



MetaFluor[®]
Analyst

MetaFluor Analyst[™] **User's Guide**

For Use with

MetaFluor[®]

*Version 7.0 for
Microsoft Windows XP[®]*

1030 2203-02

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Chapter 1

MetaFluor Analyst Overview

MetaFluor Analyst was designed for high throughput, routine work with ratiometric and non-ratiometric fluorescent dyes. It enables fast loading of image sequences, convenient region of interest (ROI) drawing, and fast plot extraction. Other features of MetaFluor Analyst include:

- Background subtraction
- Ratio and DF/F calculation with calibration
- Bleach correction
- Temporal smoothing
- Image plane filters including Fourier domain filtering, which enables selective measurement of mitochondrial derived fluorescence.

The main window of MetaFluor Analyst contains the workplace, the menu, and the toolbar. On the workplace unlimited number of Image Windows and graphs can be present simultaneously. The software is controlled by the user through the menu system: using the main menu and toolbar of the main window, while certain functions can be accessed by right-clicking active windows.

Experiments, or image sequences, created in MetaFluor are opened in image windows. Image windows contain each frame of an image sequence in sequential order. Each Image Window displays one wavelength of a measurement. If an experiment file (*.inf) contains more than one wavelength, each wavelink sequence is opened in a separate image window. These image windows are automatically linked together — linked image windows share ROIs and can be processed together (for example — parallel filtering, truncating, ratio calculation, overlaying, IMD coloring).

Using this Guide

The following sections provide information and instructions on using MetaFluor Analyst:

- | | |
|------------------|---|
| Section 1 | Introduction provides an overview of the functionality of MetaFluor Analyst, as well as hardware requirements. |
| Section 2 | Image Window provides an overview of Image Window features. |
| Section 3 | Menu Commands and Toolbar contains descriptions of the Menu and toolbar commands available in MetaFluor Analyst. |
| Section 4 | Graph Window explains how to use the graphing and charting features of MetaFluor Analyst. |
| Section 5 | Preferences explains how to use set default preferences to use in MetaFluor Analyst. |
| Section 6 | Advanced Image Processing details advanced filtering and processing techniques available in MetaFluor Analyst. |

Hardware Requirements

Operation system	Microsoft Windows 9X, NT, 2000 (NT or 2000 recommended)
Hardware	<ul style="list-style-type: none">• Hard drive space 10 MB (for executable with all dlls)• CPU Pentium class• Memory—256MB recommended
Video	True color (24 or 32 bit; high resolution recommended)

Chapter 2

Image Window

Experiments, or image sequences, created in MetaFluor are opened in image windows. Image windows contain each frame of an image sequence in sequential order. Each Image Window displays one wavelength of a measurement. If an experiment file (*.inf) contains more than one wavelength, each wavelength sequence is opened in a separate image window. These image windows are automatically linked together — linked image windows share ROIs and can be processed together (for example — parallel filtering, truncating, ratio calculation, overlaying, IMD coloring).

A sample Image Window is shown below:

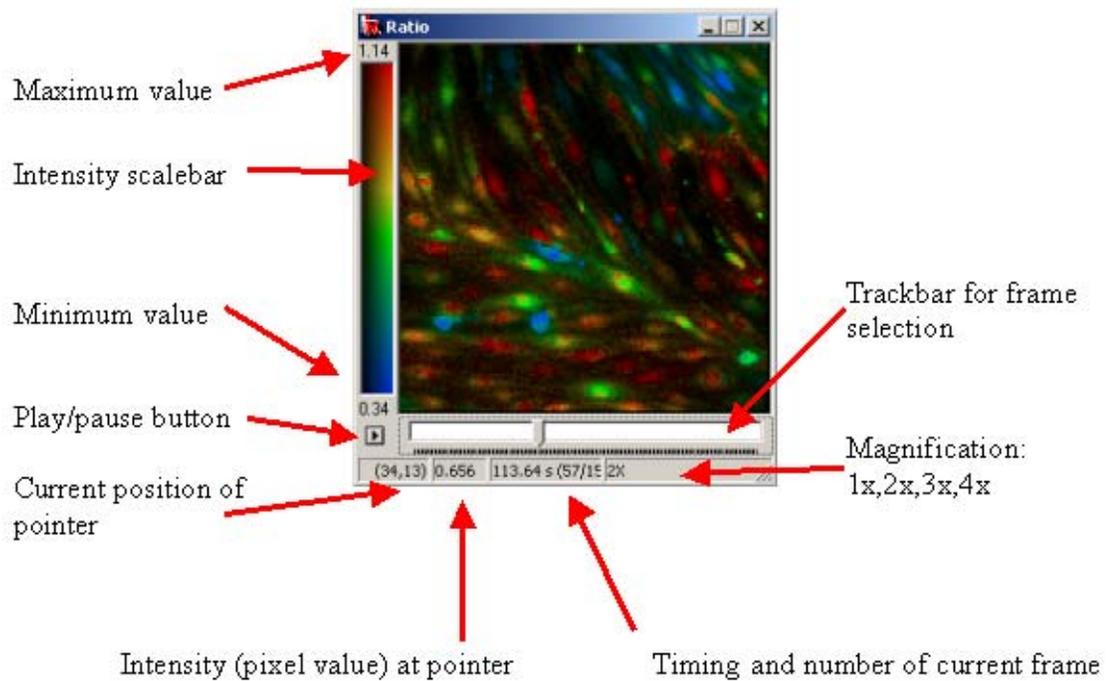


Image Window Features

Intensity Scalebar

Displays the intensity levels of the selected frame. Right-click within the Intensity Scalebar to choose from the following options:

- Copy ScaleBar to Clipboard – copies a bitmap of the Intensity Scalebar to the clipboard.
- Grayscale – displays the image in grayscale.
- Pseudocolor – displays the image with an arbitrarily assigned color display.
- IMD color – displays the image in Intensity Modulated Display (IMD) mode. IMD color is only available with IMD images.

Play/Pause Button

Press to start playback of image sequence. Press again to pause. Playback is looped back at the end.

Set the playback frame rate in the Misc tab of the Preferences dialog box .

Trackbar

To move between frames, drag the slider on the trackbar, use the scroll button on your mouse, or press the arrows keys on your keyboard.

Image

Right-click within an image to choose from the following options:

- Zoom – Select from the following magnification levels: 1x, 2x, 3x, or 4x
- Gradient – Select from the following image colors: grayscale, red, blue, green, or IMD coloring. IMD color is only available with IMD images.
- Set Scaling – Opens Set Scale dialog box.
- Attach IMD Intensity – Opens the Attach IMD Intensity dialog box.
- Attach overlay image – Opens the Attach overlay image dialog box.
- Overlay Text – Opens the Overlay Text dialog box.
- Copy image – Copies bitmap of image to the clipboard.
- Copy image with all ROIs – Copies bitmap of image to the clipboard with ROIs included.
- Show image info – Shows image information of the active image. The image information can be edited, copied, and pasted into *.ia files.
- Erase ROI – Deletes active ROIs.
- Erase all ROIs – Deletes all ROIs.

Working with the Image Window

Opening an Image

Image windows contain each frame of an image sequence in sequential order. Each Image Window displays one wavelength of a measurement. If an experiment file (*.inf) contains more than one wavelength, each wavelength sequence is opened in a separate image window. These image windows are automatically linked together — linked image windows share ROIs and can be processed together (for example —parallel filtering, truncating, ratio calculation, overlaying, IMD coloring). There can be multiple sets of linked Image Windows in the workplace.

The timing of the measurement is loaded from the *.inf file. If this file contains zero clock, then only the images after the last zero clock are loaded. If only one wavelength is loaded, the recording time saved in the image is taken as the timing of the measurement, with the first frame as t=0s. A timing of 1 frame per second is assumed during bitmap import.

New Image Windows can be created by duplicating an active one using the *Duplicate Image Window* command in the Edit menu. If the duplicate is linked to the original, it will inherit any existing ROIs. This command is useful to create a backup of an experiment before doing any image processing.

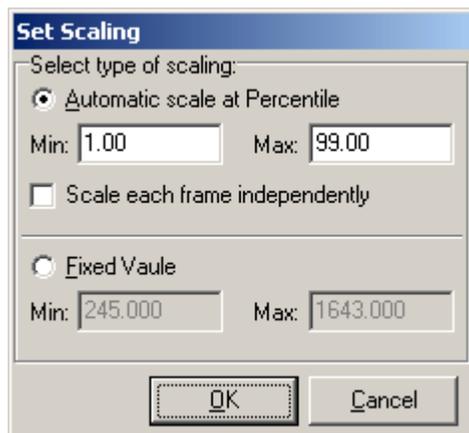
The contents of an Image Window (with ROIs and image information) can be saved into and loaded from the MetaFluor Analyst format (*.ia). This format does not support multiple wavelength — each wavelength must be saved separately.

The pixel values of an image sequence are stored as 16-bit signed integer values (range: -32768 – 32767). Image Windows can be switched into fixed point real mode using the *Convert to* command in the Calculate menu. This command stores images as 16 bit signed integer values, but they display as real values in a range of $-32768/\text{factor} - 32767/\text{factor}$, with $1/\text{factor}$ precision. The image is automatically switched to real mode during certain calculations, like DF/F, ratio. For best results, the factor of fixed point real image storage has to be optimally set in the **Preferences** dialog box according to the dynamic range of calculations. All functions work on both integer and real values.

