Travel to New Dimensions- LSM 700

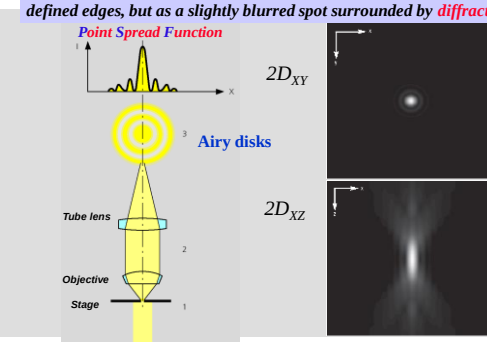
Sensitivity,
Flexibility and
Ease of Use

Innovative High-End
Laser Scanning Microscopes
from Carl Zeiss

余雅倩
台灣儀器行

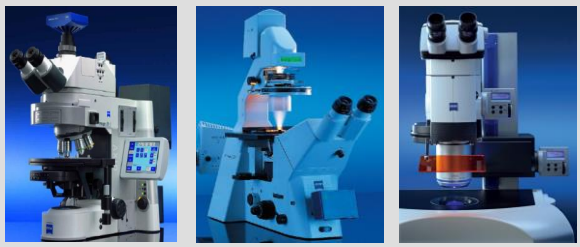
Spatial Resolution of a Light Microscope

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



Basic principle of light microscope
Different types of light microscopes

Upright Microscope | **Inverted Microscope** | **Stereo Microscope**

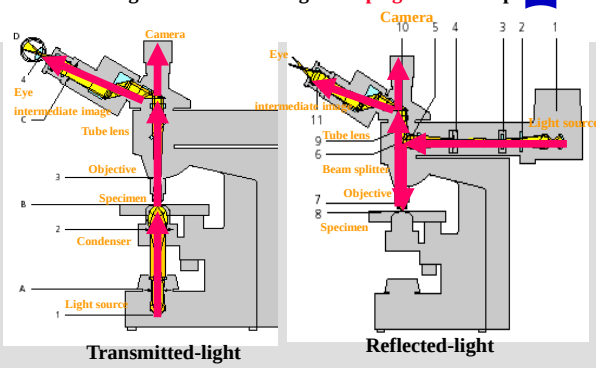


Basic principle of light microscope

Upright Microscope

- Short working distance
- Higher magnification
- Higher resolution
- Suitable for slide

Different Beam Path of Image Formation
Transmitted-light and Reflected-light in upright microscope



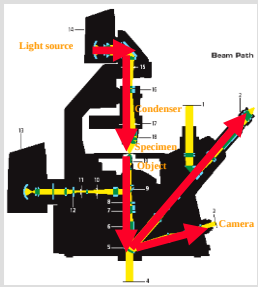
Basic principle of light microscope

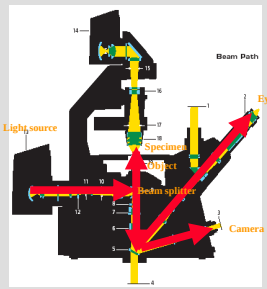
Inverted Microscope

- 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



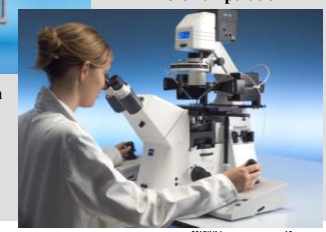


Transmitted-light **Reflected-light**

Basic principle of light microscope

Inverted Microscope

Micromanipulation



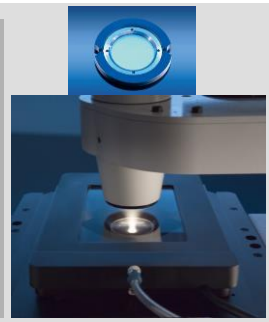
37℃, 5% CO_2 living cell incubation

The Cell Observer – New Incubation Concept

Incubation Devices

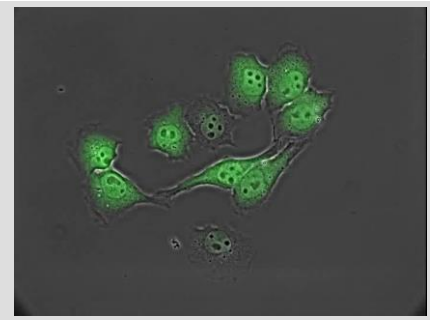
Incubator PM S1

- Compatible with petri dishes & multiwell plates
- Heating inserts: Petri dishes, 6/96 well plates
- Heatable glass surface
- Improved gas mixture delivery
- Fast changes of incubation conditions (in dynamic experiments)
- Compatible with cooling experiments
- Space-saving solution



Major tasks of a LSM

Time lapse image



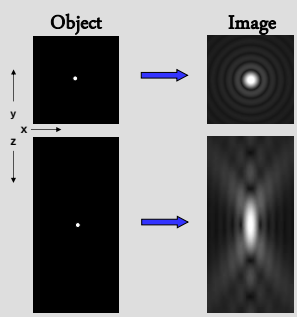
The Point-Spread-Function is a 3-dimensional function

.

