Confocal Microscopy - principles and applications

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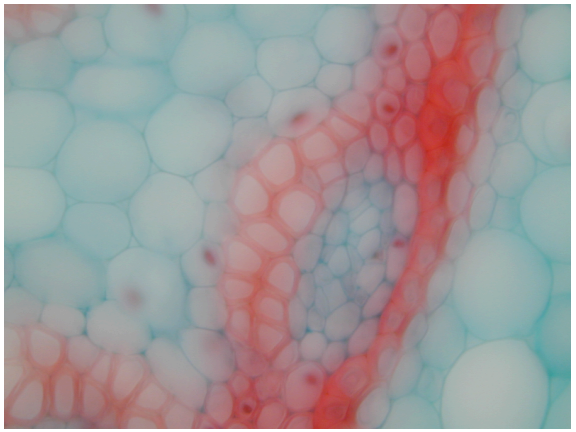
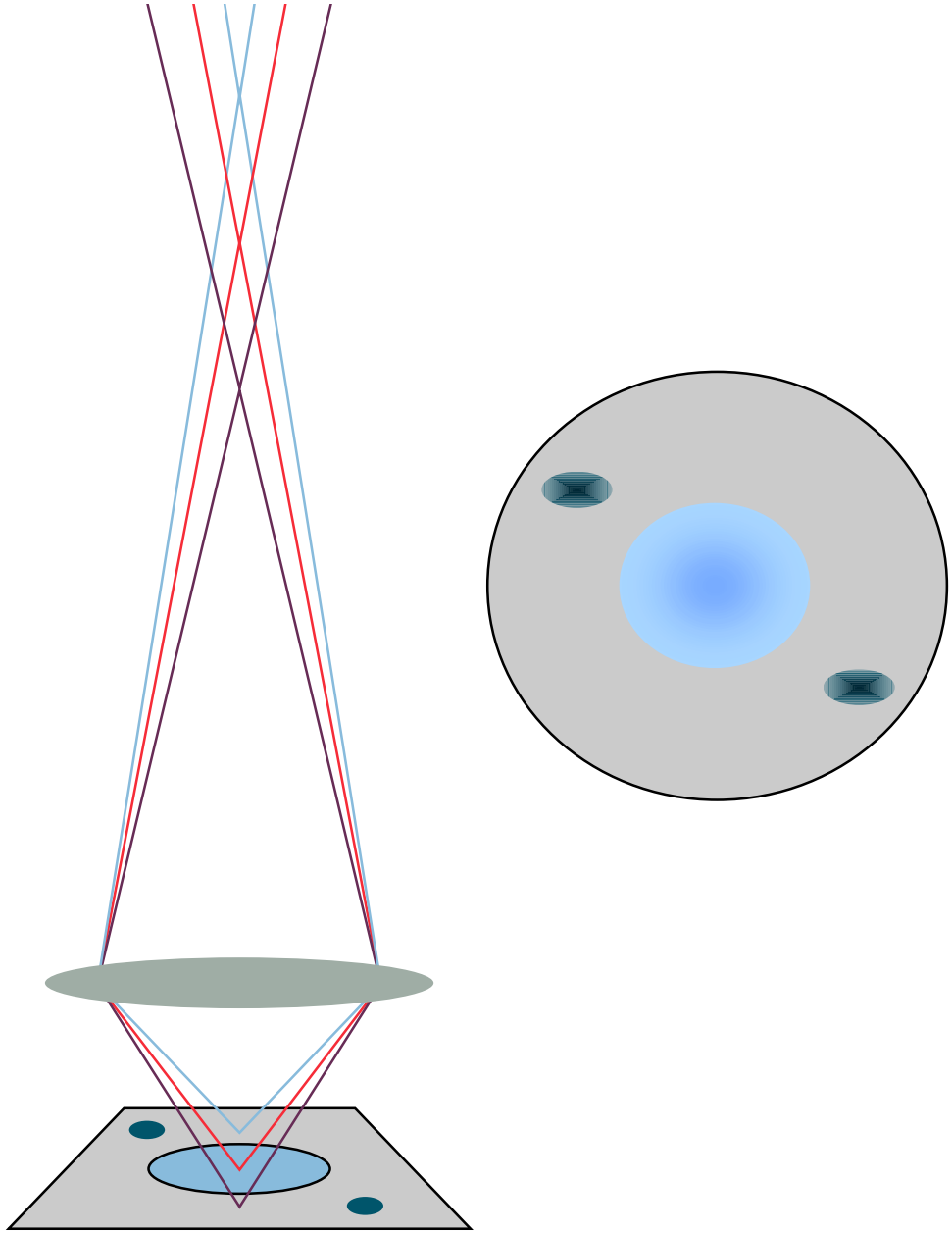
focus - standard microscopy

In standard microscopy, unless the specimen is very thin then areas of the specimen above and below the focal plane still contribute to the image as "out of focus blur"

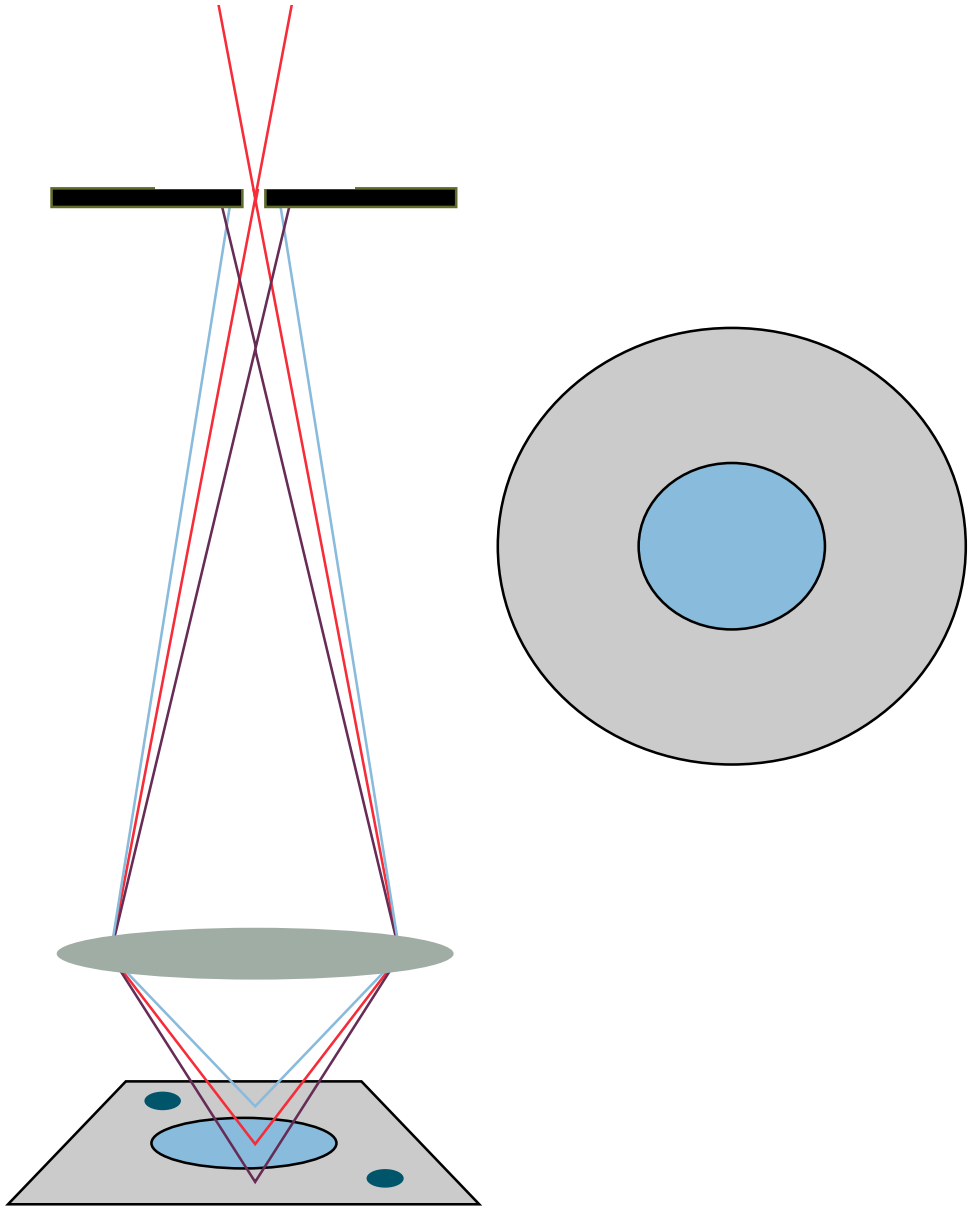




focus - confocal microscopy

In confocal microscopy, a pinhole between specimen and detector is used to select information from a single focal plane, producing a sharply focussed optical slice through the specimen.

Taking a series of optical slices from different focus levels in the specimen generates a 3D data set.

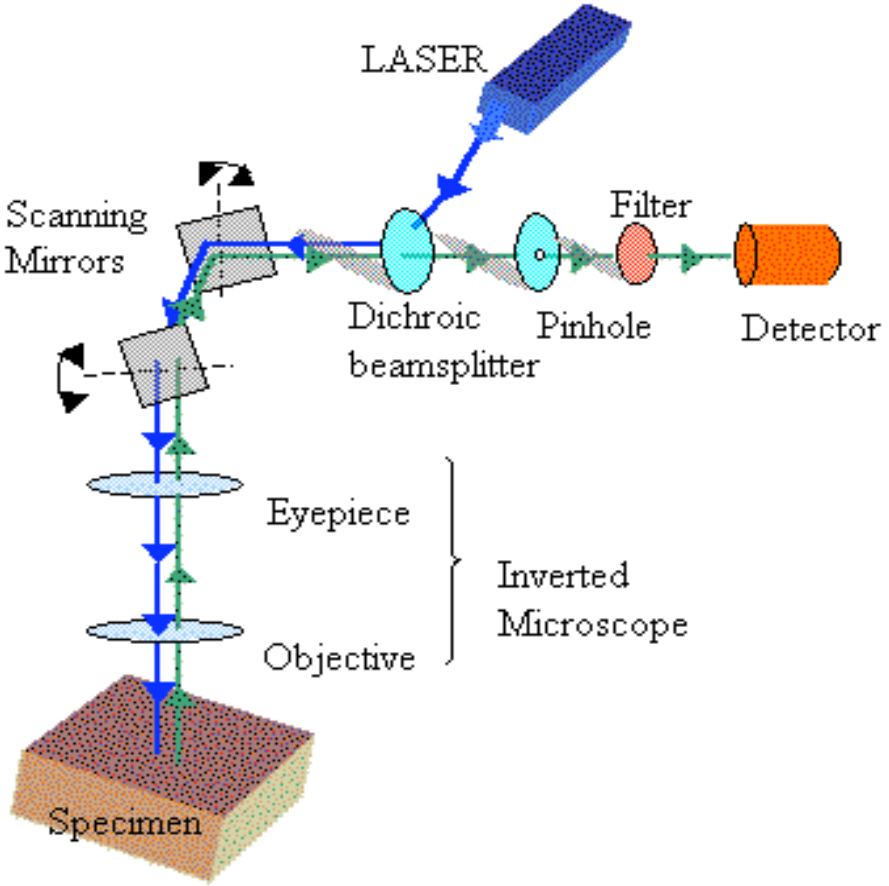


laser scanning confocal microscopy

a pair of oscillating mirrors raster scan a point of laser light across the specimen via the objective (epi-illumination) .

