Introduction to Imaging

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What can we do with imaging?

- Follow detailed cellular or tissue architecture
- Measure abundance
- Measure dynamics
- Spatial distributions
- Constrain/validate models

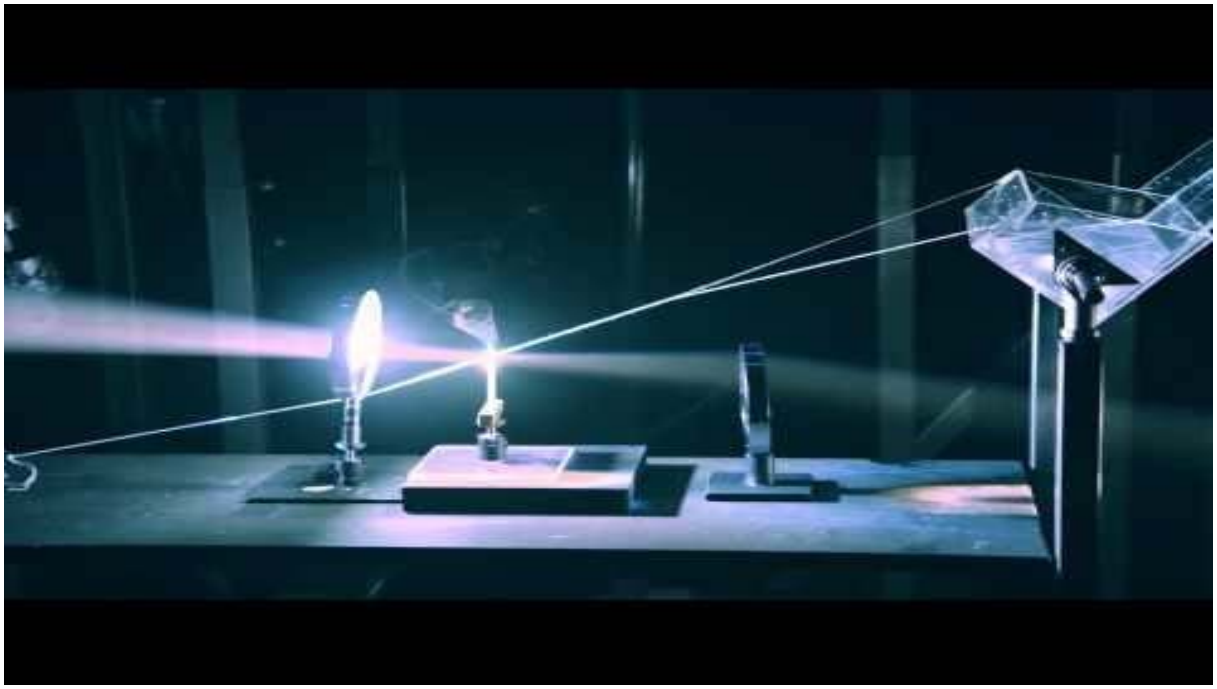
Major Imaging Functions of the Microscope

