

Introduction to Confocal microscopy

Gert-Jan Kremers
Jeffrey van Haren

Erasmus
Optical
Imaging Centre

The beginning



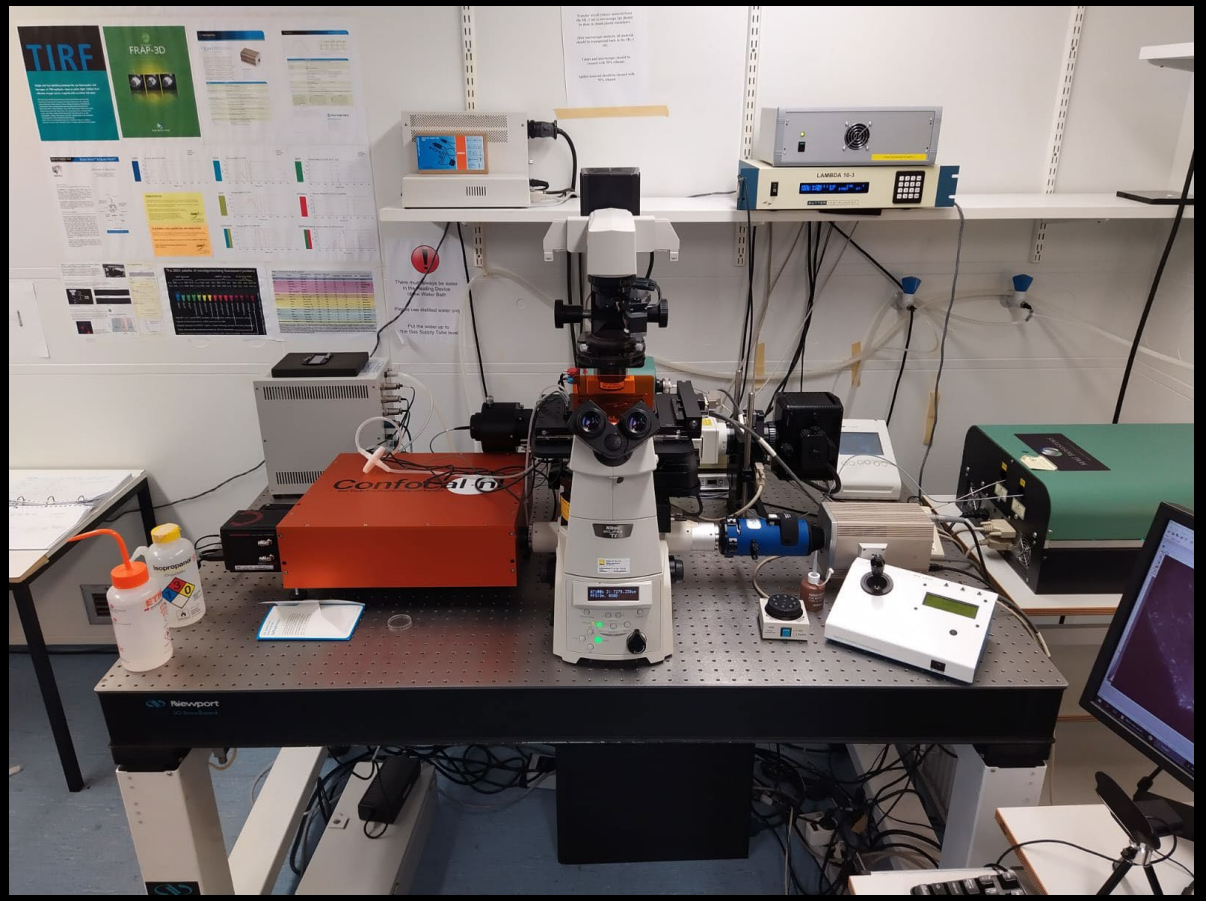
Zacherias (Hans) Jansen 1595

Van Leeuwenhoek (1632-1723) microscope

Introduction



Van Leeuwenhoek (1632-1723) microscope

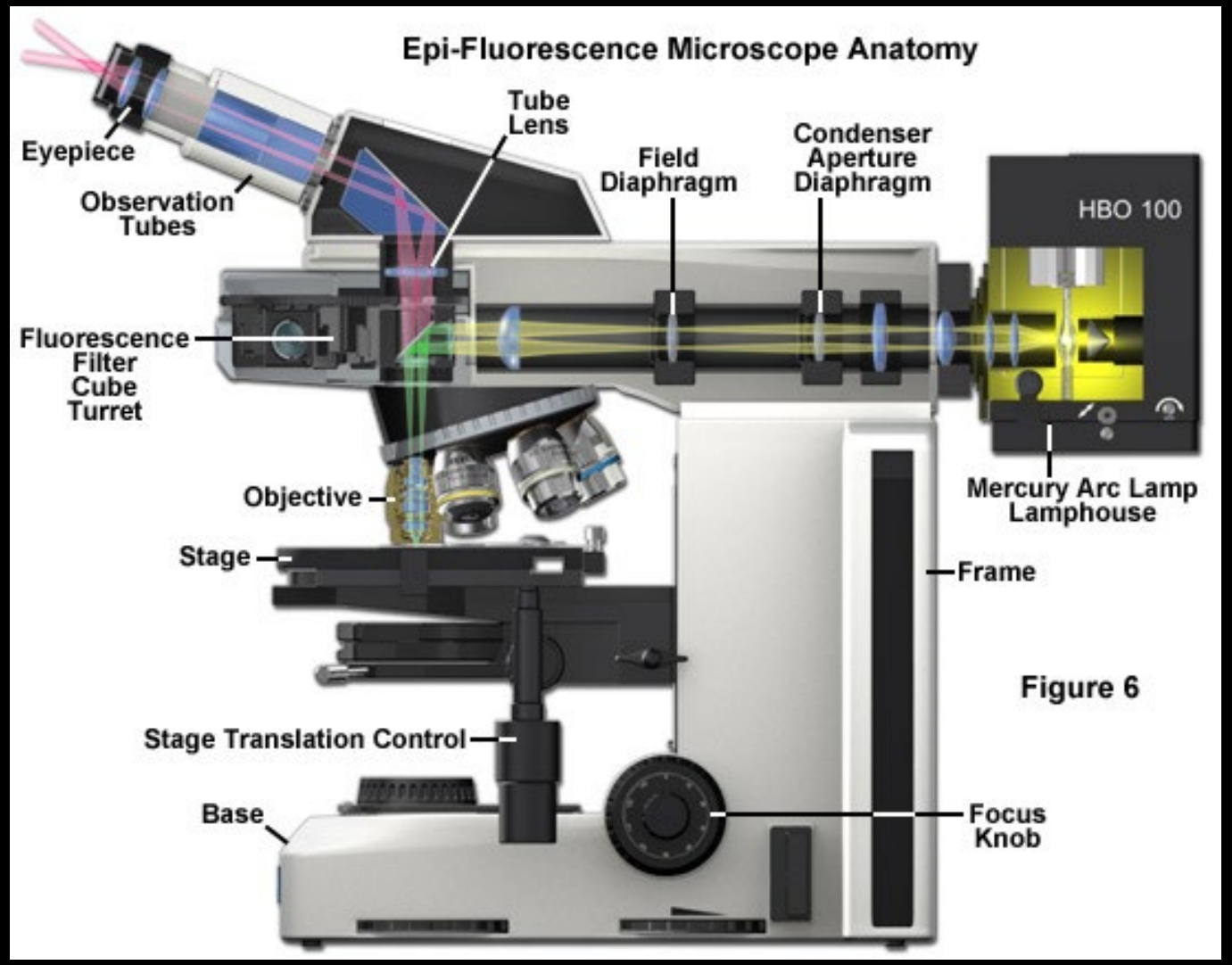


Modern confocal microscope

Introduction

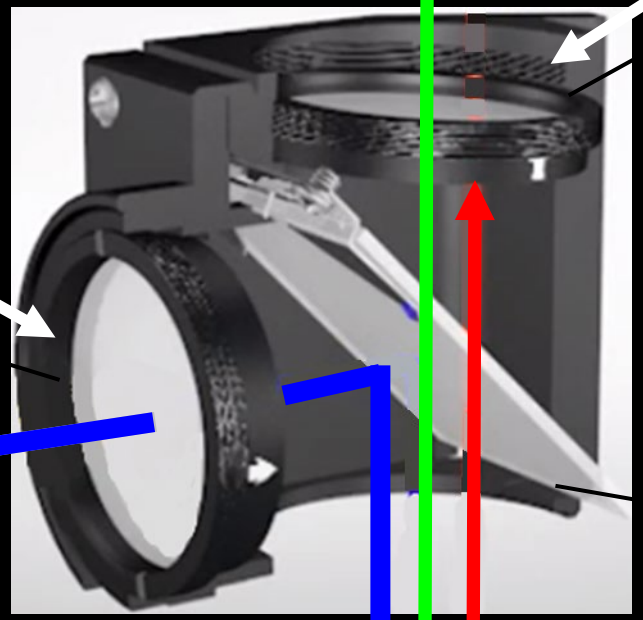
Confocal principle
Time lapse imaging
Sampling rate
Excitation
(Spectral) Detection

