MIC Microscopy Workshop 2022
Mixed Zoom / In-person Seminars

- **In Person**

Please wear a mask, your neighbor may have a medical condition!
35 sqft = 6 ft away from anybody else
Please save questions for Q. breaks
Questions from in-person audience repeated *by* speaker for Zoom

- **Zoom**

Mute your microphone
Do not talk in your microphone, in-person audience cannot hear you
Questions in chat will be relayed *to* the speaker

Presentation PDFs from MIC staff available on demand
Session is being recorded

Microscopy Workshop, Spring 2022

Vincent Schram, Ph.D.

- **LIGHT MICROSCOPY 1: TRANSMISSION AND FLUORESCENCE**
  Monday May 9, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 2: CONFOCAL, 2P AND LIGHT SHEET**
  Tuesday May 10, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 3: SUPER-RESOLUTION IMAGING**
  Wednesday May 11, B35 / GG607, 11 am - 1:30 pm

- **IMAGE ANALYSIS WORKSHOP: IMAGEJ**
  Thursday May 12, B35 / GG607, 9:30 am - 12:30 pm / 1 pm - 4 pm

- **CONFOCAL MICROSCOPY HANDS-ON**
  Friday May 20, B35 / GD922, 9:30 am - 12:30 pm / 1 pm - 4 pm