Visualization

The Image Window shows the first frame of the image sequence by default. The intensity of the image is shown by the intensity scalebar at the left side of the image. The pixel intensities between a minimum and maximum value are translated to the colors of the scalebar linearly if the image is displayed in color. These values are indicated above and below the scalebar, and can be changed using the *Set Scaling* command in the edit menu.

Set Scaling Command



Automatic scale at percentile: calculates the minimum and maximum values of intensity scalebar from the histogram of image series taking intensities at the given percentiles.

Scale each frame independently: the histogram and percentiles are calculated for each frame, instead of the whole image series.

Fixed value: Scales intensity between the entered minimum and maximum values.

Intensity scaling and coloring also have default values for the following conditions: loaded image (normal image), ratio, and DF/F image. These default values can be set in the Preferences dialog box. For example, setting the default intensity scaling and coloring of radiometric Image Windows to certain fixed values can assure that different image sequences will appear in comparable coloring in routine work.

The content of a linked Image Window can be overlaid onto the active window one using the *Attach Overlay Image* command in the Visualize menu. The overlay frame from the linked image is always the same corresponding frame as the active window.

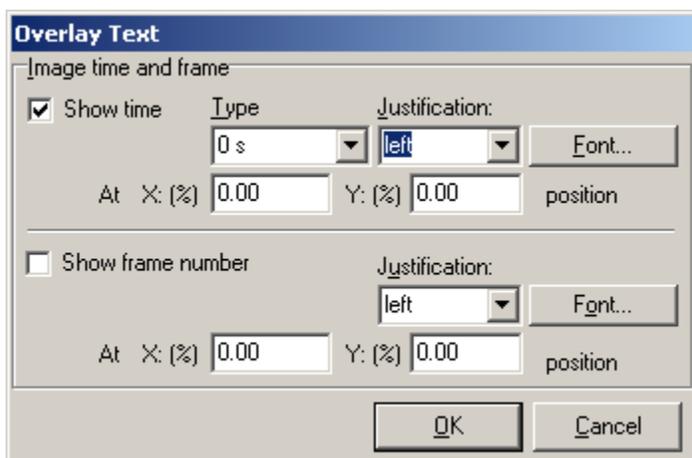
During overlaying, the image shows its original gradient and intensity values, so it does not affect stored pixel values or ROI averages.

The Intensity Modified Display (IMD) technique helps discrimination of objects in the image after ratio or DF/F calculation. If IMD coloring is enabled, the hue of the pixel is determined from the scaled pixel intensity value of the given Image Window and the brightness is determined by the attached IMD intensity image. The result of a ratio calculation automatically appears IMD colored, with the intensity (brightness) values derived from the average of the numerator and the denominator. IMD coloring can also be set up by manually attaching an IMD intensity image using the *Attach IMD Intensity* command in the Visualize menu. Use the following steps to produce an IMD colored DF/F image:

- 1 Duplicate an image using the *Duplicate* command in the Edit menu.
- 2 Use the *DF/F* command in the Calculate menu on the duplicated image.
- 3 Attach the original image as an IMD intensity using the *Attach IMD Intensity* command in the Visualize menu.

IMD coloring can be switched off by setting the *Gradient* in the popup menu of the Image Window to any other value. IMD coloring does not affect stored pixel values or ROI averages.

Overlay Text Command



Use the *Overlay Text* command in the Visualize menu to print the frame number and frame time on the image. The numbers are formatted using the *Type* and *Justification* combo boxes and the *Font* button. The position of the numbers can be set in X (%) and Y (%) fields. If the Image Window is resized, then the absolute positions of the overlaid text do not change.

Image processing

Image processing functions are accessed through menu commands and the Toolbar. If an image processing command involves more than one wavelength of an experiment (i.e. Multiple Image Windows), then the Image Window to process can be chosen from the list of Image Windows linked to the active window. The results of the command will overwrite the original image sequences, except for ratio calculation, where the result appears in a new window. If the original image is needed later, then duplicate it before processing using the *Duplicate* command in the Edit menu.

ROI drawing

Use the ROI Region of Interest toolbar commands to edit regions of interest on the active image. Select linear or area ROI editing from the toolbar then click and drag on the image to create a ROI. Double-click to set the ROI. The ROIs are visible and can be edited in any linked Image Windows. To move a ROI, select the ROI arrow button on the toolbar, then click and drag the ROI. You can also use the ROI tools to copy, paste, or delete the active ROI. Selecting or deleting a ROI also selects or deletes the corresponding plot if a Graph Window is present; deleting the plot deletes also the corresponding ROI.

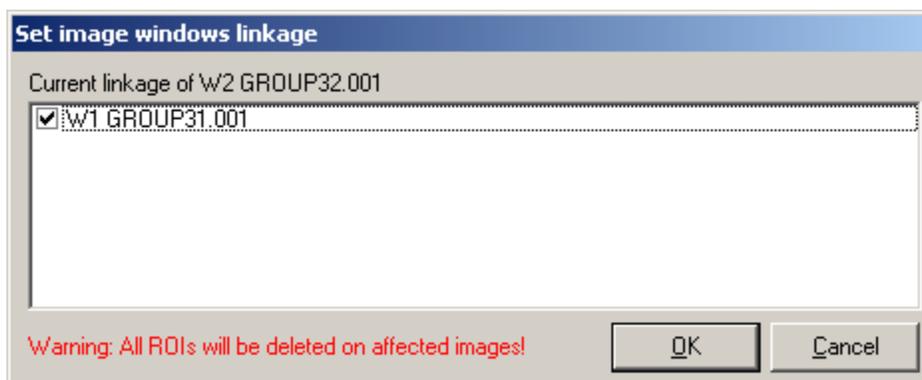
Data Evaluation

Use the Plot command in the Calculate menu to calculate a graph from the average of ROIs on a selected image. Other data evaluation commands included Correlation plot, Advanced plot/ pixelwise deviation, and Linescan Window. These commands are performed on the active ROI and running any of these commands will automatically refreshed any Image Window content including ROI settings. The color of each ROI is represented by the same color on a graph.

Setting the linkage of Image Windows

Opening a multi-wavelength MetaFluor experiment in MetaFluor Analyst opens a set of linked Image Windows that are processed simultaneously. You can also manually link Image Windows to enable them to be processed simultaneously using the *Set Linkage* command in the Edit menu. You can link separately loaded or imported Image Windows, if they have the same size and numbers of frames. Image Windows can be duplicated with or without their links using the *Duplicate* command in the Edit menu. There can be multiple sets of linked Image Windows on the Workplace.

Set image window linkage Dialog Box



The linkage of an Image Window can be changed using the *Set Linkage* command in the Edit menu. The *Set Image Windows linkage* dialog box shows all the Image Windows already linked (selected) or matching the size criteria to be linked (unselected). Other sets of linked Image Windows that can also be linked together are listed in one row.

Chapter 3

Menu Commands and Toolbar

Use the MetaFluor Analyst menu commands and toolbar to open, manipulate, and analysis experiment files created in MetaFluor.

Menu Commands

The following menu commands are used available in MetaFluor Analyst:

File menu

Open experiment

Opens a previously saved Experiment file (.inf) into an image window on the desktop for viewing, processing, or analysis.

If the image sequence descriptor (.inf) file contains zero clock, then only the images after the last zero clock are loaded. (The timing of the measurement has to be continuously incrementing, without zeroing.)

The images in an image sequence must have the same format (size and depth).

Save

Saves a modified .inf file to the .ia Image Analyst format.

Close current image window

Closes the selected window. Dependent windows such as graphs are also closed with this command.

Close all

Closes all open windows.

Export current image (series)

Choose to exports pixel values of the current selected frame or whole image series to the following formats:

.TXT file of current frame (1D array)

.TXT file of current Region of Interest (ROI) (1D array)

Movie (series of *001.bmp files for each frame)

Movie (series of *001.bmp files for each frame)

Load ROIs

Opens the Load ROIs dialog box. Use to load previously saved Regions of Interest from disk. The *.roi binary format contains corner coordinates of all ROI polygons.

Save ROIs

Opens the Save ROIs dialog box. Use to save Regions of Interest to disk. The *.roi binary format contains corner coordinates of all ROI polygons.

Save Plot Data

Runs the Plot command and saves the plots from the resulting Graph window to ASCII file in tab delimited format chart. The first column contains the timing in seconds.

Exit

Closes all open images on the desktop and quits MetaFluor Analyst.

Edit Menu

Copy image

Copies current frame of active Image Window to clipboard in the following formats:

- As image
- As image with active ROIs
- As image with all ROIs
- As 3D graph — ASCII, tab delimited

Copy plot data

Copies plots from the active Graph Window to the clipboard in ASCII, tab delimited format.

Copy all ROIs

Copies ROI polygons to clipboard in the *.roi binary format. The following choices are available:

- 4Copy active ROI
- 5Copy all ROIs

Paste ROIs

Pastes ROIs from the clipboard to the active Image Window and all linked windows, if applicable.

Delete ROIs

Deletes active ROIs.

