

The Confocal Laser Scanning Microscope Overview

Laser

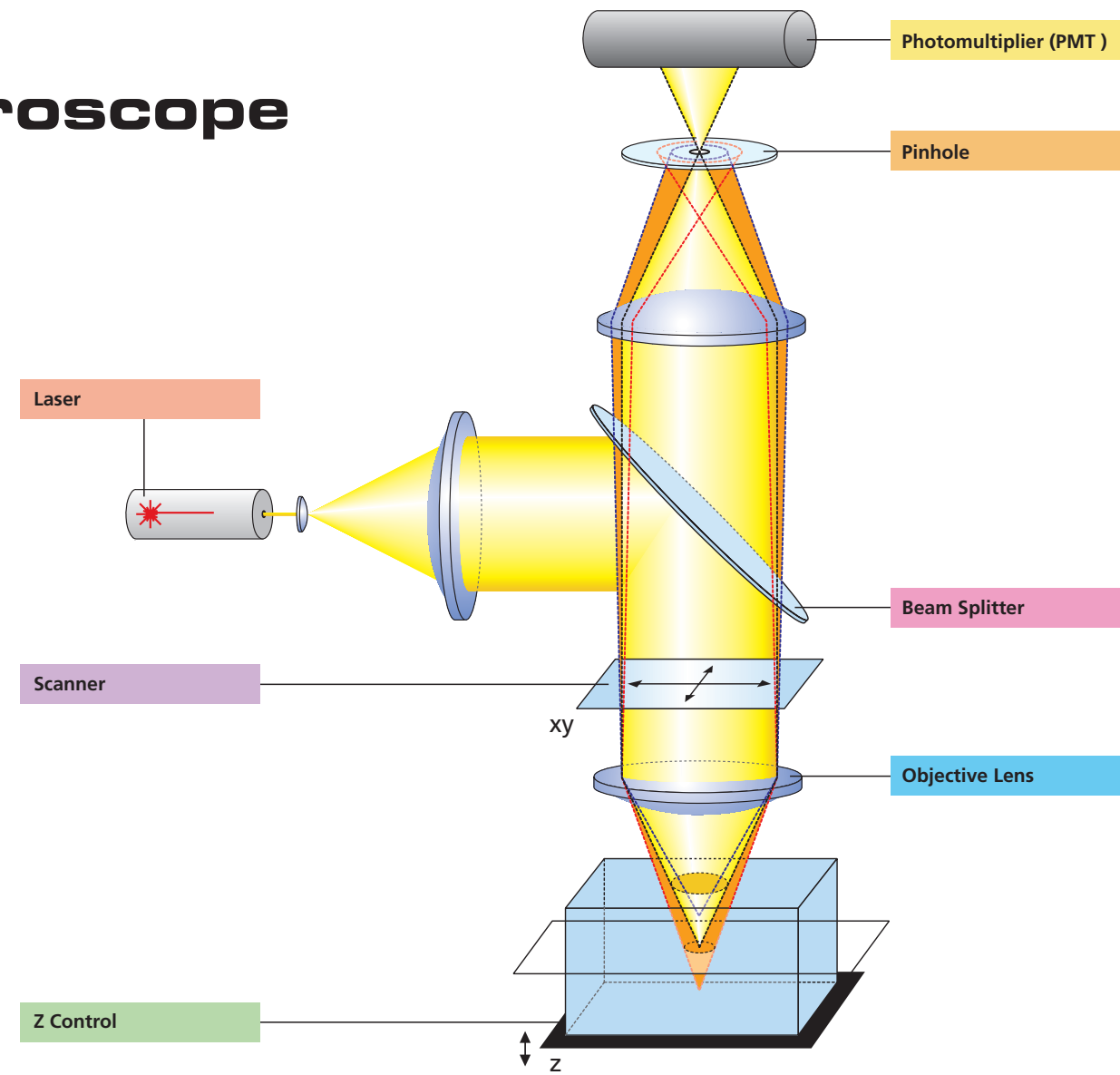
- **Light source** – projected into specimen
- **Laser power:** adjustable via attenuation device (AOTF, MOTF) and tube current setting (Ar, ArKr only)
- **Lifetime Ar, ArKr:** prolonged by using lower tube current; but laser noise will be increased, too (8 A = minimum noise)
- **Stand-by mode:** prolongs 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
- **xy 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, $\lambda = 488 \text{ nm}$: 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 $\sim 1/NA$, z resolution $\sim 1/NA^2$.
 - 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 $\leq 0.25 \times$ diameter of Airy disk (e.g.: M = 40x, NA 1.3, $\lambda = 488 \text{ nm} \Rightarrow Z = 6$).
 - 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
- **xy 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 $\lambda < 545 \text{ nm}$ and transmits light of $\lambda > 545 \text{ nm}$)
- **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)