The axial shape of the PSF is completely different from the lateral one.

The axial extension is larger than the lateral.

→A microscope has a lateral and an axial resolution.



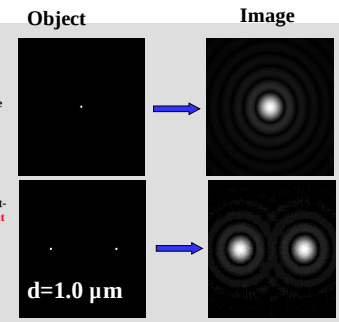
The Resolution of a Microscope is limited

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).



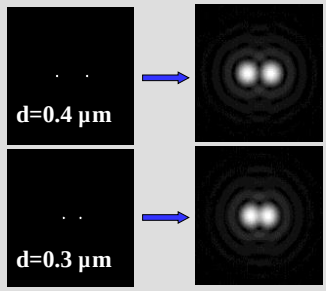
The Resolution of a Microscope is limited

Object Image

Definition

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

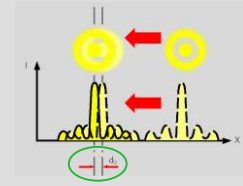
The distance between the objects is called the resolution limit.



The Resolution of a Microscope is limited

Prof. Ernst Abbe
(1840 - 1905)

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



$d_0 = \frac{1.22\lambda}{N.A._{obj} + N.A._{Cond}}$ or 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

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

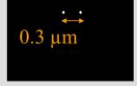
$NA = n \cdot \sin\alpha$

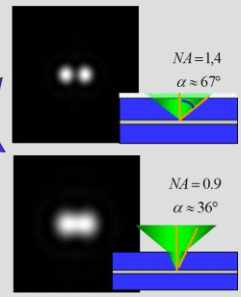
Image

Principle effect

High numerical aperture objectives have a large opening angle.

Object





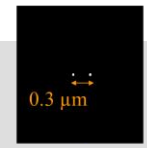
The higher the NA, the better the resolution of the microscope.

The Resolution of a Microscope depends on Numerical Aperture and Wavelength

Priciple effect

Shorter wavelengths generate smaller Point Spread Functions.

Object



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

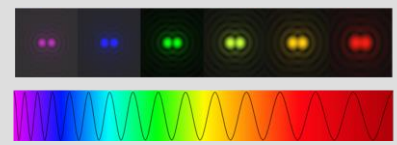
$NA = n \cdot \sin\alpha$

$NA = 1{,}4$

$\alpha \approx 67°$

Image

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

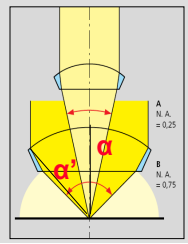


The shorter the wavelength, the better the resolution.

Basic principle of light microscope
The Numerical Aperture of Objective

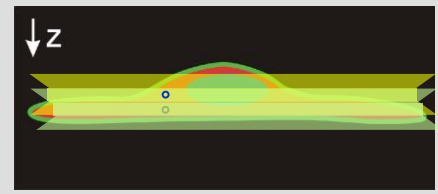
The **numerical aperture** of a microscope objective is a measure of its **ability to gather light** and **resolve fine specimen detail** at a fixed object distance.

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



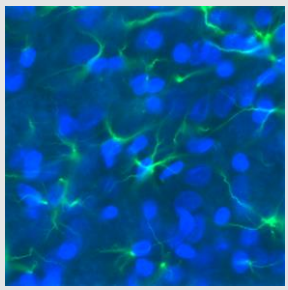
Conventional/Widefield Fluorescence

Background emission from deeper image planes

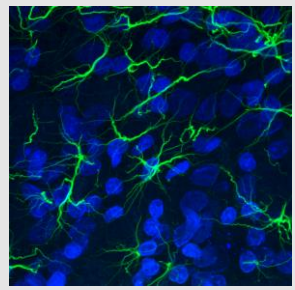


Structures which are „out-of-focus" become visible in conventional widefield-fluorescence. Because of the focal depth inherent in all objectives, they are visible as an image blur (haze, image fog).

