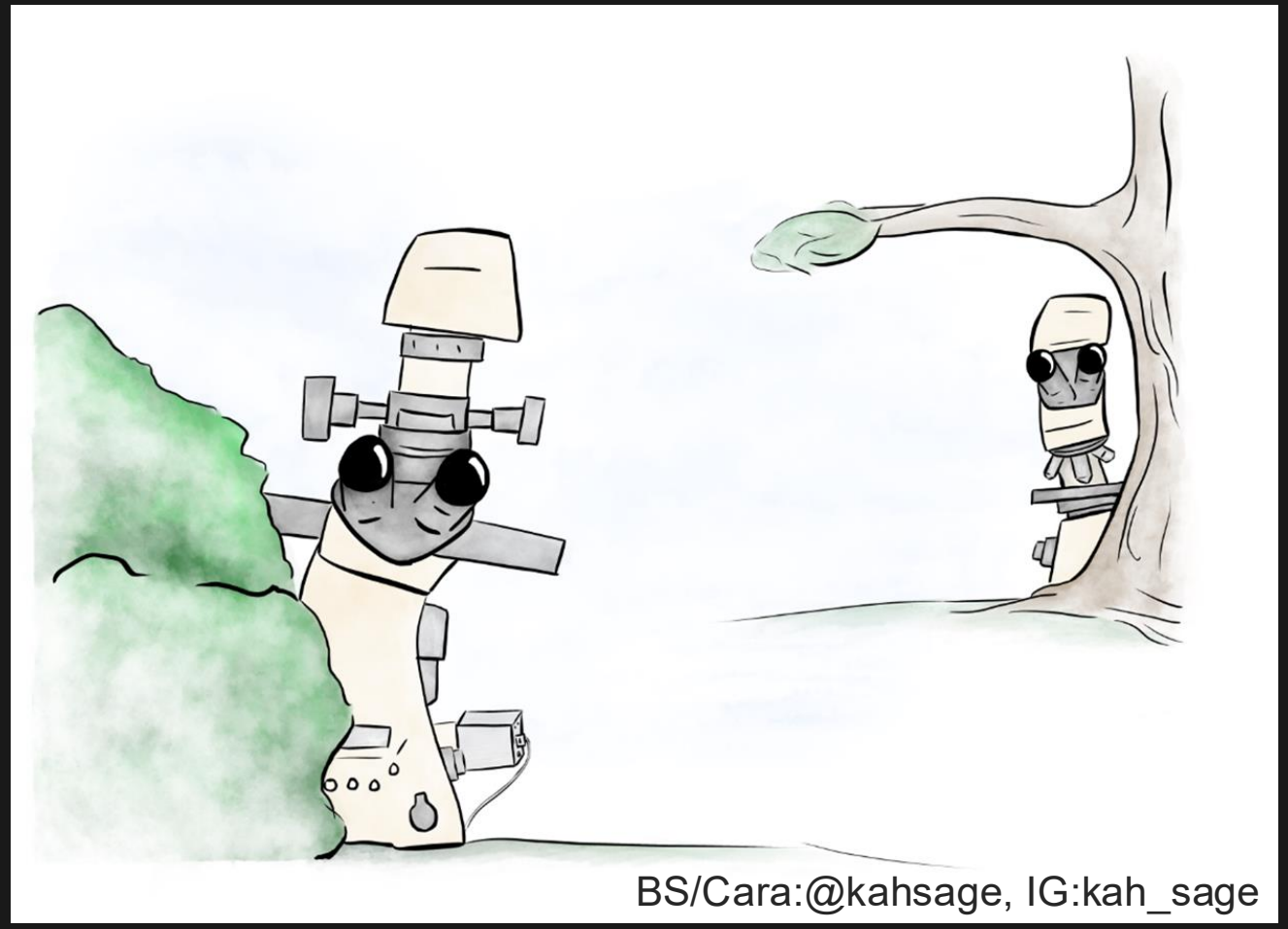
Introduction to Light Microscopy at the Center for Advanced Light Microscopy (CALM) at UCSF



https://calm.ucsf.edu

Imaging is all about:

contrast

contrast

contrast

contrast

contrast

contrast

contrast

contrast

contrast

contrast

resolution

resolution

resolution

resolution

resolution

resolution

resolution

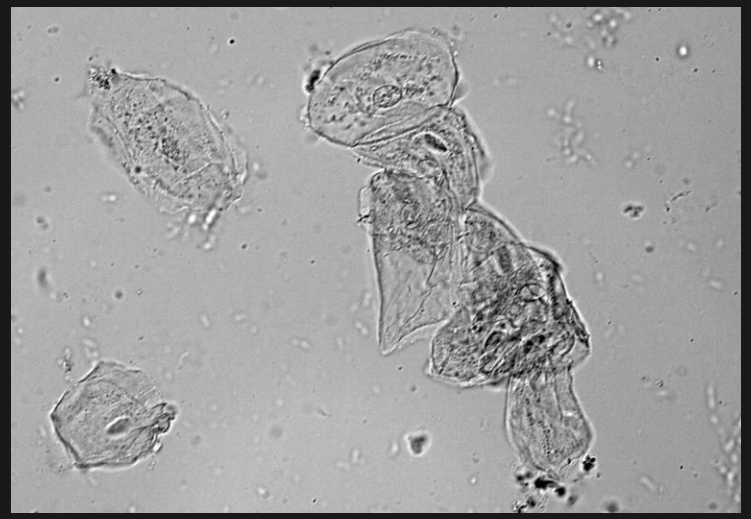
resolution

resolution

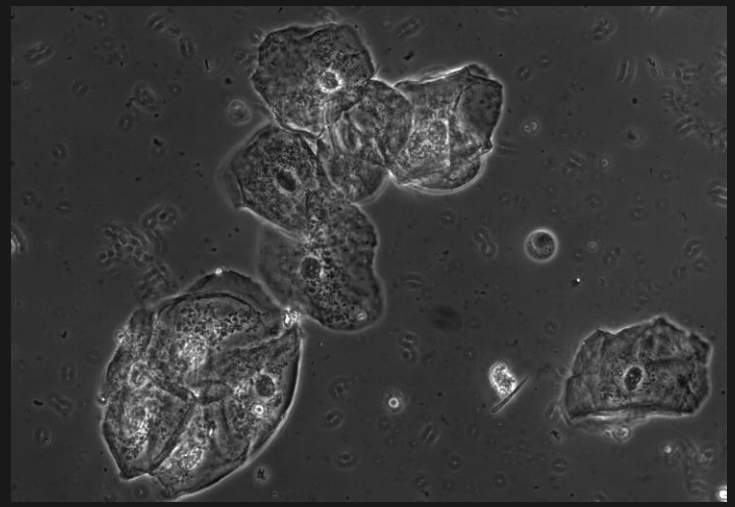
resolution

Contrast in Microscopy

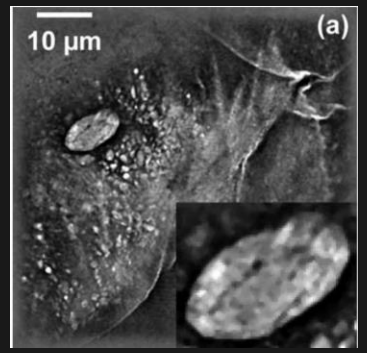
Brightfield



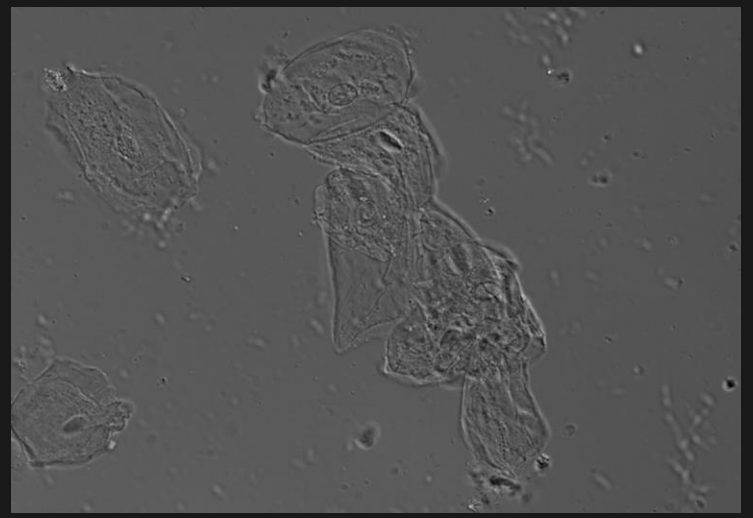
Phase Contrast

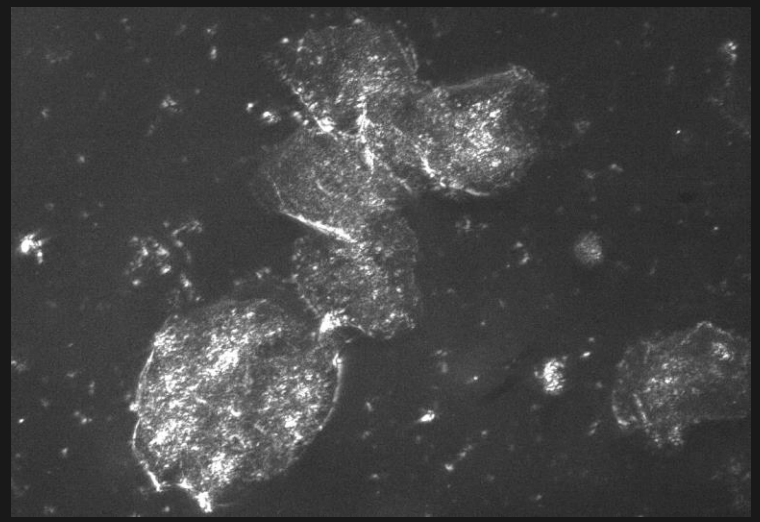


Quantitative Phase (e.g. QLIPP)



Differential Interference Contrast

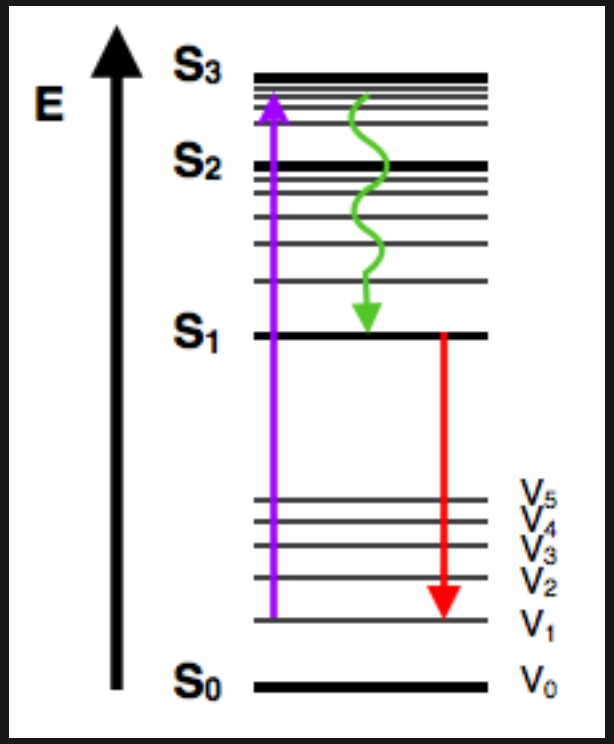




Dark Field (Reflection)

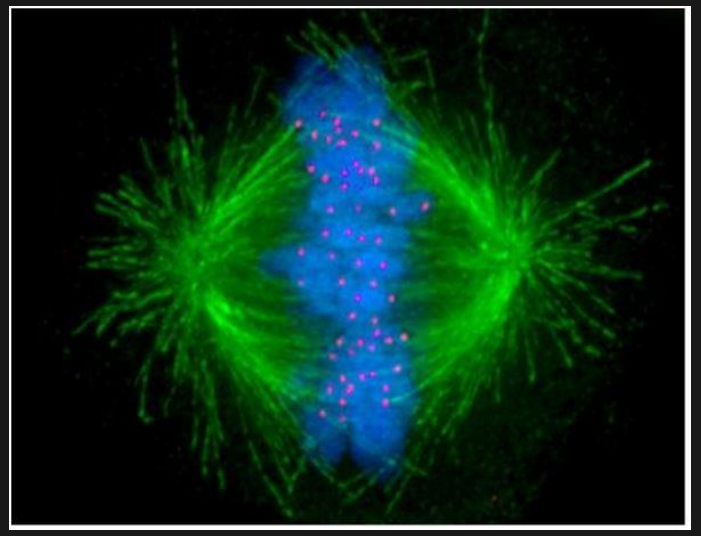
J. Biophoton. 3, No. 7, 462–467 (2010) / DOI 10.1002/jbio.200900094

