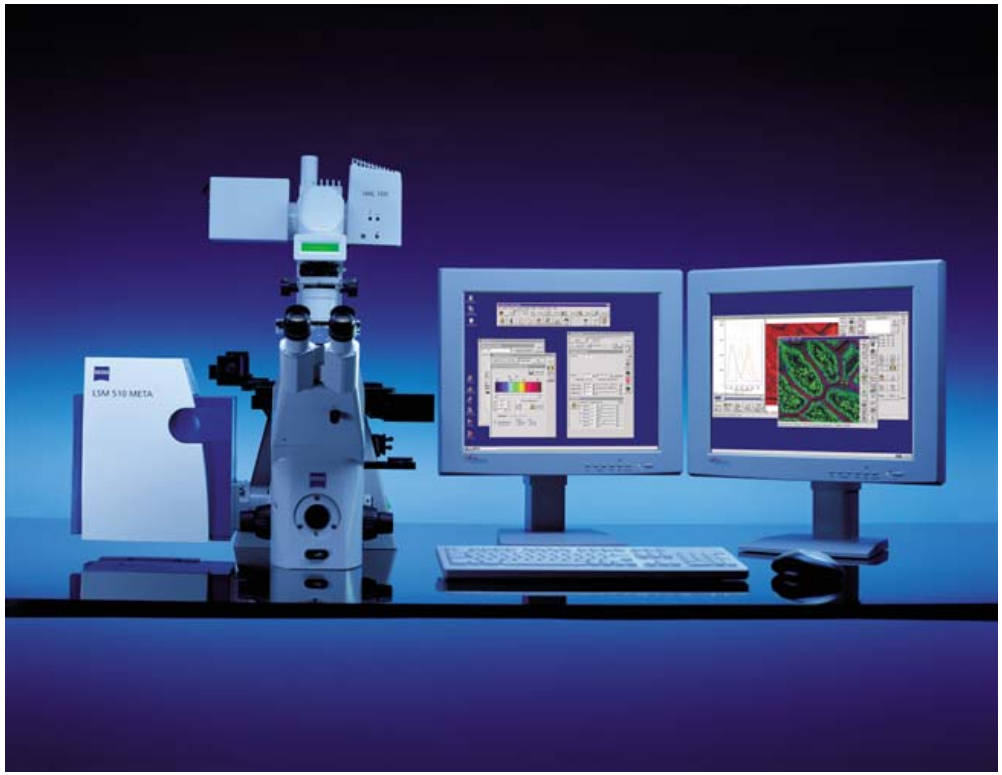
LSM 510 META

Emission Fingerprinting

Guided tour through:

- Configuration
- Defining spectral range
- Scan control settings
  - optimal pinhole size
  - detector gain, amplitude offset
  - range indicator
- Emission Fingerprinting
  1. Acquire Lambda stack
  2. Reference Spectra
     - using ROI's
     - using Spectral Database
  3. Unmix



LSM 510 META sideport configuration on Axiovert 2000 and with 2-Flatscreen monitors

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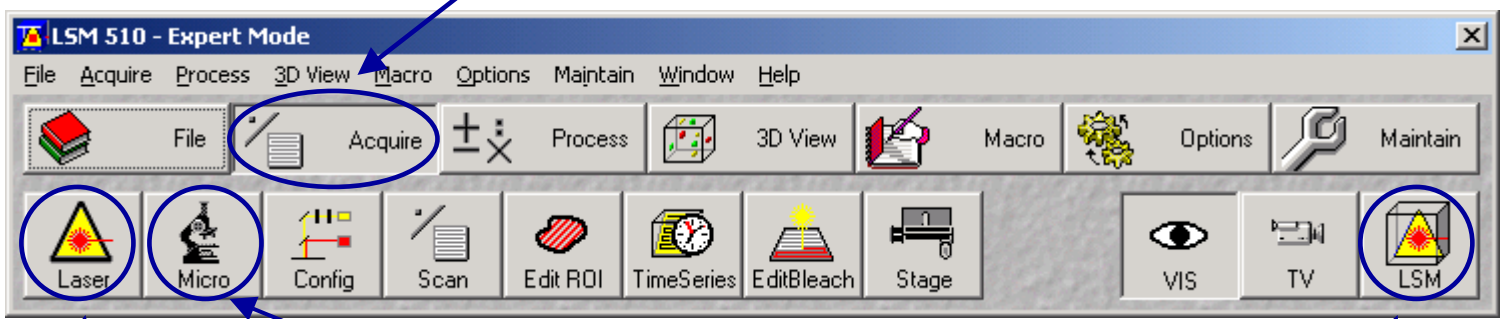
Getting started

1) Start the software

2) Select *Acquire*



3) Select *Laser*

- **switch on required Laser lines**
- **Argon laser power should be ±60 %**

4) Select *Micro*

- **Choose objective lens from pull down menu**
- **Select appropriate Filterset for fluorescence (e.g. FS01 for DAPI, FS09 for GFP)**

5) Switch to *LSM* mode

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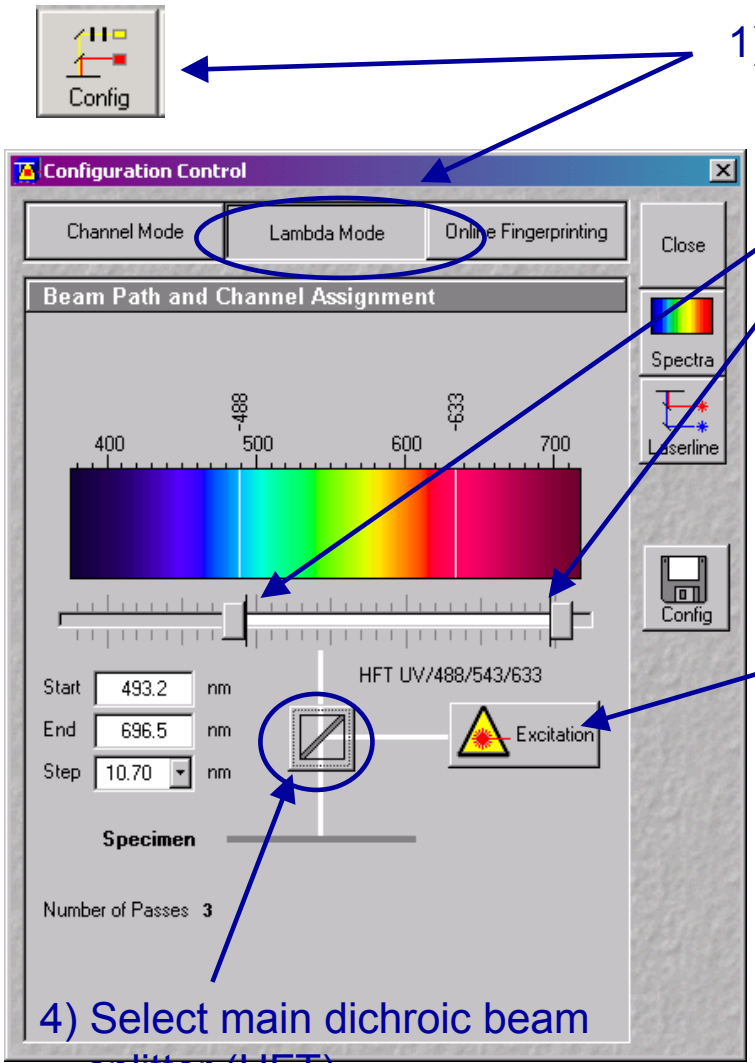
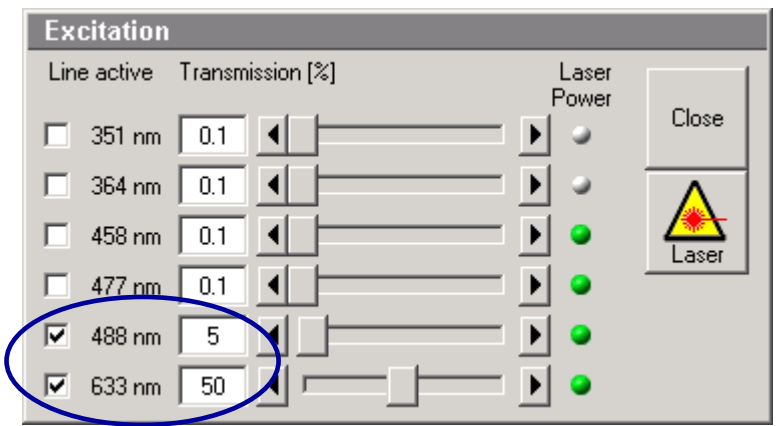
Configuration



