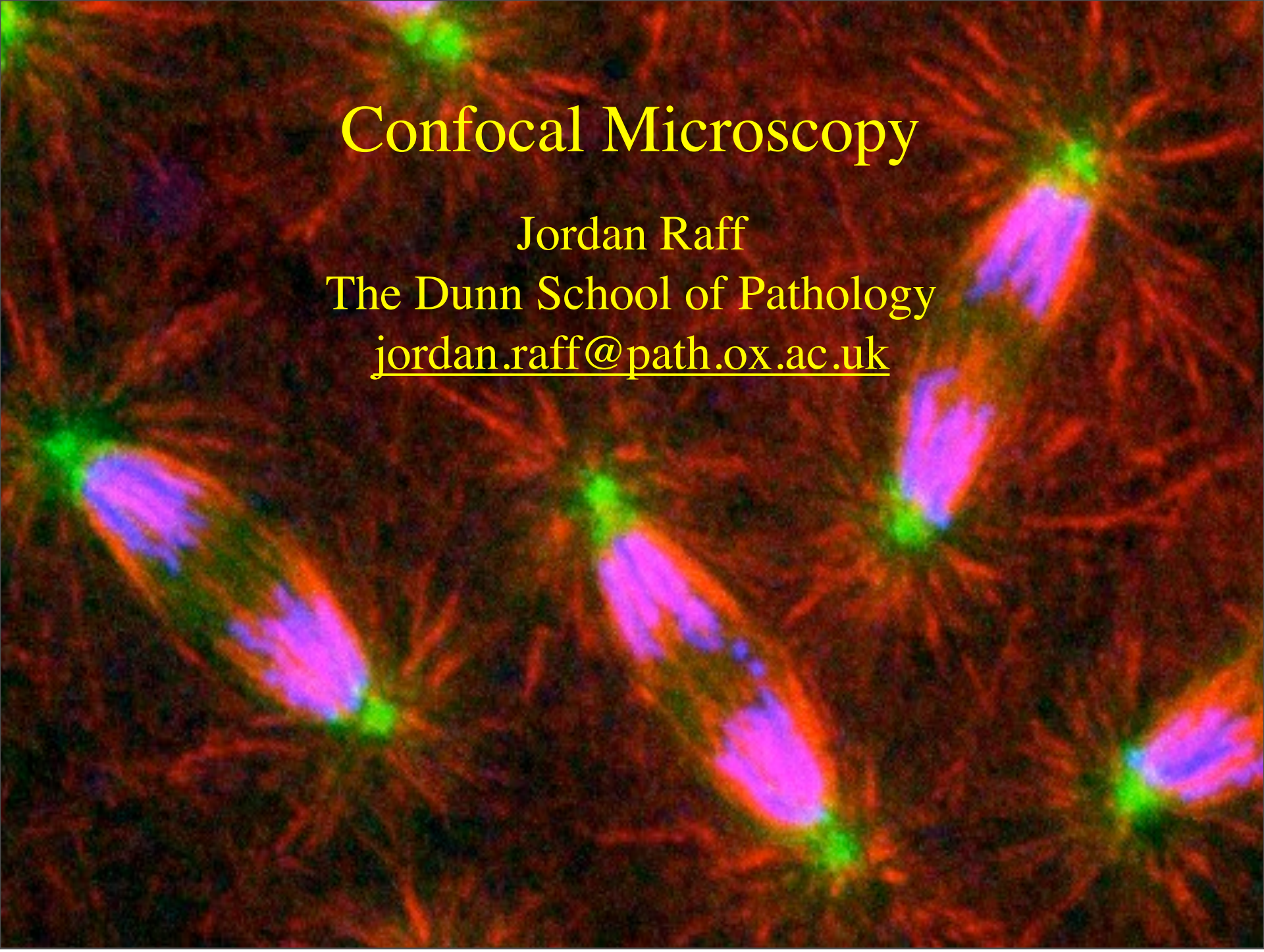
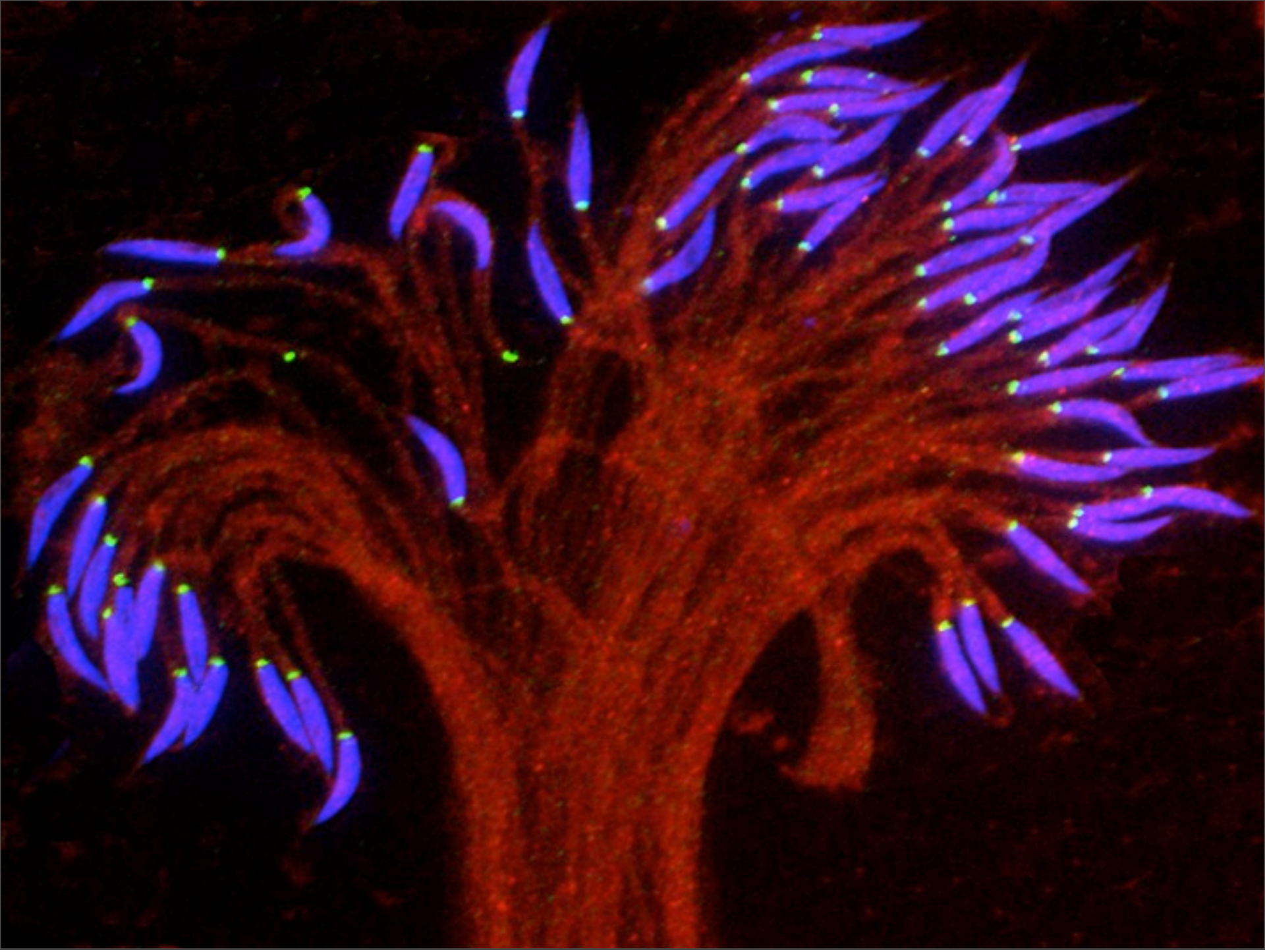


Confocal Microscopy

Jordan Raff
The Dunn School of Pathology
jordan.raff@path.ox.ac.uk



Outline of Talk

1. Confocal microscopy - some history

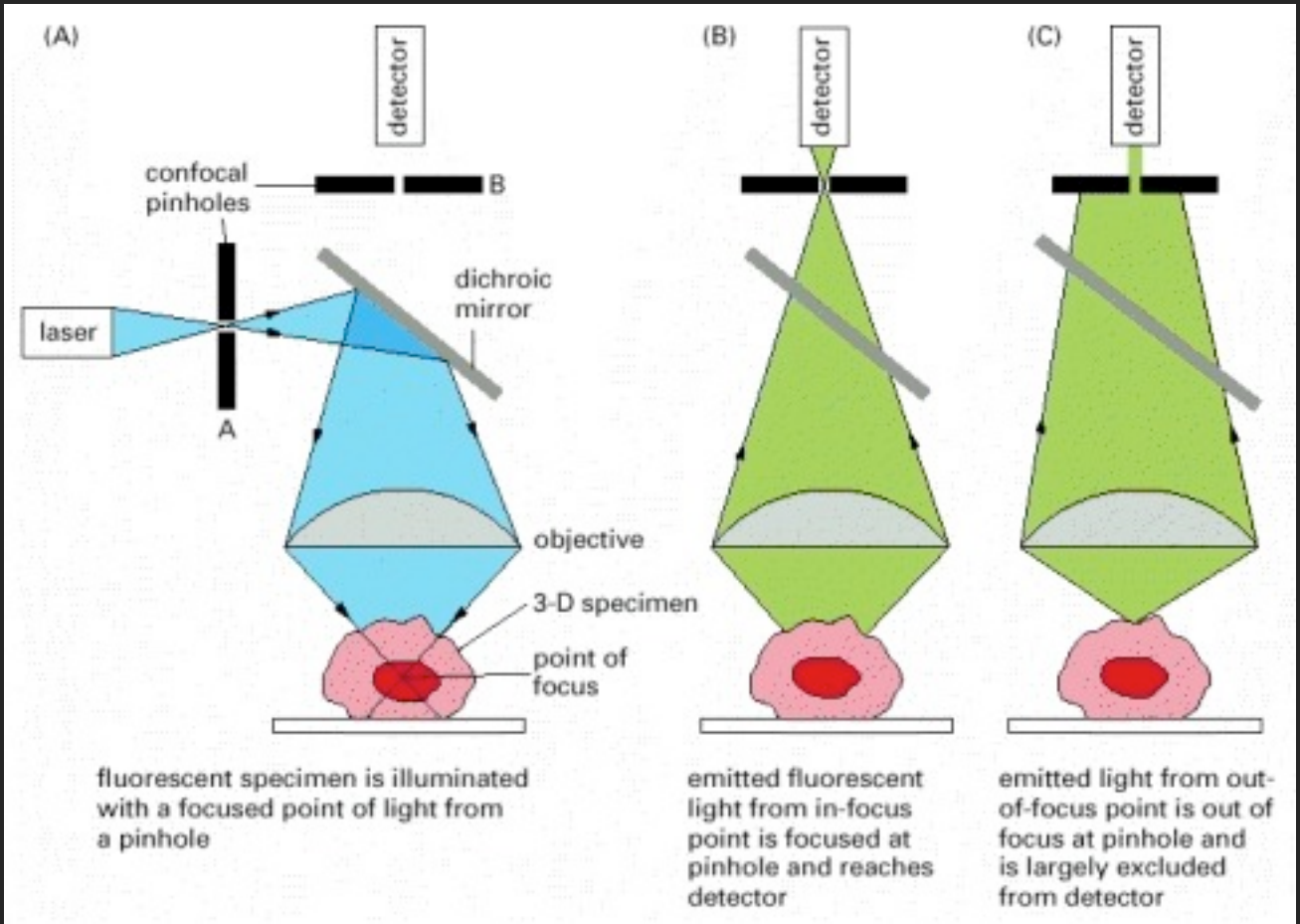
2. Laser Scanning Confocal Microscopy (LSCM)

3. Spinning Disk Confocal Microscopy

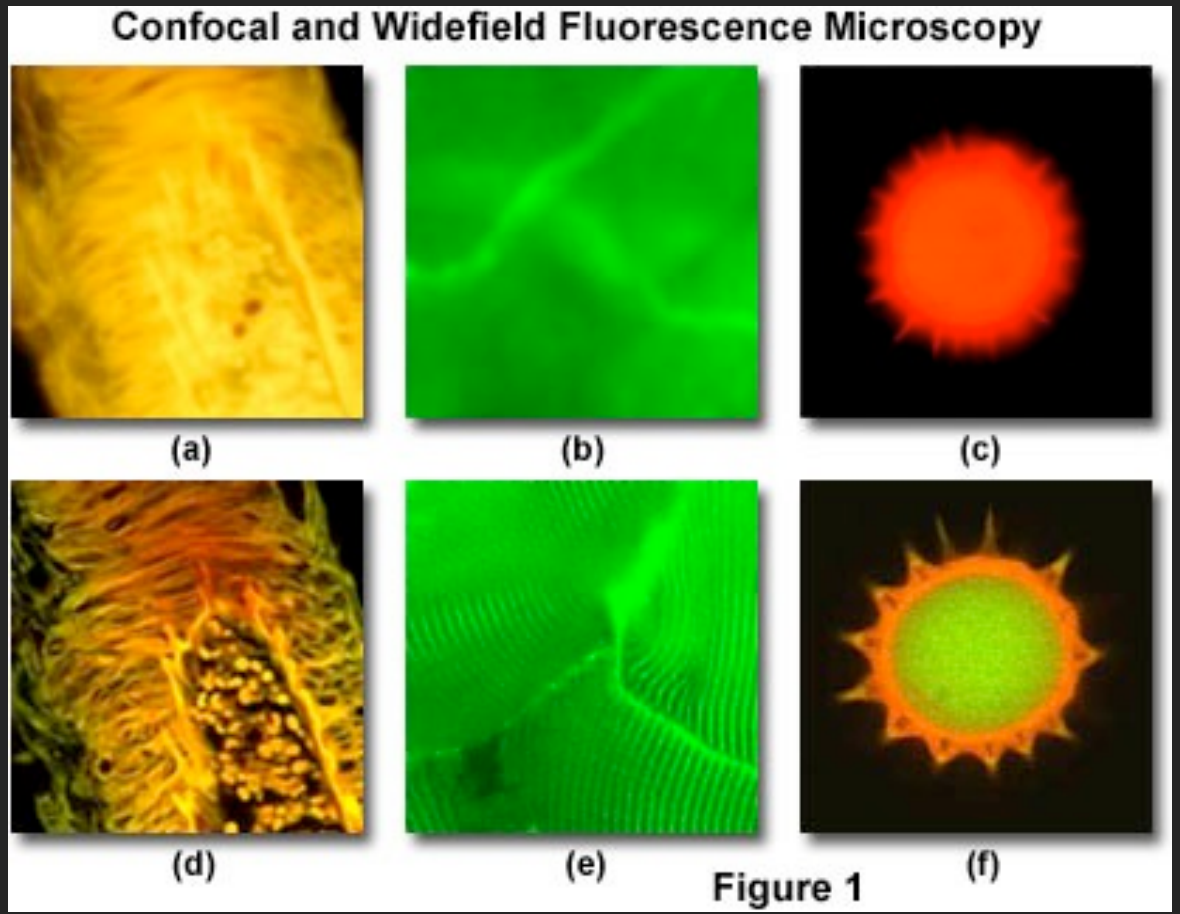
4. Widefield vs. LSCM vs. Spinning Disc

   Murray et al., Evaluating performance in 3D fluorescence microscopy. J. Microscopy (2007)

The principle of the confocal microscope



Comparison of Confocal and Widefield Microscopy



Short History of the Confocal Microscope

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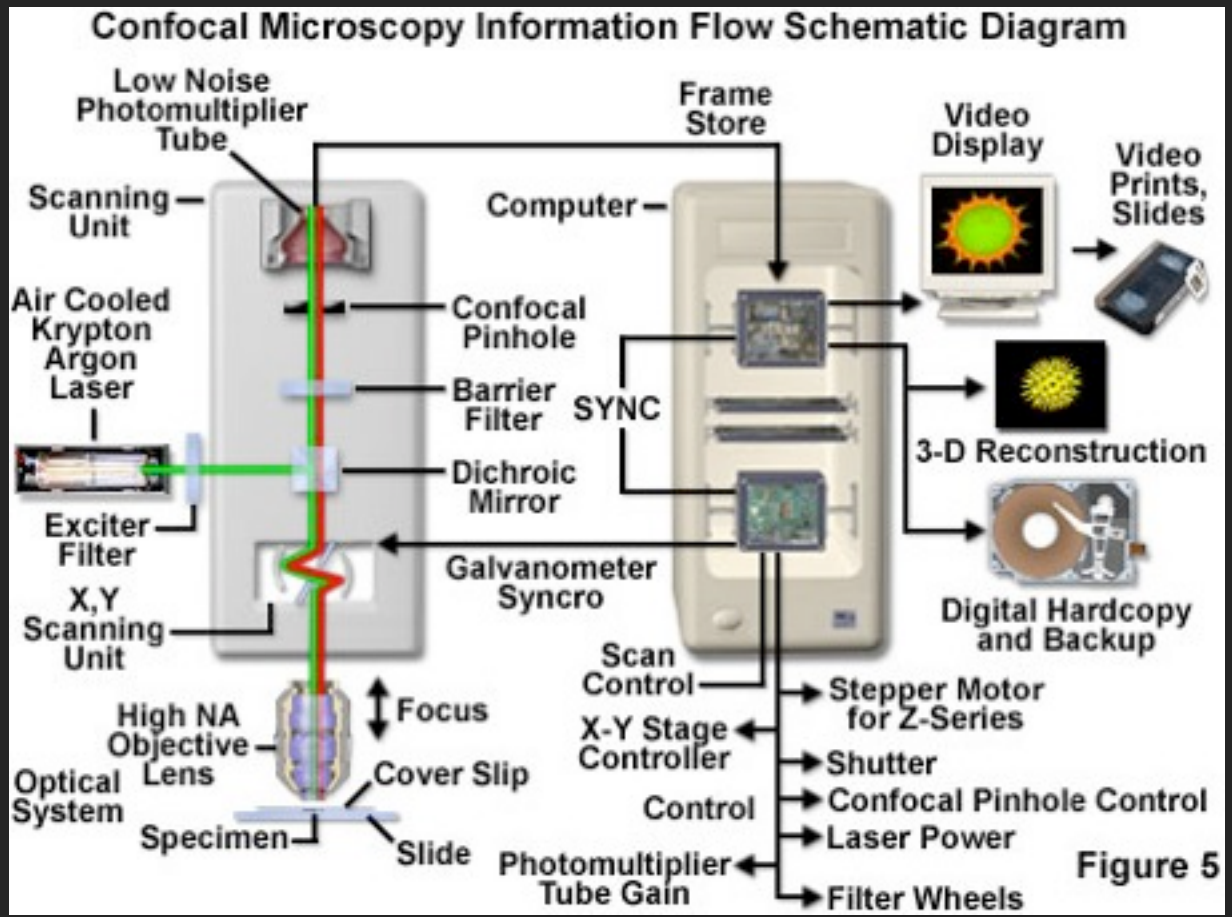
White, Amos and Wilson developed MRC 500 point scanning confocal - marketed commercially in 1987.