Fluorescence microscope



http://zeiss-campus.magnet.fsu.edu

Fluorescence lightpath



Detector

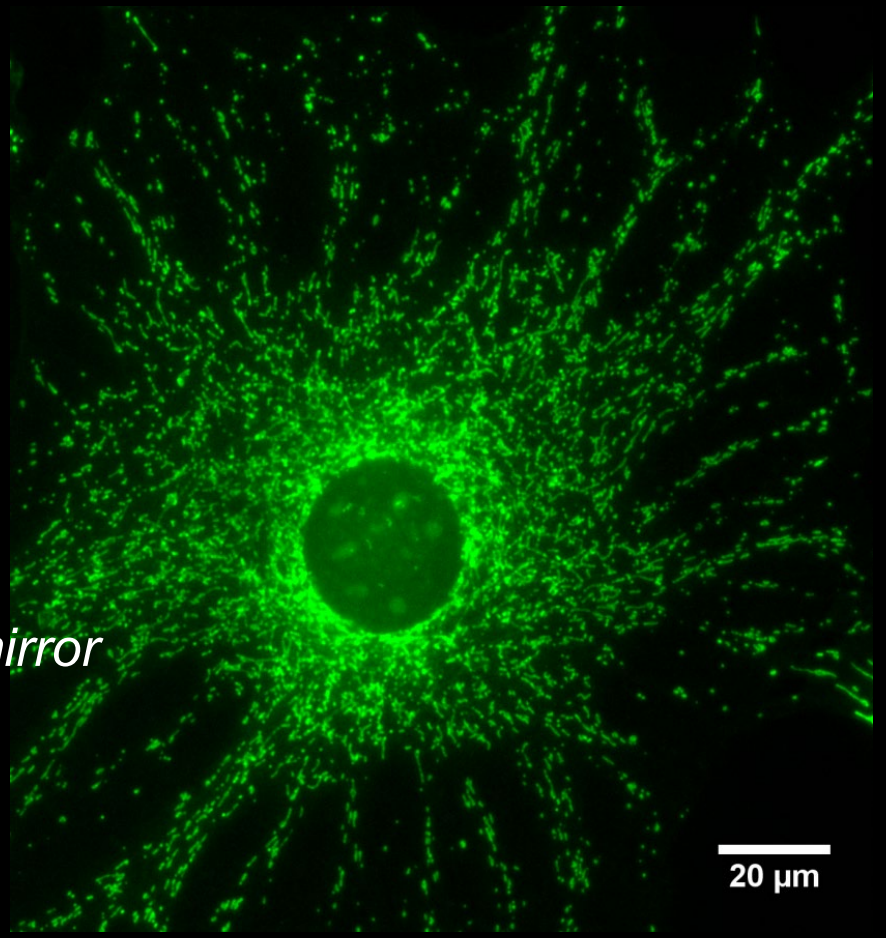
3. Emission filter

1. Excitation filter

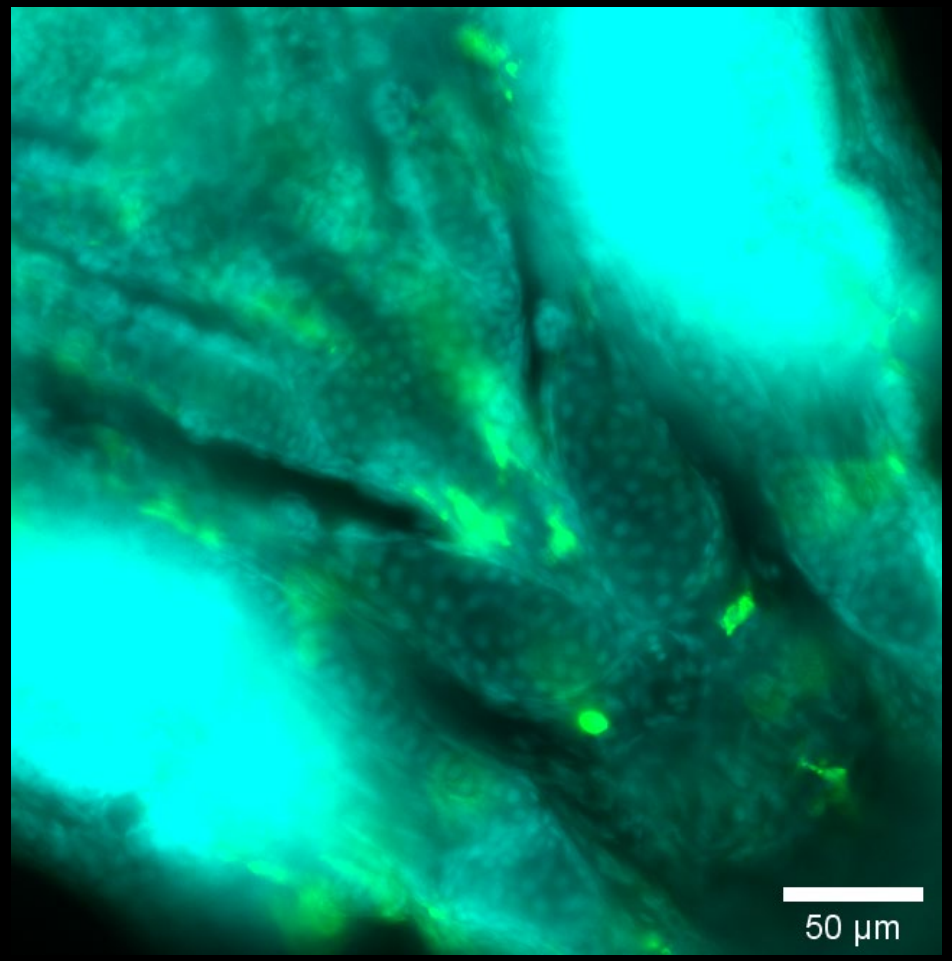
2. Dichroic mirror

Excitation light:
488nm laser line

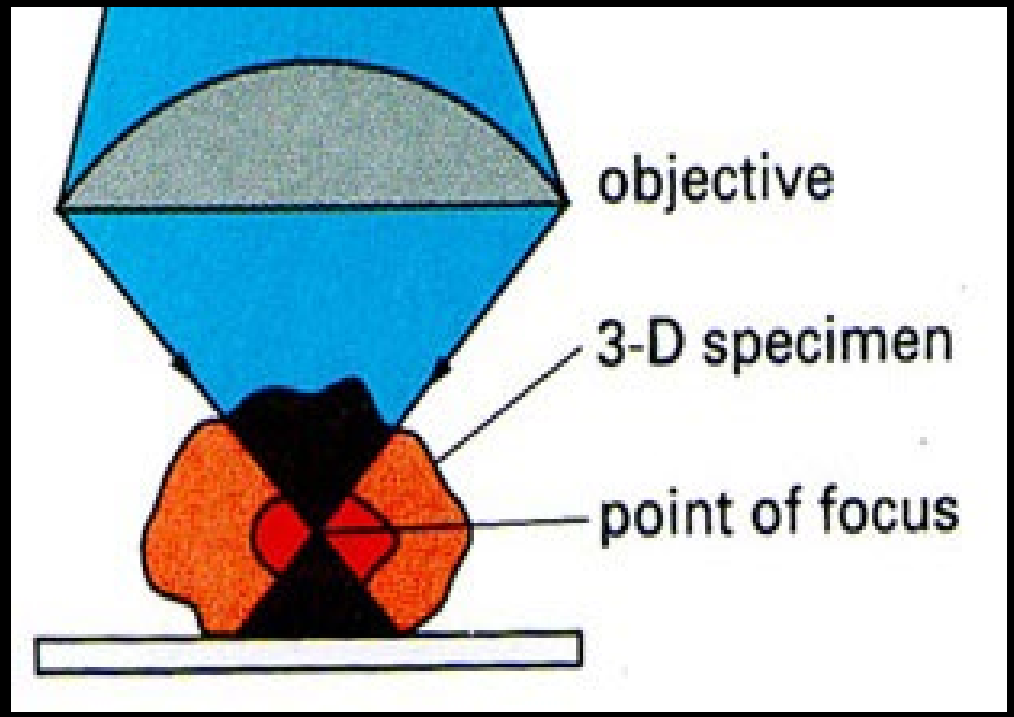
