2015

Seeing at the Nanoscale: New Microscopies for the Life Sciences

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Seeing at the Nanoscale

New Microscopies for the Life Sciences

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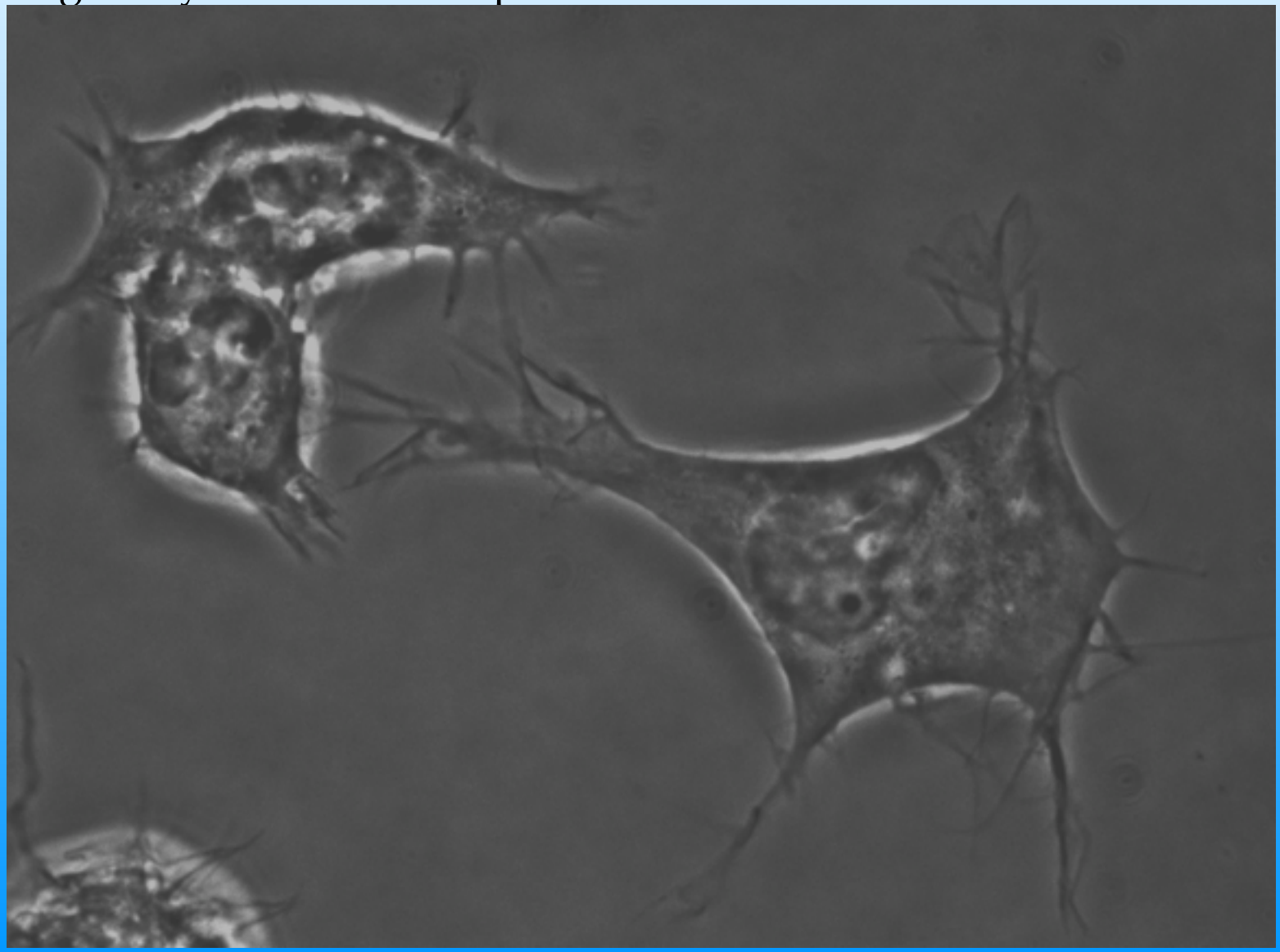
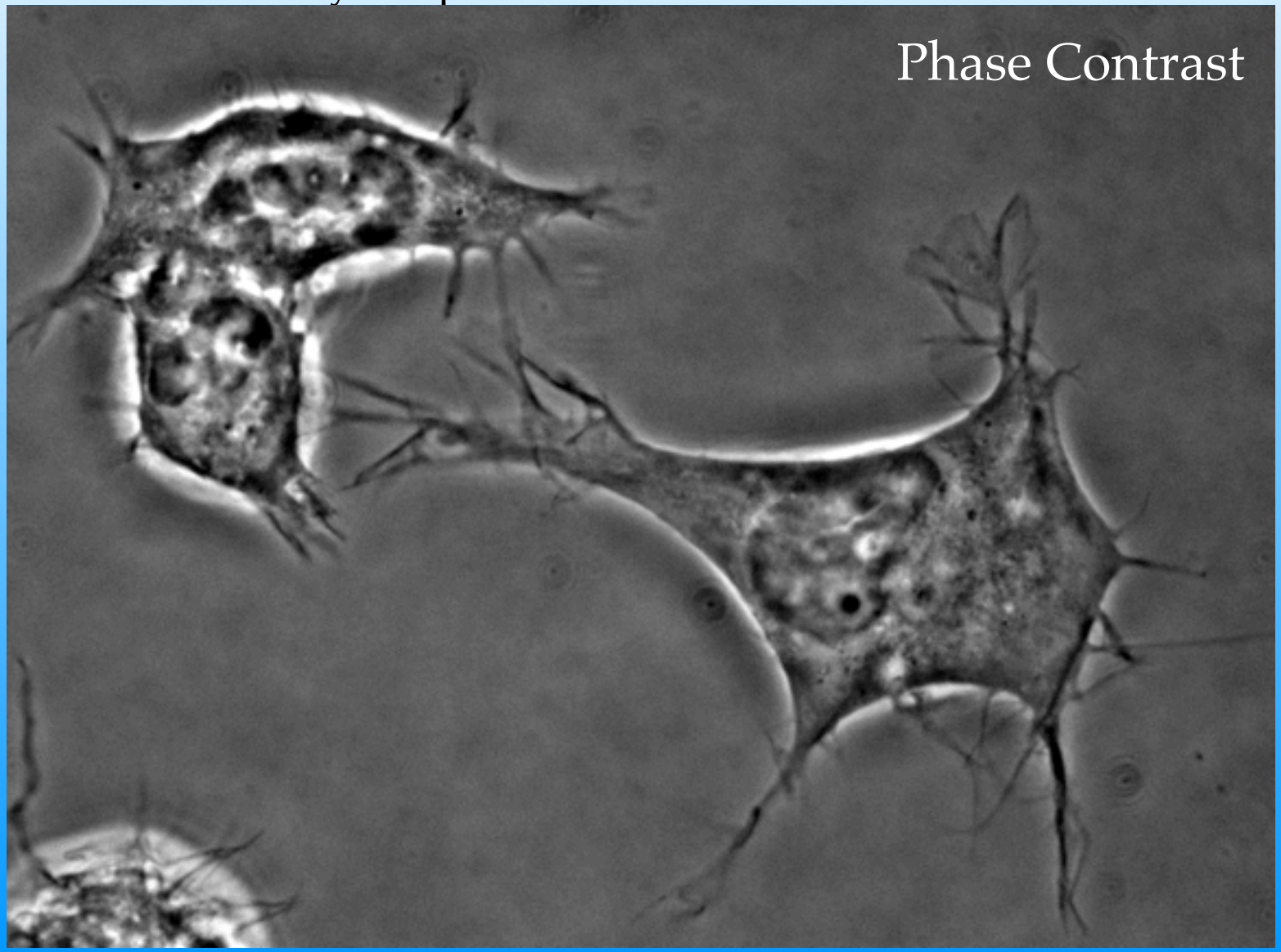
Visualizing Living Cells

Biological systems are transparent and difficult to see



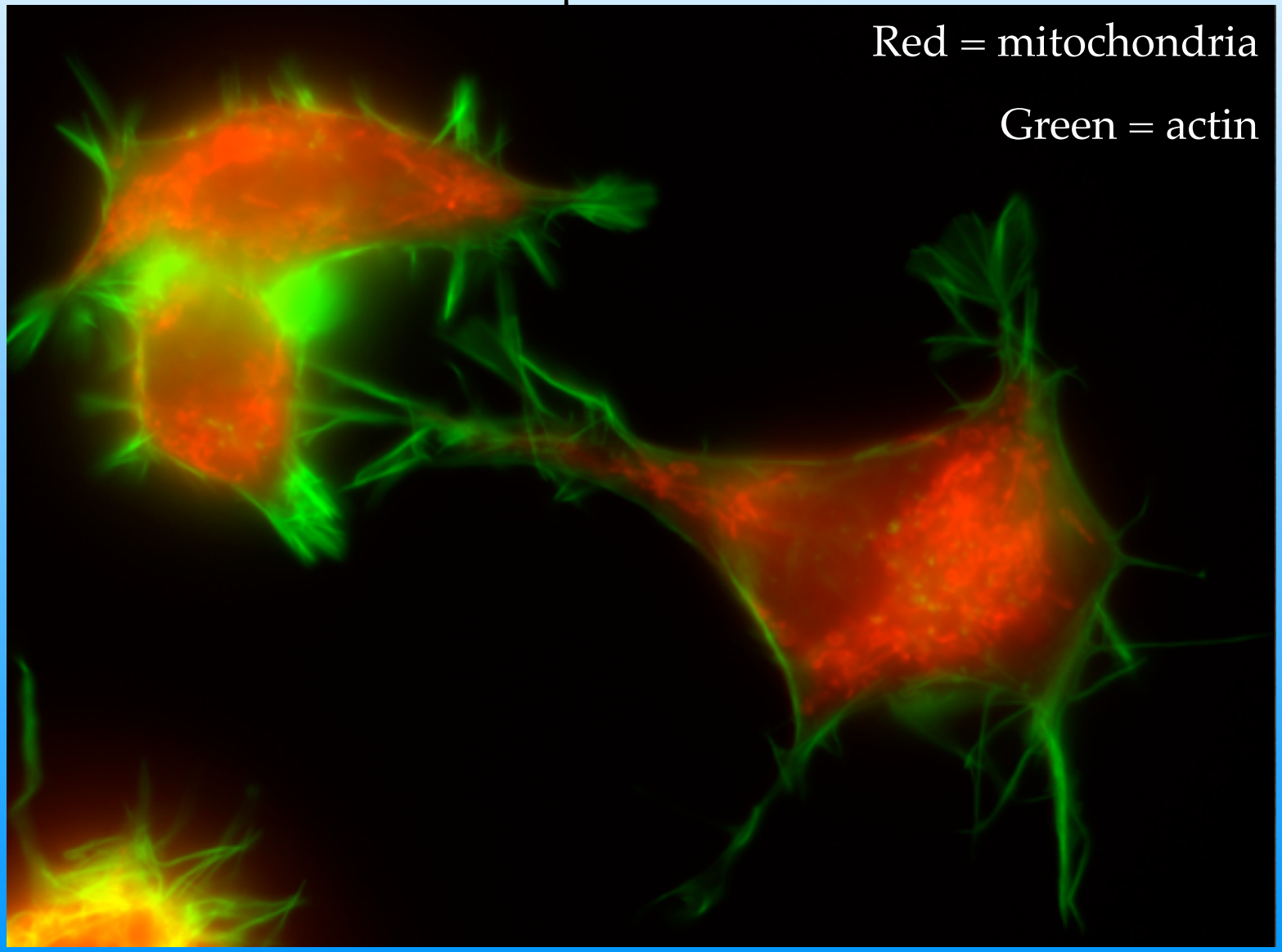
Visualizing Living Cells

We can use a variety of optical tricks to enhance the contrast



Visualizing Living Cells

We use fluorescence to see components inside cells



Principles of Fluorescence

Use a light microscope to illuminate and observe fluorescence

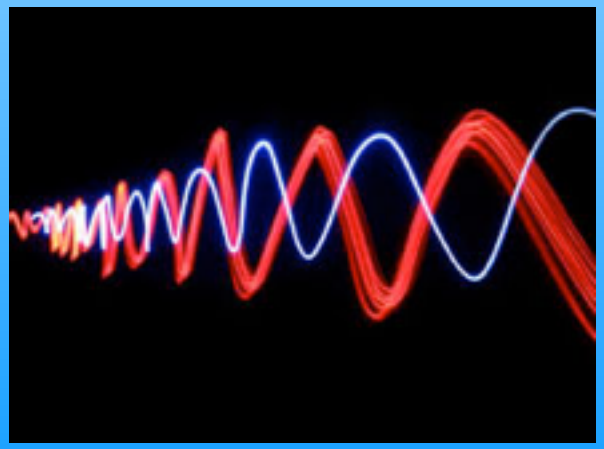
Fluorescent molecules

excites more Violet (higher energy)

emits more Red (lower energy)

High energy

Low energy

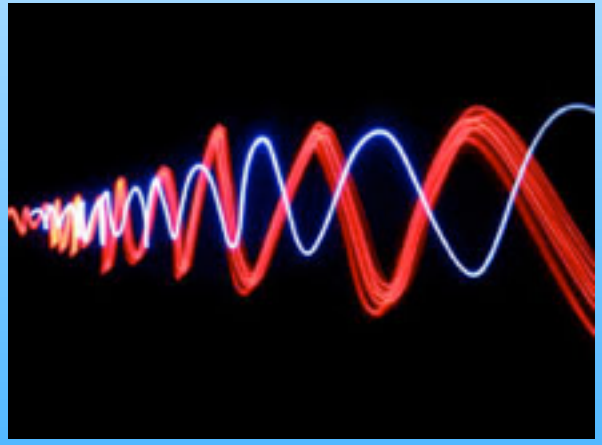


Light is a wave.
What is the speed??

Principles of Light

High energy

Low energy



Light is a wave.
What is the speed??

$c = 3 \times 10^8$ m/s

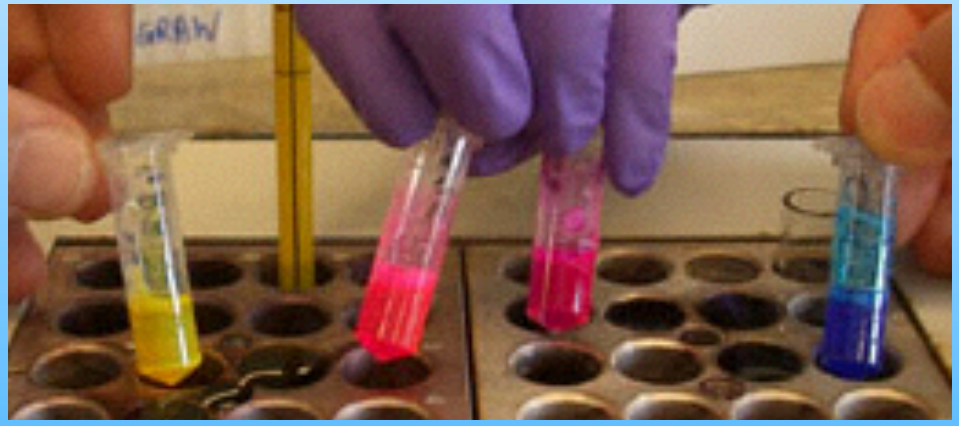
$c = f * \lambda$

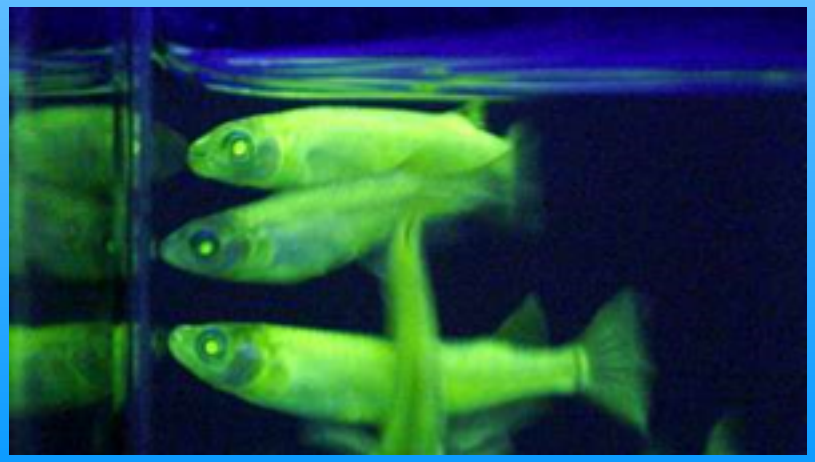
You calculate:
If the wavelength of visible light is 500 nm, what is the frequency??

