Using confocal spinning disk for high-resolution time lapse imaging

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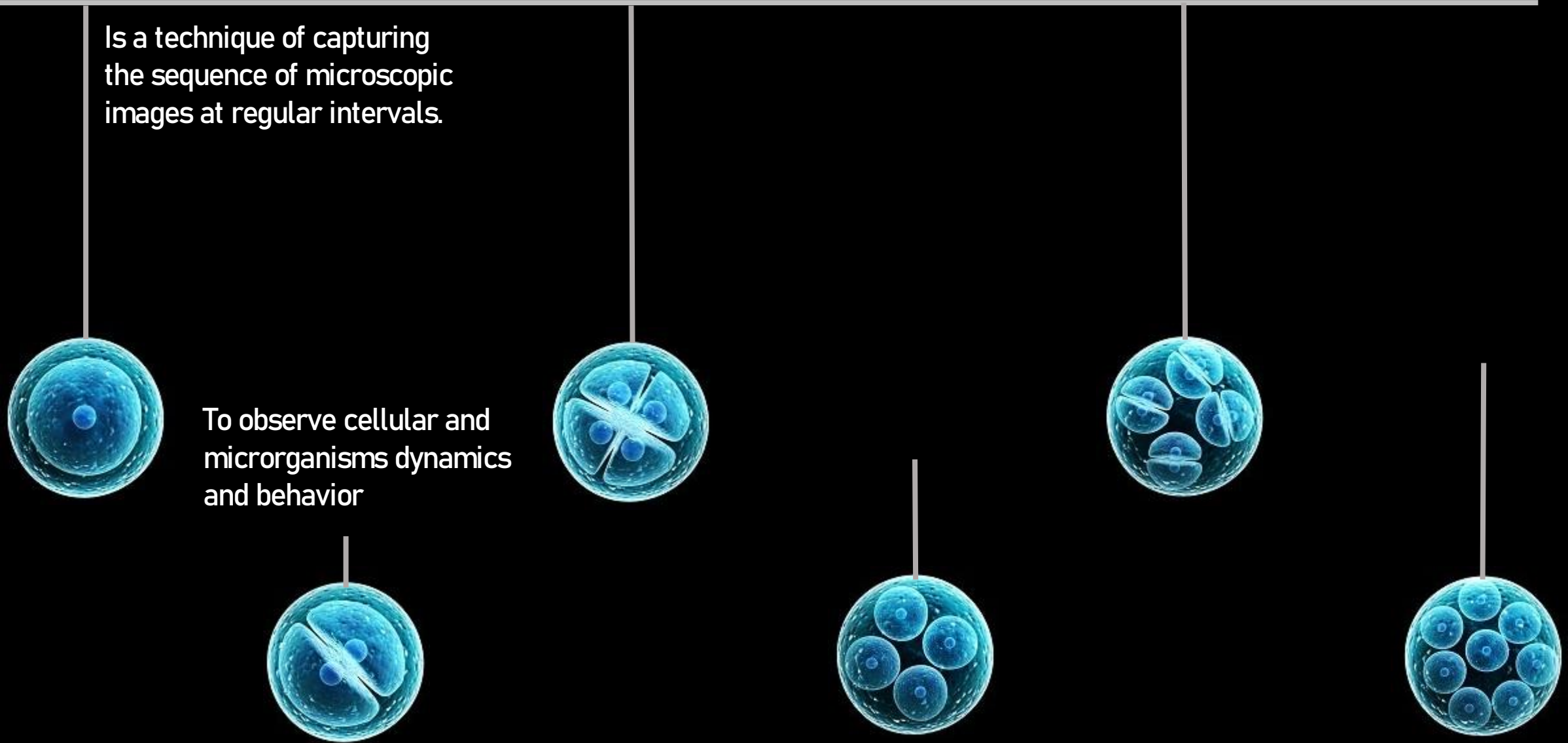
X WORKSHOP TEÓRICO-PRÁTICO DO
INFABIC
17-21 e 24-27 de outubro de 2022

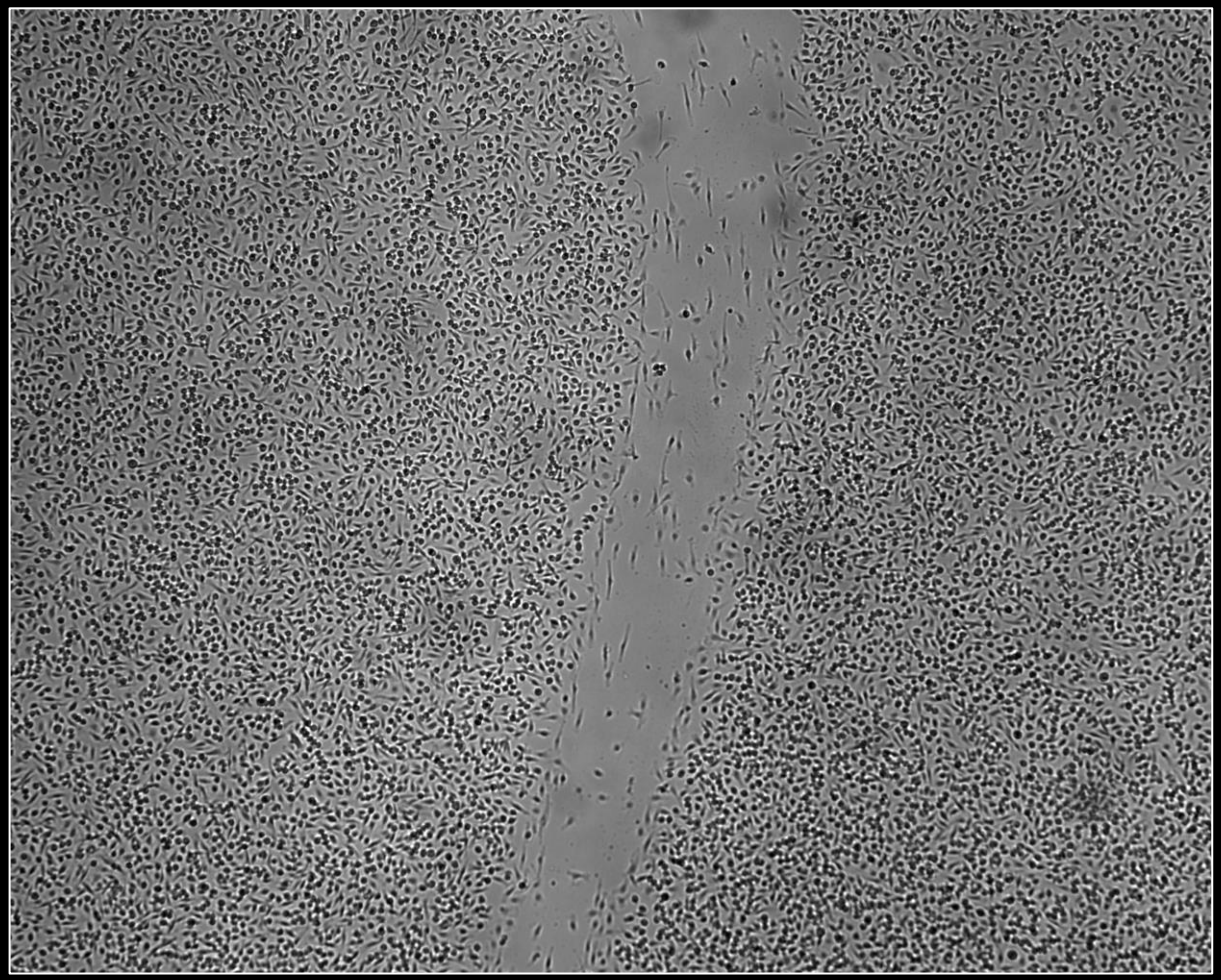
Time-lapse Microscopy (TLM)

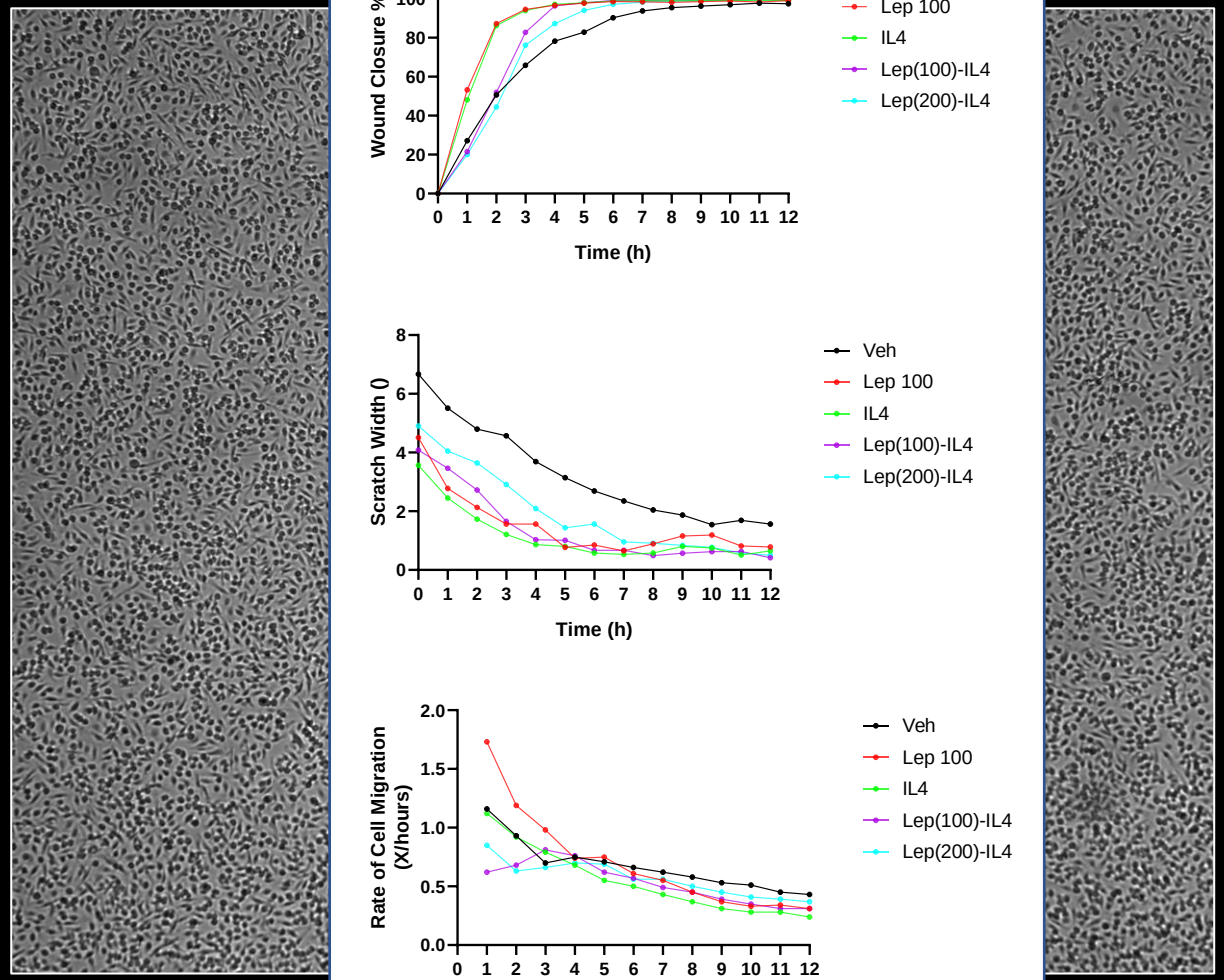
Is a technique of capturing the sequence of microscopic images at regular intervals.

To observe cellular and microrganisms dynamics and behavior



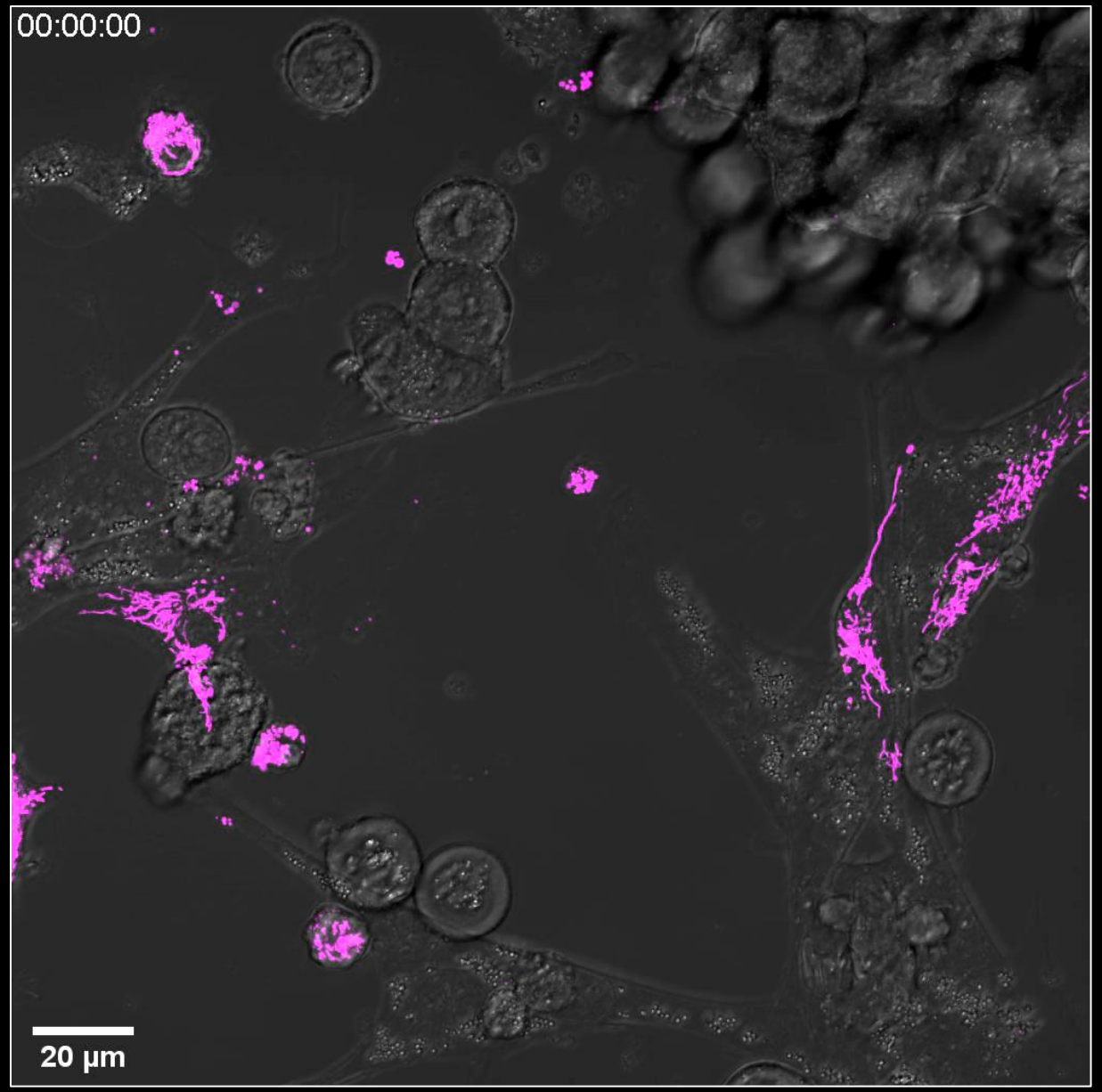
To monitor cell motility and migration...