Sample



Focus problem

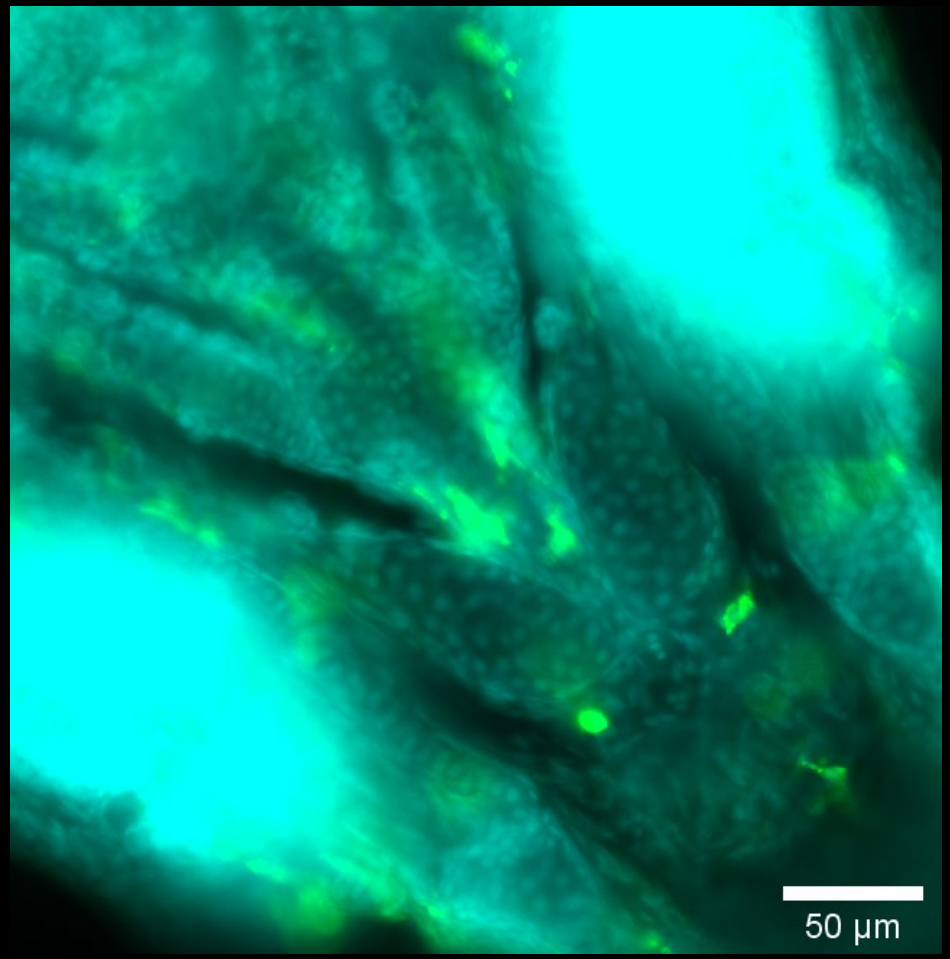


Zebrafish embryo head

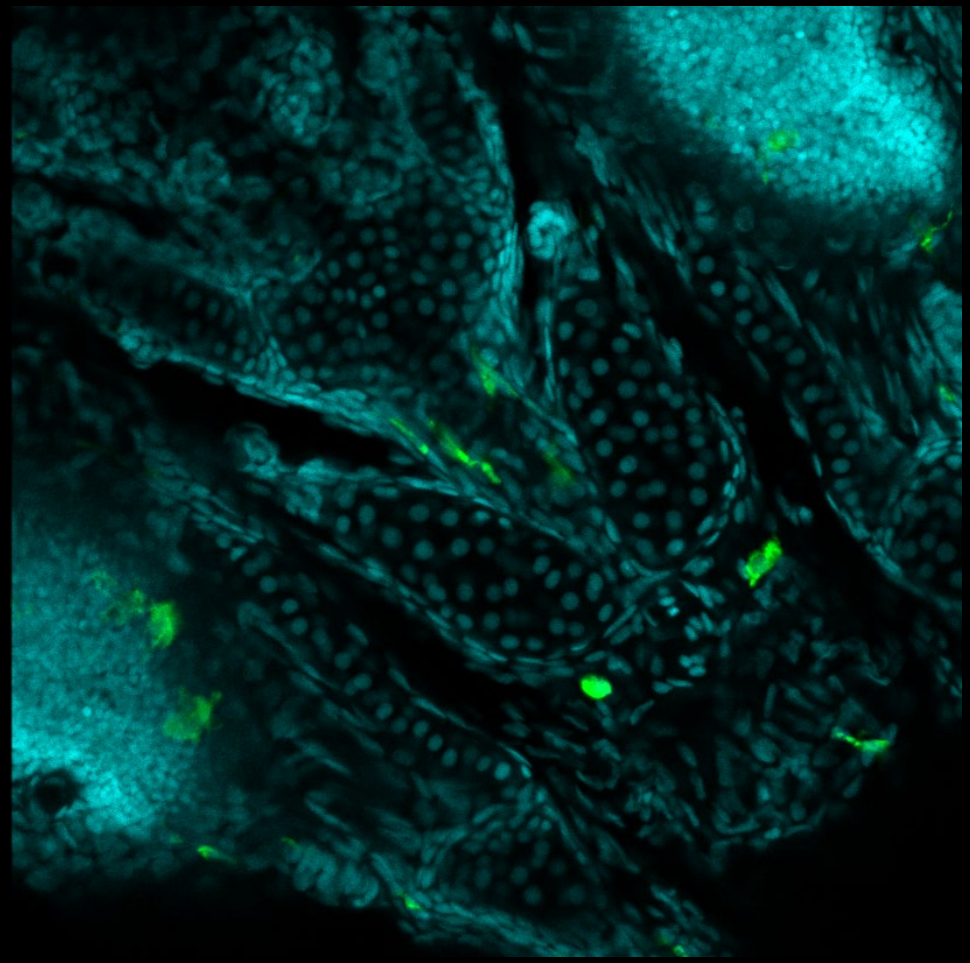


Focus blur

Confocal principle

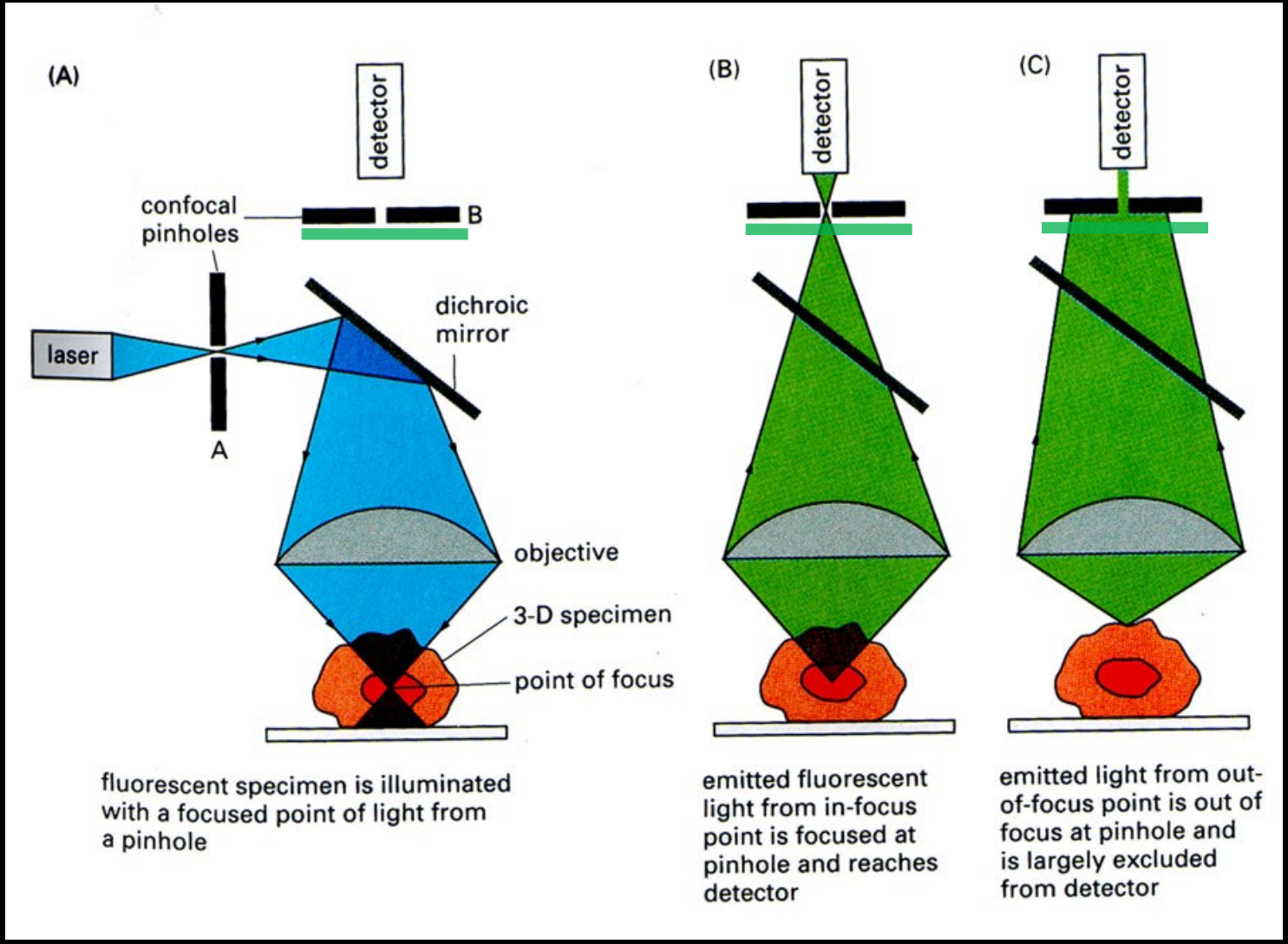


Widefield

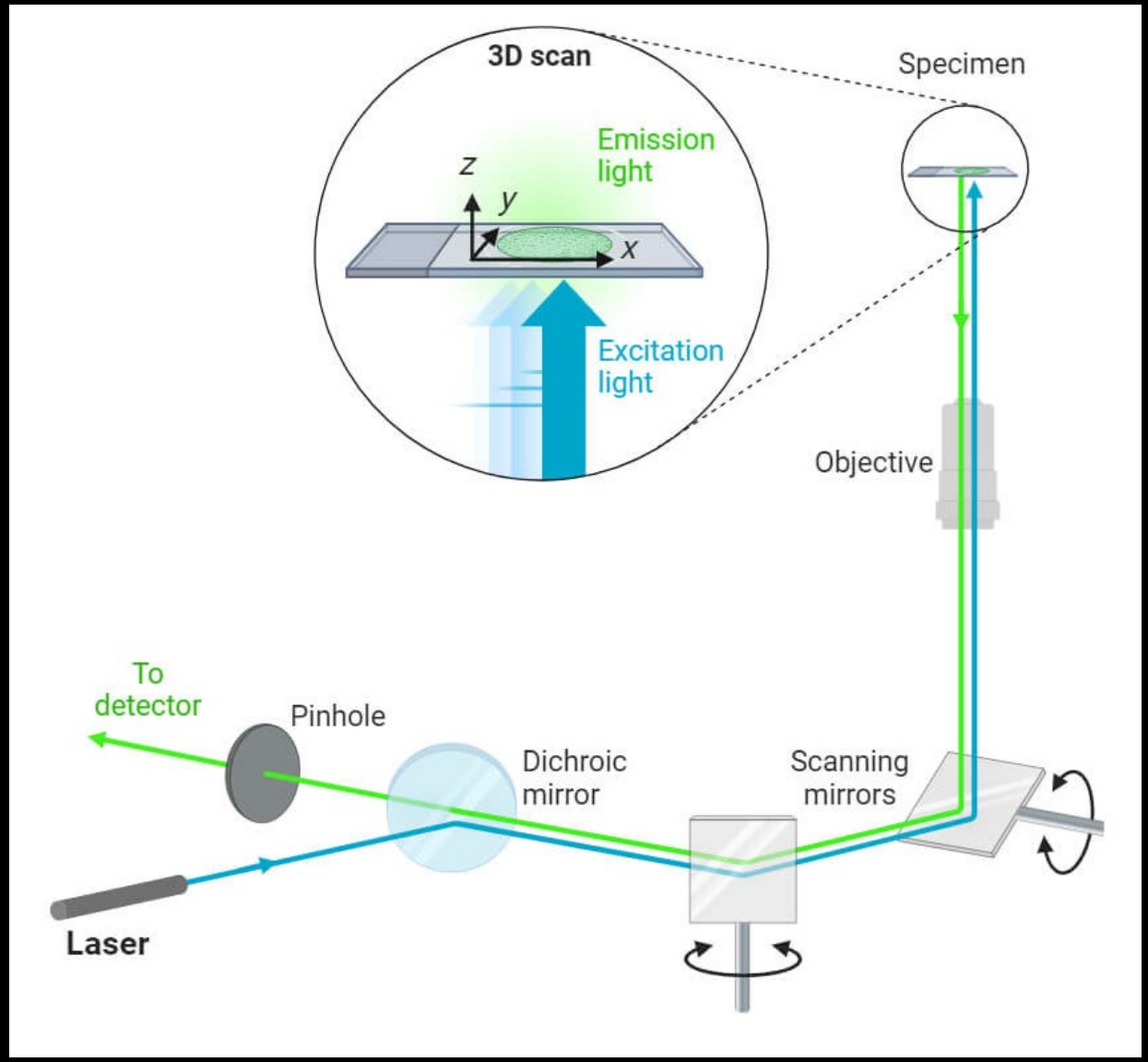


Confocal

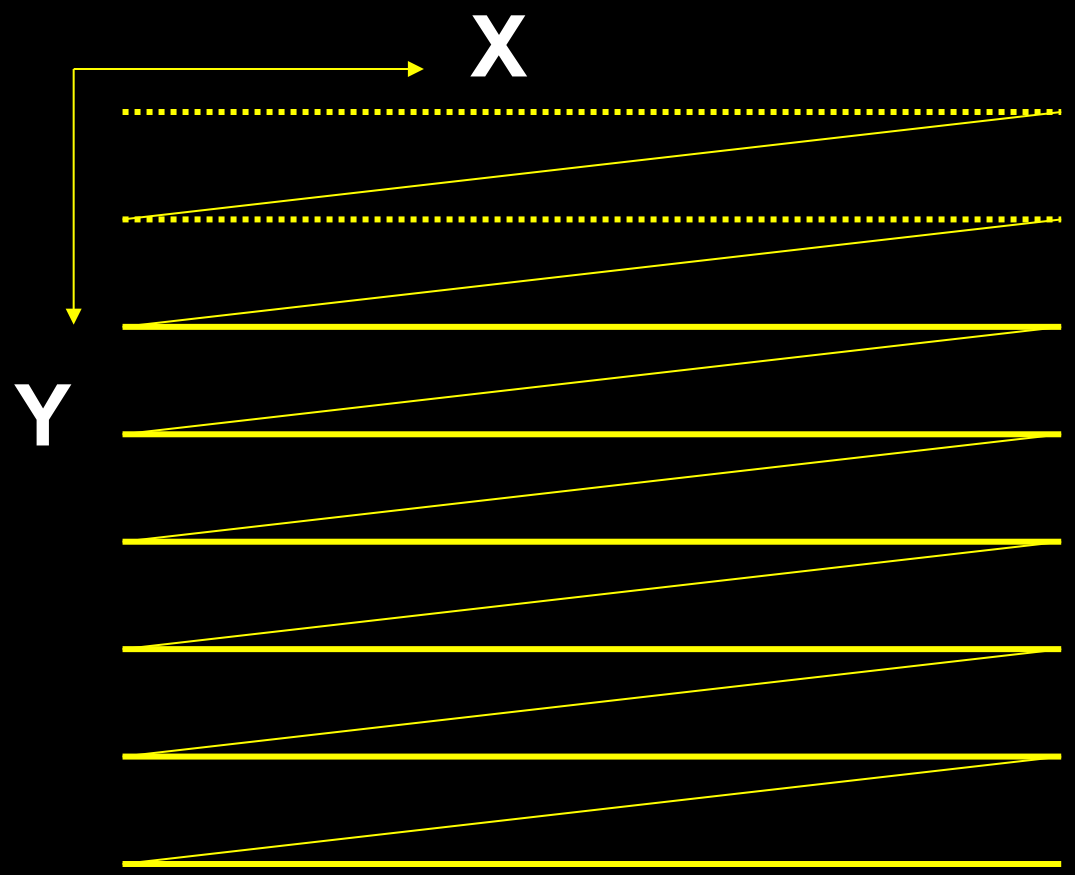
