다광자 현미경을 이용한 뇌과학 연구

박혜윤
서울대학교 물리천문학부
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생명물리 여름학교

Outline

1. Confocal microscopy
2. Multiphoton microscopy
3. Single molecule imaging of mRNA

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Optical sectioning in fluorescence microscopy

Widefield Fluorescence → Fixation and Physical Sectioning

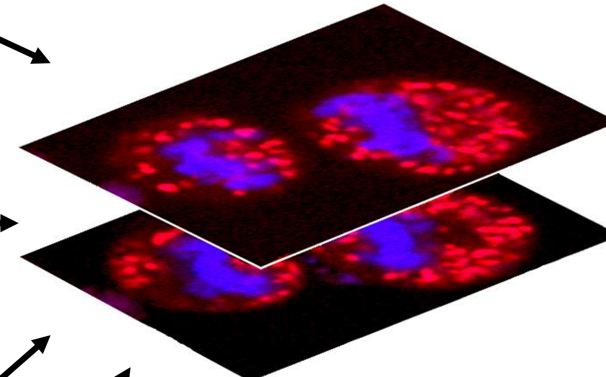
Widefield Fluorescence → Deconvolution Methods

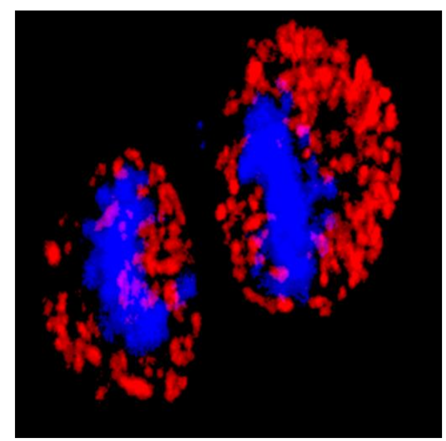
Confocal Microscopy → Confocal Aperture

(Thickness of an optical section can be < 1 μm)

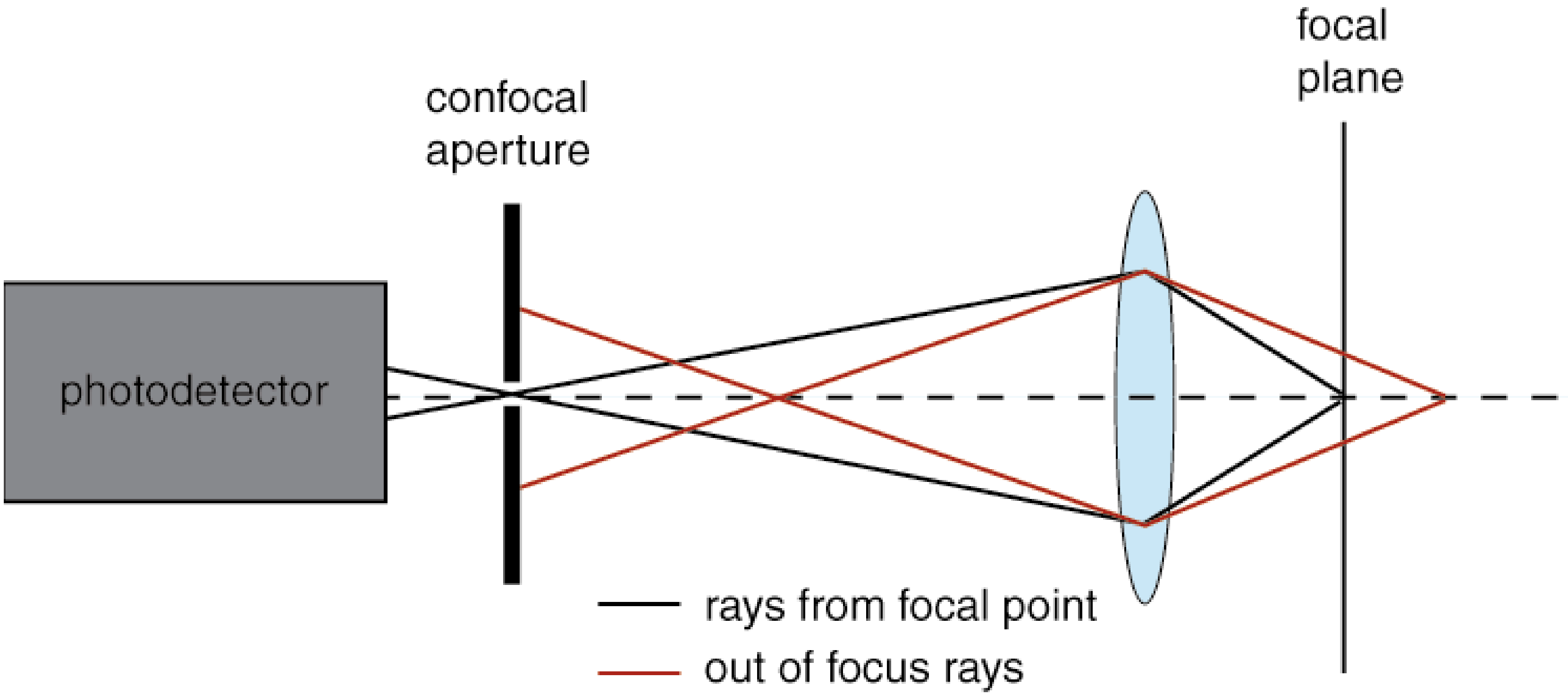
Multiphoton Microscopy → Nonlinear Excitation

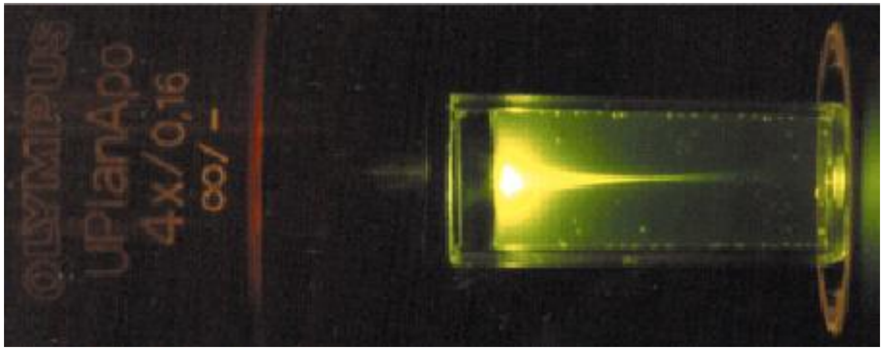
Live specimens



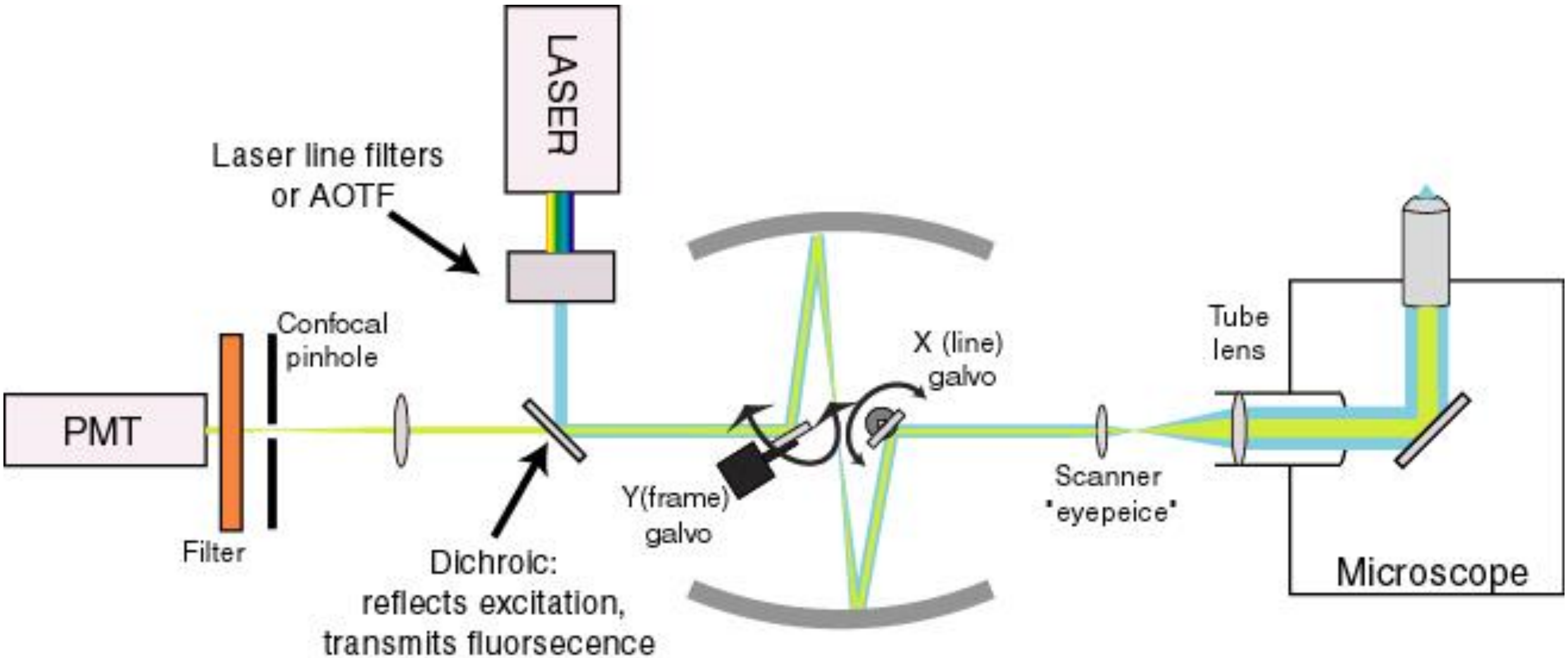


One way to achieve intrinsic optical sectioning is the use of confocal detection.





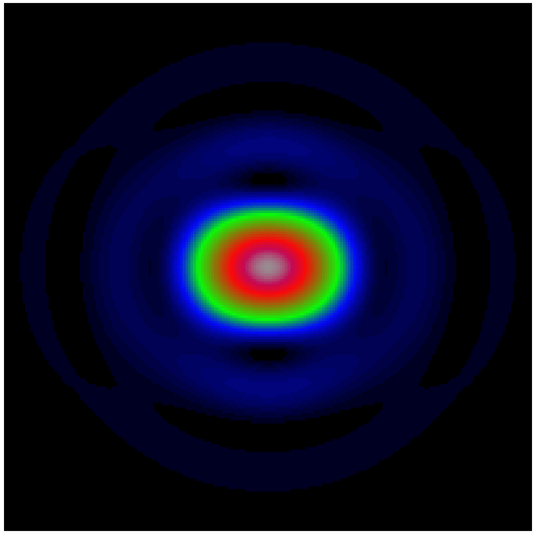
Typical confocal microscope design



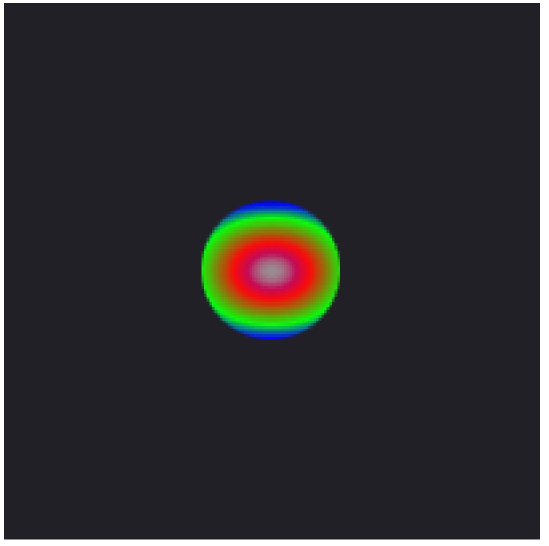
Resolution of a confocal microscope depends on:

1. Numerical aperture (NA) determines the spot size.

2. Size of the confocal aperture – The confocal image signal-to-noise ratio is optimized by a detector aperture slightly smaller than the first minimum of the Airy disk. This most efficiently balances signal collection with background rejection.



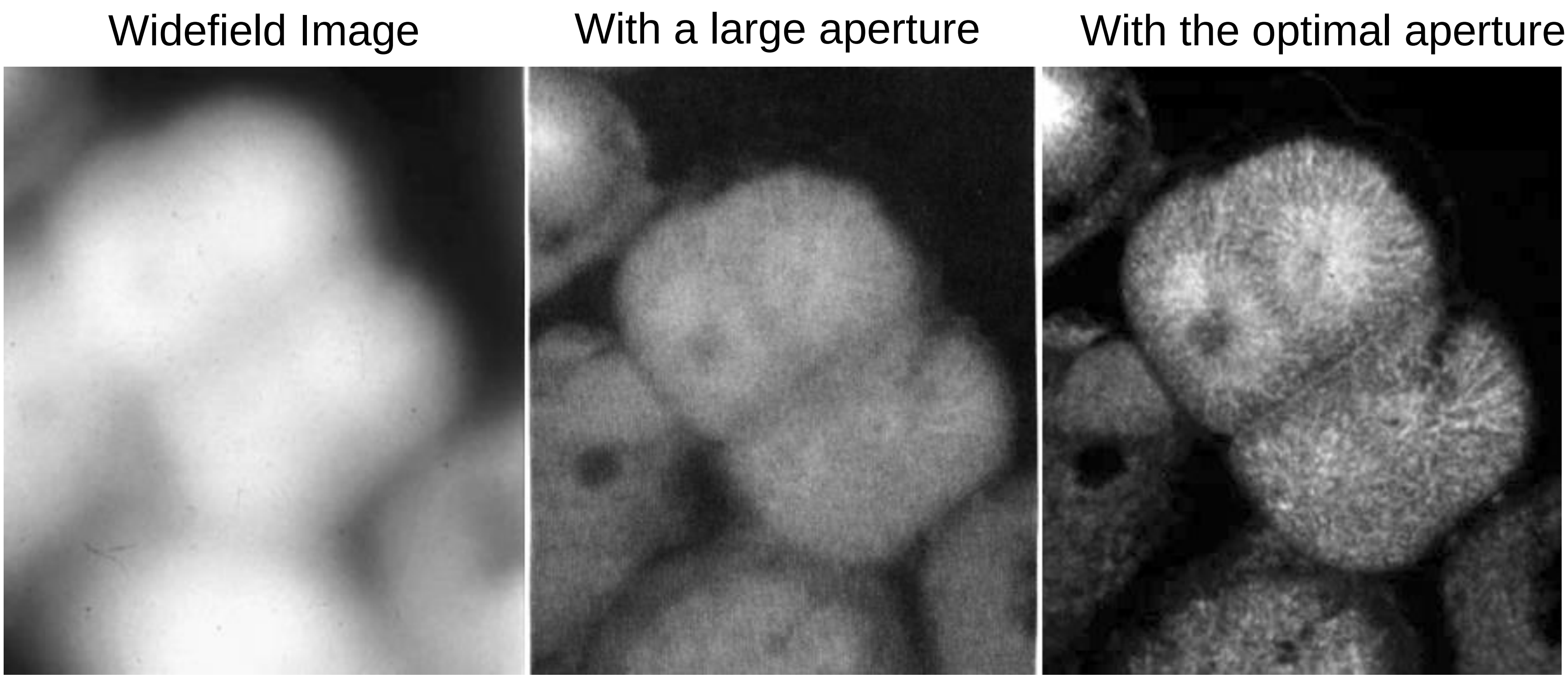
Focused illumination in the XY plane at z =0



With an optimal confocal aperture

Resolution of a confocal microscope depends on:

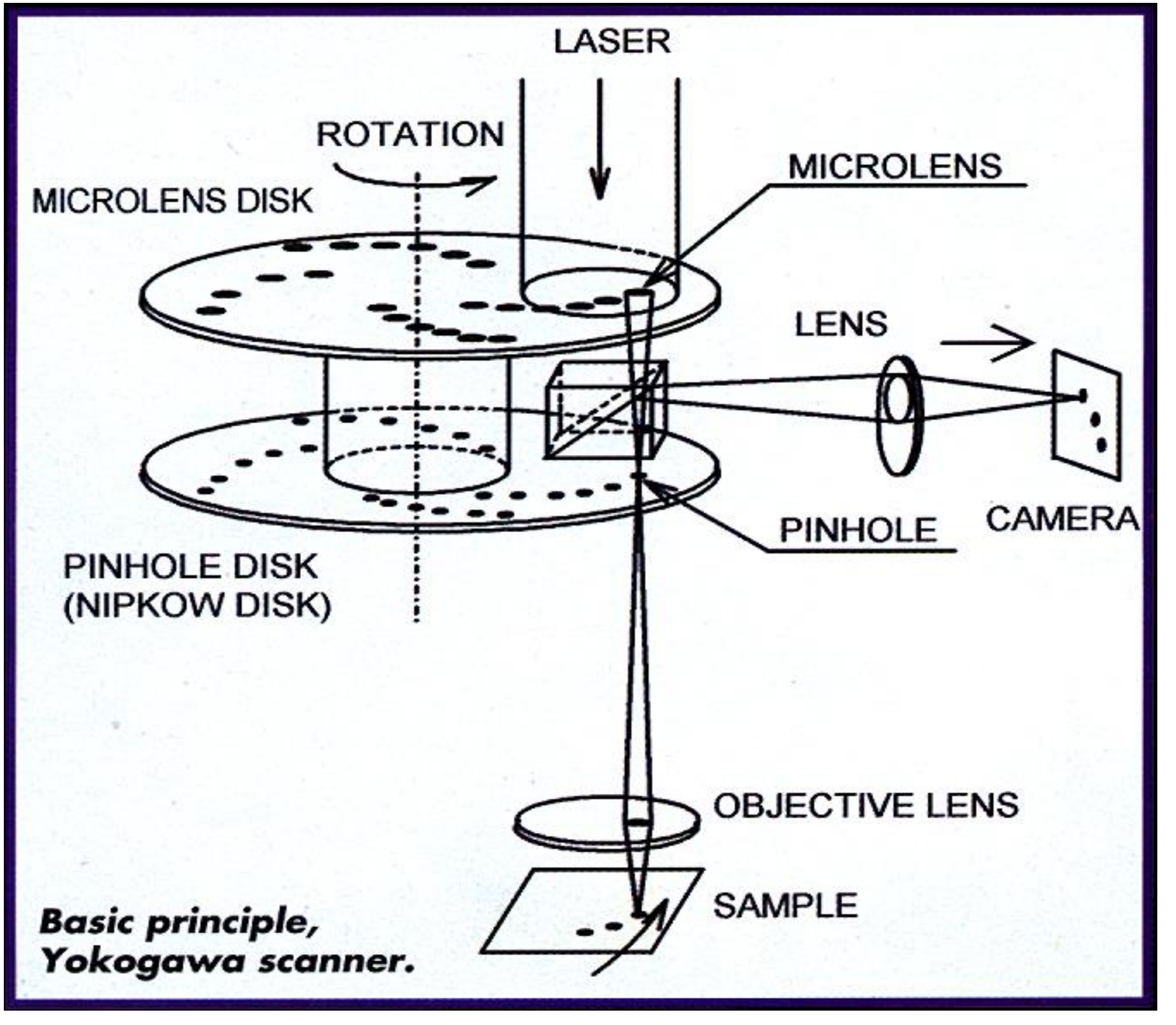
1. The NA, which determines the size of the focused spot. The objective lens magnification only changes the pixel size.
2. Size of the confocal aperture.



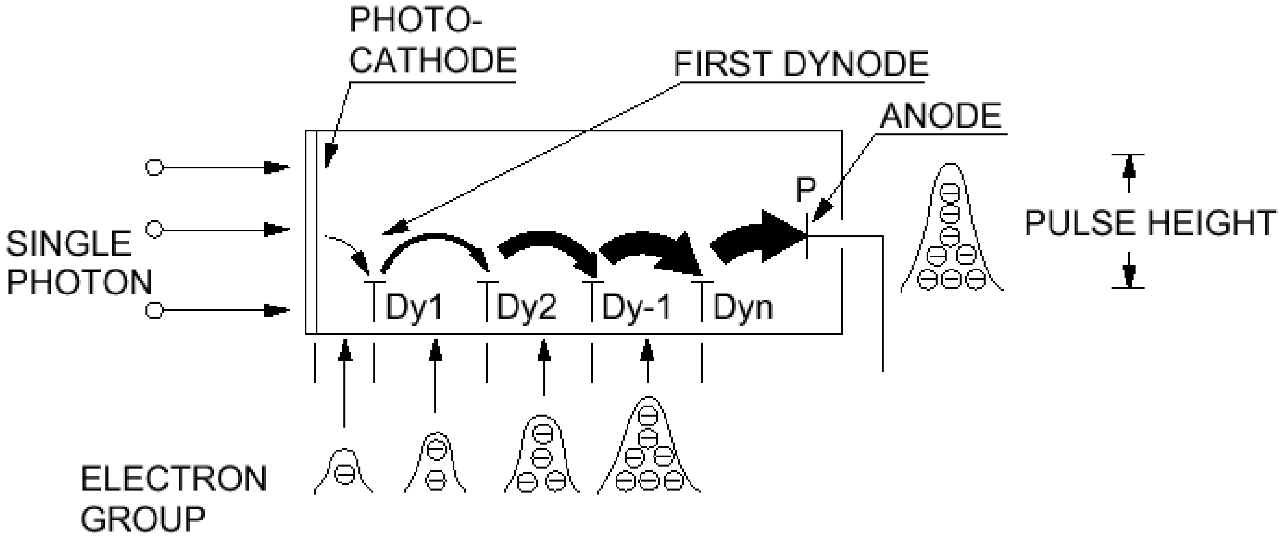
For faster scanning:

1. Resonant galvometers – run (vibrate) at one speed but it is fast. Can achieve “video rates”- 33 frames/seconds or higher. Disadvantage – can’t change the scanning speed.
2. Line scanner – scans a line on illumination across the sample. Can run at video rates. Disadvantage – not “laterally confocal”, requires a CCD camera to acquire image.
3. Nipkow disk scanners –These can image a 30 or more frames/second.

Nipkow disk


LASER
ROTATION
MICROLENS
MICROLENS DISK
LENS
CAMERA
PINHOLE
PINHOLE DISK
(NIPKOW DISK)
OBJECTIVE LENS
SAMPLE
Basic principle,
Yokogawa scanner.

Photomultiplier Tubes (PMTs) – “point detectors”



When photons strike the photocathode, photo-electrons are ejected and cascade down through the dynode chain, building in numbers. **One photon at the photo cathode results in >10^6 electrons at the anode.**

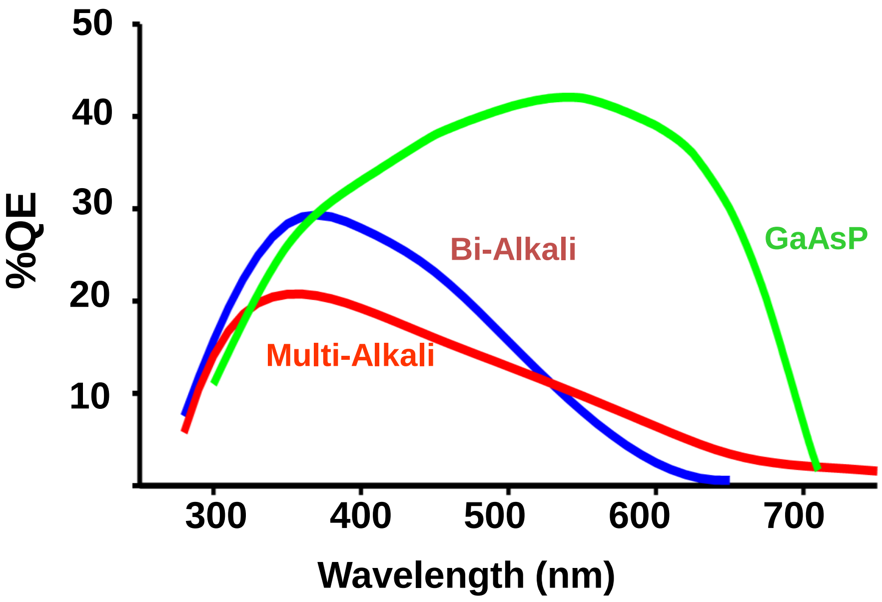
Quantum efficiency (QE) – the probability that a photon generates a photoelectron at the photocathode.

