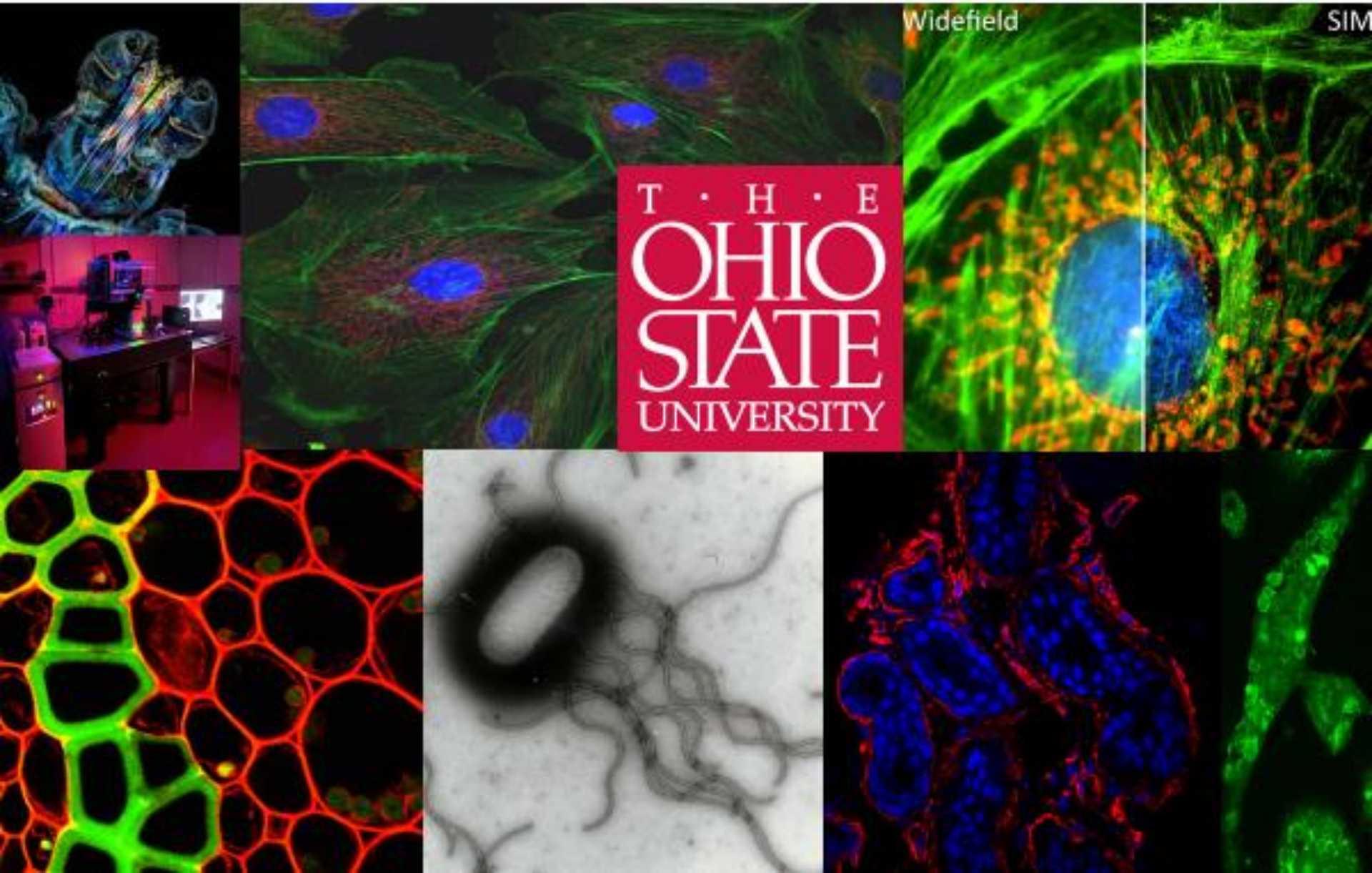
# Campus Microscopy and Imaging Facility



# FACILITY INFORMATION:

## Campus Microscopy and Imaging Facility (CMIF)
## CCC Microscopy Shared Resource (MSR)


**Dr. Paul Stoodley, Director**

**Dr. Jeff Tonniges, Sr. Research Associate**
**Kendall Gallagher, Research Coordinator**
**Brian Kemmenoe, Sr. Microscopist**

**cmif@osu.edu**

**614.292.9786**

**cmif.osu.edu**


# Why are we here?

**Facility Overview and Confocal Training Seminar**

How can I make the best use the CMIF instruments and services?

Getting Started:

Campus Microscopy and Imaging Facility (CMIF)
245 Biomedical Research Tower
460 West 12th Avenue
Columbus, OH 43210-1239

Phone 614-292-9786

# Using Our Lab

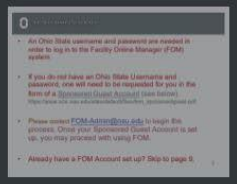
cmif.osu.edu/our-lab

# Getting Started Guide

go.osu.edu/startusingcmif


1


2



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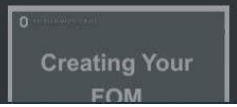



THE OHIO STATE UNIVERSITY

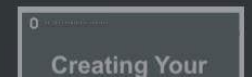
# Facility Online Manager (FOM) Guide

These CMIF/MSR instructions adapted from the OSU "Center for Electron Microscopy and Analysis" core facility with kind permission.


1


2

3




# Index

CMIF

## Campus Microscopy and Imaging Facility/Microscopy Shared Resource

## BSL2 Space Use Form

Name of Researcher _____ Date _____

Email _____ Phone Number _____

PI Name _____ PI Email _____

☐ Please check this box to confirm you have received BSL-2 training as required by The Office of Environmental Health and Safety at The Ohio State University.

☐ Please check this box if using live cells, unfixed tissue, or recombinant DNA (rDNA).

Name and organism derivation of cell line: _____

Human or Primate Material (if applicable) – IBC Approval # _____

Recombinant DNA (if applicable) – IBC Approval # _____

☐ Indicate if using rDNA with oncogenes/tumor suppressors or replication-competent viral systems (e.g., lentivirus).

**IMPORTANT: All human or primate material and recombinant DNA (plasmids, viral vectors, etc.), MUST have approval from the IBC before being brought into the Microscopy Facility.** Hazards, Risks, or Special Handling

_____

**Facility Rules:**

1. **All CMIF procedures and policies are applicable (https://cmif.osu.edu/Our-Lab-Policies).**
2. **BSL-1 or 2 materials only.**
3. **No food or drink is allowed in the facility.**
4. **All BSL-2 materials must be transported into and out of the CMIF using secondary containment that is clearly marked as biohazard. Anything brought into the laboratory must be taken out following the conclusion of the experiment.**
5. **Please fill out a new form if any of the above experimental conditions or approval numbers change.**
6. **Please leave the laboratory as clean as was found. Disinfect all areas using 10% Sodium hypochlorite with the exception of the microscope. Due to the sensitivity of the equipment, this will be performed by CMIF staff.**
7. **Notify CMIF staff of any spills immediately so that damage to the microscope may be prevented or minimized.**
8. **Failure to cleanup and/or decontaminate all laboratory areas used will incur a charge to the user.**
9. **Damage to the equipment will incur a charge to the user.**

I acknowledge these rules and will ensure compliance by my lab personnel:

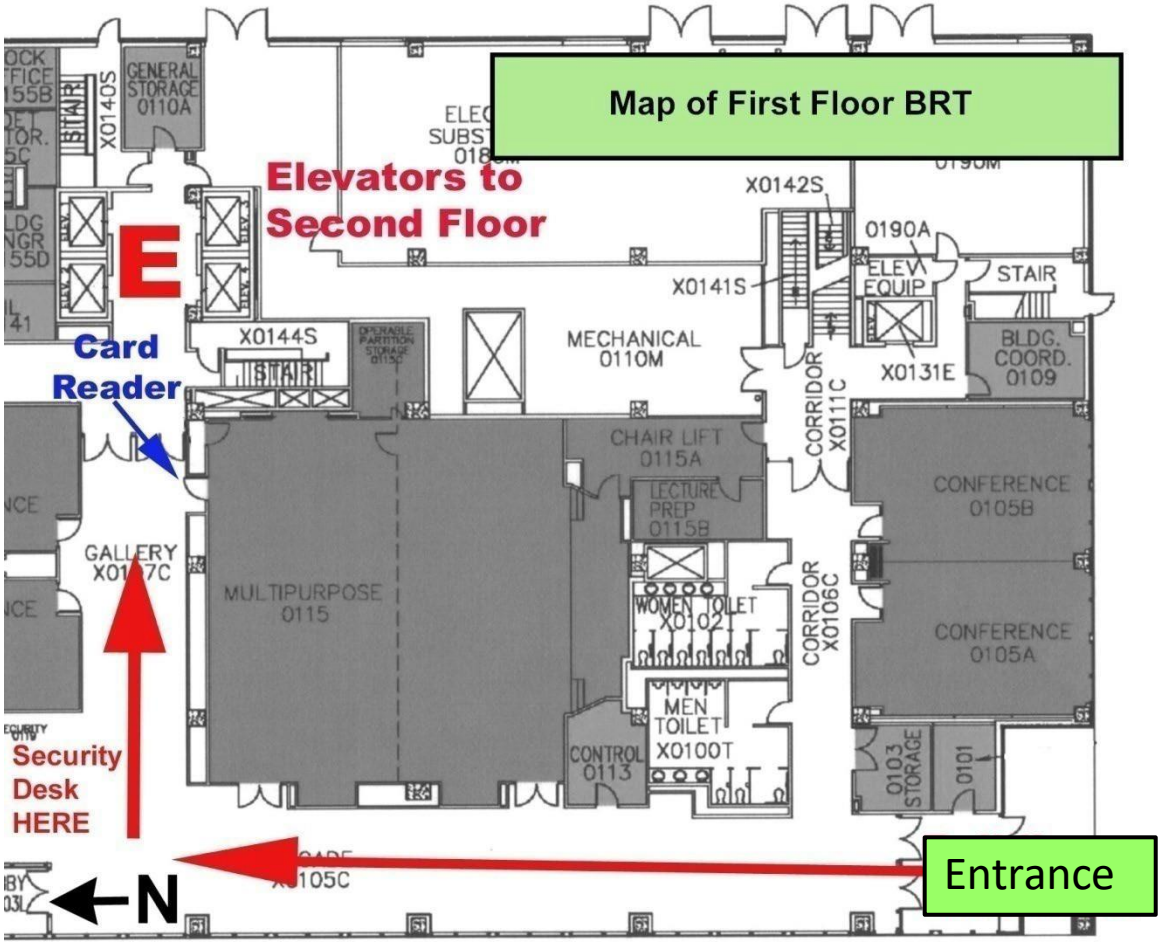
_____ _____

# CMIF Location in the BRT

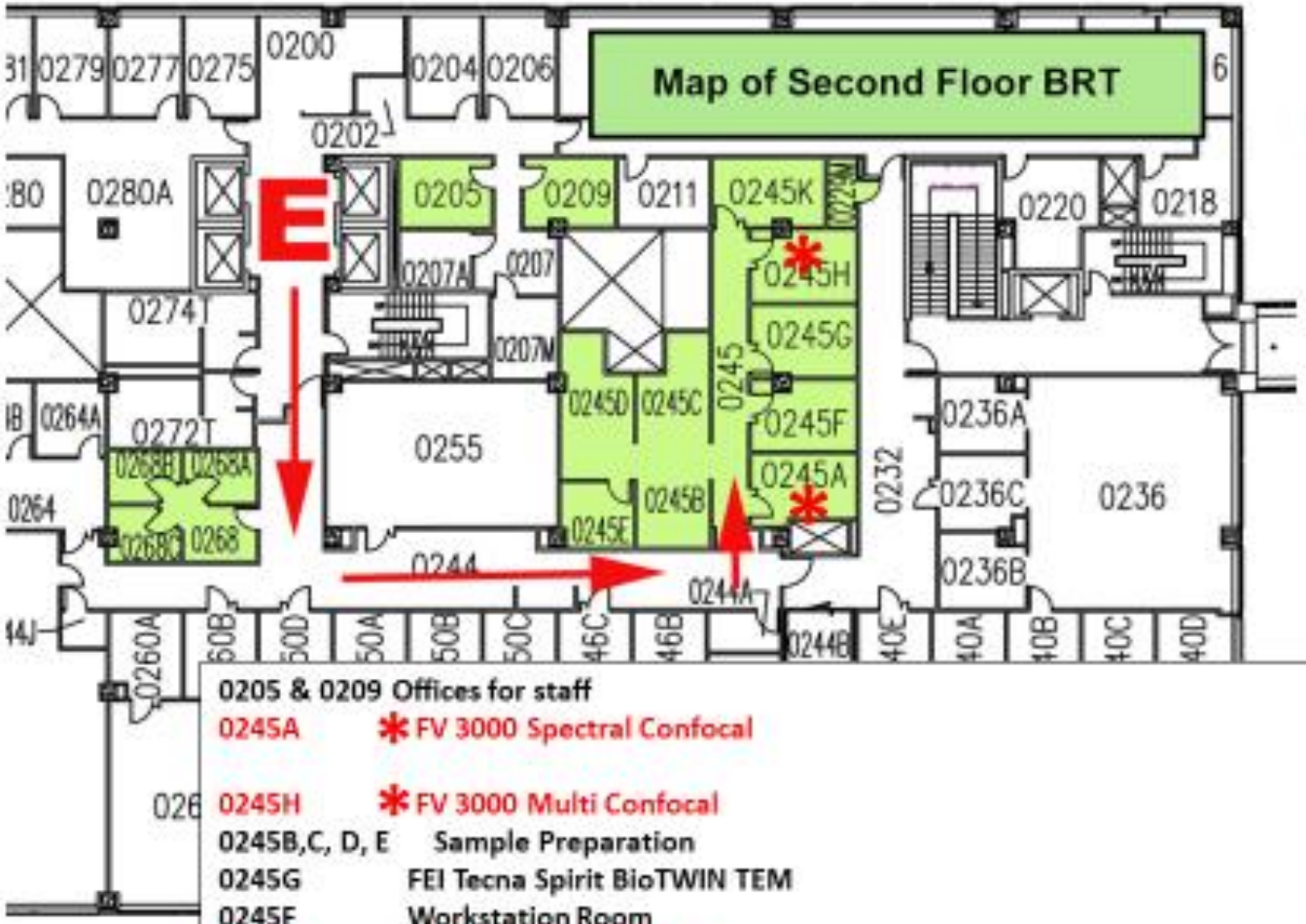
**The BRT is a secure building, and the card reader is shown in blue on the drawing. The elevators are indicated by the red E. If you do not already have ID card access, follow the steps below.**

GROUND FLOOR

There is a Security desk in the first-floor corridor where you may have to show your ID to the security guard and tell them that you have an appointment with the CMIF (rooms 245 & 205 phone 614-292-9786).



# CMIF on 2nd Floor BRT




The elevators are indicated by the red E

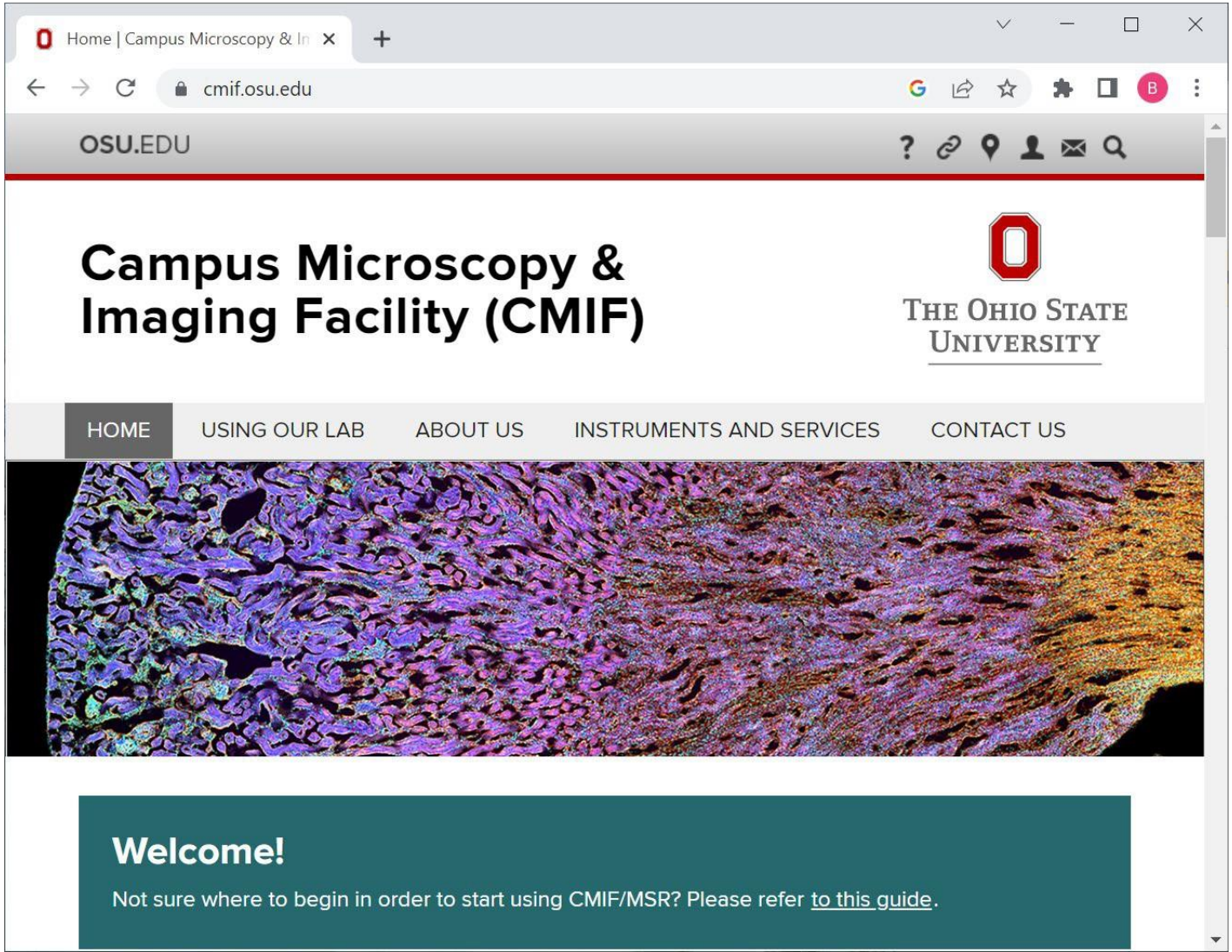
0205 & 0209 Offices for staff

| | |
|---|---|
| 0245A | * FV 3000 Spectral Confocal |
| 0245H | * FV 3000 Multi Confocal |
| 0245B,C, D, E | Sample Preparation |
| 0245G | FEI Tecna Spirit BioTWIN TEM |
| 0245F | Workstation Room |
| 0245K | FV 1000 Filter Confocal |
| 0264B | Live Cell Confocal |
| 0264A | FV 1000 Multiphoton45 Staining and preparation |
| 0268 | Super Resolution System |
| 0245K | Zeiss Axioskop, Stereo Microscope, Nuance Spectral camera |
| 0245D/E | Leica UC6 &7 Ultramicrotomes (6 is Cryo-capable) |
| 0245E | Reichert UltraCut E Ultramicrotome |

# Microscopy at OSU – what we offer

- High-end Microscopy equipment
- Training
- Consultation and support
- Image analysis software (Imaris)
- 24-hour access for experienced users
- Sample preparation for SEM and TEM

# Our Website


Home | Campus Microscopy & In
cmif.osu.edu
OSU.EDU
Campus Microscopy & Imaging Facility (CMIF)
THE OHIO STATE UNIVERSITY
HOME
USING OUR LAB
ABOUT US
INSTRUMENTS AND SERVICES
CONTACT US
Welcome!
Not sure where to begin in order to start using CMIF/MSR? Please refer to this guide.