Confocal principle

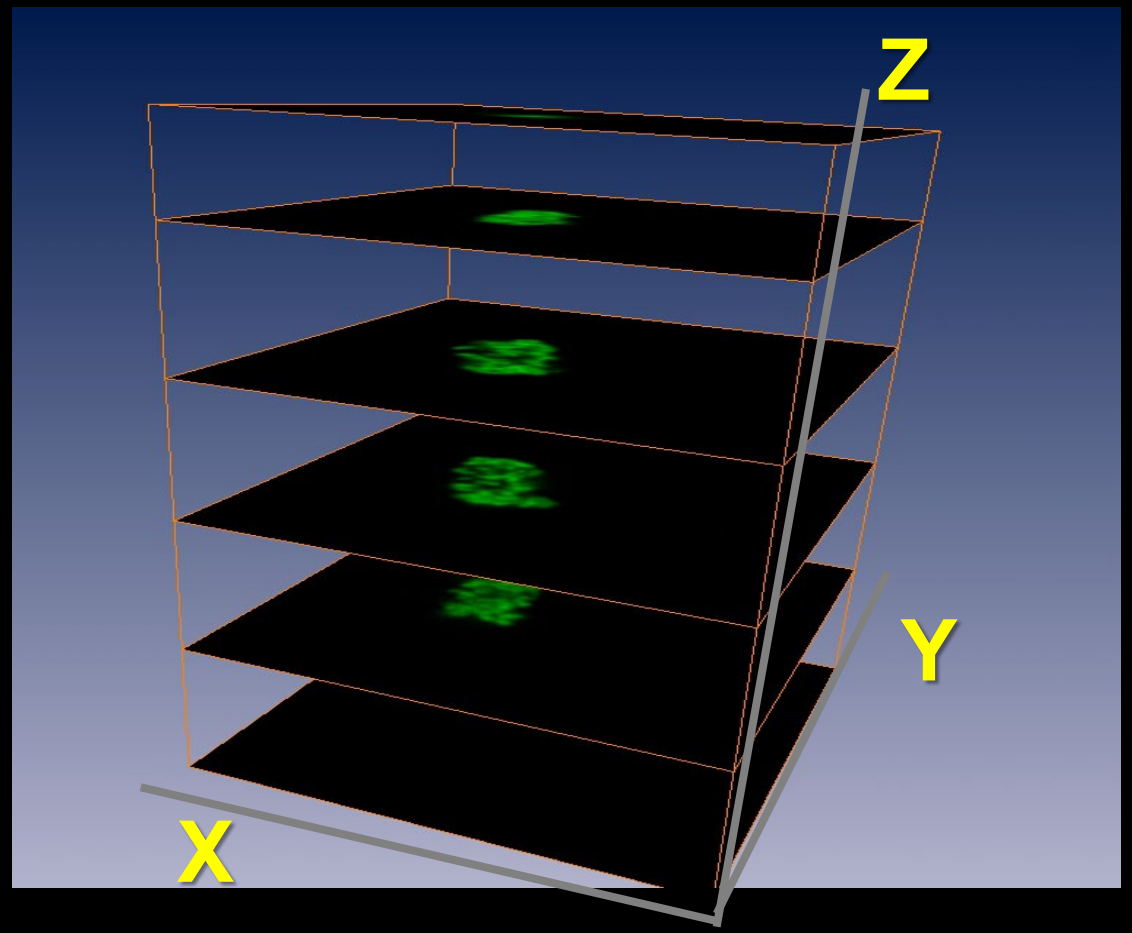


Principle of laser scanning

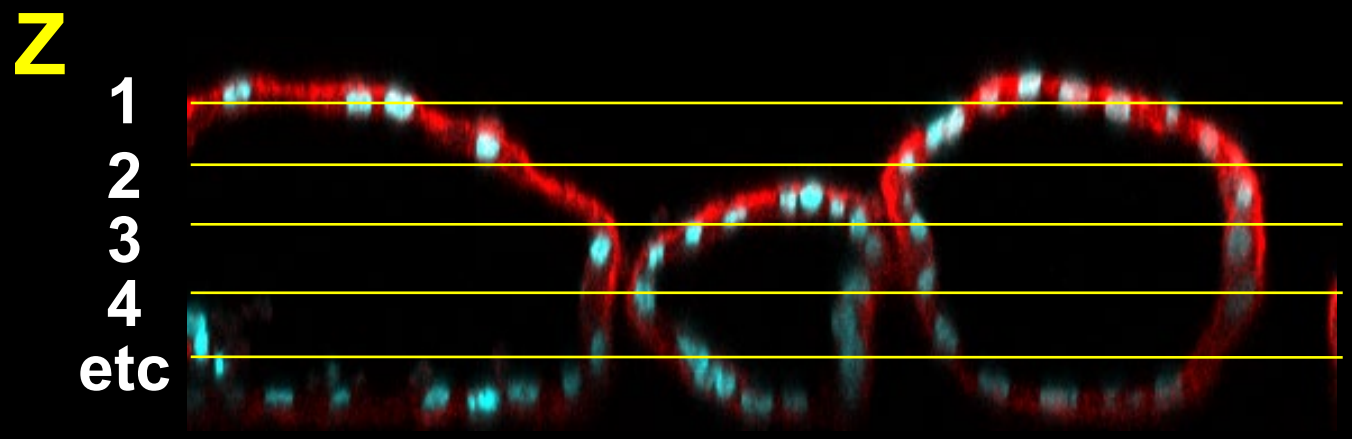


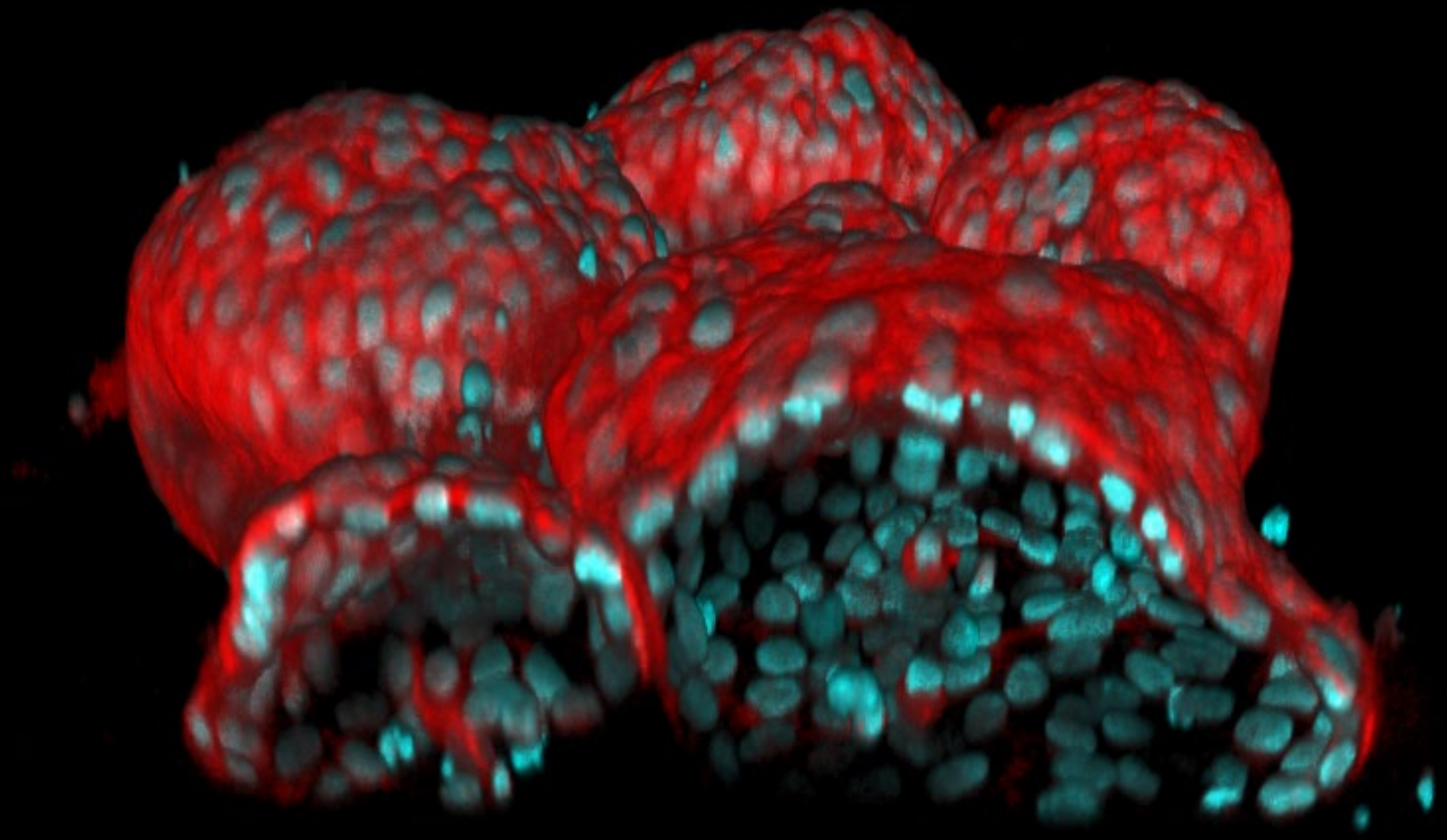
XY and Z laser scanning





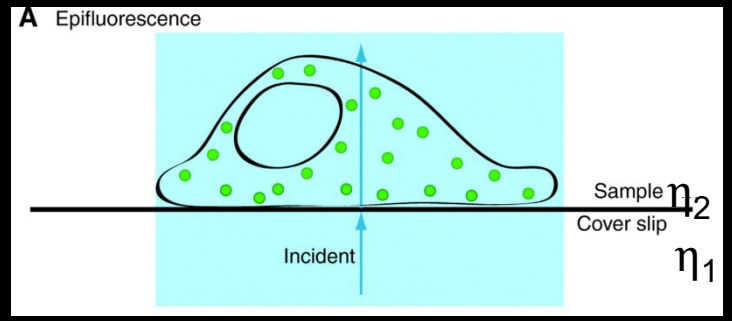
3D scanning



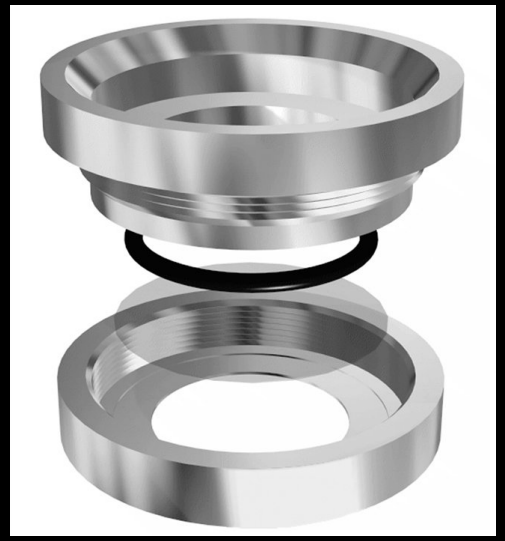


Timelapse imaging