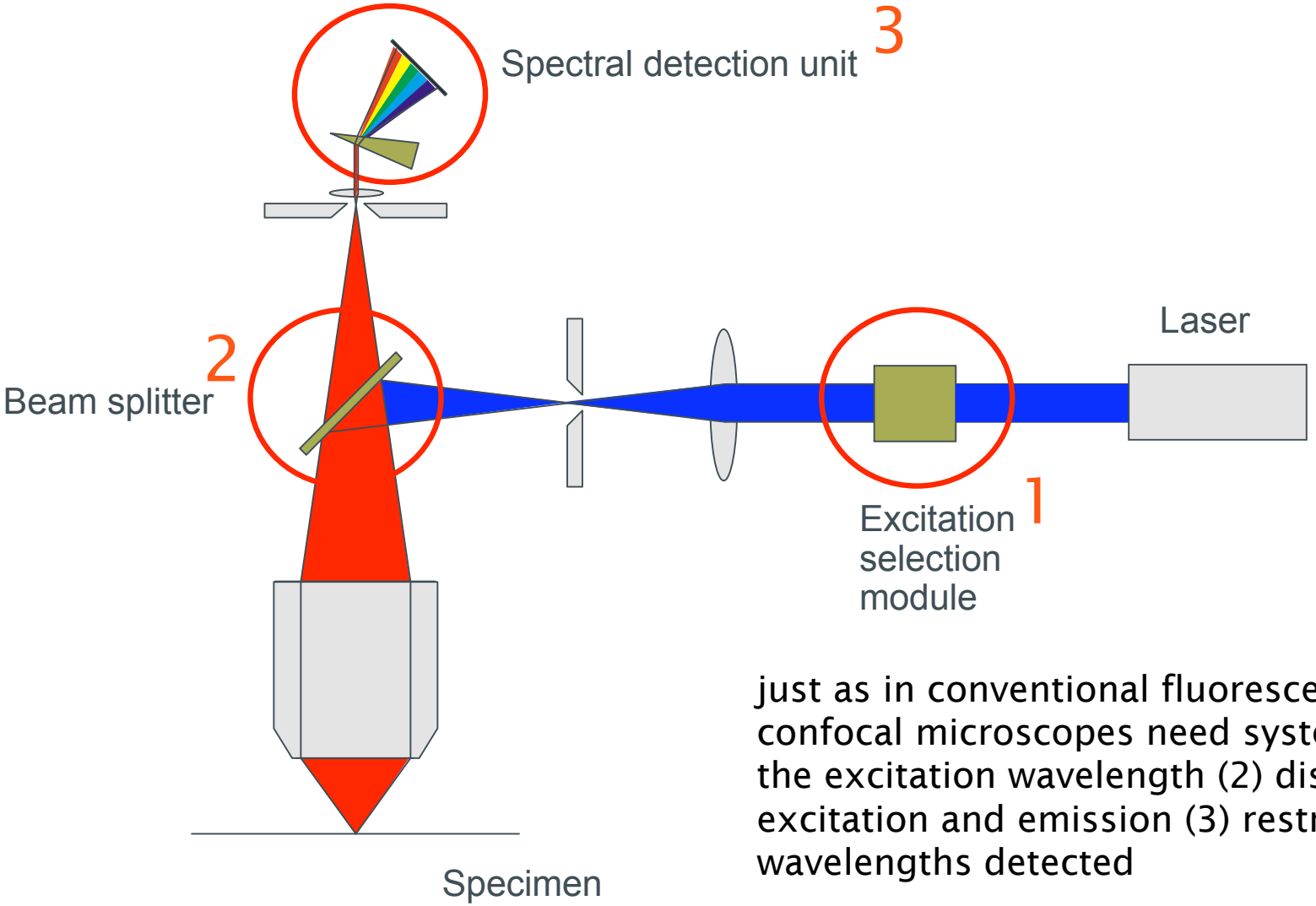
Fluorescence emitted by the specimen passes back through the mirror systems to a beam splitter which rejects any reflected excitation wavelengths and then through the pinhole to generate the optical slice.

The detector (a photomultiplier tube) simply records the brightness of fluorescence at each raster point and maps this into a 2D (XY)image.



Institute for Food research
http://www.ifr.ac.uk/materials/fractures/Confocal_microscopy.html

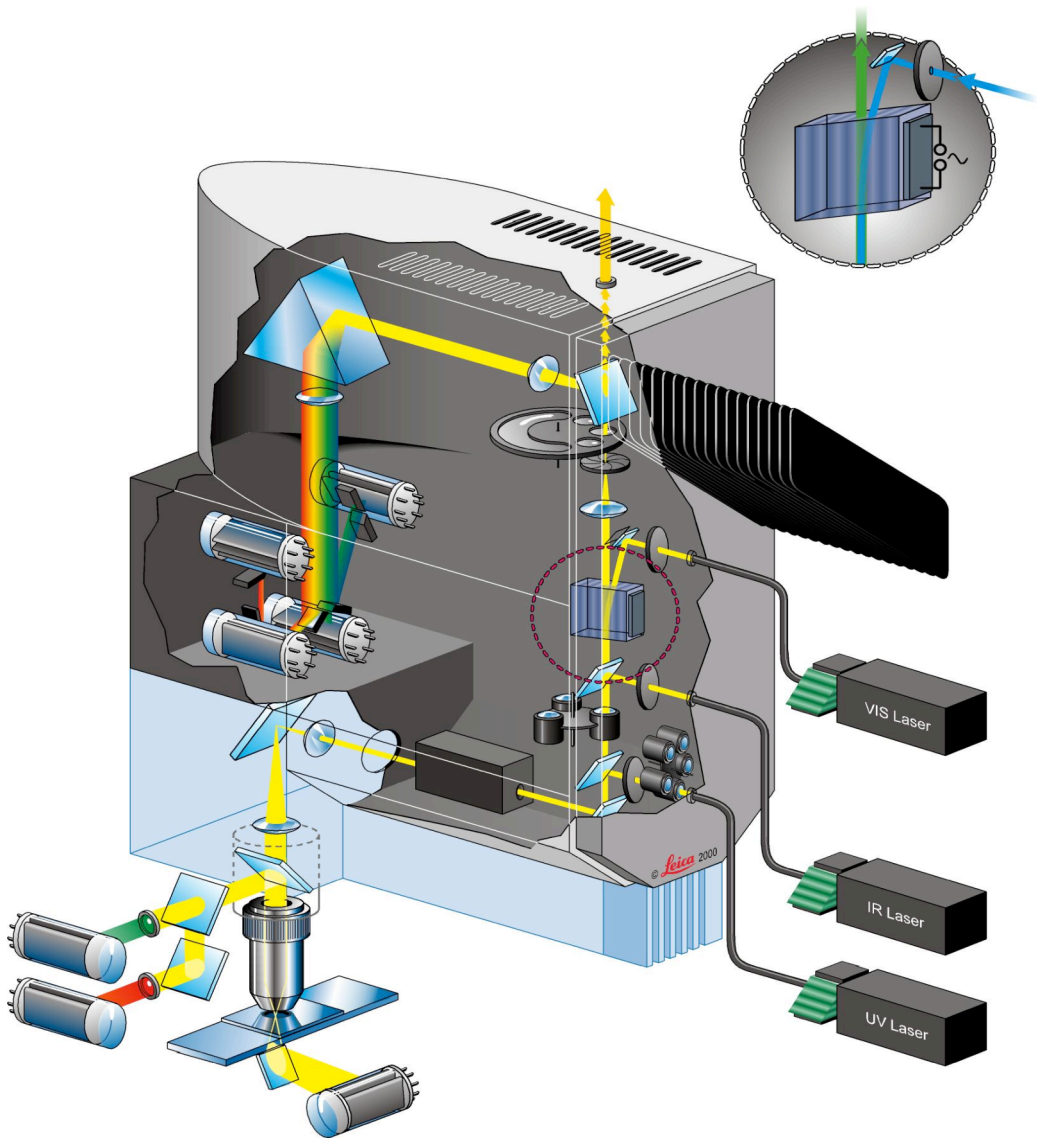
Leica wavelength selective elements



just as in conventional fluorescence microscopy, confocal microscopes need systems to: (1) select the excitation wavelength (2) discriminate between excitation and emission (3) restrict the wavelengths detected

Leica confocal microscopes are not filter based

- Acousto-optical tunable filter (SP2 & SP5)
- Acousto-optical beam splitter (SP5)
- Spectral detectors (SP2 & SP5)

