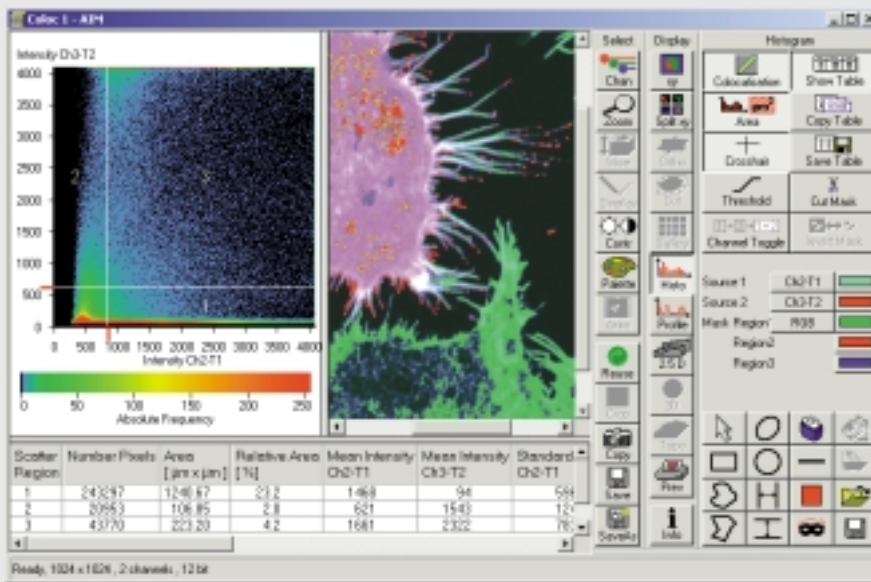
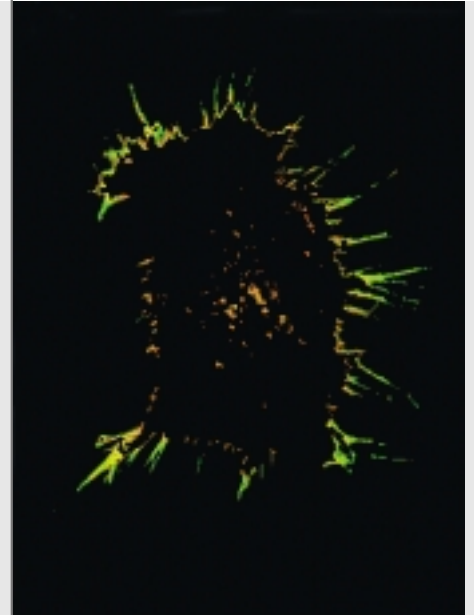


Colocalization - Analysis and Visualization

In the analysis of multichannel specimens, the colocalization of labeled structures is a problem of central importance. Frequently, colocalization serves as an indication of a suspected functional interaction. While the acquisition of multichannel images of this kind is routine, users are often uncertain about how to proceed with the analysis of the raw data, and how colocalization can be quantified reproducibly. The difficulties are even greater where the colocalization grades of different specimens have to be compared.



The Release 3.2 of the Carl Zeiss LSM Software for the first time provides a comprehensive, integrated solution for colocalization analysis. Colocalization parameters can be determined automatically or interactively with the aid of scattergrams and used for the comparative analysis of different specimens. Selected image areas of interest can be tested for colocalization separately, and colocalized structures can be visualized. Various numerical colocalization parameters are available for quantitative analysis.



Analysis of the colocalized areas in a double tagged HEK cell (green: F-actin tagged with FITC-phalloidin; red: rhodamine Red X tagging of an exogenous expressed protein tagged with c-myc).

Coloc dialog window with scattergram and quantitative parameters. Presentation of single-tagged (red and green) and colocalized image areas (purple). Extracted colocalized areas (displayed in the colors of the raw data channels)

Specimen: L. Hilenski, Emory University, Atlanta, USA).

Quantitative. Reproducible. Easy to Use.



Colocalization - the Principles

In laser scanning microscopy, two structures are said to be colocalized if the two fluorescence signals emitted by them are detected within the same confocal detection volume.

Confocal Detection Volume

is the volume within the specimen whose photon emission is registered by the detector. The size of this volume is a function of the numerical aperture of the objective, the wavelength used, and the pinhole diameter [see references]. The multiple pinhole concept of the LSM 510 provides the possibility for the confocal detection volumes of all image channels to be equalized simultaneously - an essential requirement for the 3D reconstruction and 3D colocalization analysis of multi-labeled specimens. With the LSM PASCAL, this functionality is also available in the Multitracking mode.

