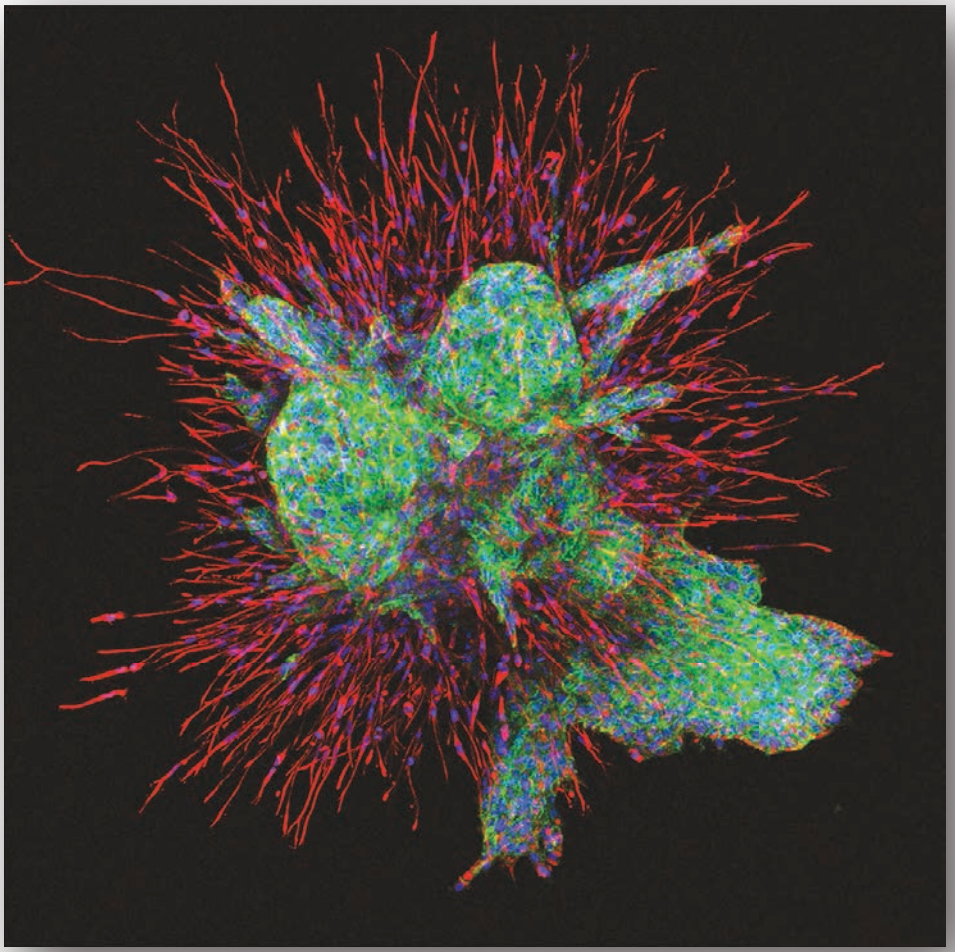
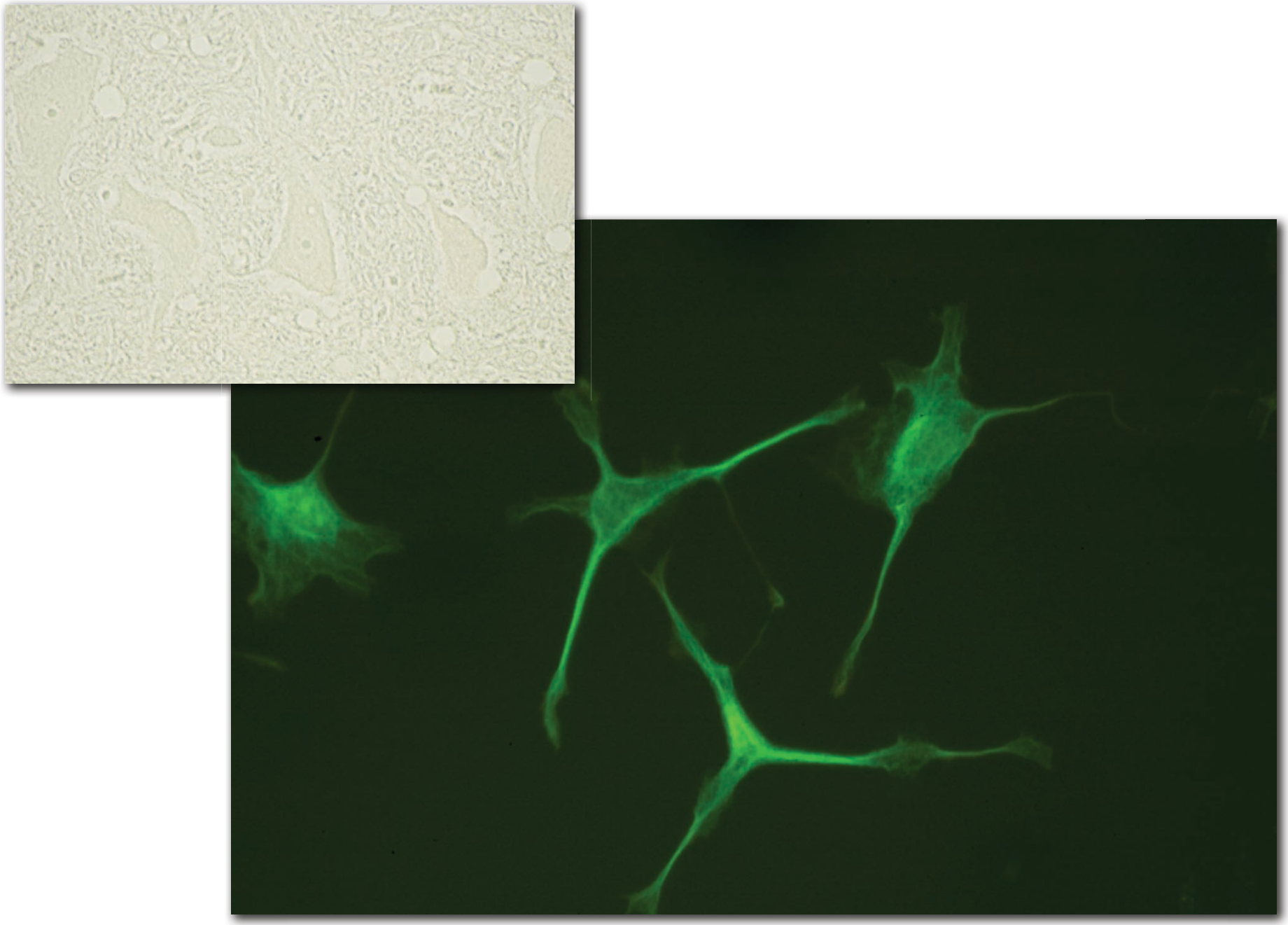
Fluorescence Photomicrography



