2024 光電聚落教育訓練課程

雷射掃描共軛焦
光譜顯微鏡原理介紹

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2024/4/19 (星期五)

台灣大學 凝態中心 奈工 電坊

「雷射掃描共軛焦光譜顯微鏡」之原理介紹

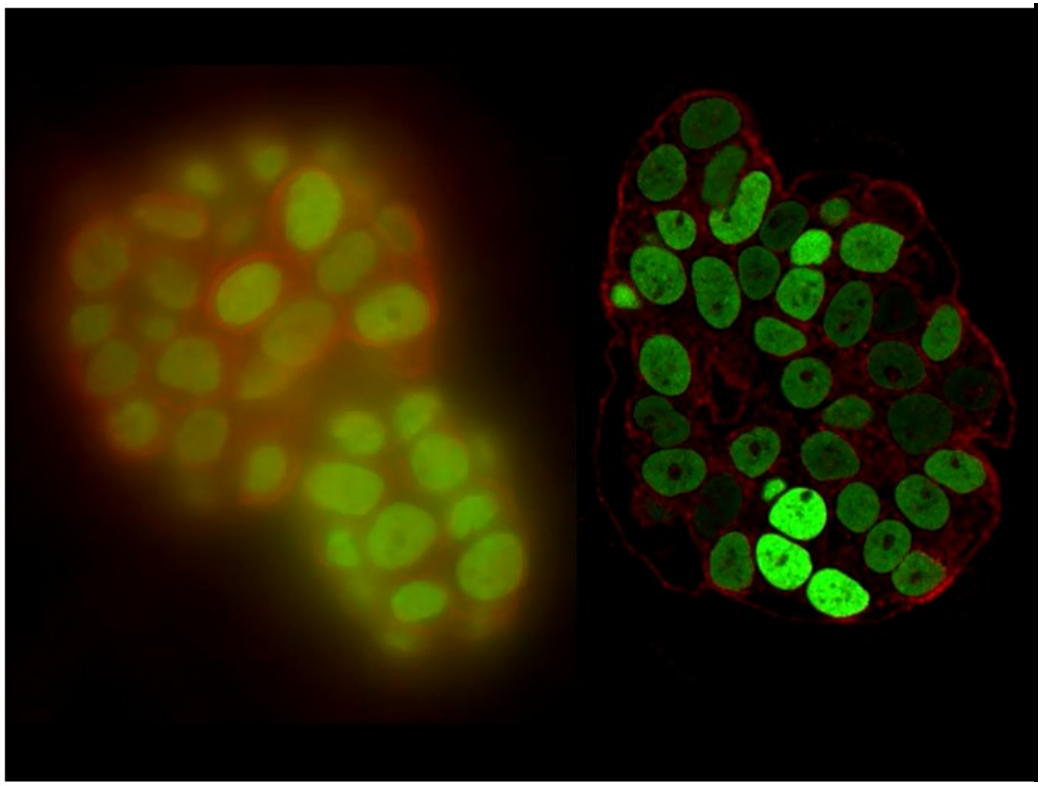
主講人：陳維良

講義內容：

1. What is a confocal microscope? How does it differ from a typical widefield optical microscope?
2. What are the main components of a typical laser scanning confocal spectromicroscope (LSCSM)?
3. How are images acquired by LSCM?
4. What are the key performance specifications of a LSCM?
5. What components determine the resolution of a LSCM?
6. How is the optimal pinhole size determined?
7. How can we measure the resolution of a LSCM?
8. Other factor affecting the performance of LSCM

WILD FIELD VS. CONFOCAL IMAGING

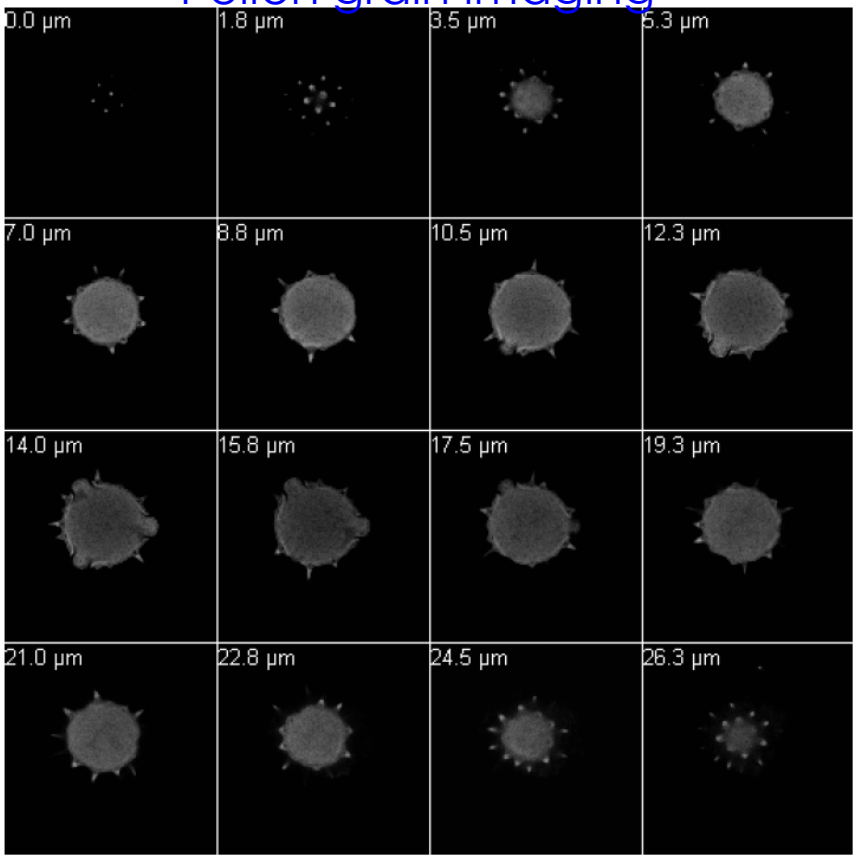
Widefield (left) vs Laser-scanning confocal (right)



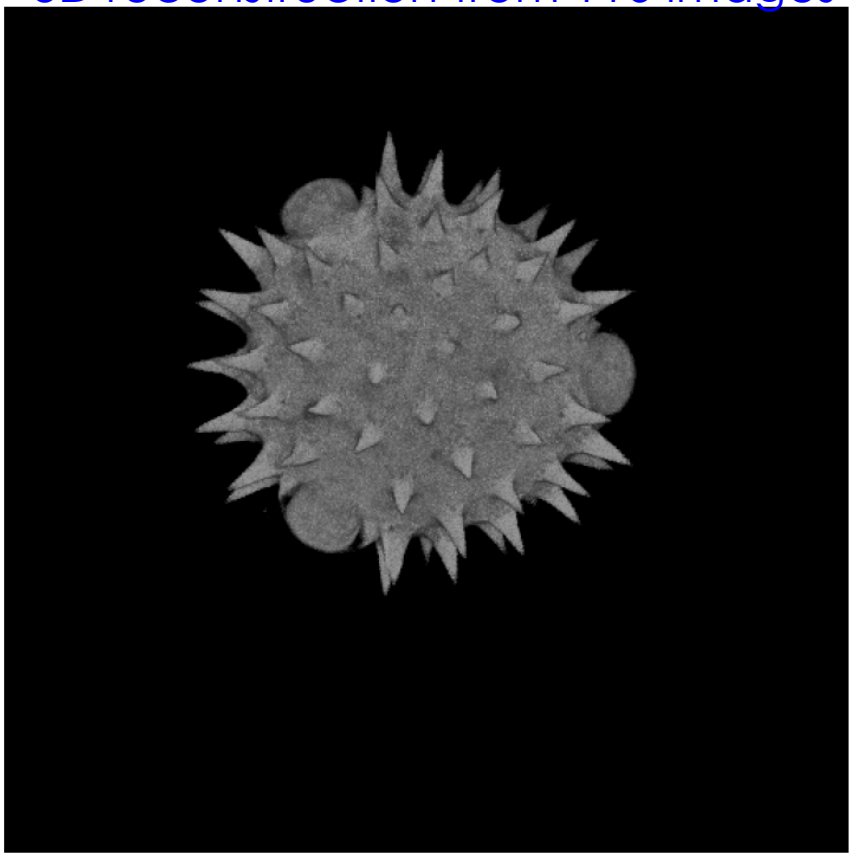
3D Culture of mammary epithelial cells (乳腺上皮細胞) (35 – 50 μm thick)
Reference: microscopysolutions.ca

CONFOCAL IMAGING AND 3D RECONSTRUCTION

Pollen grain imaging

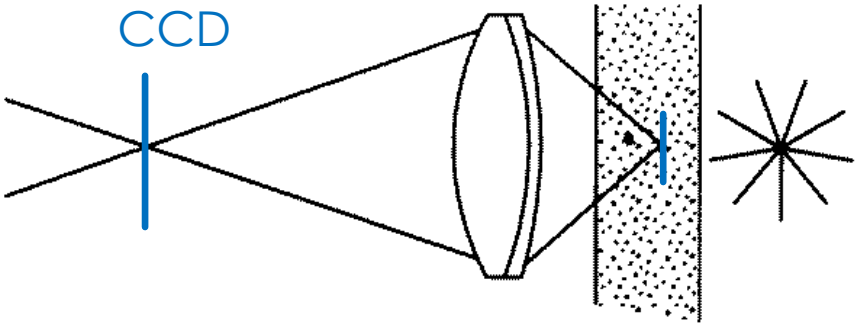


3D reconstruction from 116 images



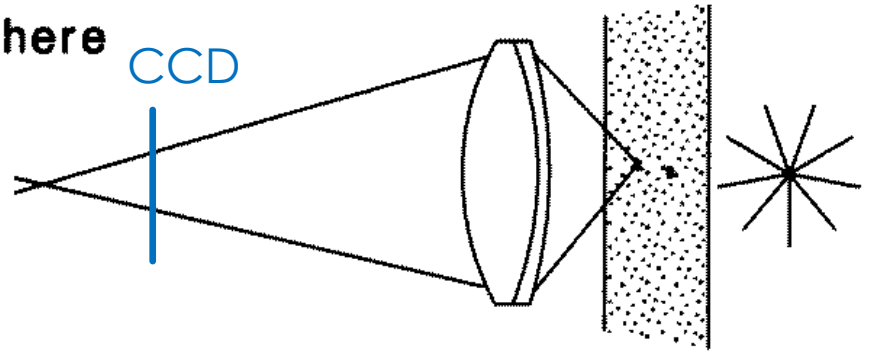
FROM WIDEFIELD TO CONFOCAL MICROSCOPE

A cell (say), in a thick sample, is imaged by a lens.



In focus

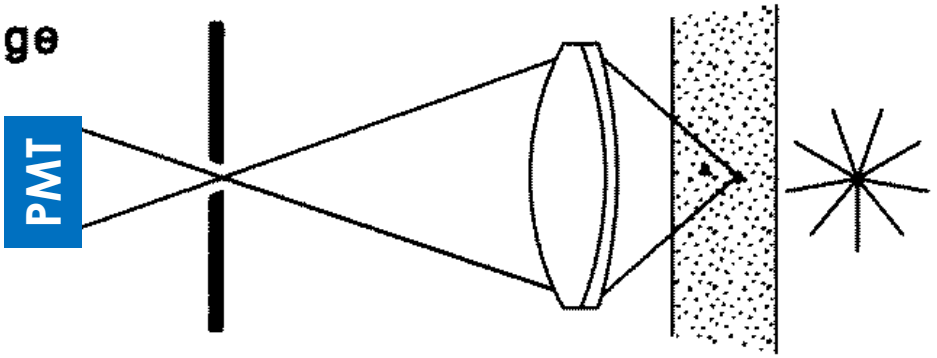
Another cell, elsewhere in the sample, is imaged at a different point.



Out of focus

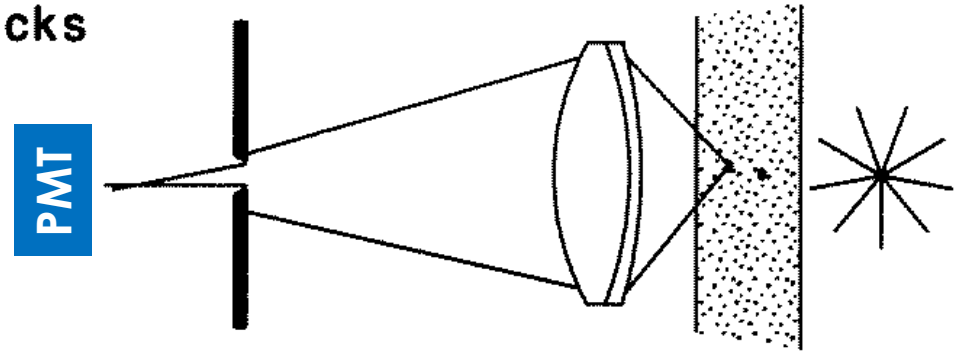
FROM WIDEFIELD TO CONFOCAL MICROSCOPE

A pinhole in image space passes all the light from cell 1.