Amos and White, Biol. Cell 2003

Simplified View of a "Point-Scanning" Confocal



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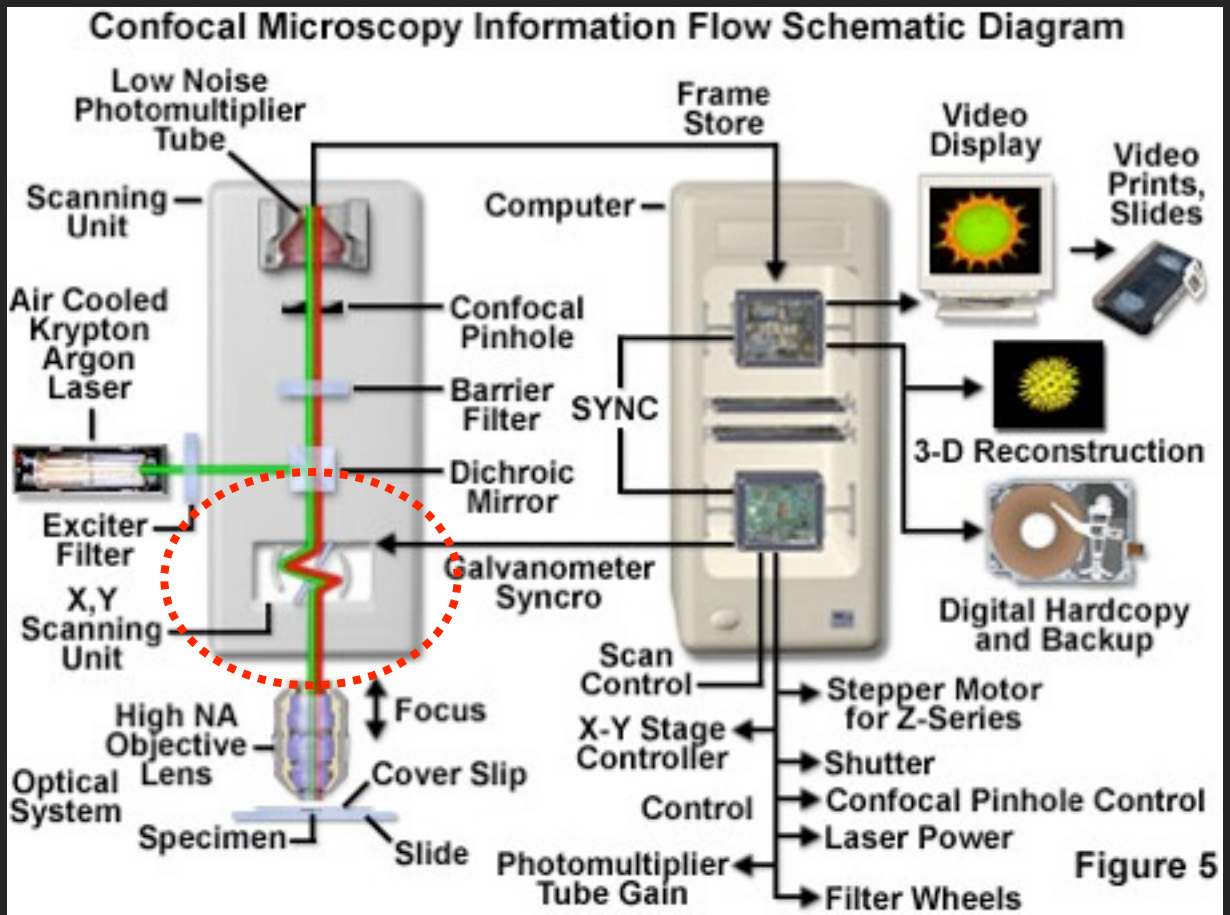
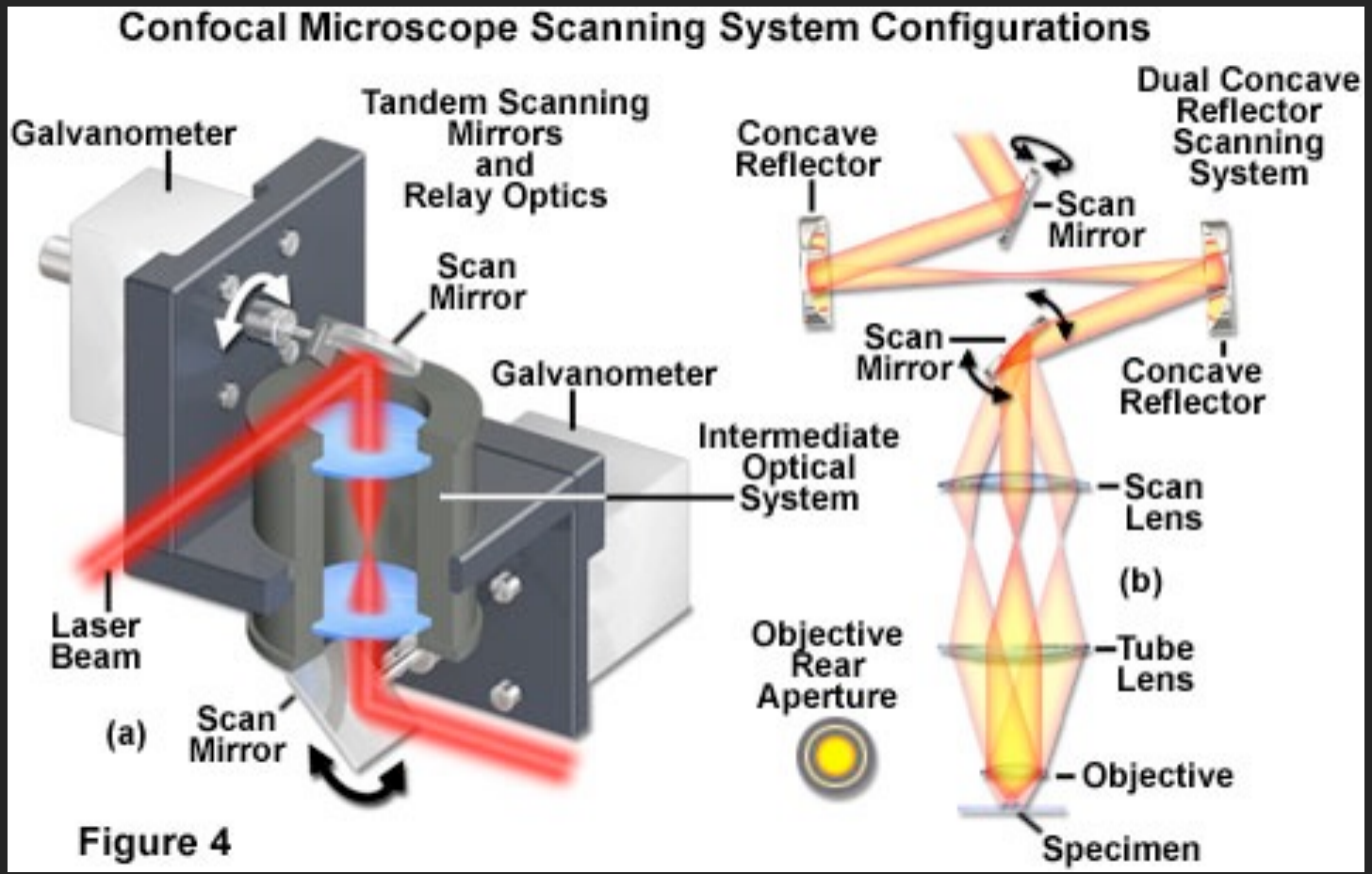
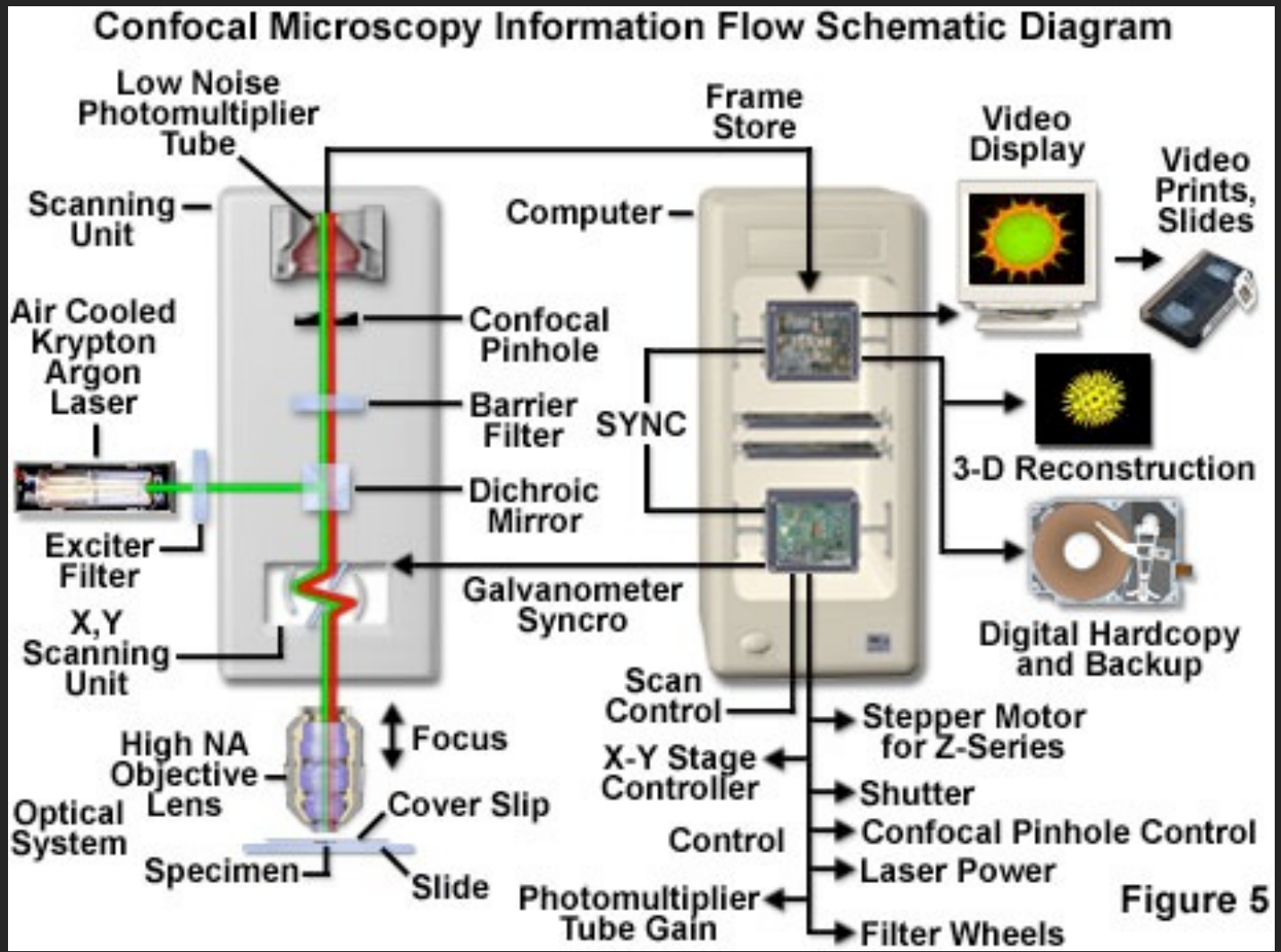


The Galvanometer/Mirror Scanning System



http://www.olympusmicro.com/primer/java/galvanometerscanning/index.html

Simplified View of a "Point-Scanning" Confocal



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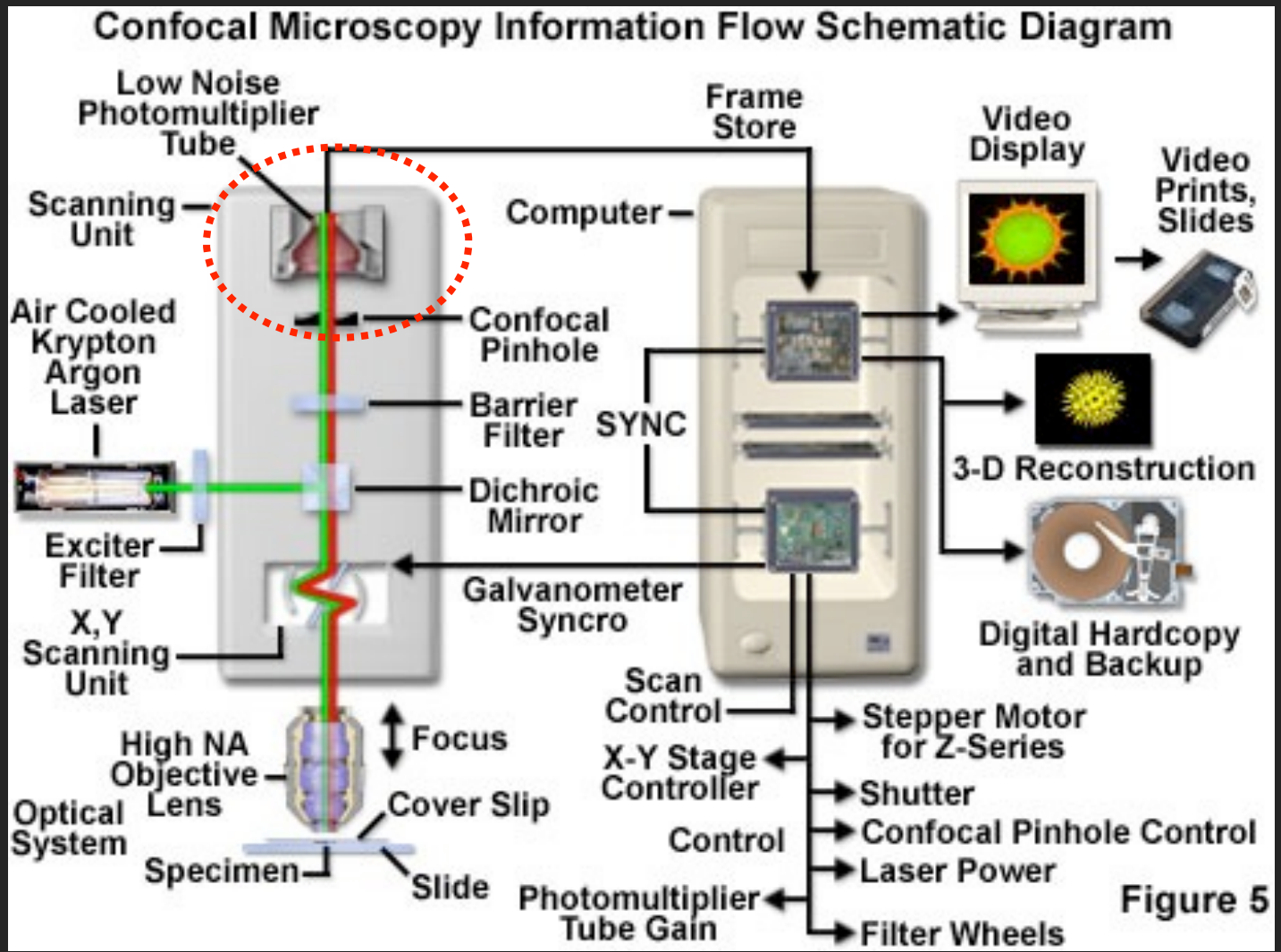
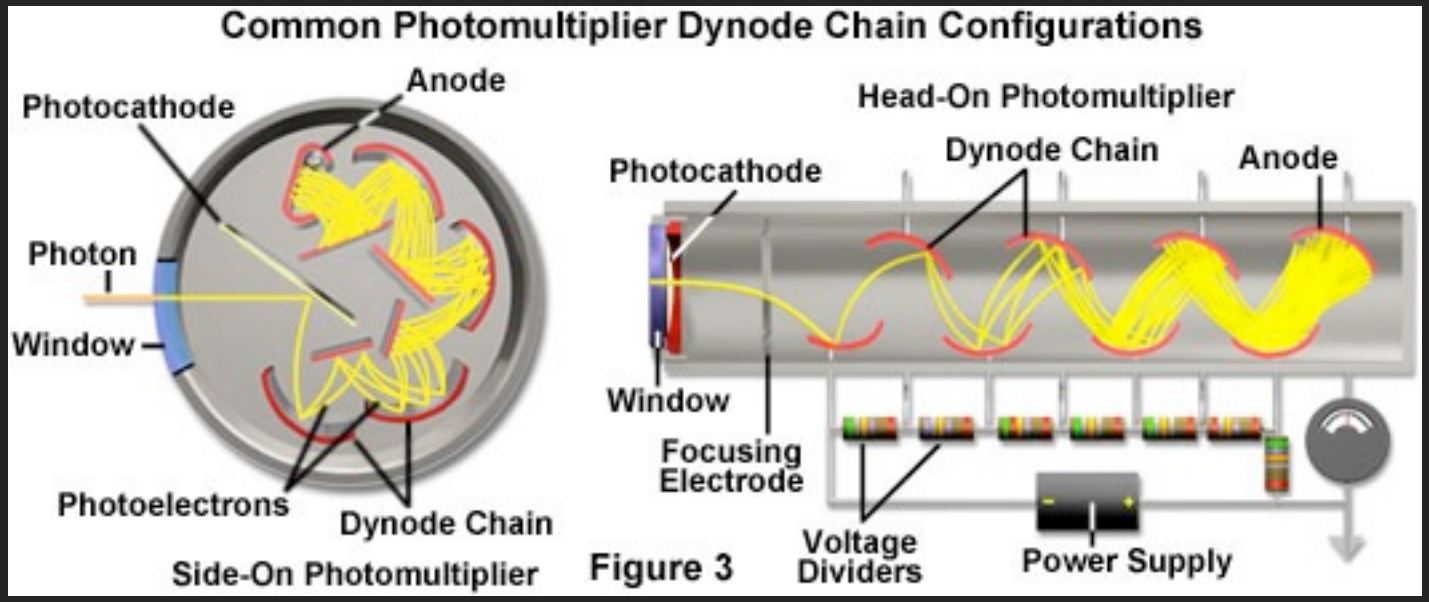