Gain - varies with PMT type, design and size of the last resistor on the voltage divider chain.

New Photocathode Types with High Quantum Efficiencies

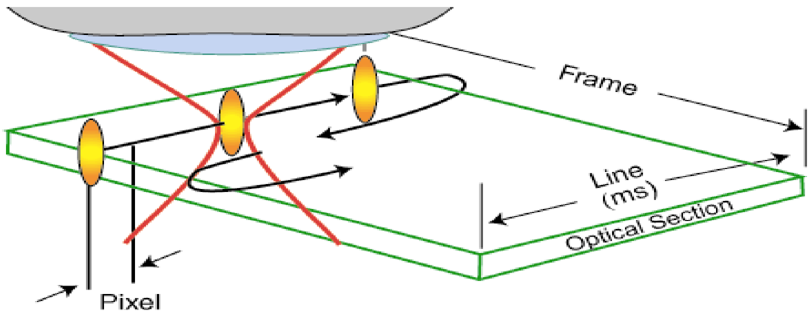
QE = probability that a photon will result in photoelectrons.

Conventional photocathode materials can reach ~30% (bialkali) and some newer photocathodes have QE's approaching 50%.



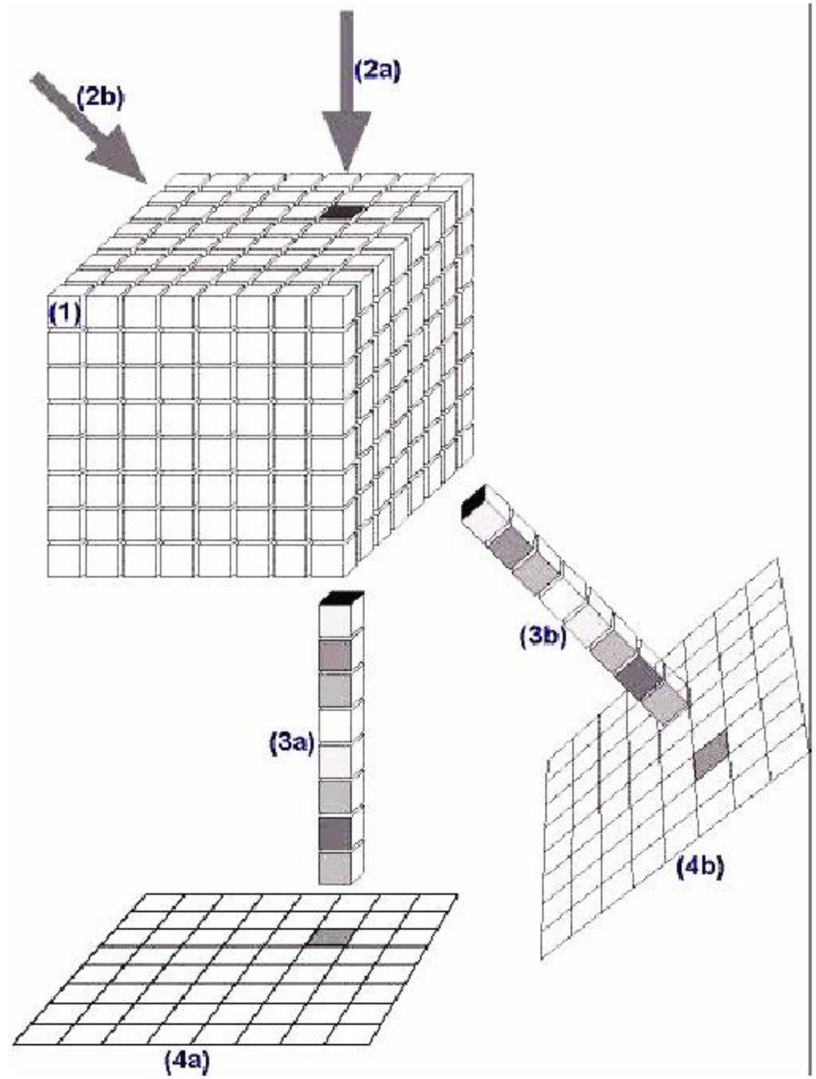
Data acquisition and 2D image formation in laser scanning microscopy

1. PMT signal is either analog integrated, digitally integrated or photons are counted for a “pixel dwell time”   This can range from ~500 ns to several μs. A line of pixels can range from 1 to 1024 pixels. The signal is typically digitized at 8 or 12 bits (256 or 16384 levels).
2. A number of lines (1 to 1024) is collected and sent to the computer RAM as well as displayed on the monitor.
3. Multiple channels (colors) can be simultaneously collected.
4. Scanning can now be carried in bi-directional mode for faster point scanning (> 2400 lines/s)



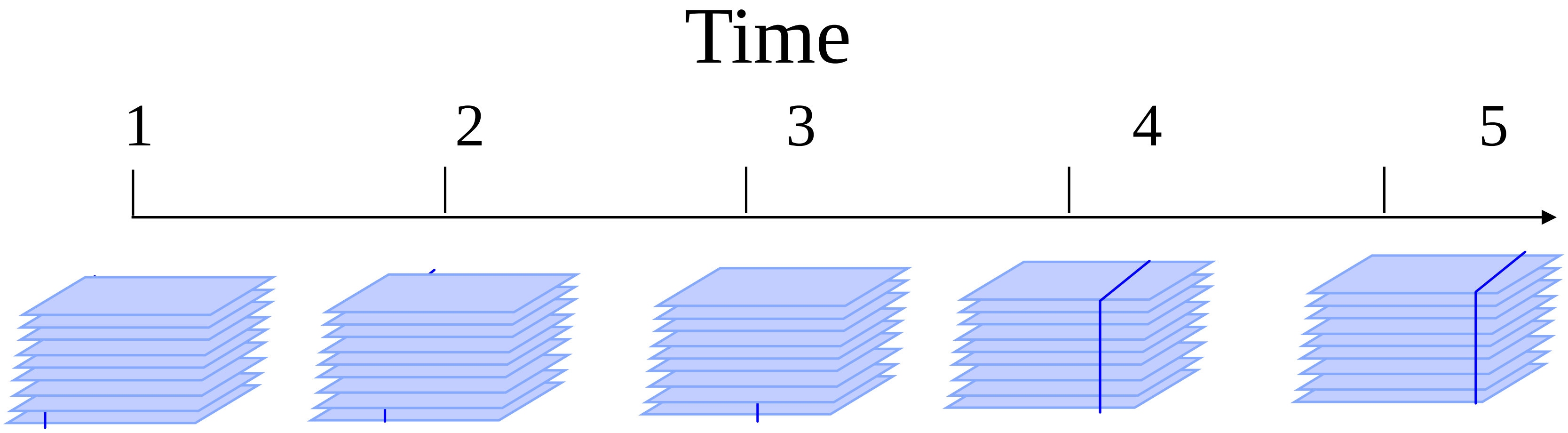
3D Images

- Z-series – acquired by stepping the focus up or down through the specimen.
- Voxels = 3D Pixels created via software



4D Imaging

- Time vs 3D sections
- Used when evaluating changes in tissue or cells
- Sometimes requires fast 3D sectioning
- Lots of data – can be difficult to evaluate



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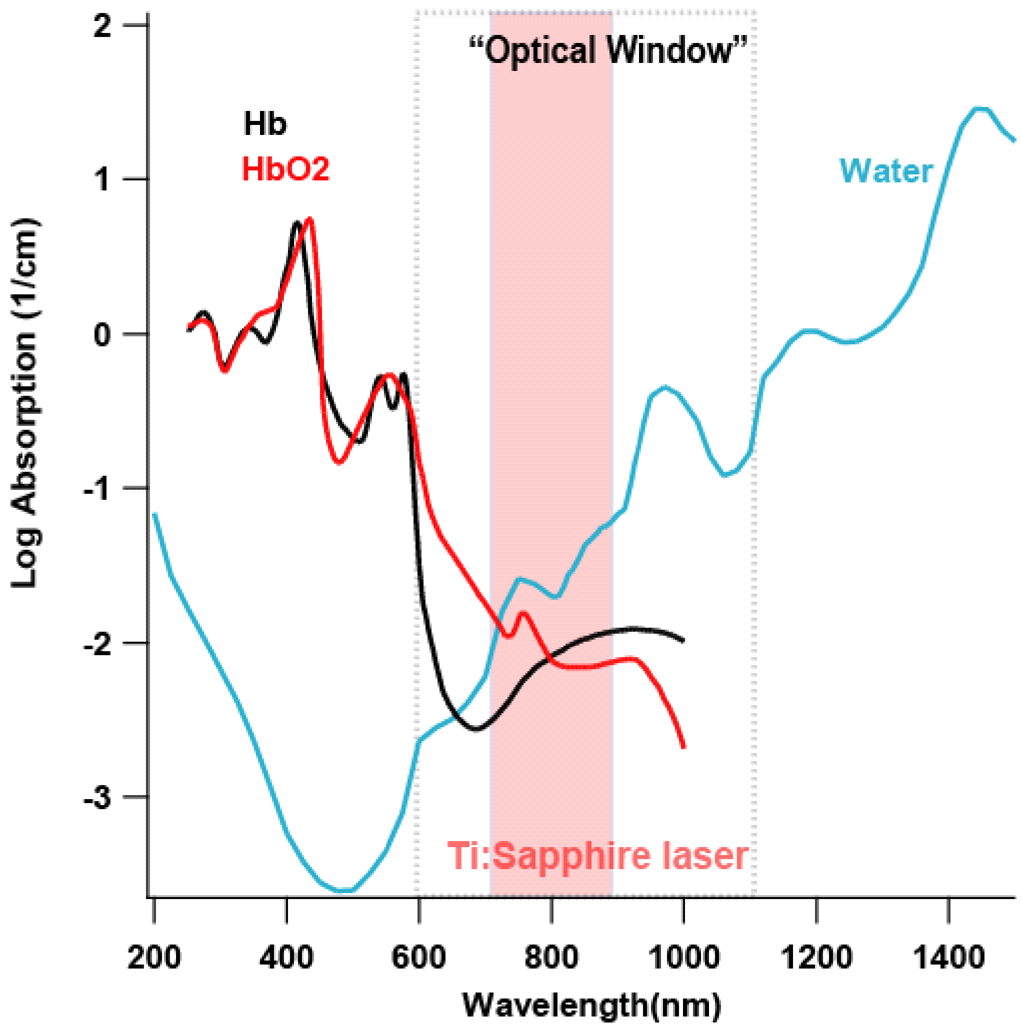
Multiphoton microscopy: tissue imaging



- Less absorption by blood and water at 600~1100 nm

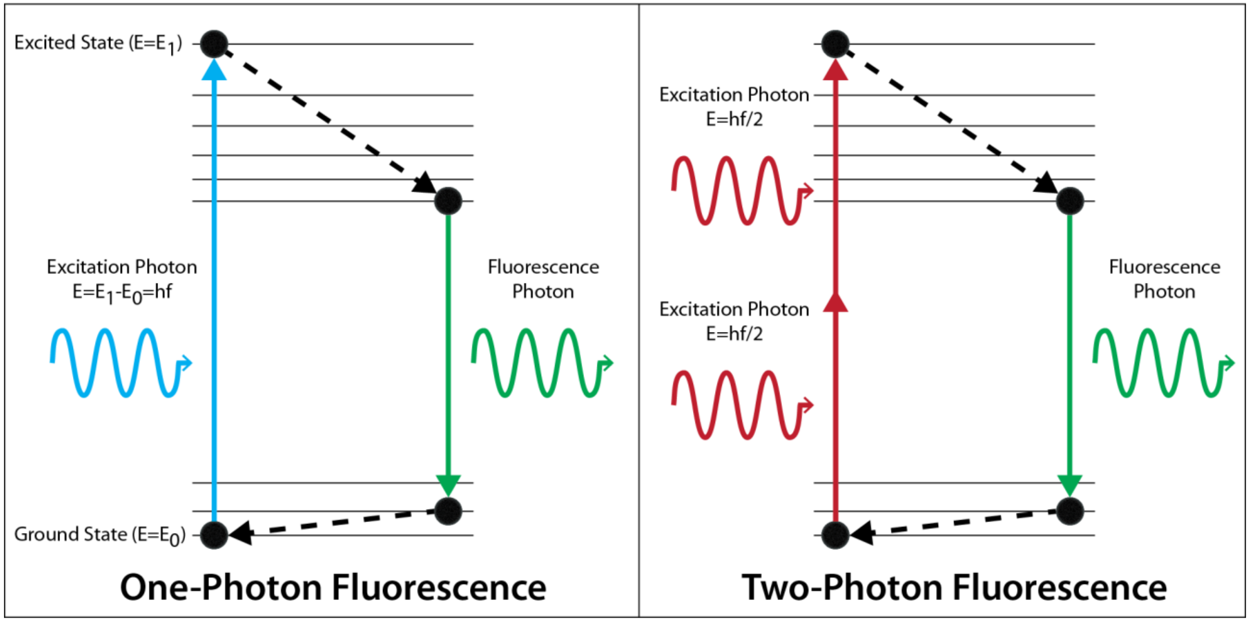
  ⇨ Near IR multiphoton excitation is highly advantageous.

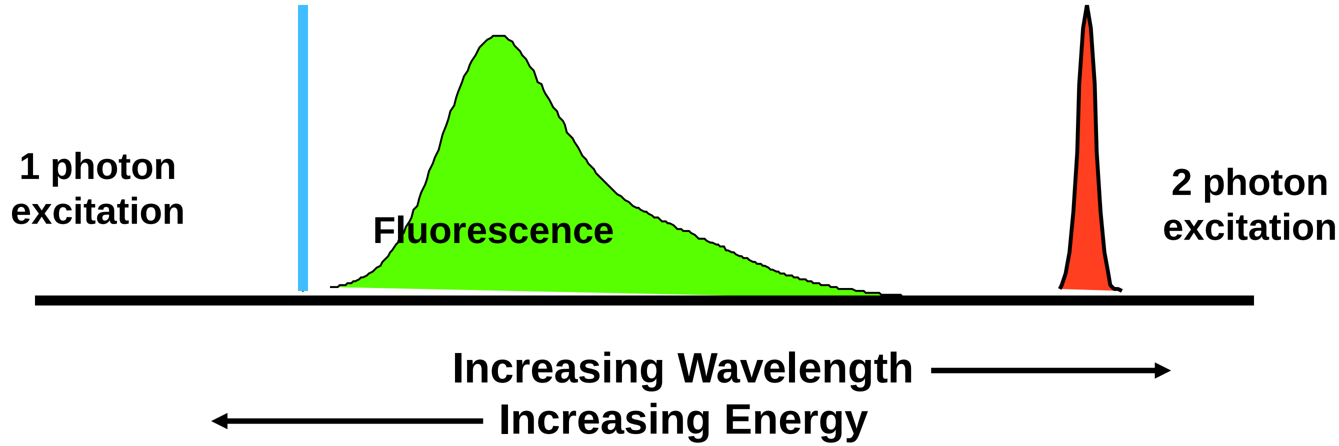
- Less scattering at longer wavelength

  (Rayleigh scattering ~ $1/\lambda^4$)

Two-photon excited fluorescence

Multiphoton excitation, based on the simultaneous absorption of photons, was predicted in 1931 by Maria Göppert-Meyer in her PhD thesis.





Two-photon excitation: Uncertainty principle

Then

$$\Delta E \, \Delta t \geq \frac{\hbar}{2}, \qquad [3.151]$$

At time *t=0*, we turn on light of frequency ***w*** on an ensemble of hydrogen atoms all in their ground state. It will however be seen that initially the atoms make transitions to several levels not obeying this constraint. As t increase, deviation ΔE from the expected final-state energy will decrease according to

$$\Delta E \sim \frac{\hbar}{t}$$

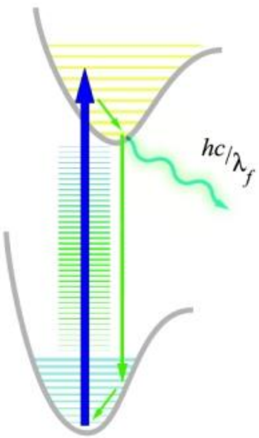
Shankar – Principles of Quantum Mechanics

Approximately $\tau = \frac{\hbar}{2E_v}$ and $\hbar = 6.6 * 10^{-15} eV * sec$

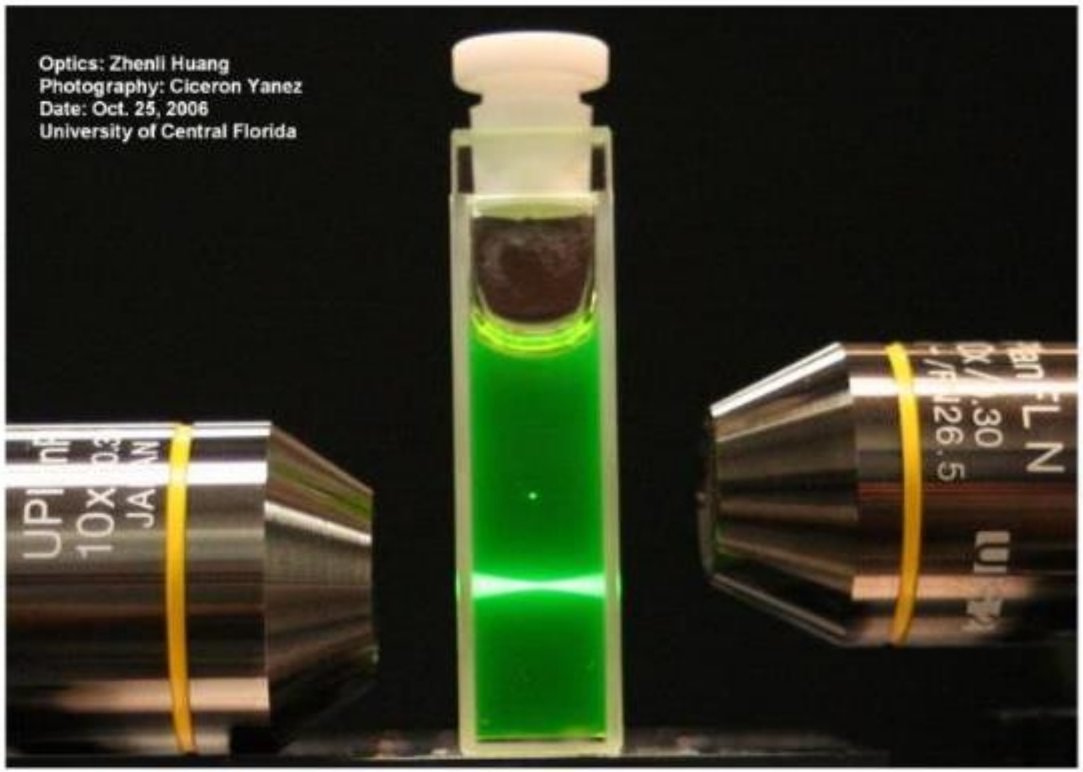
For $E_V = \frac{c}{900nm} * h$, $\tau = 2.4 * 10^{-16} sec$

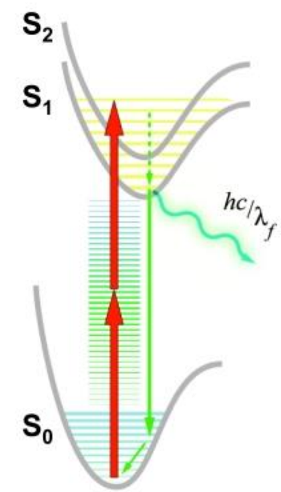
Single photon event promote a molecule to virtual state. The Uncertainty Principle allows this state for τ. If another photon strikes within this time window and excite the molecule with two photon transition → " Two Photon Absorption"

Multiphoton excitation is spatially localized



Single photon excitation (488 nm)





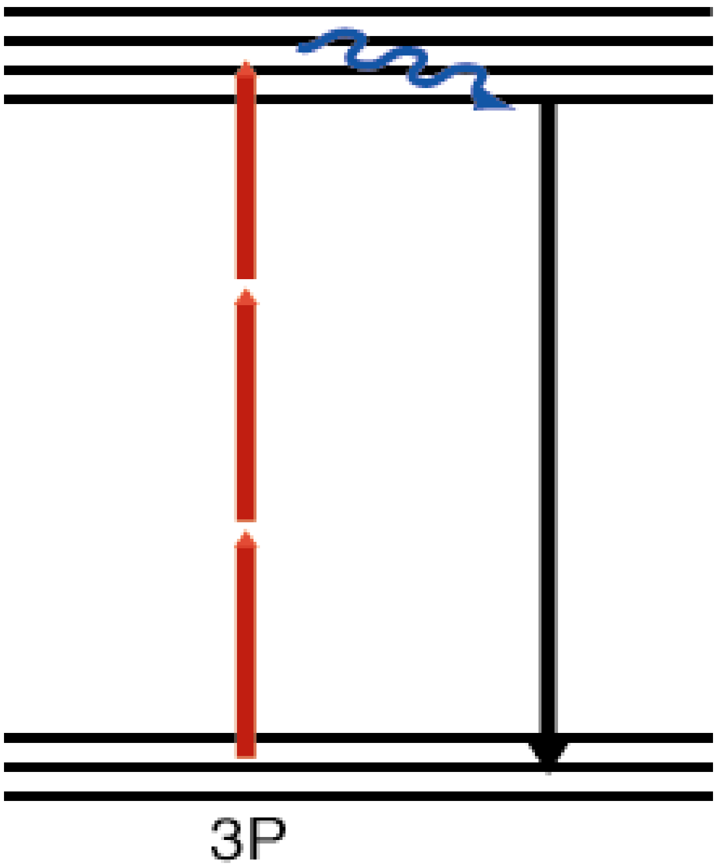
Two photon excitation (900 nm)

Denk W, Strickler JH, and Webb WW, *Science* **248**, 73 (1990)

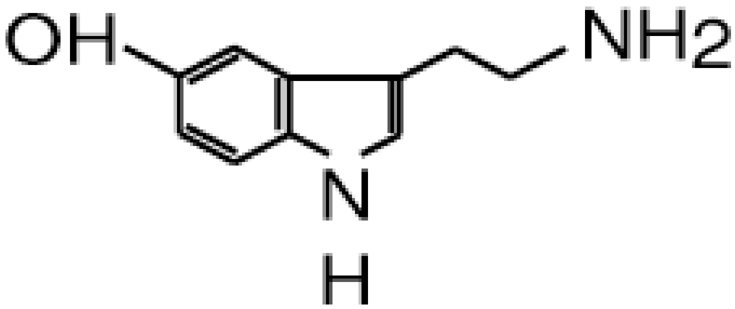
Image from http://belfield.cos.ucf.edu

Three-photon excitation

Three lower energy photons simultaneously interact with a molecule to create an excitation equal to the sum of their energies