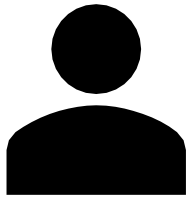
# Schedule instruments at fom.osu.edu

**All scheduling is done through FOM**

For first time users of FOM, you will need to create a profile and add a billing account (requisition) If you don't see your supervisor on the list, you can fill out the form within FOM to request they be added or contact fom-admin@osu.edu

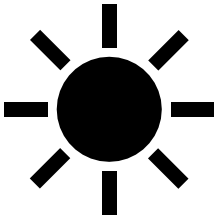
**Login with your OSU Name.#**

# Categories of FOM Users



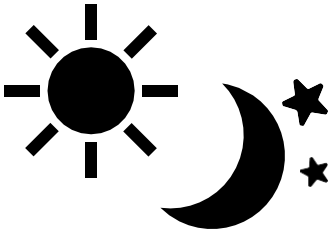
## To Be Trained

**All new users are "To Be trained"**
CMIF staff will schedule your training and email you.



## Daytime User

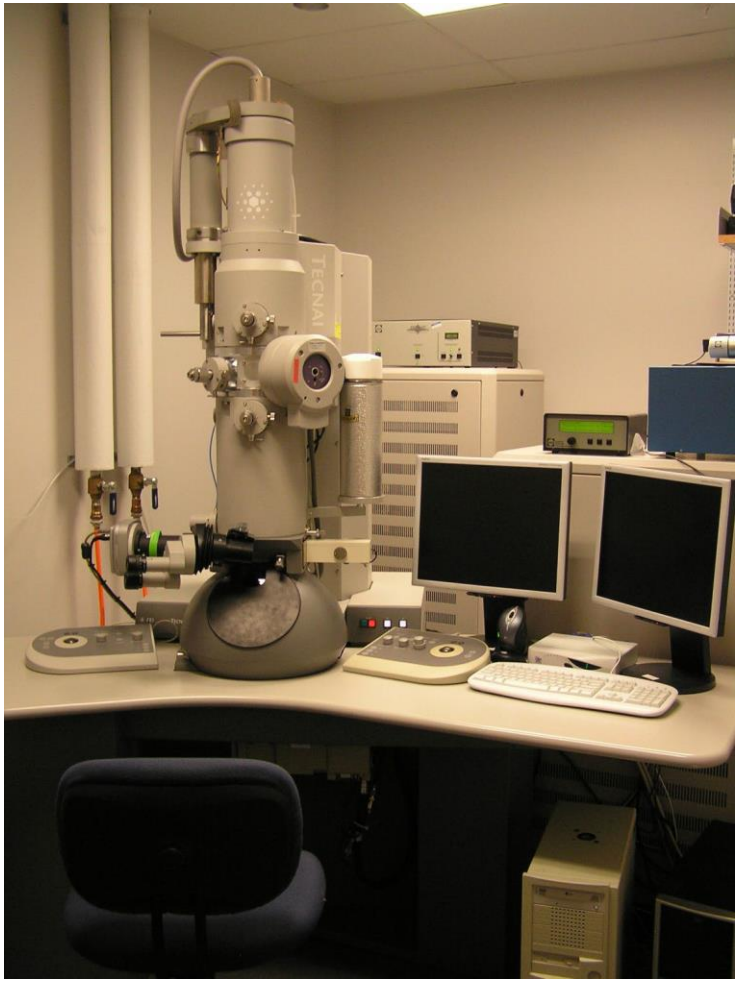
**Users who have been trained**
Can sign-up independently between 9 - 5 on weekdays.



## Anytime User

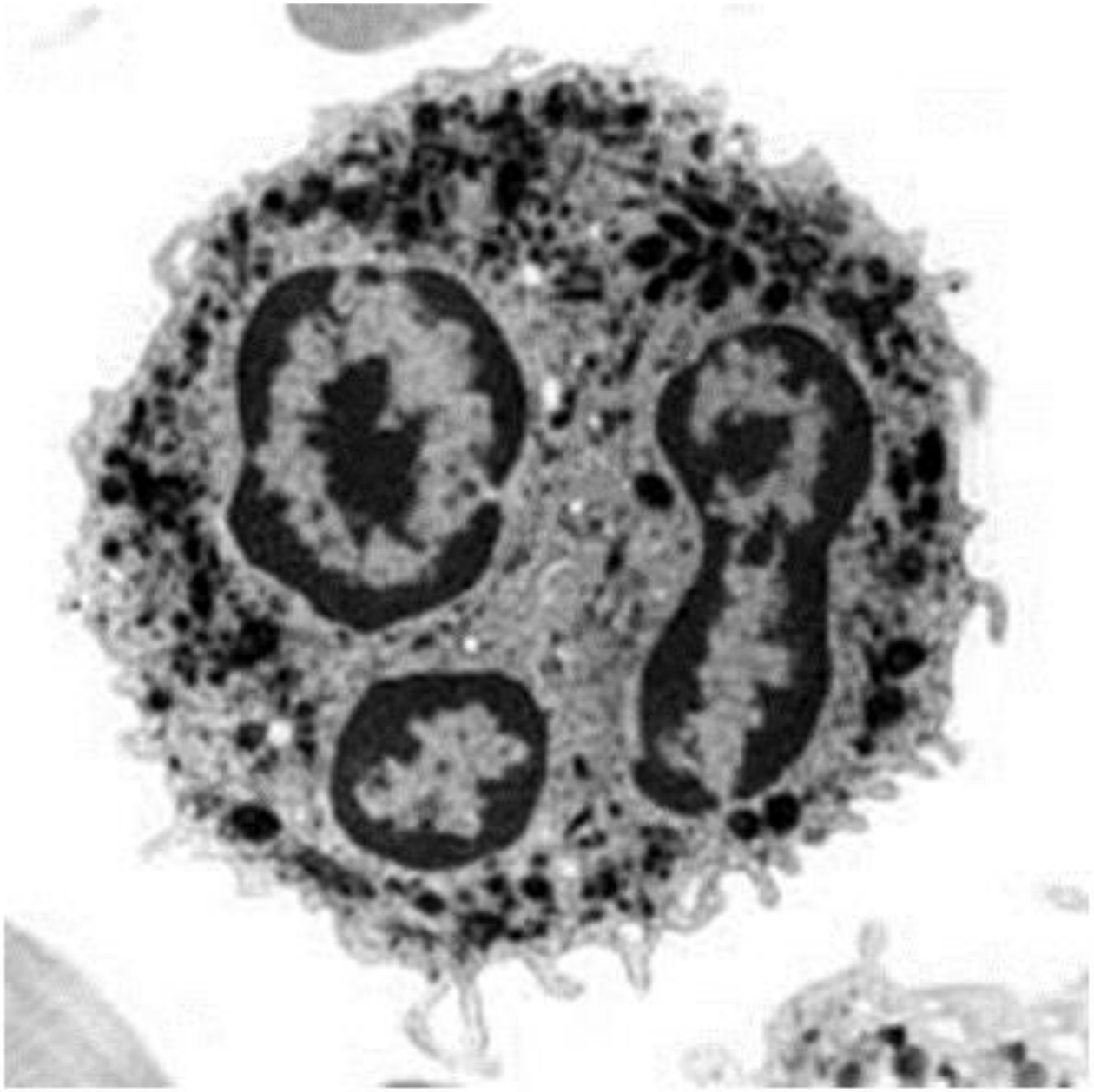
**Experienced Users with 12+ Visits Can Apply**
Can sign-up independently 24/7 with ID Card Access to CMIF

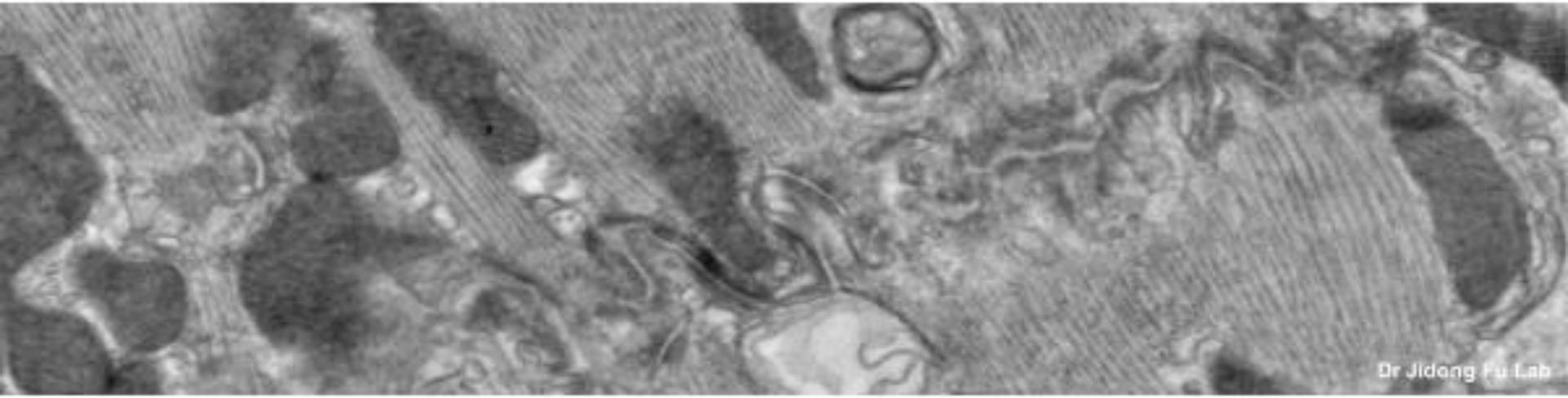
# Instruments at the CMIF

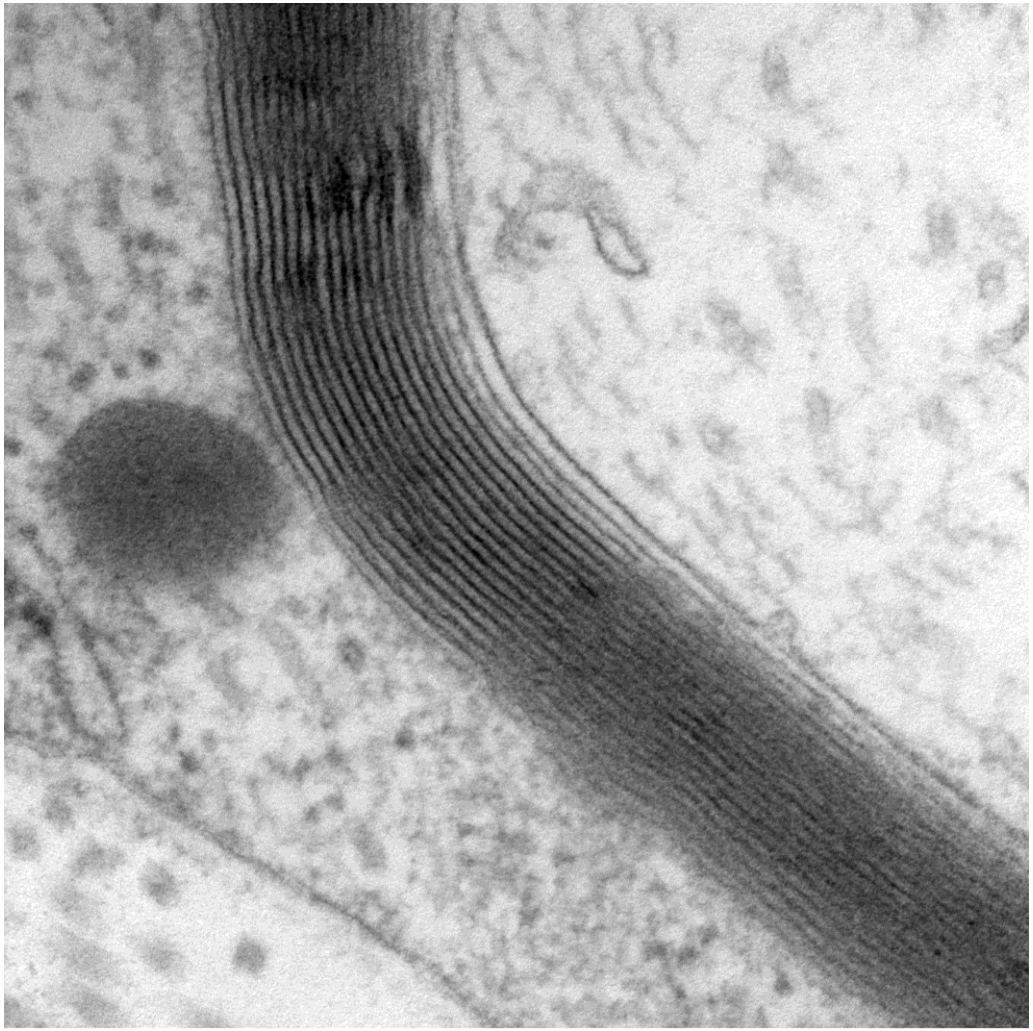


**FEI Tecnai Spirit Biotwin**

- Transmission Electron Microscope
- Critical Point Dryer (SEM prep)
- Sputter Coater (SEM Prep)
- Widefield Epifluorescence and Brightfield Microscope & Camera
- Stereo Microscope
- Confocal Microscopes
- Live Cell Confocal
- Multiphoton Microscope
- Pelco Biowave Microwave(alt. fixation)
- Super-Resolution System (SIM/STORM)
- Ultramicrotomes
- Cryostat
- Vibratome
- Image Analysis Workstation (Imaris)



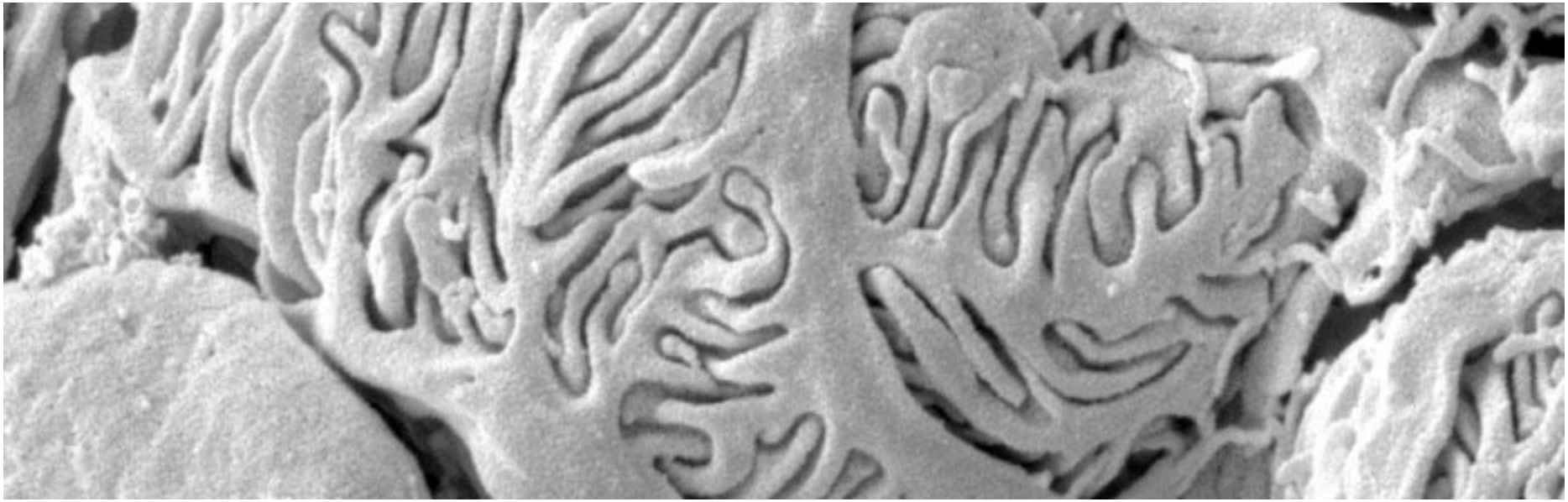

Dr Jidong Fu Lab



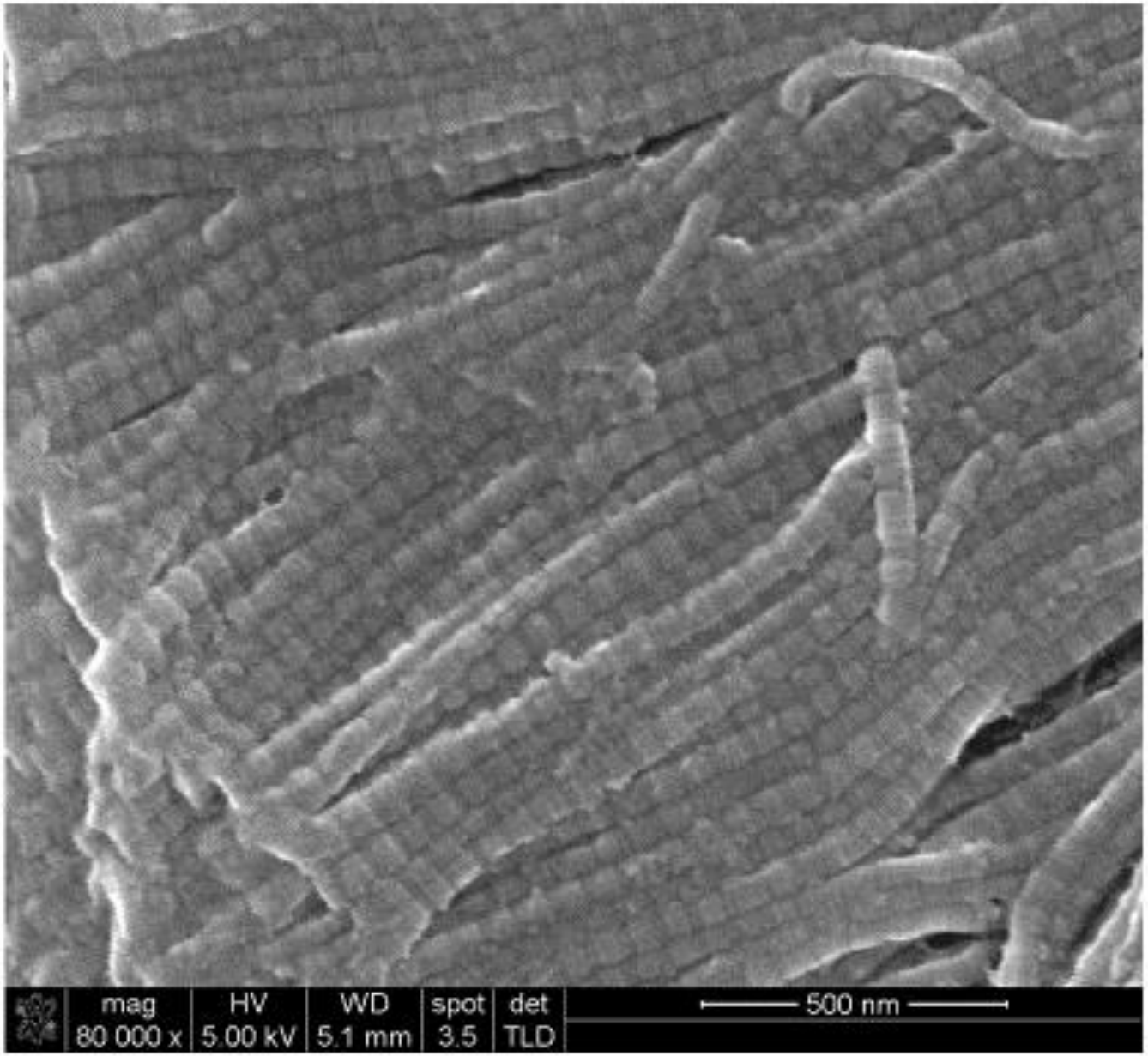
3.tif
test bright
Print Mag: 198000x @ 7.0 in
14:07 12/22/08

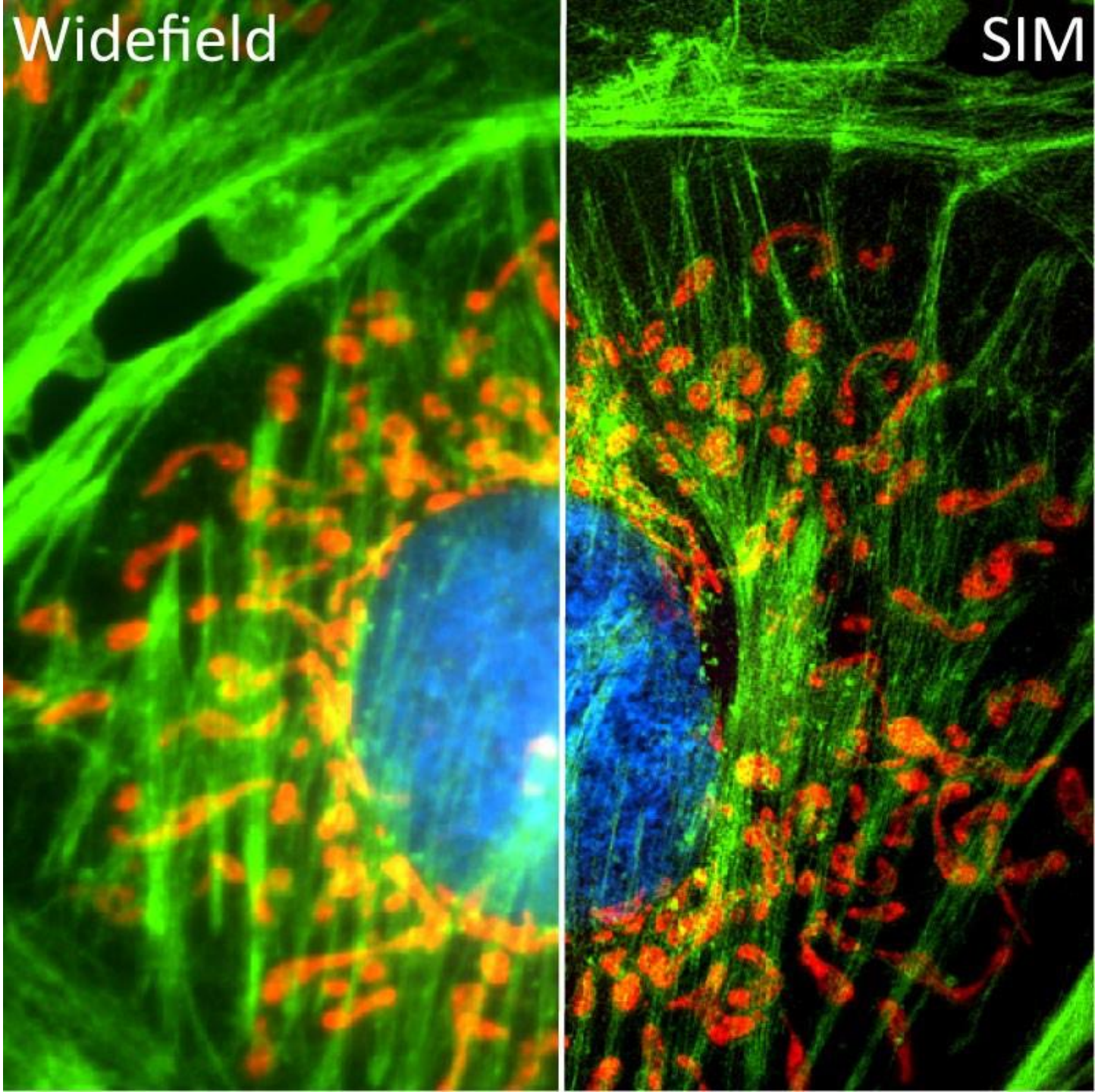
100 nm
HV=80kV
Direct Mag: 120000x
CMIF OSU

