Confocal microscopy course

7 HEC!

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Organism Biology School

Uppsala BioCenter

2014

$c = 3*10^8$ m/s

299,792,458 m/s

7.5 times around the equator in 1 second

8 minutes and 20 seconds to get from Sun to Earth

What do you want to know?

Basics

what are all these buttons I'm pushing

z-stack

mysterious stuff in the confocal, which tends to brake

Potential of confocal microscopy

Softwares

More

Leica SP8

new types of microscopy

Zeiss 510

how to make good images

how to believe what I see

sample preparation

score in amount of questions

Mohammad		Jose
Reza	6:6	Catarina
Shirin		Rita

Results of the quiz for the lecture 1 (basics in light microscopy)

1. Comments about the lecture

it was ok/good	too fast at the end
83%	17%

2. Average level of boredom (scale 0-10)

1!

3. Will the information from the lecture be useful for you

yes	maybe/some of it
75%	25%

Results of the quiz for the lecture 1 (basics in light microscopy)

4. what would you teach differently

practical examples for the basics

literature before the lecture

less time for diffraction and light behaviour

pre-make the drawings for examples

not everyone learns from drawing, videos and text in the presentation!

please be careful! origin of phantom knowledge

5. would you like to be your student

yes	maybe	no!
72%	18%	10%

6. Additional questions

do you regret drinking so much coffee during the break with no perspective of having another break?

more/less info in the lecture

CLSM

Confocal Laser Scanning Microscopy

??

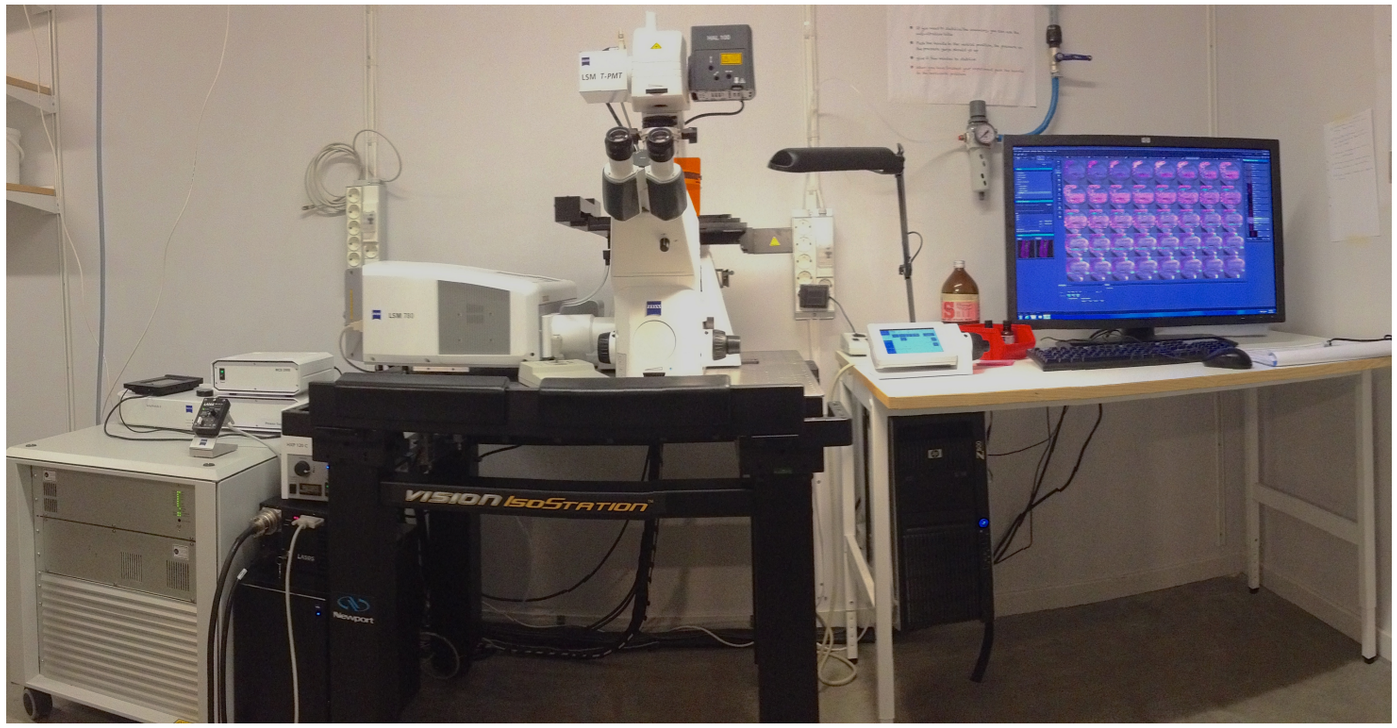
??

??

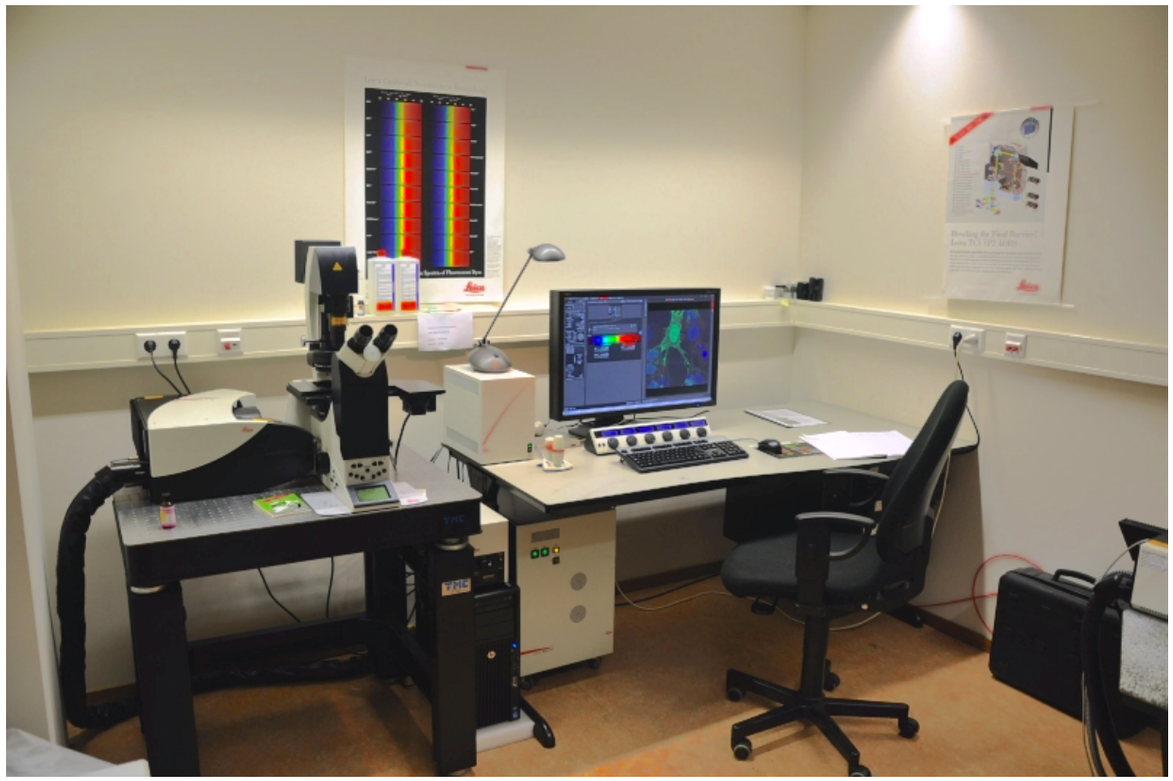
ok?

Confocal microscope anatomy

Zeiss 780

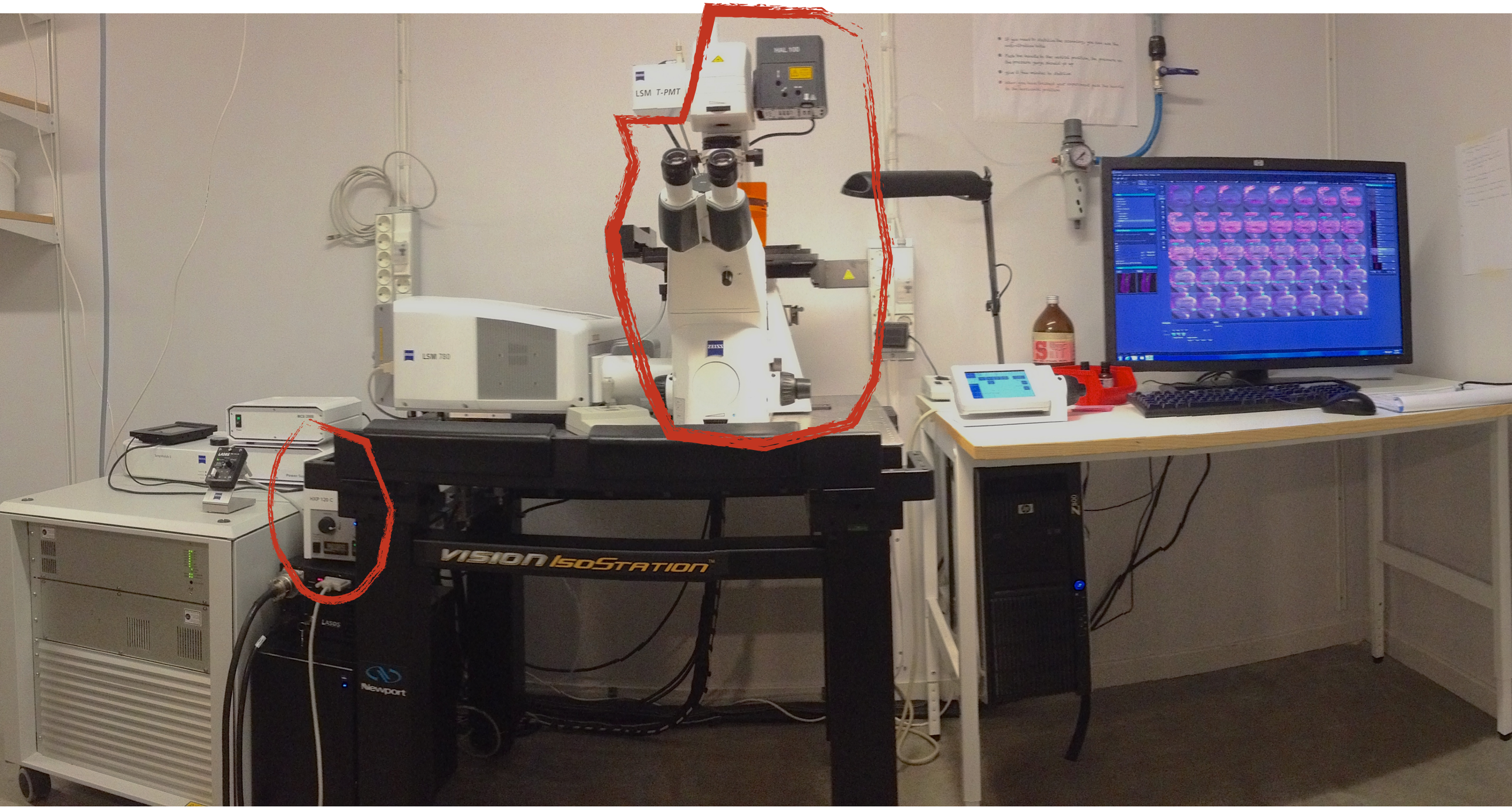


Leica SP8



Zeiss 510

Zeiss 780



= a regular epifluorescent microscope with confocal part mounted on it

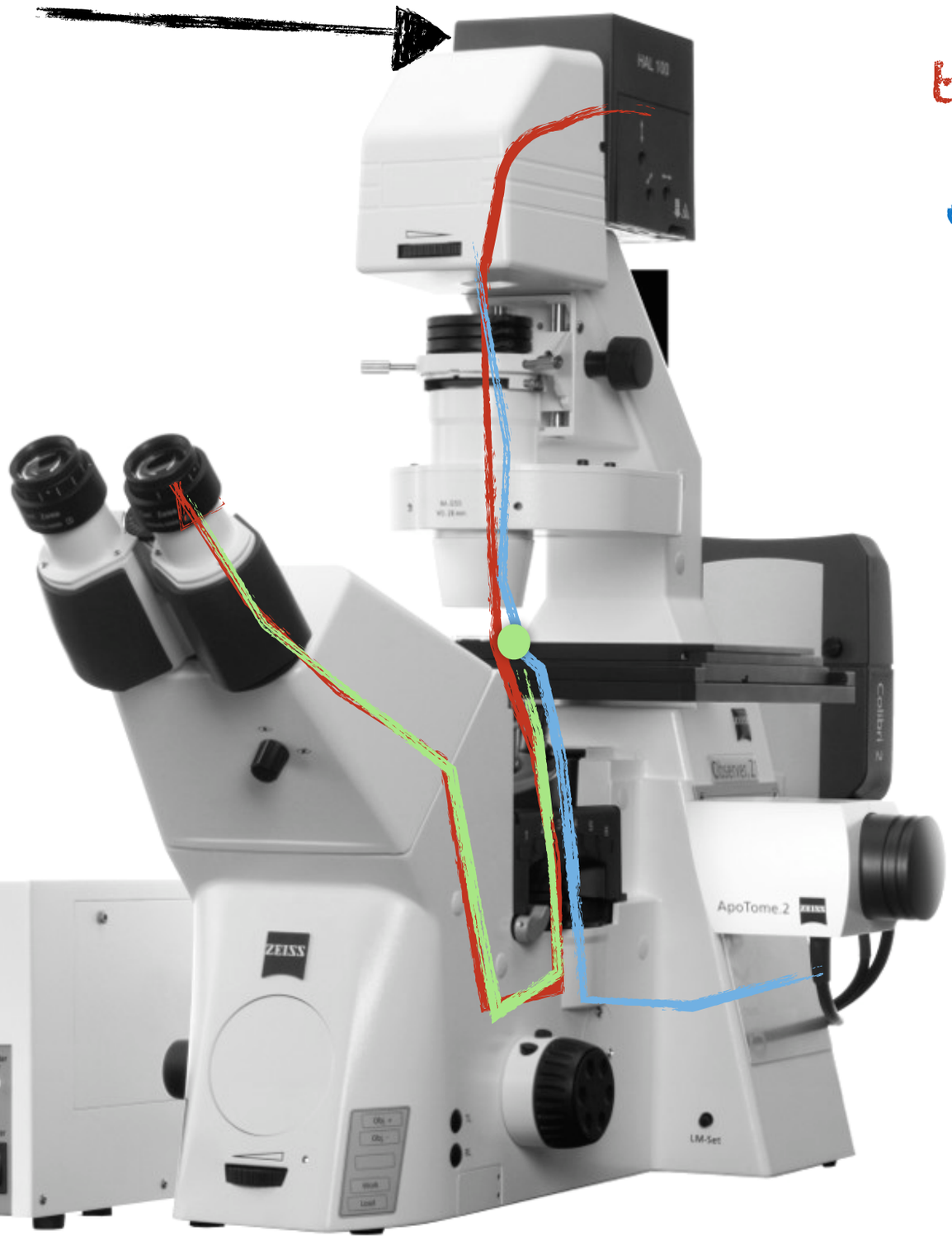
Inverted epifluorescent microscope

HAL lamp

transmitted light

UV lamp

excitation light



transmitted light

excitation light/
emission light

Upright epifluorescent microscope

1. please draw a path for transmitted light

2. please draw a path for excitation/emission light

3. why doesn't transmitted light mix up with excitation/emission light?

4. how is excitation/emission light directed inside the microscope?

1. what light does the UV lamp emit?

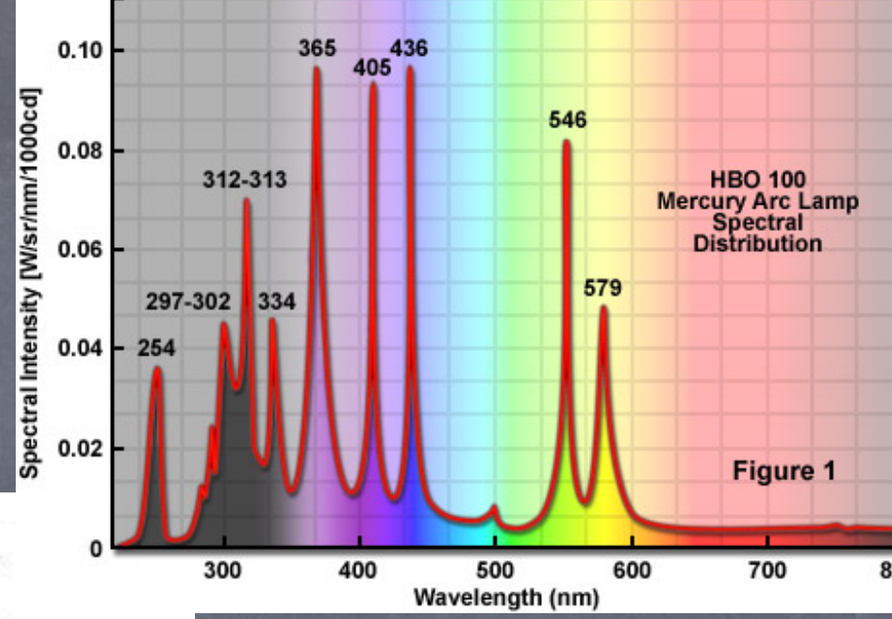
2. what will happened when it passes throughout the filter?