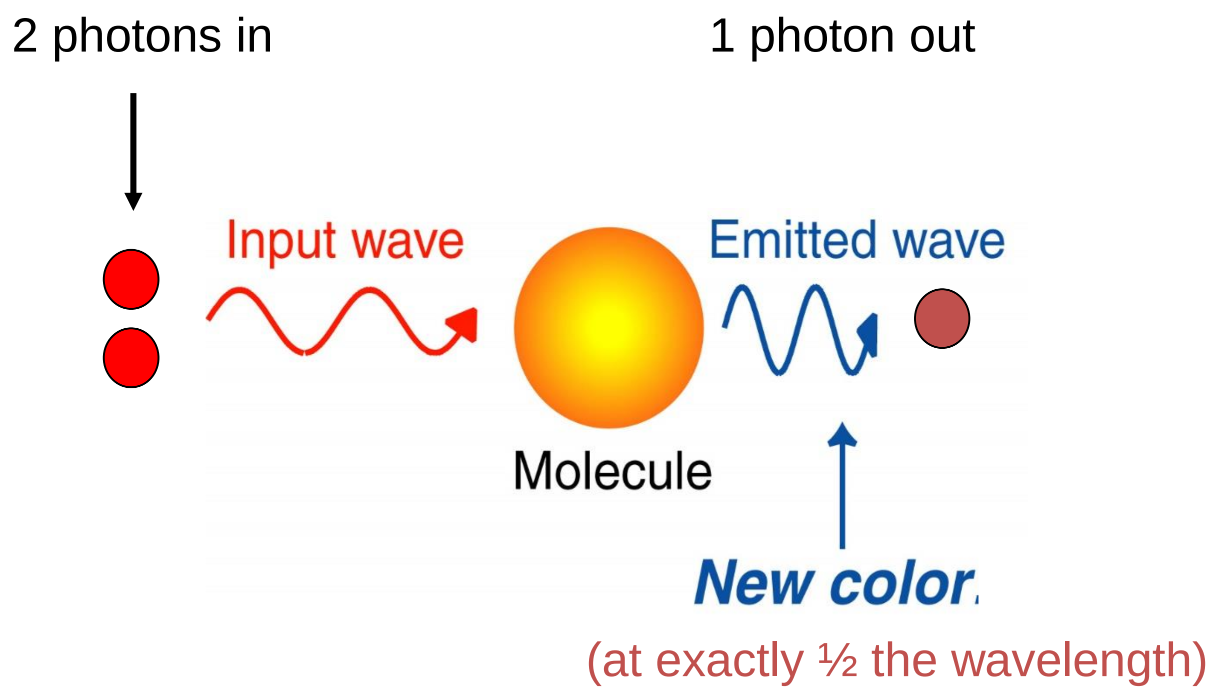
e.g. 5-HT



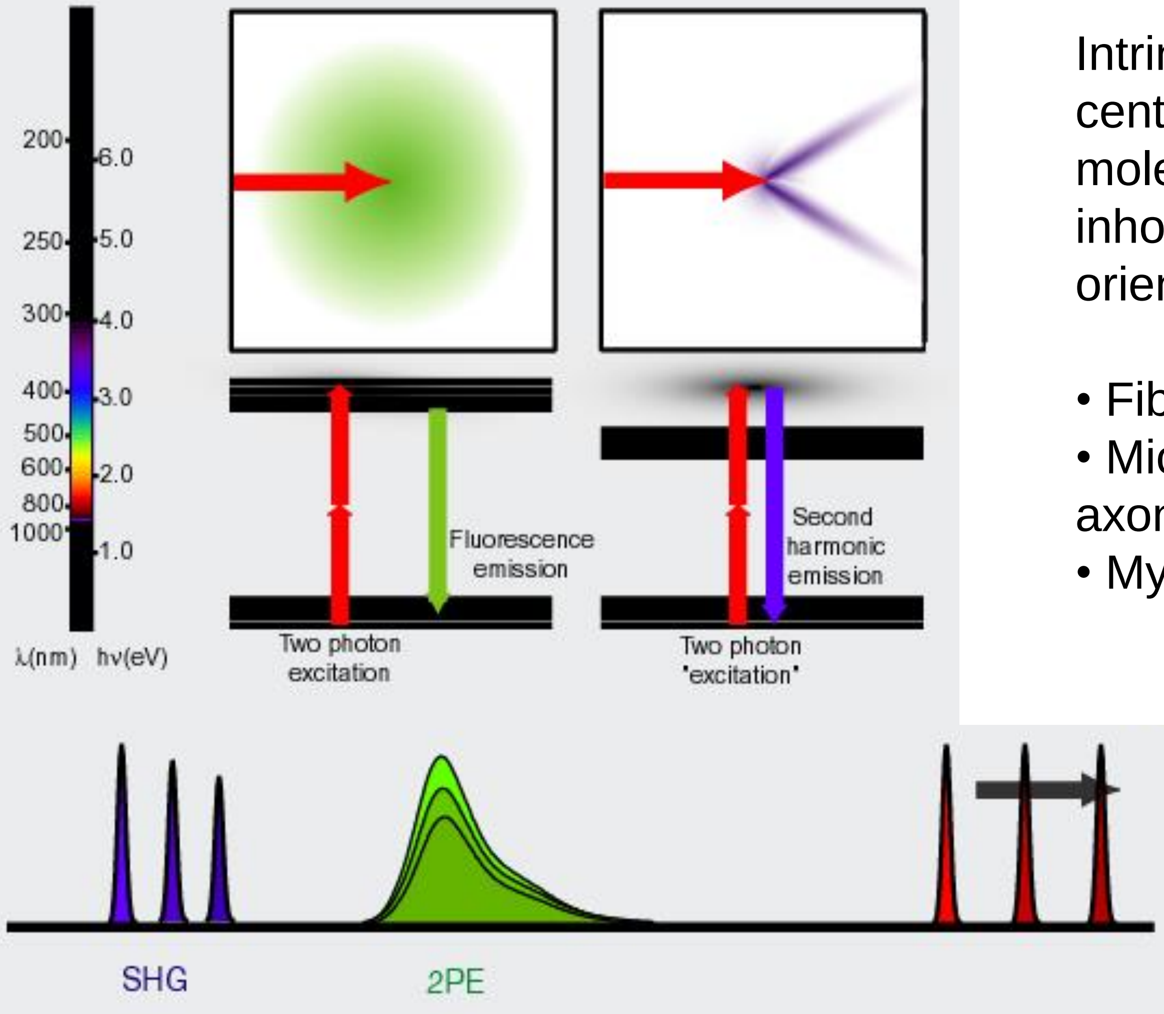
750 nm excites a
250 nm transition

Second harmonic generation (SHG) is the coherent form of nonlinear scattering --- 2 photons of incoming light are directionally scattered as a single photon at a wavelength exactly ½ the fundamental wavelength.

e.g. 800 nm is converted to 400 nm light



2P fluorescence vs 2P harmonic generation (SHG)

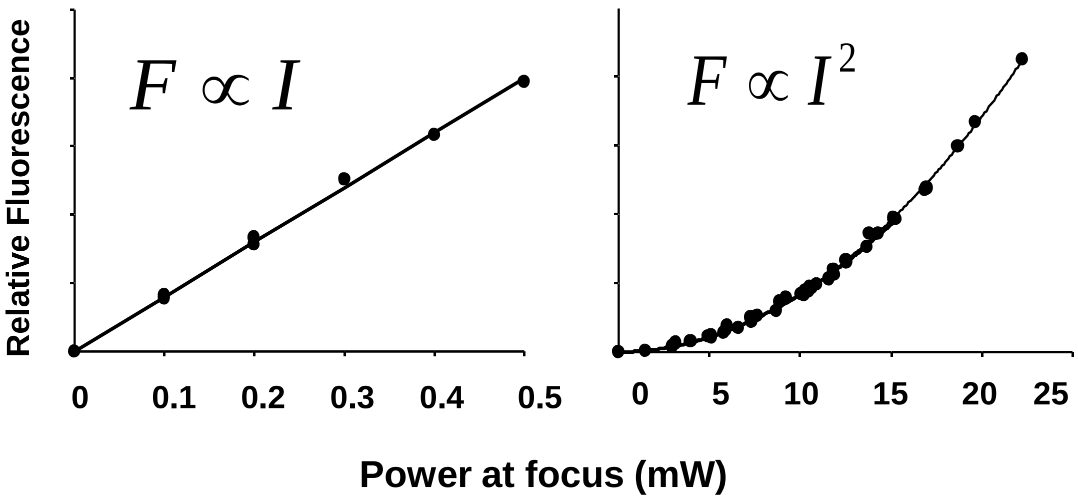


Intrinsic signal from non-centrosymmetric molecules and inhomogeneously oriented tissues

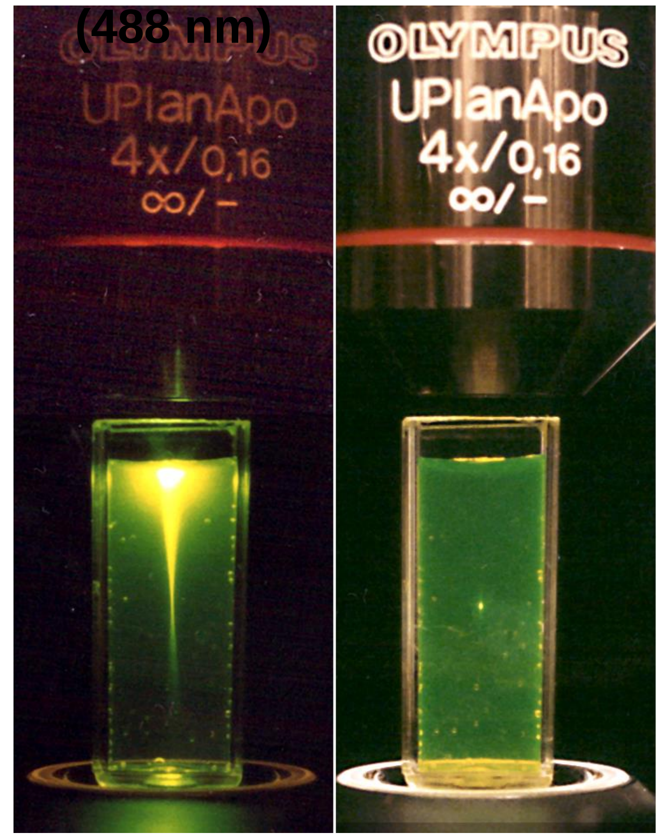
- Fibrillous collagen
- Microtubules in nerve axon bundle
- Myosin

Two photon excitation is spatially localized

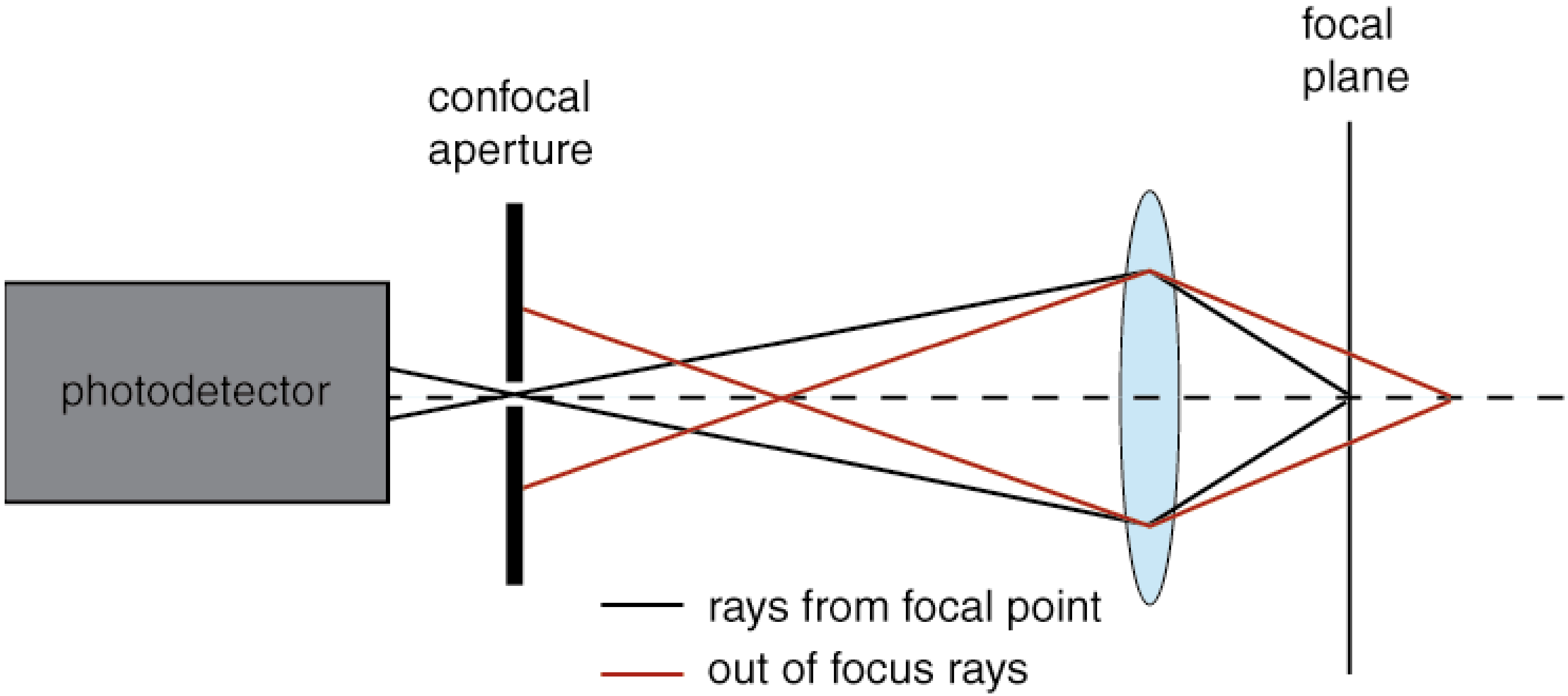
Because two photons arriving at the same time are required for excitation, the fluorescence depends on the square of the intensity, rather then being linearly proportional.

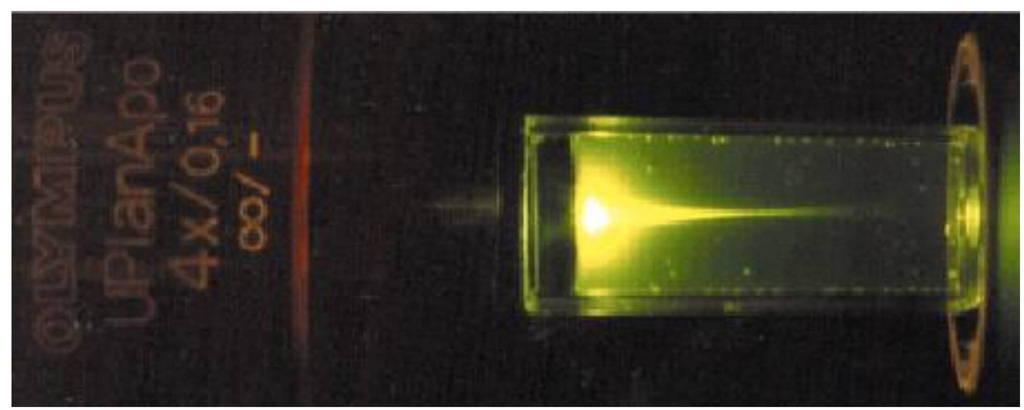


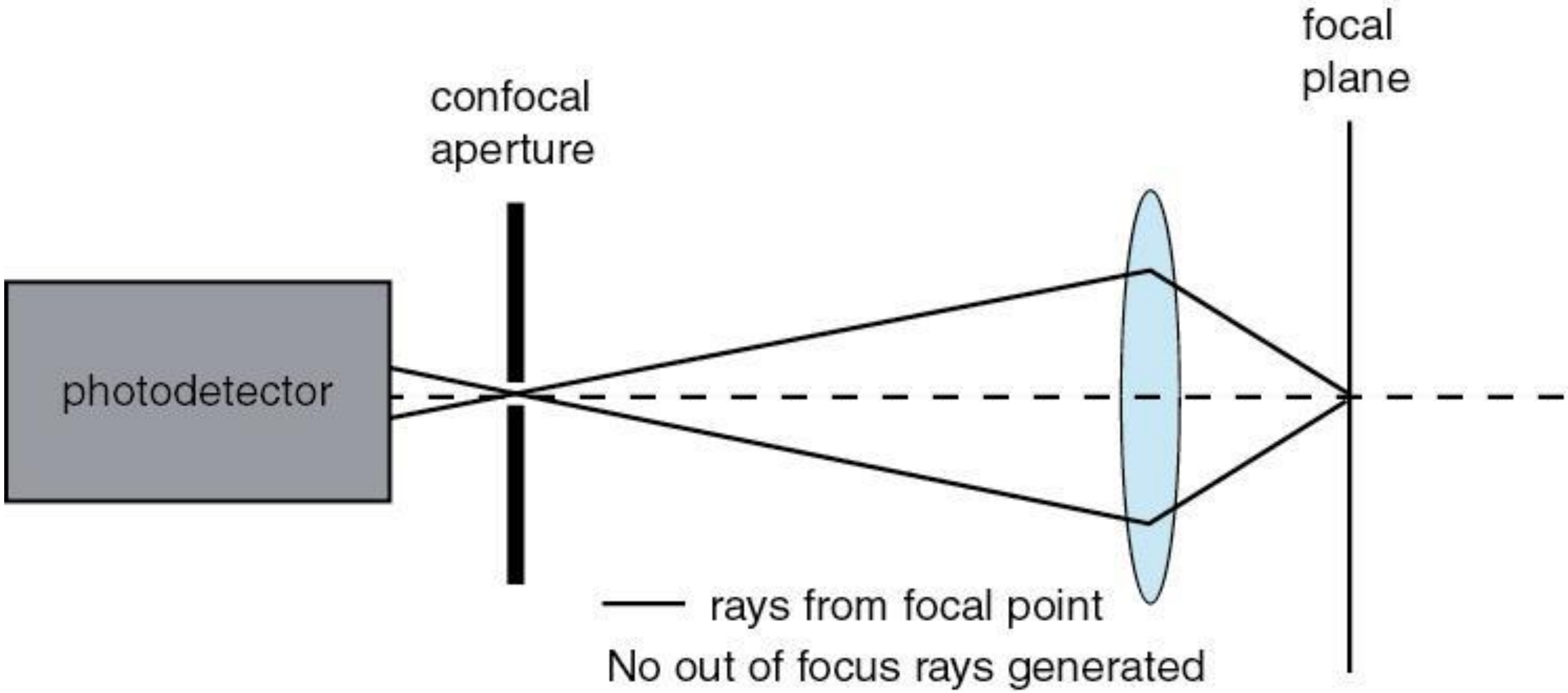
Single photon excitation (488 nm) | **Two photon excitation (900 nm)**

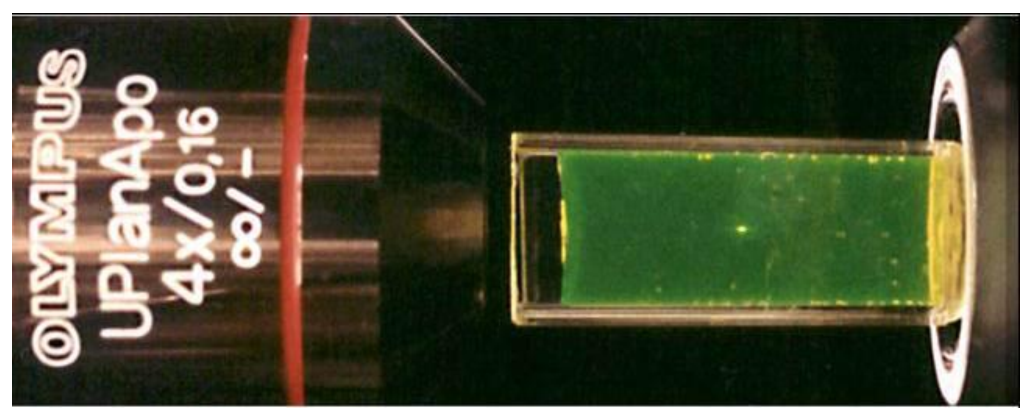


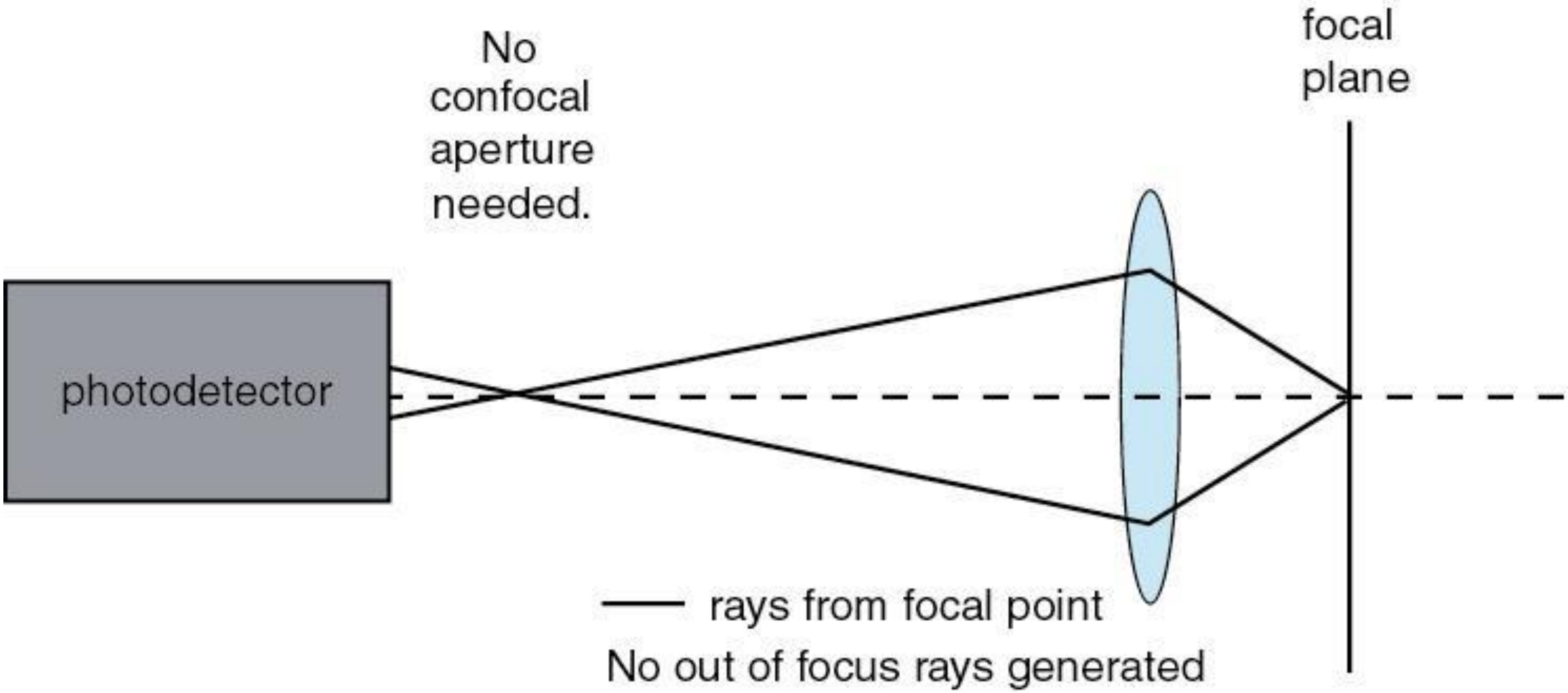
Excitation is only appreciable at the focal point (at “normal” imaging intensities – the mW range for most molecules).