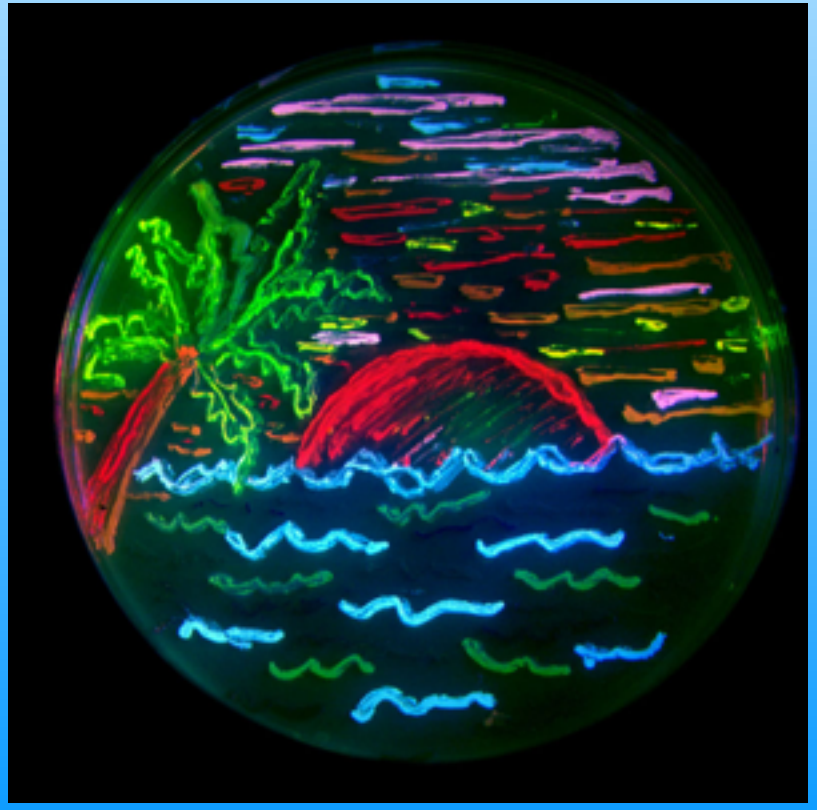
Common Fluorophores

Rhodamine, Fluoroscein, Cy-dyes, Alexa-dyes

Green Fluorescent Protein and numerous derivatives (won Nobel Prize in Chemistry 2008)







Fluorescence - How it works

Excitation

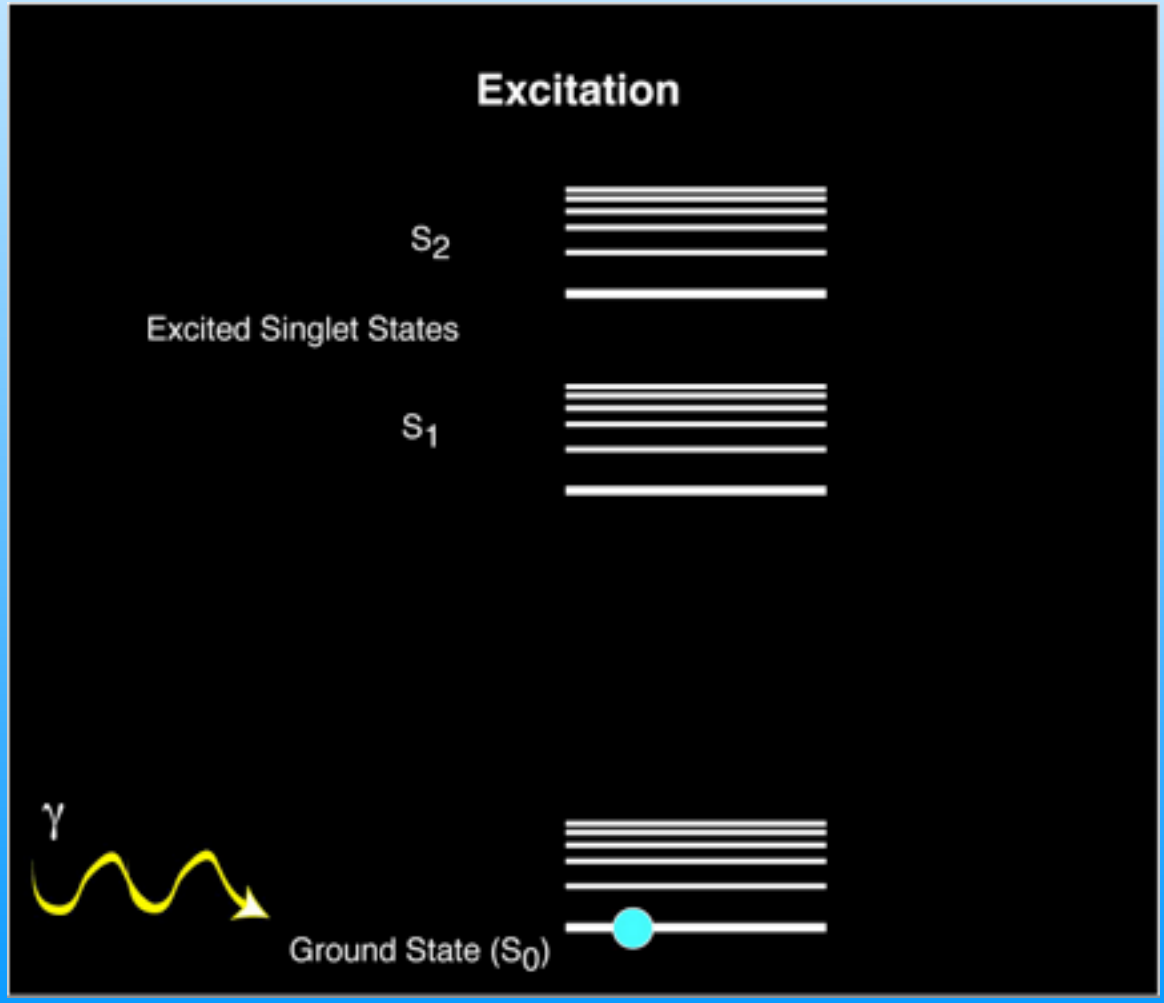
10^{-15} s

Vibrational Relaxation

10^{-14} - 10^{-11} s

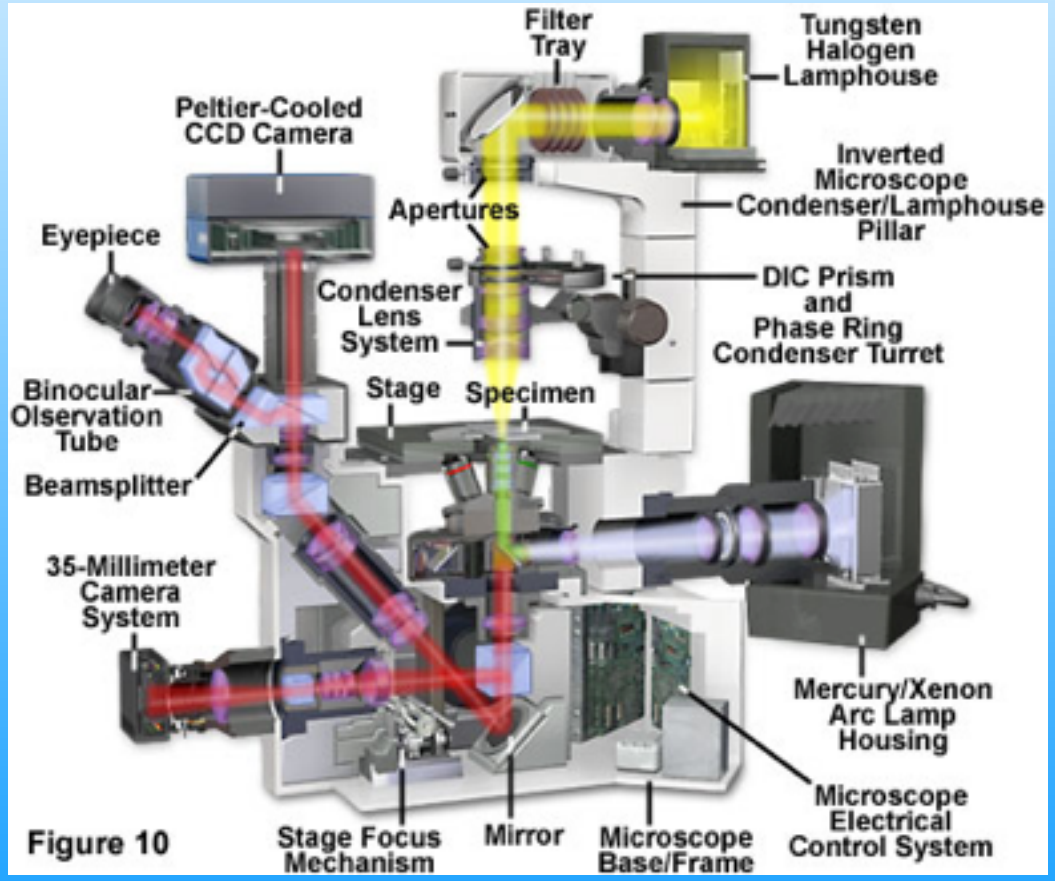
Fluorescence

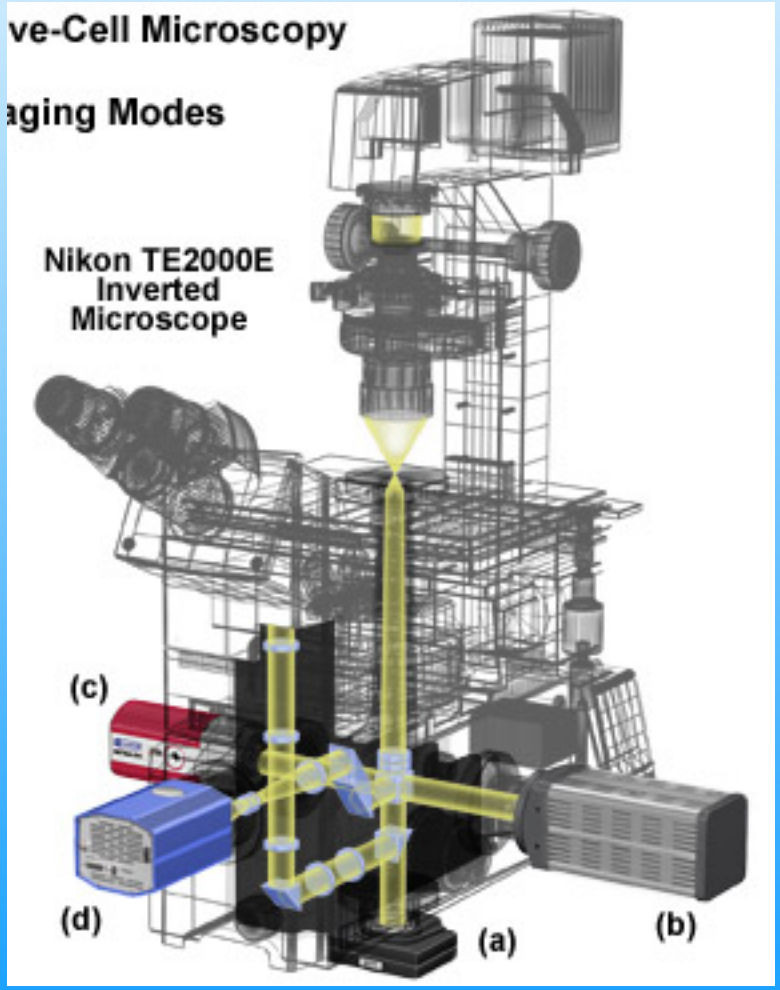
10^{-9} - 10^{-7} s



Fluorescence microscopy

Anatomy of modern inverted microscope





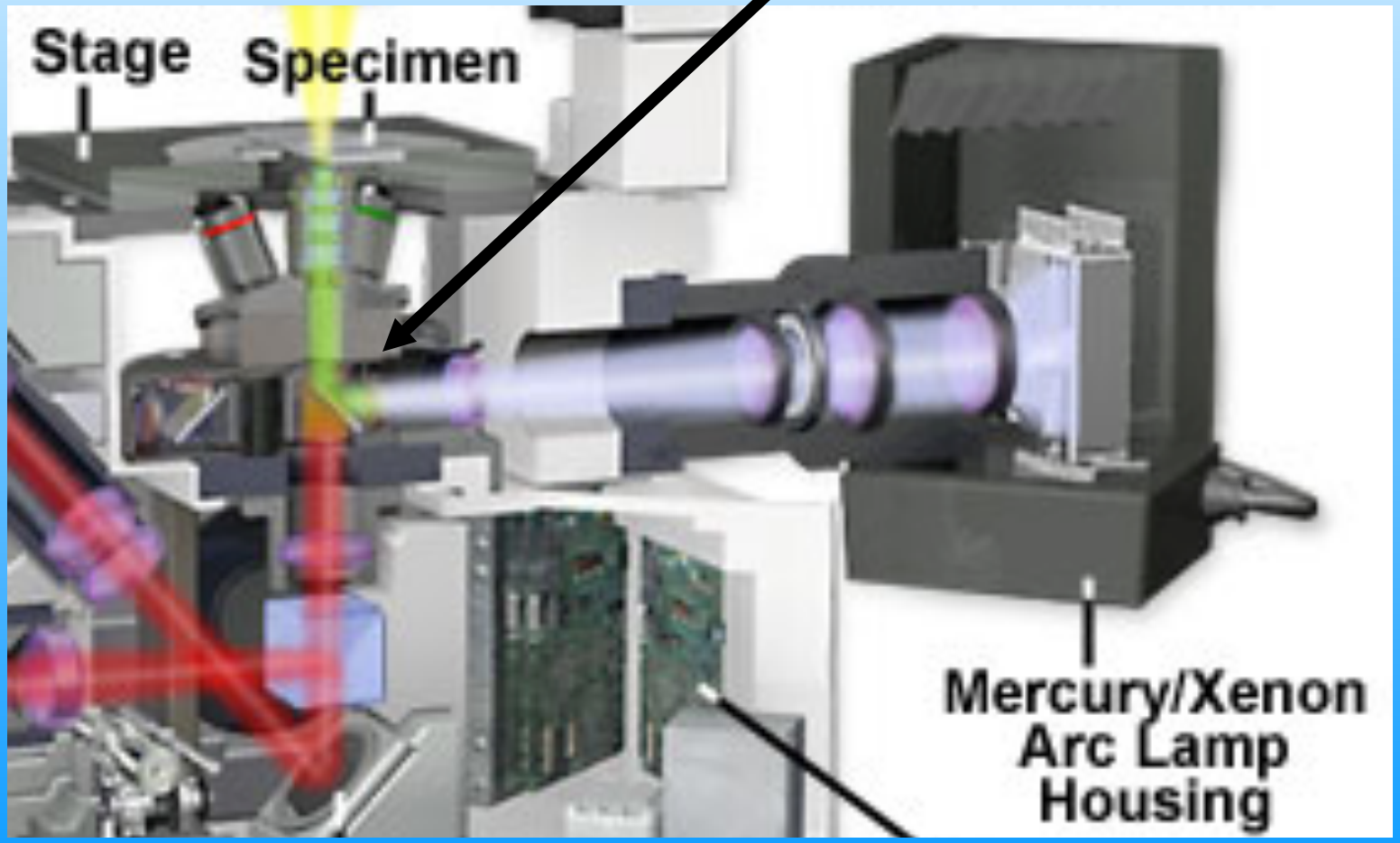
Olympus Microscopy Resource Center website