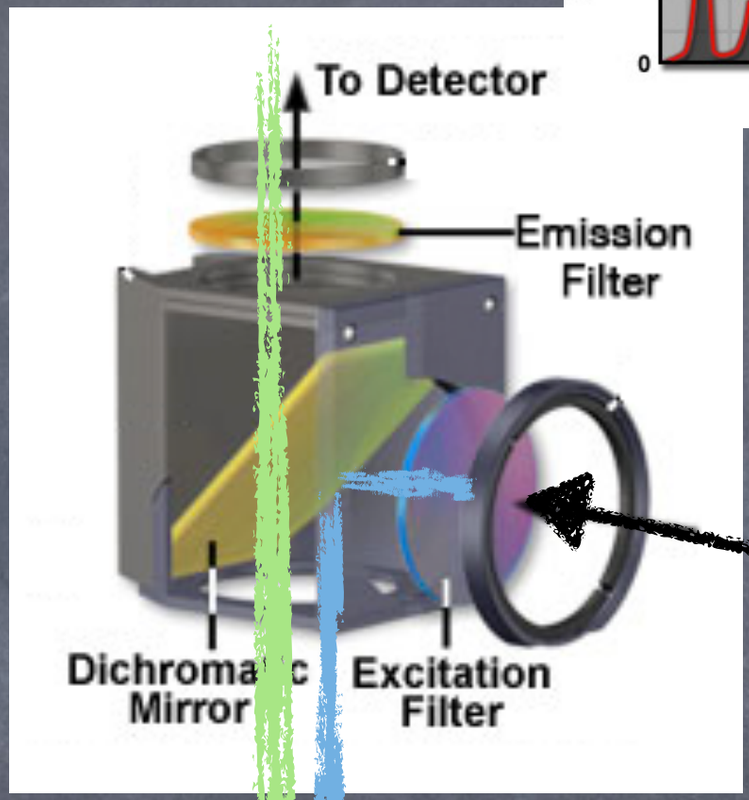
3. what happens to it on the dichromatic mirror? where will it go next?

4. where does the emission light go?

5. what happens to it on the mirror?

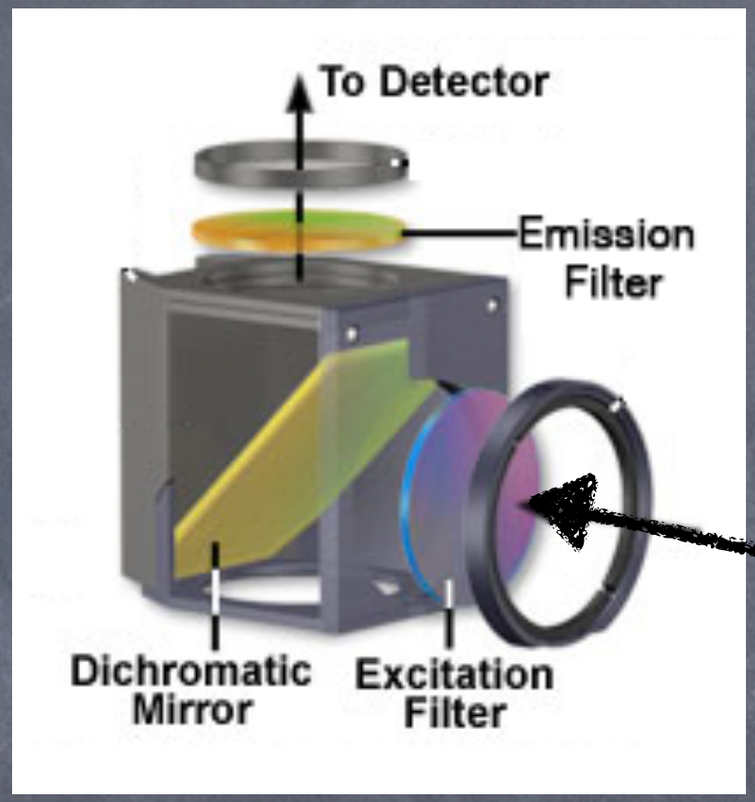
6. what is the emission filter for?



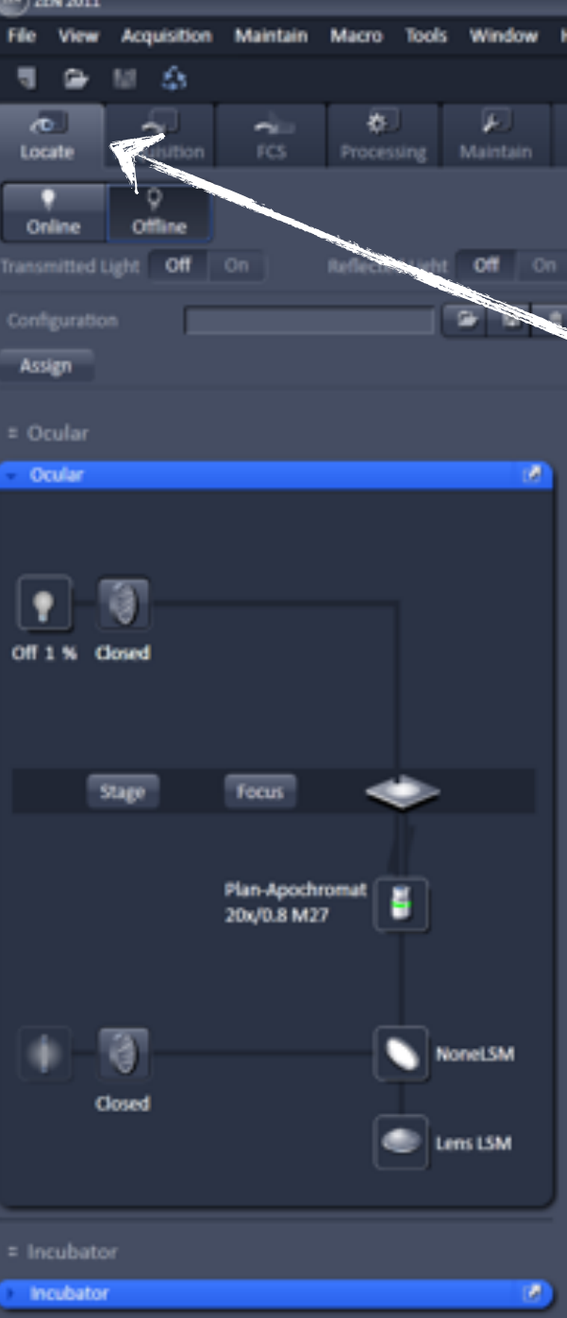


7. what part of the sample will be actually illuminated?

8. what part of the sample will be visualised on a CCD camera?



we use ZEN 2011 software



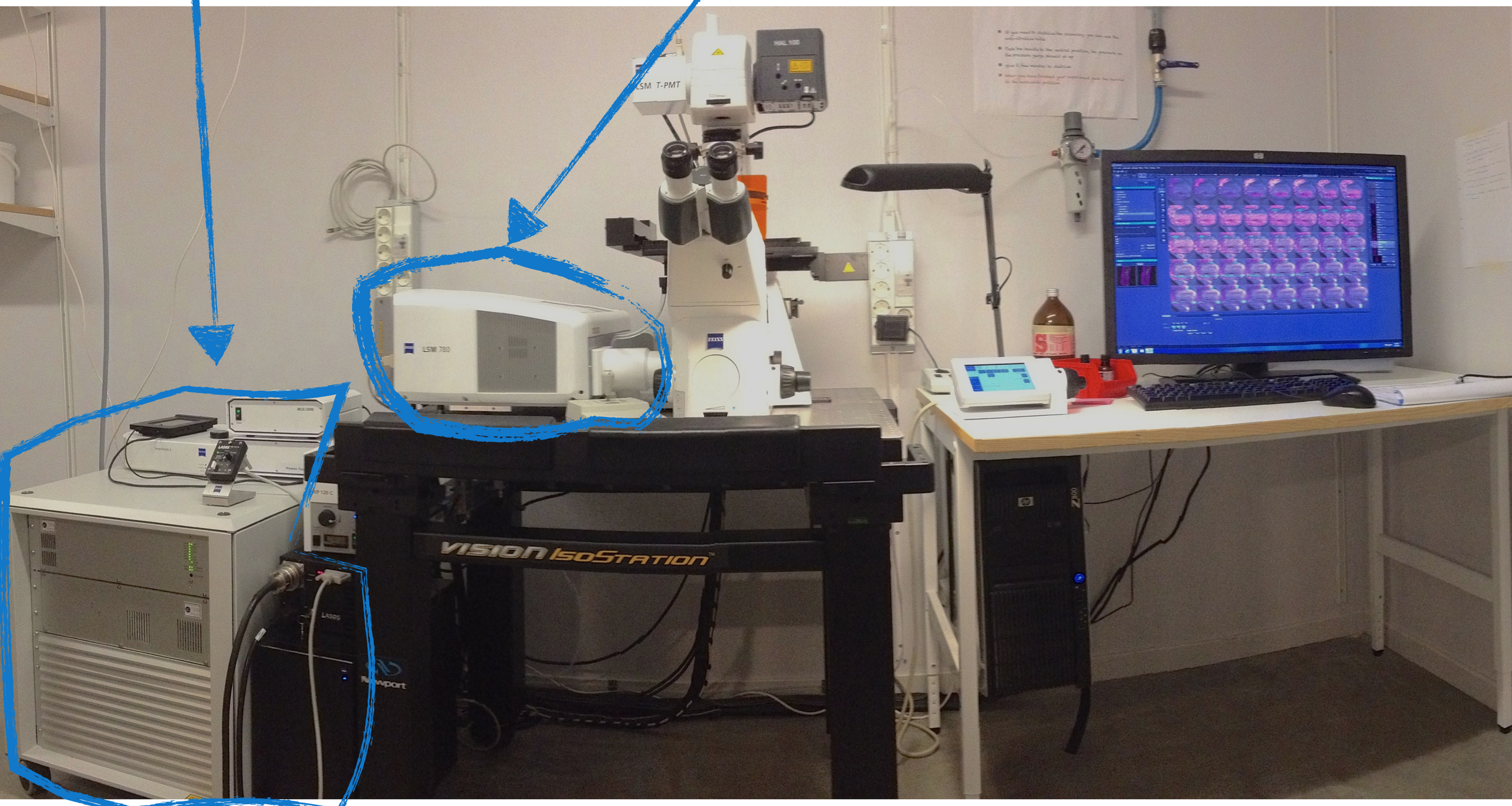
"Locate" tab allows you to use ONLY the epifluorescent microscope.

it is handy to find and focus on the place you want to image with confocal

Zeiss 780

and it all starts here

this is where magic happens



A confocal microscope

Light source

Lasers

Light amplification by stimulated emission of radiation

L
oscillation
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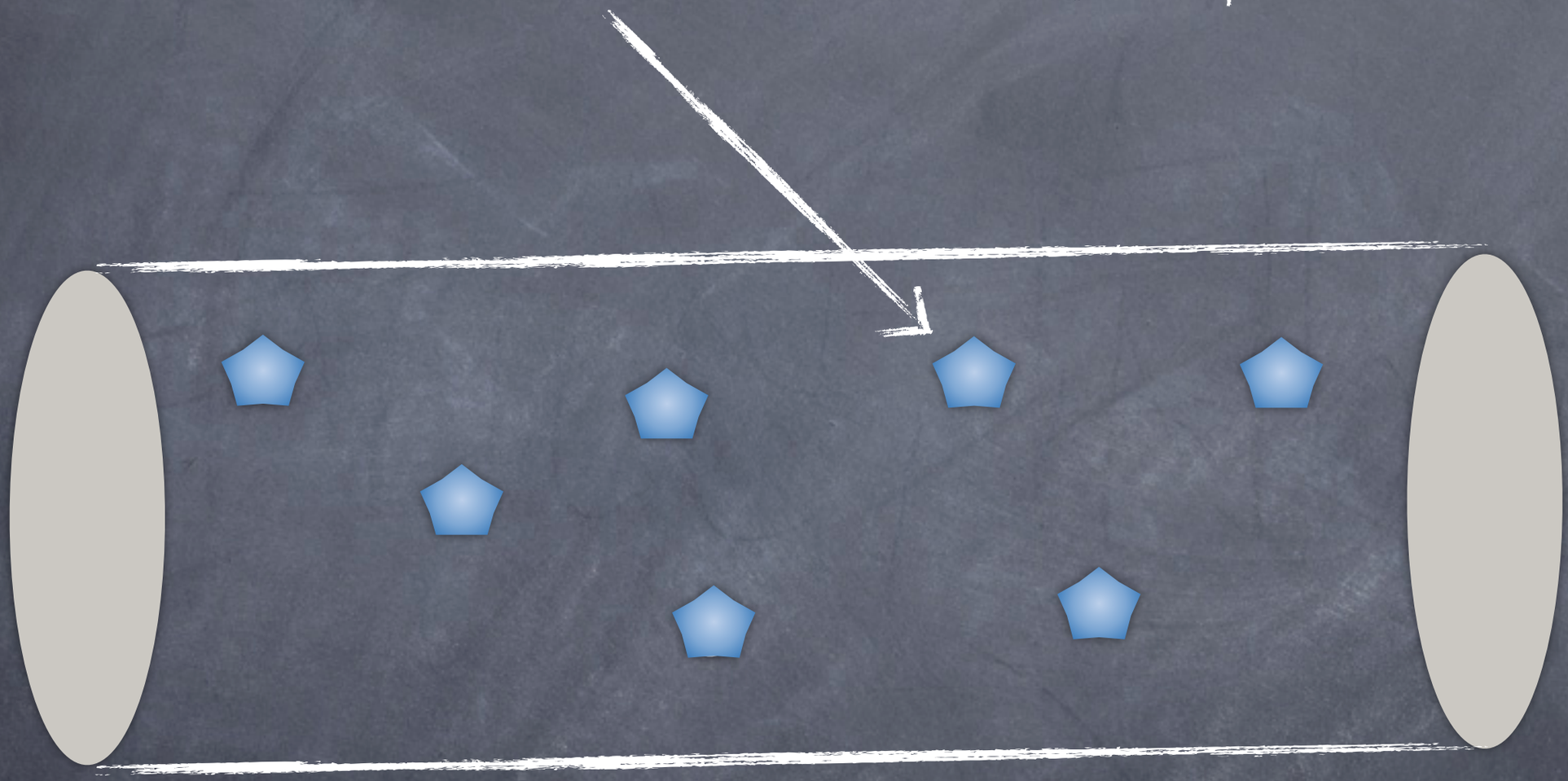
Lasers

lasers contain "gain" medium (can be gas, liquid, solid body, plasma)

