LASER SCANNING
CONFOCAL MICROSCOPY
PRACTICAL CONSIDERATIONS
OUTLINE

V. Important Parameters
- Pixel dwell time
- Zoom/Pixel number
- Digital sampling/Pixel size
- Pinhole size
- Scan mode

VI. Practical Considerations
- Light efficiency
- Signal to noise ratio
- Resolution in practice

VII. Summary
• How much time signal is collected at every pixel
• Very small values, normally in microseconds range
• Defined either directly or indirectly by scan frequency and image size
• Example: 512x512 pixels, 400 Hz - dwell time $4.9 \times 10^{-6}$ sec
• Important characteristic for signal intensity
DWELL TIME

Dwell time: 50 µs  Dwell time: 6 µs  Dwell time: 1.6 µs
Pixel size is defined by zoom and pixel number

To change pixel size
1. Change zoom - image size changed, number of pixels constant
2. Change number of pixel - image size constant, pixel size changed
SCANNING MECHANISM
ZOOMING/PIXEL NUMBERS

• Zooming: change the mirror deflection range (=Amplitude of scanner input signal)
  Zoom in: smaller field of view; higher energy deposition (bleaching)
• Pixel numbers: change the speed of the scanner
  • Large pixel numbers $\rightarrow$ lower scan speed
**DIGITAL SAMPLING**

**CAMERA BASED DETECTION**
- Fixed physical pixel size (e.g. Sony Interline chip 6.45 µm)
- Adaptation: Increase of effective pixel size via binning (CCD camera)

**POINT SCANNING DETECTION**
- No physical pixel size
- Pixel size is determined by sampling (zoom, number of pixels per image)

Digital Image
Nyquist–Shannon sampling theorem

If a function $x(t)$ contains no frequencies higher than $B$ hertz, it is completely determined by giving its ordinates at a series of points spaced $1/(2B)$ seconds apart.

In other words, a bandlimited function can be perfectly reconstructed from an infinite sequence of samples if the bandlimit, $B$, is no greater than $\frac{1}{2}$ the sampling rate (samples per second). function can be perfectly reconstructed from an infinite sequence of samples if the bandlimit, $B$, is no greater than $\frac{1}{2}$ the sampling rate (samples per second).

Light microscopy:

Bandlimit: Resolution Limit
Sampling frequency) $1/2 \ast$ resolution limit

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DIGITAL SAMPLING

Wavelength: 488nm; NA=1.4  
Rayleigh criterion: 212 nm
PIXEL SIZE

33 nm
65 nm
130 nm
260 nm

12 MB
t=64x

3 MB
t=16x

0.8 MB
t=4x

0.2 MB
t=1x
PIXEL SIZE

- Nyquist sampling is as good starting point
- Oversampling can result in better images but increases probability of photobleaching and decreases temporal resolution.
- Undersampling increases temporal resolution and decreases image file size.
- Sampling frequency should match the biological question
Closing the pinhole increases the z-sectioning capabilities because light from out of focus planes is suppressed, but also decreases the SNR/contrast (less photons are detected also from the focal plane).
SPECTRAL OVERLAP

- Spectral overlap depends on the emission-spectra of the fluorophores.
- Amount of bleed through depends on the spectral overlap the concentration of the fluorophores and the excitation intensity.
Sequential scanning : minimizes bleedthrough, less temporal resolution
Simultaneous scanning : optimizes temporal resolution
V. Important Parameters
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VII. Summary
LIGHT EFFICIENCY

Photons emitted by a single fluorophore

- How many photons can one obtain from a typical fluorophore in confocal microscopy?

- How does can we influence the photon flux?

- Why is the photo flux important for (Laser scanning confocal) microscopy?
LIGHT EFFICIENCY
PHOTONS EMITTED BY A SINGLE FLUOROPHORE

\[ k_a = \sigma * I \]

\[ k_b = \frac{1}{\tau_F} \quad \tau_F = \text{fluorescent lifetime} \]

Photon output (steady state)
\[ = k_b [S_1] \]

\[
\frac{[S_1]}{[S_{tot}]} = \frac{k_a}{k_a + k_b}
\]
LIGHT EFFICIENCY
PHOTONS EMITTED BY A SINGLE FLUOROPHORE

1 mW @488 nm focused to a radius of 0.25 μm
(e.g. objective NA 1.25)

\[ I = 5.1 \times 10^5 \text{ W/cm}^2 = 1.25 \times 10^{24} \text{ photons/(cm}^2\text{s}) \]

