Garbage in = Garbage out

Seeing is believing – pitfalls in image acquisition

Outline

Planning of the imaging experiment:
Which system will be the best for my question?

Optical system – not just the microscope!

Aberrations introduced by the system … and you

How to prepare the sample?

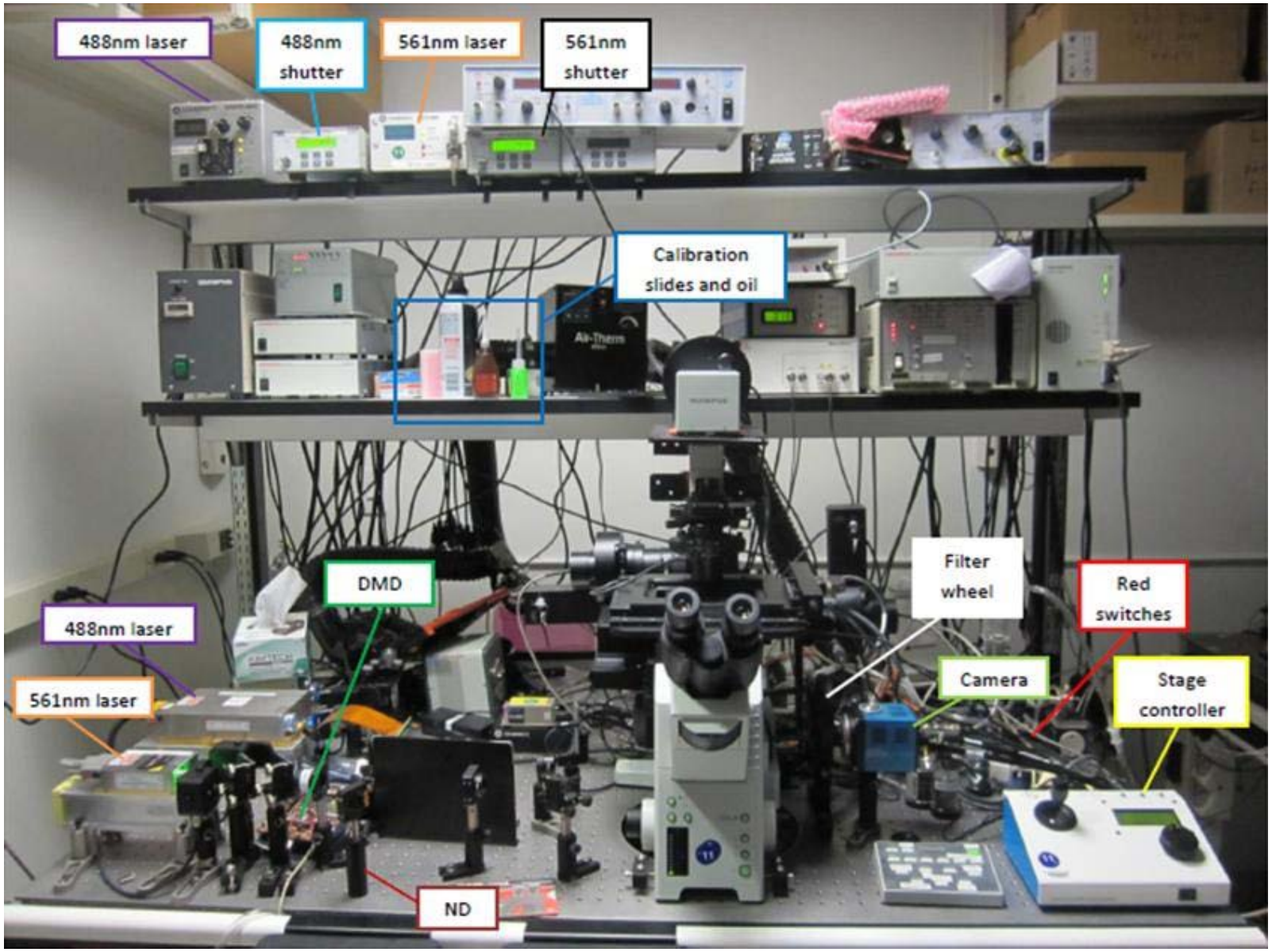
Which controls do I need to be sure about the results?


Diffraction
Sample - YOU
Noise
Aberrations
Digitization

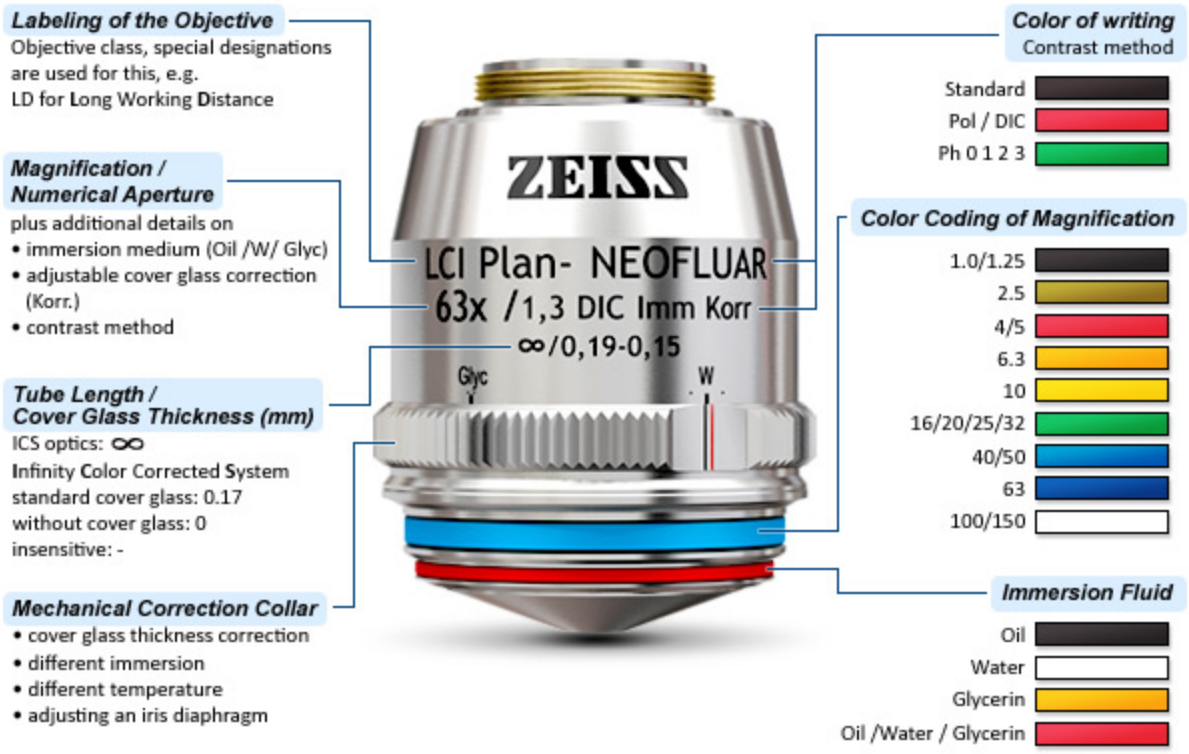
Which system will be the best for my question?

- Widefield
- Single point confocal
- Spinning disk confocal
- Slide scanner
- Temp and CO_2 control
- 2Pi

The optical system



Objectives



http://www.microscopyu.com

NA

Numerical Aperture (also termed **Object-Side Aperture**) is a value (often symbolized by the abbreviation **NA**) originally defined by Abbe for microscope objectives and condensers. It is given by the simple expression:

Numerical Aperture (NA) = n × sin(μ) or n × sin(α)

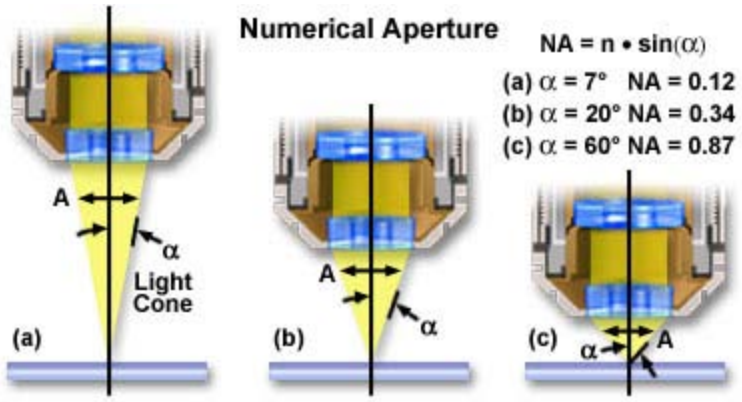
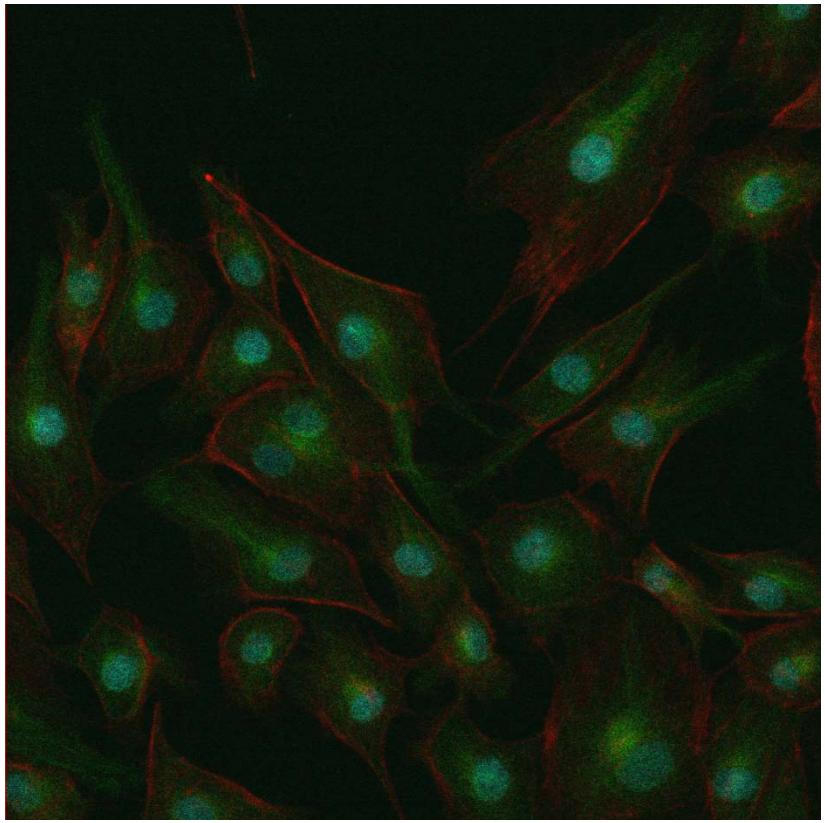


