Praktikum zur Fluoreszenz- und Konfokalmikroskopie

Winter Term 2012/13

non-confocal

confocal section

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Current Equipment

8 Instruments = 3 Confocal Microscopes + 1 Spinning Disc + 1 Live Imaging Unit + 1 Deconvolution Microscope + 1 Microdissection + 1 Apotome

- LSM Meta 2005
- LSM 510 1999
- LSM710 2011
- Spinning Disc 2012
- Olympus Cell-R 2008
- Axiovert200M Apotome 2005
- Deltavision 2010
- Leica LMD 2009
Light is an electromagnetic wave: radiation (direction and speed) and wave properties (intensity and wavelength).

Geometric Optics And Wave Optics

Refraction (Brechung) and Reflection
Interference, Diffraction (Beugung), Polarisation,
Types of Light

- **Monochromatic**
- **Linearly polarized**
- **Collimated** (coaxial paths of propagation through space – indep. of $\lambda$, phase or polarization)
- **Coherent** – same phase

- **Polychromatic**
- **Nonpolarized**
- **Non-collimated = Divergent**
- **Non-coherent**
Refraction

Refraction and Refractive Index
(measure for optical density):

Air: 1.0003
Water: 1.3333
Silica glass: 1.459
Immersion oil: appr. 1.52
Diamond: 2.417

Refraction varies by frequency
Lenses, Focus and Aberrations

Reason: lens failures - glass inconsistencies, partial reflection (sample thickness!), Optical solution: aspheric lenses (cheaper is apertures)

Can be longitudinal (as shown) and lateral (perpendicular to focal point)

 Reason: prism-effect at lens edges
Optical solution: achromatic or apochromatic lenses (2 types of glass) – Fluorescence!!

Other aberrations include: Curvature of field / Distortion (fish-eye) / astigmatism
The Microscope

1. Illumination
2. Objectives
3. Detection

http://micro.magnet.fsu.edu/primer/anatomy/bh2cutaway.html
Types of microscopes and Illumination

Reflected or Incident Light (Auflicht)

Transmitted Light (Durchlicht)

Also for thick and intransparent specimen
Simple Geometry of a Microscope

Virtual image seen by eye

Remark: Camera will record the primary image!
Objectives

**Labeling of the Objective**
Objective class, special designations are used for this, e.g. LD for Long Working Distance

**Magnification / Numerical Aperture**
plus additional details on
- immersion medium (Oil/W/Glyc)
- adjustable cover glass correction (Korr.)
- contrast method

**Tube Length / Cover Glass Thickness (mm)**
ICS optics: ∞
Infinity Color Corrected System standard cover glass: 0.17 without cover glass: 0 insensitive: -

**Mechanical Correction Collar**
- cover glass thickness correction
- different immersion
- different temperature
- adjusting an iris diaphragm

**Color of writing**
Contrast method
- Standard
- Pol/DIC
- Ph 0 1 2 3

**Color Coding of Magnification**
- 1.0/1.25
- 2.5
- 4/5
- 6.3
- 10
- 16/20/25/32
- 40/50
- 63
- 100/150

**Immersion Fluid**
- Oil
- Water
- Glycerin
- Oil/Water/Glycerin

Dipping objectives-physiology

Coverslips / Tools

• # 0: 0.08 – 0.13mm
• # 1: 0.13 - 0.16 mm
• # 1.5: 0.16 - 0.19 mm
• # 2: 0.19 – 0.25 mm

Usually glass, permanox plastics can also be used. Conventional TC plastics not useful for fluorescence applications (absorption!) For live imaging use glass-bottom dishes (expensive) or chamber slide (cave: working distance)

Resolution

Definition: the smallest distance between two points that can be displayed

\[ d = \frac{0.61\lambda}{NA} \]

Resolution thus depends on:
1. The wavelength of light that reaches the objective
2. Numerical Aperture \((N_A)\) \(\rightarrow\) Property of the objective
3. Immersion medium (part of \(N_A\) calculation)
<table>
<thead>
<tr>
<th>Material</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>1.0003</td>
</tr>
<tr>
<td>Water</td>
<td>1.333</td>
</tr>
<tr>
<td>Glycerin</td>
<td>1.4695</td>
</tr>
<tr>
<td>Paraffin oil</td>
<td>1.480</td>
</tr>
<tr>
<td>Cedarwood oil</td>
<td>1.515</td>
</tr>
<tr>
<td>Synthetic oil</td>
<td>1.515</td>
</tr>
<tr>
<td>Anisole</td>
<td>1.5178</td>
</tr>
<tr>
<td>Bromonaphthalene</td>
<td>1.6585</td>
</tr>
<tr>
<td>Methylene iodide</td>
<td>1.740</td>
</tr>
</tbody>
</table>

Numerical Aperture (NA) = \( n \cdot \sin \mu \)

Figure 2

\( NA = (n) \sin(\mu) \)

(a) \( \mu = 7^\circ \) NA = 0.12
(b) \( \mu = 20^\circ \) NA = 0.34
(c) \( \mu = 60^\circ \) NA = 0.87

http://micro.magnet.fsu.edu/index.html
Resolution

!!!!!!Magnification identical !!!!!!
Essential Wave Properties

- **Wavelength (nm):**
- **Amplitude – Intensity:**
- **Phases**
- **Diffraction**

Diffraction Pattern with main and side maxima

http://www.sgha.net/articles/diffraction.jpg

Spatial Resolution – Rayleigh Criterion
(regular light microscopy)

Counts for transmitted and reflected light microscopy
Axial Resolution

• Axial Resolution is worse than lateral: minimum distance two diffraction images of “points” can approach each other along the z-axis

\[ z_{\text{distance}} = \frac{2 \lambda n}{(N_A)^2} \]

• Z shrinks inversely proportional to the 2\textsuperscript{nd} power of the \( N_A \)

• Ratio of lateral to axial resolution is:

\[ 3.28 \times n / N_A \]
Depth of Field

The axial range, through which an objective can be focused without any appreciable change in image sharpness, is referred to as the objective **depth of field** = thickness along the z-axis where an object in the specimen appears focused !! – almost only dependent on NA !

