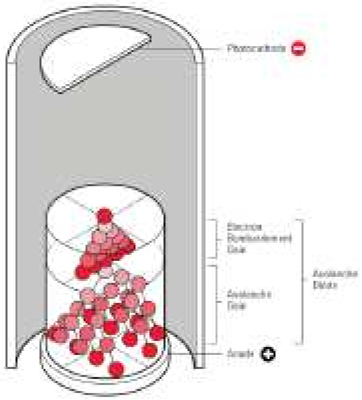
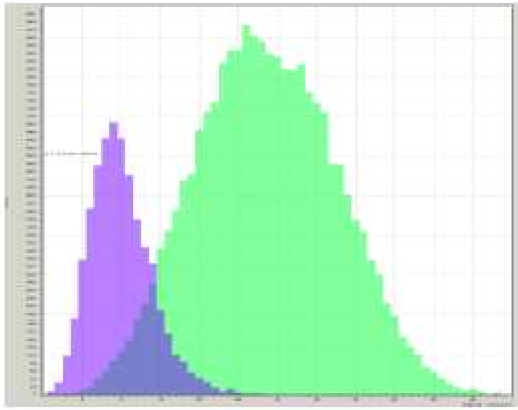
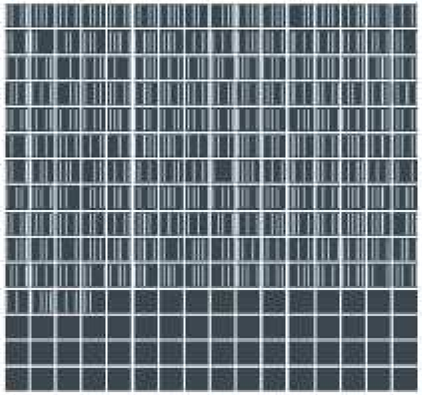
Living up to Life







HyD Information Overview

Standard and Photon Counting mode secret's reveals

- Information collected by Marco Meijering (February 2012)

- Large dynamic range
- Improved cell viability
- High-speed imaging
- Single photon counting
- Open upgrade path

Differences explained

Differences between PMT and HyD

Differences between **analog imaging** and **photon counting**

PMT

- This technique is often called; Anode Current Measurement **("analog imaging")**
- Integration is achieved **by charging a capacitor**
- The charge is measured and digitized afterwards
- **QE is 2-3 times lower** than Leica HyD (15-22%)

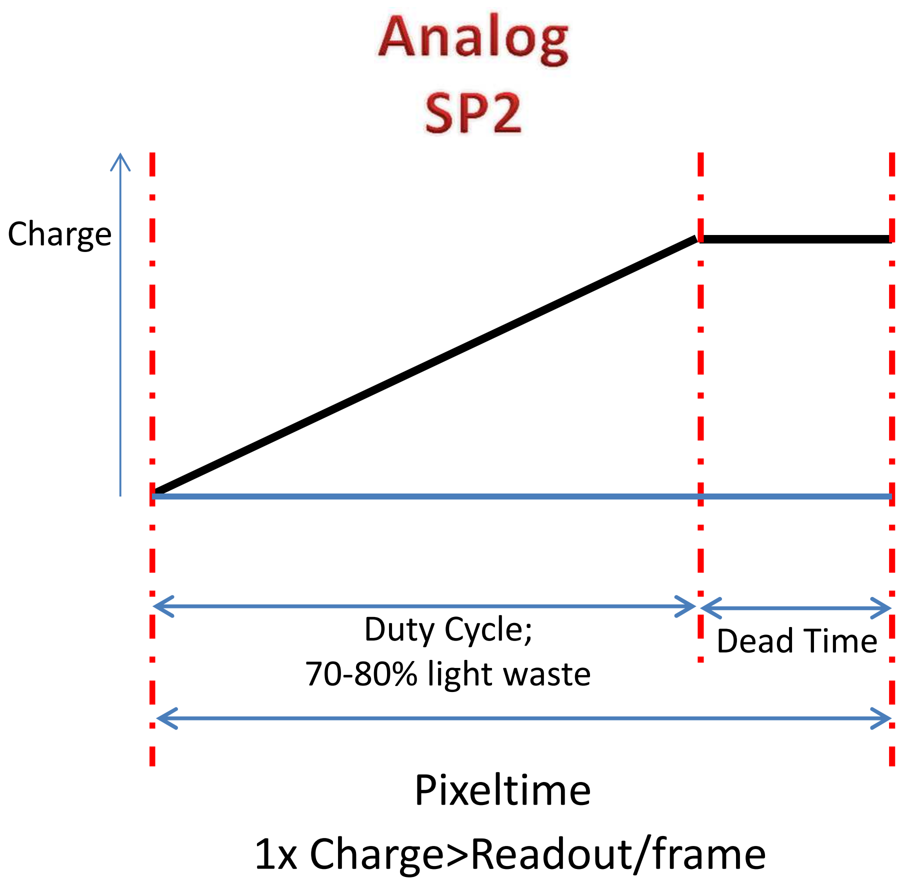
HyD

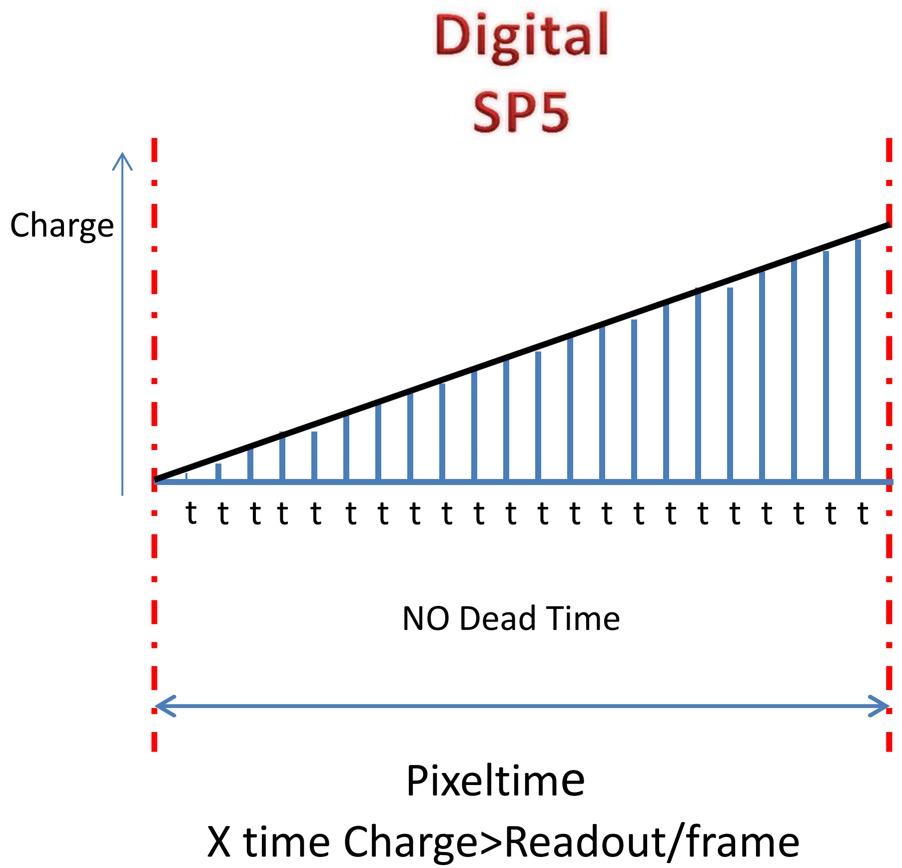
- Photons are registered individually as binary events **("Photon counting")**
- These are **continuously read out** (at MHz frequencies or faster) and **summed up digitally at the end of each pixel time.**
- Thus, digitization occurs at the **lowest possible photon level**. No charging or discharging of capacitors is needed.
- **QE ~45%@500 nm**

Quantum efficiency: Capability of detector to translate photons into electrical signals

Pixel Analog vs Digital readout

- Pixel integration / averaging

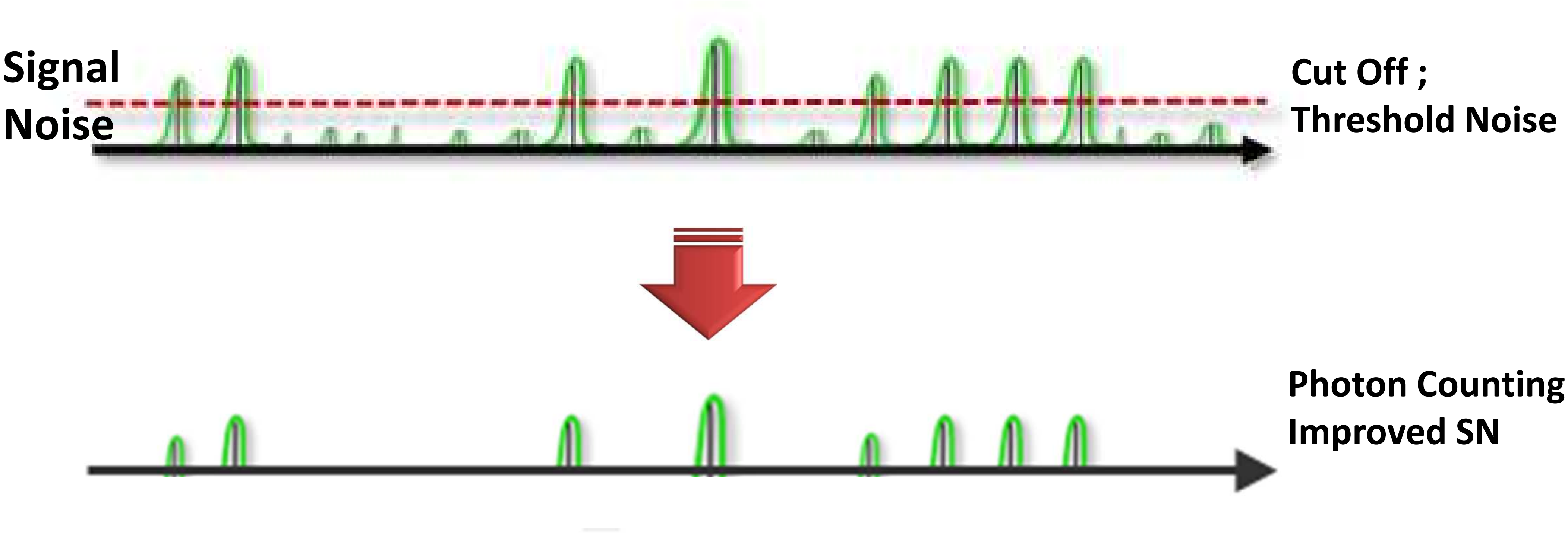




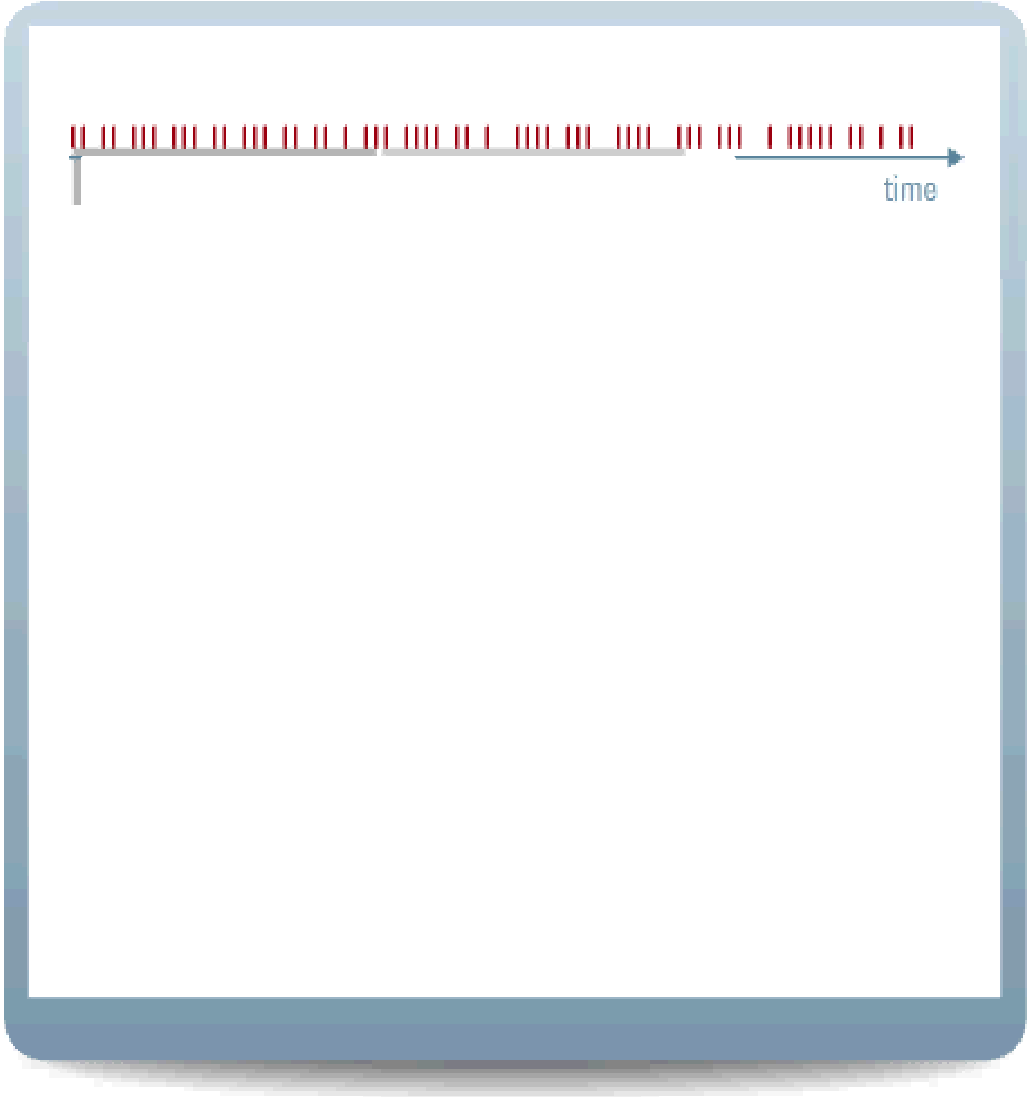
The SP5 uses a special variant of this principle by reading out its PMT at 40 MHz and averaging over the samples acquired over time.

Photon counting: Improved Signal/Noise

Thresholding separates noise from signal
Intrinsic noise gating



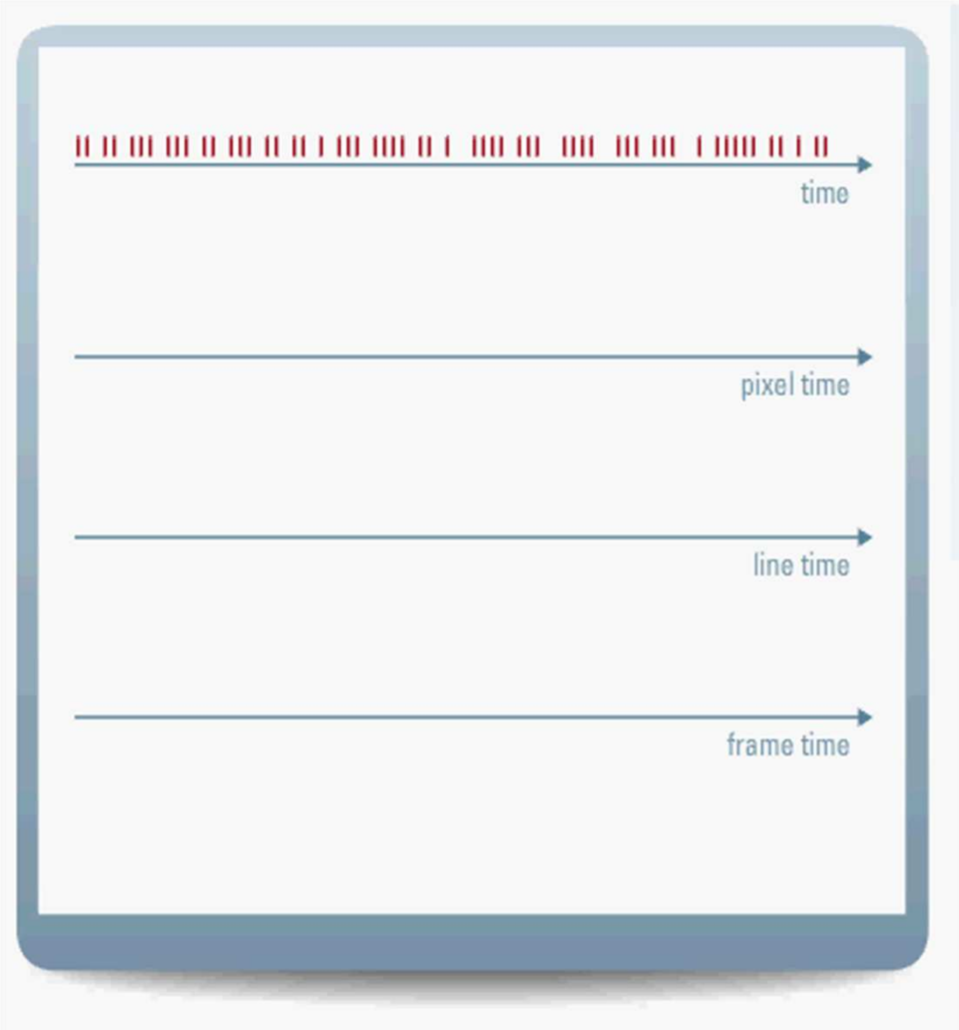
Photon Counting; HyD



Photon counting for confocal imaging

Photon counting works by thresholding electrical pulses and treating them as binary events (photon or no photon). The read-out of photons is done sequentially. The arrival time of a photon pulse in relation to scanner internal clock signals determines which pixel, line and frame the photons are assigned to. In a scanning device such as a confocal microscope this information is available by default.