Set linkage

Opens the Set Image Windows linkage dialog box. Shows linked Image Windows and enables you to manually link Image Windows so they can be processed simultaneously.

Duplicate image windows

Duplicates Image Window. The new Image Window can be linked or unlinked to the original.

Truncate frames

Opens the Truncate Frames dialog box for cutting or truncating frames.

Merge image series

N/A

Set Image Scaling

The intensity of the image is shown by the intensity scalebar at the left side of the image. The pixel intensities between a minimum and maximum value are translated to the colors of the scalebar linearly if the image is displayed in color. These values are indicated above and below the scalebar, and can be changed using the *Set Scaling* command.

Calculate Menu

DF/F

Calculates DF/F normalization for each pixel (normalized to the change from the first frame). The result is stored as real values. The Image Window intensity scaling and coloring changes to the DF/F preset

(see Properties dialog box).

Ratio

Runs dialog box for entering numerator and denominator for ratio calculation from the linked Image Windows to the active one. The result is stored as real values.

Calibrate

Enables you to run calibration equations on selected ROIs.

Convert to

Sets the pixel format:

- Integer (default): signed, 16 bit (-32768 - 32767)
- fixed point real, 16 bit, the place of the decimal is determined by a factor given in the Preferences dialog box.

Linescan

Linescan Windows contains a time-space (or linescan) diagram. This diagram consists of pixel values along a linear ROI projected onto the y-axis in every time point (x-axis).

Linescan Window can be created from an Image Window, which contains an image sequence and defined ROI(s), by using the *Linescan* command in the calculate menu. The X-axis of the diagram is the timing of the measurement in seconds; the Y-axis is the projected pixel intensities along the actual linear ROI.

Plot

Graphs can be created from Image Windows that have defined ROIs using the *Plot* command in the Calculate menu. The X-axis of the graph represents the timing of the measurement in seconds; the Y-axis represents the average values of pixel intensities covered by the ROI.

Advanced Plot

Pixelwise deviation : Similar to Plot, but plots not the average of pixel intensities over a ROI, but the standard deviation of pixel intensities. Can be used for determination of punctuate pattern versus smooth (however working with highpass Fourier filters gives usually better results).

Correlation

The correlation window compares the pixel values of the two wavelength channels by assigning a point in the graph at the intensity value B and intensity value A coordinate for each pixel of a given ROI.

Filter Menu

Note: See Section 6, *Advanced Image Processing*, for more information about the Filter menu commands.

Running median

Filters the image sequence by running a median filter in time over each pixel sequence.

Running average

Filters the image sequence by running a average filter in time over each pixel sequence. Places the average in the middle of the given window. Deletes window/2 frames from the beginning and end.

Bleach correction

Fixes photobleaching or leakage of fluorescent dye. It does this by manipulating the data by the extrapolation of a fitted exponential function on a part (baseline) of the trace.

Temporal derivation

Calculates temporal derivative by convolution of image in time with the given column vector kernel. Can

be used for any kind of convolution process in time (temporal filtering). The result is multiplied by the given time factor.

Subtract background

Removes image background data based on a number of variables.

Absolute value

Takes the absolute value of pixels.

Custom Affin Transformation

Manual UnMove

Manually correct image movements that occurred during measurements.

2D Fourier

Configure a filter that acts as a user defined optical transfer function

- **Setup DFT filter:** See DFT filter designing in chapter
- **Run DFT filter:** Runs the previously configured DFT filter on the active Image Window

2D Median

Used for noise reduction and extracting or suppressing certain image details.

2D Fixed Kernel Convolution

Makes kernel convolution filtering in image plane, frame by frame with some predefined low- and high-pass filter kernels.

2D Custom Kernel Convolution

Makes kernel convolution filtering in image plane, frame by frame with user-defined kernel.

Visualize Menu

Attach IMD Intensity

Sets the color-coding of actual Image Window to intensity modulated pseudocolor coding (IMD) by asking for a linked Image Window for intensity values. The pseudocoloring (hue) shows the pixels values of the active image; the brightness shows the pixel values of attached image. IMD coloring can be switched off by setting the **gradient** in the popup menu of the Image Window to any other value. Does not affect stored pixel values or ROI averages.

Attach overlay image

Overlays the actual Image Window content with a chosen, linked Image Window content (always frame-by-frame). The overlaid image shows in its original gradient and intensity values. Change the original image settings to affect overlaid image. Does not affect stored pixel values or ROI averages.

Overlay Text

Enables you to print the frame number and frame time on the image. The numbers are formatted using the *Type* and *Justification* combo boxes and the *Font* button. The position of the numbers can be set in X (%) and Y (%) fields. If the Image Window is resized, then the absolute positions of the overlaid text do not change.

Options Menu

Colors

Stores the ROI coloring. 16 colors are cycled during ROI drawing, set the custom colors to modify ROI coloring. The color of an existing ROI cannot be changed, only the newly created ones are affected.

Preferences

Opens the Preferences dialog box.

Window Menu

Cascade

Displays image windows stacked and cascading from the top left to the bottom right of the screen.

Tile

Displays image windows edge to edge.

Minimize All

Minimizes all open image windows to the bottom of the workspace.

Restore All

Restores all minimized images.

Show Image Info

Shows image information of the active image. The image information can be edited, copied, and pasted into *.ia files.

Show ROI Info

Shows the parameters of the active ROI.

Help Menu

About

Opens splash screen with version and copyright information about MetaFluor Analyst.

Toolbar

Tools available on the MetaFluor Analyst toolbar represent the most commonly used menu commands. See the menu command description for more information about a specific tool. The following tools are available:

Tool	Menu	Command
	File	Open Experiment
	Edit	Set linkage
	Edit	Duplicate image window
	Edit	Truncate frames
	N/A	ROI selection and movement
	N/A	Drawing of linear ROI
	N/A	Drawing of area ROI

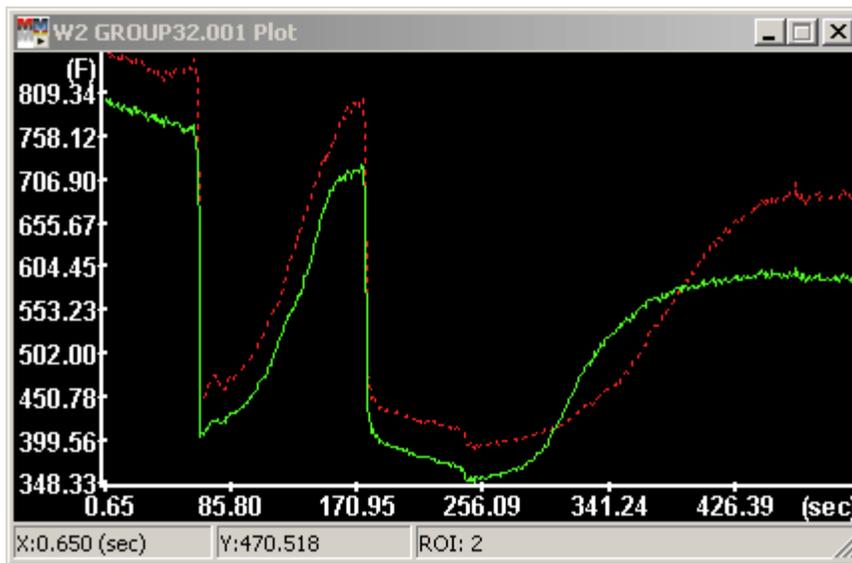
Menu Commands and Toolbar

Tool	Menu	Command
	Edit	Copy active ROI
	Edit	Paste ROI
	Edit	Delete active ROI
	Calculate	Plot
	Calculate	Correlate
	Calculate	Linescan
	Filter	Subtract background
	NA	Subtract background with the active ROI set for subtraction frame-by-frame
	File	Save plot data
	Edit	Copy plot data

Chapter 4

Graph Window

Graphs can be created from Image Windows that have defined ROIs using the *Plot* command in the Calculate menu. The X-axis of the graph represents the timing of the measurement in seconds; the Y-axis represents the average values of pixel intensities covered by the ROI. An (F) sign at the end of y-axis indicates that if the plot was created from a normal intensity-type (non radiometric) Image Window. A (R) sign indicates it was created from a radiometric Image Window. A sample Graph Window with created from a normal intensity-type (non radiometric) Image Window is shown below:



Right-click within Graph Window to choose from the following options:

- Show as DF/F of intensity view – calculates DF/F normalization trace-by trace. If the signal-to-noise ratio of the image sequence is poor, DF/F normalization of plots rather than pixels is favourable. This is an in plot calculation and does **not** affect image data. A (DF/F) sign at the end of the y-axis indicates that this mode is active.
- Resample when saving or copying data – resamples the data along the x-axis during clipboard or file output.
- Plot Cross Section – Creates a new Graph Window with ROIs (by their numbering) on the X-axis, and plot values in the current pointer position on the original plot corresponding to each ROI.
- Copy Plot Data – Copies plot data to clipboard in tab delimited, ASCII format for use in spreadsheet editors.
- Save Plot Data – Saves plot data to clipboard in tab delimited, ASCII format for use in spreadsheet editors.
- Erase Active Plot And ROI – Deletes plot below the pointer and deletes the corresponding ROI.