Crosstalk

A general requirement for colocalization analyses is the acquisition of multichannel images that are free from crosstalk, i.e. overlaps. Two kinds of crosstalk can be distinguished: emission crosstalk and excitation crosstalk.

Emission Crosstalk

This term is used if the emission signals of the dyes partly overlap, while the dyes are excited by different laser lines. Images acquired in the Multitracking mode of the LSM 5 family can be depended on to be free from this kind of crosstalk.

Excitation Crosstalk

This means that one of the laser lines used excites two or more dyes simultaneously. Unless the emission spectra overlap, the signals can be registered simultaneously in different channels.

Emission Fingerprinting

Where a combination of emission and excitation crosstalk is present, the LSM 510 META system is required. By means of its Emission Fingerprinting feature, it can record overlap-free multichannel images even if the emission spectra of the dyes used overlap almost completely [see references].

Where Do Actin and Vinculin Colocalize?

Analysis and Visualization of Colocalized Image Regions

Cells growing adherently form specialized contacts between the cell membrane and their growth substrate. These focal adhesions are dynamic structures, at which the cell's actin cytoskeleton interacts with more than 100 different proteins while it orients itself along the focal adhesions. One of the essential intracellular structural proteins for focal contacts is vinculin. Cell regions in which vinculin and actin are colocalized can thus be regarded as markers for functional focal adhesions.

Fig. 1a: Colocalization analysis at a double tagging of actin and vinculin (green: actin tagged with Alexa 488; red: vinculin tagged with Cy3). The Image ROI in the bottom right part of the Image window defines the background. The corresponding pixels are displayed close to the coordinate origin of the scattergram. Specimen: Prof. M. Bastmeyer and D. Lehnert, University of Konstanz, Germany.

Fig. 1b: In the Threshold dialog window, the position of the crosshair can be edited. The function Set from Image ROIs determines the crosshair position according to the background ROI defined in the Image window.

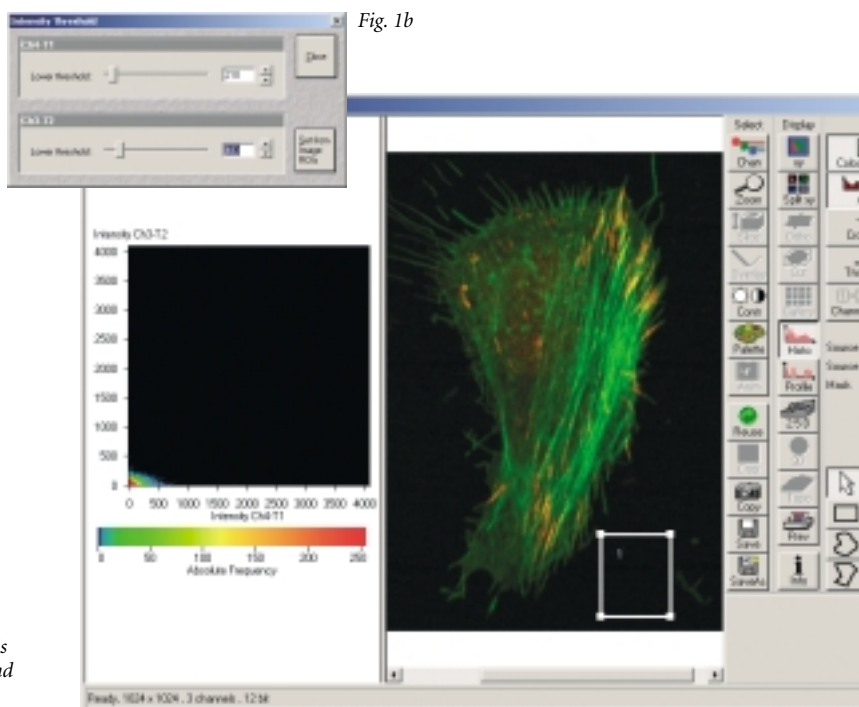


Fig. 1b

Fig. 1a

The Crosshair Function

The *Crosshair* function has a pivotal role in the visualization of colocalized image regions, as it is the tool used for defining the colocalization conditions.

