CHAPTER 6 VBA PROGRAMMING FOR LSM

CONTENTS

6 VBA PROGRAMMING FOR LSM

6.1 VBA Programming

6.1.1 General Syntax

Object oriented programming

Class Modules

6.1.2 Working with Files

I/O operations, file operations are implemented in VBA

Use of the implemented functions

Example:

```
Type CALIBRATION_PARAM
    lambda As Long
    angle As Double
    Translation As Double
End Type

Function DataFromCalibFile(strGetFile As String, calibArray() As CALIBRATION_PARAM) As Integer
    Dim hFile As Long
    Dim strLine As String
    Dim varData(1 To 3) As Variant
    Dim paramCnt As Integer

    paramCnt = -1
    'get the next free file handle
    hFile = FreeFile
    'check if file exist
    If (Len(Dir(strGetFile)) > 0) Then
        Open strGetFile For Input Access Read Shared As hFile
            If (Not EOF(hFile)) Then
                Line Input #hFile, strLine

                paramCnt = 0
                Do Until EOF(hFile)
                    Input #hFile, varData(1), varData(2), varData(3)
                    calibArray(paramCnt).lambda = varData(1)
                    calibArray(paramCnt).angle = varData(2)
                    calibArray(paramCnt).Translation = varData(3)
                    paramCnt = paramCnt + 1
                Loop
            End If
        Close #hFile
    End If

    DataFromCalibFile = paramCnt
End Function Objektstruktur
```

Use of the API function for additional functionality

6.1.3 Access to Windows API Function and external DLLs

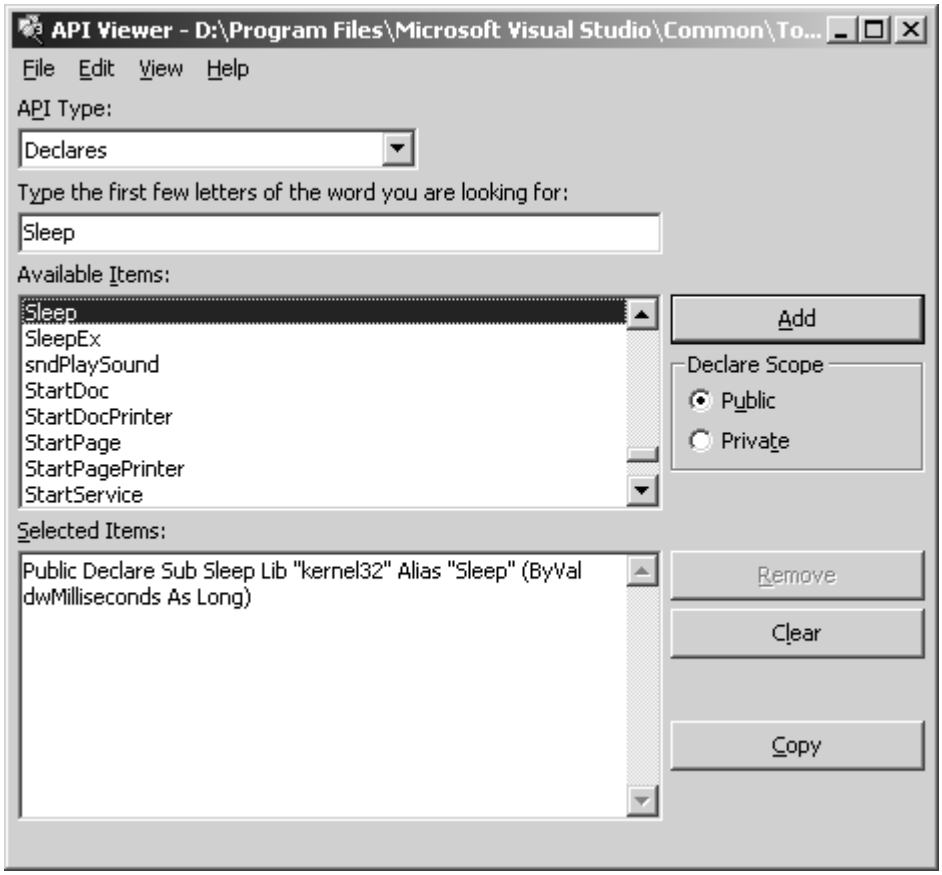
- with Type Libraries

  OLE

- with Declare Statements

  API Text Viewer

  searches WIN32API.TXT



Example:

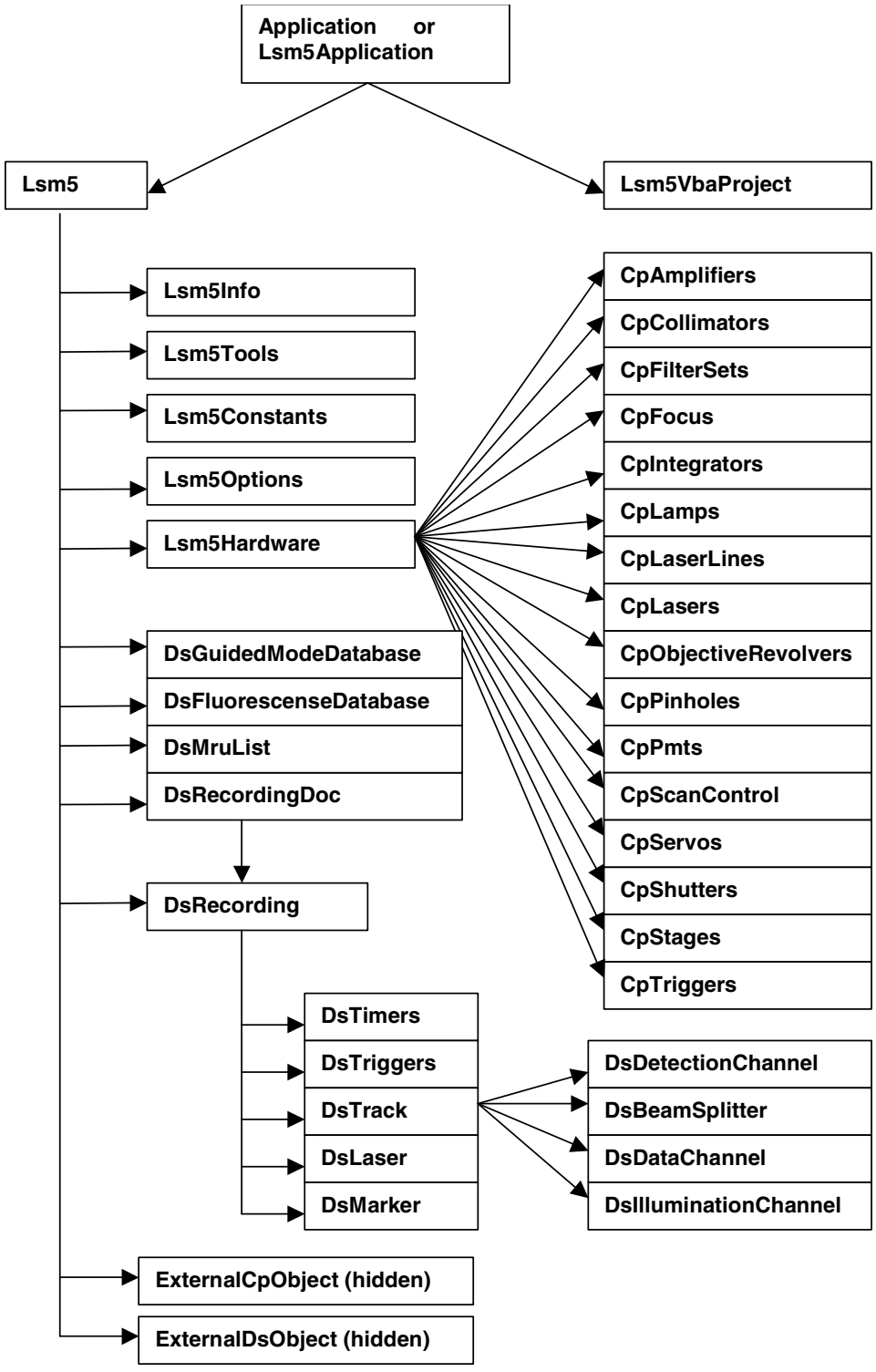
```
Declare Sub Sleep Lib "kernel32" (ByVal dwMilliseconds As Long)

Declare Function RegOpenKeyEx _
    Lib "advapi32.dll" Alias "RegOpenKeyExA" _
    (ByVal hKey As Long, ByVal lpSubKey As String, _
    ByVal ulOptions As Long, ByVal samDesired As Long, _
    phkResult As Long) As Long


Public Declare Function RegCloseKey _
    Lib "advapi32.dll" (ByVal hKey As Long) As Long


Public Declare Function RegQueryValueEx _
    Lib "advapi32.dll" Alias "RegQueryValueExA" _
    (ByVal hKey As Long, ByVal lpValueName As String, _
    ByVal lpReserved As Long, lpType As Long, _
    lpData As Any, lpcbData As Long) As Long        ' Note that if you declare the lpData  parameter
    as String, you must pass it By Value.

Public Const HKEY_CLASSES_ROOT = &H80000000

Public Const SYNCHRONIZE = &H100000
Public Const READ_CONTROL = &H20000
Public Const STANDARD_RIGHTS_READ = (READ_CONTROL)
Public Const KEY_QUERY_VALUE = &H1
Public Const KEY_ENUMERATE_SUB_KEYS = &H8
Public Const KEY_NOTIFY = &H10
Public Const KEY_READ = ((STANDARD_RIGHTS_READ Or KEY_QUERY_VALUE Or
KEY_ENUMERATE_SUB_KEYS Or KEY_NOTIFY) And (Not SYNCHRONIZE))

Public Const REG_SZ = 1                         ' Unicode null terminated string
Public Const ERROR_SUCCESS = 0&
```

```
Function FServerFromDescription(strName As String, strPath As String) As Boolean
    Dim lngResult As Long
    Dim strTmp As String
    Dim hKeyServer As Long
    Dim strBuffer As String
    Dim cb As Long
    Dim i As Integer

    FServerFromDescription = False

    strTmp = VBA.Space(255)
    strTmp = strName + "\CLSID"
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)

    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegCloseKey(hKeyServer)
    strTmp = VBA.Space(255)
    strTmp = "CLSID\" + Left(strBuffer, cb - 1) + "\LocalServer32"
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strPath = Left(strBuffer, cb - 1)
```

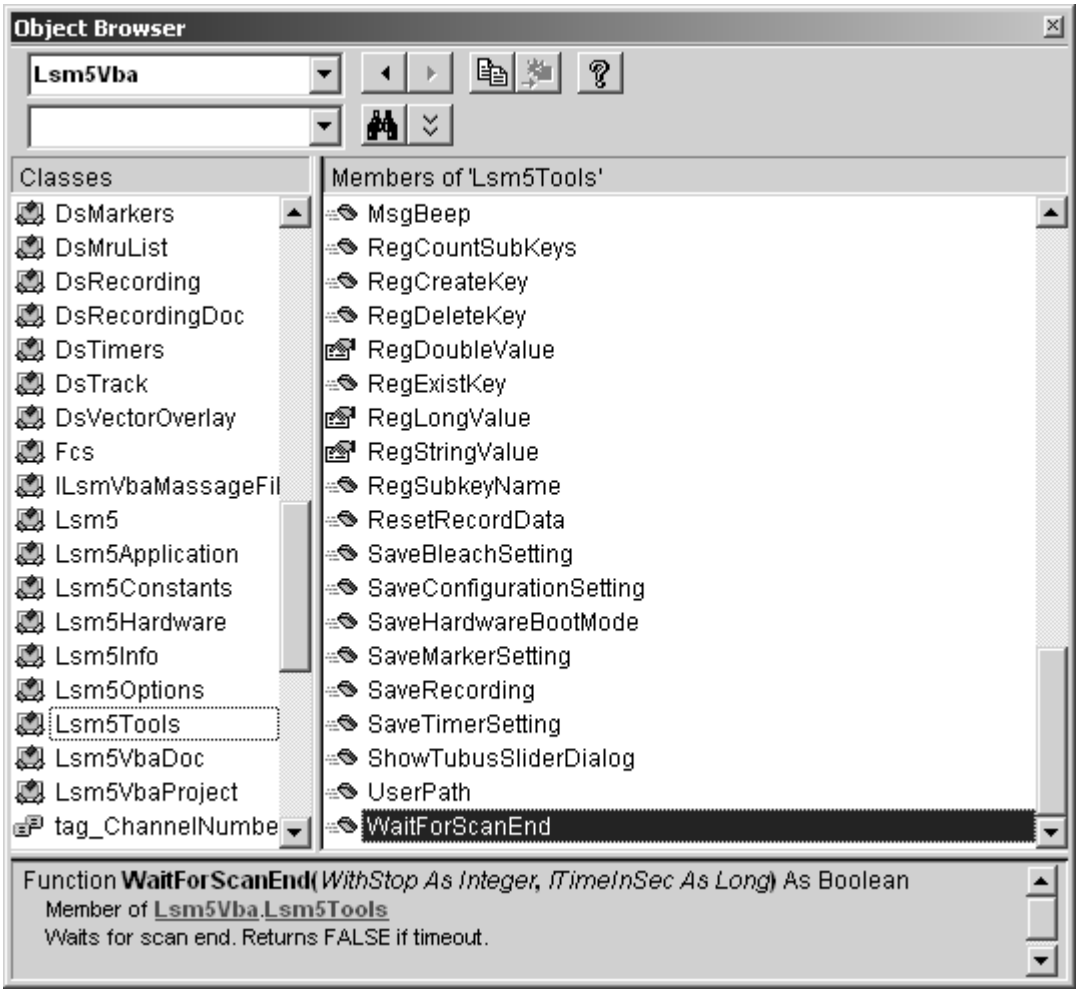
```
    lngResult = RegCloseKey(hKeyServer)
    hKeyServer = 0

    i = Len(strPath)

    Do Until (i = 0)
        If (VBA.Mid(strPath, i, 1) = "\") Then
            strPath = Left(strPath, i - 1)
            FServerFromDescription = True
            Exit Do
        End If
        i = i - 1
    Loop

error_exit:
    If (Not hKeyServer = 0) Then lngResult = RegCloseKey(hKeyServer)
    hKeyServer = 0

End Function
```

6.2 Programming for LSM

6.2.1 Object structure



To search the object structure use the object browser



6.2.2 Differences in access to LSM Hardware with Lsm5Hardware Object / DsRecording Object

Object Browser Project / Library LsmVba auswählen und Objekt Struktur untersuchen

- Access with DsRecording Object

  Status maintains at Scan Start

```
Public Sub CopyRecording(Destination As DsRecording, Source As DsRecording)
    Dim TS As DsTrack
    Dim TD As DsTrack
    Dim DataS As DsDataChannel
    Dim DataD As DsDataChannel
    Dim DetS As DsDetectionChannel
    Dim DetD As DsDetectionChannel
    Dim IlS As DsIlluminationChannel
    Dim IlD As DsIlluminationChannel
    Dim BS As DsBeamSplitter
    Dim BD As DsBeamSplitter
    Dim lT As Long
    Dim ll As Long
    Dim success As Integer

    Destination.Copy Source
    Destination.Objective = Source.Objective
    For lT = 0 To Destination.TrackCount - 1

        Set TS = Source.TrackObjectByIndex(lT, success)
        Set TD = Destination.TrackObjectByIndex(lT, success)

        TD.Collimator1Position = TS.Collimator1Position
        TD.Collimator2Position = TS.Collimator2Position

        For ll = 0 To TD.DataChannelCount - 1
            Set DataS = TS.DataChannelObjectByIndex(ll, success)
            Set DataD = TD.DataChannelObjectByIndex(ll, success)
            DataD.ColorRef = DataS.ColorRef
        Next ll
```

```
        For ll = 0 To TD.DetectionChannelCount - 1
            Set DetS = TS.DetectionChannelObjectByIndex(ll, success)
            Set DetD = TD.DetectionChannelObjectByIndex(ll, success)
            DetD.Filter1 = DetS.Filter1
            DetD.Filter2 = DetS.Filter2
            DetD.DetectorGain = DetS.DetectorGain
            DetD.AmplifierGain = DetS.AmplifierGain
            DetD.AmplifierOffset = DetS.AmplifierOffset
            DetD.PinholeDiameter = DetS.PinholeDiameter
        Next ll

        For ll = 0 To TD.IlluminationChannelCount - 1
            Set IlS = TS.IlluminationObjectByIndex(ll, success)
            Set IlD = TD.IlluminationObjectByIndex(ll, success)
            IlD.Acquire = IlS.Acquire
            lD.Power = IlS.Power
            IlD.DetectionChannelName = IlS.DetectionChannelName
        Next ll

        For ll = 0 To TD.BeamSplitterCount - 1
            Set BS = TS.BeamSplitterObjectByIndex(ll, success)
            Set BD = TD.BeamSplitterObjectByIndex(ll, success)
            BD.Filter = BS.Filter
        Next ll

    Next lT
End Sub
```

- Access with Lsm5Hardware Object

Status will be updated at Scan Start to current recording parameters

```
Private Sub FillPinholeList()
    Dim count As Long
    Dim i As Long
    Dim ObjPinholes As CpPinholes
    Dim Servos As Object
    Dim ServoX As Object
    Dim ServoY As Object
    Dim success As Boolean

    CmbPinhole.Clear

    Set Servos = Lsm5.ExternalCpObject.pHardwareObjects.pServos
    Set ObjPinholes = Lsm5.Hardware.CpPinholes
    count = ObjPinholes.count
    For i = 0 To count - 1
        success = ObjPinholes.Select(i)
        If (success) Then
            Set ServoX = Servos.pItem(ObjPinholes.Name + "X")
            Set ServoY = Servos.pItem(ObjPinholes.Name + "Y")
            If ((Not ServoX Is Nothing) And (Not ServoY Is Nothing)) Then
                CmbPinhole.AddItem ObjPinholes.Name
            End If
        End If
    Next i
    If (count) Then CmbPinhole.ListIndex = 0        ' select first pinhole

    Set ObjPinholes = Nothing
    Set Servos = Nothing
    Set ServoX = Nothing
    Set ServoY = Nothing
End Sub
```

- Access with external Lsm5Hardware Object

6.2.3 Access to hidden Interface

- When do I need to use the hidden Interface?
- How do I find the desired Object / Method?
  Open Tool / References
  Browse CP.dll search, open
  Choose Object Browser Project / Library DS or CP
  Examine Object Structure

Example:

```
Function GetLaserKind(WaveLength As String, kind As Integer)
    Dim count As Long
    Dim CpObject As Object
    Dim Lines As Object
    Dim i As Long
    Dim Success As Integer
    Dim WaveLenghtOfIndex As Long
    Dim Attenuation As Double
    Dim Enable As Integer
    Dim Name As String

    GetLaserValueMax = False

    Set CpObject = Lsm5.ExternalCpObject()
    Set Lines = CpObject.pHardwareObjects.pLaserLines

    count = Lines.lCount
    For i = 0 To (count - 1)
        Success = Lines.bLineInfo(i, WaveLenghtOfIndex, Attenuation, Enable, Name)
        If (Success And (WaveLenghtOfIndex = WaveLenght)) Then
            kind = Lines.AttenuatorType(WaveLenght)

            Set Lines = Nothing
            Set CpObject = Nothing
            Exit Function
        End If
    Next i

    Set Lines = Nothing
    Set CpObject = Nothing

End Function
```

6.2.4 Access to scanned pictures

Example:

```
Function ChannelIndexFromChannelName(ChannelName As String, ChannelIndex As Long)
    Dim num As Long, num1 As Long, num2 As Long
    Dim i As Long
    Dim Success As Integer
    Dim DetectionChannel As DsDetectionChannel
    Dim LsmInfo As Lsm5Info
    Dim Recording As DsRecording

    ChannelIndexFromChannelName = False

    Set Recording = Lsm5.DsRecording
    Set LsmInfo = Lsm5.Info
    If (LsmInfo.NumberOfPmtsInSystem(num, num1, num2) = True) Then
        For i = 0 To (num + num1 - 1)
            Set DetectionChannel = Recording.DetectionChannelOfActiveOrder(i, Success)
            If Success Then
                If (StrComp(DetectionChannel.Name, ChannelName) = 0) Then
                    ChannelIndex = i
                    ChannelIndexFromChannelName = True
                    GoTo exit_function
                End If
            End If
        Next i
    End If

exit_function:
    Set LsmInfo = Nothing
    Set Recording = Nothing
End Function
```

```
Function GetAveragePixel(ScanDoc As DsRecordingDoc, ChannelName As String, mean As Double)
    Dim line As Variant
    Dim x As Long, xmax As Long
    Dim y As Long, ymax As Long
    Dim spl As Long
    Dim bpp As Long
    Dim Sum As Double
    Dim Channel As Long

    GetAverageScanLineParams = False

    If (ChannelIndexFromChannelName(ChannelName, Channel) = False) Then
        Exit Function
    End If

    'check for valid doc
    If (ScanDoc Is Nothing) Then Exit Function
    Sum = 0
    xmax = ScanDoc.Recording.SamplesPerLine
    ymax = ScanDoc.Recording.LinesPerFrame
    For y = 0 To ymax - 1
        line = ScanDoc.ScanLine(Channel, 0, 0, 0, spl, bpp)

        For x = 0 To spl - 1
            Sum = Sum + line(x)
        Next x
    Next y
    mean = Sum / (xmax*ymax)

    GetAverageScanLineParams = True
End Function
```

6.2.5 Backup Recording

```
.
.
.
Dim Recording As DsRecording
Dim BackupRecording As DsRecording
Dim ScanDoc As DsRecordingDoc

Set Recording = Lsm5.DsRecording
'create a backup recording
Set BackupRecording = Lsm5.CreateBackupRecording
Success = BackupRecording.Copy(Recording)

Recording.ScanMode = "Line"
Recording.FramesPerStack = 1
Recording.StacksPerRecord = 1
Recording.ScanDirection = eSingleForeward
Recording.SpecialScanMode = "NoSpecialScanMode"
Recording.TimeSeries = False
Recording.ZoomX = ZOOM_X
Recording.ROTATION = ROTATION
Recording.Sample0X = OFFSET_X
Recording.Sample0Y = OFFSET_Y
Recording.SamplesPerLine = IMAGE_SIZE
Recording.StartScanTime = 8

Success = SetChannelDetection(ChannelName, 1, AmpOffset, 100)
Set ScanDoc = Lsm5.StartContinuousScan

.
.
.

Lsm5.StopScan
'restore the backup recording
Recording.Copy BackupRecording
Set Recording = Nothing
Set BackupRecording = Nothing
```

6.2.6 Events

- What are Events good for?

- Mouse Events
- Scan state events
- Hardware Events

```
Private Sub Lsm5VbaDoc_SystemEvent(ByVal EventNr As Long, ByVal Param As Variant)
    Dim x As Long
    Dim y As Long
    Dim z As Long
    Dim t As Long
    Dim c As Long
    Dim tmp As Long
    Dim pt As POINT
    Dim dsDoc As DsRecordingDoc

    'get the active Recording Document
    Set dsDoc = Lsm5.DsRecordingActiveDocObject
    If (dsDoc Is Nothing) Then Exit Sub
    If (Not dsDoc.IsValid) Then Exit Sub

    If (EventNr = eImageWindowLeftButtonDownEvent) Then
        If dsDoc.GetCurrentMousePosition(c, t, z, y, x) <> 0 Then
            'do something
                    .
                    .
                    .


        End If
    End If

End Sub
```

CHAPTER 7 ROUTINE MODE AND TOOLS

CONTENTS

7 ROUTINE MODE AND TOOLS

7.1 Routine Mode

7.1.1 General

The Routine Mode of the LSM-FCS software permits the fast and easy acquisition of scanning images by using time-tested **Standard Examination Methods** or by **User Defined Examination Methods**.

Standard Examination Methods are included in the LSM-FCS software package and must only be activated once during the first application of the routine mode.

User Defined Examination Methods are methods which were already created and optimized in the Expert Mode. If **User Defined Examination Methods** are also to be used in the Routine Mode, they must be exported in the Routine Mode first. Apart from **User Defined Examination Methods**, stacks and time series can also be exported to the routine mode in addition to simple frames.

Images scanned in the Routine Mode are displayed in the same image windows as in the Expert mode. The tools for image processing and the image display functions are identical.

☞ In the Routine mode, the FCS measurement functions are not available.

7.1.2 Starting the Routine Mode

- Start the LSM-FCS program (see chapter 4).

☞ If you want to change from the Expert Mode to the Routine Mode and vice versa, close all the windows first.

- Activate the **Scan New Images** button in the **LSM-FCS Switchboard** menu.
- Click on the **Start Routine Mode** button.
  - The **Routine Mode** window will appear.

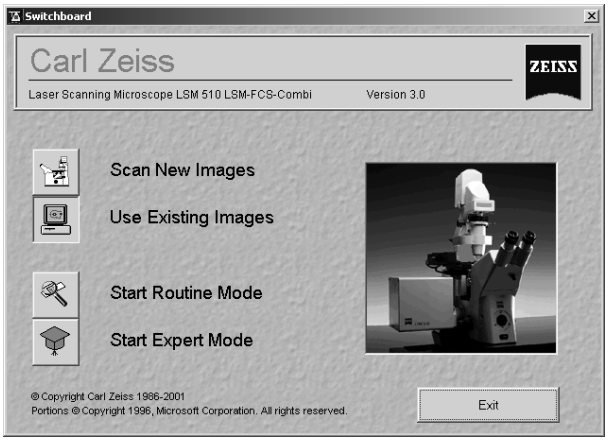


Fig. 7-1 LSM-FCS Switchboard menu

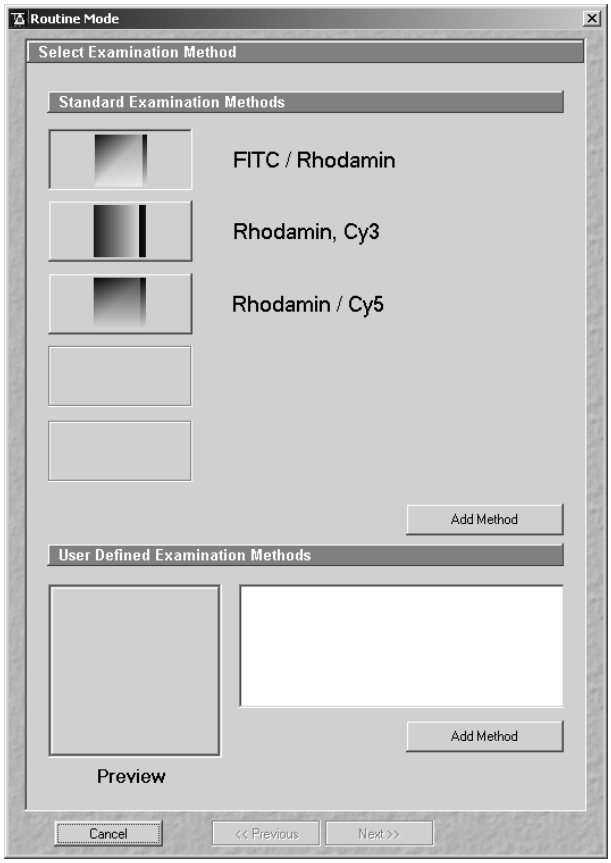


Fig. 7-2 Routine Mode - Select Examination Method window (first application)

In addition to the three standard examination methods contained in the LSM-FCS software package, any required number of further examination methods can be exported from the Expert Mode to the Routine Mode.

The maximum of five, freely selectable methods can be assigned directly to the **Standard Examination Methods** buttons.

The three standard examination modes are only available if channel 1 is a Non-META PMT channel.

The maximum of five, freely selectable methods can be assigned directly to the **Standard Examination Methods** buttons.

- Click on the **Add method** button.
  - The **Select Method To Add To Standard** window appears on the screen.
- Use the checkboxes in the **Methods** frame to select / deselect a table.
- Click on the **Select All** button to select all current methods.
- Click on the **Clear** button to deselect all current methods.
- Exit the **Select Method To Add To Standard** by clicking on the **Close** button.

All the other methods required must be activated in the **User-Defined Examination Methods** selection box.

7.1.2.1 Activate User-Defined Examination Methods in the Routine Mode

Requirements:

The examination method to be imported has been exported from the Expert Mode to the Routine Mode using the **Export RM** function in the **Options** menu (see chapter 4).

- Start the Routine Mode.
- Click on the **Add method** button.
  - The **Select Method To Add To List** window appears on the screen.

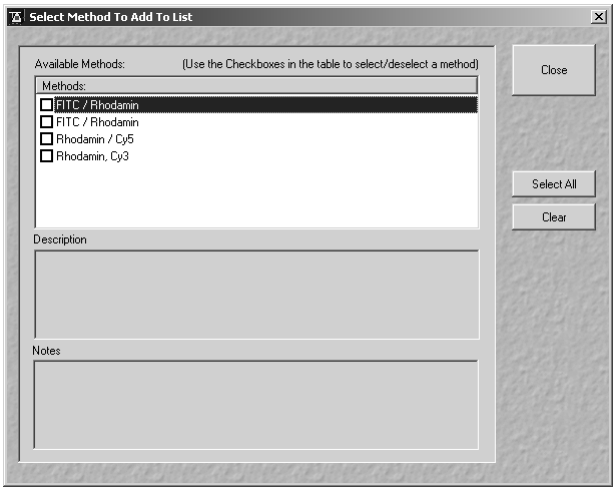


Fig. 7-3 Select Method To Add window

The method taken over from the Expert Mode appears, with the entered name, in the **Methods** panel of the **Select Method To Add To List** window, though without an icon. The method can now be activated as **Standard** or **User-Defined Examination Method**.

- Click on the **Add Method** button.
  - The **Select Method To Add To List** window appears on the screen.
- Use the checkboxes in the **Methods** frame to select / deselect a table.
- Click on the **Select All** button to select all current methods.
- Click on the **Clear** button to deselect all current methods.
- Exit the **Select Method To Add To List** window by clicking on **Close**.
  - The **Routine** window appears on the screen again. The method appears in the list of **User Defined Examination Methods**.
  - An overview image (Preview) for the selected method and the name of the scan mode used are displayed in the **User Defined Examination Methods** panel.

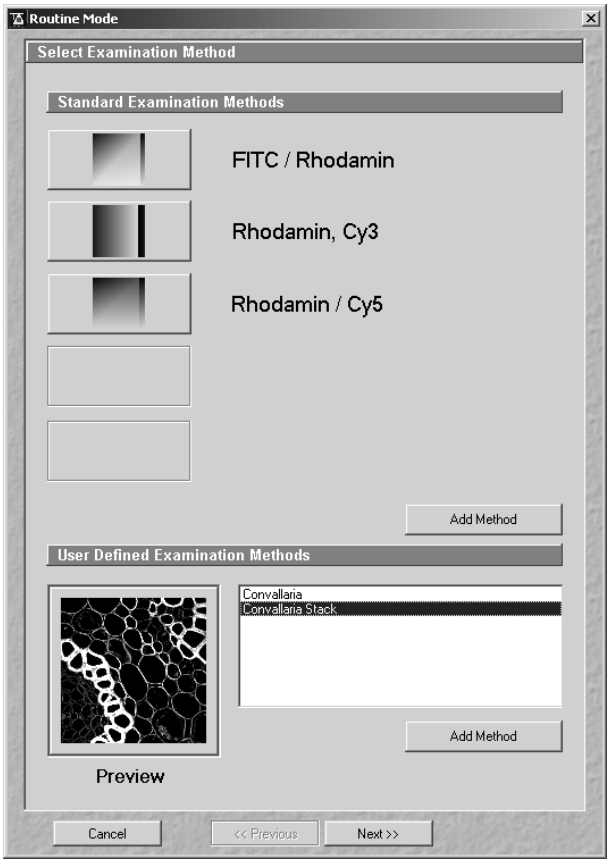


Fig. 7-4 Routine Mode - Select Examination Method window (user defined method activated)

7.1.3 Application of Examination Methods in the Routine Mode

The application of the Routine Mode is menu-controlled. The windows required for the creation of an image are limited to a minimum, and are opened one after the other using the button bar at the bottom of each window.

Lines, frames, stacks, time series and a combination of stacks and time series (4D: XYZT) can be recorded.

Additional windows will be displayed for the creation of stacks and time series. Lambda Stacks cannot be created in the Routine Mode.

The selected scan mode is displayed in all windows (except the **Microscope Setup** window) for checking purposes. It is possible to change to the next or previous window.

The buttons at the bottom of the relevant windows have the following functions:

Cancel button	Cancels the procedure, the Routine mode is finished.
<< Previous button	Activation of the previous window.
Next >> button	Activation of the next window.
Finish button	Ends the Routine mode; this button is active only in the **Image Acquisition** window.

7.1.3.1 Acquisition of a Frame

(1) Selection of the examination method

(a) Standard Examination Methods

- Click on the button of the required standard examination method, e.g. **FITC / Rhodamine**.

In case the current system has a META detector in channel 1 one has to define a method first for the routine mode.

– The **Routine Mode - Microscope Setup** window will appear.

(b) User Defined Examination Methods

- Click on the name of the examination method in the selection box of the **User-Defined Examination Methods** panel, e.g.: **Convallaria**.
- Then click on the **Next** button.

– The **Routine Mode - Microscope Setup** window will appear.

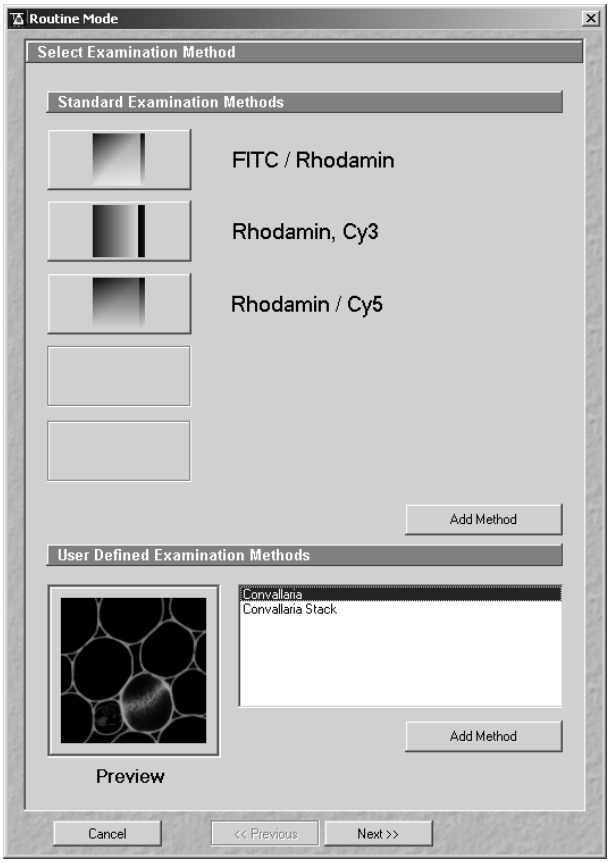


Fig. 7-5 Select Examination Method window

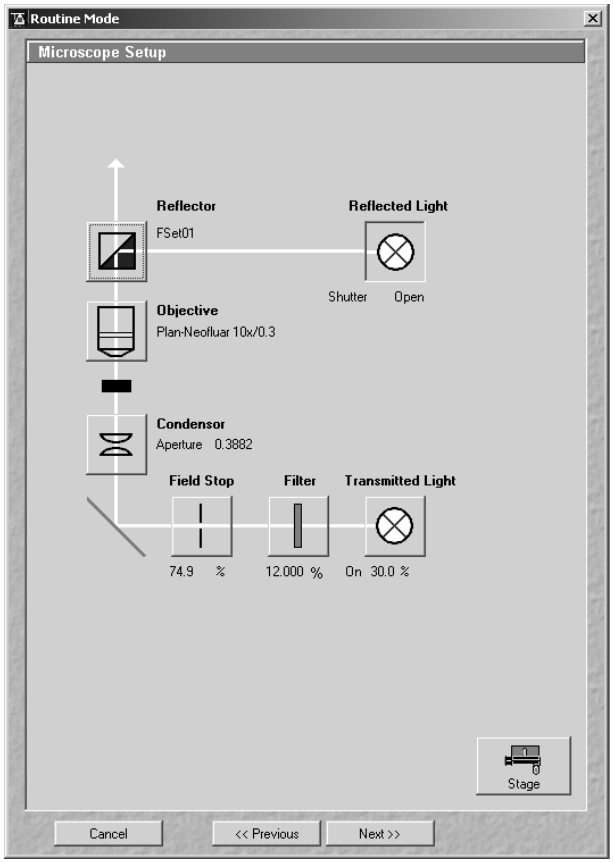


Fig. 7-6 Microscope Setup window

(2) Routine Mode - Microscope Setup window

- Prepare your specimen for examination in the same way as in the Expert mode, (see chapter 4).
- Clicking on the **Stage** button enables you to open the **Stage and Focus Control** window and to perform motorized focusing and specimen positioning.
- Set the microscope parameters for transmitted light or reflected light by clicking on the appropriate graph (see chapter 4).

The **Stage and Focus Control** window is identical to that of the Expert Mode and is handled in the same way (see chapter 4).

The **Stage** button is also available in the **Image Setup, Image Stack Setup** and **Time Setup** windows.

- When you have completed your microscope settings, click on the **Close** button to close the **Stage and Focus Control** window.
- Click on the **Next** button.
  - The **Routine Mode - Image Setup** window will appear.

(3) Routine Mode - Image Setup window

When the **Image Setup** window is opened, all the parameters of the selected examination method are set automatically:

- **Image Size**
- **Noise Reduction** corresponds to **Speed**
- **Integration Time** corresponds to **Pixel Time**
- **Data Depth**; **Scan Direction** (bi-directional setting is taken over, but is not useful and will not be displayed)
- **Zoom**, **Rotation** and **Offset** are exported with the values **1**, **0** and **0**
- **Beam Path** configuration (also Multitracks)
- **Contrast** corresponds to **Detector Gain**
- **Brightness** corresponds to **Ampl. Offset**
- **Optical Slice Thickness**
- **Excitation**
- **Average**

The parameters for Ratio and Bleaching cannot be exported to the Routine Mode.

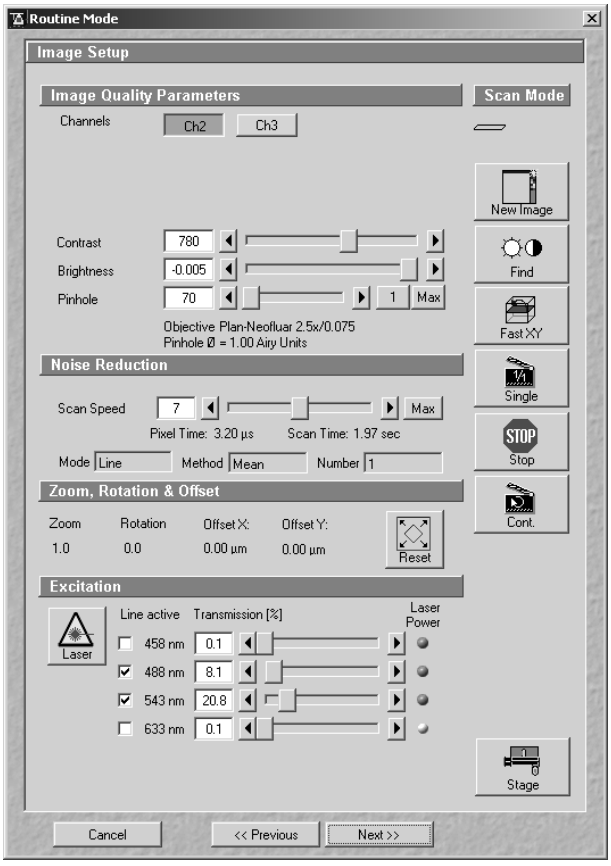


Fig. 7-7 Image Setup window

(a) Image Quality Parameters panel

The setting possibilities of the **Image Quality Parameters** panel permit the image quality to be optimized online (continuous scan procedure).

Optimization is performed track by track (if several tracks are used) and separately for each detector (channel), as in the Expert mode.