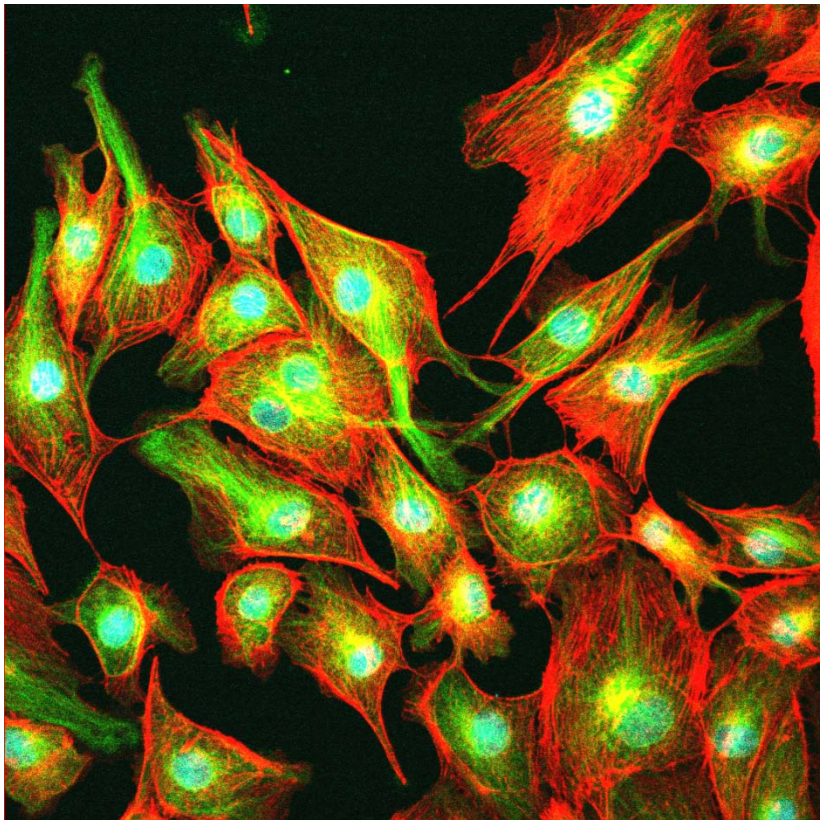
Figure 1

http://www.microscopyu.com

NA determines intensity

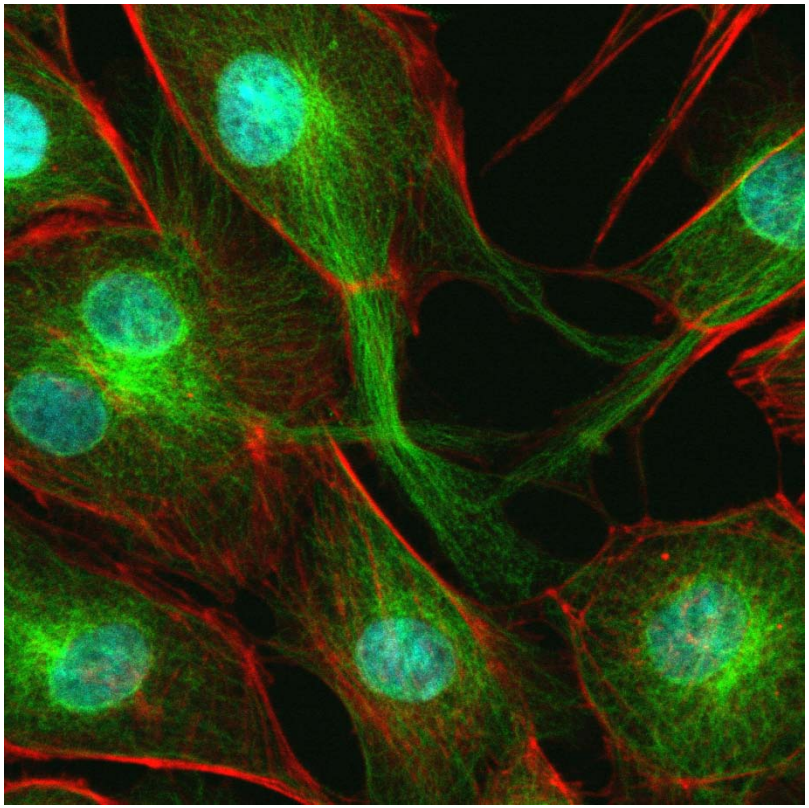


10x/0.3 PlanApo, zoom 2.5

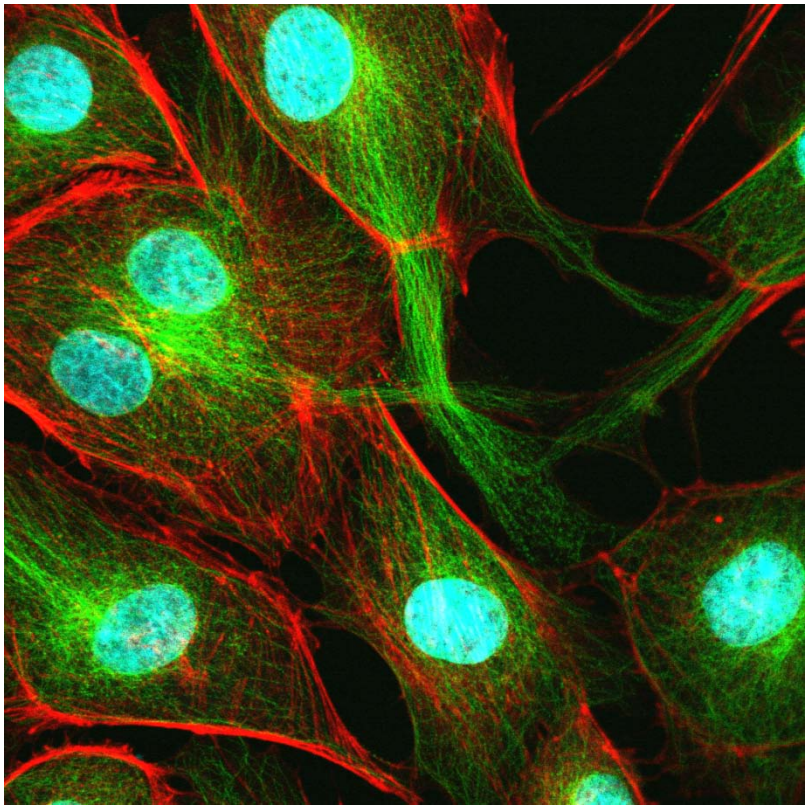


25x/0.8 PlanApo

NA determines intensity

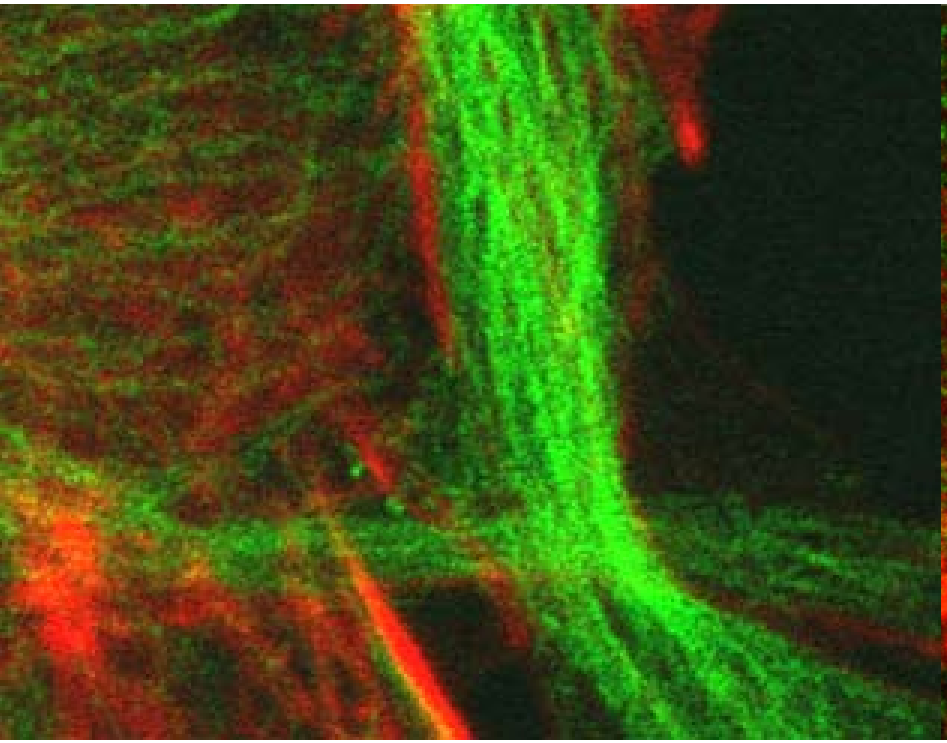


25x/0.8 PlanApo, zoom 2.52

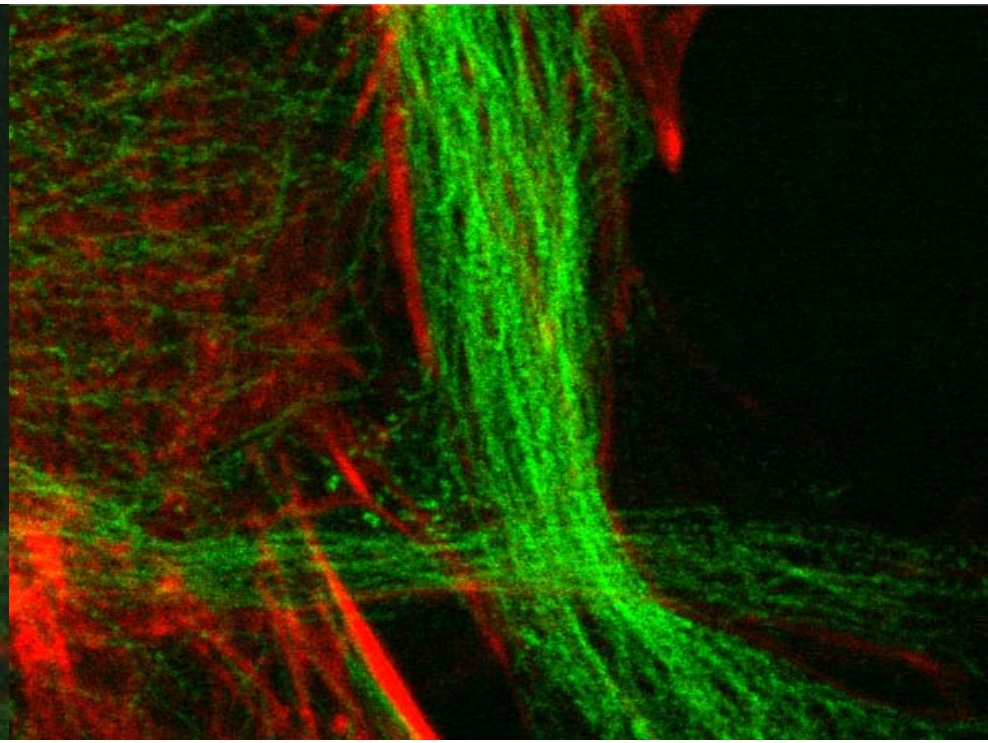


63x/1.4 PlanApo

NA determines resolution

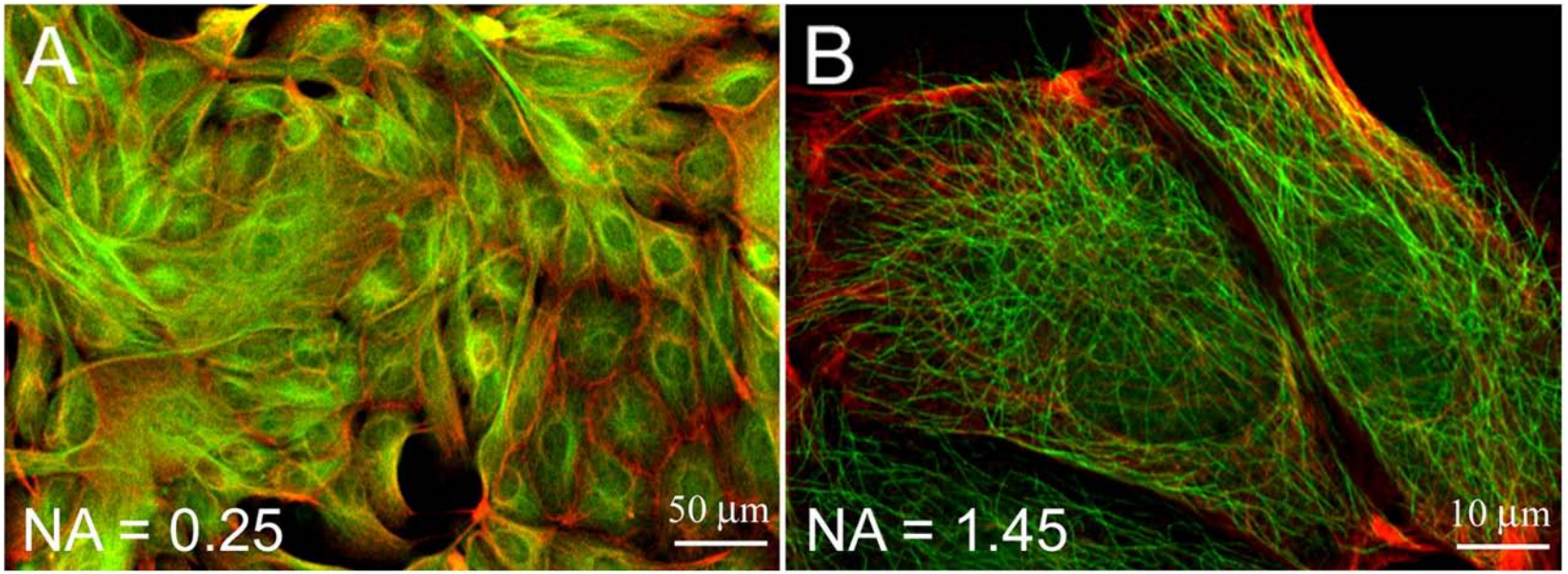


25x/0.8 PlanApo, zoom 2.52



63x/1.4 PlanApo

High resolution images are necessary for colocalization studies



It is important to consider how resolution will affect colocalization analysis. We consider two fluorochromes to be "colocalized" when their emitted light is collected in the same voxels (3D pixels). If the distance separating two labeled objects is below the resolution limit of the imaging system, they will appear to be colocalized. Thus, users may "see" colocalization using a low resolution imaging system where a higher resolution system might achieve a visible separation of labels that are in close proximity but are not actually colocalized

North A J J Cell Biol 2006;172:9-18

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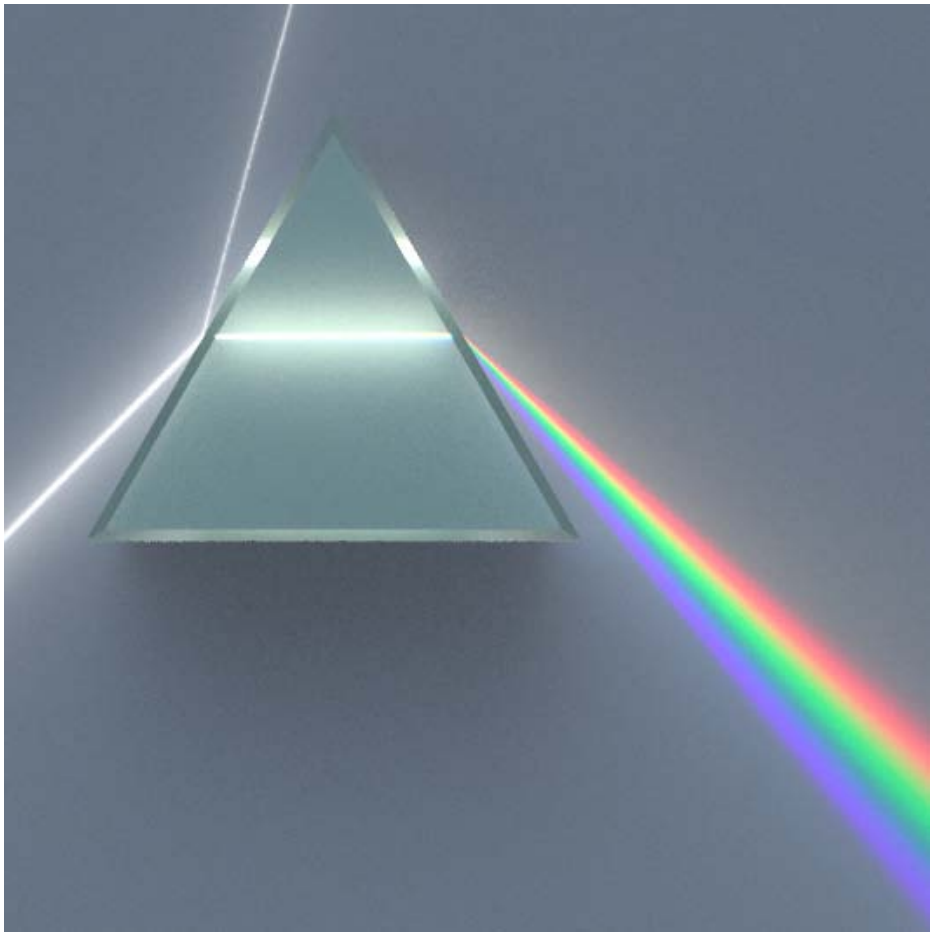
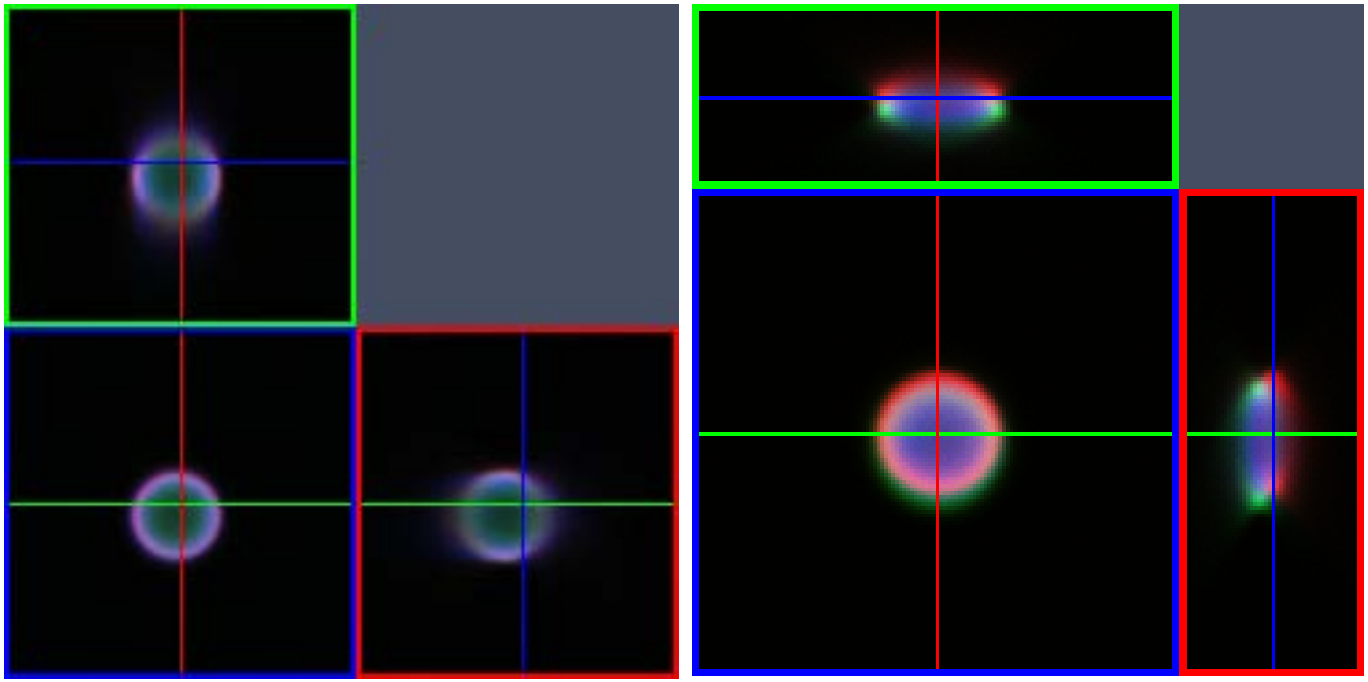
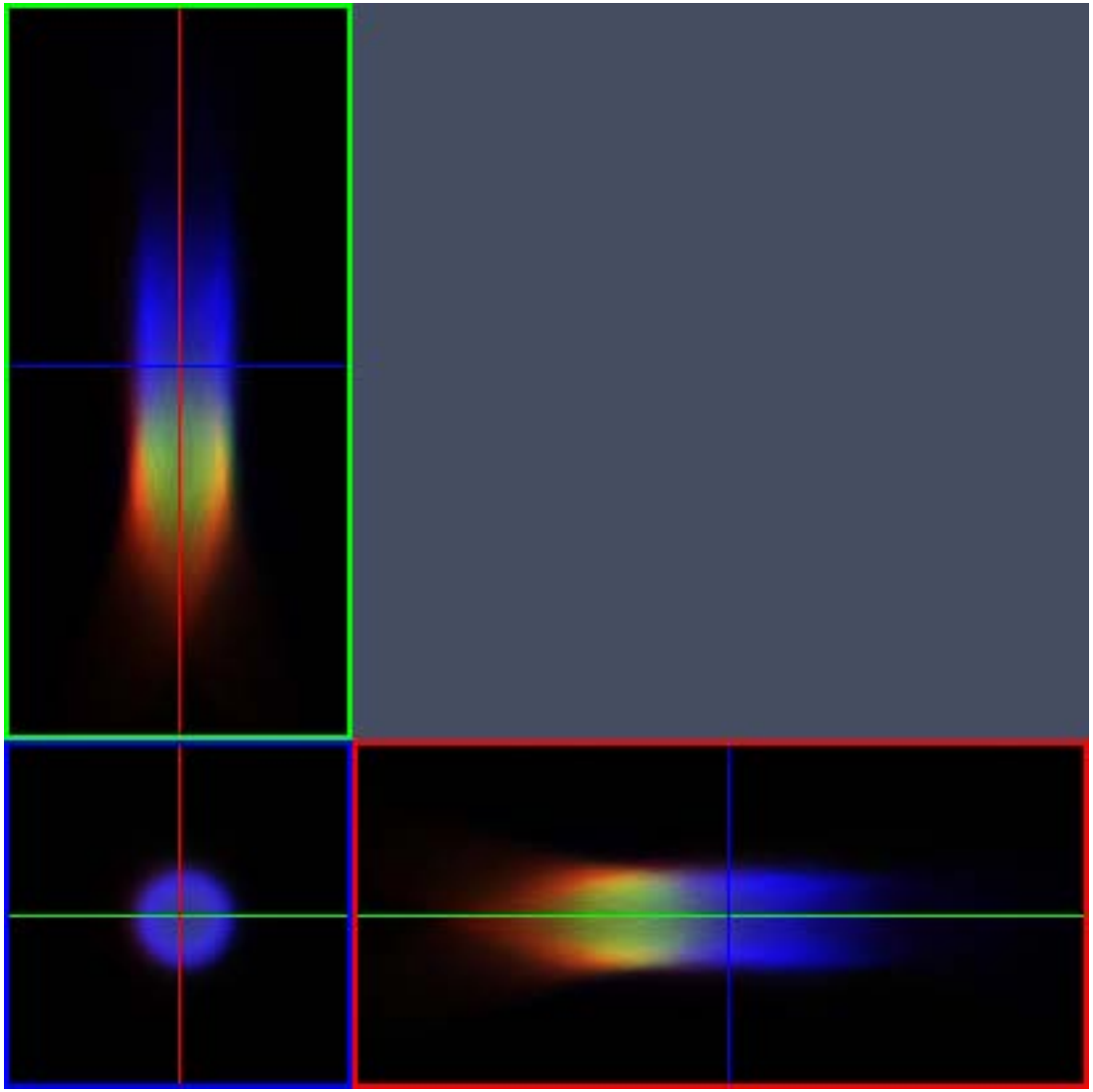
Chromatic aberrations



