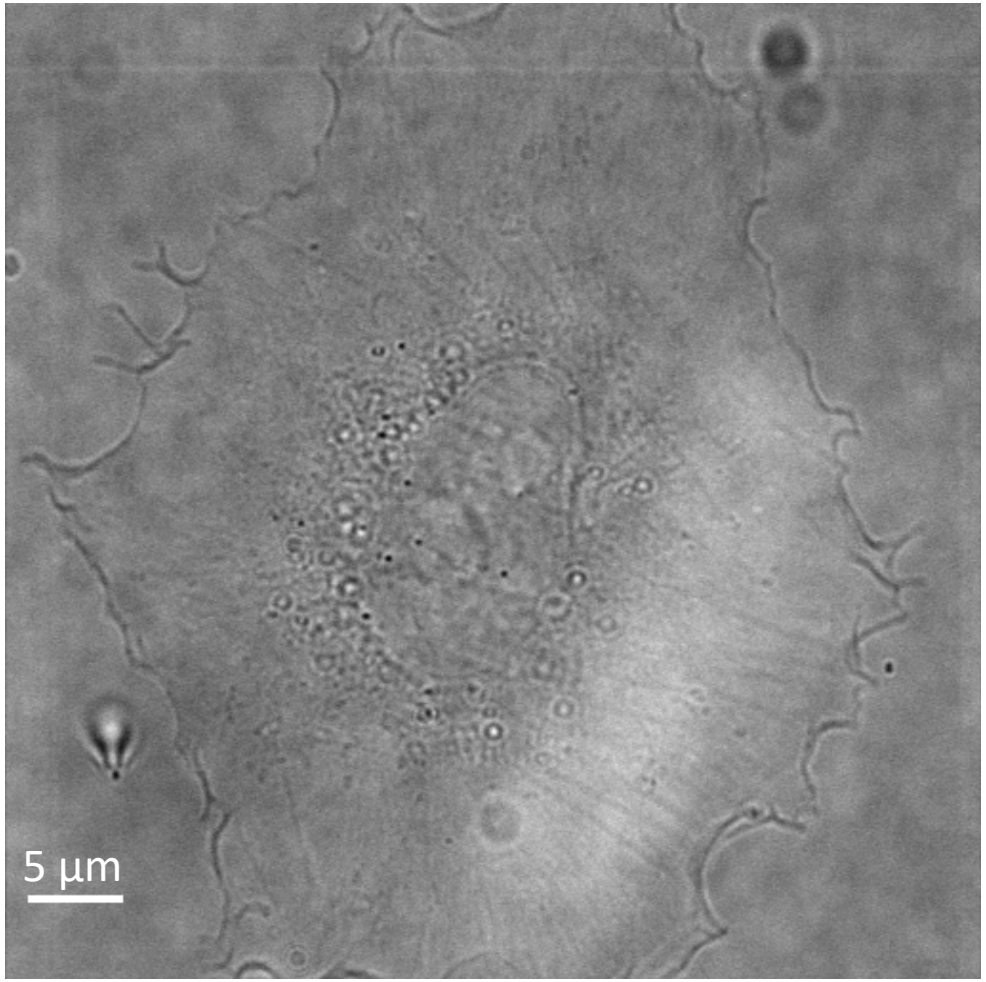
# Técnicas fotônicas de diagnóstico – microscopia de fluorescência

# Bright Field (BF) and Electron Microscopy (EM)

A Köhler, Zeitschrift für wissenschaftliche Mikroskopie, 1893

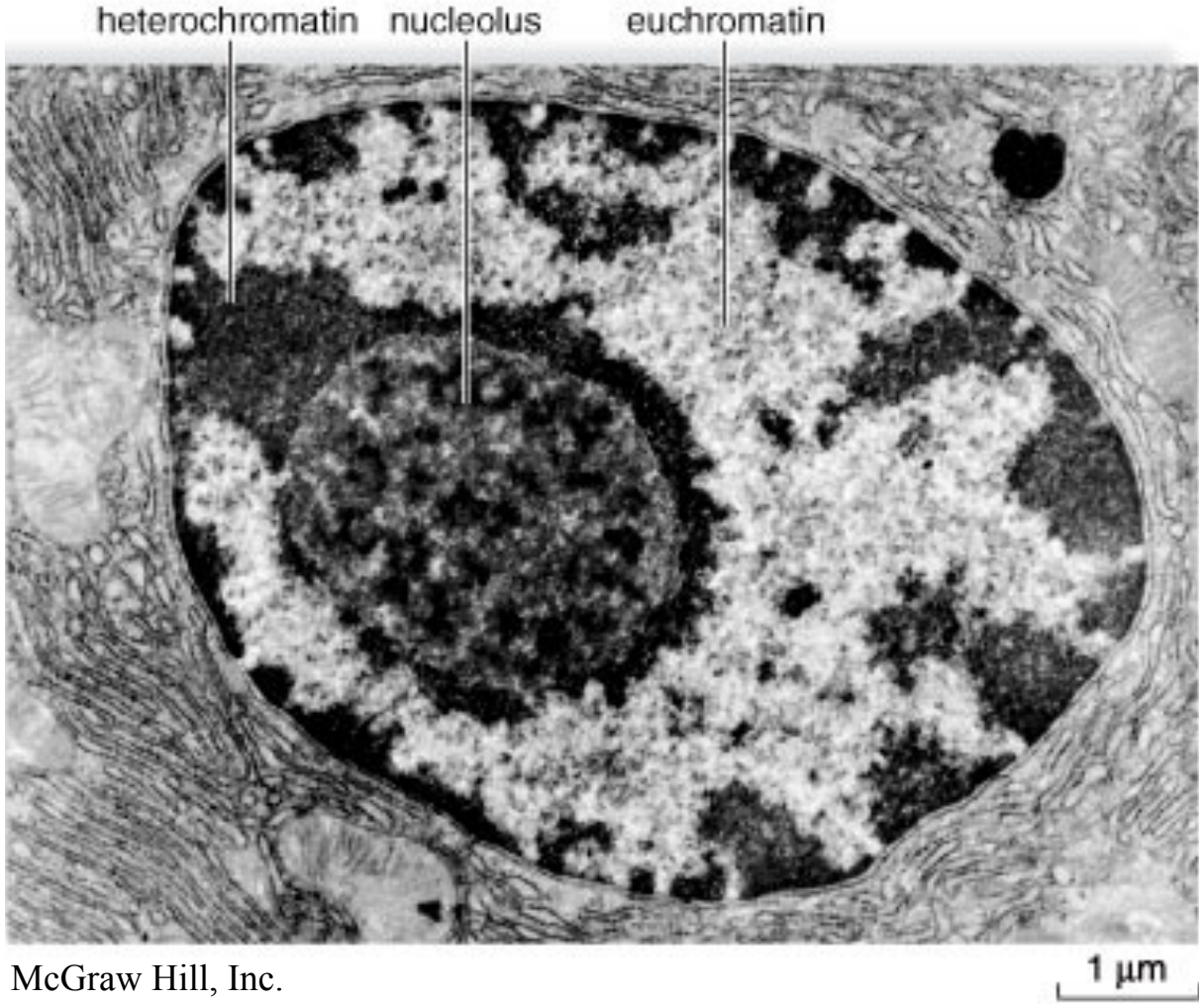



- Non invasive
- Live cells

- Lack of contrast
- Lack of specificity
- Low resolution

**(~1900)**

M Knoll, E Ruska, Zeitschrift für Physik, 1932




McGraw Hill, Inc.

- Resolution (~1 nm)
- Contrast

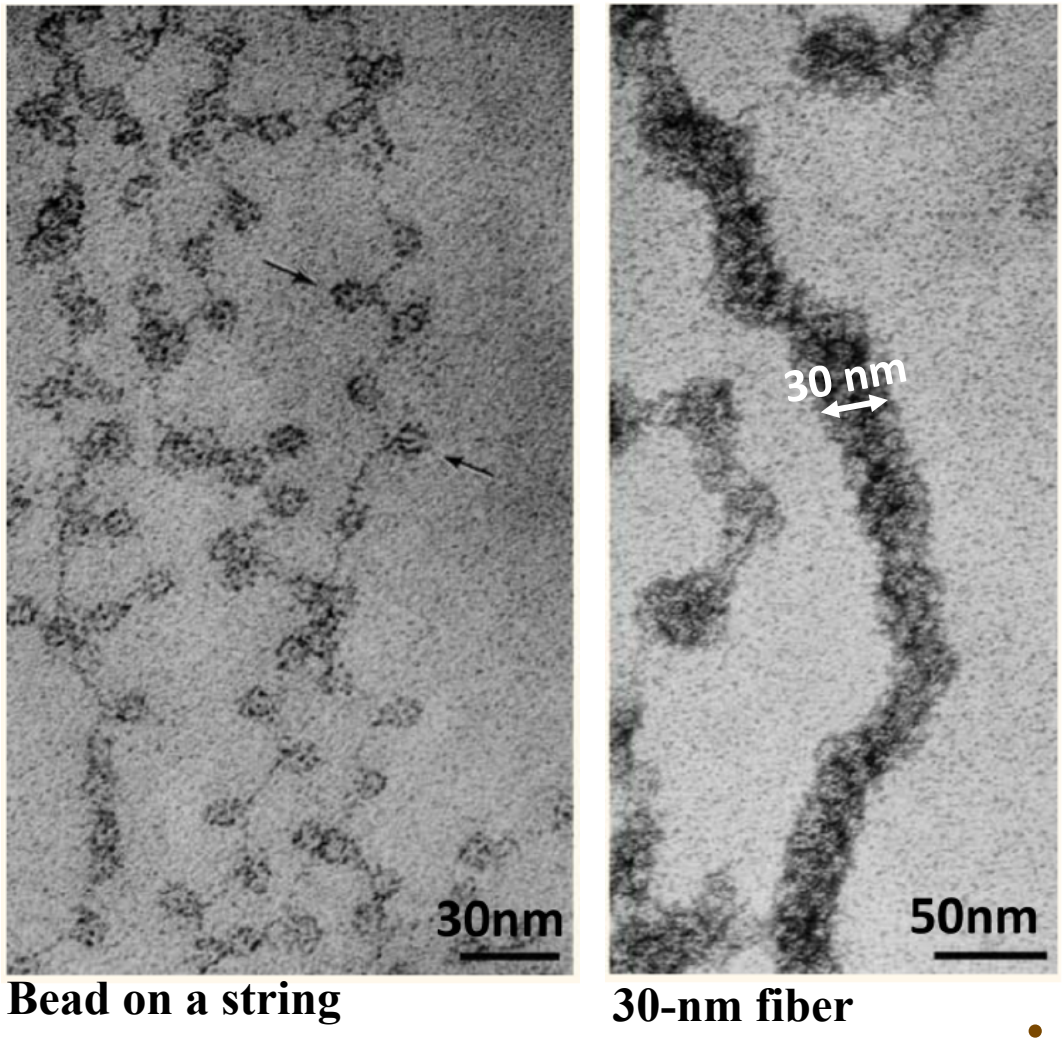
- Lack of specificity
- Fixed samples

**(~1940)**

# Scanning Electron Microscopy (SEM)

D Attack and KCA Smith, Pulp Paper Canada, 1956

## SEM images of chromatin in vitro




**Bead on a string** **30-nm fiber**

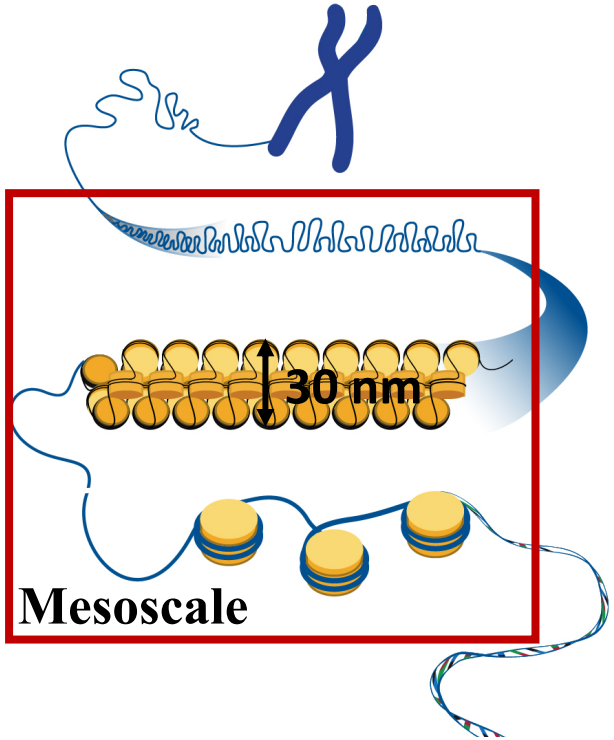
Olins et al., Nature Reviews Molecular Cell Biology, 2003

**(~1980)**

P Oudet et al., Cell, 1975

Finch et al., PNAS, 1976

Woodcock et al., J. Cell Biol., 1984




- **Resolution (~1 nm)**
- **Contrast**
- **Lack of specificity**
- **Fixed samples**

# Fluorescence Microscopy

JFW Herschel, Philos Trans R Soc London, 1845

GG Stokes, Philos Trans R Soc London, 1852

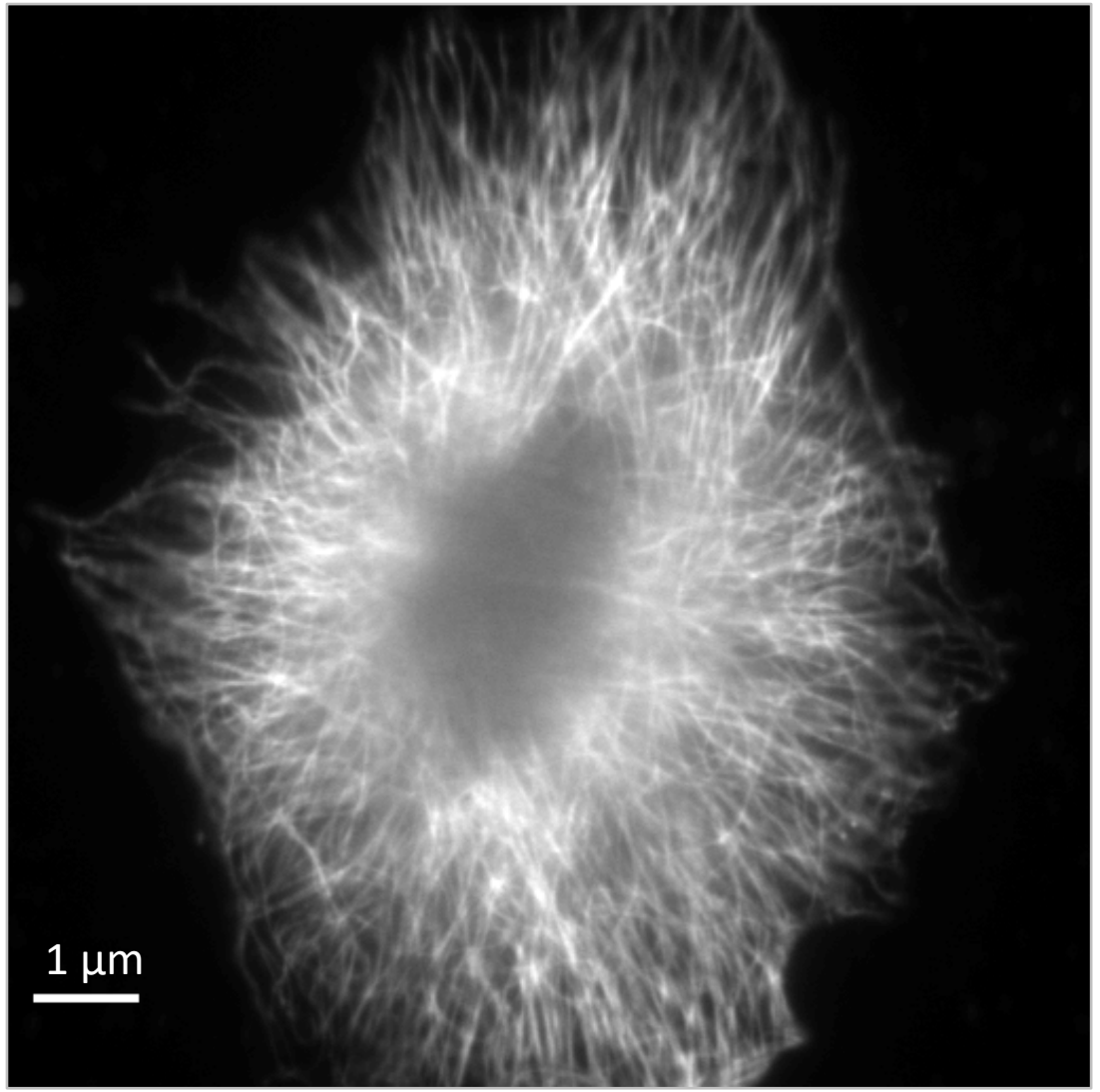
OD Heimstadt, Z wiss Mikr, 1911

AH Coons et al., J Immunol, 1942

DC Prasher et al., Gen, 1992.

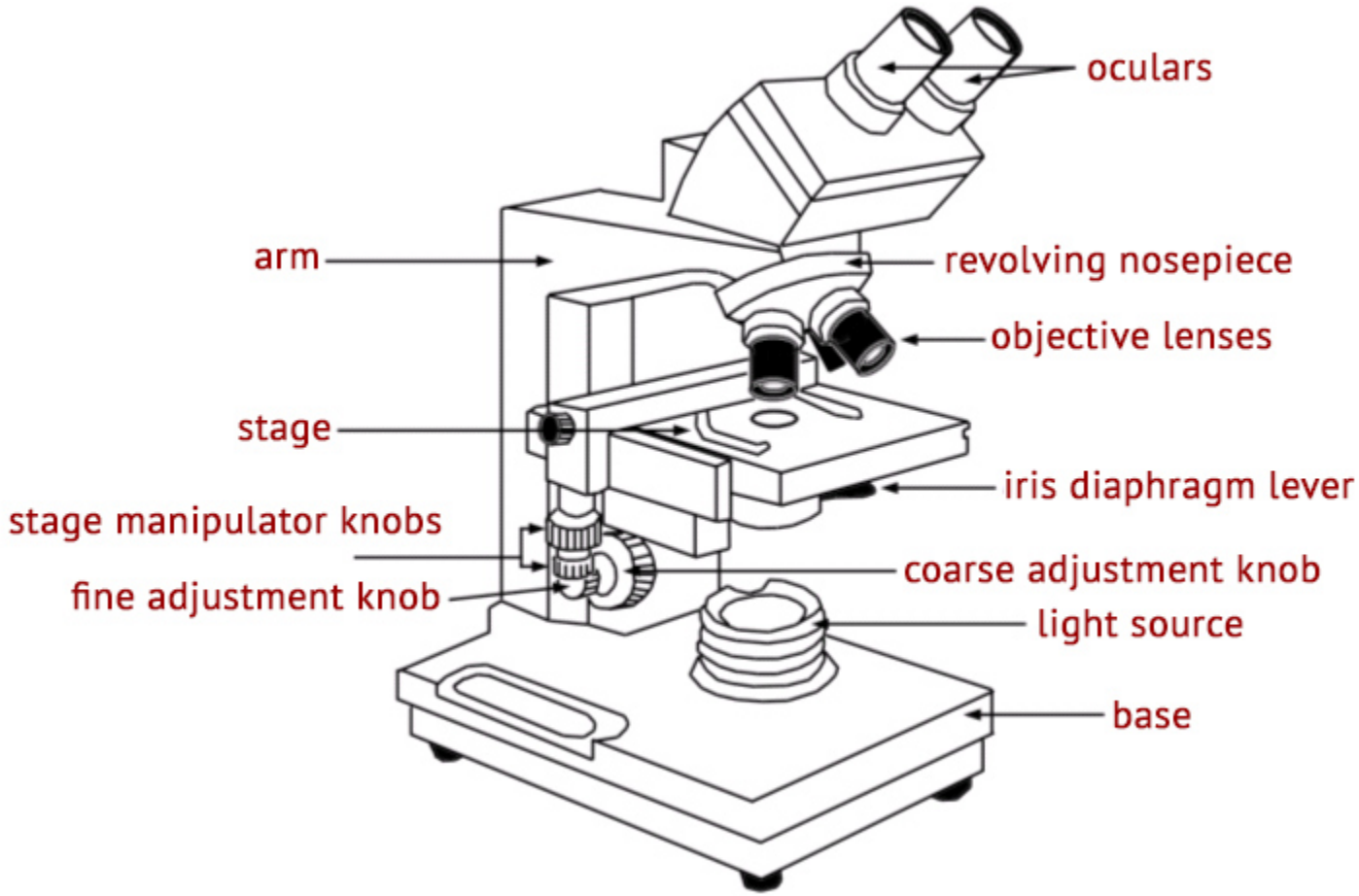
**(~1950)**

**Microtubules**



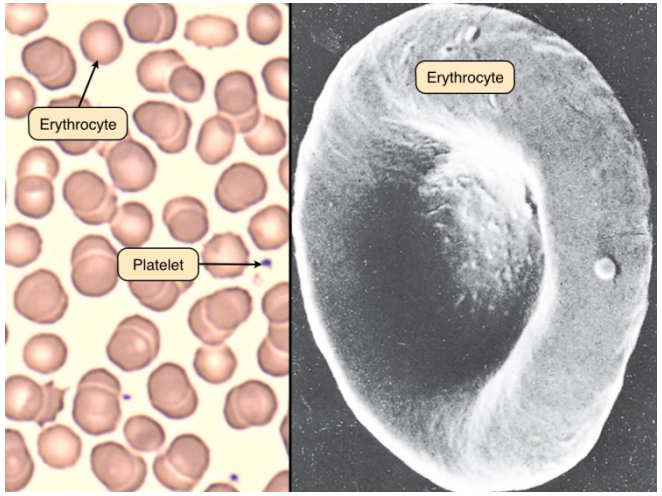

- **Specificity**
- **Contrast**

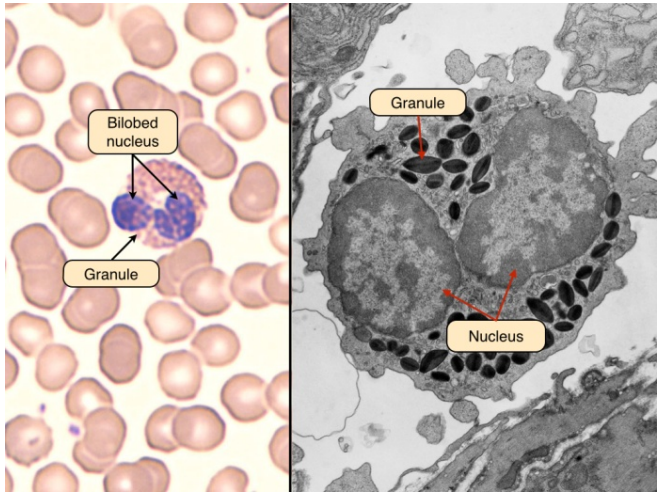
- **Low spatial resolution (~250 nm)**

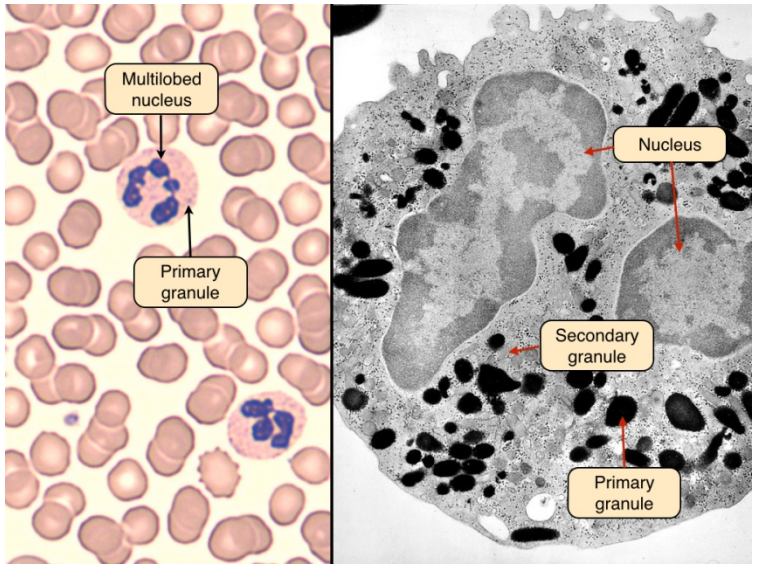



https://www.philpoteducation.com/mod/book/view.php?id=779&chapterid=1031#/

# Técnicas de microscopia - resolução




http://medcell.med.yale.edu/systems_cell_biology/blood_lab.php

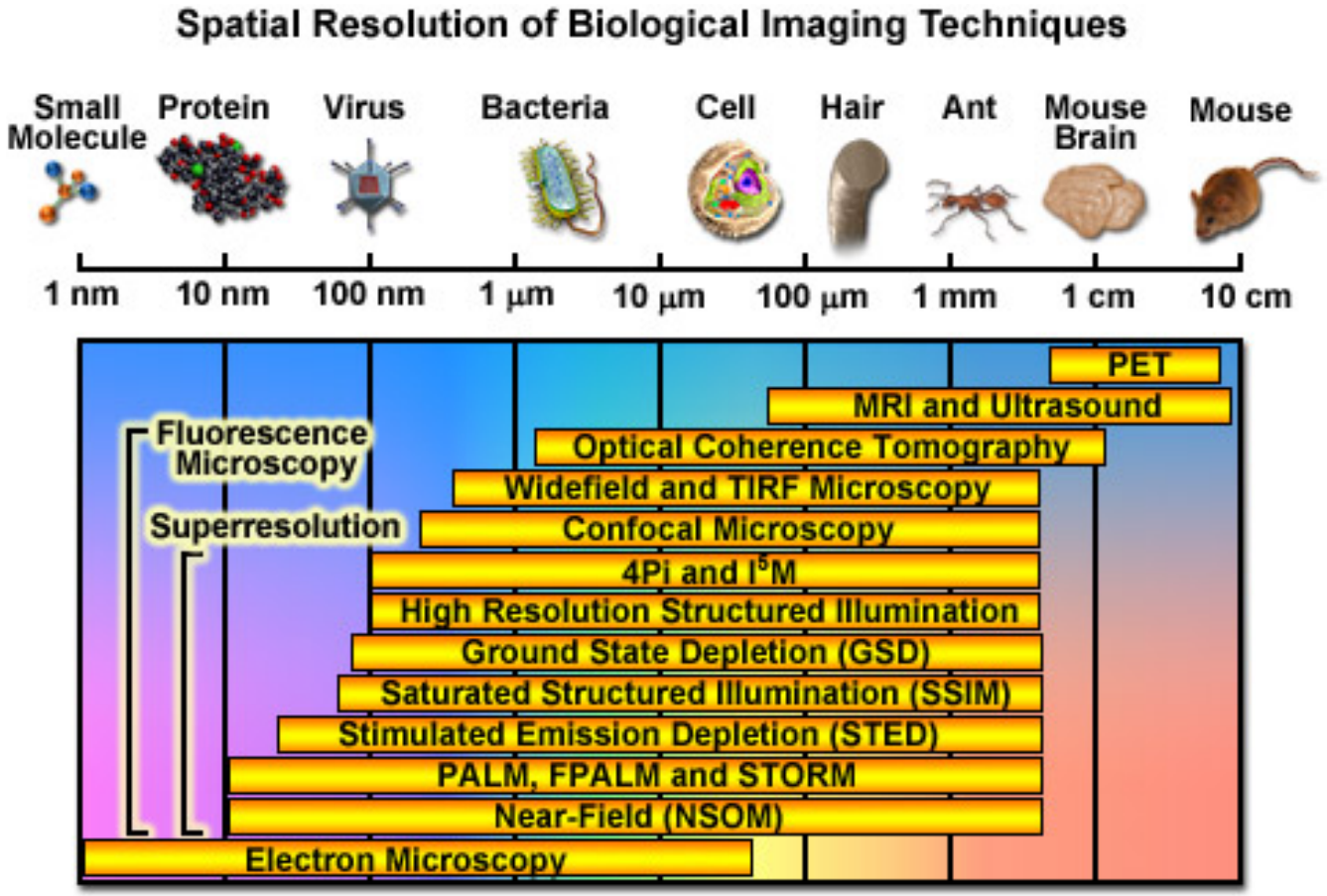
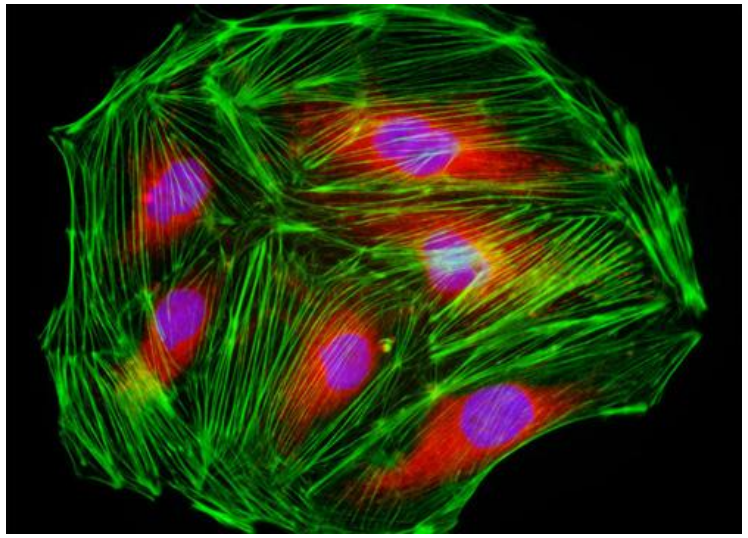
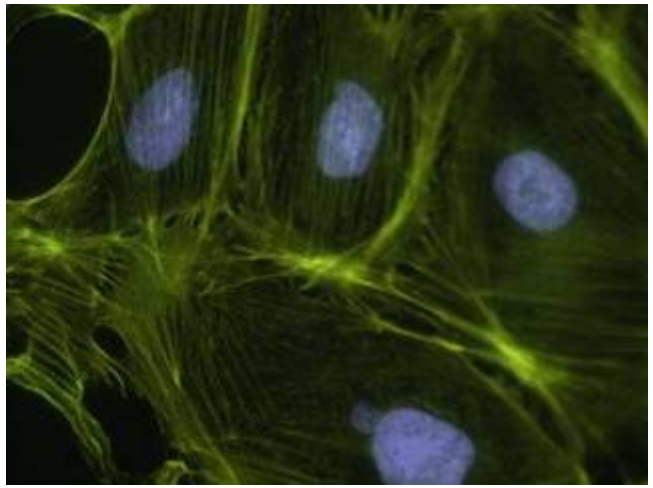