Nikon MicroscopyU website

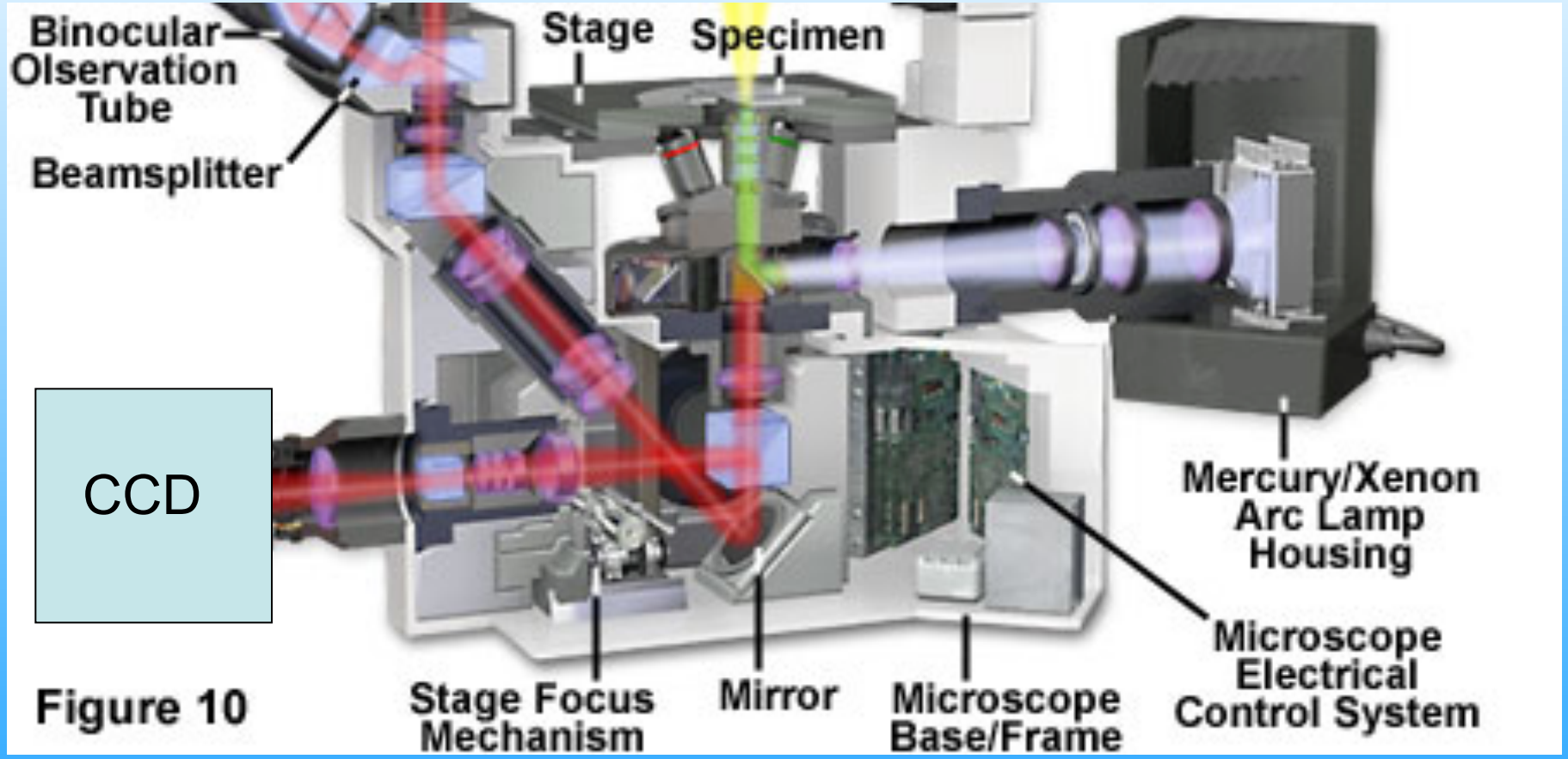
Fluorescence microscopy

Epi-illumination path

Fluorescence Cube with filters and dichroic



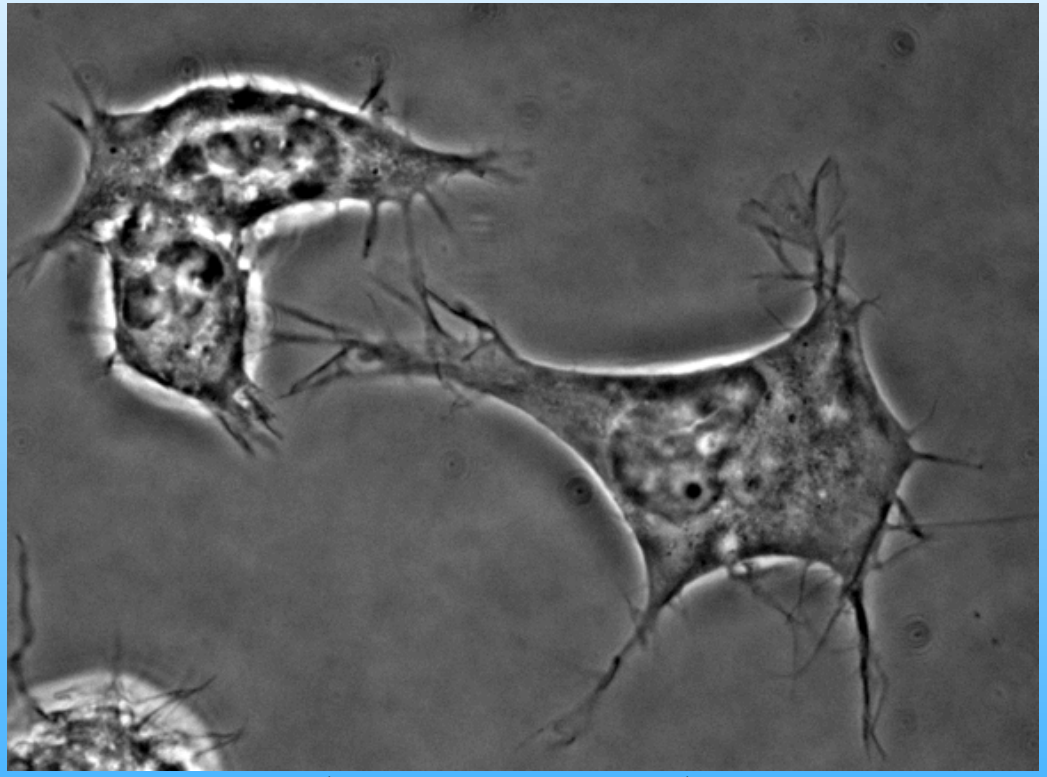
Fluorescence microscopy - Modern Detection



Charged-coupled device (CCD) camera

Each pixel detects photons, which are translated to a number that is displayed as a grey value on a computer

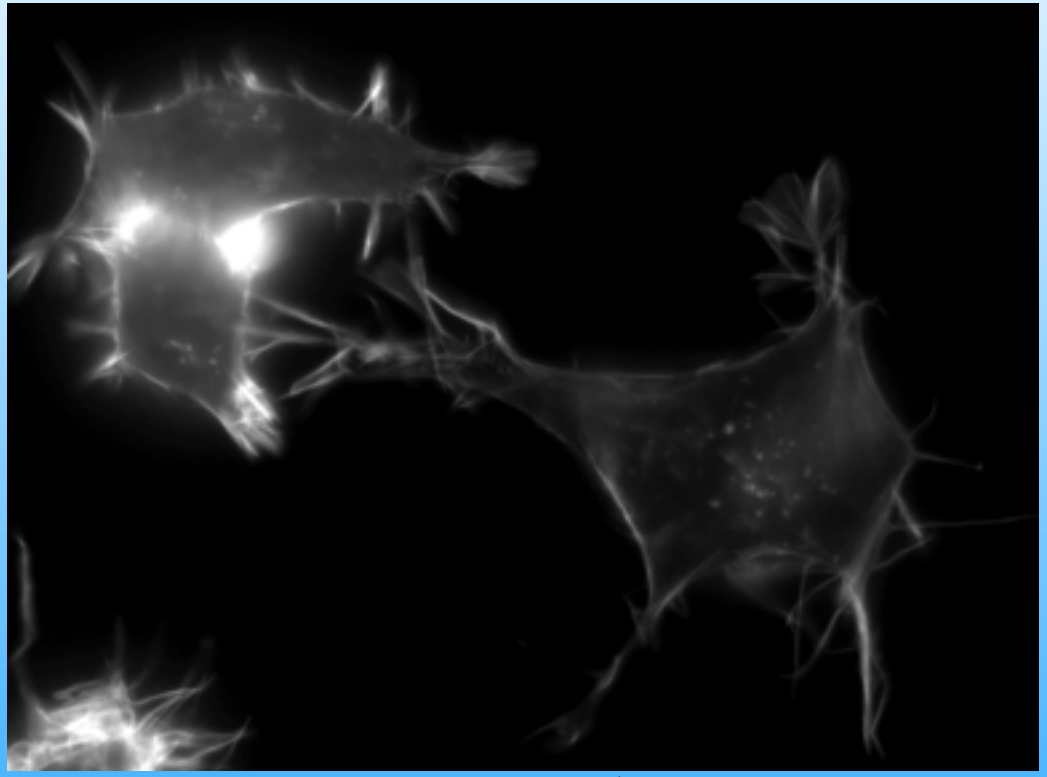
Fluorescence microscopy - Multiple Colors



Transmitted light microscopy (phase contrast)

CCD is black and white, so we switch filter sets to see different colors

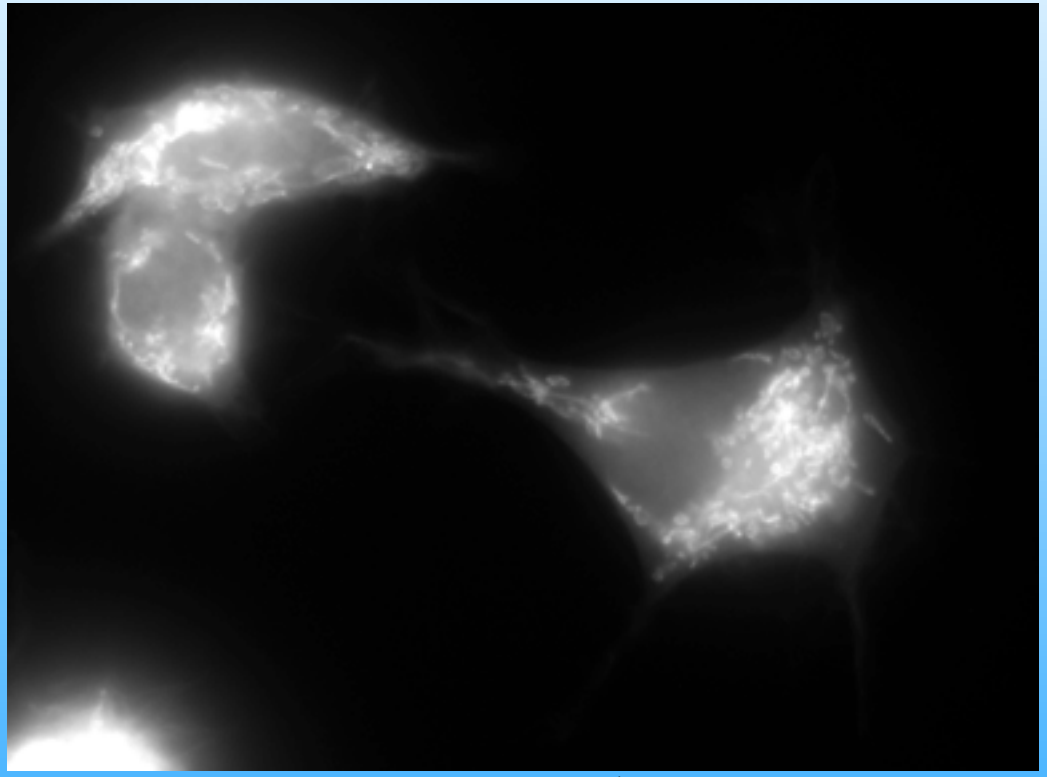
Fluorescence microscopy - Multiple Colors



Fluorescence microscopy with green emission filter set

CCD is black and white, so we switch filter sets to see different colors

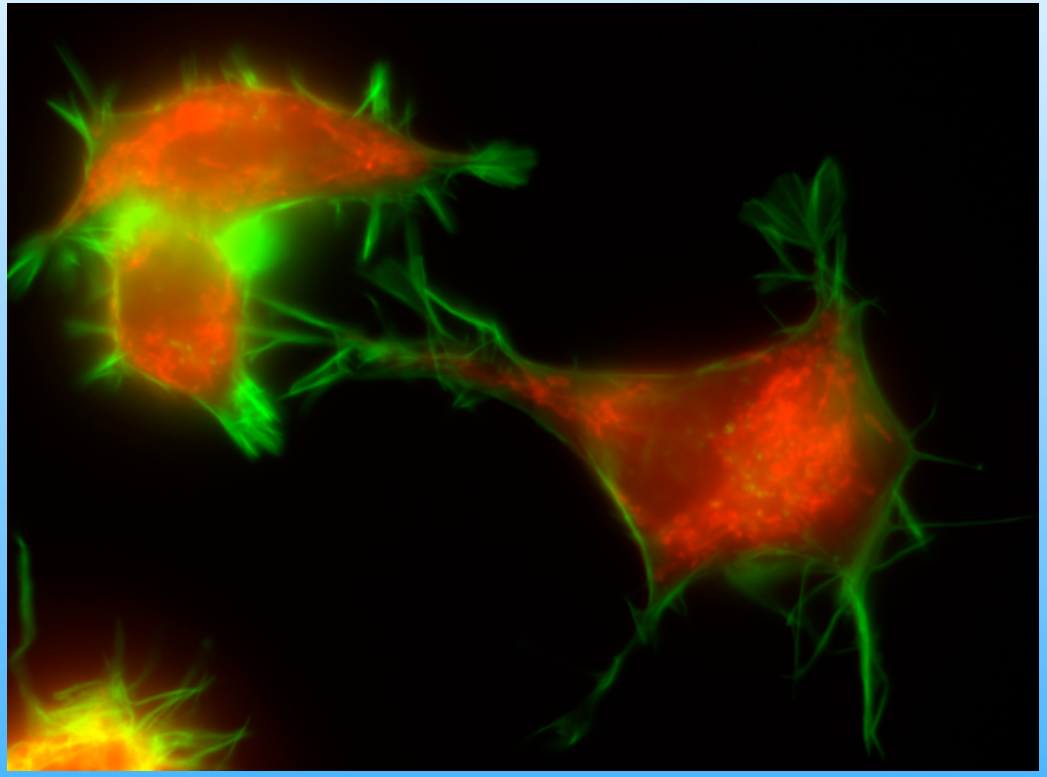
Fluorescence microscopy - Multiple Colors



Fluorescence microscopy with red emission filter set

CCD is black and white, so we switch filter sets to see different colors

Fluorescence microscopy - Multiple Colors



False color red and green overlay

CCD is black and white, so we switch filter sets to see different colors

Most two-color imaging is not simultaneous, but rather sequential

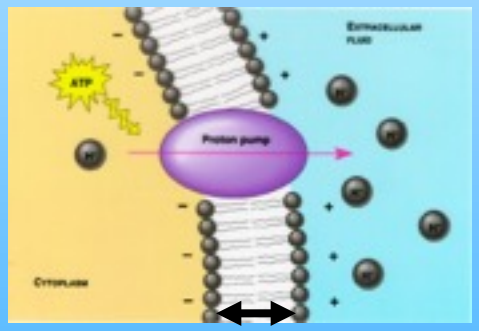
Nanoscale in Biology

Proteins (2-5 nm)

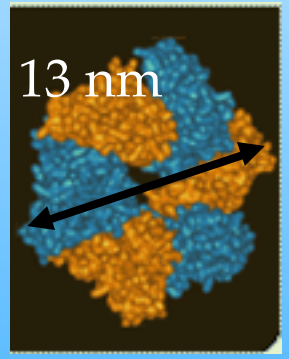
Protein, DNA, RNA filaments (2-25 nm)

Molecular complexes (5-25 nm)

Membranes (4 nm thick)

