Living up to Life
Introduction to Confocal & Multiphoton Microscopy
Introduction to Confocal Microscopy

- Basics of Light Microscopy
- Principles of Fluorescence
- Optical Resolution
- Confocal microscopy principle
- Some important aspects
- Basic Multiphoton Microscopy
- Multiphoton Microscopy Application
- Super resolution: STED
Basics of Light Microscopy
World of small structures
To see the Micro-cosmos

We need a Microscope

- Magnification: The way to see small detail
- Resolution: The way to distinguish between small detail
- Contrast: The way to see resolved and magnified detail
To see the Micro-cosmos

- Human eye : 100000 nm
- Simple magnifier : 10000 nm
- **Optical microscope** : 200 nm
- Electron microscope : 0.5 – 3 nm
- Scanning Probe microscope : 0.1 – 10 nm
WideField Microscopy

- Simultaneous illumination and observation of an extended area.
- Image formed contains in focus and out of focus information
- No scanning procedure necessary
- Standard light sources are sufficient
Principles of Fluorescence
Contrast using Fluorescence

- Only specific structures are stained and images
- Unwanted structures remain are not visible
- Detail can be seen even if smaller than resolution limits
- With the advent of special dyes, staining of living cells is now possible
Fluorescence
Jablonski Diagram

Energy

488nm
Stokes-Shift
525nm

Absorption

Excited Energy levels

Emission

Ground Energy levels

Prof. Alexander Jablonski, 1935
Fluorescence

Dichroic spectral position
Fluorescence Filter Block
Prof. Ploem’s invention
Fluorescence Filters

- Short Pass Filter (a) - Pass in UV, Block in IR
- Long Pass Filter (b) - Block in UV, Pass in IR
- Dichromatic Beam Splitter (c) - Reflect in UV, Pass in IR
- Wide Band Pass Filter (a) - Block in UV, Pass in IR
- Narrow Band Pass Filter (b) - Block in UV, Pass in IR
- Short Pass and Long Pass Filter Combination SP-LP (c) - Block in UV, Pass in IR
Optical resolution
Optical Resolution Limited by Diffraction

\[ D_0 = 1.22 \times \lambda / NA \]
Objective NA

Point Spread Function (PSF)
Fluorescence Resolution

Res = 0.61*λ / NA

Example:
63X Oil N.A. 1.4
Resolution = 210nm

credit: http://www.microscopyu.com/tutorials/
Fluorescence in a thick specimen

Lack of contrast due to flare
Confocal microscopy principle
Confocal Microscopy

- Patented by Marvin Minsky in 1957
- Elimination the out of focus flare observed in fluorescence in thick sections
Conventional vs Confocal

Confocal

Standard Fluorescence

http://www.atto.com/Carv/CarvSkinSection.htm

Basement membrane labeled with cy2 (green)
Neurons labeled with cy3 (red)
Confocal Principle

Laser

Objective

Excitation Pinhole

Excitation Filter

Emission Pinhole

PMT

Emission Filter

Emission Pinhole
Why Laser?

- Intense light source confined to small beam size to increase the signal and to allow easy focus on the diffraction limited spot.

- As different fluorescence dyes have different spectral characteristics, many laser lines are required.
Scanning Galvanometers

Point Scanning

Laser in

Laser out

To Microscope

Laser in
Scanning could be really fast!!!

- **Resonant Scanner** for fast imaging at high z-resolution, dynamic measurement at high speed (imaging of Ca\(^{2+}\) transients, movement imaging, Kinetic measurements)
- Lines: 8000 lines/sec unidirectional
  - 16000 lines/sec bidirectional
- Frames: 25 frames/sec at 512x512 pixels
  - 250 frames/sec at 512x16 pixels
Resonant Scanner

Fish embryo (Medaka)
Flow of red blood cells and migration of macrophages.
Macrophage: YFP and RFP (1st & 2nd ch)
Red blood cells: TLD

Courtesy of Clemens Grabher and Jochen Wittbrodt (EMBL), Heidelberg, Germany
Arabidopsis thaliana
First channel: Cell wall in reflection.
2 & 3 channel: Monitoring mitochondrial (GFP-green) and plastid (autofluorescence-red) movement.

