Spinning disk microscopy

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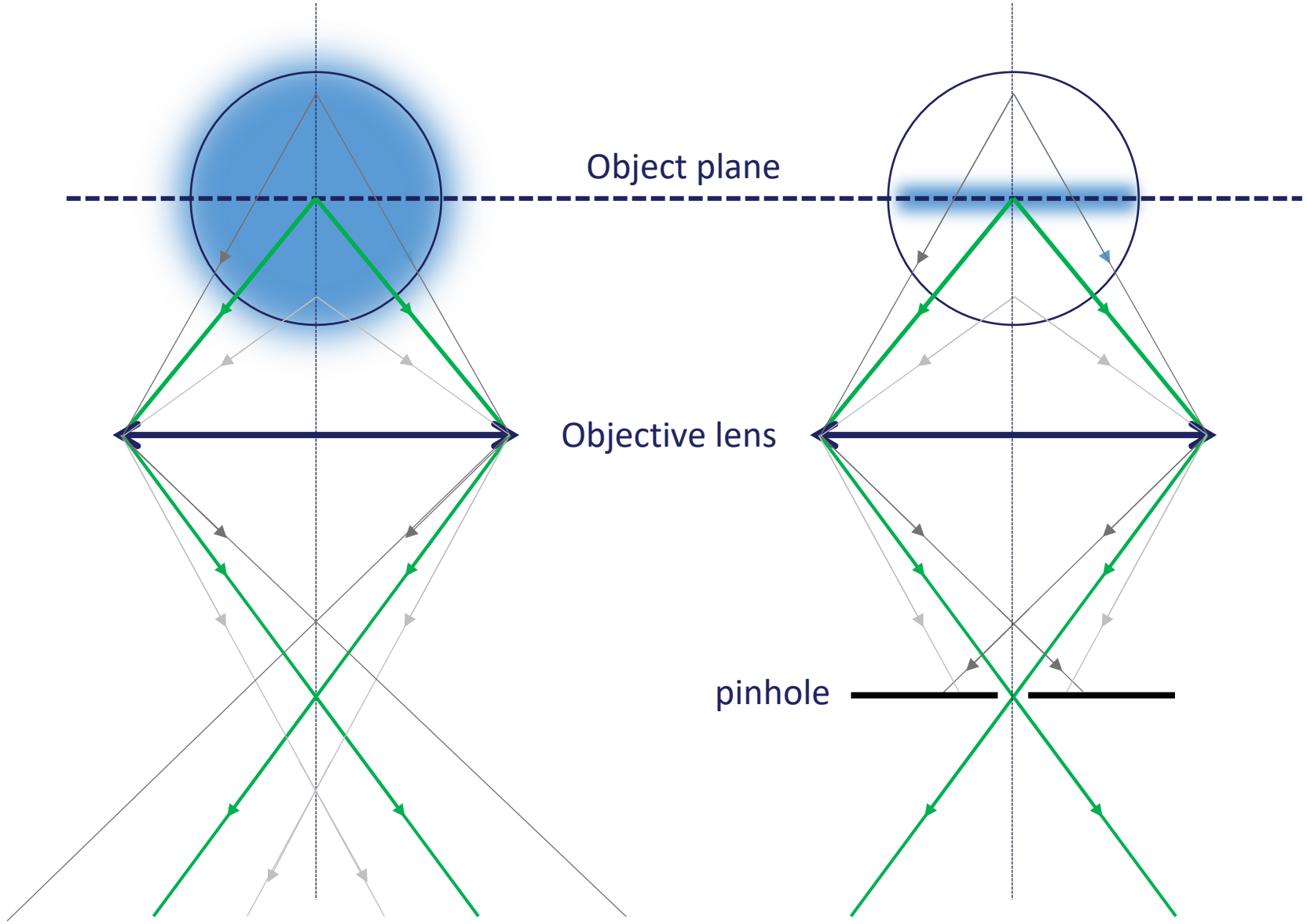
IMG

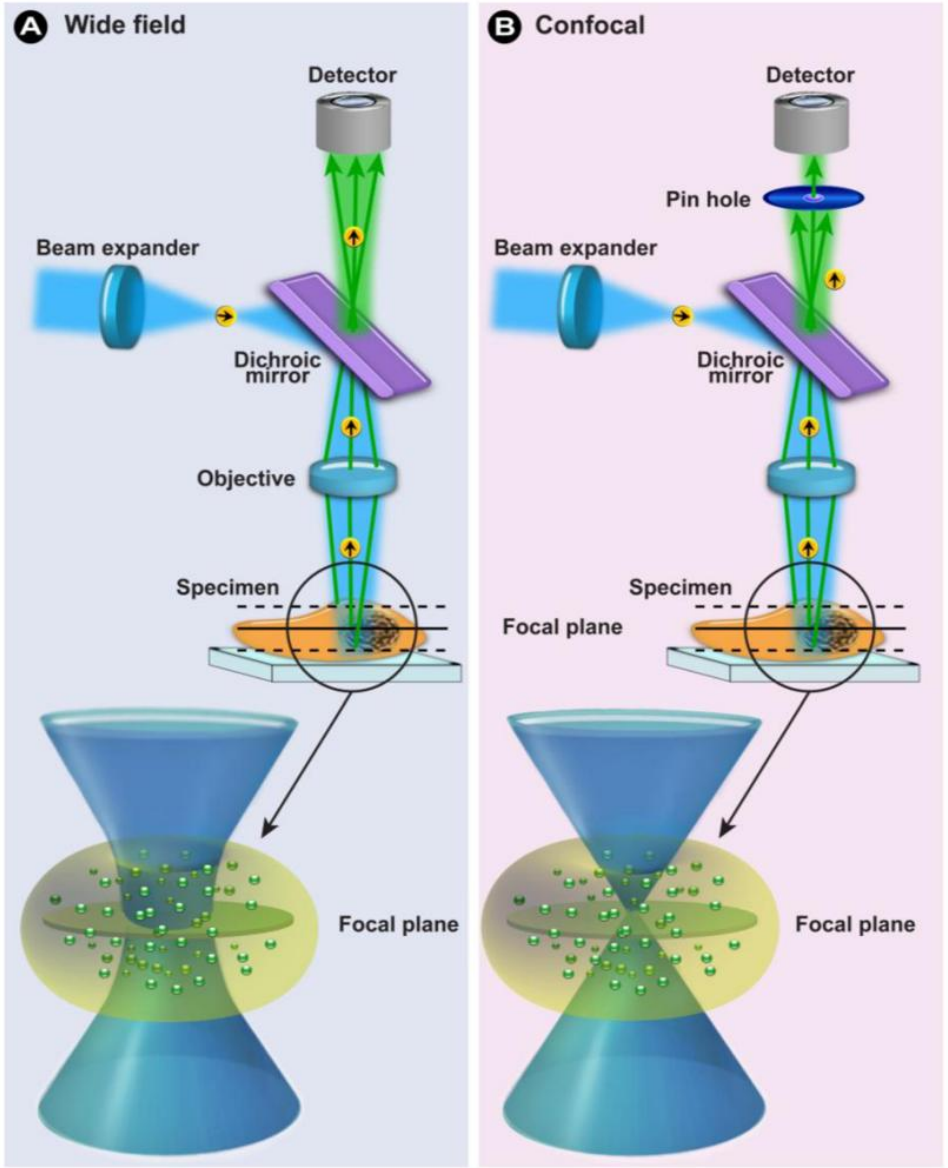
- Confocal microscopy
  - Laser scanning confocal
  - Spinning disk confocal
- Advantages/disadvantages
- Conclusion

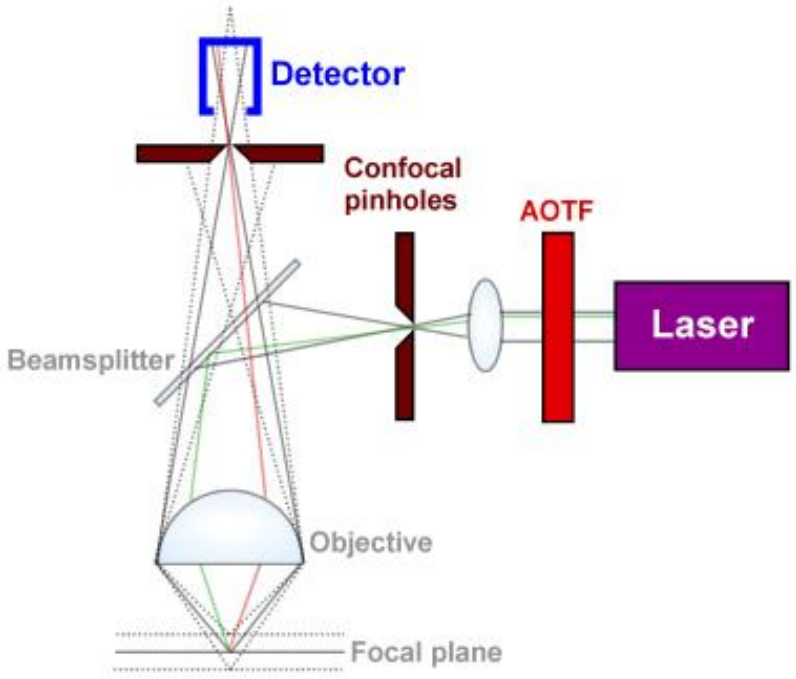
Widefield vs. Confocal microscopy



Courtesy of Ivan Novotný, LMCF, IMG CAS

Widefield vs. Confocal microscopy

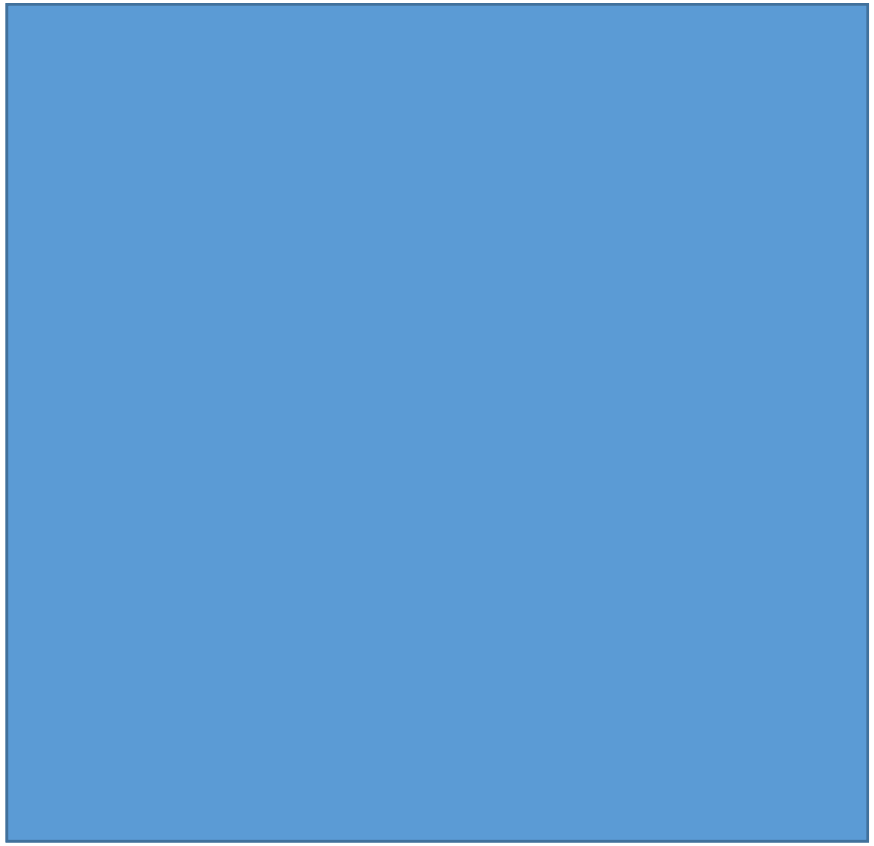


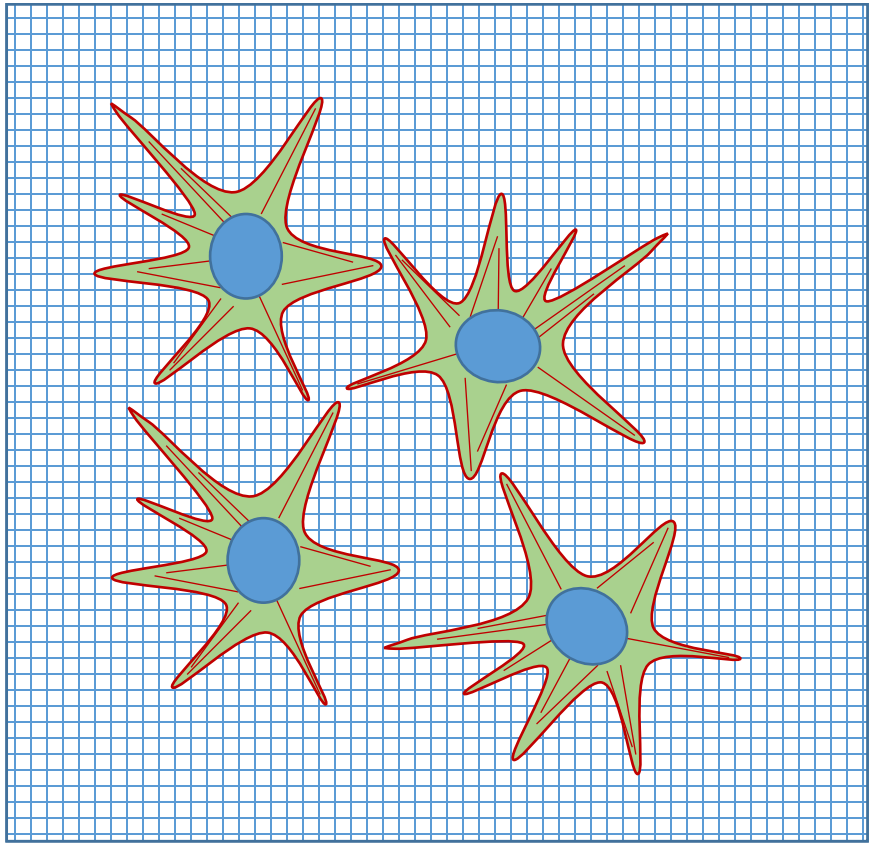


- dramatically increased contrast by removal of *out-of-focus* haze
- **optical sectioning** by confocal microscope

