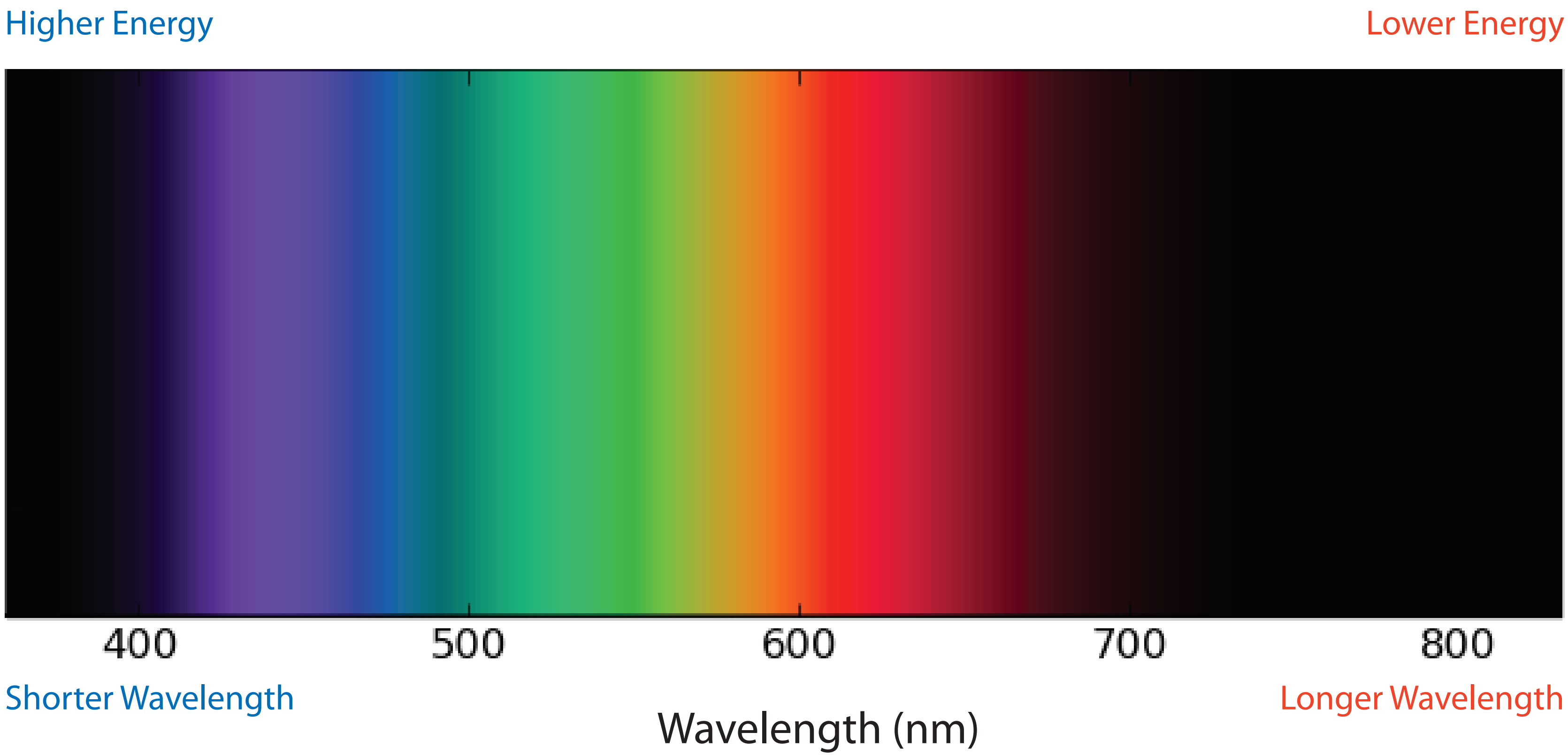
FRET Live-Cell Imaging and Quantitation Summer Workshop, 2015

Fluorescence Microscopy : Fundamentals

Philbert S. Tsai

The Visible Spectrum of Light



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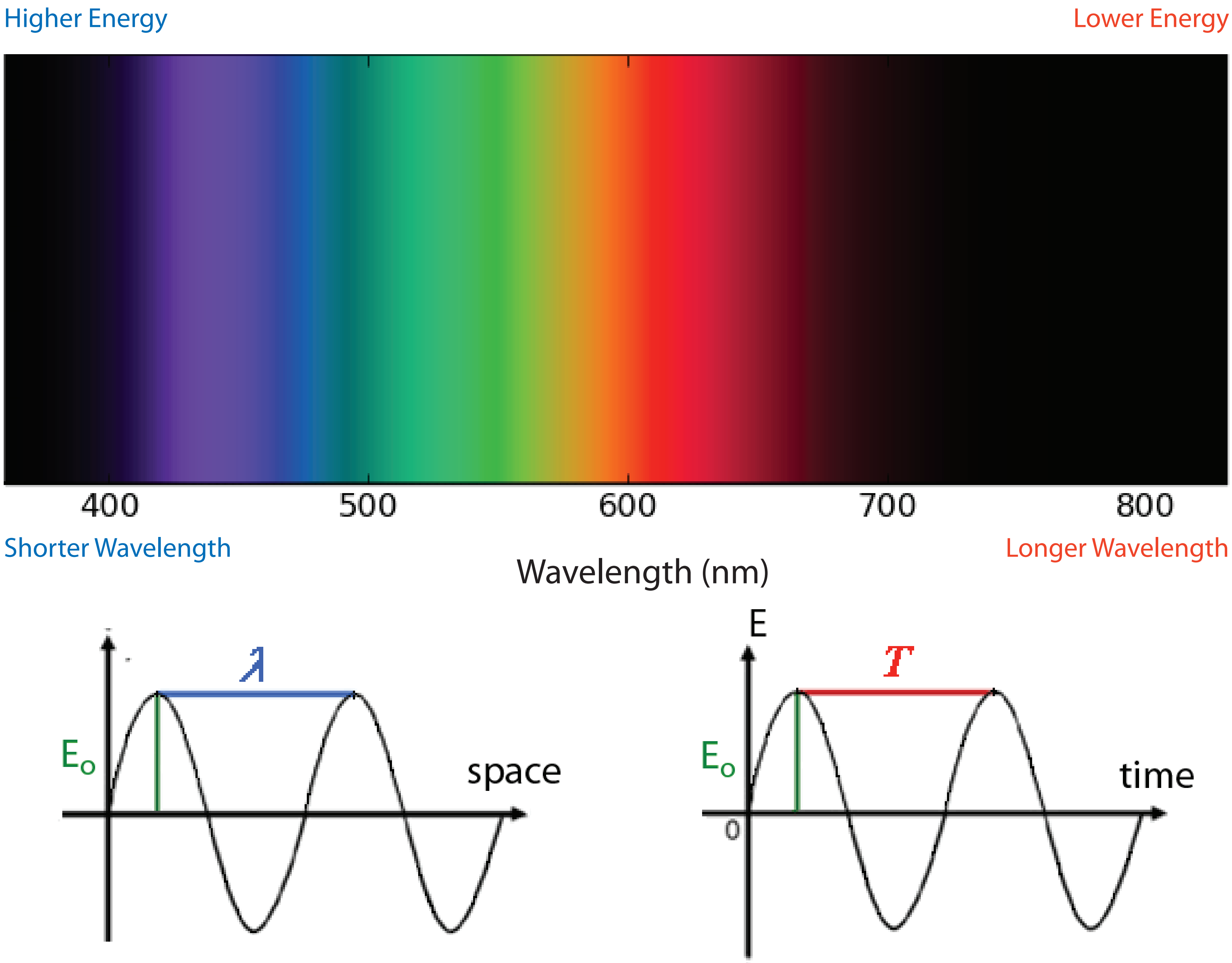

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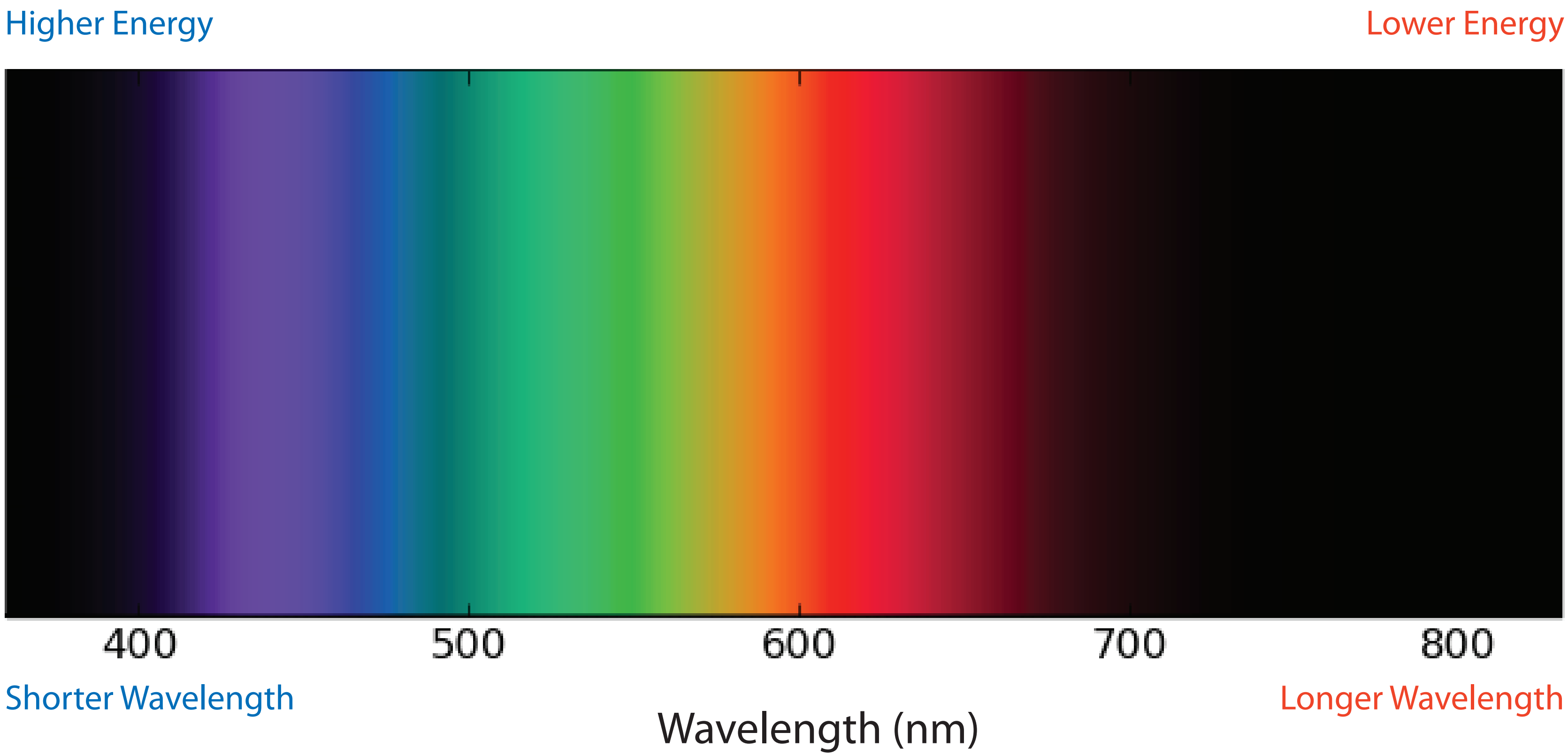

$E = h * f$ (Photon Energy = Planck's Constant * Frequency)

$E = h * c / \lambda$

h = Planck's Constant = $6.626 * 10^{-34}$ J*s

c = speed of light = $3 * 10^{8}$ m/s

λ = wavelength

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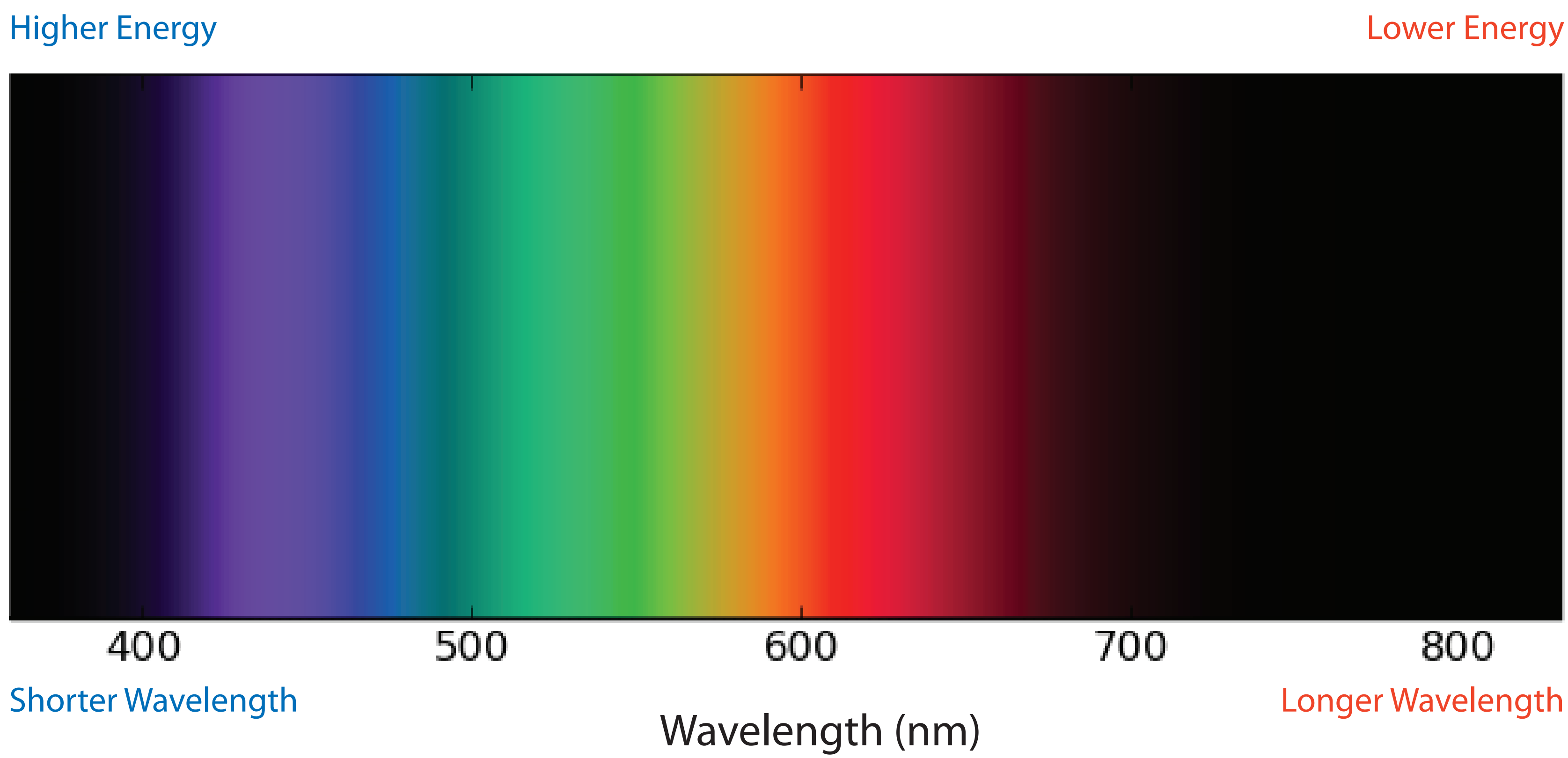
The Visible Spectrum of Light



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c = speed of light = $3 * 10^{8}$ m/s

λ = wavelength

1 photon @ 546 nm

$E = 3.64 * 10^{-19}$ J*s

so:

1 Watt = 1 Joule/sec

$= 2.7 * 10^{18}$ photons / s

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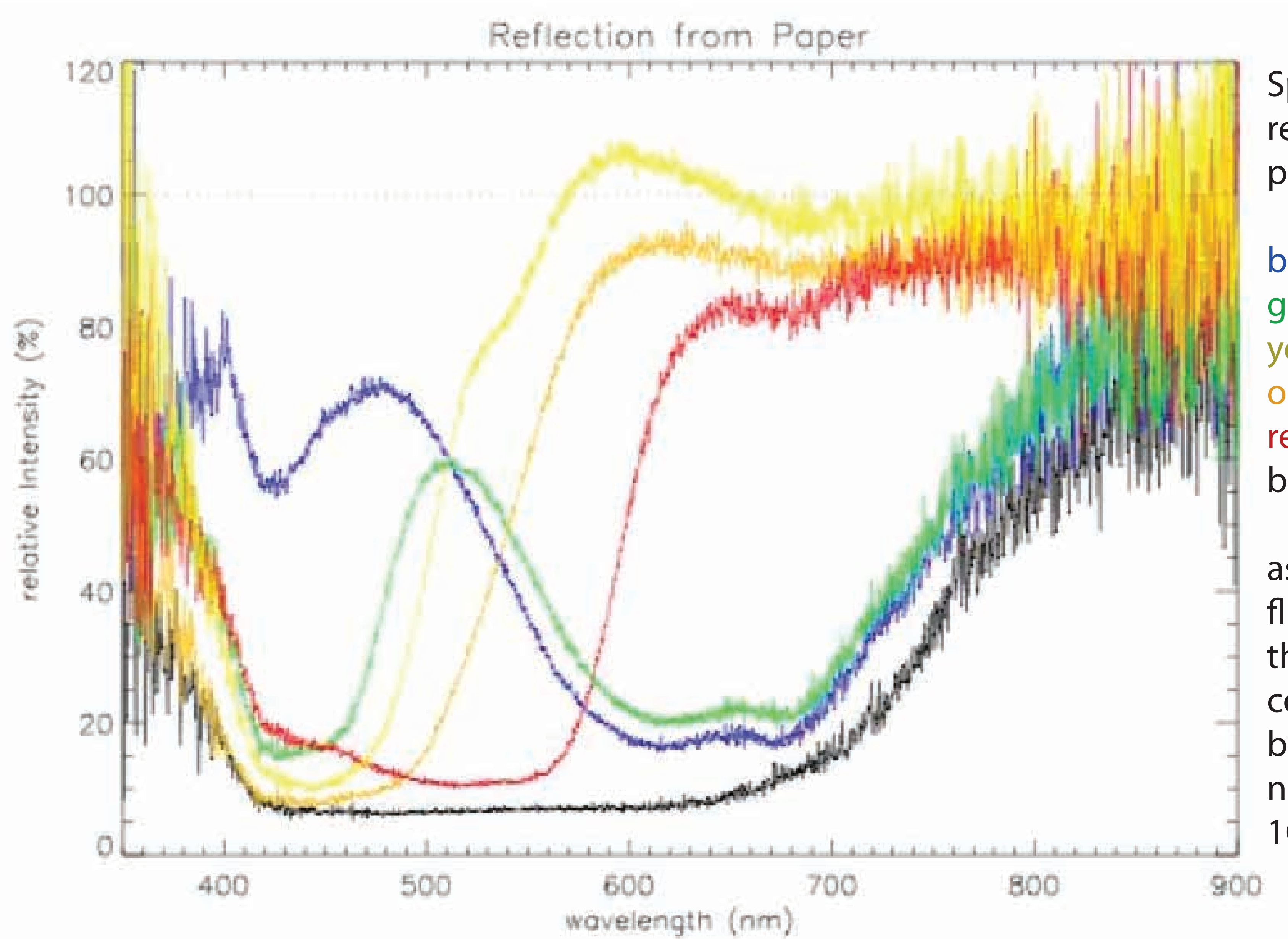
Reflectance spectra from colored paper



Spectra of sunlight reflecting off of color papers appearing :

blue
green
yellow
orange
red
black

aside from slight fluorescence in the yellow paper, colors operating by reflectance only never peak above 100%

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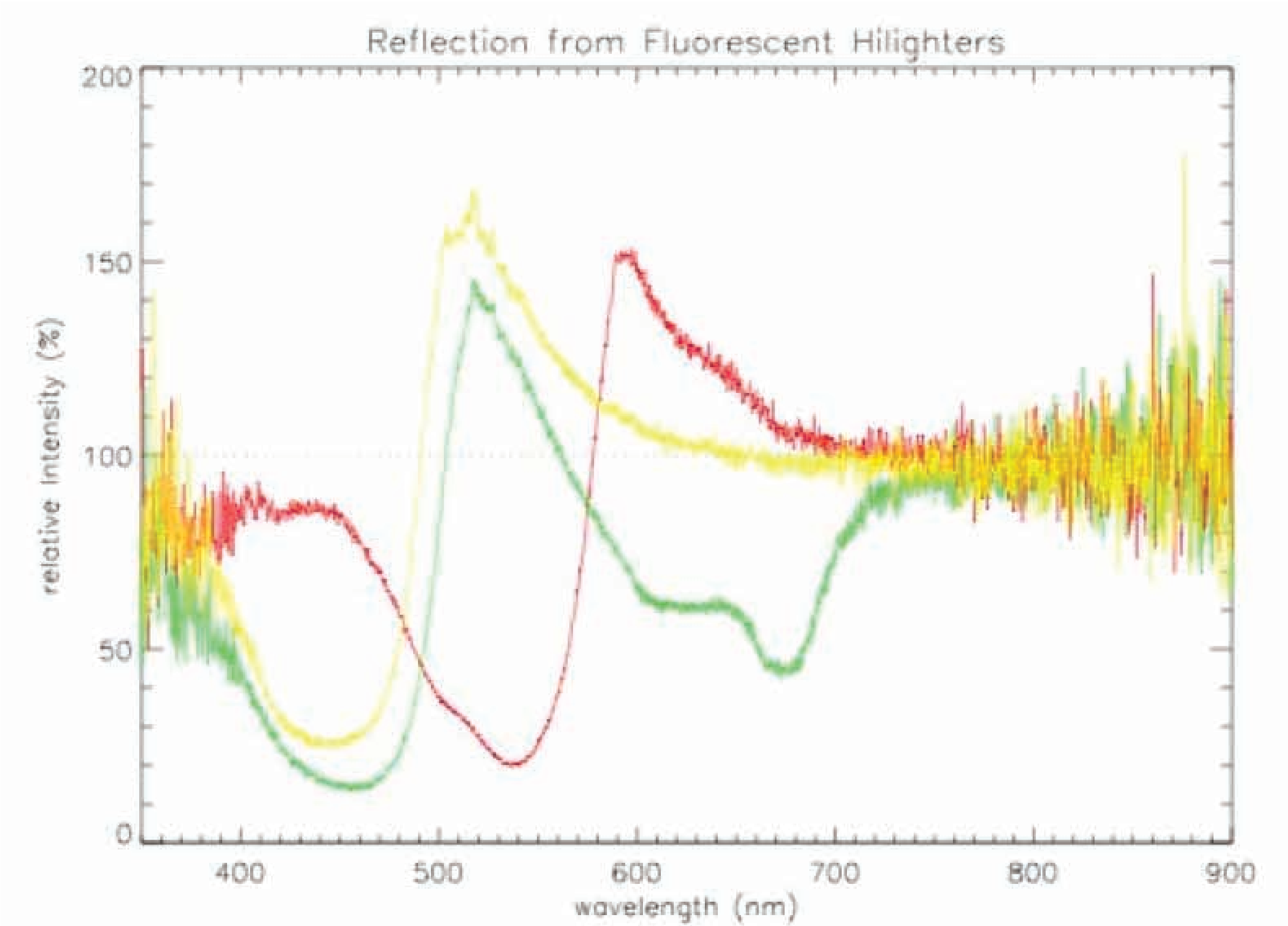
Original image source unknown

Emission spectra for fluorescent markers (highighter pens)



Fluorescent markers convert light from lower wavelengths to higher wavelengths.

green highlighter
yellow highlighter
pink highlighter

All three highlighters have emiisions that exceed the 100% that would be possible from reflection alone.

Original image source unknown

Fluorescence

Luminescence that is caused by the absorption of radiation at one wavelength followed by nearly immediate re-radiation usually at a longer wavelength.

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Microscopy

An unfortunate series of compromises

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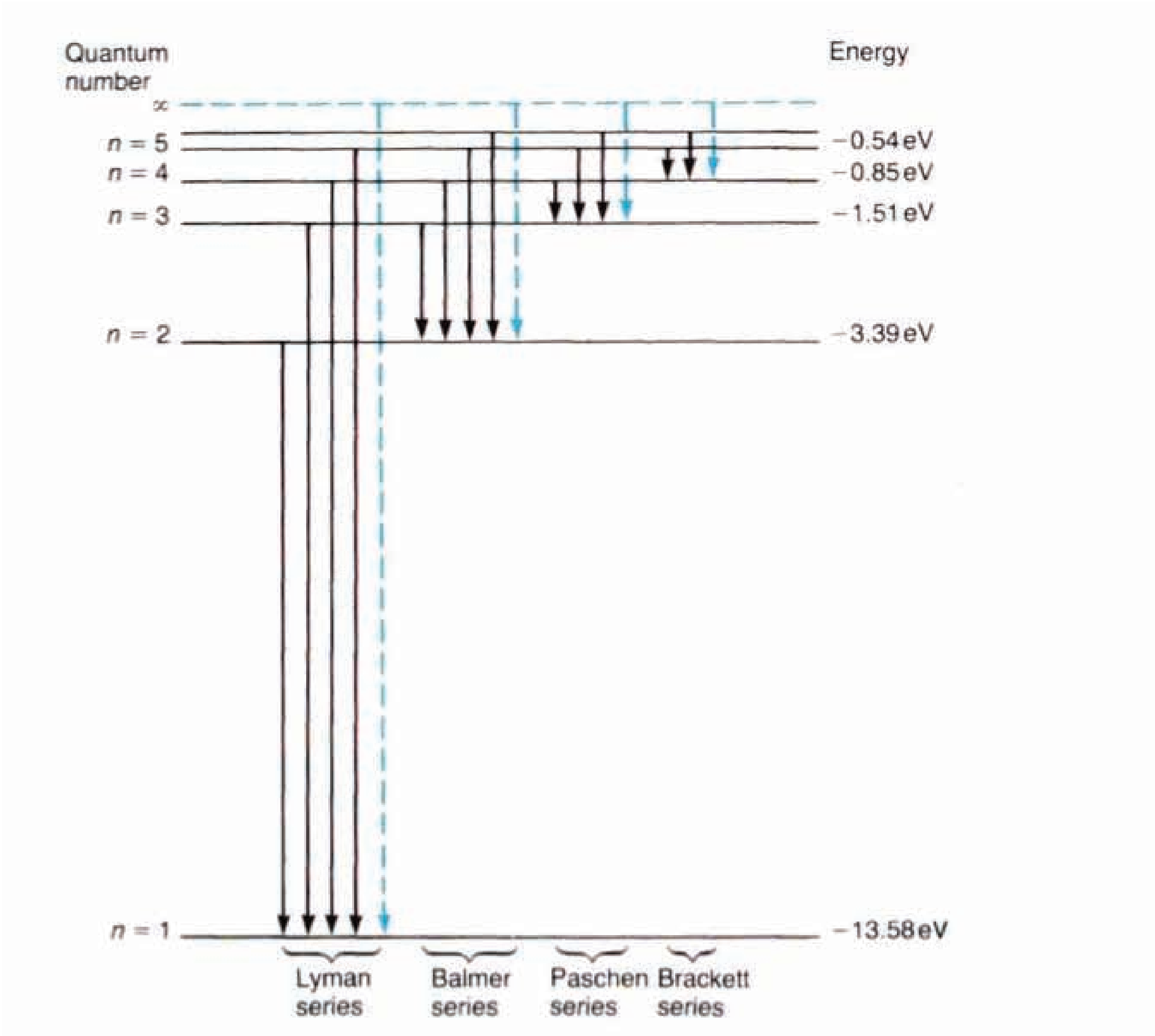
Microscopy

An unfortunate series of compromises that we are forced to make to look at very small objects.

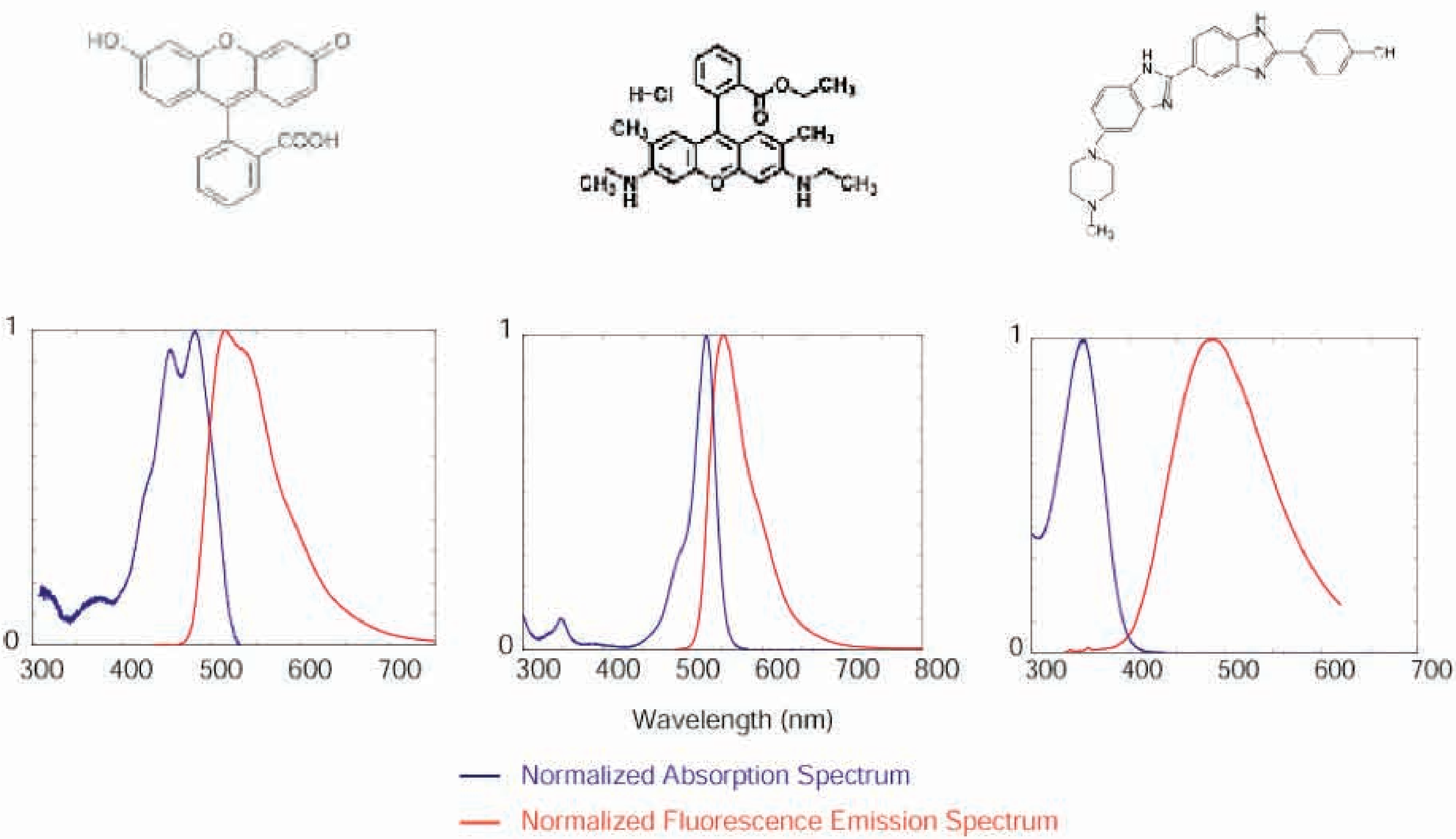
- Resolution
- Specificity
- Signal to Noise
- Speed
- Uniformity
- Phototoxicity
- $$$$

Energy levels for atomic spectra of hydrogen



Absorption & emission spectra of fluorescent organic dyes

Fluorescein | Rhodamine 6G | Hoechst 33258



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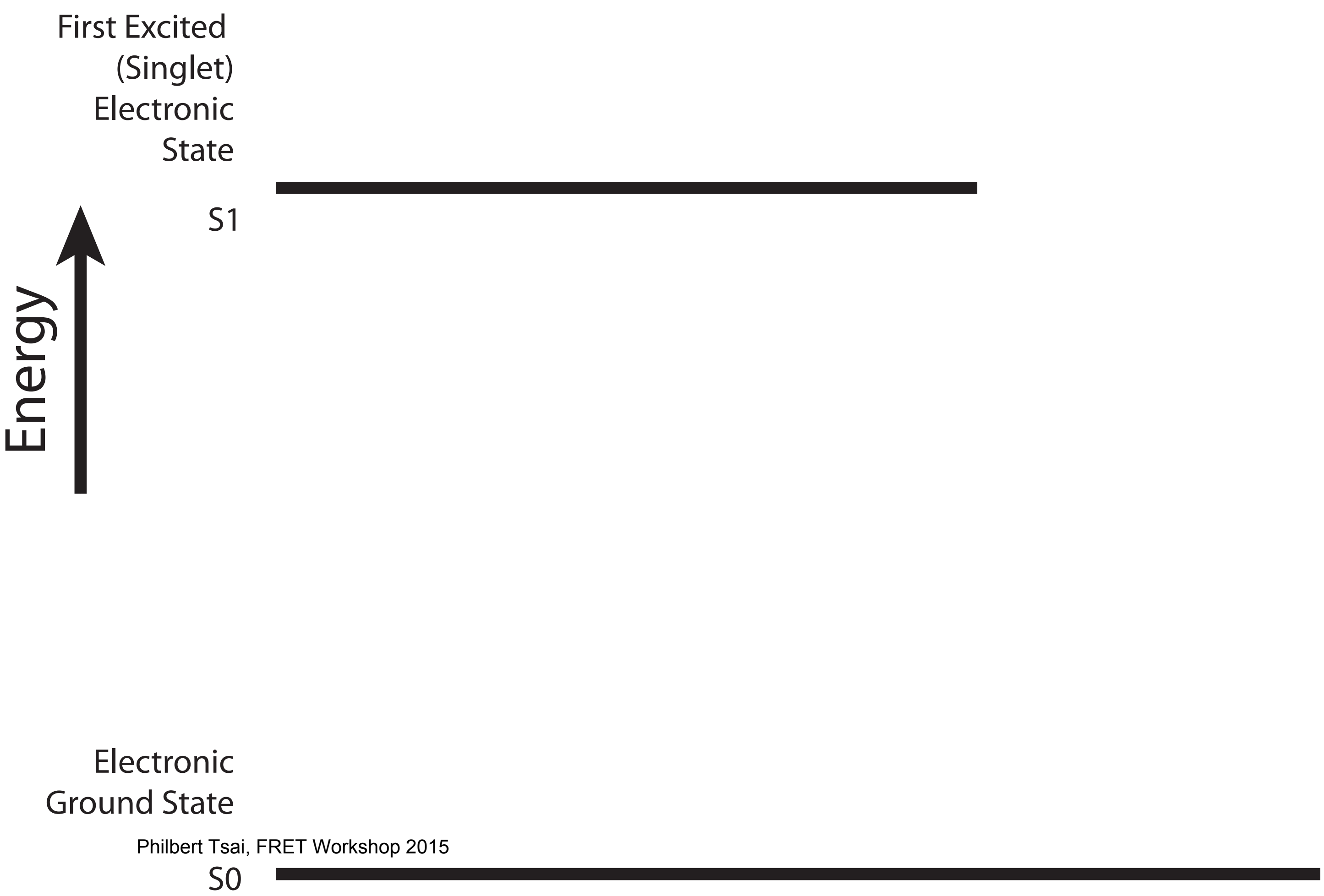
Jablonski Energy Diagram



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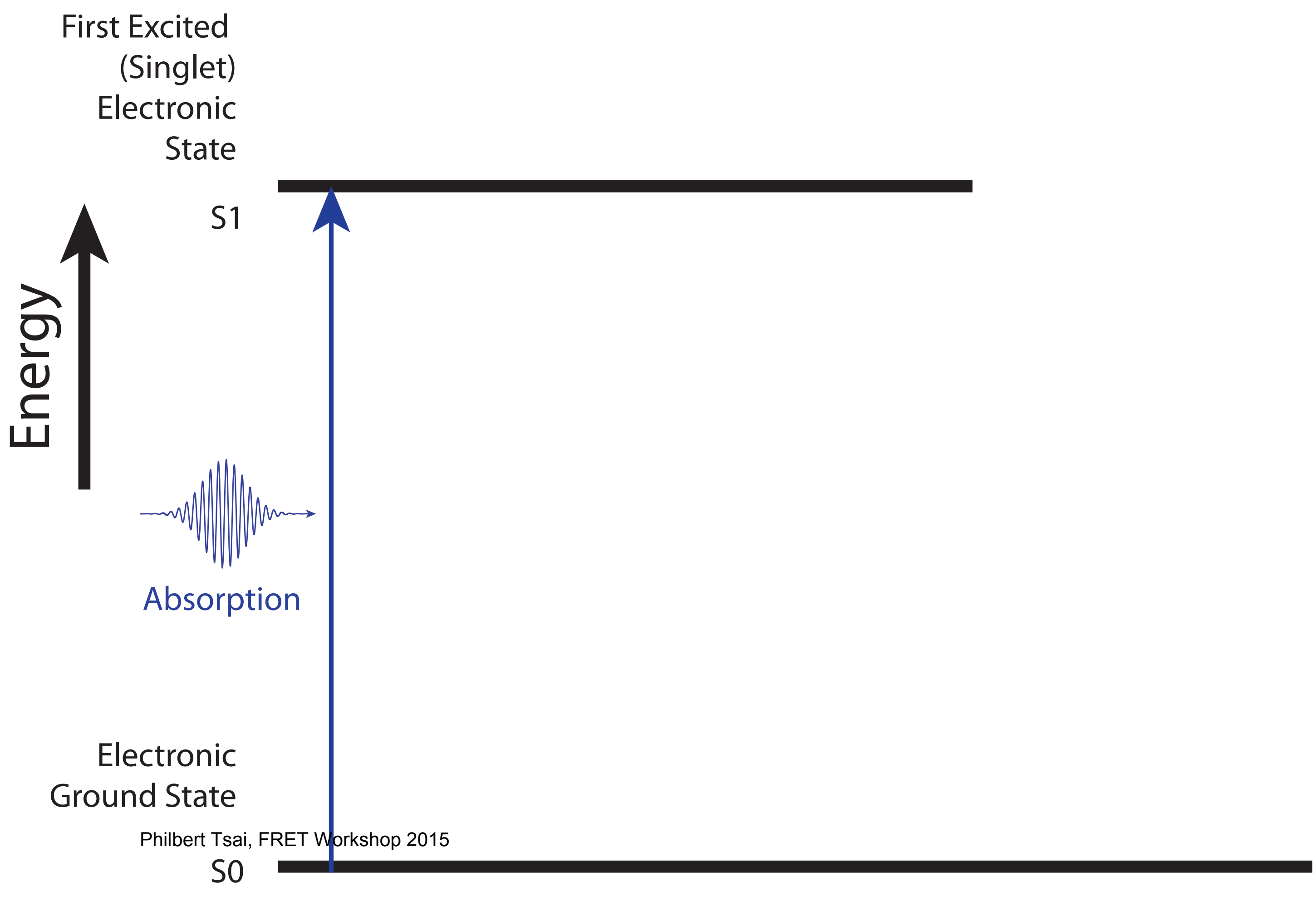

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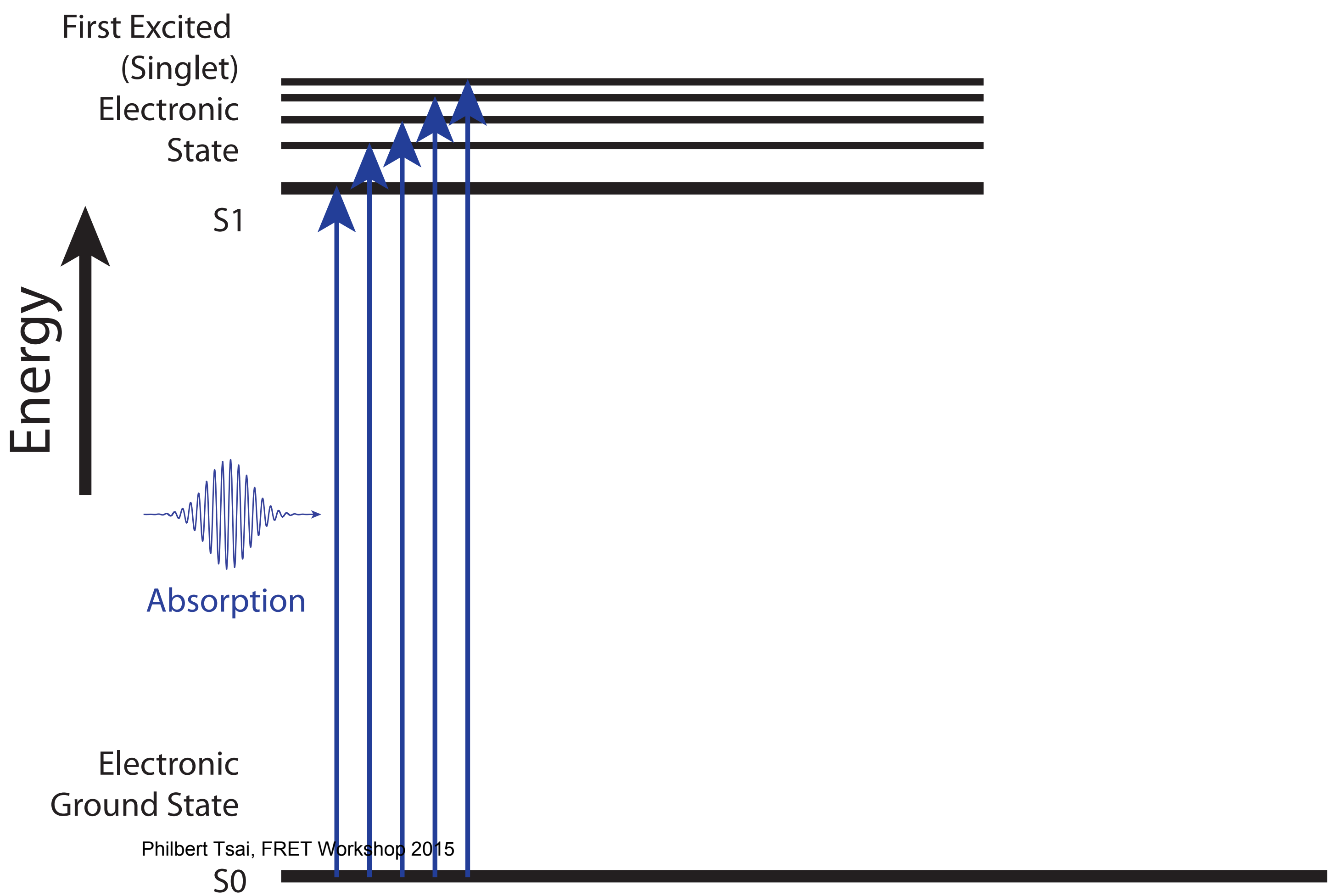

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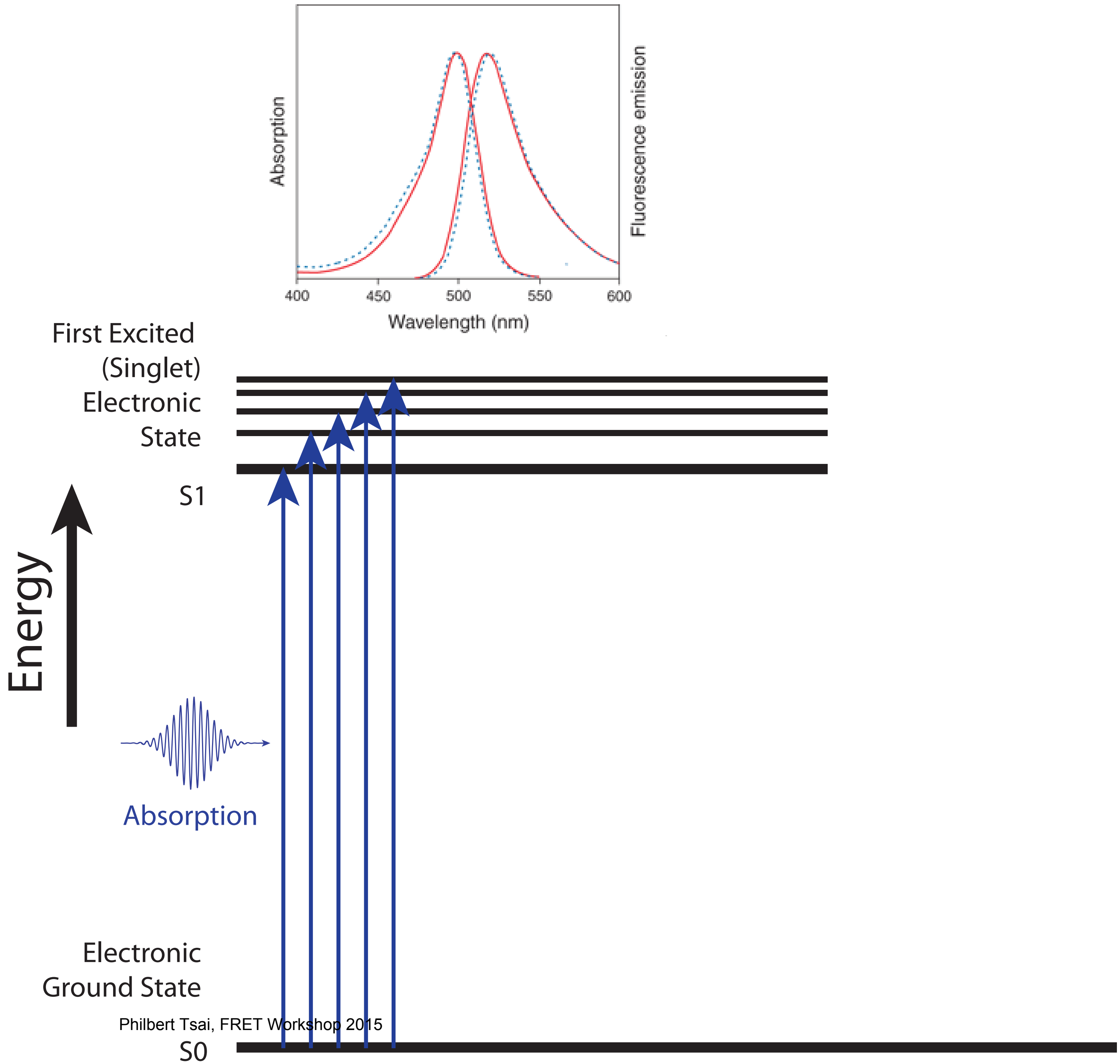

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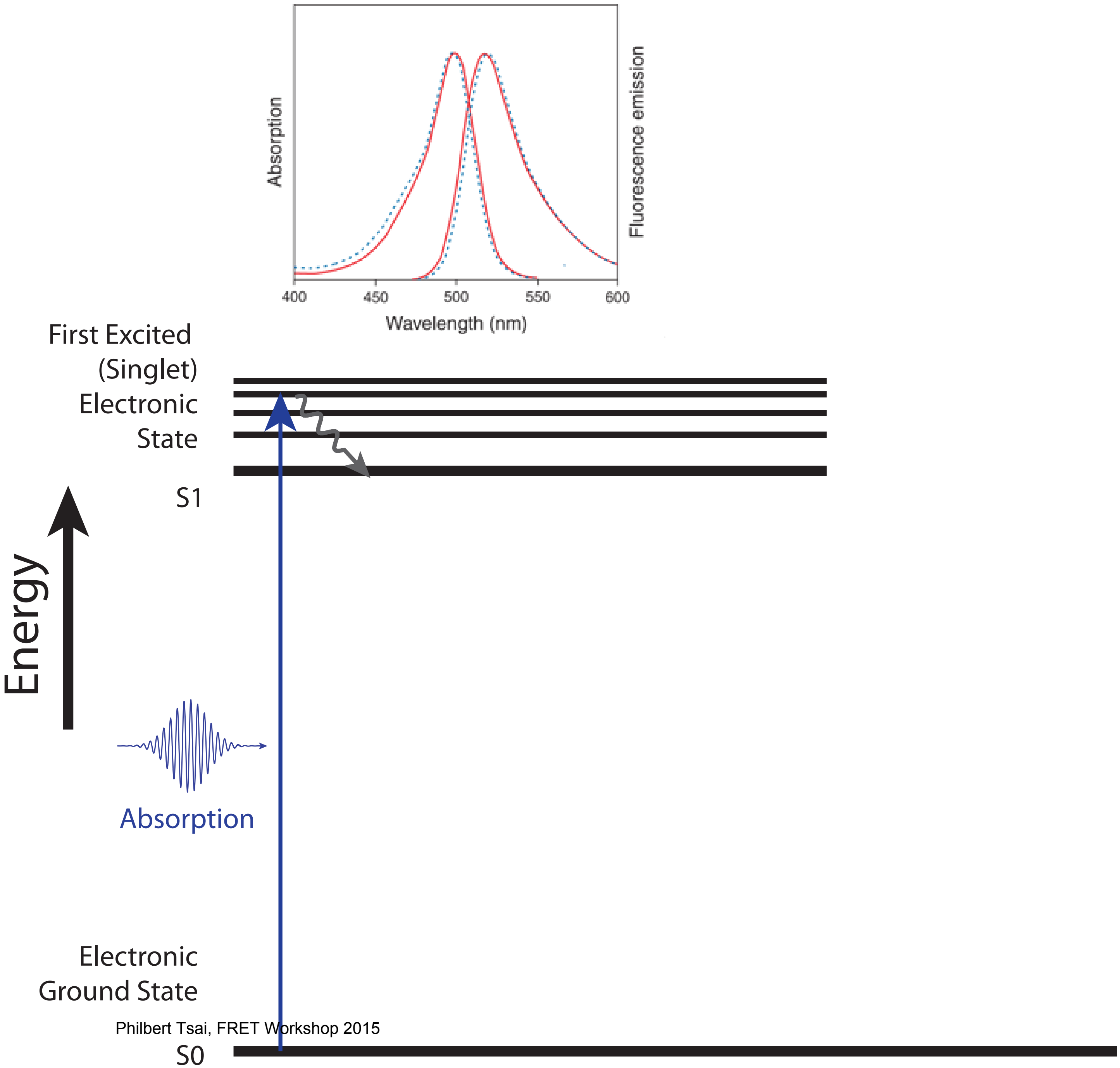

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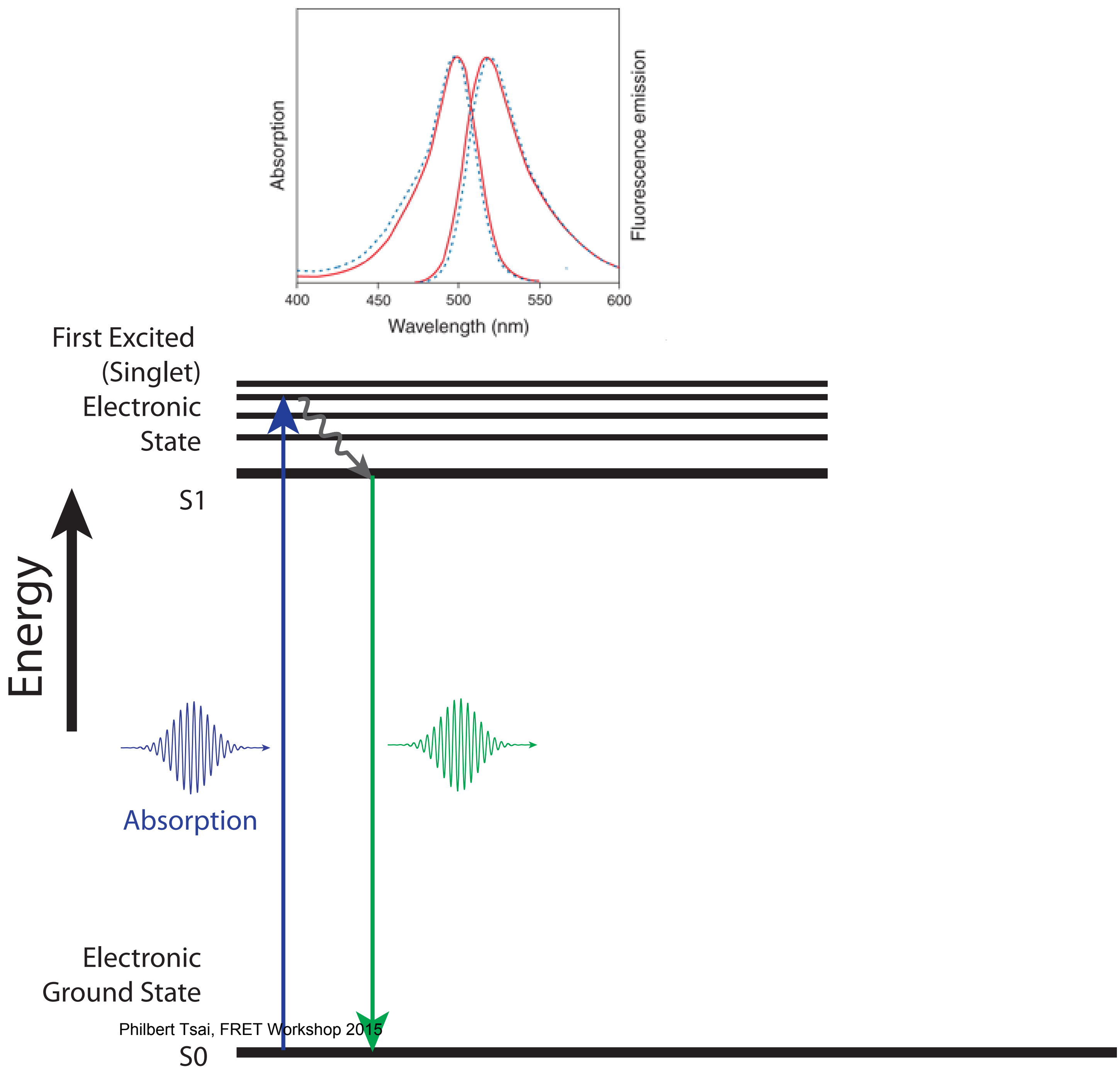

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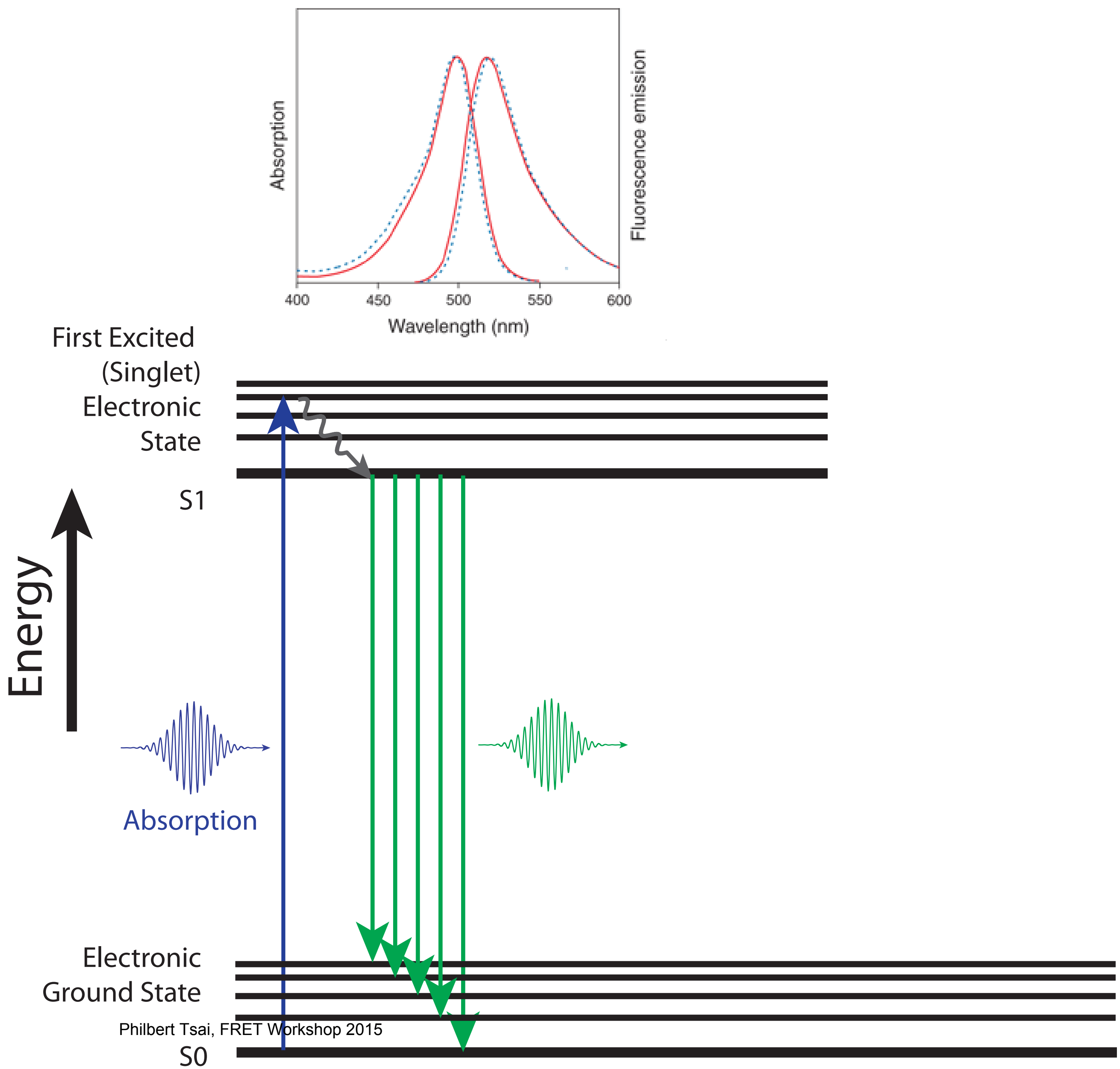

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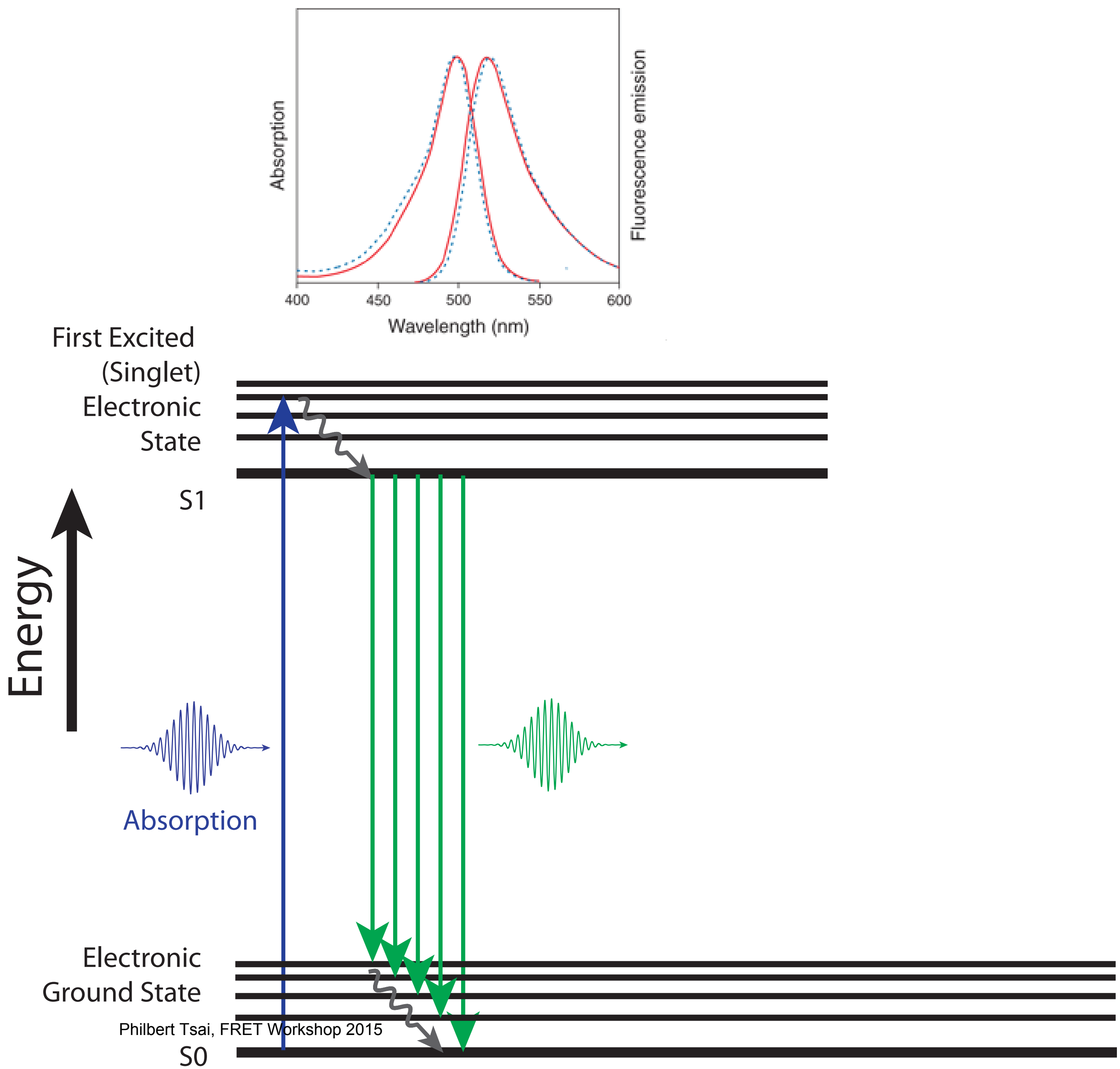

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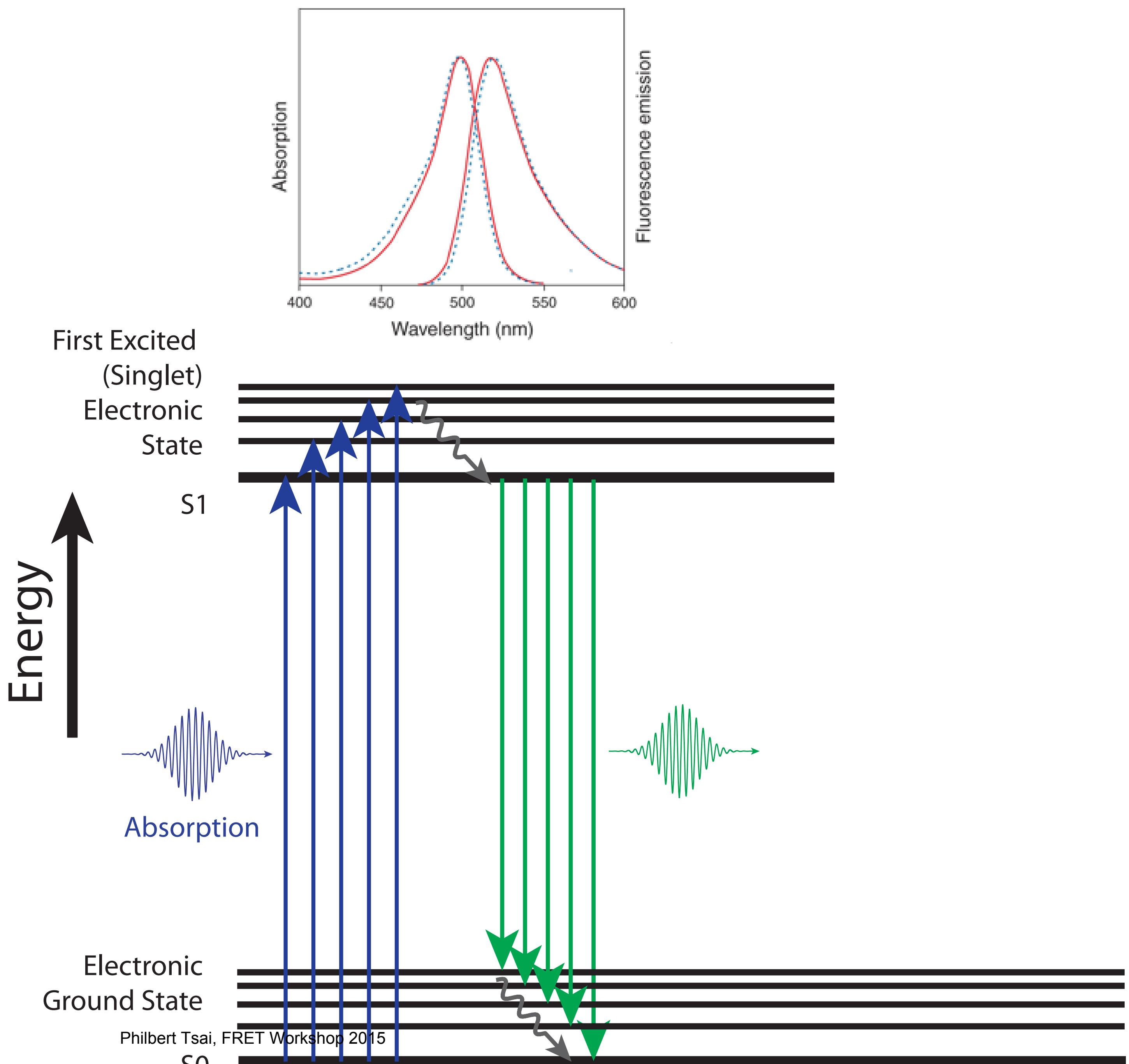

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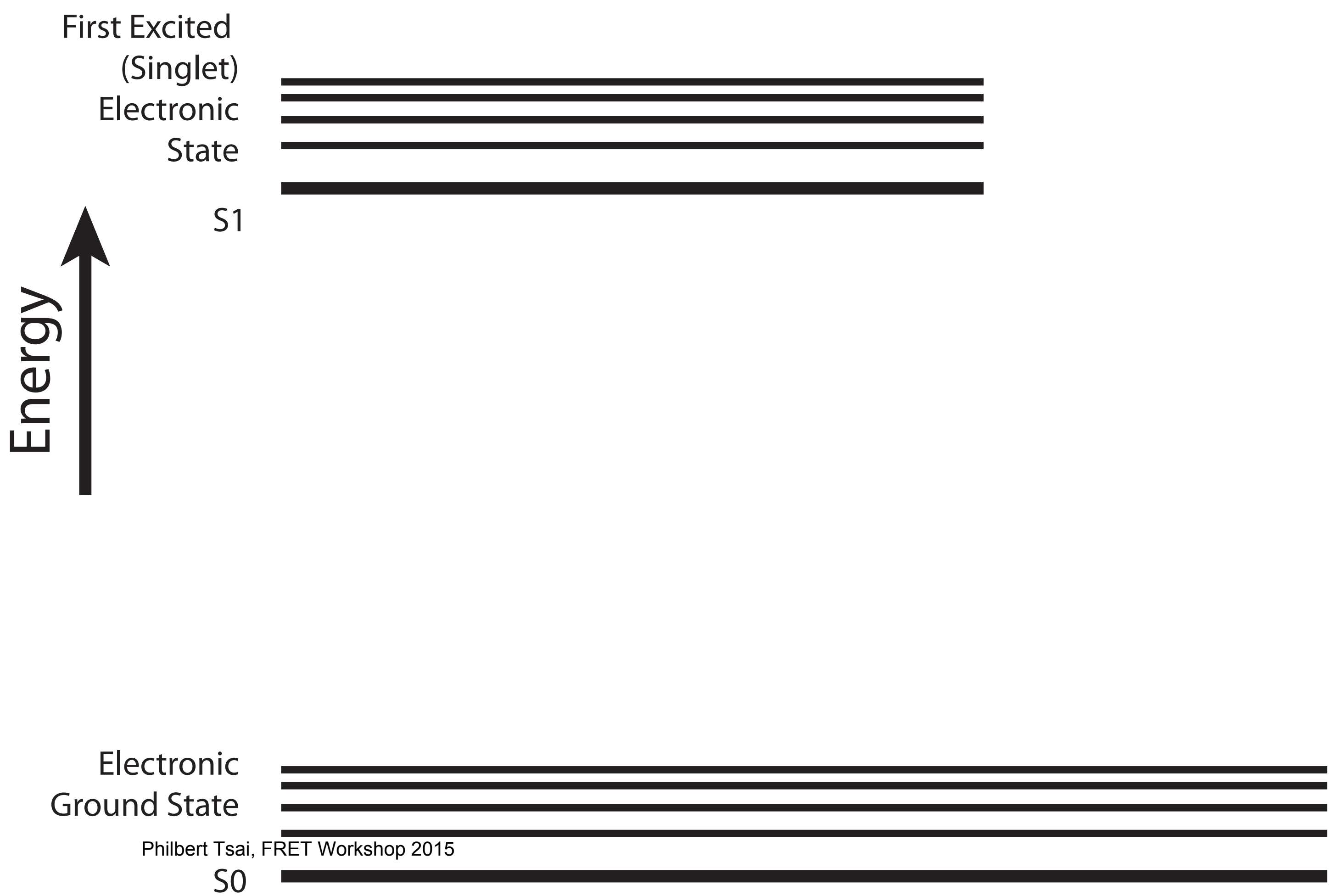

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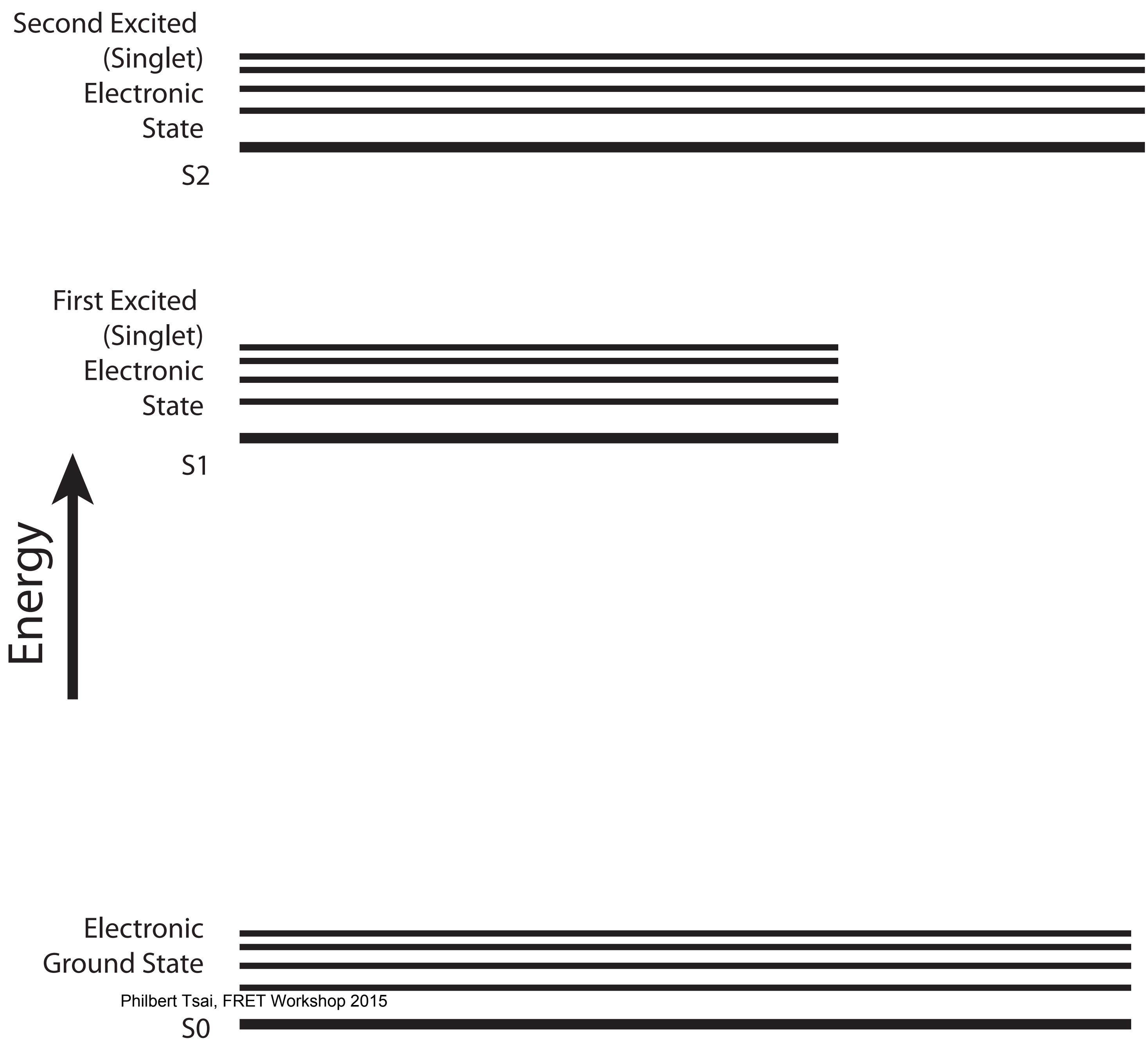

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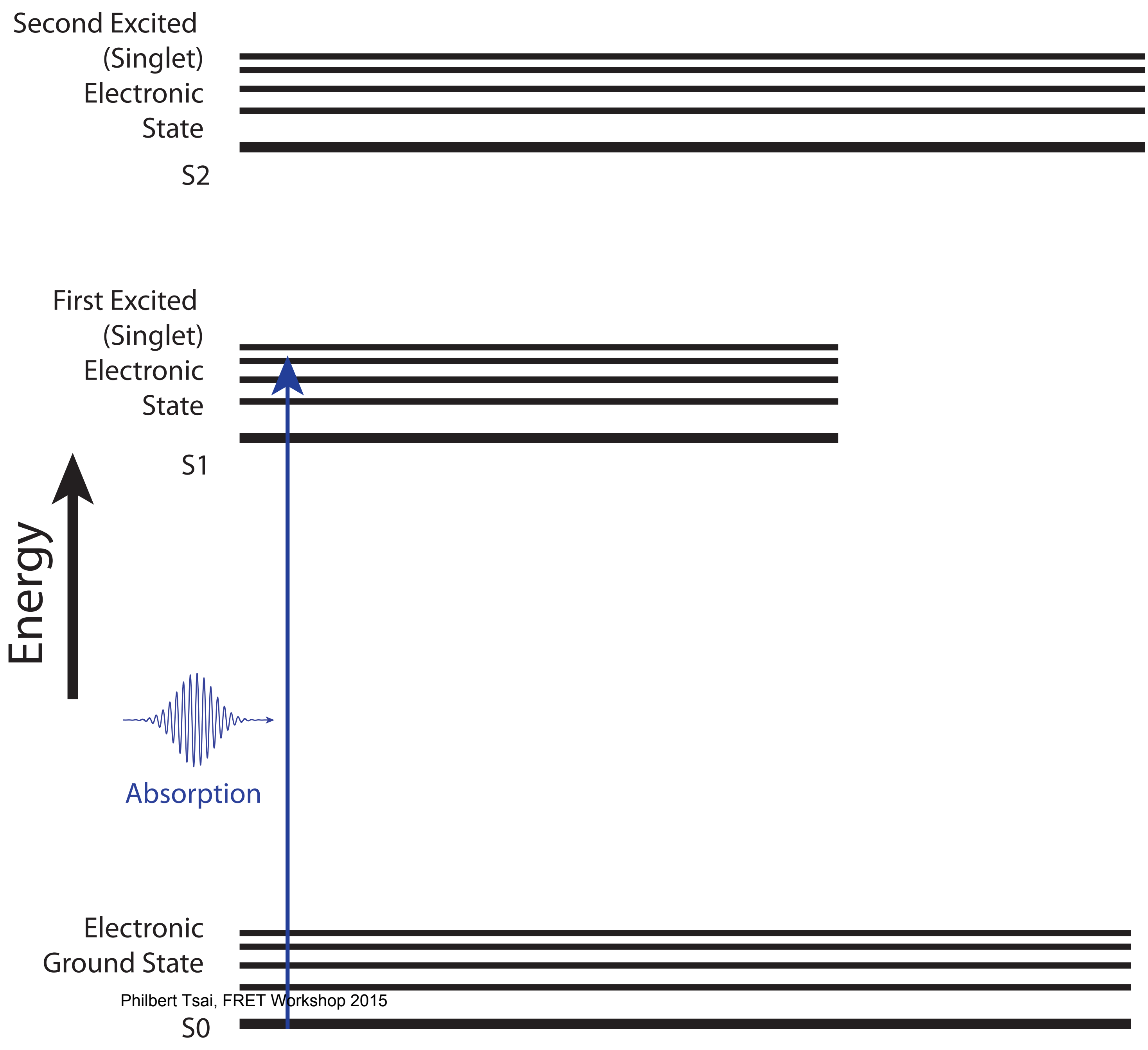