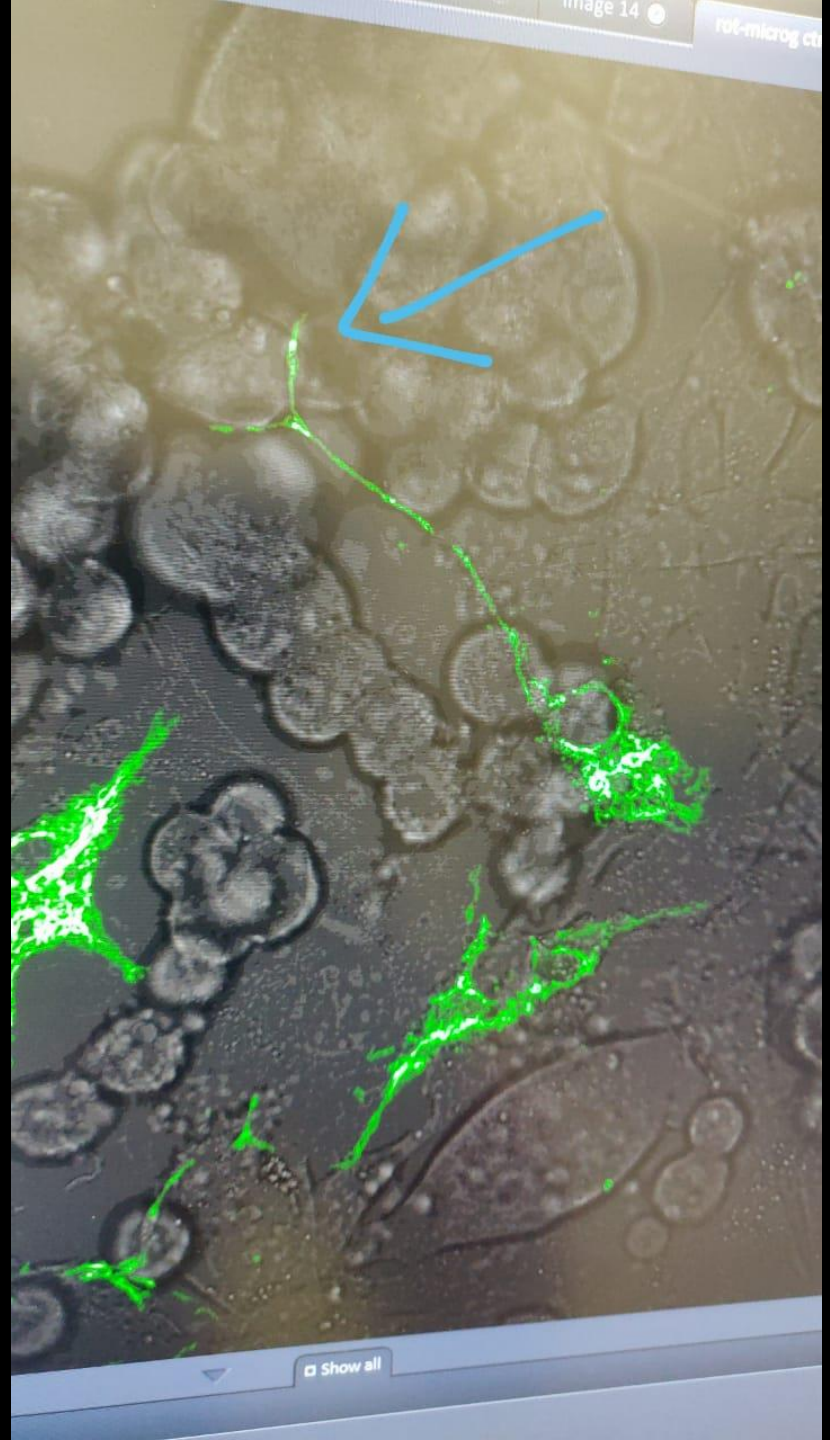


To visualize and characterize cell-cell contacts...

MITOCHONDRIA FROM MICROGLIA NEURONAL CULTURE

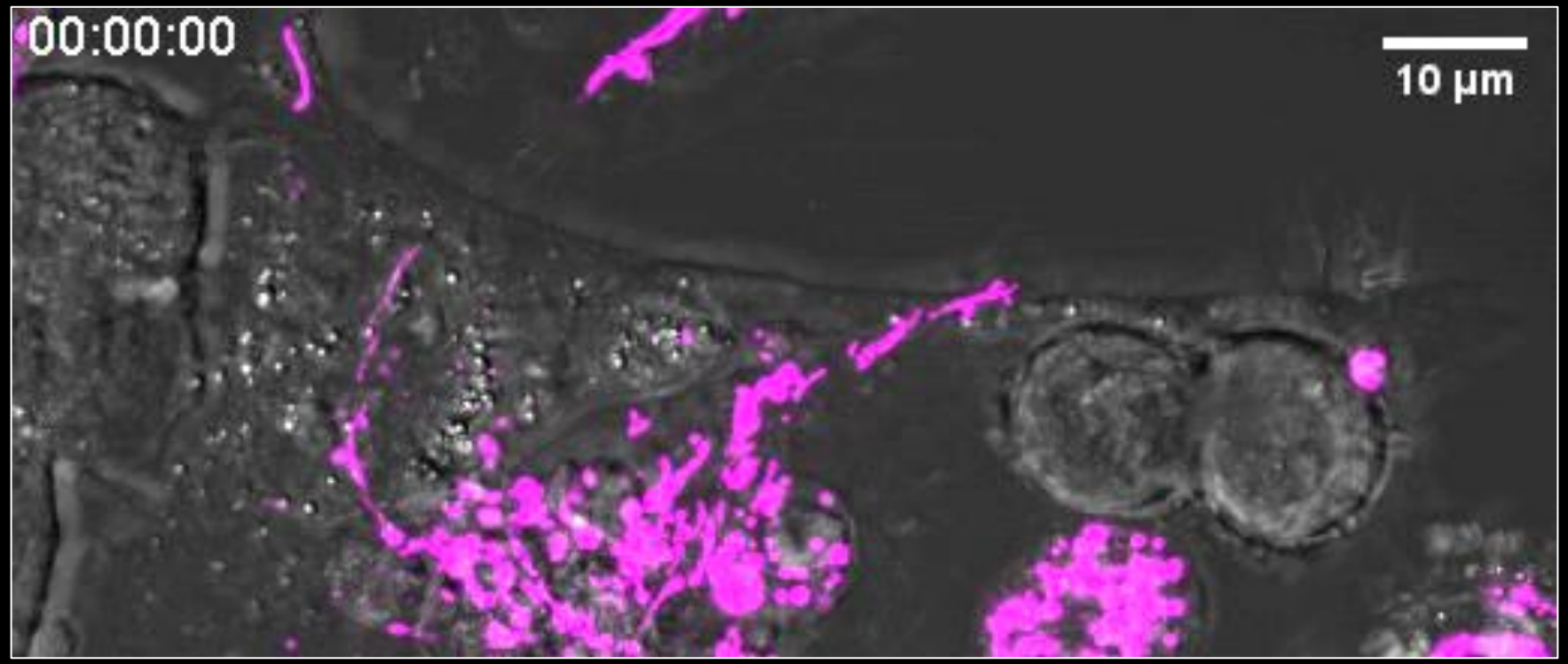


To visualize and characterize cell-cell contacts...



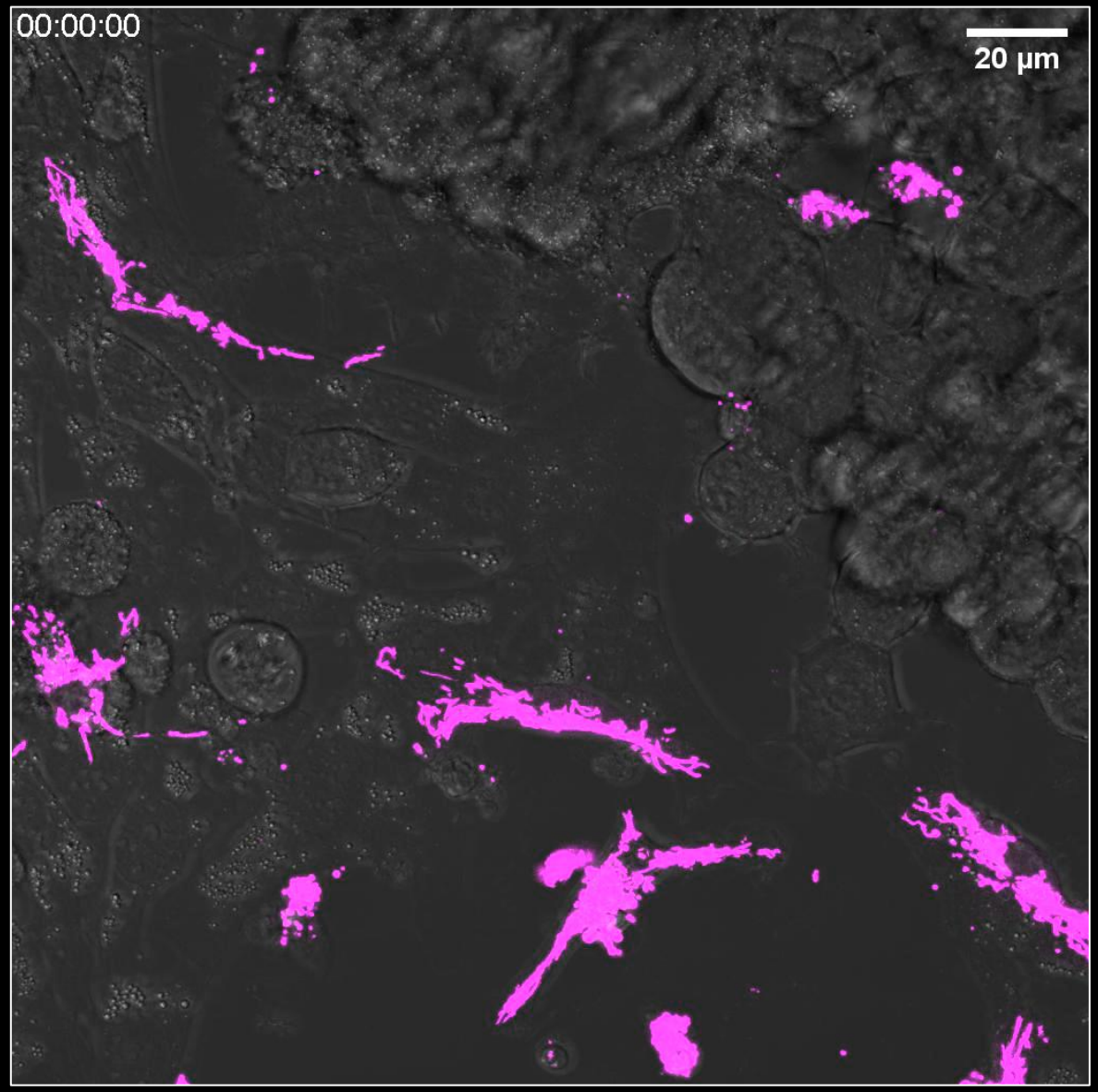
To monitor chemotaxis...

MITOCHONDRIA FROM MICROGLIA NEURONAL CULTURE



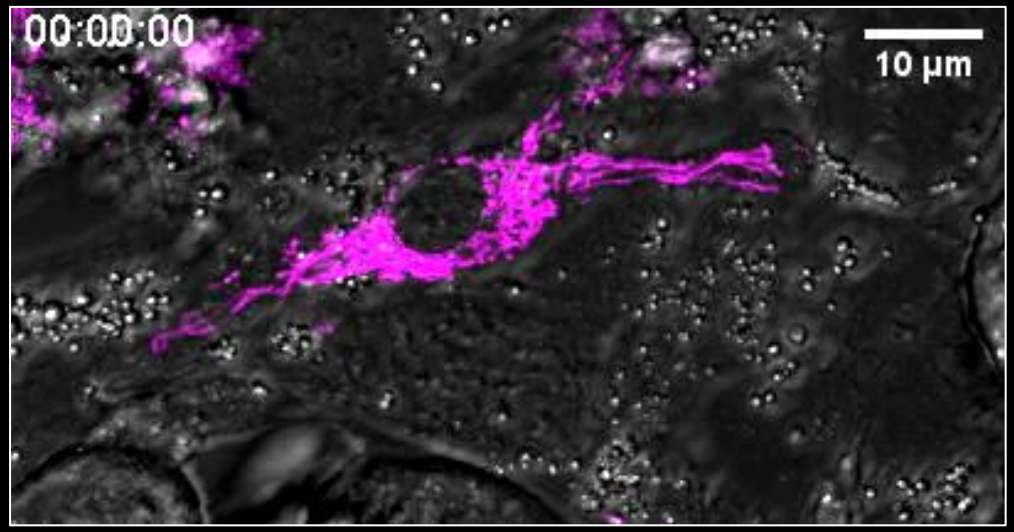
To visualize vesicles release and their content, the effect of drugs...