Fluoresceine: \( \varepsilon = 80 \, 000 \, \text{l/mol}^{-1}\cdot\text{cm}^{-1} \)

\[ \text{Cross section/molecule} = 3.06 \times 10^{-16} \, \text{cm}^2/\text{molecule} \]

\[ k_a = \sigma \cdot I = 3.8 \times 10^8 \text{ s}^{-1} \]

\[ k_b = \frac{1}{\tau_F} \]

\[ \tau_F = 4.5 \, \text{ns} \Rightarrow k_b = 2.2 \times 10^8 \text{ s}^{-1} \]
LIGHT EFFICIENCY
PHOTONS EMITTED BY A SINGLE FLUOROPHORE

\[
\frac{[S_1]}{[S_{tot}]} = \frac{k_a}{k_a + k_b}
\]

very fast

\[S_1\]

\[k_a\]

\[k_b\]

\[S_0\]
**LIGHT EFFICIENCY**

PHOTONS EMITTED BY A SINGLE FLUOROPHORE

Photon output: \( Q_e \cdot k_b \cdot [S_1] = 0.9 \times 0.63 \times 2.2 \times 10^8 \text{ s}^{-1} = 1.3 \times 10^8 \text{ photons/s} \)

\[ k_a = \sigma \cdot I = 3.8 \times 10^8 \text{ s}^{-1} \]

\[ k_b = \frac{1}{\tau_F} \]

\[ \tau_F = 4.5 \text{ ns} \Rightarrow k_b = 2.2 \times 10^8 \text{ s}^{-1} \]

512*512 pixel / s = 3.8 \mu s/pixel \Rightarrow \sim 500 \text{ photons/pixel} (for fluoresceine excited with 488 nm)
LIGHT EFFICIENCY
PHOTONS EMITTED BY A SINGLE FLUOROPHORE

Single Fluorophore (Fluoresceine):
512*512 pixel / s = 3.8 µs/pixel ➞ ~ 500 photons/pixel

But losses due to
- Geometry (fluorophore emitting in all directions)
  - NA 1.4 ➞ 40% efficiency
  - NA 0.3 ➞ 5% efficiency
- Scattering
- Optical elements
  - Objective
  - mirrors, filters
  - pinhole
- Detector efficiency 10-30%

Each fluorescent molecule contributes on the order of only one photoelectron/pixel/sweep
Signal to Noise

No Noise

Gaussian Noise: 1 STDV

Gaussian Noise: 2 STDV

Noise reduces Modulation/Contrast ➔ Decreases Resolution
NOISE REDUCTION

Strategies to reduce image noise

• reduce scan speed
  increases pixel dwell time = more photons

• Averaging
  • line averaging
  • frame averaging
  • Integrate

• Photon output
  • increase laser intensity
  • Limitation: fluorophore saturation
BLEACHING

Excitation
- Cone of light
- Bleaching occurs above and below focal plane
- z-sampling affects bleaching
Bleaching Mechanisms

Photon output: \(1.3 \times 10^8\) photons/s = \(1.3 \times 10^8\) cycles/s

High energy density
High cycle time
⇒ High bleaching probability

- Photochemistry of bleaching poorly understood.
- Differs from fluorophor to fluorophor
- Main bleaching route: Triplet state
  - Absorb another photon
  - Energy exchange with triplet oxygen, which becomes highly reactive singlet oxygen
TRIPLET STATES

Ensemble of molecules: 60% are trapped in triplet state →
decrease of fluorescent intensity by 60%
BLEACHING

Parameters affecting bleaching probability
- Fluorophore
- Laser intensity
- Pixel Dwell time
- Sampling frequency

Bleaching ↔ (Photon)Noise
LASER SCANNING CONFOAL MICROSCOPY OUTLOOK

Resolution

- Point Spread Function
- Nyquist Sampling
- Optical Slice thickness
LASER SCANNING CONFOAL MICROSCOPY
POINT SPREAD FUNCTION

Figure 3

Axial and Lateral Point Spread Functions

Figure 5
Example: $\lambda=500\,\text{nm}$; $n=1.518$; $\text{NA}_{\text{Obj}}=1.4$

- wf xy-plane resel=217 nm
- conf. xy-plane resel=157 nm
- conf. axial resel=580 nm

bead: 170 nm  
FWHM: 234 nm  

$\text{FWHM} \approx \text{resel}$
SPHERICAL ABBERATIONS

Figure 3: Specimen Image Distortion in Aqueous Media

Figure 6: Confocal Point Spread Function Contour Plots

Figure 5: Immersion Objective Contrast Transfer Functions
RESOLUTION

• **Physical Resolution**
  – Parameters affecting resolution
    • Wavelength
    • NA of objective
    • Refractive index
    • Pinhole size
  – Difference in lateral and axial resolution

• **Digital resolution (Sampling)**
  – Nyquist sampling
  – Optical zooming
  – Number of pixels
## LASER SCANNING CONFOCAL MICROSCOPY

### SUMMARY

**Widefield microscopy**
- Detector efficiency: 60%-80%
- Detector noise: 4 RMS electrons/pixel
- Pixel Dwell time: 10-1000 ms
- Optical sectioning: no
- Background suppression: no

**Laser scanning confocal microscopy**
- Detector efficiency: 3%-12%
- Detector Noise: < 0.01 RMS electrons/pixel
- Pixel dwell time: 1-100 µs
- Optical sectioning: yes
- Background suppression: yes
SUMMARY

- Decrease pinhole size
- Less photons
- Decreases SNR, contrast and thereby resolution
- Increases Resolution (mainly along z-axis, but also in the xy-plane)