Chromatic aberrations



Chromatic aberrations



Microscope Objective Optical Correction Factors

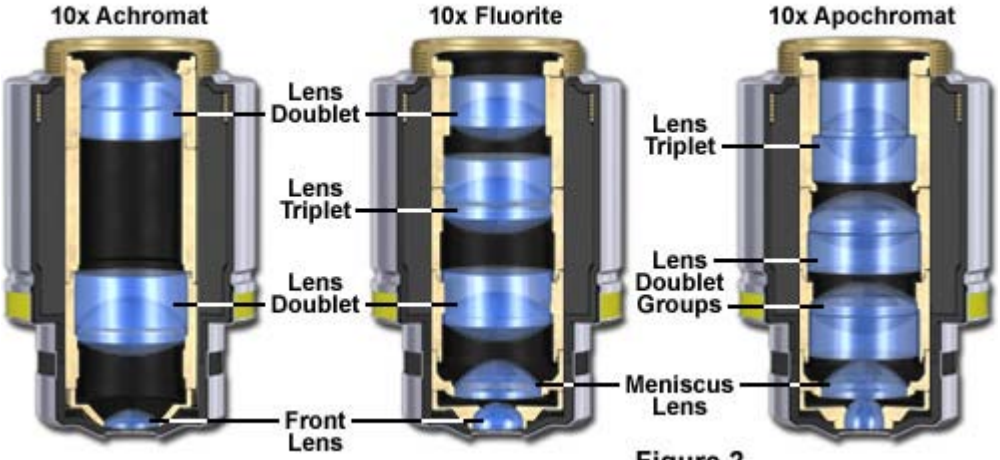


Figure 2

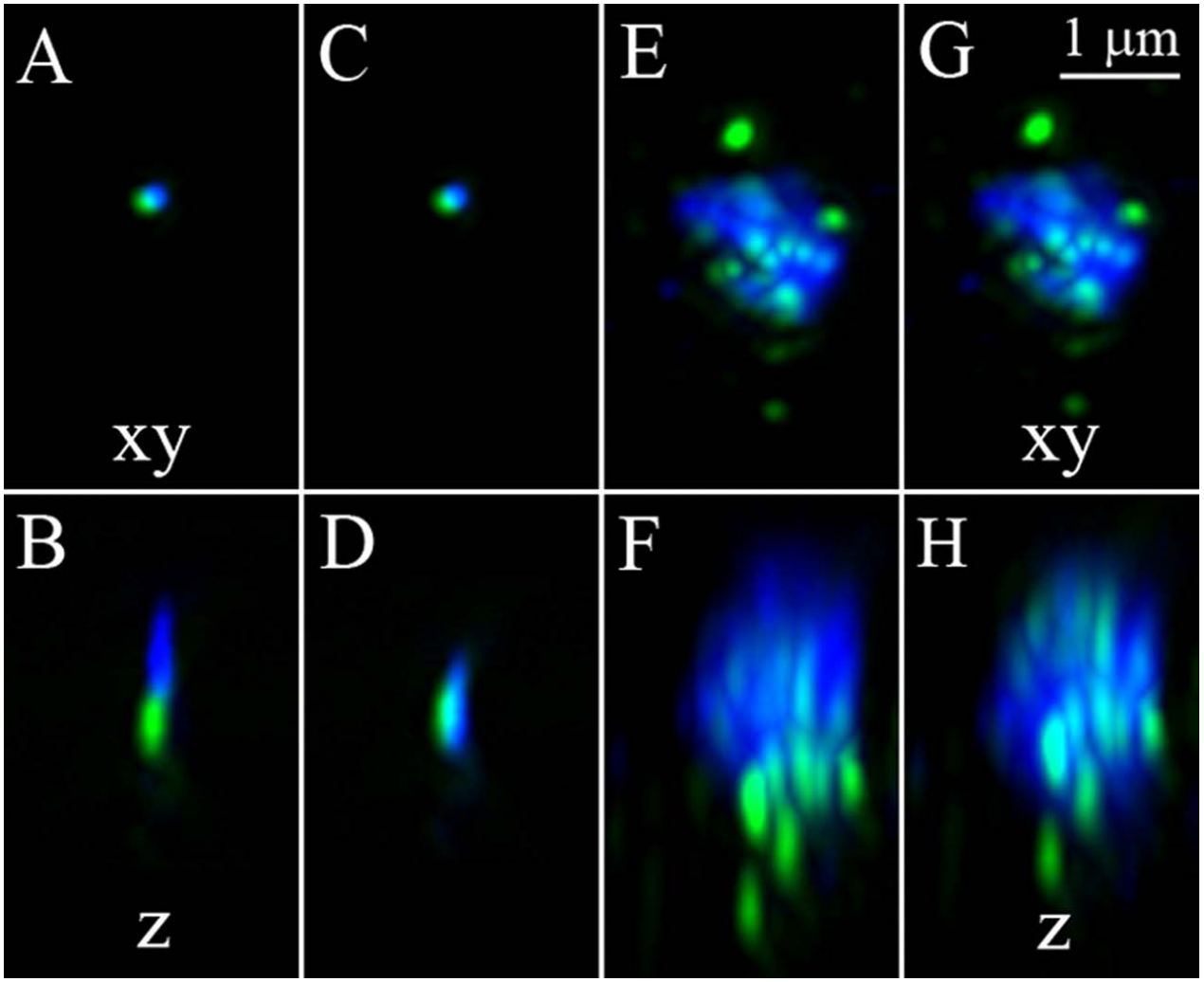
Microscope Objective Correction for Optical Aberration

Objective Specification	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

dark blue, blue, green, and red

http://www.microscopyu.com

The effect of chromatic aberrations on images



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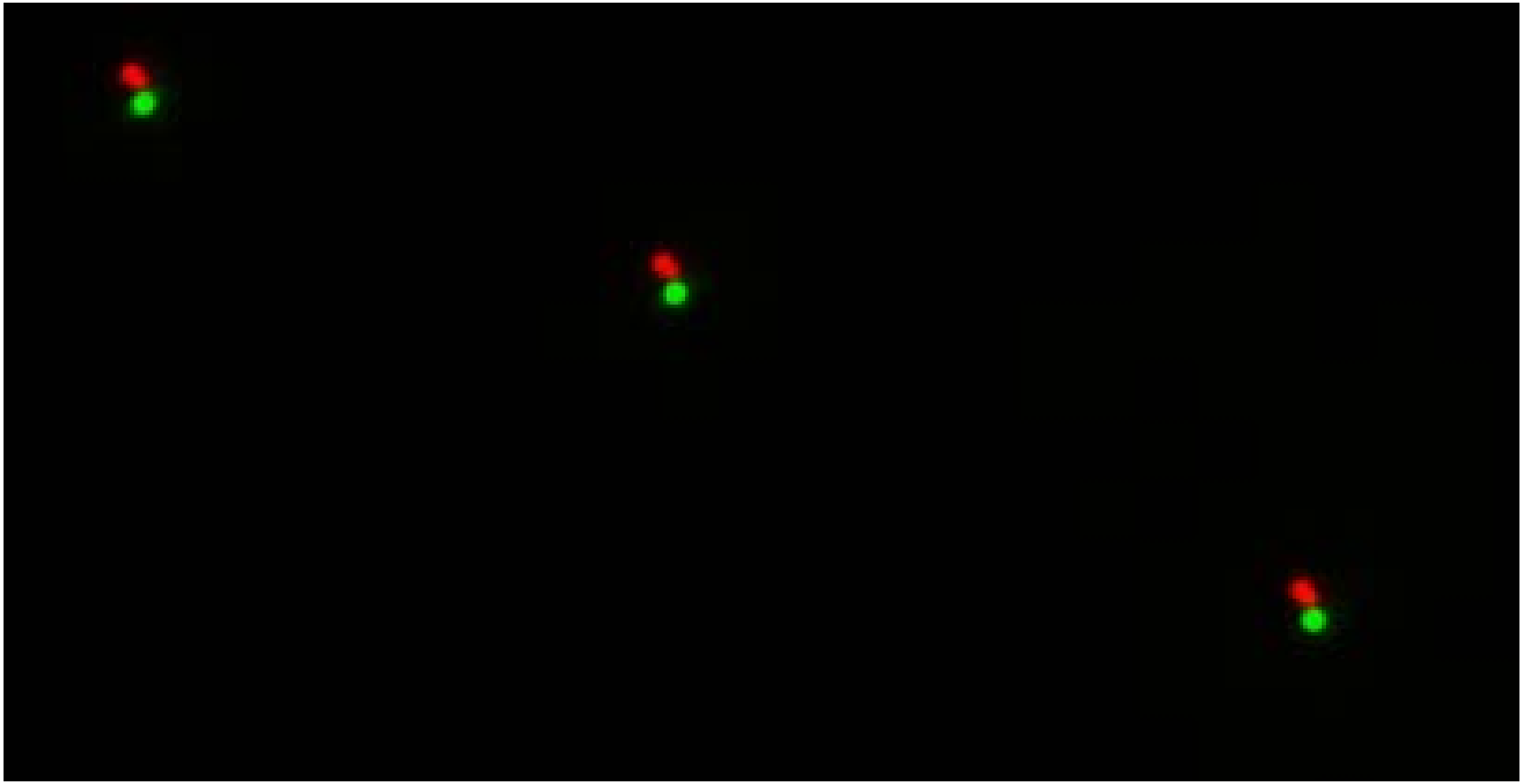
What went wrong???



Take-home message I

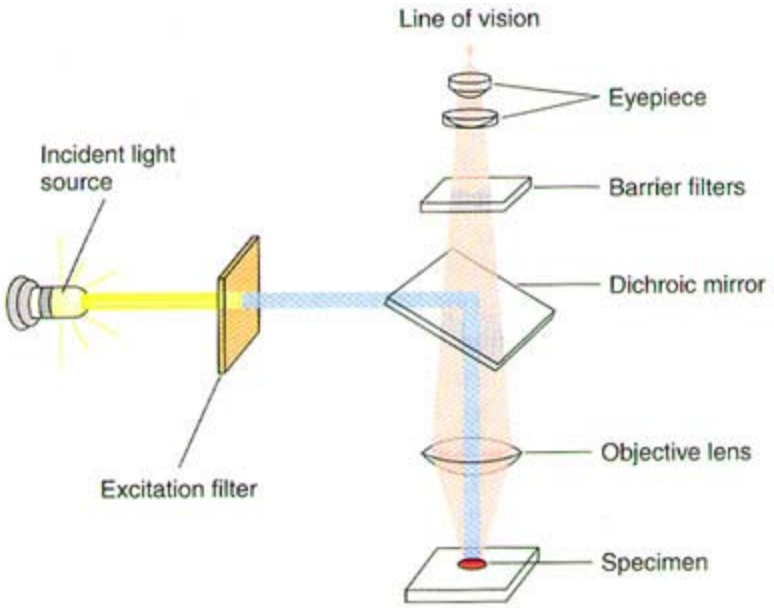
1. Carefully check the available objectives and choose the best one suiting your imaging experiment

2. Understand the language the objective is talking to you

3. Be aware of chromatic aberrations – use proper controls

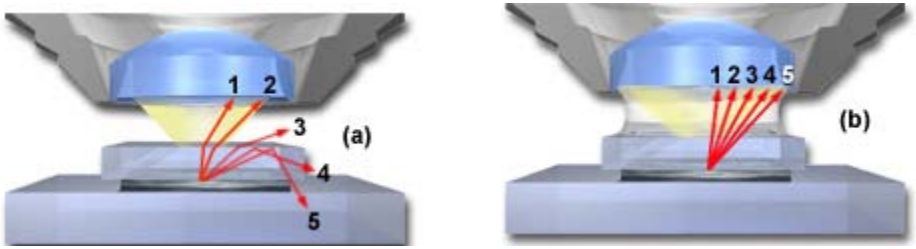
4. Check for optical elements in the imaging pathway that could change the information

