Laser

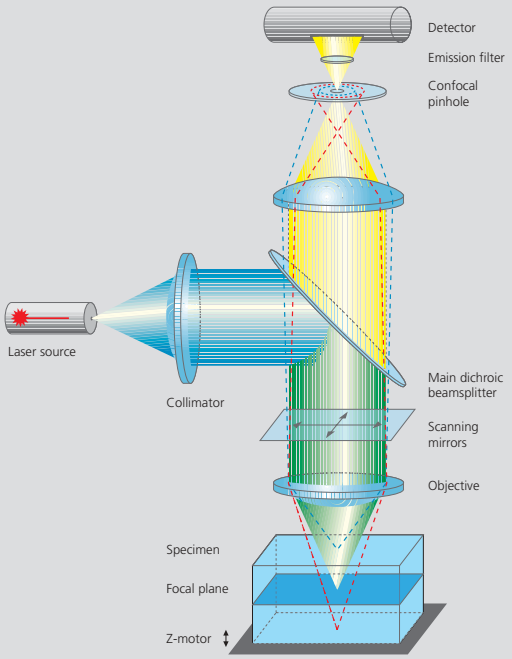
- **Light source** – projected into specimen
- Laser power: adjustable via attenuation device (AOTF, AOM, MOTF) and tube current setting (Ar)
- Lifetime Ar: prolonged by using lower tube current; but laser noise will be increased (8 A = minimum noise)
- Stand-by mode: prolongs laser lifetime; not suitable 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 objectiv 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 amplification: 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")
- HFT: separates excitation and emission light
- NFT: effects spectral division of (different) fluorescence emissions (e.g. NFT 545: reflects light of λ < 545nm and transmits light of λ > 545nm)
- BP, LP, KP: determines bandwidth of fluorescence emission for the respective channel (e.g. LP 505: λ ≥ 505 nm → detection)

Objective Lens

- **Optical image formation** – determines properties of image quality 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)

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

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