Confocal Principle

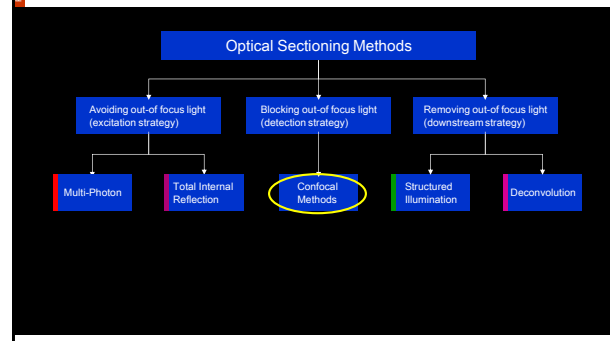


Widefield



Confocal

General Optical Sectioning Methods
General Overview

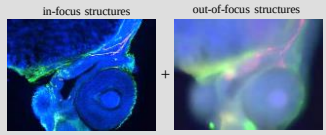


Imaging of 3-dimensional objects
The fundamental problem

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.

in-focus structures + out-of-focus structures



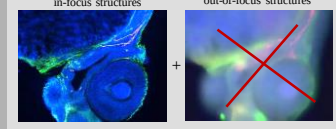
Imaging of 3-dimensional objects
The fundamental problem

Conventional Images

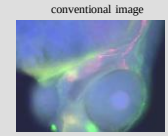
Conventional images of 3-dimensional objects always contain light from structures, which are in focus and light from structures which are not in focus.

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

in-focus structures + out-of-focus structures

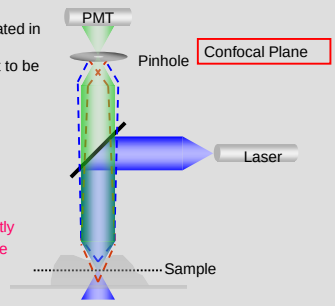


conventional image



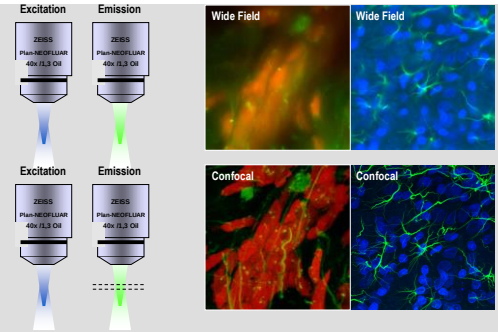
The confocal principle

A minute diaphragm, situated in a conjugated focal plane, prevents out of focus light to be detected.



The pinhole diameter directly controls the thickness of the **optical section .**

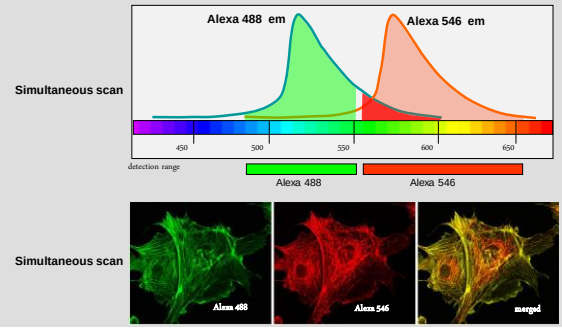
Confocal Laser Scanning Microscopy
Optical sectioning: elimination of out-of-focus light



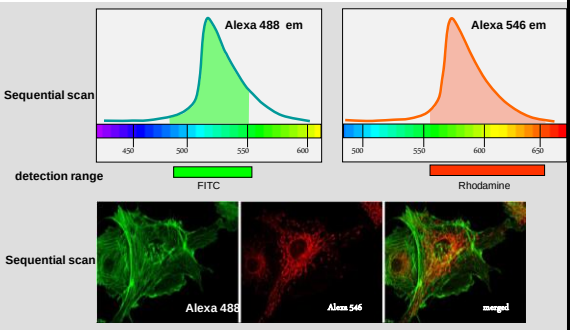
The Comparison Between the LSM and the Conventional Light Microscope


	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)

Multiple staining - the crosstalk problem



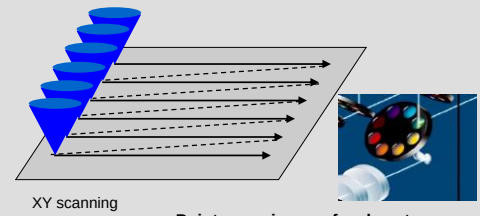
Emission Crosstalk - way around with Sequential image acquisition