In focus
Signal passes pinhole

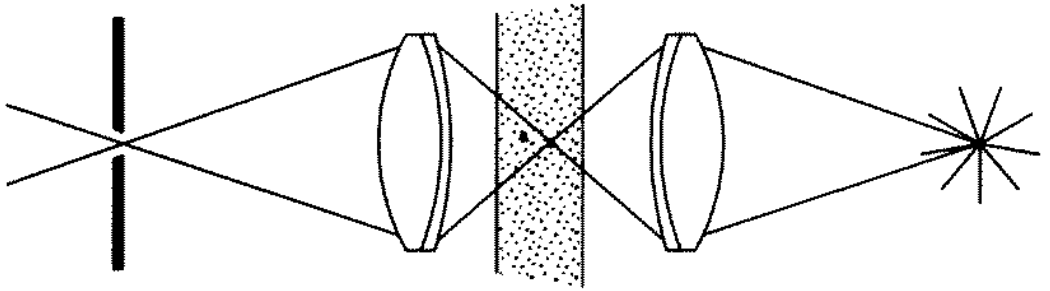
The pinhole blocks most of the light from cell 2.



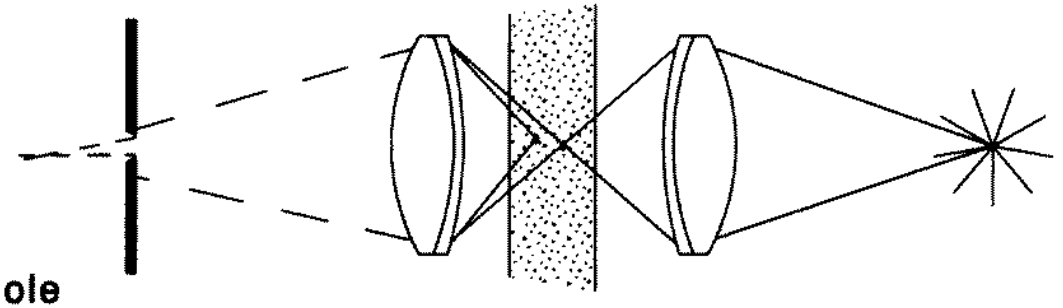
Out of focus
Blacked by pinhole

FROM WIDEFIELD TO CONFOCAL MICROSCOPE

A point source of light, CONFOCAL with cell 1 and the pinhole, selectively illuminates cell 1.



The confocal light source gives even less light to cell 2, and most is blocked by the pinhole



FROM WIDEFIELD TO CONFOCAL MICROSCOPE

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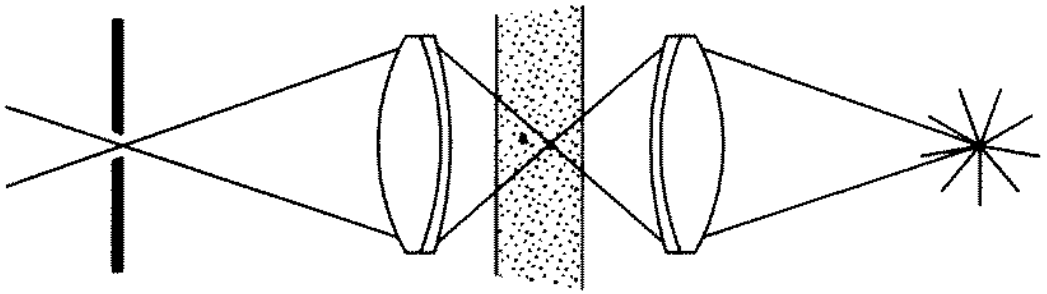
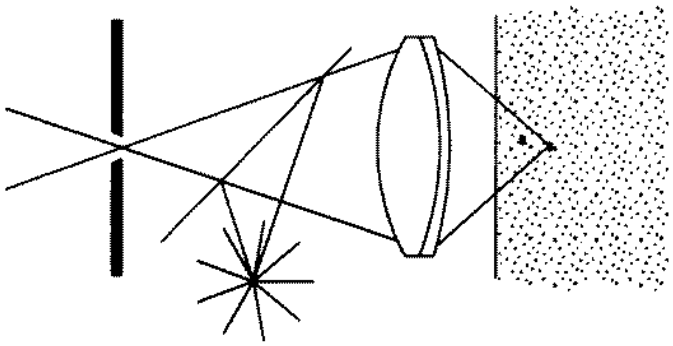
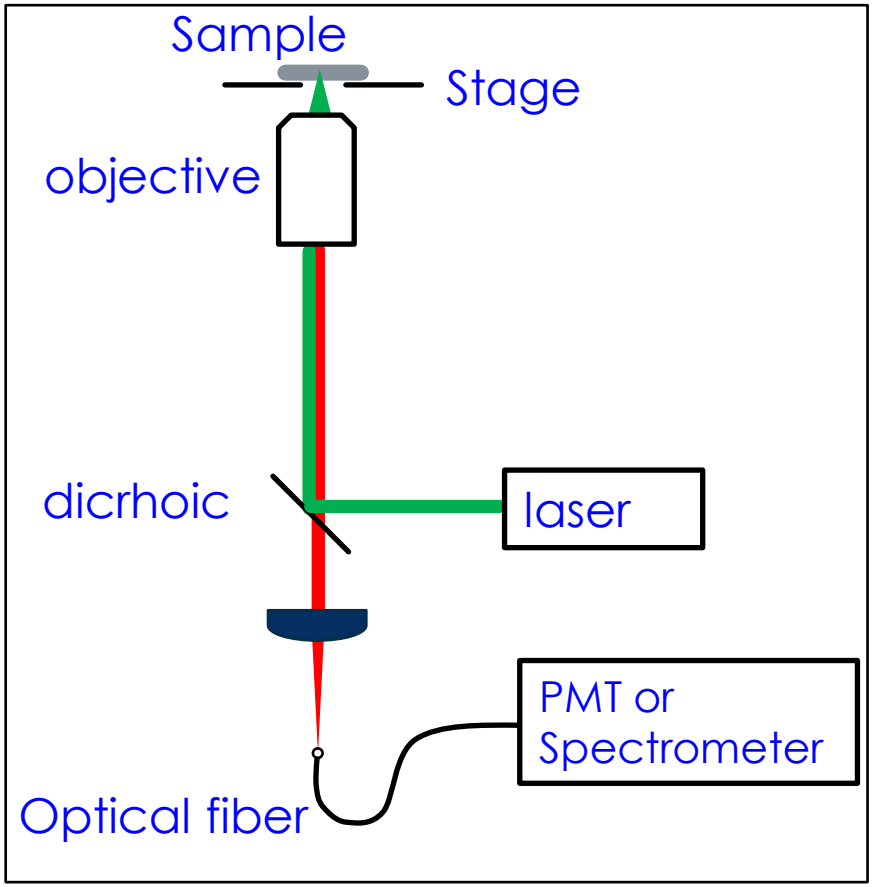


A beam splitter allows the confocal microscope to be epitaxial.



CONFOCAL ILLUMINATION AND DETECTION

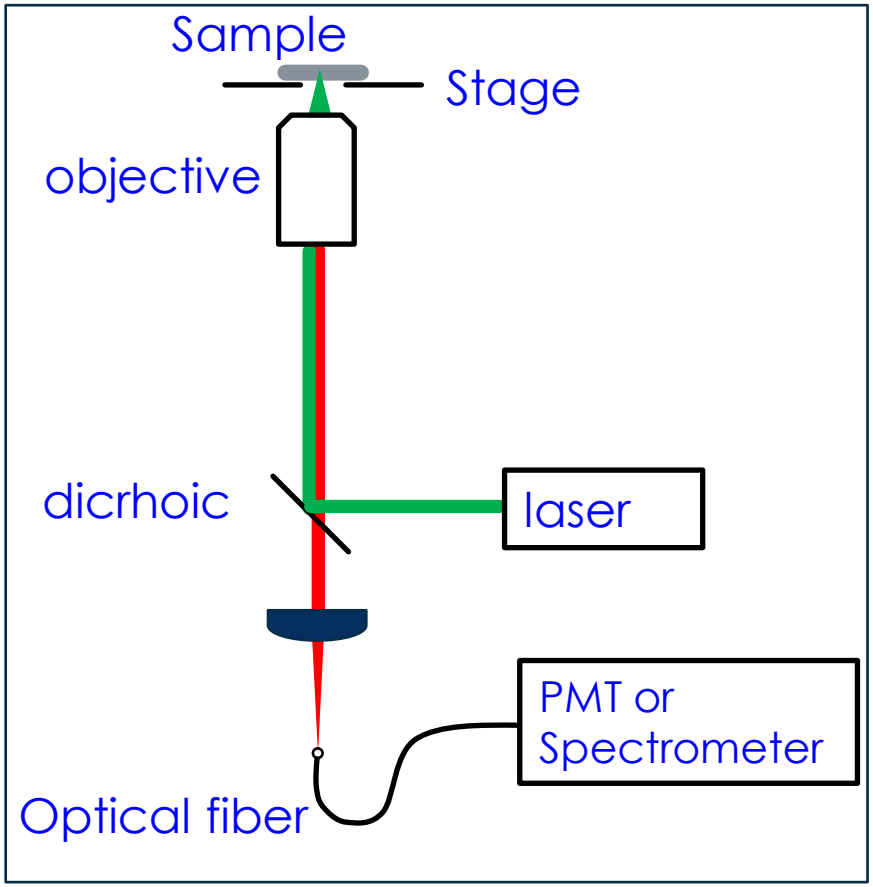
Single point confocal detection



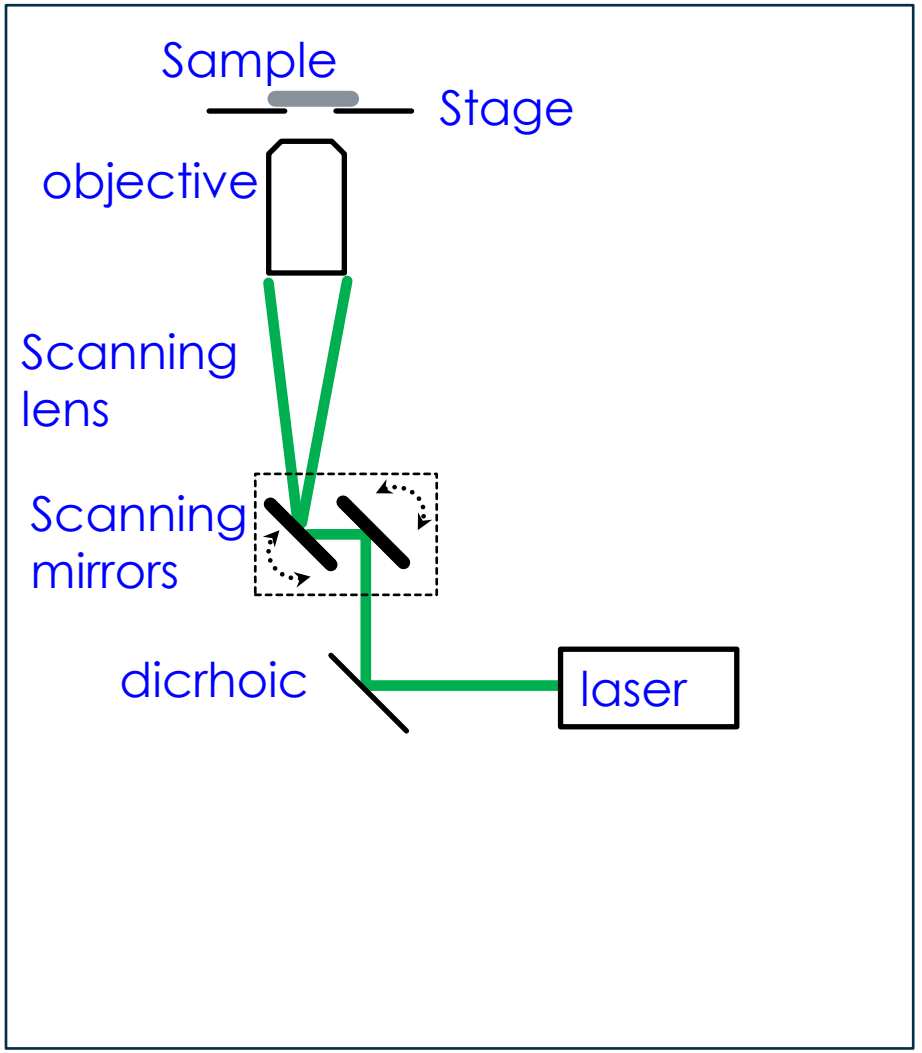
- Same objective used for focusing excitation and collecting emission
- Optical fiber diameter determines the pinhole size
- Image can be formed by moving the sample stage scanning the laser excitation
- **How do we scan the laser beam instead?**