**Depth of Focus** = the thickness of the image plane itself. Largely dependent on Magnification !

<table>
<thead>
<tr>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>Depth of Field (mm)</th>
<th>Image Depth (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>0.10</td>
<td>15.5</td>
<td>0.13</td>
</tr>
<tr>
<td>10x</td>
<td>0.25</td>
<td>8.5</td>
<td>0.80</td>
</tr>
<tr>
<td>20x</td>
<td>0.40</td>
<td>5.8</td>
<td>3.8</td>
</tr>
<tr>
<td>40x</td>
<td>0.65</td>
<td>1.0</td>
<td>12.8</td>
</tr>
<tr>
<td>60x</td>
<td>0.85</td>
<td>0.40</td>
<td>29.8</td>
</tr>
<tr>
<td>100x</td>
<td>0.95</td>
<td>0.19</td>
<td>80.0</td>
</tr>
</tbody>
</table>

\[
\Delta f = \lambda / 2 \cdot NA^2
\]

\(\Delta f\) = depth of field  
\(\lambda\) = wavelength of light (emission)  
\(NA\) = numerical aperture
Light gathering power of objectives

- $F_{\text(epi)} = 10^4 \times (\text{NA}^4/M)^2$

Means e.g. $40x /1.3 \gg 63x /1.4 > 100x /1.4$

Increasing #of pixels or mag. decreases intensity per pixel !!

Decreasing pixel size by 2 also decreases S/N by 2 -> decreases resolution (binning!) BUT increases intensity- BUT also decreases exposure time! -> “survival time”
Detection Systems Overview
Detection – Digital Cameras

- cMOS (complementary metal oxide semiconductor)

- CCD (Charge-coupled-device)
Detection

Digital Cameras: Photons elicit electron hole pairs (photoelectric effect) – charge converted to voltage – this analogue signal is amplified and converted into a binary image (AD-conversion)

**Digital Coding:**

Data Depth = levels of grey
1 bit: 0, 1
2 bit: 00, 01, 10, 11

**BINNING:**
- speed increase by sampling 4, 9, 16 pixels as one
- reduction of memory size
- shorter illumination times

Fig. 9: Bit depth and grey levels in digital images.

Fig. 10: Creation of a digital image.
Nyquist-Sampling Theorem – or how many pixel do I need for a resolution representative image?

**NYQUIST SAMPLING CRITERION:** the rate of sampling must be at least 2-fold the sample frequency to be able to reconstruct the analog signal from a digitalized one. Sampling frequency is limited by the pixel size of the chip!

Calculation – the resolution at 550nm with an objective 100x, NA=1,4 calculates to 230nm -> magnified by a factor of 100 = 23µm -> on the chip the image must be large enough to cover 2 pixels -> required pixel size is 11,5 µm!

\[
R \times M = 2 \times \text{pixel size}
\]

½ inch chip is usually 6,4mm x 4,8mm: minimum # of pixels horizontally = 6400 / 11,5 calculates to 557 pixels

Lower mag objectives usually need more pixels for optimal resolution on the CCD-chip (high resolution microscopes usually equipped with no more than 1,3 Megapixel cameras)
Imaging - Triangle of Frustration

temporal resolution

spatial resolution

sensitivity

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Live cell Microscopy
Optimal environmental conditions

Temperature
- Heating stages
- Heatable mounting frame
- Warm air (incubation)
- Objective heater

Oxygen content
- Controlling the $O_2$ content
  - Specially sealed incubation system
  - Displacement of $O_2$ by nitrogen

pH-value
- Controlling the pH-value
  - Incubators with adjustable $CO_2$ concentration

Humidity
- Increasing the relative humidity
  - Humidifier system
  - Humidifier modules
  - Membrane covers

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Fluorescence

Stokes (1852) – Jablonski (1935)

1) Molecule absorbs Light = Energy
2) Excitation of electrons
3) Relaxation of energized electrons
4) Emission of fluorescent Light of higher wavelength than exciting light
Fluorescence

Stokes Shift

\[ \lambda_{\text{Em}} > \lambda_{\text{Exc}} \]
Principle of Fluorescence

- Molecules capable to fluoresce = Fluorophores
- Excitation with light of proper wavelength lifts electrons from basal (S₀) to excited levels S₁; each of these energy levels is itself divided into several possible vibrational states of the molecule
- Emission free conversion to lowest state energy level (IC = Internal Conversion)
- Two possible ways:
  - Intersystem Crossing (ICS): Conversion to T1 triplet state without any radiative emission and return to S₀
  - Return to energy state S₀ by emission of a photon (Energy difference = \( \lambda \)) → Fluorescence
1 Photon vs. 2 Photon Excitation

1-photon excitation

2-photon excitation

exciting photon
emitted photon
Quantum Efficiency

- Only emitted light is relevant for fluorescence detection in microscopy – intersystem conversion processes equals to loss of fluorescence efficiency
- Quantenausbeute (quantum yield or quantum efficiency [QE]) in steady state:
  \[
  \text{QE} = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}}
  \]
- QE is the essential for a fluorophore to qualify for optimal use in microscopy

A good fluorophore can be excited up to \(10^6\) times before it is „dead“
Factors affecting QE