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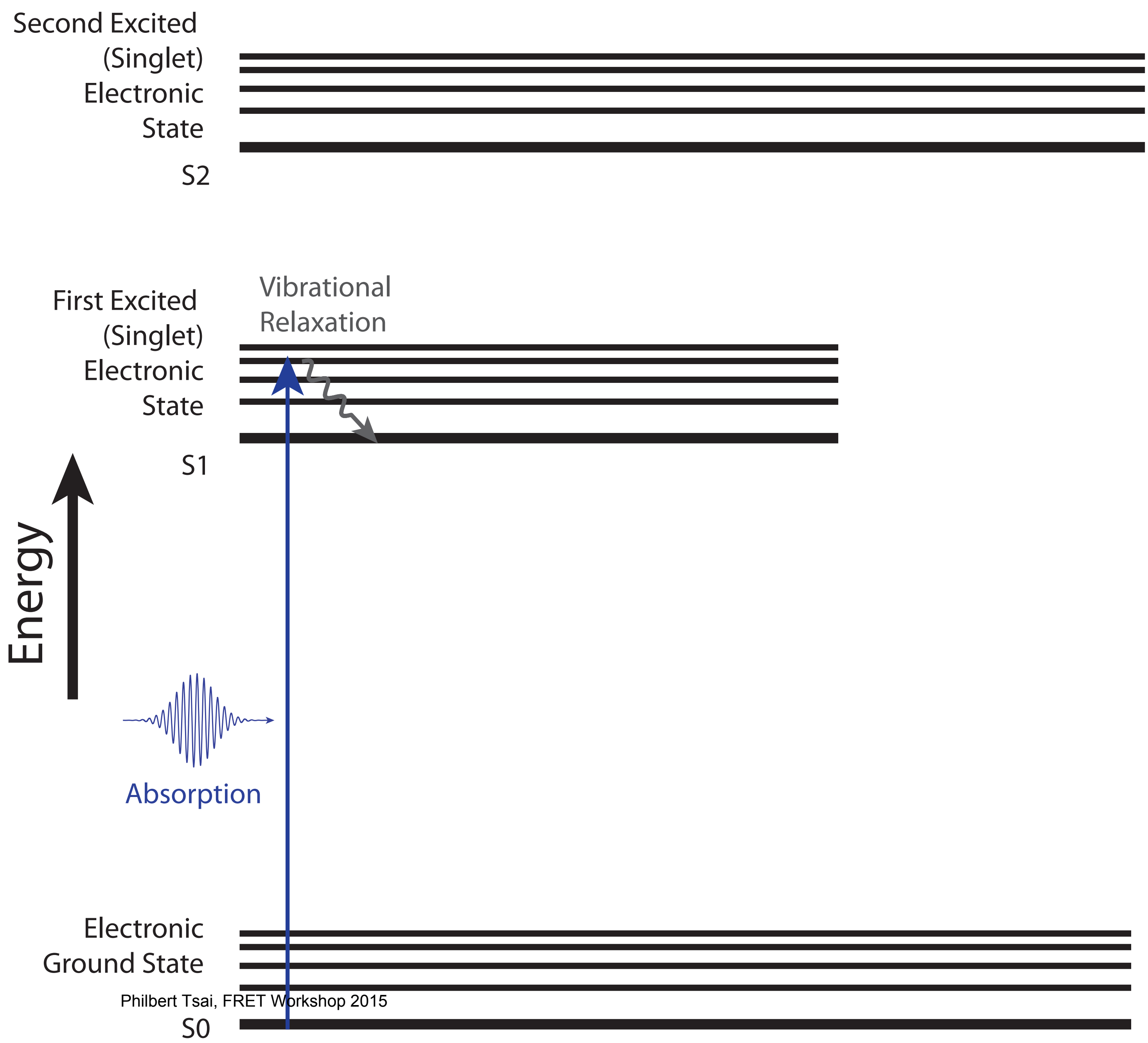

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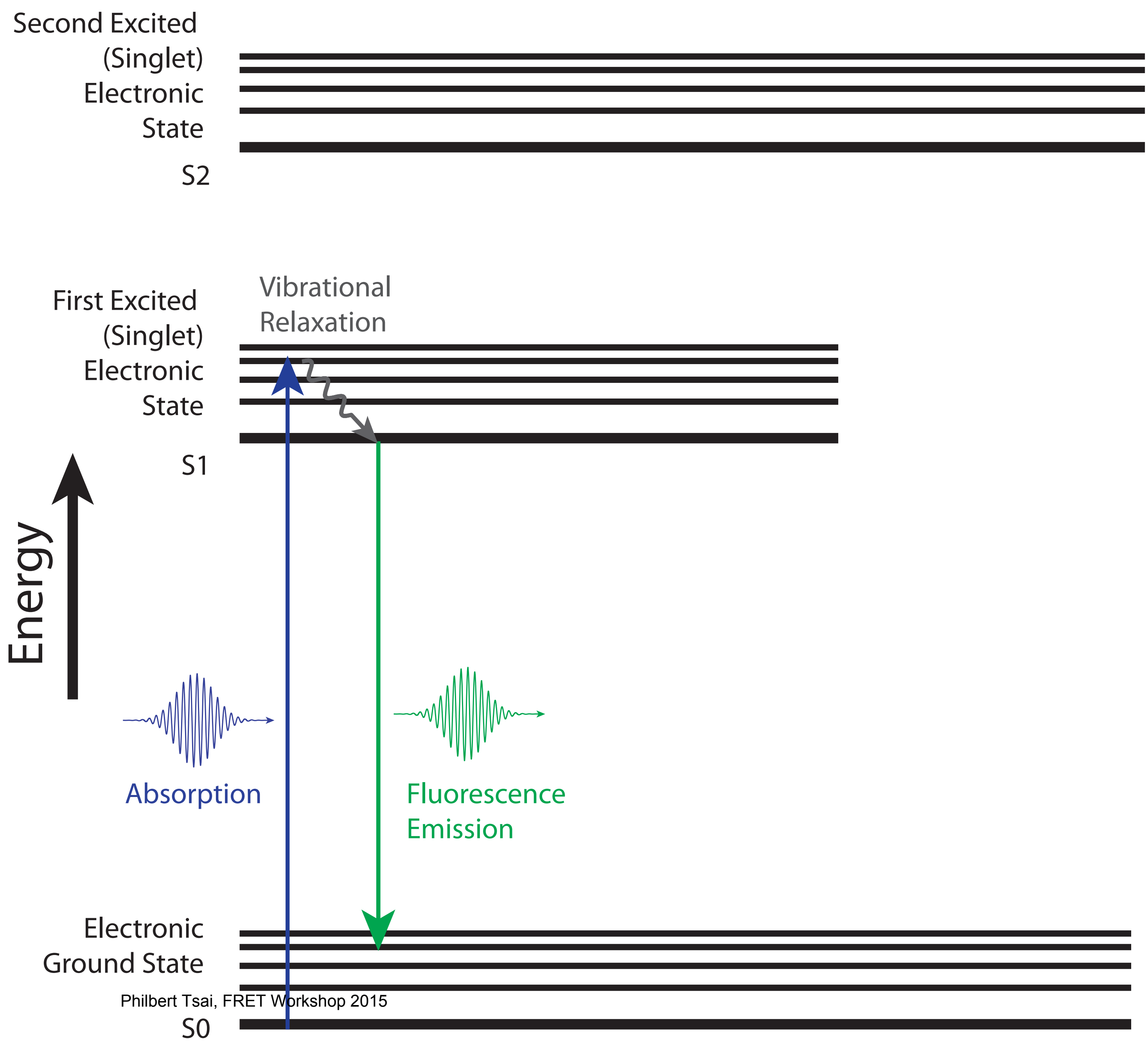

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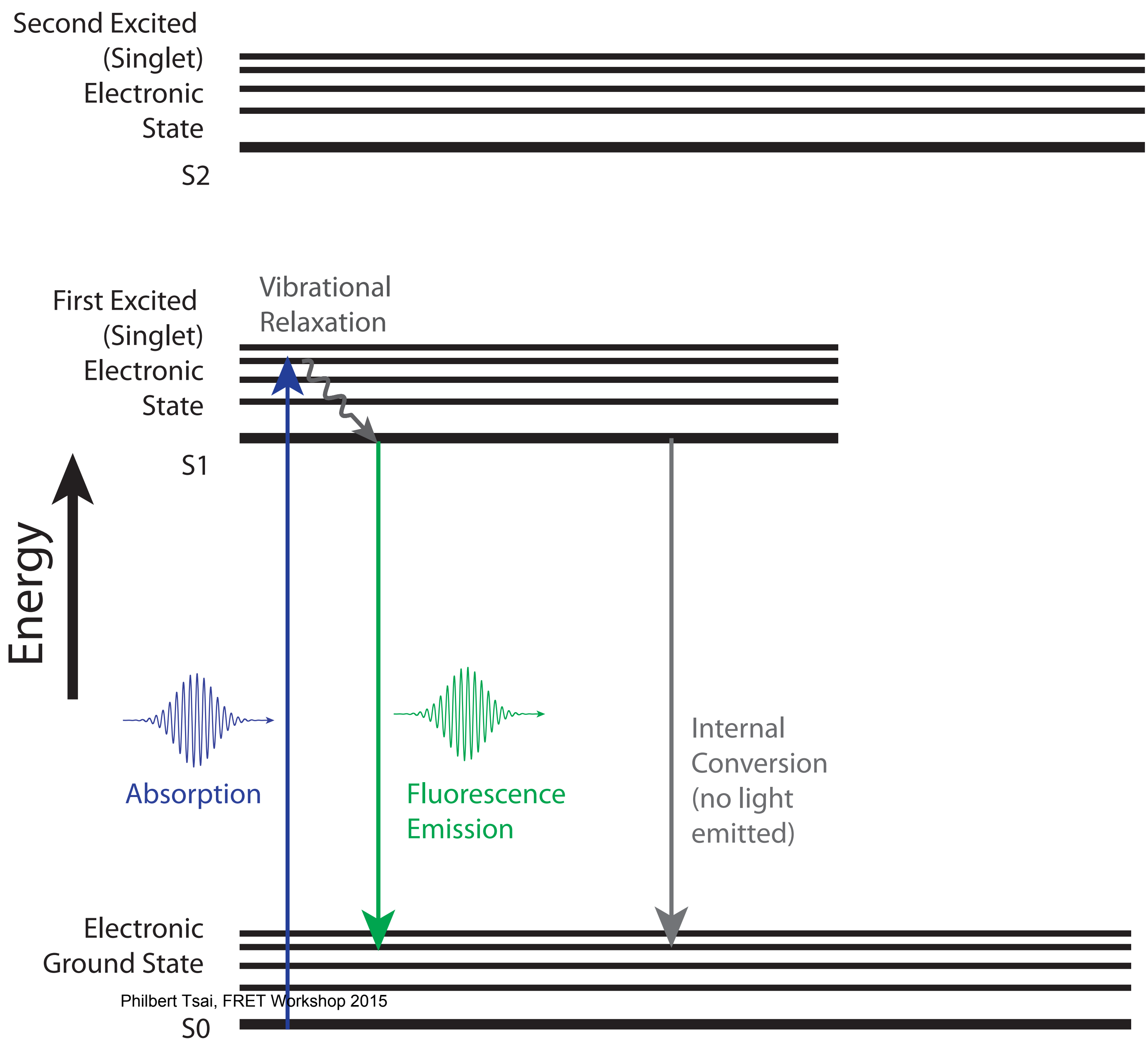

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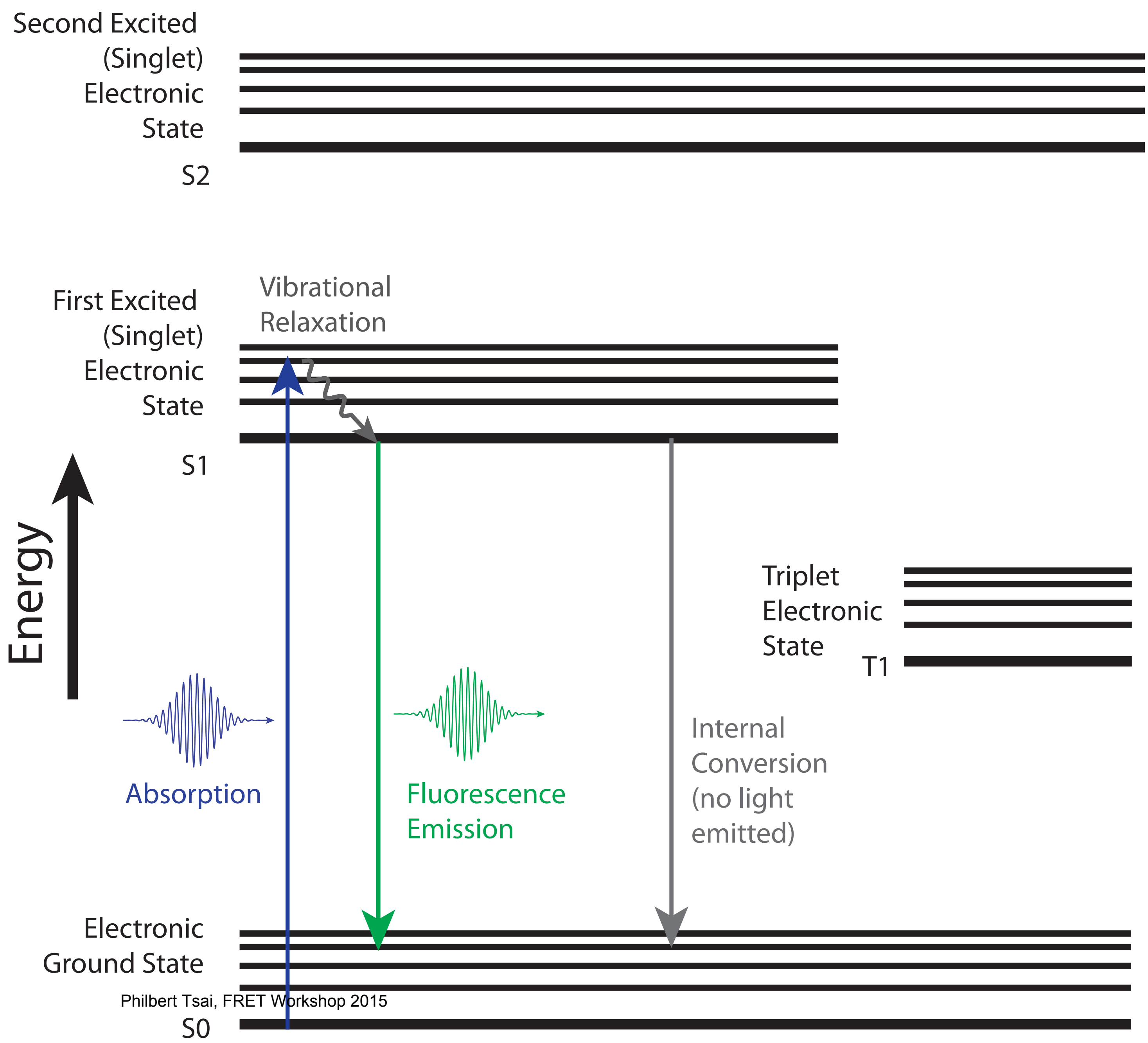

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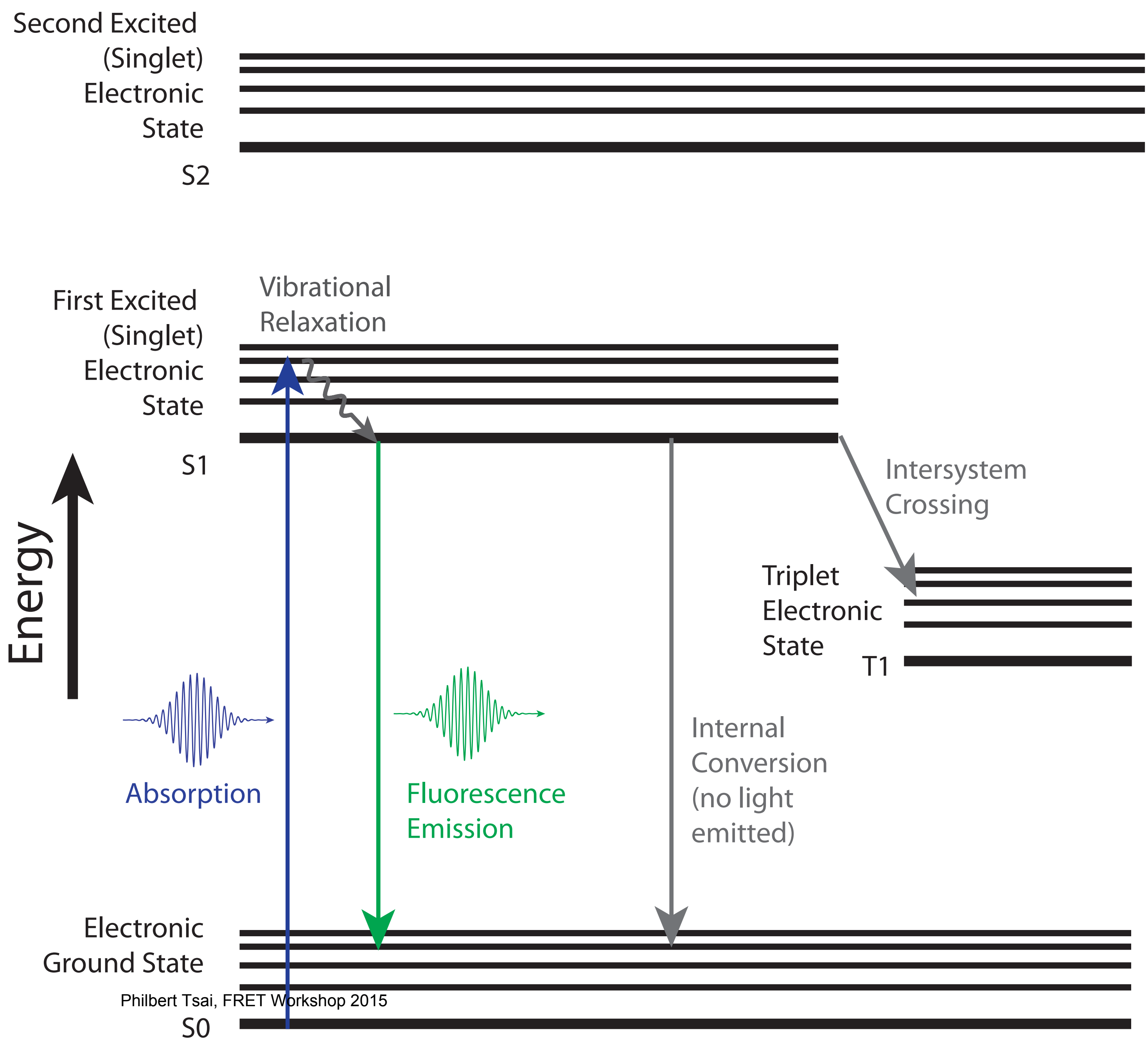

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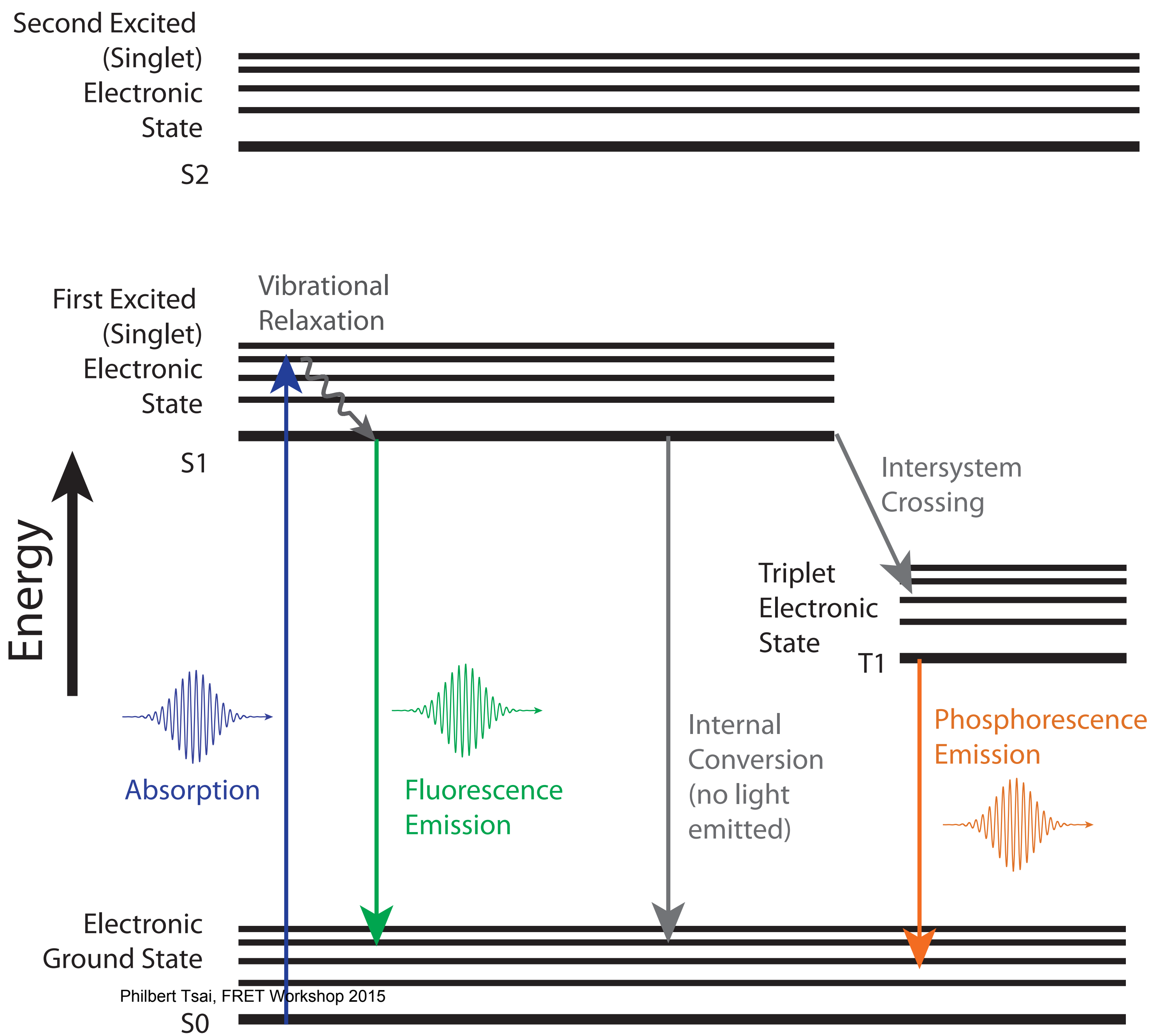

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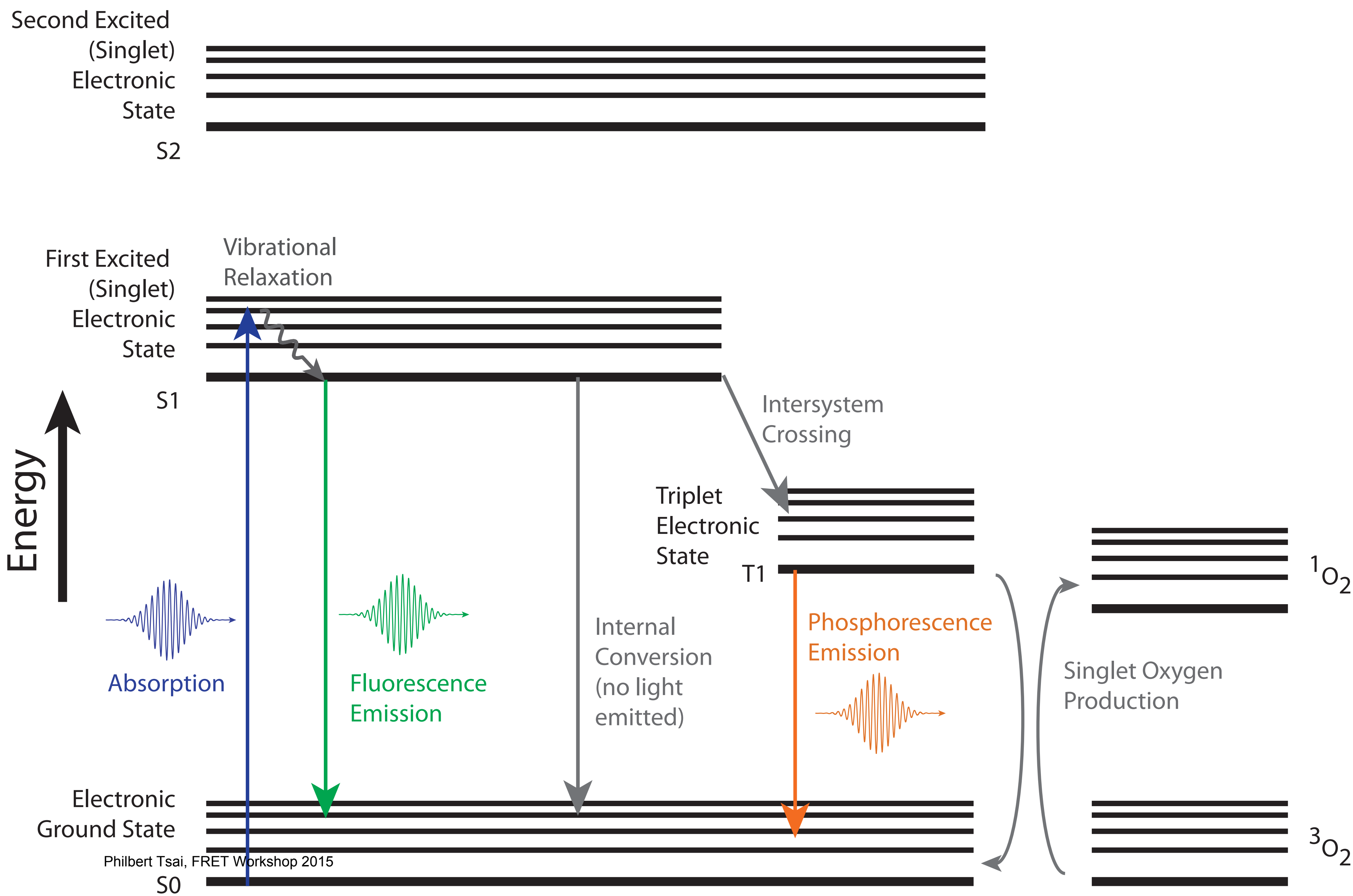

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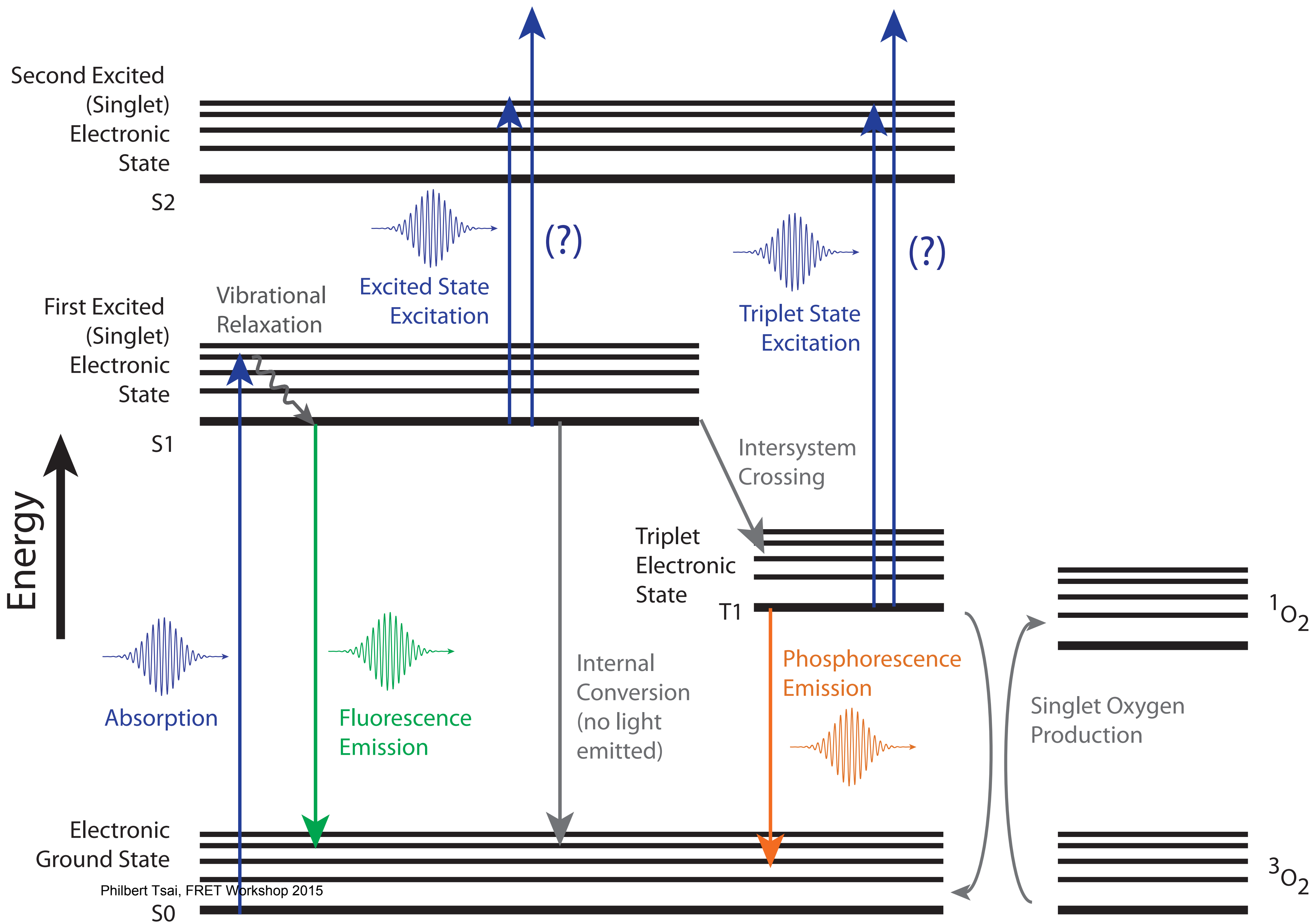

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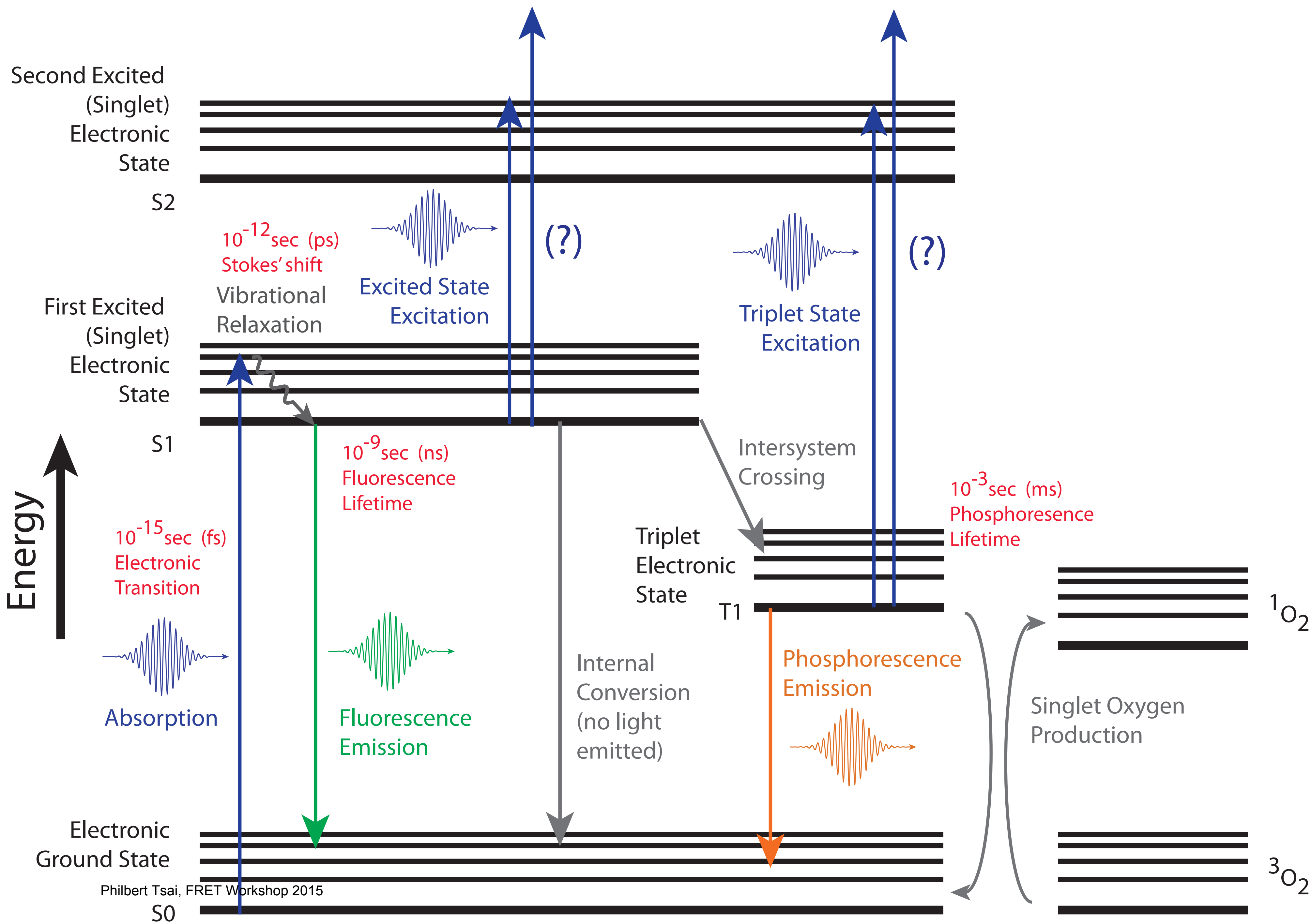

Upright Microscope : Epifluorescence Light Path

Figure adapted from http://zeiss-campus.magnet.fsu.edu

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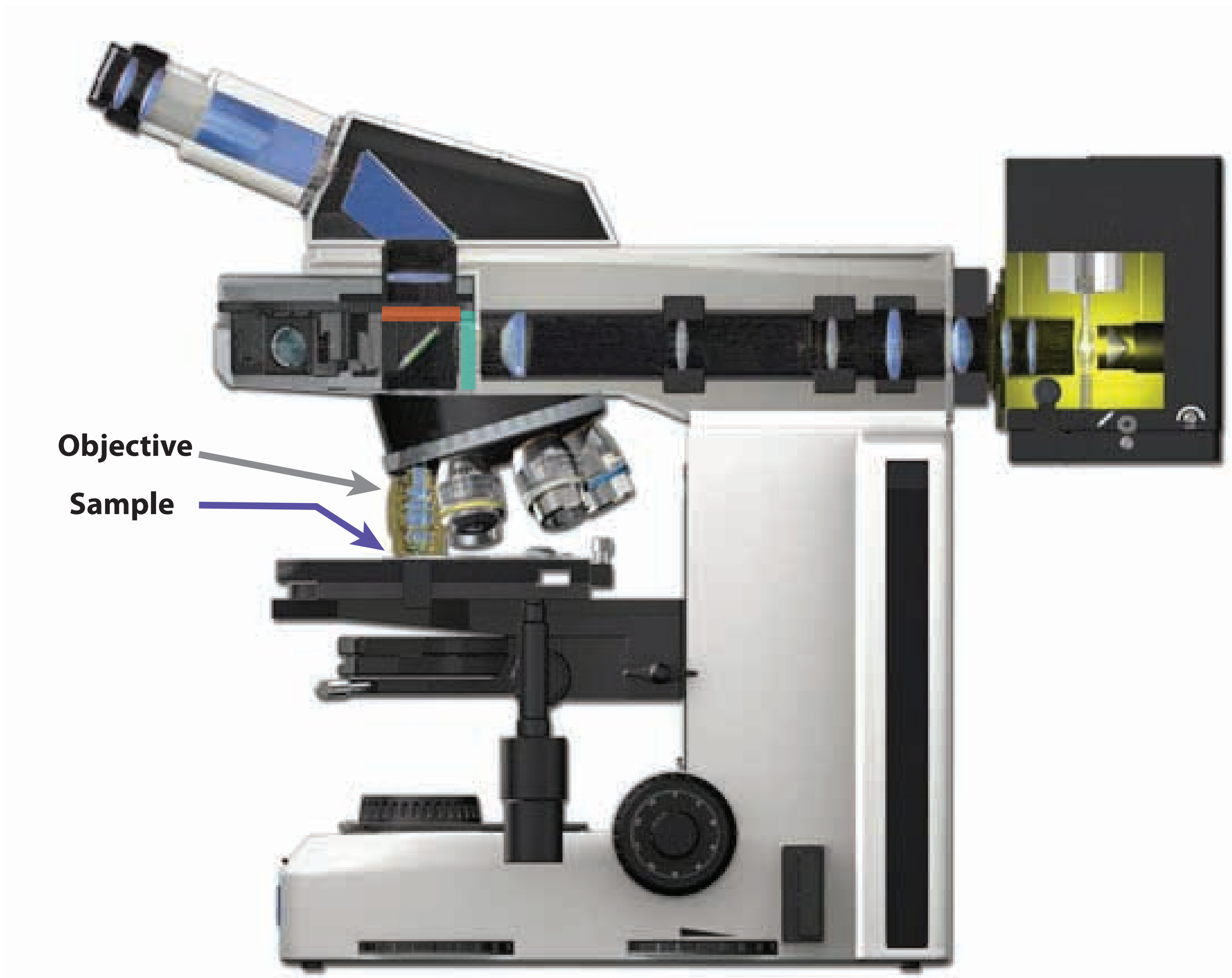

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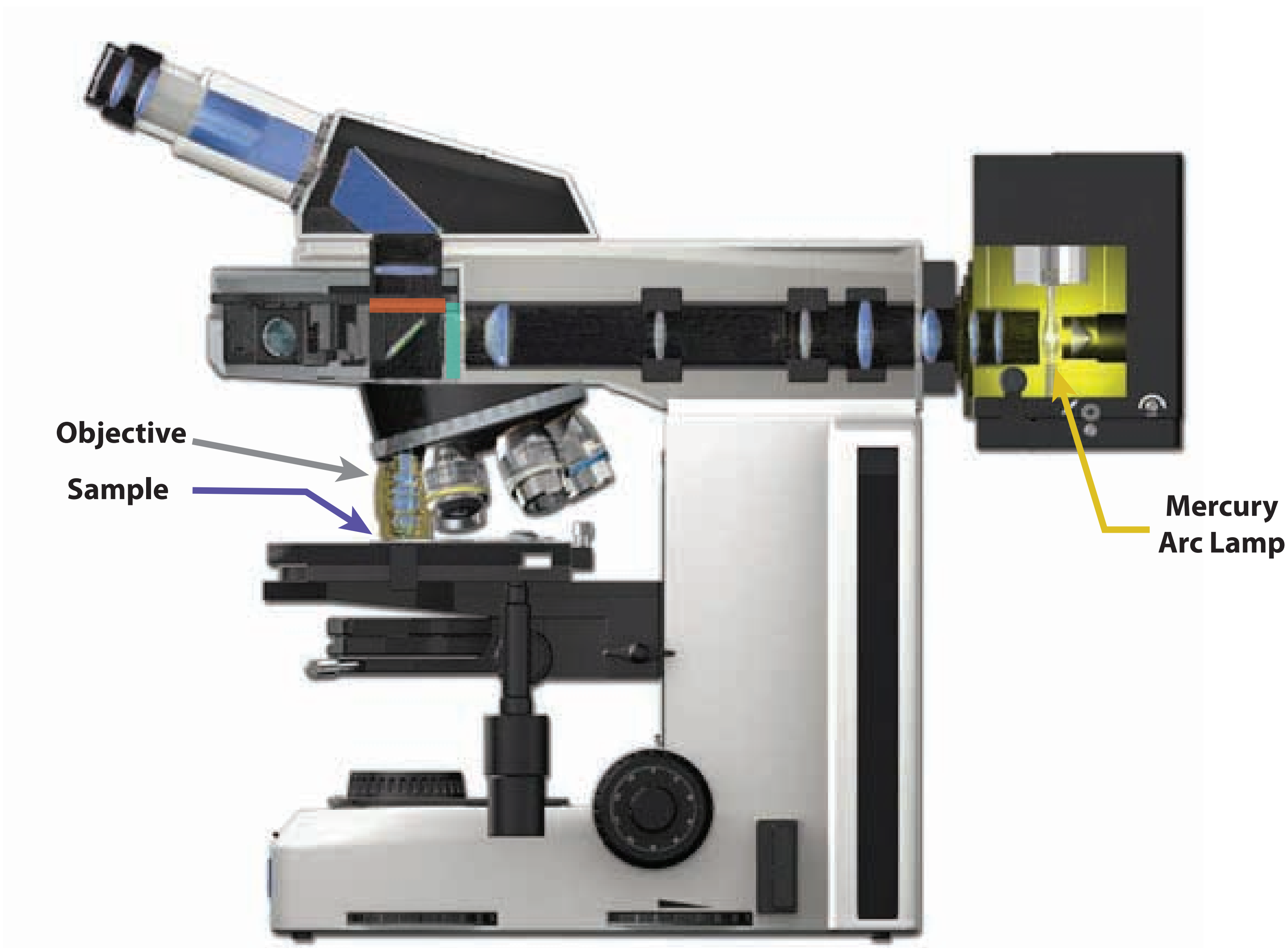

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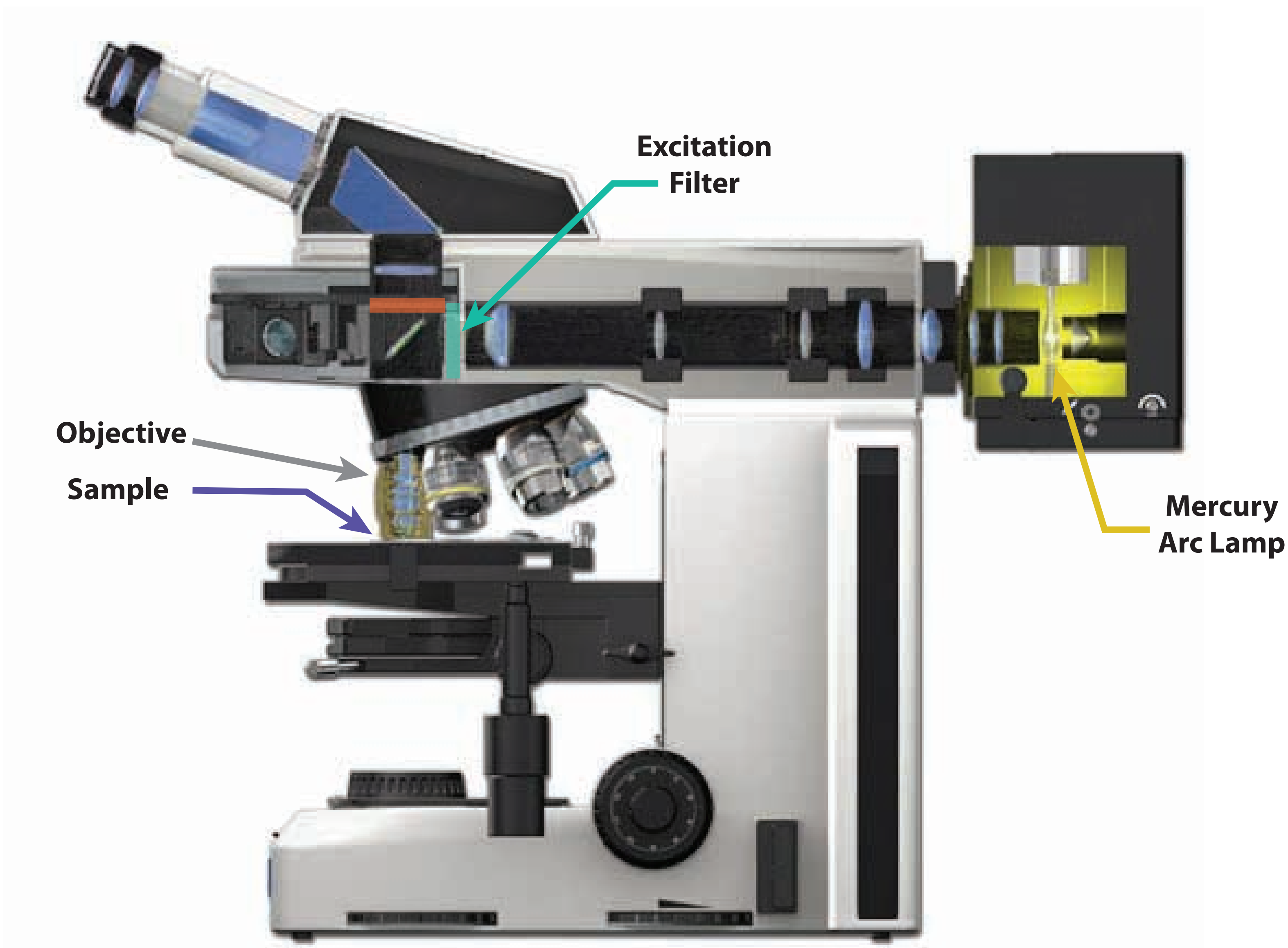

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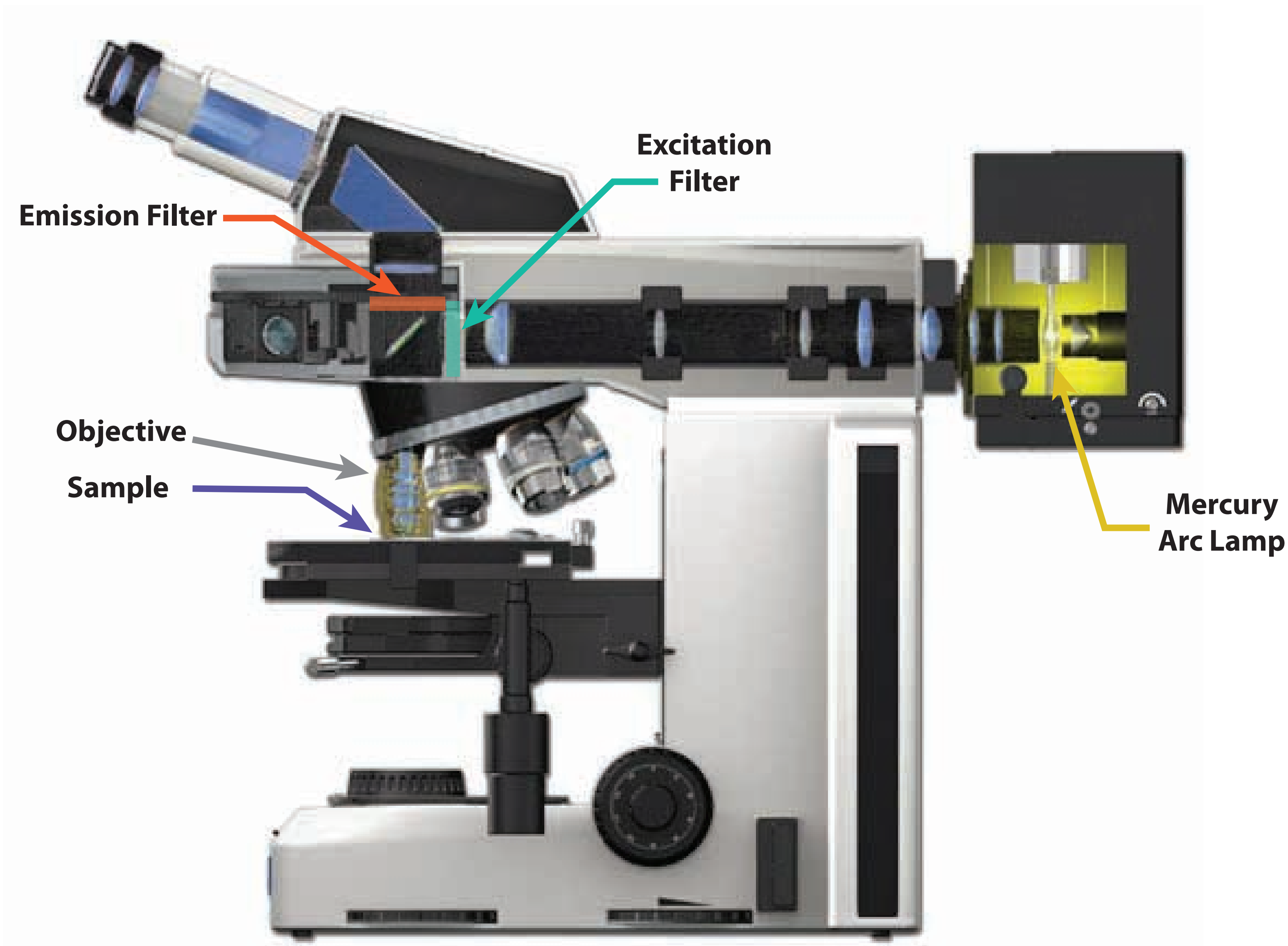

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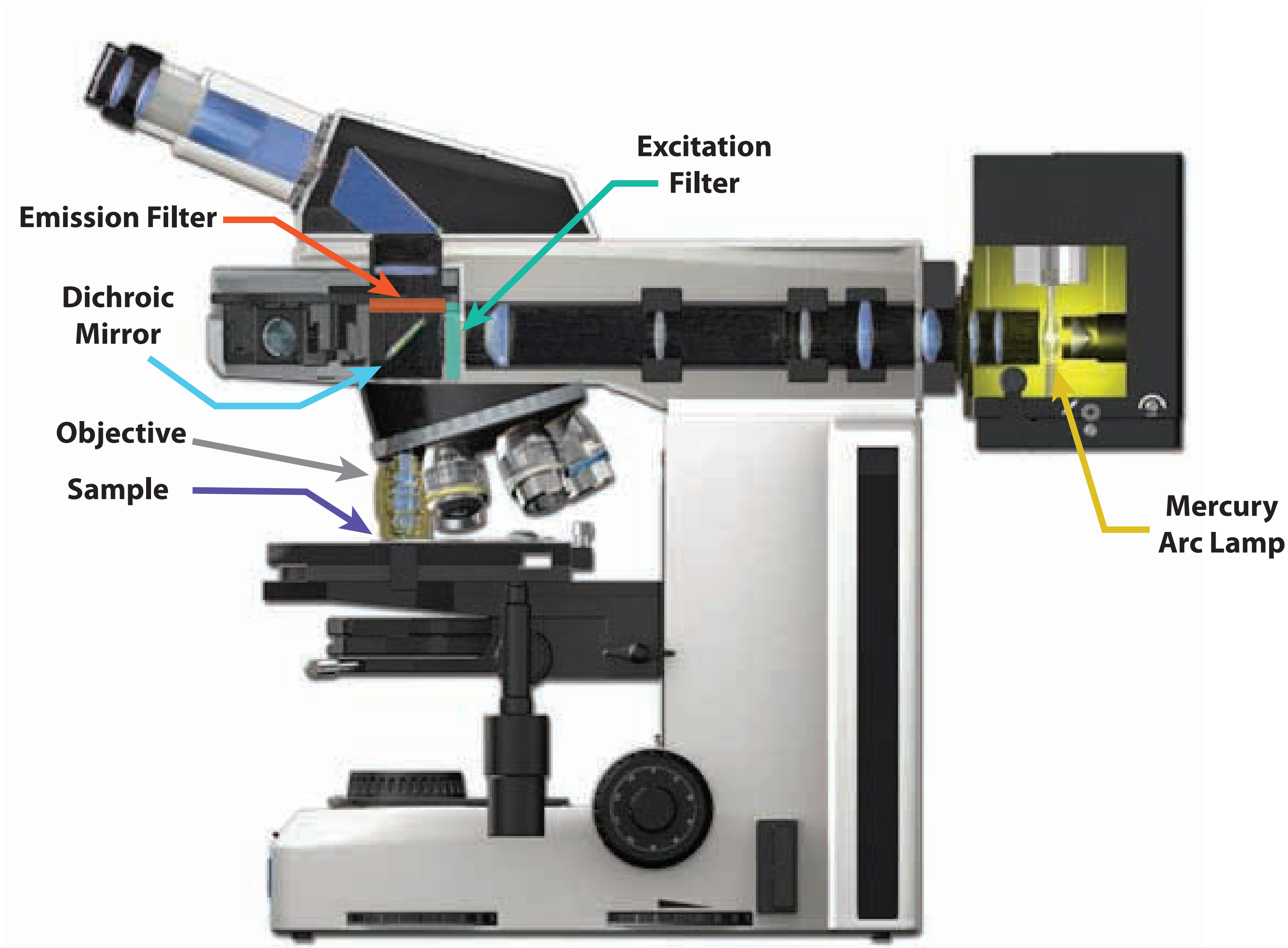

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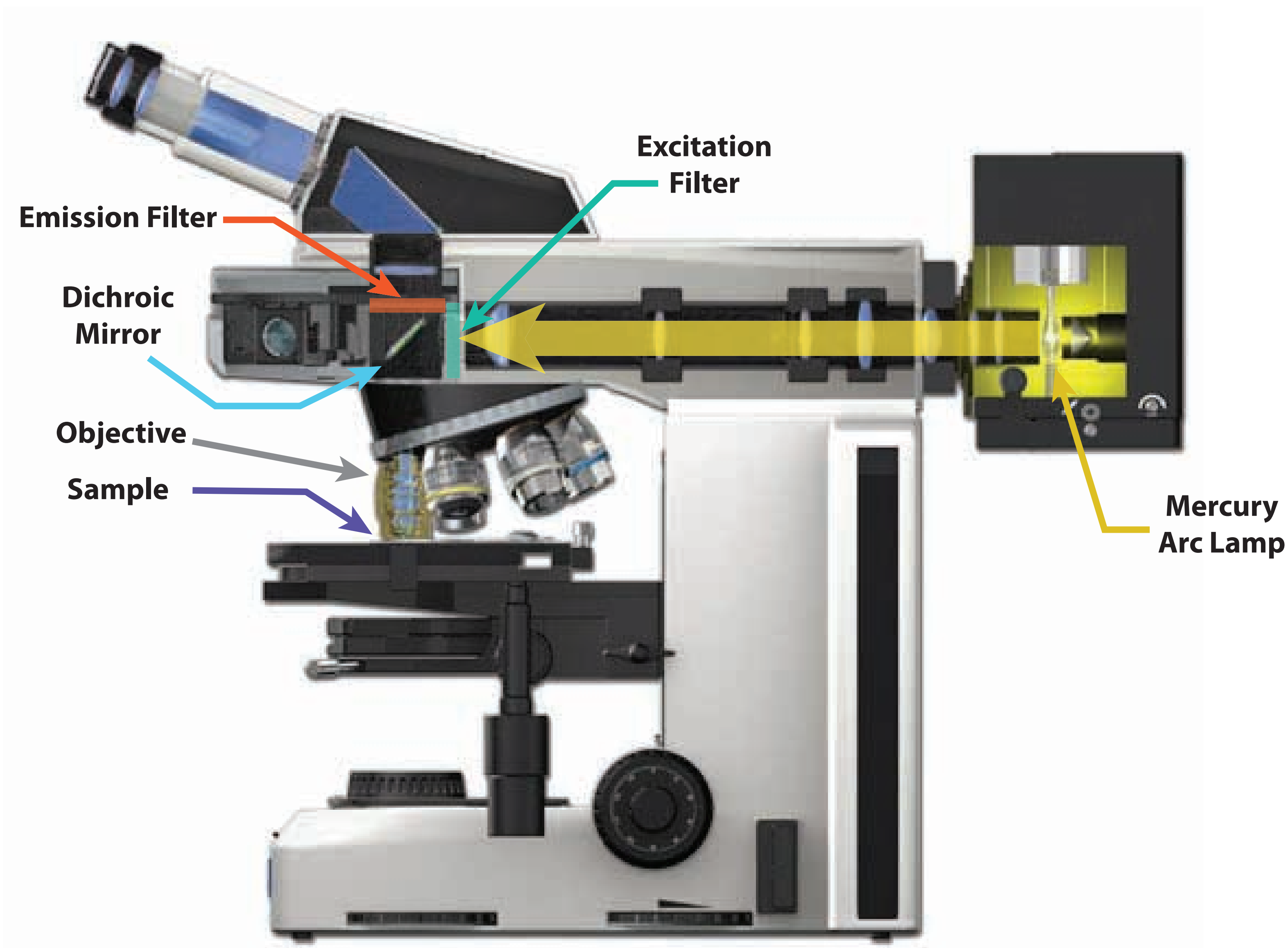

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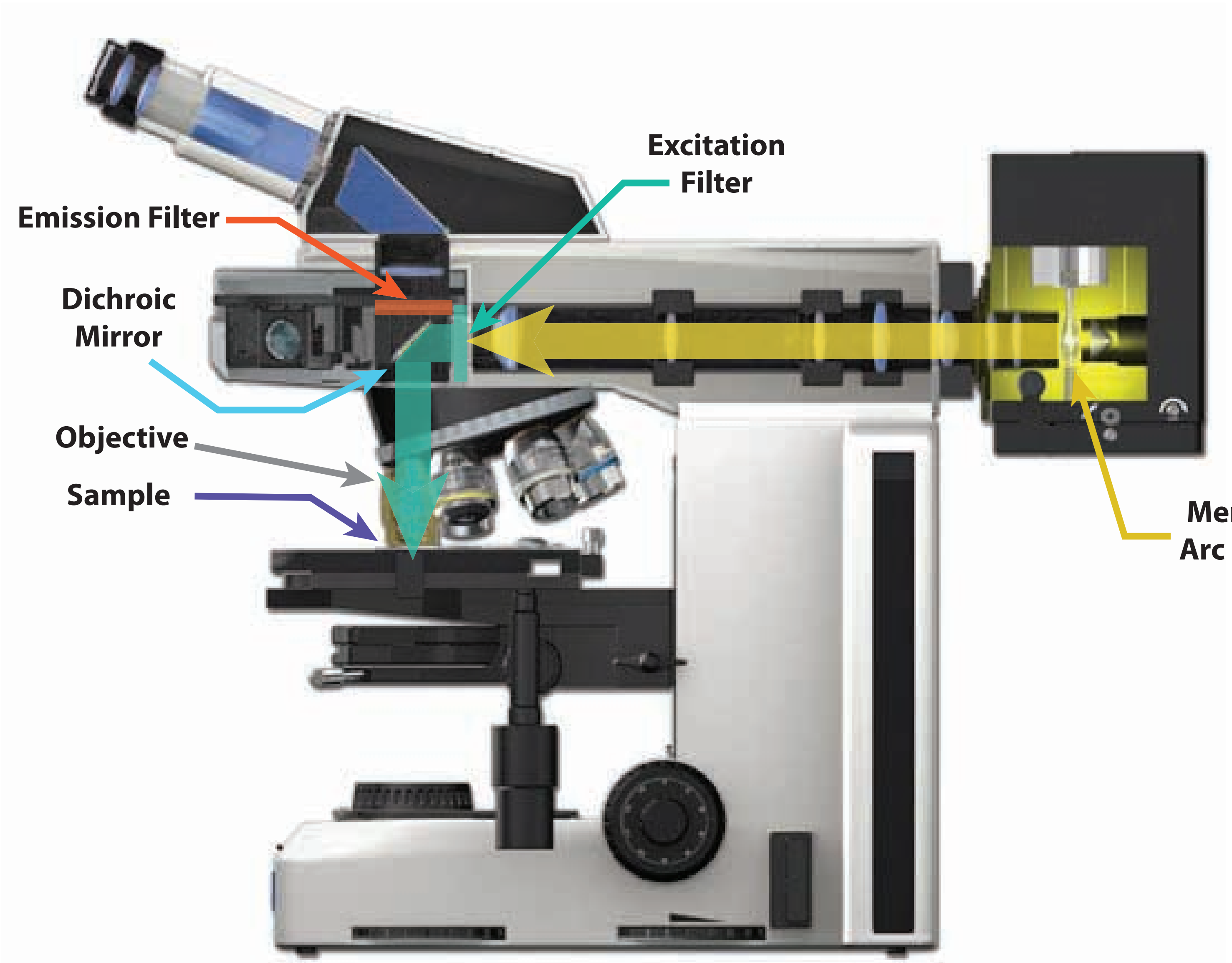

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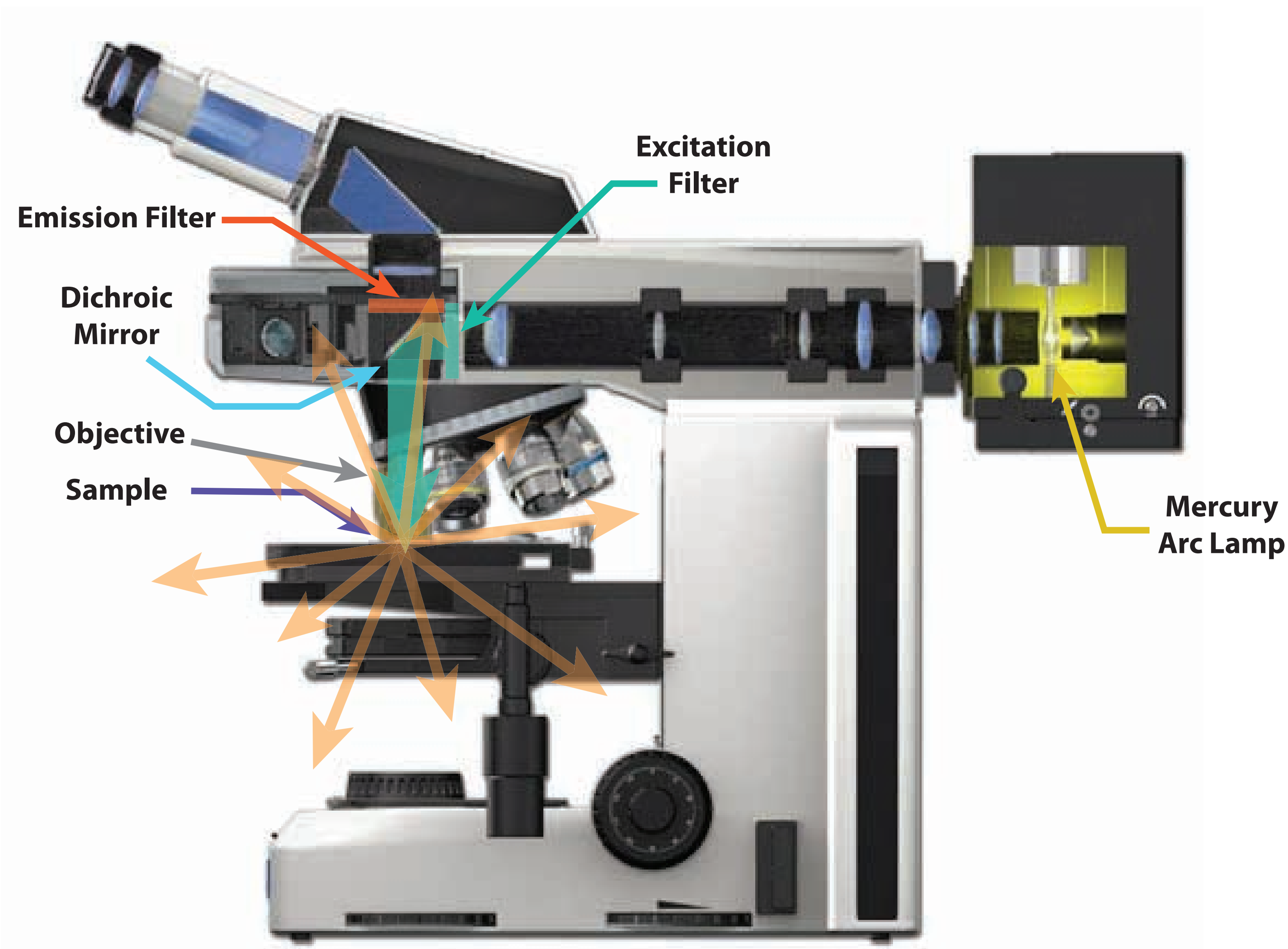

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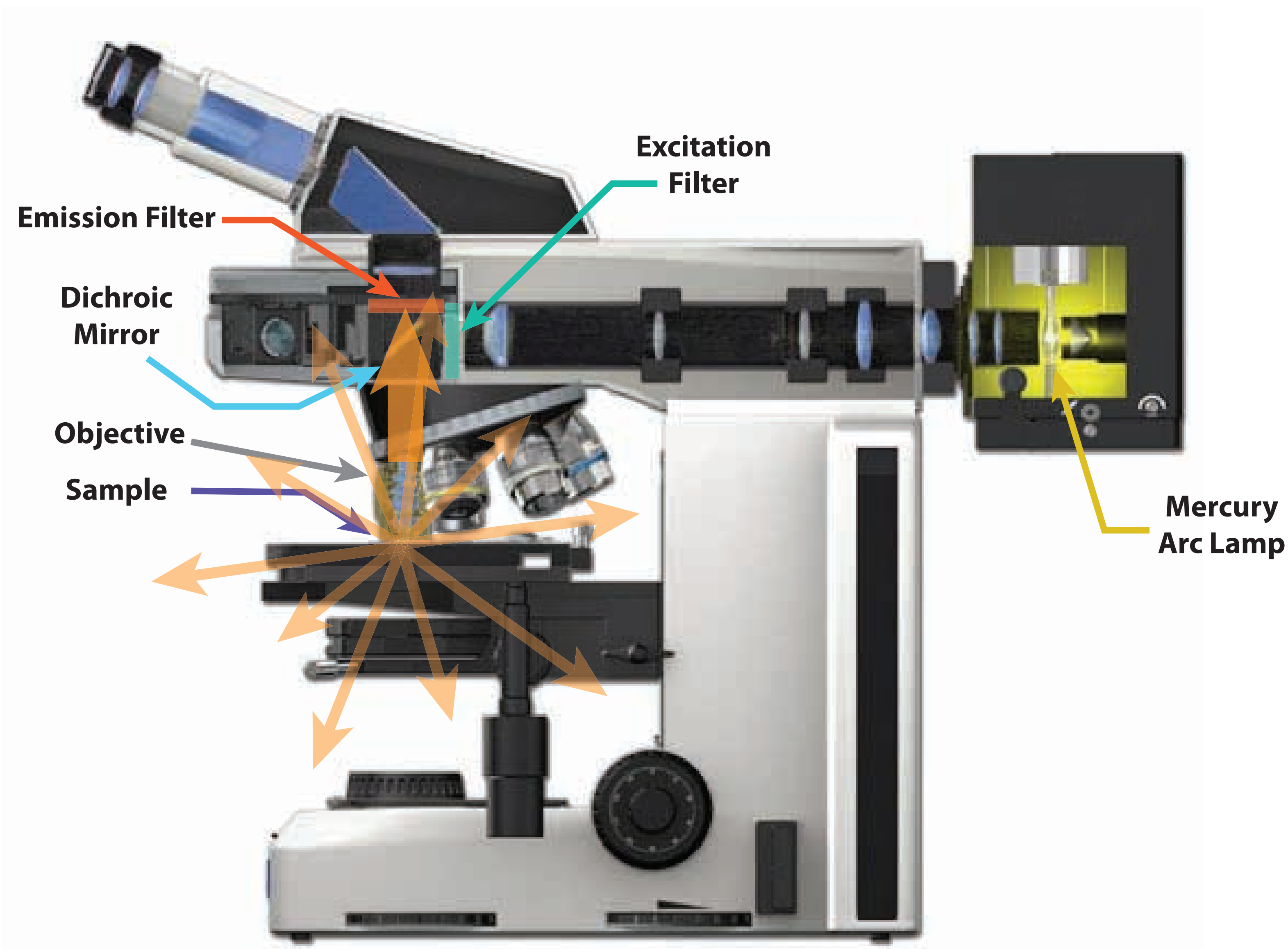

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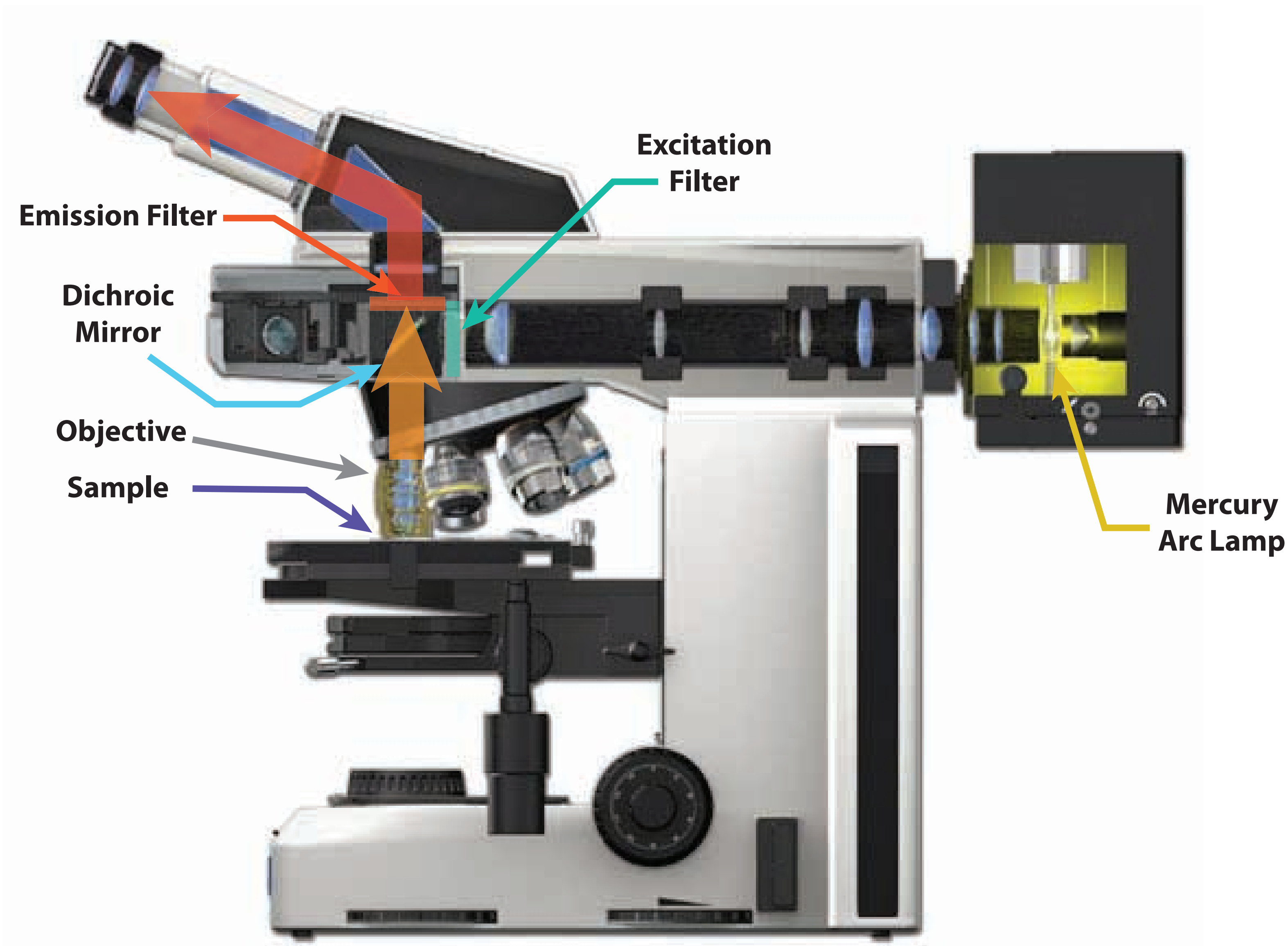

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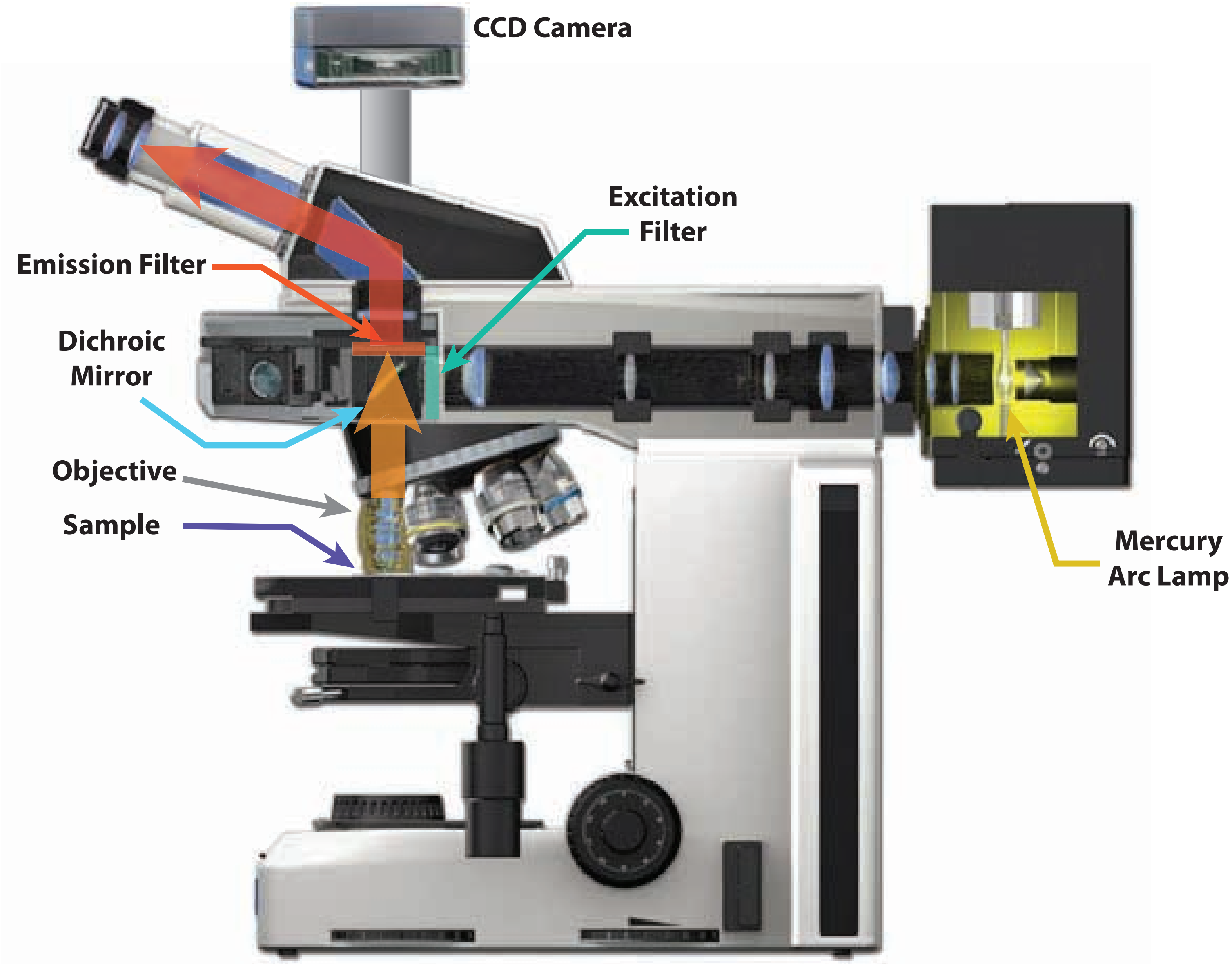