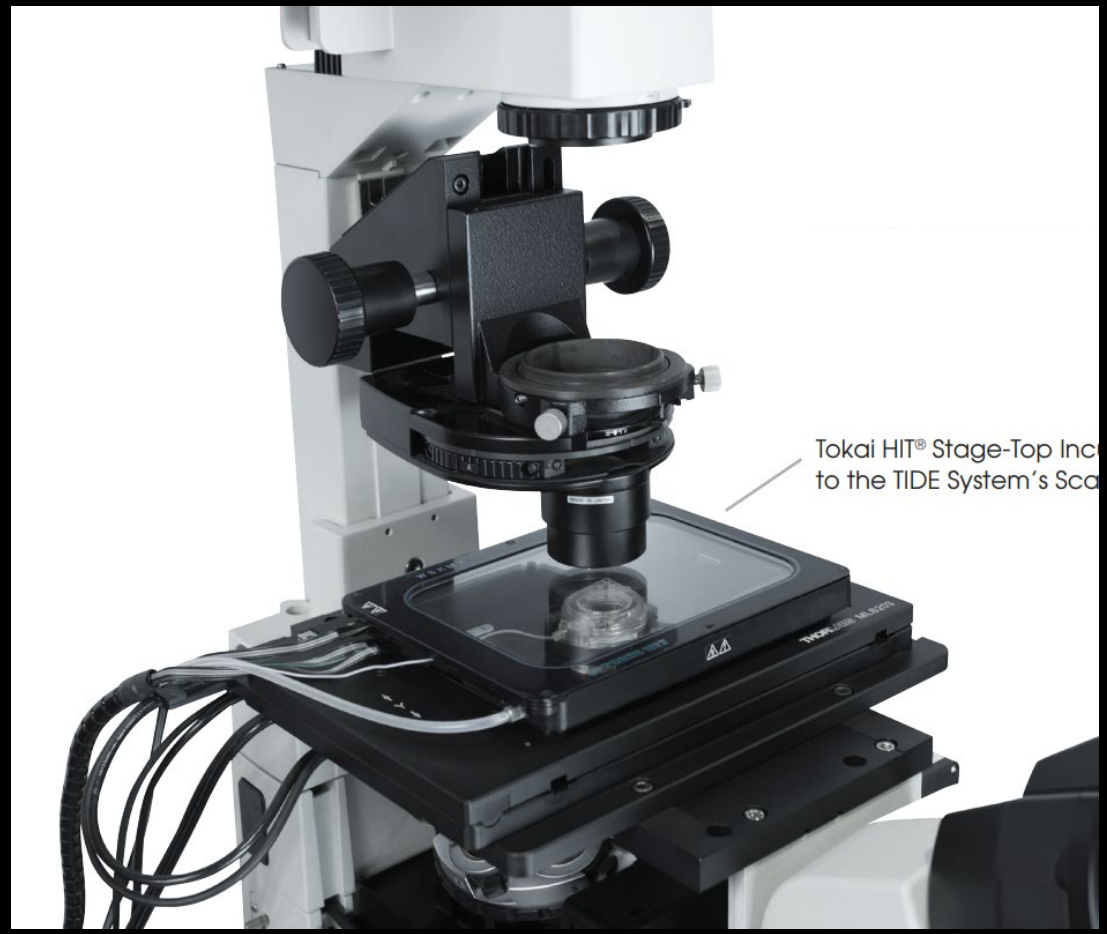
37°C / 5% CO2
High humidity





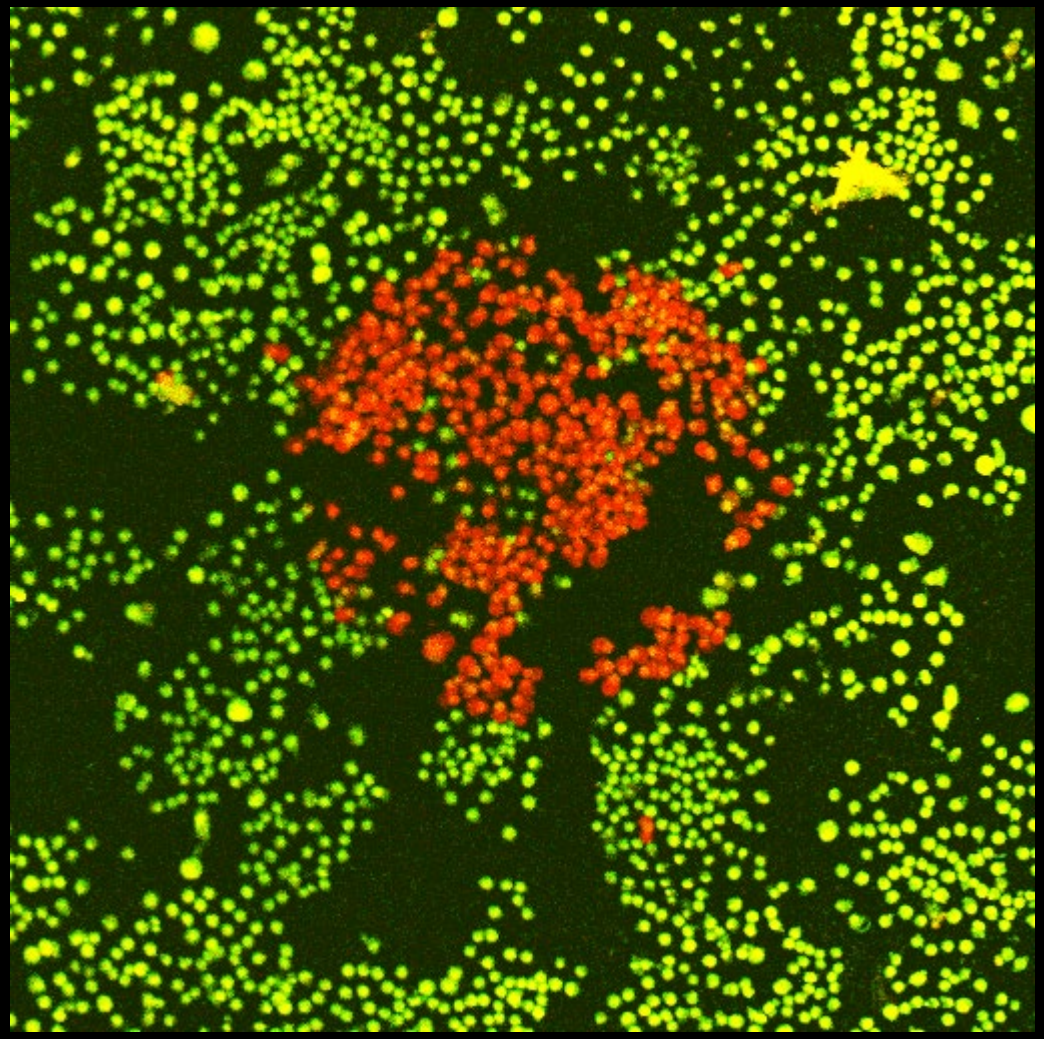


https://www.thermofisher.com



https://www.thorlabs.de/images/Brochures/Thorlabs_TIDE_Brochure.pdf

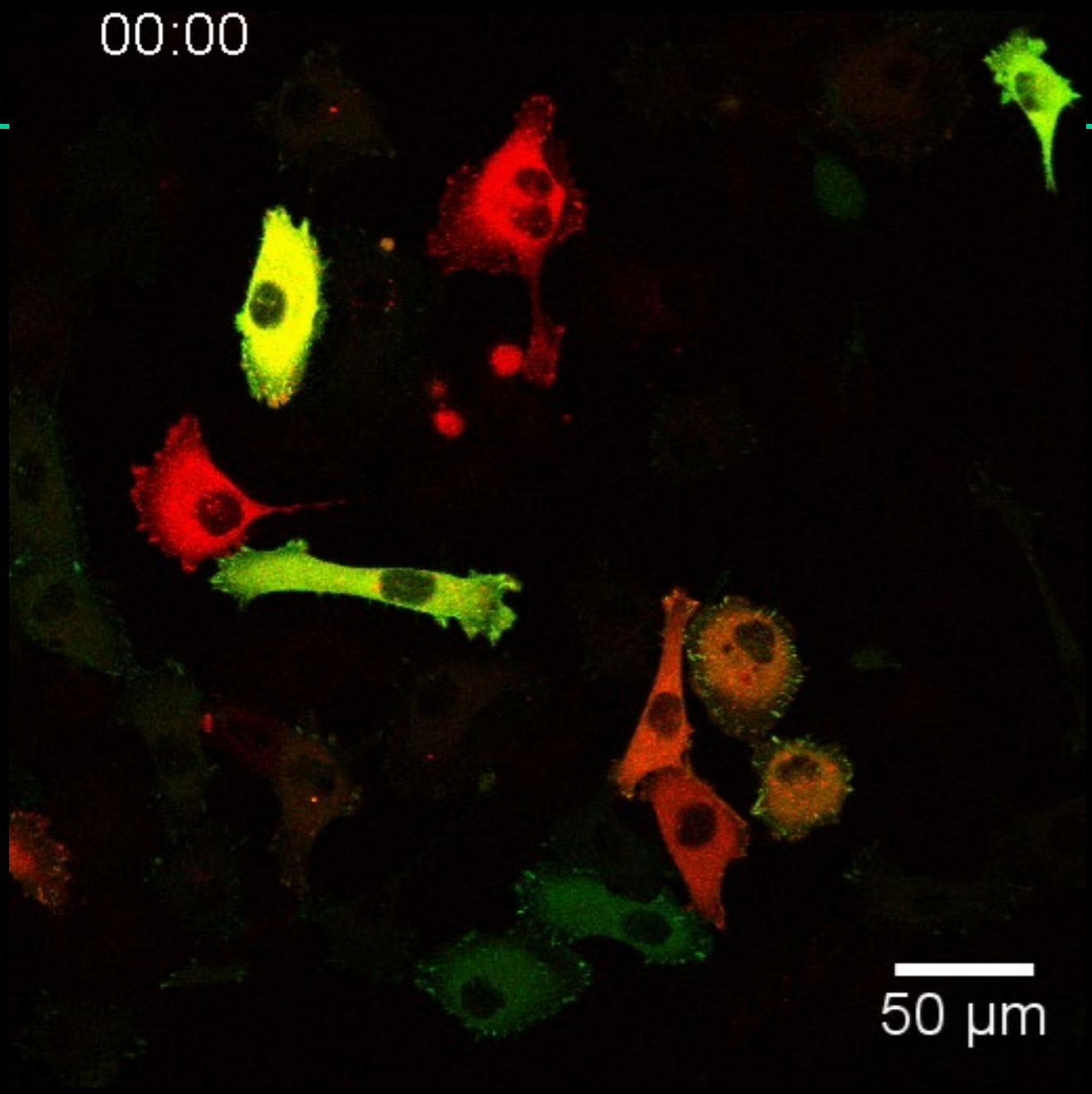
First attempt (1996) to follow cells in time