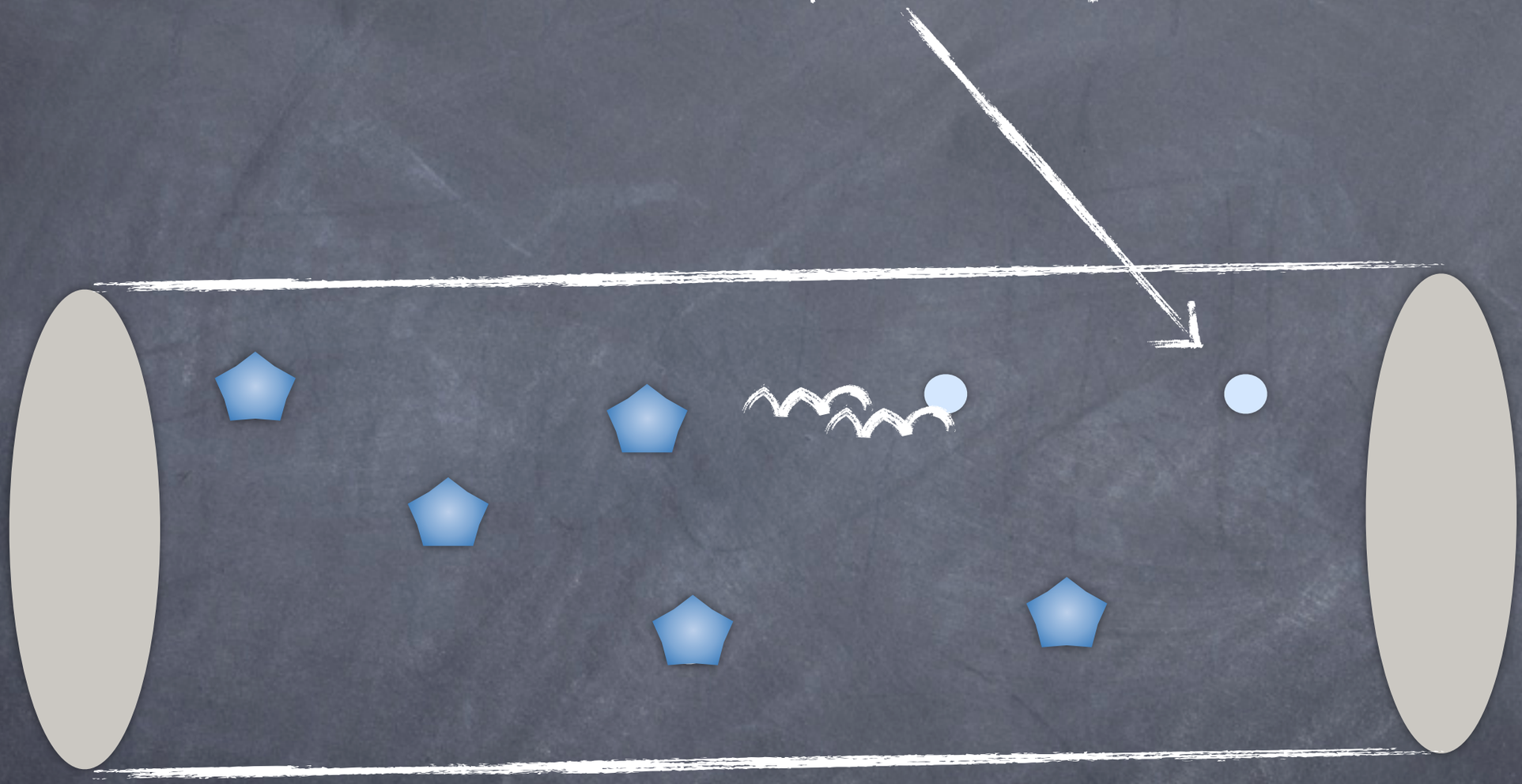


this process is called "pumping"

atoms of the "gain" medium are excited by another light source or electric field

Lasers

some atoms of the gain medium can spontaneously relax and emit a photon



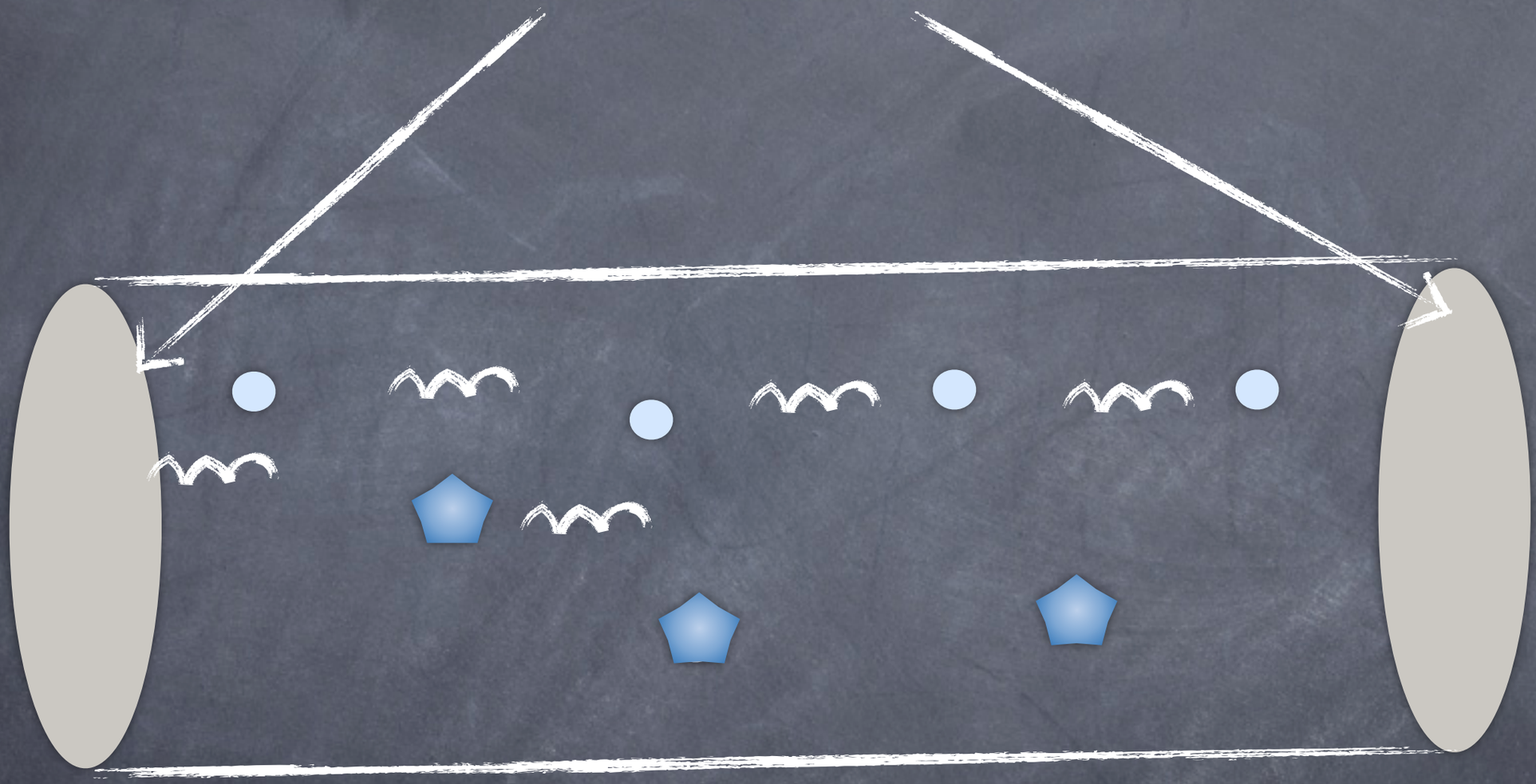
if this photon bumps into another excited atom

another atom will relax and emit identical photon

now there are two photons to bump into excited atoms!

Lasers

the gain medium is trapped between to mirrors which will reflect the photons onto excited atoms



the amount of the excited atoms in the "gain" medium is maintained by a light source or electric field