- Magnify
- Resolve features
- Generate Contrast
- Capture and Display Images

Everything comes back to the basics

Physics of light

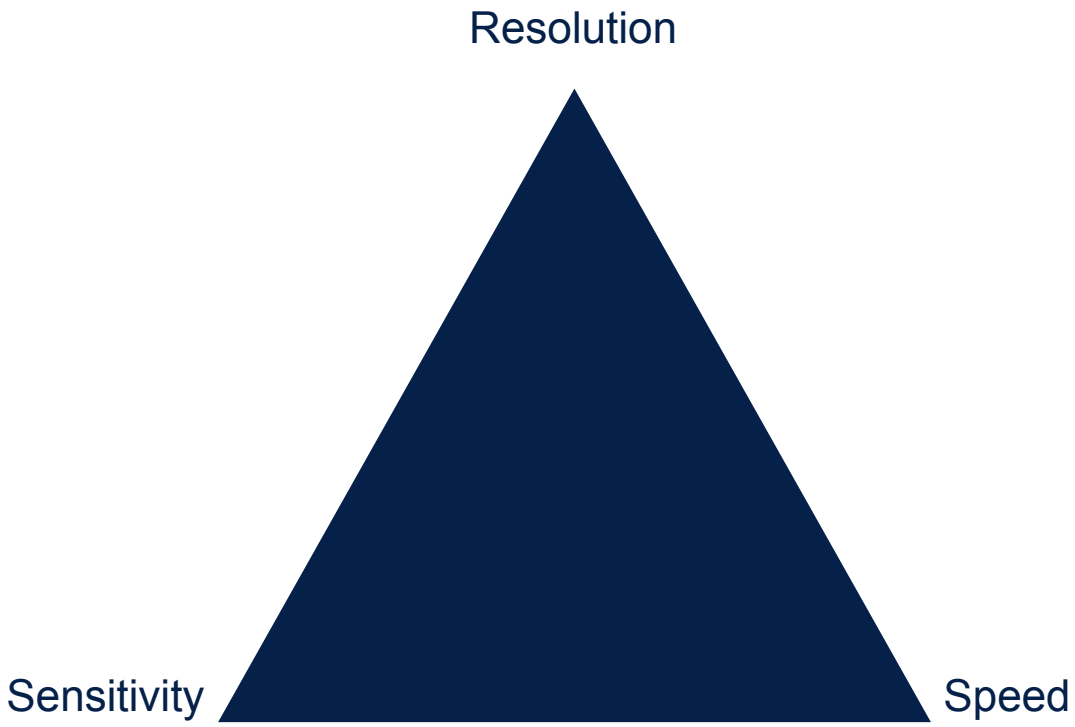
- Refraction
- Diffraction
- Reflection
- Scattering
- Absorption



We can't change the laws of physics

Imaging Trade-offs

- Resolution
- Speed
  - Temporal resolution
- Sensitivity
  - Signal to Noise Ratio
  - Depth



The objective lens controls the microscope properties

Key parameters

- Numerical Aperture
- Working Distance
- Corrections
- Magnification

Resolution

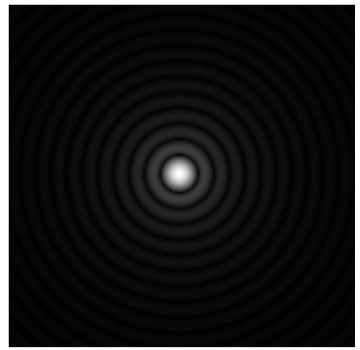
The shortest distance between two points on a specimen that can still be distinguished

Magnification ≠ Resolution

Resolution of the Microscope

limited by the point-spread function

- Microscope objective collects a limited cone of light from the sample
- This limits the resolution achievable by the microscope
- Resolution can be measured by the blurring of a point object → the point-spread function



Numerical Aperture

NA = n sin α

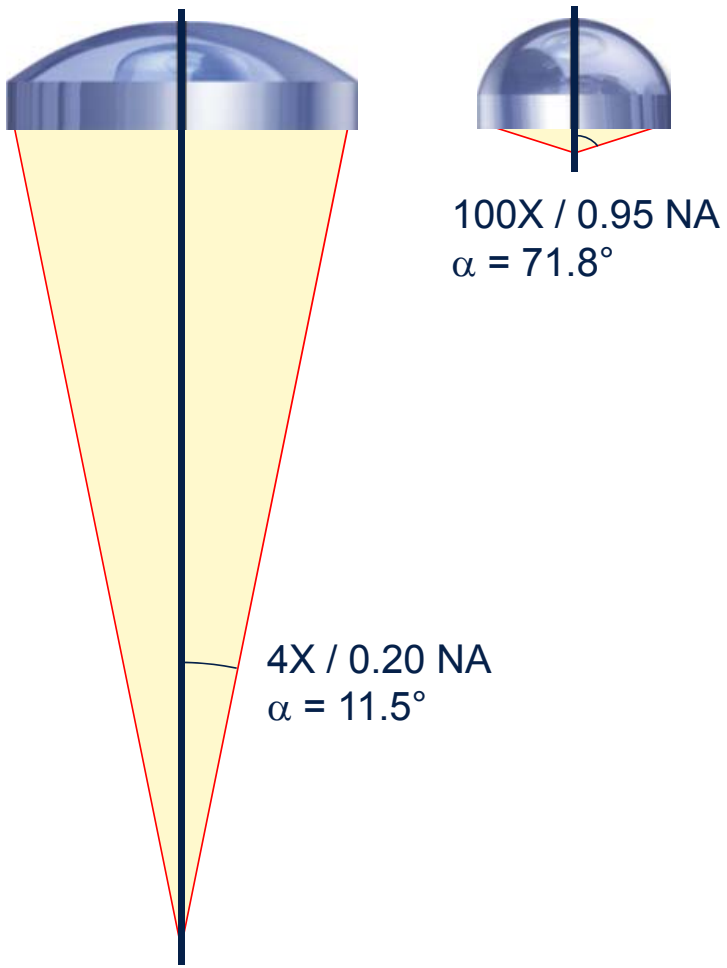
The objective NA controls:

- Light gathering power

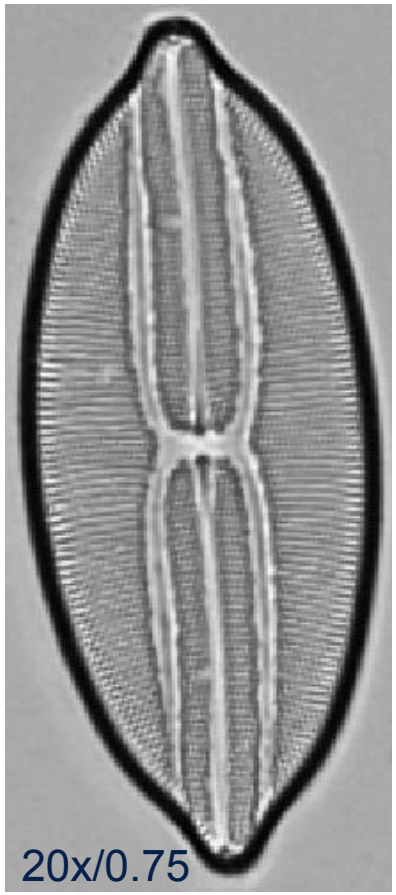
  $\propto NA^2$

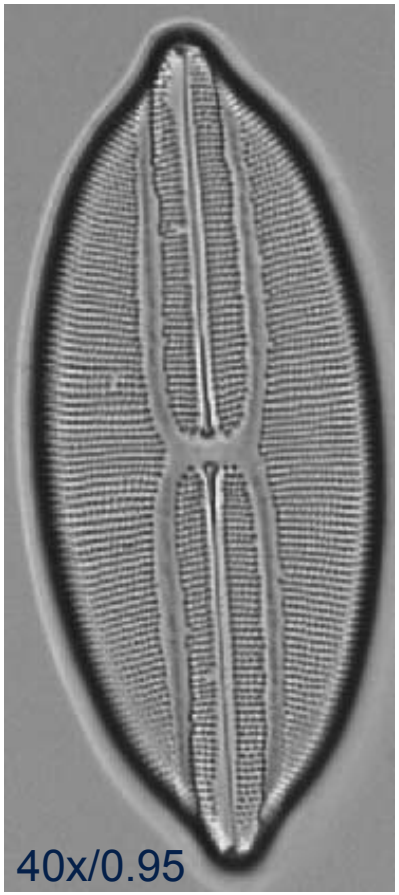
- Resolution

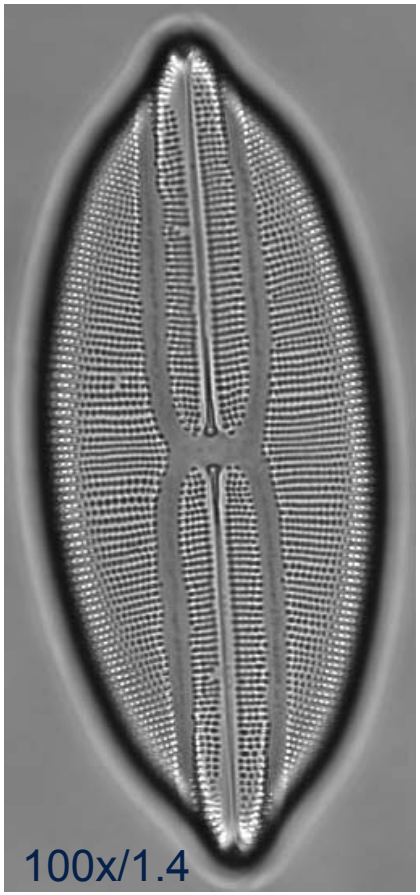
  $= 0.61\ \lambda / NA$



NA and Resolution







Sensitivity

Sample side

- Fluorophores
  - Number
  - Brightness
  - Photostability
- Phototoxicity
- Signal to Noise Ratio

