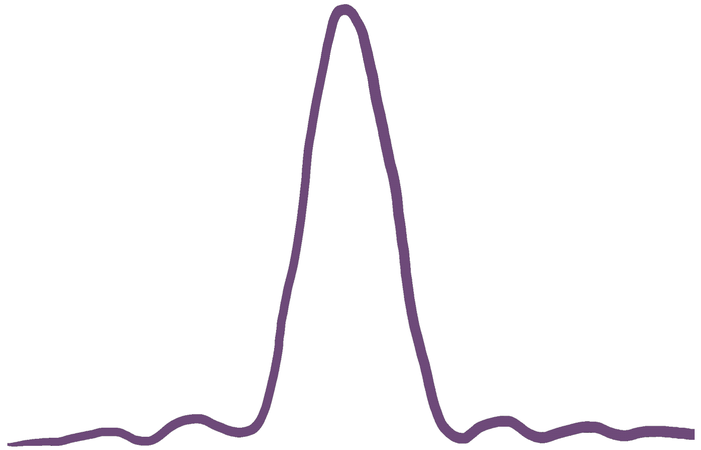
Introduction to Quantitative Fluorescence Microscopy



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BoBiAC

Boston Bioimage Analysis Course | 2025

CITE

Core for Imaging Technology & Education

HARVARD MEDICAL SCHOOL

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Slides & feedback from Team CITE & IAC!

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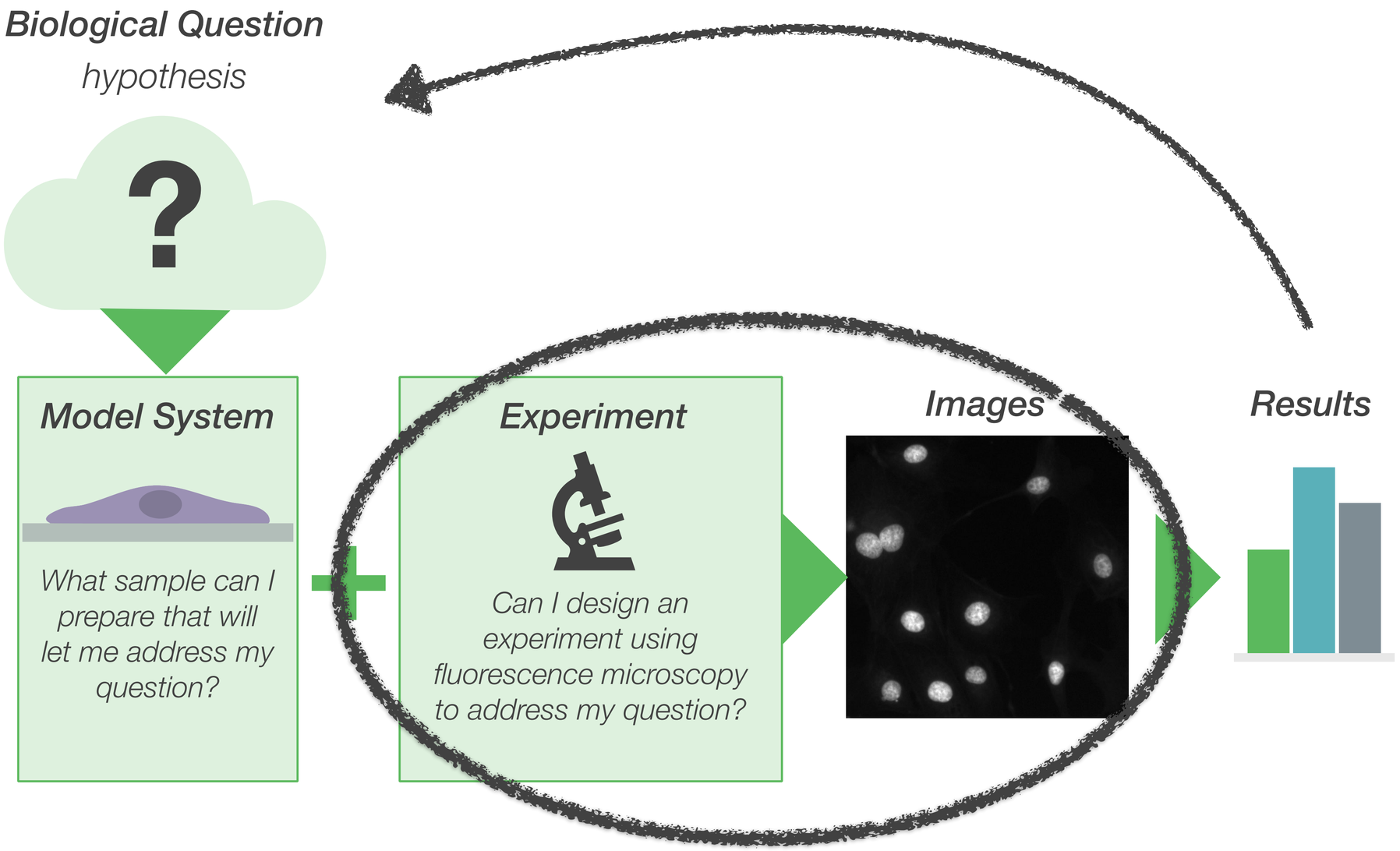
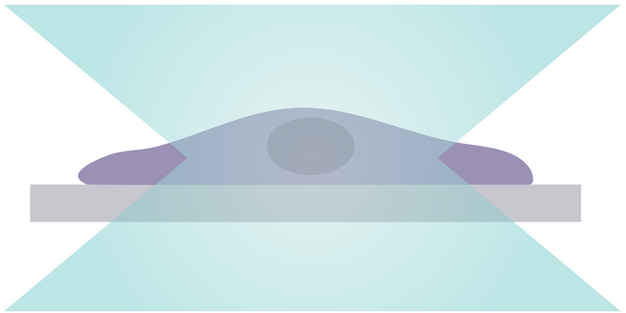

Biological Question
hypothesis
?
Model System
What sample can I prepare that will let me address my question?
Experiment
Can I design an experiment using fluorescence microscopy to address my question?
Images
Results

Fluorescence Microscopy Techniques

Fluorescence Microscopy

widefield



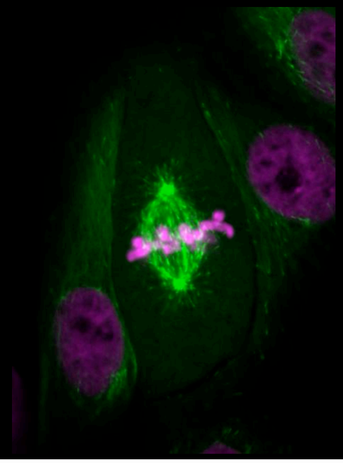
optical sectioning:

- confocal
- total internal reflection
- multiphoton
- structured illumination
- light sheet

Why ***fluorescence microscopy***?