THE LASER

All the animations and explanations on
www.toutestquantique.fr

Lasers

please discuss, what could it mean, that laser medium emits identical photons

light is of the same wavelength

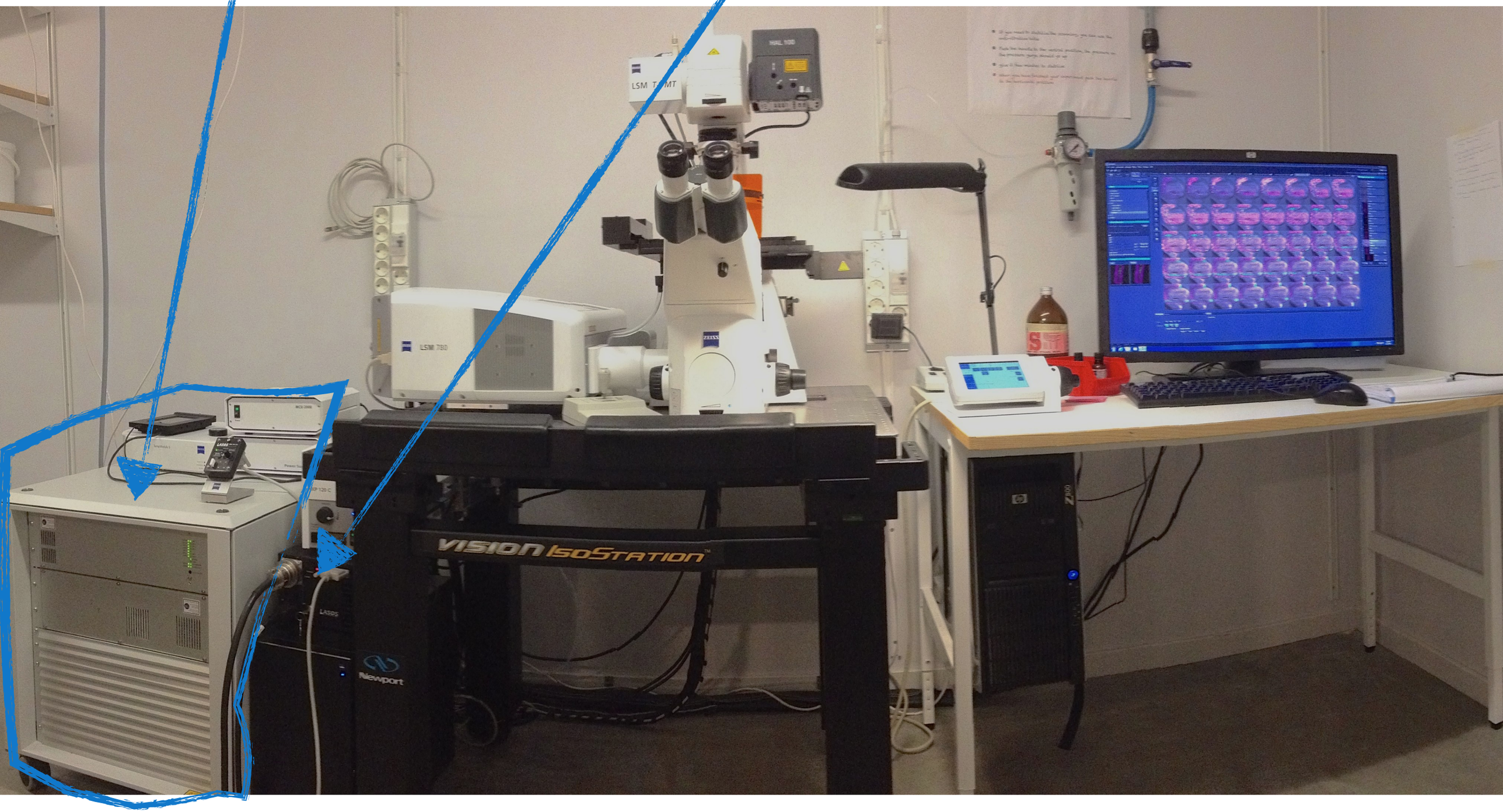
light is of the same phase coherent

coherency => collimated light

Zeiss 780

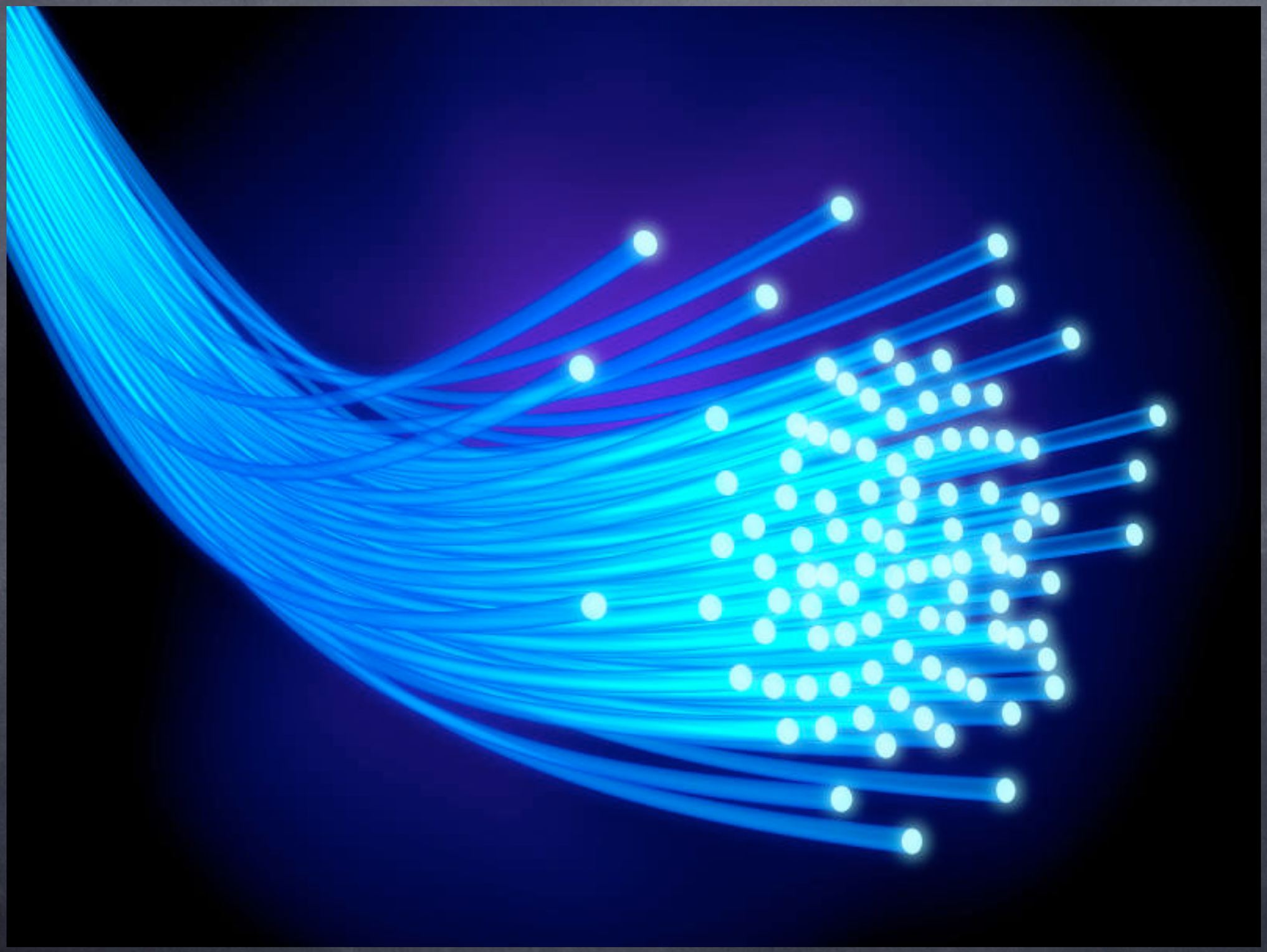
405, 561, 633 nm Lasers

Ar Laser

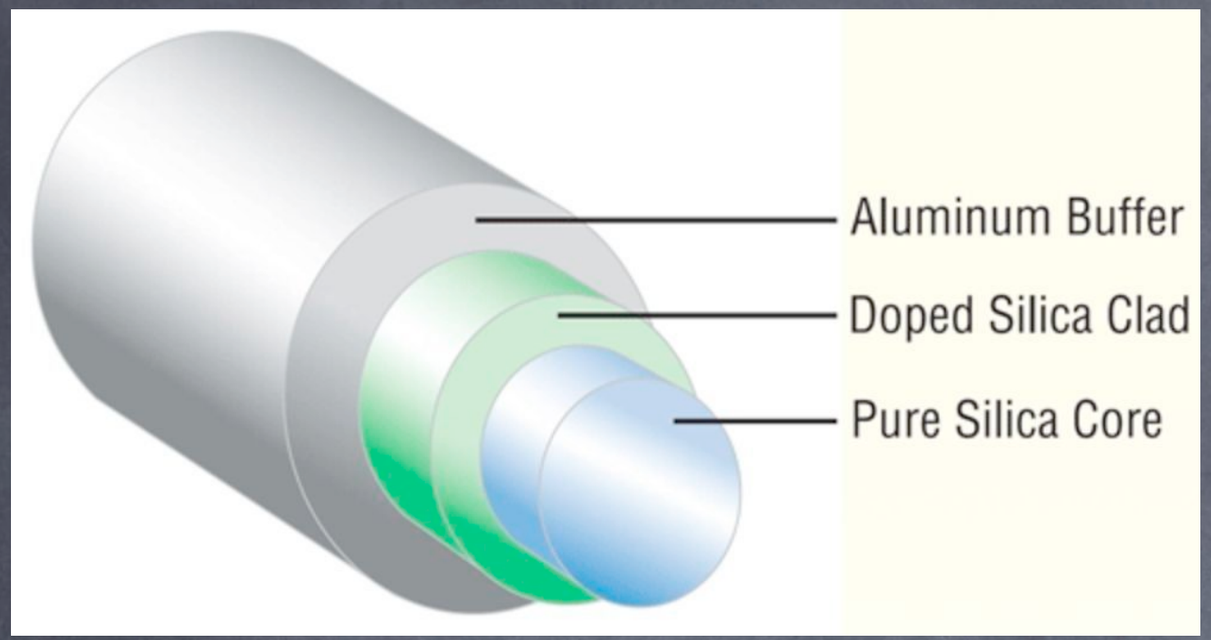


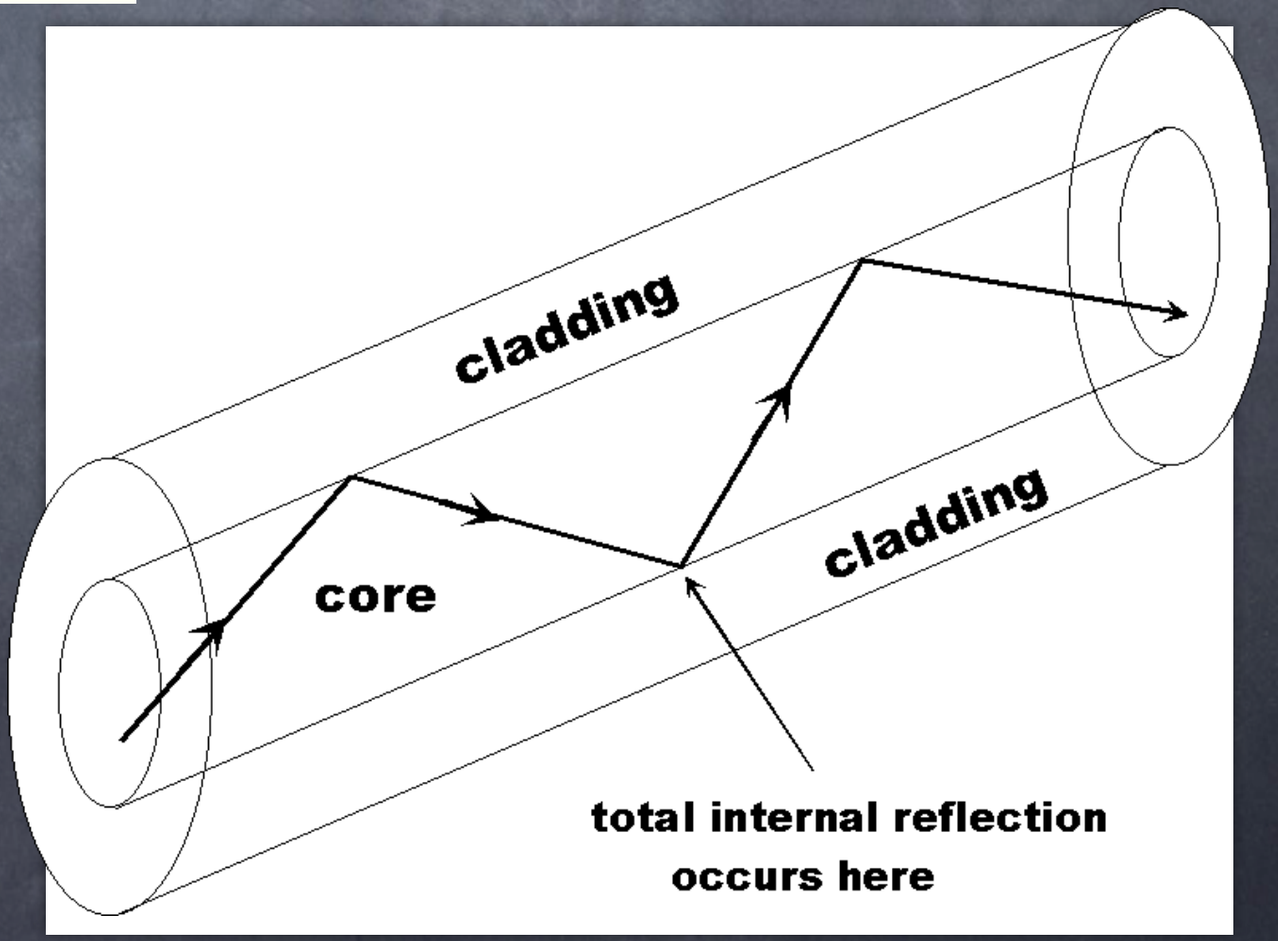
A confocal microscope

Optical fiber

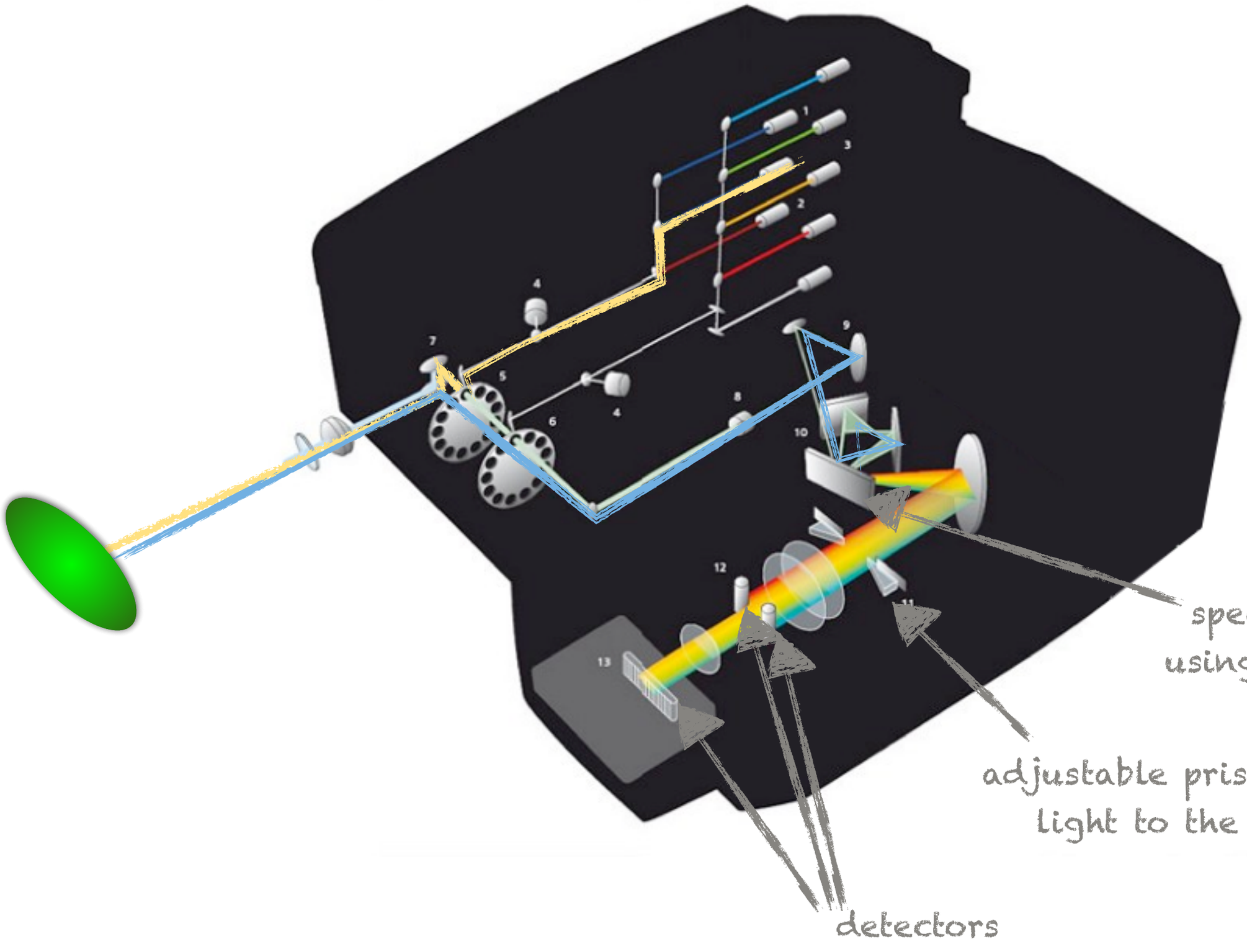


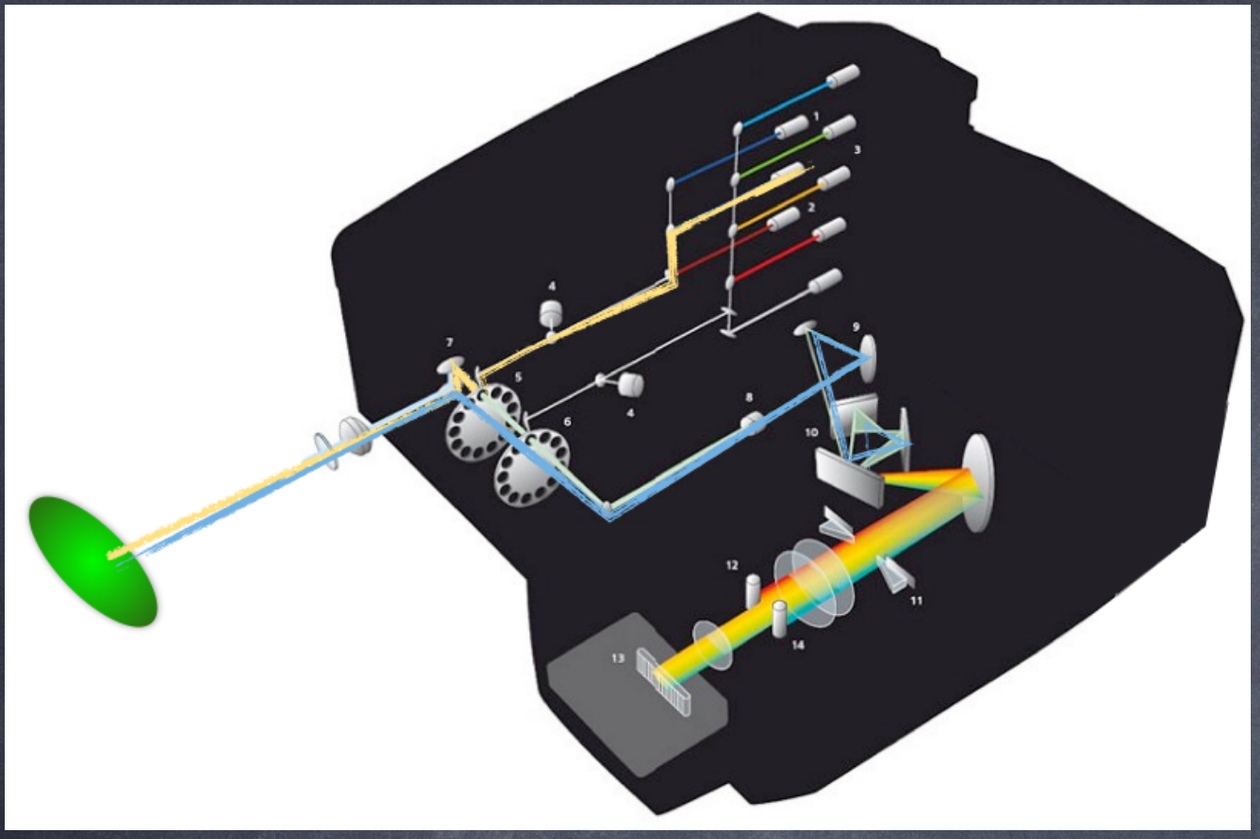
Optical fiber

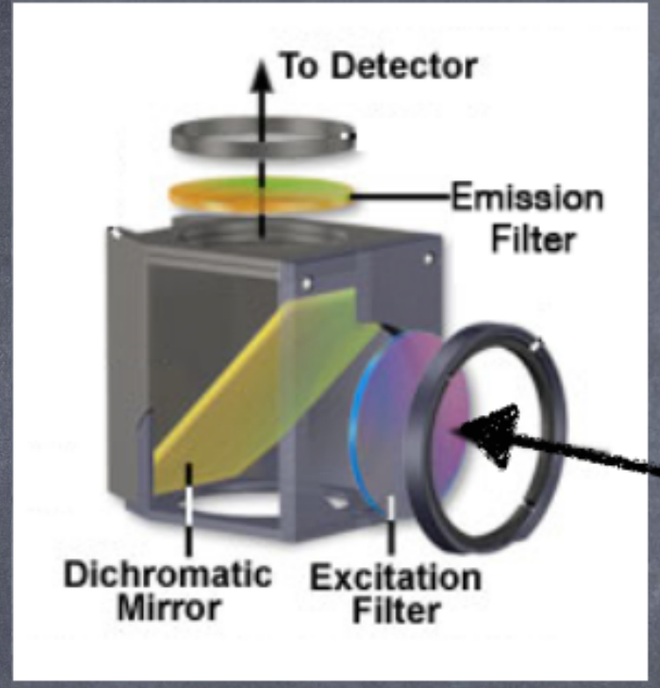


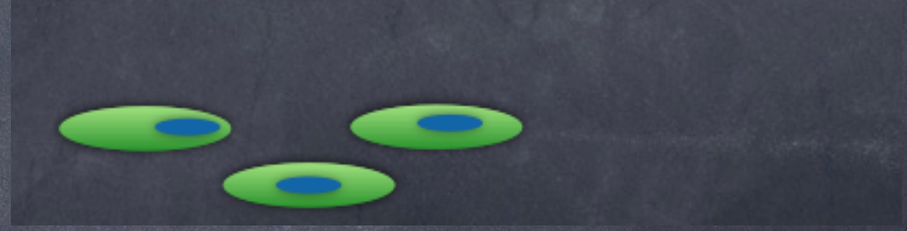


Insides of our confocal









1. what is a difference between light emitted by a laser and light emitted by a UV lamp?

2. does it need an excitation filter?

3. what happens to it on the dichromatic mirror? where will it go next?

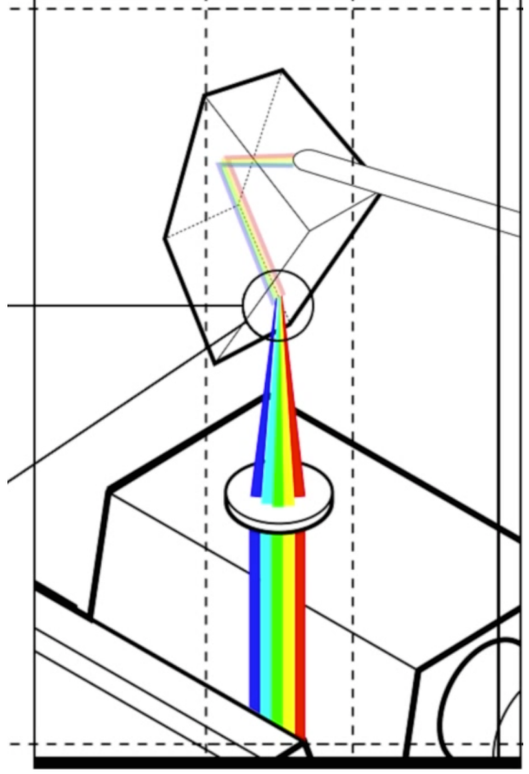
4. where does the emission light go?

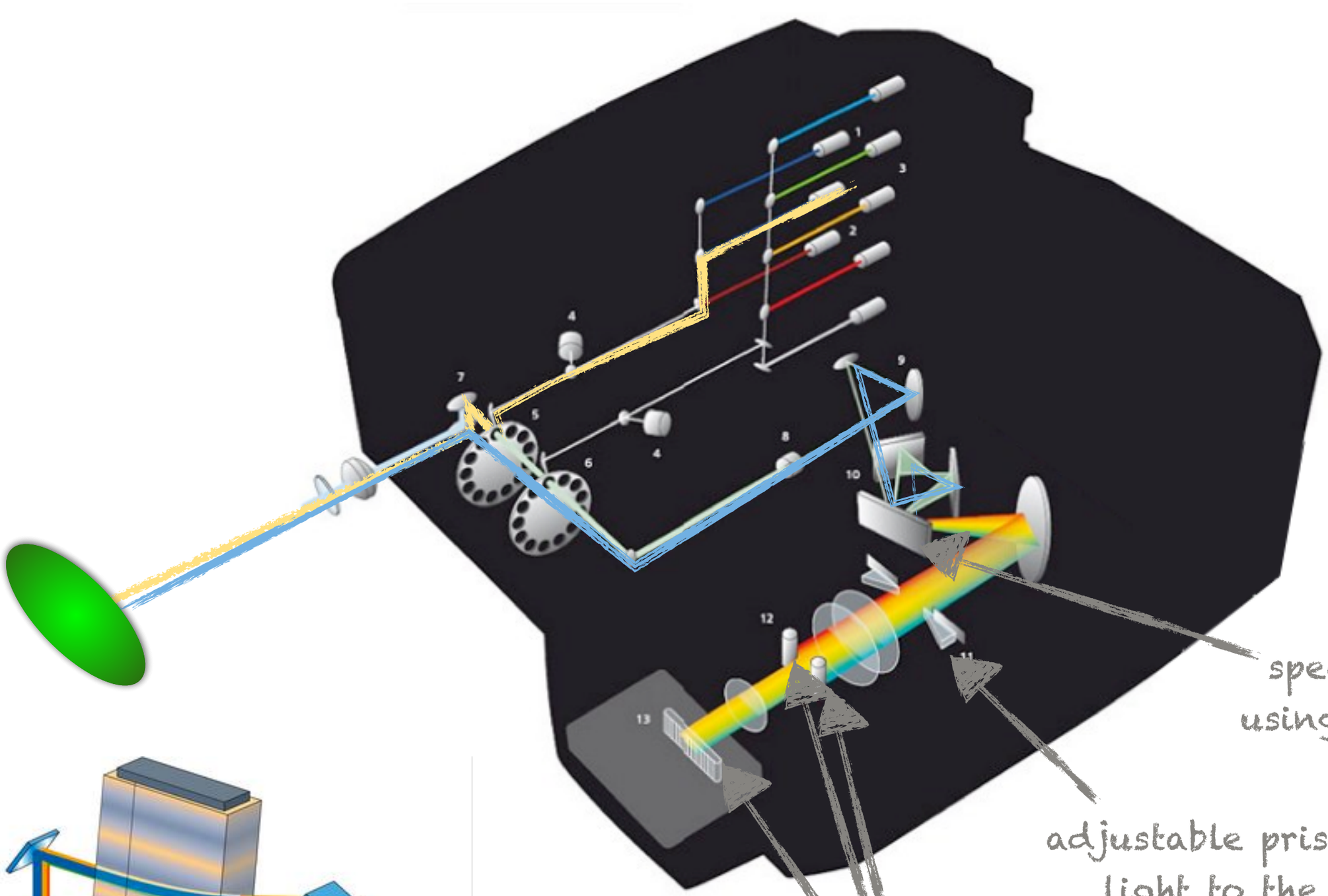
6. is there an emission filter in the confocal?

