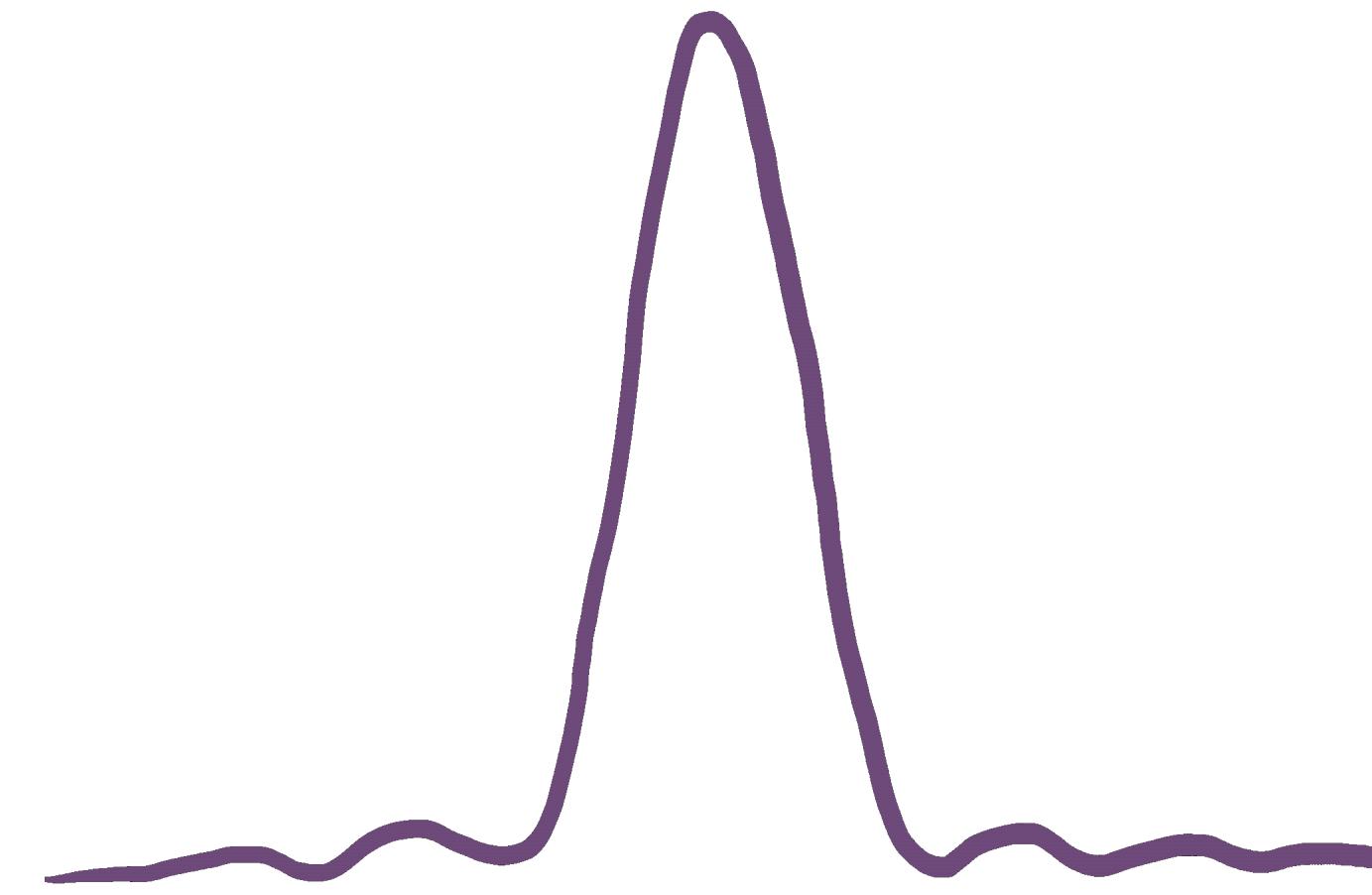


# Introduction to Quantitative Fluorescence Microscopy



**Eva de la Serna, PhD**  
Advanced Microscopy Postdoc Fellow

# Slides & feedback from Team CITE & IAC!



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## *Biological Question*

*hypothesis*



### *Model System*



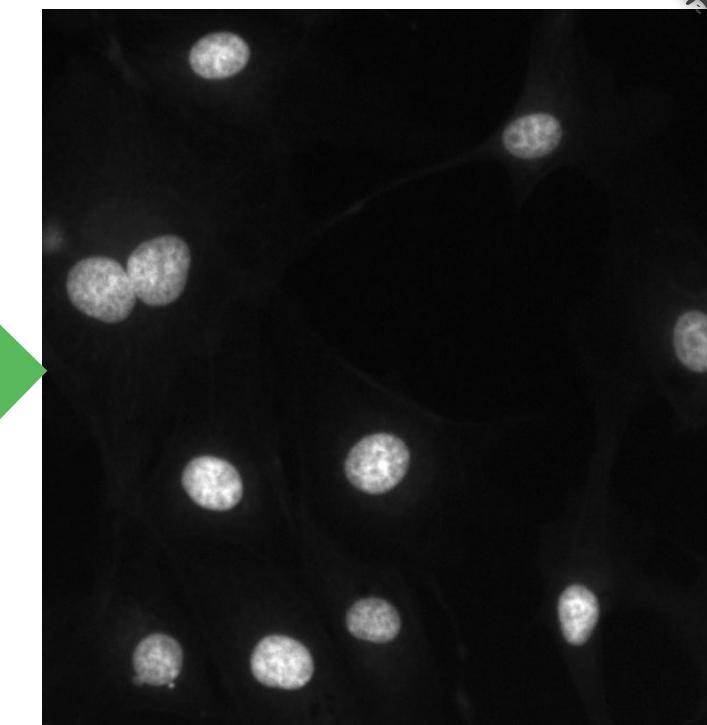
*What sample can I prepare that will let me address my question?*

### *Experiment*



*Can I design an experiment using fluorescence microscopy to address my question?*

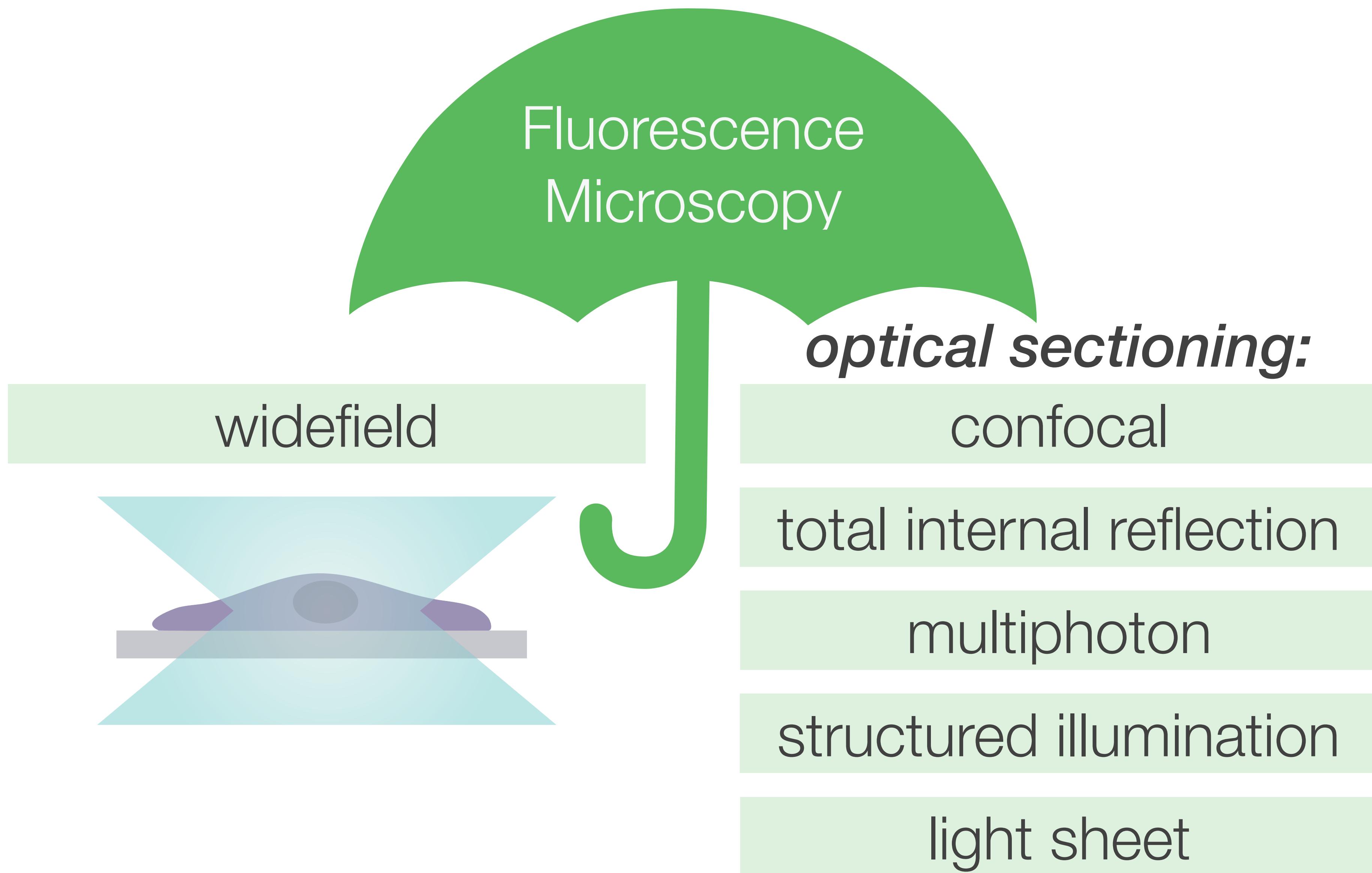
### *Images*



### *Results*

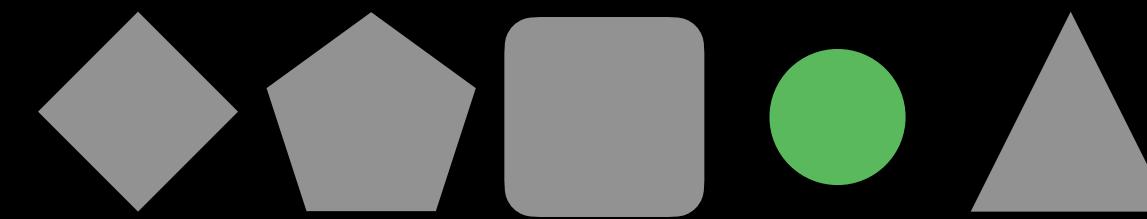


# Fluorescence Microscopy Techniques

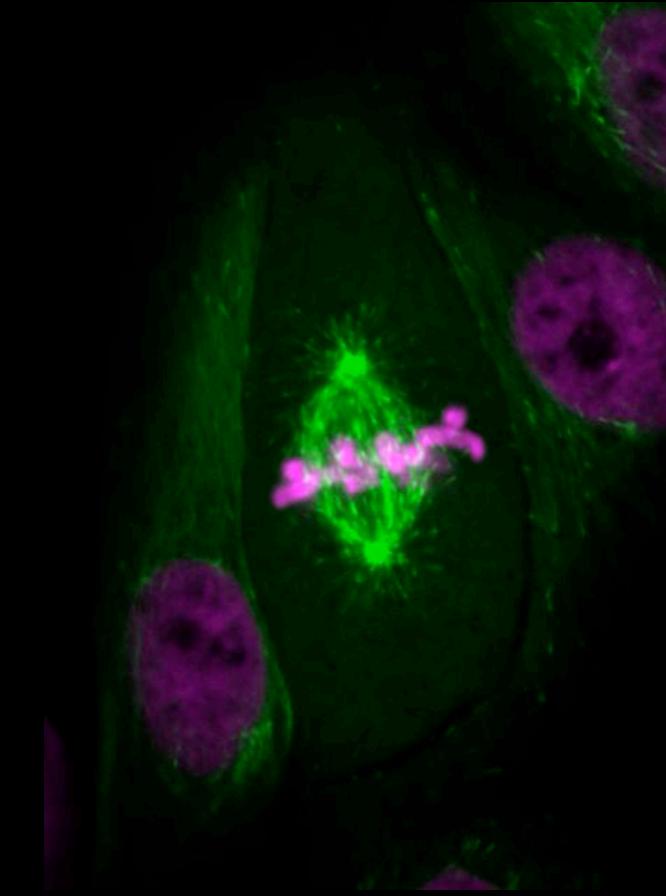


# Why *fluorescence microscopy*?

Specific



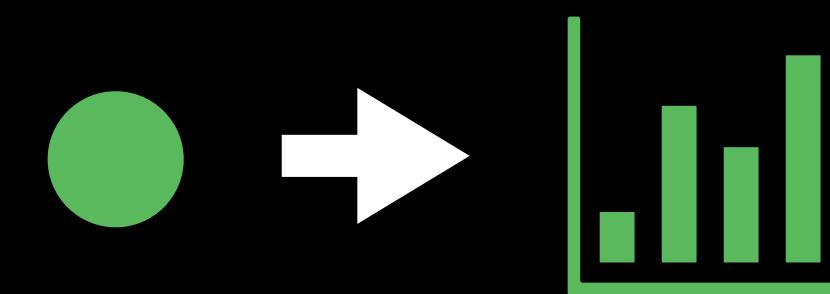
Live-cell Compatible



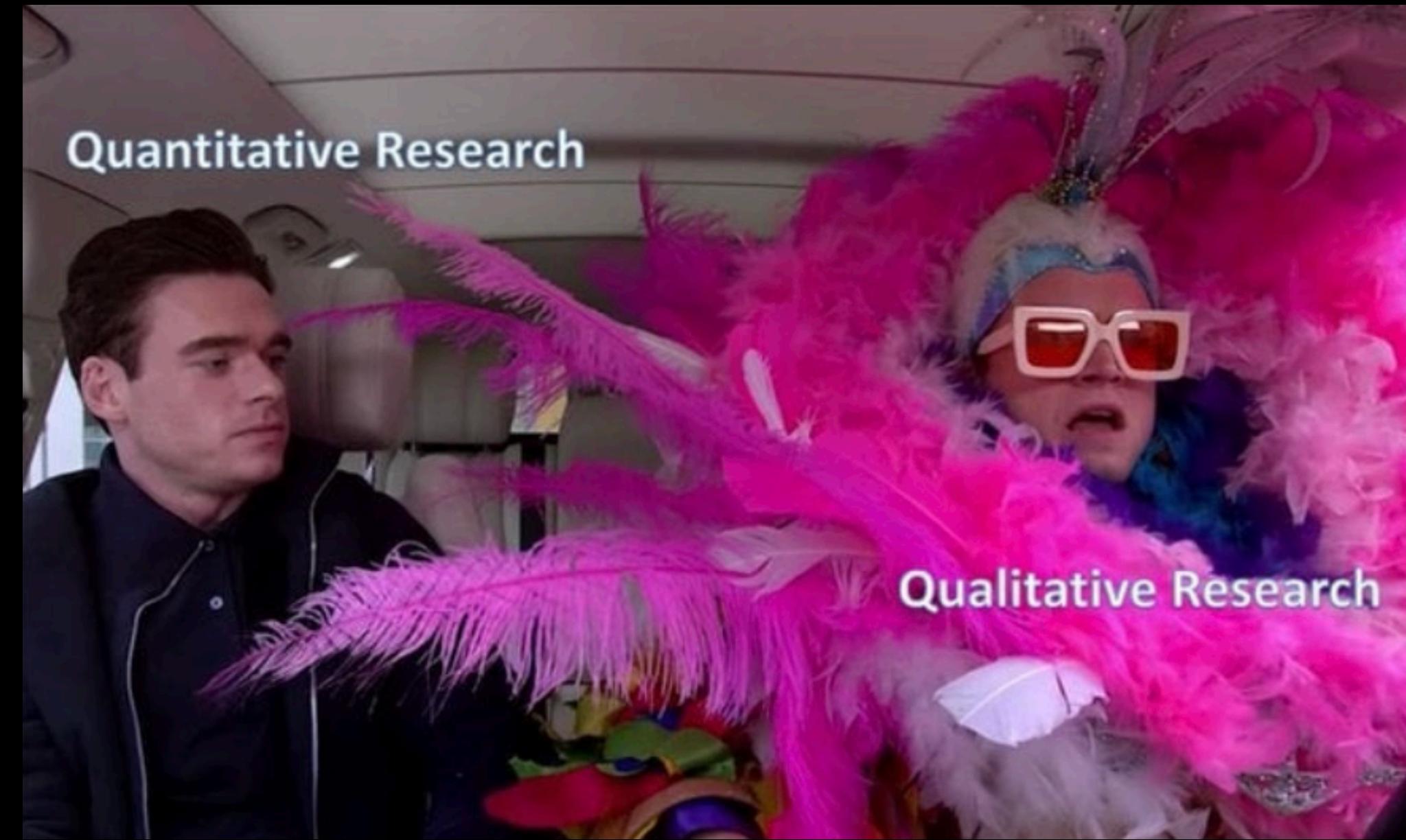
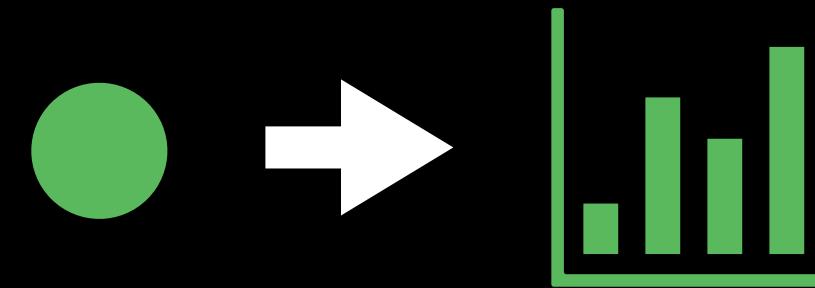
Sensitive