The optical system



Lodish H, et al. Molecular Cell Biology, p.140

Optical system - not only the microscope…



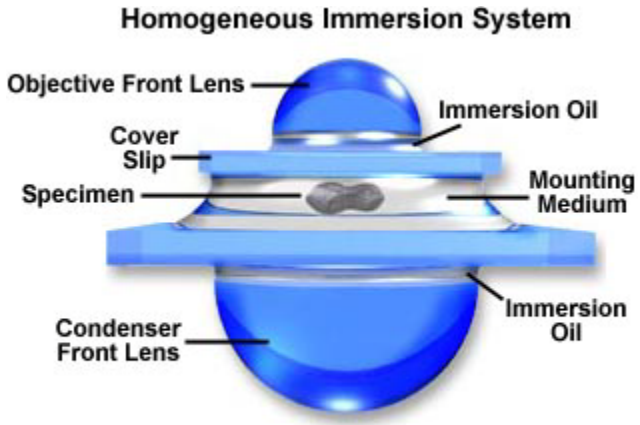
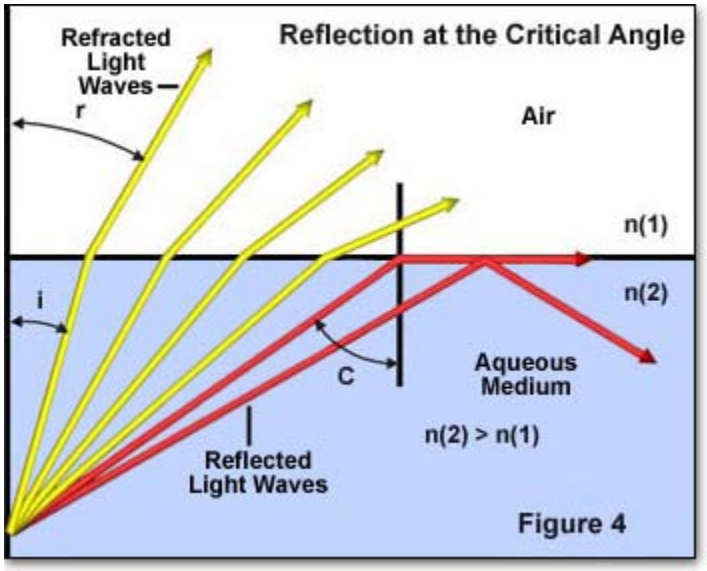
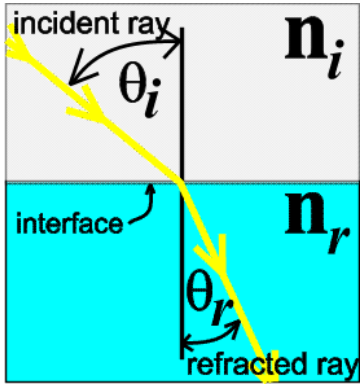


Figure 2

http://www.olympusmicro.com

Refraction





$$n = c/v$$

SNELL'S LAW

$$n_1 \sin\Theta_1 = n_2 \sin\Theta_2$$

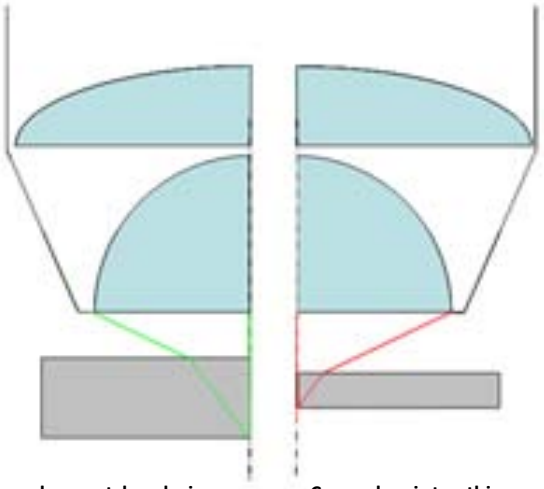
$$\frac{\sin\Theta_1}{\sin\Theta_2} = n$$

Material	Refractive Index
Air	1.0003
Water	1.33
Glycerin	1.47
Immersion Oil	1.515
Glass	1.52
Flint	1.66
Zircon	1.92
Diamond	2.42
Lead Sulfide	3.91

Material	Blue (486.1 nm)	Yellow (589.3 nm)	Red (656.3 nm)
Crown Glass	1.524	1.517	1.515
Flint Glass	1.639	1.627	1.622
Water	1.337	1.333	1.331
Cargille Oil	1.530	1.520	1.516
Carbon Disulfide	1.652	1.628	1.618

http://www.olympusmicro.com

Coverslip thickness, position of the sample in relation to the coverslip

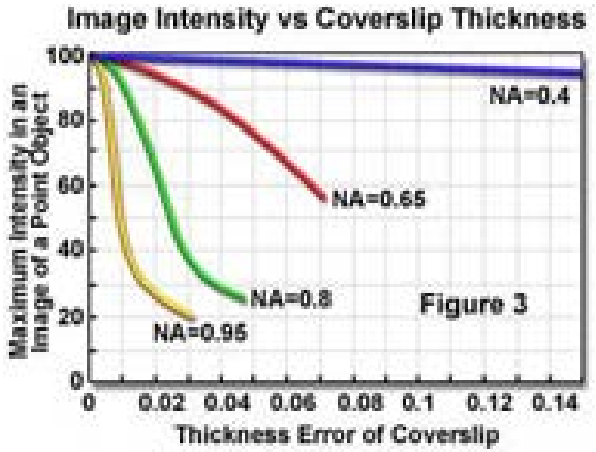


Cover glass matches design thickness 0.17mm

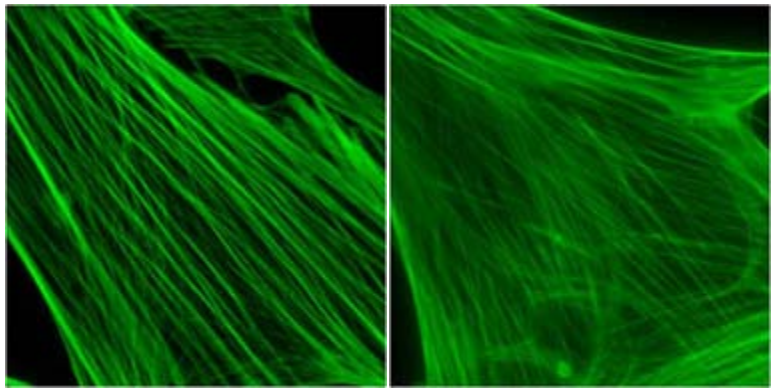
all rays meet at object point (at lower surface of the cover glass)

Cover glass is too thin

Focal plane get shifted
Paraxial and marginal rays do not meet at object point anymore (spherical aberration)
This effect mainly depends on ray angle / numerical aperture and immersion medium



The thickness of this medium *must be added* to the thickness of the cover glass. A specimen which is located deep in the medium will have a larger "effective" cover glass thickness than a specimen which is located right beneath the cover glass. A calculated (ideal) cover glass thickness 0.17mm is therefore a good compromise, even if the "real" cover glass is thinner. And yes, the refractive index of the mounting medium also plays a role.



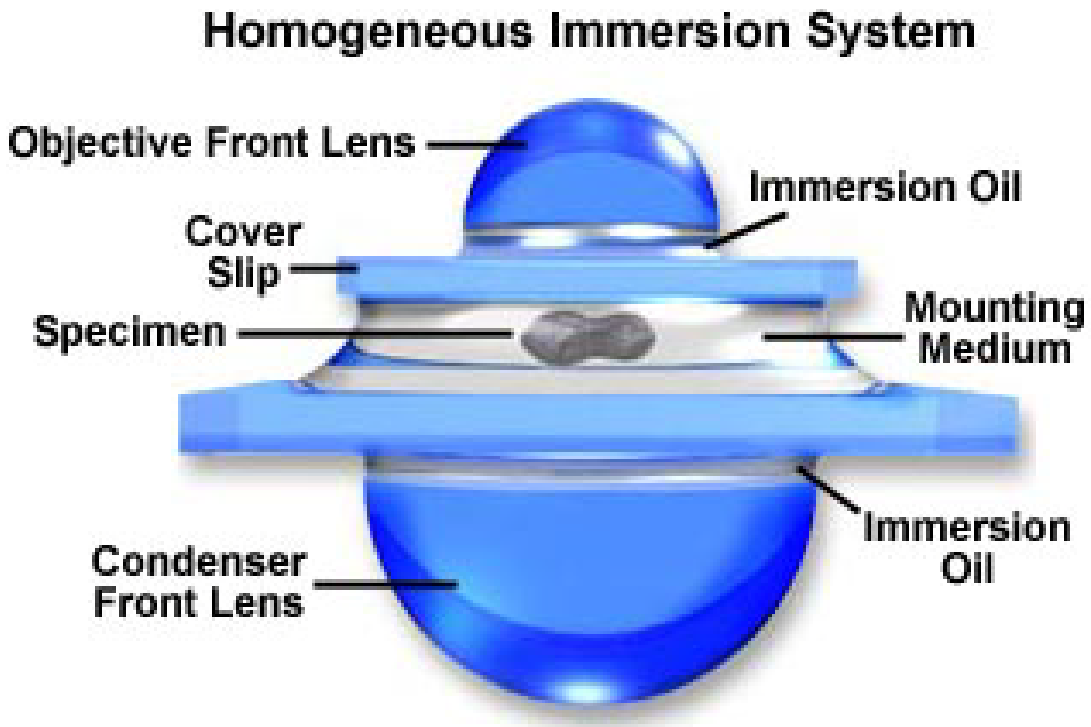
Coverslip #1.5 - ~ 170nm Thickness

Coverslip #2 - ~ 200nm Thickness

Axio Observer, Plan Apo 40x/0.95
Indian Muntjac fibroblasts stained with Alexa Fluor 488 conjugated to phalloidin
Coverslip #2 - ~ 200nm Thickness

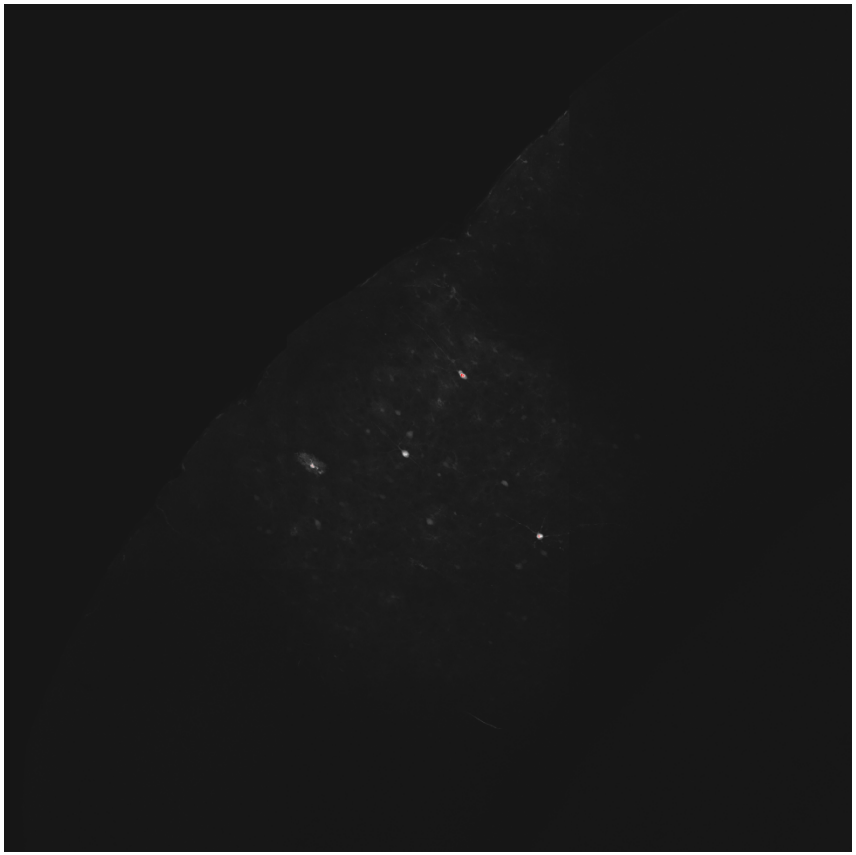
Compliments of Dr. Michael Davidson/FSU

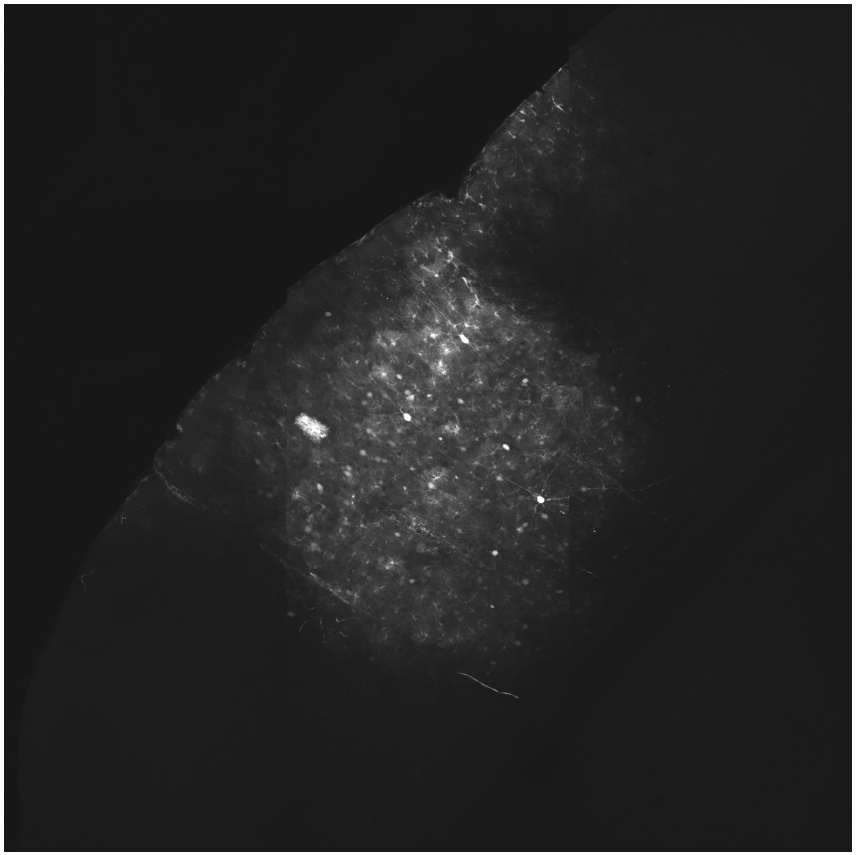
Optical system - not only the microscope…



http://www.olympusmicro.com

It really matters...