Plots are identically colored with the corresponding ROIs. Deletion of a ROI deletes the corresponding plot, and deletion of a plot deletes the corresponding ROI. A Graph Window remains linked with the Image Window used to create it, so any change of ROIs or pixel values refreshes the plot. If the plot does not refresh after an image processing command, click the ROI with the ROI arrow. To force a refresh. If this does not work, close the graph and create a new one.

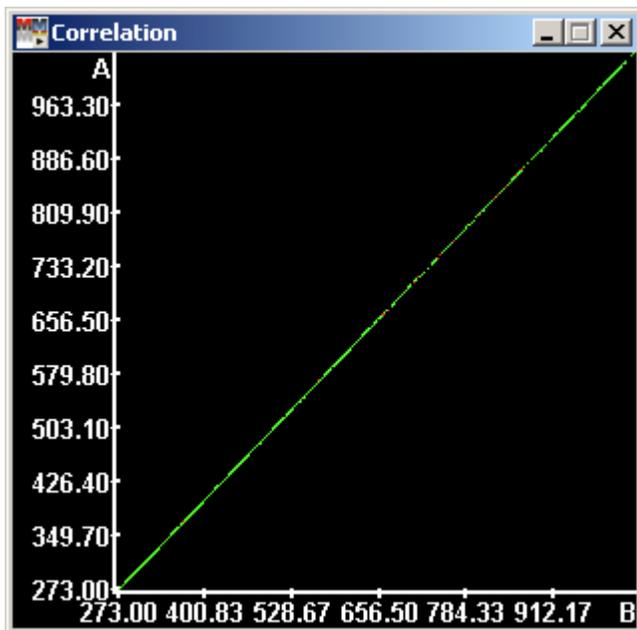
The axes of the graph are scaled automatically between the extremities of the plot data. The appearance of the graph cannot be changed, except for sizing the Graph Window.

The Graph Window was designed only for a short look at the data, and helping in the selection of correct ROIs (e.g.: manual selection for healthy cells). The plots has to copied into a spreadsheet program or plotting software for further processing. This is done by right-clicking a graph and selecting *Copy plot data*, then pasting into a spreadsheet program. Saving into ASCII files is also supported (*Right-click/Save plot data*). The output format is tab delimited ASCII, the first column contains the time values in seconds, and the remaining columns are the plot y-values in the order of ROI numbering. There is no header row. Plots also can be copied as bitmaps.

The timing of a measurement is often affected by the limited speed of camera or data storage system, so however the exact time of the exposition is recorded, the interval between image acquisition can change. This makes it difficult to average multiple measurements in a spreadsheet editor in a row-by-row base. For this reason, data resampling was implemented in the Image Analyst. This can be enabled by right-clicking the graph and selecting *Resample when Saving or Copying*. If enabled, the plotted data is resampled in time, using linear interpolation between the recorded points, yielding time points divisible by the given value in seconds at *Preferences dialog box / Data Saving / Resample / X Data resample interval*. In the *Preferences* dialog box the default value of the *Resample When Saving or Copying Data - Popup menu point* can be also set. This option affects only the copied or saved data, not the one appearing in the plots, nor the image data.

Correlation Graph

The correlation window compares the pixel values of the two wavelength channels by assigning a point in the graph at the intensity value B and intensity value A coordinate for each pixel of a given ROI. A sample correlation window is shown below:



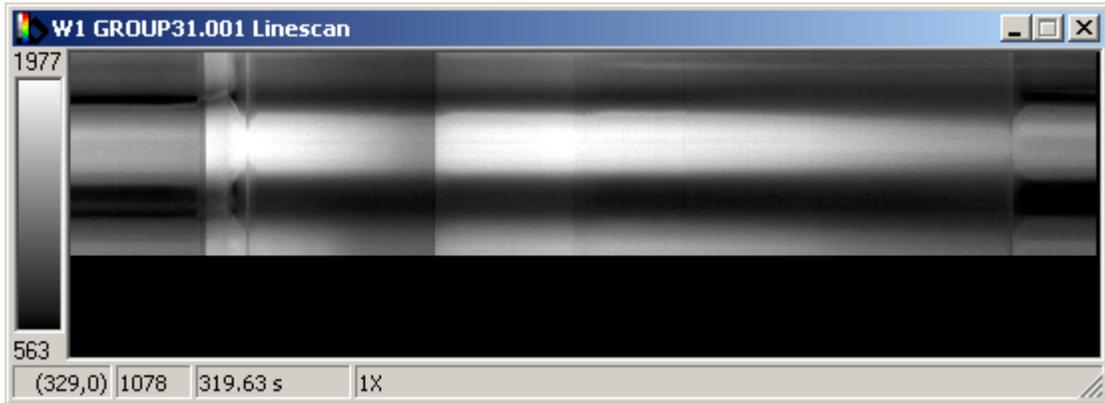
Correlation Graphs can be created (using the Correlate command in the Calculate menu) from a set of linked Image Windows, where ROIs are present. The Correlation Graph is a kind of Graph Window, so its usage and popup menu system is similar. For statistical analysis the data has to be copied into the appropriate statistical software.

Note: Correlation Graphs are useful for the investigation of colocalization of two different fluorescent dyes in the same subcellular structures. If the two dyes are colocalizing, then the same pixels are bright for both dyes. Conversely, the same pixels are dark for each dye. This means there is some correlation between the pixel intensity values, which appear in the graph as a line originating from the origin. If there is no correlation the points are scattered in the graph.

Linescan Window

Linescan Windows contains a time-space (or linescan) diagram. This diagram consists of pixel values along a linear ROI projected onto the y-axis in every time point (x-axis).

Linescan Windows can be created from an Image Window, which contains an image sequence and defined ROI(s), by using the *Linescan* command in the calculate menu. The X-axis of the diagram is the timing of the measurement in seconds; the Y-axis is the projected pixel intensities along the actual linear ROI. A sample Linescan window is shown below:



The content of a Linescan Window can be resampled by right-clicking in a Linescan window and selecting *Resample*, however in this case the appearing diagram is affected by the resampling. Right-click in a Linescan window to view additional options.

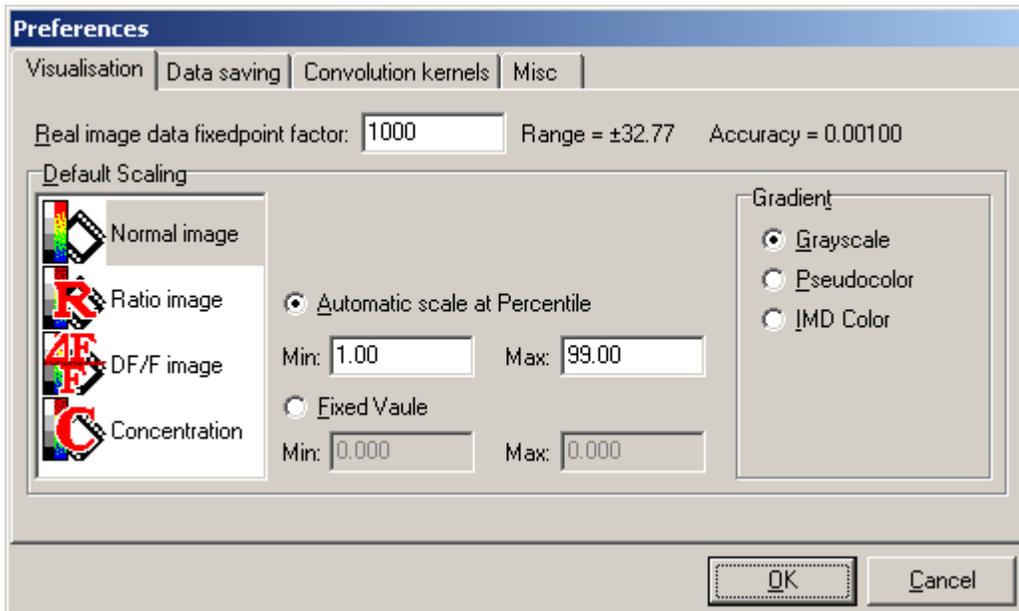
Note: The Linescan command is useful for visualization of wave-like propagation of signals (together with temporal derivation). On time-space diagrams, the stabile structures are drawn straight, while the dislocating are drawn curved or sloped paths. The characteristics or speed of movement can be determined in this way. However, further work or other software is needed to determine slopes. The *Edit / Copy Image / as 3D graph* – menu command can be used for exporting data through the clipboard to other software.

Chapter 5

Preferences dialog box

This section contains information about the Preferences dialog box. To open the Preferences dialog box, Select Preferences from the Options menu.