(Potentially)  
Quantitative



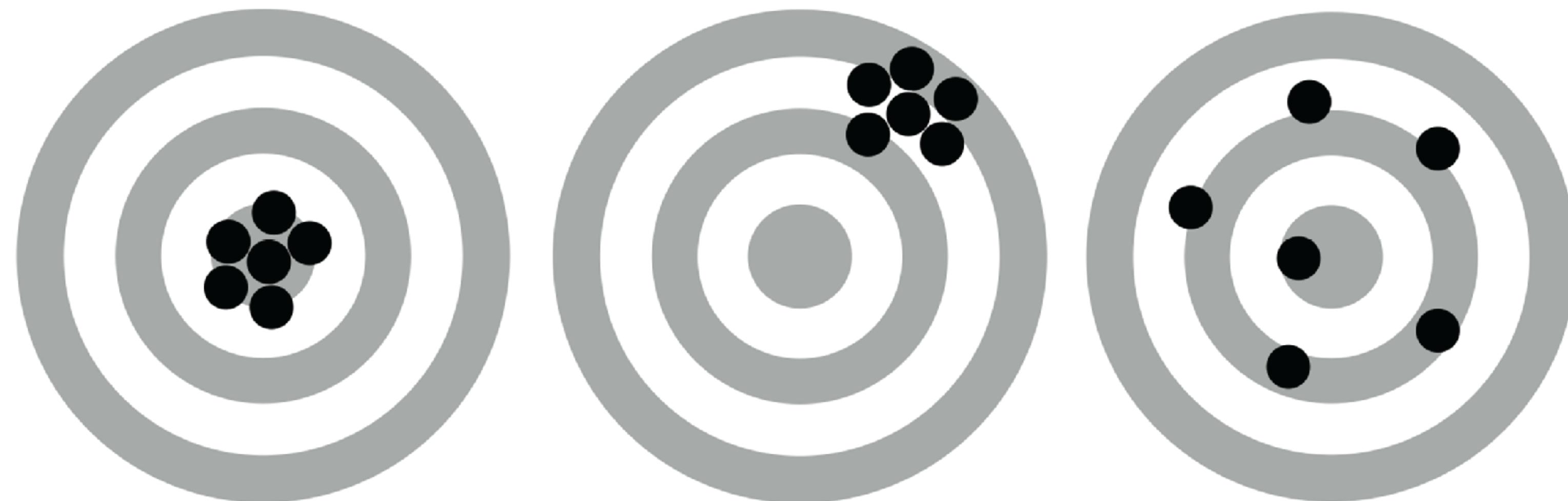
(Potentially)  
Quantitative



# 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

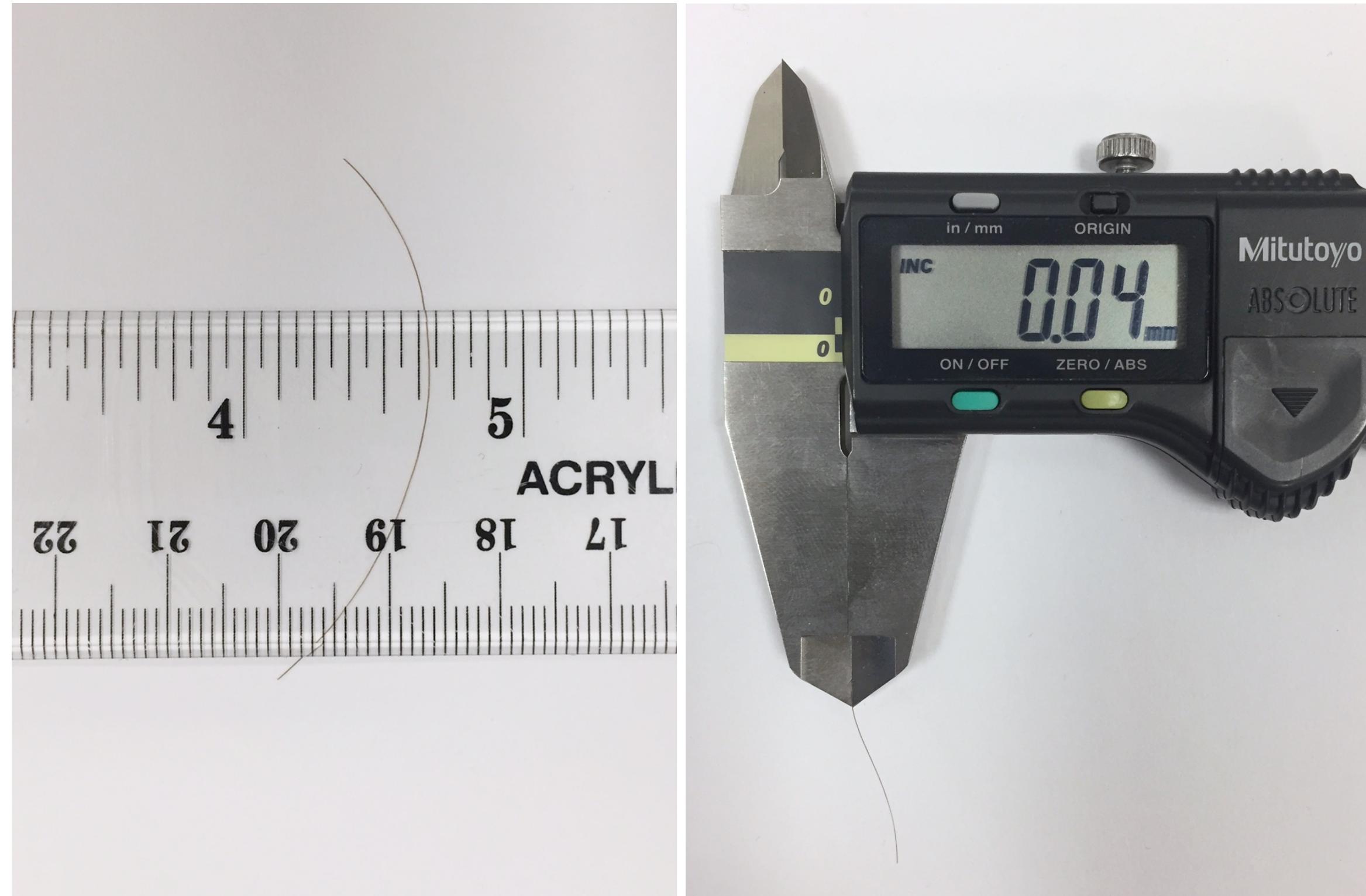
100.00+/-50.00

imprecise

low confidence in individual measurements



# The tool limits accuracy & precision of measurements



# Tools in Microscopy

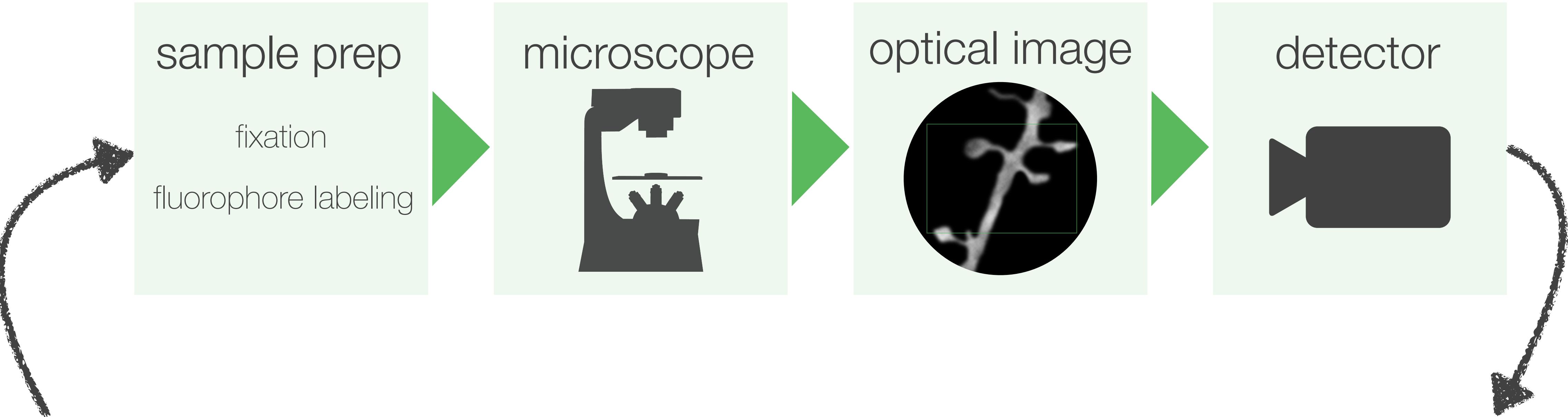


Microscope



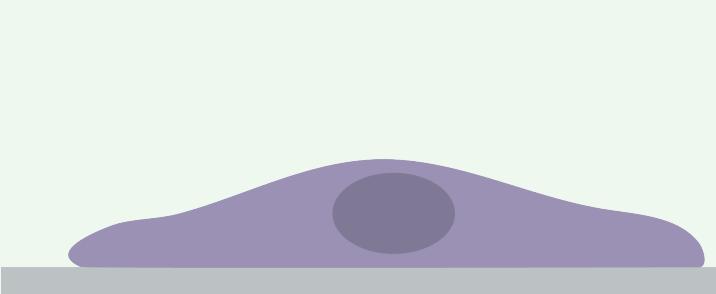
Detector





ground truth

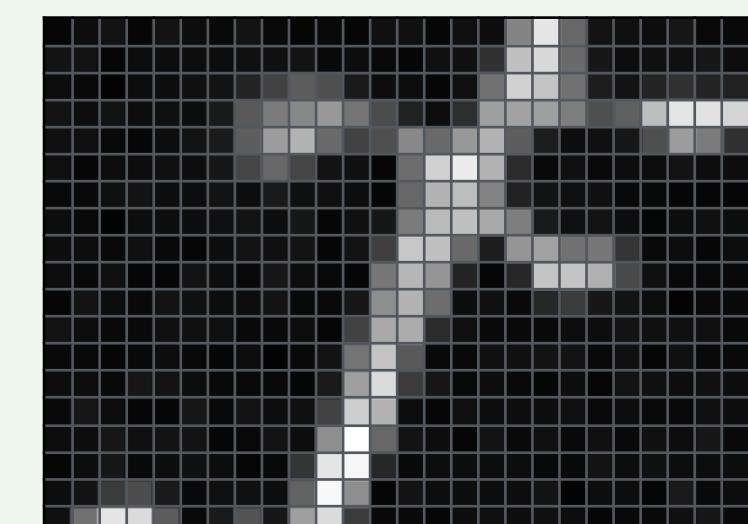
specimen



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

measurement

digital image



# Factors that can limit accuracy and precision in fluorescence microscopy

---

resolution

sampling

signal to noise ratio (SNR)