Spherical aberrations

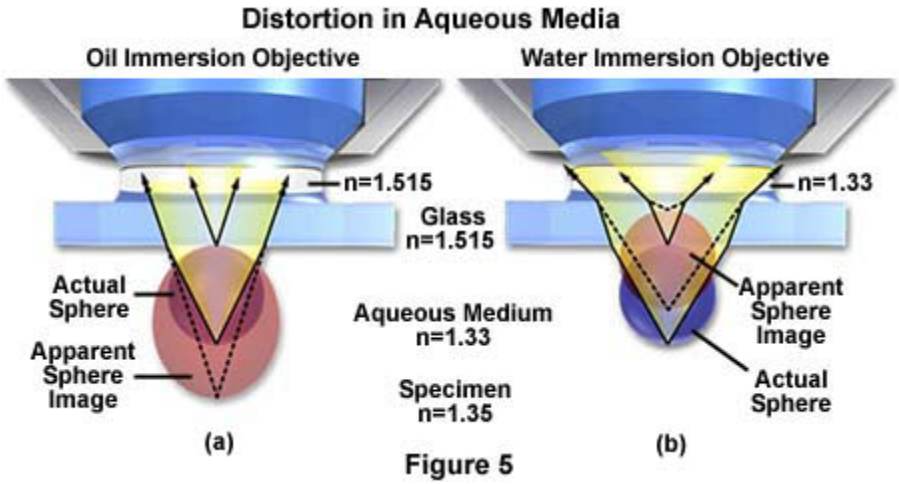


Figure 5

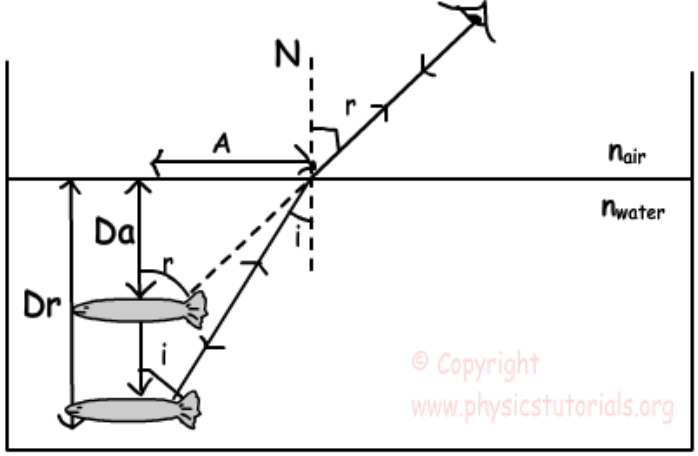
Another problem with imaging tissue samples is the limited range of resolution and illumination intensity exhibited by oil immersion objectives when examining specimens at high magnification. Immersion objectives typically lose their excellent imaging properties at depths exceeding 10 microns into the sample when it is covered by a coverslip and bathed in aqueous solution. This problem is illustrated in Figure 5(a) where ray tracing indicates that a sphere in aqueous media is distorted into an apparently elongated oval when using an oil immersion objective to image the sample through immersion oil having **n** = 1.515.

The same sphere remains spherical when using a water immersion objective, even though the imaging rays must still pass through the glass coverslip with a refractive index equal to 1.515. Water immersion objectives also eliminate spherical aberrations that are often produced when viewing specimens through an aqueous solution. These advanced objectives also have an increased axial resolution that often equals the theoretical limits for the numerical aperture and they produce exceptional contrast, resolution, and provide a higher intensity of illumination.

$$Da = \frac{Dr \cdot n_{observer}}{n_{object}}$$

where; Da is the apparent depth,
Dr is the real depth
$n_{observer}$ is the refractive index of the medium of observer
n_{object} is the refractive index of the medium of object.

Real Depth= Apparent depth x RI medium/RI immersion



Da=Apparent depth
Dr=Real depth
i=angle of incident ray
r=angle of refracted ray

Snell's Law;

$n_{water}.\sin\theta_i = n_{air}.\sin\theta_r$

$$n_{water} = \frac{\sin\theta_r}{\sin\theta_i}$$

for small angels $\sin\theta i = \tan\theta i$ and $\sin\theta r = \tan\theta r$

writing down the tangents of the angle n_{water} becomes;

$$n_{water} = \frac{\tan\theta_r}{\tan\theta_i} \qquad \text{where } \tan\theta i = \frac{A}{Dr} \text{ and } \tan\theta r = \frac{A}{Da}$$

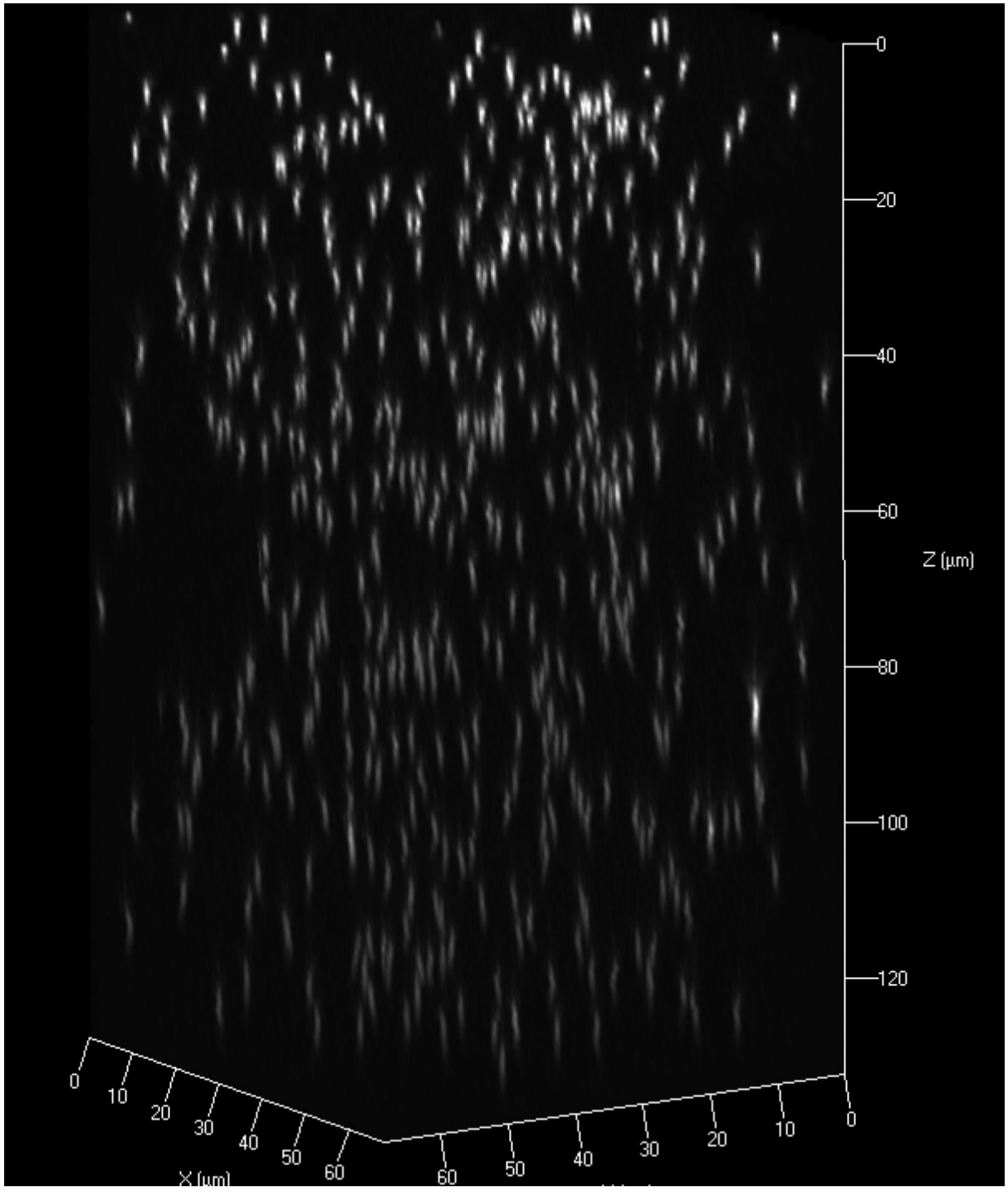
$$n_{water} = \frac{Dr}{Da}$$

$$Da = Dr.\frac{n_{observer}}{n_{object}}$$

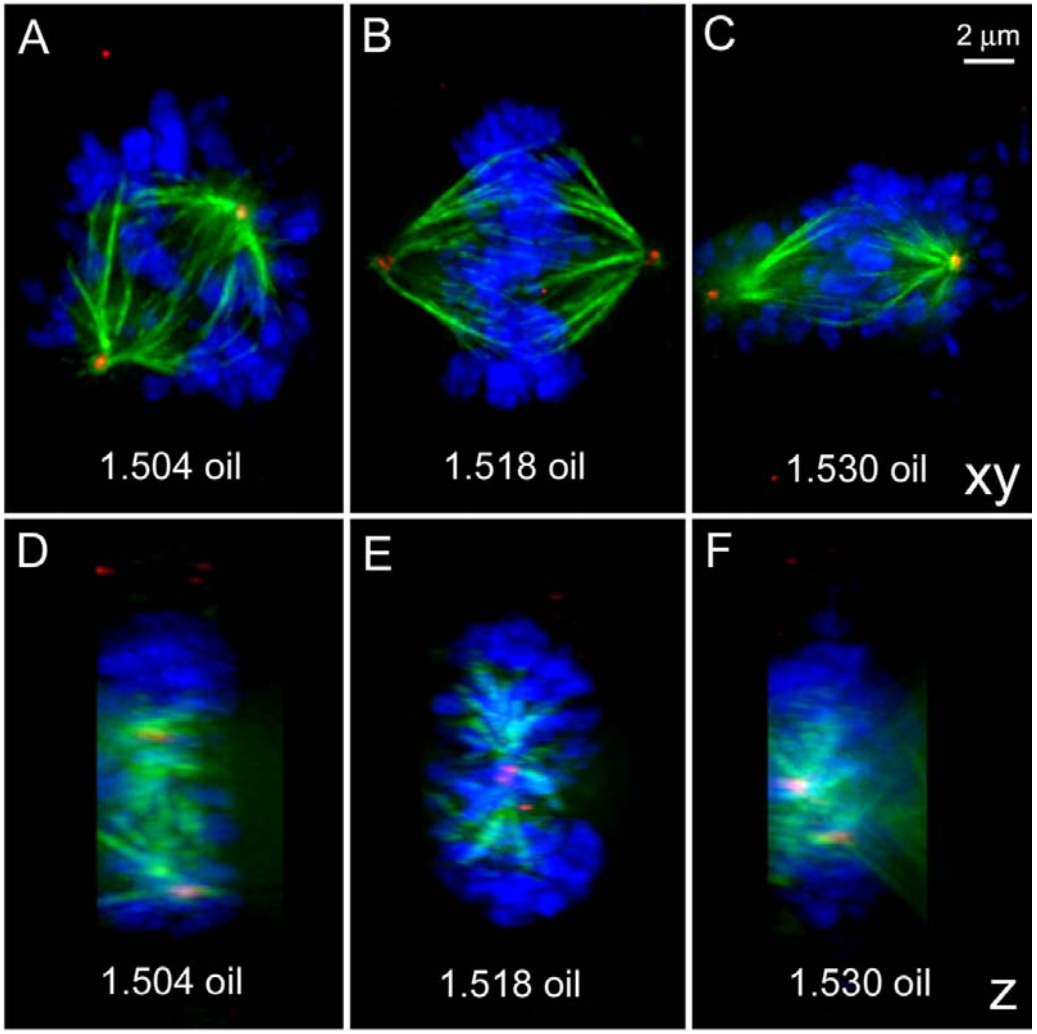
where; Da is the apparent depth,
Dr is the real depth
$n_{observer}$ is the refractive index of the medium of observer
n_{object} is the refractive index of the medium of object.

Real Depth= Apparent depth x RI medium/RI immersion

http://www.physicstutorials.org


0
20
40
60
80
100
120
Z (µm)
0
10
20
30
40
50
60
60
50
40
30
20
10
0
X (µm)

The effect of spherical aberrations on images



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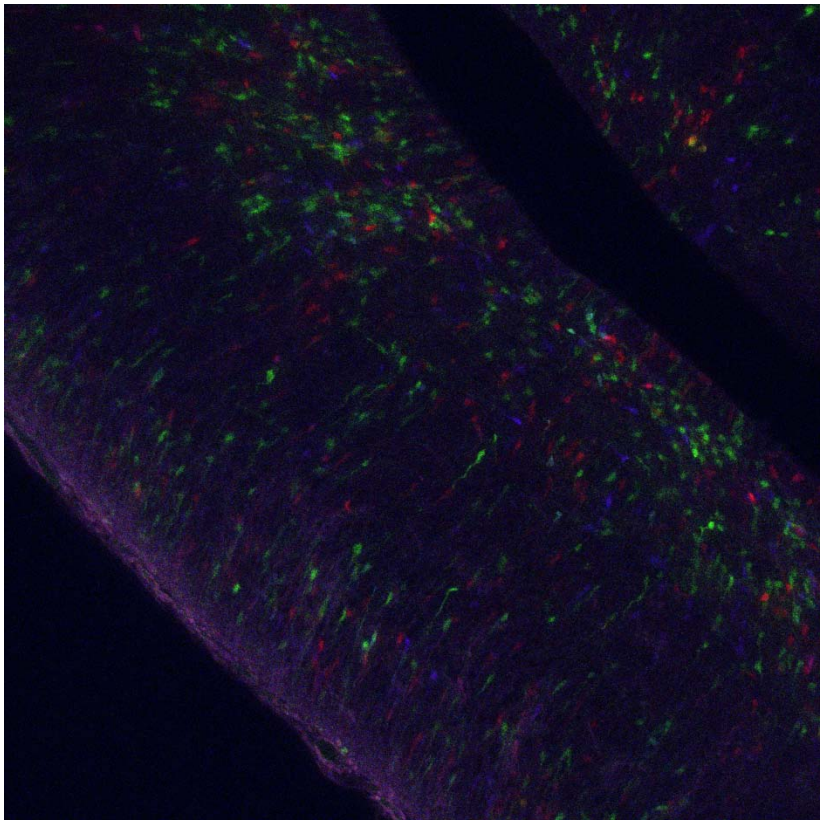
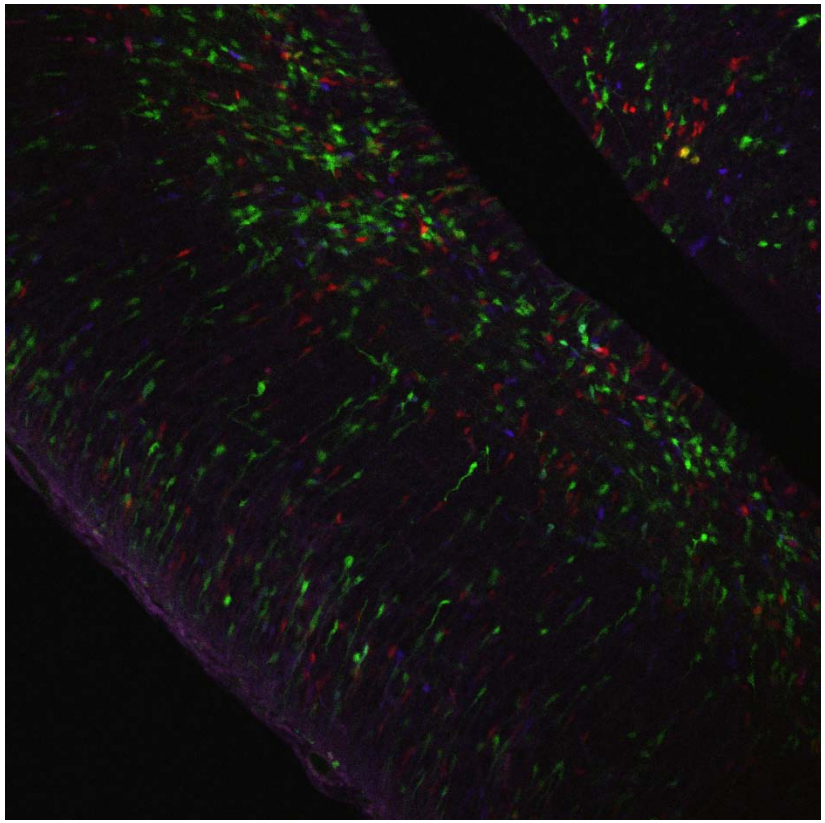
The effect of spherical aberrations on images