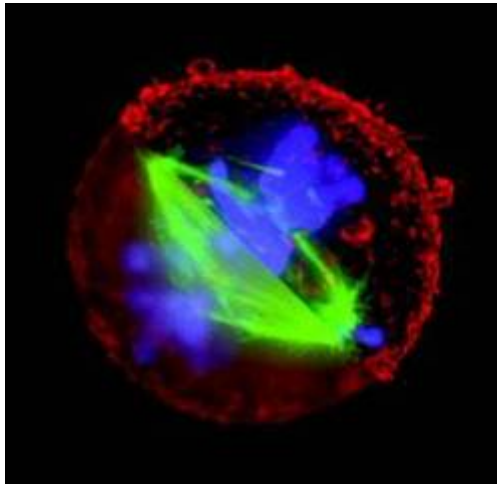

Figure 1

http://zeiss-campus.magnet.fsu.edu/print/superresolution/introduction-print.html

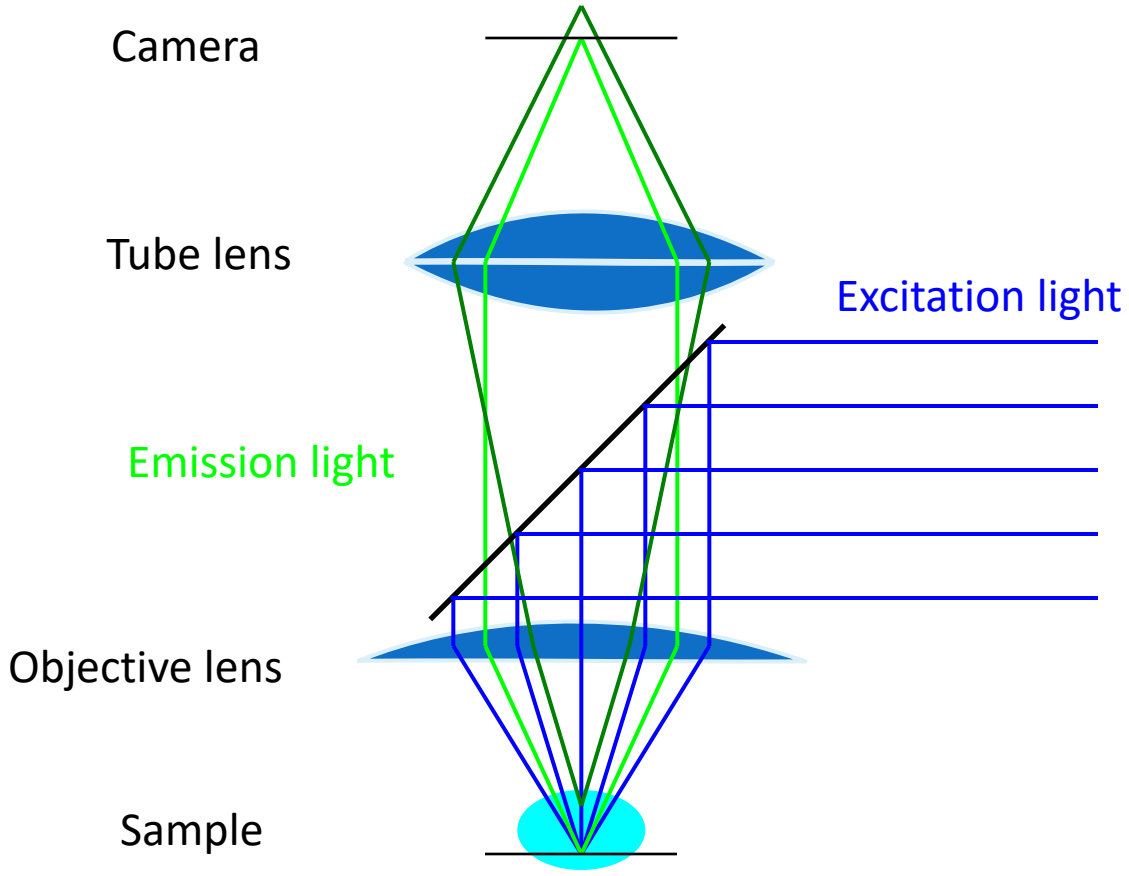
# Microscopia de fluorescência





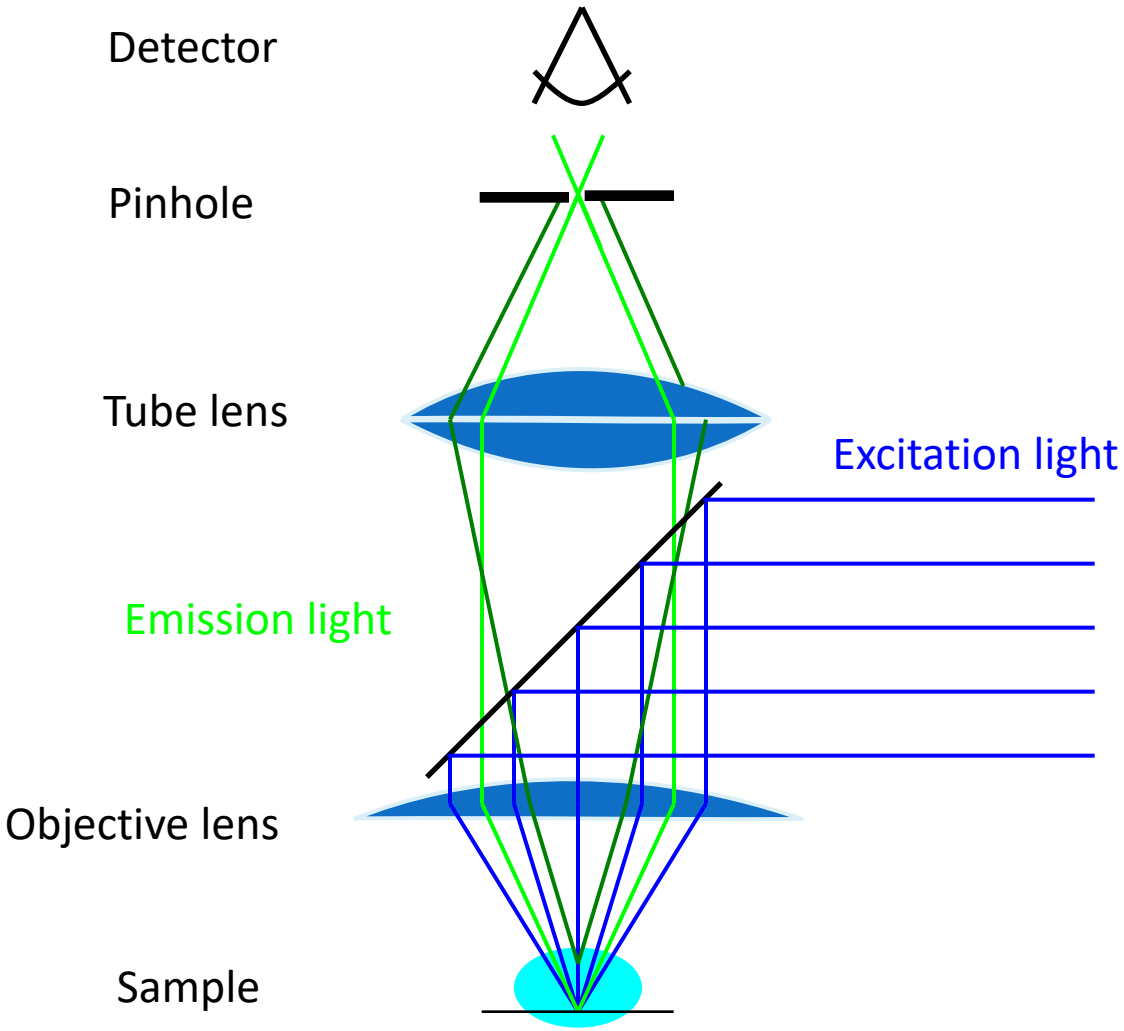


# Microscópio de fluorescência



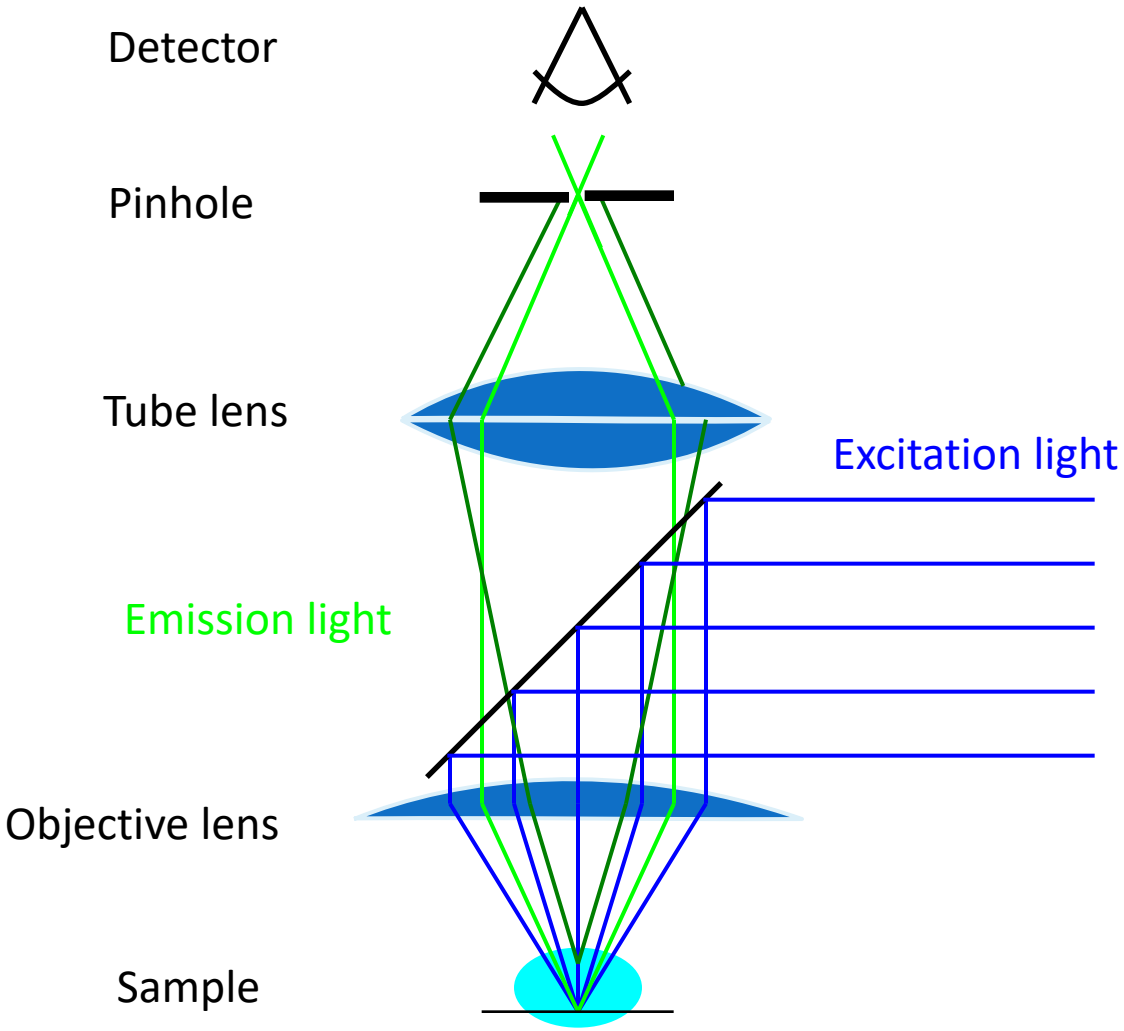

Problema: fluorescência é emitida em todo o caminho óptico na amostra

# Microscópio confocal




Varredura da excitação ponto-a-ponto para formar a imagem.

# Microscópio confocal



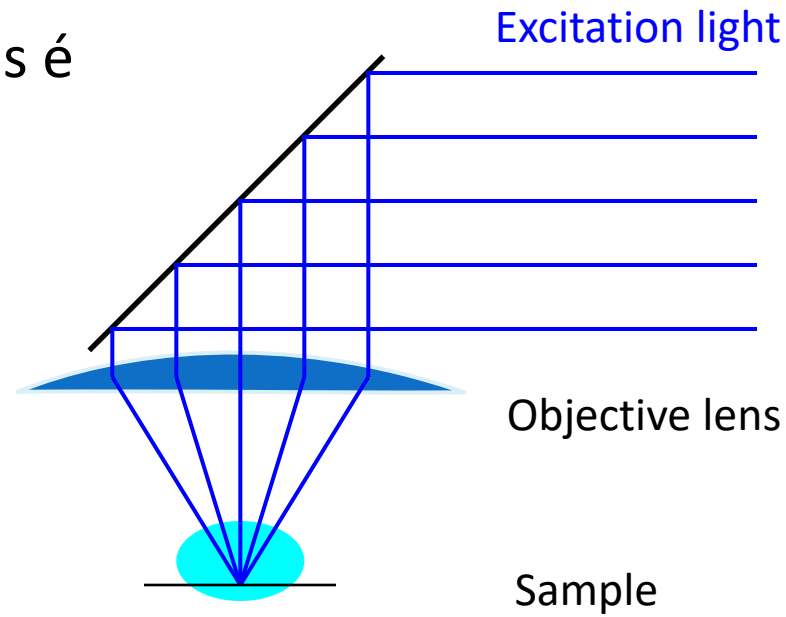

Varredura da excitação ponto-a-ponto para formar a imagem.

# Fonte de luz

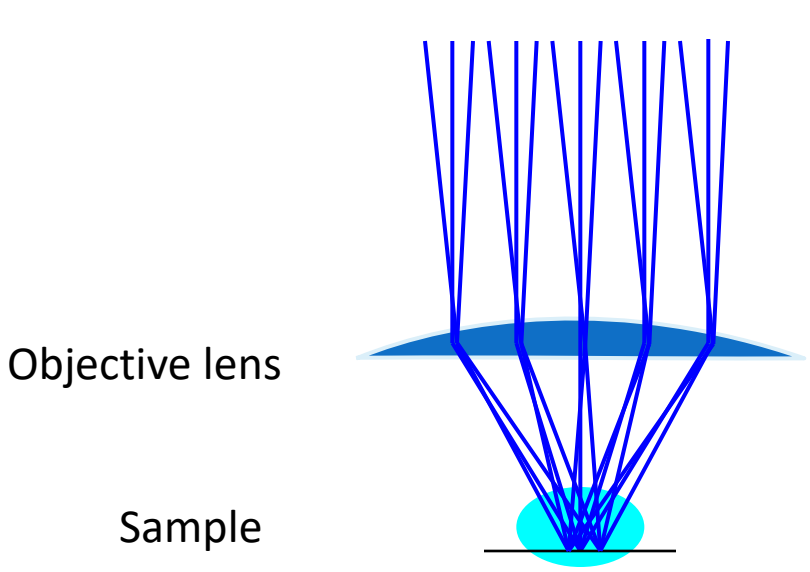
Luz de excitação deve ser focalizada em um “diffraction limited spot”

Poderia ser empregado lâmpada, mas é pouco eficiente

Laser: perfeitamente colimado e alta potência



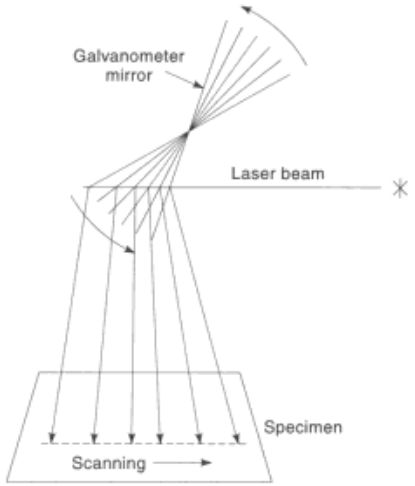
# Varredura



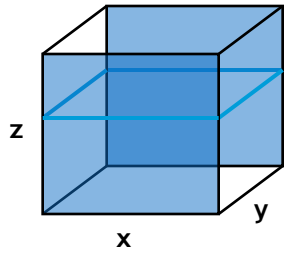

Modificação do ângulo de entrada do laser altera sua posição no plano da amostra

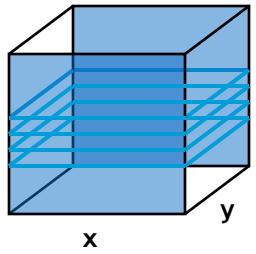
O ponto de emissão muda, então se deve assegurar a coincidência espacial do pinhole.

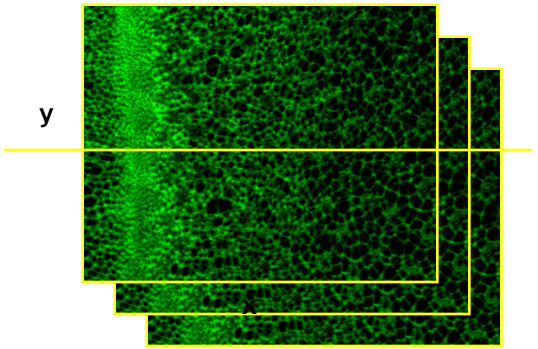
# Varredura laser



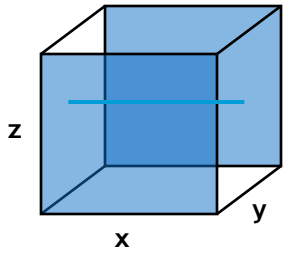

**xy scanning**

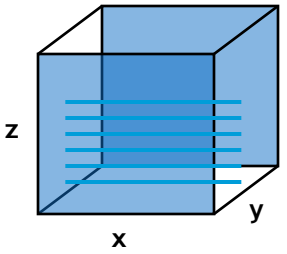


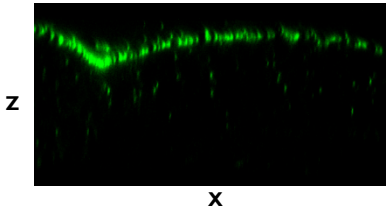




**single section** **z series**




**xz scanning**

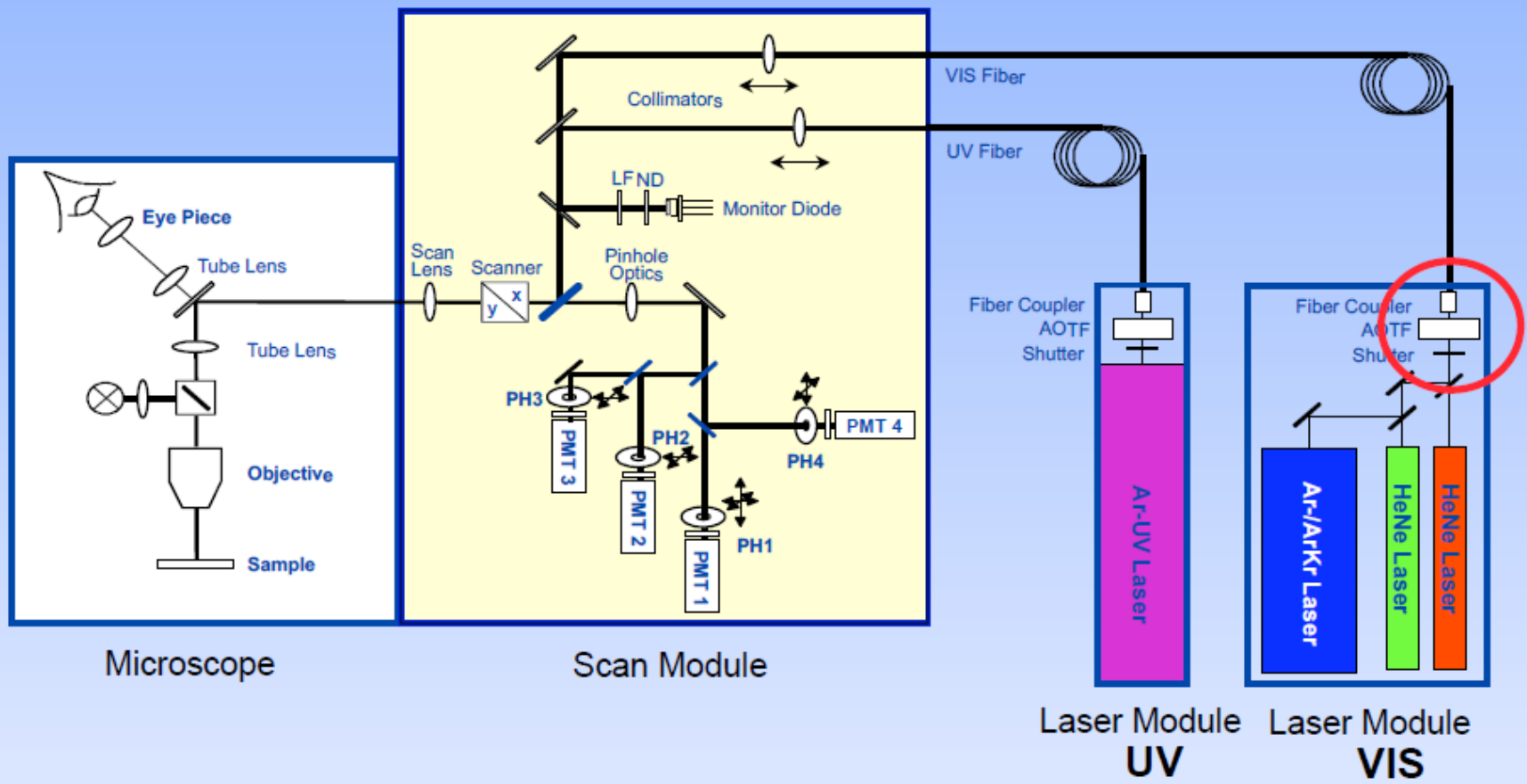
# Confocal Laser Scanning Microscopy

## How Does a CLSM Work?

ZEISS

*Optical Beam Path*

| | |
|---|---|
| AOTF | Acousto Optical Tunable Filter |
| PMT | Photomultiplier |
| PH | Variable Pinhole |



# Widefield fluorescence imaging

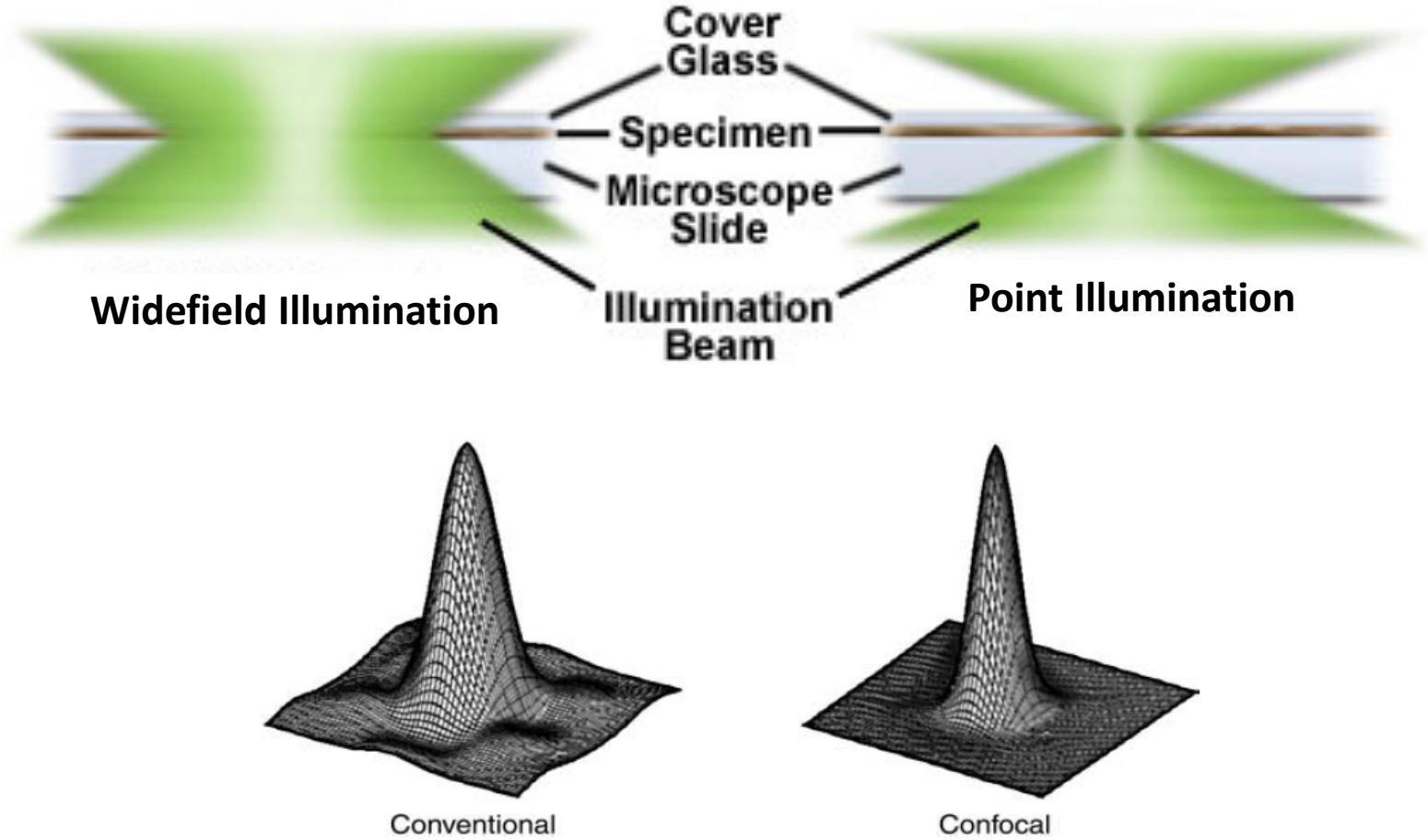
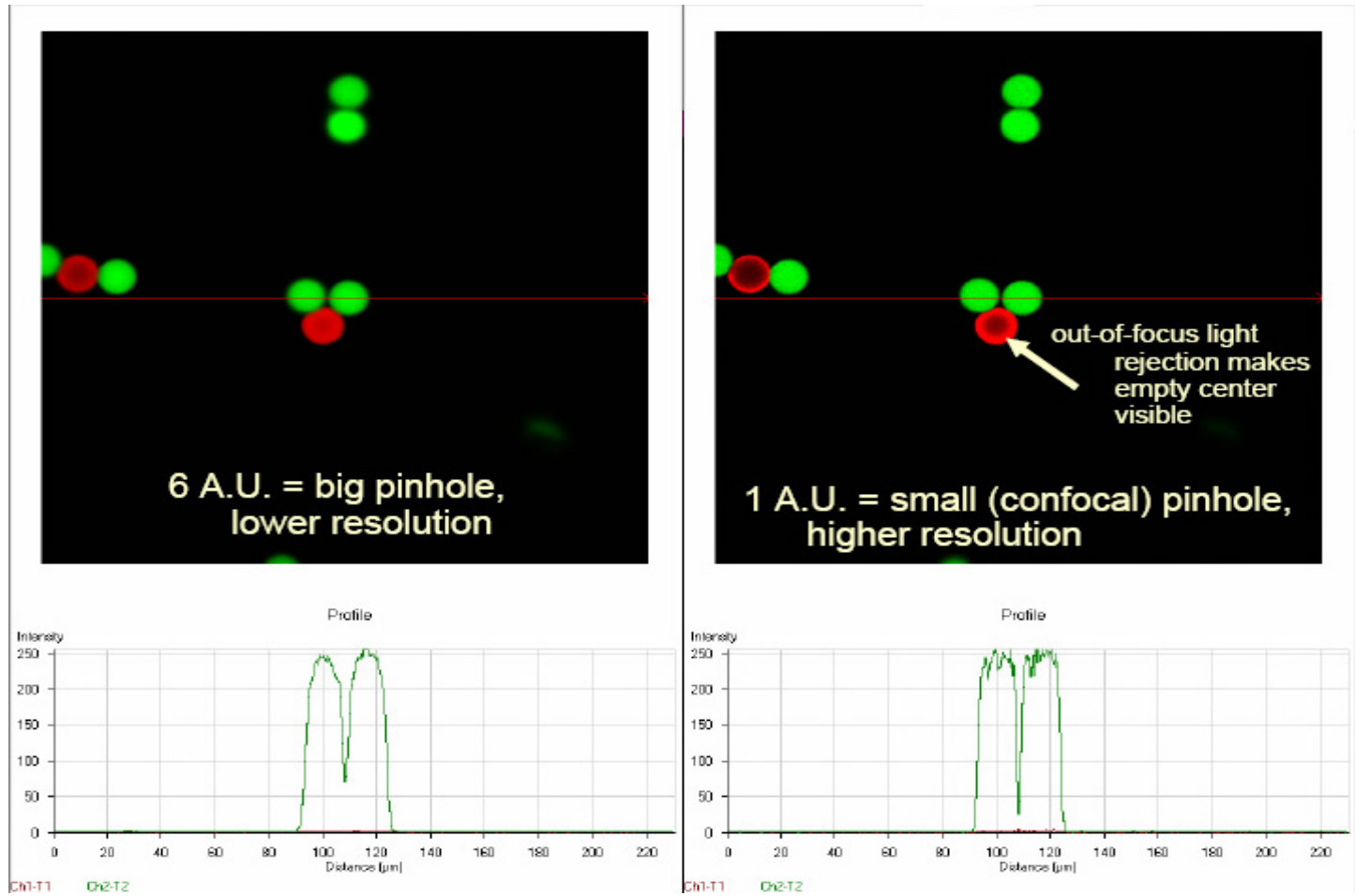


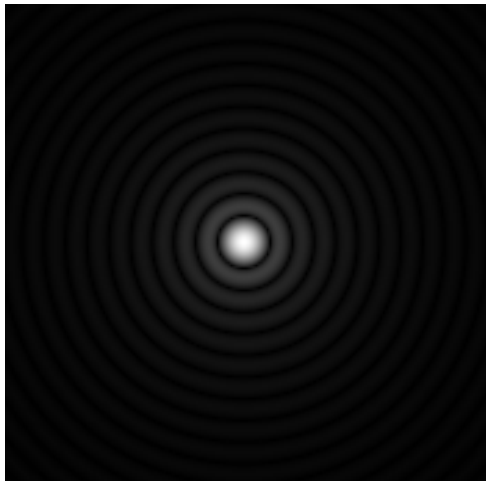

FIGURE 10.2 The point spread functions of conventional and confocal microscopes showing the improvement in lateral resolution that may be obtained in the confocal case.