7. what part of the sample will be actually illuminated?

8. what part of the sample will be visualised on the detector?

Leica has alternative solutions

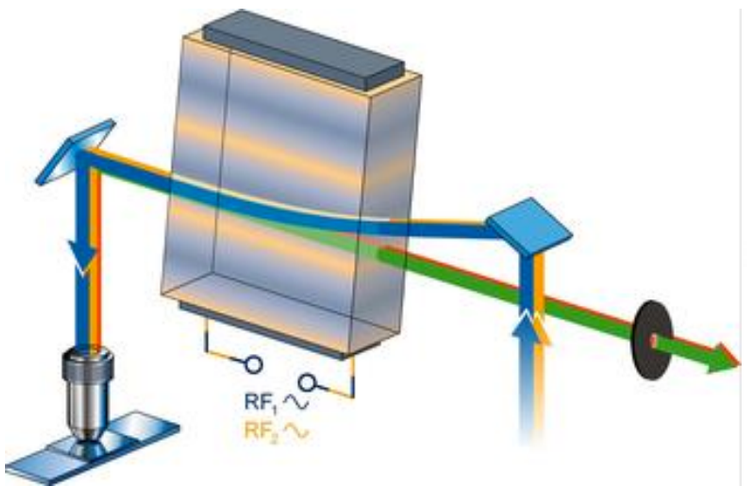




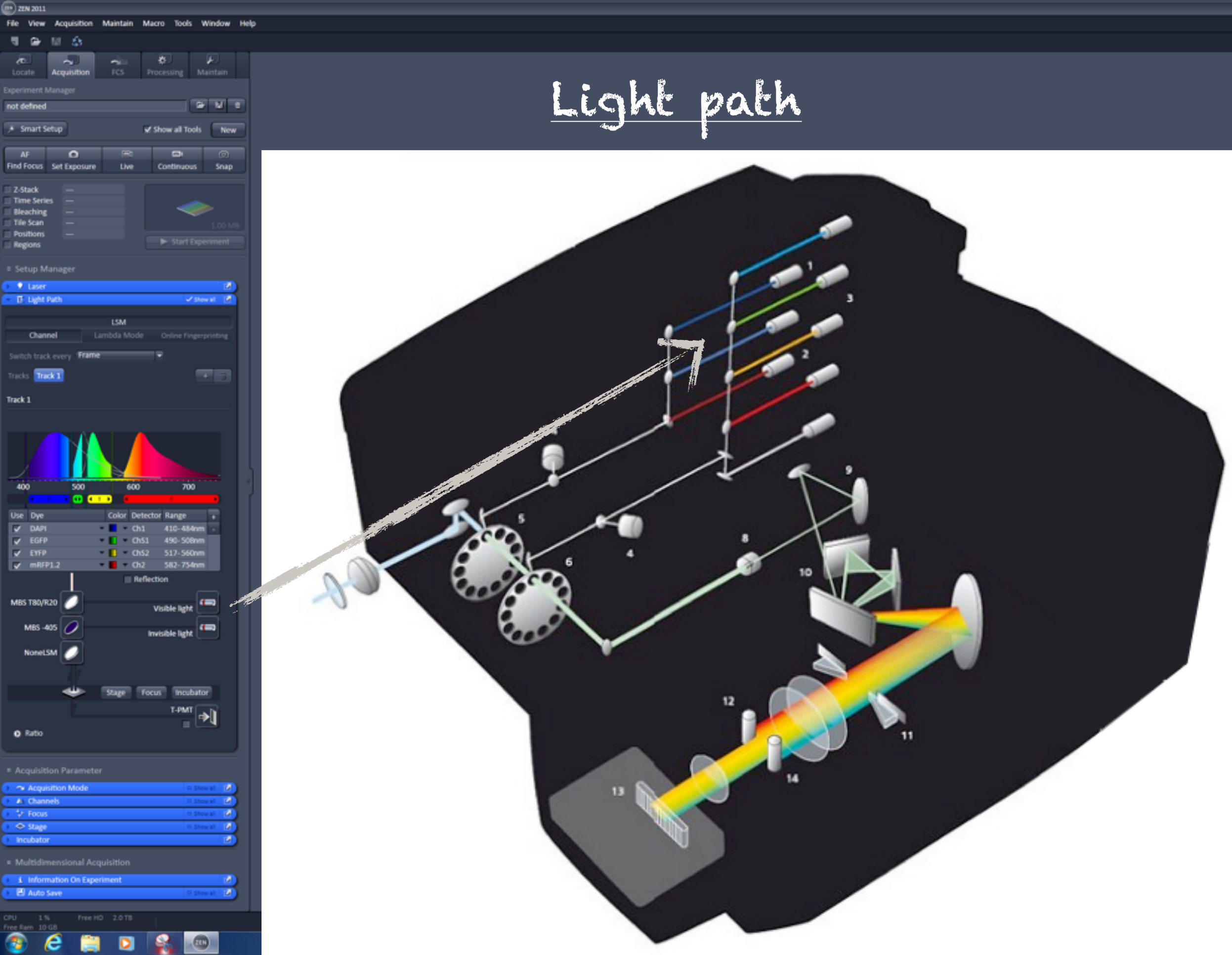
spectral dispersion
using diffraction grit

adjustable prisms targeting
light to the detectors

detectors



Light path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Light path


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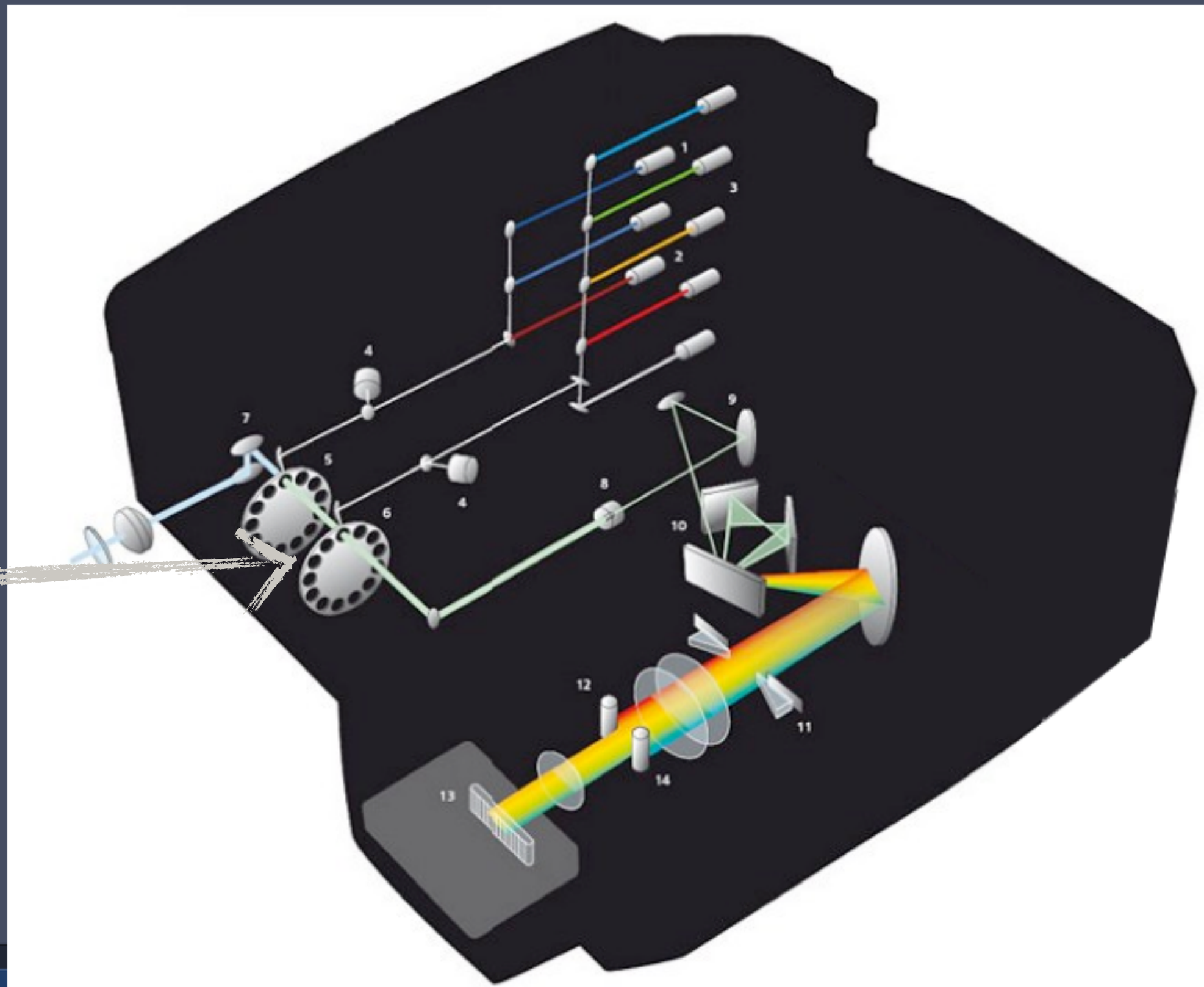
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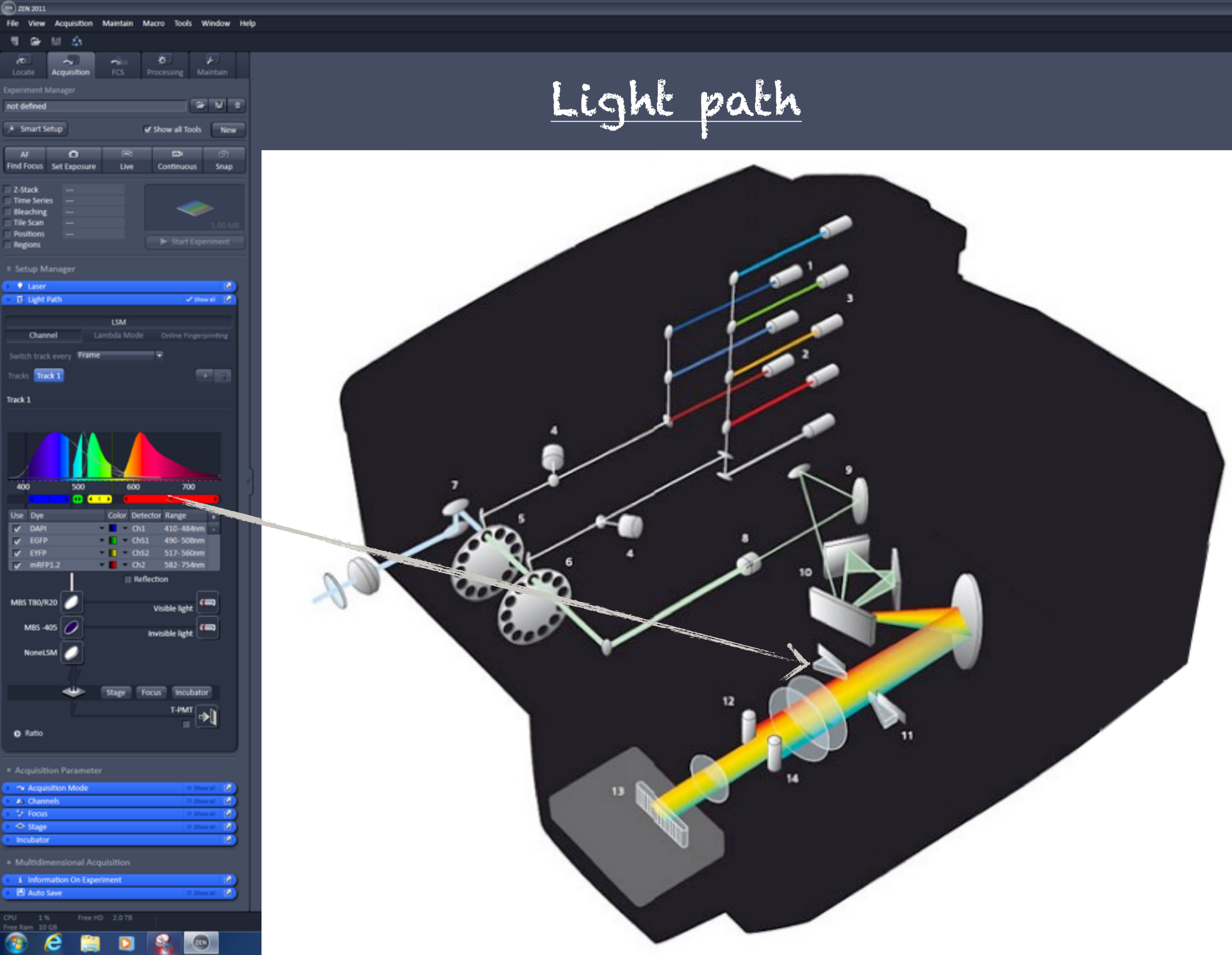
Light path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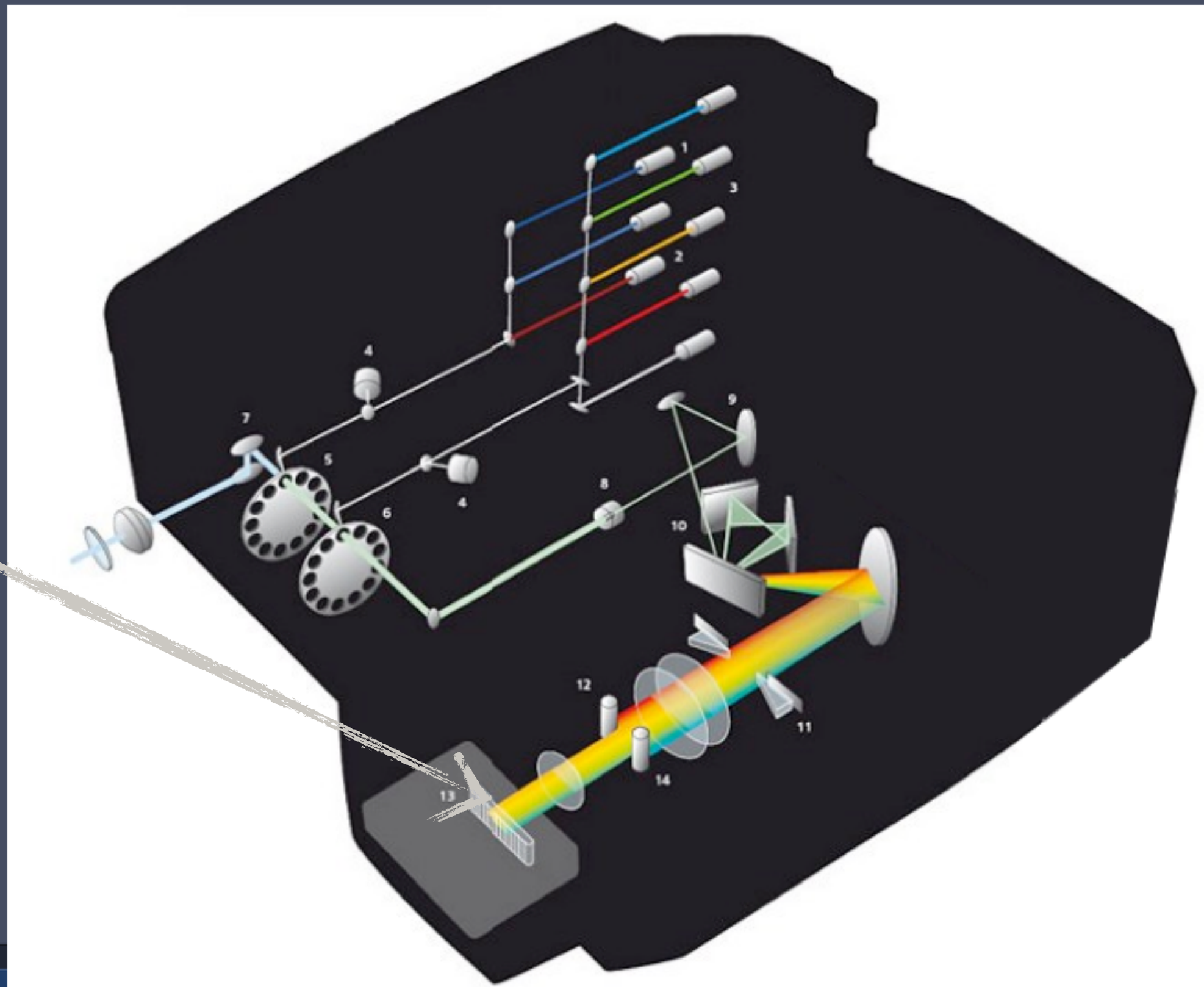
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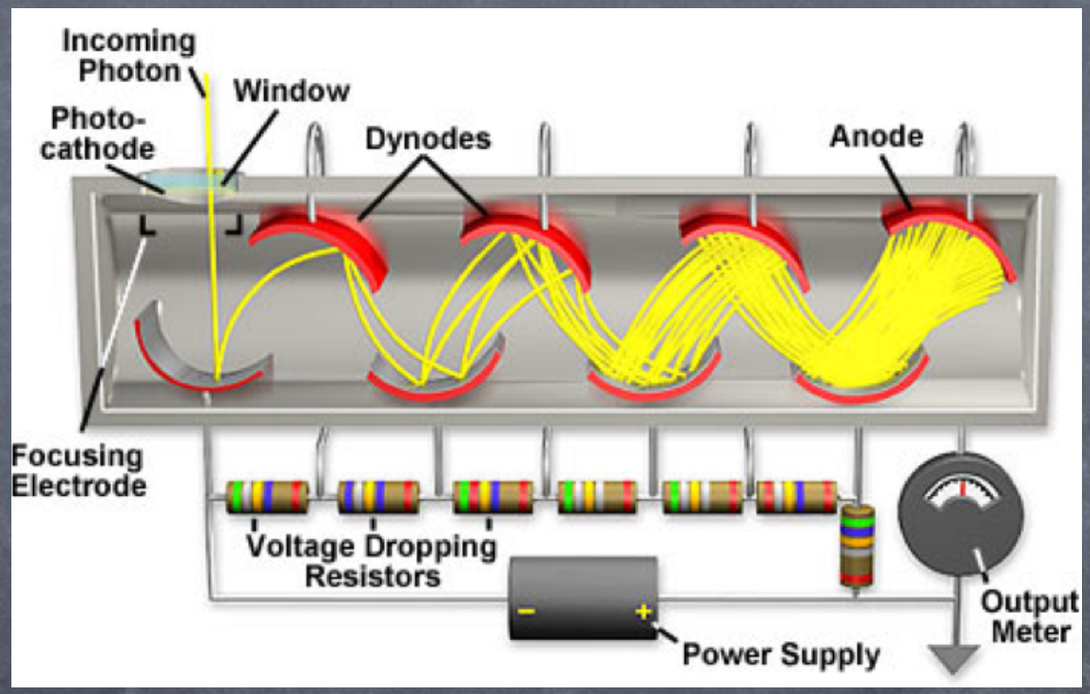
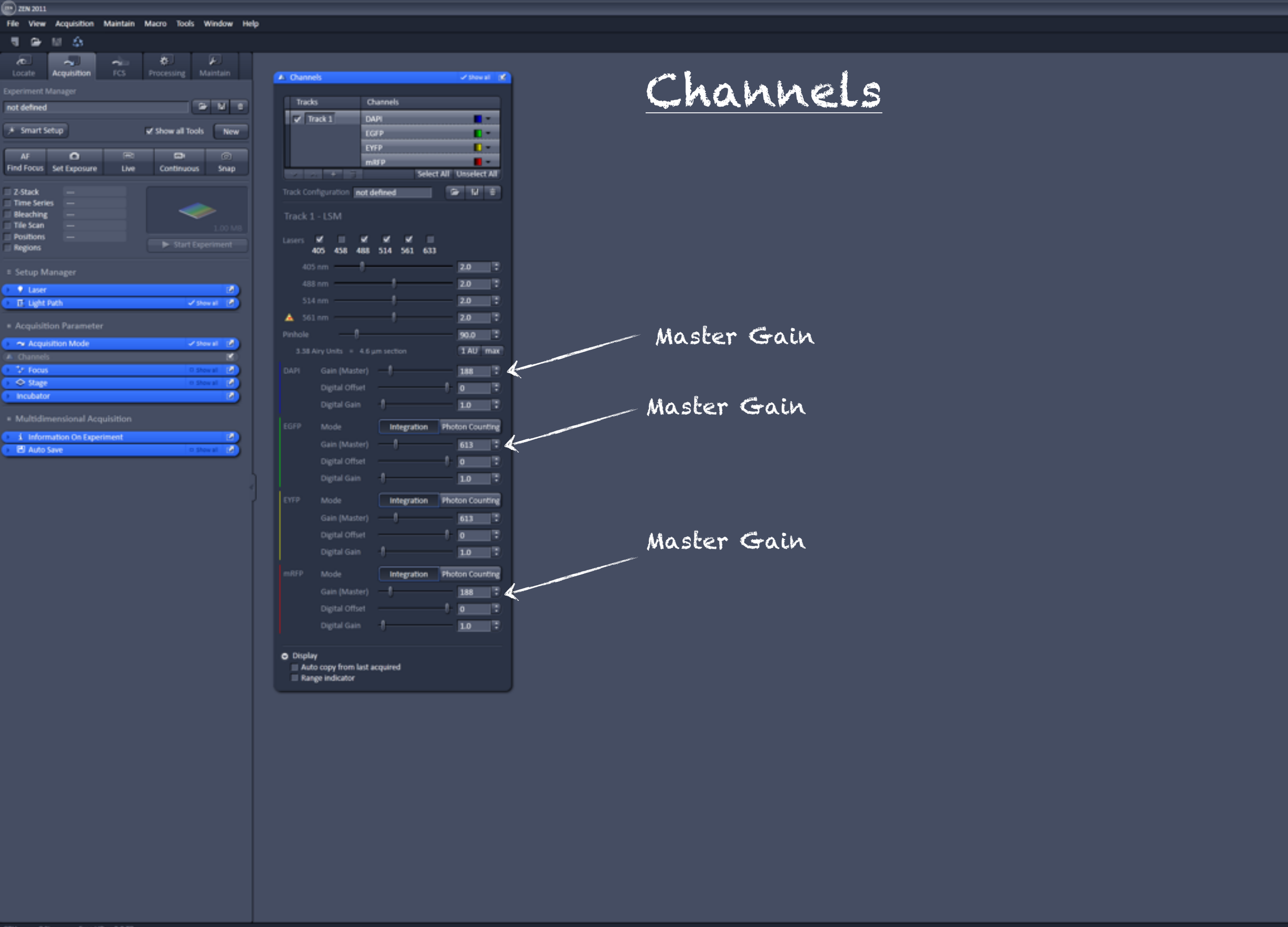
Detector = PMT