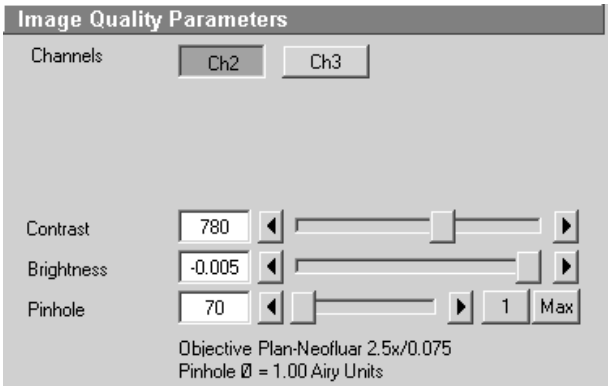


Fig. 7-8 Image Quality Parameters panel

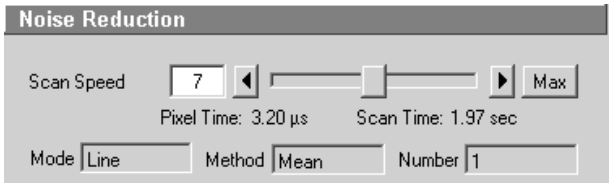


Fig. 7-9 Noise Reduction panel

(b) Noise Reduction panel

In the **Noise Reduction** panel, the scan speed and, depending on it, the scan time can be influenced.

The longer the scan time, the better the noise suppression.

- Use the **Scan Speed** slider to set the slowest acceptable **Scan Time**.

Average Mode, Method and **Number** are taken over from the Expert Mode and displayed, but they cannot be edited.

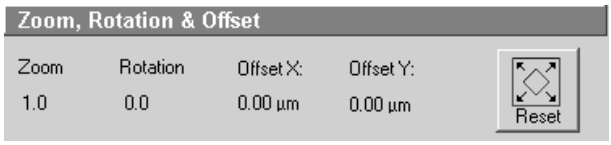


Fig. 7-10 Zoom & Orientation panel

(c) Zoom, Orientation & Offset panel

The set zoom, rotation and offset values are displayed in the **Zoom & Rotation** panel.

If the Crop function is used in the Image window, it can be deactivated again using the **Reset** button.

- Click on the **Reset** button to deactivate the **Crop** function.

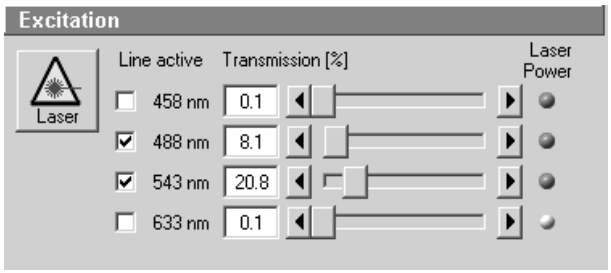


Fig. 7-11 Excitation panel

(d) Excitation panel

In the **Excitation** panel, the wavelength used (**Line Active** check box), the relevant transmission value (**Transmission** slider with input box) and the on / off laser status are displayed.

The laser status display indicates whether the laser has been switched on for the activated wavelength (green - on; gray - off).

If required, further wavelengths can be activated via the **Line Active** ☑ check box.

The laser setting is performed in the same way as in the Expert Mode (see chapter 4).

If the laser required for the activated wavelength is not switched on, the relevant message will be provided on the screen in a special window.

- In such a case, open the **Laser Control** window by clicking on the **Laser** button and set the relevant laser to **On**.

(e) Function buttons in the Routine Mode - Image Setup window

The selected scan mode is displayed at the top right of the **Image Setup** window for checking purposes.

The buttons arranged below have the following functions:

New Image button	Opens a new image window
Find button	Triggers single scan with automatic optimization of image brightness and contrast.
Fast XY button	Triggers continuous scan with maximum speed. This function should be used to a limited extent and only for a short period of time.
Single Scan button	Triggers single scan.
Stop Scan button	Stops the current scan procedure.
Contin. Scan button	Triggers continuous scan.
Stage button	Opens the **Stage and Focus Control** window.

(f) Start scanning

- Click on **Single Scan** or **Contin. Scan** to trigger the scanning procedure.
- If required, optimize the default parameters in the **Excitation**, **Zoom, Orientation & Offset**, **Image Quality Parameters** and **Noise Reduction** panels (using continuous scan).
- Click on **Stop Scan**.
- Click on **Next**.
  - The **Routine Mode - Image Save, Print & Export** window appears.

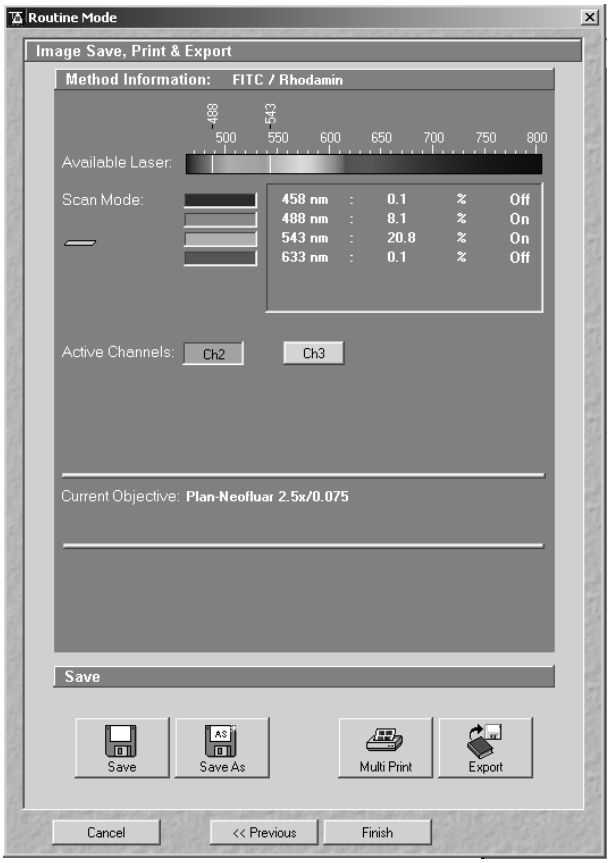


Fig. 7-12 Image Save, Print & Export window

(4) Routine Mode - Image Save, Print & Export window

The **Routine Mode**- **Image Save, Print & Export** window is used for data backup:

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Multi Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

- Click on **Save** or **Save As** to store the acquired image.
- Click on **Finish** to exit the Routine Mode and return to the **LSM-FCS Switchboard** menu.

7.1.3.2 Acquisition of a Z Stack

For the acquisition of a Z Stack, the **Routine Mode - Image Stack Setup** window showing the Z parameters is additionally displayed. The parameters correspond to those of the imported Examination Method and, if necessary, have to be matched to the specimen under examination.

- Load the required stack examination method from the **Routine Mode - Select Examination Method** window.
- Open the **Routine Mode - Microscope Setup** and **Routine Mode - Image Setup** windows one after the other by clicking on **Next**. Check the settings and change them, if required (see section 7.1.3.1, page 7-7).
- Click on the **Next** button.
  - The **Routine Mode - Image Stack Setup** window becomes visible.

- You can take over the offered parameters by clicking on the **Start** button.
  - The image stack will be recorded.
- The scan in progress can be stopped at any time by clicking on the **Stop** button.

If Z-settings must be changed, proceed as described in the following.

(1) Routine Mode - Image Stack Setup window

The buttons arranged on the right in the **Image Stack Setup** window have the following functions:

New Image button	Opens a new image window.
Start button	Triggers scanning of a stack.
Stop Scan button	Stops the current scan procedure.
XYscan button	Triggers single XY-scan.
XYcont button	Triggers continuous XY-scan.
Stage button	Opens the Stage and Focus Control window.

The definition of the stack parameters in the **Image Stack Setup** window is made in accordance with the exported Examination Method. If the exported method was created using the HRZ 200 fine focusing stage, the Z-parameters are defined via the **Line Sel** / **Range** function of the **Hyperfine Z Sectioning** panel.

Otherwise, use the **Mark First/Last** function for the definition of the Z-parameters.

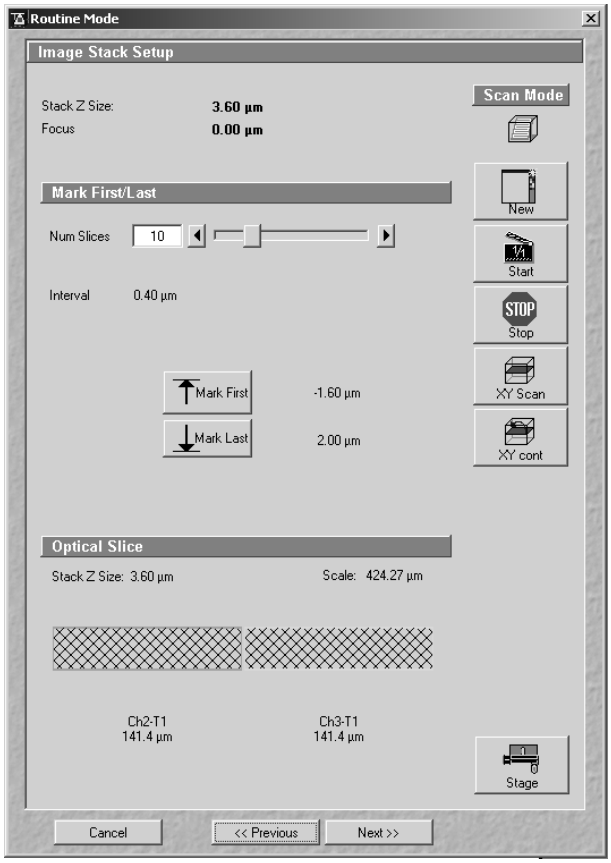


Fig. 7-13 Image Stack Setup window (Mark First/Last)

(a) Mark First/Last function

The parameters of the Z Stack to be created are also displayed in the window and can be changed online.

Stack Z Size: Optical indication, i.e. the optical beam moves over this (displayed) Z-thickness (online). The stage (nosepiece) is moved in such a way that the stack size, dependent on the refractive index, is achieved optically.

Focus Position: Mechanical indication, i.e. if the refractive index changes, the value of the focus position also changes in relation to the "0" value (online).

Z Sectioning: Setting of **Number of Slices**, **Interval** and **Current Slice** via sliders / arrow buttons.

Mark First/Last: Determination of the Z-value for the first and last XY-image of the stack, combined with manual focusing or **Stage** control.

Optical Slice: The **Optical Slice** window displays the following information:
Black:
Stack Z Size (µm) = intervals x (number of slices - 1)
Optimal Interval = depending on the objective used and the pinhole diameter setting
Red and other colors:
Presentation of the actual data set by the operator helps to optimize stack creation.

For redefining the parameters of a Z Stack, use the setting functions of the **Mark First/Last** panel.

The optimum stack size (definition of the upper and lower limits of the stack) is determined by focus adjustment during a continuous scan.

- Click on the **XYcont** button.
  - A continuous XY-scan of the set focus position will be performed.
- Use the manual focusing drive or the **Stage and Focus Control** window to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.

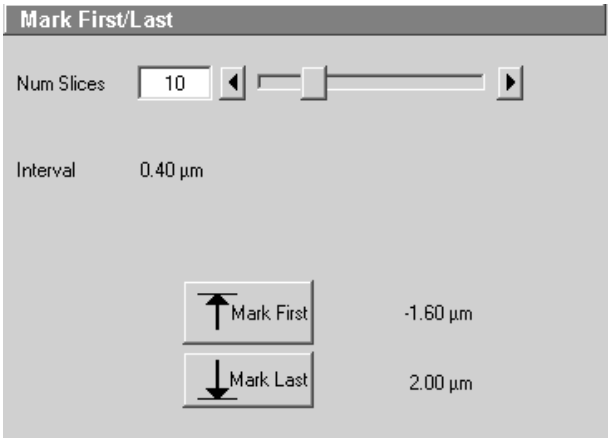


Fig. 7-14 Mark First/Last panel

- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- The **Num Slices** slider enables you to set the number of slices. The limits of the Z Stack remain constant; the interval is matched accordingly.

The changes made are visualized online in the graphic display of the **Optical Slice** panel.

- Click on the **Start** button to start the recording of the Z Stack.

In case the upper and lower limits of the stack have been switched round, automatic matching will be performed by the software, since the nosepiece of the Axiovert 200 M always moves from top to bottom.

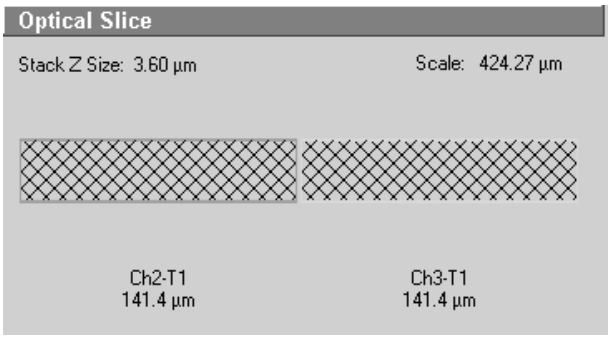


Fig. 7-15 Optical Slice panel

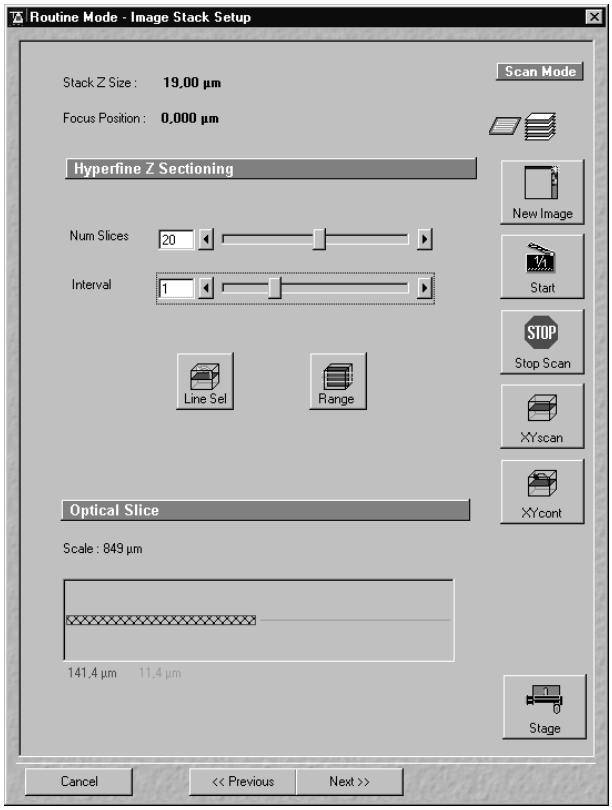


Fig. 7-16 Image Stack Setup window (Line Sel / Range)

(b) Line Sel / Range function with HRZ 200

Activation of this function is only possible if the HRZ 200 fine focusing stage has been connected.

The HRZ 200 can be controlled via software (see **Stage** - Expert Mode, chapter 4).

The accuracy of the HRZ 200 regarding the step width in the Z-direction lies in the range of 10 nm.

The HRZ 200 allows stacks to be produced considerably quicker than via the focus.

The focus position remains unchanged.

The optimum stack size is determined with the help of the **Line Sel** and **Range** functions:

- Use the slider or the arrow keys to set the number of slices for the Z Stack.
- Use the slider or the arrow keys to set the size of the interval.

Num Slices and **Interval** can be varied independently of each other within the HRZ 200 work range of ±100 µm.

For the definition of the stack limits, proceed as follows:

- Click on the **Line Sel** button.
  - An XY-scan of the current slice is performed. The cutline is displayed in the image center. The **Line** toolbar is displayed on the right edge of the Image window.

Use the **Line** toolbar to define the position, shape, width and color of the cutline in the Image window.

The following function buttons are available in the **Line** toolbar:

Function	Description
Arrow selection button 	Activates the mouse pointer for the selection, positioning and size modification of the cutline in the Image window. Size modification: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button. Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.
Line arrow button 	Generation of a straight cutline in any direction in the Image window.
Opened free shape curve button 	Generation of an open, free shape curve (spline) in the Image window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.
Line button 	Selecting the line width of the cutline.
Color button 	Selecting the color of the cutline.

- Click on the **Line arrow** button or the **Opened free shape curve** button in the **Line** toolbar.
- Define a straight line or a free shape curve (spline) as the cutline for the XZ scan.

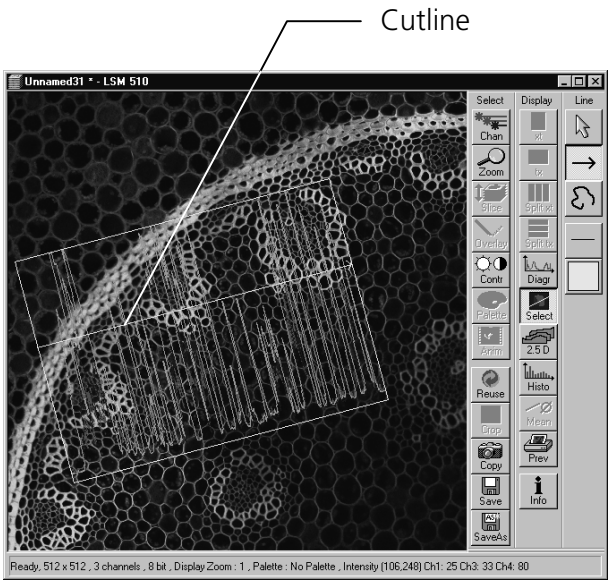


Fig. 7-17 Image window (Line Sel)

- Then click on the **Range** button.
  - The XZ-scan will be performed and displayed in the image window. At the same time, the position of the current slice is shown with a green line and the positions of the first and last slice with two red lines.

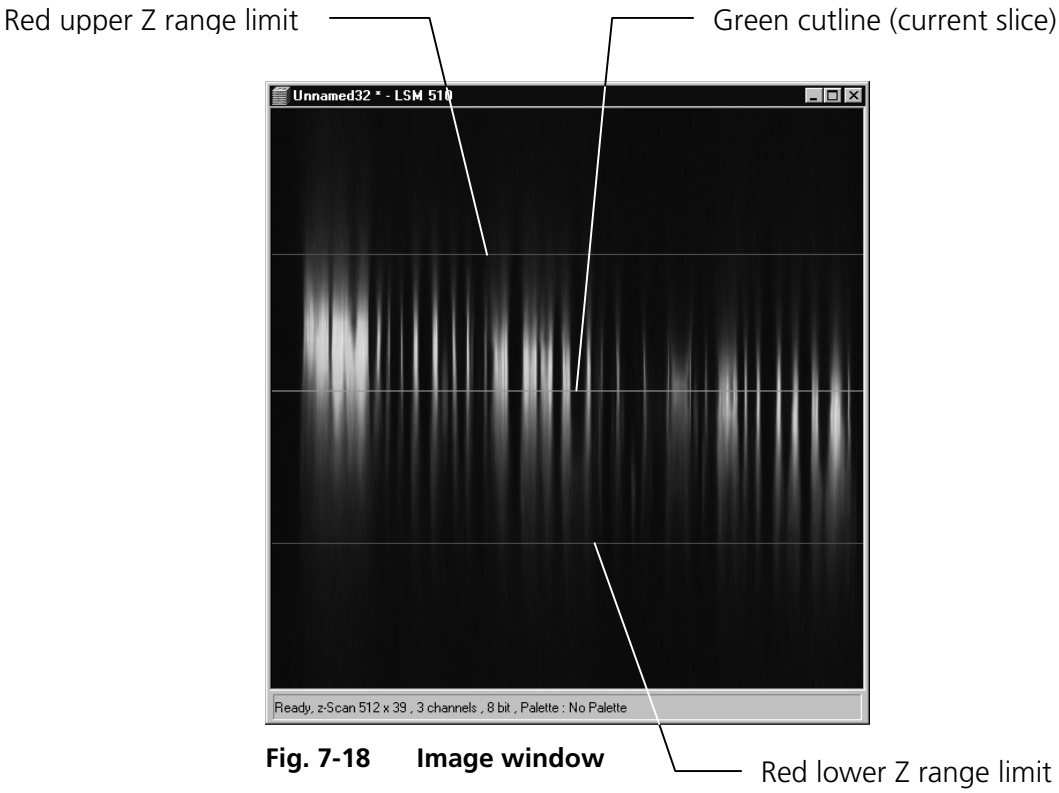


Fig. 7-18 Image window

- Moving the green line (current slice) enables you to change the current focus position (moving the stage or nosepiece in the process). If the green line (Current Slice) is shifted after the creation of **Range**, the focus position will change (the HRZ 200 remains in the center position).
- Shifting one of the red lines enables you to change the stack size. The red lines (stack limits) can only be changed symmetrically to the Current-Slice position within the HRZ 200 work range.

☞ Since the HRZ 200 moves from bottom to top during the creation of the Z Stack, top and bottom of the Axiovert 200 M have been switched round.

- A click on the **Start** button will start the recording of the Z Stack.
- When recording of the Z Stack is completed, click on **Next**.
  - The **Routine Mode - Image Save, Print & Export** window appears.

(2) Routine Mode - Image Save, Print & Export window

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Multi Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

- Click on **Save** or **Save As** to store the acquired stack image.
- Click on **Finish** to exit the Routine Mode and return to the **LSM-FCS Switchboard** menu.

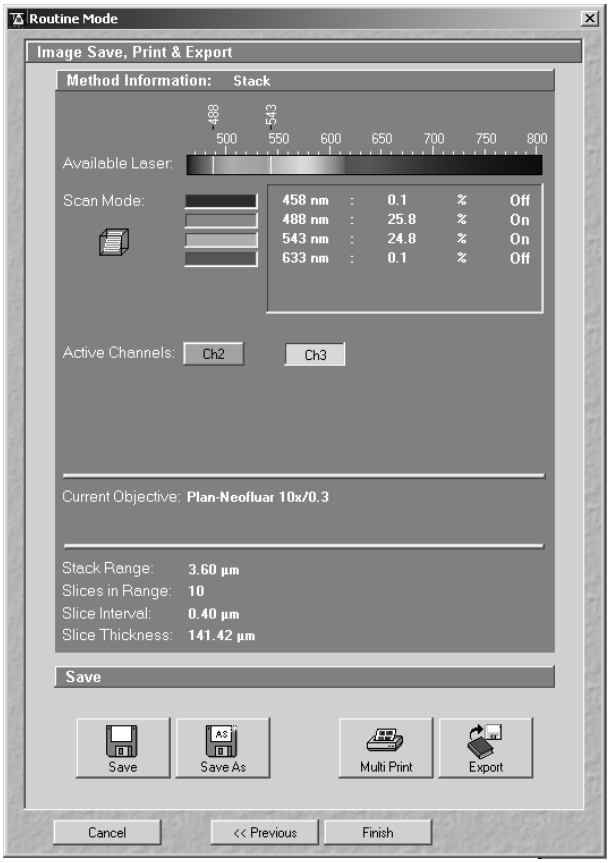


Fig. 7-19 Image Save, Print & Export window

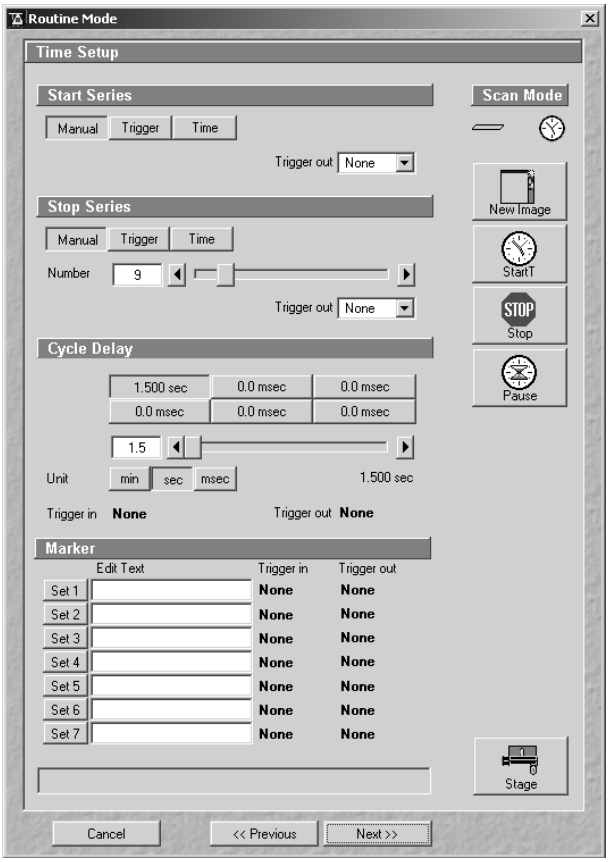


Fig. 7-20 Time Setup window

7.1.3.3 Acquisition of a Time Series

A time series is recorded in the Routine mode in the same way as a frame.

Furthermore, the Time settings are displayed in the **Routine Mode - Time Setup** window. The parameters set here correspond to those of the imported examination method and must be matched, if required.

The time series can be triggered / ended only in the way it was defined in the imported method.

- Load the required time series examination method from the **Routine Mode - Select Examination Method** window.
- Open the **Routine Mode - Microscope Setup** and **Routine Mode - Image Setup** windows one after the other by clicking on **Next**. Check the settings and change them, if required (see section 7.1.3.1, page 7-7).
- Click on the **Next** button.
  - The **Routine Mode - Time Setup** window becomes visible.

The following functions are available on the right side of the this window:

New Image button	Opens a new image window.
Start T button	Starts the time series.
Stop button	Stops the entire time series. A current scan is canceled.
Pause button	Interrupts the time series. The button labeling changes to **Resume**. A current scan is performed until its end. Pressing the **Pause** button again will resume the time series.
Stage button	Opens the **Stage and Focus Control** window.

(1) Start Series panel

Depending on the imported examination method, the time series can be started and ended manually, via trigger or by entering a time (computer time). In the **Start Series & Stop Series** panel, the relevant mode is displayed. Switching between manual, trigger and time control is not possible in the Routine mode.

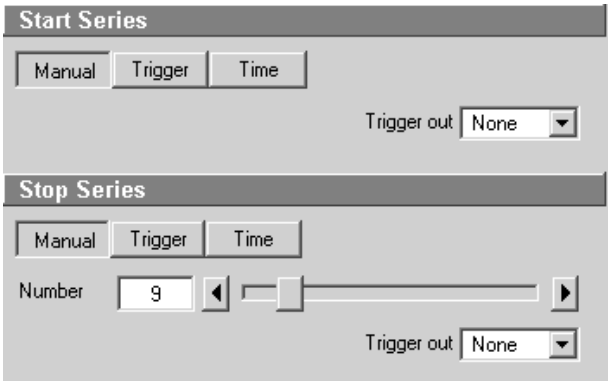


Fig. 7-21 Start & End panel

(a) Manual start of a time series

- You can take over the offered parameters by clicking on the **Start T** button.
  - The time series is recorded and displayed in the image window.
- Click on the **Stop** button to end the time series.

(b) Starting a time series via trigger

- The **Start T** button must be pressed first to start a time series via trigger.
  - **Waiting for Trigger** will be displayed in the status line.
- Then the relevant trigger key of the Trigger Control must be pressed to start the first scan procedure of the time series.
- The time series is ended by pressing the relevant trigger key.

If the number of cycles entered under **Number** has been processed without a trigger impulse having been given to end the procedure, the time series is finished.

If a trigger signal is given before the cycles have been processed, the time series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The time series can now be continued via a new trigger signal or ended via **Stop**.

(c) Starting a time series via the time

- To start a time series via the time, the **Start** button must be pressed first.
  - **Waiting for Start Time** will be displayed in the status line. The time series is started when the starting time has been reached.
  - The time series is interrupted when the end time has been reached.

If the entered **Number** of cycles has been processed, the time series is finished.

If the number of cycles has not yet been processed, the time series is only interrupted. **Waiting for Start Time** is displayed in the status line. The time series can now be continued by entering a new start time or finished via **Stop**.

The end time for the time series can be changed online.

(d) Input of the number of scan cycles

The currently set number of scan cycles is displayed in the **Number** input box of the **Start & End** panel. This number can be changed, if required.

- Set the required **Number** of scan cycles using the sliders or the arrow buttons.

(2) Cycle Delay / Time Interval panels

Depending on the Examination Method exported from the Expert Mode, the time series interval is defined either as a **Cycle Delay** or **Time Interval**. Accordingly, either the **Time Delay** panel or the **Time Interval** panel is displayed in the **Routine Mode - Time Settings** window.

Time Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

The **Scan Interval** panel permits the intervals to be activated and changed.

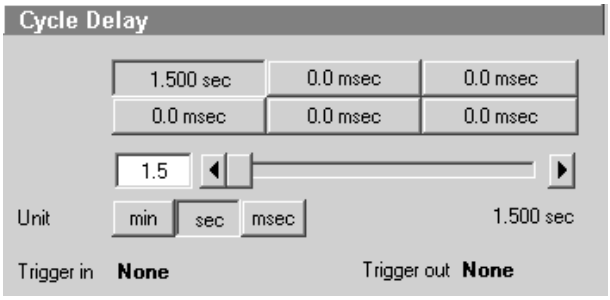


Fig. 7-22 Time Delay panel

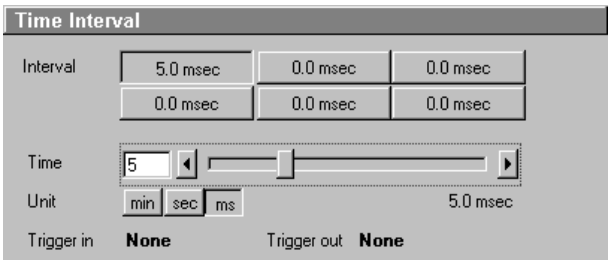


Fig. 7-23 Time Interval panel

The following functions are available:

Interval buttons	Activation of the interval set for the time series for the relevant button.
Time input box / arrow buttons / slider	Determination of the interval for the currently selected time button.
Unit buttons	Selection of the time unit: **min**, **sec** or **ms**.
Trigger in field	Display of the trigger keys (1-4) for the in signal.
Trigger out field	Display of the trigger keys (1-4) for the out signal.

- The interval to be used during the time series is set to a default value by activating a **Time** button.

The time displayed on the time buttons can be changed as follows, if required:

- Activate a **Time** button with a click of the mouse.
- Use the slider (arrow buttons or input box) near **Time** to set the required interval. The set time will be displayed online on the button. Select the required time unit by clicking on the relevant **Unit** button.

You can assign different times to all the six **Time** buttons.

- Changing to another interval is possible by activating another **Time** button during a current time series.

☞ If the interval change was made via trigger key in the imported examination method, the assigned **Time** button will be activated on pressing the displayed trigger key.

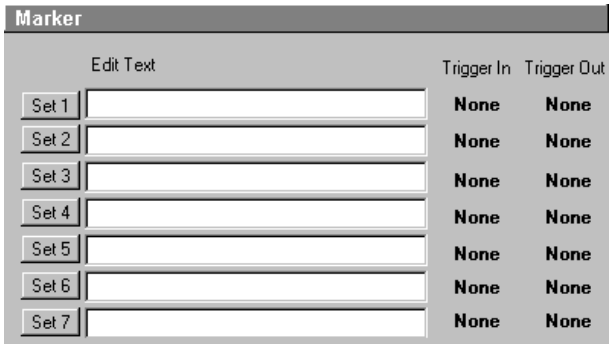


Fig. 7-24 Marker panel

(3) Marker panel

The setting of a marker permits information about the moment in the current time series and any required comment to be assigned to the current scan. The time indication is set automatically, while comments must be defined before.

The markers (red squares) are visible in the Image window if the **Gallery** button (**Display** toolbar) is activated.

On storage of the image, all the markers, including the time indication and the comments, are stored along with the image contents.

The following functions are available:

Set 1-7 button	Setting of a marker during the scan procedure.
Edit Text input box (1-7)	Entry of the comments for the marker.
Trigger in field (1-7)	Display of trigger keys (1-4) used to set the marker.
Trigger out field (1-7)	Display of trigger keys (1-4) for the out signal.

- Click once in the edit text box of the required marker. Enter the comments via the keyboard. Then click outside the editing box to close this box.
- Clicking on one of the **Set 1** to **7** marker buttons will set a marker for the current scan.

(4) Recording a time series

- Set the relevant parameters for time control in the **Start Series, Stop Series**, **Time Delay** and **Marker** panels.
- Start the time series by clicking on the **Start T** button.
- If you use Trigger Control, press the relevant trigger key to start the time series with the first scan procedure.
- Use the **Set 1** to **Set 7** buttons to set markers during the scanning procedure which will allow you to evaluate interesting scanning images later.
- End the time series by clicking on the **Stop** button.
- Click on **Next**.

– The **Routine Mode** - **Image Save, Print & Export** window appears.

☞ It is also possible to record a Z Stack in the form of a time series (4D Image).

(5) Routine Mode - Image Save, Print & Export window

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

The also available **Save** and **Save As** buttons permit scanned images to be stored in a database (see chapter 4).

- Click on **Finish** to exit the Routine Mode and return to the **LSM-FCS Switchboard** menu.

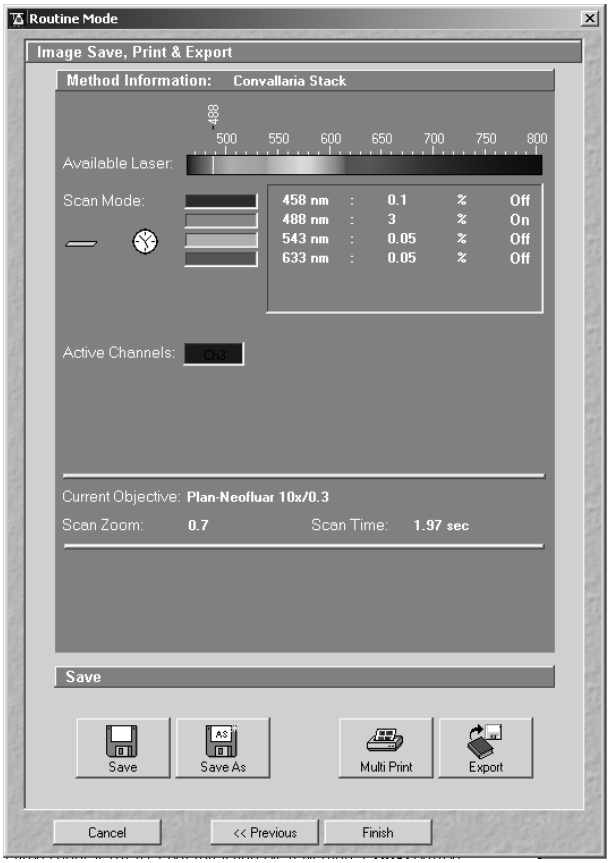


Fig. 7-25 Image Save, Print & Export window

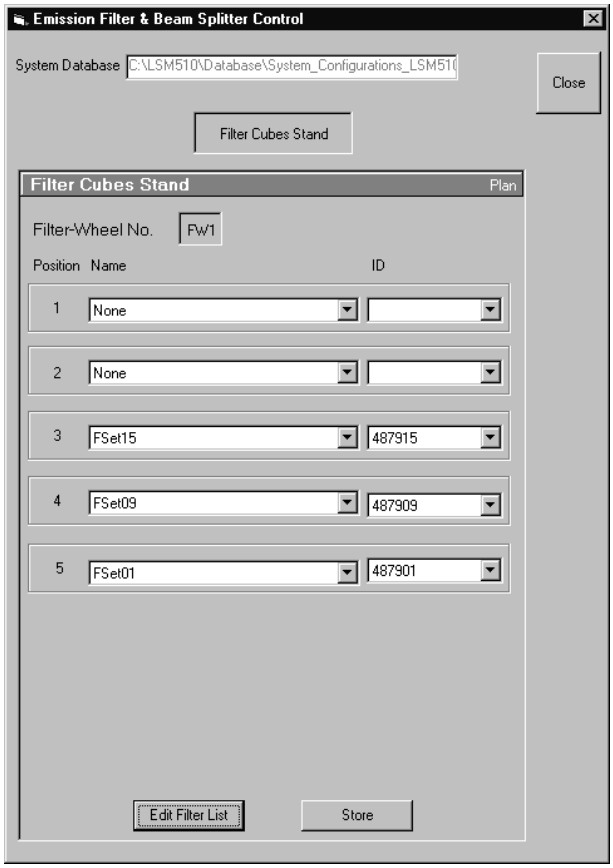


Fig. 7-26 Emission Filters & Beam Splitter Control window

7.2 Tools

7.2.1 Change Filters

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret.

- Close the LSM-FCS software program.
- Insert the new filter module in the reflector turret.
- Double-click on the **Change Filters** icon on the desktop.
  - The **Emission Filter & Beam Splitter Control** window appears on the screen. The name of the currently used database is displayed in the **System Database** box, with the filter type being indicated below for checking purposes.
  - The **Filter Cubes Stand** panel shows the **Filter-Wheel No.** and the filter positions available.
  - Use the **Name** and **ID** selection boxes to enter the filters installed in the individual positions of the filter wheel.
- Open the **Name** (or **ID**) selection box of the relevant filter position and select the new filter set from the list.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

☞ All available filter sets have to registered in the filter list (see **Edit Filter List** function, next page).

Edit Filter List

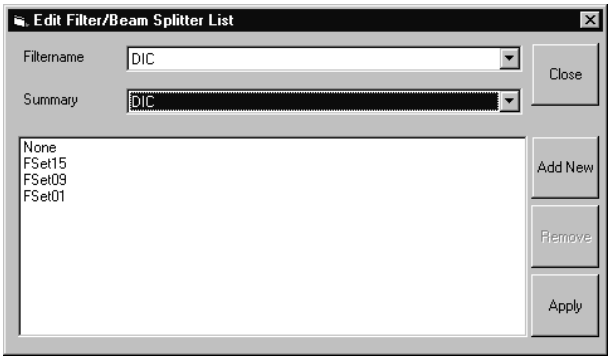


Fig. 7-27 Edit Filter/Beam Splitter List window

The **Edit Filter List** function permits updating of the filter data in the software after a change of filters on the stand.

- Close the LSM-FCS software program.
- Double-click on the **Change Filters** icon on the desktop.
- Click on the **Edit Filter List** button in the **Emission Filter & Beam Splitter Control** window.
  - The **Edit Filter/Beam Splitter List** window is opened.

This window permits a list of the most frequently used filter sets to be compiled.

- Click on the arrow button in the **Filtername** list box to open it.
- Select the filter set which shall be included in the list.
- Click on the **Apply** button.

The selected filter set is included and displayed in the list (below the **Sumary** list box).

This filter set is now also available in the **Name** selection boxes of the **Filter Cubes Stand** panel and can be assigned to a filter wheel position.

To remove a filter set which is no longer needed from the list, proceed as follows:

- Click on the name of the filter set concerned in the list box of the **Edit Filter/Beam Splitter List** window.
- Click on the **Remove** button. The filter set is deleted from the list and is then no longer available in the **Filter Cubes Stand** panel of the **Emission Filter & Beam Splitter Control** window.

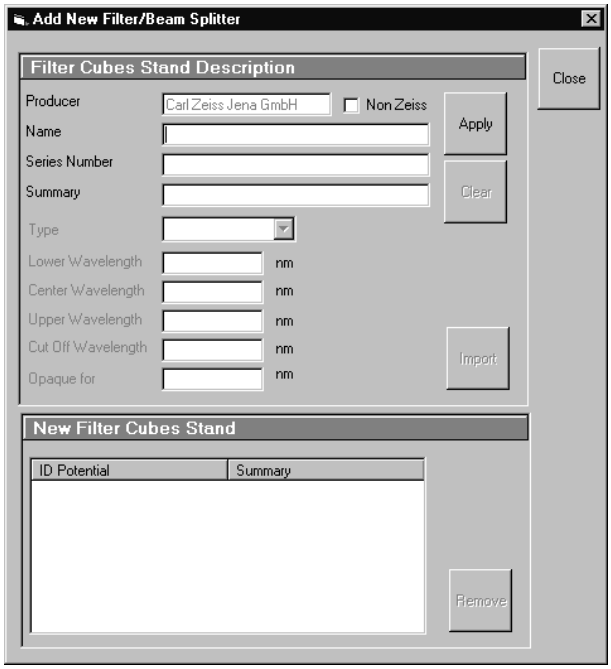


Fig. 7-28 Edit Filter/Beam Splitter List window

Add New

This function permits new filter sets to be added to the database.

For this, proceed as follows:

- Click on the **Add New** button on the **Edit Filter/Beam Splitter List** window.
  - The **Add New Filter/Beam Splitter** window is opened.
- Enter the data of the new filter set in the **Filter Cubes Stand Description** panel, then click on the **Apply** button.

The new filter set is stored in the database and included in the **New Filter Cubes Stand** panel. You can now activate the filter for a filter wheel position using the procedure described above.

☞ If you have activated the **Non Zeiss** check box, filter sets from other manufacturers can also be included in the database.