Courtesy of Prof. Dr. D. Menzel, Institut für Zelluläre und Molekulare Botanik
Zellbiologie der Pflanzen, Bonn University.
Resonant Scanner

Lines

- 8000 lines/sec unidirectional
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Frames

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Fish embryo (Medaka)
Flow of red blood cells and migration of macrophages.
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Courtesy of Clemens Grabher and Jochen Wittbrodt (EMBL), Heidelberg, Germany
Resolution: Widefield vs Confocal

**Res** = $0.61\frac{\lambda}{\text{NA}}$

**Res(xy)** = $0.4\frac{\lambda}{\text{NA}}$

**Res(xz)** = $0.45\frac{\lambda}{n(1-\cos\alpha)}$

Formulas by Kino
Resolution Widefield vs Confocal

Conventional

Confocal

Credit: Prof. A. Diaspro

\[
\text{Res} = 0.61 \times \lambda / \text{NA}
\]

\[
\text{Res} = 0.4 \times \lambda / \text{NA}
\]
Diffraction Limit:

\[ FWHM_{xy} = \frac{0.4 \lambda}{n \cdot \sin \alpha} \]

- xy resolution: \(~ 250\text{nm}\)

\[ FWHM_z = \frac{0.45 \lambda}{n(1 - \cos \alpha)} \]

- z resolution (confocal): \(~ 550\text{nm}\)
*Drosophila melanogaster* (larvae)

Green: Nuclei (RNA binding protein), Alexa 488; Red: Axons, Cy 3; Blue: Axon endings (of MJ94-positive neurons), Cy 5

Courtesy: Dr. Christoph Melcher, Research Center Karlsruhe
Some important aspects
Choose the right objective

Needs for confocal imaging:

- High aperture
- High colour correction
- Flat field
- Long working distance
- High transmittance
- Variety of coupling media (oil, water, glycerol)

i.e. Plan Apochromat & Plan-Fluotar
Match the refractive index

• Avoid mismatch of refractive index!

• Select objectives according to the sample preparation

• Oil objective gives the best confocal effect only when the specimen is close to the coverslip

• Matching the refractive index between immersion medium and the specimen gives better Z-resolution. (e.g. Water, Glycerol)
Match the refractive index

Muscle tissue embedded in glycerol/water
recorded stack: 100um
Courtesy of Dr. Günther Giese, MPI, Heidelberg
Match the refractive index

Data obtained from xyz-series of 10 μM FITC in glycerol/water (80/20%) mounted on cavity slides. 1.3 GlycCorr = glycerol objective with numerical aperture of 1.3; 1.32 Oil = oil objective with numerical aperture of 1.32.
Note the slow drop in intensity observed for the use of the glycerol lens in comparison to the strong intensity drop observed for the use of the oil lens.
Coverslip thickness

- Use coverslip with thickness = 170um
- 15um mismatch will kill ½ of resolution
- Water/Glycerol objectives provide correction collar for coverslip thickness compensation.

No. 0  – 0.085 to 0.13 mm thick
No. 1  – 0.13 to 0.16 mm thick
No. 1.5 – 0.16 to 0.19 mm thick
No. 1.5H – 0.17 to 0.18 mm thick
Format Size

- The Scan format is adjustable for Confocal microscope (e.g. 512 X 512, 1024 X 1024)

- The format size must be big enough in order to truly represent the information recorded by your objective.
Sampling Theory

- **The Nyquist Theorem**
  - Nyquest theory describes the *sampling frequency* (\( f \)) required to represent the true identity of the sample.
  - *i.e.*, how many times must you sample an image to know that your sample truly represents the image?
  - In other words to capture the periodic components of frequency \( f \) in a signal we need to sample at least \( 2f \) times.

- Nyquist claimed that the rate was \( 2f \). It has been determined that in reality the rate is \( 2.5f \) - in essence you must sample at least 2 times the highest frequency.

- For example in audio, to capture the 22 kHz in the digitized signal, we need to sample at least 44.1 kHz.
Matching the pixel size with the resolution of the objective