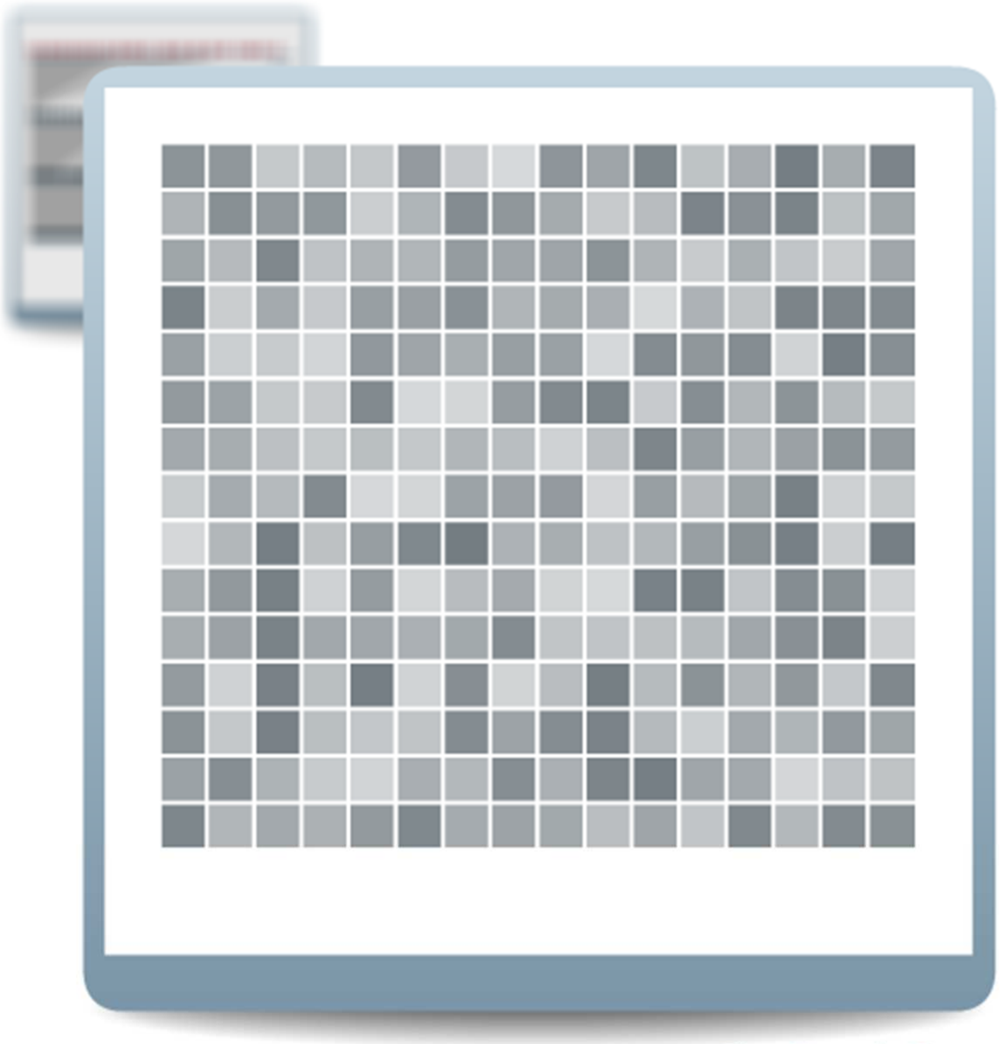
Photon Counting; HyD



One photon – one gray value

The photons assigned to individual pixels are displayed as a color-coded image. A photon counting image represents a spatial map of signal intensities.

Photon Counting; HyD

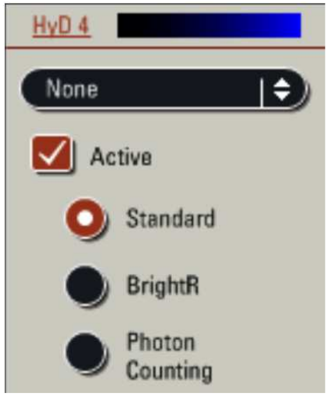


Signal Intensity Map

Signal intensity map for quantitative imaging

Due to the nature of photon counting a direct link between pixel intensity and the number of molecules exists. Knowledge of the molecular brightness (photons per molecule per unit time) allows the use of a photon counting image as a concentration map for monitoring of biochemical reactions and molecular stoichiometries.

HyD LASAF operation modes



Standard

- **Operating mode for image acquisition.**
- **Can set the Gain of the detector as usual with PMTs**
- **Mapping absolute photon counts to a look-up table means one has to introduce a scaling factor**

BrightR

- **Specifically for dynamic samples with dark and bright in one image.**
- **In this operating mode, the Gain should be set to the lowest possible value.**

Photon Counting

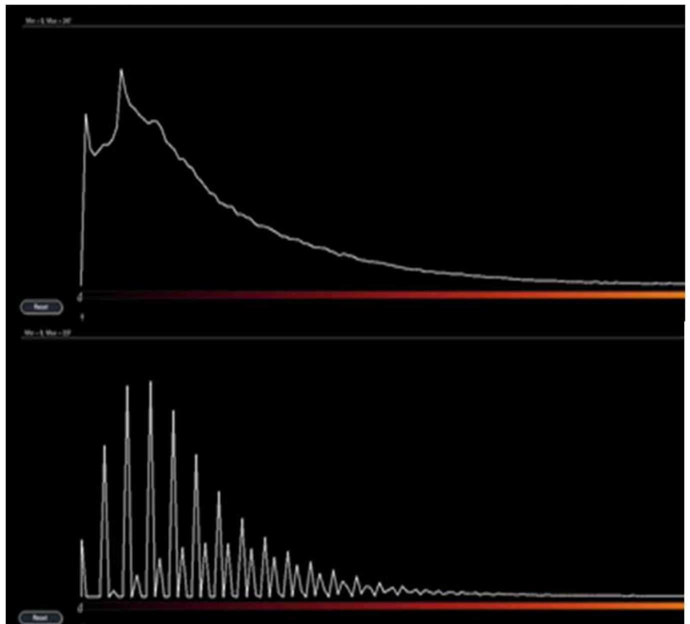
- **Direct translation of photon counts into grey value**
- **No further amplification**
- **In this operating mode, the Gain of the light signals is set to a fixed value in order to ensure constant detection conditions for photon counting.**

Histogram visualization

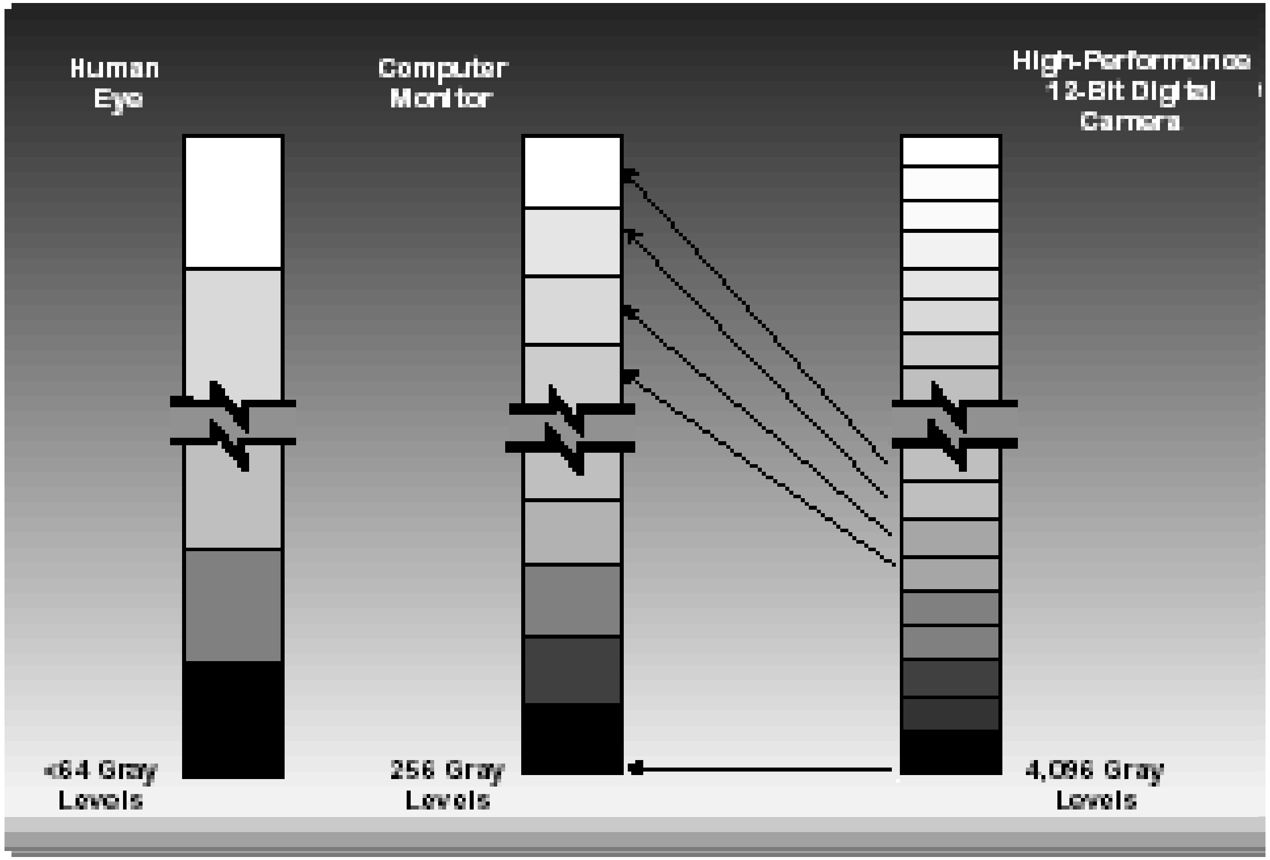
Histograms

- In order to maximize image quality and quantifiability we need a detector with
  - o–Efficient photocathode
  - o–Simple internal geometry
  - o–Photon Counting instead of anode current measurement
  - o–Digital Gain rather than varying detector voltage

This has a few consequences on the histograms

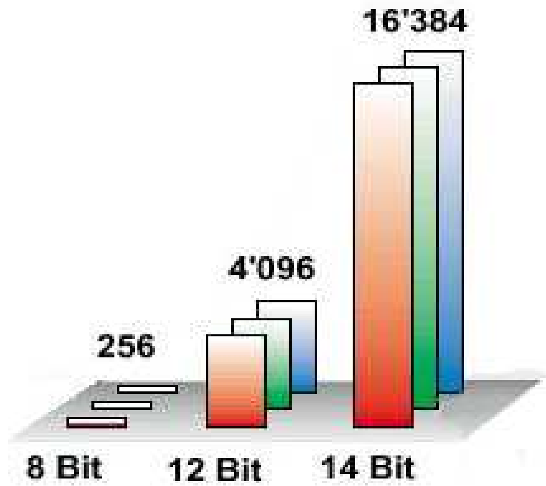


Imaging and dynamic range



•The 4096 shades of grey that constitute a 12 bit image can be rescaled and mapped to the computer in different way's in order to bring out fine details.

•Each 16 th level is mapped to the monitor so that the screen displays the full contrast range from black to white



Imaging and dynamic range

$$DR = \frac{maximum\ signal}{detector\ noise + electronic\ noise} = \frac{N_{sat}}{N_{noise}}$$

$$DR_{dB} = 20 \log\left(\frac{N_{sat}}{N_{noise}}\right)$$

HyD as a photon counting detector has REAL 16-bits, whereas analog PMT detectors have well sampled noise

How much dynamic range can you actually salvage in a digital image?

Bit Depth	Grayscale Levels	Dynamic Range (Decibels)
1	2	6 dB
2	4	12 dB
3	8	18 dB
4	16	24 dB
5	32	30 dB
6	64	36 dB
7	128	42 dB
8	256	48 dB
9	512	54 dB
10	1,024	60 dB
11	2,048	66 dB
12	4,096	72 dB
13	8,192	78 dB
14	16,384	84 dB
16	65,536	96 dB
18	262,144	108 dB
20	1,048,576	120 dB

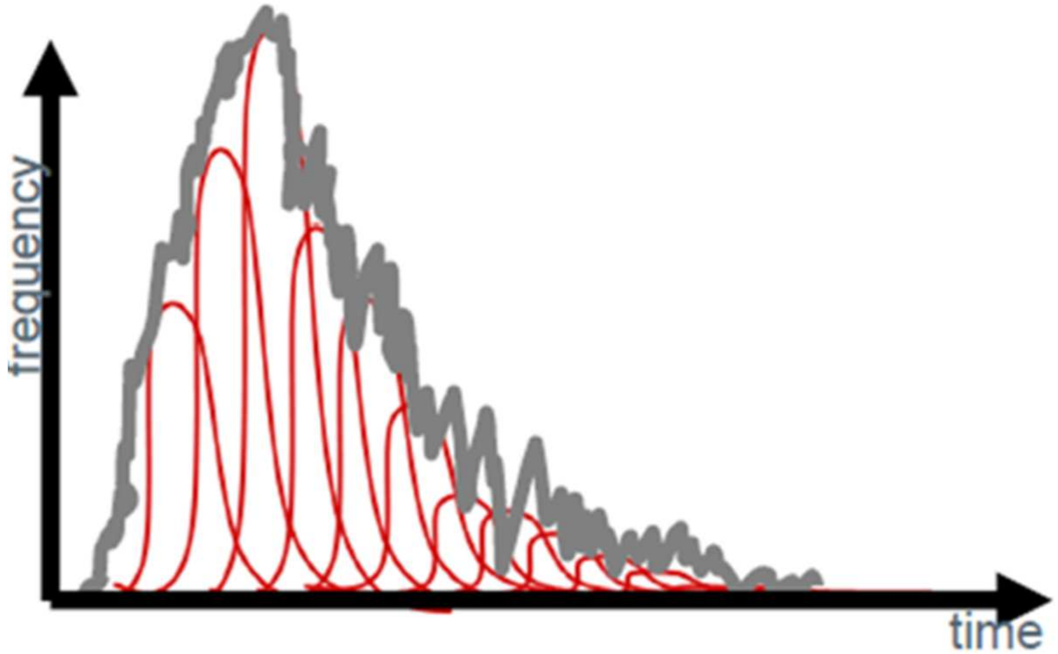
source: Hamamatsu

Leica offers 16-bit imaging => You can store up to 96 dB in one image

High dynamic range

Histograms of conventional PMTs

So, why do I see that many more grey levels with my “old” PMT?