# Confocal Pinhole



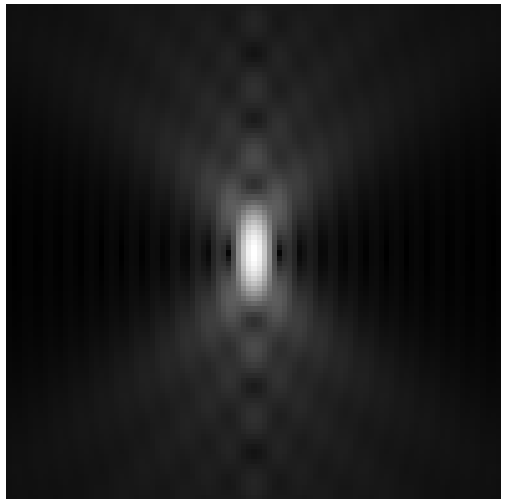
# Pinhole - diâmetro

Resolution is limited by the point-spread function

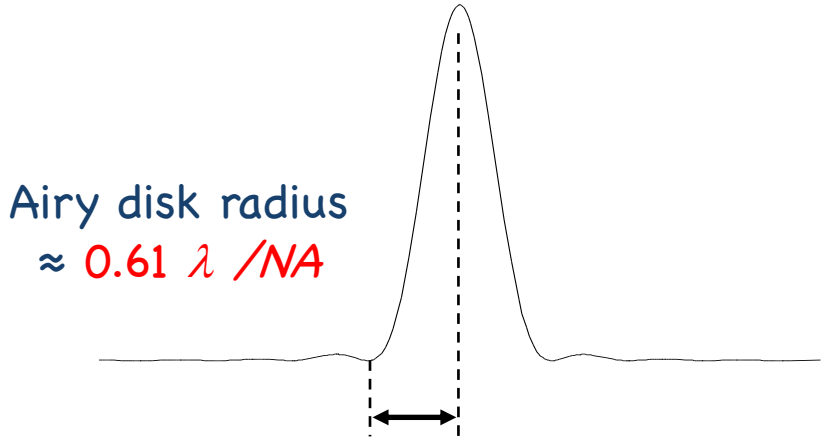


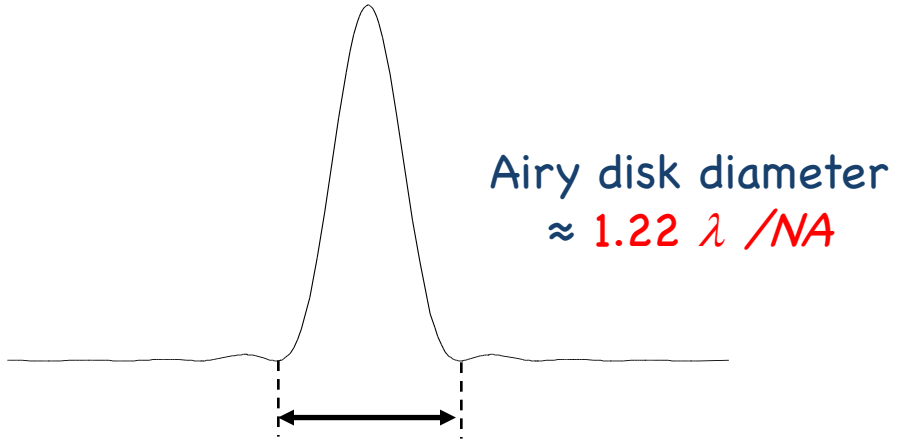
Y

X

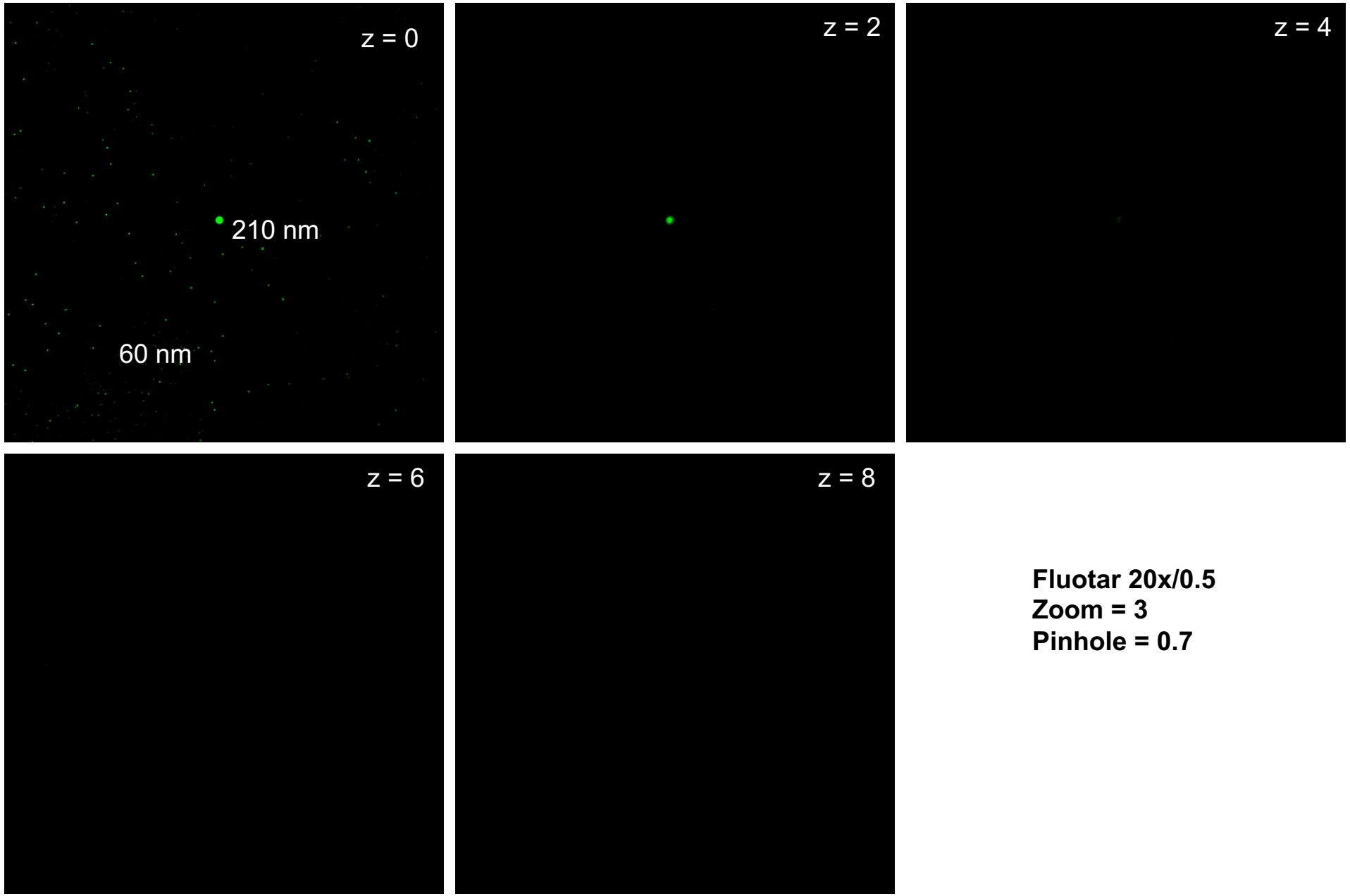


Z

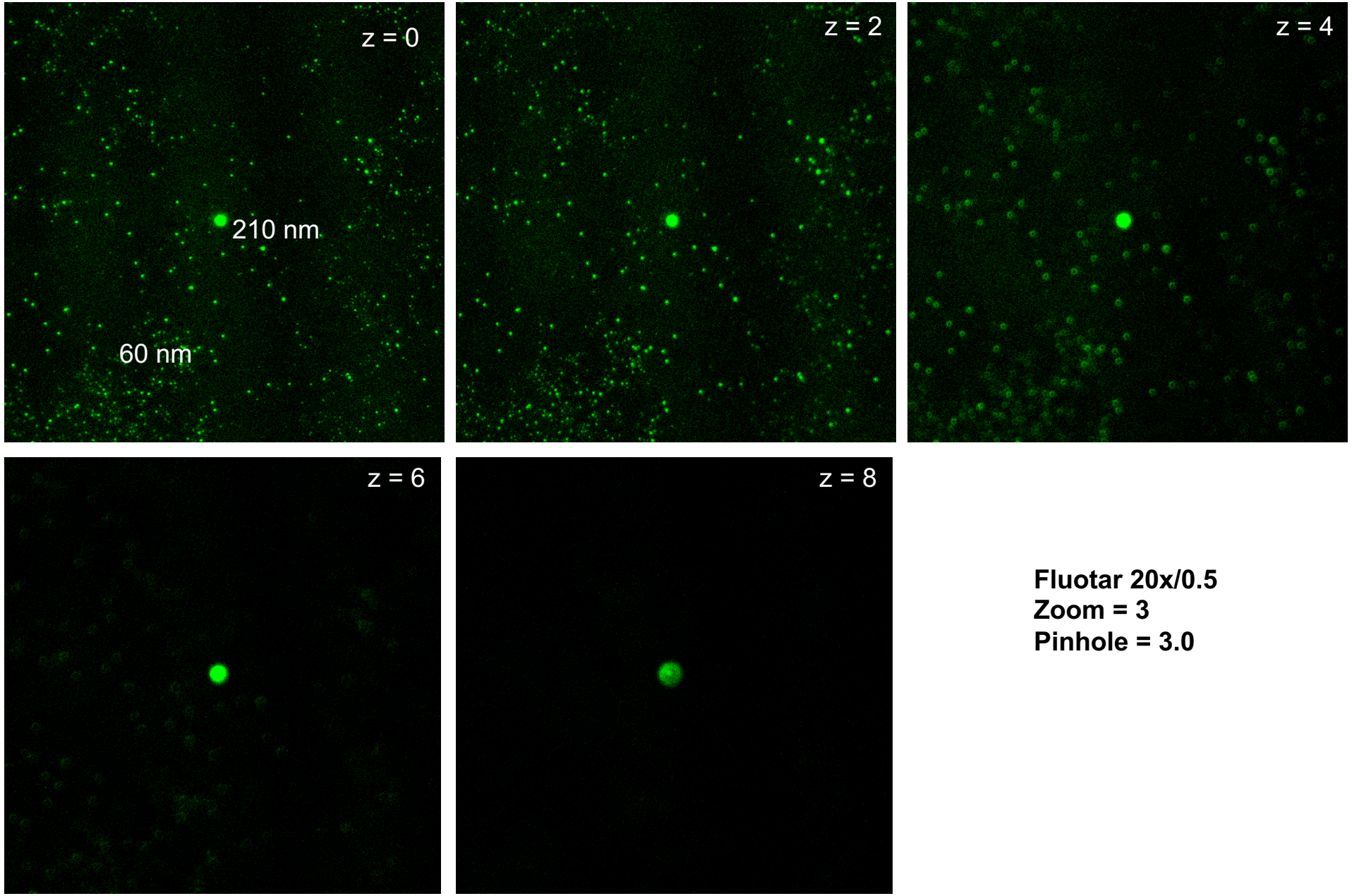



Width of point spread function at pinhole:
Airy disk diameter × magnification of lens




**Fluotar 20x/0.5**
**Zoom = 3**
**Pinhole = 0.7**




**Fluotar 20x/0.5**
**Zoom = 3**
**Pinhole = 3.0**

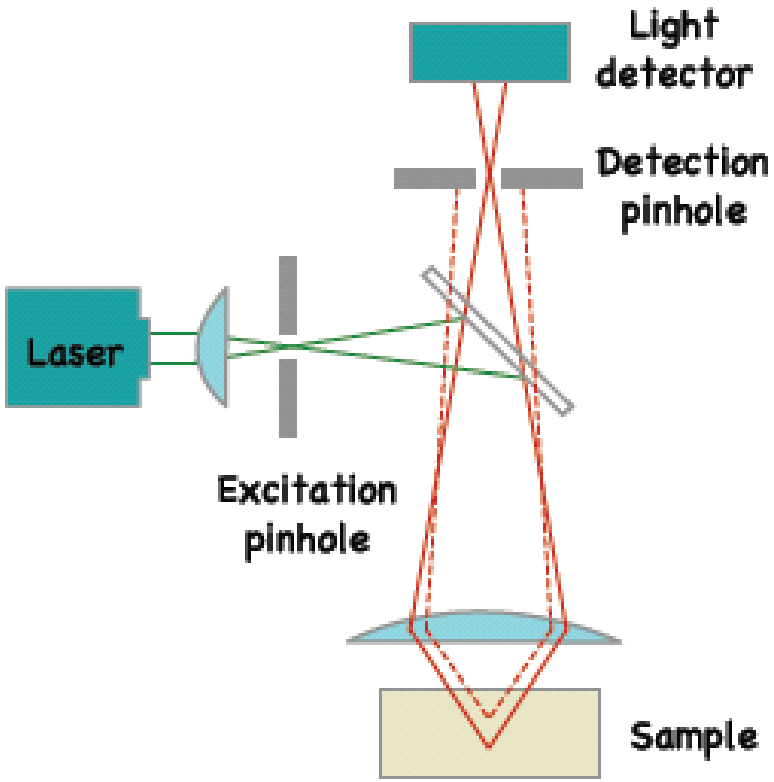
# 3D fluorescence microscopy

Acquire a "focal series" (stack) of images

**Problem:**
Each image contains out-of-focus blur from other focal planes

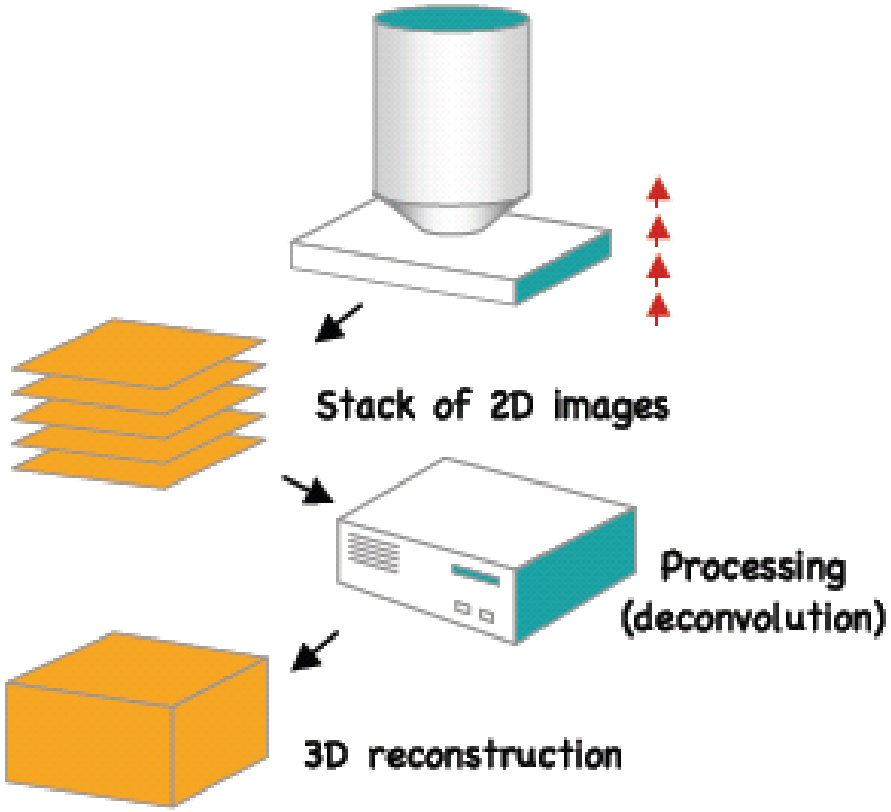
## Approach 1

Physically exclude the blur
Example: confocal microscopy




## Approach 2

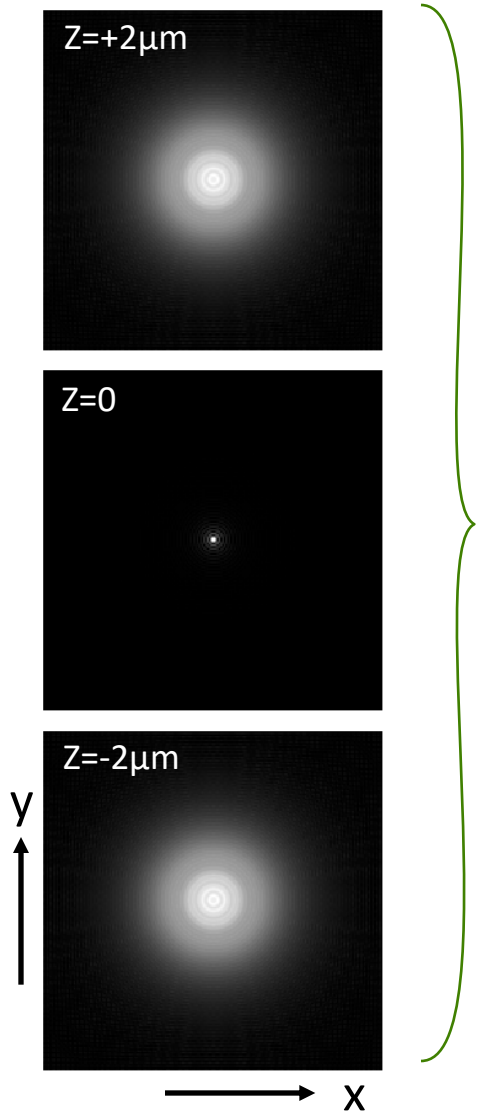
Remove the blur computationally



# The Point Spread Function (PSF)

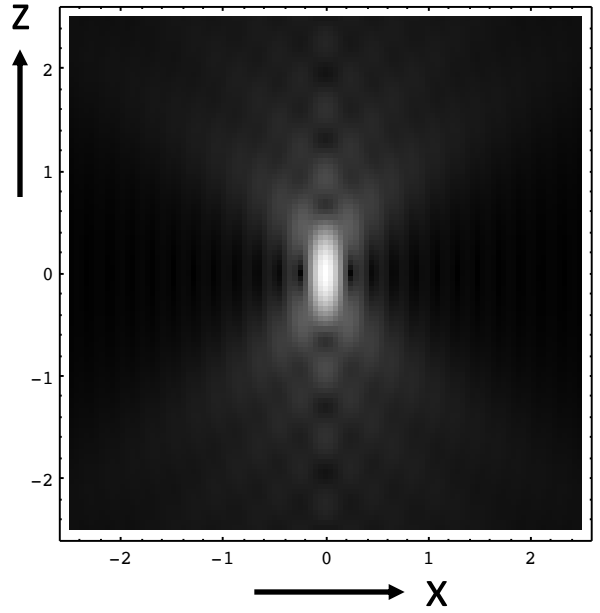
## The image of a point object

### 2D PSF
### for different defocus

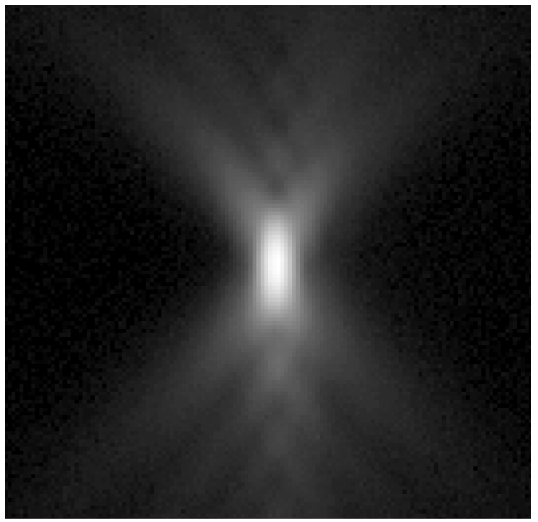



### 3D PSF

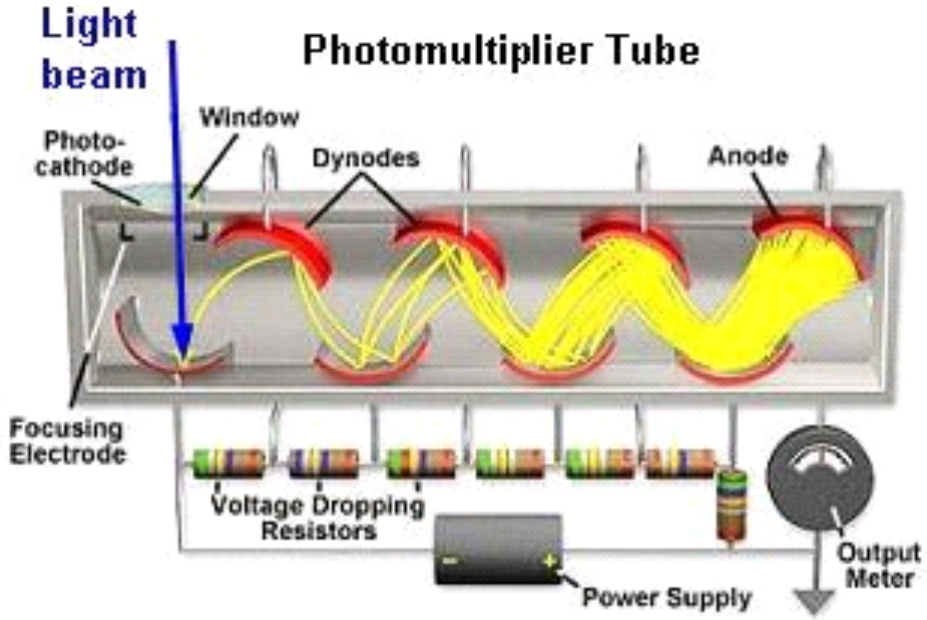
#### Calculated

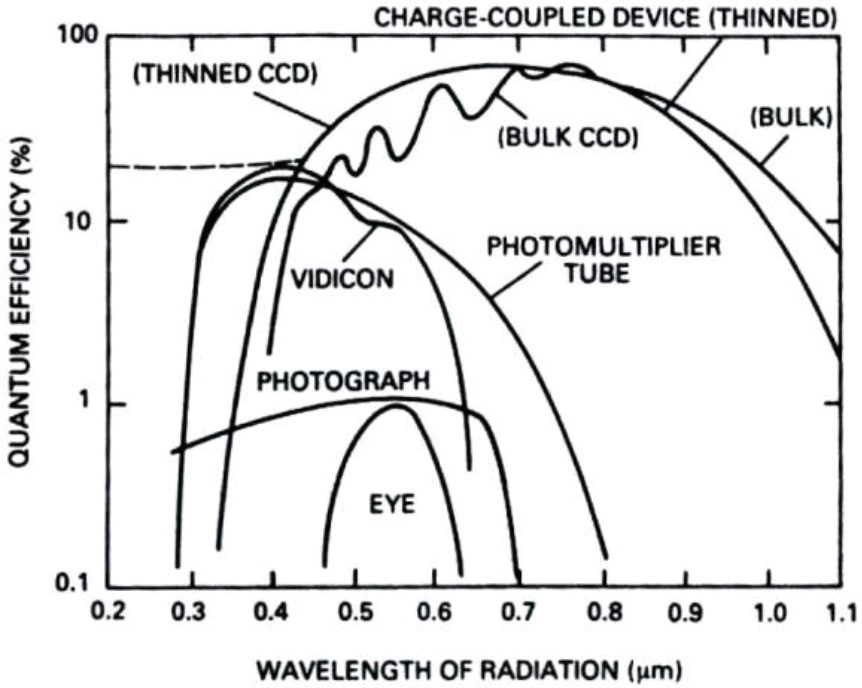


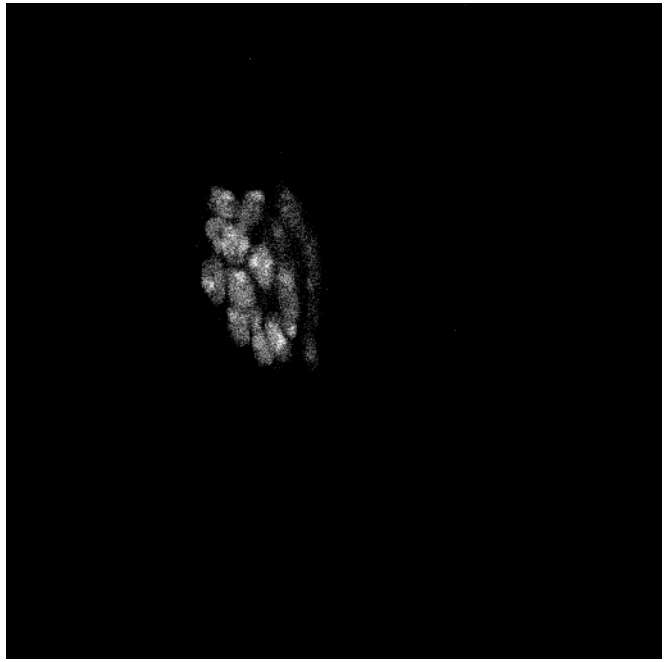
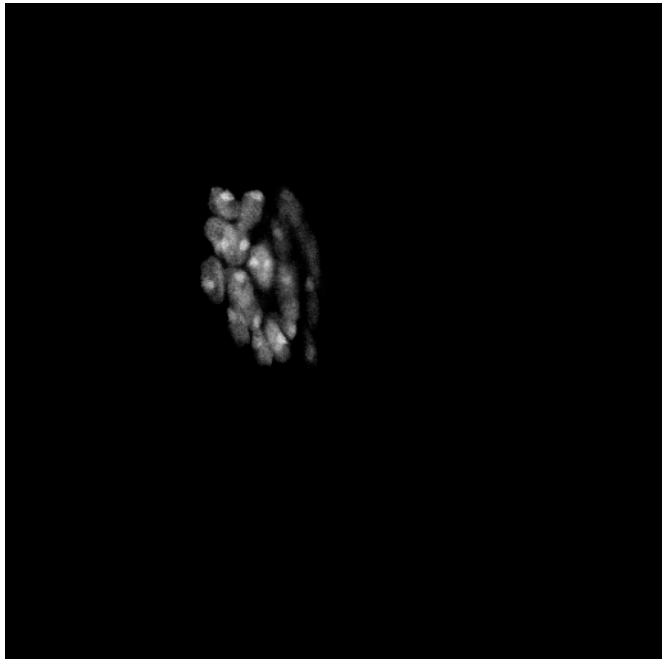
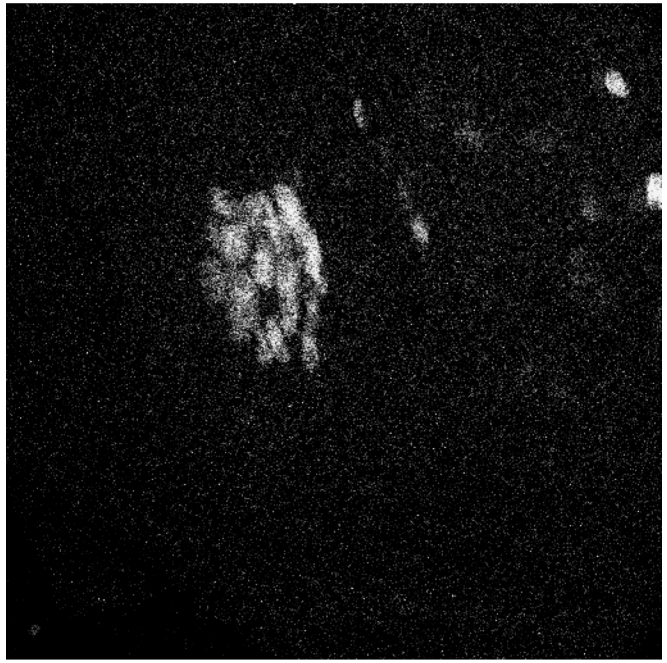
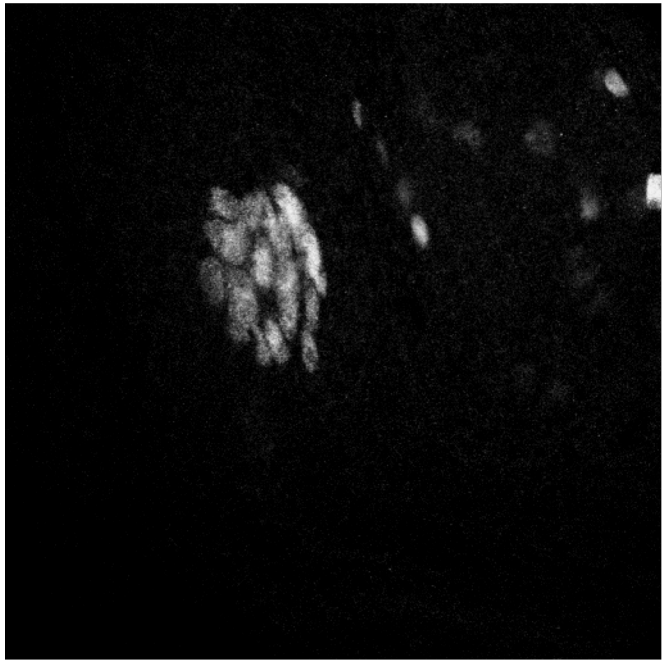

#### Measured



# Detectors - PMTs

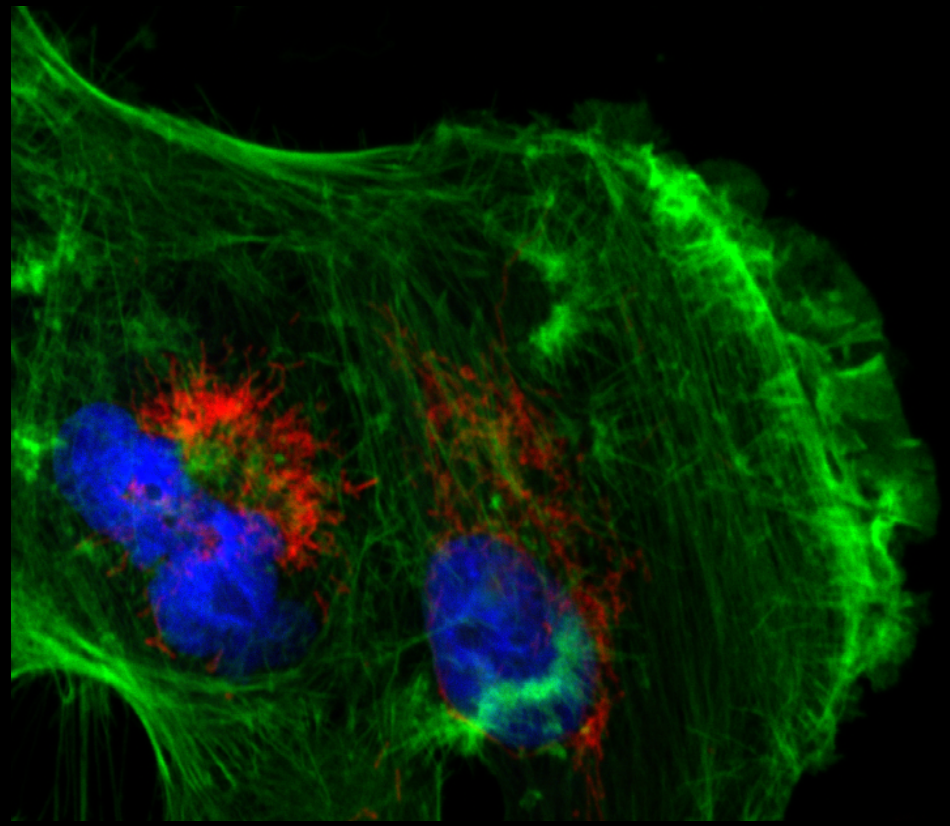


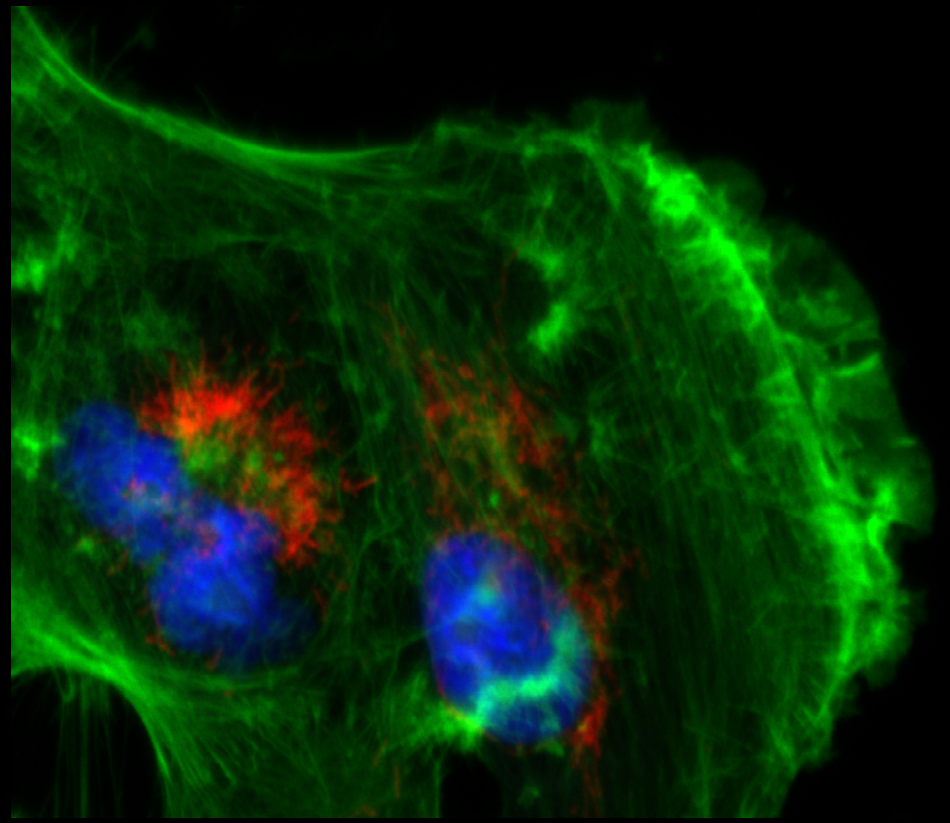
| | 1 scan | 16 scans |
| --- | --- | --- |
| **Medium gain**<br>Laser 488nm 80%<br>PMT 800V |  |  |
| **High gain**<br>Laser 488nm 10%<br>PMT 1000V |  |  |