Daily House Keeping

- Assn Four – due Monday & Wednesday
- Assn Five – Due March 2
- Assn Six – Blog & Poster
- Midterm- average grade 79.5%
- Final Exam – March 10



Fluorescence

Short λ energy is used to excite a sample that then emits longer λ energy

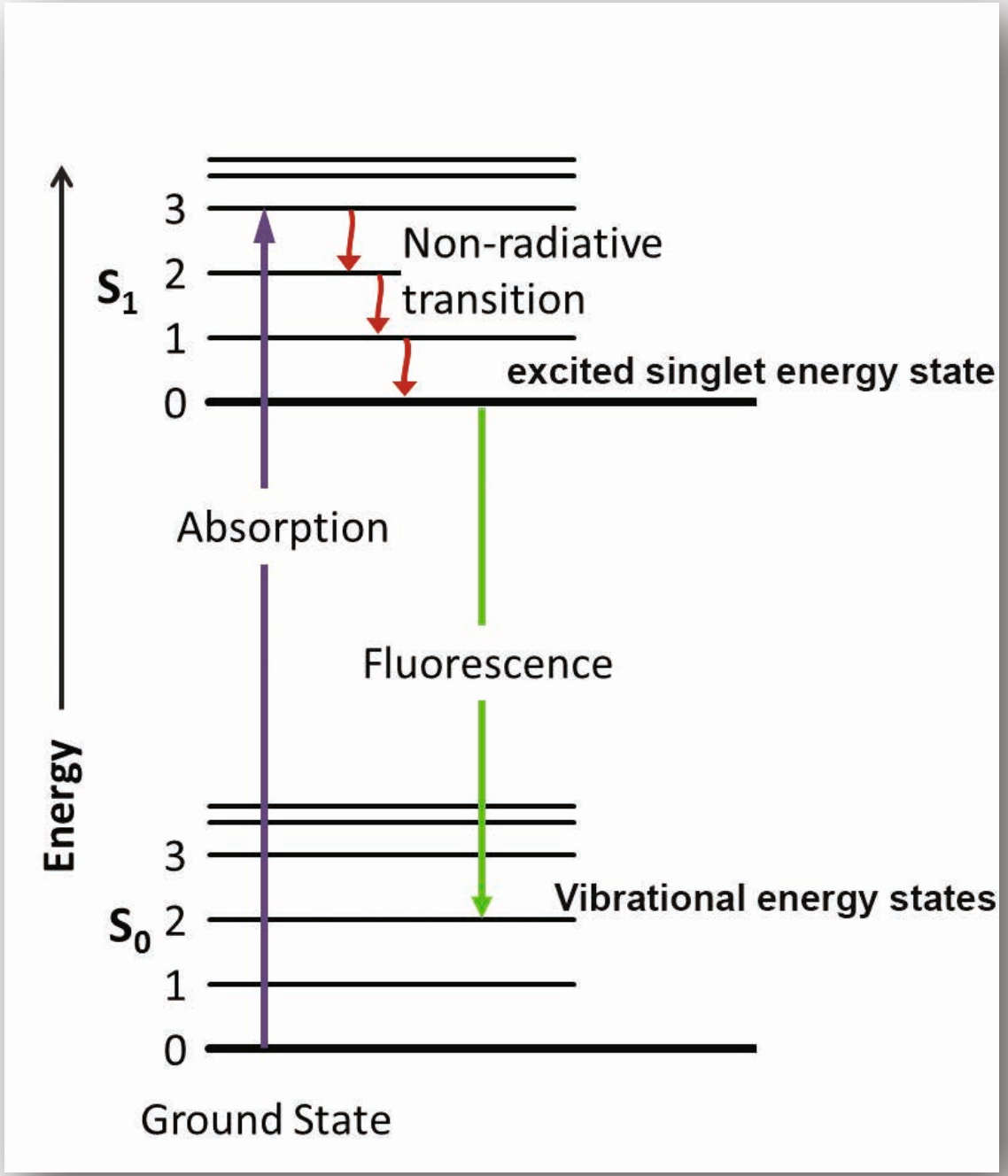
Sir Gabriel Stokes -1852

Stokes' Shift

Vibrational energy is lost as electrons go from and excited state to a ground state.

