IMARISCOLOC

Operating Instructions

Manual Version 1.0   January 2003

the image revolution starts here.

Copyright

This document contains proprietary information protected by copyright. No part of this document may be reproduced, translated, or transmitted without the express written permission of Bitplane AG, Zurich, Switzerland.

☎　　If you find any information missing in this manual or need additional support, please contact us:

Bitplane AG
Badenerstrasse 682
CH-8048 Zurich
Switzerland

Telephone	+41 1 430 11 00
FAX	+41 1 430 11 01
Email	support@bitplane.com
Web address	http://www.bitplane.com

Notice

Information in this book is subject to change without notice and does not represent a commitment on the part of Bitplane AG. Bitplane AG is not liable for errors contained in this book or for incidental or consequential damages in connection with the use of this software.

Contents

What is ImarisColoc ?

In fluorescent microscopy, colocalization describes the presence of two fluorochromes at the same physical location. ImarisColoc provides extended functionality for the analysis and visualization of colocalization in multi-channel data sets. It provides an automated selection of colocalization based on a method introduced by Costes & Lockett at the NIH, NCI/SAIC.

ImarisColoc processes 2D, 3D and in conjunction with ImarisTime also 4D data sets. It operates on two channels simultaneously and measures the degree of overlap of the two channels.

With Coloc, the user can determine and display locations within the data set with common information from all channels, e.g., to illustrate where all types of proteins can be found simultaneously or to detect low-density tissues in all channels.

The desired range for signal analysis can be limited by defining intensity thresholds or sets of intensity values for each of the involved channels. All voxels falling inside every of these channel-specific intensity limits represent a colocalization. They are emphasized in the view and statistics are calculated and displayed. When the selection is changed, the numeric and visual feedback happens in real-time.

The result of the colocalization as a whole can be saved as a separate channel, which can then be processed and viewed as any originally acquired channel in IMARIS. Multiple colocalization images are easily combined into a single view in ImarisSurpass.

ImarisColoc is an optional module within the IMARIS program and is not available as a standalone program.

Main screen

When selected in the IMARIS menu bar, the ImarisColoc main screen is displayed.

The screen consists of the following areas:
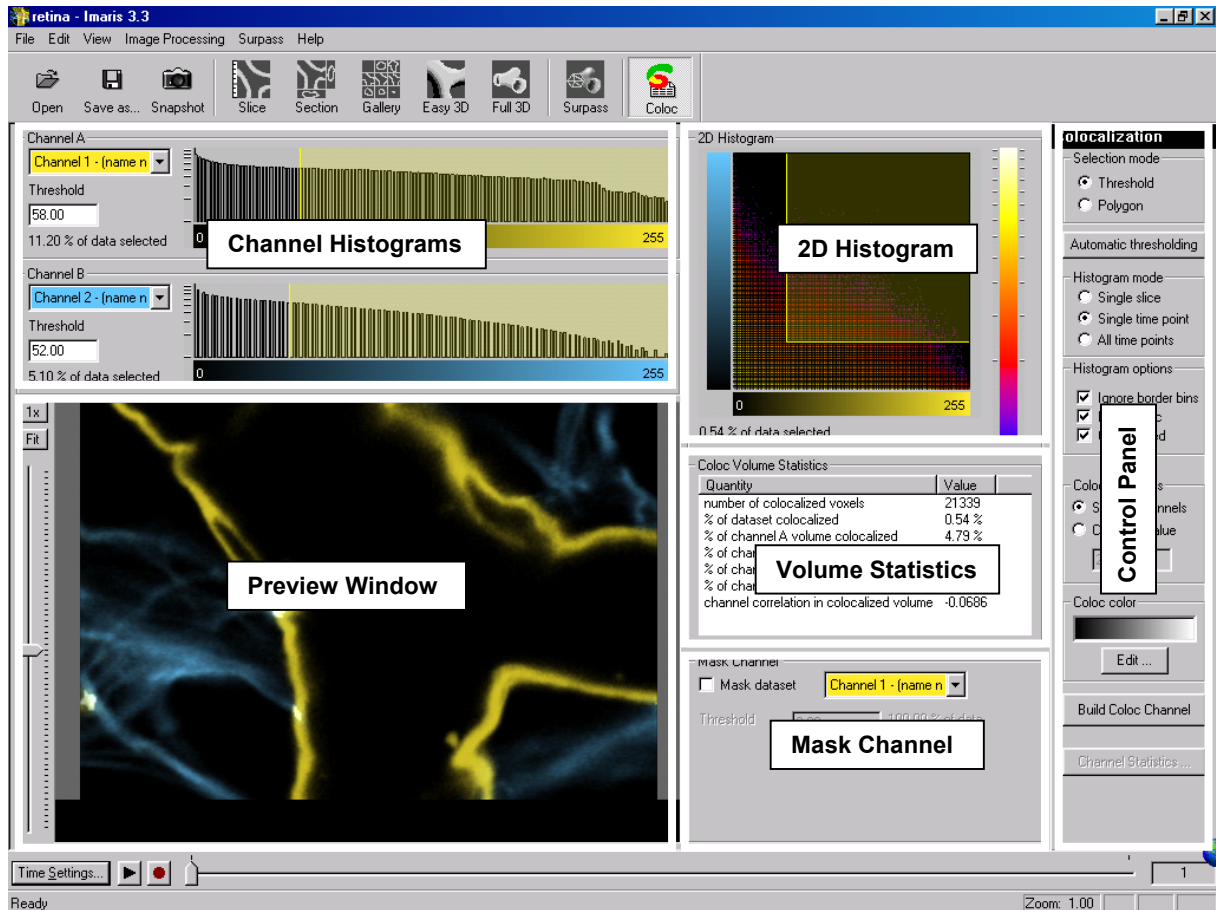
Figure 1: ImarisColoc main screen

Preview Window

This area visualizes the data set together with a preview of the colocalized region. It displays a **Slice** representation of the data set, i.e. a 2D view from the top in the z-axis direction.

A sliding bar with a handle allows inspecting and displaying the single slices in the data set along the z-axis.

The preview window updates - in real time - the display of the colocalized region based on the actual settings of the colocalization parameters.