Visualization Tab



Real image data fixedpoint factor

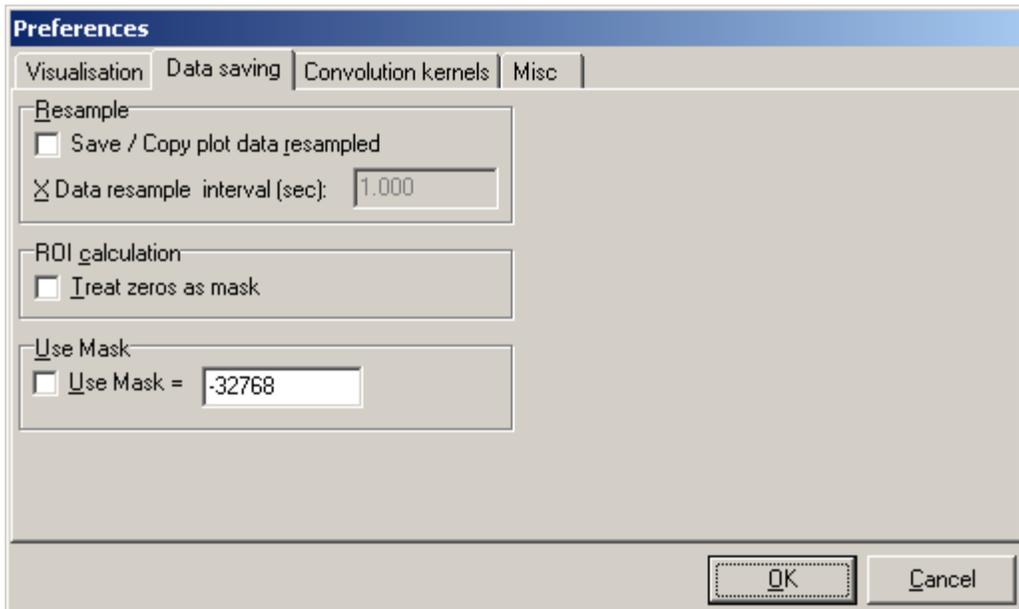
Factor for determination of the place of the decimal at fixed point real storage on 16-bits.

Default Scaling

Set the default values of Image Window intensity scaling in some common conditions: Normal image, Ratio image, DF/F image, and Concentration. Use the following procedure to set default scaling:

- 1 Select the type of Image Window by left clicking on the list on the left.
- 2 Set scaling properties.
- 3 Set the coloring for the selected Image Window type.

Data Saving Tab



Resample

- **Save / Copy plot data resampled:** Select for resampling plot data by default during saving or copying to clipboard. (See in details at Graph Windows section.)
- **X Data resample interval (sec):** Gives the base of resampling — each new data point will be in a time point divisible with this value.

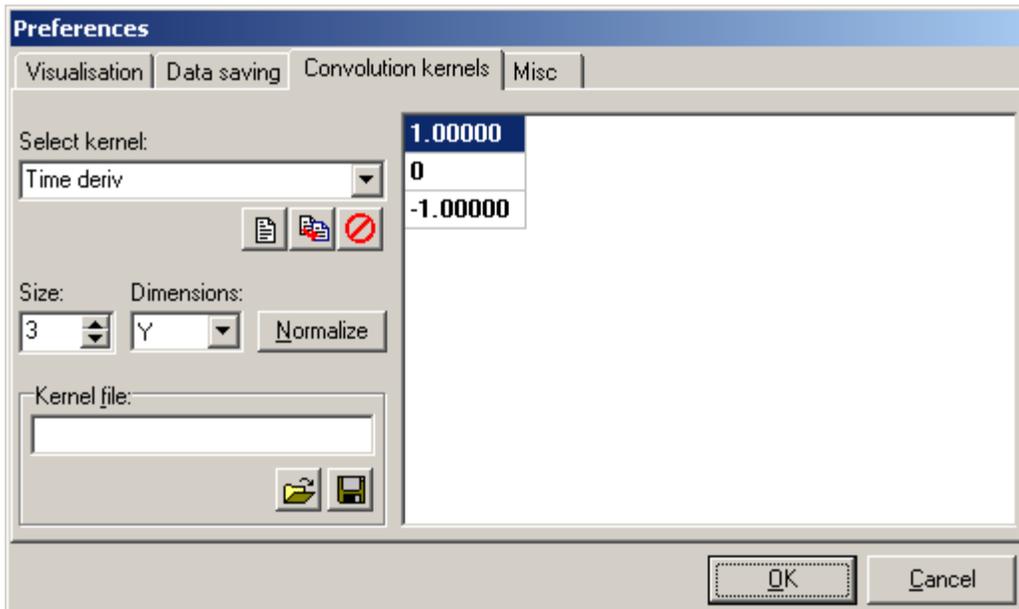
ROI calculation

Treat zeros as mask: If selected than the software neglects (assumes masked) pixels with zero value during ROI average calculation. Use for calibrated images(to exclude out of calibration pixels).

Use mask

If selected, the pixels with the given value are masked at visualization, appearing in black. (Use with pseudocoloring).

Convolution kernel Tab



Select kernel

Kernels are stored based on their names. To browse kernels use the *Select kernel* field. The selected kernel appears in the edit region of the dialog box. Kernels are stored in a binary file (*.ckr) listed in the *Kernel file* field. The changes are automatically written in the kernel file upon clicking *OK* button. To open another kernel file, or save in a new name, use the icons below the *Kernel file* field.

Kernel icons below the *Select kernel* field:

- **New kernel:** First enter a new name into the *Select kernel* field, then click *New Kernel* button. A new 3x3 nullmatrix kernel is created.
- **Copy kernel:** First enter a new name into the *Select kernel* field, then click *Copy Kernel* button. The active kernel is copied, and appearing in the new name in the list.
- **Delete kernel:** Click *Delete Kernel* button to delete selected kernel.

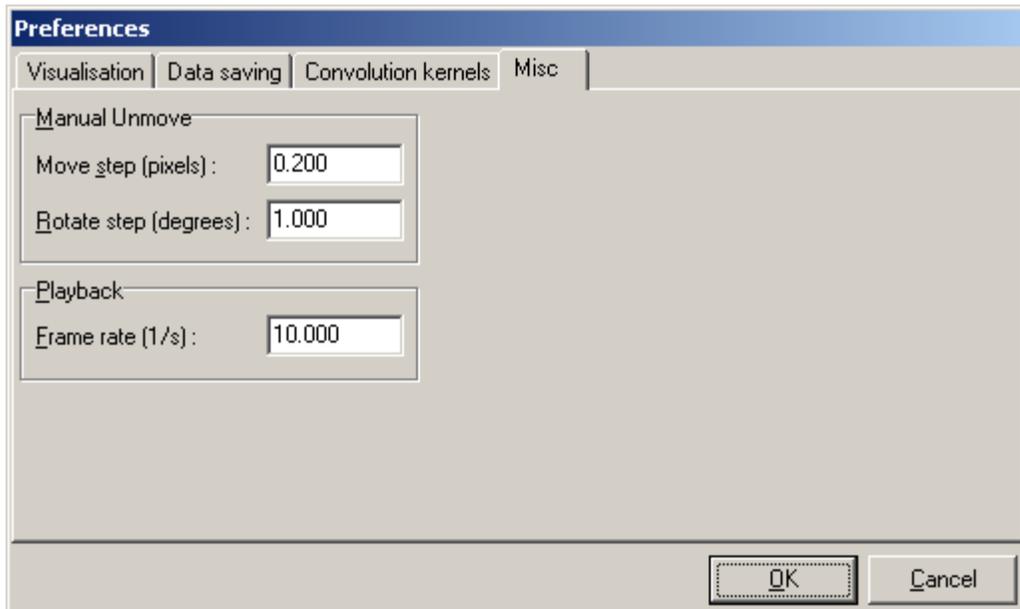
Size, Dimensions

MetaFluor Analyst uses quadrature (XY), column (Y), and row (X) matrices as kernels with the size given at the *Size* field.

Normalize

Divides the values of the kernel with the sum of absolute values in the kernel.

Misc Tab



Manual Unmove

For setting the step sizes during manual unmovement:

- **Move step:** X and Y direction step size in pixels, for fine movements it has to be smaller than 1.
- **Rotate step:** Rotation step size in degrees, should be smaller than 1 for fine movements.

Playback

Set the playback rate of frames in the Image Window (in frames per second).

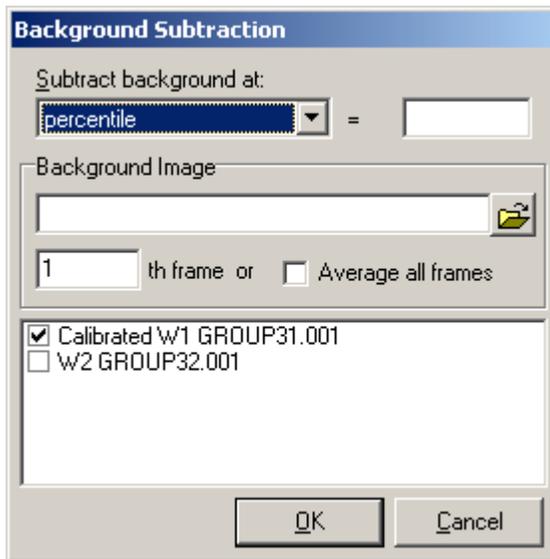
Chapter 6

Advanced Image Processing

This section contains advanced image processing techniques available in MetaFluor Analyst.