- Quenching by collision with other molecules
- **Static Quenching**: when a complex is formed between the fluorophore and a quenching molecule
- **Fluorescent energy Transfer (FRET)**: radiation-free transfer of energy from an excited donor molecule on to an acceptor molecule (can also be used for dynamic association studies- see later). Occurs preferentially in multi-colour applications – cave: keep fluorophore concentrations as low as possible.
  - Works only over a limited narrow spatial neighborhood in the range of 20 – 70 Angström
  - Emission spectra of Donor and Excitation spectra of Acceptor molecules must overlap significantly
- **Photobleaching**: Interaction with light– ROS – can lead to photochemical changes in molecule structure and in worst case to loss of fluorescent properties -> is being used for dynamic analyses
Fluorophores

- We divide two large groups:

  - **Primary fluorophores** (includes autofluorescence): endogenous fluorescence of a probe; sometimes unwanted background interference
    - Chloroplasts in plant tissue, collagen, elastin, fibrilllin, flavin in animal tissue
    - Autofluorescent proteins: GPF, YFP, CFP, FRP

  - **Secondary Fluorophores**: molecules that require other molecules by
    - A) binding to antibodies
    - B) binding to cell-intrinsic components (DAPI-DNA)
Fluorescent Dyes

- Commercially available from many companies

- Usually planar polycyclic organics with conjugated double bonds

Fluorescein
Upon oxidation the fluorescent properties are autocatalytically formed during maturation through a cyclization reaction between residues buried deep within the shielded environment of the barrel.

Thr65-Tyr66-Gly67

Also visit: http://gfp.conncoll.edu/ and http://www.olympusconfocal.com/java/fpfluorophores/gfpfluorophore/index.html
Single stainings

Counter-Stains

Double – to multiple -colours
**Organelles**

- **Mitochondria (rot) + Lysosomes (grün)** (MitoTracker – Lysotracker)
- **Golgi**
- **Endosomes**
- **ER (membrane stains like DiOC6, ConA)**
- **Nuclei (Syto, Yopro, Topo, histone-FP)**
- **Cytoskeleton (Phalloidin, Taxol)**

FUNCTIONAL ANALYSES

Apoptosis  TdT(terminal deoxy transferase)-mediated dUTP-X nick end labeling
Annexin 5 (detects phosphatidylserin on surface / Live-Dead Kits from Molecular Probes)
Cell cycle  (BrdU, Fucci Cell Cycle Sensor)

Enzyme Activities  (alk-Phosphatase)

Fluorosensors  (cAMP)

Ionic and pH Indicators  (FURA, Indo, pHrhodo)
Quantum Dots

“Whatever hue want”

QD = Colloidal Semiconductor nanocrystals

Different Sizes -> different fluorescence spectra (the larger – the redder

• Very high quantum yield
• less bleaching
• nearly no quenching effects

-> preferable for in vivo imaging

BUT: may be toxic and are not „cleared“

Usually a broad excitation peak and a symmetric, very narrow, emission peak (single light source can be used to excite more dots)

http://www.evidenttech.com/home-page.html

http://bme240.eng.uci.edu/students/07s/yokabe/main.htm
SAMPLE PREPARATION
conventional Workflow
(In)direct Immunofluorescence

1) FIXATION
2) PERMEABILIZATION
3) BLOCKING NON-SPECIFIC SITES
4) LABELING WITH ANTIBODIES
5) MOUNTING
IMMUNOFLUORESCENCE-MICROSCOPY

- Direct IF
  - Advantage: Detection more specific, since omission of unspecific Signals of secondary antibody.

- Indirect IF
  - Advantage: Signal amplification, due to binding of multiple secondary AB's to primary AB's.

Alt.: Direct IF

**Diagram:**

1. **Antigen**
2. **Fix**
3. **Permeabilise**
4. **Primary AB**
5. **Wash**
6. **Secondary AB**
7. **Wash**
8. **Embed**
9. **Microscope**

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**Legend:**
- **Antigen**
- **Fix**
- **Permeabilise**
- **Primary AB**
- **Secondary AB**
- **Wash**
- **Embed**
- **Microscope**

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Advantage direct IF: Detection more specific, since omission of unspecific Signals of secondary antibody.
Advantage indirect IF: Signal amplification, due to binding of multiple secondary AB’s to primary AB’s.
Sample preparation

Theoretically it’s a Western blot – in situ on cells.
What’s different:
Cells grow or have to be fixed on any kind of „support“ = which should be optically transparent
Fixation needed
Permeabilisation for antibody access
Antibodies coupled to fluorophors
Detection -> Microscope

„Support“ – Material:
Suspension cells:
Cytospin
Adhesion Slides (BioRad): special coating
Poly-Lysine coated Coverslips: rarely suitable for suspension cells
Adherent Cells:
Glas-Coverslips
Poly-Lysin-coated Coverslips
Filter-Inserts (porous Polycarbonat-Filter)
Chamber-Slides / Chambered Coverglass
FIXIERUNG:
variable—should be suited for antigen characteristics (Localisation, Structure, Conformation) and primary antibodies;
Usually protocol can be interrupted AFTER fixation is completed – cells on ice (in PBS [Phosphate-buffered-saline –
50mM Phospatpuffer, pH 7.2, 150 mM NaCl]).

MetOH:
not for integral membrane proteins; it’s mere protein-Precipitation!! Should be cool to cold at –20oC; minimizes lipid extraction and improves fixation quality; is a quick procedure and should not last too long – 3 to 5 min to ensure structural integrity. Often in combination (2:1 oder 1:1) with Acetone; Usually cells are already permeabilized.

!! Perfect for cytoskeletal structures !!

Formaldehyde/Para-Formaldehyde:
some antibodies don‘t like it; Concentration-range: 1-4 % (para-f. Sometimes up to 8 %); Room-Temperature! Over-fixation nearly impossible -10-30 min sufficient; After fixation freely reactive groups need to be blocked: 100mM glycine/PBS or 50mM NH4SO4/PBS or 50mM NH4Cl/PBS.

!! Perfect solution for soluble proteins, membrane antigens !!
-sometimes cytoskeletal integrity can be compromized.