- To remove an new filter set from the database, select it with a click of the mouse in the **New Filter Cubes Stand** panel and then click on **Remove**.
- Click on **Close** to close the **Add New Filter/Beam Splitter** window.

- Click on **Close** to close the **Edit Filter/Beam Splitter** List window.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

When you start the LSM-FCS software, the filter data are updated.

7.2.2 Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the LSM-FCS software program. This function should preferably be performed by authorized service personnel.

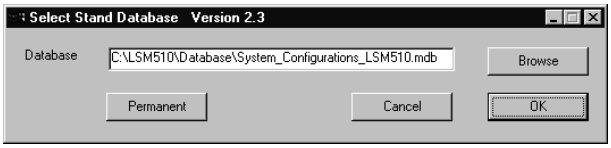


Fig. 7-29 Select Stand Database ... window

If this is not possible, proceed as follows:

- Close the LSM-FCS software program and double-click on the **Stand Select** icon on the desktop.
  - The **Select Stand Database** window appears on the screen. The currently used database is displayed in the **Database** box.

- Click on the **Browse** button to activate the new database.
  - The **Open** window appears on the screen.
- Select the directory where the new database is stored.
- Click on the name of the database (file extension: ***.mdb**) and then on the **Open** button.
  - The **Open** window is closed and the name of the new database appears in the **Database** box.
- Click on the **Permanent** button. The **Select Name** window appears.
- Select the relevant stand icon from the **Icon** list box and click on **OK**. The **Select Name** window is closed and the desktop icon is updated.
- Then click on the **OK** button in the **Select Stand Database ...** window to accept the new settings and to close the window. (Clicking on **Cancel** will cancel the procedure.)
  - After the next restart of the LSM-FCS software program, the new database will be automatically read in.

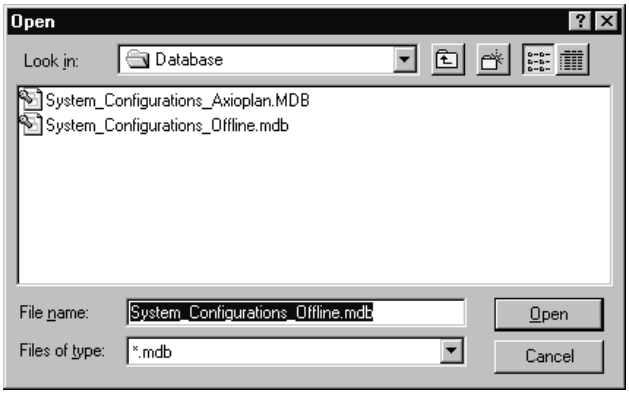


Fig. 7-30 Open window

Fig. 7-31 Select Name window

7.2.3 LSM Image Browser

The **LSM Image Browser** permits images to be loaded, imported, exported and printed quickly without having to open the LSM-FCS software. The **LSM Image Browser** can be used without dongle.

When images are opened, image processing functions of the LSM-FCS software are available to a limited extent (**Chan**, **Zoom**, **Contr**, **Palette**, **Copy**, **Save**, **Save As**, **xy**, **Split xy**, **Prev**, **Info**).

- Click on the **LSM Image Browser** icon on the desktop of the PC. The **Zeiss LSM Image Browser** main menu is opened.

Fig. 7-32 Zeiss LSM Image Browser main menu

The following function buttons are available:

New button	Opens a new database.
Open button	Opens an existing database.
Save button	Saves the current image.
Save As button	Saves the current image under a new name
Import button	Imports images.
Export button	Exports images.
Full Screen button	The current image is displayed on the full screen. Deactivation of the function with a click of the mouse.
Multi Print button	Several images are printed on one page.
RAM button	Use of the RAM memory for image display.
DISK button	Use of the hard disk as storage medium for image display.
Exit button	The **Zeiss LSM Image Browser** main menu is closed.

The functions **New**, **Open**, **Save**, **Save As**, **Import, Export** and **Multi Print** correspond to those of the Expert Mode of the LSM-FCS software and have already been described in chapter 4.

7.2.4 LSM Image Examiner

The **LSM Image Examiner** can be used without having to open the LSM-FCS software. However, this requires the installation of the relevant dongle. The **LSM Image Examiner** provides all the functions of the **LSM Image Browser,** plus the 3D functions and selected **Process** functions of the Expert Mode of the LSM-FCS.

When images are opened, a large scope of the image processing functions of the LSM-FCS software is available (for further details see chapter 4).

- Click on the **LSM Image Examiner** icon on the desktop of the PC. The **Zeiss LSM Image Examiner** main menu is opened.

Fig. 7-33 Zeiss LSM Image Examiner main menu

In addition to the buttons of the **LSM Image Browser** mentioned above, the following function buttons are available in the lower row of the **Zeiss LSM Image Examiner** main menu:

Projection button	One single projection or a series of projections can be calculated after rotation of the data package about the X, Y or Z axis.
DepthCod button	The depth information contained in a sequence can be colored with the colors of the rainbow.
Stereo button	Stereoscopic images can be generated.
Ratio button	Permits two channels to be linked into a new channel by the creation of a ratio.
Copy button	Permits one channel each of an existing image to be copied and stored as a new image.
Filter button	Permits the subsequent processing of scanned images via the integrated filters.
Interpolate button	Permits the continuous contrast and brightness change in a stack or time series through interpolation between the starting and end values.
Shift button	Produces a congruent image with relation to the pixels of the various channels.

Duplicate button	Permits images (including Z Stacks and Time Series) to be duplicated completely.
Contrast button	Permits the subsequent modification of contrast and brightness of the stored image.

These functions correspond to those of the Expert Mode of the LSM-FCS software and have already been described in chapter 5.

CHAPTER 8 3D FOR LSM 510

CONTENTS

8 3D FOR LSM

8.1 Overview and Explanations

8.1.1 The Image Sequence

The "3D for LSM-FCS" handles image sequences generated by the Zeiss LSM-FCS software. This can be three-dimensional image data or a time sequence of two-dimensional images (slices). Each slice (as well as the sequence) can consist of up to eight channels. An image sequence consists of a series of individual (2D) images and has a name that designates the entire sequence. In general an image sequence is handled as a single object in the system. Individual channels or slices can be addressed.

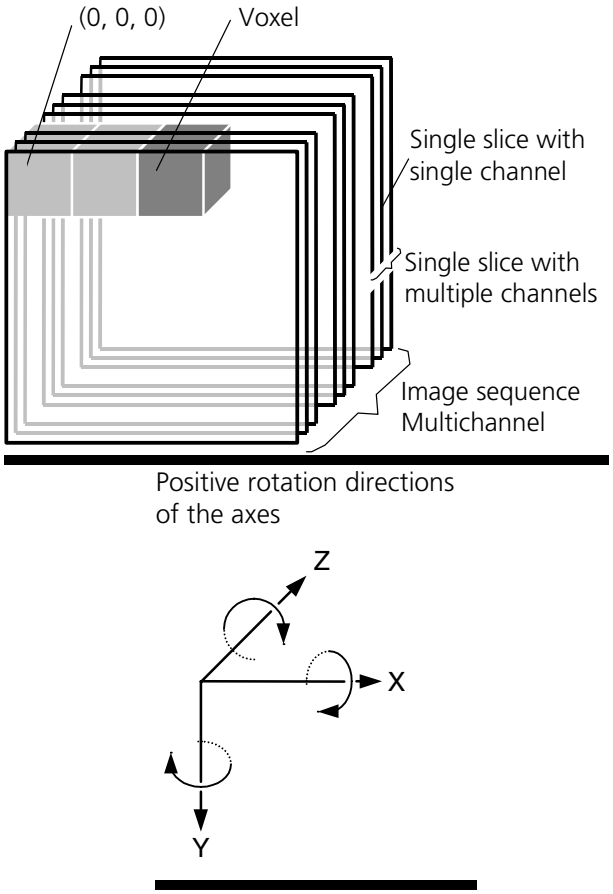


Fig. 8-1

The following terms and definitions apply for the "3D for LSM-FCS" software.

– An image sequence is a number of individual sequential images (usually called slices in the dialog boxes), the spacing between which is equal.

– Image sequences can contain up to 12 bit of image data (per channel).

– A sequence (slice) can consist of up to eight channels.

– The maximum size of an image sequence is limited by the provided memory of the operating system.

– A voxel is the smallest element of an image sequence (the equivalent of a pixel in a 2D image). All voxels in a given image sequence are the same size.

– The coordinate system originates in the left upper front corner of the image sequence. This point has the coordinates 0, 0, 0.

– All angles are positive for rotations to the right in the direction of the positive coordinate axis (right-handed coordinate system).

– A slice is an individual image in a sequence of images. The numbering of the slices starts with "1".

Image sequences can consist of several channels. Most functions and the **Display window** are providing buttons to select all or a subset of channels stored in the selected image sequence. The **Output** image sequence will only get those channels which are selected on the input side. The button selects all channels in the image sequence to be used clicking with the left mouse button on it.

Clicking with the left mouse button on any of the number buttons toggles the state of this single channel.

Clicking with the right mouse button on any of the number buttons selects this single channel exclusively. All other channels are deselected.

8.1.2 The Image Properties

Every image sequence has its own set of properties. They contain the scaling and the scaling units. The scaling and its units are required for 3D reconstruction and measurement. If a sequence of LSM-TIFF images is read in, the image properties are loaded automatically from the file header and allocated to the image properties of the new image sequence.

8.1.3 Memory Usage

All images shown in the **Gallery** are currently loaded in the system memory of the operating system. Some functions need additional temporarily used memory during their execution.

If the memory is running low delete some images from the **Gallery**. If the images are needed afterwards they must be saved to disk first. Normally all functions produce a new result (output) image sequence. In order to save some memory, other image sequences currently presented in the **Gallery** can be selected as result position. The output image is overwritten by entry execution of a function.

8.2 User Interface

8.2.1 Introduction

This section describes the following main components of the system:

Main window — **Main window** with the **Menu**, the **Tool bar** and **Gallery**. All general system functions are located here.

Gallery — Normally several images are required in order to accomplish a particular task. These images are displayed in reduced size to provide an overview and facilitate selection. This area is located just below the **Tool bar**.

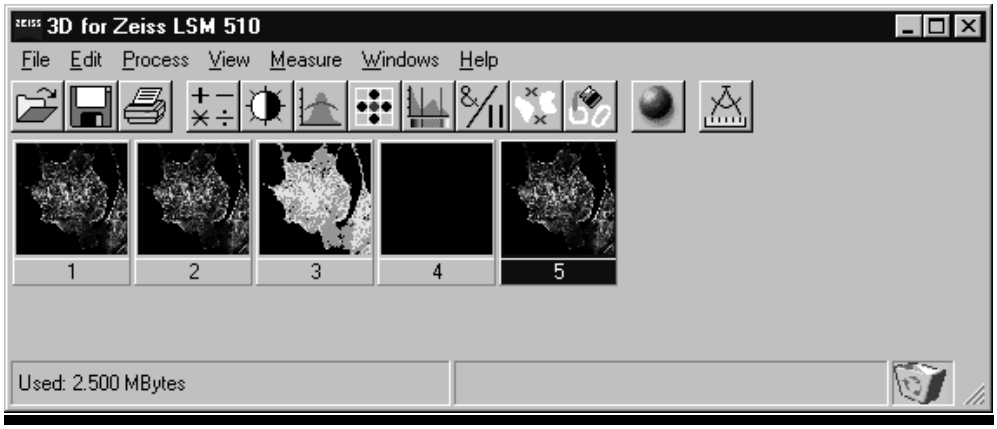


Fig. 8-2

Tool bar — This menu shows all image processing functions.

Display window This window is used to display image sequences.

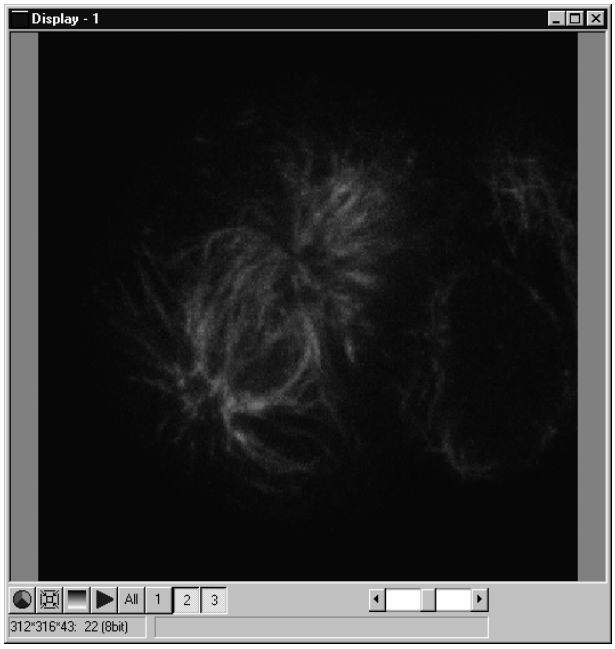


Fig. 8-3 Display window

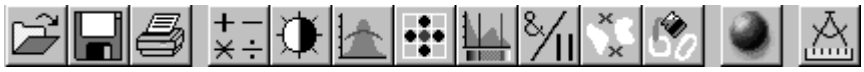
Dialog boxes All dialog boxes provide three buttons. Pressing the **OK** button executes the function with the defined parameters and closes the dialog window. Selecting the **Cancel** button does not execute the function, restores the parameters, and closes the dialog window. Pressing the **Apply** button executes the function with the defined parameters; the dialog window will stay opened.

8.2.2 Main Window

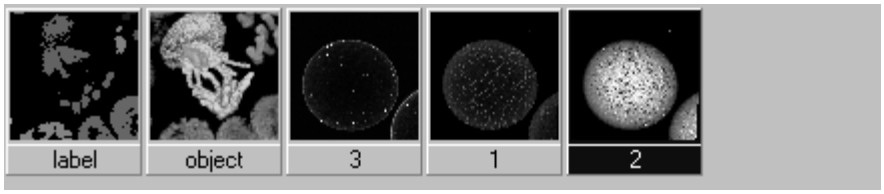
The **Main window** includes:

the **Menu**

the **Tool bar**



and the **Gallery**



File Menu

Open Image Opens a file selector dialog to load an image sequence.

Save Image As Opens a file selector to save an image or image sequence.

Save Display As Saves the currently shown contents of the Display window as a single colour image.

Print The printer parameters can be set with this tool. The standard Windows printer dialog is opened.

Exit Terminates the application.

Edit Menu

Copy	Copies the contents of the **Display window** to the clipboard.
Edit Channels	Allows to add or to remove channels to a single or multichannel image.
Delete All Images	Deletes all images and image sequences from the memory.

Process Menu

 **Arithmetics**	Adds or subtracts the grey values of two image sequences (**Add**, **Subtract**).
 **Contrast**	Enhances the contrast and brightness of an image sequence (**Interactive**, **Automatic**, **Linearize**).
 **Smooth**	Smoothes an image sequence.
 **Morphology**	Performs morphological operations on image sequences (**Erode**, **Dilate**, **Open**, **Close**).
 **Segment**	Segmentates an image sequence to propose measurement (**Interactive**, **Automatic**).
 **Boolean**	Combines two image sequences by Boolean operations (**And**, **Or**, **Not**, **Xor**, **Mask**).
 **Scrap**	Selects or deletes objects of a defined size.
 **Fill Holes**	Fills holes in objects.

View Menu

Set Channel Colour The colour and the weight of the single channels can be defined.

Properties The properties of the image (e.g. scaling, use laser etc.) are displayed.

Render Calculates 3D reconstructions of an image sequence (Surface, Alpha).

Measurement Menu

Automatic Object Measures geometrical and densitometrical features (**General**, **Object Features**, **Volume Features**, **Condition**).

Windows Menu

Arrange All Arranges the windows automatically.

Display The current image is displayed in this window.

Help Menu

Content Opens the help for the software.

About 3D for LSM-FCS Displays status and release message of the software.

Tool Bar

This bar provides buttons with iconized images of nearly all functions. Clicking on one of the buttons will open a dialog window to define the function parameters. Selecting an entry from the menu alternatively can activate the same functions. Placing the cursor on a tool bar button will show a short description, if the window is activated.

Gallery

The **Gallery** is used as an overview of the images available in memory and their contents. It is located just below the **Tool bar**. Each small image represents a sequence. The middle slice of each image sequence is shown. The status bar of each image shows the name. The name might be a number or a string.

Every image sequence has its own channel colour assignment (see **Display window**). When an image is copied the channel colour assignment is copied too. Drag and drop techniques can be applied to copy images or define the function parameters **Input** and **Output** using the **Gallery** thumbnails.

- Position the cursor on an image in the **Gallery**.
- Press the left mouse button.
- Hold the mouse button down and move the mouse to the destination position.
- At the destination release the left mouse button, the destination image will be overwritten.

To delete an image, drag it, move it to the wastebasket, and drop it.

8.2.3 Display Window

This window is used to display an image sequence, regardless of size or type. To show multiple channel sequences each channel could have its own base colour. The user can set these colours and the weighting for each channel by pressing the corresponding button  at the bottom of the window. To display a different image or image sequence, it can be dragged from the **Gallery** and dropped to the **Display window**.

The image can be displayed in full size (one pixel on the screen represents one pixel of the image) or in a zoomed size. To zoom the display view click and hold down the right mouse button on the window border and resize the window. The aspect ratio of the image will not be changed. Clicking on the button  resets the **Display window** to a full size view of the image (see above).

The title bar shows the currently displayed sequence name. The status bar displays the size of the current sequence and the selected slice on the left. On the right the cursor position within the window and the corresponding intensity (grey) value of each channel is shown.

The **Display window** can be closed without any effect to the image processing functions. If no **Display window** is opened select the entry **Display** in the **Window menu**.

The scroll bar at the lower right of the window enables to show the images in a sequence. The range reaches from one to the maximum slice provided by the current sequence.

To start the automatic animation of an image sequence start the Player tool by clicking on the button  . The colour selection for the channels can be activated by clicking on the button  . A colour image can be displayed as a grey value image by clicking on the button  .

Player

This function plays back the sequential images of an image sequence.

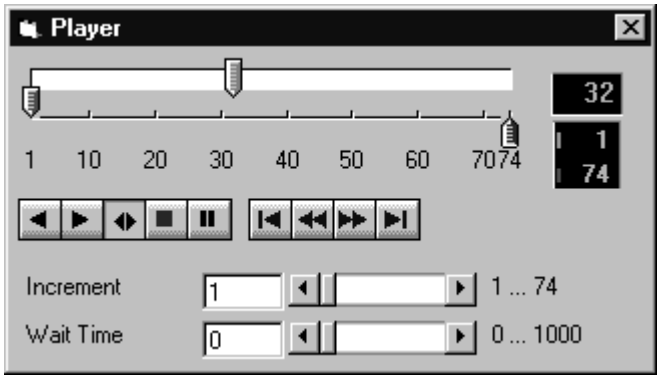


Fig. 8-4

The image sequence is displayed in the **Display window**. The display process is working as a background task; other functions can be executed while the player is running. There are several ways to stop the player:

by closing the player window

by pushing the red Stop button of the player window (the window remains open)

by closing the image window.

The **Increment** parameter specifies whether each sequential image (1) should be displayed or whether some sequential images should be skipped during display. The value 2 skips one image for every sequential image displayed, in other words, it displays only every second image.

The parameter **Wait Time** states the delay in milliseconds between two successive sequential images. The maximum display speed depends mainly on the hardware. The sequential images are always displayed in their entirety, regardless of the set delay.

Control Element of the Player

The three arrow shaped controls on the scale show the start slice and the currently displayed sequential image. The values (positions) can be changed using the mouse. Press and hold the left mouse button and move the pointer to the desired position. The set values are shown in the numerical windows at right.

 Start slice

 Currently displayed sequential image

 End slice

The buttons in the left group start and stop playback of an image sequence.

 Reverse playback

 Forward playback

 Play forward and then backward again (jojo)

 Stop playback

 Pause playback

The buttons in the middle group control the settings of the current sequential image.

 Reset to start slice.

 Single step backward (1 sequential image each regardless of Increment).

 Single step forward (1 sequential image each regardless of Increment).

 Set to end slice.

Increment Image increment.

Wait Time Displays delay between two images (in milliseconds).

Set Channel Colour

This function sets the colour and weight for the channels.

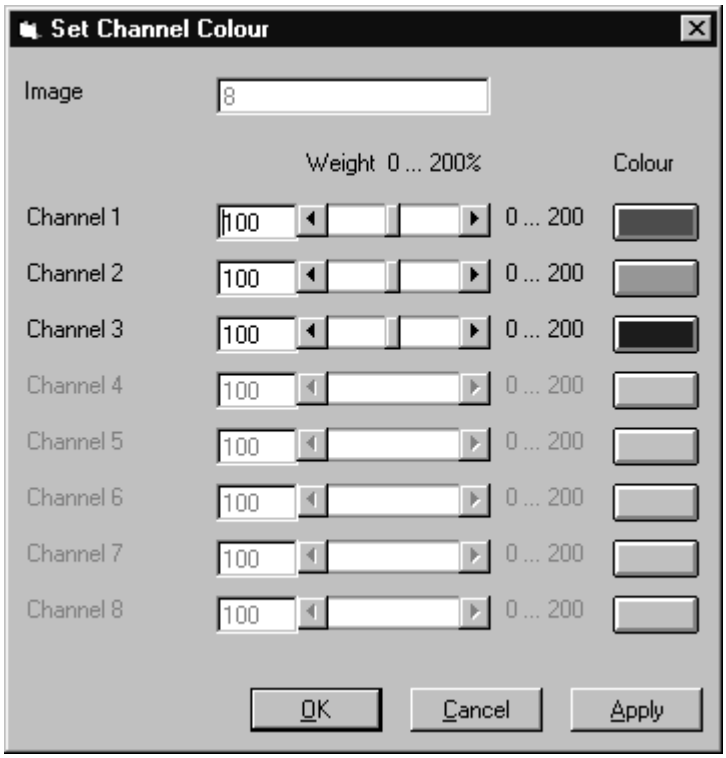


Fig. 8-5

Each image sequence can get its own colour definitions. All functions will inherit the colour definition from the **Input** sequence to the **Output** sequence. By default the colours are set to 100 % weighting and the pure base colours (red, green, blue) are defined.

The weight can be any value between 0 % and 200 %. The colour can be redefined by clicking on the coloured button on the right of the dialog. The standard Windows colour selection dialog is opened. The solution is done by clicking on one of the colours or by entering appropriate numbers in the corresponding edit boxes.

Pressing the **OK** button will close the colour selection dialog and update the **Display window** immediately.

Only those channels, which are available in the image sequence, can be defined.

Parameters:

Image	Image sequence to edit
Weight	Colour weighting for each channel
Colour	Base colour for each channel

8.3 Functions

8.3.1 Functions in the File Menu

Open Image

This function reads a Zeiss LSM-FCS (*.lsm), Zeiss LSM TIFF (*000.tif) or Carl Zeiss Vision (*0.img) image sequence from a disk or network drive.

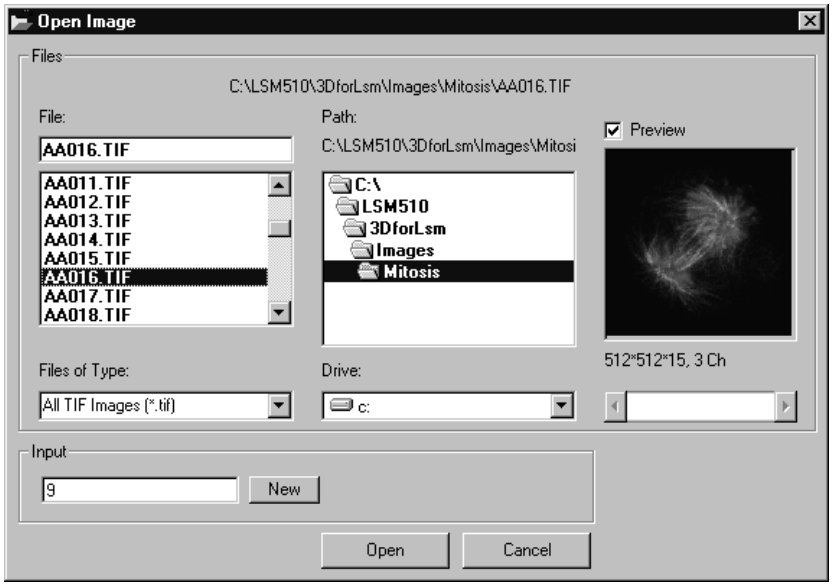


Fig. 8-6

The individual files of a Zeiss TIFF image sequence are read and saved as an image sequence in image memory. In addition, the image properties are read out of the TIFF files and allocated to the image sequence **Input**.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

In case of choosing the TIFF-format in the **Files of Type** box, three number characters are always expected before the dot in the filename extension. The first number must be 000 at the end of the filename. From a complete sequence only this file is listed in the dialog, if "LSM TIF Images (*000.tif)" is selected in the **Files of Type** box. To view all TIFF files "All TIF Images (*.tif)" in the **Files of Type** box must be selected. This selection enables to start with a different file than with the very first (named *000.tif) at the end of the filenames three number digits.

Currently the Carl Zeiss Vision file format "KE Images (*0.img)" is supported. Two files per channel are saved.

Carl Zeiss Vision image sequences must have a number digit at the end of the base filename. They are used to indicate the different channels in a multichannel sequence. The numbering starts with zero (0). If a sequence is saved in the Carl Zeiss Vision format the numbers are generated automatically. To load such an image sequence "KE Images (*0.img)" in the **Files of Type** box must be selected.

The window incorporates the usual file selection controls. The bottom half displays a selection of the image properties that are stored in the image sequence.

Parameters:

BaseName Base name of the TIFF files (image sequence) to be loaded. Only the letters before the first number are stated.

Input Name of the resulting image in which the image sequence will be saved.

Save Image As

This function saves an image or image sequence to disk or network drive.

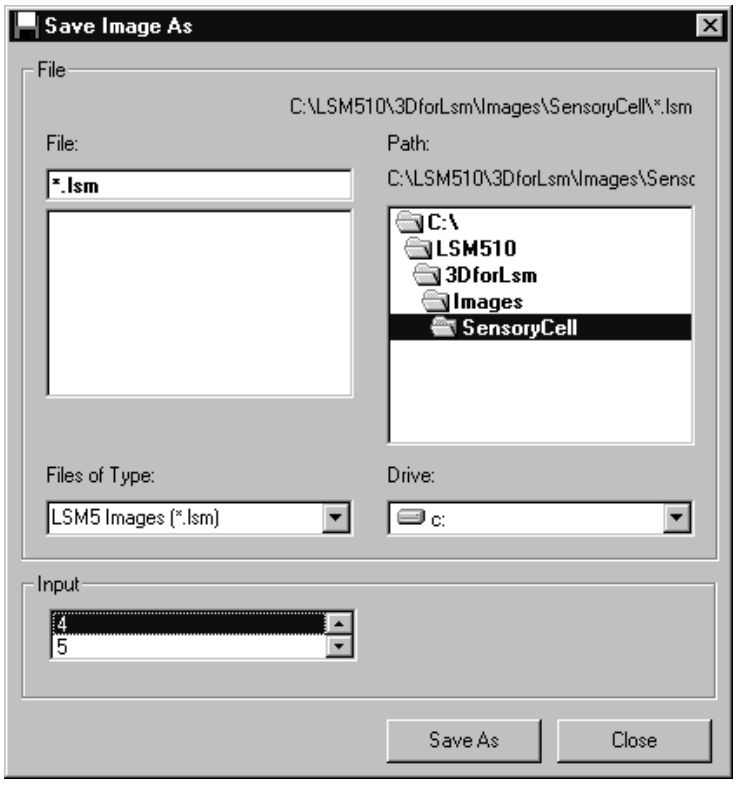


Fig. 8-7

All the files in the current directory that have the selected image format are listed in the **File Name** list box.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

Use the list box **Files of Type** to select the image format. Currently the LSM-FCS image format (*.lsm) and the Carl Zeiss Vision file format "KE Images (*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (*0.img)", two files per channel are saved. On one hand the Carl Zeiss Vision type image sequence file, on the other hand the file with the image properties. One pair of files is written per channel. They are numbered automatically, starting with zero. A one number digit is added to the end of the filenames. The two files share the same filename but have different filename extensions (*.img and *.3d).

The content of the **Gallery** is shown in the **Input** section. The selection of the sequence to save is done by highlighting one of the provided names or by drag and drop from the **Gallery**.

Parameters:

Input Name of the image sequence to be saved

Filename Name of the file to be used on disk

Save Display As

This function saves the current **Display window** contents to a disk or network drive.

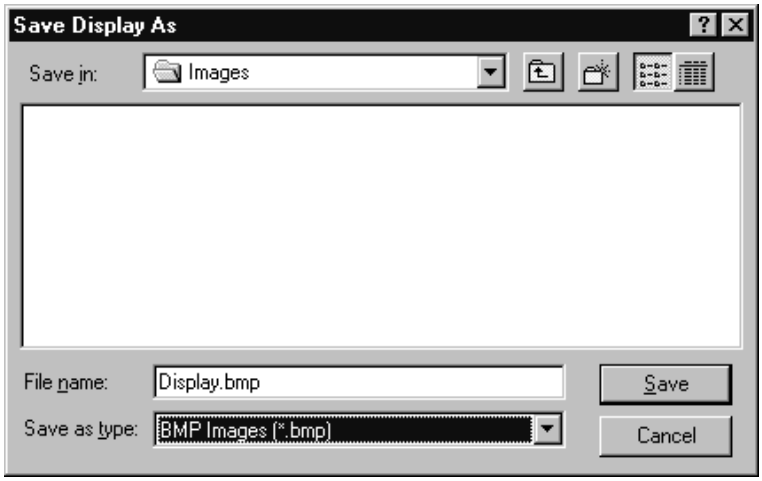


Fig. 8-8

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account, the image is saved without any zoom.

The image is saved as a true colour image with 24-bit resolution. From the **Save as Type** list box one of the provided formats can be selected.

Parameters:

None

Print

This function prints the current **Display window** contents.

The standard Windows print dialog is opened.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

Parameters:

None

Exit

This function terminates the application completely.

All images and image sequences shown in the **Gallery** will be deleted from the memory. Save those images which might be used for any further processing.

Parameters:

None

8.3.2 Functions in the Edit Menu

Copy

This function copies the current **Display window** contents to the clipboard. No dialog is shown.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account; the image is copied without any zoom.

The image is copied as a true colour image with 24-bit resolution. Afterwards the contents can be pasted to any other Windows application.

Parameters:

None

Edit Channels

This function allows to add or to remove channels to a single or multichannel image.

On the **Add Channel** tab sheet the channels of (different) **Input** sequences can be defined to add (combine) channels to an **Output** sequence.

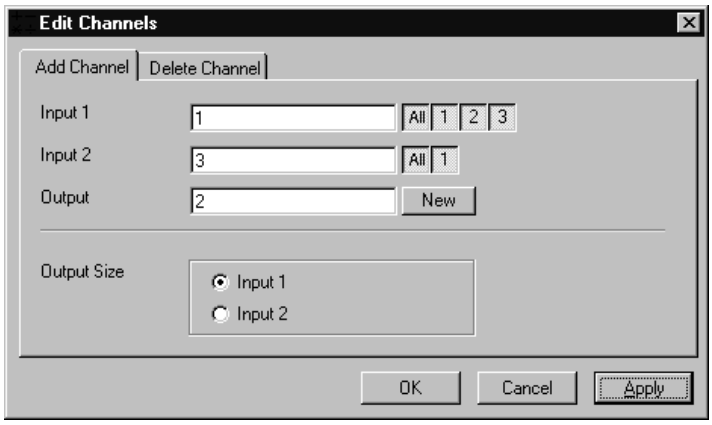


Fig. 8-9

This operation is useful to add a segmented channel (or any other result of a function) to the original image sequence. The selected channels of **Input 1** and **Input 2** are copied to **Output**. The maximum number of channels in an image sequence is eight.

If the image sequences do not have the same extents **Output Size** defines which input is taken as a reference. This selection also defines the properties for scaling and units in the output image sequences.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Output size	Defines source image sequence for size, scaling, and units

On the **Delete Channel** tab sheet channels of the **Input 1** image sequence can be selected to delete channels.

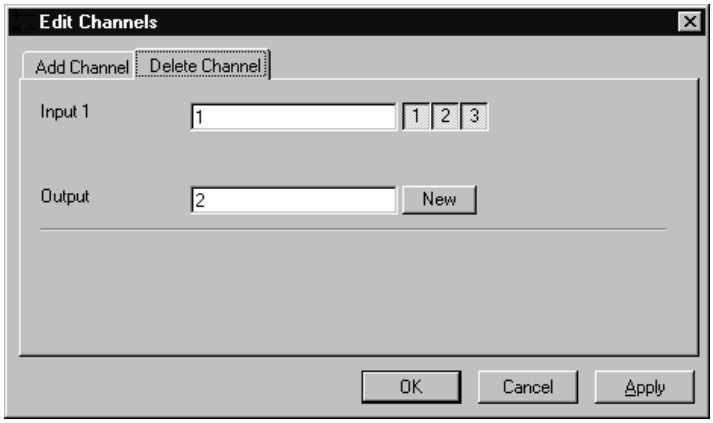


Fig. 8-10

This operation might save time and memory for further processing if not all channels are needed.

Only the selected channels of **Input 1** are copied to **Output**.

Parameters:

Input 1 Input image sequence

Output Output image sequence

Delete All Images

This function deletes all images and image sequences from the memory (**Gallery**).

The function is used whenever a completely new image sequence should be processed. In order to drop the images item by item to the wastebasket all of them can be deleted by a single function.

If any image or image sequence is needed for further use save them first.

Parameters:

None

8.3.3 Functions in the Process Menu

Arithmetics - Add

This function adds two image sequences.

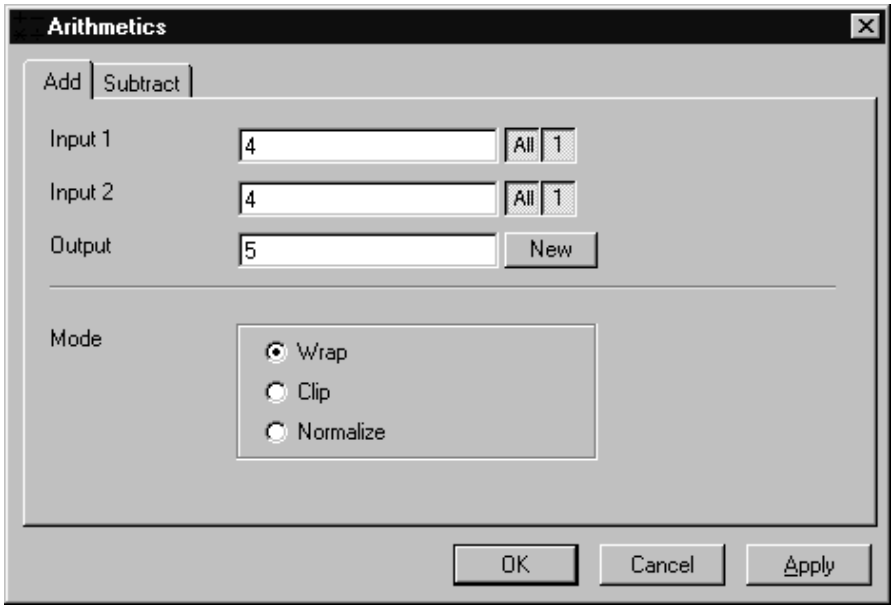


Fig. 8-11

The **Add** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function adds the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be greater than 255 (4095). The parameter **Mode** determines how a range overflow is handled:

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is greater than 255 (4095), the value 256 (4096) is subtracted from it.
2 - Clip	Grey values which exceed 255 (4095) are replaced with 255 (4095).
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize

Arithmetics - Subtract

This function subtracts two image sequences.

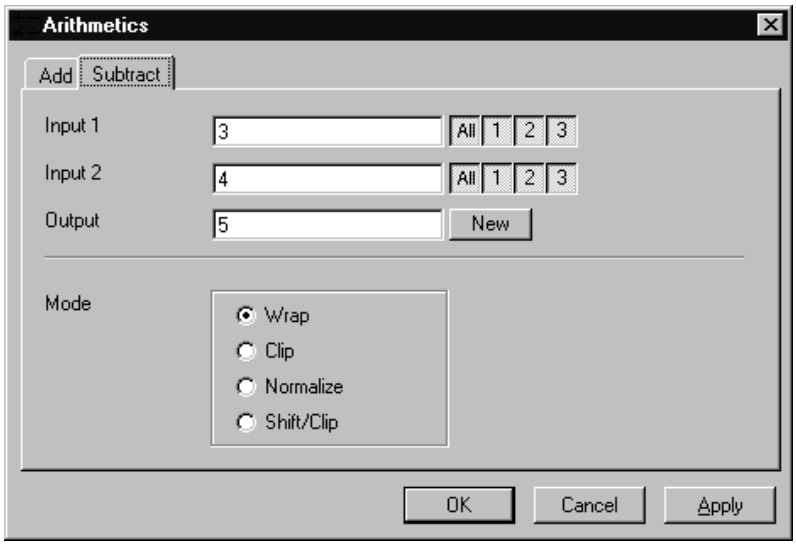


Fig. 8-12

The **Subtract** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function subtracts the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be less than 0. The parameter **Mode** determines how a range overflow (negative values) is handled.

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is less than 0, the value 256 (4096) is added to it.
2 - Clip	Negative values are set to 0.
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).
4 - Shift/Clip	128 (2048) is added to the difference, then negative values are set to 0. Values greater than 255 (4095) are set to 255 (4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize 4 - Shift/Clip

Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.

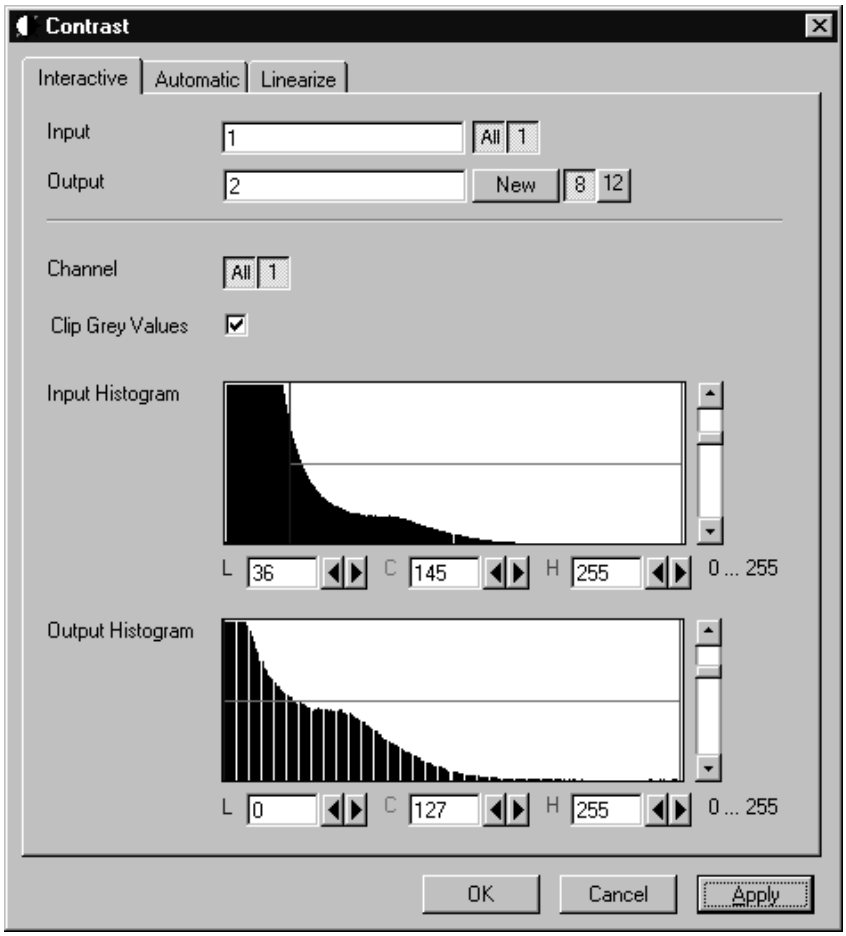


Fig. 8-13

The **Interactive** tab sheet of the **Contrast** dialog window must be selected.

