**Microscopy from Carl Zeiss**

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### The Confocal Laser Scanning Microscope

**Laser**
- Light source – projected into specimen
- Output power: adjustable via intensity device
- Wavelength: 488 nm (example)
- Variable by using laser tube current
- Power output will be increased by about 2.5 x minimum
- Laser line: can be chosen via selection device (AOTF, MOTF)
- NA dependent on fluorescent dye: Generally, the shorter the wavelength, the higher the resolution

**Optics**
- Focusing the specimen in epi-fluorescence mode using the binocular
- Objective: most suitable for microscopy
- More details!
  - Use objective with higher numerical aperture (NA): spherically corrected
  - Increase excitation energy (laser power); but pay attention to bleaching, saturation and phototoxic effects

**Z-Motor**
- Focusing the specimen – acquisition of image stacks or z sections
- Standard: fixed distance between two optical slices (step size)
- Optional: stepper step size 0.5 x optical slice thickness
- Dependent on objective:
  - Objective 40 x, NA 1.3, \( \lambda = 488 \text{ nm} \) => \( Z = 4.56 \text{ focus} \)

**Scanning Mirrors**
- Scanning unit – moves focused laser beam across specimen line by line
- Resolution: maximum resolution can be achieved if laser line is perpendicular to the pixel line (e.g.: Objective 40 x, NA 1.3, \( \lambda = 488 \text{ nm} \) => \( Z = 4.56 \text{ focus} \))

**Scanning Speed**
- Defining a number of pixels per second
- depends on fluorescent dye. Generally, the shorter the wavelength, the higher the resolution
- Example: Objective 40 x, NA 1.3, \( \lambda = 488 \text{ nm} \) => \( Z = 4.56 \text{ focus} \)

**2D/3D Laser Scanning (Light)**
- Laser scanning: point-by-point detection of photons emitted/ reflected by the respective specimen detail
- Parameters: "Detector Gain" = PMT high voltage, "Amplifier Gain" = solid state amplifier
- Calibration: "Amplifier Offset" on image background (black level)
- Detector Gain: according to scanned image (object), setting aid = "Range Indicator" = difference (in units %), goal: least number of overmodulated (red, Gain) and undermodulated (blue, Offset) pixels

**3D Laser Scanning (4D)**
- Depth discrimination – confocal aperture to prevent detection of out-of-focus light (optical sectioning)
- Achromatic: determines thickness of optical slice; optimum slice thickness = 1 / 2 x diameter of Airy disk

**Focal Plane**
- Focal plane: distance between two optical slices (step size)
- Dependent on fluorescent dye: Generally, the shorter the wavelength, the higher the resolution

**Pinhole Aperture**
- Pinhole diameter: 1 Airy unit = best trade-off between depth discrimination capability and efficiency

**Confocal Pinhole**
- Depth discrimination – confocal aperture to prevent detection of out-of-focus light (optical sectioning)
- Achromatic: determines thickness of optical slice; optimum slice thickness = 1 / 2 x diameter of Airy disk

**Pinhole Diameter**
- Pinhole diameter: 1 Airy unit = best trade-off between depth discrimination capability and efficiency

**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**
   - Activated LSM mode (select manual tube slider or button "LSM")
   - Open window "Configuration control"
   - Select a predefined configuration from list (Single Track)
   - A disk on: Apply - automatically sets up the system: laser lines, attenuation, emission filter, beam splitter (HP NT), pinhole diameter, detector settings (channels, gain, offset)

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

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**How to Enhance Image Quality**

More signal!
- Change to higher pixel dwell times by reducing scanning speed
- Use "Average" method: Calculation of "Sum" or "Mean" value of pixels of consecutive "Line" or "Frame" (Sum)
- Increase bandwidth of emission filter (e.g. LP instead of BP)
- Enhance 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): spherically corrected
- Increase "FrameSize" = number of pixels per line x lines per frame, e.g. 1024 x 1024 or 2048 x 2048 (max: 4 x 2)
- Optimize scan zoom, i.e. pixel size 0.25 x diameter of Airy disk
- Increase dynamic range (change from 8 to 12 bit per pixel)

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

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**We make it visible.**