Glutaraldehyde: only a few antibodies like it; blocking of excess with reducing agent NaBH4.

EGS (ethylene-glycol-succinimide): preferred for a mix staining of membrane and cytoskeletal structures; rarely used – not well known.
Fixation Chemistry

Formaldehyde:
Note — commercial 37% FA contains up to 10% of MetOH

Glutaraldehyde
The free aldehyde groups introduced by glutaraldehyde fixation cause various problems. These include non-specific binding of proteinaceous reagents, notably antibodies, and a direct-positive reaction with Schiff’s reagent.

http://publish.uwo.ca/~jkiernan/formglut.htm
PERMEABILISATION:
For antibody diffusion.
usually: PBS + 0.1-0.5 % TritonX-100 (3-5 min)

BLOCKING:
Mostly used: BSA or Gelatine (0.5 – 1%); for many antibodies blocking does not increase staining efficiency -> „may not help, but doesn't hurt either“-> coffee break😊.

PRIMARY-AB:
Like in Western-needs higher titer. Rule of Thumb: 10 times the concentration that worked in Western (Western 1:1000 \( \rightarrow \) IF 1:100).
Incubation: Room-Temperature; 45-60 min; if weak: either o/n 4oC or 2-3 hrs. at 37oC.
Double-Stainings: Mind species cross-reaction!

WASHING:
3-5 x PBS for 5-10 min

SECONDARY AB:
Species-and Isoform-Specificity as in western; fluorophore-coupled.
*Termed INDIRECT* Immunofluorescence (iIF) . In case the primary antibody is directly coupled: Direct Immunofluorescence (dIF) -> is more specific, though usually weaker since one antigen can only bind 1-2 antibodies, whereas secondary antibodies amplify the signal up to factors of 10.
Incubation: Room Temperature; 45-60 min; if weak: either o/n 4oC or 2-3 hrs. at 37oC.
Double-Stainings: Mind fluorophore cross-emission!

After 2nd washing step: chance for DNA-counterstaining with DAPI, Hoechst (violet) or Propidium-Iodine (Red; broad; also stains RNA-> RNAse treatment recommended) or other DNA-binding dyes (SYPRO, YOPRO, TOTO – some cell-permeable, some not).
**EMBEDDING:**
Your last washing step should always be water – to get rid of salt -> crystals (light and crystals -> rainbow😊)

Dry!

Embedding Media: Miscellaneous (see below)
Thin layers only – too thick -> focus depth -> resolution and focussing capacity decreases with imaging depth!
Some contain „Anti-Fading“ reagents – decrease bleaching effects, some, however, can also influence fluorophore efficiencies.
Embedding Media

• Note: refractive index of media should be as close to that of glass and immersion medium

• Some media change RI when hardening - Mowiol

• Basis: water, glycerine (RI: 1.41), polyvinyl alcohol

• Keep as this as possible – avoid air bubbles (scattering!)

• Fingernail polish as sealant may quench GFP

• Don’t use VectaShield or ProLong with quantum dots-quenching

• Antifade agent p-phenylenediamine (Vectashield) quenches cyanine dyes (Cy2, Cy3..)

• Crystal Mount quenches some red dyes (phycoerythrin, phycocyanine)

• ProLong is good for Alexa dyes – can affect autofluorescent proteins
Check-List

- Only use #1.5 (0.17mm) GLASS coverslips – best transmission and refractive index closest to objective and immersion oil (Correction rings objective?)
- Check thickness of support – long distance objectives?
- Embed your samples – mounting media prefered with RI close to glass and immersion medium. Antifading agents-interference with fluorophores?
- Free „floating“ samples should be sealed for immersion-medium objectives (shear forces) – sealants: nailpolish (acetone!); VALAP; self-hanrdening silicone (tooth cement)
- Counter-stain necessary? – check with fluorophores portfolio used!
- Check dyes for cross-excitation/emission and note autofluorescence (plants, media....)!
**FLUORESCENCE MICROSCOPY**

1. **First barrier filter:** lets through only blue light with a wavelength between 450 and 490 nm.

2. **Beam-splitting mirror:** reflects light below 510 nm but transmits light above 510 nm.

3. **Second barrier filter:** cuts out unwanted fluorescent signals, passing the specific green fluorescein emission between 520 and 560 nm.

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*Figure 9-12. Molecular Biology of the Cell, 4th Edition.*
Light Sources / 1

**Halogen Lamps**
- High power
- 200-500h
- Peak intensities
- Needs alignment

**Mercury Arc Lamps**
- < power than mercury
- 1500-3000 h
- Uniform spectrum
- Needs no alignment

**Xenon Lamps**
- Ultralong lifetime
- Cheap
- Narrow spectra (lack betw. 530 and 580nm)
- Individual bulbs – user specs.
- Weaker emission intensities

**LEDs**

[Images of light sources and emission spectra]

http://www.olympusmicro.com/
Light Sources / 2

Laser

- Light amplification by stimulated emission of radiation (energy (from pumping) absorption -> photon -> stimulation of absorption -> photon, photon..., -> resonator for long ways)
- Medium for amplification can be gas (HeNe, Ar, Kryptone...) or solid state (Al2O3-rubene, corunde, titan-sapphire...)
- Can be continuous wave (cw) or pulsed (photonic packages down to fs)

PROPERTIES

- COHERENT Light: means waves maintain the same phase relationship while traveling
- Laser light is also monochromatic (one wavelength) and polarized (E-vectorial propagation in parallel planes)
Types of Filters

• Longpass-filter

• Shortpass-filter

• Bandpass-filter
Longpassfilter

e.g. Longpass 420:
Number defines cut-off wavelength. This number is selected at the „cut-on point“ and will always be specified at 50% of transmission.
Shortpassfilter

e.g. Shortpass 500:
Number defines wavelength up to which transmission occurs. It defines the „cut-off point“ at 50\% transmission.
Bandpassfilter

e.g.: Bandpass 465/70 (alternatively: 430-500)
70 = Bandwith: defines broadness of the peak at 50% of transmission
465 = median wavelength – arithmetic average of cut-of wavelengths (Cave: often not identical with peak maximum)
Strahlenteiler = Beamsplitter

- Splits Excitation and Emission Light
Full Cube assembly Bandpass

31001 (Chroma)

http://www.chroma.com/
Problem 1: cross-excitation

a fluorophore is not just excited by wavelength at its peak value, but also by wavelength at certain range around the peak, which can extends into the area used by other fluorophores.