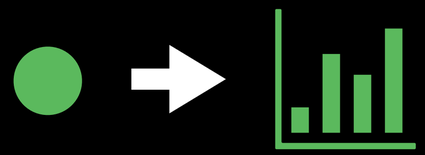
Specific

Live-cell Compatible

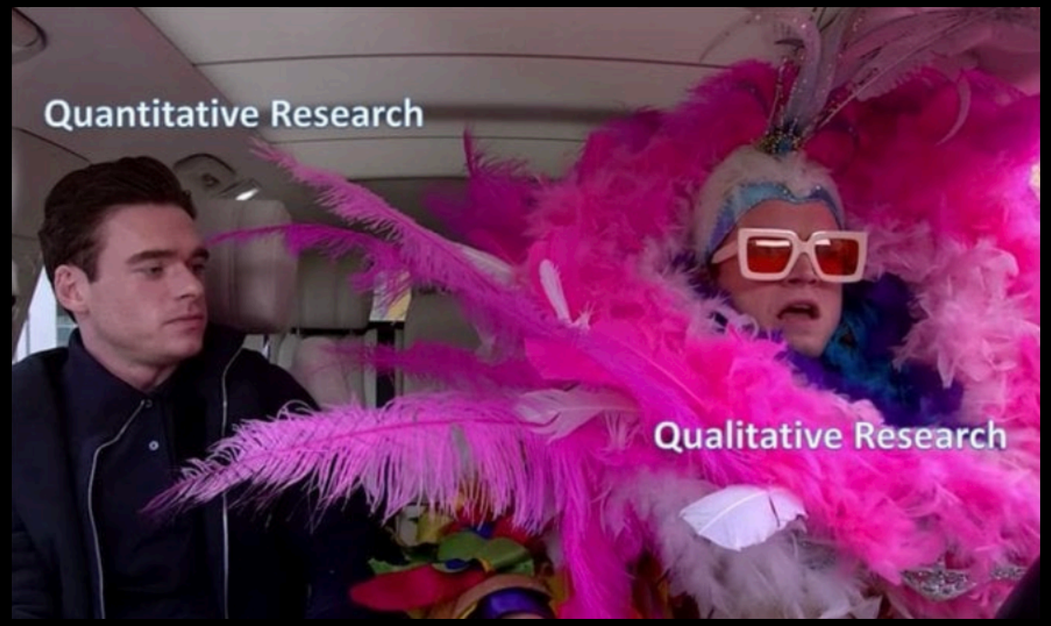


Sensitive

(Potentially)
Quantitative



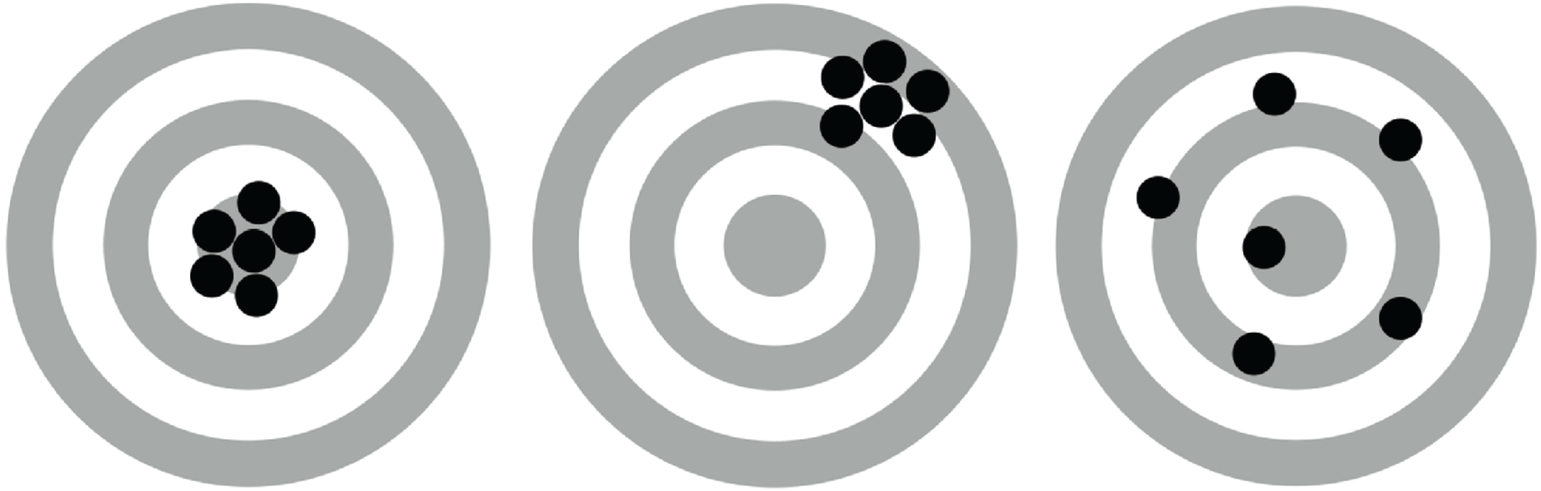
(Potentially)
Quantitative



To make a ***quantitative*** measurement, you need to think about…

Accuracy: The agreement between a measurement and the ground truth.

Precision: The uncertainty / repeatability of a measurement



Ground Truth: 100.00

150.00+/-0.01

inaccurate

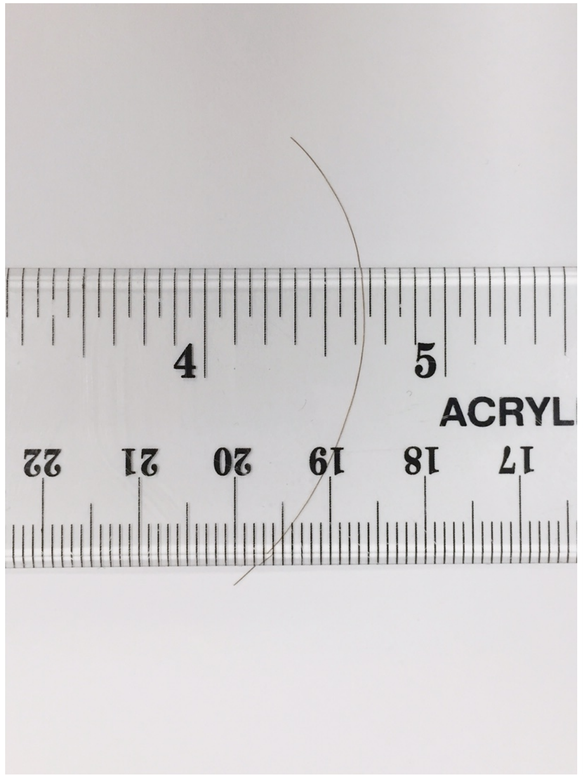
just plain wrong

100.00+/-50.00

imprecise

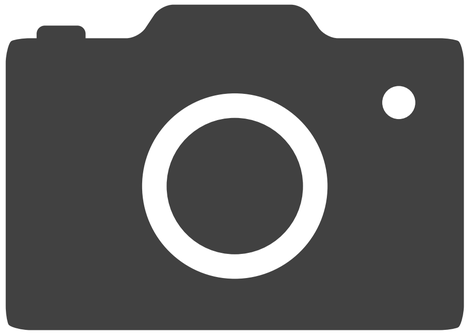
low confidence in individual measurements

The tool limits
accuracy & precision of measurements



Tools in Microscopy

Microscope



Detector

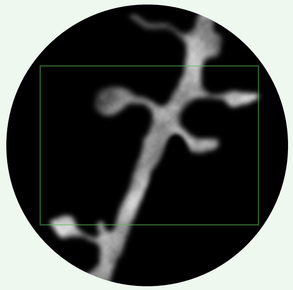
sample prep

fixation

fluorophore labeling

microscope

optical image



detector

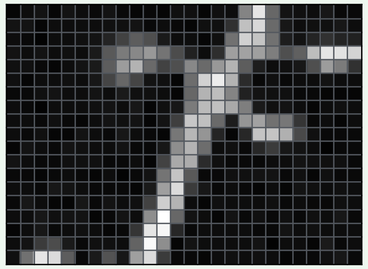
ground truth

measurement

specimen

All of these steps get you farther from the ground truth.

digital image



Factors that can limit accuracy and precision in fluorescence microscopy

resolution

sampling

signal to noise ratio (SNR)

background

resolution

What does resolution even mean???