MITOCHONDRIA FROM MICROGLIA NEURONAL CULTURE

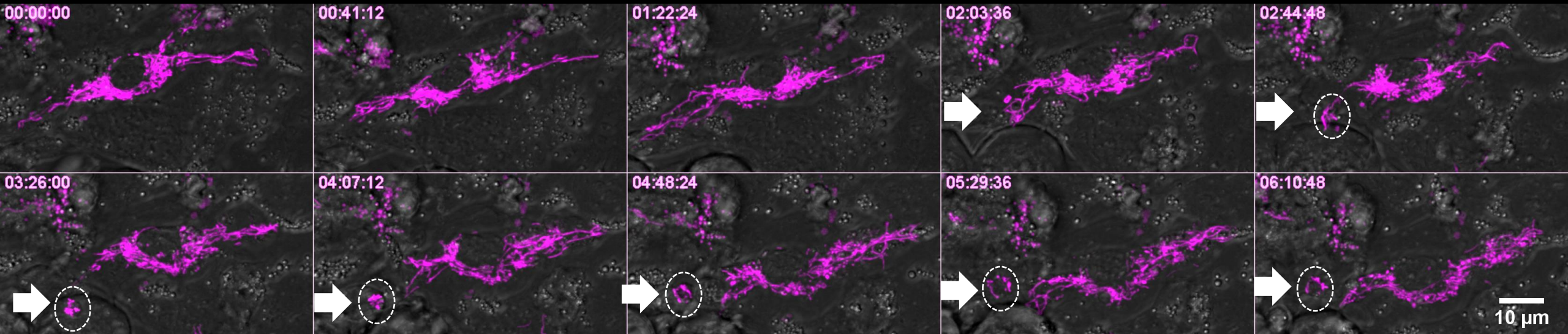


MITOCHONDRIA FROM MICROGLIA
NEURONAL CULTURE

A

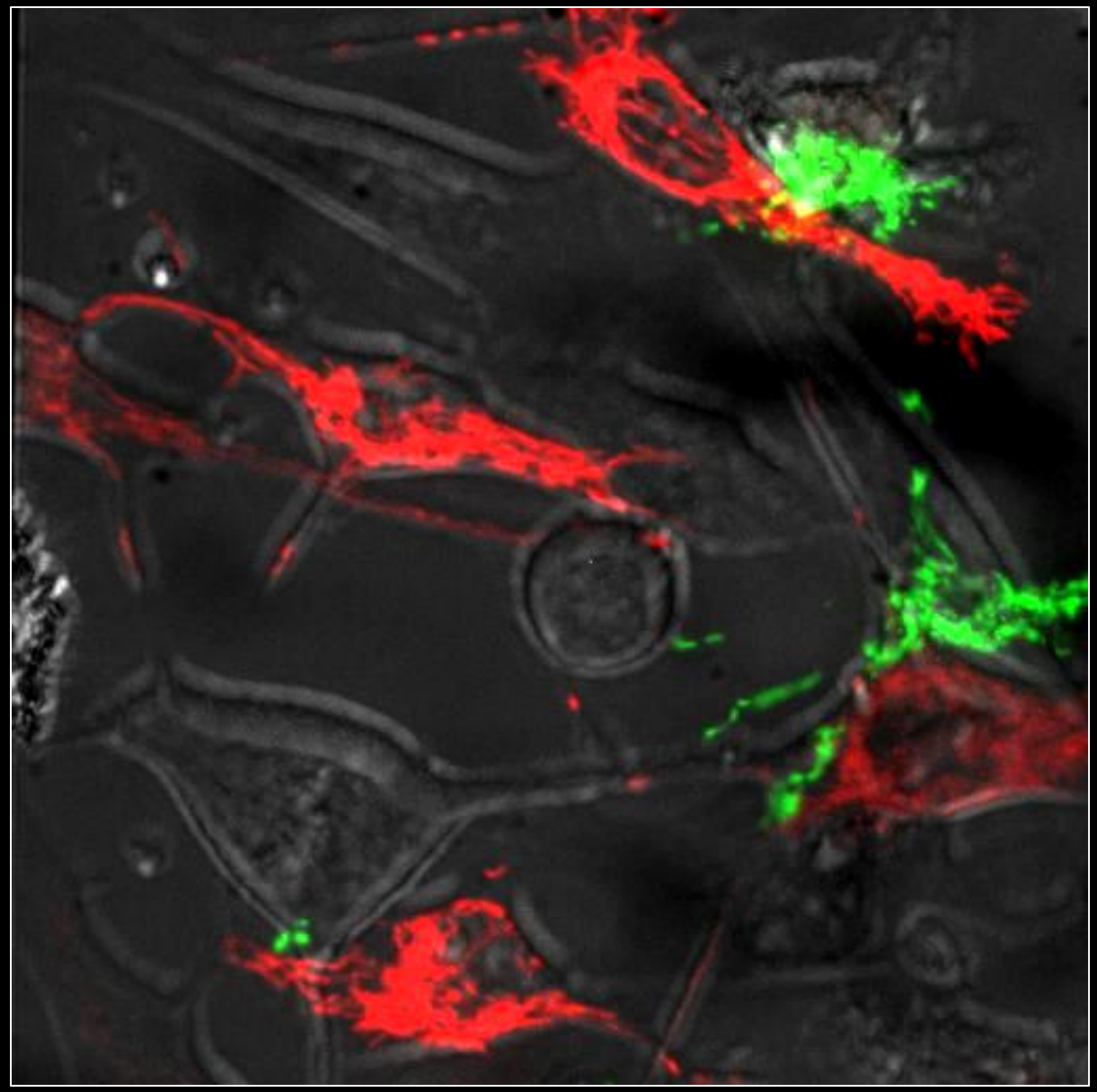


B



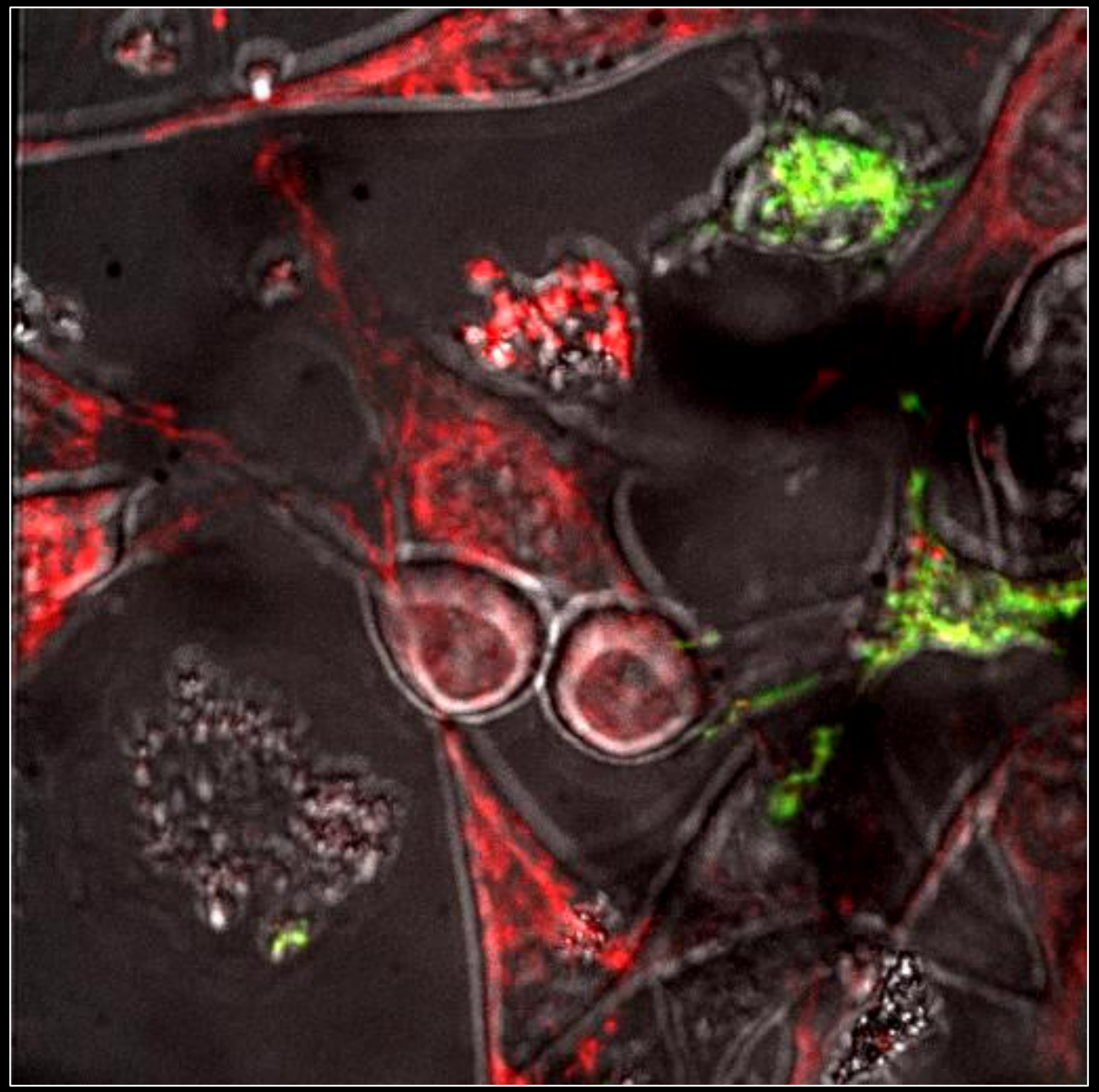
To observe cell death...

Mitocôndria de células tumorais em vermelho
Mitocôndria de macrófagos (BMDM) em verde (Pham animals)



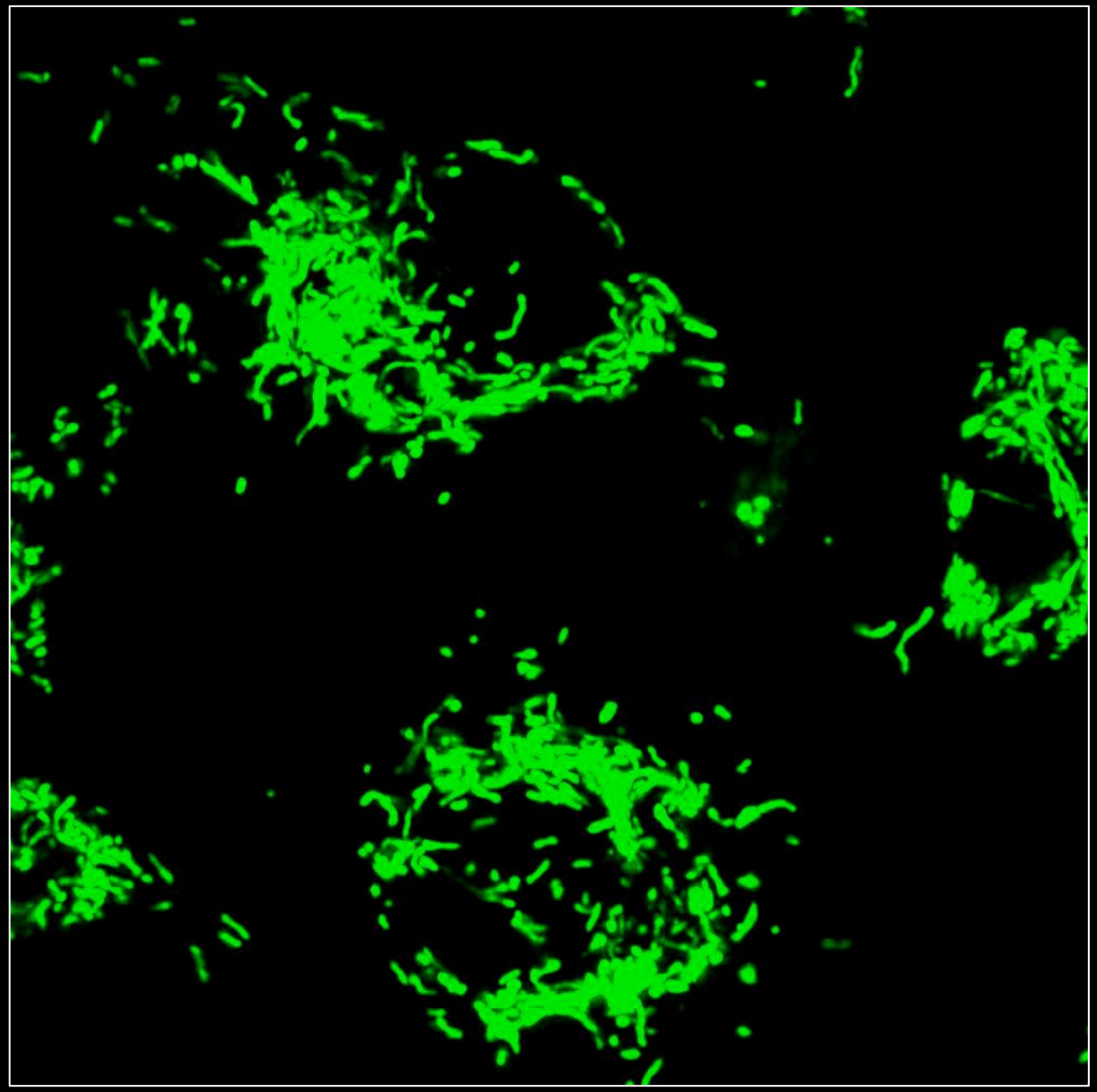
To observe cell division...

Mitocôndria de células tumorais em vermelho
Mitocôndria de macrófagos (BMDM) em verde (Pham animals)



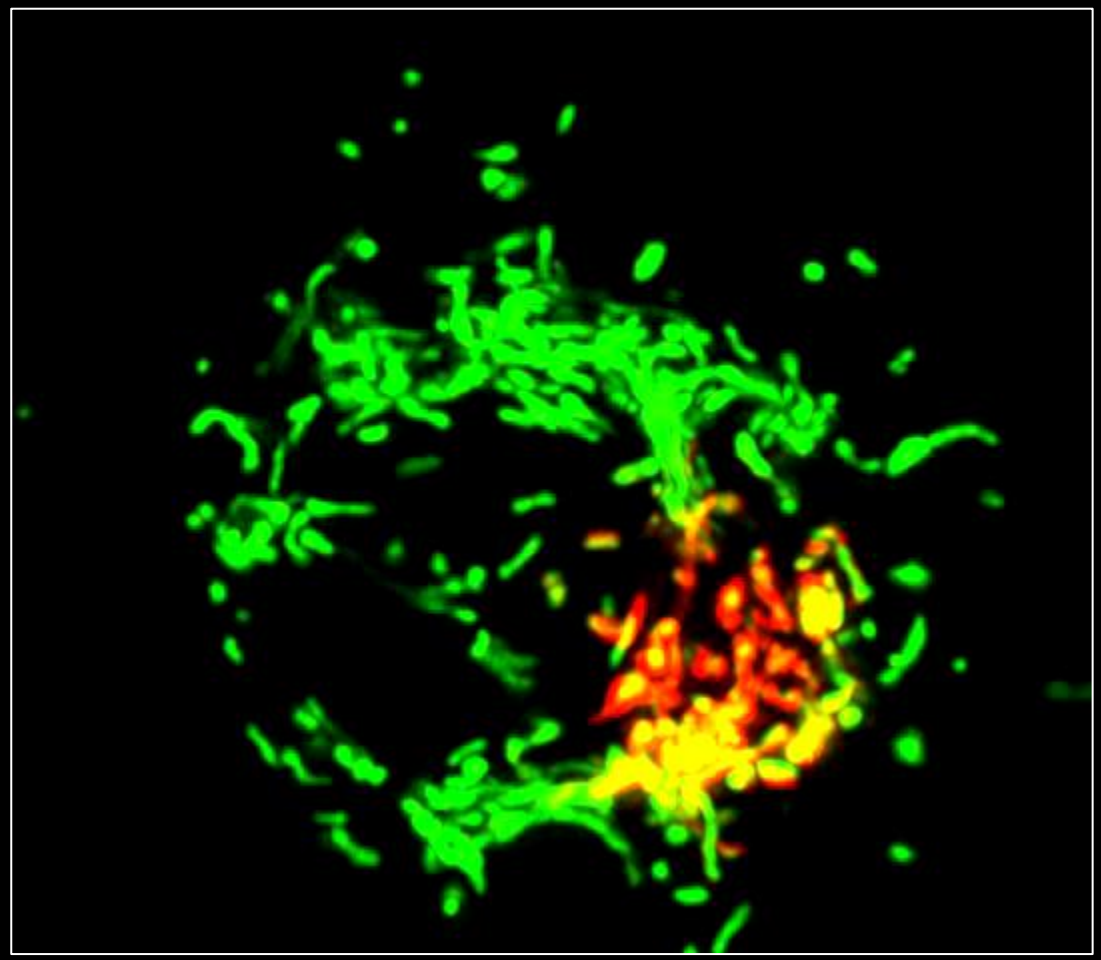
To measure mitochondrial dynamics...

Macrophages Dendra2



To measure mitochondrial dynamics…

Macrophages Dendra2 (Photoconvertible protein)



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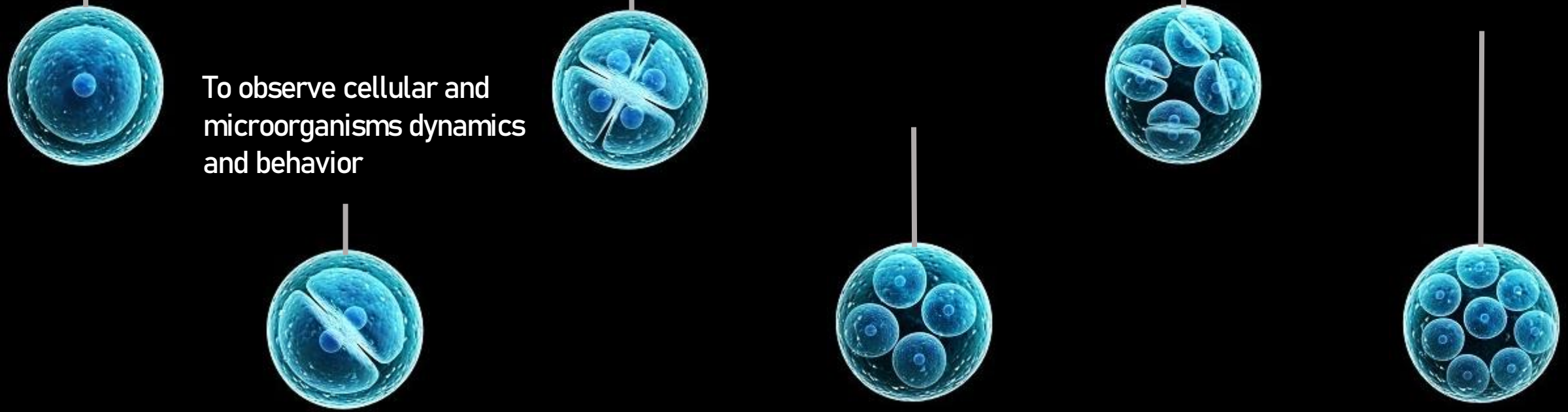
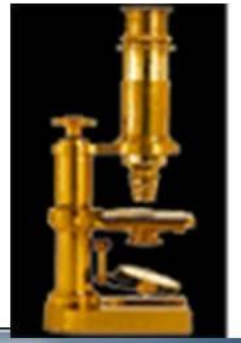
Requires:
Inverted microscope, cell incubator, sealed transparent box that maintains the temperatura, humidity and gas pressure...

