Fluorescence

Quantitative Microscopy Course
CBA 2012

Dirk Pacholsky
This lecture contains images and information from the following internet homepages:

http://micro.magnet.fsu.edu/primer/index.html
http://www.microscopyu.com/
Brief introduction to Fluorescence
Definition:
Fluorescence is the emission of light by a substance that has absorbed light or other Electromag. radiation. Form of photoluminescence. Usually: emitted light has a longer wavelength, and therefore lower energy than absorbed radiation. Emission of light happens in time scale of nano second – so to speak immediately

Compared to Phosphorescence:
- specific type of photoluminescence related to fluorescence. Unlike fluorescence, a phosphorescent material does not immediately emit light. Absorbed radiation may be re-emitted for up to several hours after original excitation. (wikipedia ;) )
Examples of fluorescent probes

Principle of fluorescent microscope

Excitation-Emission filter cube
Fluorescence: the Spectra

X axis: $\lambda$ in nm vs Y-axis: Intensity or probability of event that A) fluorophore absorbs the light for excitation (dashed line) and B) Fluorophore emits light (full line)

Normalized Intensity

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>QY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 488</td>
<td>0.92</td>
</tr>
<tr>
<td>Alexa 555</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Stokes Shift

- Ex peak at 100% $\rightarrow$ em peak at 100%, ex 20% $\rightarrow$ em 20%, same range of emission
- Stokes shift: gap between ex-peak and em peak = (loss of energy)
  $\rightarrow$ important for separation of excitation and emission light in microscope etc

Other important features of fluorophores:

- Extinction coefficient: absorbtion efficiency of a photon at particular wavelength
- Quantum yield: proportion of photons emitted at $\lambda$ em to those absorbed at $\lambda$ em
Parameters for fluorescence efficiency

- **Extinction Coefficient**
  - $\varepsilon$ refers to a single wavelength (usually the absorption maximum)
  - A measure of how efficiently a substance absorbs light of a certain wavelength

- **Quantum Yield**
  - $Q_f$ is a measure of the integrated photon emission over the fluorophore spectral band
  - The ratio of photons emitted to photons absorbed
Applications for fluorescent probes

- Proteins using antibodies
- Receptors using conjugated ligands
- DNA
- RNA
- Lipids
- Lectins to detect proteoglycans and glycolipids
- Cytoskeleton
- Organelles
- “Tracers” for cells and fluids
- Viability, proliferation
- Ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Na^+, K^+, Cl^−, …)
- ROS
- pH
- Membrane potential

See appendix for more information
The human eye perceives app. 400-700 nm

Visible Light Spectrum

Has a general resolution down to 150-300 μm

So we need a tool to see smaller things or more of the spectral range

→ Microscope (Objective
→ Camera/Film
Why Fluorescence microscopy

➔ You see only what you stain
Microscopical techniques

**Brightfield**
- low contrast for thin or translucent specimen
- staining to enhance contrast needed (histochemical staining)

**Phase contrast**
- contrast via optical element (Phase ring)
- intracellular structures can be seen
- good for cell culture applications
- negative: halos around cell bodies
- combining with other techniques is generally poor (e.g. overlay with fluorescent image)
DIC/Nomarski
- contrast via polarized light & optical element Wollaston prism
- gives a (fake) topographical view
- excellent for combination with fluorescence and histochemical staining

Fluorescence
- contrast via fluorescent staining
- special optical elements are needed (filter cubes)
- high resolution, high contrast, good for quantification (area + intensity)
- Staining is sensitive - it can fade.
The fluorescent microscope

Human eye perceives 400-700 nm
Camera/detector will do better

Filtercube with band-pass-filter to choose wavelength for Excitation and Emission, including a special (dichroic) mirror.

Mercury (Xenon) arc lamp spectrum: 300-800 (1300) nm
Objective projects image of specimen via Tube lens to Primary image plane. Eyepiece magnifies this image. *

* infinity corrected microscopes have parallel light beams between Objective and tube lens → space for different optical elements for different microscopical techniques

Old microscopes had „tube lengh“ of spec length & specific objectives which ´produce´ not convergent Light: vs

Objectives are not interchangeable

! Eyepiece/Ocular : set it to „0 – dot“ for Relaxed viewing (w/out glasses, lenses)
The Objective or Lens

The heart of a microscope may contain up to e.g. 12 lenses

Specification and Identification
- Magnification (enlargement)
- Numerical aperture (resolution)
- Immersion medium (should fit to embedding medium)
- corrections (spherical; chromatical)
- working distance
- tube length (infinity or 160 mm)
- coverslip thickness
A microscope magnifies a specimen with a certain resolution.