Hardware side

- Camera or detector choice can determine
  - Sensitivity
  - Speed
  - Sampling
- Objective choice

Cameras

sCMOS

- Large sensor size (2k x 2k)
- Smaller pixel size
- Fast
- Fixed pattern noise

EMCCD

- Smaller sensor size (512 x 512)
- Larger pixels
- Slower
- More sensitive than sCMOS for dim samples

The Chinese Menu View of Imaging

Imaging Method

- Widefield
- Laser-scanning Confocal
- Spinning Disk Confocal
- Multi-photon Confocal
- Light-sheet

Contrasting Technique

- Fluorescent Proteins
- Brightfield, Phase, DIC
- Immunofluorescence
- Physiological Dyes
- FRAP/Photoactivation
- FRET/FLIM

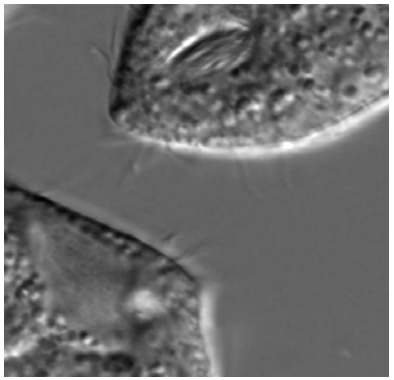
Experimental Requirements

- Time Lapse
- 3D
- Multi-wavelength
- Multi-point

Widefield Imaging

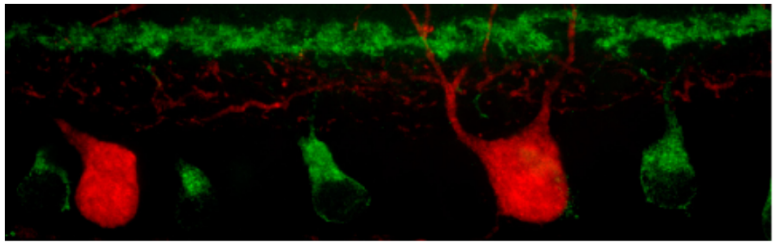
Transmitted Light

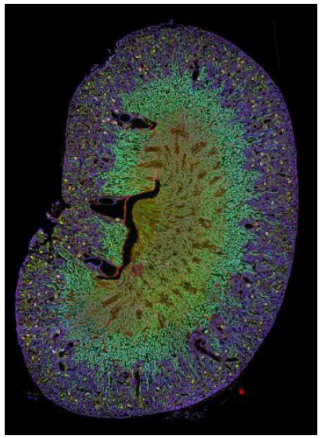
- Brightfield imaging
- Phase contrast
- DIC