Background Subtraction

Select Subtract Background from the Filter menu to open the Background Subtraction dialog box, as shown below:



Subtract background at

Options for type of background to subtract:

- percentile – the background is calculated for each frame, from the histogram of the frame: the intensity at the given percentile. (For subtract percentile calculated from the histogram of the whole image sequence, set a minimum value at *Scale Intensity* dialog box of Image Window, and subtract the value appearing below the scalebar as **value**.)
- value – user defined value to subtract. Use negative number here to add background.
- ROI average by frames – Subtracts the average of pixel intensities covered by the given ROI, calculated frame-by-frame.
- ROI average by measurement - Subtracts the average of pixel intensities covered by the given ROI, calculated for the whole image sequence.
- Image – Subtracts the image(s) entered in the *Background Image* field.

Background Image

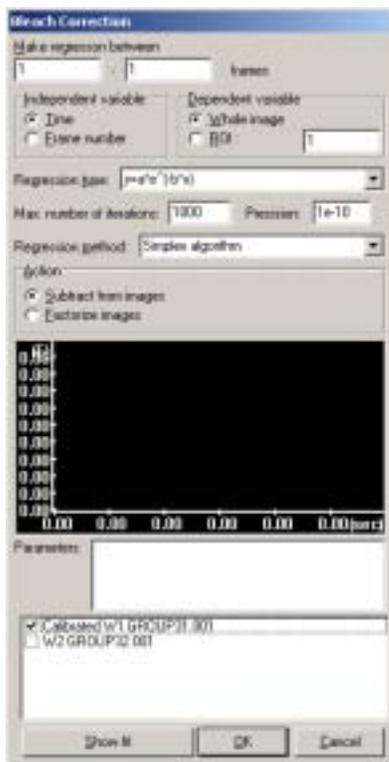
Active if the *Image* option is set in the *Subtract Background at* field. Enter the background file name here, or click the browse button to locate the file. Only images with same size can be subtracted. If a MetaFluor measurement (*.d.inf) file is given (with more than one channels), then images with the matching wavelength will be subtracted. If individual image or image series is entered, then it will be subtracted from any selected Image Window. Since background measurements can contain image sequences, the number of frame to subtract can be specified (in *th frame* field), or all loaded frames can be averaged for subtraction (*Average all frames*). In all other aspects, loading of measurements applies for background loading. When using MetaFluor measurements for background subtraction, all other Image Windows for background subtraction has to be selected in the list at the bottom of the dialog box.

Note: For elimination of uneven background illumination and CCD cold/hot spots, it is ideal to record and subtract a background image of a cell-free area. If there is temporal irregularity in background intensities, like external scattered light entering the microscope, or photobleaching of background dye fluorescence, subtraction of ROI average calculated frame-by-frame can correct for these potential artifacts. If the image contains a relatively large amount of background (cell free areas), then subtraction at given percentile can be also used as background subtraction

Bleach Correction

The Bleach Correction command fixes photobleaching or leakage of fluorescent dye. It does this by manipulating the data by the extrapolation of a fitted exponential function on a part (baseline) of the trace. Manipulation means that the first frame remains unchanged, and in the following frames: either the fitted function is subtracted, or the frames are factorized by the relative change from the first fitted value. In this way the drop in the trace due to photobleaching can be eliminated.

Select *Bleach Correction* from the Filter menu to open the Bleach Correction dialog box, as shown below:



Make regression between

The range of the frames--where to fit the exponential.

Independent variable

The independent variable can be the time or frame number. If the timing is irregular, then frame number based bleach correction is suggested, while if the decrease of fluorescence is due to dye leakage, the time based bleach correction is favorable.

Dependent variable

The exponential function is fitted onto the values of the dependent variable; this can be the average intensity of the whole frame, or the average of a given ROI.

Regression type

The two parameter exponential ($y=a * e^{(-b * x)}$) decays to the zero. It can be used with well-subtracted

background. The three parameter one ($y=a \cdot e^{(-b \cdot x)+c}$) serves more general purpose.

Regression method, number of iterations, precision

The Simplex algorithm works the most reliably. Regression method, number of iterations, precision should not be changed.

Action

- The difference of fitted function from its first value is subtracted from the frames
- Frames are factorized by the relative change of fitted function from its first value

Show Fit

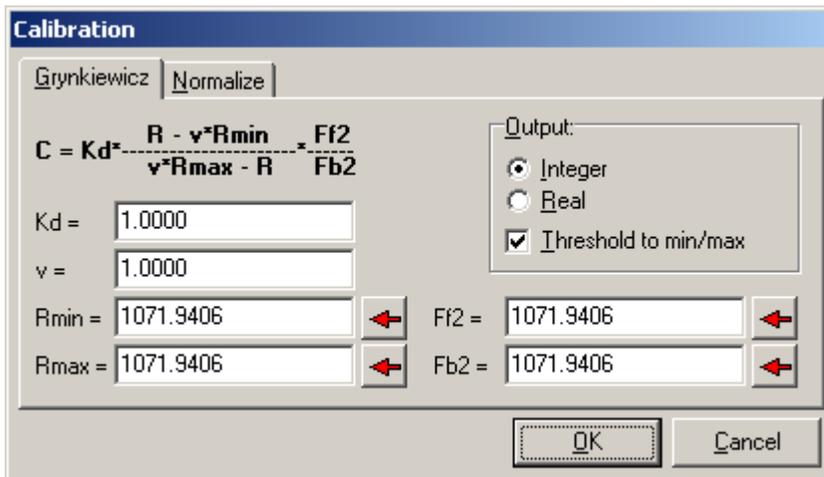
Calculates the regression, shows the parameters, and displays the results on the graph (in red).

OK

Calculates the regression and process the images sequence as specified at *Action* filed.

Calibration

Select *Calibration* from the Calculate menu to open the Calibration dialog box, as shown below:



Grynkwiewicz

Equation to calibrate ratiometric (or non ratiometric) fluorescent dyes:

$$C = K_d \frac{R - vR_{\min}}{vR_{\max} - R} \frac{F_{f2}}{F_{b2}}$$

Where the concentration (C) is calculated from the dye dissociation constant (K_d), the maximal and minimal ratio values (R_{\max} and R_{\min}), the intracellular viscosity (use 1 for water, usually between 0.6-0.8), and from the free and bound dye fluorescence at the wavelength in the denominator (F_{f2} and F_{b2}).

For non ratiometric calibration use F_{\max} and F_{\min} fluorescence intensities instead of R_{\max} and R_{\min} , taking F_{f2} and F_{b2} equal to 1.

Normalization

Proportional scaling for general purposes:

$$C = k \frac{F - F_{\min}}{F_{\max} - F_{\min}}$$

Where the concentration (C) is calculated from a arbitrary constant (k), and the maximal and minimal fluorescent intensities (F_{\max} and F_{\min}).

Entering values

To acquire calibration values from Image Windows, press the red arrow button at each field, after the corresponding ROI has been selected in the active Image Window.

Output

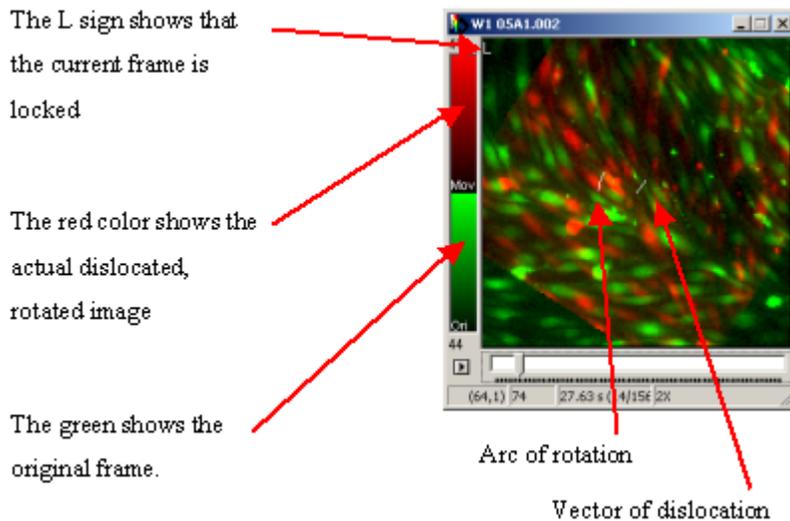
The order of magnitude of the calibrated values are highly affected by K_d and k . The result can easily be out of the range of the used 16-bit data storage type. Pay attention of output box, where the data storage type is shown of the Image Window to be calibrated. Set the data type of the calibrated Image Window (real or integer) here. For example: if calibration ratio (real values) to intracellular calcium is nM, switch output type to integer.

Threshold min/max

If selected, pixel values out of the range between R_{min} and R_{max} (or F_{min} and F_{max}) are masked by calibrating as zero. Select *Treat Zeros as Mask* at *Preferences* dialog box / *Datasaving* to enable masking during ROI calculation. Also select *Use mask (=0)* to show masking in the Image Windows.