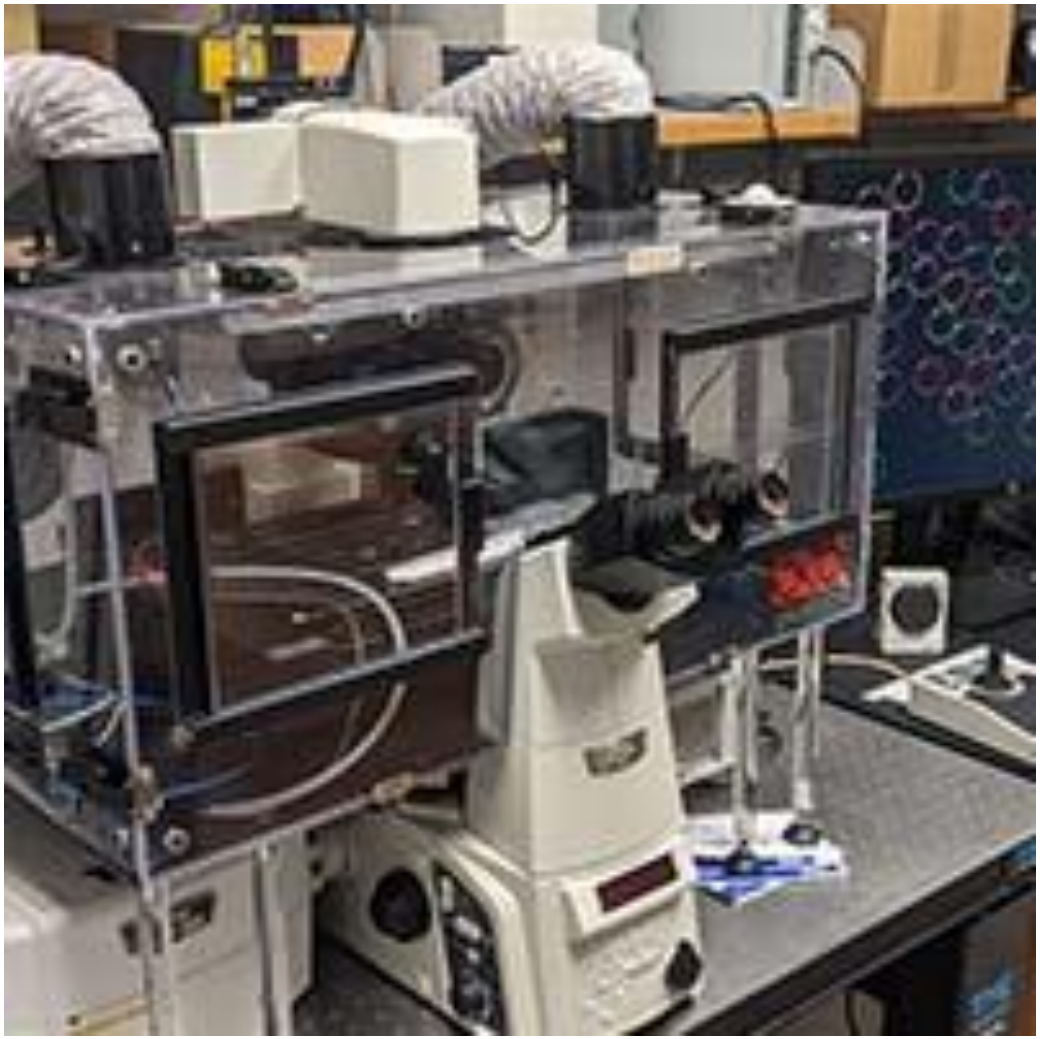
TEM - Nerve Myelin



Kidney Glomerulus (External surface) Critical-Point Dried and Sputter coated then imaged by SEM (CEMAS)

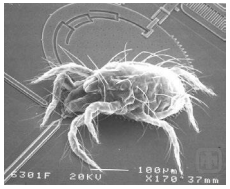

mag
80 000 x
HV
5.00 kV
WD
5.1 mm
spot
3.5
det
TLD
500 nm


Widefield
SIM



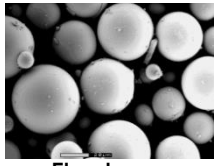
# The Scale of Things – Nanometers and More

## Things Natural



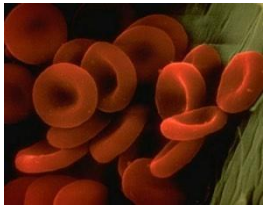
**Dust mite**
200 μm

**Ant**
~ 5 mm

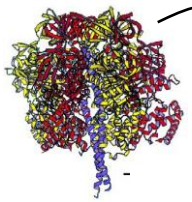


**Fly ash**
~ 10-20 μm

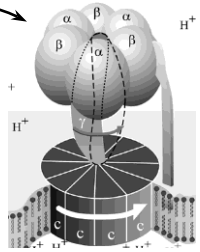
**Human hair**
~ 60-120 μm wide



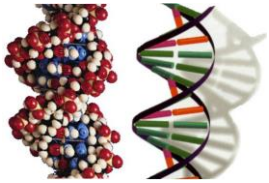
**Red blood cells**
(~7-8 μm)



~10 nm diameter

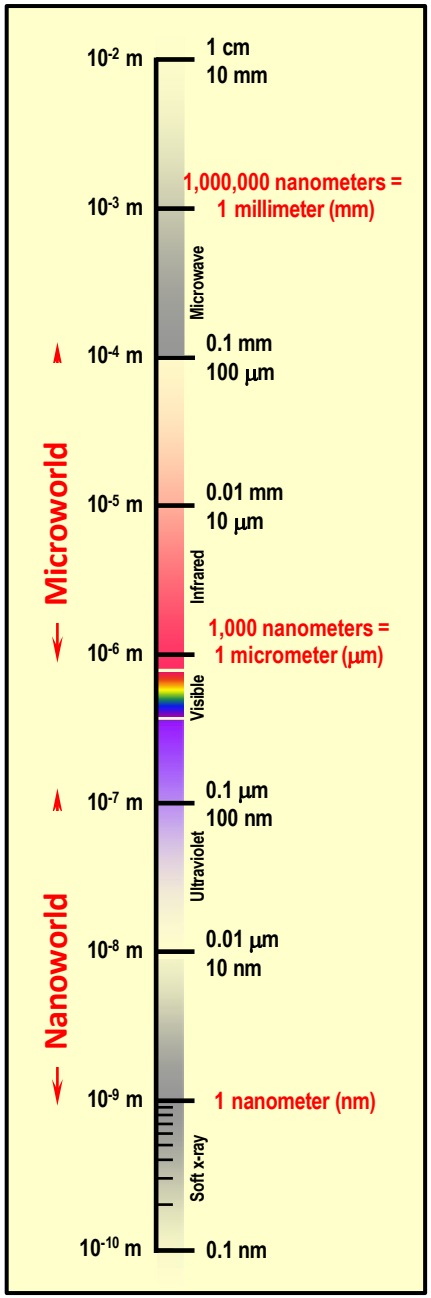


**ATP synthase**

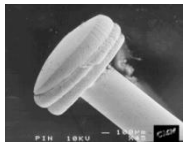


**DNA**
~2-1/2 nm diameter

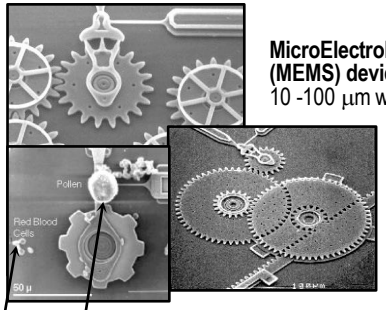
**Atoms of silicon**
spacing 0.078 nm




## Things Manmade

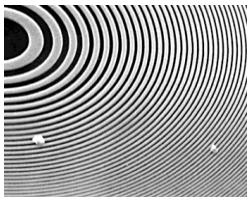


**Head of a pin**
1-2 mm

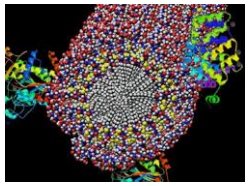



**MicroElectroMechanical (MEMS) devices**
10 -100 μm wide

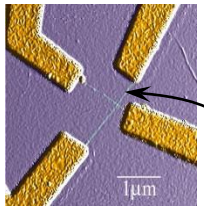
Pollen grain
Red blood cells



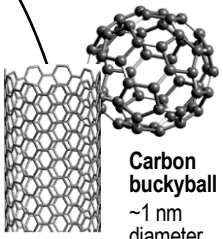
**Zone plate x-ray "lens"**
Outer ring spacing ~35 nm



**Self-assembled, Nature-inspired structure**
Many 10s of nm

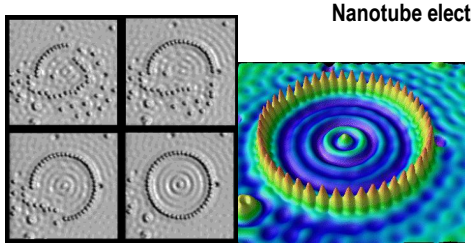



**Nanotube electrode**



**Carbon buckyball**
~1 nm diameter

**Carbon nanotube**
~1.3 nm diameter



**Quantum corral of 48 iron atoms on copper surface positioned one at a time with an STM tip**
Corral diameter 14 nm

Office of Basic Energy Sciences
Office of Science, U.S. DOE
Version 05-26-06, pmd

# CMIF Policies & Procedures

http://www.cmif.osu.edu/Microscopy-Policies

**A complete list is available on the website.**
**You will have to acknowledge that you read them.**

- **No food or drink in CMIF lab spaces.**
- **No live samples, (ONLY fixed biological samples) when using the microscopes in room 245 and there are no exceptions. Please prepare your samples outside of the microscope room either in your lab or in the wet lab in room 245.**
- **Please do not bring your own samples to the first hands-on training session since you will not be able to use them. You may bring your own samples to the second training, but the primary emphasis will be on training. Note an Exception to this policy – training on the Nikon Live Cell confocal is sometimes done using your own samples.**
- **Users are charged for instrument and technical time for hands-on training .**
- **You may be charged for last minute cancellations or no-shows.**
- **Payment is due, (Through FOM), at the first training session using FOM.**
- **Cancellations within 24 hours of the scheduled time MUST be done by the CMIF staff (contact-us)**

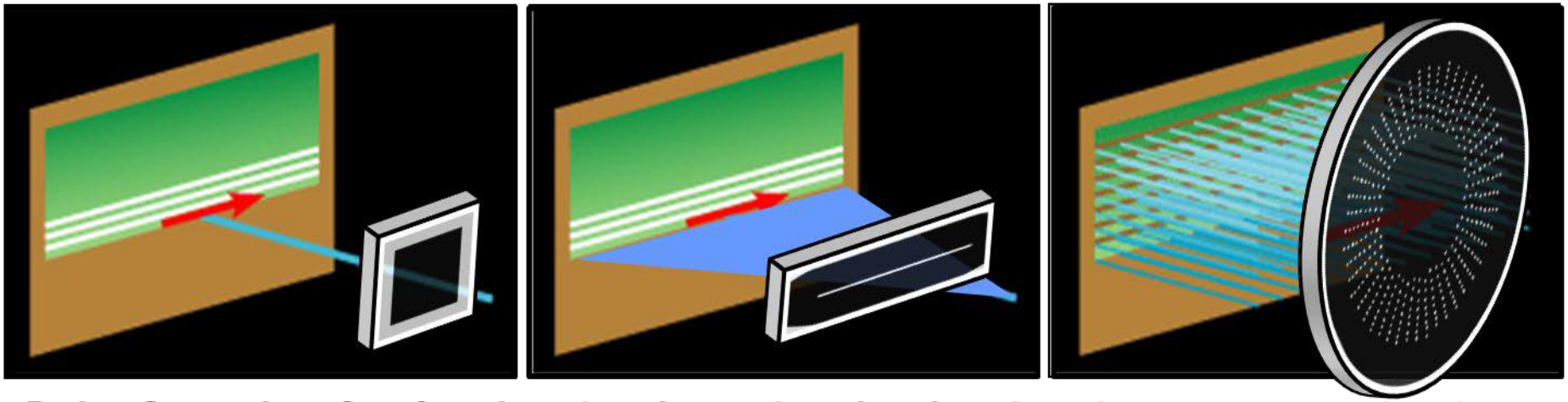
# Olympus FV1000 Filter Confocal System and FV3000 Spectral and Multi Inverted Confocals

- 4-5 Fluorescent Detectors + 1 DIC detector
- Multi FV3000 = spectral detectors and 100x lens
- Spectral FV3000 also has a 752 nm Laser
-Super corrected 60x objectives on each system

# Confocal Microscopy Types

Confocal Microscopy is an optical method that eliminates out of focus light and gives a clear optical section using only photons from the focal plane. This is accomplished by using small pinholes in front of the detector system. Single Point Scanning can use a Galvonometer or Resonant Scanner (Faster)

**Scanning methods:**



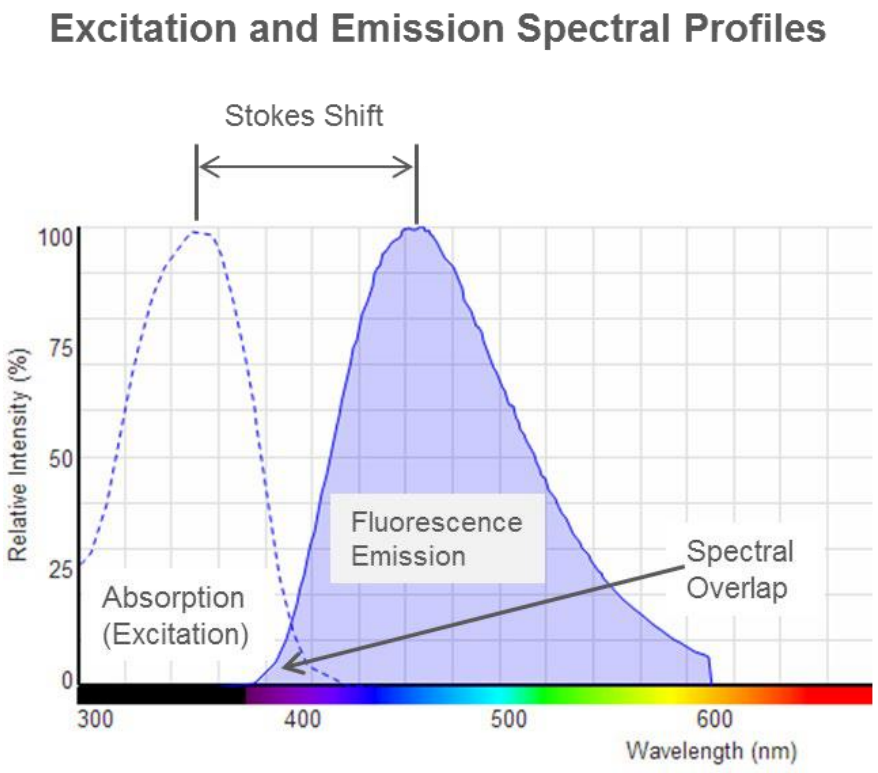
**Point Scanning Confocal**
*(single point at a time)*

**Line Scanning Confocal**
*(single line or slit at a time)*

**Spinning Disk Confocal**
*(1000 points at a time)*

# Fluorescent molecules or Fluorophores

Fluorescence is the absorption of photons by a molecule followed by the emission of photons of higher wavelength than those absorbed.




Important parameters for fluorophores:

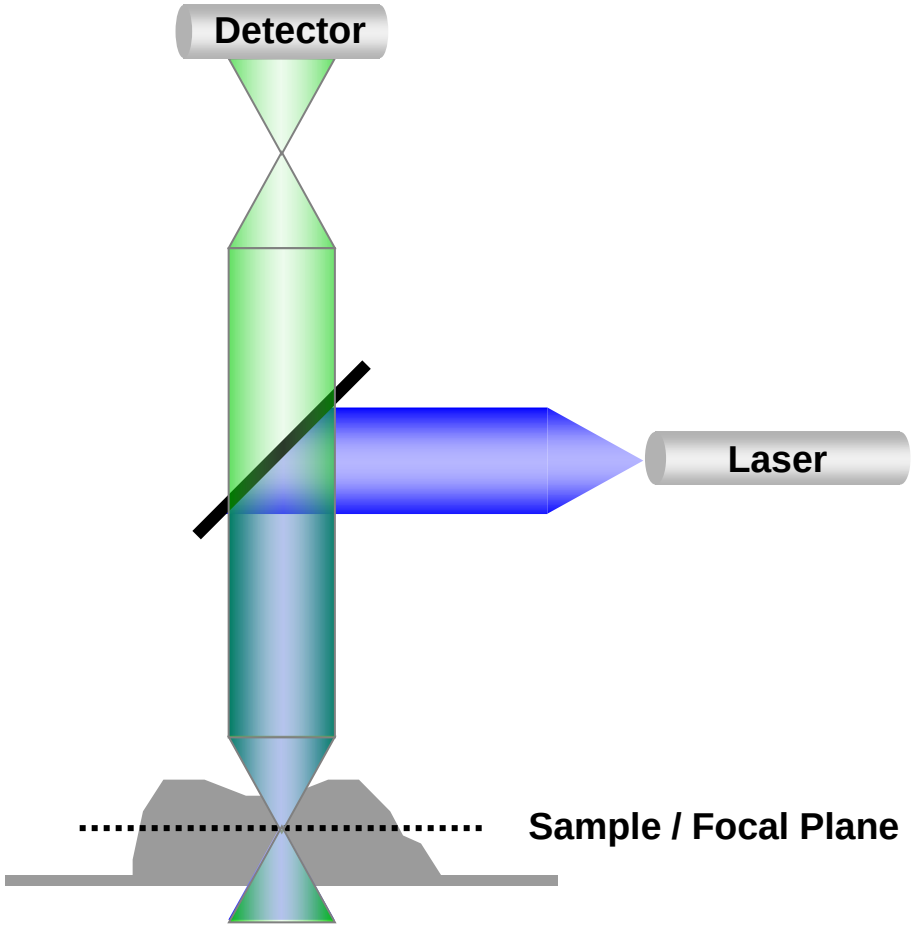
- Excitation wavelength
- Emission wavelength
- Stokes shift

**Spectra of excitation and emission – a fluorophore can be excited and can emit light over a range of wavelengths.**

**In this example, the fluorophore can be excited only weakly by light under 300nm or over 400nm and it would work with a Widefield microscope but not well on a confocal without a UV laser.**