Confocal: Point Scanning

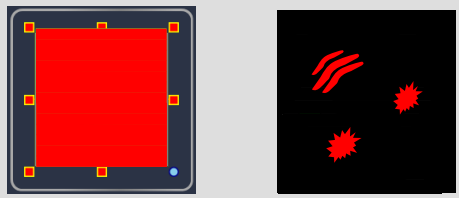
From Spot to Image

- 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

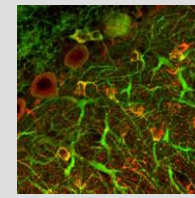


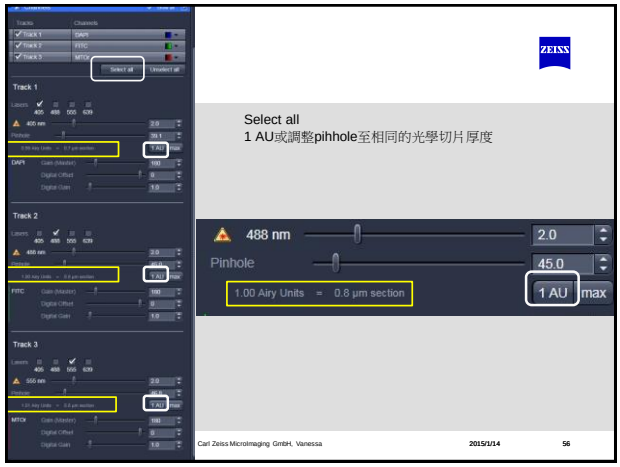
Major tasks of a LSM

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



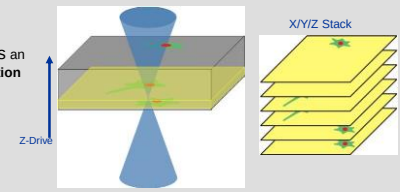




Select all
1 AU或調整pihhole至相同的光學切片厚度

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

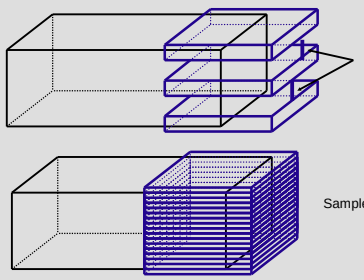
This plane represents an **optical section**



- 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

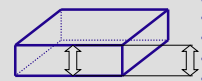


Missing Information

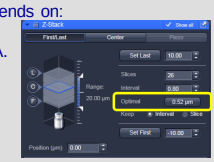
Sample bleached and much data,

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

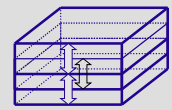
Optimal Number of sections : no missing information at minimal number of sections



Optical thickness depends on:
- wavelenght l
- objective lens, N.A.
- refractive index n
- pinhole diameter P



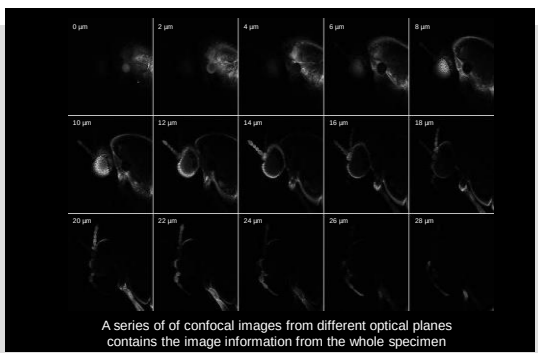
$d \sim P\ n\ l\ /\ (N.A)^2$



„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



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

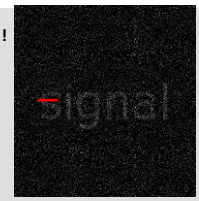
Every detail is in focus !

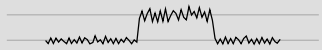
The Power of Sensitivity

Brighter Images ≠ Increased Sensitivity !

Signal to noise ratio is critical !

$$SNR = \frac{S}{N}$$



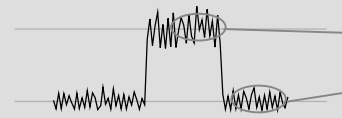


The Power of Sensitivity

Brighter Images ≠ Increased Sensitivity !

Signal to noise ratio is critical !