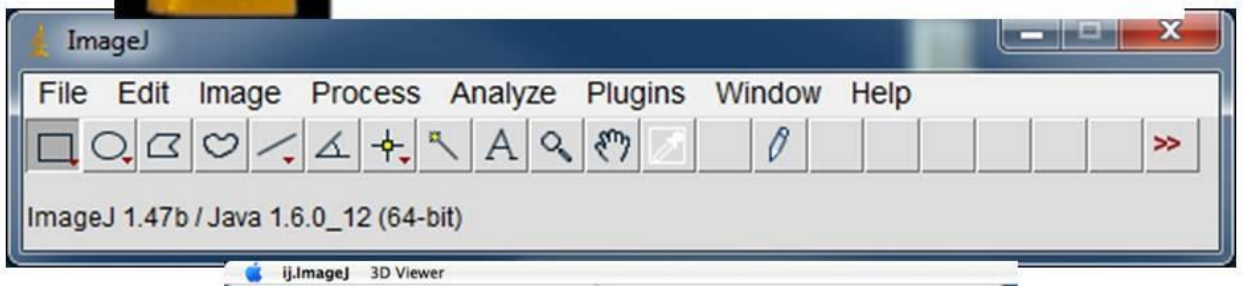
To observe cellular and microorganisms dynamics and behavior

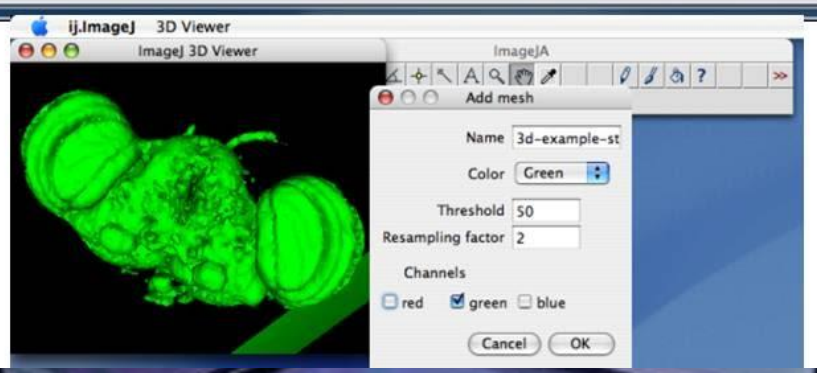


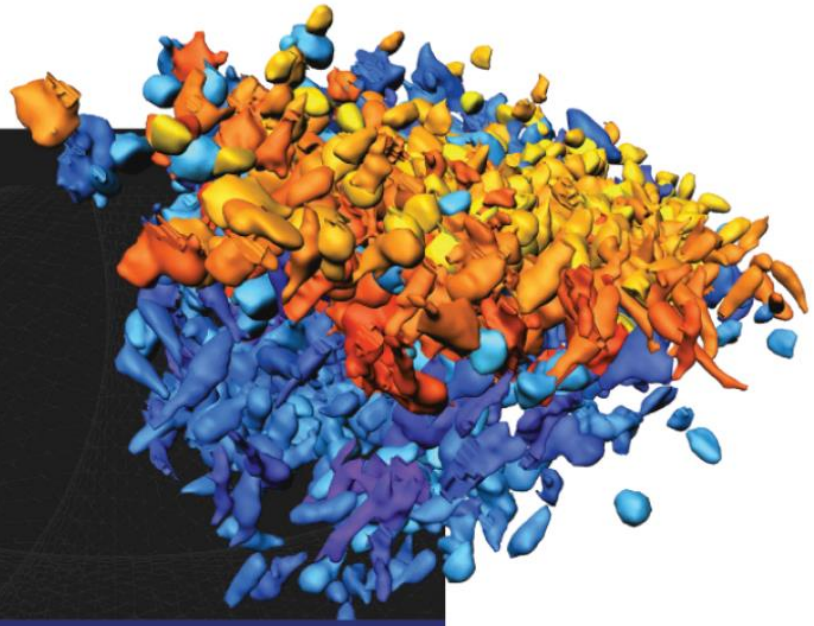


ImageJ

Image Processing & Analysis in Java







Imaris

BITPLANE
an Oxford Instruments company

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Time-lapse Microscopy (TLM)

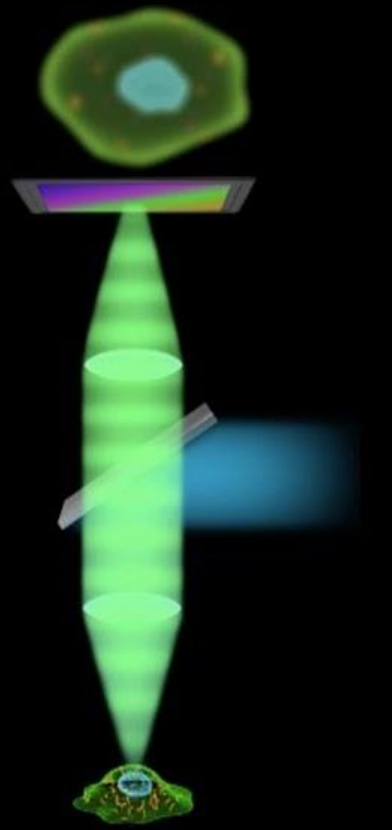
Is a technique of capturing the sequence of microscopic images at regular intervals.

To observe cellular and microorganisms dynamics and behavior

Requires:
Inverted microscope, cell incubator, sealed transparent box that maintains the temperatura, humidity and gas pressure...

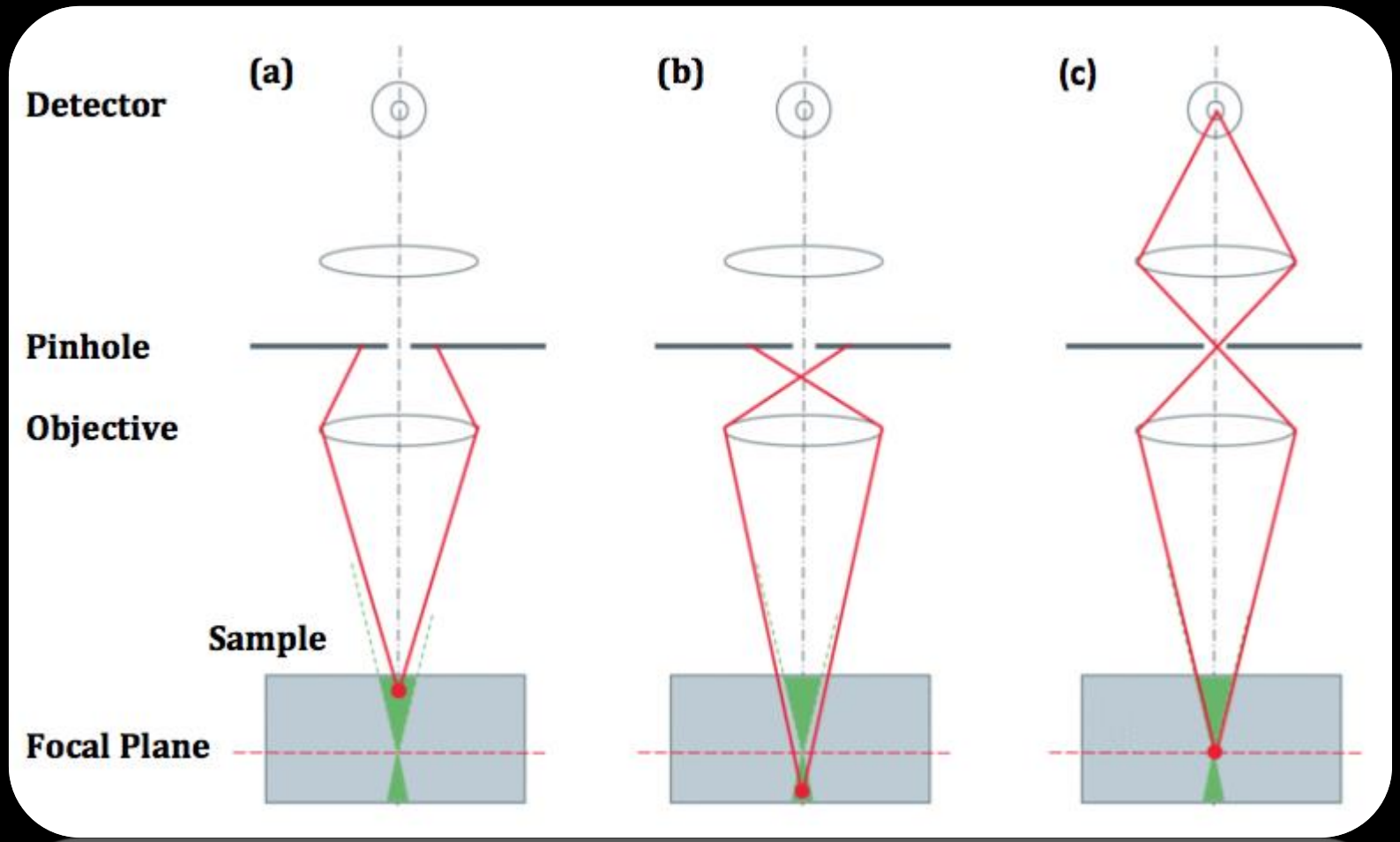
May be combined:
Multifield, Confocal, Multi-photon, 4D, bioluminescence analysis, *in toto* imaging...

Microscopes for Time-lapse



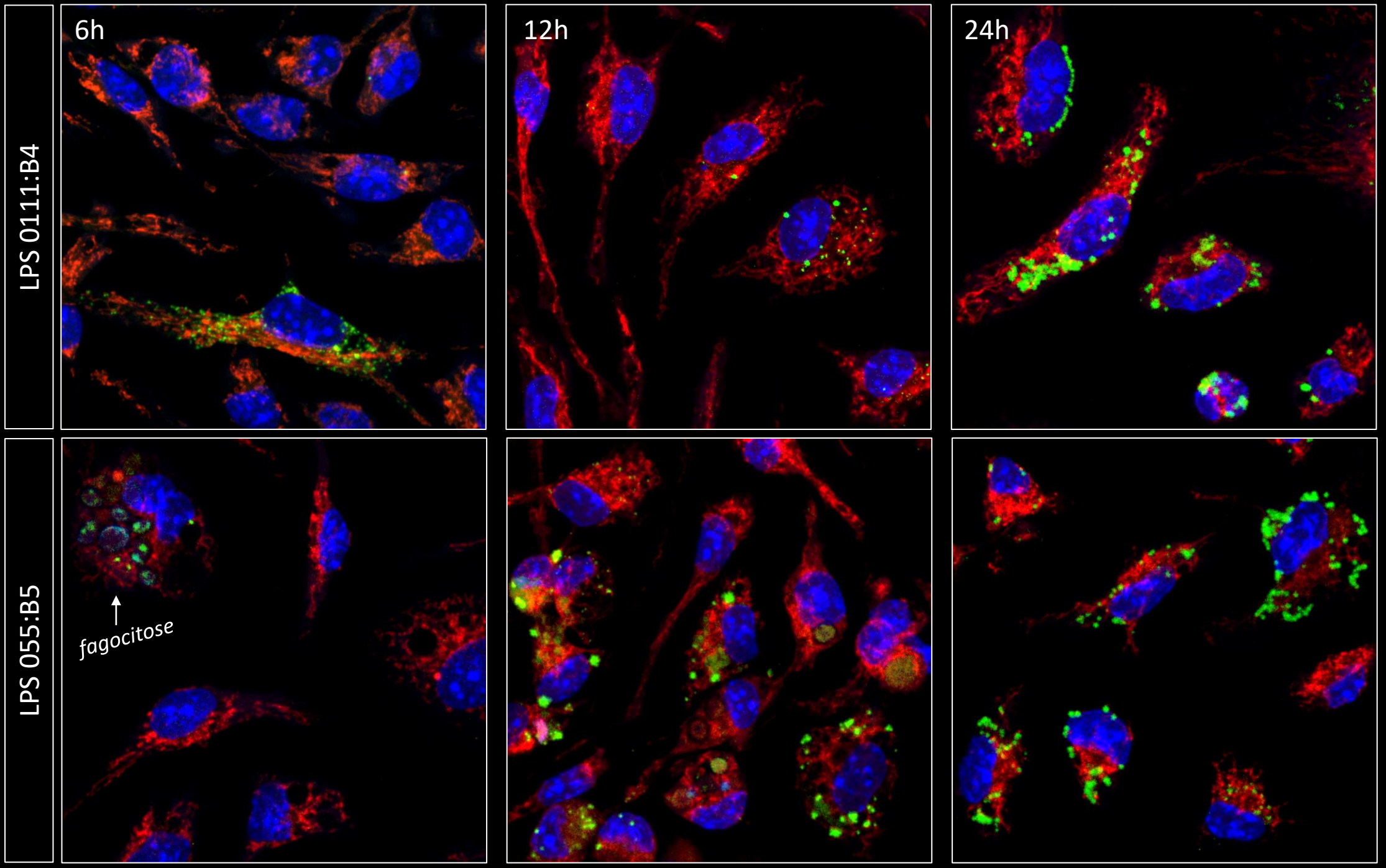
Widefield Microscope

Confocal Microscopy

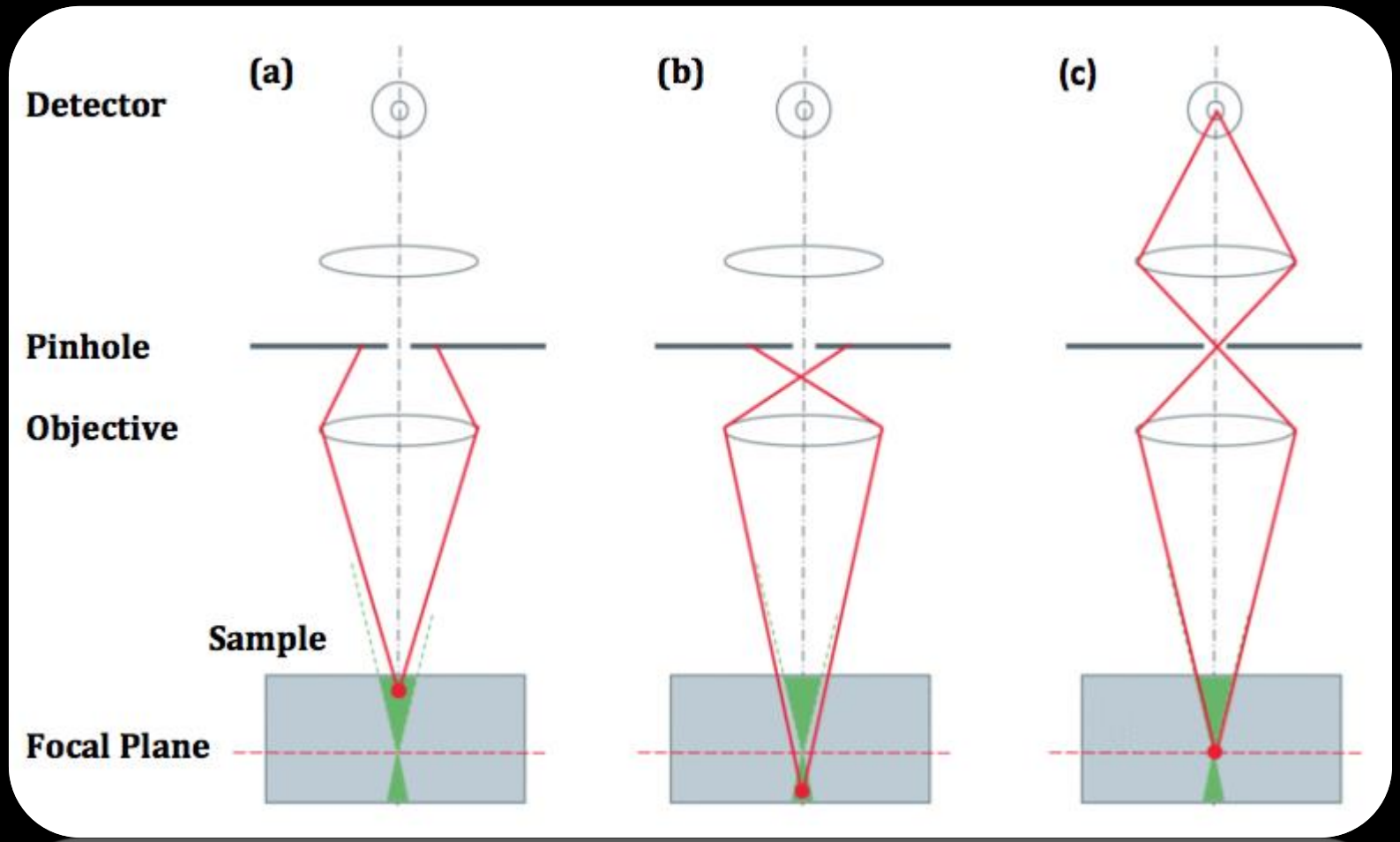


Pros:

- Can produce beautiful images
- Best optical sectioning


LPS 0111:B4
LPS 055:B5
6h
12h
24h
fagocitose
Mitotracker
Red: 200nM
Bodipy:
10uM
DAPI
LPS:
100ng

Confocal Microscopy



Pros:

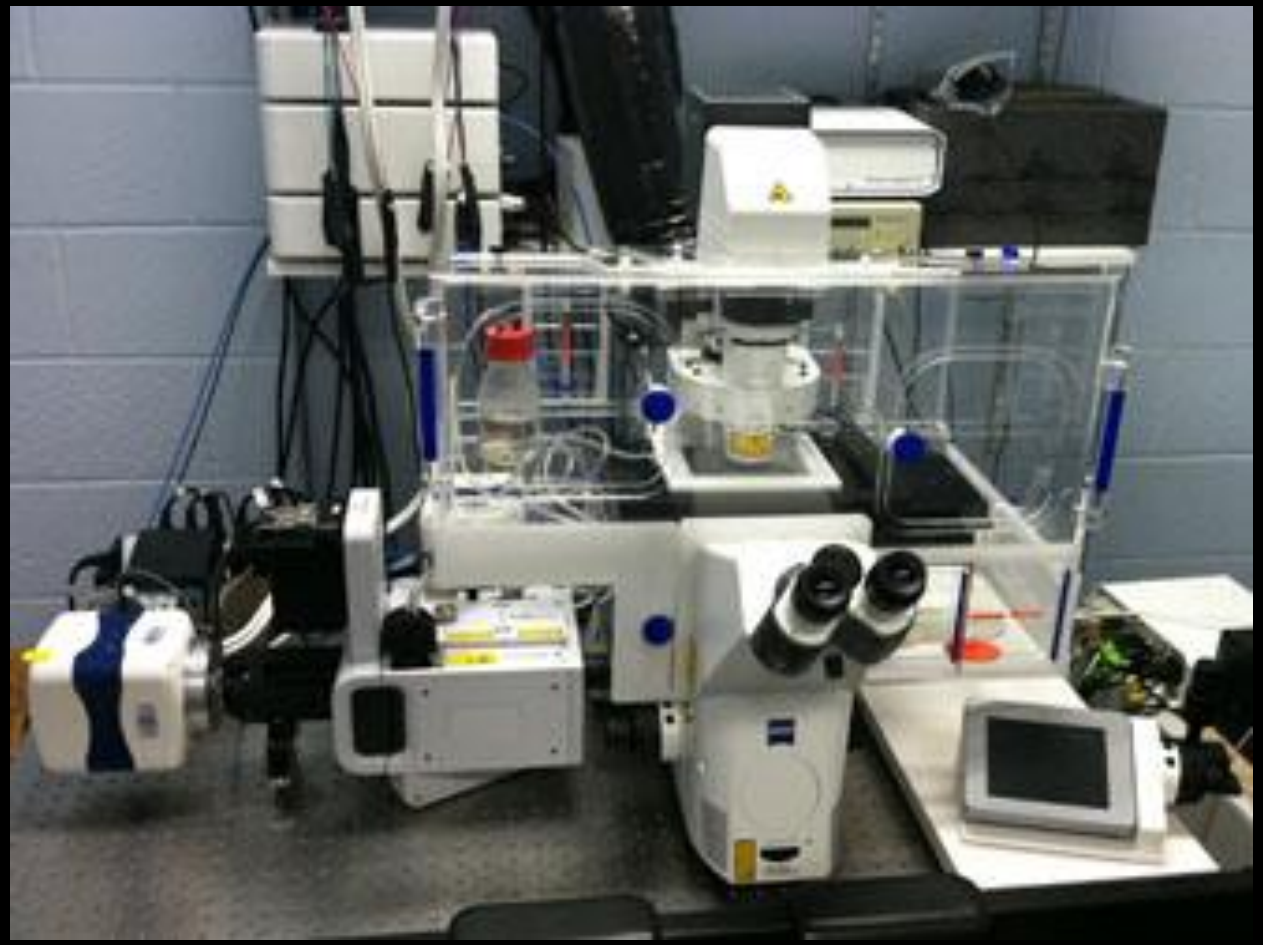
- Can produce beautiful images
- Best optical sectioning

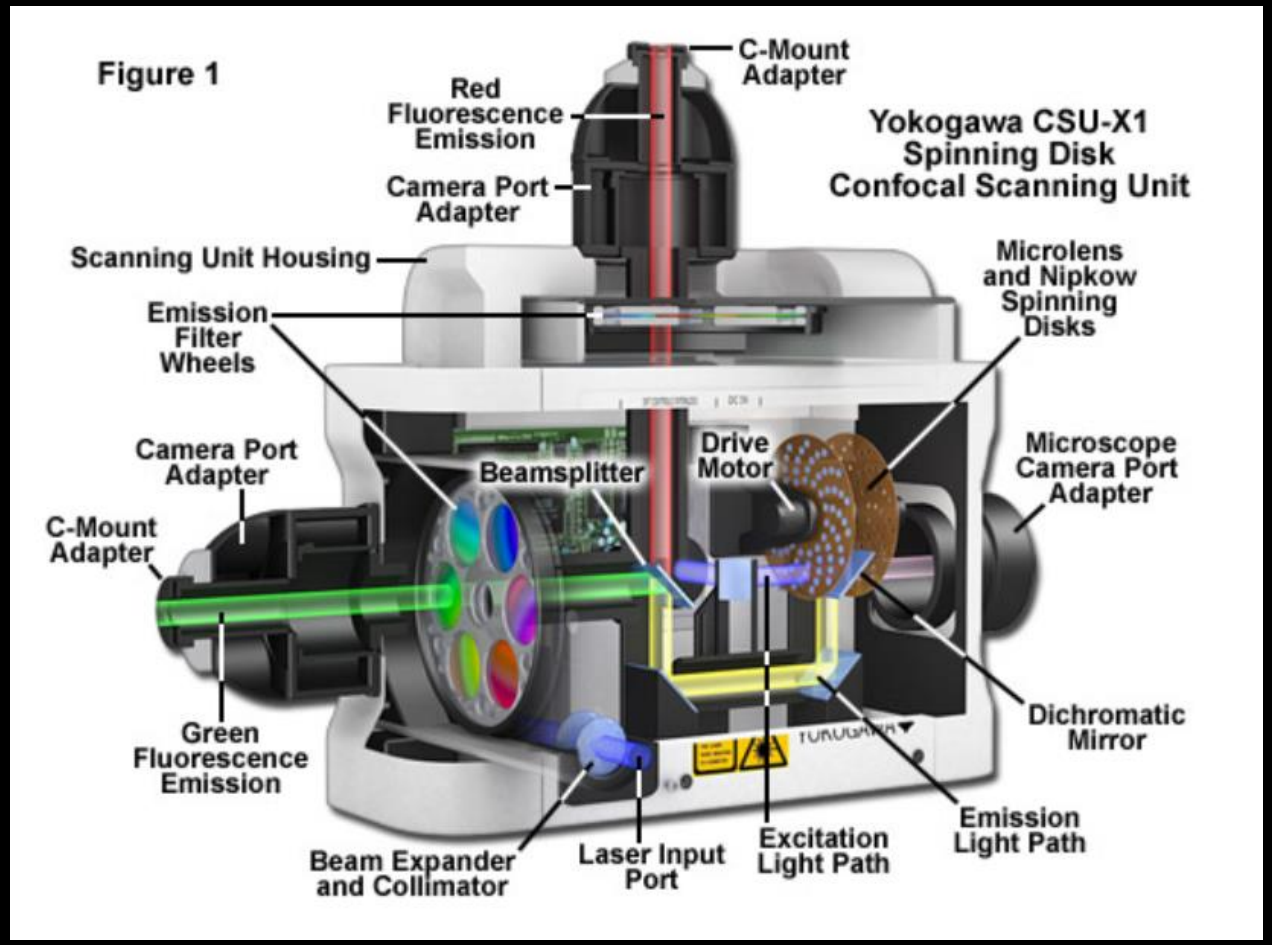
But...

- Slow (~0.5-2s per z)
- Requires blasting cells with a laser
- Photo damage
- Detector is typically a photomultiplier (PMT): detect Only 15-45% of the fluorescence signal that passes through the pinhole
- Expensive

Spinning Disk

Yokogawa Electric Corporation CSU-X1 spinning disk:
5000 ou 10000rpm → 1000 ou 2000 frames/sec





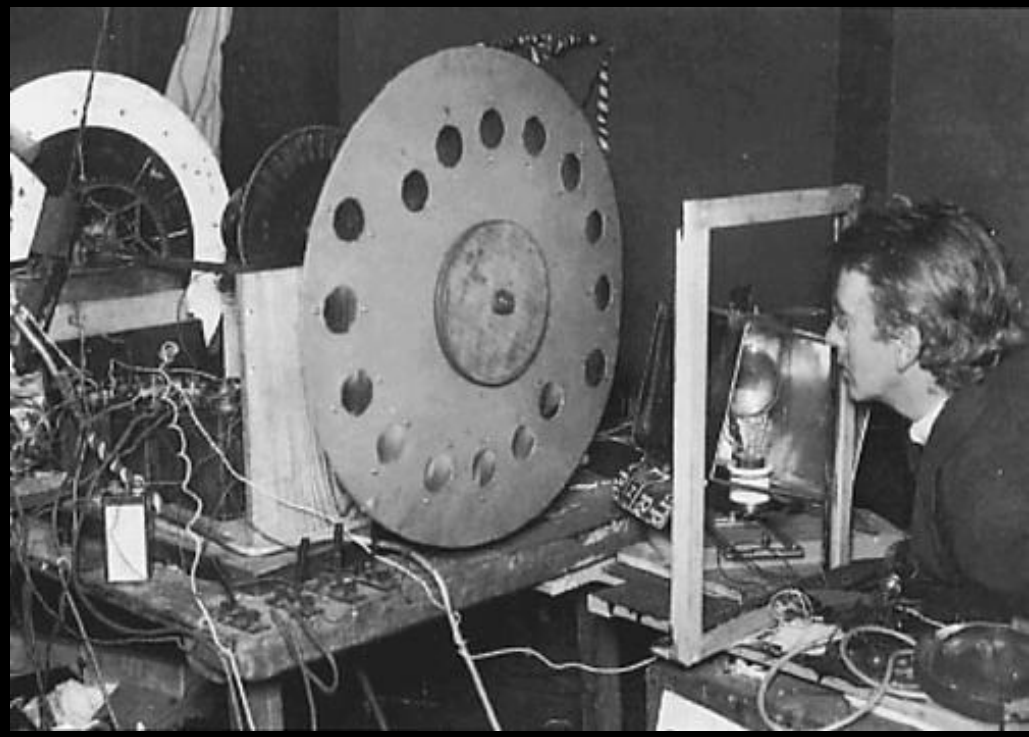
Nipkow Disk

1884

- Paul Nipkow invented the pinhole-scanning disk to turn images into signals
  - Pinholes scan across the image one line at a time.
  - 2D image → 1D signal sequence

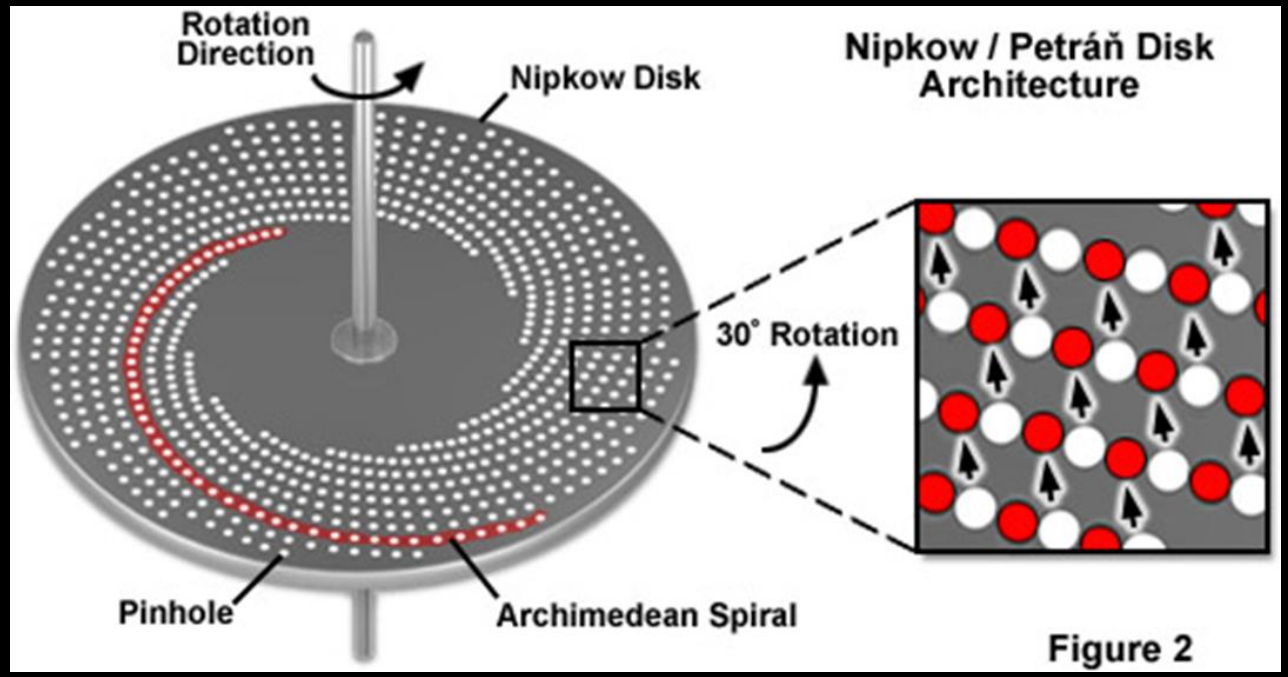
1967

- David Egger and Mojmir Petrán adapt Nipkow disk in the first Spinning Disk confocal



Petráň Disk

- 1000s of pinholes illuminate simultaneously at any point of time
- Pinholes are scanned across image multiple times per exposure
- Every part of the image is scanned by a pinhole each 30° rotation of the disk
- Imaging both thin and thick specimens in x – y or x – y – z dimensions in high resolutions
- High quantum efficiency (95% for EMCCD) Cameras can be used



Advantages of Spinning confocal

1

REAL TIME AND ULTRAFAST
30 frames/sec and in the ultrafast timescale: up to 1,000 frames/sec.

2

GENTLE
Application specific (EM)CCD câmera can produce high resolution images and image capture speeds up-to 150fps. Parallel illumination means Much less excitation light needed than LSCM.