background



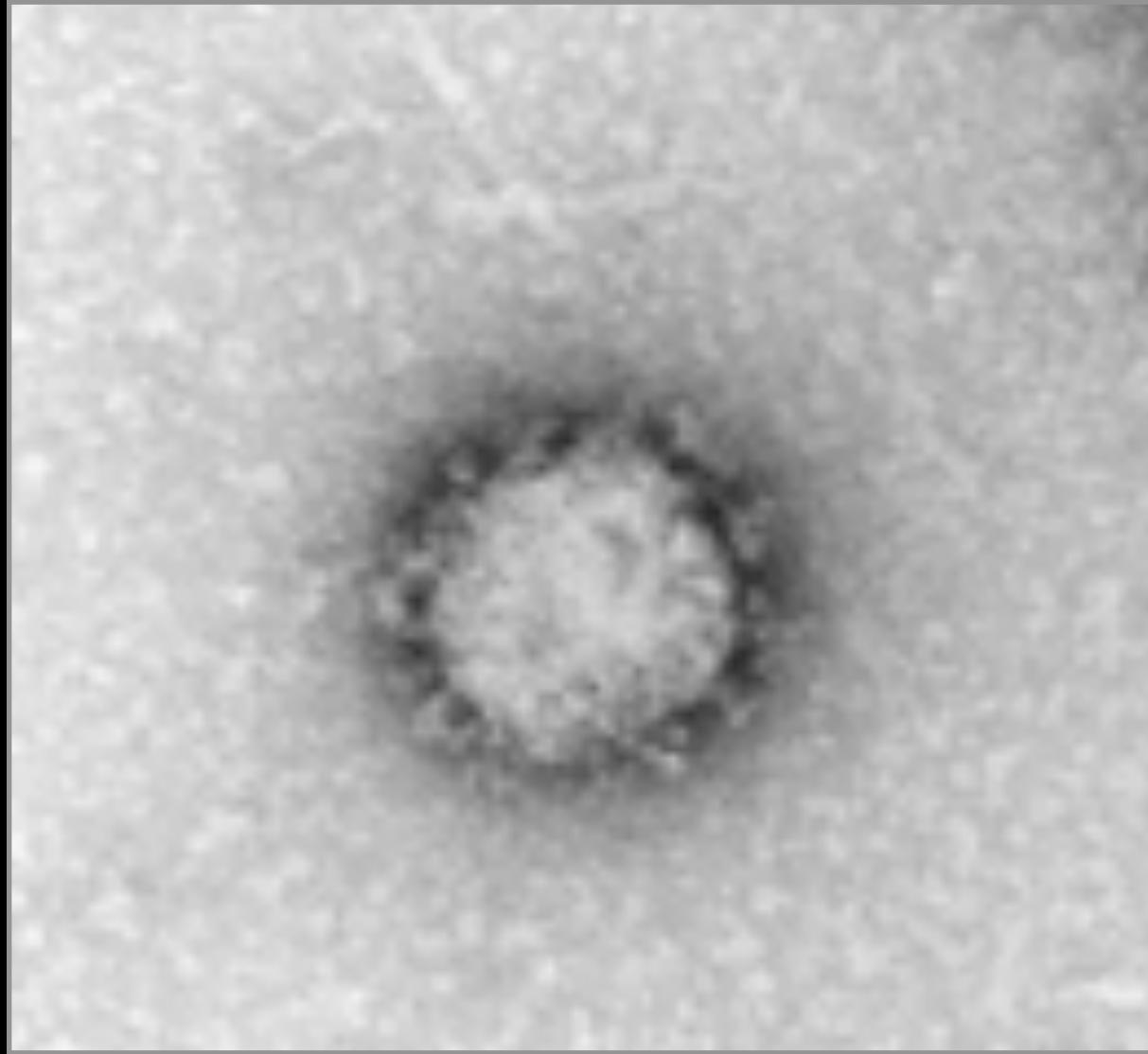
# resolution

*What does resolution even mean???*



# detection *is not* resolution

electron  
microscopy



light  
microscopy

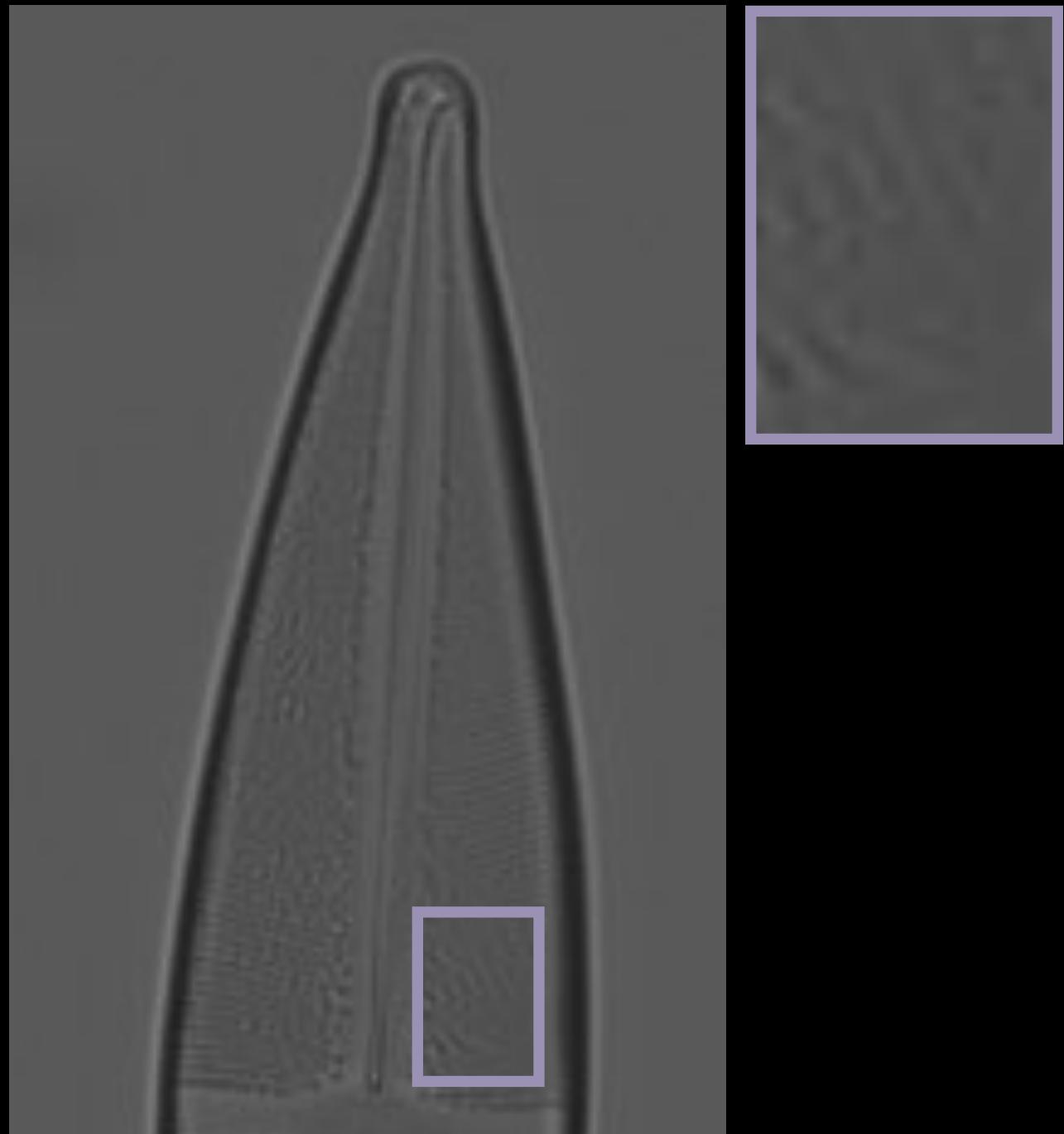


Coronavirus image: Hans R. Gelderblom, Freya Kaulbars/RKI

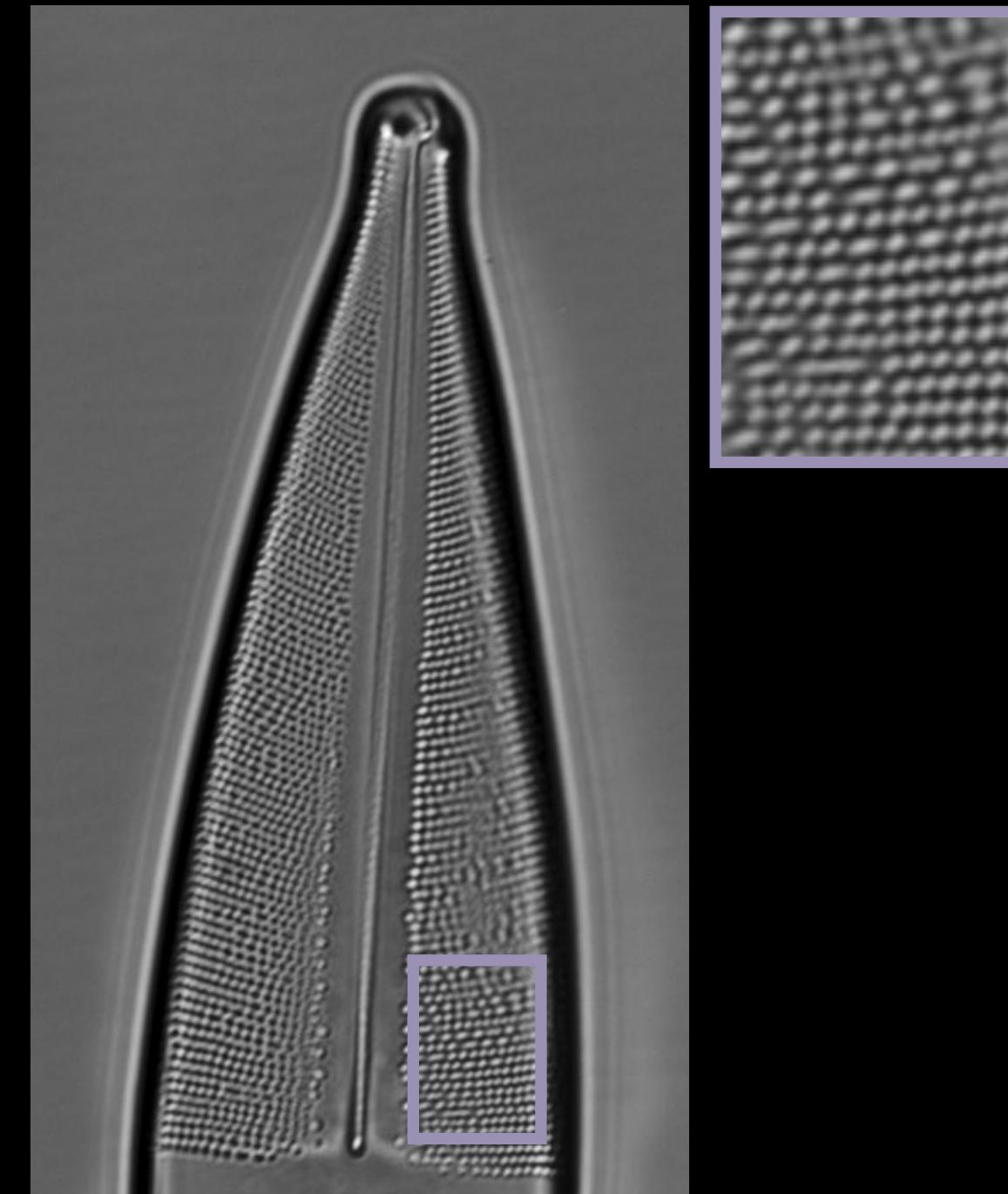
# resolution

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

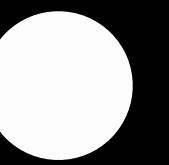
the ability to distinguish objects that are separate in the *sample* as separate from one another in the *image of the sample*

you never get to look at your sample

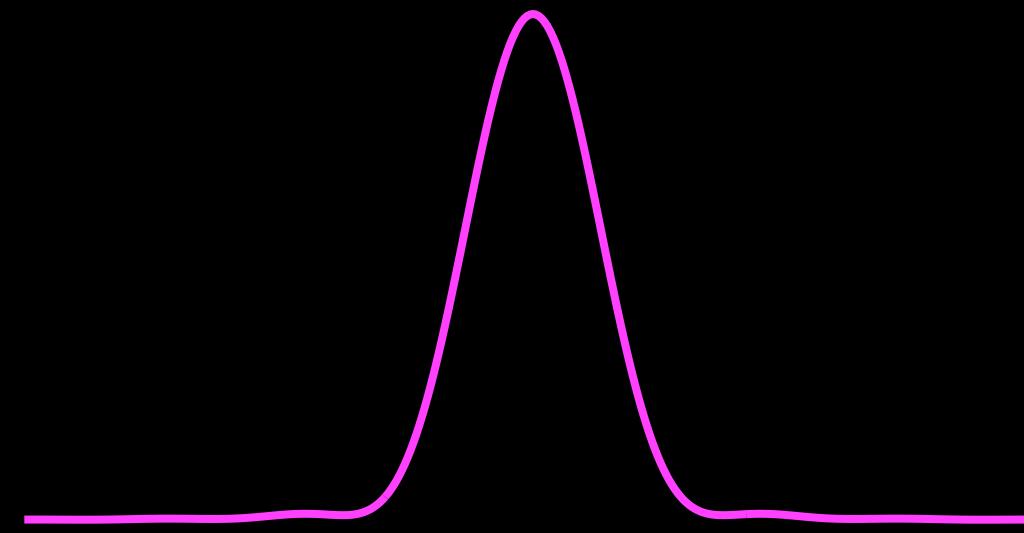
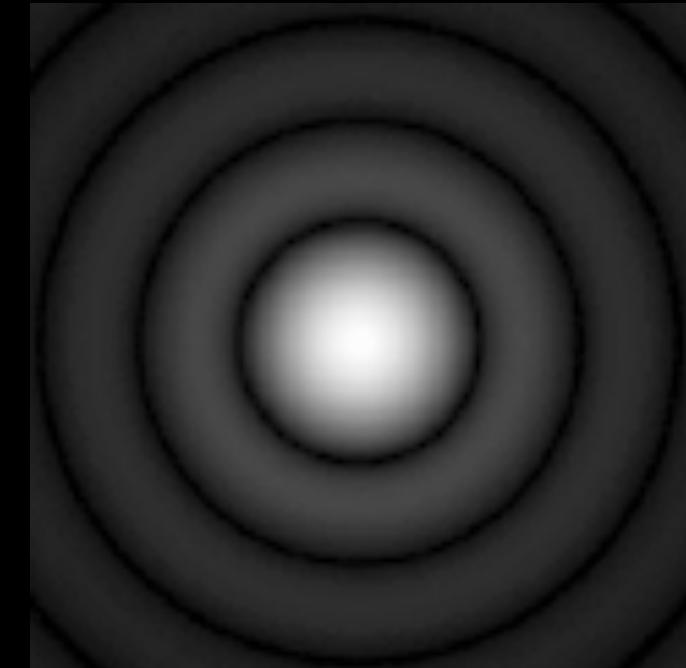
How does the *image of your sample* formed by the microscope differ from your *sample*?

# Consider a point source of light...

Expectation:



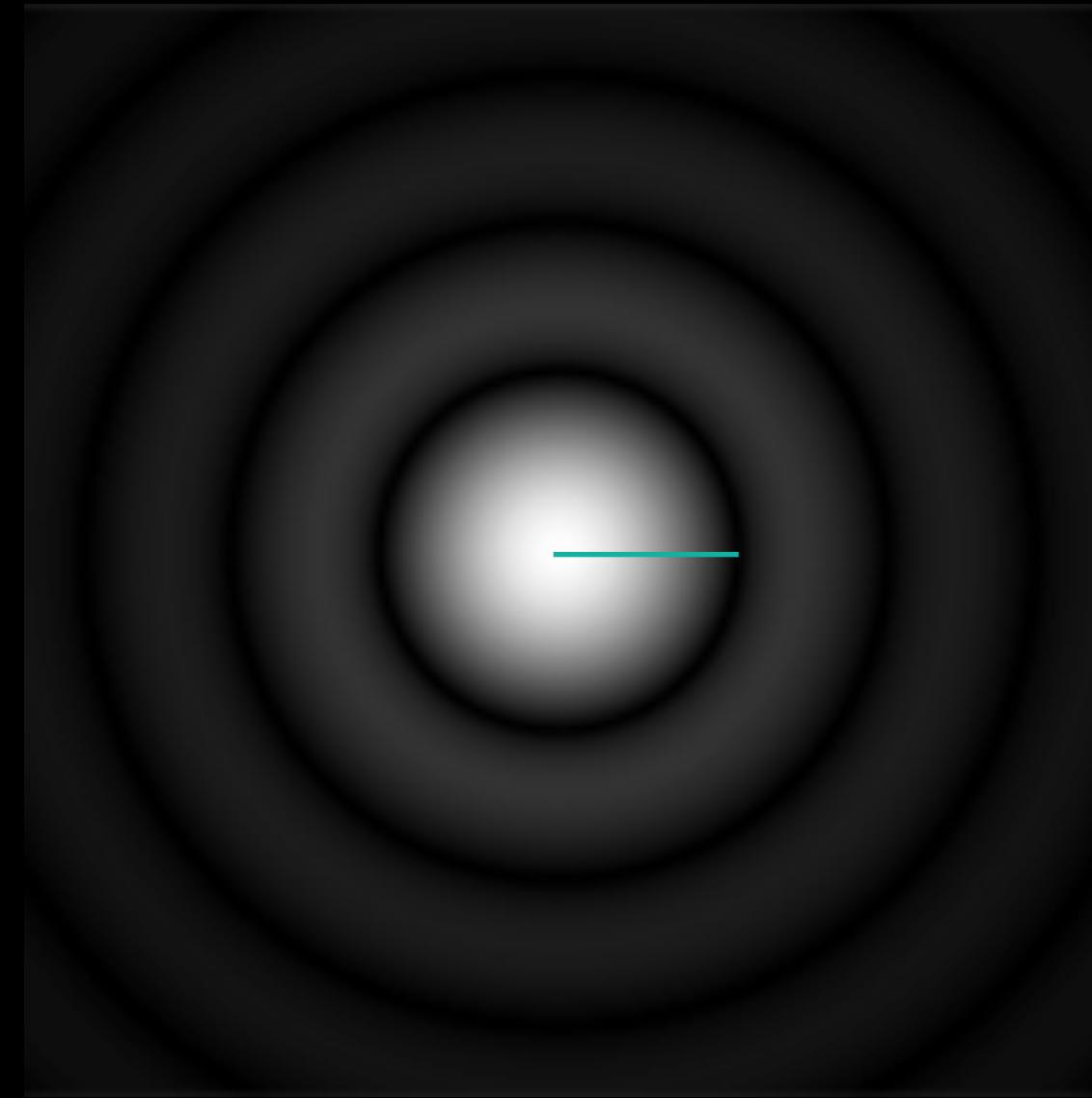
Reality:



point spread function (PSF)