Contrast in Microscopy: Fluorescence



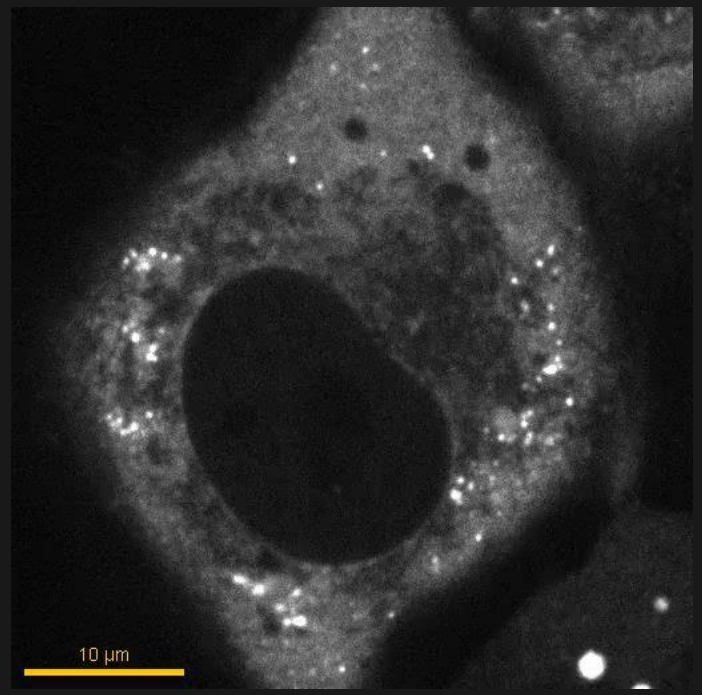
Jablonski diagram
https://chem.libretexts.org/@go/page/1769

Antibody staining

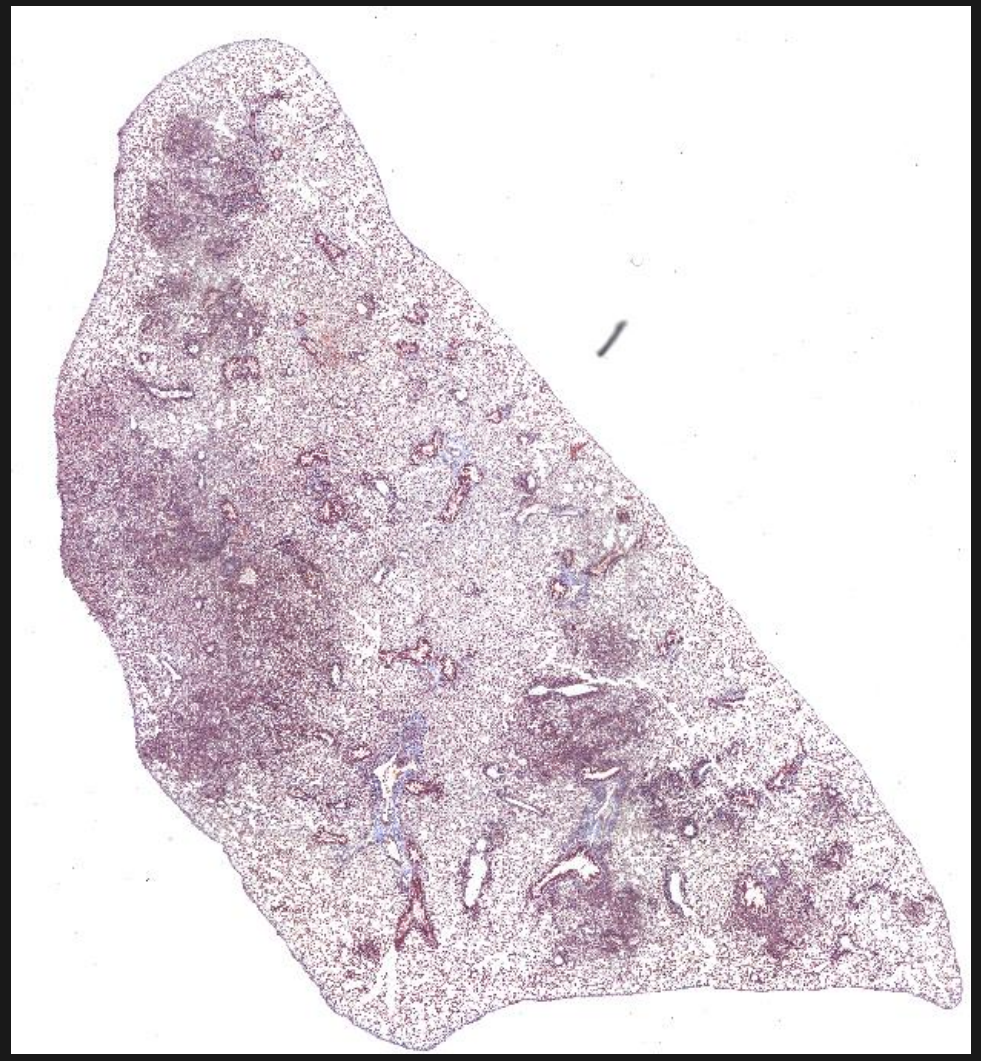


Cytoskeletal Components. (2021, January 3). University of Wisconsin-Milwaukee. https://bio.libretexts.org/@go/page/16528

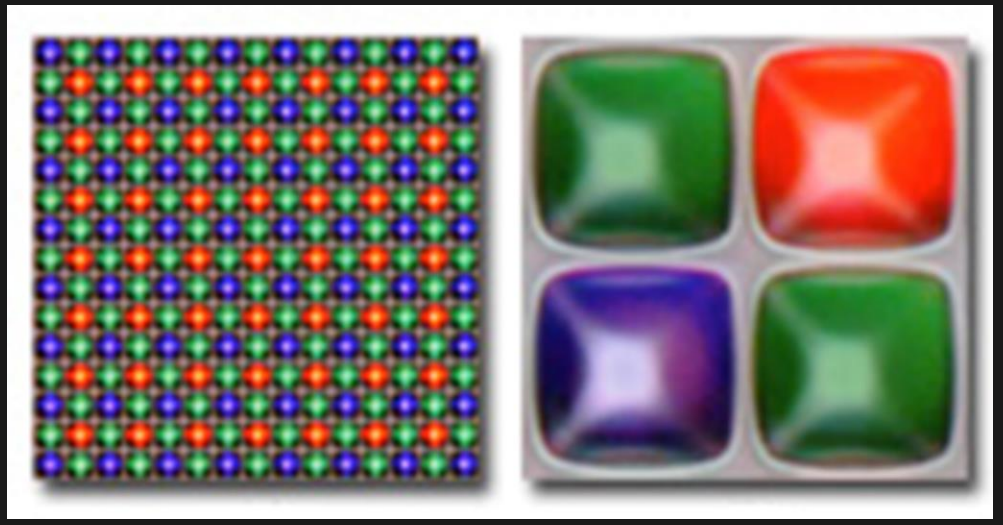
FP proteins movie



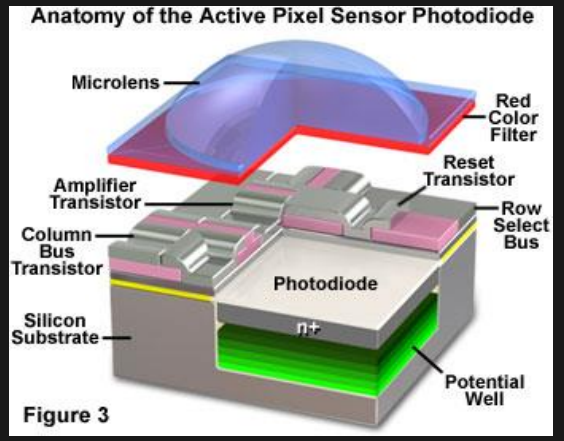
Contrast in Microscopy: Color



Weihao Zheng, PhD (Ernst Lab) taken on 6D widefield

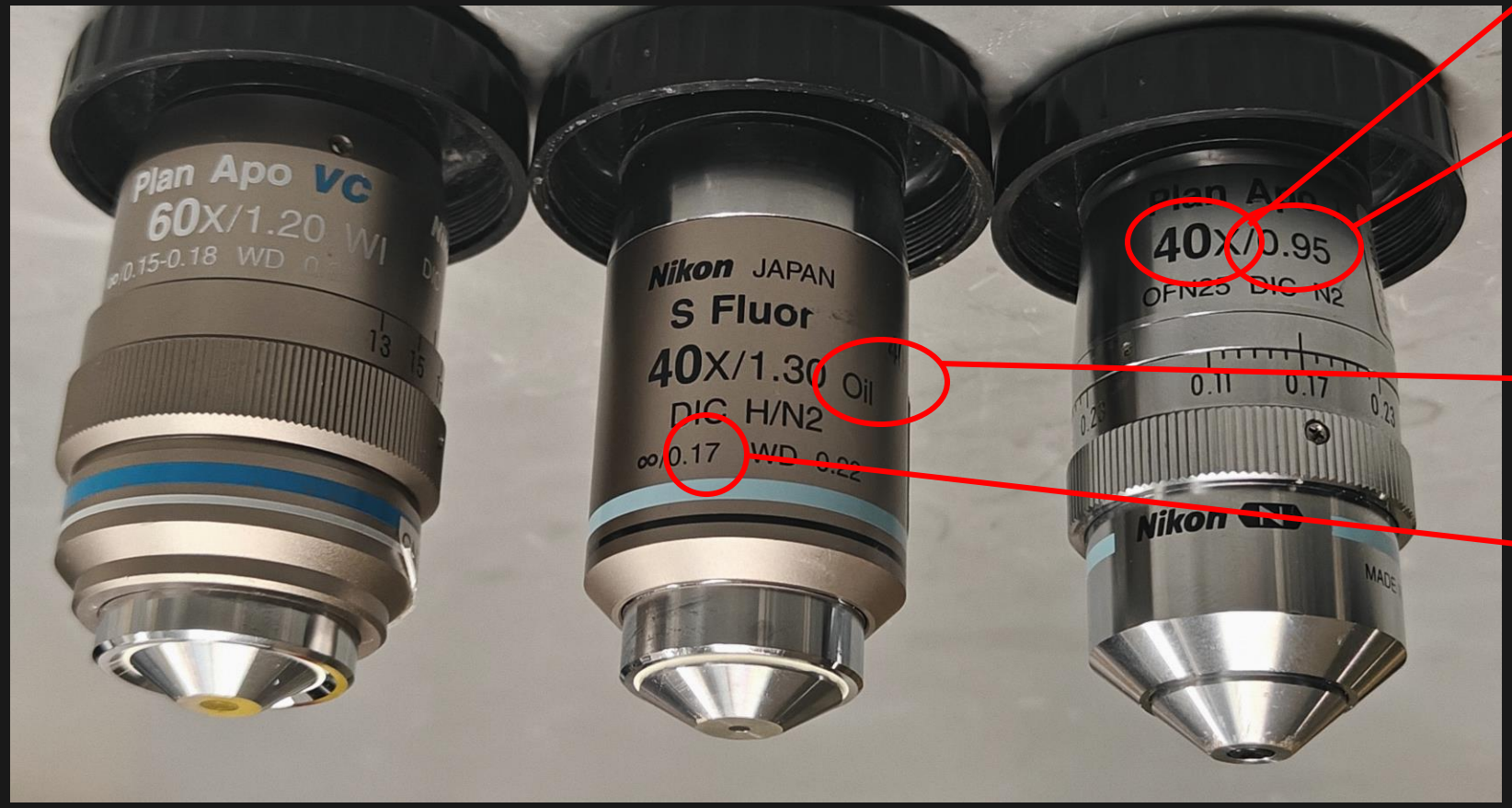


Compare to CMOS (Monochrome/ Fluorescence)



https://micro.magnet.fsu.edu/primer/digitalimaging/cmosimagesensors.html

What determines resolution?



