The Confocal Laser Scanning Microscope Overview

Laser

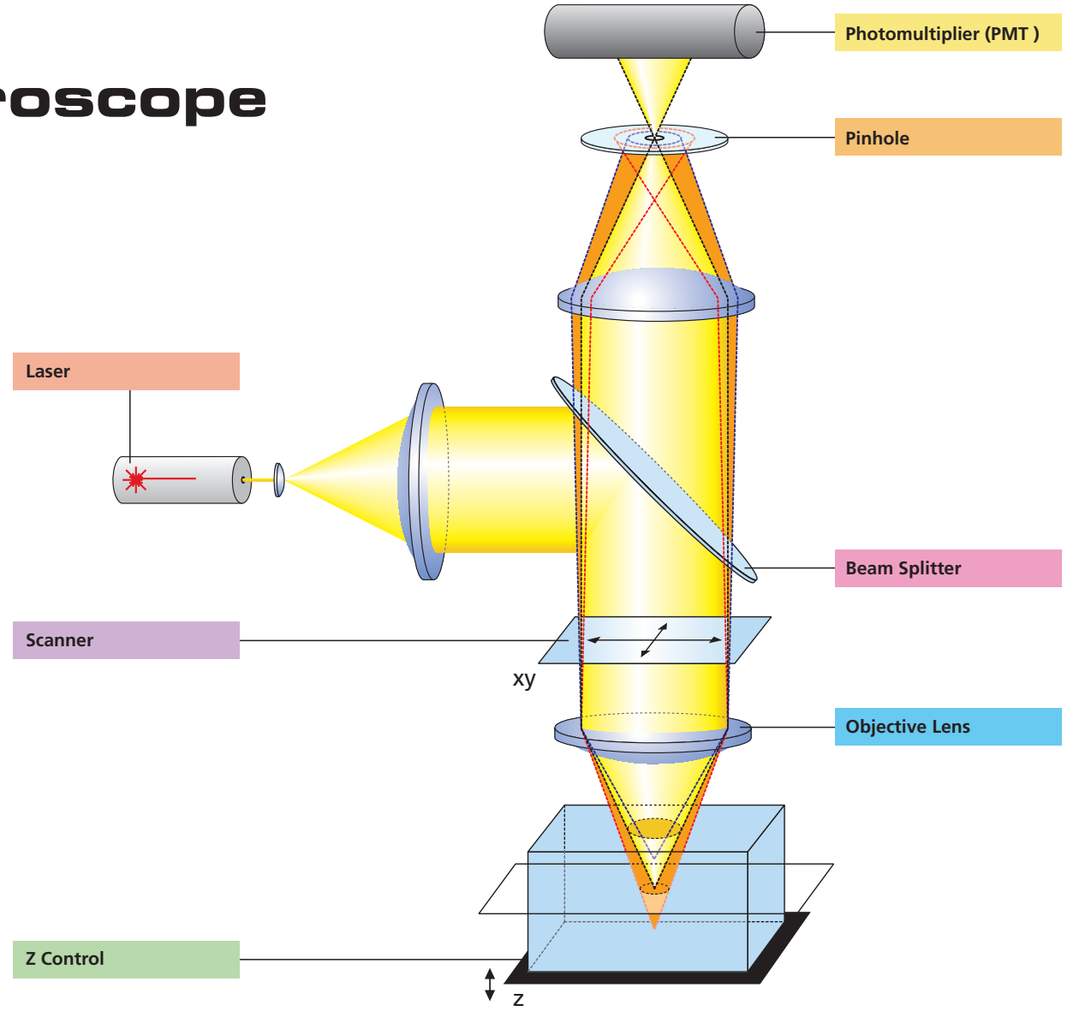
- **Light source** – projected into specimen
- Laser power: adjustable via attenuation device (AOTF, MOTF) and tube current setting (Ar, ArKr only)
- Lifetime Ar, ArKr: prolonged by using lower tube current; but laser noise will be increased, too (8 A = minimum noise)
- Stand-by mode: prolongs laser 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

Scanner

- **Scanning unit** – moves the focused laser beam across specimen line by line
- Scanning speed: defines frame rate and pixel time, i.e. time for collecting photons
- Pixel time: influences SNR of image; the longer the pixel time, the more photons per pixel, the less noise
- 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: 2.5 frames/sec (bidirectional scan ("⇄"); unidirectional scan ("→"): slower by factor 2)

Z Control

- **Focusing the specimen** – acquisition of image stacks or x-z sections
- z interval: distance between two optical slices (step size of z motor: min. 100 nm, Axioplan 2 imaging: 50 nm)
- Optimum z motor step size: 0.5 x optical slice thickness (compare: min. slice thickness for NA = 1.4, n = 1.52, λ = 488 nm: about 340 nm)
- Optional: fast z scanning stage (HRZ) = higher precision of z movement (step size 10 nm, reproducibility 30 nm, working range 200 µm)



3 Steps to get a Confocal Image

(LSM software running, lasers and HBO turned on)

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
Go to LSM mode (operate manual tube slider). Open window "Configuration control", click on "Store/Apply" and select a predefined configuration from list (Single Track). A click on "Apply" automatically sets up the system: laser lines, attenuation, filters (EF), 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 – ready!
See operating manual for scanning a stack of slices, time series etc.

How to enhance the Image Quality

(Image scanned)

1. "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.

2. "More details !"
- Use objective with higher numerical aperture (NA); x/y-resolution ~ 1/NA, z resolution ~ $1/NA^2$.
- 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.: M = 40x, NA 1.3, λ = 488 nm => Z = 6).
- Increase dynamic range (change from 8 to 12 bit per pixel).

3. "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.

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) and undermodulated (blue) pixels
- Signal amplification: First exploit "Detector Gain" slider before "Amplifier Gain" > 1

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 (EF) (→ "Acquire" – "Config")
- HFT: separates excitation and emission light
- NFT: effects spectral division of fluorescence emissions (e.g. NFT 545: reflects light of λ < 545nm and transmits light of λ > 545nm)
- EF: determines bandwidth of fluorescence emission for the respective channel

Objective Lens

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

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