1) Select *Config* and then Lambda Mode

2) Define spectral range for Lambda stack acquisition

NOTE: for specimen protection --> keep *Number of passes* low!



3) Select excitation laser lines and set transmission values

4) Select main dichroic beam splitter (HFT)

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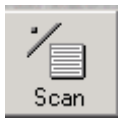
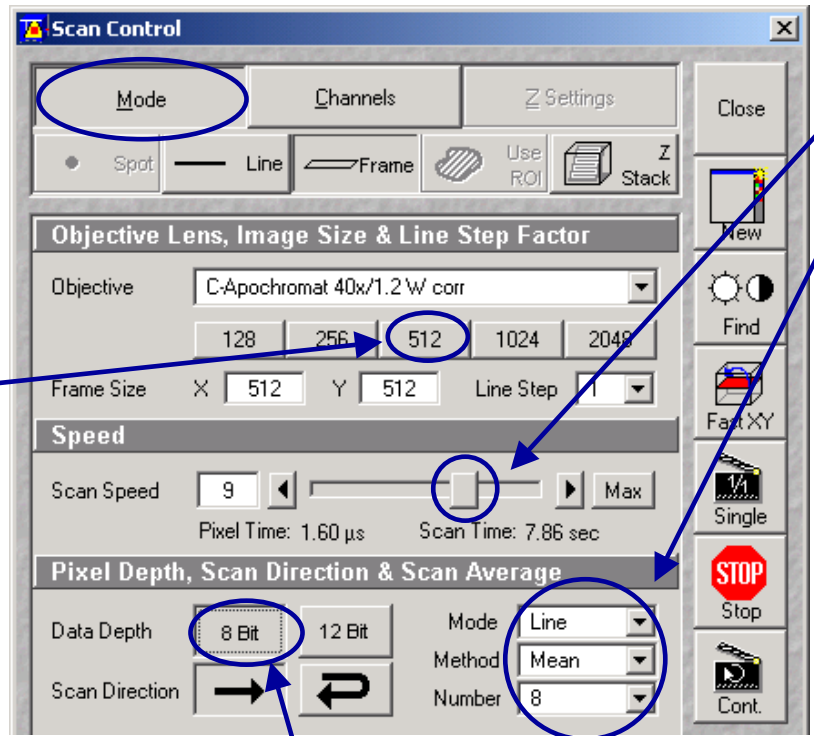
Scanning Parameters



1) Select *Mode* in the *Scan Control*



2) Select Frame size as predefined number of pixels or enter own values (e.g. 512x72)

The Number of pixels influences scanning and image resolution.

3) Enter scan speed and select *Scan average* (slower scan speed and averaging gives best signal/noise ratio)

4) Select dynamic range

8 bit yields 256, 12 bit 4096 levels

NOTE: Images for publication should be acquired using 12 bit and high number of pixels

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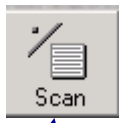
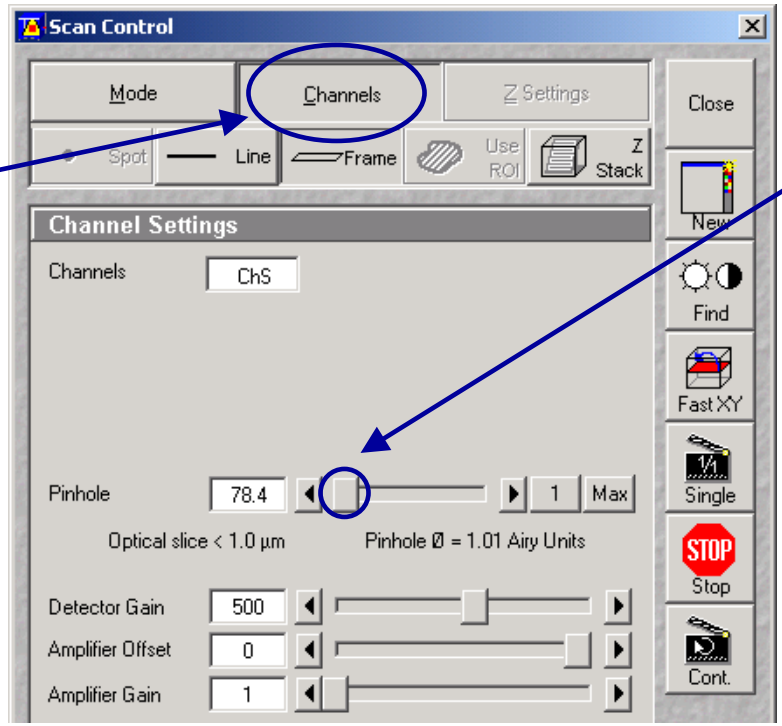
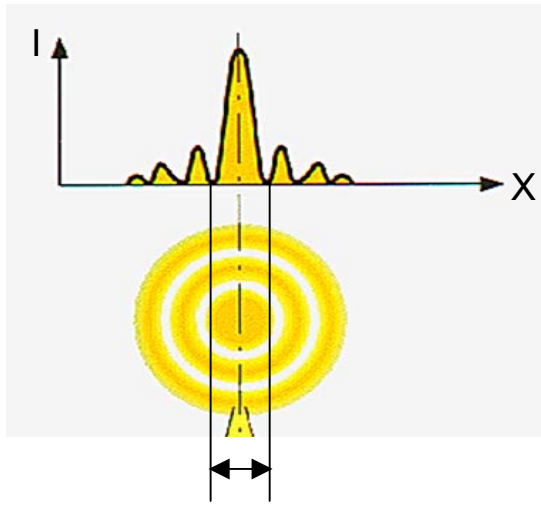
Adjusting Pinhole