**Objectives**

- 10x / high NA
- 10x / low NA
- 20x N.A. 0.75 $\rightarrow$ 0.37 $\mu$m resolution
- 40x N.A. 0.75 $\rightarrow$ 0.37 $\mu$m resolution
- 60x N.A. 0.75 $\rightarrow$ 0.37 $\mu$m resolution

Magnification without resolution is useless: empty magnification.
Lens

Numerical aperture
N.A. = n \sin \mu

\[ \mu = \frac{1}{2} \text{ of the cone angle of light emitted} \& \text{ accepted by the objective} \]

\[ n = \text{refractive index RI bw medium} \& \text{ specimen} \& \text{ objective} \]

\[ \text{Air} = 1.000 \quad \text{Oil} = 1.515 \quad \text{Water} = 1.360 \]

Resol. \( R_{xy} = 0.61\lambda/\text{NA} \) (limit is 200nm)

\( R_{xz} = 2 \lambda/\text{NA} \times \text{NA} \)

(Confocal 0.4 for 0.61 \& 1.4 for 2)
Information (light) coming from above/below focus disturbs focus information.
→ unsharp images get overlaid with sharp images from focus
→ Blurred image in total

Get rid of that “extra” to “see” only information from Focal plane by Calculations (e.g. Deconvolution) or Technique → LSM...PINHOLE
Objective - aberrations

Chromatic aberration: → color fringes
Light of different $\lambda$ coming from same point in specimen not in focus in formed image due to defraction of light within the image forming system

Spherical aberrations: → unsharp images
Different focus of paraxial and peripheral light rays

Corrections are possible
Filters for the FL-microscope
Fluorescence: Filter and dichroic mirror

**Longpass LP filter**

**White light**

LP 550
All light with \( \lambda > 550 \) passes

UV G Y R

**Shortpass SP filter**

SP 550
All light with \( \lambda < 550 \) passes

BP 505-550
All light bw 505 550 passes

Light which is not passing might be absorbed OR reflected (to other filter)

**Bandpass BP filter**

BP 505-550
All light bw 505 550 passes

**Dichroic mirror, Beamsplitter FT 550**

White light

UV G Y R

All light with \( \lambda < 550 \) passes
All light with \( \lambda > 550 \) gets reflected
Dealing with fluorescence
Excitation 350 nm excitates Blue and Green, using BP filter 400-550 collects them both. * Remember: the camera is color blind. You decide with your choice of filter what it will see.
Excitation 350 nm excites Blue and Green, using BP filter 400-480 collects only the blue. *Remember: the camera is color blind. You decide with your choice of filter what it will see.
Excitation 350 nm excitates Blue and Green, using BP filter 400-480 collects only the blue. Remember: the camera is color blind. You decide with your choice of filter what it will see.
Combining Fluorescent Dyes - Crosschecks

To avoid false positive images in Fluorescence microscopy check for

Seeing is Believing
BUT Is it true?

Unspecific backgr. by Ab) ?
cell with - w/out target X

What’s to be seen in pos/neg control
stained
unstained

Crossreact AbX with AbY?

AbX AbY(1) AbX AbY(2)

Appropr. fixation?
Fixation A) X
Fixation B)

Crosstalk/Bleeding through?

Use quality objectives, correct filter, embedding medium
Kidney sample 10µm thick, 63x/NA 1.43, focal plane app. 500nm
Widefield image and optical section using Apotome technique
Optical section 700 nm
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Widefield versus Laser Scanning
Widefield microscopy and digital camera

- all pixels of the camera will be exposed to light at once
- image is processed all pixels at once
Laser Scanning microscope and PMT

- Specimen
- Image

- scanning pixel per pixel by Laser
- photons of one pixel per time will be processed by detector
- Image processing pixel per pixel line per line
In Widefield microscopy, illumination is provided to the entire field, whereas in Laser Scanning confocal microscopy, the excitation beam is scanned point by point to achieve high-resolution imaging.
Lens Information (light) coming from above/below focus disturbs focus information. → unsharp images get overlaid with sharp images from focus → Blurred image in total

Larger N.A. Collects more photons

Information (light) coming from above/below focus disturbs focus information. → unsharp images get overlaid with sharp images from focus → Blurred image in total

Get rid of that “extra” to “see” only information from Focal plane by Calculations (e.g. Deconvolution) or Technique → LSM...PINHOLE
Illumination of Specimen in Widefield microscopy