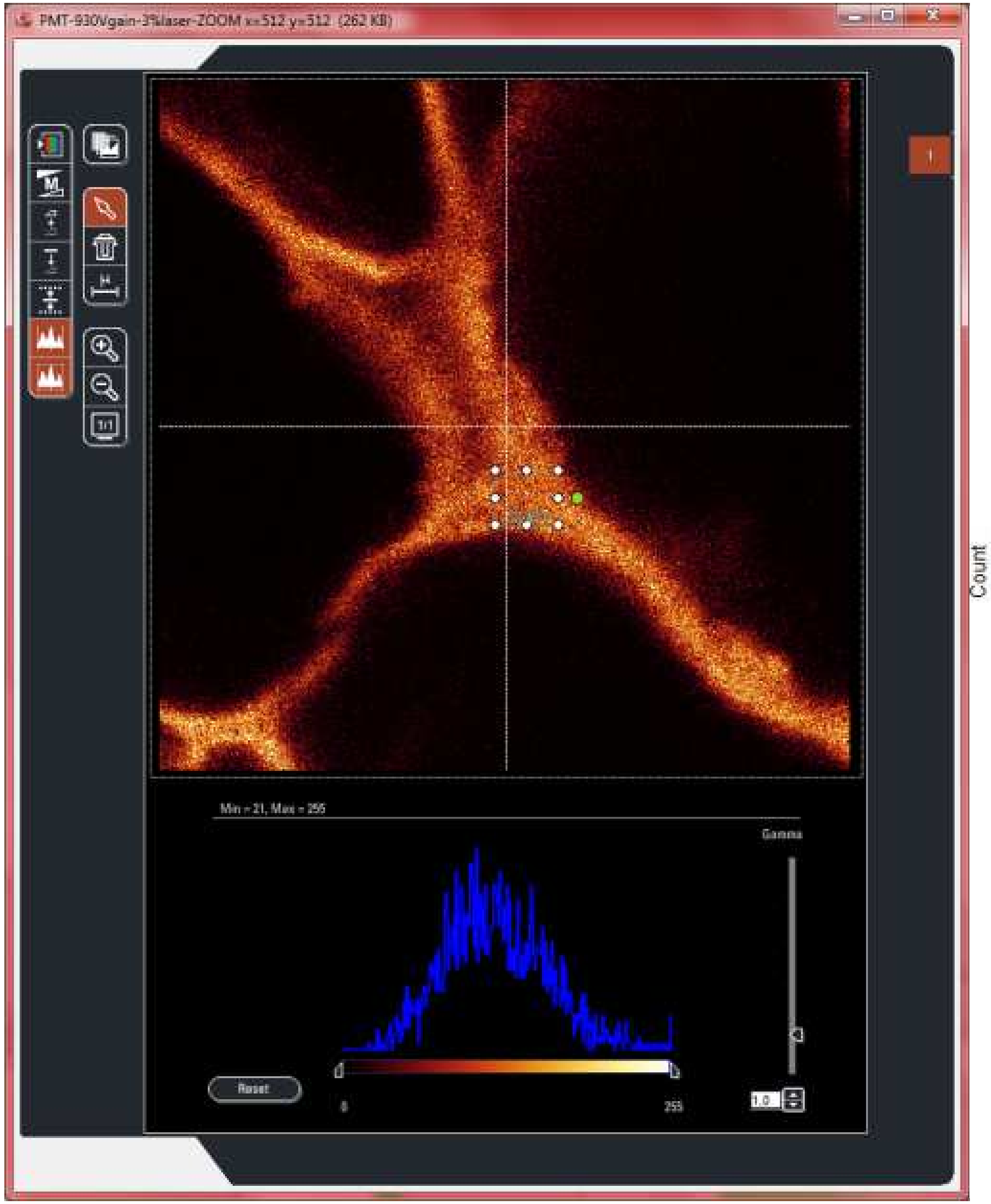


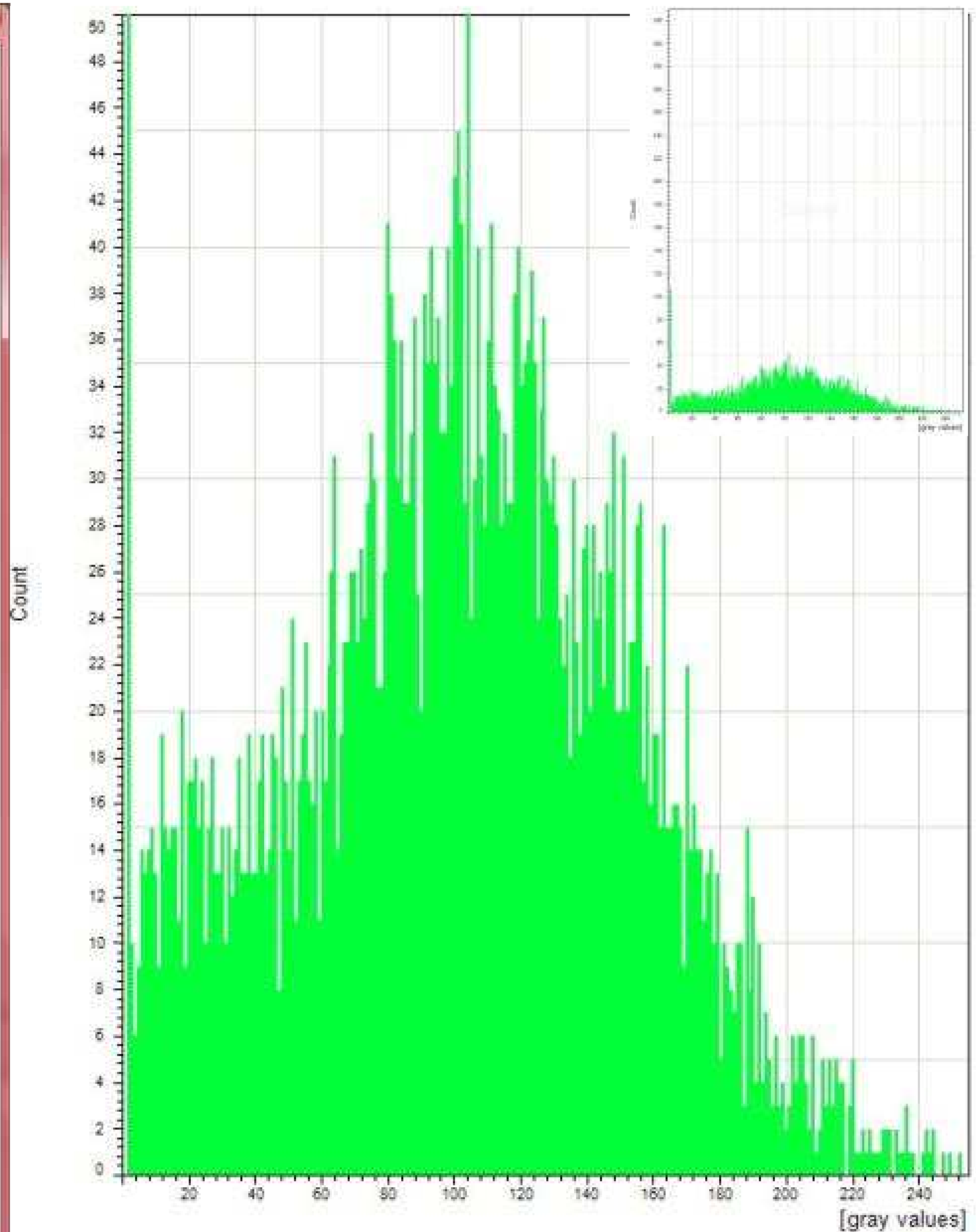
Typical PMT’s used in confocal imaging are lacking at least some of the essential properties needed for Photon Counting:

- Low dark current
- Low dead time
- Sharp single pulses
- Fast electronics
- Rapid sampling rate

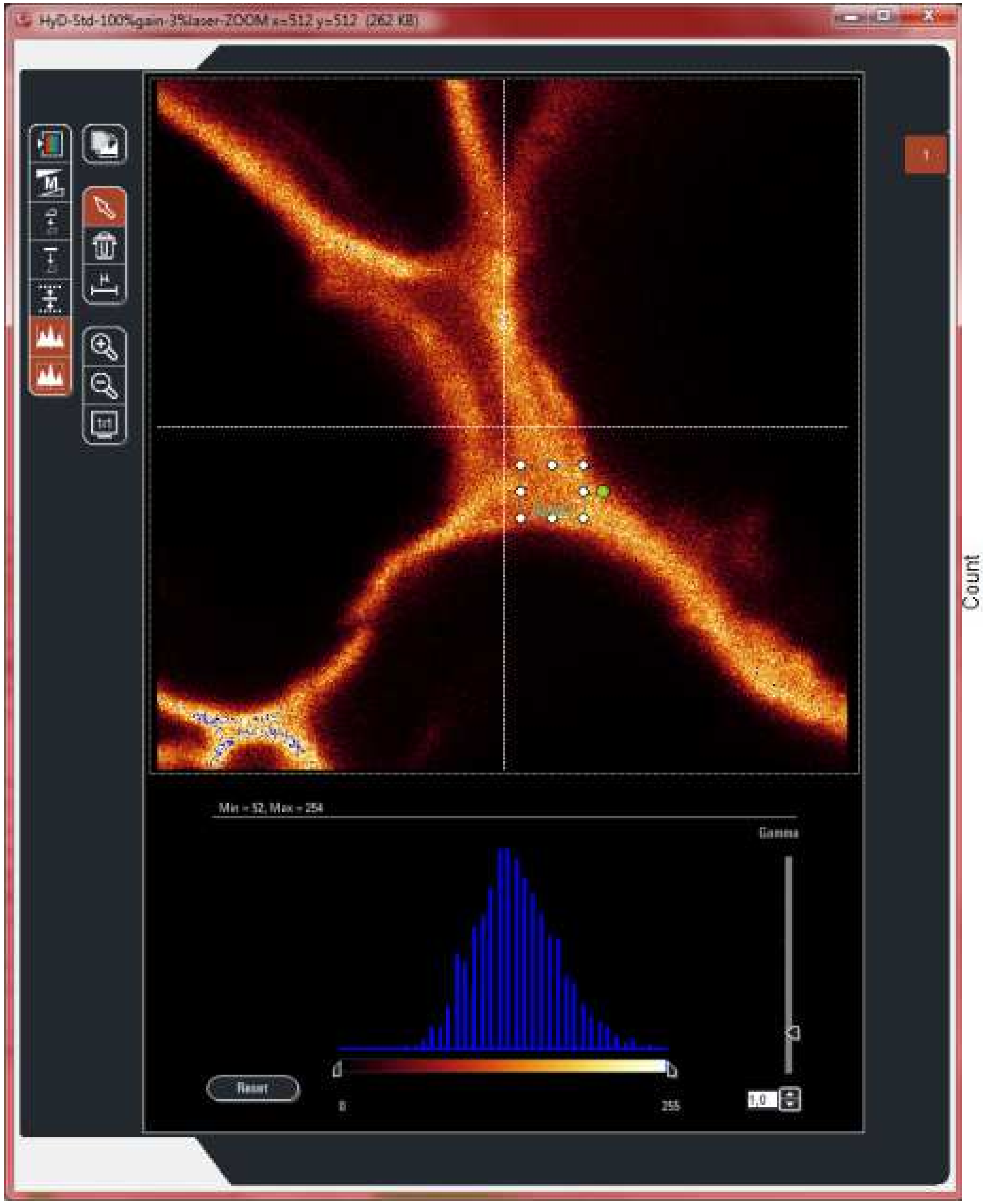
- This is the reason that the **individual photon levels** recorded by a photon counting system **degenerate to a Poisson distribution**, but you only see the **envelope** of it!
- The many grey levels exhibited by a non-photon counting system are thus an ARTIFACT.