The Photomultiplier (PMT)



http://www.olympusmicro.com/primer/java/channelpmt/index.html

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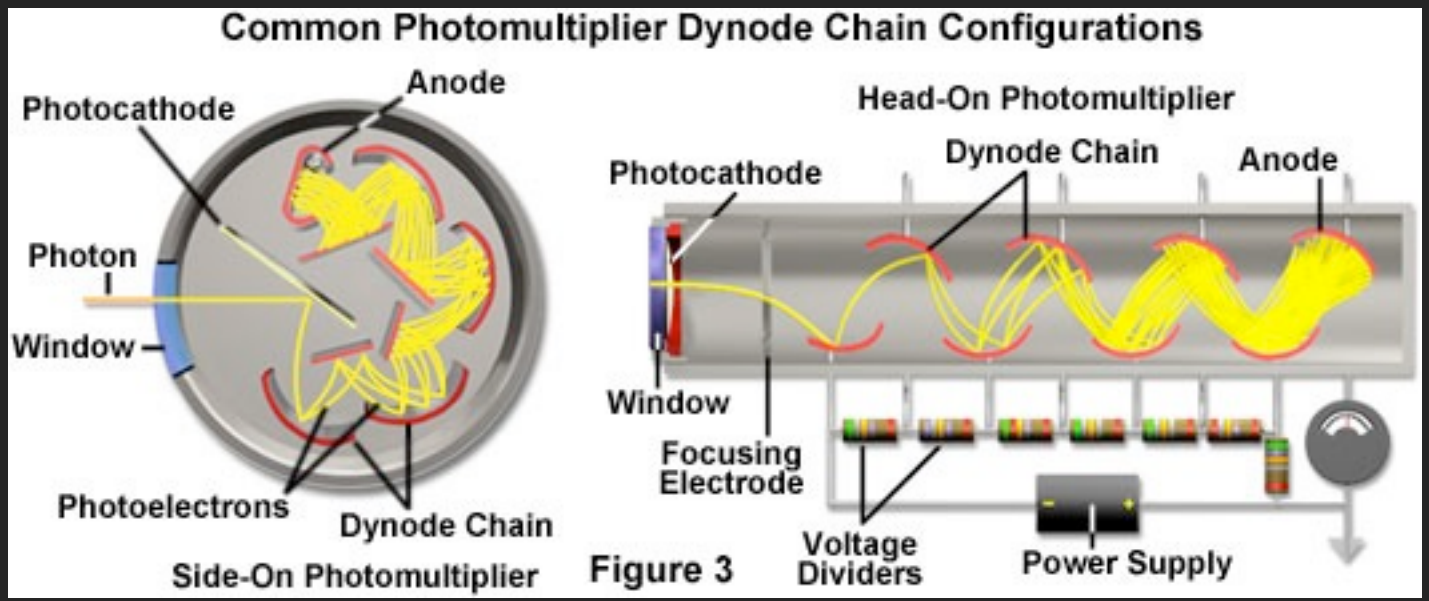
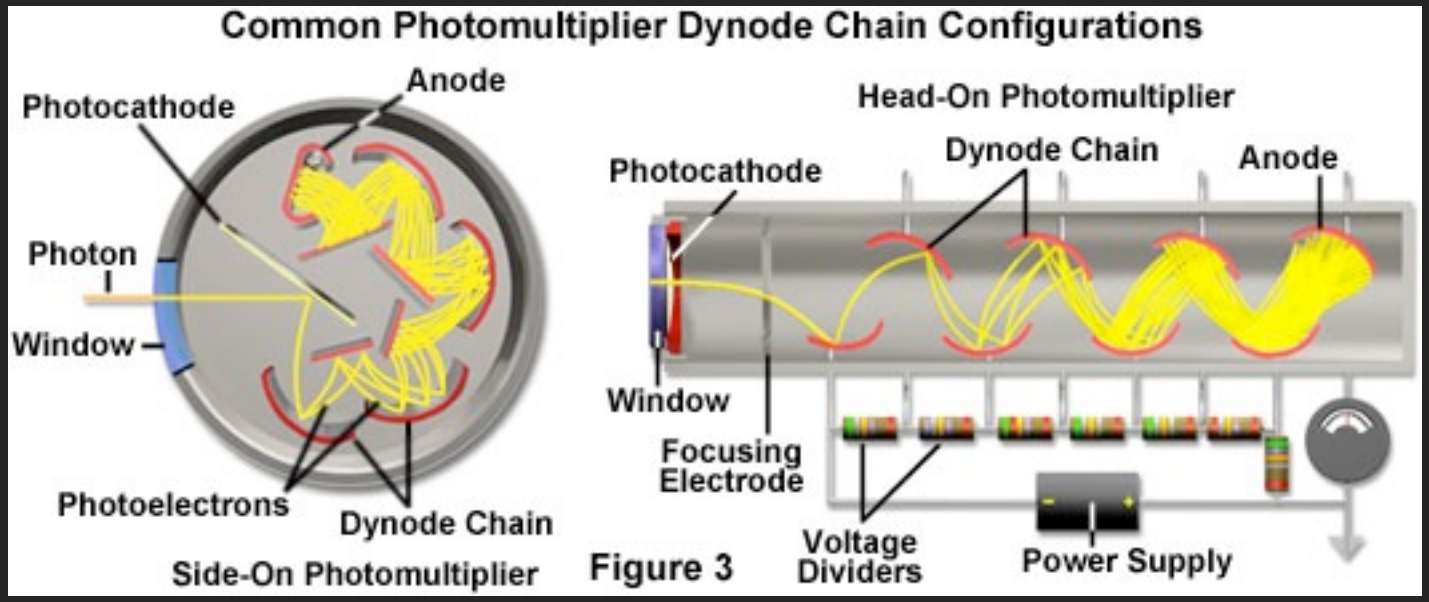


Figure 3

Very Low Noise (even without too much cooling)
Very Rapid Response
Huge Potential for Signal Amplification (~1X10^{7})

http://www.olympusmicro.com/primer/java/channelpmt/index.html

The Photomultiplier (PMT)



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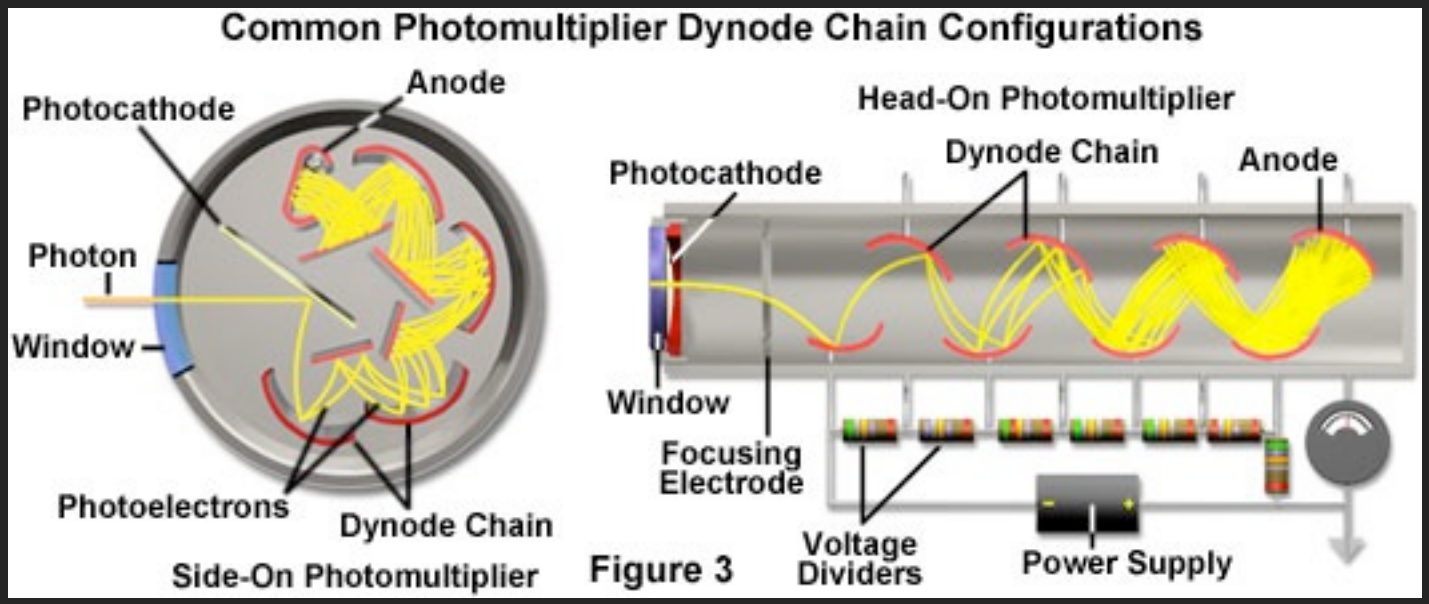
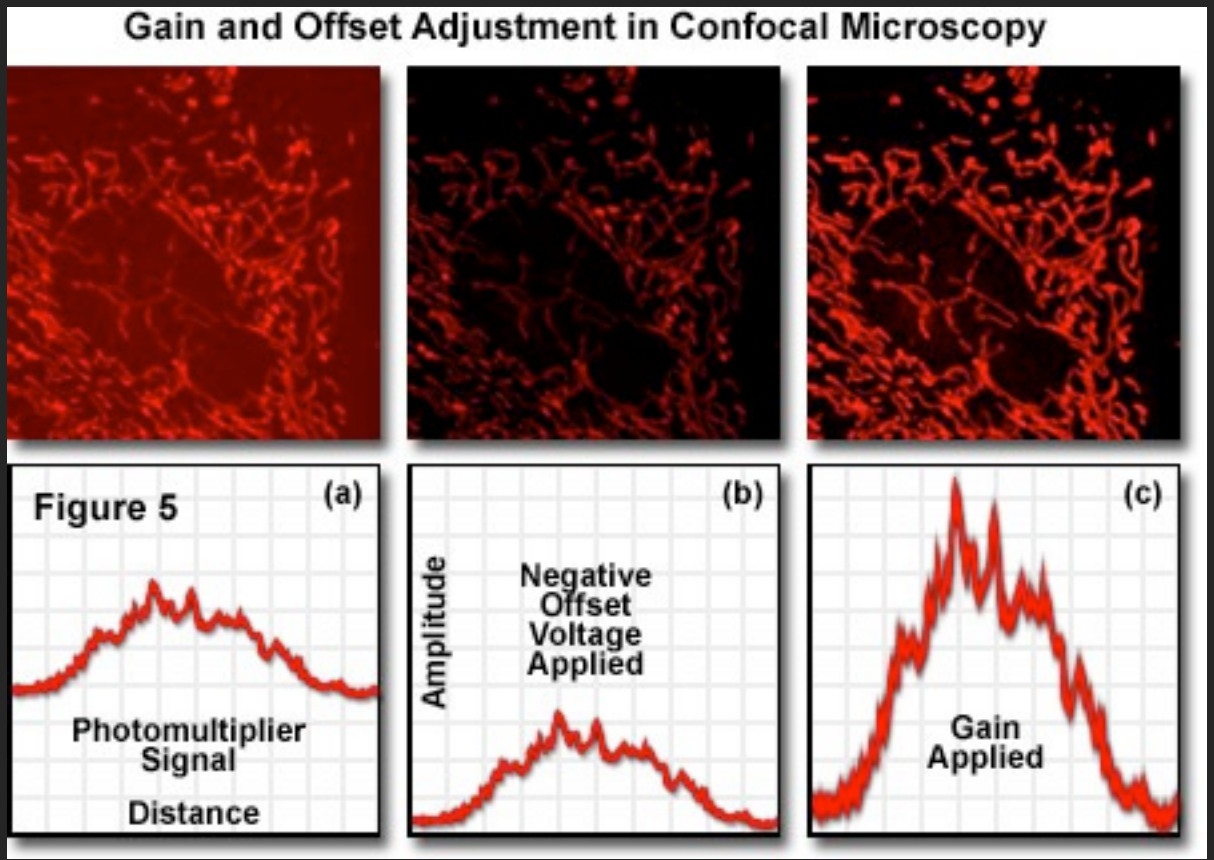


Low Quantum Efficiency (QE) (~15-40%)
GaAsP!

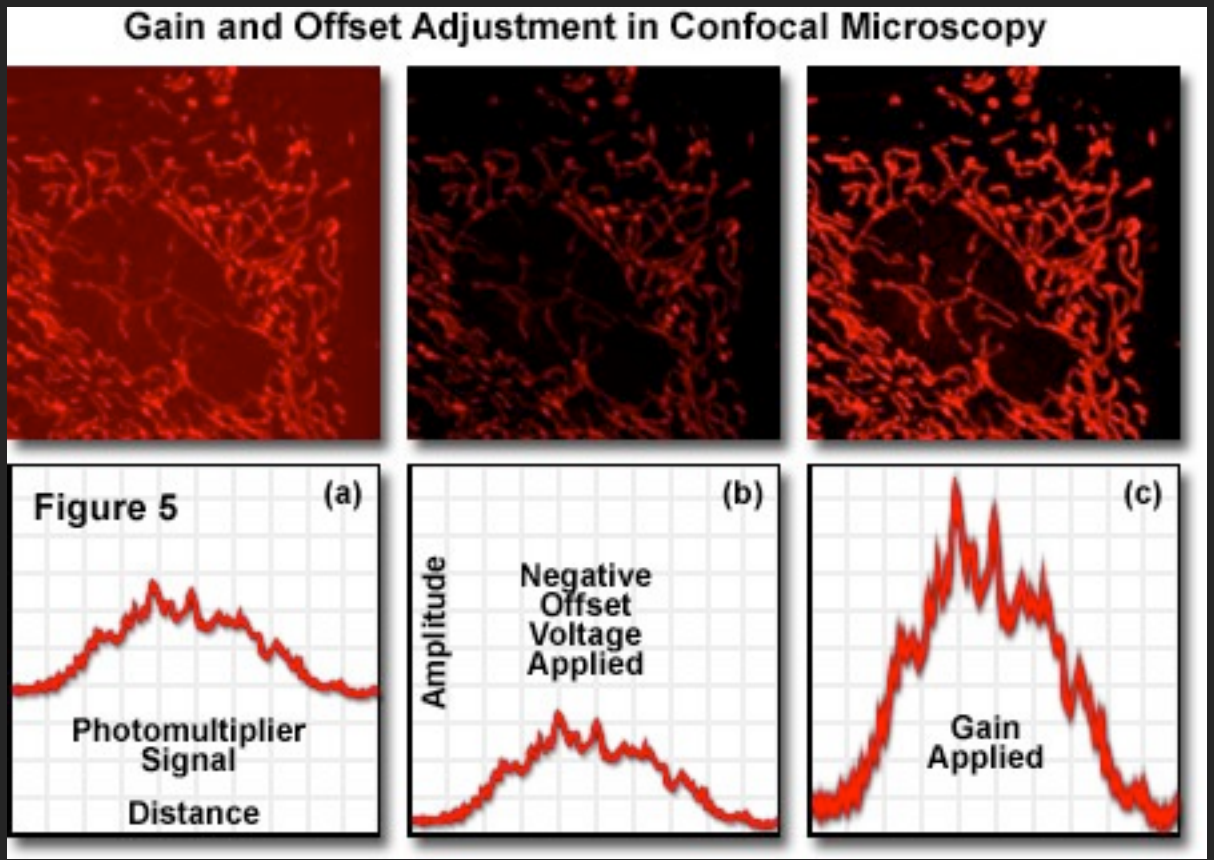
http://www.olympusmicro.com/primer/java/channelpmt/index.html

Adjusting Offset and Gain of the PMT



http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

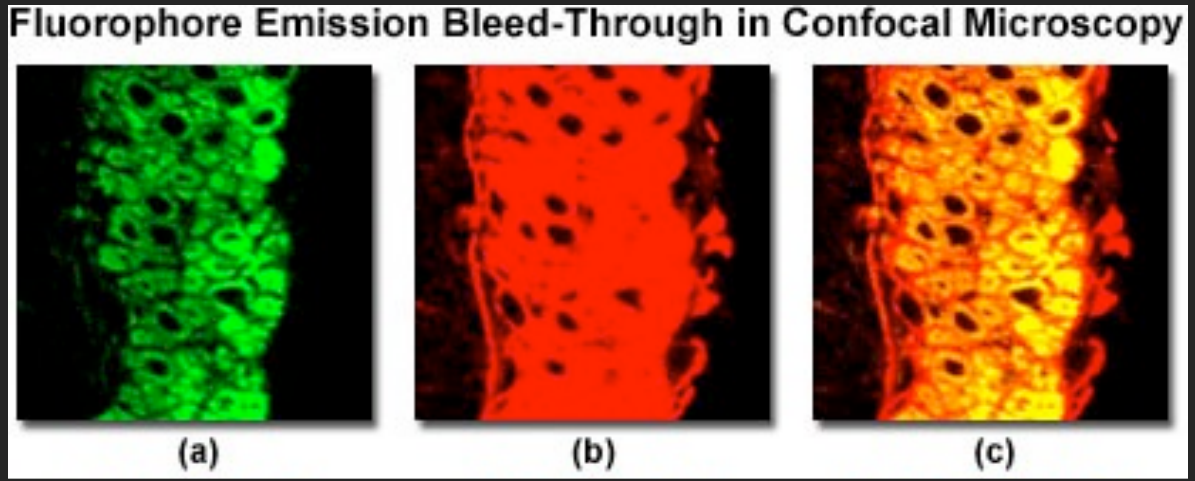
Adjusting Offset and Gain of the PMT



Beware - this is how your image will be saved!

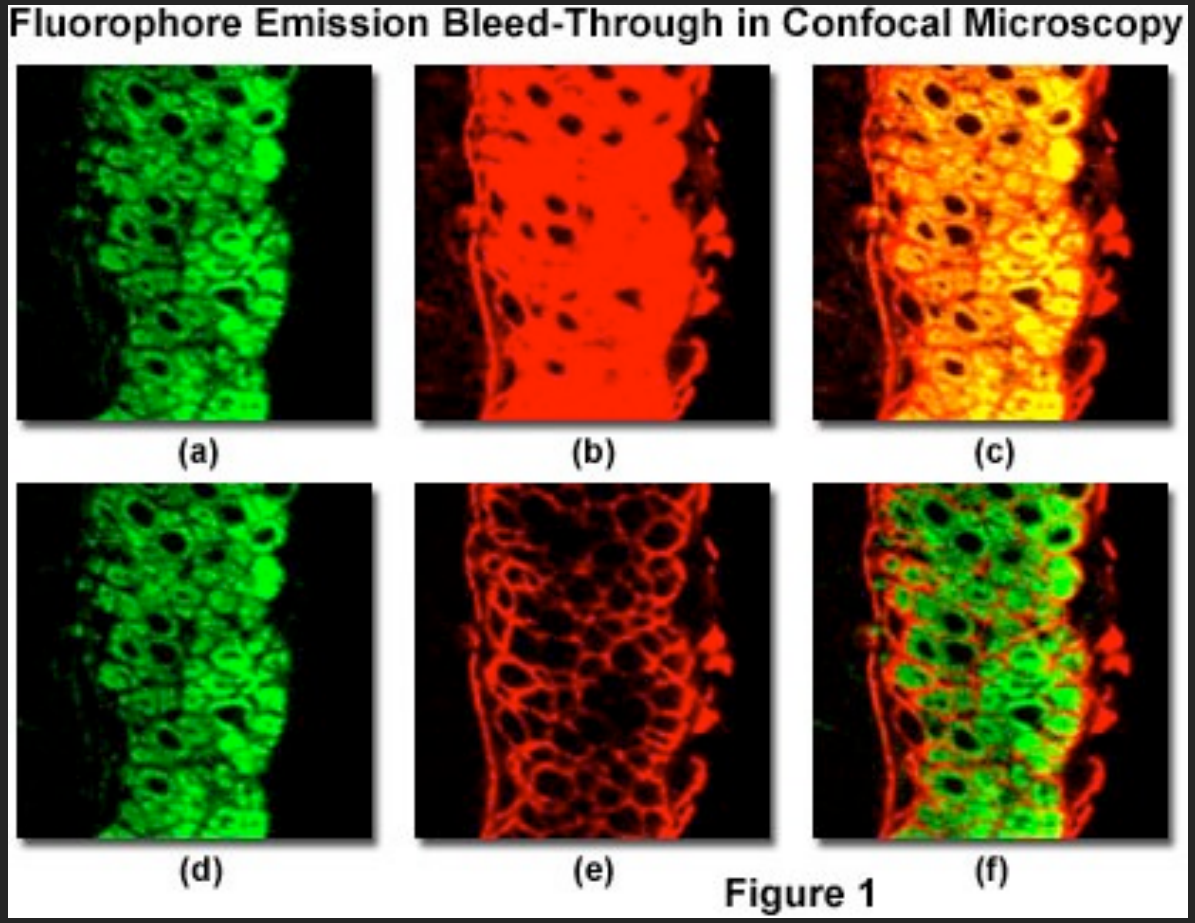
http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