The excitation is always of a lower λ and higher energy than the emission.

Jablonski Principle
Professor Alexander Jablonski in 1935 described absorption and emission of light.


Energy
S_1
3
2
1
0
Non-radiative
transition
excited singlet energy state
Absorption
Fluorescence
S_0
3
2
1
0
Vibrational energy states
Ground State

Electromagnetic Spectrum

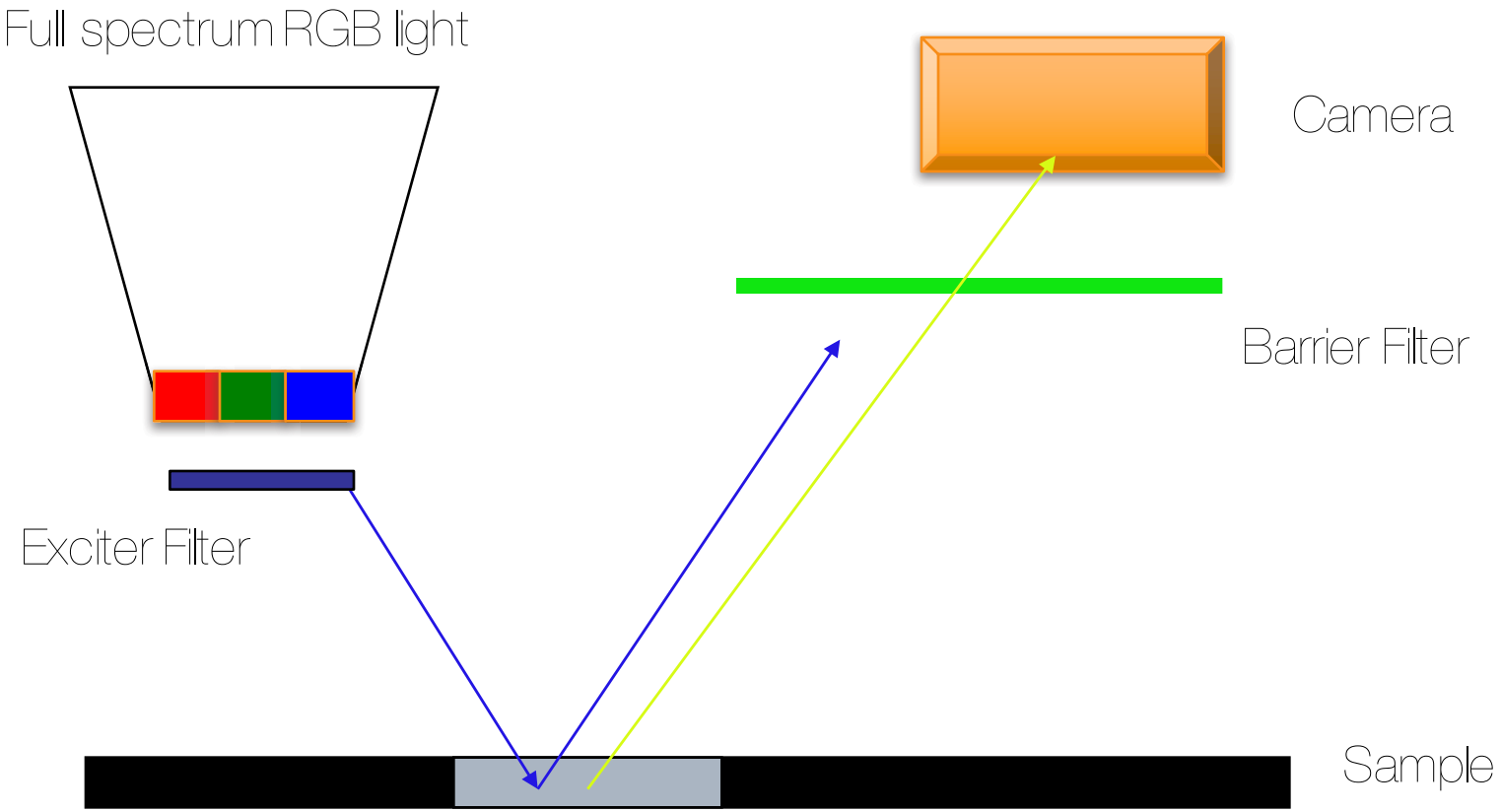
200-400nm 400 500 600 700nm 700-1000nm

ultraviolet blue green red infrared

Short λ
High energy

Long λ
Low energy

http://micro.magnet.fsu.edu/primer/lightandcolor/fluorescencehome.html

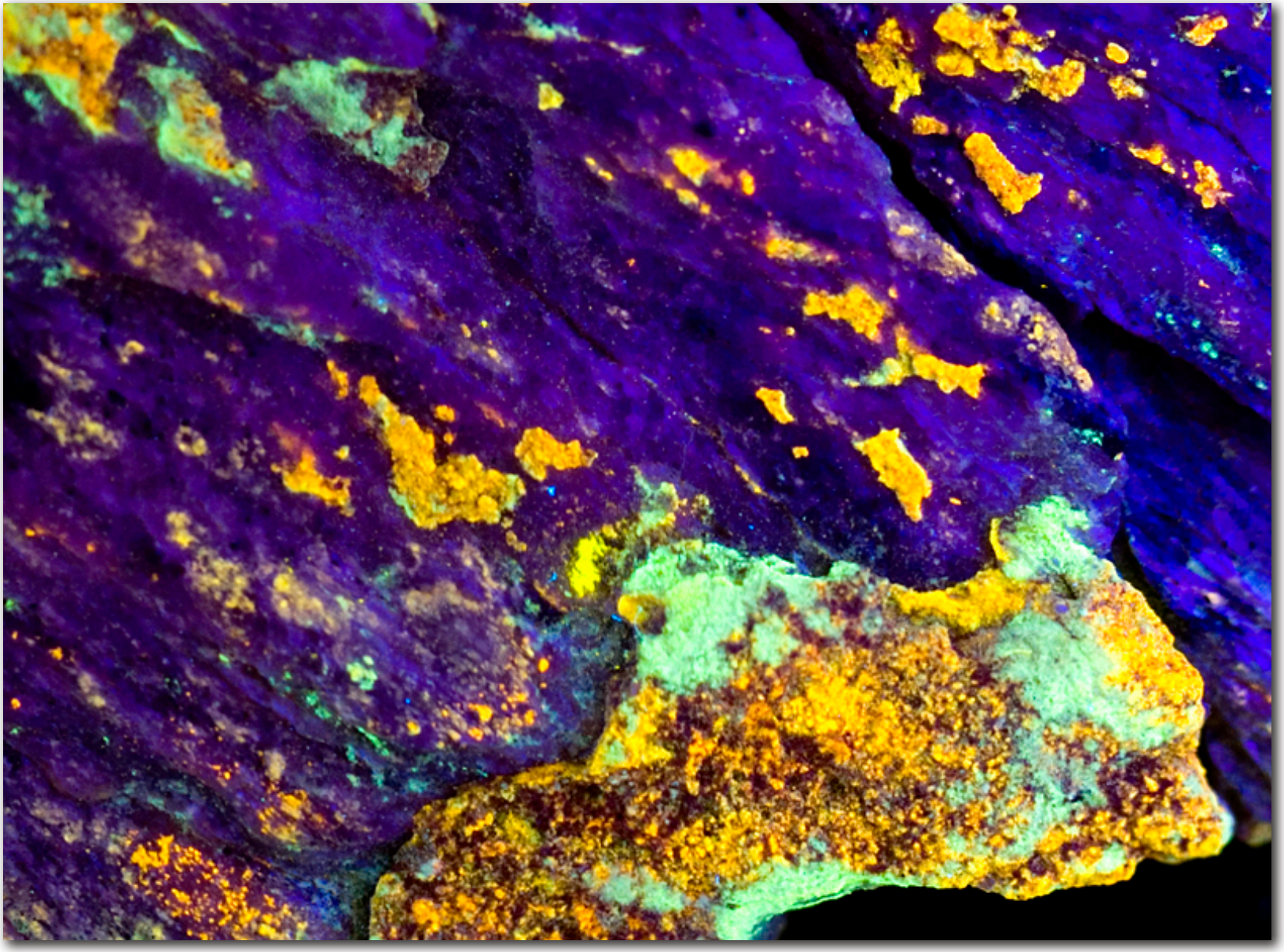

Full spectrum RGB light
Camera
Barrier Filter
Exciter Filter
Sample

What is Required?

Excitation Filter

Emission Filter

Light source



Fluorescence Microscopy

Necessary	Unnecessary
Light Source	Special Objectives*
Reflected Light Path	Kohler Illumination
Excitation Filter	Transmitted light path
Dichroic (beam splitter)	Condenser
Emission Filter	Aperture Diaphragm

Fluorescence Illumination Path

Light sources for Fluorescence

Mercury Arc Lamp

Xenon Arc Lamp

Metal Halide Lamp

Light Emitting Diodes (LEDs)

Lasers

Considerations: cost, stability, spectral output, bulb lifetime, ease of use, safety…

Choosing Filters

Filtration must be mutually exclusive.

Long pass vs. Band pass
(wide band vs. narrow band)

Dichroic Filter

(Dichromatic mirror, Beam splitter)

- Is a highly specific color filter
- In one direction filter reflects one color and transmits the other 2 primaries while traveling in the other direction, it transmits 2 primaries

Objective Selection

Special objectives are not required for fluorescence, but some are better than others.