detection *is not* resolution

electron microscopy

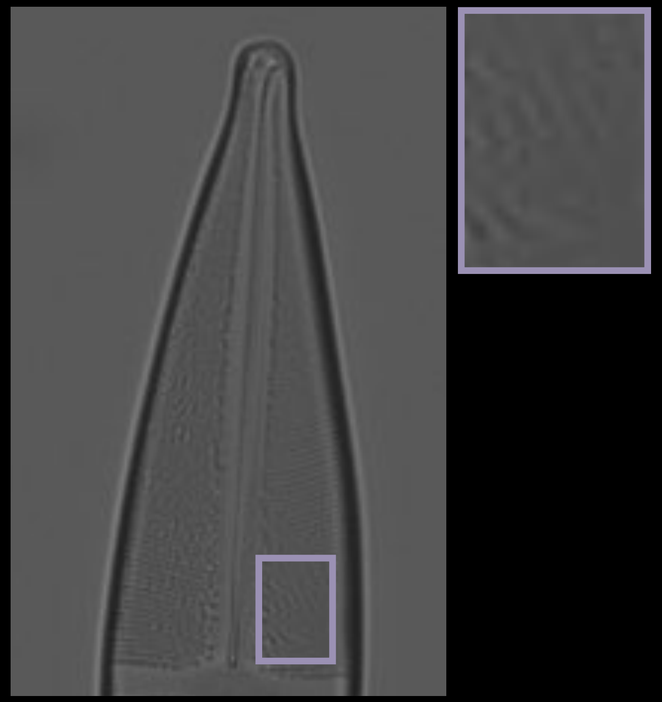
light microscopy

Coronavirus image: Hans R. Gelderblom, Freya Kaulbars/RKI

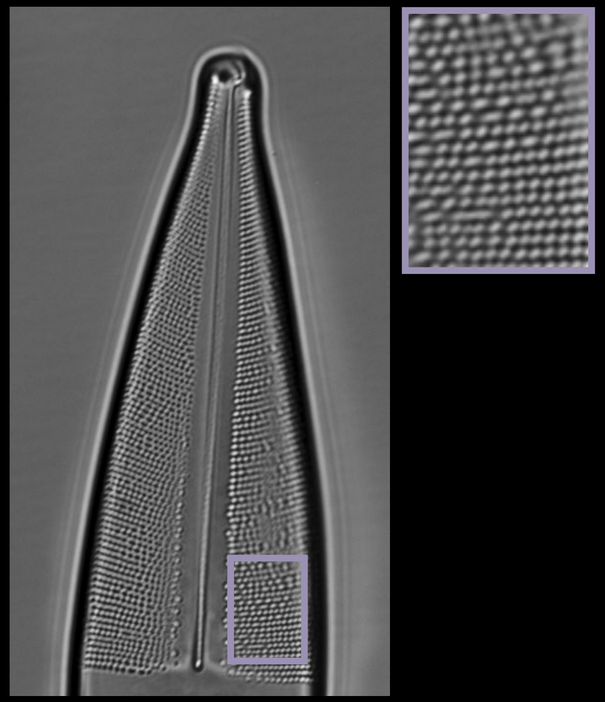
resolution

the ability to distinguish objects that are separate in the sample as separate from one another in the image of the sample

low resolution image



high resolution image



Images of *Diatoms,* Brightfield

resolution

the ability to distinguish objects that are separate in the ***sample*** as separate from one another in the ***image of the sample***

you never get to look at your sample

Resolution | Sampling | SNR | Background

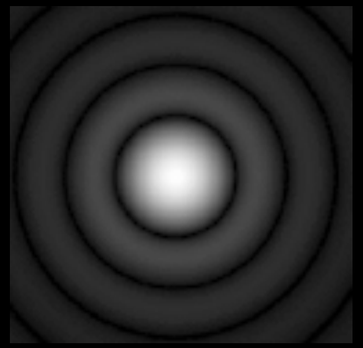
How does the *image of your sample* formed by the microscope differ from your *sample*?

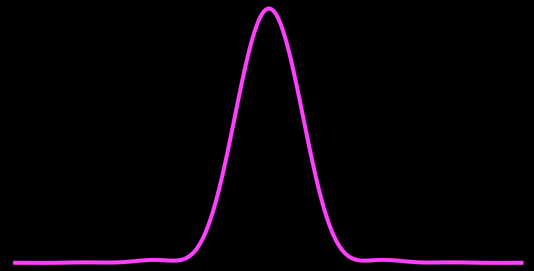
Resolution | Sampling | SNR | Background

Consider a point source of light…

Expectation:

Reality:

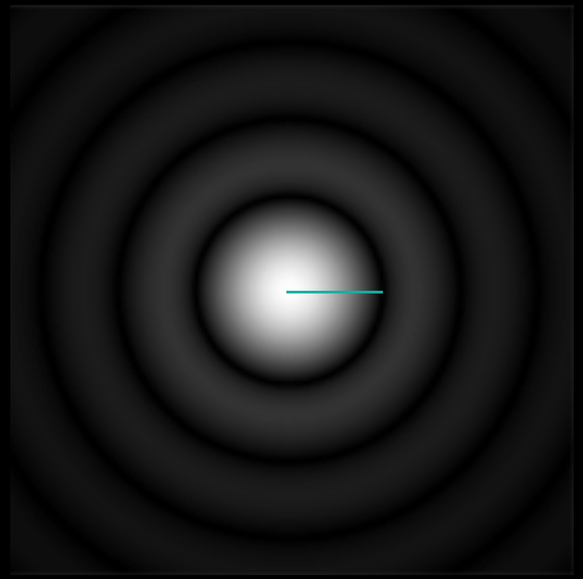




point spread function (PSF)

how big is the PSF?

lateral (xy) resolution



$$d_{min} = 0.61\lambda / NA_{obj}$$

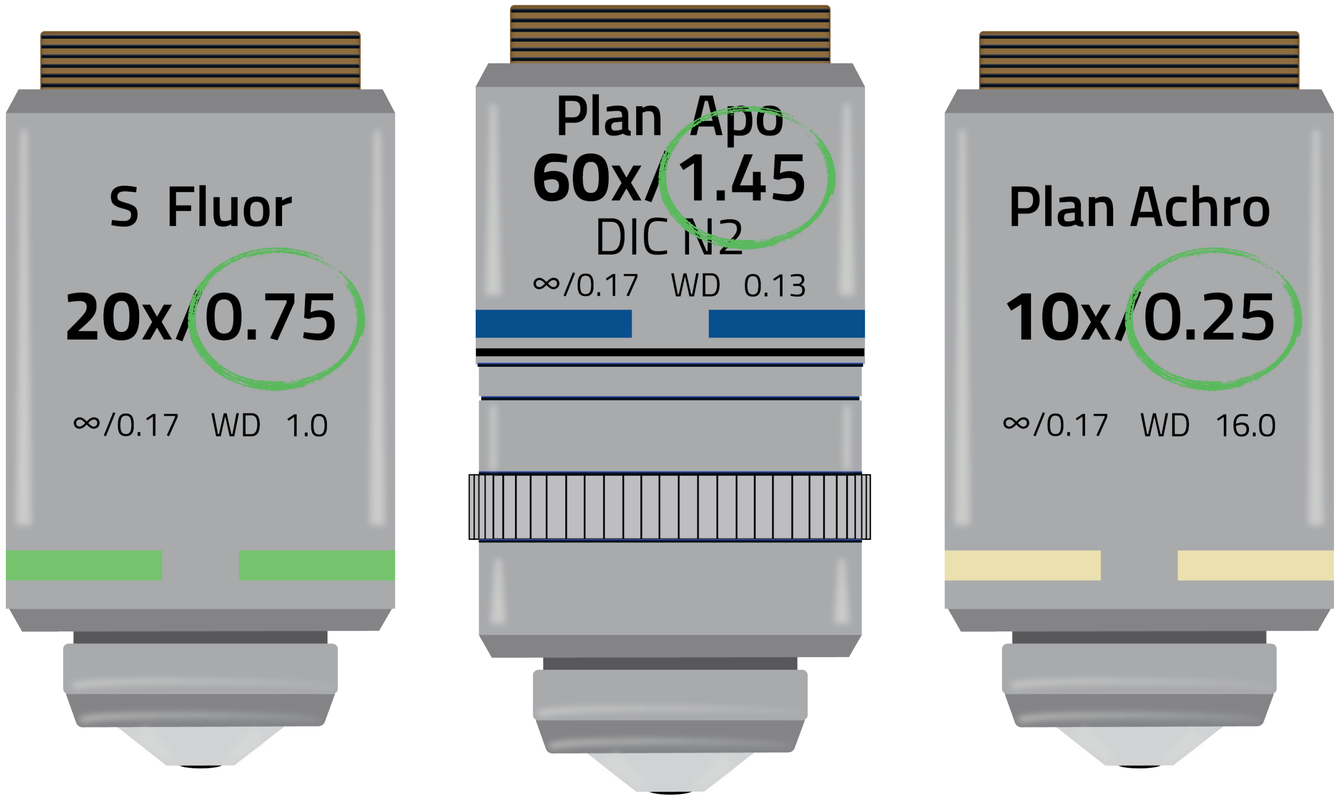
d_{min} = minimum distance between objects that can be resolved = radius of first minimum