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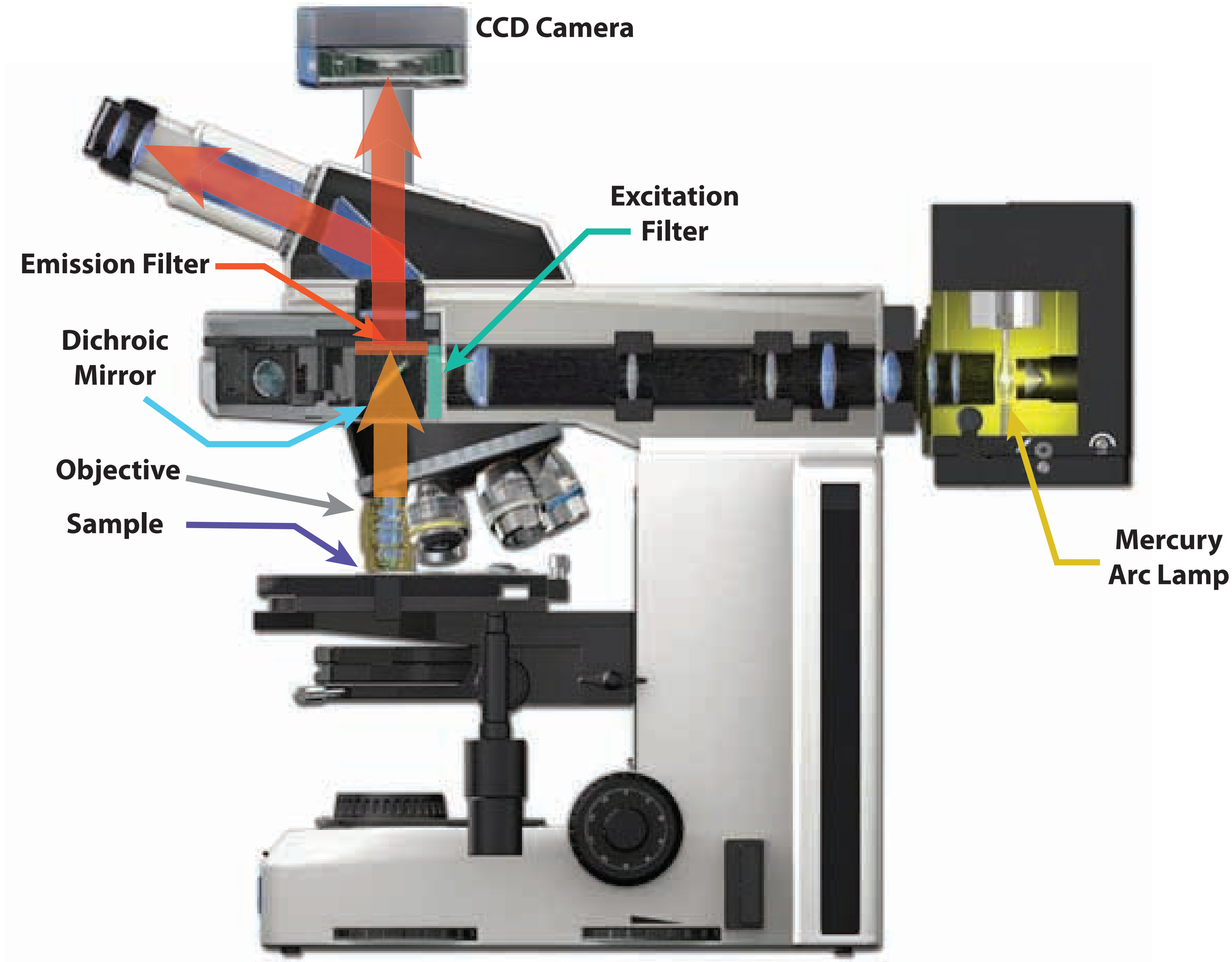

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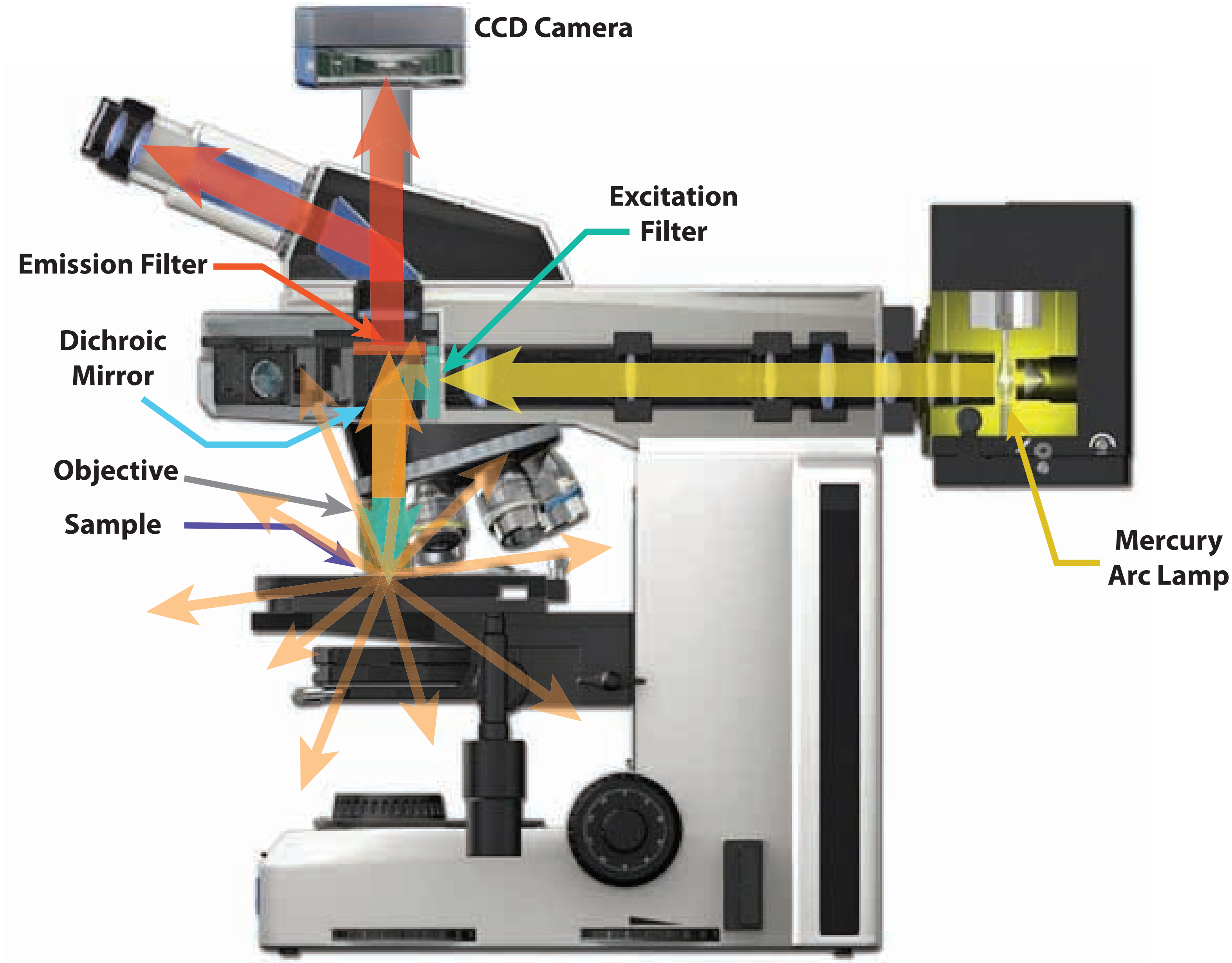

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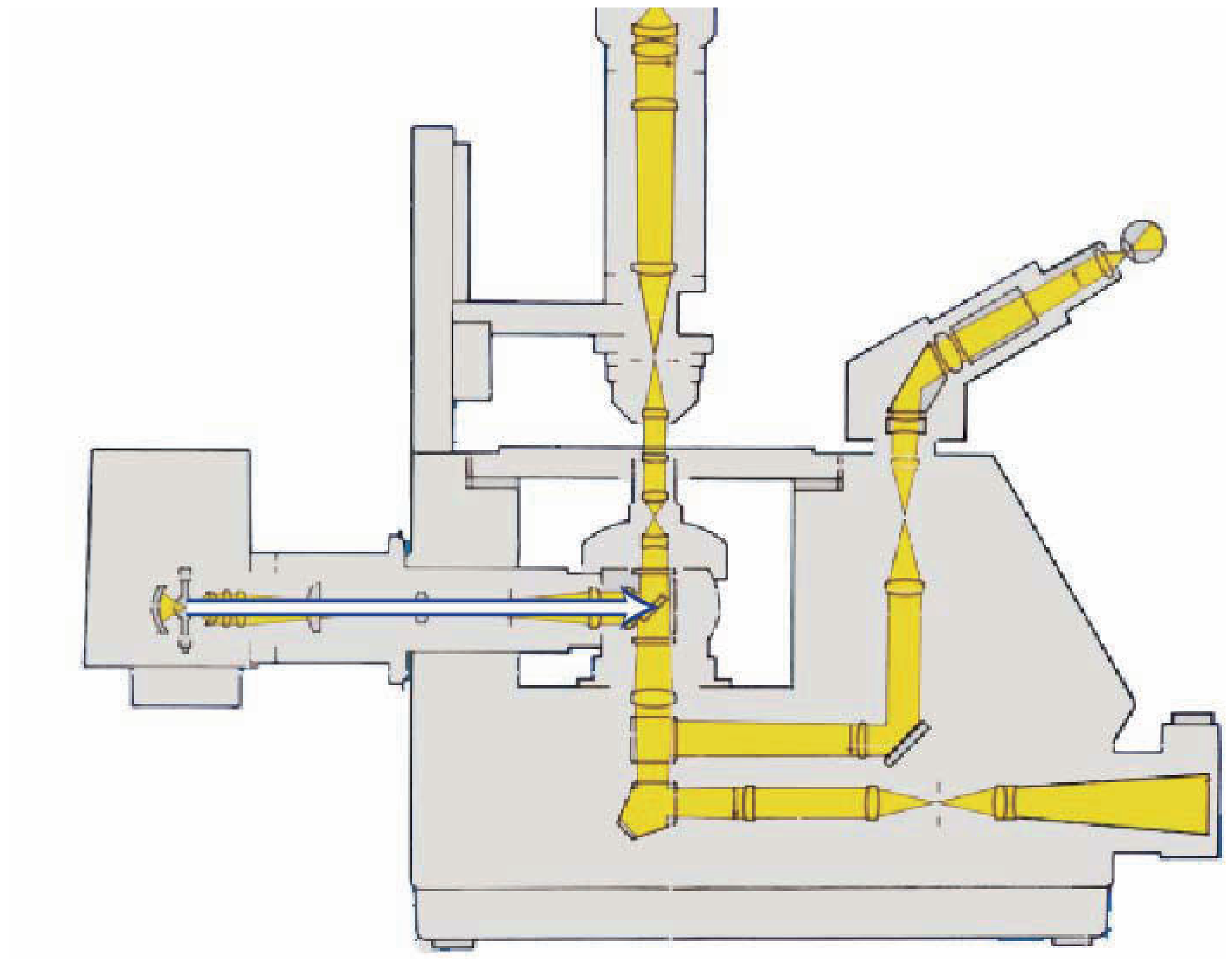
Optical schematic of an inverted epifluorescence microscope



Philbert S. Tsai, July 28,2010

Brightfield vs. Fluorescence Microscopy

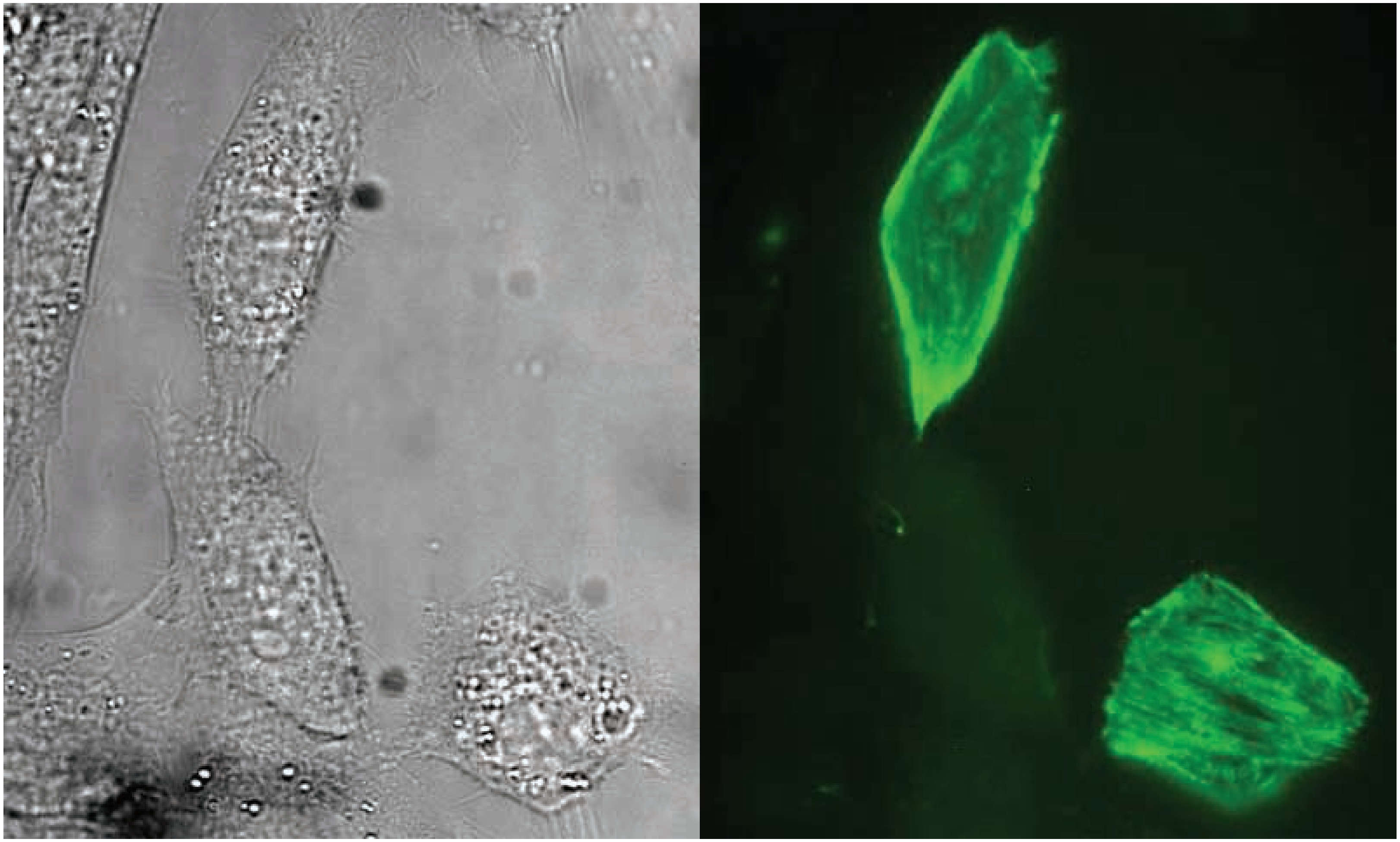
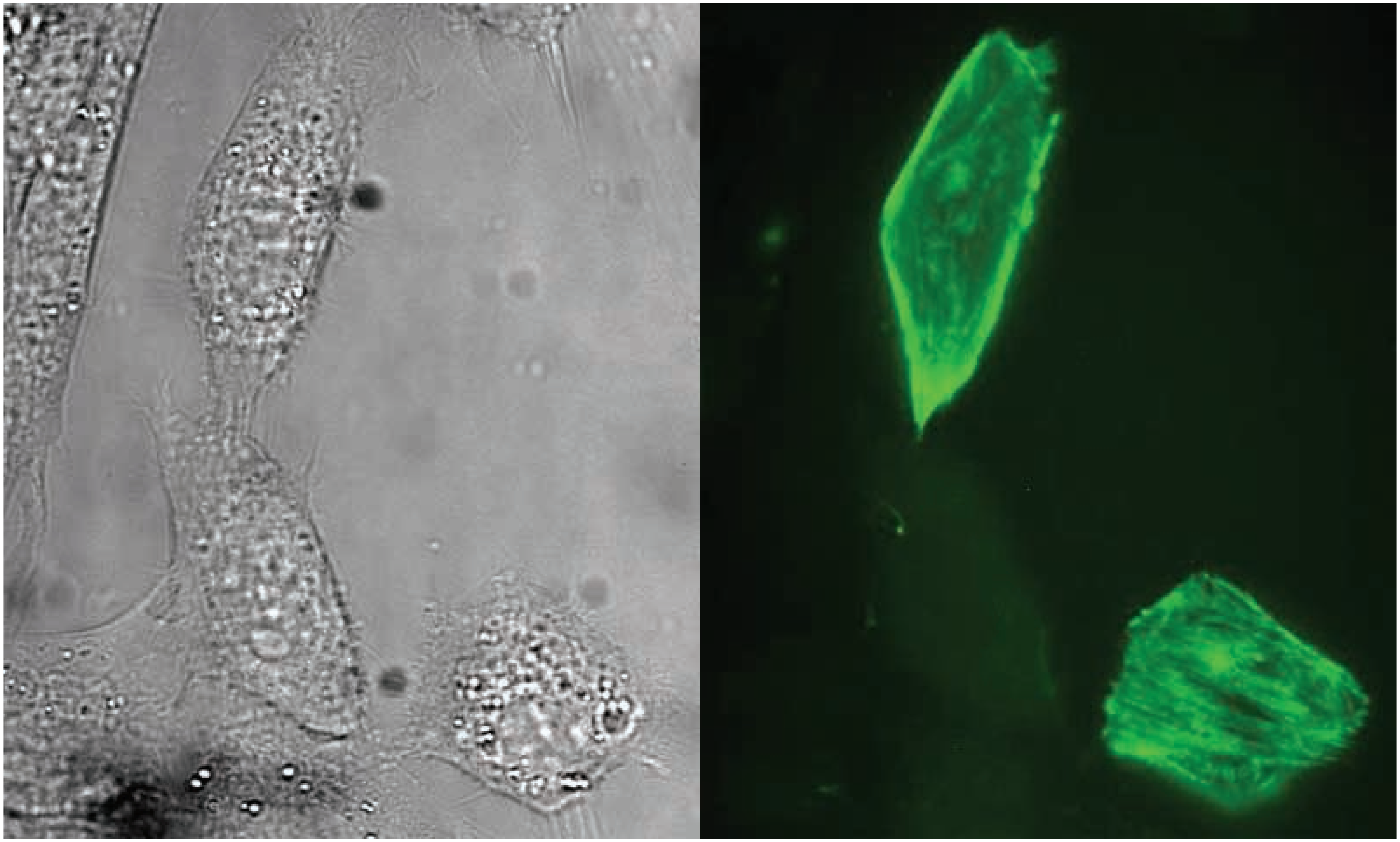


Image taken from L.W. Miller and V.W. Cornish. Curr. Opin. Chem. Biol. (2005)

Brightfield vs. Fluorescence Microscopy



Ambiguous Signal

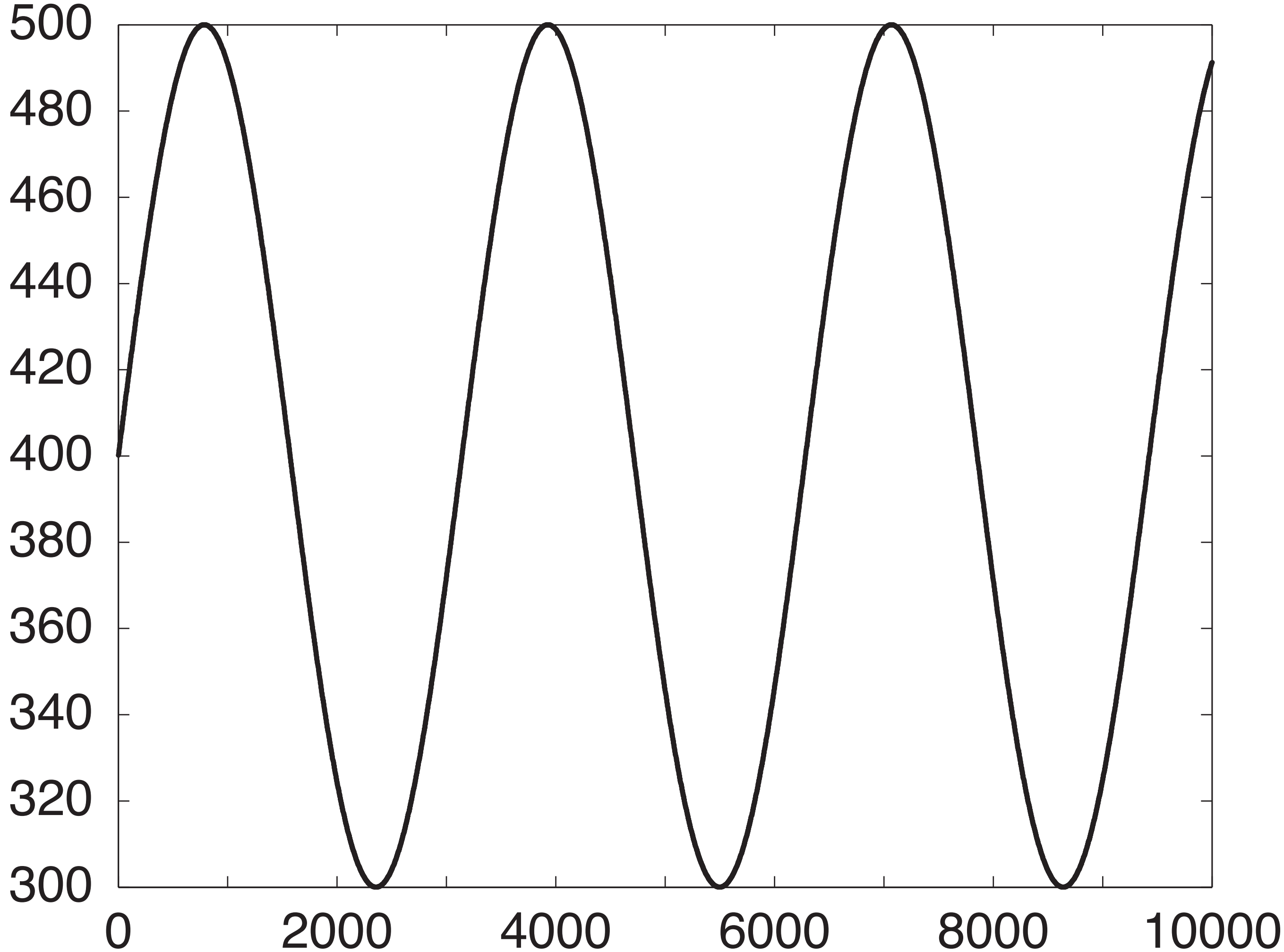
Small changes on a bright background

Cell-specific or subcellular-specific labeling

Bright signal on dark background

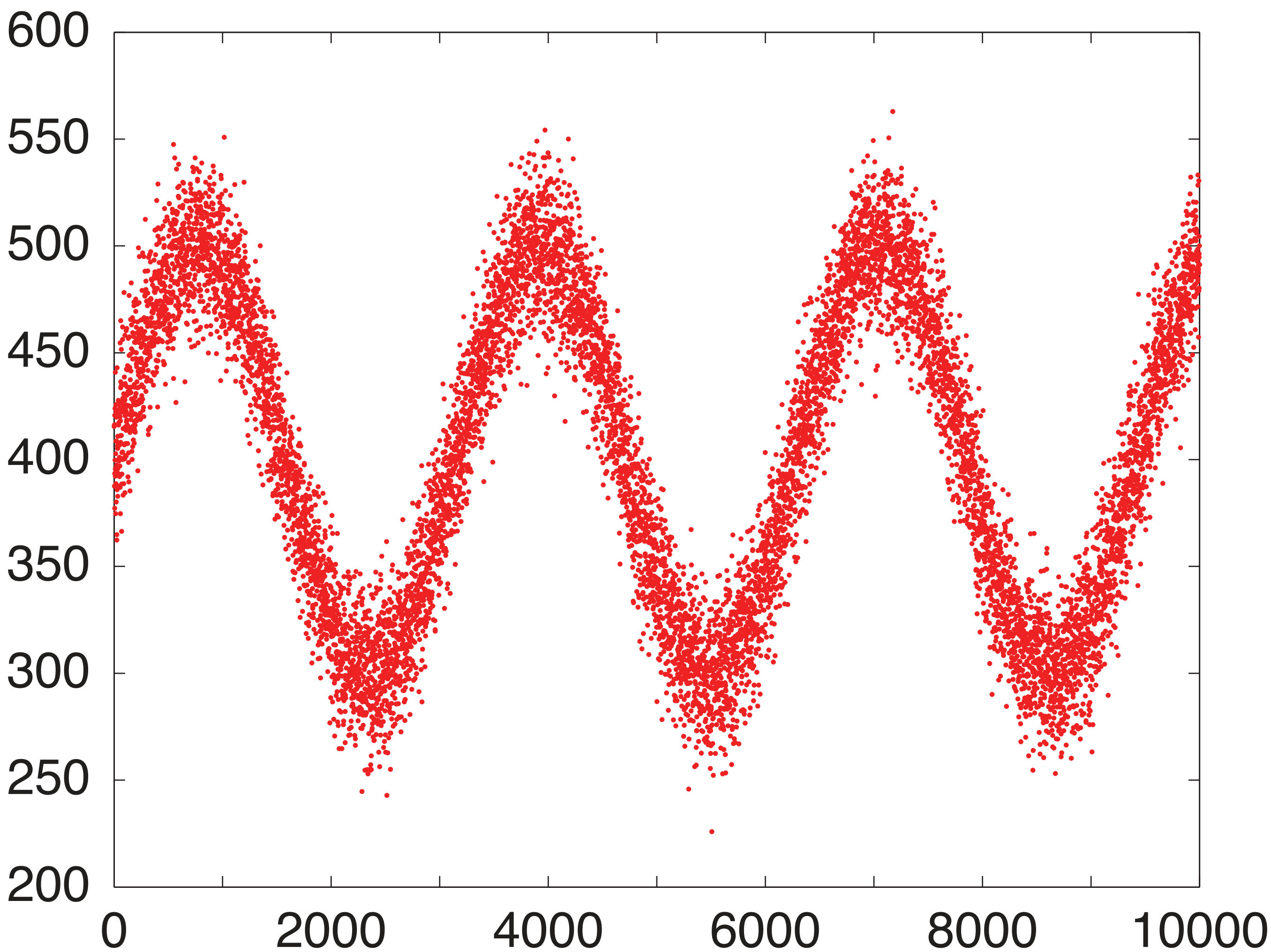
Image taken from L.W. Miller and V.W. Cornish. Curr. Opin. Chem. Biol. (2005)

The Fluorescence Advantage : Signal on a Dark Background



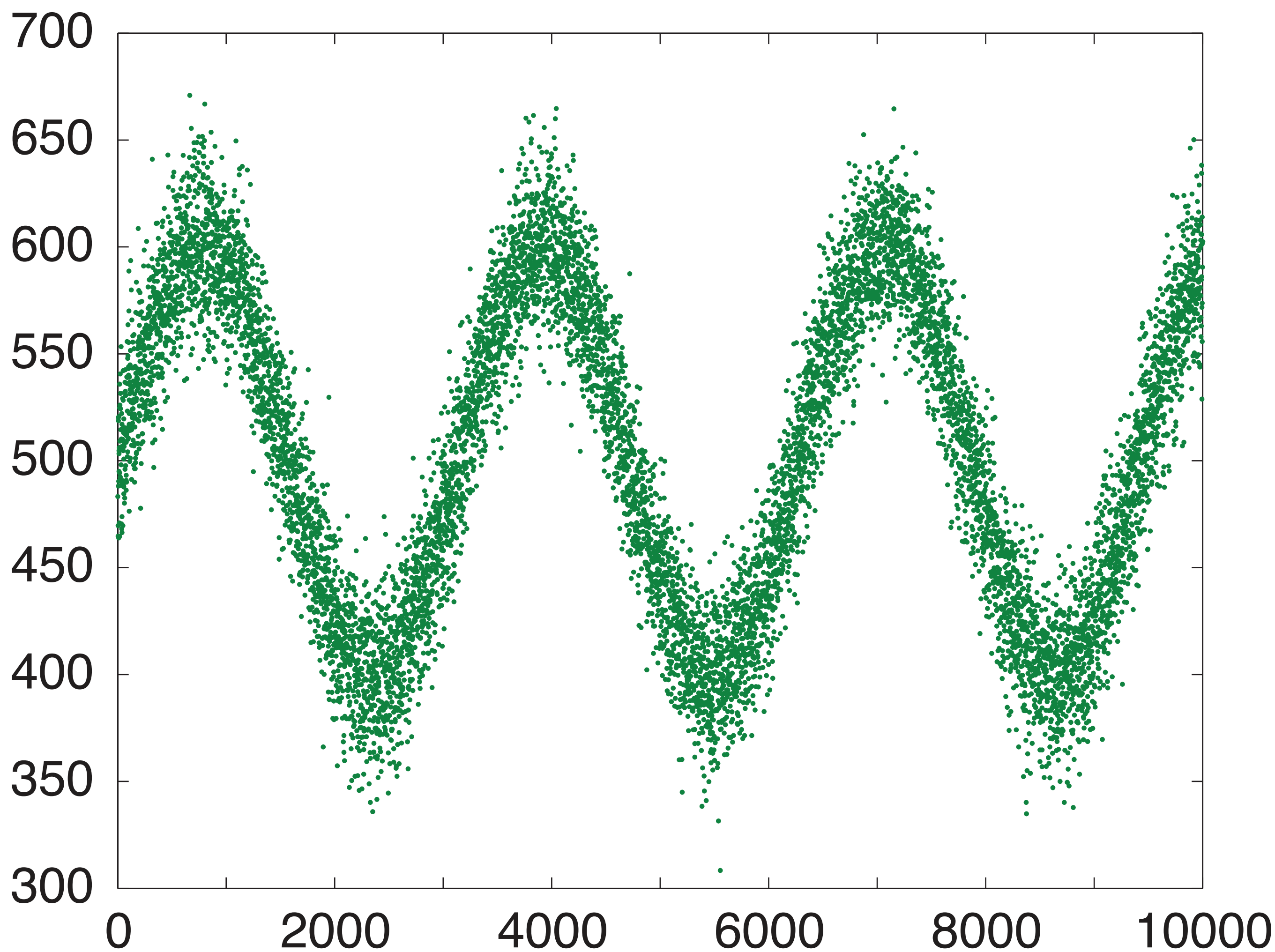
A noise-free, low-background signal

The Fluorescence Advantage : Signal on a Dark Background



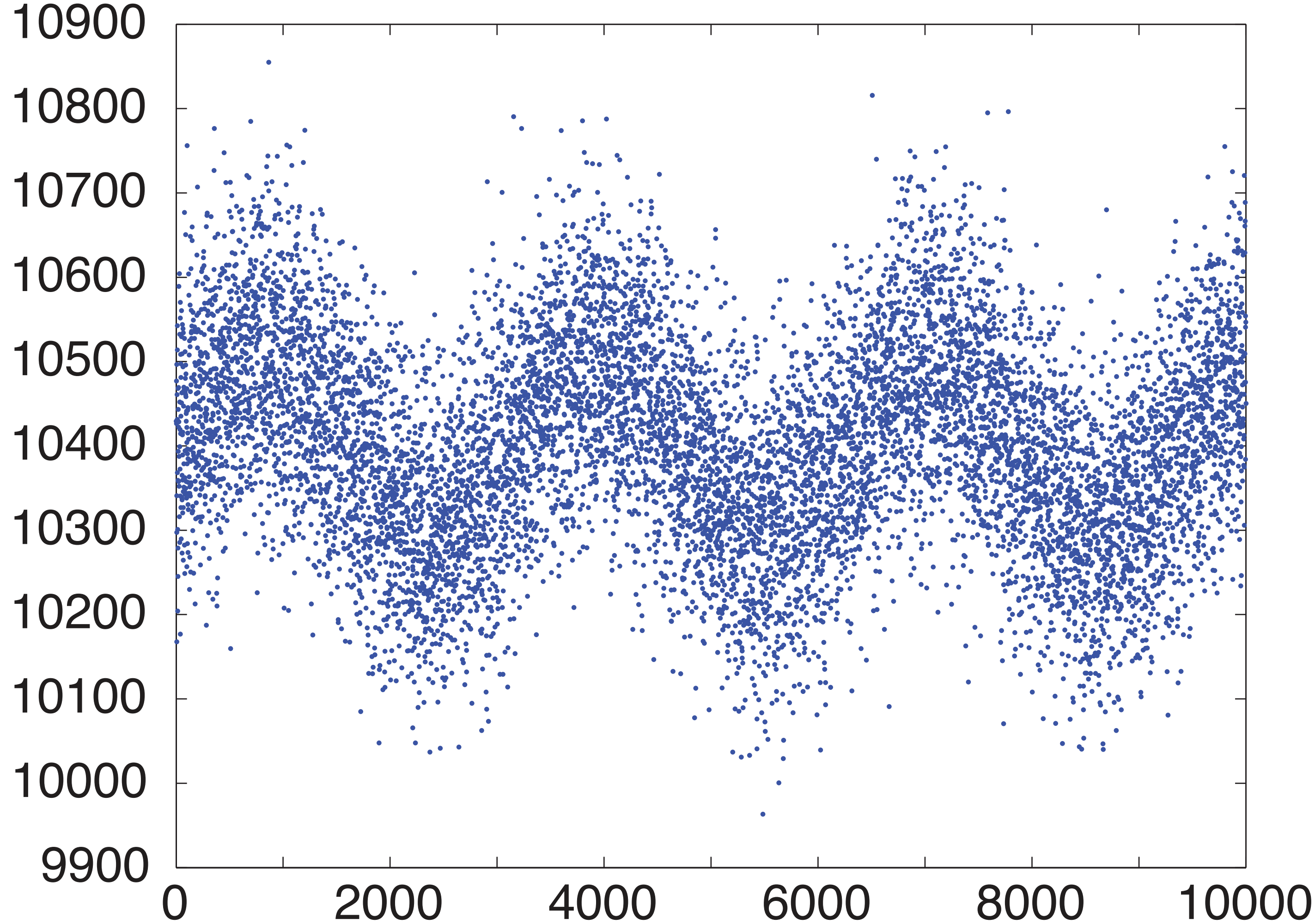
A shot-noise-limited signal with low-background

The Fluorescence Advantage : Signal on a Dark Background



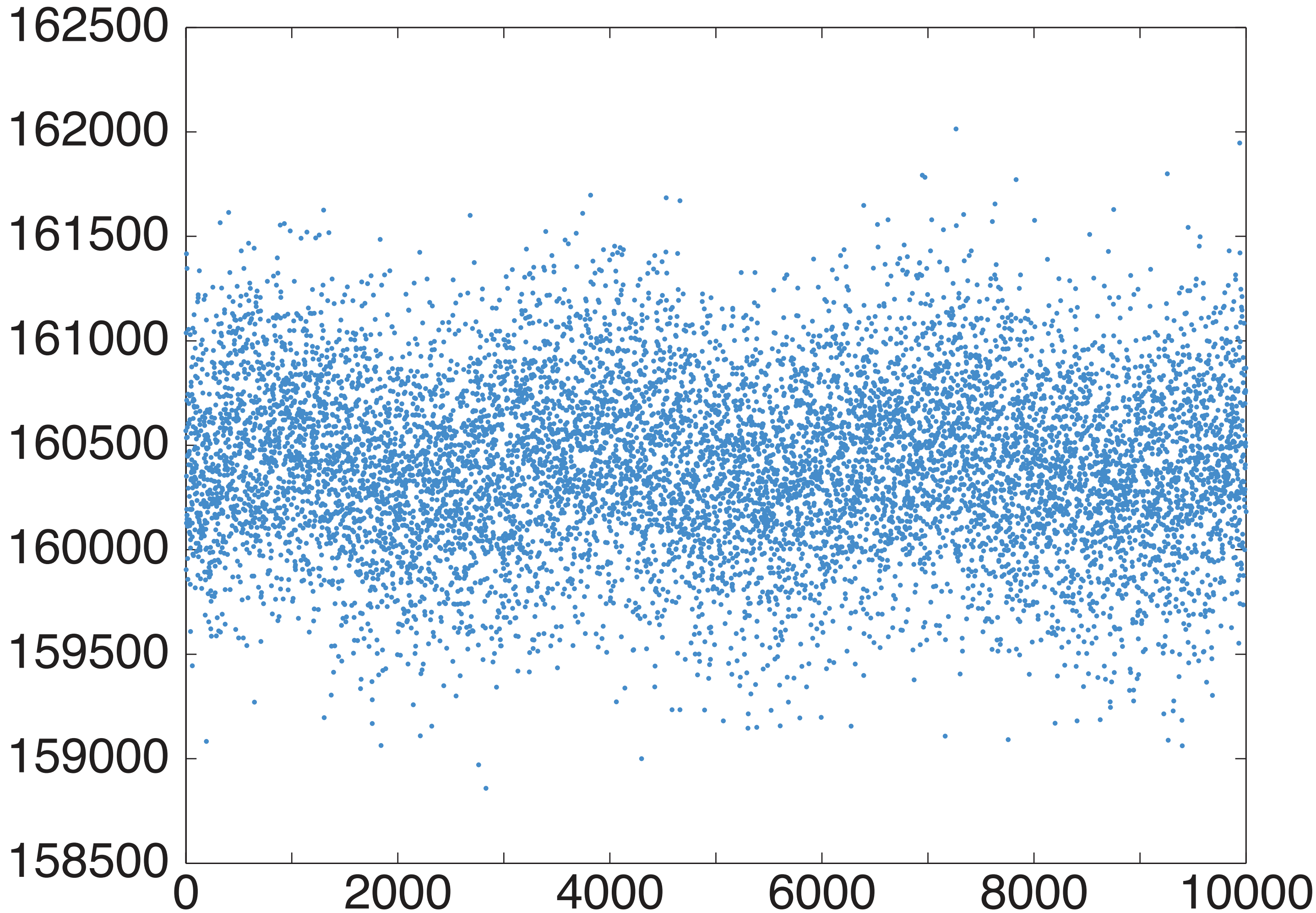
A shot-noise-limited signal with slightly higher background

The Fluorescence Advantage : Signal on a Dark Background



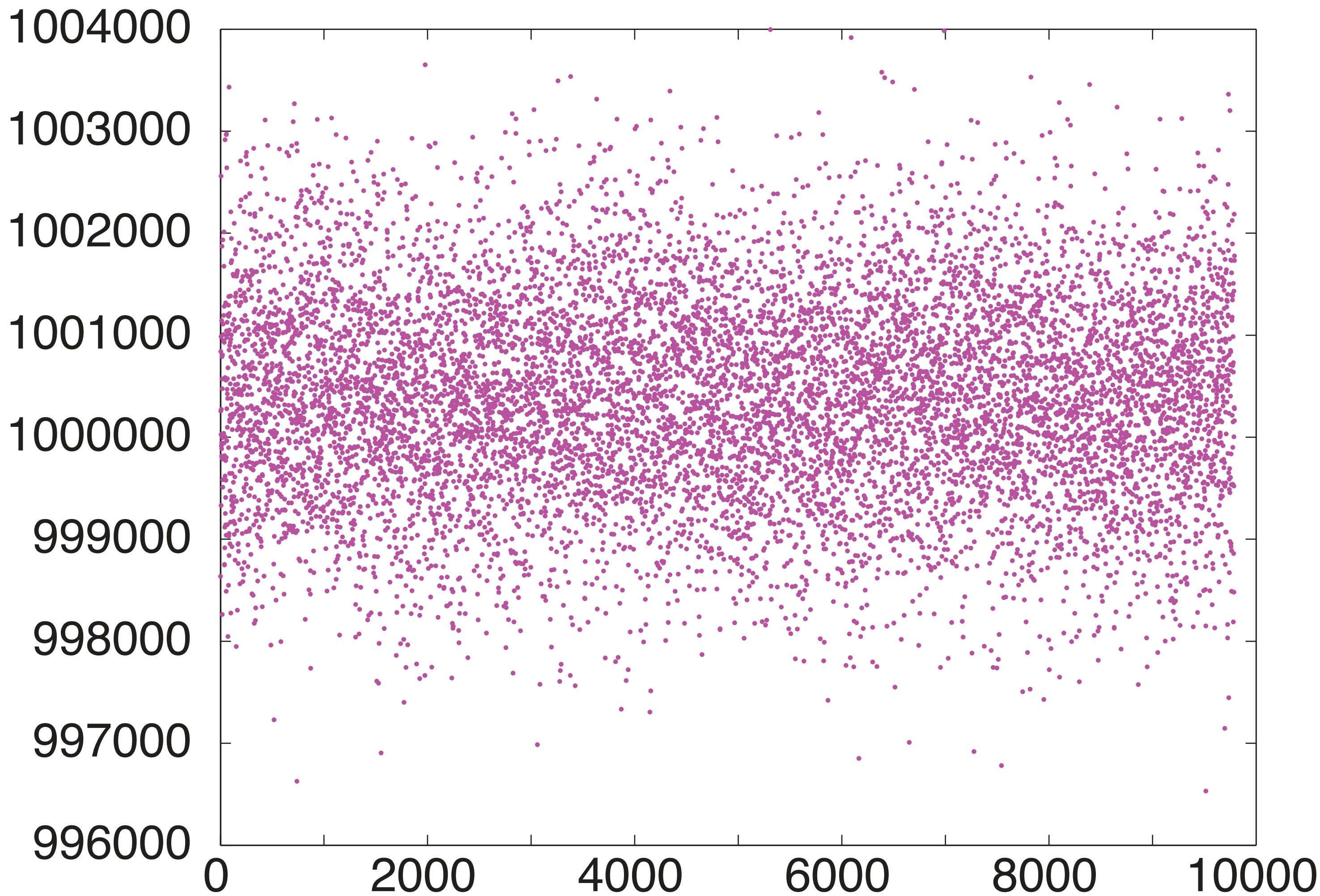
A shot-noise-limited signal with high background

The Fluorescence Advantage : Signal on a Dark Background



A shot-noise-limited signal with very high background

The Fluorescence Advantage : Signal on a Dark Background



A shot-noise-limited signal with extremely high background

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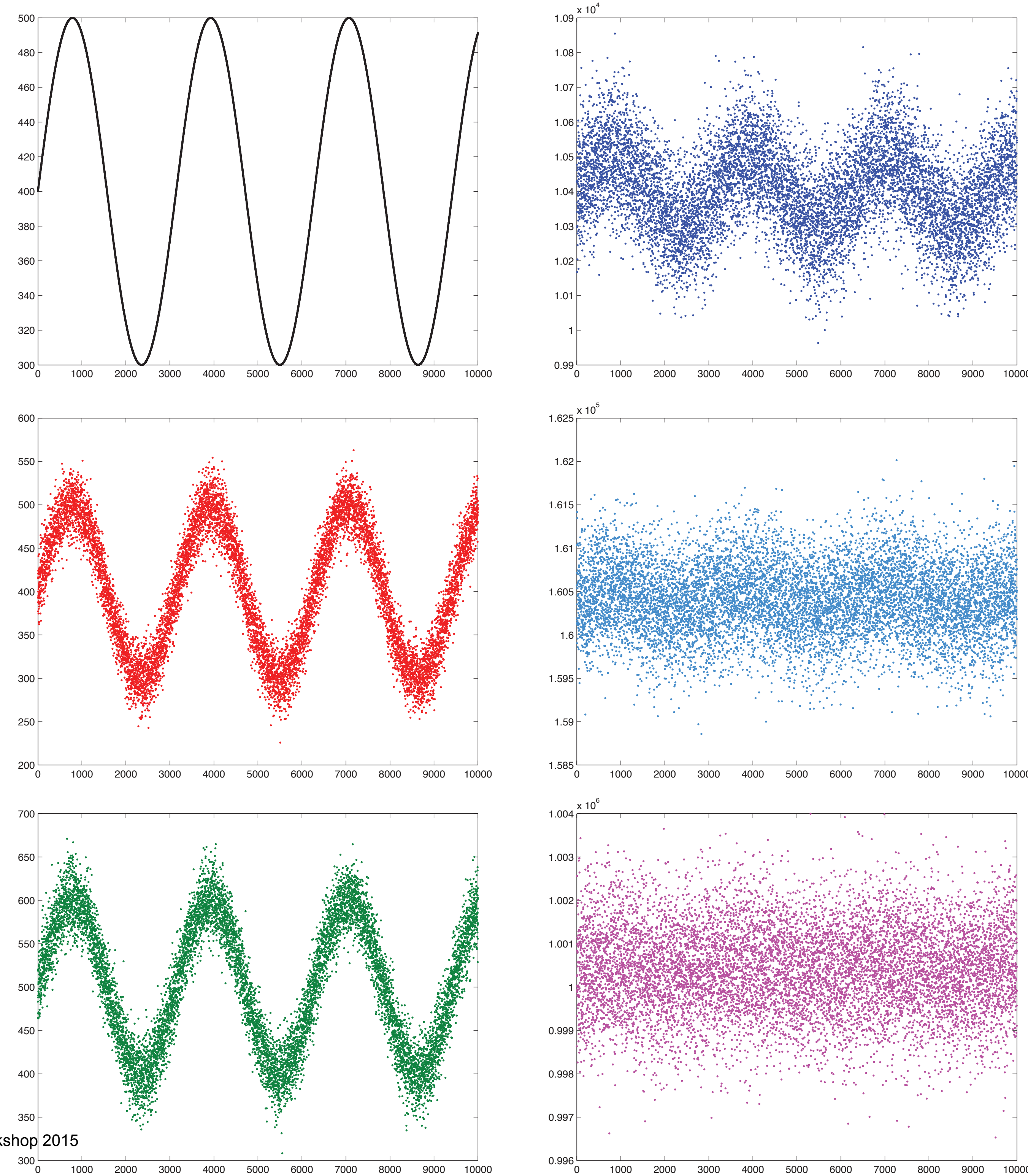

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Modern Microscope Components

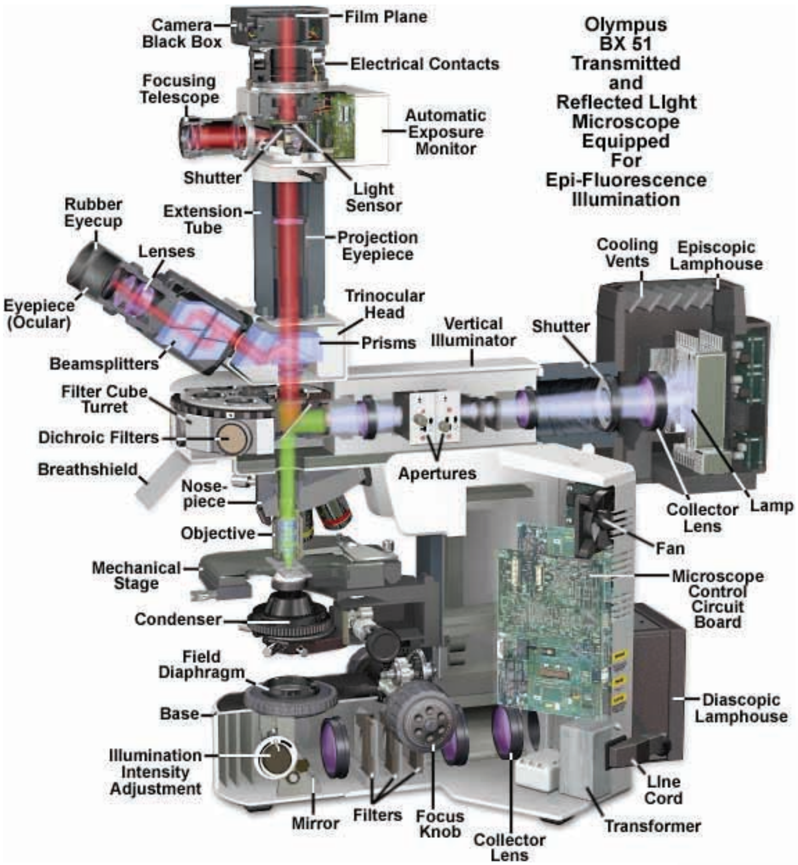

Image from Molecular Expressions webpage

+

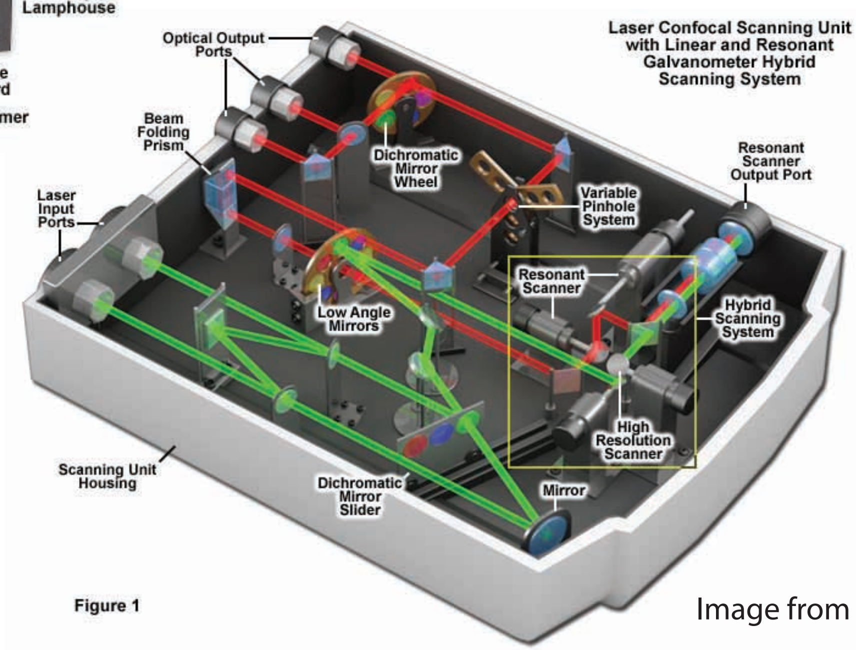

Figure 1

Image from MicroscopyU webpage

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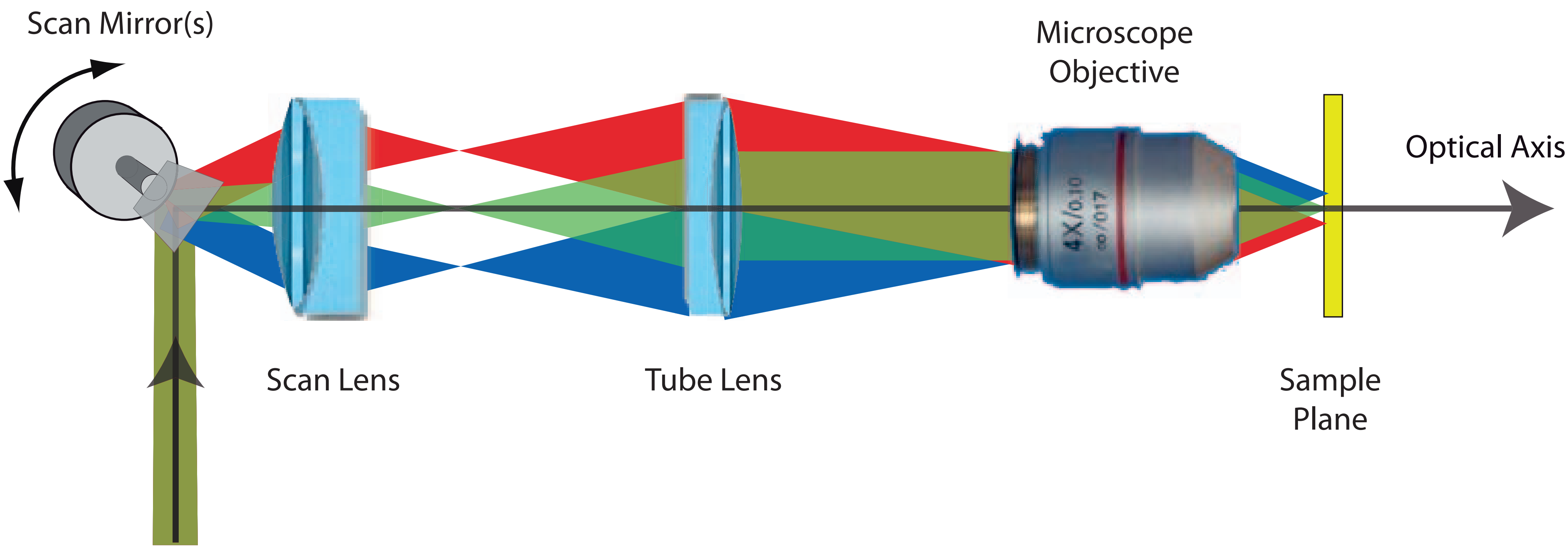
Laser Scanning Microscopy



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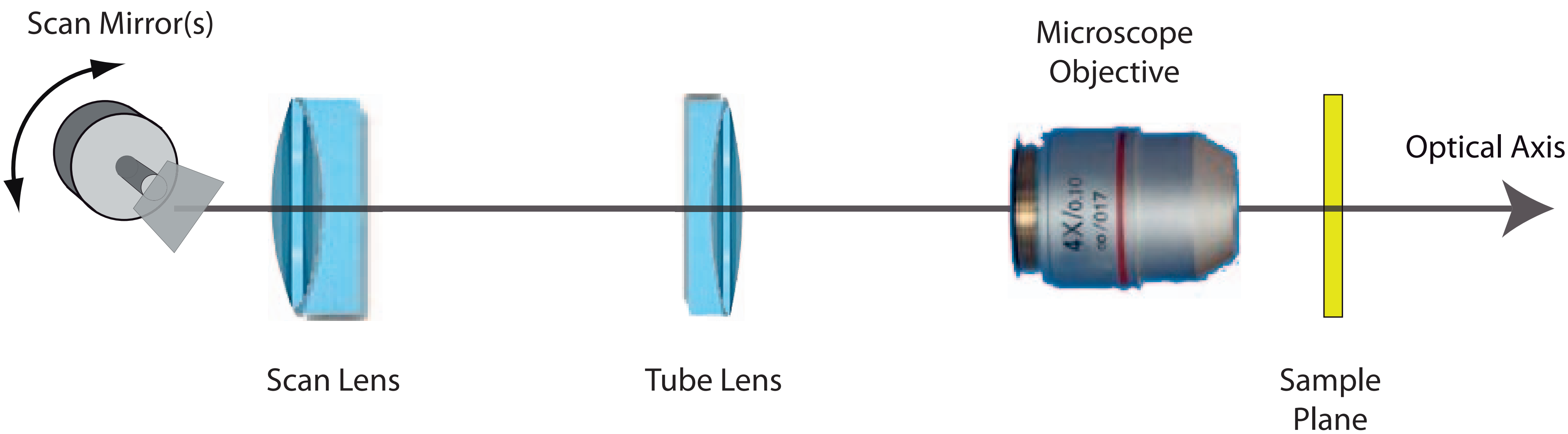

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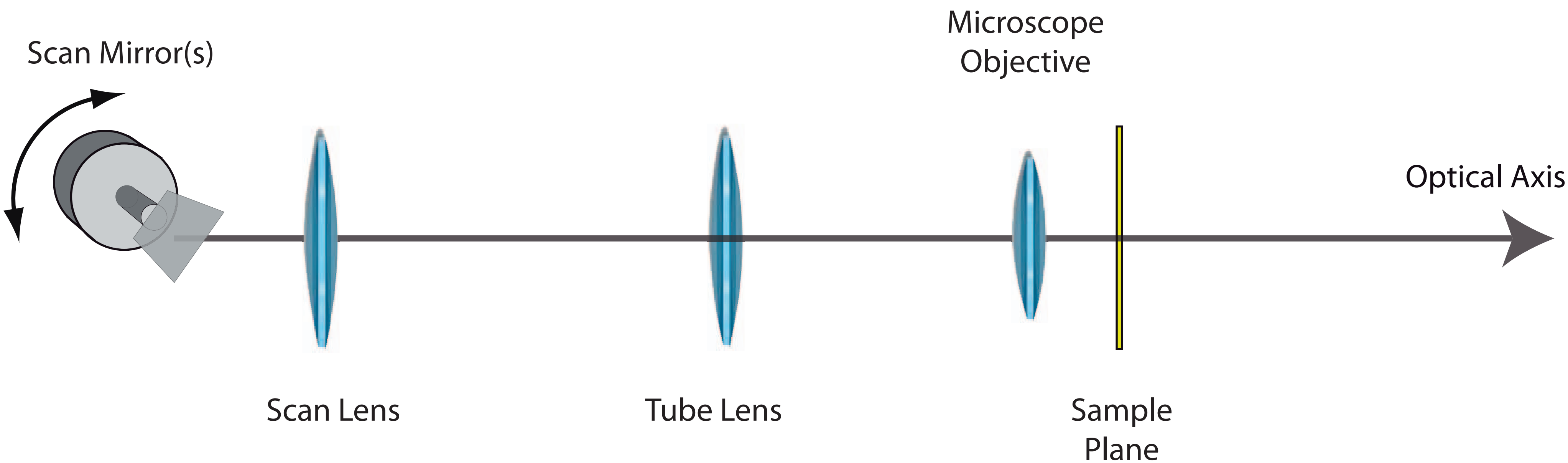

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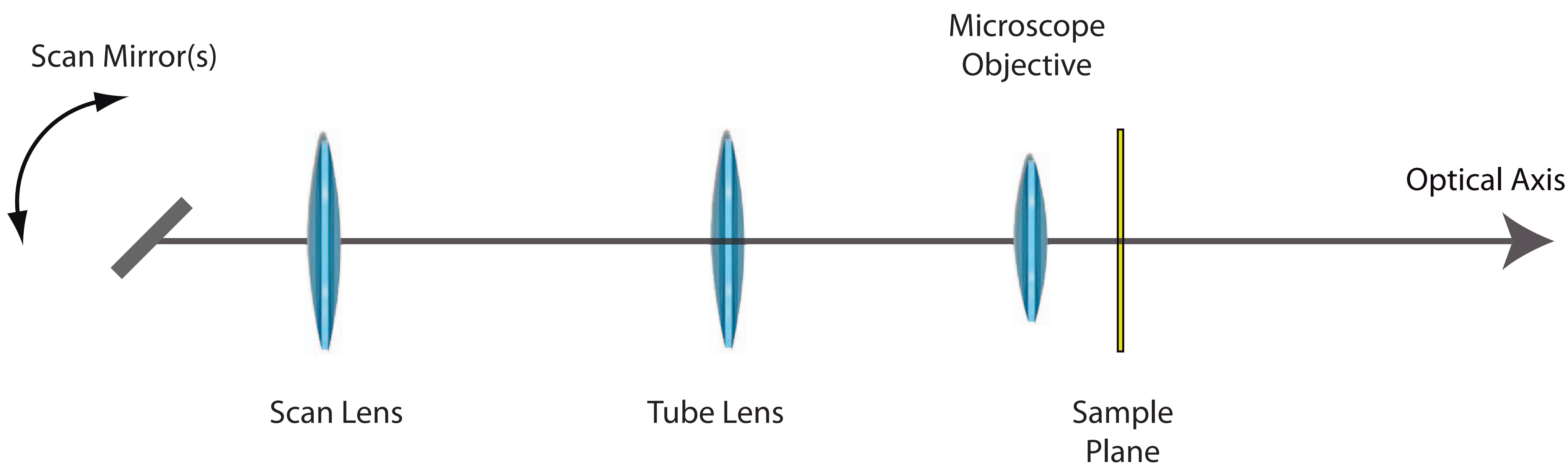

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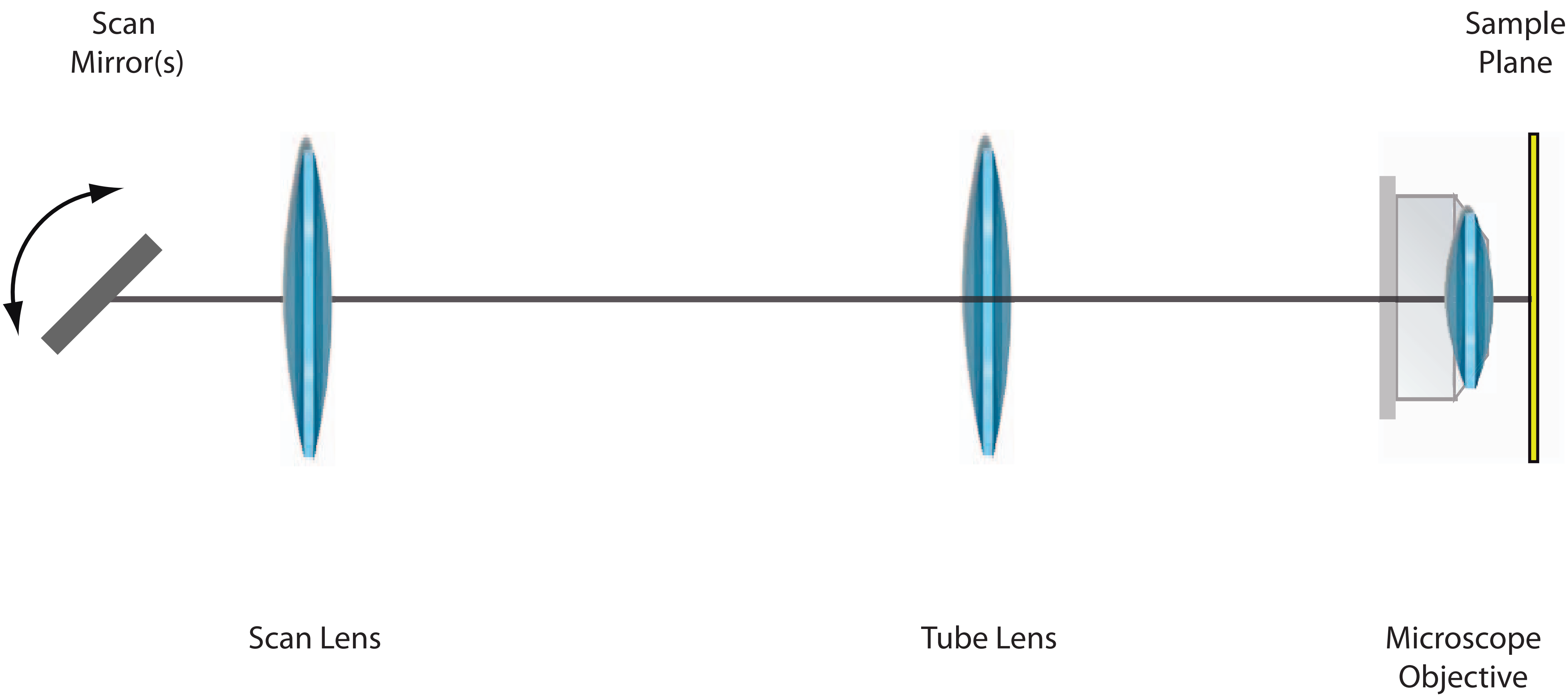

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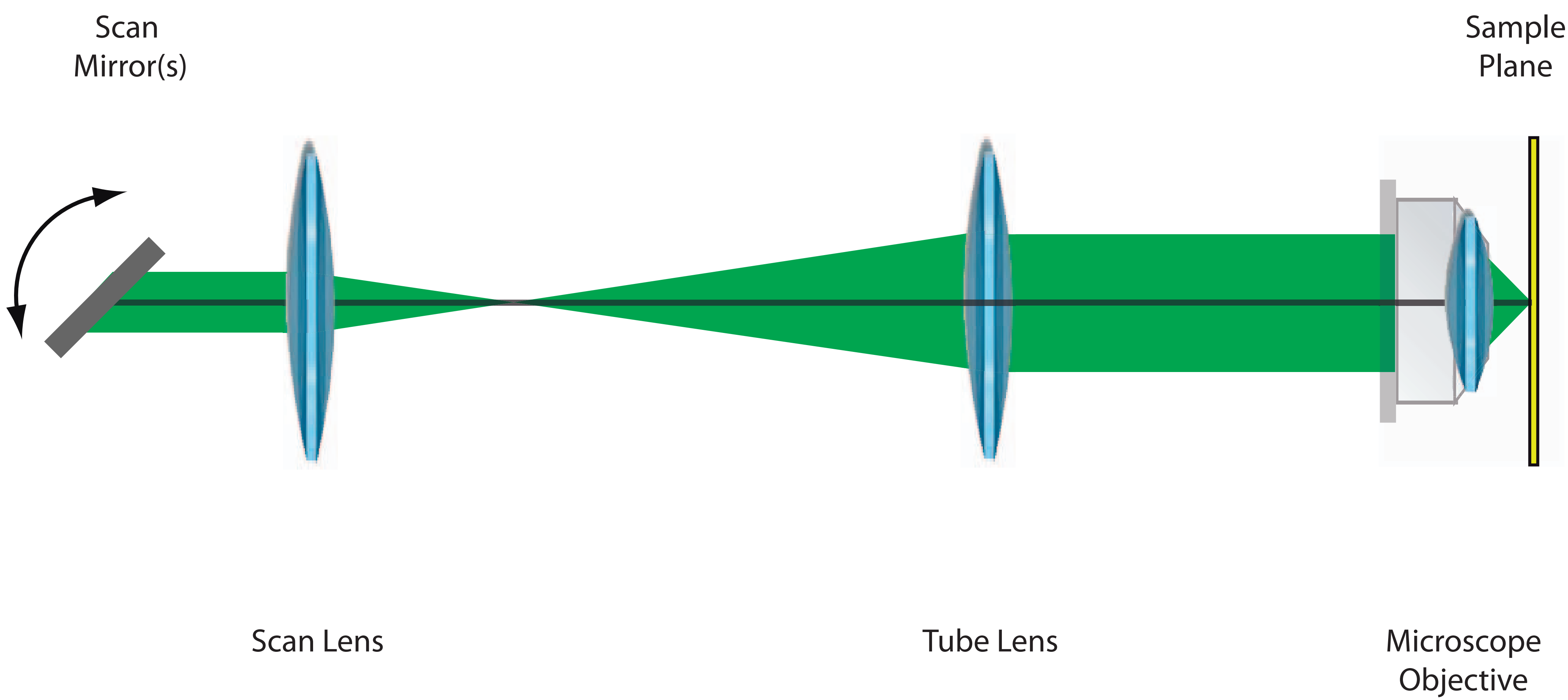

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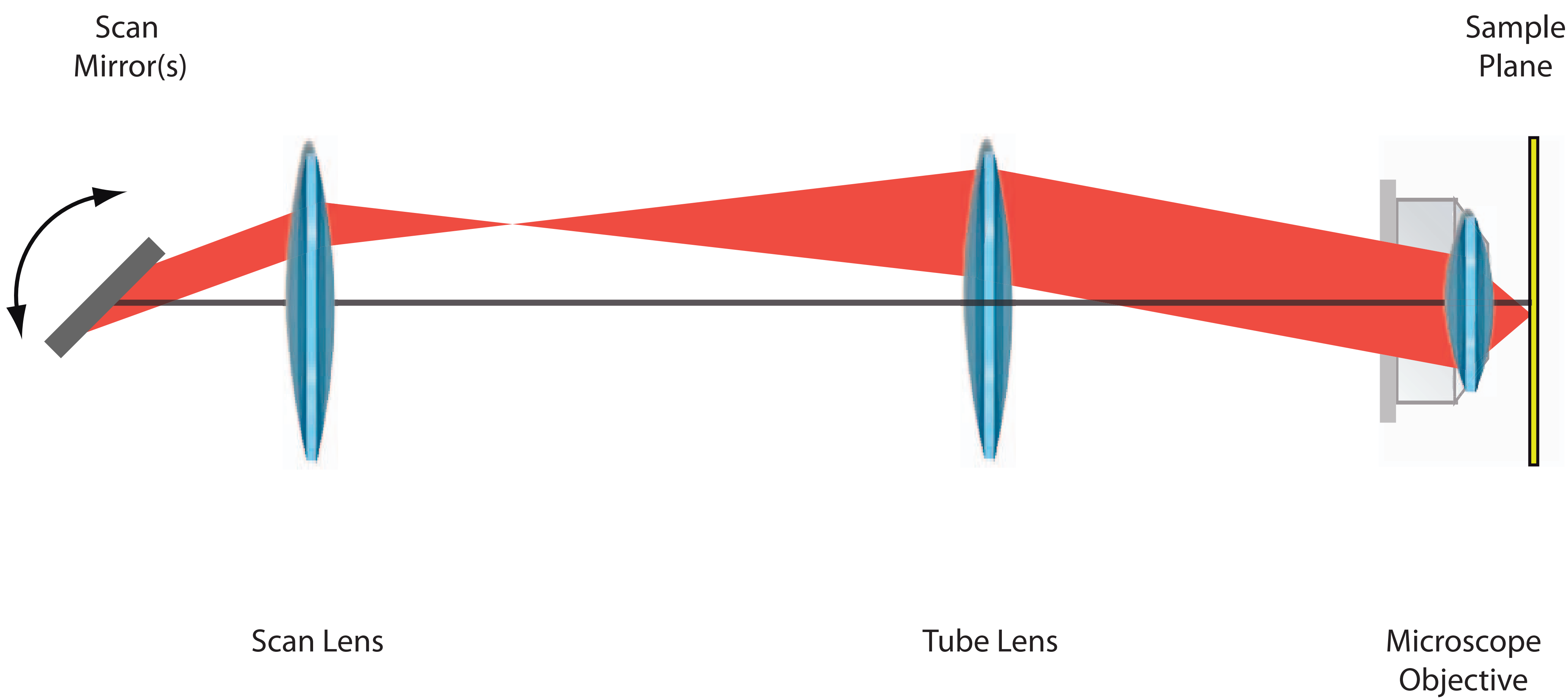
Laser Scanning Microscopy



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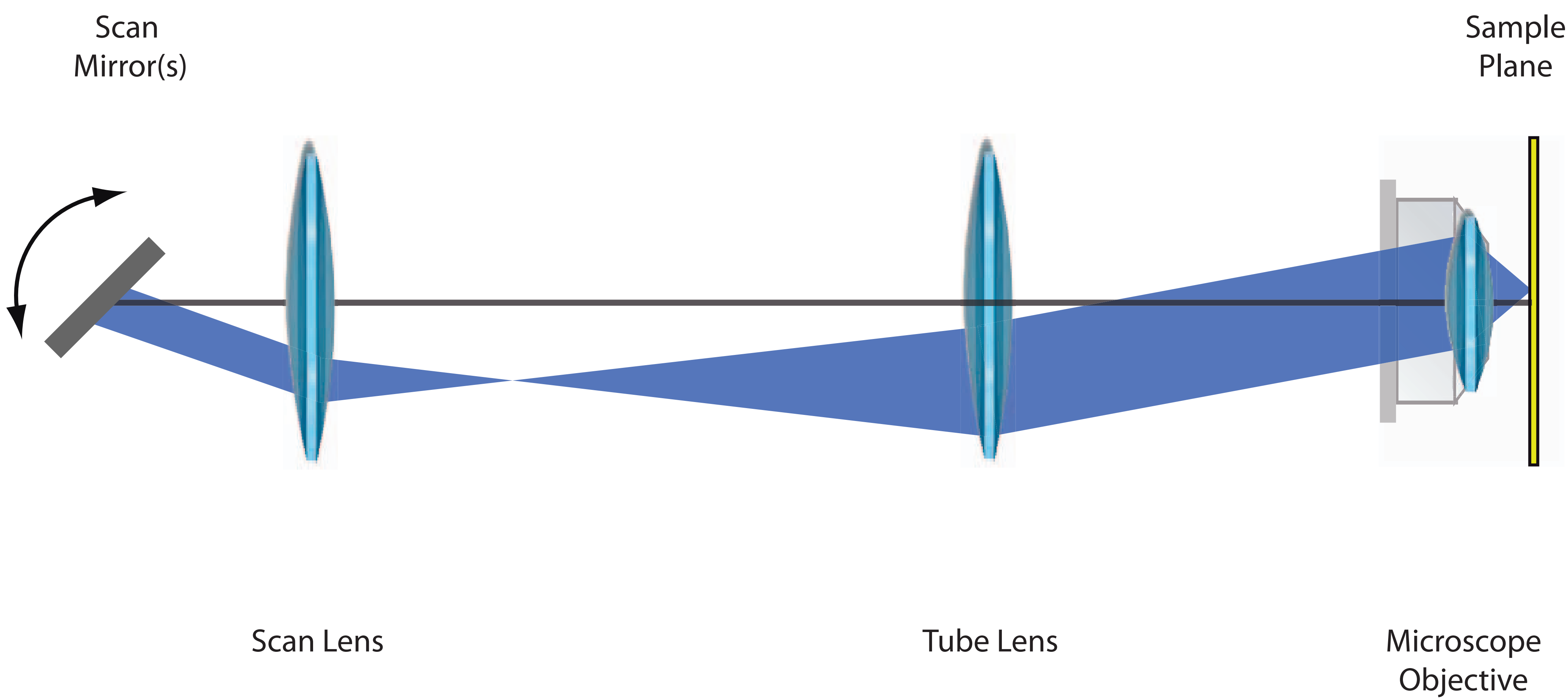
Laser Scanning Microscopy



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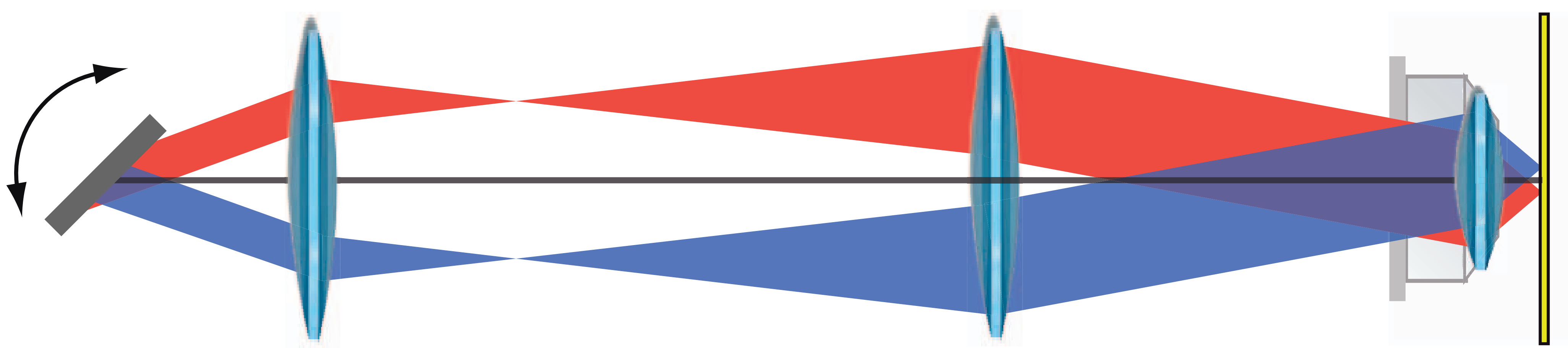
Laser Scanning Microscopy



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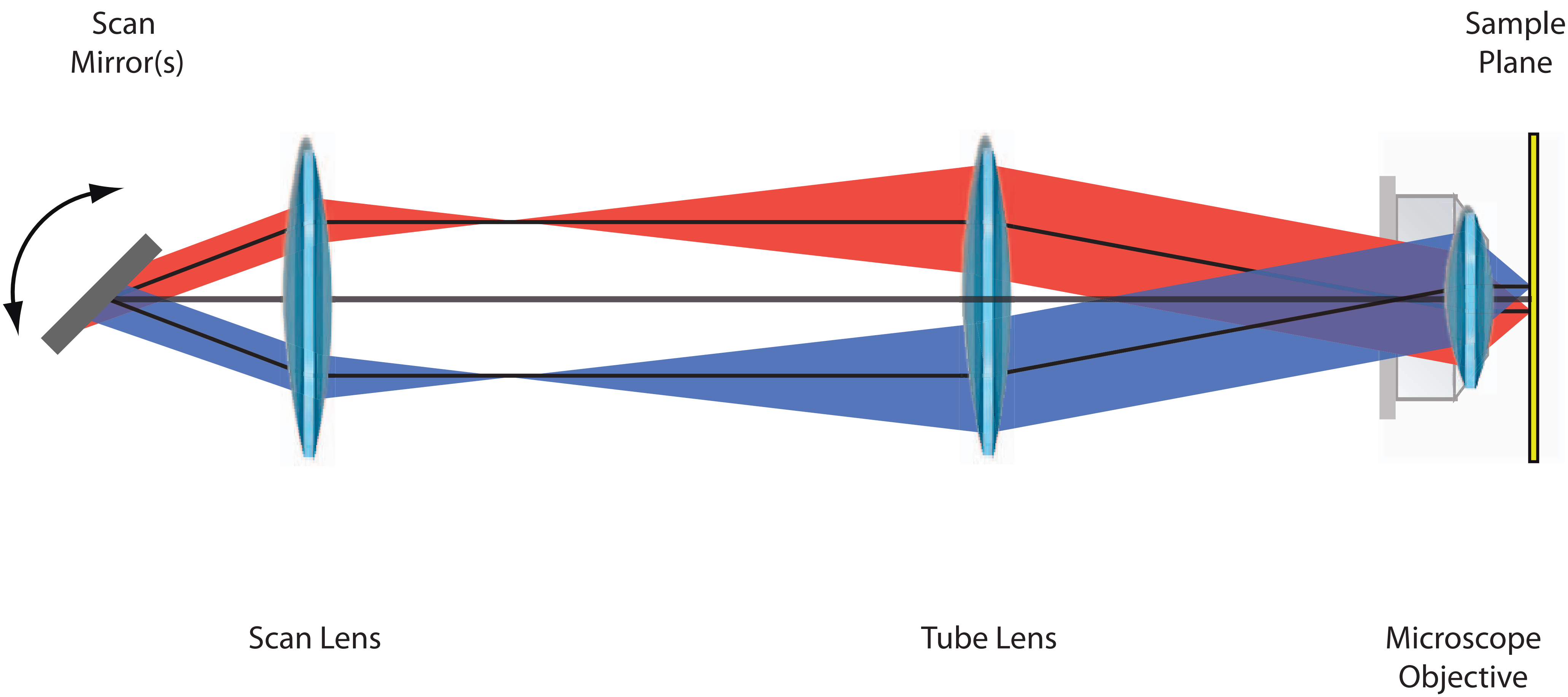
Laser Scanning Microscopy