Magnification

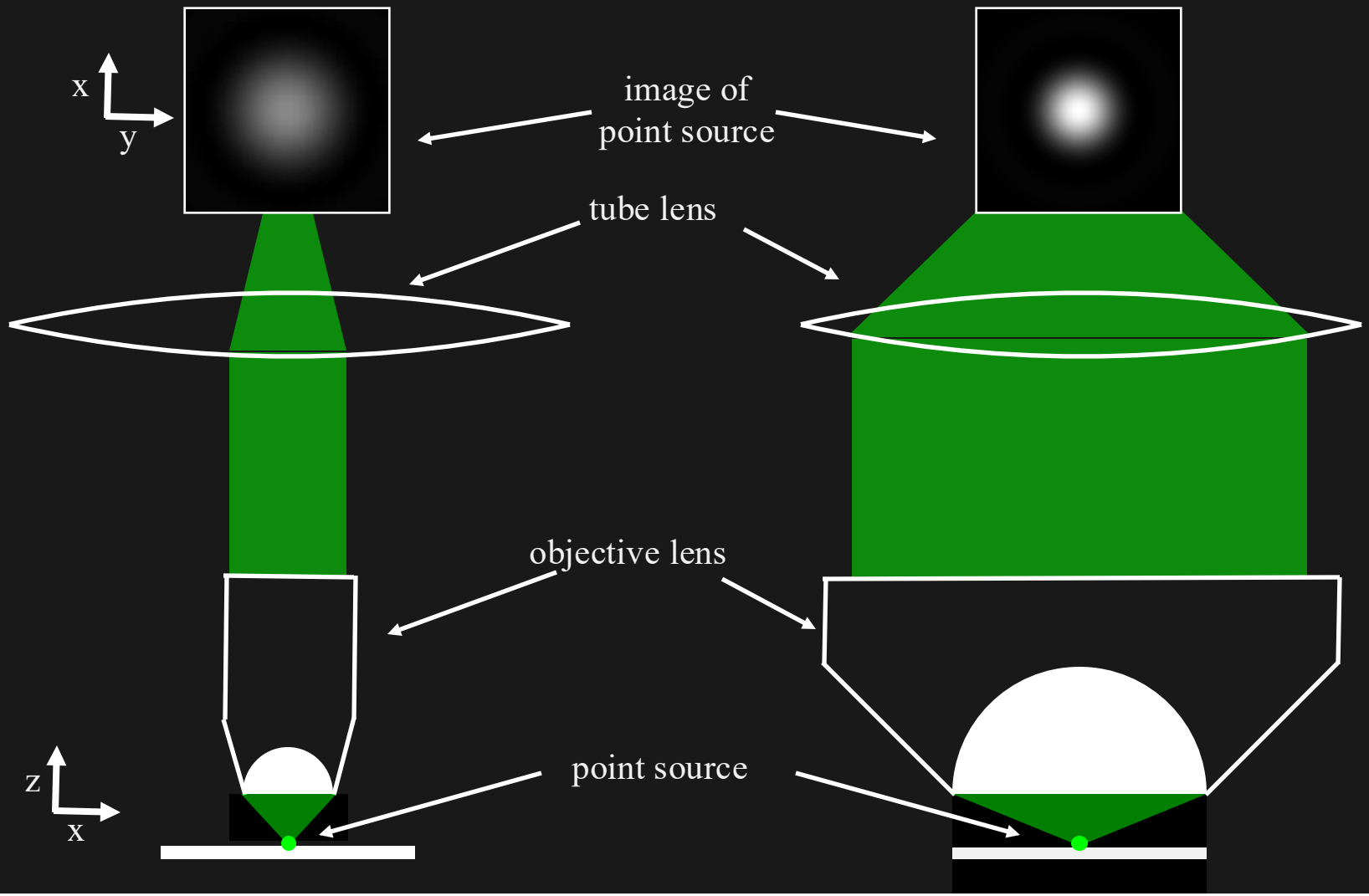
Numerical Aperture (NA):

- Light Collection Angle * Refractive Index of Immersion Medium
- Sin(α) * RI

Immersion Medium

Thickness of the coverslip (0.17mm)

Resolution: function of the objective's NA



Immersion medium limits the na

Refractive Index	
Air:	1.0
Water:	1.33
Cells:	~1.38
Silicon Oil:	1.40
Glass:	1.52
Oil:	1.51

Refractive index mismatch results in spherical aberration

Resolution ~ 0.6 * lambda / na

At 500nm max possible resolution:

Air:	300nm
Water:	225nm
Silicon Oil:	214nm
Oil:	199nm

Problem:
Beat the diffraction limit

Preventing objective lens damage

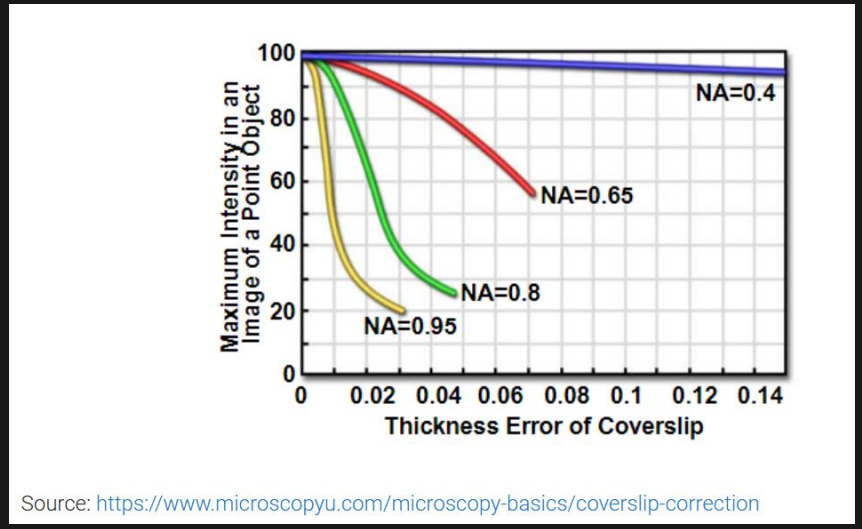
- Objective lenses are VERY expensive

- Know where the lens is in relation to the stage
  - Lower lens when switching objectives or your sample.
  - Do not use the edges of multi-well plates

- Clean off immersion media (water, oil) with lens paper and ultra clarity cleaner
  - Do not use too much oil! It will drip down the side of the lens.
  - Once you start using an oil lens, you CANNOT switch to an air or water lens unless you clean off the oil from the objective AND from your sample.

Your sample is part of the microscope!

- Objectives are designed for specific samples
- Most high na (high resolution) objectives require 0.17mm glass
- 1 mm plastic can only be used with low magnification objectives



Source: https://www.microscopyu.com/microscopy-basics/coverslip-correction

Plates and dishes for imaging

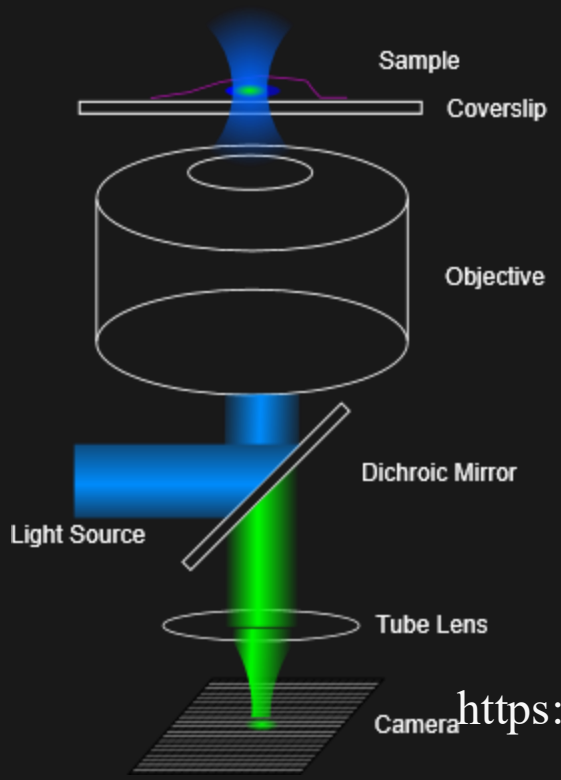
- Coverslips
  - Use 170micron (0.17mm) glass coverslips (~#1.5)
- 35mm Petri dishes
  - Mat-tek, Cellvis, Greiner bio-one, ibidi (great for finicky cells), Wilco Wells
- Chambered cover glasses/slides
  - Lab-Tek, ibidi, Cellvis, Greiner bio-one
  - (note: Mat-tek chambered slides have1 mm thick glass bottoms, so do not use for live imaging).

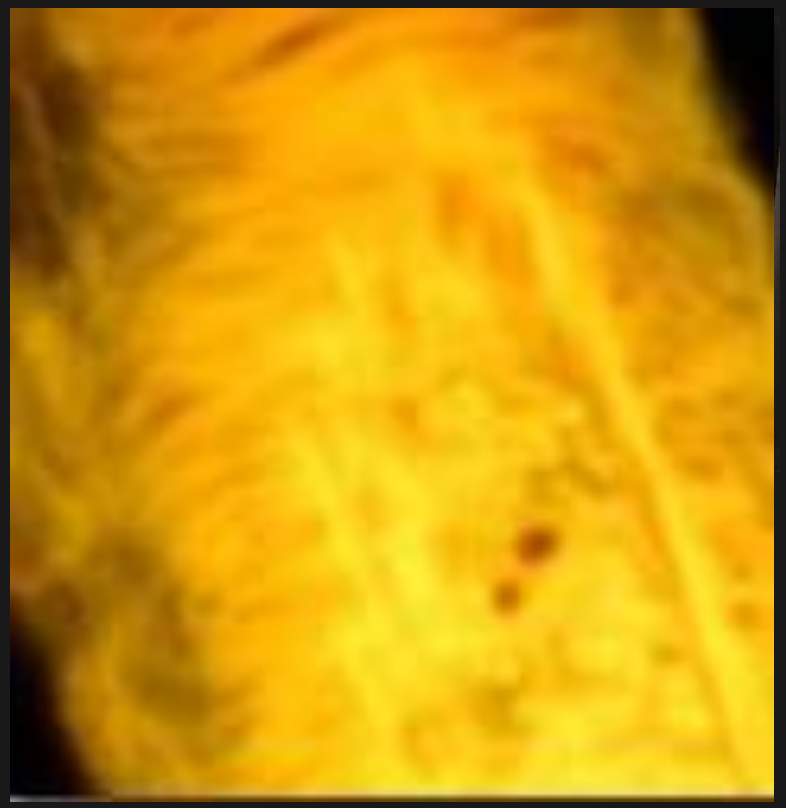
Fluorescence-
Choose the right dyes for your imaging

- Use https://www.fpbase.org/
- Choose non-overlapping fluorescent proteins and dyes that are bright and photo-stable
- Match the filters of your microscope

Talley Lambert

Epi-Fluorescence (widefield)



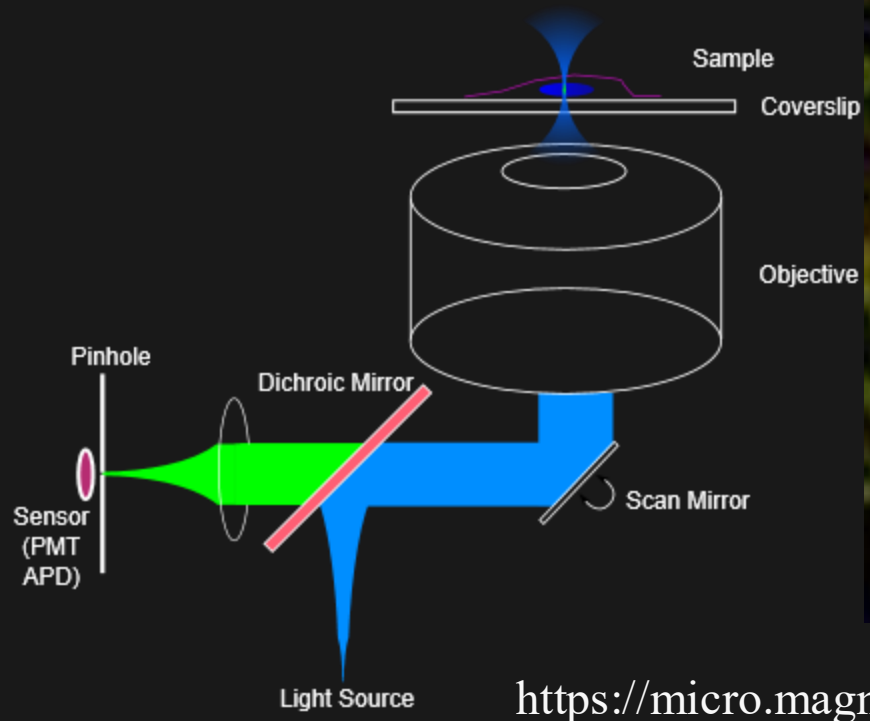


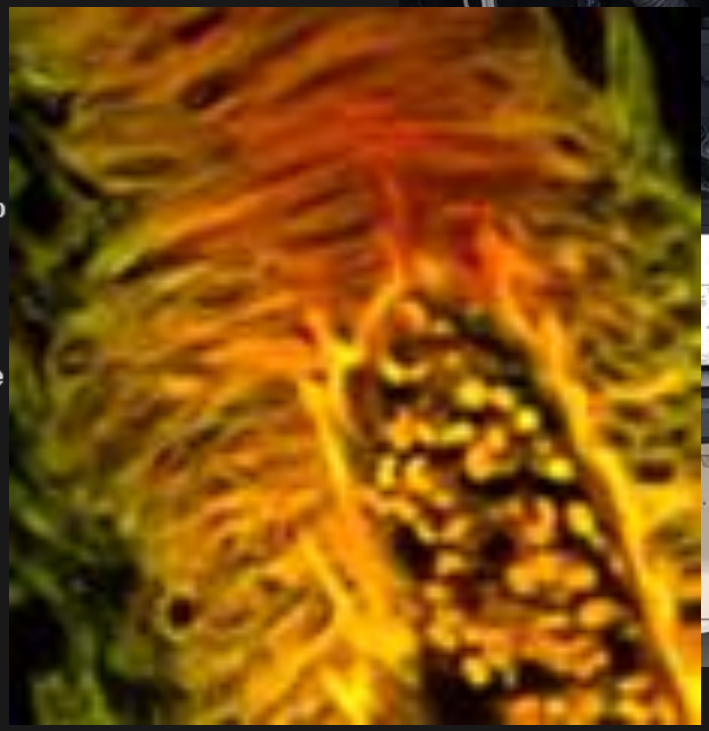
https://micro.magnet.fsu.edu/primer/techniques/confocal/confocalintroduction.html