CONFOCAL MICROSCOPY

Confocal Fundamentals

Principle, Core Applications, Rayleigh criterion, Resolution

Point Scanner

Overview, AOTF, Fiber, Detector, Image Formation

Spinning Disk

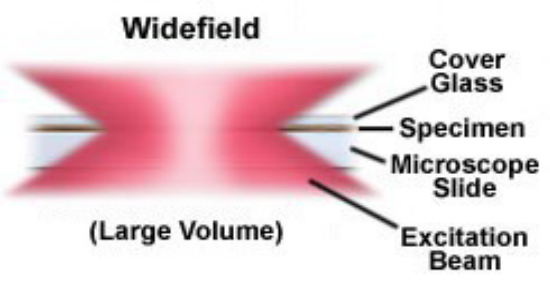
Overview, Detectors, Pros and cons

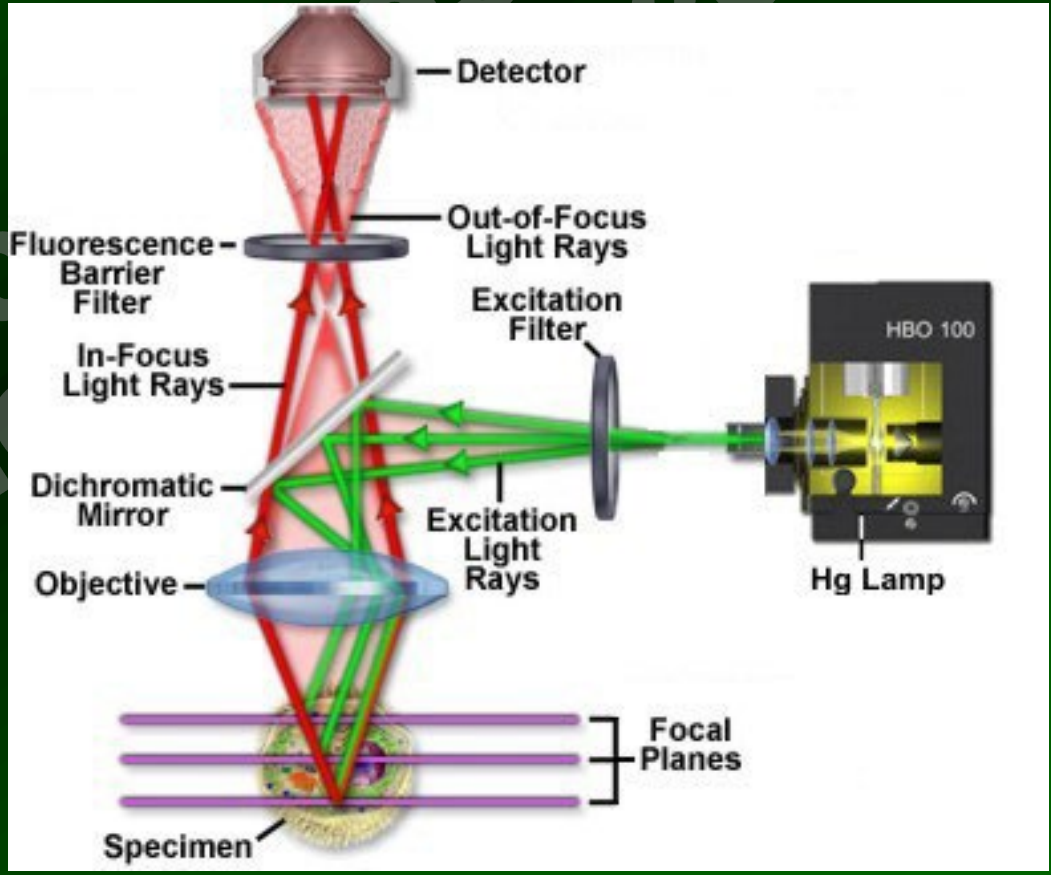
Experimental considerations

Mode of operation, Field of application, Balance and Optimize

CONFOCAL FUNDAMENTALS

Wide-Field microscope





- Wide cone of illumination

→ Full-field excitation
→ Lateral bleed-through

- Out-of-plane fluorescence

→ fluorescence blur

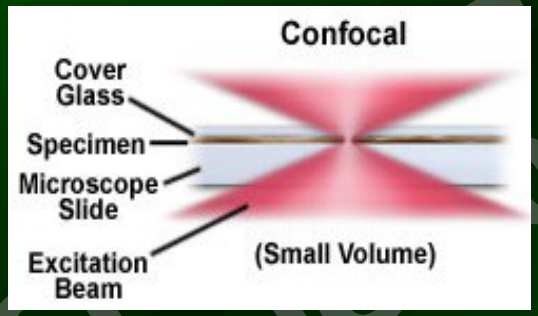
Optical sectioning

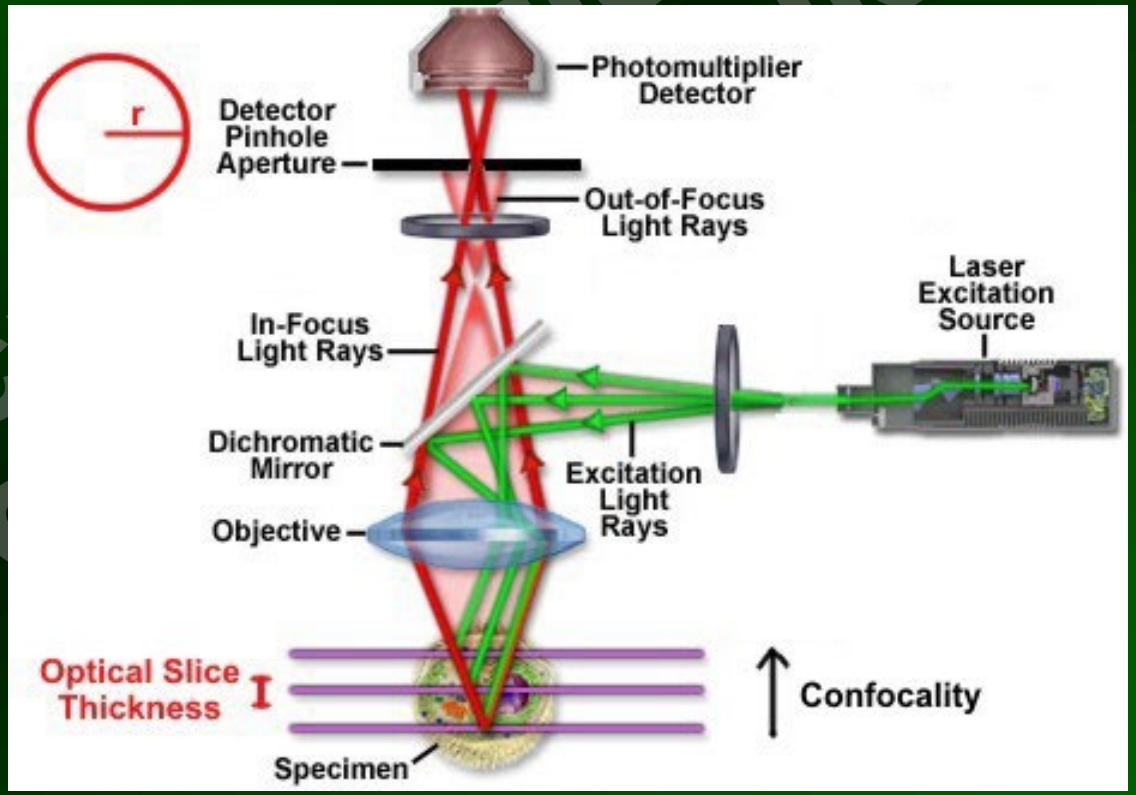
- Narrow cone of illumination

→ Localized excitation area
→ No lateral bleed-through

- Out-of-plane fluorescence

blocked by confocal pinhole

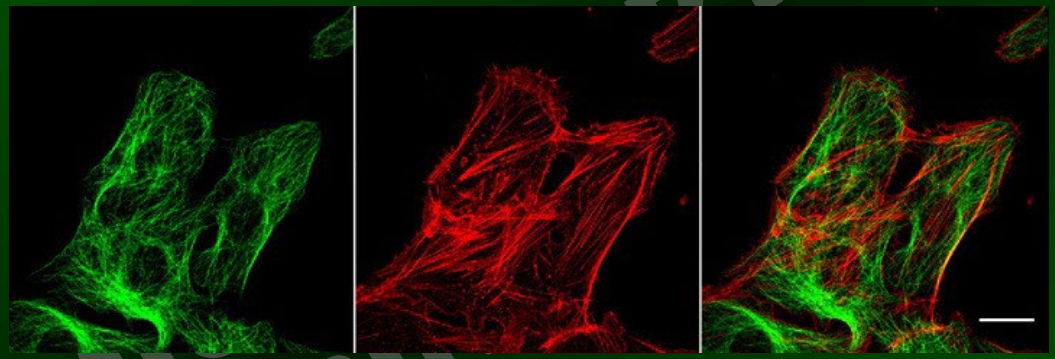


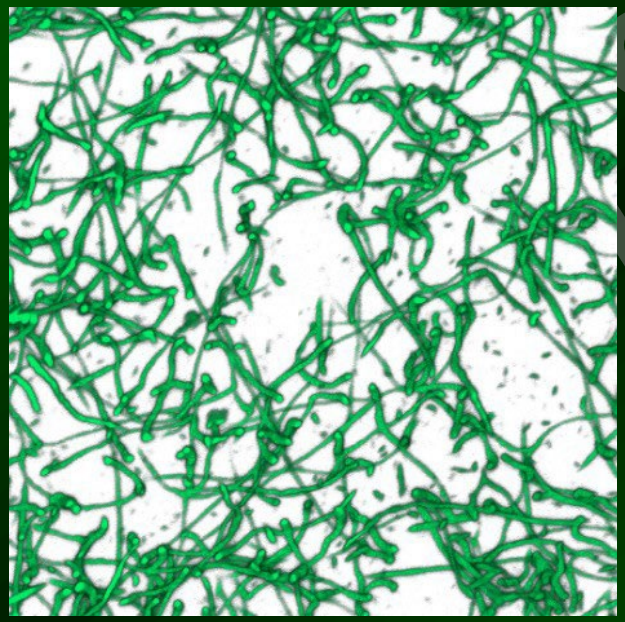


Core Applications

- **High-resolution imaging**

➔ Better spatial resolution than widefield without fluorescence blur





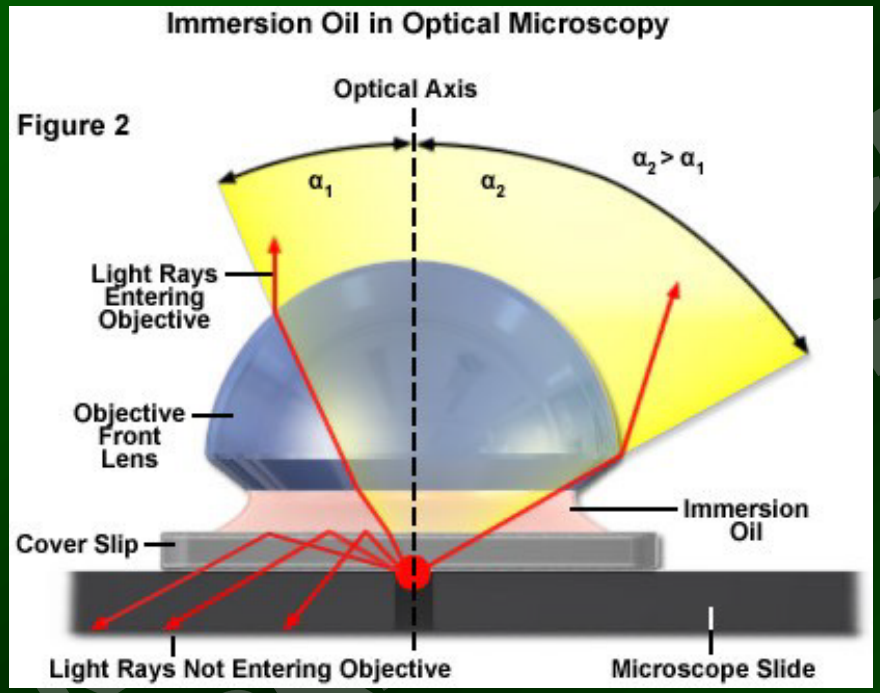
Collagen Fibers self-assembly
E. Makareeva, S. Leikin, NICHD

- **3D Imaging: z-stack**

➔ Series of images recorded at different focal planes, reconstructed as a volume in software

Resolution

- **Lateral resolution = 1.22 . λ / (2 . NA)**



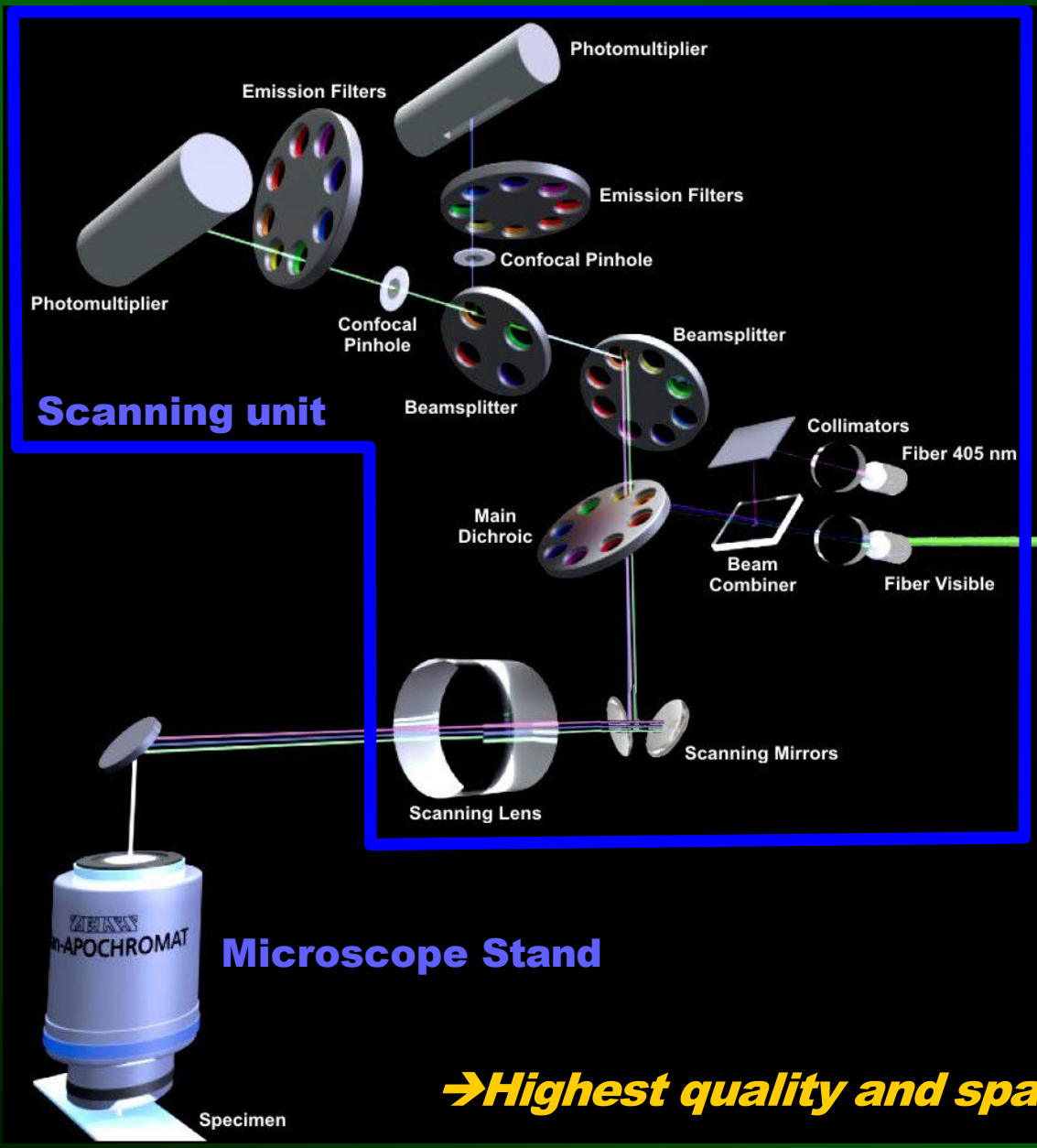
Objective/NA	d_0 (μm)
0.5x / 0.15	2.2
10x / 0.30	1.1
20x / 0.50	0.7
40x / 0.75	0.45
40x / 1.30 (oil)	0.26
63x / 1.40 (oil)	0.24
100x / 1.30 (oil)	0.26

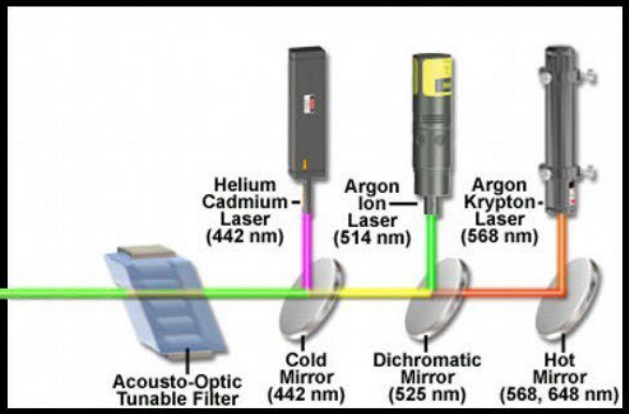
- **Axial resolution = 2 . n . λ / NA^2** → **0.5 μm at 1.4 NA**

→Resolution limiting factor = objective NA

POINT SCANNERS

Overview





Laser Launch

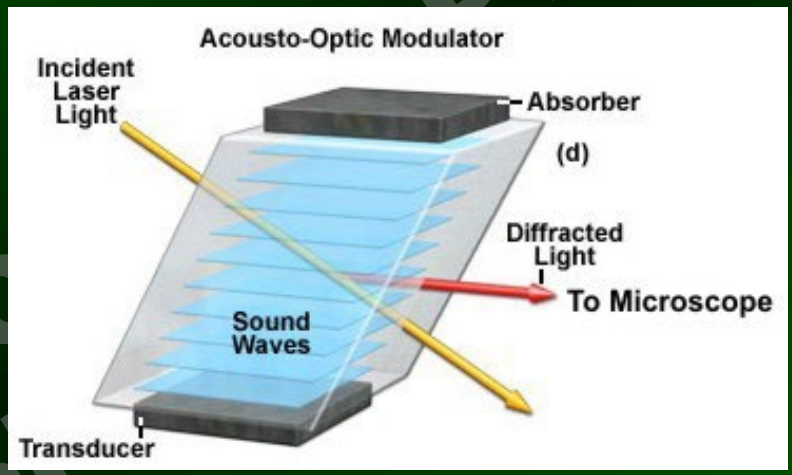
→Highest quality and spatial resolution available