To analyze the actin/vinculin specimen, the crosshair should be positioned so that the bottom left quadrant represents all pixels of the background. These pixels can be selected interactively, or automatically in the image window (Fig. 1a-c). With the interactive method, shift the crosshair in the scattergram so that the pixels of quadrants 1, 2 and 3 exclusively contain object information and no background information. The automated method computes, channel by channel, the average intensity and the standard deviation of a background ROI defined in the image window. For either channel then, the position of the crosshair corresponds to the sum of the two values (Fig. 1a and b).

Either method leads to a defined distribution of all image pixels over the four quadrants of the scattergram. With the background pixels sorted into the bottom left quadrant as described above, the single-tagged pixels of the two channels

Colocalization Analysis

Fig. 2a: Definition of colocalized areas by means of reference structures. The reference regions are marked with an Image ROI. The corresponding pixels are displayed in the scattergram.

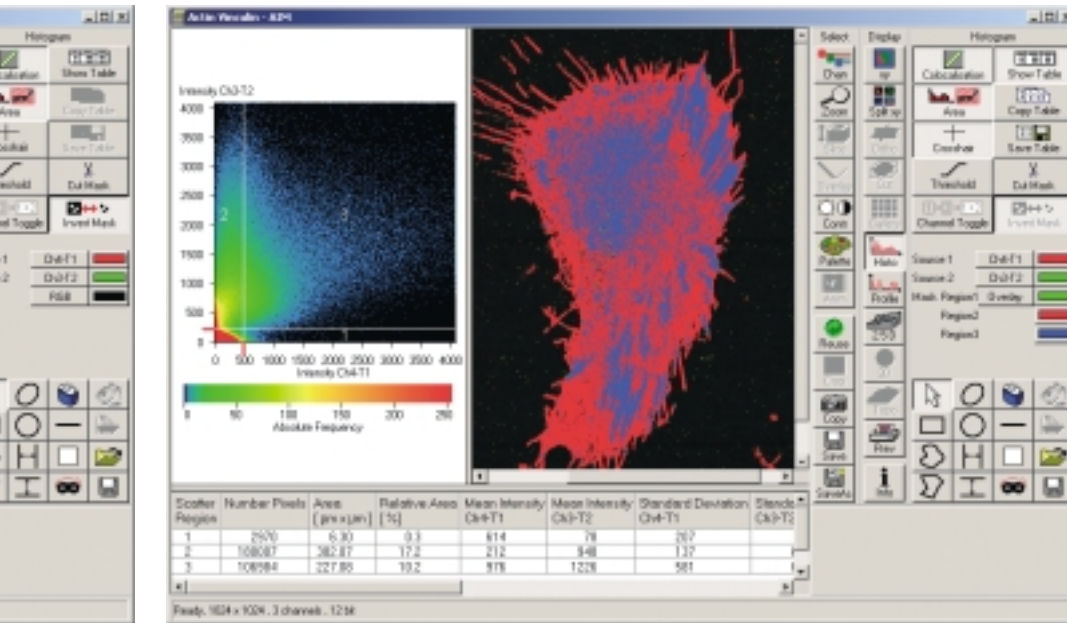


Fig. 1c

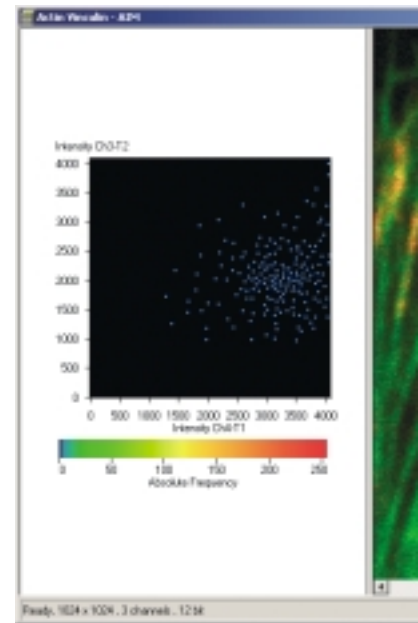


Fig. 2a

are located in quadrants 1 and 2, respectively (Fig. 1c). Pixels having an intensity above the background in *both* channels, i.e. colocalized pixels, are represented by quadrant 3. The image pixels corresponding to quadrants 1, 2 or 3 of the scattergram are then color-coded to allow fast identification of single-tagged and colocalized image areas. In the example, areas with colocalized signals of the actin and vinculin tags are marked blue. The dominating structures have widths of about 1 µm and lengths of 2-5 µm. These typical focal adhesions are mainly localized at the cell periphery. Quantitative data such as the area of colocalized pixels and other numerical colocalization parameters can be viewed with the *Show Table* function.