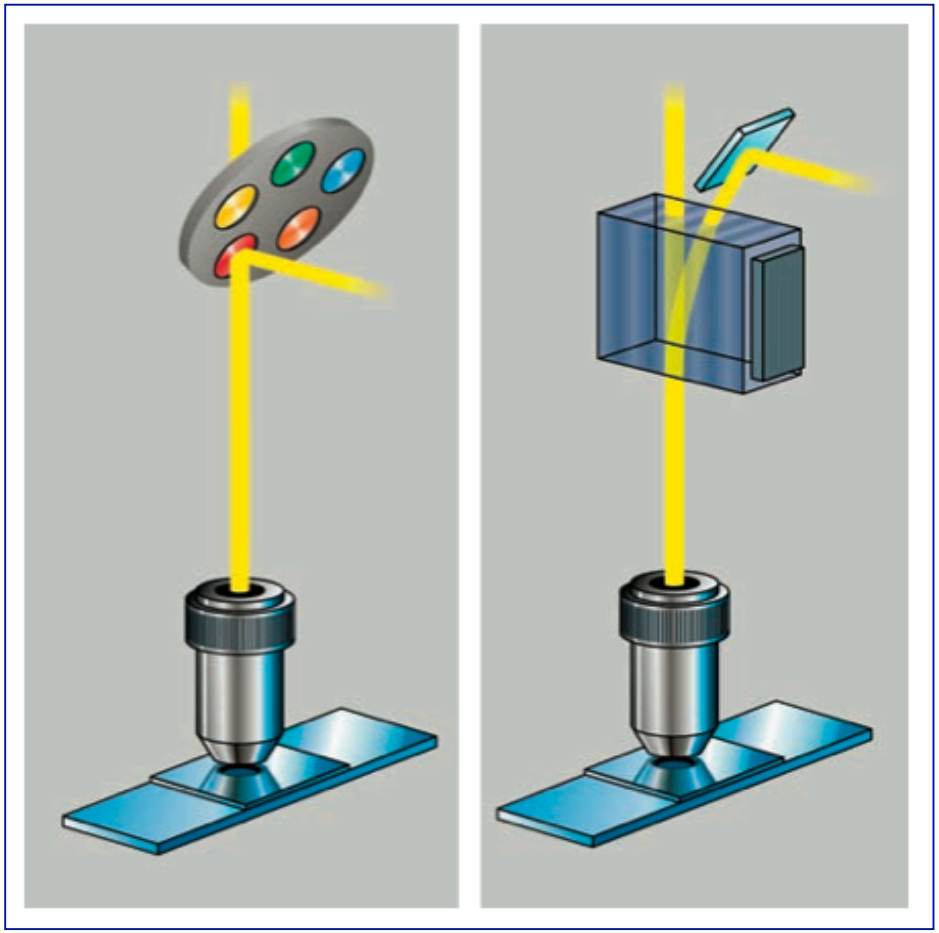


acousto-optical tunable filter (AOTF)

selects individual wavelengths from a multiwavelength output (argon) laser

Principle:

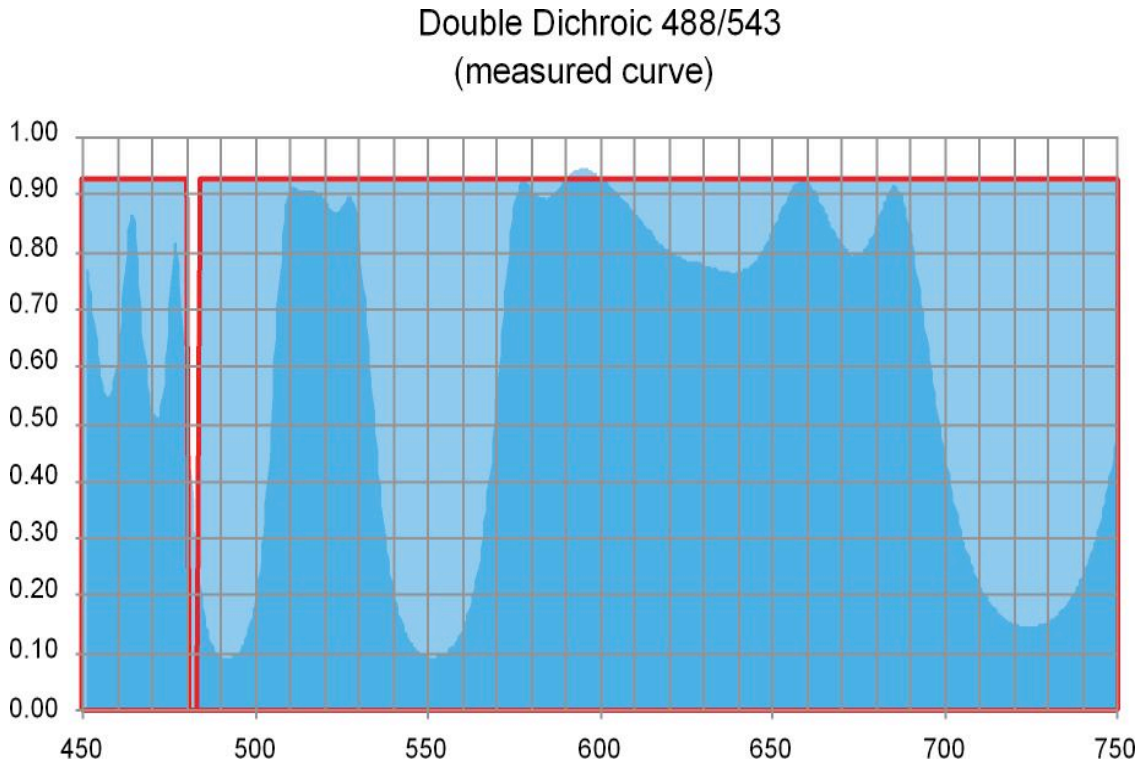
acousto optical beam splitter (AOBS)



AOBS acts like a dichroic beam splittter to separate shorter wavelength / higher energy reflected excitation from longer wavelength fluorescent emission

AOBS efficiency

AOBS shows a very clean, sharp cut off between signal transmission and rejection compared to standard optical beamsplitters



AOBS benefits

- Fast switching between channels
- Less bleed through with multiple labelling
- Higher signal transmission
- Less bleaching by optimising excitation intensity
- Easier region-of-interest scanning

spectral detection

- can detect any emission wavelength in the visible and near infra-red
- no need to buy new filters when new dyes are introduced

Leica spectral detector

fluorescence is focused into a parallel "rainbow" light path

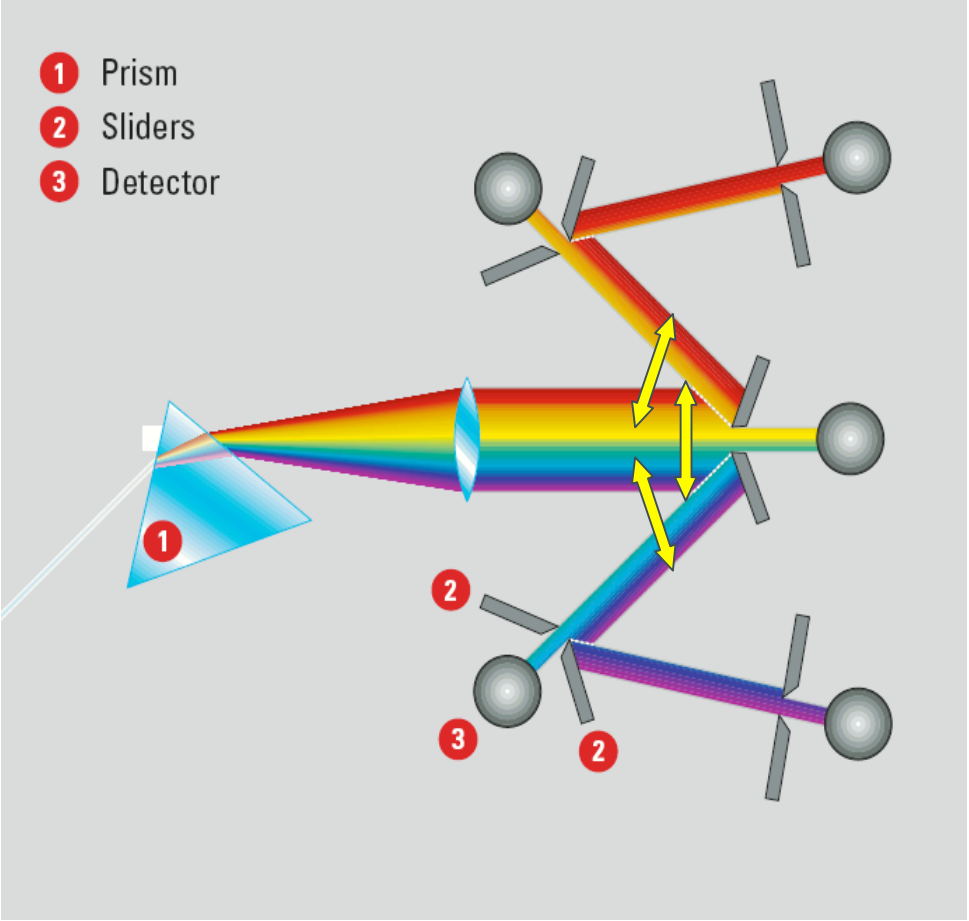
a slit edged with mirrors can be moved across the light path and opened or closed to specify which wavelengths reach the detector behind the slit

other wavelengths are deflected by the mirrors towards other detector /mirror / slit units

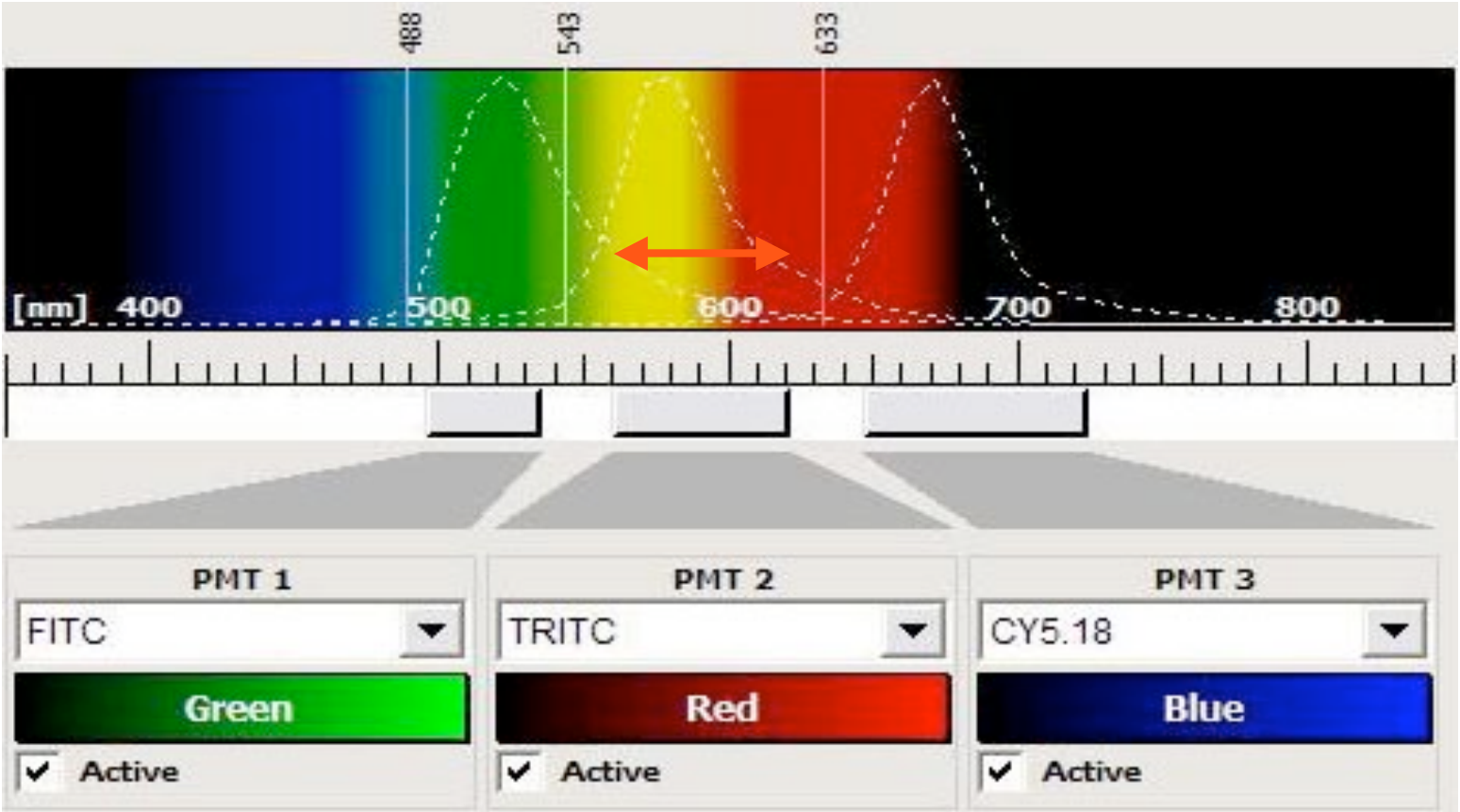
detectors simply measure brightness within the specified spectral range on a 256 level grey scale