1) Select *Scan Control* and *Channels*



2) Set pinhole size



pinhole size=1 Airy unit

1 Airy units produces best signal/noise ratio

Pinhole adjustment changes „optical slice“ (i.e.confocality)

NOTE: Pinhole 1 controls all 32 META detector elements

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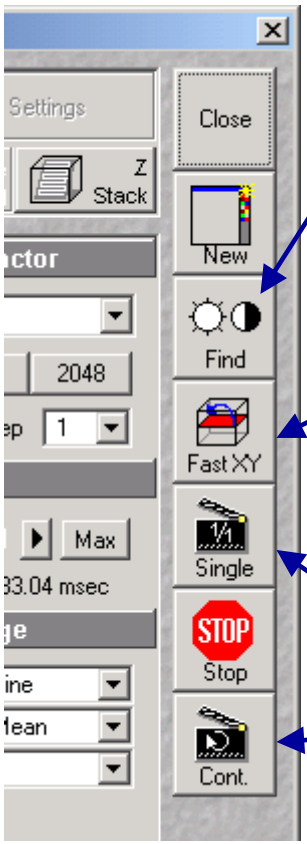
Acqusition



1) Select *Find* to automatically pre-adjusts detector sensitivity

2) Select *Fast XY* for continuous fast scanning (useful for finding and changing the focus)

3) Use *Single* or *Cont* to **Start Acquisition...**

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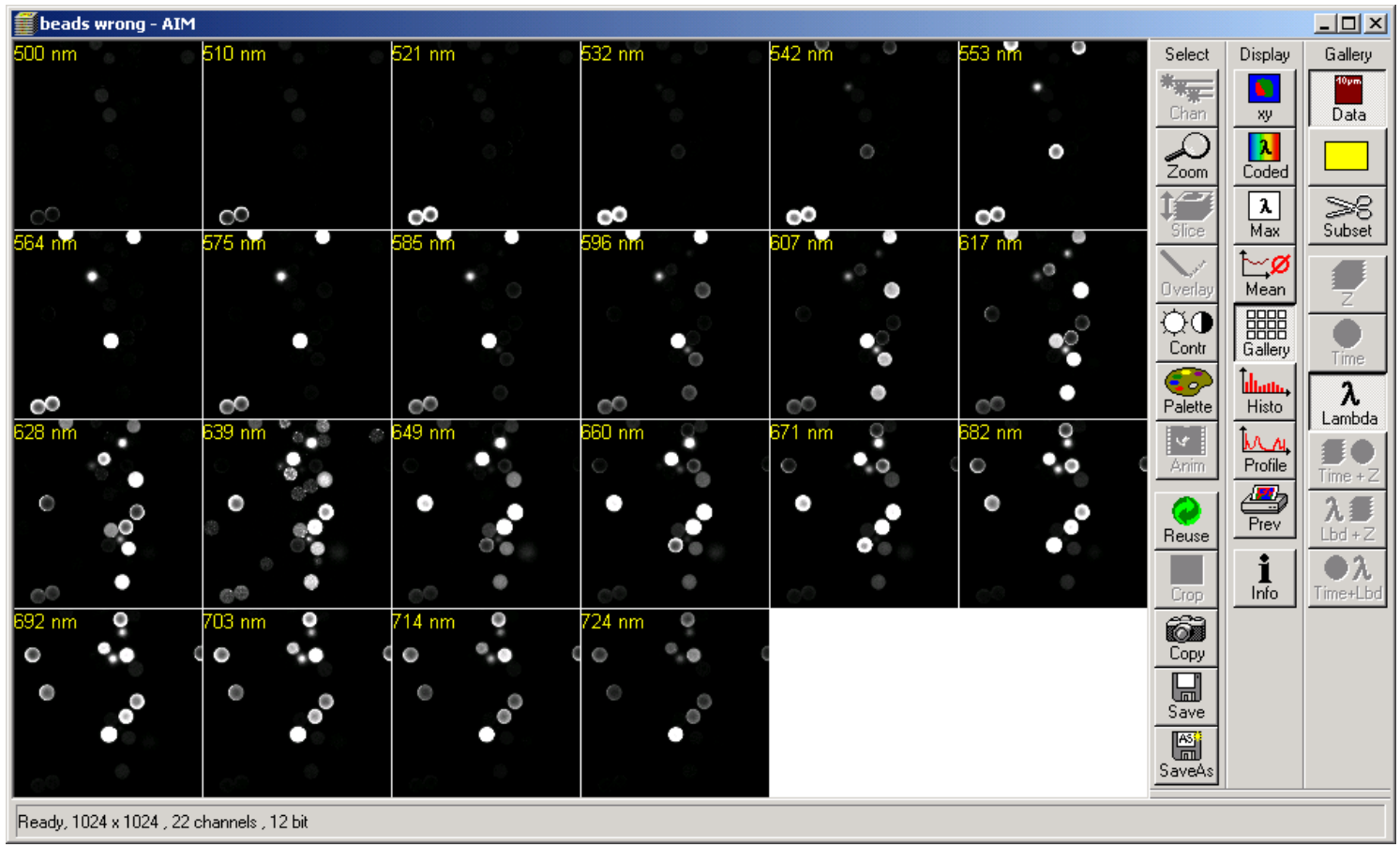
Emission Fingerprinting- Lambda stack

...

Lambda stack (22 elements, 3 passes)



(Gallery Mode display)