10 images, 30 min between images

Green= living cells (Syto16)
Red = dead cells (PI)

Gert van Cappellen



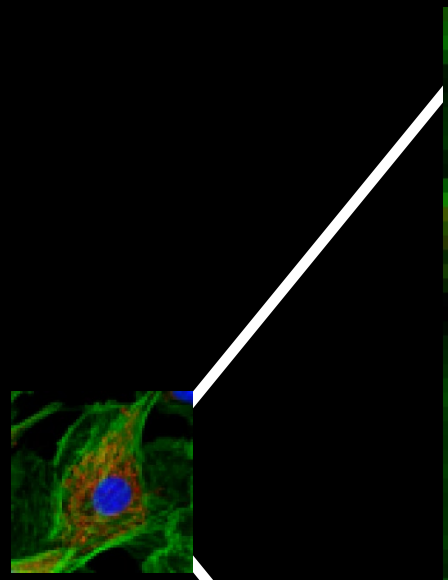
MDCK cells
GFP-Paxillin
mRFP-Zyxin

2 color z-stack
Every 5 min
Duration 18 hrs

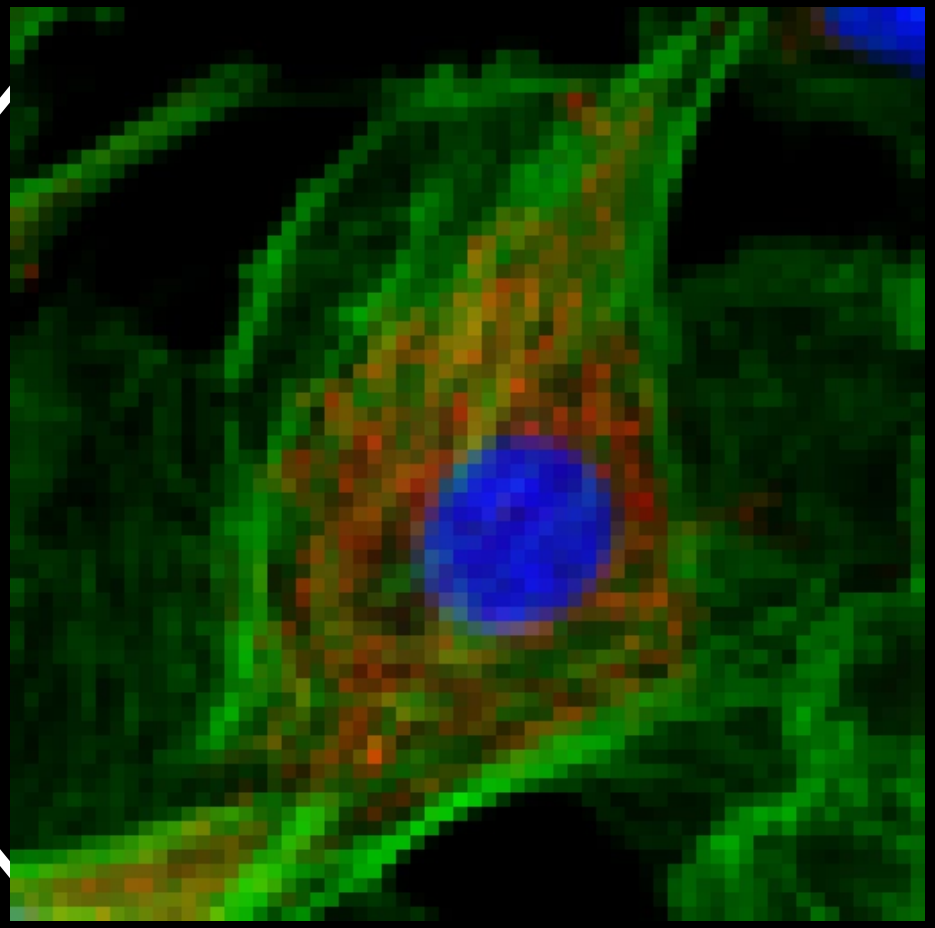
Introduction

Confocal principle
Time lapse imaging
Sampling rate
Excitation
(Spectral) Detection

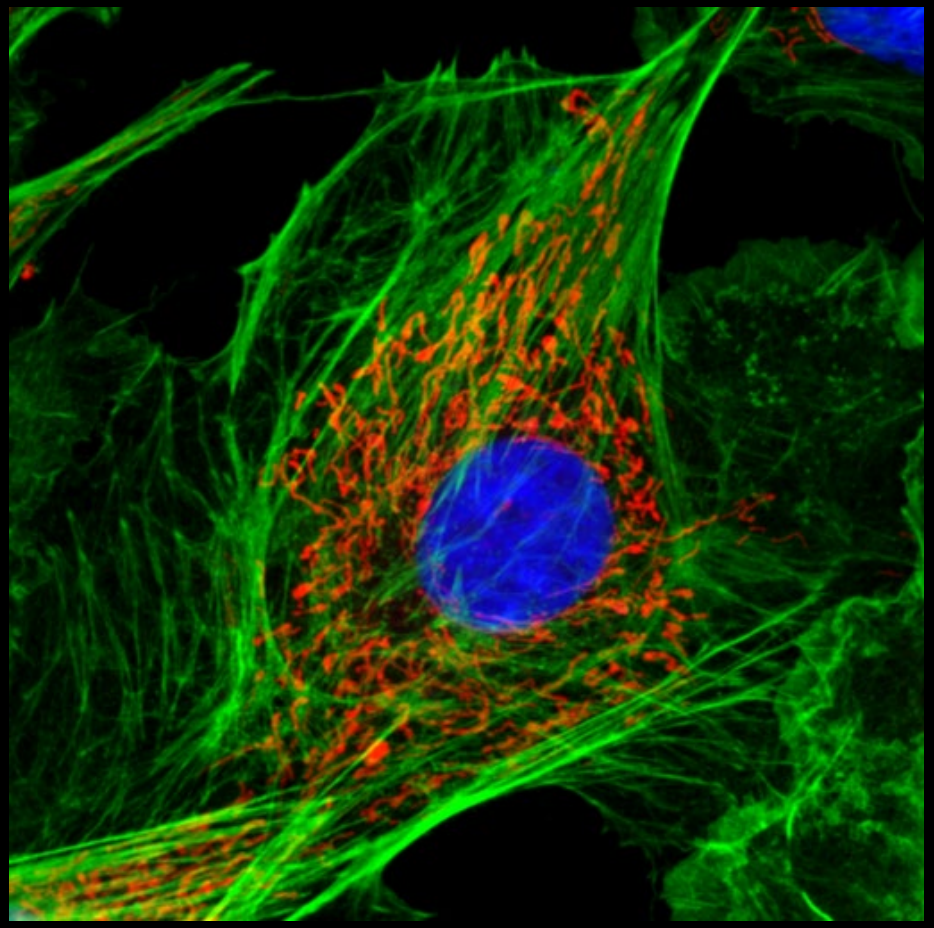
Resolution and sampling rate (pixel size)



64x64 pixels

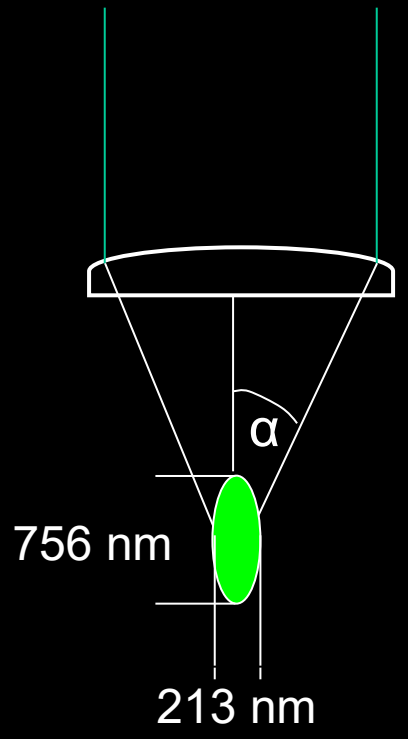


64x64 pixels



512x512 pixels

Lateral (XY) and Axial (Z) resolution



numerical aperture = refractive index * sin(α)

Lateral (XY) resolution:

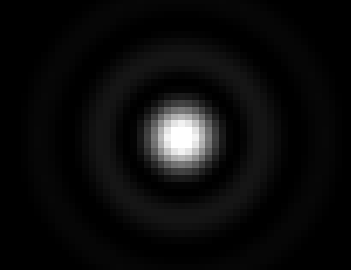
XY_{min}= 0.61*wavelength/numerical aperture

XY_{min}= 0.61*488/1.4 = 213 nm

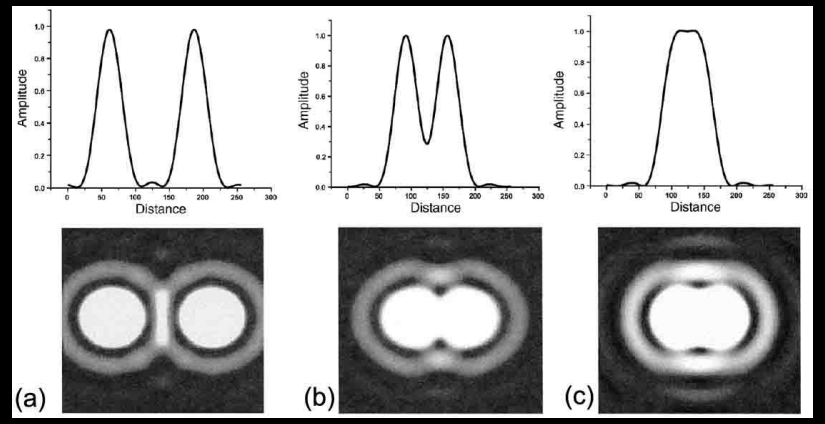
Axial (Z) resolution:

Z_{min}=2*wavelength*refractive index/$(NA)^2$

Z_{min}=2*488*1.518/$(1.4)^2$=756 nm



PS Airy disk



Rayleigh criterion

Sampling rate and resolution

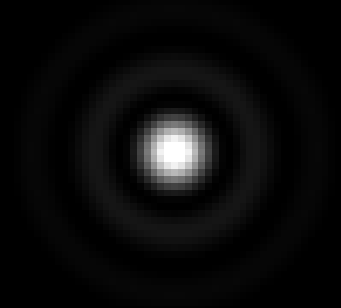
Image resolution = pixel size **vs** Optical resolution = r