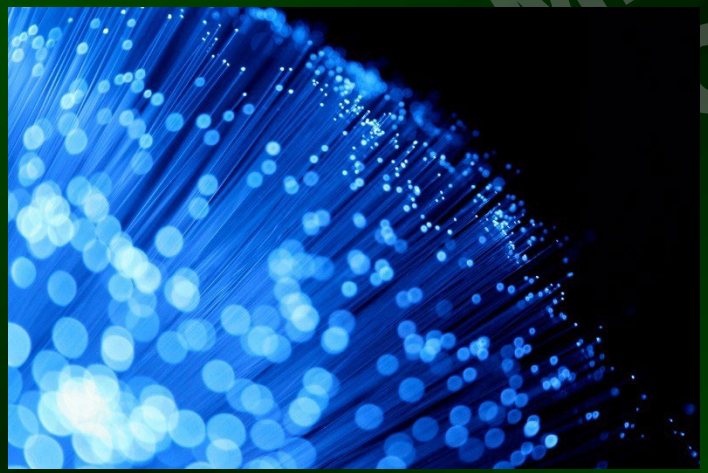
Color selection and light delivery

- **Solid-state lasers can be cycled in 10 nanosec.**

- **AOTF: Acousto-Opto Tunable Filter**

Laser line selection and attenuation
Very fast, 60% transmission
Not compatible with 2-photon





- **Optical fiber: thin strand of glass**

Smooth Gaussian beam profile
50% transmission
Not compatible with 2-photon

Detectors: Photo-Multiplier Tubes

- First photo-electron amplified by dynode chain
- Sensitive but noisy at high gain (cooling helps)
- Small dynamic range at fixed gain
- High performance GaAsP sensitive to photodamage

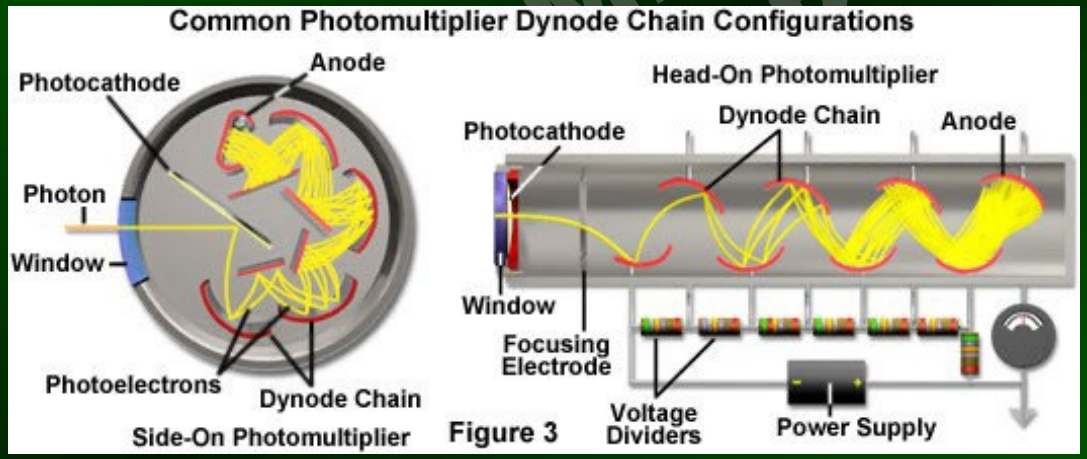


Figure 3

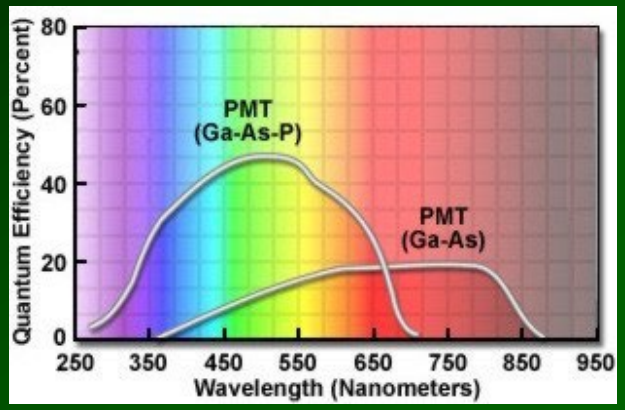
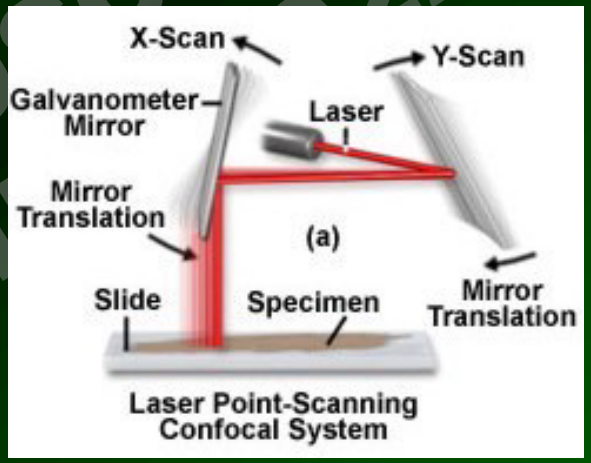


Image Formation

- Laser beam fill objective back aperture →Diffraction limited spot
- PMT records intensity
- Scanning mirrors move beam to next spot
- Confocal image = intensity map:

  $I = f(x, y)$



→ Slow, but fast resonant scanners trade speed for image quality

→ Control over scanned area = custom ROI, photobleaching

→ True optical zoom and field rotation (still 0.2 μm lateral resolution)

→ Simultaneous transmission imaging

Point Scanning Microscopes

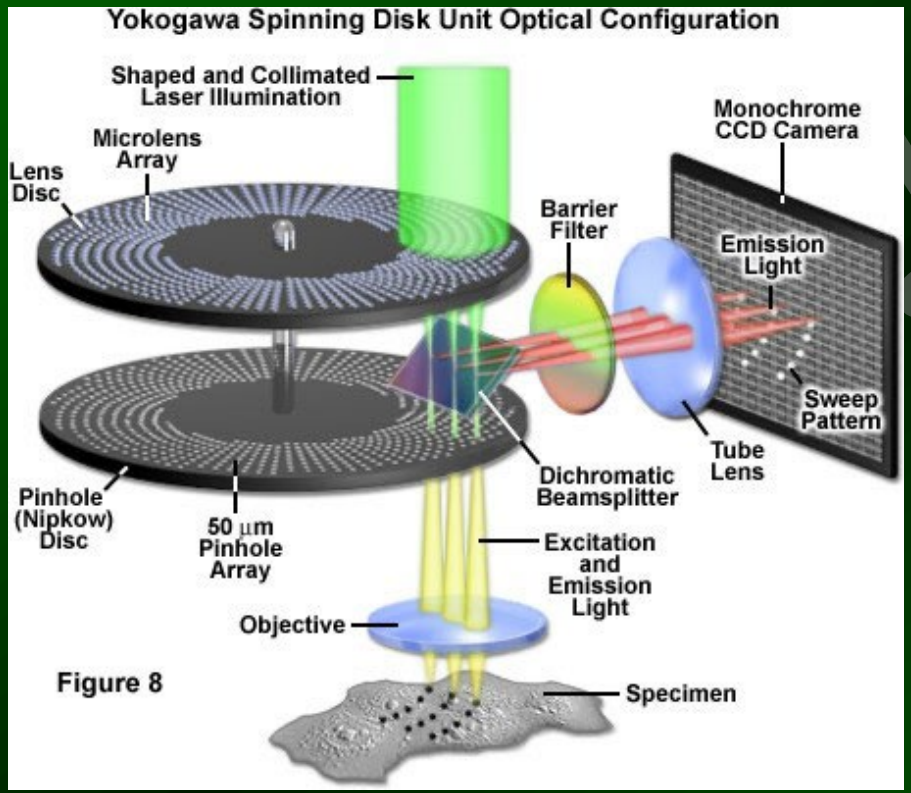
- **Pros:**
  - ***Low background***
  - **High sensitivity**
  - ***Excellent spatial resolution***
  - **No lateral bleed-through**
  - **Compatible with live cells**

- **Cons:**
  - **Slow (1-4 sec / frame)**
  - **Significant photo-damage**
  - **Requires careful calibrations**

→Best technology for high-resolution images

SPINNING DISK

Overview



- Full field illumination in confocal mode
- Manufactured exclusively by Yokogawa. CSU-X1, CSU-W1.
- Twin rotating disks