Apo 63x lens

# Confocal vs. Widefield



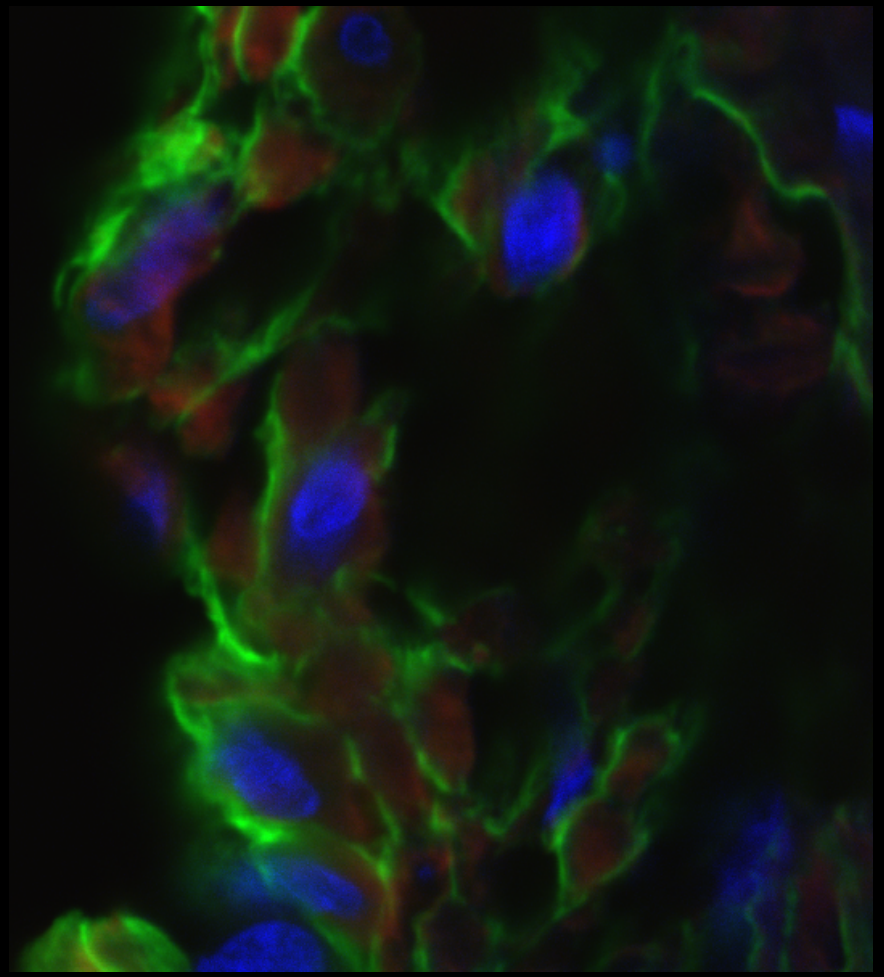
Confocal



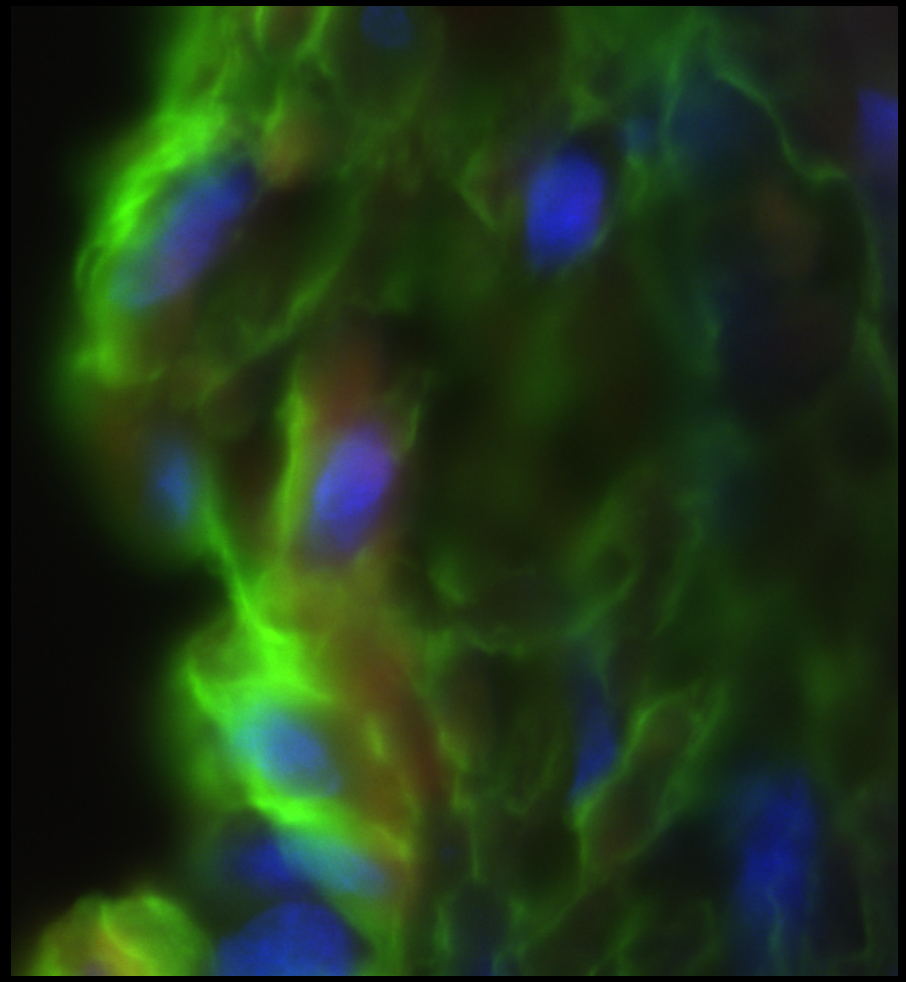
Widefield

Tissue culture cell with 60x / 1.4NA objective

# Confocal vs. Widefield



Confocal



Widefield

20 μm rat intestine section recorded with 60x / 1.4NA objective

# Marcação por imunofluorescência

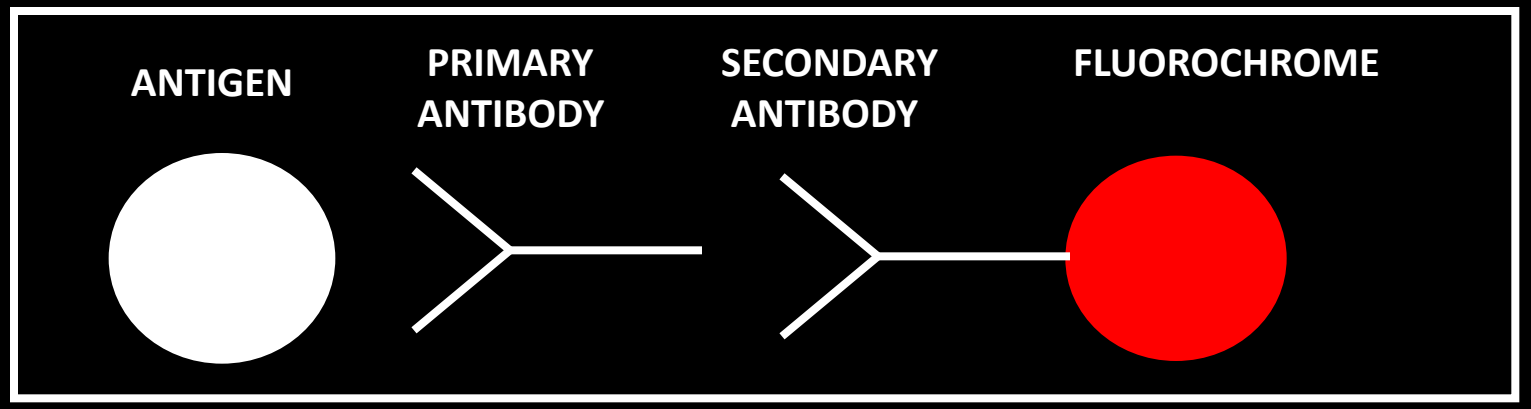
- Uso de marcadores fluorescentes para localizar e identificar biomoléculas ou padrões da expressão protéica em células.

- Anticorpo primário: ligação com o antígeno (biomolécula alvo).

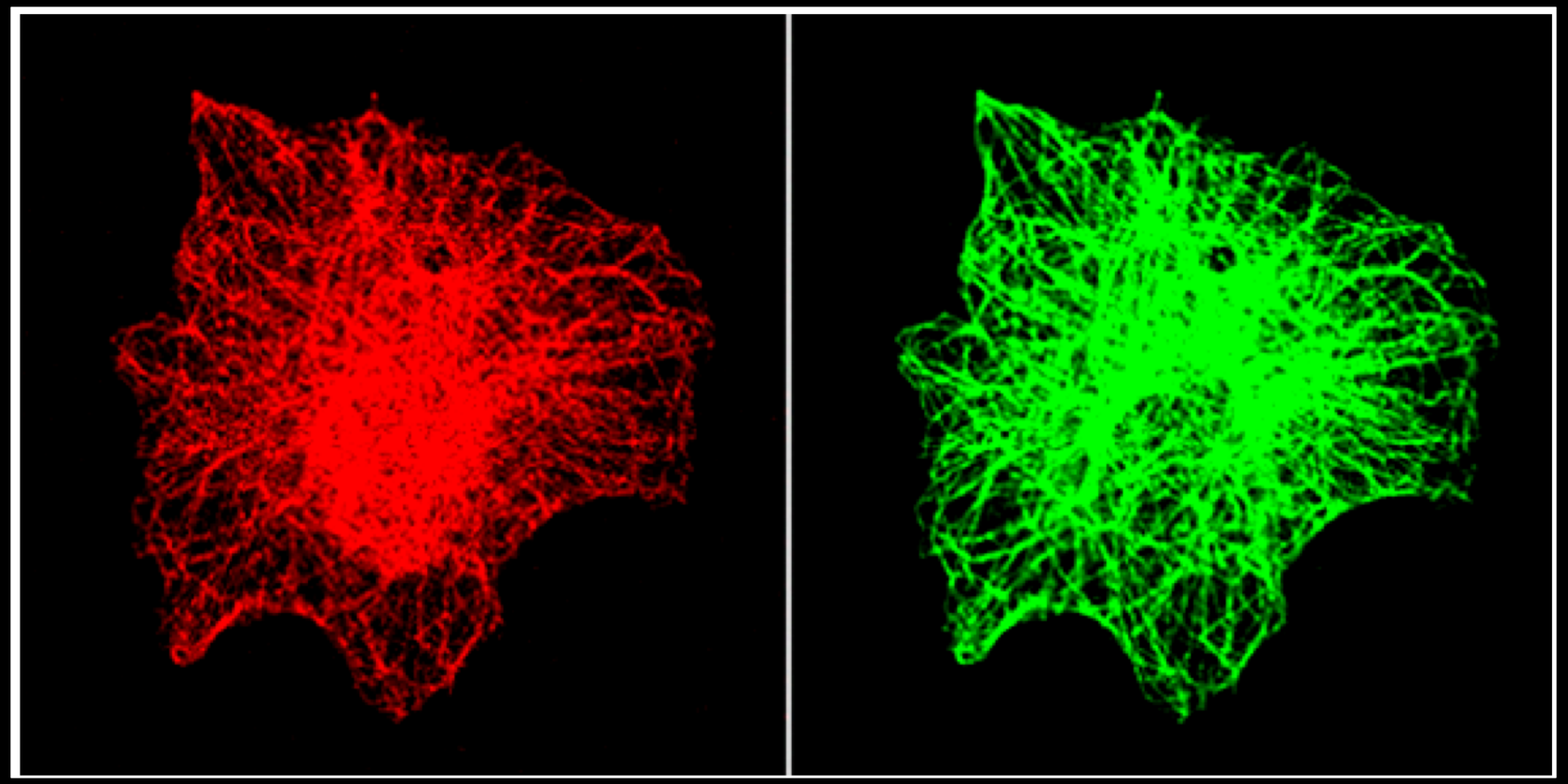
- Complexo antígeno-anticorpo é ligado ao anticorpo secundário (conjugado a um fluoróforo).

**Uso em:** 1. tecidos congelados, não fixados e em tecidos tratados com etanol.

2. células fixadas com paraformaldeído ou metanol/acetona.



Giannakakou et al., Nature Cell Biology,2000



**PRIMARY ANTIBODY**
sheep anti-p53 polyclonal

**SECONDARY ANTIBODY**
Texas Red conjugated anti-sheep

**PRIMARY ANTIBODY**
mouse anti-$\alpha$ tubulin monoclonal

**SECONDARY ANTIBODY**
FITC conjugated anti-mouse

# Direct Staining of Cell Structures

## Organelle Probes

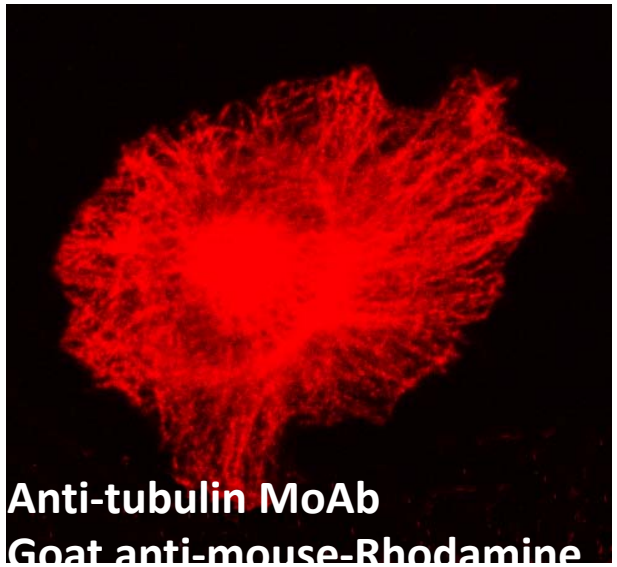
| | | |
|---|---|---|
| Mitochondria | MitoTracker | mitochondrial membrane potential |
| Lysosomes | LysoTracker | hydrolytic activity of enzymes |
| ER and Golgi | Lectin conjugates | lipid composition |

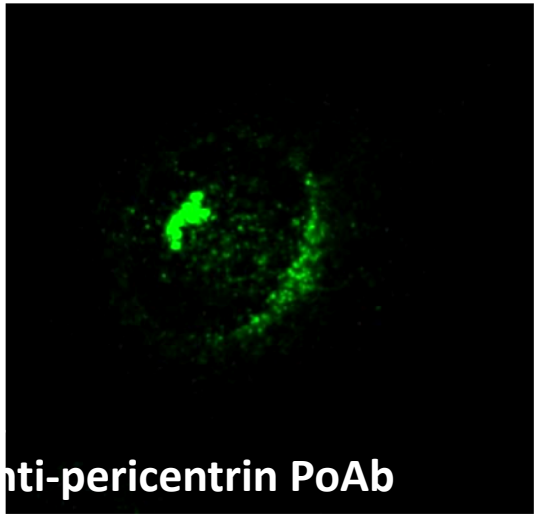
## Other Probes

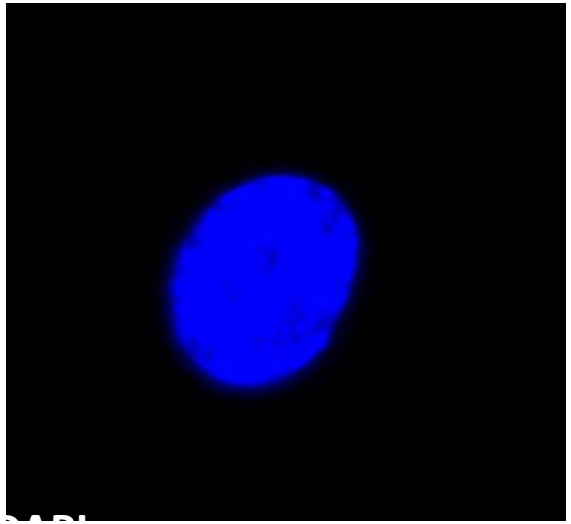
| | | |
|---|---|---|
| Stress fibers | Phalloidin-conjugaes | bind F-actin |
| Nuclei | DAPI | binds to minor groove of ds-DNA |

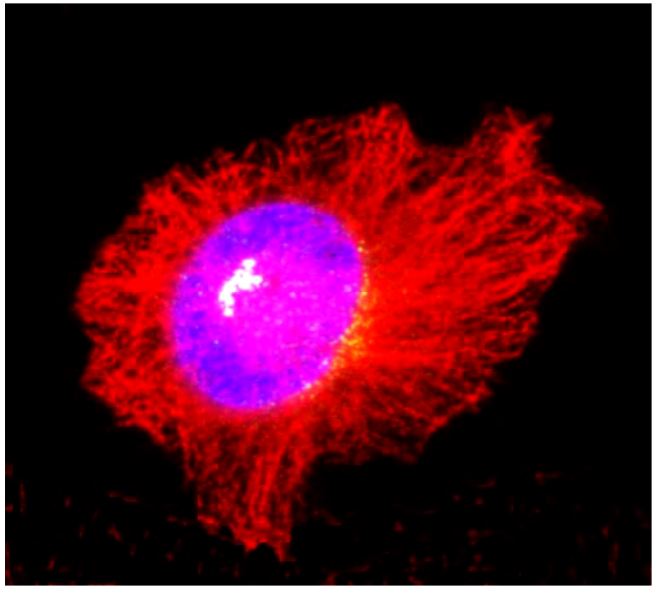
microtubules

centrosomes





nucleus

**Figure 6.** (**A**) Molecular structure and localization of the chromophoric tripeptide in *A. victoria* wild-type GFP. Notice that the tripeptide is located centrally within the β-barrel. A vast number of genetically enhanced (denoted "E", *e.g.*, EGFP) and engineered FPs [27] have been created over the pasts decades. (**B**) Anatomy of a semiconductor quantum dot (QD), which derives its fluorescent properties from the bandgap between the inner core material and the capsule shell. QDs display size dependent fluorescent properties. (**C**) Excitation and emission spectra of *A. victoria* GFP (green lines) and examples of how the size influences the fluorescent properties of QDs.

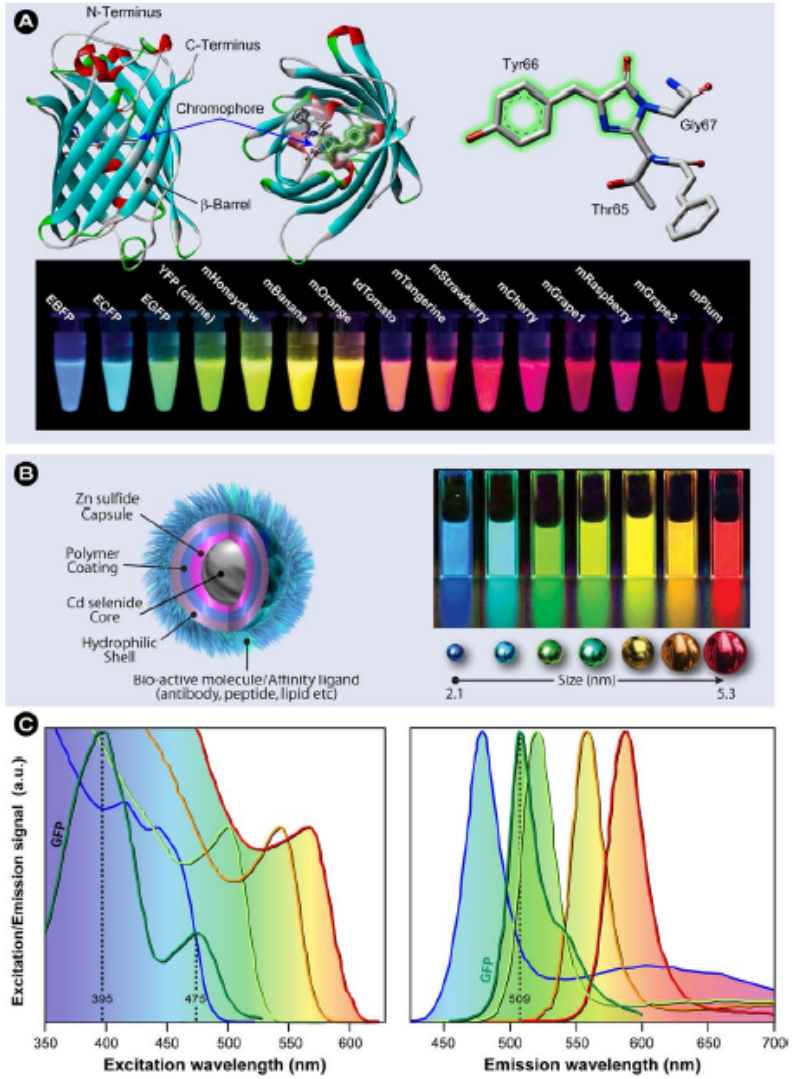
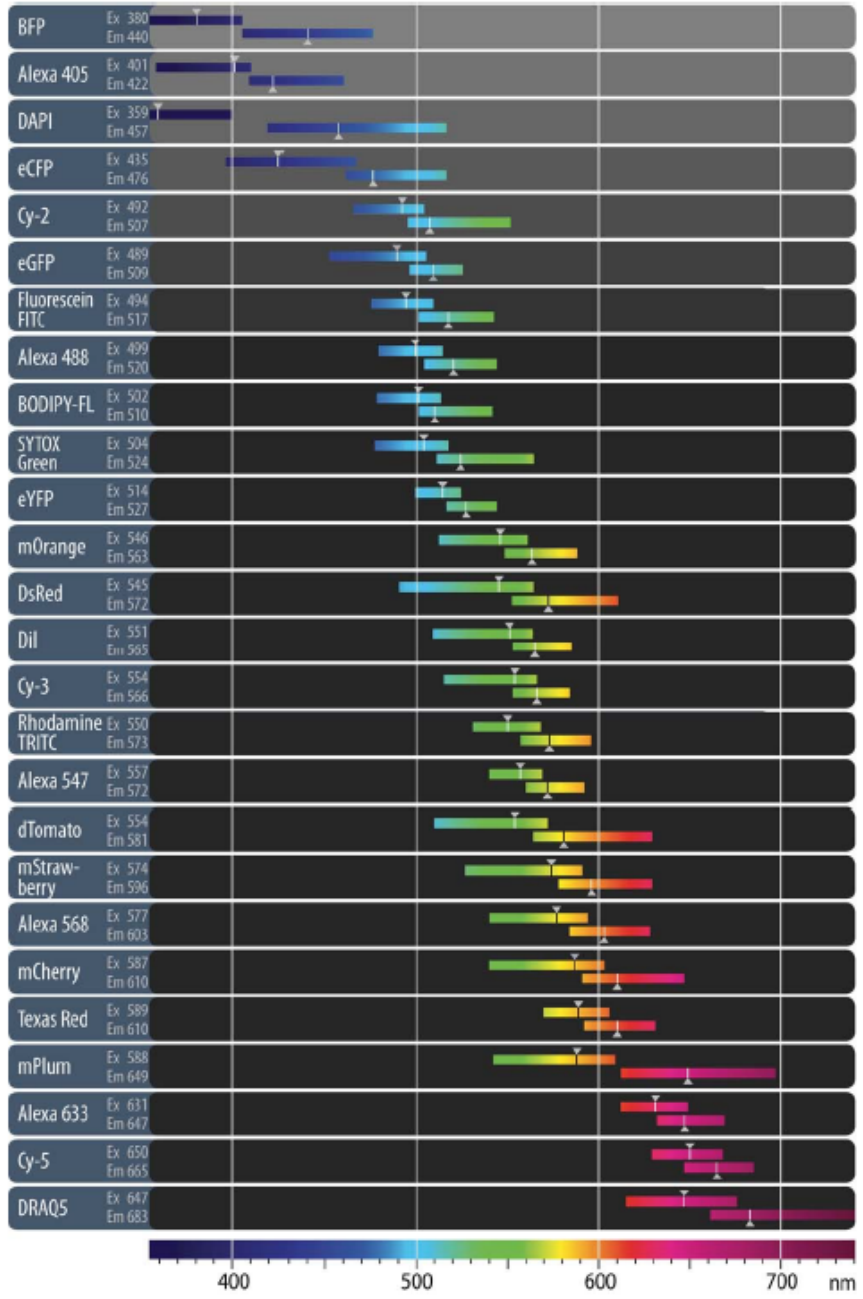



**Table 1.** Overview of the fluorescent properties of popular organic dyes and fluorescent proteins. Reproduced with permission. © 2011 Carl Zeiss Micro-Imaging GmbH.




HC Ishikawa-Ankerhold et al. Molecules 2012, 17.

| Channel | Band | Excitation laser (nm) | | | | |
|---|---|---|---|---|---|---|
| | | 405 | 488 | 561 | 642 | 785 |
| 1 | 435-505 | DAPI, BV421, Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet | | | | |
| 2 | 505-560 | BV510, PacOrange, Cascade Yellow, AF430, eFluor525, QD525 | FITC, AF488, GFP, YFP, DyLight488, PKH67, Syto13, LysoTrackerGreen, MitoTrackerGreen | | | |
| 3 | 560-595 | QD565, QD585, eFluor565 | PE, PKH26, DSRed, mOrange, Sytox Orange, Cy3 | PE, AF546, Cy3, DyLight550, PKH25, DSRed | | |
| 4 | 595-642 | QD625, eFluor625, BV605 | PE-TexRed, PI, RFP, QD625, eFluor625 | AF568, Cy3, PE-TexRed, TexRed, AF610, RFP, mCherry, PI | | |
| 5 | 642-745 | QD705, eFluor700, BV711 | PE-Cy5, PE-AF647, 7AAD, PerCP, PerCP-Cy5.5, DRAQ5, QD705 | PE-Cy5, PE-AF647, DRAQ5, 7AAD | APC, AF647, AF660, AF680, APC, Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5, PerCP, , PerCP-Cy5.5 | |
| 6 | 745-780 | QD800, BV786 | PE-Cy7, PE-AF750, QD800 | PE-Cy7, PE-AF750 | APC-Cy7, APC-AF750, APC-H7, Cy7, AF750, PE-Cy7, PE-AF750 | SSC |

# Detecção espectral

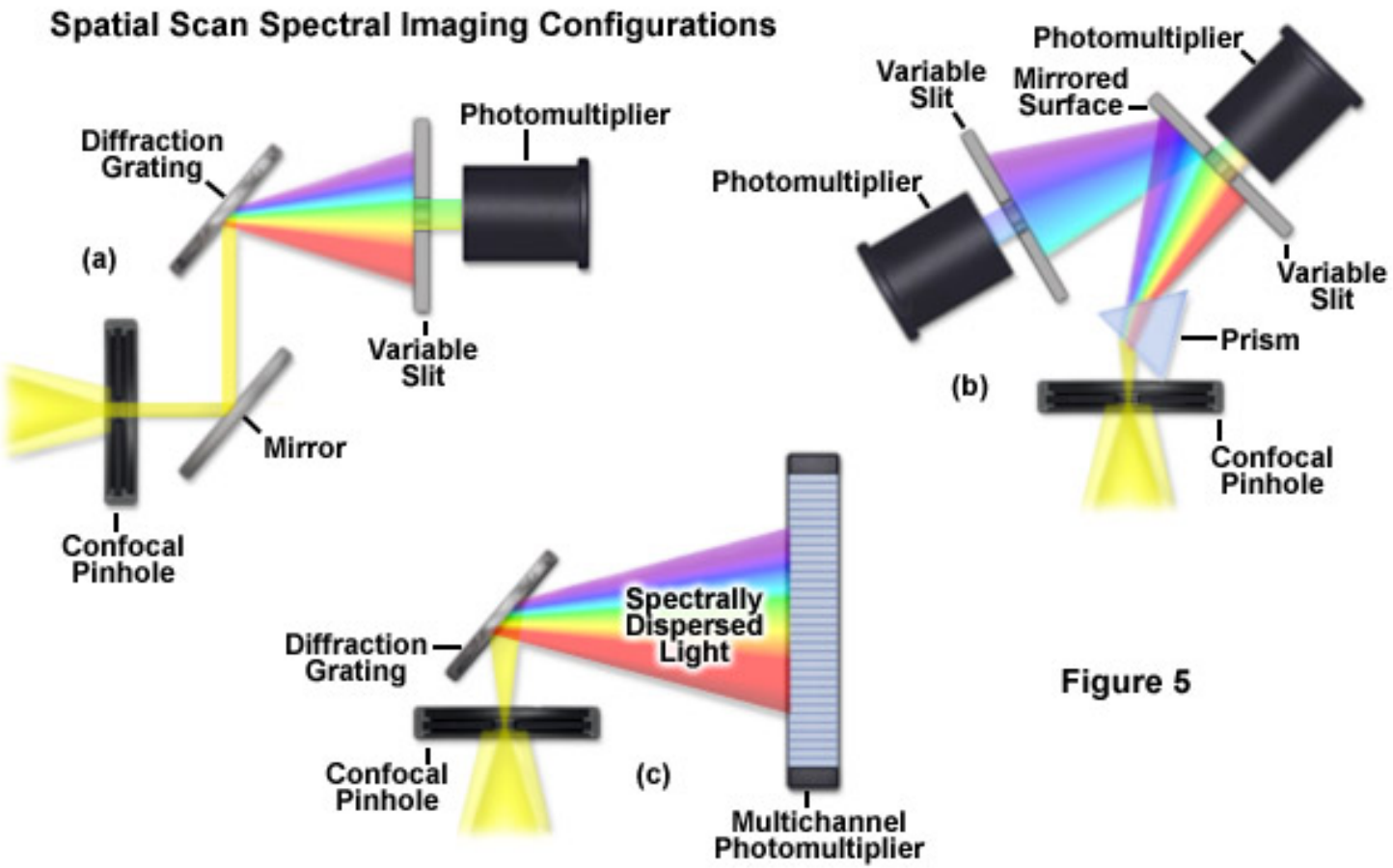
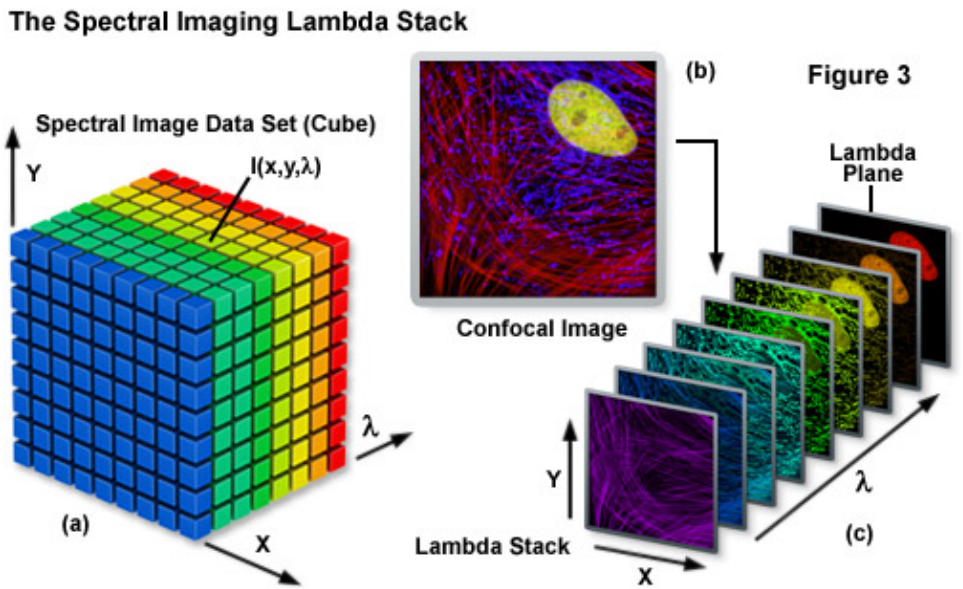



Figure 5



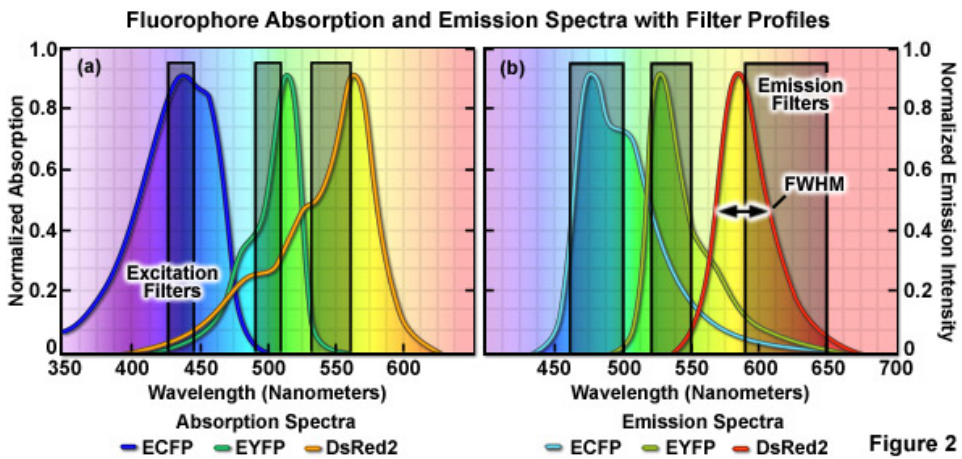
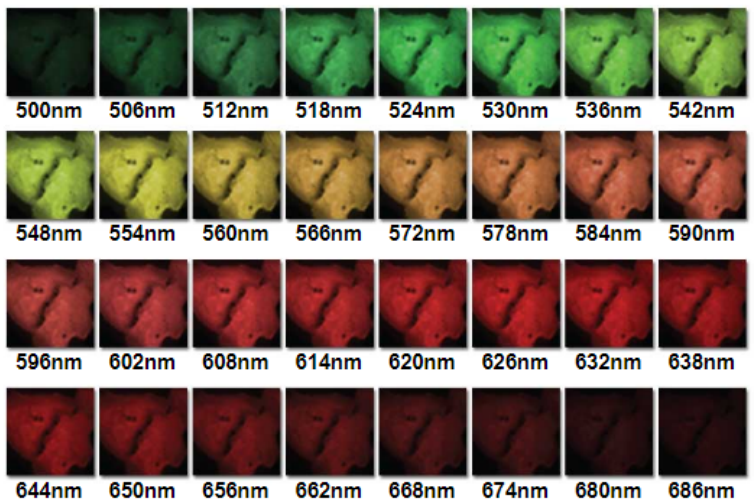