- Nr of pixels
- Field of view
  - Magnification
  - Zoomfactor

$$Pixel\ size = \frac{FOV}{Mag * Zoom * Nr\ of\ pixels}$$

- Numerical aperture of objective
- Wavelength of emission
- (Pinhole size)

$$r = 0.61 * \frac{\lambda}{NA}$$



For optimal results you need to match the image resolution and the optical resolution

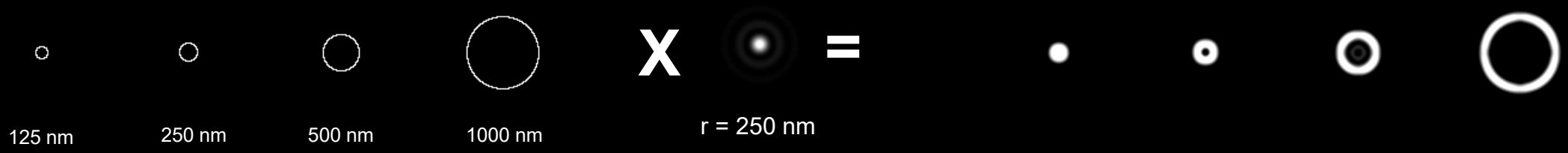
= Nyquist theorem

Sampling rate and resolution

Sample

PSF

Scanned image



Pixel size = 250 nm

Undersampling



Pixel size = 25 nm

Oversampling

Too much time

Too much bleaching

Too much data

Pixel size = 100 nm

Nyquist sampling



Excitation

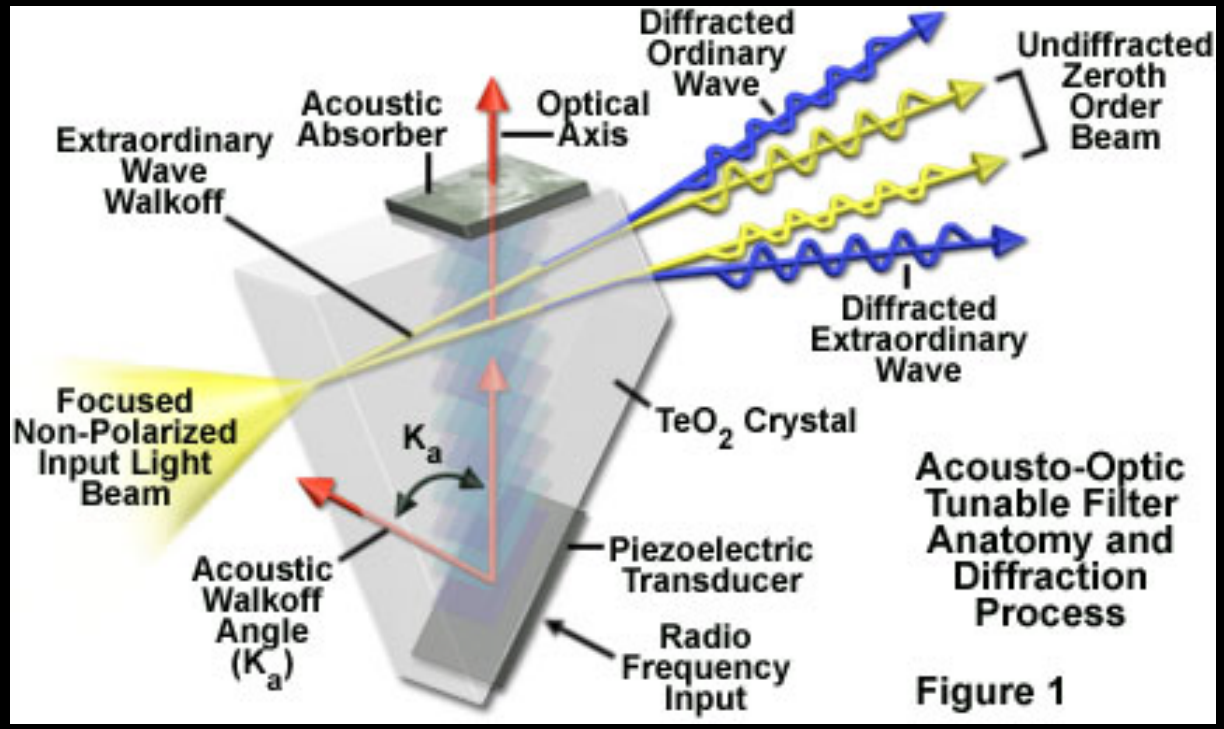


Excitation with lasers

Wavelengths:

405 – Hoechst, Dapi
458 – CFP
488 – GFP, Alexa488, Cy2
514 – YFP
(543 – dsRed, Cy3, Alexa555)
561 – mCherry, dsRed, Alexa555, Alexa594
594 – mCherry, Alexa594
640 – Cy5, Alexa647

Excitation Acoustic Optical Tunable Filter (AOTF)



Control laser power, very fast (ns)

http://www.micro.magnet.fsu.edu/primer/java/filters/aotf/index.html

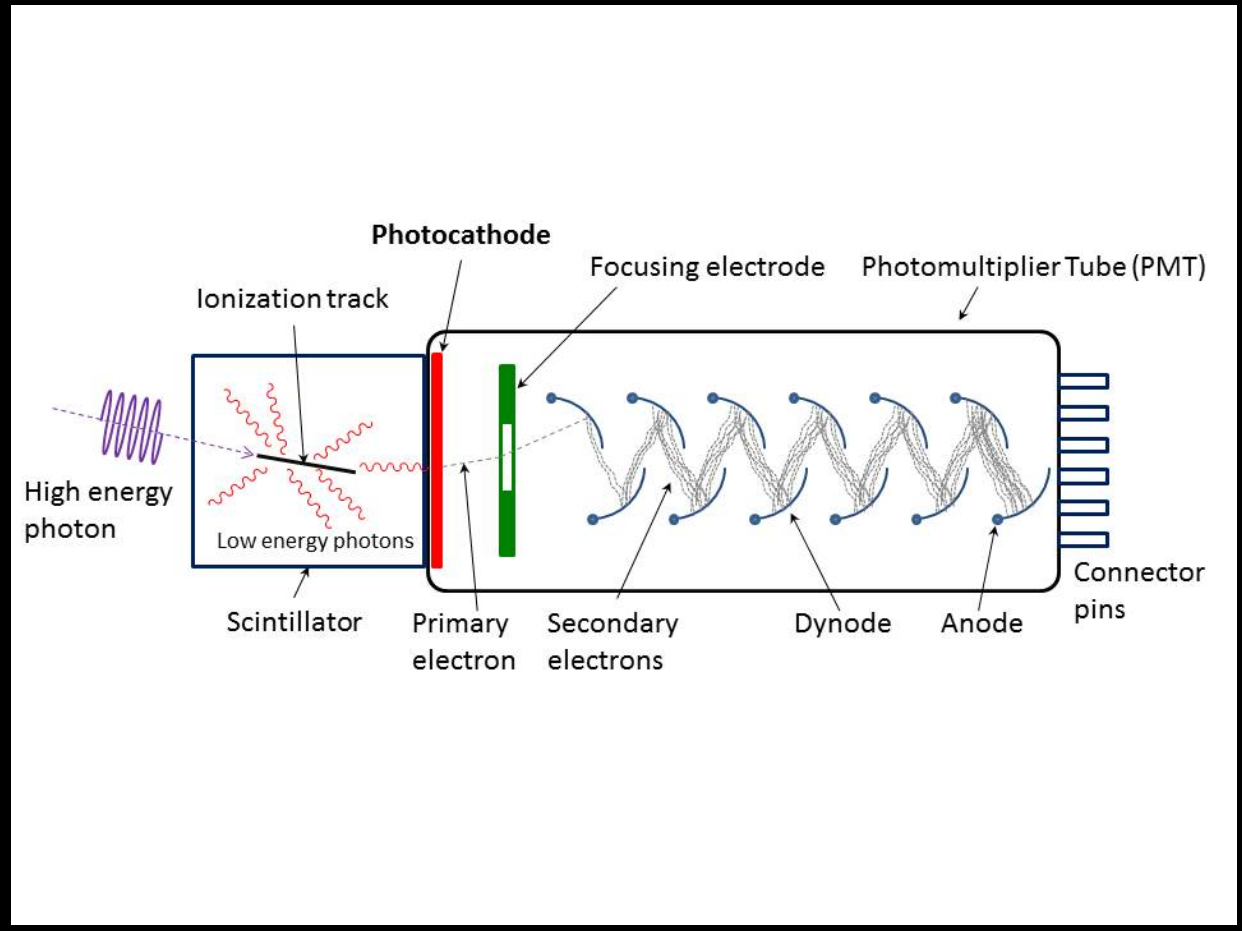
Detection







Detection photomultiplier tube (PMT)



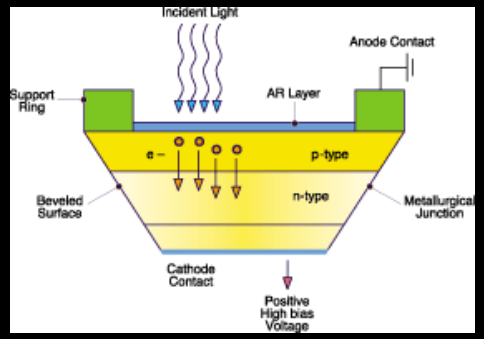
Gain increase voltage over detector

http://en.wikipedia.org/wiki/Photomultiplier#mediaviewer/File:PhotoMultiplierTubeAndScintillator.jpg

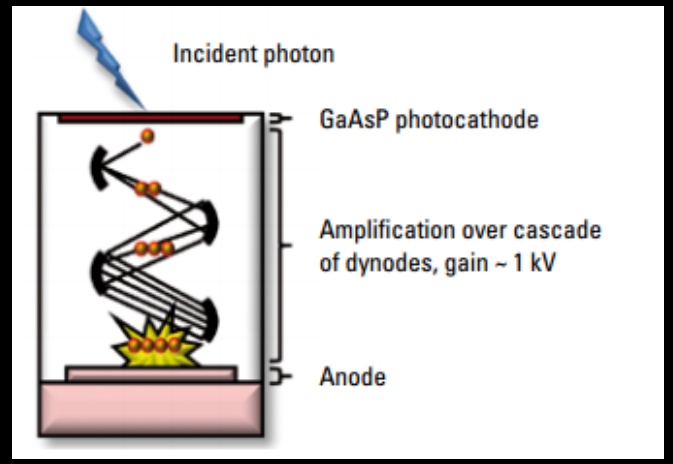
Detectors APD, hybrid, camera

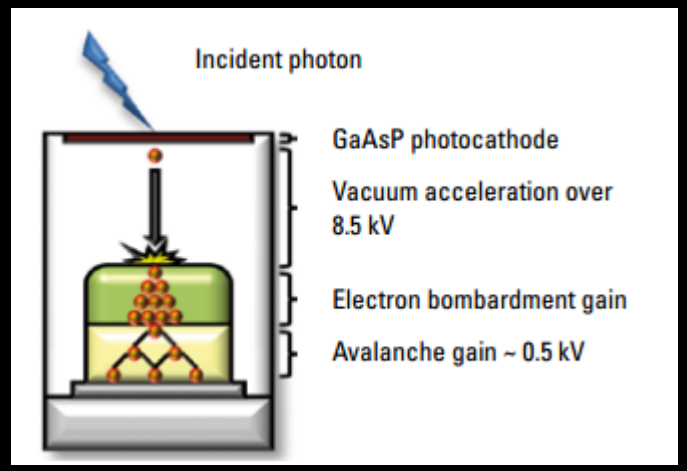
Hybrid detectors

Charge Couples Device (CCD)