<table>
<thead>
<tr>
<th>Objective</th>
<th>Wavelength (nm)</th>
<th>Nyquist sampling at minimum half resolution (nm)</th>
<th>Nyquist sampling at 2/5 resolution (nm)</th>
<th>Field diameter (μm)</th>
<th>Best scanning format minimum</th>
<th>Best scanning format at higher range</th>
</tr>
</thead>
<tbody>
<tr>
<td>10×/0.4</td>
<td>488</td>
<td>244</td>
<td>195</td>
<td>1550</td>
<td>6352</td>
<td>7941</td>
</tr>
<tr>
<td>20×/0.7</td>
<td>279</td>
<td>140</td>
<td>112</td>
<td>775</td>
<td>5556</td>
<td>6944</td>
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<tr>
<td>40×/0.85</td>
<td>230</td>
<td>115</td>
<td>92</td>
<td>388</td>
<td>3370</td>
<td>4212</td>
</tr>
<tr>
<td>40× oil/1.25</td>
<td>156</td>
<td>78</td>
<td>62</td>
<td>388</td>
<td>4968</td>
<td>6210</td>
</tr>
<tr>
<td>63× oil/1.40</td>
<td>139</td>
<td>70</td>
<td>56</td>
<td>246</td>
<td>3540</td>
<td>4425</td>
</tr>
<tr>
<td>100× oil/1.40</td>
<td>139</td>
<td>70</td>
<td>56</td>
<td>155</td>
<td>2230</td>
<td>2788</td>
</tr>
<tr>
<td>20× water/1.0</td>
<td>195</td>
<td>98</td>
<td>78</td>
<td>601</td>
<td>6164</td>
<td>7705</td>
</tr>
<tr>
<td>20× water/0.95</td>
<td>205</td>
<td>103</td>
<td>82</td>
<td>601</td>
<td>5863</td>
<td>7329</td>
</tr>
</tbody>
</table>
Diffraction Limit:

\[ FWHM_{xy} = \frac{0.4\lambda}{n \cdot \sin \alpha} \]

- xy resolution: \( \sim 250\text{nm} \)

\[ FWHM_z = \frac{0.45\lambda}{n(1 - \cos \alpha)} \]

- z resolution (confocal): \( \sim 550\text{nm} \)
Question?
MULTIPHOTON MICROSCOPY BASICS
Basic principle of multiphoton excitation

**Single photon**

- 1 photon is absorbed by a fluorophore
- From fundamental state to excited state

**Two-photon excitation**

- Simultaneous absorption of 2 photons for excitation of a fluorophore with the half energy
Basic principle of multiphoton excitation

Advantages of Multiphoton excitation

- Greater penetration depth due to lower scattering
- Excitation restricted to focal plane – no volume bleaching
- Optical sectioning properties without use of pinhole
- Reduced phototoxicity due to spatial confinement, ideal for living cells
- Photoactivation or photobleaching in a diffraction limited volume
Confocal microscopy vs. Multiphoton microscopy
The depth limit

- Achievable depth: ~ 300 – 500 µm

- Maximum imaging depth depends on:
  - Available laser power
  - Tissue properties
    - Density properties
    - Microvasculature organization
    - Cell-body arrangement
    - Collagen / myelin content
  - Collection efficiency (NDD vs internal PMT)
Two-photon: excitation probability

\[ n_a \propto \delta \left( \frac{P_{\text{avg}}^2}{\tau f^2} \right) \left( \pi \frac{\text{NA}^2}{hc \lambda} \right)^2 \]

- \( n_a \): probability of excitation
- \( \delta \): excitation cross section
- \( P_{\text{avg}} \): average power incident light (peak power)
- \( \tau \): pulsewidth
- \( f \): repetition rate
- NA: Numerical aperture
- \( h \): Planck’s constant
- \( c \): Speed of light
- \( \lambda \): Wavelength

Given the formula, MP excitation is favoured when we have:

- Molecules with large cross-section
- High peak power
- High-NA objective lenses
Examples of cross section in 2P excitation

The best excitation is not always the double of 1P excitation
TP cross-section of standard FPs

Blab et al., 2001
Examples of cross section in 2P excitation

Bestvater et al.
Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging
### Multiphoton excitation of selected dyes