Figure 2

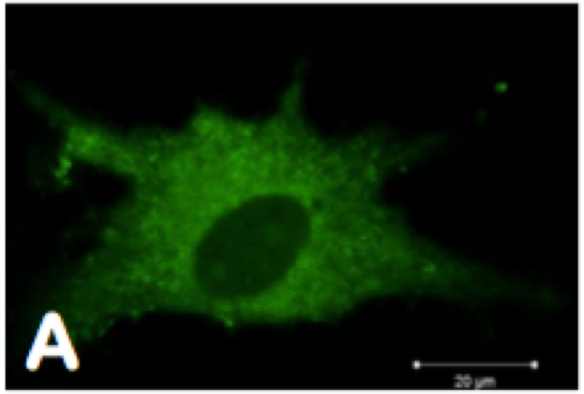
*Figure 4 - 32-Channel Spectral Image Lambda Stack Acquisition*

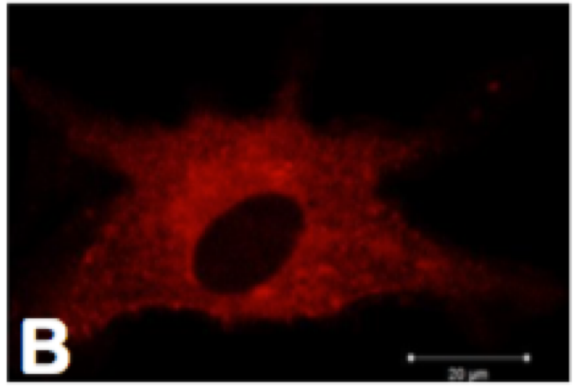


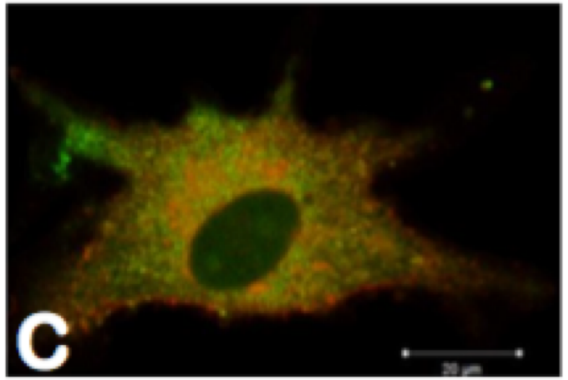
# Co-localização: distribuição celular do fotossensibilizador

Mitocôndrias

Photodithazine



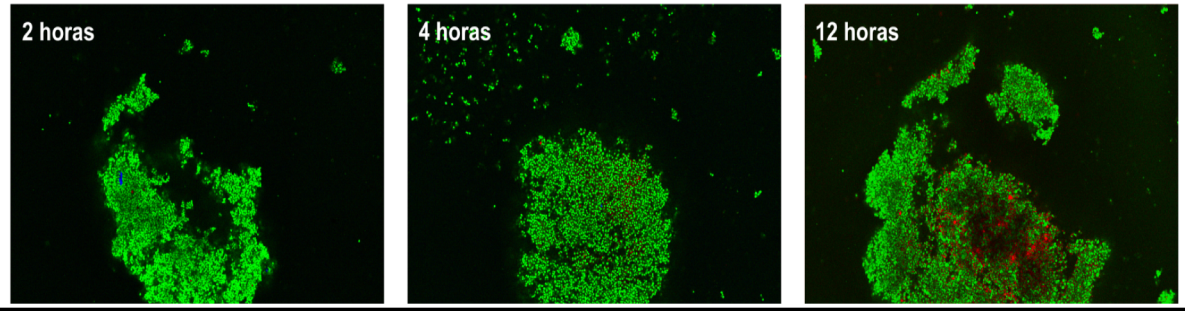
# Cinética em microscopia confocal

**Incubação com Photodithazine 50 µg/mL (Escala: 1 mm)**

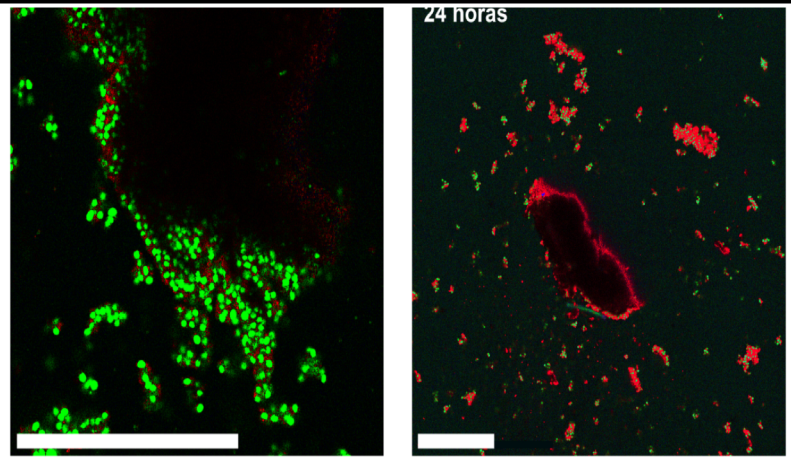

**Alta incorporação do Photodithazine nos tumores de melanoma, mesmo para menores tempos de incubação.**

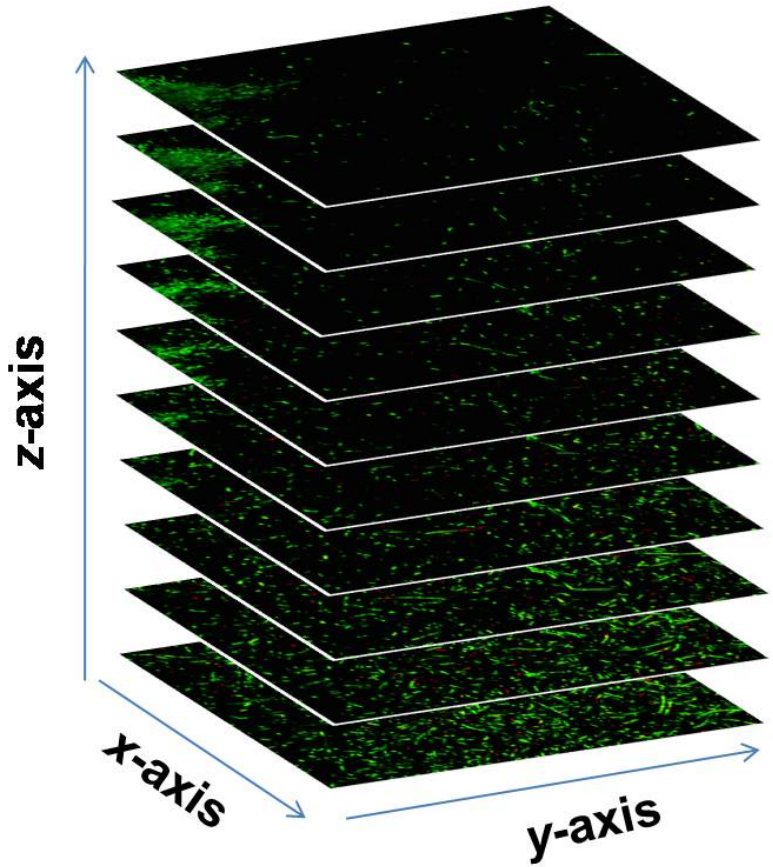
# Cinética em microscopia confocal

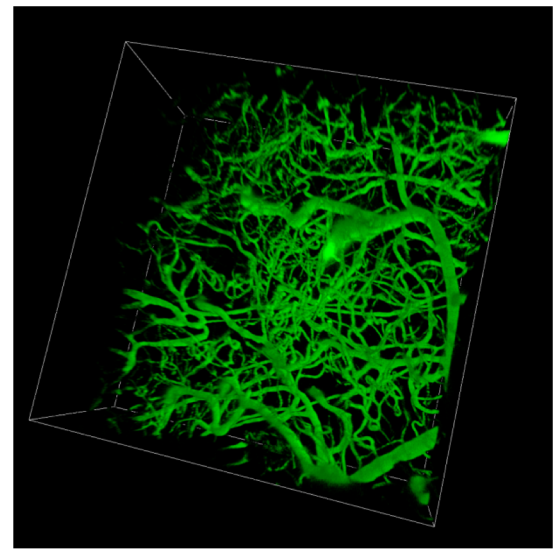
**Incubação com Photogem 50 µg/mL (Escala: 1 mm)**

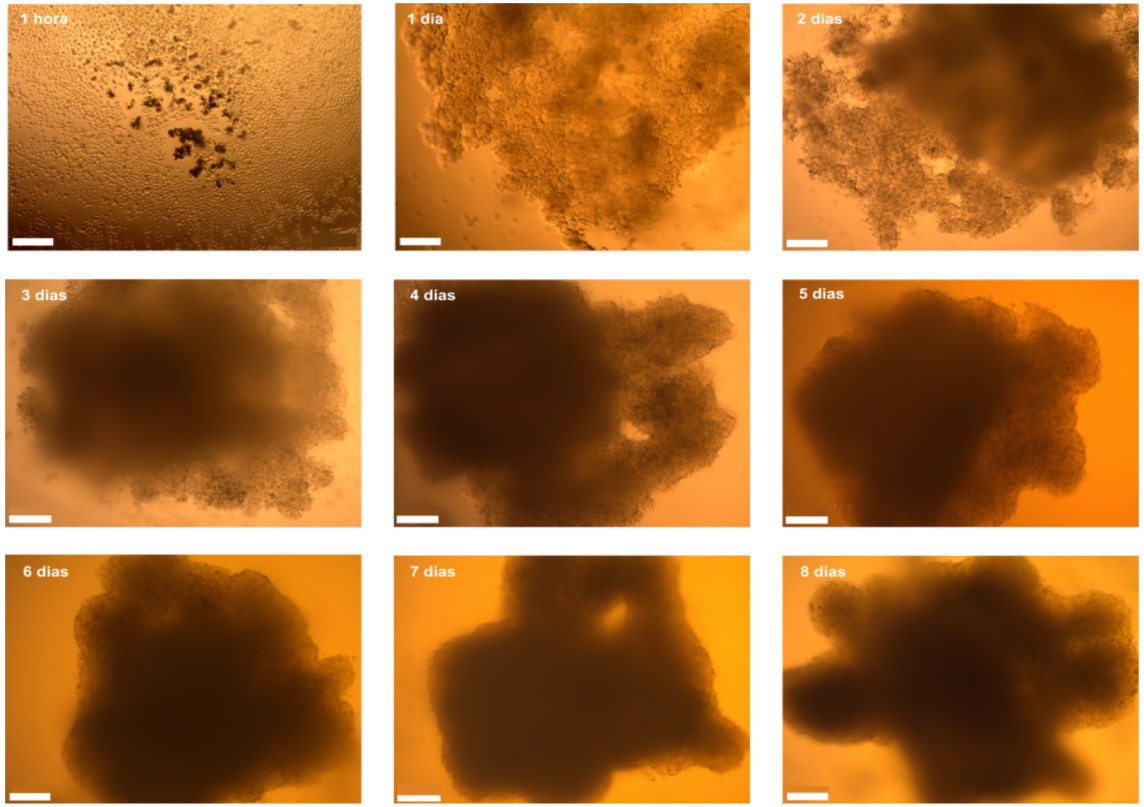


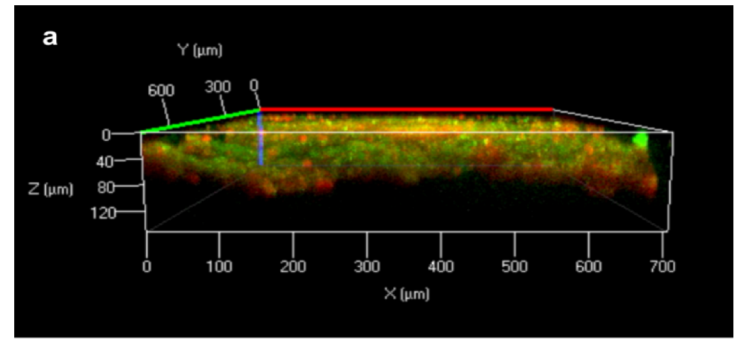

**Incorporação significativa do Photogem nos tumores de melanoma em maiores tempos de incubação**

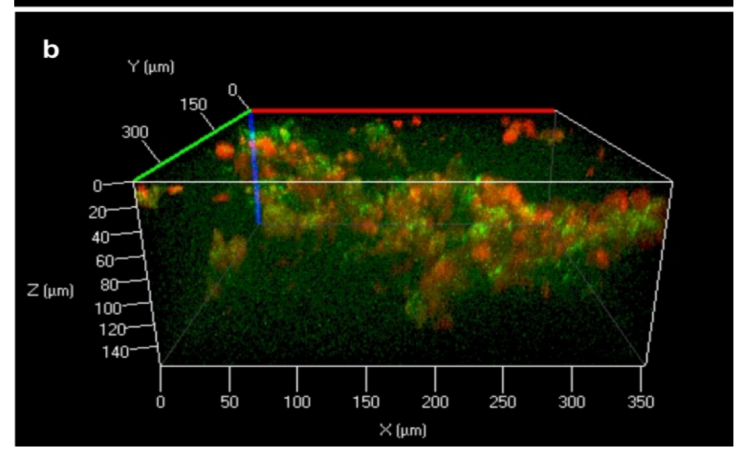



z-axis
x-axis
y-axis



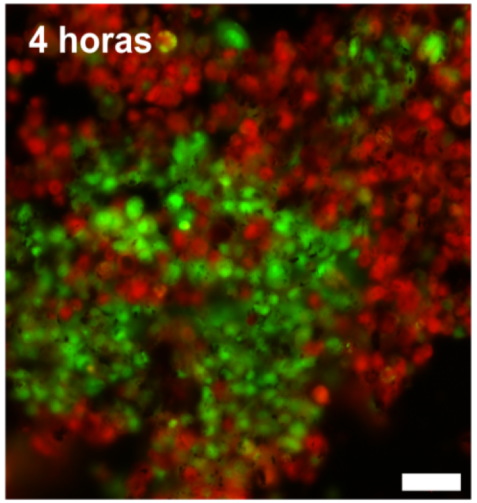

1 hora
1 dia
2 dias
3 dias
4 dias
5 dias
6 dias
7 dias
8 dias

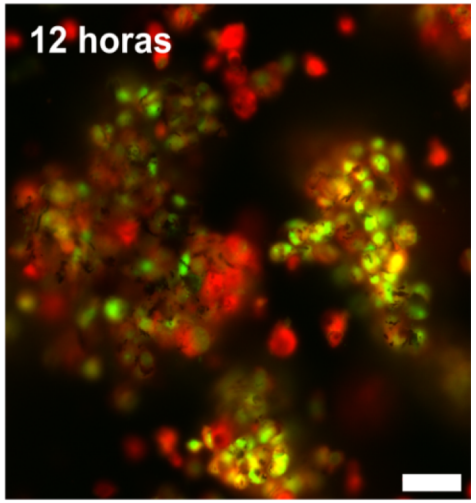

a
Y (µm)
600
300
0
0
40
80
120
Z (µm)
0
100
200
300
400
500
600
700
X (µm)


b
Y (µm)
0
150
300
0
20
40
60
80
100
120
140
Z (µm)
0
50
100
150
200
250
300
350
X (µm)

# Distribuição do Photogem

**Incubação com Photogem 50 µg/mL (Escala: 50 µm). Aumento de 40 vezes.**

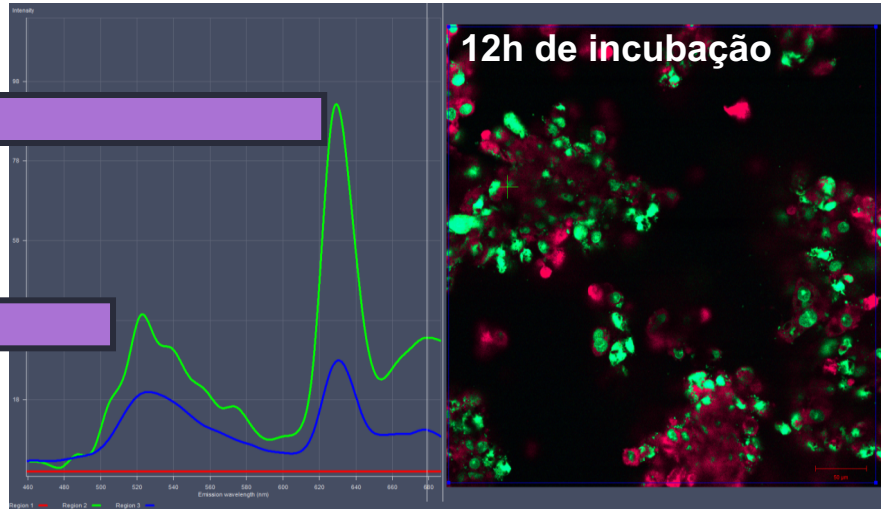



**Distribuição heterogênea mesmo em maiores tempos de incubação.**

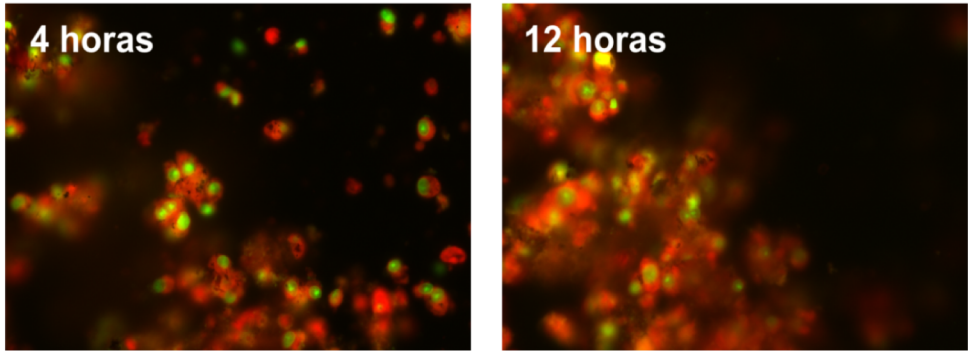
**Pico 620nm:** Photogem

**Pico 530nm:** Laranja de acridina



# Distribuição do Photodithazine

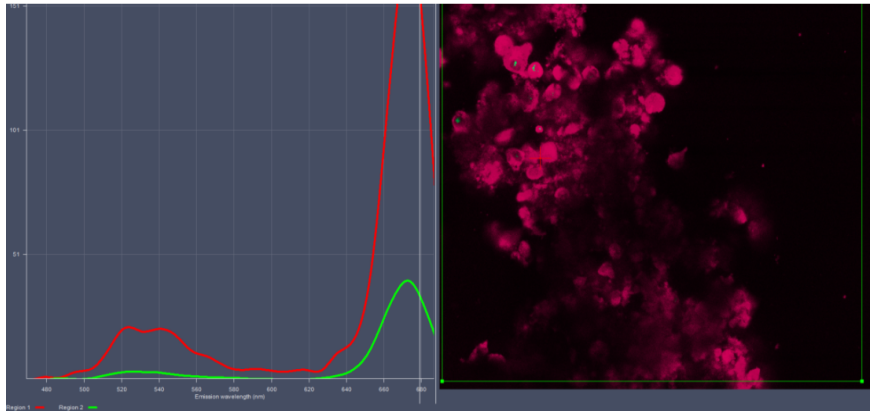
**Incubação com Photodithazine 50 µg/mL (Escala: 50 µm). Aumento de 40 vezes.**




**Distribuição homogênea do Photodithazin**

**Photodithazine melhor para TFD em melanoma. Mais hidrofóbico que Photogem – melhor distribuição. Confocal análise qualitativa. Quantificação intracelular.**

**Photodithazine**



# Quantificação de fotossensibilizador intracelular

Cultura 2D

| Fotossensibilizador | Tempo de incubação (horas) | Concentração de FS intracelular (µg/mL) |
|---|---|---|
| Photodithazine® | 4 | (3,00 ± 0,05) |
| | 8 | (3,24 ± 0,05) |
| | 16 | (5,70 ± 0,11) |
| | 24 | (3,45 ± 0,06) |
| Photoge | 4 | (1,52 ± 0,05) |
| | 8 | (2,71 ± 0,50) |

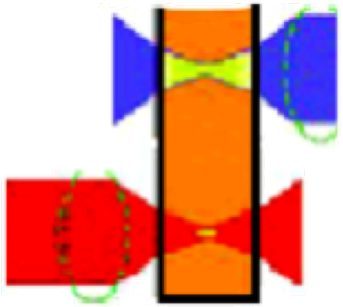
**Maior acúmulo de Photodithazine nos tumores de melanoma (3D).**
**Tempo de incubação para os experimentos de TFD de 16 horas – *uptake* de aproximadamente 5,34%.**

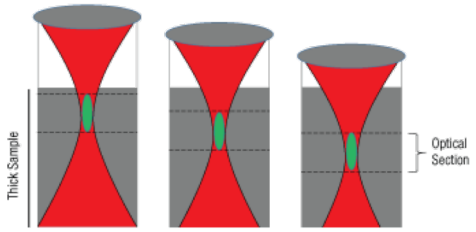
Cultura 3D

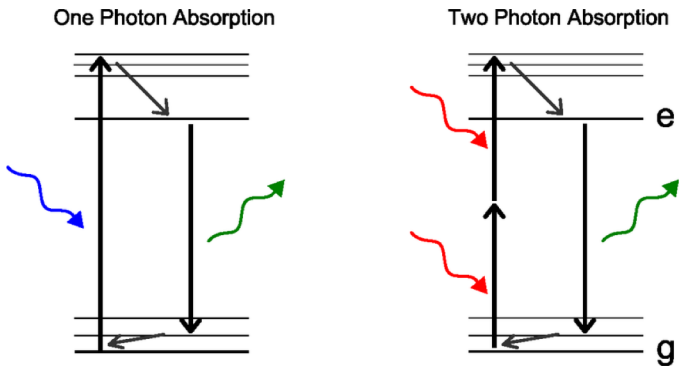
| Fotossensibilizador | Tempo de incubação (horas) | Concentração de FS intracelular (µg/mL) |
|---|---|---|
| Photodithazine® | 4 | (0,82 ± 0,02) |
| | 8 | (1,60 ± 0,03) |
| | 16 | (2,67 ± 0,04) |
| | 24 | (1,90 ± 0,03) |
| Photogem® | 4 | (0,25 ± 0,03) |
| | 8 | (0,80 ± 0,10) |
| | 16 | (1,08 ± 0,12) |
| | 24 | (1,97 ± 0,19) |

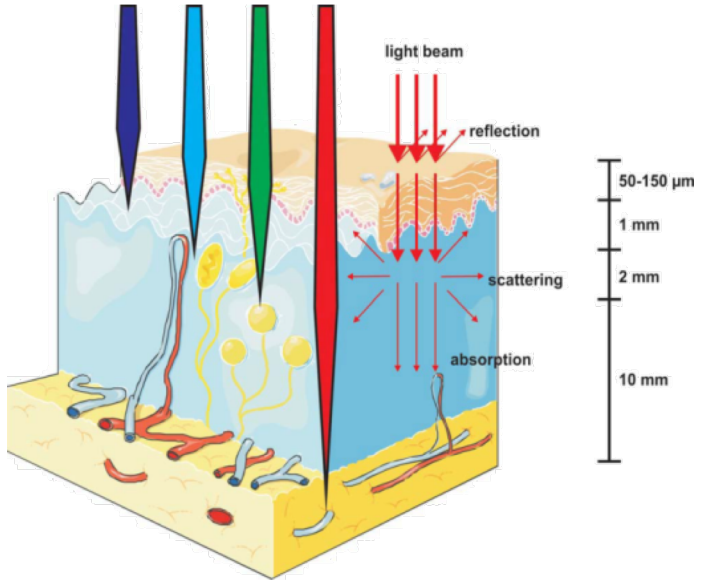
# MICROSCOPIA MULTIFÓTONS

K. Konig, 2008





# Förster Resonance Energy Transfer

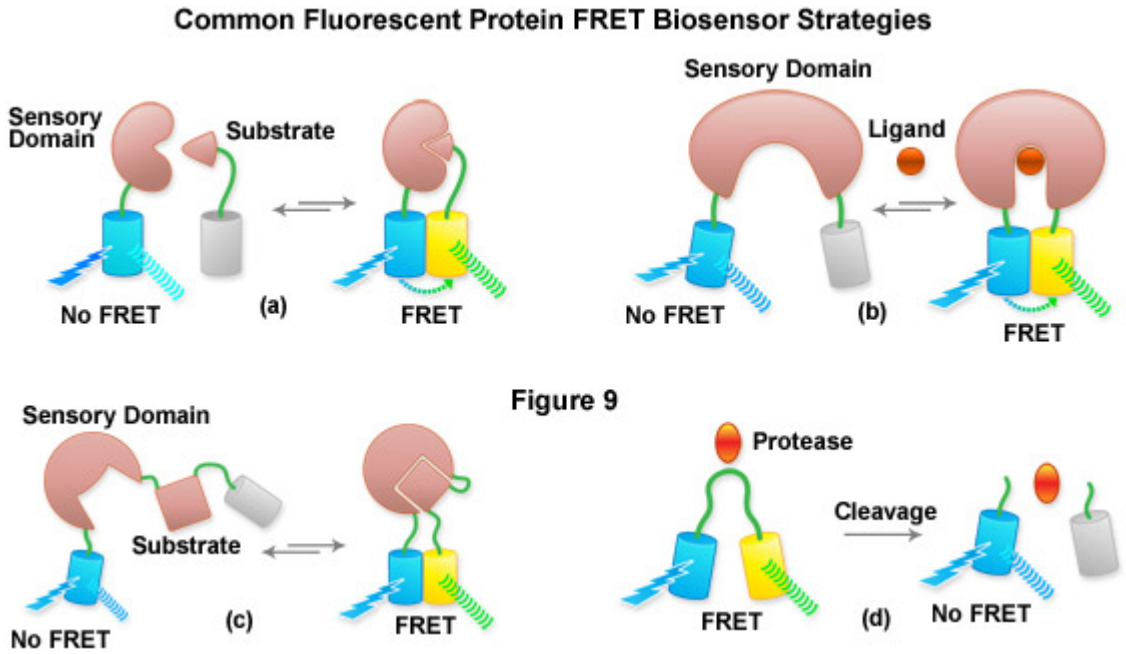



Figure 9

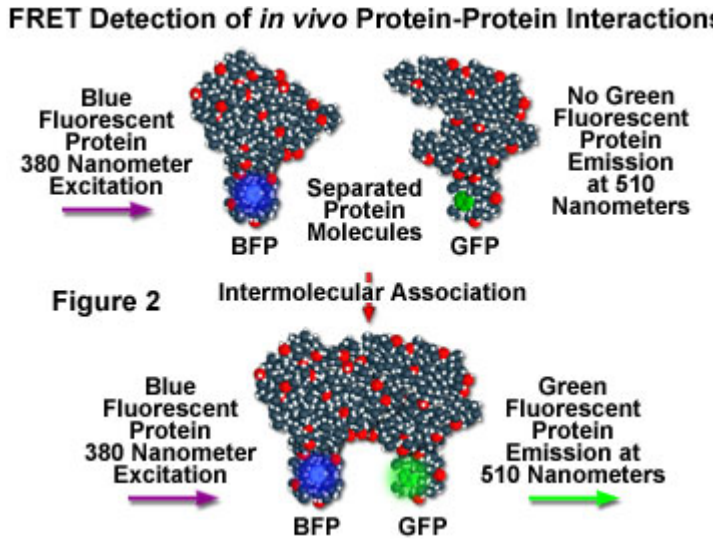



Figure 2

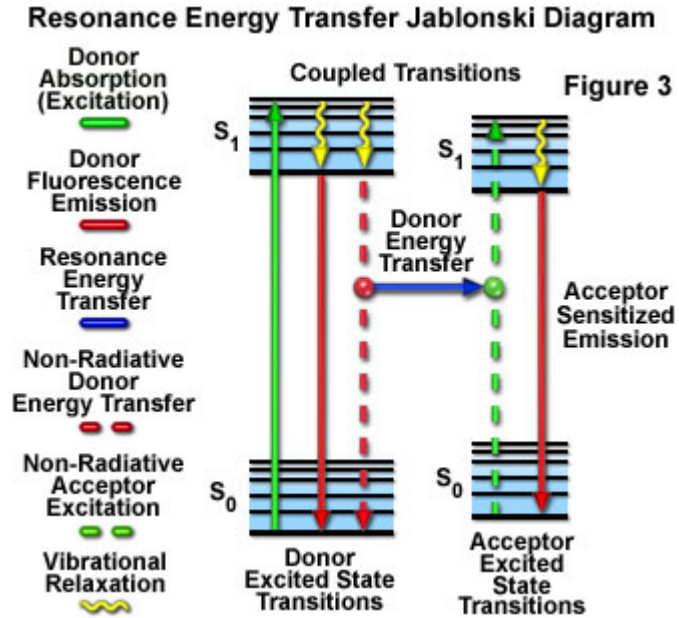



Figure 3

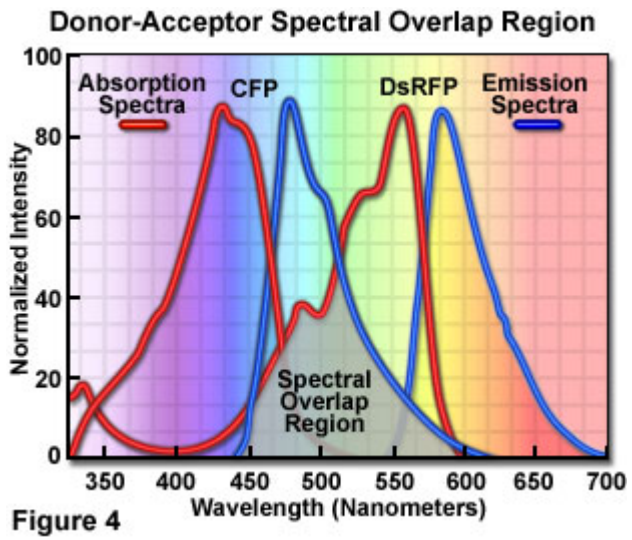



Figure 4

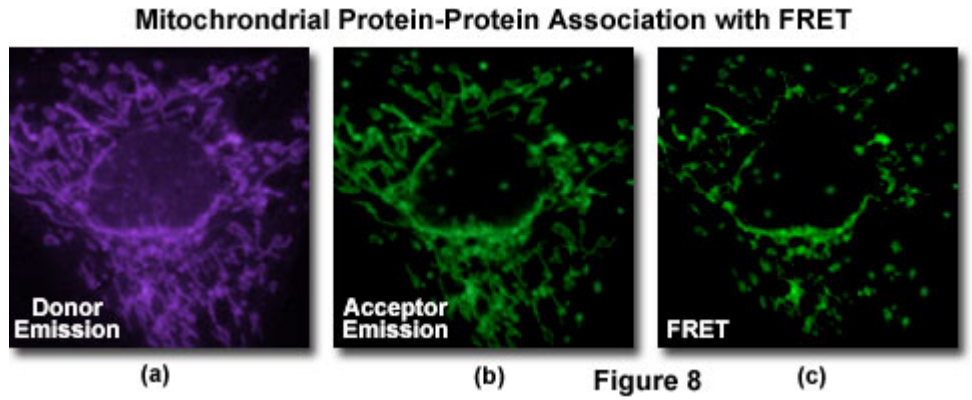
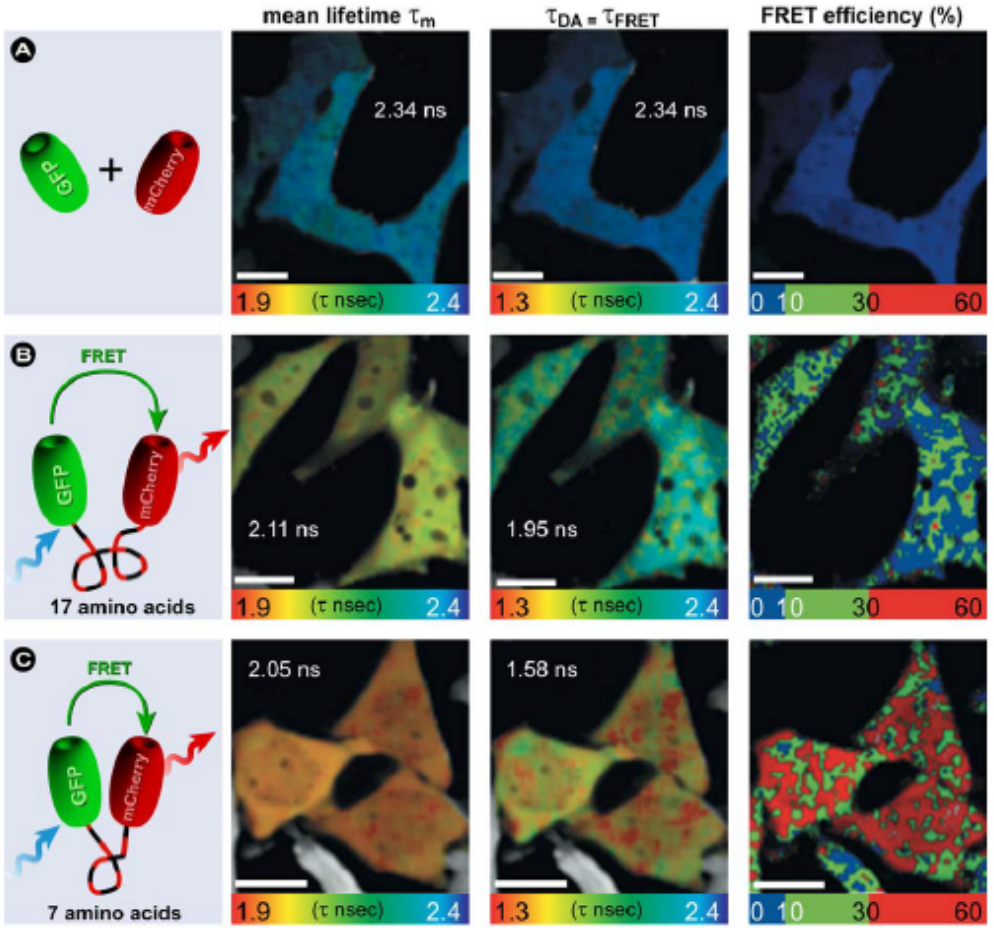



Figure 8

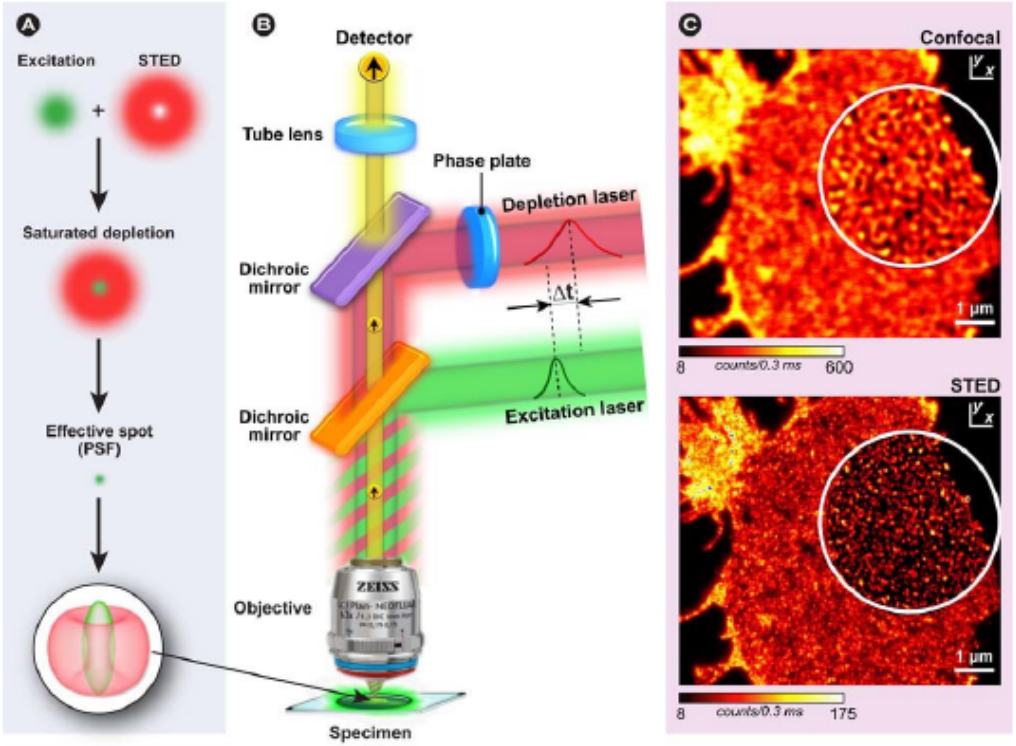
https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/fret

**Figure 23.** *In vivo* multiphoton FLIM-FRET measurements. Living HeLa cells co-expressing either unfused, free EGFP and unfused, free mCherry (**A**), or GFP-coupled directly to mCherry through a 17-amino-acid linker (**B**), or GFP-coupled directly to mCherry through a 7-amino-acid linker (**C**) were imaged by using a multiphoton scanning microscope. For each panel, the spatial distribution of the mean fluorescence lifetime ($\tau_m$) and of the fluorescence lifetime of the donor molecules interacting with the acceptor ($\tau_{DA}$) is shown throughout the cells. The FRET efficiencies were calculated for each pixel from Eq. 24 × 100%. Color scale shown covers the range of $E_{FRET}$ values from 0% to 60%. Bars, 10 μm. Adapted from [217] with permission. © 2007 John Wiley & Sons.




