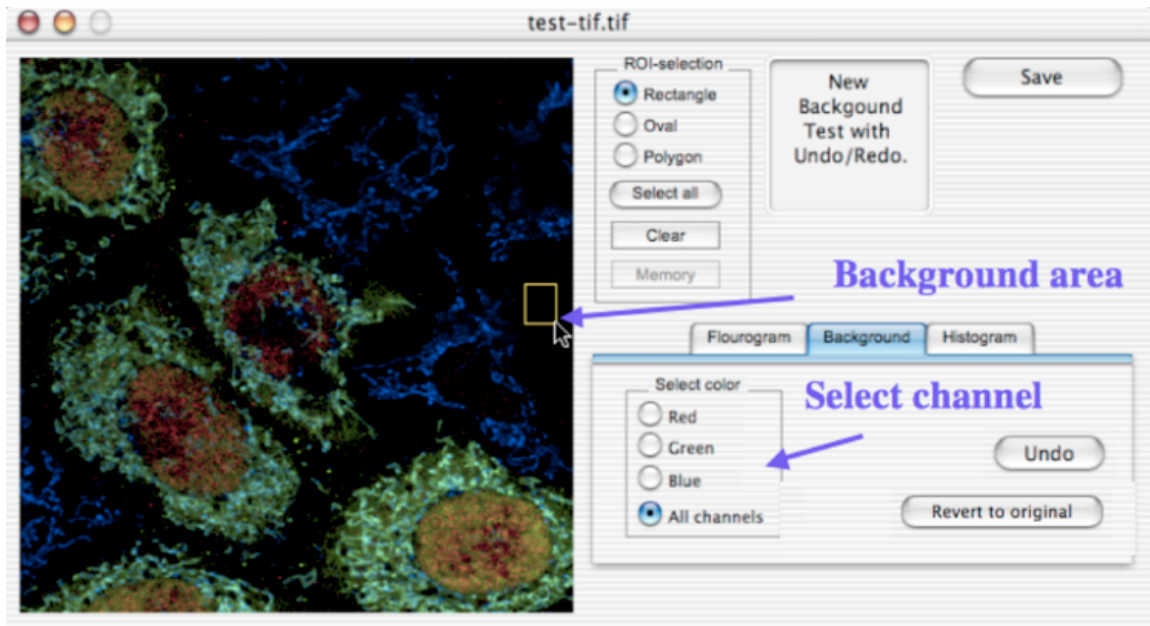
To evaluate the background handling you can toggle the view using the **Undo** button. To re-process the background, press **Undo** and specify another background area.

When ready, press the **Fluorogram tab** to exit the background removal mode. If you at any stage would like to revert back to the original imported image, press the **Revert to original** button.

> Note: The Revert to background button can be used at any stage if you wish to start working again with the imported image.

Defining a region of interest (ROI)

After import the image will be displayed in the upper left part of the window. The window will be resized to fit the image.

> Note: The application detects the size and resolution of the screen and uses this information to maintain the ability to process images even on small screens. Typically, very large images will be automatically cropped when viewed on small displays such as on laptop computers.

Rectangular region

Under **ROI selection**, check the button named Rectangle (it is checked by default). On the imported image, drag the mouse diagonally to create a rectangle (Figure 8).
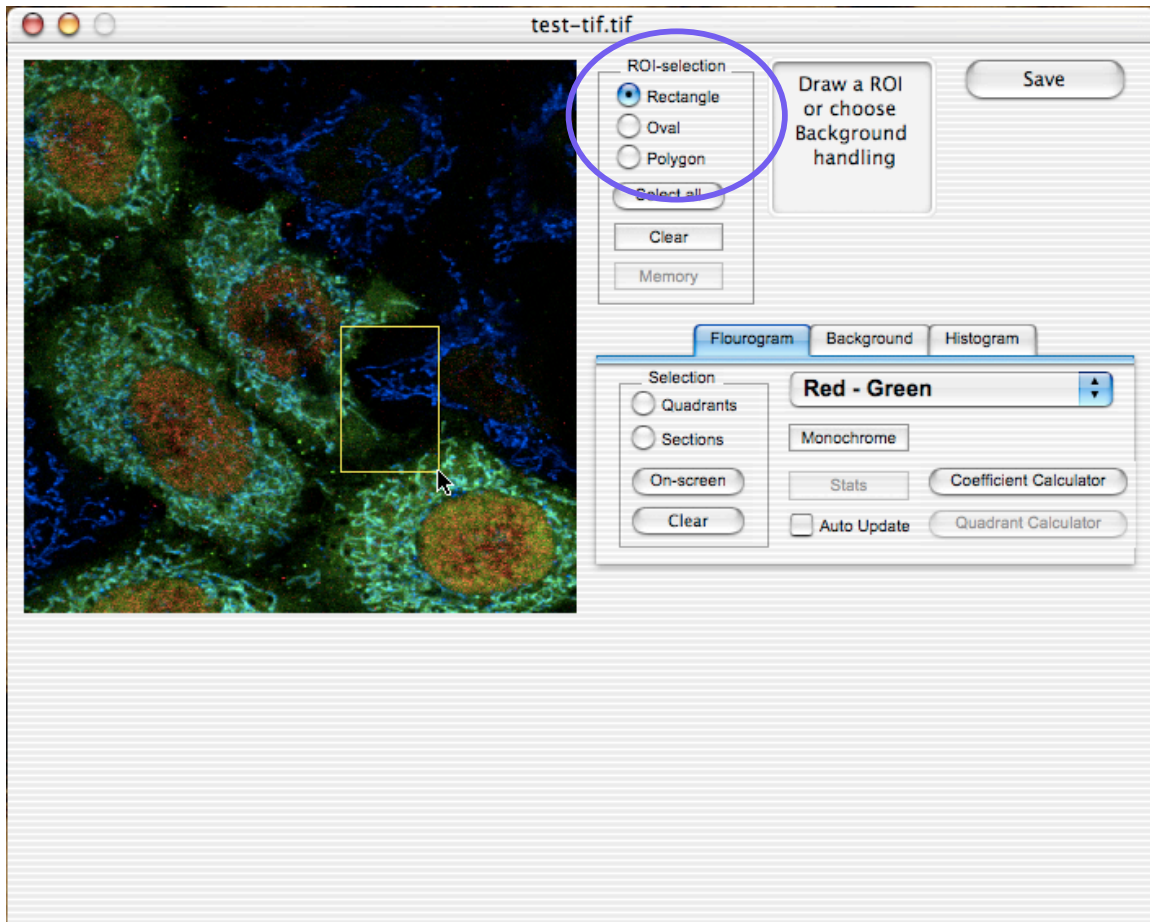
Figure 8: Create a rectangular ROI. To create an oval ROI, check the button named Oval instead.

Oval region of interest

Under **ROI selection**, check the button named Oval. On the imported image, drag the mouse diagonally to create an oval shape.
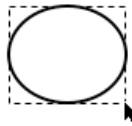
Figure 9: Tips if you feel the oval is drawn too low and to the right. Draw the oval shape by dragging the mouse as specifying an outlining rectangle.

Polygon-shaped ROI

Activate the Polygon-mode under the Advanced button. Click with the mouse to create a polygon ROI on the imported image. Close the polygon by double-clicking.

Note: A polygon can have no more than 20 corners.
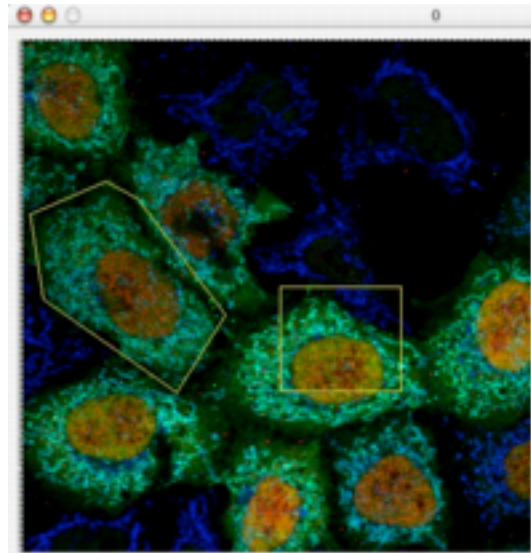
Figure 10: Create Polygon ROI using the mouse.

After defining the ROI

The ROI will be automatically projected in the lower area of the window.
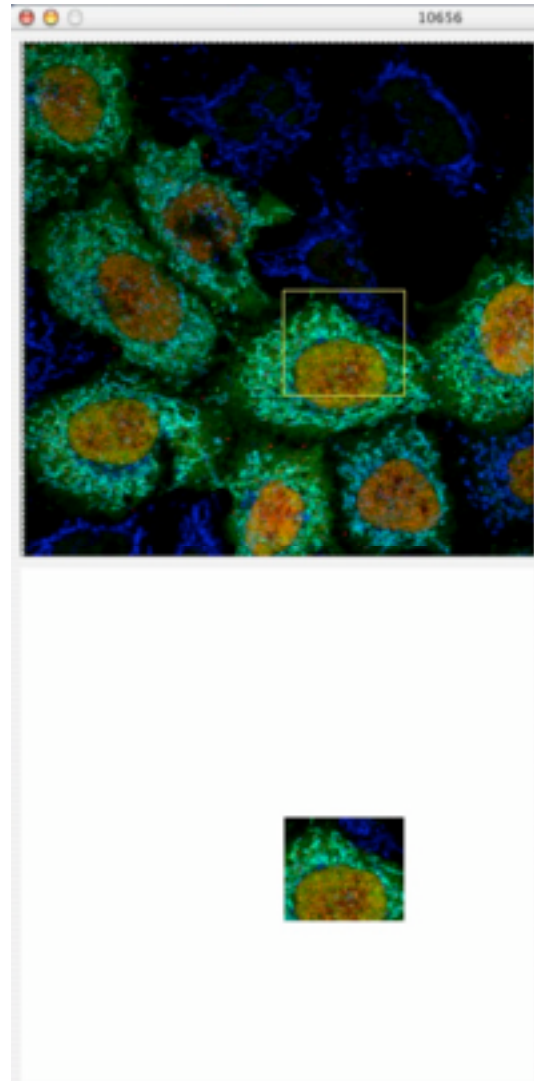
Figure 11: The lower area contains the image information that will be used for further processing.

Image analysis

Choose a two-color mode to analyze

Select a ROI (see above).
Three color-modes are available: Blue-green, red-green, and blue-red.
Select mode from the **Pop-up menu** in the Fluorogram tab. The analysis area displays a scatter plot diagram with the x and y axes corresponding to intensities of all pixels within the ROI.