The original channels can be switched on or off using the channel visibility editor. The user can zoom and pan the view with the middle and right mouse buttons, respectively.
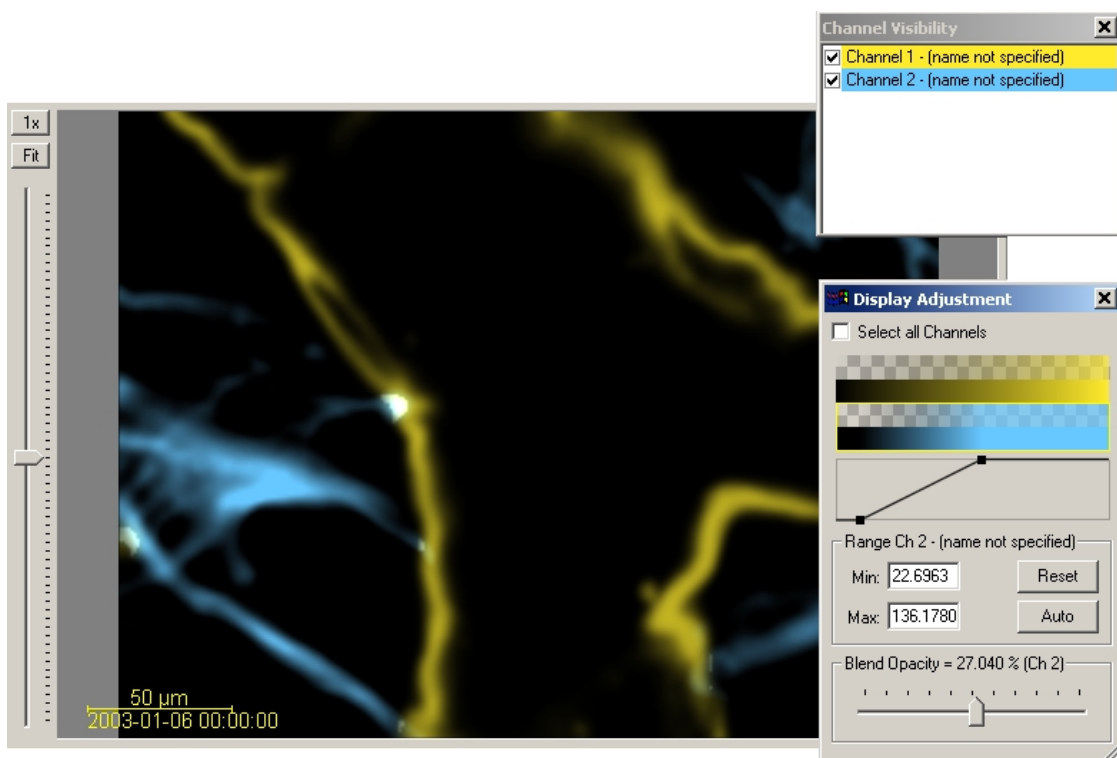
Fig. 2: Preview window with Channel visibility and Display adjustments

Channel Histograms

This area allows the selection of the two channels for the colocalization analysis. The intensity histograms are displayed for the currently selected channels.

With most data sets, checking the **Ignore border bin** and **Logarithmic** options (see "Histogram options" on page 11) allows a more representative histogram display.

The threshold for each channel can then be defined by dragging the yellow line directly in the histogram or by entering the value in the threshold field.
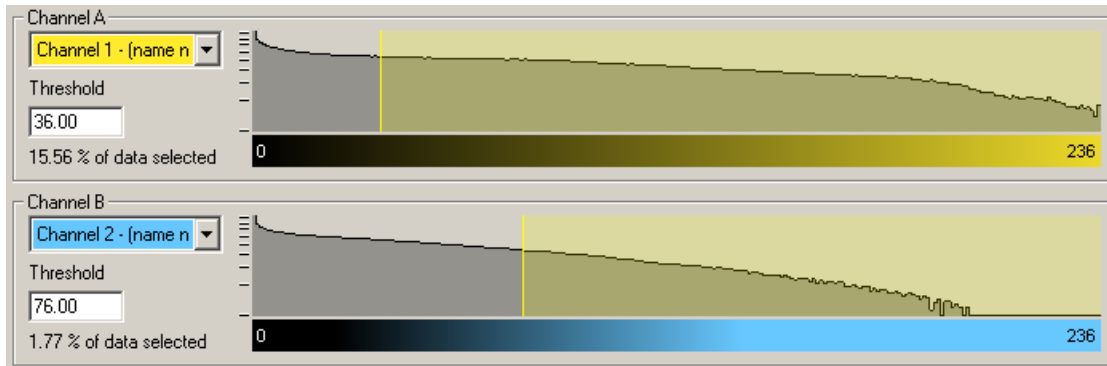
Fig. 3: Channel selection and intensity Histograms

2D Histogram

This area displays a two-dimensional intensity histogram of the selected channels. It reflects the distribution of pairs of voxel intensities occurring in the two selected channels.

The range of intensity pairs considered as colocalized can be defined on the histogram…

- either as channel thresholds, visible in form of a rectangle

- or, if selected, as an intensity area in form of a free configurable polygon.
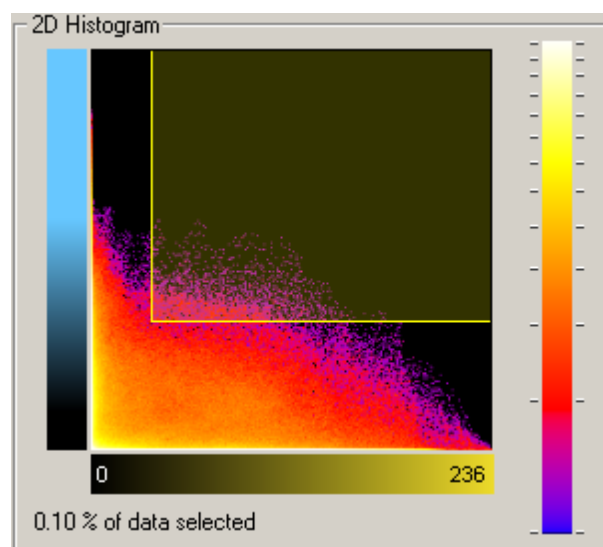
Fig. 4: 2D Histogram

Volume Statistics

This area displays statistics about the resulting colocalized volume. The display adapts instantly to all modifications in the selection.

Coloc Volume Statistics	
Quantity	Value
number of colocalized voxels	21339
% of dataset colocalized	0.54 %
% of channel A volume colocalized	4.79 %
% of channel B volume colocalized	10.52 %
% of channel A material colocalized	4.63 %
% of channel B material colocalized	9.22 %
channel correlation in colocalized volume	-0.0686

Fig. 5: Volume Statistics

Mask Channel

This feature allows the user to define a third channel as a masking area for the entire analysis.

A masking channel is e.g. a third microscope channel acquired with settings that allows defining a region of interest by simple intensity thesholding.

All voxels outside of the region of interest defined by the mask channel are ignored for the colocalization analysis. The volume excluded by the mask appears hatched in the Preview window.