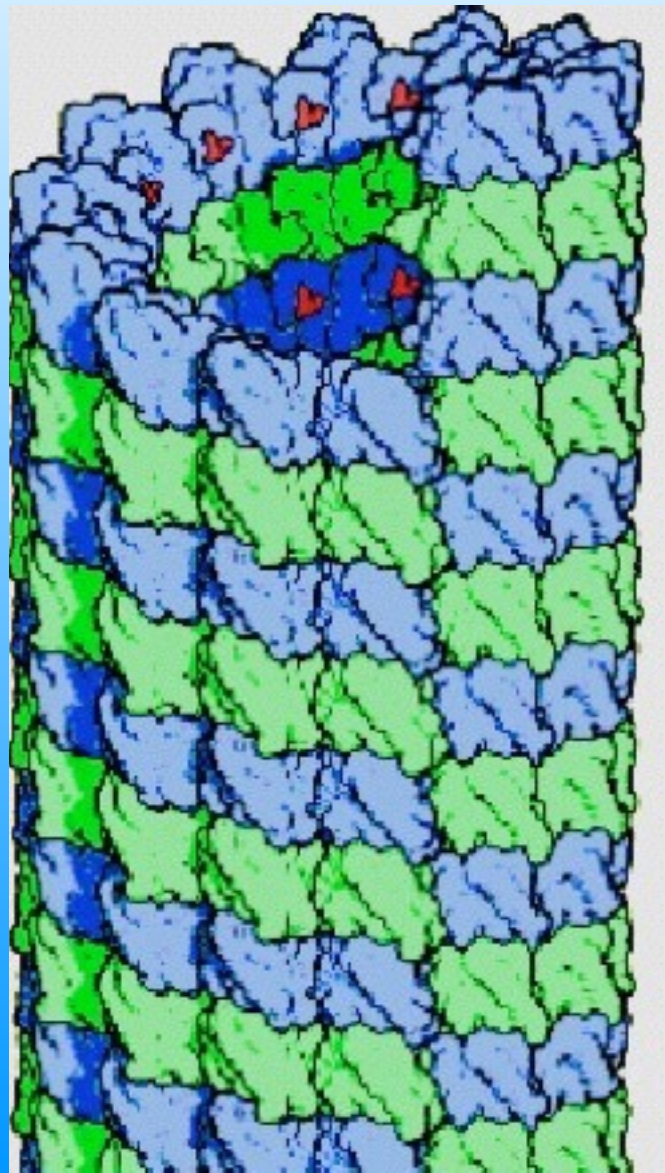


4 nm



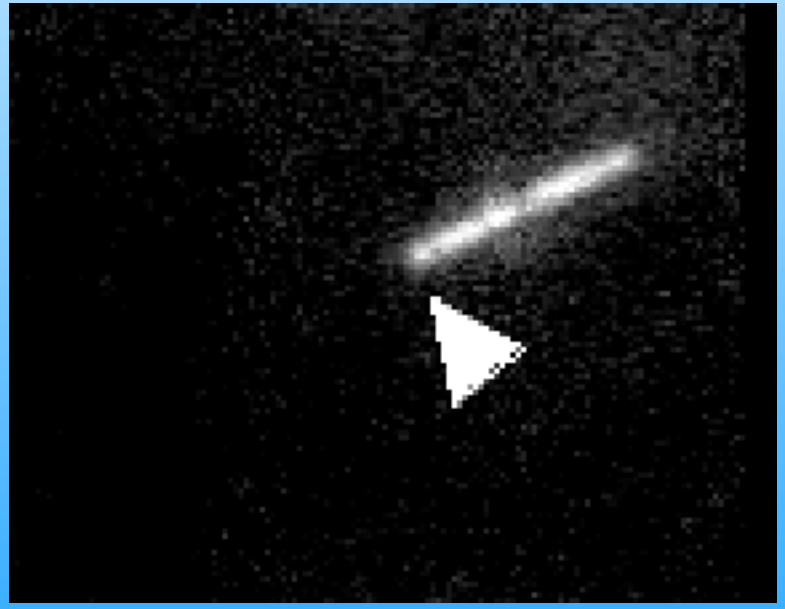
Vale, et. al. Cell 2003

25 nm

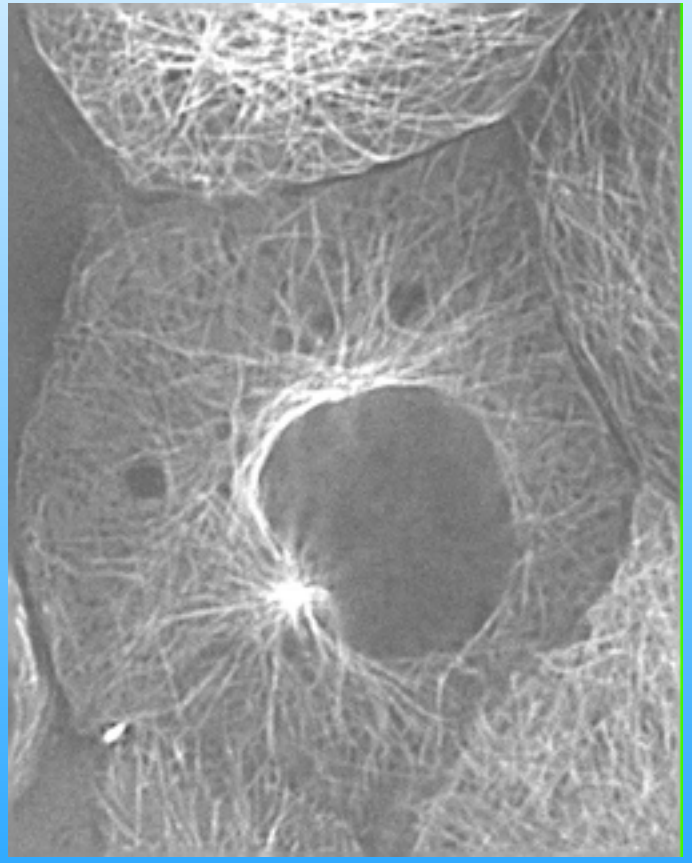


Visualizing the Nanoscale

Attaching fluorescent molecules to these objects allows us to see them and watch their dynamics



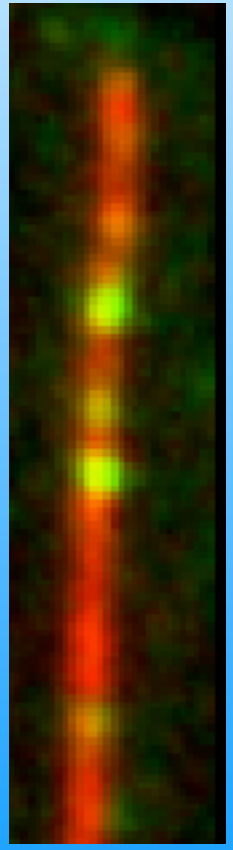
Microtubules outside of cell,
Ross Lab

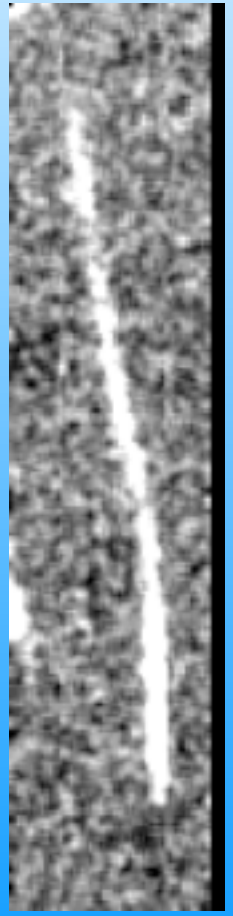


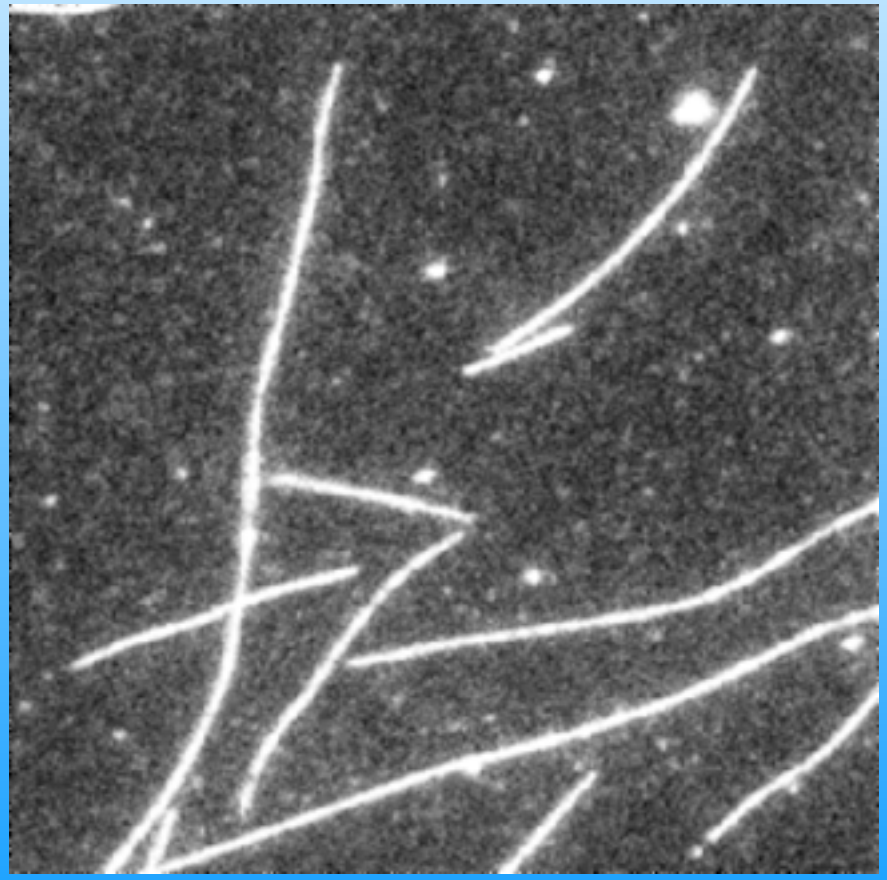
Microtubules inside of cell,
Wadsworth Lab

Visualizing Single Proteins

Attaching fluorescent molecules to these objects allows us to see them and watch their dynamics







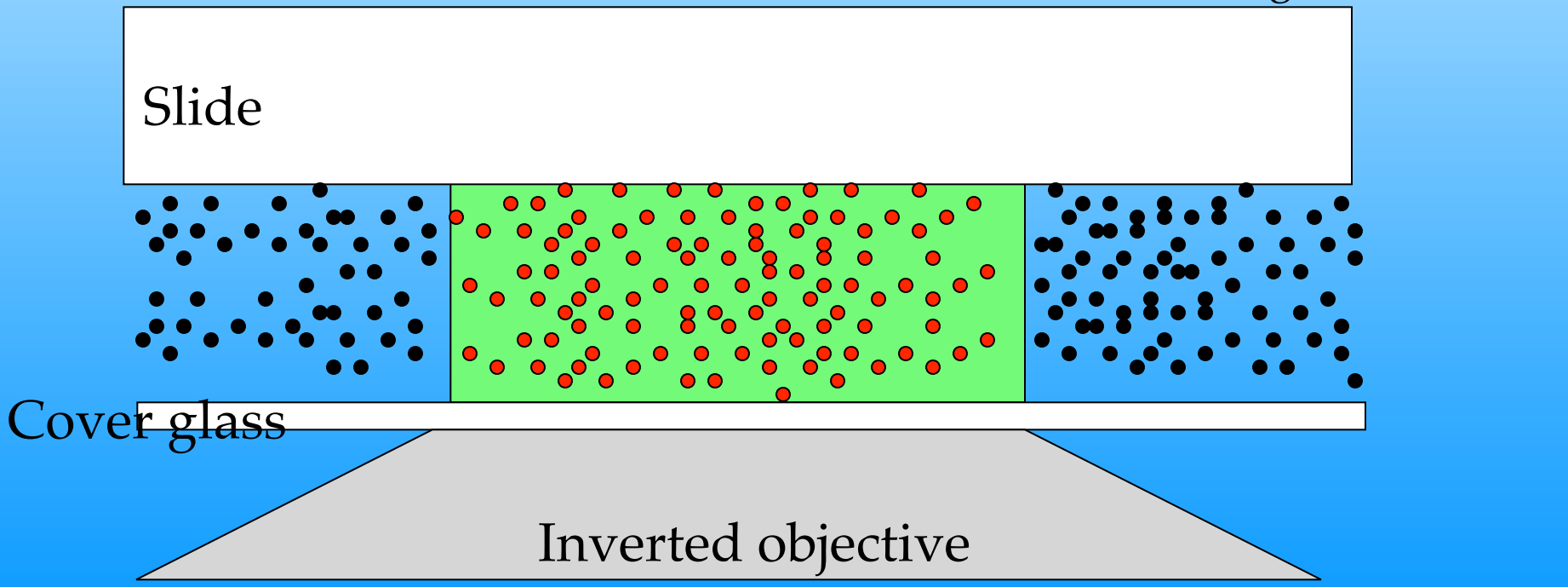
How do we Visualize Single Molecules?

When you illuminate a sample in epi-fluorescence, a rather large volume is illuminated

Causes background fluorescence

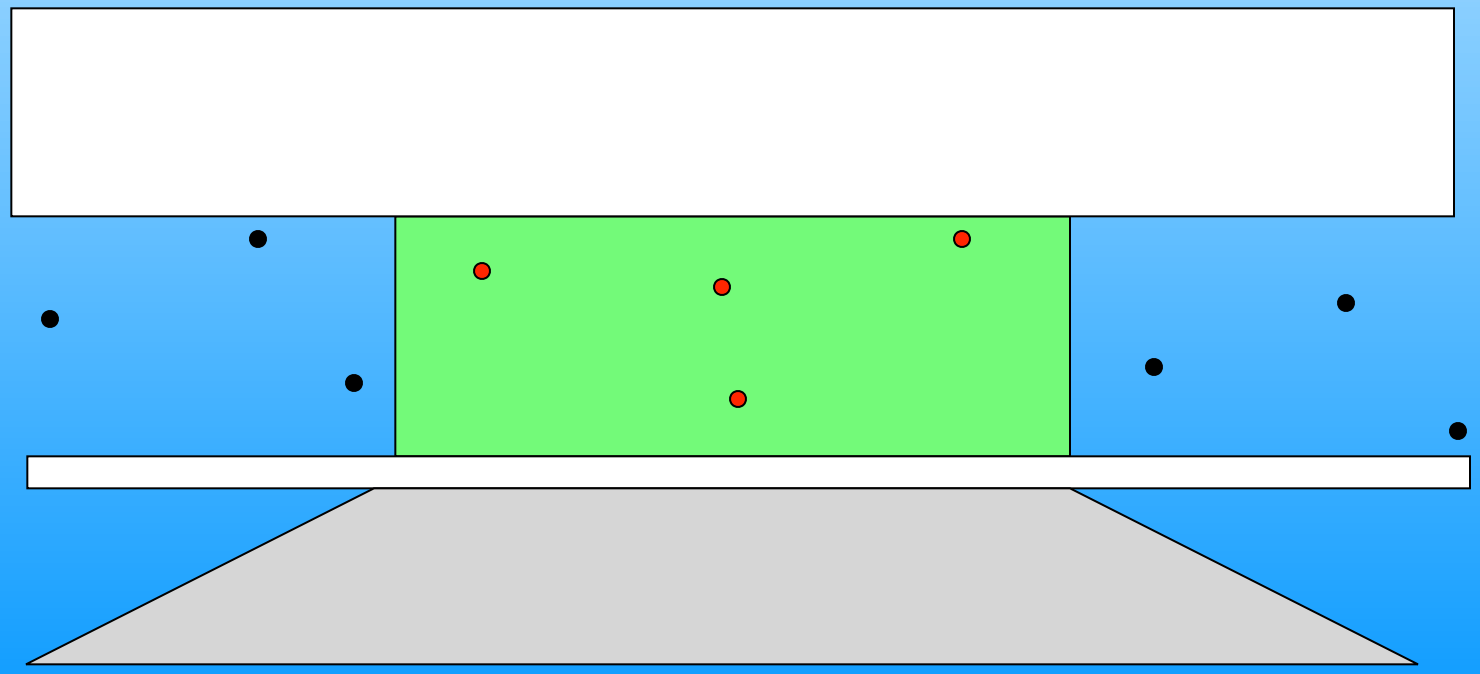
Many molecules in the field

How can we see single molecules?



