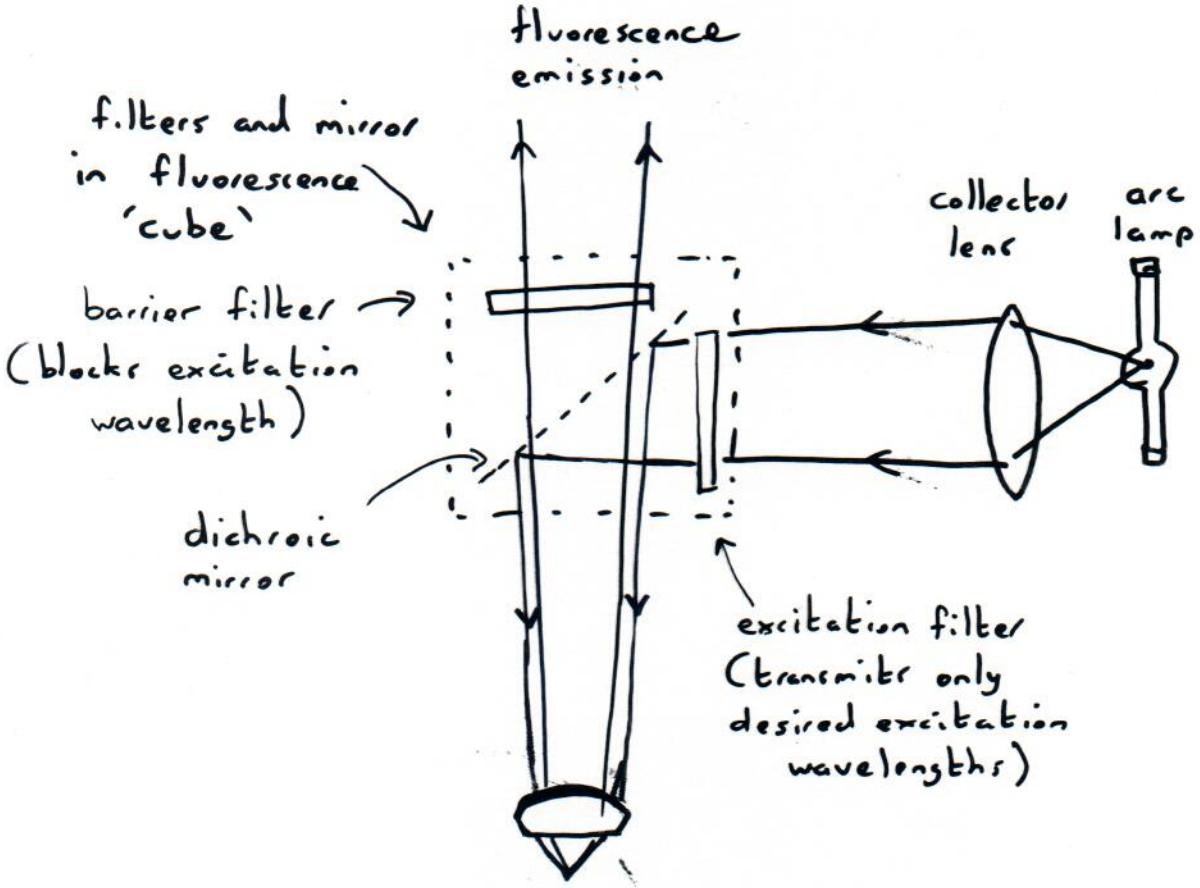
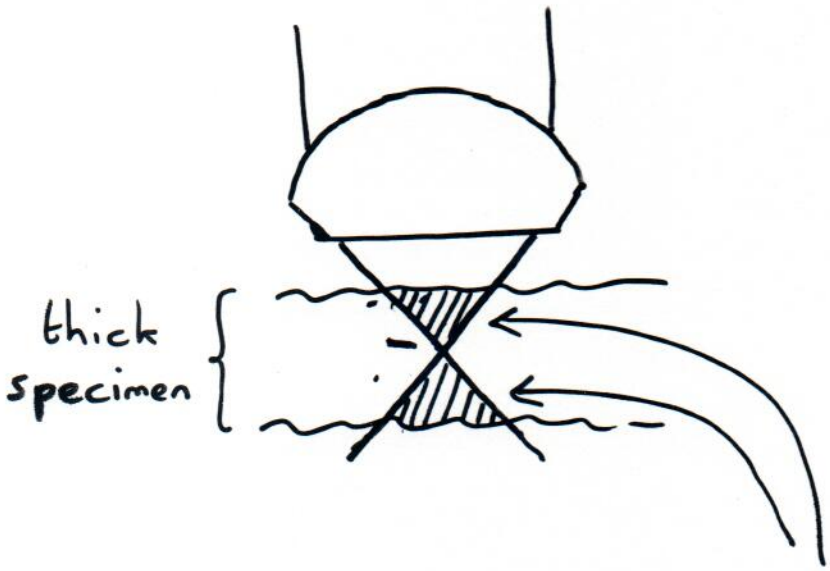
Conventional ('wide-field') epi-fluorescence microscope