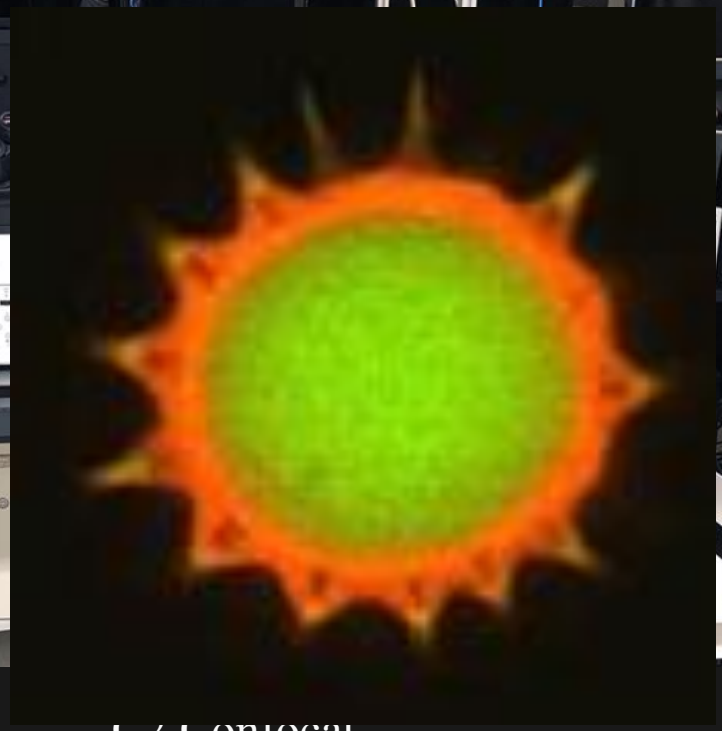
Problem:
Out of Focus Fluorescence

QLIPP TimeLapse

Confocal Microscopy







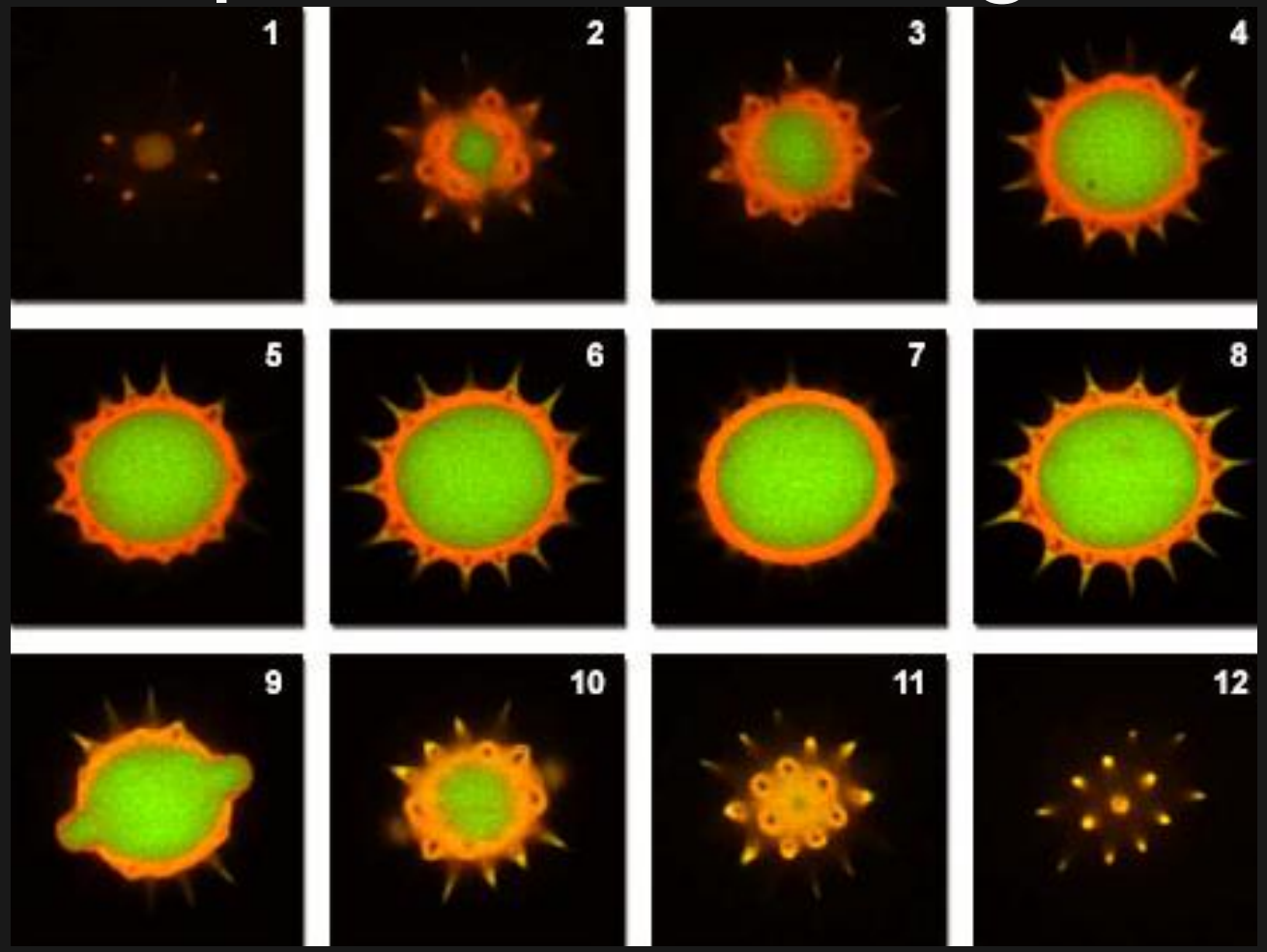
C2 Confocal

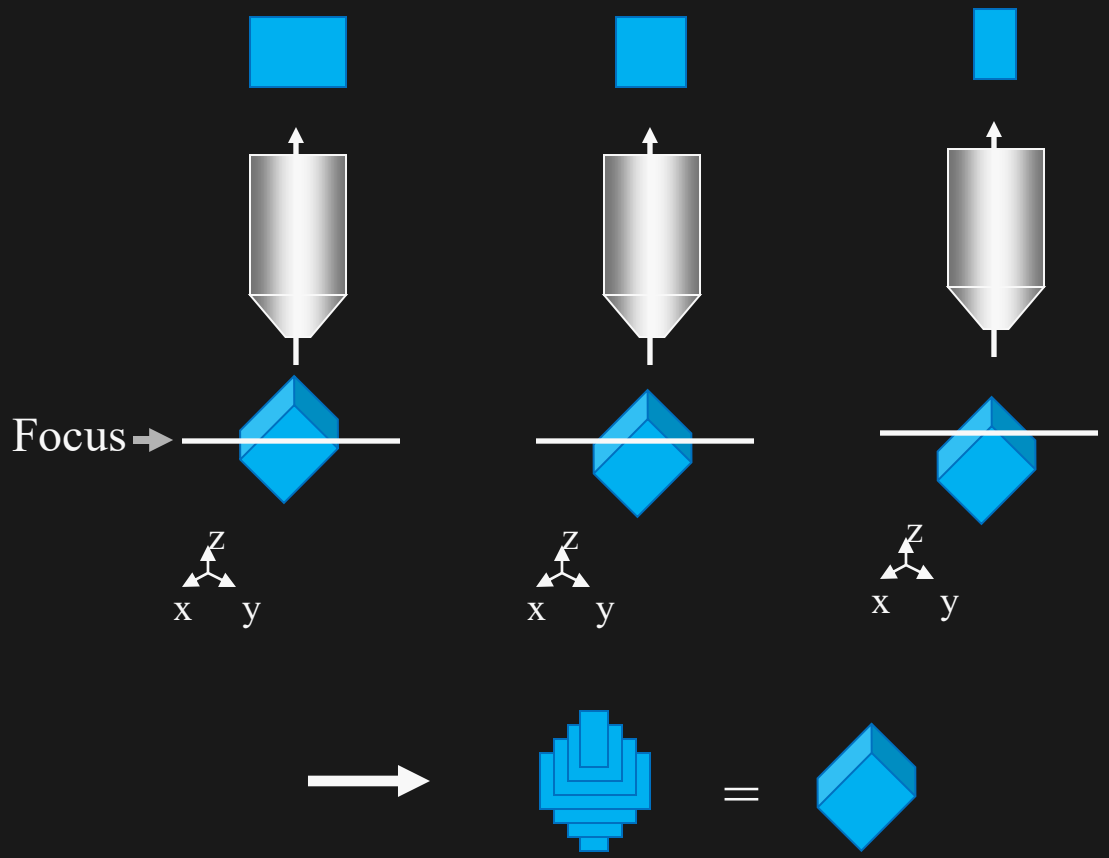
https://micro.magnet.fsu.edu/primer/techniques/confocal/confocalintroduction.html

Problem:

Slow, photobleaching

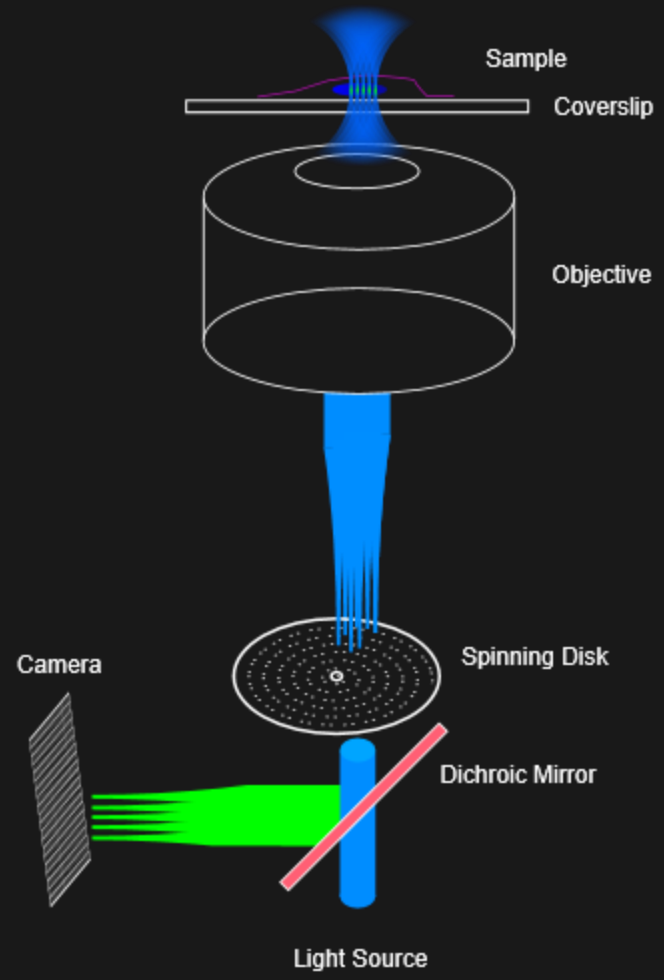
Optical Sectioning enables 3D imaging



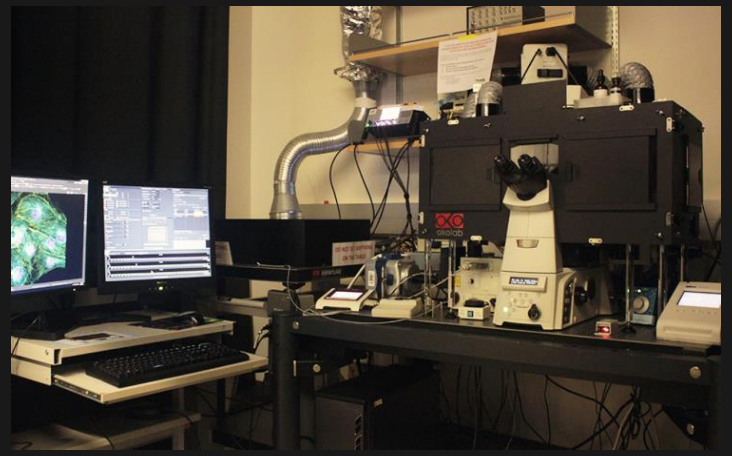


https://micro.magnet.fsu.edu/primer/techniques/confocal/confocalintroduction.html