<table>
<thead>
<tr>
<th></th>
<th>780 nm</th>
<th>820 nm</th>
<th>1064 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Wall Stain</strong></td>
<td>Calcofluor White 440/500-520</td>
<td>Calcofluor White 440/500-520</td>
<td>Ethidium Bromide 518/605</td>
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<tr>
<td><strong>Nucleic Acid Stains</strong></td>
<td>DAPI 350/470, Hoechst 350/460</td>
<td>DAPI (885,970 3P), Hoechst 350/470</td>
<td>Propidium Iodide 530-615</td>
</tr>
<tr>
<td><strong>Feulgen</strong></td>
<td>Feulgen 480/560</td>
<td>Feulgen 480/560</td>
<td>Feulgen 480/560</td>
</tr>
<tr>
<td><strong>Cell Viability</strong></td>
<td>Fluorescein Di Acetate 495/520</td>
<td>Fluorescein Di Acetate 495/520</td>
<td></td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td>Indo 1 (720, 885 3P) 430-365 /400-480</td>
<td>Fura 2 (720) 340-380/512</td>
<td></td>
</tr>
<tr>
<td><strong>Calcium Green/Texas Red (770)</strong></td>
<td>488/530, 596/620</td>
<td>Calcium Green 488/530</td>
<td></td>
</tr>
<tr>
<td><strong>Protein Conjugates</strong></td>
<td>AMCA 431/498</td>
<td>FITC 490/525</td>
<td></td>
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<tr>
<td><strong>Gene Expression</strong></td>
<td>GFPuv 395/509</td>
<td>S65T (860) 488/507</td>
<td>S65T 488/507</td>
</tr>
<tr>
<td><strong>Mito Tracers</strong></td>
<td>Rhodamin 123 507/529</td>
<td>Rhodamin 123 507/529</td>
<td>Rhodamin 123 507/529</td>
</tr>
<tr>
<td><strong>Vacuolar Tracer</strong></td>
<td>FM4-64 515/614</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipid Tracer</strong></td>
<td>Nile Red 485-530 /526-605</td>
<td>Dil (12, 16) 549/565</td>
<td></td>
</tr>
<tr>
<td><strong>Neuronal Tracer</strong></td>
<td>DID (760-780) 430/535</td>
<td>Lucifer Yellow 430/535</td>
<td>Evans Blue 550/610</td>
</tr>
</tbody>
</table>
Advantage of Multifoton vs Confocal for imaging scattering tissue

- No confocal pinhole necessary
- Detectors as close as possible to the specimen enables scattered photons to be collected

→ Much higher photon collection efficiency compared to confocal microscopy

2 NDDs architecture

- Different dichroics available to separate pairs of fluorochromes
TCS SP5 MP: NDDs

**Highest photon collection efficiency**

- Detectors directly behind Objective, RLD
- Detectors directly behind Condensor, TLD

**Advantage:**

- Scattered fluorescent photons can also be collected
- Special dichroic allows simultaneous acquisition of fluorescence and IR-SGC
- Protected by Leica patent US 6,831,780 B2
Photon Collection Efficiency - Internal vs. NDDs

Mouse brain slice: ~ 20 µm (center plane)
Detection range: 500 – 550 nm
PMT: 950 V
Objective: 20 x 1.0 W
Excitation: 920 nm, power level identical

Mean intensity image: internal 20
                        RLD 52
                        TLD 58
MULTIPHOTON MICROSCOPY Application
In vivo imaging – Thick specimen

Mounted artery of the mouse

Imaging
Excitation at 890nm

3 channel acquisition:
• Autofluorescence of elastin (blue),
• Syto13 for nuclei of cells in the vascular wall (green/white),
• Eosin auto-fluorescence (red);

Imaging depth 650µm.

Preparation
Common carotid arteries from mice are carefully dissected, excised, and stored in Hanks’ balanced salt solution (HBSS, pH 7.4).

Courtesy of Marc van Zandvoort, Biophysics, Univ. of Maastricht, Netherlands
Confocal vs. Two photon – depth penetration

Eye of zebrafish embryo (stained with DAPI)

Image size (xz): 125 μm x 125 μm - Objective: 63x 1.2 Water - Detection window: 400nm – 500nm

Ex: UV / 365 nm
PMT: 360V

Ex: IR / 780 nm
PMT: 360V
Introduction of a photoactivated marker allows for:

- Studying cell lineage
- Organell dynamics
- Protein trafficking
- Compartment connectivity

photoactivation/ ~conversion typically achieved with high intensity flash of 405 nm
# Photo-activatable / switchable FPs

<table>
<thead>
<tr>
<th>FP</th>
<th>Type</th>
<th>2 PE (nm)</th>
<th>Fluorescence</th>
<th>Reference</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>paGFP</td>
<td>photoactivation</td>
<td>750</td>
<td>green / red</td>
<td>Patterson</td>
<td></td>
</tr>
<tr>
<td>KAEDE</td>
<td></td>
<td>730</td>
<td>green / red</td>
<td>Miyawaki</td>
<td></td>
</tr>
<tr>
<td>KikGrR1</td>
<td></td>
<td>760</td>
<td>green / red</td>
<td>Miyawaki</td>
<td></td>
</tr>
<tr>
<td>PS-CFP</td>
<td></td>
<td>800</td>
<td>cyan / green</td>
<td>Lukyanov</td>
<td></td>
</tr>
<tr>
<td>mEOSFP</td>
<td></td>
<td></td>
<td></td>
<td>Wiedemann</td>
<td></td>
</tr>
<tr>
<td>Dendra</td>
<td></td>
<td>960</td>
<td>green / red</td>
<td>Lukyanov</td>
<td></td>
</tr>
<tr>
<td>Dronpa</td>
<td>photochromic</td>
<td>780, 1010</td>
<td>green</td>
<td>Miyawaki</td>
<td>Switched on / off</td>
</tr>
</tbody>
</table>
Second Harmonic Generation: Jablonski diagram