Fluorescence

- Multi-color
- Genetically encoded tags
- Dyes
- Immunofluorescence



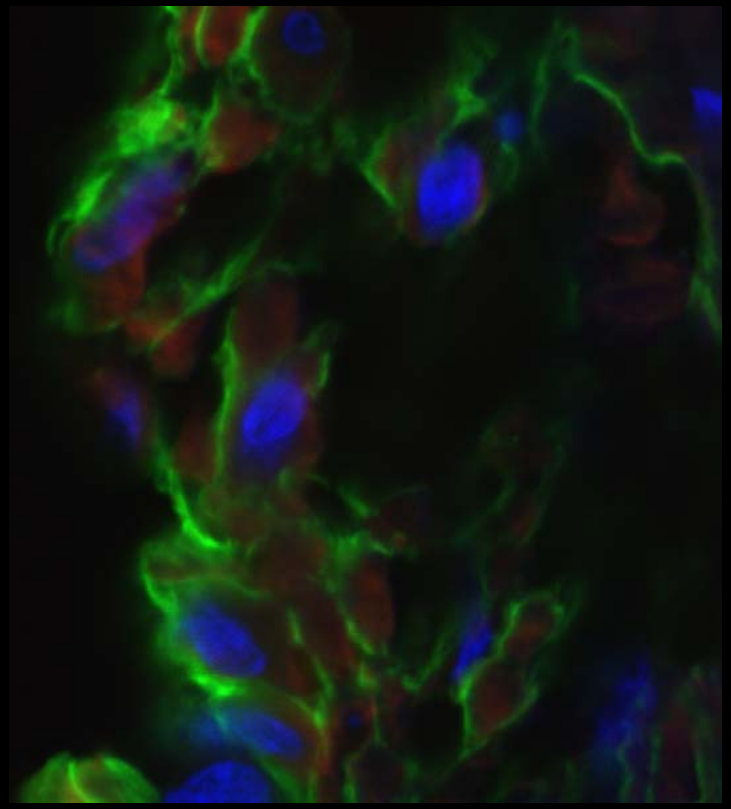


3D imaging

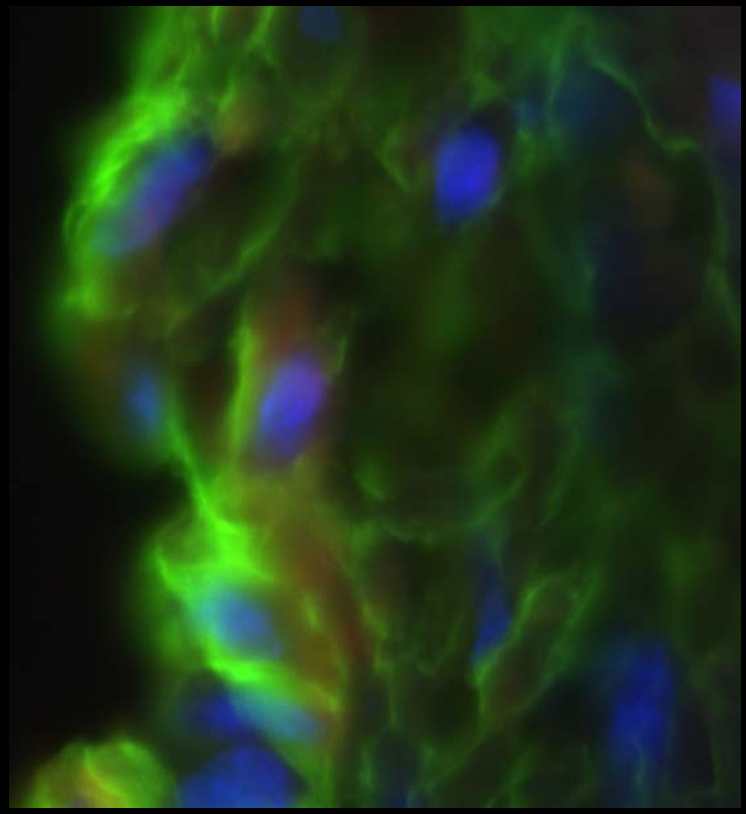
- In conventional widefield microscopy, out-of-focus light is collected at the same time as in-focus light.

- Ways to get around this:
  - Block it from reaching the detector:
    Confocal
  - Remove it after the fact (computationally):
    Deconvolution
  - Don’t excite it in the first place:
    TIRF, Multiphoton, Light Sheet