Note: The RGB Pop-up menu is located under the Fluorogram tab.
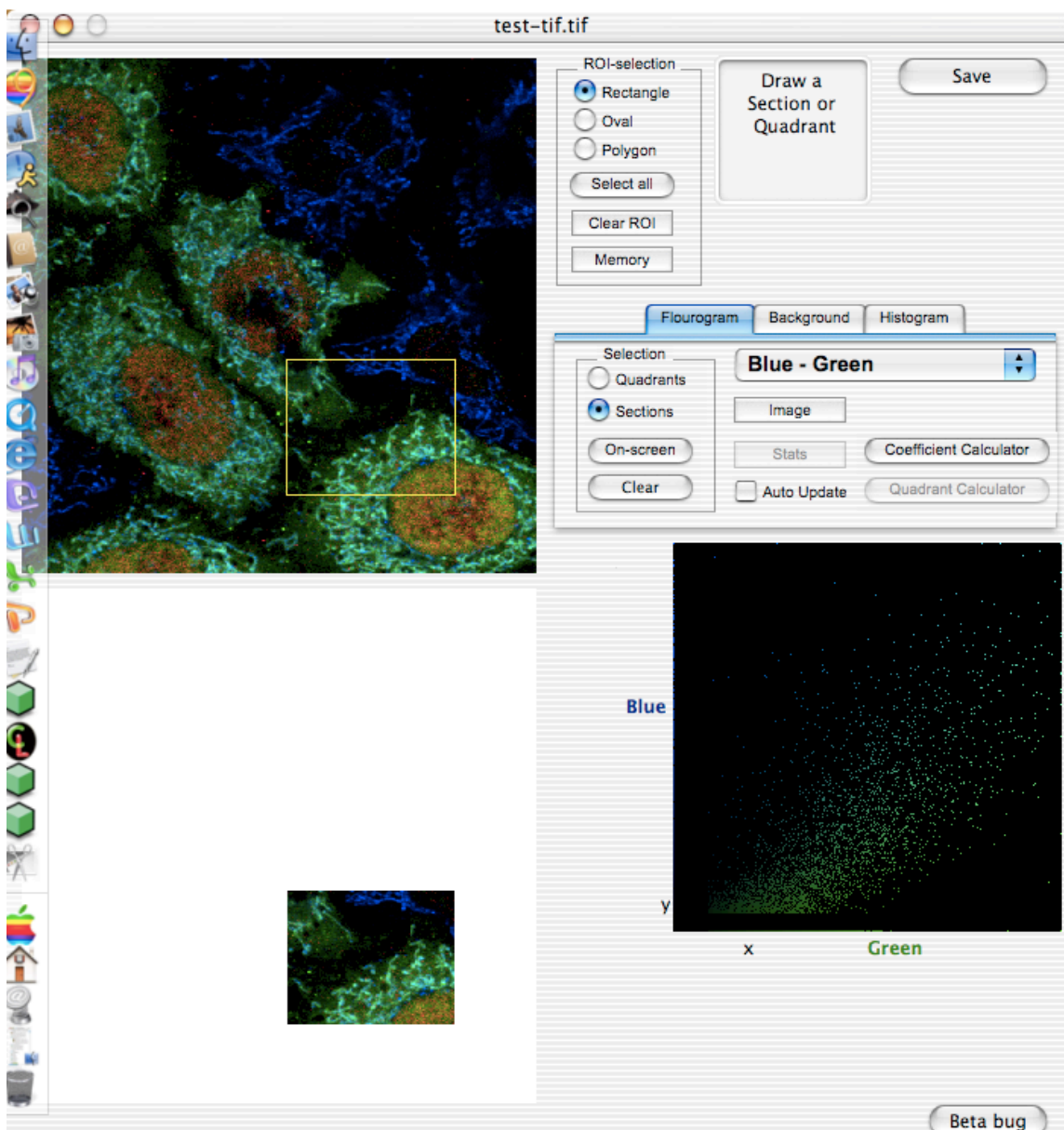
Figure 12: Blue-green mode has been selected. Pixels are plotted according to their blue and green intensities. Notice that in this example the background was removed prior to selecting the color mode.

Switch between monochrome and true-color display

CoolLocalizer can display the analysis area as either True-color mode or as a monochrome image. Toggle between the display settings by pressing the **Image** button. This setting is useful when a higher contrast is necessary.
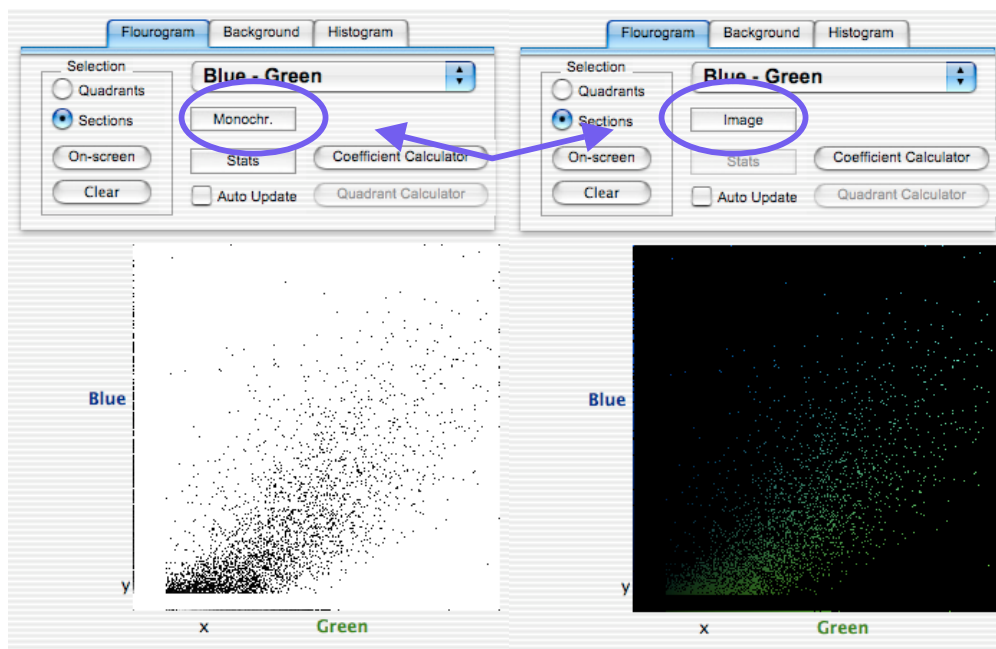
Figure 13: Use the Image button to switch between true-color mode and monochrome display settings for the analysis area.

Specify a section to analyze

To select a *Section* for further analysis, draw a rectangle in the Fluorogram (in the Analysis window). While drawing the section the position of the cursor is displayed beside the x and y-axes (see Figure 14). Repeat to specify a second section if desired.

> Note: Two rectangular sections can be kept in memory. Typically this implies the two last sections drawn.

Count pixel events within sections

After drawing a section the pixel event numbers can be counted by pressing the **Stats** button. To have the counts determined automatically, tick off the **Auto update** check box beside the **Stats** button.

> Note: It is often useful to routinely have the Auto update option selected at all times. However, it is turned off by default.

> Note: Pixel events are sometimes also referred to as *Voxels*.

Figure 14: Press the Stats button to count pixels with intensities specified by rectangular sections. The regions in this example represent 154 and 443 pixel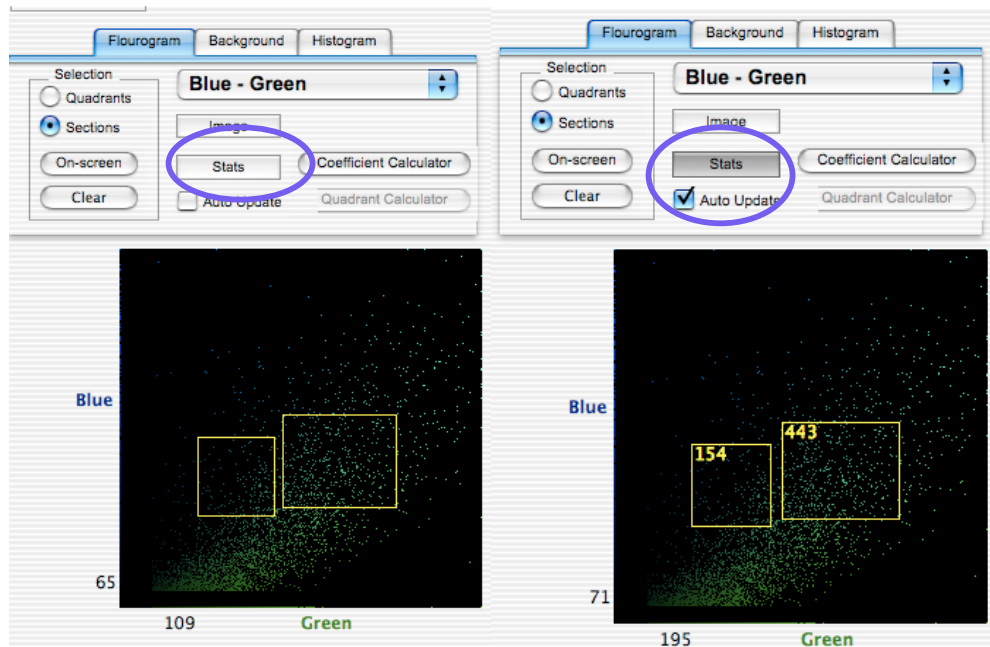 events, respectively. The numbers beside the axes (71 and 195 in this example) show the coordinates of the last cursor position.

Clear drawn sections

Typically, sections are erased by simply drawing another section. Since two sections are displayed simultaneously, the third oldest would automatically be erased.

To specifically erase drawn rectangles, press the **Clear** button.

Save analyzed sections to an image file

This function is used to quickly take a snapshot of the most recent section.

Press the **Save** button on the top right corner. You can also reach the save function from the menu bar.

Specify a file name, file format, and a destination to save the image together with and image of the ROI.