3

REDUCES PHOTOBLEACHING
Low intensity, high frequency scanning, together with High QE

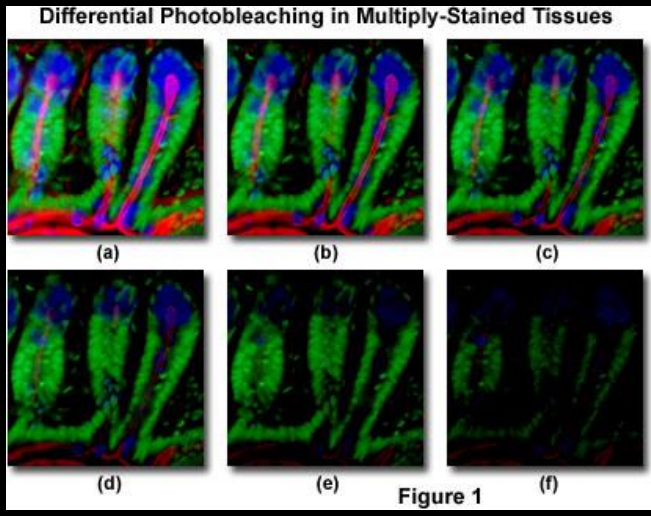


Figure 1

4

TIME LAPSE OF LIVE CELLS
In 3D/4D over long time periods

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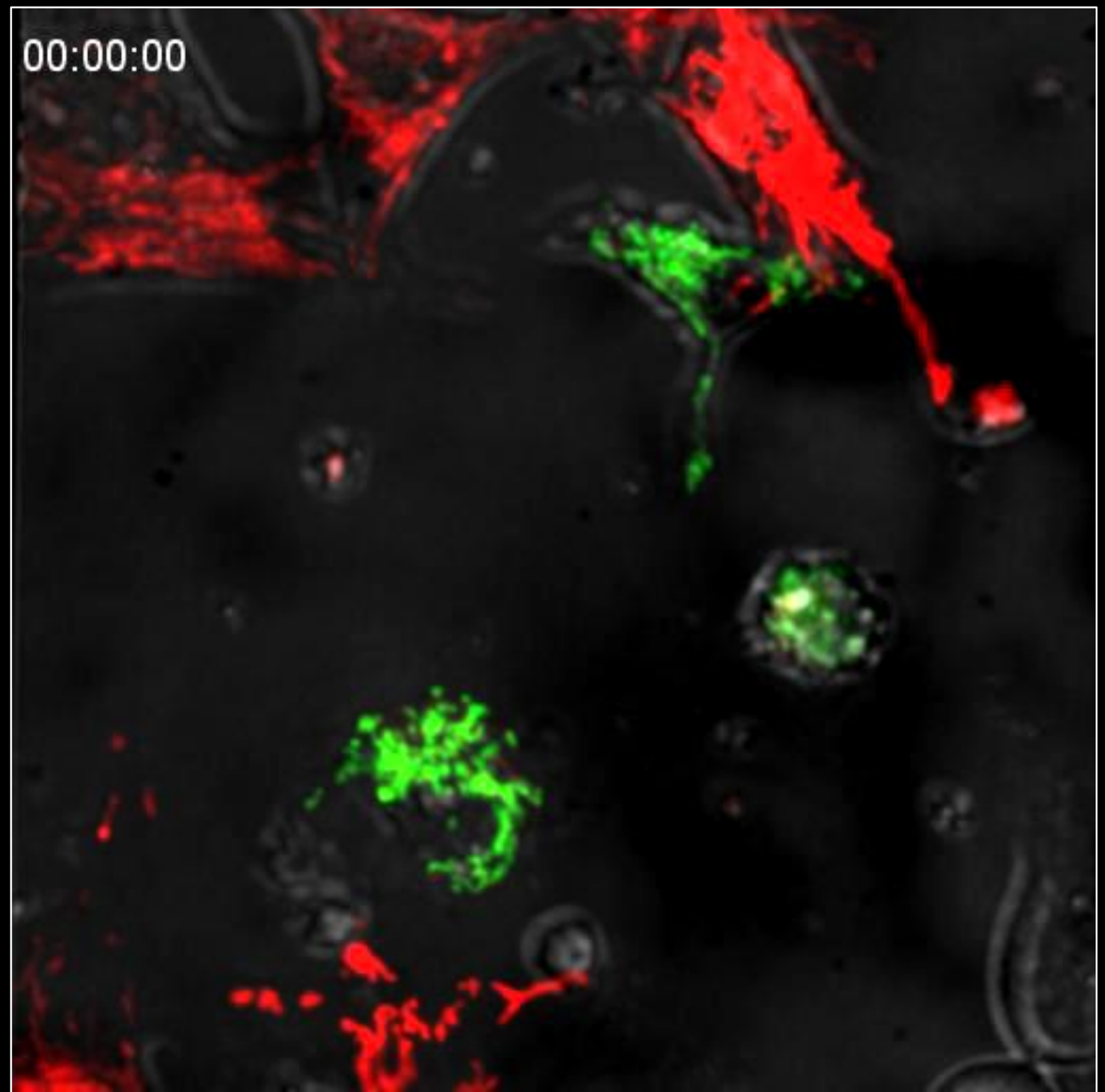
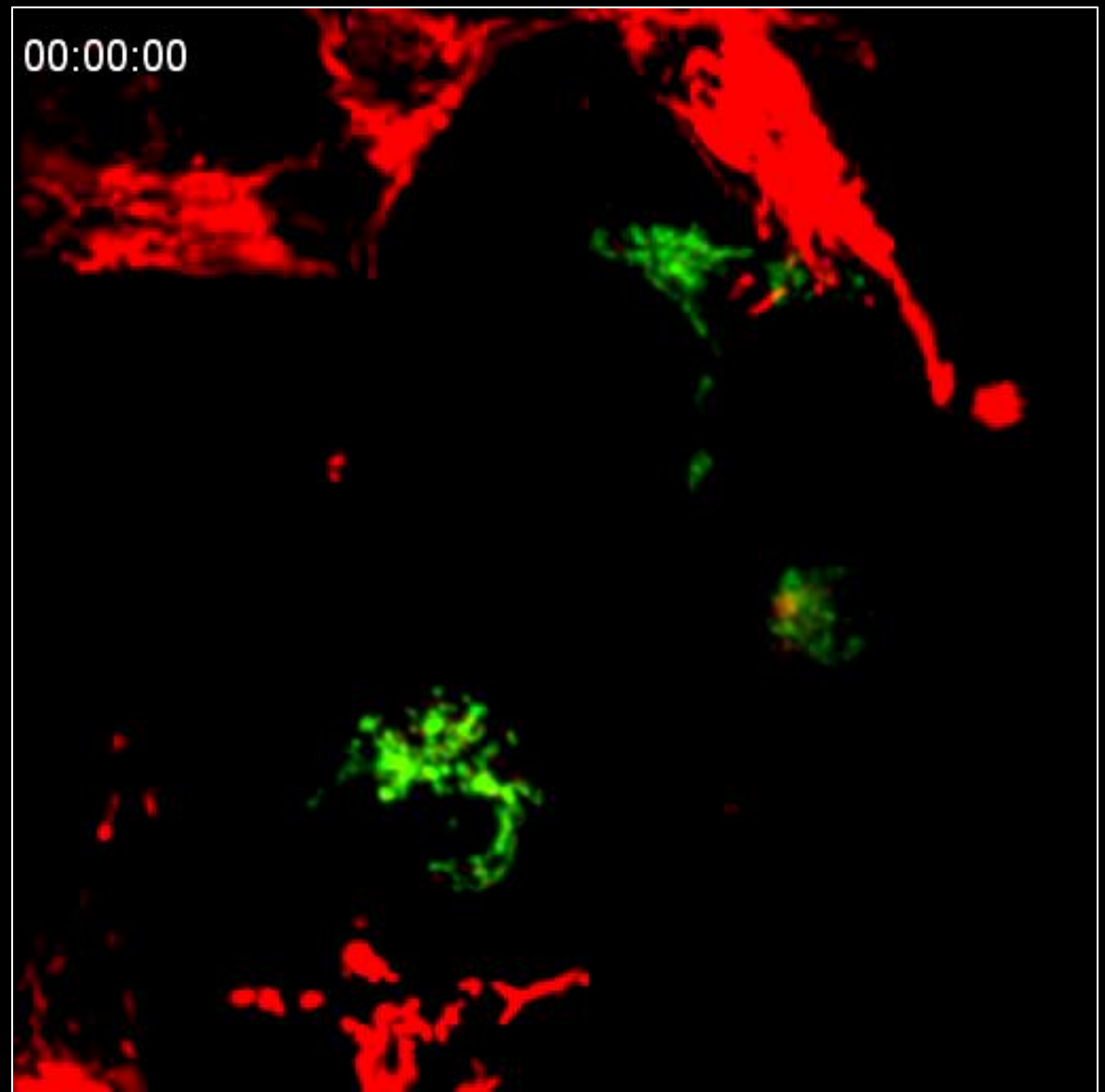
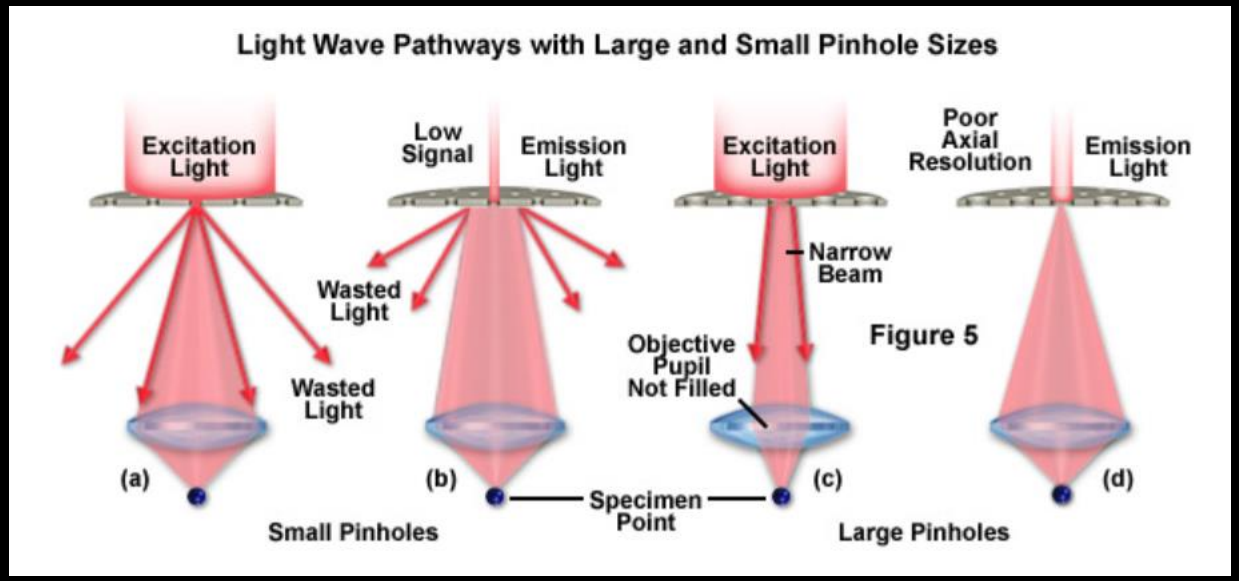
Mitocôndria de macrófagos (BMDM) em verde (Pham animals)







Optimal pinhole size is determined by size of Airy Disk and Magnification:

$$D_{optimal} = 1.2 \times M_{obj} \times \frac{\lambda_{EM}}{NA_{obj}}$$

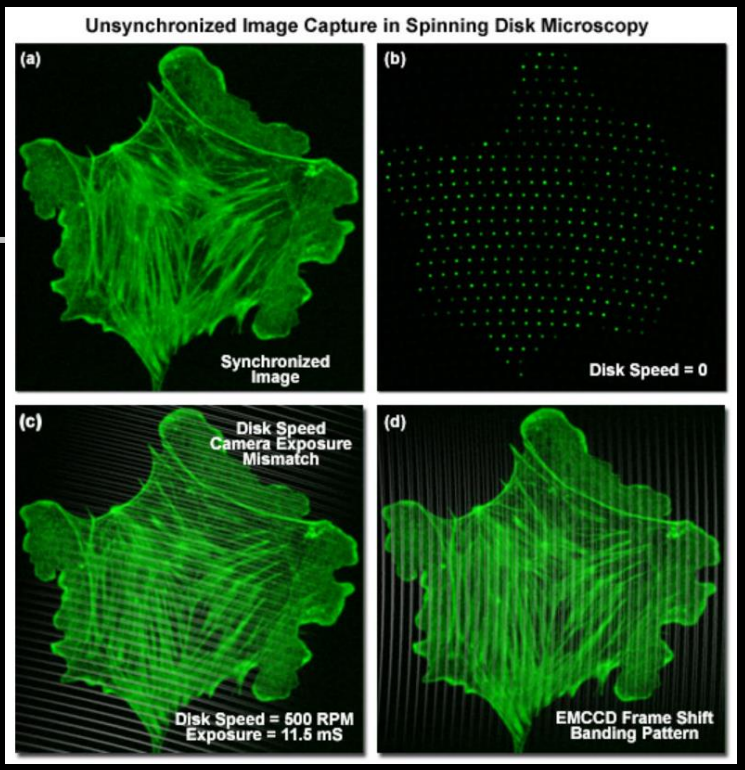
Most common pinhole sizes are: **25 µm and 50 µm**

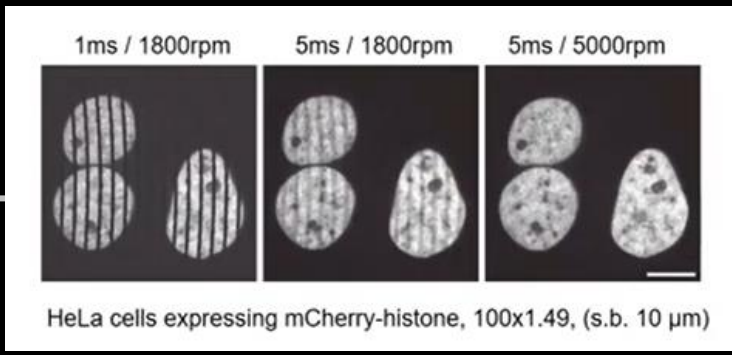
For 510nm emission (GFP):
100x, 1.4: Doptimal = 44 µm ~50 µm
60x, 1.4: Doptimal = 26 µm ~25 µm

Pinholes: Are my pinholes the right size?

Optimising Imaging Speed

- For most disks: 12 complete scans of image per full disk rotation
- Short exposure times: Exposure must be matched to whole number of scans to prevent streaking
- Long exposure times: Multiple scans begin to average out effects of streaking





Spinning Disk vs. Laser Scanning Confocal

Laser Scanning Confocal

- Best optical sectioning ar
  resolution
- Illumination and Emi
  pinholes independen
- Can choose exact emiss
  wavelength range or rang
  multiple PMTs
- Can strongly illuminate
  subregions, eg. For great detail,
  or FRAP

Only TIME-LAPSE?

Spinning Disk Confocal

lower photobleaching and toxicity:
umination power
ous acquisition

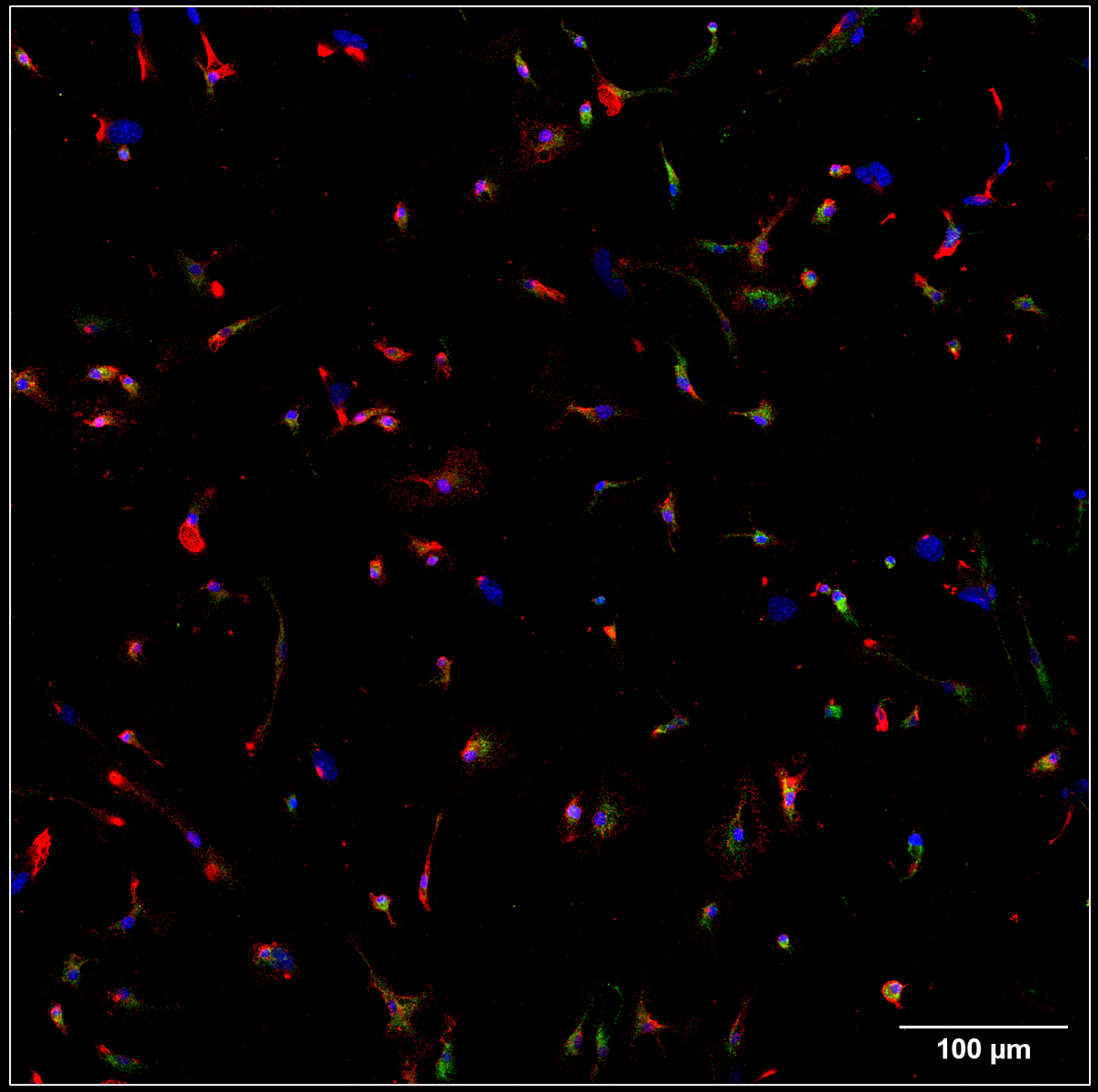
ster speed (100-1000x): <1s z stacks possible, or
ocesses in cells can be captured.