http://www.mdpi.com/1420-3049/17/4/4047/htm

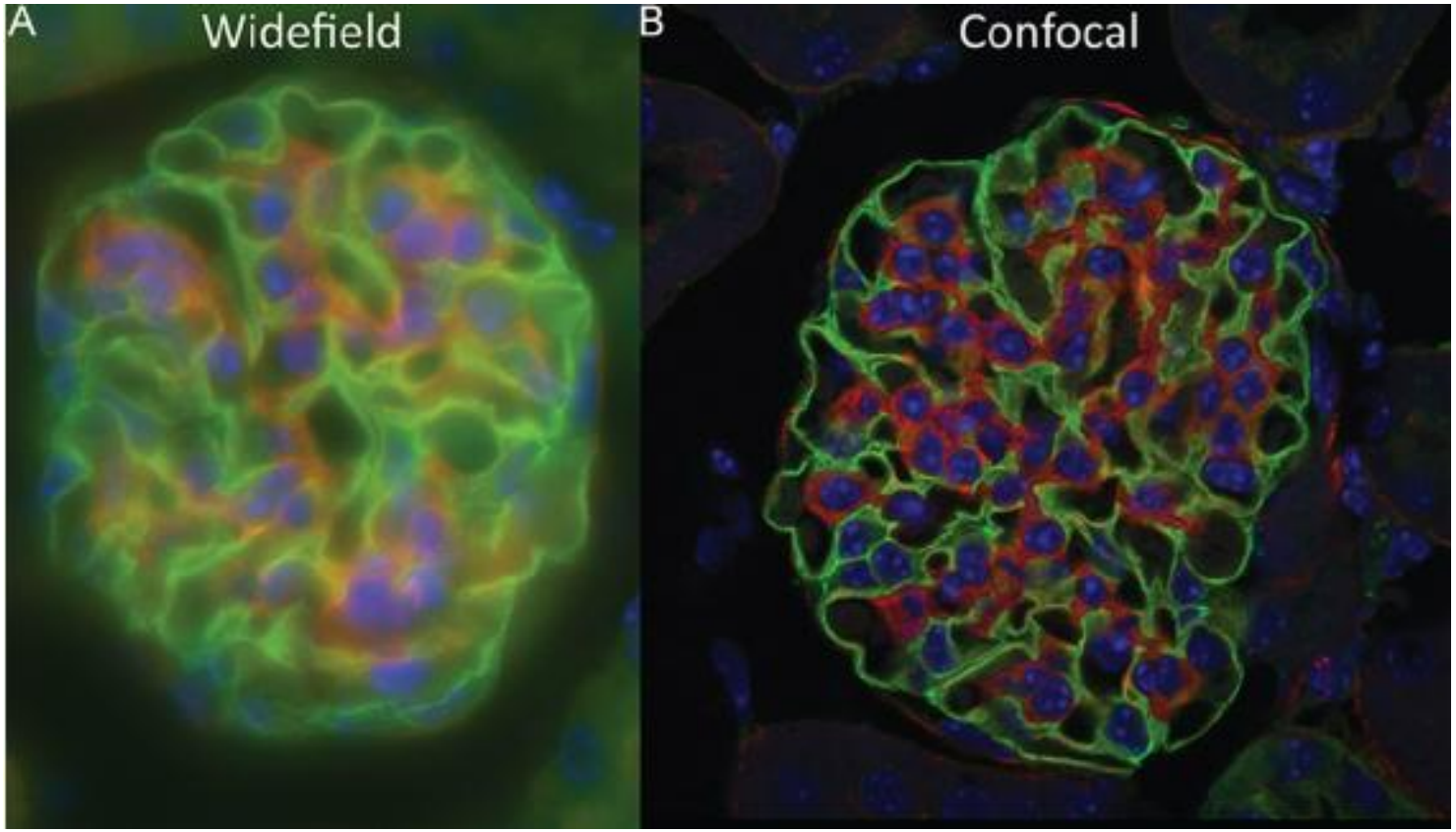
Widefield vs. Confocal microscopy





Courtesy of Ivan Novotný, LMCF, IMG CAS

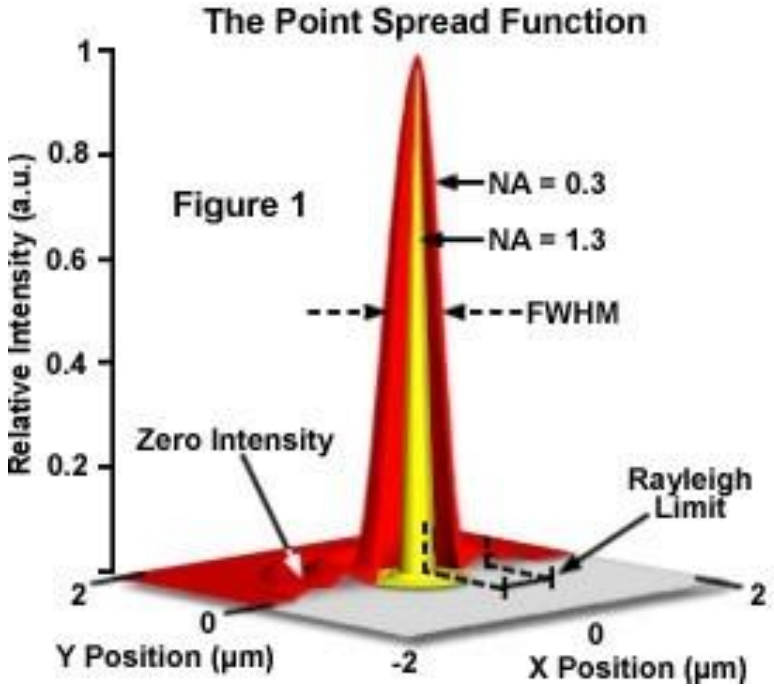
Widefield vs. Confocal microscopy



https://microscopysolutions.ca/2012/05/31/any_way_you_slice_it/

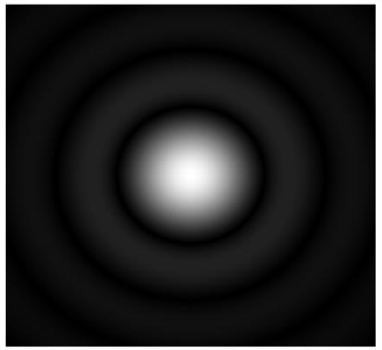
Confocal microscopy

Point spread function (**PSF**) describes the response of an imaging system to a point source or point object



- viewed in the x-y plane

- <u>lateral resolving power</u> of an objective lens can be evaluated by measuring the size of the Airy disk (FWHM)



- different shapes in *z* depending on the instrument used -> ***axial resolution***

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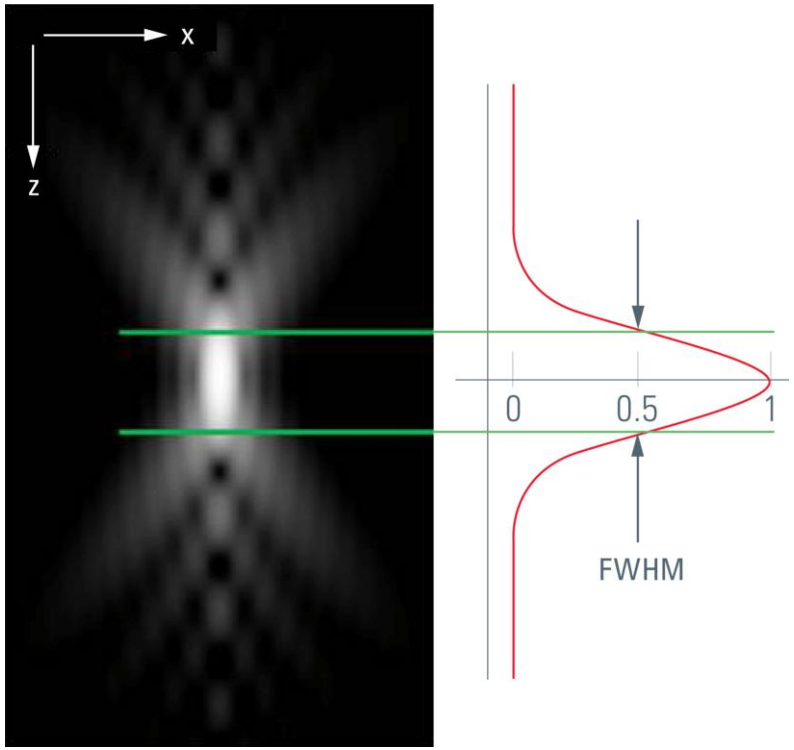
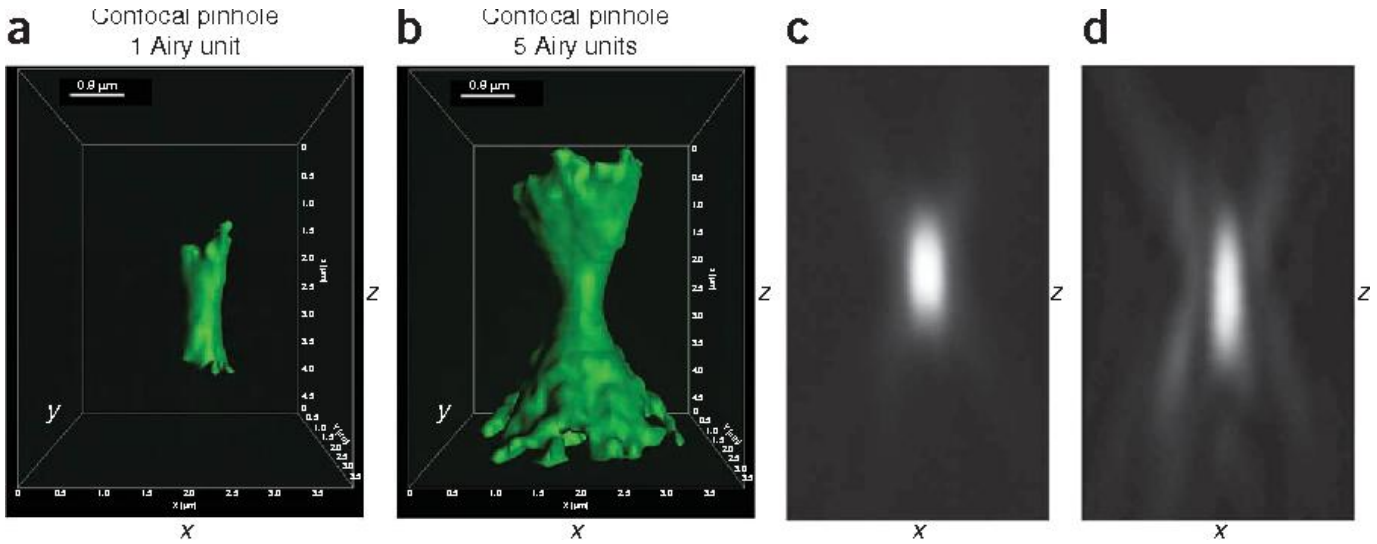




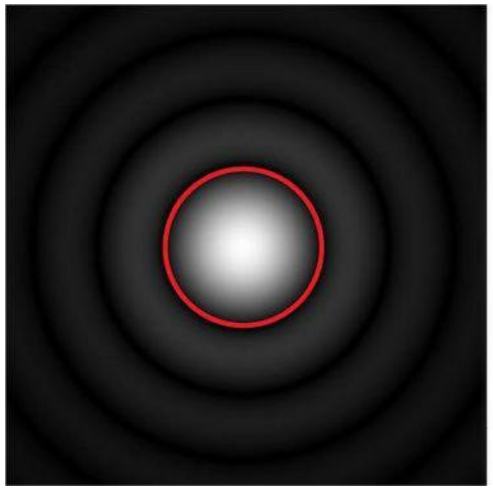
Richard W. Cole and Tushare Jinadasa and Claire M. Brown, Nature Protocols, 2011, vol. 6, 1929-1941.