Note: In the Save dialog. Use the Paste command to automatically insert the current file name. This method can be used in every dialog box throughout CoolLocalizer to quickly name a file that is to be saved
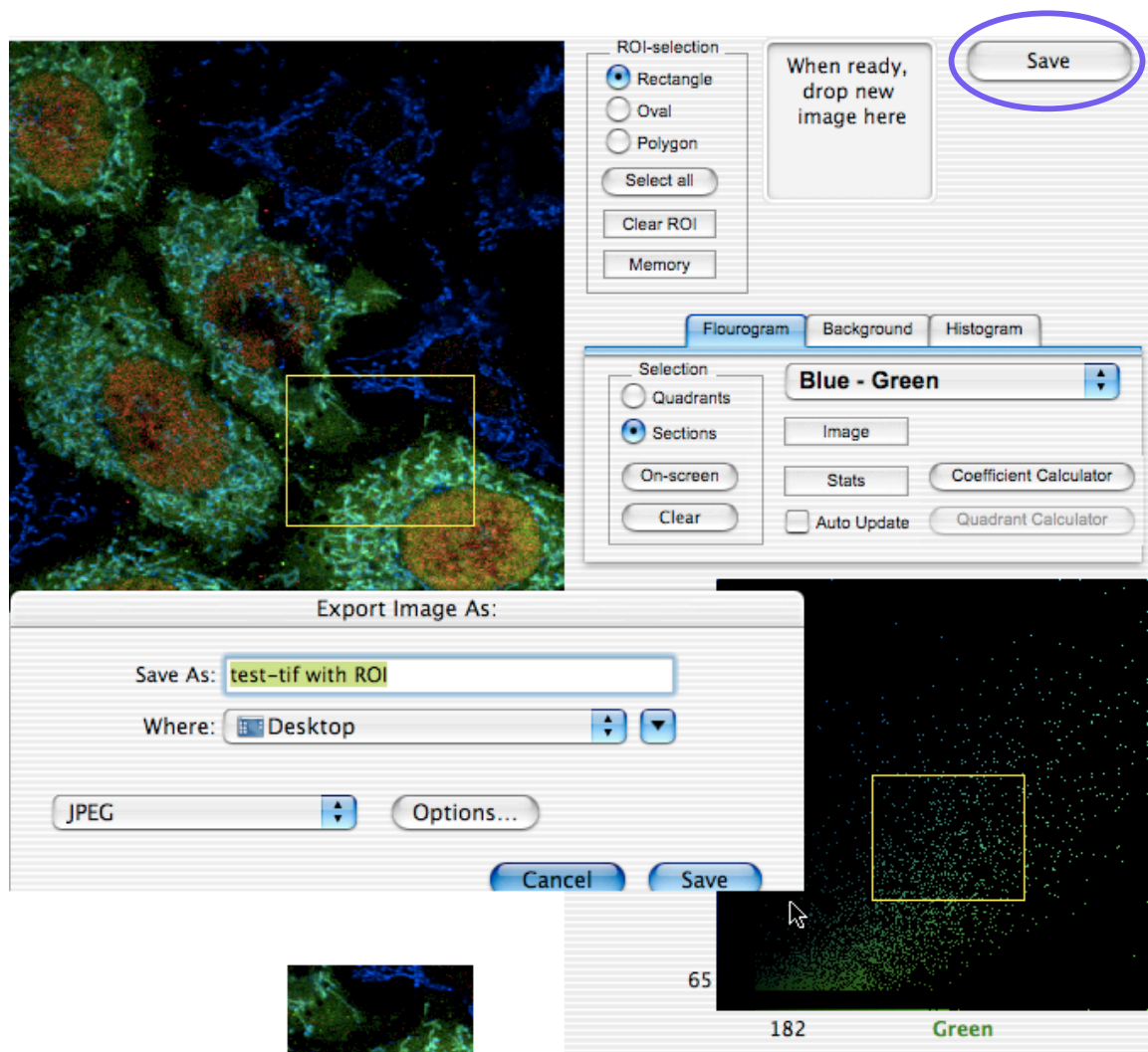
Figure 15: To document analyzed sections, use the Save function.
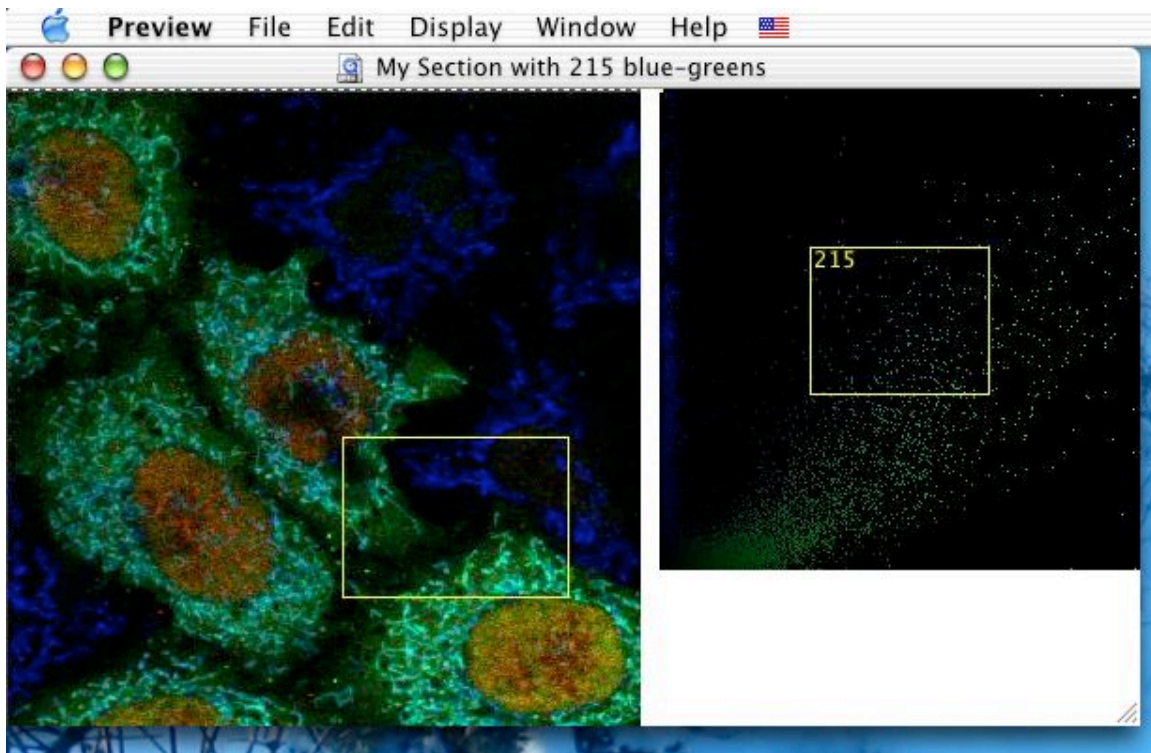
Figure 16: Saved images can be opened using most graphics software. The saved image contains the Imported image with a ROI, as well as the analysis window with the two latest sections.

Note: The Save As… function in the Manu bar is similar, but does not technically generate a screen-shot, but instead performs a clean rendition of the analyzed image, a ROI, and the most recent two sections from the Analysis window.

Clear obsolete sections

Typically, sections are erased by simply drawing another section. Since two sections are displayed simultaneously, the third oldest would automatically be erased.
To specifically erase drawn rectangles, press the **Clear ROI** button

Analyze sections in Quadrant mode

Specify grid for quadrant analysis

Quadrants are the most popular way of visualizing co-staining event for users of flow cytometry software. For microscopy, the method has several obvious advantages such as instant level management, background handling, and fully controlled quantitations.

To use the Quadrant mode, make sure a ROI is selected (see section Defining a region of interest (ROI), page 13).
Inside the Fluorogram tab, select **Quadrants.**
Click the mouse in the Analysis window to place the cross-point. A grid pattern is drawn and the numbers of pixels fallen into each quadrant are presented.
To create a new grid simply drag or click with the mouse arrow pointed to another spot on the Analysis window.
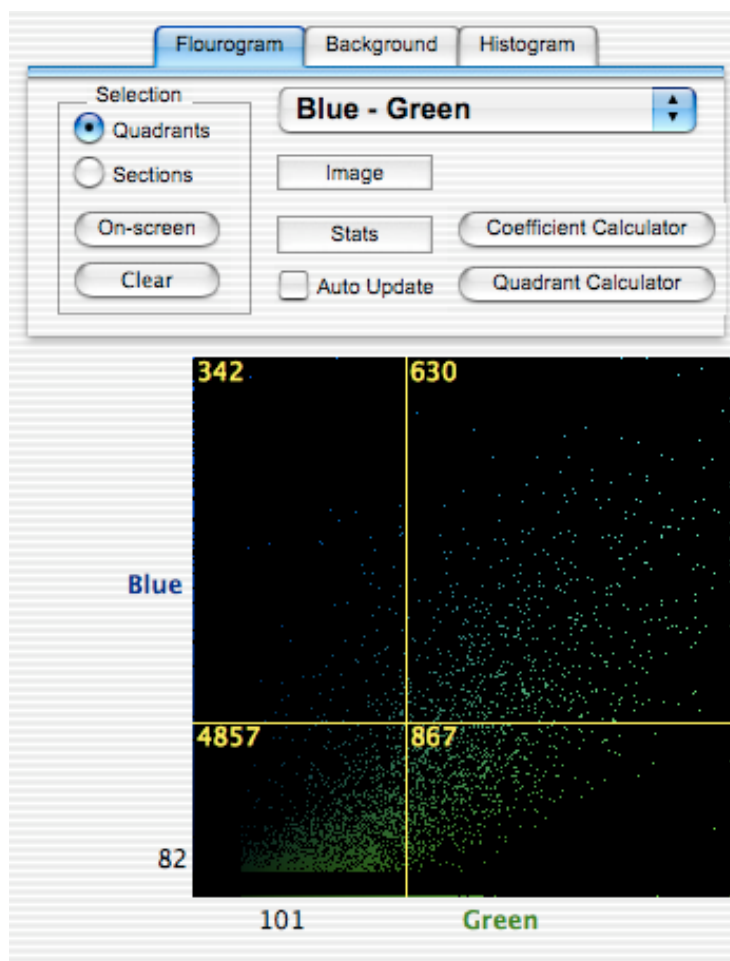
Figure 17: Quadrant mode is selected under the Advanced button. The position of the crosspoint is indicated by the coordinates displayed beside the x and y-axes. Calculated pixel events are presented in the corner of respective quadrant.

Perform Quadrant calculations

CoolLocalizer is equipped with a calculator that determines the fractions of co-localized events based on the specified grid. To access the Calculator, make sure you are in Quadrant mode and that a grid has been specified.

Press **Quadrant Calculator** button. This opens up a spreadsheet with the statistics. Percentages are calculated and indicated below the pixel numbers.

Note: Check the Auto refresh check-box if you wish to update the calculations "on the fly" while moving around the quadrant cross-point.
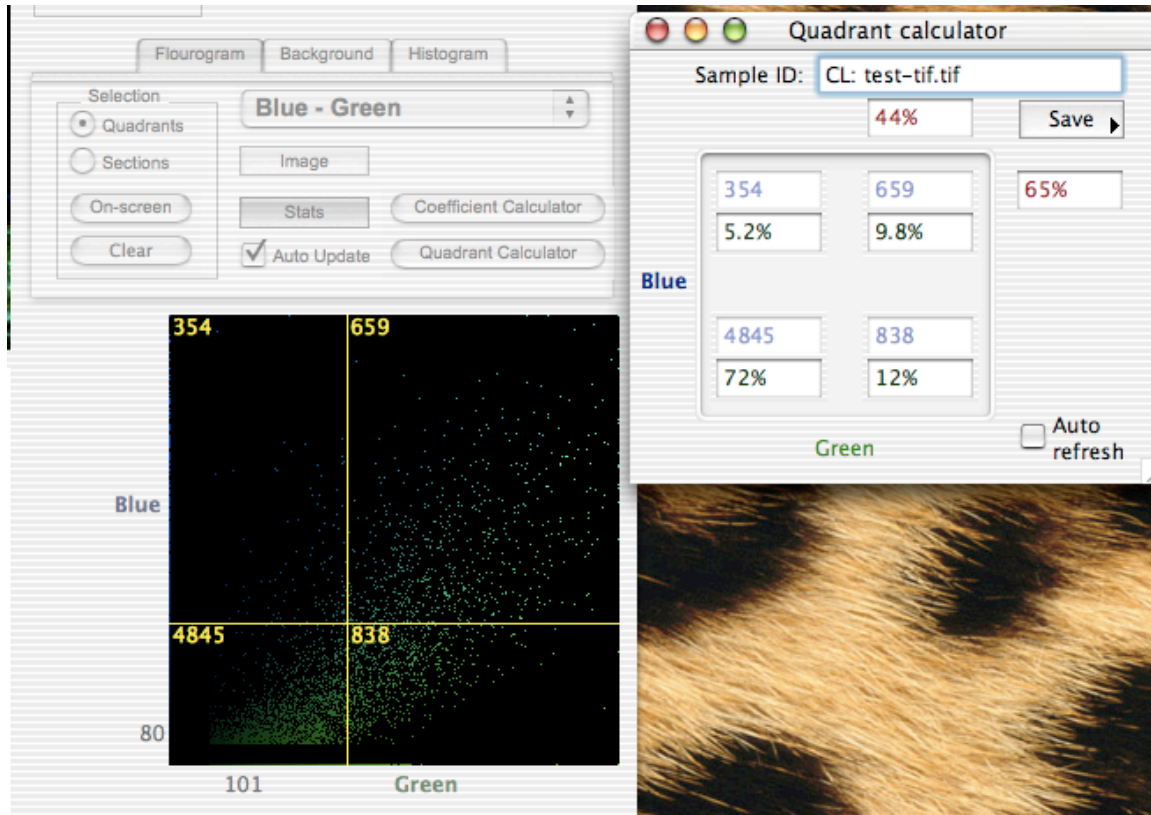
Figure 18: The function Quadrant Calculator is used to view and save quadrant quantitations. In this example, 65% of the highly blue pixel events were also determined as positive for the green channel, or 659/(659+354)*100%.