The mask channel is used in conjunction with the Automatic Threshold function. For this function, the selection of an appropriate region of interest is essential.
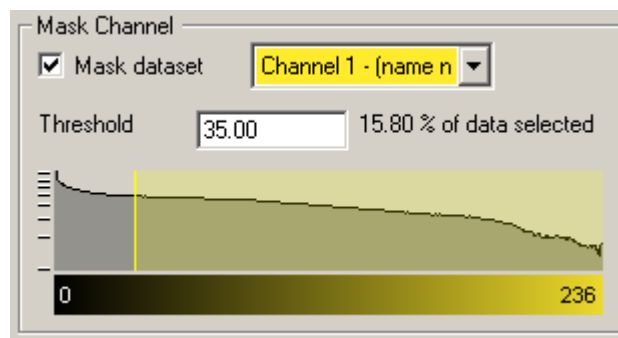
Fig. 6: Mask Channel

Control Panel

The Control panel contains several sections with all possible selection and definition tools and settings for the histograms, the thresholding, and the display as well as the buttons for the color definition and the build of the coloc channel.
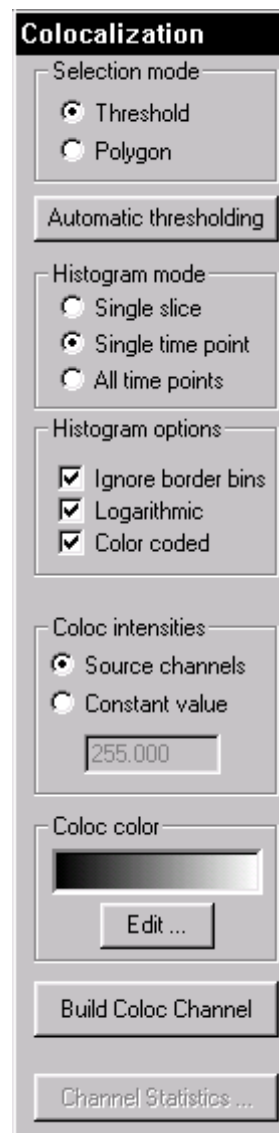
Fig. 7: Control panel

Selection mode

Threshold

The user can define, for each of the channels separately, an intensity value as a threshold. Only voxels with an intensity value above the specified threshold will be considered for the colocalization analysis.

The two thresholds are visible and can be set in four different ways:

- As a hatched region with a bolded line on the 1D histograms.

- As a hatched region with bolded lines on the 2D histogram. The region always contains the top right part of the histogram. The left and lower bolded lines define the intensity thresholds for the two channels.

- As a value in the Threshold field of each channel.

- As an active contour in the Preview window.

When using one of the methods to modify the threshold values, the other indications as well as the Preview display are updated in real-time.

Polygon

The user can define a freely shaped polygonal region whose interior defines the intensity pairs considered as colocalized.

Automatic thresholding

This button performs an automatic threshold run on both channels based on an algorithm developed by Costes and Lockett at the National Institute of Health, NCI/SAIC, which is based on the exclusion of intensity pairs that exhibit no correlation (Pearson's correlation below zero).

The automatic threshold search is done using the 2D histogram Starting with the highest intensity value, the algorithm reduces the threshold value step by step along a line shown in Figure 8 and computes the correlation coefficient of the image using only voxels with intensities below the threshold. The algorithm continues reducing the thresholds until the correlation reaches 0, thus defining the automatic threshold.

Channel A

line along which
algorithm proceeds

Correlation = 0 on
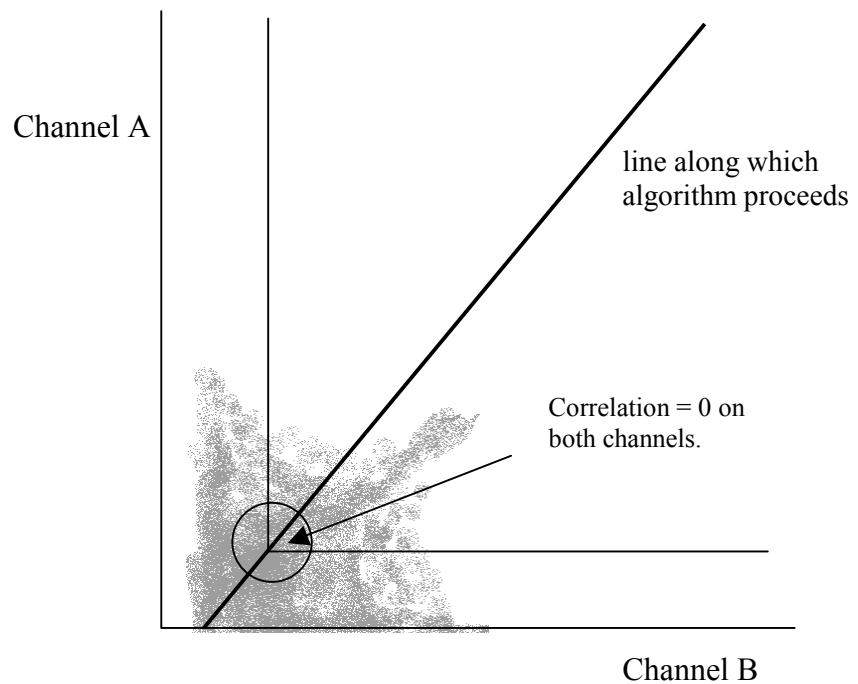both channels.

Channel B

Fig. 8: Automatic Threshold Detection

For more information please refer to the following publication:

S. Costes, E. Cho, M. Catalfamo, T. Karpova, J. McNally, P. Henkart, S. Lockett, Proceedings Microsc. Microanal. 8 (Suppl. 2), 1040-41CD, 2002

Histogram mode

The histogram mode options are used to define the calculation basis for the colocalization within the data set.

Single slice	The histograms are calculated and displayed for the current slice only.
Single time point	The histograms are calculated and shown for the entire 3D stack of the current time point. This mode allows for faster browsing through the data set along the z-axis.
All time points	The histograms are calculated and shown for an entire time data set, i.e. for all time points within the 4D data set.

Histogram options

The histogram mode options are used to define the display of the 1D and 2D histograms.

Ignore border bins	Often a lot of voxels fall into the bin with the lowest intensity. In a frequency plot this causes a massive and unnecessary downscale of all other intensity bins which are of more interest. The border bins can therefore be ignored when scaling the display of the histogram. Ignore is selected as default.
Logarithmic	The frequency plot is shown in a logarithmic scaling instead of a linear scaling.
Color coded	Applies a false color look-up table to the histogram to improve the visibility of intensity differences. The high frequencies (bins in the 2D histogram into which a lot of voxels accumulate) are shown in yellow-to-white, bins with little voxels are shown in blue-to-black.