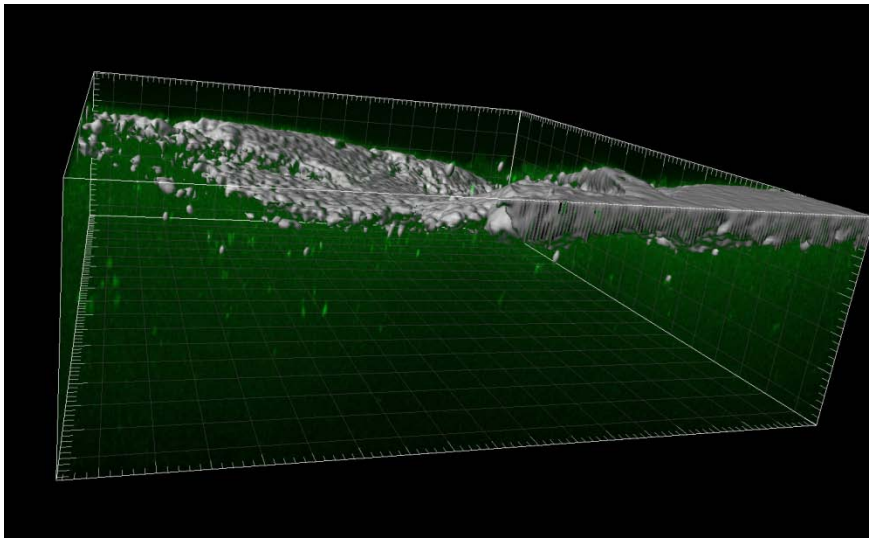


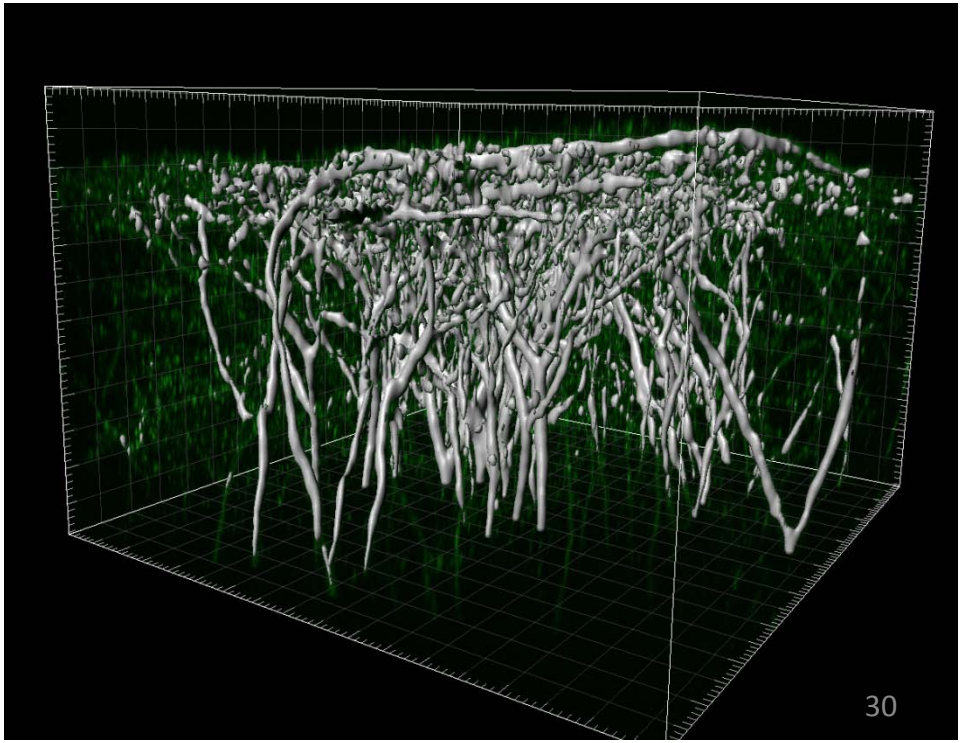
Plan-Apochromat 10x/0.3



C-Apochromat 10x/0.45 W

Matching RI – clearing to avoid scattering





Objectives – hardware corrections

Figure 1

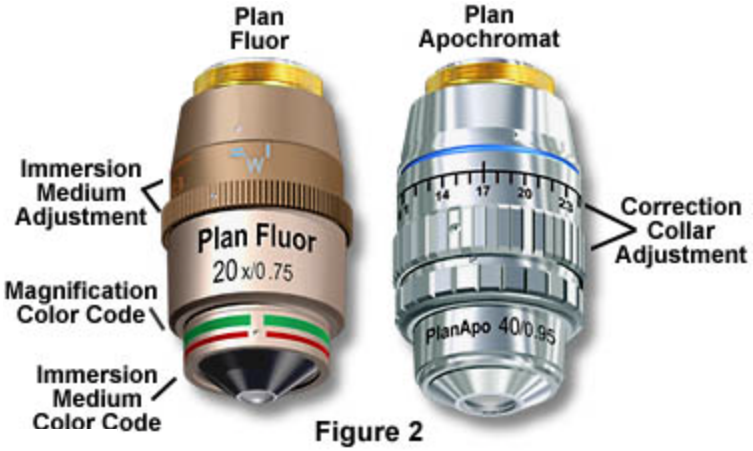
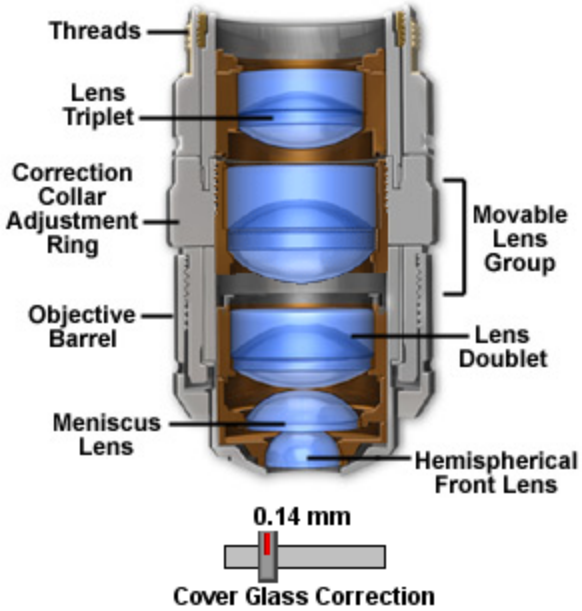


Figure 2



Take-home message II

1. Remember that optical system is not just the microscope
2. Use the right coverslips, correct for imperfections
3. Match RI to avoid spherical aberrations

Sample preparation

- controls,
- dilutions,
- fixations,
- mounting medium

An outline of the immunocytochemistry Procedure

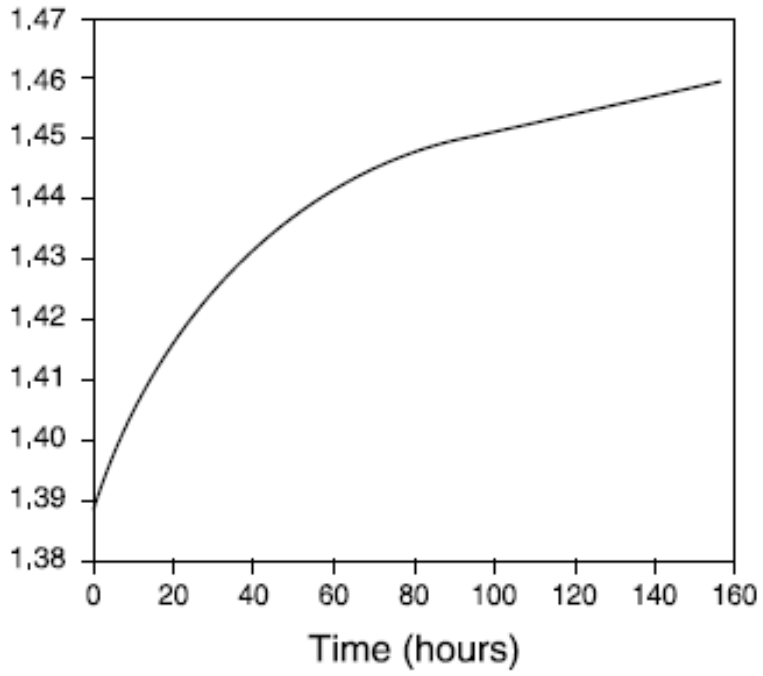
(A) Prepare samples
(B) Fix tissue or cells
(C) Embed, section, and mount tissue
(D) Block and permeabilize
(E) Rinse after block and permeabilize
(F) Incubate with 1st antibody
(G) Rinse after 1st antibody
(H) Incubate with 2nd antibody
(I) Rinse after 2nd antibody
(J) Mount coverslip
(K) Examine in microscope
(L) Evaluate results

Mounting medium

Mounting media that harden (often containing polyvinyl alcohol) are useful for long-term sample storage and are preferred for imaging using a wide-field (compound) microscope because the sample flattens as the mountant hardens.

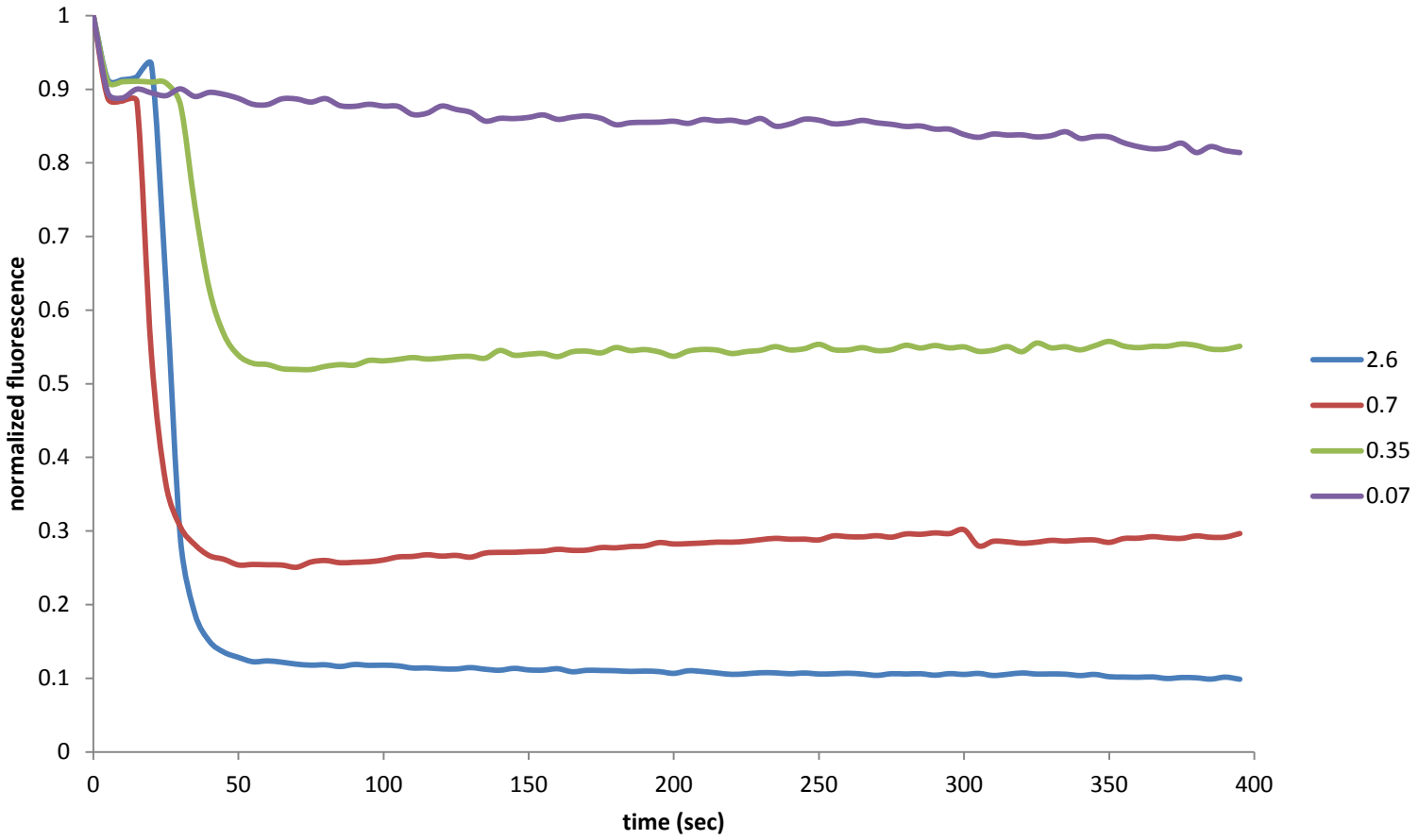
For that very reason, however, those that remain liquid (typically glycerol-based) are preferable when three-dimensional (3D) information is desired.

Mind the refractive index



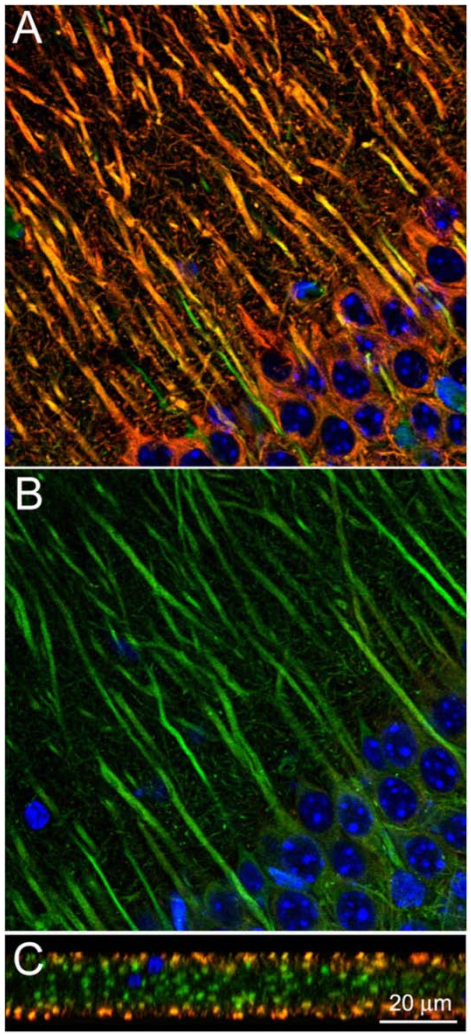
Increase in the refractive index of ProLong® Gold antifade reagent during the curing process.

GFP signal loss after fixation



Manuel Peter

Incomplete sample penetration



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The tissue was labeled with three colors: blue (DAPI); green (GFP), marking the dendrites; and red (Cy3), marking the microtubule-associated protein MAP2 by indirect antibody labeling.