- **CCD**
- **Excitation filter**
- **Fluorescence Barrier Filter**
- **In-Focus Light Rays**
- **Dichromatic Mirror**
- **Objective**
- **Light Source Pinhole Aperture**
- **Focal Planes**
- **Specimen**
Illumination of Specimen

Laser Scanning microscopy
Comparision Widefield vs LSM

Widefield images of thick specimen

LSM images of thick specimen
Airy disk or Airy Unit
AU
A point of light in the sample will not be a point of light in the image. Light originally coming from a point and passing through lenses etc. will not be a point again in the image, but rather a dot (1st maxima, Airy Disk) with several side maxima separated by mininima.

This Spreading is called Point Spread Function (PSF).

In 3D: a spheroid with "flames"

The yellow dots shall indicate infinite points, where light originally came from.
NA = (n)sin(\(\mu\))

(a) \(\mu = 7^\circ\)  NA = 0.12
(b) \(\mu = 20^\circ\)  NA = 0.34
(c) \(\mu = 60^\circ\)  NA = 0.87
Objective Magnification - NA - optical section

The maximum resolution is app. 0.15 µm lateral
0.40 µm axial

<table>
<thead>
<tr>
<th>Objective Magnification</th>
<th>NA</th>
<th>pinhole size</th>
<th>7 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>60x</td>
<td>1.40</td>
<td>0.40 µm</td>
<td>1.90 µm</td>
</tr>
<tr>
<td>40x</td>
<td>1.30</td>
<td>0.60 µm</td>
<td>3.30 µm</td>
</tr>
<tr>
<td>25x</td>
<td>0.80</td>
<td>1.40 µm</td>
<td>7.00 µm</td>
</tr>
<tr>
<td>4x</td>
<td>0.20</td>
<td>20.0 µm</td>
<td>100 µm</td>
</tr>
</tbody>
</table>

Optical slices not start/end abruptly at certain Z depth. Due to intensity distribution along optical axis, there is a continuous transition from object information suppressed and such made visible.
Laser /Diode source

Lasers

Argon Ion (Ar)
Krypton (Kr)
Helium Neon (He-Ne)
Helium Cadmium (He-Cd)
Krypton-Argon (Kr-Ar)
Diode lasers

Argon  Ar  353-361, 488, 514 nm
Krypton-Ar  Kr-Ar  488, 568, 647 nm
Helium-Neon  He-Ne  543 nm, 633 nm
He-Cadmium  He-Cd  325 - 441 nm
Diode lasers  405, 488, 635 nm etc
Filters (LSM510) or prism (LSM710)

META detector for spectral imaging

Emission filters
PMT
Dichroic mirrors
Beam splitter
Light coming from sample
Filters (LSM510) or prism (LSM710)

- Grating to disperses incoming light into its wavelength
- Spectral recycling loop
- Prism and blocker to freely choose the spectrum range to collect with the PMTs

Light coming from sample

Master pinhole

PMT

PMT

QUASAR array for spectral Imaging
The Photomultiplier tube

Light beam (photons) hits photocatode which emits photoelectron which hits dynode, which emits more electrons which reach anode to be electrical read-out.

All depend on GAIN settings

Best PMTs reach 30% efficiency...photocatode is important:
- Too thick - too much absorbance - less emission
- Too thin - photons pass through - less emission,
Side on PMT more sensitive (quantum efficiencies)
But better electron gain in end-on PMTs due to more dynodes (14 vs 9)
What is best sampling rate or pixel size?

Each pixel generates noise.
The incoming signal has to be more intense than the noise (Signal:Noise ratio).

Small pixels get less photons, but generate same/more noise like large pixels (who get more photons).

Practically: pixel size is twice as small as smallest detail to be resolved.

**LSM allows you to choose pixel size you sample with**
Imaging with LSM
Image quality - over/underexposure

Sample: 3 color staining

 Balanced imaging
over/underexposure

´palette´mode visualizing
Over/under exposure

Remember: all color is based on grey value

Red: overexposed
Blue: underexposed
Image / Channel information

original Images stores meta-data: information about pixel/voxel size, image mode, user, Laser/filter, objective used.

RE:USE this info for future application

Image channel split
free choice of color per channel
Better resolution by Pinhole size

Pinhole fully open  half closed (7 AU)  closed to 1 AU

By closure of the pinhole, light coming from above/below the focal Plane will not get collected by the detector.