false colour look up tables are used to map the intensity of each channel into arbitrary colours

our Leica SP5 has 4 spectral detectors

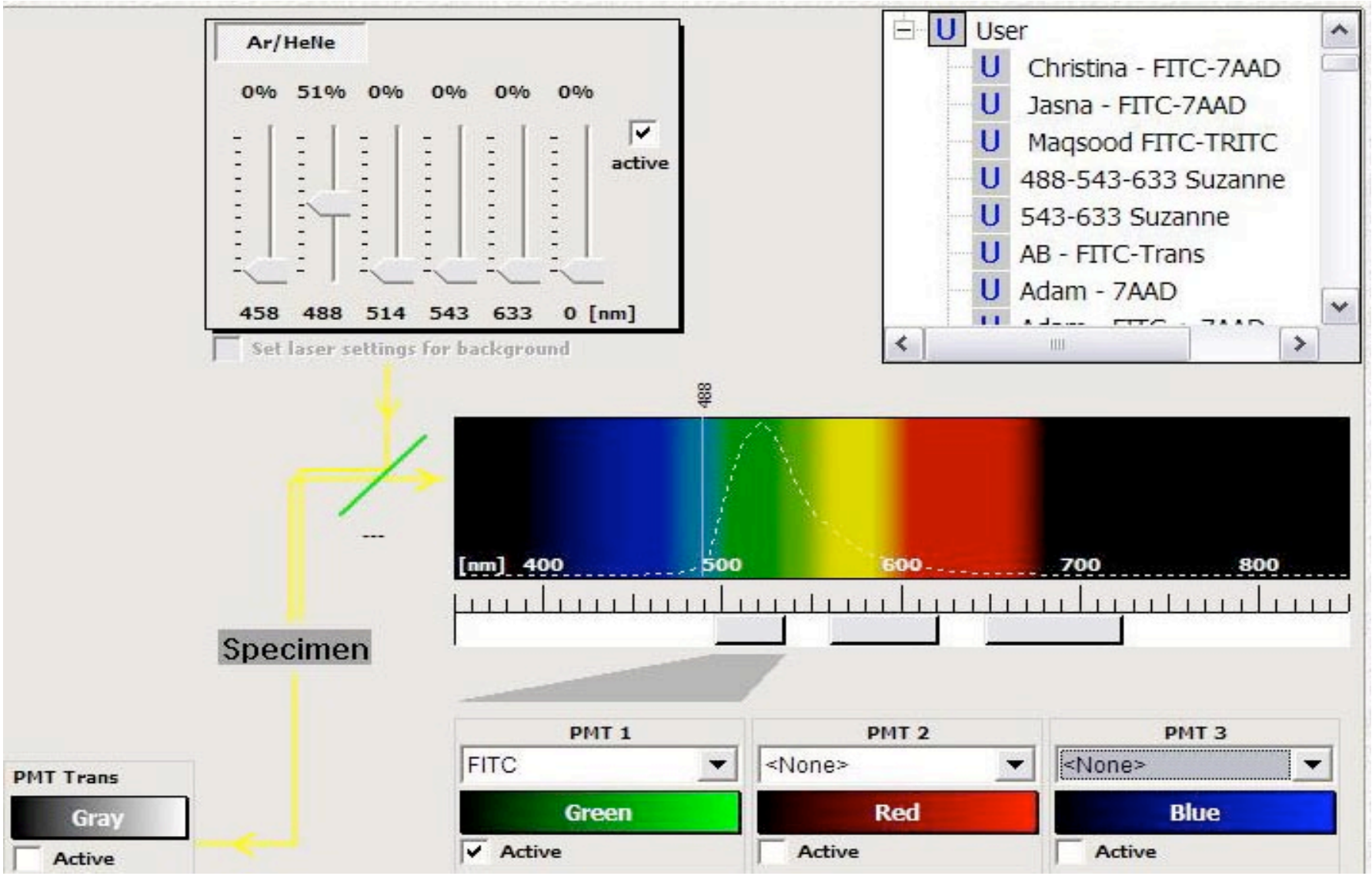


simultaneous acquisition



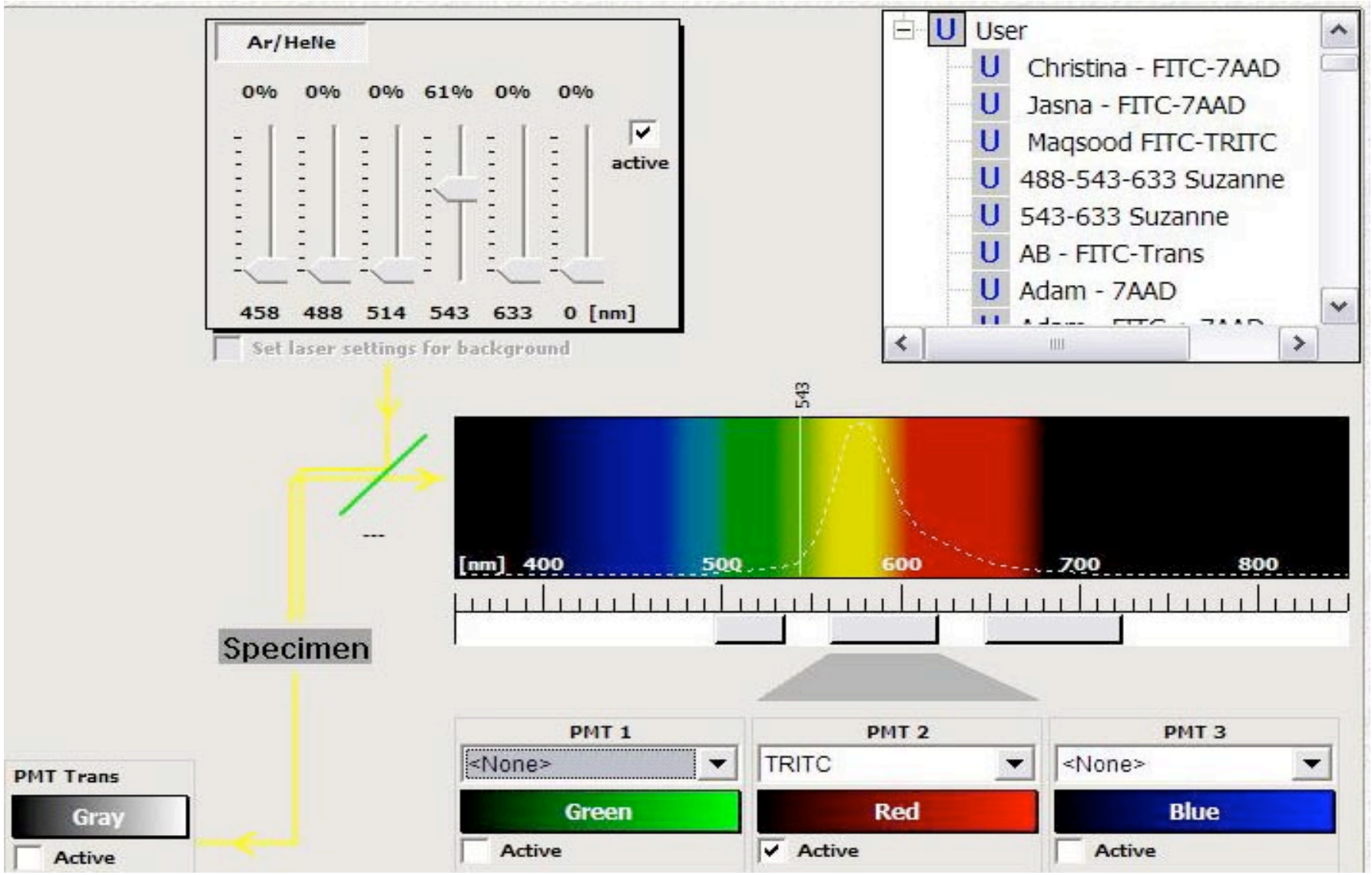
RH tail of FITC fluorescence falls within spectral region being monitored for TRITC fluorescence, ditto (to a lesser extent) for RH tail of TRITC fluorescence in CY5 detection region