Manual Unmove

Manual Unmove was designed to correct movements that happened during measurements. The stabile positioning of structures for ROI calculation in the image sequence can be ensured in this way, in the case of uniform dislocations. Each frame can be freely dislocated or rotated by the numeric keypad arrows (see popup menu). The movement of frames is linearly interpolated between the set positions; the ones after the last set position are remaining in the last set position. If a frame position is set then an "L" sign (Locked) appears on the upper left corner of the image. The first frame appears in green. The current dislocated, rotated frame appears in red, so any dislocation can be easily noticed. To apply the Manual Unmove command to an image, select Manual Unmove from the Filter menu. A sample Image Window after the Manual Unmove command has been applied is shown below:



To unmove an image after apply the Manual Unmove command, perform the following steps:

- 1 Drag the trackbar to the last correct position.
- 2 Lock the last correct frame by pressing "-" on Numeric keypad.
- 3 Go to the first dislocated frame (or if the dislocation is slow, linear, into any further position).
- 4 Correct position by using the arrow keys in Numeric keypad for dislocation ("1" and "3 for rotation). The red image has overlapping the green one.
- 5 Refine unmovement with repeating previous point in the interpolated frames.

By pressing "-" on the numeric keypad the lock on a frame can be toggled, if the frame is not locked,

then its position is interpolated between the two neighboring ones. The frame movement can be reset by pressing "*" on the numeric keypad, in this case it returns to the non dislocated position and becomes locked. You can also right-click in the Image Window to access the Unmove commands.

The unmovement can be finalized by pressing Enter or cancelled with Esc.

Filtering in Time

Filters acting in time are one dimensional filters, filtering a sequence of pixels in the same position in image plane, along the image sequence. The running average (*Filter / Running Average* menu command) and running median (*Filter / Running Median* menu command) are for noise reduction. Both are working with a window size (in frames), which can be entered in the appearing dialog box. The average or median is calculated in the given sized window from the beginning of the image sequence shifting the window position one-by-one frame to the end. The result is placed back to the middle of the window. The starting and ending, window size / 2 frames are discarded.

Temporal derivation (*Filter / Temporal derivation* menu command) is a temporal, 1 dimensional kernel convolution filter. Capable for filtering any given column vector kernel, thus can be used for not only temporal derivation, but for lowpass (or highpass) filtering in time for data smoothing. The calculated values are multiplied by timefactor, to help data to remain in the dynamic range of the 16-bit data storage (like temporal derivatives can be very small sometimes compared to the intensity values). Kernels can be designed in the Preferences dialog box.

All filtering processes are in place operations.

Temporal derivation or DF/F calculations are very sensitive for (or amplifying) noise. That is why sometimes good derivative or DF/F images can be obtained after thorough filtering in time, but also in image plane. Median filtering can be used for removing hairy, spark-like noise from the measurements.

Temporal derivation is used for enhanced visualization of propagating wave, like intracellular Ca²⁺ waves. Drawing a linescan diagram of a temporally differentiated image sequence clearly shows the wavefront of the wave. Kernels for derivation:

$$\begin{bmatrix} 1/2 \\ 0 \\ -1/2 \end{bmatrix} \quad \begin{bmatrix} -1/12 \\ 8/12 \\ 0 \\ -8/12 \\ 1/12 \end{bmatrix} \quad \begin{bmatrix} 1/60 \\ -9/60 \\ 45/60 \\ 0 \\ -45/60 \\ 9/60 \\ -1/60 \end{bmatrix}$$

Filtering in Image Plane

Filtering in image plane is used for noise reduction; however, it can also be used for extracting or suppressing certain image details. 2D median filter (*Filter / 2D Median* menu command) works in image plane with the given window size, and the result is placed in the middle of the rectangular filtering window. 2D median filtering suppresses spots appearing the images. Image plane kernel convolution filtering (*Filter / 2D Fixed* or *Custom Kernel Convolution* menu command) can serve as averaging, high and lowpass filtering, spatial derivation according to the given kernel matrix. Kernels can be designed in the Preferences dialog box. Fourier domain filtering can apply a user defined optical transfer function on the images. The result is similar to low or highpass kernel convolution, but the filter design for filtering in Fourier domain is more versatile, and the filtering spares the edges of the images. Practically by appropriate filter function design in Fourier domain the desired sized objects (image details) can be selectively amplified or suppressed. This can range from the correction for uneven background to extraction of fluorescence signal coming from small organelles, like mitochondria.

Kernels for convolution filtering:

$$\text{Averaging: } \begin{bmatrix} 1/9 & 1/9 & 1/9 \\ 1/9 & 1/9 & 1/9 \\ 1/9 & 1/9 & 1/9 \end{bmatrix} \text{ X-derivation: } \begin{bmatrix} 1/2 & 0 & -1/2 \end{bmatrix} \text{ Y-derivation: } \begin{bmatrix} 1/2 \\ 0 \\ -1/2 \end{bmatrix}$$

Or similar, larger derivative kernels like at temporal filtering.

Filtering in Fourier domain

This section of the user manual assumes the knowledge of image representation in complex Fourier domain, and optical transfer functions (See optics or system control textbooks).

This filter acts as a user defined optical transfer function (\hat{H}). The software allows to design the Fourier domain representation of the optical transfer function with the following limitations (for simplicity and manageability), assuming, that the image is quadrate, and the origin is in the middle of the image:

The transfer function does not distinguish between directions.

The transfer function does not affect phase, only amplitude, thus the transfer function is real.

Thus the transfer function imagined in 3 dimensions: as transmission factor, function of x and y spatial frequencies (wave numbers) looks like an axial symmetric object, a cone in the case of a lowpass filter.

Thus the \hat{H} is represented by a real filter function ($f_{\text{filter}}(\mathbf{k})$), which describes the filters transmission factors as a function of wave numbers. $f_{\text{filter}}(\mathbf{0})$ is at the origin, and \hat{H} is built up by circumrotating f_{filter} around the origin, ensuring the same filtering in all spatial directions:

$$\hat{H}(k_x, k_y) = f_{\text{filter}}(\sqrt{k_x^2 + k_y^2})$$

The filtering is done by the real multiplication of the complex Fourier transform of the image \mathbf{G} , and reverse Fourier transformation, that is why affects only amplitude and not phase:

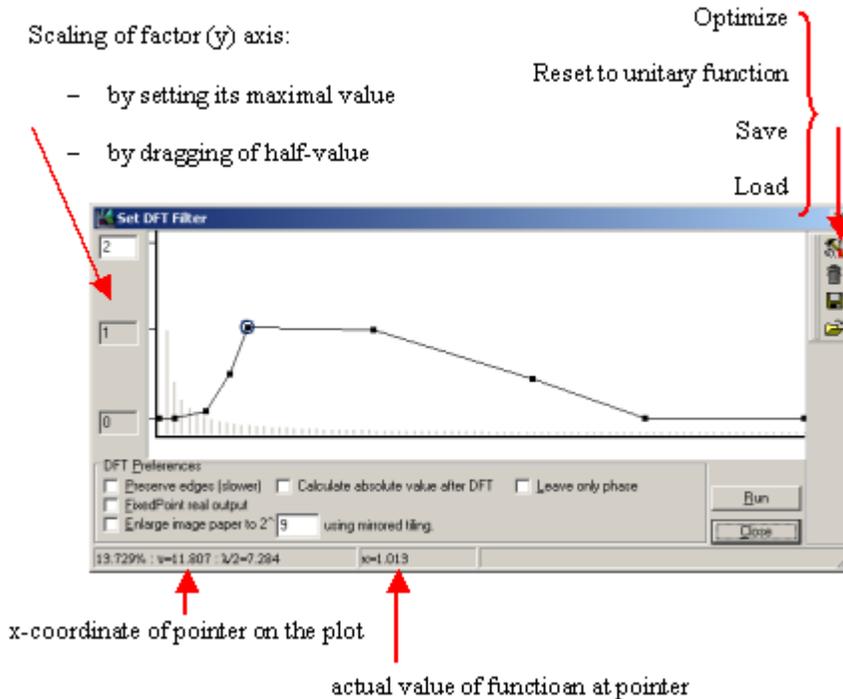
$$G' = F^{-1} \{ F\{G\} \cdot \hat{H} \}$$

The advantage of using f_{filter} for optical transfer function design is that the diagram of f_{filter} (See filter function design) clearly shows, that which sized image details are transmitted (amplified) or rejected. A filter function for transmission of given sized image details is designed for a certain size and resolution image. Using different size of images with the same filter function will different filtering.

The uniformity of the filter in different directions is satisfied only if the image is quadrate. Since this is not always the case, or if a filter function had been designed earlier for a larger quadrate image, but now only a region of the CCD was read out with the same resolution, and this sub image is intended to be

filtered similarly as the larger one, then the image has to be mapped into a larger, quadrate one for filtering. The mapping happens by tiling the large image, quadrate with the small one, placing the neighbouring images mirrored. The mirroring applies periodic features for the image, in this way the edges will not be damaged during Fourier transformation.

Filter Function Design



The *Set DFT Filter* dialog box (DFT= Discrete Fourier Transform) can be invoked by the *Filter / 2D Fourier / Setup DFT* menu command. This dialog box is for designing filter function and setting some properties of the Fourier filtering.

On the editable graph the transmission (1=100%) of filter appears as a function of spatial frequencies. Thus low frequency (large) image details are transmitted if the beginning of the plot is high (lowpass), while the high frequency (small) image details are transmitted if the end of the plot is high (highpass). The pointer position is given in the status bar of the window, for the horizontal position in relative distance from origin, percentage 100%=half image size, in spatial frequency (ν), in half of spatial wavelength ($\lambda/2$). The shown vertical position is the transmission factor at the actual spatial frequency. In the background of the plot a set of hyperbolically decreasing bars are visible, if the dialog box was invoked while an Image Window was active. The bars are showing $\lambda/2$ values corresponding to size of the active Image Window for the spatial frequencies of the graph. So high transmission factor will transmit (amplify, if >1 ; or suppress, if $=0$) image details in the same size as indicated by the bars in each position along the spatial frequency. If the filter function is zero at zero spatial frequency, then the average intensity of the image will be zero.