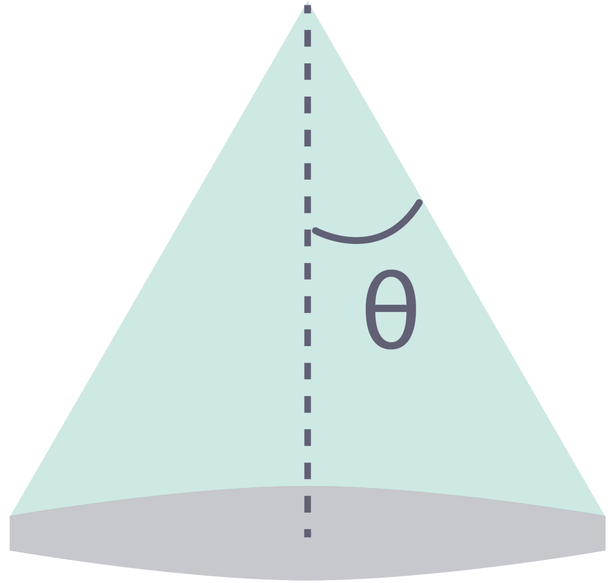
λ = emission wavelength

NA = numerical aperture

this is a property of the objective lens

Numerical Aperture (NA) is an objective lens property





$$NA = n\sin\theta$$

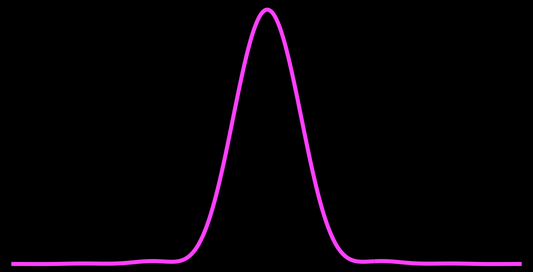
n = refractive index of immersion medium

higher NA —> more light collection

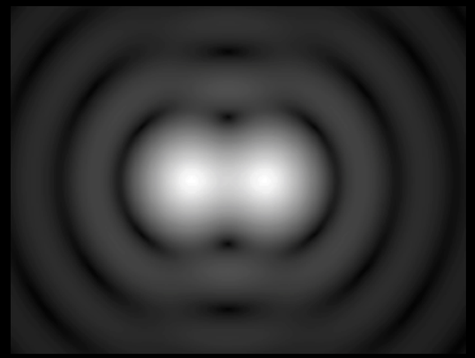
more light collection —> higher resolution

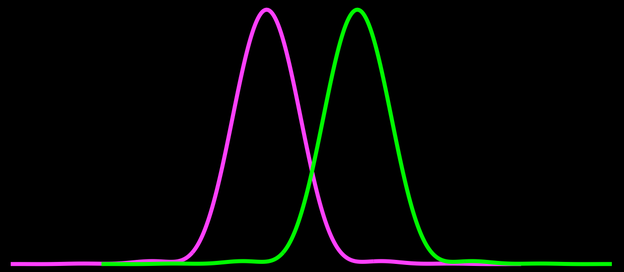
How does the PSF limit our ability to resolve 2 objects?

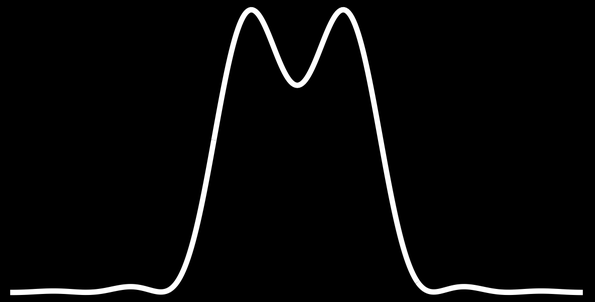
1 point source:



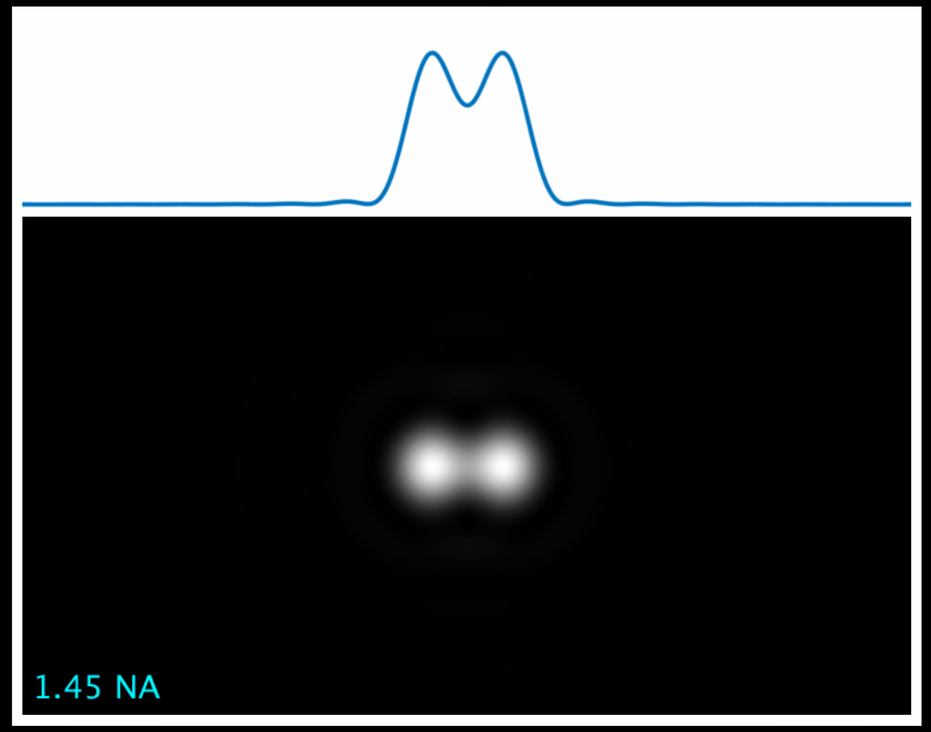
2 point sources:







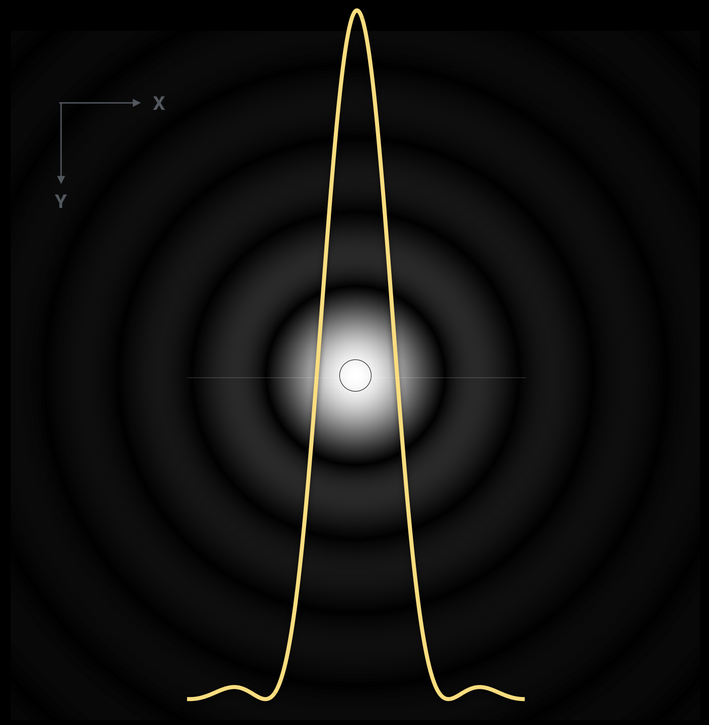
Resolution is limited by the size of the PSF

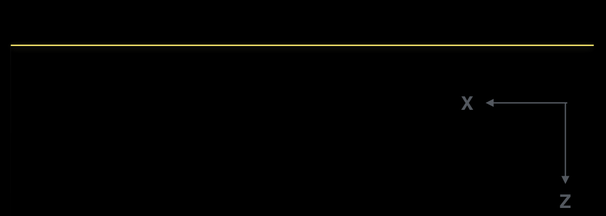


Animation: Talley Lambert

What about *axial* resolution?

the point spread function (PSF) is 3D





lateral

axial

Talley Lambert

how big is the PSF in Z?

Axial (Z)

$d_{min} = 2\lambda n/(NA_{obj})^2$

d_{min} = minimum distance between objects that can be resolved

λ = emission wavelength

NA = numerical aperture

n = refractive index of immersion media

Jennifer Waters

So far we have emphasized how theoretical resolution limit is dependent on NA…

$$d_{min,lateral} = 0.61\lambda / NA_{obj}$$

$$d_{min,axial} = 2\lambda n/(NA_{obj})^2$$

What about wavelength (λ)?