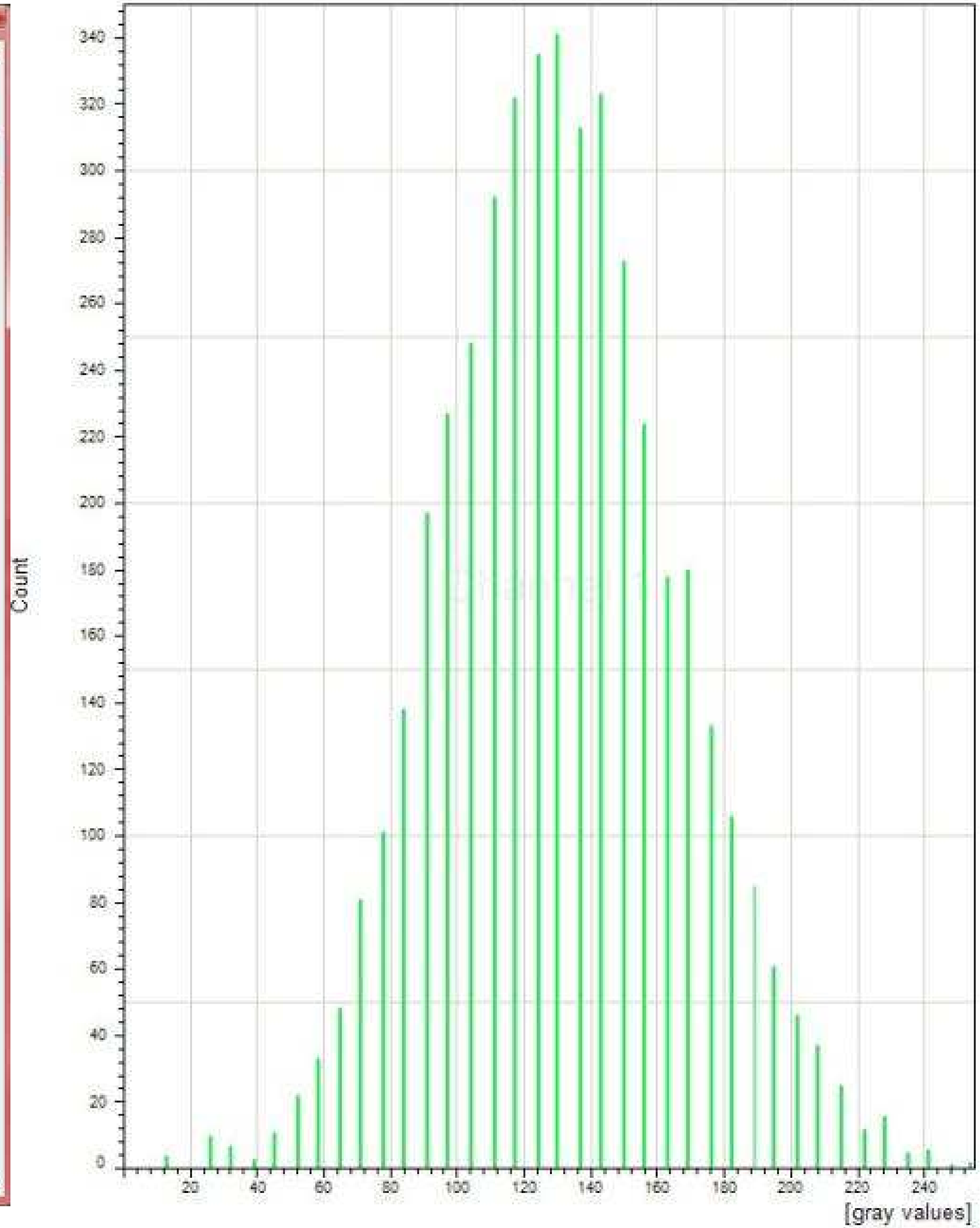
PMT 930V 3%laser zoom





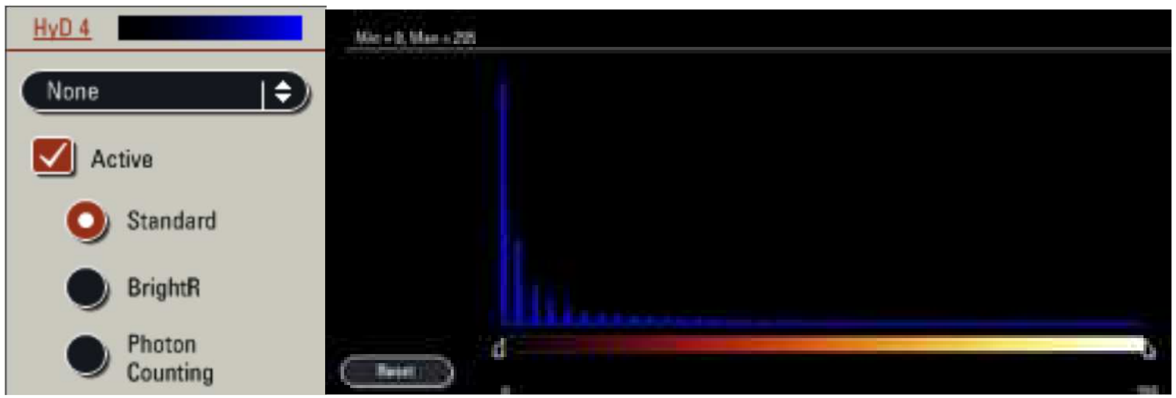
HyD Standard 100% gain 3%laser zoom

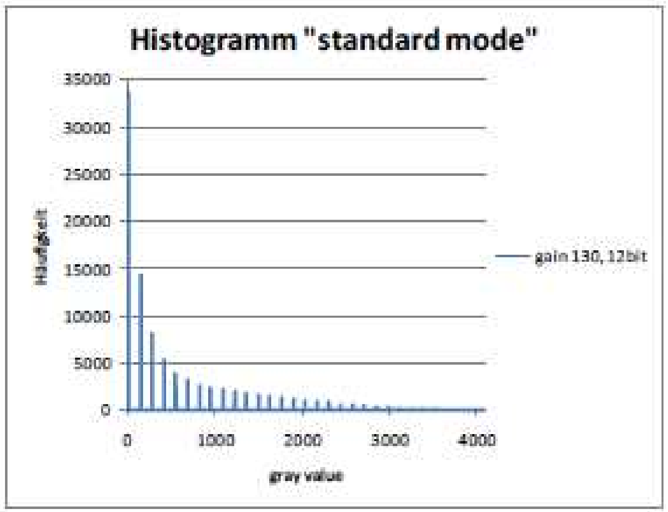




Histograms of Photon Counting with Digital Gain

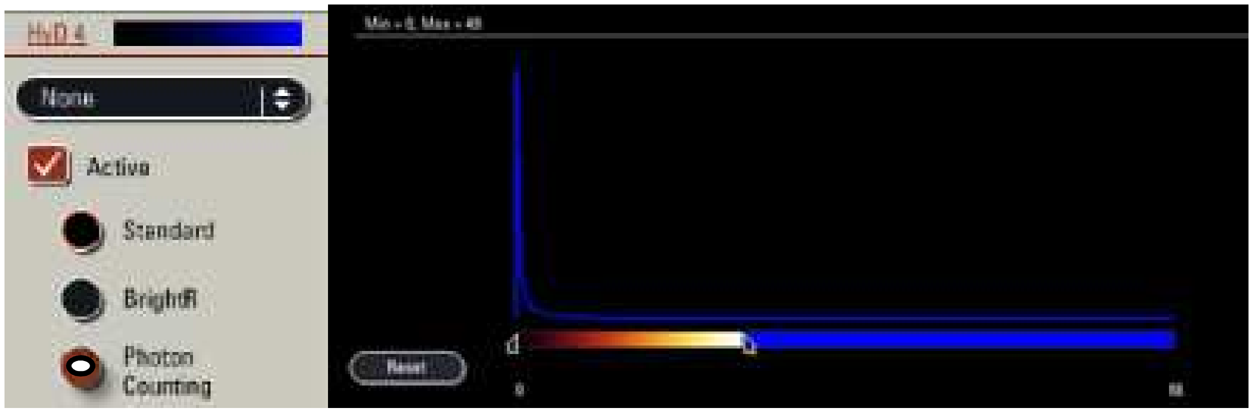
- Mapping absolute photon counts to a look-up table means one has to introduce a scaling factor.
- At the same time there can only be integer, "whole numbers" multiples of photon counts
- That's why, such a mapped image has a „spikey" look to it. This is indicative of the system's capability to really detect single photons (and for that you need high QE, good S/N, narrow single pulses from the detector, low dead time and fast electronics with high sampling frequency)

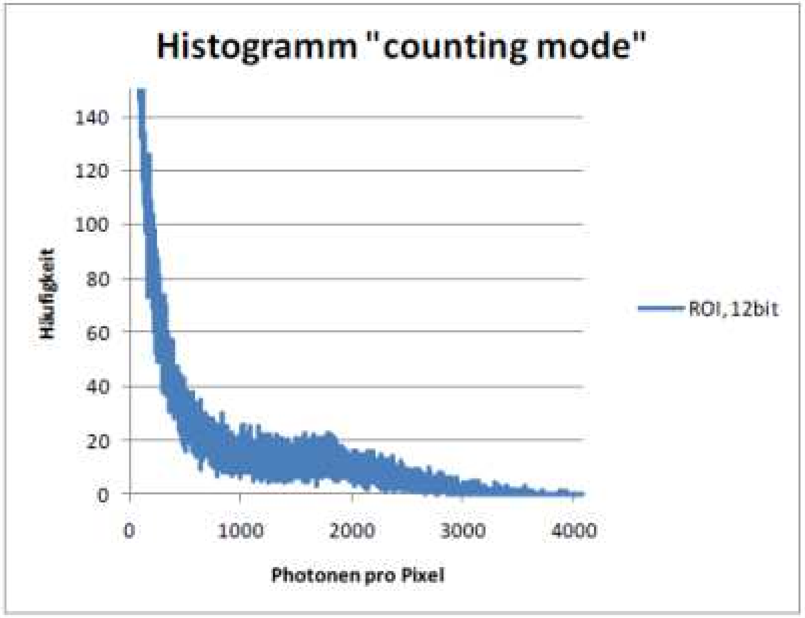




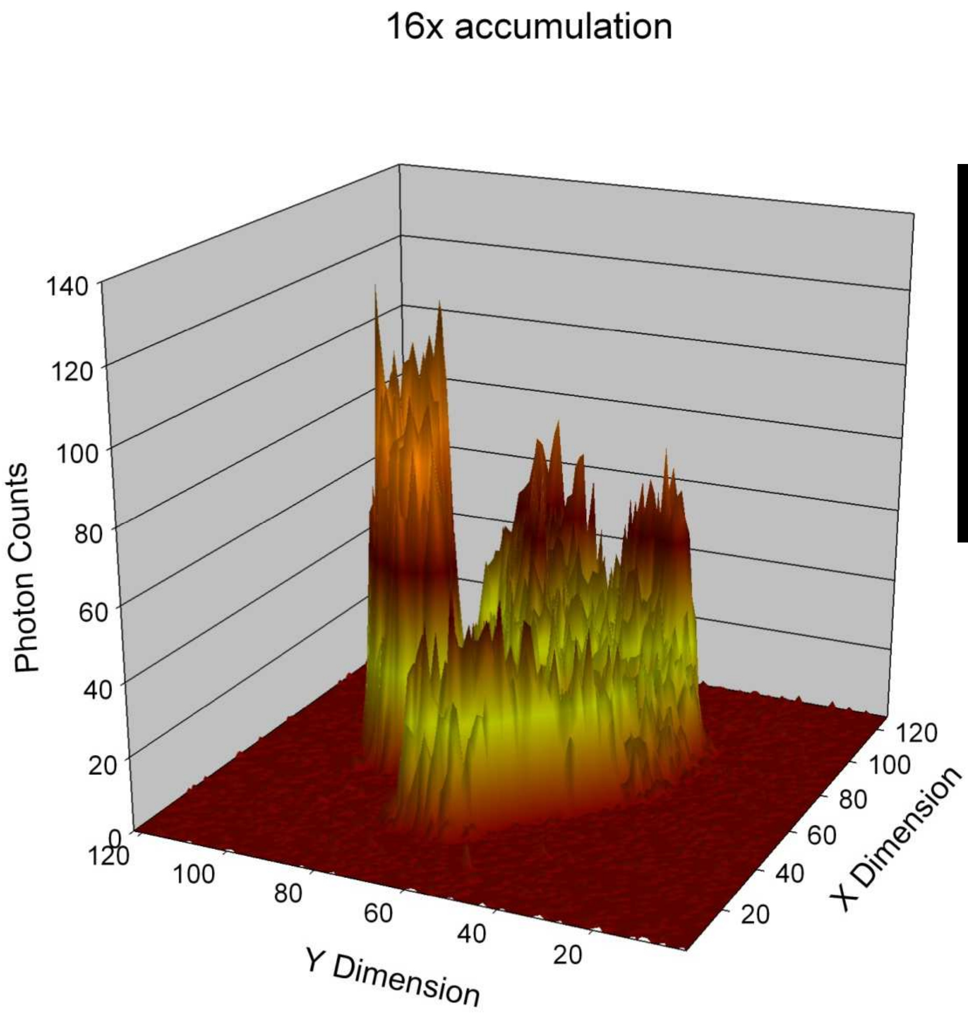
Histograms Photon Counting alone

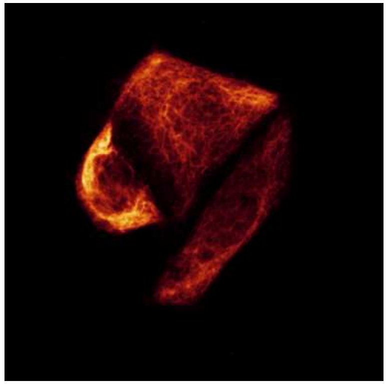
- Each photon is represented as digital number „one“
- That’s why the histogram looks continuous due to the particle nature of light and an equivalently quantized way of detection
- Unlike below the absolute numbers often are very small, as photon fluxes are typically within the Mcounts / s = MHz range
- At „normal“ microsecond pixel times this equals to a hand full of photons per pixel





Maximize dynamic resolution by photon counting





16x Accu

Detectors in detail

Leica HyD for confocal
The road to super-sensitivity

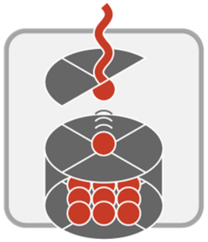
Leica MICROSYSTEMS

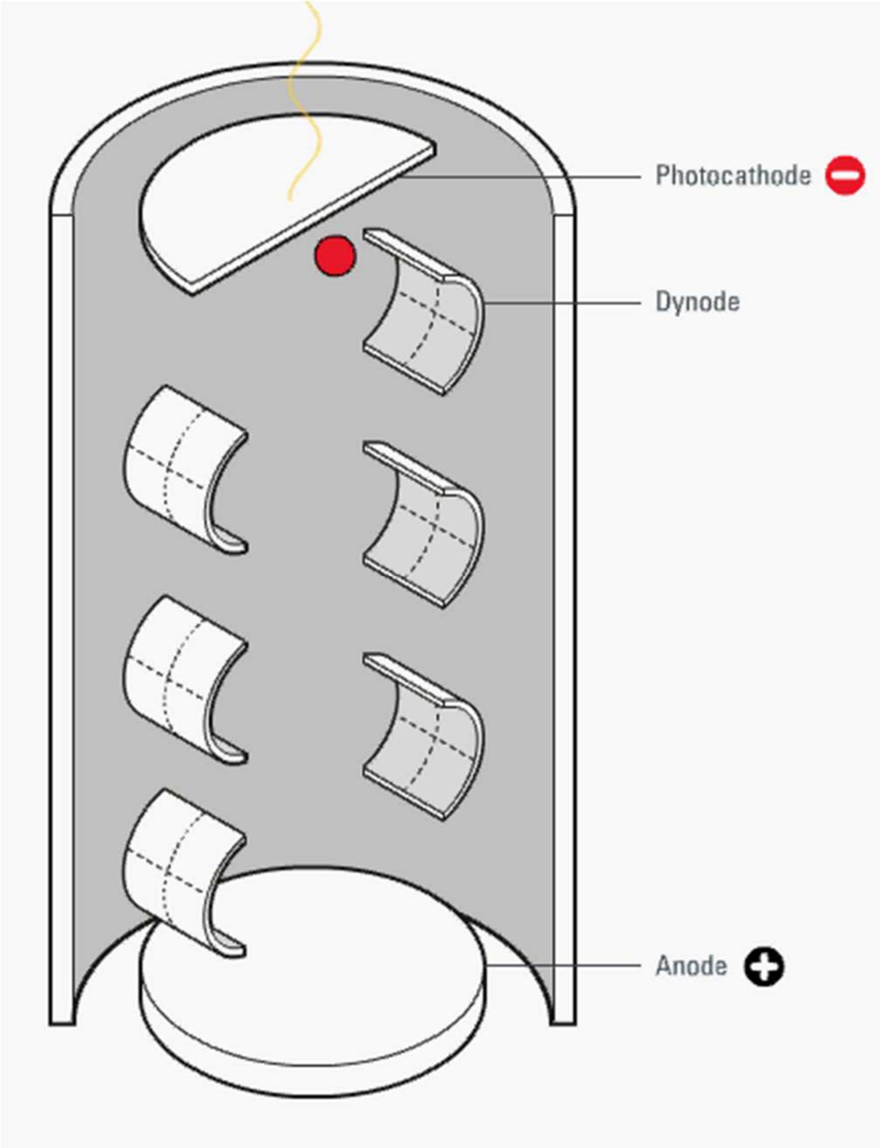
What is hybrid detection ?

Originally developed for particle physics the design goal for hybrid detection technology was a short temporal response for high intensity single photon counting. This is possible thanks to a very small transit time spread, meaning very sharp and defined pulses in response to incident photons. This is accomplished thanks to a two step architecture with one vacuum acceleration step followed by electron bombardment and avalanche gain. In the HyD implementation this architecture is complemented by a photocathode made of GaAsP material with 45 % typical quantum efficiency at 500 nm. Quantum efficiency is the percentage of photons producing primary photoelectrons.