Save calculation sheets

Two ways are available for saving Quadrant calculations.

1. **Save as Image**
   This allows for a screenshot of the image to be saved. Press the **Save** button on the **Calculation sheet**, and select **As Image.** Specify a file name, a preferred file format and location to save the image.

Note: CoolLocalizer can save images in a number of image formats including JPEG, JPEG2000, TIFF, PICT, BMP, Photoshop etc.

2. **Save as Excel sheet**
   Calculations saved using this options will be possible to handle in spreadsheet applications such as MS Excel®. Press the **Save** button

on the **Calculation sheet**, and select **As Excel sheet.** Specify a file name and destination to save the sheet.

Note: In Windows, the double-clicking the file will automatically launch Microsoft Excel. In Mac OS, the file must be opened from within Excel or by dragging it onto the Excel icon.
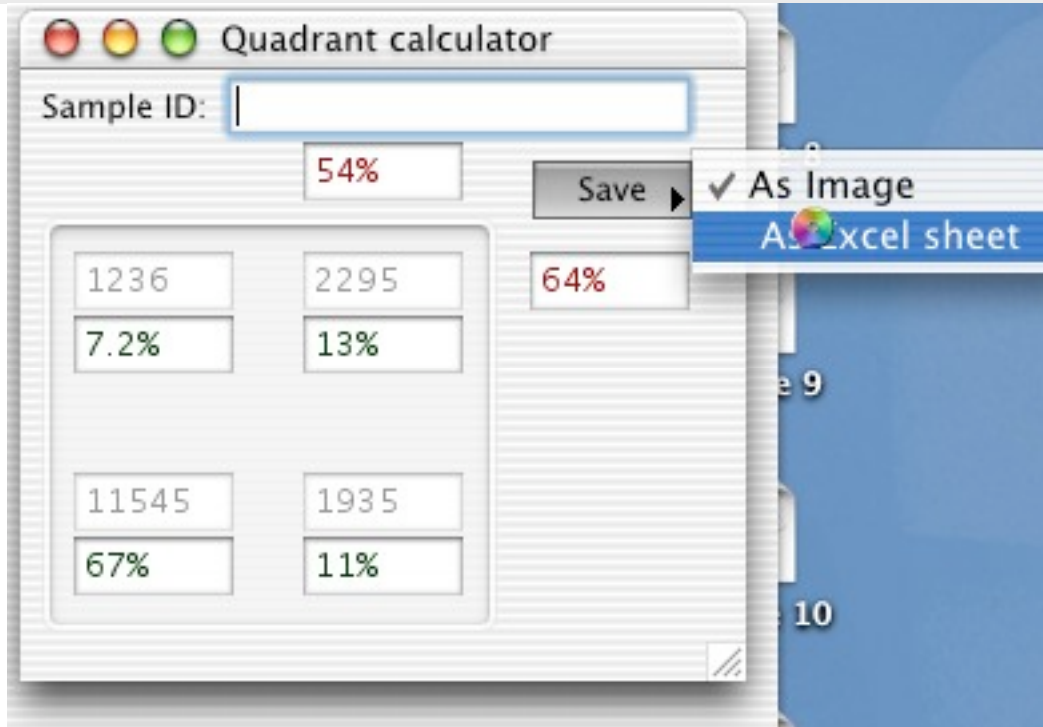

Figure 19: The quadrant calculator sheet. Use the Sample ID line to identify saved sheets.
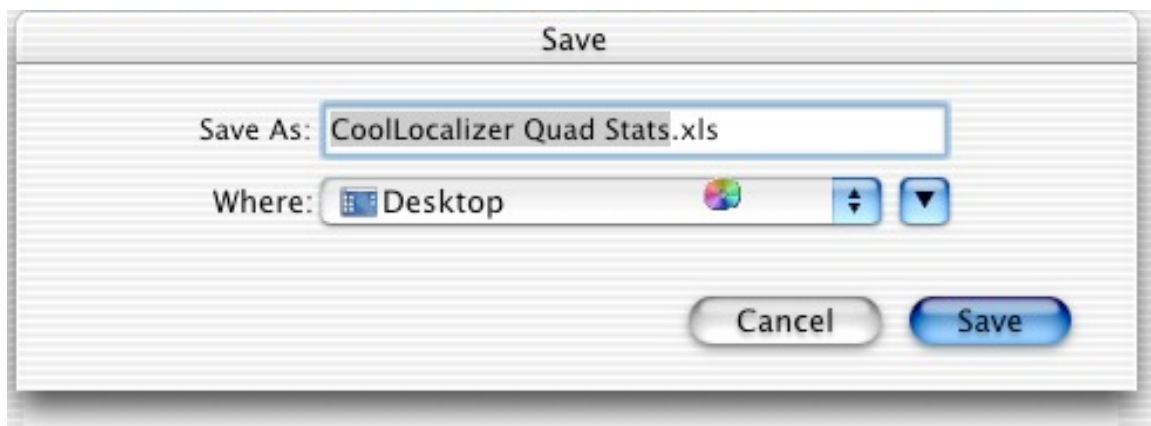

Figure 20: Save a Quadrant calculator sheet as an Image or for later access using a spreadsheet application such as Excel.
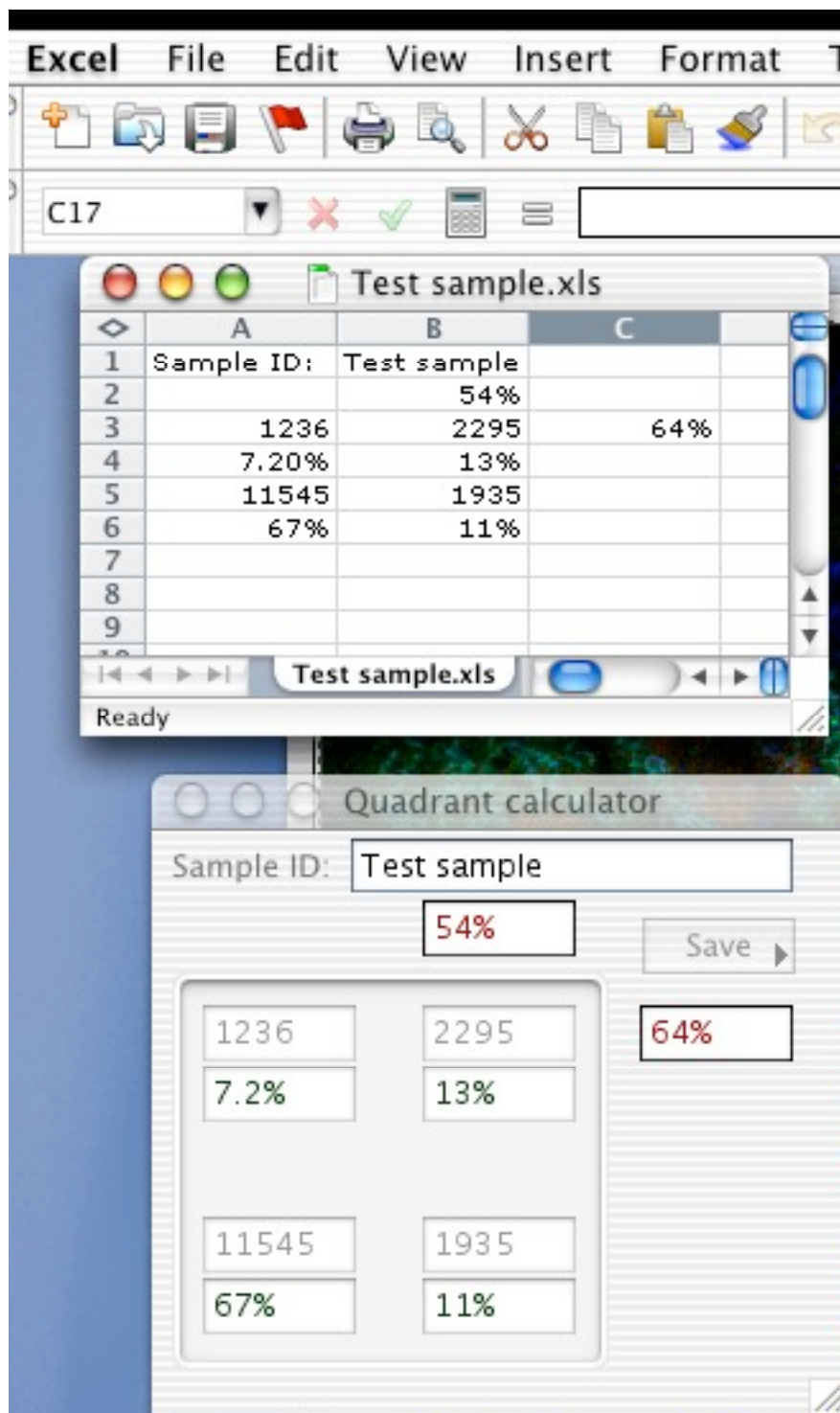
Figure 21: Opening a saved spreadsheet in MS Excel. The Sample ID is transferred along with the Quadrant stats.

Pearson's co-localization coefficients

The Pearson's method is sometimes used for measuring the degree of co-localization (Manders et al, 1993, J. Micr). The formula is described by the three examples below.

$$C(green/blue) = \frac{\sum_i Gi(coloc)}{\sum_i Gi}$$

Equation 1: *C(green/blue)* **indicates the Pearson's coefficient for how green pixels co-localize with blue.** *Gi* **indicates intensity of green pixels and** *Gi(coloc)* **intensity levels of pixels that are both green and blue.**

$$C(red/blue) = \frac{\sum_i Ri(coloc)}{\sum_i Ri}$$

Equation 2 *C(red/blue)* **indicates the Pearson's coefficient for how green pixels co-localize with blue.** *Ri* **indicates intensity of red pixels and** *Ri(coloc)* **intensity of pixels that are both red and blue.**

$$C(blue/red) = \frac{\sum_i Bi(coloc)}{\sum_i Bi}$$

Equation 3 *C(blue/red)* **indicates the Pearson's coefficient for how blue pixels co-localize with red.** *Bi* **indicates intensity of blue pixels and** *Bi(coloc)* **intensity of pixels that are both red and blue. Note that this coefficient will be different from the one described in Equation 2.**

To display the Pearson's coefficients choose the function under the Advanced button. The calculations are displayed in a new window. All six combinations are calculated. Note the positioning of the Quadrant cross-point is not a part of the formula and therefore has no effect on the coefficients.
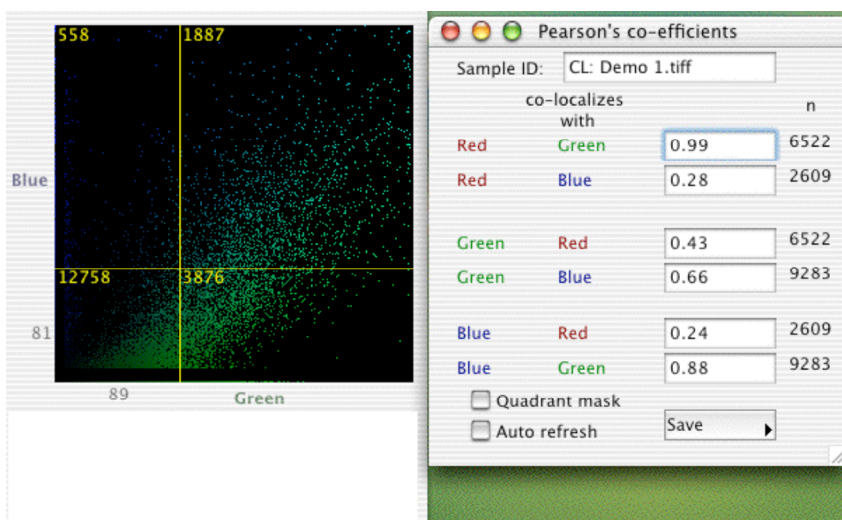
Figure 22: The Pearson's coefficients for the selected ROI. All six two-color combinations are displayed. The numbers of co-localized events used for the calculation (definition: intensity>0) are indicated in the right column. The quadrant cross-point position does not affect the calculations.