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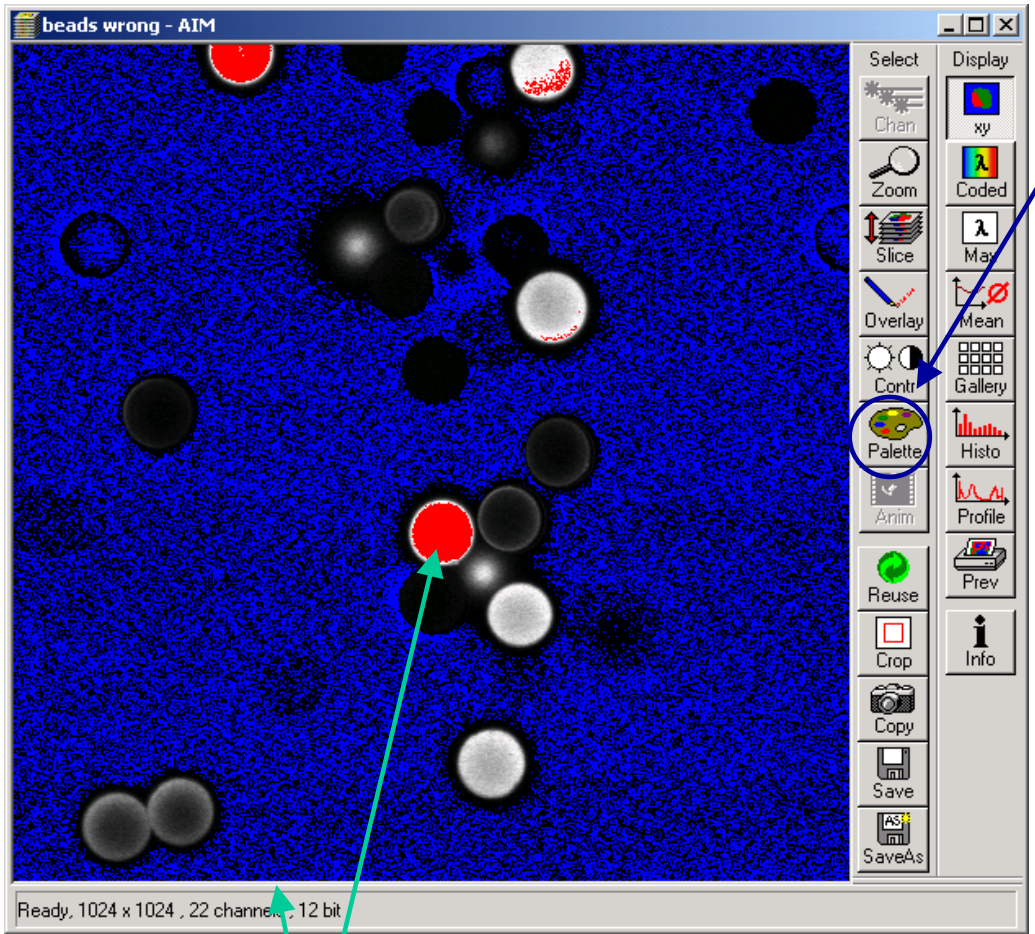
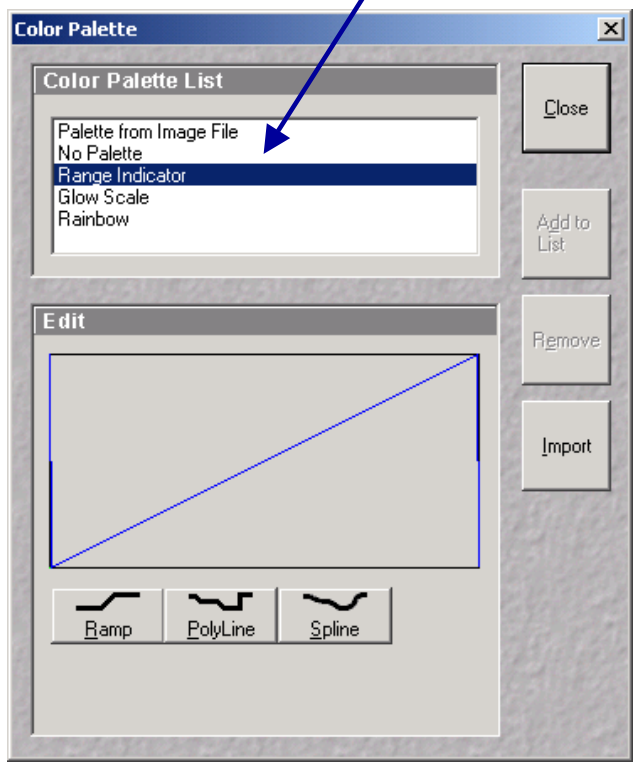
Emission Fingerprinting- Range Indicator



1) Select *Palette*

2) Select *Range Indicator*



Red = Saturation (Maximum)

Blue = Zero (Minimum)

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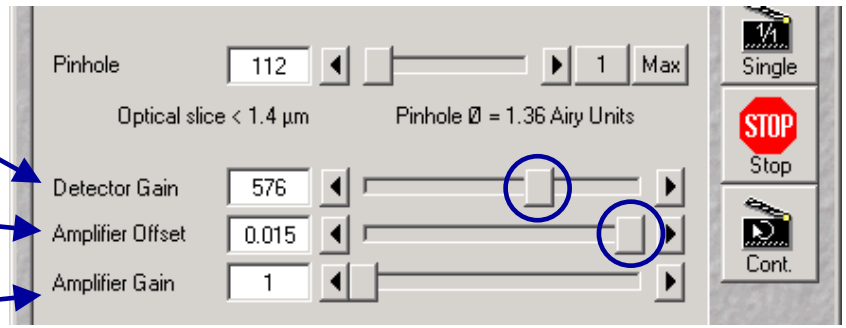
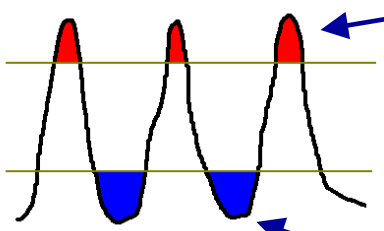
Emission Fingerprinting- Set Gain & Offset

- *Detector Gain* determines sensitivity of the detector by setting the maximum limit
- *Ampl. Offset* determines the minimum intensity limit
- *Ampl. Gain* determines signal amplification



