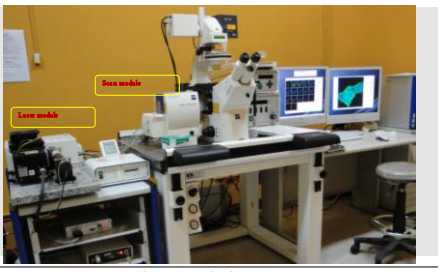
LSM 5 PASCAL

高雄榮總

LSM 5 PASCAL

高雄榮總



Basic principle of light microscope

Different types of light microscopes

Upright Microscope | Inverted Microscope | Stereo Microscope

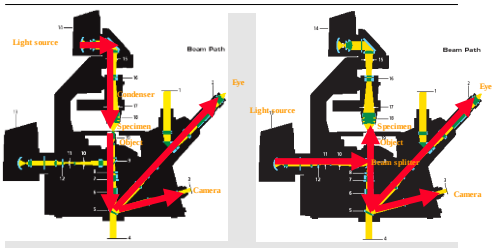
Basic principle of light microscope

Inverted Microscope

- Long working distance
- Cell incubation
- Micromanipulation
- Suitable for petri dish sample

Different Beam Path of Image Formation

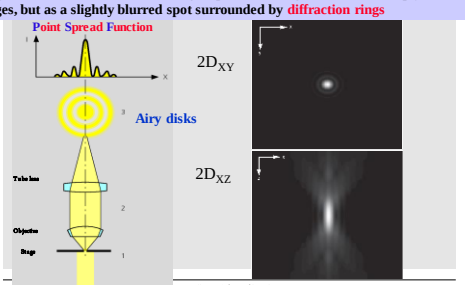
Transmitted-light and Reflected-light in inverted microscope



Transmitted-light | Reflected-light

Spatial Resolution of a Light Microscope

Objective and tube lens do not image a point as a bright disk with sharply defined edges, but as a slightly blurred spot surrounded by diffraction rings



The Resolution of a Microscope is limited

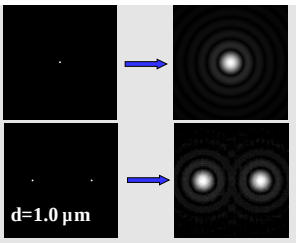
Object Image

What does that mean?

The image of a point-like structure is not a point, but a diffraction pattern with a finite extension.

This 2-dimensional pattern in the image plane is also called the Airy-disc.

In general, the image of a point-like structure is called the Point Spread Function (PSF).



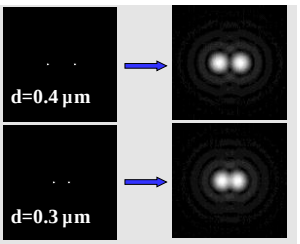
The Resolution of a Microscope is limited

Object Image

Definition

The resolution limit is reached, when two point-like objects can not be imaged as two distinct structures anymore.

The distance between the objects is called the resolution limit.



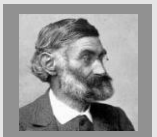
Basic principle of light microscope

Magnification alone is not enough: the resolution determines what we see.

➢ **Numerical Aperture and Resolution**

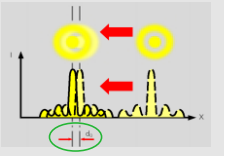
The Resolution of a Microscope is limited

The resolving power, the limit up to which two small objects are still seen separately.



Prof. Ernst Abbe
(1840 - 1905)

$d = \frac{\lambda}{2n\sin\alpha}$ (1876)



$d_0 = \frac{1.22\lambda}{N.A._{Obj.} + N.A._{Cond.}}$ more simply $d_0 = \frac{\lambda}{2N.A}$

λ = wavelength of light, e.g. 550 nm (green)

The Resolution of a Microscope depends on Numerical Aperture and Wavelength

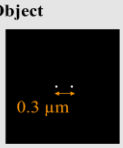
$d = \frac{\lambda}{2(NA)}$

$NA = n \cdot \sin\alpha$

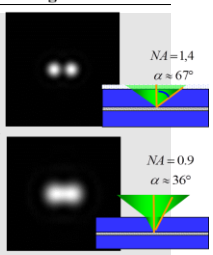
Principle effect
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

Object



Image

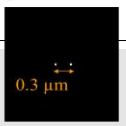


The Resolution of a Microscope depends on Numerical Aperture and Wavelength

Priciple effect
Shorter wavelengths generate smaller Point Spread Functions.