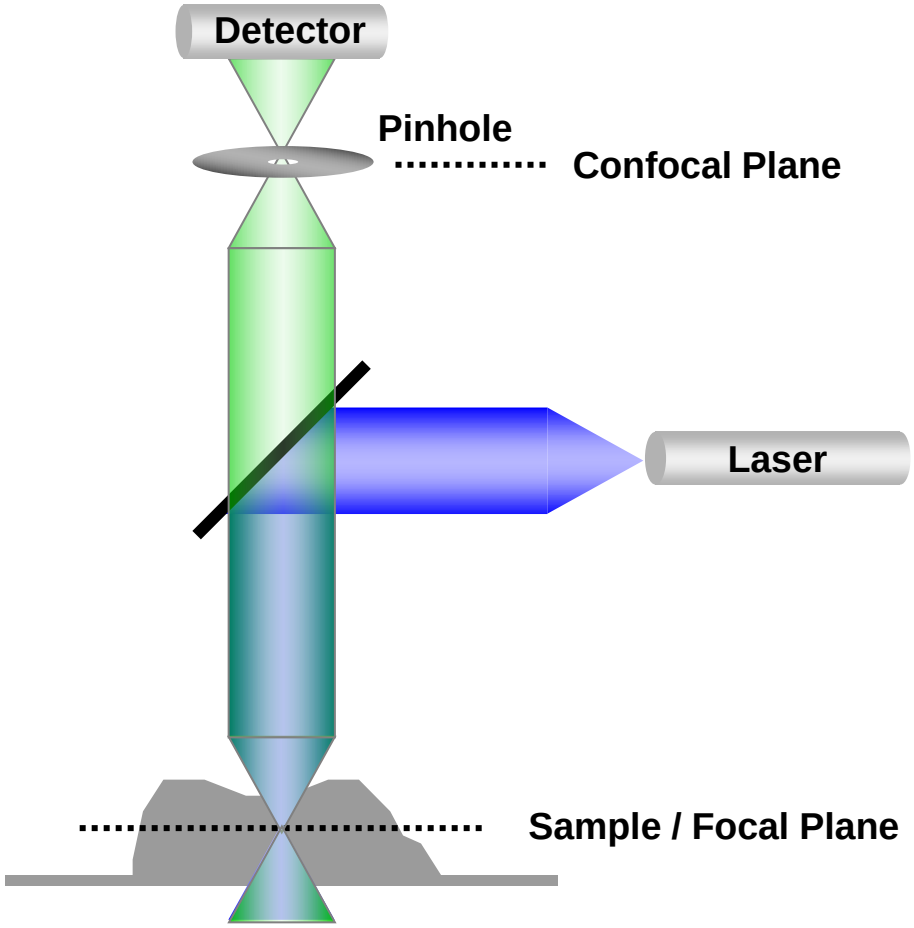
# Confocal Microscopy

- Laser excitation and sample emission separated by **dichroic**



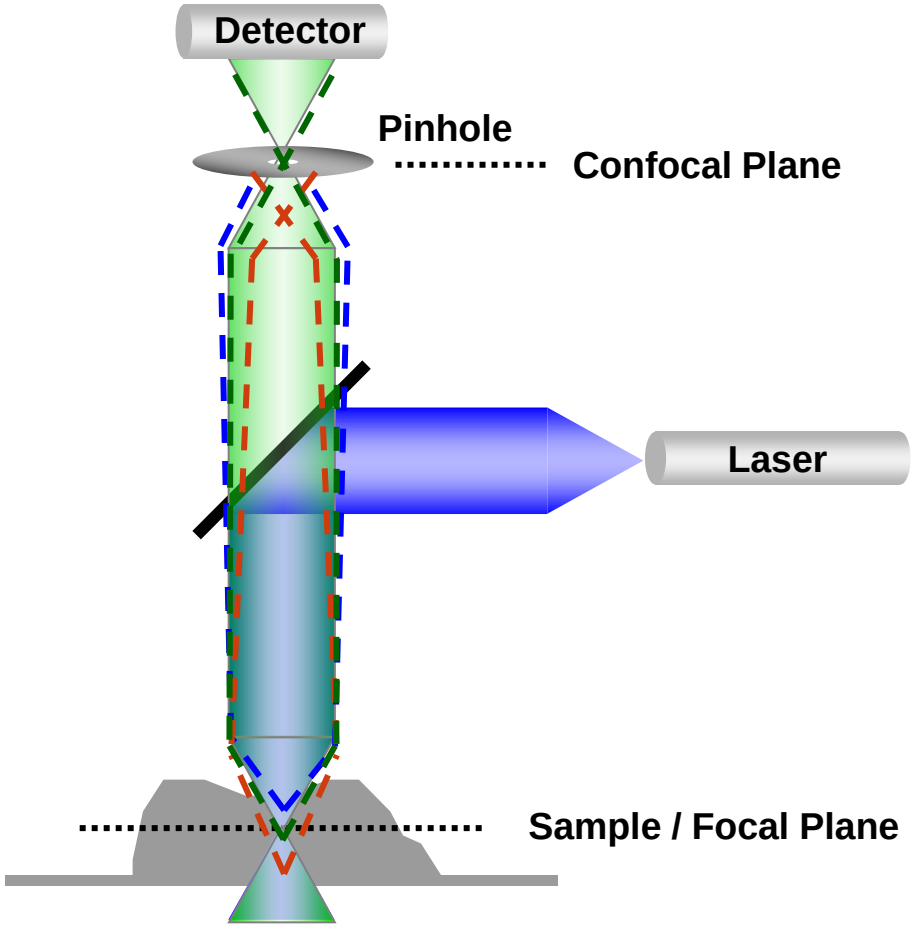
# Confocal Microscopy

- Laser excitation and sample emission separated by **dichroic**
- The **pinhole(s)** prevent detection of out-of-focus signal
  - Diaphragm situated between the detector and the focal plane



# Confocal Microscopy

- Laser excitation and sample emission separated by **dichroic**
- The **pinhole(s)** prevent detection of out-of-focus signal
  - Diaphragm situated between the detector and the focal plane
- The result is the production of an image that represents an “optical section” through the sample at the plane of focus.




The size of the pinhole is determined by the objective magnification and the wavelength of the fluorophores. This is automatically determined in the software, you should not have to

# Why Use a Confocal Microscope?

**1. Optical sectioning eliminates the out of focus fluorescence.**

**2. Obtain 3D information from cells and tissues.**
A series of optical sections can be reconstructed into a three dimensional image that can be rotated to view 3D relationships.

**3. Allows for high resolution comparison of label intensity.**
The intensity of different fluorochromes can be compared for each pixel in a digital image providing a means for semi-quantitative comparisons of label intensity.

**4. Allows for more accurate colocalization analysis.**
Since the fluorochrome signals occur in the same focal plane, the likelihood that they are in the same area of the cell is much greater.

# Why Use a Confocal Microscope?

**1. Optical sectioning eliminates the out of focus fluorescence.**

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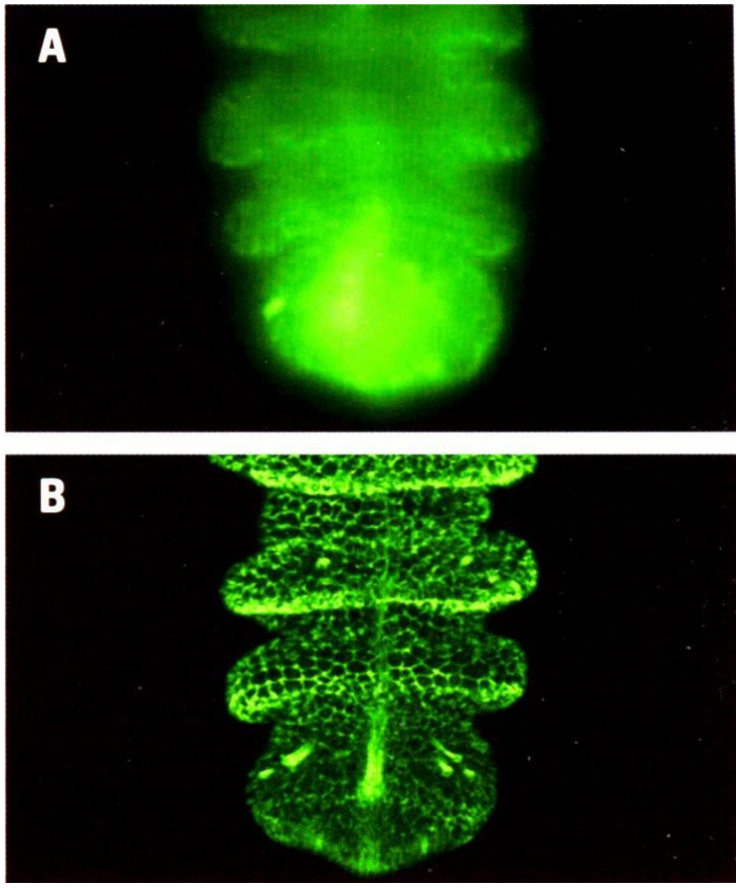
A series of optical sections can be reconstructed into a three dimensional image that can be rotated to view 3D relationships.

**3. Allows for high resolution comparison of label intensity.**
The intensity of different fluorochromes can be compared for each pixel in a digital image providing a means for semi-quantitative comparisons of label intensity.

**4. Allows for more accurate colocalization analysis.**
Since the fluorochrome signals occur in the same focal plane, the likelihood that they are in the same area of the cell is much greater.

# Why Use a Confocal Microscope?

**1. Optical sectioning eliminates the out of focus fluorescence.**



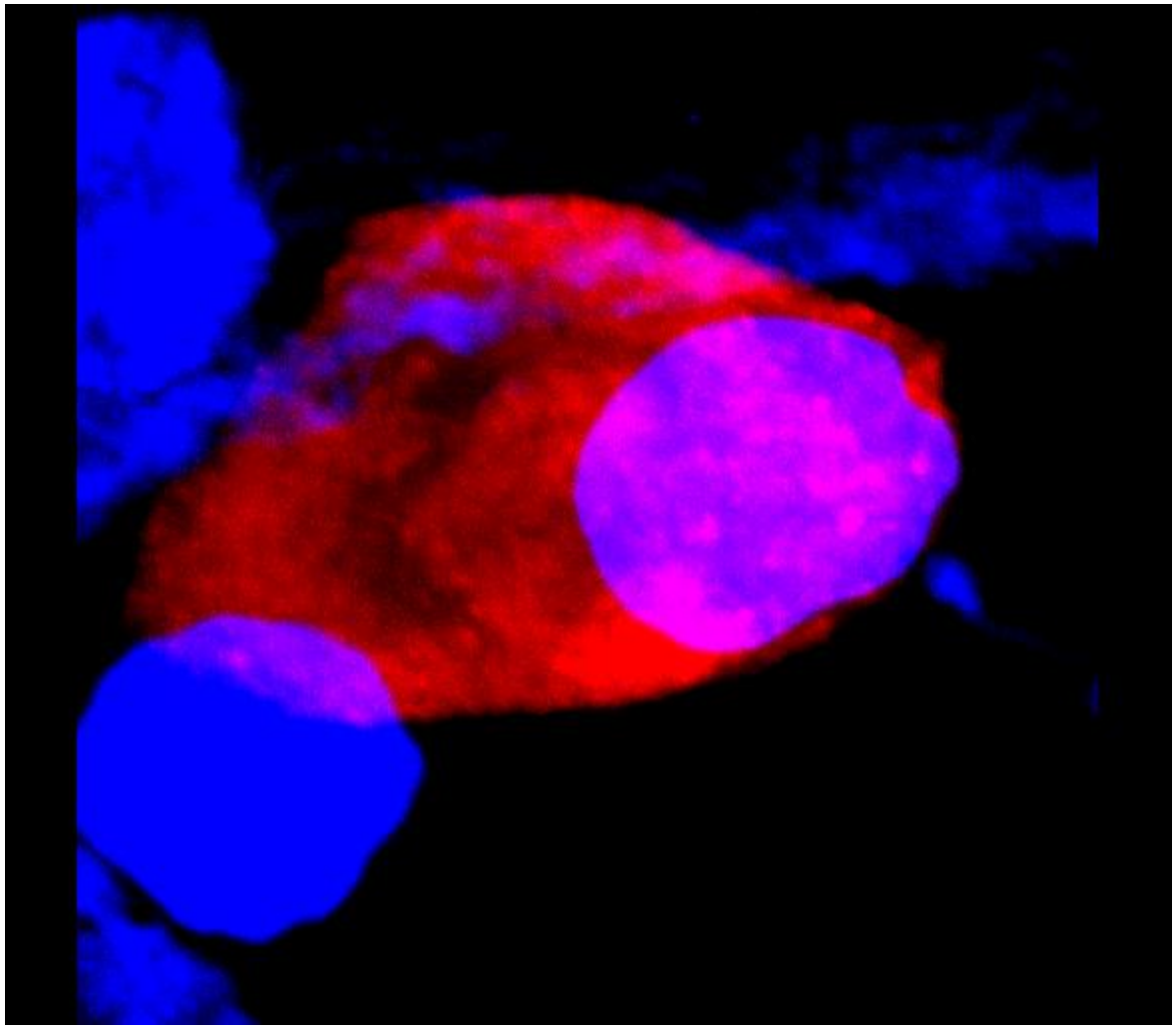

A. Wide-field fluorescence image of fruit fly embryo. Note that the out of focus fluorescence is the predominate signal. Wide-field works best for very thin sections.

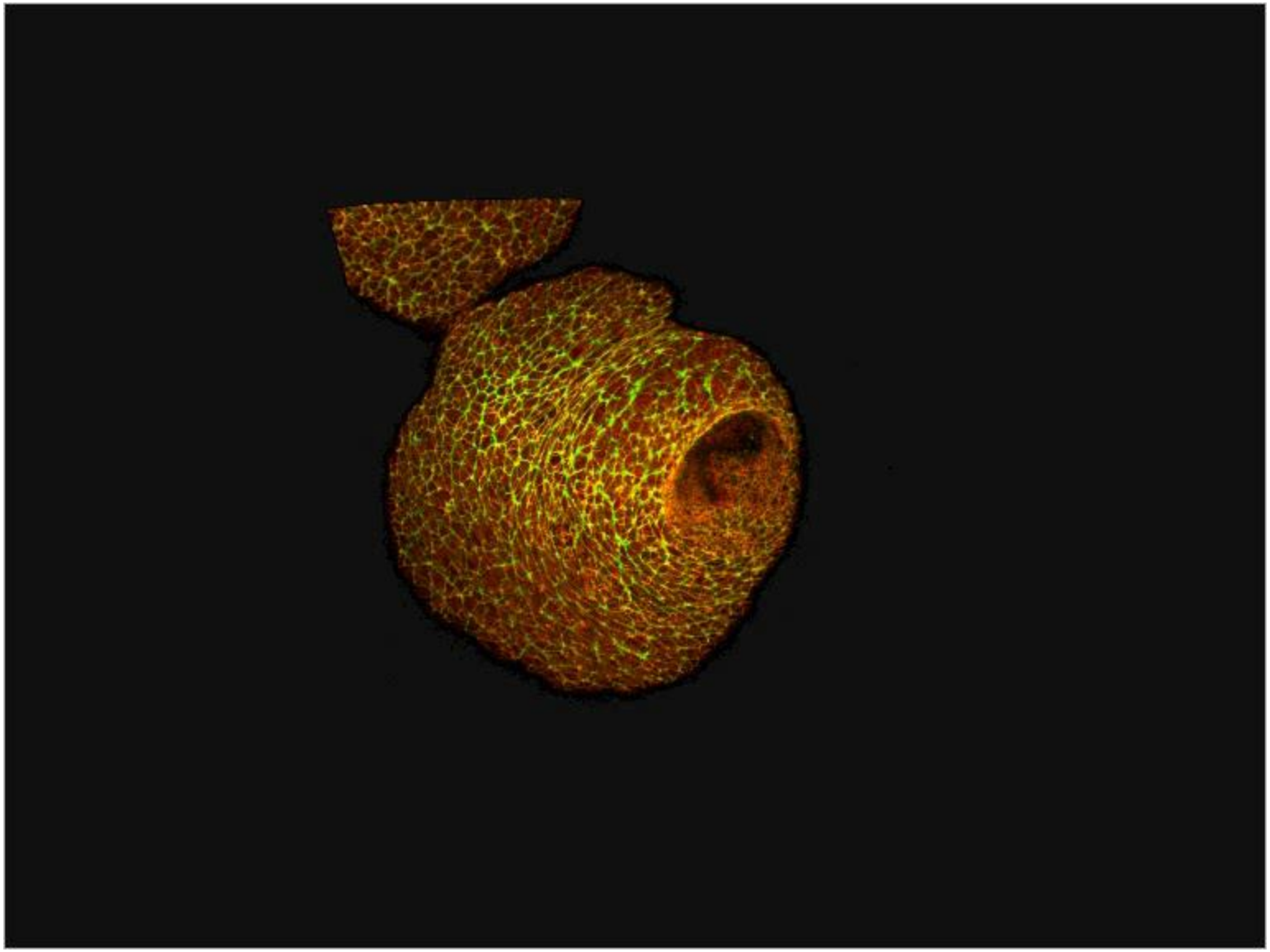
B. Confocal in-focus Image of fruit fly embryo derived from a series of confocal images – also referred to as a Z-projection.

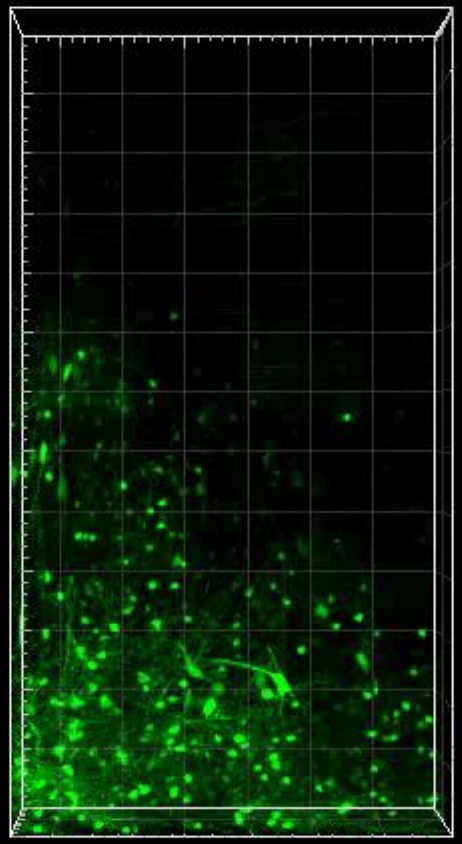
# Why Use a Confocal Microscope?

**2. Obtain 3D information from cells and tissues.**

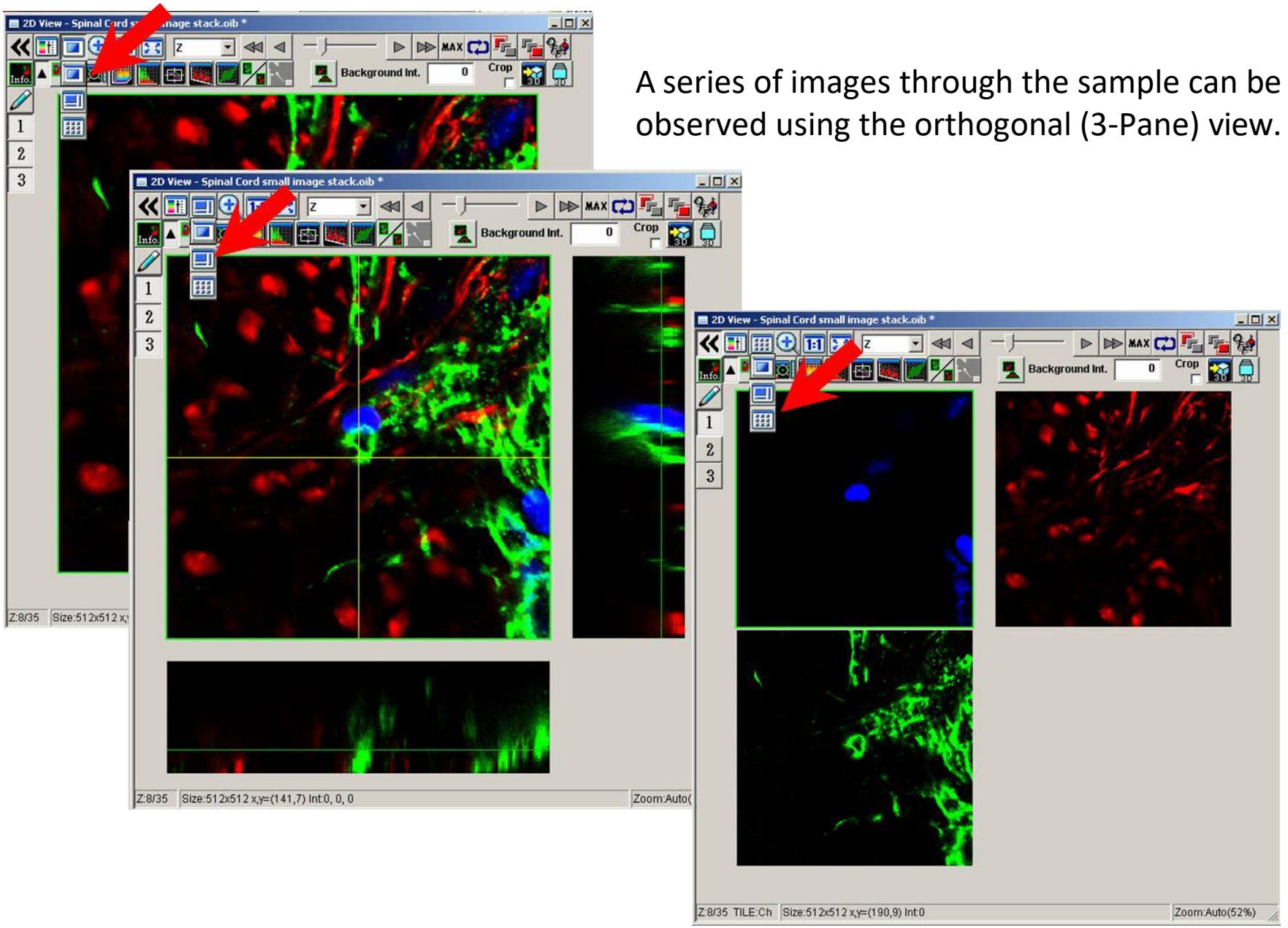
A series of optical sections can be reconstructed into a three-dimensional image that can be rearranged to get a better sense of 3D relationships.