Pearson's coefficients using the Quadrant mask method

To properly use the Pearson's method it is important to have acquired the image with low or no background for the color channels that are analyzed. This is necessary since the formula counts as positive any intensity levels that are >0 and includes values from all such pixels. Therefore, this method often results in over-estimated co-localization coefficients for many images. To circumvent this problem, CoolLocalizer is equipped with a novel and quick method of telling the formula exactly how high intensities that are required for events to be determined as positive and co-localized.

To use this method
1) Go to the Fluorogram bar and select Quadrants
2) position the Quadrant Cross-point at the location where you estimate is the cut-off for co-localized events
3) select **Coefficients calculator** button
4) check the Quadrant mask check-box shown in Figure 23.

Only two coefficients are calculated since the cut-off levels were not set for the third color not currently being analyzed. Note that this method results in a higher stringency and therefore lower coefficients for especially low-co-localized color sets.
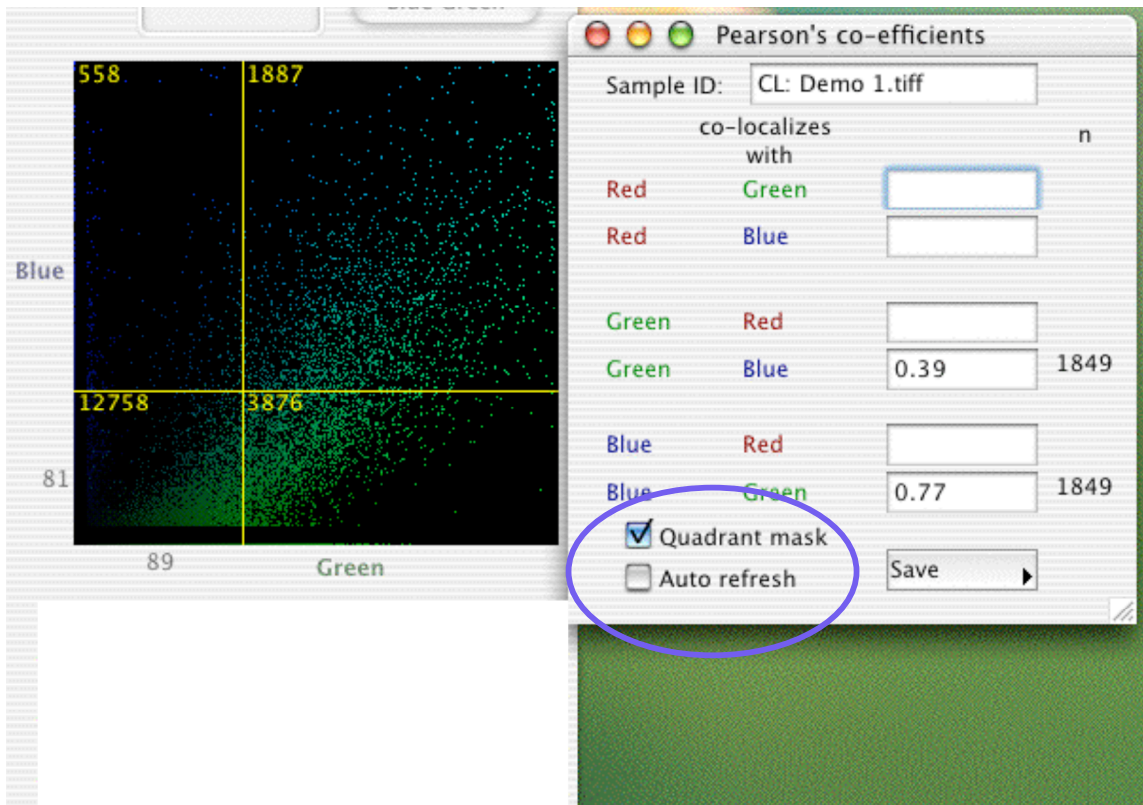
Figure 23: The Pearson's coefficients for the selected ROI using a Quadrant mask to set the requirements for positivity. Only the selected two-color combinations are possible and displayed. The numbers of co-localized events used for the calculation (definition: intensity>crosspoint levels) are indicated in the right column. Moving the cross-point will result in more or less stringent calculations.

Note: Use the Auto refresh Check-box to update the coefficients immediately when the quadrant cross-point is moved around.

Image and ROI handling

Analyze a new ROI using fixed analysis sections
The sections (or quadrants) do not need to be redrawn every time a ROI is specified. To keep the sections, enable the "auto-stats" **checkbox**. Then specify a new ROI by making either a rectangle or a polygon (as described under the section ***Defining a region of interest (ROI),*** page 13).

Recall the previous ROI from memory
The application stores the coordinates for the most recent ROI.
To recall the ROI from the memory, press the **Memory** button.

This results in the ROI to be re-drawn in the lower window, represented in the Fluorogram, and analyzed using the current mode. If the current analysis mode is Quadrants, the Fluorogram will instantly display quadrant stats using the most recent grid axes. If the "auto-stats" checkbox (see above) is enabled, sections will be kept and pixel events automatically counted when the Memory button is pressed.

Note: This function might come in handy for comparing to regions of interest on an image. First, draw a ROI, choose color mode and analysis mode (section or quadrants). Second, specify a new ROI and press Return to select the previous color mode. Hit the Memory button repeatedly to flip between the two ROI: s.

Display pixels within a section on the original image

To determine where analyzed pixel events are located on the original image, use the On-screen function. First, draw a ROI, choose color mode, and draw a Section in the Fluorogram (see *Specify a section to analyze*). Press the **On-screen** button. The image will be scanned for pixels with color intensities that fall within the specified section. Image pixels corresponding to the selected pixel events will be as *white pixels* overlayed on the originally imported image.

Note: The On-screen operation is processor intensive and is noticeably slower to perform the larger the section is. Also, polygon-shaped ROI:s take longer to analyze than rectangular ROI:s
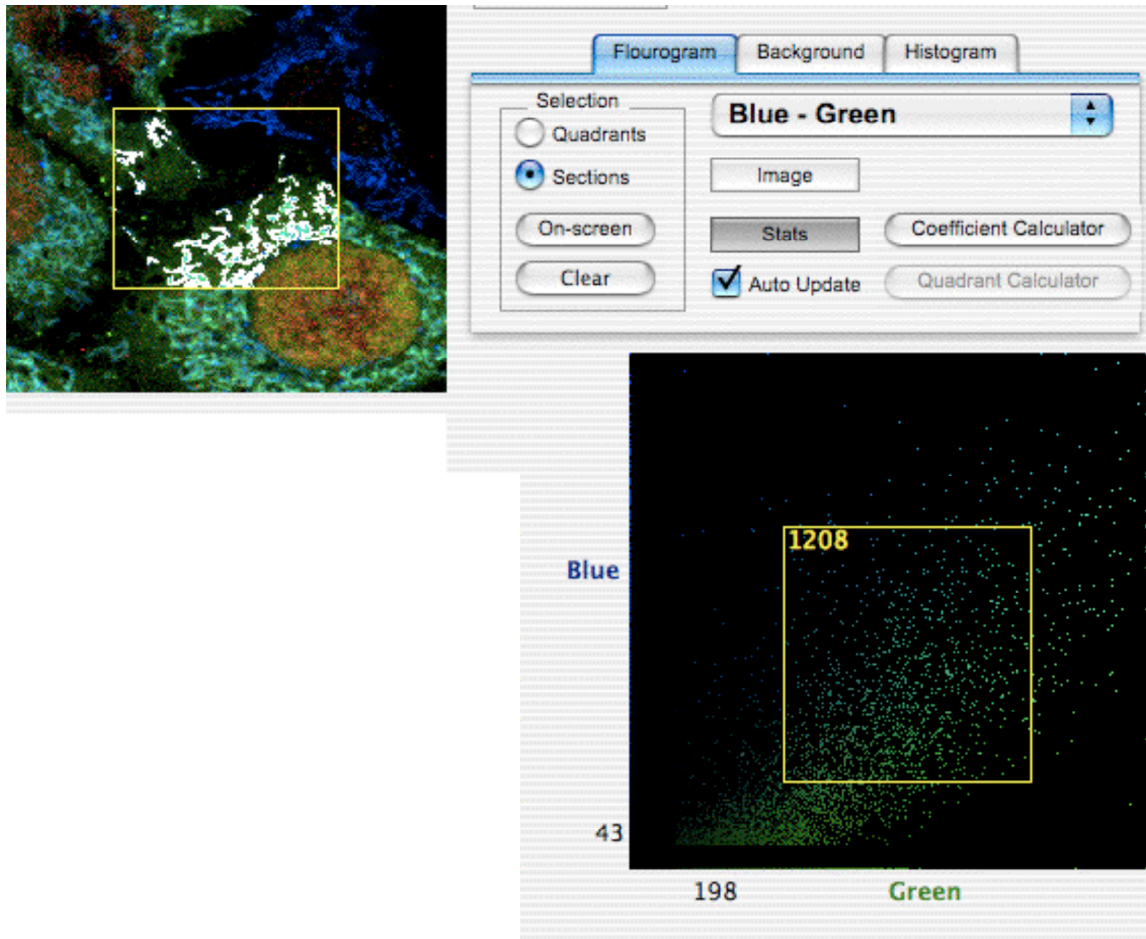
Figure 24: A section containing 1208 pixel event was analyzed using the On-screen function. In this example, the white pixels were set to represent blue-green co-localized pixel events.

The On-screen function can also be used to test the background handling. Specify sections representing pixels of expected background level and visualize them by means of the On-screen command.

Note: Only the area of an image specified within a ROI is subjected to On-screen back projection, even if there are pixels on the image (left) whose intensities would make them fall within the specified section (right).

Display pixels within specified quadrants

The On-screen function can also be used in combination with the Quadrant mode. First, draw a ROI, choose color mode, and indicate a Quadrant cross-point in the Fluorogram (see *Analyze sections in Quadrant mode* page 21).
Press the **On-screen** button. The image will be scanned for pixels with color intensities that fall within the upper right quadrant. Image pixels

corresponding to the selected pixel events will be as **white pixels** overlayed on the originally imported image. Click repeatedly on the On-screen button to display pixels represented within the other qudrants.