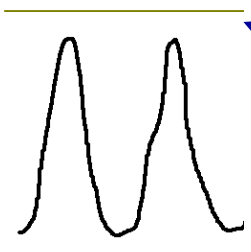


Saturation at Maximum

→ reduce *Detector Gain*

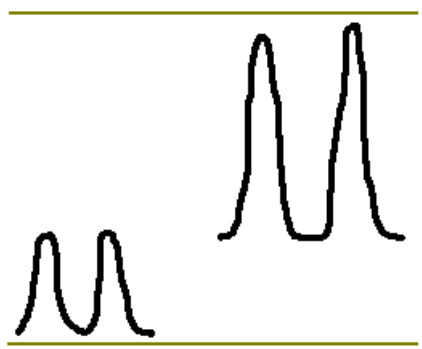
Saturation at Minimum

→ increase *Ampl. Offset*



Gain set correctly

Offset set correctly



Gain

Offset

Ampl. Gain increases whole signal, and the offset will need to be decreased

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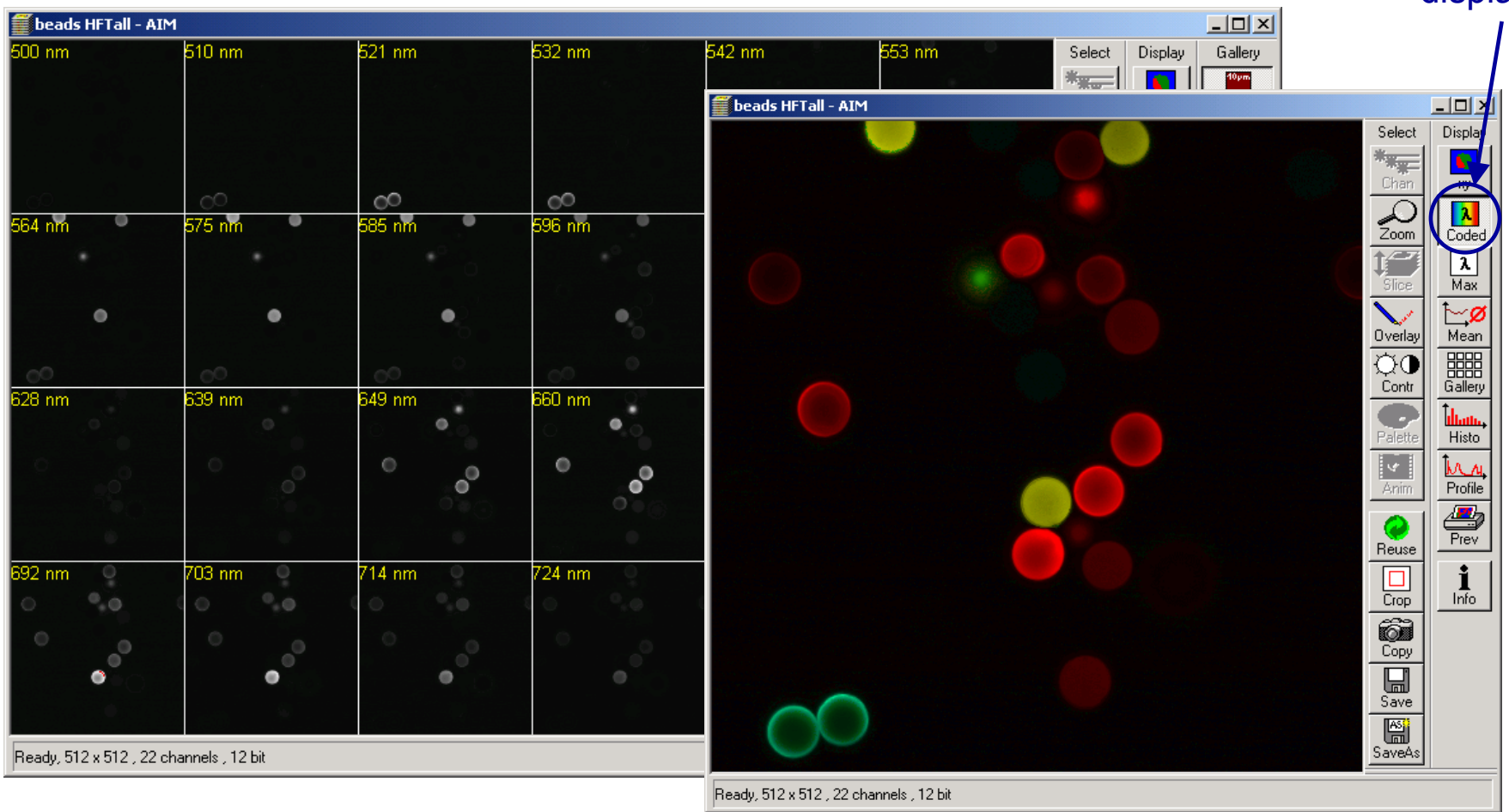
Emission Fingerprinting - correct Offset and Gain

- Increase Offset until all blue pixels disappear
- Reduce Gain until red pixels only just disappear

Lambda coded display



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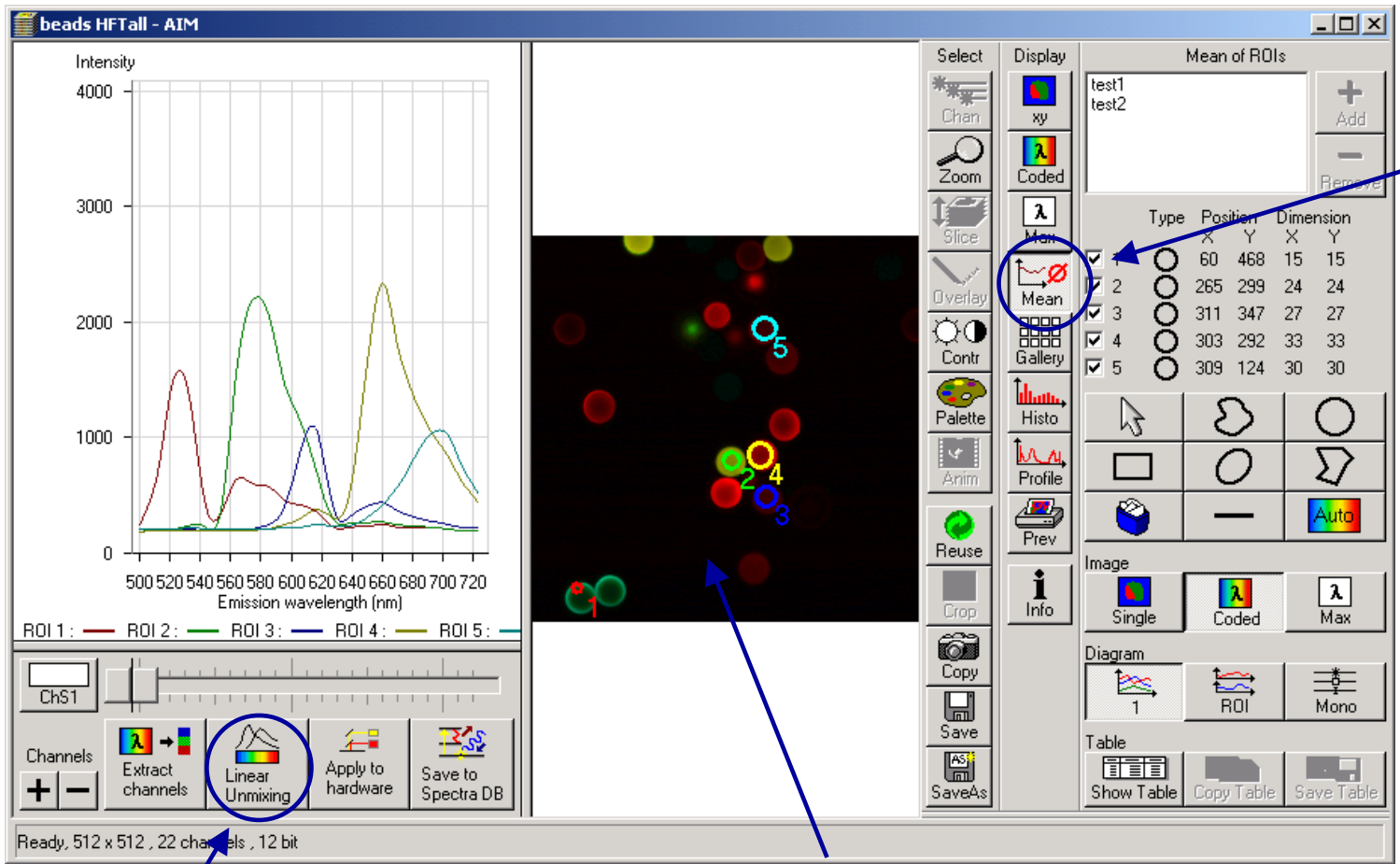
Emission Fingerprinting - using ROI's



1) Select *Mean* Mode

2) Define different Region's of Interest (corresponding spectra are displayed in graph on the left)

