Conventional ('wide-field') epi-fluorescence microscope

filters and mirror in fluorescence 'cube'
barrier filter (blocks excitation wavelength)
dichroic mirror

fluorescence emission

excitation filter (transmits only desired excitation wavelengths)

collector arc lamp
Problem with wide-field epi-fluorescence

Fluorescence generated throughout 'hour-glass' converging & diverging cones of excitation light

Out-of-focus fluorescence reduces contrast in image - wide-field microscopy has poor depth discrimination
Solutions:

0. Digital deconvolution technique

Capture digital images at 3 (or more) depths into the specimen. Process the stack out of them to correct for out-of-focus fluorescence.

Works OK, but takes long time to process.
No good for imaging fast dynamic signals.
2. **Confocal microscopy**

- **Detector (PMT)**
- **Confocal aperture (pinhole)**
- **Point source of light (laser)**
- **Objective**

- **Blue = In-focus light passing through pin-hole**
- **Black = Out-of-focus light largely blocked by pinhole**

- **Light from plane of focus**
  - Most reaches detector

- **Light from above or below plane of focus**
  - Most blocked by aperture
The 'confocal spot'

Volume of specimen through which signal is detected.

Looks like (American) football

Point-spread function (PSF).
width along x, y, z, for half-maximal intensity of point (infinitesimally small) object.

PSF improves with
↑ NA of objective
↓ wavelength
↓ confocal aperture (but image gets dimmer)

practical limit for confocal ~ 0.3 μm (x, y) - not much better than widefield
~ 0.6 μm (z) - much better
Problem

Confocal microscope only records from a single spot - how do we get an image?

A. Scan the spot, with rotating mirror

Mirror placed after eyepiece, where pupil of eye would be. Change in angle here = change in position of spot in specimen.

To set x-y scan, have 2 mirrors at right-angles.

→ fast scan in x
down in y

etc
computer 'knows' where laser spot is at all times (from drive signals to mirrors) - so can build-up complete image from series of PMT signals as mirrors scan.

Complete scan (e.g. 512 x 480 pixels) can take from 1s to <33ms, depending on design.

**Historical note**

Confocal principle patented in 1951 (Mervin Minsky) but commercial application not until 1978 (MRC 500) (after patent expired).

Depended on development of lasers, cheap computers.
Limitations of conventional confocal microscopy.

1. Although image obtained from thin 'slice', entire thickness of specimen exposed to laser irradiation - problems with photo bleaching, phototoxicity.

[Diagram of microscope focus]

2. Confocal can 'see' to only limited depth into scattering specimens (e.g. brain); depends on forming sharp laser spot in specimen, then imaging spot sharply onto confocal aperture.
Solution...

3) **2-photon microscopy**

Basis:

1 photon fluorescence

dye molecule absorbs energy of short-wavelength photon (e.g. blue) – emits slightly less energy as longer-wavelength photon (e.g. green).
fluorescence emission *linearly proportional* to intensity of fluorescence excitation

2 photon fluorescence

excite with light of about 2x the wavelength needed for regular fluorescence; i.e. each photon has only 1/2 the energy needed.
Dye molecule can absorb 2 photons at almost exactly the same time, and thus get sufficient energy to fluoresce.
fluorescence emission increases as square of intensity of fluorescence excitation
Note - 2-photon excitation is usually extremely improbable, as need 2 photons hitting molecule almost simultaneously (≈10^-18 s) e.g. about once per 1000 yrs for fluorescein molecule in sunlight.

Need the incredibly high peak energy (photon density) of brief, focused laser pulses.
why is 2-photon fluorescence useful?

regular 1-P
confocal

blue

2-P
confocal
IR

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\text{thick specimen}
\]

Get fluorescence excitation only at focal plane

[ Fluorescence declines with 4th power of axial distance from focal spot: excitation intensity \( \downarrow \) as square of distance, fluorescence proportional to square of intensity ]

2-photon imaging provides confocal sectioning without need for pinhole.
Multi-photon microscopy

Usually a synonym for 2-photon microscopy — but I can do 3-photon (even more?) imaging.

e.g. intrinsic fluorescence of cytochromes, neurotransmitters that are normally excited by UV light.

Instead of 1 photon at 330nm, excite with 3 photons at 1000nm.
Advantages

1. Only excite where you look: no out-of-focus bleaching, toxicity.

   ![1-P and 2-P diagrams]

   - 1-P: bleached volume after scanning image
   - 2-P: side view of specimen

2. Can collect as much fluorescent light as possible — even if scattered

   - Only direct photon will be focused onto pinhole of 1-P confocal.
   - Scattered photon will appear to come from here, and thus will be focused to side of pinhole and lost.

   With 2-P excitation scattered photons still carry information: they can only have come from the excitation spot.
IR light (used for 2-P excitation) penetrates into tissue (brain) with much less scattering than shorter wavelengths [why the sky is blue, and sunsets red].

IR light less damaging (lower energy, lower absorption) than UV light.

Disadvantages:

1. Cost - need special laser
2. Lower resolution: wavelength twice normal, so diffraction-limited spot twice as wide.
3. Total laser power much higher than for 1-P confocal: IR beam may cause damage.
Lasers for 2-P microscopy

Need very high intensity for 2-P excitation
But - it continuous would fry specimen

Solution - laser giving incredibly brief pulses of light

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\begin{align*}
\text{each pulse} & \quad \text{interval between pulses} \\
100 \text{fs} (10^{-13} \text{s}) & \quad 10 \text{ns} (10^{-8} \text{s})
\end{align*}
\]

ratio pulse/interval $= 10^{-5}$

so peak energy in pulse is $10^5$ greater than average power of beam

so 2-P excitation is $10^{10}$ greater $[(10^5)^2]$ than continuous beam of same average power.
Mode-locked Ti:sapphire femtosecond laser

generator pulser at < 100 fs

tunable from 700 → 1000 nm (equivalent to 1-P excitation at 350 → 500 nm)