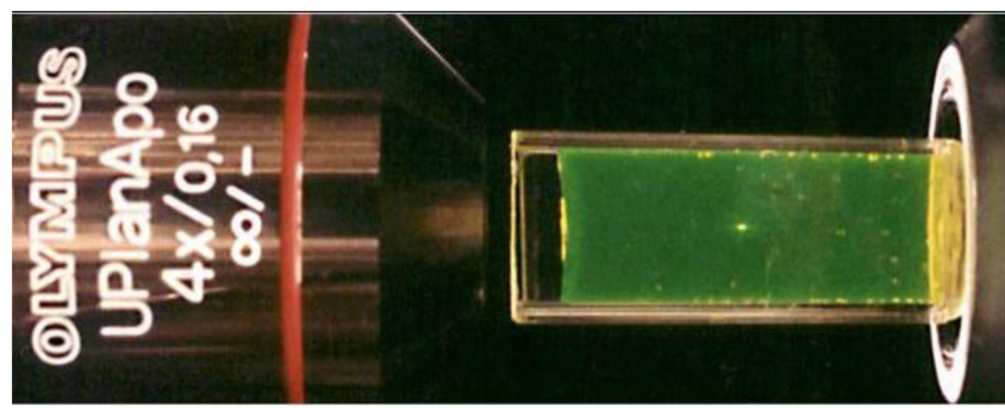

focal
plane
confocal
aperture
photodetector
rays from focal point
out of focus rays


OLYMPUS
UPlanApo
4X/0,16
∞/–

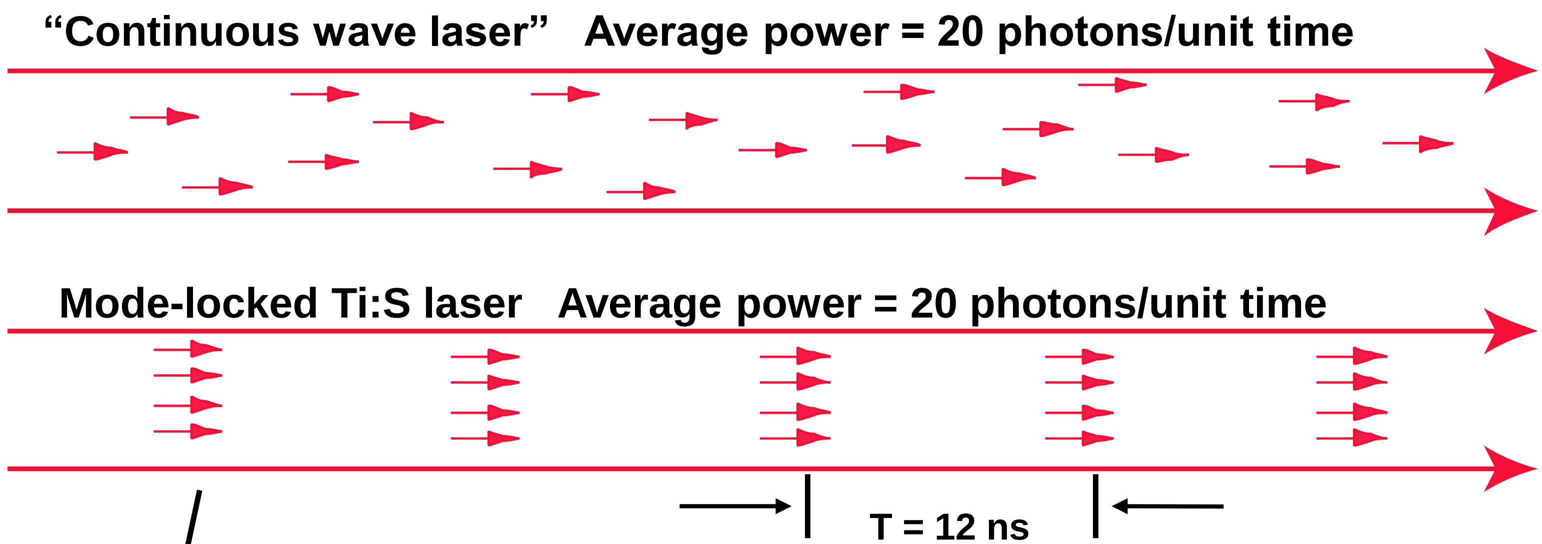

focal
plane
confocal
aperture
photodetector
rays from focal point
No out of focus rays generated


OLYMPUS
UPlanApo
4X/0,16
∞/–


No confocal aperture needed.
focal plane
photodetector
rays from focal point
No out of focus rays generated


OLYMPUS
UPlanApo
4X/0,16
∞/–

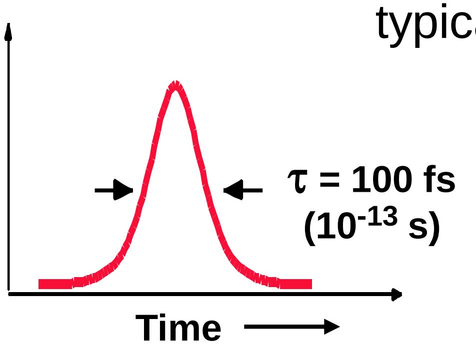
Mode-locked (pulsed) lasers make nonlinear excitation practical by greatly increasing the chance that two photons interact with the molecule simultaneously



R = laser “rep” rate

typically 80 MHz

$$F \propto \frac{\phi_F \sigma_{2P} \langle I \rangle^2}{R\tau} C$$

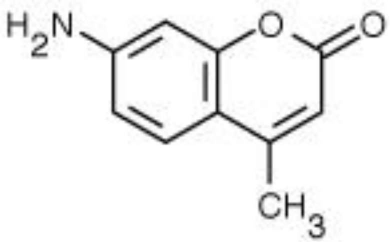


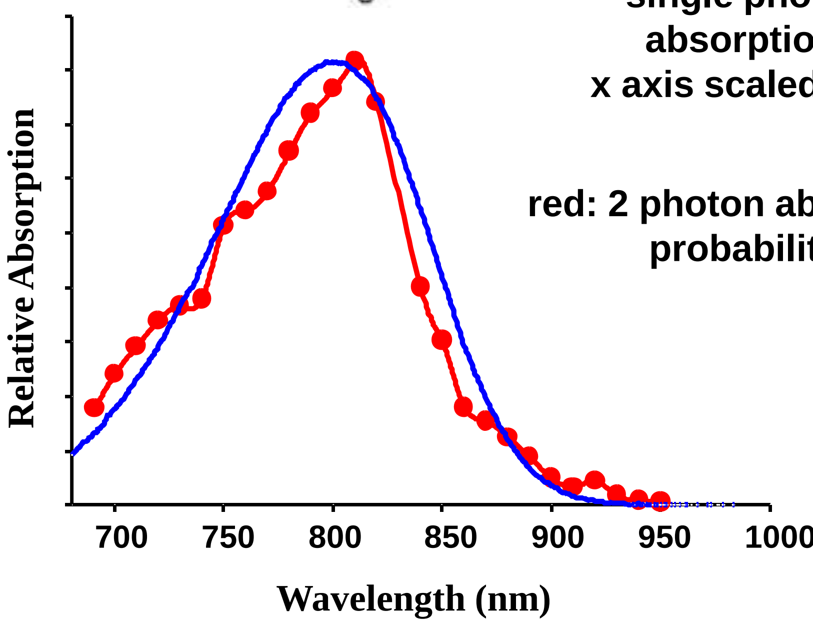
The probability of a two photon absorption is increased by 10^5 **(1/Rτ)** for the average power.

Two photon "action cross sections" are the product of the fluorescence quantum yield and the absolute two photon cross-section.

$$F \propto \frac{(\phi_F \sigma_{2P}) \langle I \rangle^2}{R\tau} C$$

For asymmetric molecules, the spectra of the 1P absorption and 2P absorption can be very similar. Example: Coumarin

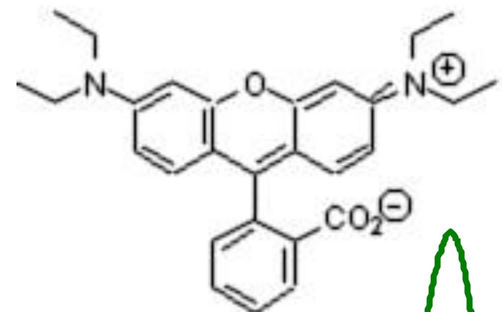


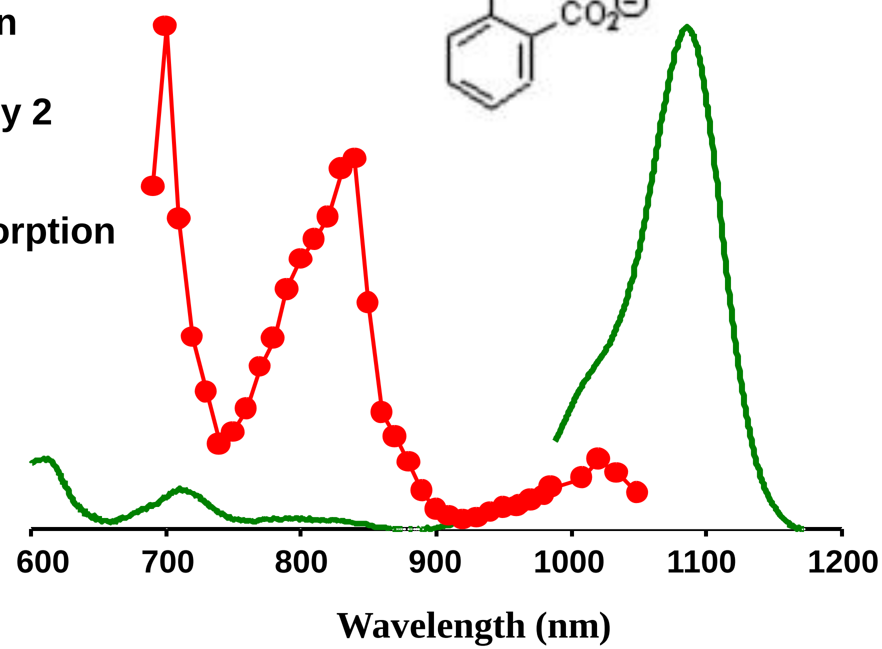


Blue and green : single photon absorption; x axis scaled by 2

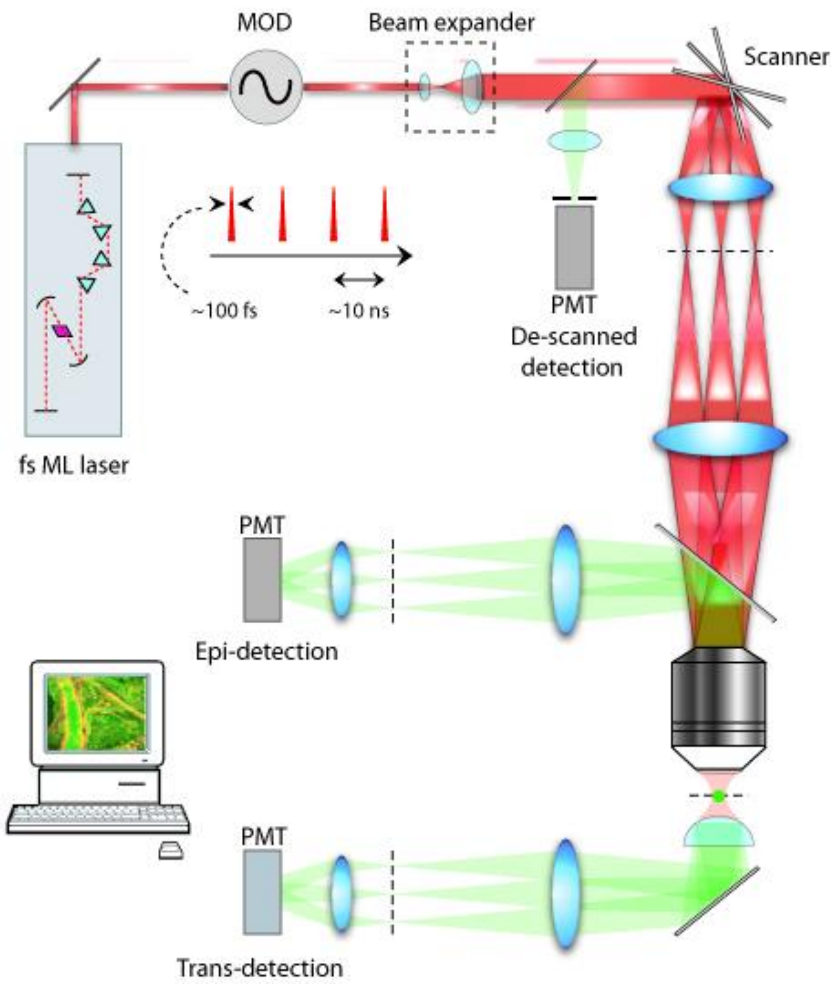
red: 2 photon absorption probability.

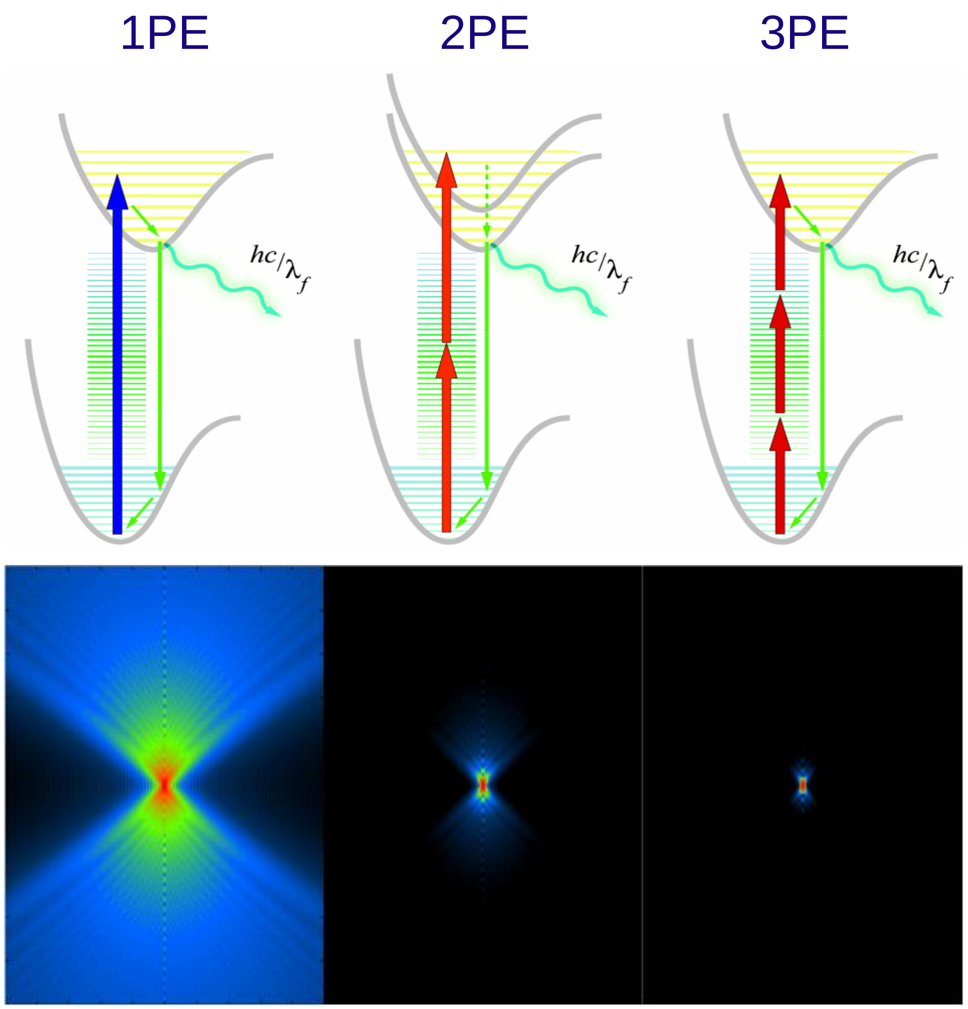
However, with more symmetric structures the spectra can be very different. Example: Rhodamine B.





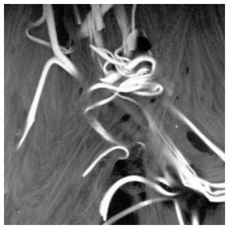
Multiphoton Excitation Laser Scanning Microscopy



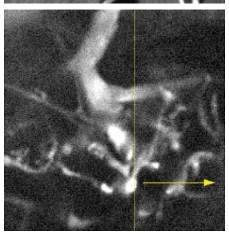


0.9 NA

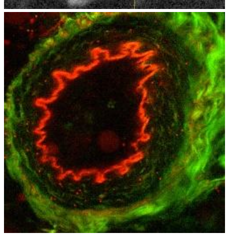
What are the advantages of multiphoton microscopy?



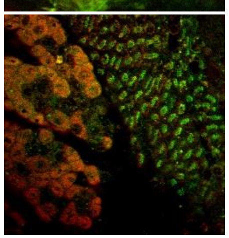
Deeper imaging into scattering specimens.
Deep = 100 to ~1000 microns depending on the tissue.
Typically 2-3 times that of confocal microcopy.



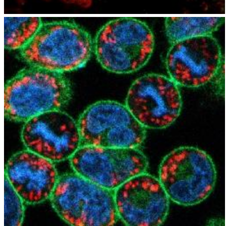
Reduced photobleaching and photodestruction in optically thick specimens.



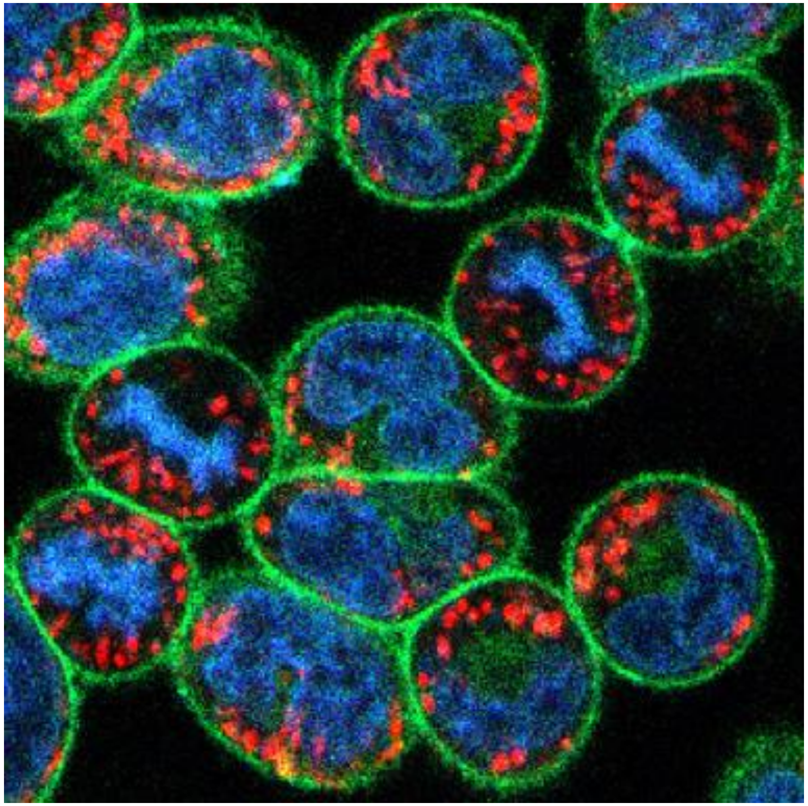
Access to nonlinear signals other than fluorescence such as second harmonic scattering.



Access to UV and deep UV molecular excitation regimes.

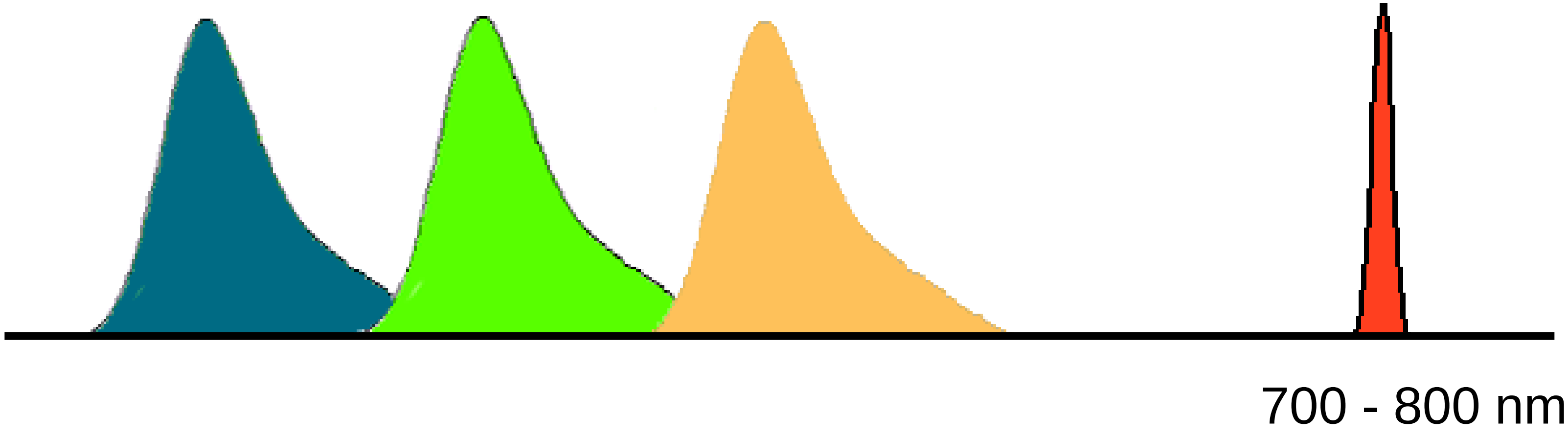


Simultaneous excitation of species that emit at widely diverging wavelengths.