3) Click *Linear Unmixing*

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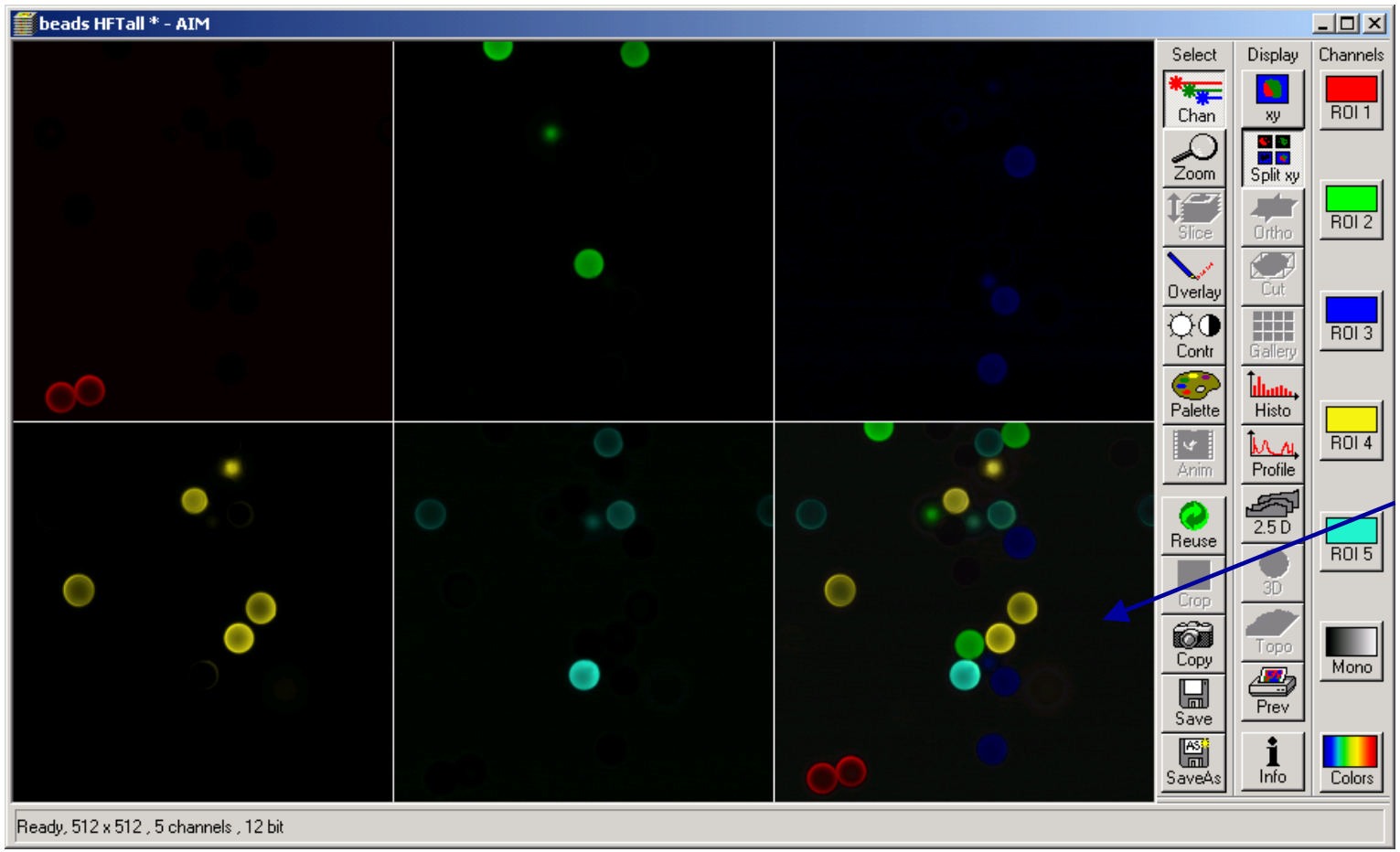
Emission Fingerprinting - using ROI's



Clear separation, no overlap

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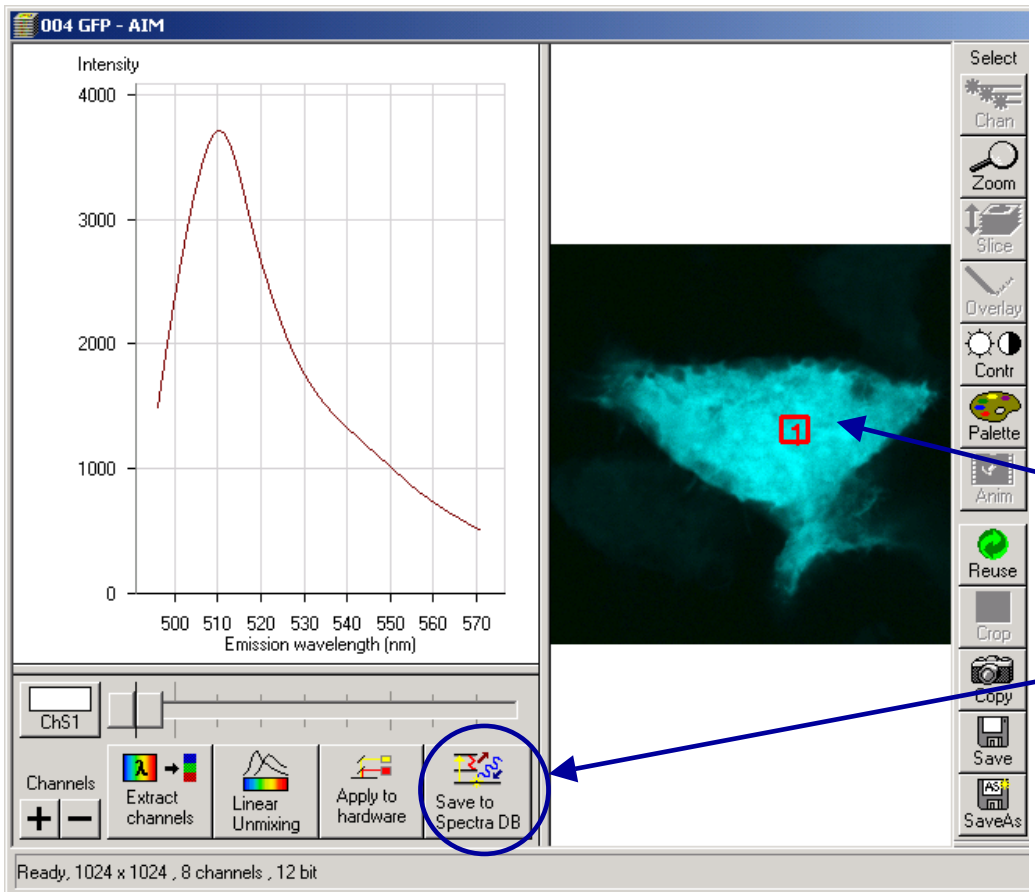
Emission Fingerprinting - using Spectral DB



If no clear spectral separation between different areas is possible, then reference spectra need to be obtained from individual controls (One fluorochrome in the sample only!)