Coloc intensities

The Coloc intensities option defines the brightness of the calculated colocalization displayed in the Preview window.

Source channels	Uses the intensities of the two channels for displaying the intensity of a colocalized voxel as the square root of the product of the intensities of the original channels.
Constant value	Displays the intensity of a colocalized voxel defined by the specified value. No distinction is made whether high-intensity or low-intensity voxels colocalize as long as they are within the selection.

Coloc color

Coloc color allows the user to specify his personal color settings for the colocalization display in the Preview window.

Edit	Clicking on the Edit button opens the Color window. The user can either select one of the predefined colors to apply on the colocalization areas or define, add, and apply his own color definitions.

Build Coloc Channel

Clicking this button calculates the colocalization channel according to the selected settings and adds it to the data set. The channel can then be saved and is part of the data set.

Channel Statistics

After building the coloc channel, its statistics become available in the Image Properties.

The Channel Statistics button displays directly the Image Properties window with the statistics for the calculated channel.

Basic Operation

The basic use of ImarisColoc in the daily workflow comprises the following steps:

1. Loading a data set
2. Selecting the channels
3. Setting the thresholds
4. Checking the statistics
5. Building the Coloc channel
6. Saving the Coloc channel

Loading a data set

Any 2D, 3D, or 4D multi-channel and/or time data set of the all types supported by IMARIS can be loaded and used for colocalization detection:
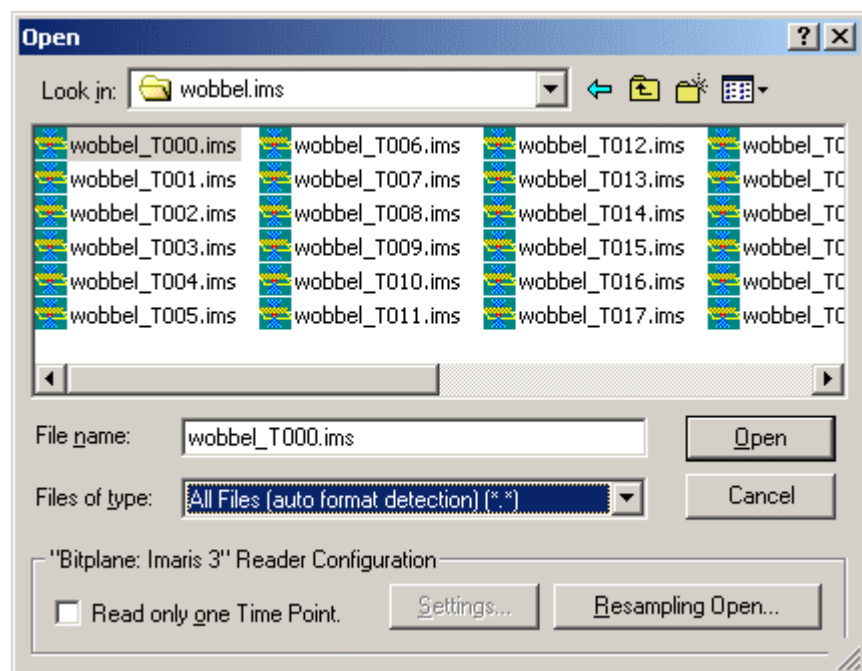

Fig. 9: File open dialog

1. In the main toolbar select *File – Open*.
   The Open box is displayed.
2. Select file type from the Files of Type pull-down menu.
3. Select a file name from the list and click **Open** or double-click on the requested file entry.
   The file is loaded.

Selecting the channels

The two channels for colocalization detection must be selected first.

1. Select channel A and channel B from the pull-down lists.

    The 1D and 2D intensity histogram displays are updated.

2. Modify the Histogram options if necessary in order to display an expressive voxel intensity representation.

Setting the thresholds

Specify Selection mode

In order to be able to set the thresholds, the user must check the Selection mode **Threshold** in the control panel.

Select Histogram mode

Check:

*   **Single Slice** for calculating the histogram for current slice only.

*   **One Time Point** to calculate the histogram for the entire 3D time point.

*   **All Time Points** for the entire 4D image.

Threshold setting methods

There are basically four different methods how the thresholds can be defined.

On the 1D Channel Histogram

1. Point with the cursor on the histogram. The cursor turns into a cross.

2. Click on the histogram to determine a threshold.

    The bolded left line of the overlaid hatched area (indicating the selected voxel intensity range for colocalization) jumps on the marked spot.

3. Click on the line and drag it to another position in the histogram.

    Effects of the modification are instantly visible in the other indications as well as in the Preview window.

4. Perform for both channels until the settings are as desired.

By entering a value

1. Click in the Threshold field of one of the channels and enter a value, then press Enter.

    The bolded left line of the overlaid hatched area (indicating the selected voxel intensity range for colocalization) jumps on the marked spot.

    Effects of the modification are instantly visible in the other indications as well as in the Preview display.

2. Perform for both channels until the settings are as desired.

On the 2D Histogram

1. Point with the cursor on the 2D histogram. The cursor turns into a cross.

2. Click on the histogram to determine the thresholds.

    The edge of the overlaid hatched area (indicating the selected voxel intensity range for colocalization) jumps on the marked spot.

3. Click on the edge and drag it to any another position in the histogram.

    Effects of the modification are instantly visible in the other indications as well as in the Preview display.

On the Preview Window

1. Click with the cursor on the image in the Preview window.

    An active contour is displayed, outlining image elements brighter than the location clicked.

2. Drag the line on the image to define the threshold of channel A.

    The selection works best when starting with brighter spots and dragging towards darker areas.

3. Press and hold the SHIFT key, click with the cursor on the image, then drag the line to determine the threshold of channel B.

    Colocalization occurs where the voxels are above threshold in channel A and channel B.
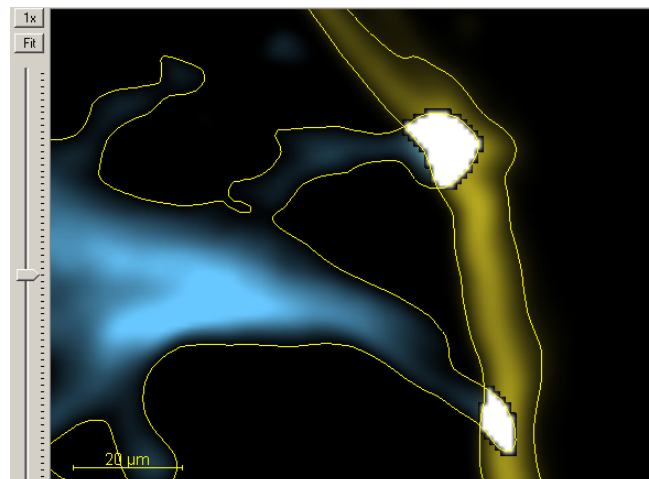

Fig. 10: Threshold selection on the Preview window

Coloc Display

The user may determine the intensity for the colocalized voxels to be shown.