Confocal vs. Widefield



Confocal

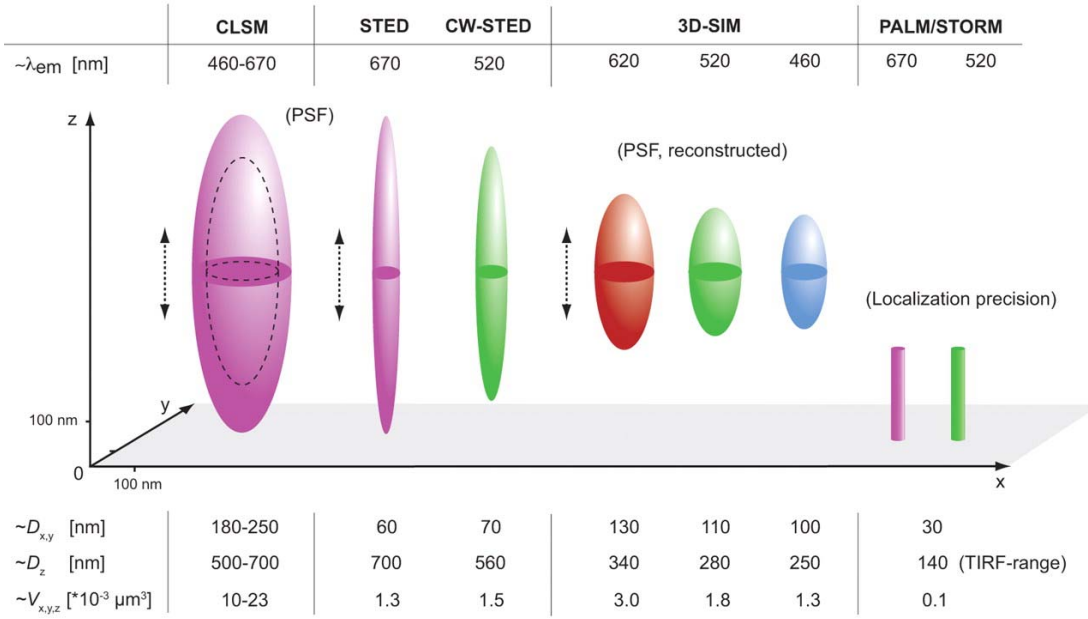


Widefield

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

Super-Resolution Imaging

- Beats the diffraction limit using various tricks
- Techniques give different resolution improvements from 1.7-fold improvement down to 20-30nm resolution
  - Expansion Microscopy
  - Structured Illumination (SIM)
  - Airyscan Confocal Microscopy
  - Stimulated Emission Depletion (STED)
  - Single Molecule Localization Microscopy (SMLM)

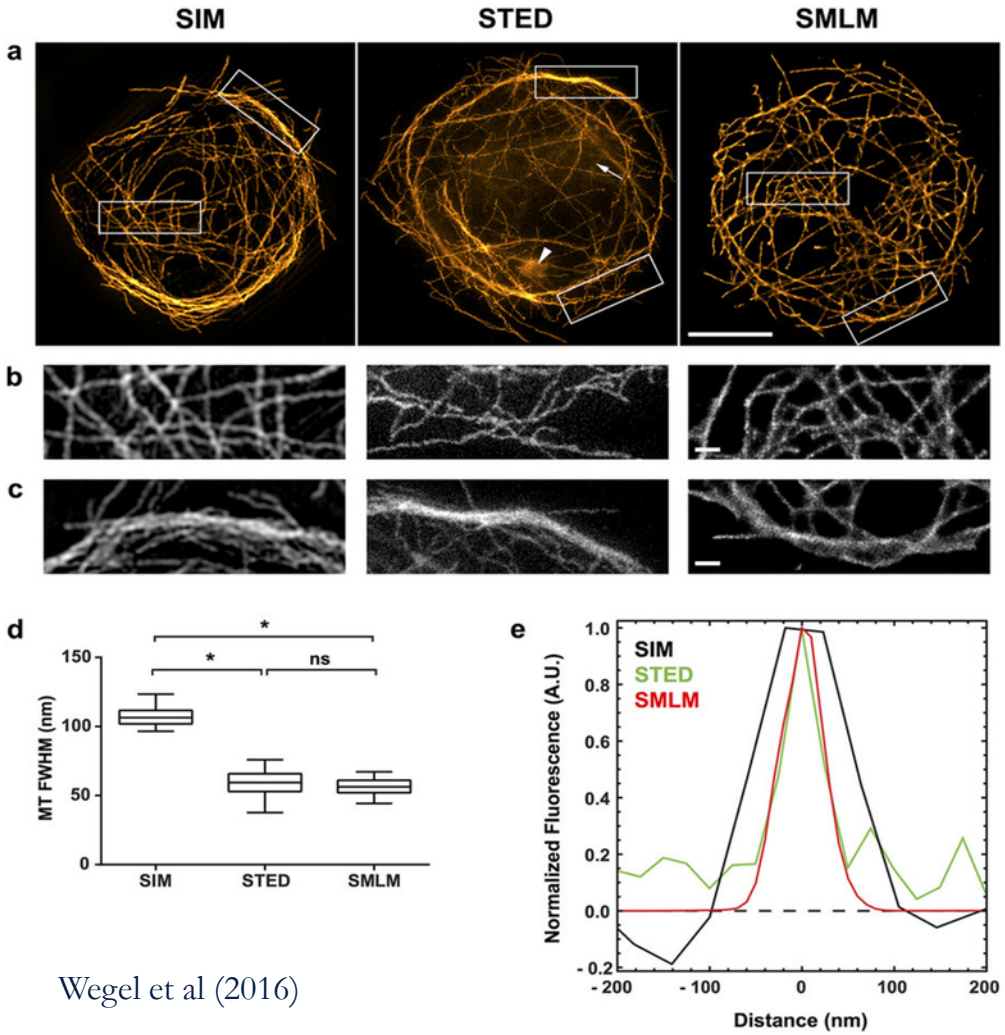


Schermelleh et al (2010)