Visualizing Single Molecules

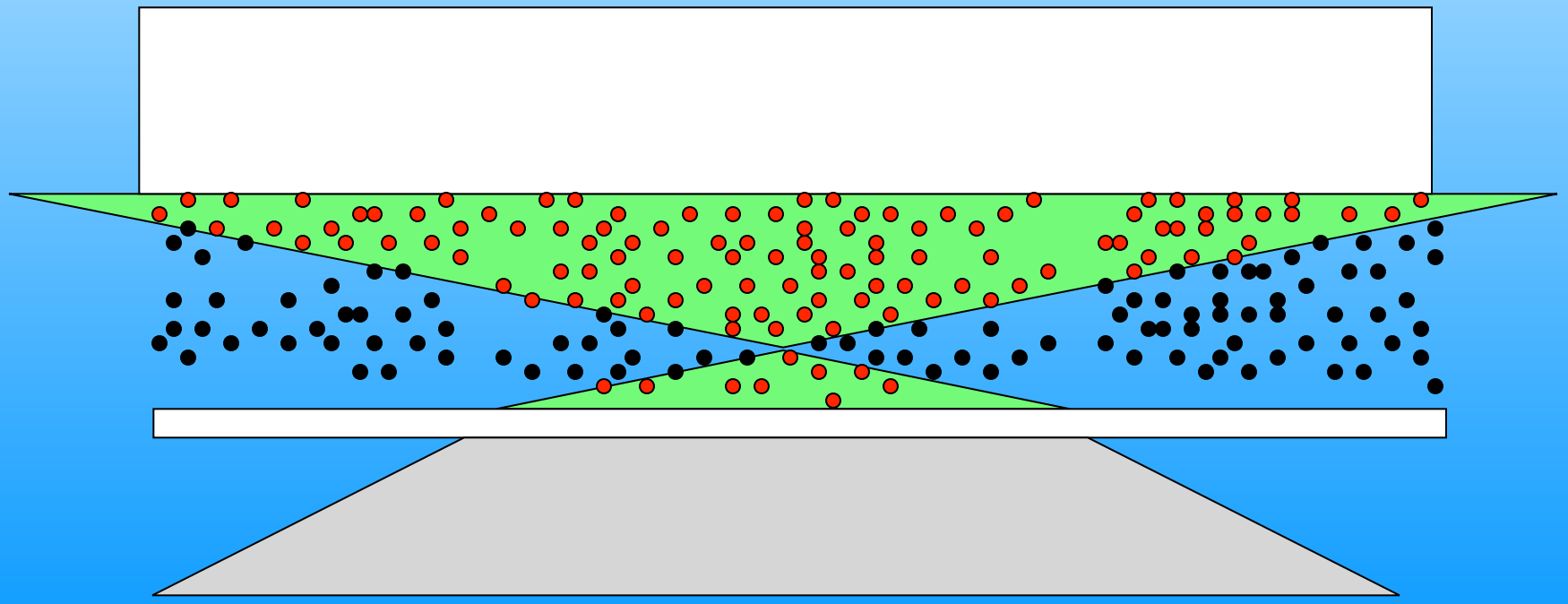
1) Dilute the sample



Visualizing Single Molecules

1) Dilute the sample

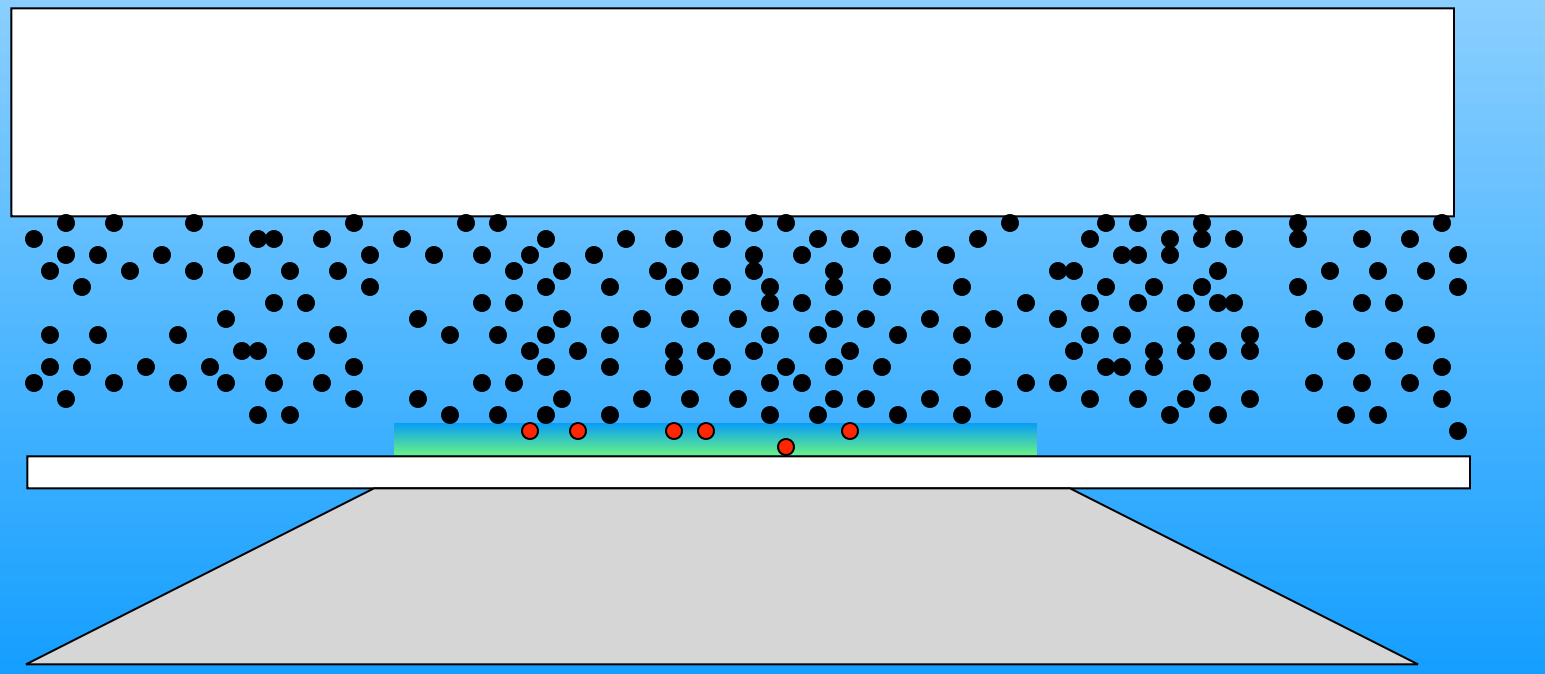
2) We could use a confocal spot with apertures to block out-of-plane fluorescence



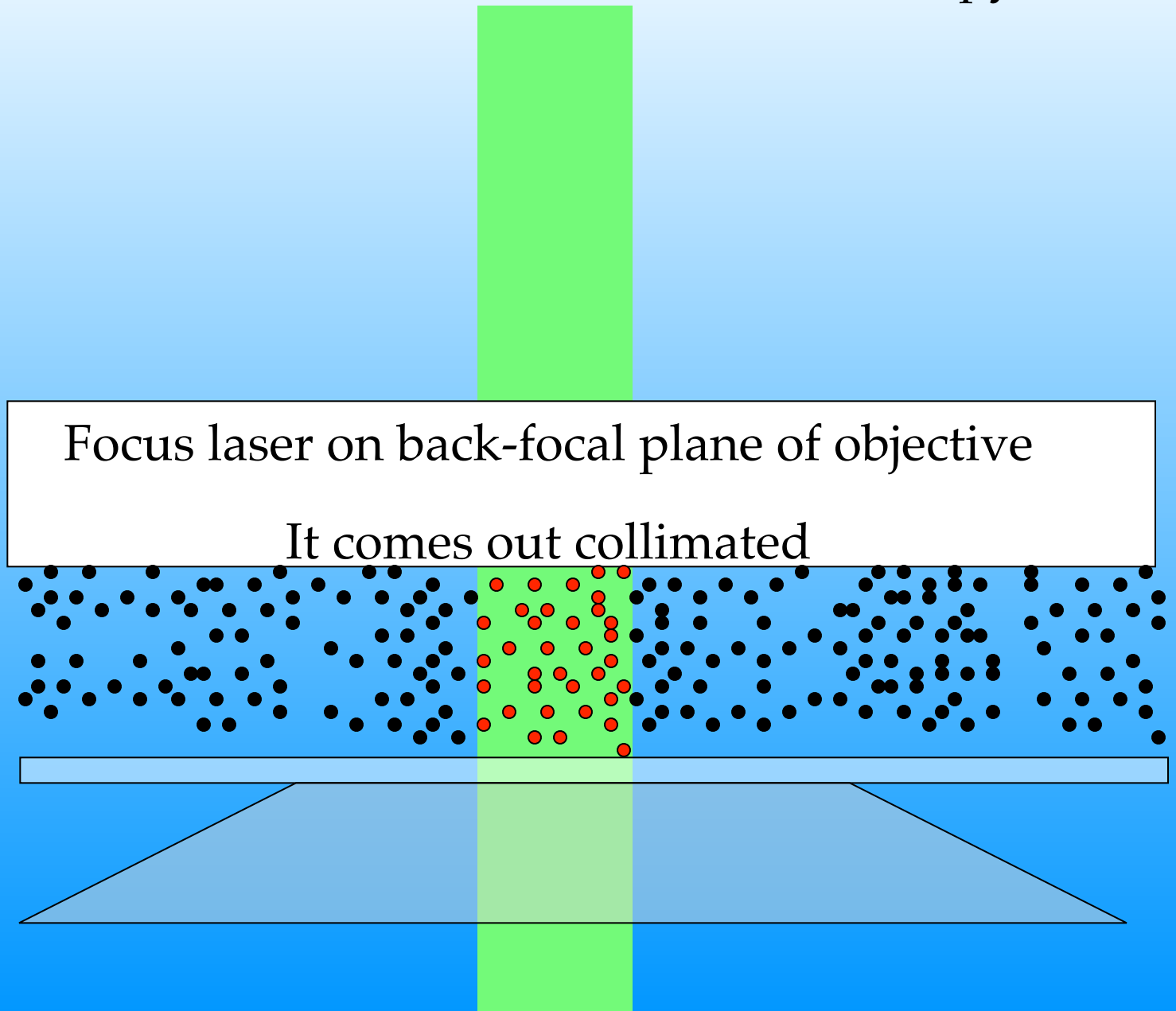
Visualizing Single Molecules

1) Dilute the sample
2) We could use a confocal spot with apertures to block out-of-plane fluorescence
3) Total Internal Reflection Fluorescence

My method of choice

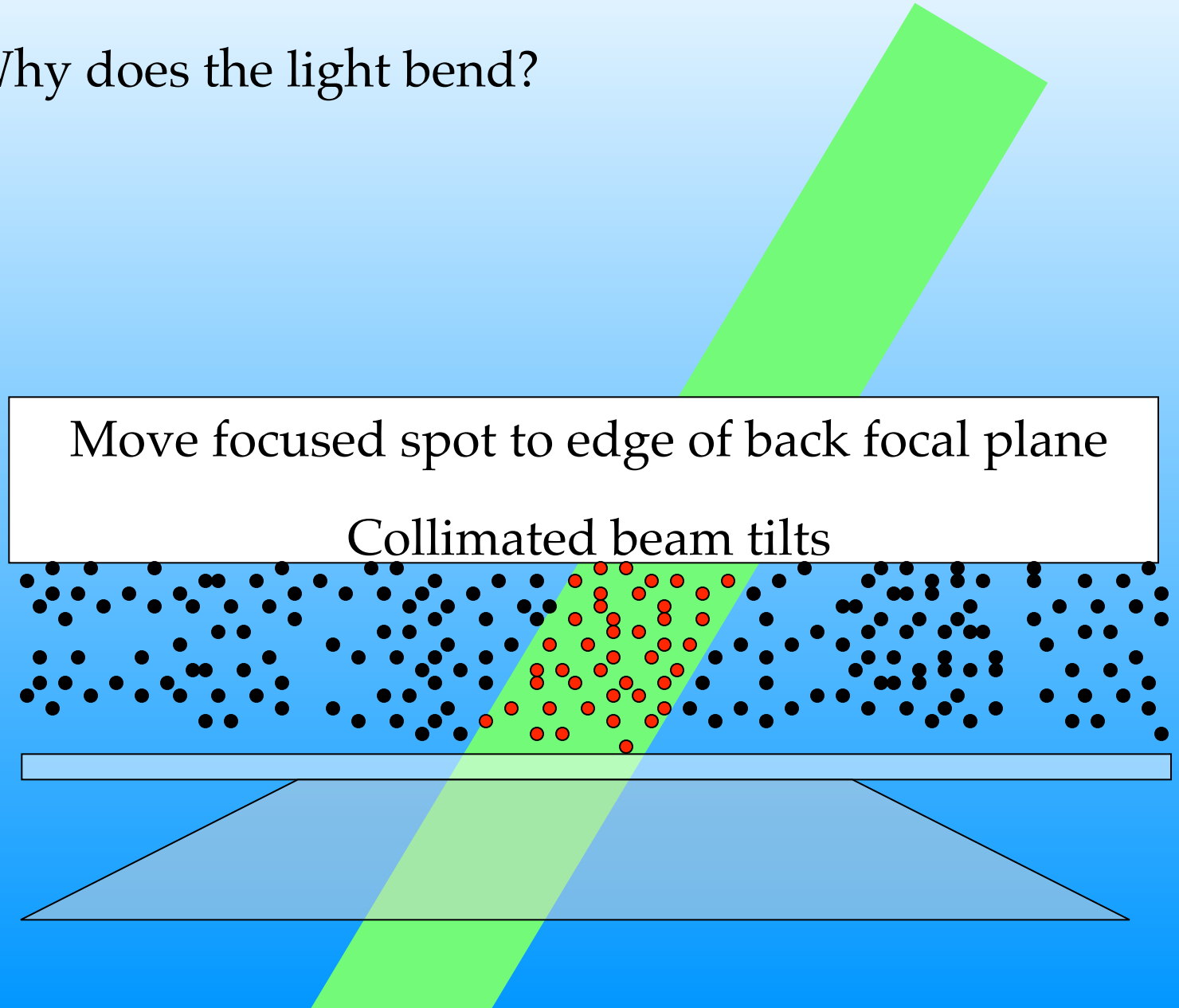


Total Internal Reflection Fluorescence Microscopy



Total Internal Reflection Fluorescence Microscopy

Why does the light bend?