# how big is the PSF?

## lateral (xy) resolution



$$d_{\min} = 0.61\lambda / \text{NA}_{\text{obj}}$$

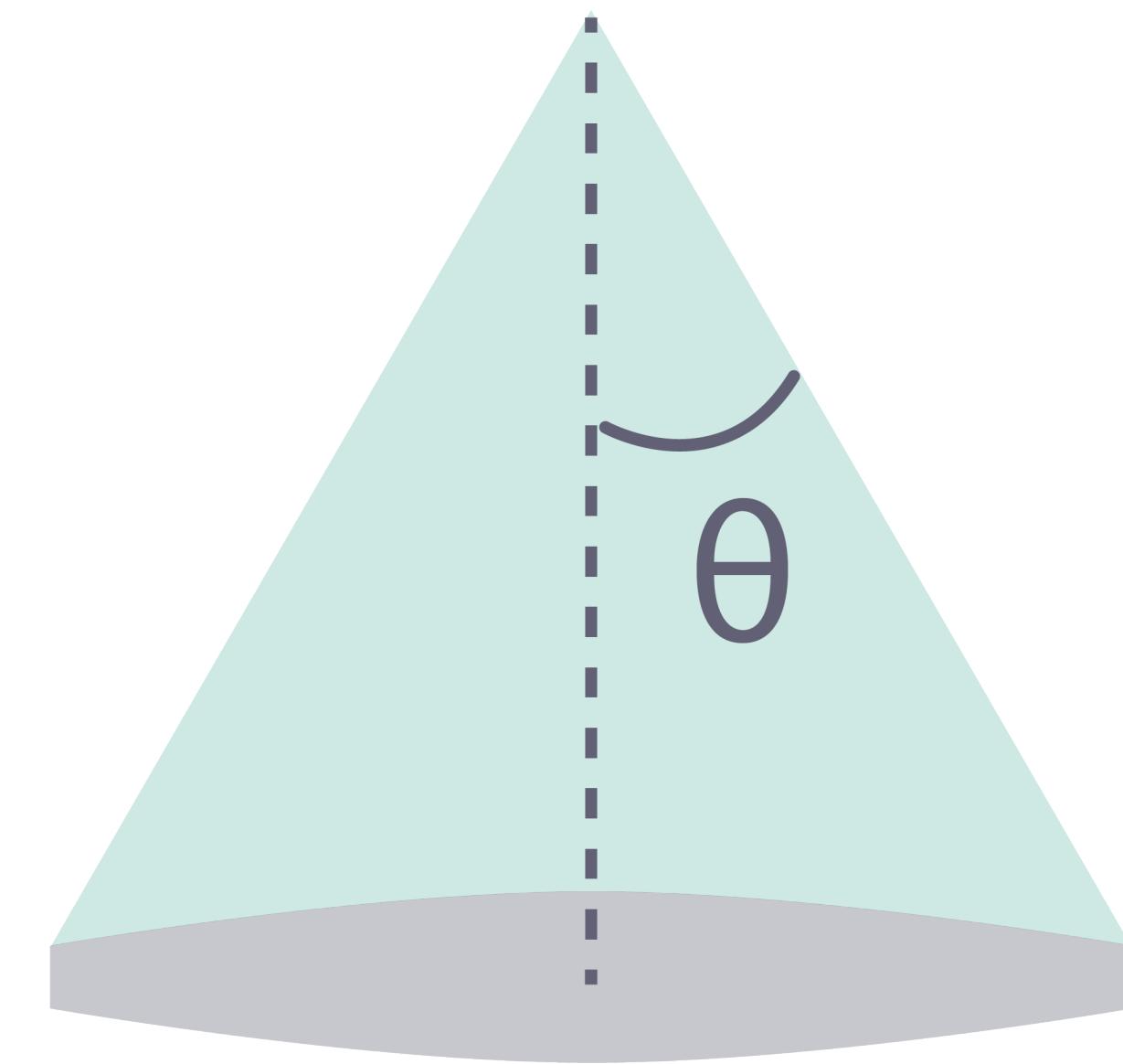
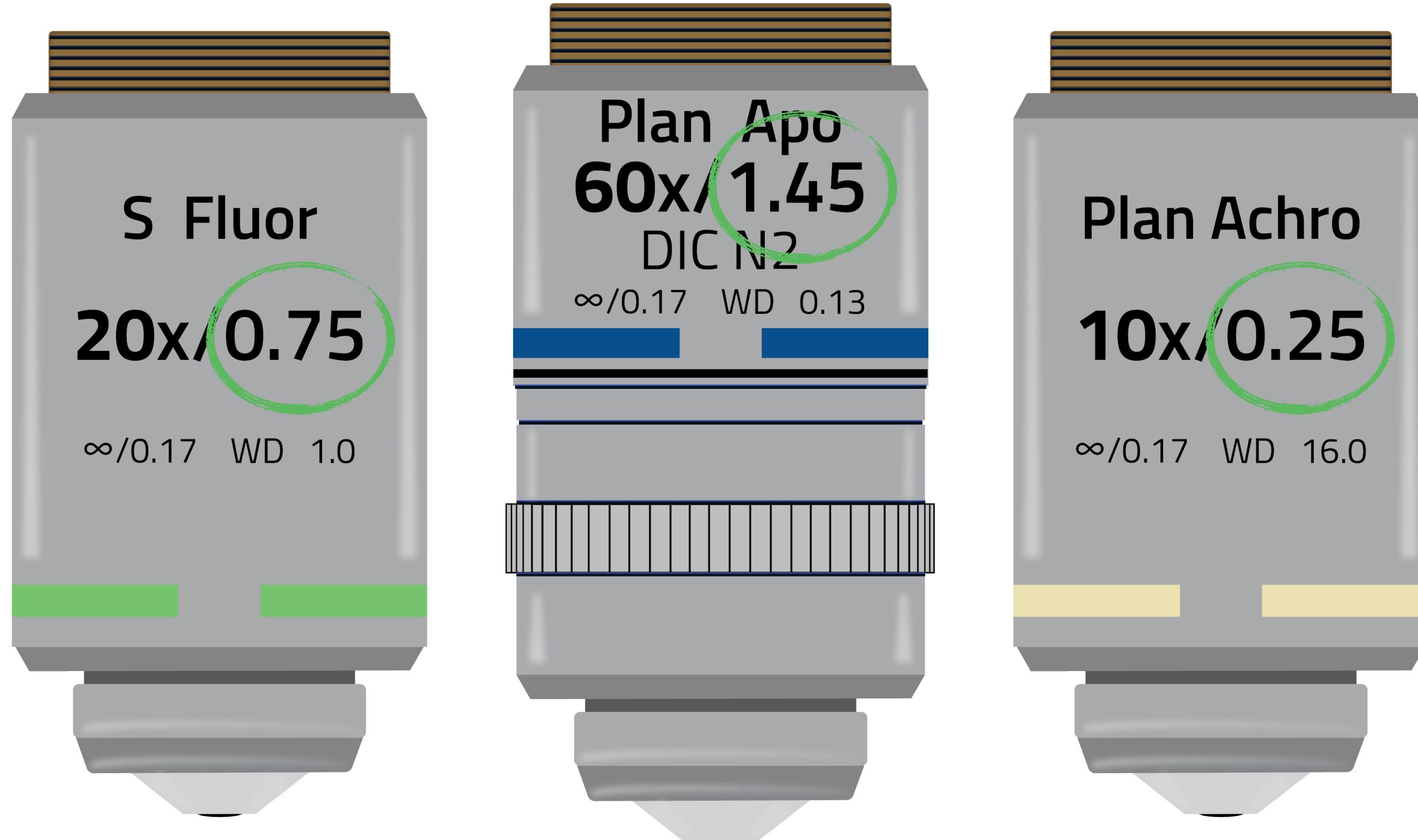
$d_{\min}$  = minimum distance between objects that can be resolved =  
radius of first minimum

$\lambda$  = 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

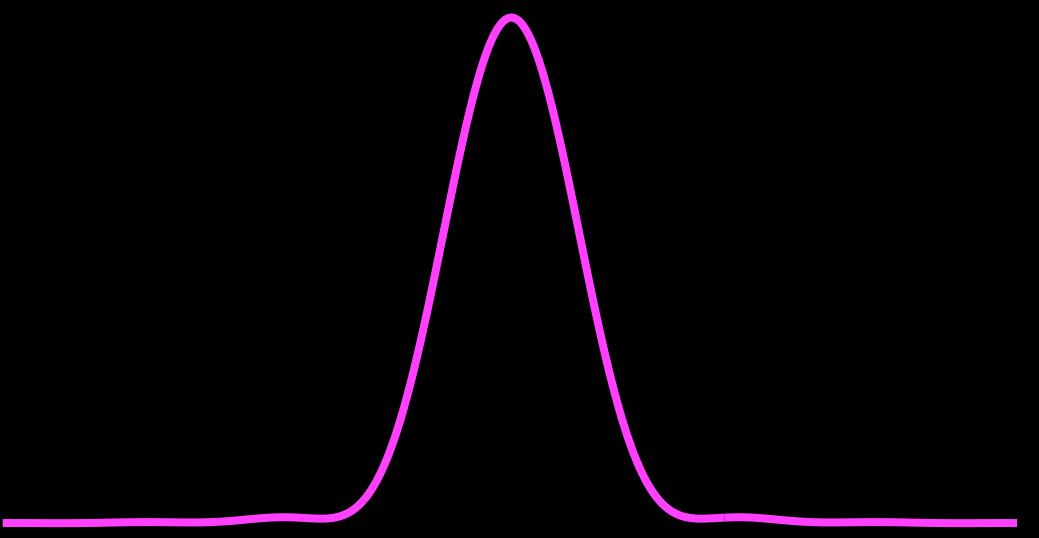
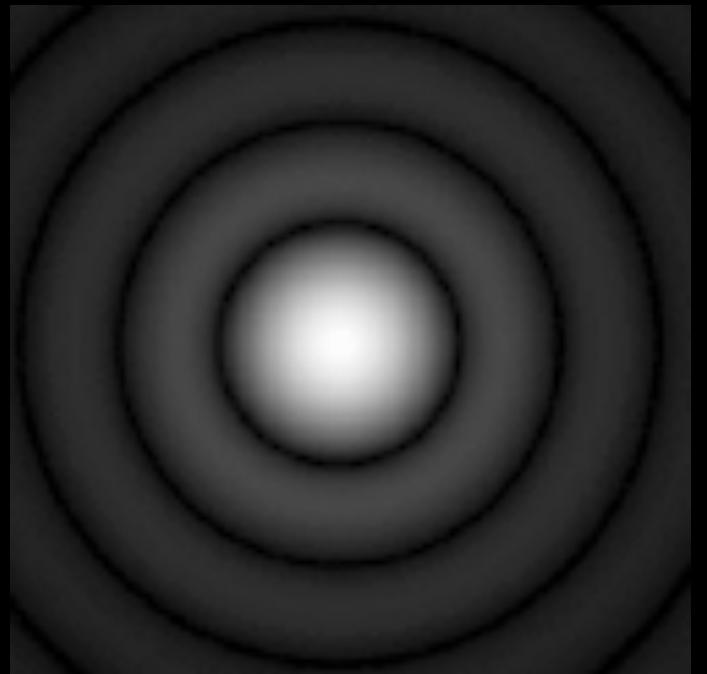
higher NA → more light collection

more light collection → higher resolution

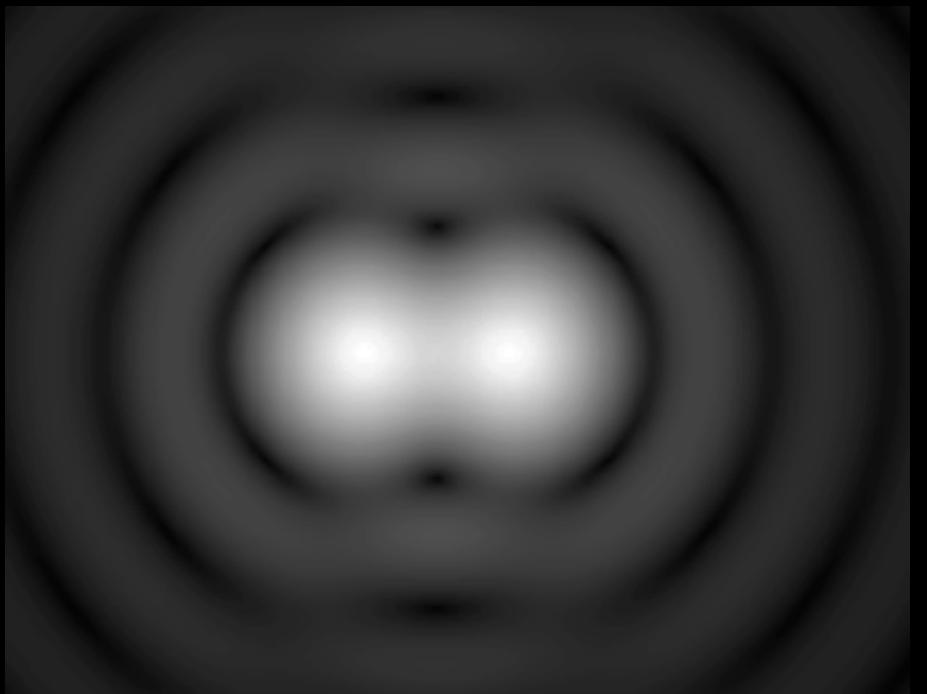


# How does the PSF limit our ability to resolve 2 objects?

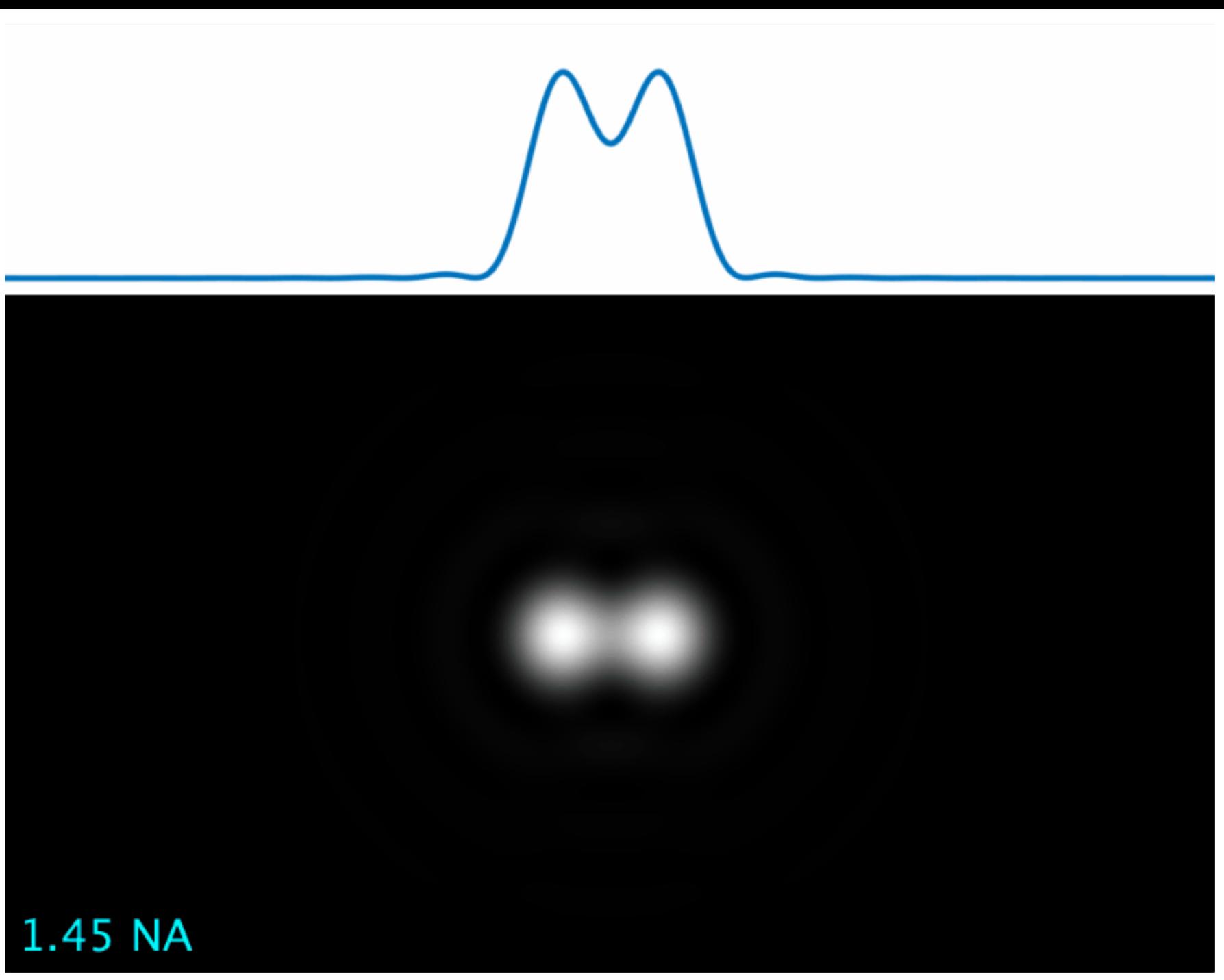
1 point  
source:



2 point  
sources:



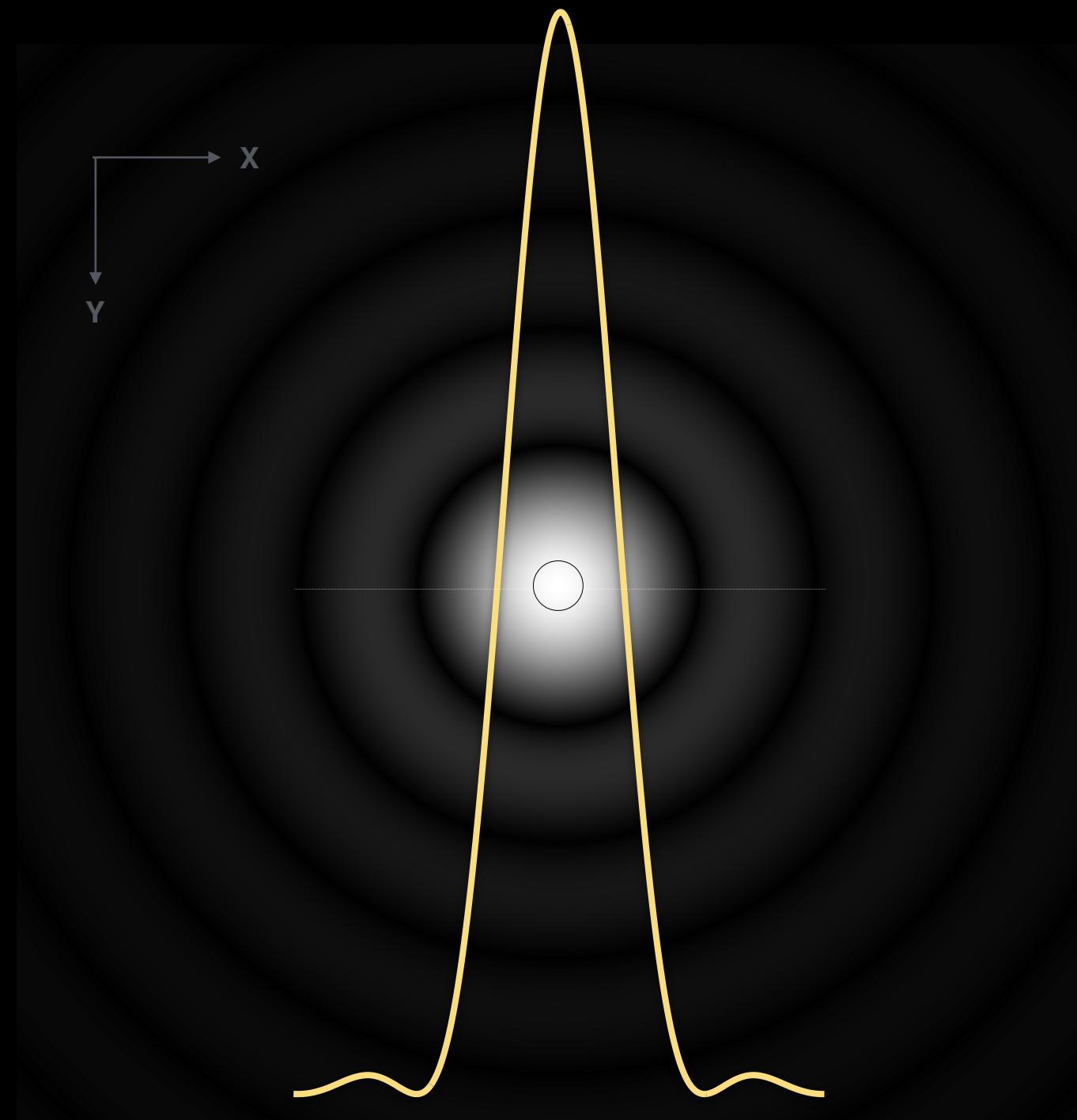
# Resolution is limited by the size of the PSF