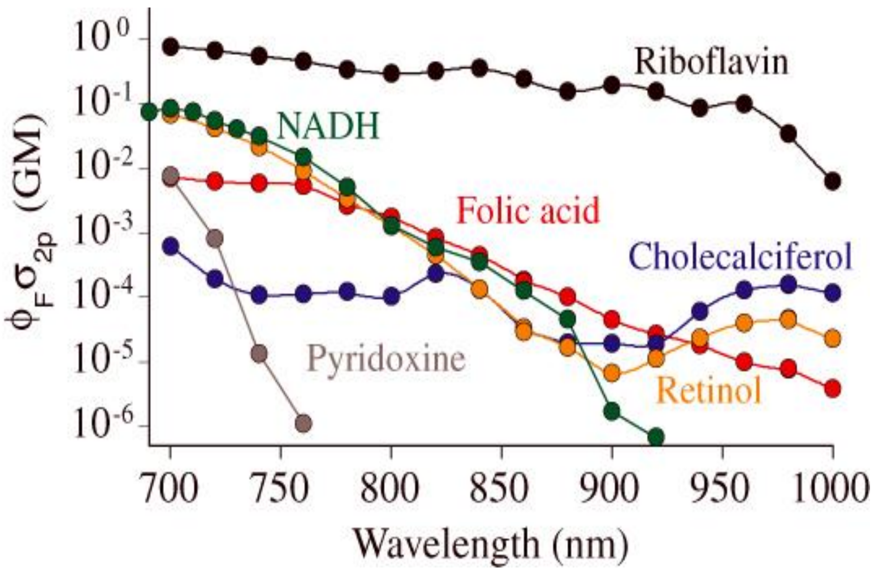


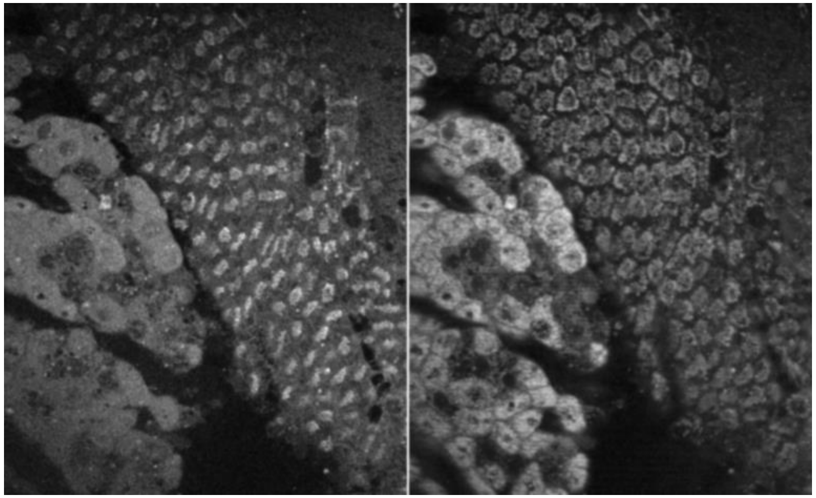
All fluorescence is emitted from the same plane and because the emissions are not “imaged” during collection, multiphoton microscopy does not suffer from chromatic aberrations.

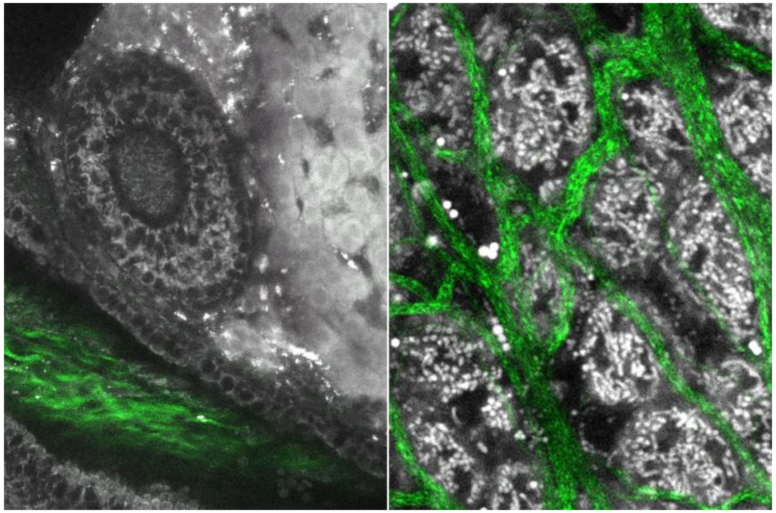
The long wavelength of excitation makes collection of separate emissions easier as well since there is no need to block laser lines in the visible.

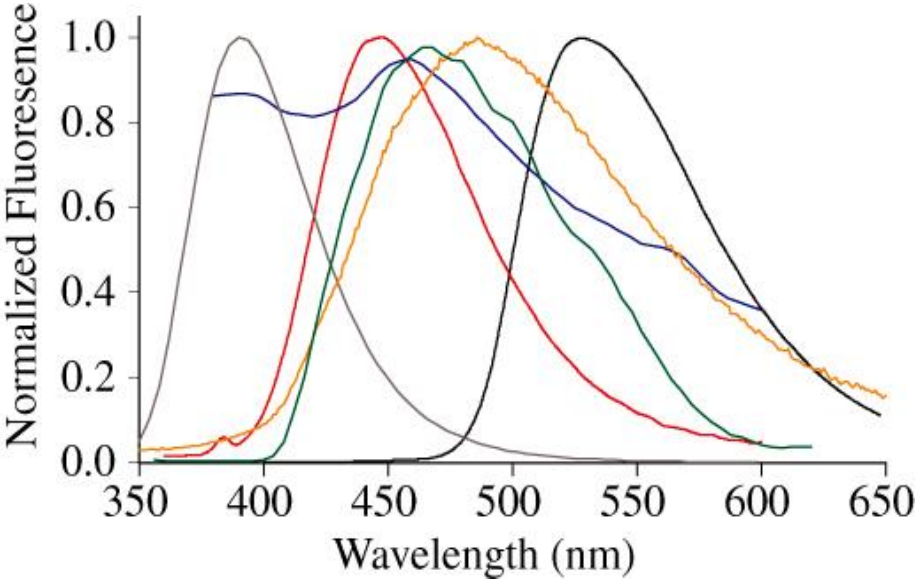


Two and three photon excited intrinsic fluorescence



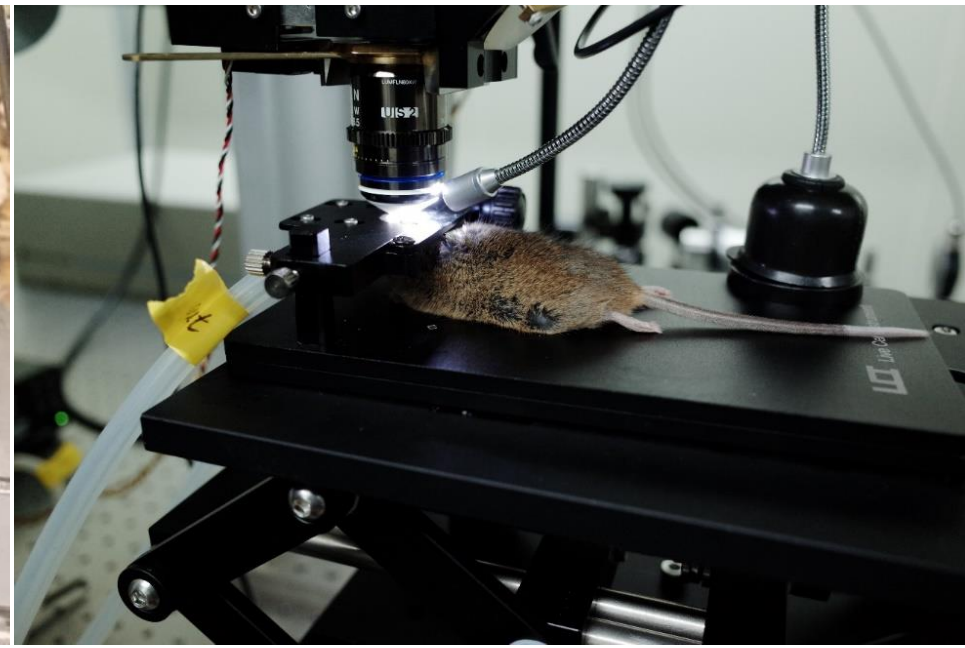






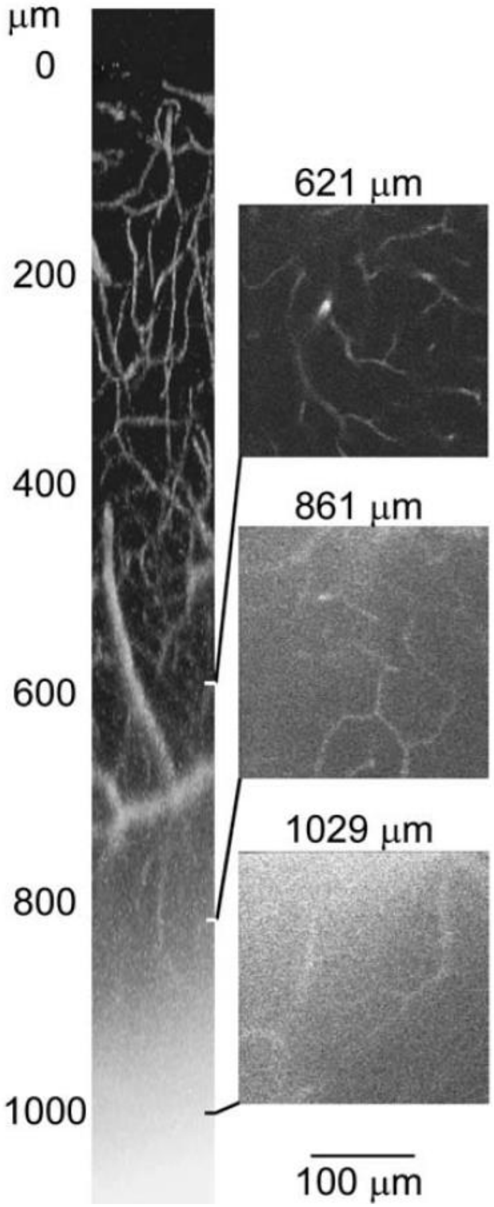
Two-photon microscopy in neuroscience

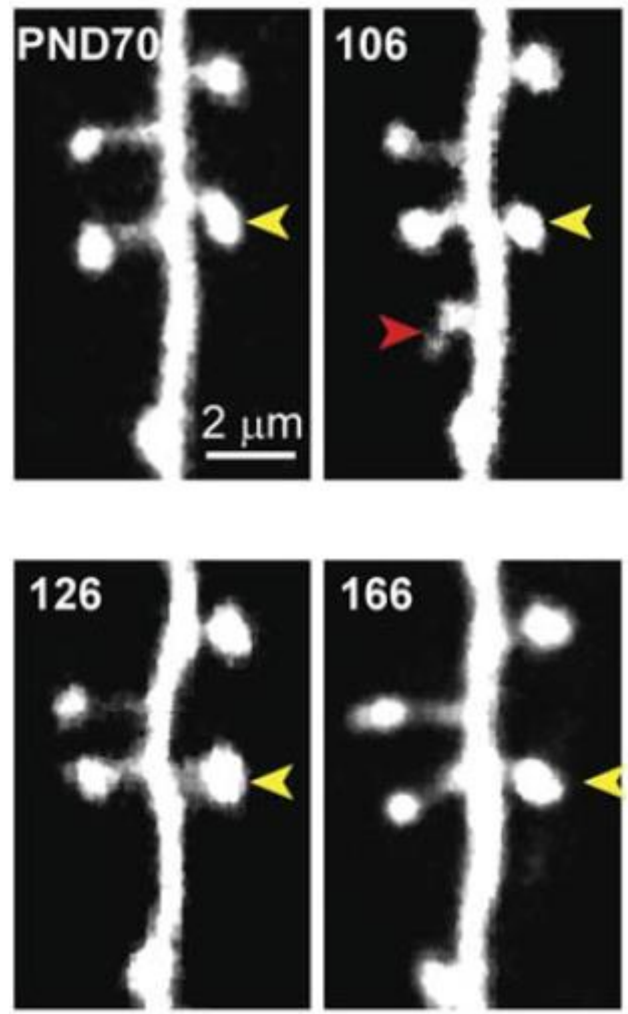
Cranial window



Two-photon imaging

Two-photon microscopy in neuroscience



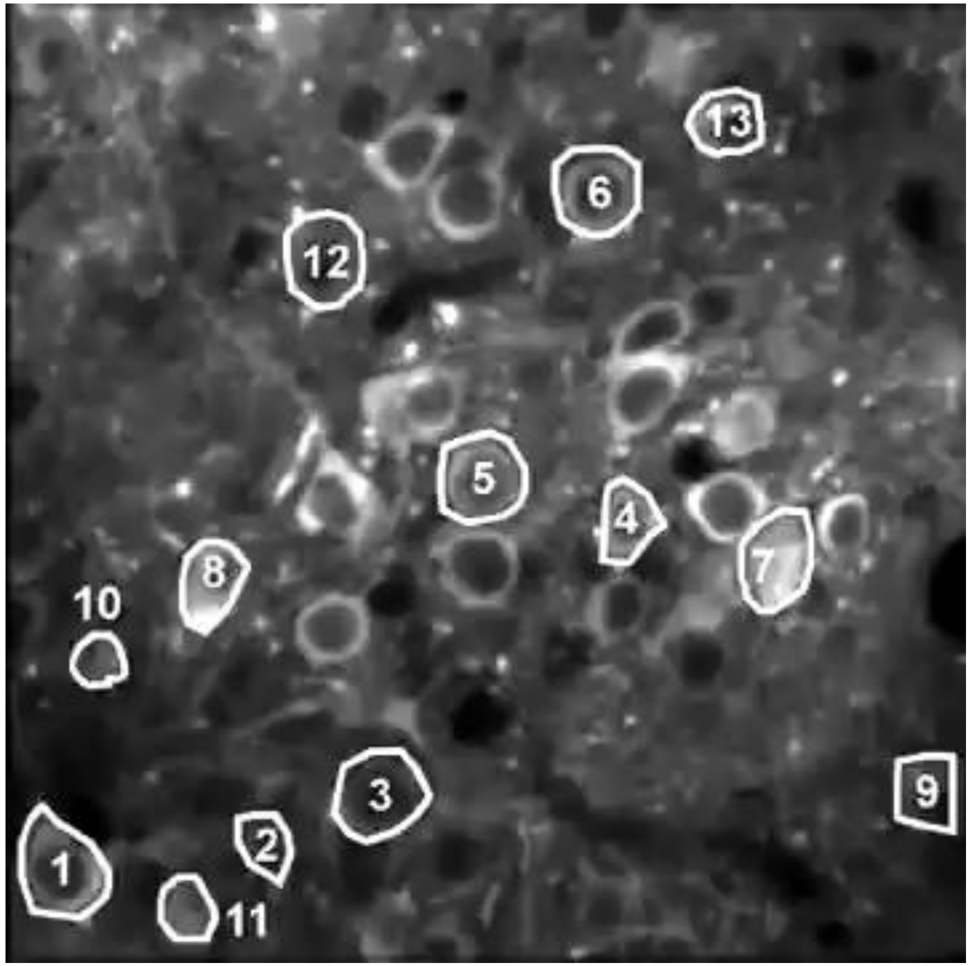


Svoboda and Yasuda, Neuron 50, 823 (2006)

Current techniques for live brain imaging

From David Tank's Lab at Princeton

Current techniques for live brain imaging



From Karel Svoboda's Lab at Janelia Research Campus of Howard Hughes Medical Institute

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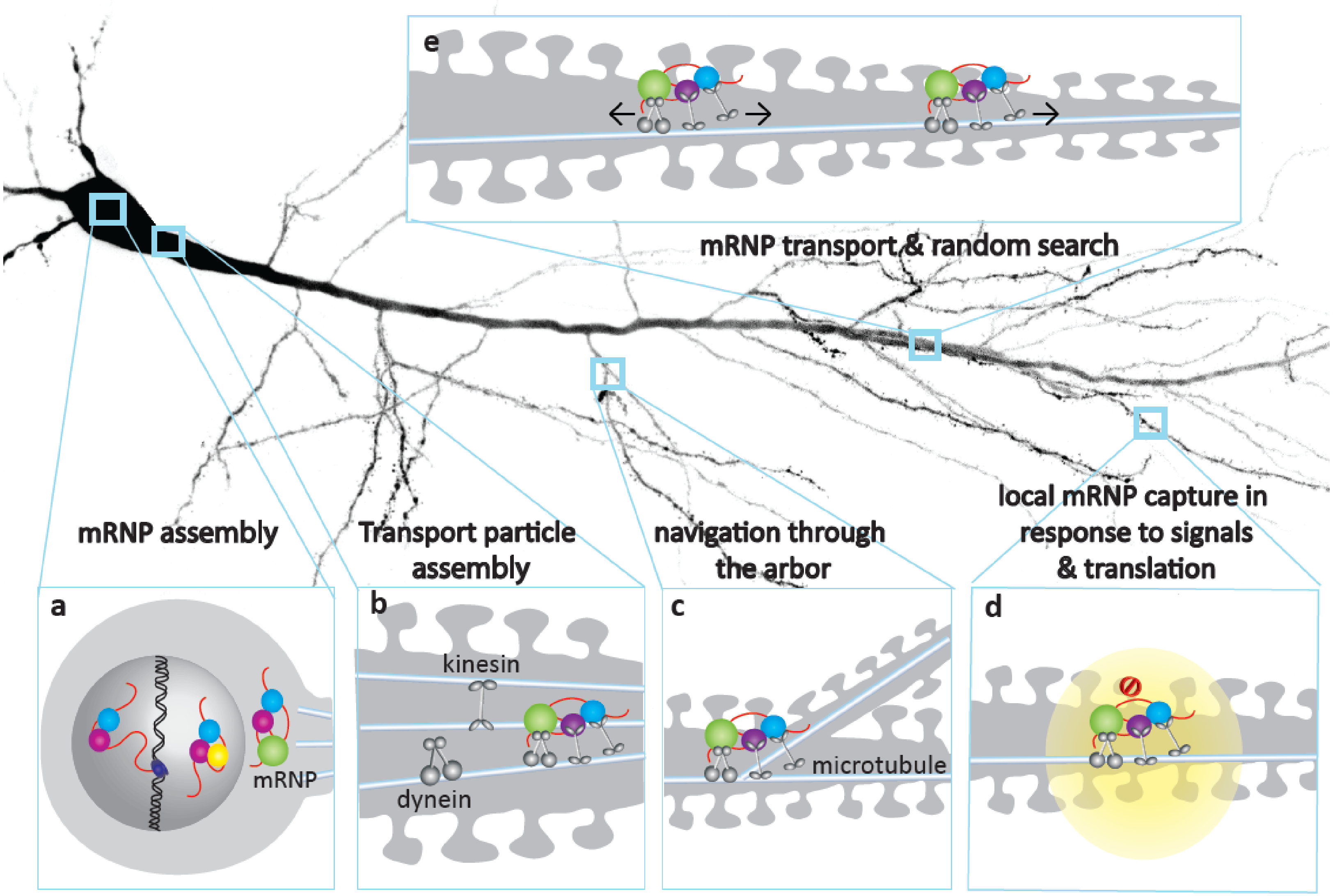
2. Multiphoton microscopy
3. Single molecule imaging of mRNA

Single Endogenous mRNA in Neurons *in vivo*



Buxbaum, Yoon, Singer, and Park, Trends in Cell Biology 25, 468 (2015)

Each Synapse is Composed of ~50,000 Proteins

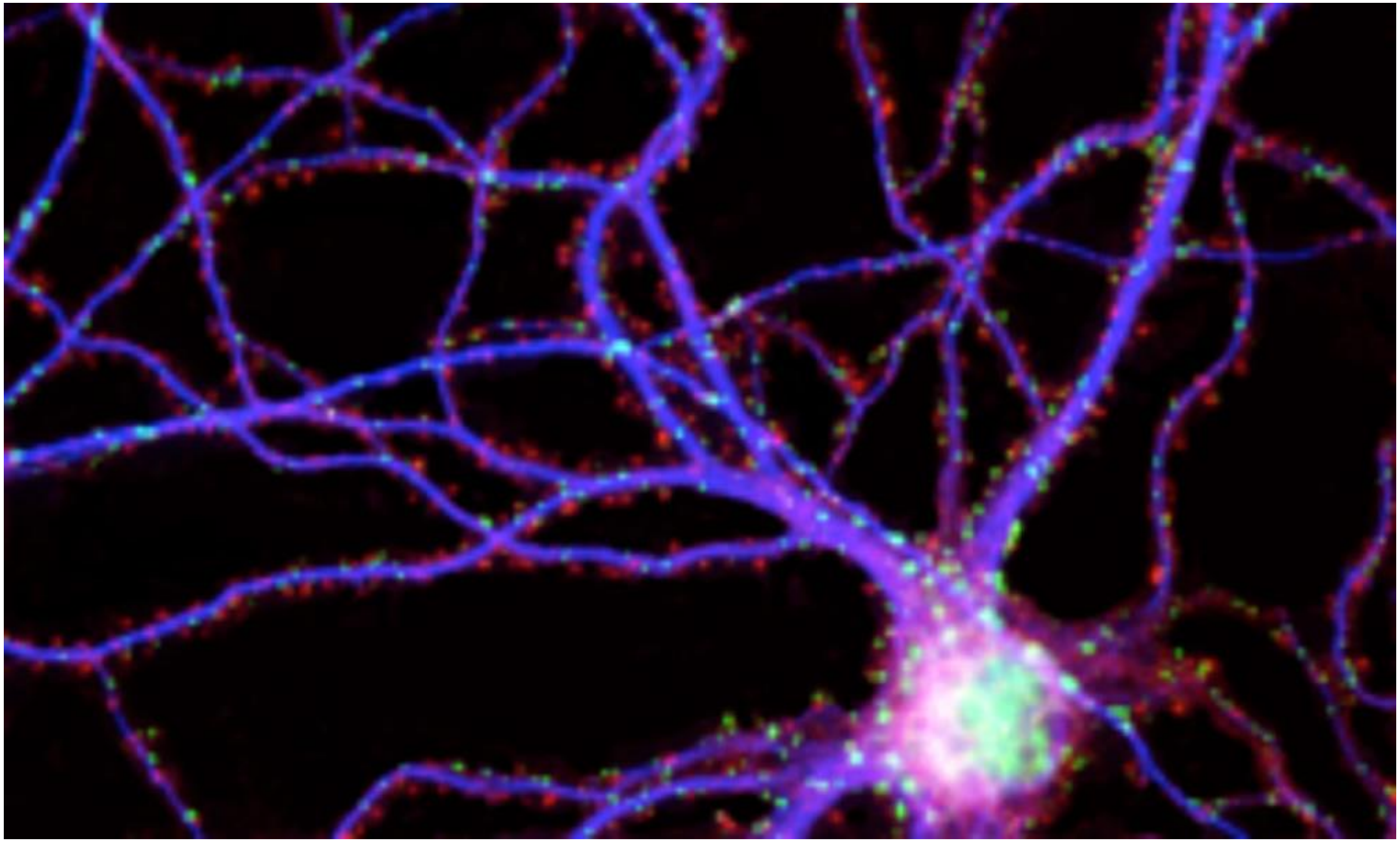
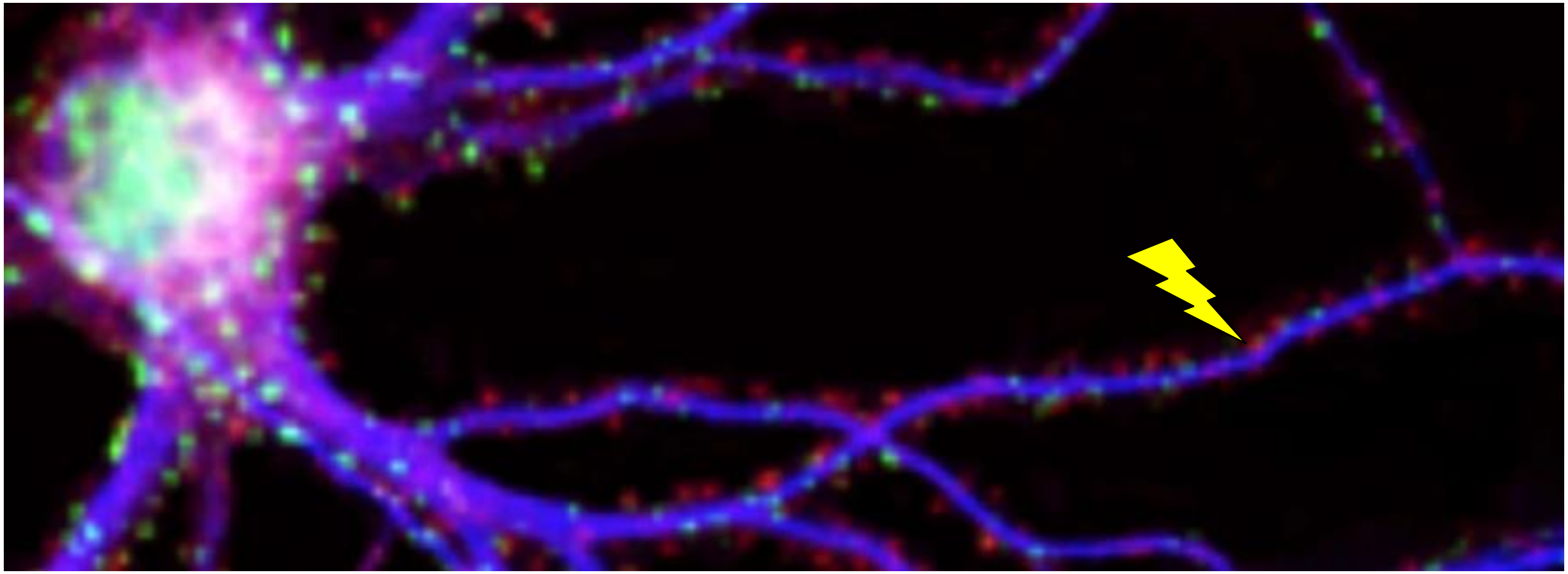


Image: Kamal Sharma/ Johns Hopkins University School of Medicine

- Average half-life of proteins : ~24 hours
- ~25,000 new proteins need to be made per synapse every day.
- ~250,000,000 proteins are made per neuron per day to maintain our memory.