➔ Microlens array focus light onto matched pinhole array

➔ Pinhole disk rejects out-of plane fluorescence

➔ Disk rotation (4 or 10k rpm) averages pinhole pattern

- Dichroic between disks projects image onto camera

Detector: EM-CCD or CMOS camera

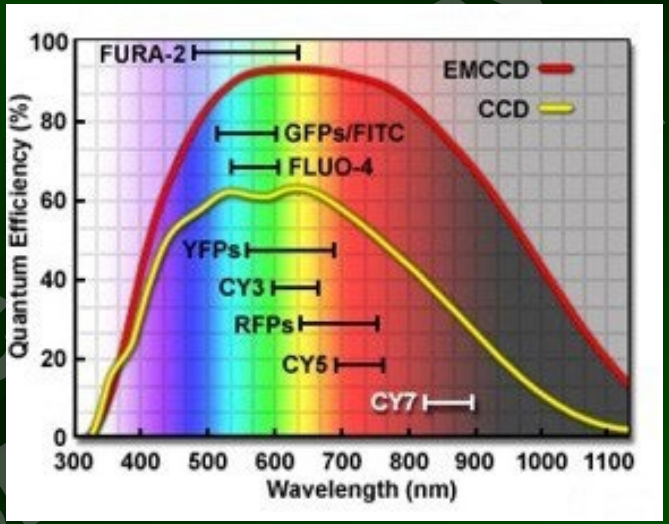
- **Electron-multiplied CCD**

Costly

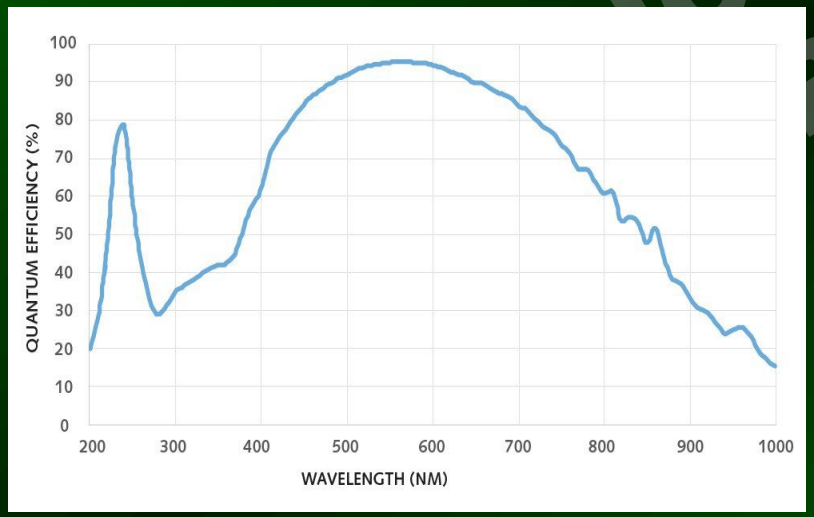
Relatively slow

Large pixels

Old technology



Photometrics Prime 95B cmos



- **CMOS camera**

Cheaper

Faster

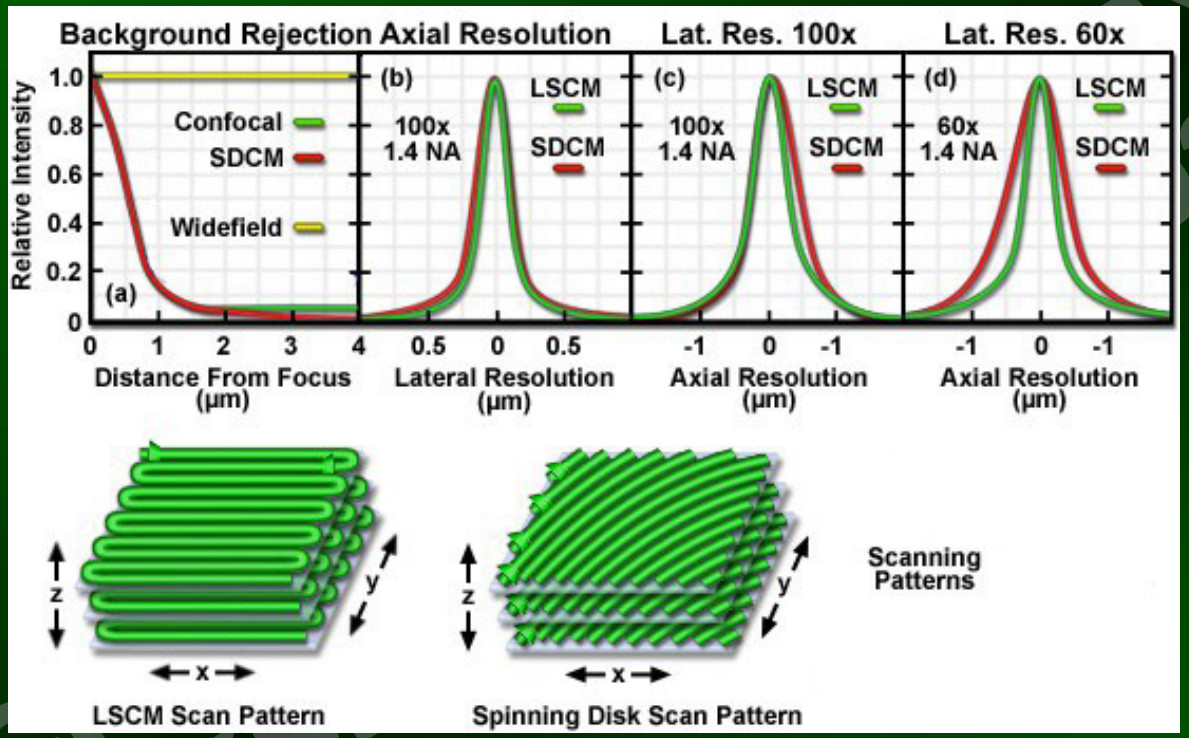
Small pixels = large field of view

As sensitive as CCD

Low floor-noise versions

➔CMOS camera better choice

Resolution

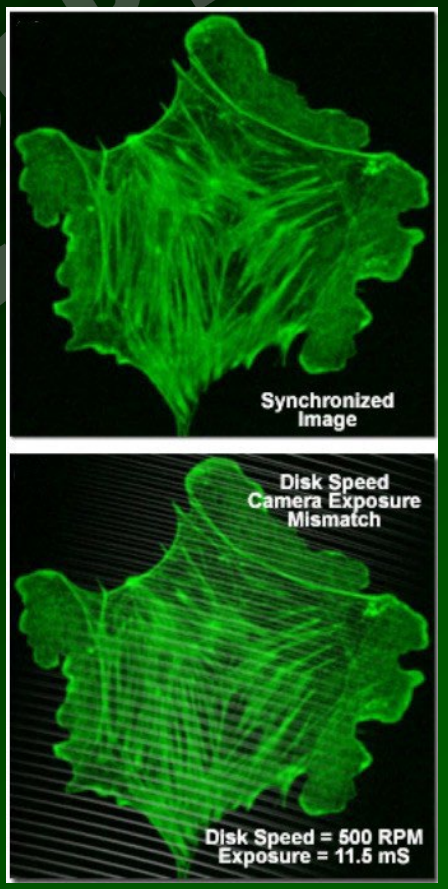


- Fixed pinhole size optimized for 100x 1.2 NA water objective
- Full-field illumination →prone to lateral bleed-through
- Super-resolution (“SoRa”) variant: 120 nm in xy, 350 nm in z

Spinning Disk Technology

- **Pros:**
  - ***Very fast***
  - **Low photo-damage**
  - **Sensitive**
  - **Mature CMOS camera technology**
  - **Modest alignments / calibrations**

- **Cons:**
  - **Lower spatial resolution than point scanners**
  - **Photo-activation requires auxiliary unit**
  - **High-speed acquisition prone to disk artifacts**

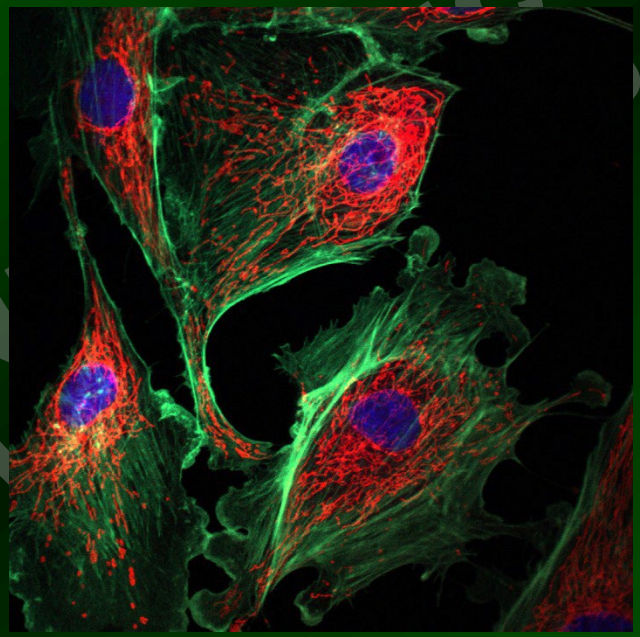


➔Best technology for high-speed, volumetric or high-throughput acquisition

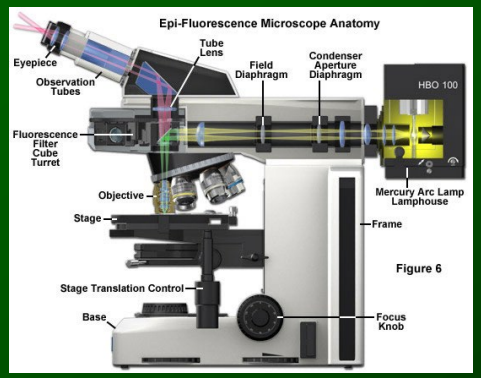
EXPERIMENTAL CONSIDERATIONS

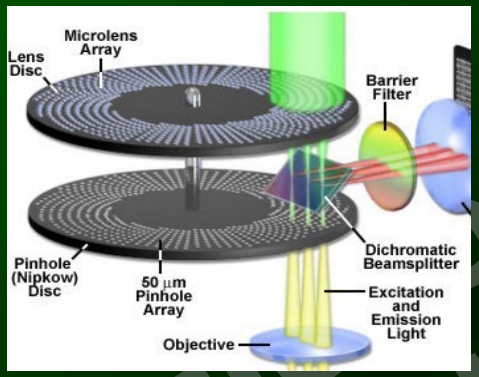
Mode of operation

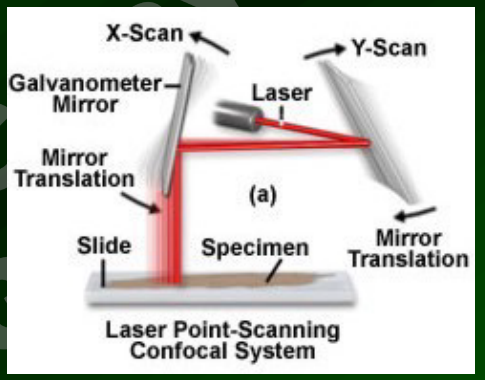
- **Confocal microscope:**
  - → **Flat images, multiple colors, z-stacks, or combinations**
  - → **Motorized stage: tile scanning, multiple locations (no immersion)**
  - → **Incubator: long term live imaging**
  - → **Hardware autofocus: required for t > 5 minutes**



Field of application







Wide-Field **Spinning Disk** **Point Scanner**

Efficiency (light throughput)

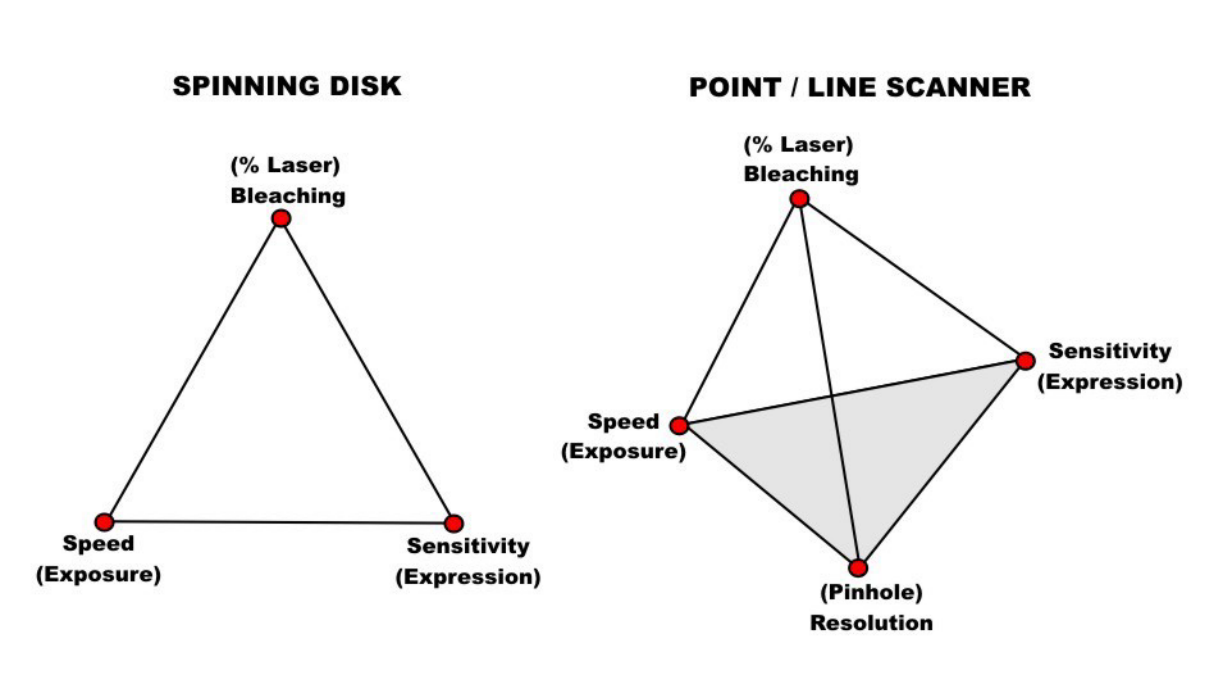
Phototoxicity

Lateral and axial resolution

Fast **Slow**

Cheap **Expensive**

Balancing Variables



- Speed, photo-damage and sensitivity mutually exclusive
- Live cells: minimal exposure, allows dark interval
- Point scanner: open pinhole (live cells)