An increase in brightness of the image does not provide better information



Sources of noise:
- Shot noise

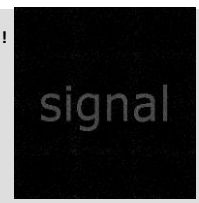
Sources of noise:
- Dark noise
- Electronic noise
- Laser reflection

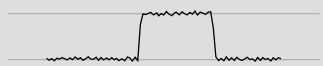
The Power of Sensitivity

Brighter Images ≠ Increased Sensitivity !

Signal to noise ratio is critical !

A decrease in noise gives cleaner images



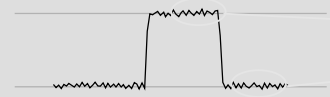


The Power of Sensitivity

Brighter Images ≠ Increased Sensitivity !

Signal to noise ratio is critical !

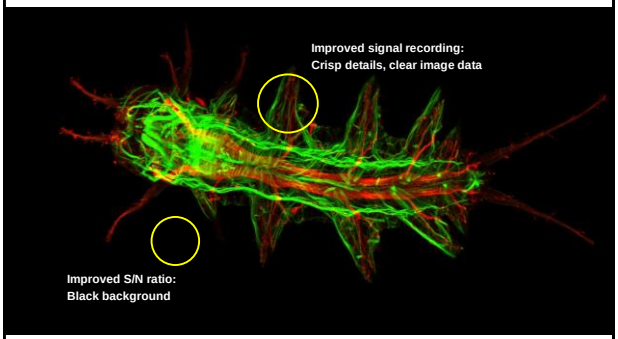
Low-noise images can be displayed with optimal brightness



Noise reduction in LSM 700:
- Reduced dark noise
- Reduced electronic noise
- Reduced laser reflection

LSM 700 in Standard Applications

- Uncompromized Image quality



LSM 700
Laser line

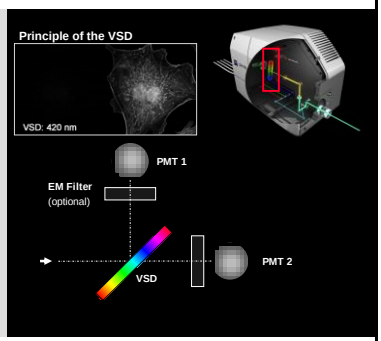
Laser line	Fluorochrome
405 nm	DAPI, Hoechst, Alexa 405, BFP,
488nm	Alexa 488, Fluo-4, FITC, eGFP
555 nm	Rhodamine, Alexa 546, 555, 568, Cy3, TRITC, DsRed, Texas Red, MitoTracker Red
639 nm	Alexa 633, Cy5..

Detectors:
2 reflection PMT detectors for fluorescence images

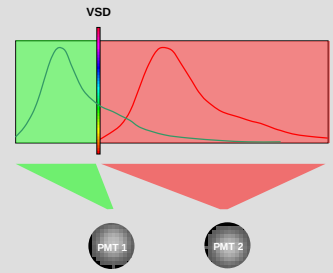
1 transmitted PMT detector for Bright Field(PH/DIC) images

LSM 700
VSD – Variable Secondary Dichroic

- VSD is a variable short pass beam splitter for splitting signals between detectors
- Positioning of VSD allows precise tuning of wavelenth at which signals are split (splitting possible between 420 and 630 nm, min. step: 1 nm)
- Enables highly light-efficient detection strategies and spectral imaging (lambda stack acquisition)
- Patented Zeiss innovation



LSM 700
VSD – the new flexible way



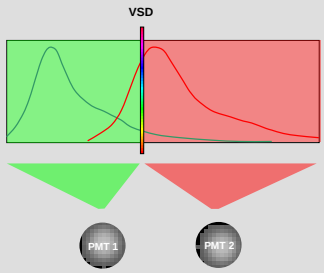
Example: Dual-color Detection of GFP and MitoTracker Orange

Approach: "New Flexible Way"

Flexible dual-color detection enabled by the new variable secondary dichroic (VSD) of the LSM 700.

Improvement: Enhanced light efficiency because no portion of the signal is excluded from the detection process.