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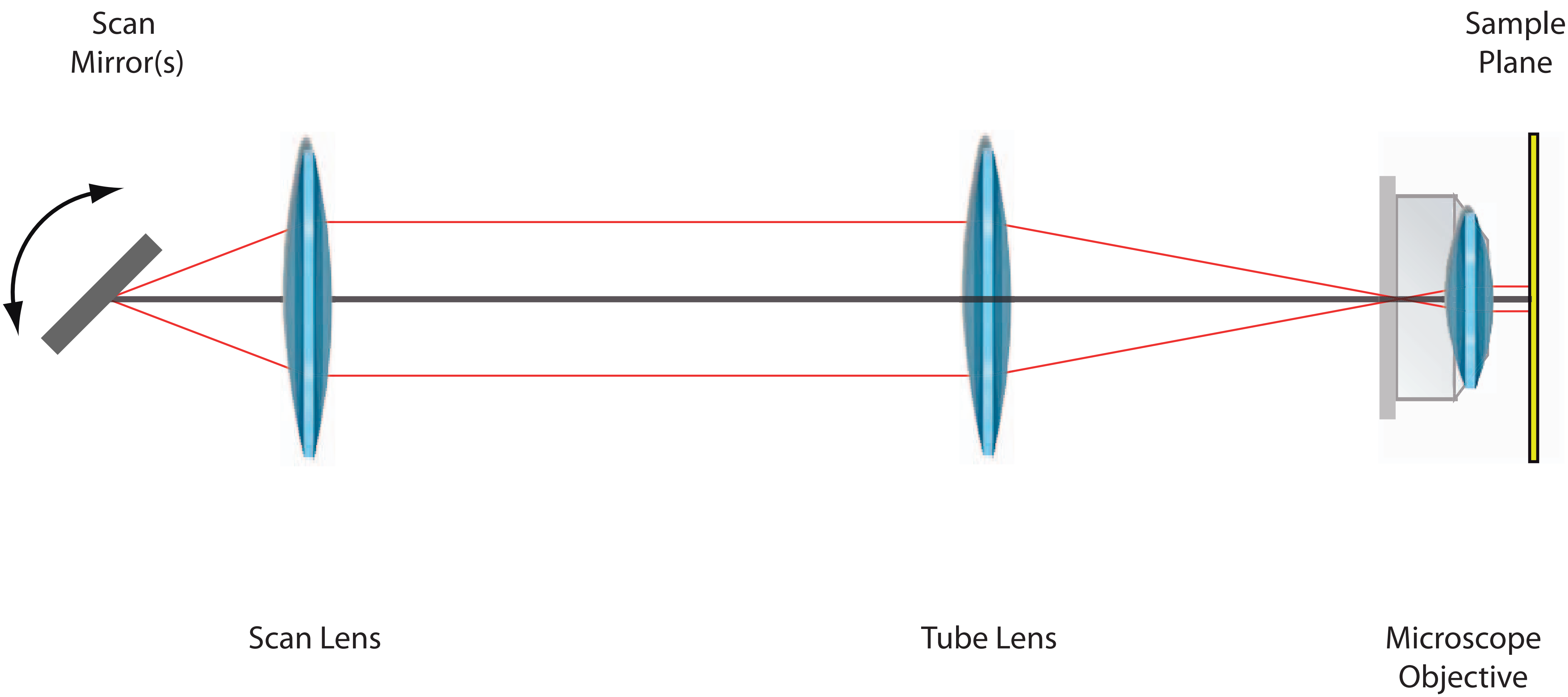
Laser Scanning Microscopy



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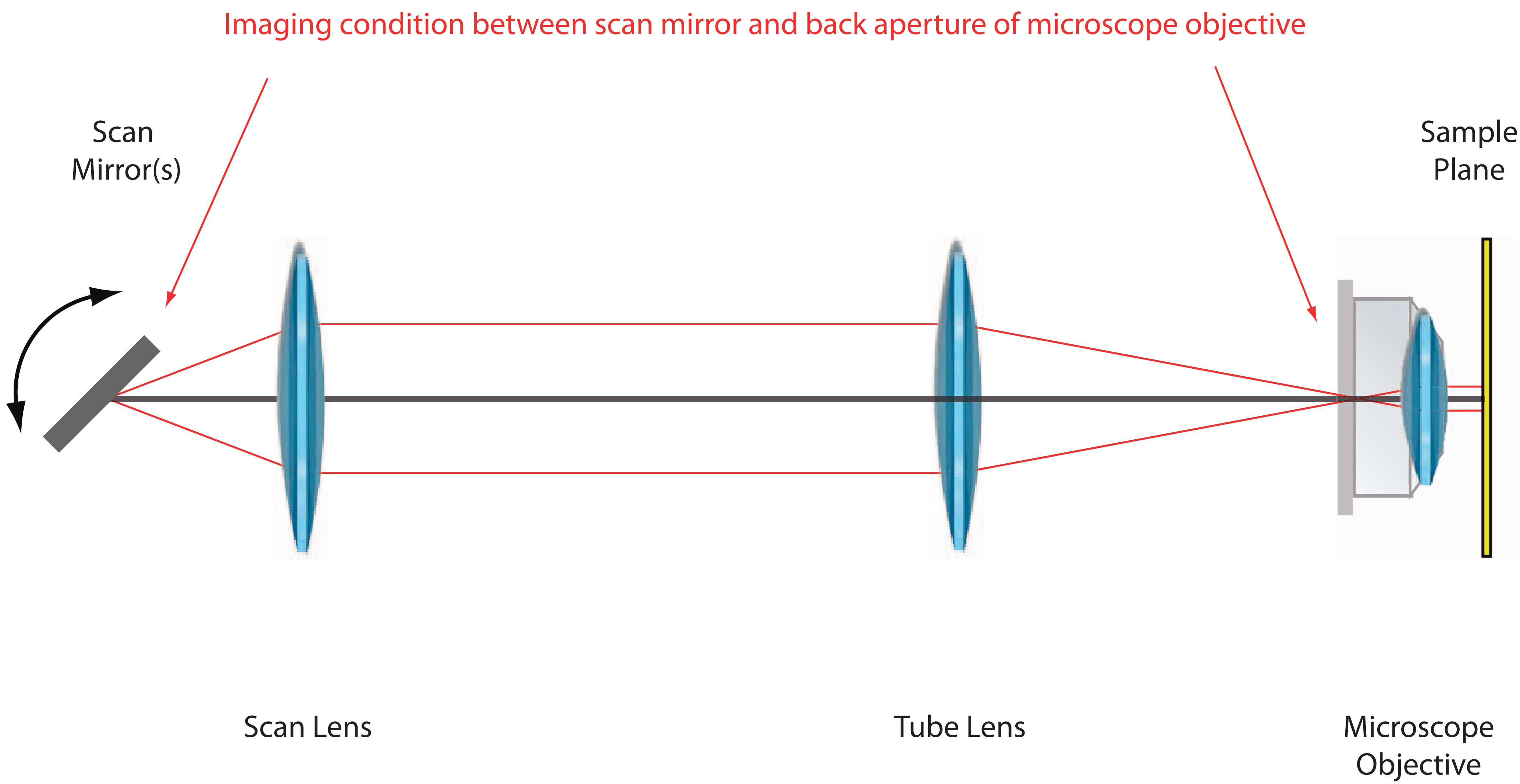
Laser Scanning Microscopy



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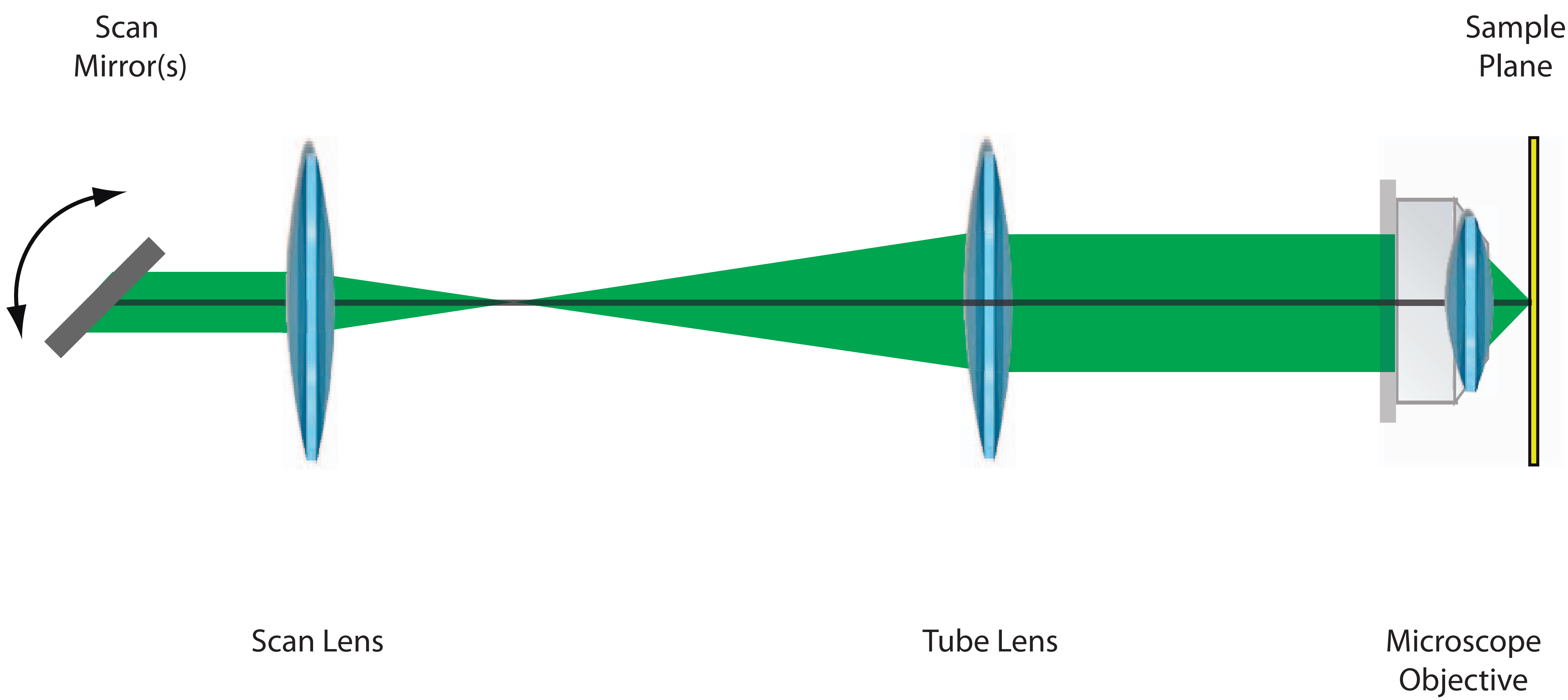
Laser Scanning Microscopy



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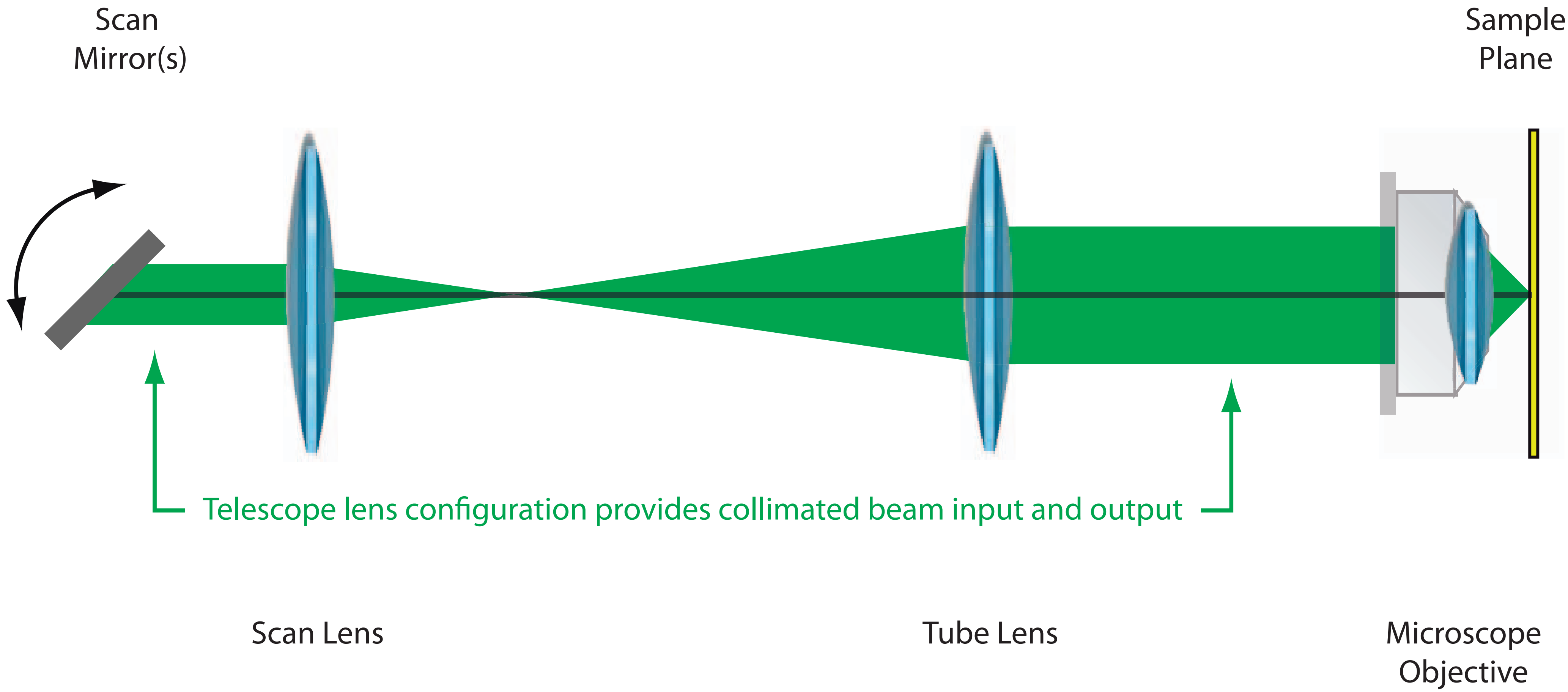
Laser Scanning Microscopy



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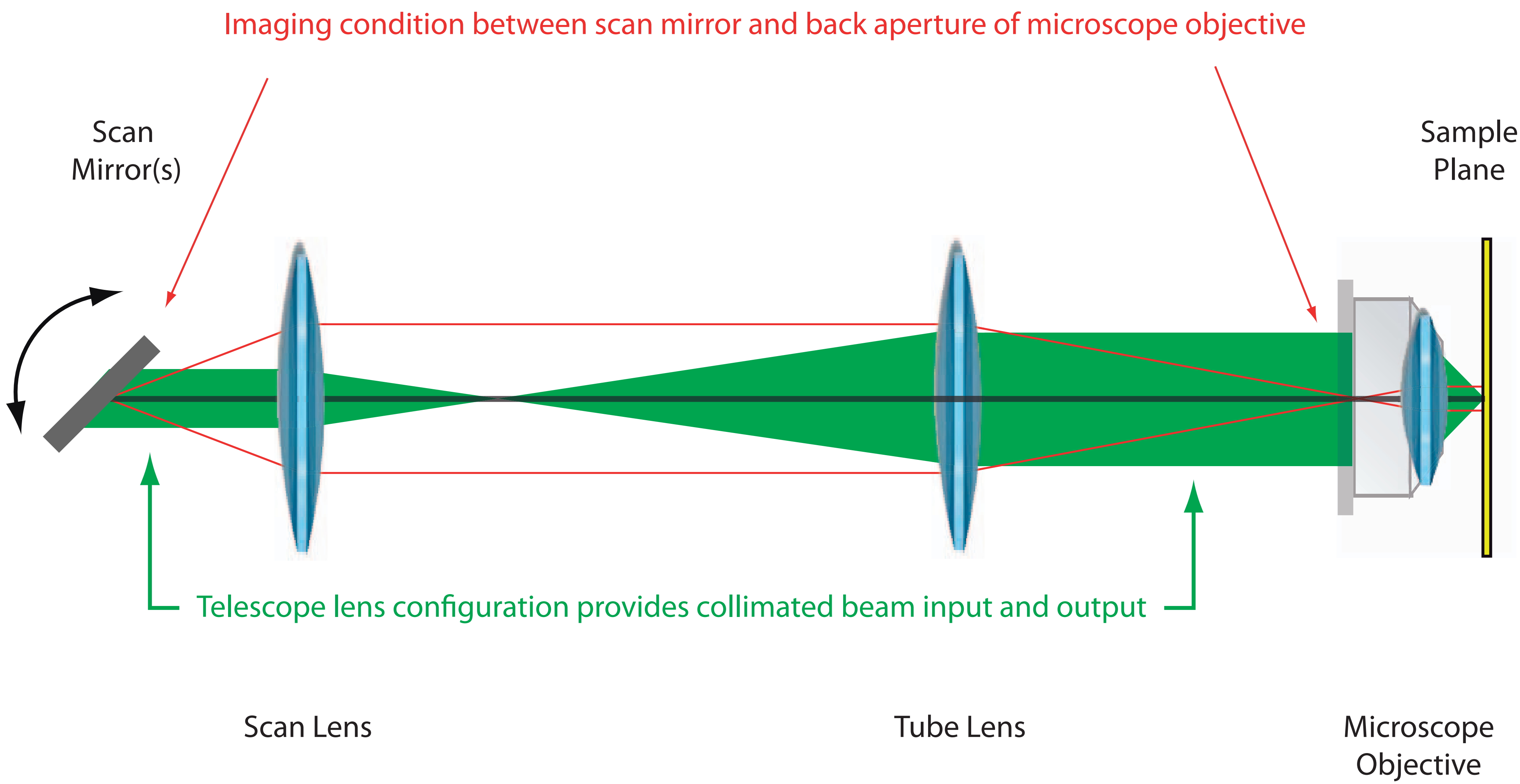
Laser Scanning Microscopy



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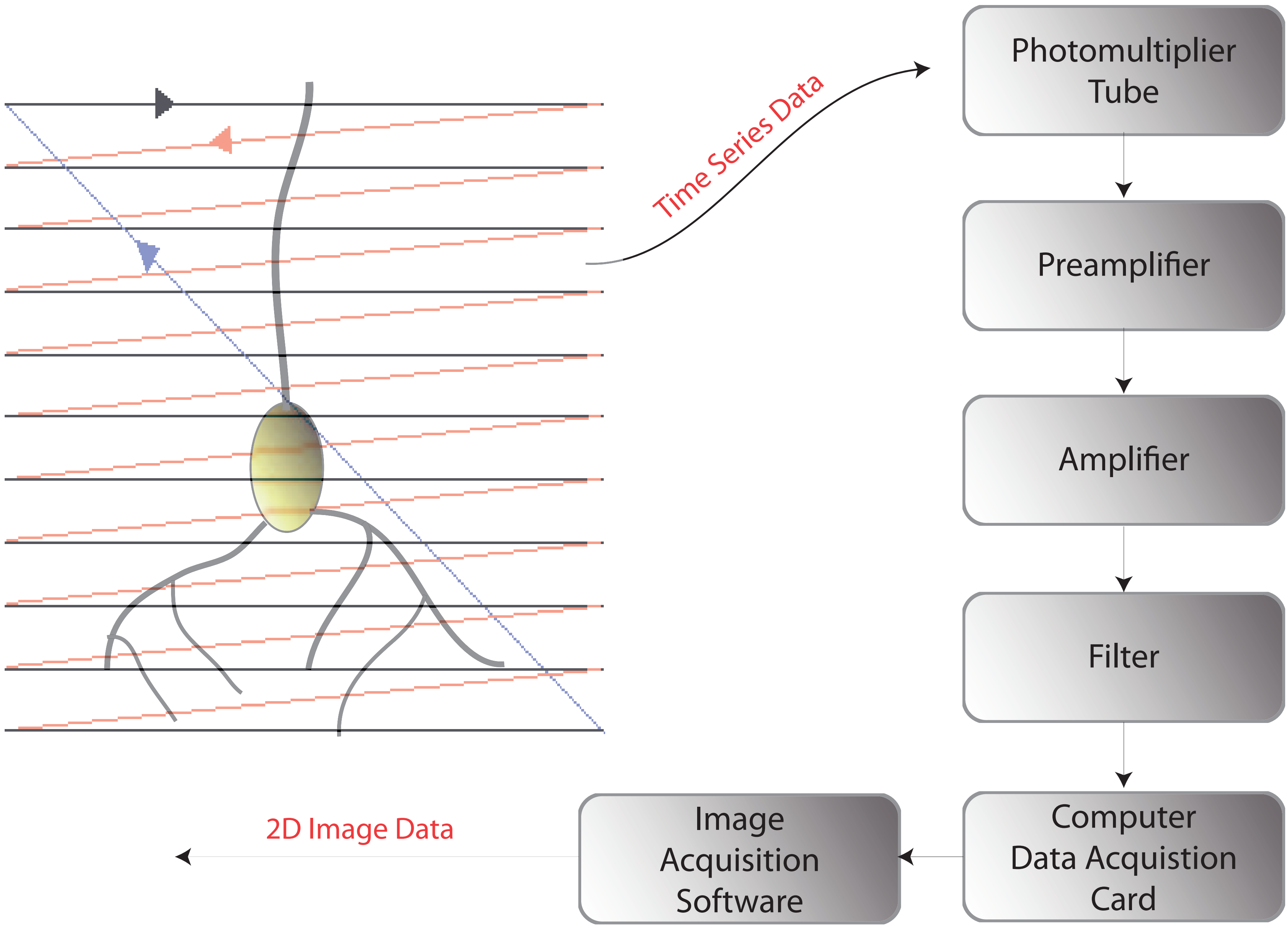
Data Acquisition

Raster Scan Imaging



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Some Relevant Parameters

Extinction Coefficient

ε = 5,000 - 200,000 M^{-1} cm^{-1} for most fluorophores

refers to the absorption at a single wavelength (typically the maximum)

Quantum Yield

Q = 0.05 - 1 for most fluorophores

refers to the integrated photon emission over the entire emission spectrum

Below saturation, total fluorescence intensity $\sim Q * \varepsilon$

Lifetime

τ = 1 - 10 ns for most fluorophores

A quick & dirty calculation of saturation

Absorption cross-section, σ

$\varepsilon = 80{,}000\ M^{-1}\ cm^{-1}$

$\sigma = \varepsilon \cdot \ln(10) / 6.023 \cdot 10^{23}$

$\sigma(\text{fluorescein}) = 3 \cdot 10^{-16}\ cm2 \cdot \text{molecule}^{-1} \cdot \text{photon}^{-1}$

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Assume 1mW of green light (around 488 nm) at the sample:

$1\ mW = 1 \cdot 10^{-3}\ J/s$

1 photon (488nm) <-> 2.5 eV $= 4 \cdot 10^{-19}\ J$

1 mW (488nm) $= 2.5 \cdot 10^{15}$ photons/s

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Epifluorescence : Illuminate 500 x 500 um area ~ $2 \cdot 10^{-3}$ cm^{-2}

Intensity = P ower / Area ~ $1.25 \cdot 10^{18} \cdot$ photons $\cdot$ cm^{-2} $\cdot$ s^{-1}

A quick & dirty calculation of saturation

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Intensity = P ower / Area ~ $1.25 \cdot 10^{18} \cdot$ photons $\cdot$ cm$^{-2} \cdot$ s^{-1}

Point Scanning : Illuminate 0.5 x 0.5 um area ~ $2 \cdot 10^{-9}$ cm^{-2}

Intensity = P ower / Area ~ $1.25 \cdot 10^{24} \cdot$ photons $\cdot$ cm$^{-2} \cdot$ s^{-1}

A quick & dirty calculation of saturation

Absorption and Fluorescence Rates

$k_a = \sigma \cdot I$

$k_f = 1/\tau$

Let x = fraction of fluorophores in the excited state

$k_a \cdot (1-x) = k_f \cdot x$ <-- in steady state

$\mathbf{x = k_a / (k_a + k_f)}$

A quick & dirty calculation of saturation

Epifluorescence :

$I = \text{Power} / \text{Area} \sim 1.25 \cdot 10^{18} \cdot \text{photons} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$

$\sigma(\text{fluorescein}) = 3 \cdot 10^{-16}\ \text{cm}^2 \cdot \text{molecule}^{-1} \cdot \text{photon}^{-1}$

$k_f = 1/\tau = 1 / 4.5\ \text{ns} = 2.2 \cdot 10^8\ \text{s}^{-1}$

$k_a = \sigma \cdot I = 375\ \text{s}^{-1}$

$x = k_a / (k_a + k_f) = 1.7 \cdot 10^{-6}$

Only ~ 2 out of every billion fluorophores is in the excited state

A quick & dirty calculation of saturation

Point Scanning :

$I = Power / Area \sim 1.25 \cdot 10^{24} \cdot$ photons $\cdot$ $cm^{-2} \cdot s^{-1}$

σ(fluorescein) $= 3 \cdot 10^{-16}$ $cm^2 \cdot$ molecule$^{-1} \cdot$ photon^{-1}

$k_f = 1/\tau = 1 / 4.5$ ns $= 2.2 \cdot 10^8\ s^{-1}$

$k_a = \sigma \cdot I = 3.75 \cdot 10^8\ s^{-1}$

$x = k_a / (k_a+k_f) = 0.63$

63% of the fluorophores is in the excited state!

Why is saturation bad?

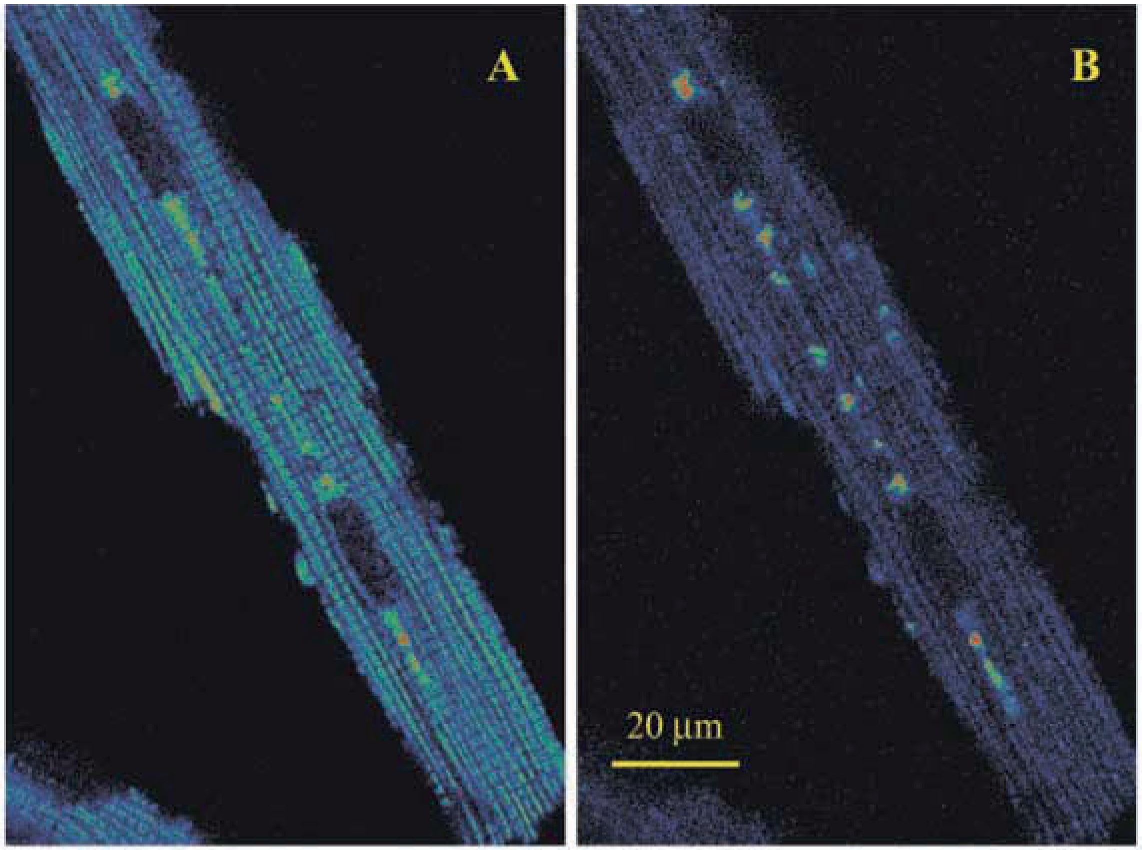
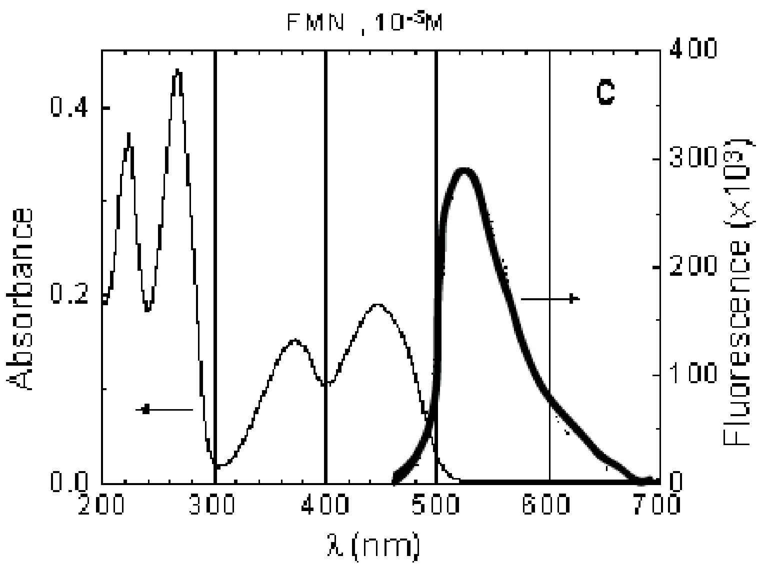
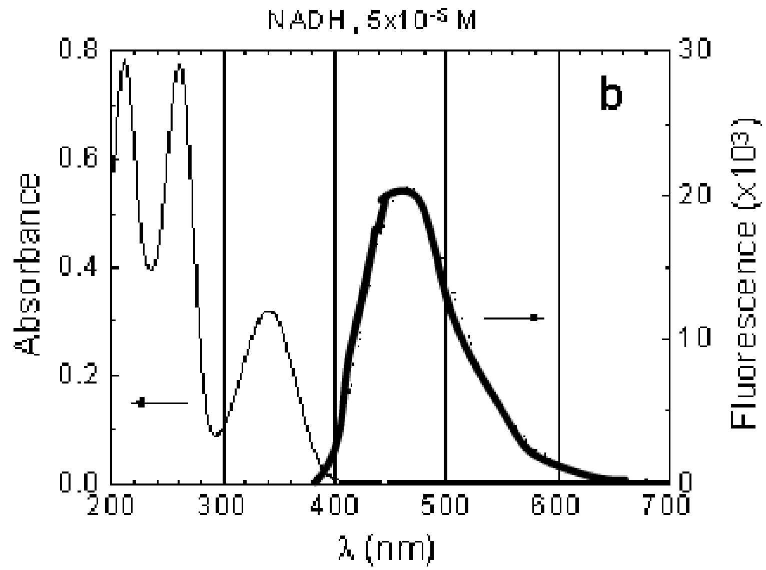


FIGURE 3 At 750 nm excitation, the autofluorescence emission of isolated cardiomyocyte is divided between PMT1 (~410–490 nm) and PMT2 (~510–650 nm), which are set up for maximal detection of the NAD(P)H and FP fluorescence, respectively. The 2P-autofluorescence image obtained at PMT1 (*A*) is much brighter than that at PMT2 (*B*), indicating a predominant NAD(P)H signal. Images shown are the average of five consecutive scans.

Taken from Huang, et. al., Biophysical Journal., 2002





web source : http://www.photobiology.com/photobiology2000/VSitas/ps_abs_sp_resd.htm

Molar Extinction Coefficients

Alexa Fluor 555	: 150000 cm^{-1} M^{-1}
Fluorescein	: 70000 cm^{-1} M^{-1}
eGFP	: 55000 cm^{-1} M^{-1}
NADH	: 6220 cm^{-1} M^{-1}

Time averaging to recover S/N

Photobleaching

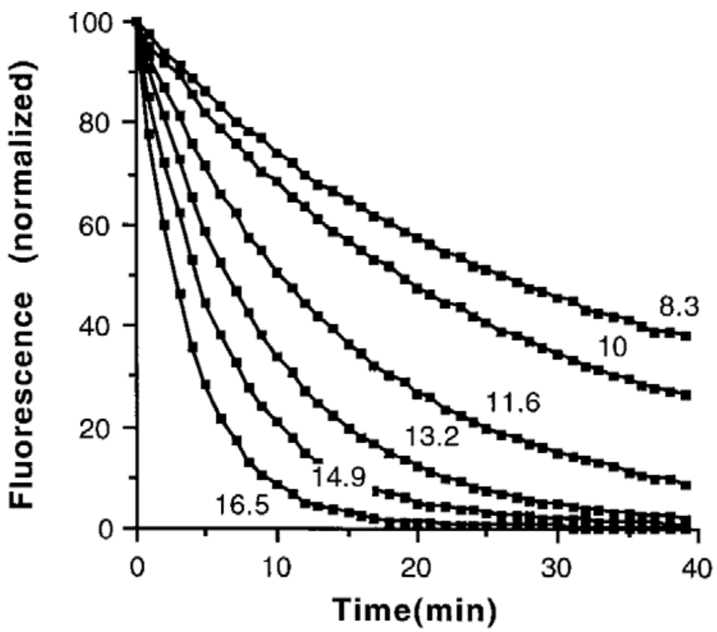
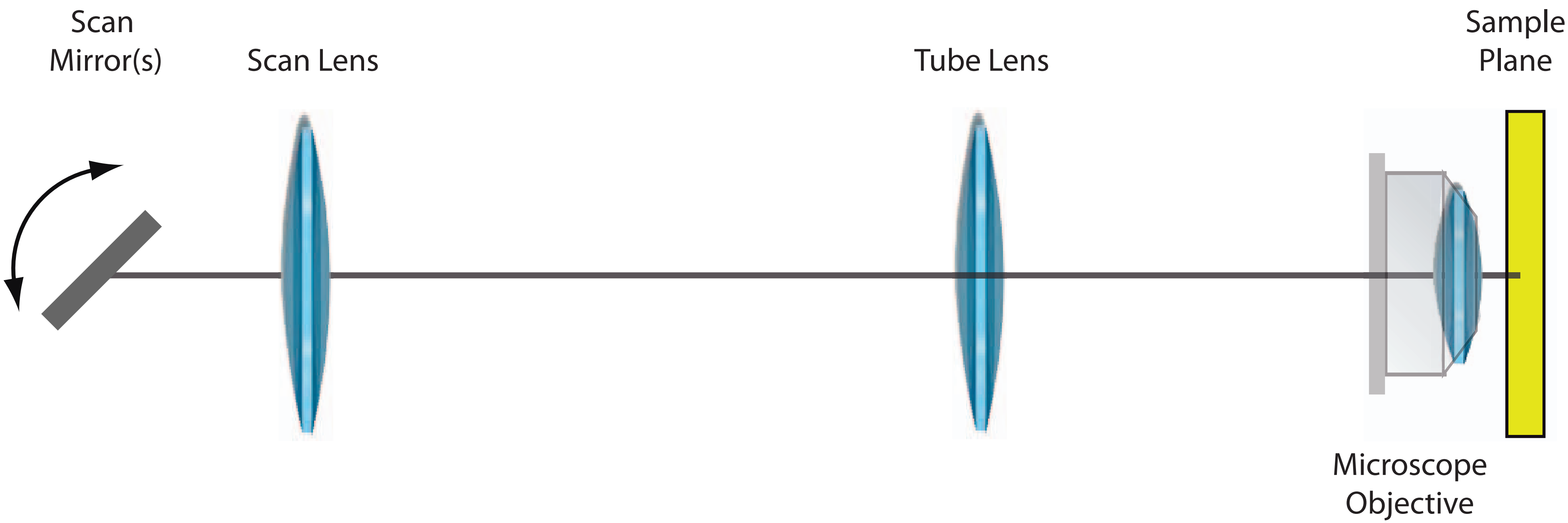


FIGURE 1 Fluorescein dextran (10 μM in pH 7.3) was suspended in ~1-μm-thick microdroplets and continuously irradiated with 710 nm mode-locked laser light at six different power levels (in mW units) while collecting images at 1-min time points.

Taken from Patterson & Piston, 2000, Biophysical Journal

Confocal Laser Scanning Microscopy



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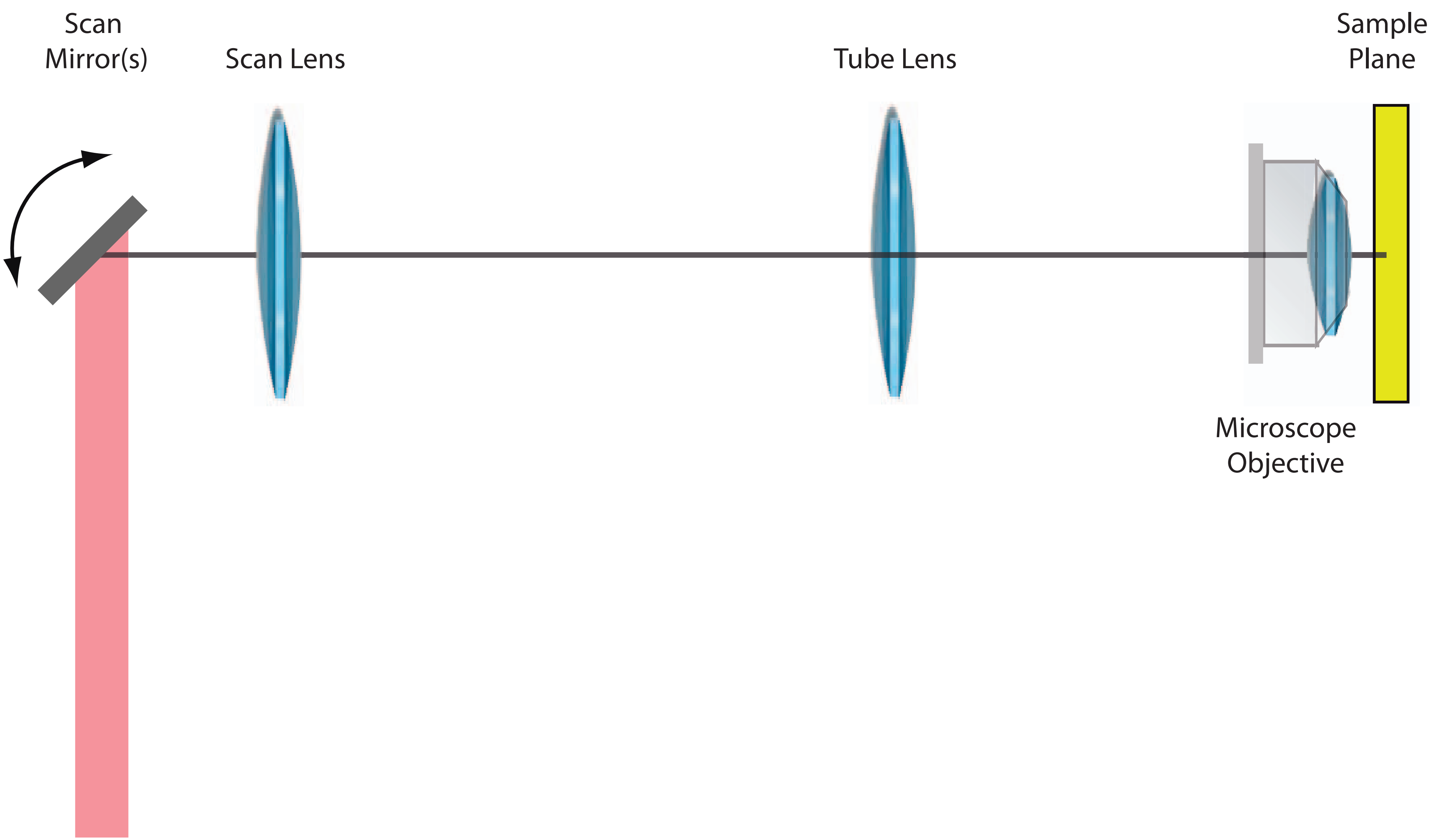
Confocal Laser Scanning Microscopy



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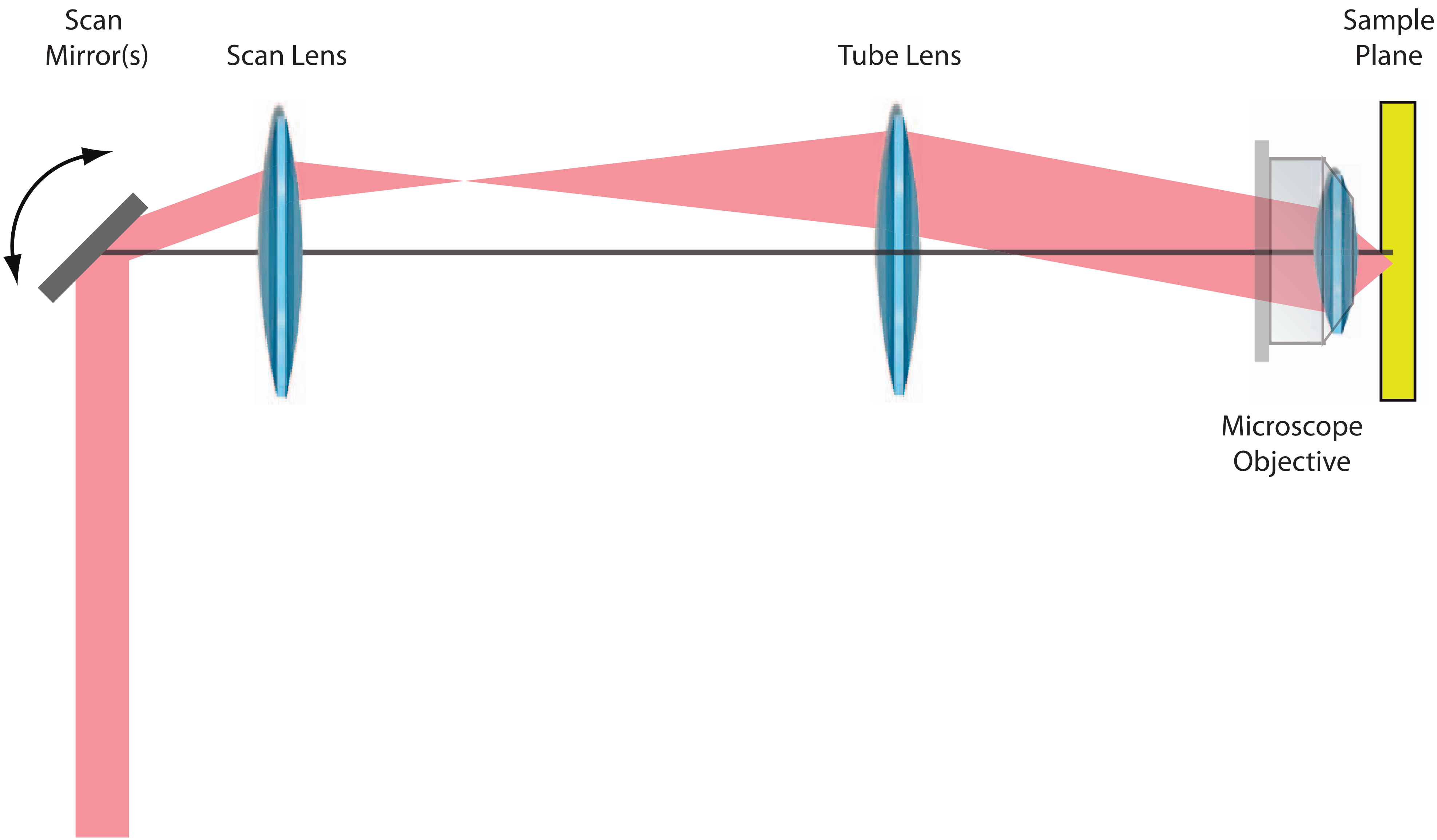

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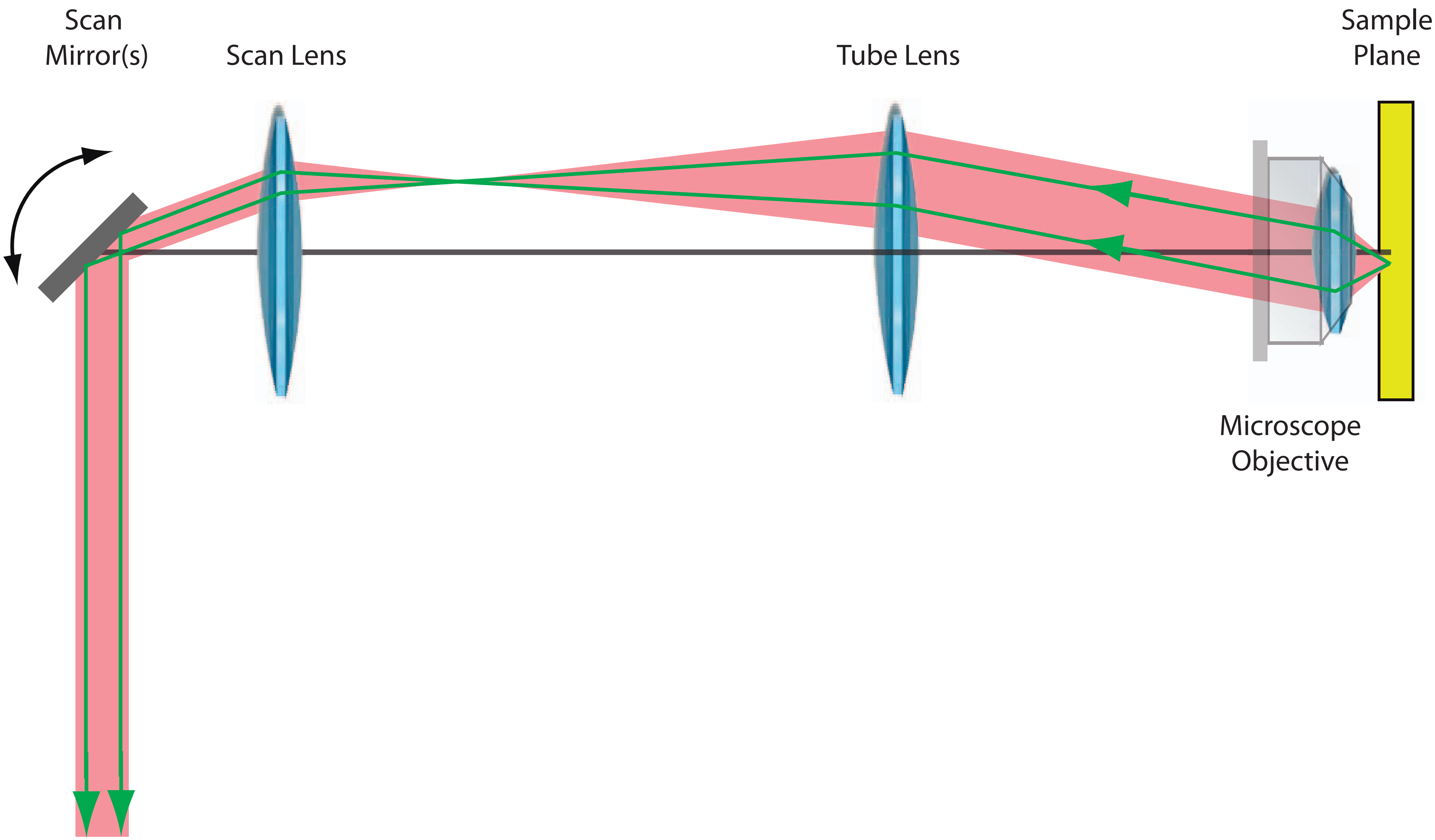
Confocal Laser Scanning Microscopy



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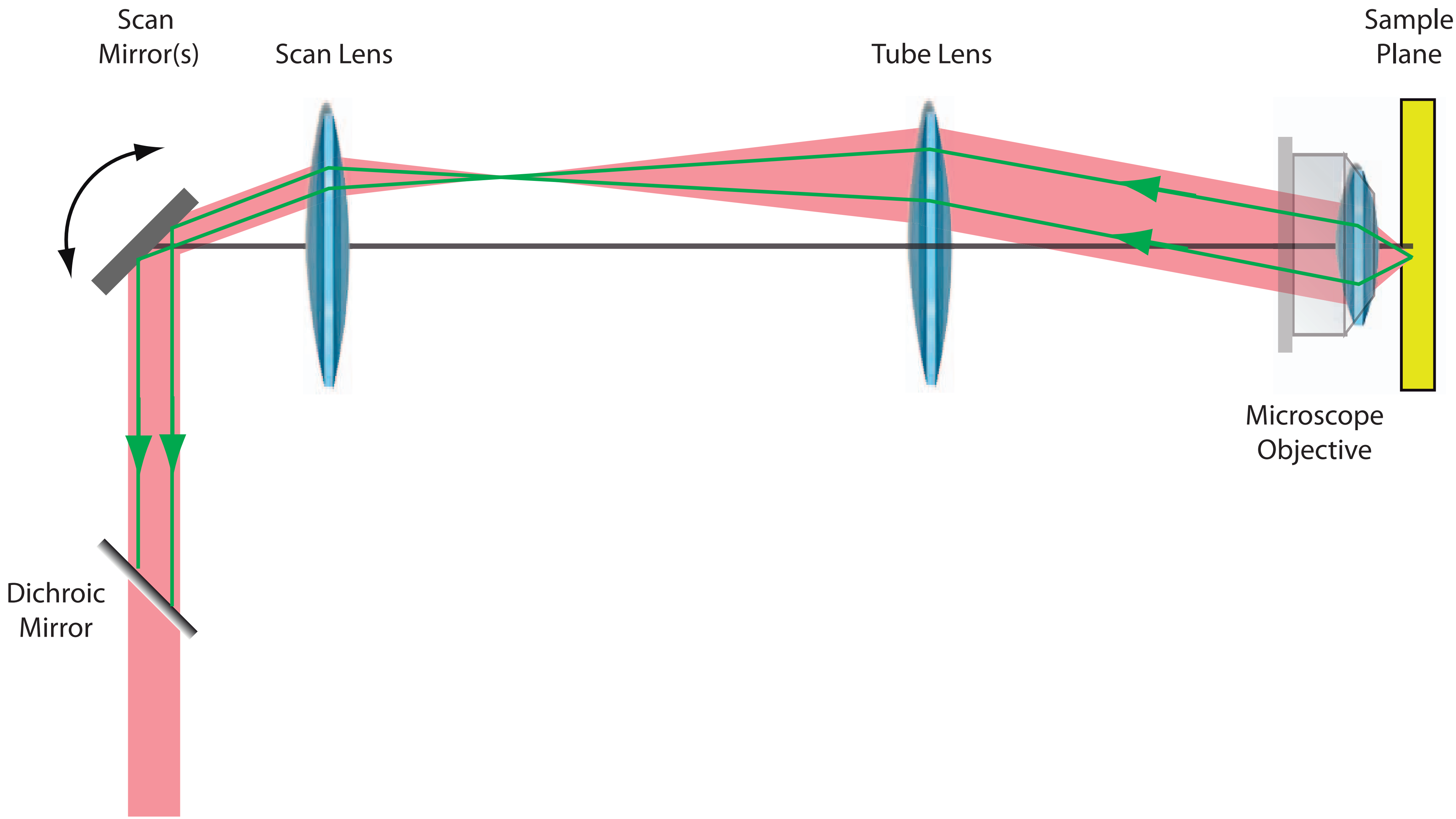

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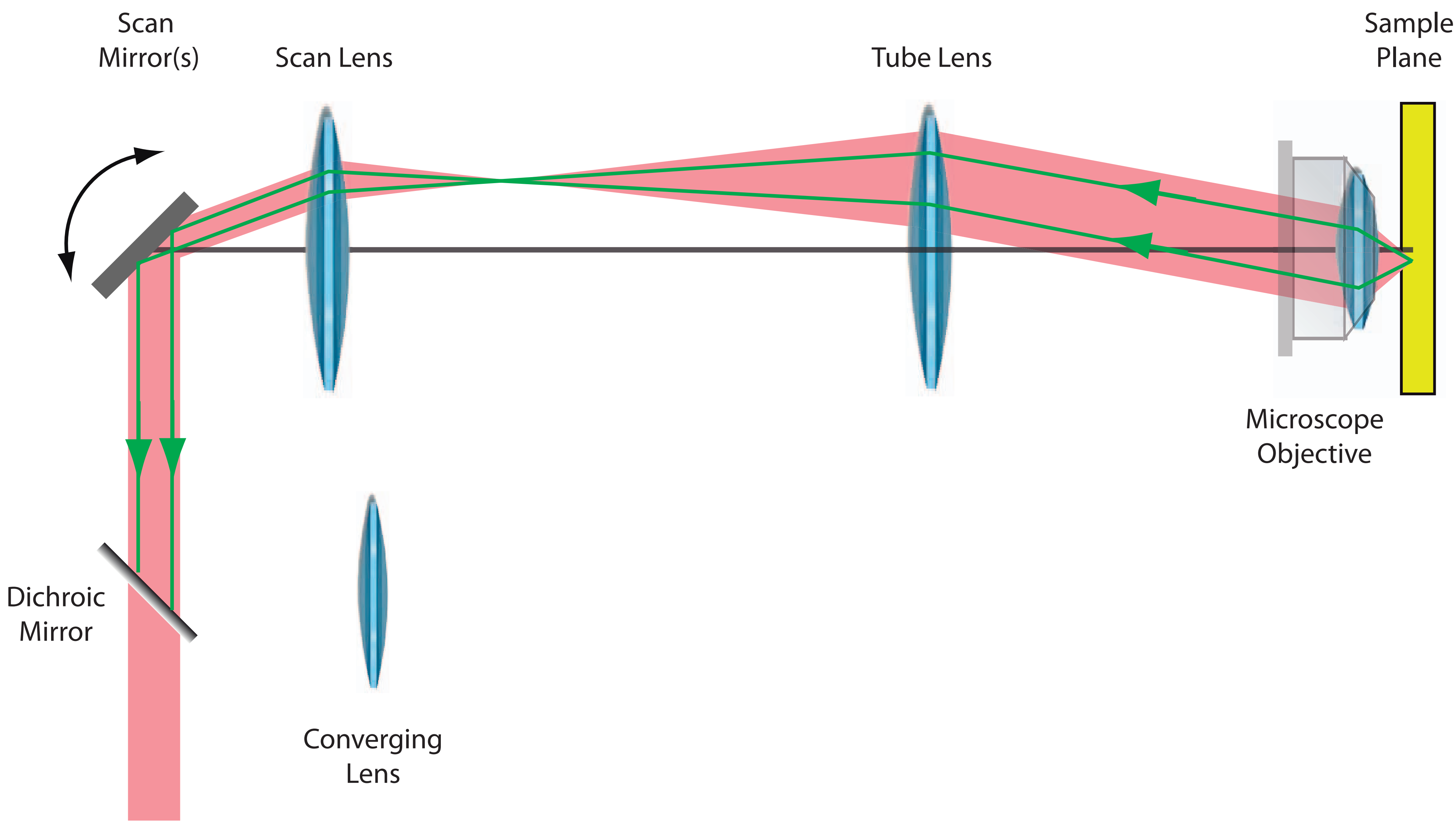

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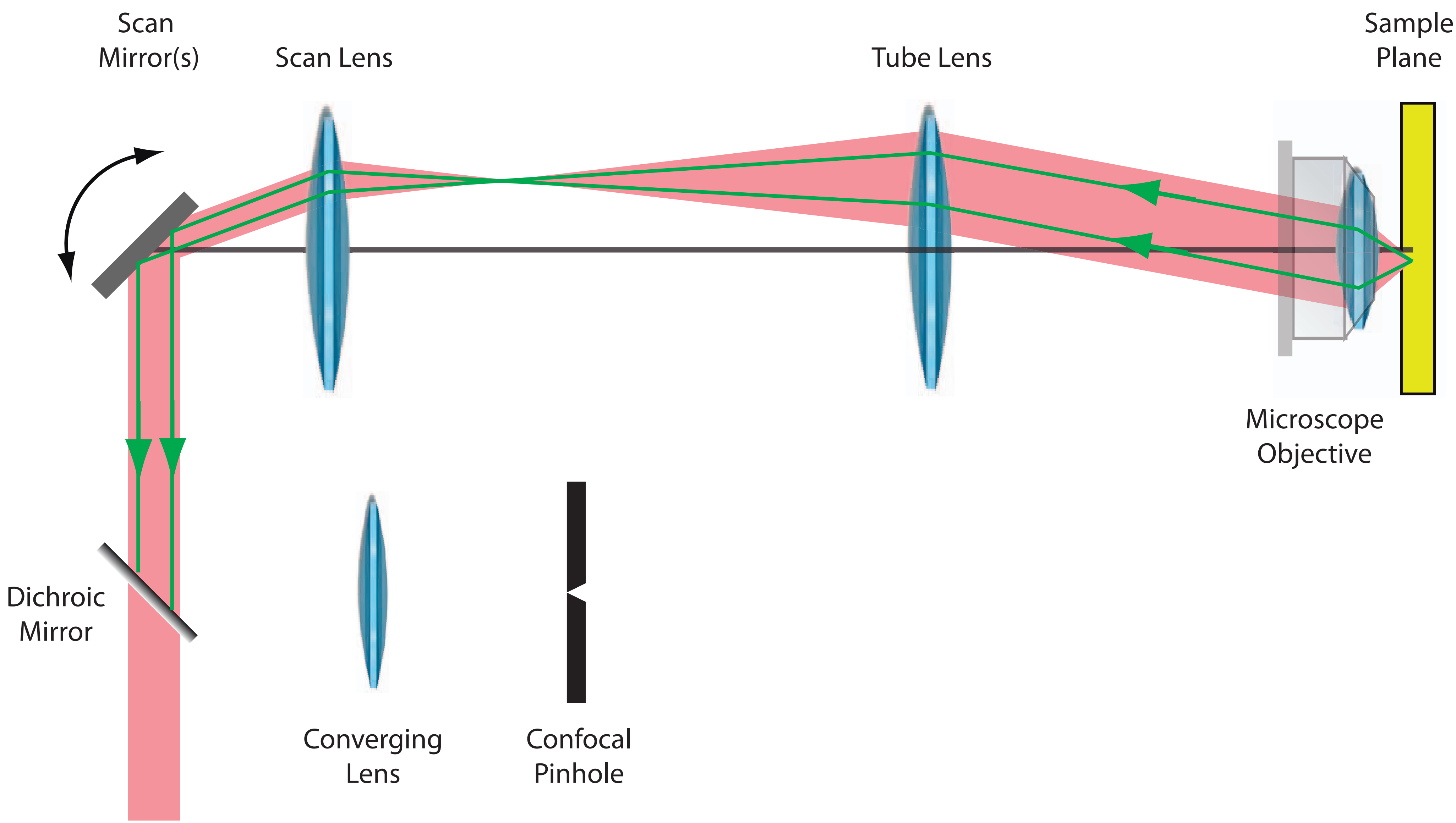

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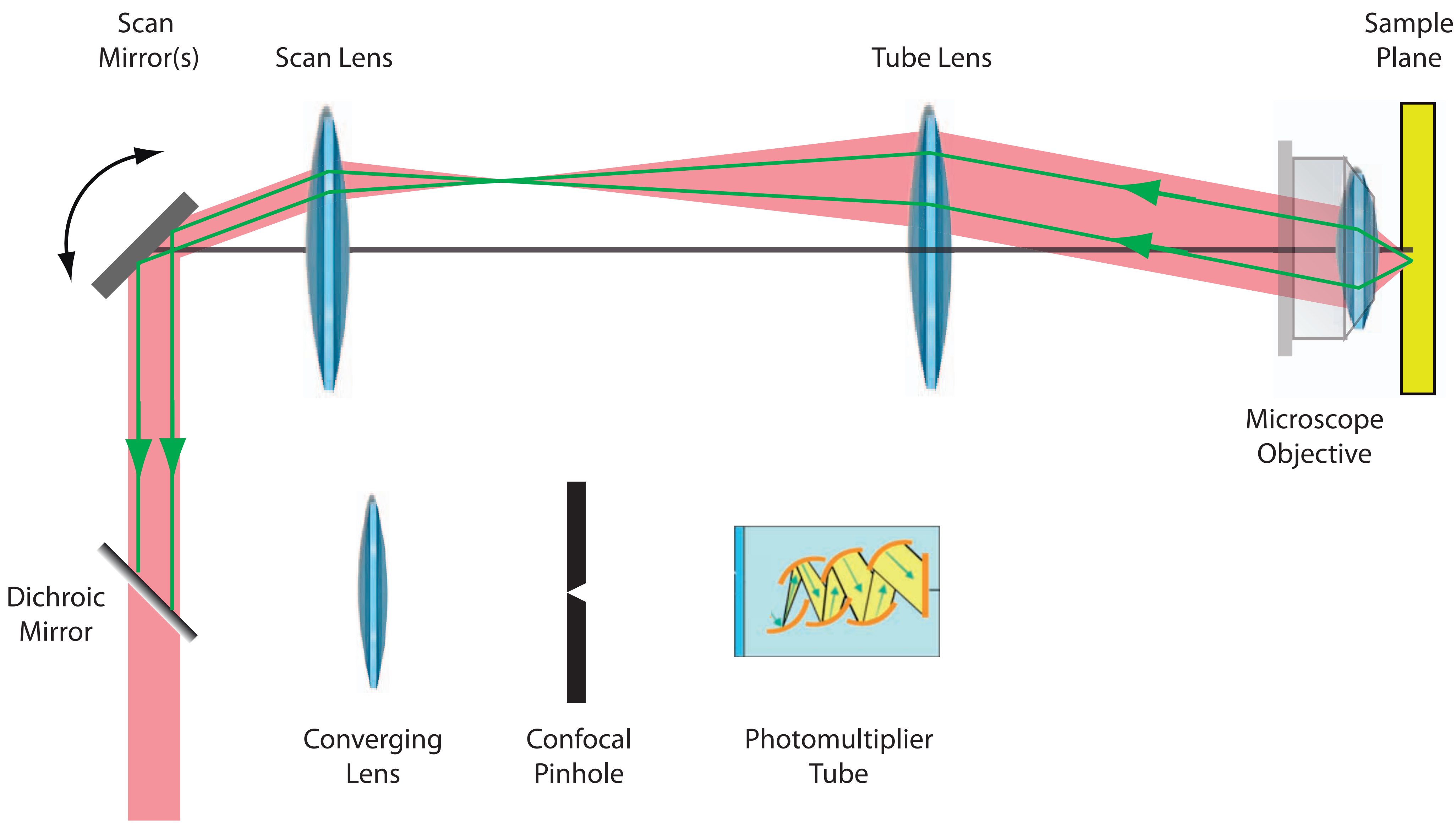

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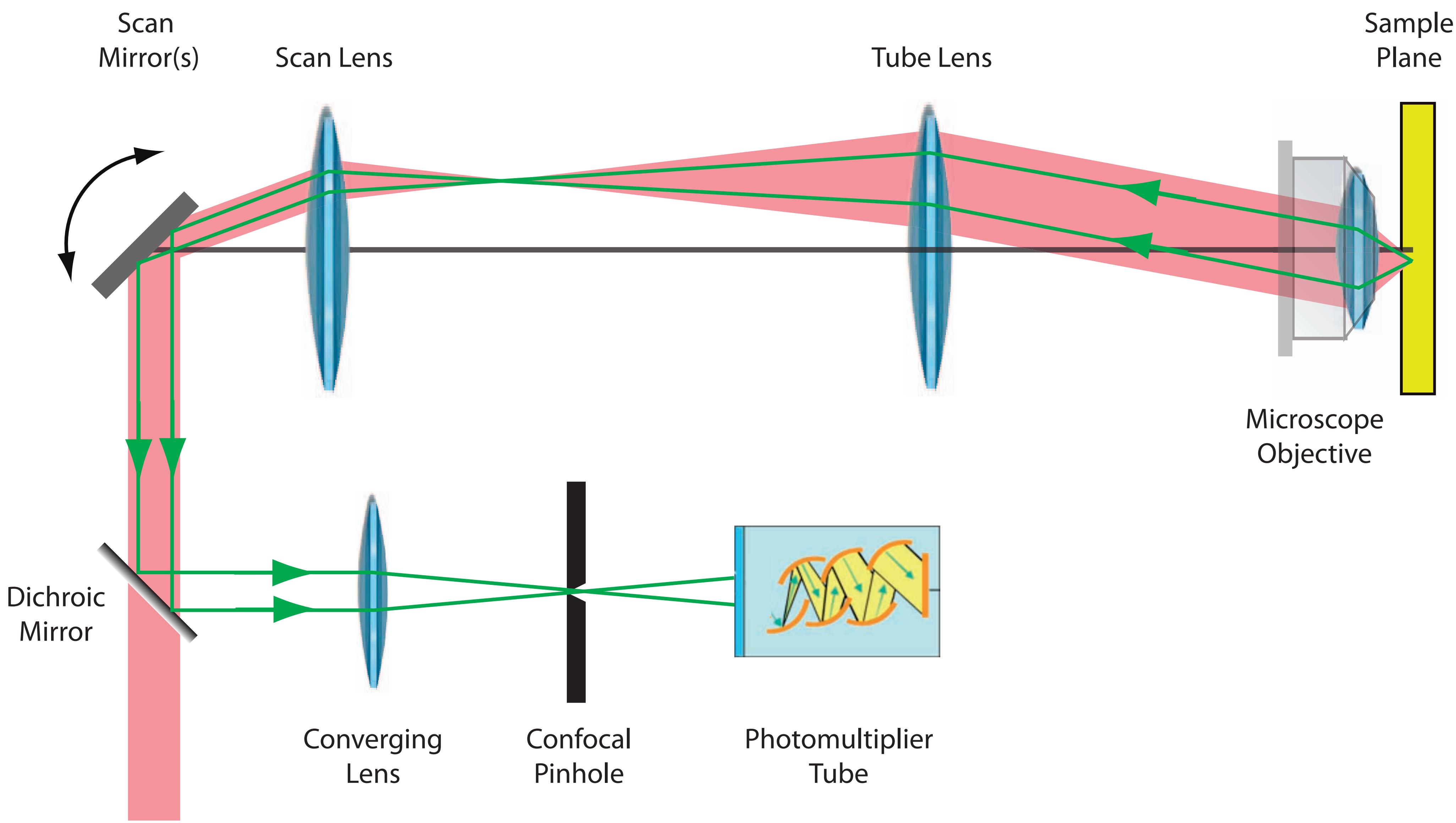

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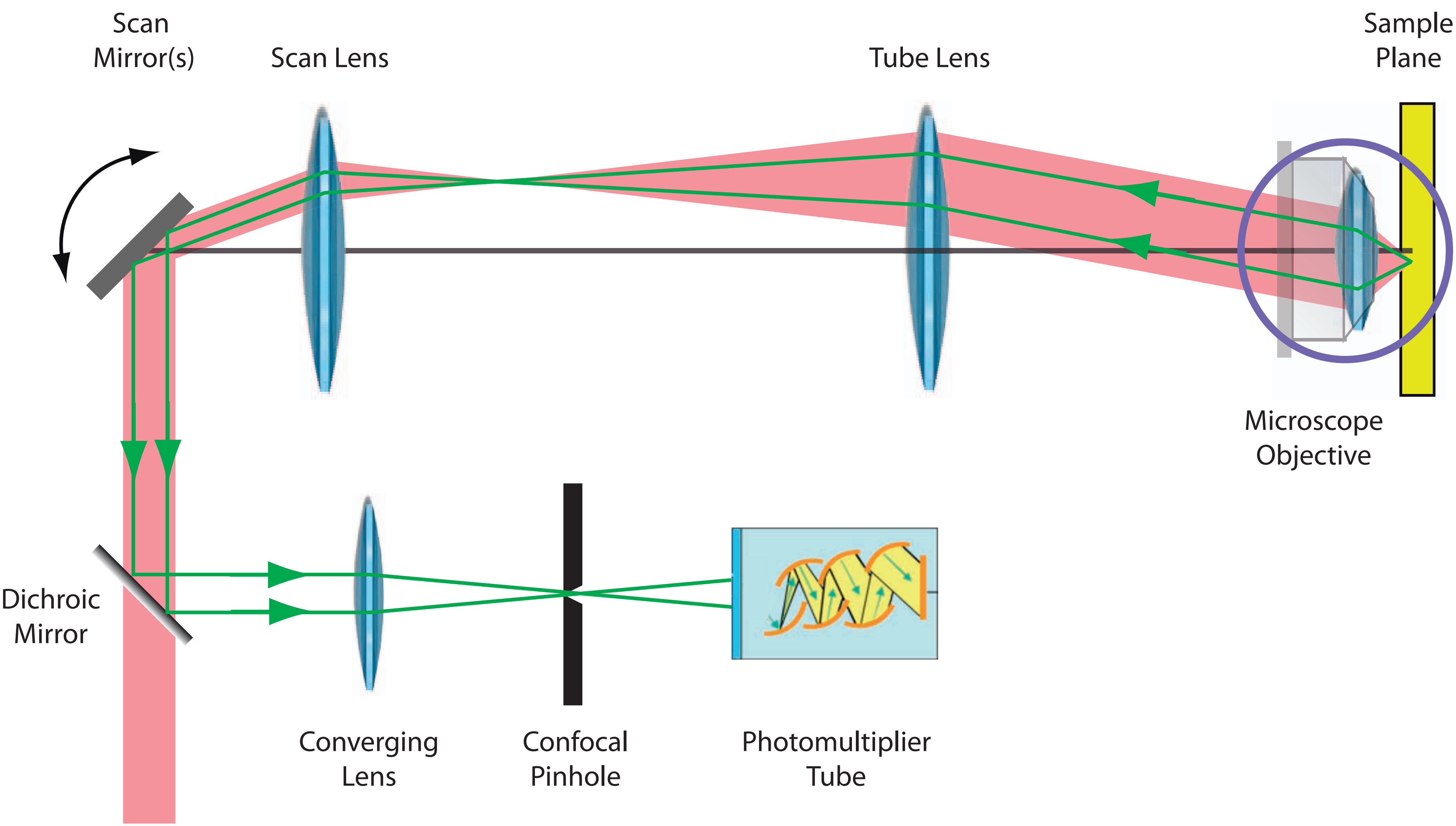

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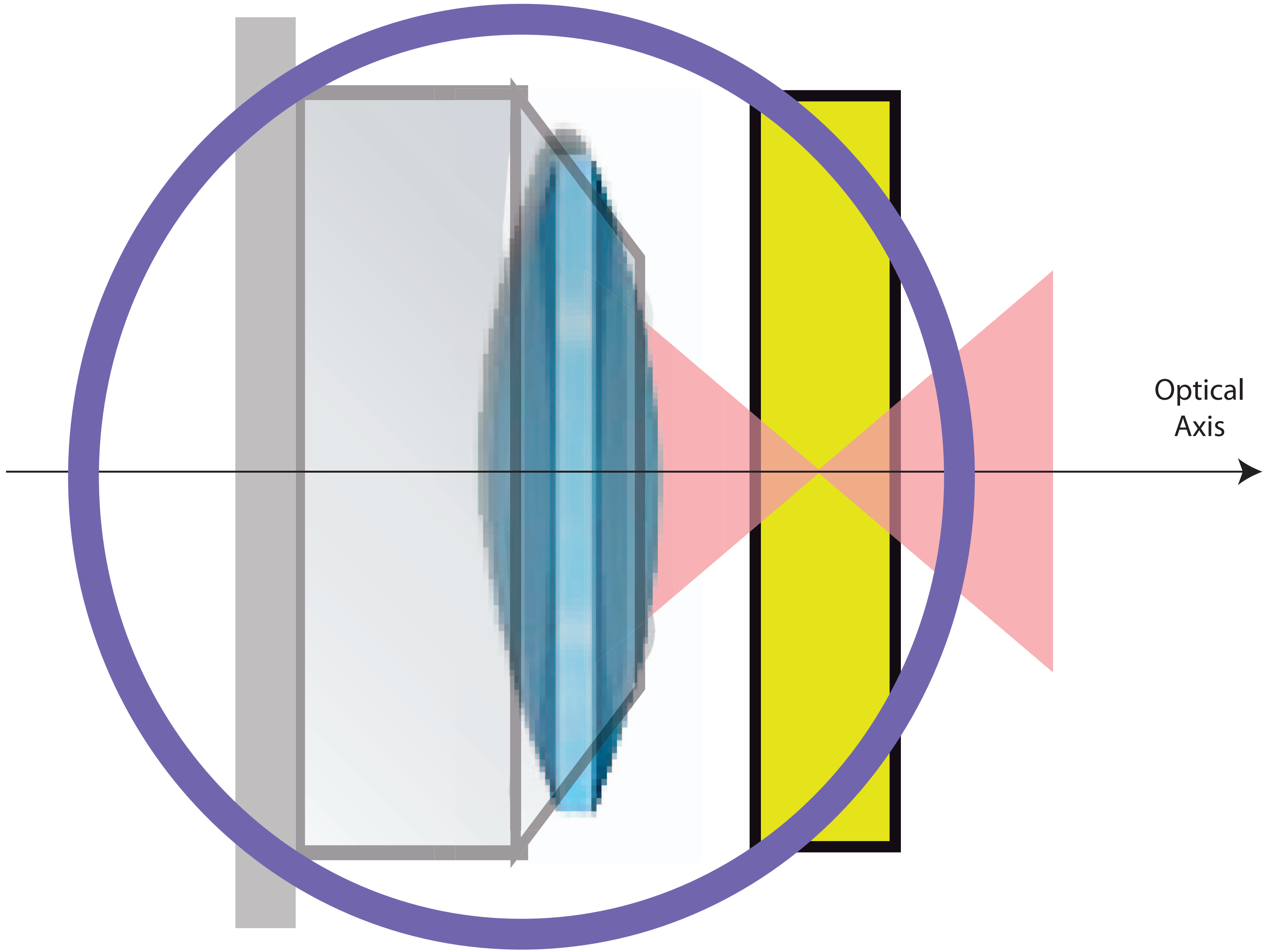