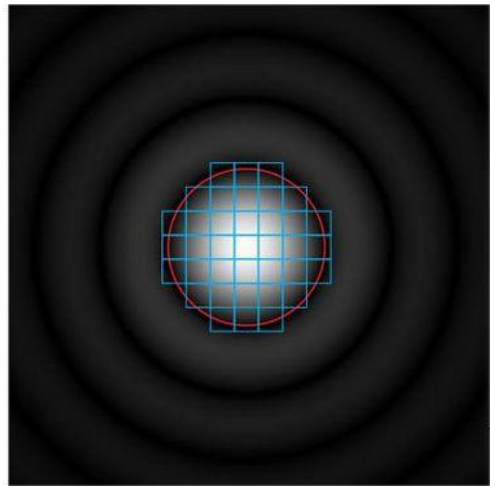
Confocal microscopy

Pinhole – light source is projected onto a tiny aperture (excitation)

– detection pinhole (emission) – *blocks out-of-focus signal*

- Confocal imaging – transmits Airy disc
  - *Closing of the pinhole is measured in Airy units, 1 Airy unit = diameter of the pinhole that passes the Airy disk*
  - *Depends on:*
    - wavelength λ
    - numerical aperture NA
    - magnification of the objective lens
    - magnification of internal optics of the microscope





https://www.leica-microsystems.com/science-lab/pinhole-effect-in-confocal-microscopes/

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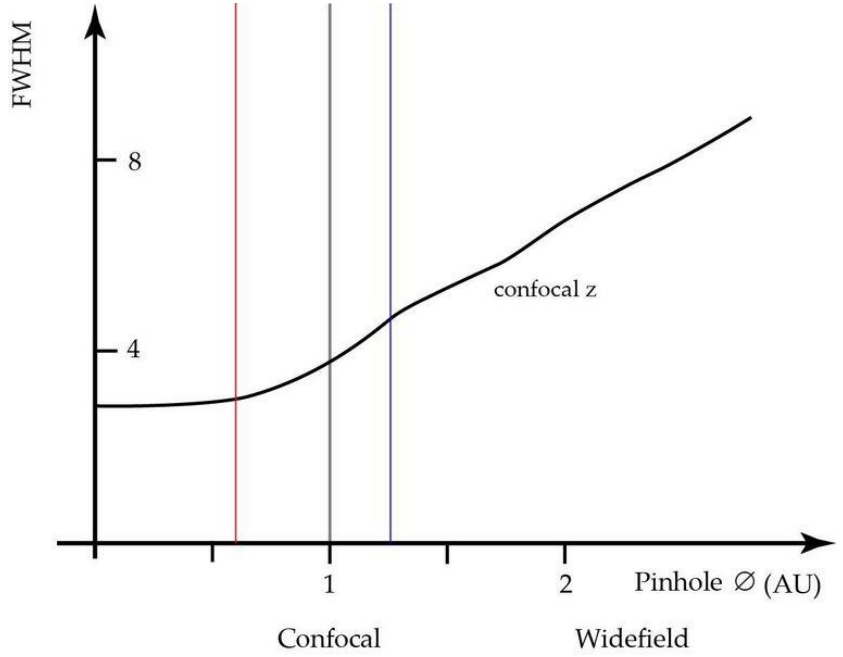
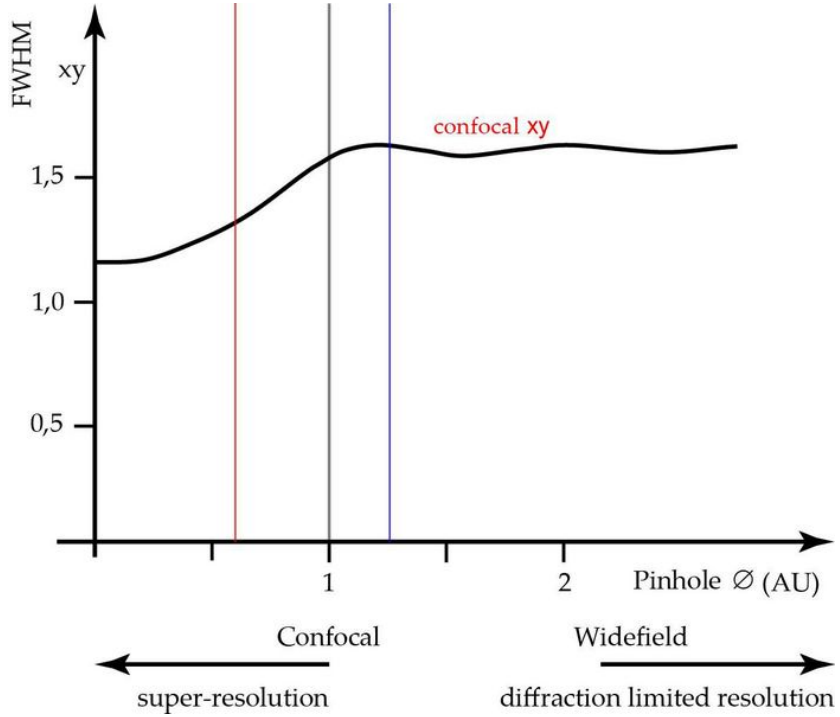




Larger pinhole – better SNR

– more extrafocal contributions

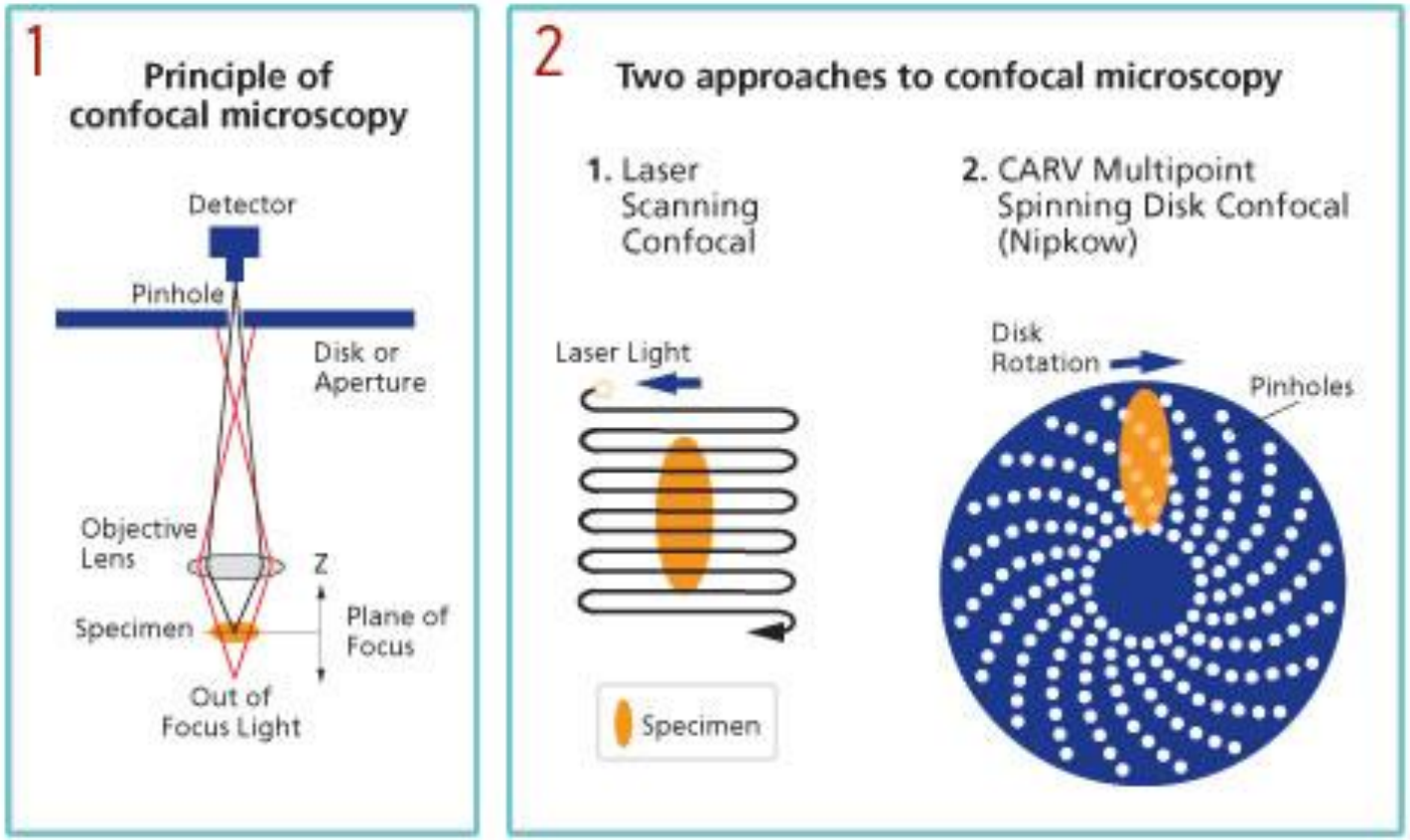
Smaller pinhole – better lateral resolution

– lower SNR

https://www.leica-microsystems.com/science-lab/pinhole-effect-in-confocal-microscopes/