Photomultiplier tube



http://micro.magnet.fsu.edu/primer/java/digitalimaging/photomultiplier/sideonpmt/index.html

Channels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Lasers
405 458 488 514 561 633
405 nm 2.0
488 nm 2.0
514 nm 2.0
561 nm 2.0
Pinhole 90.0
3.38 Airy Units = 4.6 µm section
1 AU max
DAPI Gain (Master) 188
Digital Offset 0
Digital Gain 1.0
EGFP Mode Integration Photon Counting
Gain (Master) 613
Digital Offset 0
Digital Gain 1.0
EYFP Mode Integration Photon Counting
Gain (Master) 613
Digital Offset 0
Digital Gain 1.0
mRFP Mode Integration Photon Counting
Gain (Master) 188
Digital Offset 0
Digital Gain 1.0
Display
Auto copy from last acquired
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Master Gain
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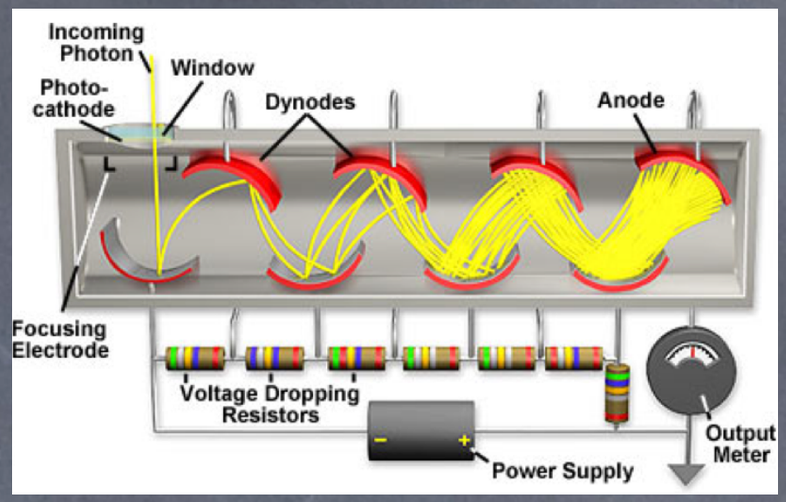
Photomultiplier tube = PMT

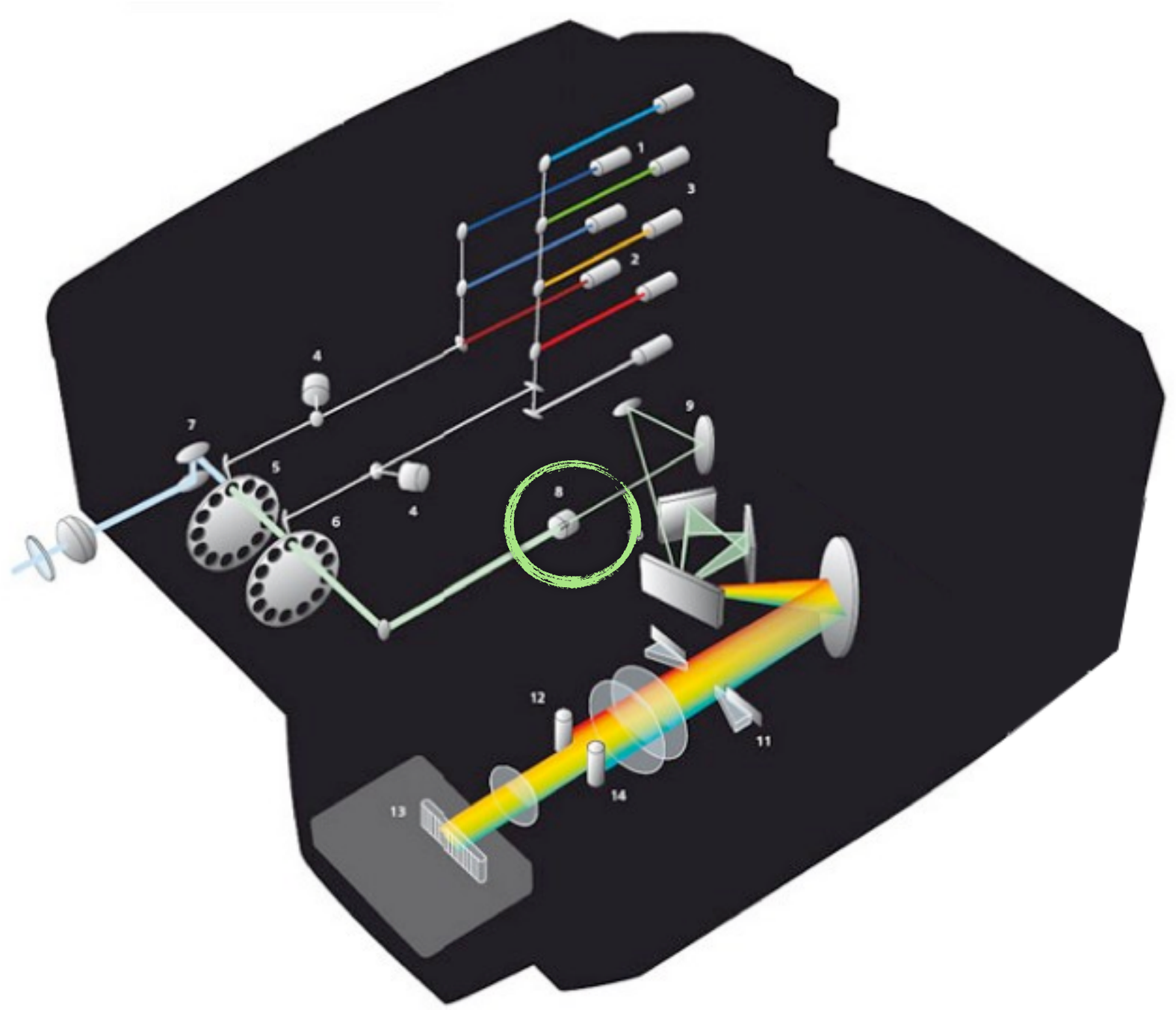


please discuss what exactly does the PMT detect:

- amount of photons?
- frequency?
- wavelength?
- energy?

please sum up how does the information you get from a PMT detector differ from what you get with a usual CCD microscope camera?

The crown jewel of a confocal



CLSM

Confocal Laser Scanning Microscopy

??

ok

??

ok

why is confocal called confocal?