UCSF

Choosing a technique

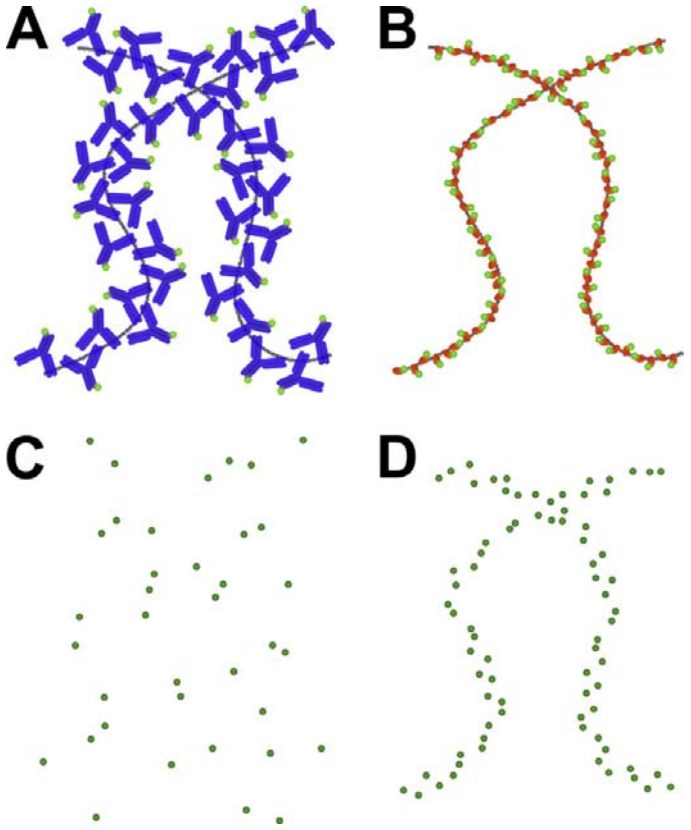
- Availability of microscope
  - At UCSF we have everything except for STED
- Sample constraints
  - Imaging depth, Fluorophore choice, signal level
- Resolution
- Temporal Resolution
  - Live vs fixed



Wegel et al (2016)

Sample Preparation Considerations

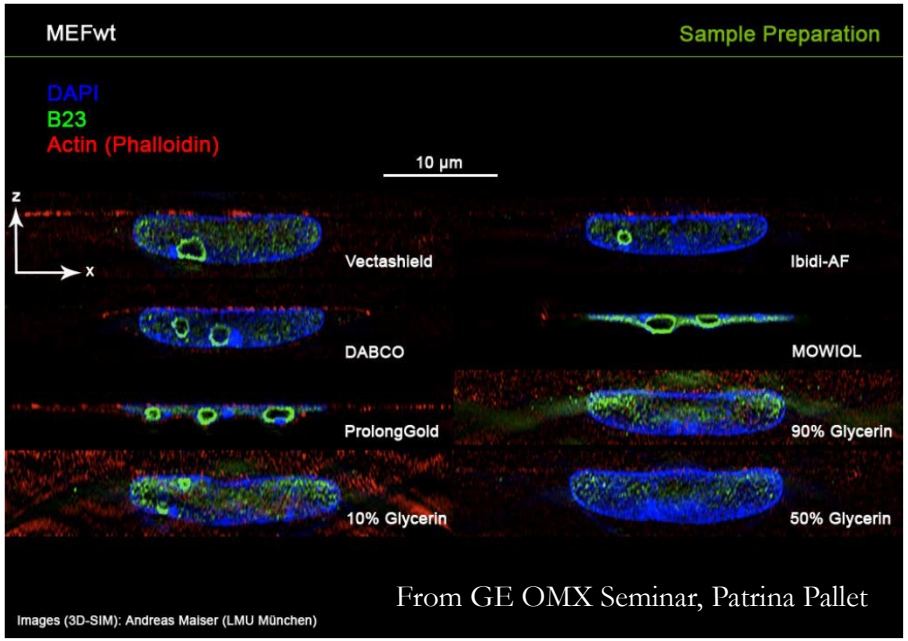
- **Optimize fixation**
  - Need low background
  - Watch for fixation artifacts
- **Optimize antibody staining**
  - Labeling density
  - Low background
- **Fluorophore Choice**
  - Photostability
  - Wavelength dependent resolution
  - SMLM requires fluorophores that can switch
  - STED has limited fluorophores that work well, choice dependent on depletion laser used