1) Acquire Lambda stack

2) In *MeanROI* Mode, draw Region of Interest

3) Save Spectra to Database

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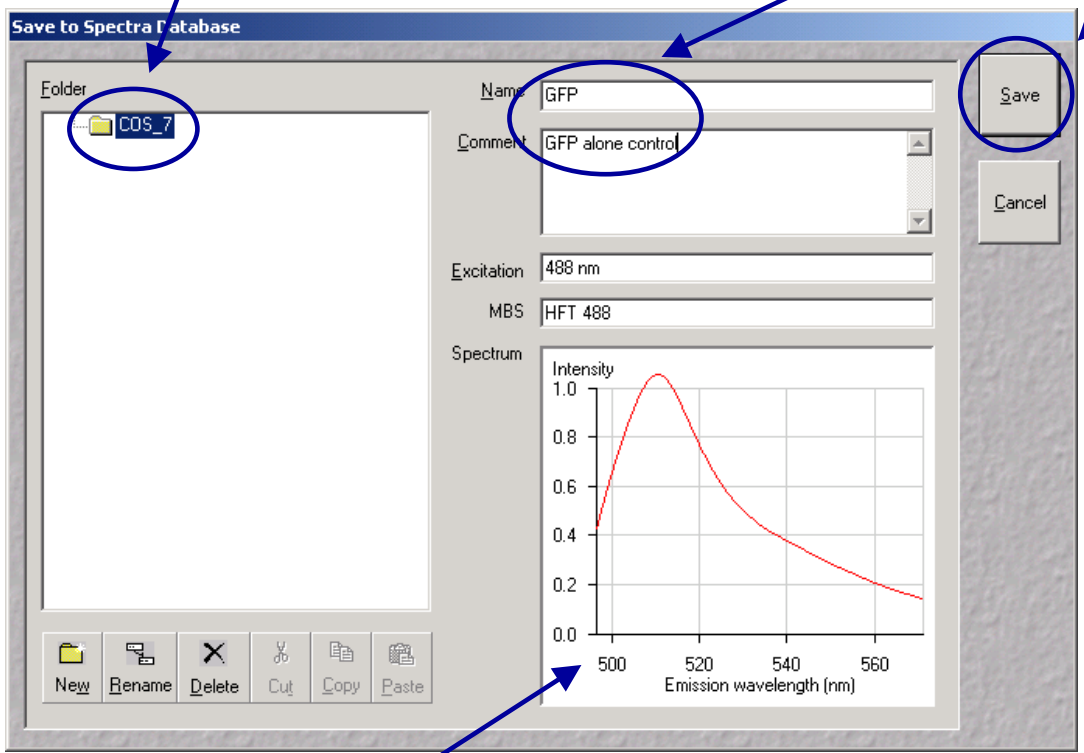
Emission Fingerprinting - using Spectral DB

4) Select existing or create new folder

5) Assign appropriate name and enter comments (optional), then *Save*



6) Repeat steps 1) - 5) for all necessary individual controls (including Autofluorescence and background)

IMPORTANT: All controls have to be obtained under the SAME CONDITIONS (i.e. same system, HFT, detector range and objective!!!)

Otherwise unmixing will produce false results!!!

NOTE: Reference spectra will be normalized to the maximum intensity and can be displayed either as spline curve or raw data points (use right mouse button for switching)

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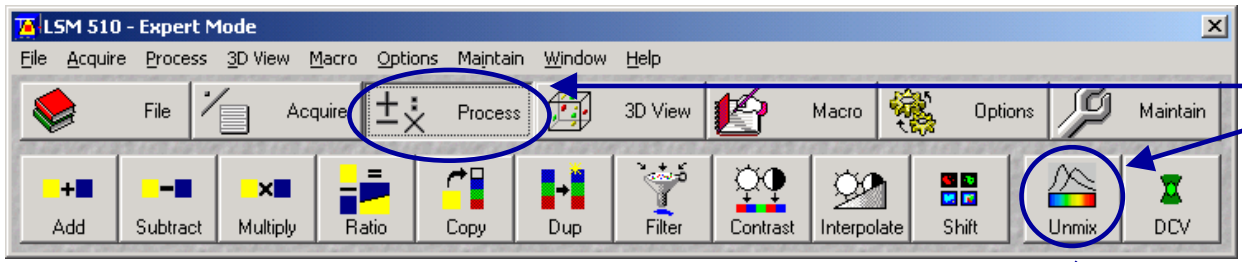
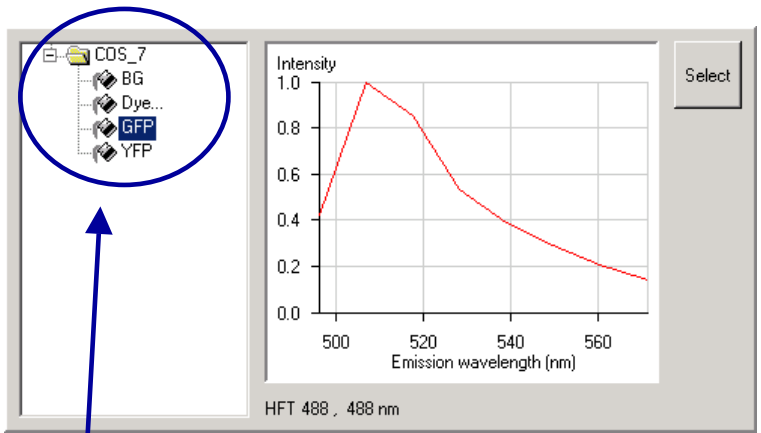
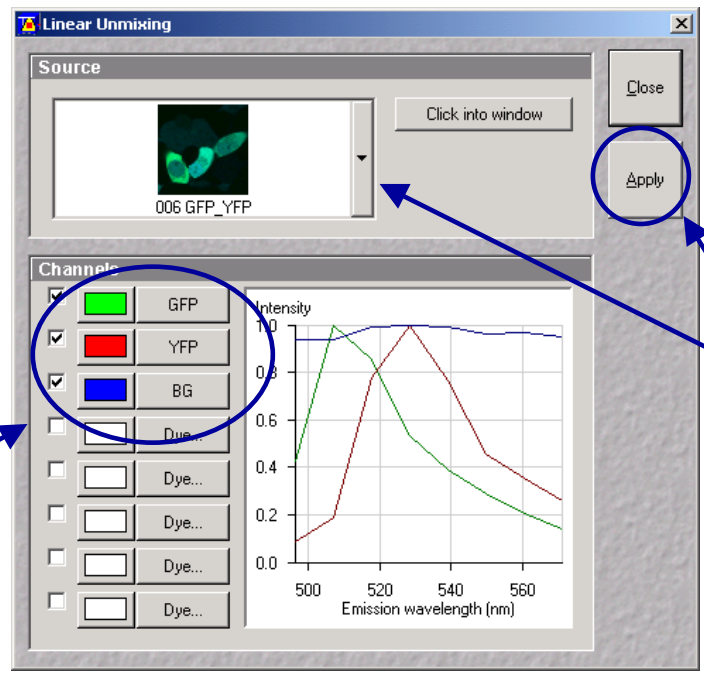
Emission Fingerprinting - using Spectral DB



1) From Main Menu, select *Process* and then *Unmix*



2) Select individual previously saved reference spectra from folder, assign channel and color



3) Select Lambda stack (Source) for unmixing, click *Apply*

...