The Problem of "Bleedthrough"



http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

The Problem of "Bleedthrough"



http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

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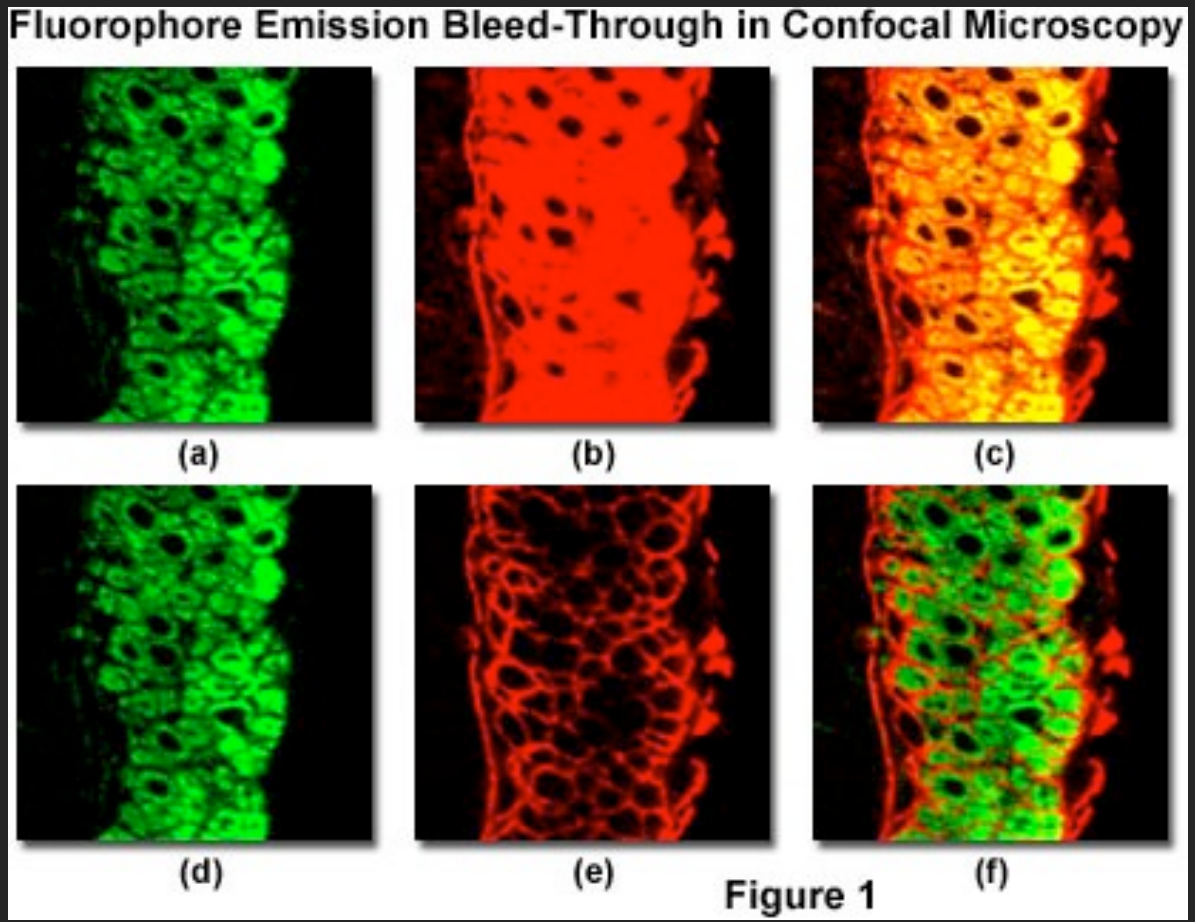
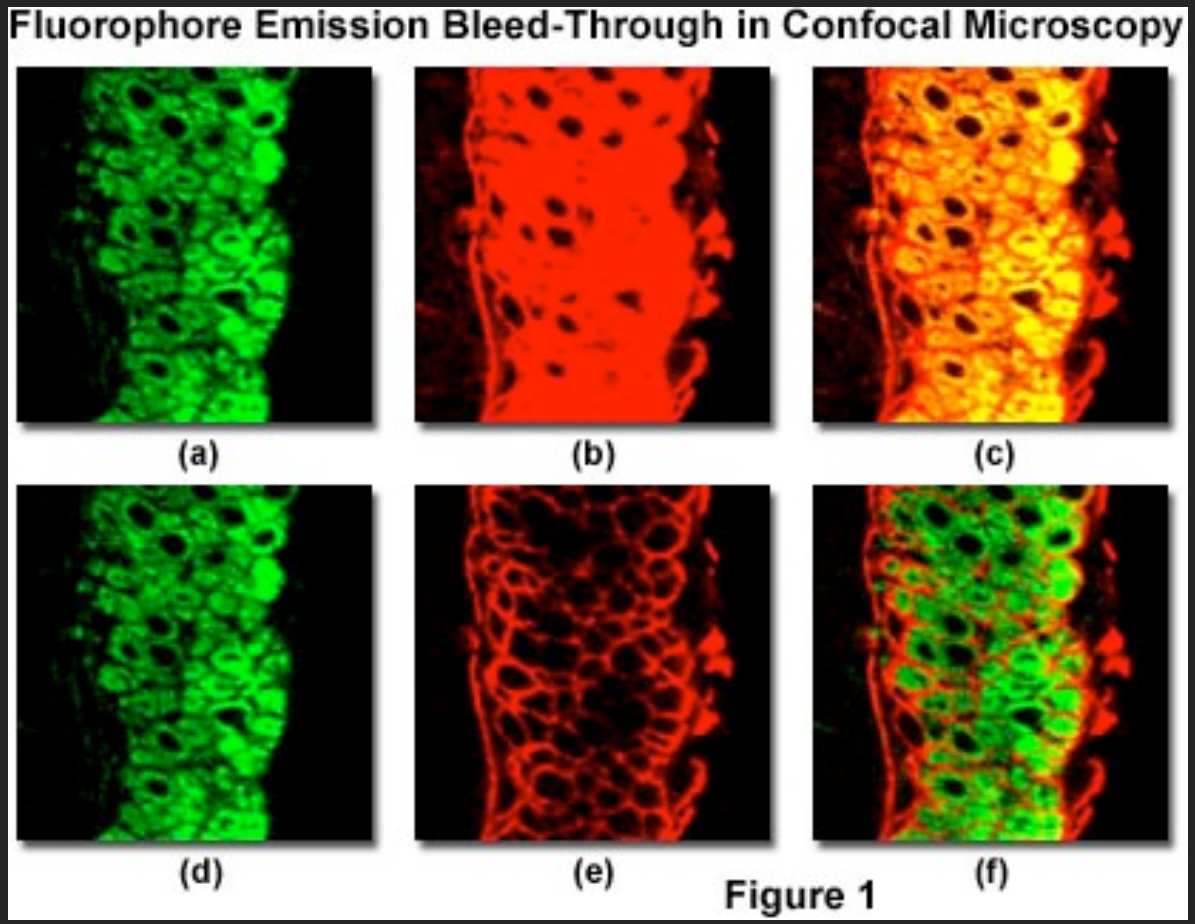


Sequential scanning

http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

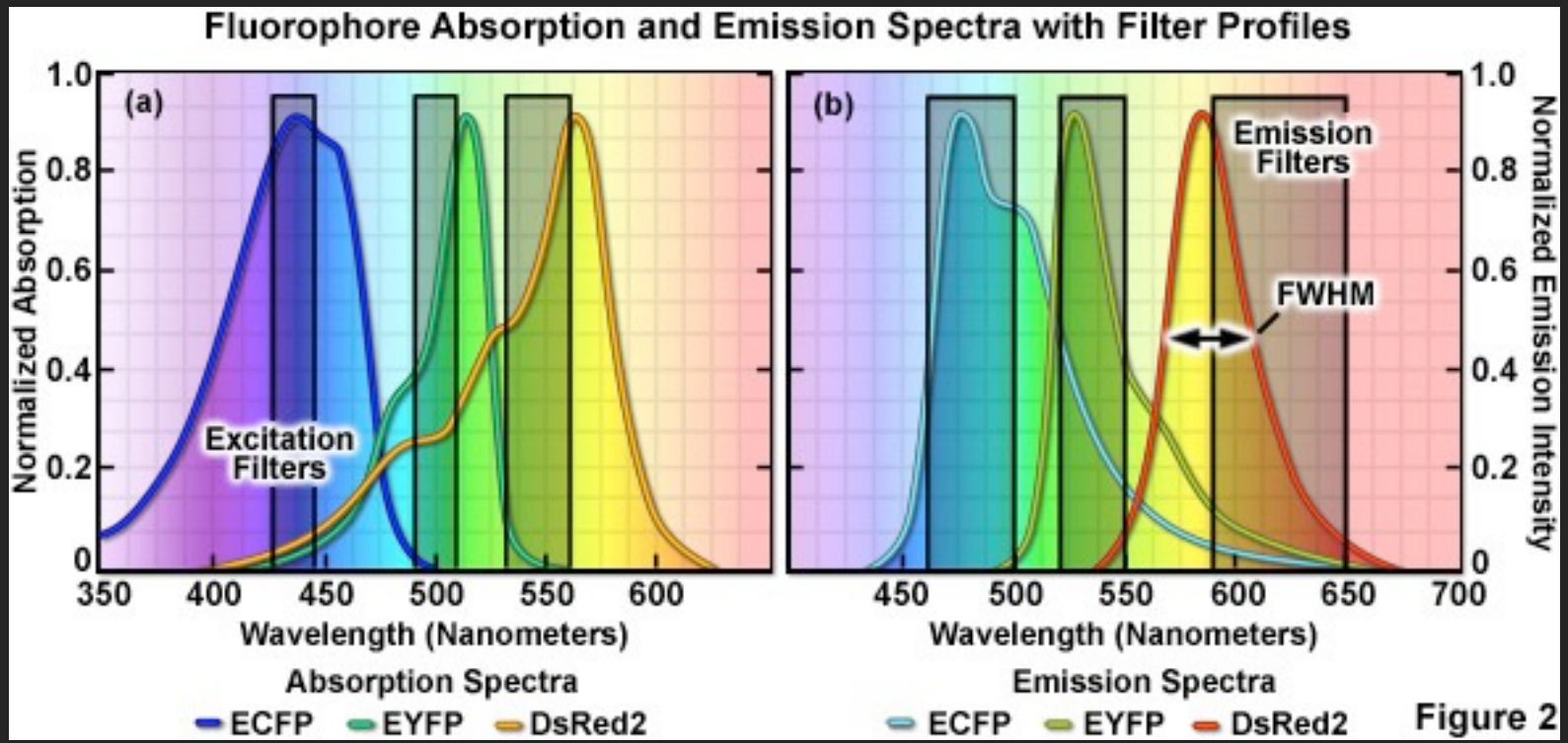
The Problem of "Bleedthrough"



Sequential scanning
- but a problem for live cells

http://www.olympusmicro.com/primer/java/confocalsimulator/index.html

Minimising “Bleedthrough”



Spectral Unmixing

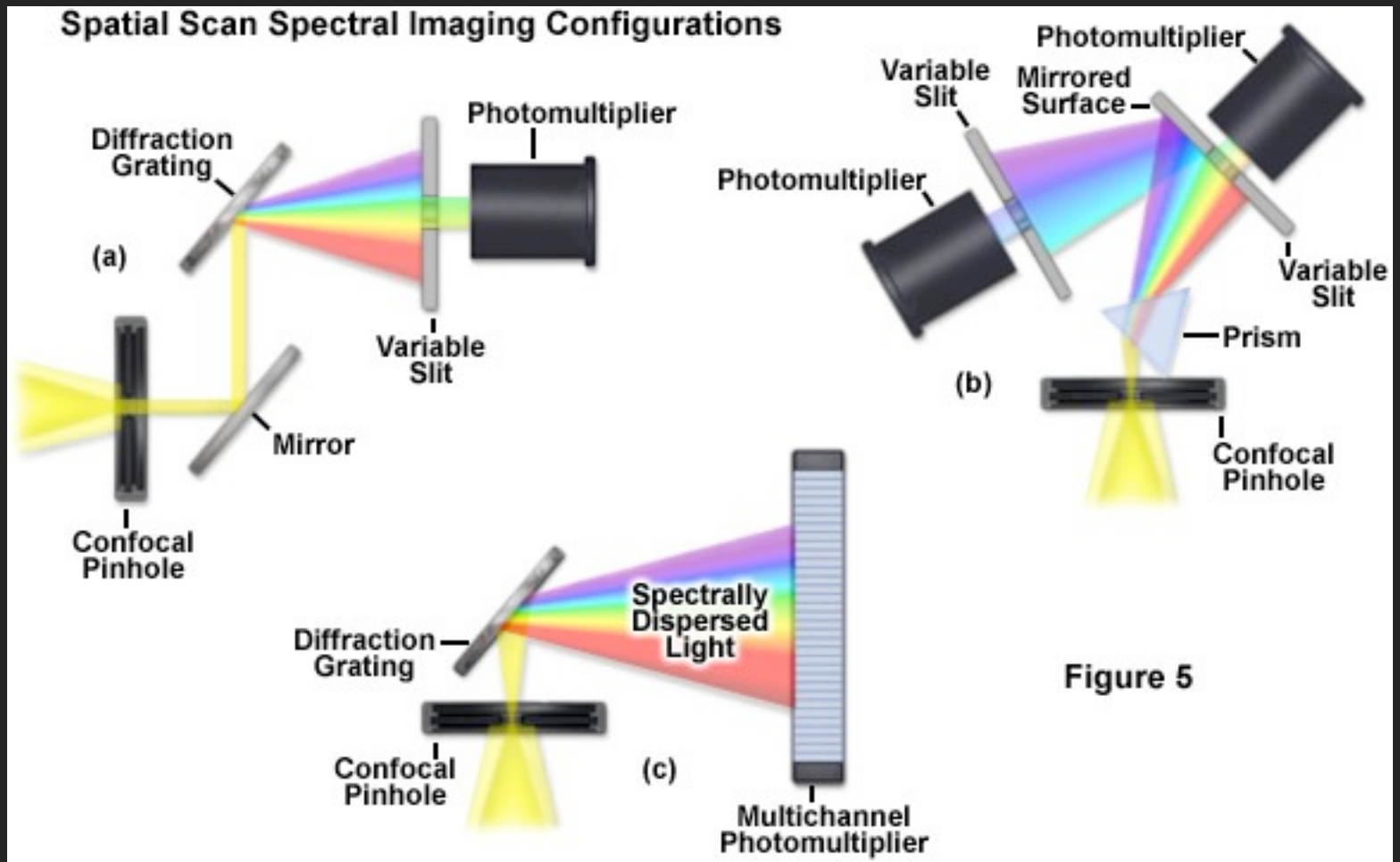
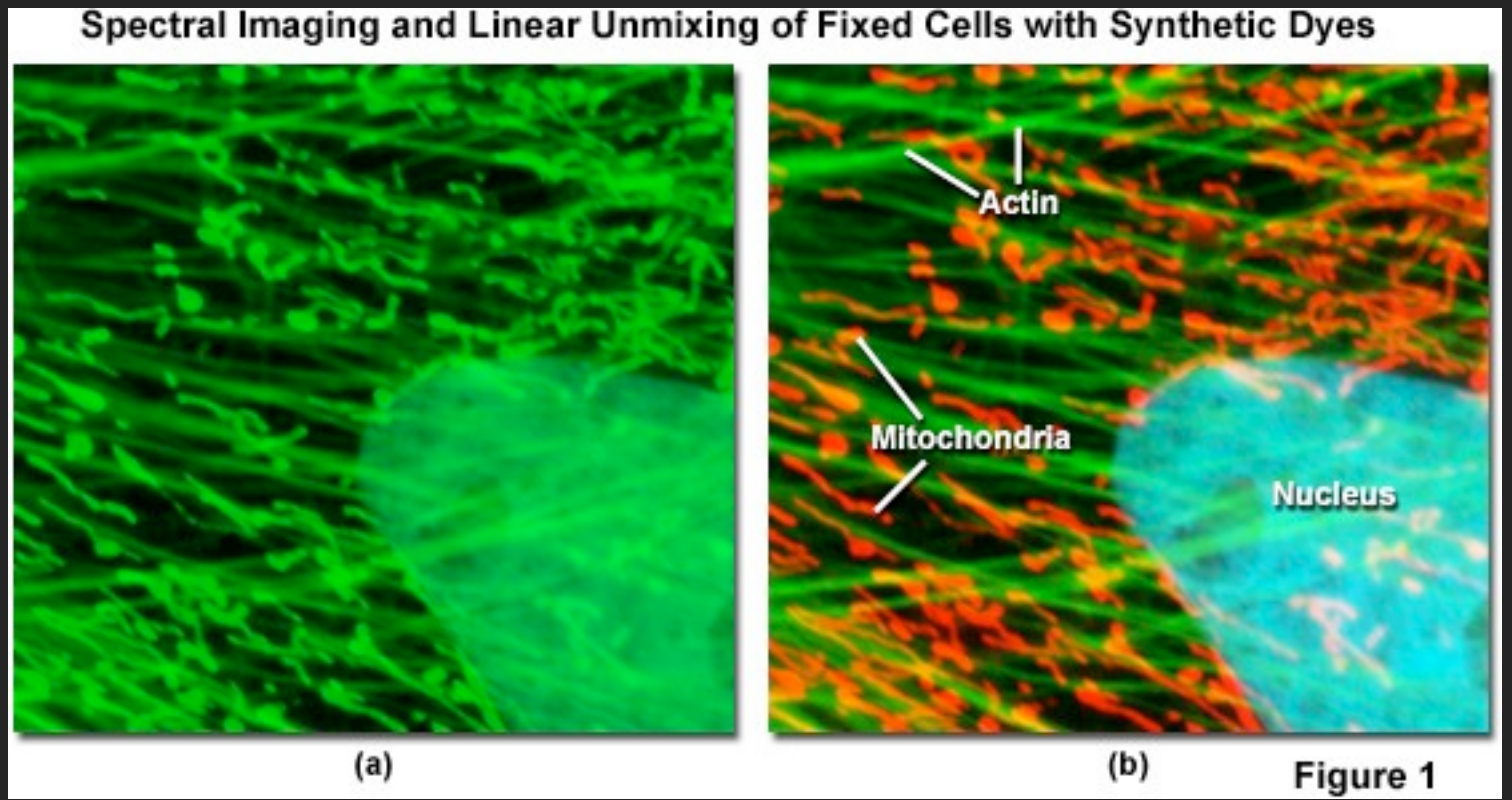