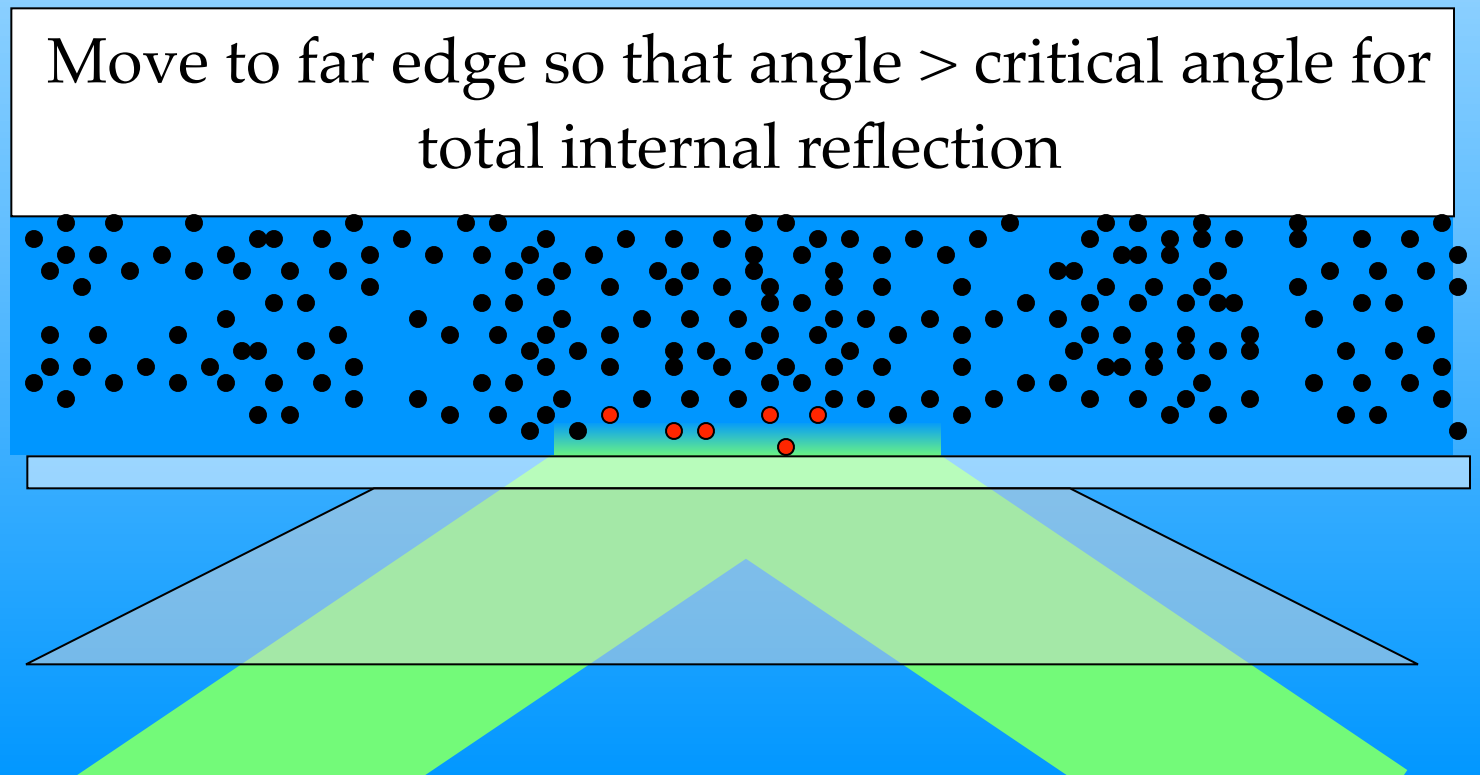


Total Internal Reflection Fluorescence Microscopy

Snell's Law says: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Calculate:

If $n_{glass} = 1.51$ and $n_{water} = 1.38$, what is the "critical angle" for total internal reflection? (When does $\theta_2 = 90°$?)



Total Internal Reflection Fluorescence Microscopy

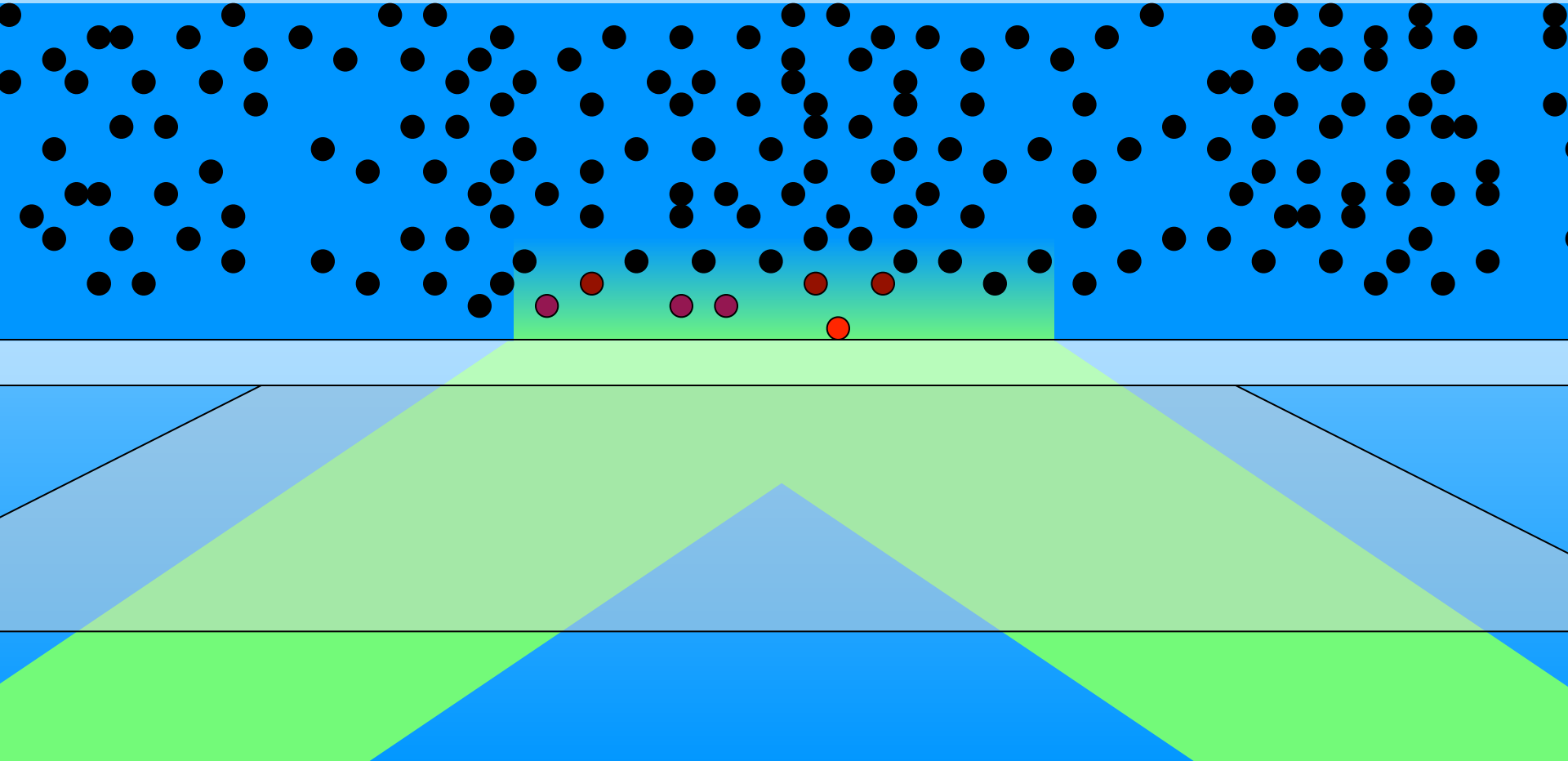
Zoom in on Evanescent Wave

Decays exponentially in z

Brighter is closer to cover glass

Only about 100 nm into sample

Only molecules within 100 nm are visible



Total Internal Reflection Fluorescence Microscopy

Zoom in on Evanescent Wave

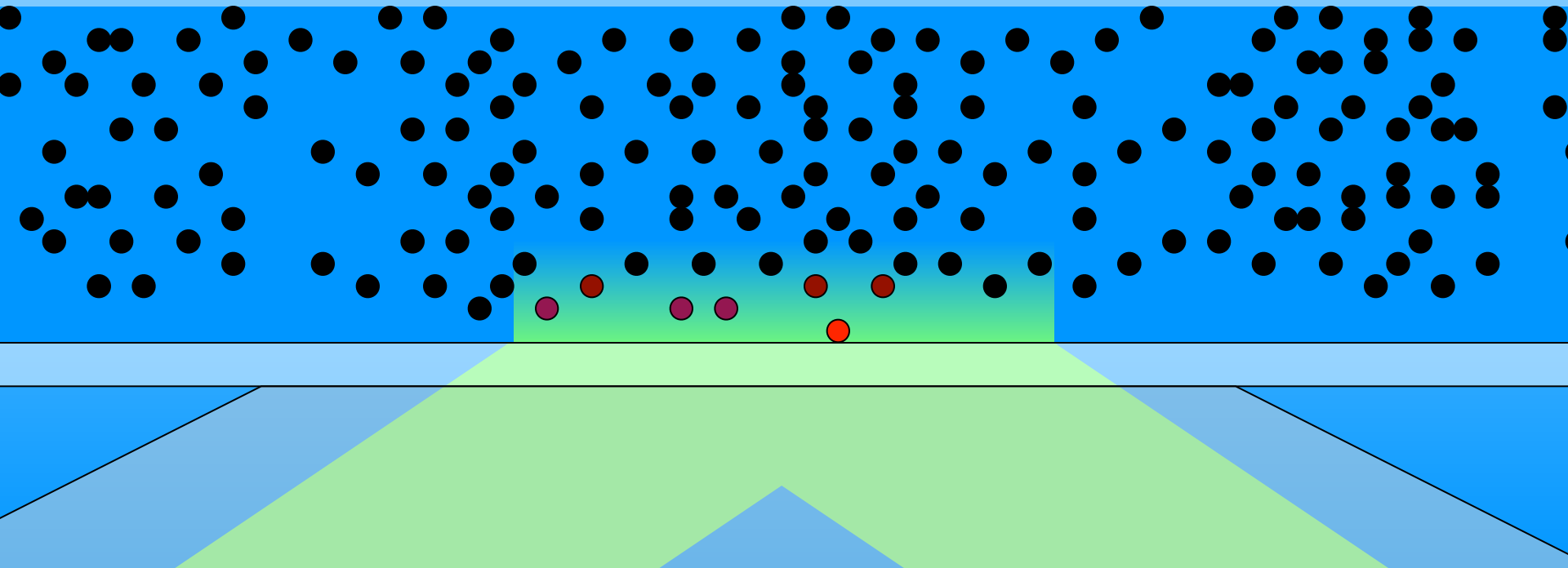
Decays exponentially in z

Only about 100 nm into sample

What does an exponential decay look like?

Plot it.

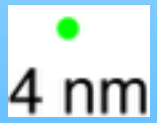
If it dies off after 100 nm, what does that tell you about the decay constant??



Resolution Limits to Imaging Single Molecules

A motor protein takes an 8 nm step, can we measure that in our single molecule assay?

Please vote by holding up the number of fingers you want to vote for: (1) Yes. (2) No.



Resolution Limits to Imaging Single Molecules

A motor protein takes an 8 nm step, can we measure that in our single molecule assay?

Ideally, the motor is only about 4 nm, so an 8 nm step should be visible

But it's not resolvable…

Resolution Limits to Imaging Single Molecules

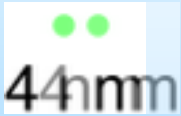
Why?? Why can't we see the step?

What is resolution??

What is the resolution of our microscope?

Resolution Limits to Imaging Single Molecules

Why?? Why can't we see the step?



What is resolution??

The quantifiable ability to resolve or distinguish between two items. In this case, the items are the images of the same molecule at two positions.

Why does it happen??

Because light is a wave, we cannot focus it infinitely well. This is a fundamental physical limit of all imaging systems.

What is the resolution of our microscope?

Distance resolvable:

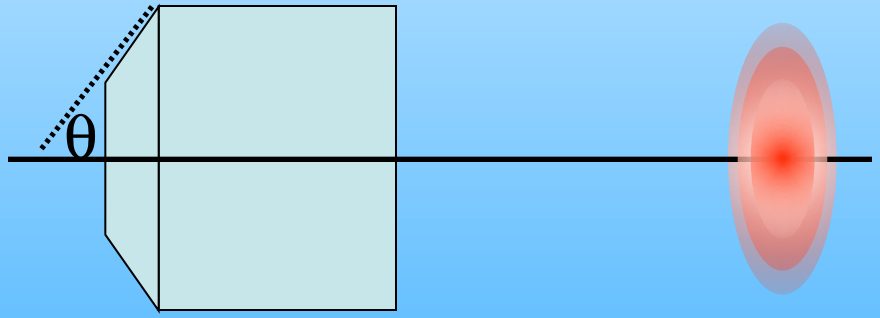
$$d = 1.22 \frac{\lambda}{2NA}$$

Microscope resolution:

Resolution Limits to Imaging Single Molecules

Why?? Why can't we see the step?

The objective diffracts the light, because it is a wave. Water waves diffract, too:

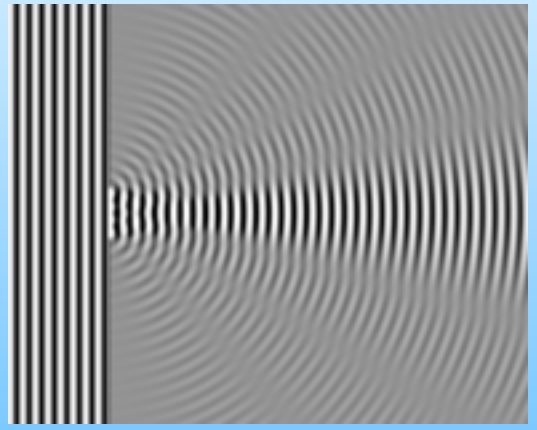


$NA = n^{*}\sin\theta_{max}$

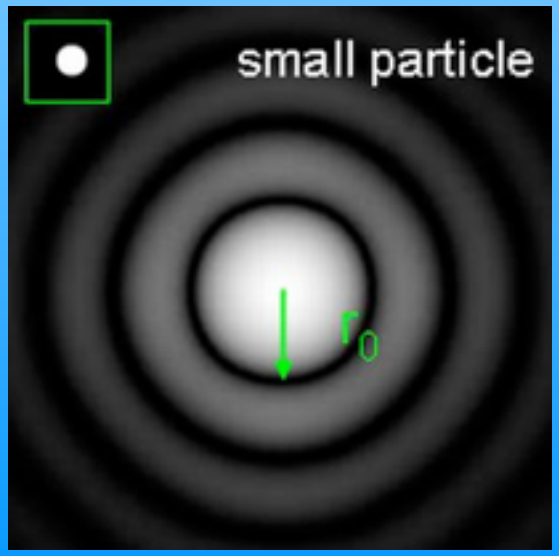
n = index of refraction

$$d = 1.22\frac{\lambda}{2NA}$$

Water waves diffracting through a hole in barrier

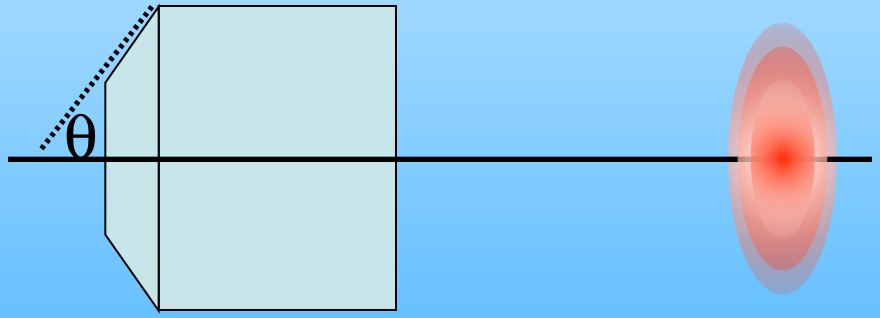


This is the diffraction limit



Resolution Limits to Imaging Single Molecules

Why?? Why can't we see the step?