A grey value range of the **Input** image sequence is scaled to another range in the **Output** image sequence. Both ranges can be edited interactively. This function is used to achieve a better view of an image sequence, or to scale a range of grey values to single value for a special coding in an image sequence. The function does not improve the result of the linear segmentation function **Segment**.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

If **Clip Grey Values** is selected, the output grey values are clipped to the **Low (L)** and **High (H)** values. If **Clip Grey Values** is not selected, output grey values beyond the **Low** and **High** value range are possible.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The selected range is marked by the borderlines in the histogram. The blue line or **L** indicates the lower boundary, the red line or **H** the upper one, **C** indicates the center of the range.

There are three ways to change the range: clicking and dragging the borderlines with the mouse.

Entering a new value in the appropriate text boxes, clicking on the buttons  or using the arrow keys from the keyboard. To alter the values within the histogram move the mouse pointer over one of the three coloured lines until the shape changes. Press and hold the left mouse button to move the line to a new position. To change the values with the arrow keys click once into the histogram. Using the left or right arrow key by its own will move the whole range. Pressing the **Shift** key additionally moves the lower boundary, the **Control** key the upper boundary.

The vertical scale of the histogram is set using the scroll bar. The units are percents of the maximum grey value distribution. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Channel	Selection of the channel numbers for the **Output** image after contrast enhancement
Clip Grey Values	Clipping of grey values to the **Low (L)** and **High (H)** output grey values boundaries
Input L	Lower boundary of grey value range **Input**
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.

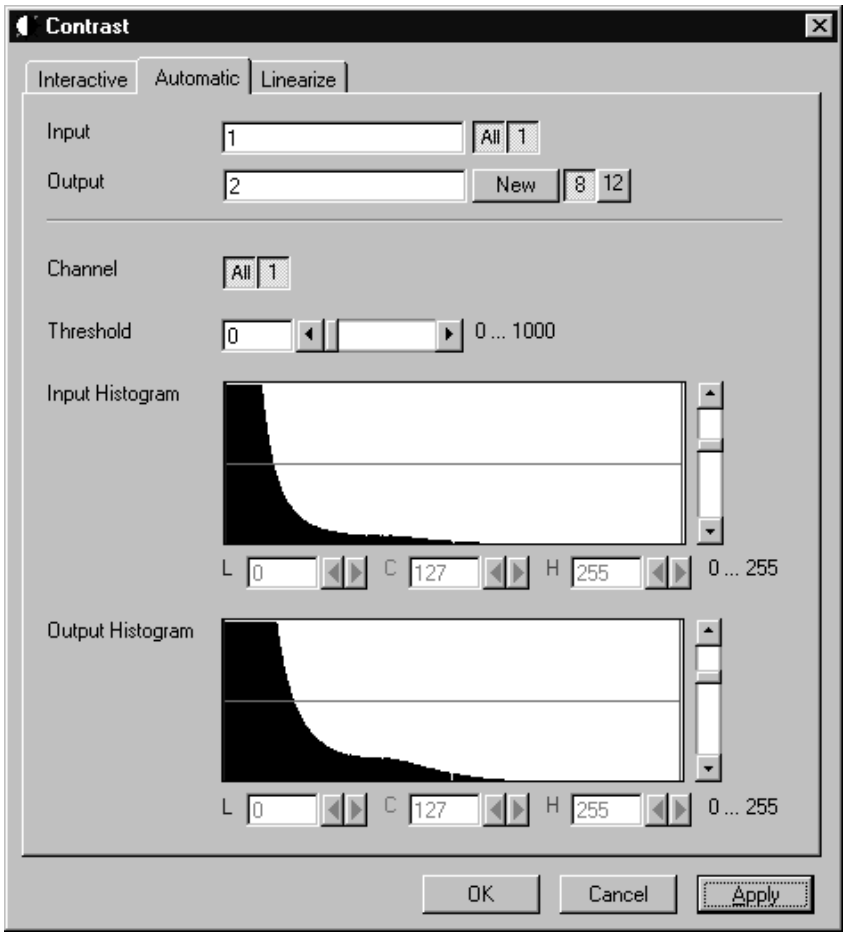


Fig. 8-14

The **Automatic** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast of an image sequence by spreading the grey value distribution over the maximum possible range. This function is used to achieve a better view of an image.

The light and dark grey value ranges with a low share of pixels are excluded from the operation by the parameter **Threshold**. The **Threshold** units are in thousandths of the total number of voxels. Using a value of 10 means that the scale interval is set so that 5/1000 of the total number of voxels on the light side, and 5/1000 of the total number of voxels on the dark side of the grey value distribution are excluded.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit.

Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

The **Output** histogram shows the resulting histogram. They are not editable. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Threshold	Exclusion value - 0...1000
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.

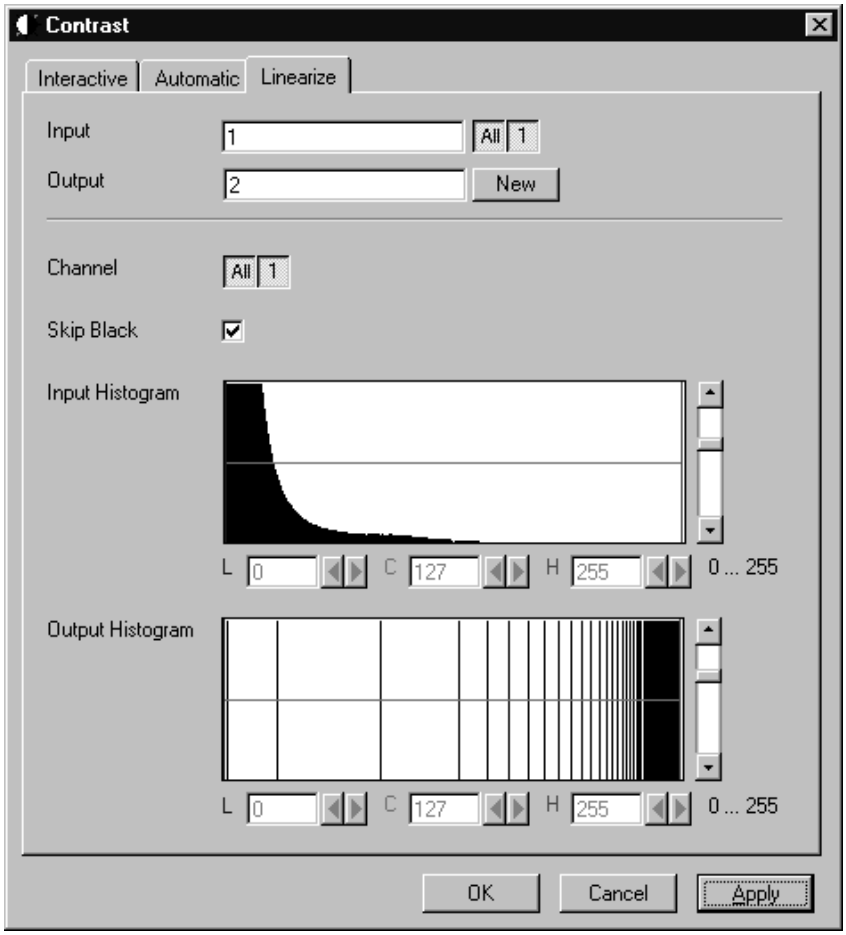


Fig. 8-15

The **Linearize** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast by linearizing the histogram of the image sequence to equal area fractions in the histogram. The areas (voxel count multiplied by grey value range) of all grey values in the **Output** histogram are the same. This function is used to achieve a better view of an image sequence. When **Skip Black** is checked the grey value 0 will not be taken into account for linearization.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the range of the result sequence. It will get only these channels which are chosen by the **Input** parameter. The grey value (intensity) resolution will be the same as the one from **Input**.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

Parameters:

Image	Input image sequence
Output	Output image sequence
SkipBlack	0 - Grey value black is ignored 1 - Grey value black is taken into account
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range Input
Input H	Upper boundary of grey value range Input
Output L	Lower boundary of grey value range Output
Output C	Center of grey value range Output
Output H	Upper boundary of grey value range Output

Smooth (Gauss)

This function performs a Gauss filter.

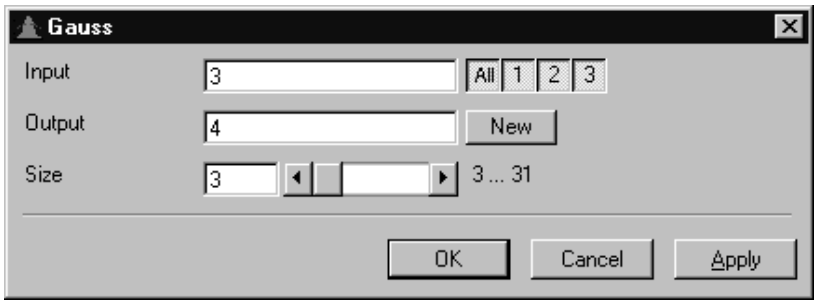


Fig. 8-16

The noise in the image sequence is reduced, the edge shape is nearly unchanged, local maxima are leveled, the dynamic range is reduced.

Image sequences should be smoothed before they are reconstructed or segmented. For most sequences a **Size** value of 3 is sufficient enough. If **Input** is a multichannel sequence, any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The grey value of every pixel is substituted by a weighted average of its surrounding neighbors. The neighbors are defined by a cube. The affected pixel is the central pixel of the filter cube. The weighted filter cube is approximated by a binomial distribution. The size of the filter cube is set using the **Size** scroll bar. Even numbers are set to the next odd value. The **Size** defines the strength of the smoothing.

Parameters:

Input	Input image sequence
Output	Output image sequence
Size	Filter size (3...31, only odd numbers)

Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.

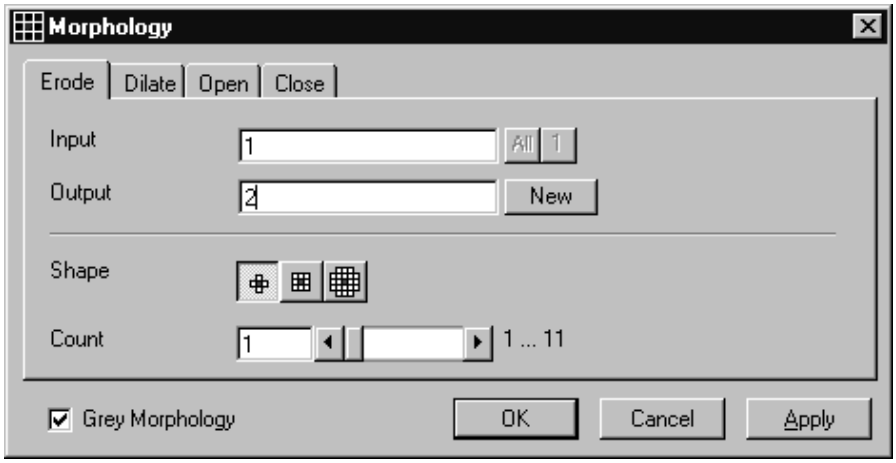


Fig. 8-17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

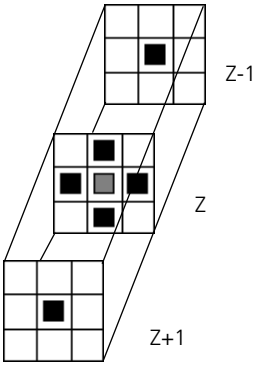
Literature

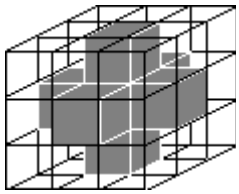
Bomans, M.; Höhne, K.-H.; Tiede, U.; Riemer, M.:
3D-Segmentation of MR Images of the Head for 3-D Display
IEEE Transactions on Medical Imaging 9, 1990, 177-183
Schiemann, T.; Bomans, M.; Tiede, U.; Höhne, K.-H.:
Interactive 3D-Segmentation of Tomographic Image Volumes
14. DAGM-Symposium Mustererkennung, Springer-Verlag 1992, 73-80

The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

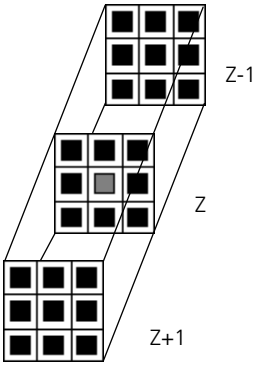
The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

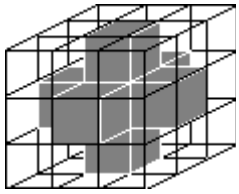
Sequential image: Volume view: Cross shape



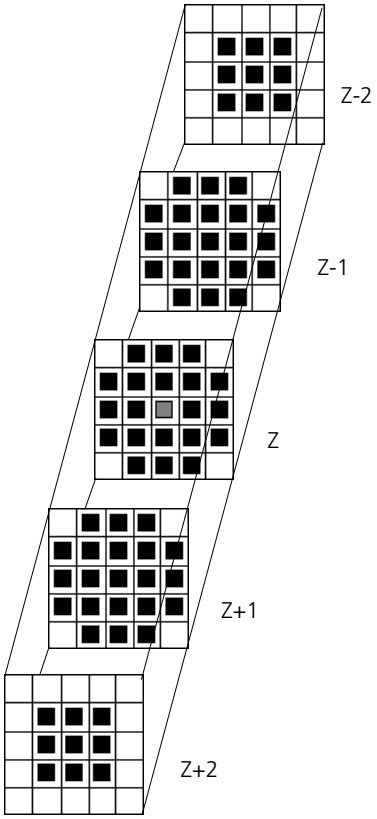


Sequential image: Volume view: Cross shape

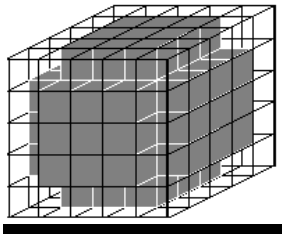




Sequential image:



Volume view:



Cube cross shape: created through application of "cube" and "cross" one after the other.

For regions (voxels) that are at the edge of the image sequence, it assumed for erosion that there are white voxels with a grey value of 255 (4095) outside the edge. For dilation, it is assumed that there are black voxels with the grey value 0 outside the image sequence.

If the **Grey Morphology** tickbox is activated, erosion sets the grey value of the central voxel to the minimum of all neighboring voxels affected by the structural element; dilation sets the grey value of the central voxel to the maximum.

If the **Grey Morphology** tickbox is not activated, the neighboring voxels are only distinguished by grey value 0 and non-0. For erosion the central voxel is set to 0 if any of the neighbors is 0. It is set to 255 (4095) if any neighbor is not 0. For dilation the central voxel is set to 255 (4095) if any of the neighbors is not 0. It is set to 0 if all neighbors are 0.

Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

The result of erosion and dilation is called opening. On the one hand, this maintains to some extent the original size of the regions while not losing the smoothing effect of erosion on the image. This name stands for the operation of reducing convex bulges in the contour of the region. Thin connections between regions are eliminated, broken borders between regions are connected, and small regions disappear.

The opposite operation (first dilation, then erosion) is called closing. Concave bulges in the contours of regions are filled in; connections are formed between adjacent regions.

The following example illustrates the operations "Open" and "Close" in two dimensions:

Open = Erosion + Dilation

Fig. 8-18

Close = Dilation + Erosion

Fig. 8-19

The "cube cross" shape was used for the operations shown.

Morphology - Erode

This function erodes structures in an image sequence.

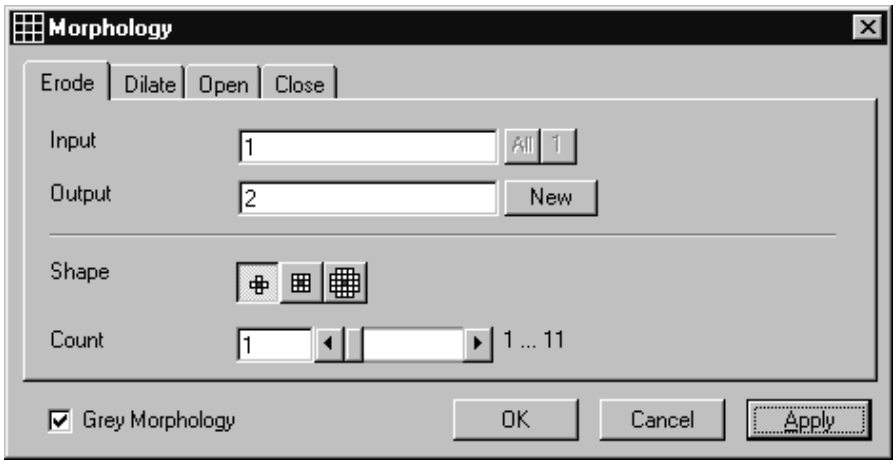


Fig. 8-20

In the **Morphology** dialog window, the tab sheet **Erode** must be selected.

Erosion makes bright regions smaller on a dark background. It also results in separation of thin connections between regions. Small regions disappear entirely.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results. The Input image sequence is eroded **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Dilate

This function dilates structures in an image sequence.

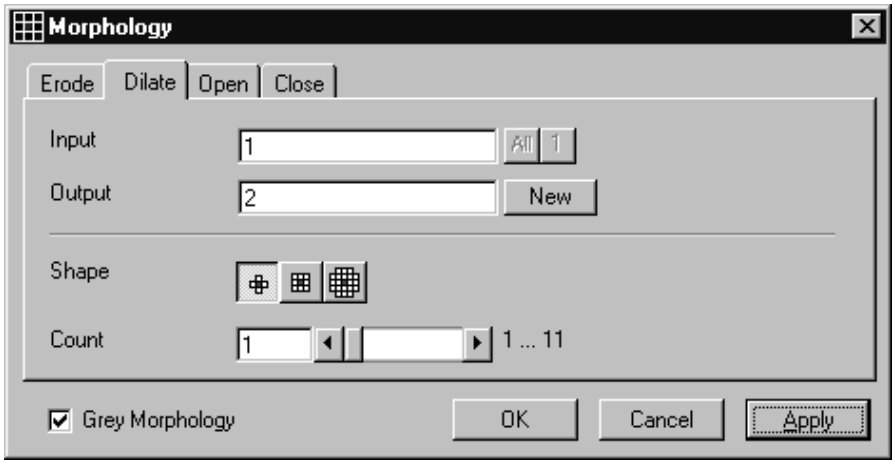


Fig. 8-21

In the **Morphology** dialog window, the tab sheet **Dilate** must be selected.

Dilation makes bright regions larger on a dark background. It also results in the filling of gaps and smoothing of small contour details.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Input** sequential image is dilated **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Open

This function carries out an opening.

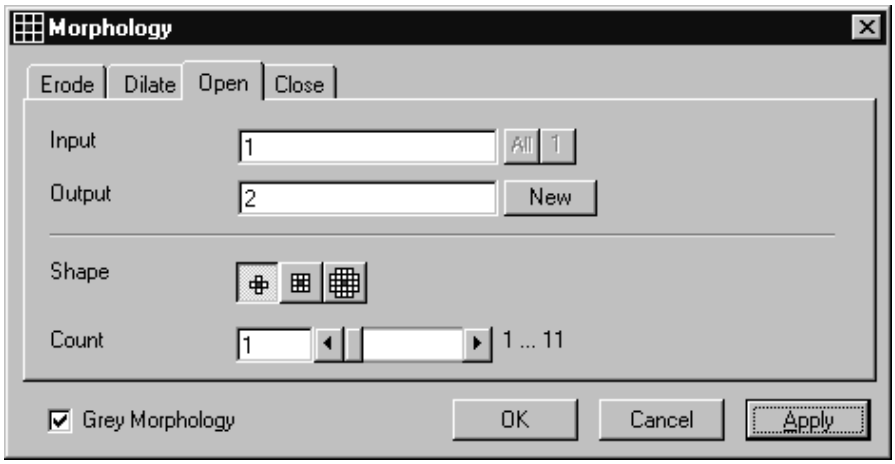


Fig. 8-22

In the **Morphology** dialog window, the tab sheet **Open** must be selected.

This function carries out an erosion followed by a dilation. For the most part, the opening maintains the original size of the regions. Thin connections between regions and small regions themselves disappear. Convex bulges in the contours of the regions are reduced. The opening is applied to the grey value image sequence **Input Count** times with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Close

This function carries out a closing.

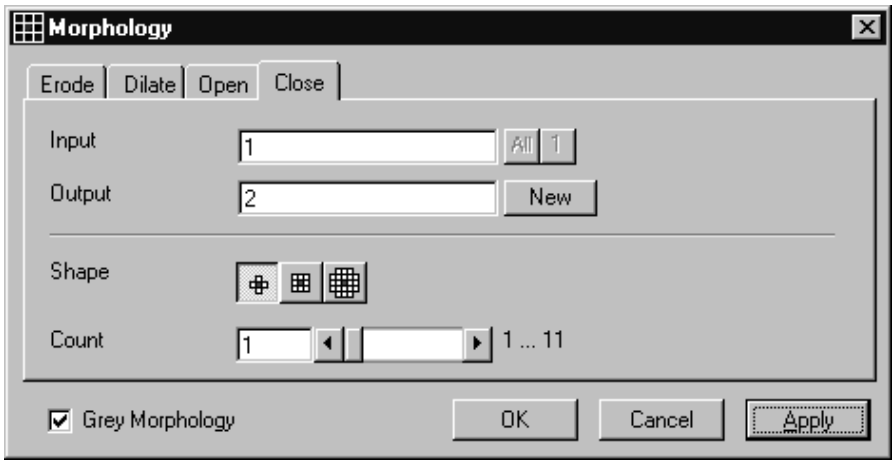


Fig. 8-23

In the **Morphology** dialog window, the tab sheet **Close** must be selected.

This function carries out a dilation followed by an erosion. For the most part, the closing maintains the original size of the regions. Connections are formed between adjacent regions; gaps and bright concave bulges in the contours of regions are filled in. The closing is applied **Count** times to the grey value image sequence Input with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:

If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Segment - Interactive

This function carries out a grey value segmentation by means of thresholding.

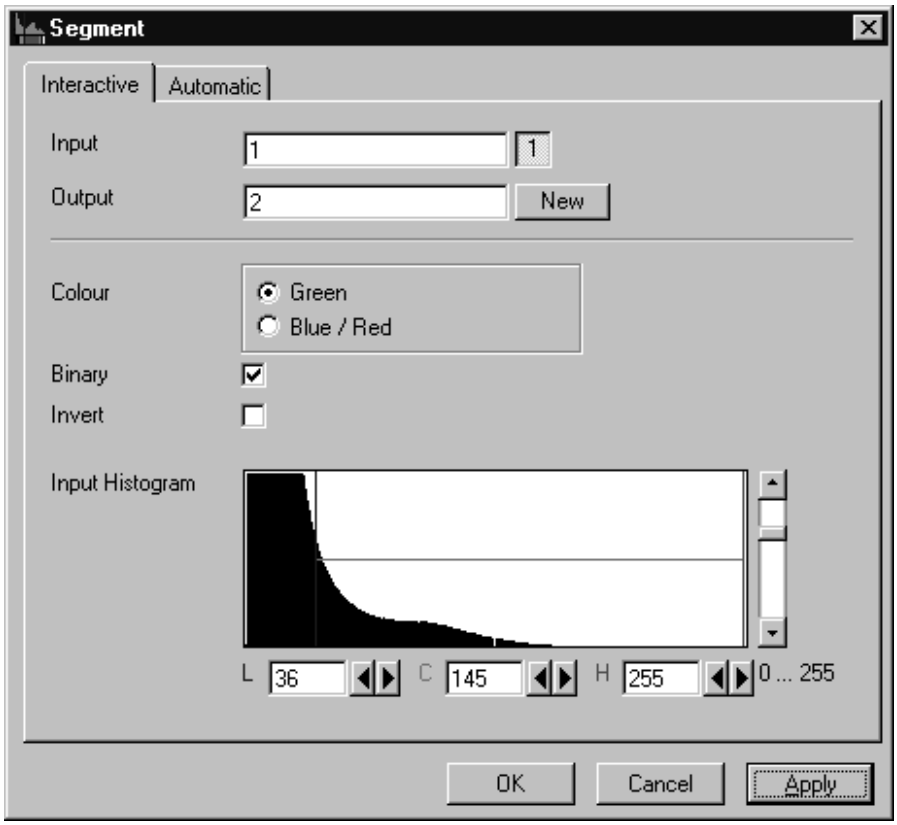


Fig. 8-24

The **Interactive** tab sheet of **Segment** dialog window must be selected.

Segmentation is especially used to generate binary regions. These are required for the measurement.

Two threshold values determine which grey value range of the **Input** image sequence is preserved and/or deleted in the **Output** image sequence. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence.

The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The thresholds **L**ow and **H**igh are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **L**ow, **C**enter and **H**igh can be set through entry in the corresponding fields.

To move the lower (**L**) and upper (**H**) thresholds at the same time, move the vertical line in the grey value histogram or set the scroll bar (**C**).

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black. The measurement function accepts both results without any difference in the results.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green
Blue/Red	Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.

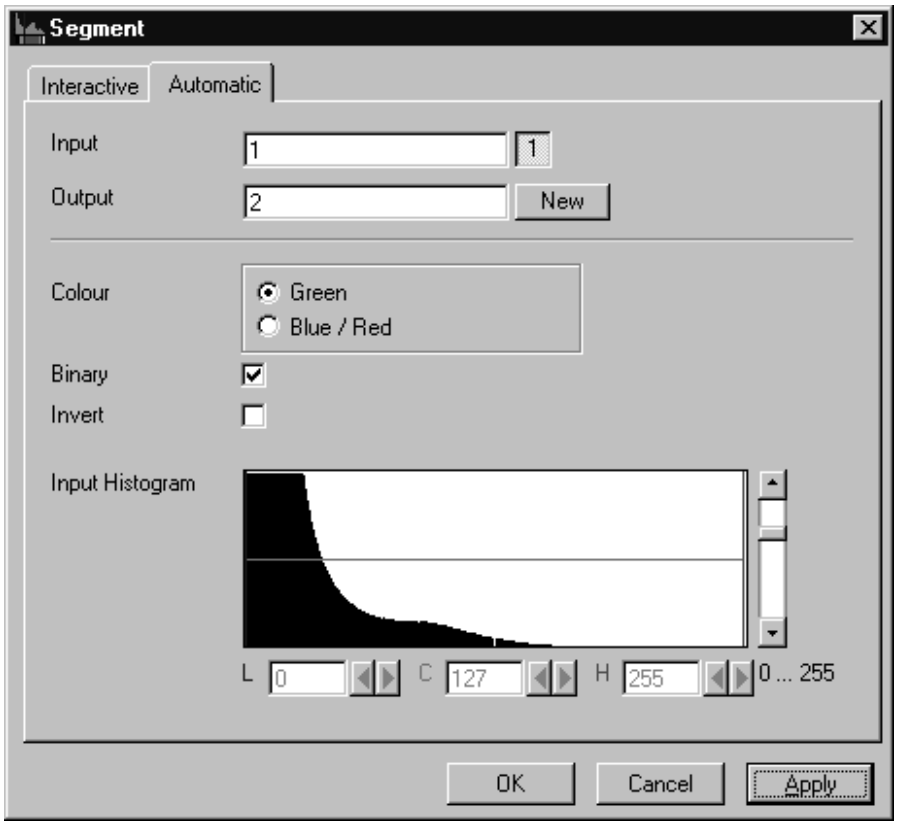


Fig. 8-25

The **Automatic** tab sheet of the **Segment** dialog window must be selected. Segmentation is especially used to generate binary regions. These are required for the measurement.

The function calculates the two strongest local minimums in the histogram of the **Input** image sequence. These values are used for the discrimination. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence. The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255 (4095)) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Boolean - And

This function carries out a bit-by-bit **And** calculation for the image sequences **Input 1** and **Input 2**.

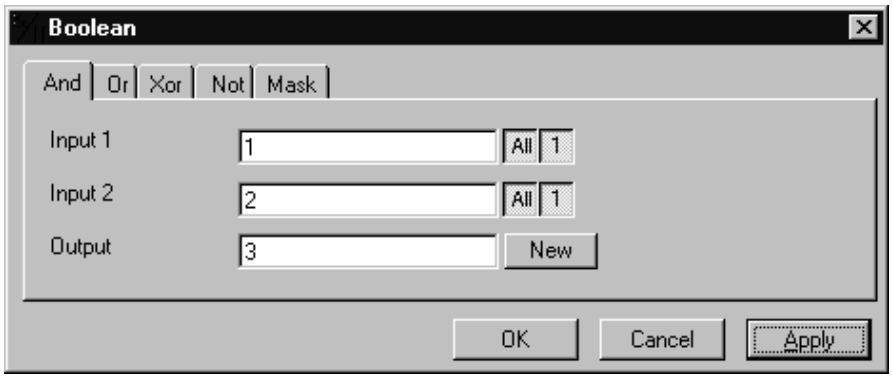


Fig. 8-26

The **And** tab sheet of the **Boolean** dialog window must be selected.

This function is especially well suited for masking images.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.

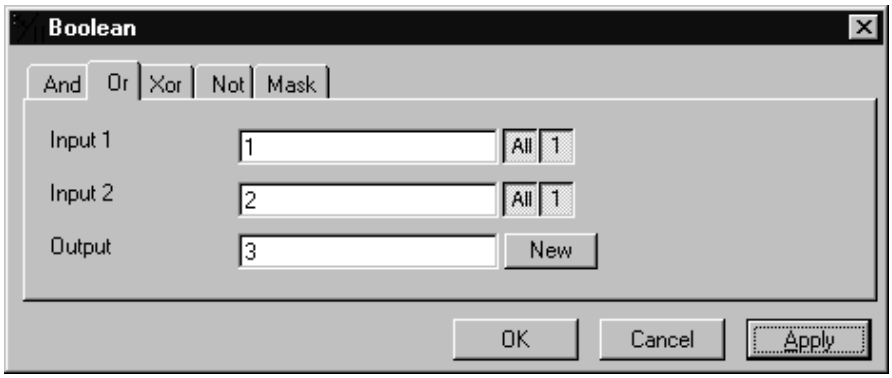


Fig. 8-27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.

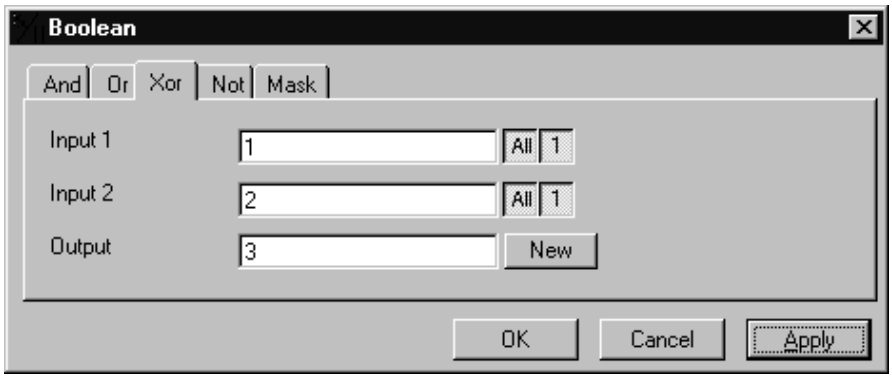


Fig. 8-28

The **Xor** option button of the **Function** option group in the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Not

This function carries out a bit-by-bit negation of an image.

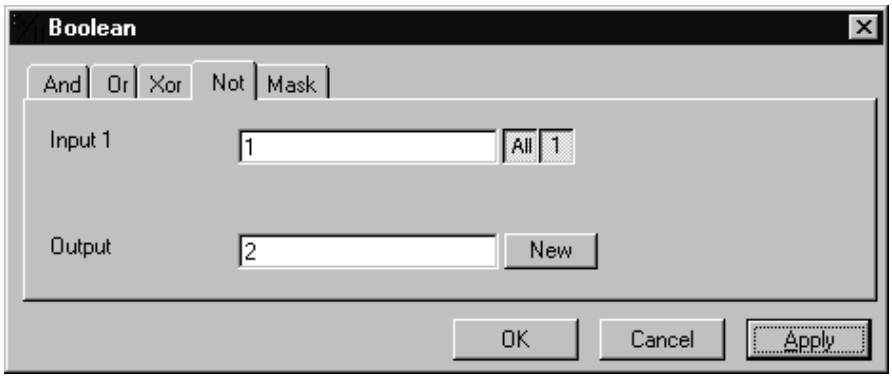


Fig. 8-29

The **Not** tab sheet of the **Boolean** dialog window must be selected.

If **Input** is a multichannel sequence any number or combination can be selected.

Parameters:

Input	Input image sequence
Output	Resulting image sequence

Boolean - Mask

This function masks a grey value image sequence.

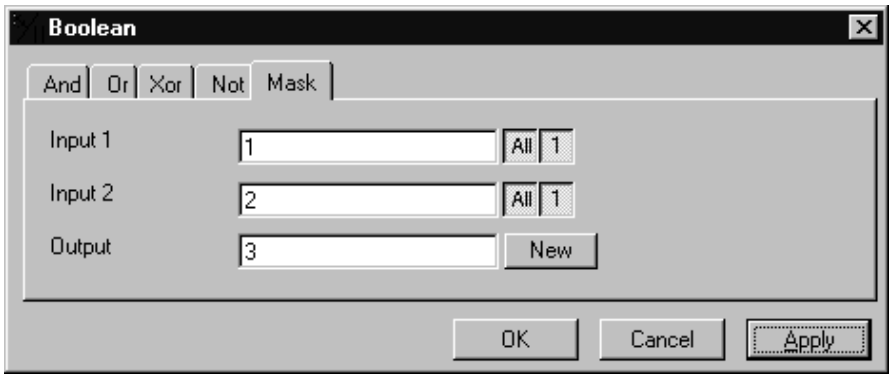


Fig. 8-30

The **Mask** tab sheet of the **Boolean** dialog window must be selected.

This function modifies the **Output** image sequence depending on the mask image sequence used.

If the grey value in **Input 2** is higher than 0, then the voxel values are copied from **Input 1** to the image sequence **Output**. If the grey value of the voxel is 0, then the voxel value of the **Output** image sequence is taken over.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 2** must be 1 or the same as for **Input 2**. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Scrap

This function deletes or selects objects in a specified size range.

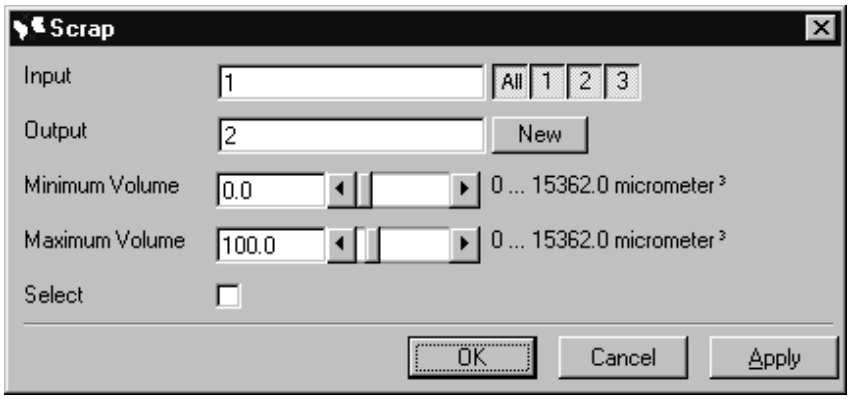


Fig. 8-31

The operation deletes or selects objects on the basis of their total volume in voxels. Objects with a volume within the range **MinVolume** to **MaxVolume** are effected.

To delete objects outside the range, the parameter **Select** must be active. If the parameter is not activated objects outside the defined volume range are deleted.

Parameters:

Input	Input image sequence
Output	Output image sequence
MinVolume	Minimum object size
MaxVolume	Maximum object size
Select	0 - Select the objects outside the size range 1 - Select the regions within the size range

Fill Holes

This function fills holes in all objects.

Fig. 8-32

All holes in objects are filled by this operation. Holes are structures, which have a grey value of 0 and are surrounded completely by voxels with a grey value not equal to 0. It is assumed that regions outside the image are black. Holes, which touch the image border, are retained.

Parameters:

Input	Input image sequence
Output	Output image sequence

8.3.4 Functions in the View Menu

Render - Surface

This function displays an image sequence according to the **gradient shading method**.

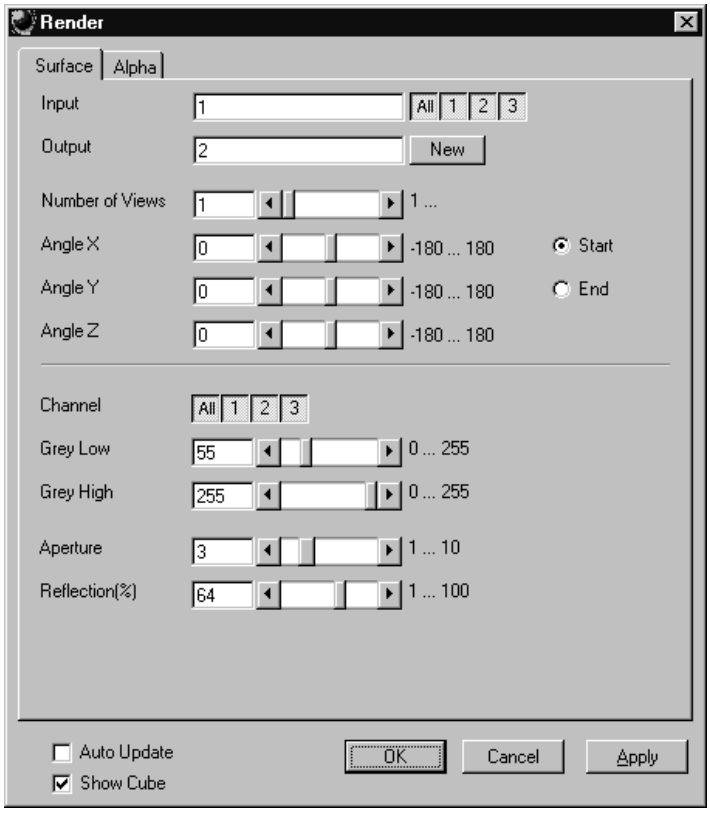


Fig. 8-33

The **Surface** tab sheet of the **Render** dialog window must be selected.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles. The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the thresholds for the channels independently is useful if the grey value boundaries of the objects differ too much in the different channels. The thresholds **Grey Low** and **Grey High** define the grey value range of the objects.

The parameter **Aperture** is a measure of the size of the highlights. Small values generate large highlights. Large values generate small highlights (similar to a spot).

Use the parameter **Reflection** to control the ratio of diffuse and reflective brightness components, i.e., the overall basic brightness compared with the highlights. When the value of **Reflection** is low, the highlights predominate; when the values are high, the region appears to be uniformly illuminated and the highlights are not so pronounced. When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

This method can be applied, if the structures in the **Input** sequence can be segmented by grey value thresholding. Because the gradient is calculated for every pixel, the **Output** appears in very fine detail.

Noisy **Input** sequences must be smoothed (function **Smooth**) before rendering, otherwise the surface appears rough.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Grey Low	Low grey value threshold of the region to be displayed
Grey High	High grey value threshold of the region to be displayed
Aperture	Measure of the extent of the highlights
Reflection	Weight of the defuse brightness components in comparison to the highlights
Auto Update	0 - Function execution is performed on **OK** or **Apply** 1 - Function execution for the current angle is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the **Display window**

Render - Surface: Method Description

This method displays the surface of structures in the **Input** sequence shaded as if a light illuminated it. The position of the light is behind the view point with parallel rays in the direction of the sequence.

The input sequence is segmented into object and background by grey value thresholding: object voxels are within the grey value range **Grey Low** to **Grey High**.

Each **Output** pixel corresponds to a point at the surface at which the ray in view direction through the **Output** pixels hits the surface. All rays are parallel.

The surface normal required for shading in this gradient renderer is the grey value gradient in the **Input** volume at the surface voxel position. It is not the geometric surface normal. The grey value gradient is determined from the grey values in a 3x3x3 cube around the surface voxel by averaging e.g. the x-gradient in y- and z-direction [4].

There is no depth cueing (far objects would appear darker).

The illumination model is a Phong model [1] (surface normal is determined for each **Output** pixel) with diffuse reflection and specular reflection. Diffuse reflection means that the surface reflects light with equal intensity in all directions. The brightness of a given surface patch depends not on the view-direction, but only on the angle between light and surface normal. Specular reflection is observed on shiny surfaces as a highlight. The light is reflected as from a mirror. The maximum intensity is observed when the view direction is the one of the mirrored light direction.

Render - Alpha

This function displays an image sequence according to the **alpha rendering method**.