- Transmission characteristics of objective
- Aberration correction = more glass, greater light loss
- High NA at lowest magnification possible

Microscope set up

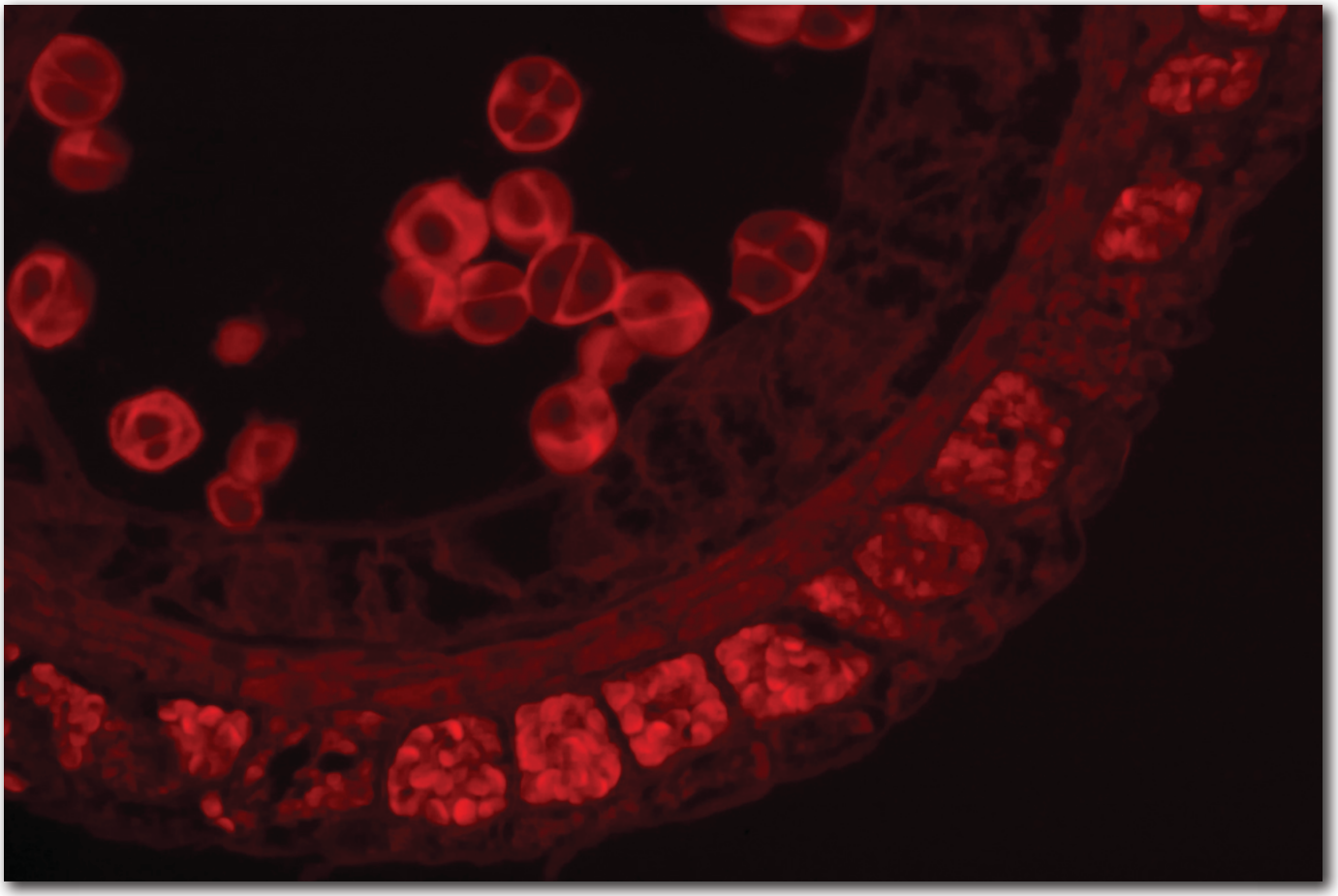
Homogeneous Illumination

Field stop set

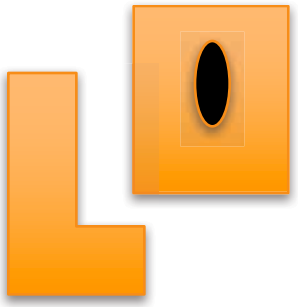
Transmitted light off

FL Filter set in place

Autofluorescence



Evaluating the Presence of a Protein

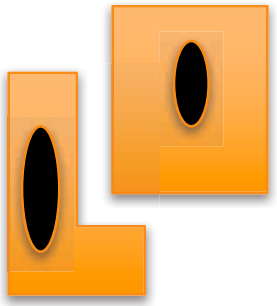


Expression or co-localization

Sometimes called Tagging, Fluorochrome, Fluorophore, Probe, or Fluoresence Dye

Antigen – Antibody
Lock & Key

Evaluating the Presence of a Protein