Thanks to the possibility to automatically determine the crosshair position, this method is particularly well suited to the standardized analysis of a number of images. With the interactive and automatic capabilities, operation is easy and reproducible.

Fig. 1c: The crosshair is positioned so that the background pixels are in the bottom left quadrant. After the Image ROI has been deleted, all image pixels are displayed in the scattergram, with the crosshair position being maintained. On switching to the Crosshair function, the pixels in the Image window are color-coded according to their positions in the scattergram. The background pixels of the bottom left quadrant are displayed in their original color, i.e. with low gray levels. The single-tagged pixels of quadrants 1 and 2 are shown red and green, respectively. Colocalized pixels of quadrant 3 are shown blue.

with the LSM 5 Family

Fig. 2b: The pixels of the scattergram representing the reference structure are selected with a freehand Scatter ROI. After the Image ROIs have been deleted, all image pixels are displayed in the scattergram. The Image window now selectively shows the focal adhesions (white).

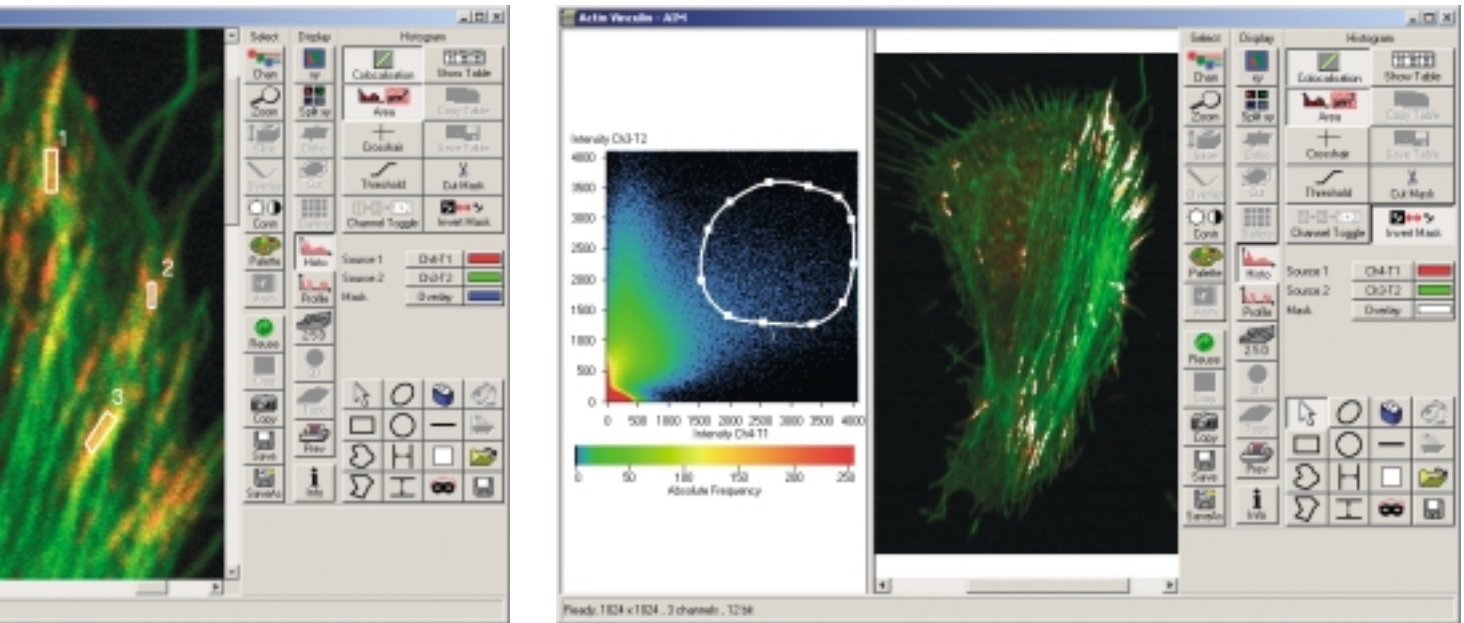


Fig. 2b

The Scatter ROI Mode

The *Scatter ROI* mode is just as important as the *Crosshair* function. It allows colocalized structures to be marked and analyzed via reference areas in the multichannel image.