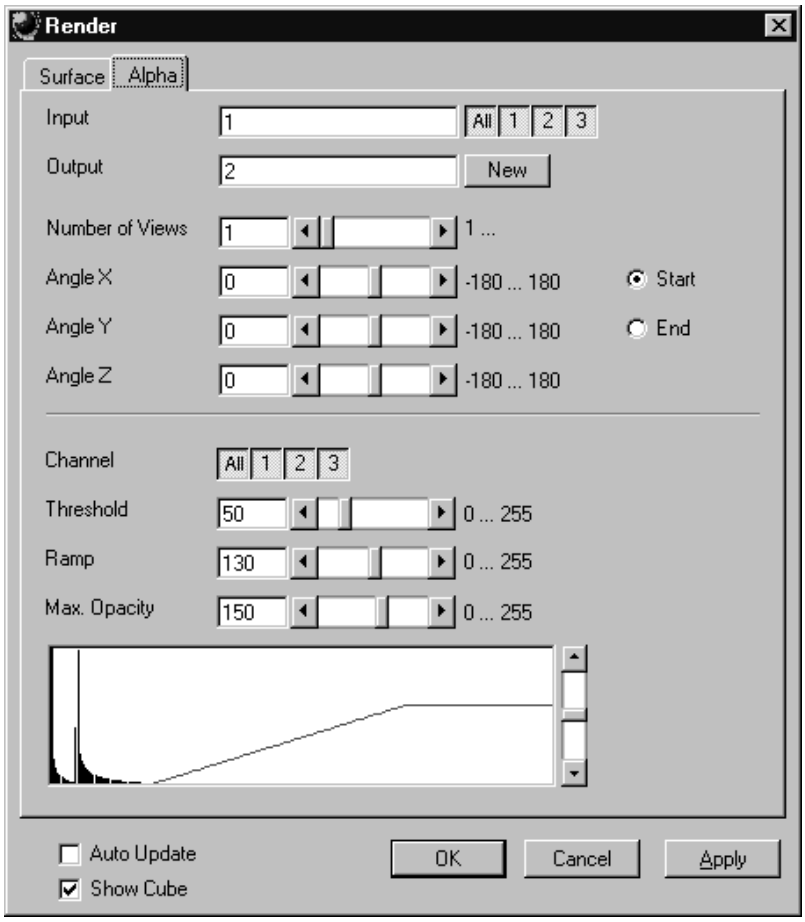


Fig. 8-34

The **Alpha** tab sheet of the **Render** dialog window must be selected.

One or more reconstructions of the input image sequence are computed according to the alpha rendering method. This type of reconstruction should be used if there is no possibility to segment the structures in the image sequence and also if the objective is to make deeply layered structures visible.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles.

The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the opacity for the channels independently is useful when the brightness and contrast of the channels differ too much. **Threshold** defines the range with no opacity. It is completely transparent. The range starts at grey value 0.

The length of slope is defined by **Ramp**. The maximum opacity value is set with the parameter **Max. Opacity**. This range ends at the maximum grey value. The **Opacity Table** shows the grey value histogram of Input with the opacity definition as a red line.

When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

1. This method can be applied, if the structures in the Input sequence are unsharp so that objects are poorly defined by their grey value.
2. In this case, the Opacity Table is defined as a ramp. Low grey values have weight 0 to suppress the background voxels. The opacity rises with increasing grey values, depending on the parameter Ramp. The value of Max. Opacity defines the weight of the high grey values. High grey values above a threshold have weight 255 to show the "object" voxels unsuppressed. Of course a smooth step can be used.
3. The result is a display with inside structures shining through. A 3D impression can be obtained by rendering with several view directions.
4. In contrast to this, a voxel renderer like the gradient renderer would display only the surface of objects that are defined by grey value-thresholds. This surface would appear shaded as if illuminated by a light.
5. The method can also be applied to visualize pronounced structures within other enclosing structures, if the structures have different grey value ranges.
6. In this case, the Opacity Table is defined as a step. Low grey values (background) have weight 0. High grey values (inside structures) have maximum weight.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Threshold	Grey value where the opacity starts rising
Ramp	Length of the opacity slope
Max. Opacity	Maximum opacity value
Opacity Table	Maximum opacity value
Auto Update	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the Display window

Render - Alpha: Method Description

Each **Output** pixel is a weighted sum of the **Input** voxels along a ray in view direction through the **Input** sequence. Each **Input** voxel has an opacity value, dependent only on its grey value. The opacity values are defined by the parameters **Threshold**, **Ramp**, and **Max. Opacity**.

Accumulation of pixels proceeds along the ray from back to front, i.e. from far pixels to near pixels. If a new pixel is added, it increases the result intensity by its grey value weighted by the opacity value, and attenuates the previously accumulated intensity according to the opacity value. Full intensity stops accumulation.

This calculation must be repeated for each pixel of the ray to generate one **Output** pixel. Then for each **Output** pixel to produce a 2D **Output** image for the selected view-angle. Then for each view-angle to produce an output sequence for **Number of Views** different view angles.

Render - References

[1] J.D. Foley,A.van Dam, S. K. Feiner, J.F.Hughes, Computer Graphics: Principles and Practice, Addison Wesley, Reading, MA, 1990.

[2] M. Levoy, Display of Surfaces from Volume Data, IEEE Computer Graphics & Applications, May 1988, 29-37.

[3] J. Ylä-Jääski, F.Klein, O. Kübler, Fast Direct Display of Volume Data for Medical Diagnosis, VGIP:Graphical Models and Image Processing 53,1991,7-18.

[4] K.H. Höhne, R. Bernstein, Shading 3D-Images from CT Using Gray-Level Gradients, IEEE Transactions on Medical Imaging, 5, 1986, 45-47.

[5] D.Gordon, R.A. Reynolds, Image Space Shading of 3-Dimensional Objects, CVGIP 29, 1985, 361-376.

8.3.5 Functions in the Measurement Menu

Measurement Concept

Measurement is based on regions (objects) in three-dimensional space. Segmenting an image sequence generates these. The image segmentation process produces a mask image that defines the region.

A region is a group of voxels that touch at the surfaces or at the edges, but not at the corners (18 voxel neighborhood).

This is illustrated by the following example. The voxels marked black in sequential image Z-1, Z, Z+1 all belong to the same region as the grey central voxel in sequential image Z. The volume view shows the neighborhood interrelationships as a 3D projection.

Sequential image: Volume view:

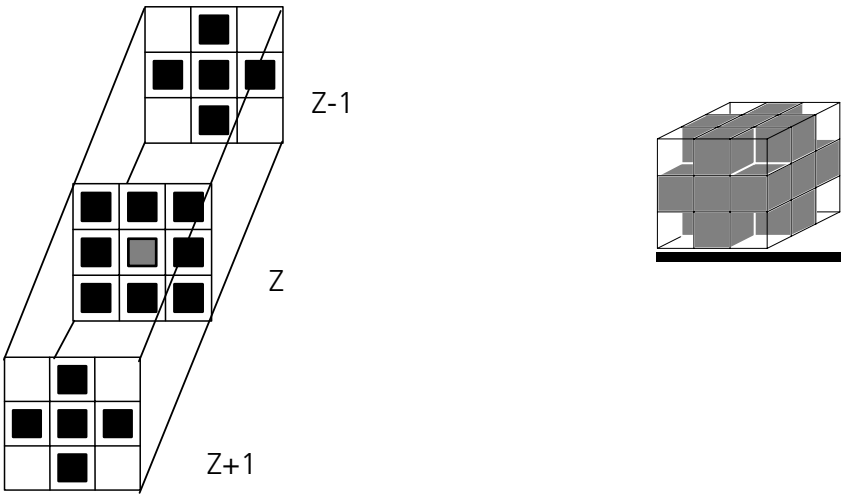


Fig. 8-35

Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition:	- Automatically from the mask image
Region validation check depends on:	- Minimum volume - Measurement condition
Feature calculation depends on	- Shape of the region - Densitometric value distribution of the region - Feature parameters

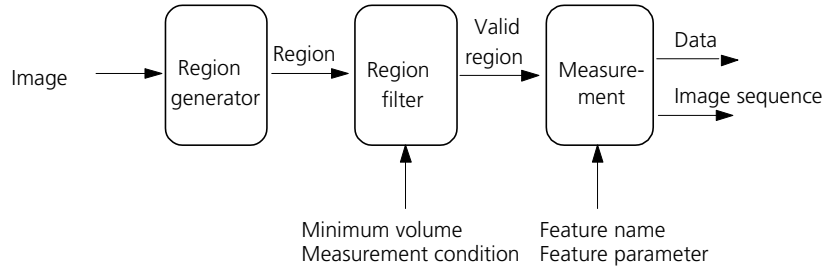


Fig. 8-36

All regions found are checked according to certain conditions. The voxel volume of each region must be equal to or greater than **MinVolume**. The measurement condition must be fulfilled. Only those regions that meet all the conditions are valid for the measurement. The region can be measured or labeled. Measurement is a process that produces data. Labeling is a process that generates an image volume.

Automatic Object Measurement – Object Features

A measurement feature describes a region characterized by a number (e.g. volume, area or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

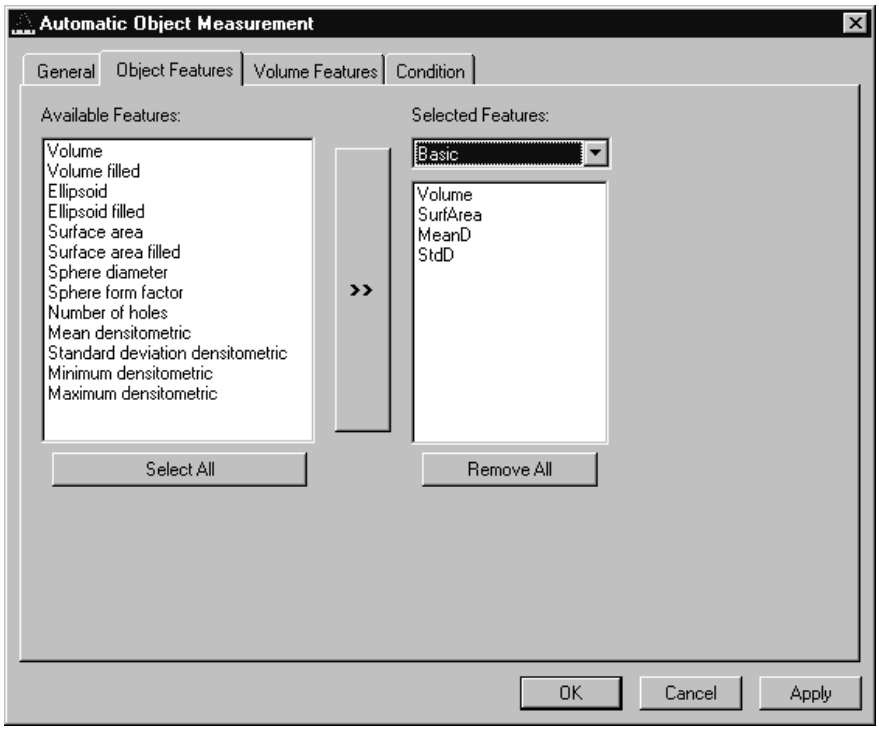


Fig. 8-37

The scalings and units are taken automatically from the assigned sequence.

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

The following sections describe all measurement features which are defined in the system.

Object Features (geometric)

If **Object Features** are selected, one set of measurement data is calculated for each object.

Group Name	Name	Description
Volume	Volume	Volume of the object.
Volume Filled	VolumeF	Volume of the filled object.
Ellipsoid	EllipseA	Length of the main axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseB	Length of the middle axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseC	Length of the minor axis of the ellipsoid with the same geometrical moment of inertia as the object.
Ellipsoid filled	EllipseAF	Length of the main axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseBF	Length of the middle axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseCF	Length of the minor axis of the ellipse with the same geometric moment of inertia as the filled object.
Surface Area	SurfArea	Surface area of the object.
Surface Area Filled	SurfAreaF	Surface area of the filled object.
Sphere Diameter	Dsphere	Diameter of the sphere with the same volume. $\sqrt{6 * VOLUMEF / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \frac{VOLUMEF}{\sqrt{SURFAREAF^3}}$
Number of Holes	Nparts	Number of holes within an object.

Object Features (densitometric)

Group Name	Name	Description
Mean Densitometric	MeanD	Densitometric mean value of an object.
Standard Deviation Densitometric	StdD	Standard deviation of the densitometric values of an object.
Minimum Densitometric	MinD	Minimum grey value of an object.
Maximum Densitometric	MaxD	Maximum grey value of an object.

Automatic Object Measurement - Volume Features

A measurement feature describes a region characterized by a number (e.g. volume, area, or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

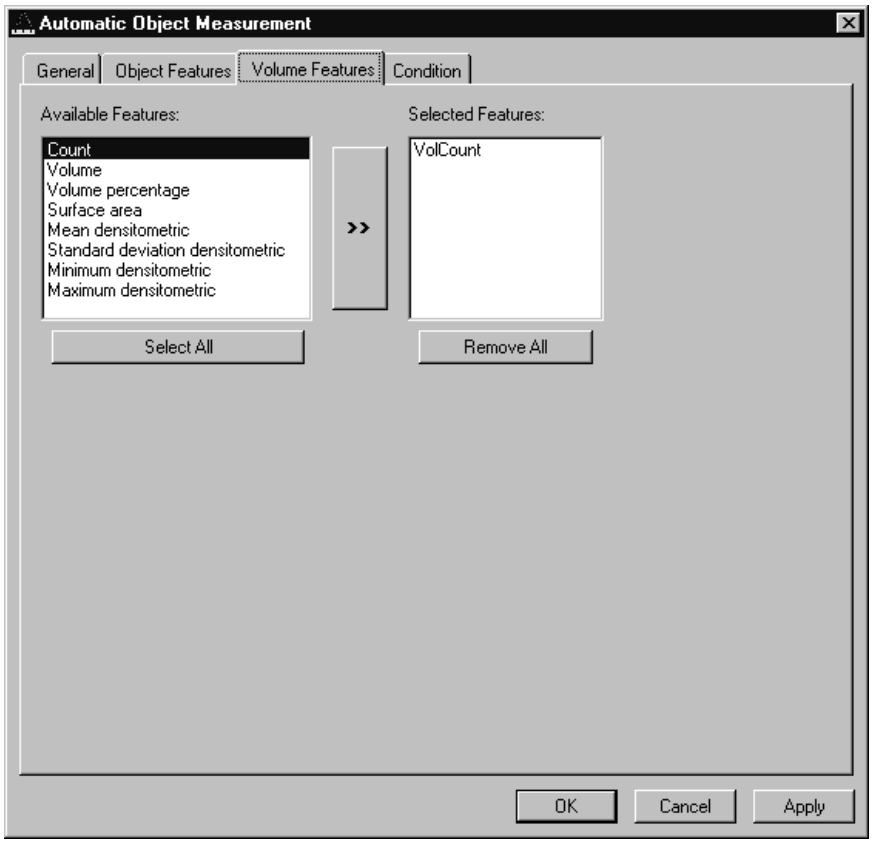


Fig. 8-38

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

Volume Features (geometric)

The volume-related measurement generates one measured value per image sequence. The following table contains the predefined volume characteristics.

Group Name	Name	Description
Count	VolCount	Number of regions measured.
Volume	VolVolume	Total volume of all regions.
Volume Percentage	VolVolumeP	Total volume of all regions, in relation to the volume of the image sequence.

Volume Features (densitometric)

Group Name	Name	Description
Surface Area	VolSurfArea	Total surface area of all regions.
Mean Densitometric	VolMeanD	Mean grey value of all regions.
Standard Deviation Densitometric	VolStdD	Grey value standard deviation of all regions.
Minimum Densitometric	VolMinD	Minimum grey value in the image sequence.
Maximum Densitometric	VolMaxD	Maximum grey value in the image sequence.

Automatic Object Measurement - Condition

The measurement conditions are used to limit the objects to be evaluated (e.g. only objects with defined minimum value). All objects are tested against the defined conditions. If the conditions are fulfilled the feature values are written to the data table.

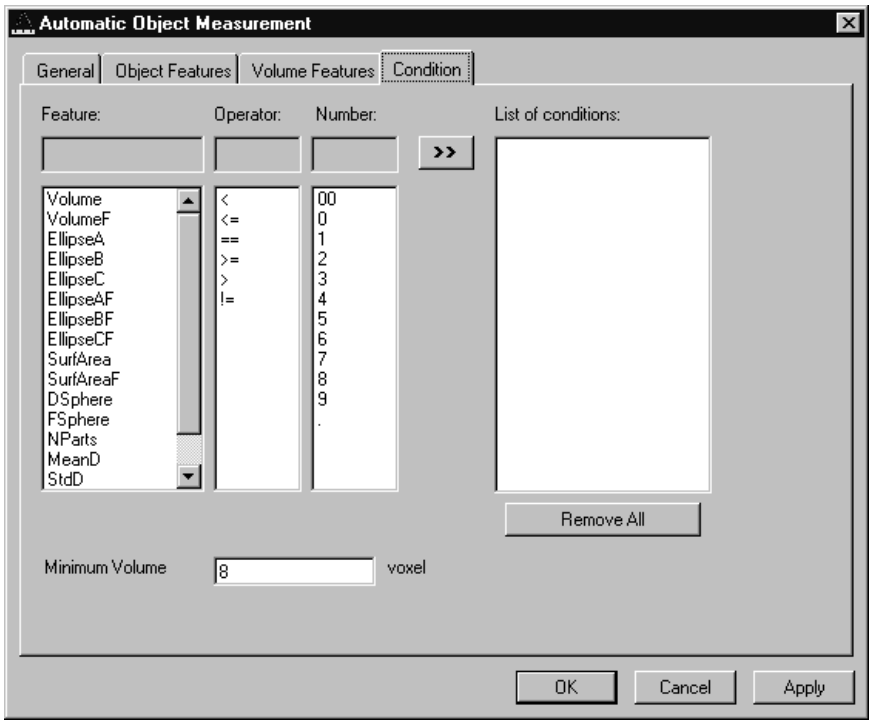


Fig. 8-39

To define the following parameter select the **Condition** tab sheet of the **Automatic Object Measurement** dialog window.

The list on the very left at the dialog shows all the measurement **Features**. The second list provides the comparison **Operators** and the next **Numbers** to define a value. This gives the possibility to compose an expression to test a feature value against a constant value. The fields above the lists will show the composed (selected) string. Clicking on the desired list entry does the selection. The button with the „>>„ characters adds this string to the **List of Conditions**. All lines of the **List of conditions** are combined with the AND expression automatically. To remove a condition line double-click on it.

The parameter **Minimum Volume** defines the minimum voxel volume for the measurement. This is an easy way to eliminate very small regions caused by noisy sequences and segmentation process.

The button **Remove All** will clear the list of defined conditions.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Feature	List of available object features
Operator	List of available condition operators
Number	List of numbers to compose the value
List of conditions	Defined condition list
Remove All	Remove all entries from the **List of conditions**
Minimum Volume	Minimum object volume in voxel

Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.

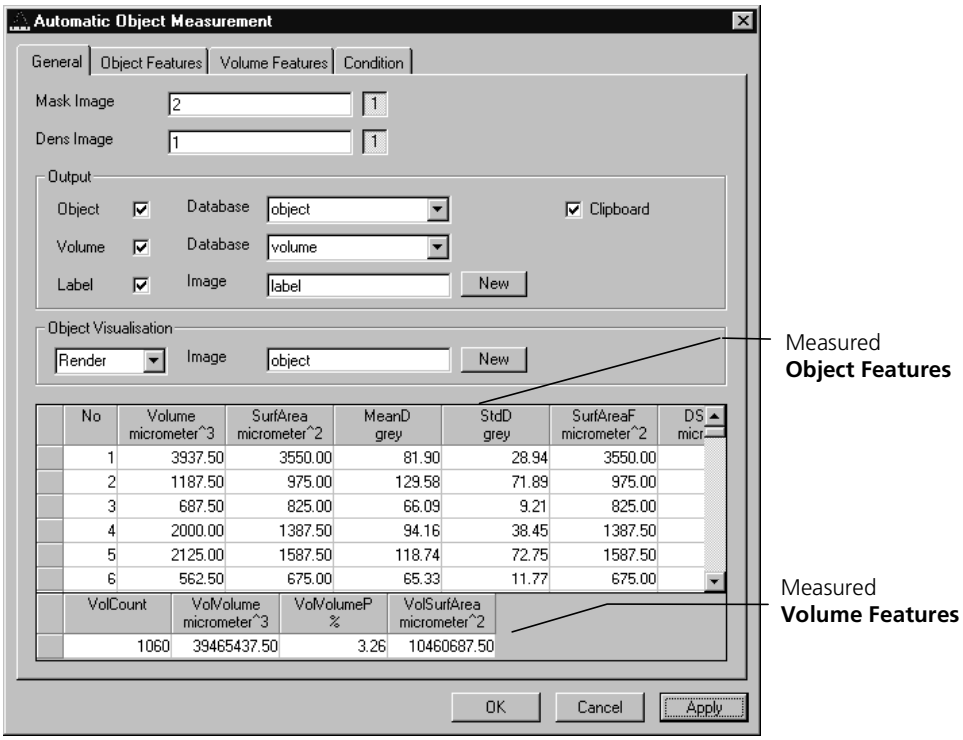


Fig. 8-40

The regions must be defined by an image sequence **Mask Image** (the objects must be separated from one another by black voxels with the grey value 0). This sequence is generated with the function **Segment**. If it is a multichannel sequence a single channel has to be chosen.

The image **Dens Image** is needed for the measurement of the densitometric features. Image sequence properties like scaling and unit are taken from **Dens Image**. A single channel of this sequence (if it is multichannel) must be selected with the buttons to the right of the parameter.

The measurement results can be stored to database files. These files are tab delimited ASCII files which can be easily imported to major Windows programs like text processing or spreat sheet application. Writing database files are independently supported for object and volume features. Activating the corresponding check boxes enables it. The name of the database is defined with the field **Database**. The files will be located in the subdirectory DATA of the main installation directory. The filename extension TXT will be added automatically.

If the check box **Label** is activated a single channel sequence will be generated. It contains all the measured objects, each object is coloured homogeneous but in different colours. To copy all measurement values to the clipboard activate the check box **Clipboard**.

A single object of interest can be visualized. Clicking on a specific row in the data grid chooses the object. By selecting a row in the data grid a new image is created with the object of interest visualized. The visualization depends on the settings in the **Object Visualisation** field. If **Render** is chosen, the object of interest is displayed with the **Surface Rendering** method. If **Mask** is chosen, the object is labelled in a pseudo colour in a new image stack.

Parameters:

Mask Image	Single channel mask image sequence that defines the objects
Dens Image	Image sequence for densitometric measurement and property source
Object	Stores measurement values of objects, including database filename
Volume	Stores volume measurement values of objects, including database filename
Label	Generates an image sequence with all objects labelled in different pseudo colours
Clipboard	Measurement values are automatically written to the clipboard

CHAPTER 9 ANNEX

CONTENTS

9 ANNEX

9.1 Beampath Configuration Guide for Fluorescence Microscopy with the LSM 5

9.1.1 Optical elements in the Configuration Control window:

All wavelength values given in Nanometer [nm].

9.1.1.1 Main Dichroic Beam Splitter (HFT)

- A **HFT XXX[/YYY/...]** deflects the indicated laser lines onto the specimen and allows the emitted fluorescent light to pass through.
  Example: HFT 458/514, HFT UV/488/543/633 (deflects also UV excitation light)
- A **HFT KP XXX** (KP = Short Pass) is a special type of a main dichroic used for IR multi photon excitation. The **HFT KP 650**, deflects laser light with a wavelength longer as 650 nm onto the specimen and allows fluorescent emission light in the visible range below 650 nm to pass through.
  Example: HFT KP 650
- A **HFT KP XXX_YYY** is a combination of a HFT YYY and HFT KP XXX used for simultaneous IR multi photon and single photon excitation.
  Example: HFT KP 700_488.

9.1.1.2 Secondary Dichroic Beam Splitter (NFT)

- The **NFT XXX** is used to split the emitted light which will be guided into separate channels. Light with shorter wavelength than XXX nm is deflected, light with longer wavelength passes the NFT. A cascade of NFTs allows to distribute the emission light to more than two channels/detectors.
- The **NFT KP YYY** solits emission light the other way round: it transmits light shorter than YYY nm and deflects above YYY nm.

9.1.1.3 Emission Filters (EF)

- A **LP XXX** (Long Pass) transmits emission light with wavelengths longer than the indicated threshold value XXX.
- A **KP XXX** (Short Pass) transmits emission light with wavelengths shorter than the indicated threshold value XXX.
- A **BP XXX-YYY** (Band Pass) transmits emission light within the indicated wavelength band.
- A **BP XXX/BB** has a transmission band for emission light with a center wavelength of XXX nm and a width of BB nm.
- The **BG 39** (Blue Green glass) blocks infrared excitation light by absorption.
- **BP ... IR** (Band Pass – Infra Red) is a band pass suitable for detection of IR excited dyes. It blocks the IR light.

9.1.1.4 Miscellaneous

- **Plates** do transmit light 100%. They are used for a correct beam guidance and will be set automatically.
- **Mirrors** do deflect 100% over the whole spectral range and can be used to guide the emission light to selected detectors.

9.1.2 Setup of Single Tracks Using Single Detectors

- Switch on the suitable lasers for excitation of the dyes in the specimen. For the UV laser and the Argon laser set the tube current of the laser to a value of app. 50% (**Excitation, Laser, Output [%]**). Example: for Alexa 488 and CY 3 switch on **Argon** (blue excitation) and **HeNe1** (green excitation).
- Activate the proper laser lines in the **Line Active** check box, set **Transmission [%]** for each active line.
  Example: Select 488 to 5% and 543 to 100%
- Select a main dichroic beamsplitter (HFT) which deflects the selected laser lines to the specimen.
  Example: HFT 488/543

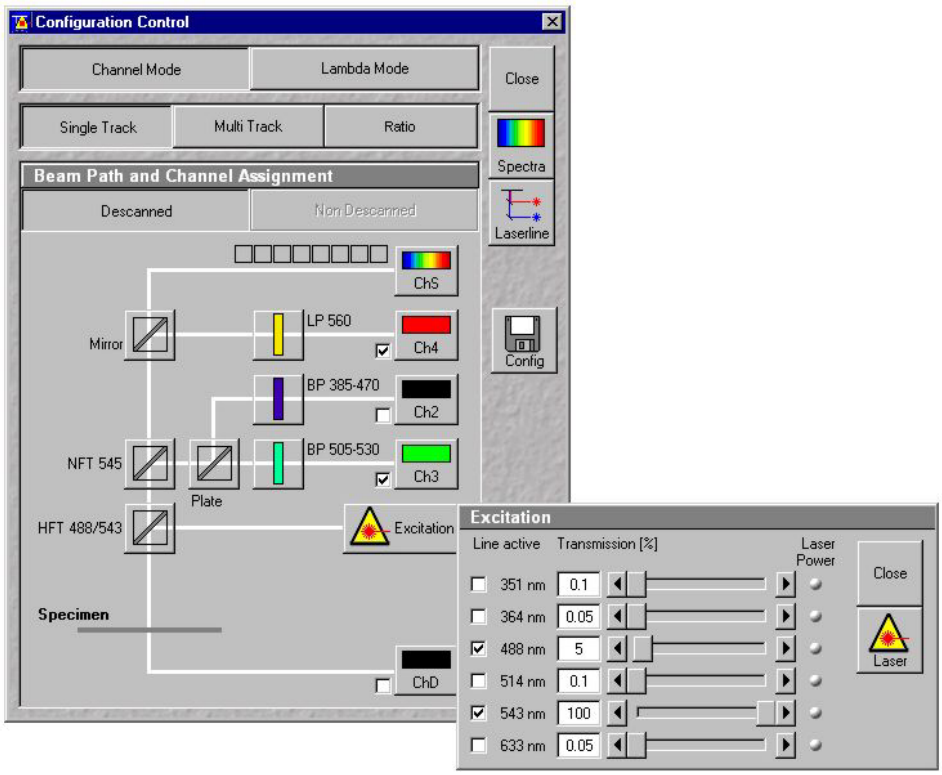


Fig. 9-1 Configuration Control window and Excitation panel

- Check the available emission filters (EM 1- 4) for transmissionof fluorescent light from the specimen, in order to identify the channels for detection.
  Example: BP 505-530 in channel Ch 3 for acquisition of green emission and LP 560 in channel Ch 1 for acquisitionof red emission

- Use the secondary beam splitters (NFT 1/2/3) to split and guide the emitted fluorescent light to the detectors (PMTs) of the selected channels (see above).
  Example: an NFT 545 in NFT 1 position will allow light longer then 545nm to pass to Ch 1 and deflects light shorter then 545 towards channel Ch 3 (if available) and channel Ch 2.
  Note: on switching from 'None' to a beam splitter in the NFT 1 position the system will automatically set 'Plate' in NFT 3 position.
- Select the proper emission filters in front of the channels and activate channels.
  Example: select LP 560 in front of Ch 1, and BP 505-530 in front of Ch 3].
- Make sure that the active detection bands do not include any of the active laser lines
  Example: do not use a BP 505-550 for detection of green emission when using the 543 nm line for green excitation

Additional hints:

- Do not forget to turn on detectors.
- Assign appropriate colors to these activated channels. Example: Ch 1 - red (for Cy3 emission, Ch 3 - green (for Alexa488 emission)
- The Spectra dialog is a big help for checking if the configuration of the beam path was successful. It shows activated laser lines and for each channel the emission range that can be "seen" by the detector indicated by the corresponding channel color. A gray bar indicates an emission range that is guided into a channel, but the detector is not turned on.
- When simultaneously detecting more than one fluorescent dye use channel Ch 1 for detection of the emission with long wavelength, then channels Ch 4 and Ch 3 (if available) for medium wavelengths and channel Ch 2 for short wavelengths.
- Use NFT 3 for separating emission into channels Ch 1 and Ch 4 and NFT 2 for separating emission into channels Ch 2 and Ch 3

9.1.3 Multitracking Configuration

Multitracking is the method of choice for multi fluorescence imaging. It has the advantage to avoid artifacts based on emission crosstalk that occurs when using simultaneous excitation and detection. Laser lines are switched very fast and channels recorded quasi-simultaneously.

The configuration of Multiple Tracks follows the same rules described above for single track configuration. The main difference is that each track is configured to excite and detected only one fluorescent dye to prevent cross talking (or two dyes with non overlapping emission spectra).

- Create a single track for both, Alexa 488 and CY 3 detection separately (see above).
- Open the Multi Track configuration window. The system displays the Single Track setup as track one.
- Add a new track.
- Click on track one, deactivate Ch 1 (red emission detection) and switch off the green laser line (543nm) in the **Line Active** check box in the **Excitation** control window.
- Click on track two, deactivate Ch 2 (green emission detection) and switch the blue laser line (488) off.
- To extend the detection band for the green light it is now reasonable to use the BP 505-550 instead of BP 505-530 in track one. This is now possible since the green laser line is turned off during detection of the green fluorescence emission.
- Use the Spectra window to check the proper settings for each individual track as described above.

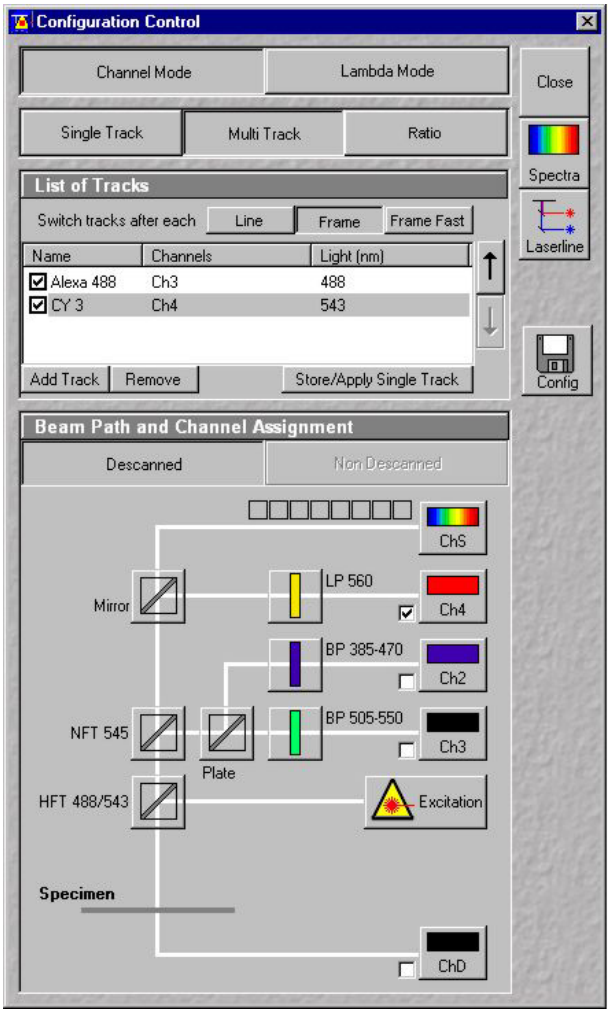


Fig. 9-2 Configuration Control window

Line and Frame Mode of Multitracking

- Settings can be used for **Line** or **Frame** wise Multitracking.
- In Line mode the lines are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. Preferred for living samples with moving objects. Acquisition time can be reduced using bidirectional scan mode.
- In Frame mode whole frames are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. This mode can be advantageous for dyes that tend to bleach and need time to recover.
- There are parameters that can be changed quickly in a line wise: Amplifier Gain, Amplifier Offset, Laser Line Attenuation,
- Any other changes of track settings of the selected tracks, e. g. different filters, dichroics or Detector Gain settings, need a bit more time to be changed, and require Frame mode.
- There is a fast Frame mode, that requires identical settings of these parameters.
- In our example it is now possible to use the BP 505-550 also in track two. There is no function of this BP in track two, but it guarantees equal settings in both tracks/channels, which now allows line wise Multitracking.

9.1.4 Beam path Configuration for Multi Photon Excitation

- The beam path configuration for multi photon excitation follows the same rules as described for a single and multi track configuration (see above).
- Use a KP 650 or KP 700 as main dichroic beam splitter to deflect the IR excitation light (700-900 nm) onto the specimen.
- On detection side, set always a BG 39 in the beam path or use a IR suitable band pass filters (BP XXX-YYY IR) to prevent a bleed through of IR excitation light to the detector.

9.2 Recommendations for excitation laser lines and emission filters of dyes

Dye	Laser line/HFT	Emission/EM
DAPI	364 or 405	> 385/420, max. at 461
EBFP	364 or 405	> 385/420, max. at 447
Hoechst	364 or 405	> 385/420, max. at 440
Fluoro-Gold	405 or 458	> 420/475, max. at 536
ECFP	405 or 458	> 420/475, max. at 501
Lucifer Yellow	458	> 475, max. at 536
EGFP	477 or 488	> 505, max. at 507/516
FM 1-43™	477 or 488	> 505, max. at 598
Alexa Fluor 488™	488	> 505, max. at 520
Calcium Green	488	> 505, max. at 531
Cy2™	488	> 505, max. at 508
DiO (DiOC18(3))	488	> 505, max. at 508
Fluo-3	488	> 505, max. at 520
Fluorescein (FITC)	488	> 505, max. at 520
Cy3™	514	> 530, max. at 566
EYFP	514	> 530, max. at 535
Oregon Green	514	> 530, max. at 535
SYTOX Green	514	> 530, max. at 536
FM 4-46	514 or 543	> 560, max. at 640
Alexa Fluor 546™	543	> 560, max. at 572
Calcium Orange	543	> 560, max. at 575
DiI (DiIC18(3))	543	> 560, max. at 565
DsRed	543	> 560, max. at 583
Tetramethylrhodamine (TRITC)	543	> 560, max. at 576
Rhodamine B	543	> 560/585, max. at 625
Texas Red™	543 or 568	> 560/585, max. at 620
Alexa Fluor 633™	633	> 650, max. at 654
Cy5™	633	> 650, max. at 666

Here you can note your specific combinations:

Dyes	Laser/HFT	EM1	NFT	EM2

Example:

Dyes	Laser/HFT	EM1	NFT	EM2
FITC/Cy3	488/543	BP 505-530	545	LP 560

9.3 Configurations Overview

9.3.1 LSM 510 META

Configuration	3 META	13 META	15 META	18 META
Main beam splitter / available laser lines	**NT 80/20 UV/488/543/633** **477/543** **488/543** **458/514** **514/633** **458** **488**	**NT 80/20 UV/488/543/633** **KP 700/488** **KP 700/543** **458/514** **458** **488** **KP 650**	**NT 80/20 UV/488/543/633** **UV/488** **458/514** **477/543** **458** **UV (375)** **488**	**NT 80/20 UV/488/543/633** **405/488/543** **405/514** **458/514** **488/543** **458** **488**
Secondary beam	**none**	**none**	**none**	**none**

splitter 1	**mirror** **545** **570** **635 VIS** **none** **KP 545** **plate**	**mirror** **490** **515** **545** **635 VIS** **KP 545** **plate**	**mirror** **490** **515** **545** **635 VIS** **KP 545** **plate**	**mirror** **490** **515** **545** **635 VIS** **KP 545** **plate**
Secondary beam splitter 2	**mirror** **515** **545** **plate**	**mirror** **490** **545** **BG39**	**mirror** **490** **545** **plate**	**mirror** **490** **515** **545**
Secondary beam splitter 3	**none** **plate** **none** **mirror**	**none** **plate** **BG39** **mirror**	**none** **plate** **none** **mirror**	**none** **plate** **none** **mirror**

9.3.2 LSM 510 Basic Configurations

Configuration	1	2	3	4
Main beam splitter / available laser lines	NT 80/20 458/514 458/543 488/543 458 488 514 477/543	NT 80/20 UV/488/543/633 458/514 458/543 458 488/548 514/633 488	NT 80/20 UV/488/543/633 477/543 488/543 458/514 458 514/633 488	NT 80/20 UV/488/568/633 488/568 488 568 633 none none
Secondary beam splitter 1	none mirror 515 545 none none* none plate	none mirror 515 545 635 VIS none* none plate	none mirror 545 570 635 VIS none* none plate	none mirror 570 635 VIS none* none none plate
Secondary beam splitter 2	mirror	mirror	mirror 515 545 plate	mirror 570 plate none
Secondary beam splitter 3	none plate none mirror	none plate none mirror	none plate none mirror	none plate none mirror

***) Position used for beam splitter NFT 610 of SNARF filter sets**

****) Position used for beam splitter NFT 450 of Indo-1 filter sets**

9.3.3 LSM 510 Upgraded Configurations - UV

Configuration	5	9	11	15	16	18
Main beam splitter / available laser lines	NT 80/20 UV/488 UV/543 458/514 488/543 UV (375) 477/543 458	NT 80/20 UV/488/543/633 UV/488 458/514 488/543 UV (375) 477/543 458	NT 80/20 UV/488/543/633 UV/488 UV/568 488/568 UV (375) 488 568	NT 80/20 UV/488/543/633 UV/488 458/514 477/543 458 UV (375) 488	NT 80/20 UV/488/543/633 UV/488 458/514 477/543 488/543 UV (375) 458	NT 80/20 UV/488/543/633 405/488/543 405/514 458/514 488/543 458 488
Secondary beam splitter 1	none mirror 490 515 545 none** none* plate	none mirror 490 515 545 570 none plate	none mirror 490 545 570 635 VIS plate none	none mirror 490 515 545 635 VIS plate none*	none mirror 490 515 545 635 VIS plate none*	none mirror 490 515 545 635 VIS none* plate
Secondary beam splitter 2	mirror	Mirror 490 none* plate**	mirror 490 none* plate**	mirror 490 545 plate**	mirror 490 545 plate**	mirror 490 515 545
Secondary beam splitter 3	none plate none mirror	None Plate None Mirror	none plate 635 VIS mirror	none plate none mirror	none plate 635 ViS mirror	none plate none mirror

***) Position used for beam splitter NFT 610 of SNARF filter sets**

****) Position used for beam splitter NFT 450 of Indo-1 filter sets**

9.3.4 LSM 510 Upgraded Configurations - NLO

Configuration	12	13
Main beam splitter / available laser lines	**NT 80/20** **KP 700/488** **KP 700/514** **KP 700/543** **458/514** **488/543** **488** **KP 650**	**NT 80/20** **UV/488/543/633** **KP 700/488** **KP 700/543** **458/514** **458** **488** **KP 650**
Secondary beam splitter 1	**none** **mirror** **490** **515** **545** **none** **none*/**** **plate**	**None** **mirror** **490** **515** **545** **635 VIS** **none*/**** **plate**
Secondary beam splitter 2	**mirror**	**mirror** **490** **545** **BG39**
Secondary beam splitter 3	**none** **plate** **BG39** **mirror**	**none** **plate** **BG39** **mirror**

***) Position used for beam splitter NFT 610 of SNARF filter sets**

****) Position used for beam splitter NFT 450 of Indo-1 filter sets**

9.4 Filter Change in the Detection Beam Path of Channels 1 and 2

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter wheels of channels 1 (upper cover cap) and 2 (lower cover cap on the right side) of the Scanning Module have a change position in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment. The filters can be rotated in their cells, and with the light path being eccentric relative to the filter center, the best transmission area of the filter for the respective wavelength or pass range can be found by rotating the filter. This is very important for the investigation of specimens of low emission.