Coloc intensities

Check:

- **Source channel** for using the intensities of the two channels for displaying the intensity

- **Constant value** for displaying the intensity of a colocalized voxel defined by the specified value.

Coloc Color

1. Click the **Edit** button.

   The color box is displayed.

2. Select any of the colors and click **OK**.

   The indication bar shows the selected color and the colocalization areas in the Preview window are colored accordingly.

3. If required, click Color definition and use the colorwash to create and add your own colors for selection.

Coloc Volume Statistics

The values are updated in real-time and refer to the entire volume data of the current time point, regardless of the settings for the Histogram mode. The values are calculated by the following formulas:

Definitions

Regions:

Data set	all data set voxels
Coloc	colocalized voxels
Object A	Voxels with channel A intensity above threshold A
Object B	Voxels with channel B intensity above threshold B

Indicators:

N	Number of voxels
SA_i	Channel A intensity of ith voxel
SB_i	Channel B intensity of ith voxel
$SA_{avg, coloc}$	Average channel A intensity in the coloc region

Number of colocalized voxels

Total count of colocalized voxels N_{coloc}.

% of dataset colocalized

Percentage of total data set voxels colocalized.

$$\frac{N_{coloc}}{N_{dataset}} \cdot 100\%$$

% of channel A volume colocalized

Percentage of channel A voxels above threshold A colocalized.

$$\frac{N_{coloc}}{N_{objectA}} \cdot 100\%$$

% of channel B volume colocalized

Percentage of channel B voxels above threshold B colocalized.

$$\frac{N_{coloc}}{N_{objectB}} \cdot 100\%$$

% of channel A material colocalized

Percentage of channel A material above threshold A that is colocalized.

$$\frac{\sum_{i \in coloc} SA_i}{\sum_{i \in objectA} SA_i} \cdot 100\%$$

% of channel B material colocalized

Percentage of channel B material above threshold B that is colocalized.

$$\frac{\sum_{i \in coloc} SB_i}{\sum_{i \in objectB} SB_i} \cdot 100\%$$

% of unmasked dataset colocalized

Percentage of channel A and channel B inside the residual mask volume that is colocalized.

$$\frac{N_{coloc}}{N_{mask}} \cdot 100\%$$

% of mask A material
colocalized

Percentage of residual channel A material inside the mask volume that is colocalized.

$$\frac{\sum\limits_{i \in coloc} SA_i}{\sum\limits_{i \in mask} SA_i} \cdot 100\%$$

% of mask B material
colocalized

Percentage of residual channel B material inside the mask volume that is colocalized.

$$\frac{\sum\limits_{i \in coloc} SB_i}{\sum\limits_{i \in mask} SB_i} \cdot 100\%$$

Channel correlation
in colocalized volume

Pearson correlation of channel A and channel B inside the colocalized region. (Note: The correlation value ranges between 1 and -1. A value of 1 represents perfect correlation, 0 no correlation, and -1 perfect inverse correlation.)

$$R_{AB,coloc} = \frac{\sum\limits_{i \in coloc}\left(SA_i - SA_{avg,coloc}\right)\left(SB_i - SB_{avg,coloc}\right)}{\sqrt{\sum\limits_{i \in coloc}\left(SA_i - SA_{avg,coloc}\right)^2 \sum\limits_{i \in coloc}\left(SB_i - SB_{avg,coloc}\right)^2}}$$

Channel correlation
in data set volume

Pearson correlation of channel A and channel B inside the entire data set volume.

$$R_{AB,dataset} = \frac{\sum\limits_{i \in dataset}\left(SA_i - SA_{avg,dataset}\right)\left(SB_i - SB_{avg,dataset}\right)}{\sqrt{\sum\limits_{i \in dataset}\left(SA_i - SA_{avg,dataset}\right)^2 \sum\limits_{i \in coloc}\left(SB_i - SB_{avg,dataset}\right)^2}}$$

Channel correlation
in unmasked volume

Pearson correlation of the residual channel A and channel B inside the mask volume.

$$R_{AB.mask} = \frac{\sum\limits_{i \in mask}\left(SA_i - SA_{avg,mask}\right)\left(SB_i - SB_{avg,mask}\right)}{\sqrt{\sum\limits_{i \in mask}\left(SA_i - SA_{avg,mask}\right)^2 \sum\limits_{i \in mask}\left(SB_i - SB_{avg,mask}\right)^2}}$$

Build Coloc Channel

Calculation and building

After its build the Coloc channel is added to the current data set.

The new channel is then available in all IMARIS views - most interestingly in Surpass.

1. Click the **Build Coloc Channel** button.

    The colocalization channel is calculated while a box is displayed showing the process.

Viewing statistics

The calculated statistics for each colocalization channel can be viewed in the Image properties.

1. In the menu bar select *Edit – Image Properties.*

    The Image Properties window is displayed.

2. Click the required channel in the menu tree*.*

    The **Name** of the channel, the **Description** of the source channels and the selection method are indicated and the Coloc Statistics are displayed.

Export statistics

The statistics can be exported.

1. In the Image Properties window click the **Export** button*.*

    The Export Coloc Statistics window is displayed.

2. Select the directory and enter the name for the file to be saved.

    The statistics are saved as .csv Excel file and can be opened directly with Microsoft Excel.

Save Coloc Channel

Before the channel is added permanently to the data set it must be saved.

1. In the menu bar select *File – Save As* or click the **Save as…** button in the toolbar.

   The **Save As** box is displayed.