# Data in 3 dimensions can better support your conclusions




A series of images through the sample can be observed using the orthogonal (3-Pane) view.

## Guidelines for Image Collection in Fluorescence Microscopy

- Choose the objective lens according to your needs - If resolution is the goal, use the objective lens with the highest numerical aperture (N.A.).
- Compose the image and make full use of the dynamic range.
- Maximize the signal to noise settings (intensity vs. black level) for all fluorescent labels. Getting a good signal to noise ratio is mostly done during sample preparation.
- Collect images with the sequential or serial image collecting mode (each fluorophore independently).
- Choose the appropriate number of pixels for your image.
- The experimental and control images for a single experiment must all be collected with the **same settings** when comparing intensity levels.
- Always save the original data in the proprietary formats (Olympus FV1000 = .oib, 3000=.oir)
- Avoid photobleaching the fluorophore.

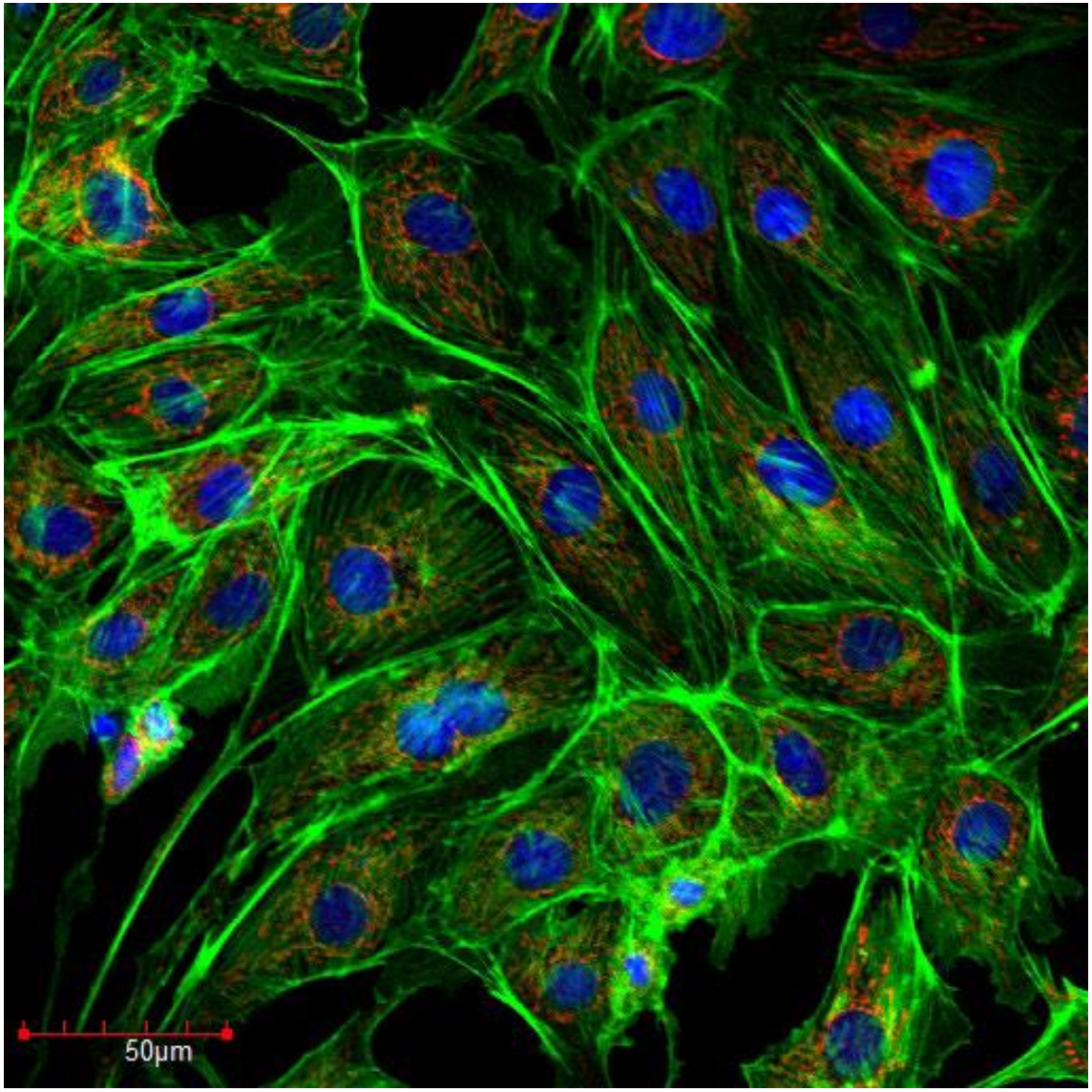
**Guidelines for Image Collection in Fluorescence Microscopy**

- Compose the image and make full use of the dynamic range.

# Image composition: Always be thinking: ‘Why am I here?’

STEP 1: Frame your subject

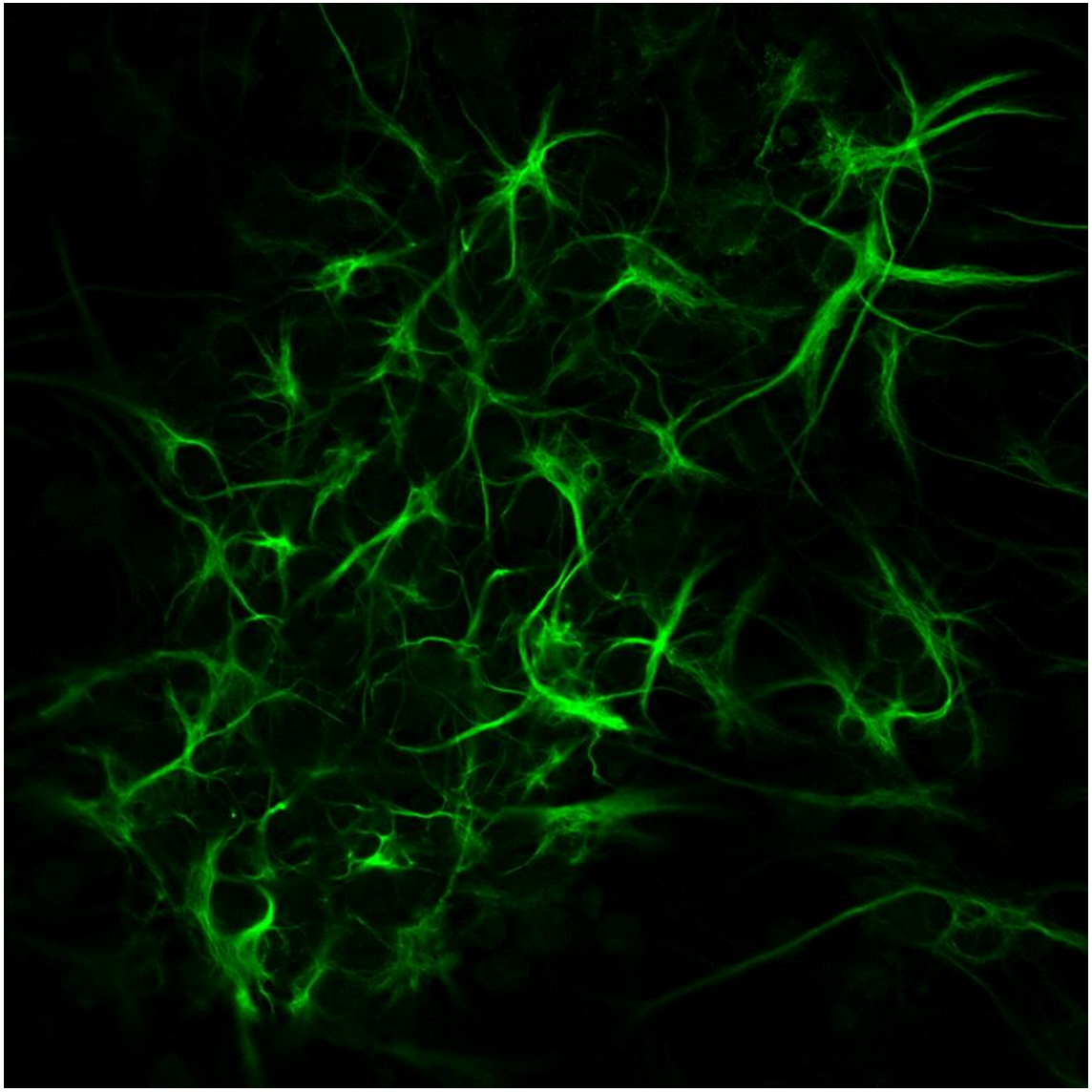
poor



# Image composition: Always be thinking: ‘Why am I here?’

STEP 1: Frame your subject

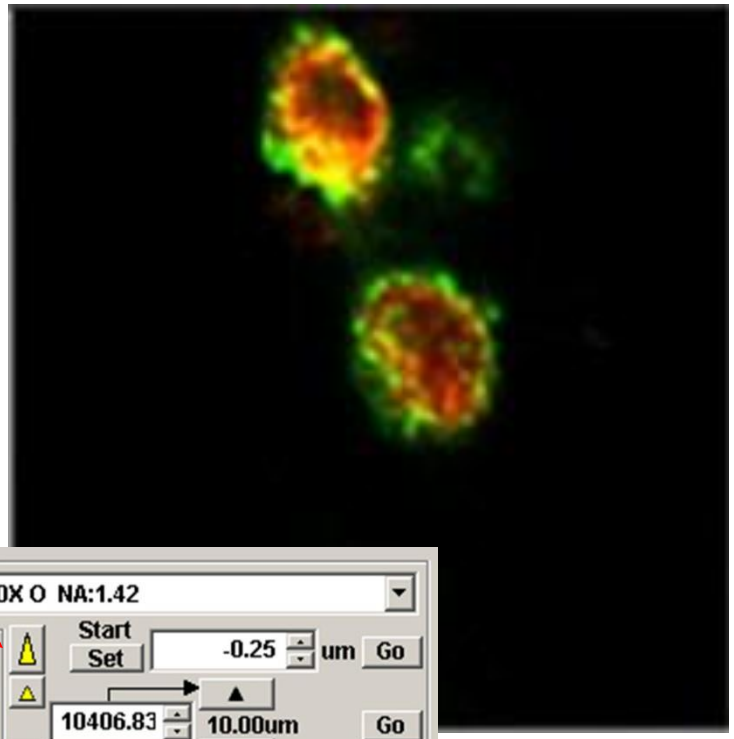
good

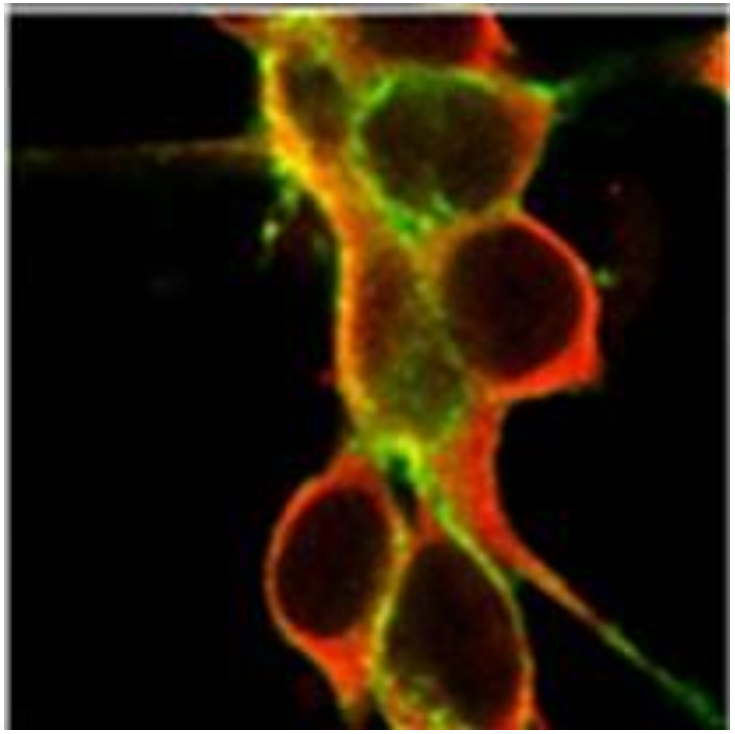


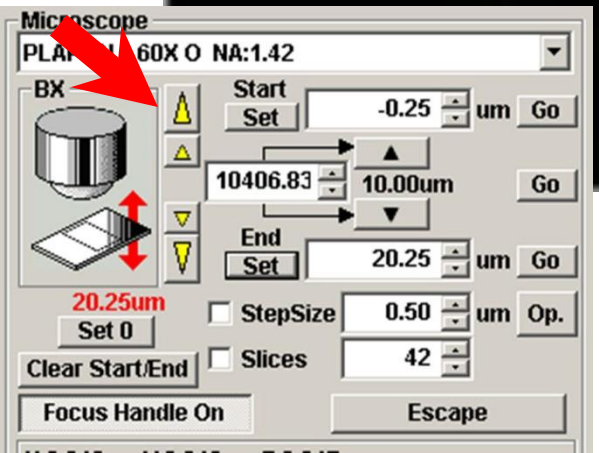
# Image composition

STEP 2: Find your focal plane

typically this will be the “brightest” area of the sample volume however, This will depend on what you’re trying to demonstrate.





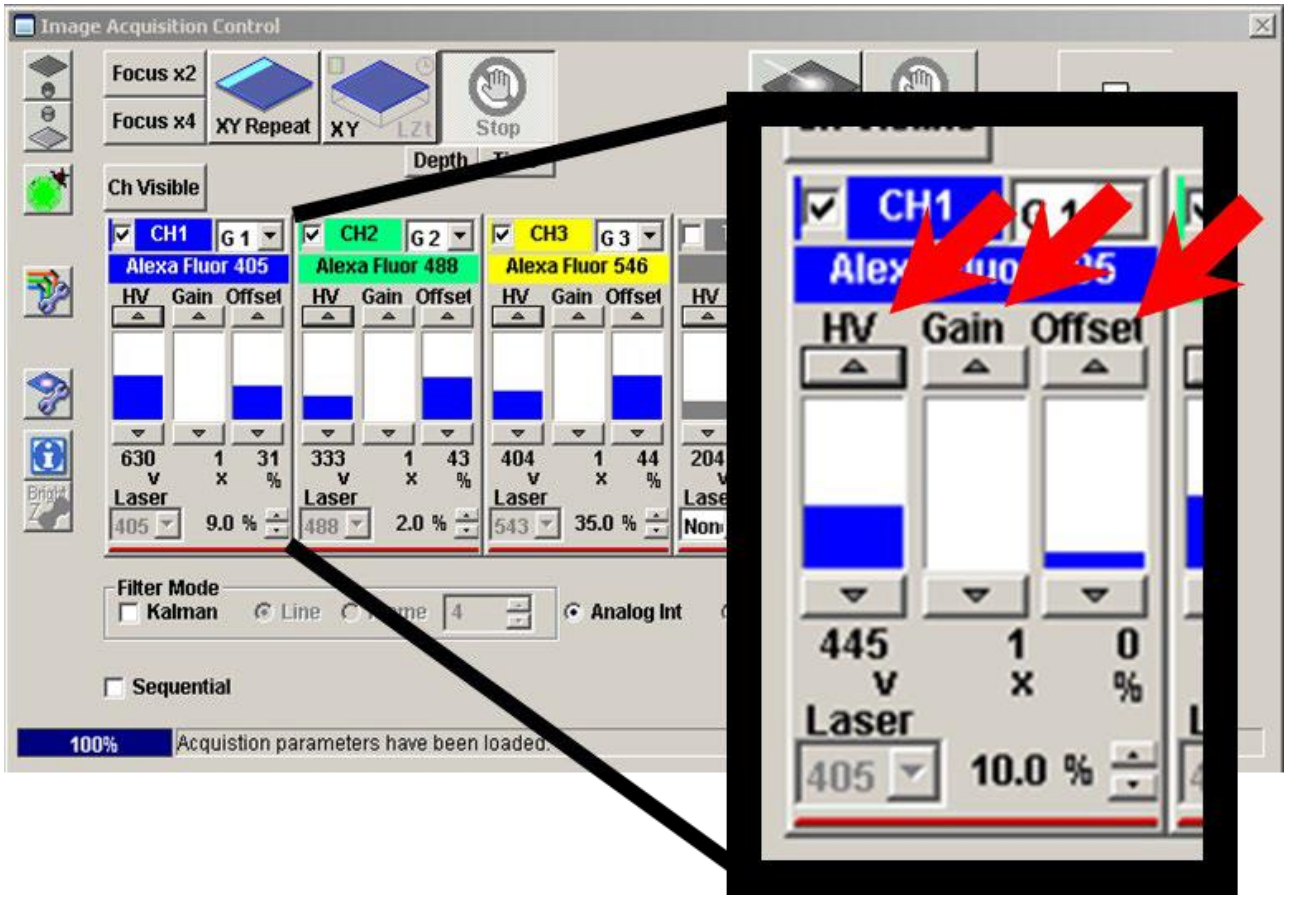



Remember to always be thinking: ‘Why am I here?’

# Image composition

STEP 3: Adjusting the brightness and contrast settings in order to maximize the signal to noise ratio.

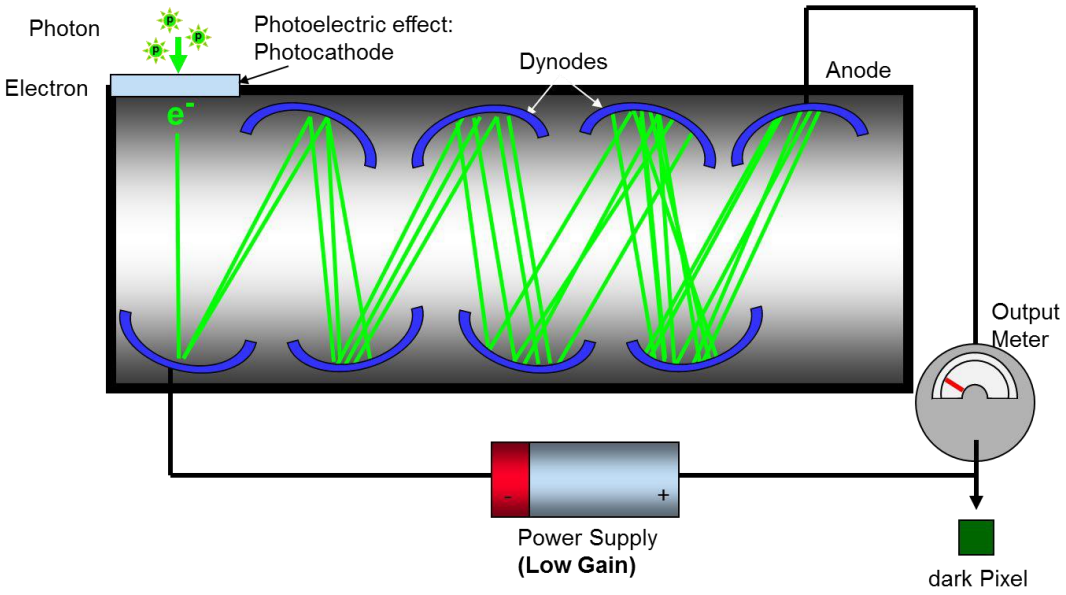
HV and offset in the Olympus software.




The GOAL is to stay within the dynamic range of the detector!