LSM 700
VSD – the new flexible way



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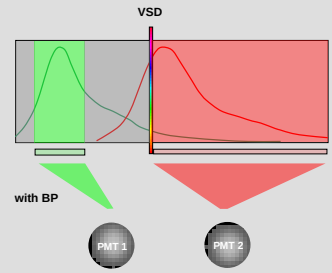
VSD – the new flexible way



Example: Dual-color Detection of GFP and MitoTracker Orange

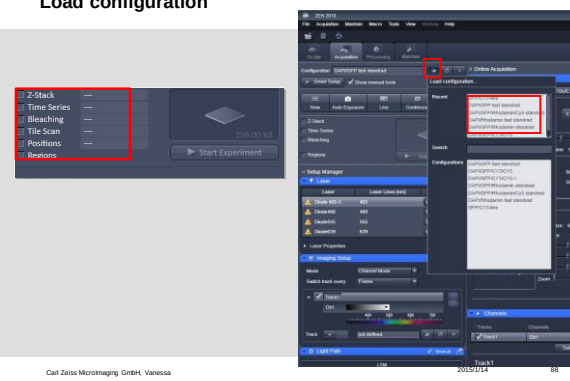
Approach: "New Flexible Way"

Flexible dual-color detection enabled by the new variable secondary dichroic (VSD) of the LSM 700.

Also possible: use of emission filters (optional) for additional specificity.

ZEN 2011 - Efficient Navigation
Powerful software for powerful LSM systems

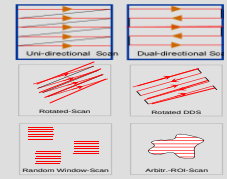
ZEN 2011
Load configuration



ZEN 2011
Load configuration

Major tasks of a LSM
Laser and scanning mirror control

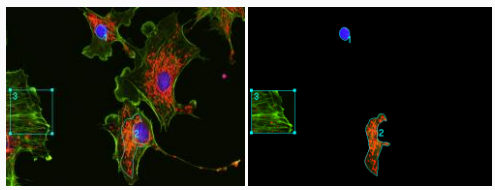
- Easy sample manipulation
- Flexible scanning strategies (1D to multiD)
  Scan Mode --- 1D, 2D, and free 2D Image



Absolut linear scanner movement:
The same dwell time for every pixel in the images
(essential for any quantitative measurements)

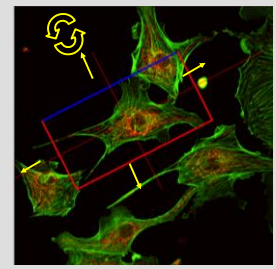
Major tasks of a LSM
Laser and scanning mirror control

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

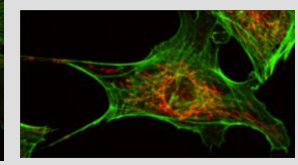


Major tasks of a LSM
Laser and scanning mirror control

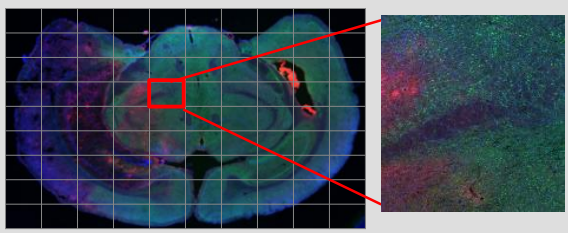
Two independent scanning mirrors



Free scan field rotation (0-360º)
Free online zooming (0.6~40x (zoom=66.7x)
Any geometry: 1x4... 6144*6144
Faster rectangular acquisition (e.g. video rate)

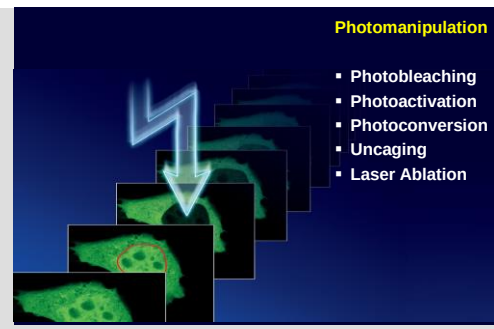


Tile scanning with motorized scanning stage
大面積高倍數掃描



40X objective, 10X9

Major tasks of a LSM
Laser and scanning mirror control
Photomanipulation for studying cellular dynamics

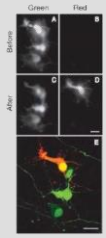


Photoconversion - Kaede

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

Ryoko Ando*†, Hiroshi Hama*, Miki Yamamoto-Hino*, Hideaki 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 Trachyphylla geoffrey

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

Photoconversion - Kaede

Photoconversion from green to red

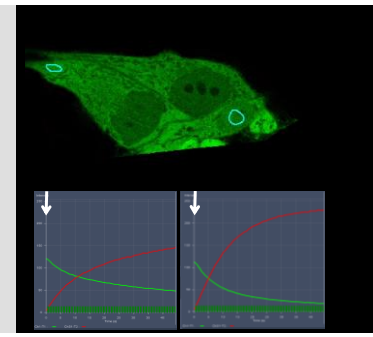
Sample

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%



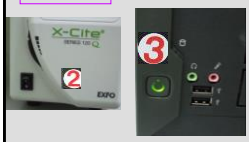
1. 開關機步驟

1.
總電源(延長線上)