The shorter the wavelength, the better the resolution.

Object



$d = \frac{\lambda}{2 \cdot NA}$

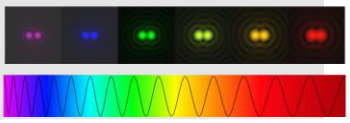
$NA = n \cdot \sin\alpha$

$NA = 1,4$

$\alpha \approx 67°$

Image

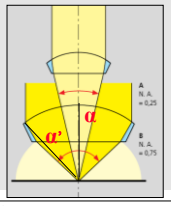
λ = 350nm 480nm 520nm 570nm 610nm 640nm



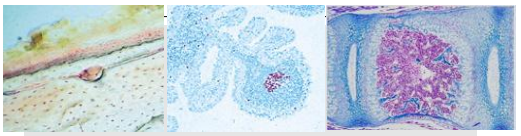
Basic principle of light microscope
The Numerical Aperture of Objective

The **numerical aperture** of a microscope objective is a measure of its **ability to gather light** and **resolve fine specimen detail** at a fixed object distance.

A. Low Magnification (10X/0.25)
B. High Magnification (40X/0.75)

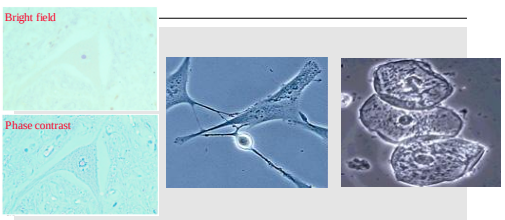


Different Beam Path of Image Formation
Bright field- Contrast depends on light absorption



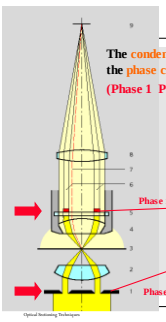
- **Bright Field is the most universal technique used in light microscope.**
- **Usually used in samples with colorimetric staining or good contrast.**
- **Color temp = 3200k (Halogen lamp) = yellow background**
  = 5500k (with conversion filter) = whiter background

Different Beam Path of Image Formation
Phase contrast

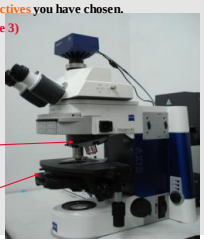


- **Usually used in samples with unstained thin sample.**
- **Phase contrast makes these tiny differences visible by the use of optical devices – i.e. it translates them into differences in intensity.**

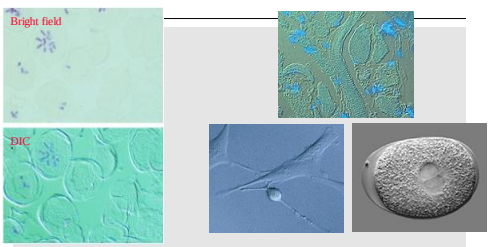
Different Beam Path of Image Formation
Phase contrast



The condenser requires one, two or three phase stops, depending on the phase contrast objectives you have chosen.
(Phase 1 Phase 2 Phase 3)

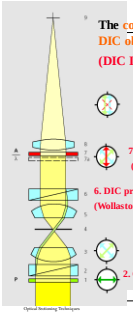


Different Beam Path of Image Formation
Differential Interface Contrast

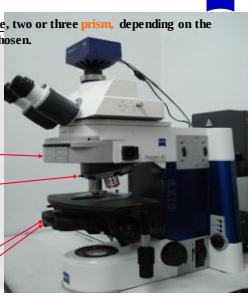


- **DIC components : 2 prism and 2 polarizer**
- **DIC is also ideal for the optical sectioning of unstained, thick objects**

Different Beam Path of Image Formation
Differential Interface Contrast



The condenser requires one, two or three prism, depending on the DIC objectives you have chosen.
(DIC I DIC II DIC III)