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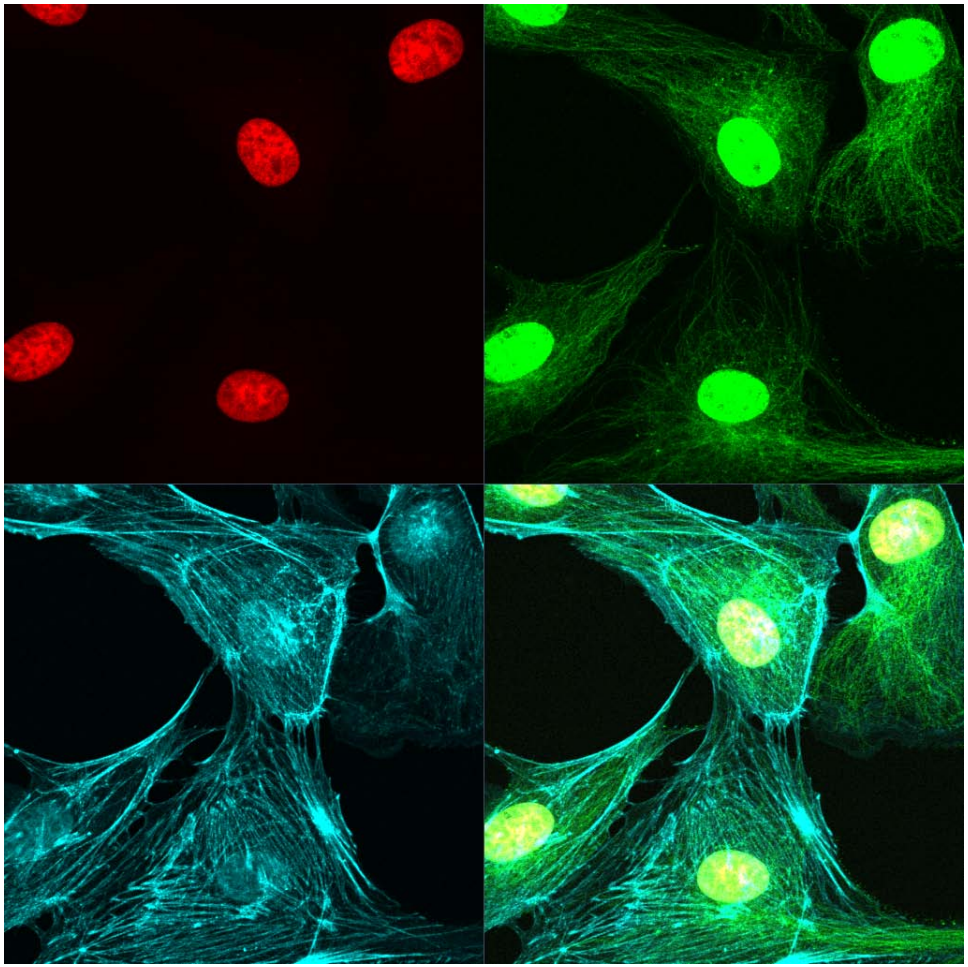
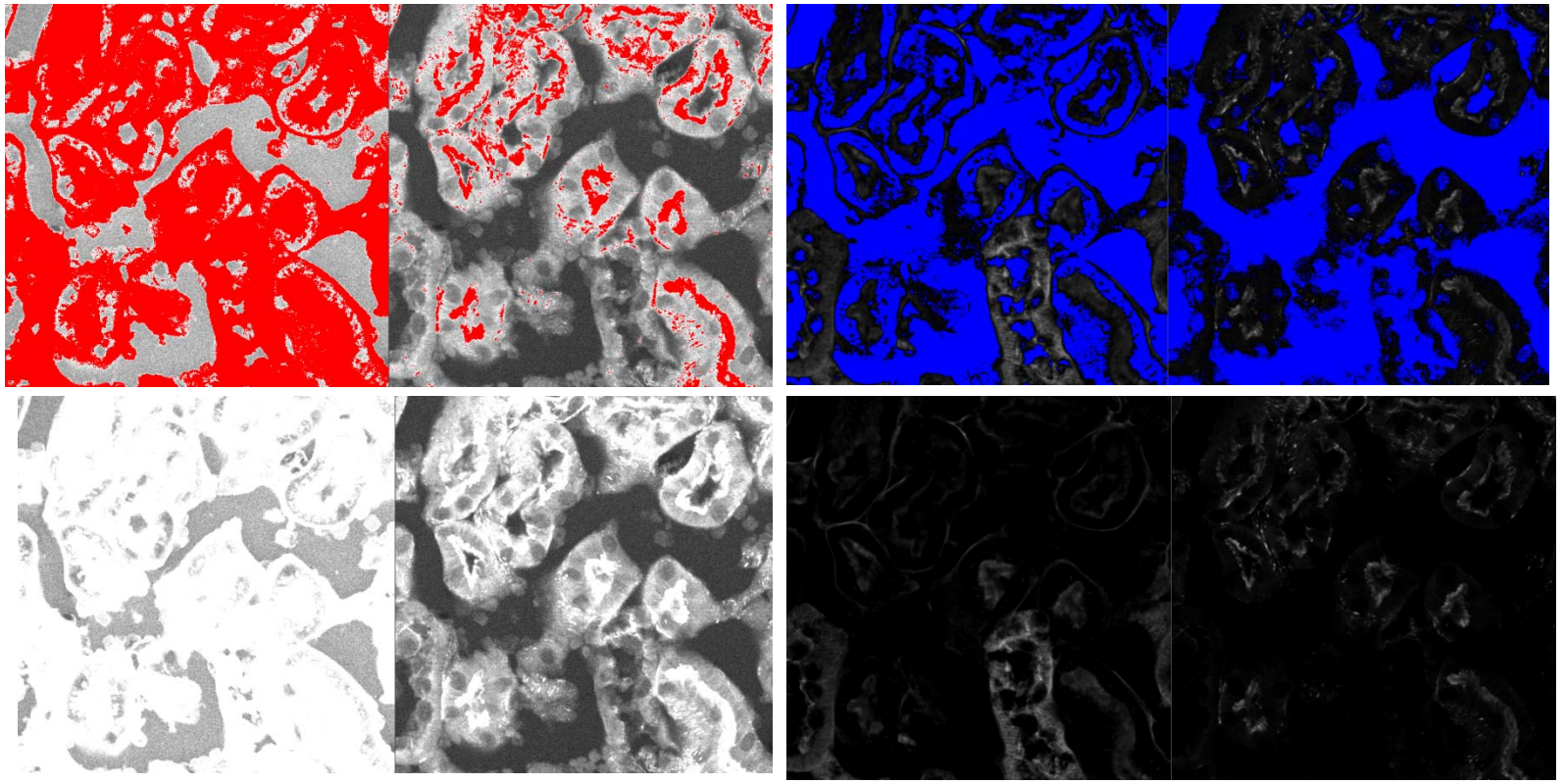
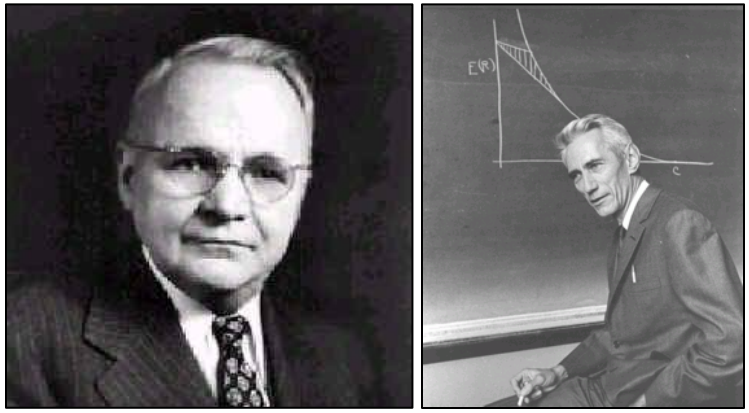
Cross talk



Saturation - offset



Sampling frequency - Nyquist-Shannon theorem



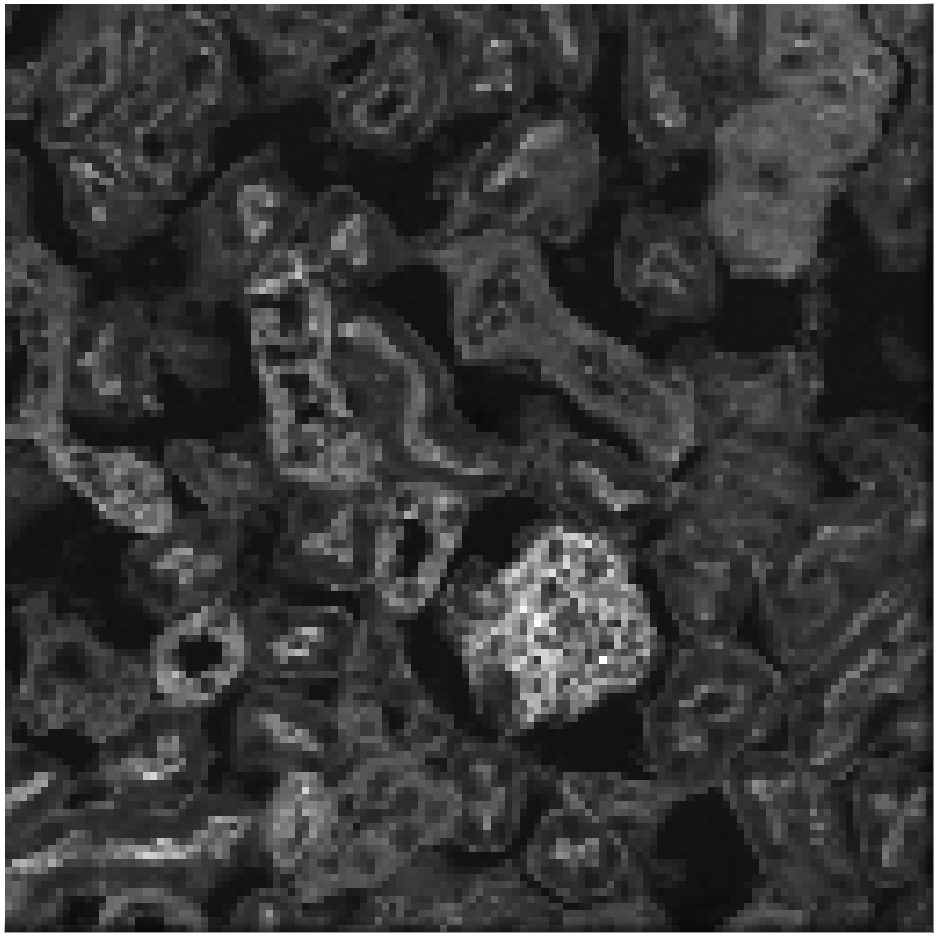
In order to get images into a form computers can manipulate they must be modified from their analog form (continuous gradients) into a digital form (step gradients) using the technique of sampling. Sampling is the key technique used to digitize analog information such as sound, photographs, and images.

Nyquist sampling (f) = d/2, where d=the smallest object, or highest frequency, you wish to record. The sampling frequency should be at least 2x the highest frequency you wish to record in your image

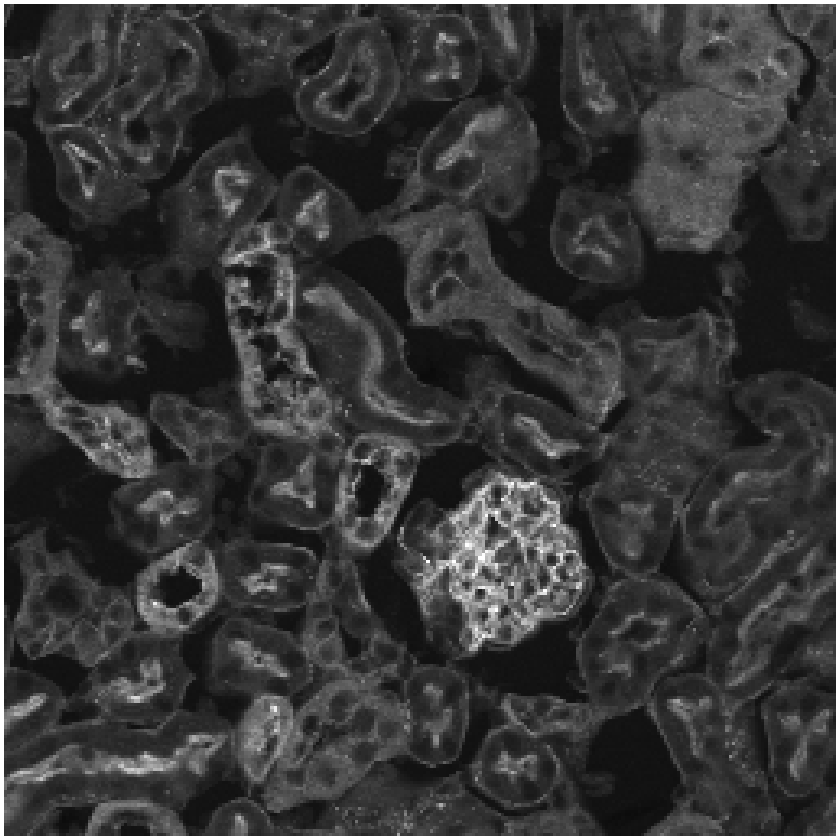
With images, frequency is related to structure size. Small structures are said to have a high frequency. Thus, the imaging sample rate (or pixel) size should be 1/2 the size of the smallest object you wish to record.

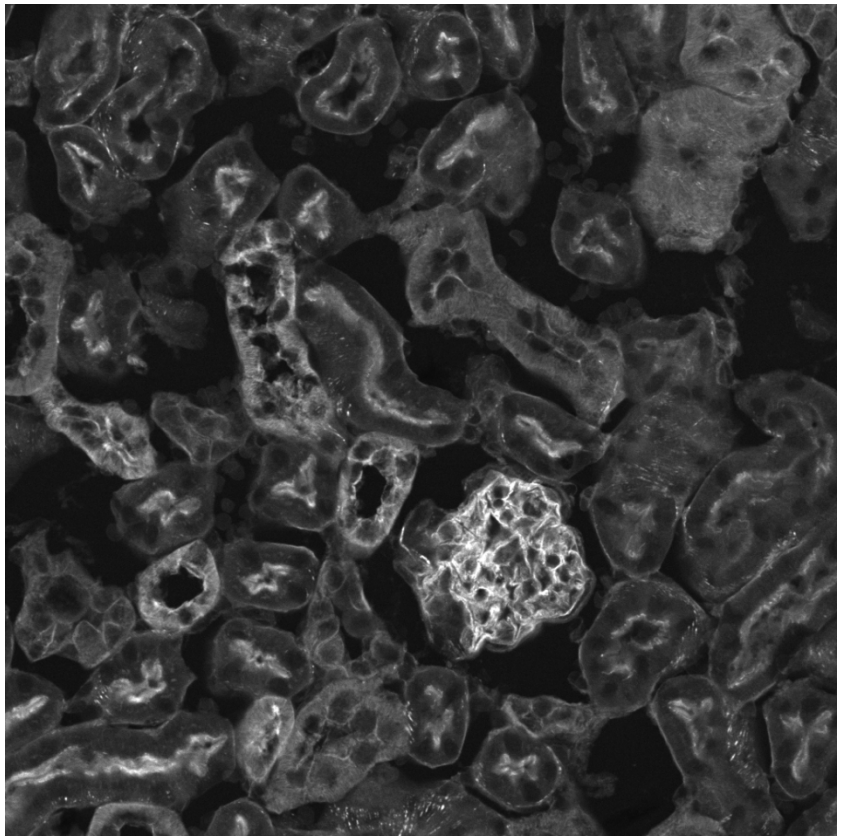
http://redwood.berkeley.edu/bruno/npb261/aliasing.pdf