COMPONENTS OF LSCM

Single point confocal detection



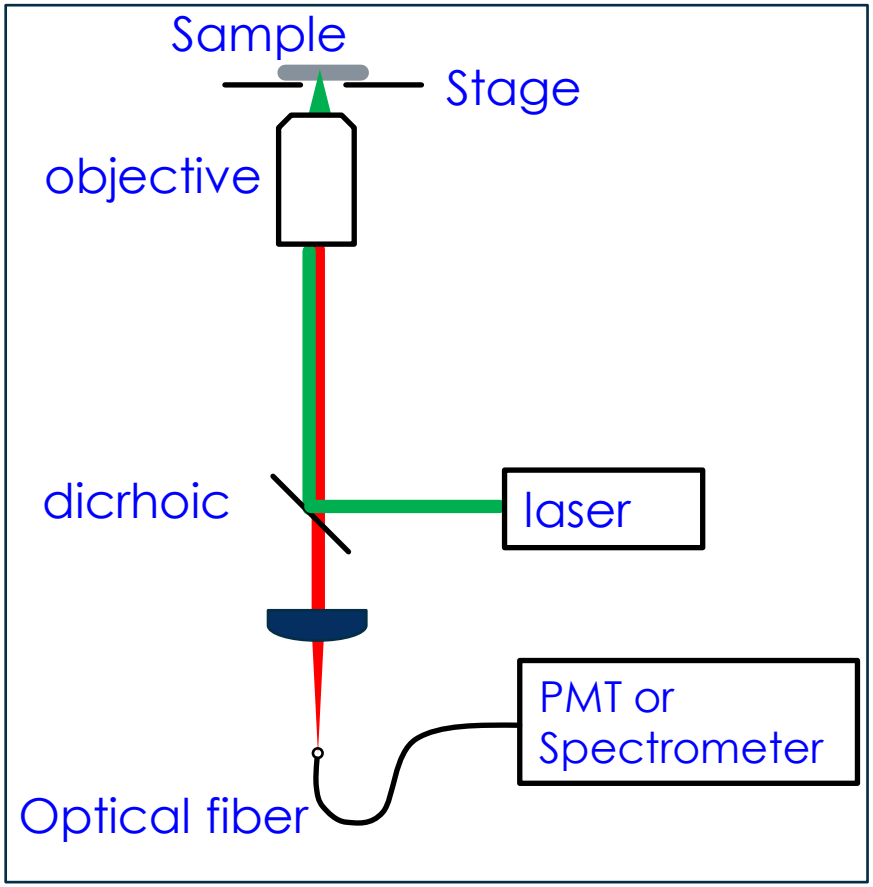
Laser scannnig confocal microscope (LSCM)



- Add scanning mirrors to scan laser beam
- **But how to ensure laser still hits the back focal plane of the objective?**

COMPONENTS OF LSCM

Single point confocal detection



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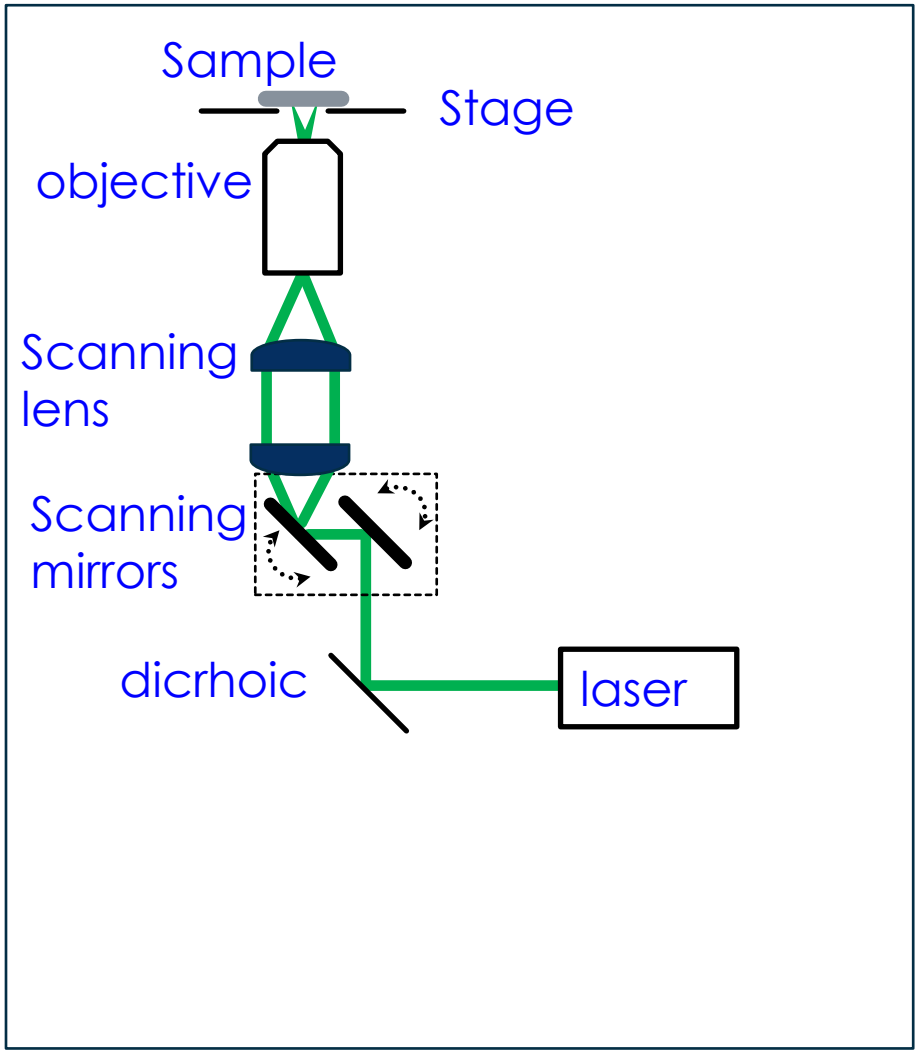
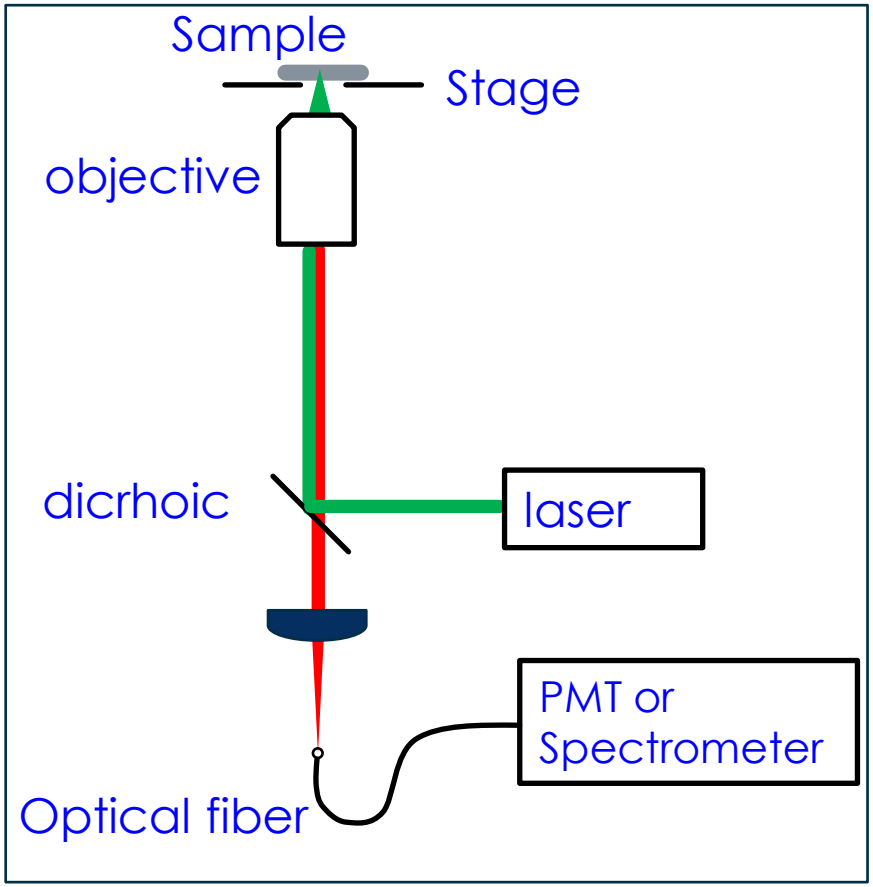


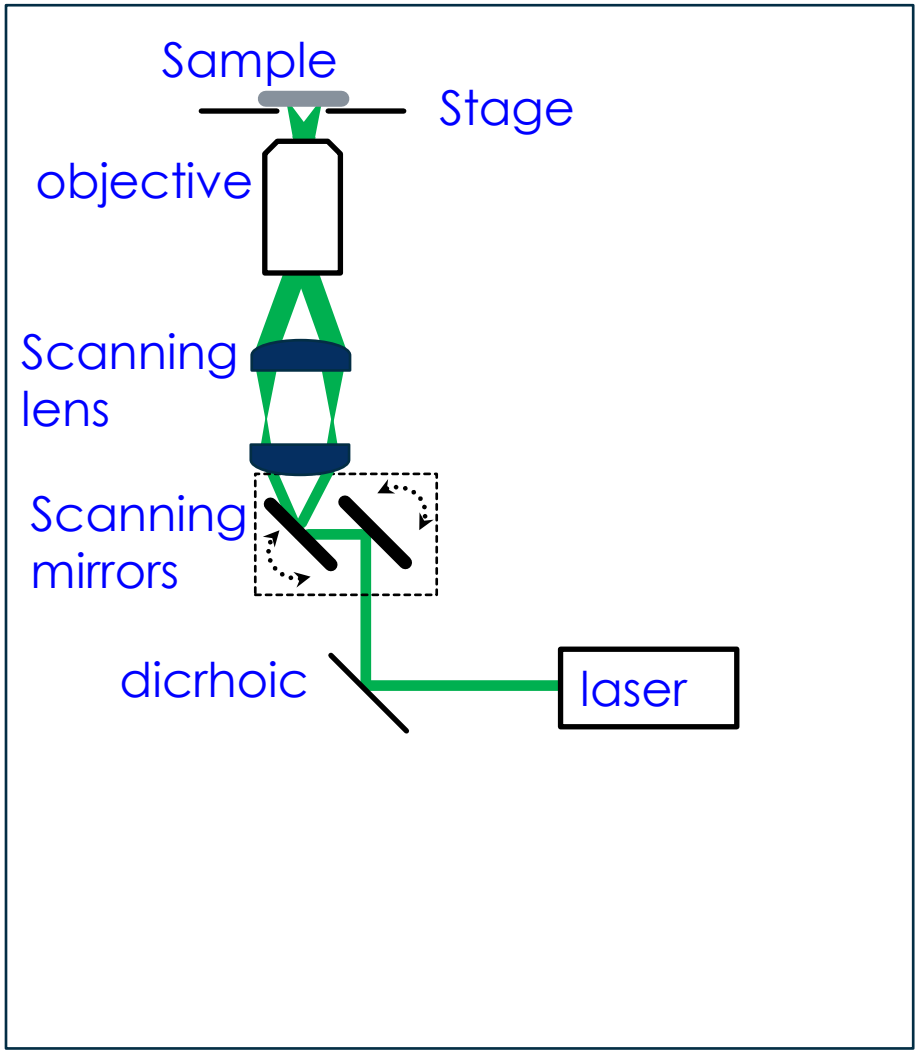
- Scanning lens ensure scan beam hit the same spot on the back focal plane of objective

COMPONENTS OF LSCM

Single point confocal detection



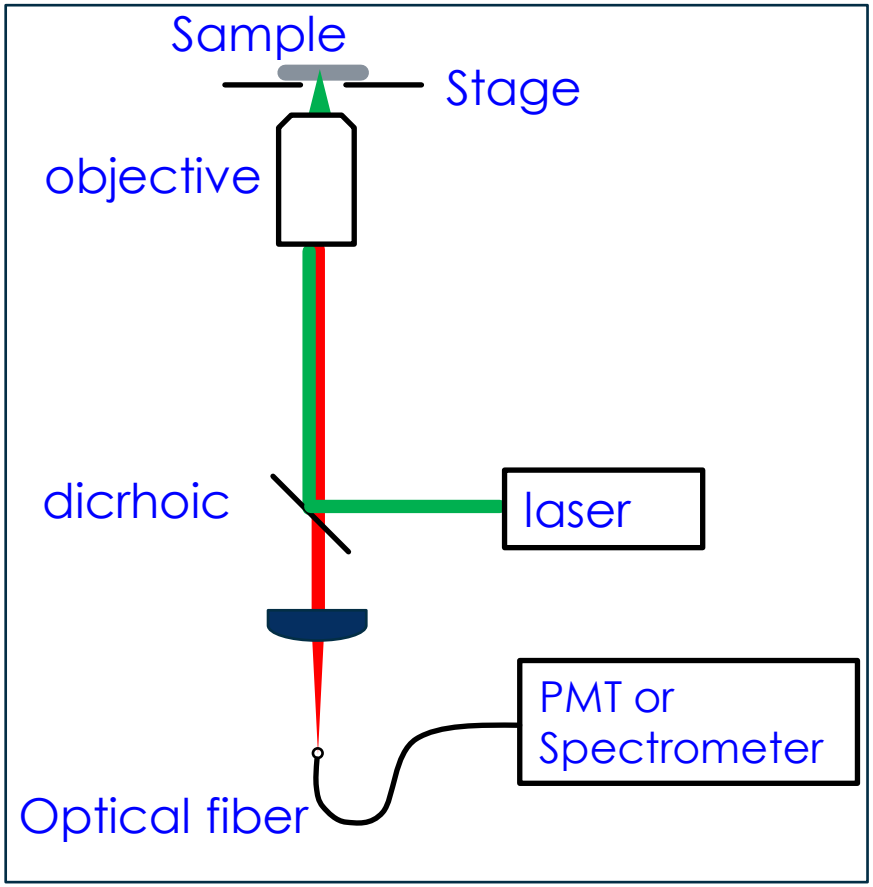
Laser scannnig confocal microscope (LSCM)