! The smaller the pinhole the smaller the actual focal plane from Which light gets collected – good staining is needed!
Image quality

Average mode: instead of "reading the specimen" only once, one can "read it" 2, 4, 8, 16 times. And average these 2 or 16 images into one.
→ Better signal to noise ratio → better image.
...BUT Bleaching might occur...

The "average" can be combined with ´pixel dwelling time´ - the time of how long the laser excites the pixel/voxel and hence how much time there is for the objective /PMT to gather emitted photons.
... BUT Bleaching might occur ...
Images taken according to Nyquist, and scan speed 7, avg 2, 63x/NA1.4

A) Zoom 1.0, 2048x2048px, scan : 16 sec
B) Zoom 2.0, 756x756px, scan: 8.0 sec
C) Zoom 4.0, 376x376px, scan : 2.8 sec
D) Zoom 4.0, 1024x1024px, scan : 7.8 sec

→ A, B – bleach window from former scan
→ C, oversampling but not better resolution
From Optical sectioning to 3D reconstruction

Optical slice from certain depth in sample

Many slices from adjacent depths

3D reconstruction of all slices

For optimal settings concerning resolution
Some parameters have to fit.
See Nyquist theorem
Problem of Fluorophore fading

Coverslip Laser

Focal Plane

Glass slide Bleached fluorophore

Scan for image 1
Scan for image 2
Scan for image 3
Scan for image 4

Every image scan excites the whole specimen
Bleaching

Bleaching before and after 100x imaging same area with Widefield microscopy. Test sample is a strong stain and so bleaching might be subtle and only clearly be see in LUT (look-up-tables)

Intensities of emission are shown in LUT
Black to white
LUT= Blue, over green, yellow, red

You might not see the subtle changes But would like to compare Intensities Between image 1 and 2?

Be aware... Have some internal standard
Gallery view of optical slices from LSM images

Part of gallery of 107 optical slides through plant stem

Subset gallery of 107 optical slides through plant stem
Slide XX to YY every 3rd
3 D information from LSM images

Orthogonal view

3D information, dashed lines in blue, red, green indicate position in ZXY and are movable

Observe that light could not penetrate material on certain areas*

3D surface reconstruction

3 dimensional reconstruction of image

Observe that light could not penetrate material on certain areas*
3D information from LSM images

Color depth coding

Overlay of all (or selected) optical slices into one merged image with colors indicating their depths

Maximum Intensity Projection MIP

Overlay of all (or selected) optical slices into one merged image
Line Scan with LSM

Whole image of kidney sample

Line scan including Z stacking
Line Scan with LSM

Line scan of fast moving particles over time

- Time point sec 100
- Events over baseline
- Time point sec 2000

One scan at a specific time point

Bidirectional scan
Scan process takes its time
(depending on frame size you intend to scan)
Laser power should be low
(to avoid cell damage)

Frame size in pixel: frames/sec
2048 x 2048 0.03
1024 x 1024 0.13
512 x 512 0.53
256 x 256 2.00
128 x 128 5.00

http://vimeo.com/4257616
Techniques for the LSM and Live Cell Imaging

Photoactivation /uncaging

**FRAP**

Fluorescence recovery after photobleaching (FRAP)

Initial condition

Laser Beam

Photobleach

Recovery

Cell

Caged

Scavenger

Activated

Fluorescence

$F_i$

$F_\infty$

$F_0$

$1/2$

Time

**CALI**

Chromophore Assisted Light inactivation

FRET Detection of *in vivo* Protein-Protein Interactions

Blue Fluorescent Protein

380 Nanometer Excitation

No Green Fluorescent Protein Emission at 510 Nanometers

Separated Protein Molecules

BFP

GFP

Intermolecular Association

Figure 2

Blue Fluorescent Protein

380 Nanometer Excitation

Green Fluorescent Protein Emission at 510 Nanometers

BFP

GFP

http://www.cellmigration.org/resource/imaging/imaging_approaches_photomanipulation.shtml
Imaging – Nyquist theorem

To sample each spatial frequency (resolved feature) at least 2.3 times gives a reasonable resolution. This is the Nyquist theorem.

Why not use more pixels and oversample?

More and smaller pixels = more background noise

More and big pixels = expensive camera

On all LSM there is a OPTIMAL button which calculates the potential best imaging settings regarding pixel size (Nyquist) and pixel dwelling time (Signal to noise).

