



Laser

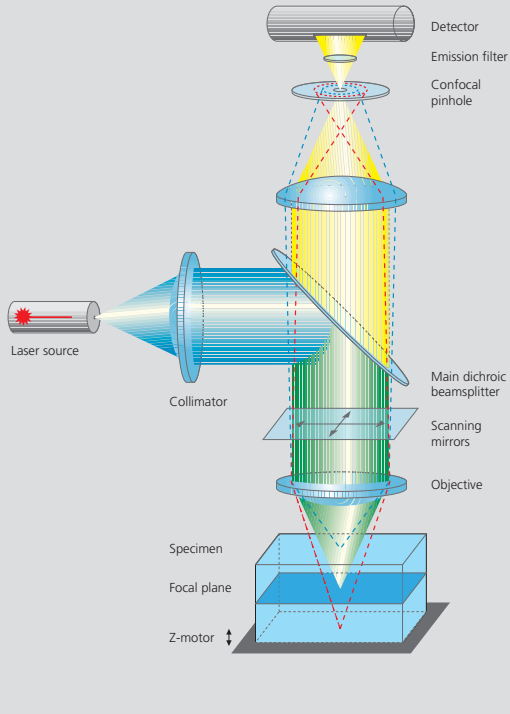
- **Light source** – projected into specimen
- **Laser power:** adjustable via attenuation device (AOTF, AOM, MOTF) and tube current setting (Ar)
- **Lifetime Ar:** prolonged by using lower tube current; but laser noise will be increased (8 A = minimum noise)
- **Stand-by mode:** prolongs laser lifetime; not suitable for image acquisition
- **Laser line:** can be chosen via selection device (AOTF, MOTF) dependent on fluorescent dye. Generally: the shorter the wavelength, the higher the resolution
- **Application goals:** (1) Protect specimen (reduction of dye bleaching and phototoxicity) by reduction of laser power. (2) Maximize fluorescence signal (higher SNR) by longer pixel dwell times or averaging

Scanning Mirrors

- **Scanning unit** – moves focused laser beam across specimen line by line
- **Scanning speed:** defines frame rate (frames/sec) and pixel time, i.e. time the specimen is illuminated
- **Pixel time:** influences SNR of image; the longer the pixel time, the more photons per pixel, the less noise in the picture; but bleaching of fluorochromes may increase
- **Pixel resolution:** maximum resolution can be achieved if pixel size is set correctly (at least 4 x 4 pixels (x, y) per smallest detail) → directly adjustable via scan zoom
- **x/y frame size:** variable from 4 x 2 up to 2048 x 2048 pixels; maximum frame rate with 512 x 512 pixels 5 frames/sec (bidirectional scan ) ; unidirectional scan  : slower by factor 2

Z-Motor

- **Focusing the specimen** – acquisition of image stacks or x-z sections
- **z-interval:** distance between two optical slices (step size of z-motor: min. 25 nm)
- **Optimum z-motor step size:** 0.5 x optical slice thickness (compare: min. slice thickness about 340 nm for NA=1.4, n=1.52, λ =488 nm)
- **Optional:** fast z-scanning stage (HRZ) fast piezo objective focus



Photomultiplier (PMT)

- **Detector** – pixelwise detection of photons emitted/reflected by the respective specimen detail
- **Parameters:** "Detector Gain" = PMT high voltage, "Amplifier Offset" = black level setting, "Amplifier Gain" = electronic post-amplification
- **Calibration:** "Amplifier Offset" on image background (object-free area), "Detector Gain" according to scanned image (object); setting aid = "Range Indicator" (→ "Palette"). Goal: least number of overmodulated (red, Gain) and undermodulated (blue, Offset) pixels
- **Signal amplifier:** First exploit "Detector Gain" slider before "Amplifier Gain" > 1

Confocal Pinhole

- **Depth discrimination** – confocal aperture to prevent detection of out-of-focus light (optical sectioning)
- **Diameter:** determines thickness of optical slice; optimum diameter: 1 Airy unit = best trade-off between depth discrimination capability and efficiency
- **x/y position:** factory-adjusted for all beam path configurations; can be modified manually (→ "Maintain-Pinhole" →)

Beam Splitter

- **Fluorescence beam path** – definable by combination of main (HFT) and secondary (NFT) dichroic mirrors and emission filters (BP = bandpass, LP = longpass, KP = shortpass) (→ "Acquire" → "Config")
- **HET:** separates excitation and emission light
- **NET:** effects spectral division of (different) fluorescence emissions (e.g. NFT 545: reflects light of $\lambda < 545\text{nm}$ and transmits light of $\lambda > 545\text{nm}$)
- **BP, LP, KP:** determines bandwidth of fluorescence emission for the respective channel (e.g. LP 505: $\lambda \geq 505\text{nm}$ → detection)

Objective Lens

- **Optical image formation** – determines properties of image quality such as resolution (x, y, z)
- **Numerical Aperture (NA):** determines image spot size (jointly with wavelength), and substantially influences the minimum optical slice thickness achievable
- **Refractive index (n):** match $n_{\text{immersion liquid}}$ with specimen mounting medium for better image quality.
- **Best confocal multifluorescence images (VIS, UV):** use water immersion objectives with apochromatic correction (C- Apochromat)

3 Steps to Get a Confocal Image

1 View specimen in VIS mode

Focus the specimen in epi-fluorescence mode using the binocular and center the part of interest, select fluorescence filter cube according to application (e.g. FITC or Cy3) via SW (window "Microscope Control"); match the field of view: change to appropriate objective magnification (consider use of correct immersion medium).



2 Load an LSM configuration

Activate LSM mode (operate manual tube slider or button "LSM"). Open window "Configuration control", and select a predefined configuration from list (Single Track). A click on "Apply" automatically sets up the system: laser lines, attenuation, emission filters, beam splitters (HFT, NFT), pinhole diameter, detector settings (channels, gain, offset). Or: Click on "Reuse" button (stored image/image database window) to restore settings of a previous experiment.



3 Scan an image

Click on "Find" button (right row in window "Scan Control") => System automatically opens image window, optimizes detector settings (matches PMT gain and offset to dynamic range of 8 or 12 bit), and scans an image.



See operating manual for scanning a stack of slices, time series etc.

How to Enhance Image Quality

More signal!

- Change to longer pixel dwell times by reducing scanning speed
- Use "Average" method: Calculation of "Sum" or "Mean" value of pixels of consecutive "Line" or "Frame" scans.
- Increase bandwidth of emission filter (e.g. LP instead of BP).
- Enlarge pinhole diameter; Note: optical slice thickness increases accordingly.
- Increase excitation energy (laser power); but pay attention to bleaching, saturation and phototoxic effects.

More details!

- Use objective with higher numerical aperture (NA); x/y-resolution ~ 1/NA, z-resolution ~ 1/NA².
- Increase "FrameSize" = number of pixels per line + lines per frame, e.g. 1024 x 1024 or 2048 x 2048 (min. 4 x 2).
- Optimize scan zoom (Z), i.e. pixel size ≤ 0.25 x diameter of Airy disk (e.g.: Objective 40x, NA 1.3, l = 488 nm => Z = 4.56).
- Increase dynamic range (change from 8 to 12 bit per pixel).

More reliability!

- Use Multitracking: very fast switching of excitation wavelengths; prevents crosstalk of signals between channels; predefined configurations available.
- Use ROI (Region Of Interest) function: significantly reduces excited area of specimen and increases acquisition rate at constant SNR; several ROIs of any shape can be defined and used simultaneously.

The Confocal Laser Scanning Microscope



We make it visible.