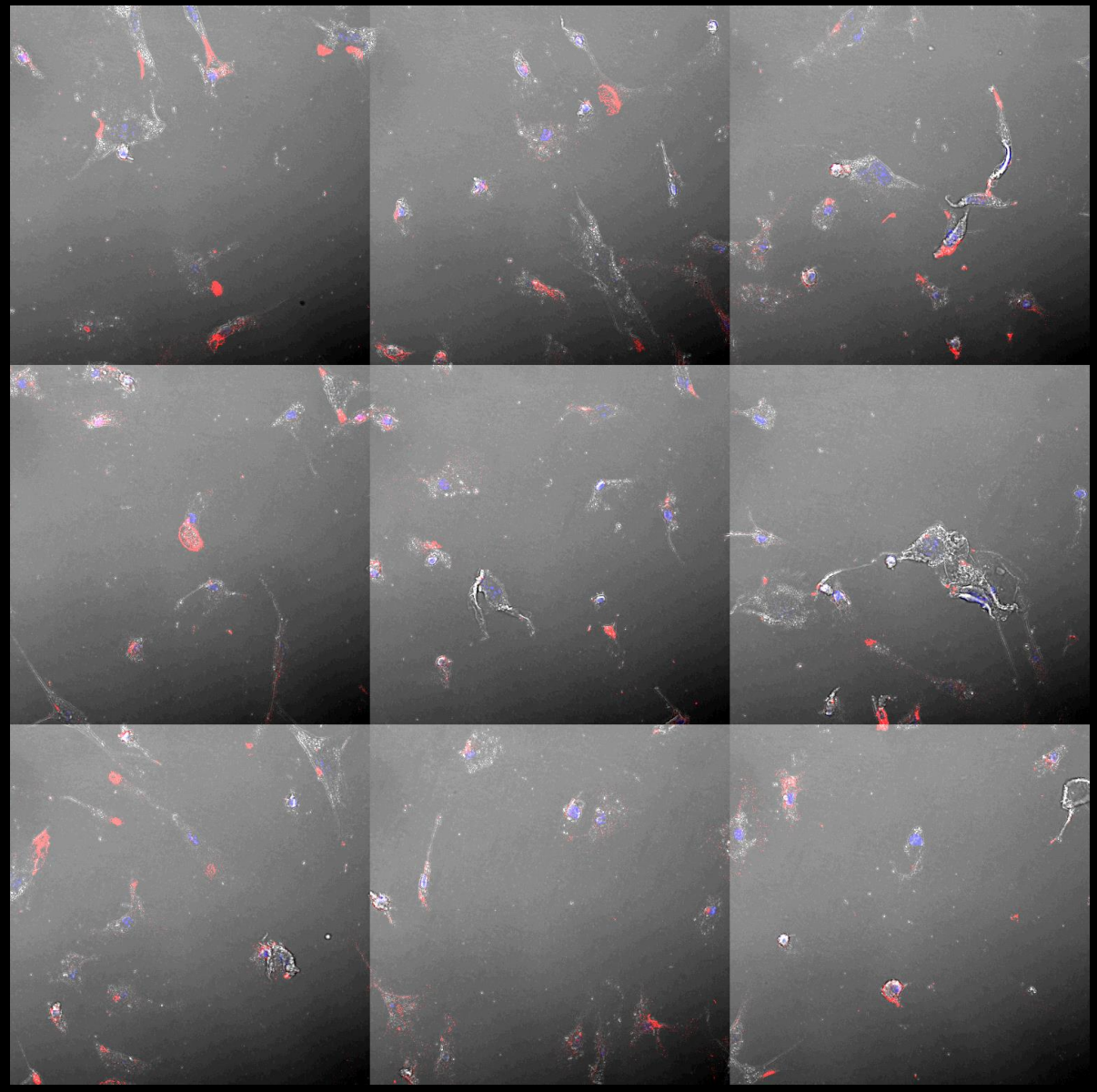
ell time per individual pixel is increased

CCD Camera

- Cheaper, and can be added to existing microscope
  easily.

Iba1 Dendra2 DAPI





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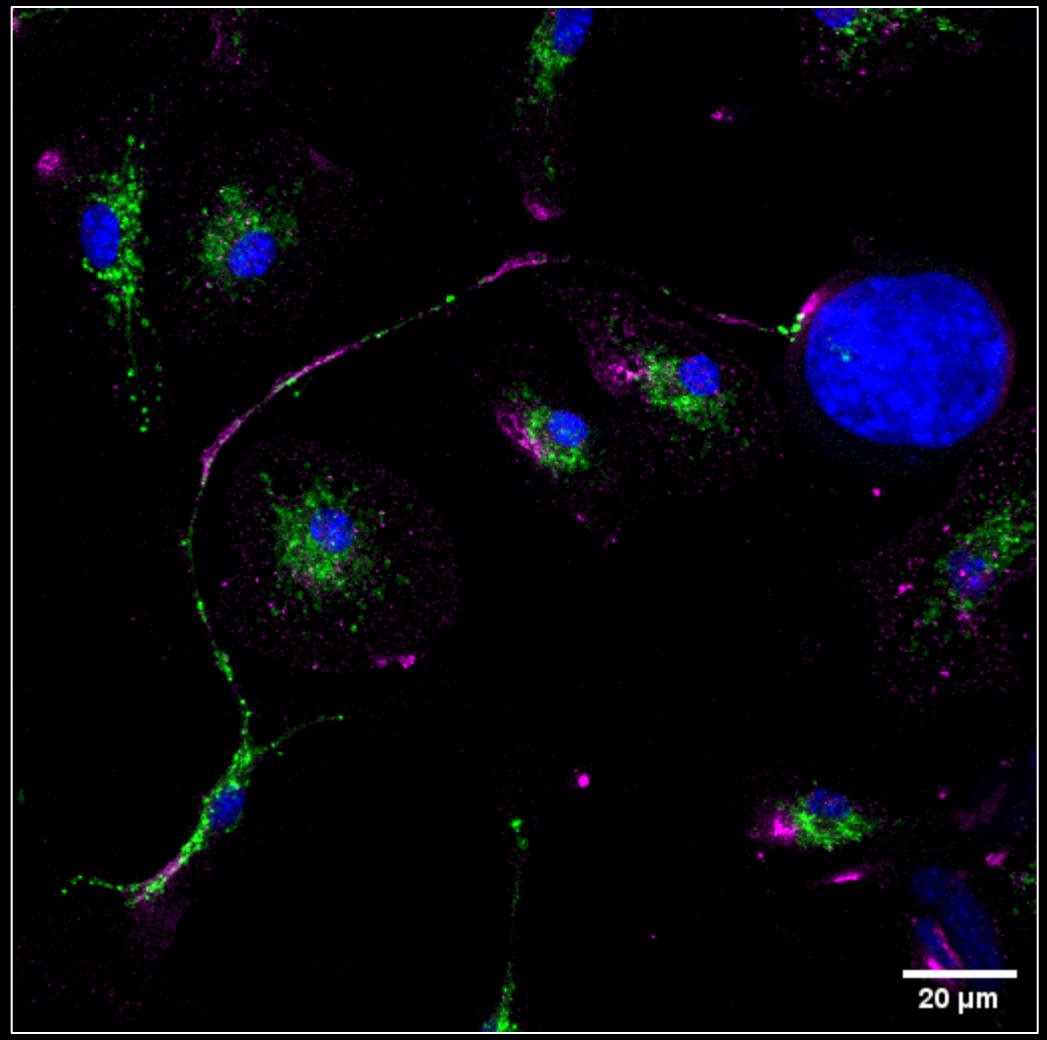
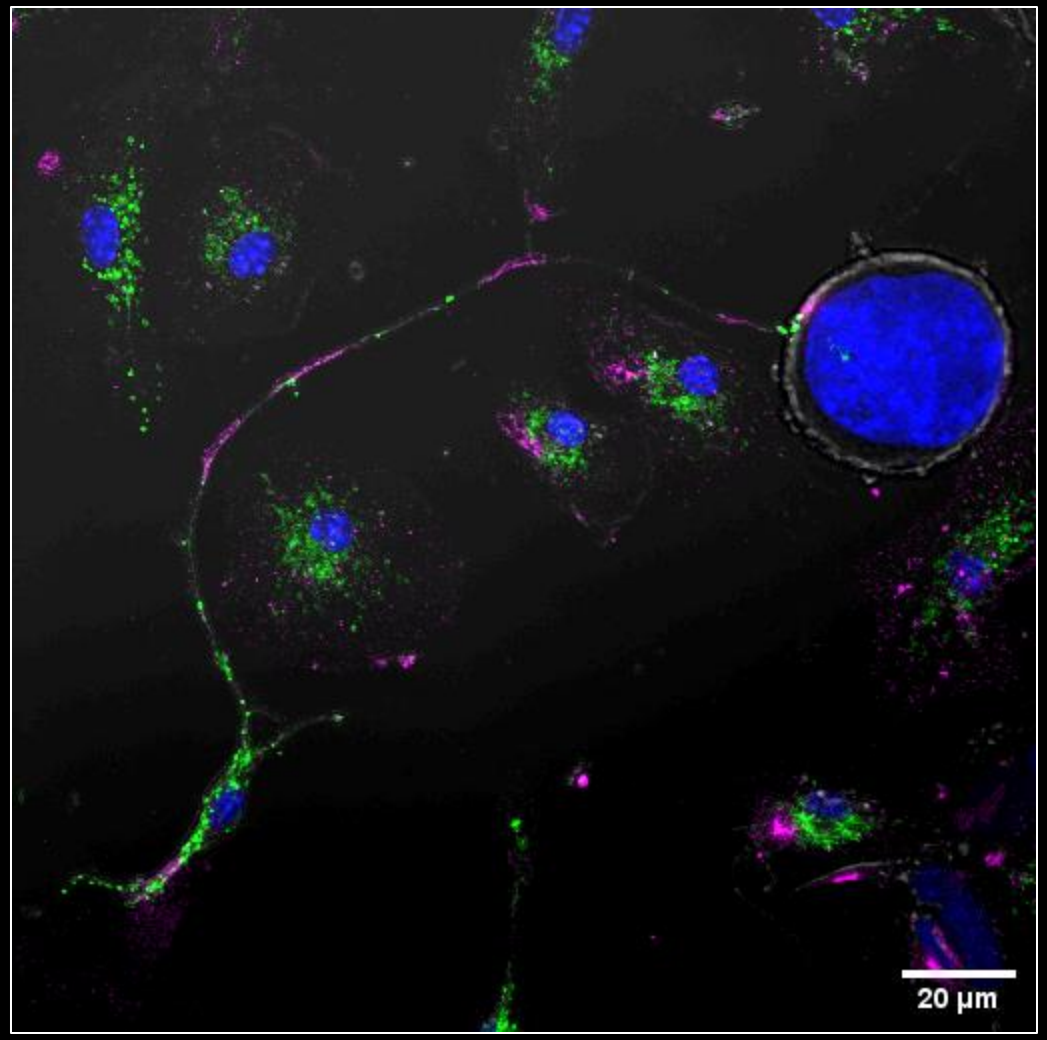
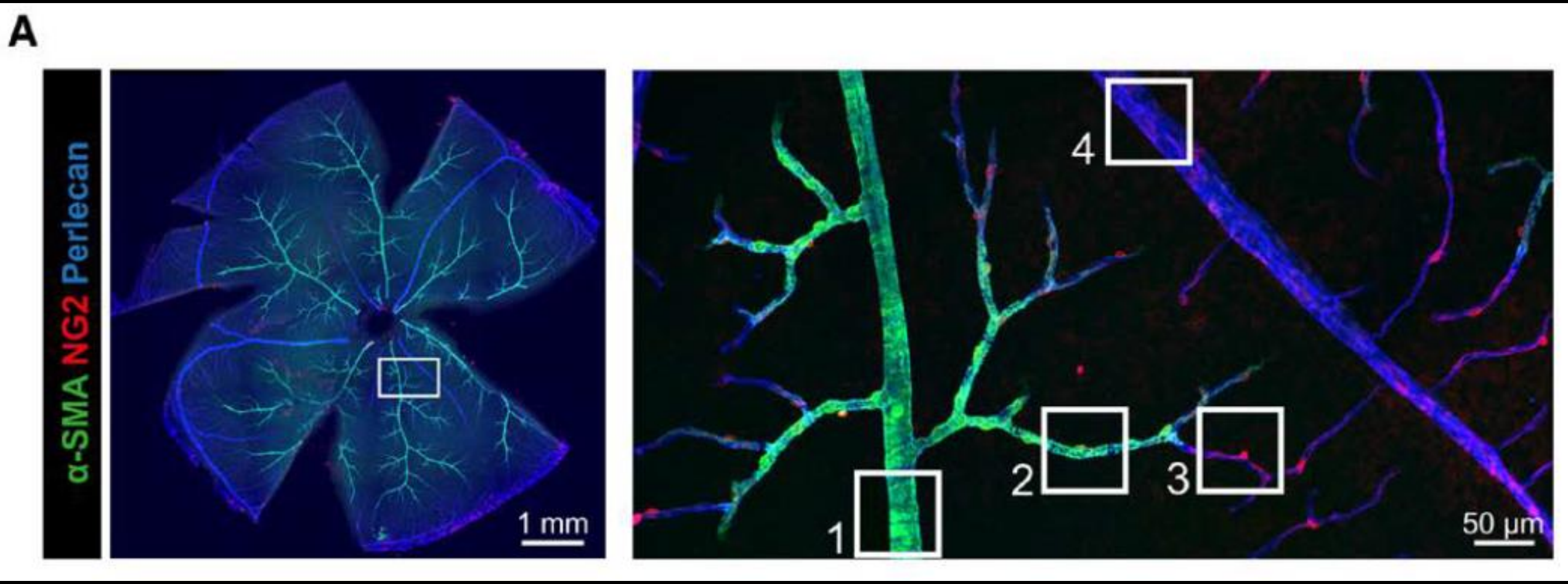