confocal = to have the same foci

1957 patent by Marvin Minsky

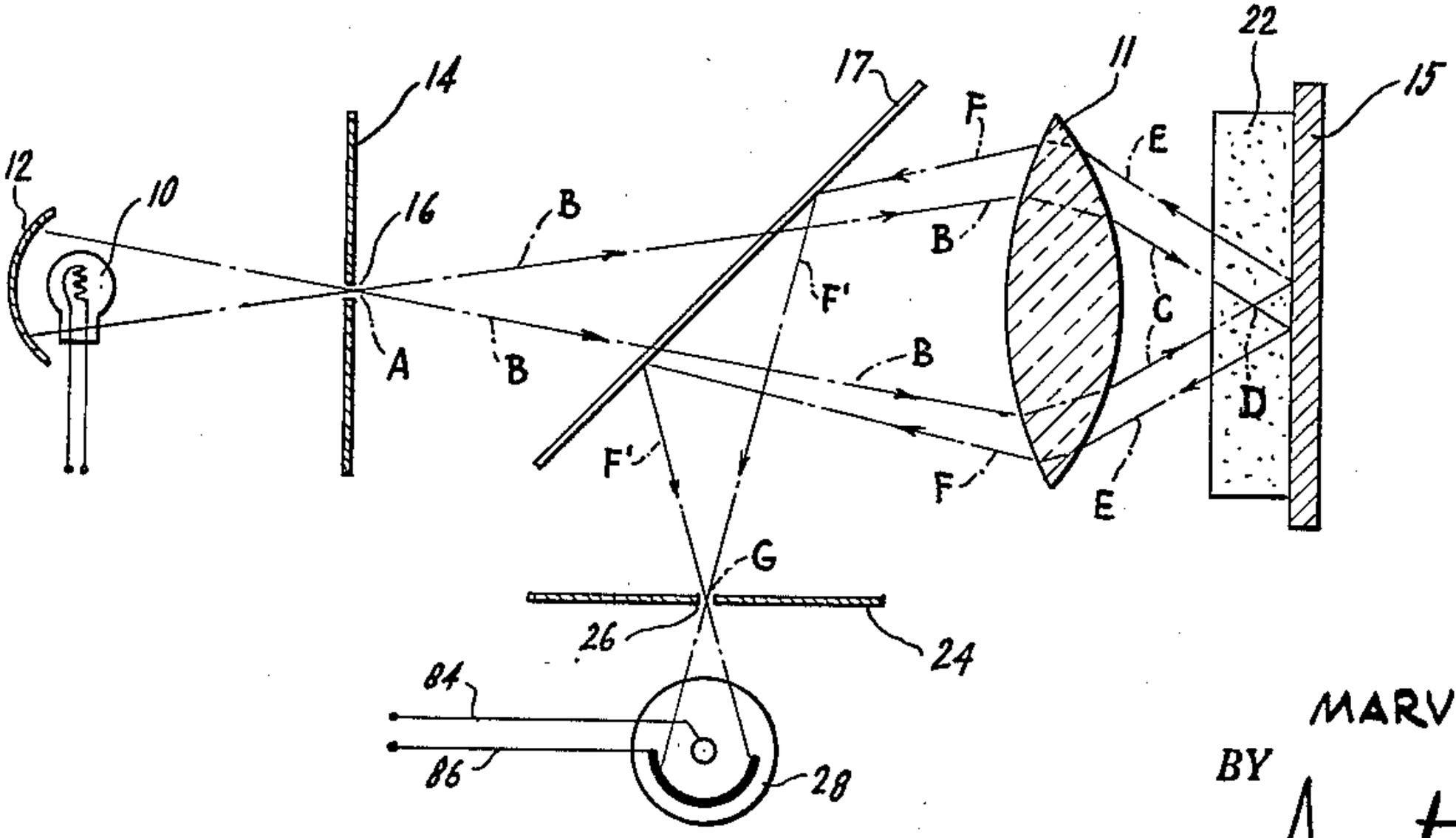
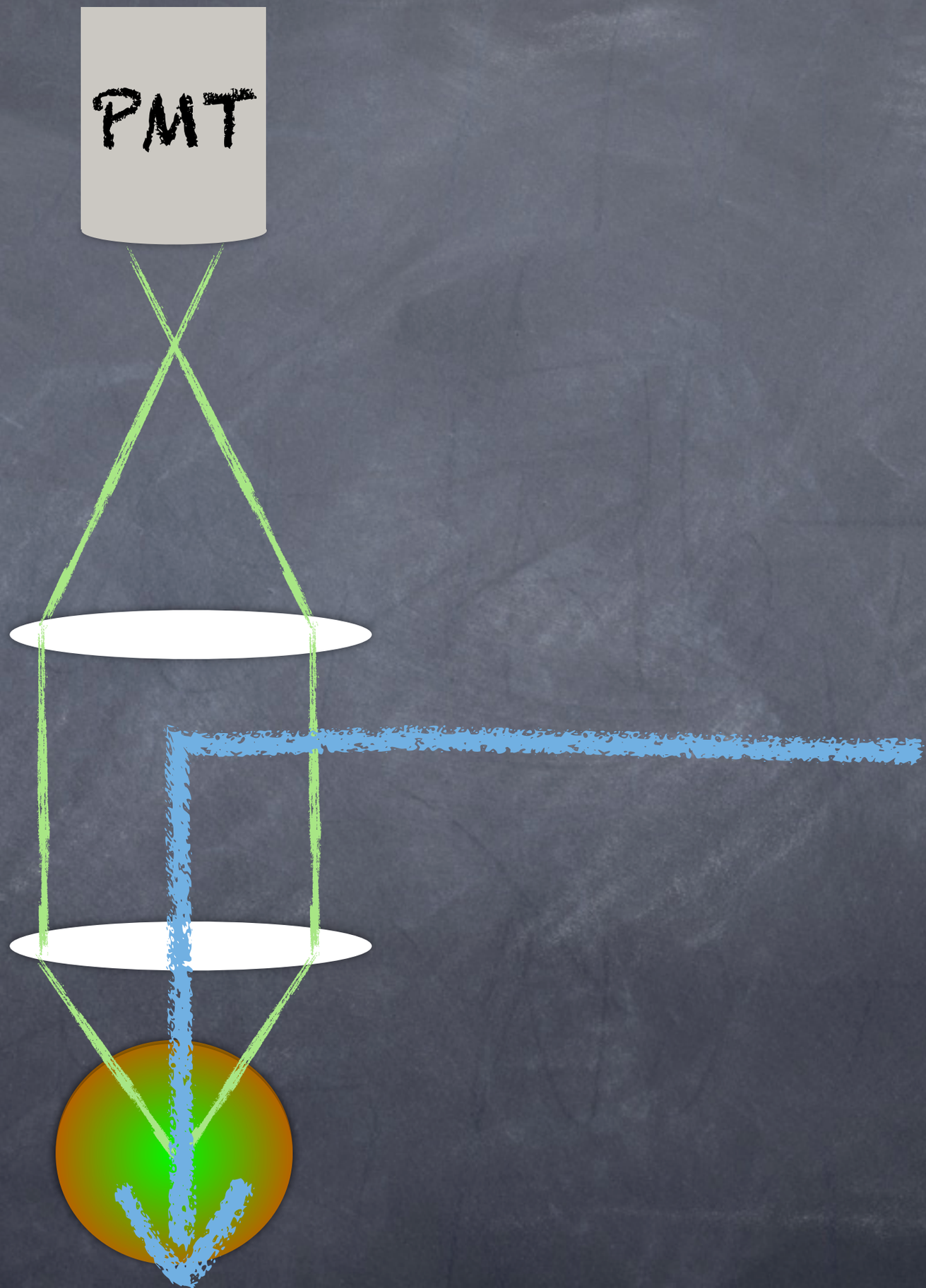
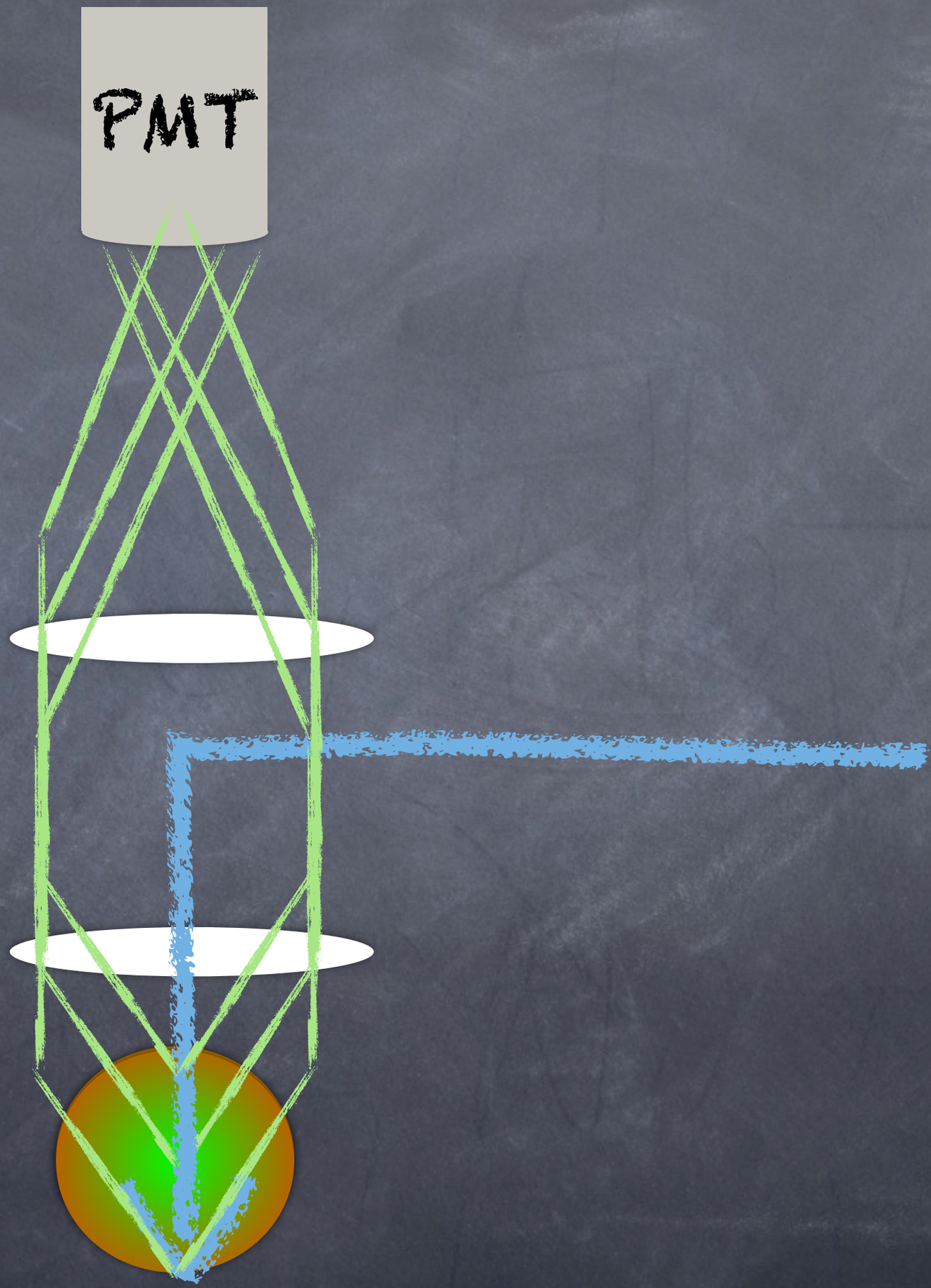
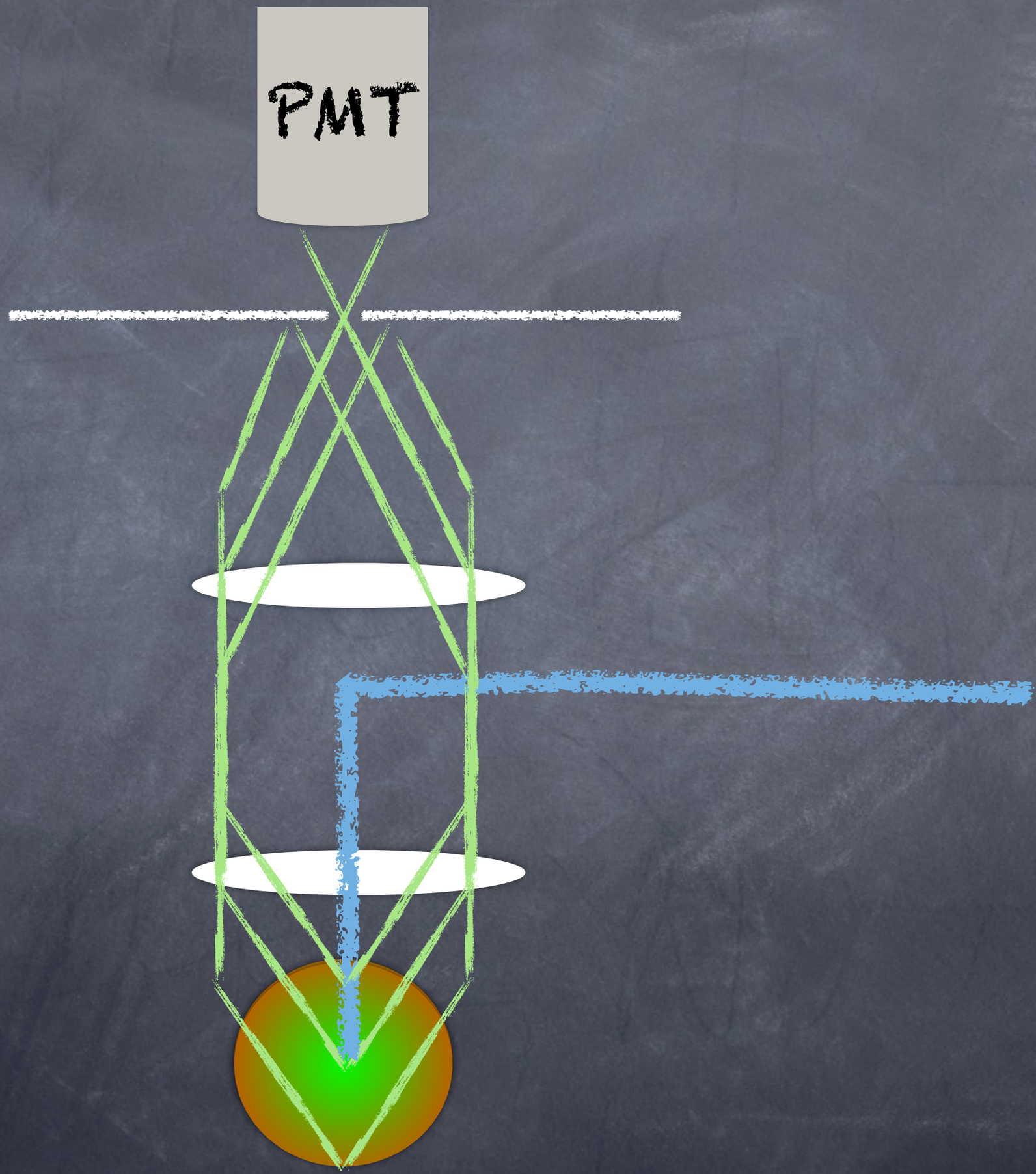


FIG. 3.

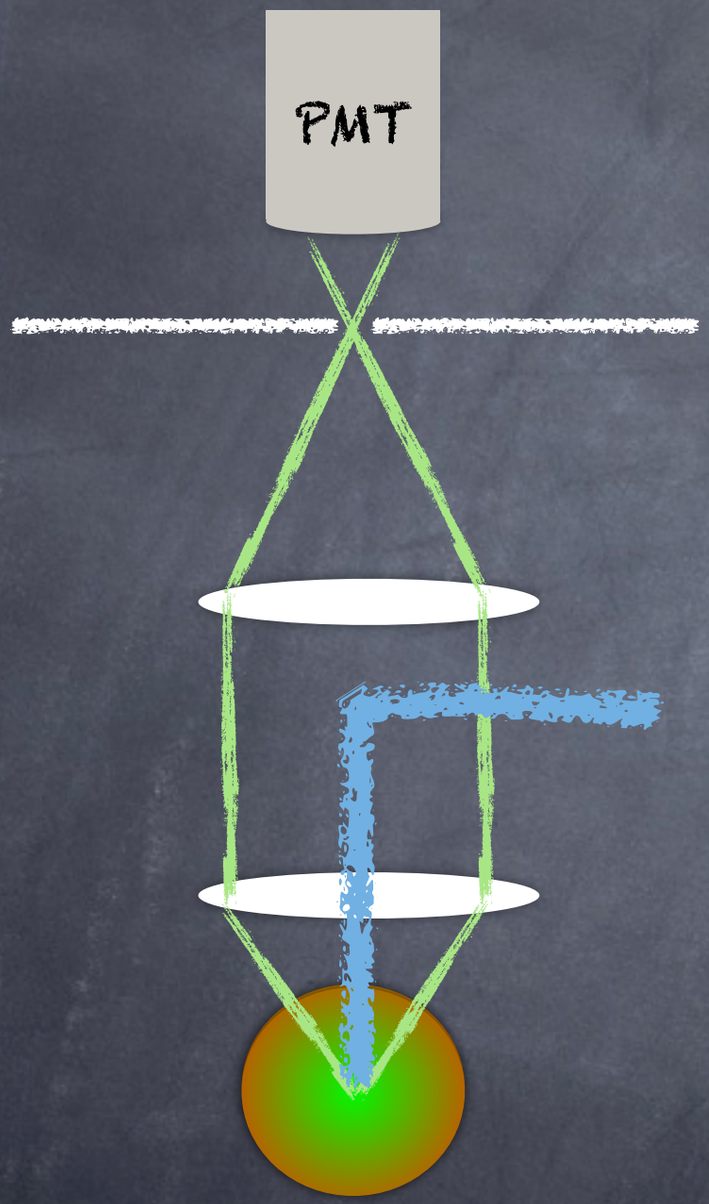
INVENTOR.
MARVIN MINSKY
BY
Amster & Levy
ATTORNEYS


PMT


PMT

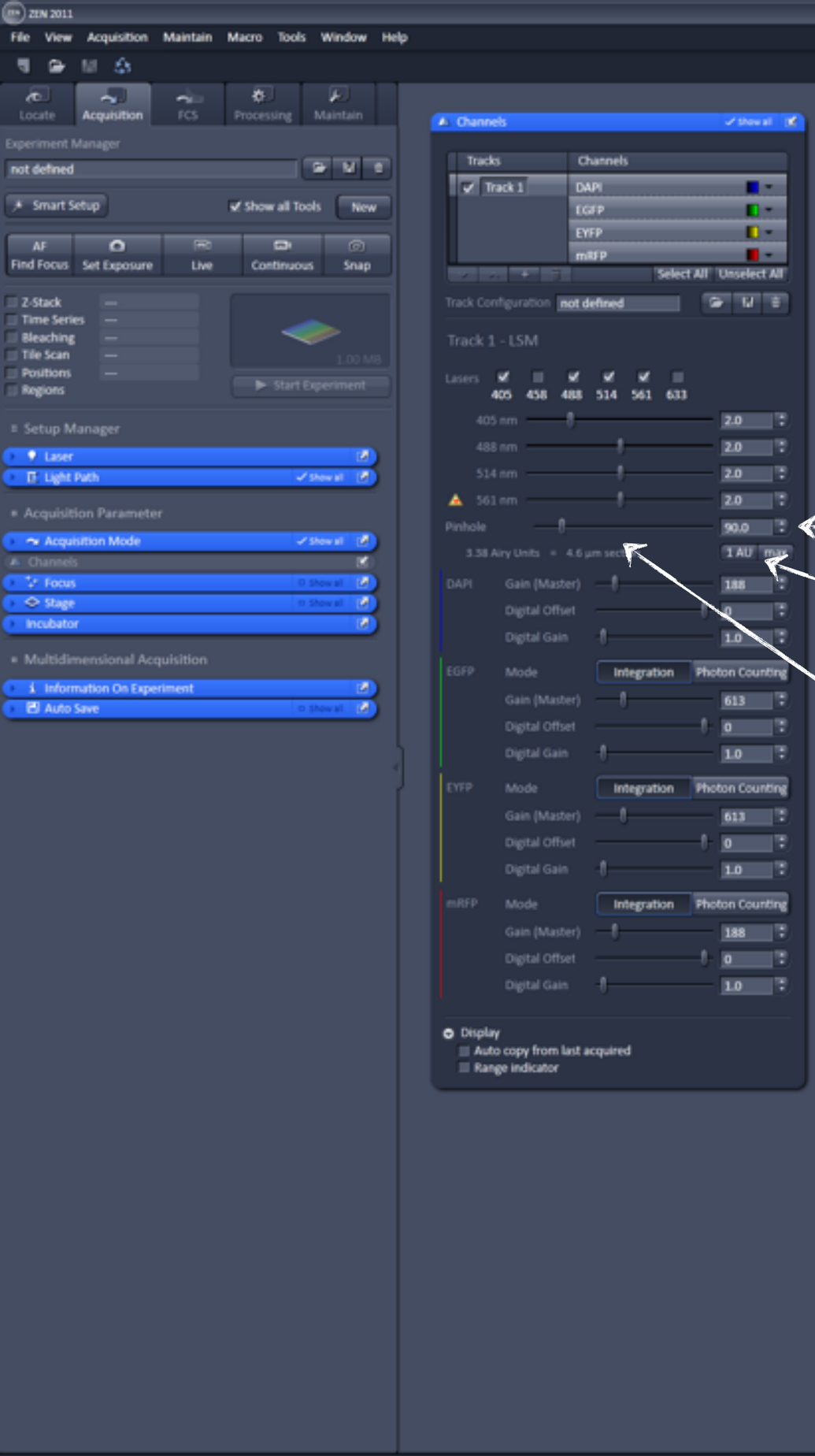

PMT

The size of the pinhole



For the best signal to noise ratio
pinhole should match the Airy disk diameter

Channels



pinhole size

1 AU is kindly calculated for you by the software

pinhole size defines thickness of an optical section

The size of an Airy disk

$$r(\text{Airy}) = 0.61\,\lambda / NA$$

please discuss:

- what is an Airy disk
- what defines it's size
- why is it good to have the pinhole of the matching diameter?