$NA = n^{*}\sin\theta_{max}$

n = index of refraction

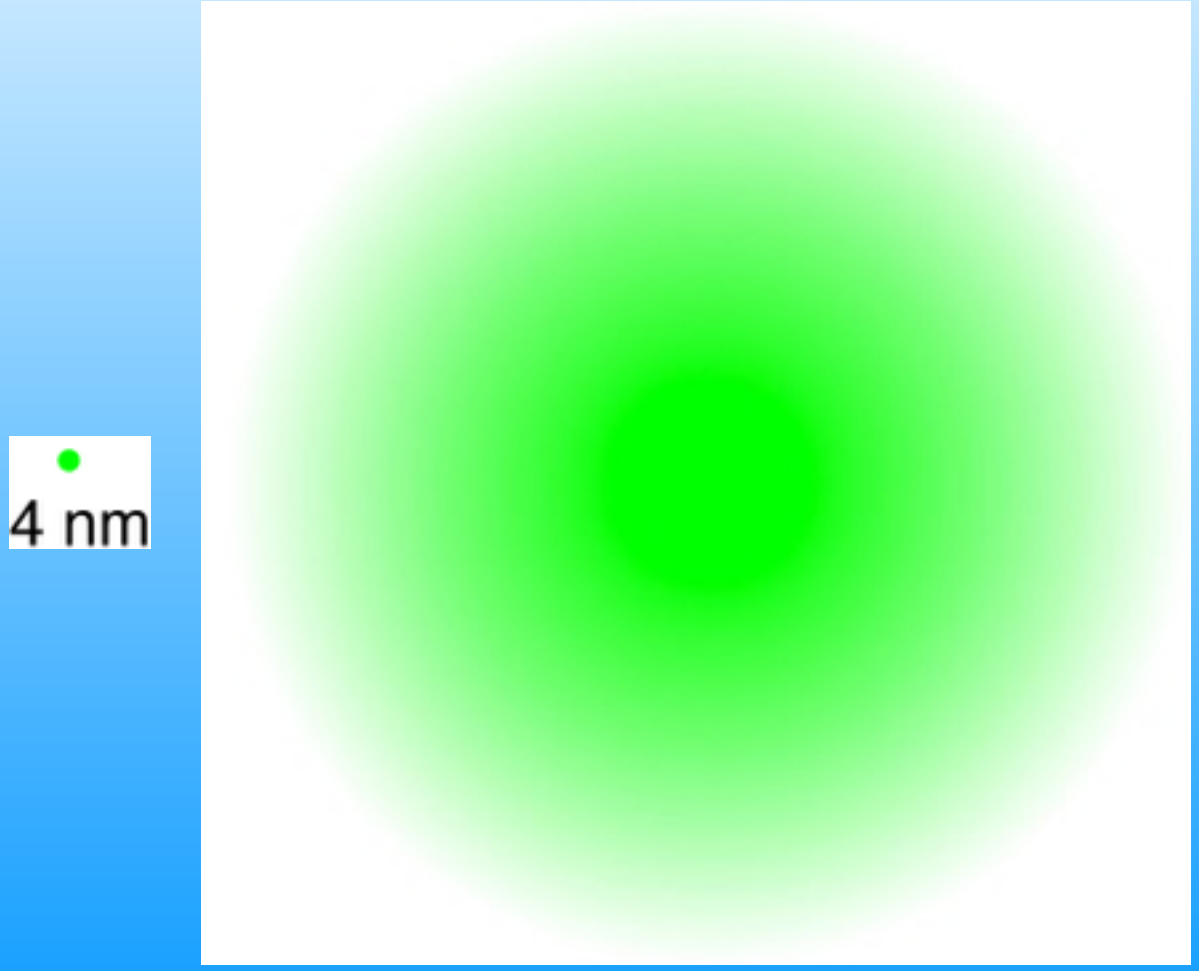
$$d = 1.22\frac{\lambda}{2NA}$$

Calculate:

What is θ_{max} if the NA of my objective is 1.49 and the index of refraction, n, is 1.51?

Resolution Limits to Imaging Single Molecules

Diffraction limited spot for a high-NA objective



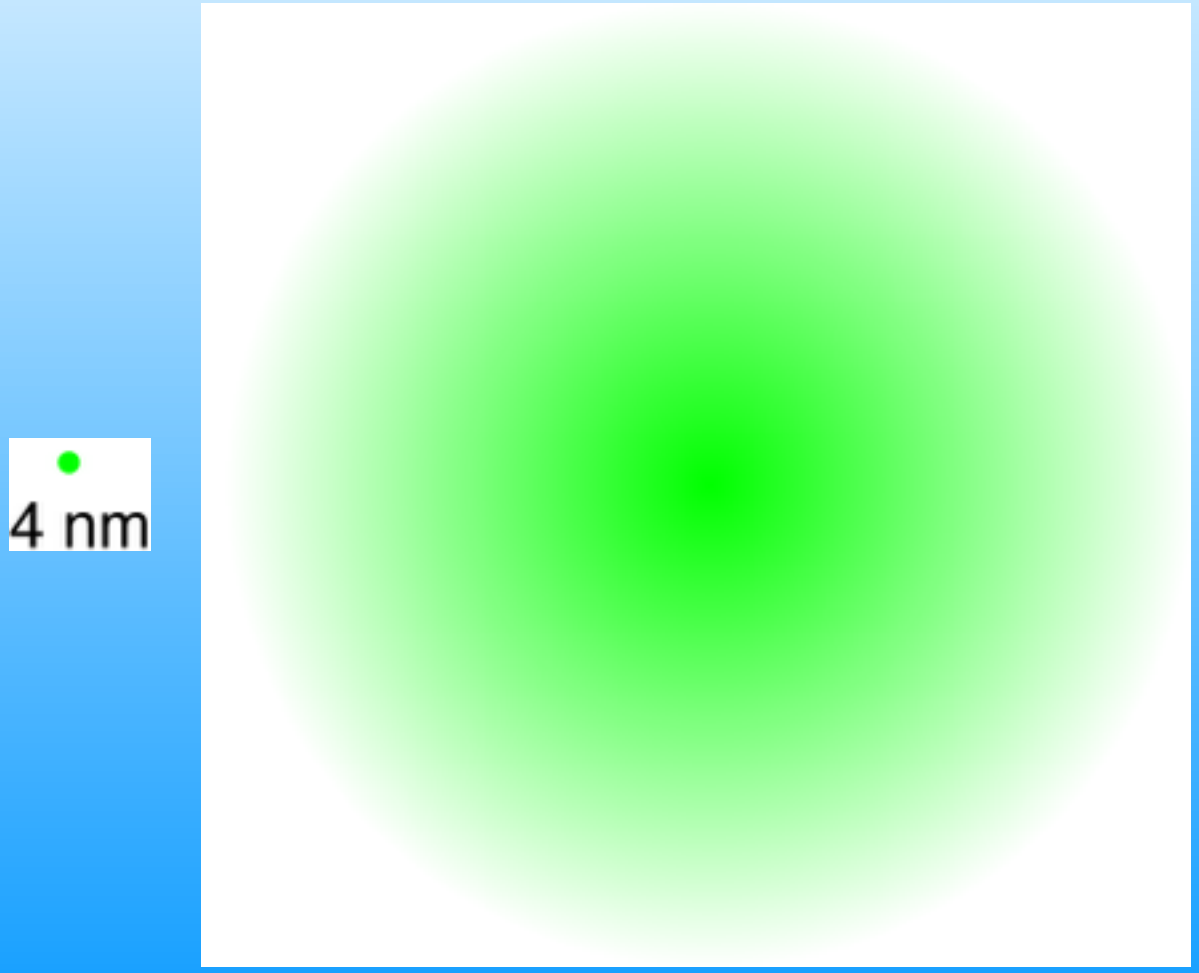
$$d = 1.22 \frac{\lambda}{2NA}$$

Calculate:

What is d if the NA of my objective is 1.49 and the wavelength of light is 508 nm?

Resolution Limits to Imaging Single Molecules

Diffraction limited spot for a high-NA objective



$$d = 1.22 \frac{\lambda}{2NA}$$

How can we improve our resolution?

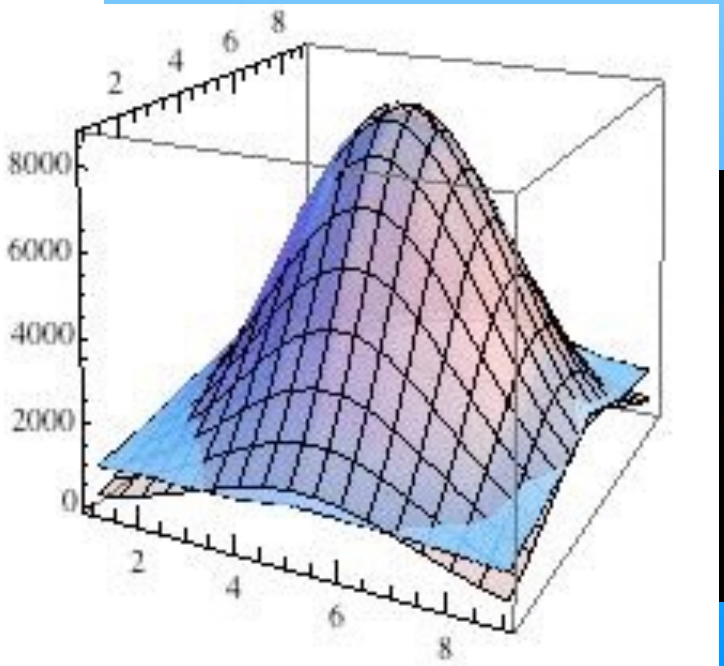
Super-resolution

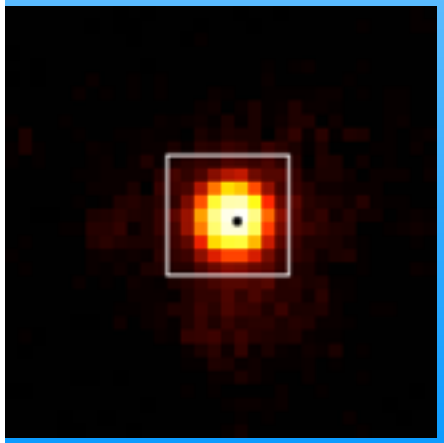
Use math tricks!

Intensity of diffraction-limited spot highest at center

Actually a Bessel function, but is well-fit by a 2-D Gaussian

Fit the shape of the intensity to find the center with high accuracy

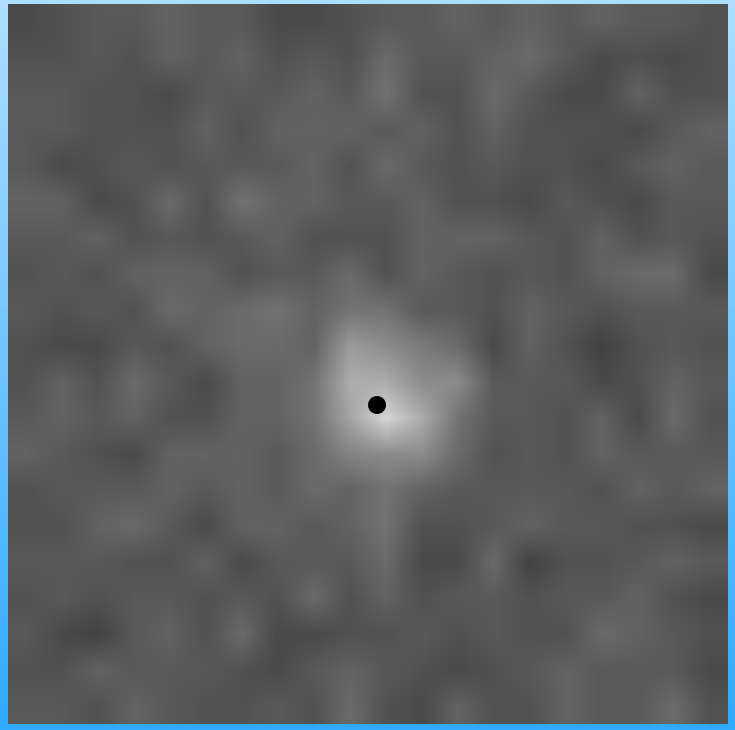


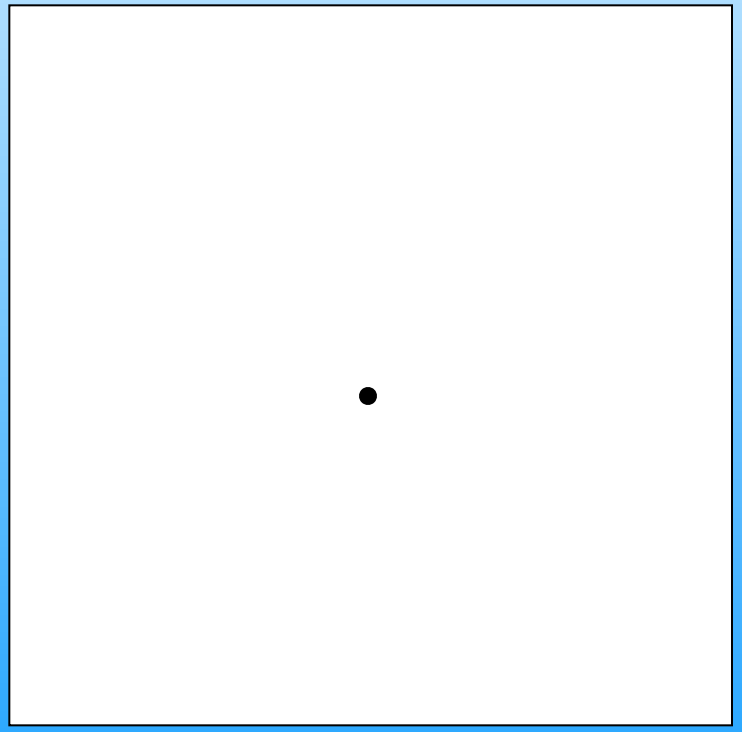


FIONA: Fluorescence Imaging with One Nanometer Accuracy

Replace the fuzzy spot with a 2-20 nm dot

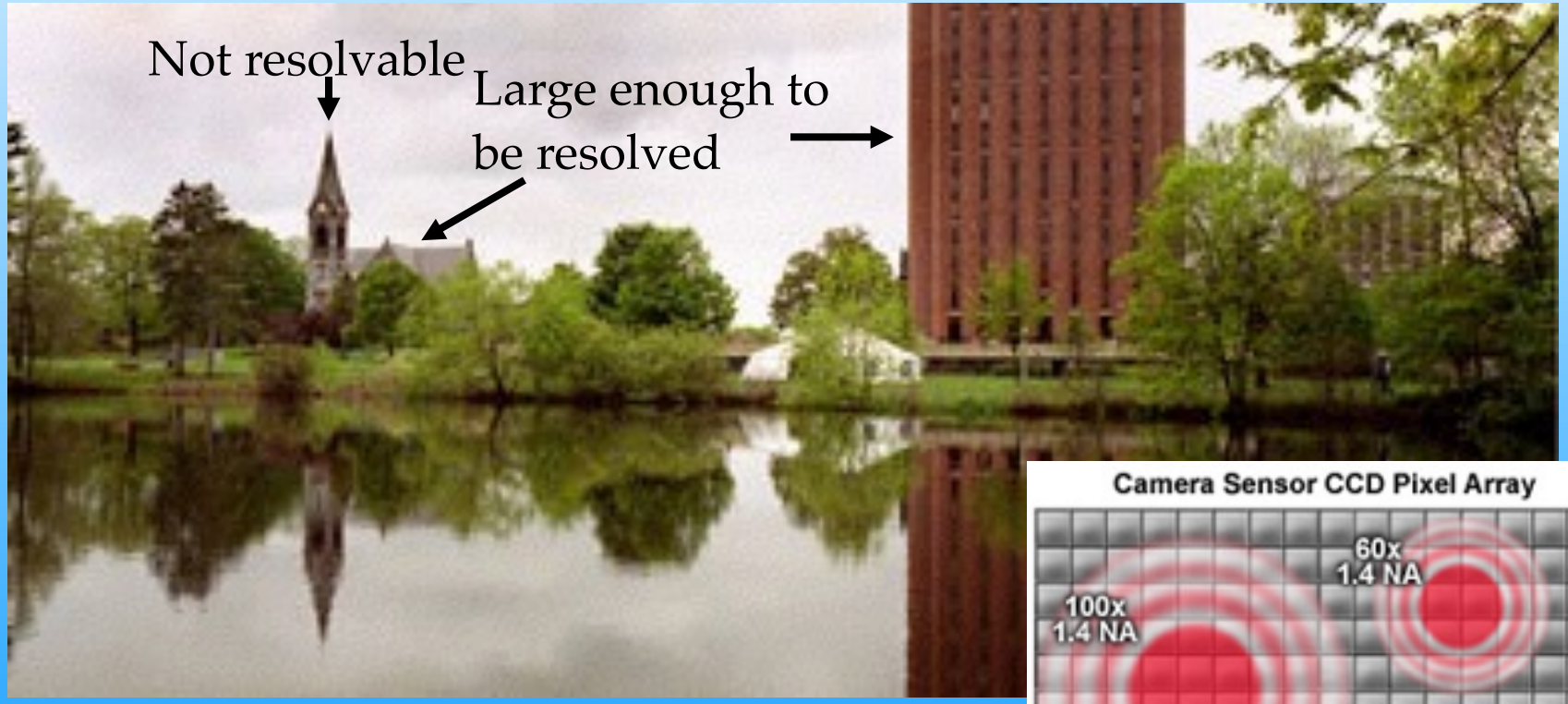
More photons (brighter spot) leads to better “resolution” and a smaller dot.