Filter change

- By software control, move filter wheel (9-3/**5**) to the change position.
- Pull cover cap (9-3/**1**) off the Scanning Module.
- Use the filter tool (9-3/**2**) to pull the filter mount (9-3/**4**) with the filter (9-3/**3**) out of the guide well.
- Change filter to suit the application.

The filter is rotatable in its mount, allowing adjustment for finding the best transmission area of the filter for the wavelength used.

- Enter the designation of this particular filter into the System Software database.

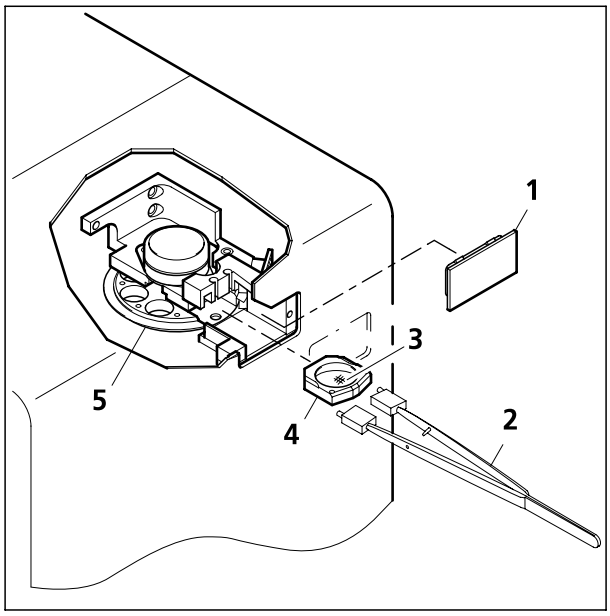


Fig. 9-3 Filter change

9.5 Detaching / Attaching the Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key

☞ The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. **No adjustment** is required after the change-over. Described below is the change-over from an Axioplan 2 to an Axiovert 200 M in baseport configuration.

⚠ **Before** the change-over, **shut down** the system as described in chapter 4 in order to avoid damage to the system and loss of data.

- Turn out both knurled-head screws (9-4/**1**) at the Scanning Module (9-4/**2**) fitted to the Axioplan 2.
- Turn out M3 hexagon socket screw (9-4/**3**) with the Allen key.
- Cautiously pull Scanning Module off the Axioplan 2 stand.
- Attach Scanning Module to the baseport of the Axiovert 200 M, minding the guide pins (9-4/**6**), and secure it with the M3 hexagon socket screw (9-4/**3**).
- Fasten Scanning Module to the baseport with two hexagon socket screws (9-4/**5**), using an offset Allen key.

☞ As the Scanning Module is heavy, weighing about 14 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (9-4/**4**) from the CAN-BUS and RS232 interface ports at the rear of the Axiovert, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS232) from the Axioplan 2, plug them into the Axiovert and secure them there.
- Switch the LSM-FCS on with the REMOTE CONTROL switch.
- Click on the **Stand select** icon to update the system database with the new database of the Axiovert 200 M microscope.
- Restart the LSM-FCS program.

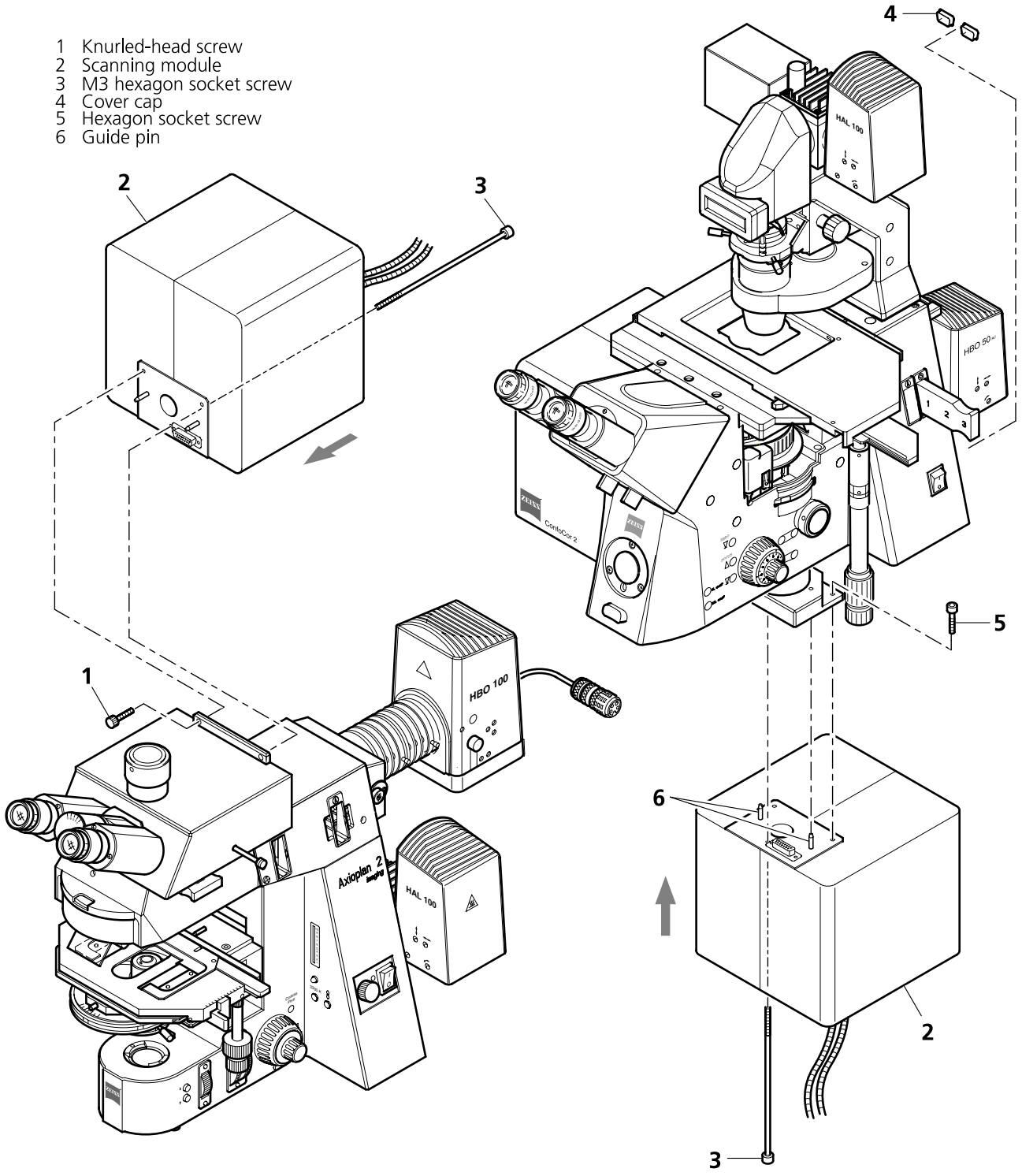


Fig. 9-4 Change-over of the Scanning Module

9.6 Hints on the Use of the HRZ 200 Fine Focusing Stage

9.6.1 General Description

The HRZ 200 fine focusing stage is a compact attachment for the Axiovert 200 M microscope stage, which allows the particularly fast and high-precision fine focusing of the object. The HRZ 200 permits fine focusing over a range of 200 µm, with the smallest step width being less than 10 nm, reproducibility better than 40 nm, and the maximum speed amounting to 10 Hz. The stage allows the use of specimens with a weight of less than 100 g.

The HRZ 200 is not used if manual coarse focusing is performed. To position the objective in relation to the optical Z-axis, the standard XY-microscope stage is used.

The HRZ 200 features a mount for standard object carriers of 76 mm x 26 mm x 1 mm and a milled-out receptacle for ∅ 36 mm x 1 mm Petri dishes.

9.6.2 Application Fields

– High-precision fine focusing and translation of the object along the optical axis.

– Fast and high-precision mounting of one-dimensional Z-line sections.

– Fast and high-precision mounting of two-dimensional R-Z-longitudinal sections.

– Fast and high-precision mounting of XY-Z-Stacks for the three-dimensional reconstruction of the object.

– Exact measurement of Point-Spread-Functions for deconvolution.

9.6.3 Additional Information on the Operation

The HRZ 200 fine-focusing stage is a high-precision, sensitive accessory for the LSM-FCS from Carl Zeiss and must therefore be treated carefully.

High mechanical stress, such as the use of specimens weighing more than 100 g or the application of pressure or knocks on the movable stage tongue, can result in damage and therefore in failure of the stage function.

To be able to fully utilize the outstanding precision attainable with the fine focusing stage, anything which could interfere with its operation, especially mechanical knocks and impact of the LSM-FCS components, should be avoided. We would recommend you to always use the actively vibration-damped Kinetics stage (available as accessory under the order number 1007 508 or 1007 512) as the base for the setup of LSM-FCS systems containing the HRZ 200 stage.

The specifications of the stage are obtained only after a heating phase of approx. 30 minutes. Furthermore, the installation conditions for the LSM-FCS system must be observed.

The maximum reproducibility (better than 40 nm) for moving to an absolute position in Z is achieved by always moving to the required position from below.

Fine focusing is performed mechanically via an inclined position of the stage tongue. Therefore, the lifting range Z at the location of the image field depends on the position of the HRZ in relation to the optical axis. This means: if the user shifts the object on the microscope stage to the right via the HRZ 200, the lift will be different from the one in the zero position of the stage (max. 200 µm) and also from the one after a shift of the stage to the left.

The HRZ has been developed to enable minute increments at a high precision. It is possible to have either a large travel range at a low precision or a low travel range at a high precision. The entire travel range of ± 100 µm can only be passed without intermittent "Leveling" if step width >1 µm is selected.

If the LSM-FCS system is equipped with a motorized scanning stage, this shift is read back to Δx and the lift is calibrated automatically if the zero position of the HRZ has been matched to the zero position of the scanning stage via an initialization run. For this, activate the **Stage** button of the **Acquire** toolbar. Then position the scanning stage in such a way that the optical axis of the microscope corresponds to the zero position of the HRZ, i.e. to the center of the specimen holder in the stage tongue. Then perform initialization by pressing the **HRZ Null** button. This step must be repeated after every new start of the system. Also see the notes on the operation of the motorized scanning stages.

If the system is equipped with a manual microscope stage, the user has the option of performing the calibration by entering the Δx shift in mm via the **Calibration** slider.

The shift is read off from the microscope stages. In the case of the manual Axiovert 200 stage, a scale is located on the right of the knob, where the 45 mm Δx shift relative to the zero position of the microscope stage can be read off. The Δx value is positive for both stages if shift from the zero position is made to the right and negative if the shift is made to the left.

On account of the inclined position of the stage tongue, the object is also shifted laterally during the fine focusing motion. This lateral shift is negligibly small if, as recommended by us, specimen carriers with thickness 1.0 mm are used exclusively. Otherwise, the marked lateral shift of the object during fine focusing can result in image distortion. For the same reason, Petri dishes without fixation ring must be used exclusively.

The nosepiece of the Axiovert stand is moved to the load position prior to switching off the LSM-FCS system and the HRZ 200 is then moved to the lowest position to avoid damage of the objective or object by a possible collision. The user must refocus after start-up of the system. Before an objective change in the Axiovert 200, the nosepiece and the microscope stage must be moved to the load position by the user, and then back to the work position to prevent the objectives from hitting the HRZ components. This is performed automatically if the objectives are changed menu-controlled via the relevant buttons of the LSM-FCS program.

The HRZ 200 for the Axiovert 200 M (000000-1013-186) can be attached to the following standard microscope stages:

mechanical stage 85 x 130 for Axiovert (451339-0000-000)

scanning stage DC 100 x 90 for Axiovert (451740-0000-000)

In the case of the last configuration, the object plane is shifted upwards so that KÖHLER illumination and classical transmitted-light microscopy will no longer be possible because the condenser cannot be moved sufficiently close to the object.

The user will not have to deal with any other restrictions.

9.7 Piezo Objective Focussing Device - MIPOS 3 SG

For upright stands Axioplan 2 imaging MOT, Axioskop 2 mot plus, Axioskop 2 FS MOT

Range: 80 µm

Minimum step size: 5 nm

Speed:

		Piezo objective focussing device	HRZ 200	Piezo / HRZ
Slices	**Step size [µm]**	**xz-lines / s**	**xz-lines / s**	
20	1	10	2.8	x 3.6
20	0.5	10	2.8	x 3.6
10	1	20	5.7	x 3.5

Objectives:

W0.8/M27; Diameter max. 29 mm => NO C-Apochromats 40x/63x

Modified Achroplan 40x / 0.8 W with reduced length to compensate for piezo height

Technical data:

part no. thread M25xo.75 ..... O-303-01
RMS (W0.8x1/36") O-304-01

motion ..... 100/80 µm
(typical value measured with -10 V to 150 V) (open loop/closed loop)

operating voltage.....-10 to 150 V

capacitance .....7.2 µF
(typical value for small electrical field strength) .....

resonant frequency..... 700 Hz
(without load / objective mass 140 g)

resolution open loop..... 0.13 nm
(measured with-103-18 amplifier)

stiffness.....1.4 N/µm

connector.....LEMOSA

cable length..... 1 m

weight..... 115 g

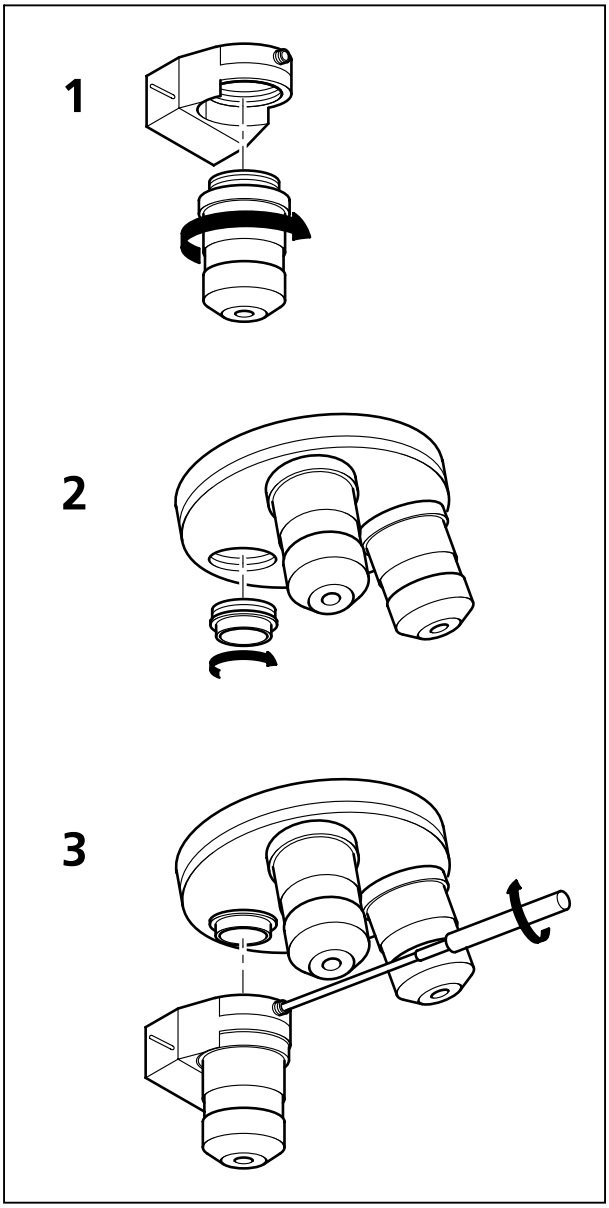


Fig. 9-5 Installation of the Piezo Objective Focusing Device

Installation:

- Screw in your microscope objective into Piezo Objective Focusing Device (see Fig. 9-5/**1**).
- Screw the thread-ring into your microscope (see Fig. 9-5/**2**).
- Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 9-5/**3**).

9.8 Specifications of Trigger-Interface LSM-FCS

Application:

With the LSM-FCS Release 2.8 you can control various actions externally using Trigger-In or force external devices to work at a defined time depending on an action using Trigger-Out during time series. These actions are: Scan-Start / Stop, Bleach, Change of Scan-Interval, end of a countdown or even a mouse-click on a button.

Interface:

Front plate Scanner-Interface (Scan-IF) inside

Electronic-Box (Scan-Control-Module) of LSM-FCS:

Connector 'User I/O', 26-pin shrunk SUB-D

Number:

4x Trigger-In, 4x Trigger-Out

Type/Voltage Range:

TTL (HCMOS), 0.0 - 5.0 V

Load:

In: 22 kOhm input impedance

Out: ± 4 mA

Trigger pulse description:

Level detection:

Low level:	0.0 - 1.0 V
High level:	3.0 - 5.0 V

Slew rate:

rising edge:	1 µs
falling edge:	1 µs

Pulse width (always positive pulses / high level):

Trigger-In:	≥ 20 ms	(Speed 10 - 5)
	≥ 31 ms	(Speed 4)
	≥ 62 ms	(Speed 3)
	≥ 123 ms	(Speed 2)
	≥ 246 ms	(Speed 1)
Trigger-Out:	ca. 100 ms	

Pulse frequency:

Trigger-In:	2x pulse width
Trigger-Out:	> pulse width

Valid edge:

Trigger-In:	Rising edge
Trigger-Out:	Falling edge

Caution:

Never apply more than 5 V or negative voltages to avoid any damage.

In and outputs are not galvanically decoupled.

Therefore proper measures for galvanic decoupling of external devices have to be taken (opto-coupler etc.).

Do not connect pins labeled ‘reserved’ (see table below). Otherwise, at least the interface can be damaged.

Pin assignment:

No.	Name	Direction	Description
1	Trig1O	Out	Trigger Output #1
2	Trig2O	Out	Trigger Output #2
3	Trig3O	Out	Trigger Output #3
4	Trig4O	Out	Trigger Output #4
5 ... 8	-	-	reserved
9	GND	-	Ground (0 V)
10	Trig1I	In	Trigger Input #1
11	Trig2I	In	Trigger Input #2
12	Trig3I	In	Trigger Input #3
13	Trig4I	In	Trigger Input #4
14 ... 17	-	-	reserved
18	GND	-	Ground (0 V)
19 ... 25	-	-	reserved
26	GND	-	Ground (0 V)

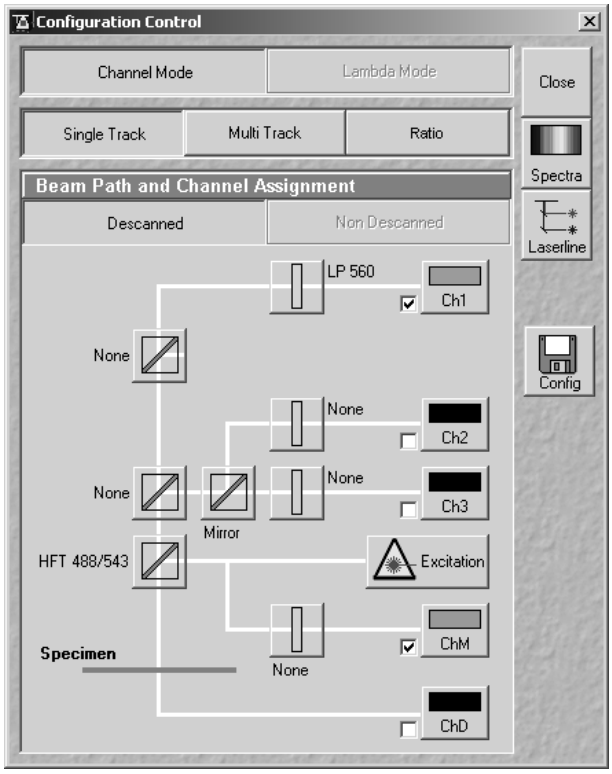


Fig. 9-6 Configuration Control window

9.9 Monitor Diode

The monitor diode is placed in the excitation ray path of the LSM-FCS behind the beam splitter combining the visible and the UV ray path and in front of the main beam splitter. Therefore it allows to check the laser input in terms of power and noise. With the attenuation filter wheel in front of the diode it is possible to attenuate the laser power reaching the diode. It is not possible to select one line out of a few excitation wavelengths to be detected by the diode.

Proceed as follows to activate the diode as a detector:

- Click on the corresponding button in the **Configuration Control** window of the LSM-FCS software (Expert Mode).
- Choose either **Frame** or **Line** scan.

- Change to the **Scan Control** window and press **Cont.**; the system will scan with the diode as a channel.
- Choose the right amplification of the signal obtained by using the special neutral density filters in front of the diode or / and by using the setting of the **Amplifier Gain** and **Amplifier Offset** value.
  (**Scan Control - Channels ChM-1**).

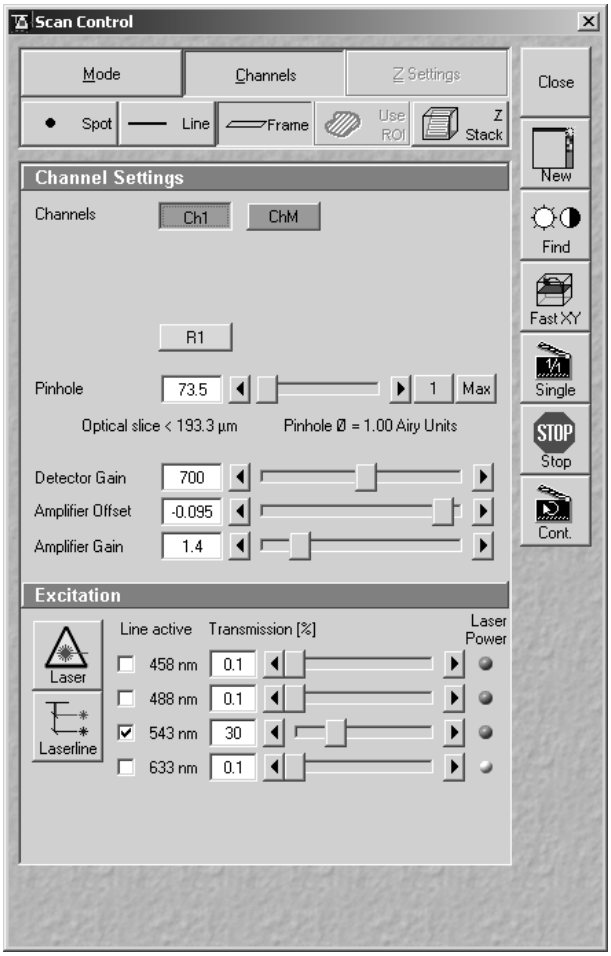


Fig. 9-7 Scan Control window

Application examples:

a) Checking the laser power

This function is not automated so far. To qualitatively measure the laser power, the diode can be used in such a way that the gray level obtained in the **Line Scan** mode at a certain setting of the whole system is stored as a text overlay together with the image (manually done by user). As the diode setting (Ampl. Gain, Ampl. Offset, ND filter) is stored together with the image, the setting is automatically reloaded when using the **REUSE** button. If deviations can be observed it is easy to set the laser power to the old value by means of the AOTF transmission.

b) Noise Reduction by Ratio

Contrary to the PMT signal, the signal of the monitor diode is not modulated by any specimen information. Thus it can be used to ratio the PMT signal to get rid of the laser noise (due to any laser as a physical fact) and thereby improve the signal to noise ratio of the fluorescence or reflectance image. The major condition which has to be fulfilled to use the monitor diode for this purpose is that the dominating source of noise is laser noise. The signal of the monitor diode will always be dominated by laser noise (independent of the power set at the laser, or the transmission set at the AOTF), whereas the dominating source of noise in the PMT signal can also be the shot noise of light (shot noise especially occurs in low light fluorescence application; as rule of thumb it can be noted that the shot noise is limiting the signal to noise ratio, if the PMT voltage has to be set to a value > 400 V).

☞ Any kind of noise which can not be observed in both channels at a time will be amplified and not reduced by the ratio process. Low or high frequency laser noise is the only source of noise which is correlated in the PMT signal and the signal of the monitor diode.

Low or high frequency laser noise is mainly introduced if the Ar, ArKr lasers are used at a tube current lower than 8 A (Ar-Vis, ArKr) or 20 A (Ar-UV) respectively.

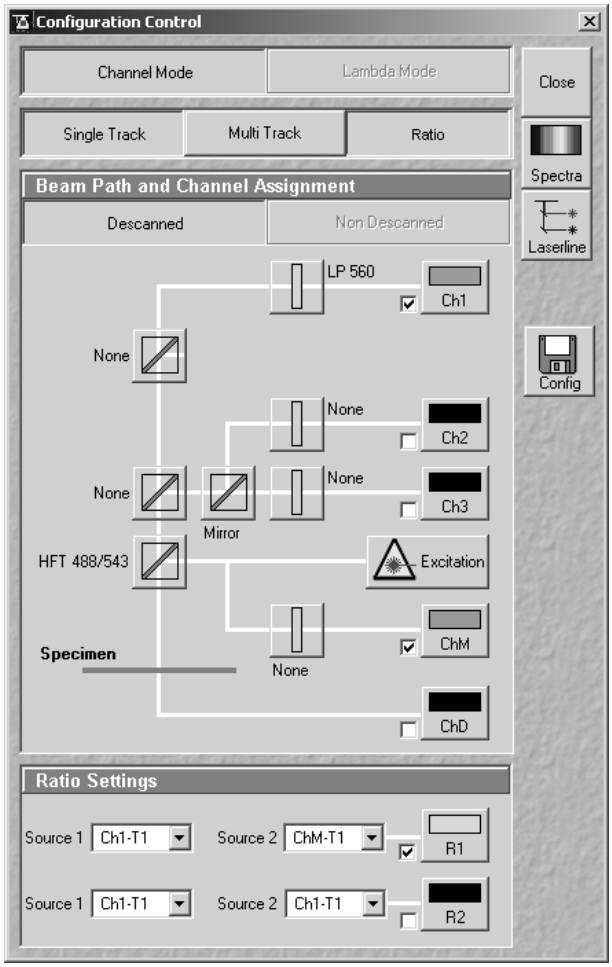


Fig. 9-8 Configuration Control window

To use the monitor diode for ratio application, proceed as follows:

- Click on the **Ratio** button.
- Activate the ratio channel R1 or R2 in the **Ratio Settings** panel in the **Configuration Control** window in addition to the monitor diode channel (ChM-1) and one PMT channel.
- Choose the appropriate PMT channel as source 1 in the **Ratio Settings** panel and ChM-1 as source 2. If this numbering is changed (inverted), the ratio image will show an inversion of gray levels if compared to the PMT image.

☞ It is not possible to do the ratio between an on-line ratio image generated with two PMT channels (as in ion-concentration sensitive ratio imaging) and the signal of the monitor diode.

The following image is an example of the reduction of correlated noise. The low frequency noise has been generated artificially.

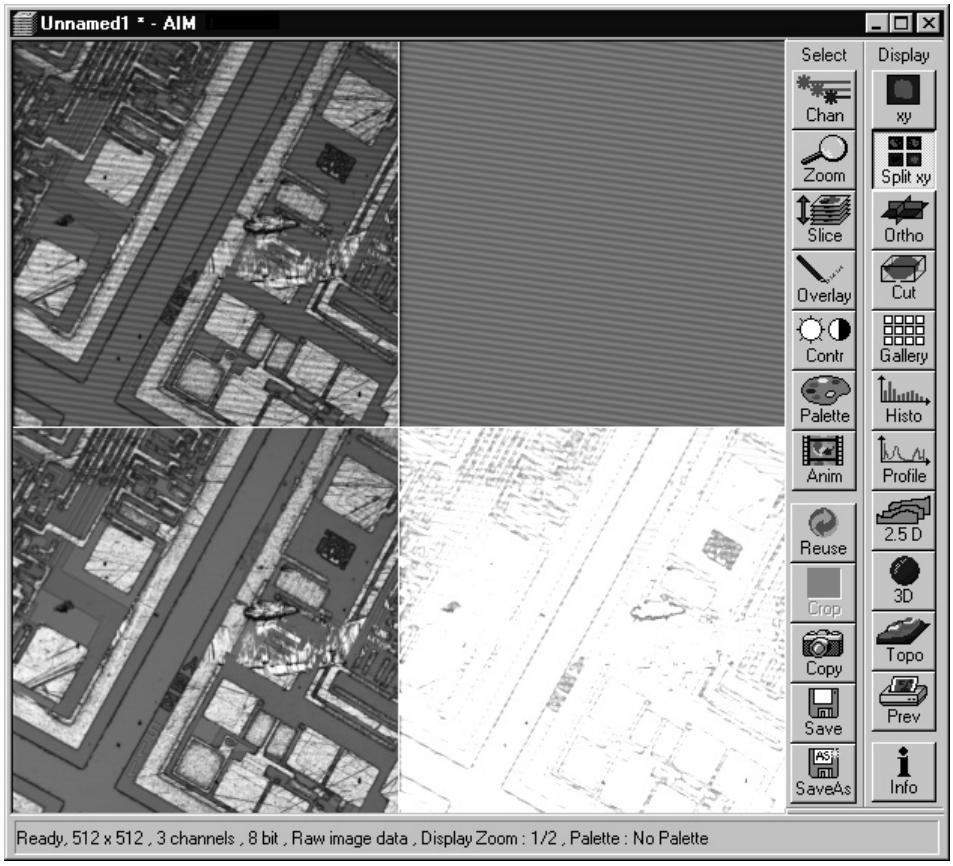


Fig. 9-9 Image window

The image in the upper left corner shows the PMT image plus noise, the image beneath this (upper right corner) shows the signal of the diode expanded to 512 x 512 pixels (noise without object information). The two images below show the ratio of the PMT and diode signal (left) and the sum of all signals (right). The sum-image does not contain any information and can therefore be neglected.

To get a ratio image like the one shown here, Detector Gain, Amplifier Gain, Amplifier Offset of the PMT channel, Gain and Offset of the diode channel, Gain and Offset of the ratio channel must be set in the correct way.

Each of the parameters summarized effects either the amplification of the ratio image, or the contrast of the ratio image, or the quality of the noise reduction.

The single steps to find the right setting of all the parameters to be set are listed in the following:

- Activate the Range Indicator.
- Adjustment of **Amplifier Offset**: the Offset of the PMT channel and diode channel have to fit to each other to guarantee the best noise reduction.

The best way to do the adjustment is the following:

- Choose different colors in the **Configuration Control** window for PMT and diode channel.
- Activate **Line Scan**.
- Switch off all laser lines in the **Excitation** window.
- Activate **Cont.**
- Set values for **Ampl. Gain** to 1 in each channel.
- Set the lines visible to the same level as close to the ground level as possible; the values you find for the Offset in each channel should be negative.

A final adjustment of the offset adjustment is done by visually evaluating the noise reduction in the ratio image. As the Offset value of the PMT channel influences the range setting of the ratio image much less than the Offset value of the diode channel, the fine tuning should be done via the PMT offset, if required.

☞ As mentioned before, the calculation of the ratio image is very sensitive to different signal offsets in the two channels used. As the offset is influenced by the scan speed as well as by the Amplifier Gain used, the offset calibration is not valid any more if the scan speed is changed, or the Ampl. Gain is set to a new value respectively. In most cases a new fine tuning is necessary. If this doesn't work, the complete calibration process has to be repeated.

Another possibility to calibrate the offset values is to set the values to -0.1 as default for both channels, then perform steps 3 and 4 and finally adjust the noise reduction by varying the PMT offset value.

If the ratio application is used and the offset has been set to the best reduction of noise in the ratio image, it is not allowed to change the offset of the PMT channel to change the reduction of background fluorescence, for example. This can be done only if the diode offset is corrected afterwards.

Adjustment of Detector Gain

The Gain of the PMT should be set with the help of the range indicator function. No 'red ' and no 'blue' pixels should occur in the image of the PMT.

Amplifier Gain

The diode signal is set to the right range (gray level between 50 and 200 - 8 bit image / 750 and 3500 - 12 bit image) with the help of gray filters and amplifier gain. The use of a lower filter density should be prioritized against the use of a high gain value.

The value of the amplifier gain of both channels (PMT and diode) should be set to one, if possible. Because of an increasing amplifier noise, parallel to the gain factor, a gain value of more than 2 should be avoided. The most important thing is to avoid pixels below the zero level and beyond the maximum range respectively.

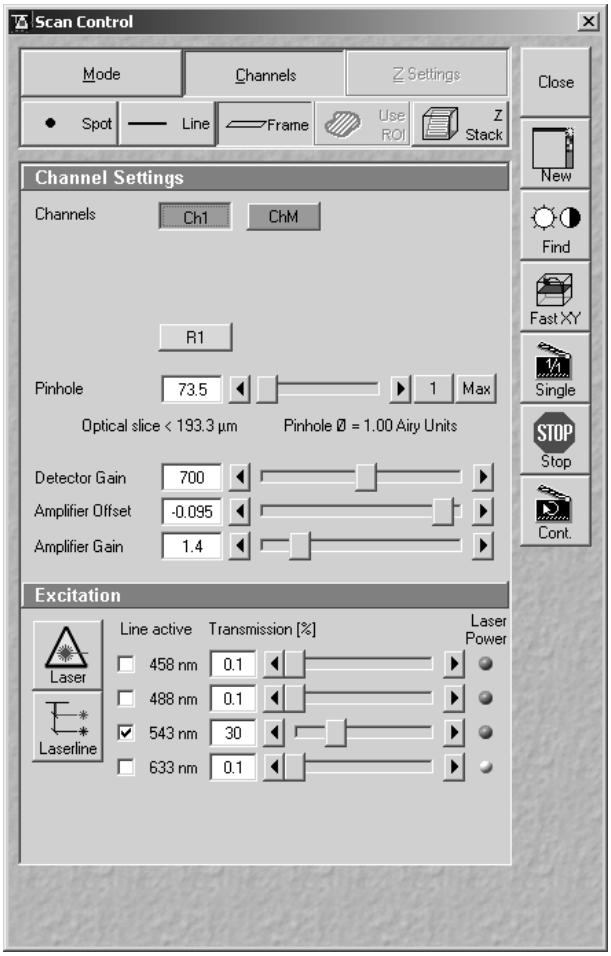


Fig. 9-10 Scan Control window

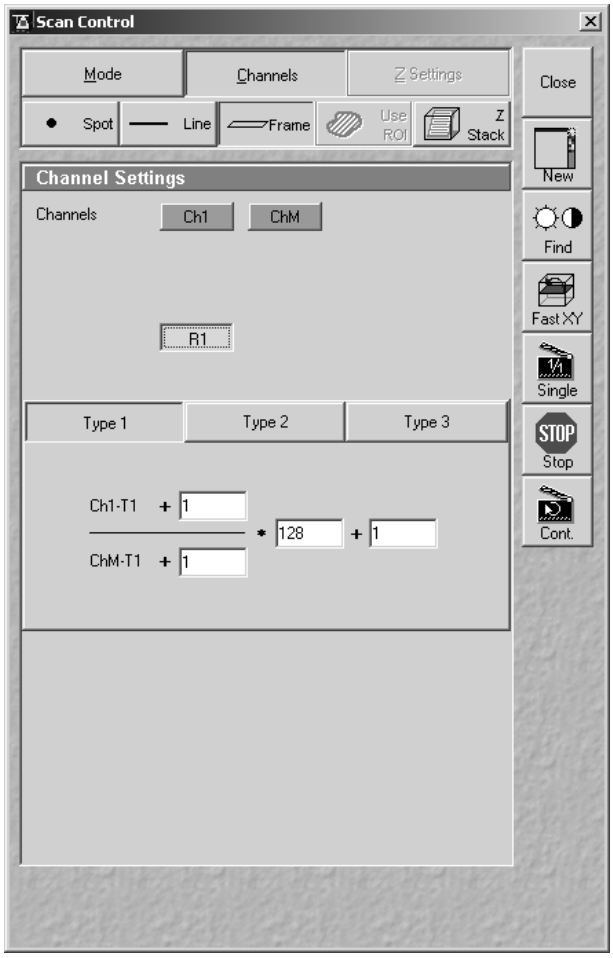


Fig. 9-11 Scan Control window

Gain and offset in Ratio channel

If the setting of the PMT channel is finished, the range of the ratio channel is adjusted by the parameters in the corresponding formula. Three types of formulas are offered when the **R1-1** button is pushed. The only formula needed for a ratio image with the monitor diode is type 1:

$$\frac{S1+n}{S2+m} * x+y$$

The values for n and m have to be zero, as well as the value for y. Any deviation from zero will decrease the contrast of the ratio image.

Only the value of x shall be influenced by the user. Dependent on the choice of data depth (8 or 12 bit), x is between 0 and 256 (8 bit) or between 0 and 4096 (12 bit).

Default settings are 150 and 3000 respectively. With the help of the range indicator the default value is changed until pixel overflow ('red pixels') is no more available

☞ Any new value can be set by hand-typing and pressing the **ENTER** key while the scan is running.

Any change in the setting parameters of PMT and diode signal will make a new Gain x in the ratio formula necessary.

If the adjustment of all parameters is finished, only the ratio image can be scanned or displayed by switching off the PMT channel and the diode channel in the **Configuration Control** window and leaving only the Ratio Channel turned on. As a result, only the ratio image is displayed; which can still be influenced by the settings in PMT and diode channel.

9.10 NLO Non Linear Optics Laser for LSM-FCS NLO

When the optional NLO laser (Titanium-Sapphire-Laser in the near infrared range - NIR) is used, some specialties in the operation of the **Laser Control** window and the configuration of the system in the **Configuration Control** window must be taken into consideration.

9.10.1 Laser Control Window

The **Laser Control** window features no remote control functions for the NLO laser.

On activation of the **On** button, the laser is not switched on directly, but the software is only informed that the laser is switched on and the laser safety shutter can be opened.

The laser wavelength to be used must be matched with the hardware and entered in the software.

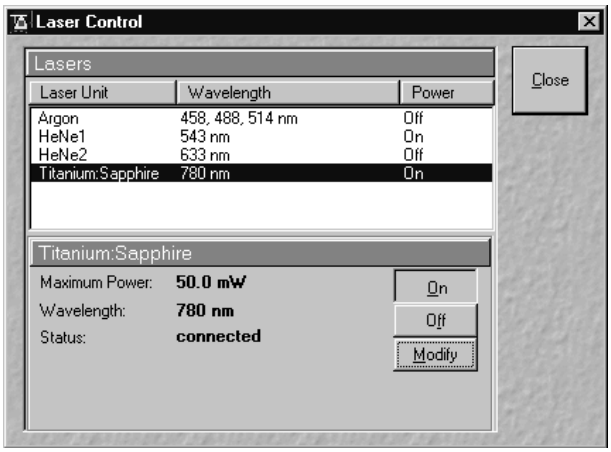


Fig. 9-12 Configuration Control window

- Allow the use of the **Titanium:Sapphire** laser with a click on the **Lasers** panel of the **Scan Control** window. The **Titanium:Sapphire** panel is displayed.
- Click on **On** to activate the laser for the software.
- Click on the **Modify** button. The **Laser Modify Control** window is displayed.
- Enter the wavelength set on the laser in the **Edit Laser Wavelength** input box (no laser tuning).
- The **Fine Tuning AO-Frequency** slider enables you to fine-tune the AO-frequency (Acousto Optical) during the continuous scanning procedure. This should only slightly influence the intensity of the signal because the automatic presetting is of high precision.
- Click on **Store** to confirm the setting. The **Laser Modify Control** window is closed and the **Laser Control** window updated.