HC Ishikawa-Ankerhold et al. Molecules 2012, 17.

**Figure 26.** Principle of stimulated emission depletion (STED) microscopy. (**A**) STED is based on shrinking the excitation focal spot by depleting the outer excited state fluorochromes through stimulated emission with a doughnut-shaped STED beam of red-shifted and Δt time-shifted light (**B**). In essence the excitation PSF is combined with the PSF of the STED depletion laser (**B**) to produce a resultant PSF that is smaller than the diffraction limit of light. (**C**) Ultra-high resolution nanopattern distribution of the antibody-tagged SNARE protein SNAP-25 on the plasma membrane of a mammalian cell imaged with confocal and STED microscopy. The encircled areas show linearly deconvolved data. STED microscopy provides a substantial leap forward in the imaging of protein self-assembly; here it reveals for the first time that SNAP-25 is ordered in clusters of <60 nm average size. Part C adapted from [288]. © 2006 IOP Publishing Ltd.




# STED microscopy

HC Ishikawa-Ankerhold et al. Molecules 2012, 17.

# Localization-based microscopy

Super-resolution microscopy

# Fluorescence Microscopy

JFW Herschel, Philos Trans R Soc London, 1845

GG Stokes, Philos Trans R Soc London, 1852

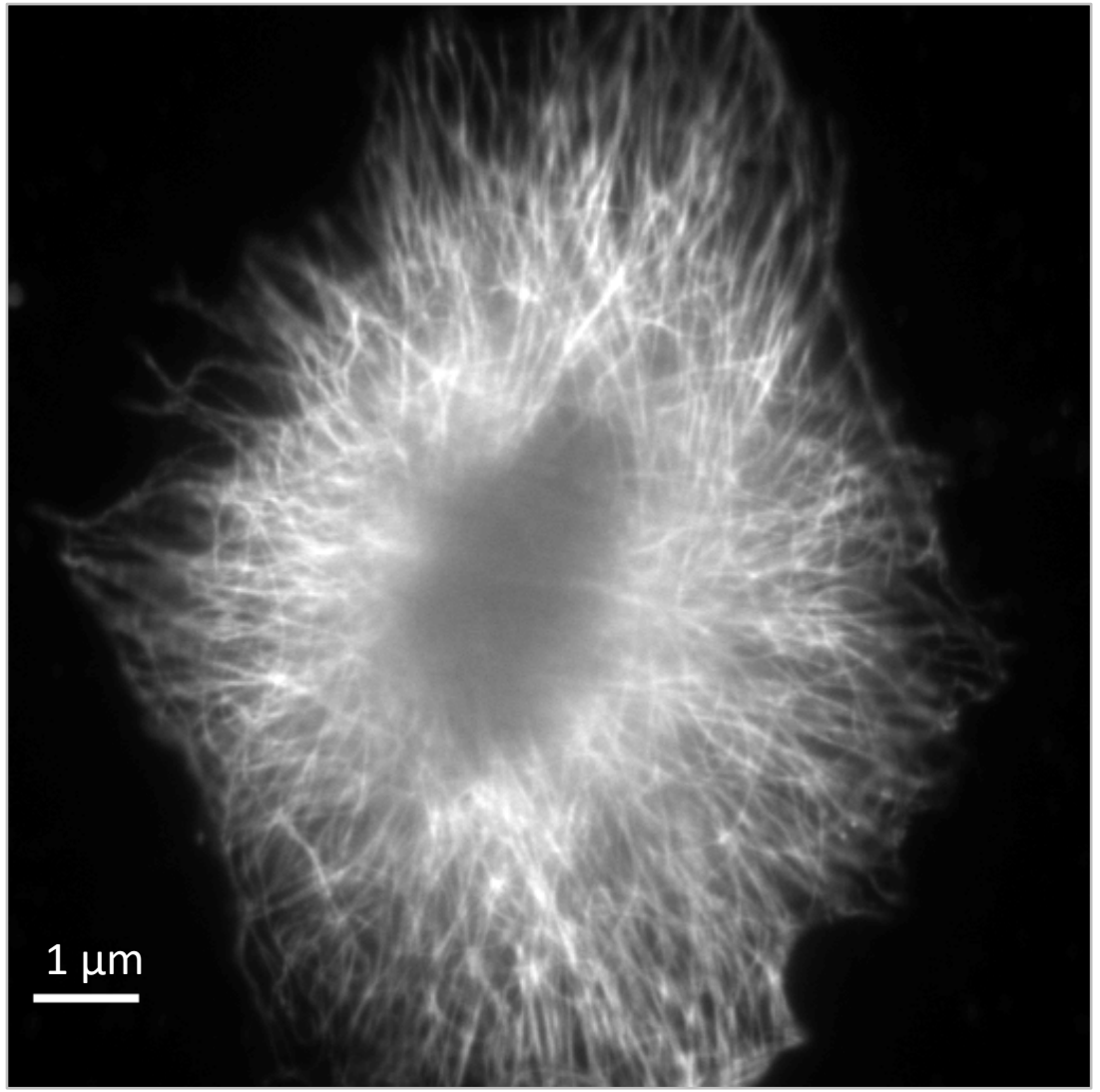
OD Heimstadt, Z wiss Mikr, 1911

AH Coons et al., J Immunol, 1942

DC Prasher et al., Gen, 1992.

**(~1950)**

**Microtubules**



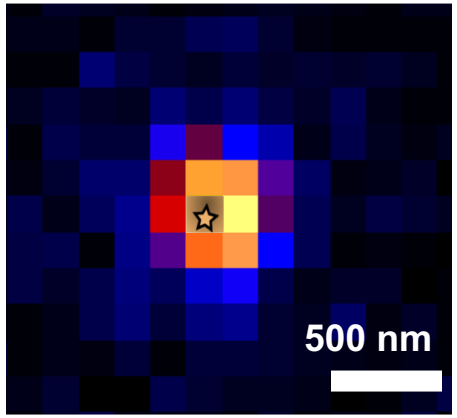

- **Specificity**
- **Contrast**

- **Low spatial resolution (~250 nm)**

# Diffraction limit

## Point Spread Function (PSF)

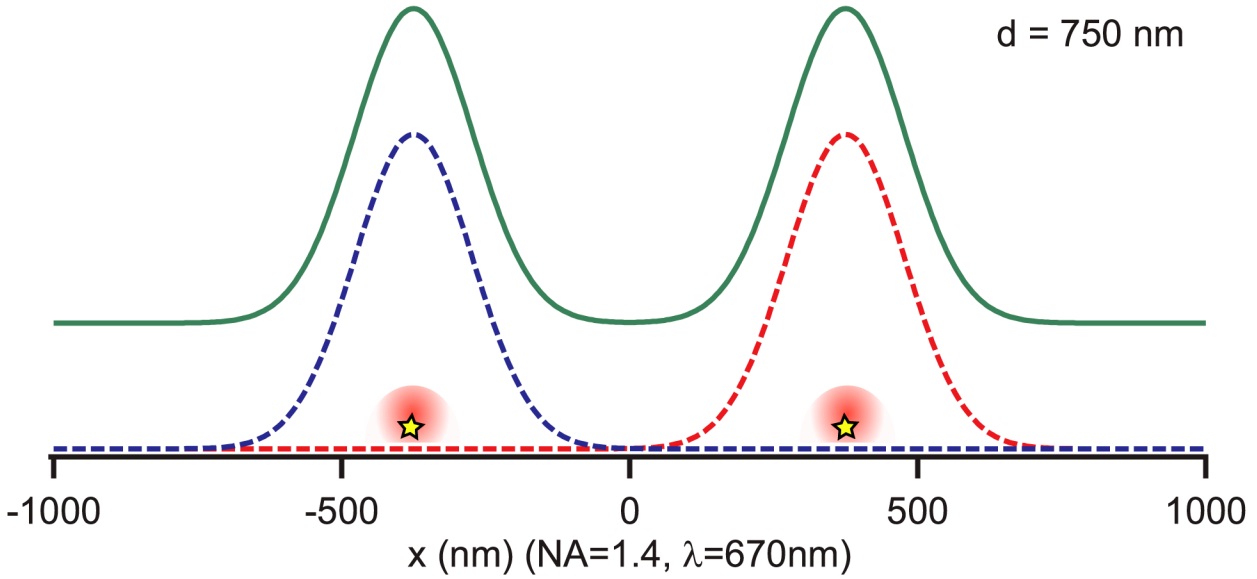
GB Airy, Transactions of the Cambridge Philosophical Society, 1835

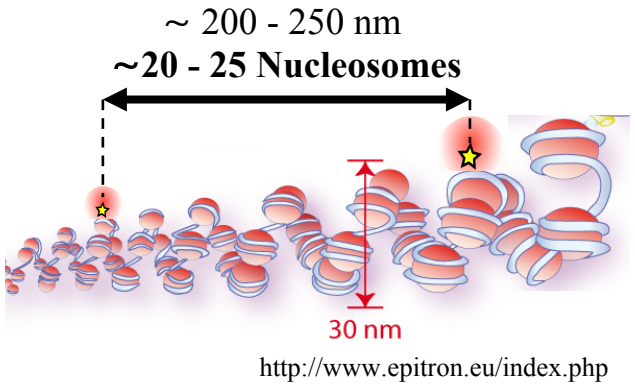



E Abbe, Arch Mikr Anat,1873

$$w_{x,y} \approx \frac{\lambda}{2NA} > 200nm$$

$$w_z \approx \frac{2\lambda\eta}{(NA)^2} > 500nm$$

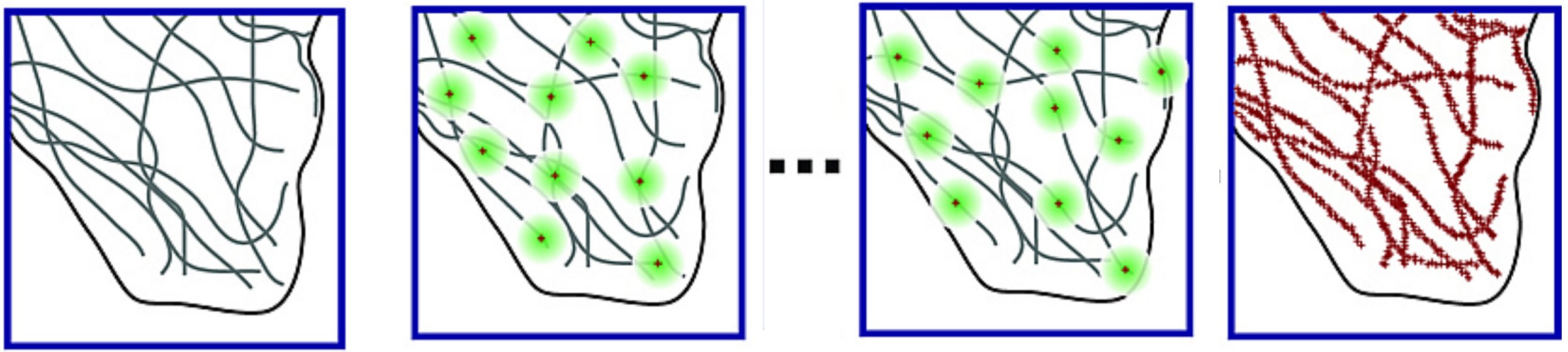



http://www.epitron.eu/index.php

# Localization-based microscopy

**Target structure** | **Localizing activated subset of probes** | **Reconstructed image**



SW Hell et al., Optics letters, 1994

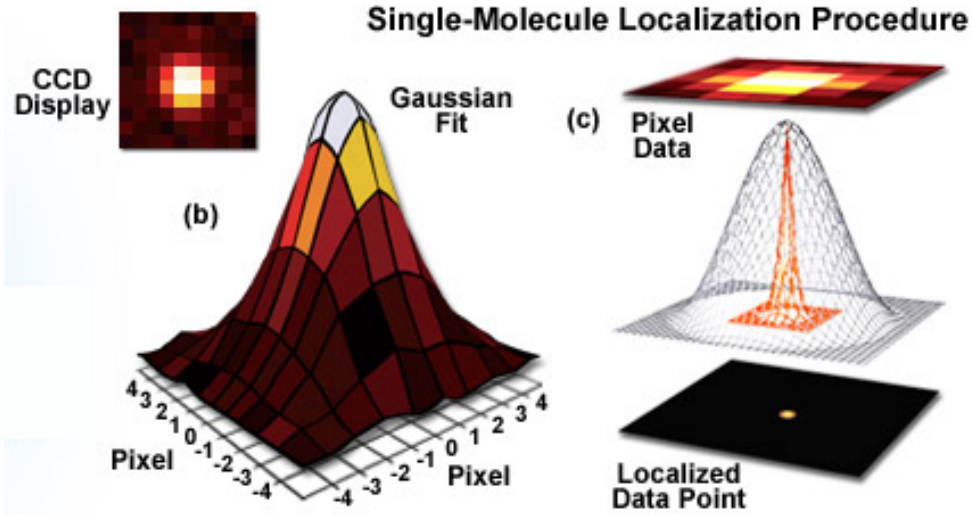
RM Dickson, WE Moerner et al., Nature, 1997

E Betzig et al., Science, 2006

MJ Rust, M Bates, X Zhuang, Nature methods, 2006

**(~2005)**

***Super-resolution microscopy**




Rust et al. Nature Methods, 2006

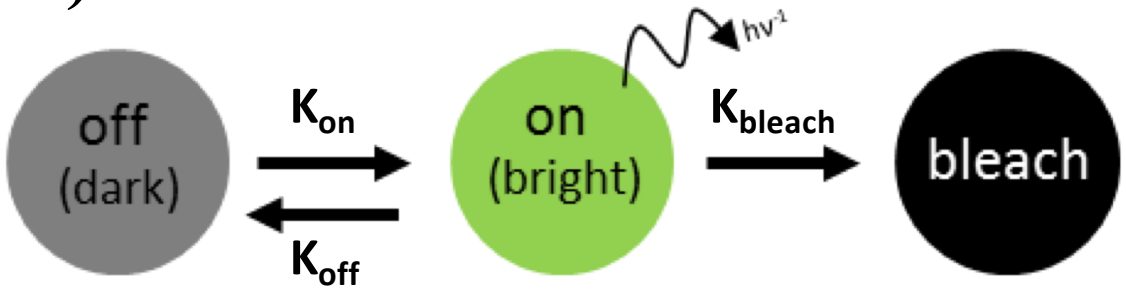
**Localization precision of ~10-50 nm**

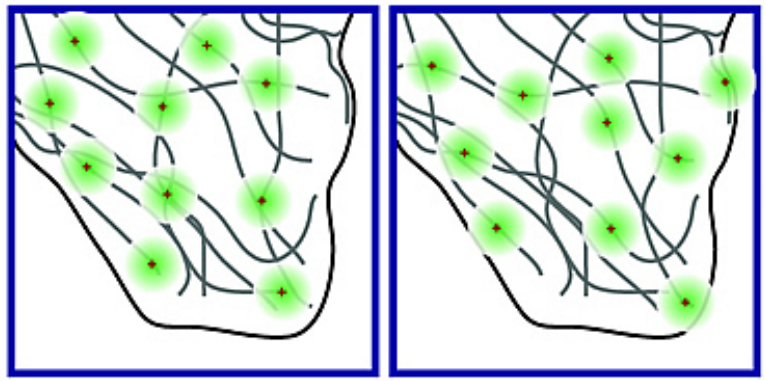
***depends on the number of collected photons**

# STORM

(~2005)

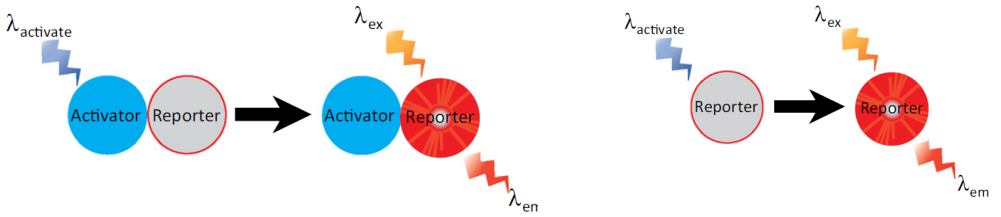
## Photoswitchable dyes



Rust et al., Nature Methods, 2006

- Medium buffer (Oxygen scavenger)
- High laser power density (~300 W/cm$^2$)
- Activator/Reporter or Reporter




Sydor et al., Trends in Cell Biology, 2015

***The quality of the image depends on the photophysical properties of the dye**

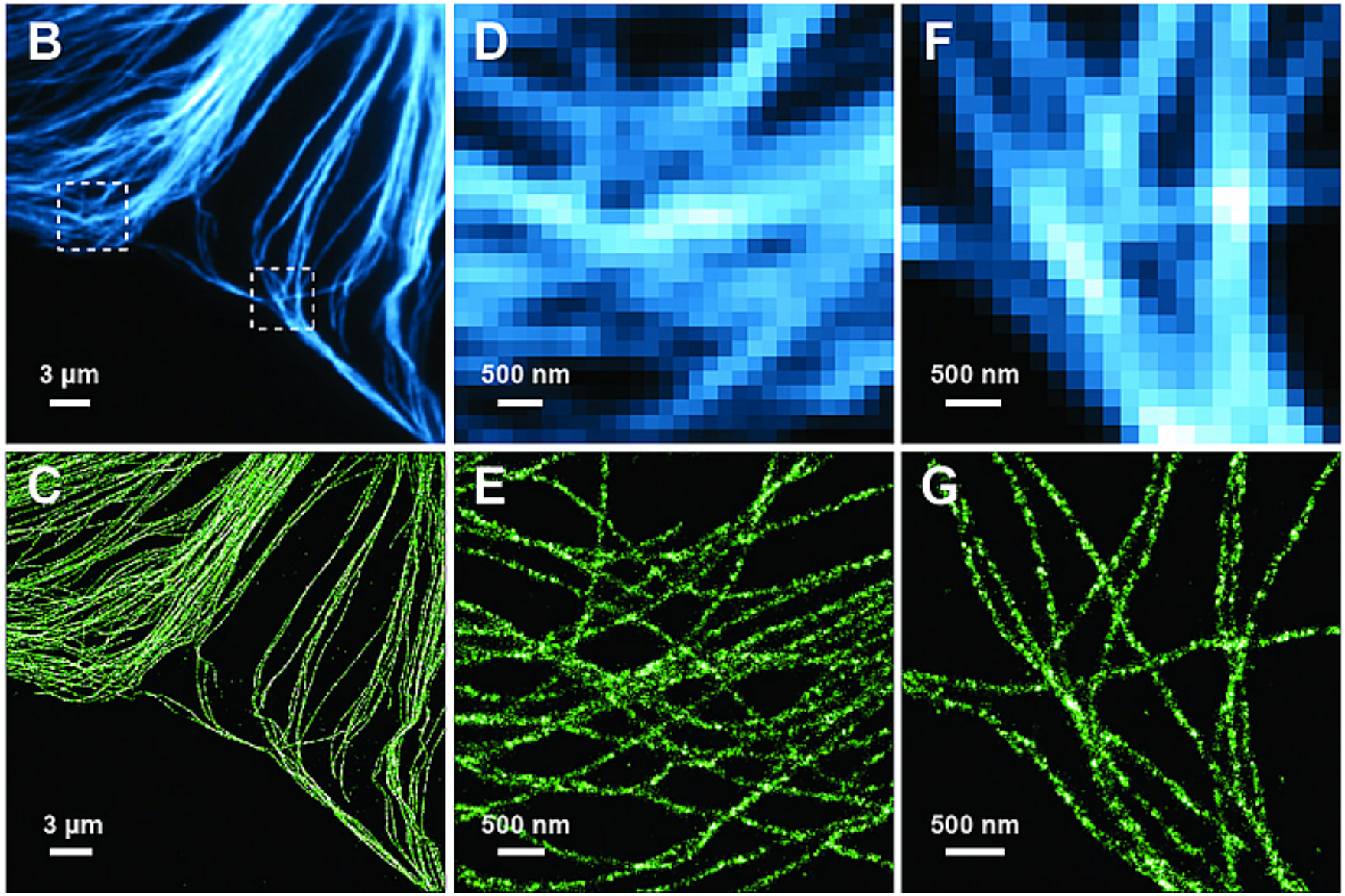
- Small ratio of: $k_{on}$ / $k_{off}$
- High quantum efficiency
- Bright
- Low photobleaching

STORM: Stochastic Optical Reconstruction Microscopy

# Fluorescence Microscopy: STORM & Conventional

**Conventional microscopy**
**~250 nm resolution**

**Localization-based microscopy**
**~20 nm resolution**



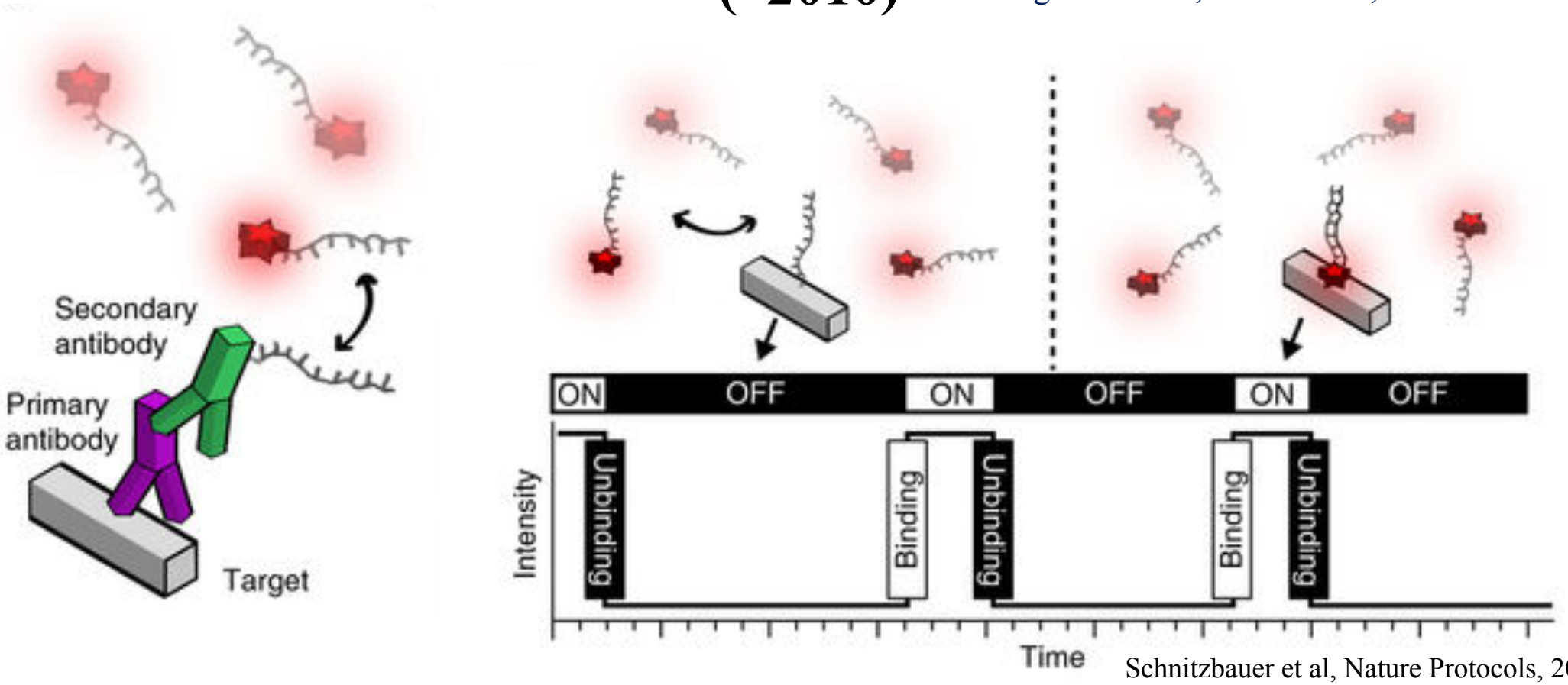

STORM: Stochastic Optical Reconstruction Microscopy

Bates et al., Science, 2007

# DNA-PAINT

## (~2010)

R Jungmann et al., Nano letters, 2010



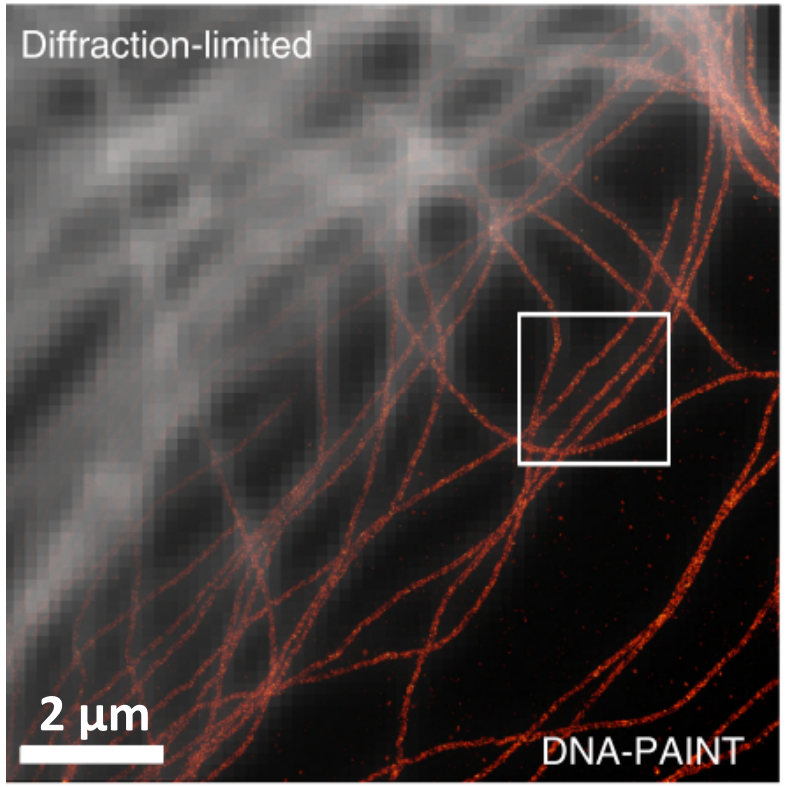

Schnitzbauer et al, Nature Protocols, 2017

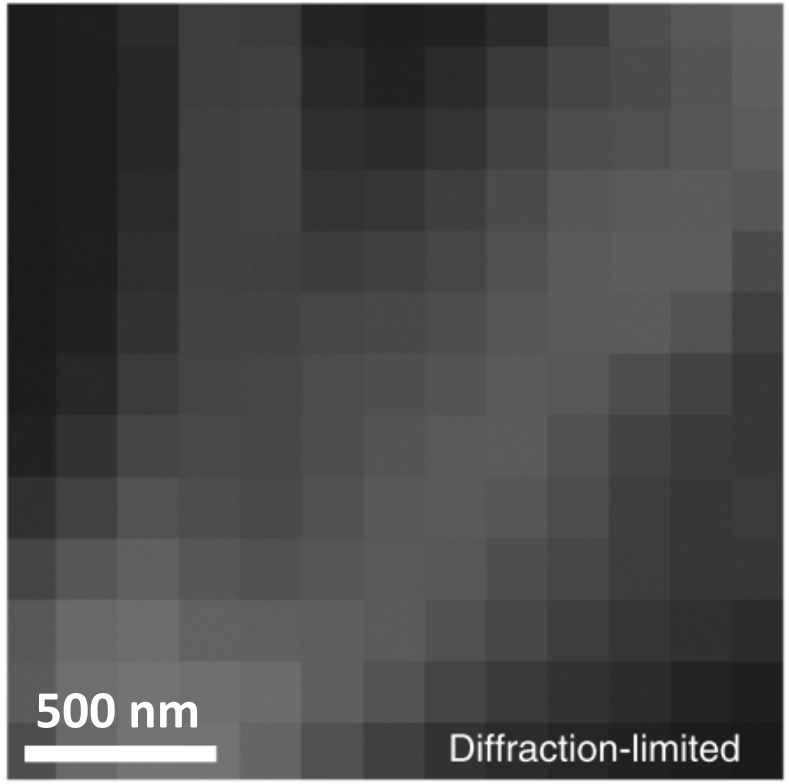
- **Does not rely on fluorophore photo-physics**
- **No irreversible photo-bleaching (low laser)**
- **Image buffer not required**