Spinning Disk Confocal



Crest V2 Confocal

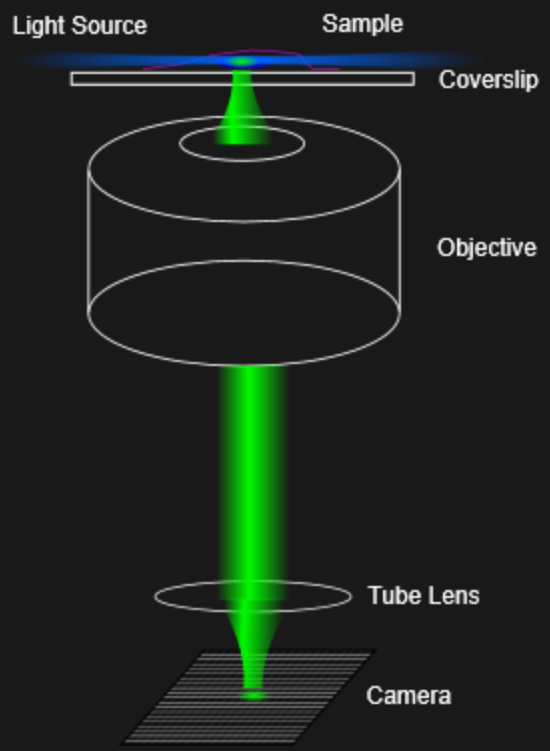


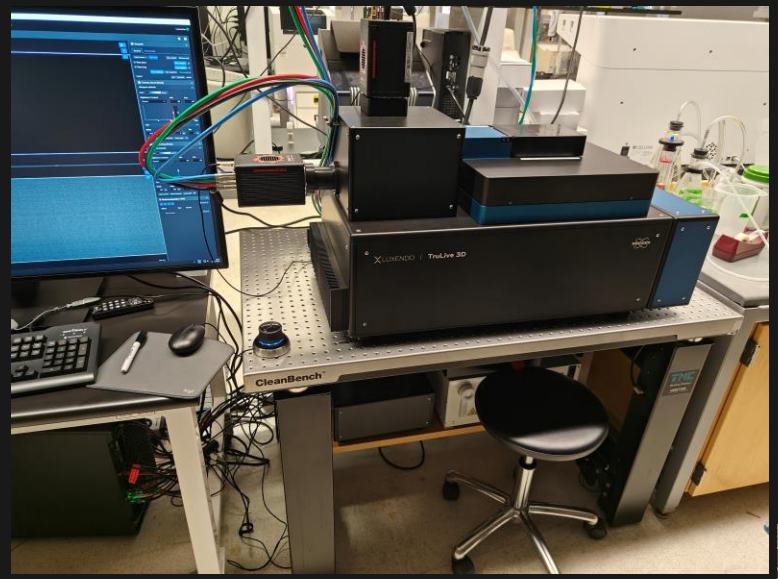
CSU22

Problem:
Photobleaching

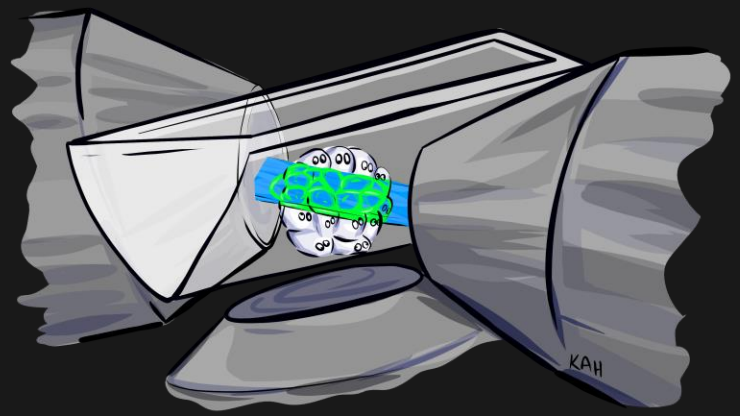
CSU-W1

LightSheet



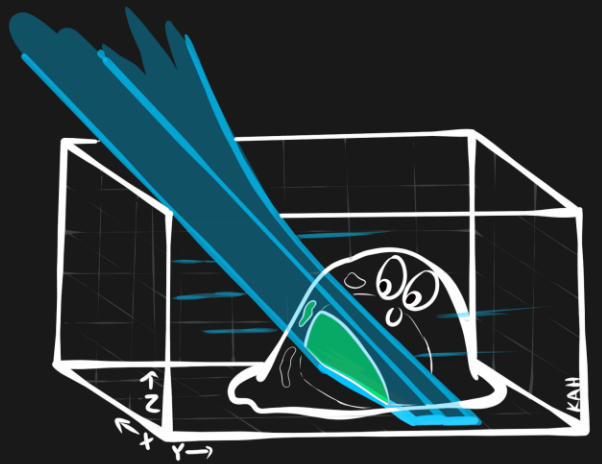


TrueLive

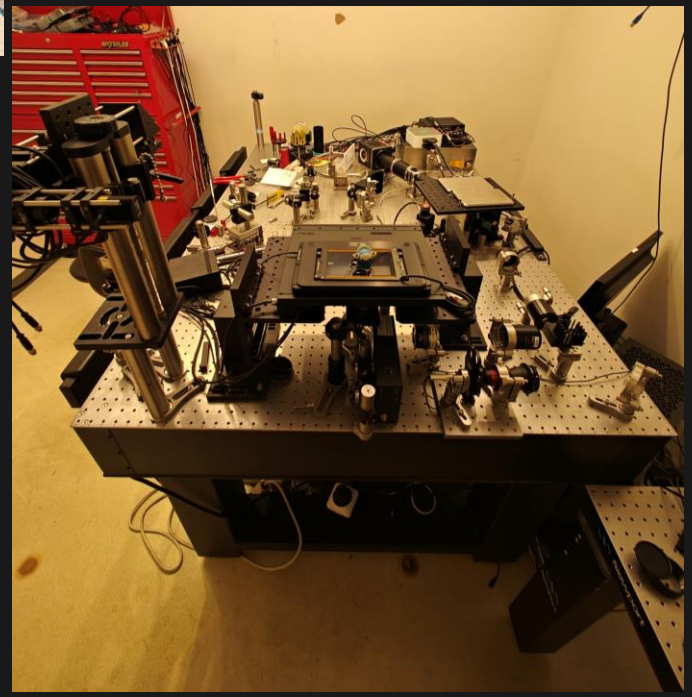


BS/Cara:@kahsage, IG:kah_sage

Contrast

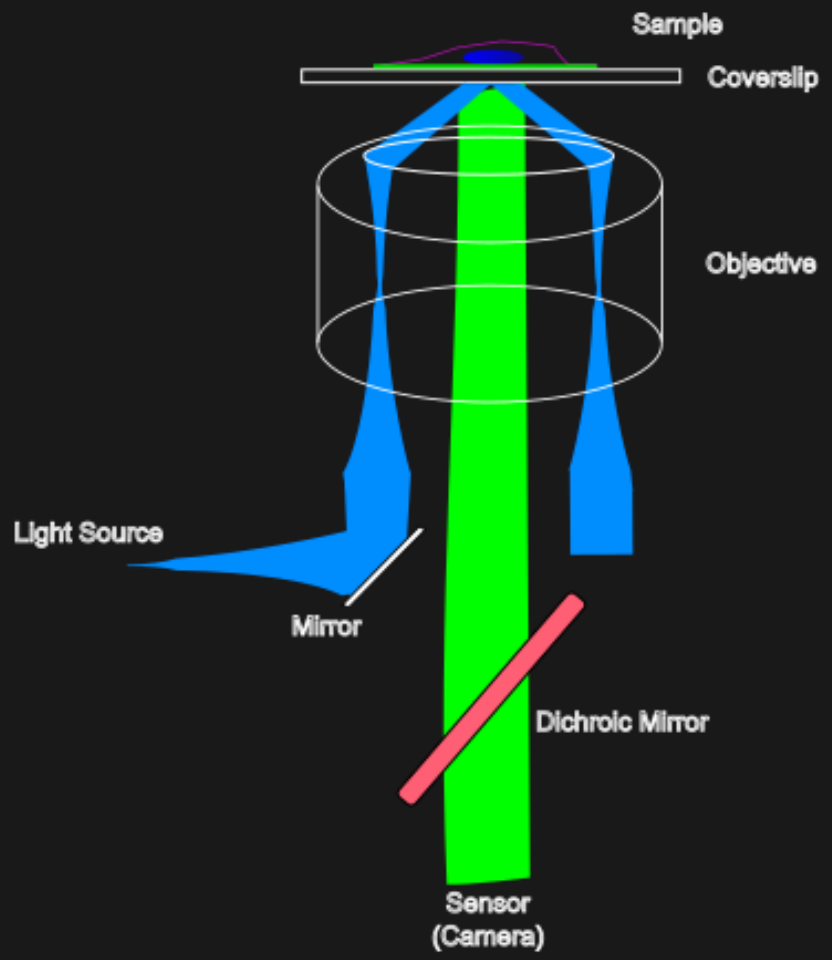


BS/Cara:@kahsage, IG:kah_sage

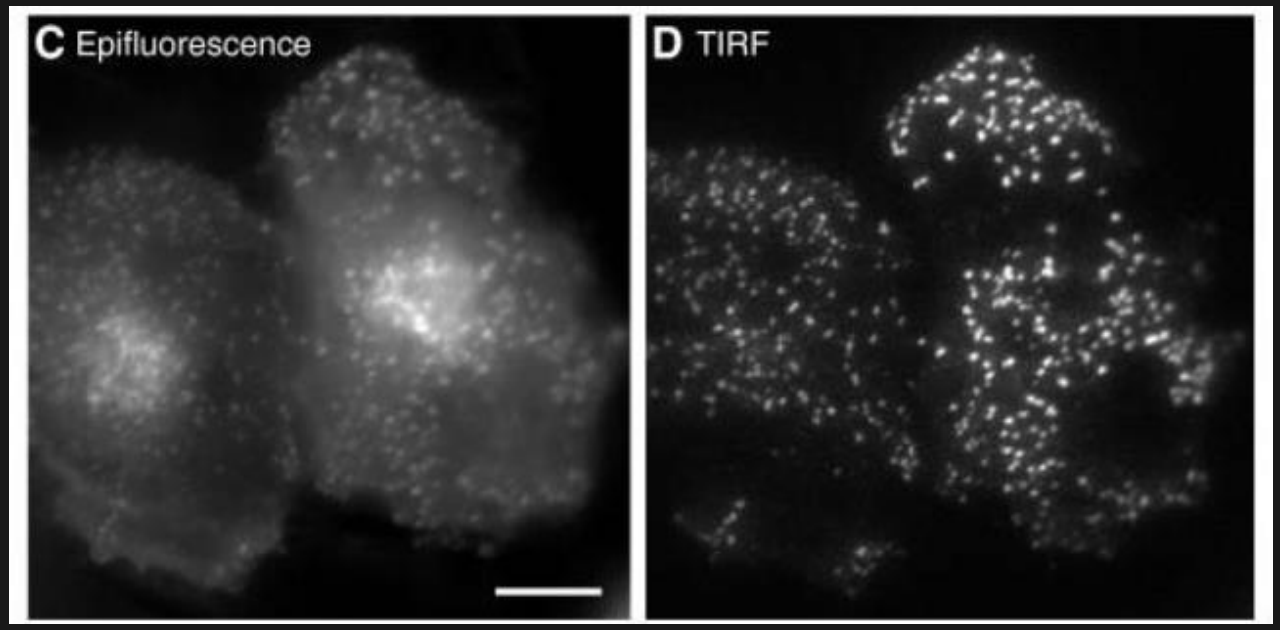


Snoutscope (SOLM)

TIRF



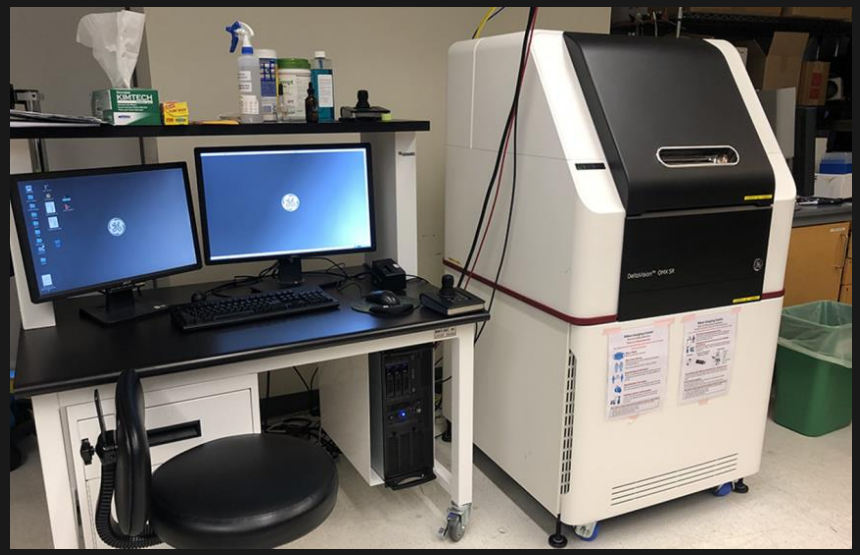
TIRF



doi: 10.1242/jcs.056218 Clathrin (clathrin light chain–GFP) in a HeLa cell

SuperResolution

Structured Illumination



OMX SR

STORM

Which microscope to use?

We will help you choose and set you up with the best microscope!

Contrast: Fluorescence versus label free

How thick is the specimen?