How to Transport 250 Million Proteins into 10,000 Synapses?

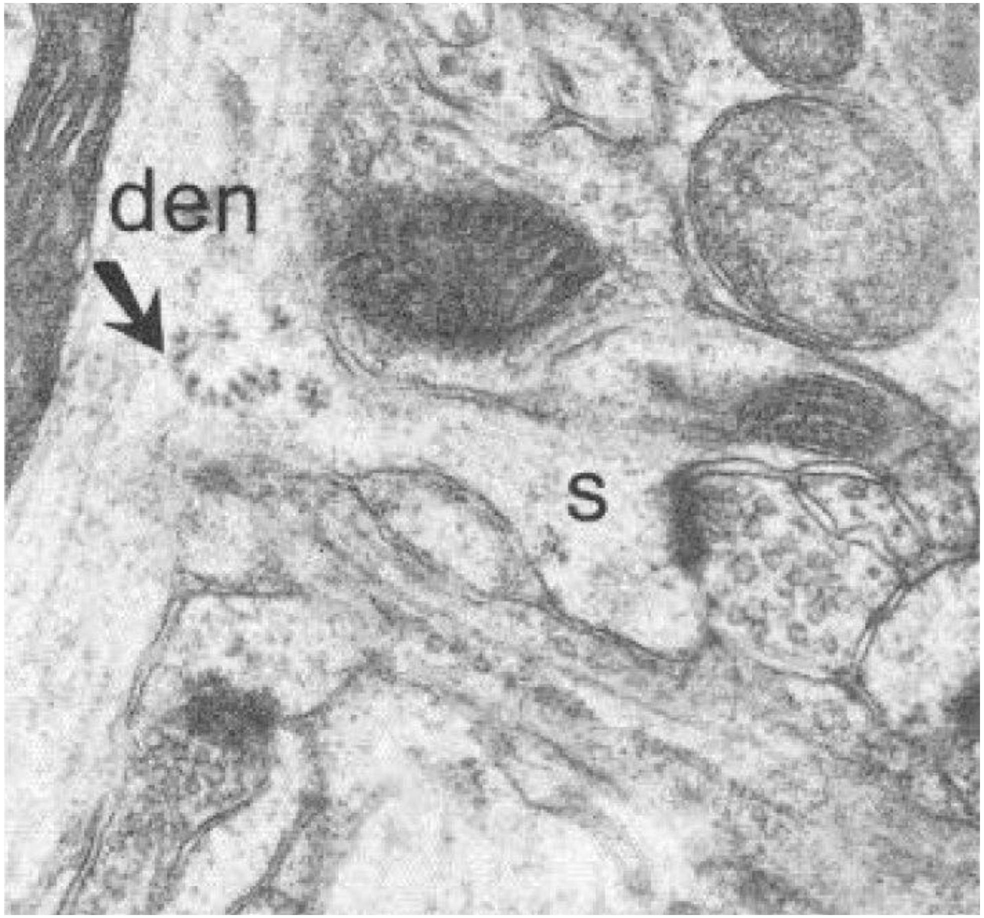


Electrical signal

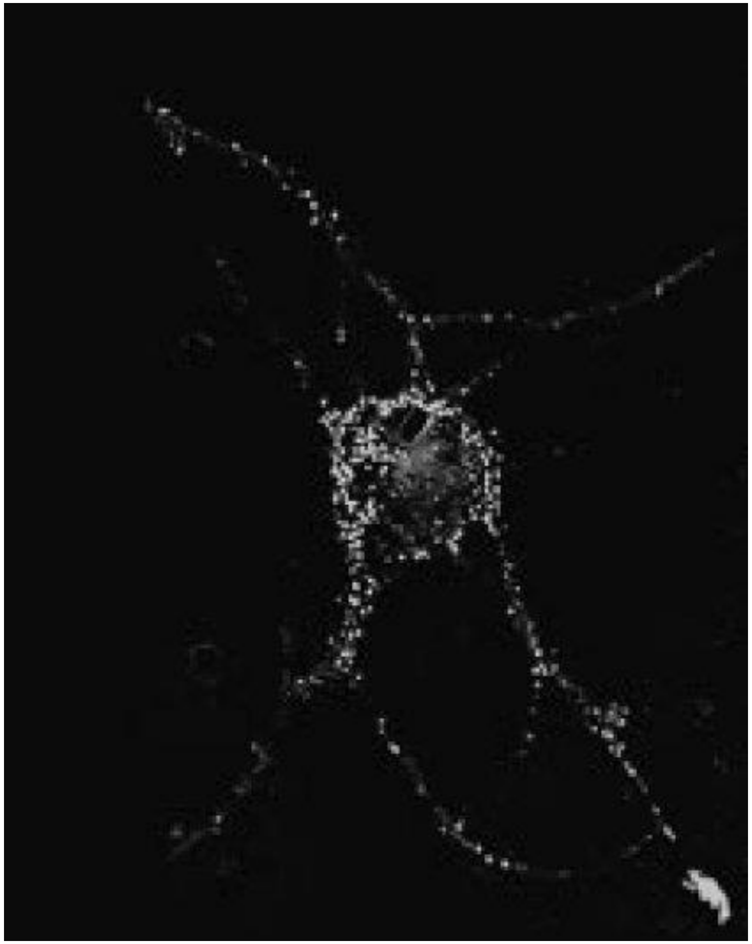
DNA → mRNA → Protein → Synaptic remodeling

Transcription   Translation

Local Protein Synthesis at the Synapse

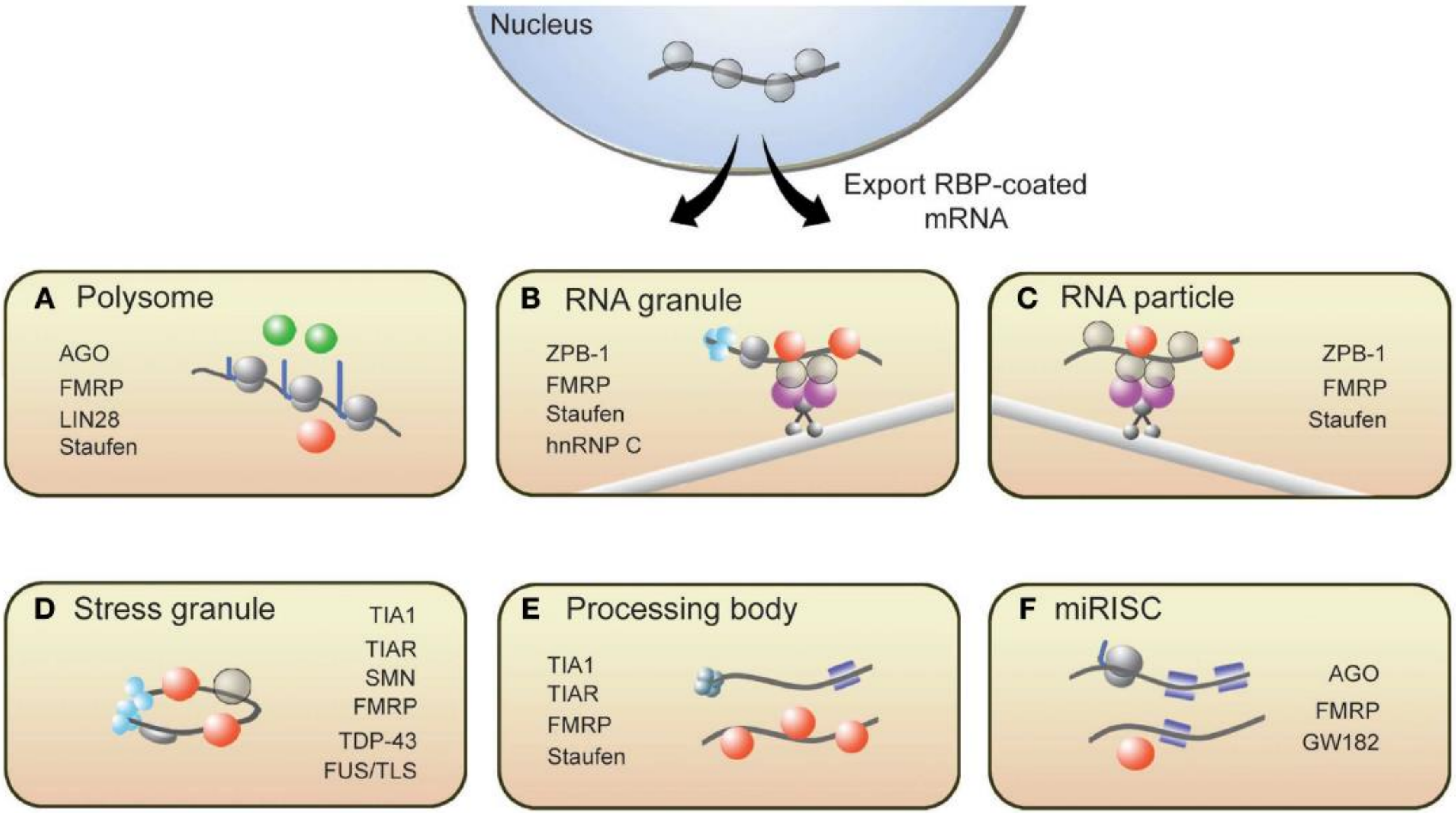


Steward and Levy, J Neuro, 2, 284 (1982)



Bassell *et al*., J Neuro, 18, 251 (1998)

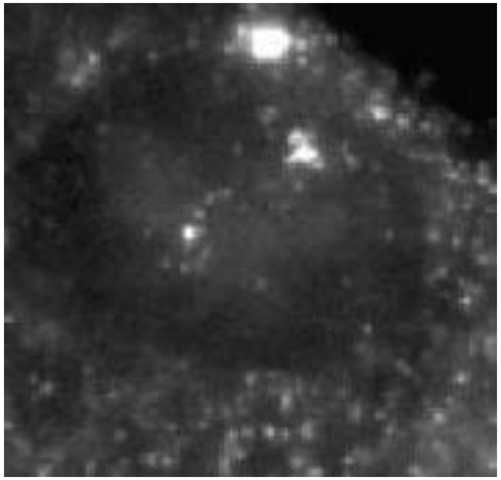
Single Endogenous mRNA in Neurons *in vivo*



Kapeli and Yeo, Front Neurosci. 6, 144 (2012)

Previous Methods for Single mRNA Imaging

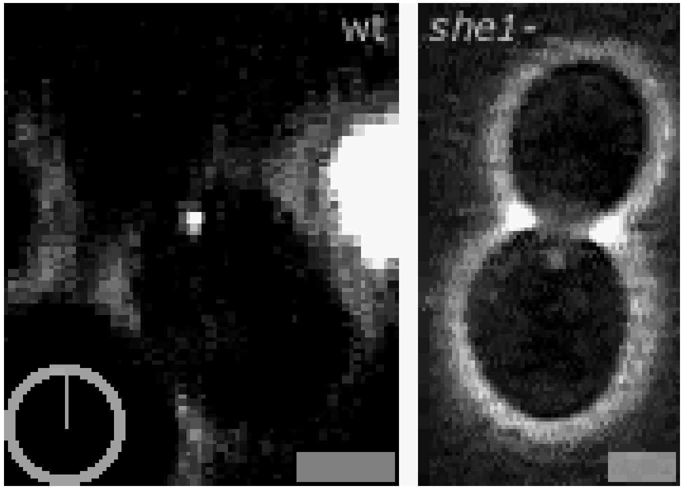
Single molecule fluorescence in situ hybridization (FISH)



Femino *et al*.,
Science 280, 585 (1998)

- Visualizes **endogenous** RNA
- Requires fixation of the specimen

MS2-GFP system for live-cell imaging of mRNA



Bertrand *et al*.,
Mol Cell 2, 437 (1998)

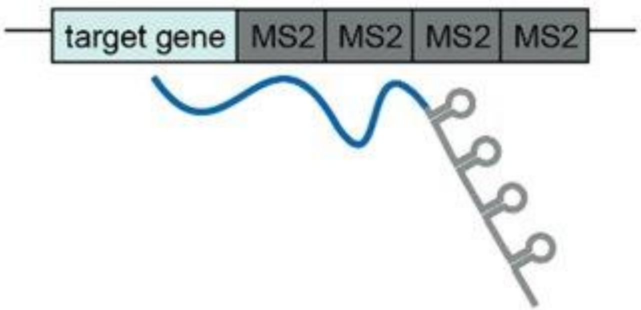
- Reveals RNA dynamics in **live cells**
- Requires transfection of exogenous reporter genes

MS2-GFP System

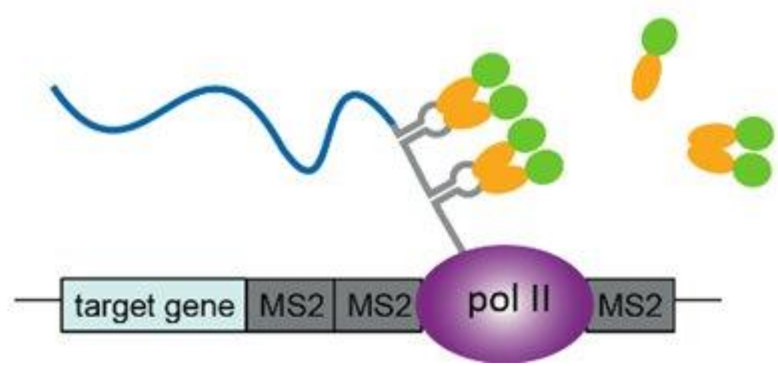
MS2-GFP system uses highly specific binding between **RNA stem-loop** and **capsid protein** from MS2 bacteriophage.

Bertrand *et al*., Mol Cell 2, 437 (1998)

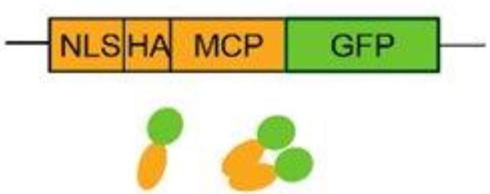
RNA tagged with MS2 binding site (MBS) stem-loops



Double transfection



MS2 capsid protein fused with GFP (MCP-GFP)

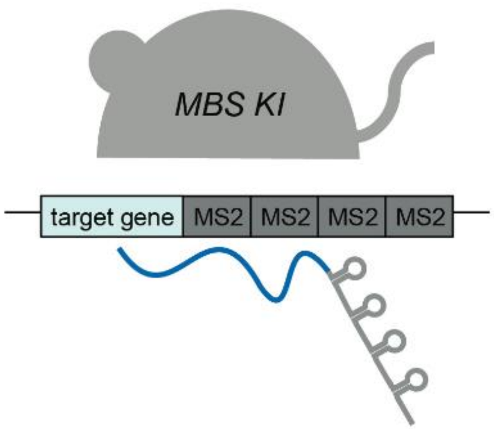


Single mRNA molecule labeled with multiple GFPs

Intravital MS2-GFP System

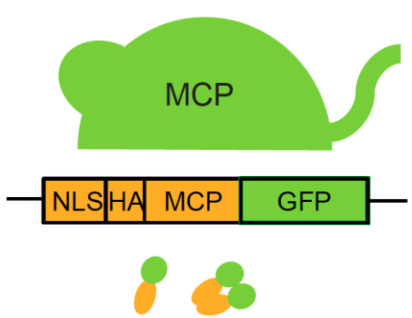
MBS knock-in mouse

Lionnet *et al*., Nature Methods 8, 165 (2011)



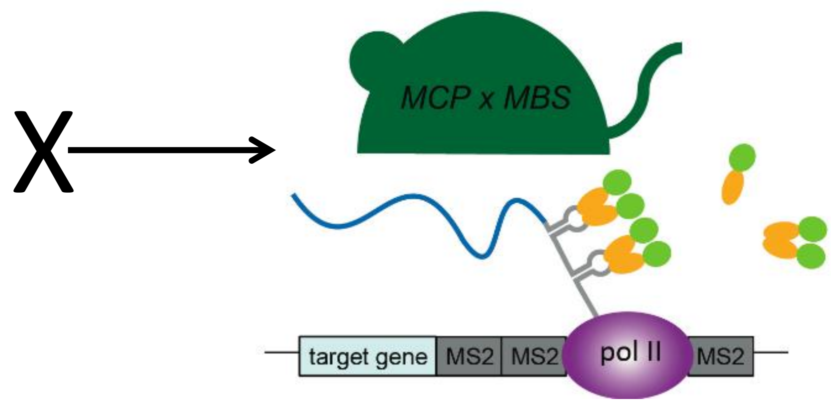
MCP mouse

Park *et al*., Science 343, 422 (2014)



24 repeats of MBS are integrated into the 3' untranslated region (UTR) of a target gene.

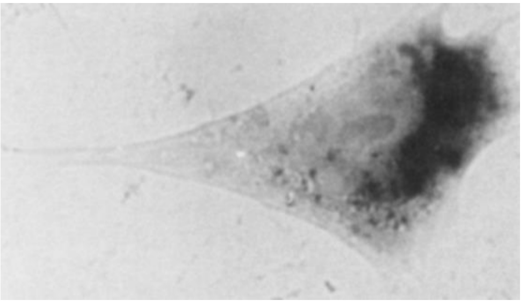
MCP x MBS hybrid mouse



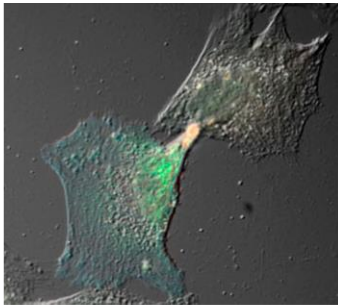
- Endogenous mRNA can be labeled with multiple GFPs.
- mRNA in primary cells and tissues can be imaged.

First Target RNA: β-actin mRNA

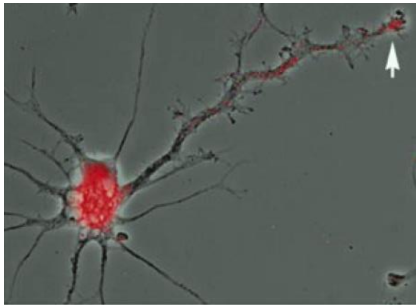
Localization of β-actin mRNA has been observed in various cell types in culture by in situ hybridization.



Embryonic Fibroblast
Lawrence and Singer,
Cell 45, 407 (1986)



Myoblast
Rodriguez et al.,
JCB 175,67 (2006)



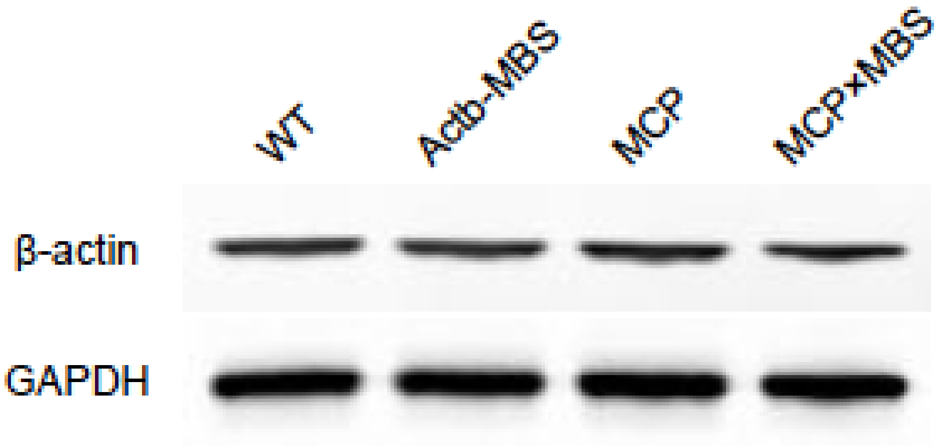
Neuron
Bassell et al.,
J Neurosci 18, 251 (1998)

***MCP x MBS* mouse ➔ Dynamics of β-actin mRNA**

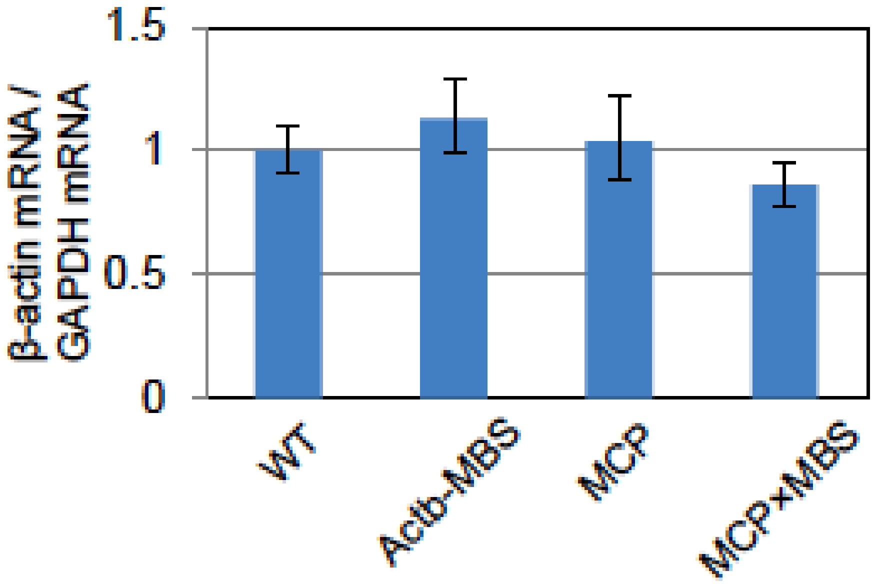
- Double homozygous mice are viable and normal.
- All endogenous β-actin mRNAs are labeled with up to 48 GFPs.