FITC TRITC excitation peaks
Problem 2: cross emission (emission bleeding through)

When emission spectra of two fluorophores overlaps, emission from one channel will extend to another channel.
Filter set for simultaneous detection of triple fluorescence

82000v2 Filtersatz von Chroma
Für DAPI, FITC, TRITC

Excitationfilter
Dichroic mirror
Emissionfilter

http://www.chroma.com/

• “Apotome”: the principle is to collect “in focus” and “out-of-focus” information using a structured illumination

http://zeiss-campus.magnet.fsu.edu/tutorials/opticalsectioning/apotome/index.html
Pseudo-confocal Microscopy- „Apotome“

- Based on an interferometric principle of „grid projection“ – Structured Illumination Microscopy
- Getting rid of blurring – depth of field information only
- Grid with a defined width inserted into the plane of the field stop into the reflected light beam path – grid lies in focal plane
- Apotome scanner permits the grid to be shifted in position upper and below focal plane
- 3 images generated and superimposed – algorithm based „deblurring“ – pseudoconfocal image
Different grids – Different sectioning

- 2 grids – Vh and Vl – for high and low magnification

Optical section thickness (μm):
- 20x/0.75 1.5
- 40x/0.75 1.6
- 40x/1.3 0.8
- 63x/1.4 0.7

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WIDEFIELD

APOTOME-MODE

63x oil, nA=1.40

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Drawbacks - Troubleshooting

- Photobleaching
- Live Samples -> no fast moving objects
- Calibration – UV; violet!
- Thickness
- Noise – spherical aberration - noise from out of focus light worse than in confocal (use cooled CCDs)
- No high transmission filters

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Apotome - Advantages

- Simple system – easy integration
- Cheap in investment / maintenance

Recent improvement:
„Vivatome“: grid on a disc
Focus info through disc, out-of-focus info reflected -> overlay. („aperture correlation“)
+ faster

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Deconvolution

Limitations to the resolution in an optical system stems from „convolution“: glare, distortion and blurriness from stray light from out-of-focus areas, especially in fluorescence microscopy cause acquisition „artefacts“. Also in confocal microscopy these artefacts may occur from optical inconsistencies in the specimen, glass, or from optical defects (inproper corrections) in objectives.

Highly sophisticated software calculations can be applied to „reverse“ these artefacts and create crispy images for better evaluation.

Why do we do it:
• Enhanced resolution in all 3 dimensions x, y, and z
• Reduction of Noise to improve S/N ratio
• Reversal or optimization of optics-based aberrations
The Point-Spread Function

For this reversal the „point-spread-function“ (PSF) is either calculated or experimentally determined and the PSF is the basis for this mathematic reversal approach.

The point spread function is the image of a point source of light from the specimen projected by the microscope objective onto the intermediate image plane, i.e. the point spread function is represented by the Airy disk pattern (see resolution). Due to diffraction, the smallest point to which one can focus a beam of light using a lens is the size of the Airy disk. PSF of a system is the three-dimensional diffraction pattern generated by an ideal point source of light. The PSF is a measure for the ability of a system to create contrast for a given resolution in the intermediate image plane. PSF of an individual objective or a lens system depends on numerical aperture, objective design, illumination wavelength, and the contrast mode.
Literature

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- http://www.coolled.com/default.htm
- http://www.embl.de/almf/ALMF/Welcome.html
- http://www.mshri.on.ca/nagy/
- http://www.svi.nl/
Thank you for your attention
Confocal Microscopy

what is different?

Johannes Koch
johannes-paul.koch@univie.ac.at
Confocal and Widefield Fluorescence Microscopy
Depth of Focus
Confocal: Functional Elements

- Illumination
- Pinhole
- Detector
- Filter
- Beam splitter
- Scanner
- Microscope
- Z drive
Light source: Extended vs Point illumination
The Pinhole I

• Light from below - focal plane is focused at a plane behind the pinhole.
• Light from above - focal plane is focused before the pinhole.
• Only the light from the focal plane is focused at the pinhole and can reach the image detector.
The Pinhole II

Pinhole diameter effects

small pinhole diameter:
- thin optical section
- high z-resolution possible
- low signal strength

big pinhole:
- thick optical section
- low z-resolution
- bright

optical sectioning possible

\[ d_{\text{Airy}} = \frac{1.22 \lambda}{NA} \cdot M \cdot 3.6 \]
A pinhole of 1 airy unit (AU) gives the best signal/noise. A pinhole of 0.5 airy units (AU) will often improve resolution IF THE SIGNAL IS STRONG.
E-cadherin

β-catenin

Desmoplakin

Tight junctions

Adherens junctions

Desmosomes
Confocal Overview II

- A point light source for illumination
- A point light focus within the specimen
- A pinhole at the image detecting plane
- These three points are optically conjugated together and aligned accurately to each other in the light path of image formation, this is **confocal**.
- Confocal effects result in supression of out-of-focal-plane light, supression of stray light in the final image
Laser I

- Monochromatic
- Linearly polarized
- Collimated (coaxial paths of propagation through space – indep. of λ, phase or polarization)
- Coherent – same phase

<table>
<thead>
<tr>
<th>Type</th>
<th>Power</th>
<th>Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diode</td>
<td>25 mW</td>
<td>405</td>
</tr>
<tr>
<td>Argon</td>
<td>100 mW</td>
<td>458, 477, 488, 514</td>
</tr>
<tr>
<td>Green HeNe</td>
<td>15 mW</td>
<td>543</td>
</tr>
<tr>
<td>Green DPSS</td>
<td>75 mW</td>
<td>561</td>
</tr>
<tr>
<td>HeNe</td>
<td>25 mW</td>
<td>633</td>
</tr>
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</table>
Laser II – How are they used?