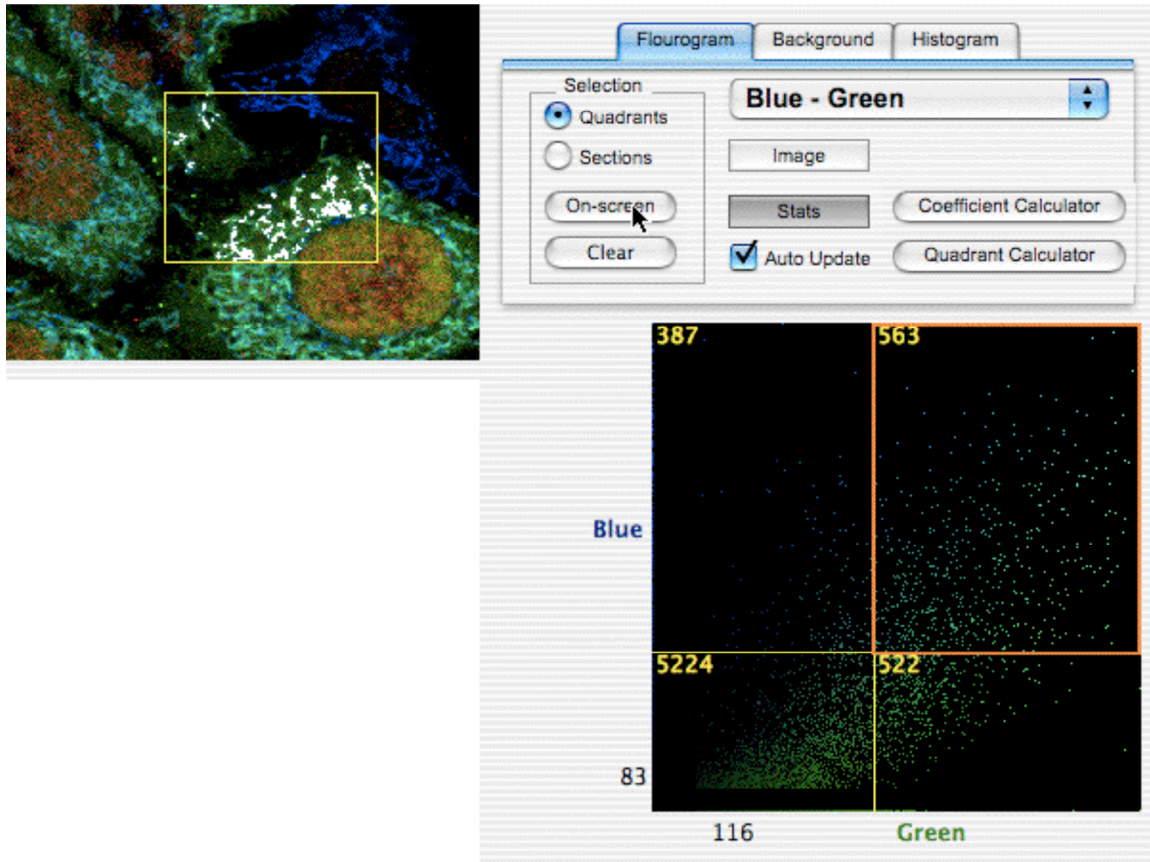Note: The qudrant that is currently represented by overlayed white pixels is high-lighted in orange.

Figure 25: The 563 blue-green pixels with intensities specified withing the upper-right quadrant are overlayed in white by using the On-screen function.

Clear overlayed white pixels after the On-screen operation

To restore the original image, press the **Clear ROI** button. Double-clicking will also erase the yellow ROI boundaries.

Analyze a single color channel and present pixel events using the histogram function

Create histograms

Specify a ROI (see page 13, *Defining a region of interest (ROI)*). Point the mouse to the **Histogram** tab (Figure 26).

Select a color channel by pressing the **Red, Green, or Blue radio-button** (**Figure** 27). This displays an analysis of the selected ROI as a histogram. The pixel intensities are plotted on the x-axis while the number of pixel events is plotted on the y-axis.

Note: The histogram represents all pixels in the ROI. Any sections drawn in the Analysis window will not be considered for the histogram representation.
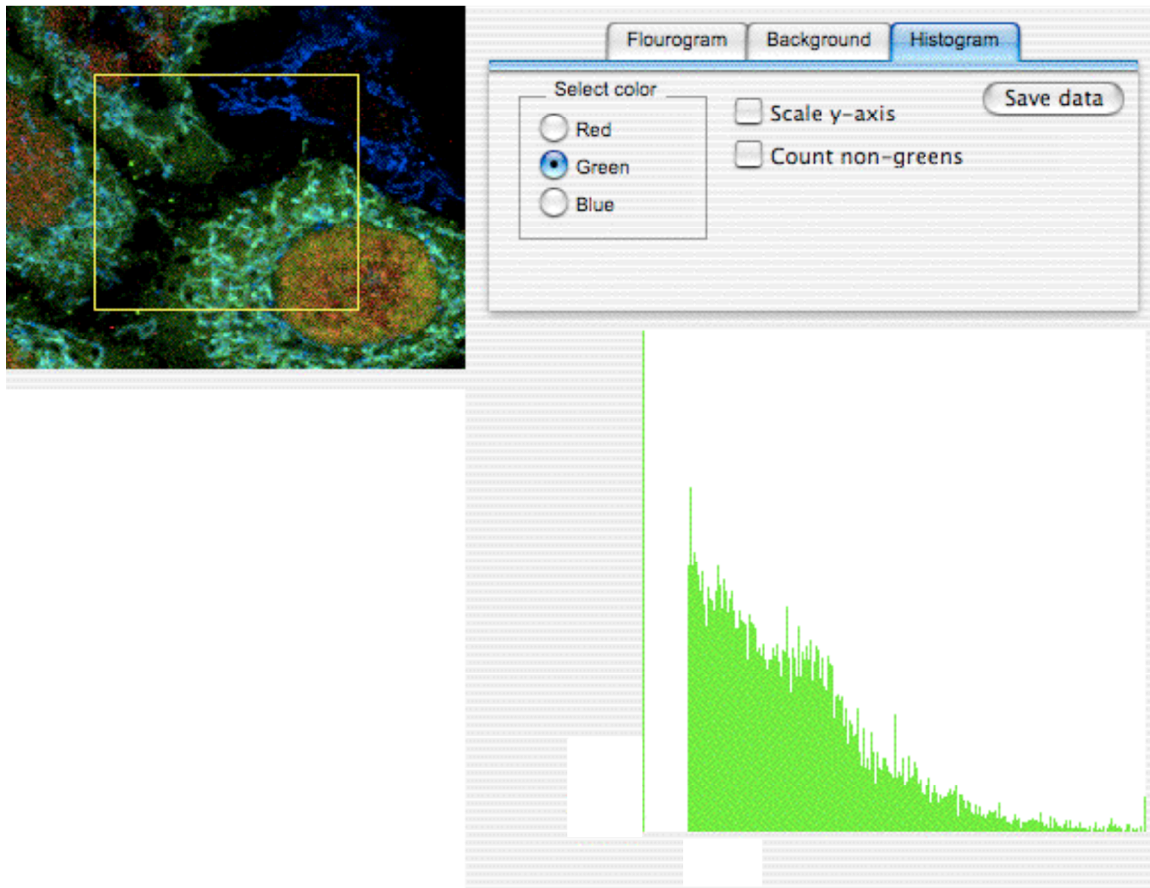

Figure 26: The histogram function is available as an option under the Advanced button menu. Specify a channel (red, green or, blue to analyze).

Perform quantitative analysis in a histogram

It is possible to calculate the number of pixel event that are below and above a specified color intensity level.
Press the mouse arrow on the histogram plot. A *divider line* is drawn vertically across the histogram. The number of pixel events on either side is indicated as shown in **Figure** 27.
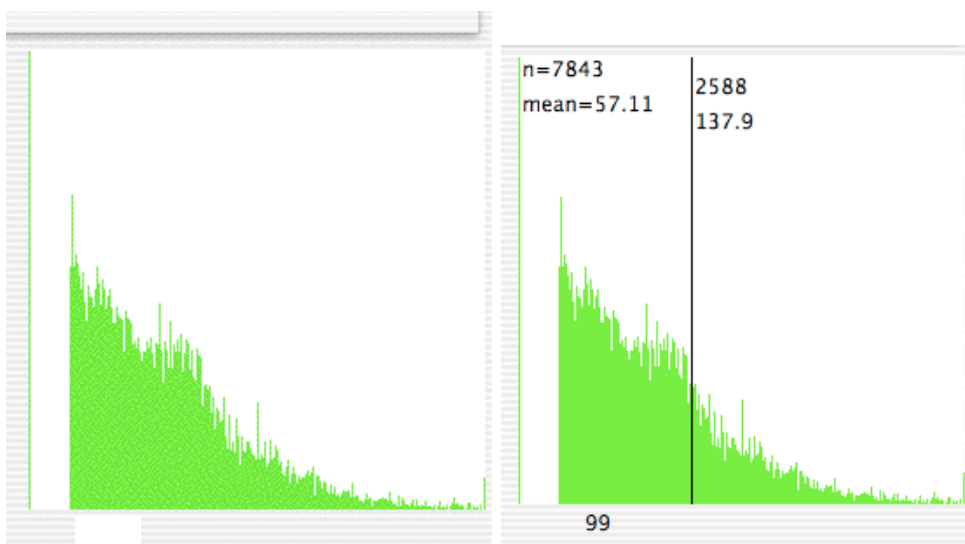
Figure 27: The pixels within the ROI displayed as a histogram. The green channel was selected. Dragging the divider line over the histogram displays the pixel intensity under the x-axis (99 in the example). The number of pixel events as well as the mean intensity are drawn in the upper part of the histogram window. As shown, the areas on both sides of the divider line are quantitated separately.

Choose to include or exclude pixel events with channel intensity of zero.

Only non-black pixel events are included in the histogram, i.e. all pixels that are plotted and counted have red, blue, or green of a level higher than background. The pixels plotted at the y-axis have an intensity level of zero for the analyzed RGB channel. Depending on the experiment it might be appropriate or not to include those pixel event in performed quantitations.
Press the **Count non-[color] check box** to include or exclude pixel event with sub-background intensity for the chosen channel.

Image analysis to reveal saturated pixels

About saturated pixels and co-localization studies

The calculation of Pearson's co'localization coefficients depends on each voxel's intensity. Saturation of pixels will lead to incorrect coefficients. It is therefore important to acquire all images carefully and avoid saturated pixels. CoolLocalizer keeps the information for all pixels even when the background levels are processed (see Define background levels, page 11).

Analyze an image or a ROI for saturation

Images can be analyzed by CoolLocalizer for the amount of saturated pixels.