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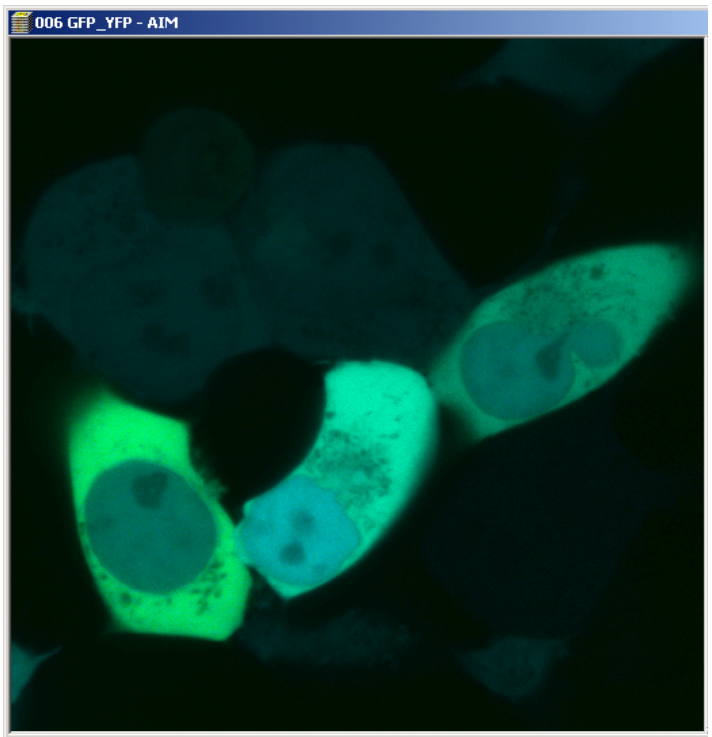
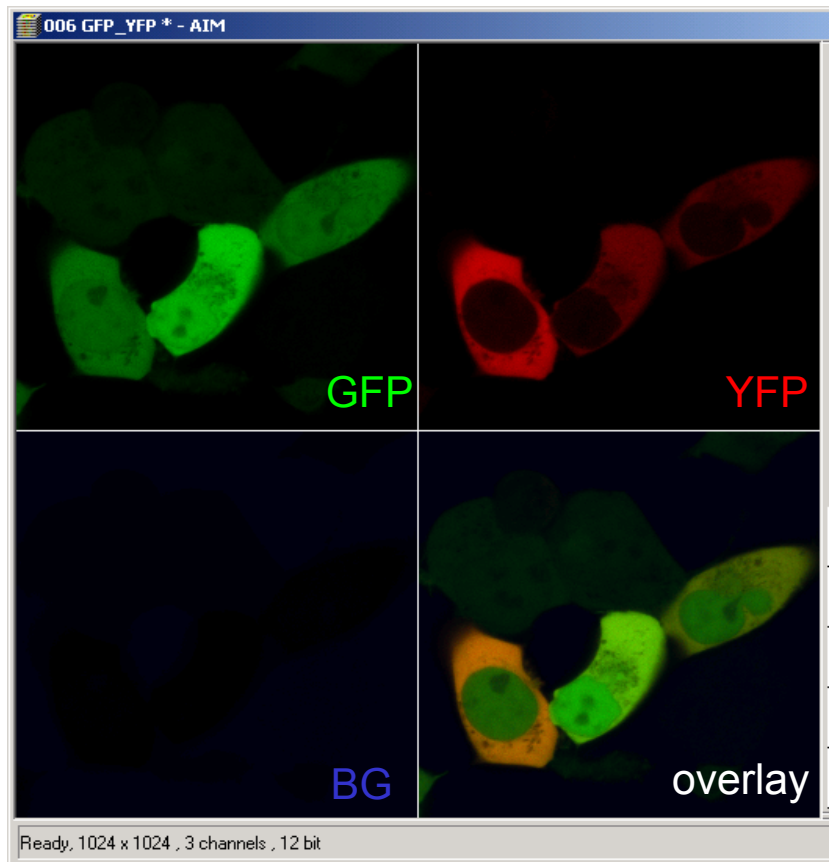
Emission Fingerprinting - using Spectral DB

Original Lambda stack...



...separation into individual channels after linear unmixing



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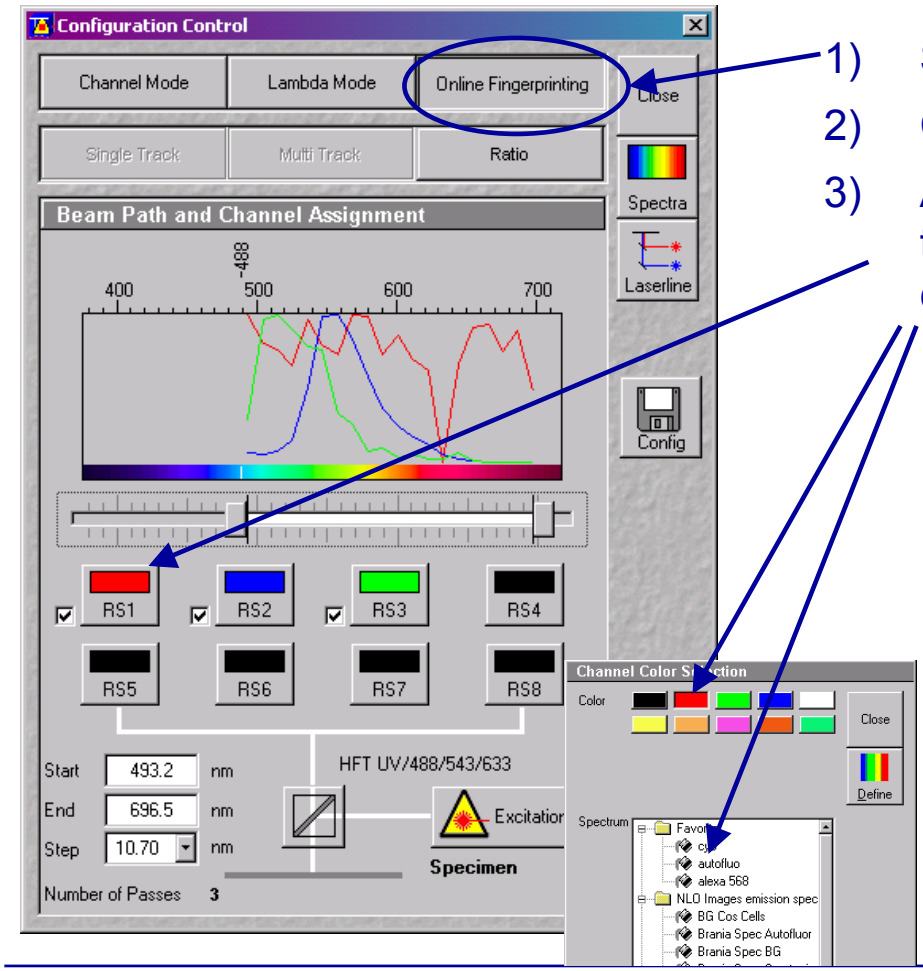
Online Fingerprinting

Emission Fingerprinting can be preformed online avoiding the accumulation of possibly unnecessary data (lambda stacks) if reference spectra are available.



1) Select *Config* and then *Online Fingerprinting*
2) Choose Beam Splitter and Laser Line
3) Assign emission spectra from the database to the channels by indicating the spectrum and a color

Starting the Scan will only produce the result of the linear unmixing procedure and no other images.

Emission Fingerprinting

What is Emission Fingerprinting?

- 3-step-method for (1) recording, (2) analysis and (3) separation of emission signals in multifluorescence imaging
- Separation of individual emissions based on the recording of spectral signatures and a linear unmixing procedure using reference spectra

What is it good for?

- Separation even of fluorochomes with widely overlapping emission spectra
- Separation of fluorochromes that are excited by the same laser line (in single-photon and multiphoton microscopy)
- Elimination of background- and autofluorescence