Confocal microscopy

Laser scanning confocal microscopy (LSCM) vs. Spinning disk confocal microscopy



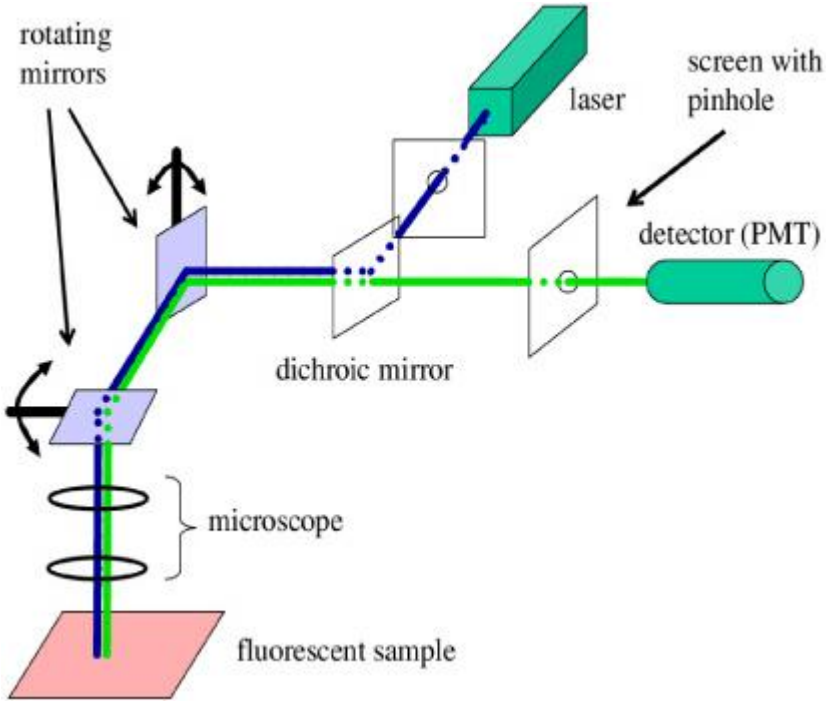
Confocal microscopy

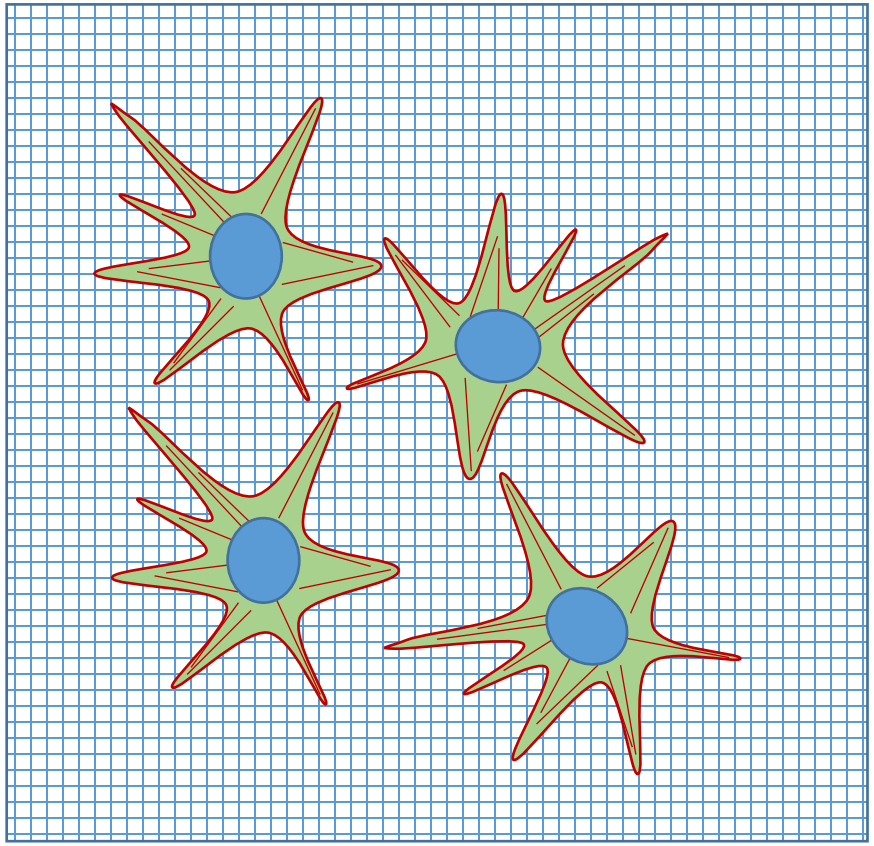
Resolution

- **geometry of the detection pinholes or slits**
- objective numerical aperture
- wavelength of emission light
- refractive index of the imaging medium between the specimen and the objective front lens

- *characteristics and thickness of the specimen*
  - issue in spinning disk microscopy - heavily stained, thick specimens
  - larger pinhole diameters for sufficient signal-to-noise ratio in the final confocal image, lower axial resolution than LSCM

Laser scanning microscope



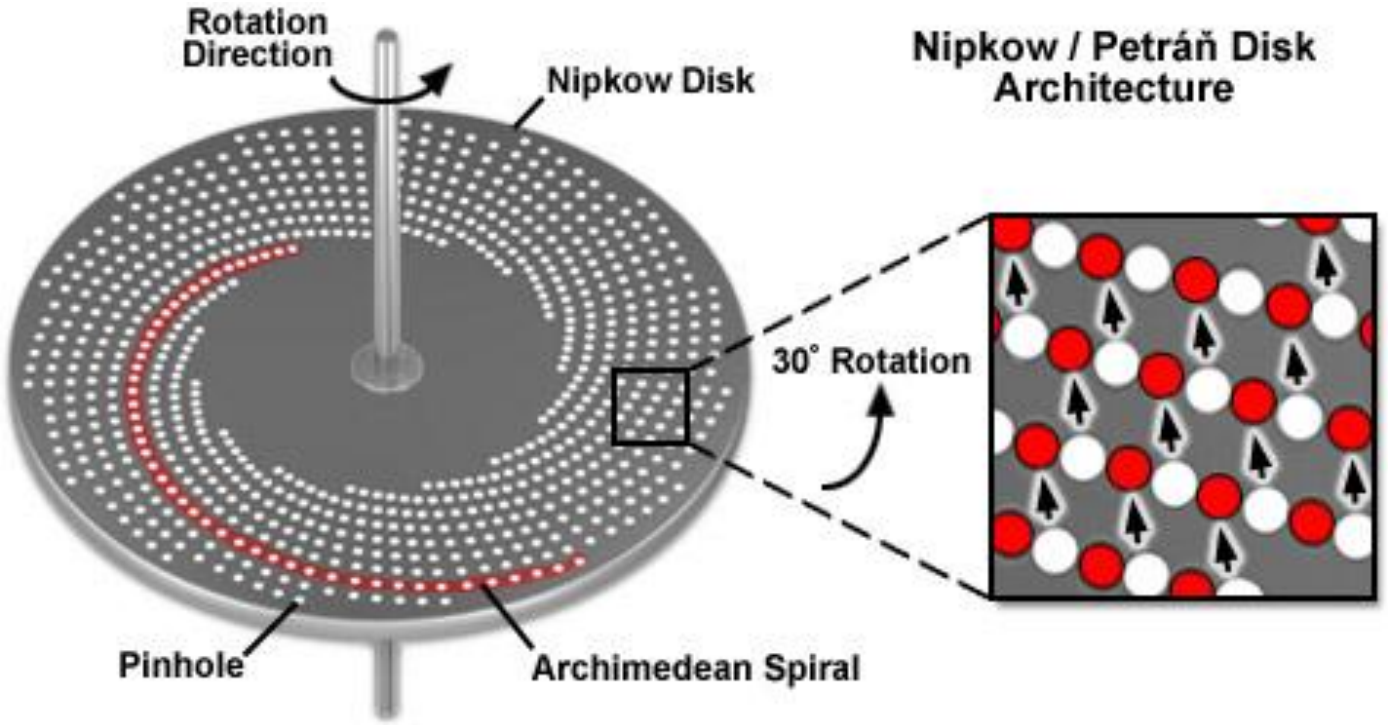


Scanning of the sample point by point using the galvo scanning mirrors

Courtesy of Ivan Novotný, LMCF, IMG CAS

Spinning disk microscope

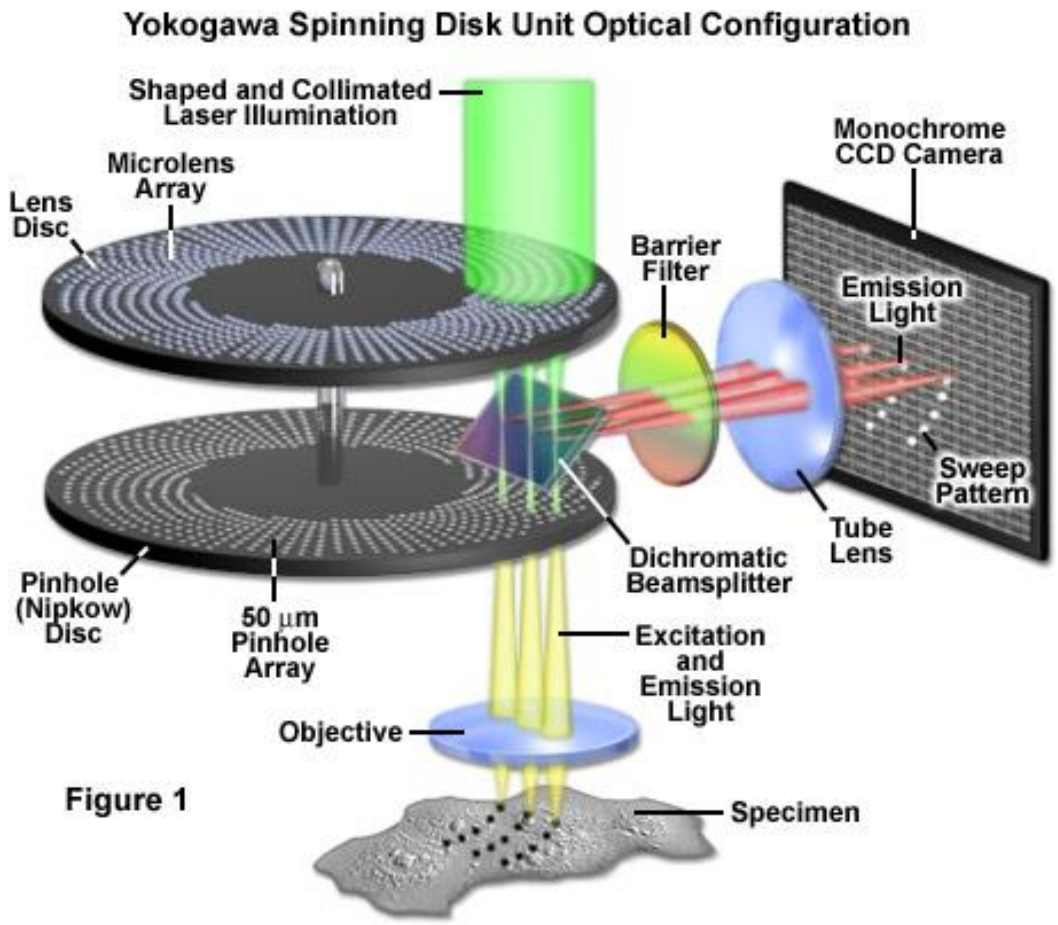
- Nipkow disk (Paul Nipkow, 1884)
  - pinholes arranged in an Archimedean spiral
  - approximately 1,000 pinholes being illuminated and returning an image at a time



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

Spinning disk microscope

- Spinning disk microscope design

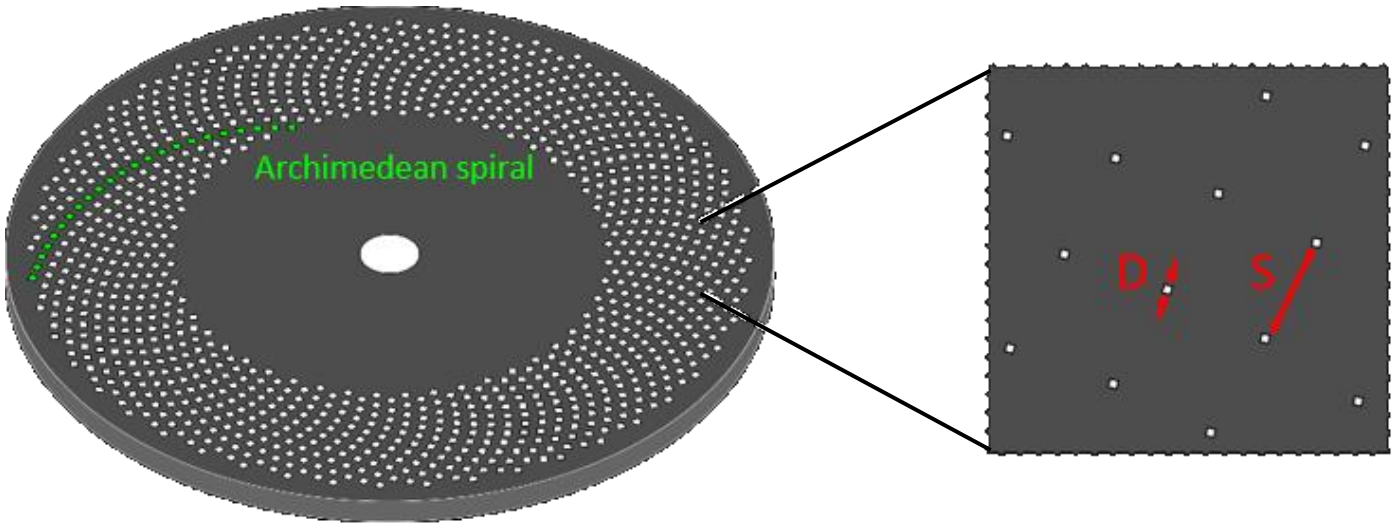


- microlens disk
- Nipkow disk (pinholes)
- light throughput approaches 40 to 60 %

- image is created with each 30° rotation of the disc
- up to 2000 images per second

- *pinhole size and spacing - a tradeoff between generating enough light to successfully image the specimen and maintaining a high degree of confocality (usually 2 sets of pinholes)*

Nipkow disk



D - pinhole diameter

S – pinhole spacing

$T = (D/S)^2$

Transmittance - proportion of incident light that passes through a disk (typically only few %)

D – determines thickness of vertical section

S – pinhole cross-talk

Example: D = 50 *µm, S* = 250 *µm*

-> pinholes will transmit 4 % of incident light

https://www.photometrics.com/learn/spinning-disk-confocal-microscopy/introduction-to-spinning-disk-confocal

Nipkow disk

Optimal pinhole size

$$D_{opt} = 1.2 \frac{M_{obj}\lambda_{em}}{NA_{obj}}$$

- first minimum of the Airy disk from a point source

- too high – reduces axial resolution
- too low – reduces image contrast, blocks emission light