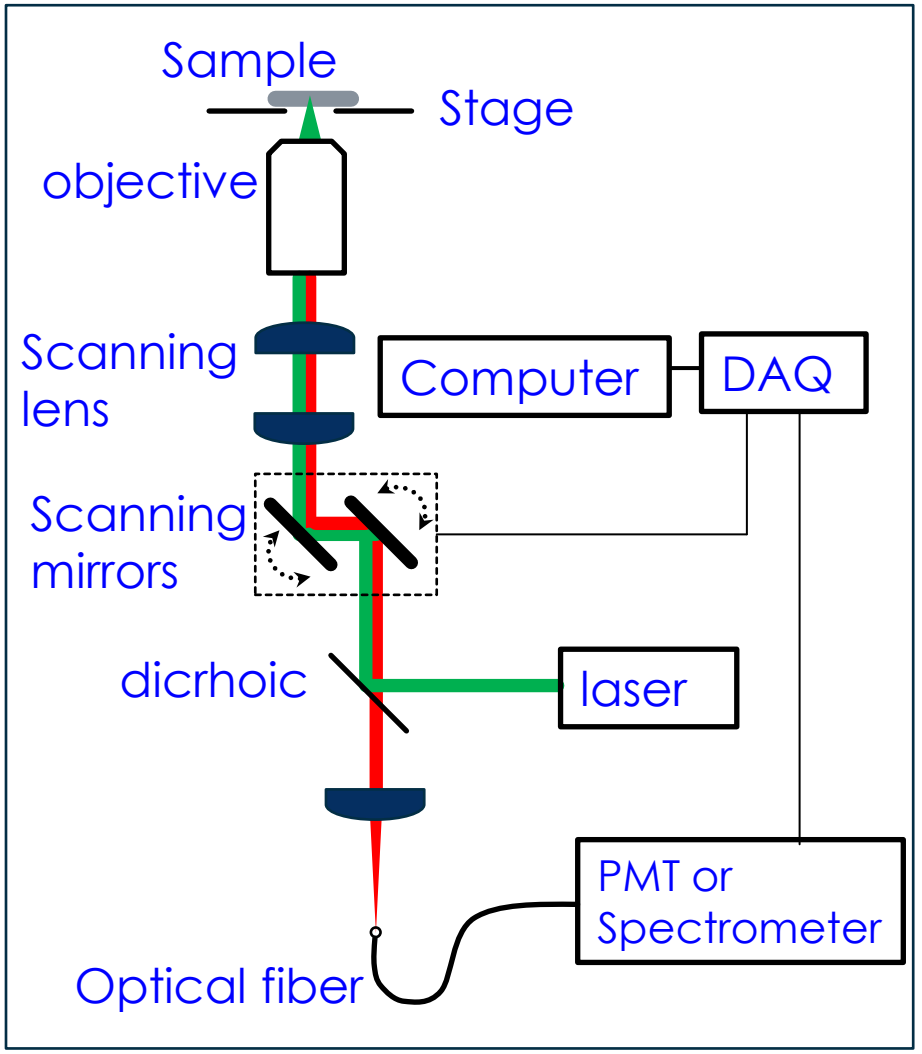
- Scanning lens ensure scan beam hit the same spot on the back focal plane of objective
- Scanning lens also expand the laser beam to fill the back aperture

COMPONENTS OF LSCM

Single point confocal detection

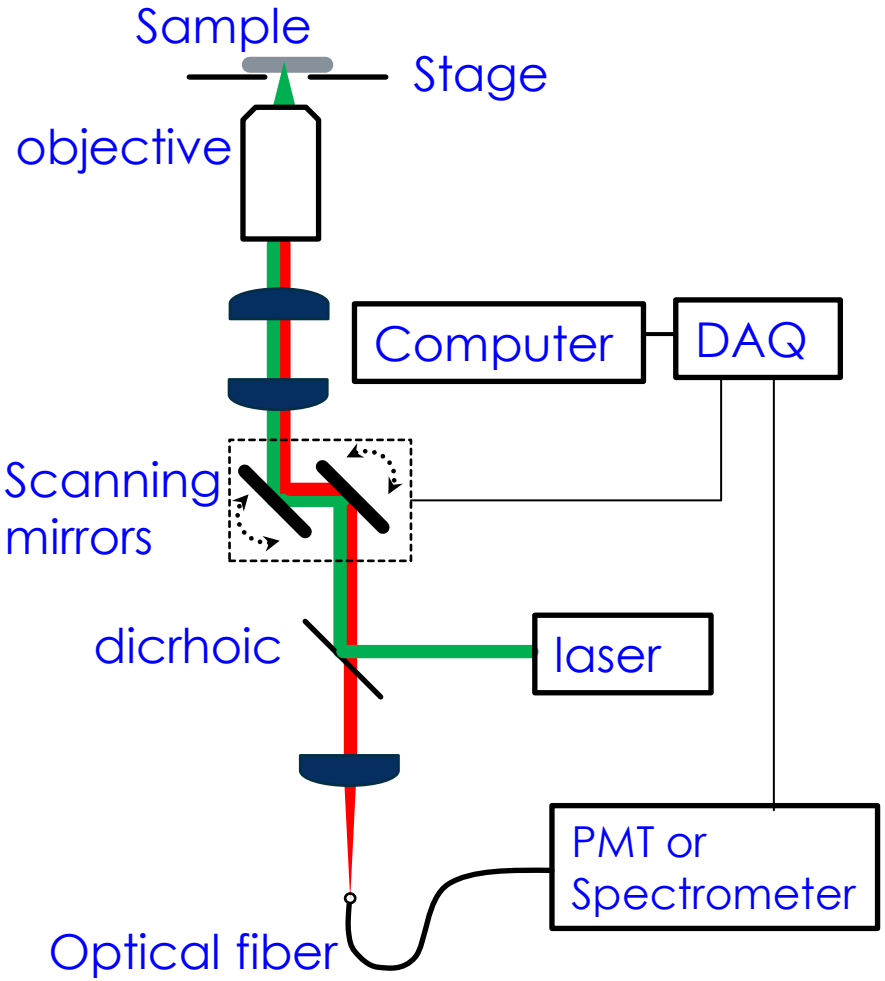


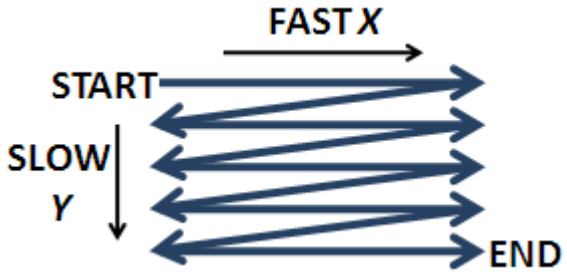
Laser scannnig confocal microscope (LSCM)



- Computer controls the scanning mirror
- Acquired PMT or spectrum signal is correlated with mirror position to form image

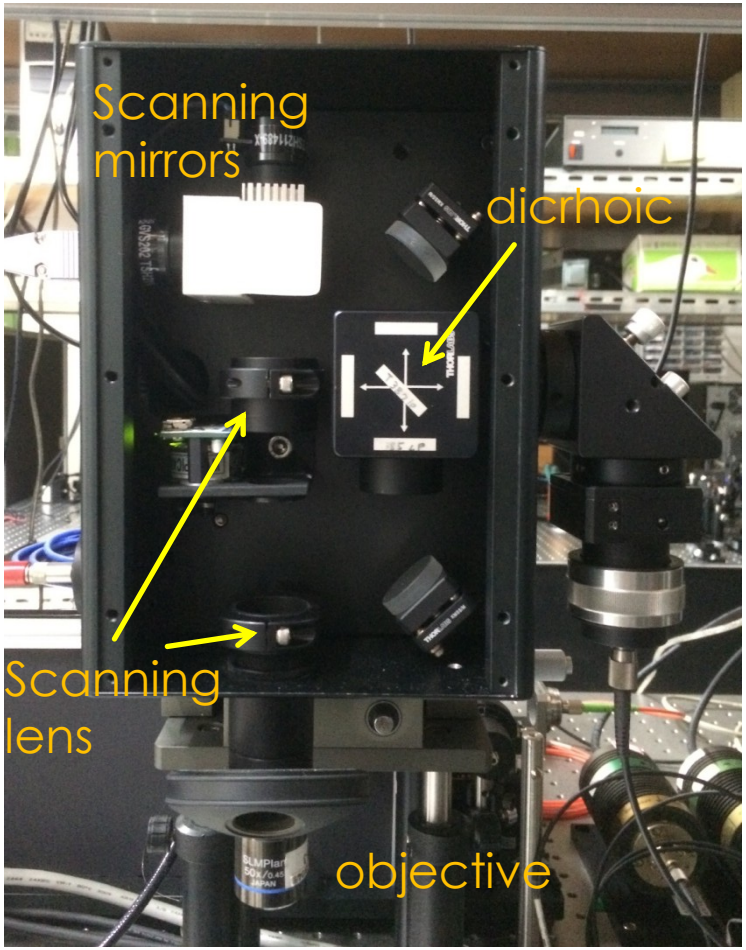
HOW ARE IMAGES ACQUIRED BY LSCM?

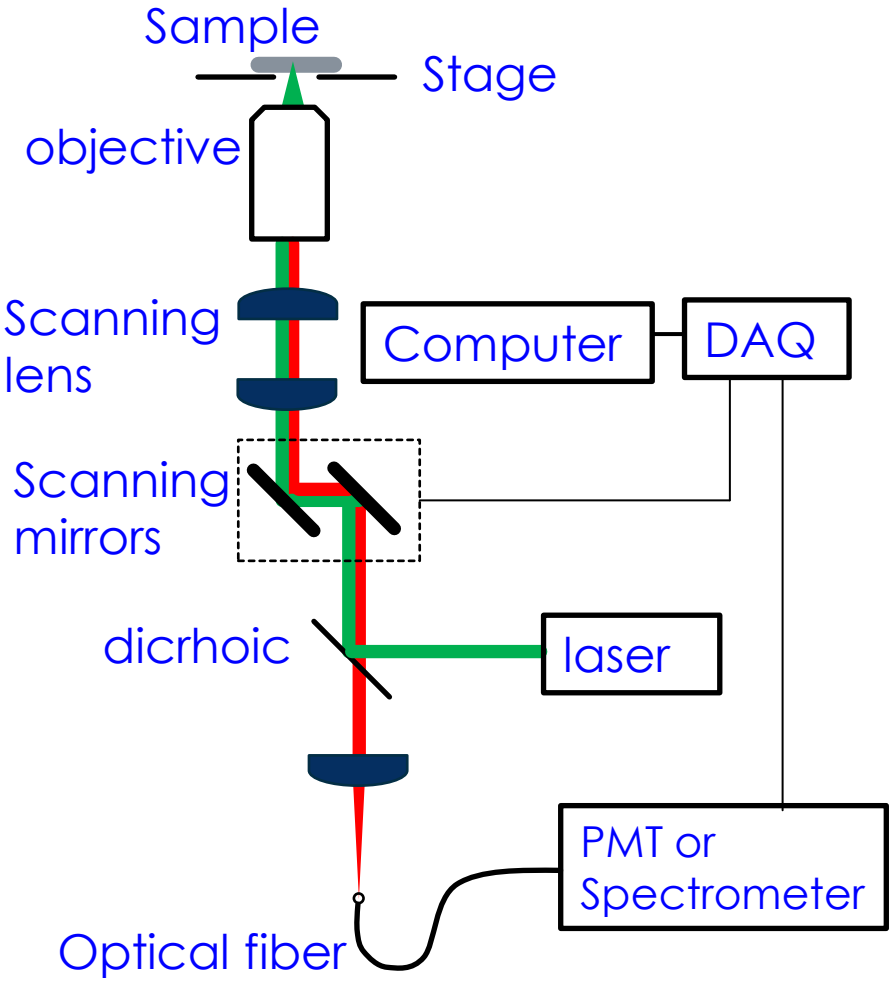




1. Computer send signal to position scanning mirror
2. Signal read in from PMT or spectrometer point by point

LSCM SYSTEM


Scanning
mirrors
dicrhoic
Scanning
lens
objective

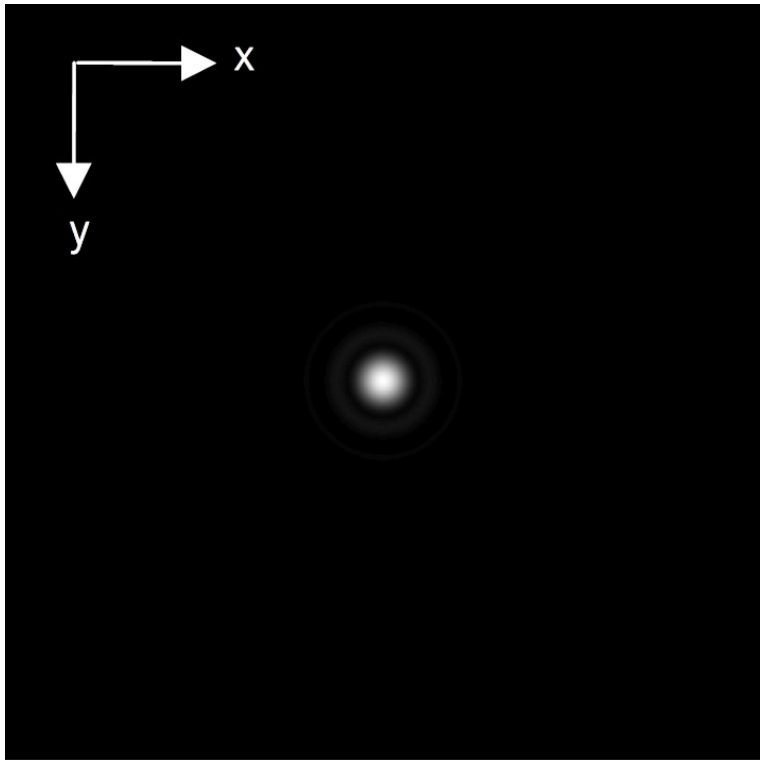

Sample
Stage
objective
Scanning
lens
Computer
DAQ
Scanning
mirrors
dicrhoic
laser
PMT or
Spectrometer
Optical fiber