In the example, use of the *Crosshair* function identified, besides the focal adhesions, spot-shaped structures near the cell nucleus as areas of colocalized actin and vinculin (Fig. 1c). These are probably contacts in the process of disintegration. The colocalization conditions selected with the crosshair obviously apply to both structures. If the structures differ with regard to the expression ratios of the two proteins, one can use the *Scatter ROI* mode to define colocalization conditions that selectively consider the focal adhesions only.

For that purpose, first the areas of three focal adhesions are outlined with an Image ROI as reference structures (Fig. 2a). The corresponding pixels are

selectively displayed on the scattergram. The area of the plot in which these pixels are located is then outlined with a Scatter ROI (Fig. 2b). After the Image ROI has been deleted in the image window, the scattergram represents all pixels of the image. The image window displays all pixels within the Scatter ROI boundary. To these pixels there apply exactly the same colocalization conditions as to the reference structure. The window exclusively displays focal adhesions. The *Show Table* function again permits direct access to the colocalized area, the degree of colocalization, and other quantitative parameters.

Are GFP and YFP Expressed at a Constant Ratio? Expression Level Analysis with the Scattergram Function

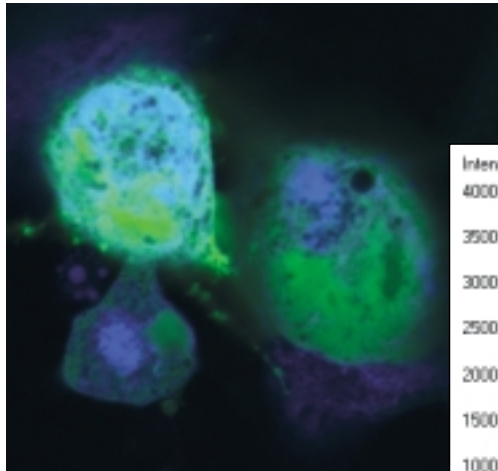


Fig. 3a *Fig. 3a: COS-7 cells, double-transfected with YFP (free) and CFP (ER localization sequence). Specimen: A. and Dr. F. Böhmer, Friedrich Schiller University of Jena, Germany.*

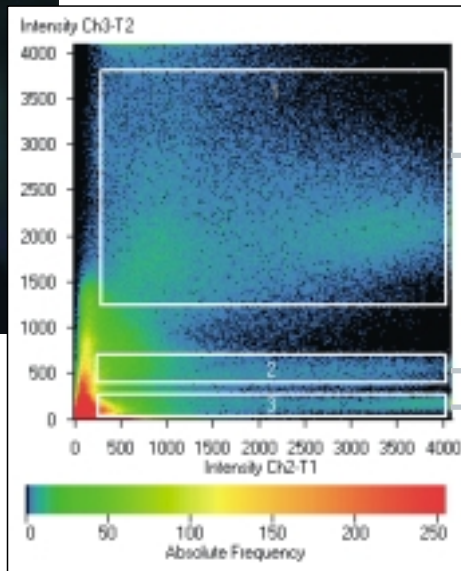


Fig. 3b

Fig. 3b: Scattergram of the double-transfected COS-7 cells. Three horizontally elongated pixel clouds are depicted with Scatter ROIs.

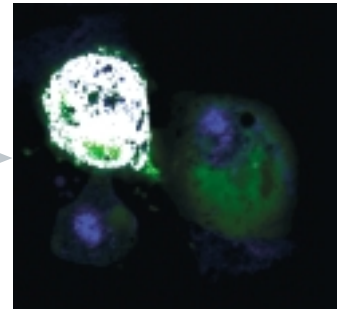


Fig. 3c

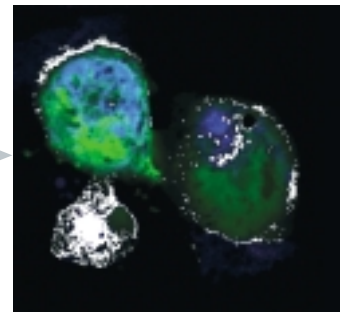


Fig. 3d

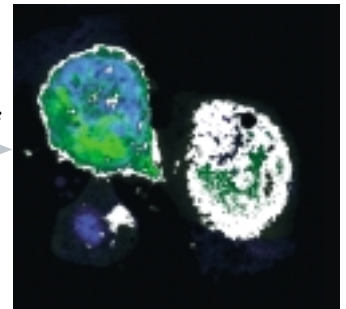


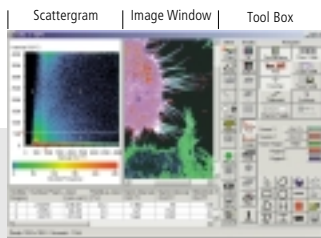
Fig. 3e

Figs. 3c - e: The cells marked white correspond with the pixels of Scatter ROIs 1, 2 and 3 of Fig. 3b. They differ with regard to their CFP/YFP expression ratio.