sequential acquisition



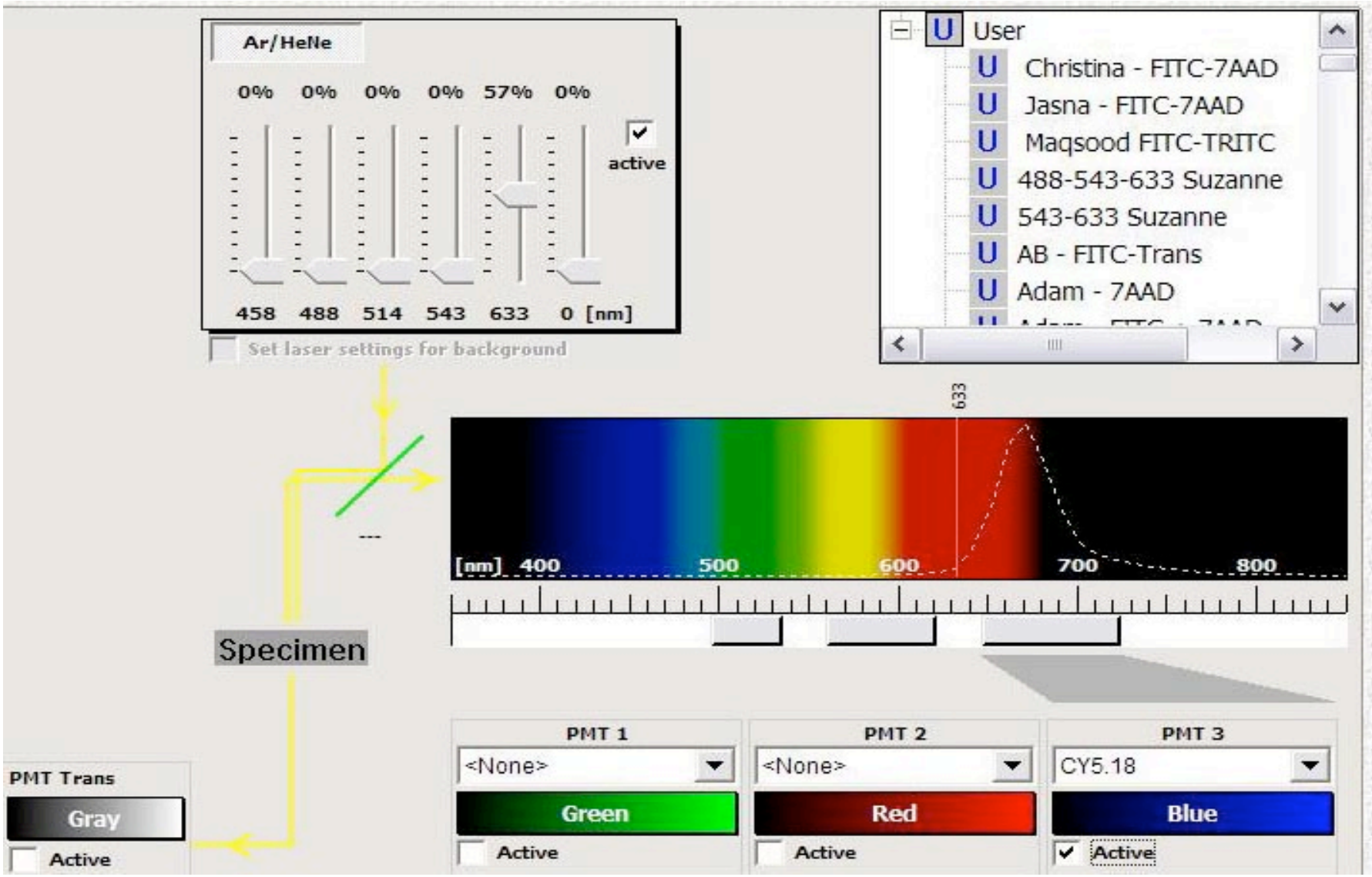
no TRITC region detection when FITC is being excited

sequential acquisition



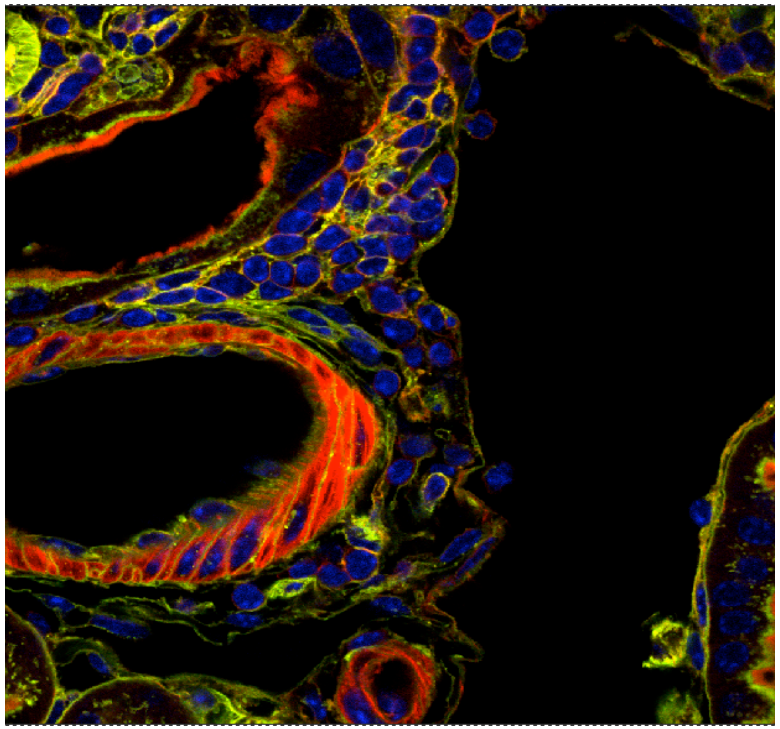
no FITC fluorescence when TRITC is being excited

sequential acquisition



no TRITC fluorescence when CY5 is being excited

simultaneous or sequential acquisition?

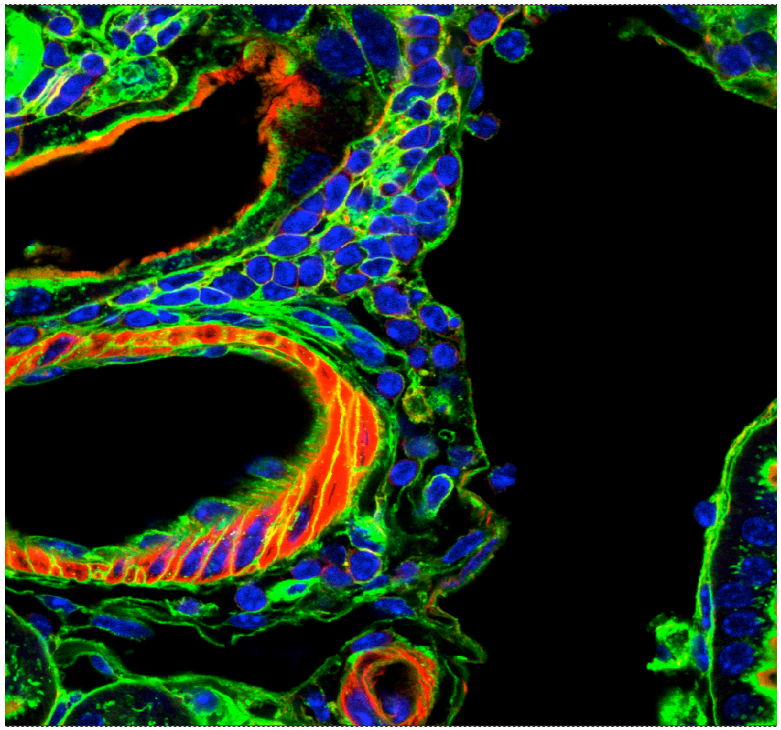


"simultaneous" acquistion:

crosstalk from Green to Red Channel

"sequential" acquistion:

no crosstalk from Green to Red Channel



images: Colin Park, Leica

Leica SP5 LSCM

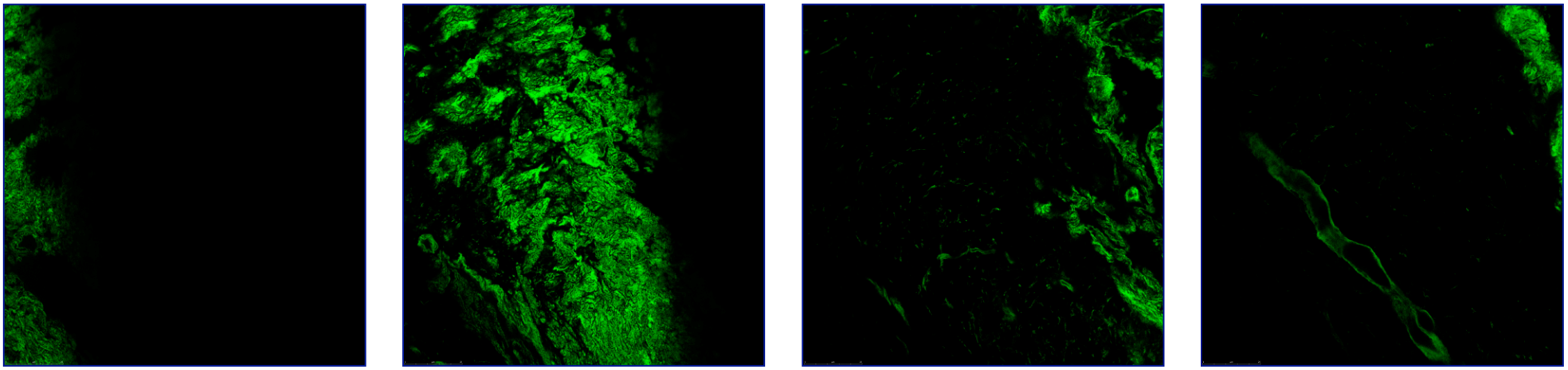
- inverted Leica DMI600 stand
- 9 laser lines (405 to 633 nm)
- 4 spectral detectors and transmitted light detector to >8000 pixel linear resolution
- objective range x5 to x100 with optical zoom
- DIC and phase contrast imaging
- environmental chamber and 5% CO_2 in air supply
- resonant scanner
- electronic stage with relocation and tile scan function
- standard fluorescence microscopy with FITC / TRITC / DAPI compatible filter cubes
- 4 MP colour digital camera

Leica SP2 LSCM

- upright Leica DM RBE stand
- 5 laser lines (405 to 633 nm)
- 4 spectral detectors and transmitted light detector to > 8000 pixel linear resolution
- objective range x5 to x100 with optical zoom, incl. x50 dipping lens
- DIC imaging
- galvo stage for fast XZ imaging
- standard fluorescence microscopy with FITC / TRITC compatible filter cubes