- Laser Modulation -> AOTFs
- No filters, filter wheels, tunable intensities, wavelengths and selection of ROIs
- Ultrasound waves work as grating and deflect specific $\lambda$ / intensity of waves regulates intensity of light

Demonstration
Laser III – How are they used?

![Laser Control Interface](image)

### Lasers

<table>
<thead>
<tr>
<th>Laser Unit</th>
<th>Wavelength</th>
<th>Power</th>
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<tbody>
<tr>
<td>Laser Diode 405</td>
<td>405 nm</td>
<td>On</td>
</tr>
<tr>
<td>Argon/2</td>
<td>458, 477, 488, 514 nm</td>
<td>Stdby</td>
</tr>
<tr>
<td>HeNe1</td>
<td>543 nm</td>
<td>On</td>
</tr>
<tr>
<td>HeNe2</td>
<td>633 nm</td>
<td>On</td>
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</table>

### Argon/2

<table>
<thead>
<tr>
<th>Maximum Power</th>
<th>Wavelength</th>
<th>Status</th>
<th>Tube Current</th>
<th>Output [%]</th>
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<tbody>
<tr>
<td>30.0 mW</td>
<td>458, 477, 488, 514 nm</td>
<td>Warming up</td>
<td>2.0 A</td>
<td>25</td>
</tr>
</tbody>
</table>
Area Scanning – Point by Point

Figure 2
Photomultiplier Tube (PMT)

+ no „false positives“
- efficiency
Optics I: Overview
Optics I: Splitting wavelengths

AOBS:

MBS:

Beam Path and Channel Assignment

<table>
<thead>
<tr>
<th>Beam Path and Channel Assignment</th>
<th>Deteced</th>
<th>Non Detected</th>
<th>Camera</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mirror</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFT 333/343</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Optics II: Selecting wavelengths

Filter based system:

Spectrometer system:

- Selectivity?
- Flexibility?
- Transparency?
- Costs?
**Optics III: Spectral Detection**

- **Grating spectrometer:**
  - Large splitting
  - Linear splitting

- **Prism spectrometer**
  - Much higher transparency
  - For all wavelengths and polarizations

---

**Demonstration**

![Diagram of beam path and channel assignment]

- **Beam Path and Channel Assignment**
  - Beam splitter
  - Confocal pinholes
  - Detector
  - Objective

- **Prism spectrometer setup**
  - Prism
  - Sliders
  - Detector

---

**Optics III: Spectral Detection**

- **Grating spectrometer:**
  - Large splitting
  - Linear splitting

- **Prism spectrometer**
  - Much higher transparency
  - For all wavelengths and polarizations
no interference from lateral stray light: higher contrast.

no out-of-focal-plane signal: less blur, sharper image.

images can be derived from optically sectioned slices (depth discrimination).

Improved resolution (theoretically) due to better wave-optical performance.
Practical Examples

CLSM + spectral detection (LSM 510 META)

CLSM for live imaging (LSM 5 Live)

Spinning disk

Multiphoton

Techniques + Problems

Superresolution
Configurations – Zeiss LSM510-710

- Up to 50 laser lines possible
- Recycling loop to improve light collecting efficiency
- QUASAR: more sensitive, down to 3nm

280000€
Live Confocal – „Pin“ slit vs Pinhole
Figure 3. A recent improvement in confocal microscopy is the Nipkow disc system, which uses multiple pinholes to scan at high speeds.
Spinning Disk II

Spinning Disk Confocal

Broad laser illumination (limited excitation wavelengths): single color acquisition is very fast, slow switching between laser lines can also limit acquisition speed.

A second array of simultaneously rotating pinholes generates confocality.

Spinning disk confocal microscopes incorporate a rotating array of microlenses to focus illumination.

CCD detector captures light from all pinholes rapidly and simultaneously.

Nipkow (spinning) disk systems enable rapid live cell imaging with significantly reduced photodamage at an intermediate cost. Photobleaching experiments are not possible.

Demonstration

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## Confocal <-> Spinning Disc

### Beams and Illumination

<table>
<thead>
<tr>
<th></th>
<th>Confocal (Point Scanner-Confocal)</th>
<th>Spinning Disc</th>
<th>Widefield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beams</td>
<td>1</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>Illumination</td>
<td>1 mW</td>
<td>0.4-0.6 μW</td>
<td></td>
</tr>
<tr>
<td>Emission rate</td>
<td>1.26 x 10^8 photons/sec</td>
<td>1.72 x 10^5 photons/sec</td>
<td></td>
</tr>
<tr>
<td>Fluorophore saturation</td>
<td>63%</td>
<td>0.09%</td>
<td></td>
</tr>
</tbody>
</table>

### Acquisition

<table>
<thead>
<tr>
<th></th>
<th>Point Scanner-Confocal</th>
<th>Spinning Disc</th>
<th>Widefield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acquisition</td>
<td>Point by point - SLOW</td>
<td>Many points - FAST</td>
<td>One frame at a time - VERY FAST</td>
</tr>
</tbody>
</table>