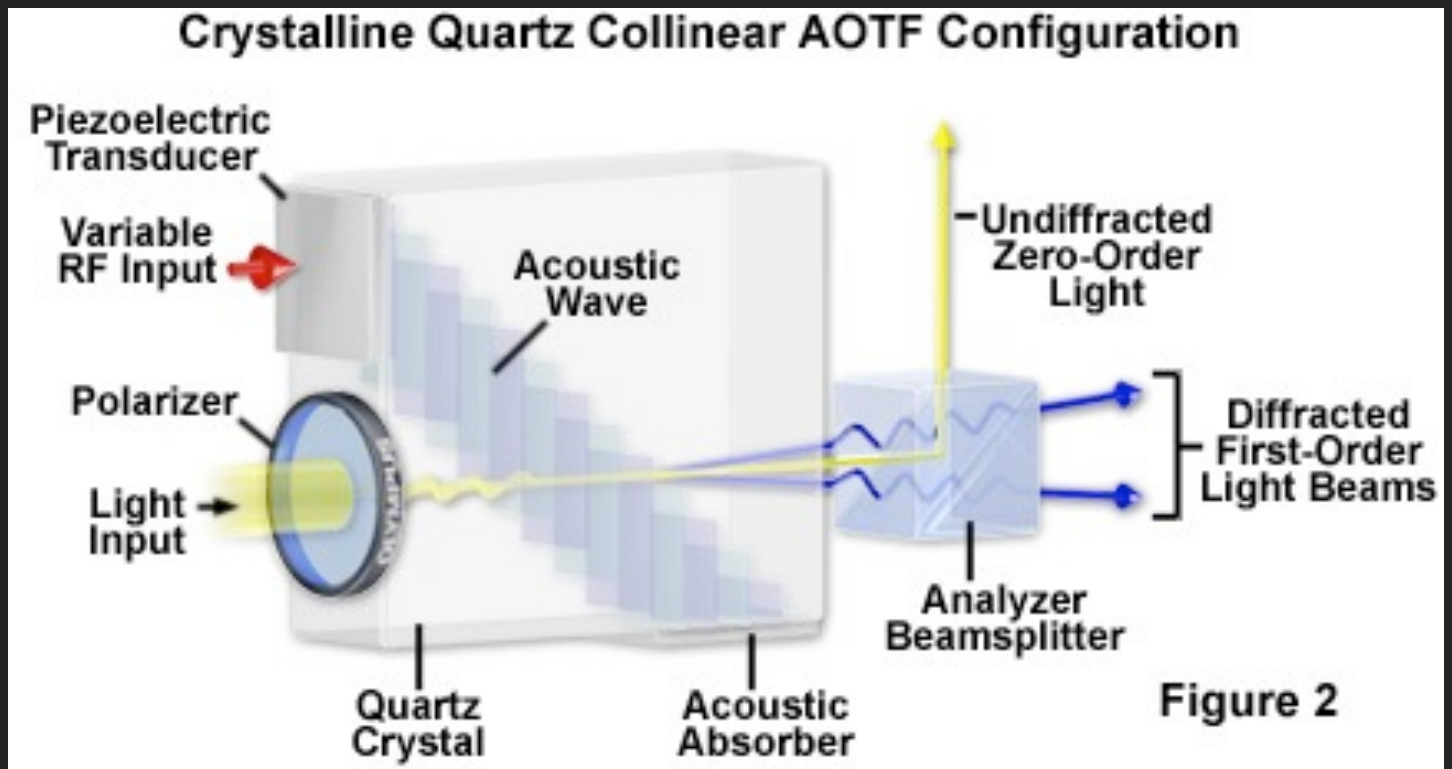
Figure 5

http://zeiss-campus.magnet.fsu.edu/tutorials/spectralimaging/linearunmixing/index.html

Spectral Unmixing

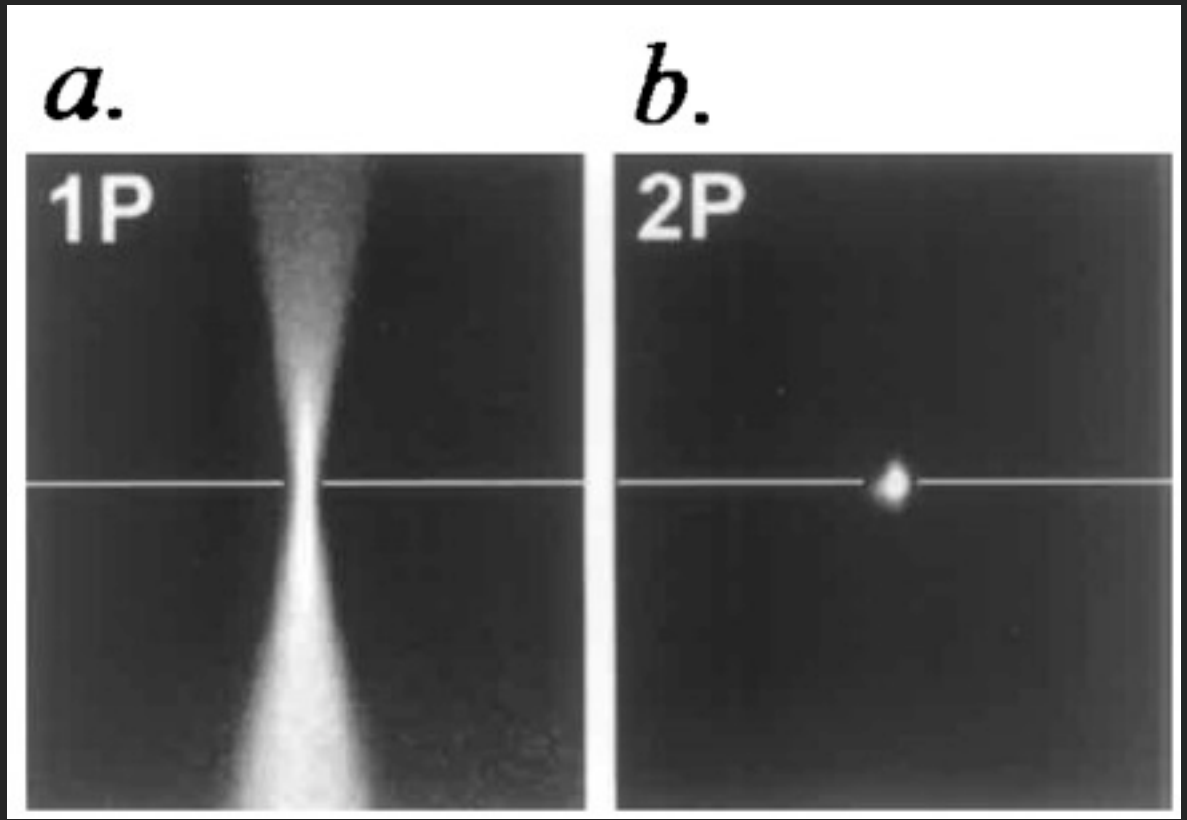


Acousto Optic Tunable Filter (AOTF)



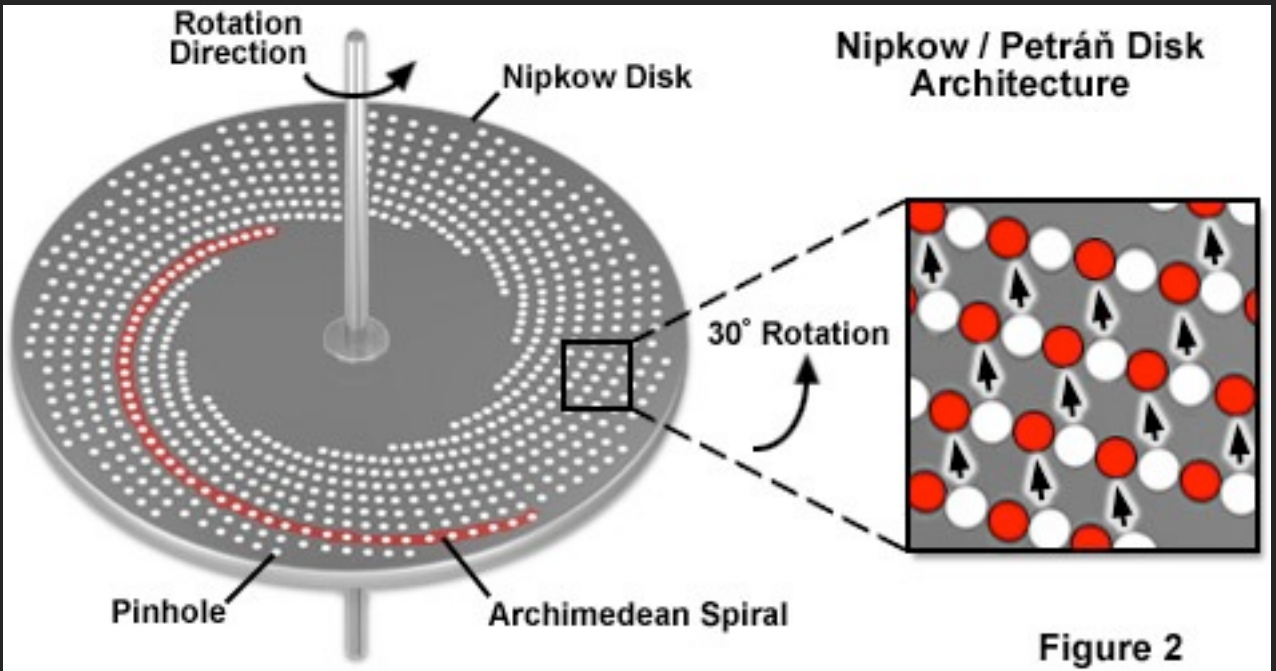
http://www.olympusmicro.com/primer/java/filters/aotf/index.html

Multiphoton Confocal Microscopy



http://www.olympusmicro.com/primer/java/multiphoton/jablonski/index.html

Spinning Disk Confocal Microscopy

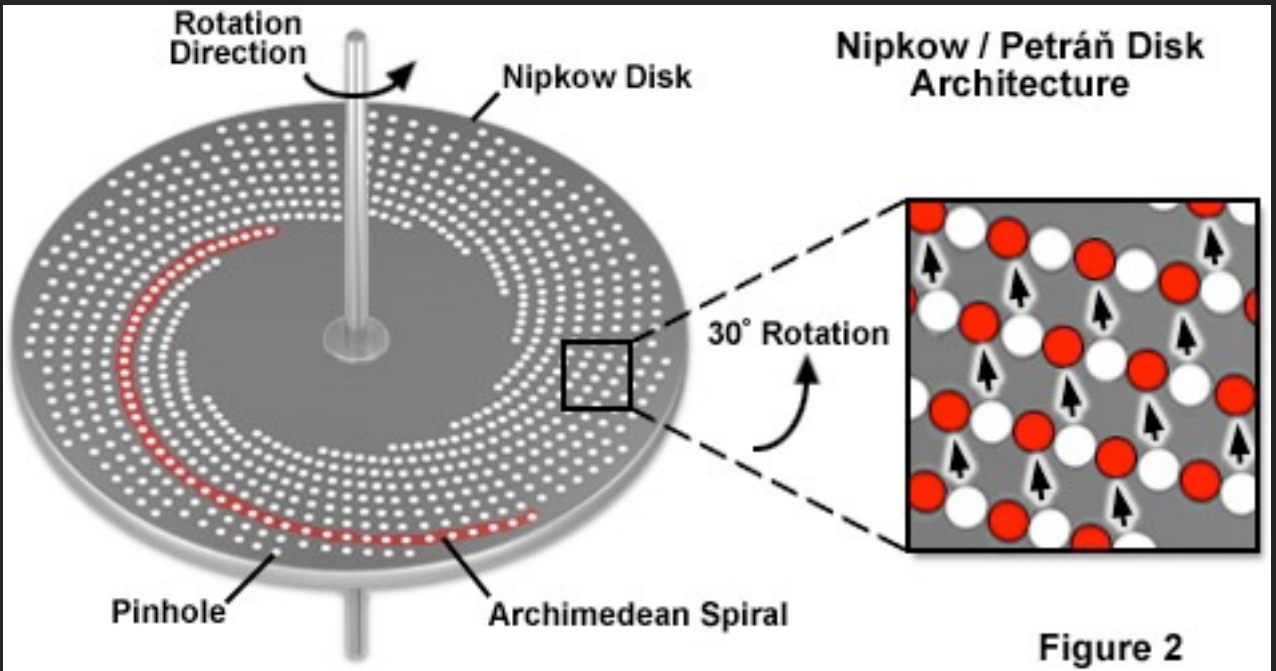


The Nipkow Disk

Paul Nipkow, 1884

Eggar and Petran, 1967

Spinning Disk Confocal Microscopy



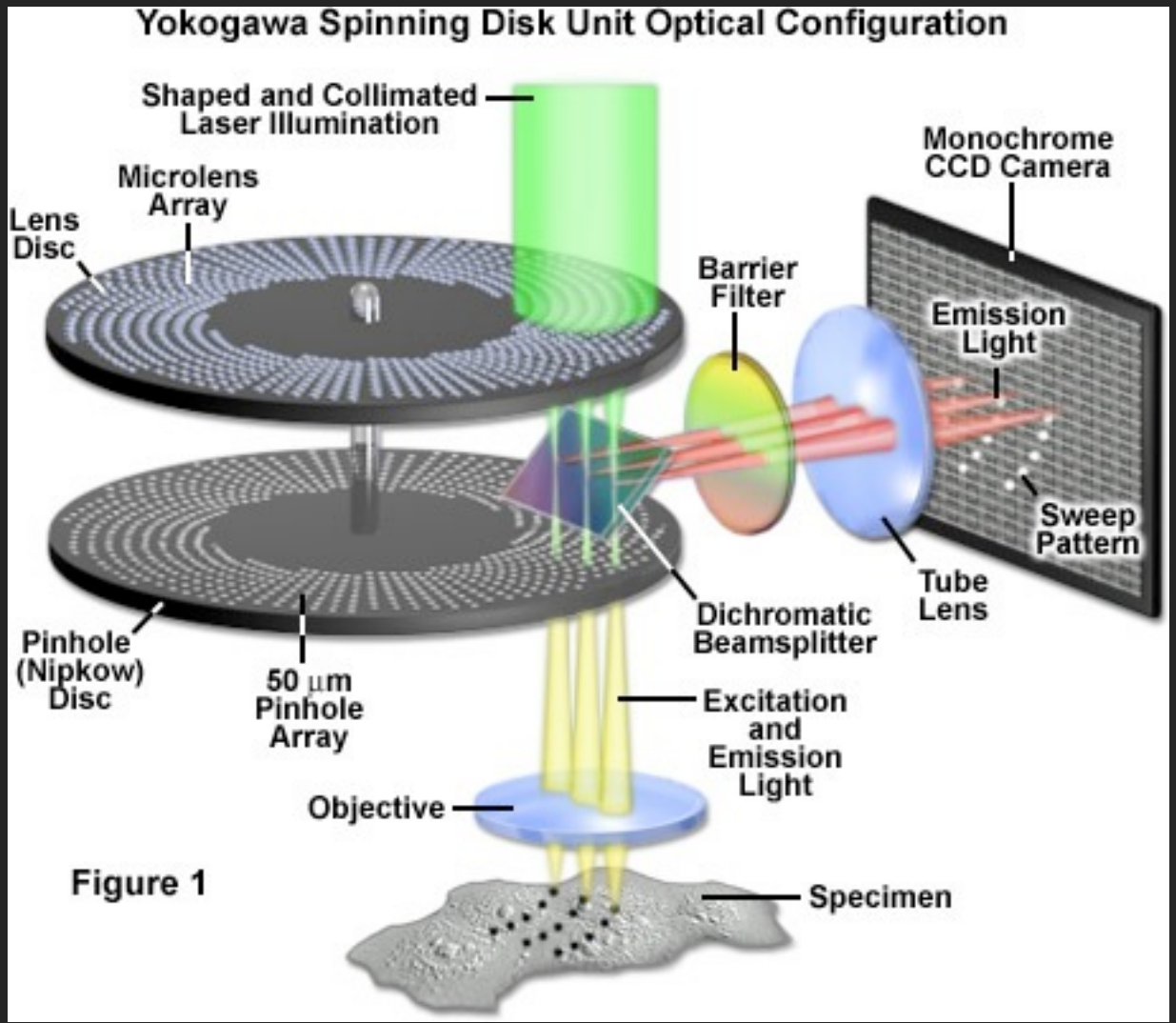
Constant Battle:

Larger pinholes - brighter image, but less “confocal”

Smaller spacing - more light gets through, but “crosstalk”

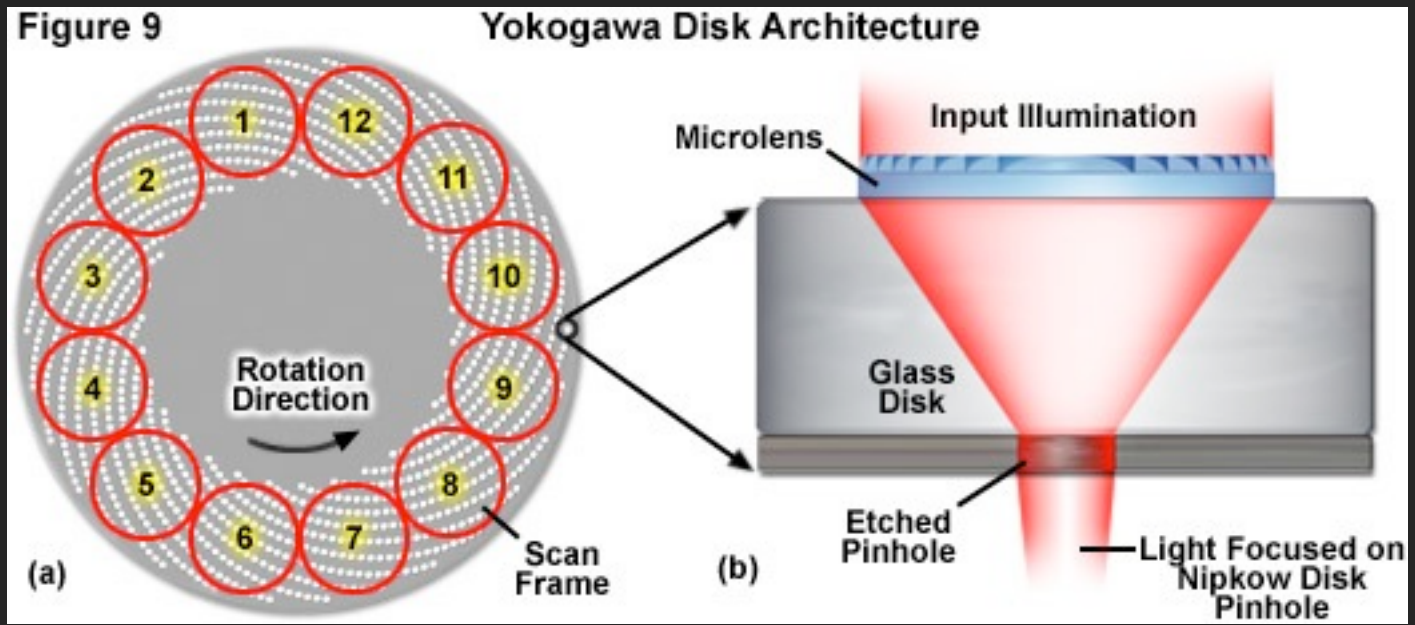
http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/microlensoptimization/index.html