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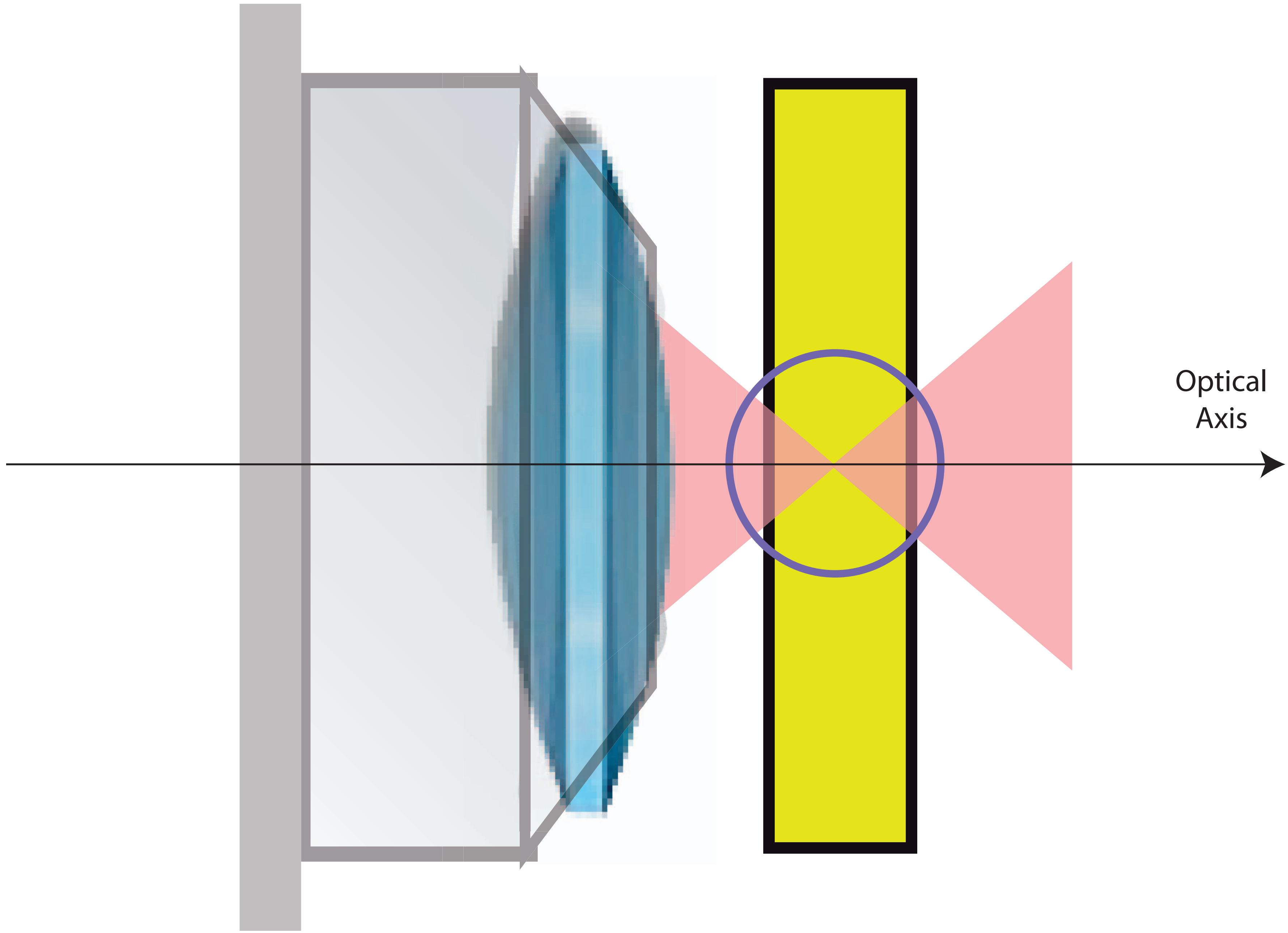

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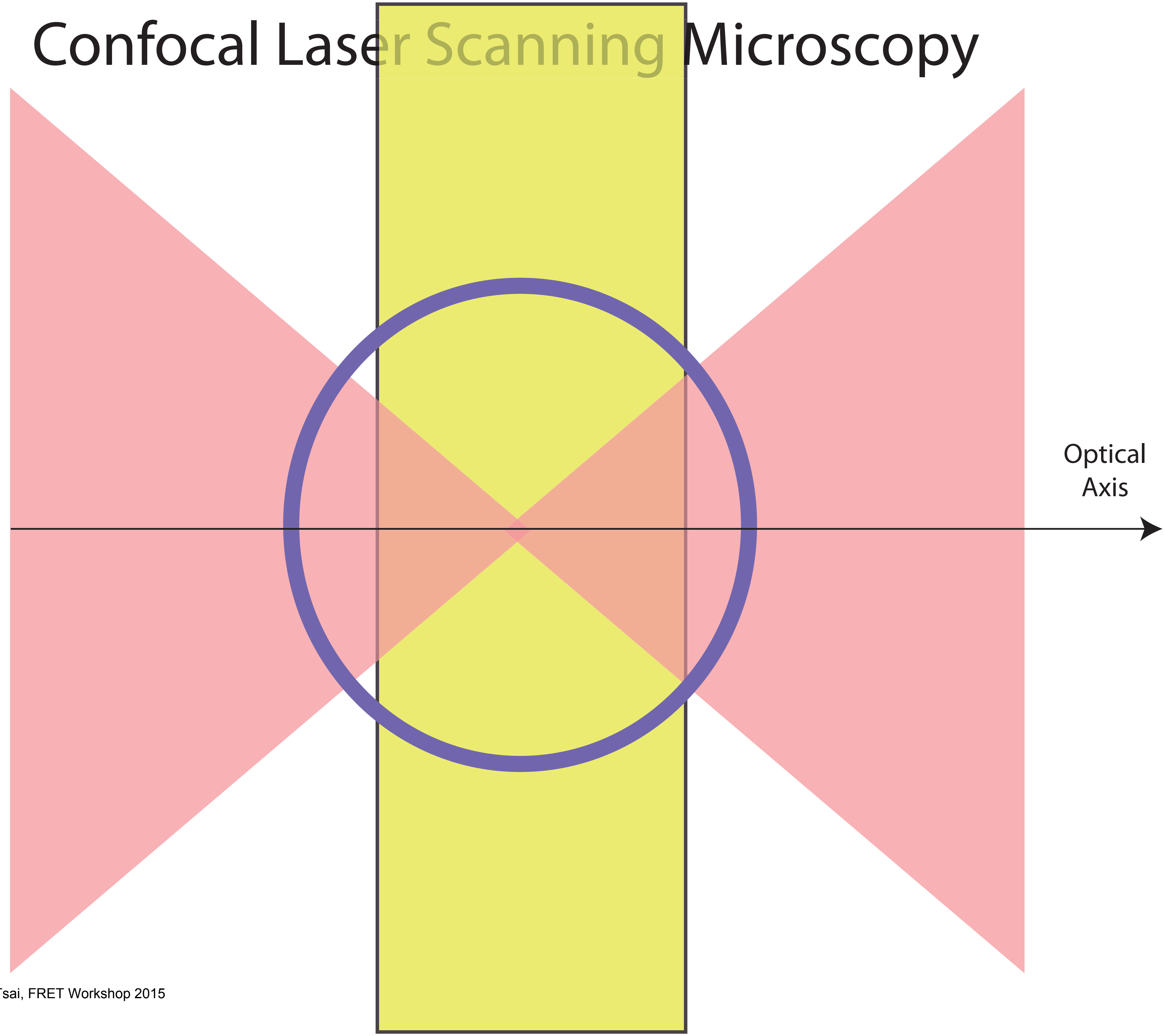

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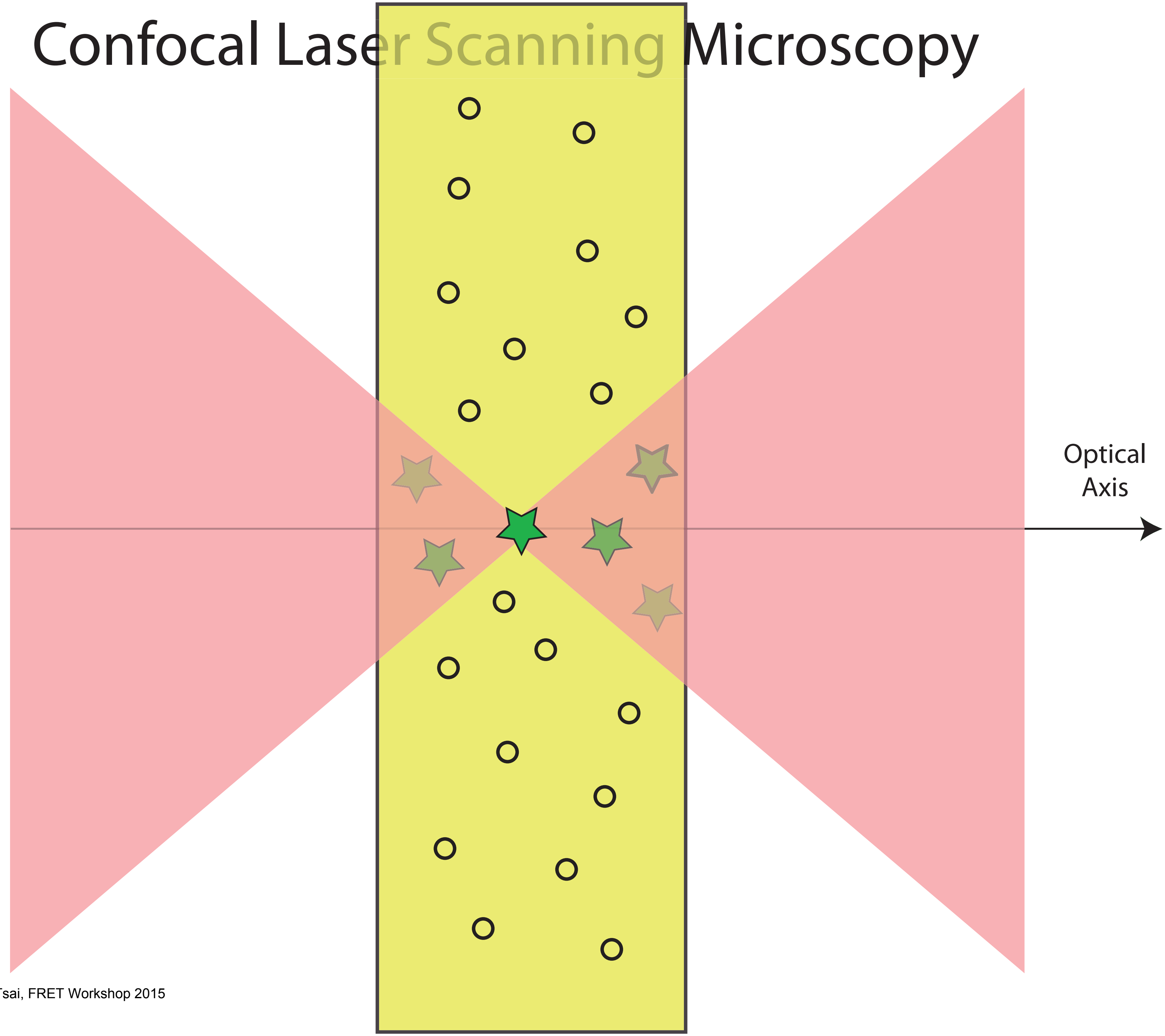

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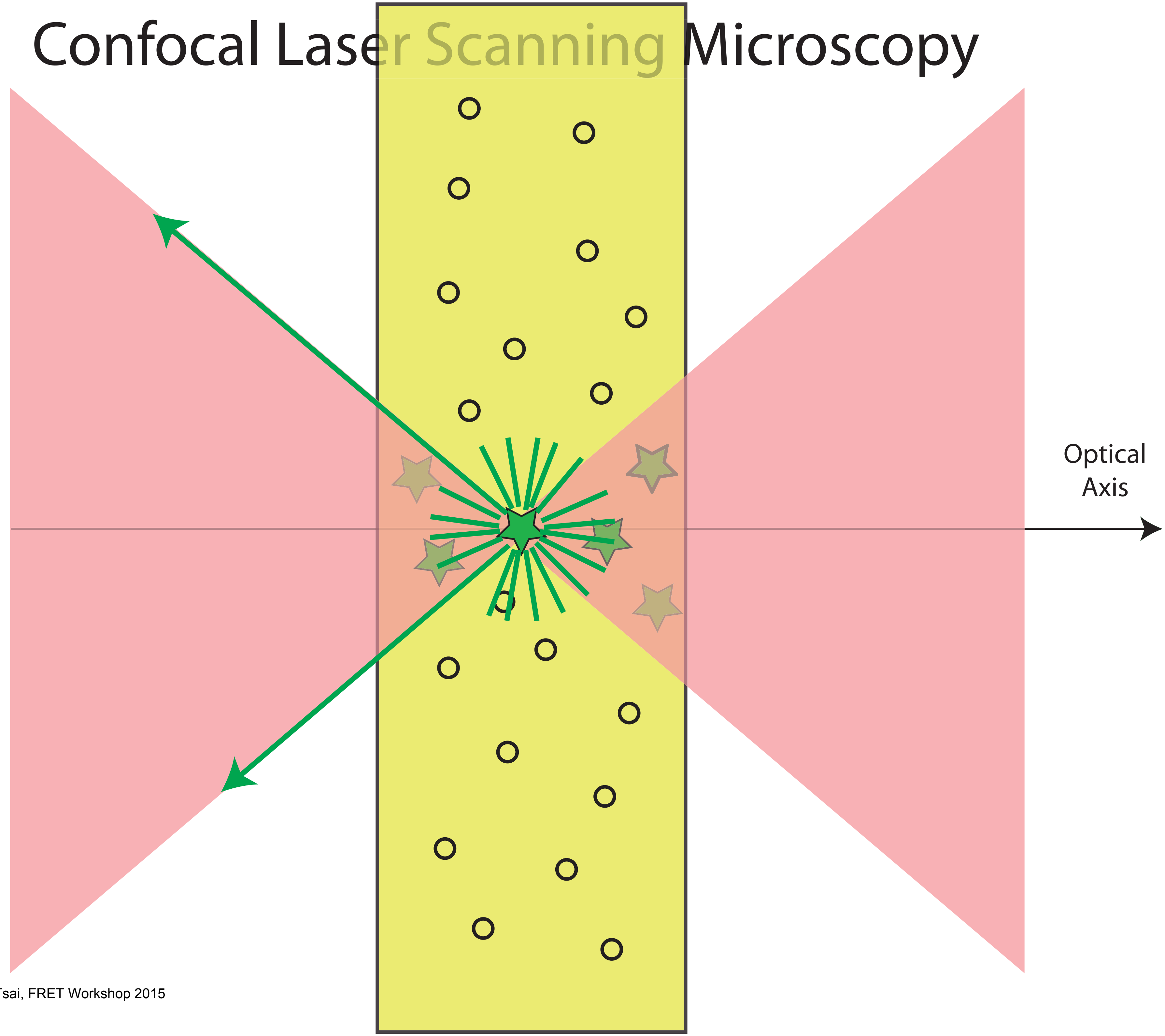

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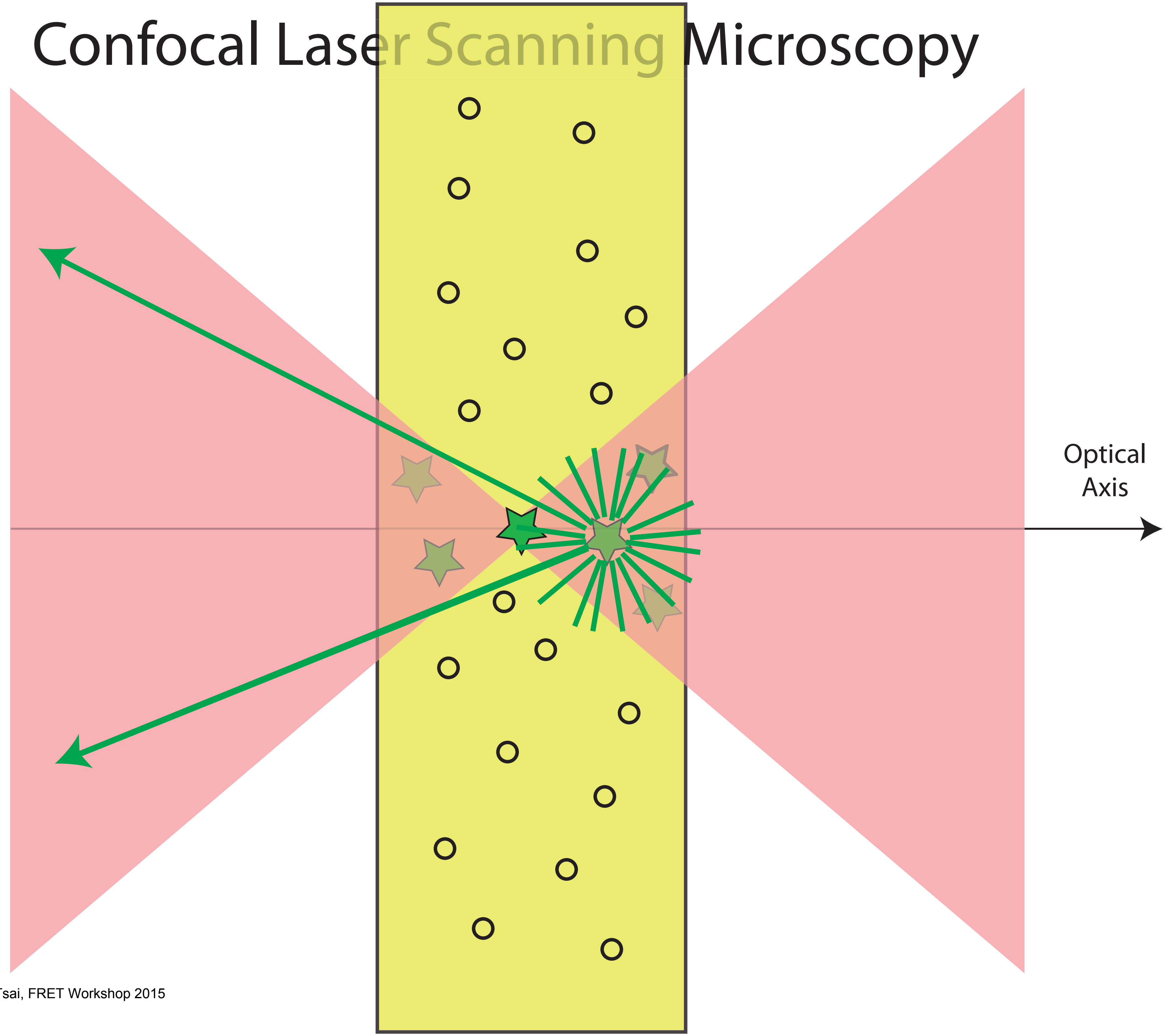

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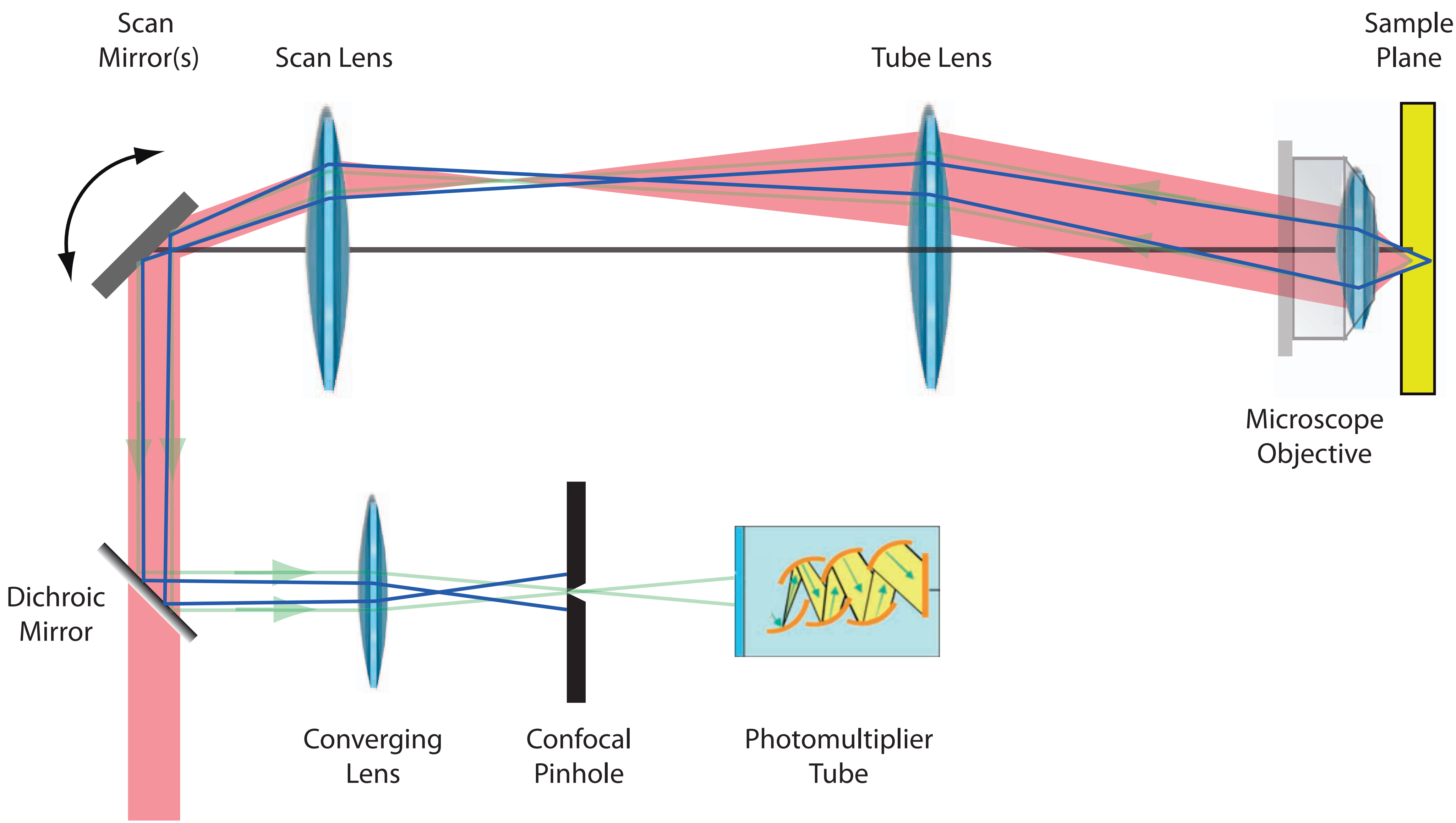

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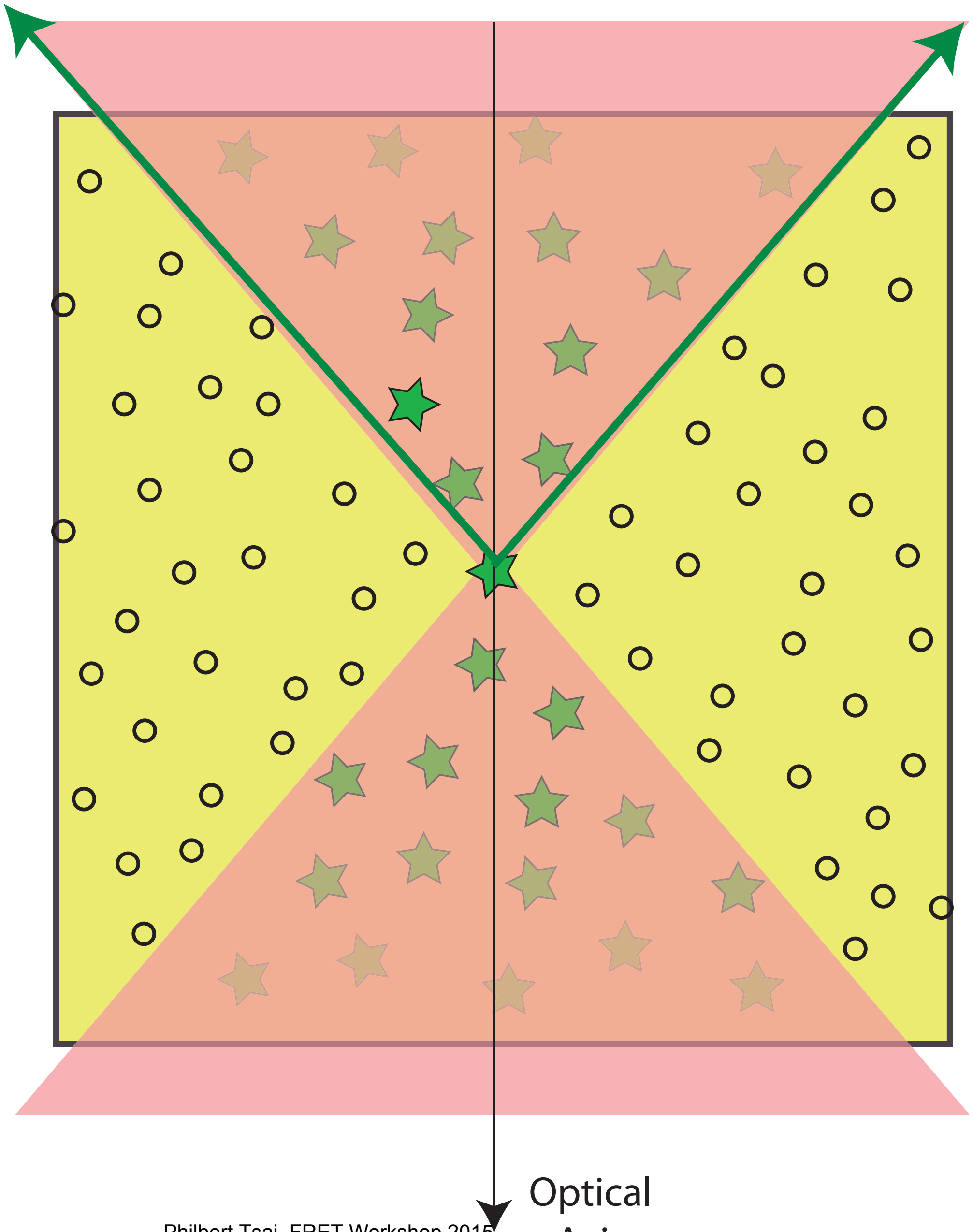

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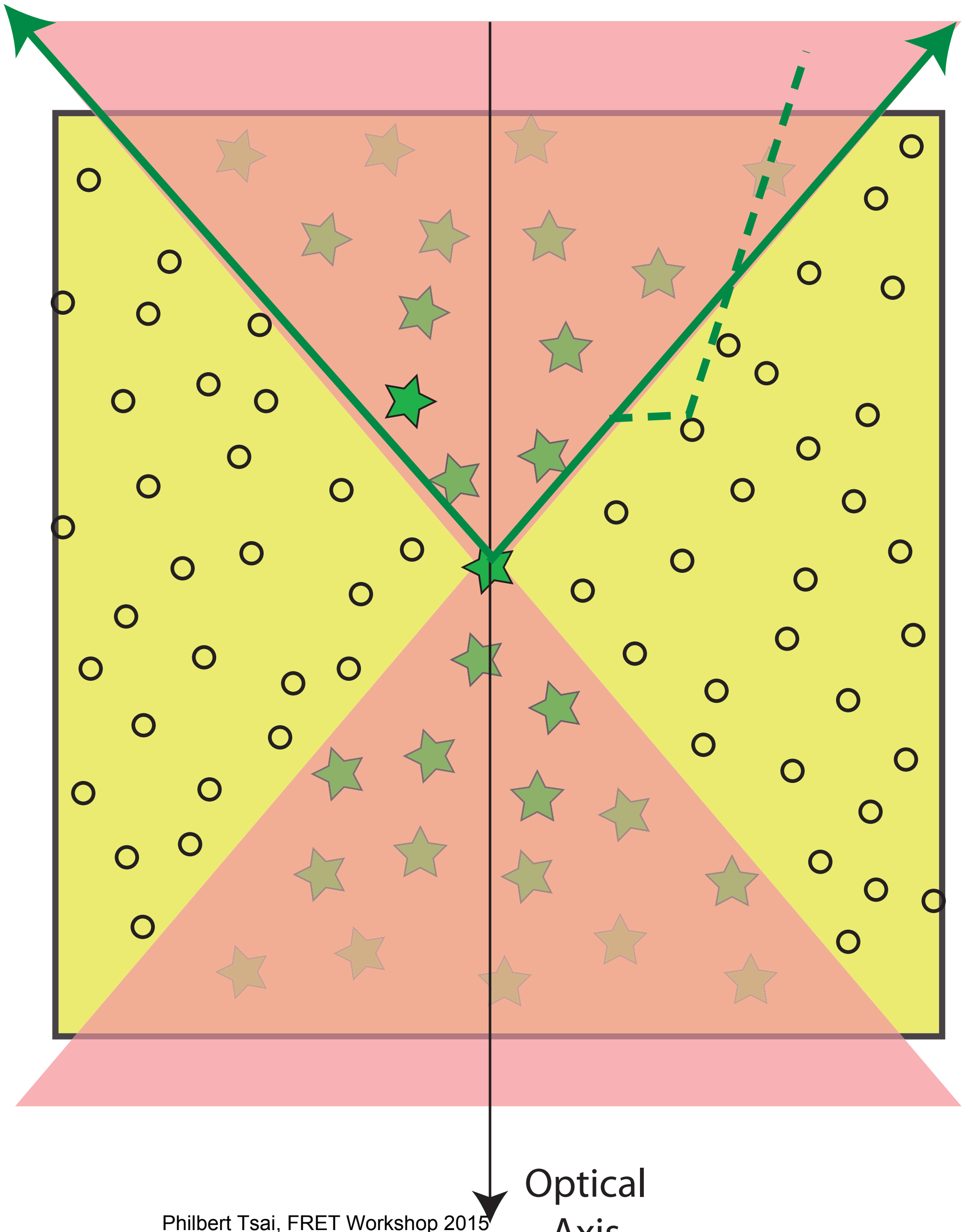
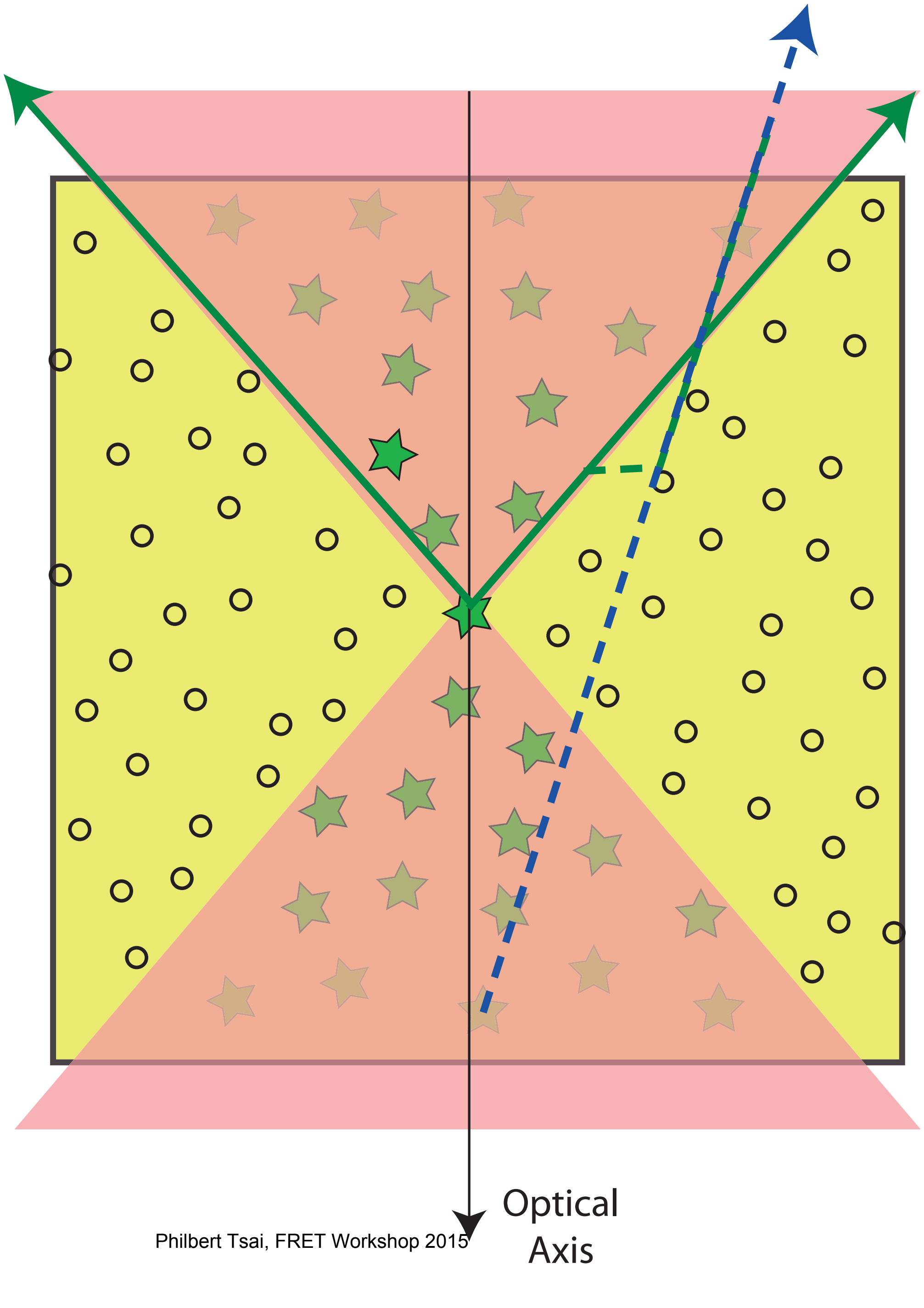


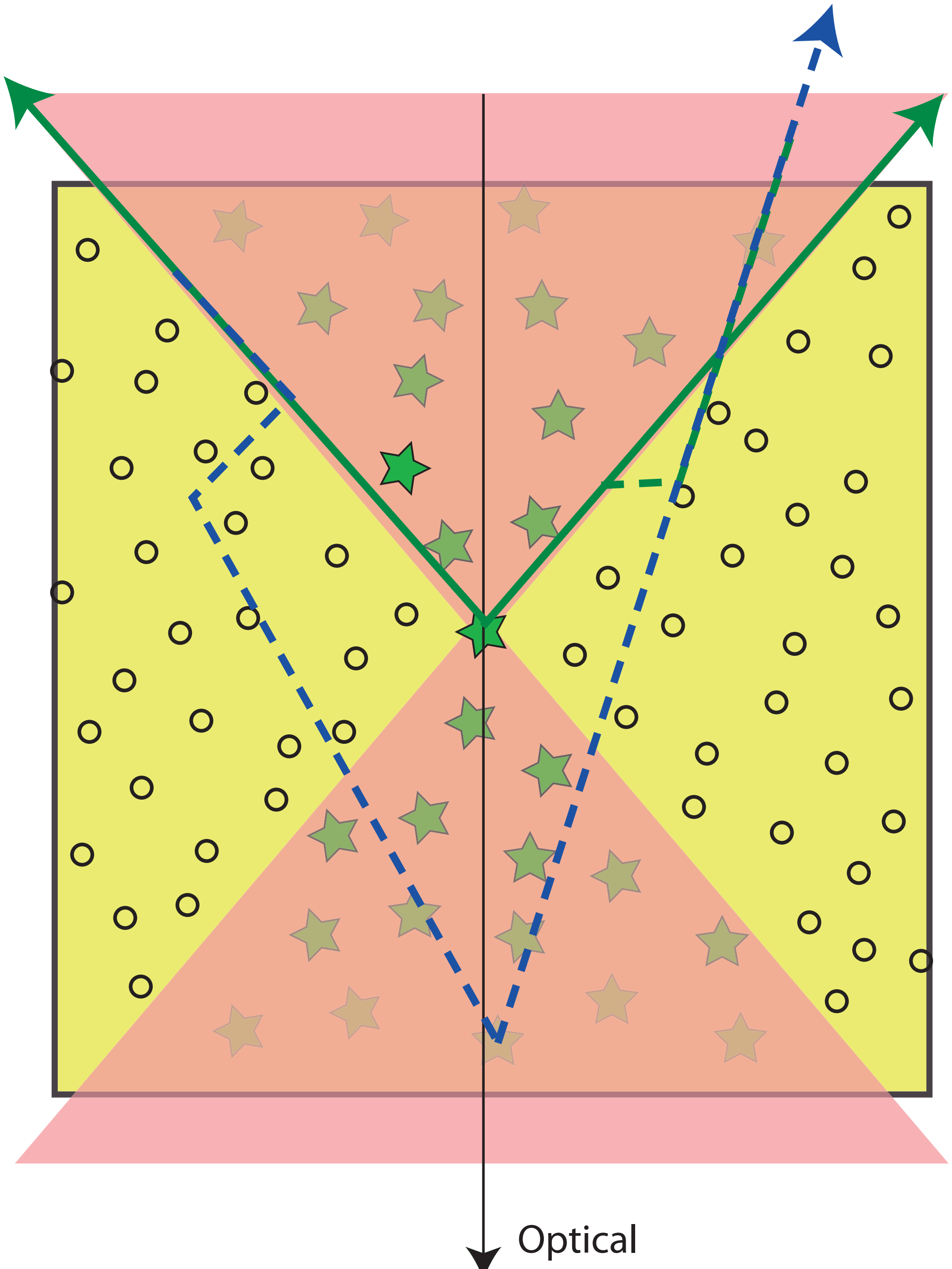
Confocal Laser Scanning Microscopy



In-focus fluorescence can scatter into the same path as out-of-focus fluorescence, and be blocked by the confocal pinhole.

This decreases the signal reaching the detector.

Confocal Laser Scanning Microscopy



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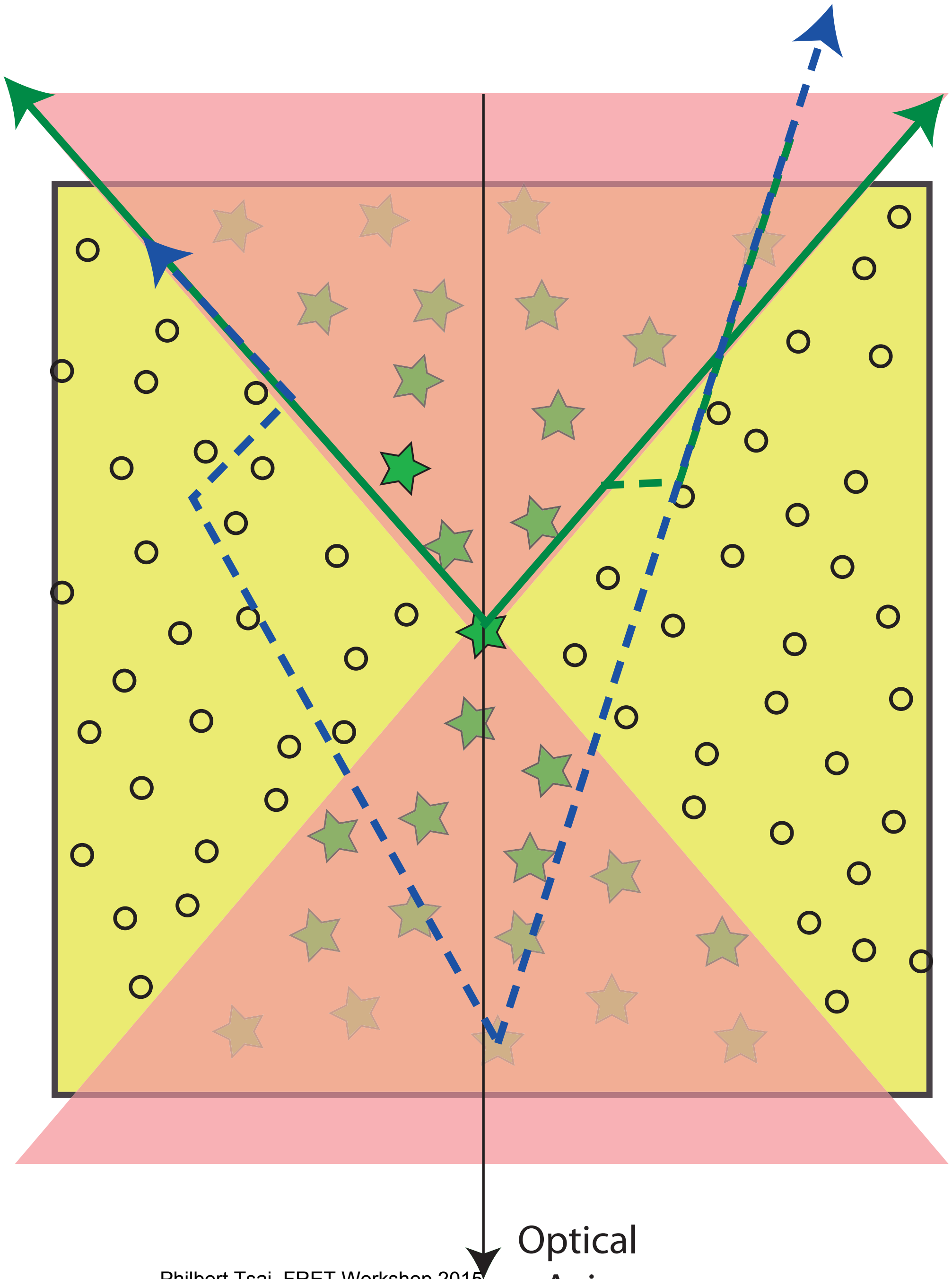
Confocal Laser Scanning Microscopy



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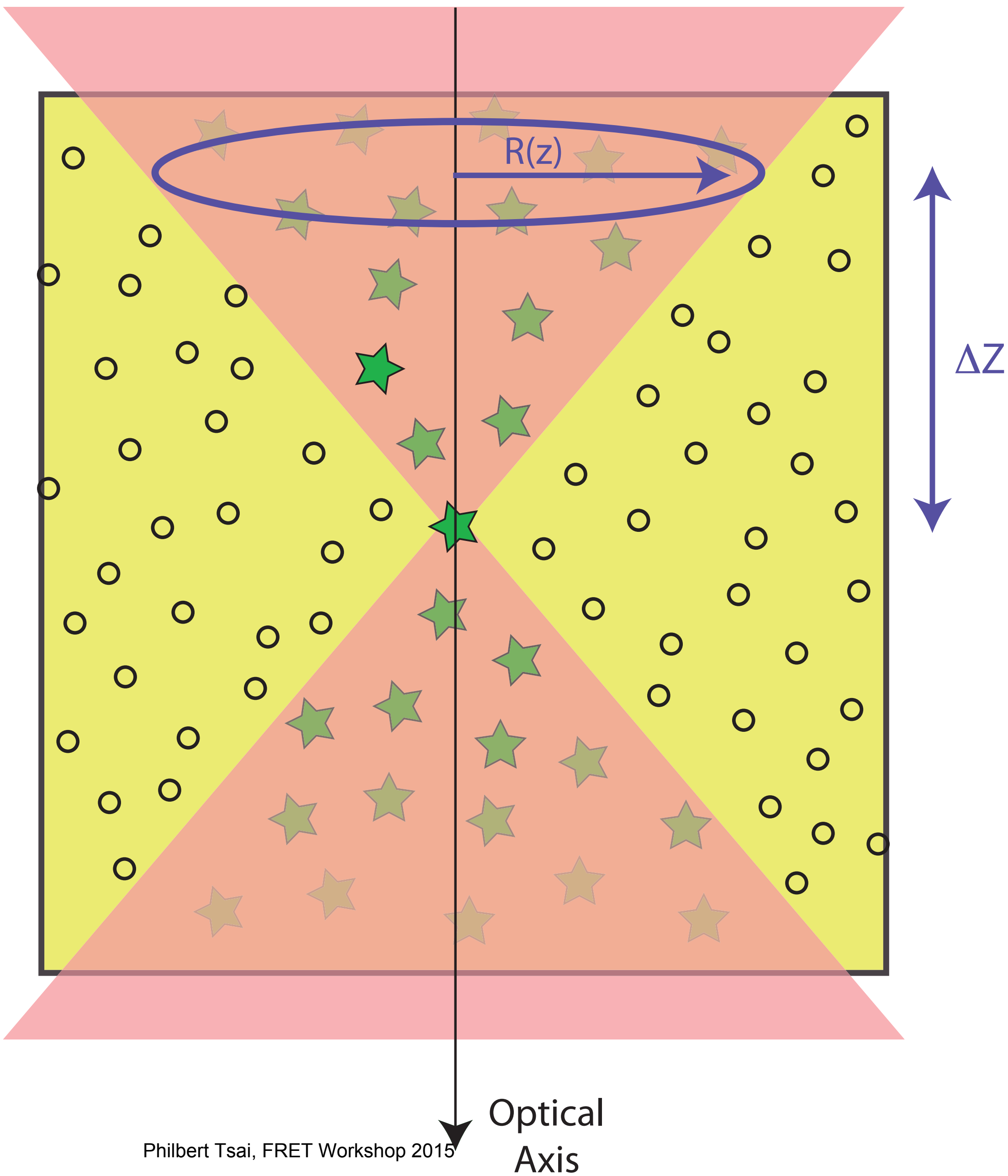
Out-of-focus fluorescence can scatter into the same path as in-focus fluorescence, and pass through the confocal pinhole.

This increased background reduces the signal-to-noise ratio.

Two Photon Laser Scanning Microscopy



R(Z) = Radius

A = Area

ΔZ = Distance from Focal Plane

I = Intensity

P = Power

F_m = Fluorescence per Molecule

σ = Dye Cross-section

F_p = Fluorescence per Plane

TWO PHOTON EXCITATION

$R(z) \sim \Delta Z$

$A \sim R^2 \sim (\Delta Z)^2$

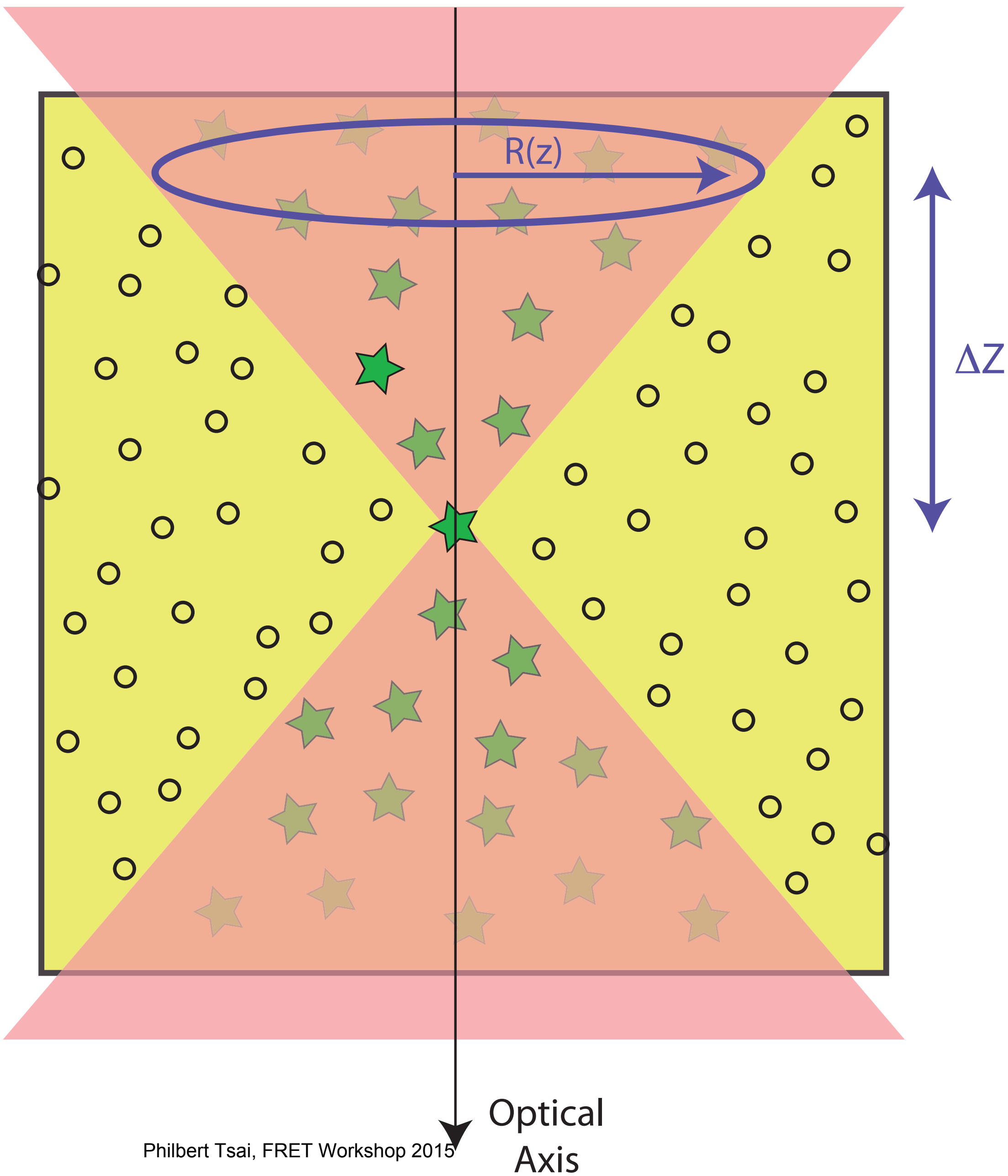
$I = P / A$

$F_m = \sigma * I^2 = \sigma * P^2/A^2 = \sigma * P^2/(\Delta Z)^4$

$F_p = F_m * A = \sigma * P^2/A \sim \sigma * P^2/(\Delta Z)^2$

For two photon excitation, the fluorescence per plane falls quadratically with distance from the focal plane !

Confocal Laser Scanning Microscopy



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F_p = Fluorescence per Plane

SINGLE PHOTON EXCITATION

$R(z) \sim \Delta Z$

$A \sim R^2 \sim (\Delta Z)^2$

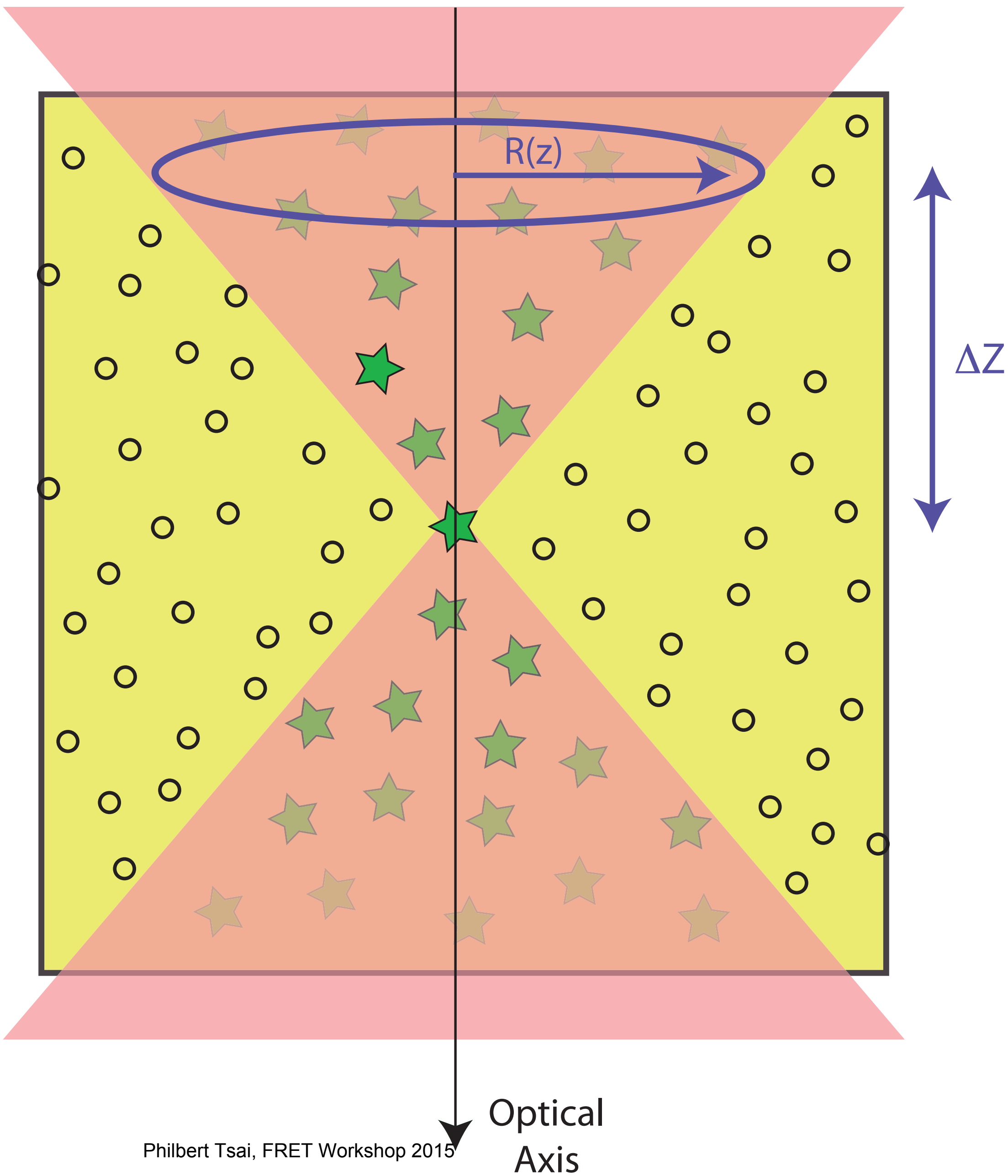
$I = P / A$

$F_m = \sigma * I = \sigma * P/A = \sigma * P/(\Delta Z)^2$

$F_p = F_m * A = \sigma * P$

For single photon excitation, the fluorescence per plane is independent of distance from the focal plane !

Two Photon Laser Scanning Microscopy



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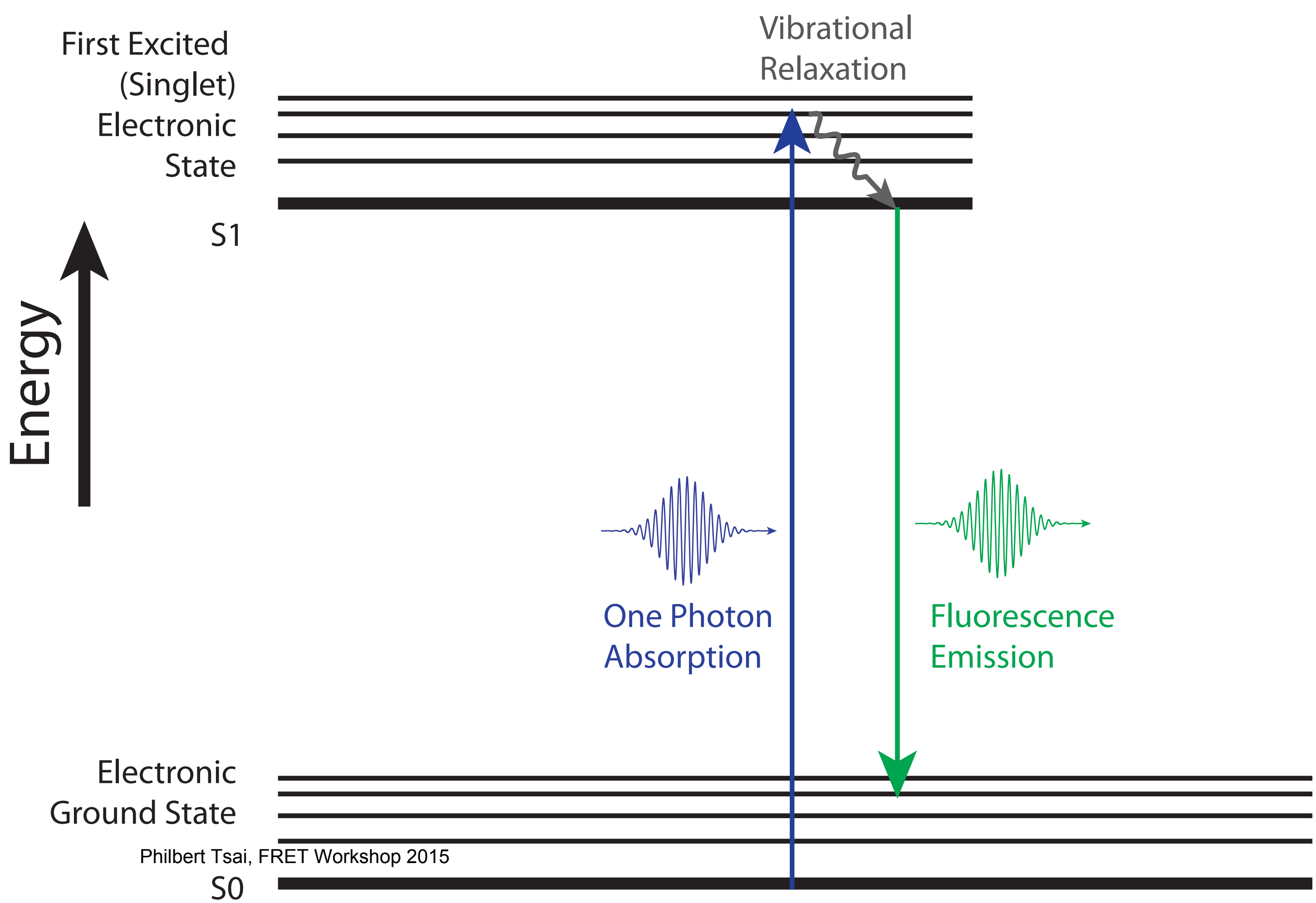
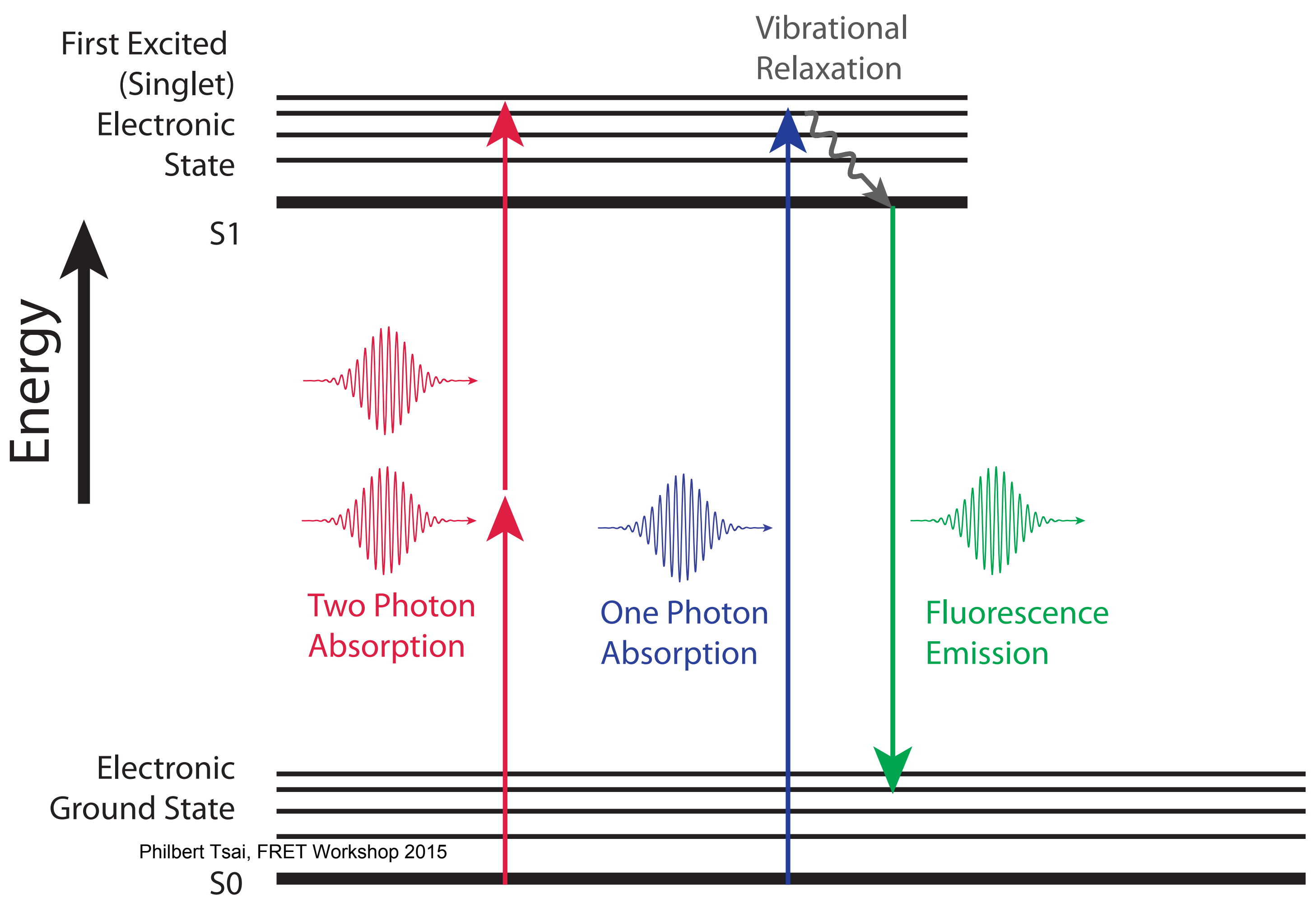
For two photon excitation, the fluorescence per plane falls quadratically with distance from the focal plane !

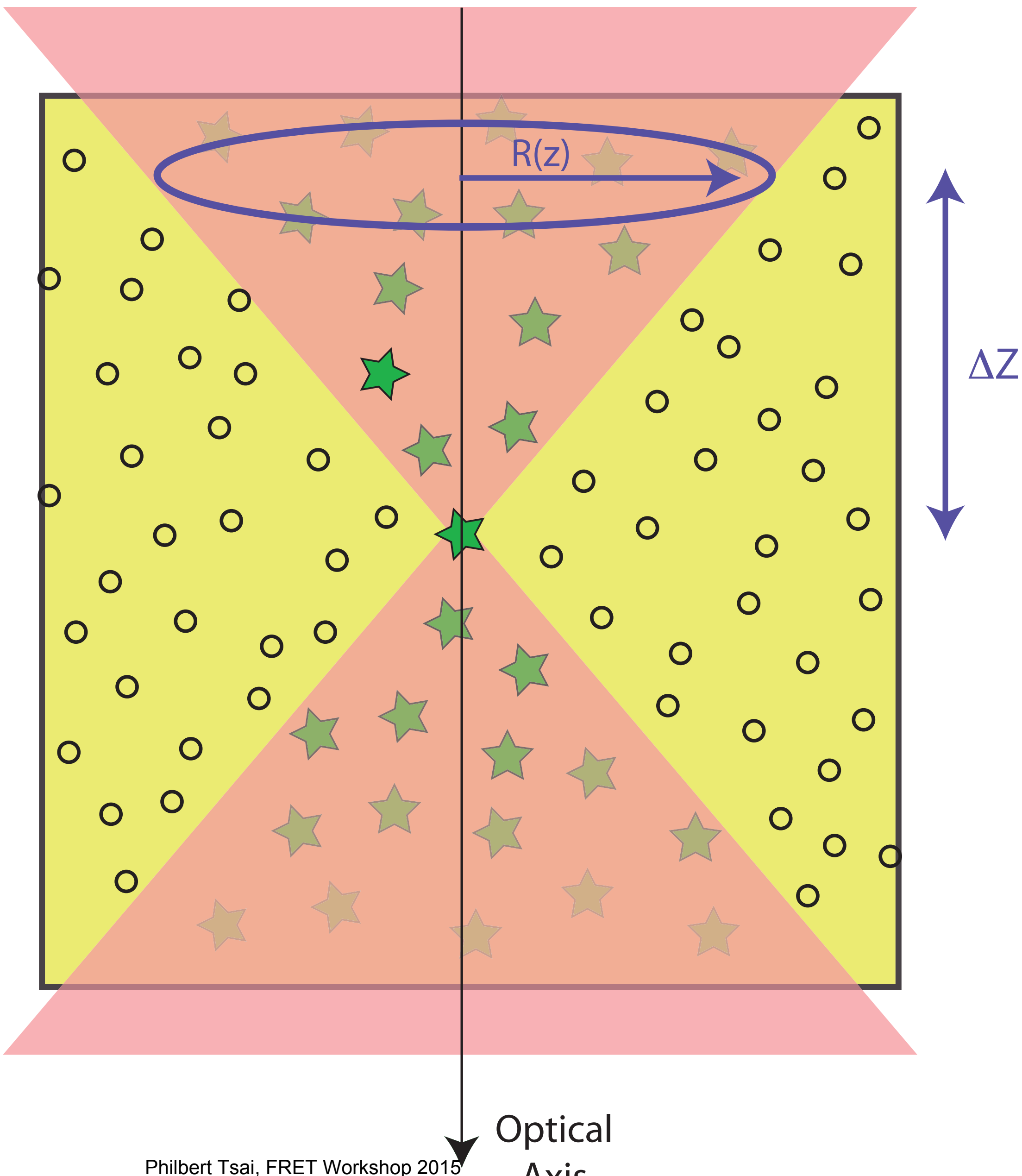
Jablonski Energy Diagram



Jablonski Energy Diagram



Two Photon Laser Scanning Microscopy



$R(Z)$ = Radius

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TWO PHOTON EXCITATION

$$R(z) \sim \Delta Z$$

$$A \sim R^2 \sim (\Delta Z)^2$$

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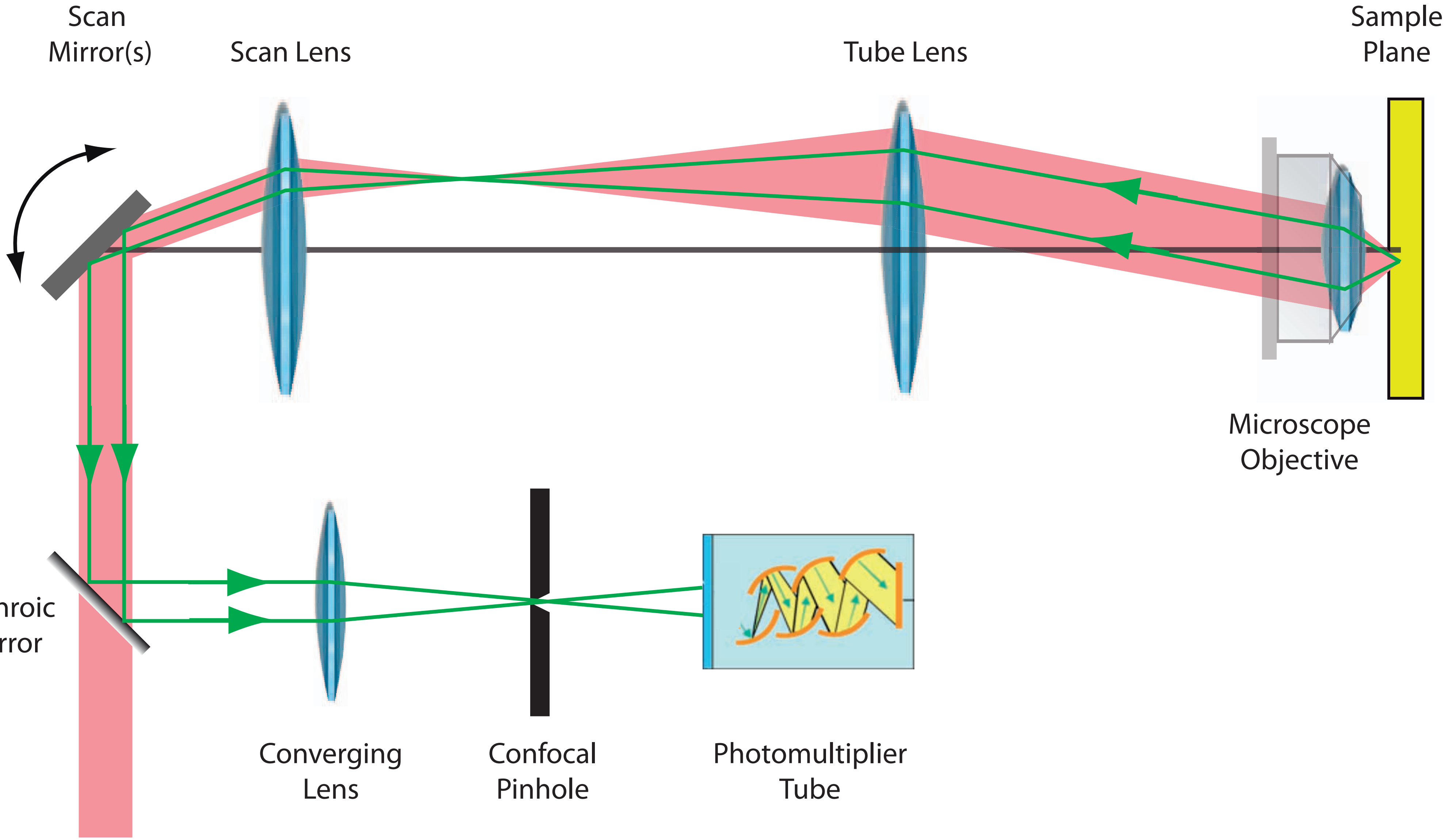
Confocal Laser Scanning Microscopy



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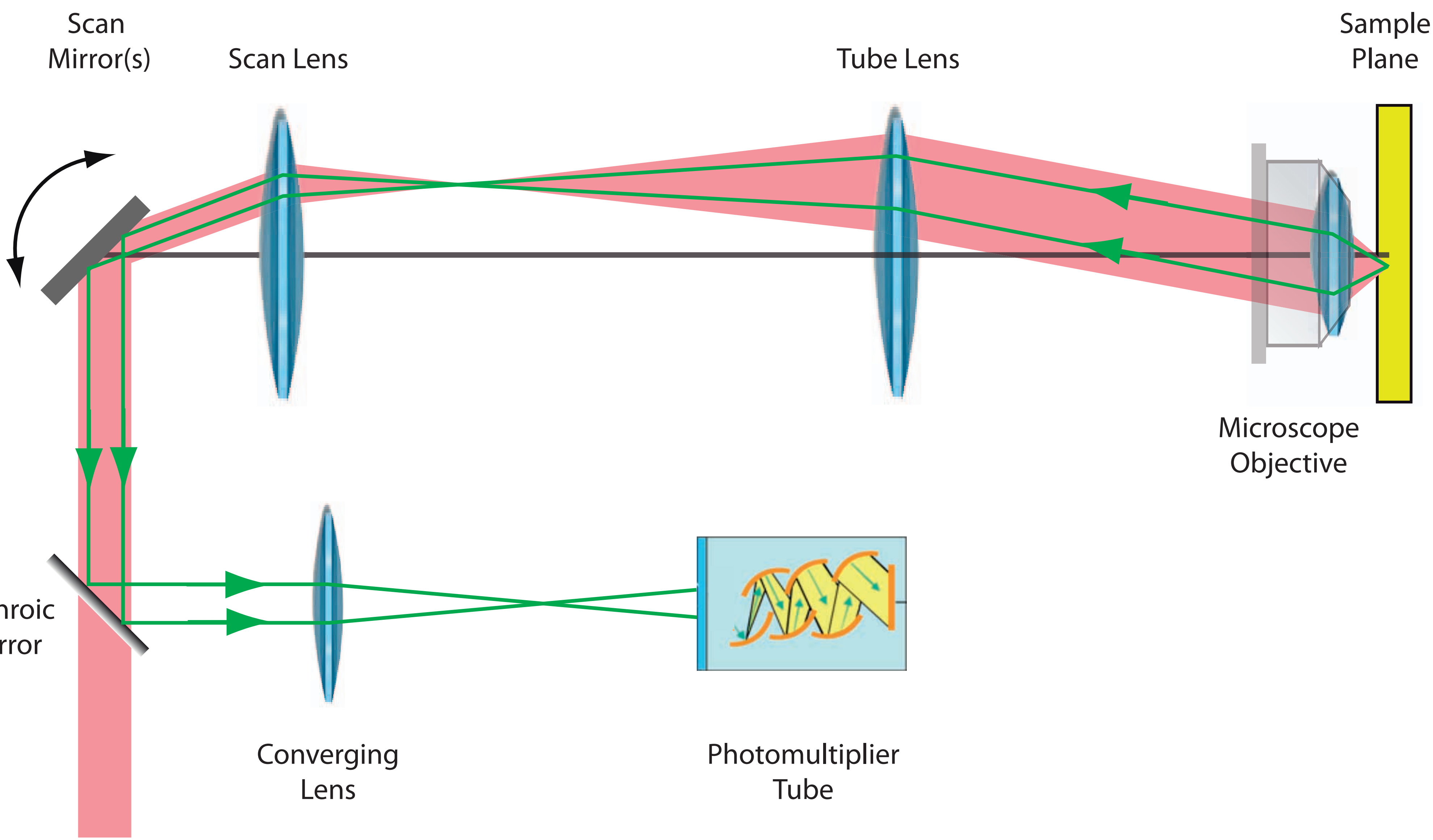
~~Confocal~~ Laser Scanning Microscopy
Two Photon



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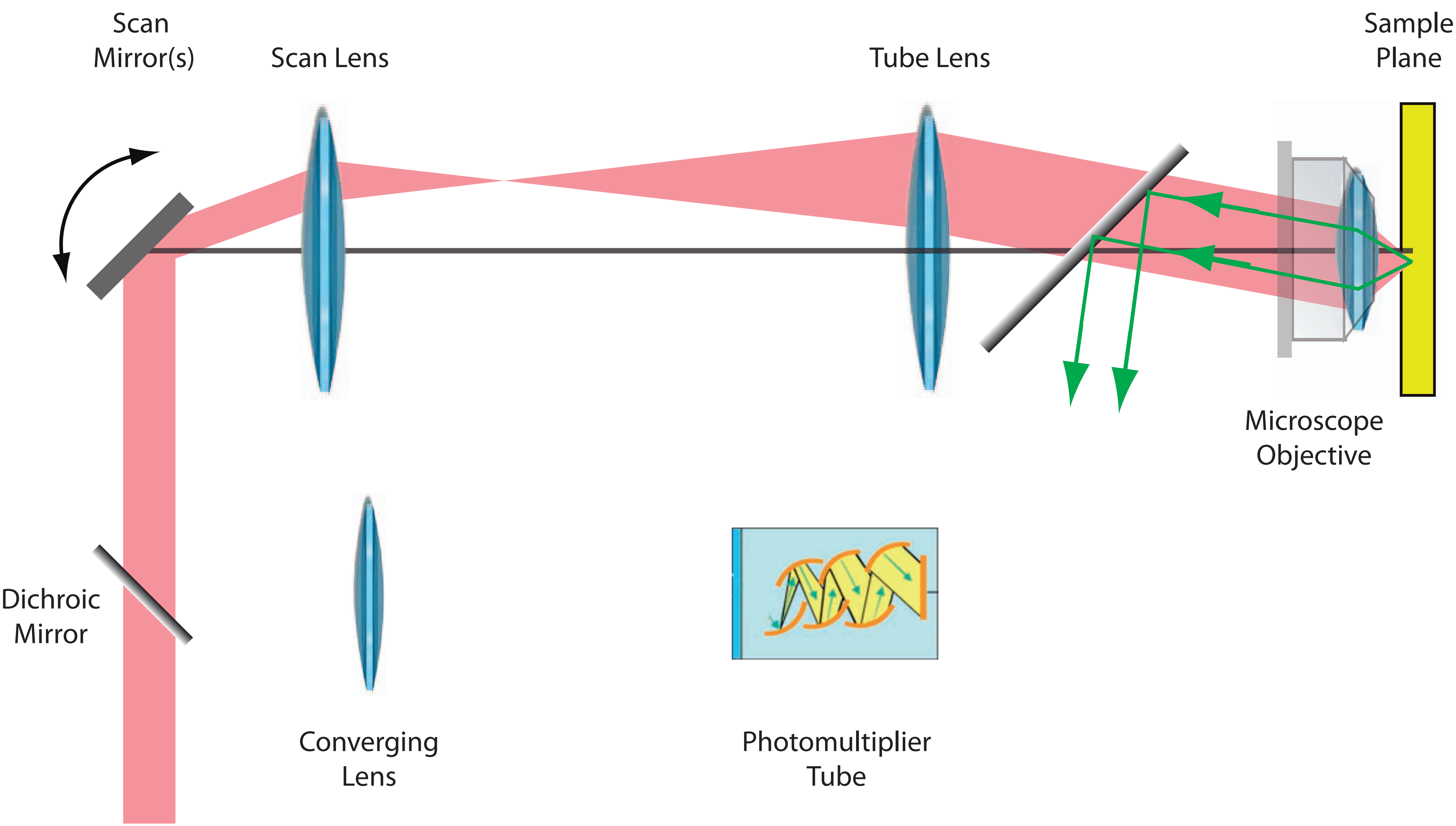
~~Confocal~~ Laser Scanning Microscopy

Two Photon



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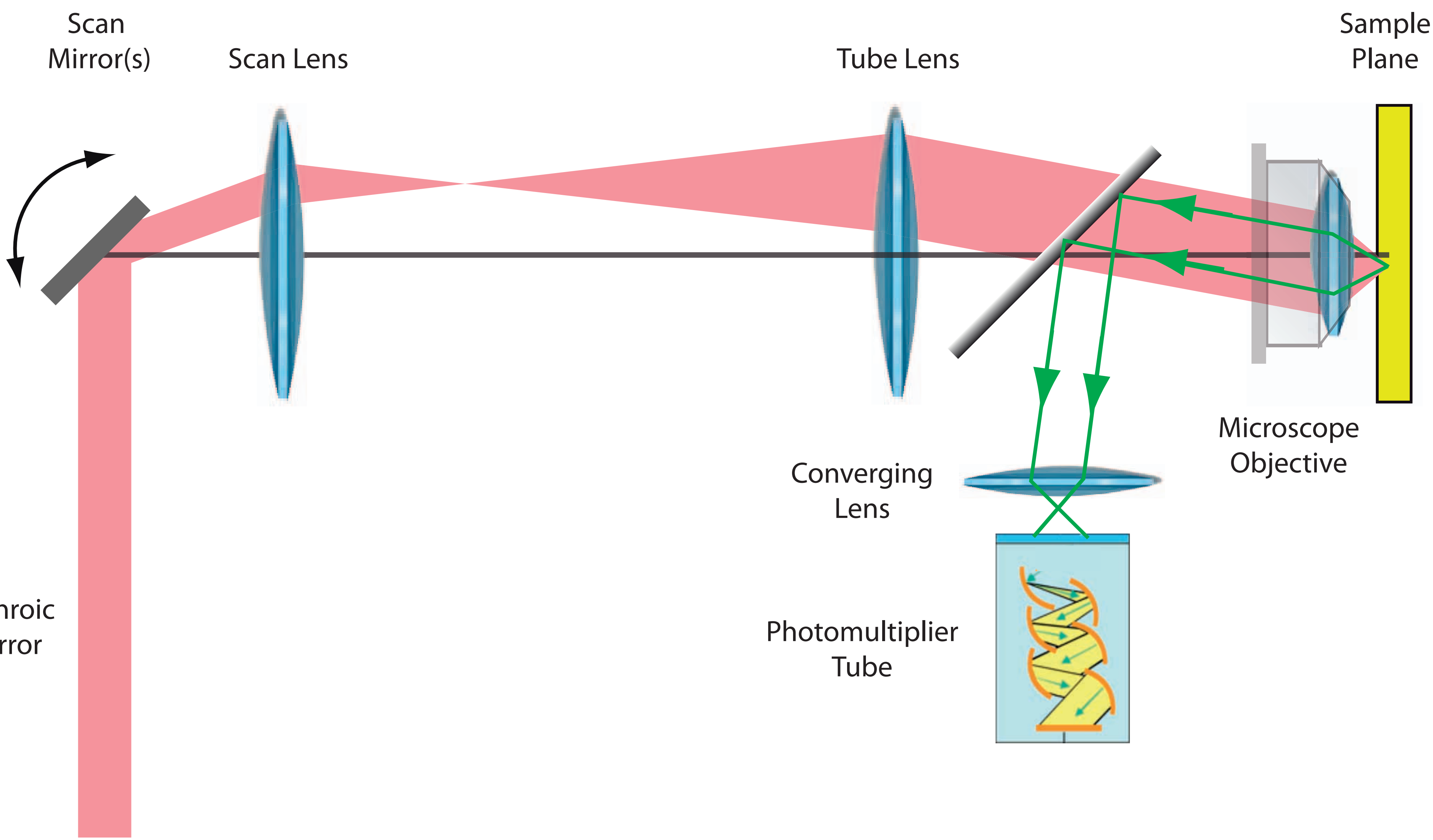
~~Confocal~~ Two Photon Laser Scanning Microscopy



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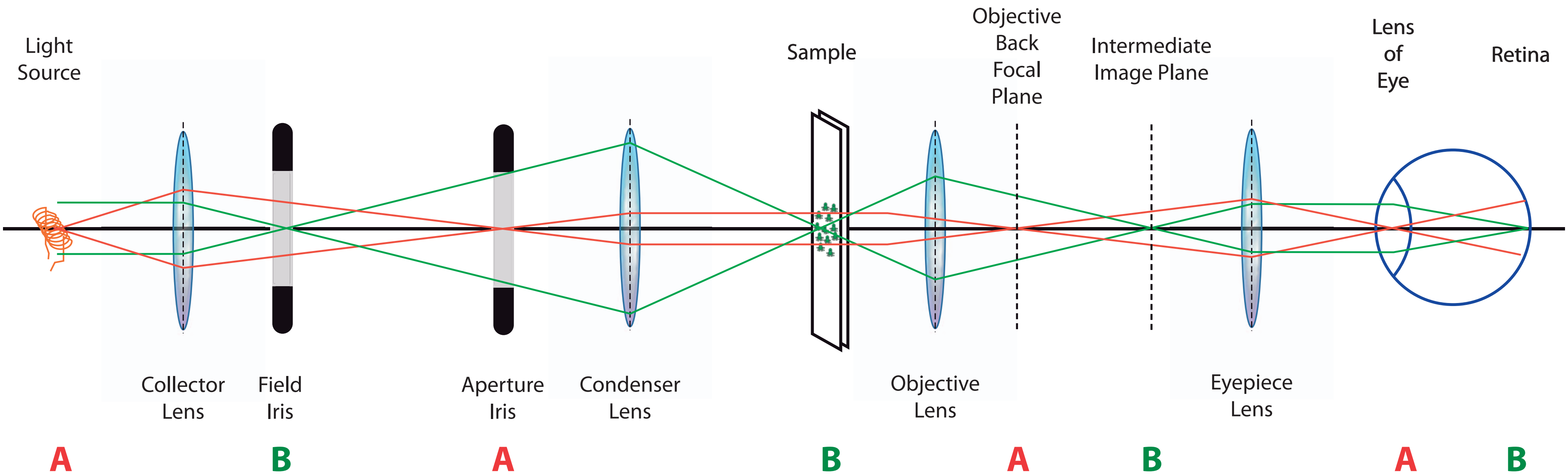
Kohler Illumination

Dual Light Paths



Illumination Conjugate Planes

Light Source
Aperture Iris
Back focal plane of objective
Front lens of eye

Sample Image Conjugate Planes

Field Iris
Sample plane
Intermediate image plane
Retina

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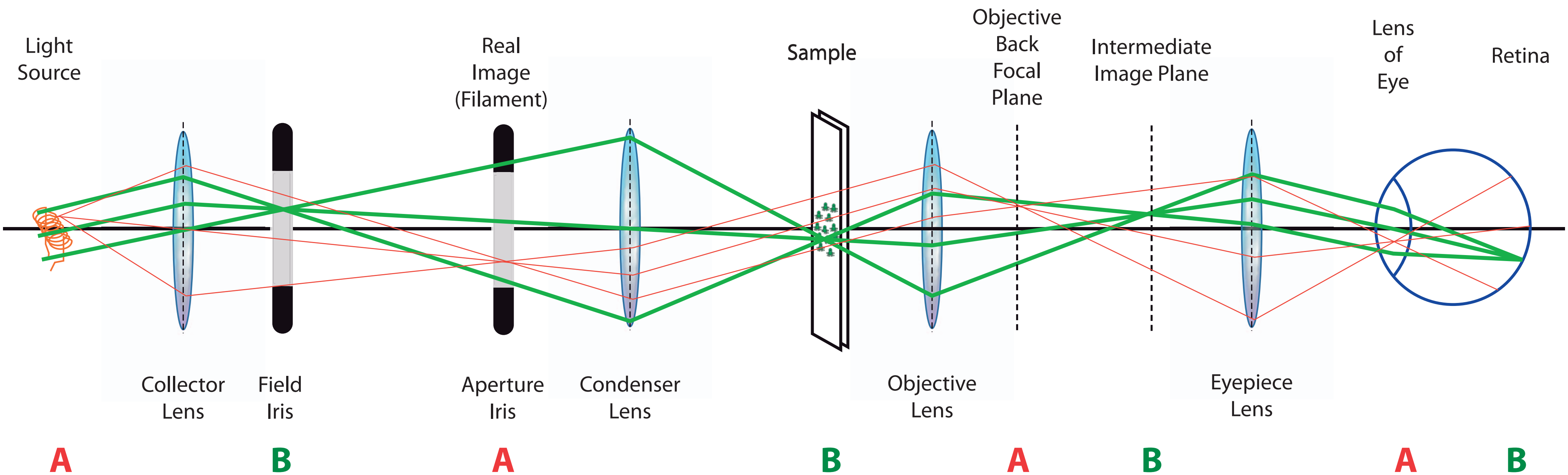
Kohler Illumination

How do we best illuminate the sample?