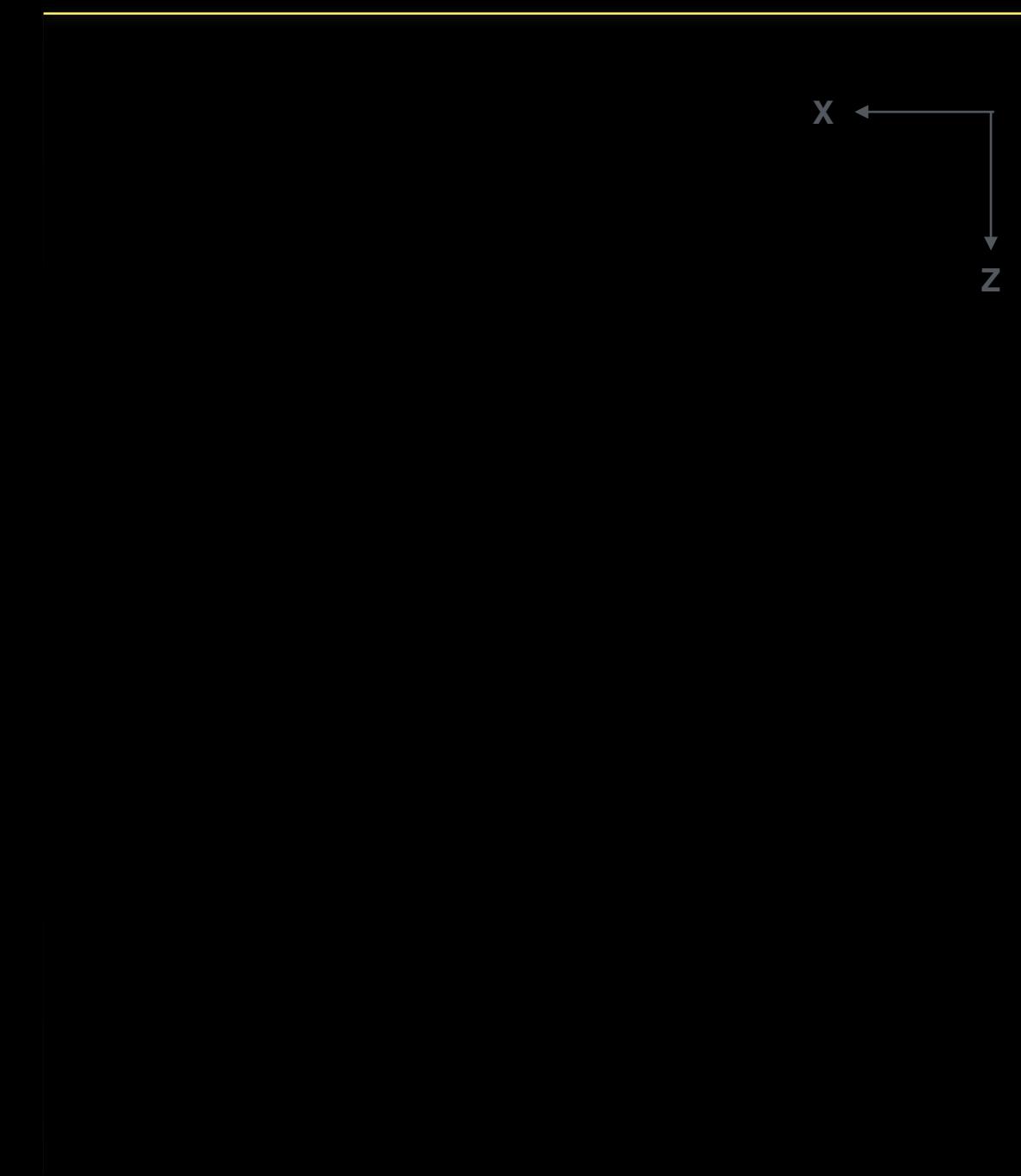
Animation: Talley Lambert

What about *axial* resolution?

the point spread function (PSF) is 3D



lateral



axial

Talley Lambert

# how big is the PSF in Z?

Axial (Z)

$$d_{\min} = 2\lambda n / (\text{NA}_{\text{obj}})^2$$



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

$\lambda$  = 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...

---

$$d_{\min,\text{lateral}} = 0.61\lambda / \text{NA}_{\text{Obj}}$$

$$d_{\min,\text{axial}} = 2\lambda n / (\text{NA}_{\text{Obj}})^2$$

What about wavelength ( $\lambda$ )?



# 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  $\theta \rightarrow$  higher resolution

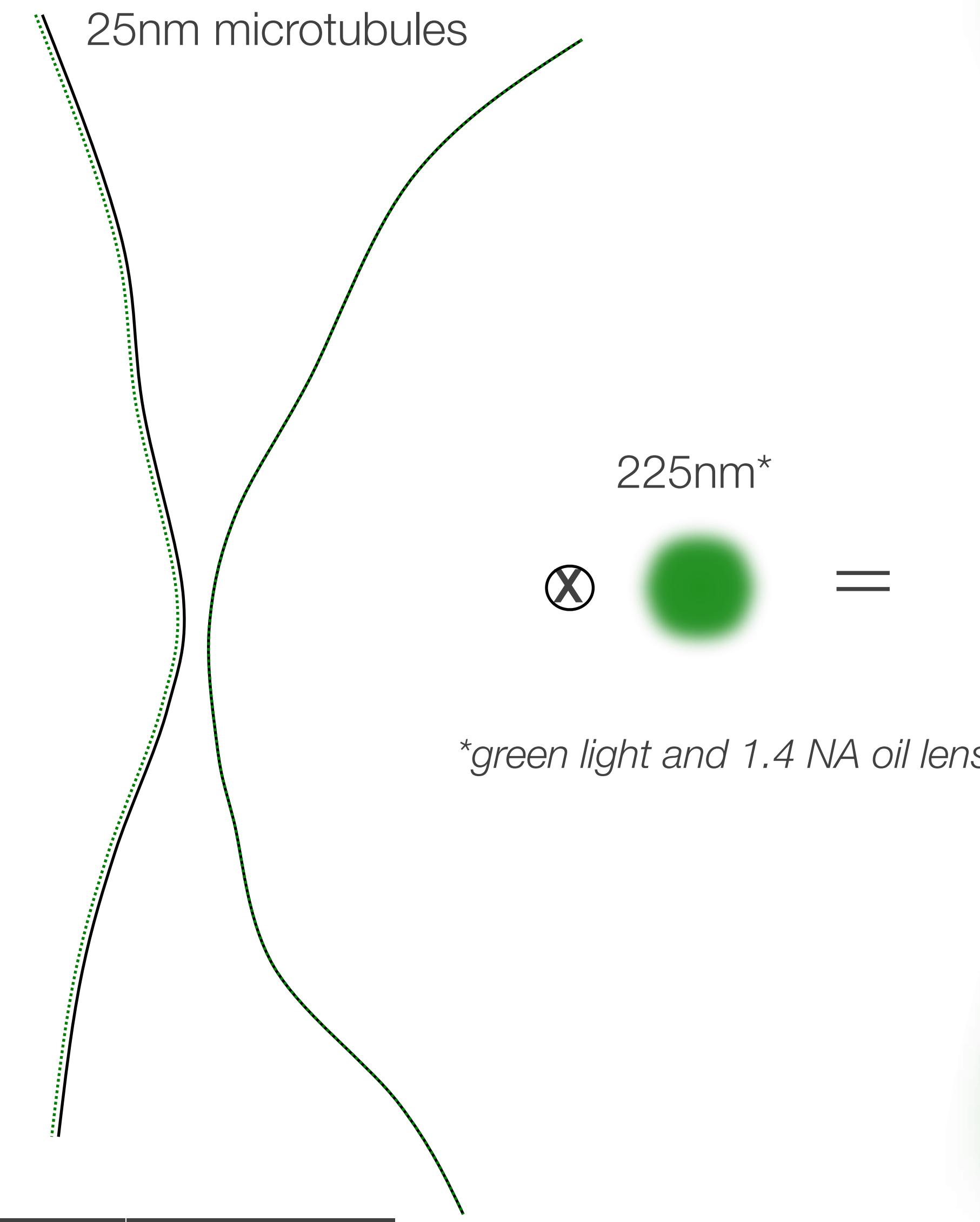


# What does the PSF mean for the image of *your* sample?

---



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



Resolution

Sampling

SNR

Background



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

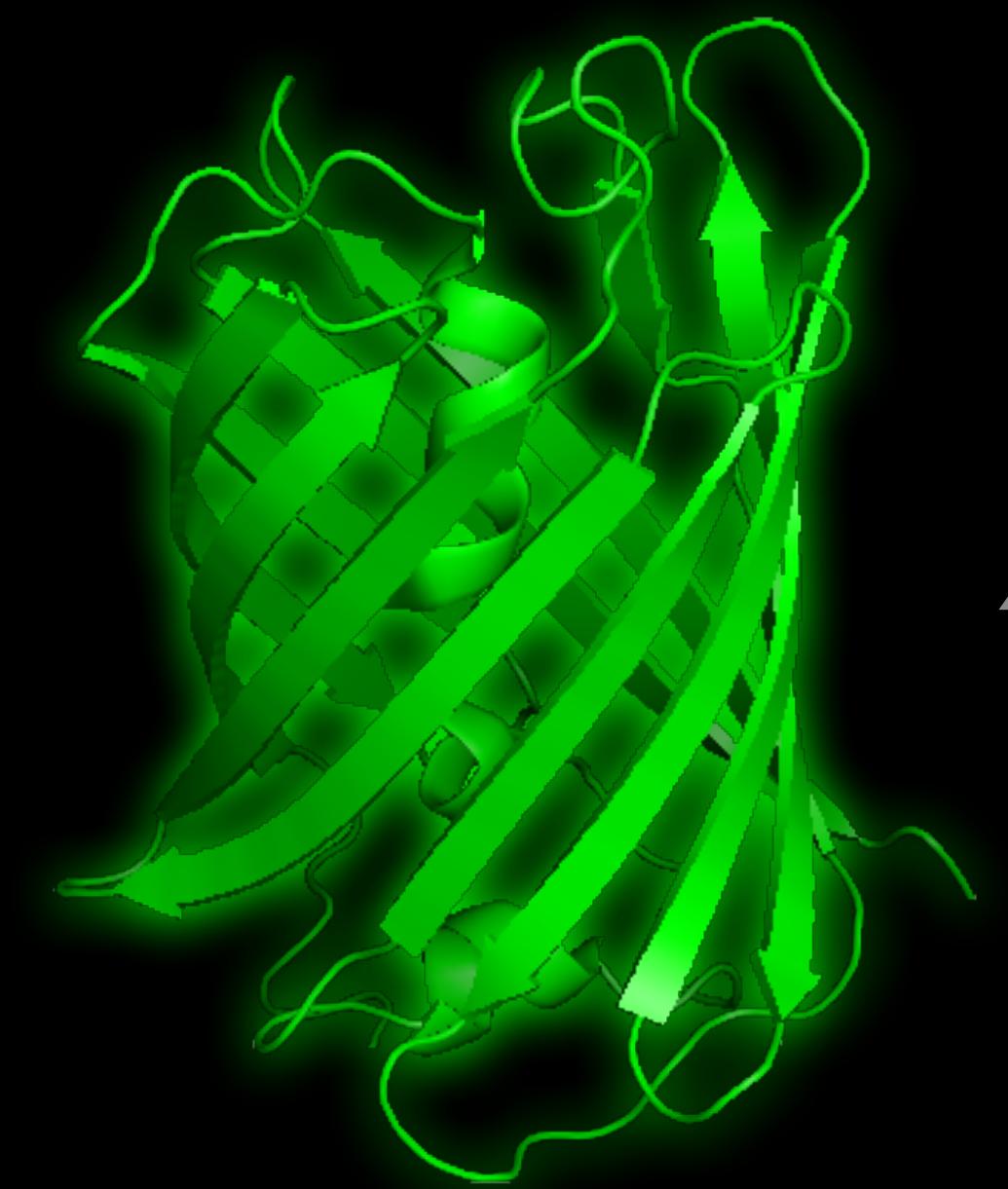
assume the resolution limit is 240 nm in XY.

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

interacting

not interacting

# Important Point

## #1

Resolution is fundamentally limited by the size of the PSF.



# sampling

Resolution

Sampling

SNR

Background



# Sampling: Selection of a subset meant to represent the whole

---

## spatial sampling: pixel size

ground truth

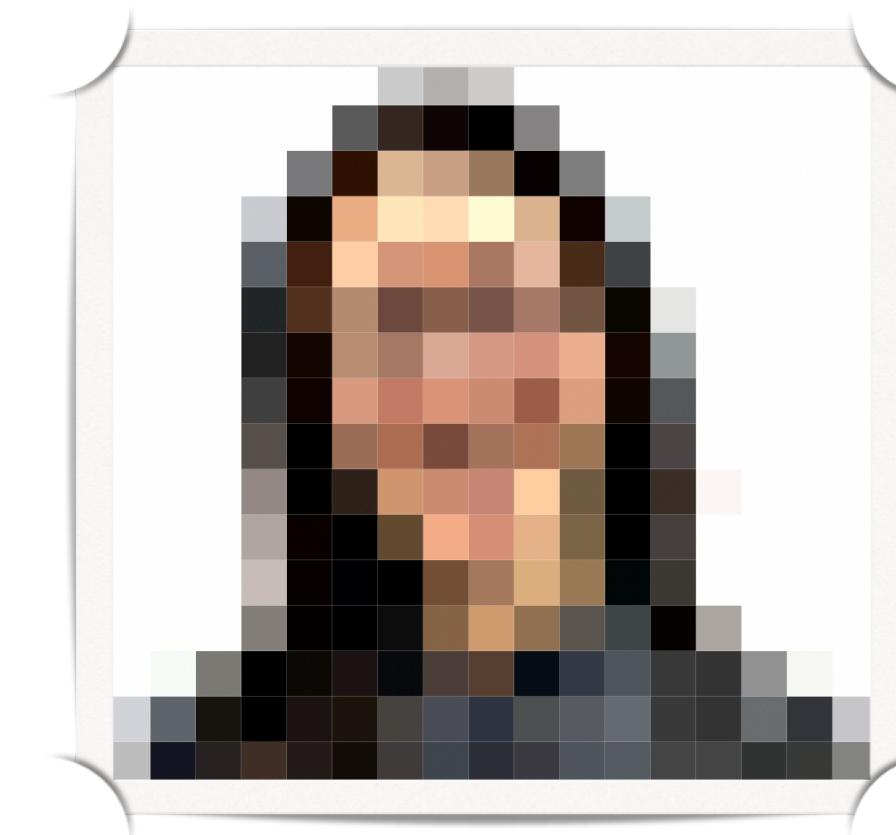
measurement

me!