Lambert and Waters (2016)

Sample Preparation Considerations

- Use #1.5 coverslips
  - High precision coverslips (#1.5H) help remove a variable, 170 ± 5 um
- Mount samples on coverslip if possible, you want the sample as close as possible to coverslip
- Do not use DAPI in the mounting media
- Choose an appropriate mounting media with anti-fade agent, non-hardening helps to preserve 3D structures
- Mount in middle of slide to make sure slide can sit flat on microscope
- Aberrations impact your results more than in normal diffraction limited microscopy



Choosing a technique

	Airyscan	SIM	STED	SMLM	Expansion
Resolution (XY)	140 nm	130 nm (λ dep)	85 nm	20-30 nm	~ 70 nm (4.5x) ~ 25 nm (20x)
Fluorophore choice	Standard fluorophores	Standard fluorophores	Limited fluorophores, depends on depletion laser	Limited Fluorophores, must be able to switch	Standard fluorophores
Multi-color	Easy	Easy	Limited	Hard	Easy
Speed	Medium	Low	Medium	Very low	Medium to Fast
Computational Requirements	Low	Medium	Low	High	Low-Medium (depends on volume)

Common Pitfalls

- Garbage in – Garbage out
- Details matter
- You need pilot experiments
  - Are your images giving you what you need?
  - Do you need to optimize your sample prep, image collection, etc?
- Watch your data size
  - Current automation makes it trivial to capture large data sets
  - Analysis of large data sets becomes the new bottleneck

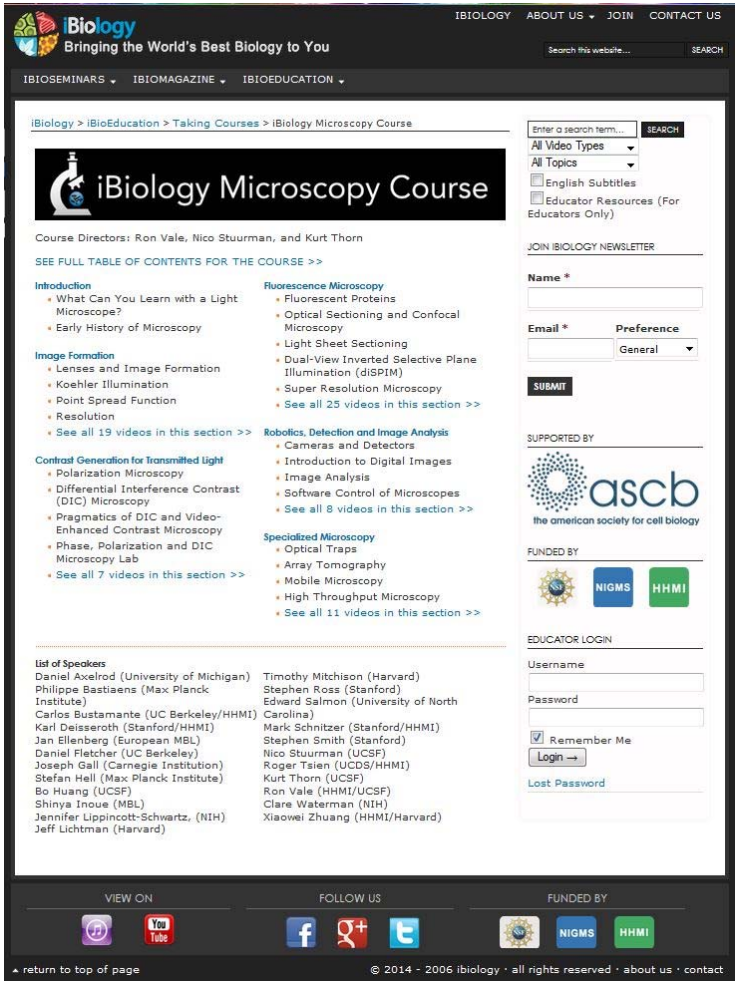
Resources

NIC Wiki

Nikon Imaging Center Wiki:

http://nic.ucsf.edu/dokuwiki/doku.php?id=start

- Links to great resources under the heading Microscopy References and Education



http://www.ibiology.org/ibioeducation/taking-courses/ibiology-microscopy-course.html