Gene Expression Level after MS2-GFP Labeling

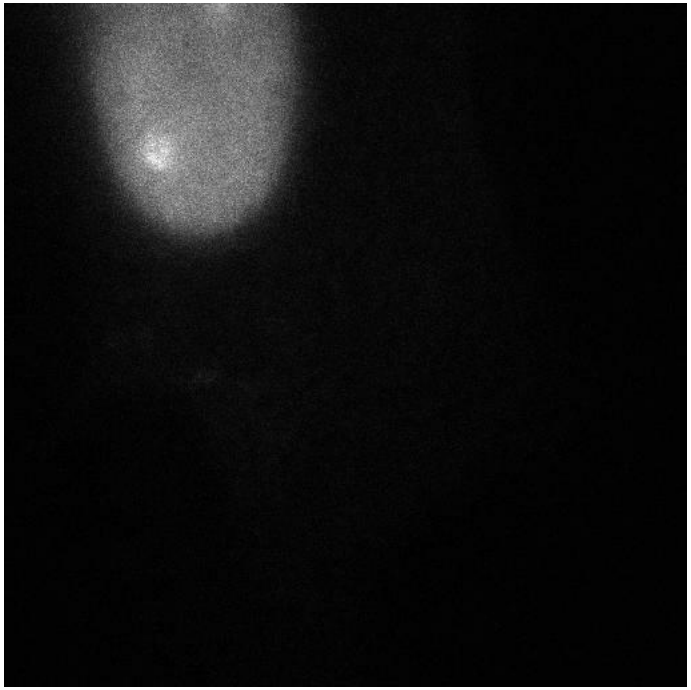
Western Blot of B
rain Lysate



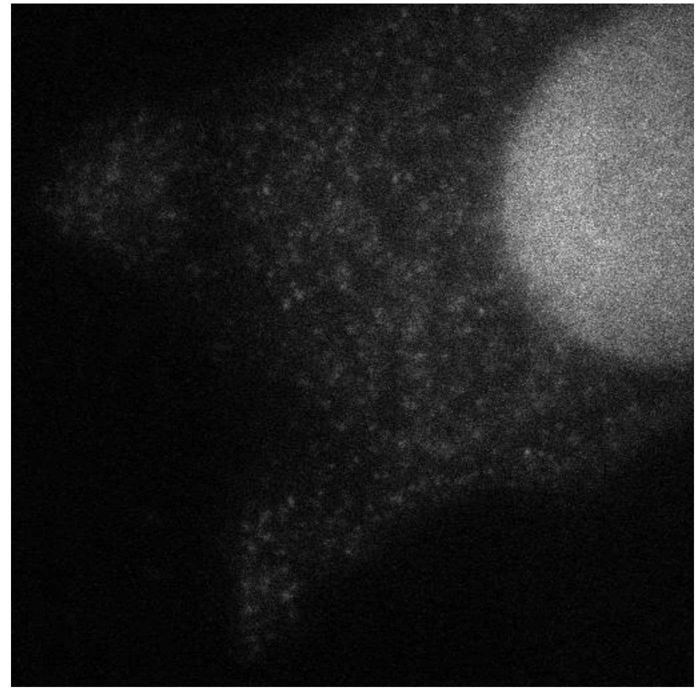
qRT-PCR Analysi
s of
Brain Lysate



MCP-GFP With and Without MBS-Tagged mRNA



MCP
Mouse embryonic fibroblast (MEF)

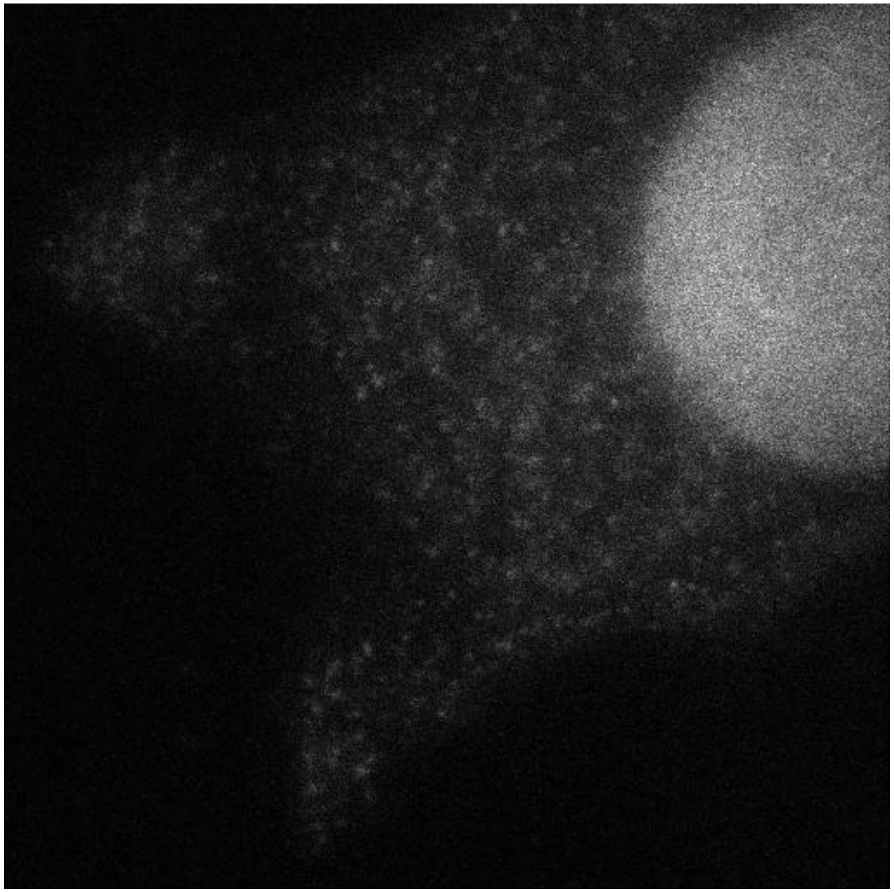


MCP x *MBS*
MEF

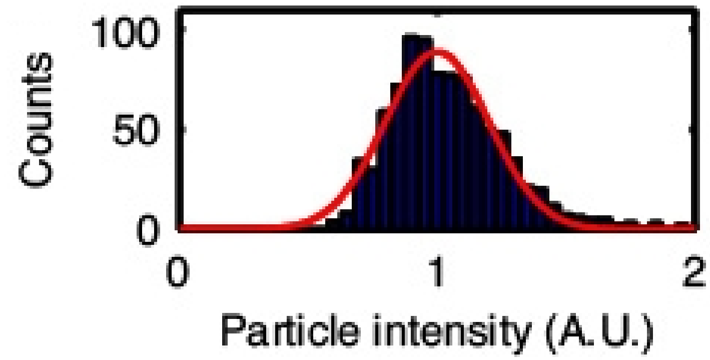
10 μm

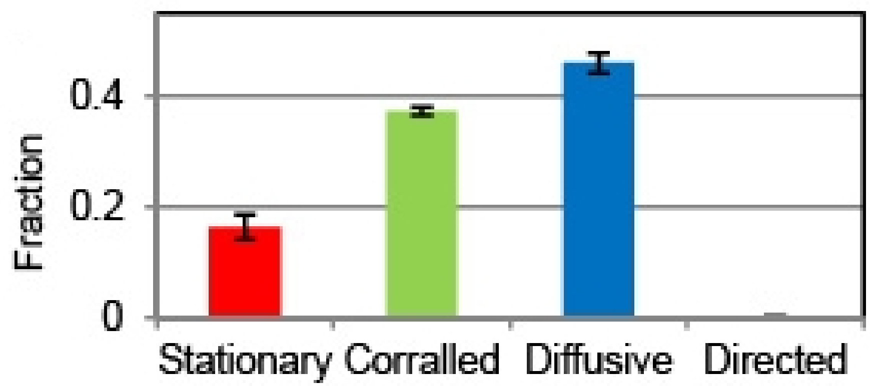
Particles in *MCP* x *MBS* mouse cells are MBS-tagged β-actin mRNA.

Single mRNA Tracking in Primary MEFs



movie played at 3 times real speed

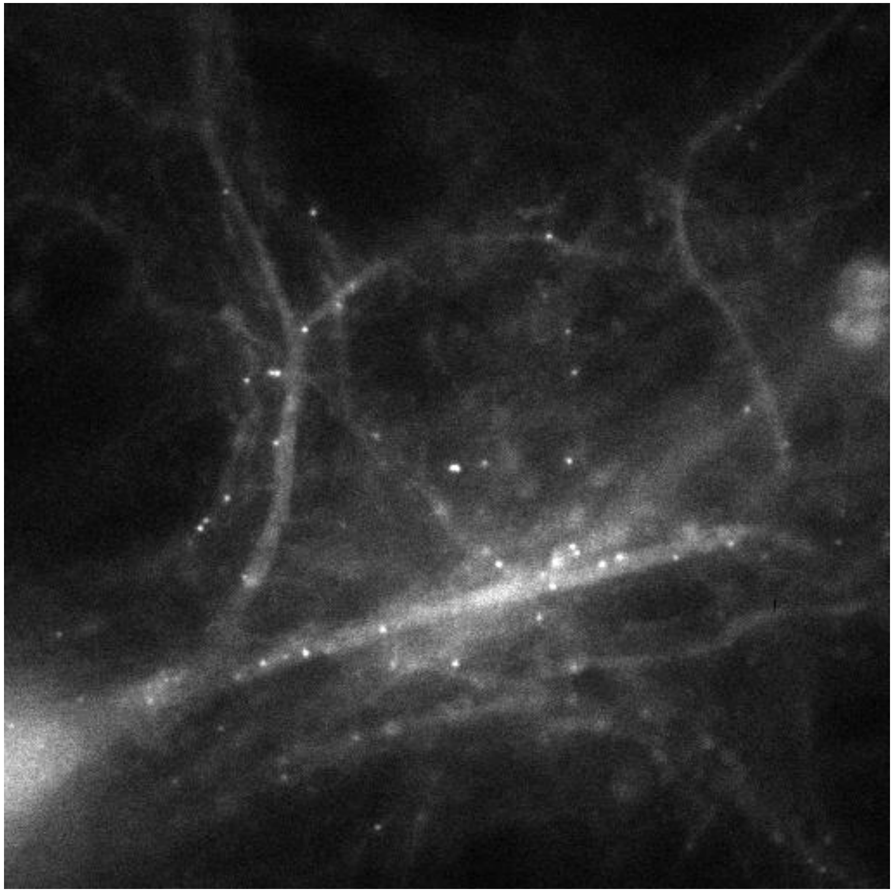




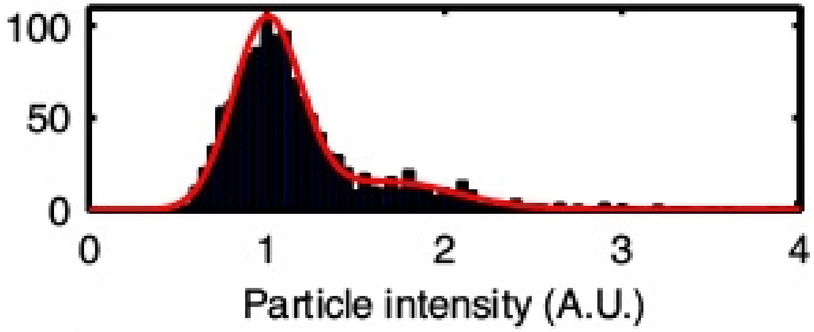
Mouse embryonic fibroblast

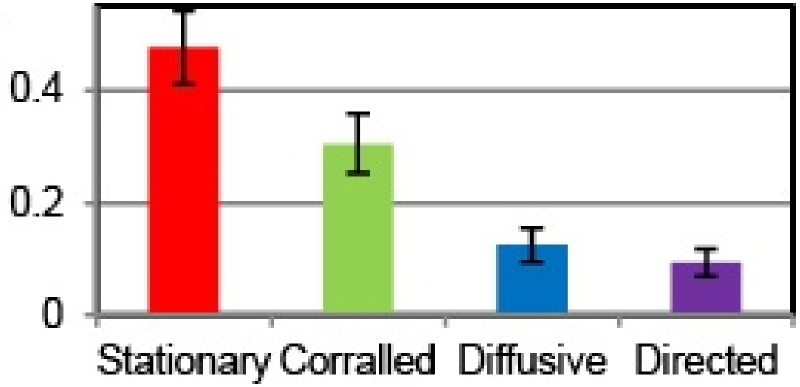
- Single mRNA
- Mostly diffusive

Single mRNA Tracking in Neurons



Neuron at 17 days in vitro (DIV)
Movie played at 6 times real speed

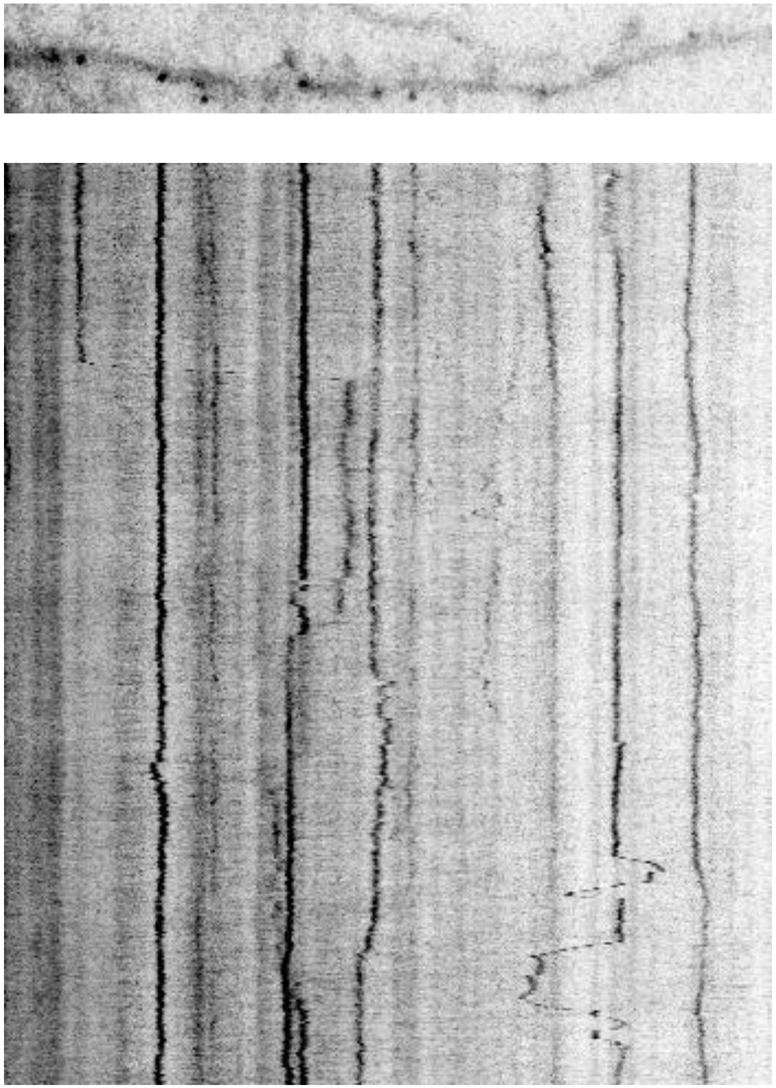


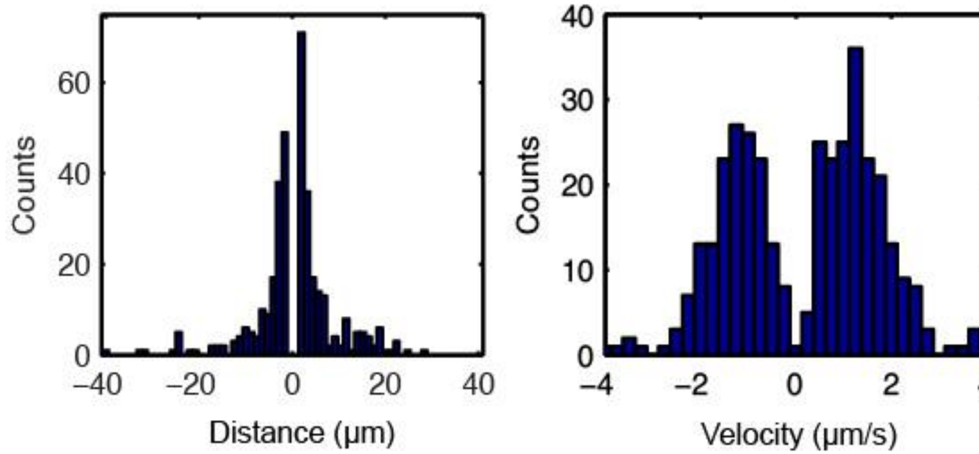


Hippocampal Neuron

- Multiple mRNAs in mRNP complex
- Stationary & directed motion

Transport of β-Actin mRNA in Neurons



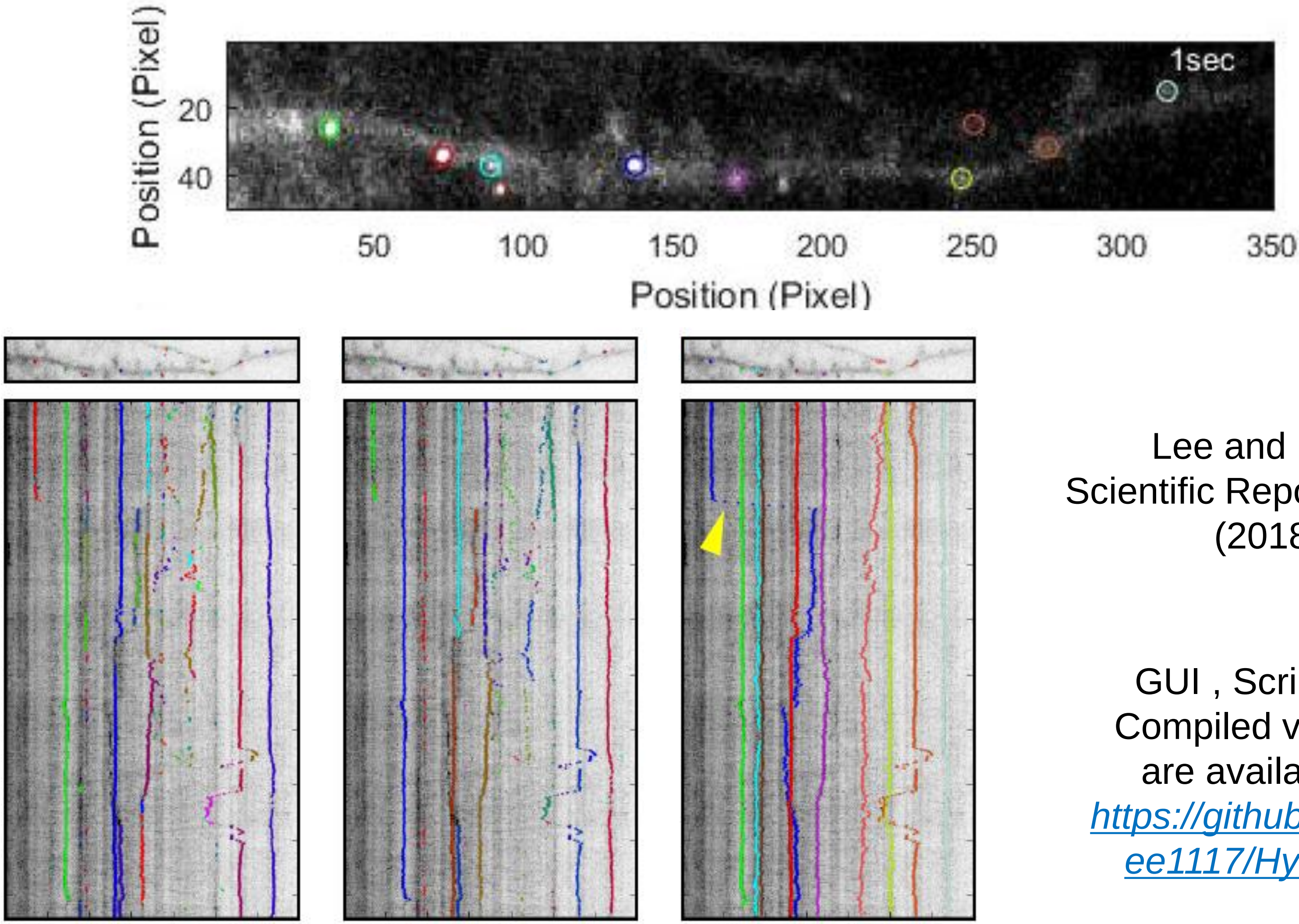


- ~ 10% of mRNPs show directed motion (mean speed: 1.3 µm/s)
- The ratio of anterograde to retrog rade motion ~ 1.2

1 min

10 μm

HybTrack: Combination of Manual and Automatic Tracking



u-track
Jaqaman et al.,
Nat Methods 5, 695 (2008)

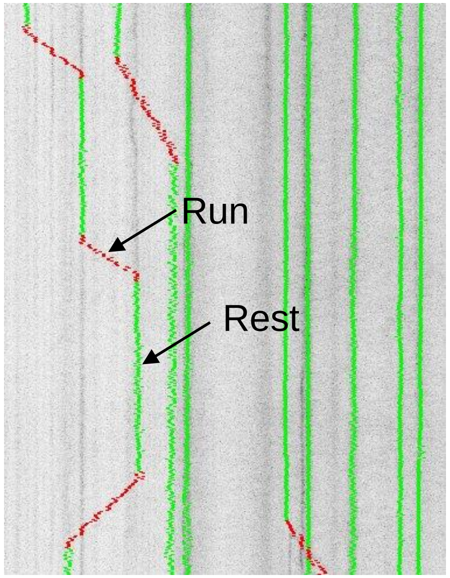
TrackNTrace
Stein et al.,
Sci Rep 6, 37947 (2016)

HybTrack

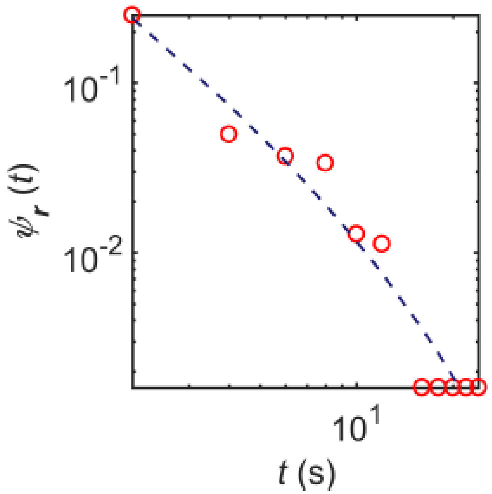
Lee and Park,
Scientific Reports 8, 212 (2018)

GUI , Script and Compiled versions are available at https://github.com/bhlee1117/HybTrack