開機1→4順序開啟
關機4→1順序關閉

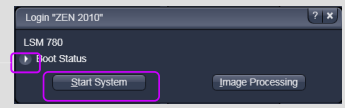
2. 螢光燈源

3.電腦電源



1.進入軟體ZEN

2.開啟軟體與硬體連結



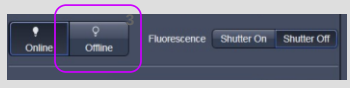
進階模式

啟動機器掃圖

*啟動軟體全功能，但不與硬體做連結，**單純分析資料、沒有要操作機器請選此項。**

3. 控制顯微鏡找到樣品焦距

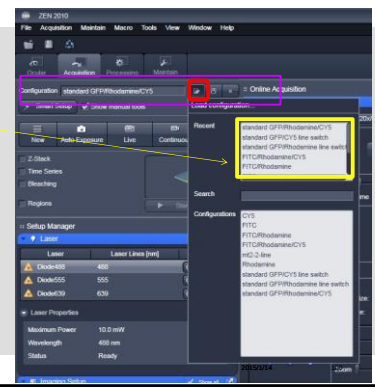
1. Online ，自目鏡觀察，找到樣品焦距。
2. 利用configuration切換各贏光濾片組與穿透光設定。
3. 確認樣品位置及焦距後切至Offline ，即可以進入LSM 影像擷取模式。



Online: 分光至目鏡
Offline: 分光至LSM ，此時目鏡無法做觀測

4. Apply Configuration Setting

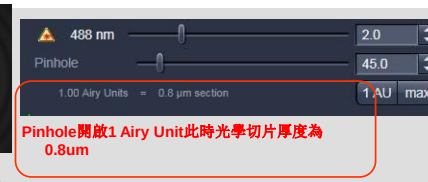
套用適合的Configuration
(選擇染劑名字)



5. Acquisition setting

Select all
1 AU或調整pihhole至相同的光學切片厚度

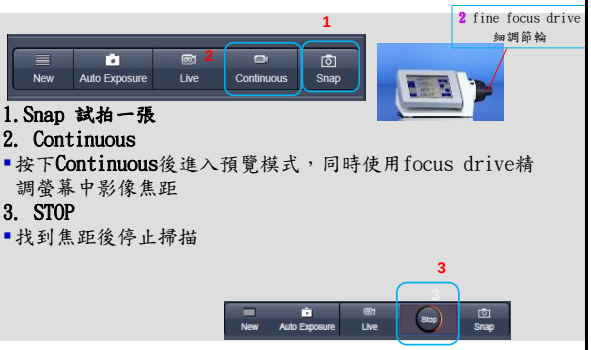
5.1 :設定適當的Pinhole大小



Pinhole開啟1 Airy Unit此時光學切片厚度為0.8um

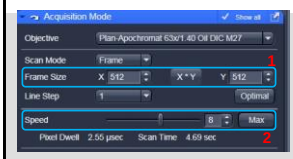
Pinhole設定:
一般選擇1AU
亦可以設定成所有channel 為相同的um厚度，例如在高倍物鏡下可以統一選擇1um 。

6. 預覽掃圖



1. Snap 試拍一張
2. Continuous
   - 按下Continuous後進入預覽模式，同時使用focus drive精調螢幕中影像焦距
3. STOP
   - 找到焦距後停止掃描

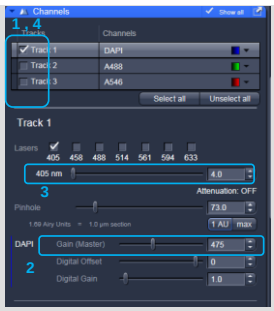
6.1 掃描參數值設定



1&2. 於frame size 512*512畫素、speed 8下進行影像快速掃描，以方便即時預覽更改參數後的結果

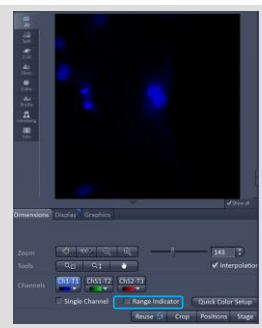
- 3. Continuous: 持續掃描，要按停止才會停止掃描
- 4. Snap:拍一張影像