Getting The Most Out Of Your Confocal

- **Objective cleanliness:**

Immersion oil hardens after a few days and diffracts light
Point scanner highly sensitive to misalignment
→ Sensitivity, lateral and axial resolution severely compromised…!

- **High-quality coverslip, #1 (tissue) or #1.5 (cells / high-res.)**

- **Match immersion fluid to mounting media (thick tissues)**

- **Steady artifact on images →close to specimen or detector**
  (shading correction = band-aid, clean it…!)

- **Highly sensitive = prone to false positive.**

Always use controls!
→ No primary w/ secondary for each immuno
→ Unstained / WT control
→ Beware of autofluorescence at high gain / high laser

TAKE-HOME MESSAGE

Confocal microscopes = spatial resolution

- **Pros:**
  - **0.2 μm lateral / 0.6 μm axial resolution (1.4 NA)**
  - **Large choice of dyes**
  - **Very sensitive**
  - **High speed (spinning disk)**
  - **Wide field of applications and specimens (cell, tissue, animals)**
- **Cons:**
  - **Optical sectioning sometimes detrimental (3D tracking)**
  - **Lower efficiency than wide-field**
  - **More expensive than wide-field**

→Best Resolution when clean objective, correct coverslip and oil

QUESTIONS

MULTIPHOTON MICROSCOPY

Multiphoton Fundamentals

Principle, MP Excitation, Resolution

Two-Photon Microscope

Overview, Light source, Pulse compressor, Non-descanned detectors

Field of Applications

Dyes, Applications, Experimental considerations

MULTIPHOTON FUNDAMENTALS

Principle

- **Tissues more transparent to infra-red light**
  →*IR excitation penetrates deeper than visible*

- **Fluorophore can be excited by absorption of two photons of half-energy (twice λ):**

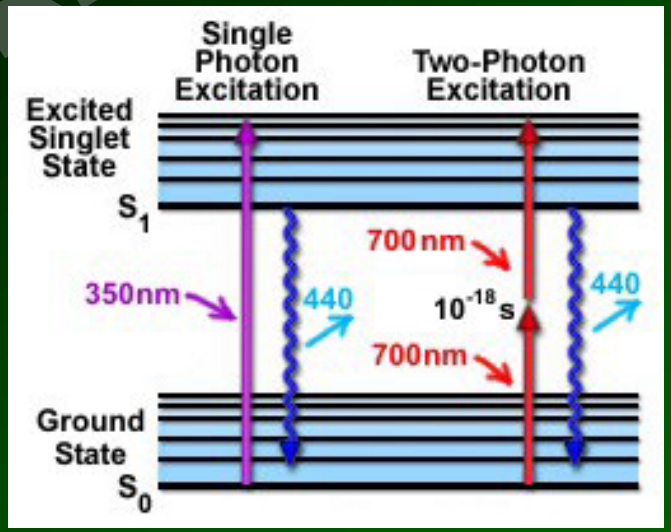
→ First photon induces highly unstable intermediate state (10^{-18} sec.)

→ Second photon within 10^{-18} sec.

Full transition

Identical de-excitation pathway

Same emission wavelength



Multiphoton Excitation

- 1-photon: Beer-Lambert Law: $I(x) = Io\ e^{-(\varepsilon \cdot I \cdot C)}$

  Absorption coefficient ε (mole / m^2), I = distance, C = concentration

- 2-photon: $I(x) = Io / (1 + \varepsilon . I . c . Io)$

  Two-Photon Absorption coefficient ε = Cross-section

  Unit = Goppert-Mayer (GM) = 10^{-50} $cm^4 . s$ / photon

➔ Emission varies with square of excitation intensity ("non-linear" optics)

➔ Requires 10^4 to 10^6 more photons than visible excitation...!

➔ 3-photon absorption possible, 10x more light (UV dyes)

➔ ***Highly inefficient process***

➔ ***Extreme photon density only possible with pulsed laser***

Lateral and Axial Resolution

- Emission varies with square of excitation intensity (NLO)

  → Decays very fast away from the focal point
  → Intrinsically confocal
  → No need for emission pinhole

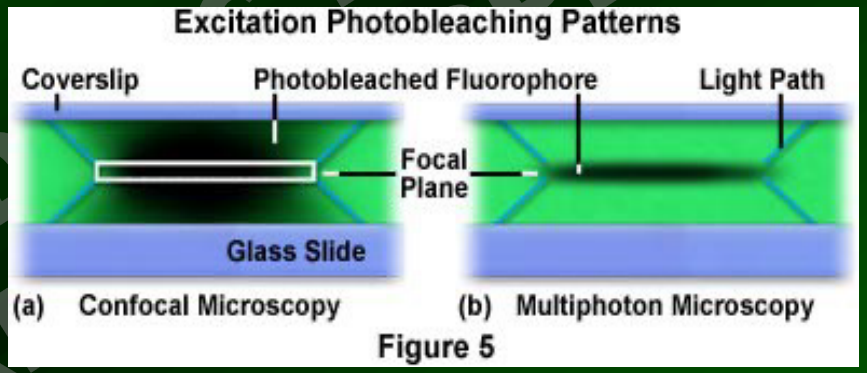
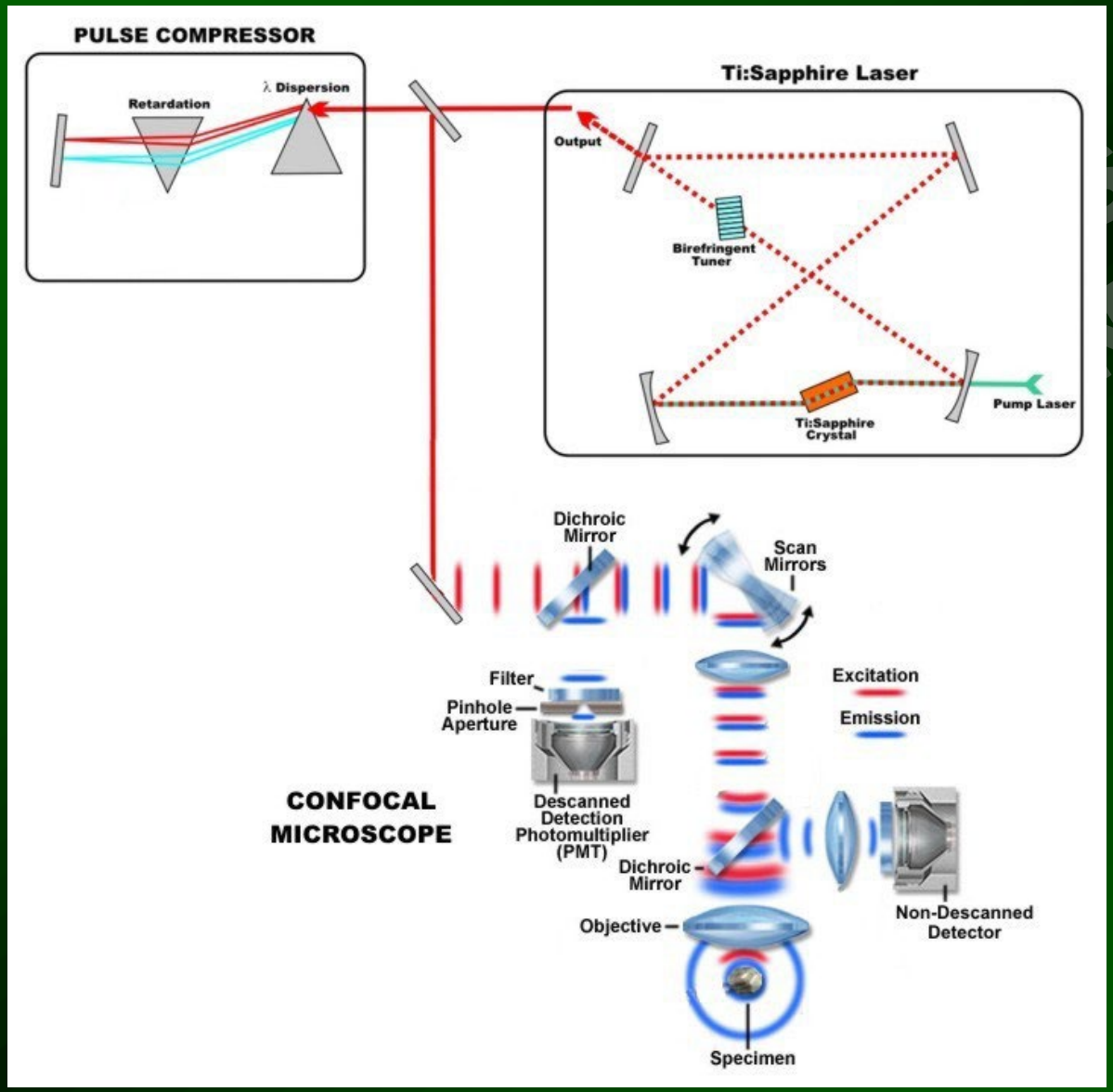