digital image of me

larger pixel size

smaller pixel size



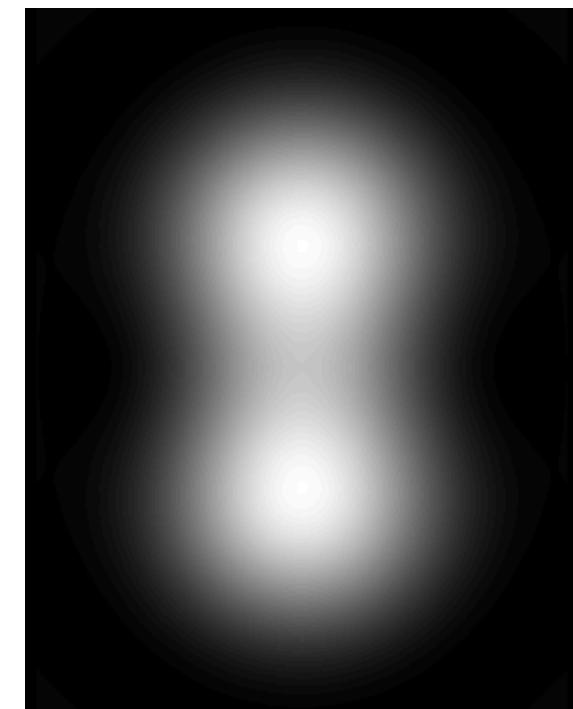
## spatial sampling

ground truth

measurement

2 fluorescent beads

digital image of the beads

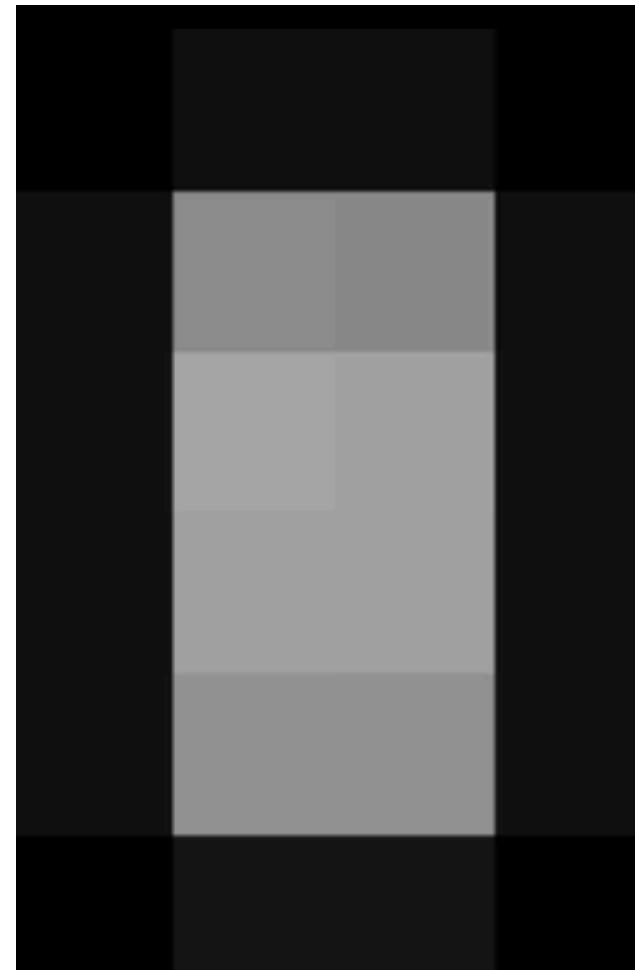


How many pixels do we  
need to distinguish 2 beads?

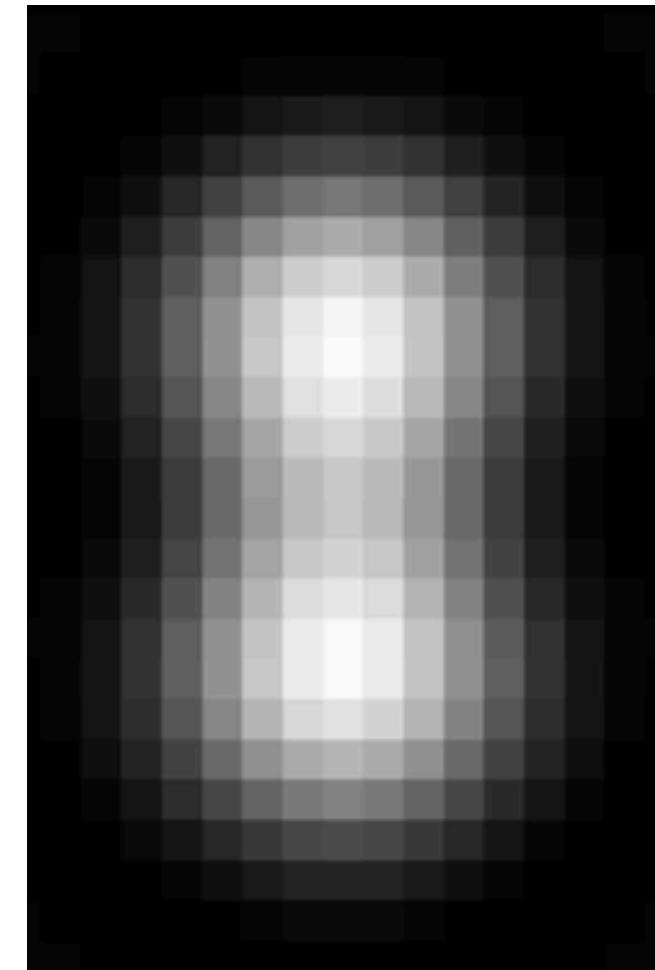
undersampling (aliasing)



Nyquist



oversampling



Images by Talley Lambert

Resolution

Sampling

SNR

Background



# Important Point

## #2

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

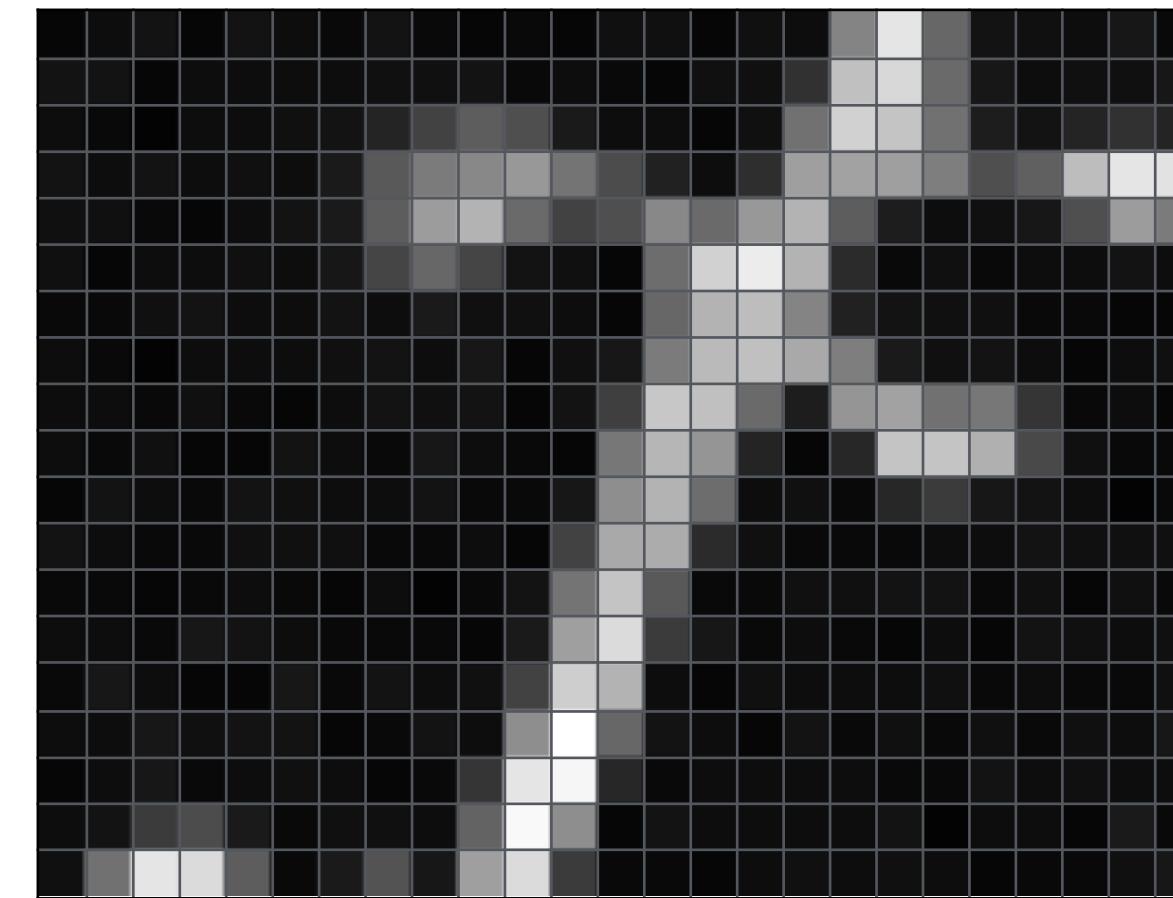


# signal to noise ratio (SNR)

Resolution   Sampling   SNR   Background



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	13	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	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	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

Resolution

Sampling

SNR

Background



# signal to noise ratio (SNR)

---

signal

photons you want to measure

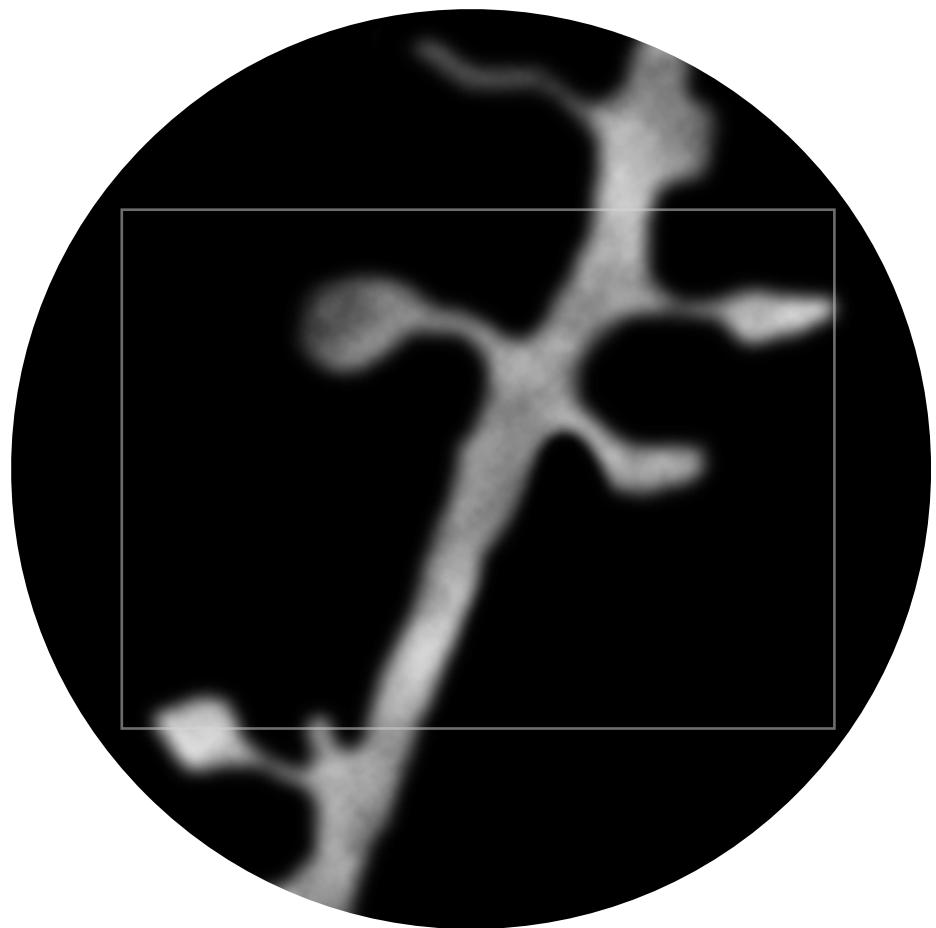
---

noise

fluctuations of measured intensity values



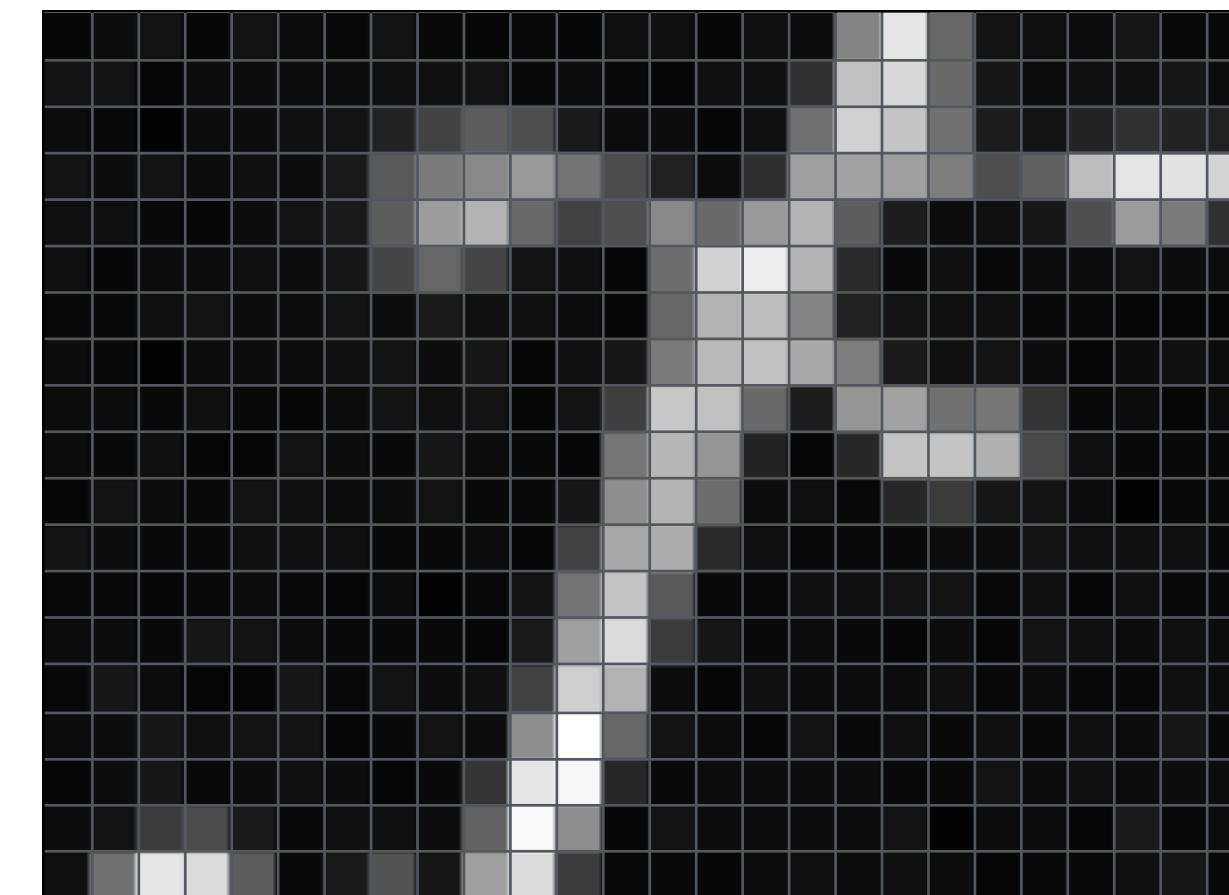
# Detector: Collect optical image photons and output digital image



detector



spatial



photons  
optical image

intensity values  
digital image

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	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	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	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	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	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	6	9	9	16	23	9			

intensity values ≠ photons!!