# What is the detector?

It helps to know the system you're using




**CMIF systems use Photomultiplier Tubes (PMT):**

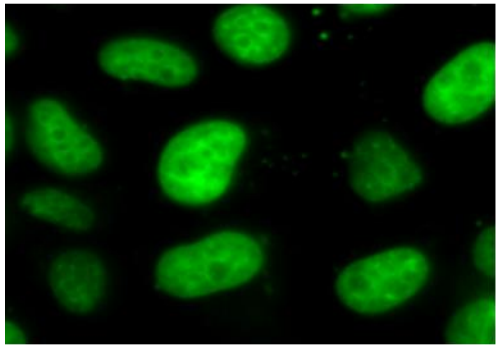
The end result is a 12 bit digital image

A single pixel in a 12-bit image is encoded by twelve '1's and '0's (binary code)

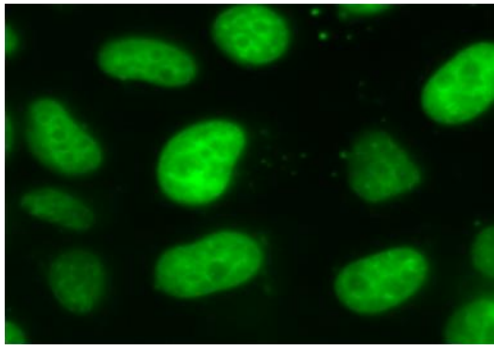
The FV 3000 confocals also have high sensitivity detectors, which need less brightness. You can use lower laser power which causes less photobleaching.

# Bit depth (grey values) is useful for intensity analysis

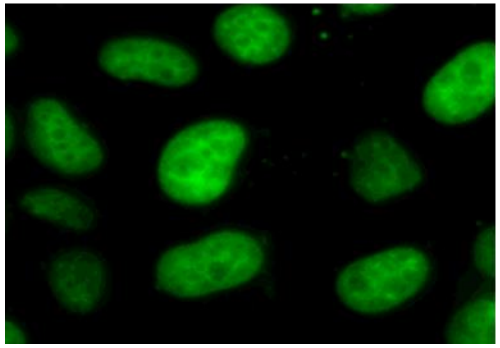
12 bits / 4096 greyvalues



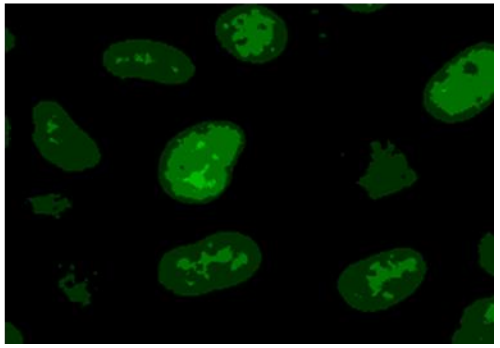
8 bits / 256 greyvalues



4 bits / 16 greyvalues



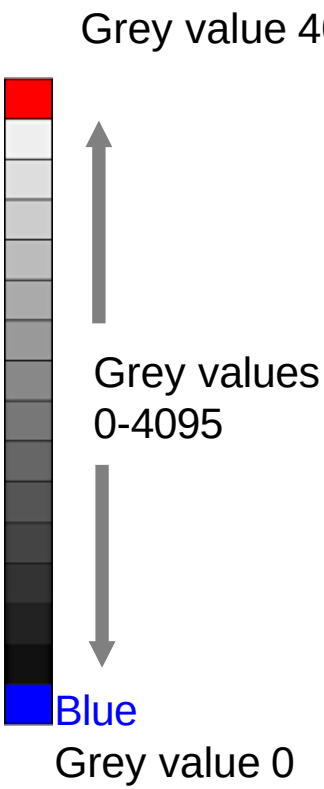
2 bits / 4 greyvalues

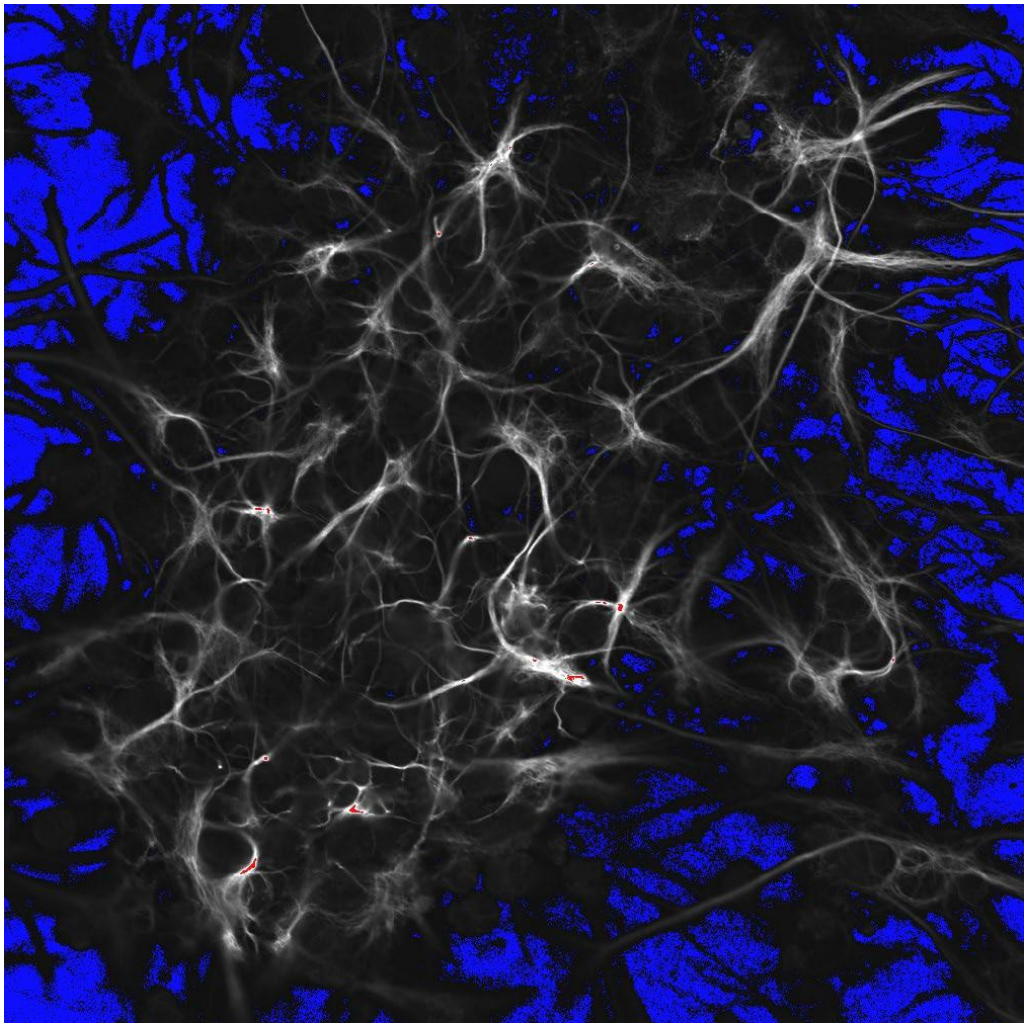


**The bit depth of the digital image yields a range of "brightness" values that each pixel can occupy. THIS is the dynamic range. The key to successful imaging is to make the most of this range without exceeding it.**

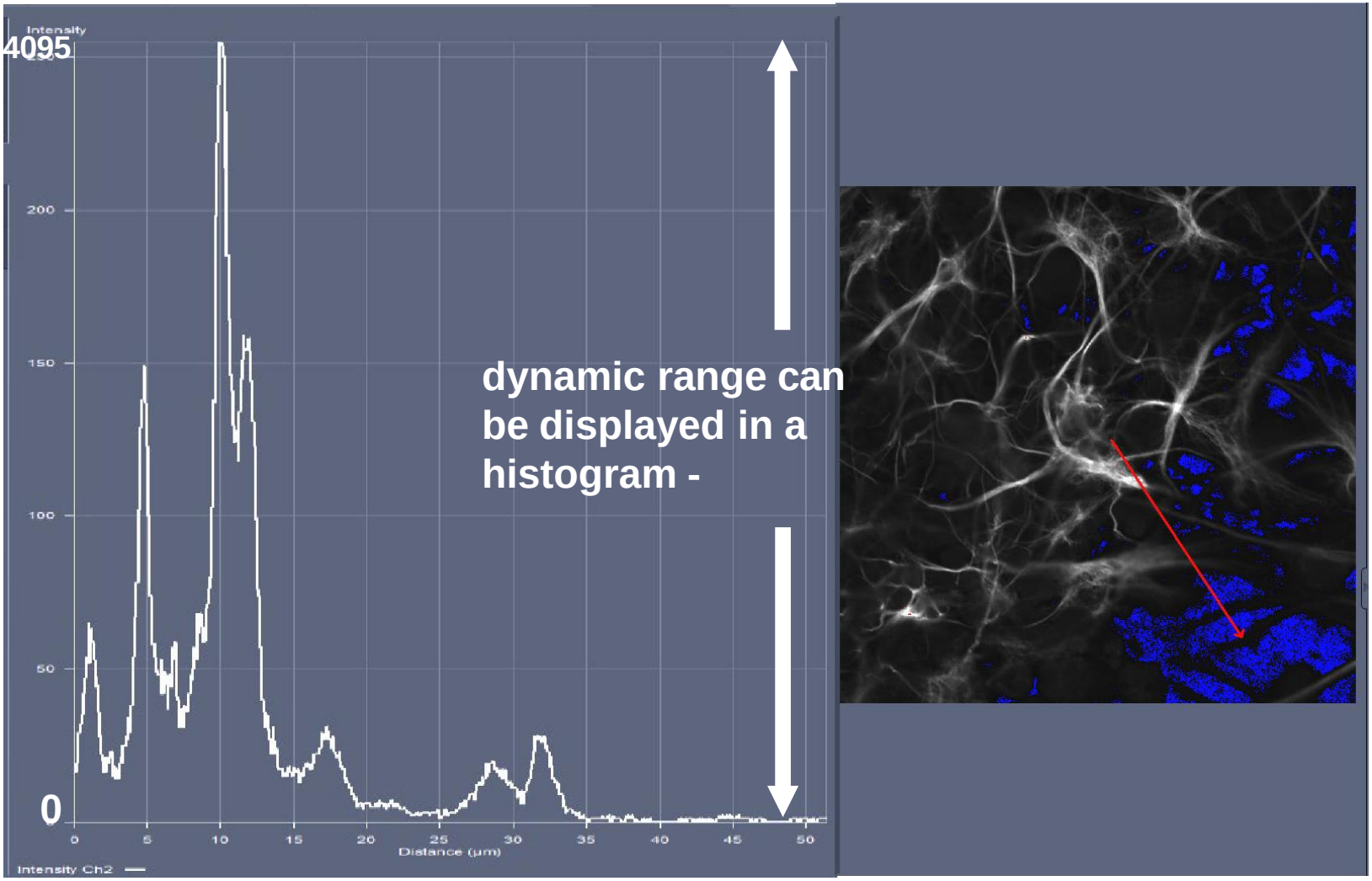
# Making the most of the dynamic range: Range indicator (hi-low pseudo-color)

**Look-up table**
**Range Indicator (8bit)**

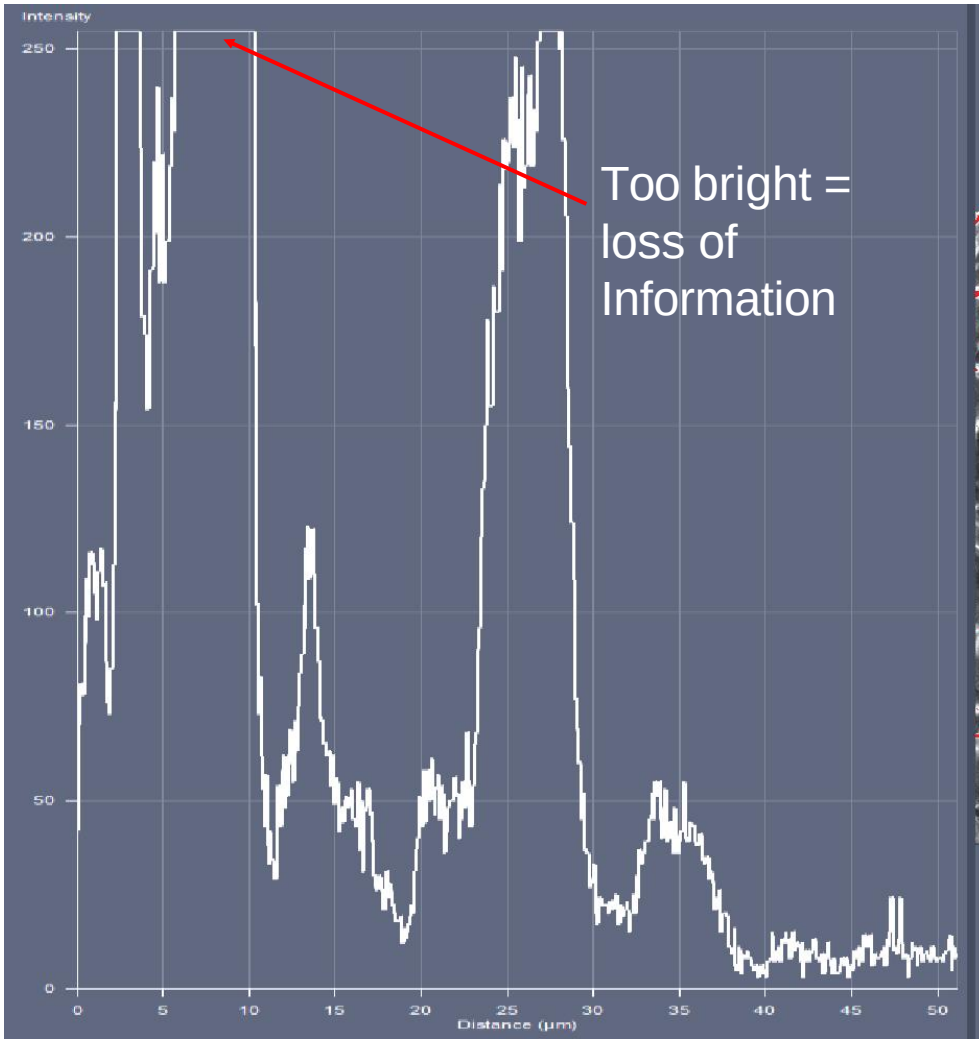


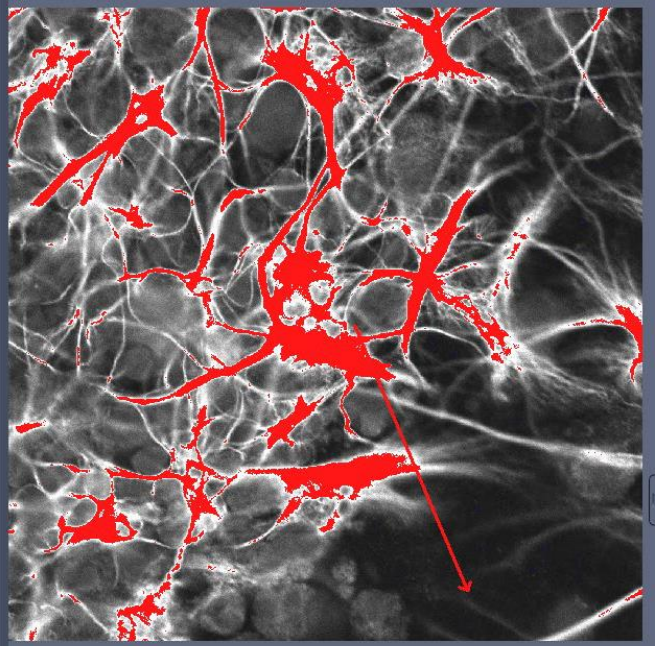



# Making the most of the dynamic range:



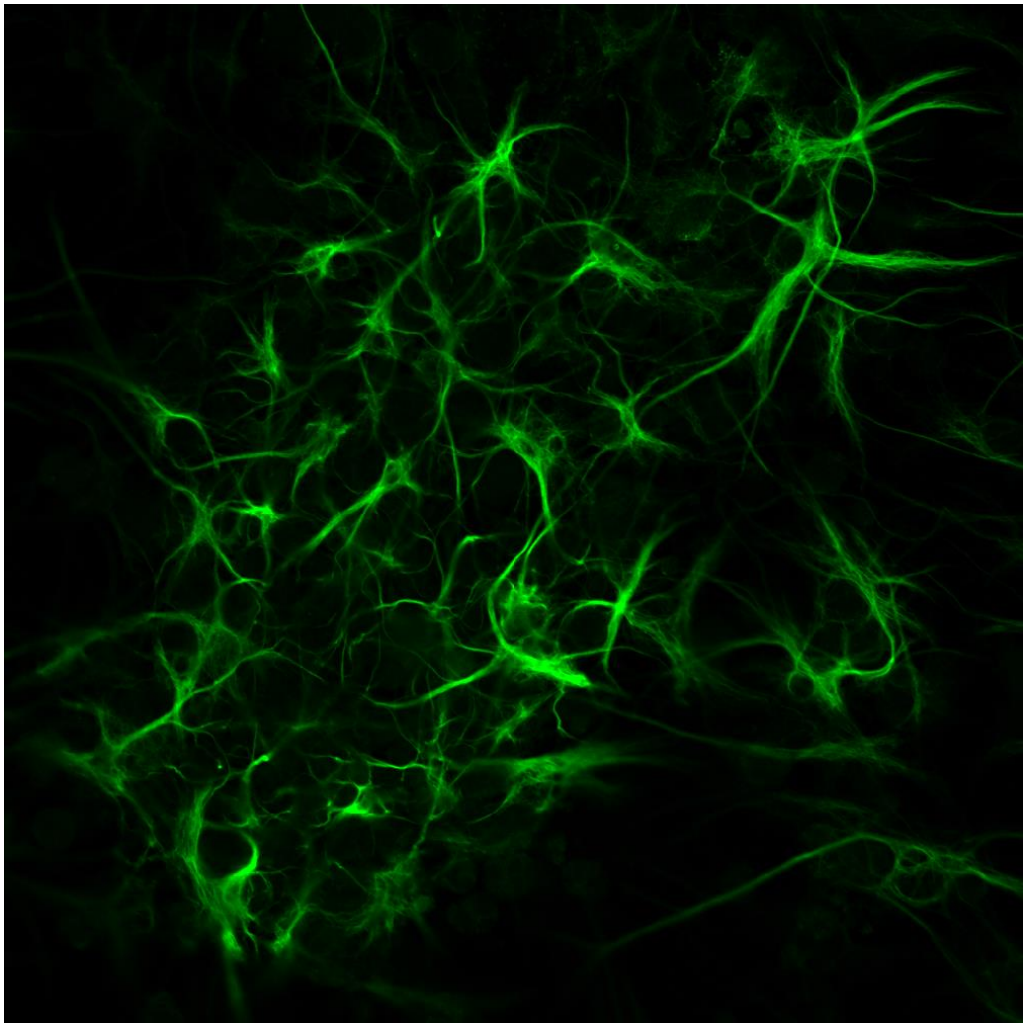
# Making the most of the dynamic range:



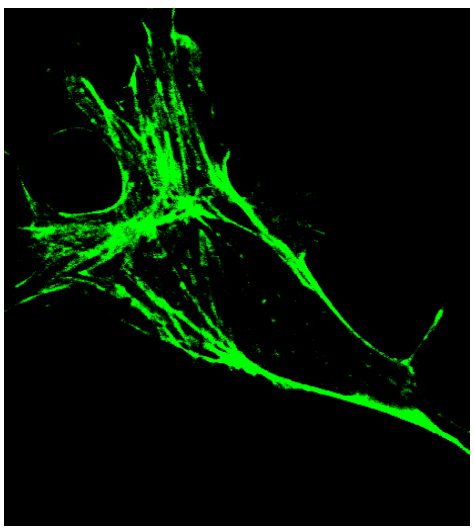
# Making the most of the dynamic range:

**Illumination:**
Not too bright,
Not too dim

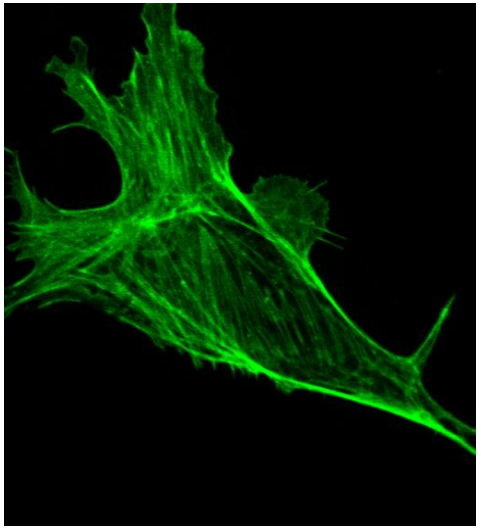


# Image composition

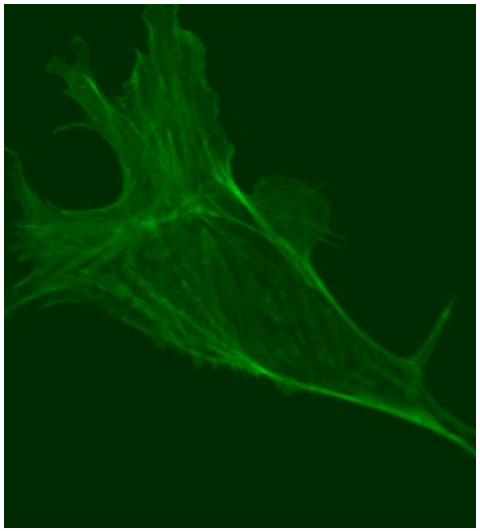
In Summary: High contrast results in LOST data and low contrast results in a bad image.