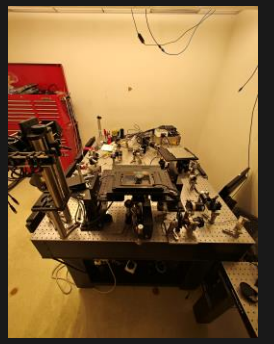
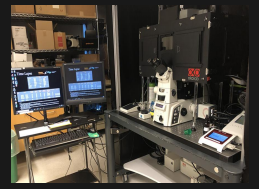
Your Question
Your Sample

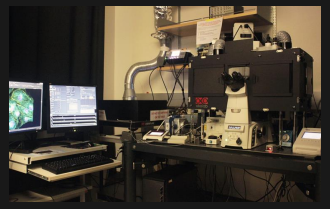
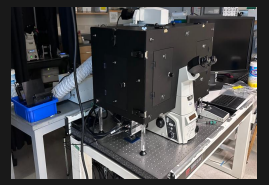
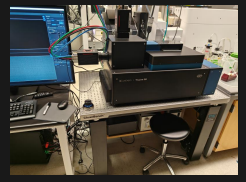
Magnification/Resolution

Speed

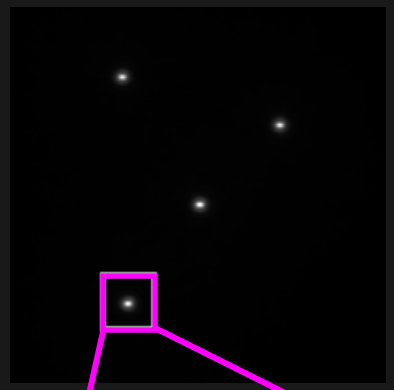
Live or fixed

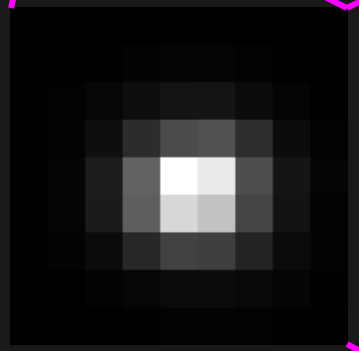


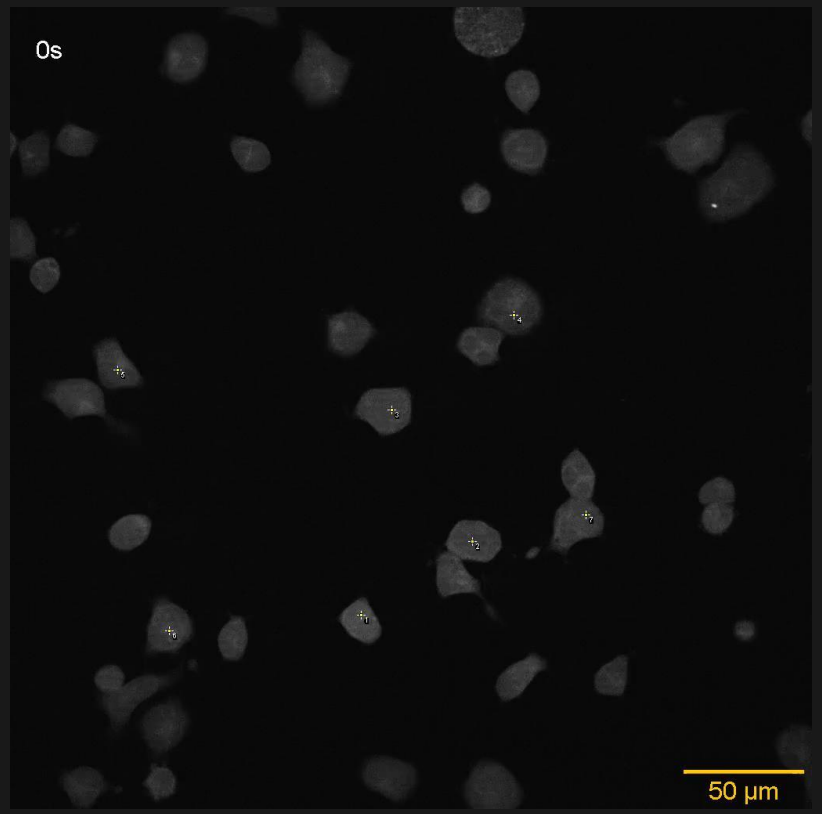
Digital images are many measurements of light intensity

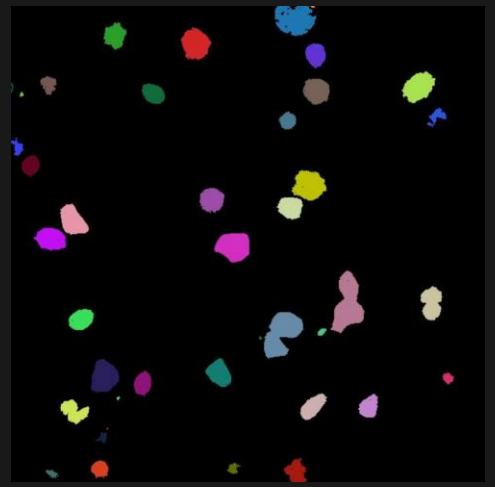




0	0	0	0	0	0	0	0	0
0	0	1	3	5	4	2	0	0
0	2	6	13	20	20	11	4	0
0	3	14	44	75	81	45	12	2
0	5	28	98	255	234	78	20	4
0	4	27	94	215	194	68	18	2
0	3	11	39	66	63	35	11	3
0	0	2	6	11	12	8	5	1
0	0	0	1	2	3	2	0	0

General workflow for image analysis



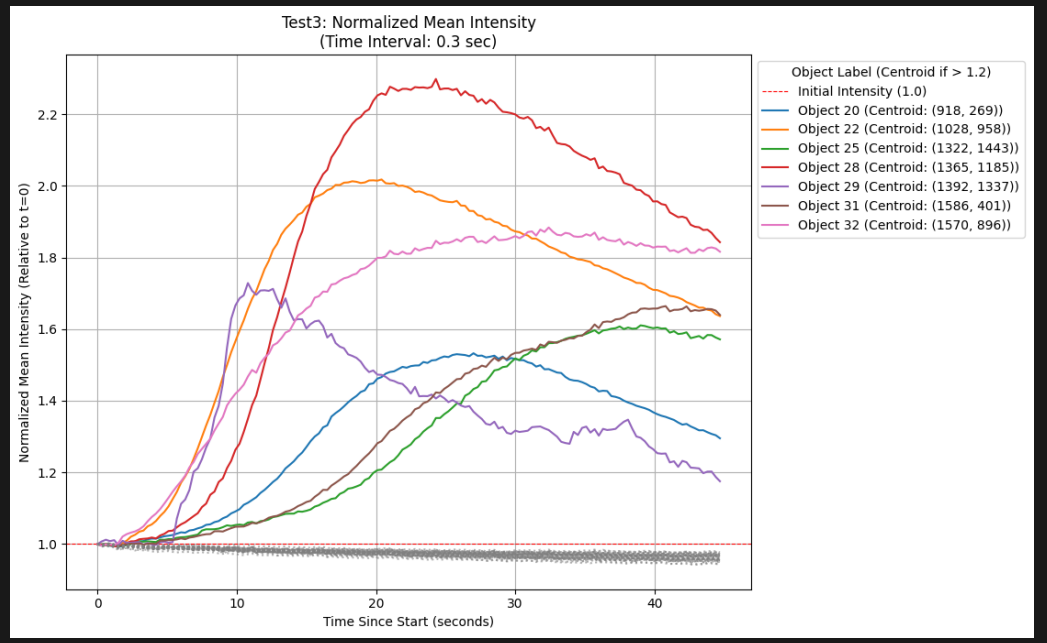


“Classical”

“AI” (Cellpose)

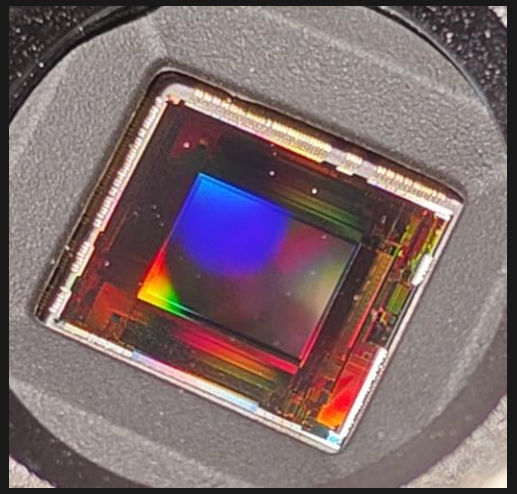
Number of cells: 126
Avg size (um^2): 269.9

Normalized intensity under each mask



Light Detectors that generate digital images

Cameras

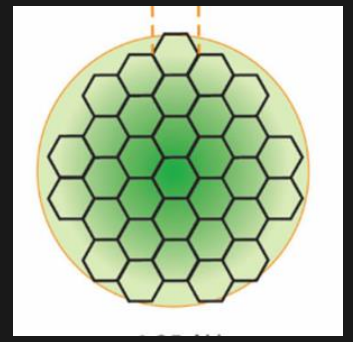


Dynamic range 10,000s

Parallel > Fast

Point detectors (PMTs, APDs)

PMT



doi:10.1007/978-1-0716-1402-0_5

Zeiss AiryScan detector

(schematic

Dynamic range 10s

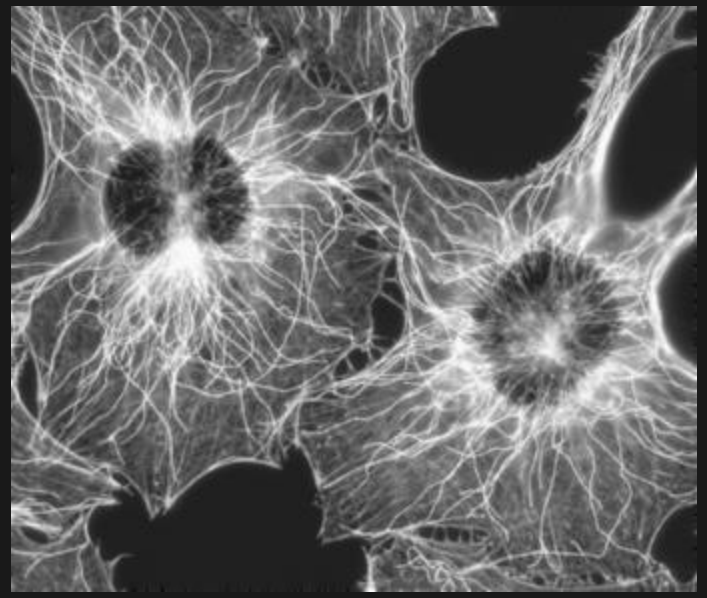
Serial > Slow

1 k x 1k, 1 second > 1 µs / voxel

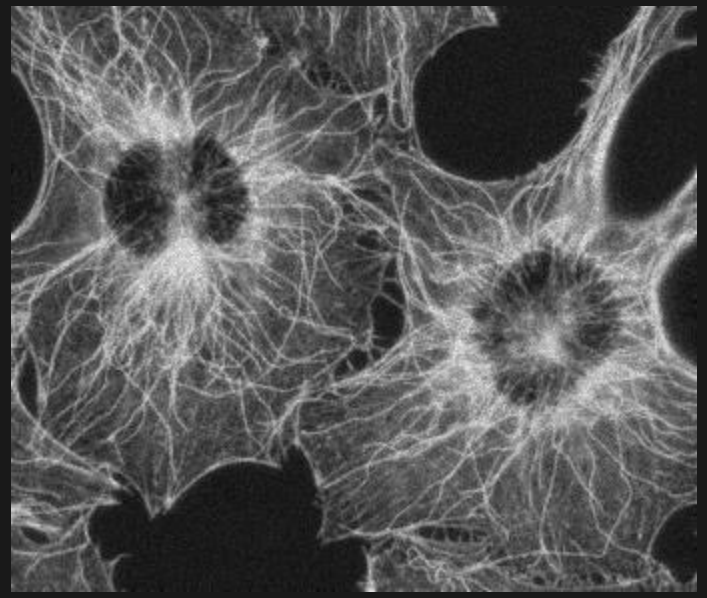
For best measurement: High Signal / Noise, How?

Problem 1: Detection and System noise: constant

Problem 2: Photons do not arrive at a constant rate



Shot noise = √(Signal)



https://helios2.mi.parisdescartes.fr/~rabergel/tvdenoise-poissonian.html

Shot noise
Poisson distribution

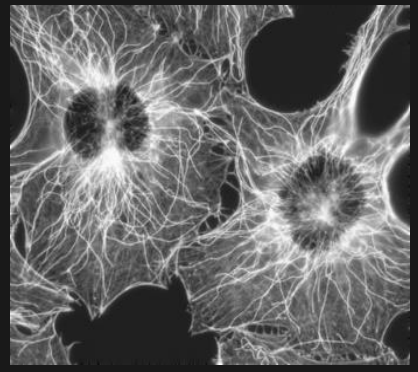
For best measurement: High Signal / Noise, How?

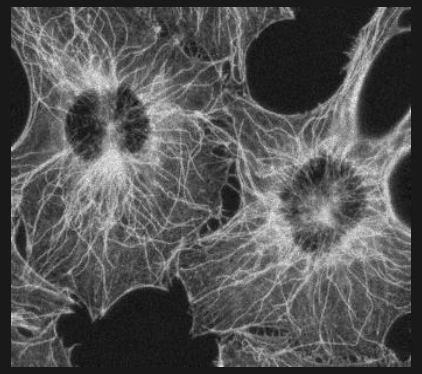
$$S / N = S / \sqrt{S} + \text{constant}$$

When constant is low (for us, most often):

S / N ratio scales with √S

So, 4x higher signal > 2x higher S/N





https://helios2.mi.parisdescartes.fr/~rabergel/tvdenoise-poissonian.html

Maximize Signal, how?