Thanks to its capability to visualize intensity distributions, the new colocalization software is excellently suited to the analysis of the ratio of fluorescence tags, e.g., as an indication of the expression ratios of certain intracellular proteins. If cells are transfected with constructs that are tagged with fluorescent proteins, the ratio of expression levels is frequently decisive for whether or not the corresponding biological function will establish. In other cases, the main point of interest may be the ratio between an endogenous cellular molecule and a transfected protein. In the example below, COS-7 cells were transfected with CFP and YFP (Fig. 3a). The question was whether the individual cells express both proteins at constant or different ratios. In the scattergram of the two-channel image, several well-separated, horizontally elongated pixel clouds are conspicuous. Whereas the vertical

position of the clouds correlates with the intensity of the YFP signal, the horizontal scatter represents the distribution of the CFP intensities. Which structures are represented by the individual pixel clouds can be conveniently determined in the *Scatter ROI* mode - simply by moving one of the drawing tools around the respective cloud in the scattergram. The corresponding image pixels are then displayed selectively in the Image window. With this procedure, each of the three pixel clouds can be assigned a cell in the Image window. With regard to the question put above, it is obvious that the three cells clearly differ with regard to their YFP expression levels, whereas the broad CFP intensity spectrum is similar for all cells.

Functions at a Glance



Scattergram: A graph in which two channels of a multichannel image are compared with one another. Pixels of both channels having identical positions in the image can be regarded as a pair. Accordingly, each pixel pair has two intensities - one for either channel. The intensities of the two channels are represented by the axes of the scatter graph. All pixel pairs of the original image are entered into the graph according to their intensity levels. If several pixels of the image are entered in the same position of the graph, this accumulation is identified by color coding (many pixels = red, few pixels = blue).

Image Window: Presents those pixels in the multichannel image that correspond to the scatter plot. Here, regions of interest can be selected for analysis in the scattergram.

Tool Box: The Coloc subdialog provides all tools required for analysis.

Source 1	Ch2-T1
Source 2	Ch3-T2

Channel Selection: If an image has more than two channels, this function allows you to select two of them for comparison in the scattergram.



Crosshair Function: Divides the scattergram into four quadrants: Background, single-tagged pixels in channel A, single-tagged pixels in channel B, and colocalized pixels. Image pixels can be presented in different colors according to the corresponding quadrants, so that colocalized and single-tagged pixels can be quickly assigned to quadrants.



Scatter ROI Mode: A mode in which subpopulations in the scattergram can be defined. The corresponding pixels are selectively displayed in the image window.

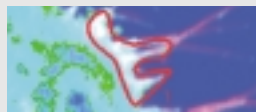


Image ROI Mode: Image regions of interest can be selected for presentation in the scattergram.



Drawing Tools: A variety of drawing and masking tools, such as freehand lines and mask inversions, are available for defining regions of interest (ROIs) in the Scatter ROI and Image ROI modes.



Save and Load Function: Crosshair position or Scatter ROIs once defined can be used for the standardized analysis of several images.



Cut Mask: This function permits the extraction of colocalized image pixels. This produces images containing exclusively the colocalized regions.



Show Table: This function allows access to numerical colocalization parameters (e.g., area and mean intensity of the colocalized regions, degree of colocalization, colocalization coefficient, Pearson's correlation coefficient, and the overlap coefficient according to Manders [see references]).



The new LSM Software from Carl Zeiss sets a standard in the quantitative colocalization analysis of multichannel images. Colocalized image areas can be extracted with a few mouse clicks. The comparison between different images under constant conditions becomes a routine procedure. In addition to the pictorial information, numerical colocalization parameters are available for quantitative analysis. Add to this the unique functions for crosstalk-free multichannel image acquisition, such as Multitracking and Emission Fingerprinting, and it becomes clear that the instruments of the Carl Zeiss LSM 5 Family are not only outstanding imaging systems of benchmark performance, but also highly useful analysis systems for biomedical research.

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Subject to change.

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