Select a ROI or choose **Select all** in the **ROI selection box**. Then go to Tool in Menu bar and activate the command **Analyze ROI** (Figure 28).
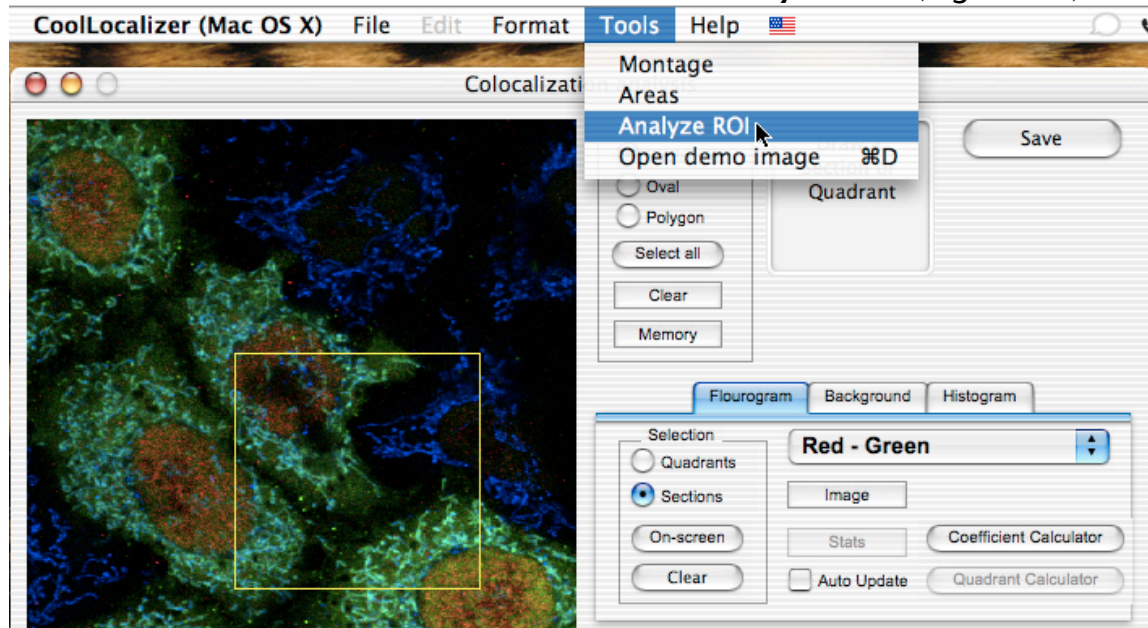

Figure 28: The Analyze ROI command is accessed from Tools in the Menu bar.
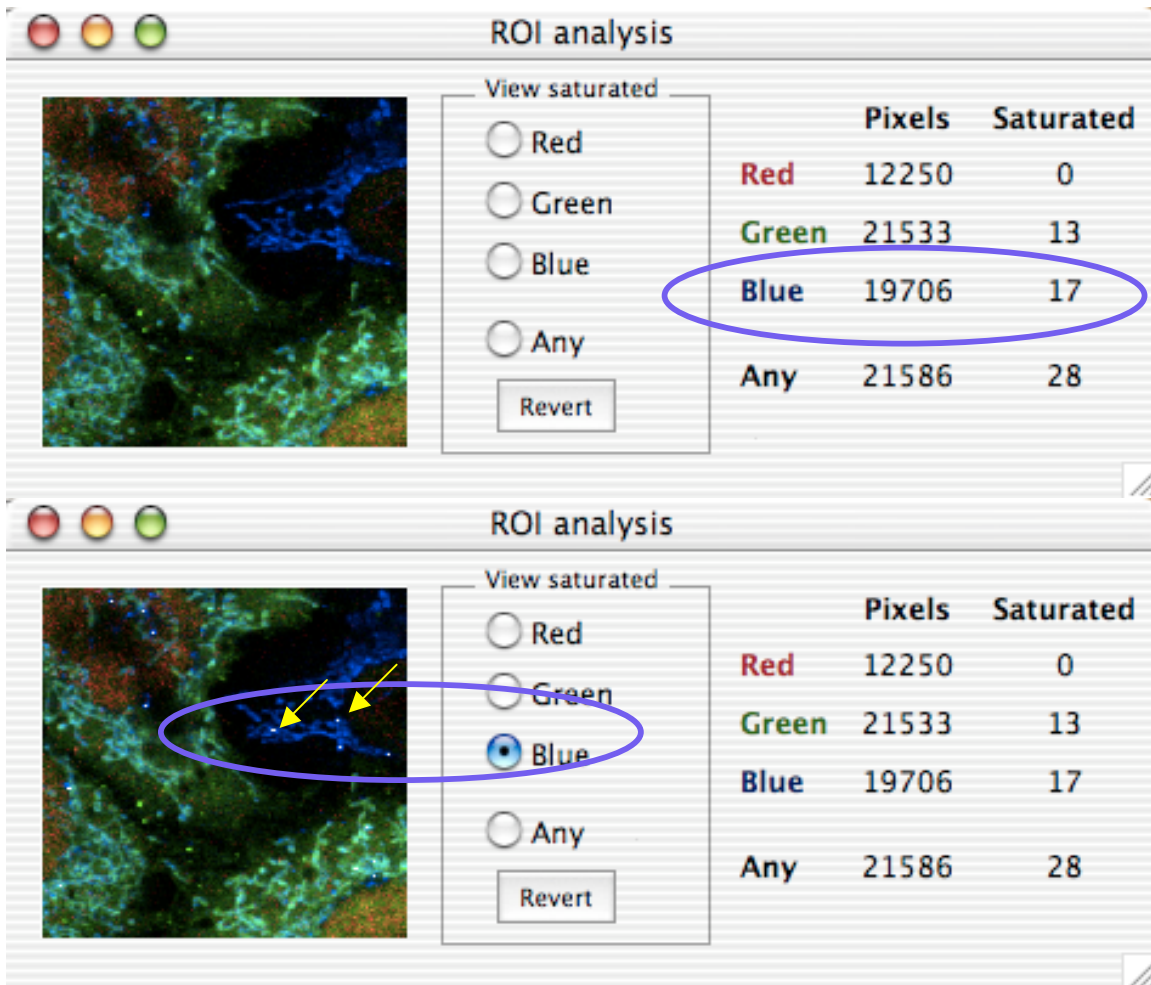
The ROI Analyzer window opens up.

Figure 29: The ROI analysis window. The ROI has been analysed for total pixels (intensity above zero) and for saturation. In this example the ROI contained 17 pixels that were saturated for the blue channel. These pixels are visualized as white in the lower fig.

The ROI analysis window displays a table with the numbers of red, green, and blue pixels, respectively. Also the number of pixels with any color (non-black) is calculated and shown in the table. The numbers of saturated pixels (intensity of 255) is also indicated.
To visualize the saturated pixels for a specific channel, press the button for the corresponding color. The saurated pixels become white. Choose **Any** to visualize all saturated pixels regardless of channel. Use the **Reset button** to clear the white overlay.

Processing of images for documentation and presentations

Using the Montage function

CoolLocalizer comes with built-in functions for quick processing of images into formats suitable for publications, documentation, as well as presentations. The traditional way to show co-localization has been three images displayed beside each other:

Green – Red – Merge (where overlapping regions turn yellow).
The Montage can produce this and other often even more illustrative images without the need for other image processing software.

Two-color montages

Two-color montages are used to display two channels and a third image which represents a merged picture of the two. To create a montage you must first specify a rectangular ROI. Only the ROI will be included in the montage.
After selecting a ROI activate **Montage** under **Tools**. You will be presented with two windows: the **Montage Setup window**, and the actual **Montage window** (see Figure 30).
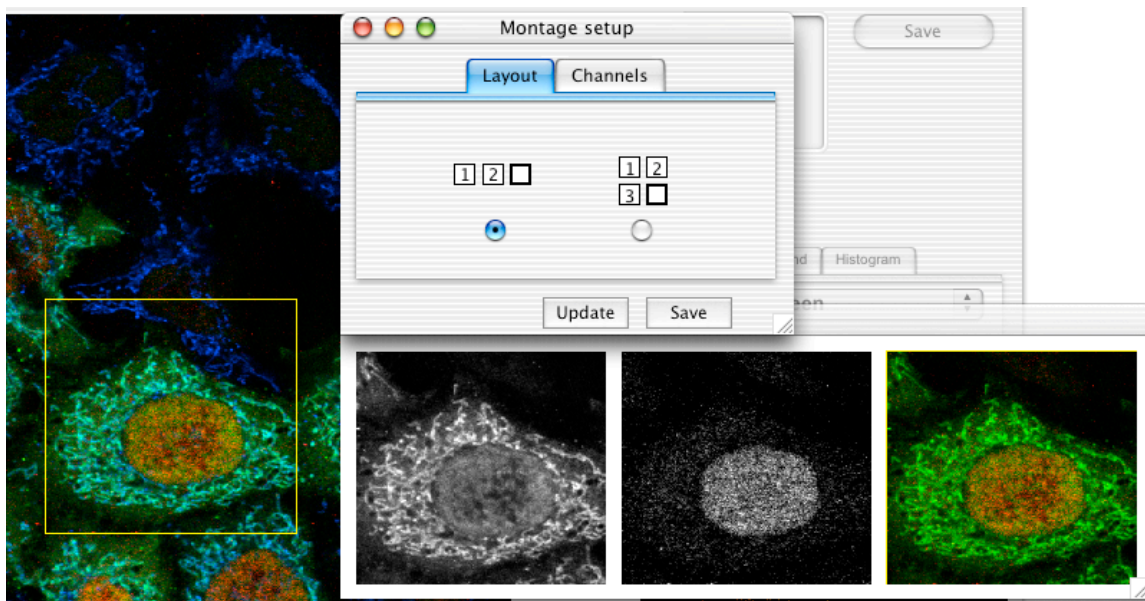

Figure 30: The Montage function is accessed from the Tools menu. The Montage setup is used to control the montage layout whereas the montage itself is displayed in a separate window below.

How to change color channels to incude in the montage

By default, the two channels will be included that are currently being analyzed. To change which colors to be represented in the montage display the **Channels** tab (Figure 31). Under this tab you can also select the order of how the sub-images as well as how the channels should be represented.
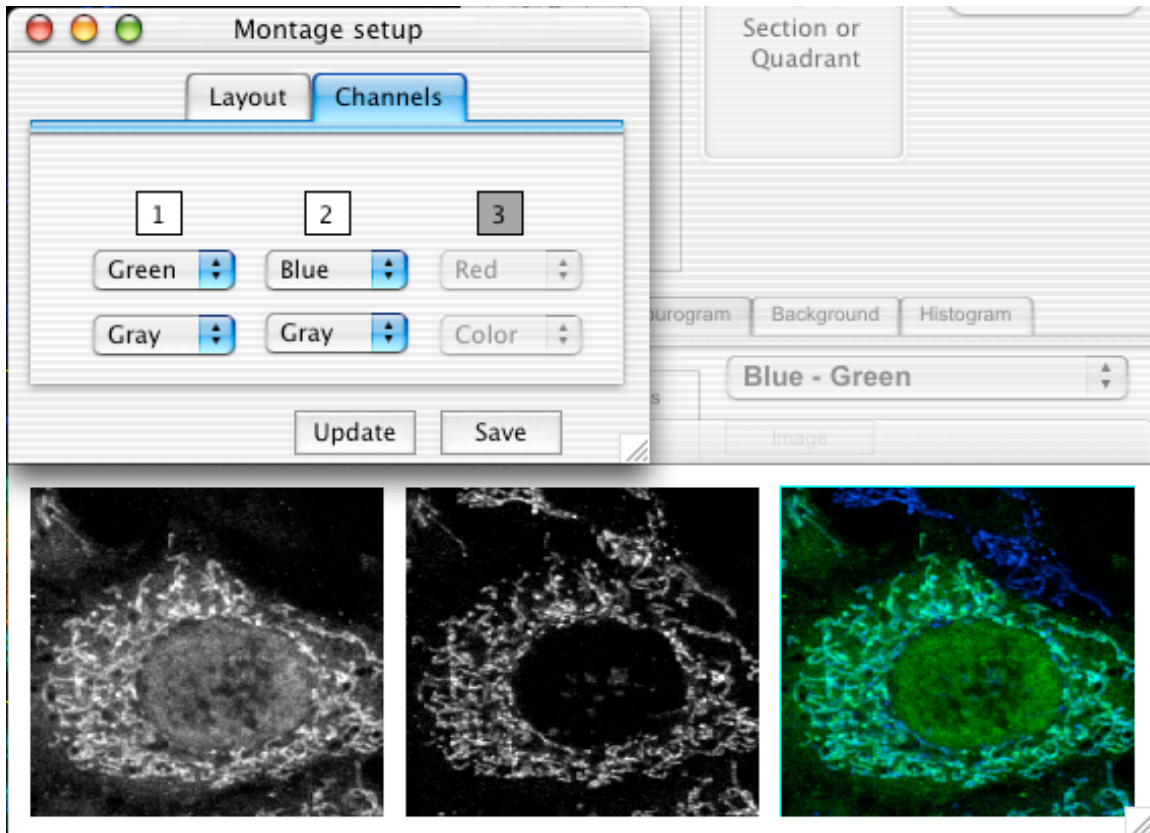

Figure 31: What channels to display and their order are controlled from the Channels tab in the Montage setup window. The Update button is used whenever a new ROI is specified in order to redraw the montage. Use the Save button to save the montage (see below).