6.2 掃描參數值設定



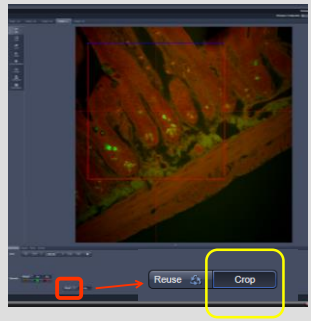
1. 依序設定每個track的掃圖參數
2. 調整PMT gain值，數值越大訊號被放大得越多，影像越亮，可使用滑鼠中鍵滾輪滑動調整。
   (建議數值600-750)
3. 調整 laser強度，數值越大，影像越亮。
4. 調整好後進行下一個track的設定，重複1~3步驟直到每個track都設定完畢。

6.3 掃描參數值設定



拍圖調整掃描條件時建議選取range indicator套色方式表現色彩，將有助於將顏色之intensity調到最佳分布。紅色表示飽和(調整detector gain和laser量)， background 深藍表示全黑(調整digital offset)
建議調整到全畫面當中訊號少部分飽和，background 部分為藍色。

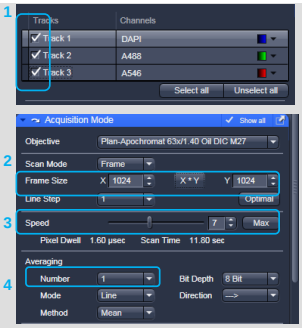
6.4 掃描參數值設定 zoom、掃描區域選擇



•Corp功能包含zoom in/ zoom out

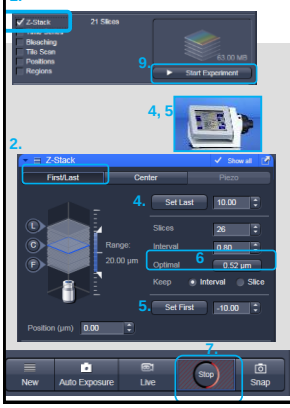
•Zoom 勿過度使用,一般不會超過3否則將造成bleach樣品的效果

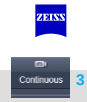
7. 正式掃圖:獲得高解析度影像



1. 將所有已經調整好參數的track都打勾
2. 選擇需要的畫素,一般需要發表須要1024*1024.
3. 調整掃圖至慢速度,高品質影像建議scan speed為5~7
4. 一般均設定1，若影像品質不佳可採用平均數次可以使影像品質提升,降低雜訊
5. SNAP拍一張,獲得漂亮的data!!

8. Z stack

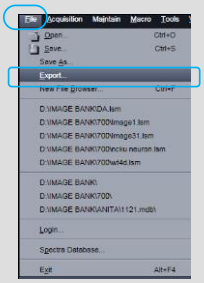


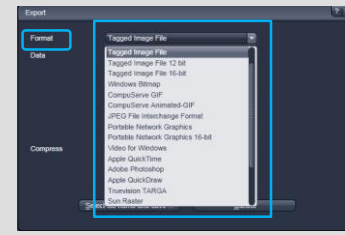


1. 進入Z stack
2. 進入 Mark First/Last
3. Continuous scanning
4. 用Focus drive找到觀察樣品厚度之最高/低點→mark first
5. 反方向轉動粗細調節輪找到欲觀察範圍的最高/低點→mark last
6. 滑鼠按下 Optimal interval建議值
7. 設定完畢後stop, 避免樣品被bleach
8. 回到掃圖設定成1024*1024，speed 7~5
9. Start experiment 開始執行Z section拍照

9.存檔—*.lsm完整檔案, 可以reuse!

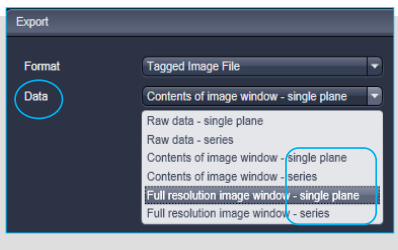
File→ Export





Format：下拉選擇欲存檔之類型（tif、jpg…），建議以tif檔儲存,減少壓縮損失，亦可儲存serial section 成動畫影片檔(.avi)

10. 輸出成圖片檔或者影片檔



•Raw data ：不含尺規,選擇要存的顏色，是否為灰階等等。

•Content of image window ：存下室窗內的影像畫面，包含尺規。

•Full resolution ：包含尺規依照拍照時的畫素存檔（建議使用!）

•建議使用Full resolution 或者Contents of image window

•single plane：單張，目前所顯示的單層/單張影像。

•series ：一系列圖，適用於Z stack ， time series和movie檔。

11.把多張Z section疊成一張

製造全景深影像: maximum intensity projection