### Detection

<table>
<thead>
<tr>
<th></th>
<th>Point Scanner-Confocal</th>
<th>Spinning Disc</th>
<th>Widefield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection</td>
<td>PMT - Medium sensitivity (but signal amplification)</td>
<td>EM-CCD - High Sensitivity</td>
<td>CCD-EMCCD - Medium-to-high sensitivity</td>
</tr>
</tbody>
</table>

### Optical Sectioning

<table>
<thead>
<tr>
<th></th>
<th>Point Scanner-Confocal</th>
<th>Spinning Disc</th>
<th>Widefield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Sectioning</td>
<td>Adjustable Pinholes - FLEXIBLE – light loss; NO CROSSTALK</td>
<td>Fixed Pinhole - INFLEXIBLE; light loss; Crosstalk possible</td>
<td>No sectioning - All light collected</td>
</tr>
</tbody>
</table>
Spinning Disc

• **Advantages:**
  - Fast acquisition with minimized energy input to the specimen
  - Cameras instead of PMTs – no scanning units necessary
  - Illumination sources from lasers extended to LEDs, metal halide lamps or even HBO
  - Preferential for live imaging

• **Disadvantages:**
  - No selectable pinholes – semi-confocal!
  - No ROIs – no FRAP, PA; PC
  - Thickness of sample restriction – fixed pinhole
Multiphoton Microscopy

Pulsed lasers
No pinhole needed

Advantage: low photo-cytotoxicity, since the long wavelength infra red laser has less toxicity than short wavelength.

Disadvantage: complicated, expensive
Multiphoton:

- label is excited only at the focus of the beam

No out-of-focus-fluorescence:

- No need of confocal aperture
- Dye bleaching and phototoxicity are limited to the plane of focus
What can you do?

Z- stack including „3D“ applications

Multicolor

Spectral Imaging

Colocalization

F-techniques

............
Z-stack
Z-stack: Reducing Dimensions
Z-stack: Animation
Z-stack: Reconstruction

- X/Y/Z image stacks are the raw data for 3D reconstruction
- The 3D VisArt menu makes it easy to create 3D views in shadow, transparency and surface projection
Objective behaviour – Imaging Thick samples

Refractive Index
Multicolor Labeling

Kern

Stützgerüst

Mitochondrien
Problem I: cross-excitation

The excitation profile of a fluorophore is not just a single line, it is a spectrum, i.e. the fluorophore can be excited by light at nm other than at its excitation peak.
Problem II: cross- emission

When emission spectra of two fluorophores overlap, emission from one channel is recorded (and attributed) to the other channel.

Example: ECFP and DsRed2 are excited by lasers (432/ 561nm), emission is recorded by two PMTs after a beam splitter operating at 550nm.

Solution: 1) Add extra filters – record DsRed2 starting from 590nm!
2) Do not record both fluorophores at the same time!
Problem III: Problem I + Problem II

When emission spectra of two fluorophores overlap, emission from one channel is recorded (and attributed) to the other channel.

Example: EGFP and DsRed2 are excited by lasers (488/561nm), emission is recorded by two PMTs after a beam splitter operating at 550nm.
The Problems: How to they look like?
Spectral Imaging

λ-scan

Define fluorophores and spectra
Spectral Imaging

Removing Autofluorescence with Spectral Imaging and Linear Unmixing

(a) HeLa Cell Expressing EGFP-Actin

(b) EGFP-Actin Signal

Figure 8
The F-techniques

- FRAP: Fluorescence Recovery after Photobleaching
- FLIP: Fluorescence loss in photobleaching
- PA: Photoactivation
- …
F-techniques - methodology

Photobleaching and Photoactivation Techniques

Figure 2

(a) FRAP
(b) FLIP
(c) PA

Kinetics

Time
Using FRAP, one can determine:

- diffusion coefficient
- immobile fraction
- binding or residence time.

**FRAP**

**A**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>pre</td>
<td>bleach</td>
<td>1.5s</td>
<td>10s</td>
</tr>
<tr>
<td>EGFP-Sec61β</td>
<td>EGFP-Sec61β</td>
<td>EGFP-Sec61β</td>
<td>EGFP-Sec61β</td>
</tr>
</tbody>
</table>

<p>| | | | |</p>
<table>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>pre</td>
<td>bleach</td>
<td>10s</td>
<td>50s</td>
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<tr>
<td>EGFP-HsPEX11β</td>
<td>EGFP-HsPEX11β</td>
<td>EGFP-HsPEX11β</td>
<td>EGFP-HsPEX11β</td>
</tr>
</tbody>
</table>

**B**

![Graph showing FRAP recovery](http://www.cf.gu.se/digitalAssets/725/725440_FRAP_recovery.gif)
iFRAP

B
GFP-Sarl FL
Prebleach  Postbleach  1h 33m  4h 40m  7h 32m  10h

C
GFP-N-2TM
Prebleach  Postbleach  3m 7s  7m 7s  15m 7s  35m 7s

D
GFP-TM-C
Prebleach  Postbleach  3m 11s  7m 11s  15m 11s  29m 11s
A part of a cell at a distance from where there is an excess of fluorescent protein is bleached with a laser at low intensity. One then images the sample just before and at constant intervals after bleaching, allowing the bleached molecules to redistribute through the cell – the loss of fluorescence is recalculated to measure the diffusion dynamics.