The Yokogawa Spinning Disk

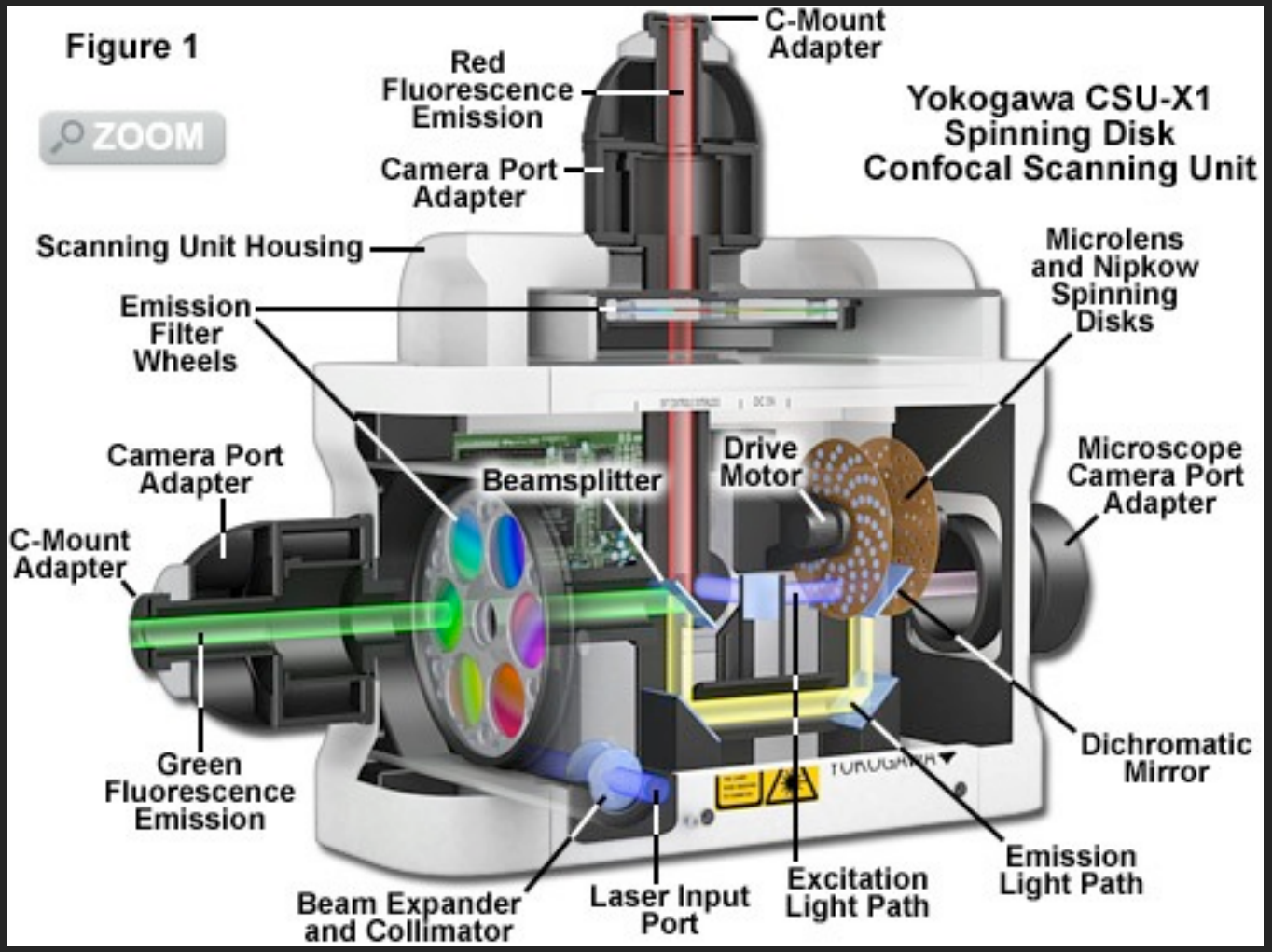


http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/index.html

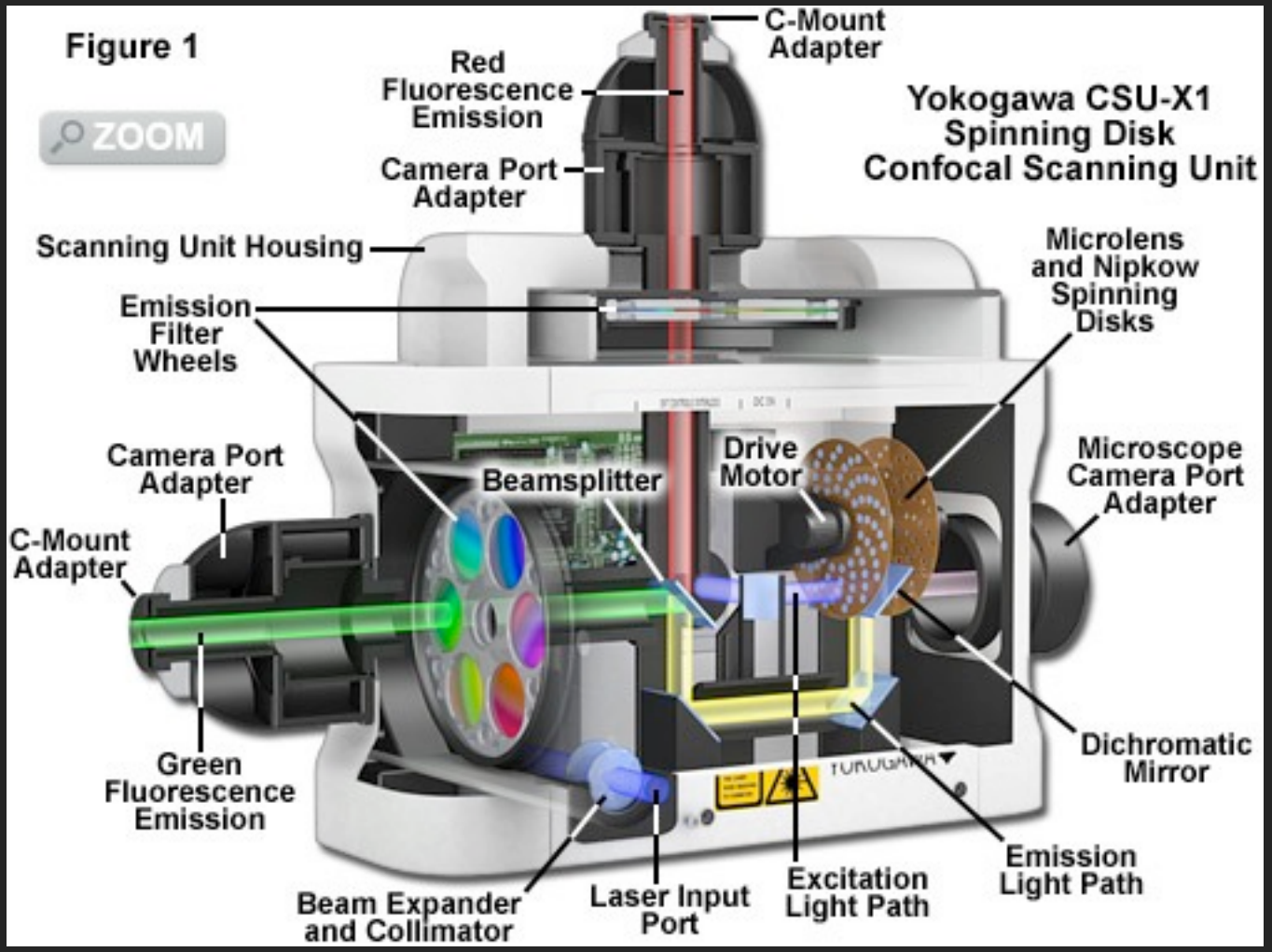
The Yokogawa Spinning Disk



The Yokogawa CSU-X1 Spinning Disk

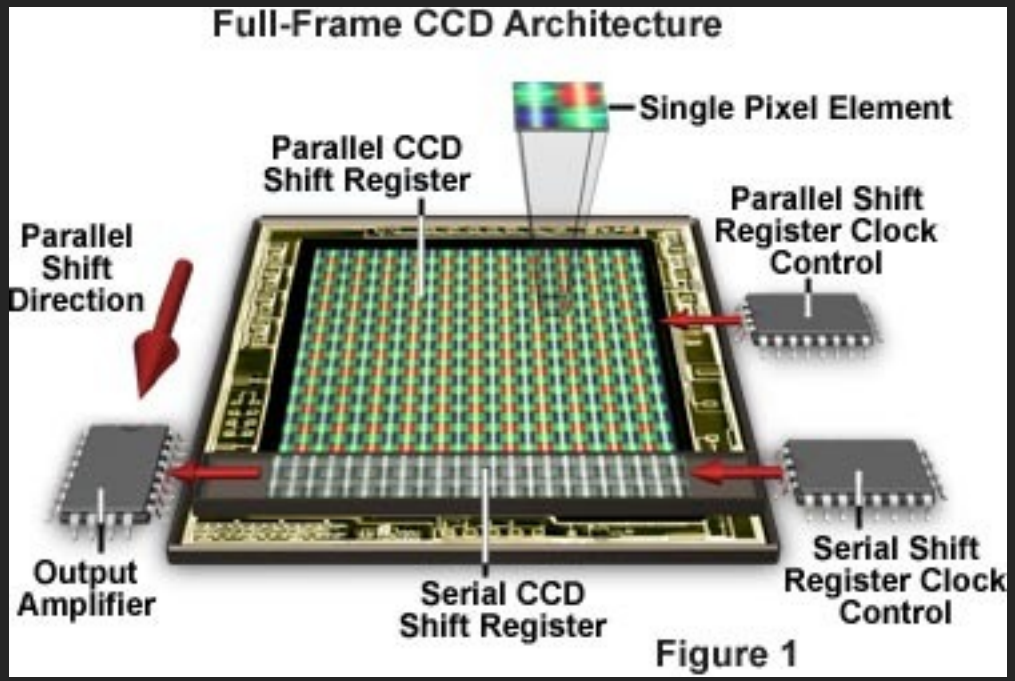


The Yokogawa CSU-X1 Spinning Disk



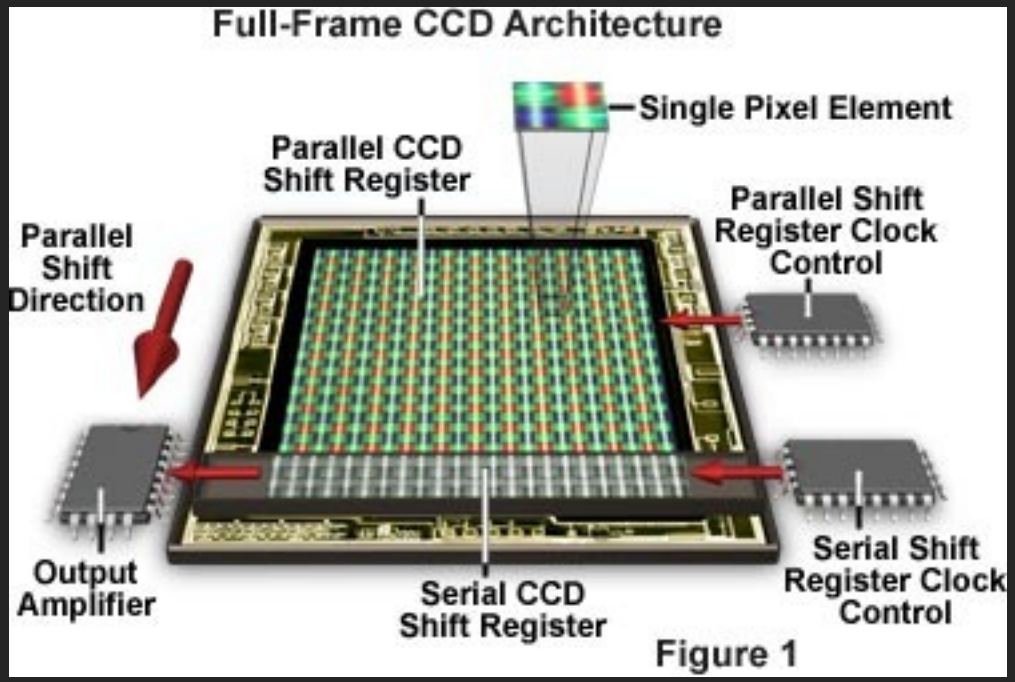
Can collect 2000 images per second

The Charged Couple Device (CCD) Camera



http://www.olympusmicro.com/primer/java/digitalimaging/ccd/interline/index.html

The Charged Couple Device (CCD) Camera

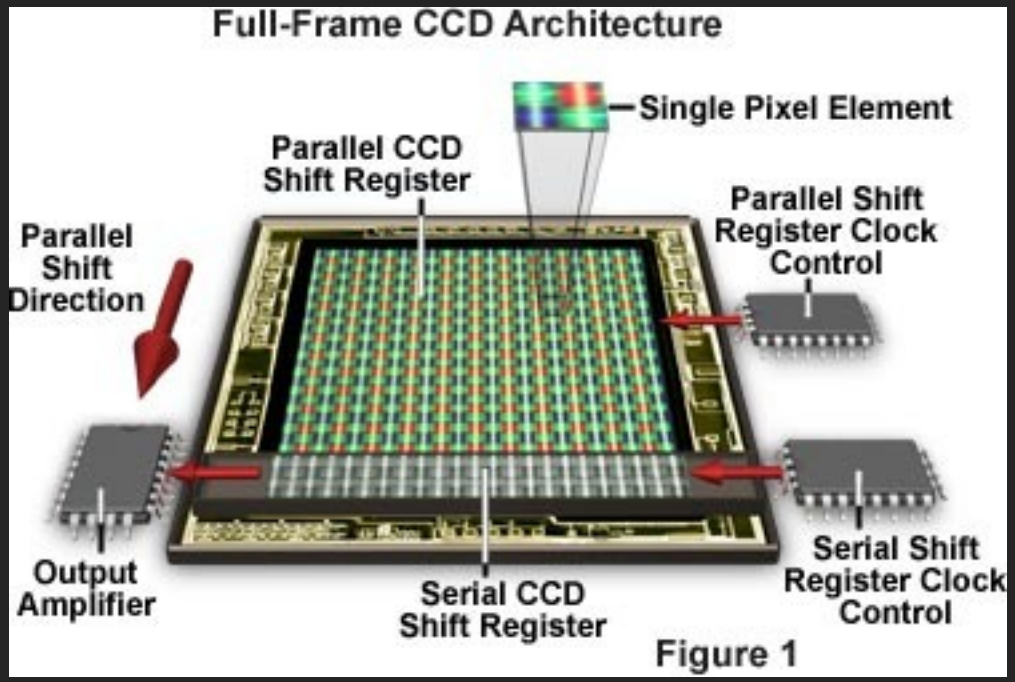


Can get very high QE - up to 95%

Can be very fast

http://www.olympusmicro.com/primer/java/digitalimaging/ccd/interline/index.html

The Charged Couple Device (CCD) Camera



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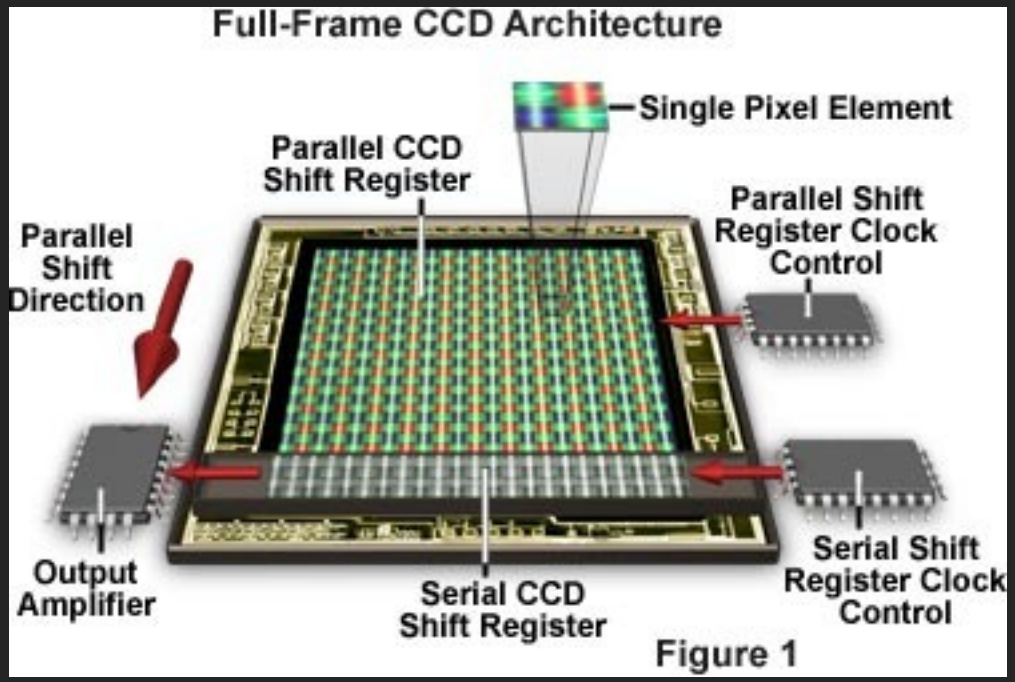
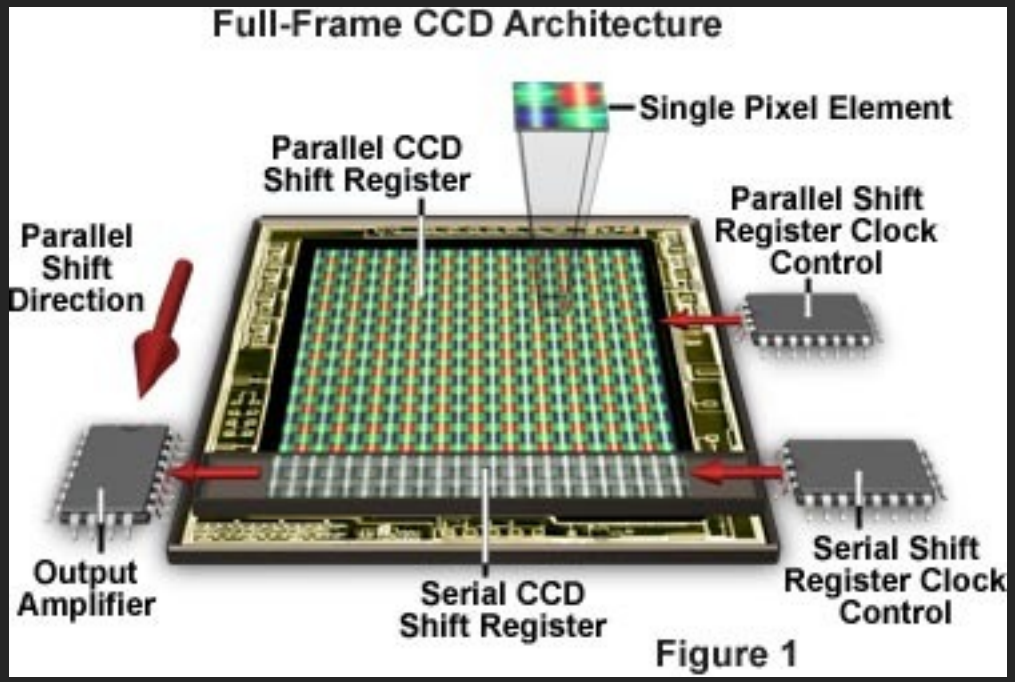


Noise!