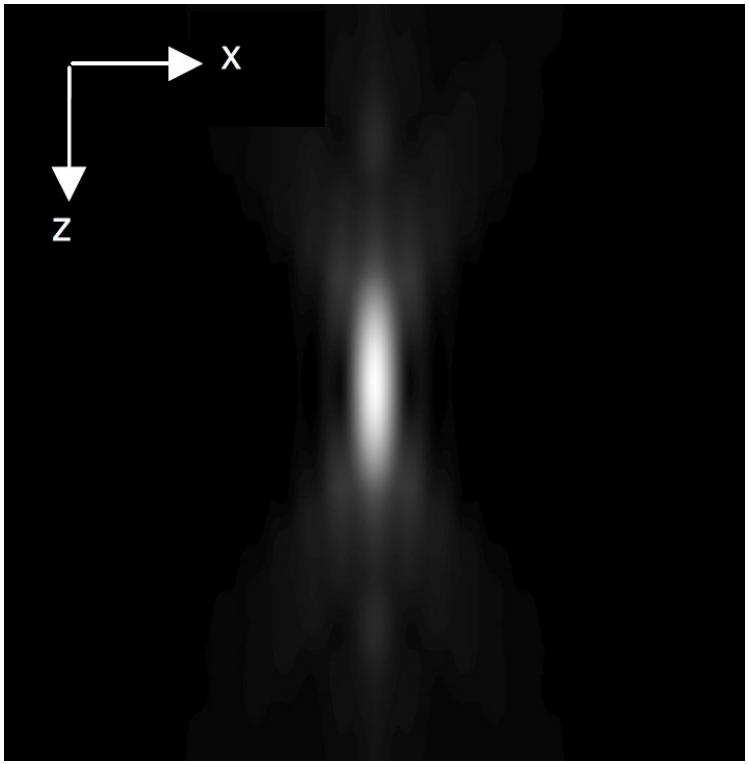
KEY PERFORMANCE SPECIFICATIONS OF A LSCM?

- Resolution
  - Lateral and axial
- Scanning speed and precision
  - Galvo speed and precision
- Scanning field size
  - Scanning lens size
- Detection sensitivity and spectral range
- Available light source / filters

RESOLUTION OF A LSCM

- Resolution determined mainly by the point spread function
- Even for perfect optics, the psf is limited by diffraction

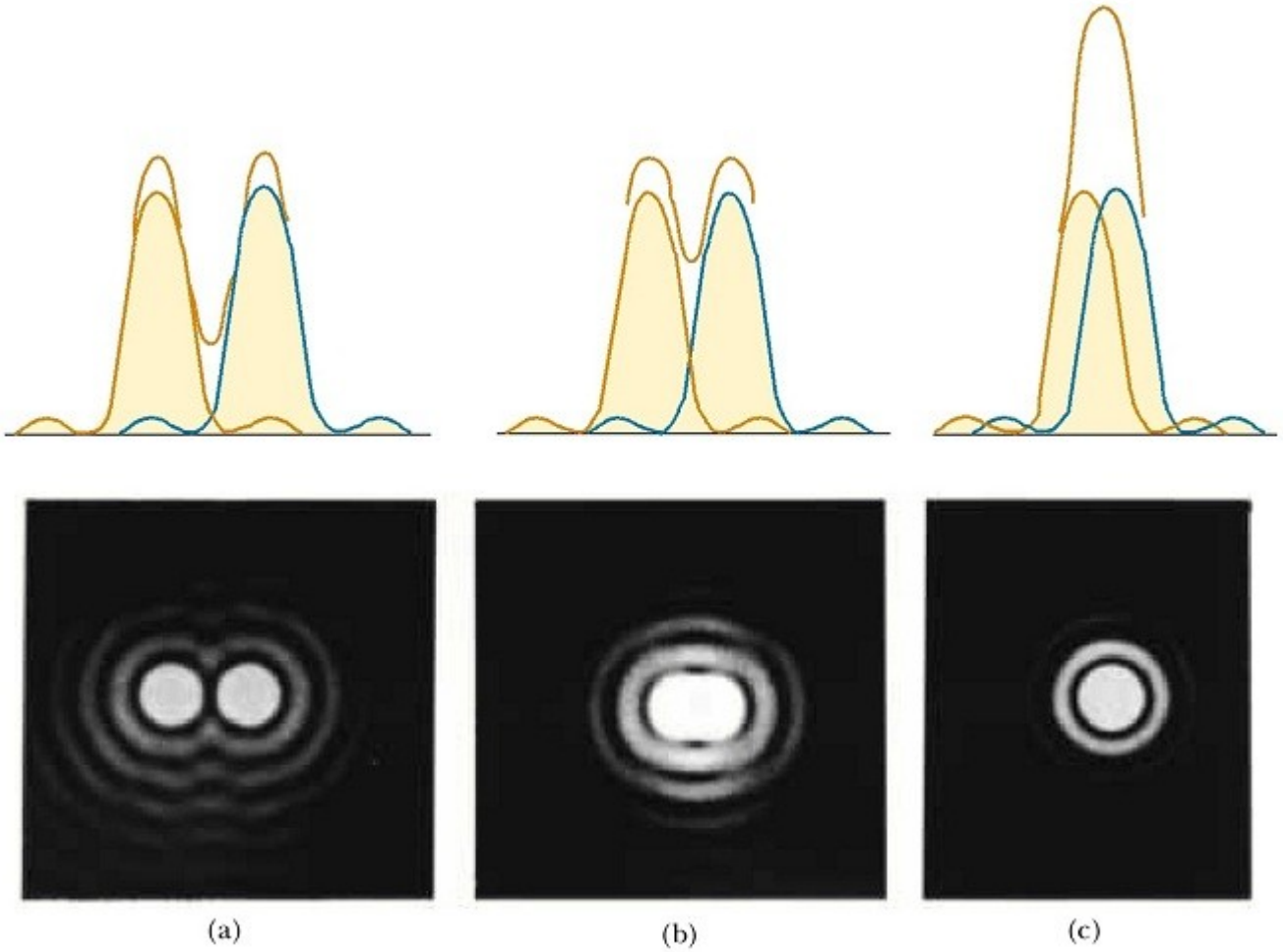




http://zeiss-campus.magnet.fsu.edu/referencelibrary/laserconfocal.html

RESOLUTION BY RAYLEIGH CRITERION

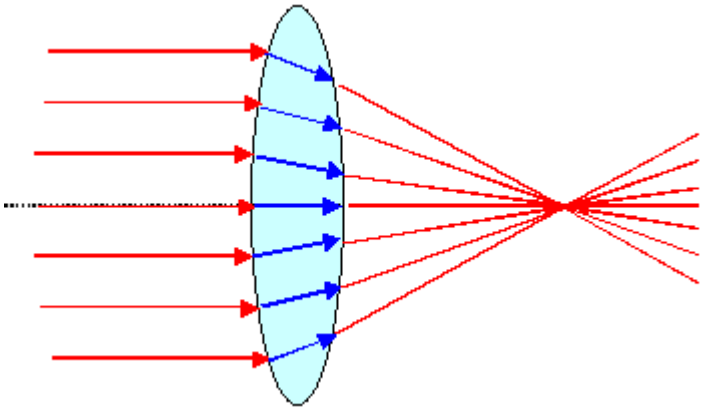
Rayleigh criterion: minimal of one peak coincide with maxima of the other peak



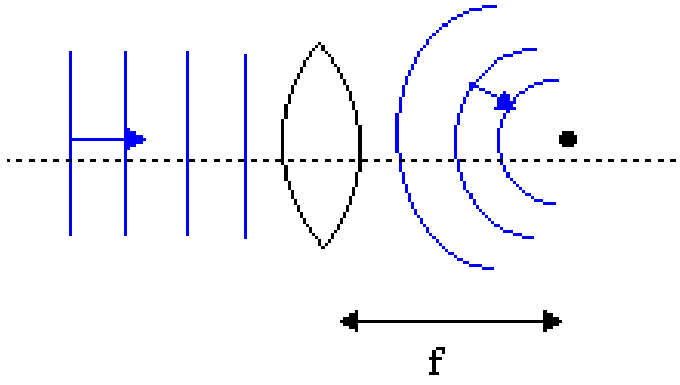
http://www.kshitij-iitjee.com/resolution-of-single-slit-and-circular-apertures

GEOMETRIC VS WAVE OPTICS

Geometric optics: infinitely small focus



Wave optics: focal point has finite size



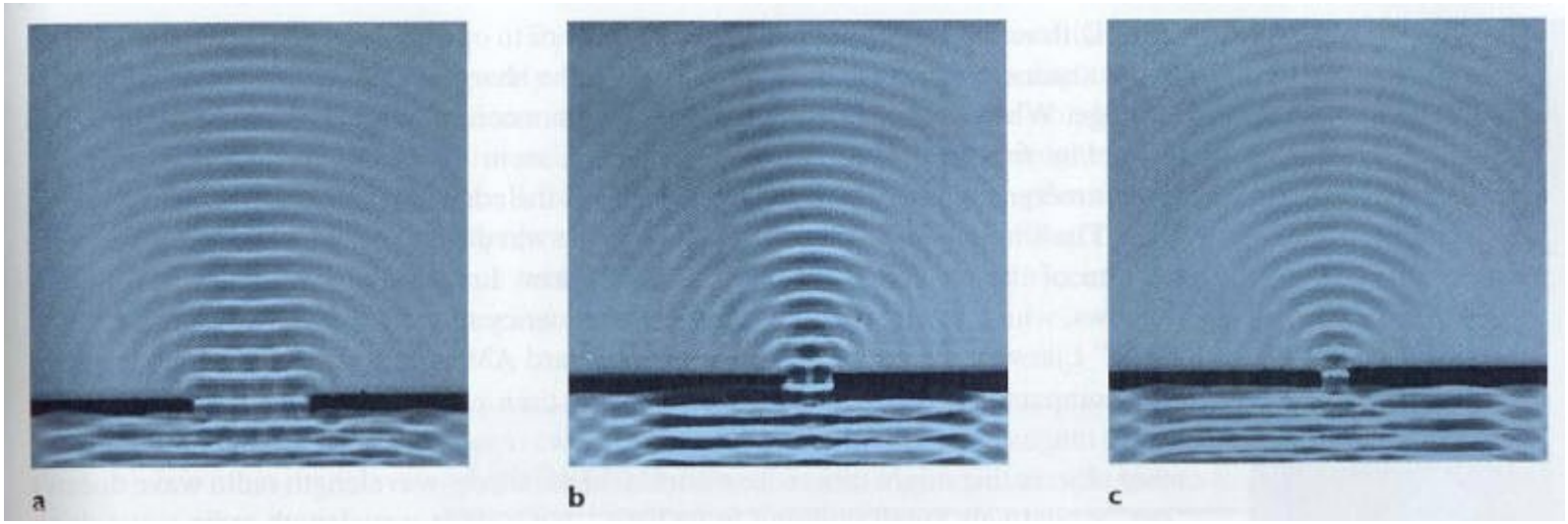
Converging lens

The smaller the focal point, the better the resolution

Reference:
https://www.tf.uni-kiel.de/matwis/amat/admat_en/kap_5/backbone/r5_1_2.html
https://www.sparknotes.com/physics/optics/geom/section3/

DIFFRACTION (繞射)

How does the minimum position change with the opening size?