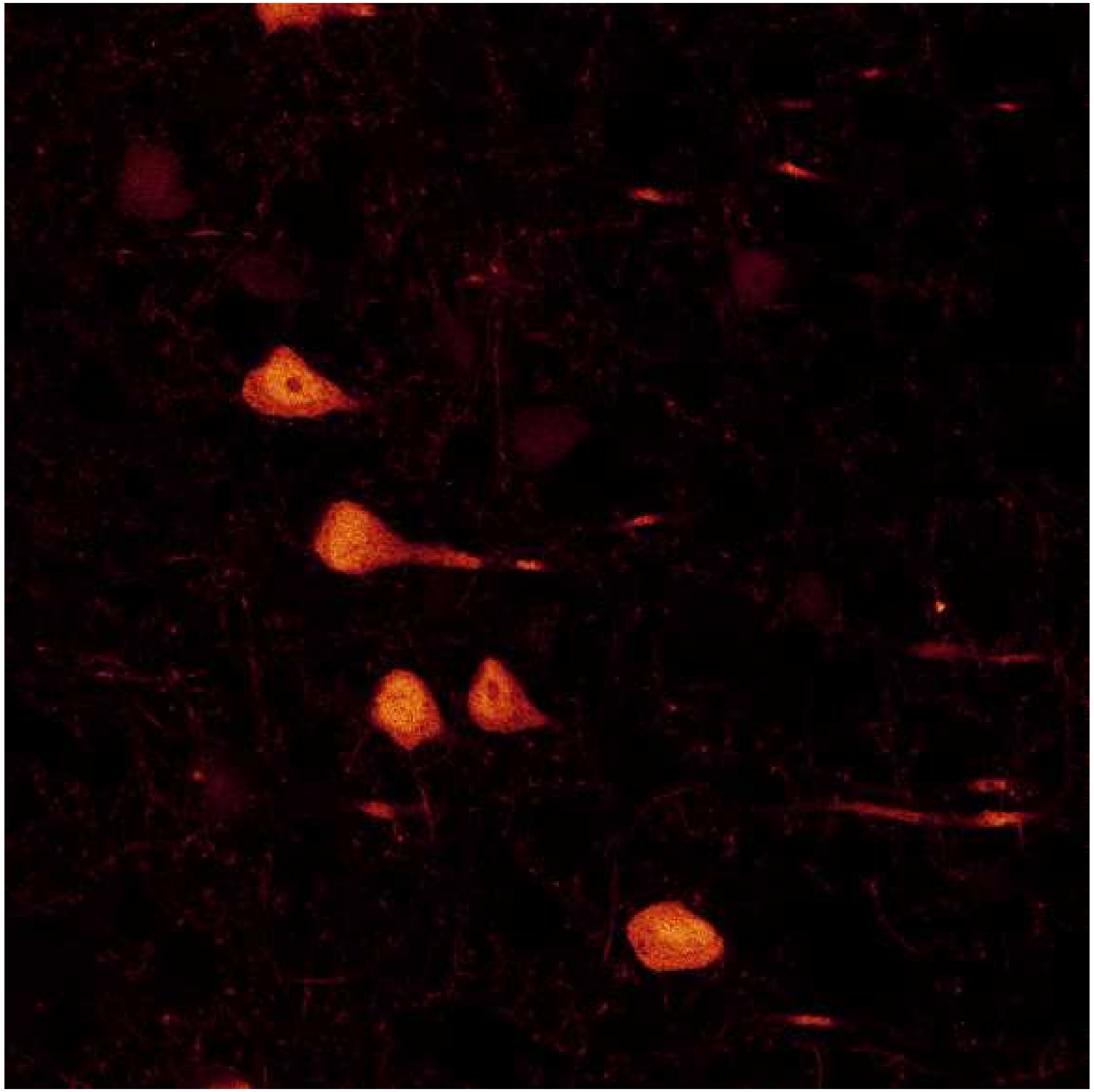
http://www.leica-microsystems.com/science-lab/step-by-step-guide-to-hybrid-detection-and-photon-counting/

Leica HyD for confocal
The road to super-sensitivity





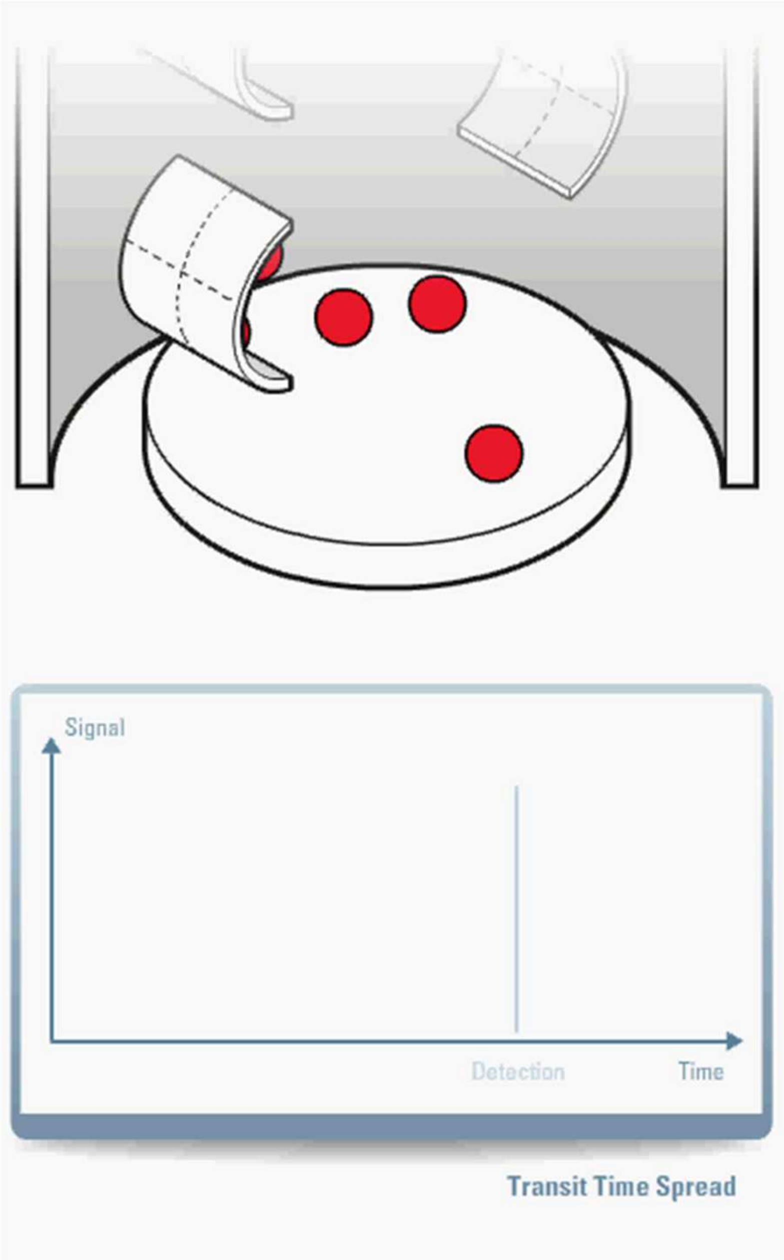
Photomultiplier technology



PMT 800 V

Leica HyD for confocal

The road to super-sensitivity

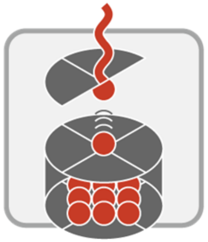


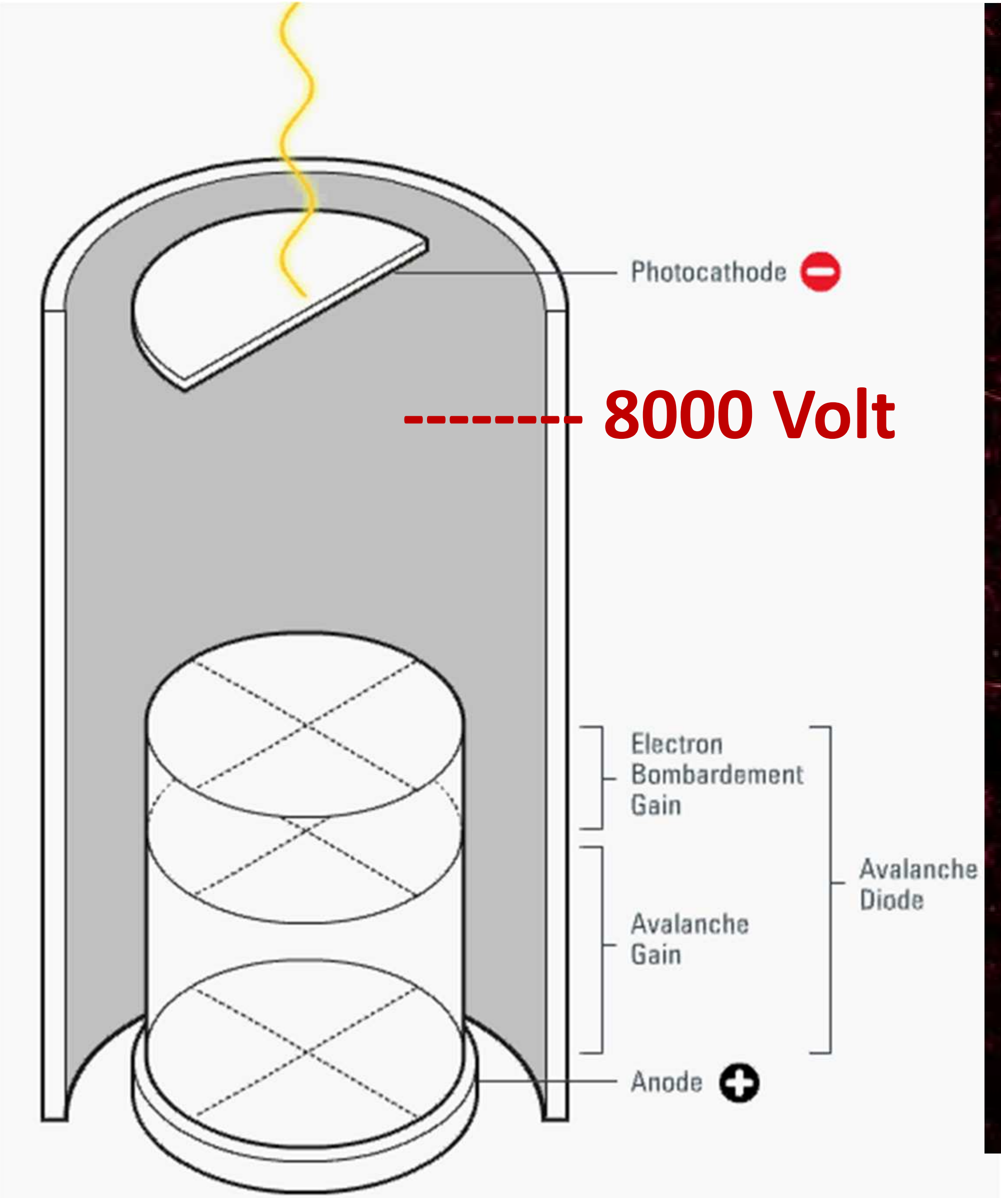
Time of flight dispersion in PMTs

Observing very closely the arrival of photoelectrons allows to understand the transit time spread of detection. Time of flight dispersion and statistical secondary electrons lead to a distribution of arrival times of single photons. Unfortunately, this means that there is a lower limit to resolving consecutive photon pulses being recognized as individual signals. Conversely, the maximal rate of photon current (i.e. intensity) is limited by the transit time spread and downstream electronics.