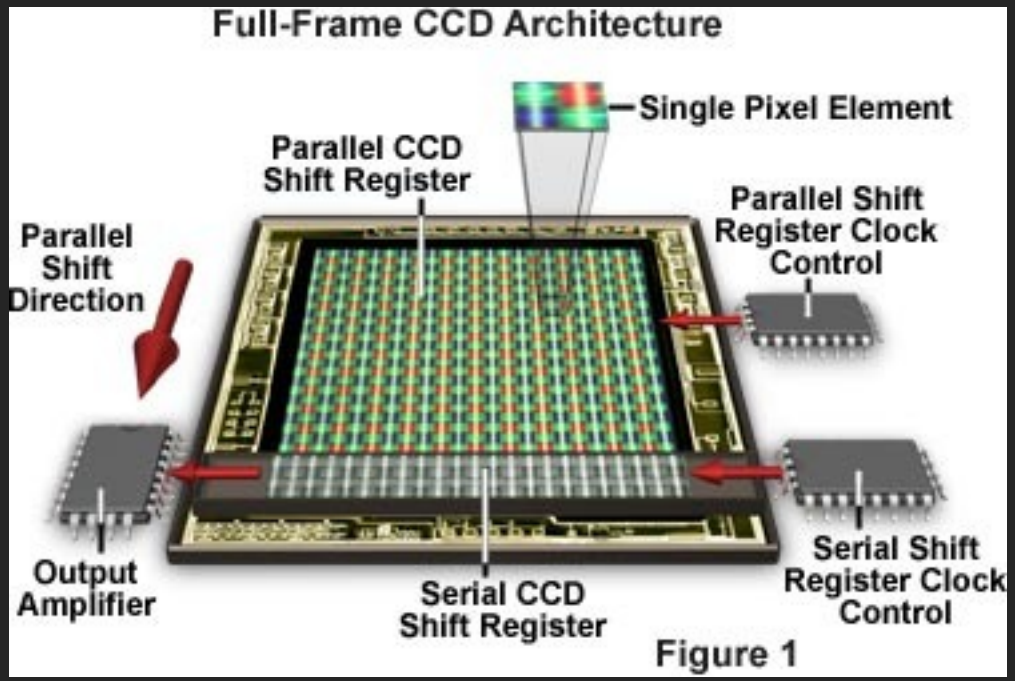
The Charged Couple Device (CCD) Camera



Noise!

Dark Noise - less of a problem at low temperature

The Charged Couple Device (CCD) Camera

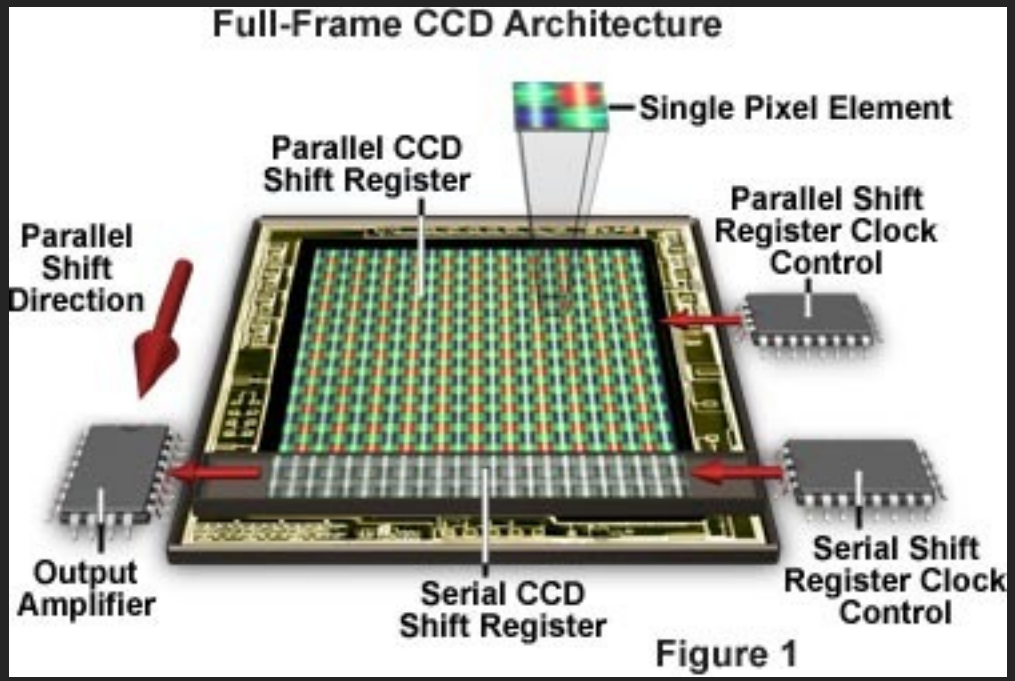


Noise!

Dark Noise - less of a problem at low temperature

Read Noise - inherent to camera, but worse at high speed

The Charged Couple Device (CCD) Camera



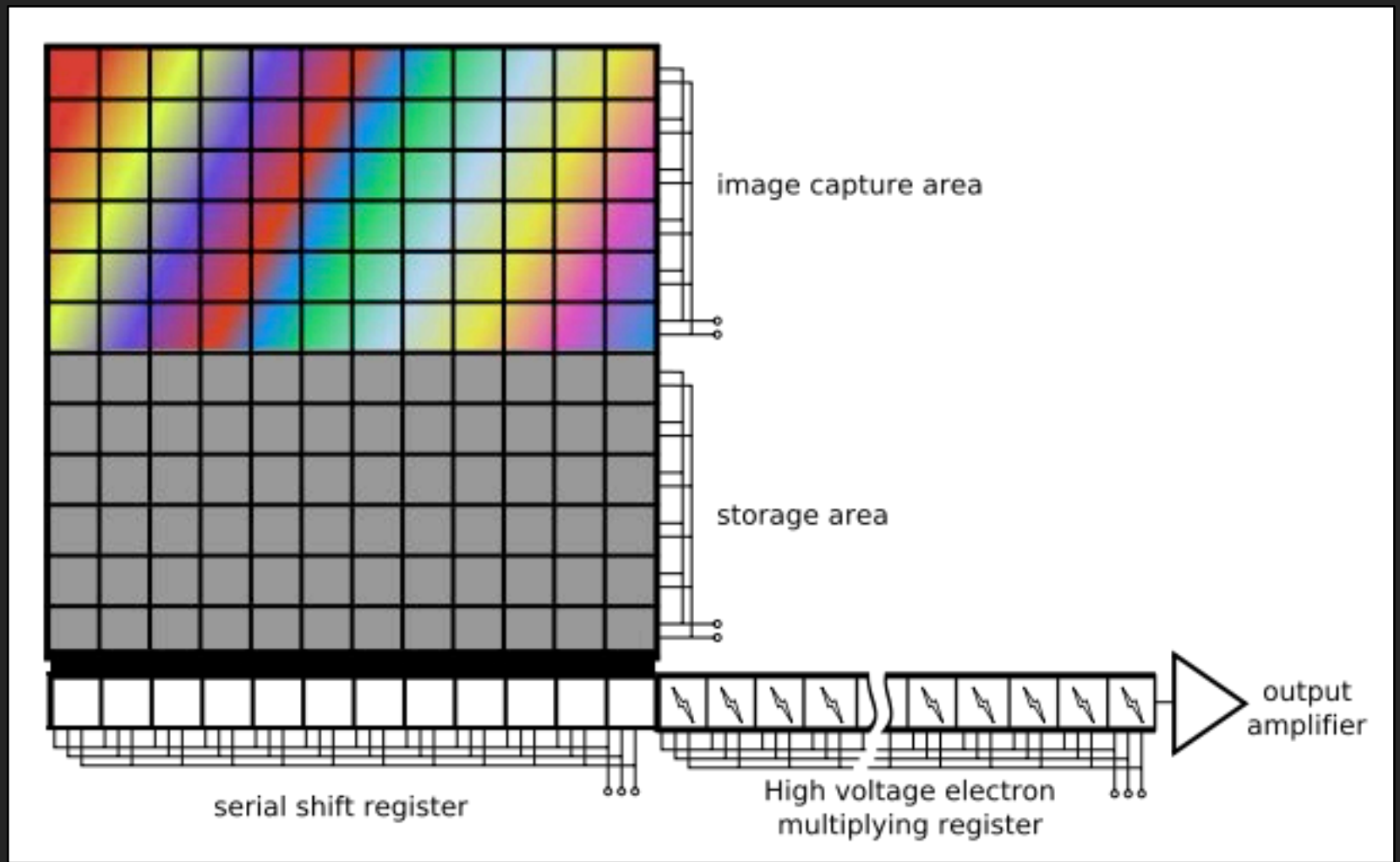
Noise!

Dark Noise - less of a problem at low temperature

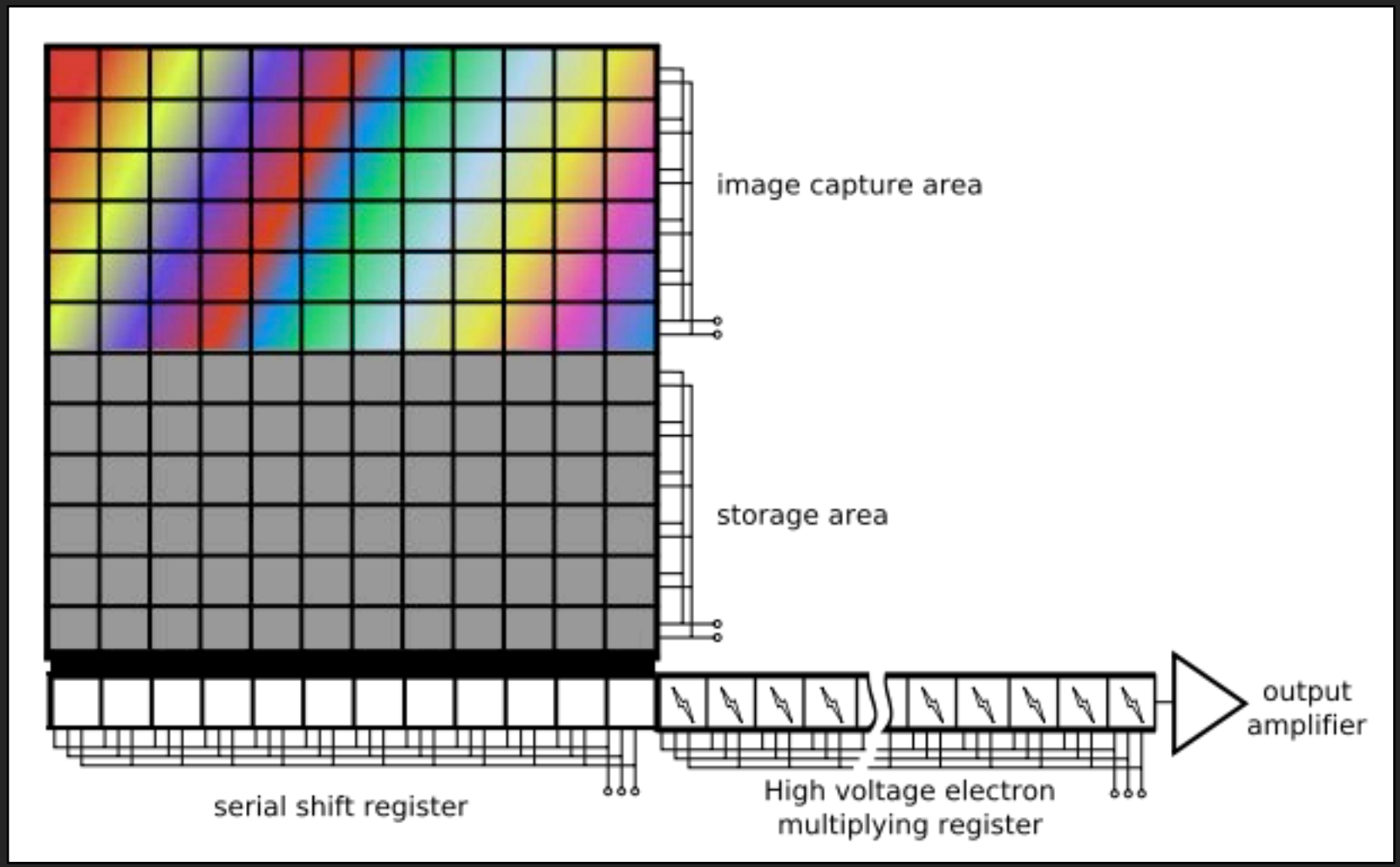
Read Noise - inherent to camera, but worse at high speed

Shot Noise - due to stochastic nature of fluorescence

The Electron Multiplying Charged Couple Device (EMCCD) Camera

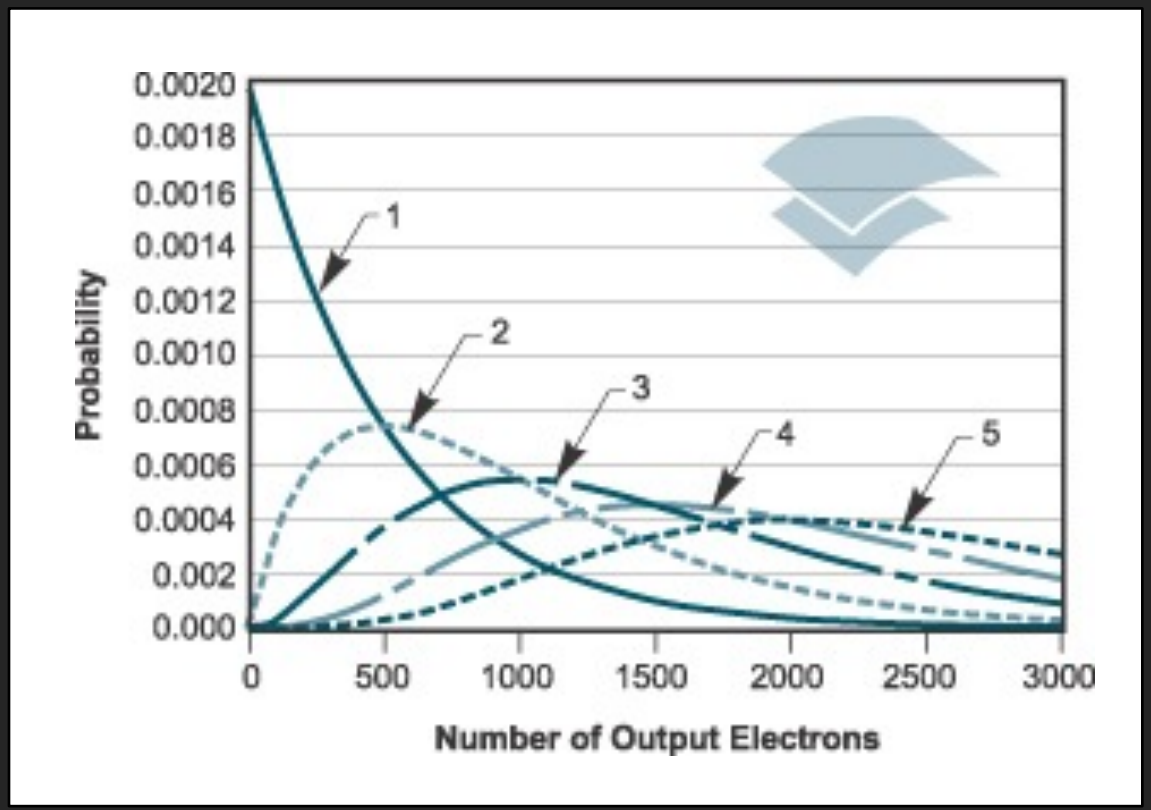


The Electron Multiplying Charged Couple Device (EMCCD) Camera



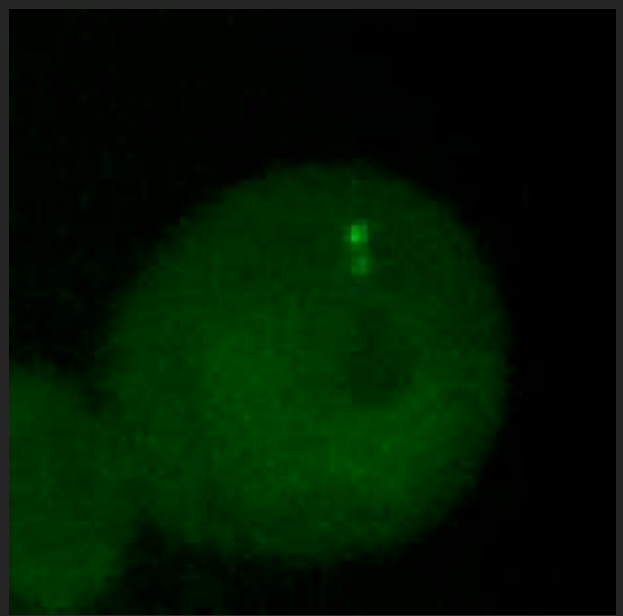
Beware!!

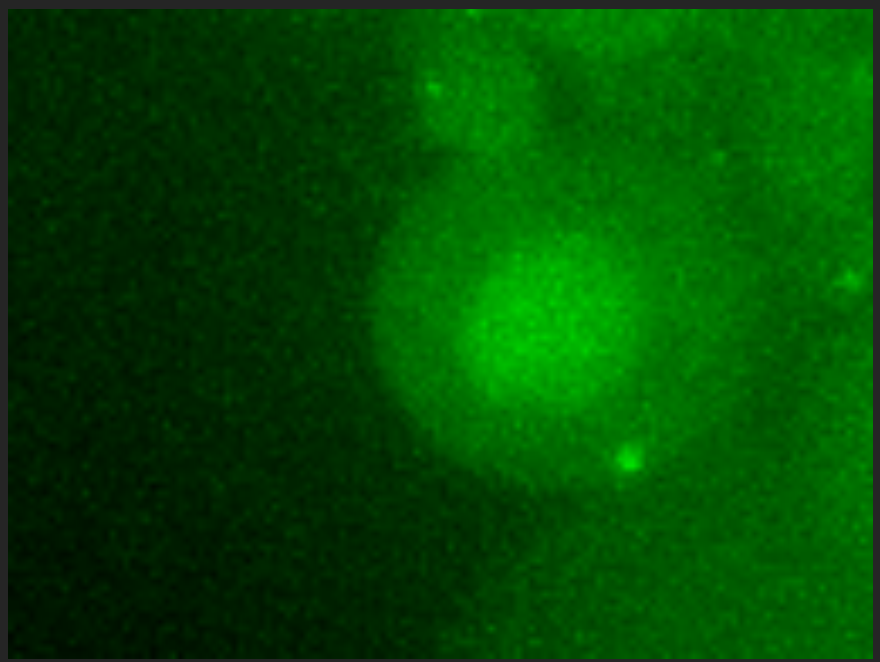
How quantitative is an EMCCD Camera?