Wavelength dependency of theoretical resolution limit

theoretical resolution limits in nanometers when using a 1.4 NA oil immersion objective lens

Wavelength	Lateral	Axial
350	153	464
400	174	531
450	196	597
500	218	663
550	240	730
600	261	796
650	283	862
700	305	929

shorter θ —> higher resolution

What does the PSF mean for the image of ***your*** sample?

each point in the specimen is **convolved** with the point spread function

225nm*

⊗

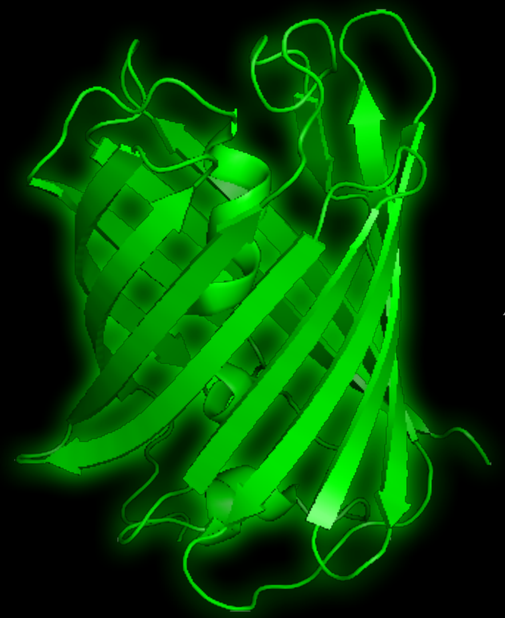
=

**green light and 1.4 NA oil lens*

let’s think about what diffraction-limited resolution means for images of a cell.

assume the resolution limit is 240 nm in XY.

proteins ~2nm
membrane 5-10nm thick
ER tubule 60-100nm in diameter
mitochondria 200-500nm in diameter



4.2nm

2.4nm

Resolution | Sampling | SNR | Background

Resolution | Sampling | SNR | Background

interacting

Resolution | Sampling | SNR | Background

not interacting

Resolution | Sampling | SNR | Background

Important Point #1

Resolution is fundamentally limited by the size of the PSF.

sampling

Sampling: Selection of a subset meant to represent the whole

spatial sampling: pixel size

ground truth — me!

measurement — digital image of me

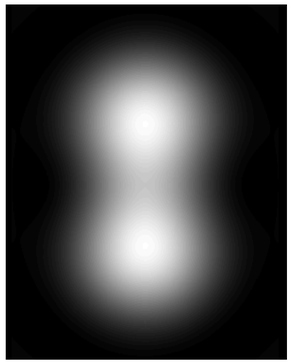
larger pixel size

smaller pixel size

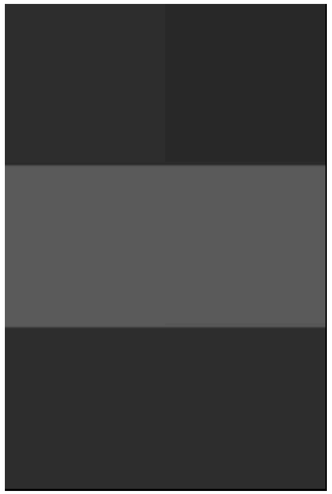
spatial sampling

ground truth — 2 fluorescent beads

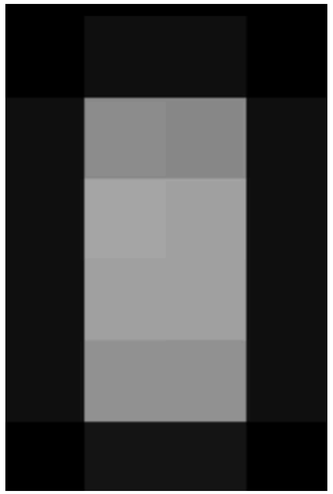
measurement — digital image of the beads



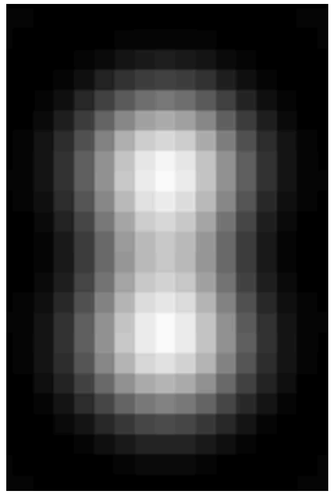
undersampling (aliasing)



Nyquist



oversampling



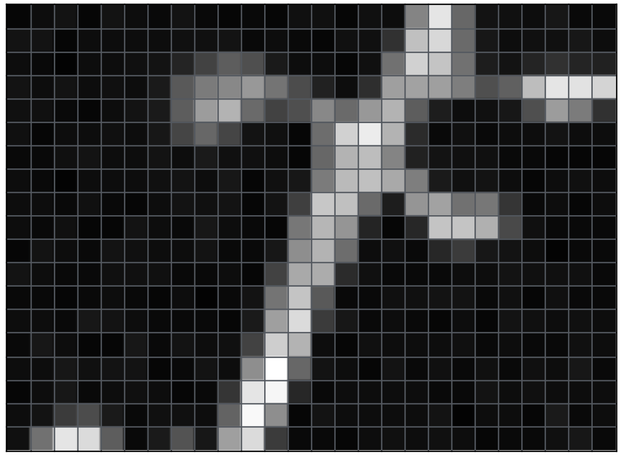
Images by Talley Lambert

Important Point #2

Improper sampling can result in lost or erroneous data. Oversampling can increase acquisition time and file size.

signal to noise ratio (SNR)

spatial



intensity

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
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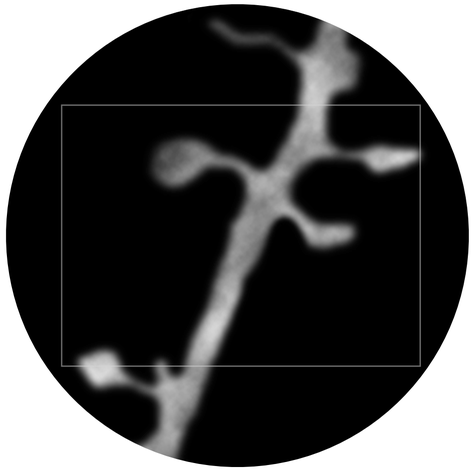
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

signal	photons you want to measure
background	additive increase in intensity values that’s not due to photons you want to measure
noise	fluctuations of measured intensity values

signal to noise ratio (SNR)

signal	photons you want to measure
noise	fluctuations of measured intensity values

Detector: Collect optical image photons and output digital image

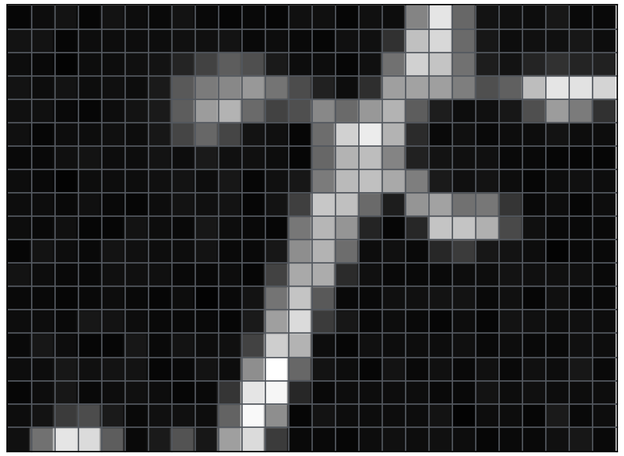


photons

optical image

detector

spatial



intensity

6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
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19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
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16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

intensity values

digital image

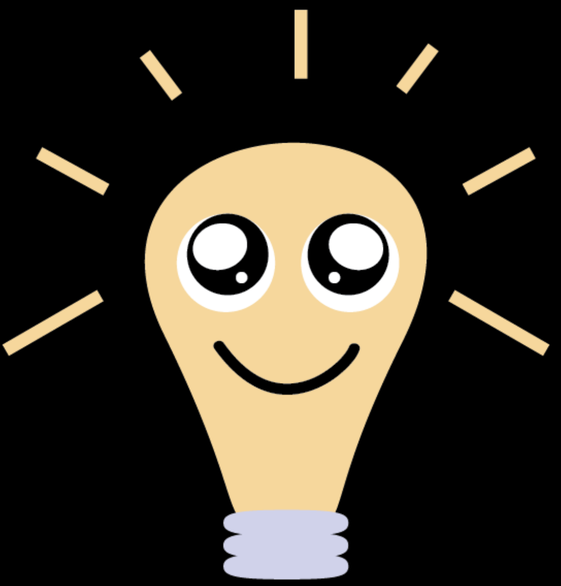
intensity values ≠ photons!!