Sometimes called Tagging, Fluorochrome, Fluorophore, Probe, or Fluoresence Dye

Antigen – Antibody
Lock & Key

Common Fluorochromes

Dapi

Fura 2

CFP

FITC

GFP

YFP

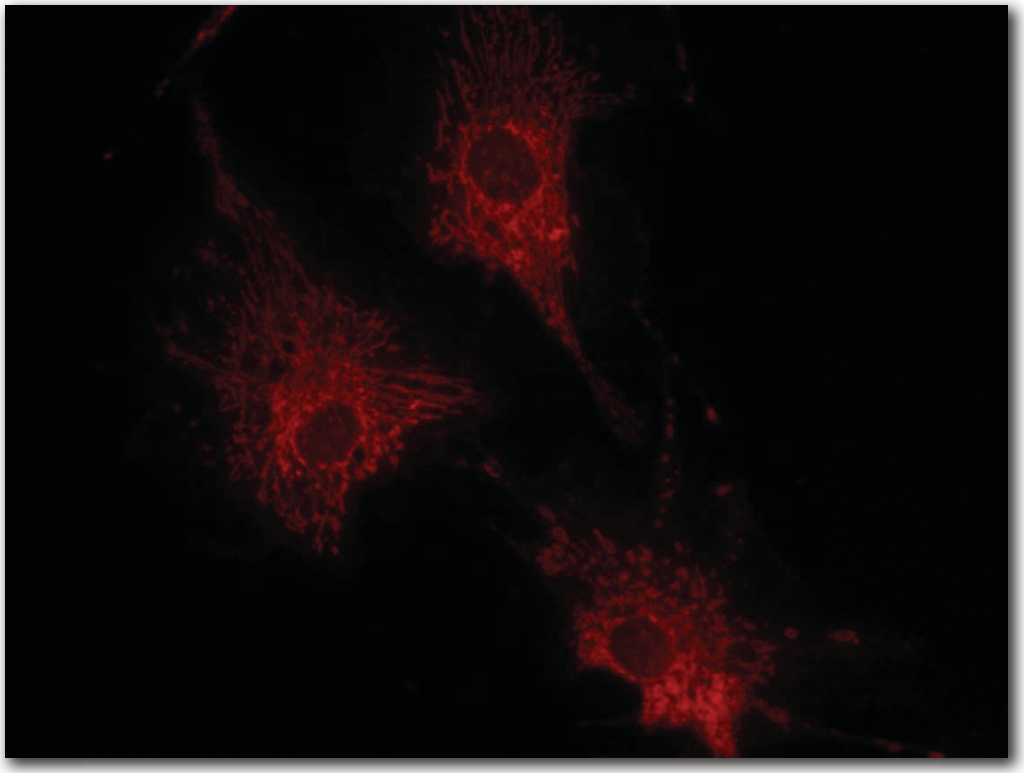
CY3

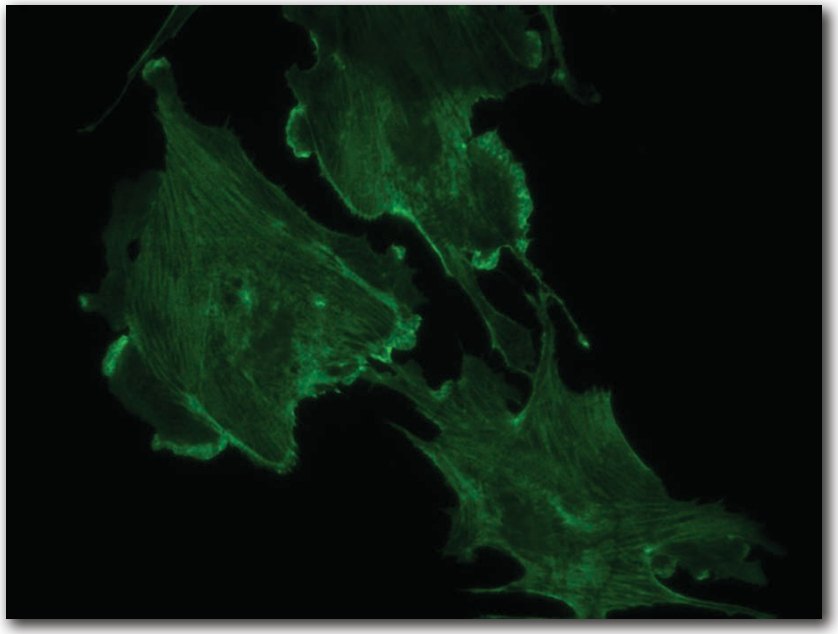
Texas Red

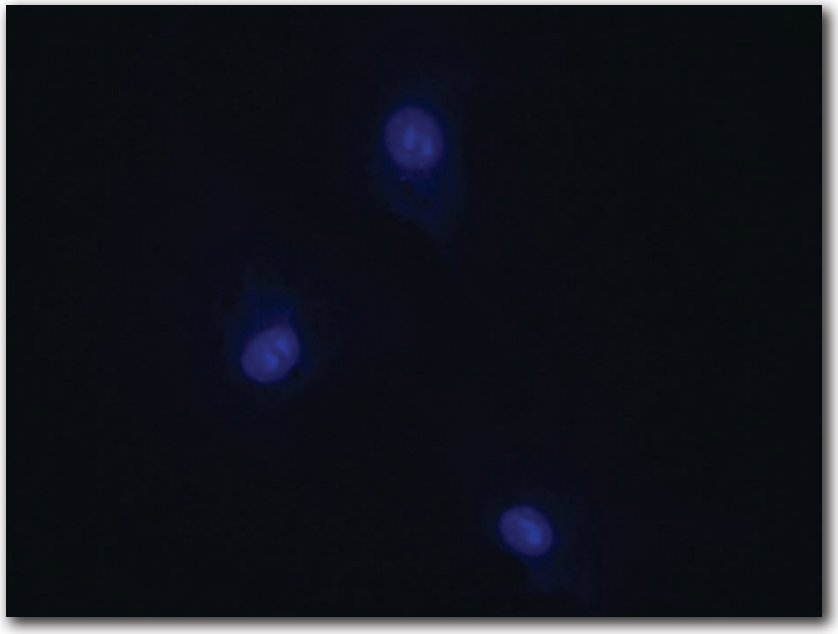
Rhodamine

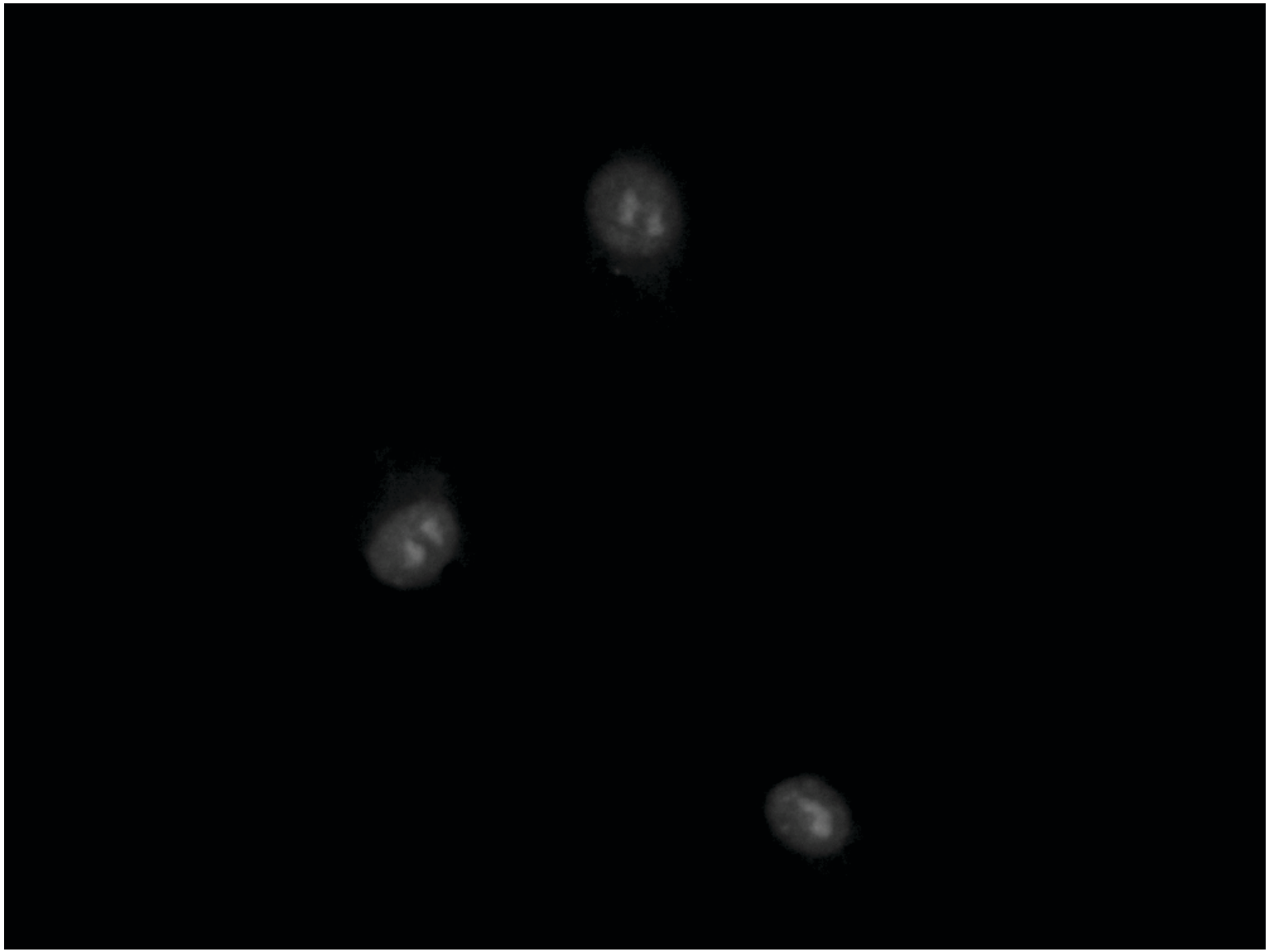
CY5.5

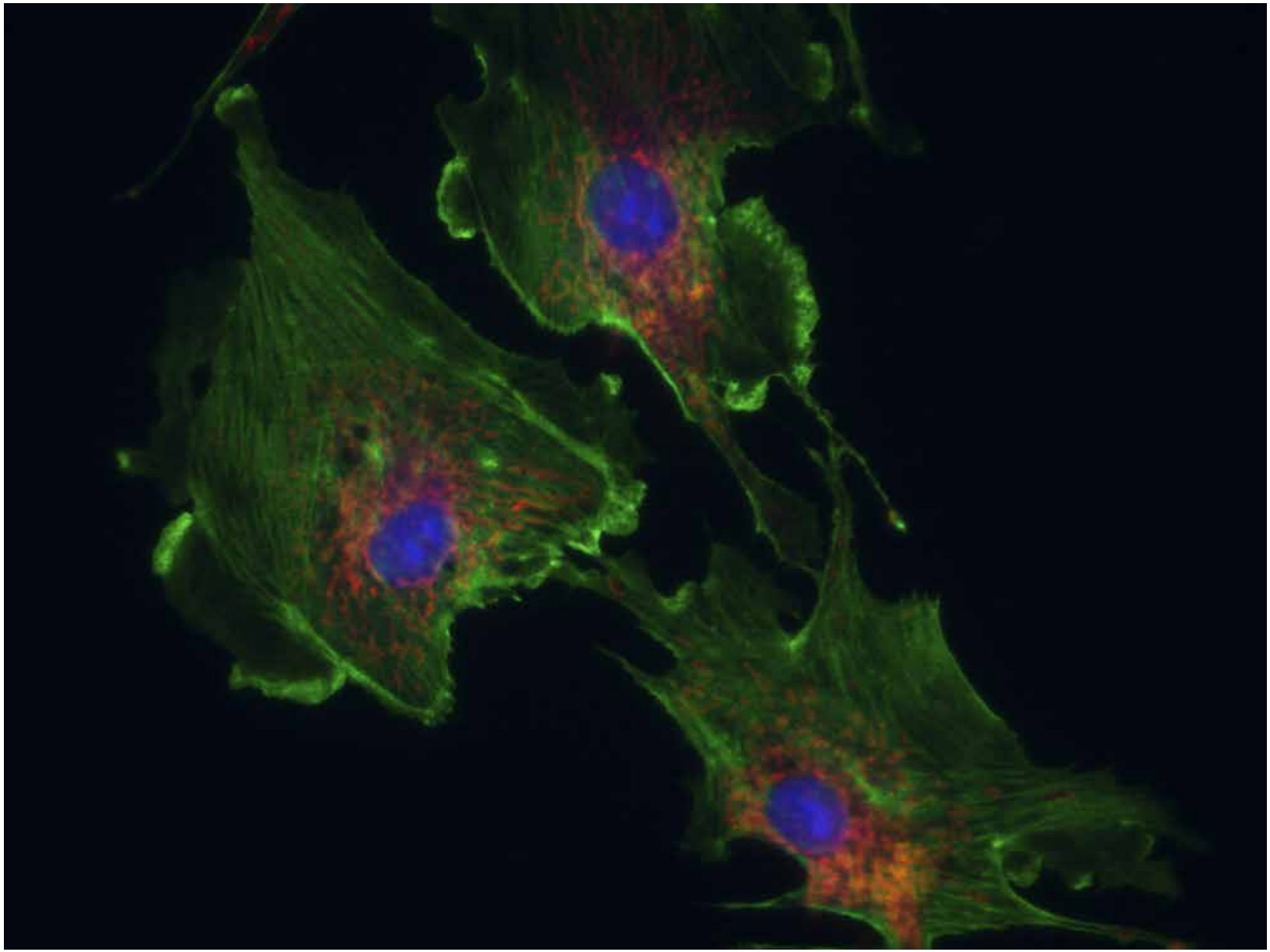
Immunofluorescence











Challenges

Low light

Long exposure

Bleaching / Quenching/Burn Out

High Contrast

Out of focus Fluorescence areas

Uneven Fluorescence across field

Signal to Noise Ratio

Exposure

Tips, Tricks & Reminders

Keep the room lights off