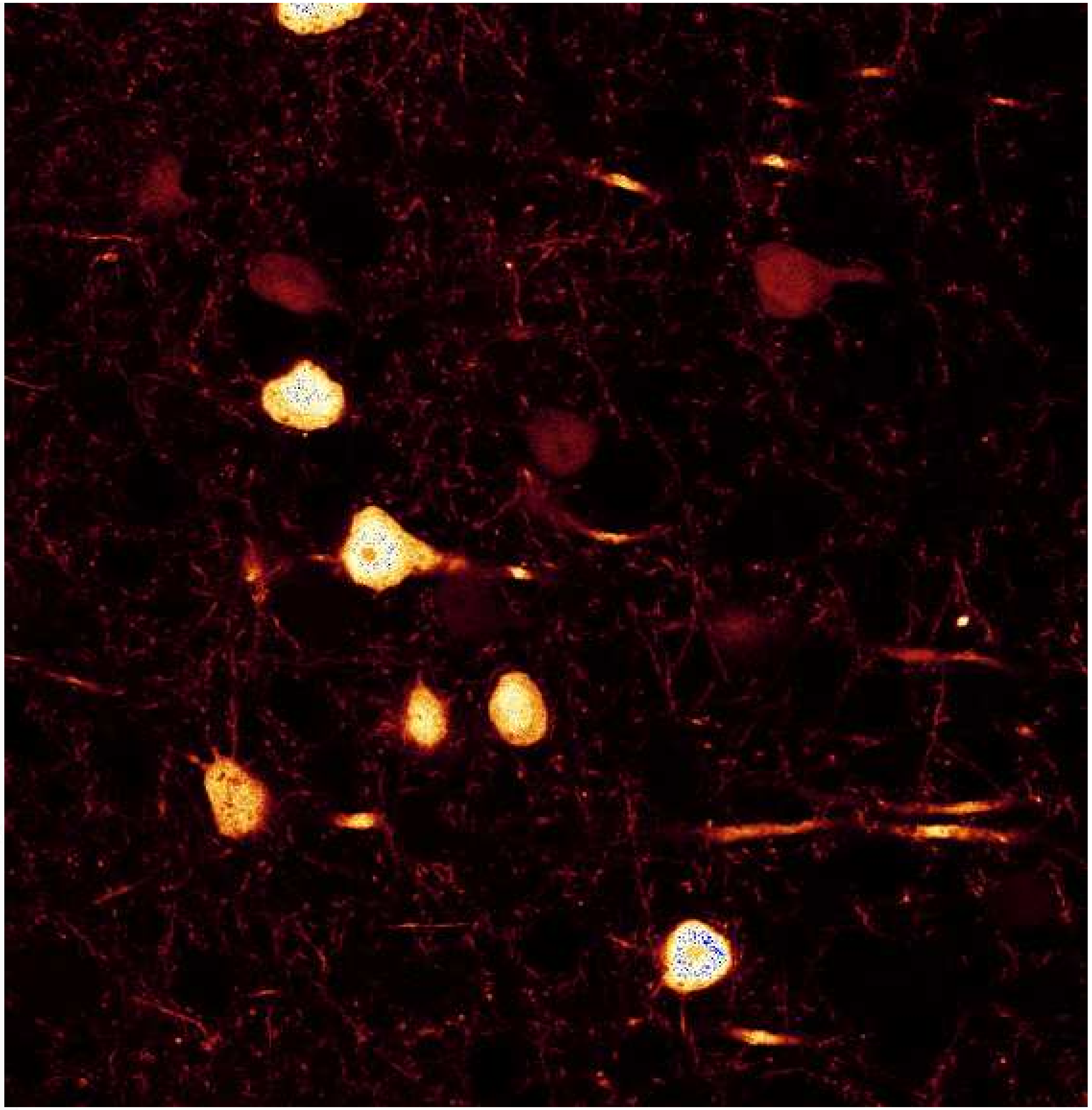
Time of flight dispersion in PMTs

Leica HyD for confocal
The road to super-sensitivity



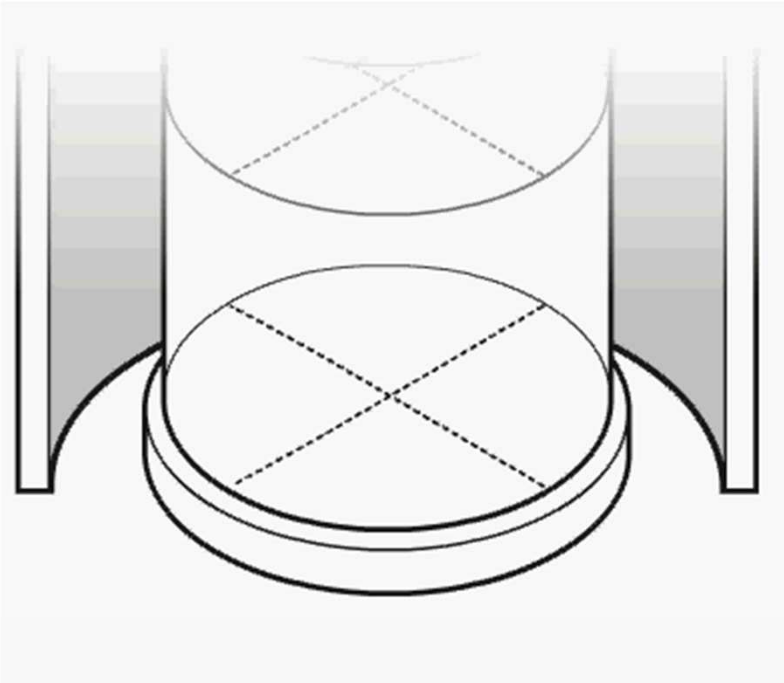


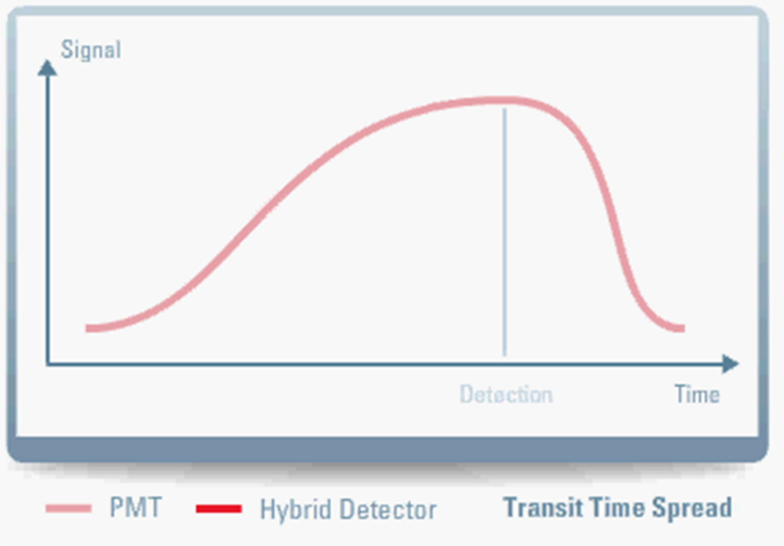
Hybrid detection technology



HyD 100

Leica HyD for confocal
The road to super-sensitivity



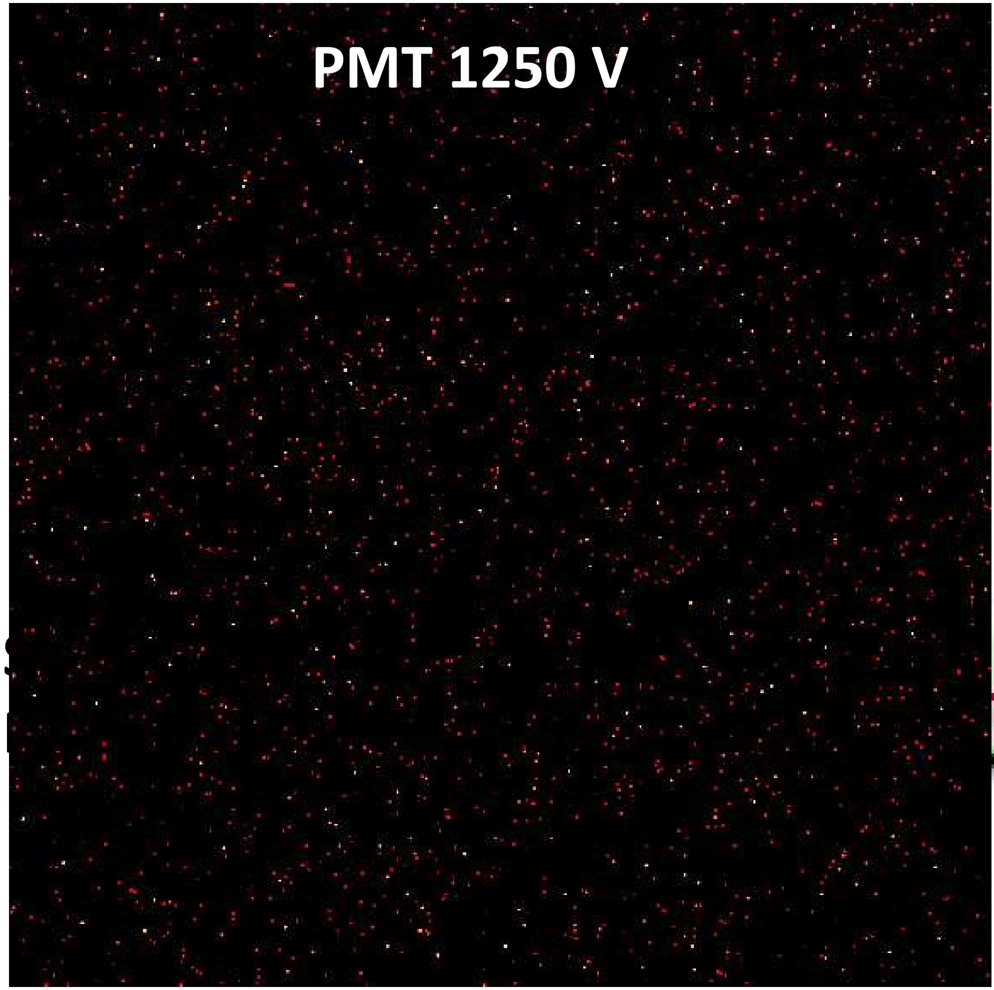


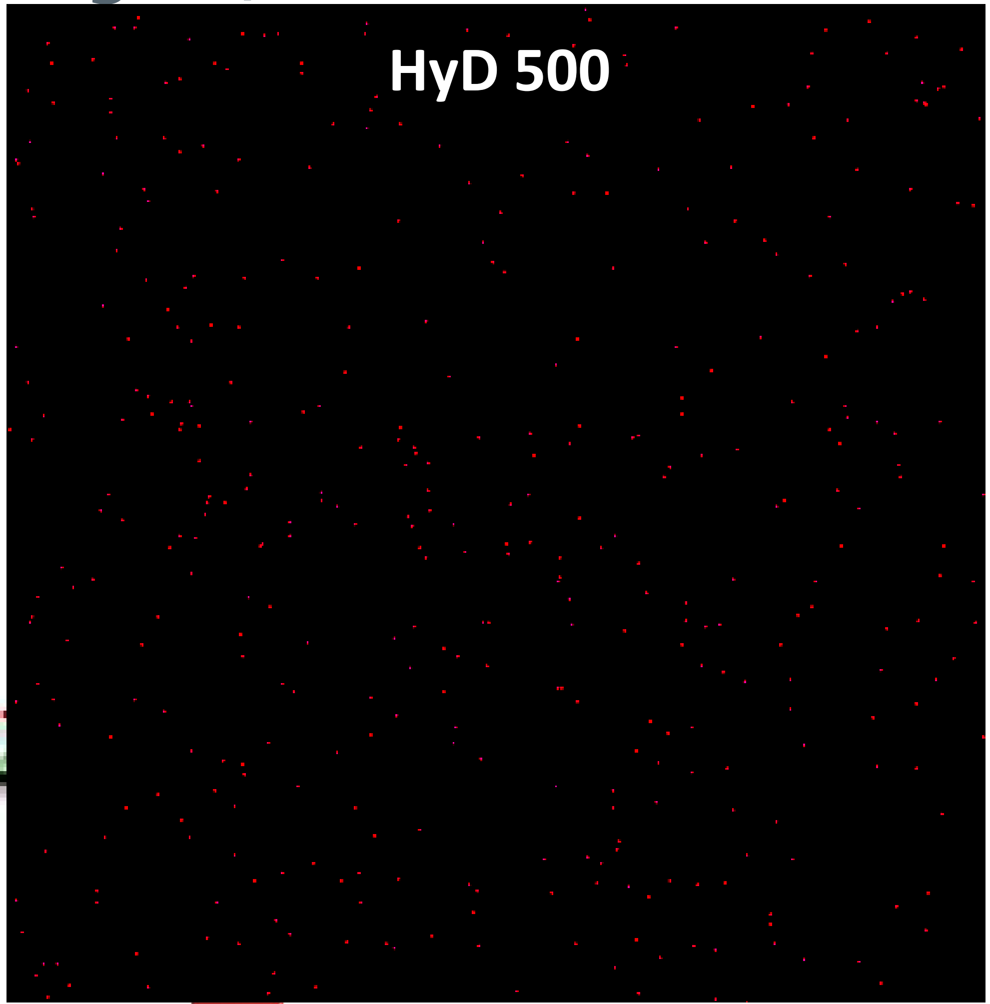
Half PMT and half APD our hybrid detectors with a **GaAsP** photocathode offer superior **sensitivity** combined with a large **dynamic range**.

- Low noise
- High dynamic range
- High sensitivity

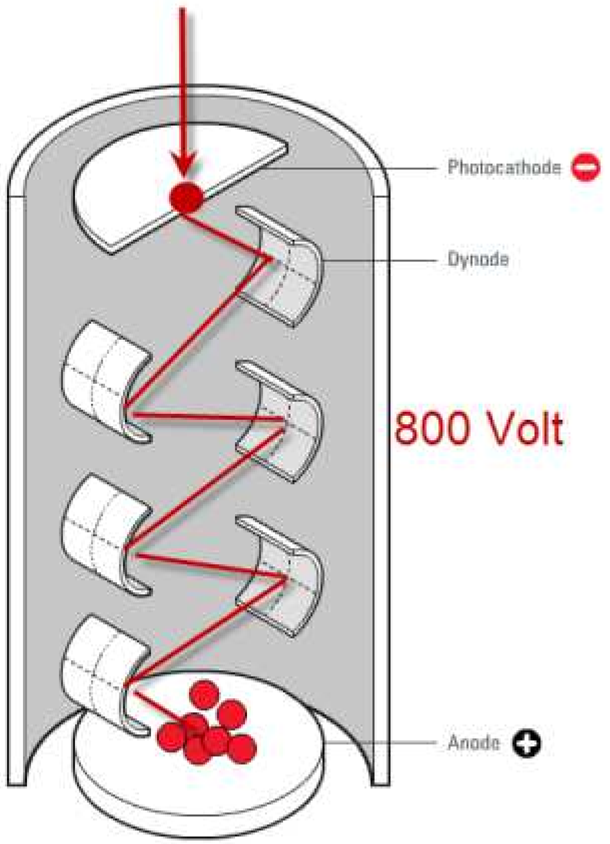
Short transit time spread in HyD

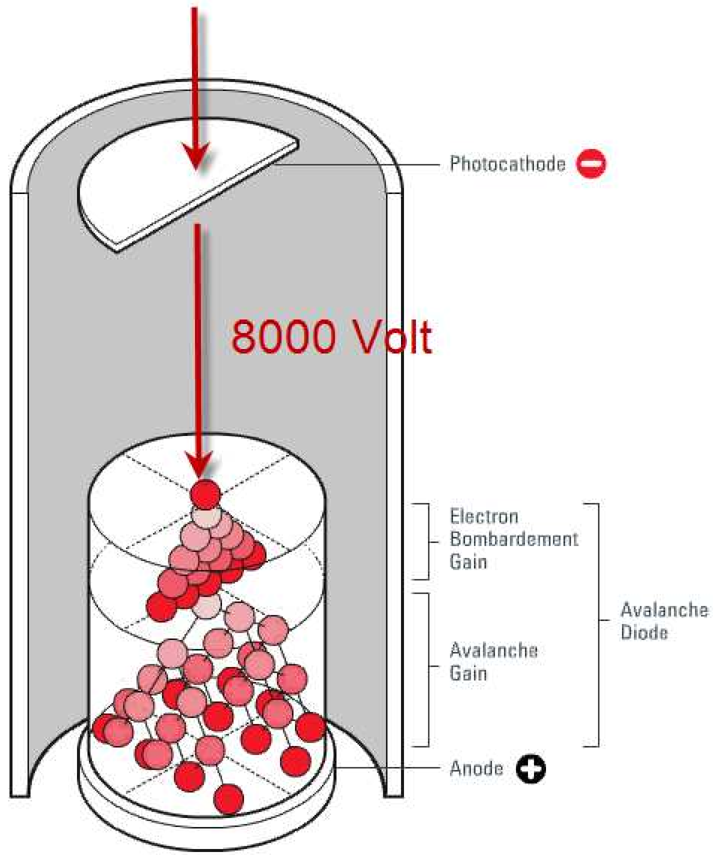
Photon counting: Improved Signal/Noise



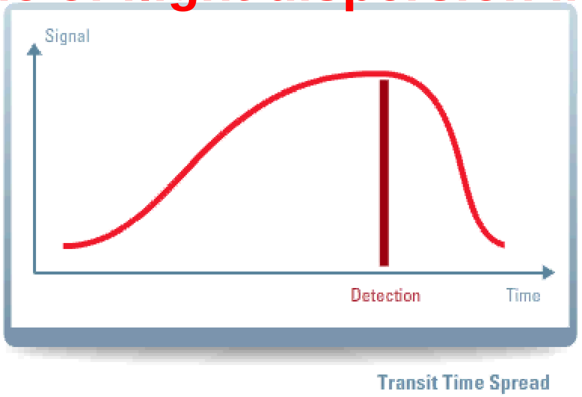


http://www.leica-microsystems.com/science-lab/step-by-step-guide-to-hybrid-detection-and-photon-counting/