Illumination Conjugate Planes
Light Source
Aperture Iris
Back focal plane of objective
Front lens of eye

Sample Image Conjugate Planes
Field Iris
Sample plane
Intermediate image plane
Retina

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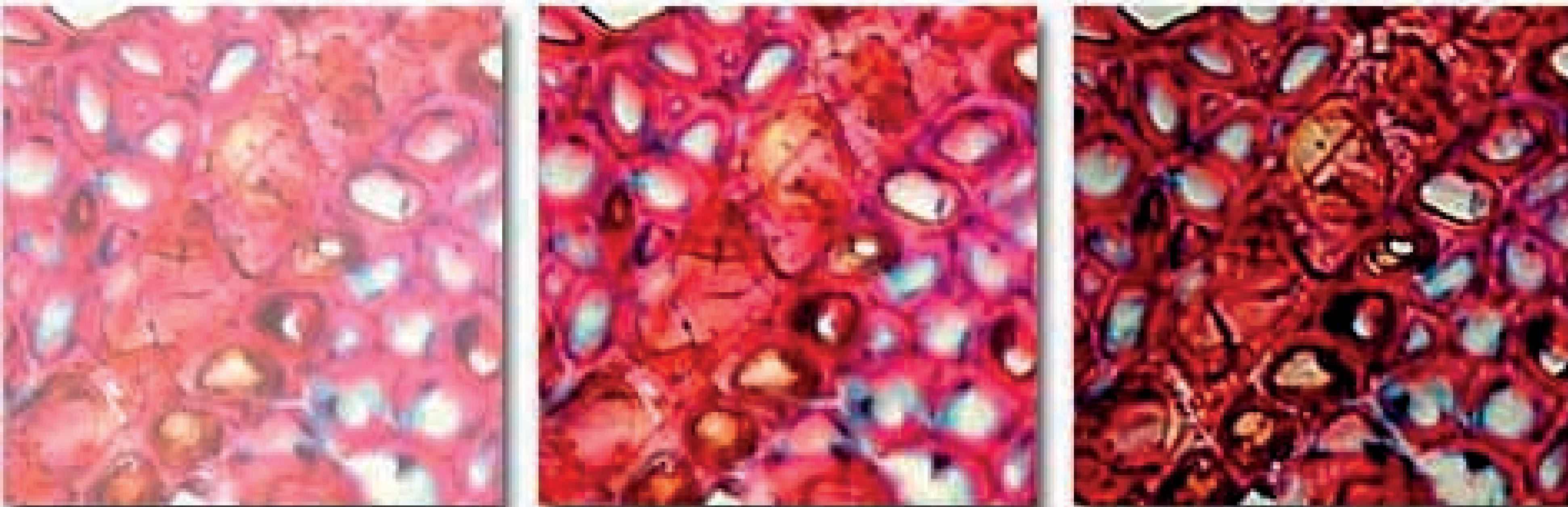
Kohler Illumination

Effect of Aperture Diaphragm on Contrast and Resolution

Photomicrograph of Plum Tree Stem infected with Black Knot Fungush



Objective NA = 0.75
Condenser NA = 0.90

Objective NA = 0.75
Condenser NA = 0.54

Objective NA = 0.75
Condenser NA = 0.18

Image take from Olympus Microscopy Resource Center Website

Kohler Illumination

Light Pathways in an upright microscope

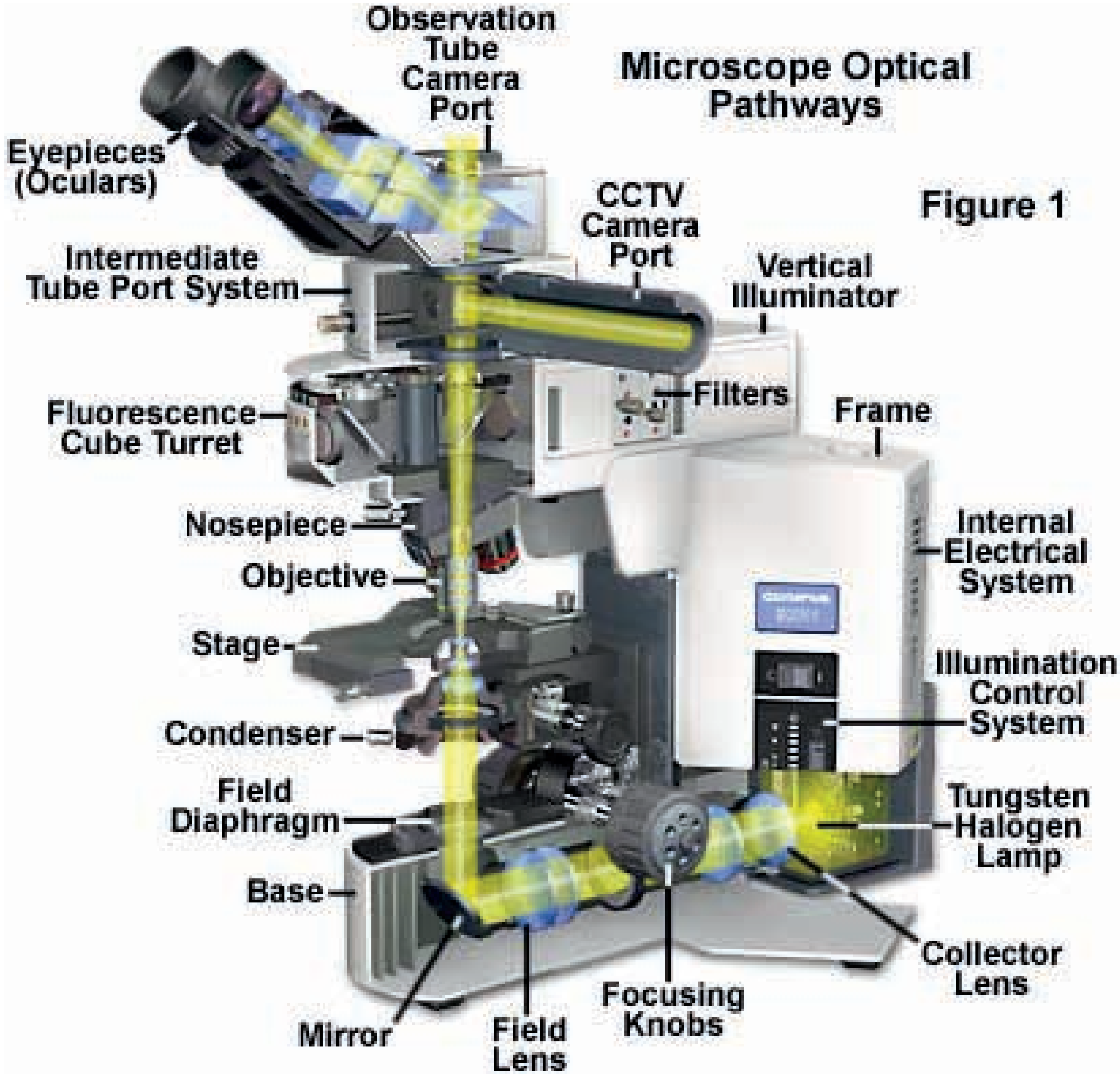


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Image take from Olympus Microscopy Resource Center Website

Kohler Illumination

Light Pathways in an inverted microscope

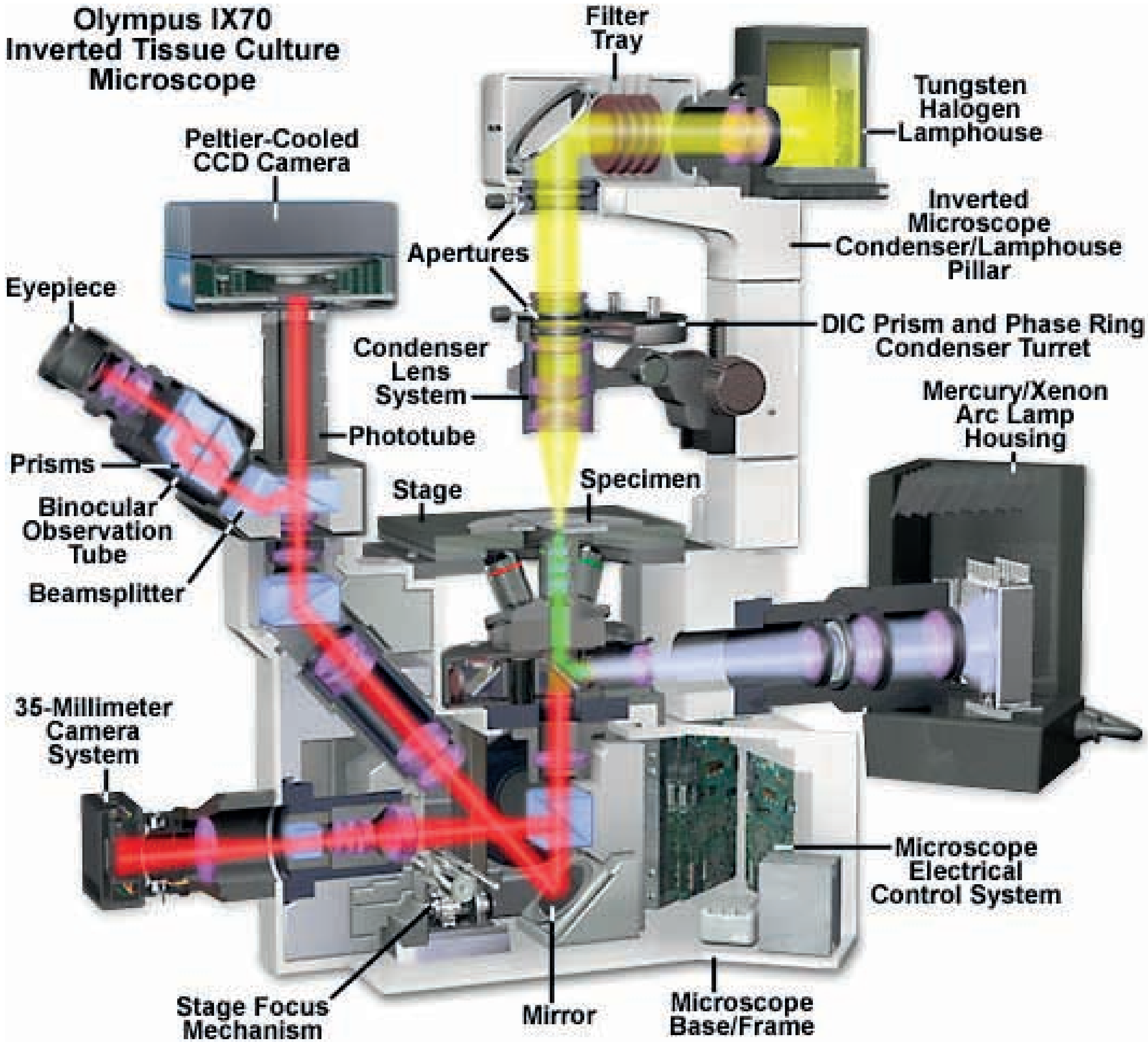

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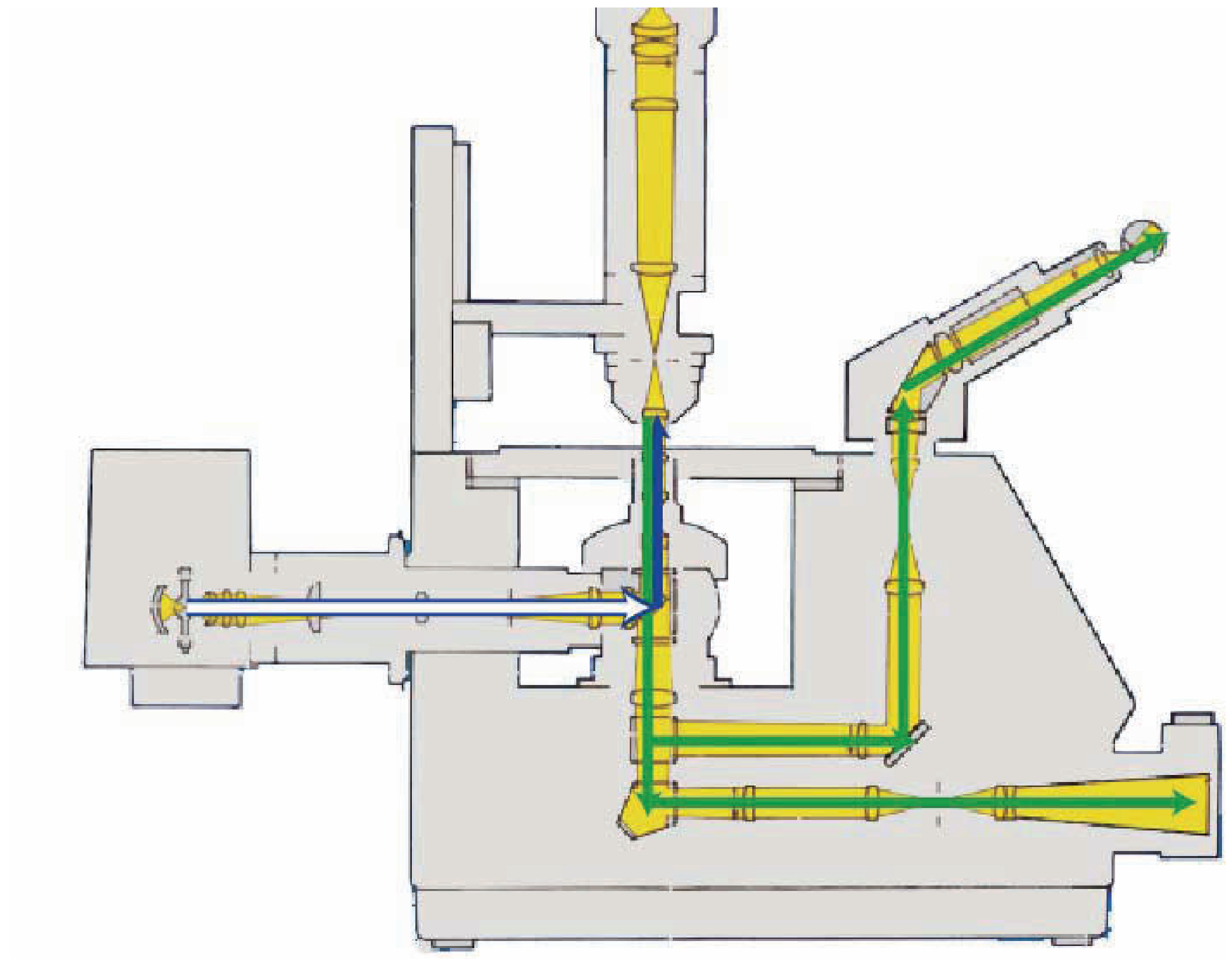
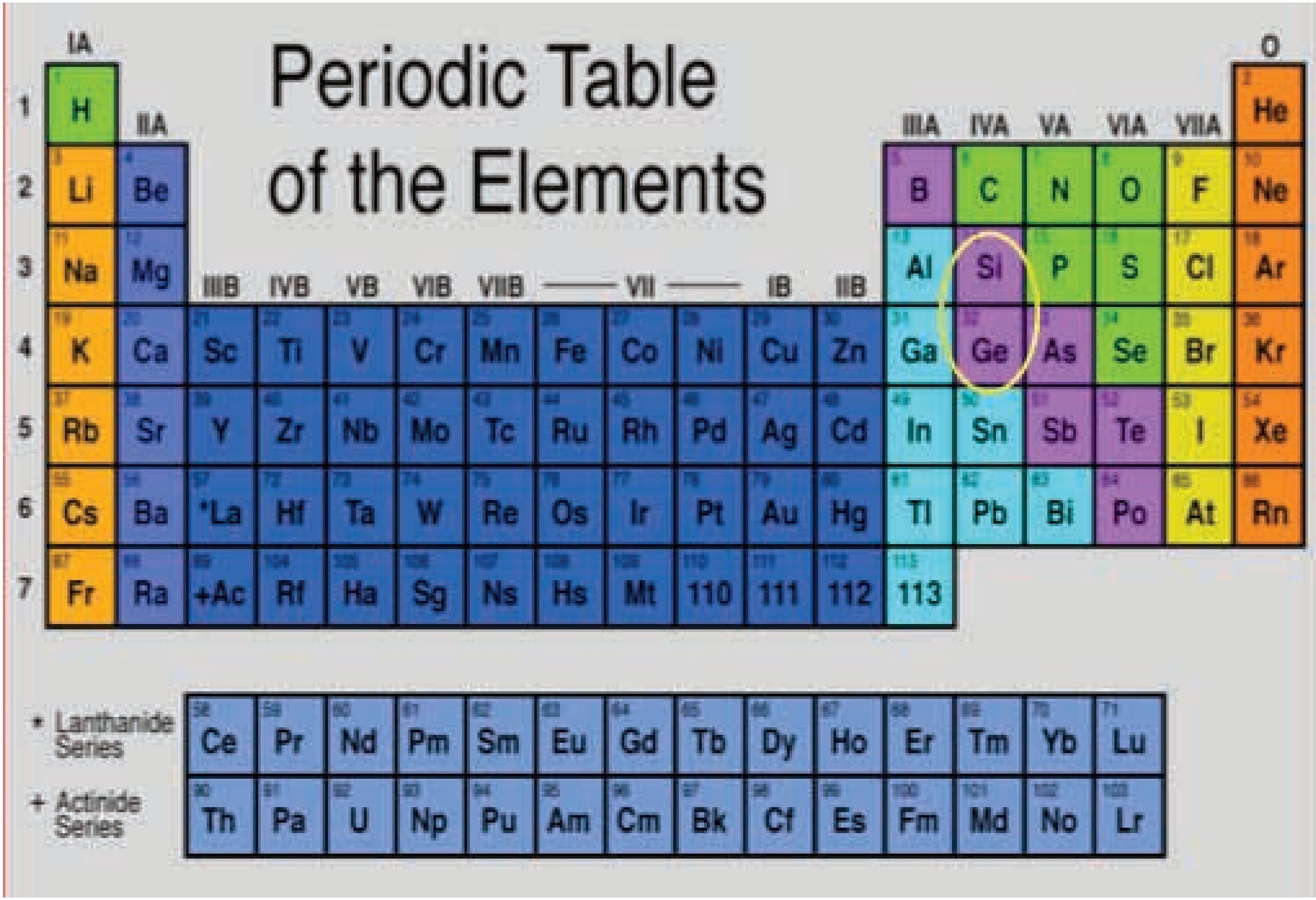
Image take from Olympus Microscopy Resource Center Website

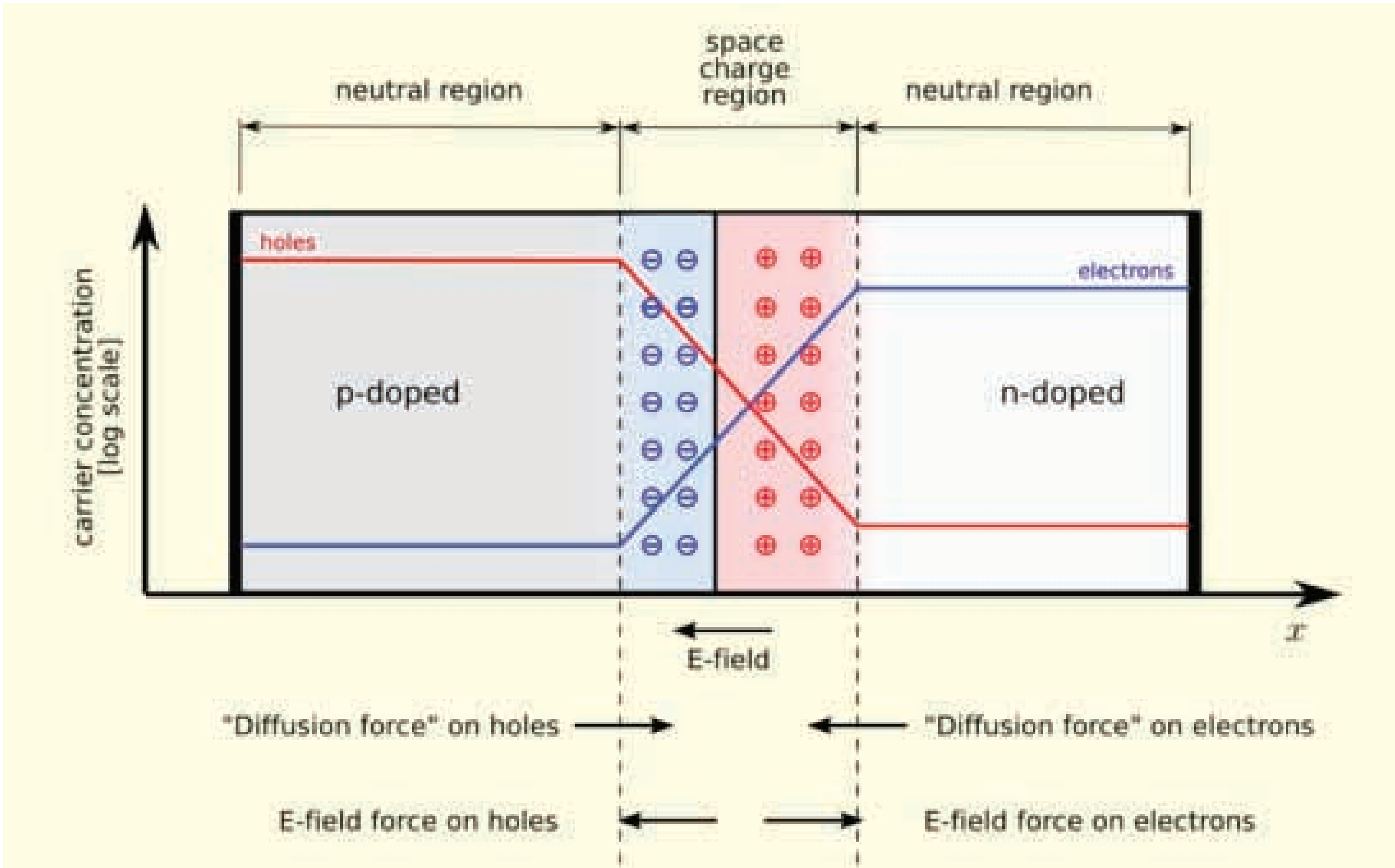
Optical schematic of an inverted epifluorescence microscope



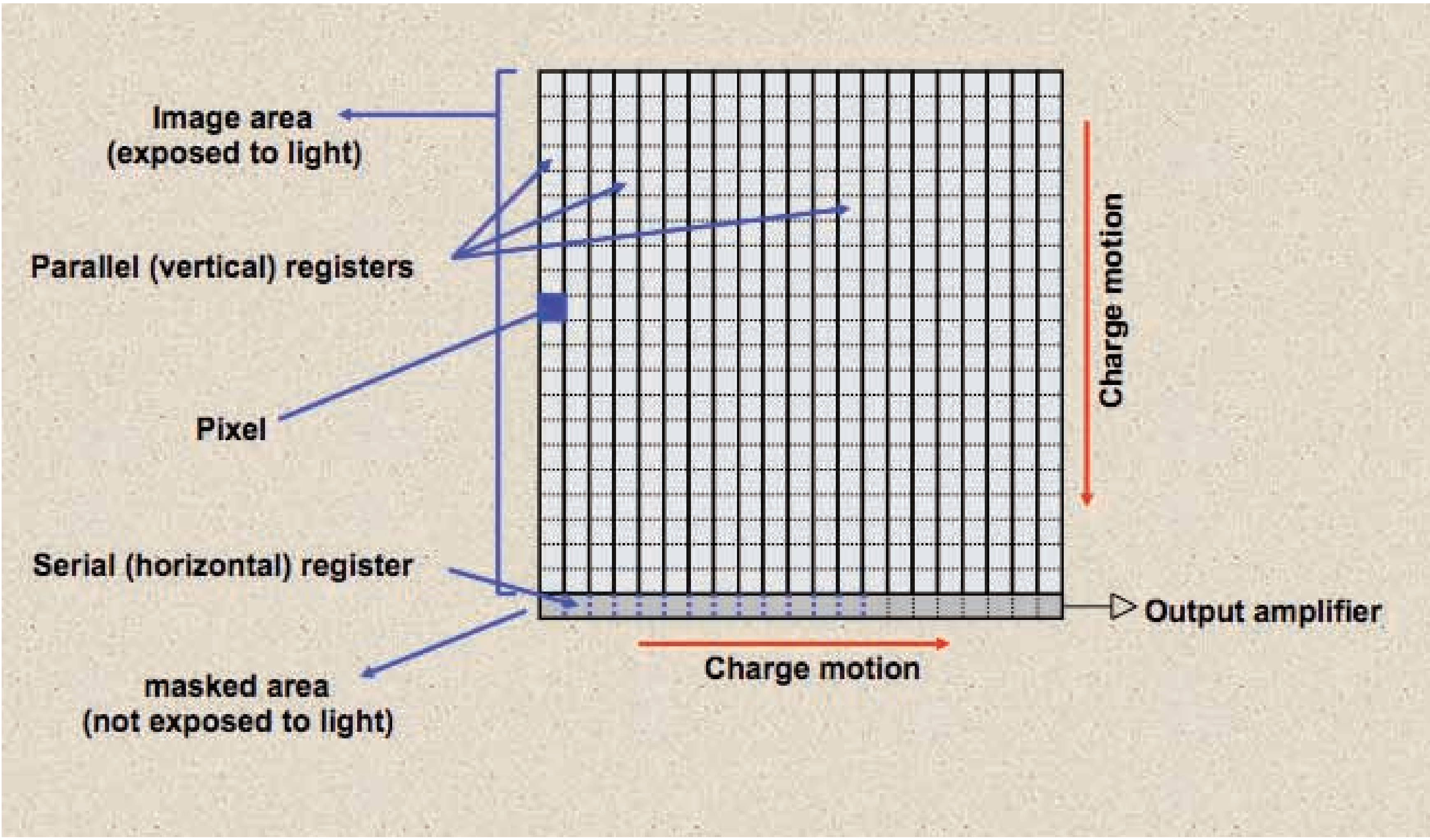
Philbert S. Tsai, July 28,2010


Periodic Table of the Elements
IA
IIA
IIIB IVB VB VIB VIIB VII IB IIB
IIIA IVA VA VIA VIIA
0
1 H He
2 Li Be B C N O F Ne
3 Na Mg Al Si P S Cl Ar
4 K Ca Sc Ti V Cr Mn Fe Co Ni Cu Zn Ga Ge As Se Br Kr
5 Rb Sr Y Zr Nb Mo Tc Ru Rh Pd Ag Cd In Sn Sb Te I Xe
6 Cs Ba *La Hf Ta W Re Os Ir Pt Au Hg Tl Pb Bi Po At Rn
7 Fr Ra +Ac Rf Ha Sg Ns Hs Mt 110 111 112 113
* Lanthanide Series
Ce Pr Nd Pm Sm Eu Gd Tb Dy Ho Er Tm Yb Lu
+ Actinide Series
Th Pa U Np Pu Am Cm Bk Cf Es Fm Md No Lr

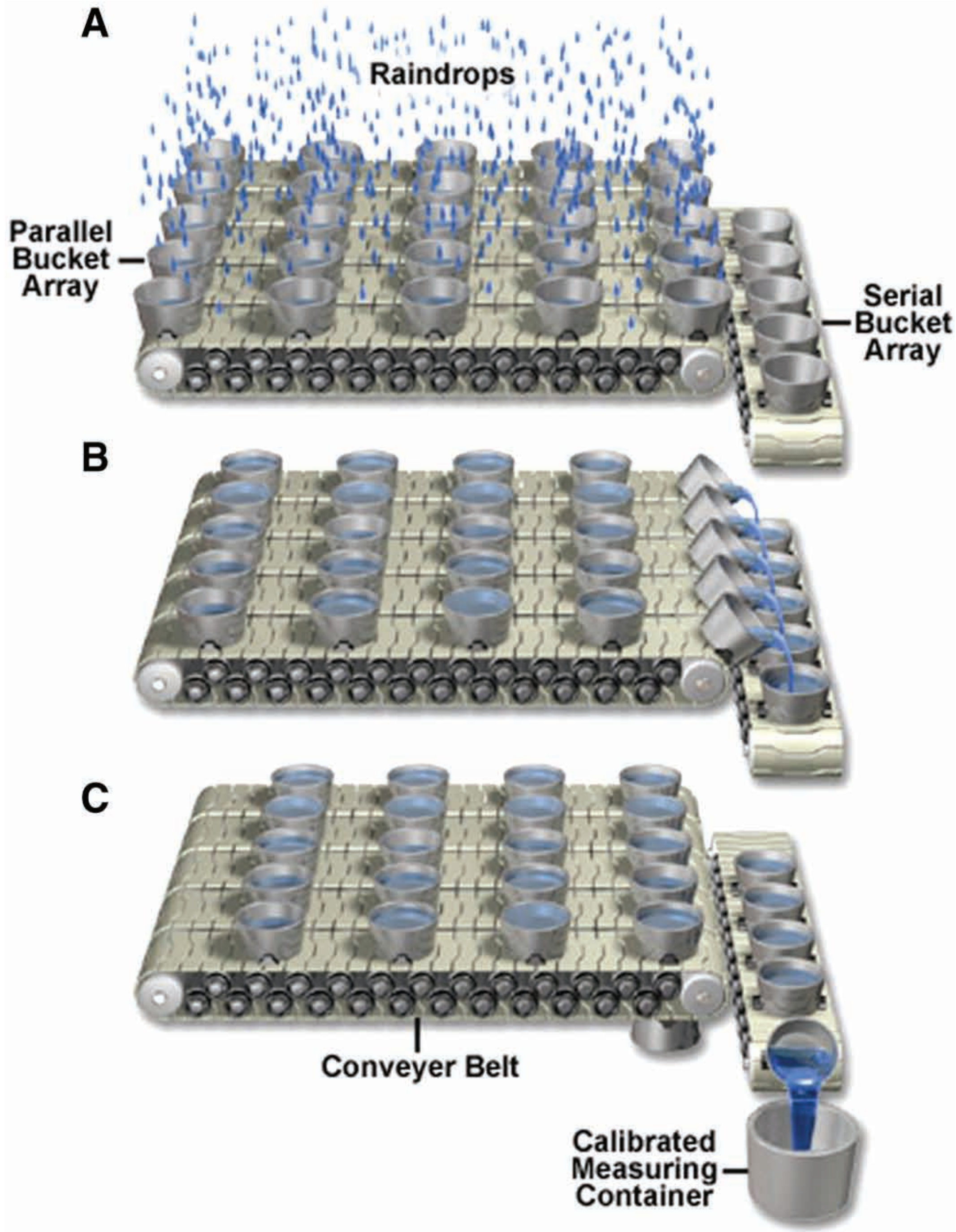
PN Semiconductor Junction



CCD Chip Layout



Charge Coupled Device (CCD) Charge Transfer



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CCD Performance Categories

- Charge generation

  Quantum Efficiency (QE), Dark Current

- Charge collection

  full well capacity, pixels size, pixel uniformity, defects, diffusion (Modulation Transfer Function, MTF)

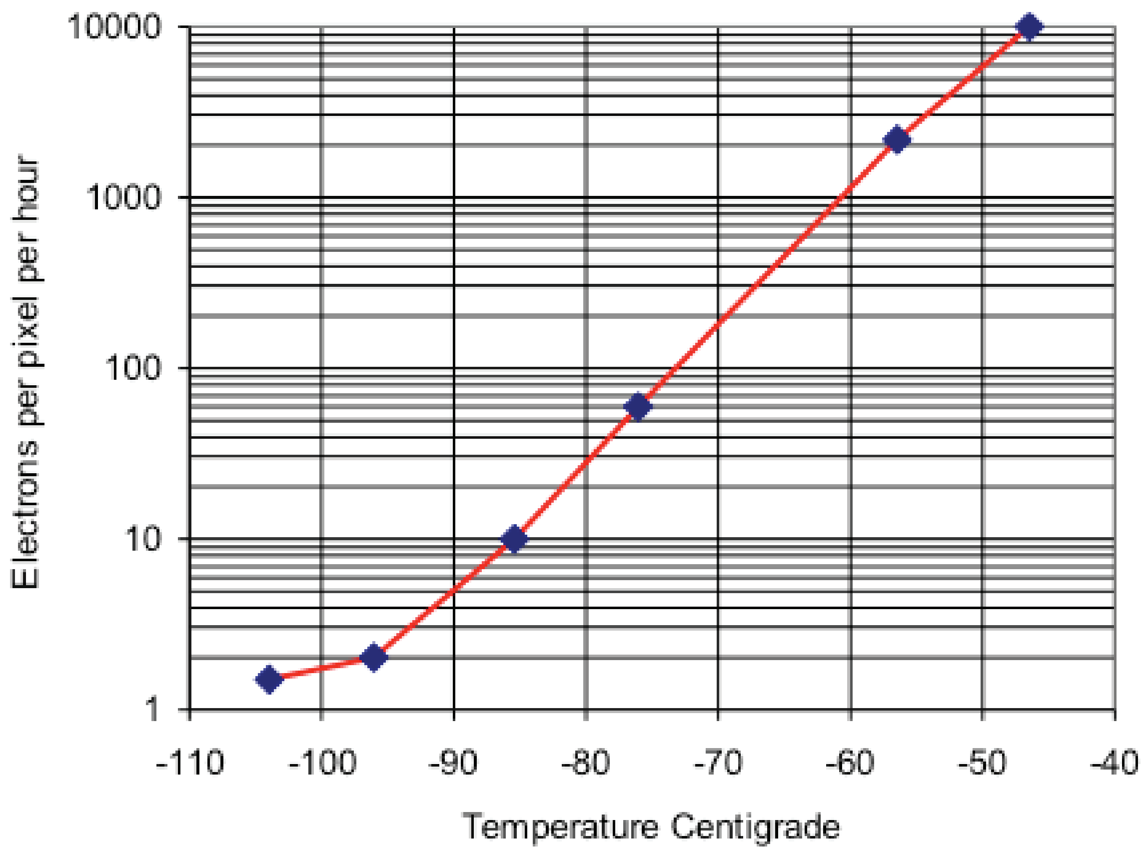
- Charge transfer

  Charge transfer efficiency (CTE), defects

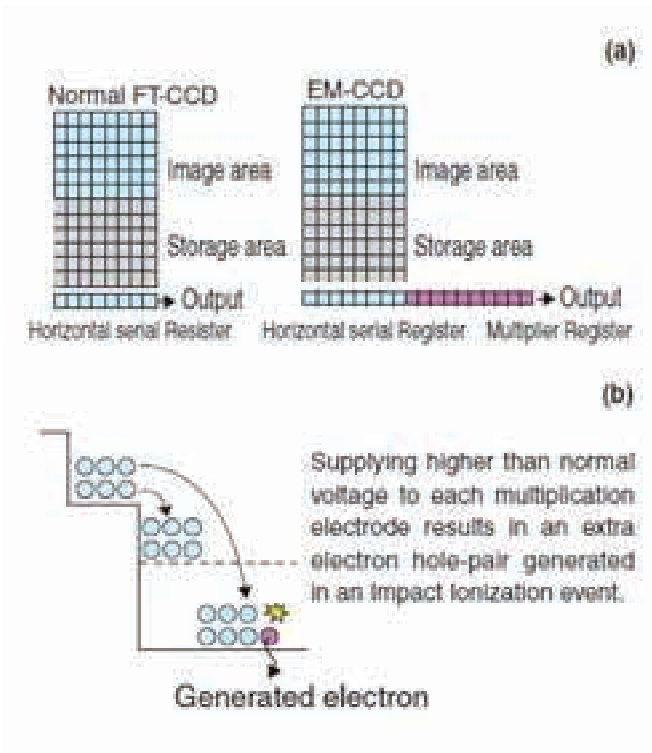
- Charge detection

  Readout Noise (RON), linearity

Temperature Sensitive of CCDs (Dark Current)

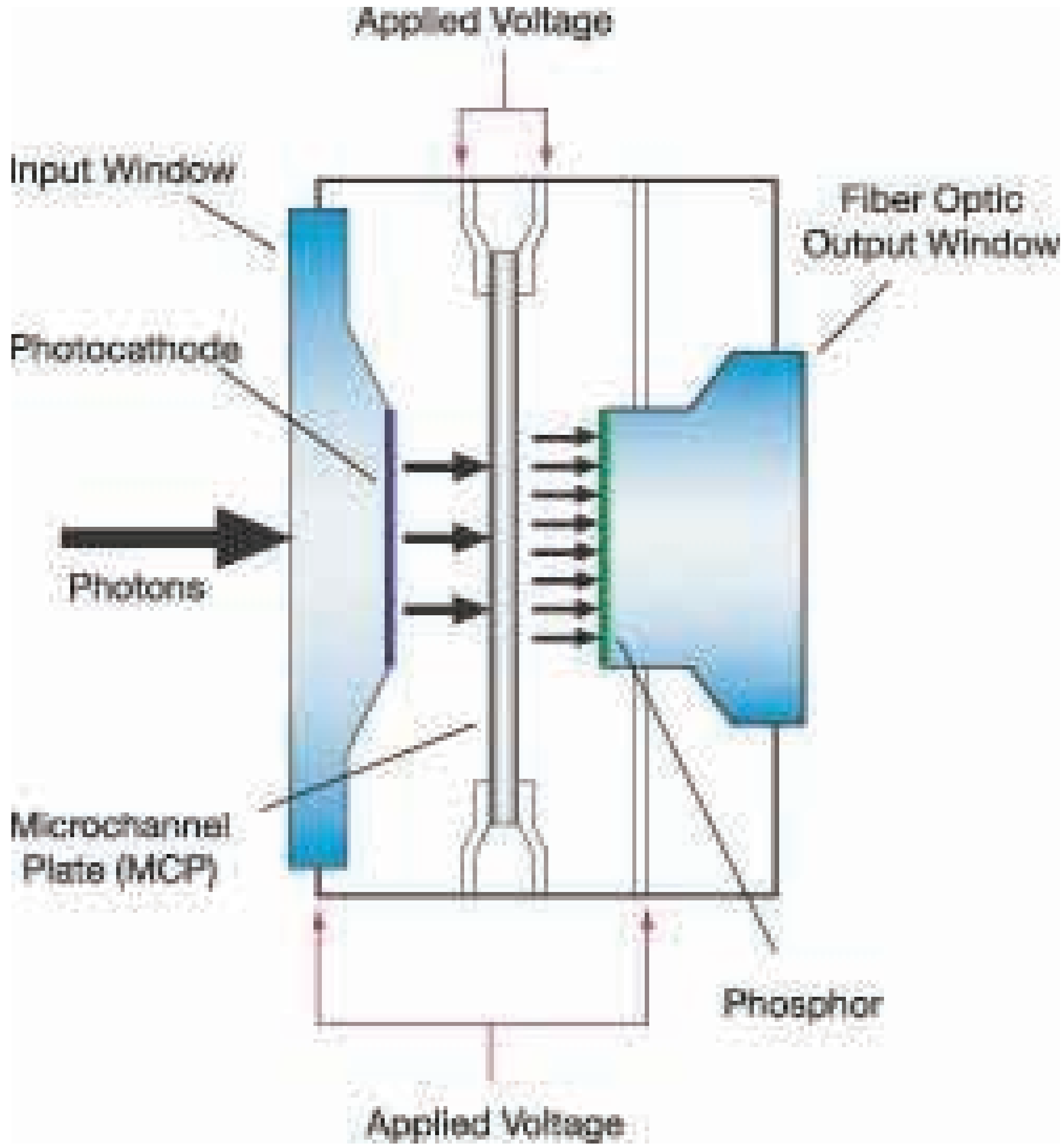


Electron Multiplied Charge Coupled Device (EMCCD)



Per Transfer Gain ~ 1.02
but over 500 transfer
Total Gain is > 1000x

Intensified Charge Coupled Device (ICCD)



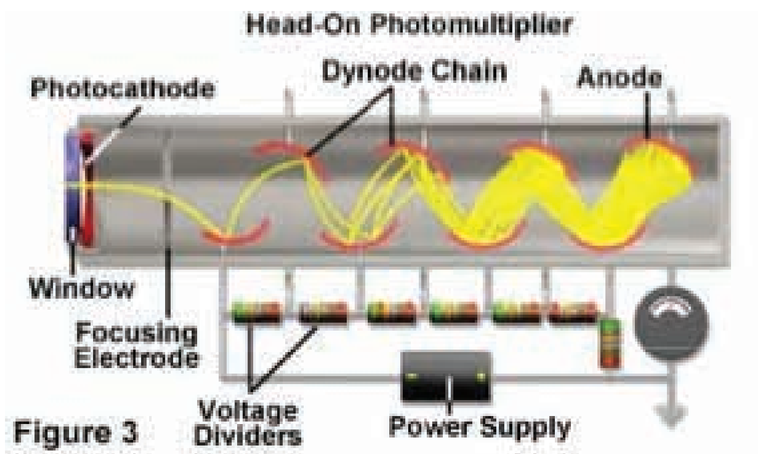
Taken from Andor.com

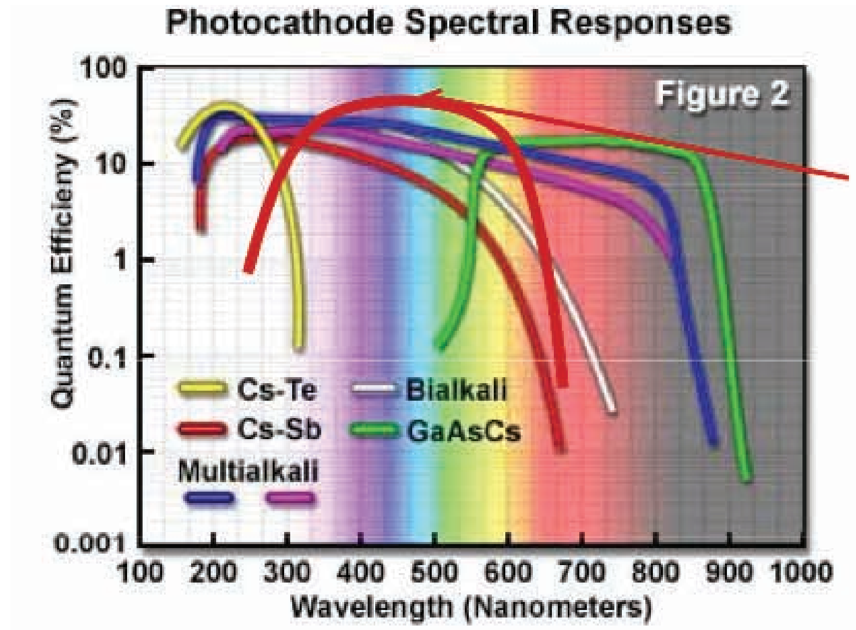
Charge Coupled Device (CCD) Technologies

Detector Type	Advantages	Disadvantages
EMCCD	Single Photon Sensitive High and broad QE Good resolution - Pixel limited Good dynamic range possible Fast or slow readout Flexible - Operate as EMCCD or CCD (*gain can be turned off*). Conventional CCD amplifier on some sensors No photocathode! Relatively affordable (broad range of pricing, largely sensor dependent)	No nano or picosecond gating (*microsecond gating available on some recent interline EMCCD sensors*) Multiplication noise (*effectively increases shot noise by x1.41*)
CCD	High and broad QE Good resolution – pixel limited Good dynamic range possible No multiplication noise No photocathode! Greater choice of sensor formats available	Read noise limited - not single photon sensitive Limited readout speed due to read noise restraints
ICCD	Single Photon Sensitive Nano and Picosecond time-resolved gating possible Fast or slow readout NIR photocathode options	QE restricted by photocathode (<50% max) Poor dynamic range – need to operate at high gains Cross-talk between channels of MCP – increased point spread function Higher multiplication noise Artefacts, e.g. halo, chickenwire Inflexible – no 'CCD mode' Expensive Damage susceptible – longevity issues

Taken from emccd.com

Photo Multiplier Tube (PMT)





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Taken from Hamammatsu

Photo Multiplier Tube (PMT)

Side-On New Compact Type Photomultiplier Tubes

Type No.	Remarks	Spectral Response: Range (nm)	Spectral Response: Peak Wave-length (nm)	Ⓐ Photo-cathode Material	Ⓑ Window Material	Ⓒ Outline No.	Ⓓ Dynode Structure / No. of Stages	Socket / Socket Assembly	Ⓕ Maximum Ratings: Anode to Cathode Voltage (Vdc)	Ⓕ Maximum Ratings: Ⓖ Average Anode Current (mA)	Cathode Sensitivity Luminous Min. (μA/lm)	Cathode Sensitivity Luminous Typ. (μA/lm)
R6350	For UV to visible range, general purpose.	185 to 650	340	Sb-Cs	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	20	40
R6351	Synthetic silica window type of R6350	160 to 650	340	Sb-Cs	Q	2	CC/9	E678-11U/Ⓔ	1250	0.01	20	40
R6352	High sensitivity variant of R6350	185 to 750	420	BA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	80	120
R6353	Low dark current bialkali photocathode	185 to 680	400	LBA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	30	70
R6354	For UV range	160 to 320	230	Cs-Te	Q	2	CC/9	E678-11U/Ⓔ	1250	0.01	—	—
R6355	For UV to near IR range, general purpose	185 to 850	530	MA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	80	150
R6356	High sensitivity variant of R6355	185 to 900	600	MA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	140	250
R6357 *	High sensitivity variant of R6356, Meshless type	185 to 900	450	MA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	350	500
R6358	Low dark current variant of R6356	185 to 830	530	LMA	U	1	CC/9	E678-11U/Ⓔ	1250	0.01	140	200

Taken from Hamammatsu

Photo Multiplier Tube (PMT)

Cathode Sensitivity			Anode to Cathode Supply Voltage (J)	Anode Characteristics (K)								Notes	Type No.
Blue (5-58) Typ. (µA/lm-b)	Red/White Ratio Typ. (H)	Radiant Typ. (mA/W) (I)	(Vdc)	Anode Sensitivity: Luminous Min. (A/lm)	Anode Sensitivity: Luminous Typ. (A/lm)	Anode Sensitivity: Radiant Typ. (nm) (I)	Current Amplification Typ.	Anode Dark Current (After 30 min.) Typ. (nA)	Anode Dark Current (After 30 min.) Max. (nA)	Time Response: Rise Time Typ. (ns)	Time Response: Electron Transit Time Typ. (ns)		
5	—	48	1000	50	300	3.6×10^5	7.5×10^6	0.5	5	1.4	15	Photon counting type: R6350P: 10cps Typ.	R6350
5	—	48	1000	50	300	3.6×10^5	7.5×10^6	0.5	5	1.4	15		R6351
10	—	90	1000	100	700	5.2×10^5	5.8×10^6	1	10	1.4	15		R6352
6.5	—	65	1000	100	400	3.7×10^5	5.7×10^6	0.1	2	1.4	15	Photon counting type: R6353P: 10cps Typ.	R6353
—	—	62 (a)	1000	—	—	1.8×10^5 (a)	3×10^6	0.5	5	1.4	15		R6354
6	0.15	45	1000	100	600	1.8×10^5	4×10^6	1	10	1.4	15		R6355
7	0.3	60	1000	400	2500	6×10^5	1×10^7	1	10	1.4	15		R6356
13	0.4	105	1000	1000	2000	4.2×10^5	4×10^6	2	10	1.4	15		R6357 *
7.5	0.15	70	1000	300	700	2.5×10^5	3.5×10^6	0.1	1	1.4	15	Photon counting type: R6358P: 20cps Typ.	R6358

Taken from Hamammatsu

GaAsP PMTs

Metal package PMT with Cooler

Photon Counting Head H7421 Series

Heatsink with fan (A7423) sold separately

The H7421 series are photon counting head devices containing a metal package photomultiplier tube having a GaAsP/GaAs photocathode and a thermoelectric cooler. The thermoelectric cooler reduces thermal noise generated from the photocathode which also offers a high quantum efficiency, allowing measurement to be made with a good S/N ratio even at very low light levels.

The H7421-40 has high sensitivity on wavelength from 300 nm to 720 nm. The H7421-50 is sensitive over a wide spectral range from 380 nm to 890 nm. The photomultiplier tube is maintained at a constant temperature by monitoring the output from a thermistor installed near the photomultiplier tube and regulating the current to the thermoelectric cooler.

Product Variations

Type No.	Spectral Response	Features
H7421-40	300 nm to 720 nm	GaAsP photocathode, QE 40 % at peak wavelength
H7421-50	380 nm to 890 nm	GaAs photocathode, QE 12 % at peak wavelength

Specifications

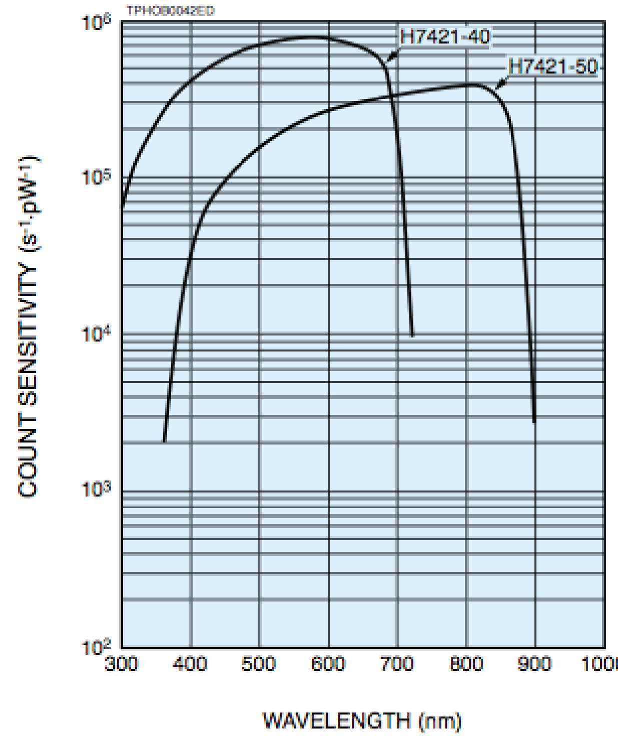
Parameter		H7421-40	H7421-50	Unit
Input Voltage		+4.5 to +5.5		V
Max. Input Voltage for Main Unit		+6		V
Max. Input Current for Main Unit		50		mA
Max. Input Voltage for Thermoelectric Cooler		2.6		V
Max. Input Current for Thermoelectric Cooler		2.2		A
Effective Area		φ5		mm
Peak Sensitivity Wavelength		580	800	nm
Count Sensitivity		7.8×10^5	3.9×10^5	$s^{-1} \cdot pW^{-1}$
Count Linearity *1		1.5×10^6	1.5×10^6	s^{-1}
Dark Count *2 *3	Typ.	100	125	s^{-1}
	Max.	300	375	
Pulse-pair Resolution		70		ns
Output Pulse Width		30		ns
Output Pulse Height *4	Typ.	3.6		V
	Min.	3.0		
Recommended Load Resistance		50		Ω
Signal Output Logic		Positive logic		—
Operating Ambient Temperature		+5 to +35		°C
Storage Temperature		-20 to +50		°C
Weight		340		g

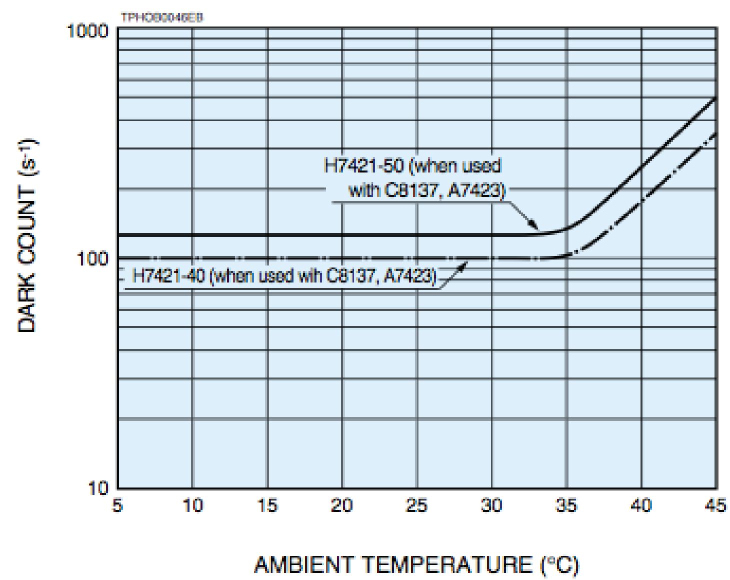
*1: Random pulse, at 10 % count loss
*2: PMT setting temperature 0 °C, used with C8137, M9011 and A7432
*3: After 30 minute storage in darkness
*4: With input voltage +5 V, Load resistance 50 Ω and Coaxial cable RG-174/U (450 mm)

Cooling Specifications

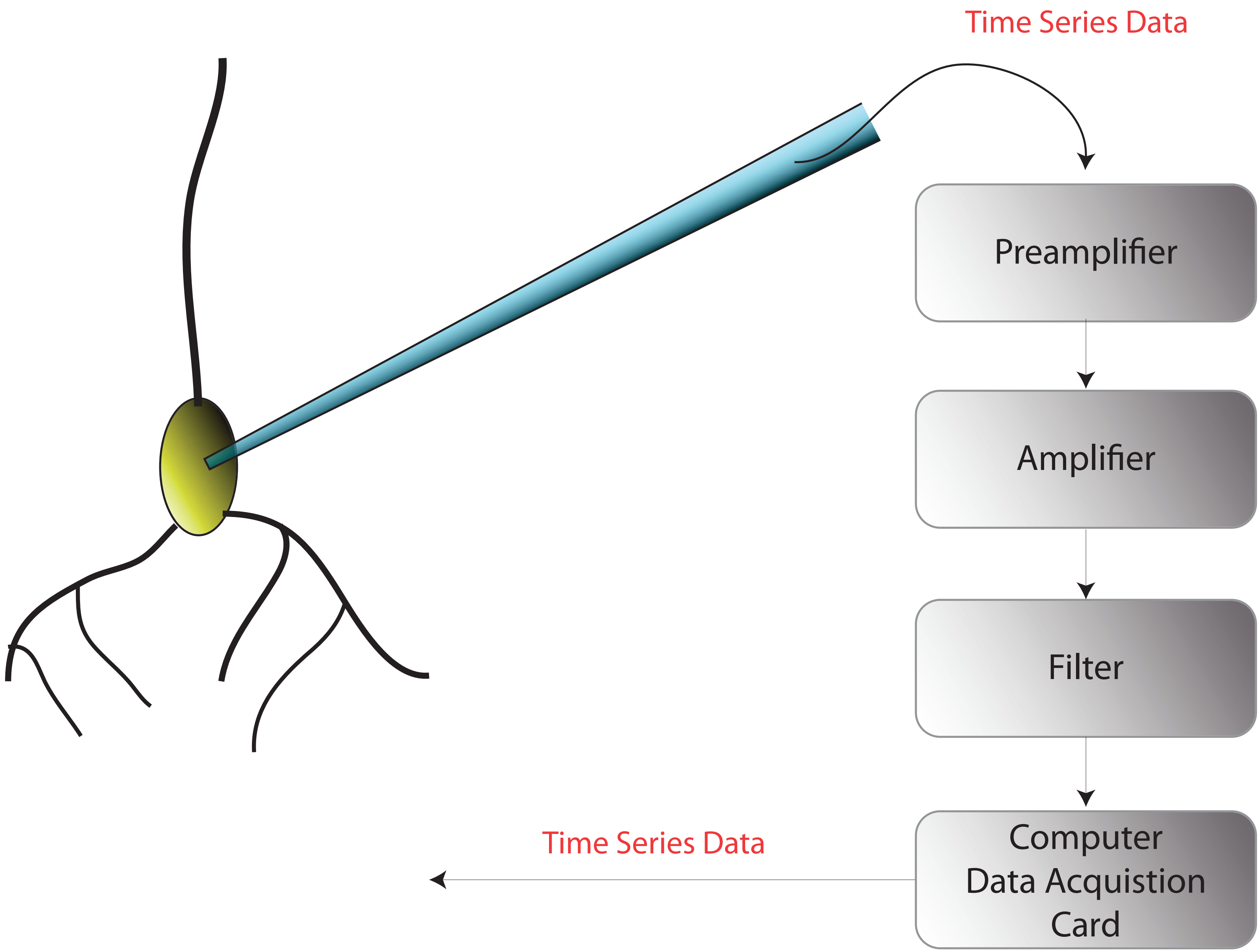
Parameter	H7421-40/H7421-50	Unit
Cooling Method	Thermoelectric cooling	—
Max. Cooling Temperature (ΔT) *5	35	°C
Cooling Time	Approx. 5	min

*5: Input current to thermoelectric cooler = 2.0 A





Data Acquisition



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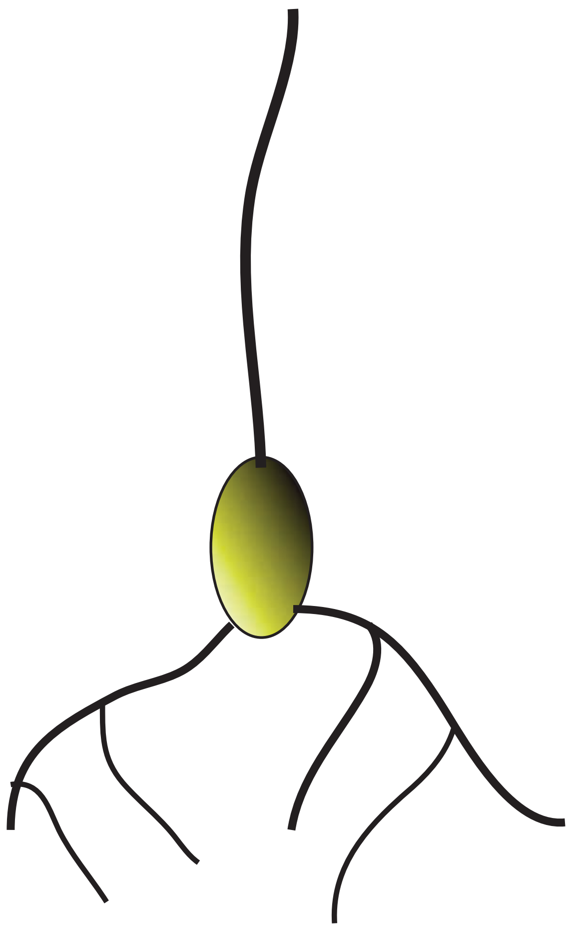
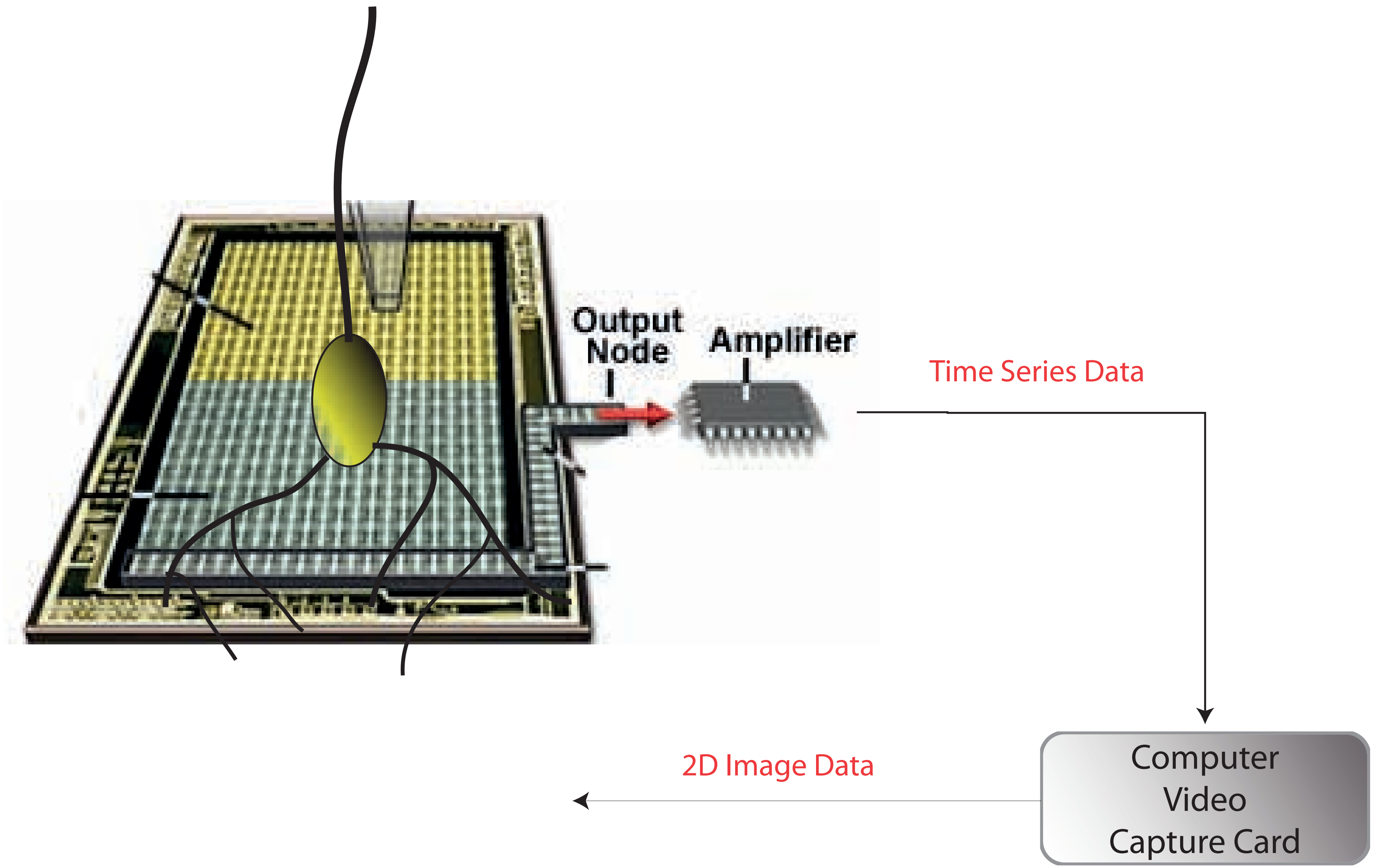
Data Acquisition



Data Acquisition

CCD Camera Imaging



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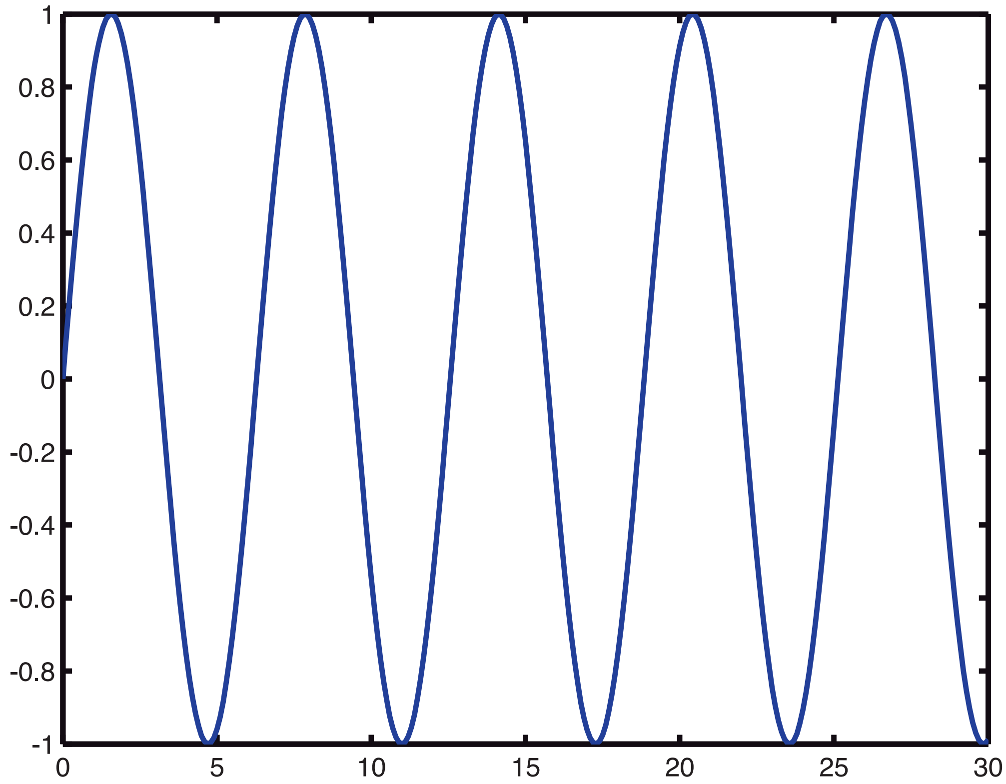
Nyquist Sampling Theorem

How often should you collect data?