Find your sample in brightfield before switching to fluorescence.

Close the reflected light shutter when you are not looking at or taking a picture of sample (if no shutter, cover your sample w/ lens cap.)

It is not necessary to white balance the camera.

Tips, Tricks & Reminders

- Binning
- Gain
- Lower Monitor Brightness to help with seeing
- Use a grayscale camera and pseudocolor
- Three shot camera


Episcopic Illumination
100W
Exciter Filter
high
Low Dispersion
Emission or Barrier
Dichroic Mirror
Imaging Solutions
- Gain
- Binning
- Use N.D. filters for viewing
- Use Grayscale camera
- Use Shutter
- Close FS. more
- Critical Exposure
- Low Brightness
- Low Contrast Recording
- Quenching
- Bleaching
- Photo Bleaching
- Extinguish
- Burn out
- High Contrast
- Z. Exposure
Remove Pol Filter
Filters
- Mutually exclusive
- Broad or Narrow Band
Imaging Problems
- Low Light
- High Contrast
- Short Duration Event
- Focus of Location of Fluoresc. Reaction
gamma
1.4
0
255

Safety Concerns

Do not touch bulb.

Turn Mercury lamp on before microscope, camera and computer.

Warm up Bulb time

On for the day/off for the day

Log use for lamp lifetime (most power supplies have counter)

Mercury lamps have a 200-300 hour lifespan.

- Flicker, Arching, instability, risk of explosion