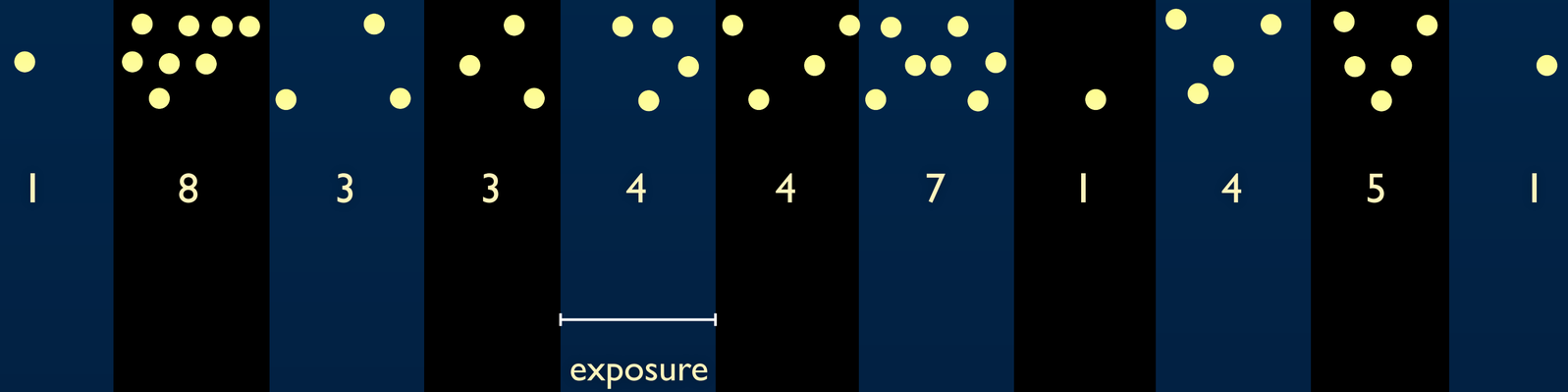
We send optical image photons to the detector…

…but do they all arrive at the same time?

exposure

Modified from Talley Lambert





Modified from Talley Lambert

Resolution | Sampling | SNR | Background

noise

Poisson Noise is caused by the stochastic arrival of optical image photons at the detector

signal to noise ratio (SNR)

signal	photons you want to measure
noise	fluctuations of measured intensity values

We will ***always*** have Poisson noise.

what can we do to increase our SNR?

Important Point #3

Poisson noise is a fundamental limitation in quantitative microscopy.
Maximizing signal increases SNR.

Is Poisson Noise the only source of noise?

What happens if we send no light to the detector and take an image?

noise

detector noise

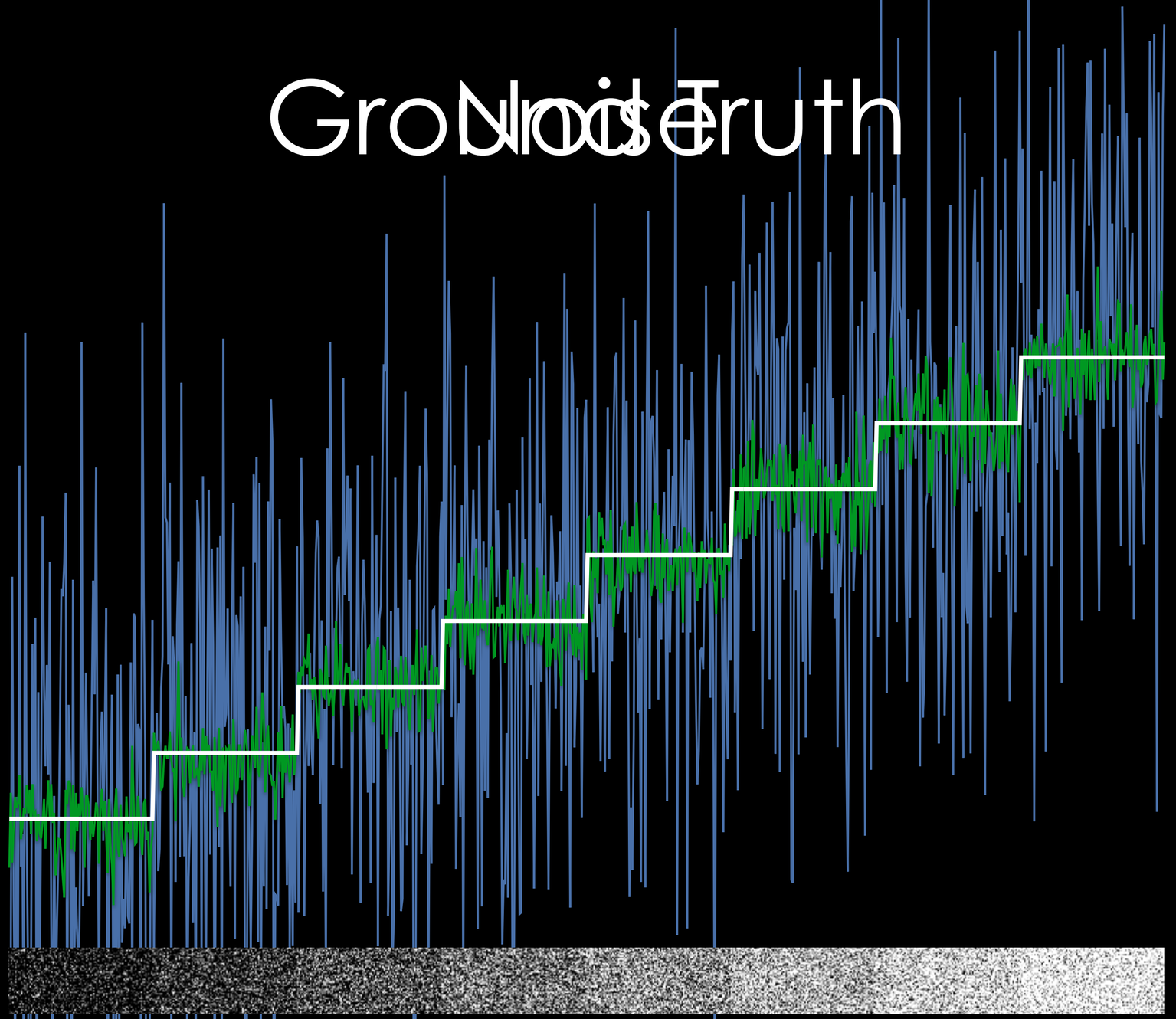
signal to noise ratio (SNR)

signal	photons you want to measure
noise	fluctuations of measured intensity values

Poisson noise

detector noise

How does SNR impact your measurements?