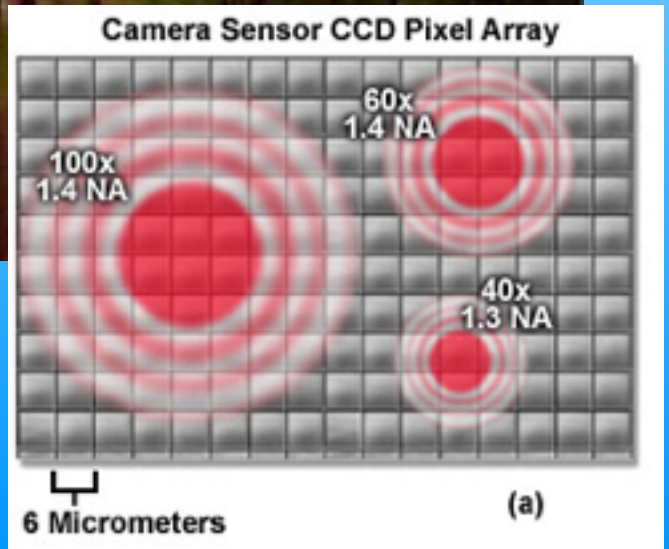




Effect of pixel size on resolution

Here, resolution is limited by pixel size - not fundamental properties of light





Gaussian fitting gives better than 1 pixel accuracy

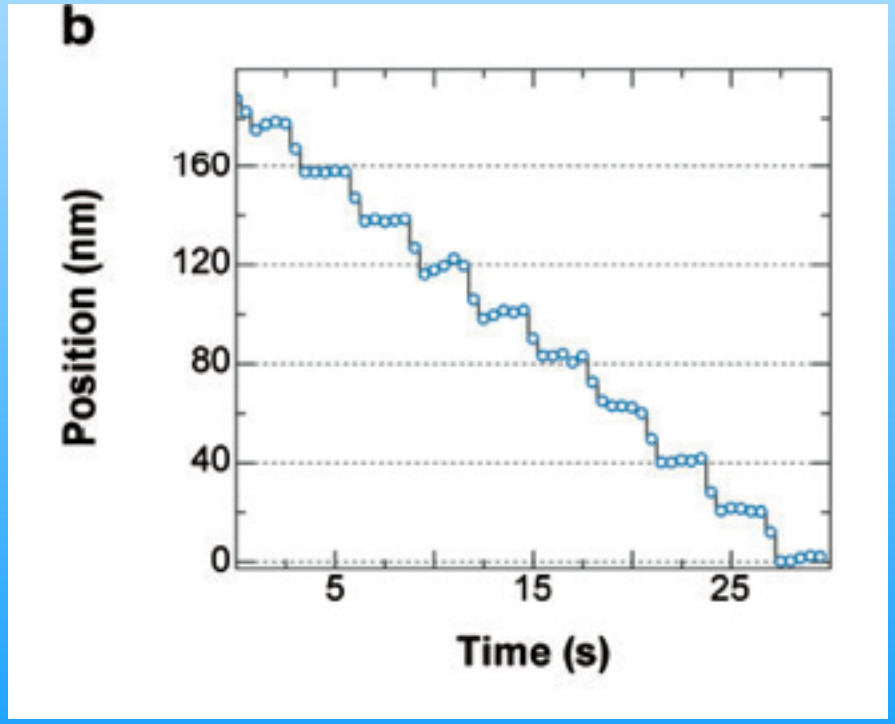
FIONA: Fluorescence Imaging with One Nanometer Accuracy

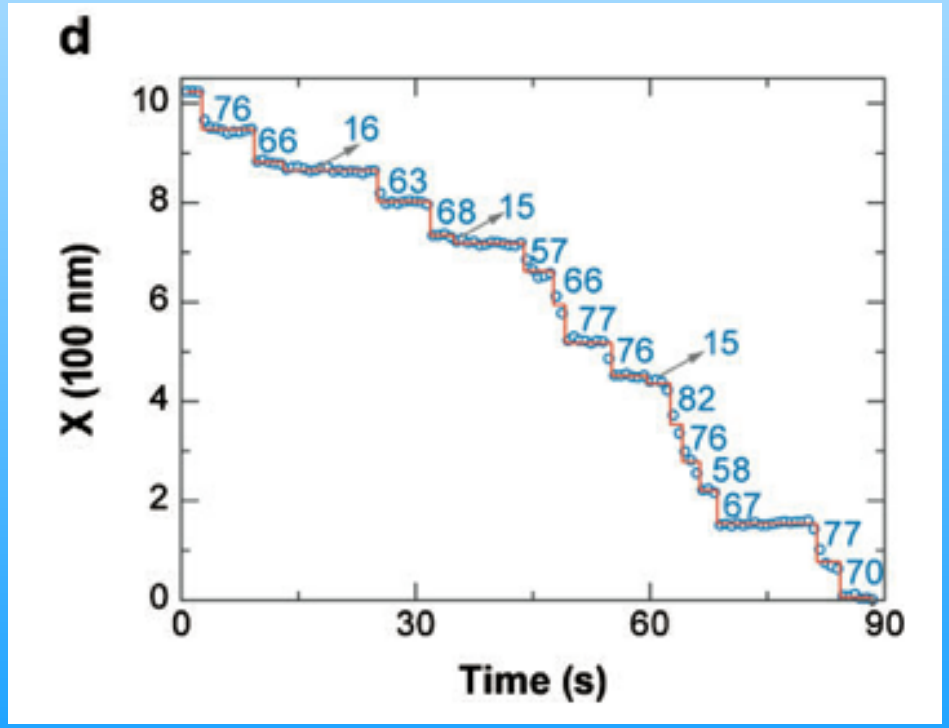
Follow the motor for multiple frames

More photons, better fitting

New Fluorescent Tools for Watching Nanometer-Scale Conformational Changes of Single Molecules

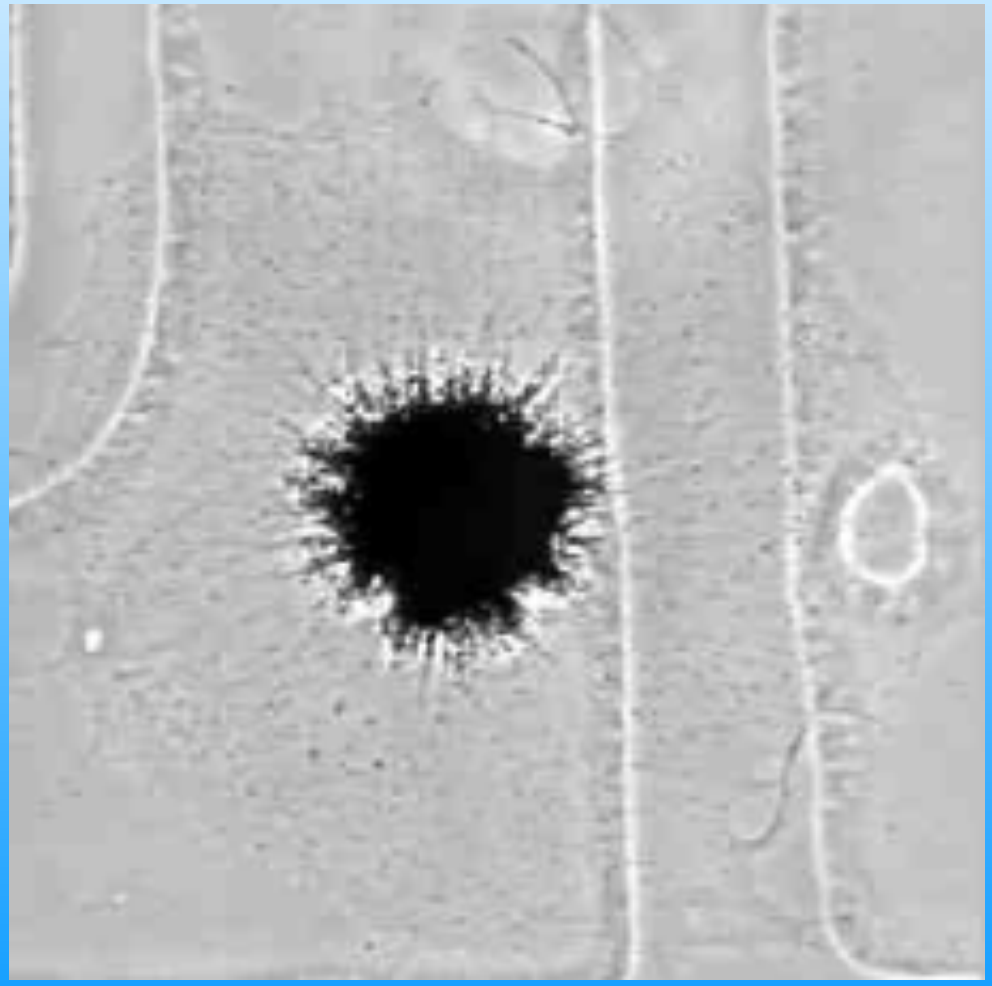
Erdal Toprak[1] and Paul R. Selvin[1,2]





The diffraction limit is broken!

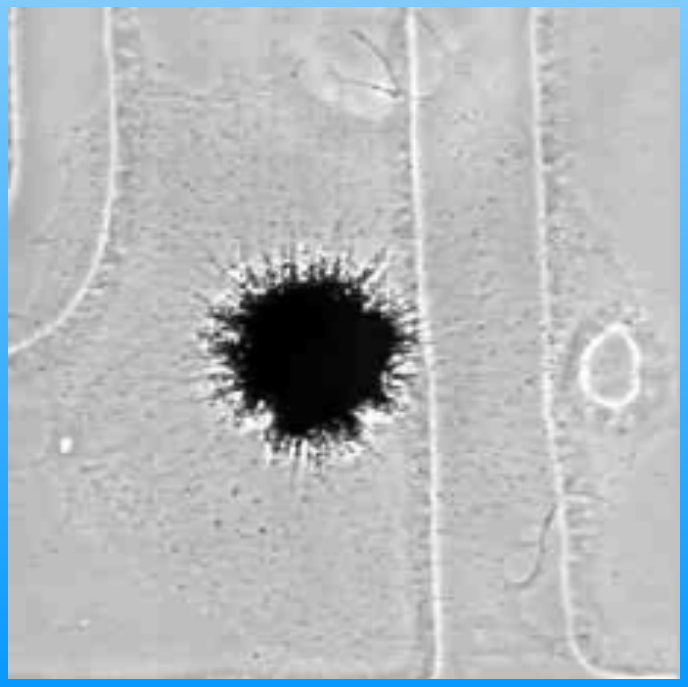
Example I: Micro-parasol



Black Particles: melanosomes

Dark pigment-filled vesicles (10-100 nm).

Bringing it back to the cell: Micro-parasol

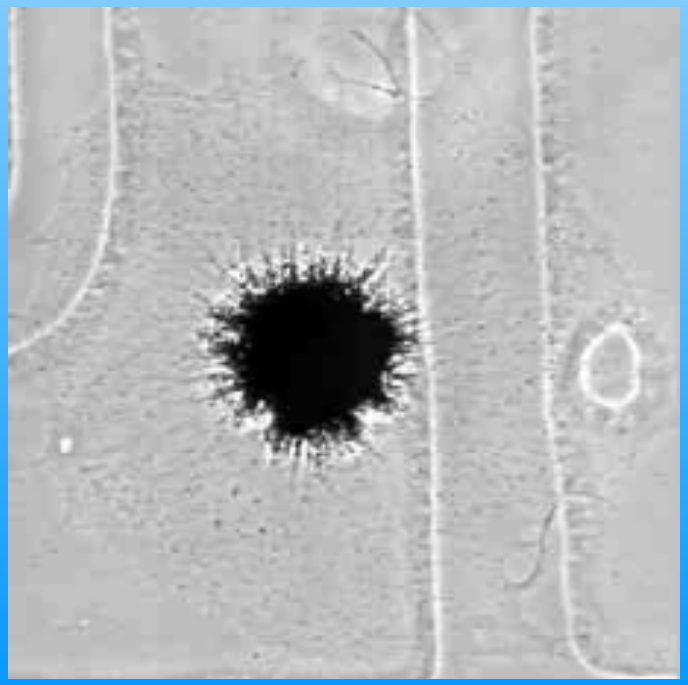


Black Particles: melanosomes

Dark pigment-filled vesicles (10-100 nm).

Protect your cells' nuclei from harmful UV rays

Bringing it back to the cell: Micro-parasol



Black Particles: melanosomes

Dark pigment-filled vesicles (10-100 nm).

Protect your cells' nuclei from harmful UV rays.

Moved into position by motor proteins taking 8 nm steps!

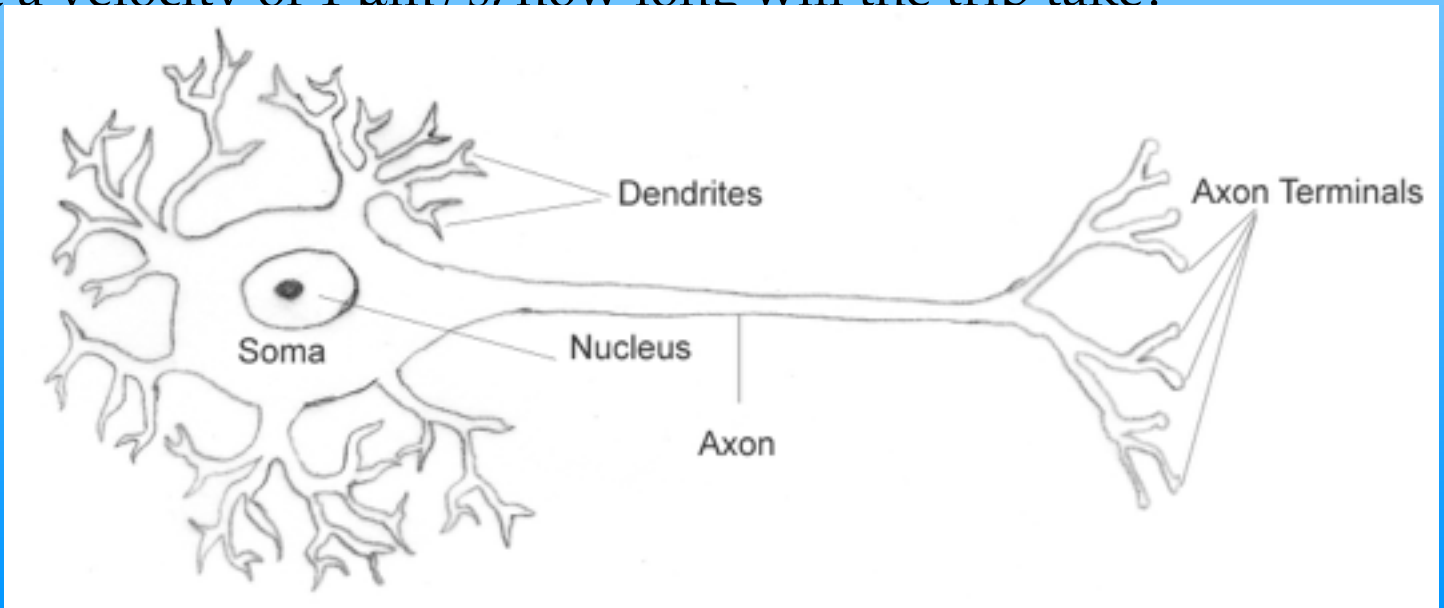
Example II: Motor Transport inside Cells: From nanometers to meters

Some neuronal cells can be up to 1 m long to connect your toes to your spinal cord

Motor proteins transport goods up and down the axon taking 8 nm steps.

How many steps do they need to take to go from the cell body (soma) to the axon terminals, 1 m away?

If they travel at a velocity of 1 μm/s, how long will the trip take?



Summary

Molecular biology exists, organizes, and engineers on the nano-scale

Amazingly, these organizational principles are at the core of all animals from bacteria and yeast to people and elephants!

New optical techniques requiring math tricks are required to see these nano-processes

Thank you for your attention!