Undersampling

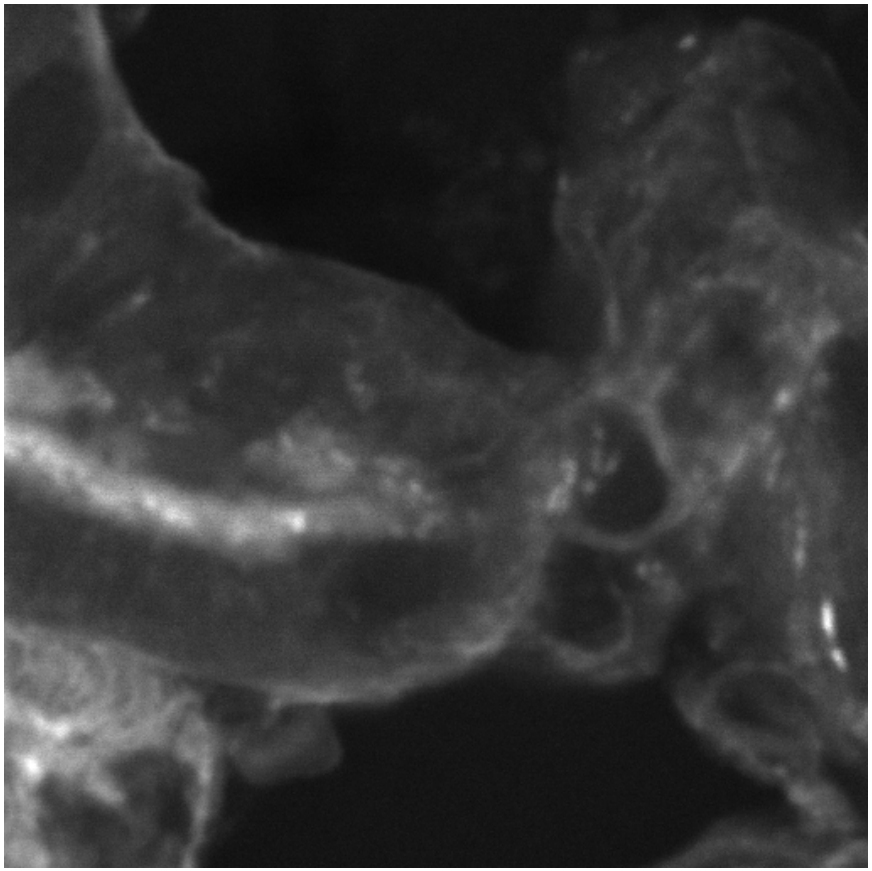


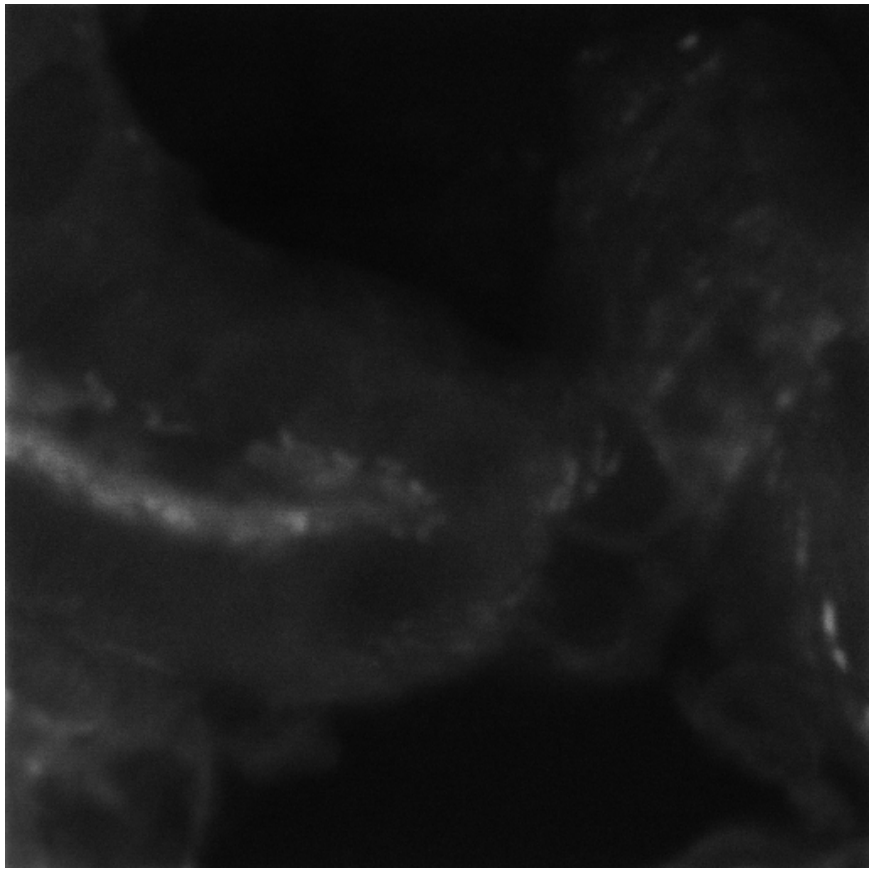
Undersampling



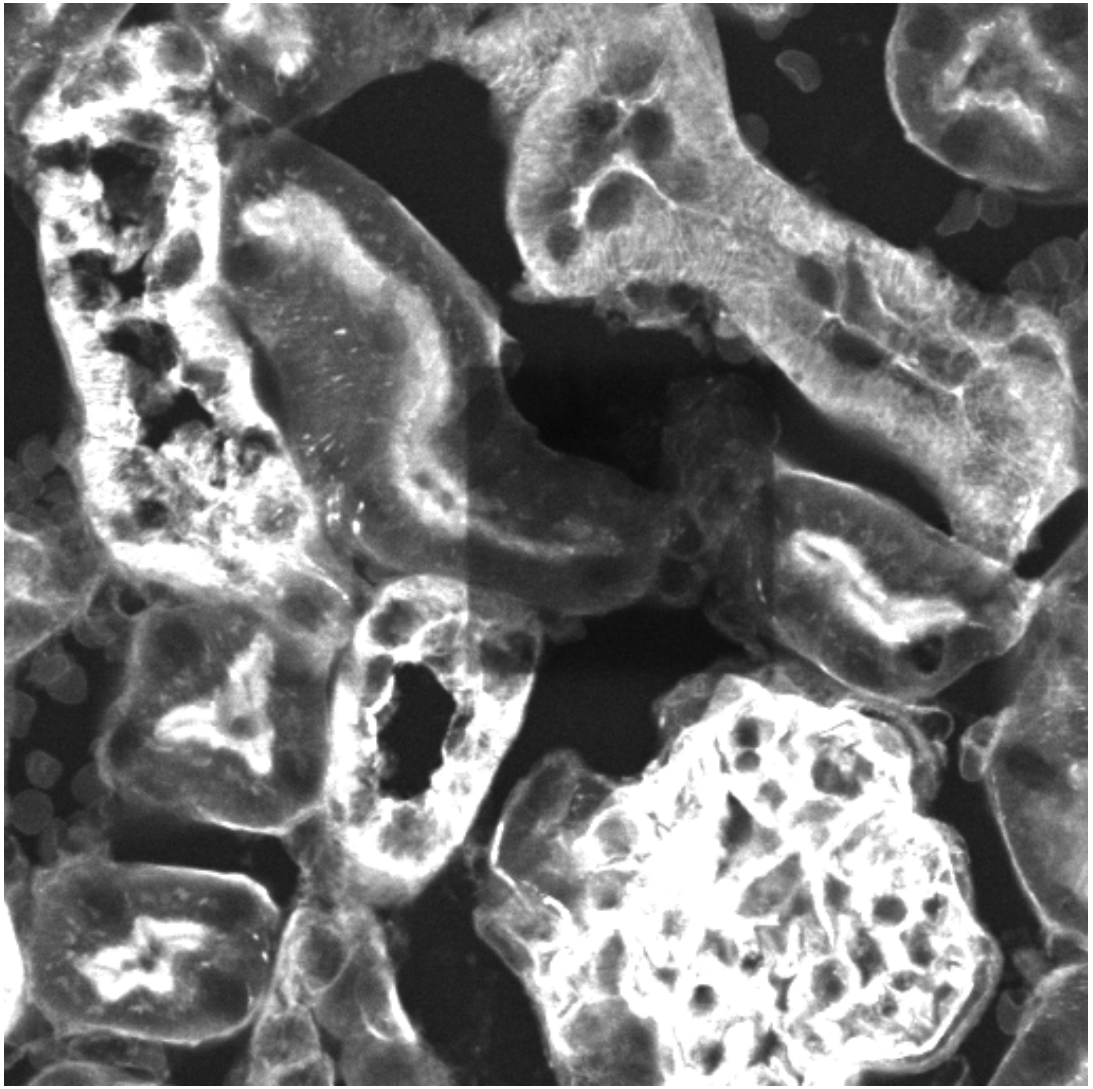


Bleaching

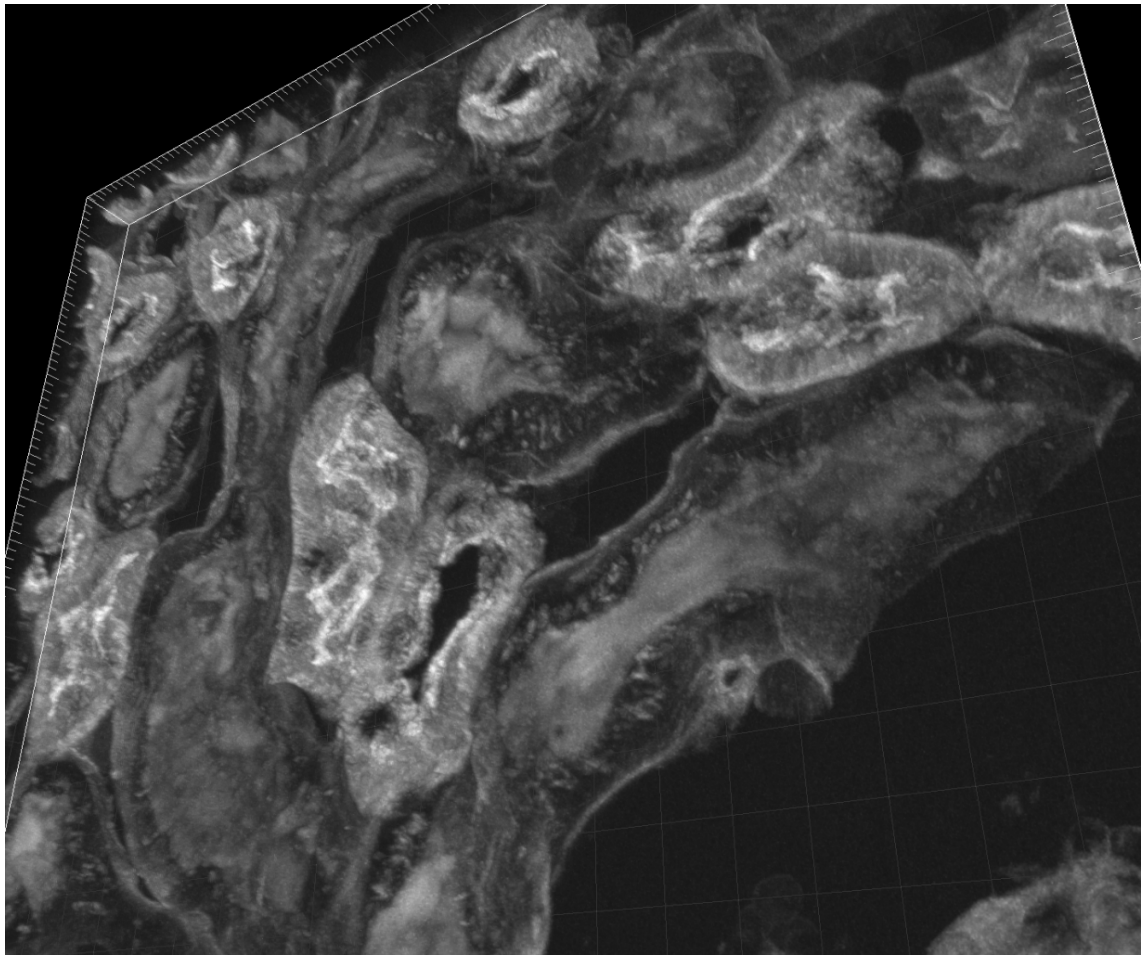




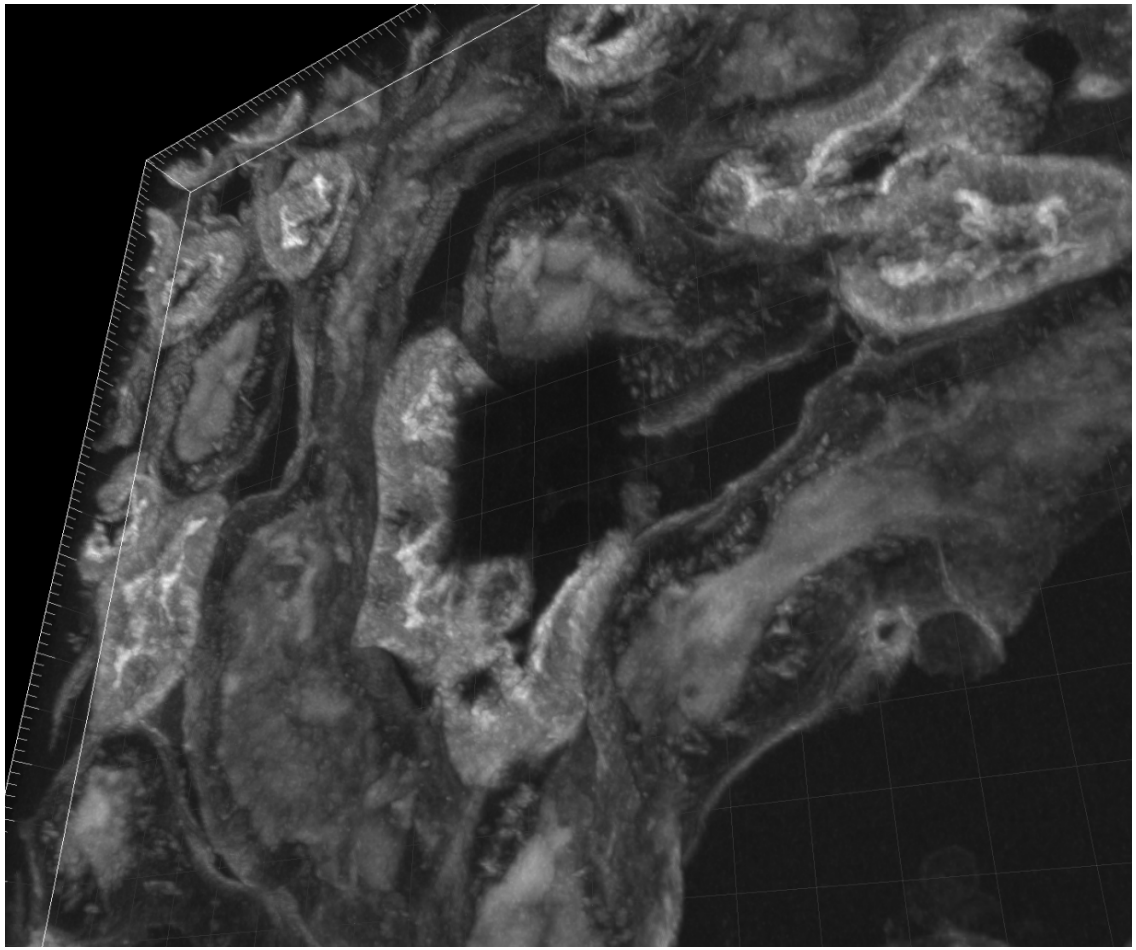
Bleaching



Bleaching



Bleaching



Low number of repetitions

Take-home message III

1. Think about technical requirements before setting up the experiment
2. Choose the best imaging system that will suit your experiment
3. Think and MAKE all necessary controls
4. Remember the sample is part of the optical system
5. Set-up the acquisition parameters so that you get data that can be analyzed
6. Controls
7. Clean the "optics" – think aberrations
8. Save the raw data
9. If possible employ as much of automatic acquisition as possible


Like

Quality in = Fame out