Figure 5

- Higher λ →lower resolution than visible confocal

TWO-PHOTON MICROSCOPE



Point scanner only,
no wide-field / SD 2P

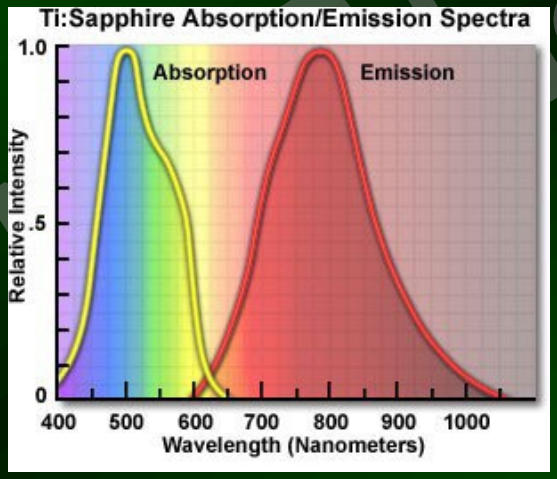
Two-photon Light Source

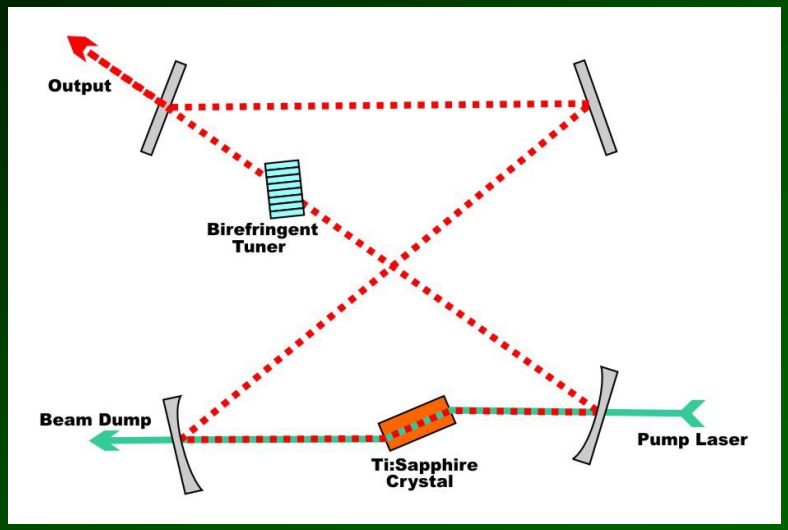
- **Ti-Sapphire laser:**

Pulsed laser, 100 femtoseconds, 80 MHz → x10^5 peak energy

- **CW power = 1-3 watts, instant up to 450 kW**

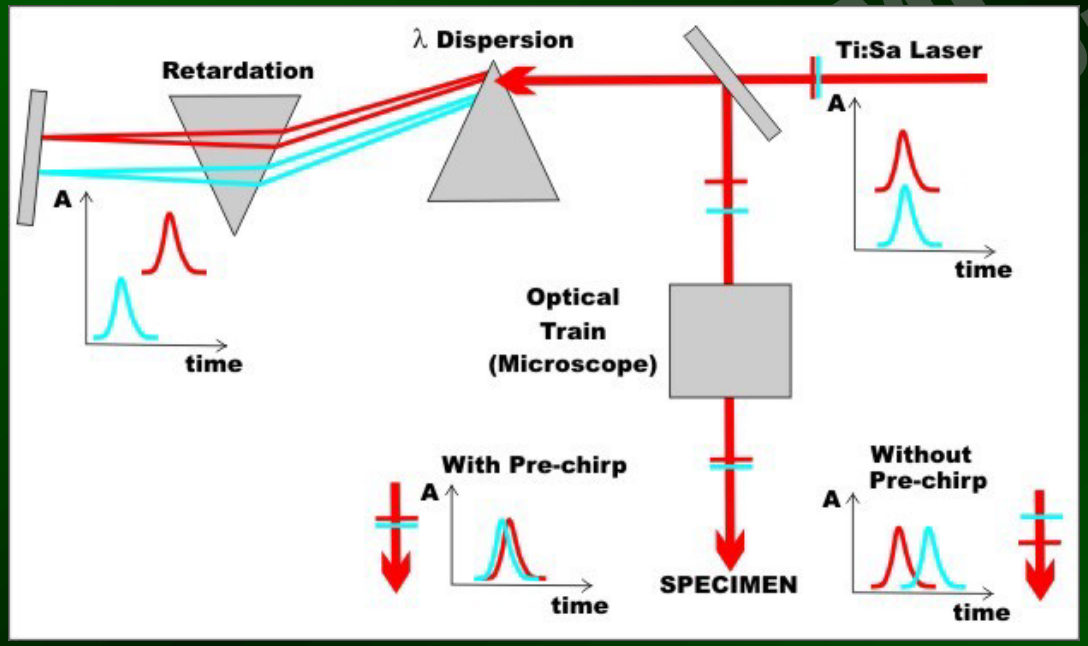
- **Ti^{3+} in sapphire crystal has broad absorption and emission**
  - **→ Tunable laser, 650-720 to 950-1050 nm**
  - **→ "Mode-locked" operation, no rapid λchange**





Pulse Compressor ("Pre-chirper")

- Output of Ti:Sa laser not strictly monochromatic $\Delta\lambda$= 1 – 5 nm
- Speed of light in media = c / n, and n decreases with higher λ
  - ➔ Longer wavelengths travel faster in glass (ultra-fast optics)
  - ➔ Glass in optical train spread pulse by retarding shorter λ
  - ➔ ***Excitation efficiency depends on square of instant photon density…!***



- Pulse compressor: retard higher λ w/ thicker glass (glass vs. air difference)
- Prism translation = retardation adjusted for shortest pulse at specimen = brightest image

➔ ***Always adjust compensation on 2P confocals***

Non-Descanned Detector

- **Ballistic photons:**

Travel in straight lines, acquired

- **Non-ballistic photons:**

Scattered by turbid specimen (or optics)

- **Non-descanned detector:**

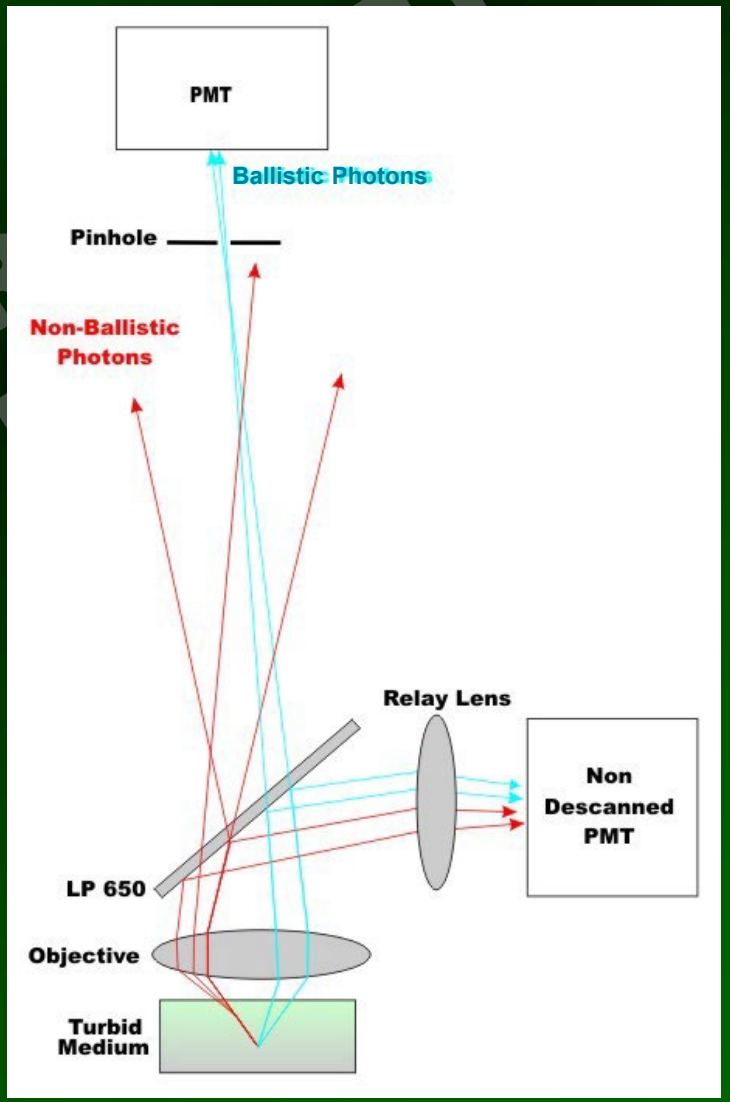
PMT close to specimen

➔ Wider aperture than descanned

➔ Useable only in 2P (SP filter)

➔ Higher efficiency when scattering specimen (deep)

➔ No pinhole



➔ Considerable improvement with scattering sample (thick tissue)

IMPLEMENTATION

Multiphoton Dyes

- **2P absorption spectra difficult to establish**
- **Wider absorption than visible → Crosstalk**
- **Many fluorescent dyes absorb in 2P mode, but not always efficiently**

→ **Alexas**

→ **DAPI, FITC, Rhodamin**

→ **Most fluorescent proteins: tissues / intra-vital**

Blue/Cyan dyes
Alexa 350 (780 nm-800 nm)
Hoechst (780 nm-800 nm or 900-1100 nm)
DAPI (780-800 nm or 900 nm-1100 nm)
CFP (800 nm -900 nm)

Green dyes
Oregon Green (800 nm-860 nm)
Alexa 488 (800 nm-830 nm)
GFP (840 nm-900 nm)
BODIPY (900 -950 nm)
FITC (750 nm-800 nm)
DiO (780 nm-830 nm)

Yellow Dyes
YFP (890 nm-950 nm)

Orange dyes
DiA (800 nm-860 nm)

Red dyes
DiI (830-920 nm)
Rhodamine B (800 nm-860 nm)
Alexa 568 (780 nm-840 nm)

Applications

2P useful only with turbid -i.e. thick- specimen

➔ **Thick tissue section, fixed (virus injection, transgenic anim…)**

➔ **Do not combine 2P with cleared tissues…!**

➔ **Live tissue section (electrophysiology)**

➔ **Live animal imaging (heavy experiments)**

Experimental Considerations

- **Choice of dyes**

Cross-talk more a problem than in visible

Multiple NDDs outfitted with filter cubes →limited filter selection

→Pre-chirp adjustment critical (adjust for max. intensity)