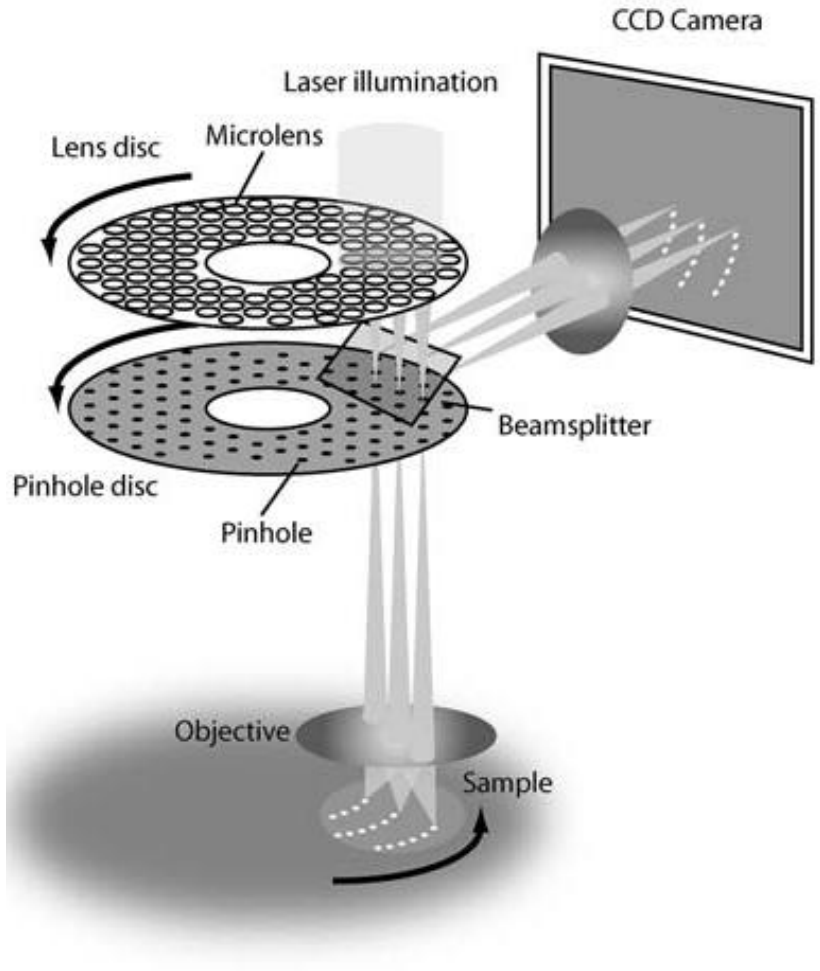
Example: GFP (λ_{em} = 509 nm), 60x 1.4NA oil objective -> 26.2 µm
100x 1.4NA oil objective -> 43.6 µm

https://www.photometrics.com/learn/spinning-disk-confocal-microscopy/introduction-to-spinning-disk-confocal

Microlens disk

Disk with microlenses in the place of pinholes

- focuses illumination light through the pinholes of the primary disk
- transmittance may be <u>improved by an order of magnitude</u>



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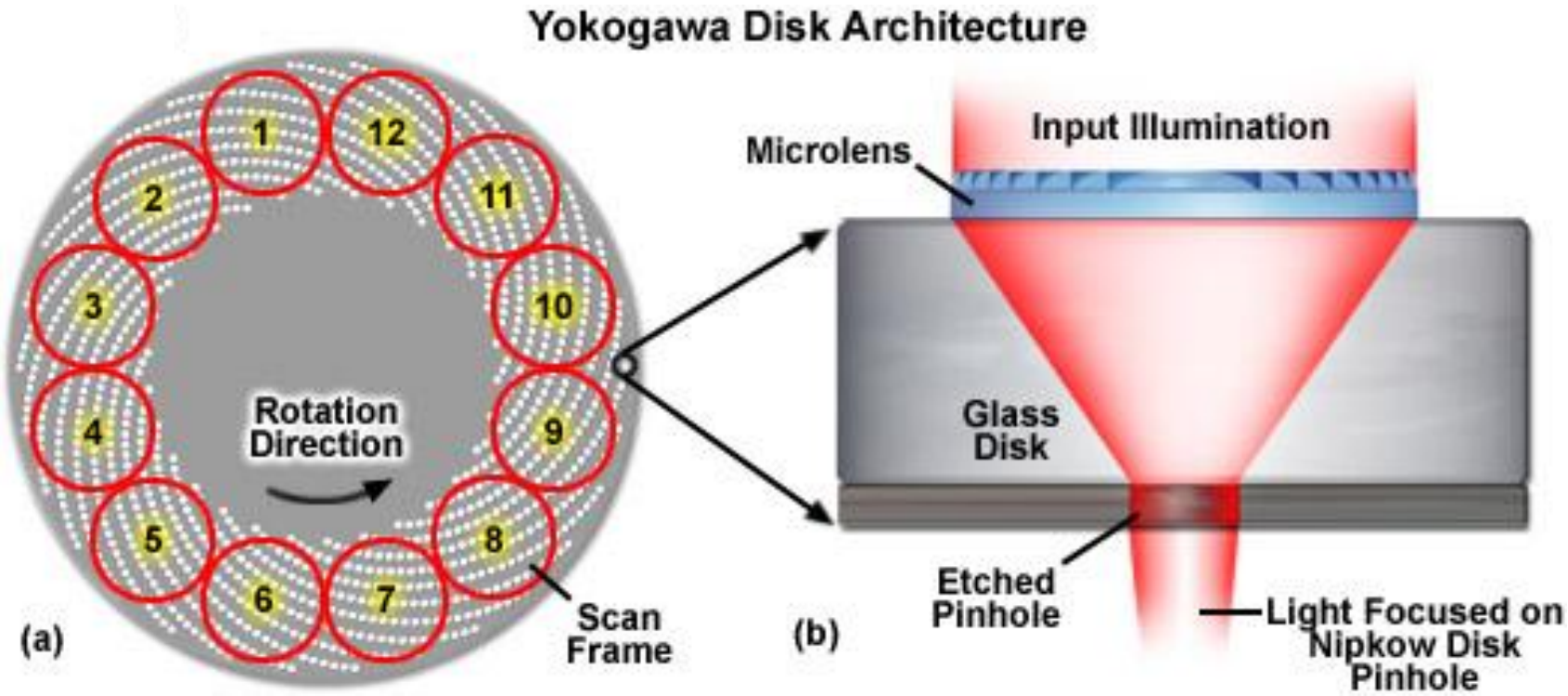
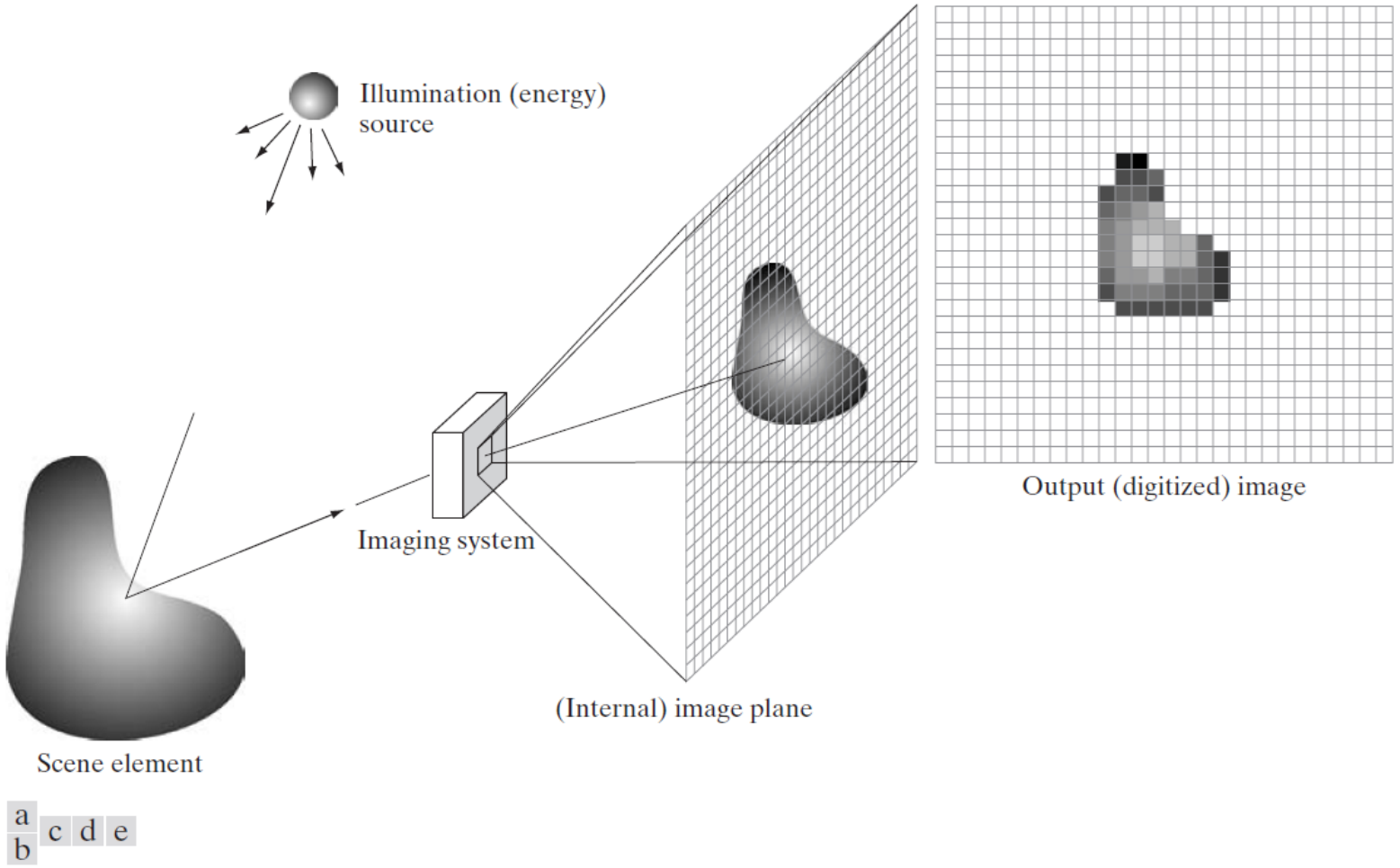
Microlens disk



- single scan of the specimen - every 30-degree rotation
- 360-degree rotation of the disk = 12 frames, or 2,000 images per second at the highest disk speeds

Cameras

- Sensor arrays in digital cameras
- In sensor arrays each picture element consists of a **photodetector**