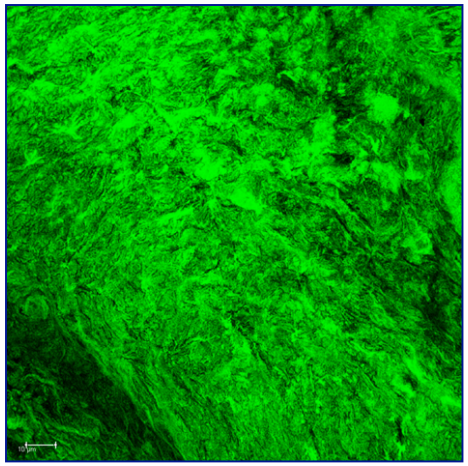
confocal image types

(1) animated Z stack



playing back the Z series as a movie can show the relative positions of fluorescent signals but does not reveal the "whole picture"

image shows collagen staining on skin basement membrane

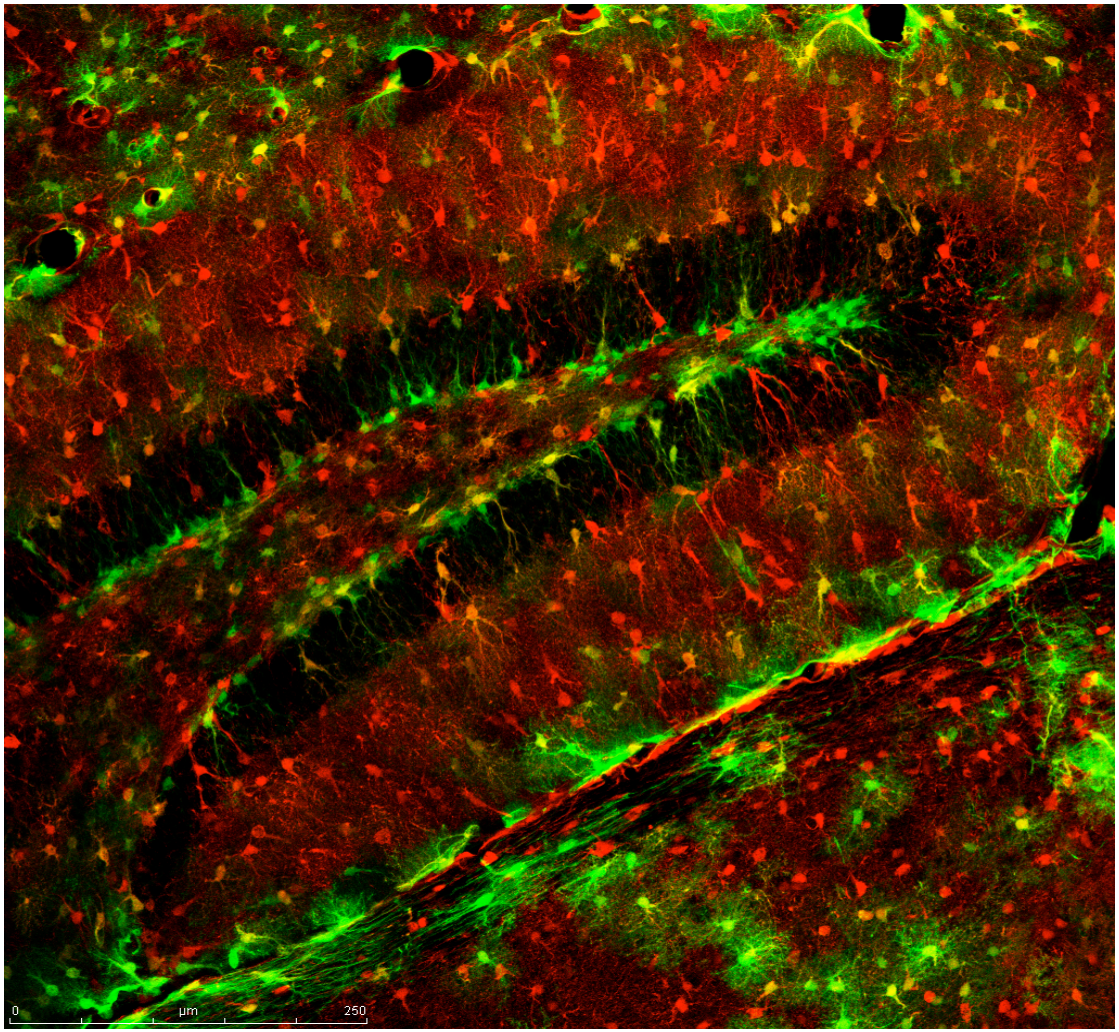


(2) maximum Projection

creating a 2D image, pixel by pixel using the brightest value for that pixel in the Z series for each colour channel

recreates the "whole picture" as a sharply focused image

it is useful where structural elements span multiple Z planes as it allows them to be visualised as a whole - eg. neurons in a brain slice

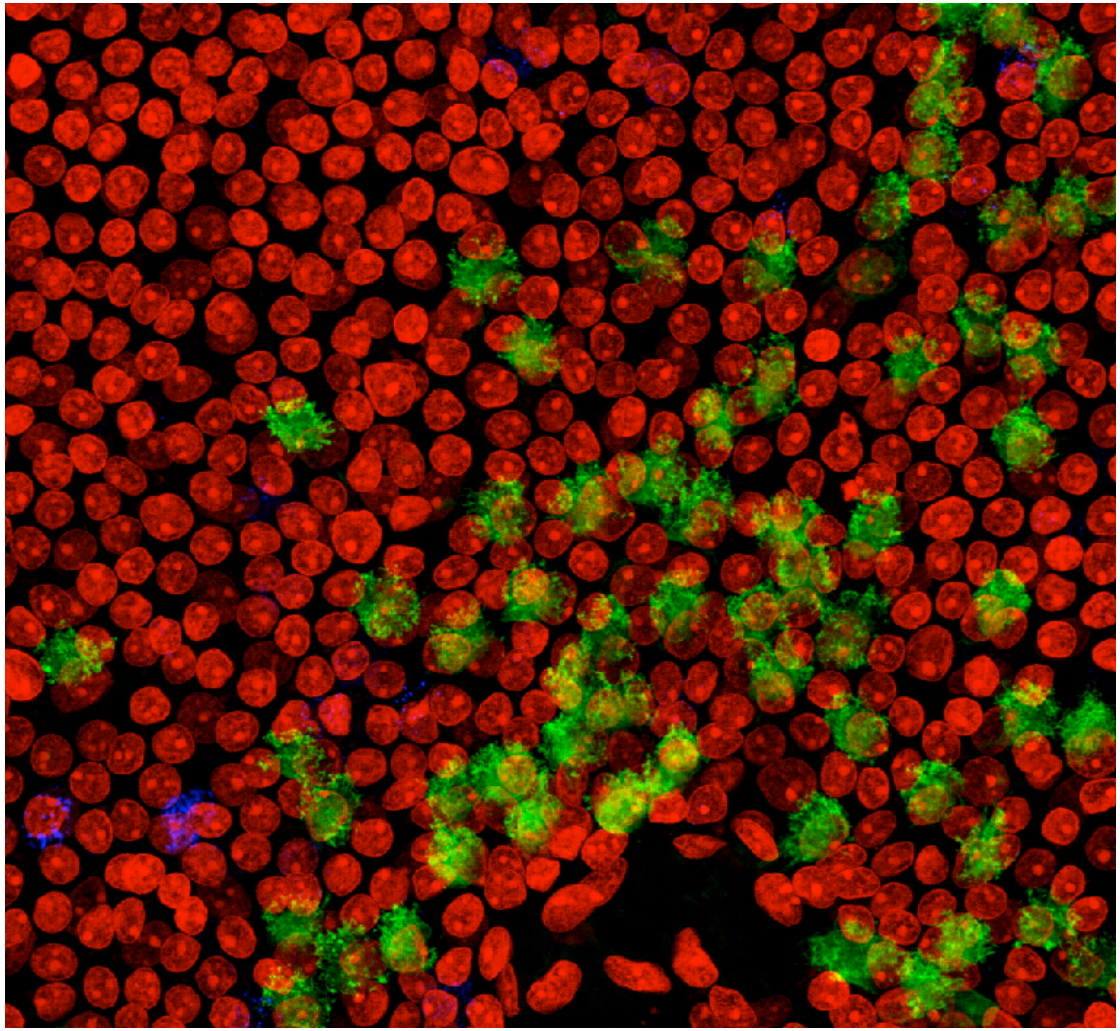


(2) maximum projection

a disadvantage of maximum projection is the loss of spatial information in the Z plane

in this image of bronchial epithelial cells, the maximum projection reveals that cilia (green) occur on a subset of cells and that a smaller and different subset are mucin (blue) producing goblet cells

however, there is no way to tell that the cilia are at the luminal surface, the blue in the middle of the cell and the nuclei (red) are basal