- **NDDs very sensitive to ambient light →light-proof enclosure**

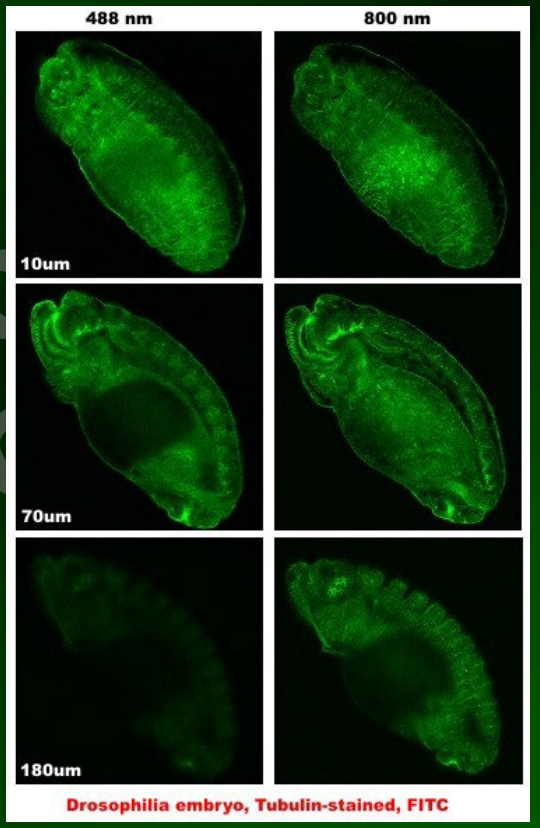
- **Live tissue / animal requires paraphernalia (perfusion, heat, gases, etc…)**

- **Photodamage: careful at the surface, ramp power inside tissue**

(depth correction also useful in visible)

- **Require IR-optimized objective and optics**

TAKE -HOME MESSAGE



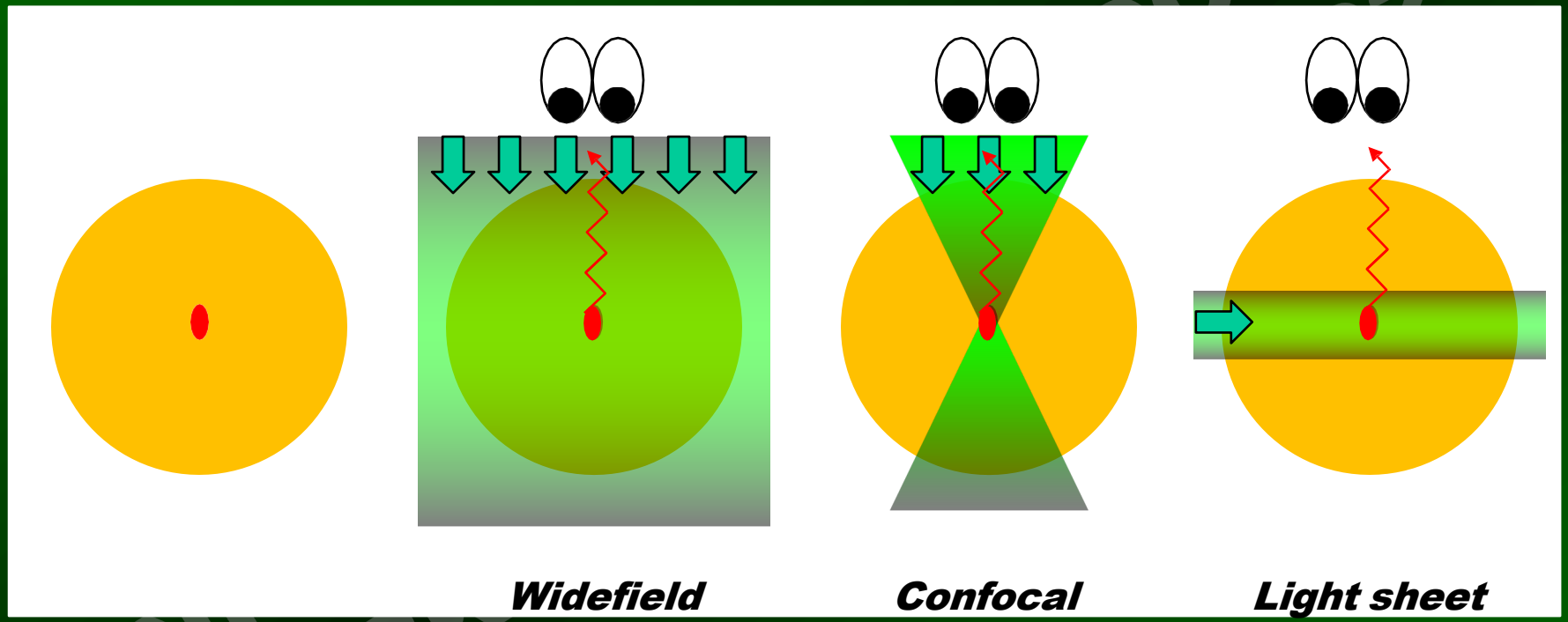
- **Multi-photon is NOT better than visible**
  - → **Similar or worse resolution**
  - → **Inefficient excitation**
  - → **Broad absorption spectrum = crosstalk**

- **Useful only for *deep imaging***
  - → **IR light penetrates *deeper***
  - → **NDDs collect scattered photons more efficiently**
  - → **Intrinsically confocal: no pinhole = cheaper microscope**
  - → **Pulsed IR laser expensive**

→2-photon = penetration

QUESTIONS

LIGHT SHEET MICROSCOPY



- Optical sectioning: side illumination with thin layer of light
- Separate optical paths for excitation and detection

Light sheet illumination

- **Advantages:**

Low bleaching, highly efficient
Very fast (CMOS camera)

- **Problems:**

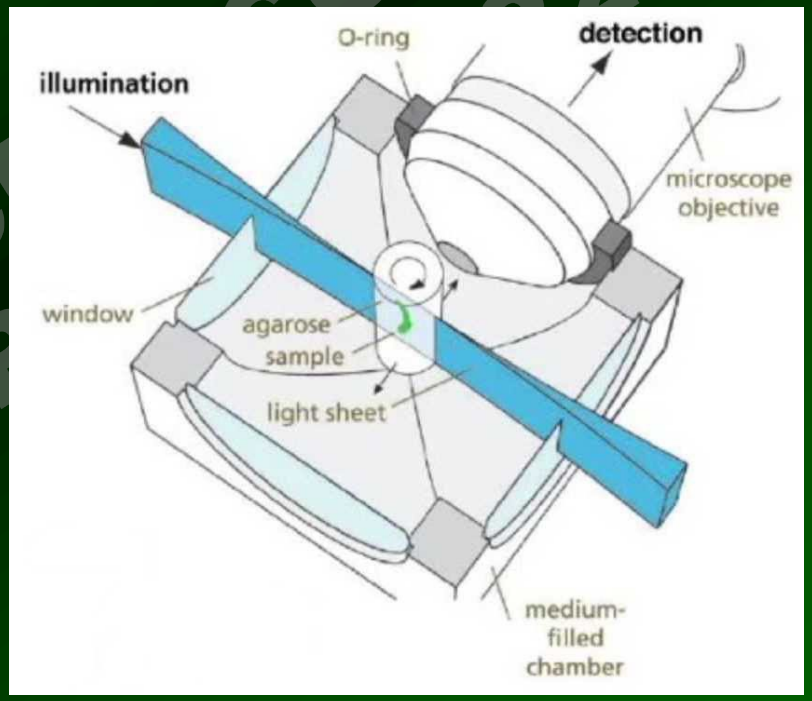
Limited optical sectioning: 2-10 μm
Limited xy resolution (working distance)

Dense object creates stripes
→ dual-side or tilted excitation

Clearing method labor-intensive and induce morphology changes

Complex specimen mounting

Zeiss controls crucial patents →little competition



Large Specimen: Zeiss Z1

- **Optical clearing required**

- **Clearing reagent:**

FocusClear, Clarity, Scale, Cubic…

Choice of clearing method critical…!