Resolution

Sampling

SNR

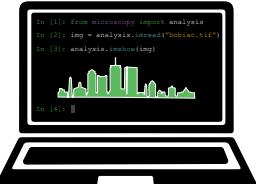
Background

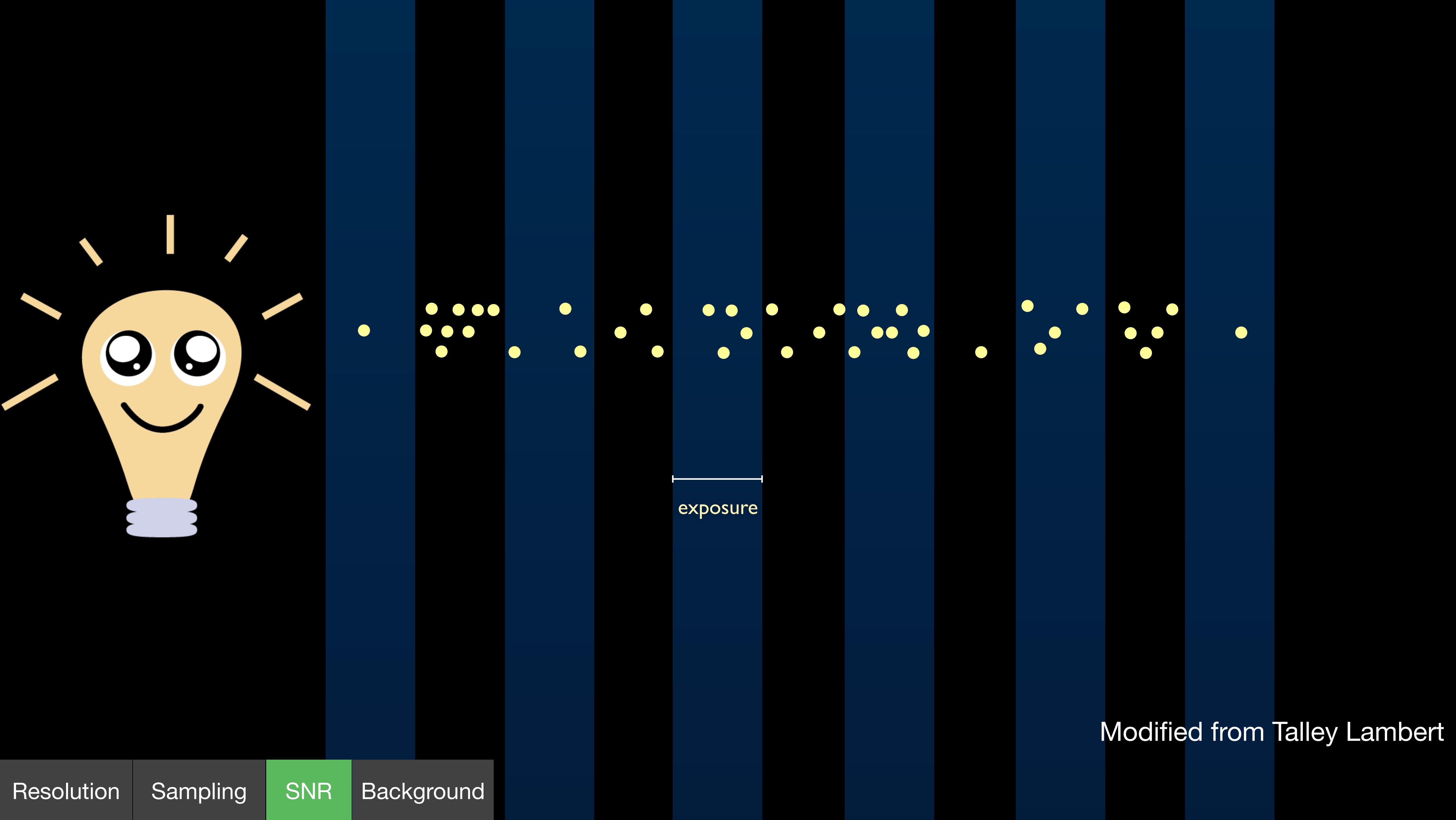


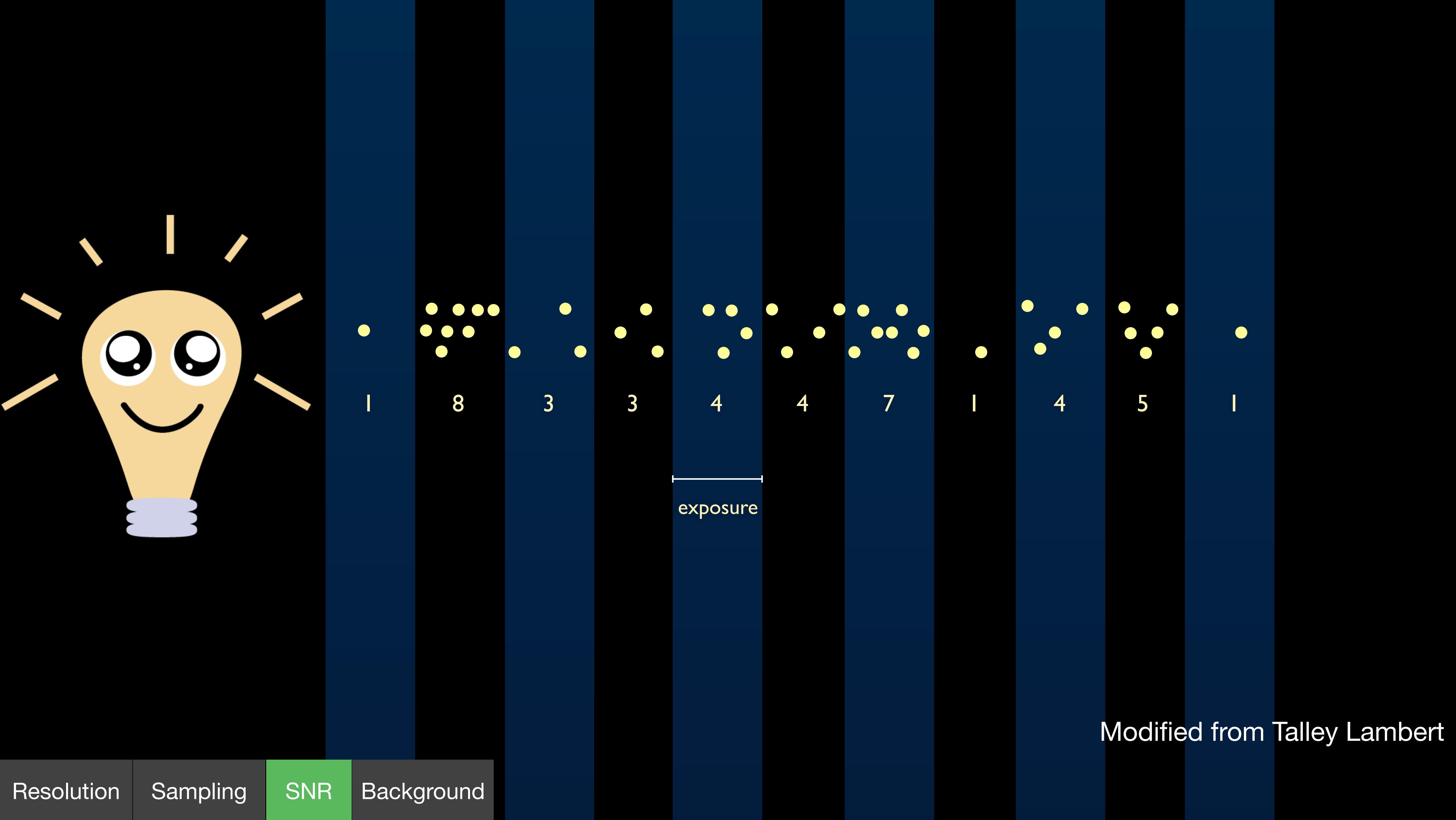
We send optical image photons to the detector...

---

...but do they all arrive at the same time?







noise

---

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



# signal to noise ratio (SNR)

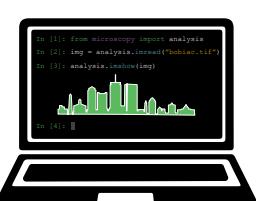
signal

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?

---

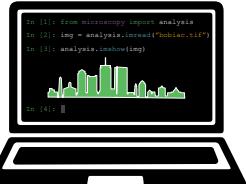


# What happens if we send no light to the detector and take an image?

---

noise

detector noise



# signal to noise ratio (SNR)

signal

photons you want to measure

noise

fluctuations of measured intensity values

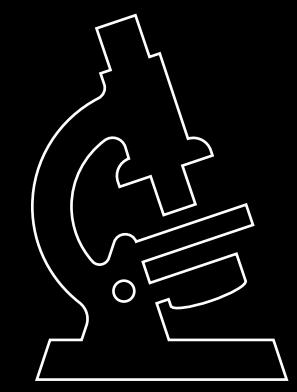
Poisson noise  
detector noise



# How does SNR impact your measurements?

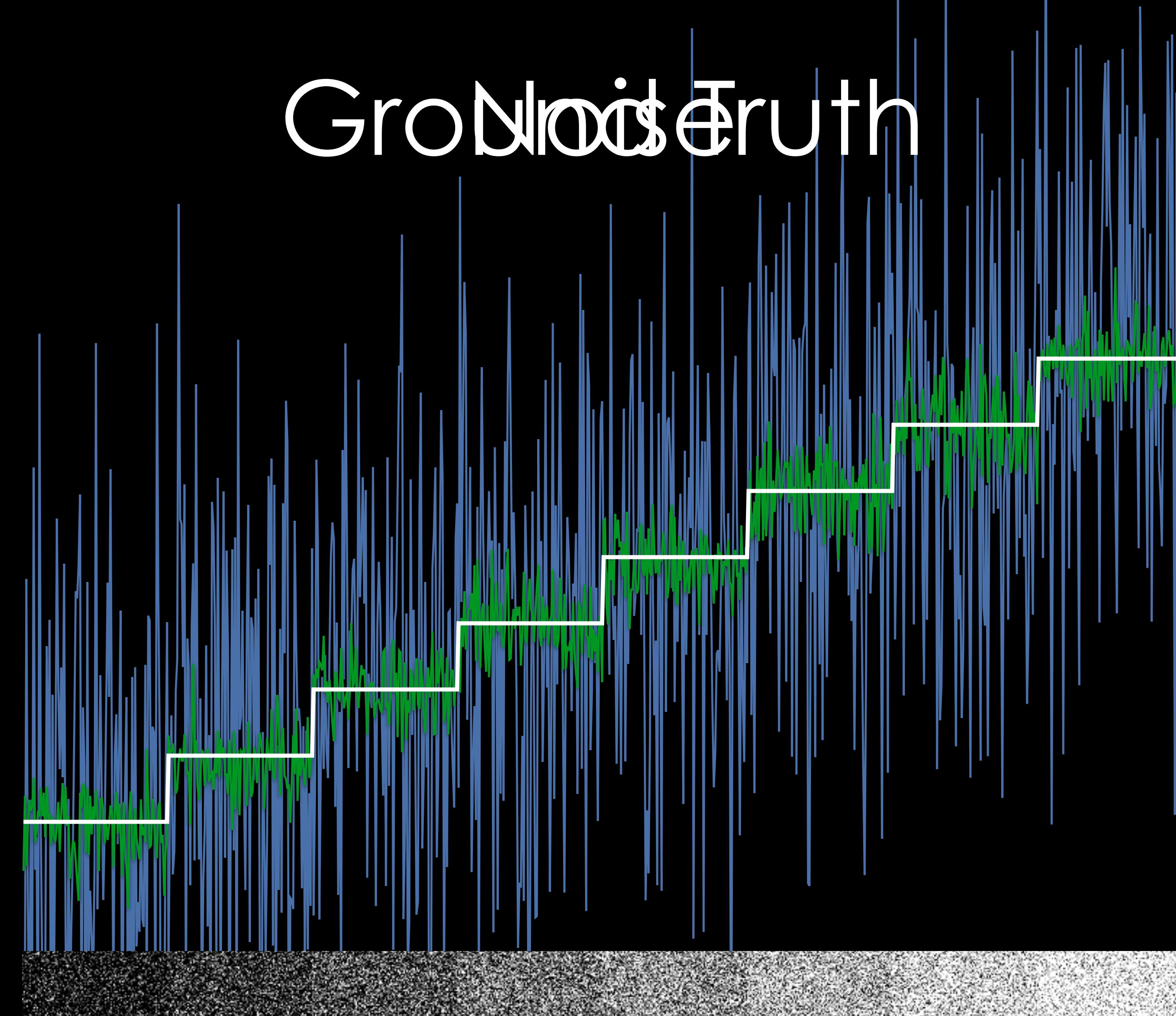
---





$\mu$ Courses

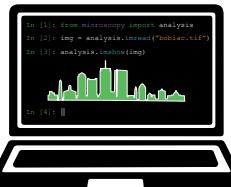
# GroNoisEruth



# Important Point

## #4

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



# background

Resolution

Sampling

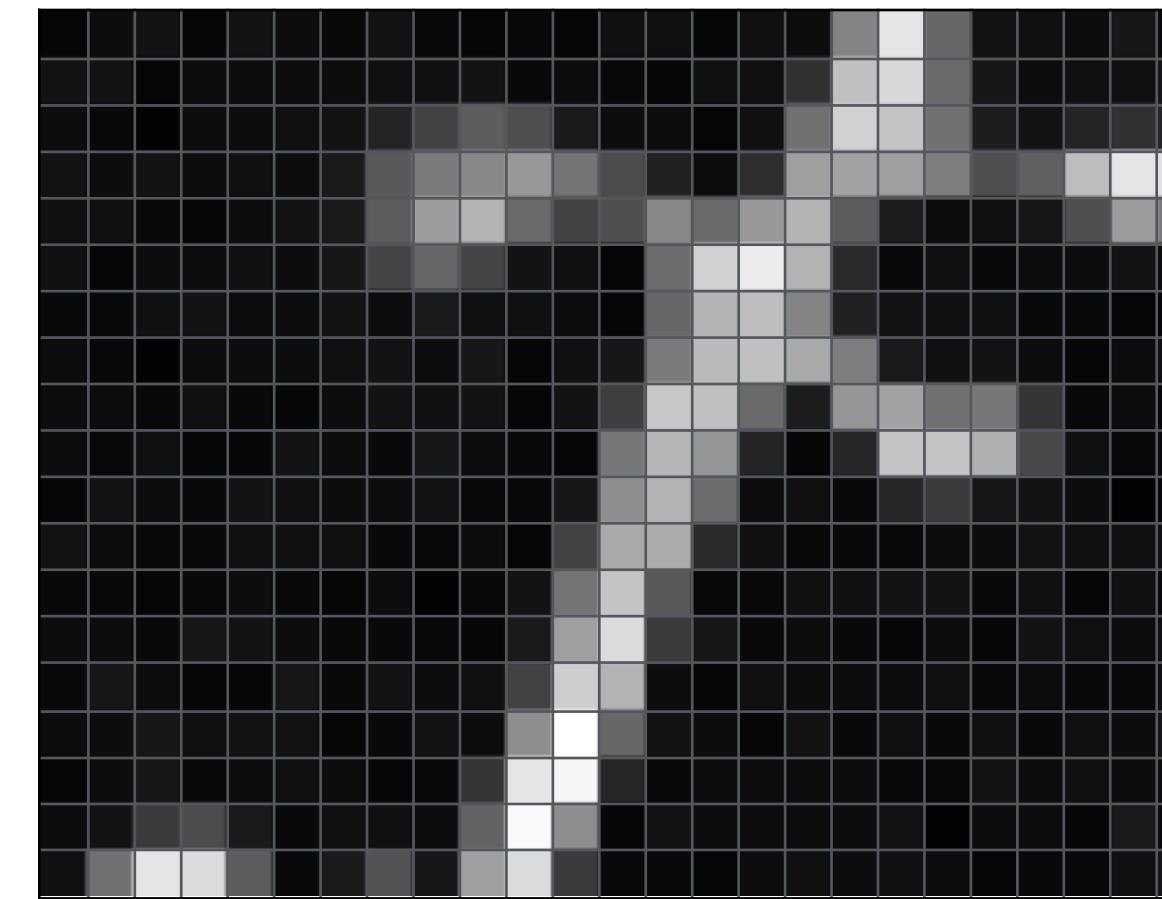
SNR

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	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