High Contrast



Normal Contrast



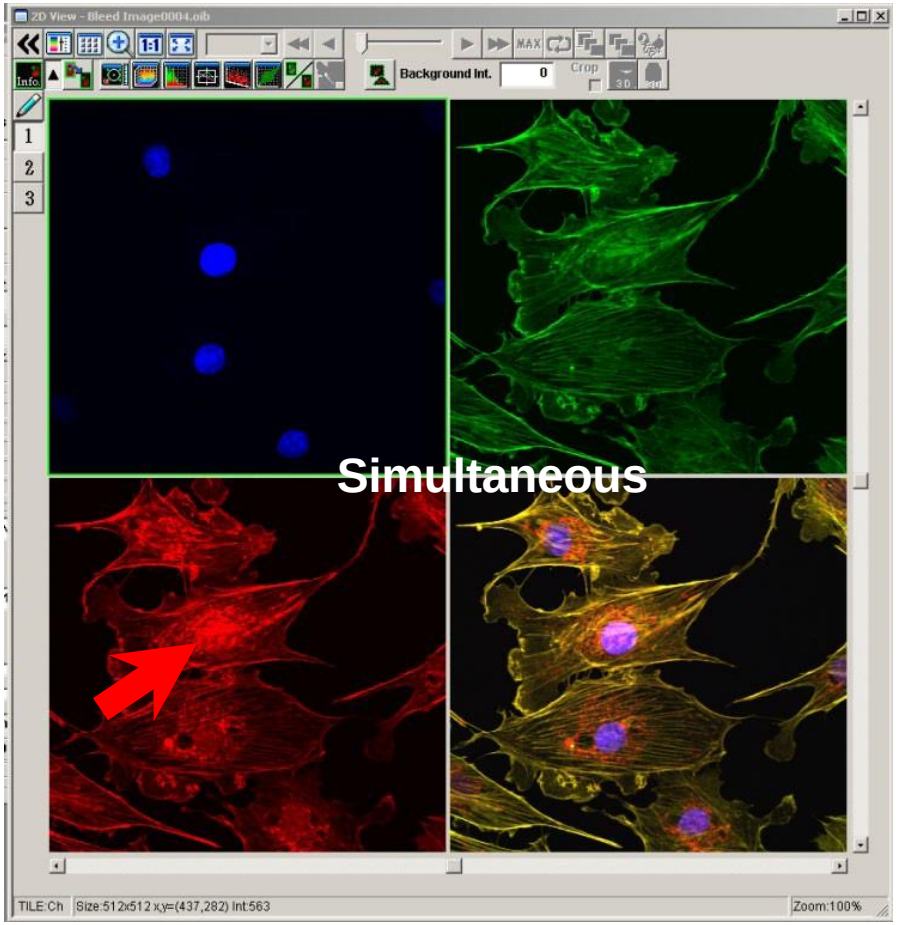
Low Contrast

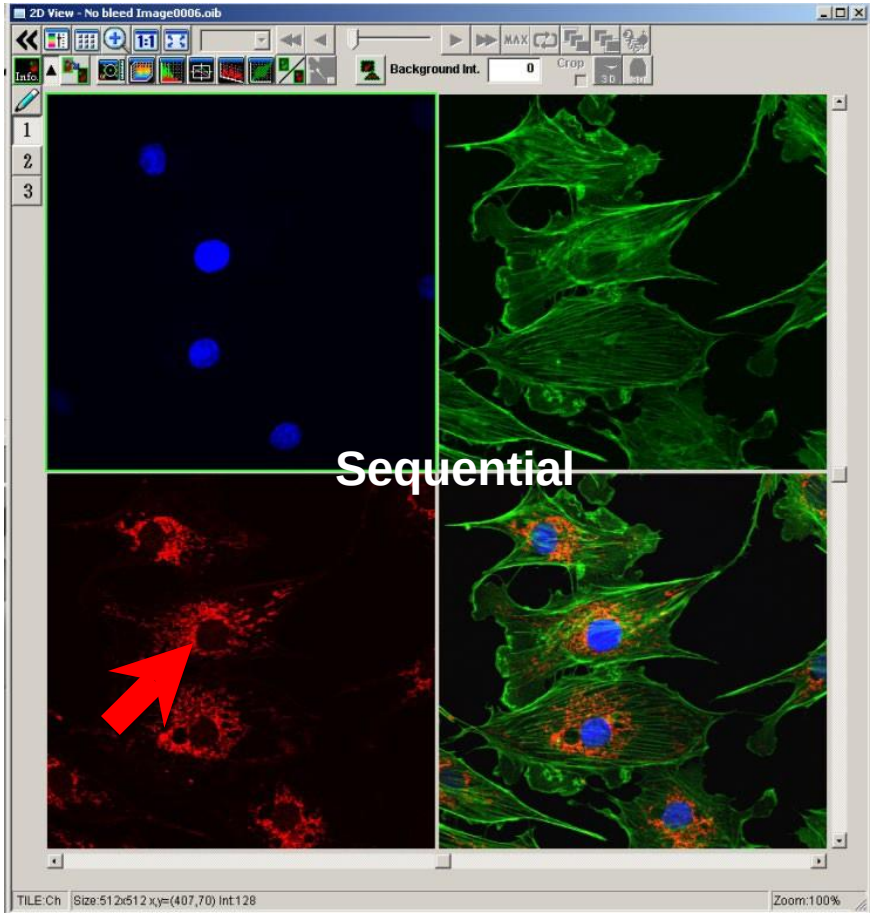
**Guidelines for Image Collection in Fluorescence Microscopy**

- Collect images with the sequential or serial image collecting mode (each fluorophore independently)

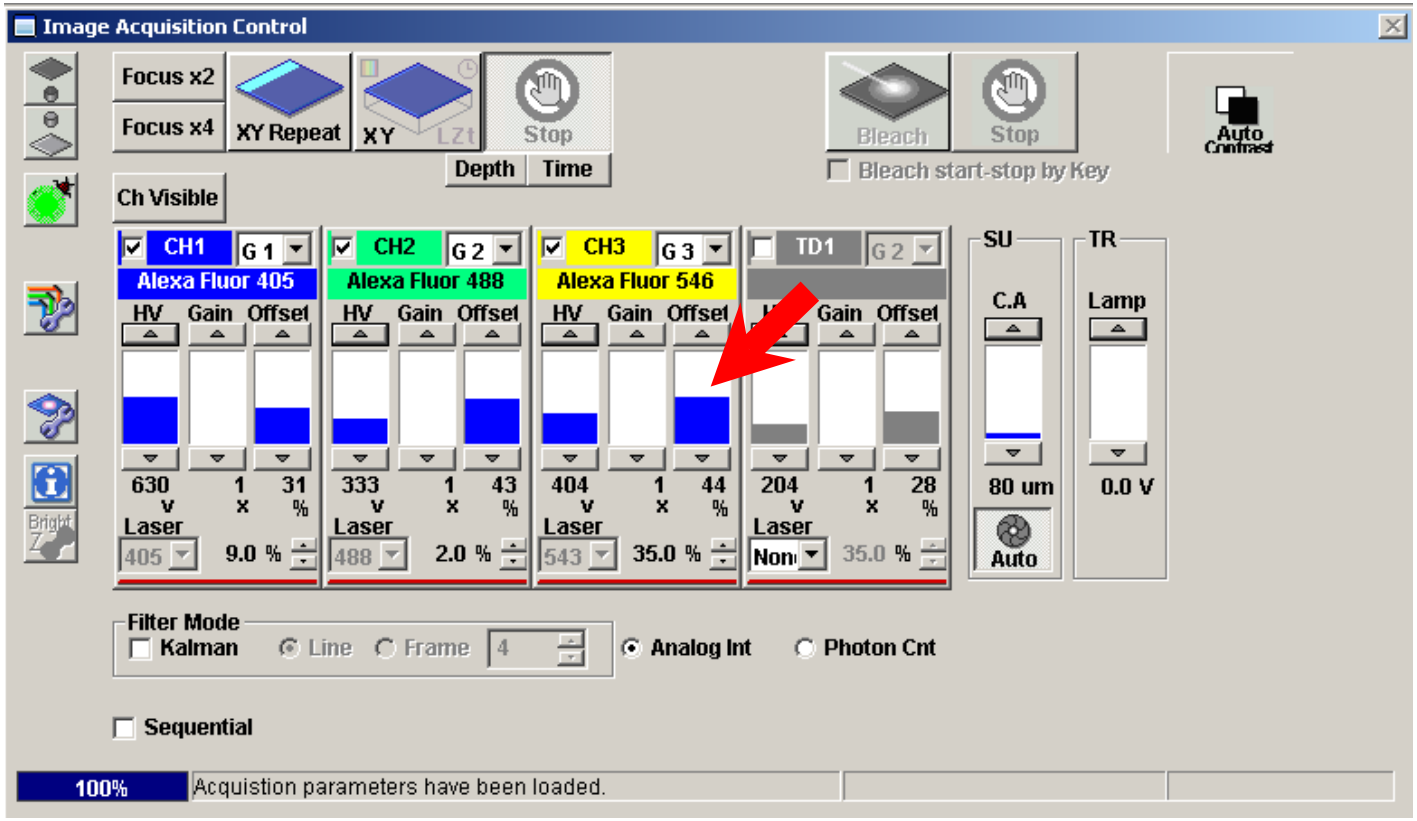
# Simultaneous versus sequential

Images collected simultaneously can result in bleed-through from the one channel (green arrow) to another (red arrow left).
Images collected sequentially show the appropriate signal distribution.



# Voltage/HV and offset settings can indicate a low signal to noise ratio



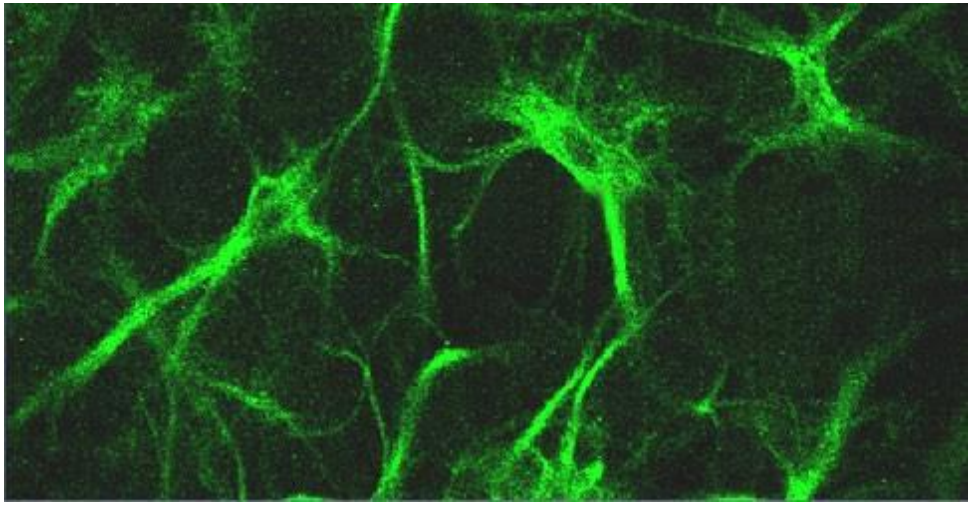

If the Voltage is higher than 700 you should try to make some improvements.
If the Offset is higher than 35%, you should try to make some improvements.
Always check that “Sequential” imaging is selected!

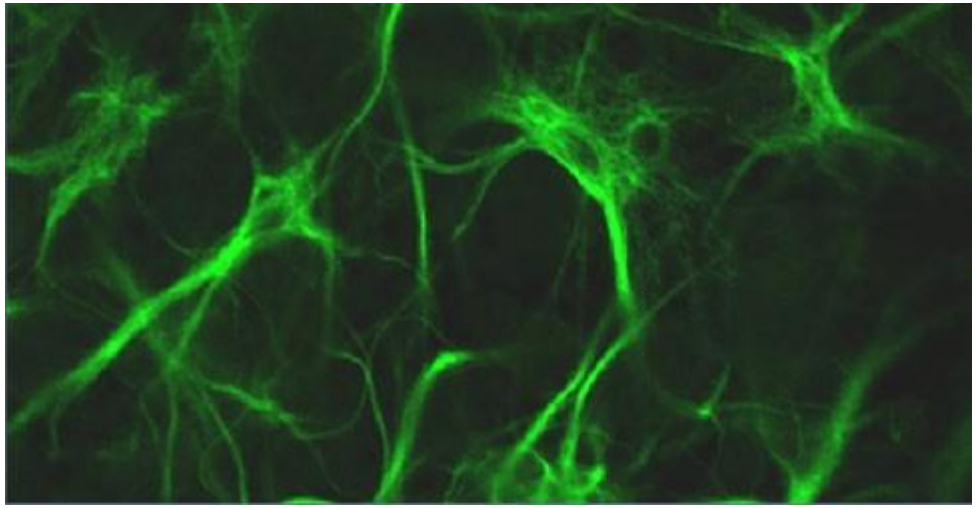
# Optimize signal to noise ratio:

“Bad” Image

- Increase laser power
- Scan more slowly (increase pixel dwell time)
- Apply Averaging
- Brighter Sample
- Higher N.A. Objective
- “Better“ detector

“Good” Image



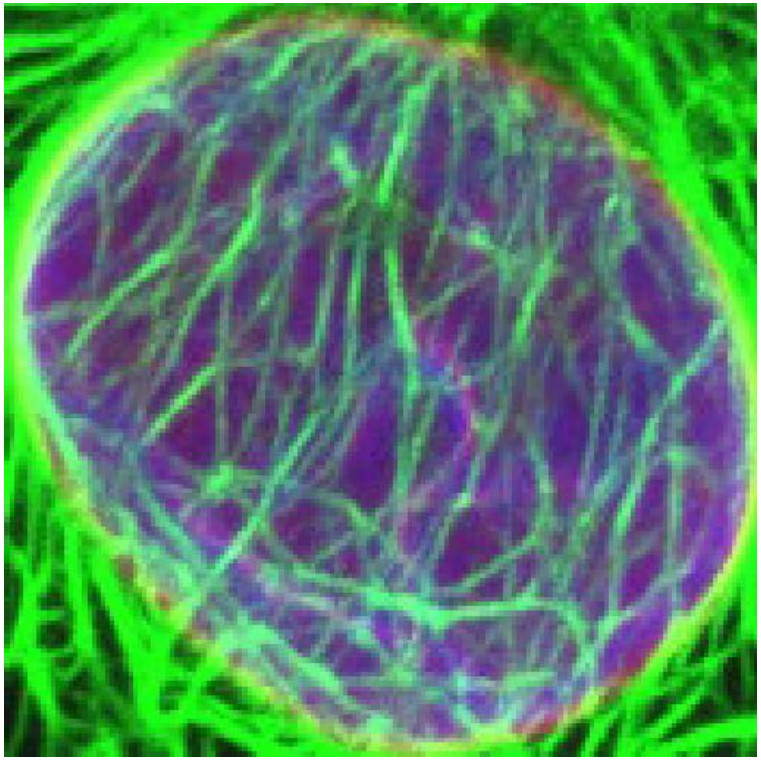


**Guidelines for Image Collection in Fluorescence Microscopy**

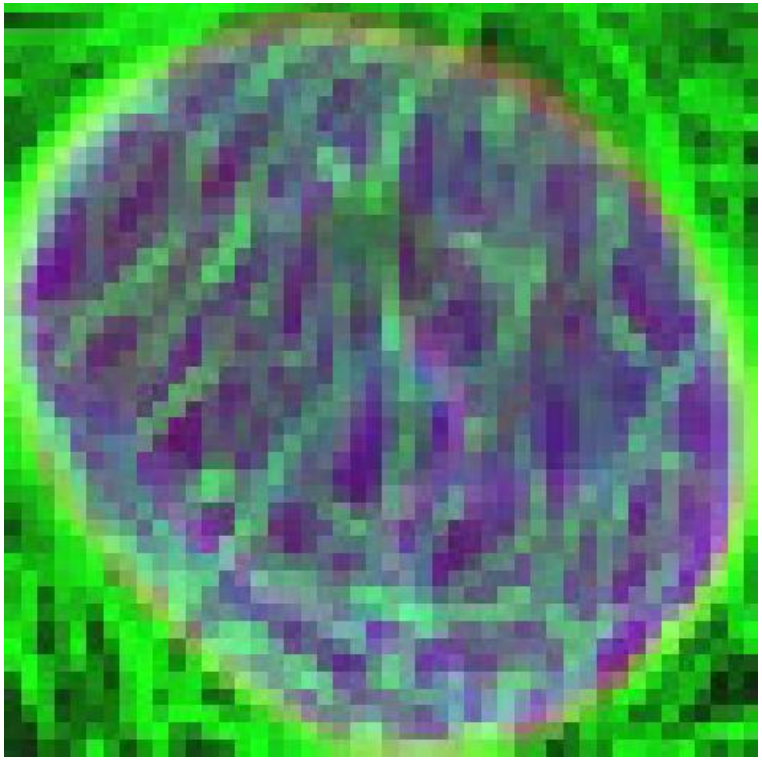
- Choose the appropriate number of pixels for your image.

## **Use the right pixel density to show the appropriate detail -** the option for Olympus is 512x512 or1024x1024

Pixel number ok (512x512)

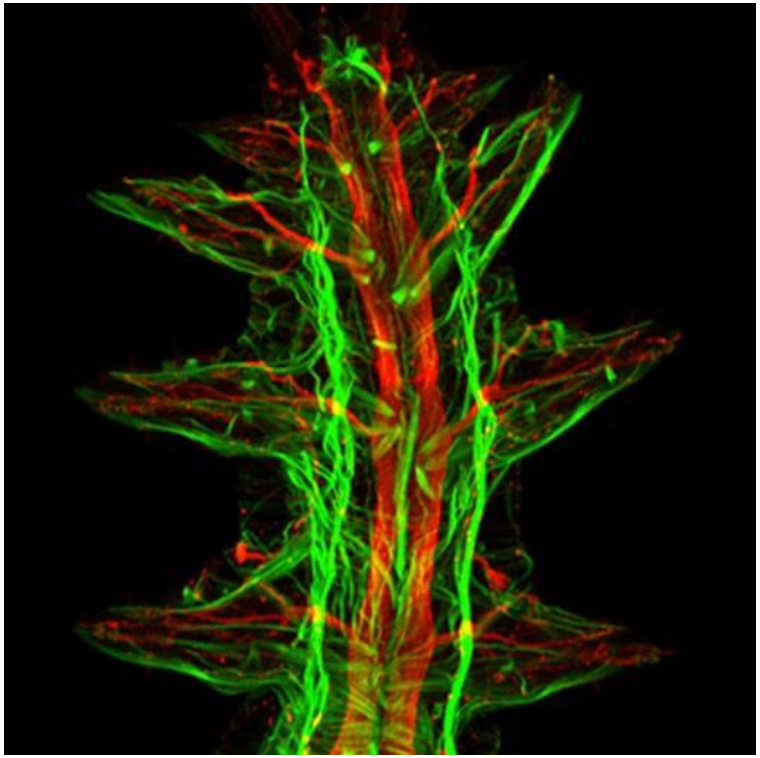


Pixel number too low (256x256)

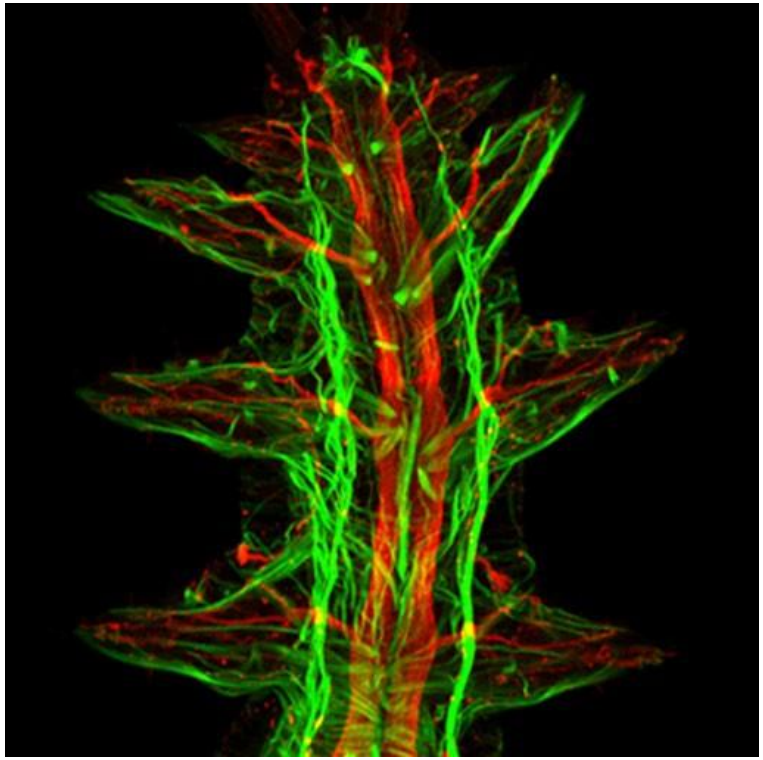


# Using too many pixels wastes time and photobleaches your sample!

Pixel number ok (512x512)



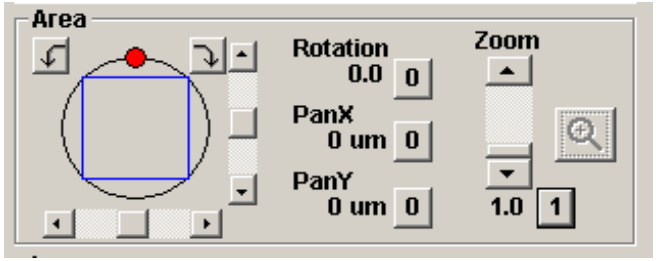
Pixel number too high (1024)



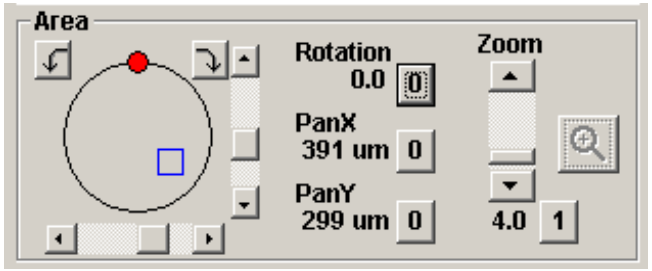
The exception? if you plan to enlarge  your image such as for a poster.