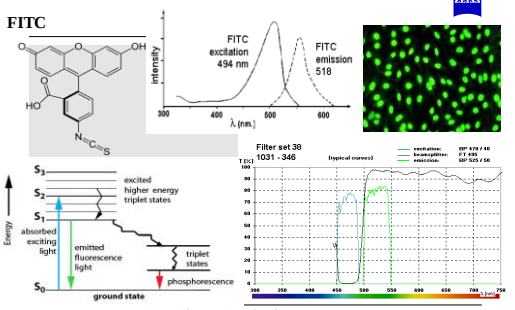


Objectives list and application field

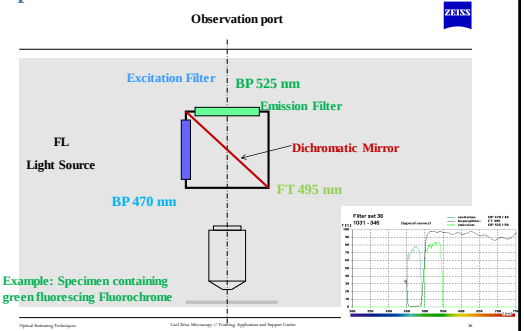
	BF	Phase	DIC	FL	glass cover slide	Plastic dish
Plan-Neofluar 10X/0.3 Ph1	v	v		v	v	v
LD Plan-Neofluar 20X/0.4 corr Ph2	v	v		v	v	v
LD Plan-Neofluar 40X/0.6 corr Ph2	v	v		v	v	v
Plan-Neofluar 40X/1.3 oil	v		v	v	v	
Plan-Neofluar 100X/1.3 oil DIC	v		v	v	v	

Different Beam Path of Image Formation
Fluorescence Dye

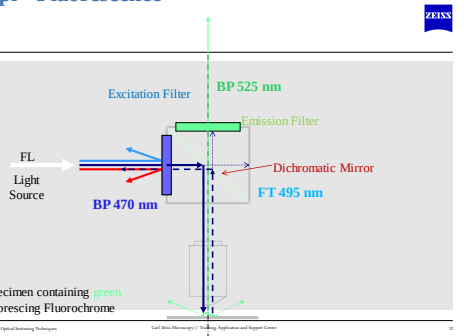
FITC



Epi - Fluorescence



Epi - Fluorescence

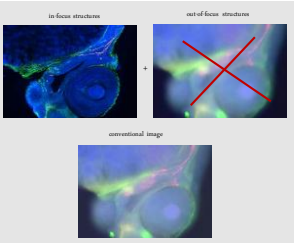


Imaging of 3-dimensional objects
The fundamental problem

Conventional Images

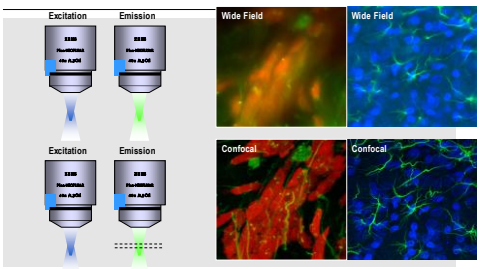
Conventional images of 3-dimensional objects always contain light from structures, which are in focus and light from structures which are not in focus.

This out-of-focus light blurres the structures from the focal plane and reduces the contrast and resolution.



Vanessa_Yu Take an Instrument Company

Confocal Laser Scanning Microscopy
Optical sectioning: elimination of out-of-focus light

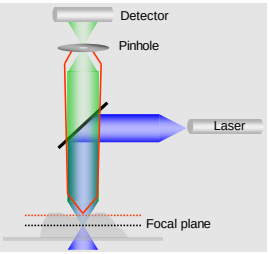


Vanessa_Yu Take an Instrument Company

Point scanning confocal microscopes
Confocal principle

Spot Detection

This pinhole simply blocks fluorescence light, which originates from above or below the focal plane of the objective.



Point scanning confocal microscopes
Confocal principle

Spot Detection

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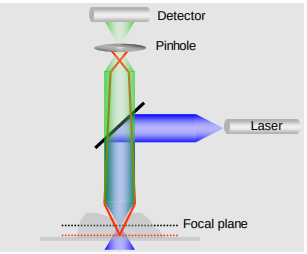
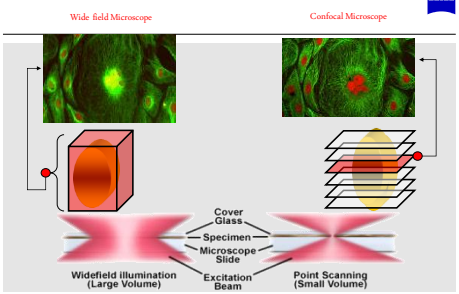