Resolution | Sampling | SNR | Background

Talley Lambert

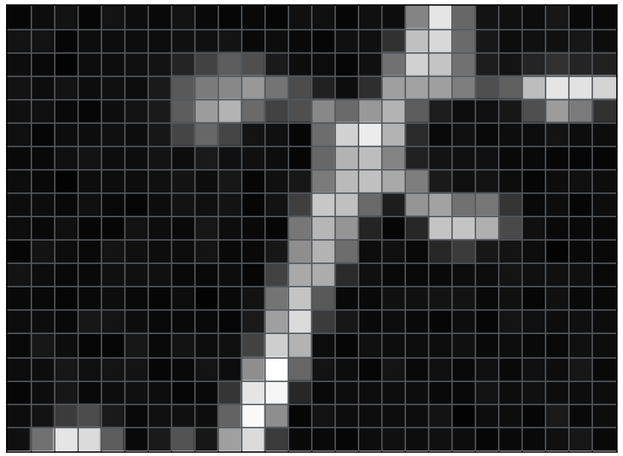
Important Point #4

Image SNR determines the minimum detectable signal and minimum detectable change in signal, which together affects achievable resolution.

background

Signal, background, & noise

spatial



intensity

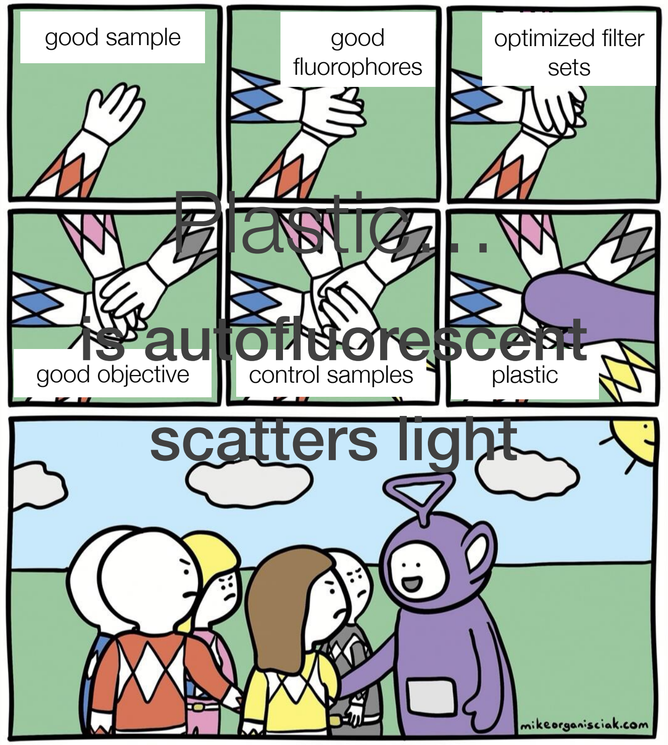
6	13	19	6	19	13	9	19	9	6	9	6	16	16	6	16	13	132	229	103	19	16	13	23	9	9
19	19	6	13	13	13	13	16	16	19	9	13	9	6	16	16	49	192	216	106	23	13	16	16	23	13
13	9	4	13	13	16	19	36	66	93	79	26	13	13	6	16	113	209	196	113	29	19	36	49	36	33
19	13	19	13	16	13	26	89	123	136	152	116	76	33	13	46	159	162	159	126	79	96	189	229	226	212
16	16	9	6	13	19	26	93	156	179	106	66	79	136	106	152	179	93	29	13	16	23	79	156	123	49
16	6	13	13	16	13	23	69	103	69	19	16	6	109	209	236	179	43	9	16	9	13	13	19	13	13
9	9	16	19	13	13	19	13	26	16	16	13	6	103	179	189	132	33	19	16	16	9	9	6	6	6
13	9	4	13	13	13	16	19	13	23	6	16	23	123	186	192	169	126	26	16	19	13	6	13	16	13
13	13	9	16	9	6	13	19	16	19	6	19	63	199	192	106	29	149	162	113	119	53	9	13	6	13
13	9	16	6	6	19	13	9	23	13	9	6	119	182	149	36	6	39	196	196	176	73	16	9	9	9
6	19	13	9	19	16	13	13	19	9	9	23	142	179	109	13	16	9	39	59	23	19	13	4	9	9
19	13	9	9	16	16	16	9	9	13	6	66	169	172	43	16	9	9	9	13	13	19	16	16	16	9
9	9	6	9	13	9	6	13	4	9	19	116	196	89	9	9	16	16	19	19	9	16	6	16	9	9
13	13	9	23	19	13	9	9	9	6	26	159	219	59	23	9	13	9	6	13	6	19	16	13	16	13
9	23	13	6	6	23	9	19	13	16	66	206	179	13	6	16	13	13	13	16	9	13	9	9	16	13
13	13	23	16	19	19	6	9	19	13	142	255	103	19	13	6	19	9	16	9	16	9	16	13	23	9
6	13	23	9	13	16	13	6	9	53	229	246	39	9	13	13	13	13	9	9	19	13	16	13	13	13
13	19	59	76	26	9	16	16	13	99	249	142	6	19	13	13	13	13	19	4	13	13	6	26	9	13
16	113	229	219	93	9	26	83	23	159	219	59	9	9	6	13	16	13	16	13	6	9	9	16	23	9

signal	photons you want to measure
background	additive increase in intensity values that’s not due to photons you want to measure
noise	fluctuations of measured intensity values

Examples of choices that increase background…

imaging with plastic

"Friends don't let friends image through plastic."



Plastic…

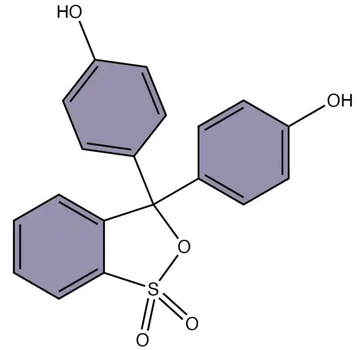
is autofluorescent

scatters light

using mounting medium w/DAPI

you can't wash out the unbound DAPI!

using imaging medium w/phenol

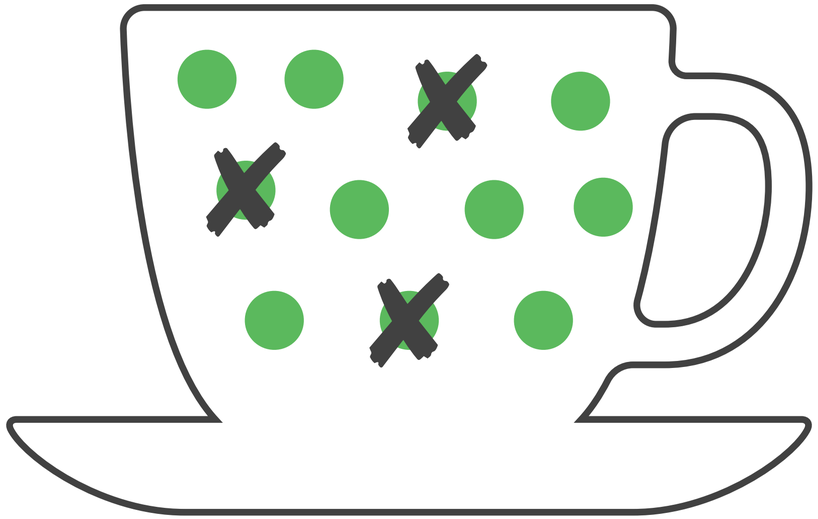


Phenol Red

Why is high background problematic?

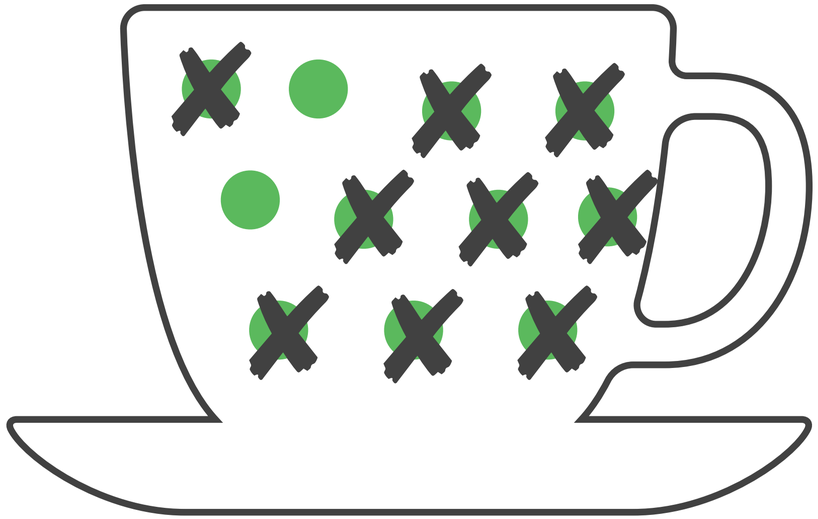
photon

background subtracted



high signal

low background



low signal

high background

Modified from Jennifer Waters

Important Point #5

Background decreases SNR.