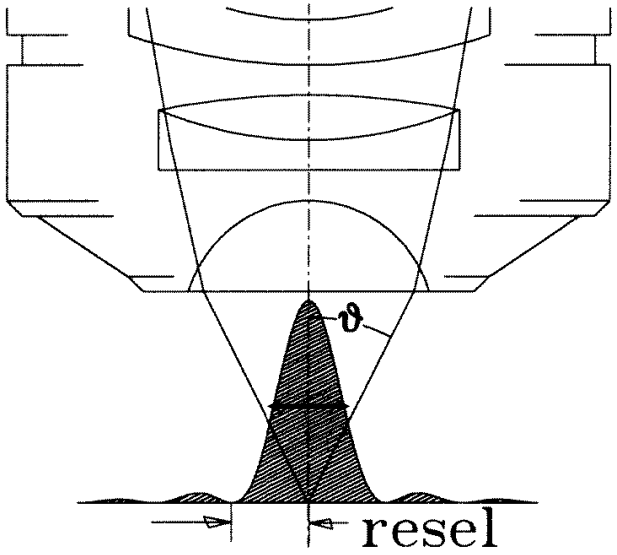


Reference: http://electron6.phys.utk.edu/light/1/Diffraction.htm

FACTORS THAT DETERMINES THE RESOLUTION OF A LSCM

- Objective numerical aperture (NA)
- Excitation and detection wavelength
- Pinhole size

$$r_{resel} = 0.61\lambda/NA$$
$$NA = n \sin\theta$$

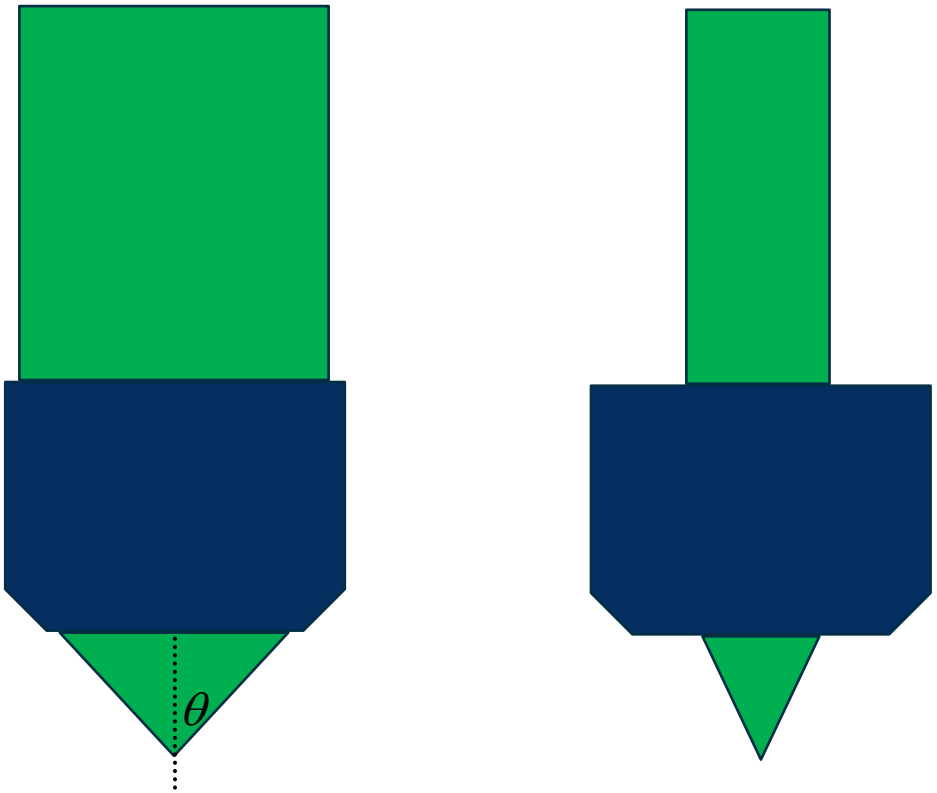


Rayleigh criterion for resolution: minimal distance resolvable between two points is r_{resel}, corresponds to a 26% dip

resel=resolution element
FWHM = 0.84 * resel

For 50x NA=0.8 objective with λ=405nm,
Resel = (0.61)(405nm)/0.8 = 300 nm

FILLING THE OBJECTIVE PUPIL

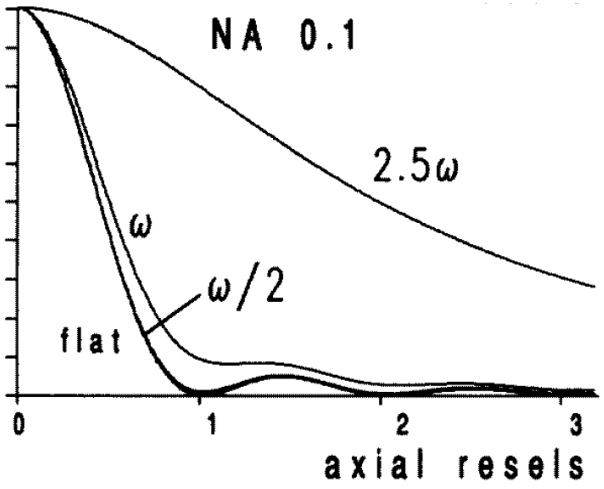


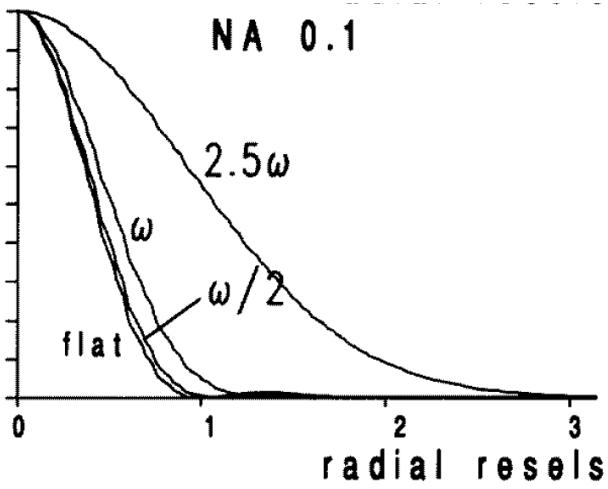
$$NA = n \sin\theta$$

- Under-filling of pupil can lead to lost of resolution (effectively low NA)
- Over-filling of pupil loses laser power

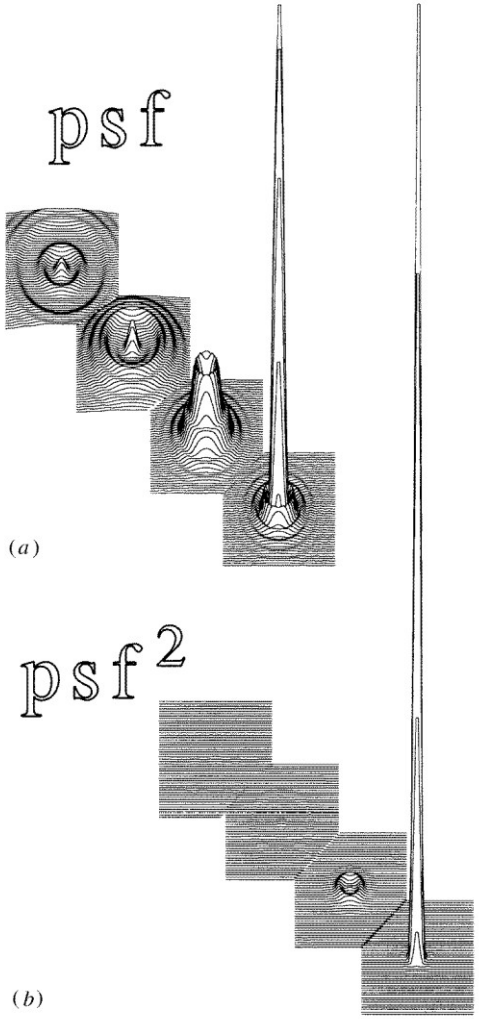
FILLING THE OBJECTIVE PUPIL

- ω = radius of Gaussian laser beam containing 86% of light
- Plot of psf for 4 pupil size compare to laser beam waist ω
- Too much under-filling of pupil can lead to lost of resolution





CONFOCAL RESOLUTION



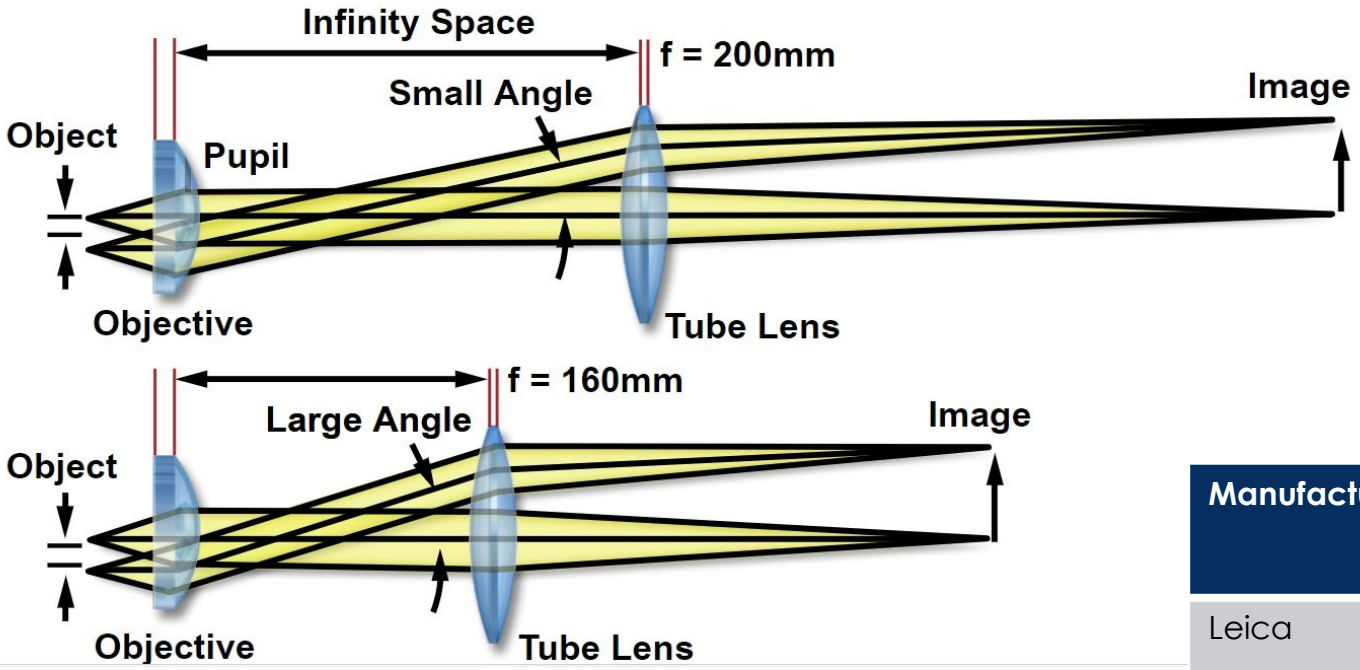
- Point excitation + point detection leads to better resolution
- Point spread function (psf) at the focal plane and planes parallel to it for (a) wide field (b) confocal
- Ideal confocal resolution based on Rayleigh criterion (26% dip):

$\Delta r_{conf} = 0.44\lambda/NA$ (lateral resolution)

$\Delta Z_{axresel} = 1.5n\lambda/NA^2$ (axial resolution)

Actual resolution depends on pinhole size

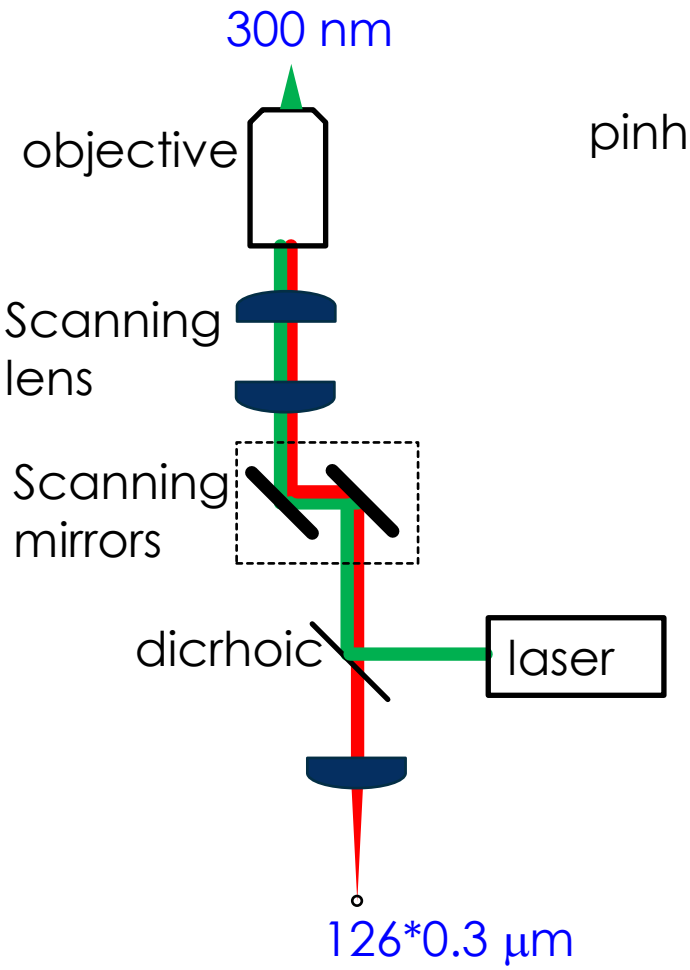
MAGNIFICATION FOR INFINITY OPTICS

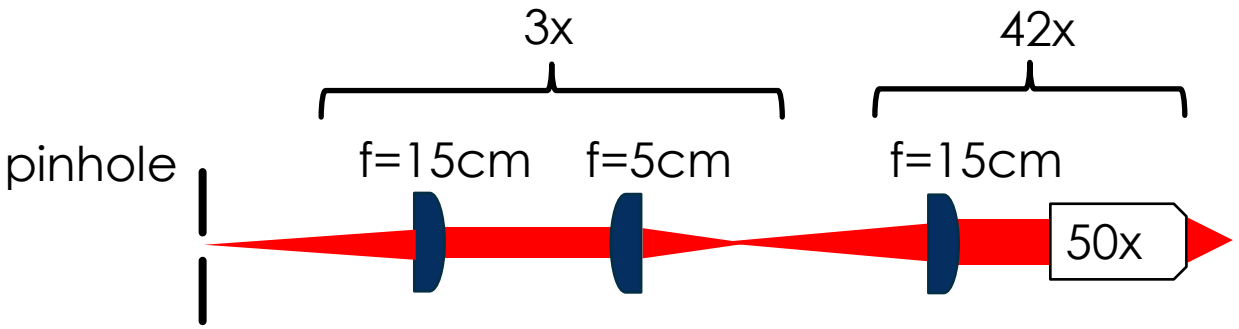


Manufacturer	Tube Lens Focal Length (mm)
Leica	200
Nikon	200
Olympus	180
Zeiss	165

- Magnification (f/f_{obj})depends on tube lens focal length
- Objective and tube lens distance does not change magnification
- Tube lens focal length differs for different manufacturer

HOW BIG SHOULD THE PINHOLE BE?