Edit the plot in the following way:

- 1 Double-click to add a new point to the plot.
- 2 Click and drag point to move.
- 3 Right-click, select *Delete* to delete a point.

Toolbar Icons

- Optimization
- Reset plot to unitary function

- Save — saves into own, binary format (*.flt)
- Load — loads previously saved *.flt format

DFT Preferences

Preserve Edges

Maps the image with the mirrored tiling (described at *Filtering in Fourier domain*) into a double sized image during filtering — in this way Fourier transformation does not cause artifactual stripes appearing at the edges of the images. Double sized image means four times of calculation at DFT, thus it slows down processing.

Real DFT output

The result appears as fixed point real values.

Enlarge image paper to 2^n ... using mirrored tiling

Maps the image with the mirrored tiling (described at *Filtering in Fourier domain*) into a given sized window during filtering. Select this option for filtering non-quadrate images, or smaller images than that for the filter function was designed. For fast DFT transformation 2^n sized images are optimal.

Calculate absolute value after DFT

Calculates absolute value of pixel intensities after filtering. Select this option for filtering with highpass characteristics, if the filtered image sequence is for ROI average calculations. Loss of low (and zero) frequency components renders the image zero mean, even during local, ROI calculations. However absolute value calculation applies halos around objects, but makes ROI averaging feasible.

Leave only phase

After Fourier transformation the complex amplitudes are set to unitary, while the complex phase is left intact. The filtering continues normally with the multiplication by the transfer function and reverse Fourier transformation.

Run

Runs the filter on the active Image Window, separately on each frame. Same as the *Filter / 2D Fourier / Run DFT* menu command.

Sharpening images (2D blind deconvolution):

Microscope optics (as every real optics) has low pass optical transfer characteristics. This phenomenon causes blurring of the image and can be considered as a limitation of the resolution. Images can be sharpened by designing a filter function where the higher spatial frequencies are amplified for compensation of the loss of the high frequency components. Very high spatial frequencies (>50% of half image) are carrying mostly noise; that is why these frequencies should be suppressed rather than amplified.

Extraction of mitochondrial fluorescence for calcium imaging [1]:

Mitochondria are appearing as thin filamentous structures over the bulky cytosolic staining in high-resolution fluorescent images of the calcium sensitive, classical chelator dye rhod-2 or its derivatives loaded to monolayer cell cultures (this dye preferentially loads mitochondria, however not completely selective, stains also the cytosol). With the filter function design it is possible to design a highpass filter, which transmits the high frequency (small) details of mitochondria, while rejects the low frequency (bulky) details of cytosol. Use the absolute value option for gaining images for ROI calculations.

The theory is the following:

The fluorescent image ($\mathbf{G}(\mathbf{x},\mathbf{y})$) is assumed to consist of the sum of fluorescence deriving from the mitochondria (\mathbf{G}_m) and the cytosol (\mathbf{G}_c):

$$G = G_m + G_c$$

This can be done, because the focal plane of wide-field fluorescence microscopy is thick enough to image both mitochondria and cytosol together. The Fourier transformation of the image keeps the sum structure due to its linear nature:

$$F\{G\} = F\{G_m + G_c\} = F\{G_m\} + F\{G_c\}$$

The goal of Fourier transformation is that $F\{\mathbf{G}_m\}$ and $F\{\mathbf{G}_c\}$ are separated in the wave number space, i.e. mitochondrial fluorescence (fine details) are represented at high, whereas that from the cytosol at low wave numbers. Thus an appropriate optical transfer function separates mitochondrial derived fluorescence from the cytosolic one.

Use mitofilter.flt for 512x512 pixel images acquired by a 100x optics (0.15 μm /pixel).

[1]: Ákos A. Gerencsér and Vera Adam-Vizi, Selective, High-Resolution Fluorescence Imaging of Mitochondrial Ca^{2+} Concentration, Cell Calcium 2001; 30.5: 311-321

Optimization of Filter Function for Image Detail Extraction

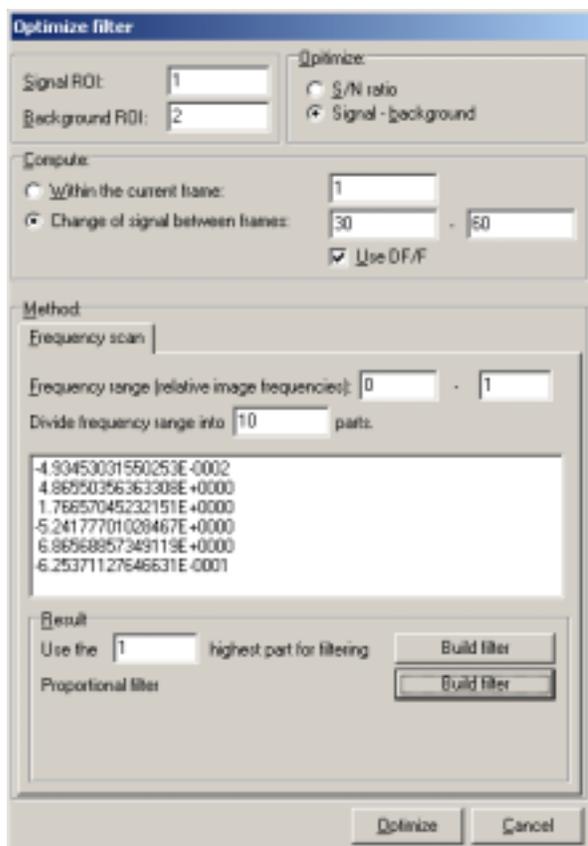
Image detail extraction (like the discussed Mitochondrial Calcium measurement) is based on the transmission of spatial frequencies carrying the important information, while rejecting the rest. For the best results the optimally designed filter function is essential. Finding the best filter function is largely empiric, manual work, the *Optimize filter* dialog box gives help in this.

This routine scans a range of spatial frequencies by bandpass filters in the given range, while determining the level of image detail extraction by comparing intensities in different ROIs on the filtered images. A new filter function can be built from the result of the scan, which probably needs also some further manual enhancement, but gives the sketch of the filter function.

To start optimization dialog, perform the following steps:

- 1 Draw two ROIs on the image — one over an area rich in details to extract, and one over an area, which is rich in details to reject.
- 2 Start *Set DFT Filter* dialog box (*Filter / 2D Fourier / Setup DFT* menu command), and set up preferences (like enabling image enlargement and absolute value calculation). The filter function will be deleted, so save it if needed.
- 3 Start *Optimize filter* dialog box, by pressing optimize icon in the top right corner. The *Optimize filter* dialog box opens, as shown below:

-
-
-



Signal/Background ROI

Set the ROI number of details to extract in *Signal ROI* field, and set the ROI number of details to reject in *Background ROI* field

Optimize

The routine will optimize the ratio of Signal over Background (*S/N ratio*) or the difference: *Signal – Background*.

Compute

In this field the input frames have to be entered. There are two choices:

Calculate within the current frame: Calculates the parameter given at *Optimize* within the actual frame in the actual Image Window. Use this if there are no fluorescence increases in the image sequence.

Change of signal between frames: Calculates the change of parameter given at *Optimize* between the given two frames. Use this for extraction details brightening up upon a stimulus. Select *Use DF/F* to compare DF/F normalized changes of Signal and Background.

Method (Frequency scan)

The routine scans spatial frequencies in the range of *Frequency range* fields (use numbers between 0 and 1) with bandpass filters dividing the range *into Divide frequency range into number* parts.

Result

New filters can be built either by taking the union of the *Use the* number most optimal bandpass filters, or by building a proportional filter assigning 1 transmission to the best bandpass.

Optimize

Press *Optimize* button at the bottom to run optimization. A series of bandpass filter will run through the filter function plot, generating results in the output of *Method* box. The Image Window content is left intact. Build a new filter function by pressing the *Build filter* buttons of *Result* box.

Optimizing in situ mitochondrial calcium measurement:

Use a high-resolution image sequence of rhod-2 fluorescence, recorded during stimulation, evoking calcium transient:

- Set the signal ROI for the mitochondrium rich peripheral area of the cell
- Set the background ROI to the nucleus of the cell.
- Open *Set DFT filter* dialog. Select *Enlarge image*, if the recorded image is not quadrate. Select *Absolute value*.
- Open *Optimize* filter dialog.
- Enter minotochondrial ROI for *Signal*, and nuclear for *Background*.
- Select *Signal – Background* for optimization.
- In the *Compute* box: select *Change of signal*; Enter a frame number from the baseline, and one from the stimulus evoked fluorecence peak (where both nuclear and mitochondrial fluorecence rise is present). Select *Use DF/F*.
- In the *Method* box: scan range can be 0-0.5, dividing into 10 parts, however other setting should be also tried.
- Run optimization and build filter.