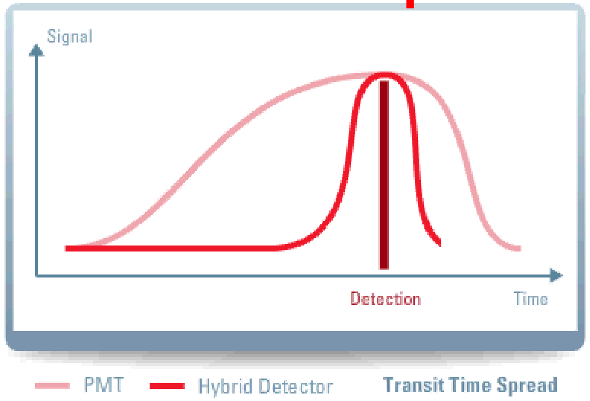




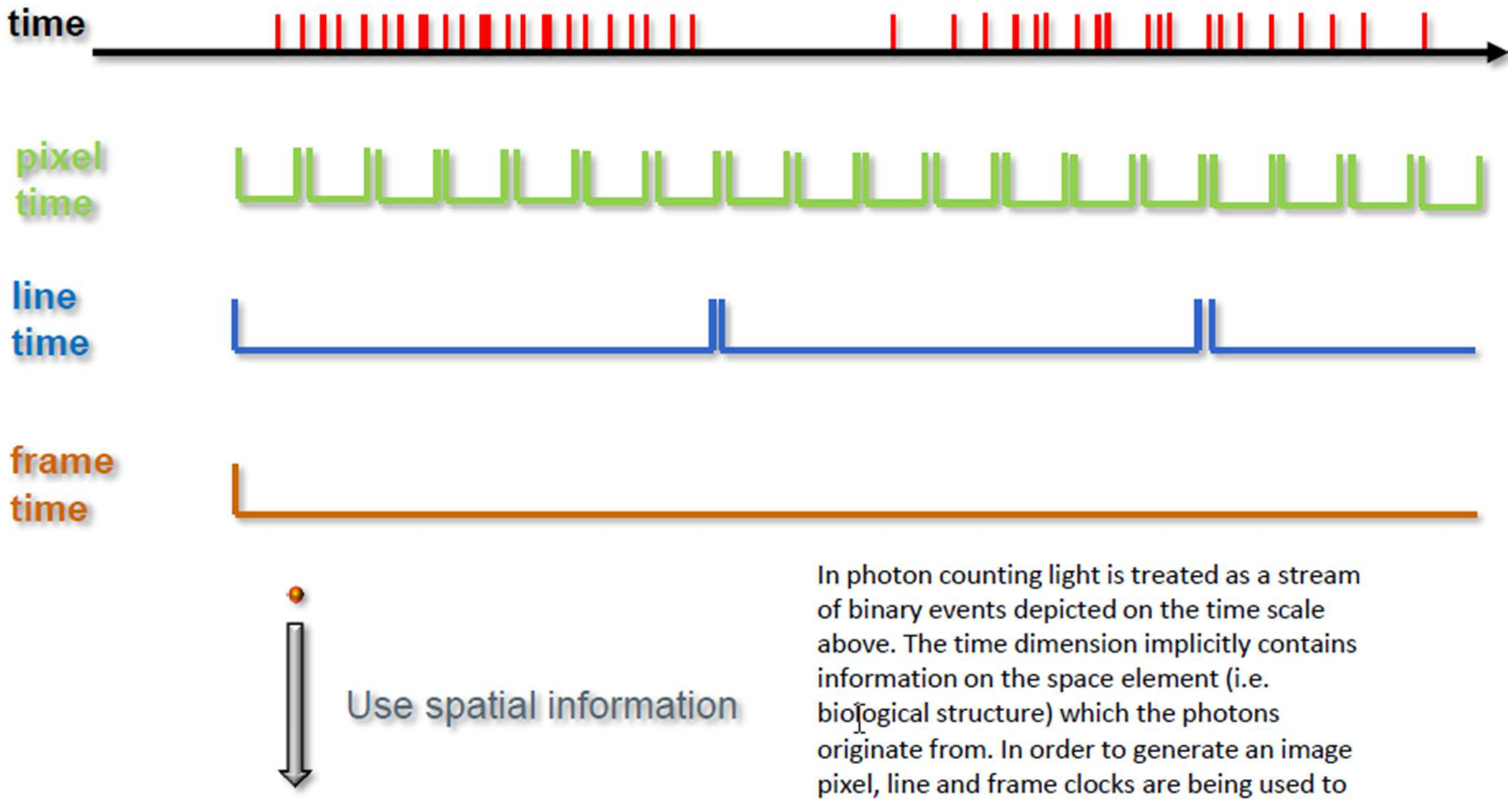
Time of flight dispersion in PMT's



Short transit time spread in HyD

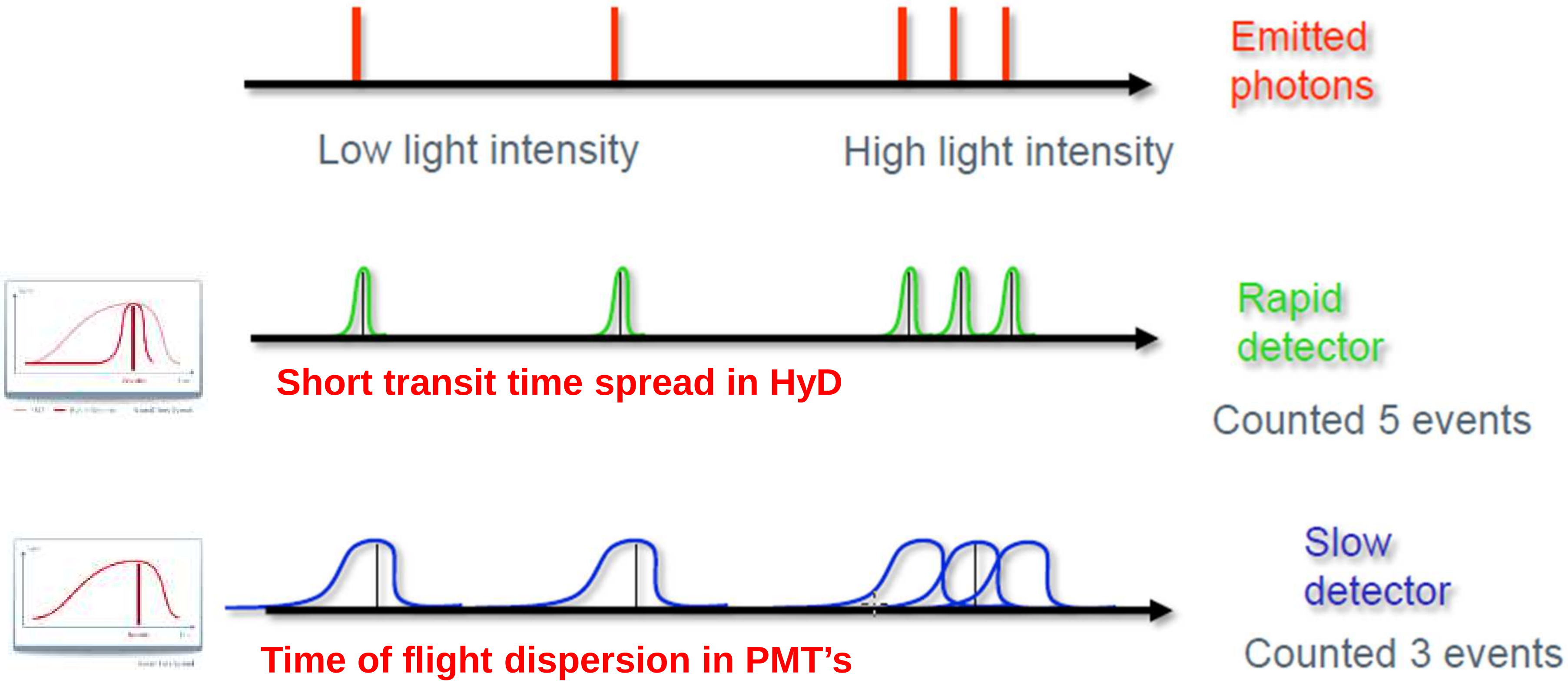


Let's take a look at photon counting and how you do imaging with it



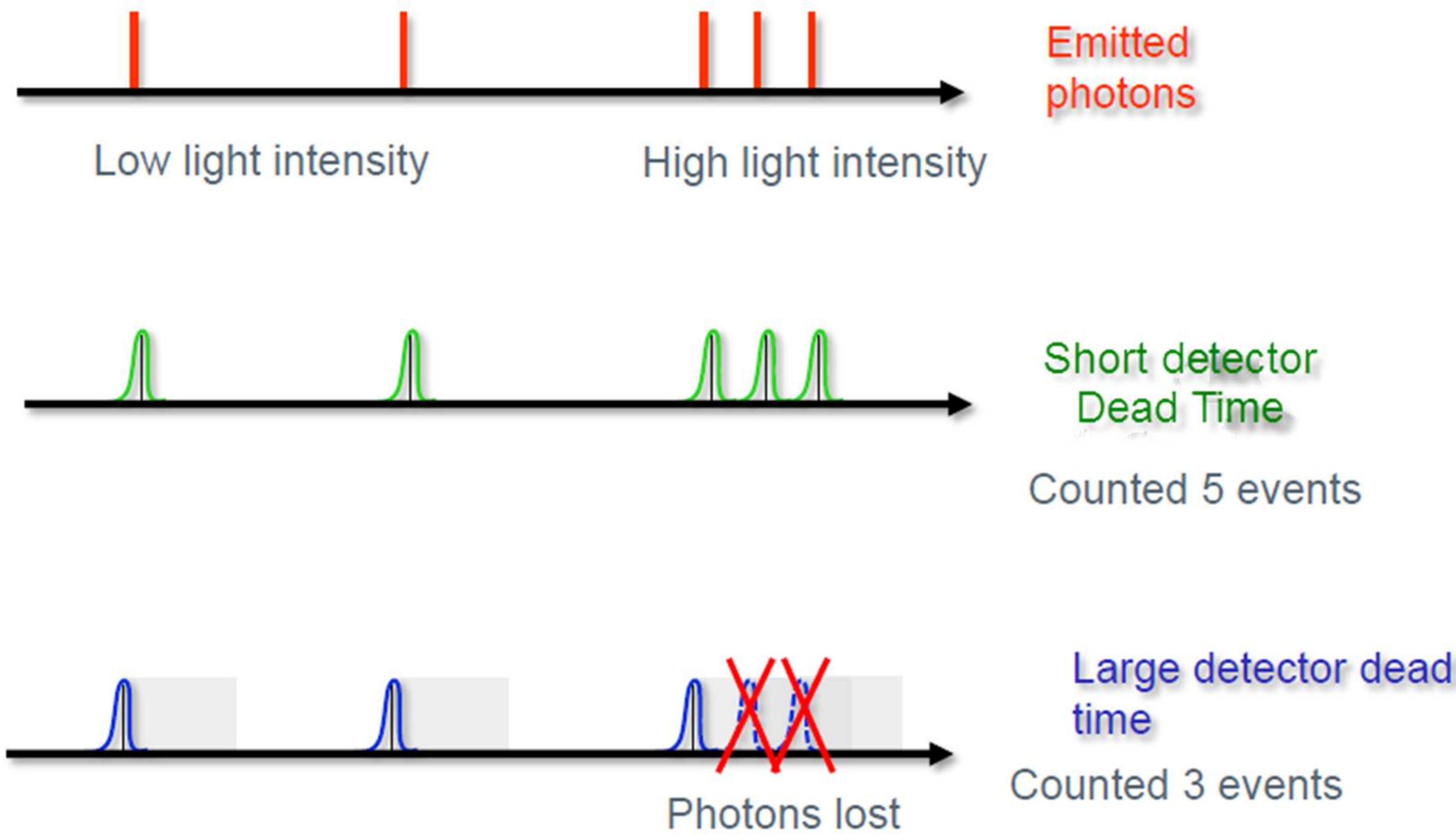
In photon counting light is treated as a stream of binary events depicted on the time scale above. The time dimension implicitly contains information on the space element (i.e. biological structure) which the photons originate from. In order to generate an image pixel, line and frame clocks are being used to sort the photons into pixels.

Photon counting: fast detector



Small transit time TTS

Photon counting: detector dead time

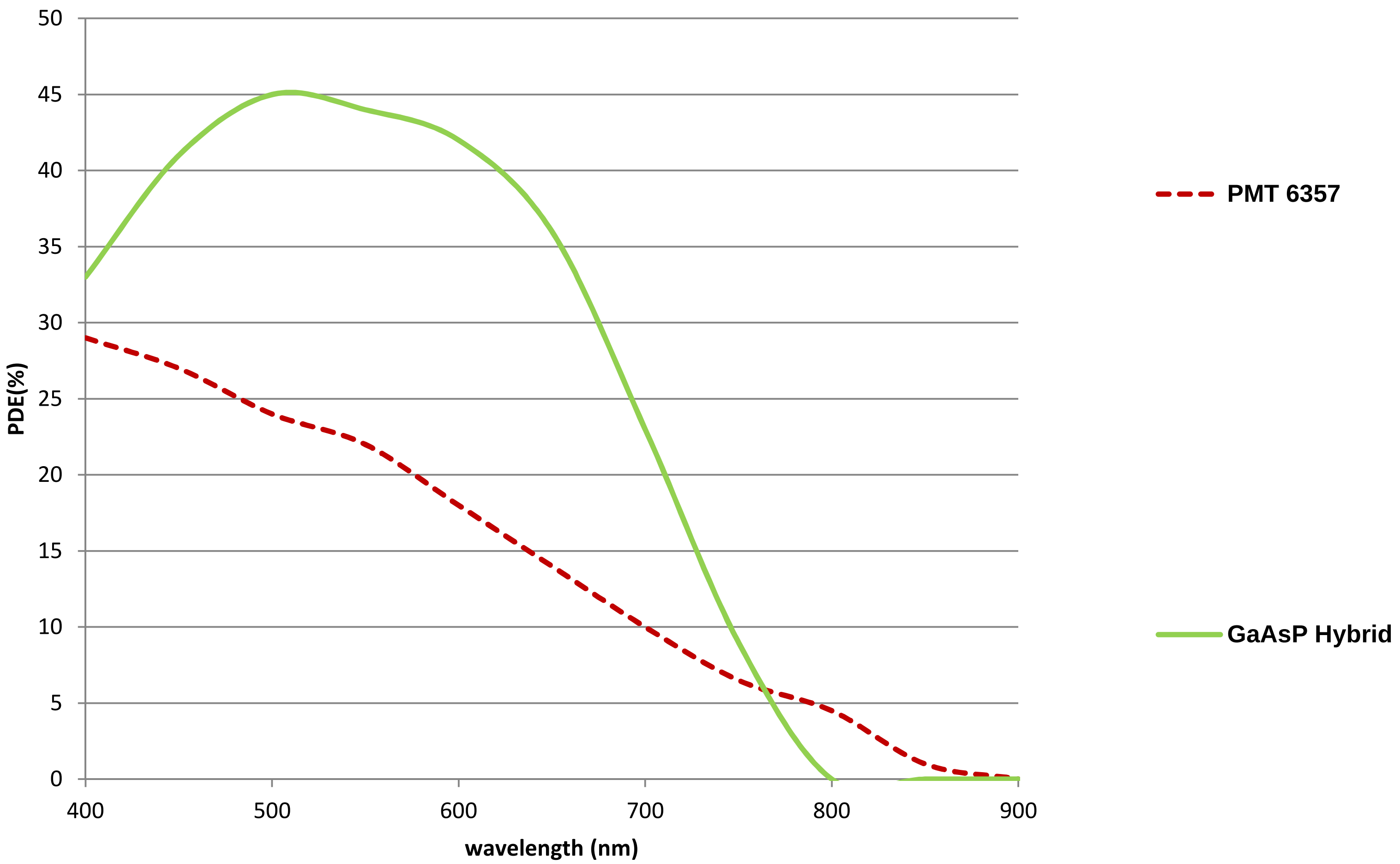


Small dead time

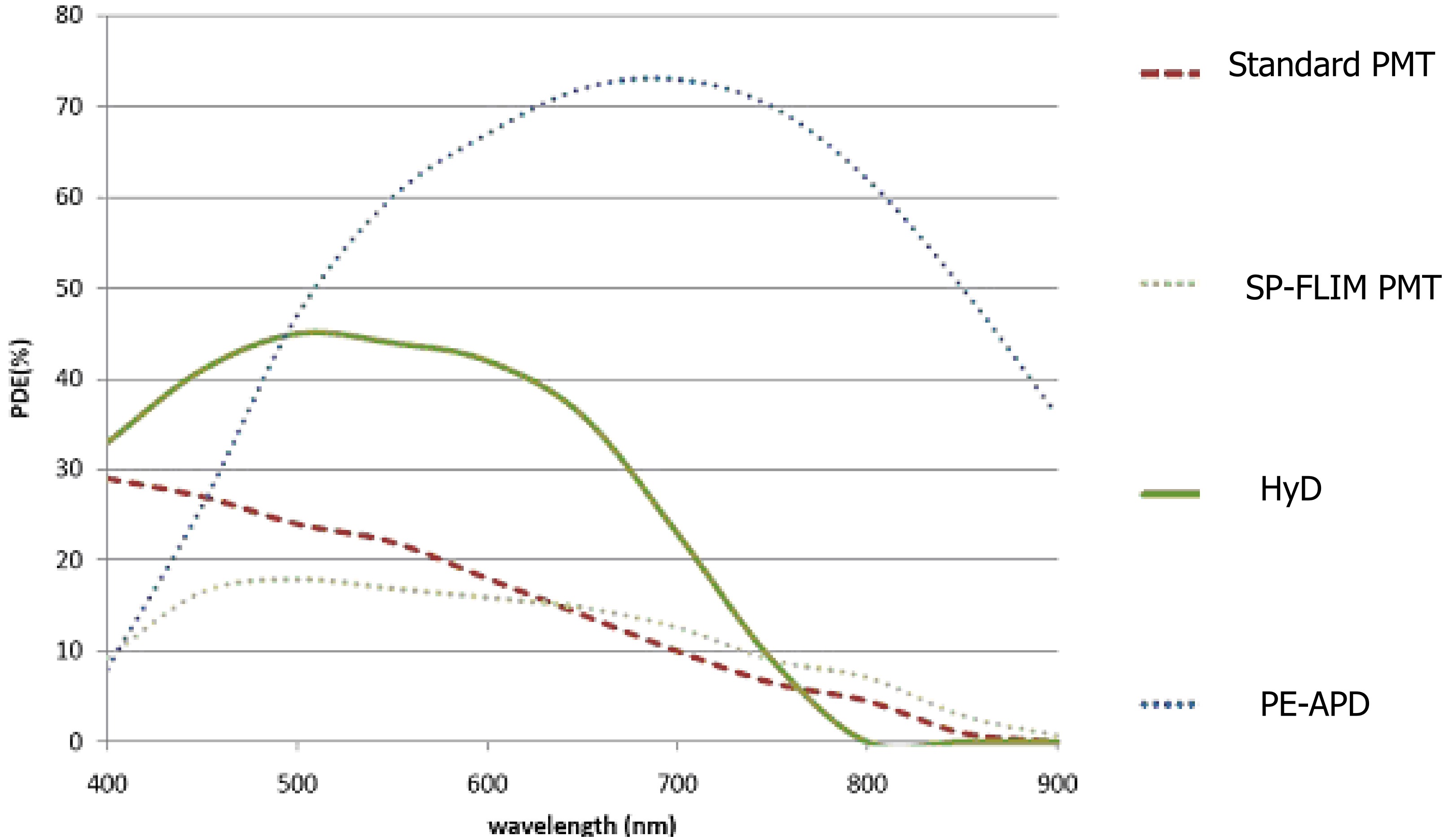
Quantum efficiency

Quantum Efficiency
The ratio of hole-electron pairs or photoelectrons to the number of photons received by a photo detector.