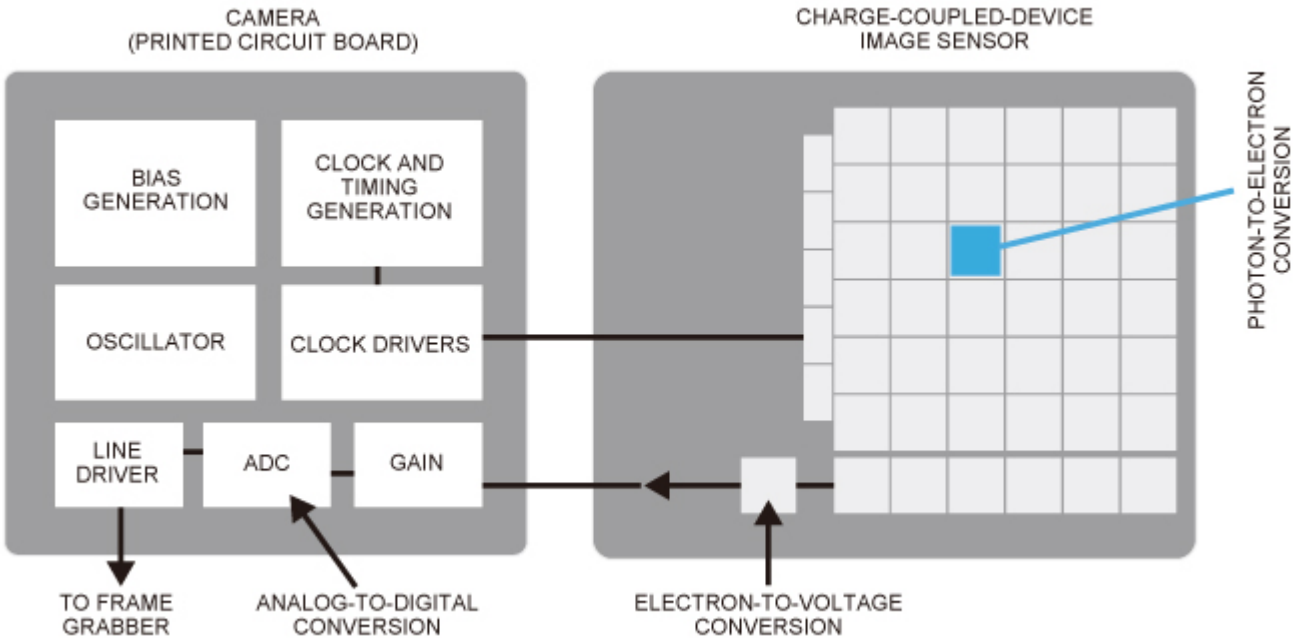


a b c d e

Rafael C. Gonzales, Richard E. Woods: Digital Image Processing, 3rd edition

Cameras

- *Charge-coupled device* **(CCD)** - photoelectric sensor array

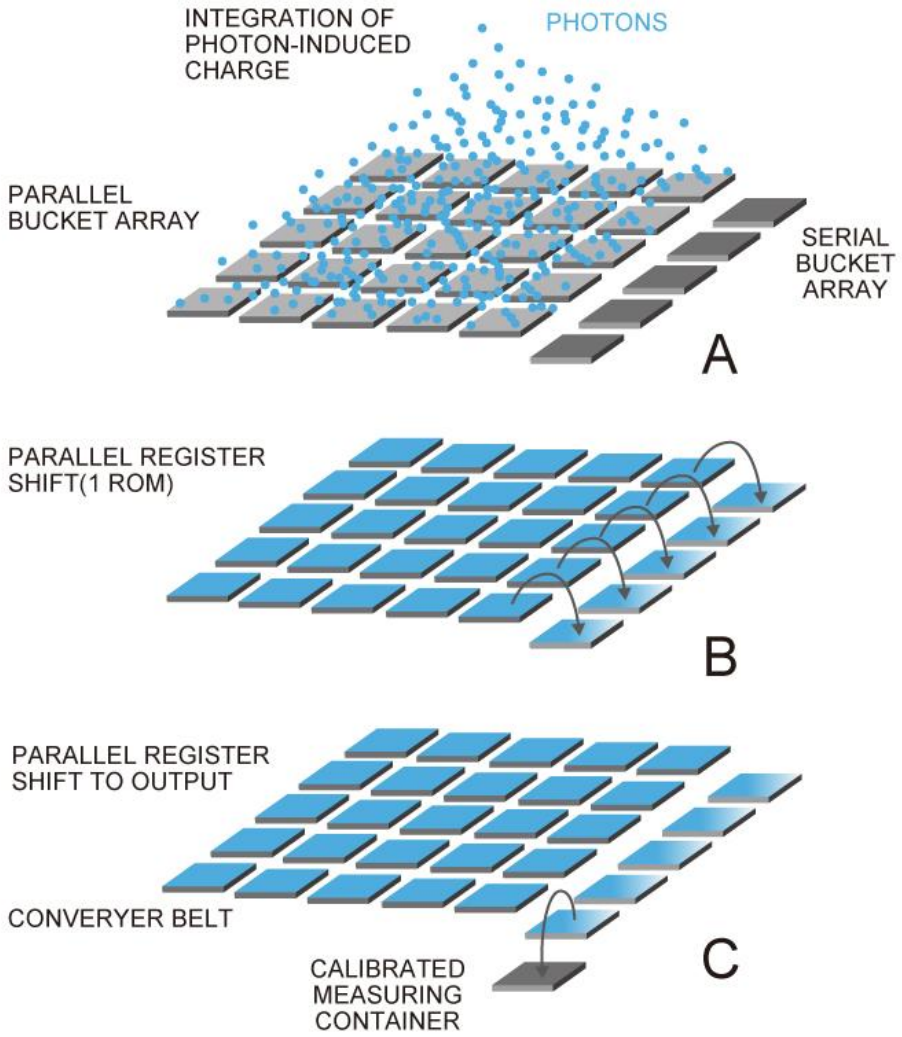


- Each pixel is composed of *photodiode and potential well*
- Light hitting the sensor is converted into electric charge

https://camera.hamamatsu.com/jp/en/technical_guides/visual_guide/index.html

Cameras

- *Charge-coupled device* **(CCD)** - photoelectric sensor array

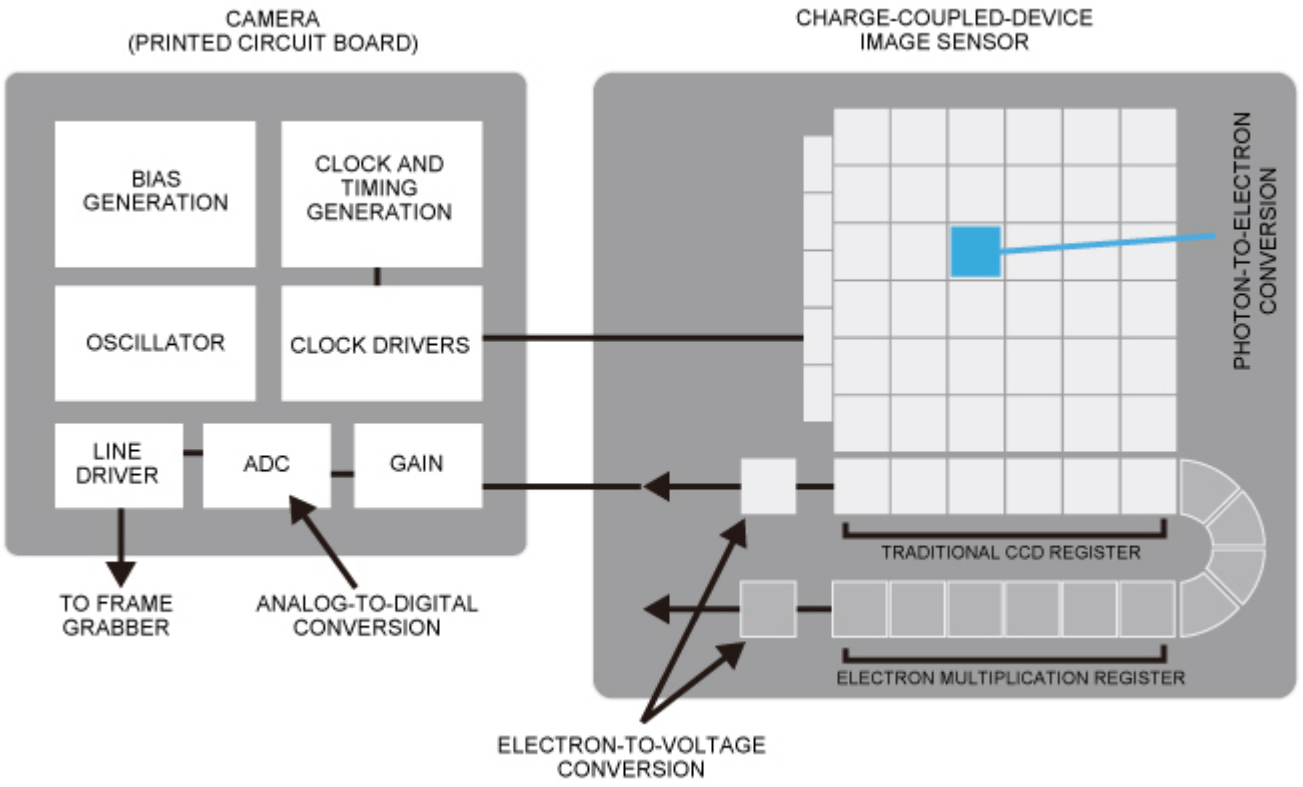


- Each pixel is composed of *photodiode and potential well*
- Light hitting the sensor is converted into electric charge
- A control circuit causes each capacitor to transfer its contents to its neighbor (operating as a **parallel shift register**)
- The charge is gathered pixel-by-pixel—**serially**—into a container at the end of the relay, conversion into voltage -> sequence of voltages
- The speed of image acquisition is limited

https://camera.hamamatsu.com/jp/en/technical_guides/visual_guide/index.html

Cameras

- *Charge-coupled device* **(CCD)** - photoelectric sensor array



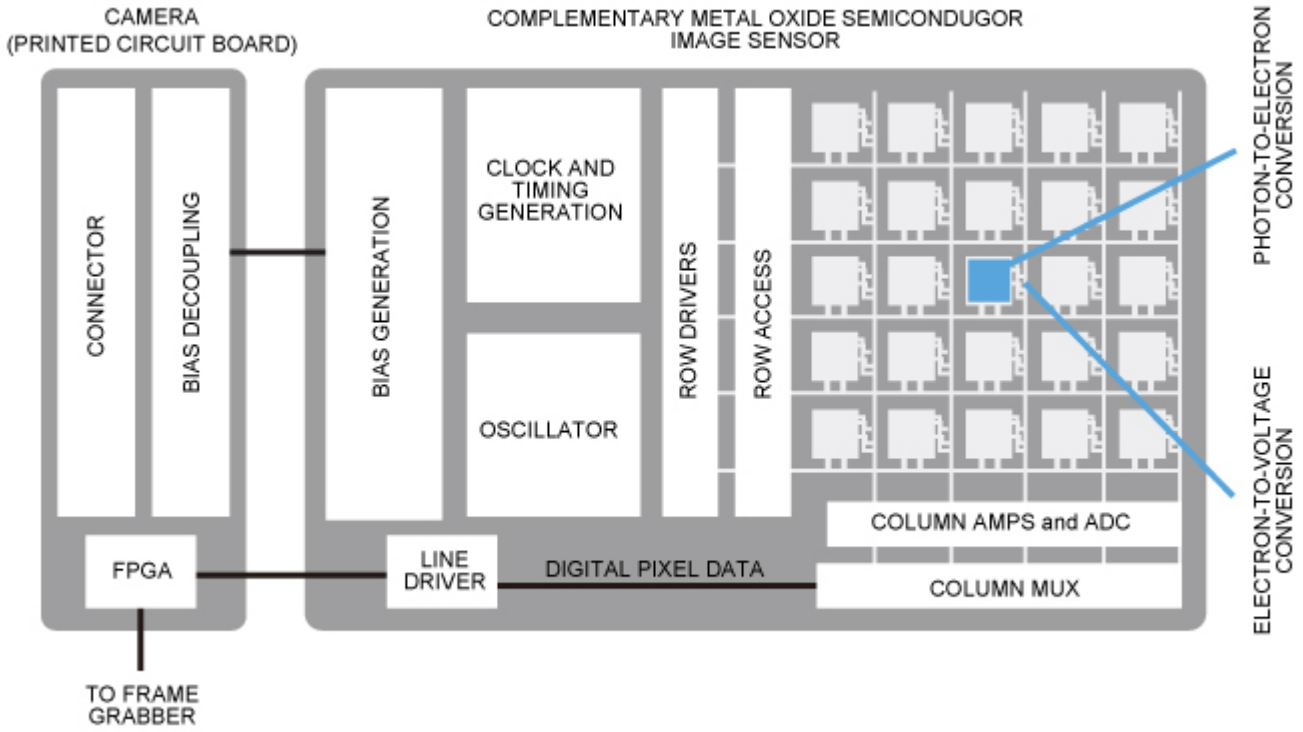
- **EM-CCD** (electron multiplying CCD) sensors have an additional component - the multiplication register, that multiplies the photoelectrons before the readout of the sensor
- Additional source of noise, lower quantum efficiency
- Back-illuminated > 95% QE

-> *High sensitivity, advantageous for low light conditions*

https://camera.hamamatsu.com/jp/en/technical_guides/visual_guide/index.html

Cameras

- *Complementary metal-oxide-semiconductor* **(CMOS)** – photoelectric sensor array