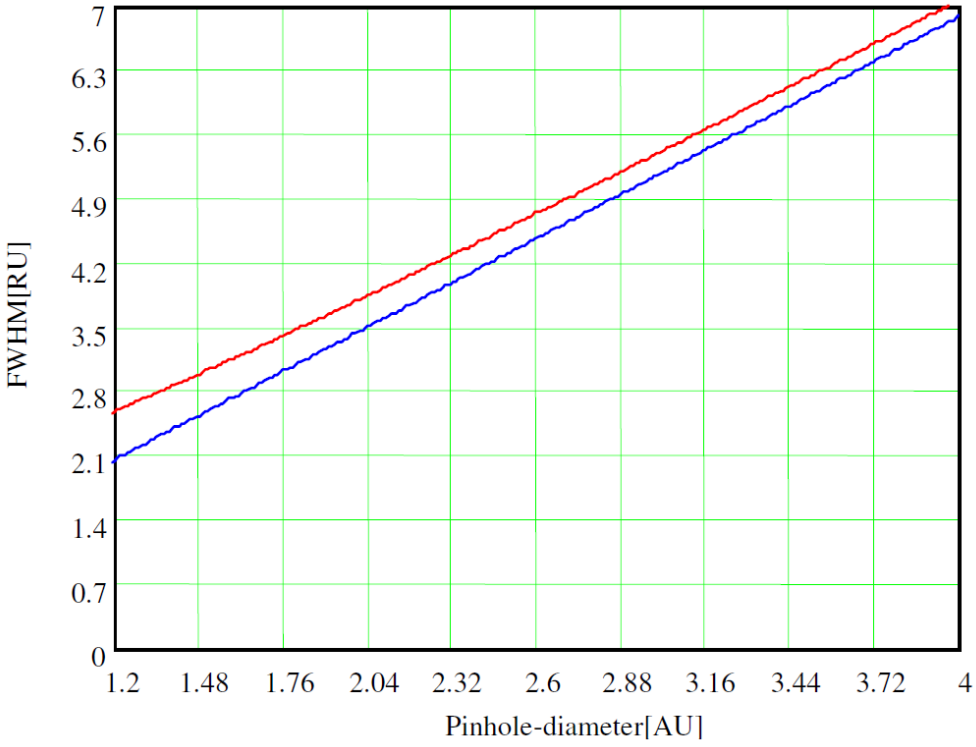


Determine how 1 resolution element gets Mangnified at the pinhole position:
resel at pinhole = (3)(42)(resel at sample)

For 50x NA=0.8 objective with λ=405nm,
Resel at pinhole = 126*0.3 μm ~ 40 μm

- A pinhole smaller than one resel does not improve resolution, it just loses light

HOW AXIAL RESOLUTION CHANGES WITH THE PINHOLE SIZE?



- Red curve shows how axial resolution changes with pinhole size
- NA = 0.6
- n=1
- λ=520 nm

1AU = $1.22\lambda/NA$
1 RU = $n\lambda/NA^2$

Reference: Zeiss Confocal Principles

HOW CAN WE MEASURE THE RESOLUTION OF A LSCM?

- Lateral resolution:
  - Scan image of an object (fluorosphere) with size below optical resolution
- Axial resolution:
  - Axial scan of a small fluorescent object
  - Axial scan of a mirror (detect reflection)
  - Axial scan of a fluorescent thin plane
- Often easier to measure FWHM = 0.84*resel

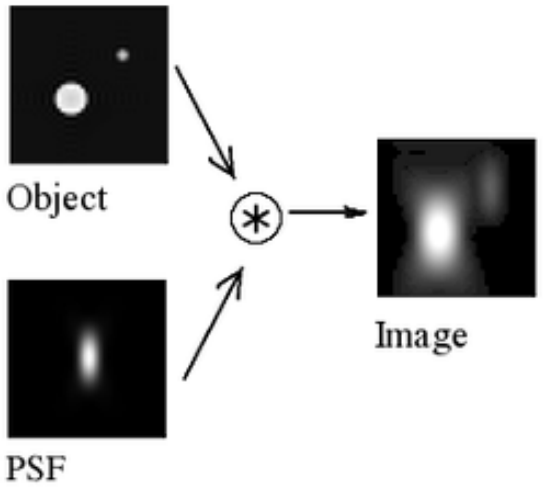
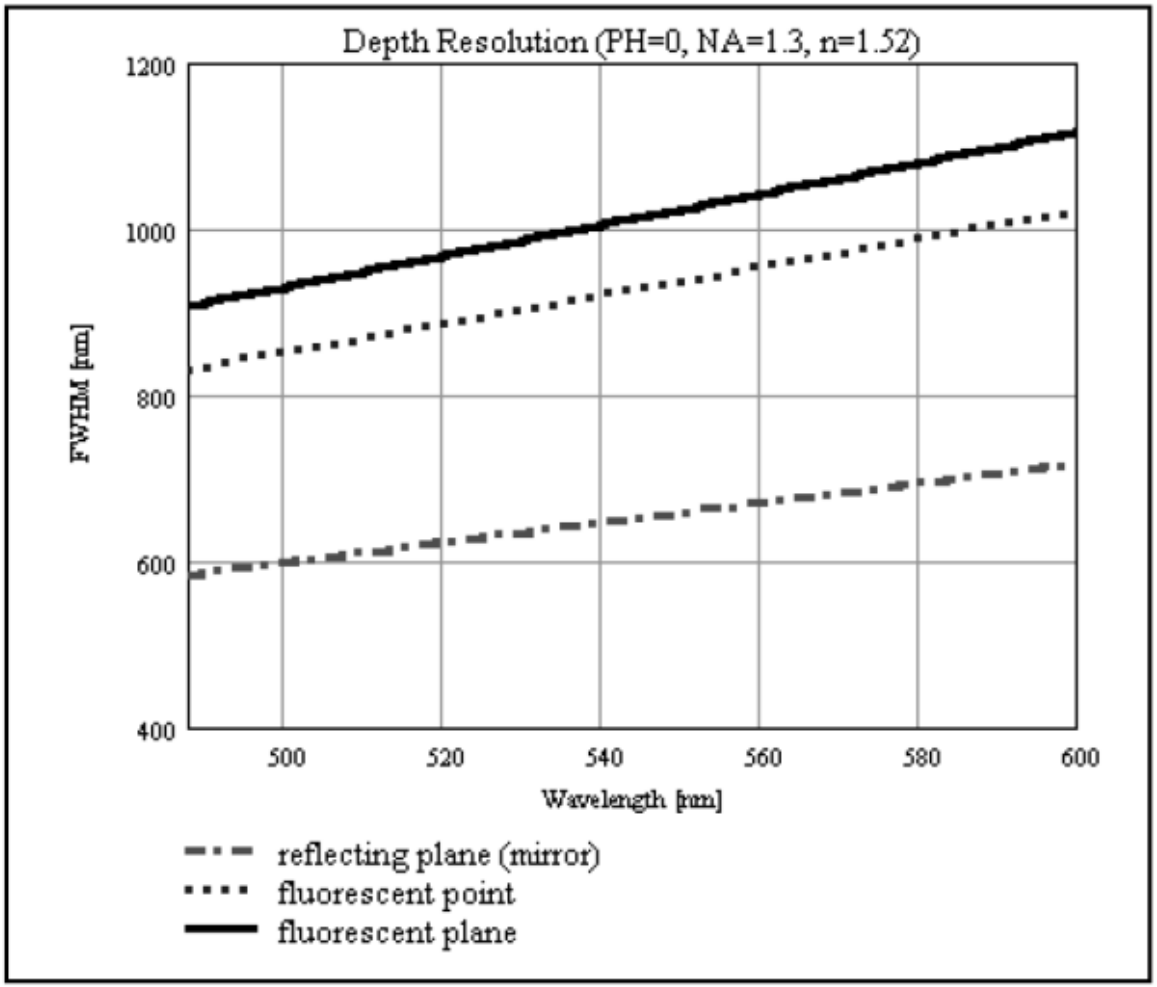