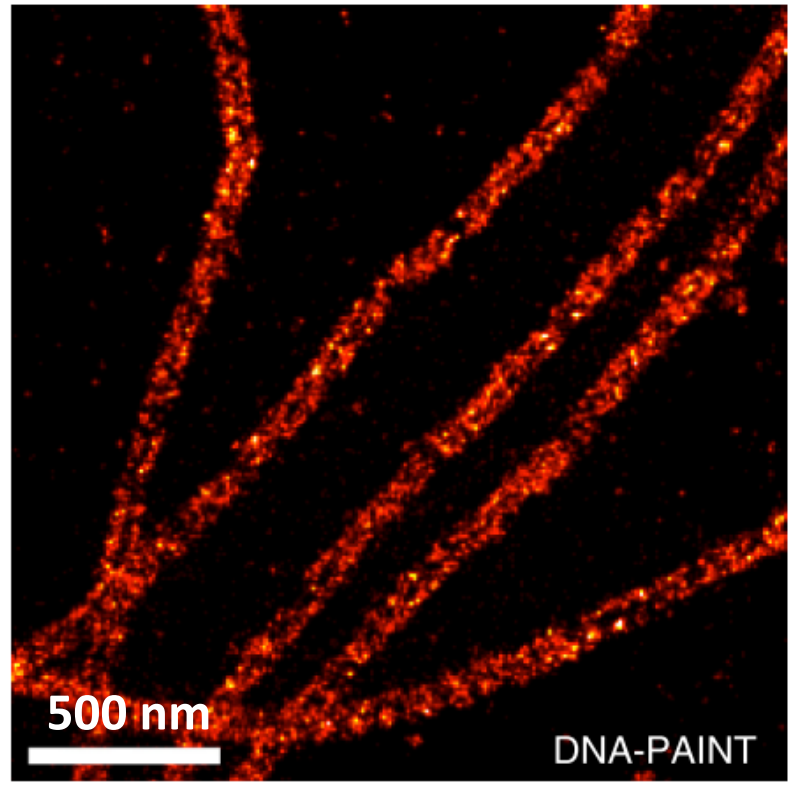
- **Requires long exposure times (100 – 300 ms)**
- **Time consuming**

DNA-PAINT: DNA-based Point Accumulation for Imaging in Nanoscale Topography

# DNA-PAINT



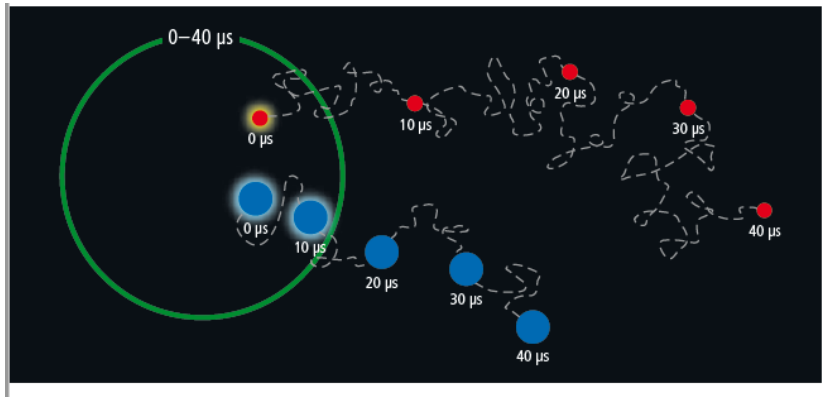




Schnitzbauer et al, Nature Protocols, 2017

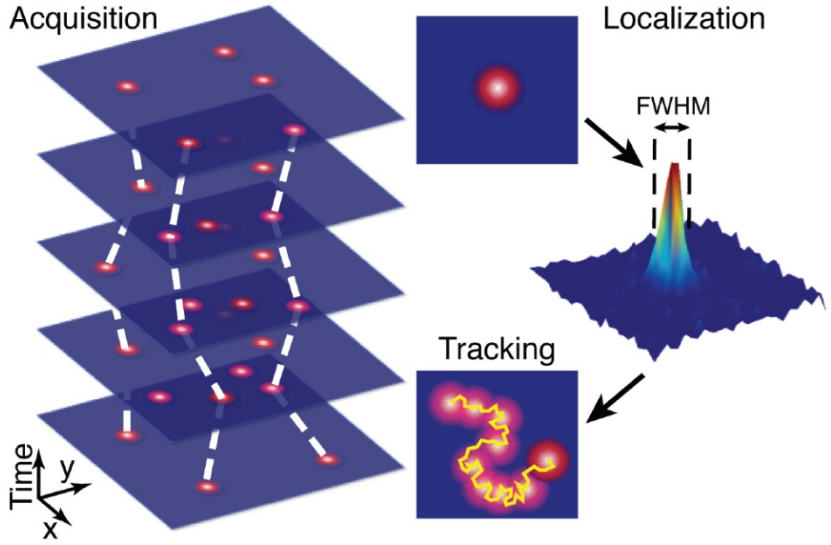
DNA-PAINT: DNA-based Point Accumulation for Imaging in Nanoscale Topography

# Fluorescence correlation spectroscopy

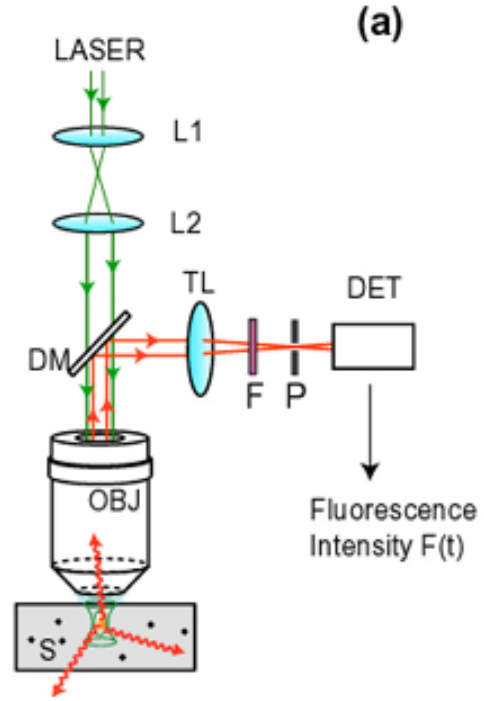


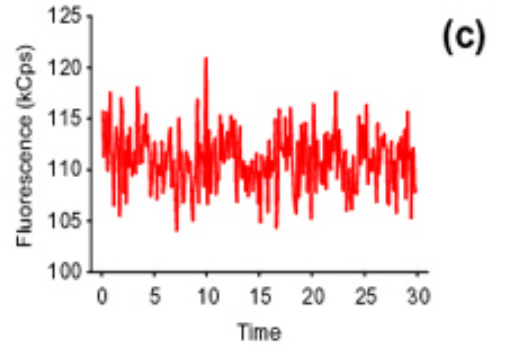

www.research.nki.nl

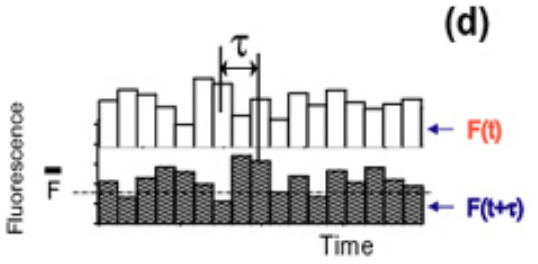
Single molecule tracking

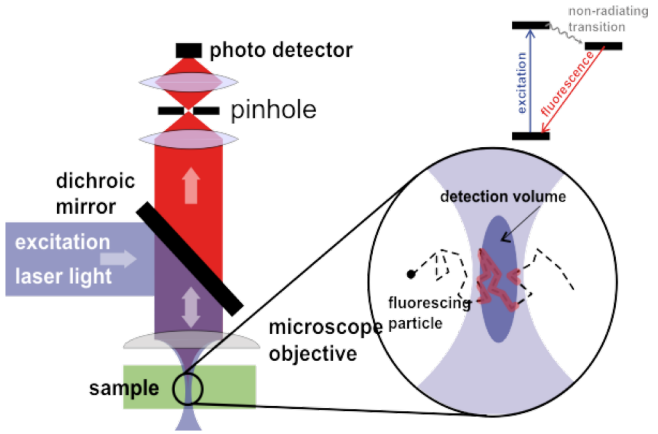


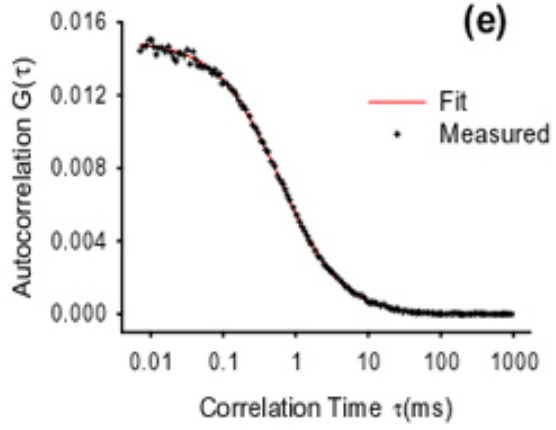

Manzo and Garcia-Parajo, Rep Prog Phys 78(12), 2015



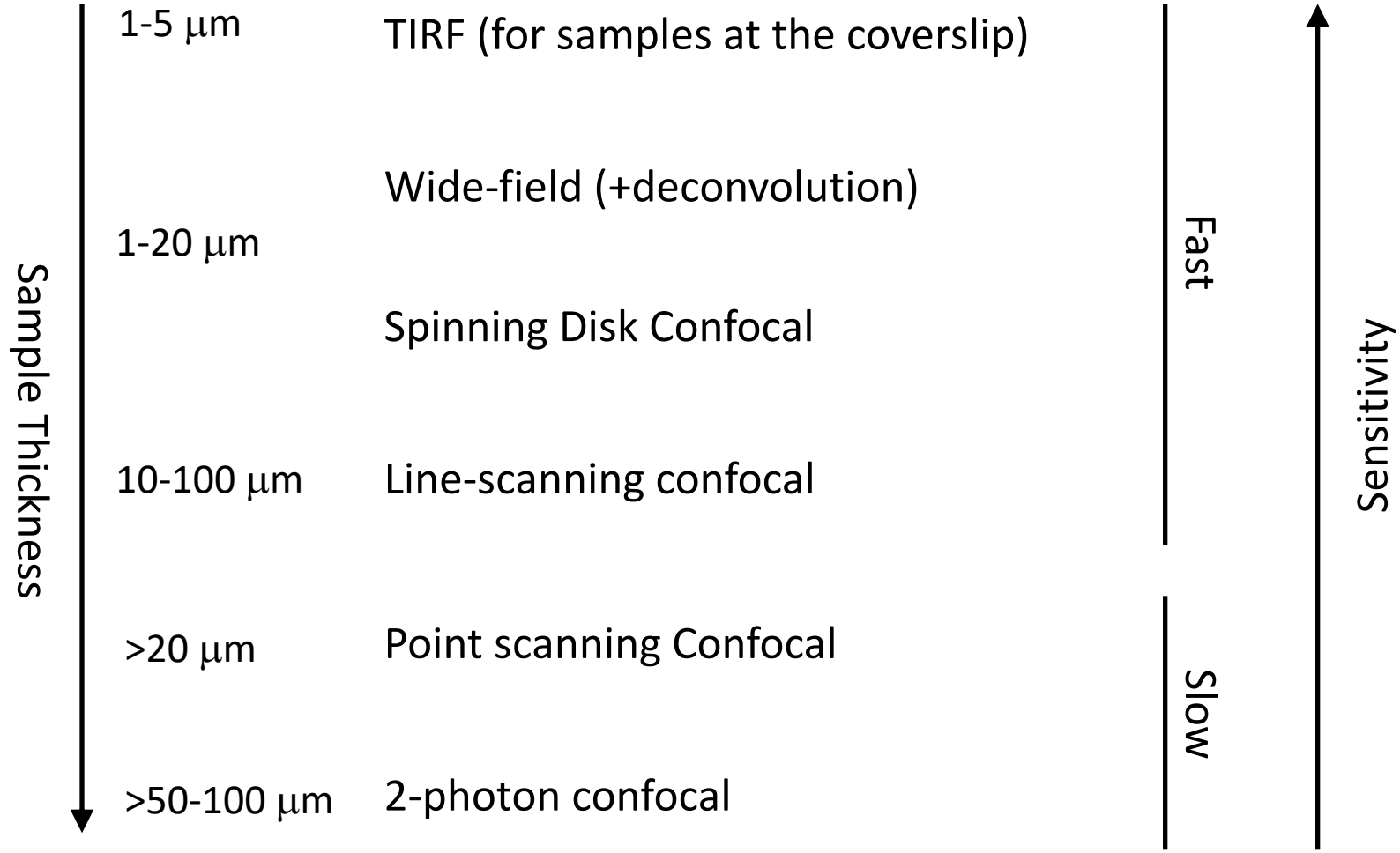



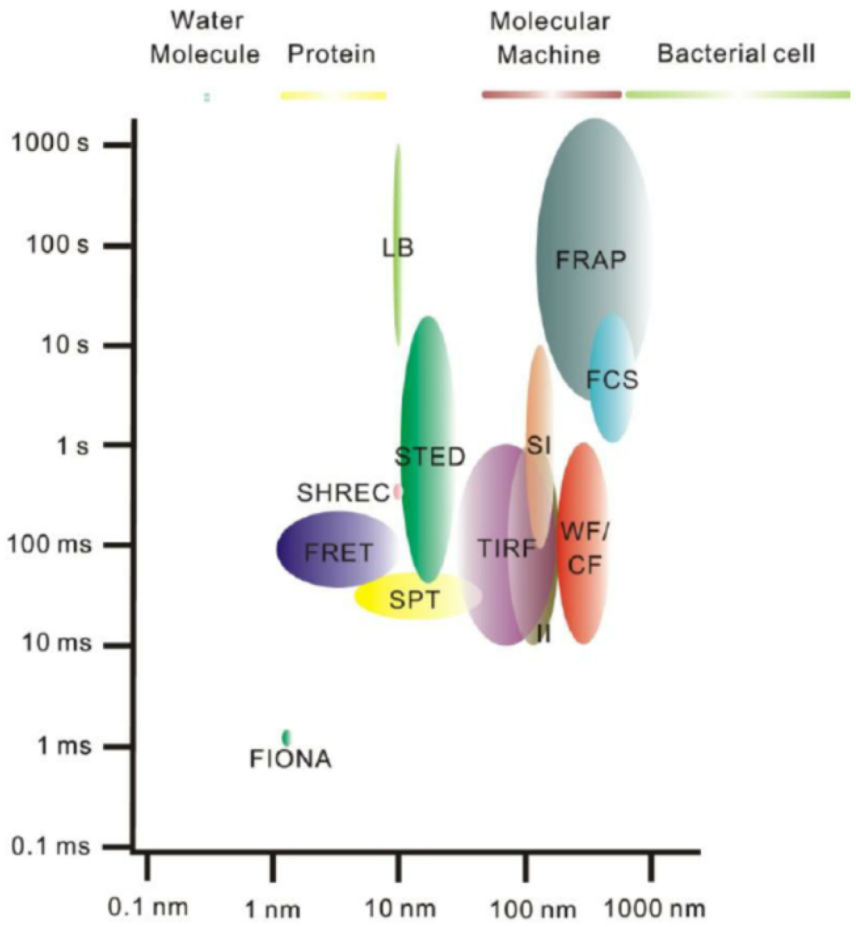



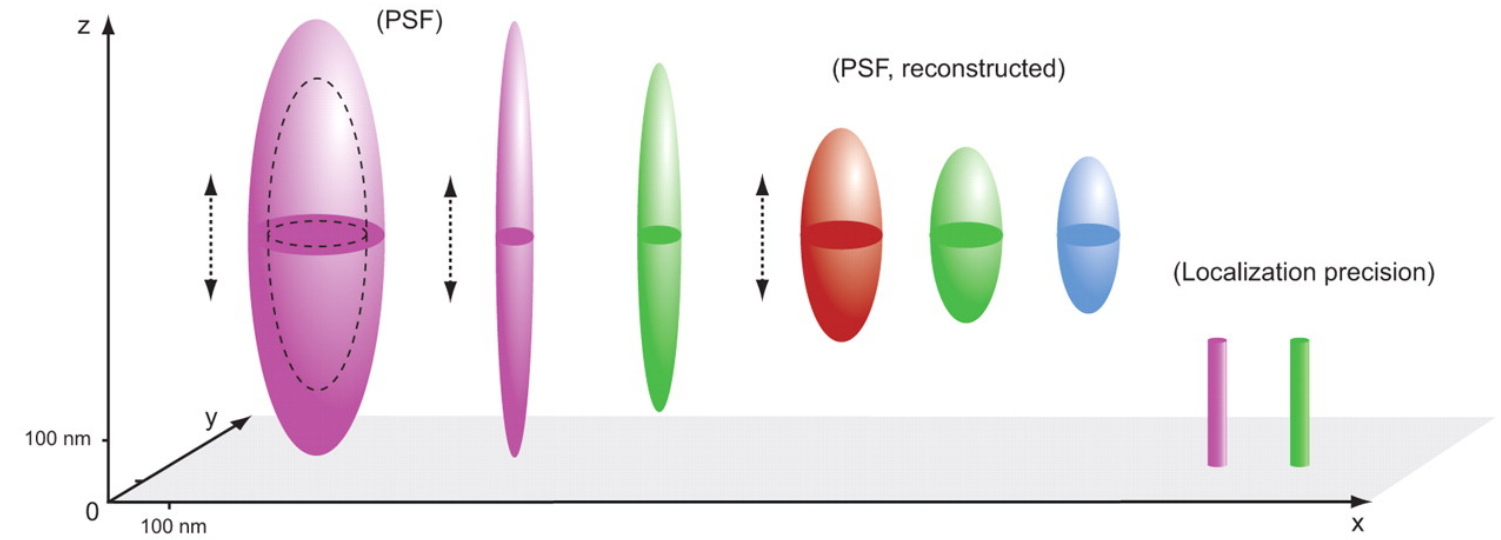



# Which imaging technique should I use?





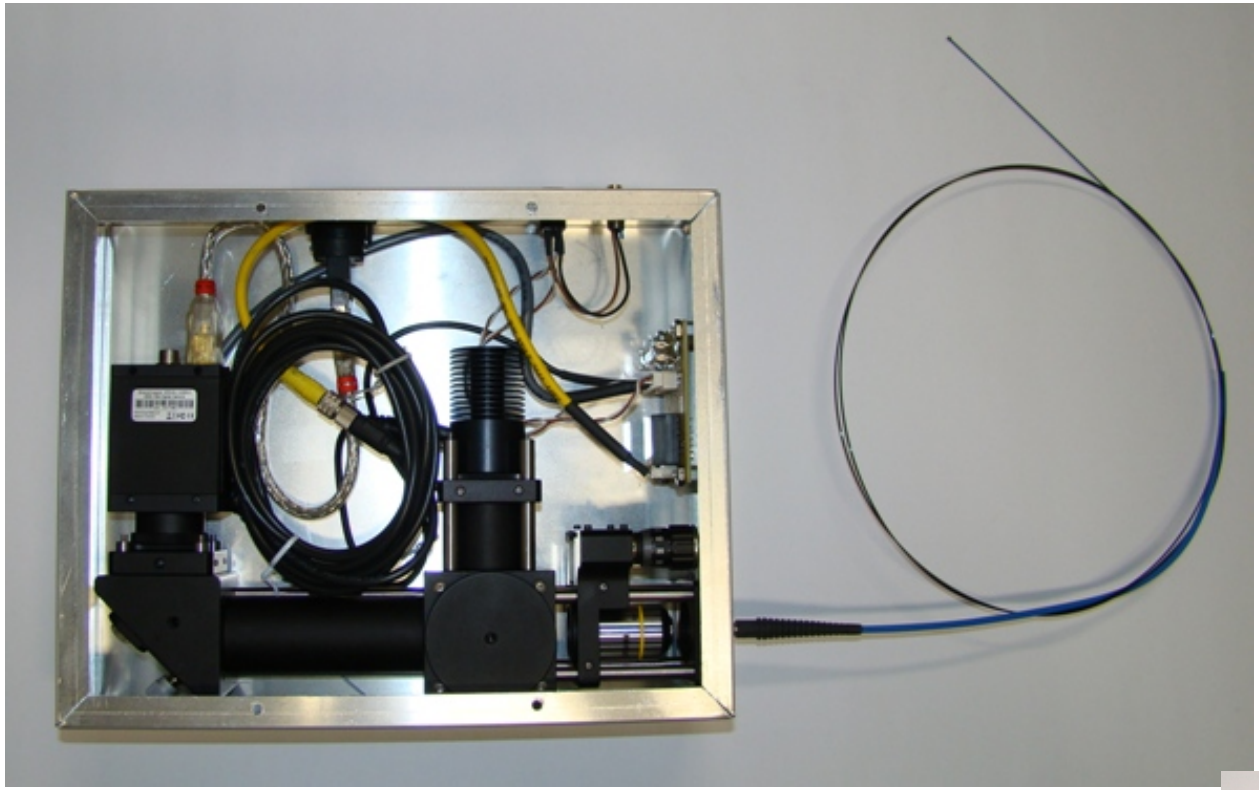

| | CLSM | STED | CW-STED | 3D-SIM | | | PALM/STORM | |
|---|---|---|---|---|---|---|---|---|
| ~$\lambda_{em}$ [nm] | 460-670 | 670 | 520 | 620 | 520 | 460 | 670 | 520 |



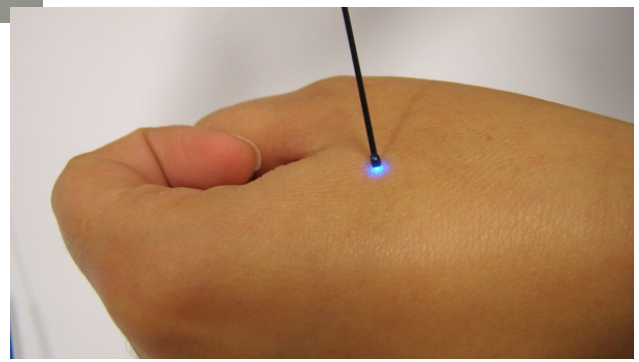

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ~$D_{x,y}$ [nm] | 180-250 | 60 | 70 | 130 | 110 | 100 | 30 |
| ~$D_z$ [nm] | 500-700 | 700 | 560 | 340 | 280 | 250 | 140 (TIRF-range) |
| ~$V_{x,y,z}$ [*$10^{-3}$ µm$^3$] | 10-23 | 1.3 | 1.5 | 3.0 | 1.8 | 1.3 | 0.1 |

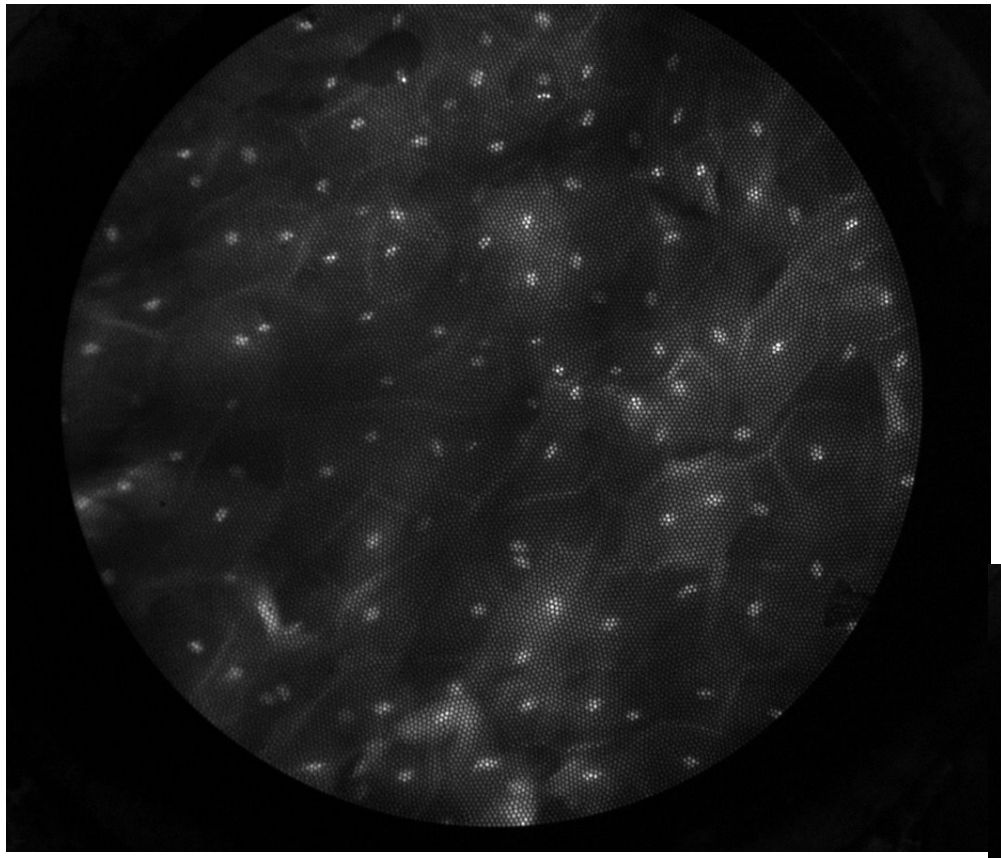
Chiu, Sheng-Wen and Leake, Mark C. Int J Mol Sci 12(4):2518-42, 2011

# Sistema de microscopia a fibra



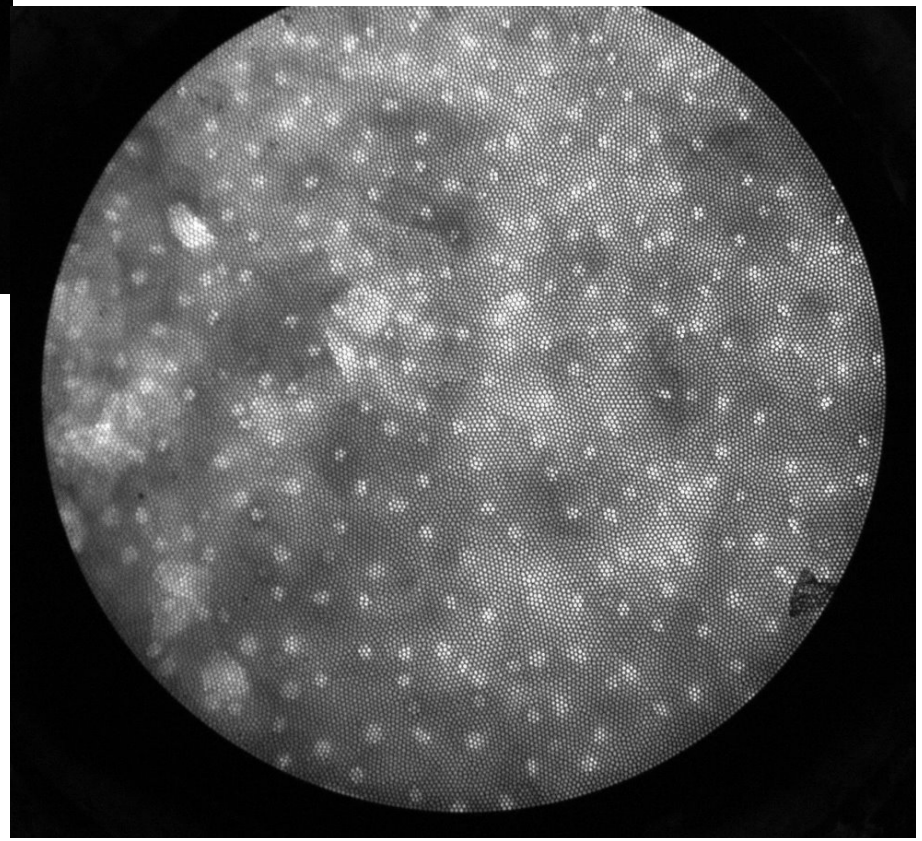
T. Muldoon,
M.C. Pierce,
R Richards-Kortum