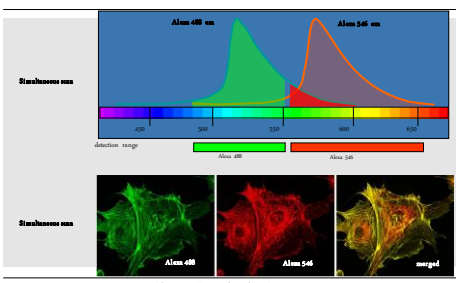
The Comparison Between the LSM and the Conventional Light Microscope


	Wide Field Microscope	Laser Scanning Microscope
Light Source	Mercury or Xenon Lamp	Laser
Illuminated Field	Wide Field	Spot
Image Acquisition	Parallel, Frame at Once	Sequential, Pixel wise
Signal Separation	Dichroic Beam Splitter, Emission Filter	Beam Splitter Cascade, Emission Filter
Detector	Eye or CCD Camera	Diffraction limited by pinhole → Photomultiplier (PMT)

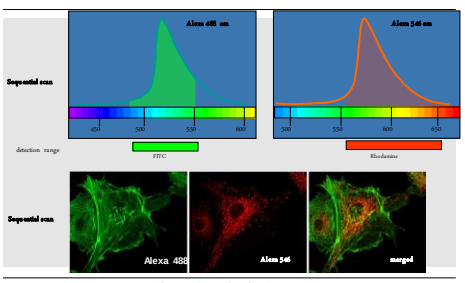
The advantages of confocal microscope - small excitation Volume



Multiple staining - the crosstalk problem



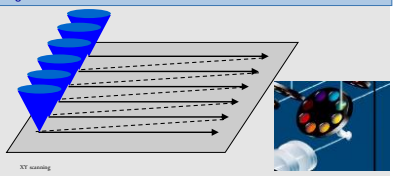
Emission Crosstalk - way around with Sequential image acquisition



Confocal: Point Scanning

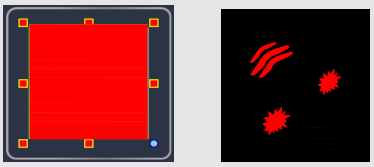
From Spot to Image

- To get a 2 dimensional image from the specimen, the excitation spot has to be moved over the specimen
- The scanning mirrors move the excitation beam in a line wise fashion



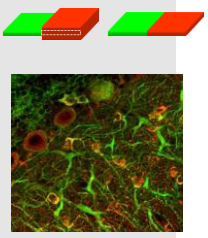
Point scanning confocal systems

Confocal: Point Scanning
Sequential image acquisition



Major tasks of a LSM
Colocalization in Confocal Microscopy

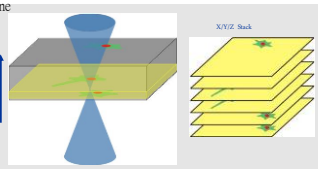
- Acquisition of Crosstalk free images required
- Occurrence of two fluorescent emission signals inside the same detection volume
- Identical size of detection volumes for different color channels required
- Intensities and position of the signals inside the detection volume may vary



Major tasks of a LSM
Optimal optical sectioning in thick tissue Z stack

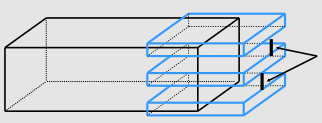
From Spot to Image Plane

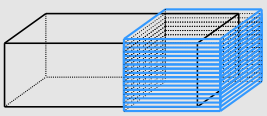
This plane represents an **optical section**



- 3 D information is acquired by moving the excitation focus not only in XY direction but also in Z direction
- The result is a **3 D data stack** consisting of number of XY images representing different optical sections from the specimen

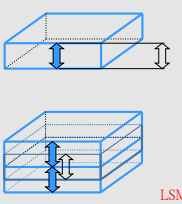
Major tasks of a LSM
Optimal optical sectioning in thick tissue Z stack
Number of sections





Major tasks of a LSM
Optimal optical sectioning in thick tissue Z stack

Optimal Number of sections : no missing information at minimal number of sections



Optical thickness depends on:

- wavelenght l
- objective lens, N.A.
- refractive index n
- pinhole diameter P

$d \sim P\ n\ l\ /\ (N.A)^2$

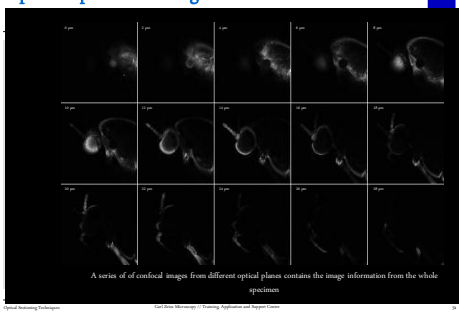
„Nyquist-" or Sampling- Theorem

slices overlap by the 50% of their thickness

LSM software: One click for best resolution

Major tasks of a LSM

Optimal optical sectioning in thick tissue Z stack



A series of of confocal images from different optical planes contains the image information from the whole specimen

Major tasks of a LSM

Optimal optical sectioning in thick tissue

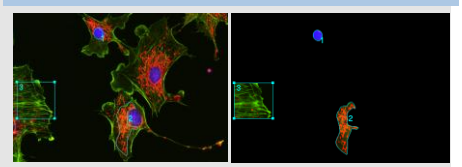
- An overlay (maximum projection) of these single images results in an image with an **enhanced depth of focus**
- This image contains all information from the specimen

Every detail is in focus !

Major tasks of a LSM

Laser and scanning mirror control

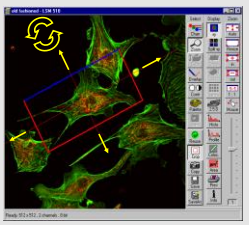
Real Regions of Interest (rROI)
Irregular shaped areas
Up to 99 areas simultaneously
Sample irradiation only during data Acquisition (beam blanking)
No photobleaching in surrounding areas



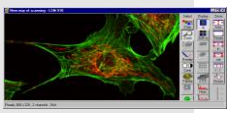
Major tasks of a LSM

Laser and scanning mirror control

Two independent scanning mirrors



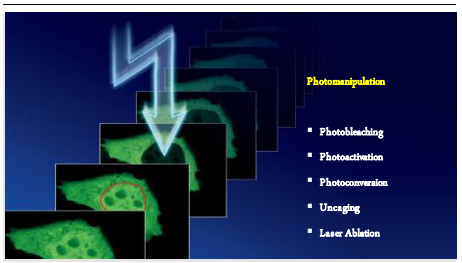
Free scan field rotation (0-360°)
Free online zooming (crop)
Any geometry: 1x4... 2048x2048
Faster rectangular acquisition (e.g. video rate)



Major tasks of a LSM

Laser and scanning mirror control

Photomanipulation for studying cellular dynamics



LSM for interaction & measurement –

FRAP: Fluorescence Recovery after Photobleaching

Sample

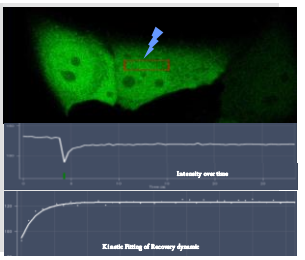
Cell culture stable expressing cytoplasmatic GFP

Imaging with 488 Laser (0.5%)

Bleaching with 488 Laser (100%)

Recovery at ½ time of 600ms

Imaging rate 400ms / frame

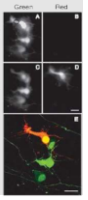


Photoconversion - Kaede

An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein

Ryoko Ando*†, Hiroshi Hama*, Miki Yamamoto-Hino*, Hideki Mizuno*, and Atsushi Miyawaki*†

PNAS | October 1, 2002 | vol. 99 | no. 20 | 12651-12656



Kaede = maple leaf (jap.)

New fluorescent protein from the stony coral Trachyphyllia geoffroy

Includes Tripeptide which acts as green chromophore that can be converted to red

Conversion highly sensitive to irradiation with UV light

Example: allows to delineate a single neuron in a dense culture

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Photoconversion - Kaede

Photoconversion from green to red

Sample

Cell culture stable expressing cytoplasmatic KAEDE

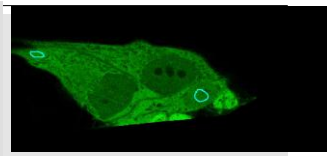
Convertible Fluorescent Protein (from green to red)

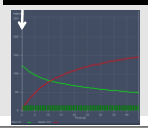
Recorded time: 45 sec

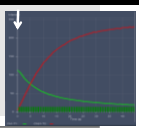
Conversion: 405 Laser

Left region: 50%

Right region: 100%







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Thank you for your attention!!