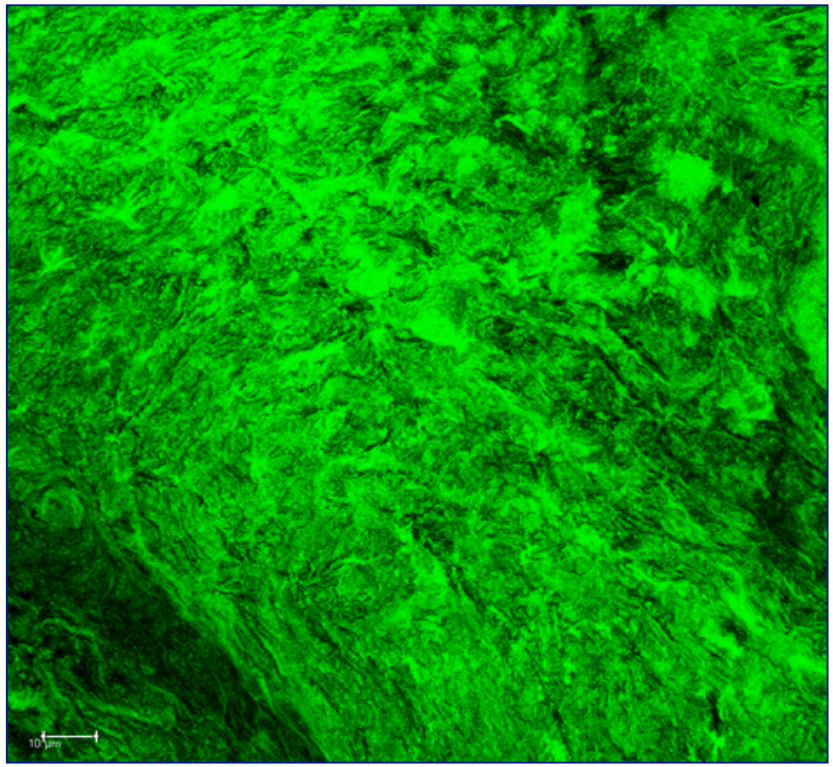


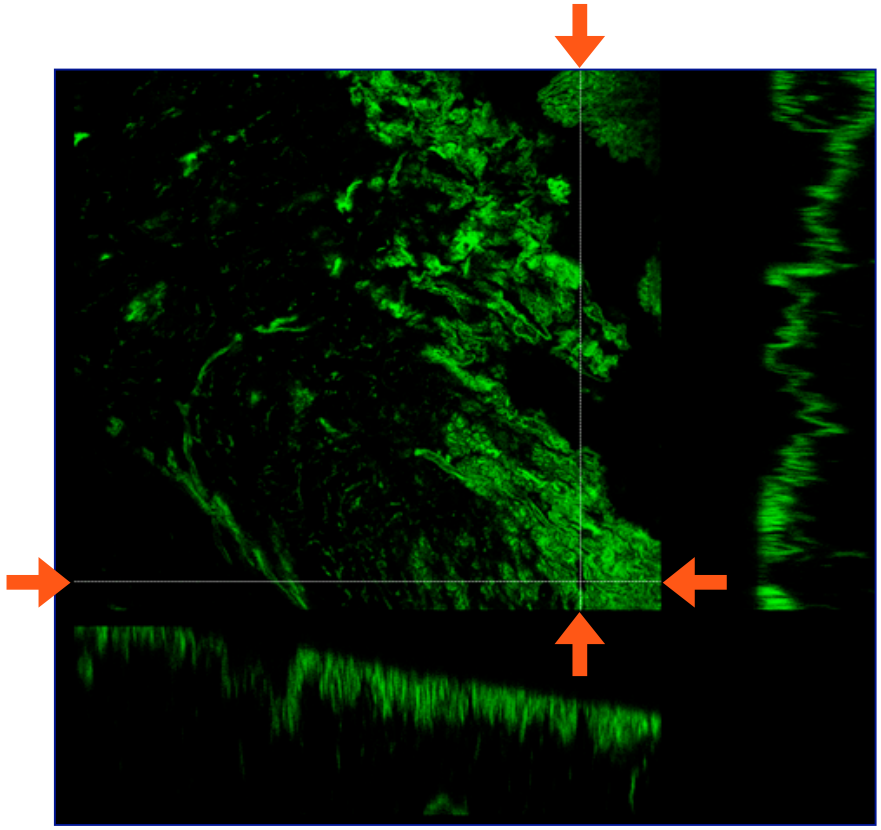
(3) rotated projection

since the Z series is a 3D dataset, it is possible to calculate what it would look like if viewed from any other arbitrary angle

it is therefore possible to create image series that show the sample rotating around any axis

(4) Z section

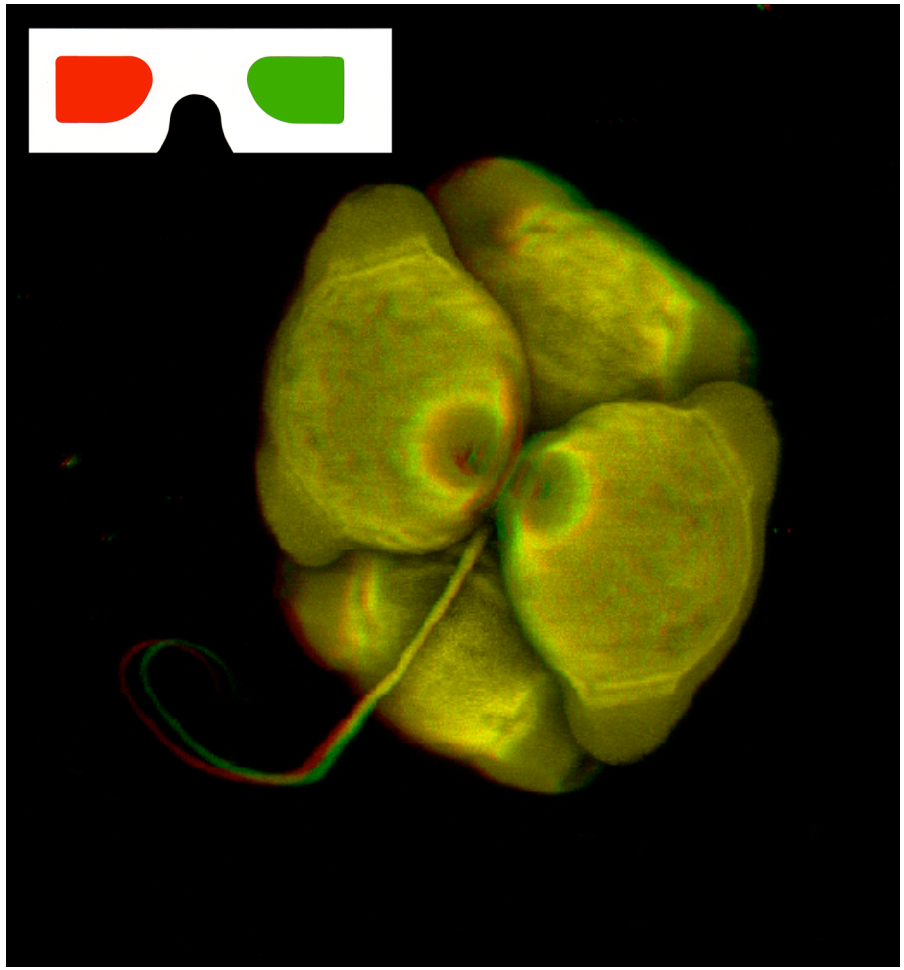




since the Z series is a 3D dataset, it is possible to calculate what it would look like if sliced across at any position

image shows the topology of skin basement membrane revealed by collagen staining: on the left, a maximum projection (no topology evident): on the right, Z sections through the positions indicated by the faint white lines reveal a convoluted surface

(5) 3D viewing



rotating a single channel Z series to the left and right by about 8-10 degrees, and mapping one image to green, the other to red produces an image that can be viewed in 3D

image shows willowherb pollen

application types

resonant scanning

Standard scan mode:

512 x 512 pixel scan = 2-3 fps

Resonant scan mode:

- true confocal scanning
- up to 1024 x 1024 pixels
- up to 5 channels
- 512 x 512 pixel scan = 25 fps
- 512 x 32 pixel scan = 250 fps
- 512 x 1 pixel scan = 16,000 fps
- useful for calcium imaging, muscle contraction and other rapid process imaging

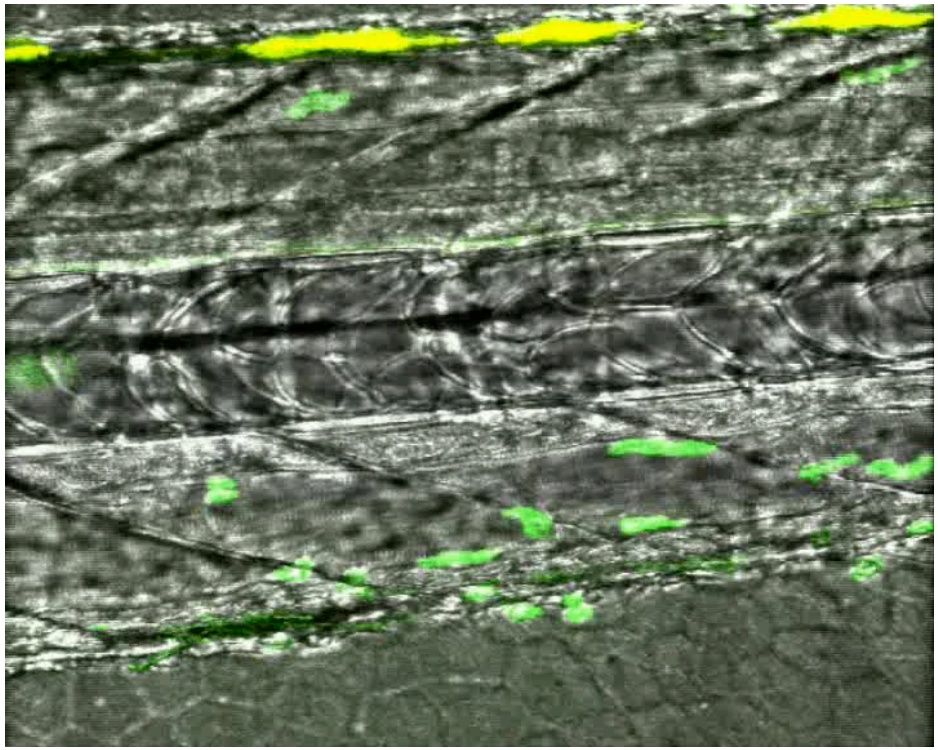
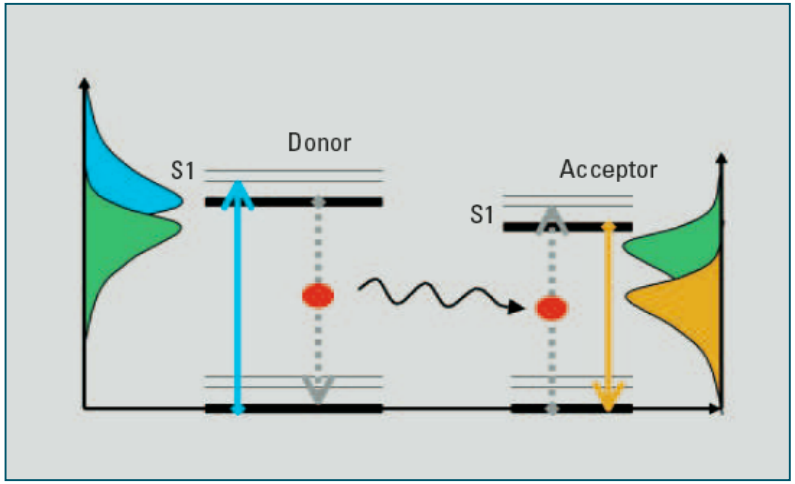