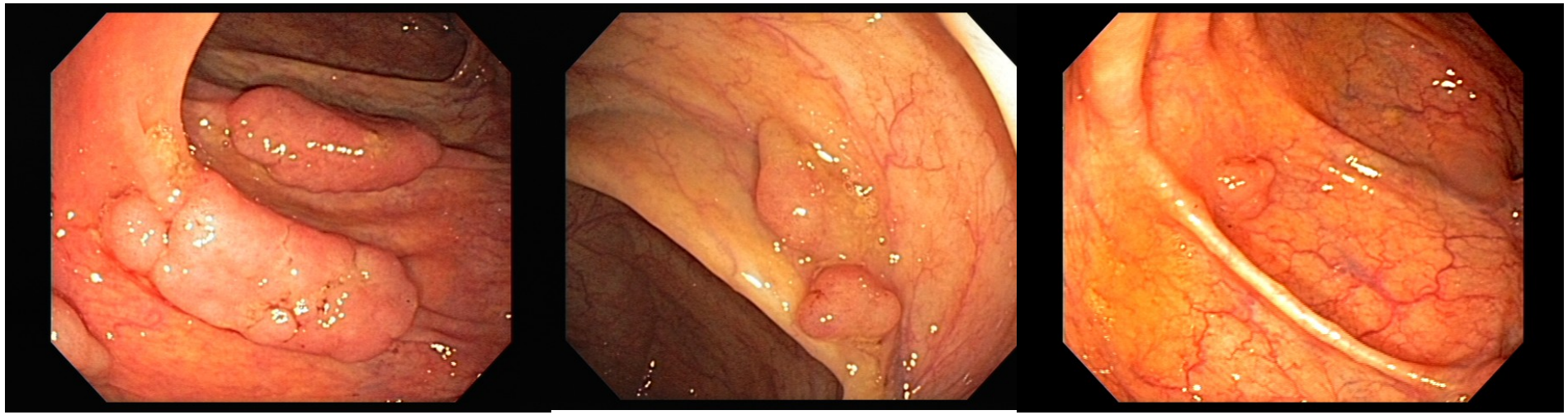


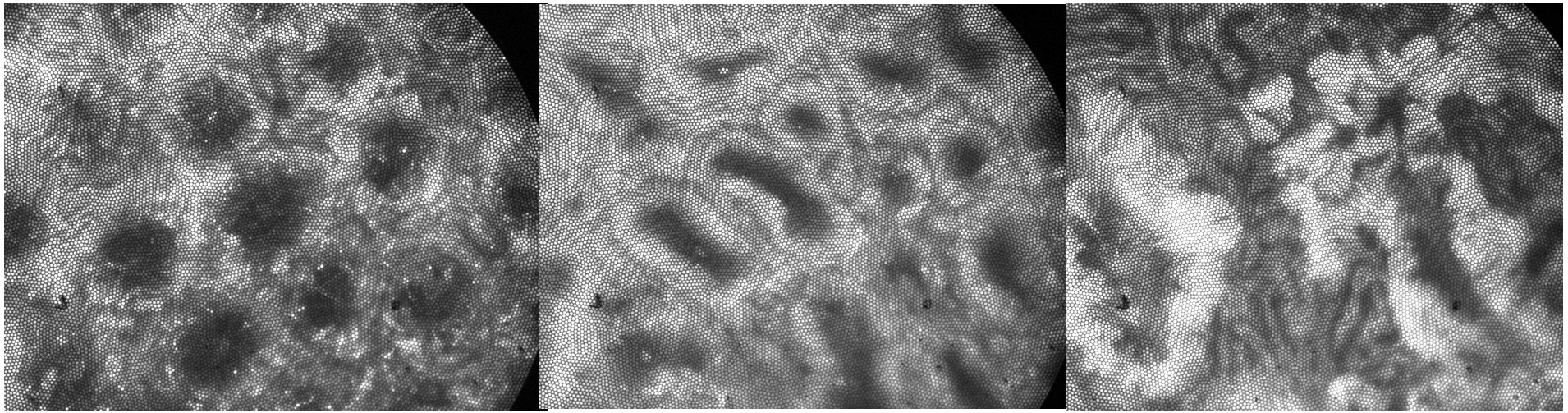
Mucosa bucal

Gengiva



# Pólipo adenomatoso – colo retal



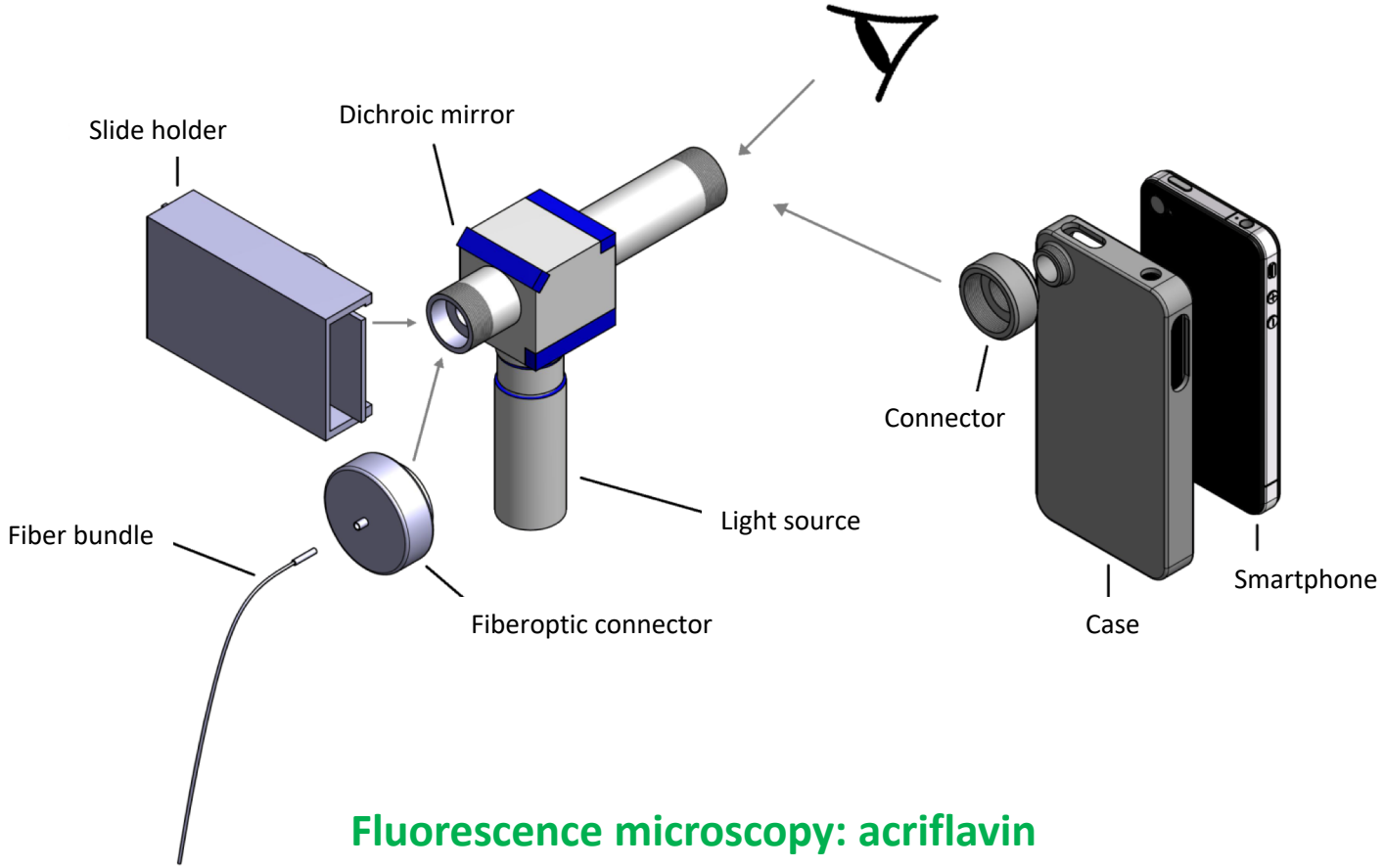


Mucosa normal

Pólipo adenomatoso

Pólipo adenomatoso

# Microscope coupled to smartphone




**Fluorescence microscopy: acriflavin**

# Dual configuration microscope

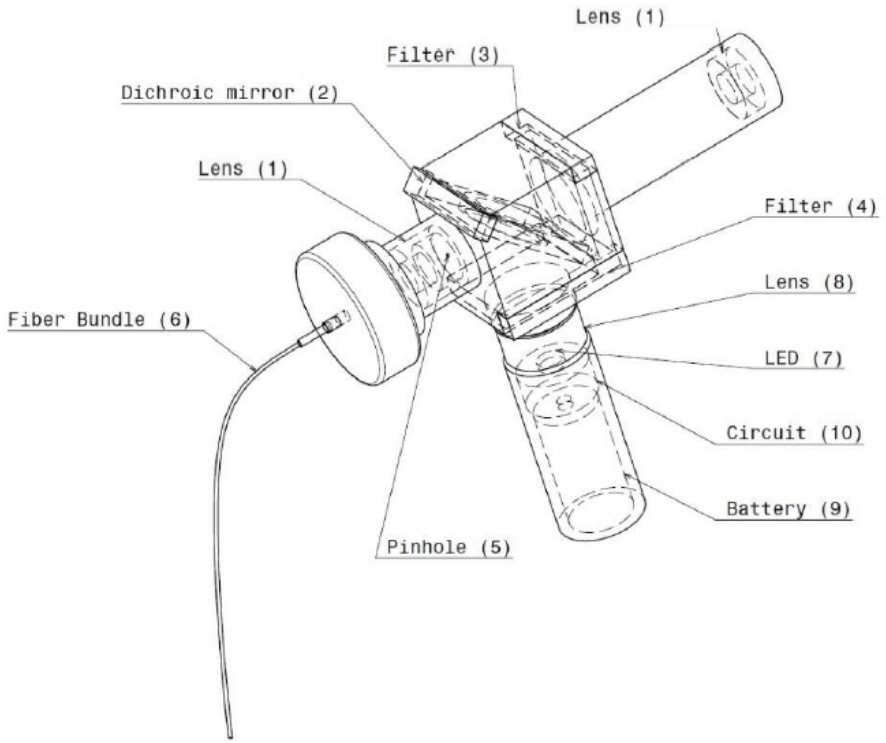



Figure 1: View of the complete system high resolution portable microendoscope assembly.

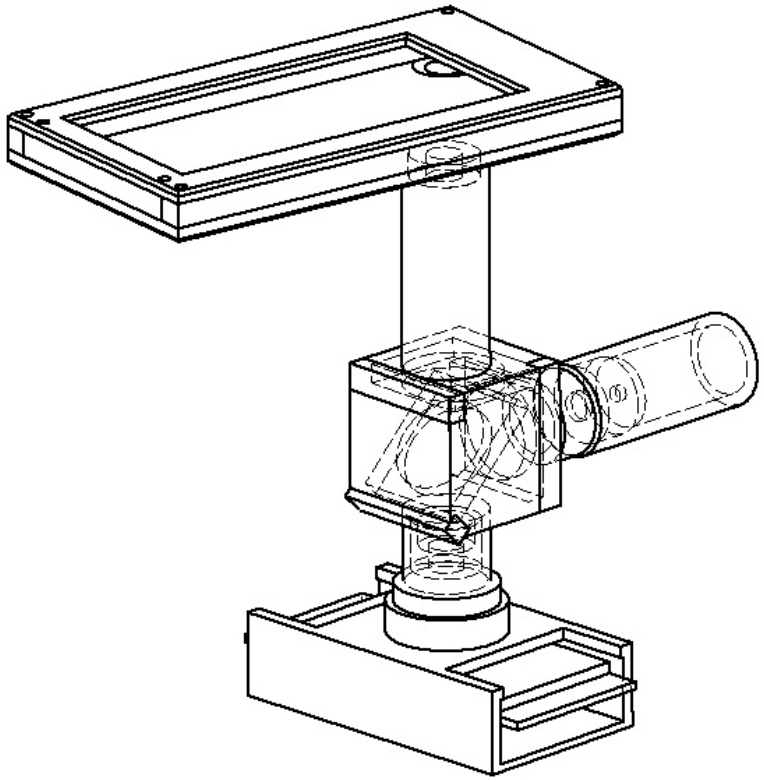
Fiber bundle IGN-08/30 (Sumitomo Corp, Japan)

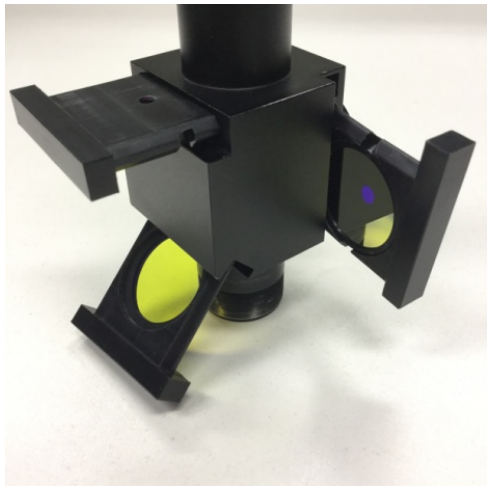
Royal Blue Rebel Luxeon LED (Philips, Netherlands)

Rechargeable 3.7V battery (TrustFire 18350, EUA)

PA Gómez-García et al., *Proc. of SPIE* Vol. 9531 953149-3 (2015)

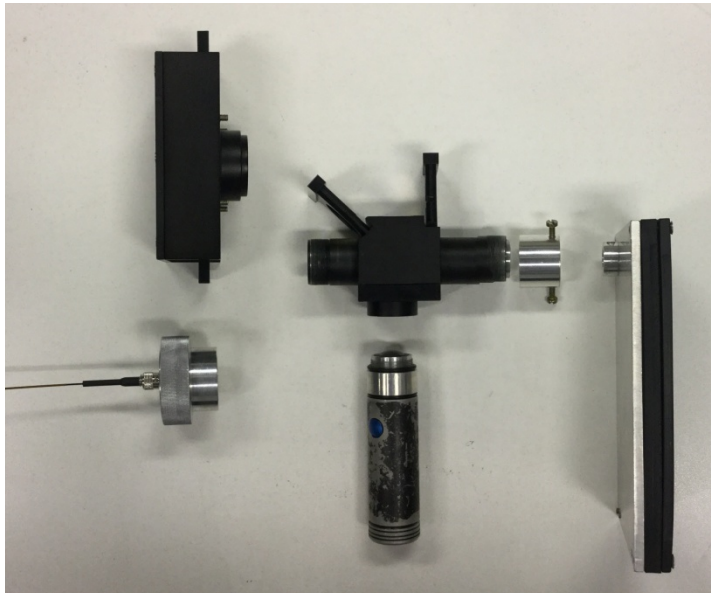
# Dual configuration microscope





PA Gómez-García et al., *Proc. of SPIE* Vol. 9531 953149-3 (2015)

# Main characteristics



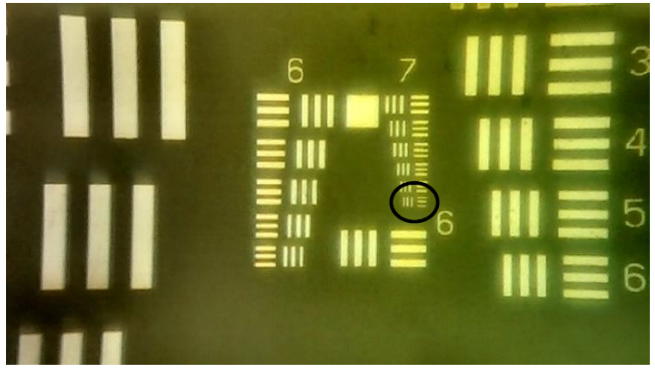
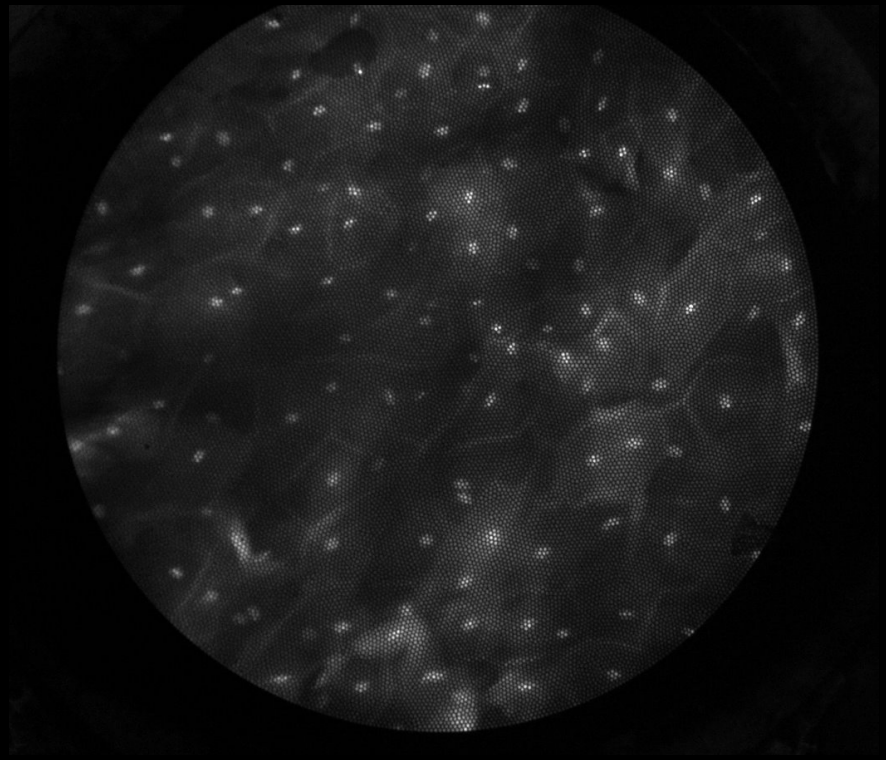


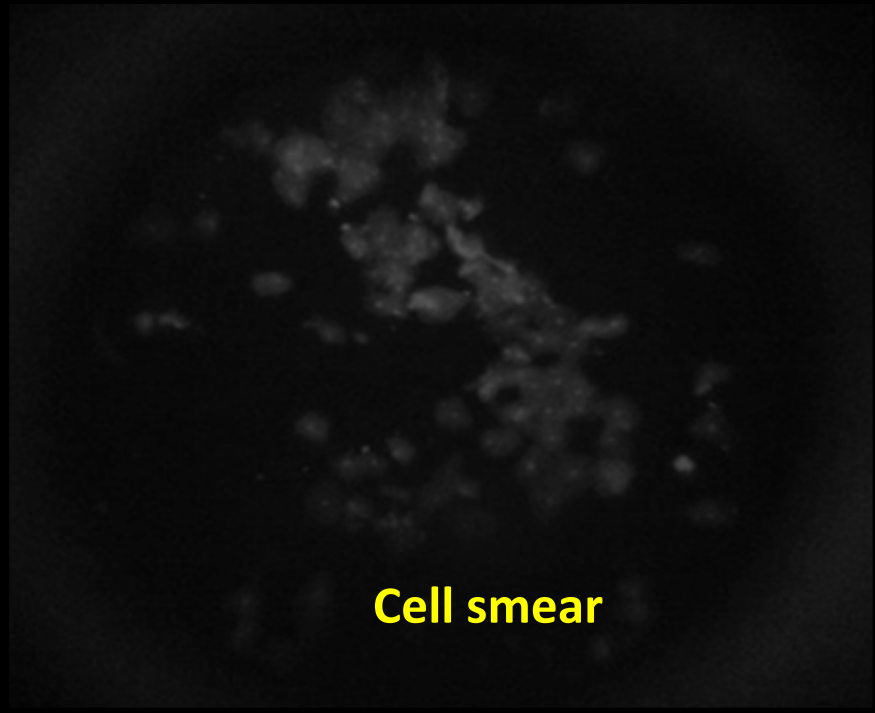

Figure 2: Image of a USAF 1951 chart taken with the system, where the smallest distinguished lines are on the 6th element of the 7th group. Each of those lines has a width of 2.19 µm.

Table 1: Main characteristics of the system.

| Property | Value |
|---|---|
| Resolution | ~2 µm |
| Magnifying power | 150X |
| Characteristic size | 120 mm |
| Weight | 0.25 kg |
| Main material | Aluminum |
| Penetration (mucosa tissue) | ~50 µm |
| Field of view | Ø 1.5 mm |
| Resolution (with fiber bundle) | ~4 µm |
| Field of view (with fiber bundle) | Ø 850 µm |

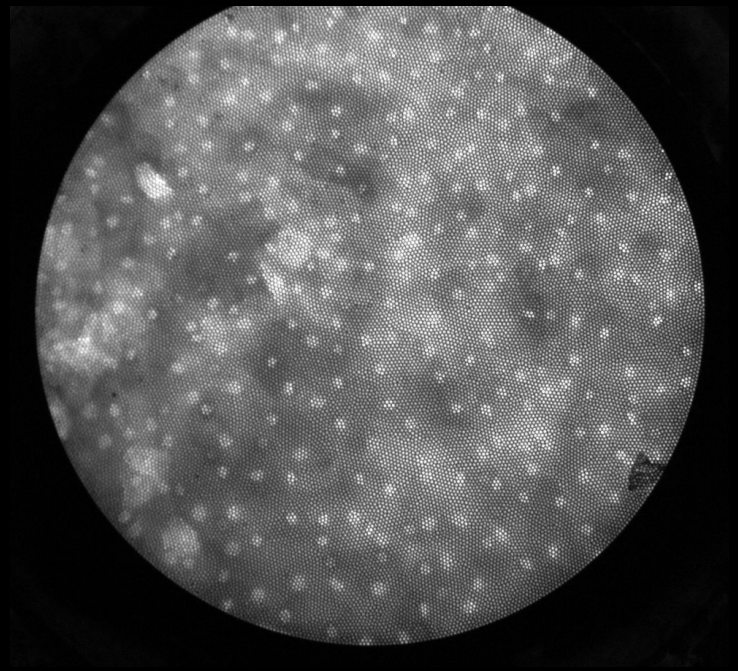


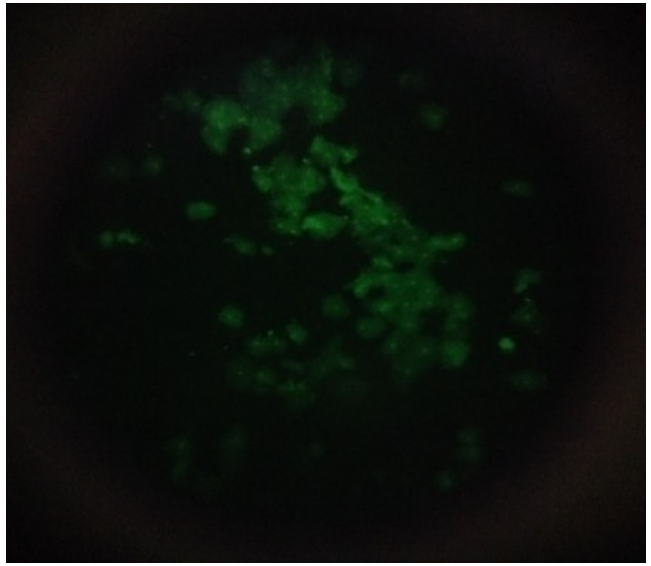
Mucosa bucal

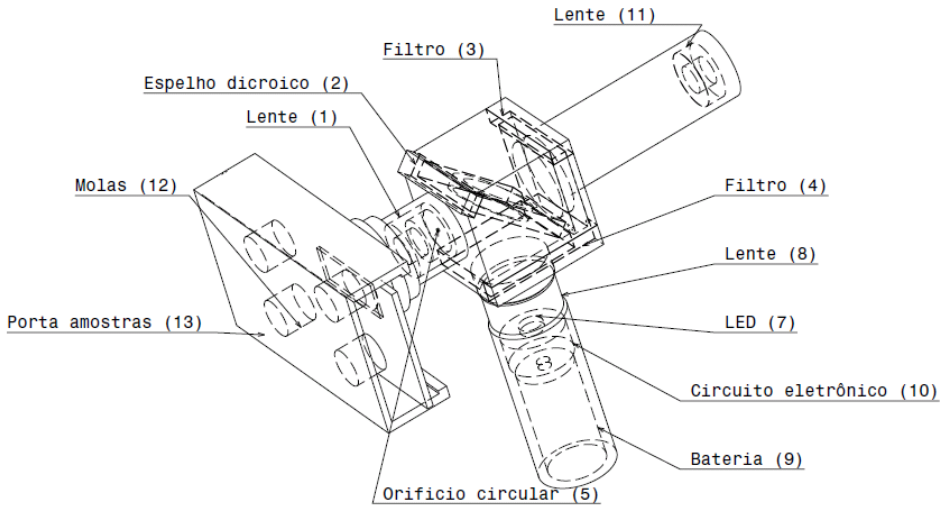


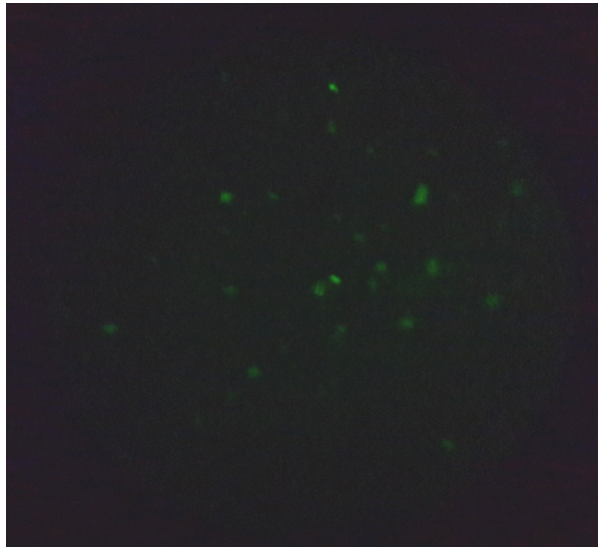
Cell smear

Gengiva

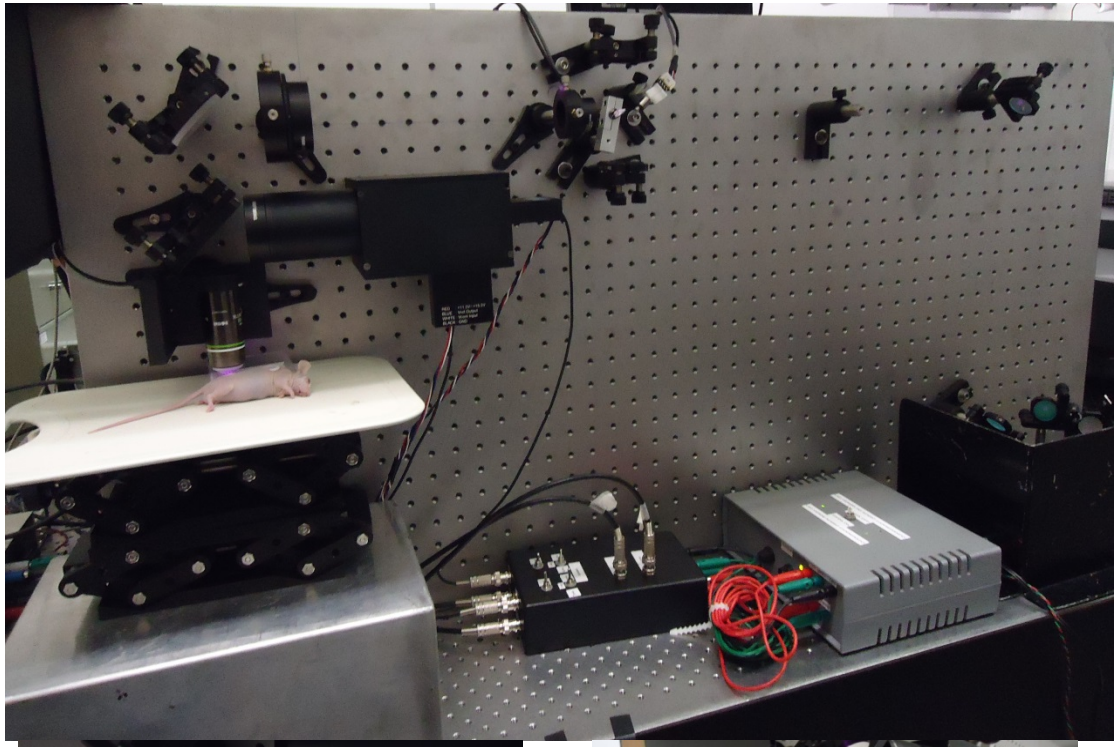


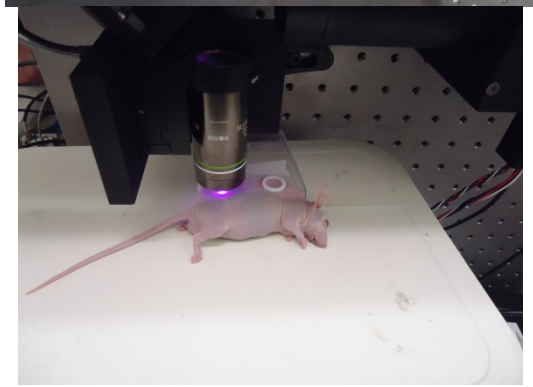


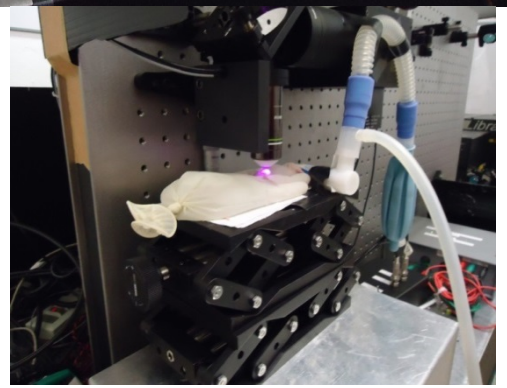

Lente (11)
Filtro (3)
Espelho dicroico (2)
Lente (1)
Molas (12)
Filtro (4)
Lente (8)
Porta amostras (13)
LED (7)
Circuito eletrônico (10)
Bateria (9)
Orificio circular (5)



# Microscópio Multifótons



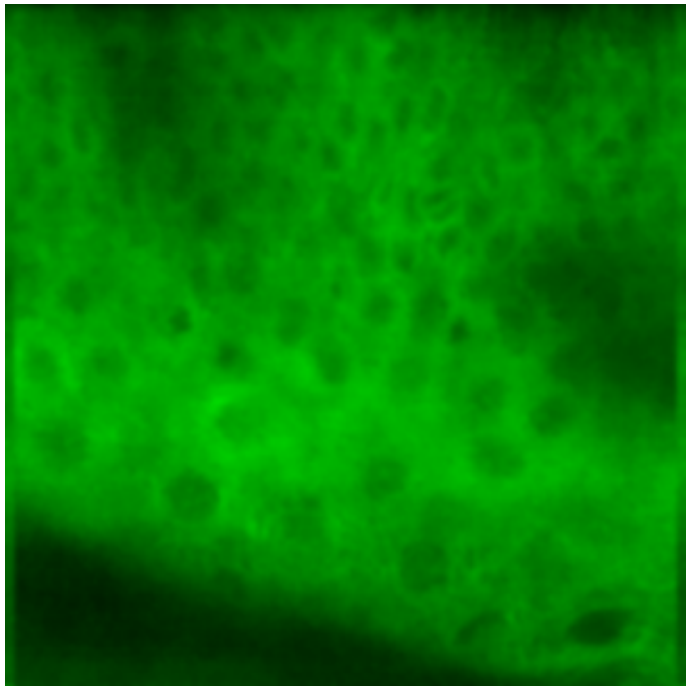




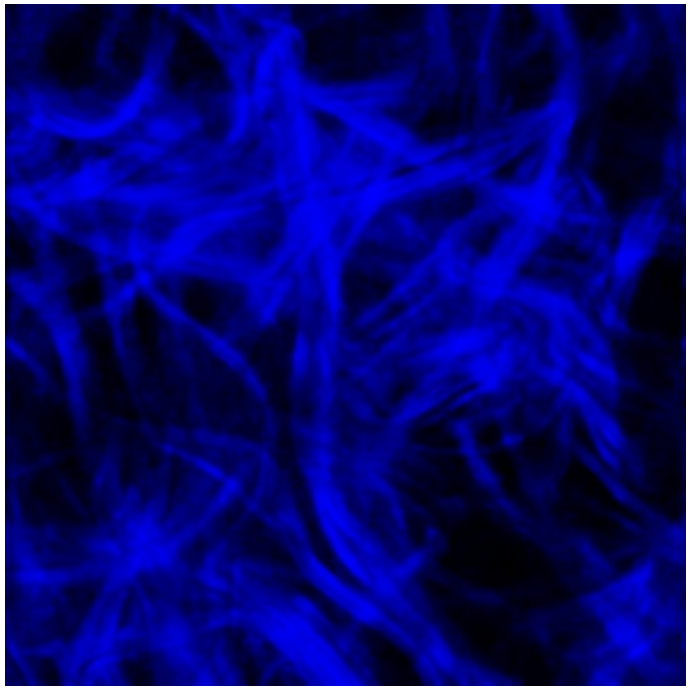
# *Aplicações*

Primeiros testes amostras *ex vivo*:

Pele de rato Wistar



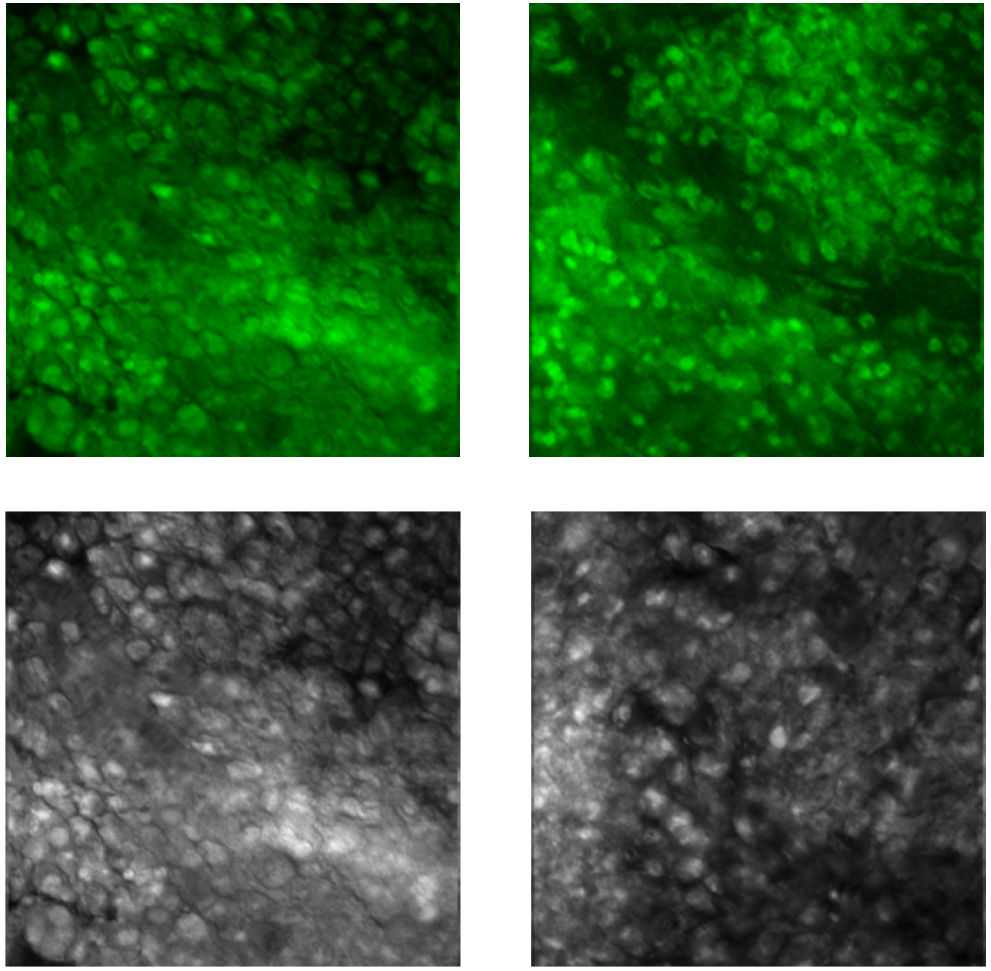
Tecido epitelial (queratinócitos ); z ≈ 15 μm; 100 x 100 $\mu m^2$



Fibras de colágeno; z ≈ 35 μm; 100 x 100 $\mu m^2$

# *Aplicações*

Primeiros testes cultura de células de tumor de Erlich em membrana corioalantóica:



100 x 100 $\mu m^2$