- **Specimen mounting:**

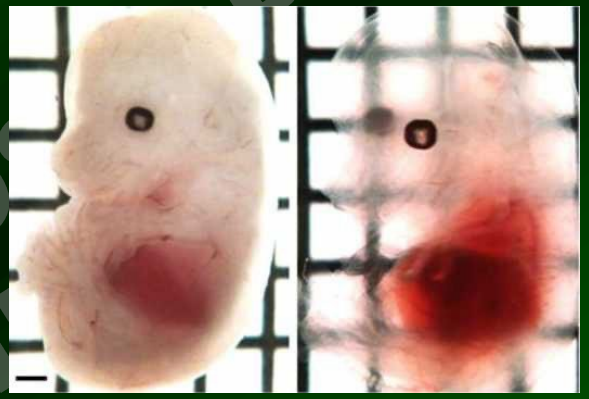
Agarose embedding in syringe

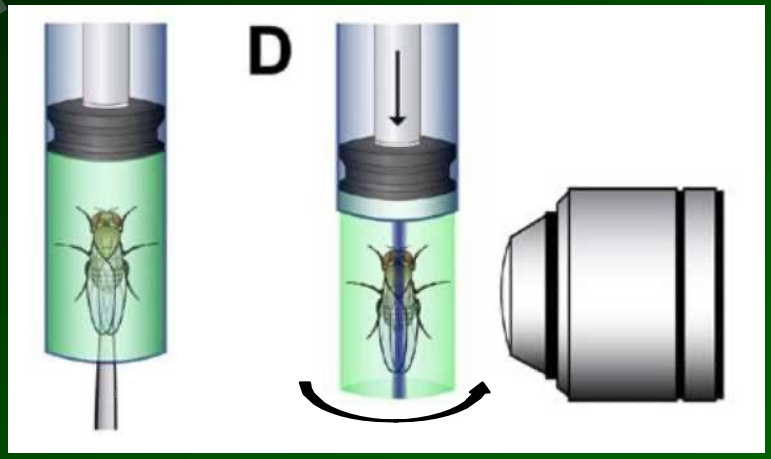
Agarose cylinder mounted on xy stage

- **Stationary imaging plane → motorized stage, limited speed**

- **5 μm sectioning, 25x lens:**

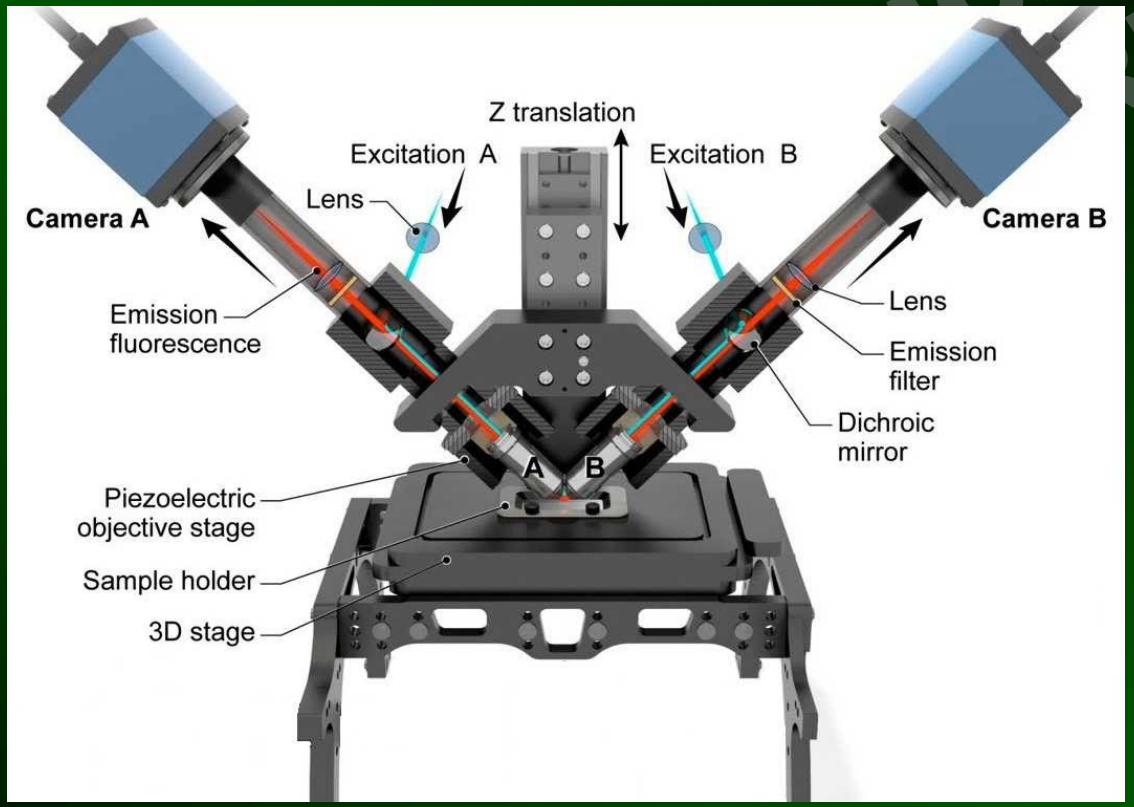
→ 100-1000 μm: embryo, whole organ (mouse brain), small organism (zebra fish, drosophila…)

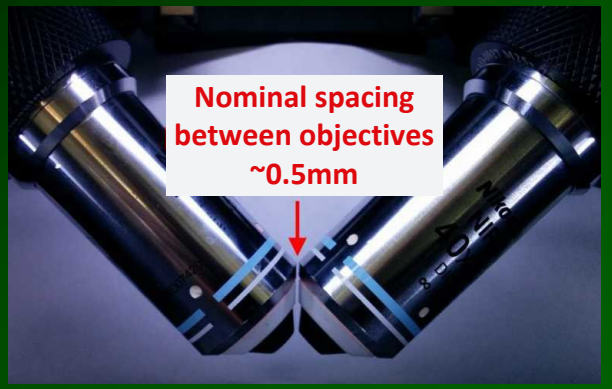




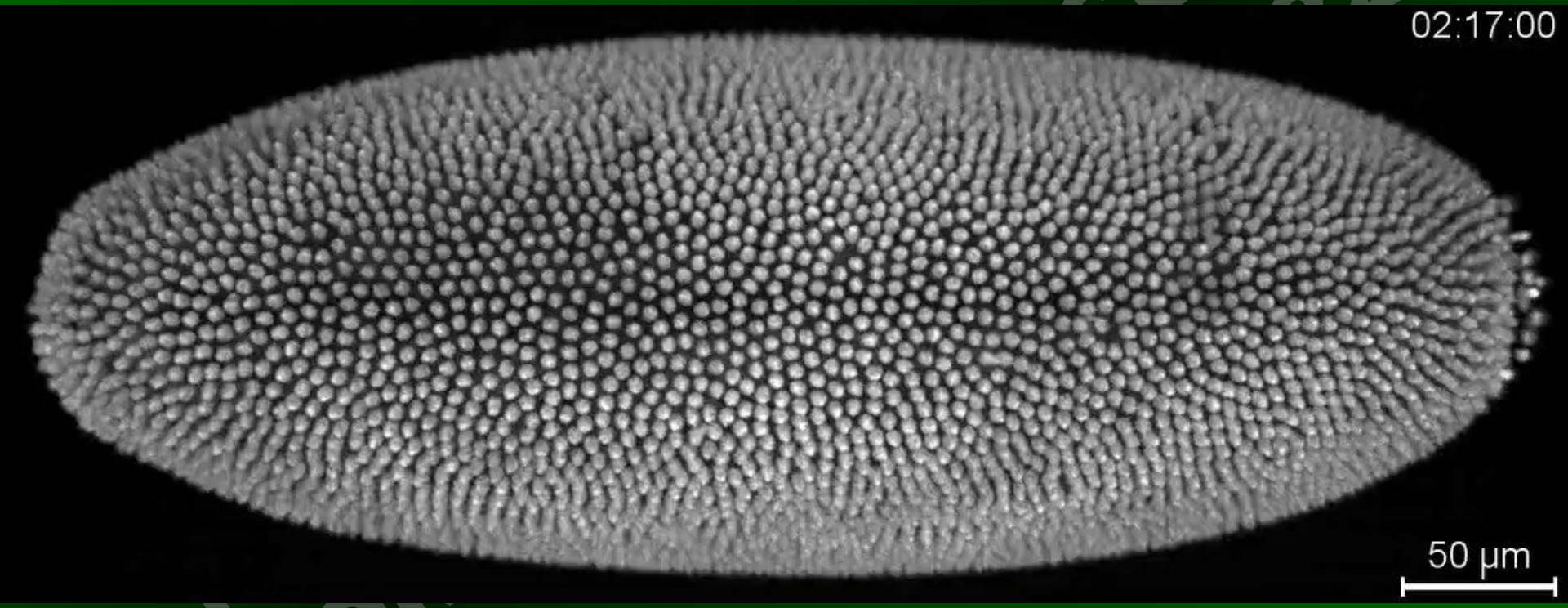
Small specimen: di-SPIM

- Developed at NIH by Hari Shroff, commercialized by ASI (add-on)
- Symetrical, alternating, dual-side illumination and imaging
- Stationary specimen, optical scanning = very fast
- High vertical, medium x-y resolution = 5 – 100 μm specimen



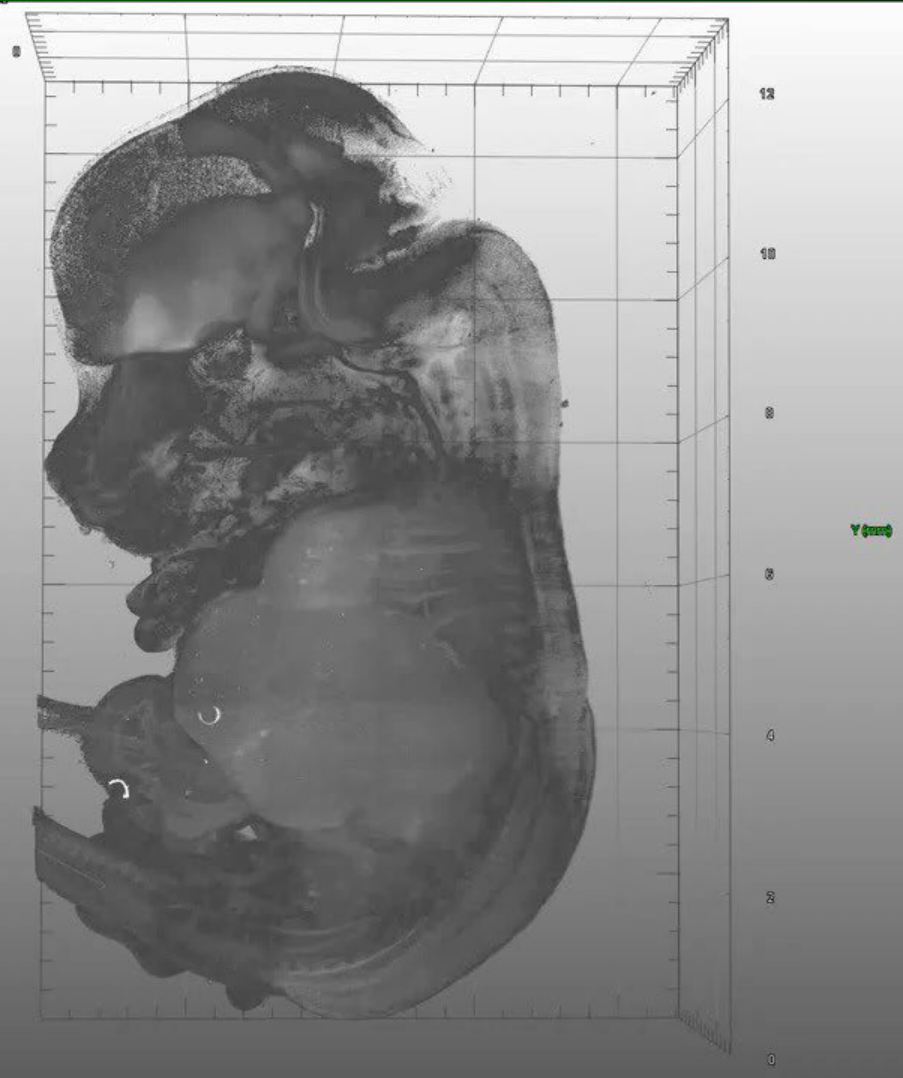


Example: Drosophila Development



Drosophila melanogaster, Histone - RFP, Philip Keller, HHMI / Janelia Farms

Example: Mouse Embryo



Thy1 EGFP M line mouse w/ prop iodine, O.Efimova, National Research Center, Moscow

TAKE-HOME MESSAGE

- Low resolution = large specimen
- Require transparent specimen (cleared = fixed)
- Optical clearing difficult and induces morphological changes (shrinkage / expansion)
- Volume reconstruction CPU and data intensive
- Derivatives technologies (di-SPIM, lattice light sheet) offer high spatial resolution, under development

→Light sheet = speed (high-volume acquisition)

QUESTIONS