Nyquist Sampling Theorem

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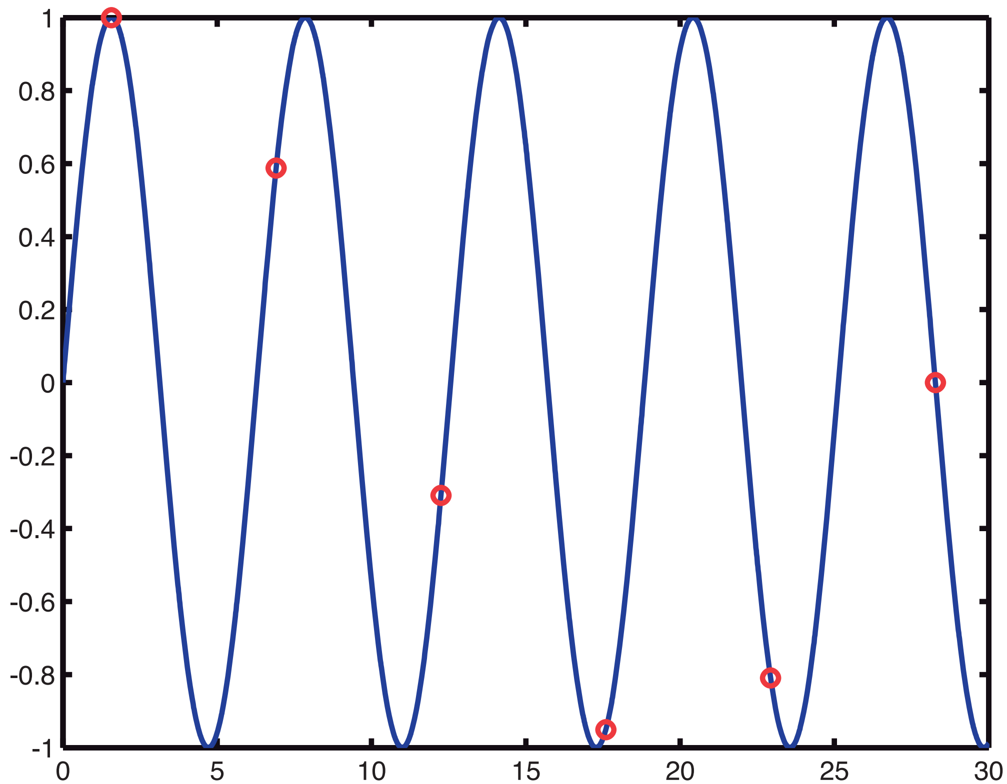

Nyquist Sampling Theorem

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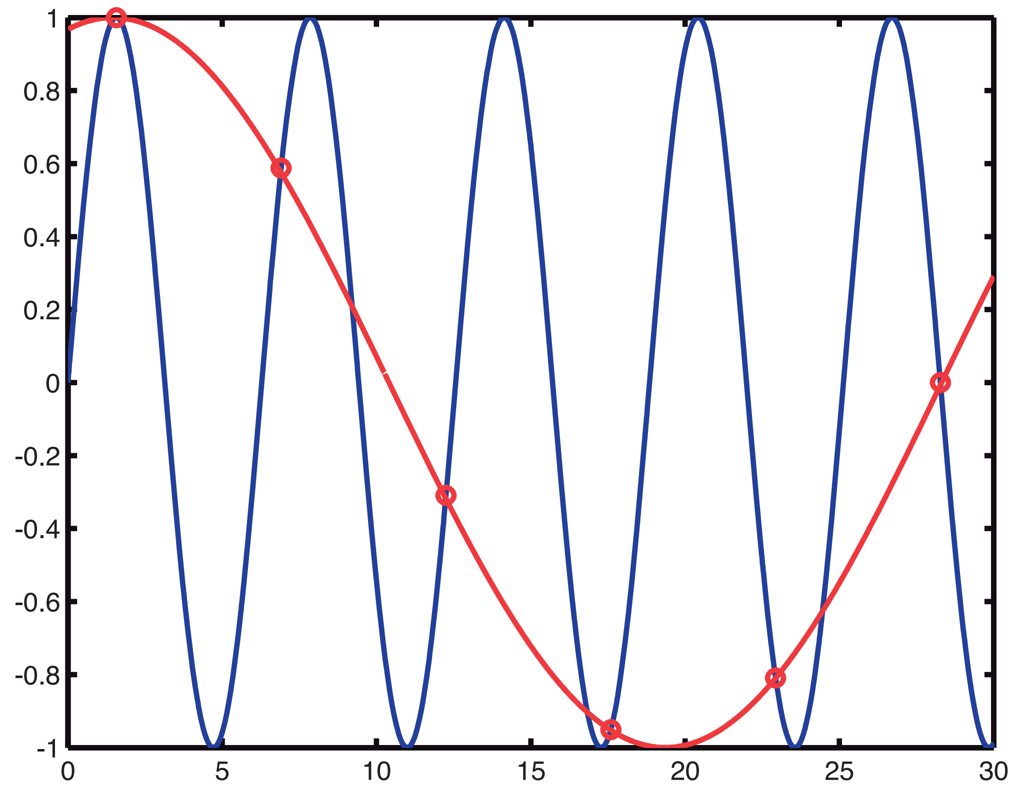

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Nyquist Sampling Theorem

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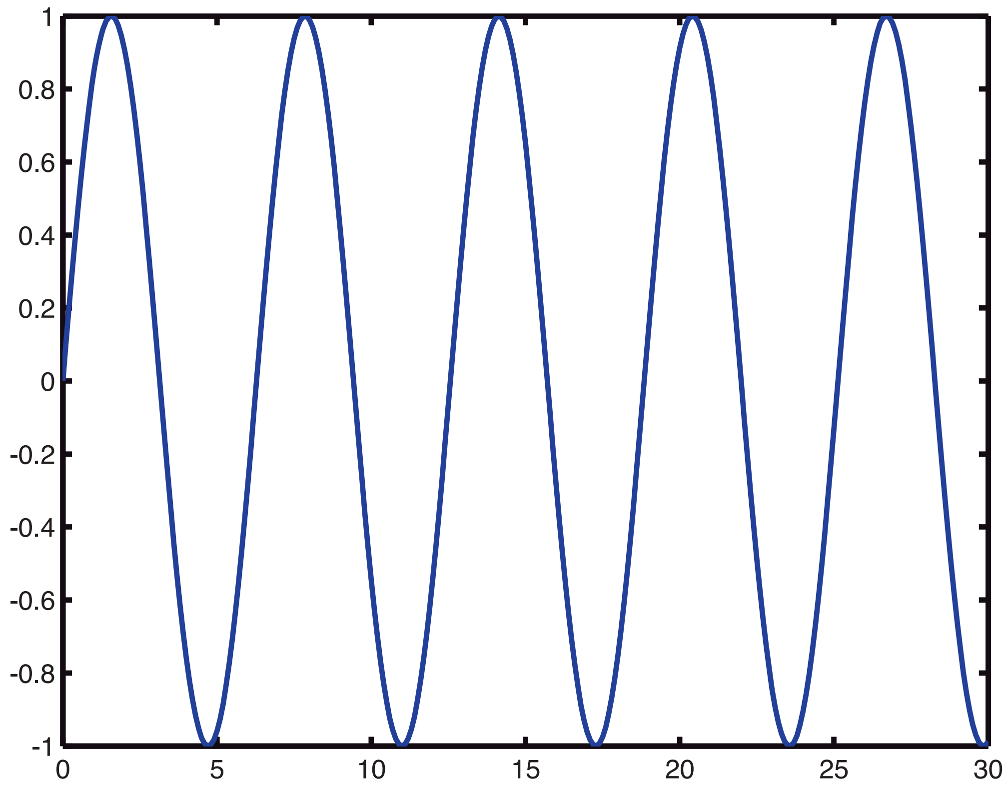

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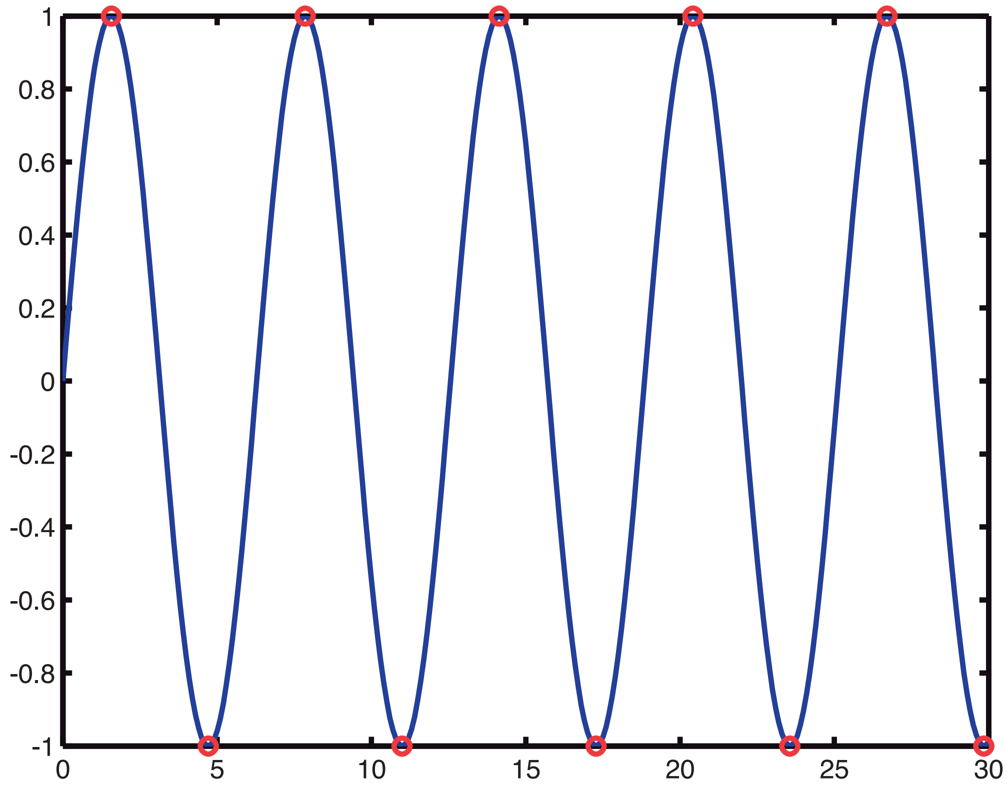

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Nyquist Sampling Theorem

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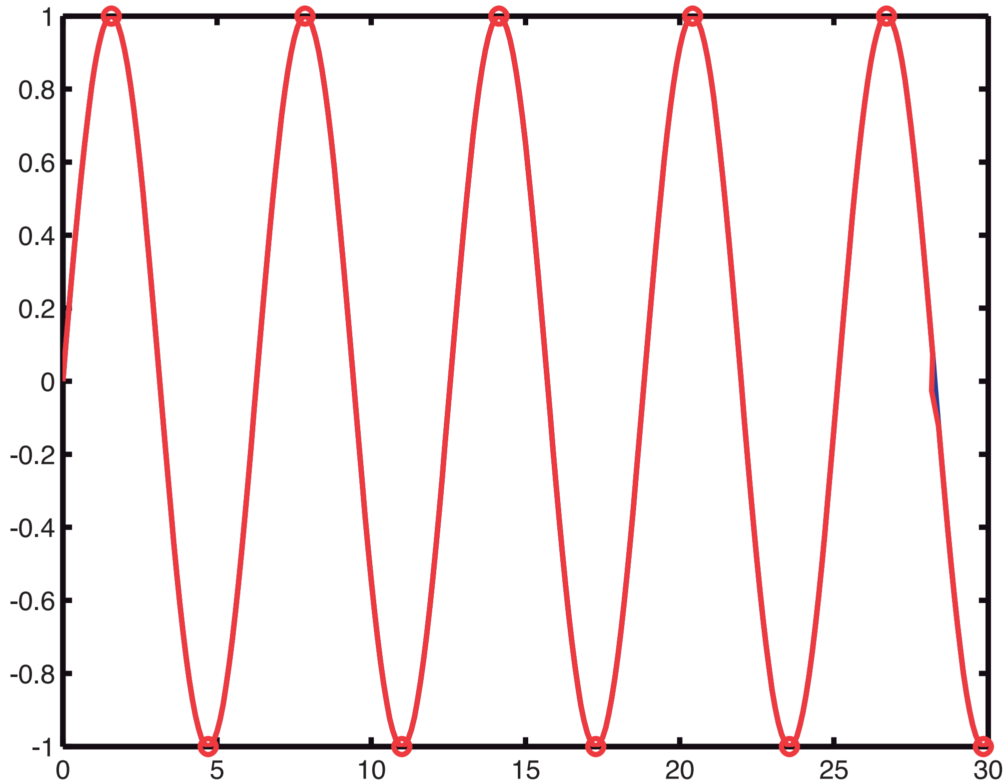

Nyquist Sampling Theorem

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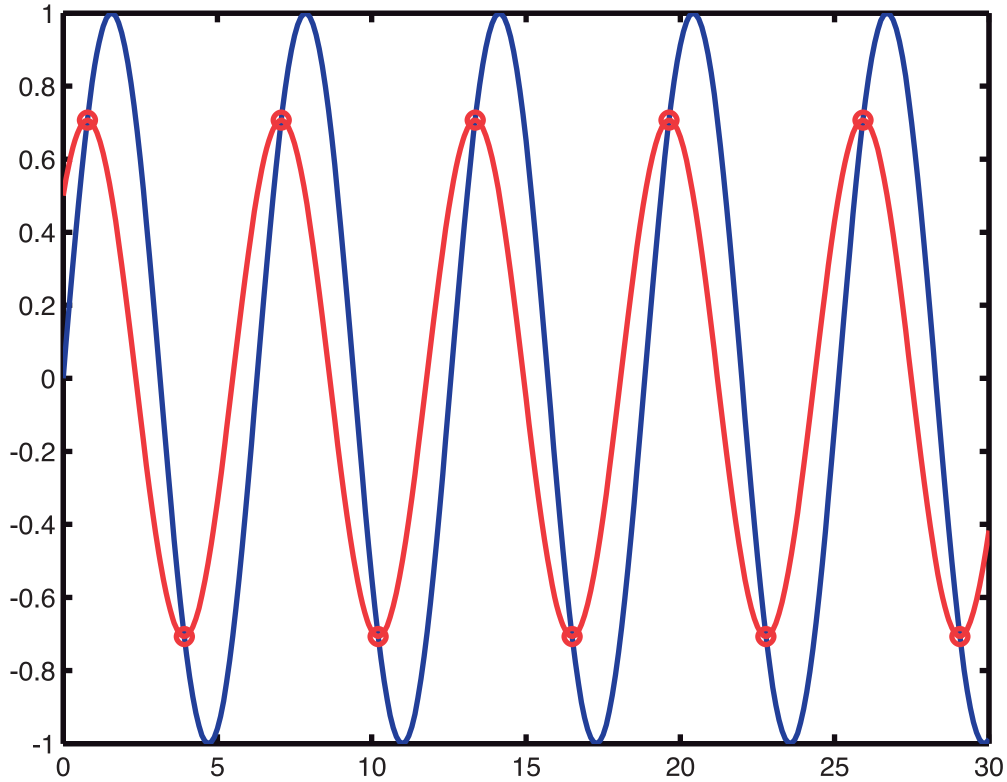

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Nyquist Sampling Theorem

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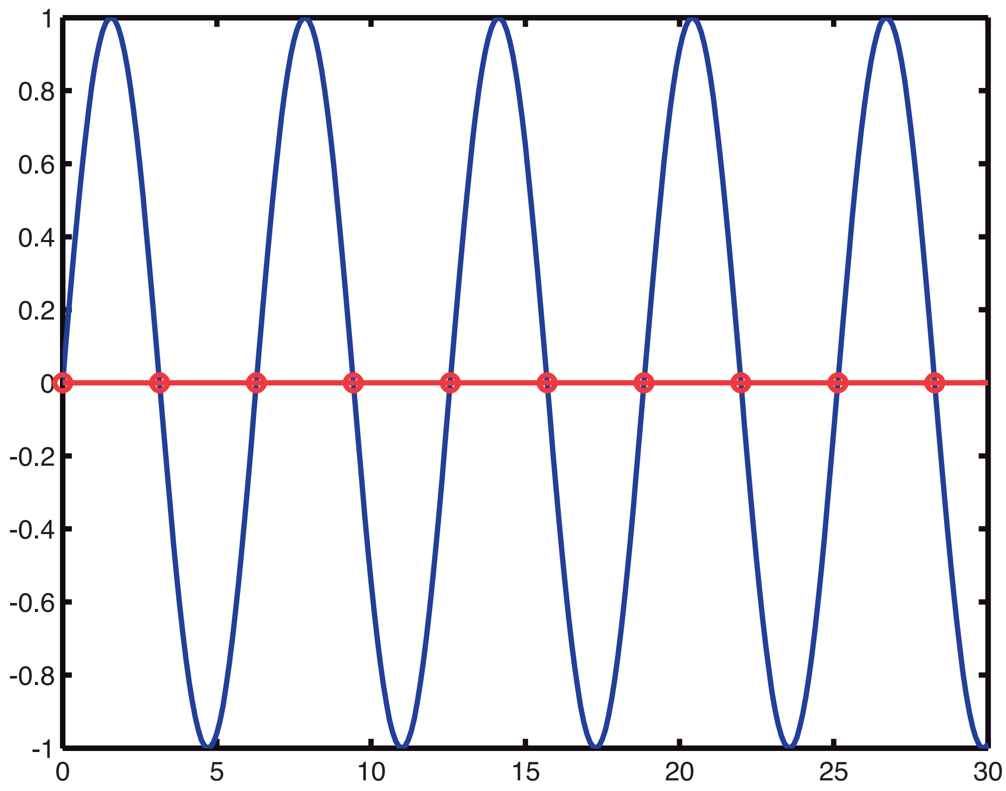

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Nature of Light

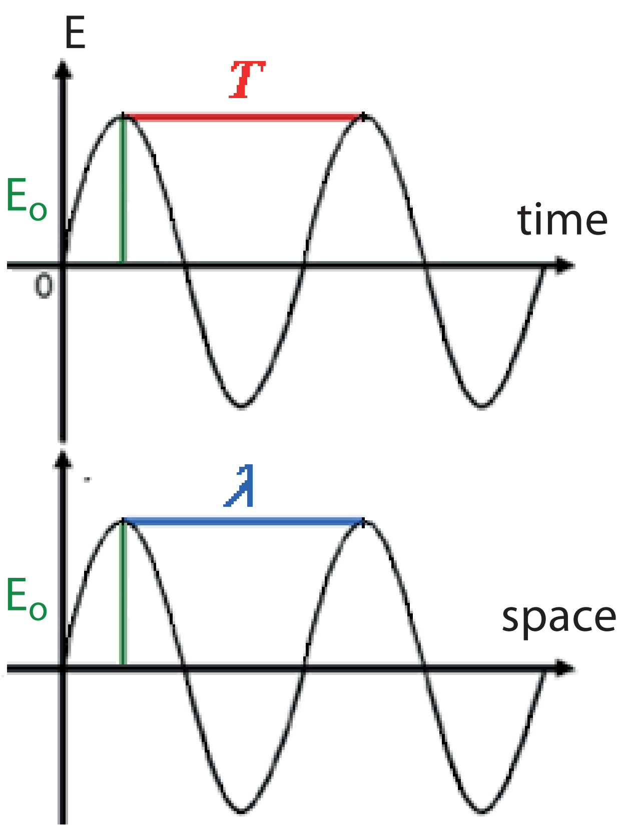
Light can be described as a traveling electromagnetic wave

$$E(r, t) = E_o \sin(k \cdot x - \omega \cdot t + \phi)$$

$\omega = 2\pi \cdot f$ angular frequency

$f = 1 / T$ frequency

$k = 2\pi / \lambda$ wave number



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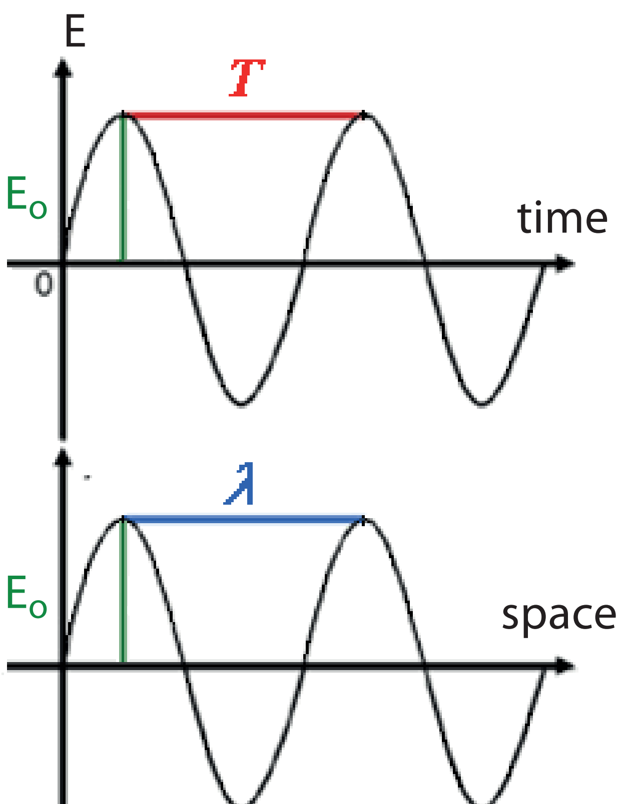
$k = 2\pi / \lambda$ wave number

which is a solution to the wave equation:

$$\frac{\partial^2 E}{\partial x^2} = \frac{1}{c^2}\frac{\partial^2 E}{\partial t^2}$$



c = speed of light in vacuum

$$c = f \cdot \lambda = \omega / k$$

$$I = \text{Intensity} = (c \cdot \varepsilon_o \cdot n / 2) \cdot |E|^2$$

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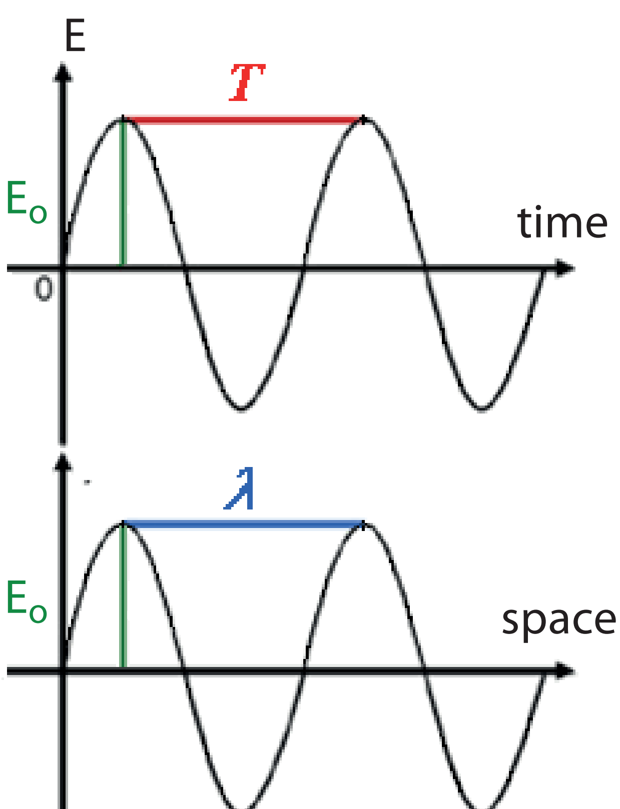
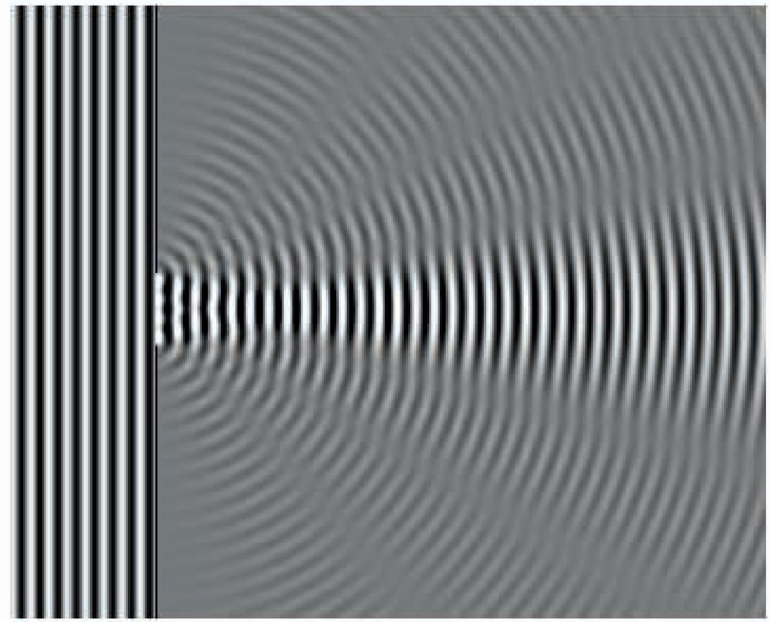
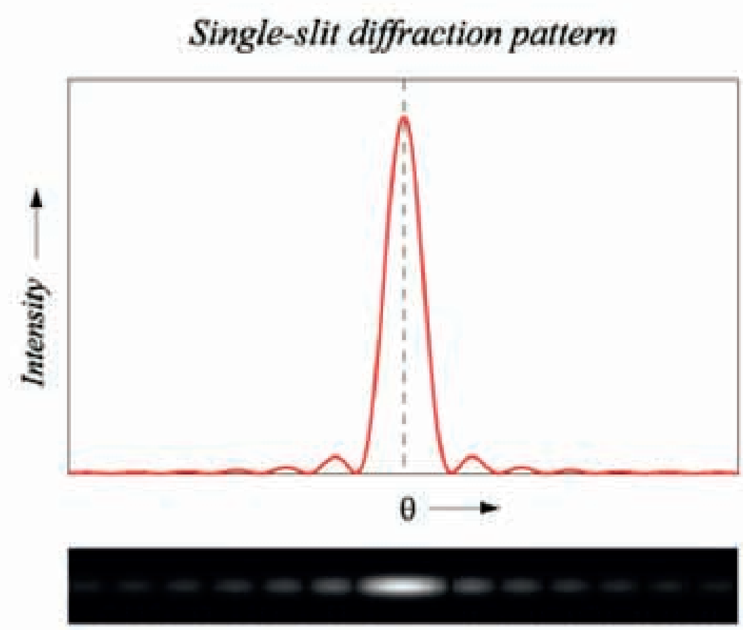
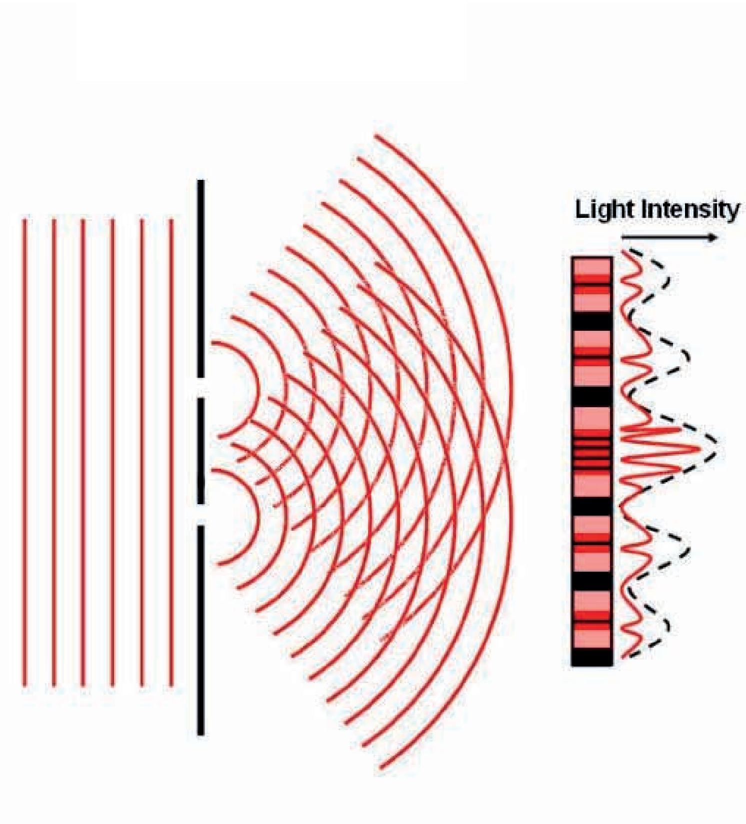




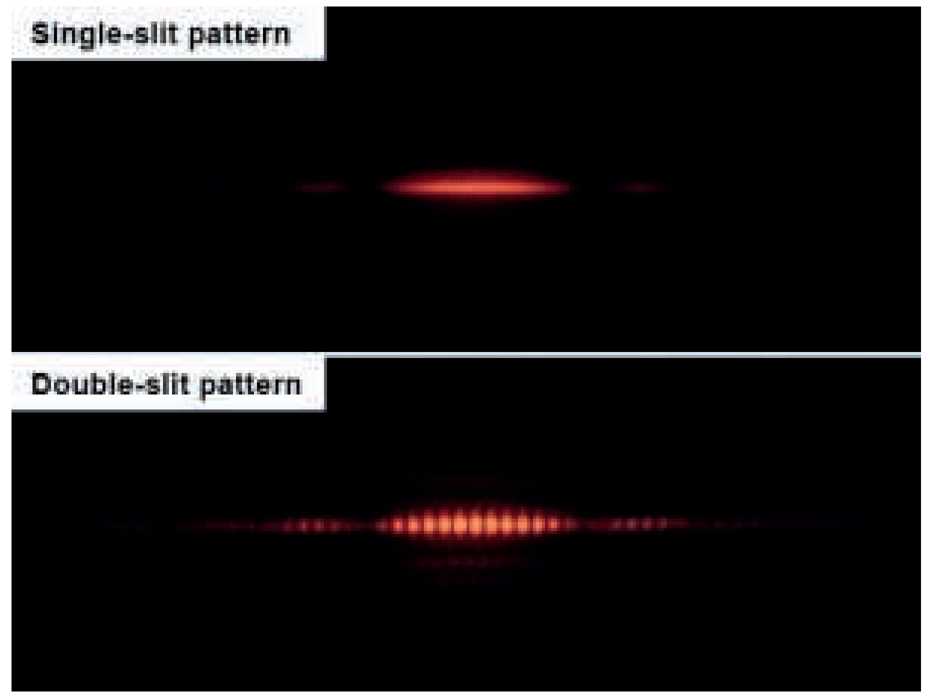


Nature of Light

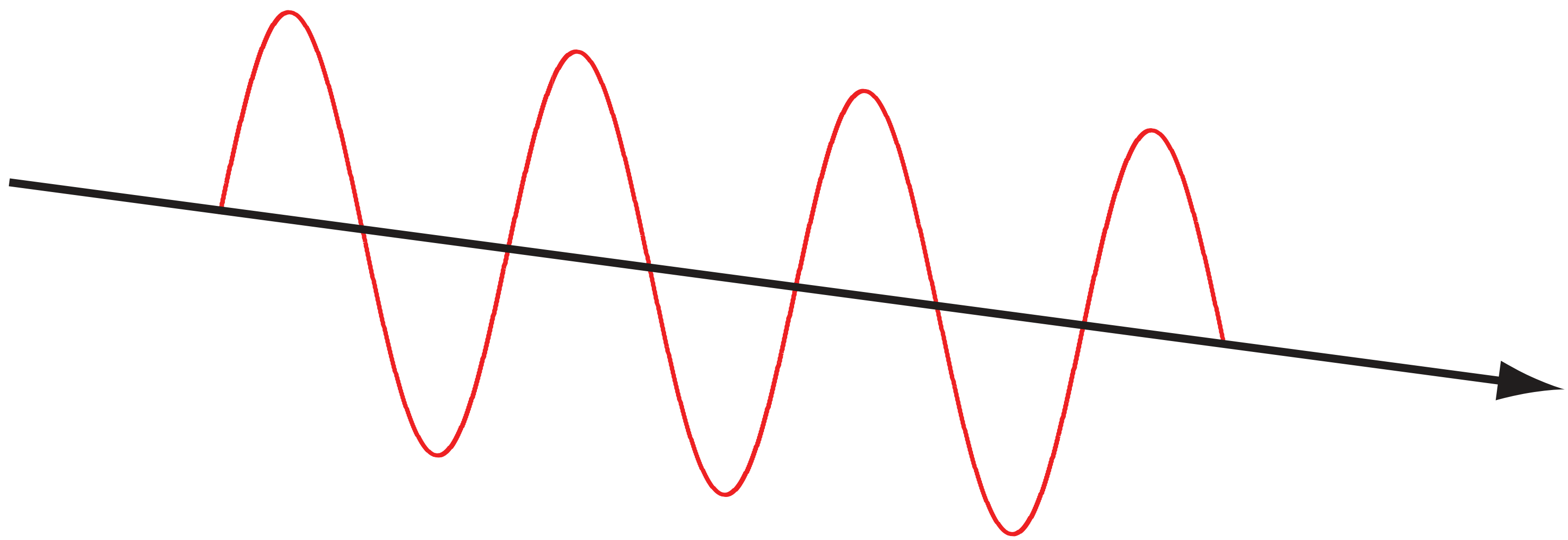
Light can be described as an traveling electromagnetic wave



Double Slit
Diffraction Experiment



Linearly Polarized Light



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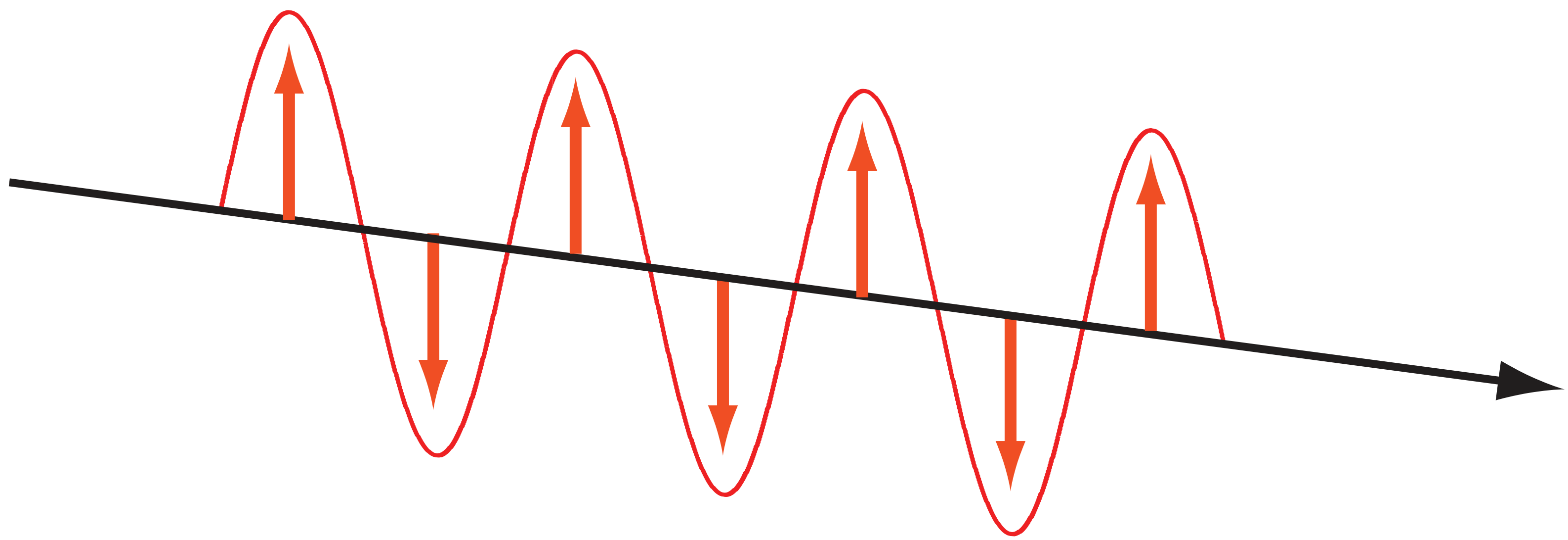
Linearly Polarized Light



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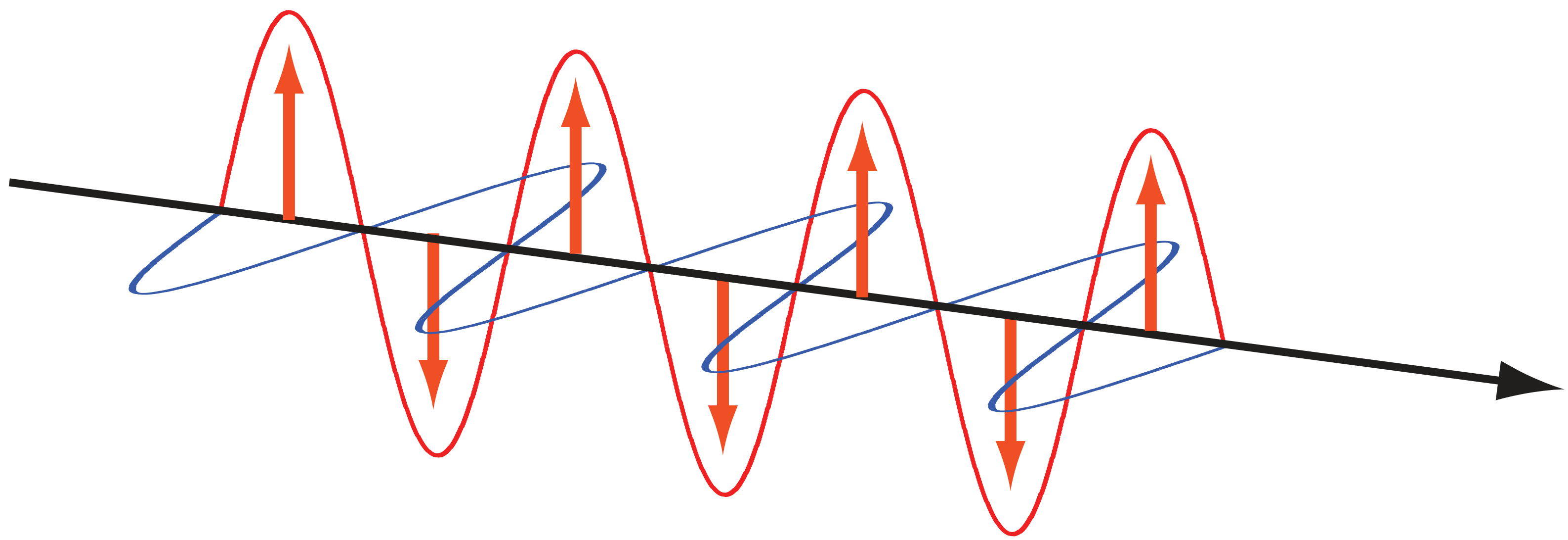

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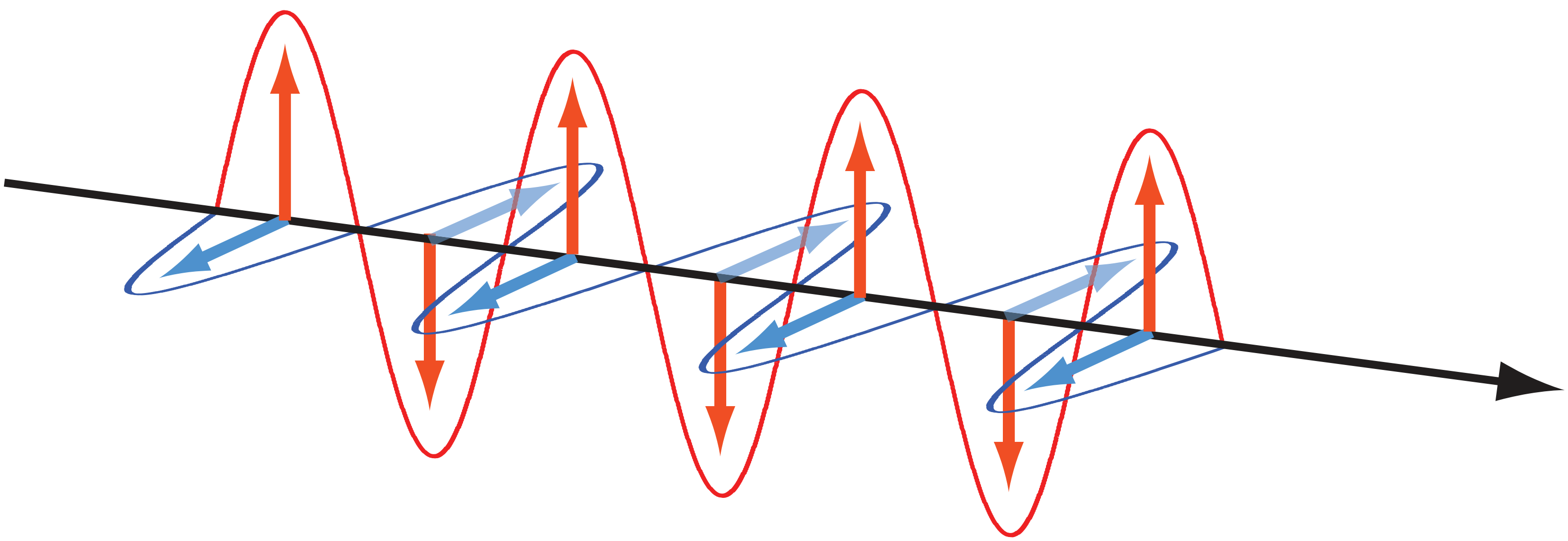
Linearly Polarized Light



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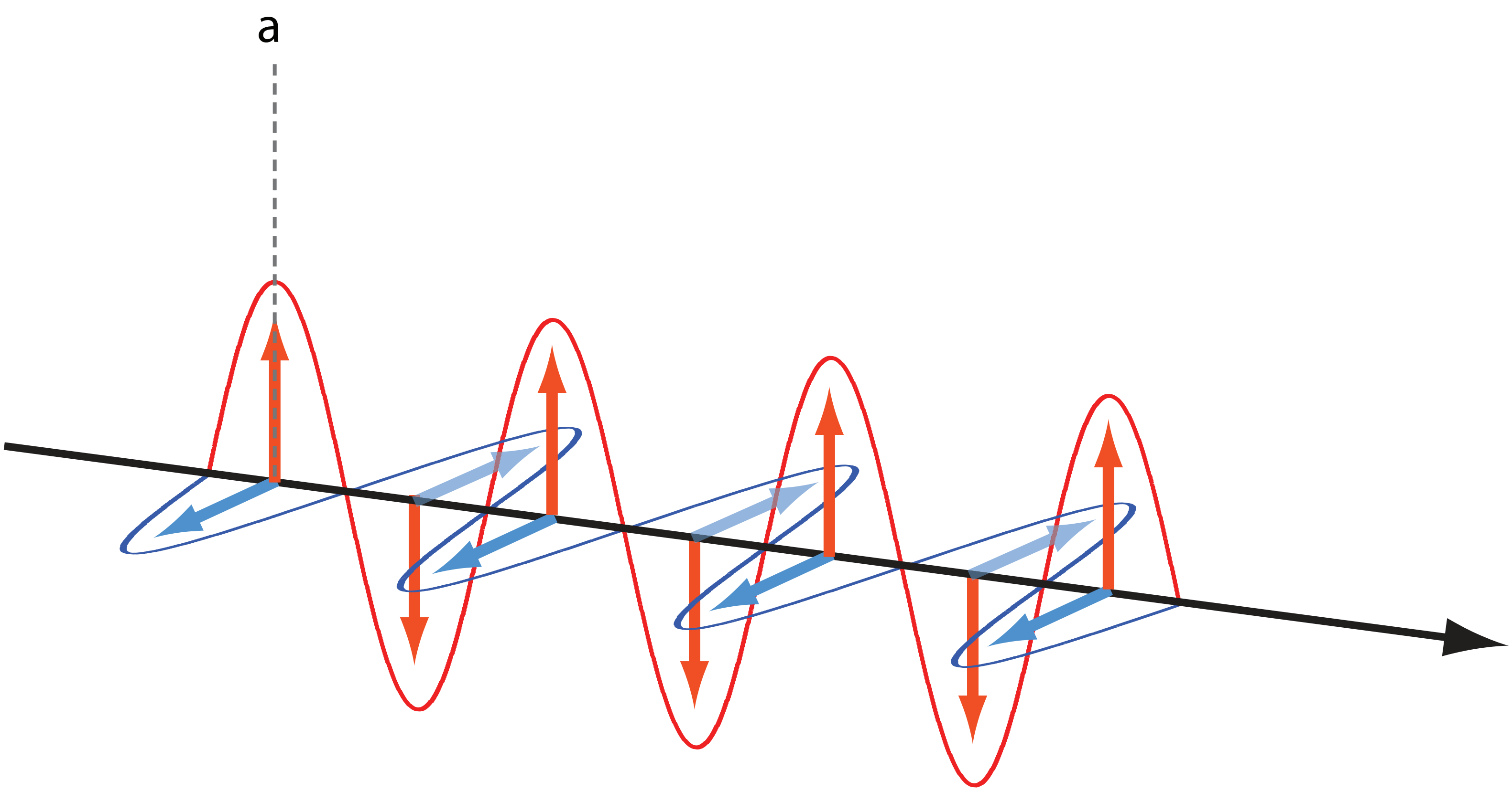
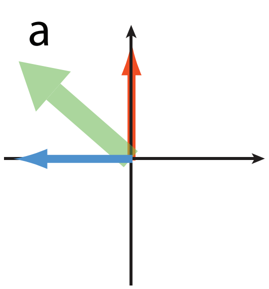
Linearly Polarized Light





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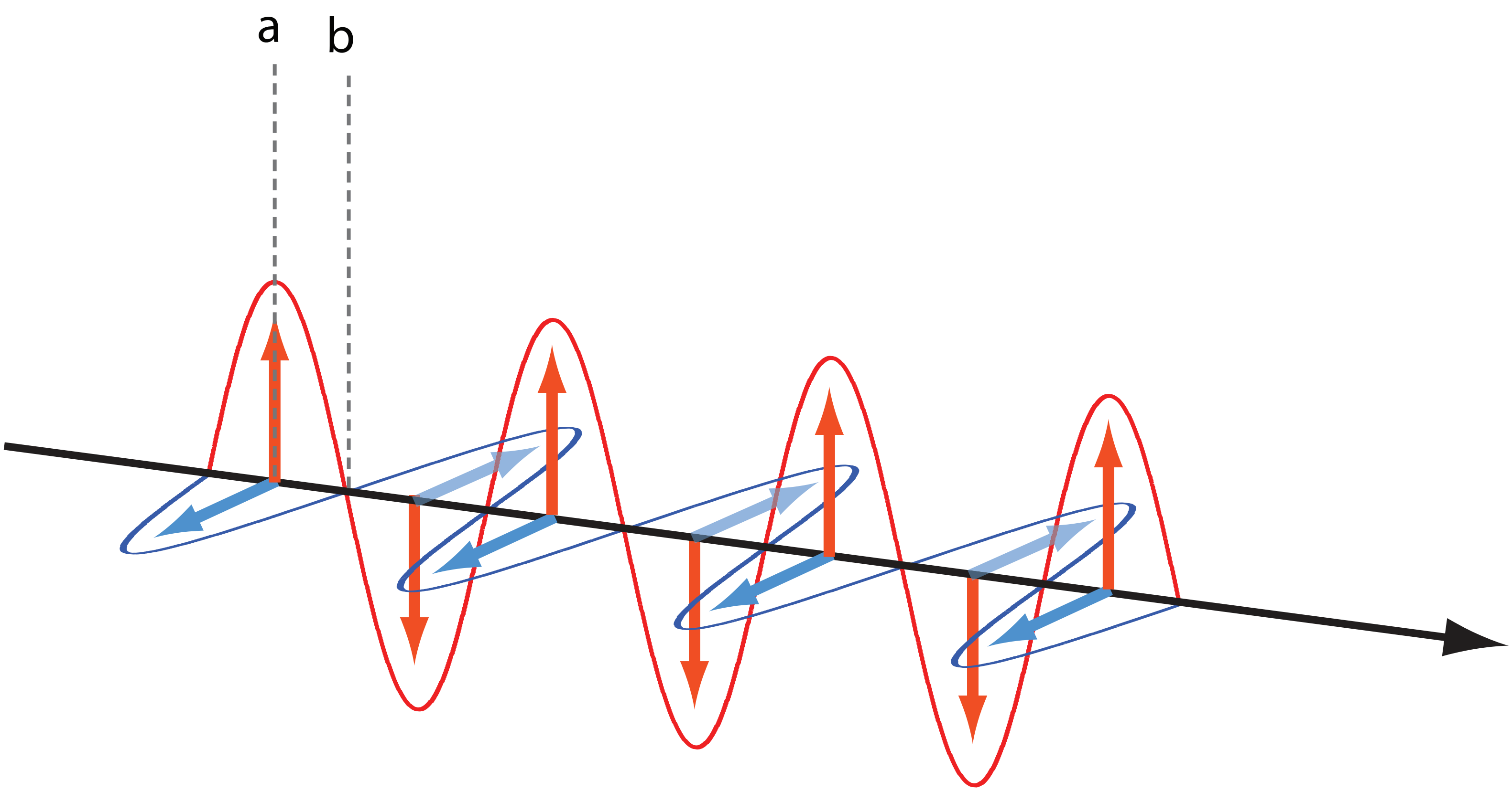
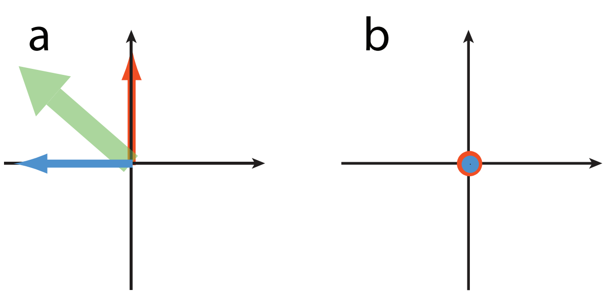
Linearly Polarized Light





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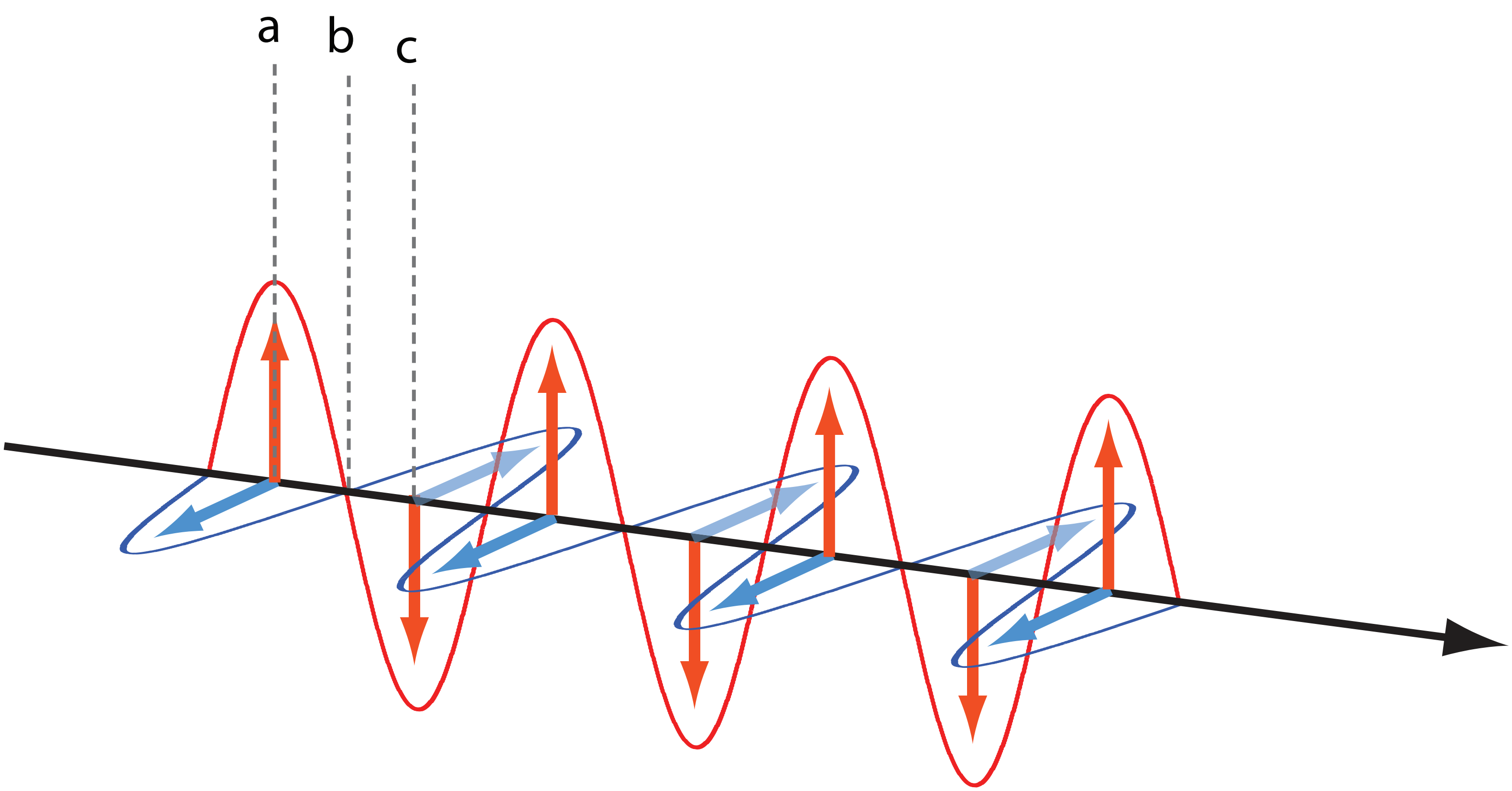
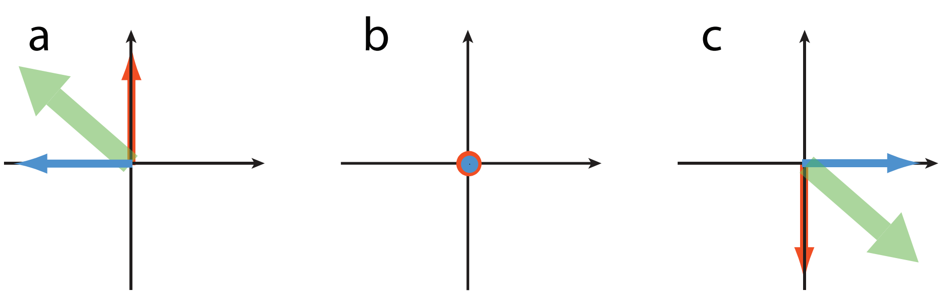
Linearly Polarized Light





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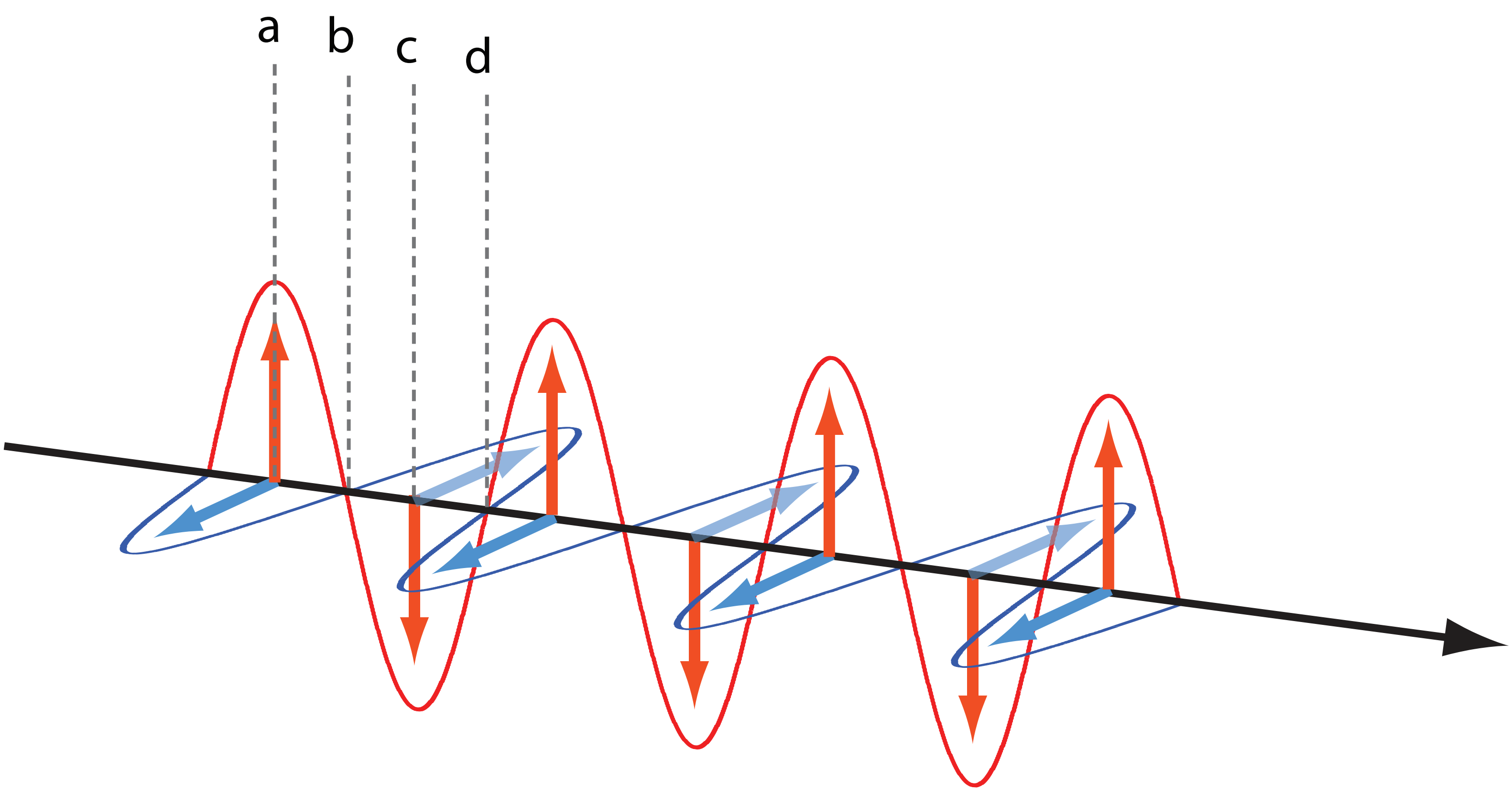
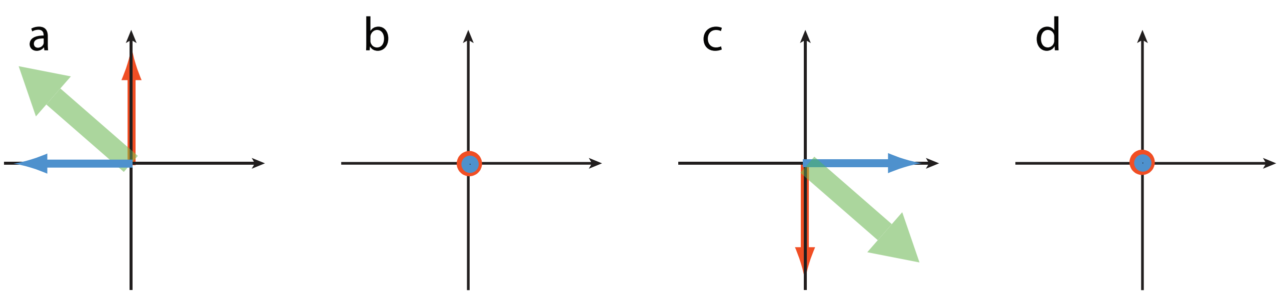
Linearly Polarized Light





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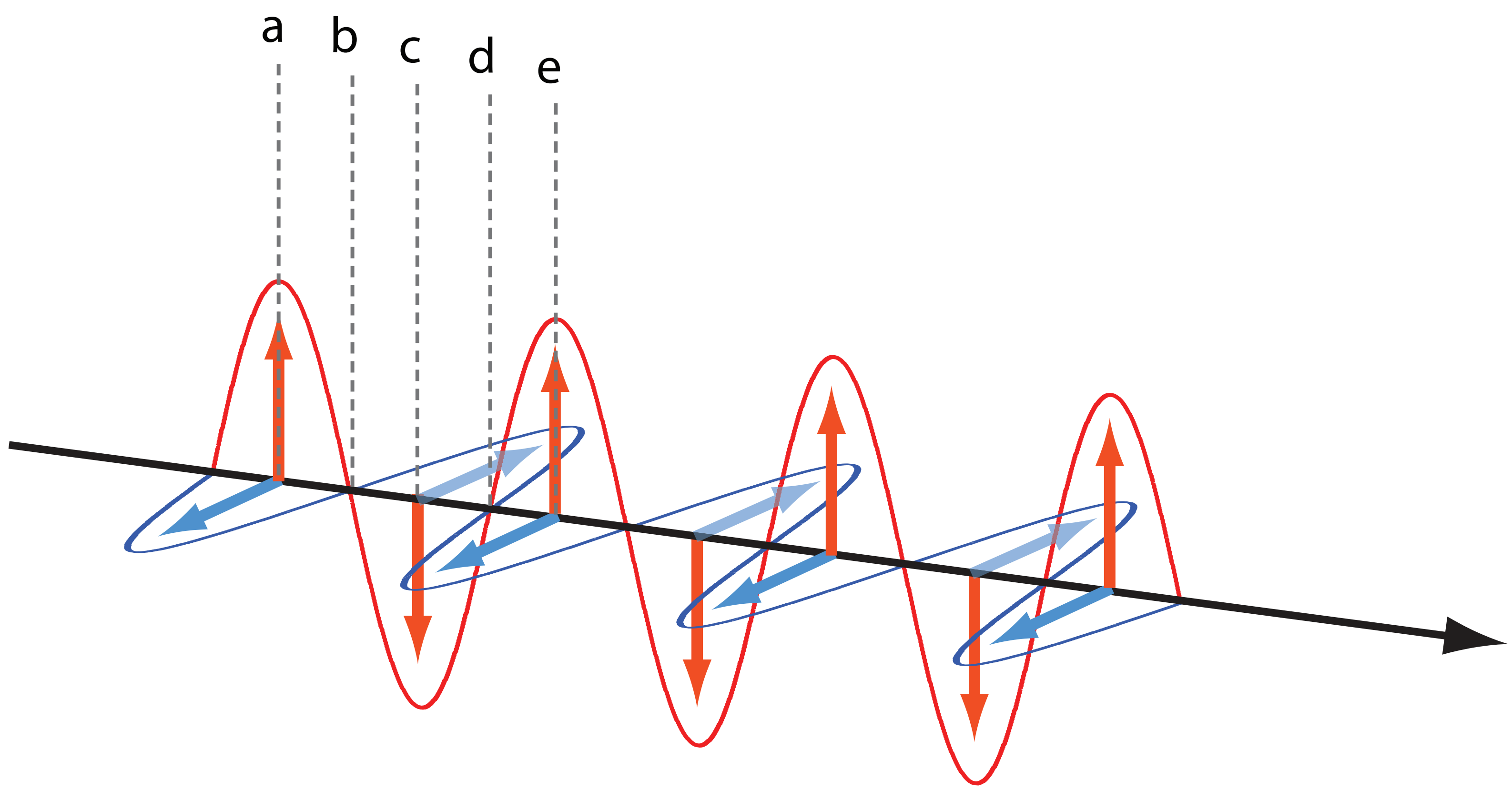
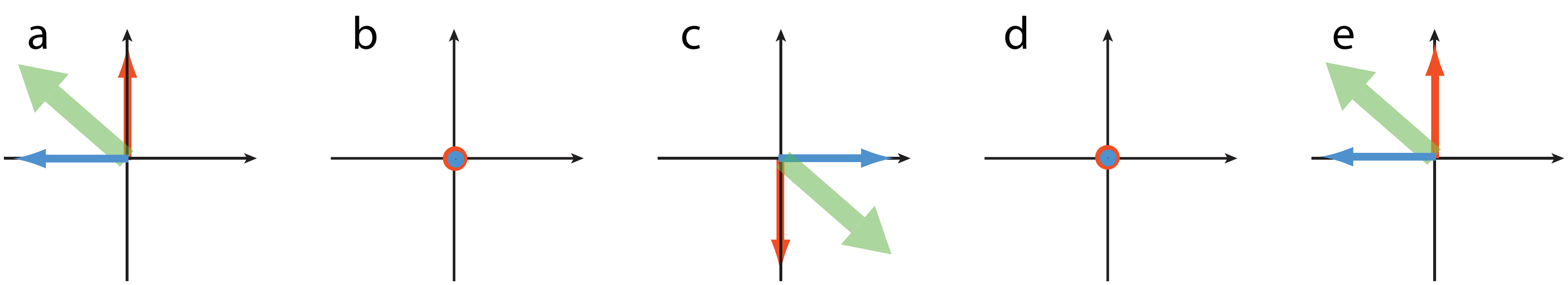
Linearly Polarized Light





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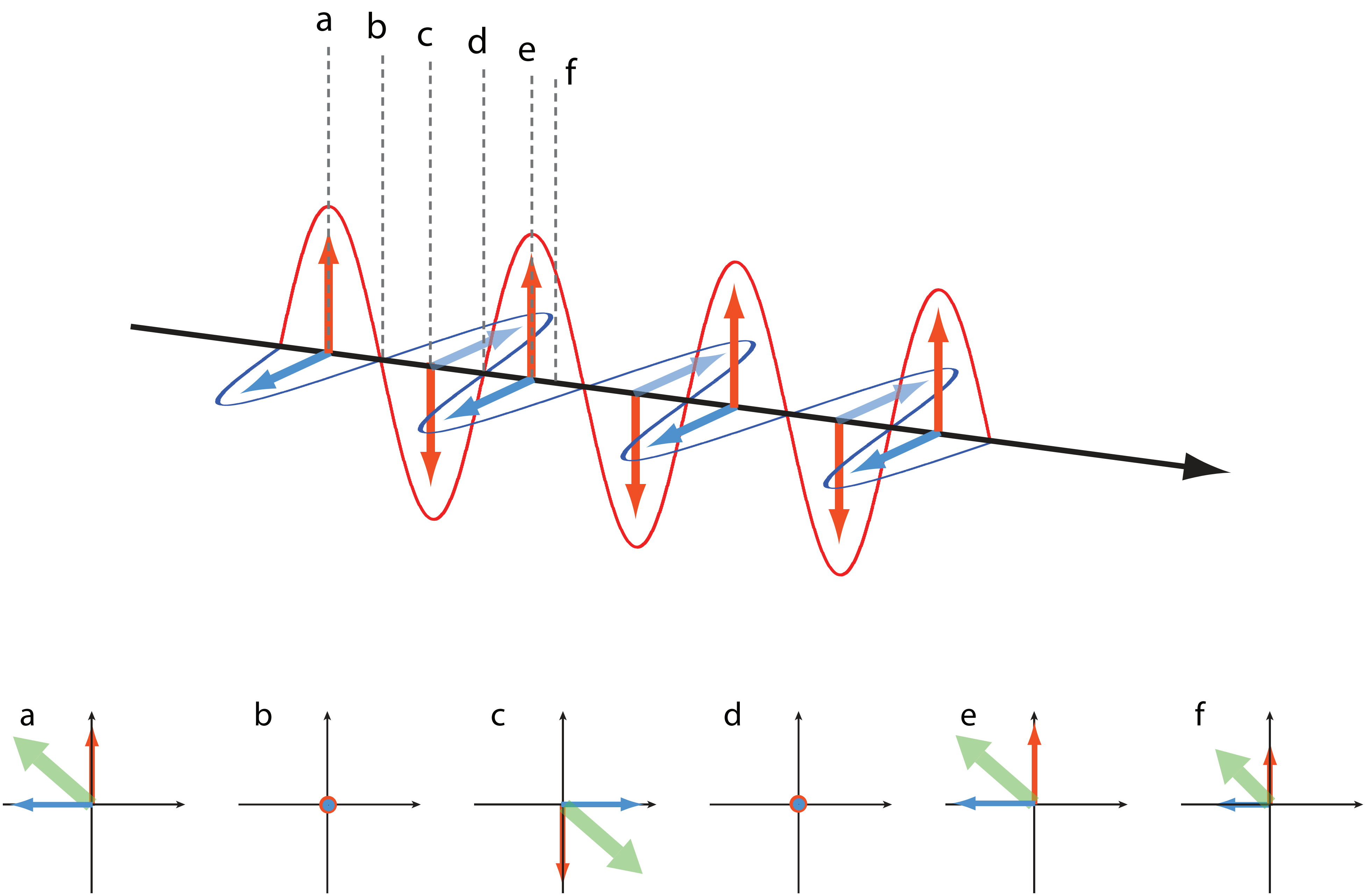
Linearly Polarized Light



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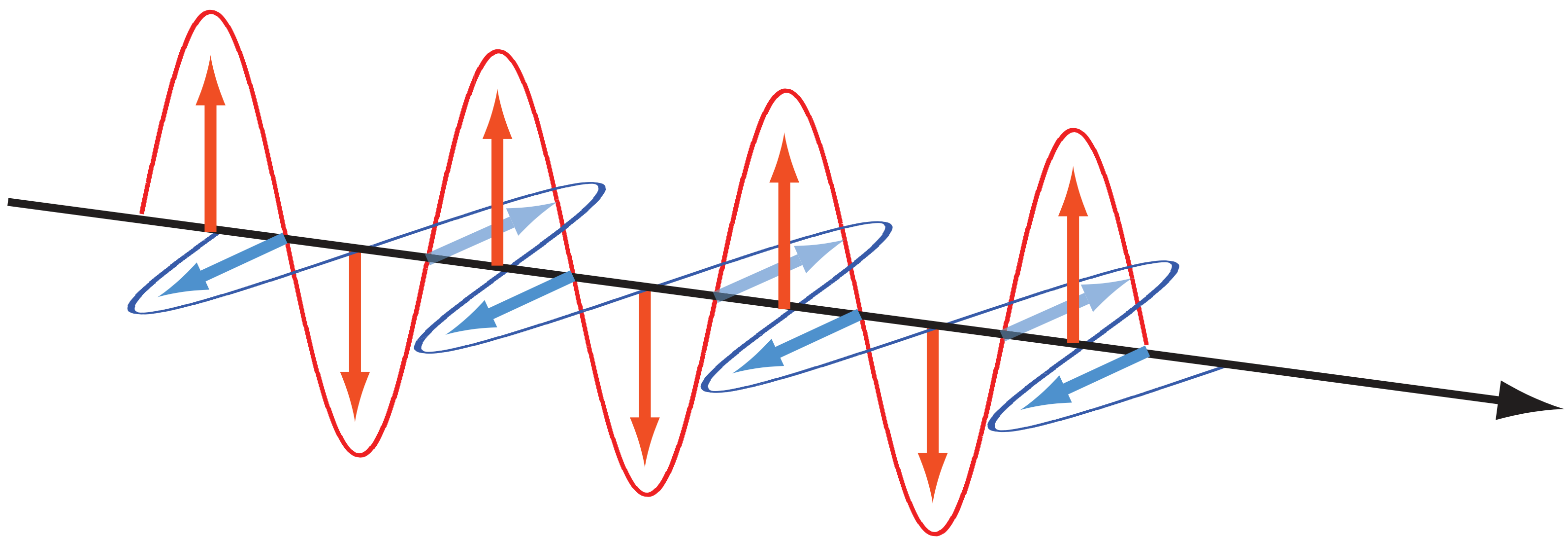
Circularly Polarized Light



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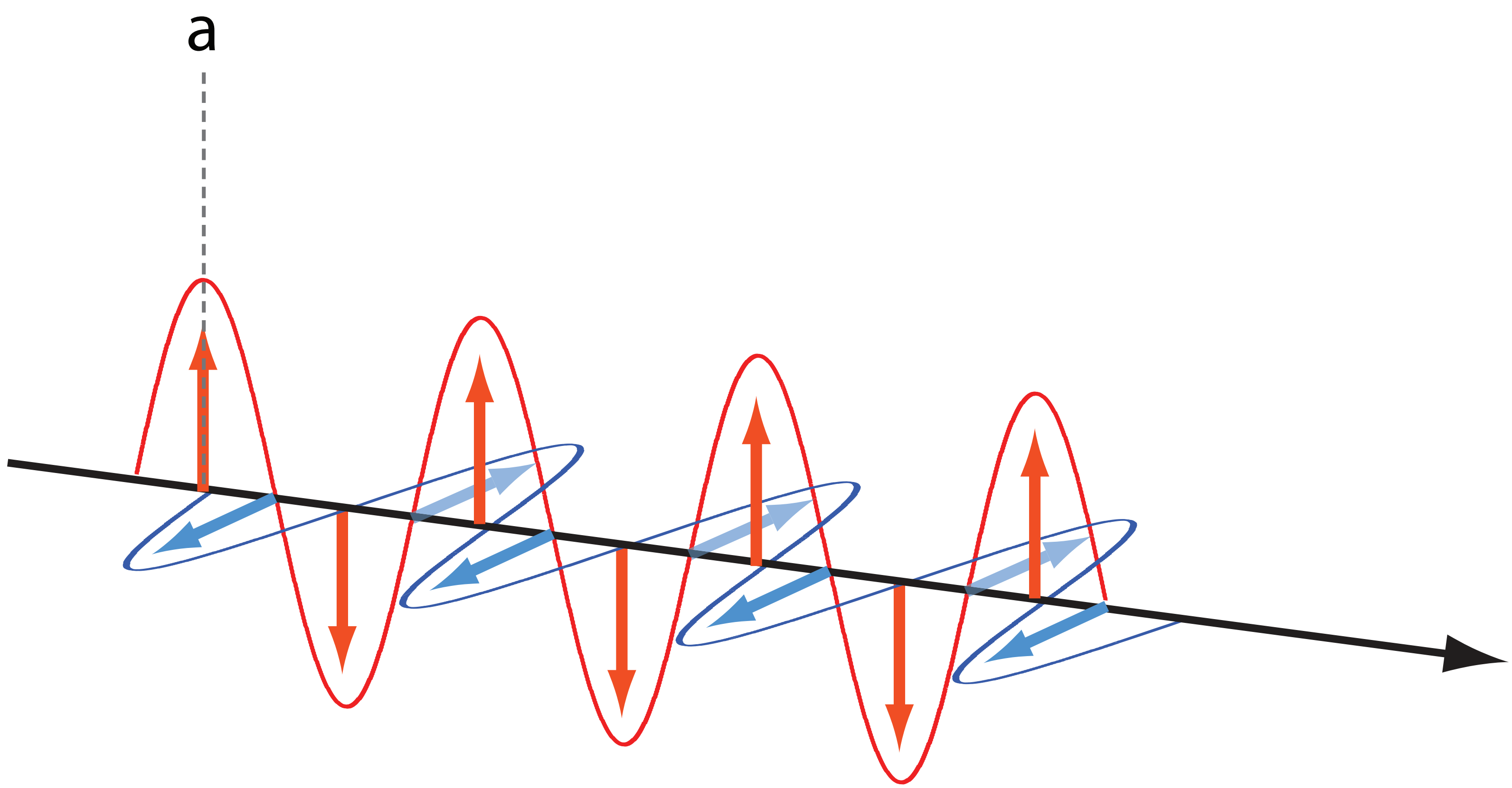
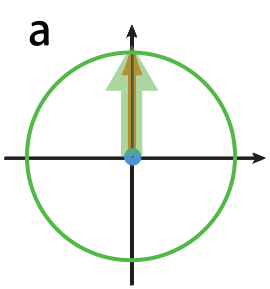
Circularly Polarized Light





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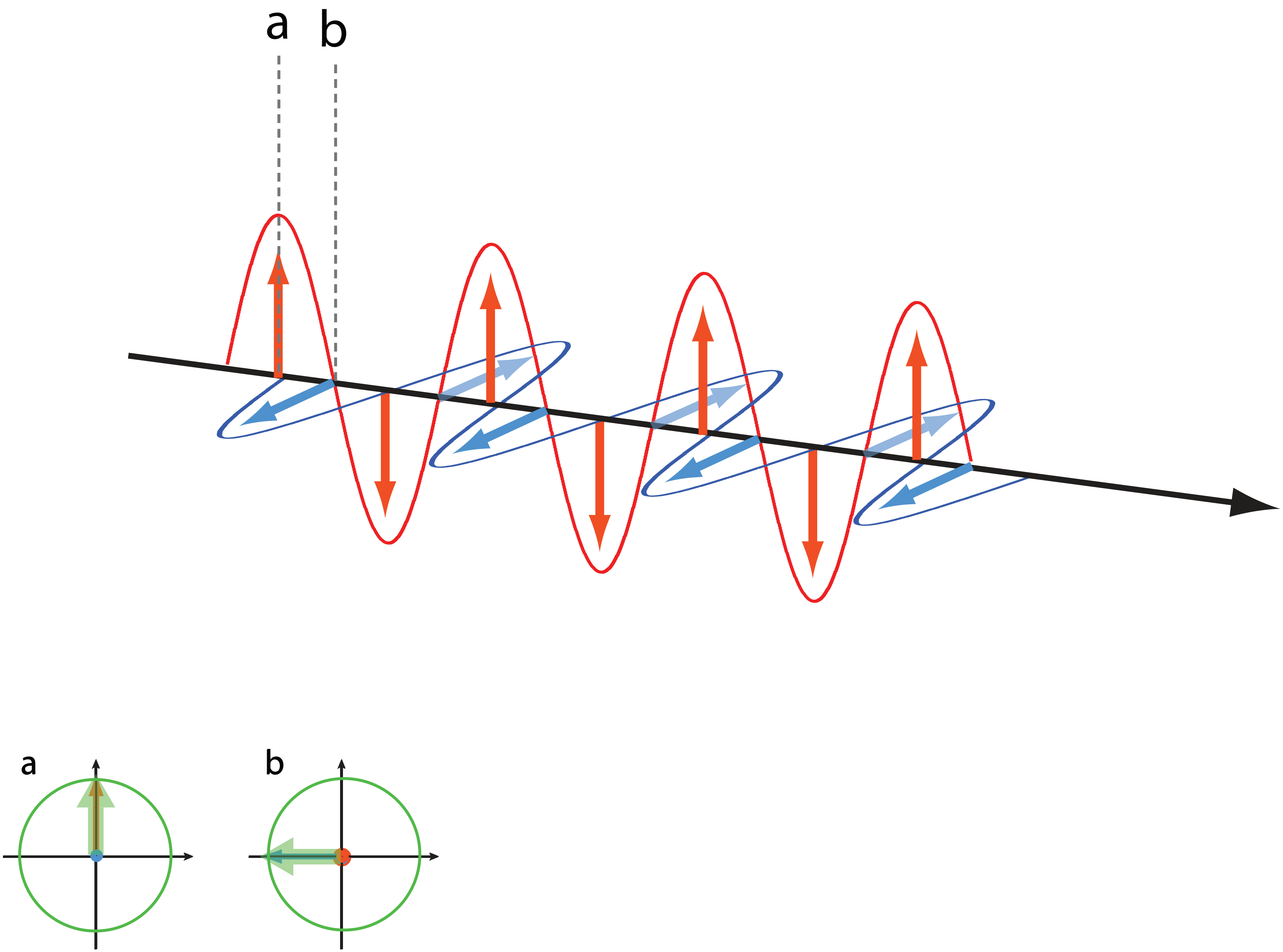