Three-color montages

A three-color montage is used to illustrate three analyzed channels plus a merged image. To create a three-color montage, display the **Layout** tab and select the three-color mode symbol (see Figure 32).
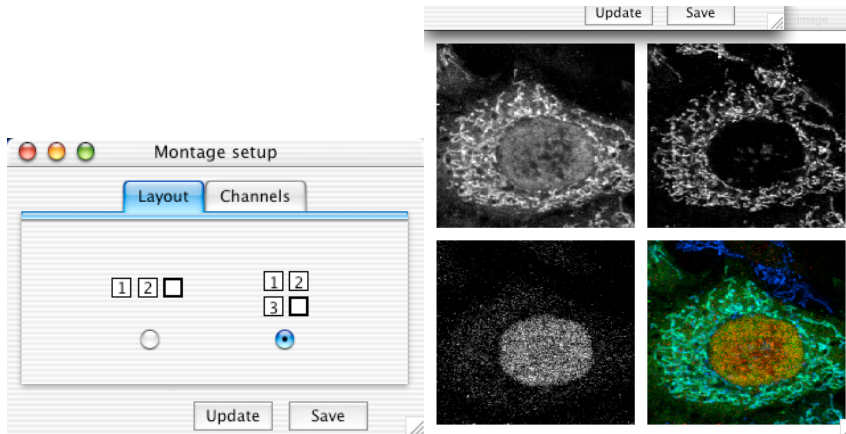
Figure 32: Switch between two-color and three-color montages from the Layout tab in the Montage setup window. Here the three-color mode was selected and the montage dosplays all three RGB channels as well as a merged image.

Display the channel images in gray-scale or color.

Often it is preferred to display the channels in gray-scale. This method shows the highest contrast for the viewer and is therefore the method created by default. However, it is very common to depict fluorescence microscopy images in color.

To change the way the montage shows the channels from grayscale to color, first display the **Channels** tab. Under respective channel selector (shown by a number corresponding to its position) use the pop-up to select **color**.
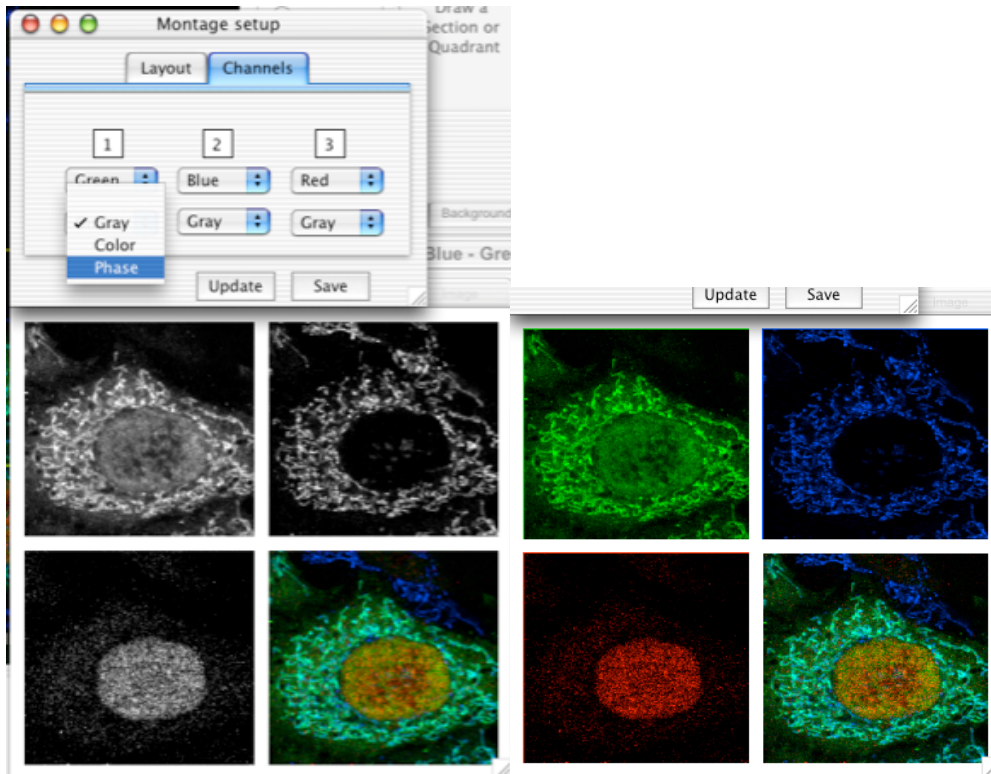
Figure 33: Use the color-mode pop-up menu under the respective channel selector to switch between grayscale (left) and color (right).

Montages containing phase-contrast images

For obvious reasons phase-contrast has no place in colocalization analyses. However, CoolLocalizer can be used to create montages from images where one of the channels is a phase-contrast picture. A phase-contrast image will be displayed in grayscale even in the montage's merged sub-image. Phase-contrast images can be included in both two-color and three-color montages.
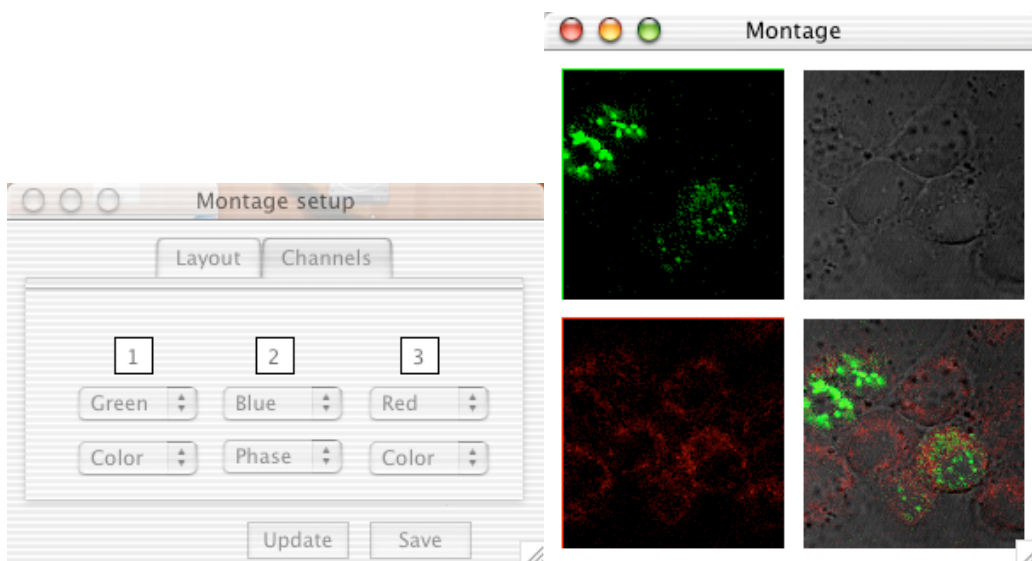
Figure 34: To create montages from images containing a phase contrast picture in one channel, select Phase in the color-mode Pup-up menu (see also Figure 31). The image in this example contains green red channels and a phase contrast image in the third (using the blue) channel.

Appendix 1

Why acquire sequentially?

The purpose of this manual is to guide the user through CoolLocalizer and not serve as a tutorial for confocal microscopy. However, this paragraph contains some tips on the purpose behind aqcuiring sequentially. Sequential acquisition is preferred when the used chromophors overlap in their emission's spectra and therefore "bleed" over into one of the other channels. Such artifacts are especially problematic for co-localization purposes as pixels containing 'bleed-through" information will falsely be perceived as co-localized events.
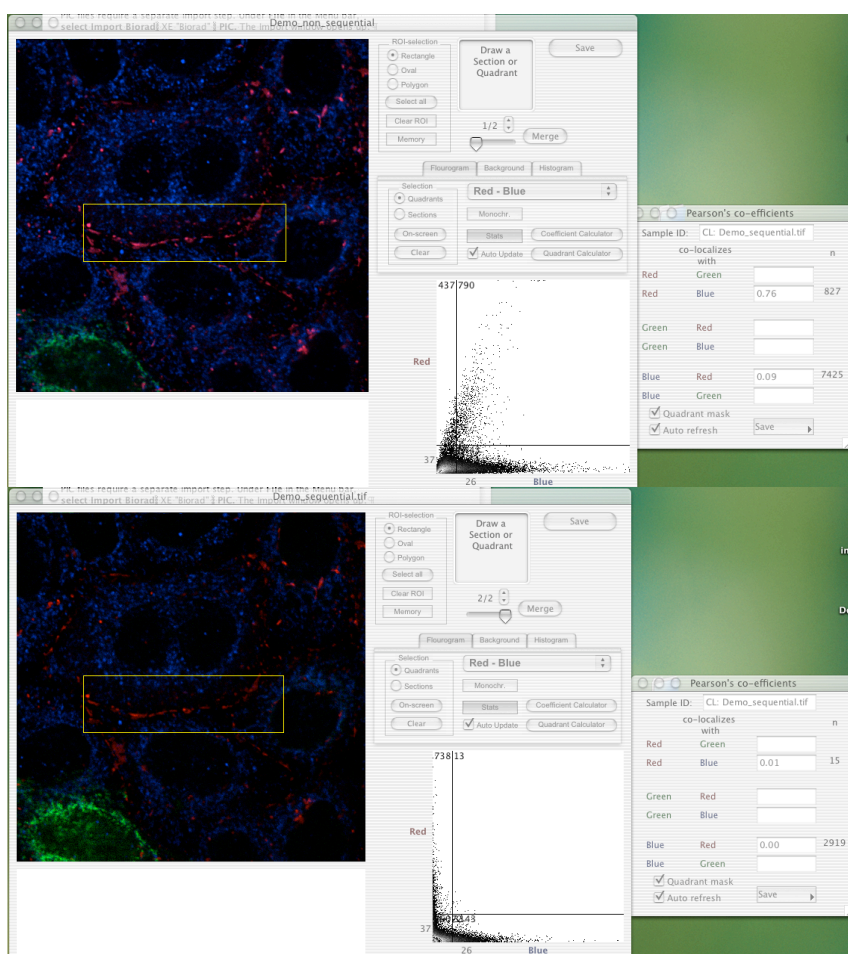

Figure 35: Sequential aqcuisition. The upper image is acquired by all three channels simultaneously whereas the lower image is composed of three channels aqcuired in sequence. Note how the red areas (Cy3) appear also to be stained with the dye represented in the digital image by blue (Cy5). Both the fluorogram and the co-localization coefficients indicate co-localization, which is merely a result of "bleed-through" artifacts.

Contact info

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Notes