Drawbacks:

- Probably long times to wait for a scan
- Your sample might be bleached

Find a compromise between Image quality needed versus Sample robustness.
A Multiphoton microscope gives you the opportunity to get images from deep (e.g. 500 nm) within (living) tissue, whilst photodamaging imaged volume only*.

A Multiphoton microscope is a point scanning system which excites fluorophores within the Focus volume* only. Therefore you collect emission light from this volume only, enabling you to acquire optical slices, without the use of confocal pinholes.

Beside this, one is able to photomanipulate tissue/cells within a very small volume.
Why use 2-Photon microscopy?

"See deep and excite to the point"
Why use 2-Photonmicroscopy? – see deep

Dendrites of cortical projection neurons of a transgenic mouse expressing YFP via the thy1 promoter. This high-resolution image of the dendritic processes to a depth of 430 µm was made using multiphoton excitation of 920 nm in the living animal. Specimen provided by Stephen Turney, MCB, Harvard University, USA

3-dimensionally constructed images of neurons expressing EYFP in the cerebral neocortex of a mouse under anesthesia. Cross-sectional images down to 0.7 mm from the surface can be observed after attachment of a special adapter to the specimen.

Objective: LUMPlanFL 60xW/IR

Hiroki Waki, Tomomi Namoto, and Junichi Nabekura
National Institute for Physiological Sciences, National Institutes of Natural Sciences, Japan

app. 450µm

app. 600µm
Why use 2-Photon microscopy? – see deep

NIR light: 700-1100nm travelling through specimen to focal plane will not scatter and disperse* as much as light of shorter $\lambda$ (350-633 nm for FL microscopy)

$\rightarrow$ excitation of fluorophores in greater depth

Problem: different fluorophores need its own NIR Laser?

Solution: Laser can be tuned from e.g. 690 to 1040 nm, fluorophores have wide excitation range in 2PM

*(due to different refractive indices of the various components in specimen)
Why use 2-Photon microscopy? – focus spot only

How is this achieved?
Why use 2-Photon microscopy? – focus spot only

Multiphoton                      LSM

Two-Photon event only in focus volume
→ All emission light is directly from focus

Resolution is similar (or worse) to LSM
→ 0.3x1µm ellipsoid (high NA objective)

Penetration depth depending on specimen and optical parameter but might be up to nearly 1mm

These features will be important for various live cell imaging techniques

Ex~(P_{avg}/A)^2=I^2
Ex~P_{avg}

That’s why Multiphoton is also named NonLinear
Chance for 2PM event drops drastically with distance to focus
Why use 2-Photon microscopy? – focus spot only

Laser of LSM scans through specimen

Laser of 2PM scans through specimen

Excitation/ emission and photodamage/heat

occurs within specimen
also outside the focal plane

occurs within specimen
only in the focal plane
Theory for 2PM: $\lambda \sim E$ - The Energy of a Photon

$$E = \frac{hc}{\lambda} \cdot \text{eV} / 1.6 \times 10^{-19} \text{ J}$$

- $h$: Planck Constant: $6.626 \times 10^{-34} \text{ J} \cdot \text{s}$
- $c$: speed of light: $299,792,458 \text{ m/s}$
- $\lambda$: wavelength in nm
- $\text{eV}$: electron Volt: $1.6 \times 10^{-19} \text{ J}$, gain of energy when an unbound electron is accelerated by an electrostatic potential difference of 1V

$1 \text{eV} = 1 \text{p} \ 400 \text{nm} = 2 \text{p} \ 800 \text{nm} = 3 \text{ eV}$

<table>
<thead>
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<th>nm</th>
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<tr>
<td>12,39</td>
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<tr>
<td>6,20</td>
<td>200</td>
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<td>1500</td>
</tr>
<tr>
<td>0,74</td>
<td>1600</td>
</tr>
</tbody>
</table>
Theory for 2PM: How to excite (Tryptophan)

Single-photon
1 photon, 280 nm
4.5 eV

Two-photon
2 photon, 580 nm
2.13 eV x2
4.26 eV

Three-photon
3 photon, 840 nm
1.47 eV x3
4.41 eV

This virtual state is VERY short
0.01 fsec
(10^{-17} sec)

2-PM hypothesis introduced by Maria Göppert-Mayer, doctoral thesis 1931
What is the chance that 2 photons hit the same fluorophore at almost the same time?
→ a matter of time and area

Time → the virtual state
→ Δt of intermediate virtual state = 10 attosec (10⁻¹⁷ s)
→ 1 attosecond (10⁻¹⁸ s) is the time window
→ light travels 3 hydrogenatoms within 1 as ...