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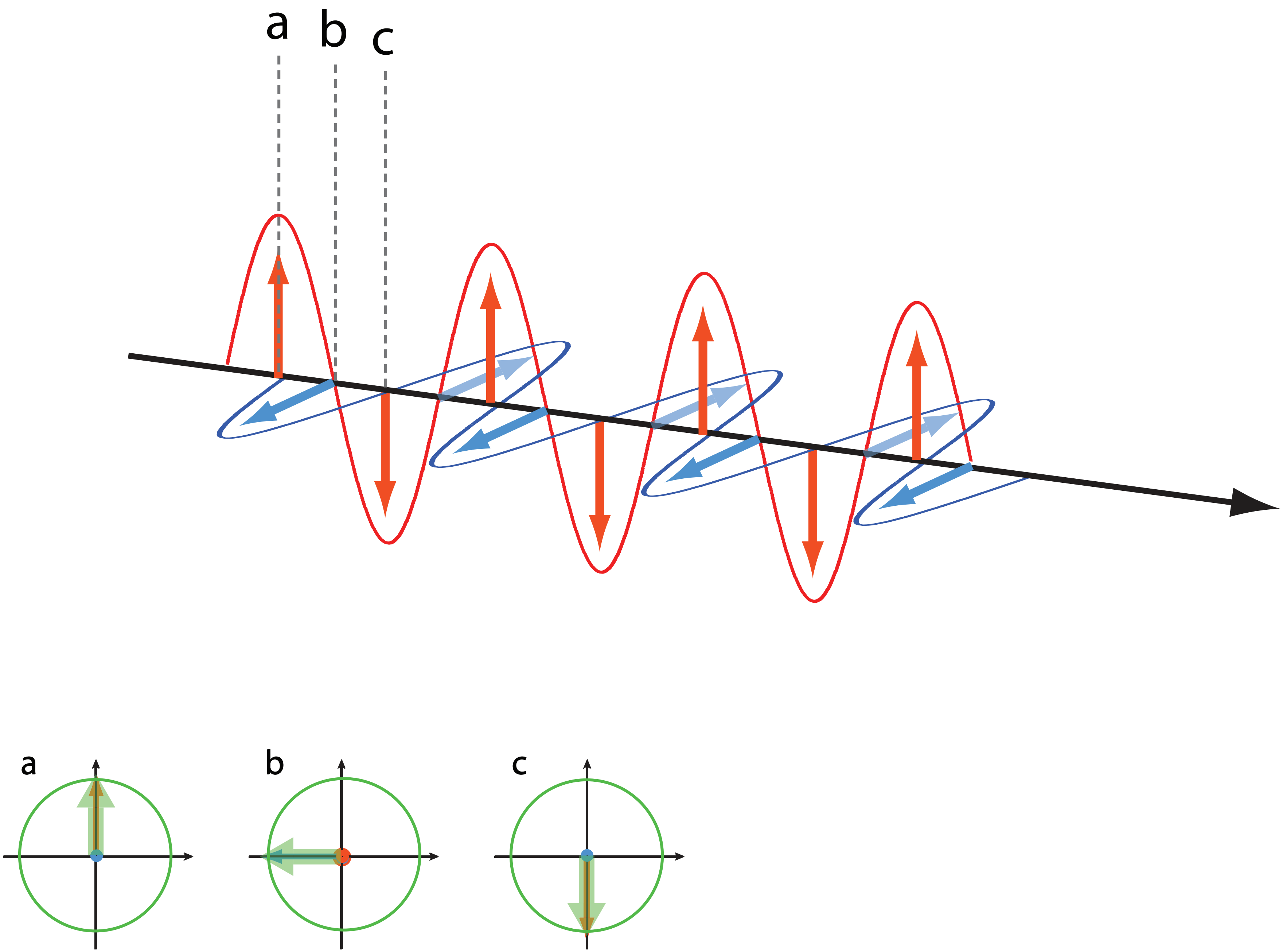

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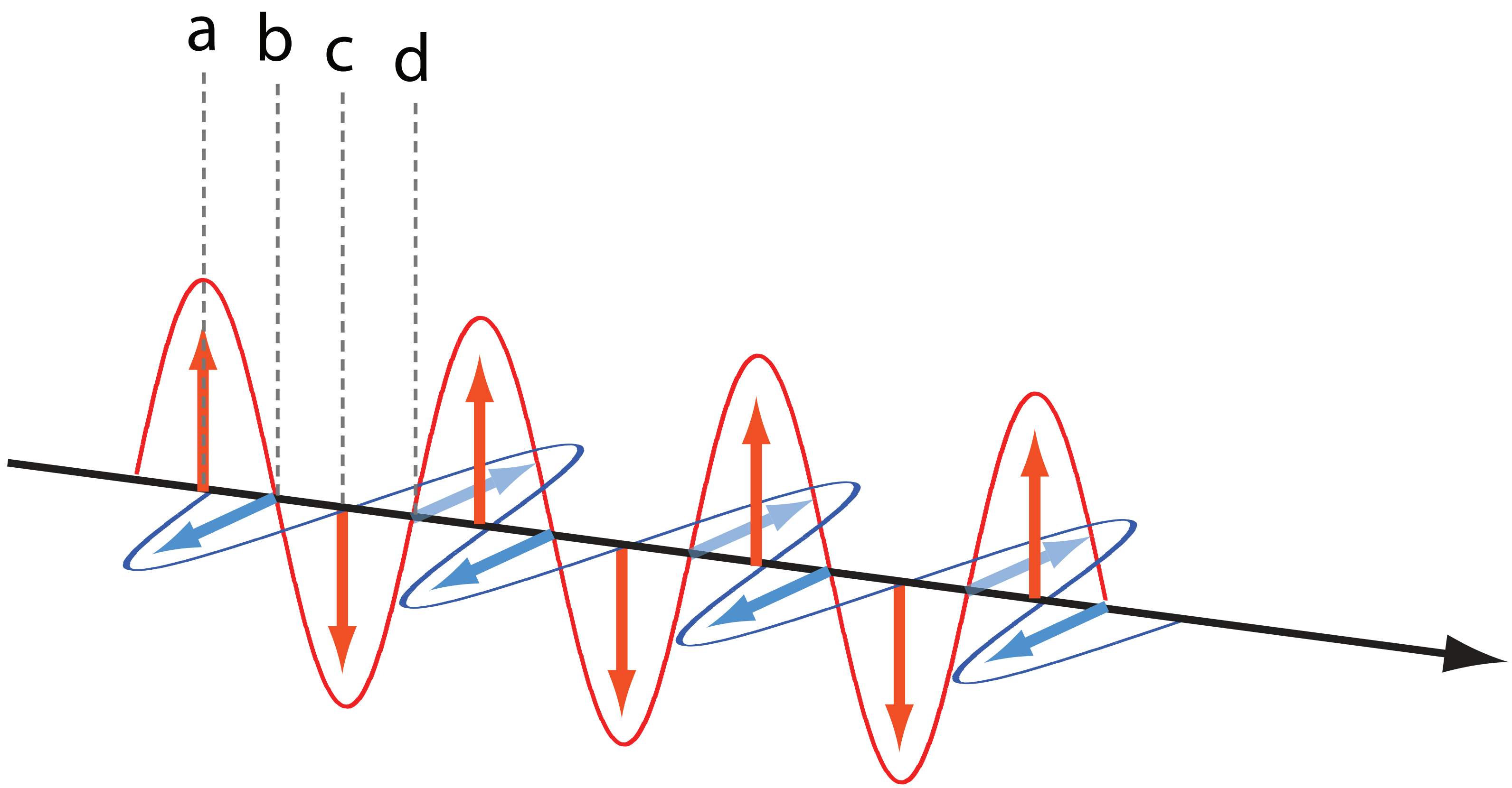
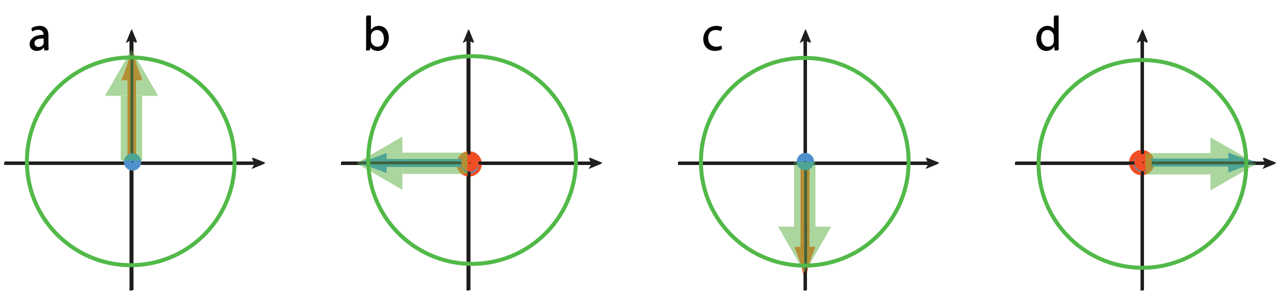
Circularly Polarized Light





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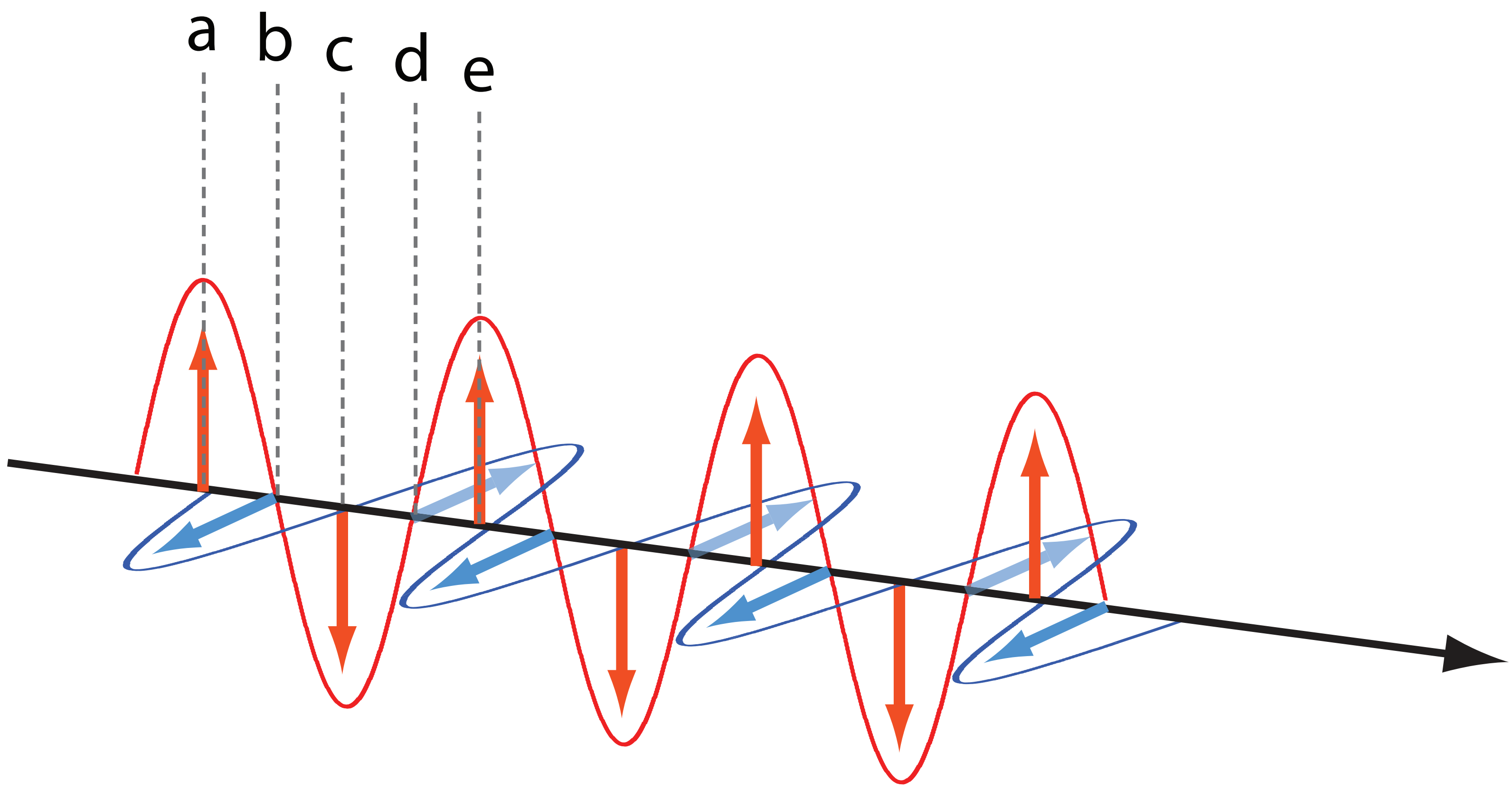

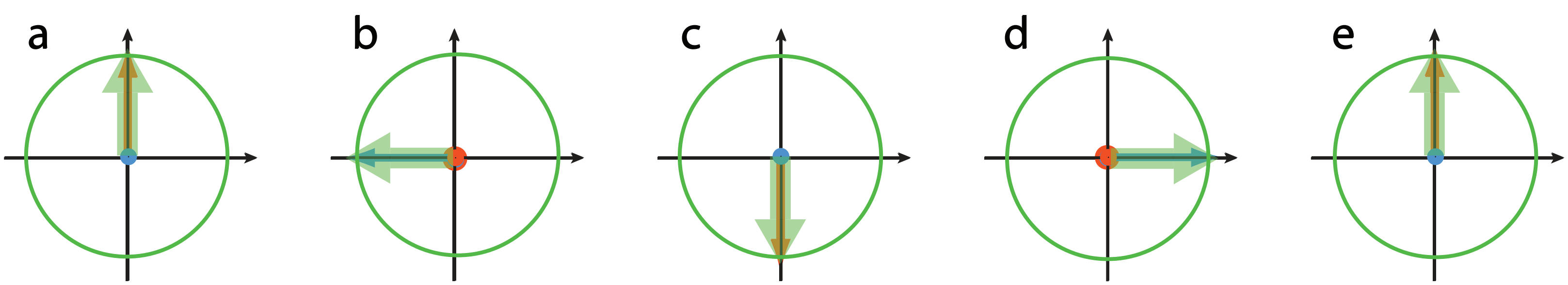

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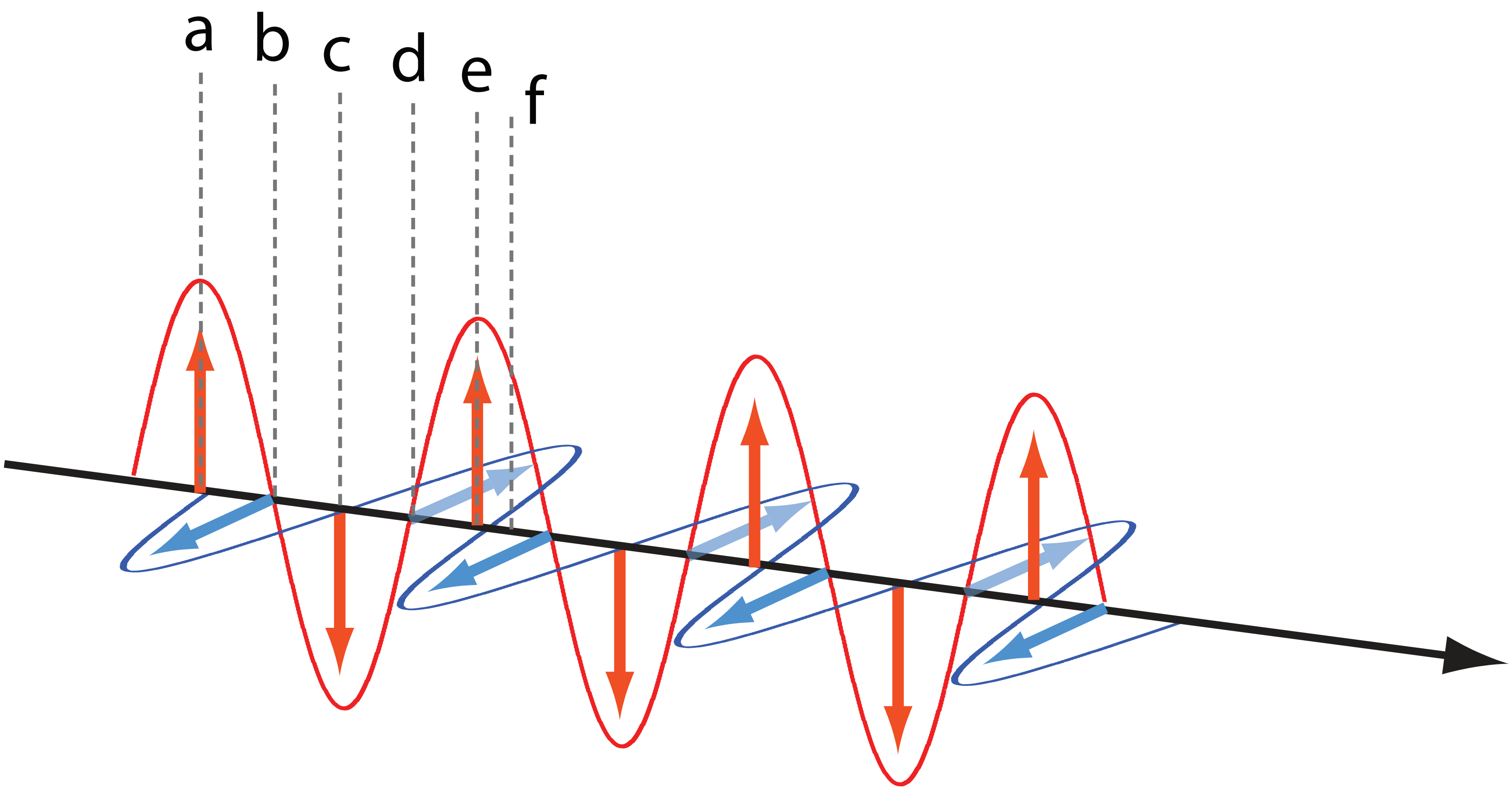
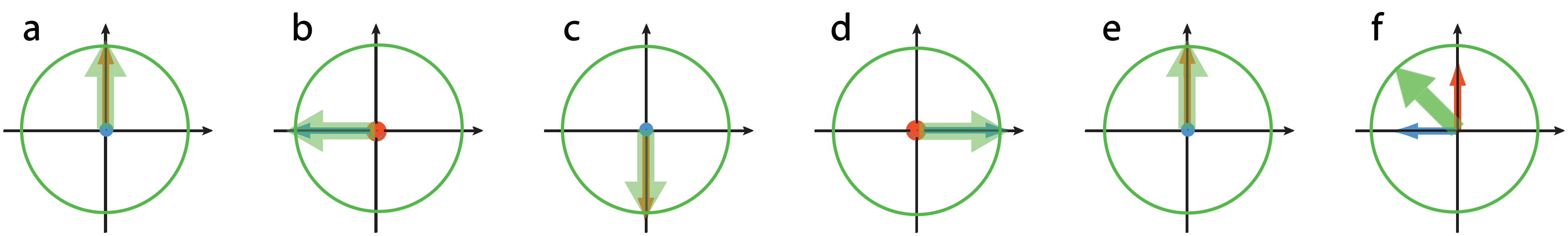
Circularly Polarized Light





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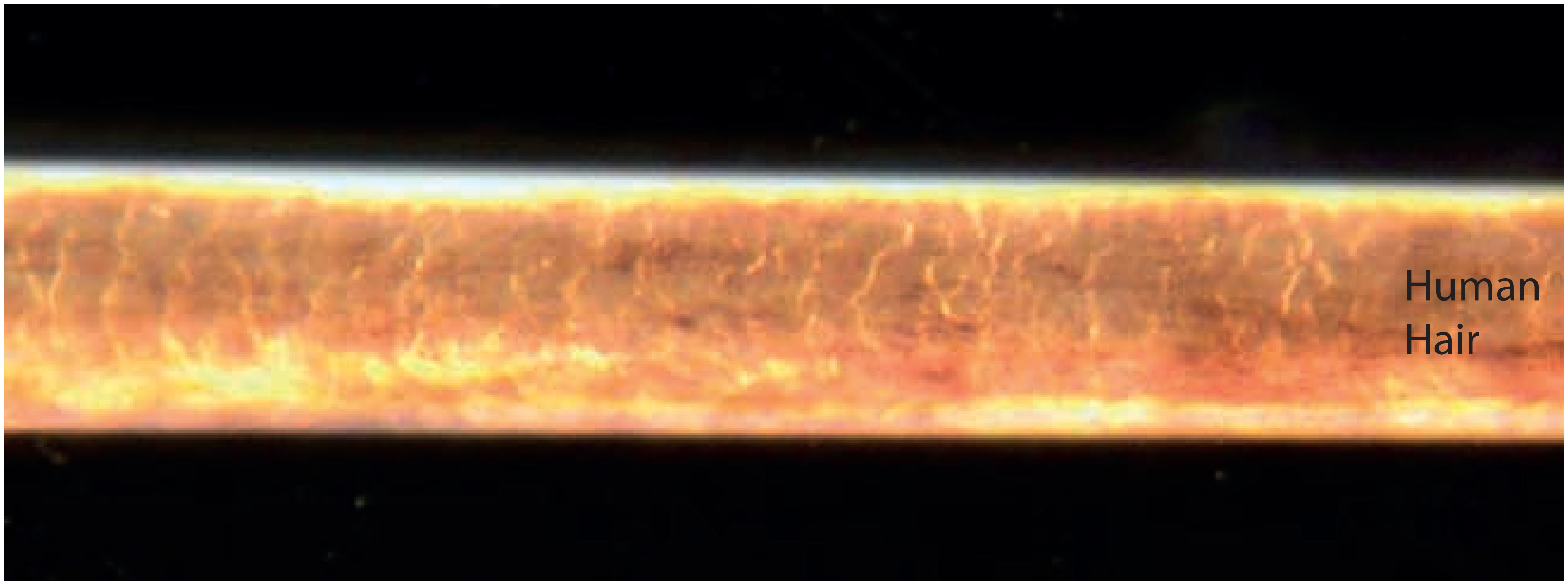
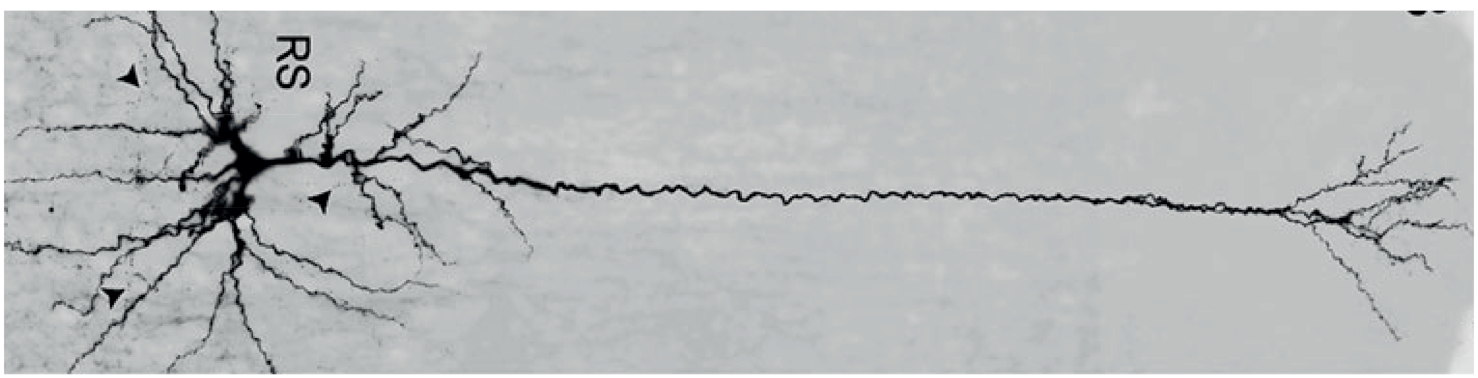
Sense of Scale

100 μm



20 μm



Pyramidal
Neuron

7 μm ▪ Red Blood Cell

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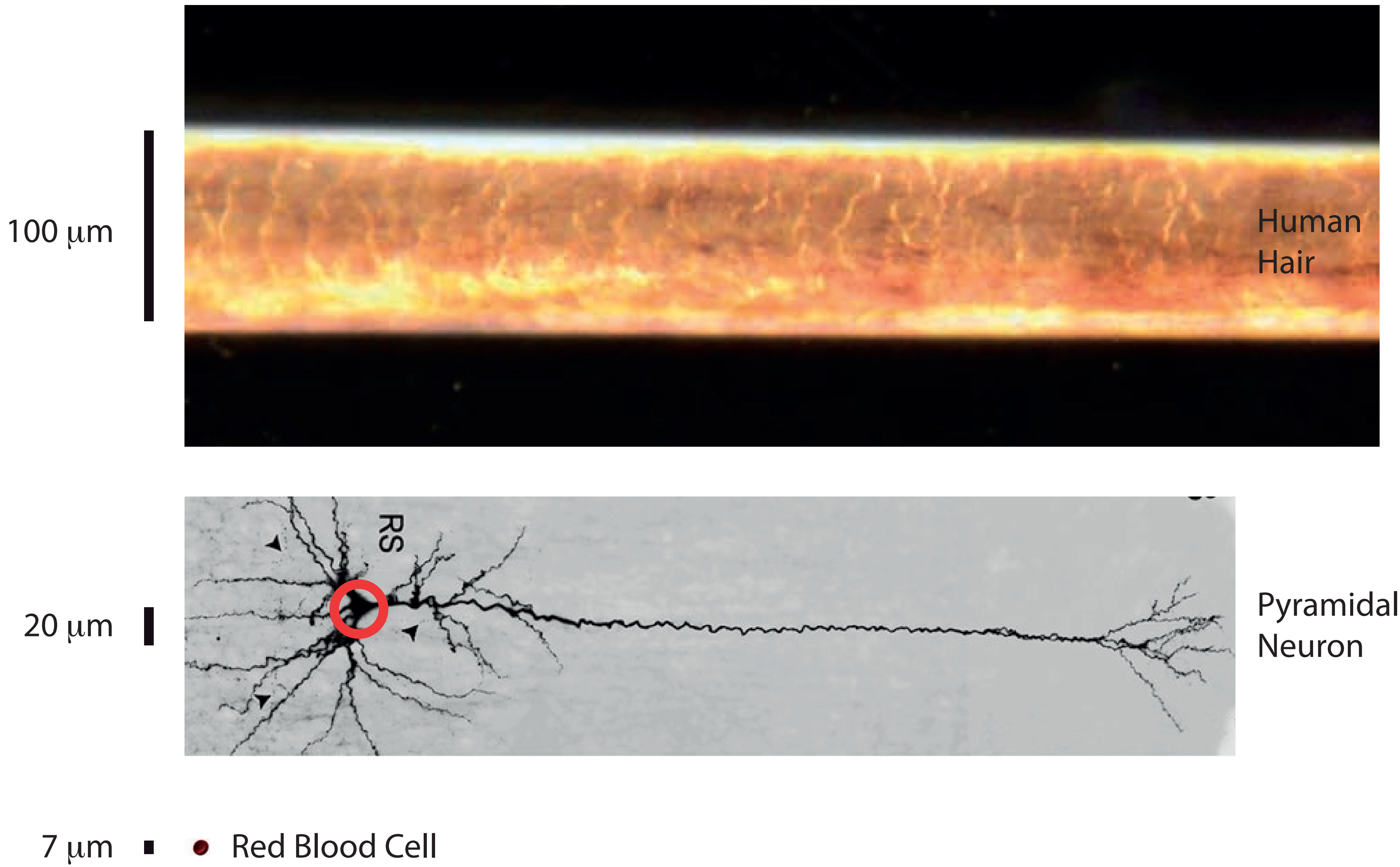
Sense of Scale



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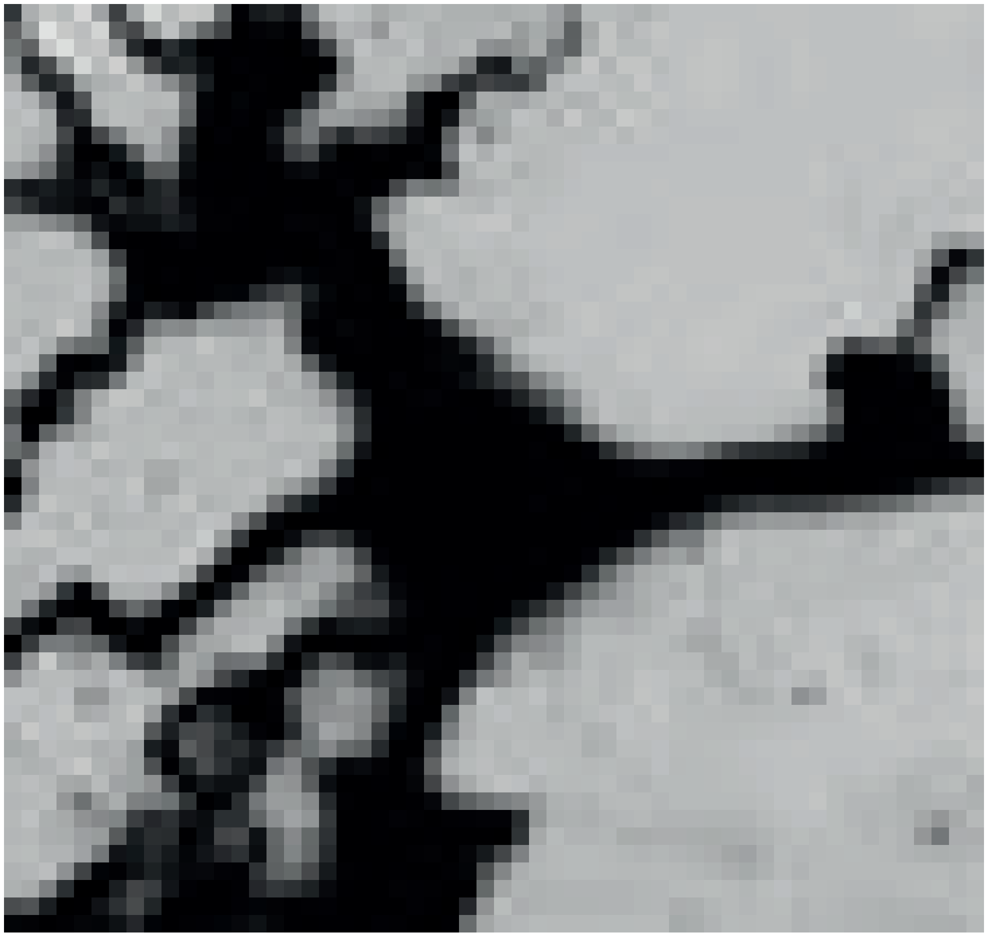
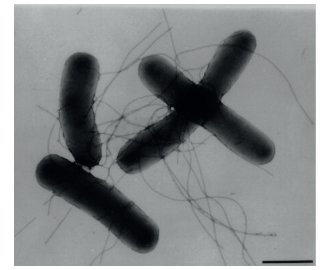
Sense of Scale

Pyramidal Neuron Cell Body (~10 μm)

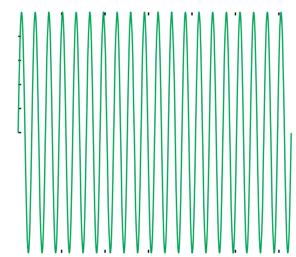


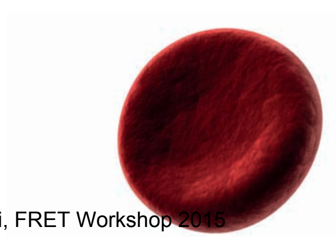
20 μm

Bacterium (1 x 5 μm)



20 cycles of green light
(λ = 0.5 μm)





Red Blood Cell (7 μm)

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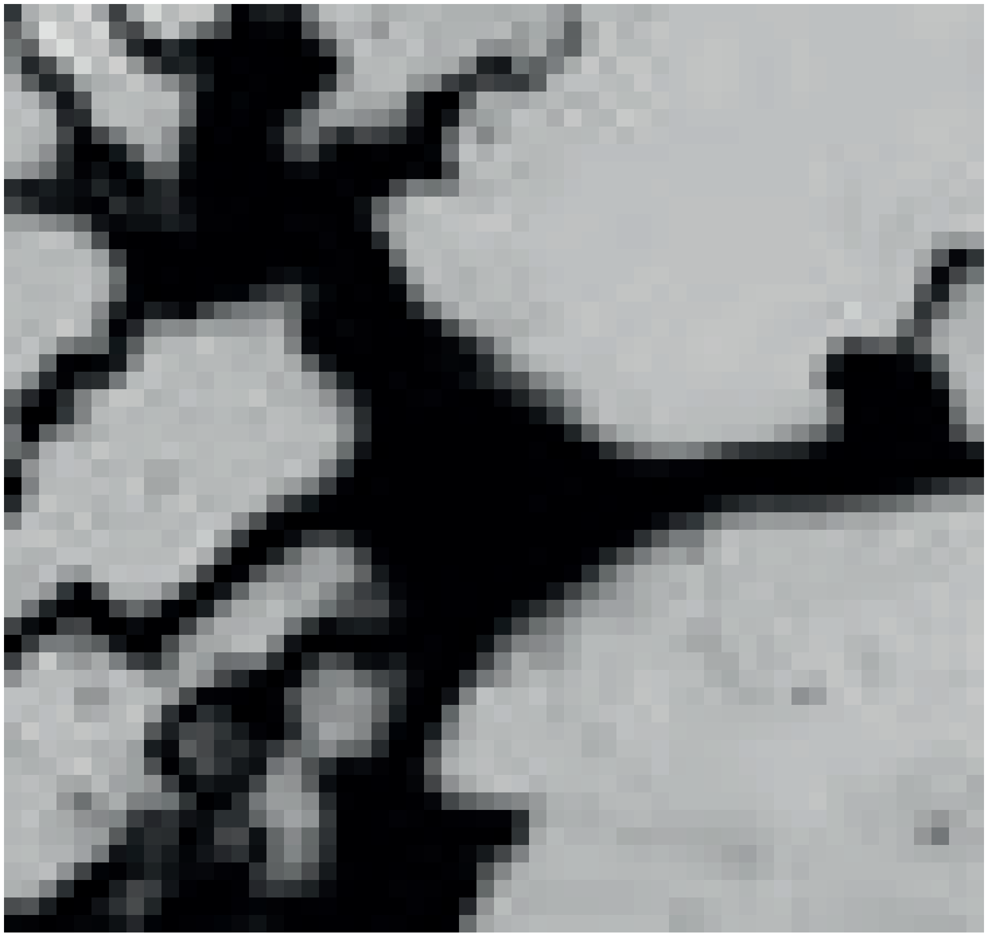
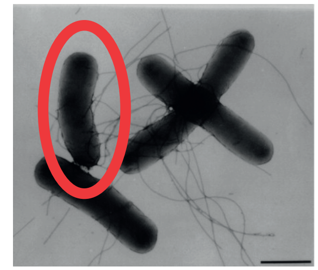
Sense of Scale

Pyramidal Neuron Cell Body (~10 μm)

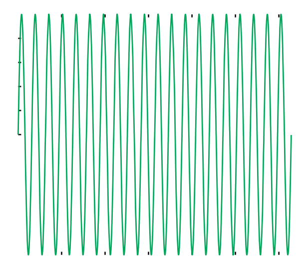


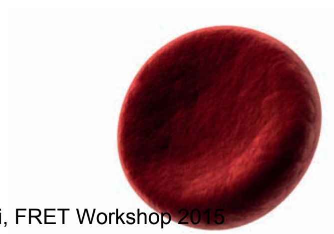
20 μm

Bacterium (1 x 5 μm)



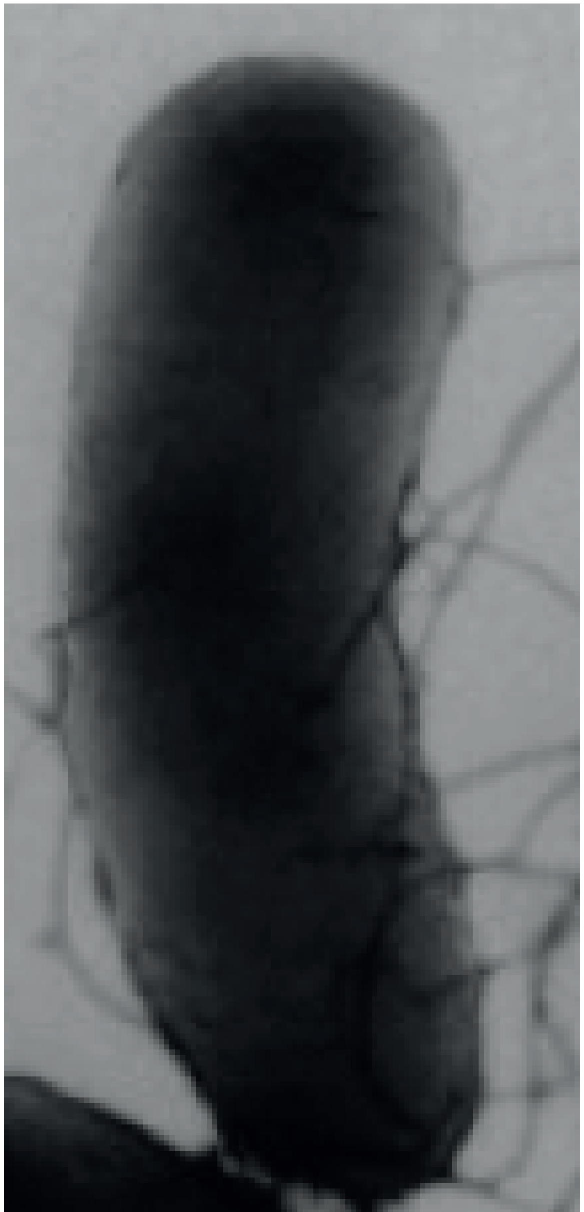
20 cycles of green light
($\lambda = 0.5$ μm)





Red Blood Cell (7 μm)

Sense of Scale



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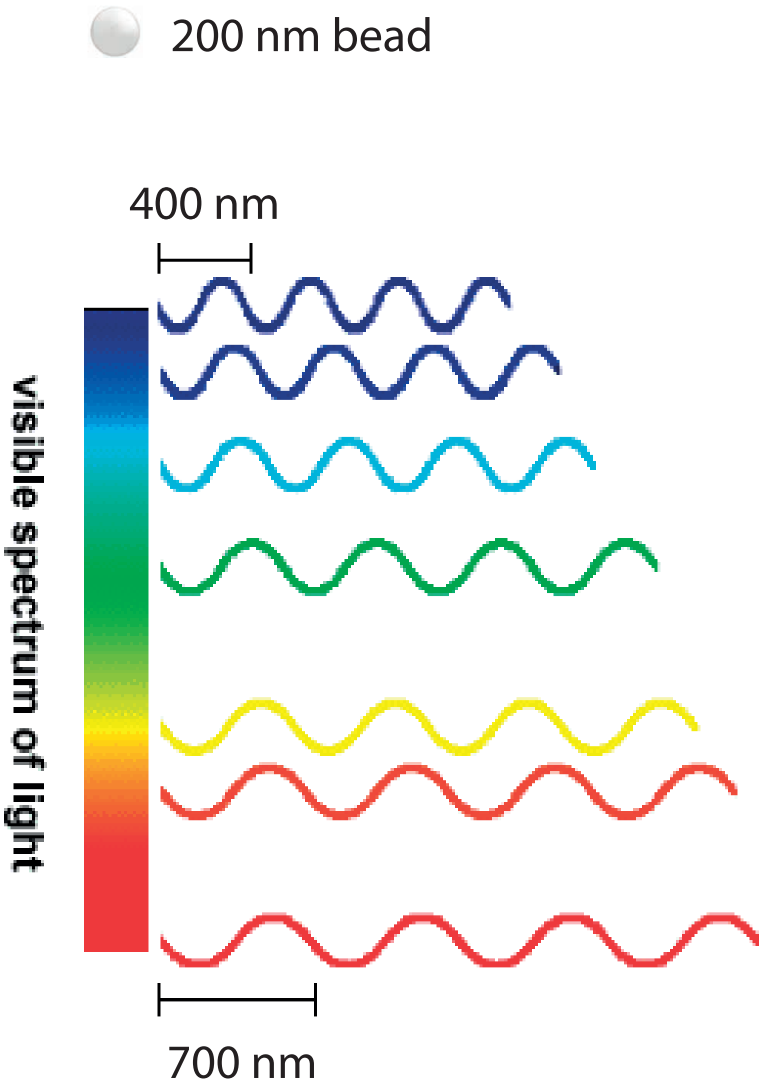
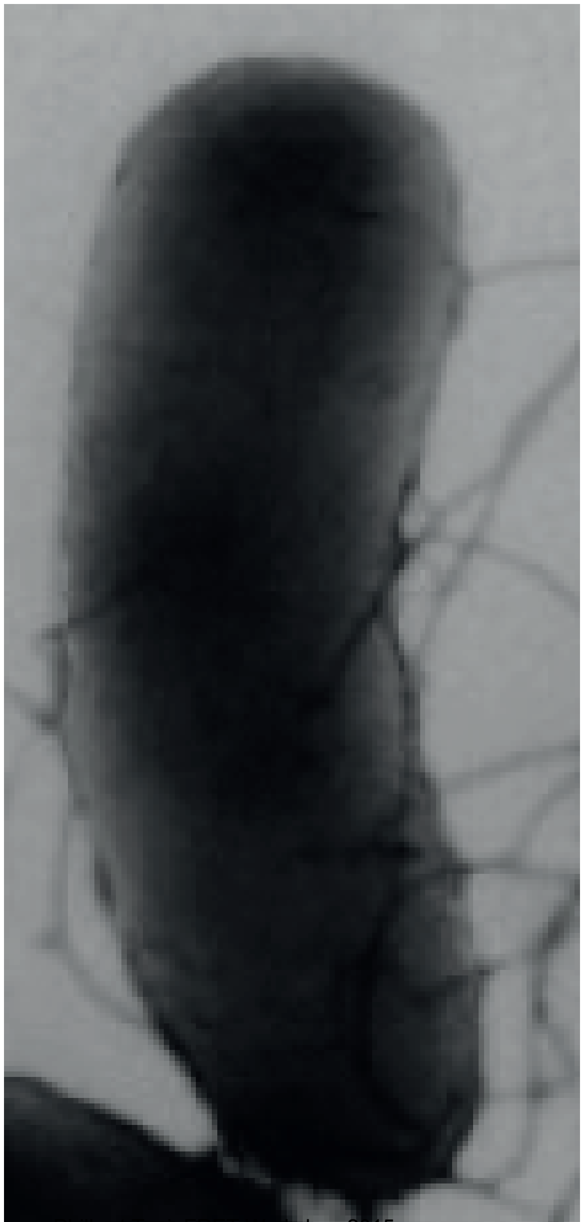
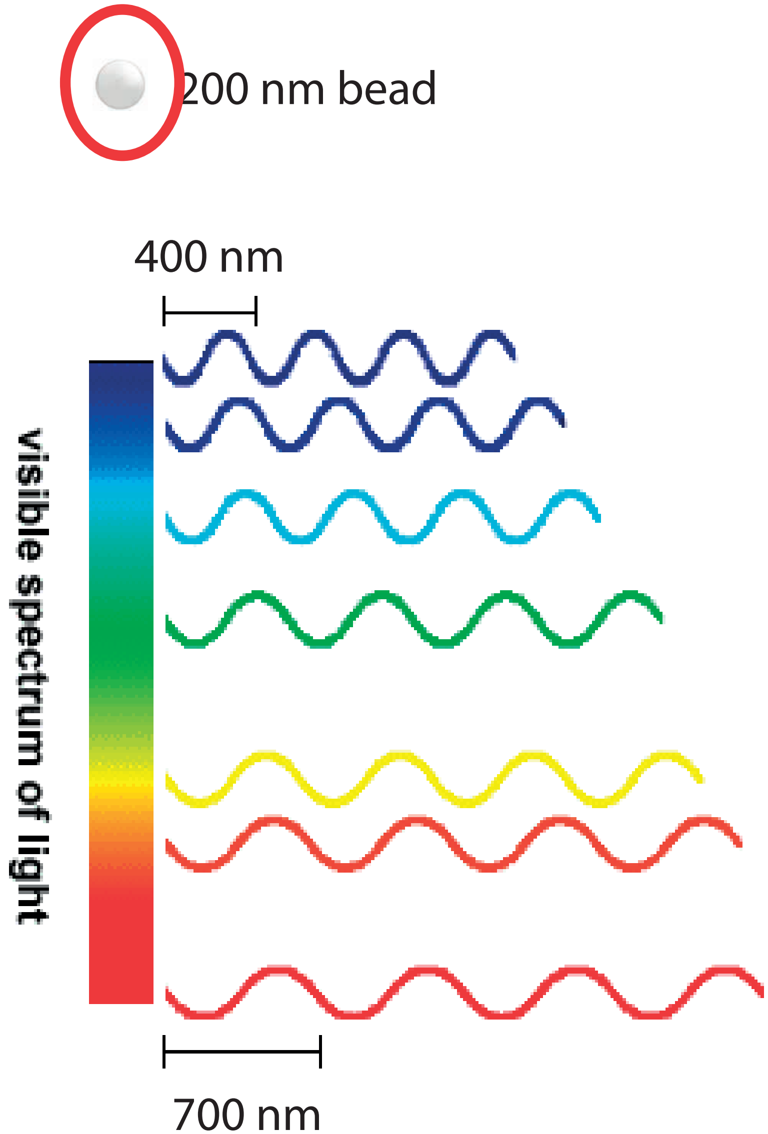


Sense of Scale





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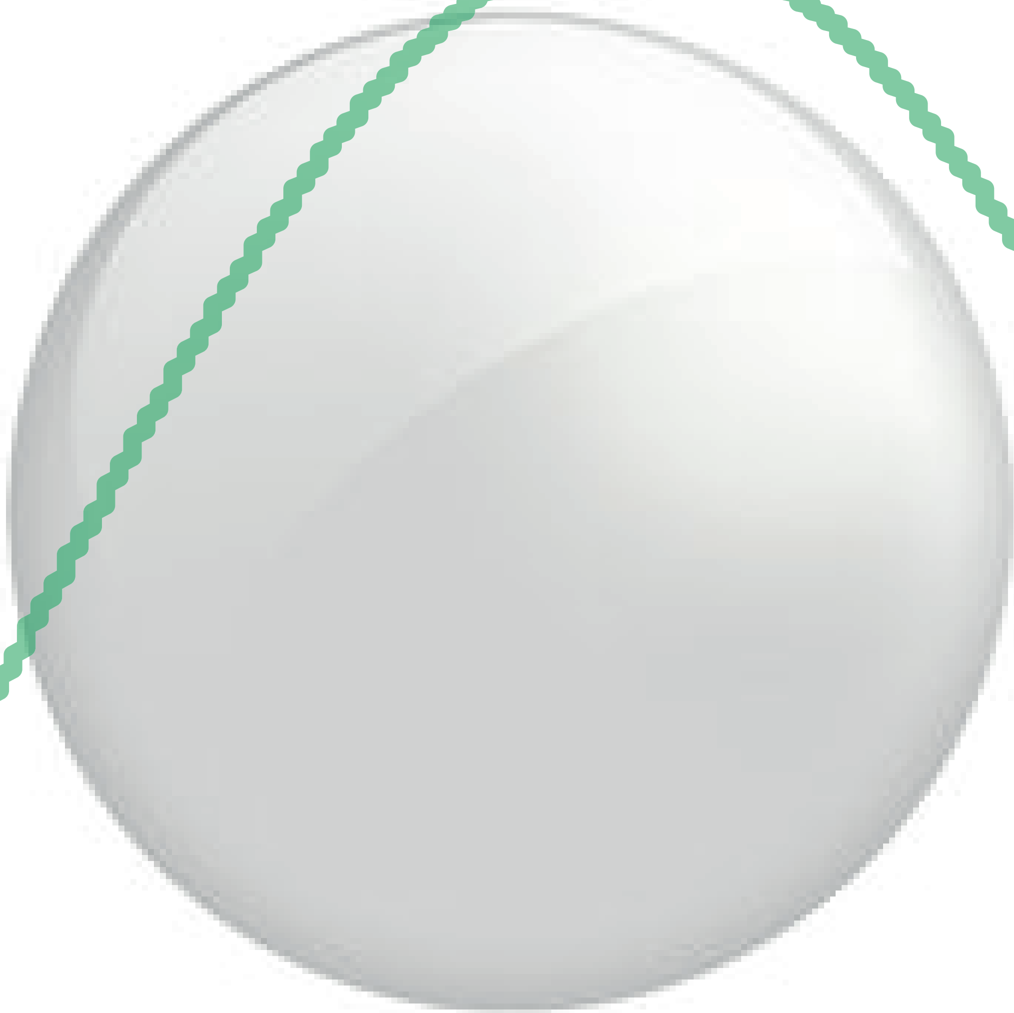
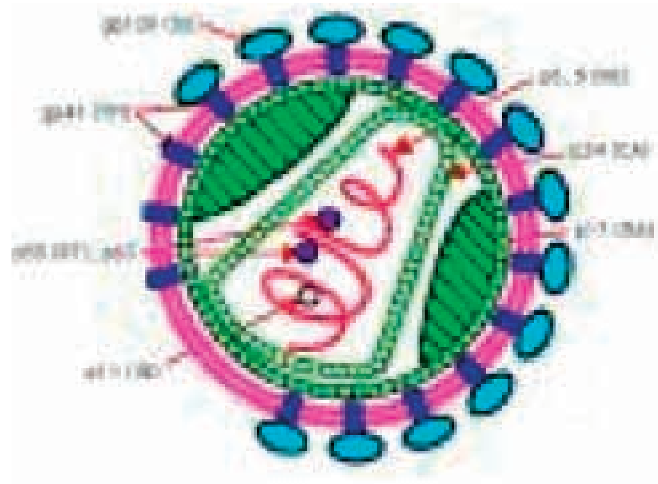
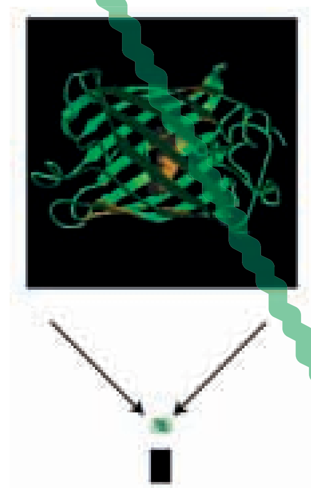
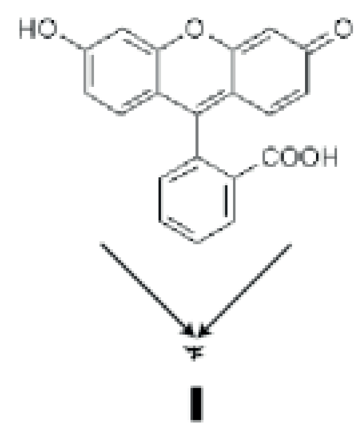
Sense of Scale





75 nm Virus Particle





200 nm bead

4 nm
Green Fluorescent
Protein

2 nm
Fluorescein
Molecule

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Modern Microscope Components

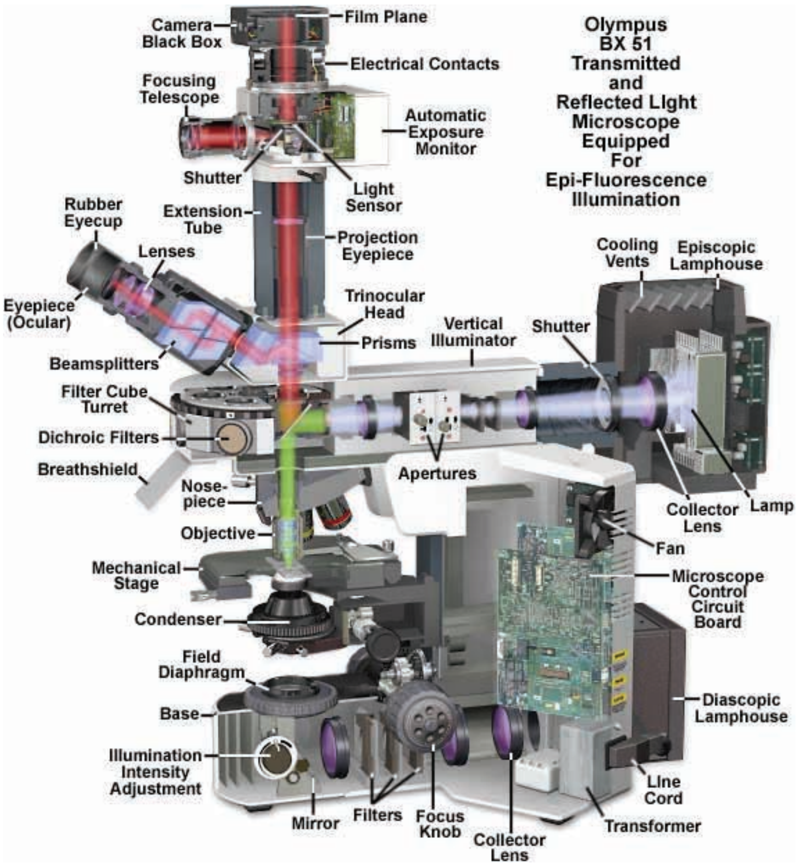

Image from Molecular Expressions webpage

Modern Microscope Components

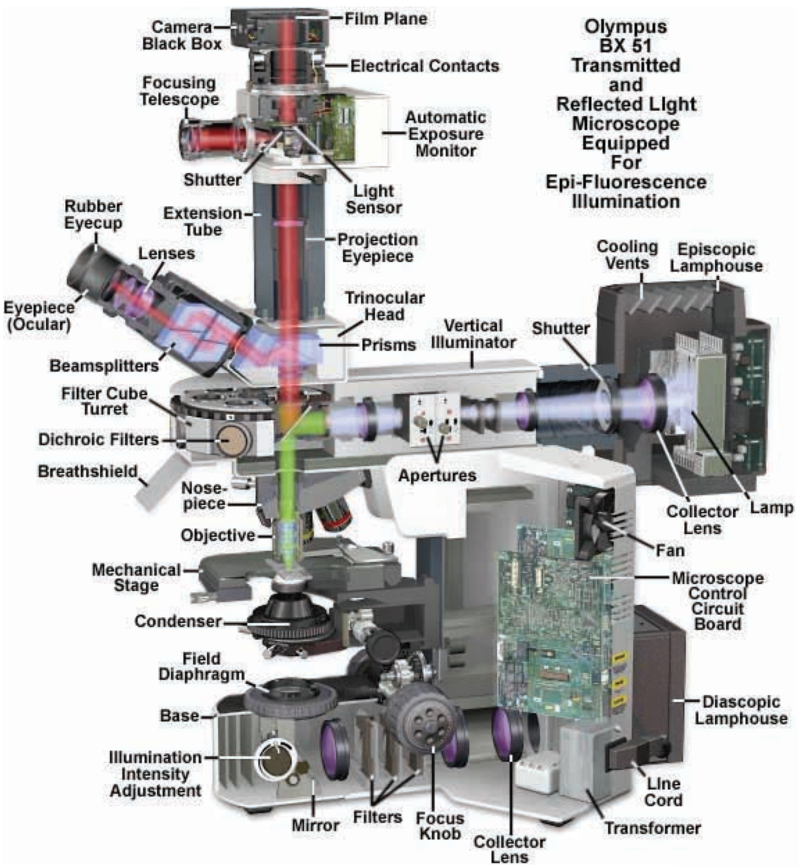

Image from Molecular Expressions webpage

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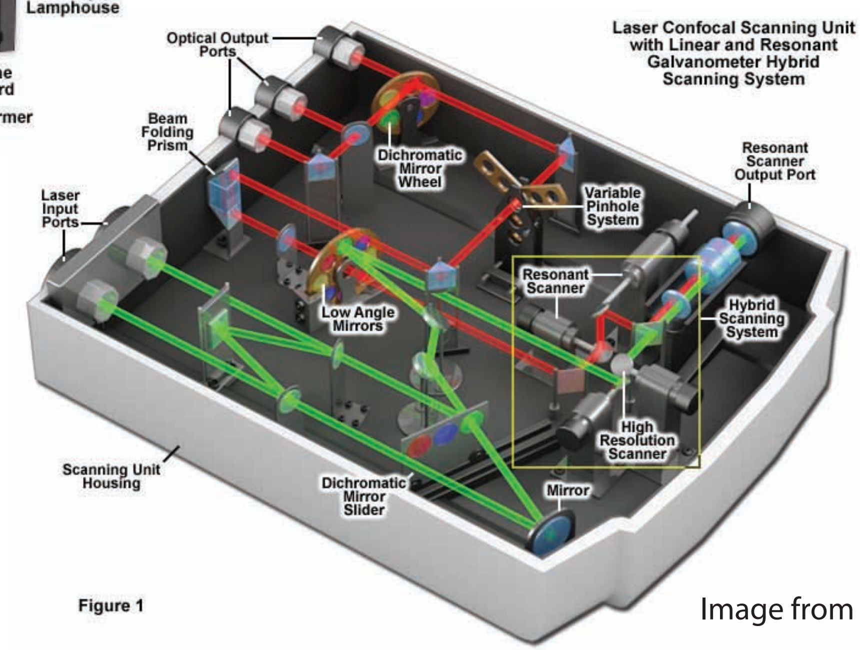
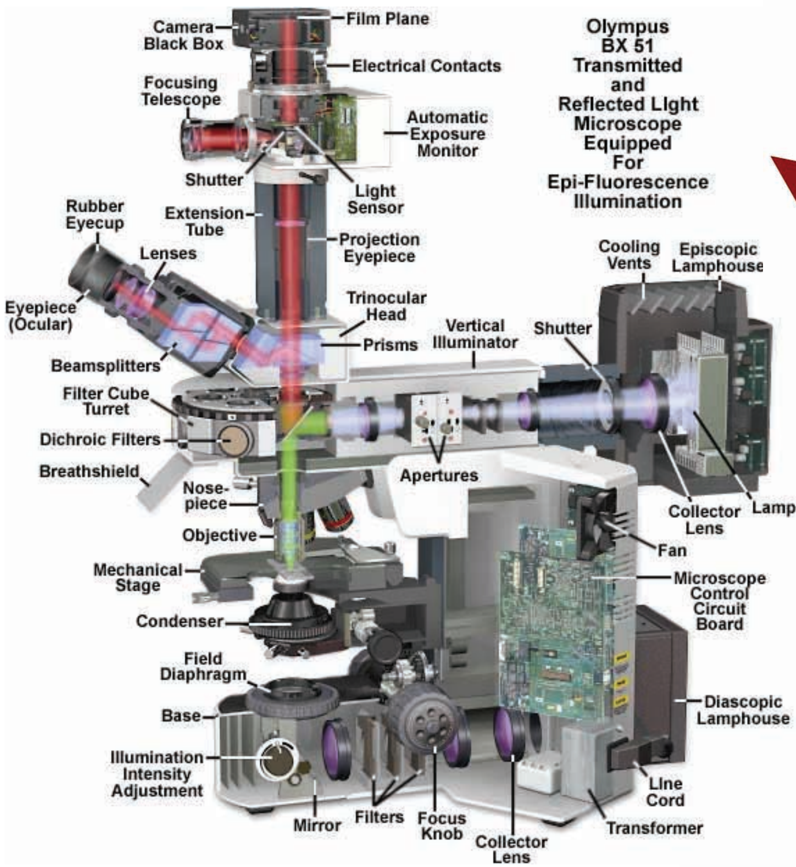


Image from MicroscopyU webpage

Modern Microscope Components



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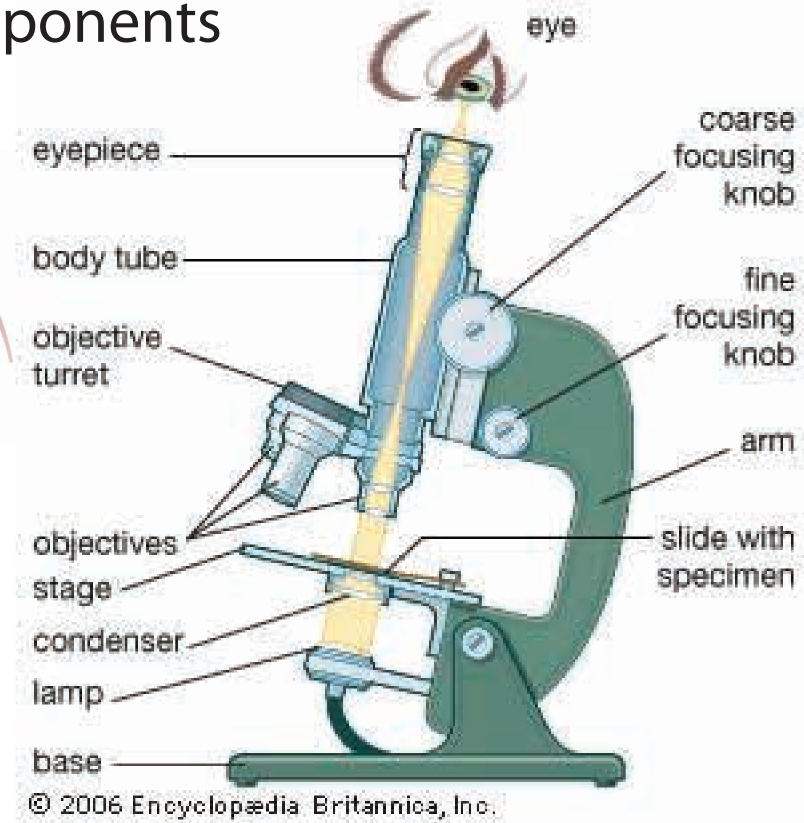

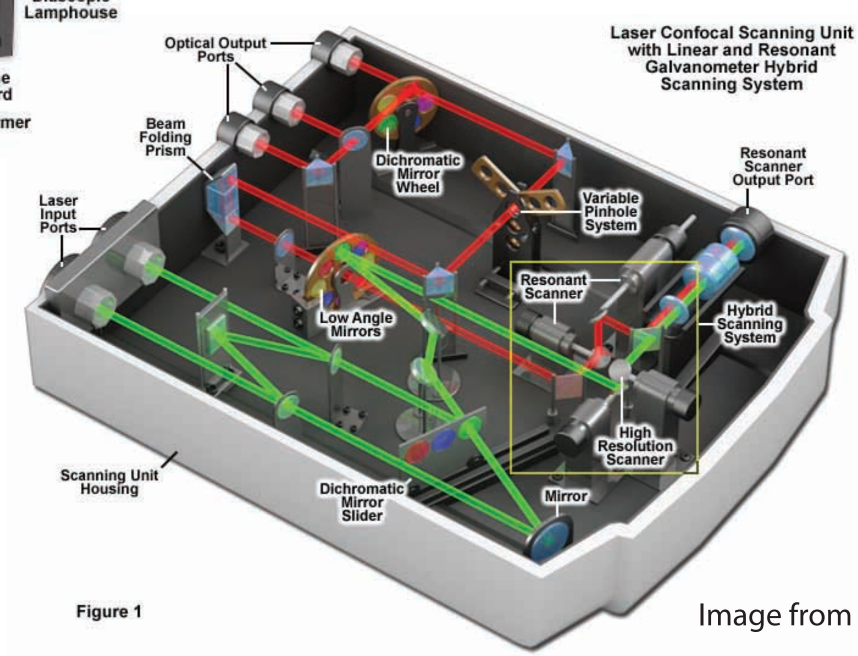

Image from MicroscopyU webpage

Kohler Illumination

Light Pathways in an upright microscope

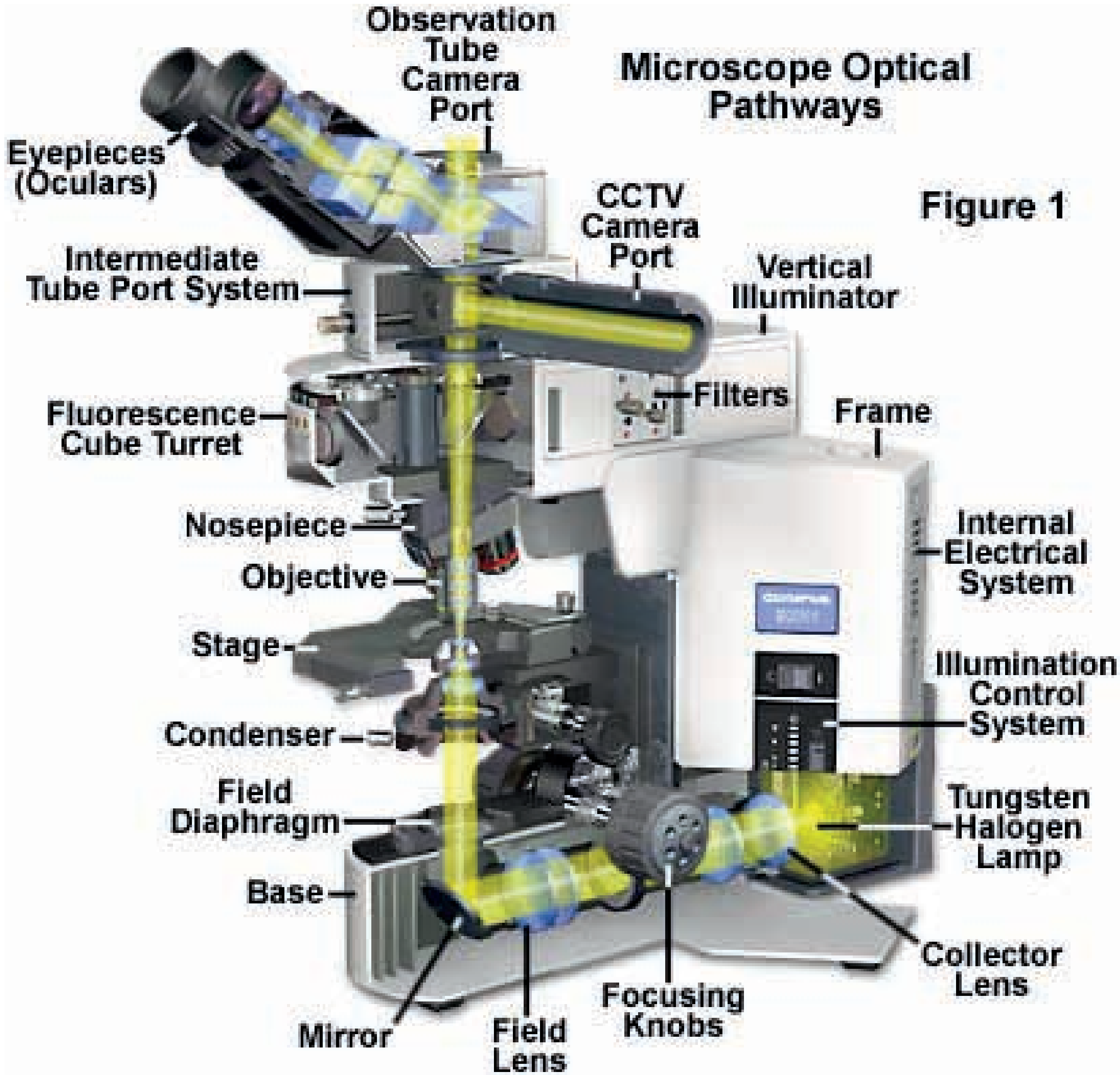

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Image take from Olympus Microscopy Resource Center Website

Kohler Illumination

Light Pathways in an inverted microscope

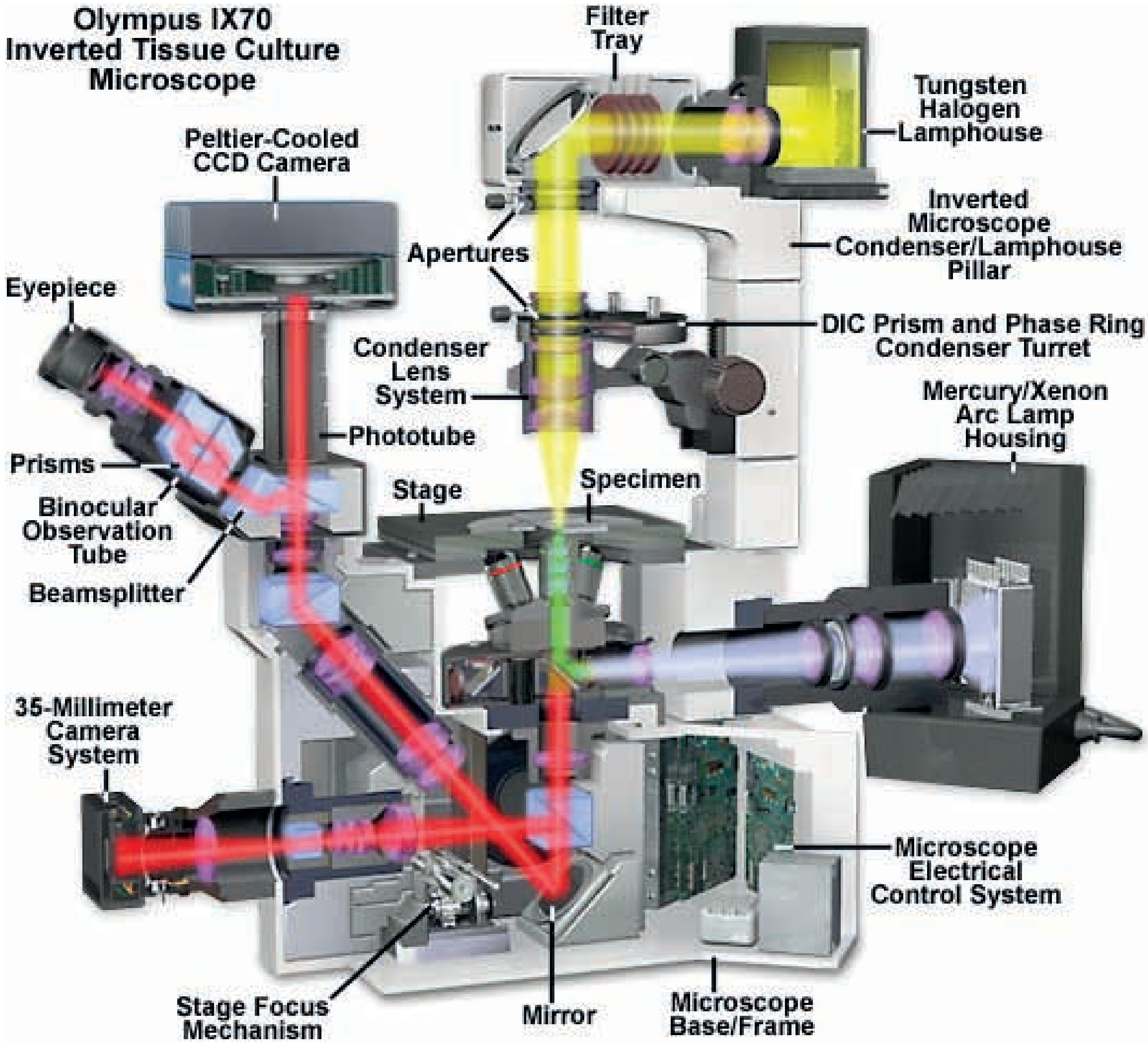

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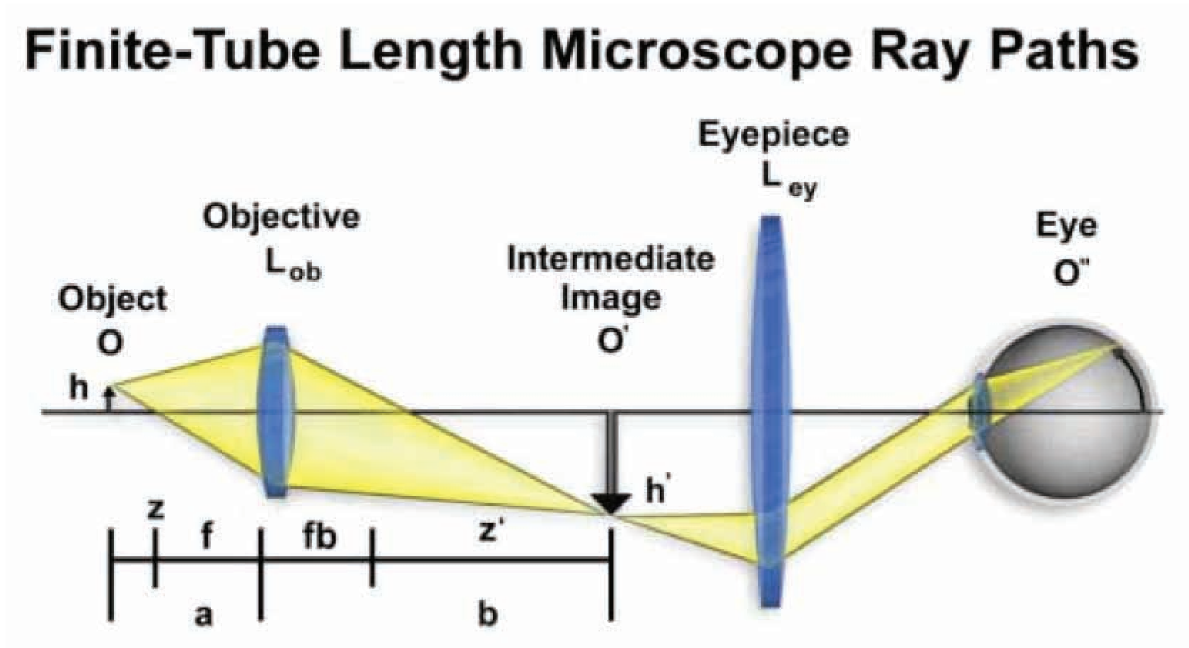
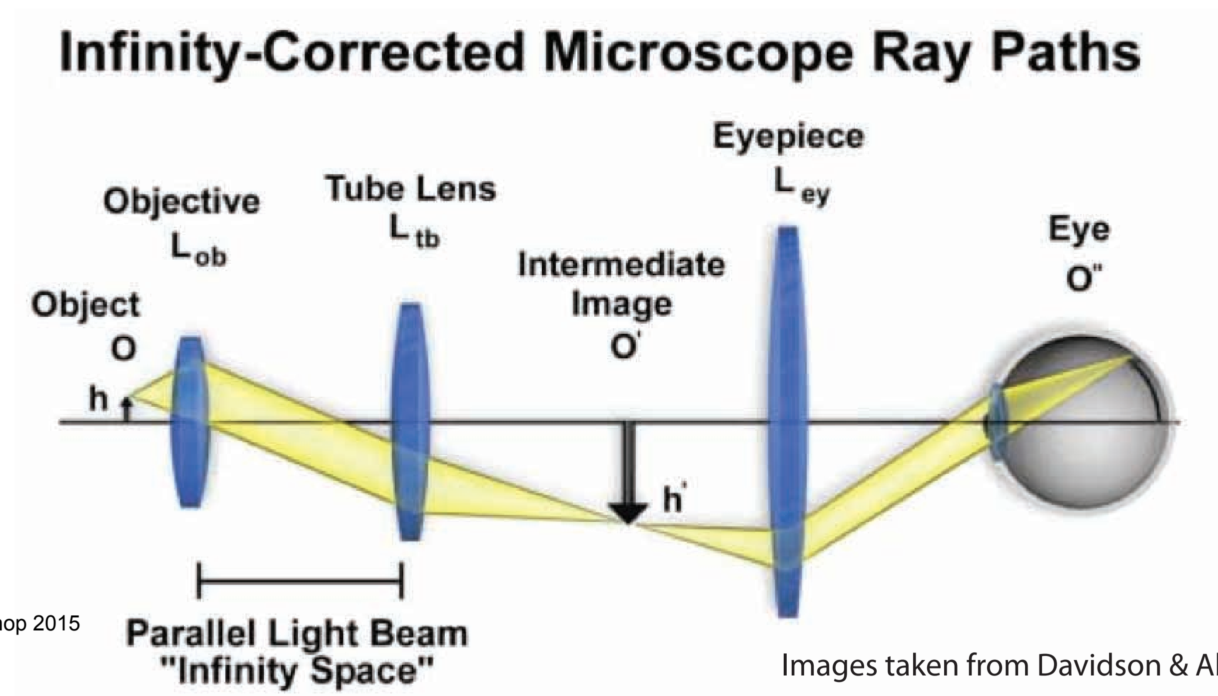
Image take from Olympus Microscopy Resource Center Website

Infinity-Conjugate vs. Finite-Conjugate Microscopes





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Images taken from Davidson & Abramowitz, Optical Microscopy

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