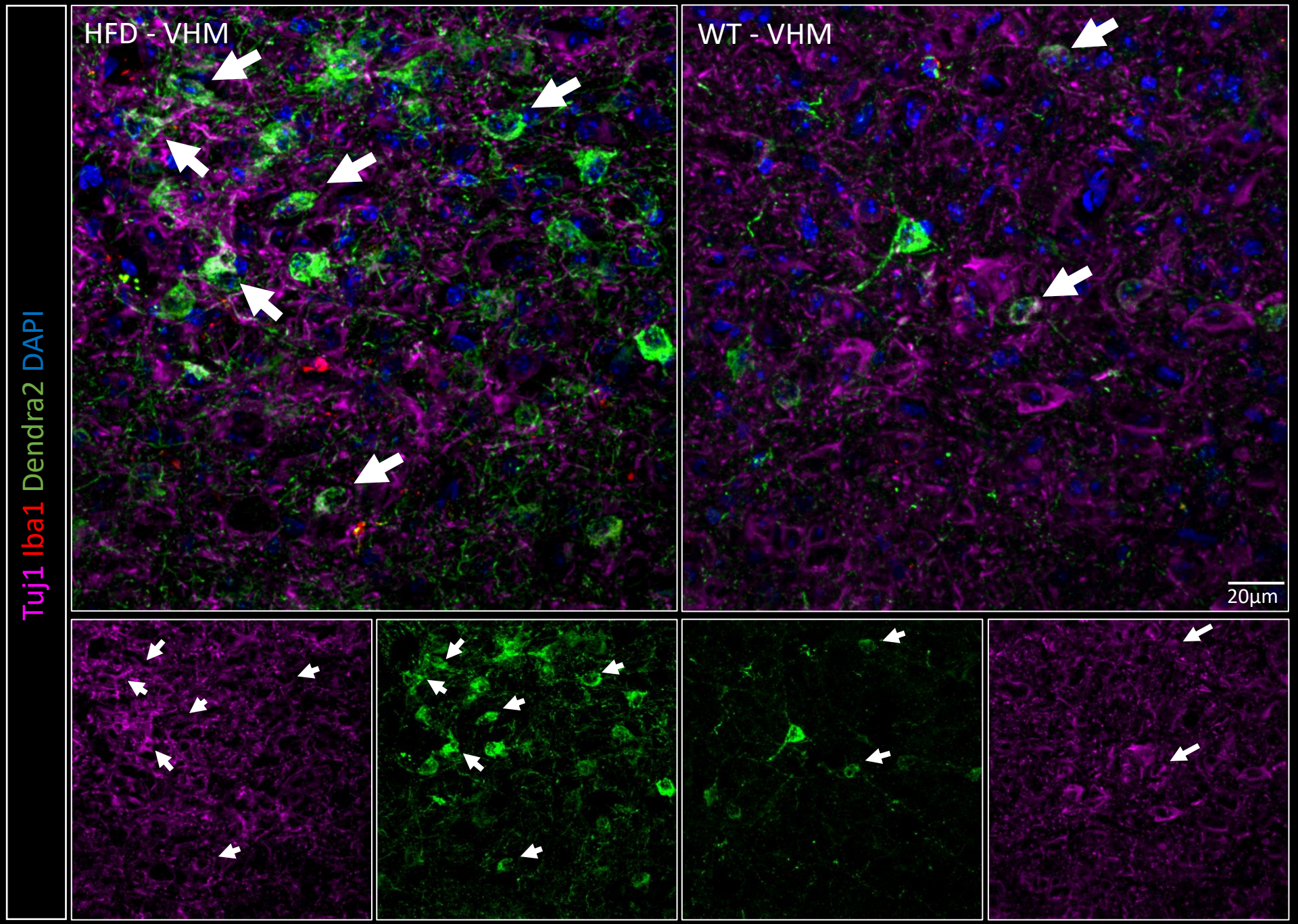




Ratelade et al., 2019

in vivo:

Pham mouse
after 12wks on HFD

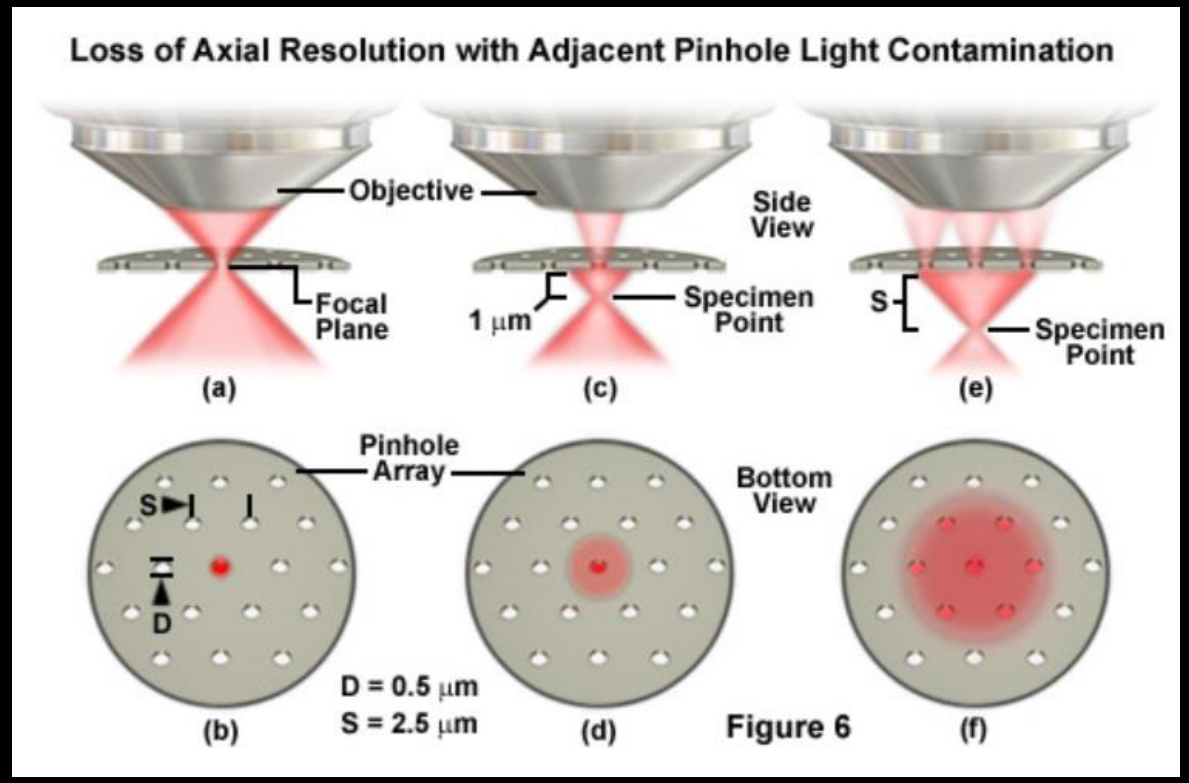


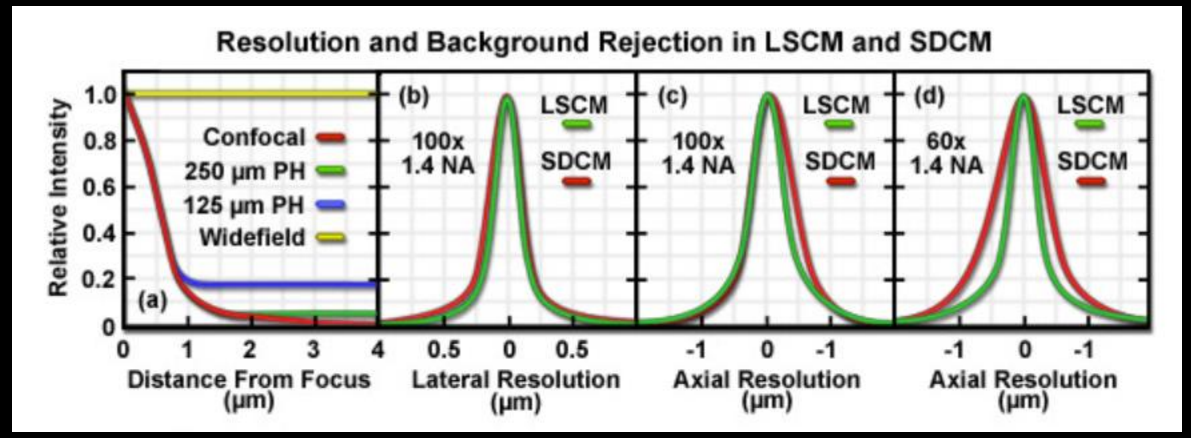
Disadvantages to using SDCM

1. Pinhole crosstalk

Pinhole Crosstalk

- Closer pinholes means more transmission
- ...but also crosstalk: out-of-focus light passing through adjacente pinholes
- This leads to background "haze" that worsens with sample thickness





Disadvantages to using SDCM

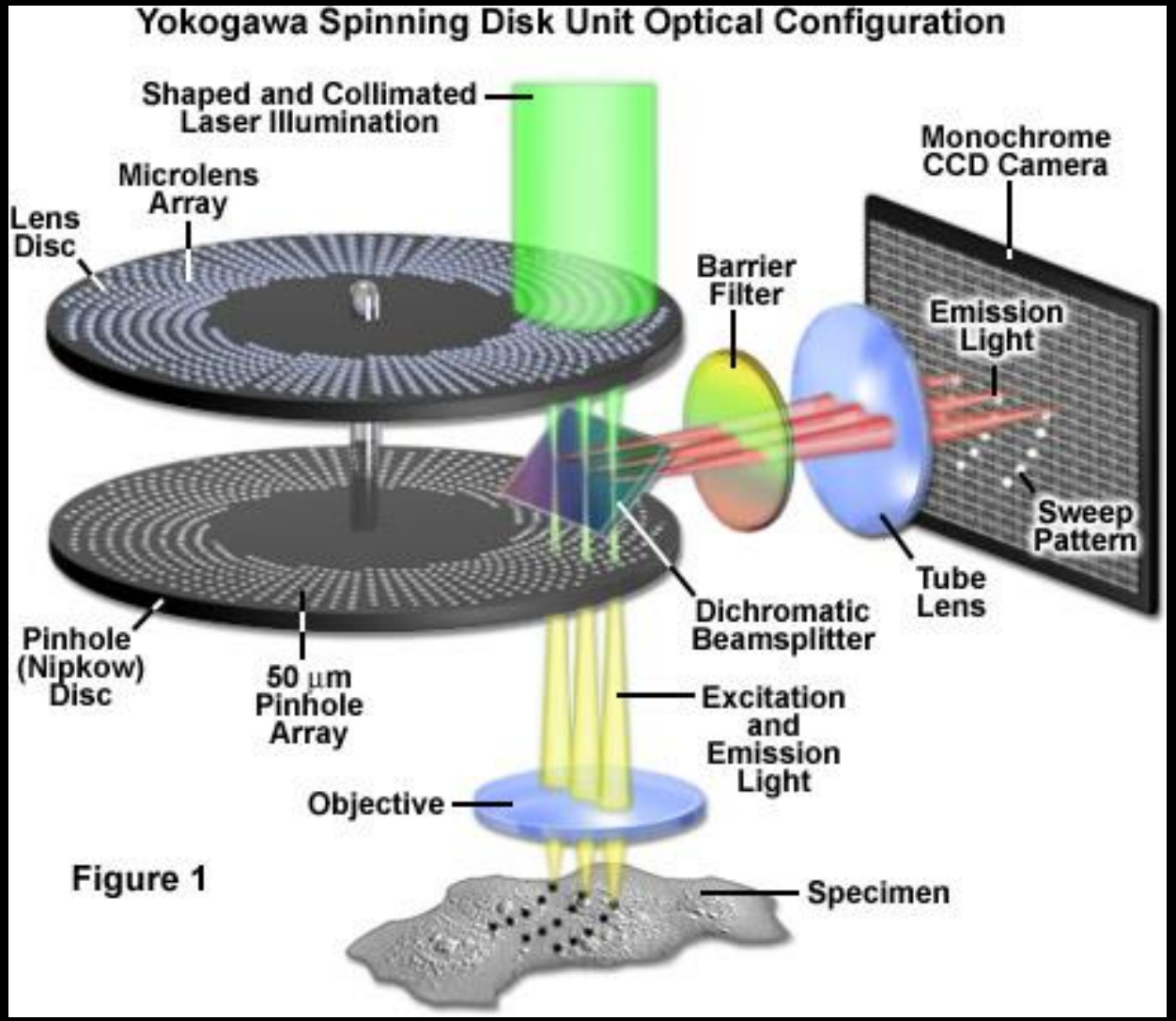
1. Pinhole crosstalk
2. Low level of light transmission

Transmittance: How Much light reaches the sample?

- Most light is blocked by the disk
- Transmittance ratio depends on size and spacing of pinholes:
- $T_{pinholes} = (D/S)^2$
- For typical values of D = 50µm, S = 250 µm,
- $T_{pinholes} = 4\%$

→ Need Strong illumination → Laser

Microlens Spinning Disk System



- Transmission of excitation light can be vastly increased with a second disk:

  A disk with a microlens array matching the pinhole array focuses excitation light through pinholes.

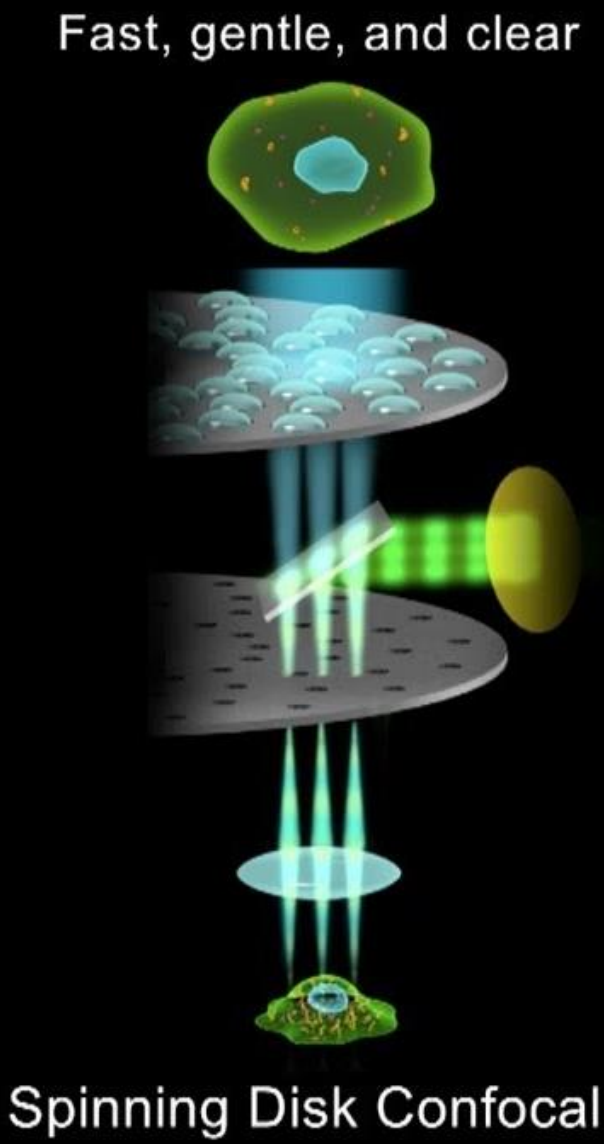
- Transmittance for excitation is increased up to 10x.

Disadvantages to using SDCM

1. Pinhole crosstalk
2. Low level of light transmission
3. In some instruments, 90% of the illumination light does not pass through the disk
4. Do not have the ability for experiments that require photobleaching or photoactivation

Conclusion

- Spinning disk confocal microscopy can significantly enhance the contrast and axial resolution of thin samples.
- Imaging can be performed at high speeds for large fields of view.
- Spinning disk confocal is kinder to sample than widefields or laser scannning confocal.
- There are a few adjustments and alignments needed to set up a spinning disk, but setup is typically straightforward.



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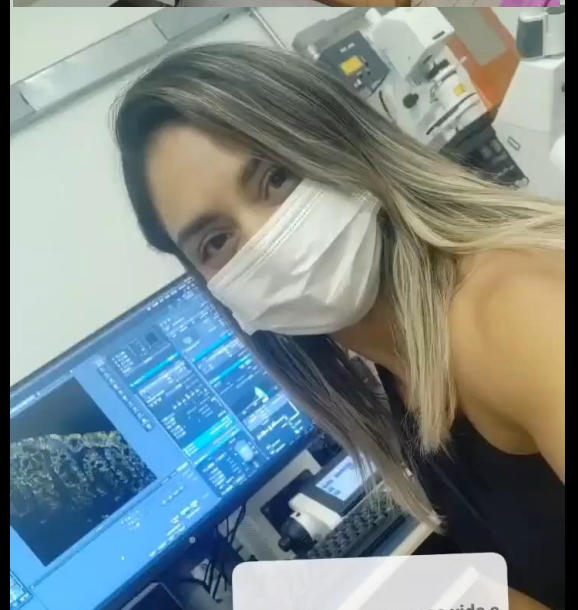
EXPERIMENTAL MEDICINE RESEARCH CLUSTER
EMRC

FAEPEX
UNICAMP


IT'S FRIDAY


Ousada Atrevida
Nicolas e Victor
FALTA POUCO PRA ESSA SEMANA LOUCA TERMINAR!
FORÇA NA PERUCA, GALERINHA!


INFABIC




Ah, se teus olhos pudessem ver
REALIDADE!