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

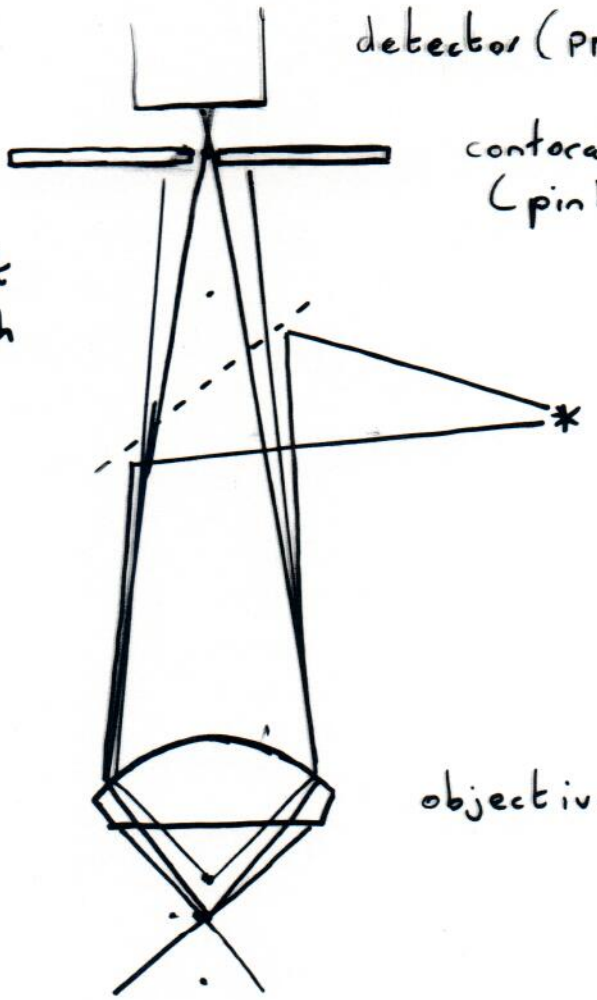
Solutions:

① Digital deconvolution techniques

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

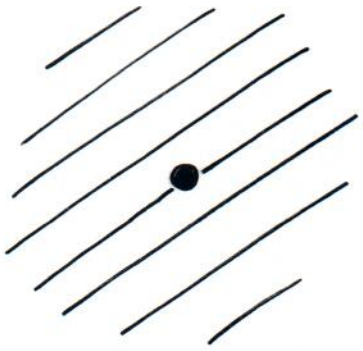
Works OK, but takes long time to process. No good for imaging fast dynamic signals.

② Confocal microscopy



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

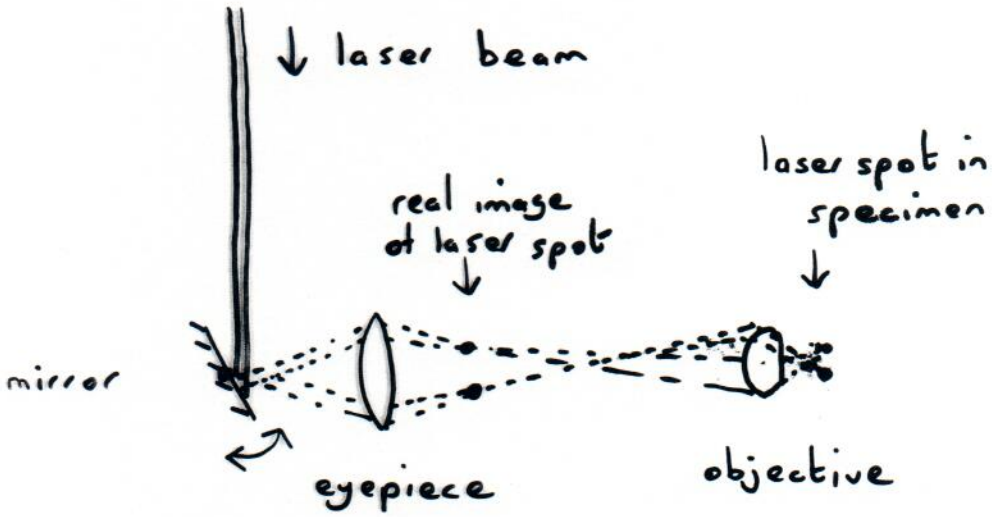
light from above or below plane of focus

most blocked by aperture

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

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 (eg 512 × 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 ~1 (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 photobleaching, phototoxicity.

② Confocal can 'see' to only limited depth into scattering specimens (eg 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 or longer-wavelength photon (eg green).

fluorescence emission linearly proportional to intensity of fluorescence excitation

2 photon fluorescence

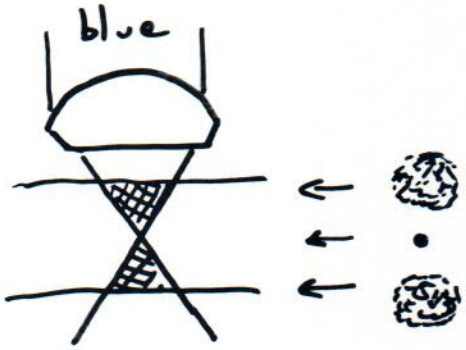
excite with light of about 2× the wavelength needed for regular fluorescence: i.e. each photon has only ½ the energy needed.

Dye molecule can absorb 2 photons at almost exactly the same time, and thus get sufficient energy to fluoresce.

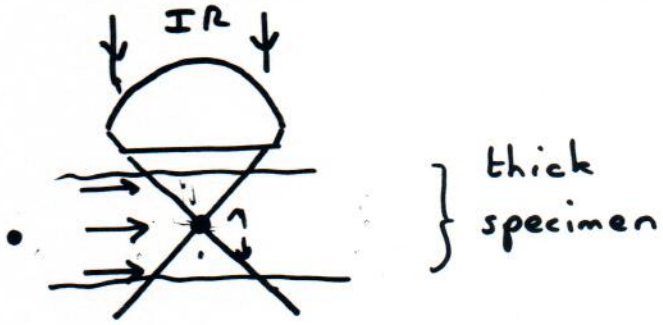
fluorescence emission increases as square of intensity of fluorescence excitation

why is 2-photon fluorescence useful?

regular 1-P confocal



2-P confocal



Get fluorescence excitation only at focal plane

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

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