2. Select the directory and enter the name for the file to be saved or confirm the suggestion.

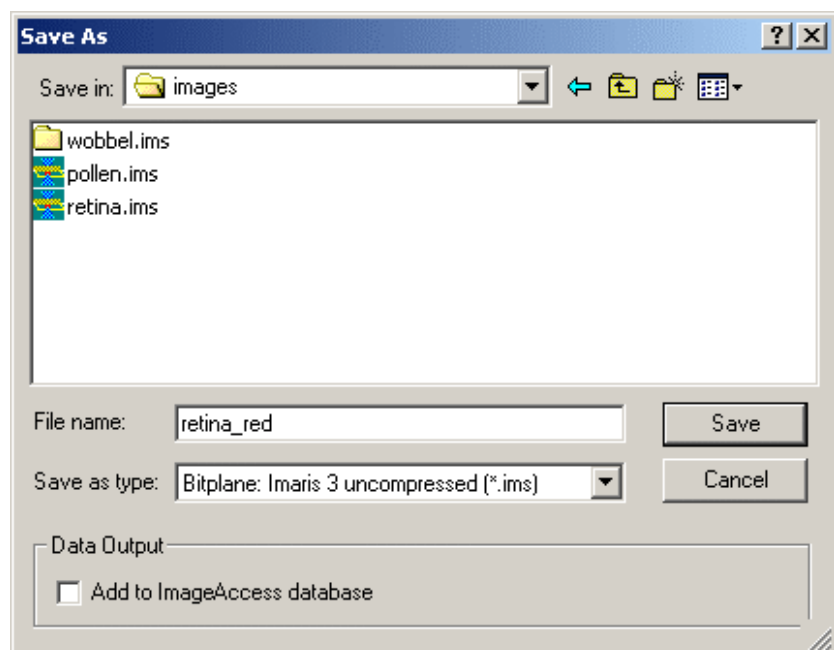3. Select the requested file format and click **OK**.

   The data set is saved.

Fig. 11: Save As dialog

Advanced Operation

Polygon Mode

Instead of using the basic threshold mode for defining the area where a colocalization will be determined, the user can define a freely shaped polygonal region.

The polygon can be drawn on the 2D histogram, when the Selection mode **Polygon** is checked.

This method is especially useful for eliminating channel bleed through regions along the border of the lower intensity bins.

Drawing methods

After checking the Polygon mode, the overlaid threshold rectangle in the 2D histogram is converted into a polygon with four corner points. The user can now start to modify this polygon or to draw a completely new one.

The initial point of the polygon is visible as black filled.

Setting points

Press and hold the **SHIFT** key. The cursor turns into a cross with a little "+" sign. Click on the 2D histogram with the left mouse button to place a point (vertex). Any additional point connects with lines to the initial point and the last point inserted.

Inserting points in lines

Press and hold the **SHIFT** key. The cursor turns into a cross with a little "+" sign. Point on the line between two vertices and click on the line with the left mouse button to add a point.

Automatic point setting

Press and hold the **CTRL** key. The cursor turns into a cross with a little "+" sign and a stack symbol. Drag the cursor around. New vertices are added automatically at each change of cursor position.

Moving points

Point on one of the vertices, left-click and drag the vertex around to the desired location. The polygon changes its shape.

Moving lines

Point on a line between two vertices, left-click and drag the line around to the desired location. The polygon changes its shape.

Moving the polygon

Point somewhere beside of the polygon edge, left-click and drag the polygon around to the desired location.

Deleting points

Point on one of the vertices. Press the **DEL** key to delete the vertex.

Deleting the polygon

Point somewhere beside of the polygon edge and press the **DEL** key to delete the entire polygon.

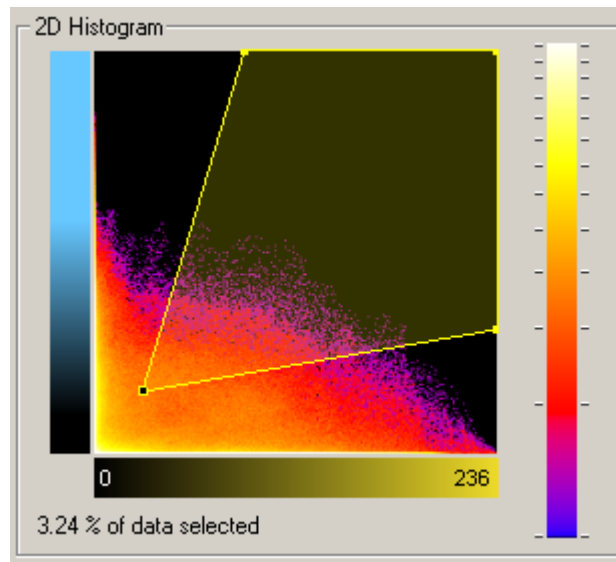Fore more information select *Help – Keys Help* in the menu bar.

Figure 12: Typical polygon selection for channel bleed through

Mask Channel and Automatic Threshold

Automatic thresholding produces only relevant results, if an adequate mask is defined (please refer to the publication by Costes and Lockett, mentioned on page 10).

1. In the Mask channel area select the channel to be used from the pull-down list.

2. In the Mask channel area check the Mask dataset field.

    The 1D histogram of the mask channel is displayed and the Threshold field is activated.

3. Point with the cursor into the histogram and click to set the threshold.

    A bolded line marks the threshold in the histogram and the corresponding value is indicated in the threshold field.

    The masked volume of the data set appears hatched in the Preview window.

4. Drag the cursor in the histogram to modify the threshold.

    Effects of the modification are instantly visible in the threshold field as well as in the Preview window.

5. When the mask is set as required, click the Automatic thresholding buttons to calculate the thresholds. Build the colocalization channel by clicking the Build Coloc Channel button.

Time Settings

For a detailed description of the Time option please refer to the IMARIS manual (ImarisTime is an optional module of IMARIS and may be not available).

There are no time-dependent parameters for the colocalization analysis. The Preview window always displays the currently selected time point of the data set. The values in the Statistics window always refer to the volume of the current time point. However, when the Build coloc channel button is pressed, the coloc channel and statistical values for all time points are calculated.

Examples

Visualize the Coloc Channel in IMARIS

The colocalization channel can be visualized like any other channel in IMARIS. In the following examples, a coloc channel was built on the retina data set.

Example 1

Visualize and adjust the Coloc channel in IMARIS Easy 3D.

1. After building the Coloc channel, click on the **Easy 3D** button in the IMARIS menu bar.

2. Check the desired **Rendering mode** parameters and adjust the **Settings** if necessary, then click the **Calculate** button.