Area → the fluorophore
→ quite small target

**Problem:** Light can not travel faster than speed of light
**Solution:** More photons are needed (high density of photons)

We need a million times more photons than in single photon fluorescence and ‘good’ objectives
Theory for 2PM: More photons please

**Problem:**
1 million times more photons?
This Laser is dangerous and will burn holes in glas
not mention the sample...

**Solution:** A weak Laser with high photon intensity pulses
→ low average power (0.3 - 2.5 W)
→ high power (30-300 kW) pulses 50-100 fs wide
→ pulse frequency 80 Mhz (1 pulse/ 12.5 ns)
Even this Laser is still dangerous when used!

**Problem:** Many fluorophores but one Laser
**Solution:** To excite a wide range of fluorophores the laser is
tuneable for e.g. 700-1040 nm
Important to consider in multiphoton microscopy:

**short pulse of high intensity light is needed**

High (ultrashort*) energy pulse → needed for 2 Photon excitation

**BUT**

Low average laser intensity → not to fry the specimen

* If pulse spreads - intensity drops
And 2PM effect will not take place
Principle of 2PM

- Objective
- Aperture of objective
- Specimen
- Focal plane of objective (depth of focus), light is focused here
Principle of 2PM

Laser pulse far from focal plane
NO incident of two photons hitting one fluorophore
Laser pulse photons more concentrated
still away from focal plane
NO incident of two photons hitting one fluorophore
Focused Laser pulse reaches focal plane with photons in temporal and spatial proximity.

High probability that 2 photons hit one fluorophore within 10 attosecond.
The lucky ones emit fluorescence like they were hit by 1 high energy photon instead of 2 low energy photons.

**Excitation / emission occurs only in Focal plane /spot**
Laser pulse leaves focal plane, NO incident of two photons hitting one fluorophore.
Laser pulse disperses in tissue
NO incident of two photons hitting one fluorophore
Exitation events in the Multiphoton microscope using fs-Laser pulses

**REMEMBER**

Excitation / emission occurs only in Focal plane /spot confocal image without a pinhole
Recapitulate:

- NIR Laser to reach deep
- Excitation of ´normal´ fluorophores via 2P effect
- NIR is tuneable over range e.g. 690 nm – 1040 nm
  - 2P is only happening in focal volume
  - Ex/Em/photodamage only at focal volume

Applications:

Living animals
Manipulation of ´precise´ small volumes
Multiphoton microscopy
Objectives and
Detectors

Light must come in to depth
Light must get collected from the depth
Bring back home the Photons

Laser → **Objective** → Excitation

Emission → **Objective** → Detector

Low NA  High NA
Multiphoton microscopy objectives and detectors

Each NDD houses 2 emission filtercubes
Loss of emission light: NDD vs LSMD I

Alexa 488, MaiTai 780nm, 5% (quite high), spectral range emission 500-550nm, no/open pinhole, digital gain etc for NDD (no over/under exposure)
Go deep without 2 Photon microscopy

A method called Optical Clearing is available
Making visualization depth of e.g. 1 mm possible
Using light of 300 – 633 nm for excitation

Dendrites of cortical projection neurons of a transgenic mouse expressing YFP via the thy1 promoter. This high-resolution image of the dendritic processes to a depth of 430 µm was made using multiphoton excitation of 920 nm in the living animal. Specimen provided by Stephen Turney, MCB, Harvard University, USA.

app. 600µm
Optical Clearing

**Problem**
Biological tissue: poor light transmission due to interface lipid:aqueous (PM: in/ex-cellular fluids)

**Solution**
Replace aqueous fluids with something which matches Refractive Index (RI) of lipids.
→ Penetration of light into the tissue increases,
→ scattering of light decreases.

Optical Clearing Agents (OCAs): aromatic hydrocarbons
→ water insoluble but soluble in EtOH or MetOH.
→ each clearing is preceded by dehydration (Et/MetOH)
→ benzyl-alcohol-benzoate (BABB) (excellent)
→ Methyl salycylate (wintergreen oil) (very good)
→ Thiodiethanol (TDE) (good)
→ Glycerin (poor clearing)
→ OCAs have usually a refractive index of around 1.5, hence matching RI of glass, and immersion oil.

*Ya-Yuan Fu et. al. MicroVascular Research 80 2010*
Go deep with LSM

[Image of optical micrographs showing tissue samples at different depths with various mounting media: PBS 60x wi, PBS 40x oil, Glycerol, TDE, BABB.]