Photons > Digital Numbers

Photon Conversion Factor

Digital Number Ranges:

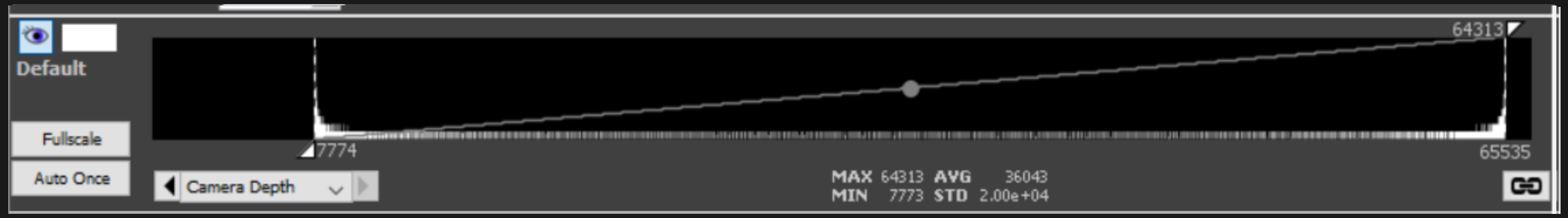
8-bit : 0-255

12-bit : 0-4,095

16-bit : 0-65,535

Offset: ~100

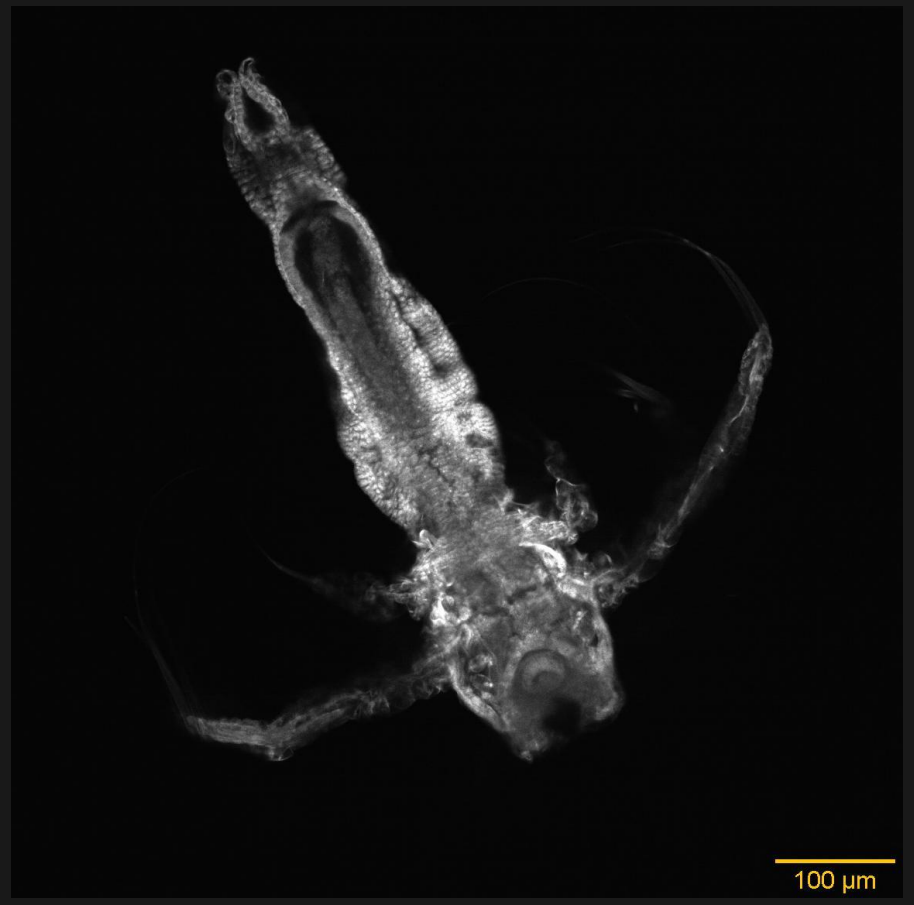
Cameras are linear to ~90%



Maximize Signal, how?

Computer display dynamic range: < 8-bit

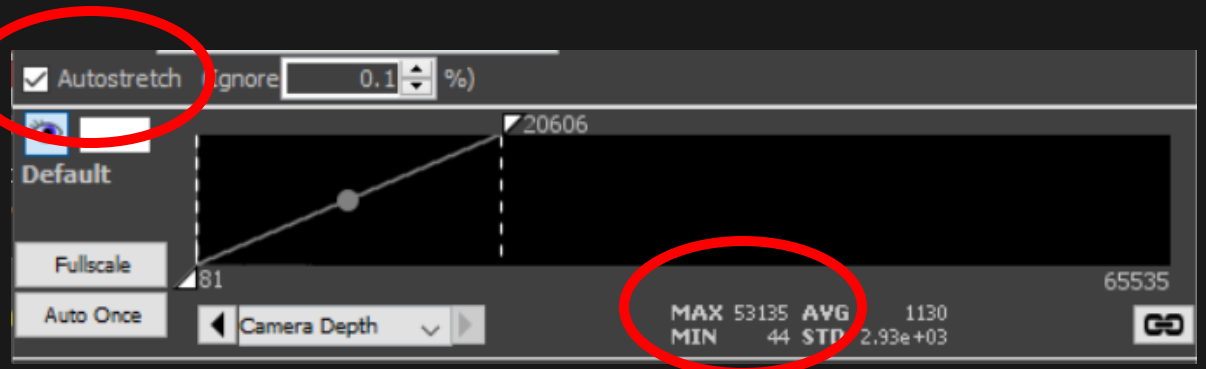
Don’t trust what you see:

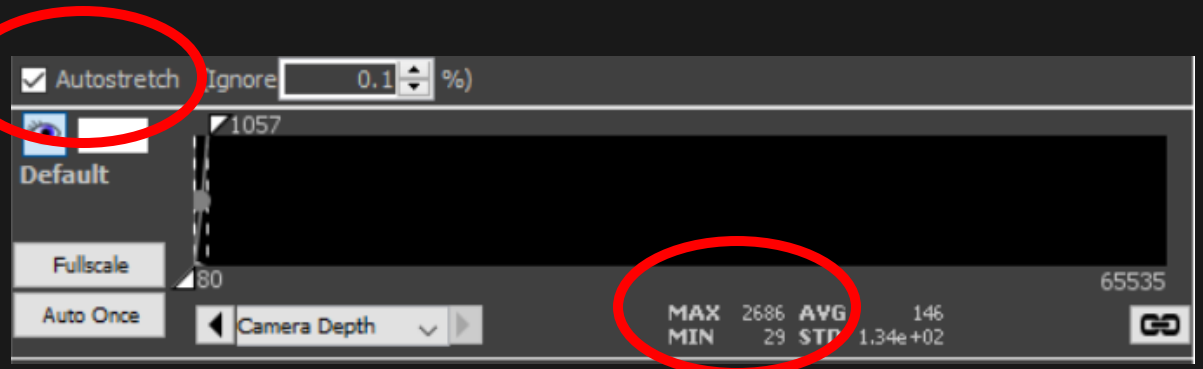


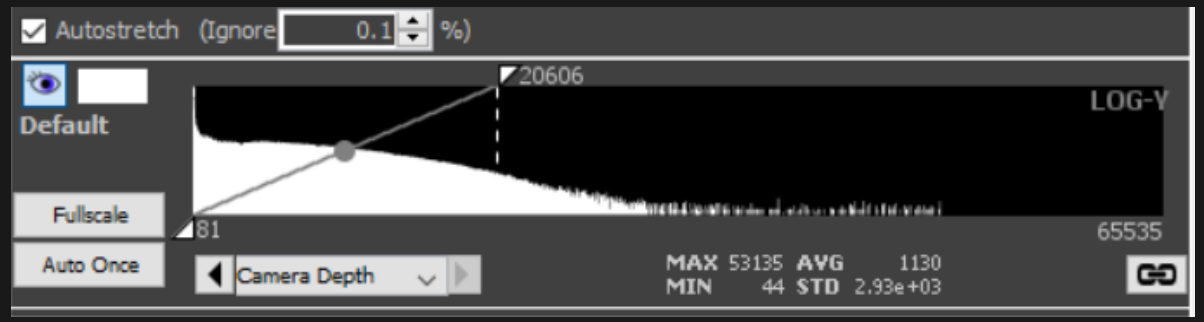
Maximize Signal, how?

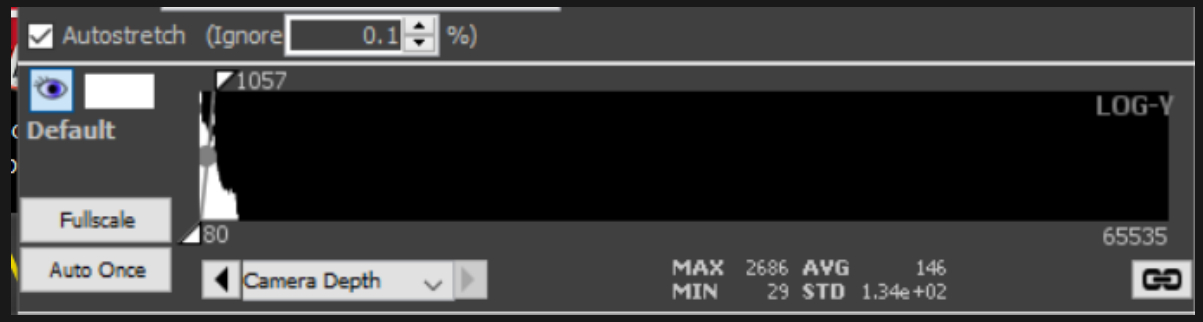
Computer display dynamic range: < 8-bit

Trust Histograms and Pixel Values:



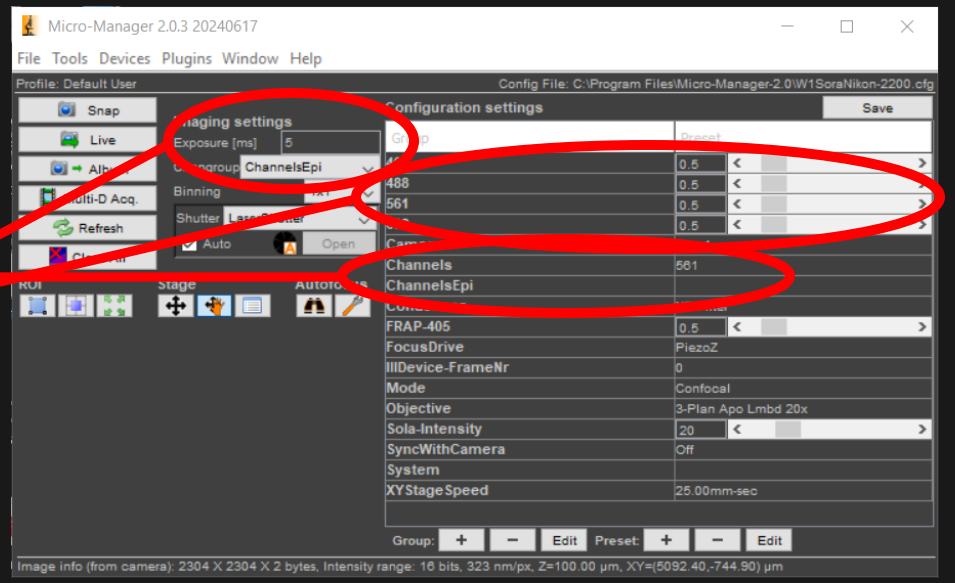






Maximize Signal, how?

Cameras:
- Choose the best illumination settings
- Increase illumination power
- Increase exposure time

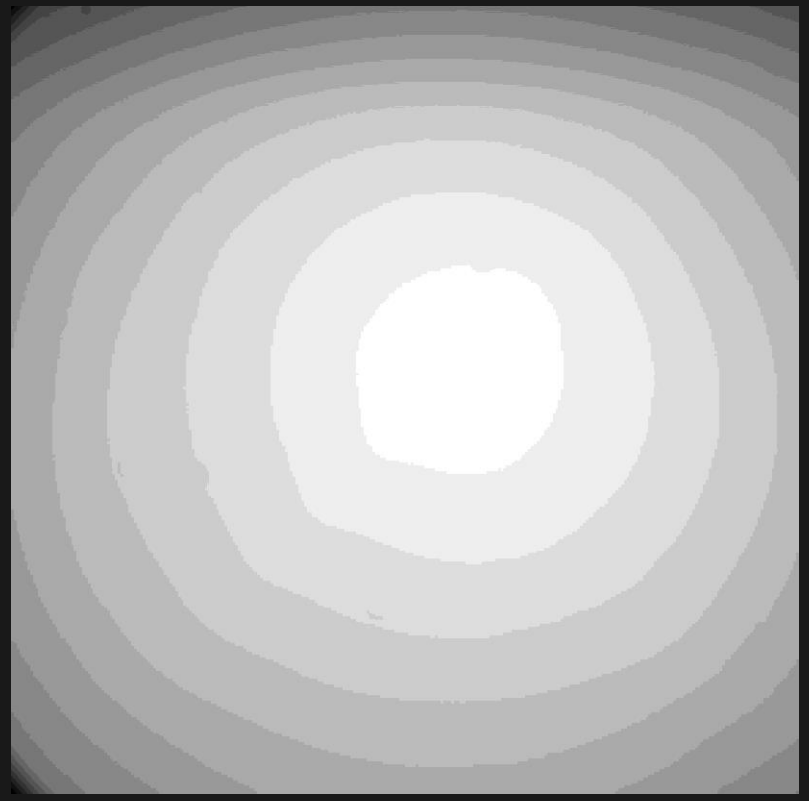


Point Detectors:
- Choose the best illumination settings
- Increase illumination power
- Slow scan speed (increase dwell time)
- Average