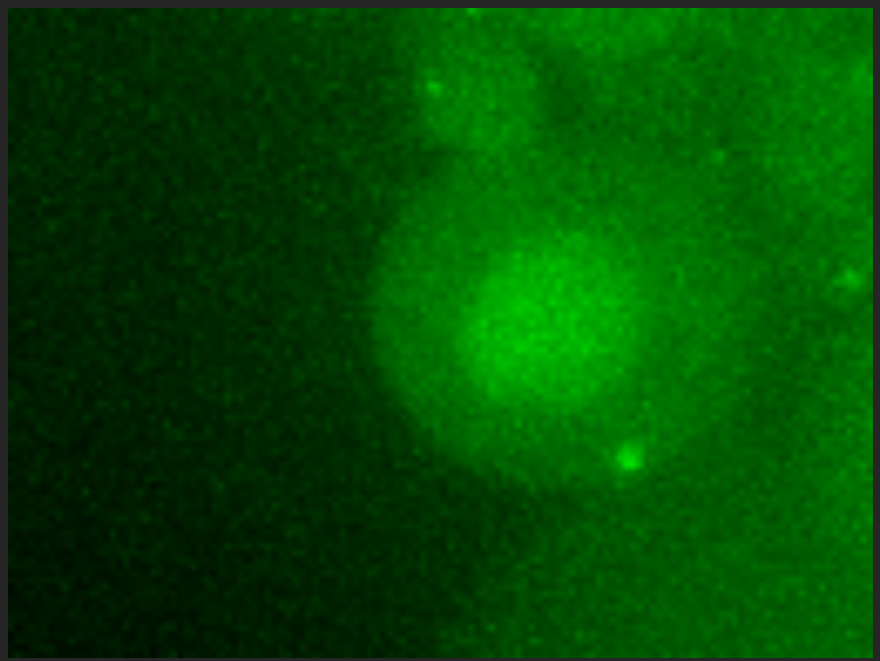
The Electron Multiplying Charged Couple Device (EMCCD) Camera

The Electron Multiplying Charged Couple Device (EMCCD) Camera





The Electron Multiplying Charged Couple Device (EMCCD) Camera



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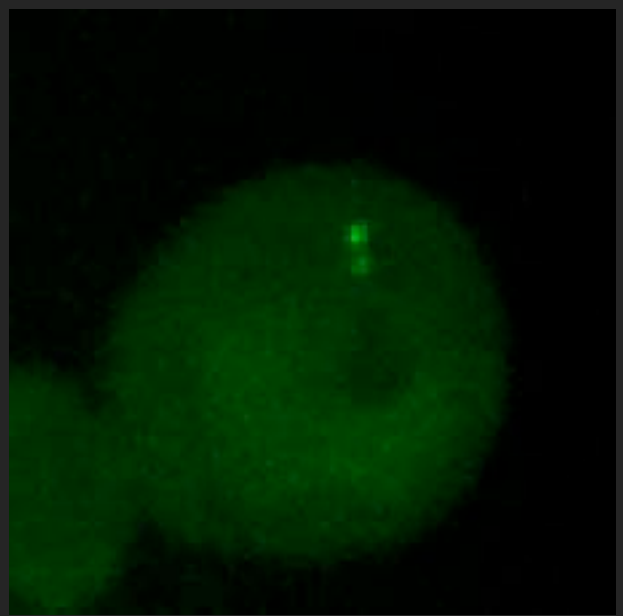
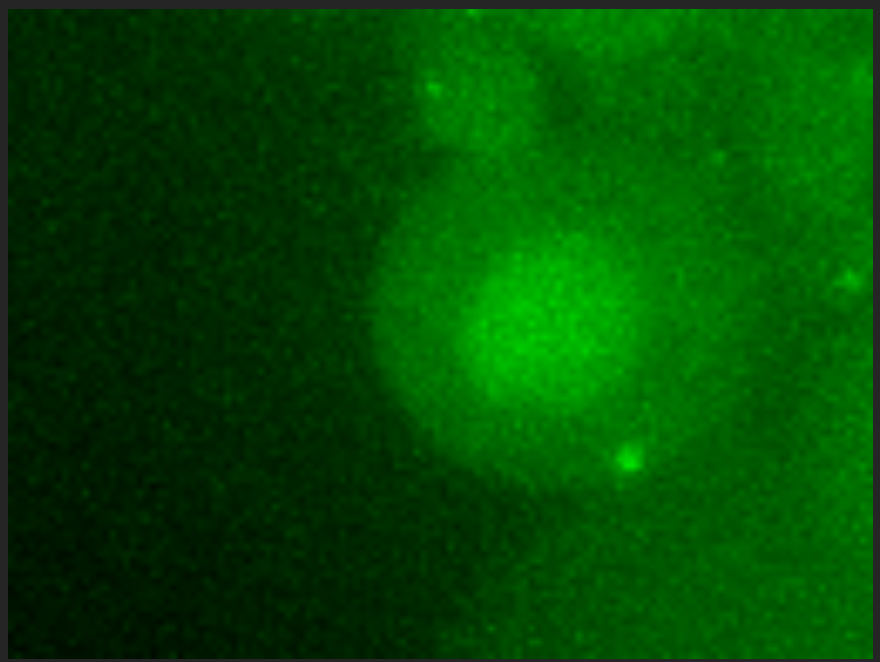
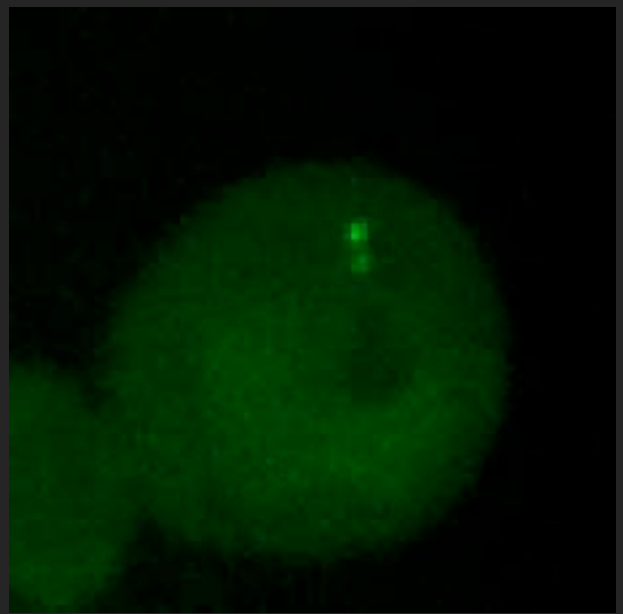




The Electron Multiplying Charged Couple Device (EMCCD) Camera



Understanding Zoom!

Point Scanning vs. Spinning Disk

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK
Flexibility	Good	Poor

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good
Pretty Pictures	Unbeatable!	Pretty damn good!

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK
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Pretty Movies	Good if process slow	Unbeatable!

Point Scanning vs. Spinning Disk

Speed	Slow (secs)	Fast (100msec)
Sensitivity	OK	OK
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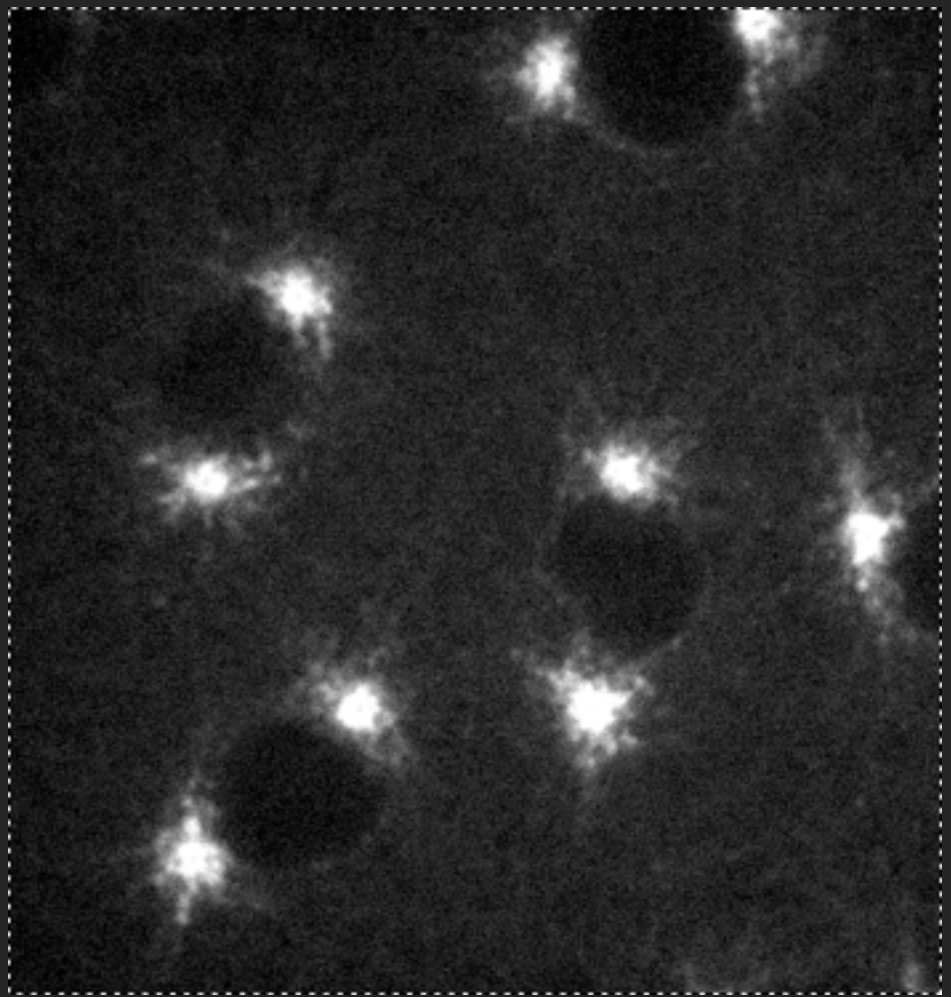
Pretty Movies	Good if process slow	Unbeatable!

Murray et al., Evaluating performance in 3D fluorescence microscopy. J. Microscopy (2007)

Example of fast imaging - single plane

Example of fast imaging - single plane

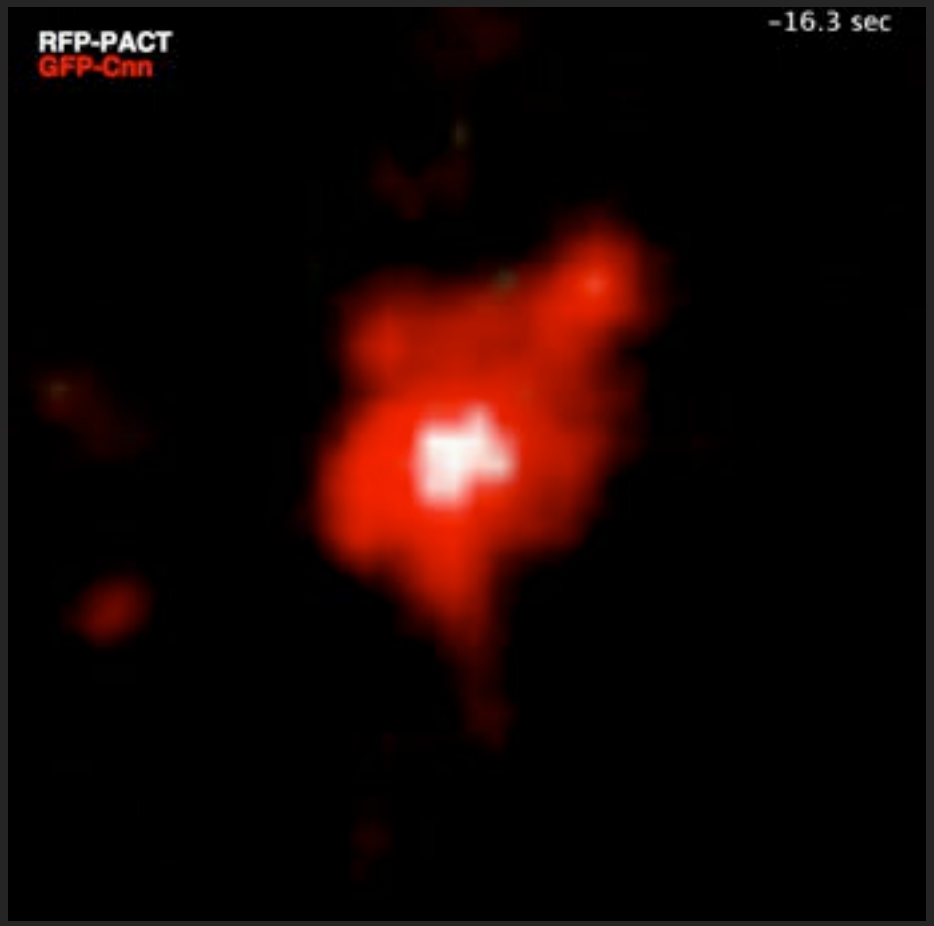


Example of FRAP on Spinning Disk

Centrioles

Cnn

Example of FRAP on Spinning Disk



Centrioles

Cnn

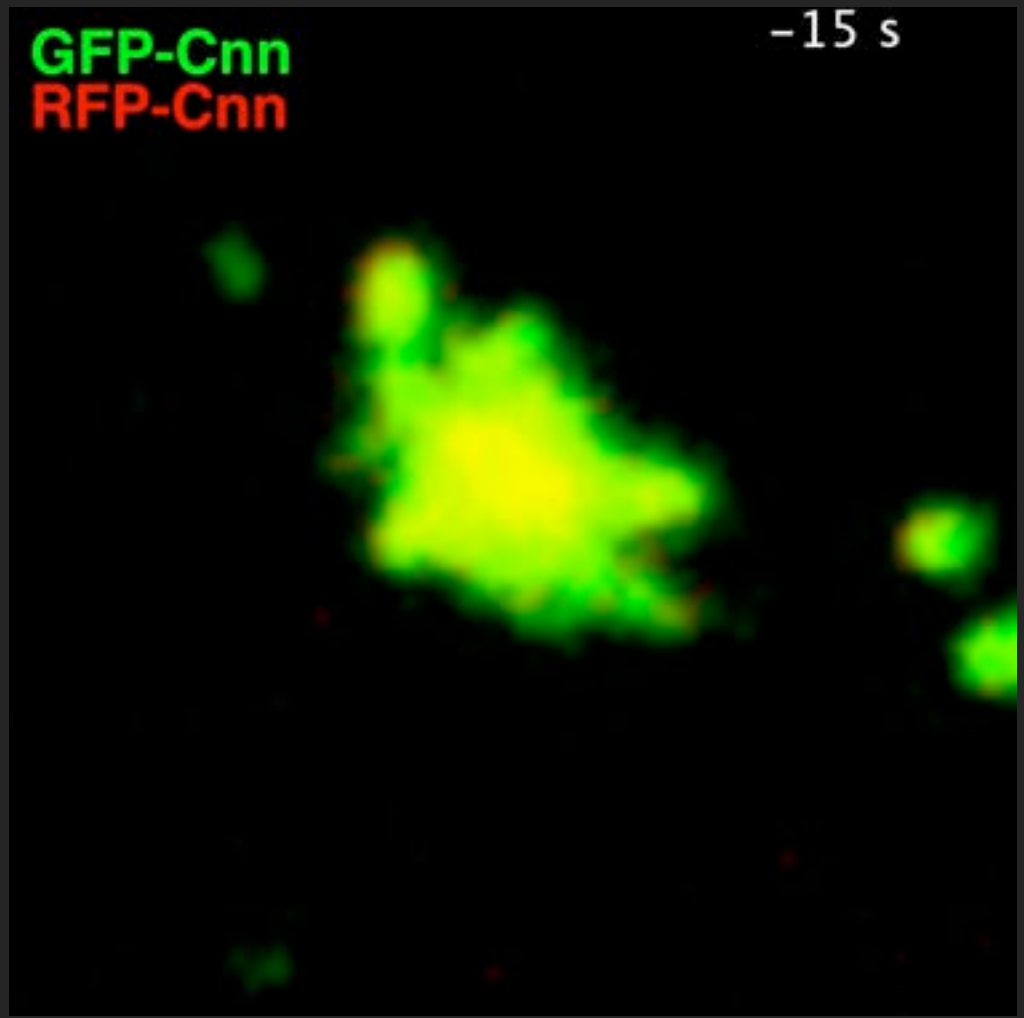
Example of FRAP on Spinning Disk

Centrioles

Cnn

Another example of FRAP on Spinning Disk

Another example of FRAP on Spinning Disk

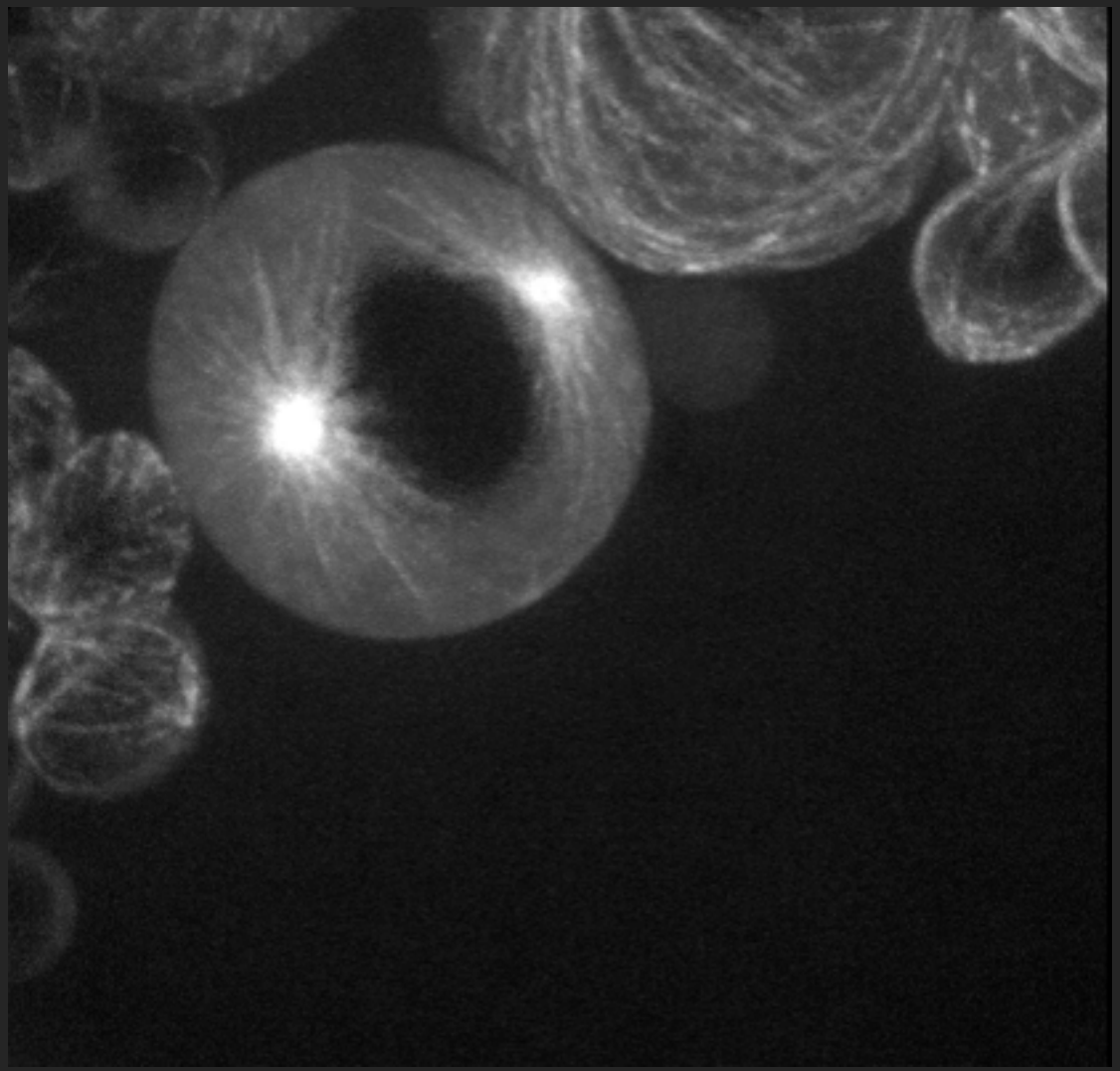


Another example of FRAP on Spinning Disk

Cell division in brain stem cells (neuroblasts)

Microtubules

Cell division in brain stem cells (neuroblasts)



Microtubules

Cell division in brain stem cells (neuroblasts)

Microtubules

Identifying proteins required for centrosome duplication

Centrosomes

Centrosomal Antibody

Identifying proteins required for centrosome duplication