# OPTICAL ZOOM

A




B



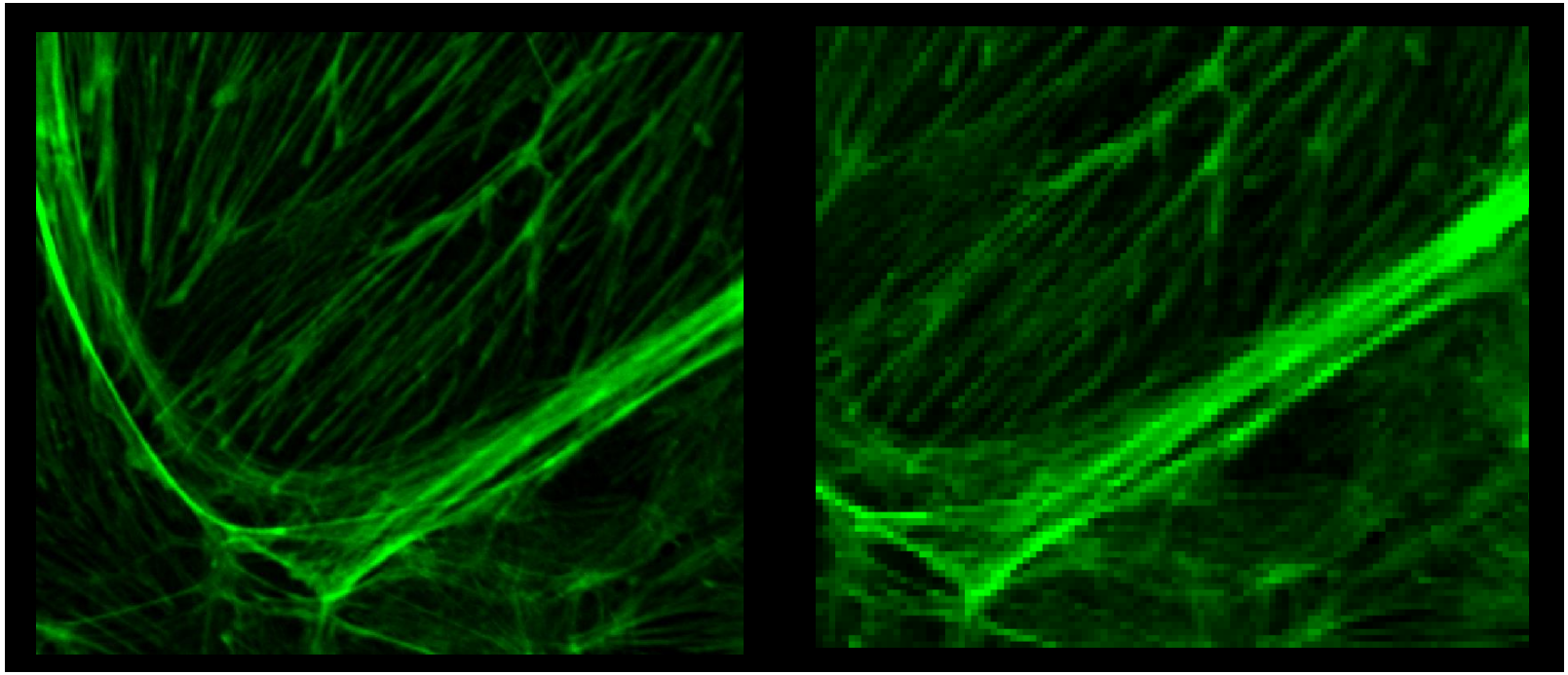

An optical zoom (left) contains the same number of pixels in a smaller area and is therefore superior to a digital zoom. (right)

A zoom of 4x using a 40x objective lens roughly translates to a magnification of 160x.

- A zoom greater than 6 begins to degrade the image and the resolution suffers. Consider using a higher magnification objective.

***Nyquist sampling, the pixel should be at least two times smaller than the smallest features that you expect to see in your specimen. Assuming a Rayleigh Criterion resolution of 200 nanometers, the pixels should be smaller than 100 nanometers. Larger ones produce undersampling, reducing the recorded brightness of small features.***

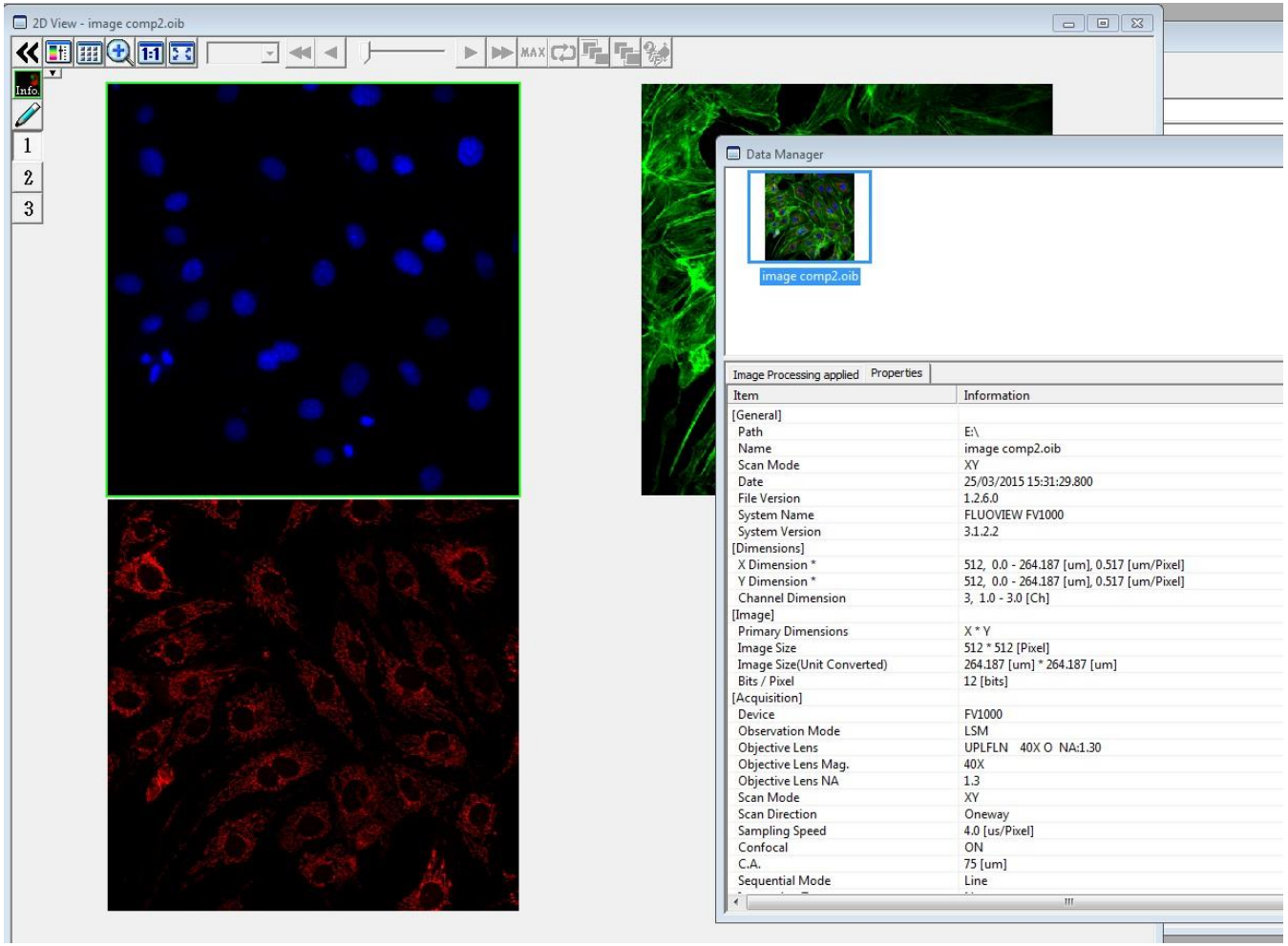
It's good practice to use the optical zoom in case you should ever need a zoomed in view.



**Guidelines for Image Collection in Fluorescence Microscopy**

- Always save the original data in the proprietary formats (Olympus = .oib (Fv1000), or .oir (Fv3000)

# Maintaining data integrity – DO NOT MAKE CHANGES TO ORIGINAL DATA




METADATA: Valuable information about the image. This information is lost when saving in any other format! IMPORTANT:Don't delete original data!

**Guidelines for Image Collection in Fluorescence Microscopy**

- Avoid photobleaching the fluorophore.

# Fading of fluorophores from exposure to light occurs because of two phenomena.

1. Quenching. The reduced output of fluorophore (fluorochrome) in the presence of oxidizing agents, such as salts, heavy metals or other fluorophores (environmental conditions). This can be controlled. Note: Hemoglobin is a quenching agent!

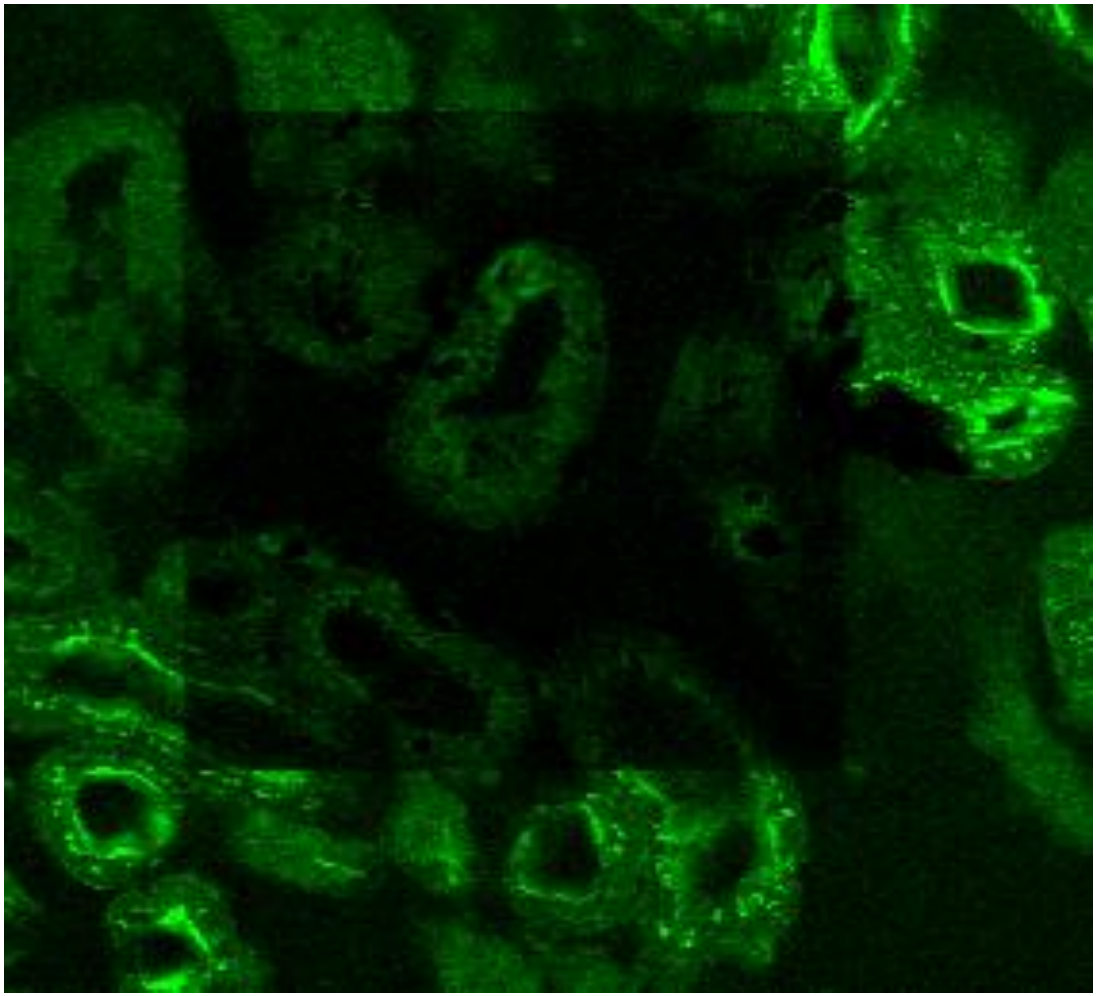
2. Photobleaching. The reduced output of a fluorophore due to damage to the fluorophore molecule. Can be worse in the presence of molecular oxygen.

   Anti-fading agents are useful for eliminating oxygen or oxidizing agents. Can be in mounting media such as Prolong Gold.

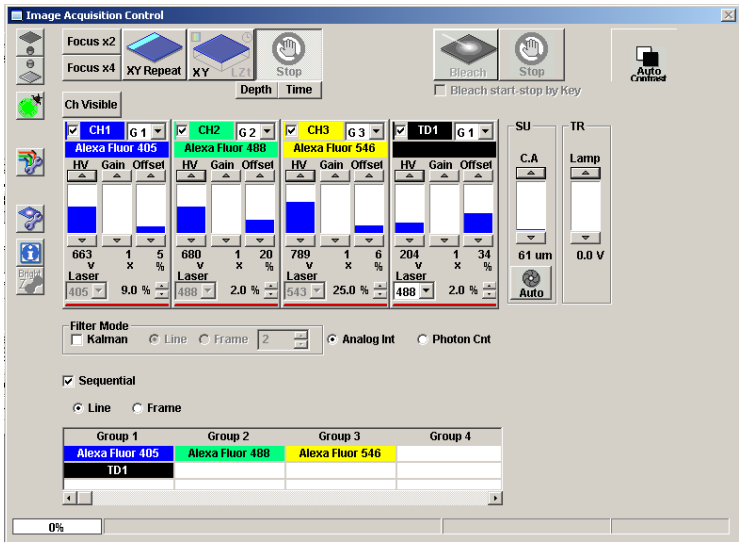
   Newer dyes (e.g. Alexa fluor, dylight, Atto dyes) are much more stable and do not generally require using antifade agents.

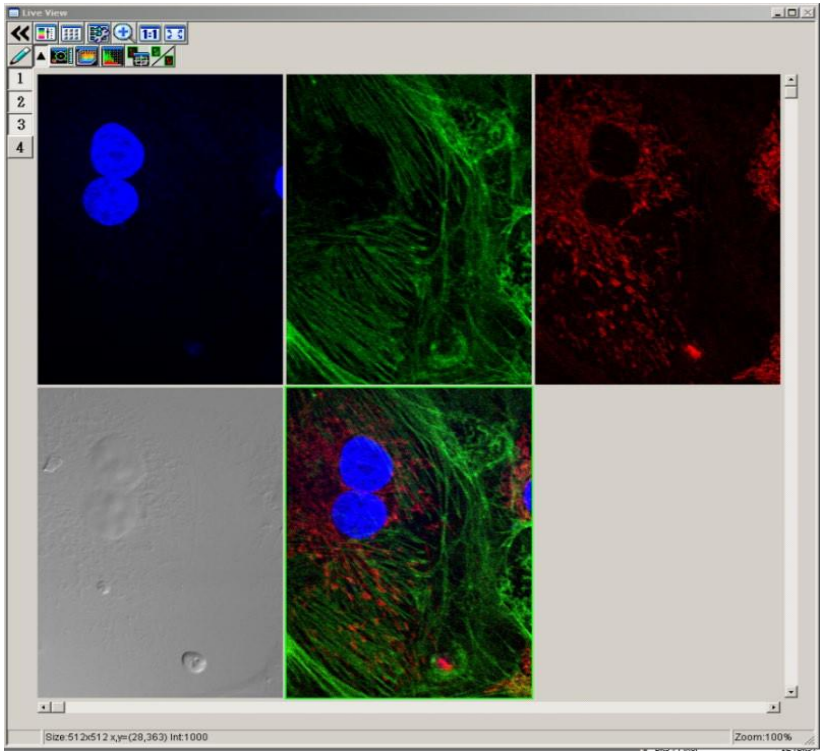
# Photobleaching –

The loss of fluorescence signal is irreversible. The extent of photobleaching is dependent on the duration and intensity of exposure to excitation light. Higher optical zoom increases Photobleaching.



# Transmitted Detector for Widefield DIC imaging




Differential interference contrast (DIC) does not require the presence of a fluorescence molecule.
Caveat – the sample has to be thin enough so the light can pass through (transmitted). There are several optical elements that need to be aligned in order to obtain a good DIC image.

This type of contrast imaging is useful in the following circumstances:

- you do not observe a fluorescence signal in your sample. A DIC image will prove to your advisor/committee/reviewer that there is actually a sample present.
- You perform an experiment with the expectation that only a subset of the sample will be labelled – i.e. transient transfection.

# CONTROL control control control control….

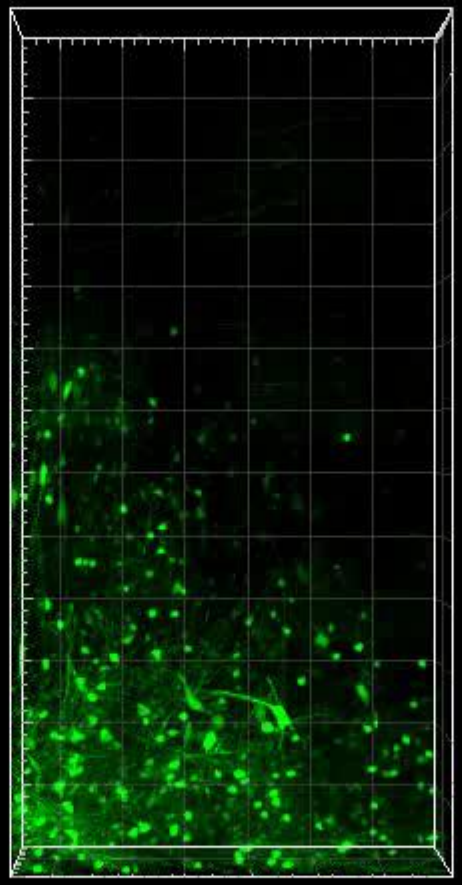
Sample with no fluorescence - control for auto-fluorescence.
– fluorescence present in the sample without you having to put it there. A common issue for tissue samples, less for cells.

Controls for each fluorophore you use.
Single color controls
Example for an immuncytochemistry experiment:
-primary antibody control
-secondary antibody control

Controls help you troubleshoot when the experiment doesn’t work and also prevent embarrassing errors!

# At The end of your imaging session

- Shut down the software and leave the microscope clean and in the same configuration as you found it.
- DO NOT EVER GET YOUR IMAGES FROM THE SYSTEM COMPUTERS!!. Retrieve your images from one of the workstations. Locations: BRT 245F, 245K & 268
- Do most image manipulations at the workstation, or on the Freeware.
- We keep your data only for 24 hours. We cannot guarantee that the computer hard drive will not fail. This has happened in the past. If you store your data on the system computer, you may lose it.
- Copy images to a USB flash card/thumb drive and then archive your original data.
- We suggest having your archived files somewhere where you will not be tempted to change them.



# Ready to publish?

**Please support the CMIF by including the following:**

We acknowledge resources from the Campus Microscopy and Imaging Facility (CMIF), and the OSU Comprehensive Cancer Center (OSUCCC) Microscopy Shared Resource (MSR) at the Ohio State University with NIH S10 OD025008 and NIH NIC P30CA016058.

**Current text available on website**

# Have you saved our contact info?