- SHG involves virtual transitions in which no energy is absorbed
- 2 photons „simultaneously“ scattered, resulting in „frequency doubling“
- In contrast 2PE involves absorption (real transition) and excitation of molecules
- \[ \text{SHG} = \frac{\lambda_{\text{incident}}}{2} \]
- Predominantly forward-directed emission

- i.e. *trans*-detection
  - High NA condensor (1,4 oil)
  - 2nd objective (on DMI)
  - backscattered possible

- Visualize well-ordered structures:
  - Collagen fibers
  - Microtubules
  - Muscle myosin
  - Membrane potential via dyes
SHG images

SHG combined with fluorescence:
Collagen fibrils (SHG, grey), Macrophages (Fluorescence, green & red)

Striation pattern of murine heart
Question?
STED: Technology and Applications
Why super-resolution?

We want to study details!

- beyond the diffraction limit
- with standard dyes/FPs
- inside cells/organisms
Why super-resolution?

The purpose of STED microscopy is to increase lateral (xy) resolution in fluorescence microscopy over classical Abbé Limits:

\[ FWHM_{xy} = \frac{0.4 \lambda}{n \cdot \sin \alpha} \]

- \( xy \) resolution: \( \sim 250 \text{nm} \)

\[ FWHM_z = \frac{0.45 \lambda}{n(1 - \cos \alpha)} \]

- \( z \) resolution (confocal): \( \sim 550 \text{nm} \)
STED Microscopy: The Principle
STED microscopy – the concept


XY resolution of 70nm!!!
STED microscopy – the concept

the focal spot

Excitation and fluorescence emission

Excitation and stimulated emission
Confocal super-resolution

STED microscopy allows optical sectioning

- penetration depth depends on refractive index matching
- best results at coverglass
- routinely good results achieved at 20µm
- deeper imaging possible
3D capability
Oregon Green 488 Vimentin

Maximum projection confocal

Maximum projection STED

z-pos: 0.9 µm
z-pos: 2 µm
z-pos: 4 µm
z-pos: 6 µm
Application

Confocal

STED

Enhanced detail on PTK2 cells, microtubules
Application

Neuromuscular synapses

Drosophila neuromuscular synapses. LiprinProtein, stained with ATTO 647N; 2048 x 2048 pixels
Courtesy of Stephan Sigrist, Wuerzburg, Germany
Some working dyes for TCS STED

<table>
<thead>
<tr>
<th>Dye</th>
<th>Vendor</th>
<th>Laser lines</th>
<th>2 color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abberior STAR 635</td>
<td>Abberior</td>
<td>640/750</td>
<td>recommended</td>
</tr>
<tr>
<td>Atto 647N</td>
<td>ATTO-TEC</td>
<td>640/750</td>
<td>recommended</td>
</tr>
<tr>
<td>Atto 655</td>
<td>ATTO-TEC</td>
<td>640/750</td>
<td></td>
</tr>
<tr>
<td>Atto 665</td>
<td>ATTO-TEC</td>
<td>640/775</td>
<td></td>
</tr>
</tbody>
</table>
Sample preparation

- STED is fully compatible with all standard staining procedures for fluorescence microscopy (FISH, direct IF, indirect IF ...).

- Stay as close as possible to your original staining protocol.

- Be aware of the size of the label.

- If you want to work deep inside the samples match the refractive index.

<table>
<thead>
<tr>
<th>Mounting media</th>
<th>RI</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDE</td>
<td>1.514</td>
<td></td>
</tr>
<tr>
<td>ProLong Gold</td>
<td>1.46</td>
<td>Hardening, variation in batch quality</td>
</tr>
<tr>
<td>Mowiol +/- 2.5% DABCO</td>
<td>1.5</td>
<td>Hardening</td>
</tr>
<tr>
<td>86% glycerol + 4% NPG*</td>
<td>1.452</td>
<td></td>
</tr>
<tr>
<td>86% glycerol + 2.5% DABCO**</td>
<td>1.452</td>
<td></td>
</tr>
</tbody>
</table>