Applications: e.g.: checking the continuity of membrane systems (ER, Golgi).
“Optical Highlighters”

PHOTOACTIVATION

PHOTOCONVERSION

PHOTOSWITCHING

© Josef Gotzmann
Overview

**Optical Highlighter Fluorescent Proteins in Action**

- (a) Photoactivation 405 nm
- (b) Photoswitch ON
- (c) Photoswitch OFF
- (d) 543 nm
- (e) 488 nm
- (f) Photoswitch ON
- (g) Photoconversion 405 nm
- (h) Plaque Fusion
- (i) Annular GJ
- (j) 1 μm
- (k) 0.2 μm

Figure 5
Photoactivation (and yet another F-technique…)

**Fluorescence Decay**

After photoactivation

Mito-paGFP

GFP active

Merge channels

GFP inactive

RFP active

GFP Photoactivation 405nm, msec

Decay

Freely diffusible proteins

Concentrated not diffusible proteins

No decay

Normalized mean fluorescent intensity in percent

Time in seconds

Fluorescence decay

Fluorescence gain
**PA-GFP:** photoactivation (PA) none ->green

**Kaede:** green-> red photoconversion (PC) irreversible; tetrameric and hard to couple to biological samples

**Kindling red:** none->red PA upon green light (less damage)

**Dronpa:** on-off photoswitchable protein – 490nm ->off / 400nm ->on

**Dendra2:** monomeric; green->red PC; by blue light (also for imaging unconverted!)

---

**Table 2. Physical properties of useful optical highlighter fluorescent proteins**

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Color of spectral class</th>
<th>Excitation peak (nm)</th>
<th>Emission peak (nm)</th>
<th>Brightness1</th>
<th>pKa2</th>
<th>Association state3</th>
<th>Chromophore</th>
<th>Filter set4</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>PA-GFP (N)5</td>
<td>Green</td>
<td>400</td>
<td>515</td>
<td>2.7</td>
<td>4.5</td>
<td>Weak dimer</td>
<td>SYG</td>
<td>DAPI/FITC</td>
<td>Patterson and Lippincott-Schwartz, 2002</td>
</tr>
<tr>
<td>PA-GFP (P) **</td>
<td>Green</td>
<td>504</td>
<td>517</td>
<td>13.8</td>
<td>4.5</td>
<td>Weak dimer</td>
<td>SYG</td>
<td>FITC/GFP</td>
<td>Patterson and Lippincott-Schwartz, 2002</td>
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<tr>
<td>PS-CFP2 (N)</td>
<td>Cyan</td>
<td>400</td>
<td>468</td>
<td>8.6</td>
<td>4.3</td>
<td>Monomer</td>
<td>SYG</td>
<td>CFP</td>
<td>Chudakov et al., 2004</td>
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<tr>
<td>PS-CFP2 (P)</td>
<td>Green</td>
<td>490</td>
<td>511</td>
<td>10.8</td>
<td>6.1</td>
<td>Monomer</td>
<td>SYG</td>
<td>FITC/GFP</td>
<td>Chudakov et al., 2004</td>
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<tr>
<td>PA-mRFP1 (P)</td>
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<td>578</td>
<td>605</td>
<td>0.8</td>
<td>4.4</td>
<td>Monomer</td>
<td>QYG</td>
<td>TxRed</td>
<td>Verkhusha and Sorkin, 2005</td>
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<tr>
<td>tdEos (N)</td>
<td>Green</td>
<td>506</td>
<td>516</td>
<td>55.4</td>
<td>5.5</td>
<td>Tandem dimer</td>
<td>HYG</td>
<td>FITC/GFP</td>
<td>Nienhaus et al., 2006</td>
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<tr>
<td>tdEos (P)</td>
<td>Red</td>
<td>569</td>
<td>551</td>
<td>19.8</td>
<td>5.5</td>
<td>Tandem dimer</td>
<td>HYG</td>
<td>TRITC</td>
<td>Nienhaus et al., 2006</td>
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<tr>
<td>Dendra2 (N)</td>
<td>Green</td>
<td>490</td>
<td>507</td>
<td>22.5</td>
<td>6.6</td>
<td>Monomer</td>
<td>HYG</td>
<td>FITC/GFP</td>
<td>Gurskaya et al., 2006</td>
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<tr>
<td>Dendra2 (P)</td>
<td>Red</td>
<td>553</td>
<td>573</td>
<td>19.3</td>
<td>6.9</td>
<td>Monomer</td>
<td>HYG</td>
<td>TRITC</td>
<td>Gurskaya et al., 2006</td>
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<tr>
<td>KFP1 (P)</td>
<td>Red</td>
<td>580</td>
<td>600</td>
<td>4.1</td>
<td>NA</td>
<td>Tetramer</td>
<td>MYG</td>
<td>TRITC/DsRed</td>
<td>Labus et al., 2002</td>
</tr>
<tr>
<td>Dronpa (P)</td>
<td>Green</td>
<td>503</td>
<td>518</td>
<td>80.8</td>
<td>5.0</td>
<td>Monomer</td>
<td>CYG</td>
<td>FITC/GFP</td>
<td>Ando et al., 2004</td>
</tr>
</tbody>
</table>

*Table of physical properties for the monomeric and tandem dimer optical highlighters. *Common literature FP abbreviation. 1Product of the molar extinction coefficient and the quantum yield (mM×cm)−1. 2Literature values except as noted. 3Recommended common filter sets and custom FP sets available from aftermarket manufacturers. For specialized applications, we suggest choosing filter combinations that closely match the spectral profiles (see Shaner et al., 2005). 4Native conformation. **Photoactivated or photoconverted conformation.
Superresolution I

i.e. breaking the **diffraction**-limit of a microscope

<table>
<thead>
<tr>
<th>physically</th>
<th>mathematically</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REversible Saturable Optical Fluorescence Transitions</strong> (STED, GSD, SSIM, …)</td>
<td>PALM STORM</td>
</tr>
<tr>
<td>4-Pi</td>
<td></td>
</tr>
<tr>
<td>Near-Field</td>
<td></td>
</tr>
</tbody>
</table>
Superresolution II - RESOLFT

REversible Saturable Optical Fluorescence Transitions

~20nm resolution in x/y, „only“ 100nm in z
Blinking fluorophores is the pre-requisite for this technique
(Fluorescent)-PhotoActivationLocalization Microscopy (PALM)
(Eric Betzig, Berkeley)

http://www.youtube.com/watch?v=RE70GuMCzww