Factors that can limit accuracy and precision in fluorescence microscopy

resolution

sampling

signal to noise ratio (SNR)

background

Together, what does this mean for you at the microscope?

practical considerations

photons available from sample

x

% photons detected

=

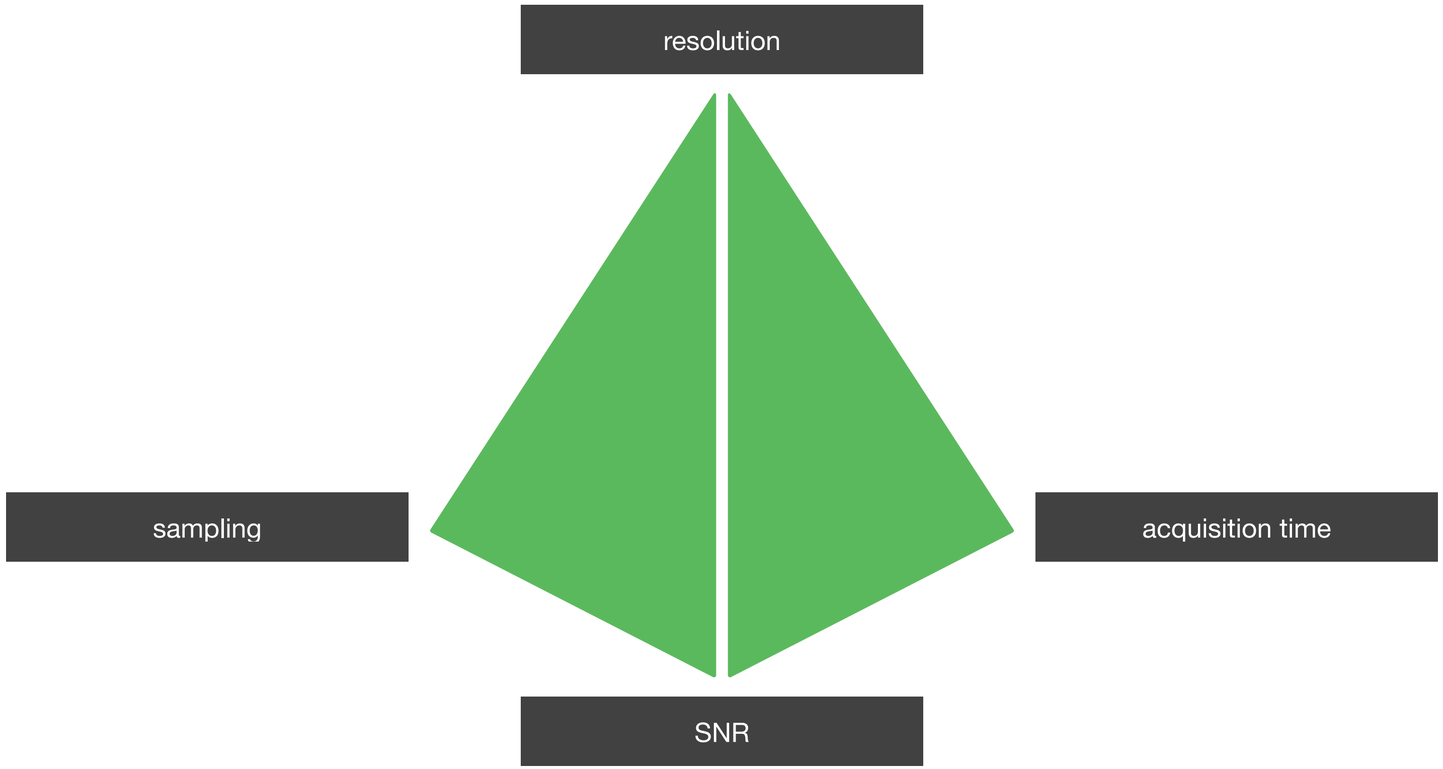
Photon Budget

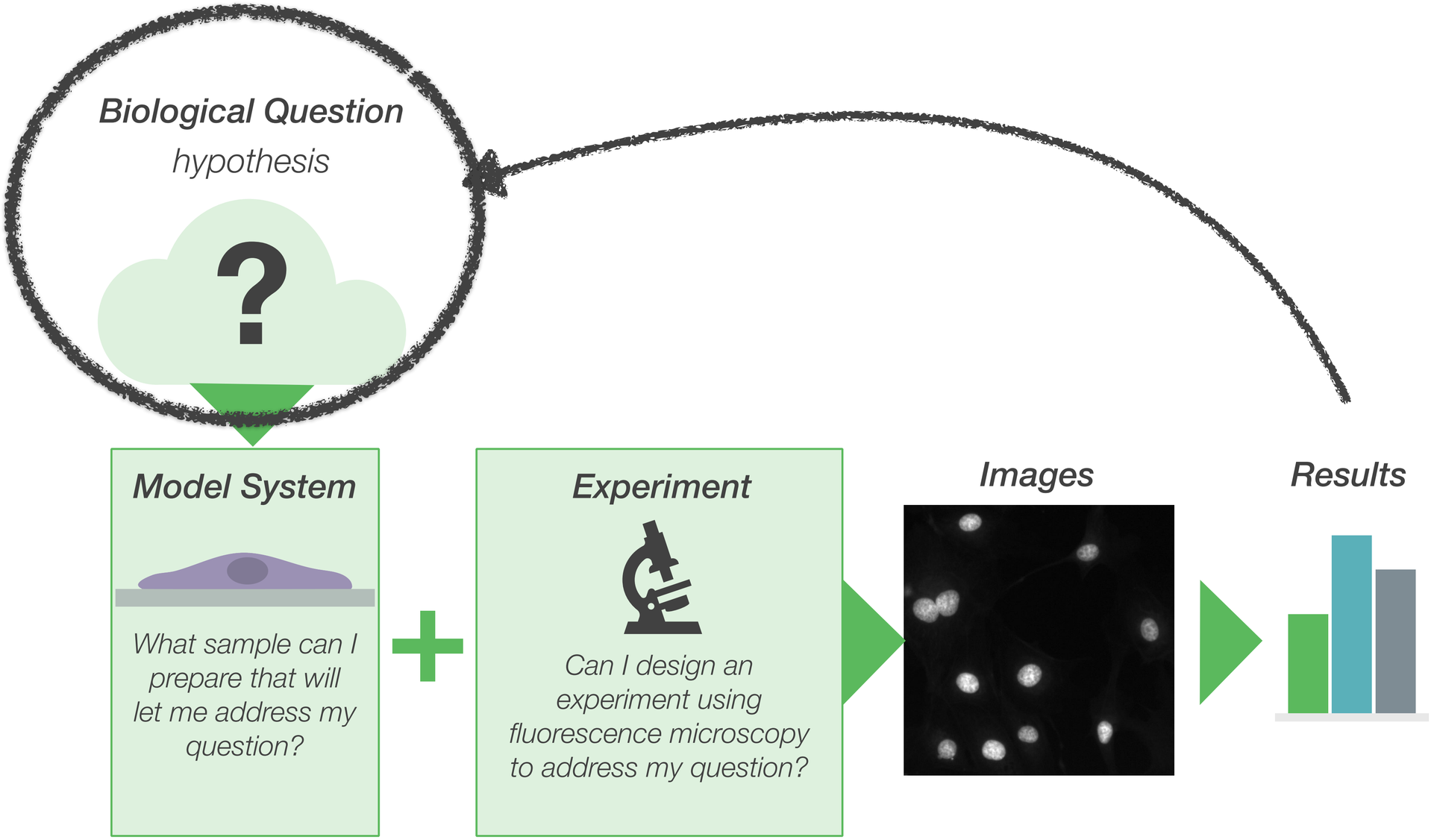
You have a limited number of photons.

How will you spend them?

The reality: You need to compromise at the microscope

prioritize…




Biological Question
hypothesis
?
Model System
What sample can I prepare that will let me address my question?
Experiment
Can I design an experiment using fluorescence microscopy to address my question?
Images
Results

Important Point #6

You must make compromises. Make educated & deliberate ones!

You need to validate your methodology

To be an excellent scientist, you need to convince yourself that something wrong **isn't** happening with your experiment.

Important Point #7

Always validate your methodology with control experiments.


Now what?

Resources anywhere in the world

microtutor

an interactive, remote course on fundamental principles in optical microscopy

microtutor.globalbioimaging.org

µcourses

youtube.com/microcourses

short, informative videos on microscopy concepts

Questions?