Image reference: https://en.wikipedia.org/wiki/Point_spread_function

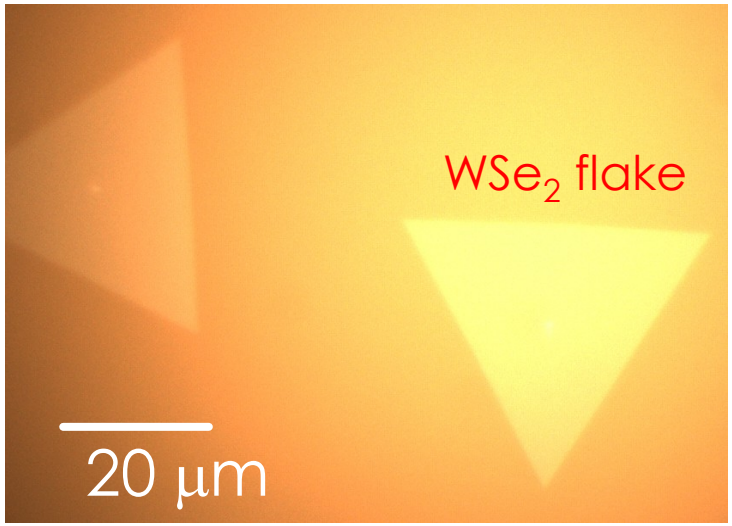
SAMPLE AFFECTING RESOLUTION

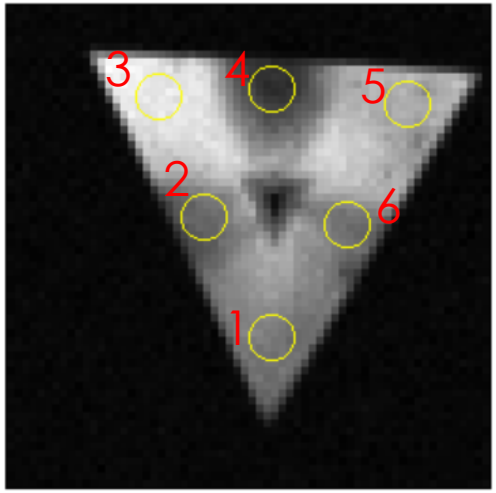


c) Variation of wavelength

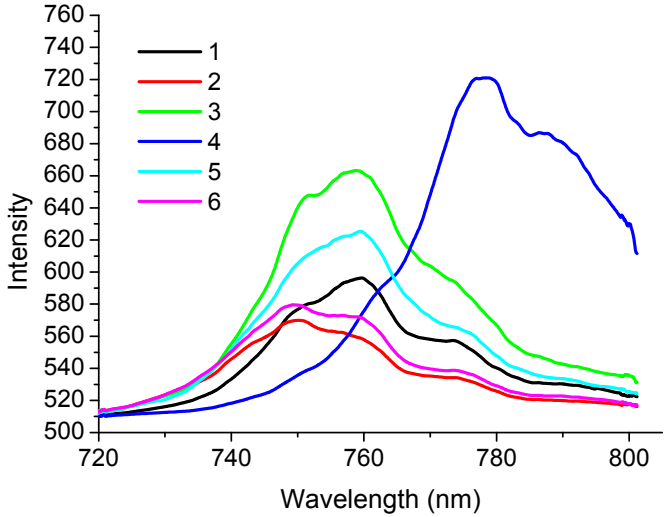
From Zeiss Confocal Principles

LSCM APPLICATION: SPECTRAL MAPPING

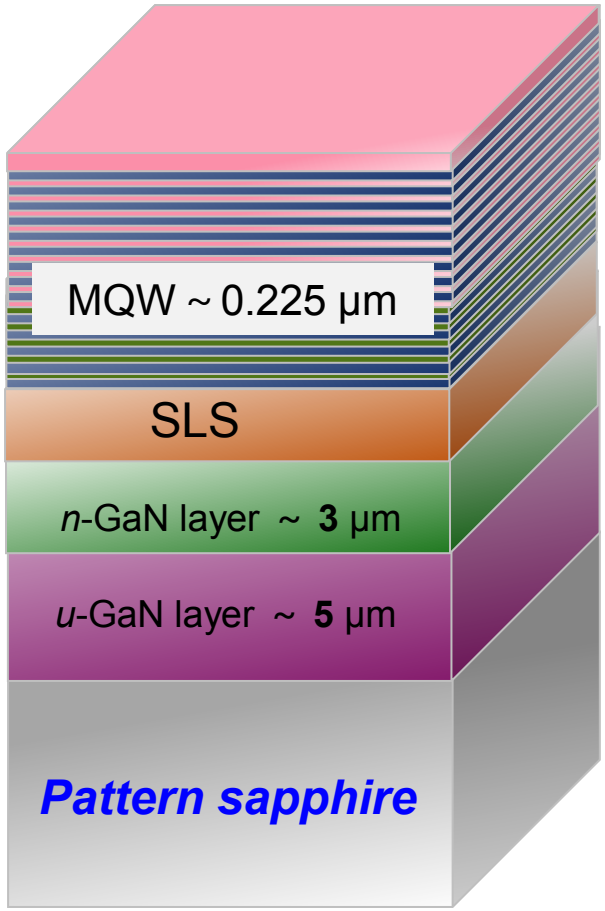


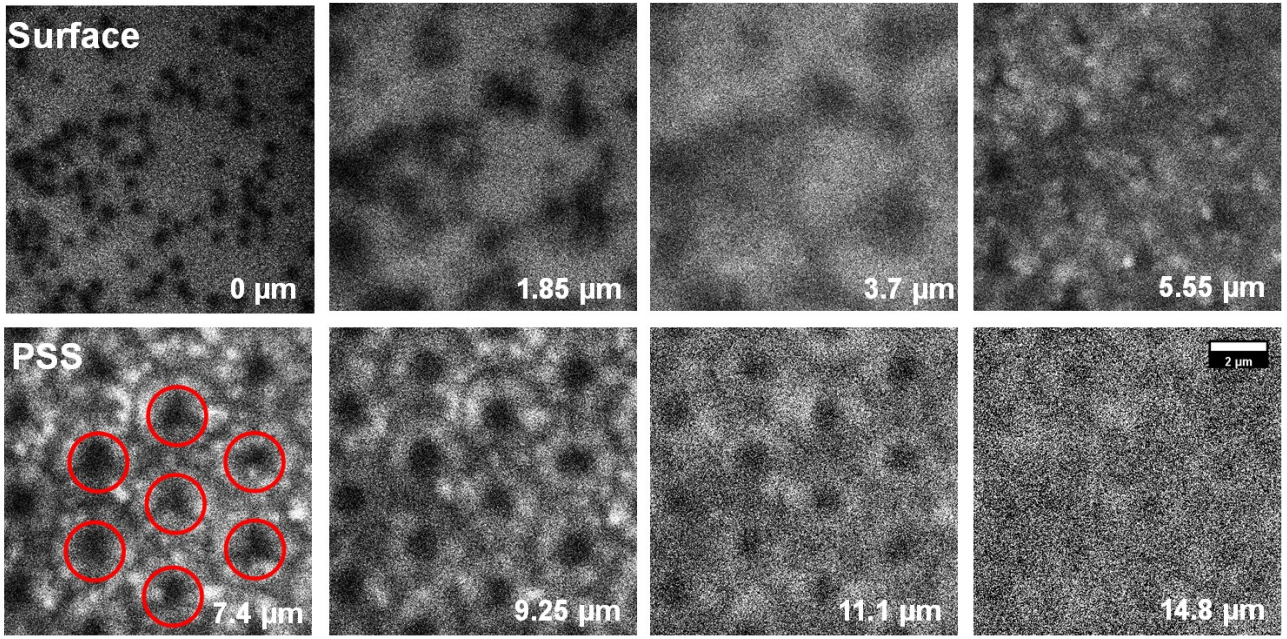


- PL spectral mapping of WSe_2 flake show position dependence in PL spectra



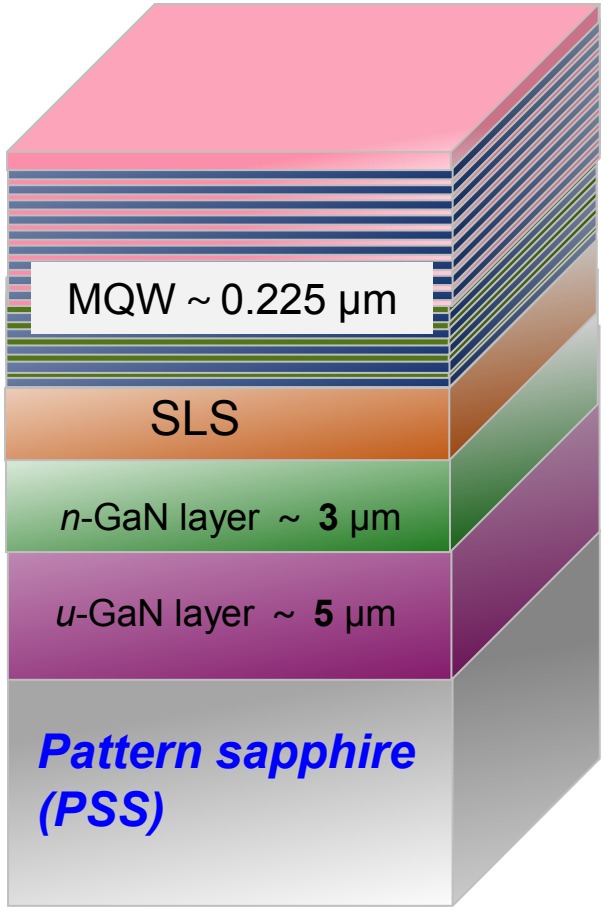
LSCM APPLICATION: DEPTH RESOLVED RAMAN MAPPING



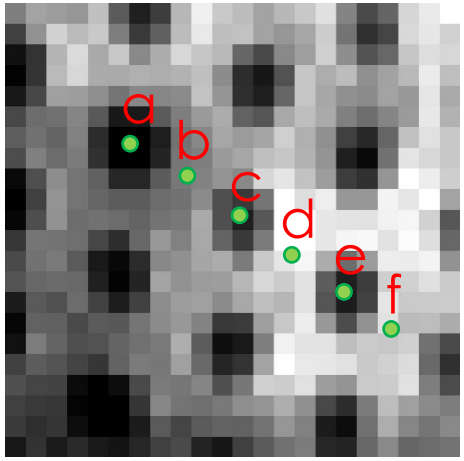


- Depth resolved mapping of GaN E_2(high) Raman line

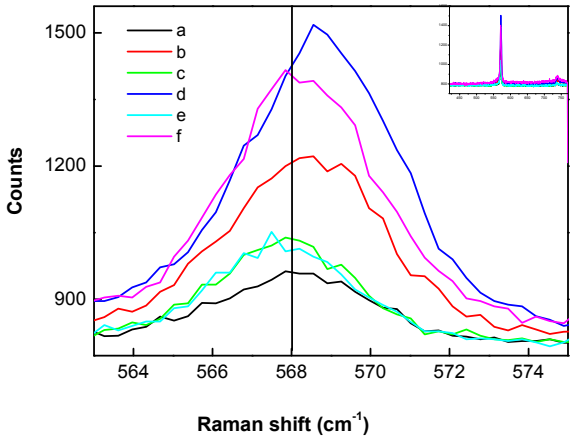
Depth Resolved Raman Mapping of LED



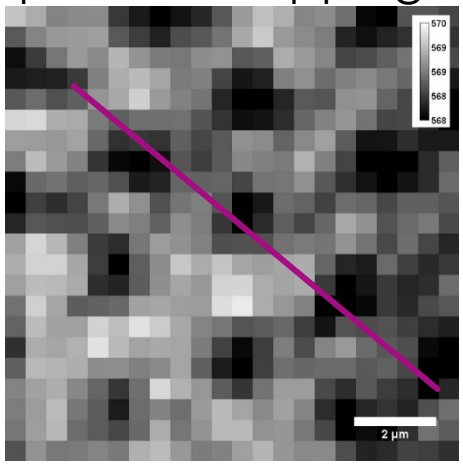
E_2(high) intensity mapping



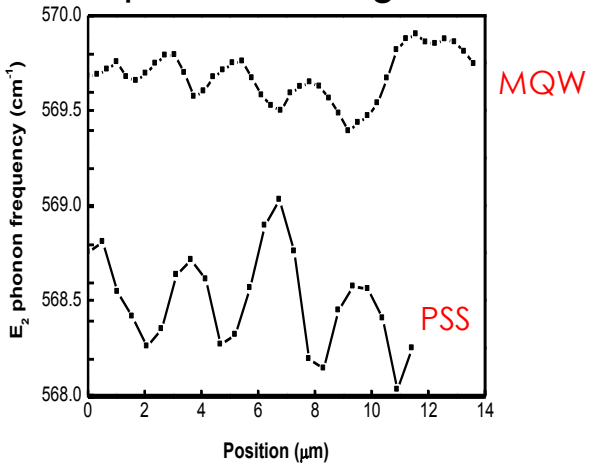
The Raman at different position



E_2(high) peak position mapping

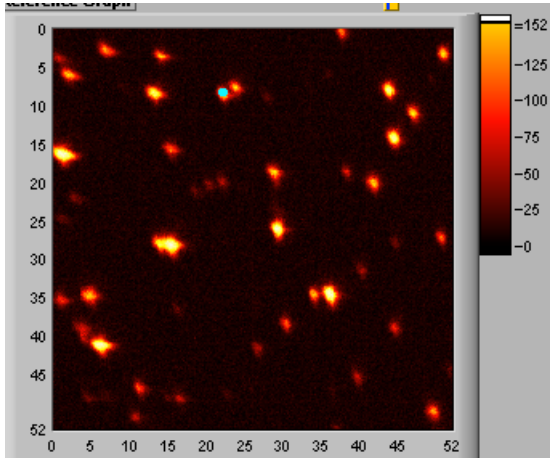


Raman frequencies along red lines

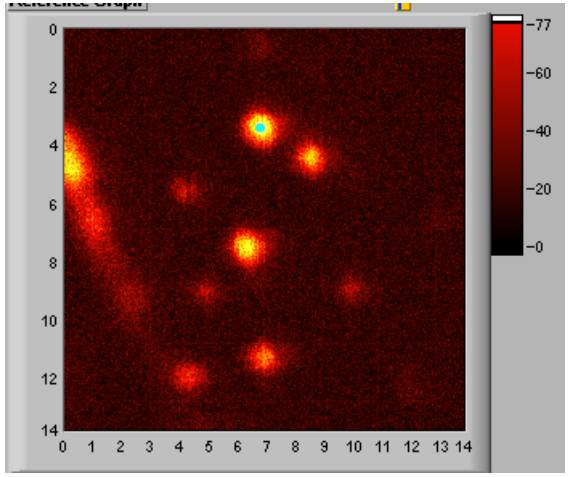


LSCM APPLICATION: TRPL OF NANO STRUCTURE

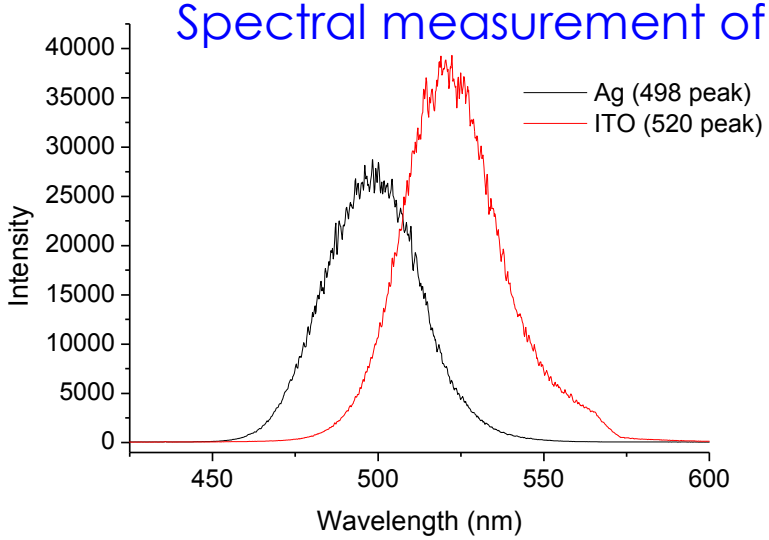
Rod on Ag



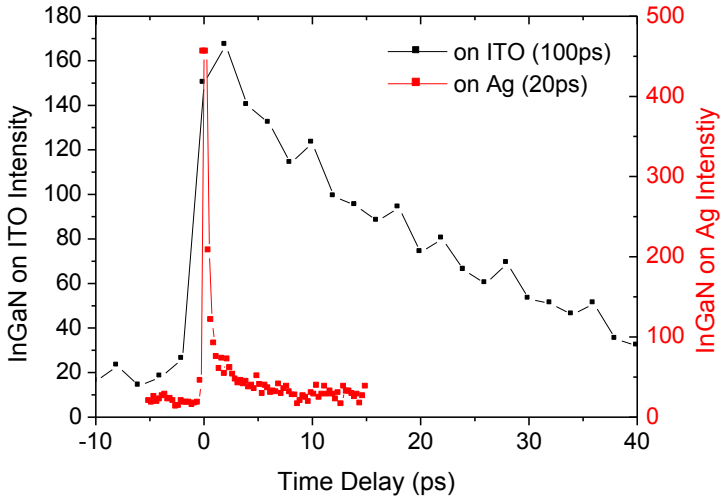
Rod on ITO



Spectral measurement of single rod



Lifetime measurement of single rod



WHAT I HOPE YOU LEARNED:

- How confocal microscope obtain optical sectioning
- The main components of a LSCM and how they affect the performance of LSCM
- How to better integrate LSCM into your research