Quantifying images: Flatfield correction

Correct for uneven illumination and detection

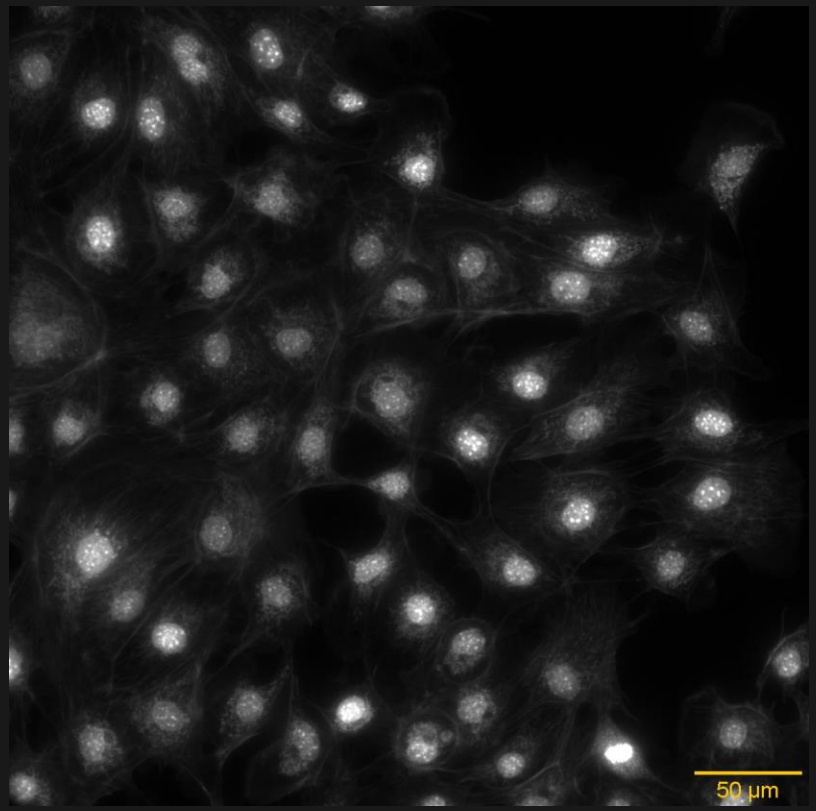
1. Take “Perfect” image of homogeneous sample: “Flatfield” image



Quantifying images: Flatfield correction

Correct for uneven illumination and detection

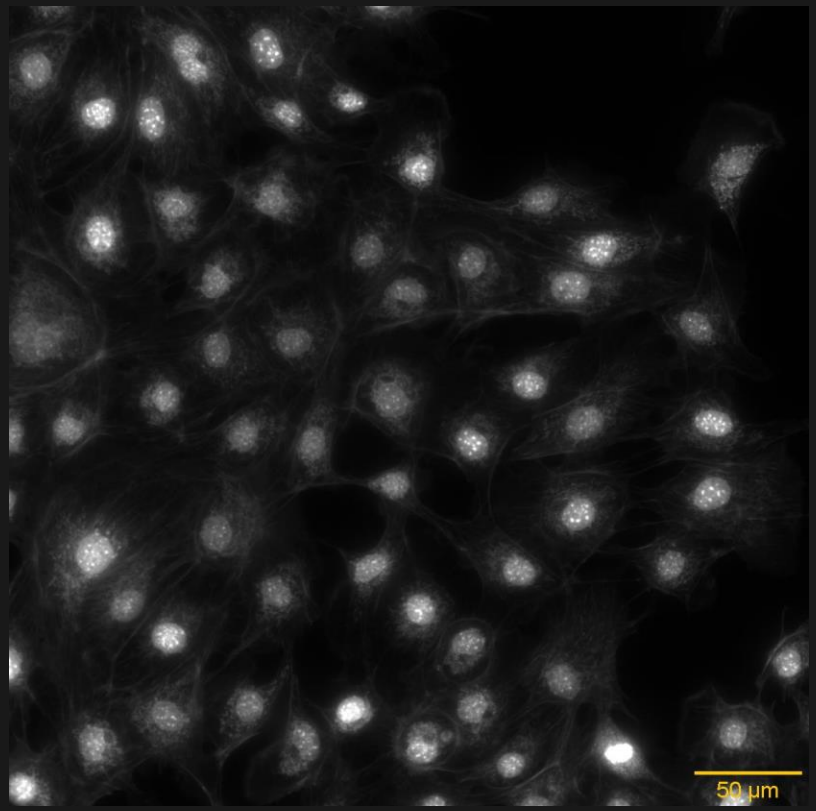
2. Image = Object * Flatfield + Background



Quantifying images: Flatfield correction

Correct for uneven illumination and detection

3. Object = (Image – Background) / Flatfield



Saving images: Preserve your pixel values

Usually good: “Native” format (.czi, .nd2, .ome.tiff)
16-bit Tiff

For presentation only: .jpg
.gif
.avi
.mp4
.png

“Lossless”
Versus
“Lossy”

When comparing images:

- Ensure all display settings (brightness/contrast, min/max, gamma) are the same
- Declare non-linear settings (gamma)

CALM Wiki

More resources:

Microscopy:

https://www.ibiology.org/online-biology-courses/microscopy-series/ (iBiology)

Bio-Image analysis:

https://www.ibiology.org/online-biology-courses/bioimage-analysis-course/ (iBiology)

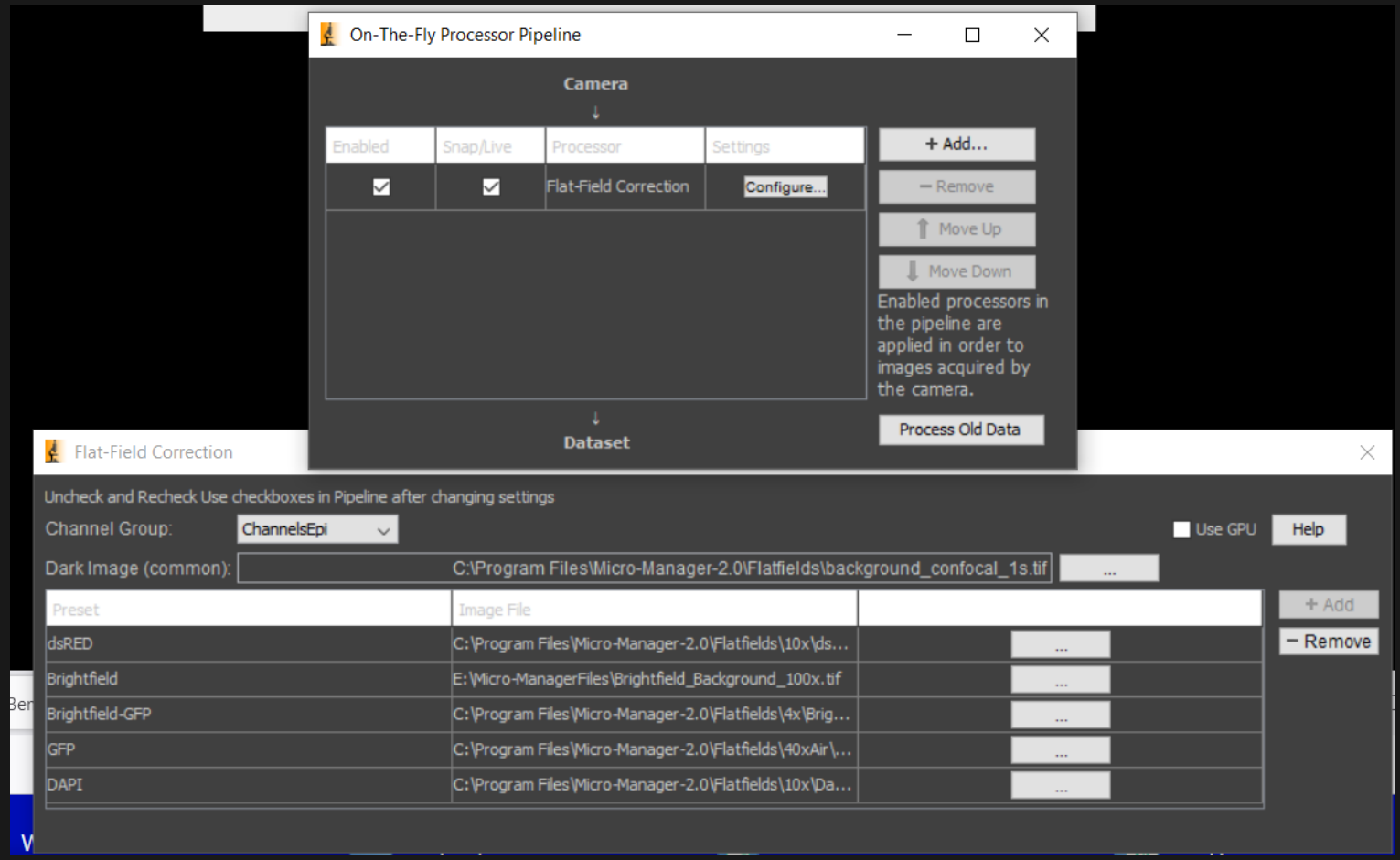
https://www.youtube.com/watch?v=QDS5t7oZH-c (Napari / Python)

https://www.youtube.com/playlist?list=PL5ESQNfM5lc7SAMstEu082ivW4BDMvd0U (Fiji)

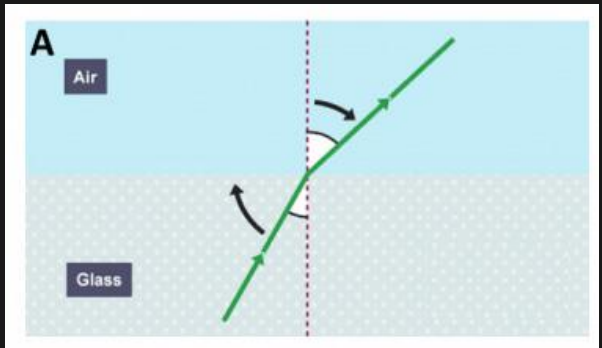
https://bioimagebook.github.io/ (General introduction, ebook)

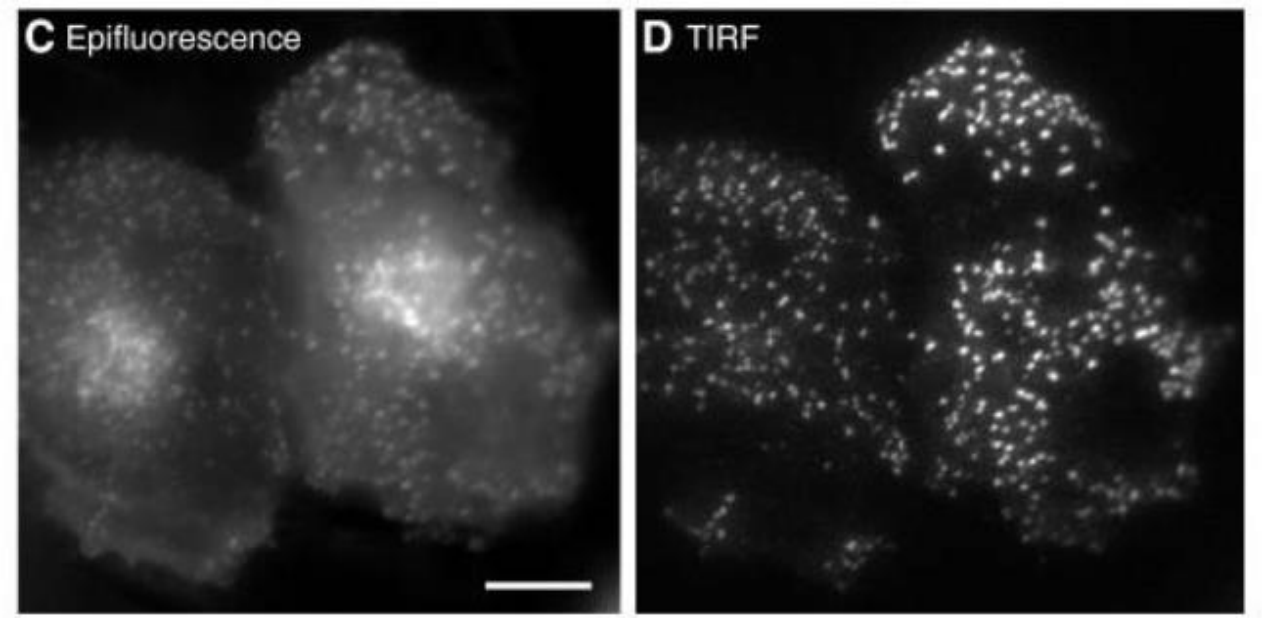
Quantifying images: Flatfield correction

Correct for uneven illumination and detection



Total Internal Reflection (TIRF)





doi: 10.1242/jcs.056218 Clathrin (clathrin light chain–GFP) in a HeLa cell

https://www.teledynevisionsolutions.com/learn/learning-center/scientific-imaging/what-is-tirf-microscopy/https://www.teledynevisionsolutions.com/learn/learning-center/scientific-imaging/what-is-tirf-microscopy/
http://www.pages.drexel.edu/~bio/cores/cic/tirf-microscopy.html
http://www.bbc.co.uk/bitesize/guides/zctmh39/revision/1