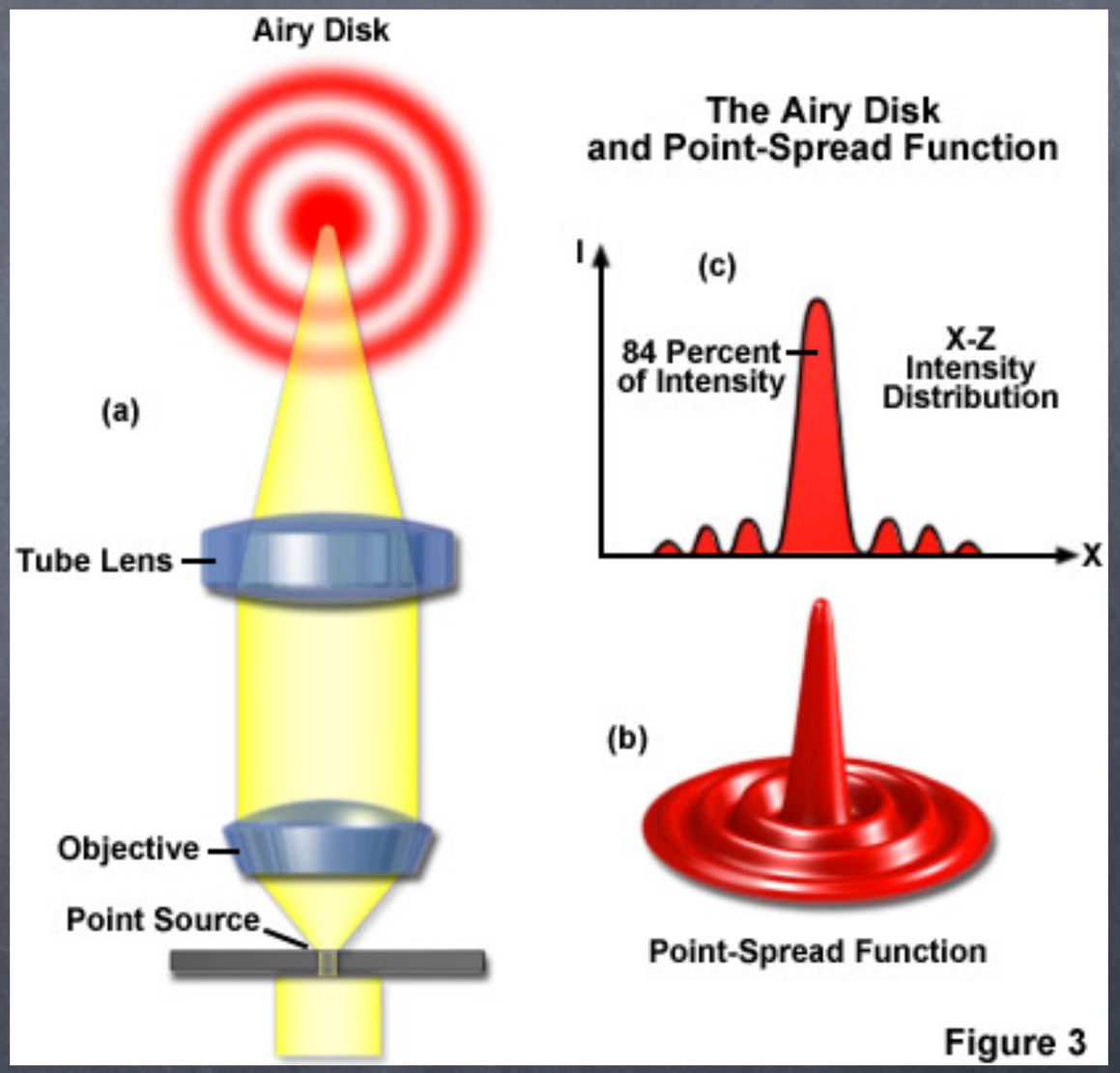
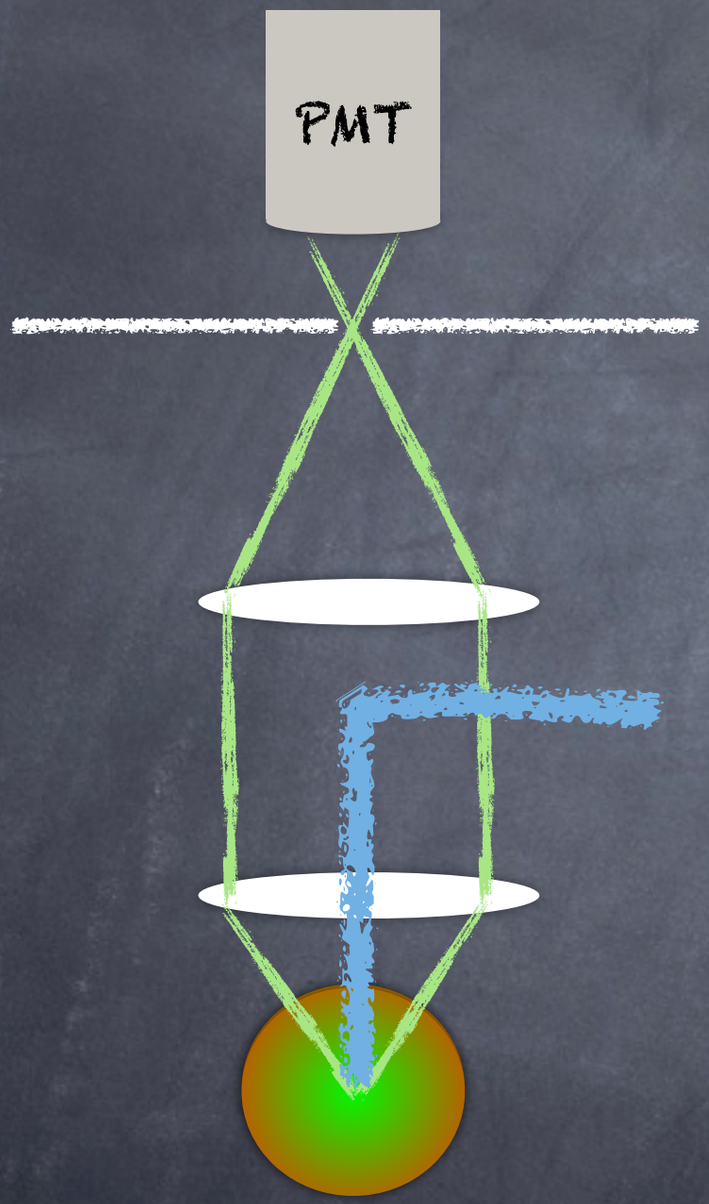


Figure 3

The size of the pinhole



please discuss:

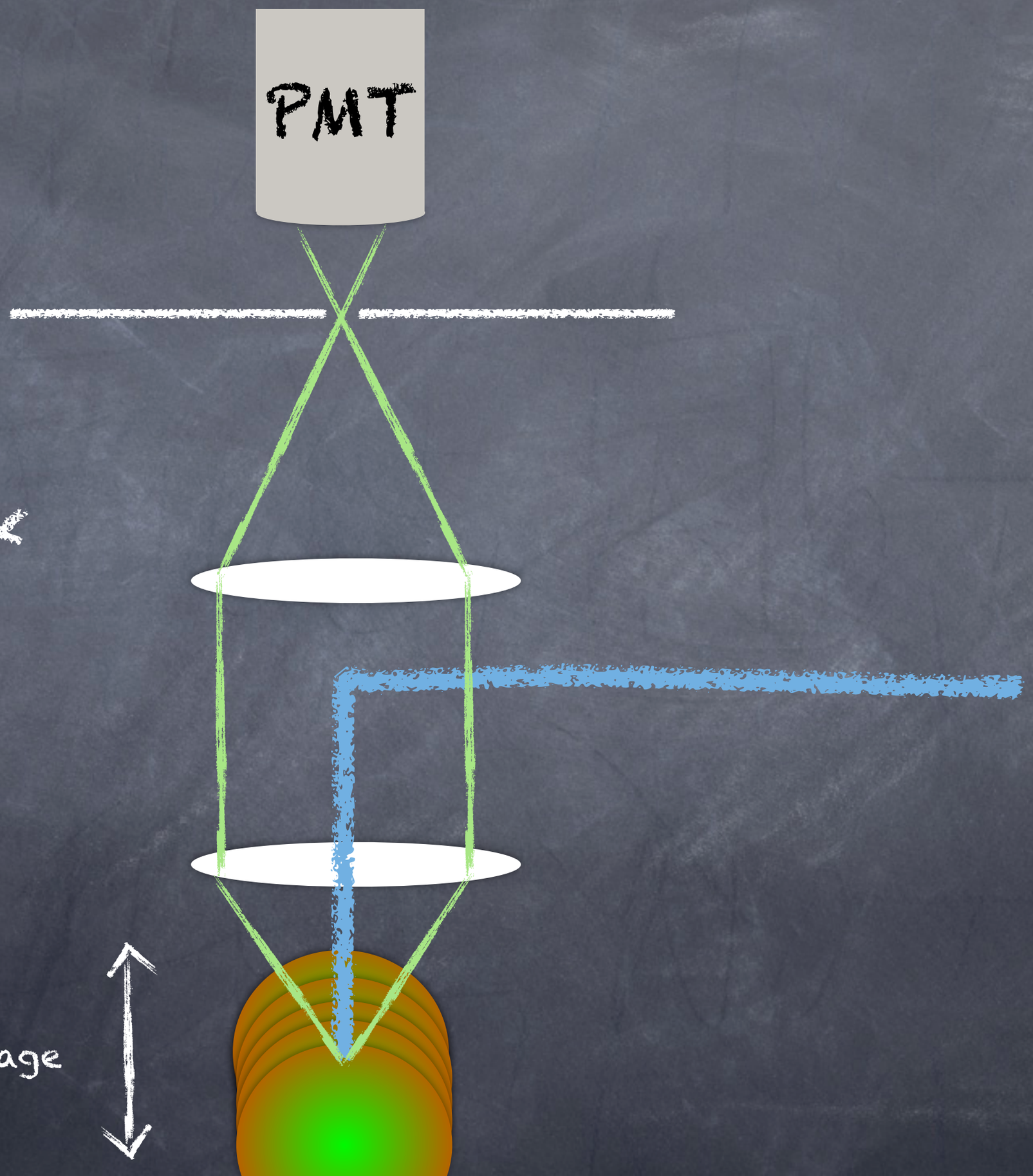
- what would happen if you set pinhole smaller than 1 AU?

- what would happen if you set pinhole size larger than 1AU?

PMT

z-stack

z-motorized stage



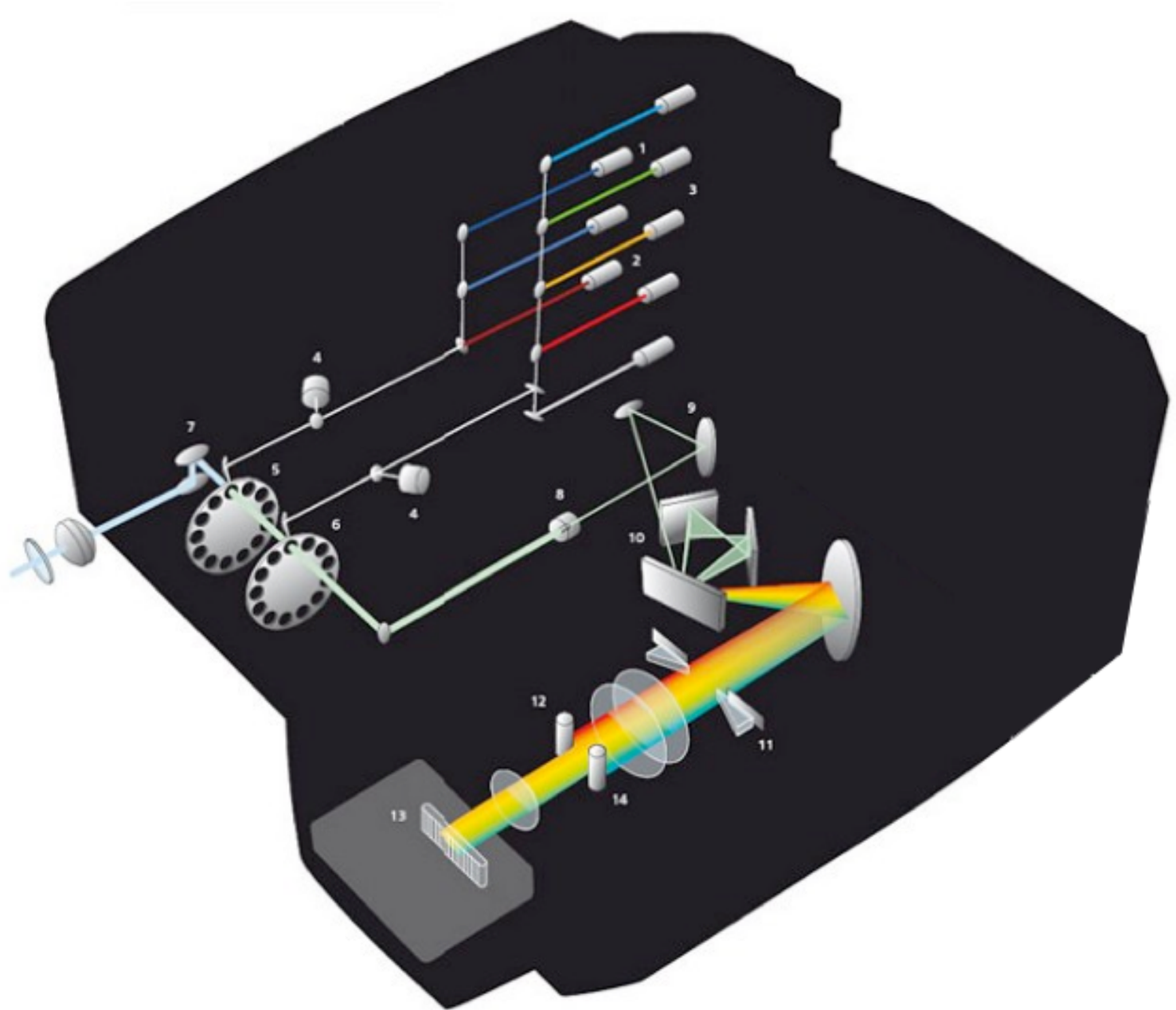
Z-stacks


ZEN 2011
File View Acquisition Maintain Macro Tools Window Help
Workspace Config
Locate Acquisition FCS Processing Maintain
Experiment Manager
not defined
Smart Setup
Show all Tools
New
AF Find Focus
Set Exposure
Live
Continuous
Snap
Z-Stack
21 Slices
Time Series
Bleaching
Tile Scan
Positions
Regions
5.25 MB
Start Experiment
Acquisition Parameter
Acquisition Mode
Channels
Focus
Stage
Incubator
Multidimensional Acquisition
Z-Stack
Information On Experiment
Auto Save
Z-Stack
Show all
First/Last
Center
14.3
-14.3
Set Last
Range 20.00 µm
Slices 21
Interval 1.00
Optimal 15.27 µm
Keep Interval Slice
Set First -10.01
Position (µm) 0.00
Optimize Sectioning and Step
Ch1
30.5 µm
Match Pinhole
Optimal
Undo
X:Y:Z = 1
Correction
Refractive Index Correction 1.00
Auto Z Brightness Correction
Use Correction
Add
Remove
Remove all
Move to
Position
0.00 µm
Load ...
Save ...
Spline interpolation
Extrapolate
Enable Test
CPU 0 %
Free HD 2.0 TB
Free Ram 10 GB

CLSM

Confocal Laser Scanning Microscopy

ok ok ?? ok



Acquisition mode

speed of scanning

bi-directional scanning is twice faster

you can rotate area of scanning,
zoom in and out

CLSM

Confocal Laser Scanning Microscopy

ok?