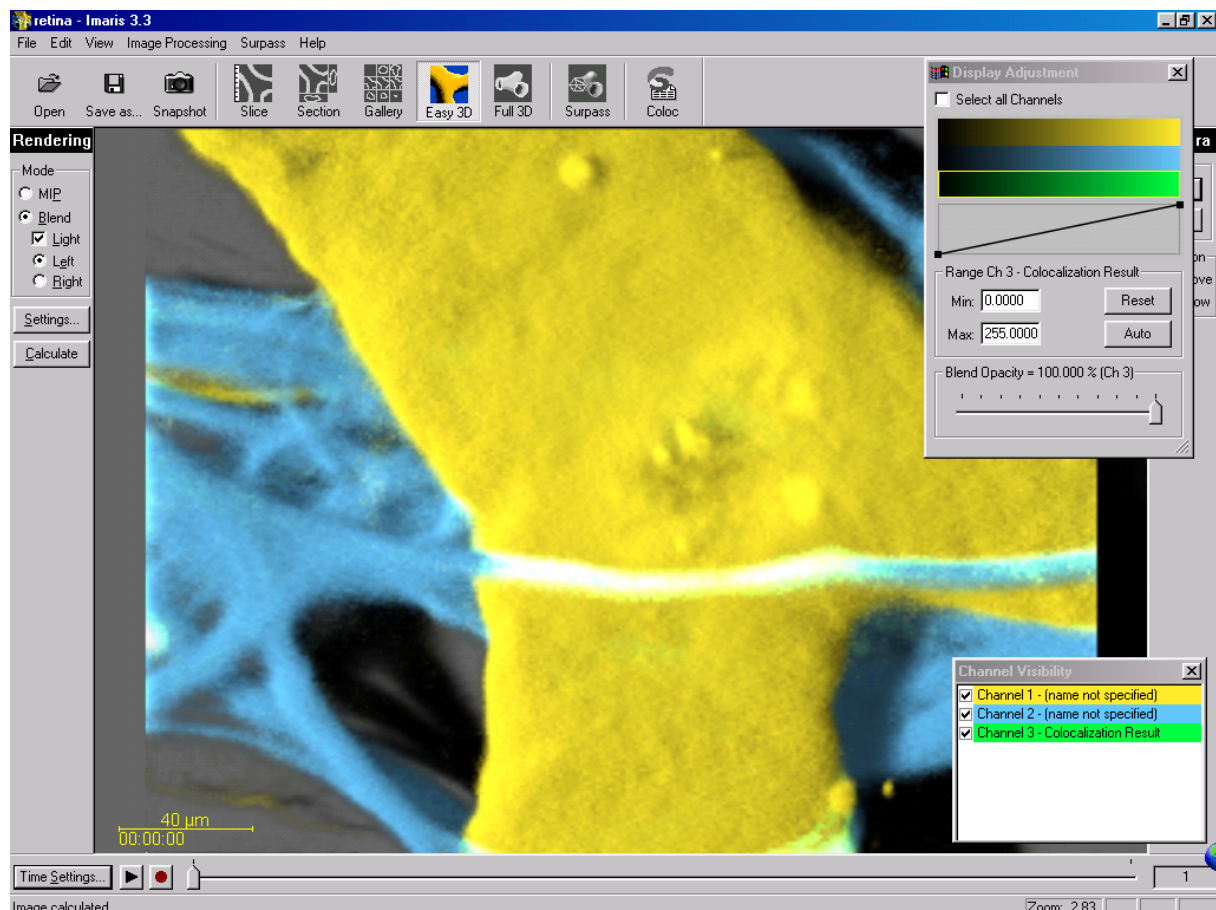   The 3D image of channels is calculated and displayed.

Figure 13: Easy 3D representation of all three channels

As the colocalized region always lays inside the initial channels, making the initial channels invisible or more transparent allows a better view of the colocalized region.

3. In the IMARIS menu bar select *Edit – Channel Visibility.*

4. In the Channel Visibility window uncheck one of the two original channels, then click the **Calculate** button again.

    The 3D image of the original and the Coloc channel is calculated and displayed.

5. In the IMARIS menu bar select *Edit – Display Adjustment.*

6. In the Display Adjustment window select the Coloc channel and adjust its settings.

7. In the Display Adjustment window select the remaining original channel and adjust its settings. In this example the channel is set as partly opaque.

8. After each modification, click the **Calculate** button again to calculate the result.
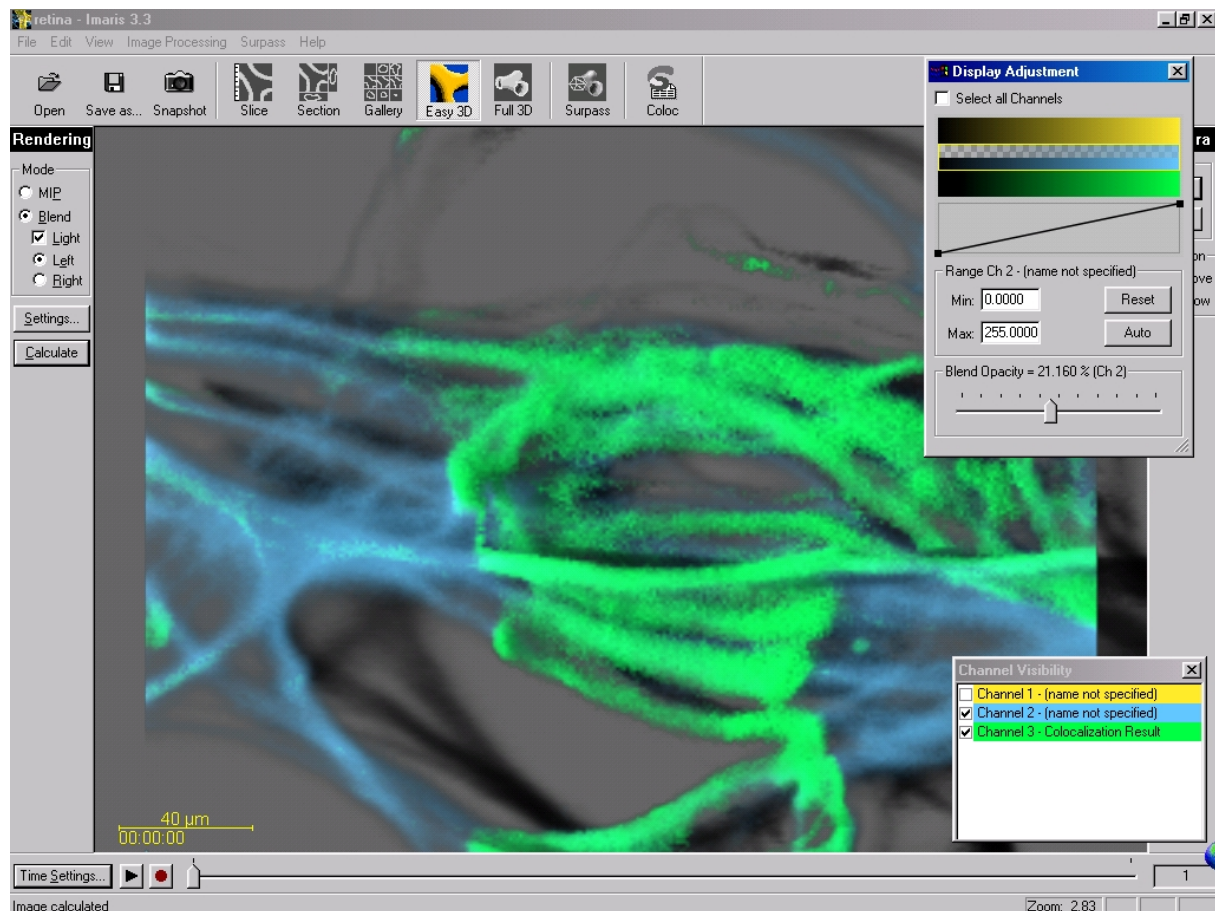

Figure 14: Easy 3D representation of the Coloc channel with one original channel

Visualize the Coloc Channel in SURPASS

The colocalization channel can be visualized like any other channel in Surpass (Surpass is an optional module of IMARIS and may be not available).