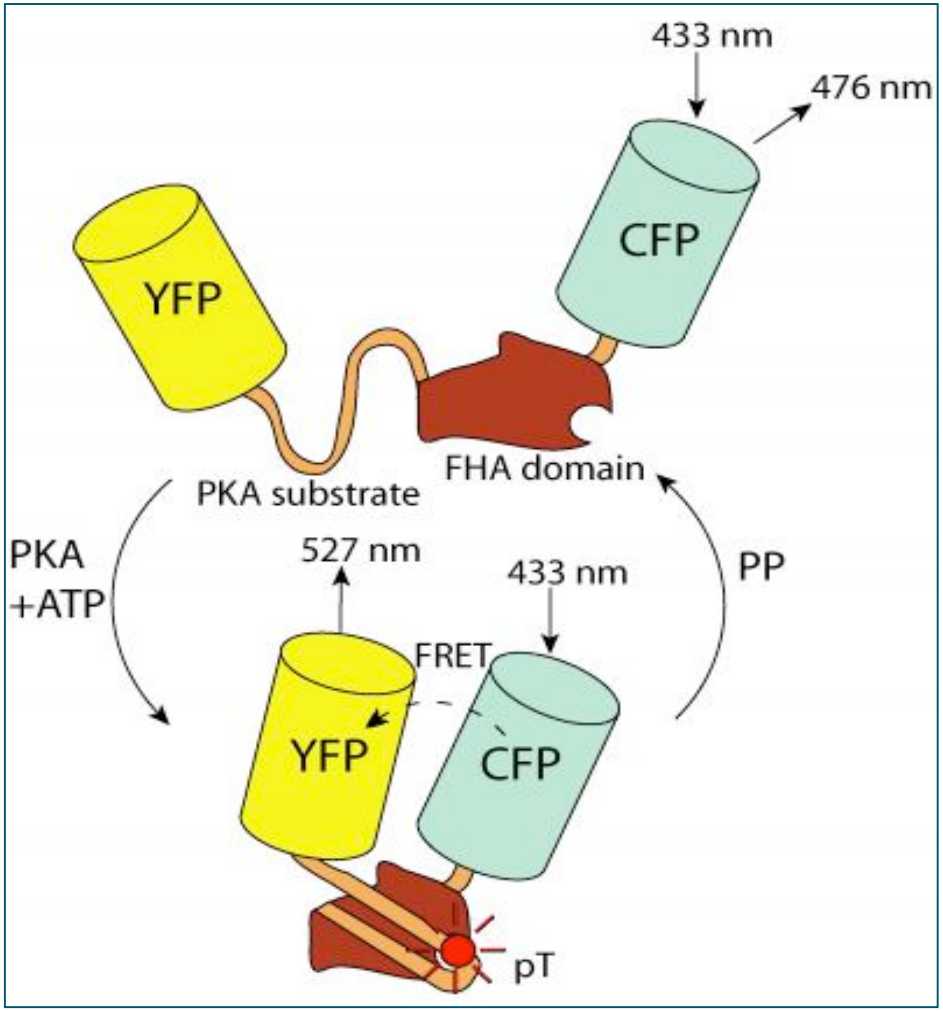
image: Leica Microsystems

FRET- fluorescence resonance energy transfer

a method to study inter-molecular interactions at the 1-10nm scale by transfer of energy between flurochromes

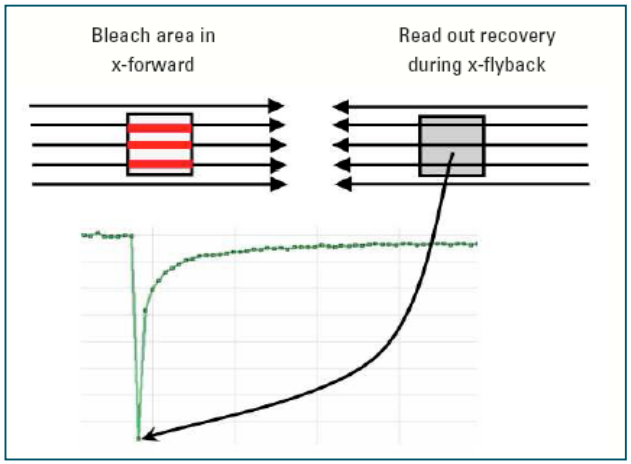
if donor and acceptor flurochromes are physically close enough, the energy emitted by the donor is not released as donor fluorescence but excites acceptor fluorescence





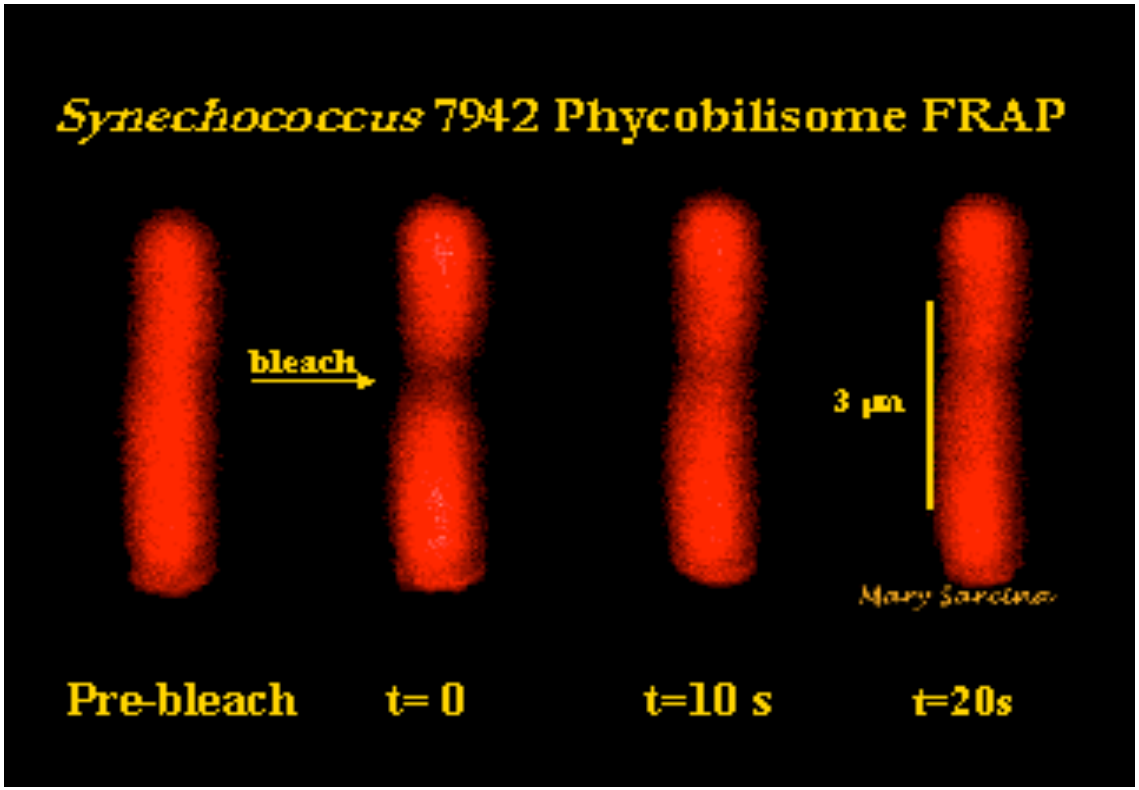
images: Leica Microsystems and AAAS

FRAP fluorescence recovery after photobleaching



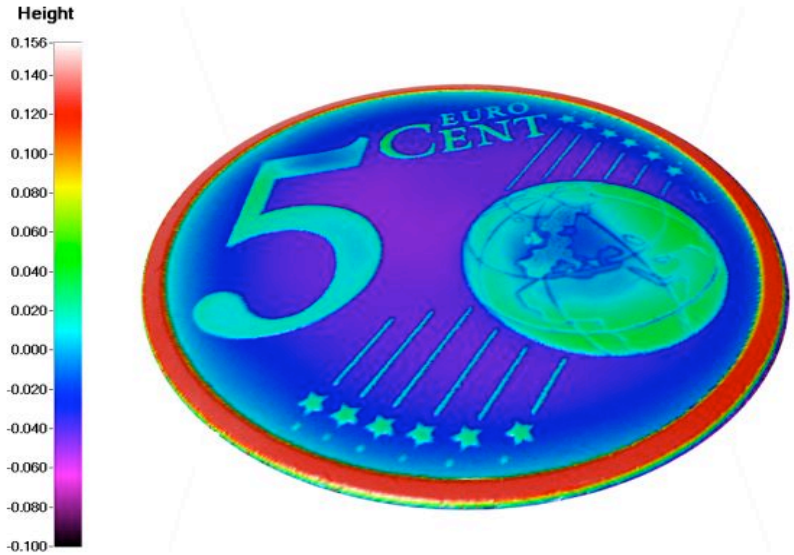
a method to study the rate of diffusion of fluorescent molecules in fluid media (cytoplasm, membranes etc.)

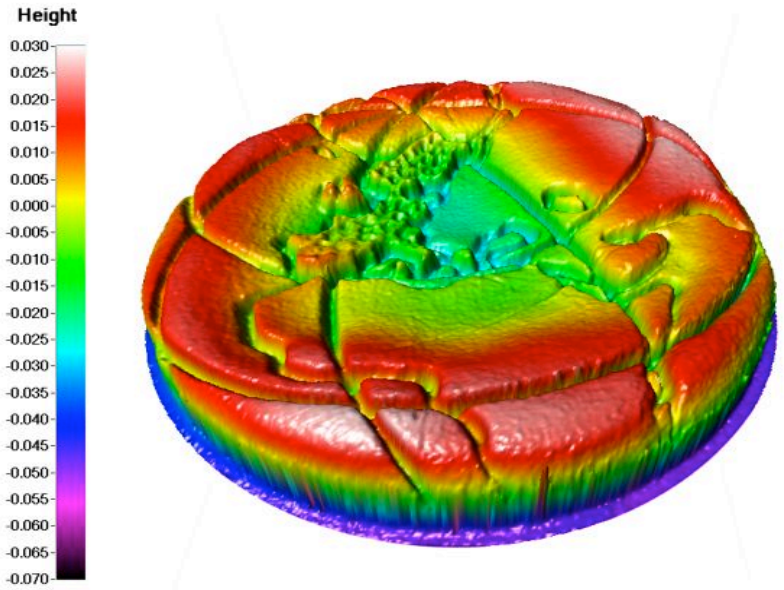
fluorescence in a specified region of interest is bleached by intense excitation and the rate at which fresh fluorescence diffuses back into the bleached region is determined



images: Leica Microsystems, BBSRC Imaging Facility, Daresbury

reflectance mode





images: TaiCaan Technologies