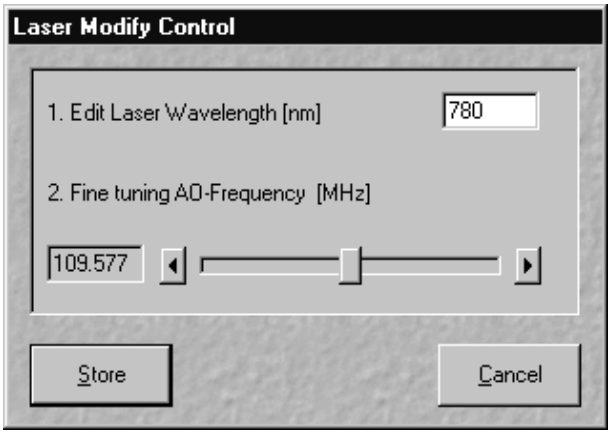


Fig. 9-13 Laser Modify Control window

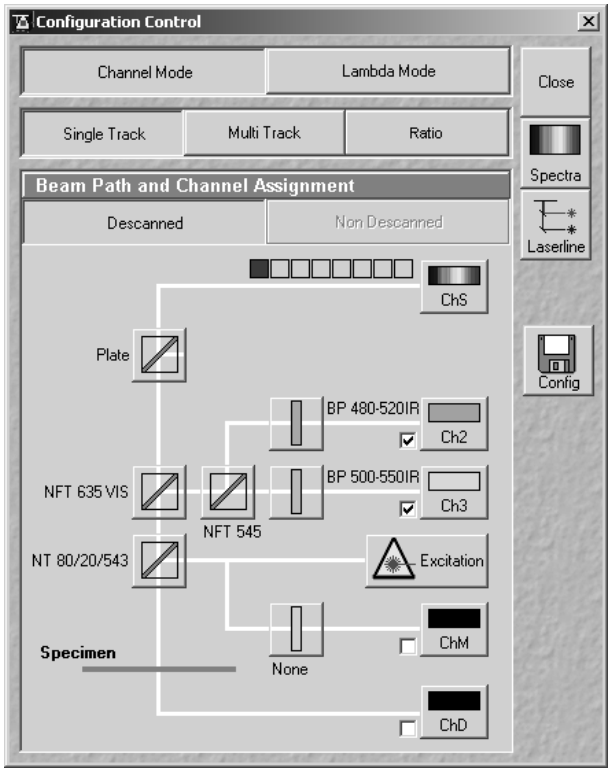


Fig. 9-14 Configuration Control window

9.10.2 Configuration Control Window

Application of the NLO laser requires special main dichroic beam splitters and the relevant emission filters to be activated in the **Configuration Control** window.

NLO-excited signals can be detected in every channel by taking care on the blocking of the NIR laser excitation light.

The following filters are especially designed for detection of NLO-excited fluorescence signals:

HFT KP 680
main dichroic beam splitter reflecting NIR excitation longer than 680 nm,
transmission for shorter wavelengths

HFT KP 680 / 488 (514, 543)
main dichroic beam splitter reflecting NIR excitation longer than 680 nm,
transmission for shorter wavelengths and reflecting 488 nm excitation for simultaneous detection of NIR and VIS excitation

KP 685 (short pass filter)
transmitting wavelengths shorter than 685 nm

BP 500-550 IR
special bandpass for NLO blocking also in the NIR range (extension IR),
can be used without additional BG 39

BG 39
highly efficient block filter for NIR, for combination with normal emission filters without IR extension

9.10.3 Pinhole and Collimator Settings

- The pinhole can be fully opened for maximum detection efficiency due to the focal excitation capabilities of the NLO effect (see **Scan Control** window, **Channel Settings**).
- In the **Pinhole & Collimator Control** window, the **NIR** collimator can be used to align the overlap of the excitation planes within the object for VIS as well as NIR excitation light wavelength in the **Collimator** panel (see **Maintain** menu, **Pinhole** button).

9.11 Non Descanned Detection (NDD)

The application of Non-Descanned Detection with the LSM 510 is only useful in combination with the optional NLO laser.

The Non-Descanned Detection modules can be used on the reflected or transmitted-light beam path or simultaneously on both beam paths. This means that the maximum of four NDD channels can be configured. If two NDD channels have been assigned to the transmitted-light beam path, no transmission PMT can be implemented.

In Non-Descanned Detection, the radiation emitted by the specimen is conducted directly on the relevant detector without passing the scanner mirror again.

Non-Descanned Detection is set and configured in the **Configuration Contro**l window by activating / deactivating the buttons **Descanned Detection** and **Non-Descanned Detection** while the NDD module is being connected.

- Click on the **Non-Descanned Detection** button to change to Non-Descanned Detection.
- Configure the NDD channels analog to the Descanned Detection mode.

The configuration of multitracks is also possible for NDD applications, though not in combination with the standard channels.

- Pull out or push in the pushrod (9-16/**1**) to close or open the shutter for the HBO 100 illuminator.

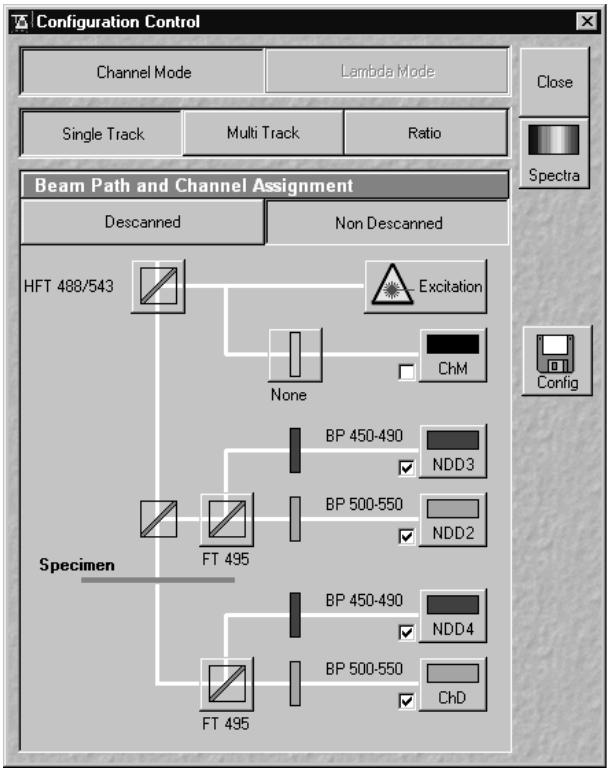


Fig. 9-15 Configuration Control window

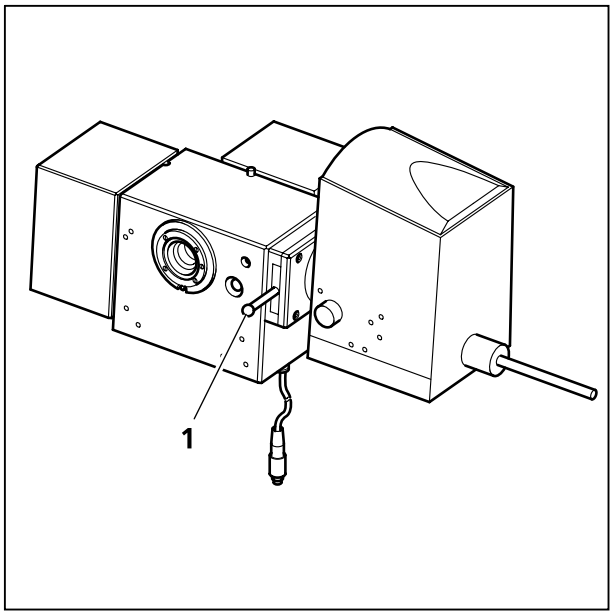


Fig. 9-16 Non descanned detection module with HBO 100 lamp

9.12 Description of the Raw Data Format for FCS

The data format is an exact representation of the dual channel photon trace within the limits given by the digitalization. This means that the data are recorded without losses within these limits.

The basic idea is to record the time between subsequent pulses from the detector in units of elapsed clock cycles (run length encoding). However, the format is modified to conserve space at high count rates and to be capable to handle dual channel data.

Data will be recorded in 16 bit words. Whenever a pulse is detected or the counter counting the clock cycles overruns 255 (FF hex), a word will be recorded. The word will contain information how many clock cycles elapsed (*1 ... 255*) since the last word had been recorded and additionally what happened in the four cycles of data generation *bt1 ... bt4*.

The recorded word has the following structure:

Bit	meaning
0 (LSB) ... 7	clock counter value (starting at 1) during the triggering event trigger events are pulse recordings or counter overruns zero is reserved and only transmitted at the end of the measurement
8	1, if pulse recorded in channel 1 during cycle bt1; else 0
9	1, if pulse recorded in channel 2 during cycle bt1; else 0
10	1, if pulse recorded in channel 1 during cycle bt2; else 0
11	1, if pulse recorded in channel 2 during cycle bt2; else 0
12	1, if pulse recorded in channel 1 during cycle bt3; else 0
13	1, if pulse recorded in channel 2 during cycle bt3; else 0
14	1, if pulse recorded in channel 1 during cycle bt4; else 0
15 (MSB)	1, if pulse recorded in channel 2 during cycle bt4; else 0

Table 1 Structure of the recorded word

We hope the two examples will make these statements clearer. The tables show part of the running pulse train (from left to right) divided into clock cycles. "1" in the corresponding box indicates that a pulse arrived in this cycle. The "counter" row shows the counter readings. The lowest row indicates when the word has been recorded.

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						...							
CH 2					1							...							
counter	120	121	122	123	0	0	0	1	2	3	4	...	254	255	0	0	0	1	2
							⇑ W1										⇑ W2		

Table 2 Example 1

Example 1

The following words will be recorded:

- at W1:
  high byte: 00011001(bin) = 19 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 197B (hex)
- at W2:
  high byte: 00000000(bin) = 00 (hex); low byte: 255 (dec) = FF (hex); resulting word = 00FF (hex)

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						...			1				
CH 2												...				1			
counter	120	121	122	123	0	0	0	1	2	3	4	...	254	255	0	0	0	1	2
							⇑ W3										⇑ W4		

Table 3 Example 2

Example 2

The following words will be recorded:

- at W3:
  high byte: 00010001(bin) = 11 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 117B (hex)
- at W4:
  high byte: 00100100(bin) = 36 (hex); low byte: 255 (dec) = FF (hex); resulting word = 36FF (hex)

The clock runs at a clock rate of 20 MHz. This means a maximum data rate of 10 Mbyte/s. If no pulses are recorded, the clock rate drops to approx. 155 Kbyte/s according to counter overflows.

The first 30 bytes of the raw data file contain the comment "ConfoCor_2_-_Raw_data_file_1.0" and have to be ignored.

9.13 List of Key Words

L

M

N

O

P

T

U

W

X

Z

Multiphoton Laser Scanning Microscopy

Using the Zeiss LSM 510 NLO

Urechis caupo
Serotonin positive nerve cells

Written by Mary Dickinson, PhD
Biological Imaging Center
California Institute of Technology
February 2002

ZEISS

CONTENTS

10 MULTIPHOTON LASER SCANNING MICROSCOPY

10.1 Preface

Safety Considerations when using ultrafast lasers coupled to a microscope.

Users operating ultrafast lasers have to observe all of the precautions specified in the Operating Manual for the laser and caution should be exercised when using the laser.

All users should be familiar with risks and good safety practices before access to the laser is granted.

Users must not look directly into the laser beam. Direct eye contact with the output beam from the laser will cause serious damage and possible blindness.

Every precaution should be taken to avoid exposing skin, hair or clothing to the laser, as this may cause burns.

Beware of reflected laser light and remove jewelry before working with the laser.

Avoid using organic solvents near the laser.

Protective housings should remain in place, when the laser is in use.

Safety signs have to be posted to inform people that lasers may be in use.

10.2 Introduction to Multiphoton Laser Scanning Microscopy

Multiphoton laser scanning microscopy (MPLSM) has become an important technique in vital and deep tissue fluorescence imaging. In MPLSM, fluorescent molecules are excited by the simultaneous absorption of two or more near infrared (NIR) photons. Multiphoton excitation has a quadratic dependence, producing excitation only at the focal plane; thus, out-of-focus fluorescence does not contribute to image background, and photodamage outside the plane of focus is greatly reduced. In practical terms, MPLSM makes it possible to acquire images with a high signal-to-noise ratio by using a wavelength that is less harmful to live cells. The use of NIR light makes it possible to image deeper in the specimen, due to less scatter and absorption of the incident light. However, multiphoton excitation depends on some special criteria that differ from those needed for single photon excitation events. Here we will provide a simplified explanation of the physics of multiphoton excitation.

10.2.1 Multiphoton excitation – How does it work?

In single photon excitation, a fluorescent molecule or fluorochrome (also called a chromophore) absorbs a high energy photon of light within a certain wavelength range and then, within nanoseconds, releases a photon of longer wavelength (lower energy). The absorption of a photon results in the excitation of the molecule, by displacing an electron within the molecule from the ground state to an excited state. Thus, for a single photon excitation event, excitation is directly proportional to the incident photon flux of the source, since each photon has an equal probability of exciting a molecule in the ground state. As the molecule relaxes back to the ground state, some energy is lost through non-radiative exchange (heat or vibration within the molecule), but the rest is shed as a photon of light.

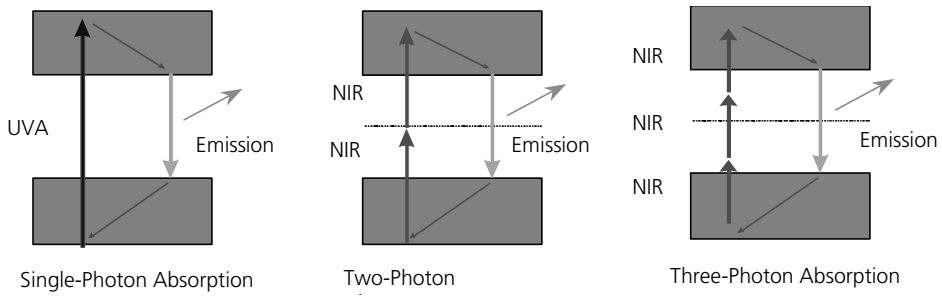


Fig. 10-1 The principle of multiphoton excitation

The energy loss accounts for the Stokes shift seen between the excitation and the emission wavelength and explains why the emission maxima is always of a lower energy, more red-shifted, from the excitation maxima. Multiphoton excitation of the fluorochrome is induced by the combined effect of two or more, lower energy, NIR photons. As a rule of thumb the energy of the two photons is roughly half the energy of the photons needed for single photon absorption, although there are clear exceptions to this rule. Multiphoton excitation can be achieved by two photons of the same or different wavelengths, but with a single laser source, two photons of the same wavelength are used.

The probability of multi-photon excitation is proportional to the incident photon flux density which is the intensity squared (I^2), because a quasi-simultaneous absorption of two photons is necessary. It follows, that for three-photon excitation, the probability of three-photon absorption is the intensity cubed (I^3). The emission characteristics of the excited fluorochrome is unaffected by the different absorption processes (Fig. 10-1).

While this appears conceptually simple, two difficulties, at the level of the fluorochrome, confound our understanding of this process. First, it is difficult to predict whether a molecule will efficiently absorb the two lower energy photons simultaneously. Drastic differences in multiphoton absorption between different molecules have been identified and it is difficult to predict by the structure of a molecule how well it will efficiently absorb simultaneous low energy photons, although some theories are emerging (see Albota et al., 1998; Rumi et al., 2000 for review). Second, the wavelengths for maximum multiphoton excitation are very difficult to predict. Deriving the multiphoton excitation wavelength maximum is clearly not as simple as doubling the single photon excitation wavelength maximum. Both of these criteria must be measured and are reflected in the multiphoton cross-section (usually referred to as δ) for a given fluorochrome (see Xu, 2000 for review).

The cross-section data indicate how well the molecule absorbs multiphoton energy at different wavelengths of NIR light. What is both interesting and perplexing about this data is that several molecules that all emit green light, i.e. excited at roughly the same wavelength via single photon absorption, can have multiphoton excitation maxima that are very different. For instance, although Fluorescein and GFP both emit green light, the multiphoton cross-section peak for Fluorescein is 770-790 nm, but is centered around 900 nm for GFP (S65T) (Xu, 2000) whereas, the single photon excitation maxima for these two molecules are both around 470-490 nm. These questions represent an intense area of investigation for physicists and chemists who specialize in multiphoton absorption (see Section 10.3.5 Choosing fluorescent probes for MPLSM).

10.2.2 Increased signal-to-noise, enhanced vitality, and deep optical sectioning in MPLSM

One of the greatest benefits of multiphoton excitation is that excitation is practically limited to the focal plane. This effect increases signal-to-noise and decreases phototoxicity. In single photon excitation, the excitation of a dye is directly proportional to the average power (Ex ~ P_{avg}). Thus, excitation takes place in the whole cone of focus and optical resolution is accomplished by using a confocal aperture. For multiphoton excitation, however, excitation of the dye is proportional to the squared intensity (Ex ~ I^2), as mentioned above. For a focused beam, intensity (I) can be described as average power (P_{avg}) divided by the cross-sectional area of the beam (A), so for multiphoton excitation, the excitation is proportional to the average power divided by the area of the beam, squared (Ex ~ $[P_{avg}/A]^2$). Thus, as the beam diameter becomes smaller (such as at the focal plane) excitation is increased and excitation out of the plane of focus becomes highly improbable and falls off with the axial distance from the focal plane with the power of 4. This explains why multiphoton excitation is mainly limited to the focal plane (Fig. 10-2). Moreover, the cross-sectional area of the beam is dependent on the NA of the objective. Objectives with a larger NA can focus light to a smaller beam waist, which is why high NA objectives are preferred for multiphoton excitation microscopy.

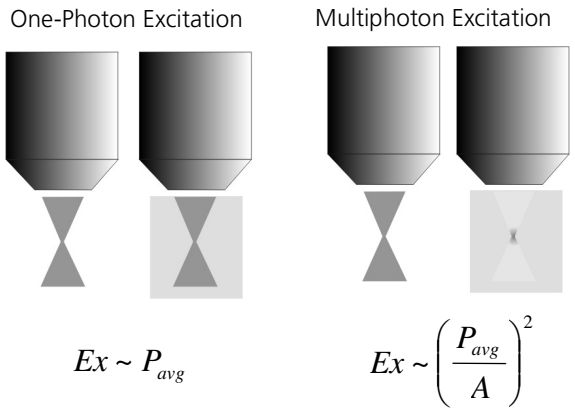


Fig. 10-2 One-photon vs. Multiphoton excitation. Two images of an objective are shown for each example. In each case, the first indicates the shape of the focused beam after passing through the objective. The second indicates the fluorescence that would be observed if the beam was focused through a cuvette containing a homogeneous solution of fluorescent dye.

Since out-of-focus fluorescence, which usually contributes to background noise in the image, is created inefficiently, MPLSM can be a better technique for imaging fine structures masked by background noise. Optical sectioning can be performed without the use of the pinhole to eliminate out-of-focus fluorescence and nearly all of the fluorescence produced at the plane of focus can be used to make the image. Although a pinhole is not normally needed using MPLSM, it is possible to use the confocal pinhole together with multiphoton excitation to prevent highly scattered photons from reaching the detector and to improve optical sectioning. This can be done on an LSM 510 NLO by carefully adjusting the collimation lens and the z-position of pinhole 1 (Refer to the alignment protocol in subsequent sections).

It is tempting to think of the decrease in background signal as an increase in resolution. This is a common misconception. In fact, due the longer excitation wavelength the optical resolution along the optical axis is worse in comparison to the resolution in a classical confocal LSM. Objects obscured by background fluorescence, may appear brighter or more defined using MPLSM, but this is not due to an increase in resolution, but rather a reduction in background noise, resulting in better contrast.

The lack of out-of-focus excitation greatly reduces bleaching, and therefore, photodamage, throughout the sample. This reduces damage caused by repeated or slow scans; however, photobleaching at the focal plane is still present. In fact, some reports indicate that bleaching may be accelerated at the plane of focus using multiphoton excitation (Patterson and Piston, 2000). Some essential, endogenous molecules within the cell can absorb UV or visible range photons (such as NAD, FADH etc.), which can destroy and deplete these molecules. Thus, it can be safer for vital imaging to use an excitation source outside of the visible range like a NIR laser. A profound example of this effect is seen in a comparative study performed by Squirrell and colleagues (Squirrell et al. 1999), where the vitality of cleavage stage hamster embryos was assessed after repeated exposure to visible range laser light and pulsed 1047 nm laser light. In these experiments, confocal imaging resulted in arrested cellular division and embryo lethality, whereas imaging using multiphoton microscopy resulted in much less embryo lethality and better data collection. In fact, at least one embryo imaged in this way was able to develop into a completely normal adult hamster named laser, illustrating the strength of this technique.

The use of NIR light has the additional benefit of being able to penetrate deeper into tissue than visible wavelength light. Compared to confocal microscopy, excitation can be achieved in deeper positions of the specimen and more data along the z-axis can be obtained. However, particularly in deep tissue imaging, care must be taken to recover as many emission photons as possible. While the incident NIR light has an advantage over visible range excitation sources, the photons that are emitted are at visible wavelengths and have the potential to be scattered or absorbed by the tissue. To improve the efficiency of collection, high numerical aperture (NA) objectives should be used, although it is often difficult to obtain lenses that have NAs above 1 with a working distance longer than 250 µm. In addition, non-descanned detectors (NDDs), which collect photons at a point closer to the specimen and do not require the emission to be focused back through the scan mechanism, can be used to improve deep tissue imaging by improving the collection efficiency of scattered photons

10.2.3 Drawbacks of using NIR light for microscopy

Although the use of NIR photons has many advantages, there is a distinct disadvantage to this mode of excitation.

Absorption of NIR light by water and some other particular molecules (such as melanocytes or condensed particles such as calcium carbonate crystals) can create dramatic local heating effects within the sample. This effect increases as the overall power from the laser is increased at the sample. It is very important to use minimized power levels to reduce the effects of local heating. For live samples, power levels above 6 mW may disturb cell replication (König et al. 1996) or even cause cells to explode, as in the case of melanocytes in zebrafish embryos. Effects of local heating can not only damage cells, but can also contribute to image artifacts (see Section 10.3.6. Samples your mother should have warned you about). As you will see in the next section, optimizing the pulse length at the specimen can improve multiphoton excitation without raising the average power.

10.2.4 Achieving Efficient Multiphoton Excitation using Ultrafast lasers

In order to achieve efficient two- or three-photon excitation, the photons must collide with the molecule simultaneously. For single-photon excitation, a continuous wave laser with a continuous photon flux can be used because the probability of excitation is directly proportional to the photon flux or average power of the source. Increasing the laser intensity (turning up the power) increases the photons delivered to the sample, increasing excitation until all of the molecules are saturated. To deliver enough photons to achieve simultaneous absorption of two NIR photons using a continuous wave laser would require enormous power. Ultrafast lasers improve the efficiency of multiphoton excitation by delivering photons in pulsed "wave packets". The high peak intensity needed for multiphoton excitation is created by concentrating photons into very brief pulses which are delivered to the sample over and over again at a rapid rate, about every 13 ns, to ensure efficient dye excitation. Instead of a steady flux of photons bombarding the fluorochrome one after another, multiple photons collide with the molecule simultaneously. This process has the advantage of delivering a high peak intensity, to satisfy the I^2 or I^3 requirement for two- or three-photon excitation, without using enormous amounts of average power.

Many lasers are now available that can produce ultrashort pulses at high repetition rates. Titanium-sapphire lasers, for instance, are capable of producing ~100 fsec pulses over a broad tunable wavelength range (690 nm-1064 nm) with a high repetition rate, ~80 MHz. Similarly, solid state, doubled neodymium doped yttrium lithium fluoride (Nd: YLF) lasers, emitting 1047 nm, 175 femtosecond pulses at 120 MHz have also be used for multiphoton excitation.

Ti: Sapphire lasers are probably the most popular lasers because of the wavelength range that is available. These lasers can operate in both a continuous wave (CW) mode or in a mode that emits pulsed light. Lasers operating in this latter mode are said to be mode-locked (ML), which refers to the fact that the laser is locking in different frequencies together to form a pulse of a particular bandwidth.

The use of ultrafast mode-locked lasers for multiphoton excitation requires to consider several additional factors which are not necessary for continuous-wave lasers used for single-photon excitation. The length of the laser pulse (referred to as the pulse length or pulse width) (τ), the peak intensity produced at the focal plane (I_{peak}), the average power of the laser at the specimen (P_{avg}), the cross-sectional area of the beam (A), and the pulse frequency (F_p) or repetition rate are all important factors for achieving and maintaining efficient multiphoton excitation in the sample.

Raising the average power of the laser, P_{avg} (controlled by the Acousto-Optic-Modulator, AOM), will raise the peak intensity. However, raising the average power will also increase the amount of heat generated in the sample, which may damage vital processes or disrupt cellular structures.

The pulse frequency, F_p, is determined by the design of the laser and is not easily manipulated. For the Coherent Mira 900 Ti: Sapphire laser, Fp is equal to ~76 MHz.

The cross-sectional area of the beam, A, is an important term to consider when dealing with light focused through an objective. Simply put, reducing the cross-sectional area of the beam, for instance by focusing the beam through an objective, increases the intensity at the point of focus or in other words increases the amount of photons per area as we saw above.

The exact dimensions of A will vary greatly depending on the properties of the objective. In general, higher magnification, higher NA objectives will reduce A, similar to the way such objectives increase resolution. However, other considerations such as the transmission efficiency of the objective and the amount of dispersion produced by the objectives must also be considered as this will reduce P_{avg} or increase respectively. In addition, spherical aberration caused by the objective and sample and the diameter of the beam at the back aperture of the objective can also affect A. Thus, empirical determination of the best objective is often relied upon. (see Section 10.3.4 Objectives recommended for multiphoton excitation).

The pulse length, τ, is a measure of the duration (length) of the pulse of photons being delivered to the sample, measured at the full width, half maximum. Increasing the pulse length, decreases I_{peak}. As mentioned above, ultrafast lasers are able to supply short pulses with a high duty cycle. Unfortunately, the pulse length is very difficult to measure, especially through a microscope objective. However, it is possible to make such measurements and users should refer to Wolleschensky et al. (1998) for details. The LSM 510 NLO fiber-delivery system contains a pre-chirping unit that is used to compensate for Group Velocity Dispersion (GVD) along the optical path. This system is capable of producing 120-200 fsec pulses at the sample, but the fiber imposes considerable restrictions on the peak power, the available wavelength range and the ease of tunability. Several factors that effect the pulse length are discussed below.

10.2.5 Optimizing the Peak Intensity without Frying the Samples

Although raising the average overall power is the easiest way to increase the peak intensity, excessive heat can destroy both live and fixed samples, as well as produce unwanted imaging artifacts. Assuming that one is using a laser with a fixed pulse frequency and a high magnification, high NA objective optimized for multiphoton microscopy (see Section 10.3.4 Objectives recommended for multiphoton excitation), the pulse length becomes the most important term to minimize.

The Spectral Bandwidth refers to the spectral frequencies of the pulse. Each short pulse produced by the laser has a broad spectral band centered around the selected wavelength. Spectral bandwidth and pulse length are inversely related. The wider the bandwidth, the shorter the pulse.

The Coherent Mira 900F produces pulses ranging from 70-150 fsec with a bandwidth range from 6-13 nm.

GVD is a temporal broadening of the pulse length as the pulse travels through normal dispersive media such as glass (see Diels and Rudolph, 1994 for more discussion). The pulse length becomes broader in dispersive media because the red shifted frequency components, with respect to the center wavelength, travel faster than the blue shifted frequency components. Pulses with a broader spectral bandwidth are more susceptible to the effects of GVD, as there is a greater difference in wavelength, and thus, velocities within the pulse (Fig. 10-3). In addition, the amount of dispersion is related to the thickness of the dispersive media (see Wolleschensky et al. 2001 for review). When possible, thinner glass optics and lenses are used along the routing path.

Within the laser, a prism pair is used to adjust the spectral bandwidth of the laser pulses. However, outside the laser, glass elements within the microscope and scan module, within the objective, and along the routing path, can lengthen the pulse. In this case, the broader the spectral bandwidth, the more broadening of the pulses will be caused by GVD. Thus, for direct-coupled systems without the use of a prechirping unit, it is advantageous to adjust the laser so that the bandwidth of the pulse is at a minimum. For instance, if the bandwidth is less than 7 nm, the pulse length at the sample will be approximately 300 fsec. If the bandwidth is 12 nm, the pulse becomes substantially broadened by the same dispersive elements so that the pulse length is about 700 fsec at the specimen.

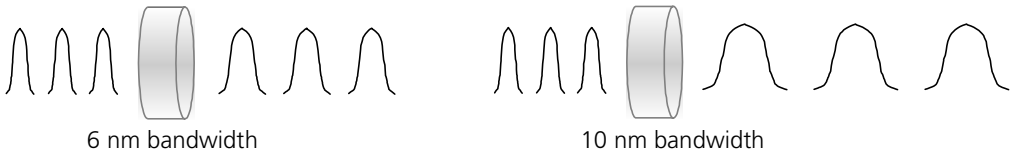


Fig. 10-3 Effect of GVD on short pulses with a broad bandwidth. The broader the bandwidth , the more the pulse is stretched. Longer wavelengths travel faster through the glass than shorted wavelengths in the pulse. Thus, a positively chirped pulse is broadened.

It is possible to compensate for GVD along the routing path and in the microscope to preserve the pulse length to the sample. By using a pulse stretcher/fiber combination, as is the case with the LSM 510 NLO GDC fiber delivery system, a pulse length of 120-200 fsec can be preserved at the sample (see Wolleschensky et al., 2001).

This system consists of a 5 W Verdi/Mira 900F laser system, a Grating Dispersion Compensator (GDC), a 3 meter single-mode fiber and a Post-fiber compressor unit (PFC). In this system, 100-150 fsec (10^{-12} nm) pulses exit the laser and enter the GDC. The grating compensator within this unit applies a negative chirp to the pulses and stretches the pulses to 6 psec. As the pulses pass through the 3 meter fiber, the 6 psec pulse becomes compressed due to GVD within the fiber and exits the fiber as a 3 psec pulse. Seven passes through the post-fiber compressor, which contains additional dispersive media, further compress the pulses as they enter the micro-scope so that at the sample, the pulses are now back to being chirp free at a pulse length of about 120 fsec.

This system enables the user to achieve efficient excitation of the fluorochrome with minimal power, by minimizing the pulse length and maximizing the peak intensity using low average power. Although effective at preserving the pulse length, this system has several limitations. First, due to self phase modulation, the fiber has an upper limit for the deliverable peak power (5.5 kW for a 120 fs pulse at a repetition rate of 76 MHz and at an average power of 50 mW). In a direct-coupled system the full power of the laser (~750 mW) can be sent to the sample resulting in peak powers greater than 80 kW, although this is rarely needed. Second, the fiber has a wavelength range of 750-950 nm for a 10 W pump laser and 750-900 nm for a 5 W pump laser. This limits the available wavelength range of the laser which is 680-1000 nm. Third, the laser can be difficult to align through the pulse stretcher, especially for novice users. Despite these limitations, this system provides an ideal imaging configuration for users who are concerned about keeping the average power low while imaging or placing the scan module on different microscope stands.

10.3 Using the LSM 510 NLO direct coupled system: Practical considerations for optimal imaging

10.3.1 Coupling the Coherent Mira 900F to the LSM 510 NLO

Coupling an ultrafast laser to a laser scanning microscope is a fairly simple matter, if several key considerations are understood. Typically, the routing kit for coupling the LSM 510 NLO to the Coherent Mira 900F will consist of the following components: a beamsplitter optic, used to send a small percentage of laser output to a Rees Analyzer; an Acousto-Optic Modulator (AOM), used to control the intensity of the beam and for beam blanking (allowing for region of interest (ROI) and bi-directional scanning and for performing Auto-Z-Brightness correction); two 45° mirrors for directing the beam to the microscope on the table; and a 1 meter lens. In the case of an upright system, the beam is brought up into the scan head via a periscope, whereas for an inverted microscope, the height of the beam will be adjusted by a smaller periscope positioned in place of the second turning mirror. These devices ensure that the beam remains parallel to the table when horizontal and enters the scan head at the proper height (see diagram). The 45° routing or turning mirrors are adjustable and will be used routinely to peak the coupling of the beam into the scan head. The 1 m lens is used to expand the beam to fill the back aperture of the objective. It is mounted in a fixed position by a trained service engineer during installation and should not be adjusted by the user (Fig. 10-4).

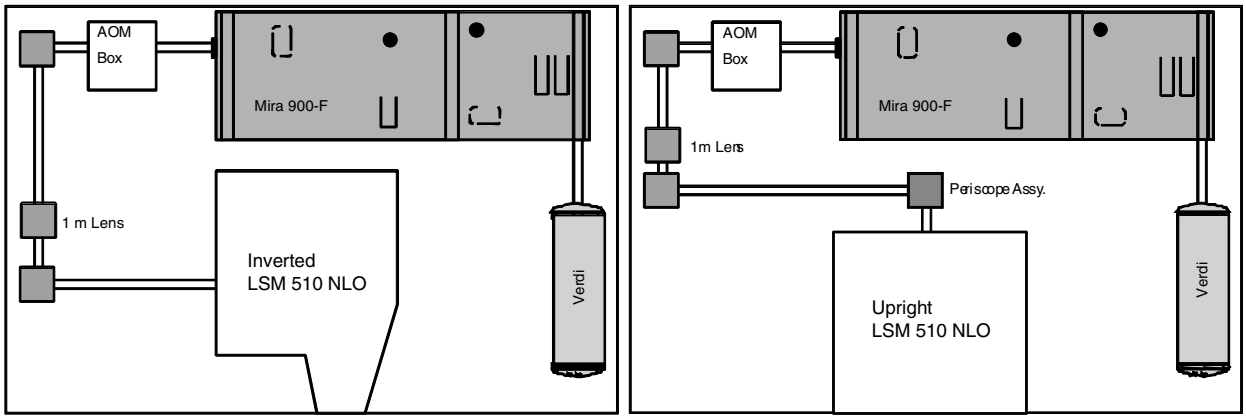


Fig. 10-4 Typical alignment scheme for the LSM 510 NLO direct-coupled system using and inverted (left) or upright (right) microscope.

10.3.2 Using and tuning the Coherent Mira 900F – A simplified protocol for direct-coupled LSM 510 NLO systems

We have developed a simplified tuning protocol for the Coherent Mira 900F in order to preserve alignment of the beam into the scan head while tuning. This protocol will cover tuning over most of the usable wavelength range of the Mira (~720-950 nm), but slight adjustments in the alignment may be necessary for tuning the complete range (~700-990 nm).

10.3.2.1 Turn on procedure

Turn on the system by switching the key on the Verdi Controller Box from STANDBY to ON. Turn on the TEC chiller that cools the Verdi baseplate and Ti: Saphire crystal. Allow the system at least 30 minutes to warm up. The laser should be left in the standby position, unless the laser will not be used for a very long period of time (> 2 weeks). If the laser is completely shut down, the system will require at least an hour to warm up. Turn on the Mira 900F controller box using the switch on the back panel. Turn on the IST-Rees Spectrum Analyzer and oscilloscope.

☞ Tuning and mode-lock can be achieved without a Rees Analyzer, however it is recommended that this is included on all systems in order to adjust the bandwidth of the pulse accurately and in order to adjust the AOM frequency to match the output wavelength.

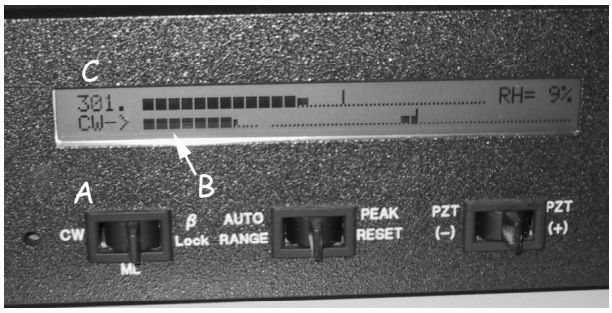


Fig. 10-5 The Mira Controller display panel. (A): CW-ML-ß lock switch to initiate mode-lock. (B): Indicator for CW break-through. In CW mode, the range would be full, whereas for a laser in mode-lock, the range would be empty. The laser here is partially mode-locked with CW breakthrough as in Fig. 10-7/B. (C): The power indicator from the Optima system in arbitrary units.

After the laser has warmed up, switch the CW-ML-ß lock switch to the ML position (Fig. 10-5/**A**). Make sure that the Rees

Analyzer is adjusted so that the center wavelength is visible on the oscilloscope screen (a blinking reference marker should be seen) by adjusting the up and down buttons (indicated by arrows) on the Rees Analyzer control box (Fig. 10-6).

A bell-shaped curve should appear at the center wavelength, indicating good mode-locked operation (Fig. 10-6, Fig. 10-7/**A**). If the laser is partially mode-locked, but has some CW break through exiting the cavity, a spike will appear along with the curve (Fig. 10-7/**B**). If the laser is not mode-locked at all, a sharp spike will be seen (Fig. 10-7/**C**).

A bell-shaped curve that has a broad noisy line, as in Fig. 10-7/**D**, indicates that Q-switching is occurring.

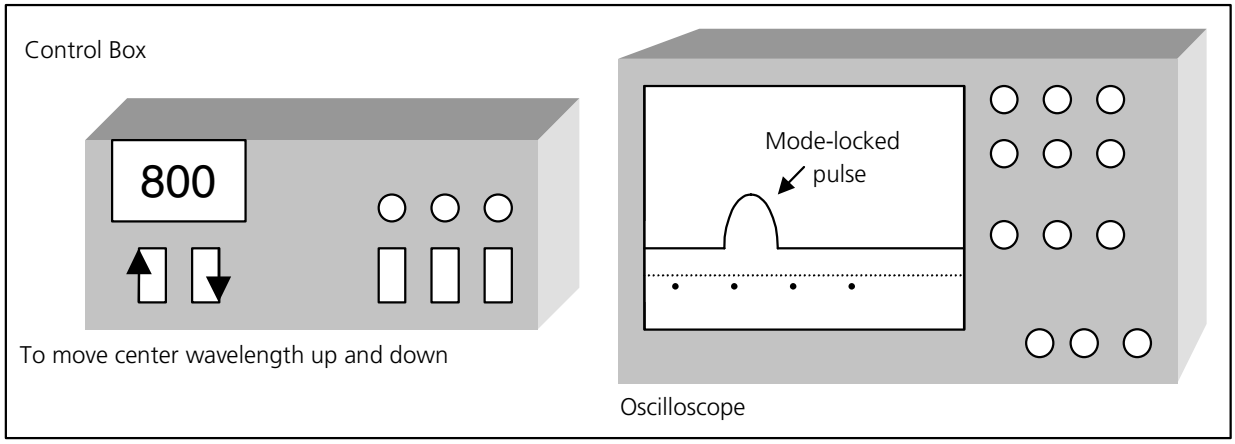


Fig. 10-6 Schematic of a IST Rees Spectrum Analyzer

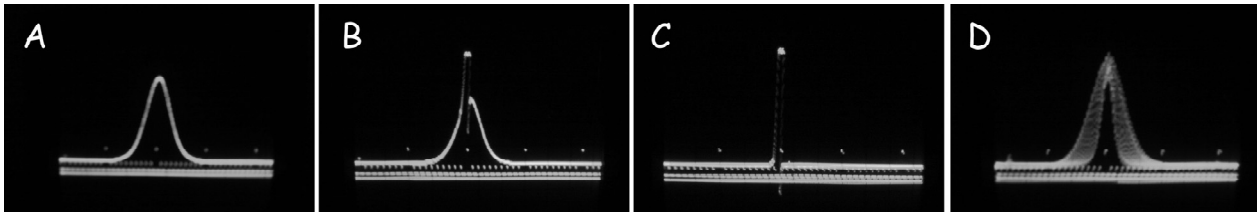


Fig. 10-7 Examples of common oscilloscope beam profiles for the Coherent Mira 900F. A, stable mode-locked operation. B, partially mode-locked operation with CW breakthrough. C, pure CW operation indicating no mode-lock. D, Q-switching indicating that the slit aperature is too small to reinforce stable mode-lock.

10.3.2.2 Achieving stable mode-lock

If a stable, mode-locked pulse does not appear at start-up (Fig. 10-7/**B-D**), adjustments should be made to stabilize mode-lock. Every attempt should be made to establish a stable mode-lock at system start up and shut down, and mode-lock should always be established before tuning. This will help preserve the alignment of the laser. Simple adjustments in the slit width may correct the problems seen in Fig. 10-7/**B** or Fig. 10-7/**D**. CW breakthrough often results when the slit opening is too large. Try closing the slit slightly. This can also be adjusted by turning BP2 in the counter-clockwise direction, but adjustments in the slit width should be made first. Q-switching often results when the slit width is too small, so opening the slit slightly should eliminate this problem. When imaging, CW breakthrough will lead to gaps in the image since the laser is going in and out of mode-lock while the scanner is in motion, whereas Q-switching can lead to wavy lines being formed in the image due to power fluctuations (see Fig. 10-8 for description of the Mira controls).