Example 2

Create an Iso Surface of the Coloc channel add the two initial channels as volume rendering.

1. After building the Coloc channel, click on the **Surpass** button in the IMARIS menu bar.

2. In the Object area click the **Add New Iso Surface** button.

3. In the Iso Surface Setup window check and select the Coloc channel.

4. Select the desired parameters. In this example, we set the threshold just below the lowest intensity values in the histogram.
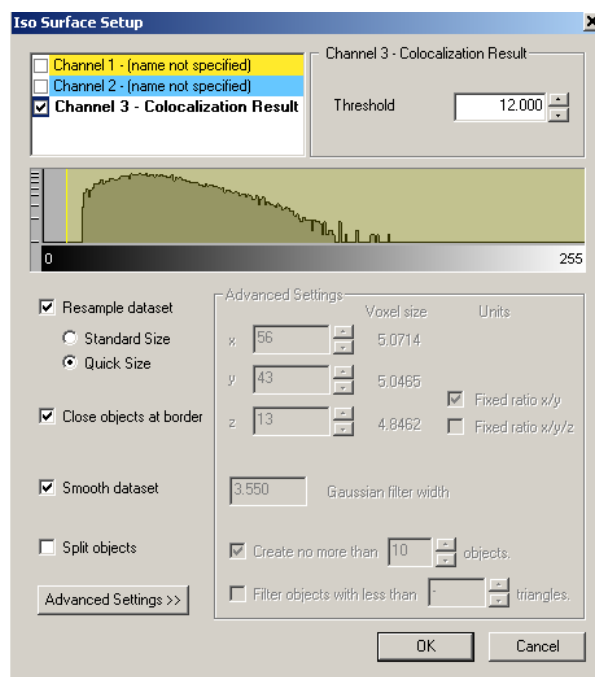

Figure 15: Iso Surface settings for colocalized channel

5. In the Object area click the **Add New Volume** button.

6. In the IMARIS menu bar select *Edit – Channel Visibility.*

7. In the Channel Visibility window uncheck the coloc channel.

   The Surpass Volume object displays only the two initial channels.

8. In the IMARIS menu bar select *Edit – Display Adjustment.*

9. In the Display Adjustment window select the two initial channels and adjust their settings, e.g. decrease the blend opacity.
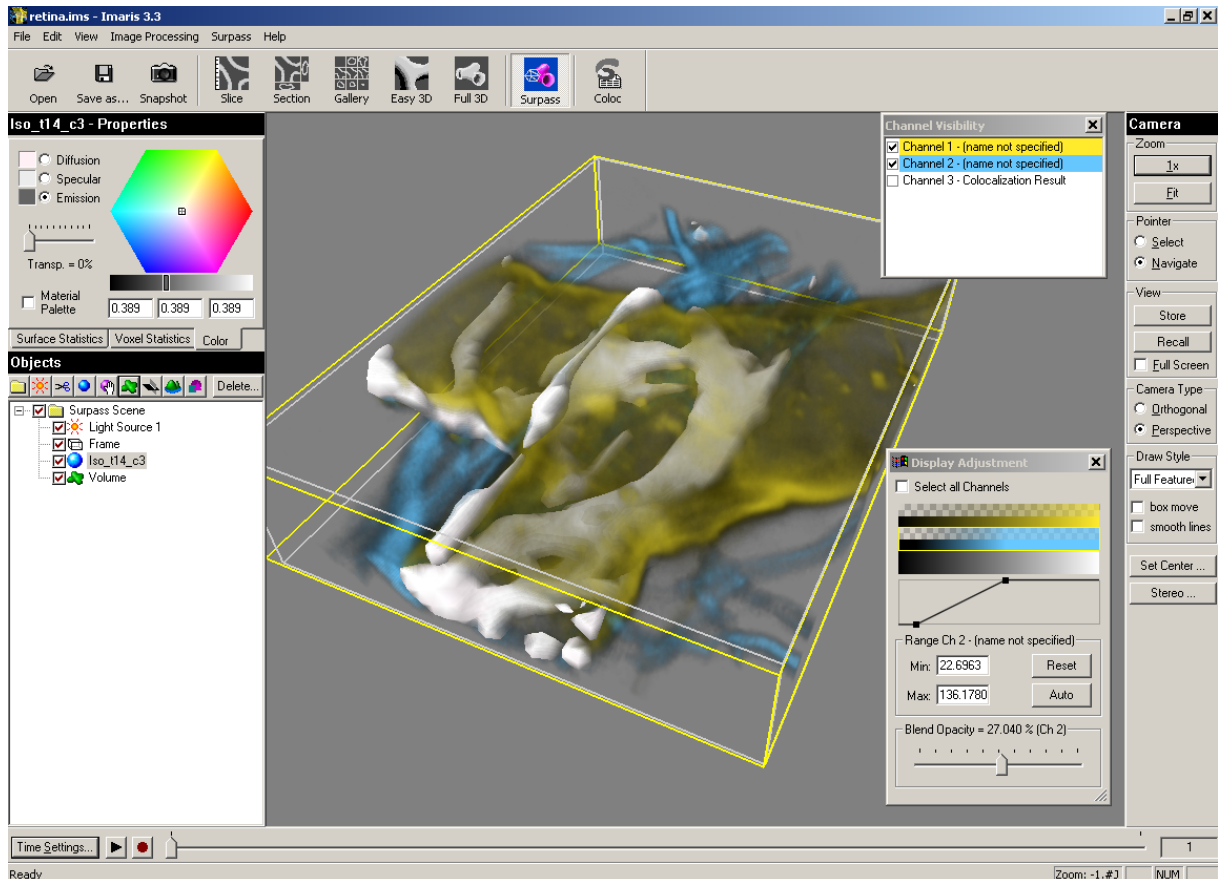

Figure 15: Iso Surface of colocalized region and volume rendering of initial data set