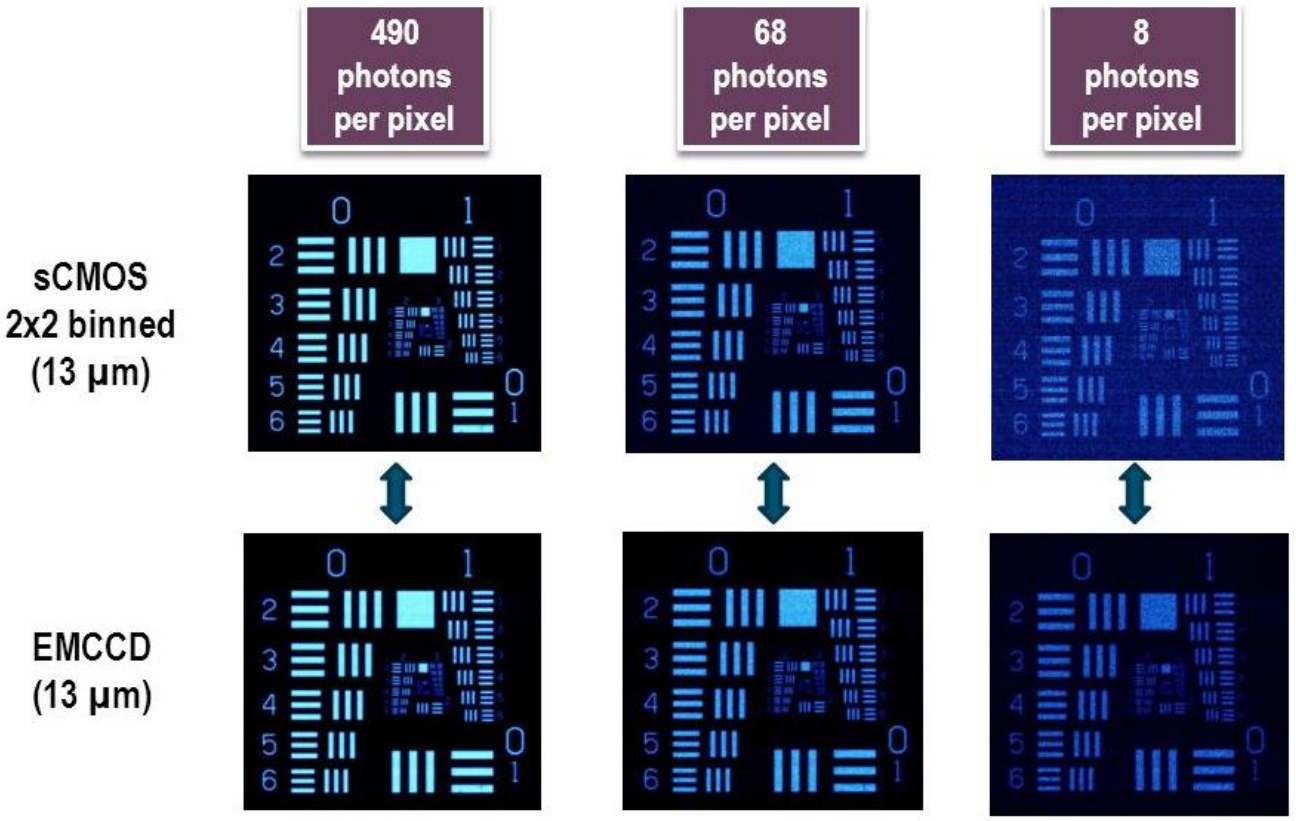


- Each pixel of a CMOS image sensor is composed of a *photodiode-amplifier pair*
- Light hitting the CMOS sensor is converted into photoelectrons, photoelectrons are converted into voltage at each pixel **in parallel**
- Typically captures a row at a time (rolling shutter), or entire sensor array simultaneously (global shutter)

https://camera.hamamatsu.com/jp/en/technical_guides/visual_guide/index.html

Cameras

- *Complementary metal-oxide-semiconductor* **(CMOS)** – photoelectric sensor array



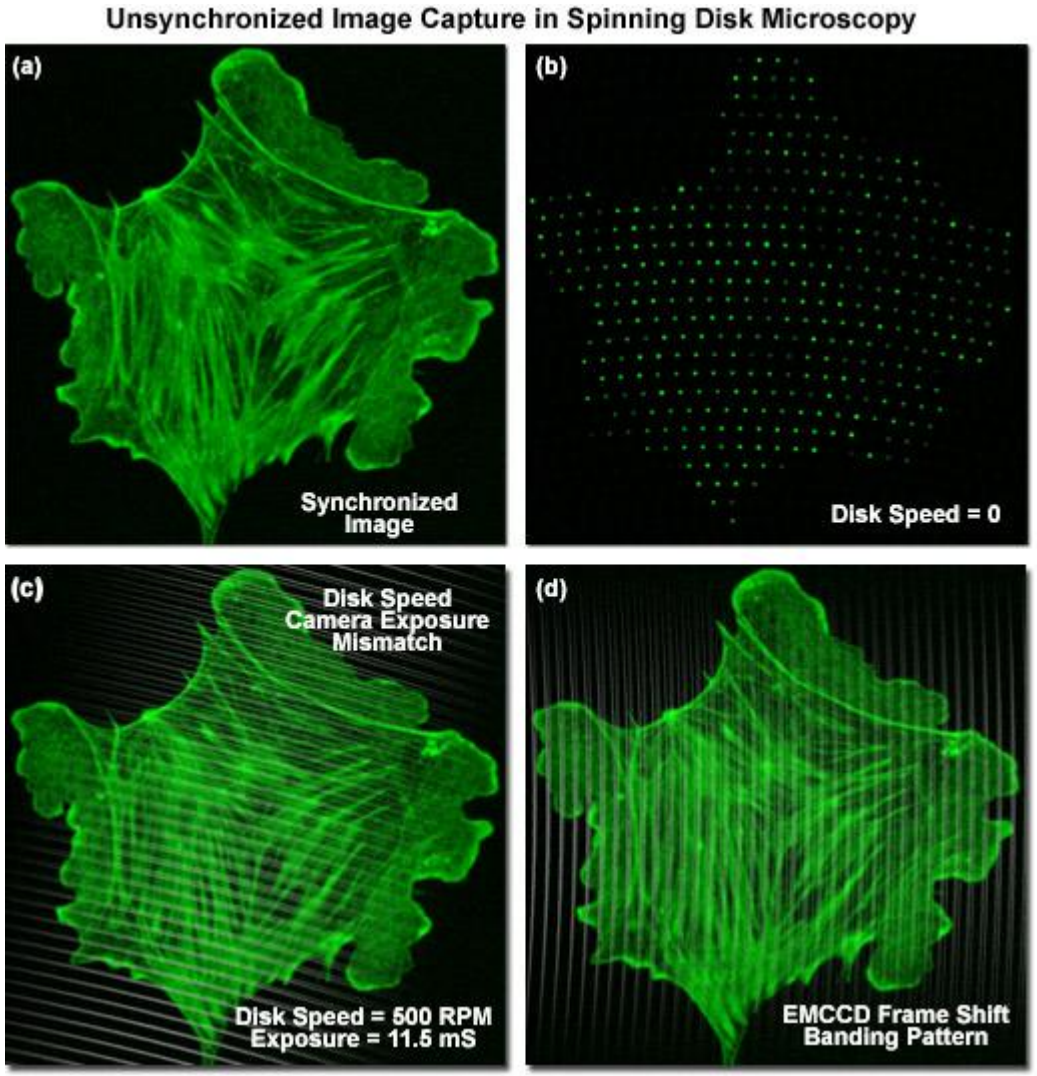
- **sCMOS** (scientific CMOS) next-generation sensors
- Low noise, high quantum efficiency
- Can capture data in a global-shutter mode over all the pixels or rectangular subsets of pixels, and can also operate in a rolling-shutter mode
- Back-illuminated: 95% QE

-> Better temporal resolution due to higher acquisition frame rates
-> Larger fields of view due to larger chip sizes
-> Better spatial resolution due to smaller pixel sizes

https://andor.oxinst.com/

Spinning disk microscope

- Lightsource – lasers (not necessarily)
- Detector – cameras (EMCCD, sCMOS)
  - the disk rotation speed must be adjusted to match the camera exposure time (problem for very short exposure times)
  - the frame shift of accumulated charge during EMCCD readout might conflict with the disk rotation speed
  - Usually two cameras – <u>dual color imaging</u>



Spinning disk microscope

Dragonfly 500

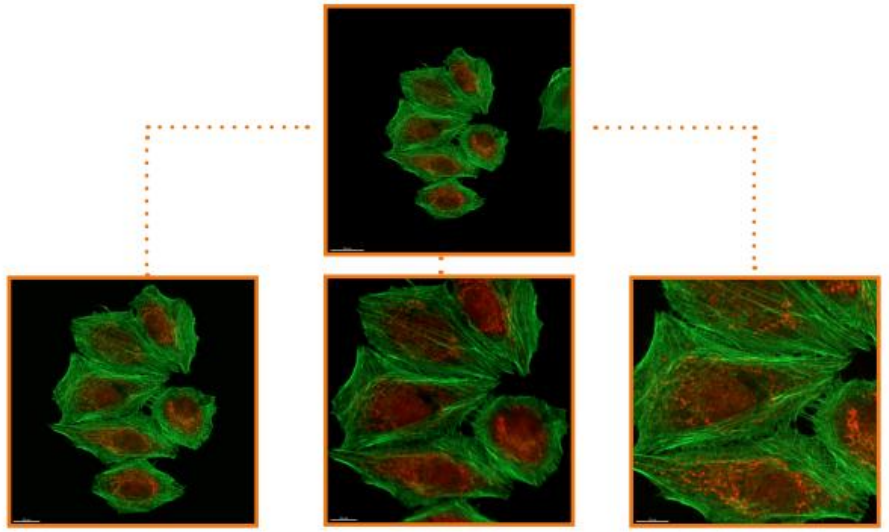
http://www.andor.com/microscopy-systems/dragonfly

Spinning disk microscope

Dragonfly 500

Laser Range	Confocal Speed	Aperture	Camera Zoom	Illumination Zoom	Pinholes Ø
400 - 800 nm	400 fps	22 mm	1x | 1.5x | 2x	1x | 2x | 4x | 6x	40 µm | 25 µm

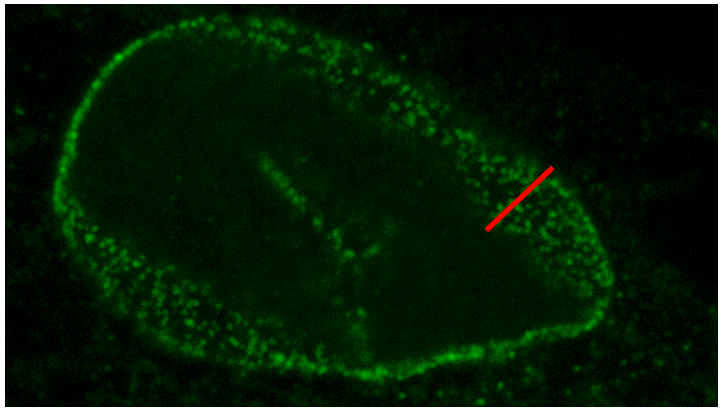
- Microlens enhanced dual disk
- 2 cameras
  - Zyla 4.2 sCMOS (up to 400 confocal fps 2048x128, pixel size 6.5 µm)
  - iXon 888 EMCCD (single photon sensitive, pixel size 13 µm)
- Borealis – patented illumination (multimode fibre and correction optics)
  - High degree of uniformity
- Camera zoom
  - Enables to reach Nyquist sampling with high NA objectives