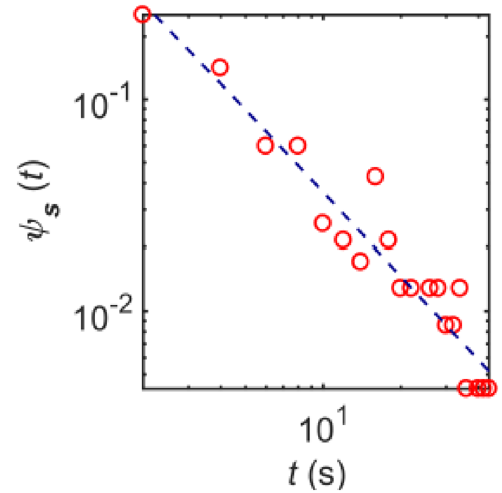
Neuronal mRNA Transport: Lévy Walk Model

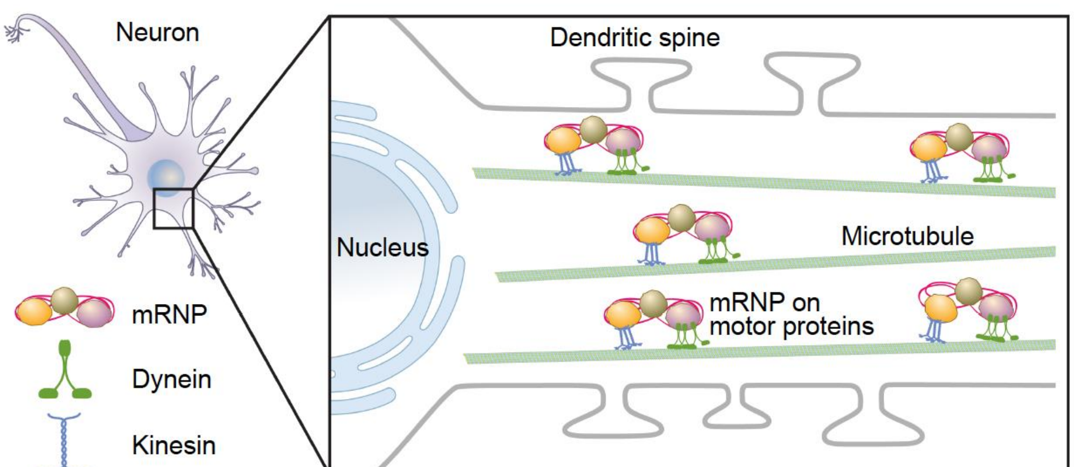


Run time distribution

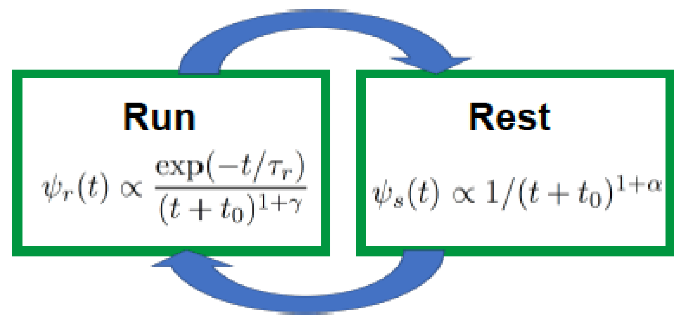


Rest time distribution



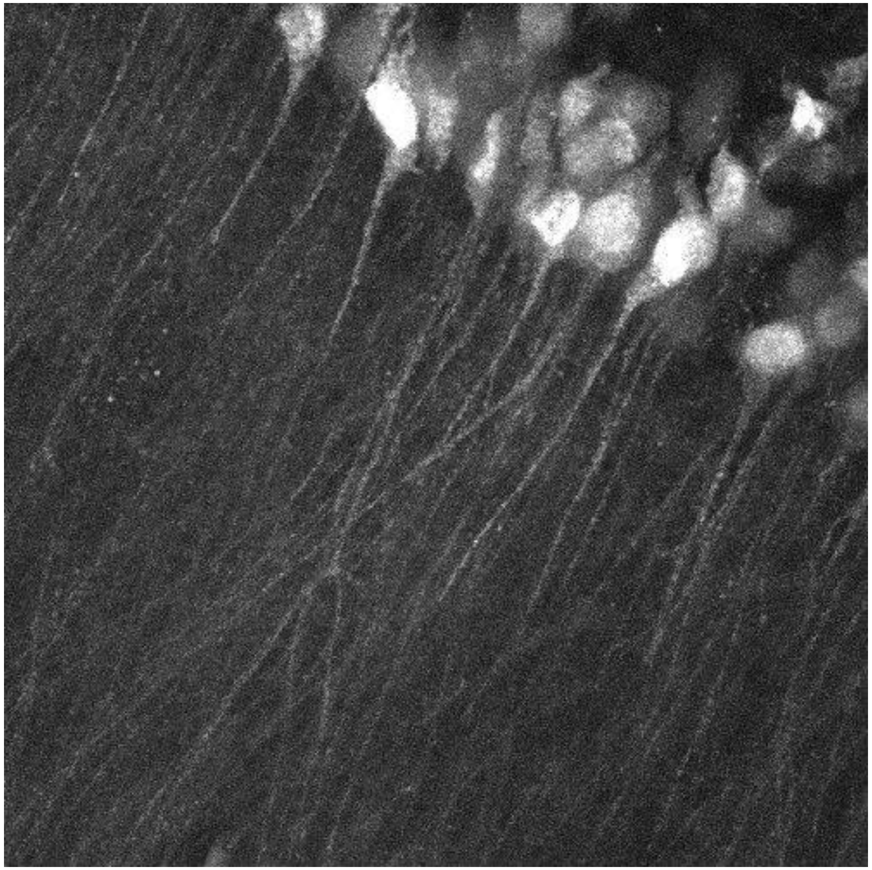


(collaboration with Prof. Jae-Hyung Jeon)

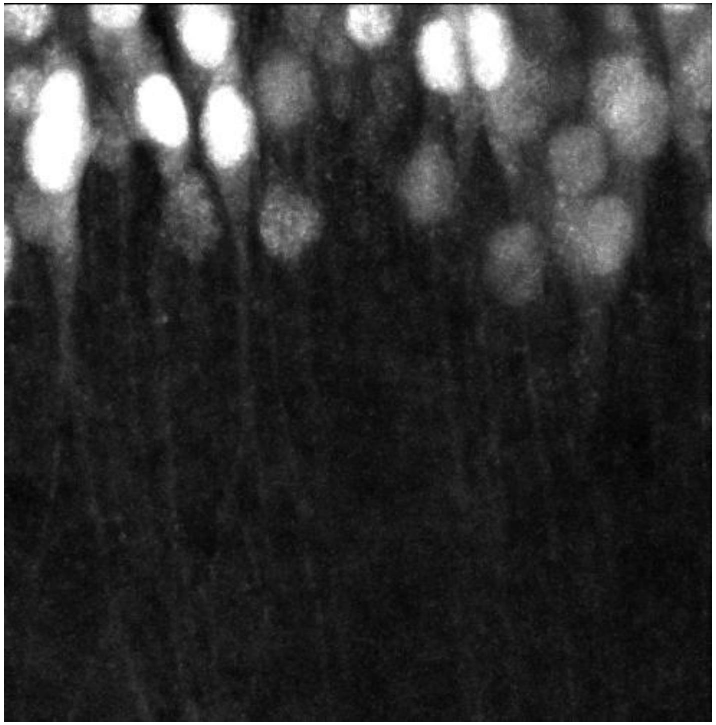


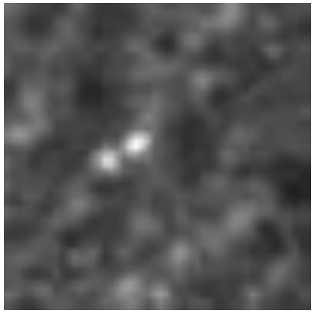
Song, Moon, Jeon, and Park, Nature Communications 9, 344 (2018)

Acute Brain Slice of *MCP* x *MBS* Mouse



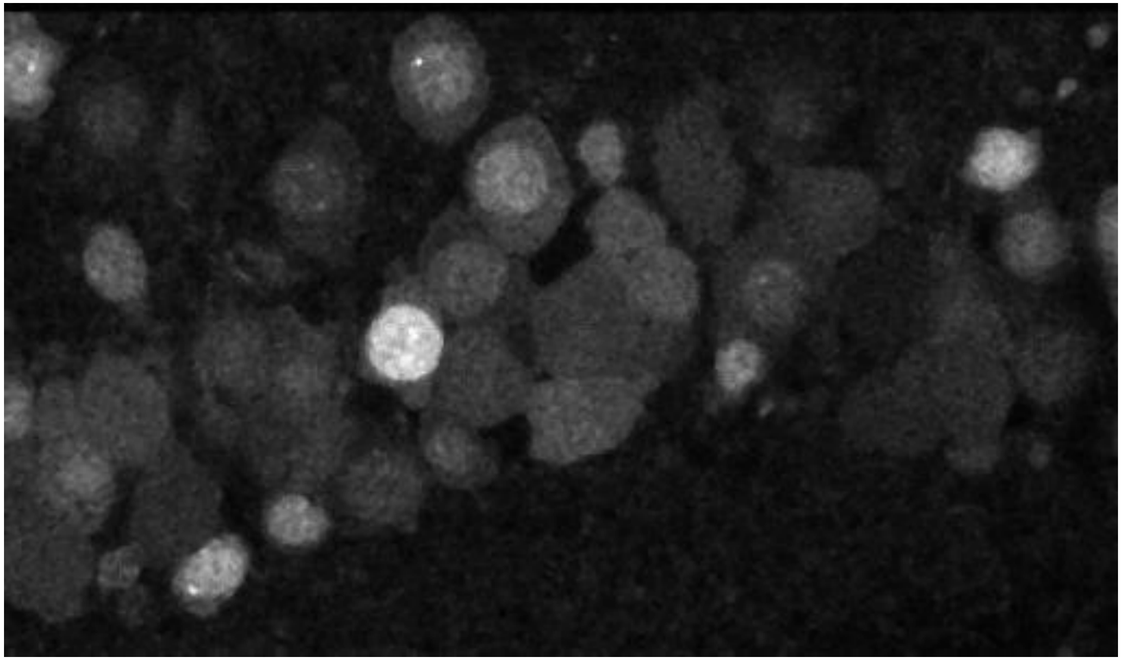
CA1 region of hippocampus
Acute brain slice, 1 µm z-sections
Multiphoton microscopy



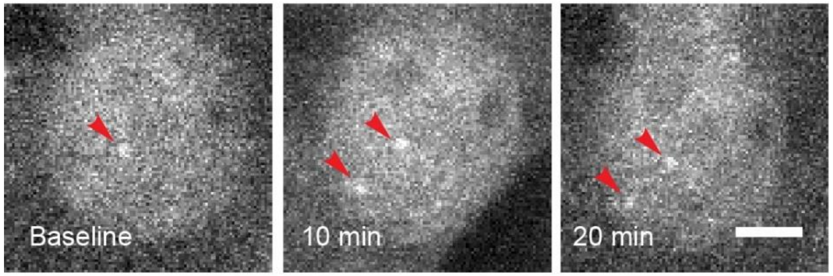


Time-lapse
1 min interval

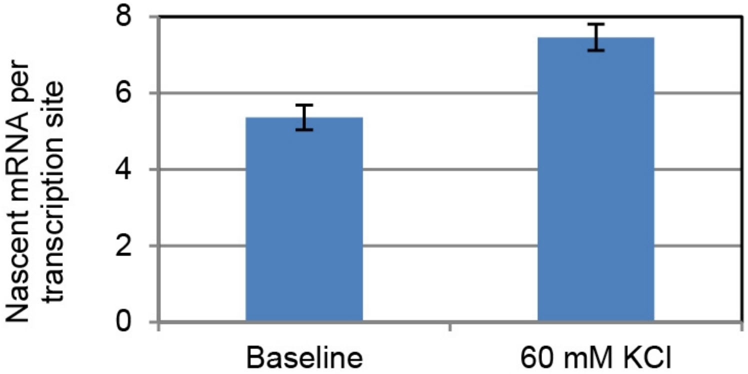
Immediate Early Transcription of β-Actin mRNA



CA1 region of
hippocampus
0.5 µm z-sections



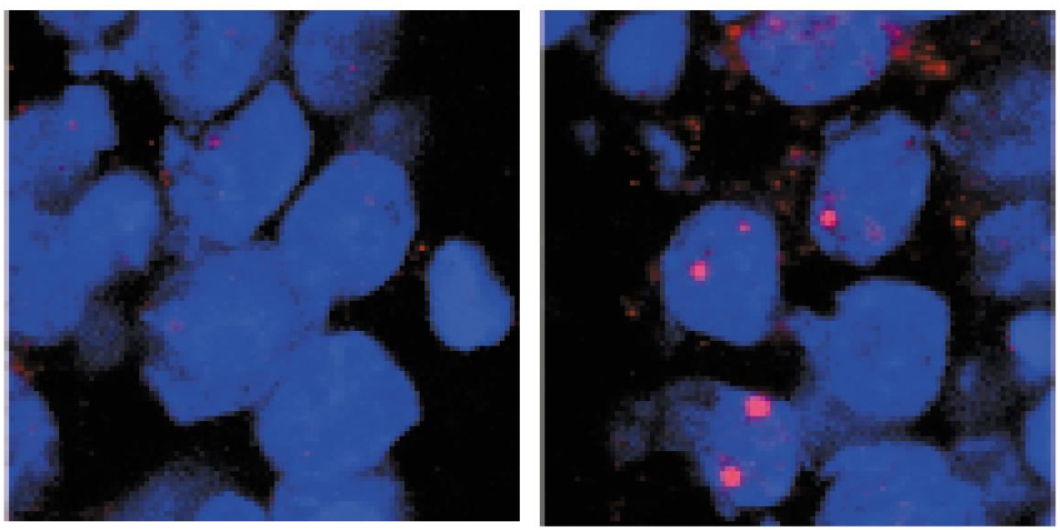
60 mM KCl for 3 min



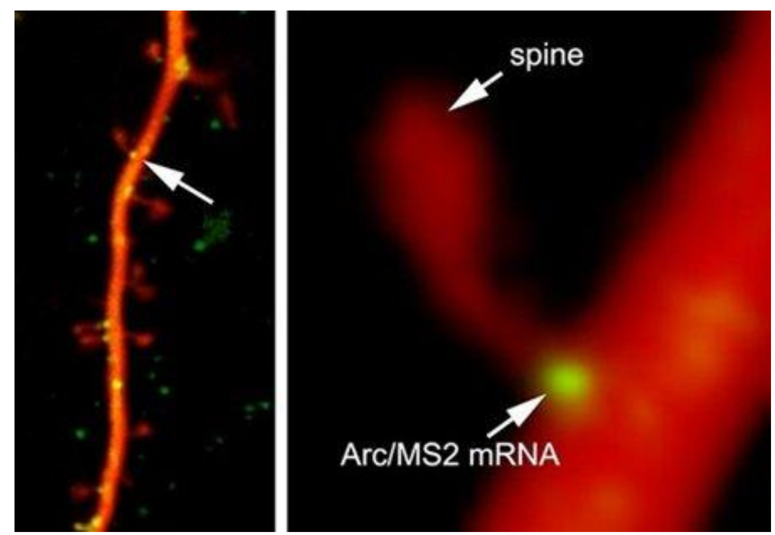
Park *et al*. Science 343, 422 (2014)

Imaging Endogenous Arc mRNA

- Immediate early genes (IEGs) : *c-Fos, Arc, Egr-1(Zif268)...*
- *Arc* is required for long-term memory consolidation.
- Fluorescence in situ hybridization (FISH) of *Arc* mRNA is widely used to ide ntify memory trace cells.
- *Arc* mRNA is localized to activated synaptic sites.



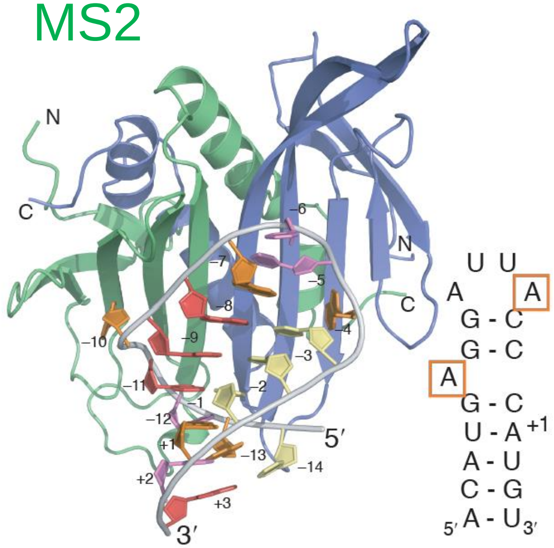
Guzowski *et al.*, Nat Neurosci 2, 1120 (1999)



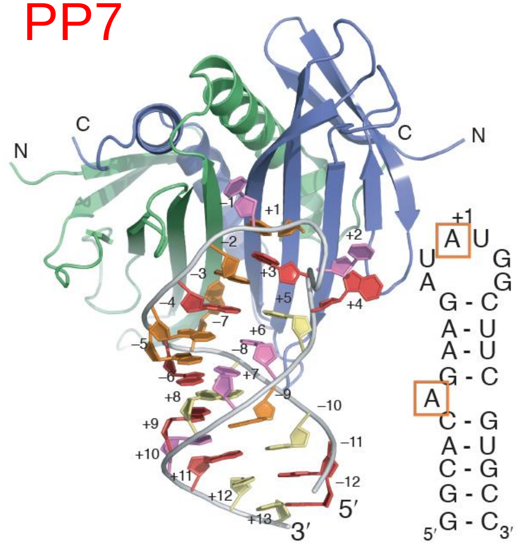
Steward *et al.*, Front Mol Neurosci 7, 101 (2015)

Labeling Endogenous Arc mRNA with the PP7 system

MS2



PP7



MS2 and PP7 capsid proteins bind to unique RNA stem-loops.

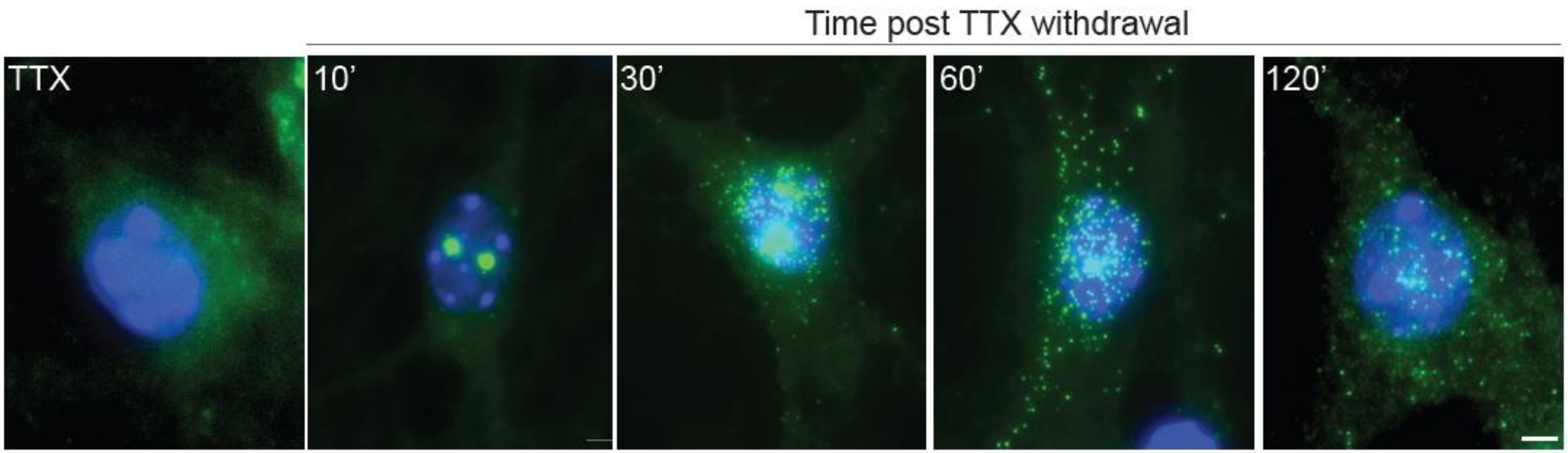
Chao *et al.*, Nature Struct Mol Biol, 15, 103 (2008)

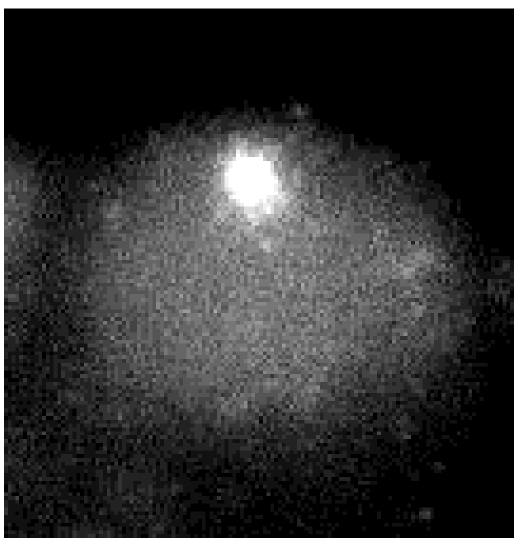
Arc – PBS knock-in mouse

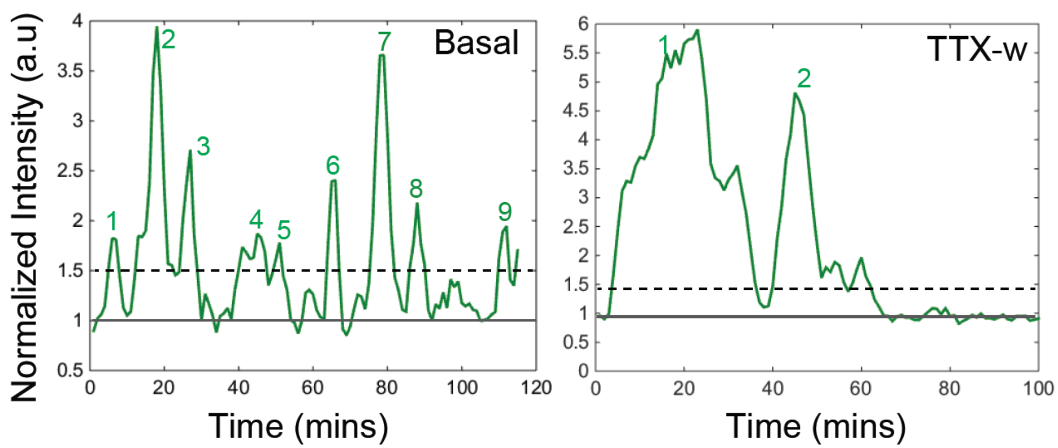
24x PBS cassette is inserted at 250 bp downsteam of the stop codon in the endogenous *Arc* gene.

Das, Moon, Singer and Park, Science Advances 4, eaar3448 (2018)

Time Course of Arc Transcription







Neuronal activity induces bursting of Arc transcription.

Das, Moon, Singer and Park, Science Advances 4, eaar3448 (2018)

Summary

- New mouse models are developed for imaging endogenous mRNA in live cells and tissues.
- In fibroblasts, β-actin mRNA molecules are predominantly transported by diffusion.
- In neurons, β-actin mRNA transport follows Lévy Walk.
- Neuronal activity induces bursting of Arc transcription.
- Dendritic transport of Arc mRNA is independent of neuronal activity.
- Next challenge: Visualization of single mRNA in live mouse brain.

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Janelia Research Campus, HHMI

- Dr. Sulagna Das
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