If a mode-locked pulse is not evident after start-up, be sure that the laser has had ample time to warm up. If pure CW operation is seen (Fig. 10-7/**C**), adjustments in laser alignment will need to be made. First make SMALL adjustments up and down with the BRF micrometer. Often a mode-locked beam profile will appear. Then you can stabilize the beam at this wavelength and make small adjustments in the alignment to center the beam at the wavelength of interest (see below: Tuning the MIRA 900F to a new wavelength). If a bell-shaped curve does not appear, try opening and closing the slit a quarter rotation. Next, make small adjustments in BP2, both in the clockwise and counterclockwise direction.

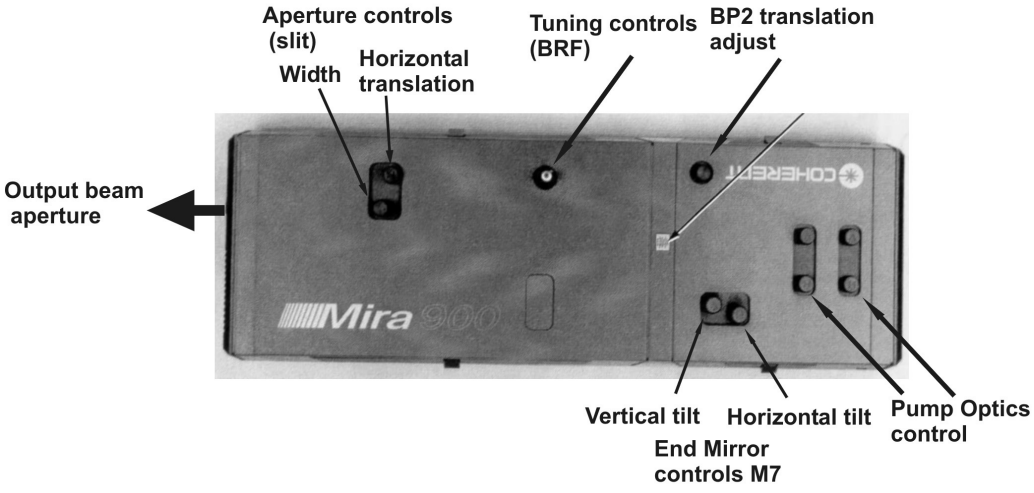


Fig. 10-8 Mira 900F controls

If all adjustments fail and a mode-locked beam cannot be found, set the BRF to the desired wavelength by locating the CW spike on the oscilloscope (Fig. 10-7/**C**). Next, open the slit to allow maximum power through to the output coupler. Then rotate BP2 to allow less glass in the path and until the laser stops lasing. (The Optima power reading on the Mira will be reduced to the baseline, Fig. 10-5/**C**). Note the position of the mark on the BP2 knob and then rotate the knob back to add glass four full rotations. Close the slit until a mode-locked beam profile appears. Maximize the power by making very small adjustments with the M7 mirror (usually only horizontal adjustments are necessary).

Re-adjust the opening of the slit apparatus to ensure stable mode-lock. At this point, since the slit width has been fully opened and then closed again, it may be necessary to make SMALL adjustments in the horizontal position of the slit. While monitoring the power level reading on the MIRA output controller, slide the slit translation knob back and forth to find the optimum position for the slit. The optimum position will result in a higher power reading. Re-adjust the slit width to achieve stable mode-lock.

CAUTION: This horizontal position of the slit should only be adjusted after the slit has been opened completely to re-tune the laser and only small adjustments should be necessary. Large adjustments in the position of the slit indicate that the alignment of the laser is not optimal and, as a result, the laser may not be aligned into the AOM. Realignment of the laser and the AOM will require the help of trained service engineers.

10.3.2.3 Tuning the MIRA 900F to a new wavelength

Once a mode-locked beam profile is stabilized, one can easily tune to a new wavelength. To do this, turn the BRF in the direction required to move to the desired wavelength. You will see the curve on the oscilloscope move. The shape of the curve may begin to change. The bell-shaped curve may begin to get flatter or taller, or spikes may appear in the curve (Fig. 10-7/**B**), or the single line of the trace may begin to look wavy (Fig. 10-7/**D**). Adjusting the slit width and/or BP2 will restore stable mode-lock as indicated above. It is recommended that you make small adjustments in the wavelength using the BRF and then optimize the mode-lock and then to repeat this process until you have arrived at the desired wavelength.

Once the beam profile is centered over the desired wavelength, again optimize mode-lock by adjusting BP2 and the slit width. Small changes in M7 may help to recover more power and produce a more stable mode-locked beam. In some cases, maximum power output will only be achieved if small adjustments in the fine-tuning controls for the pump laser are made (rear-most pump optics control knobs). Balance changes in the rear pump optics with M7.

☞ DO NOT MAKE ANY ADJUSTMENTS IN THE COURSE ALIGNMENT OF THE PUMP BEAM USING THE FORWARD PUMP OPTICS CONTROL KNOBS.

10.3.2.4 Adjusting the bandwidth of the pulse

For direct-coupled systems using no pre-chirping unit the bandwidth of the pulse should be minimized to limit the effect of GVD along the routing path and through the microscope and the objective. Therefore, the final adjustment to the mode-locked beam should be to lower the bandwidth of the pulse as much as possible by adjusting BP2 (usually removing glass) and opening the slit. Fig. 10-9 shows an actual trace from the oscilloscope of a direct-coupled system. The pulse is mode-locked and centered at 800 nm. To estimate the bandwidth, the full-width, half-maximum (FWHM) is used. For this trace the bandwidth appears to be approximately 6 nm, which is ideal for imaging with a direct-coupled system.

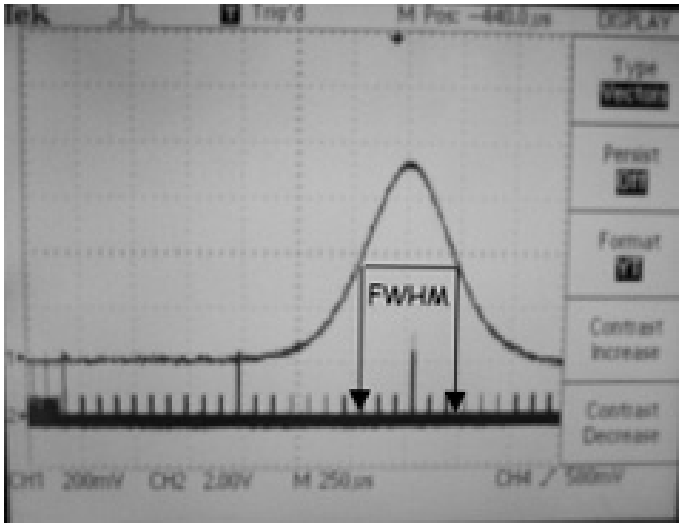


Fig. 10-9 The beam profile of a mode-locked laser output. The bandwidth of the pulse is the full width at half maximum (FWHM) of the bell shaped curve. In this case the bandwidth is approximately 6 nm, which is ideal for direct-coupled systems.

10.3.3 Alignment of the Coherent MIRA 900F into the LSM 510 NLO scan head

The alignment of the laser through the AOM and into the scan head is critical for producing high quality images using multiphoton excitation. Below are two protocols that can be used to optimize alignment of the beam into the scan head.

10.3.3.1 Quick alignment protocol

After the system is installed and aligned by a qualified service engineer, it is often the case that only small alignment adjustments are necessary. For instance, proper alignment should be verified after the laser is tuned to a new wavelength. Below is a quick and easy protocol that can be used to center the beam into the back aperture of the objective lens for optimum image quality.

Confirm that the correct wavelength for the laser is set in the laser control panel in the software. The wavelength can be changed by selecting the modify option. Refer to the Rees analyzer or the BRF setting to determine the wavelength of the MIRA 900F.

Start by scanning a sample that is normally excited by the wavelength of the external laser. For instance, a pollen grain slide (Carolina Biological) or a Fluocells sample (Molecular Probes) can be used. (NOTE: This procedure may cause bleaching of the sample.)

Adjust the laser power and gain so that the sample is visible in the image acquisition window.

Scan the sample using the fast XY scanning mode.

Using the signal intensity as a guide, peak the alignment of the beam. Begin by using the alignment mirror closest to the laser (first turning mirror). Make improvements using both alignment pins on the mirror mount. (NOTE: a small hex wrench (Allen key) may be needed to adjust alignment). When the best signal intensity is obtained by tweaking the mirror closest to the laser, now make improvements by tweaking the mirror closest to the microscope scan head. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

After alignment, it may be necessary to optimize the AOM (Acousto-Optic Modulator) frequency, if an AOM is used. Open the modify window in the laser control panel. Confirm that the correct wavelength is set for the wavelength of the external laser. With the sample in place and while scanning in the Fast XY mode, slide the AOM frequency slider to the right and left to obtain the brightest signal. A peak should be found within the range of the slider. If not, the beam may be misaligned through the AOM. If this is the case, contact a qualified service engineer. NOTE: Confirm alignment of the beam into the scan head using the above protocol before modifying the AOM frequency.

As a final verification of proper alignment, check the alignment of the beam onto the back aperture directly by viewing the projected beam without the objective in place. To do this, place a white card on the stage (Fig. 10-10/**A**) or lens paper on the specimen holder for an inverted microscope.

Remove one objective, select 50-100 % transmission, HFT KP 680 or 700, and Fast XY scan. When the system is scanning, a round, red spot should appear on the card (Fig. 10-10/**B**). Seeing this beam may be aided by using an IR viewer, especially at longer wavelengths.

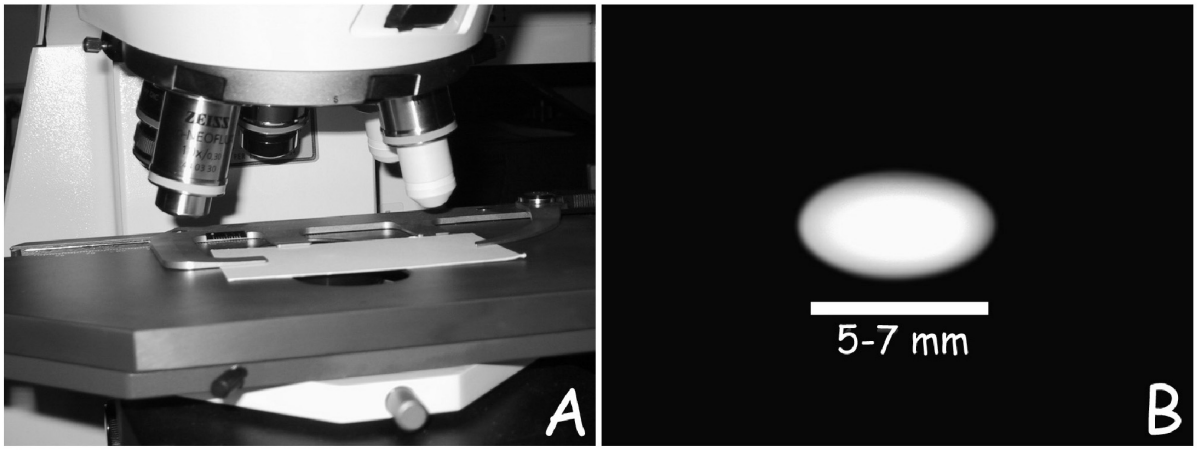


Fig. 10-10 (A) Setup for checking the beam alignment; (B) Optimal shape of the NIR laser spot

Ideally this spot should be 5-7 mm in diameter and should be completely round and uniform. If the beam appears to be clipped (flat on one side), small adjustments in the routing mirrors can improve the alignment (see above). Shadows in the illuminated spot may indicate dust or debris on the routing optics or in the scan head (check all visible elements for debris, then contact a service engineer if the problem persists).

If proper alignment cannot be established using this protocol or if the image quality appears to be poor, use the advanced alignment protocol to peak alignment.

10.3.3.2 Advanced alignment protocol

When the system is installed, the service engineer will establish alignment of the laser beam into the scan head by verifying the overlay between the NIR laser and one of the VIS lasers. Although routine use usually does not usually require a complete realignment with the scan head, tuning the laser over large wavelength increments or changing or cleaning the optics in the laser can cause gross changes in beam pointing. If this occurs, it is best to use the following protocol to re-establish proper alignment with the scan head. For this protocol, a partially reflective grid slide is needed (Part number 474028-0000-000, Test Grid Specimen for LSM). Care must be taken to use low amounts of laser power when using this slide or the grid can be easily burned.

Begin by confirming that the correct wavelength for the laser is set in the laser control panel in the software. The wavelength can be changed by selecting the modify option. Refer to the Rees analyzer or the BRF setting to determine the wavelength of the MIRA 900F.

Next, place the partially reflective grid slide on the microscope stage. Bring the grid slide in focus using the 10x Plan-Neofluar objective and transmitted light. Configure the scan head as follows (Fig. 10-11):

This configuration is designed to use the PMTs to detect the reflection of the NIR and VIS laser light off of the grid slide so an image of the grid is made on the screen. For this protocol, the 543 nm line has been chosen. The HFT KP 700/488 beam splitter is used to avoid sending too much 543 nm laser light to the reflective slide. A KP 700/543 beam splitter can be used, but care must be taken not to burn the slide. Alignment using this procedure will ensure that the NIR and VIS lasers overlay on the combining mirror when they enter the scan head.

To perform the alignment, set the laser power for both lasers at 3 % (1 % for a MIRA 900F with a 10W pump laser) and adjust only the gain to improve the intensity of the signal.

WARNING: Too much laser power can cause damage to the grid slide! Be sure attenuation is set before scanning.

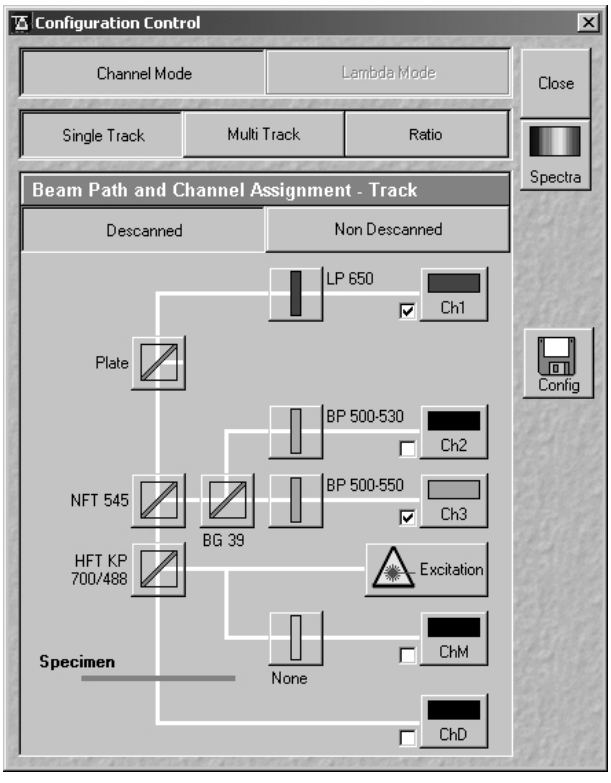


Fig. 10-11 Configuration for testing the overlay of the grid reflection images gained with the vis (543 nm) laser and the NIR laser

Begin scanning using the FAST XY mode. An image of the grid should appear on the screen. If not, adjust the gain for each channel so that the output from both channels is visible. The two colors represent the reflection of the 543 nm line (green) and the reflection of the NIR laser (red) on the metallic grid surface. The focus may also need to be adjusted in order to get the optimal output from both channels. If the red image is out-of-focus when the green image is in focus, the collimating lens should be adjusted so that both beams are focused on the same spot. To adjust the collimator, open the Maintain panel in the LSM 510 software. While the laser is scanning, click on the pinhole button. At the bottom of this panel, there is a slider to adjust the NIR collimator.

☞ DO NOT ADJUST THE VIS COLLIMATOR.

Adjustments in the NIR collimator should improve the focus of the NIR beam. If no signal at all is detected in the red channel, turn up the laser transmission in small increments and begin peaking alignment.

☞ Note that once alignment is improved, the laser intensity will need to be attenuated to avoid burning the slide. If no red signal at all can be found, contact a service engineer.

Once both beams are in focus, begin optimizing the overlay of the grids by first adjusting the routing mirror that is closest to the laser. Make small adjustments using both alignment screws while watching the effect on the monitor screen. Adjustments of this mirror will increase the intensity of the red signal. Readjust the gain as needed. After optimizing the alignment using this mirror, begin adjusting the mirror that is closest to the scan head (NOTE: A small hex wrench [Allen key] may be needed to adjust alignment). Adjustments in this mirror will improve the alignment of the NIR overlay on the VIS overlay. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

10.3.3.3 Tips on maintaining alignment with the scan head

Since the NIR beam is aligned into the scan head through mirrors and free space, it is possible that any alteration in these routing components can effect proper alignment. Below are some tips to maintaining good alignment:

- Use the simplified tuning protocol in Section 10.3.2.3 to tune to different wavelengths
- Do not move the position of the 1m lens or the beamsplitter used to send the signal to the Rees analyzer. Even small adjustments of these components may cause significant changes in the alignment of the routing optics.
- If the laser is cleaned or optics within the cavity are changed, note the position of the output of the beam before adjustments are made and realign the beam to this position when the laser is optimized. An aperture stand supplied with the laser is useful for this.
- If recurring alignment drifts or power fluctuations in the laser are observed, consider floating the optical table and/or regulating the room temperature more closely. Changes in the temperature of the table or optical components can often cause drift in alignment of the laser and routing optics.
- Avoid tuning the laser in increments larger than 50 nm without peaking the alignment.

10.3.4 Objectives recommended for Multiphoton Excitation

For nonlinear microscopy, objectives should be optimized for the following parameters: high transmission in the NIR and the VIS-wavelength range, long working distance with a high numerical aperture, limited pulse broadening due to GVD, a uniform GVD across the pupil of the objective for the excitation wavelength range and a small propagation time difference (PTD). As we have already discussed, low transmission in the NIR can lower the average power at the sample, thus lowering the peak intensity.

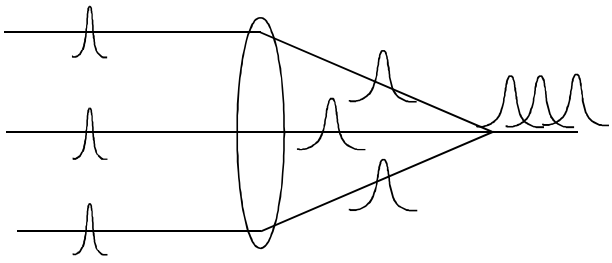


Fig. 10-12 Schematic pulse transformation by a singlet lens showing the influence of chromatic aberrations on the peak intensity due to Propagation Time Difference (PTD).

Long working distance objectives with a high numerical aperture are clearly favored for deep imaging so that the beam can be focused deep into the tissue and the emission photons can be collected efficiently. Objectives should have limited GVD to reduce the chance that short pulses will be lengthened en route to the sample, which will again reduce the peak intensity.

In addition to GVD, chromatic aberration of objectives leads to pulse distortions. Specifically a radius-dependent group delay is introduced (Kempe et al., 1993; Netz et al., 2000). Therefore, different radial portions of the beam across the pupil of the objective arrive at different times at the focal region and cause a temporal broadening of the pulse. This results in lower peak intensity in the focal region (Fig. 10-12). This effect is also referred to as PTD.

Table 1 summarizes the important characteristic parameters for objectives recommended for nonlinear microscopy and for the optics inside the LSM 510 NLO including the microscope stand. The dispersion parameters were calculated based on the material data and the thickness of each optical element on axis. For comparison, the dispersion parameter for the optics within the LSM 510 NLO including the microscope stand and the AOM is 7500 $fsec^2$.

The variation of the dispersion parameter for different beams across the pupil of the objective is listed in the forth column. It can be seen from both parameters that the pulse broadening is nearly independent of the particular objective used and the position of the beam across the pupil of the objective. The pulse broadening due to the GVD of the different objectives and the LSM 510 NLO including the microscope stand and the AOM was calculated for 100 fs pulses at a wavelength of 800 nm and is listed in column 5.

The pulse broadening due to PTD is listed in the last column. The PTD was calculated for the whole optical setup, since it depends on the chromatic aberrations of the complete system including the LSM 510 NLO and the microscope stand for a wavelength of 800 nm. The PTD effect is negligible if the pulse length is not much shorter than 100 fsec. It can be seen that the special IR corrected objectives show a smaller PTD in comparison to the standard UV/VIS corrected objectives.

Objective	Working Distance [mm]	Dispersion Parameter $[fsec]^2$		Pulse broadening factor of 100 fsec pulse	Max. PTD [fsec] (*)
		On axis	Variation		
IR-Achroplan 40x/0.8 W	3.61	1714	± 20	1.14	-3
IR-Achroplan 63x/0.9 W	2.00	1494	± 15	1.11	-9
Plan Neofluar 40x/1.3 oil	0.20	2328	± 30	1.23	9
Plan Apochromat 20x/0.75	0.61	1531	± 10	1.12	10

Table 1 Summary of specific parameters for objectives recommended for 2-Photon applications. All data were measured at 800 nm. (*) Propagation time difference (PTD) is calculated for the whole optical setup including the LSM 510 NLO and the microscope stand.

Negative values indicate that pulses at the edge of the pupil are delayed with respect to pulses traveling on axis.

With regard to transmission, objectives for biomedical applications traditionally have been optimized for UV and visible range imaging. Thus, many are not corrected for transmission of NIR or IR light. The transmission of an objective depends strongly on the design of the antireflection coating. Typical transmission curves for two different objectives are shown below. The Achroplan series is coated with UV/VIS-antireflection coating giving optimal performance from 350 nm to approximately 800 nm. In contrast, the IR-Achroplan series has a VIS/IR coating that enables high transmission from 420 nm to 1400 nm. The Achroplan objectives are best for multiphoton applications, where excitation is below 850 nm and the fluorescence from the specimen is in the ultraviolet or lower visible wavelength range.

These criteria describe the imaging parameters for many ion indicators, nuclear dyes such as DAPI or Hoechst, Blue Fluorescent Protein, and Fluorescein or Alexa 488. The IR-Achroplan objectives are best for multiphoton applications requiring an excitation wavelength greater than 850 nm and where the fluorescence emission is above 420 nm (Fig. 10-13). These latter criteria apply to many dyes, such as DiI, and many of the fluorescent proteins, such as CFP, GFP, YFP, and dsRed. However, some important fluorochromes can be imaged well with either objectives.

Rhodamine, for example, has a multiphoton absorption peak at 840 nm, and like Alexa 568 it can be excited well at 800 nm. Thus, the examples are given to indicate ideal imaging criteria; however, non-optimized parameters can often be used to produce high-quality images.

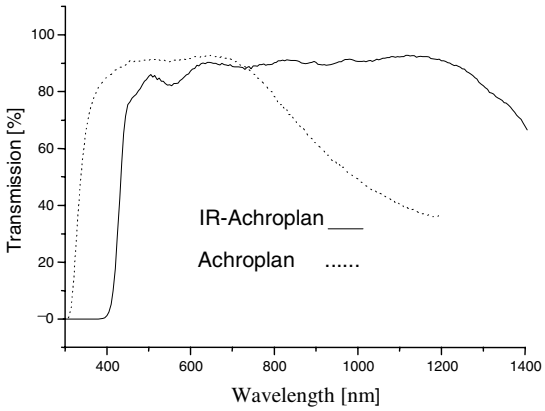


Fig. 10-13 Typical transmission curves of an IR-Achroplan and an Achroplan.

While the Achroplan objectives clearly provide the best transmission curves with the longest working distance, other objectives have proven to be useful for multiphoton imaging. For instance, the Plan-Apochromat objectives have favorable properties for multiphoton imaging as Table 1 indicates. In addition, the C-Apochromat 40x W/NA 1.2 and its 63x counterpart have proven to be very effective objectives, perhaps due to their high NA and collection efficiency. Moreover, these objectives have a 0.23 and 0.25 mm working distance, respectively, and can be very useful for collecting large 3-D data sets. Unfortunately, these objectives begin to decline in transmission at around 840 nm, so they are not optimal for dyes with more red-shifted excitation wavelength absorption maxima, but can often be used without concern for the lost photons

10.3.5 Choosing Fluorescent Probes for MPLSM

Presently, there are thousands of different fluorochrome derivatives and conjugates that can be used to study a variety of biological events. Many of these fluorochromes have been optimized for single-photon excitation and scientists are just beginning to concentrate on developing optimized fluorochromes for multiphoton imaging applications (see Albota et al, 1999). Furthermore, it is difficult to predict whether or not a particular dye will be useful for multiphoton imaging and many of us have relied on trial and error while a greater understanding of the physical principles of multiphoton excitation is evolving. That being said, there are some important parameters that one can use to help predict which dyes may be best for a particular application.

Similar to the way one would choose a fluorescent probe for conventional fluorescence microscopy, the best fluorochromes for multiphoton imaging are the ones that efficiently absorb light, reliably emit fluorescence, and are photostable. Values such as the multiphoton cross-section and the fluorescence quantum yield are important to consider when choosing a fluorochrome.

The multiphoton cross-section is a measure of how strongly a fluorochrome absorbs photons at a given wavelength and is expressed in units of 10^{-50} cm^4 sec/photon or 1 GM (Goeppert-Mayer unit, after the scientist who first predicted multiphoton absorption, Maria Goeppert-Mayer). A list of cross-sections can be found in Xu (2000) or at

www.uga.edu/caur/dyechart.pdf

The web-site shows a collection of excitation cross sections for various dyes. Currently available dyes have multiphoton cross-section values ranging from 40-200 GM (see Xu, 2000).

These values are only an indication of how well a dye will perform in biological imaging using MPLSM. A one to one correlation between the cross-section and dye performance cannot always be drawn. Measurements of multiphoton cross-section are usually performed with the dye dissolved in organic solvents or at non-physiological conditions and the local environment of a dye may influence absorption and emission characteristics. Also, these values are determined at an optimal pulse-width that cannot always be achieved at the level of the specimen. Although the cross-section is a valuable first approximation of how a dye will perform, trial and error provides the truest test for a particular application.

The multiphoton cross-section not only provides information about how well a particular molecule is excited by pulsed NIR light but also indicates the multiphoton absorption peak, a value that has been surprisingly difficult to predict. While it may seem logical that the two-photon absorption peak should be twice the one-photon peak, this has not proven to be the case. An emerging rule of thumb is that the peak is usually shorter (more blue-shifted) than twice the one-photon absorbance peak (Xu, 2000). However, one should also keep in mind that the brightest signal may not be obtained at the predicted excitation peak but at the point where the excitation peak and the power peak of the laser overlap.

Blue/Cyan dyes
Alexa 350 (780 nm-800 nm)
Hoechst (780 nm-800 nm or 900-1100 nm)
DAPI (780-800 nm or 900 nm-1100 nm)
CFP (800 nm-900 nm)
Green dyes
Oregon Green (800 nm-860 nm)
Alexa 488 (800 nm-830 nm)
GFP (840 nm-900 nm)
BODIPY (900-950 nm)
FITC (750 nm-800 nm)
DiO (780 nm-830 nm)
Yellow Dyes
YFP (890 nm-950 nm)
Orange dyes
DiA (800 nm-860 nm)
Red dyes
DiI (830-920 nm)
Rhodamine B (800 nm-860 nm)
Alexa 568 (780 nm-840 nm)

Table 2 Recommended multiphoton excitation wavelengths for common dyes.

For tunable Ti: Sapphire lasers, there is a peak output at or near 800 nm, with less average power produced at lower and higher wavelengths; as a result, it is possible to excite a given dye at a non-optimal wavelength by using more power. However, more power may also mean more heat or bleaching, so one should be careful to optimize the signal-to-power ratio.

In addition to how well a molecule absorbs light, the best molecules to choose are the ones that also efficiently release light as the molecule relaxes back to the ground state. The fluorescence quantum yield is a measure of the proportion of emission photons that are shed per excitation event.

In general, this value is the same for one- or multiphoton excitation and values for common fluorochromes can be found in Haugland (1996).

As mentioned above, photostability is an important factor when choosing a dye. Photobleaching is minimal in out-of-focus regions using MPLSM, but bleaching still occurs at the point of focus. In fact, some dyes that are relatively stable using one-photon excitation, have more rapid bleaching rates using MPLSM (Patterson and Piston, 2000). This phenomenon is not fully understood, so the bleaching rate of dyes of interest should be tested for each application. In cases where rapid bleaching occurs, we have found that ProLong (Molecular Probes, Eugene, OR) has helped stabilize the signal in fixed samples.

Table 2 is a rough guide of fluorochromes recommended by the Biological Imaging Center at Caltech. Currently, there is no comprehensive reference for all dyes excited by two- or three-photon excitation for the user to refer to for information on the peak absorbance wavelength or relative brightness of a given dye.

The information given below is based on empirical observation and does not reflect a serious, scientific investigation of all dyes, nor does it indicate the exact optimal excitation wavelength for each dye in question. Instead, this list is offered as a rough guideline of what is known to work for various imaging applications and the user is encouraged to empirically determine which dyes are most useful for their own applications. Clearly the success of any fluorescence imaging experiment relies on how well the target is labeled. The same is true for MPLSM.

10.3.6 Samples your mother should have warned you about

While multiphoton excitation has many advantages, there are also some disadvantages to using high intensity NIR or IR light for fluorochrome excitation. Probably one of the biggest disadvantages is the heat that is generated via the absorption of NIR photons by water. The local heating that is caused can affect vital cellular processes, such as cell replication (König et al., 1996) and other growth abnormalities in sensitive specimens. Thus, great care should be taken to maximize PMT settings so that minimal power levels can be used while imaging. Particular care should be taken when imaging cells or specimens that contain molecules that absorb light such as melanin.

In some cases, the absorption of NIR photons can cause dramatic local heating effects and cause cells to explode. This effect has been seen frequently in zebrafish embryos, but can be avoided by blocking melanin production with chemical agents, such as PTU or by using albino embryos.

Other molecules, such as salt crystals, can also focus light within tissues and intensify heating effects. This can also lead to imaging artifacts, perhaps due to signal produced via second harmonic generation (SHG). In Fig. 10-14 Calcium Carbonate crystals in the inner ear of the frog have produced blotchy artifacts.

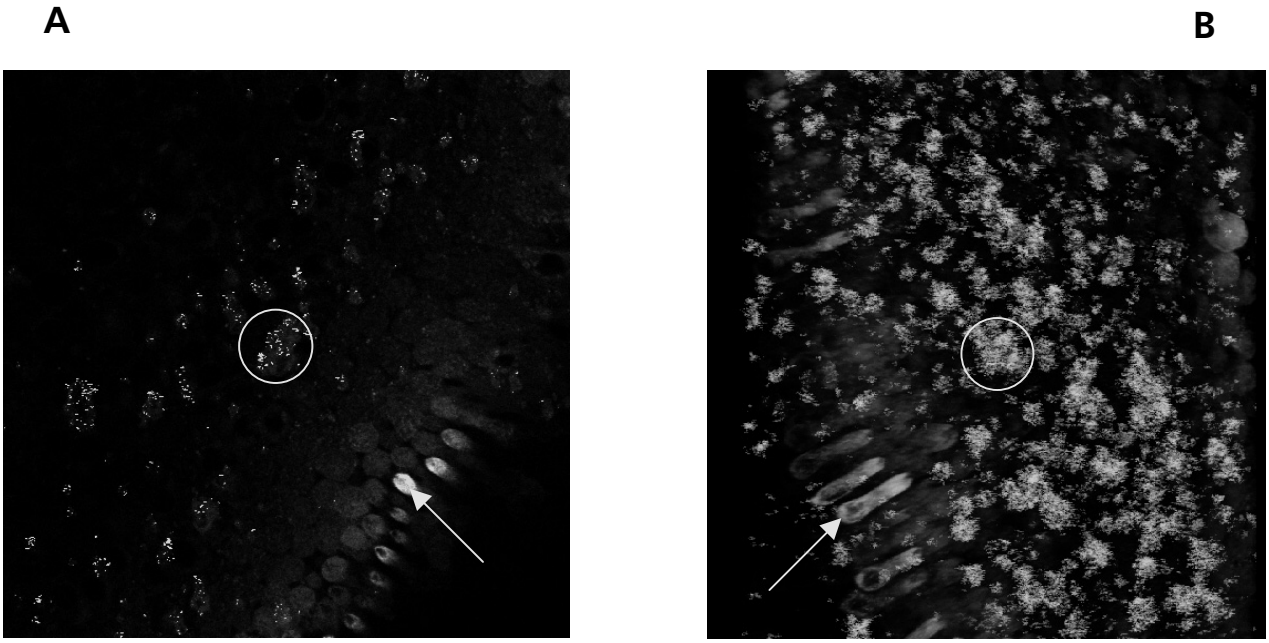


Fig. 10-14 Multiphoton image (A) and stack of images (B) of the frog otolith. Cells have been fluorescently stained by soaking the freshly prepared specimen in FM164 (arrow). Signal produced by the artifact is shown inside the circle. Sample courtesy of Bill Roberts, University of Oregon.

The artifacts completely obscure the three-dimensional reconstruction shown in Fig. 10-13/**B**.

This effect is not specific to Calcium Carbonate and has been seen in other samples, such as those with crystals of salt from evaporated sea water (data not shown).

Artifacts can also occur in fixed tissue. Local heating effects can cause bubbles to form in the mounting media or induce localized photochemistry in some mounting medias. It is important to test all mounting medias before use for multiphoton imaging. Fig. 10-15 shows an image where a large artifact is produced in an image using multiphoton excitation. In this example, Epon-araldite resin, generally used for EM embedding, was used. This is particularly bad for multiphoton imaging as it quickly becomes heated using focused NIR light.

Similar effects can been seen if oil and water are mixed, such as on the top of a coverslip when different immersion objectives are used on the same sample. Similar to the way water sizzles in a frying pan, steam will be produced from the focused heating of the oil and image artifacts result. Usually, these types are artifacts are dynamic as the water evaporates or the oil breaks down and the artifact may grow as you image for a longer time. It is important to clean the coverslip of your samples well with ethanol to dry off the water before mounting in oil or to clean off the oil before mounting in water.

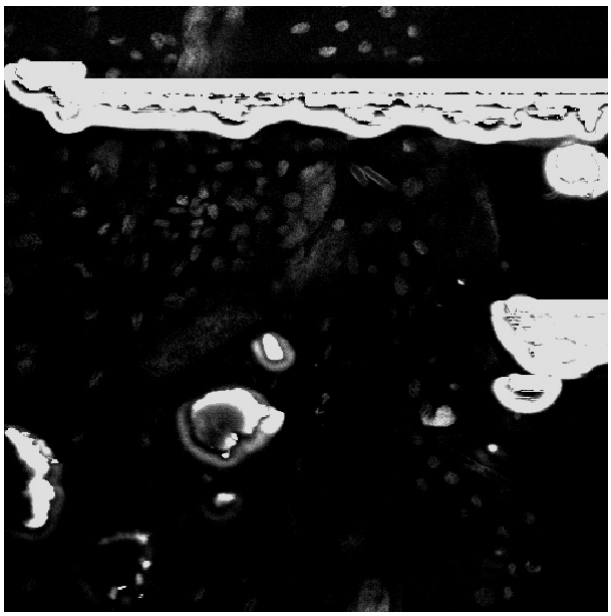


Fig. 10-15 Bubbles have formed in the mounting media of a fixed sample of a moth embryo. The embryo was labeled with DiA, but the specific signal is obscured by the artifact produced. Sample courtesy of Patty Jansma, University of Arizona.

10.4 Troubleshooting Checklist

Several key things can go wrong when imaging that will result in poor or no image being produced. Below is a checklist to help the user produce the best images possible.

10.4.1 No image being produced

Laser mode-lock:

Check to see if the laser is turned on and that it is mode-locked. Be sure that the ML-CW-ß-lock switch on the MIRA controller is set to ML. There should be no filled boxes next to the CW——— on the MIRA controller LED readout. If the readout shows CW □□□□□□□ then the laser is not mode-locked and no multiphoton excitation can occur. Similarly, there should be a nice bell-shaped curve on the oscilloscope from the Rees Spectrum Analyzer when the laser is mode-locked. A sharp spike anywhere on the Rees Analyzer display is an indication of CW operation. To eliminate CW breakthrough, optimize mode-lock as described in Section 10.3.2.

AOM not on or too little laser power:

Check to see if the laser is turned on in the laser control panel and that the box is checked next to the percent transmission slider. If no image is seen and the laser is on, check the gain on the PMT and then increase the laser power. Determine if the wavelength of the laser matches the wavelength setting on the AOM. Check to see if increasing the percent transmission increases the power to the sample (use the monitor diode) in a linear way. If not, the AOM settings may need to be reset using the "linearize AOTF" macro.

Inefficient fluorochrome excitation (see 10.3.5):

Use a control slide to determine if the problem is in the sample. If excitation is seen with other samples, optimization of the laser wavelength may be necessary.

Laser power to the sample. Verify that the laser is focused through the objective by placing a white card on the microscope stage and removing the objective.

A bright red spot approximately 5-7 mm wide should be seen on the white card.

If no light is seen, then the beam may not be focused into the scan head or the shutter of the AOM may be closed. Check to see if there is a normal level of power going into the scan head. If no beam can be found entering the scan head, the shutter or the AOM may not be working and a service engineer should be called. If there is a beam after the 1 m lens, there may be poor alignment going into the scan head. Report this to a service engineer or attempt realignment under the guidance of a service engineer or expert user. Partial illumination of the card reflects poor alignment and the beam should be realigned into the scan head.

Non-linear power attenuation:

If changing the percent transmission leads to non-linear power attenuation, the AOM can be linearized using the AOTF fit macro. By opening this macro and following the instructions, one can verify the linearization of any of the laser lines and then run a linearization protocol to reoptimize the settings. Be sure the laser is set to the appropriate wavelength before running this macro. Choose only the NIR wavelength to optimize the AOM attenuation for the Ti: Sapphire laser.

10.4.2 Poor image quality

Lines or spaces across the image:

This can result from an unstable mode-lock. CW breakthrough appears as blank lines in the image. Q-switching will result in wavy lines in the image. The laser must be adjusted to produce stable mode-lock. Check the output from the Rees Spectrum Analyzer and the Optima on the Laser power box. Re-adjust the laser to a stable mode-lock is produced (see 10.3.2.2).

Poor fluorescence sensitivity:

This can result from many factors or a combination of factors. These include the following:

Correct wavelength (see 10.3.5):

Be sure that the laser is set at the best wavelength for the fluorochrome being examined. Empirical testing may be necessary to determine the best wavelength. Tuning to a different wavelength may also help to reduce autofluorescence from some tissues.

Poor Beam Alignment into the scan head (see 10.3.3.2):

Remove the objective and look at the reflection of the beam onto a white card. The beam should be round and even without any flat edges. Peak the alignment of the beam into the scan head.

Poor transmission through the objective (see 10.3.4):

Many objectives are corrected for work with UV and visible range excitation and have poor transmission of photons in the near IR.

Poor power output from the laser (see 10.3.2):

Using a power meter check to see if the power output for the laser is in a normal range. The laser is supplied with details about the optimum power output. A very poor power output may be a sign of misalignment of the laser and will affect mode-locking. To peak the alignment, first try to make small adjustments in M7. This will usually restore power to a peak level. You may also need to readjust the BP2 GVD prism and/or the slit width to mode-lock the laser. Make cautious and conservation changes as it is possible that re-alignment of the laser may uncouple the laser from the AOM.

☞ DO NOT CHANGE THE HORIZONTAL POSITION OF THE SLIT.

Bandwidth too large (see 10.3.2.4):

Minimal bandwidth (6-8 nm) is preferred for this system. Although the laser is capable of producing a mode-locked pulse of up to 12 nm, a pulse with a band width this large will be subject to significant Group Velocity Dispersion (GVD) and result in a longer pulse length at the sample than a pulse with a shorter bandwidth. A longer pulse length at the sample will result in less excitation per unit of average overall power.

Sliders/polarizers in the path:

DIC sliders and polarizers in the path can reduce excitation and emission sensitivity. Remove sliders or slide them to an open position.

Poor sensitivity in fixed samples:

Fixatives and mounting medias can sometimes limit the sensitivity of fluorescence detection or increase autofluorescence reducing the signal-to-noise ratio. For multiphoton excitation, the mounting media can also reduce the peak intensity of the laser deep into the sample. Empirical determination of the best fixative and mounting media for a particular application may be necessary.

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