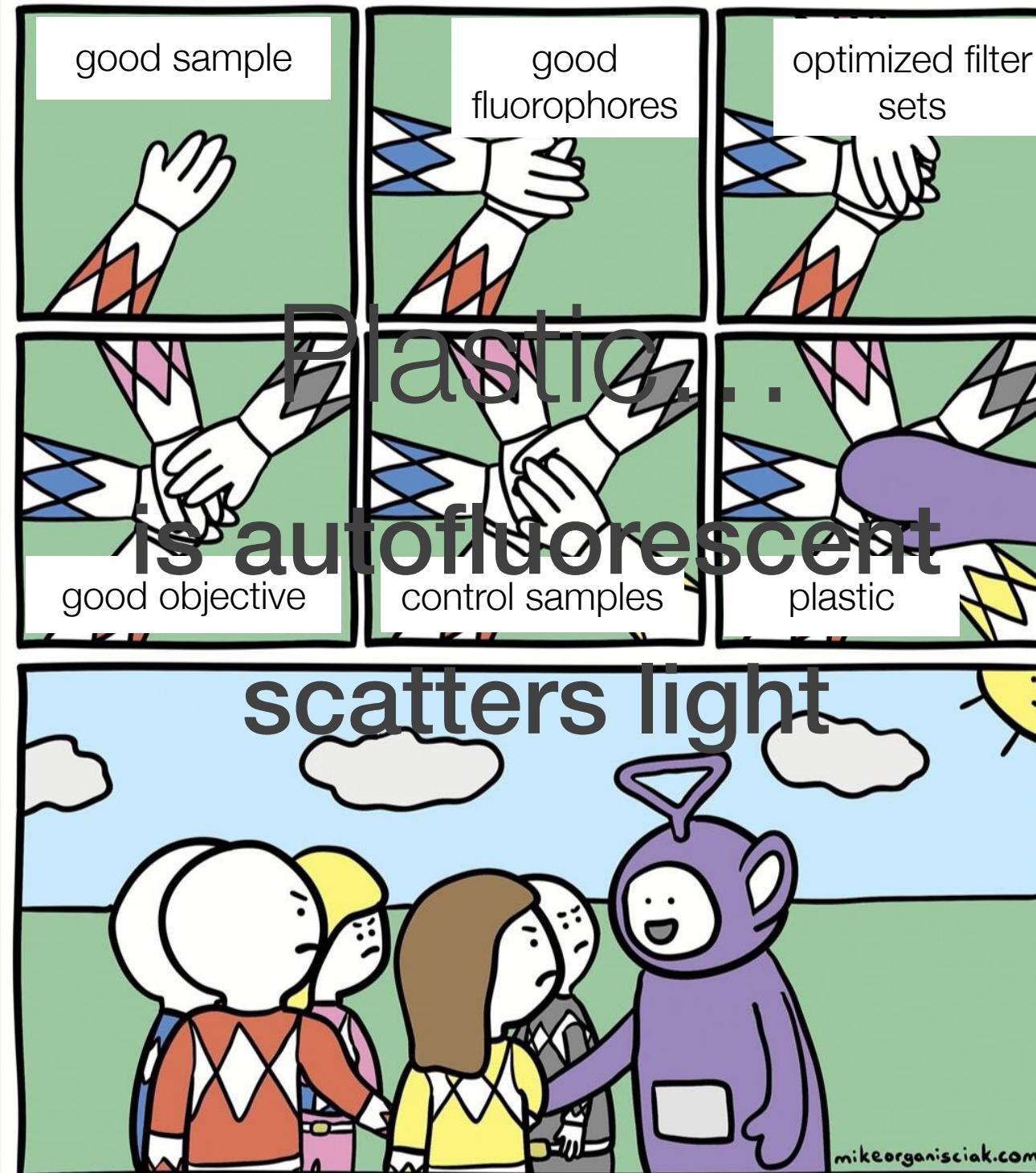
fluctuations of measured intensity values



# Examples of choices that increase background...

## imaging with plastic

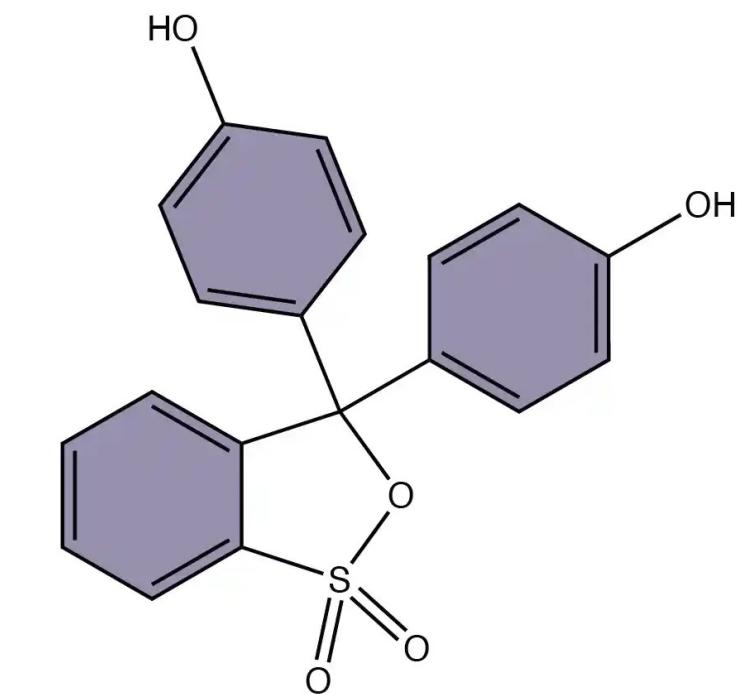
“Friends don’t let friends image through plastic.”



## using mounting medium w/DAPI



## using imaging medium w/phenol

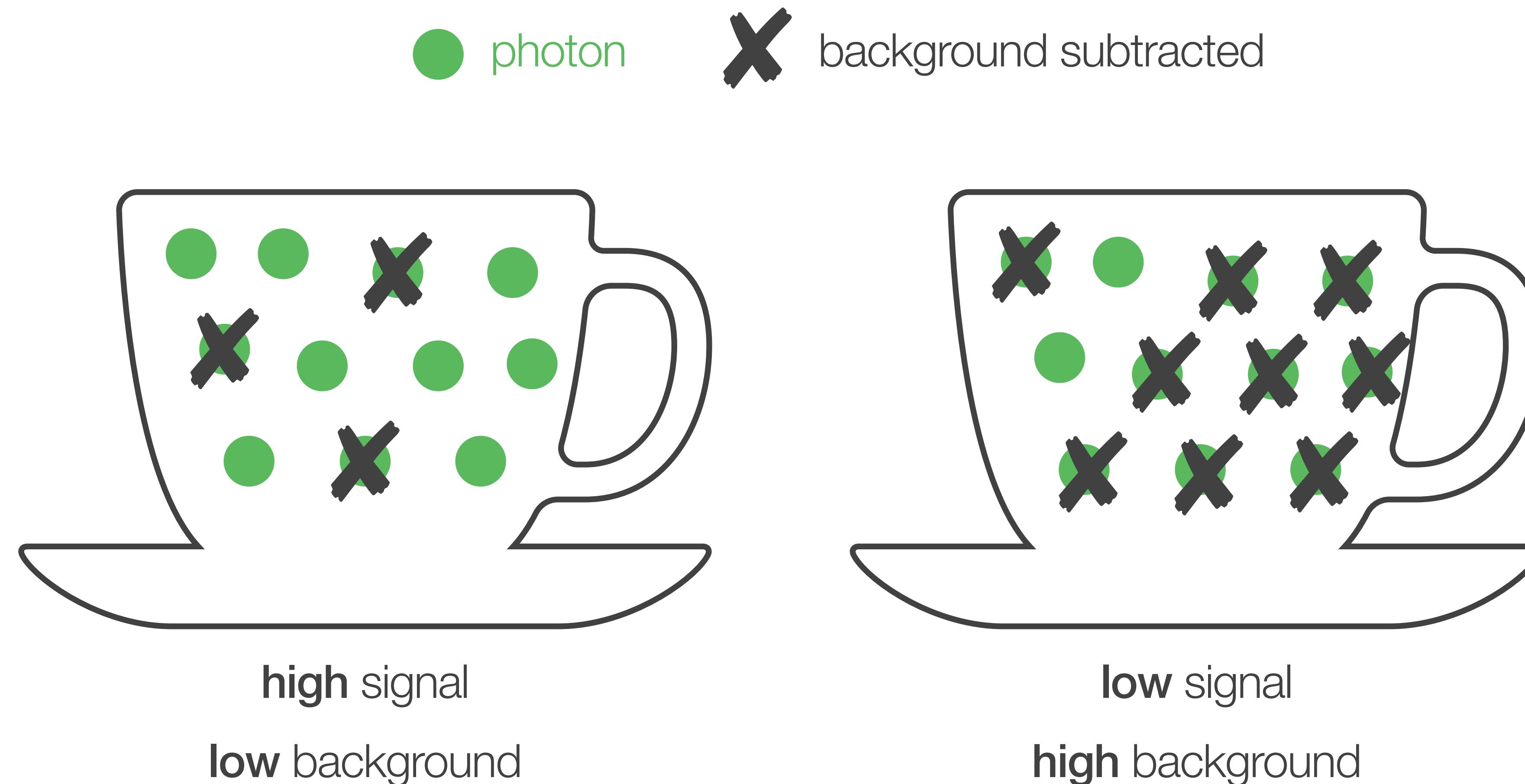


Phenol Red

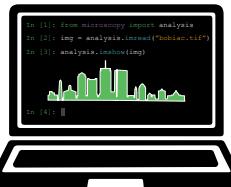
you can't wash out the unbound DAPI!



# Why is high background problematic?



Modified from Jennifer Waters



# Important Point

## #5

Background decreases SNR.



# Factors that can limit accuracy and precision in fluorescence microscopy

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resolution

sampling

signal to noise ratio (SNR)

background



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

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practical considerations



photons available  
from sample

x

% photons  
detected

=

Photon Budget

You have a limited number of photons.

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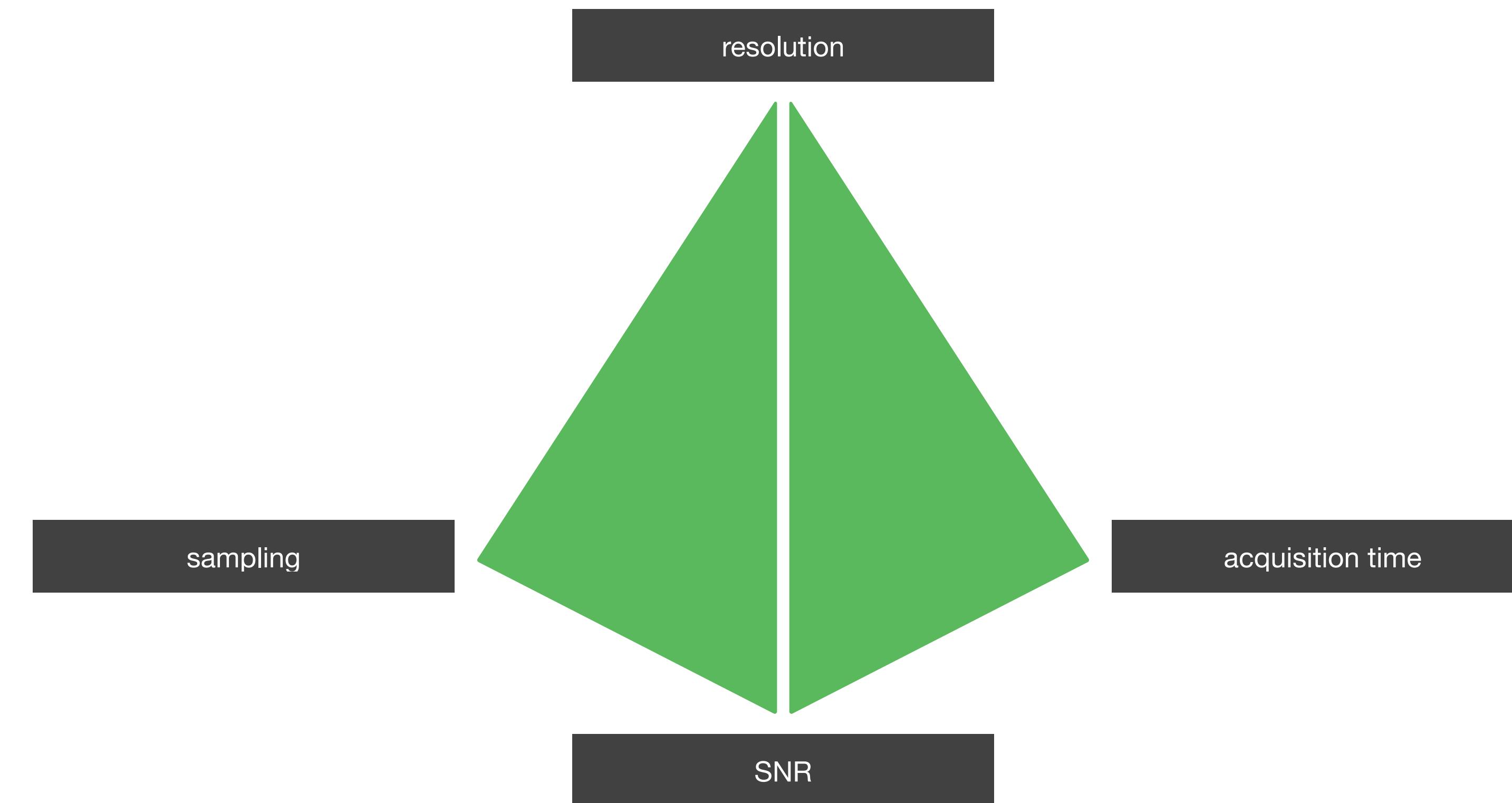
How will you spend them?

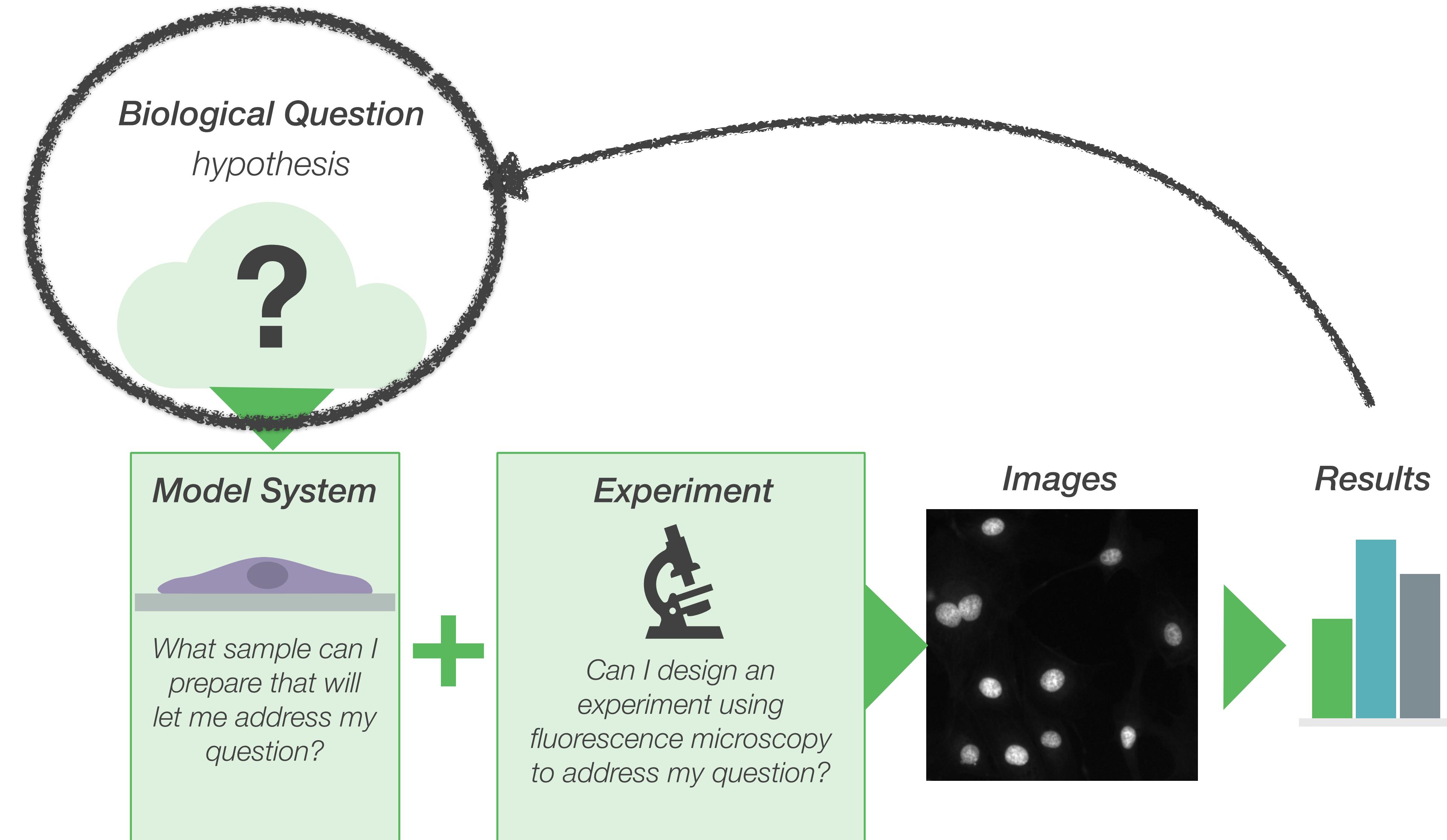


# The reality: You need to compromise at the microscope

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prioritize...





# Important Point

## #6

You must make compromises. Make educated & deliberate ones!



# You need to validate your methodology

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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

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an interactive, remote course on fundamental principles in  
optical microscopy

[microtutor.globalbioimaging.org](https://microtutor.globalbioimaging.org)



[youtube.com/microcourses](https://youtube.com/microcourses)

short, informative videos on microscopy concepts



# Questions?