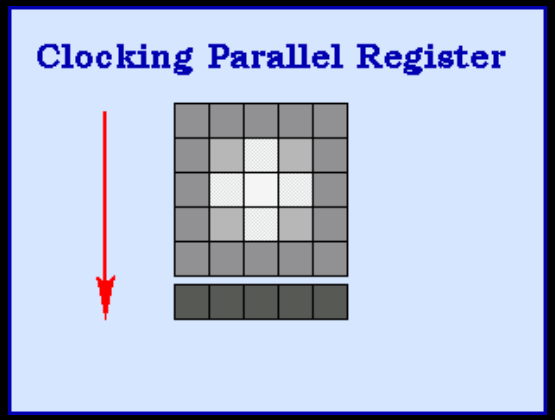
Avalanche Photo Detector (APD)

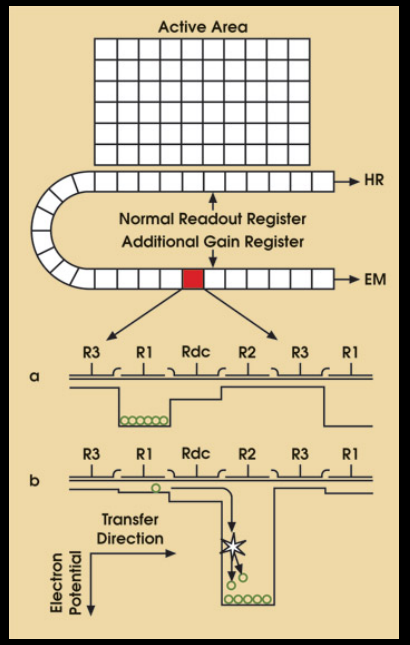




GaAsP layer







Difference between detectors

PMT - very fast/slow, high dynamic range,
low noise, less sensitive

APD - most sensitive, low dynamic range

Hybrid - very sensitive, better dynamic range

CCD - very sensitive, whole image, fast, noisy

sCMOS – sensitive, whole image, very fast, noisy

Spectral detection

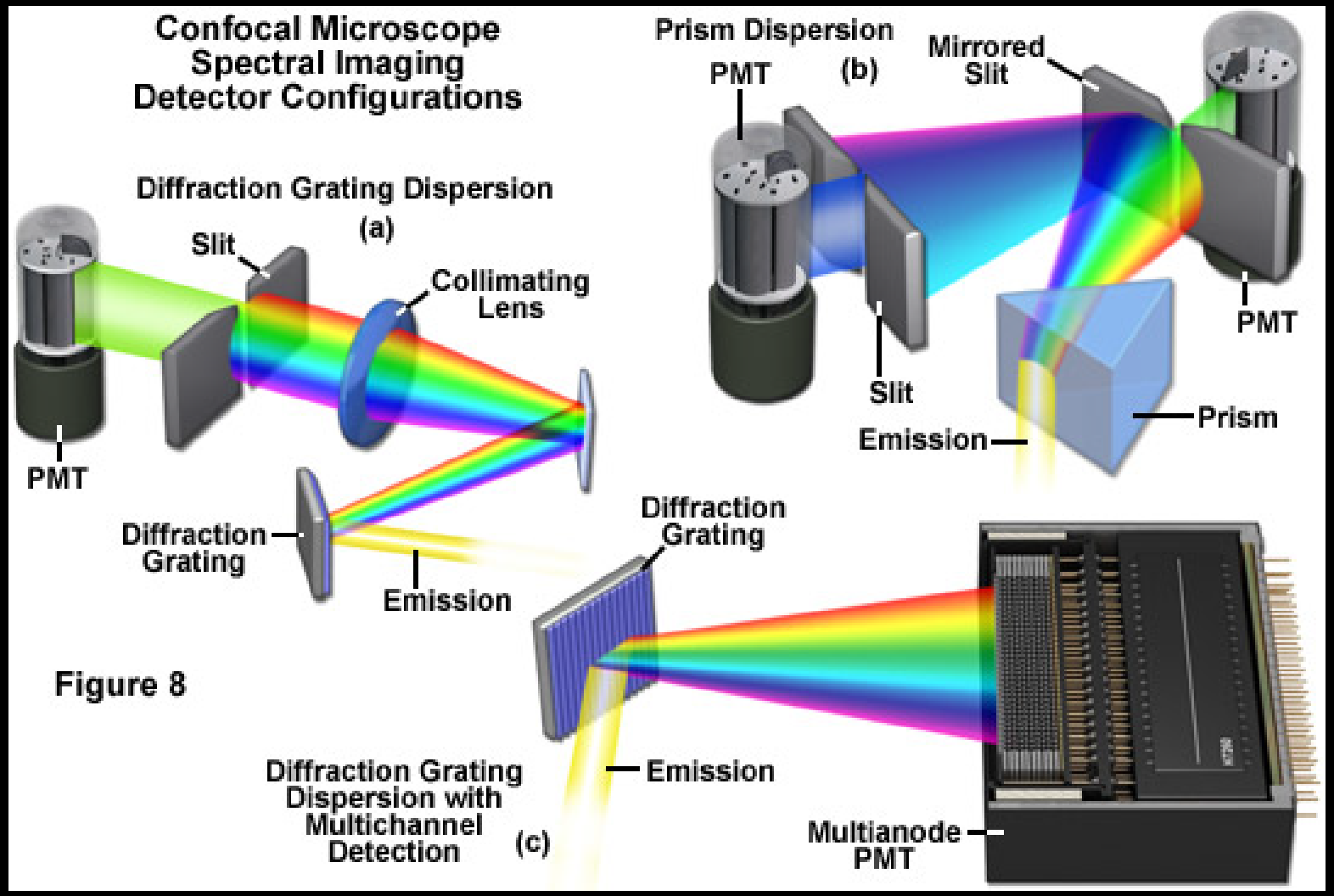
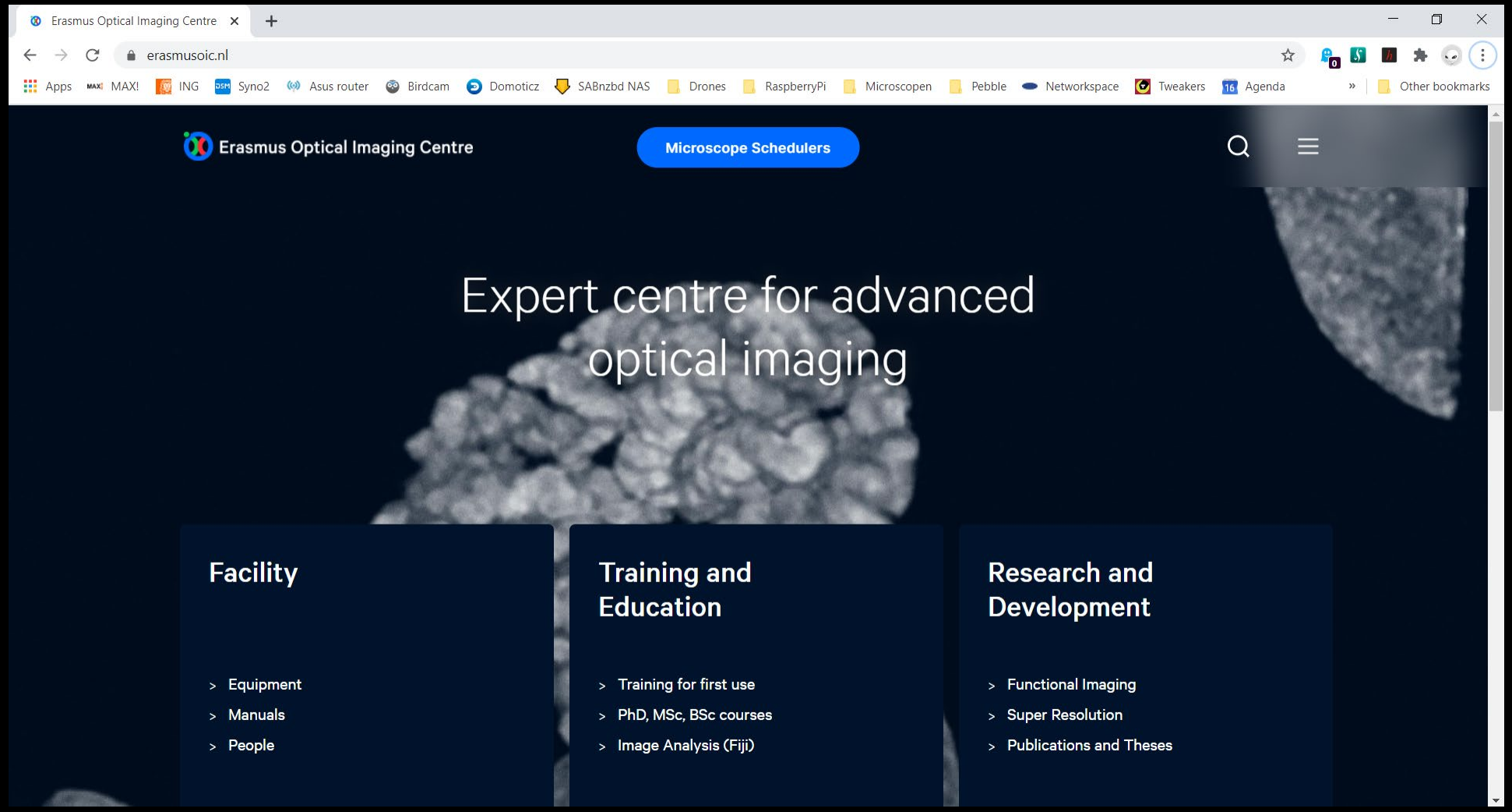


Figure 8

http://www.microscopyu.com/articles/confocal/spectralimaging.html

Erasmus Optical Imaging Centre (OIC)

erasmusoic.nl



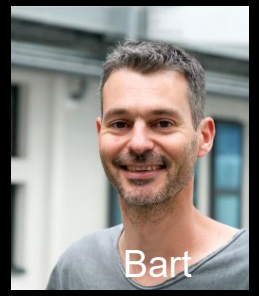
The “OIC” team


Tsion


Martijn


Alex

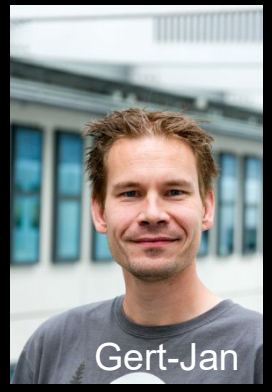

Marla


Bart


Ilse


Johan


Jeffrey


Gert-Jan


Adriaan


Gert

Erasmus Optical Imaging Centre (OIC)



erasmusoic.nl

www.erasmusmc.nl/oic

Expert centre for advanced imaging