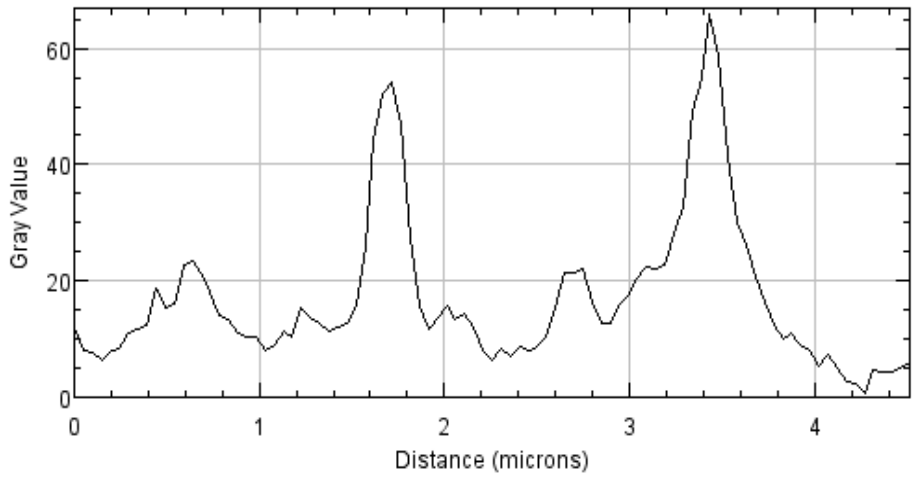


http://www.andor.com/microscopy-systems/dragonfly

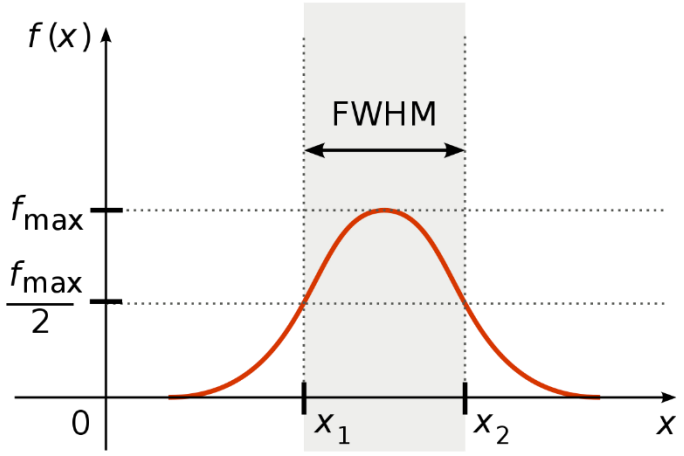
Spinning disk microscope

Dragonfly 500





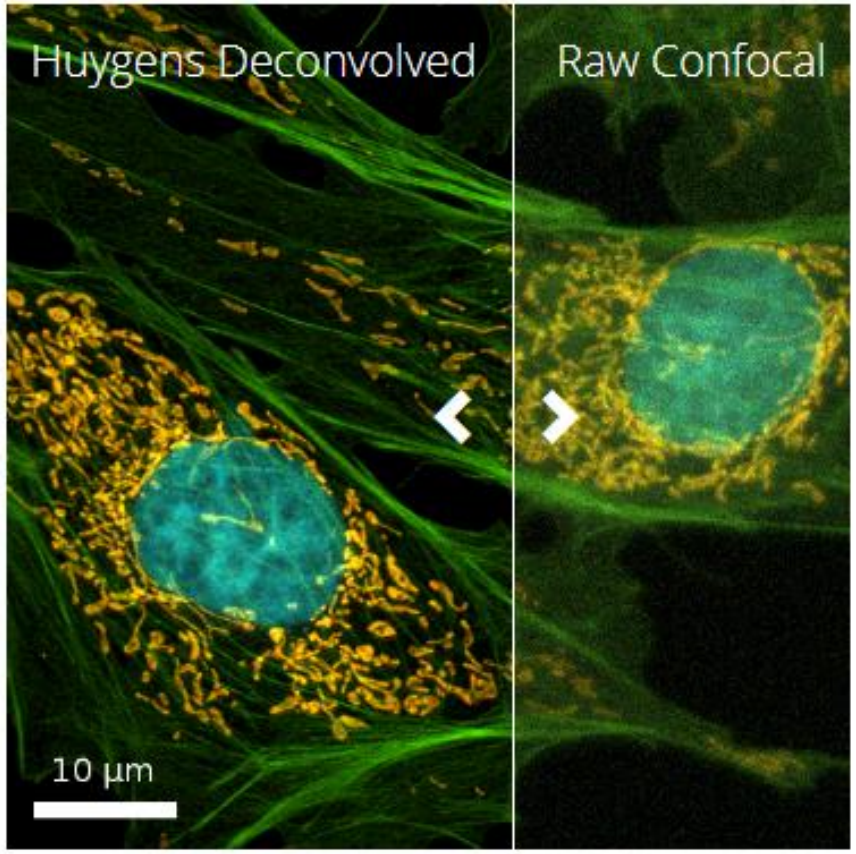
- Confocal resolution
  - *Lateral **238 nm** (typical FWHM)*
  - *Axial **523 nm** (typical FWHM)*



http://www.andor.com/microscopy-systems/dragonfly

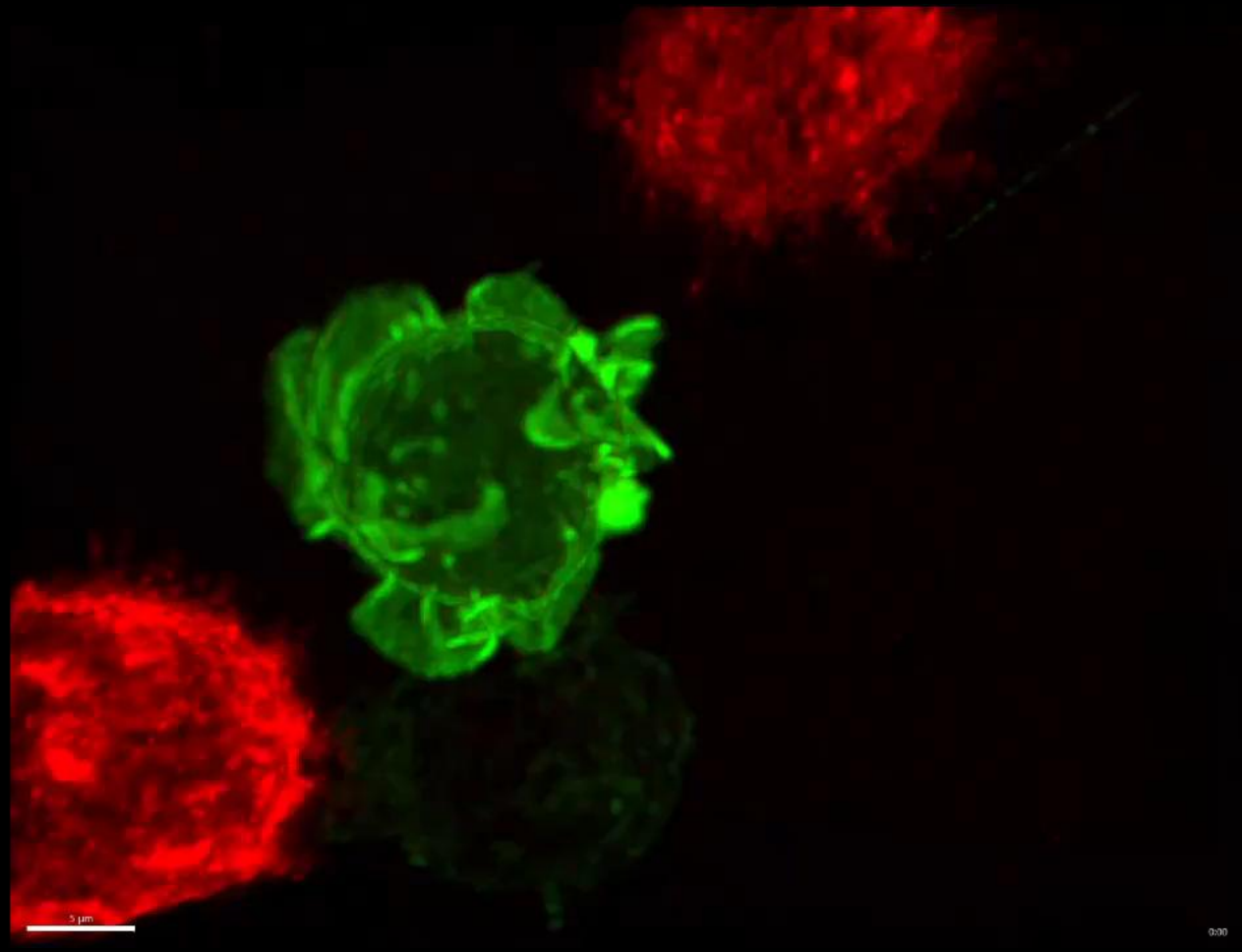
Deconvolution

- Image restoration – recovery of an image that is degraded by convolution (optical system)
  – corrects systematic error of blur (loss of contrast in smaller features)



https://svi.nl/Deconvolution

Spinning disk microscope

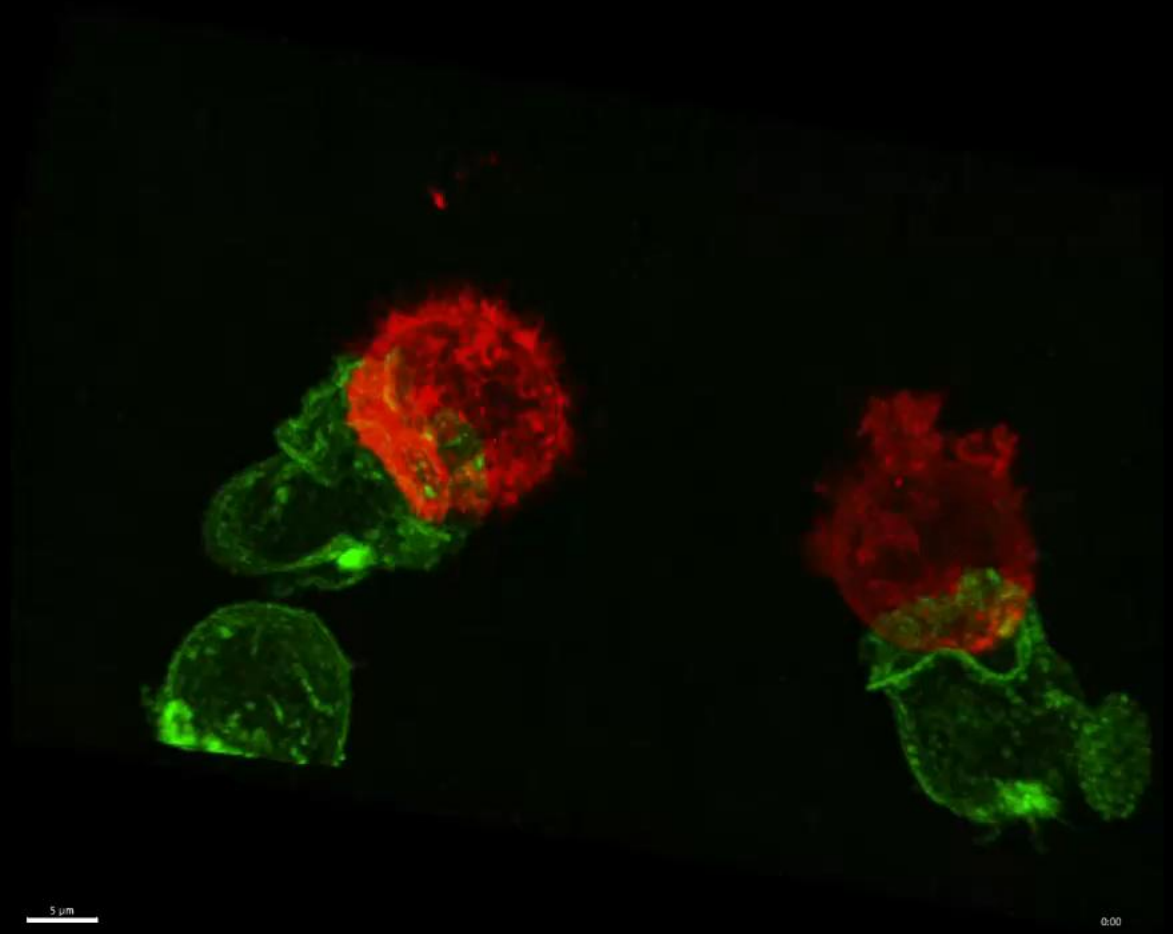


G: Jurkat T-cells

R: Raji B-cells

Courtesy of Ondřej Ballek, LMCF, IMG CAS

Spinning disk microscope



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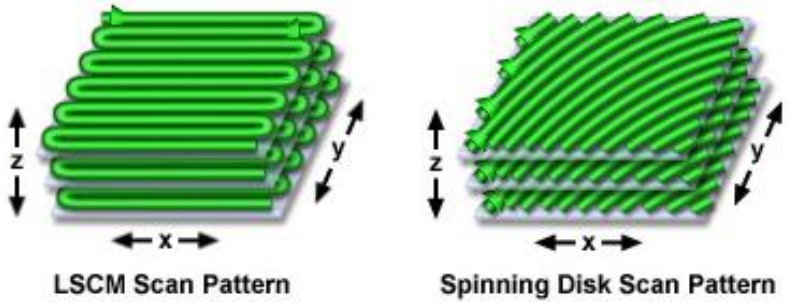
Courtesy of Ondřej Ballek, LMCF, IMG CAS

Conclusion

Advantages/disadvantages

(+)

- Speed (fast scanning, cameras, ~1000 pinholes at once)
- Photobleaching (lower illumination intensity)
- Pixel dwell time (higher, better image quality)
- Illumination (no strict requirement for lasers)



LSCM Scan Pattern

Spinning Disk Scan Pattern

Conclusion

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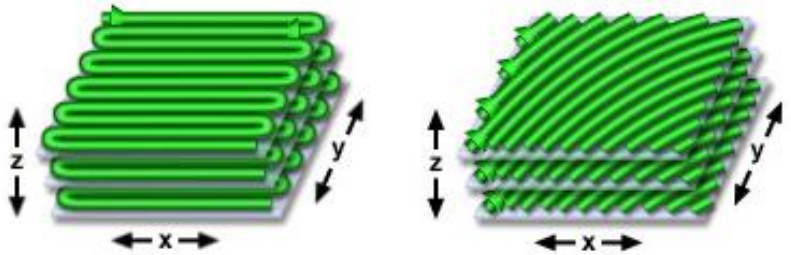

LSCM Scan Pattern Spinning Disk Scan Pattern

(–)

- Pinhole size (not adjustable – can use magnifying tube lens, usually lower axial resolution)
- Pinhole crosstalk (out-of-focus light through adjacent pinholes, reduces axial resolution)
- Level of light transition (lower, light throughput 40 - 60%)
- Field uniformity (usually lower, requires correction)

Thank you for your attention!