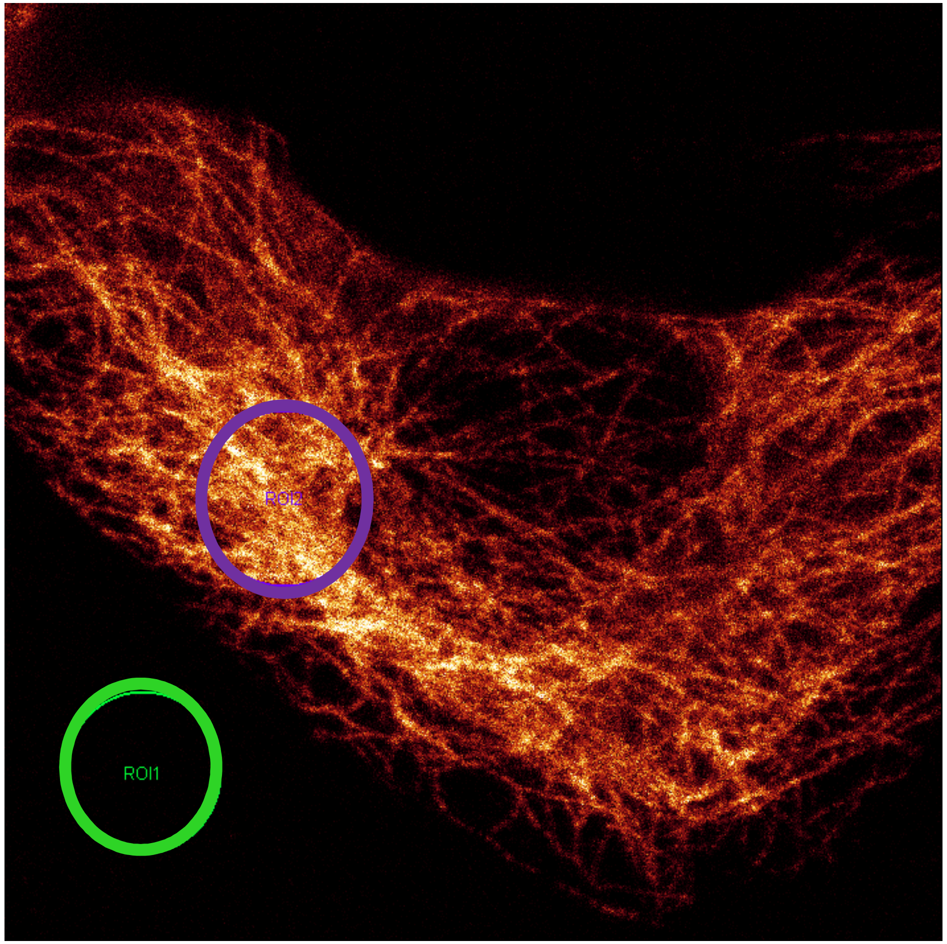
High quantum efficiency

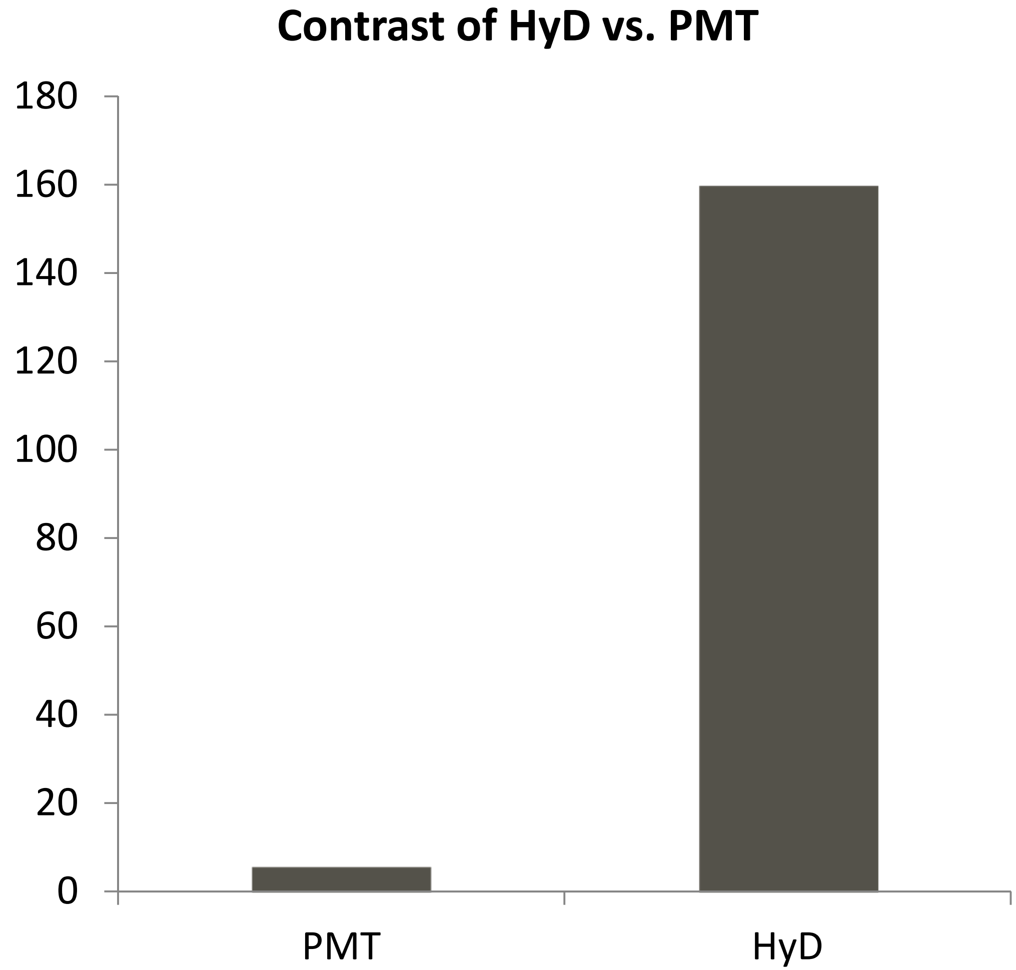


Huge quantum efficiency

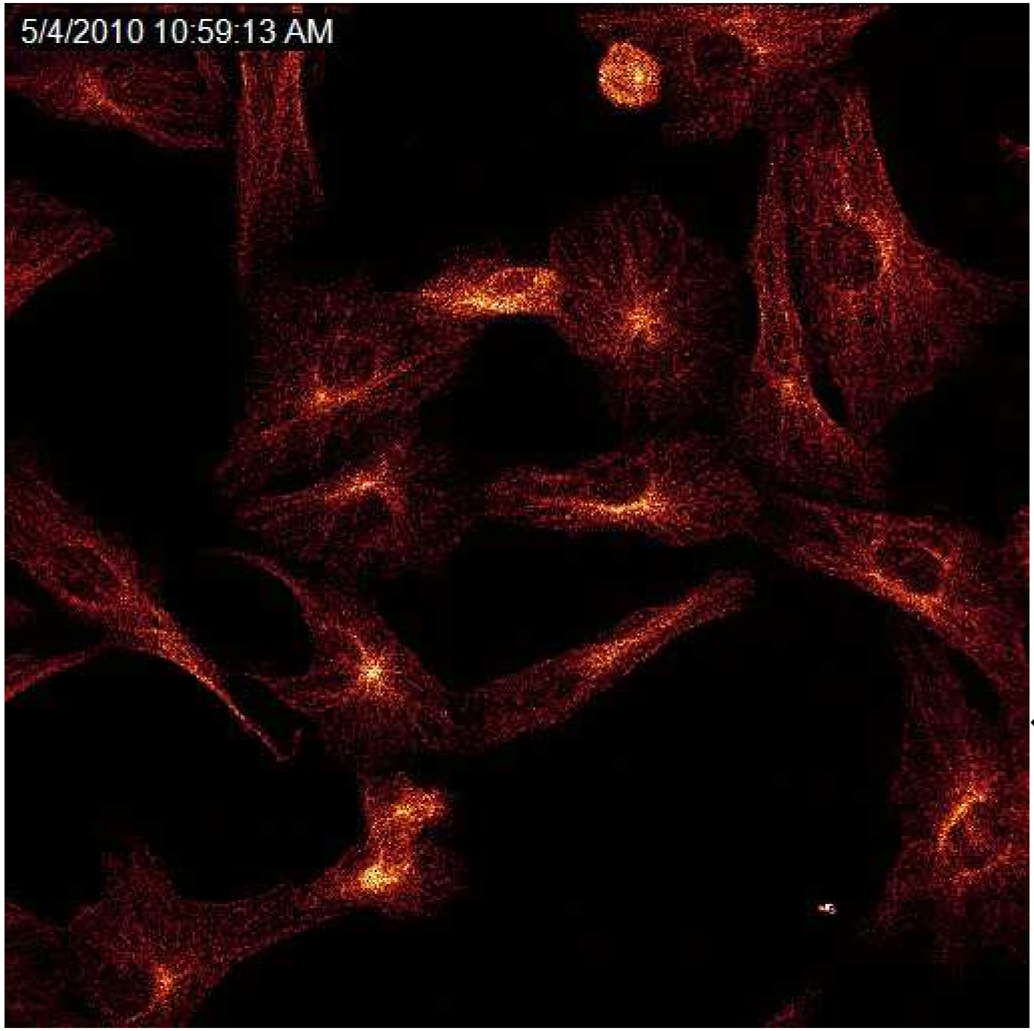


Improved contrast of HyD vs. PMT





Fast super-sensitivity with tandem scanner

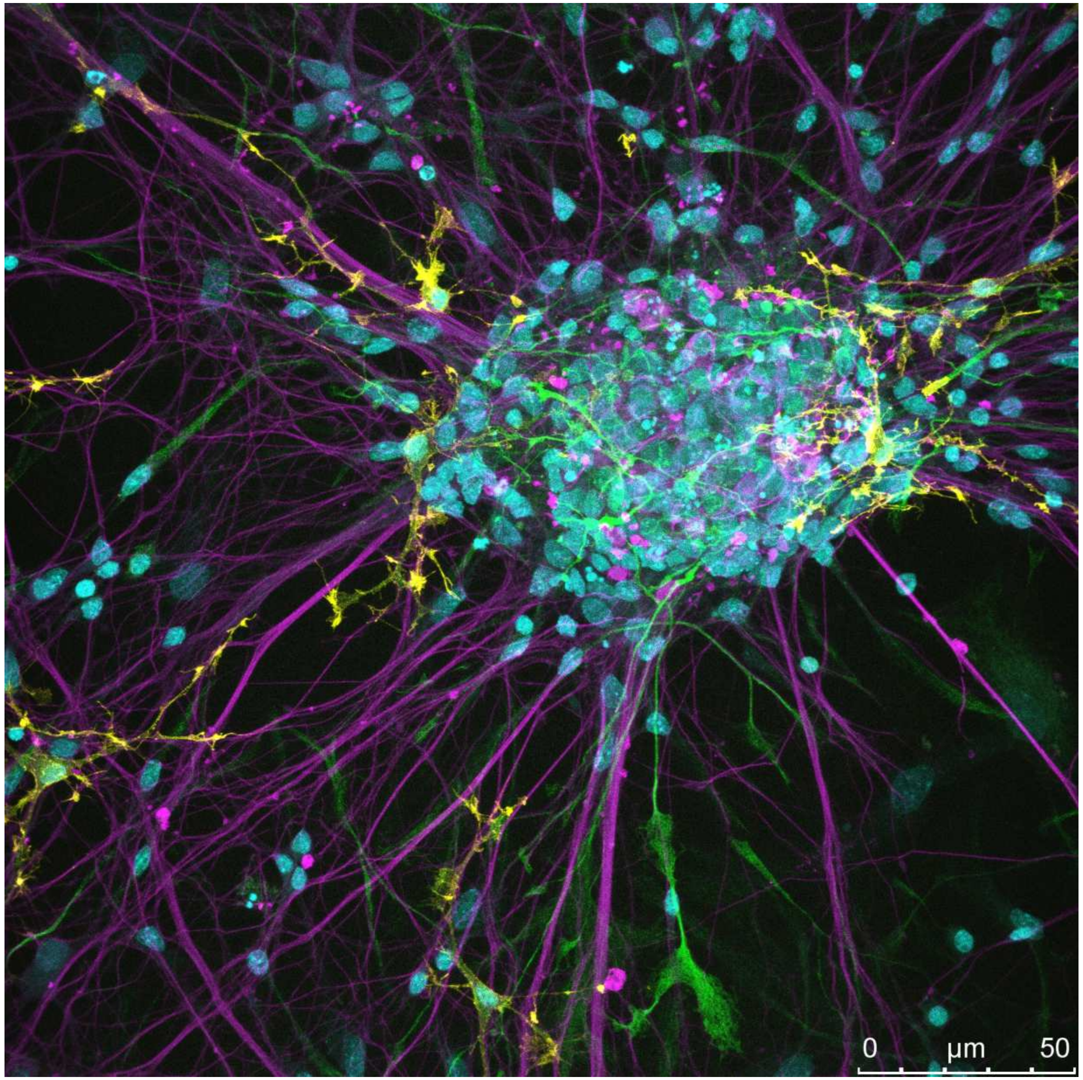


HyD

Tubulin-Atto647N
Ex 633 nm
Tandem scanner
512x512
29 fps

FOV up to ~1 mm @ 10x objective

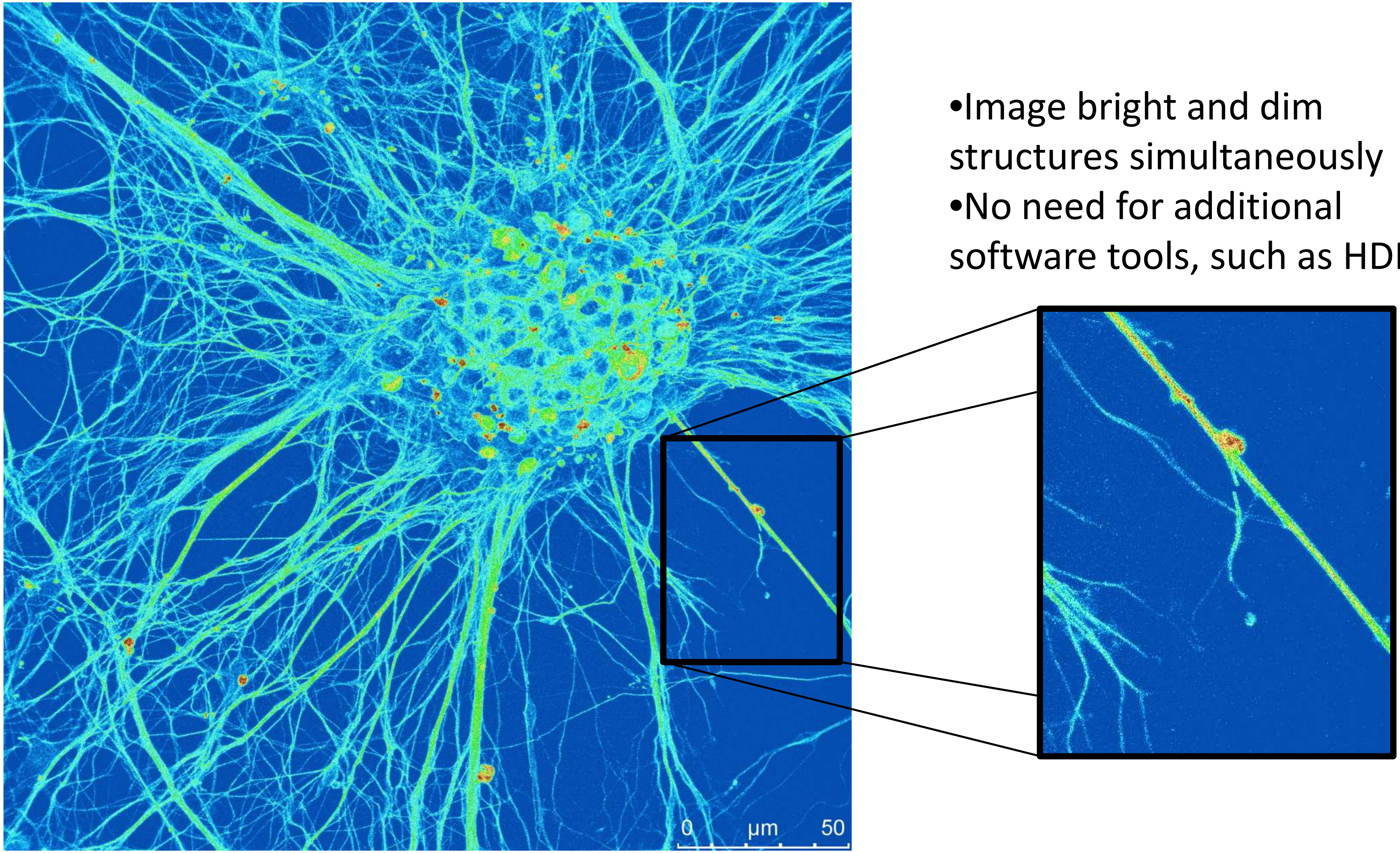
High dynamic range of detector 1/2



- Image bright and dim structures simultaneously
- No need for additional software tools, such as HDR

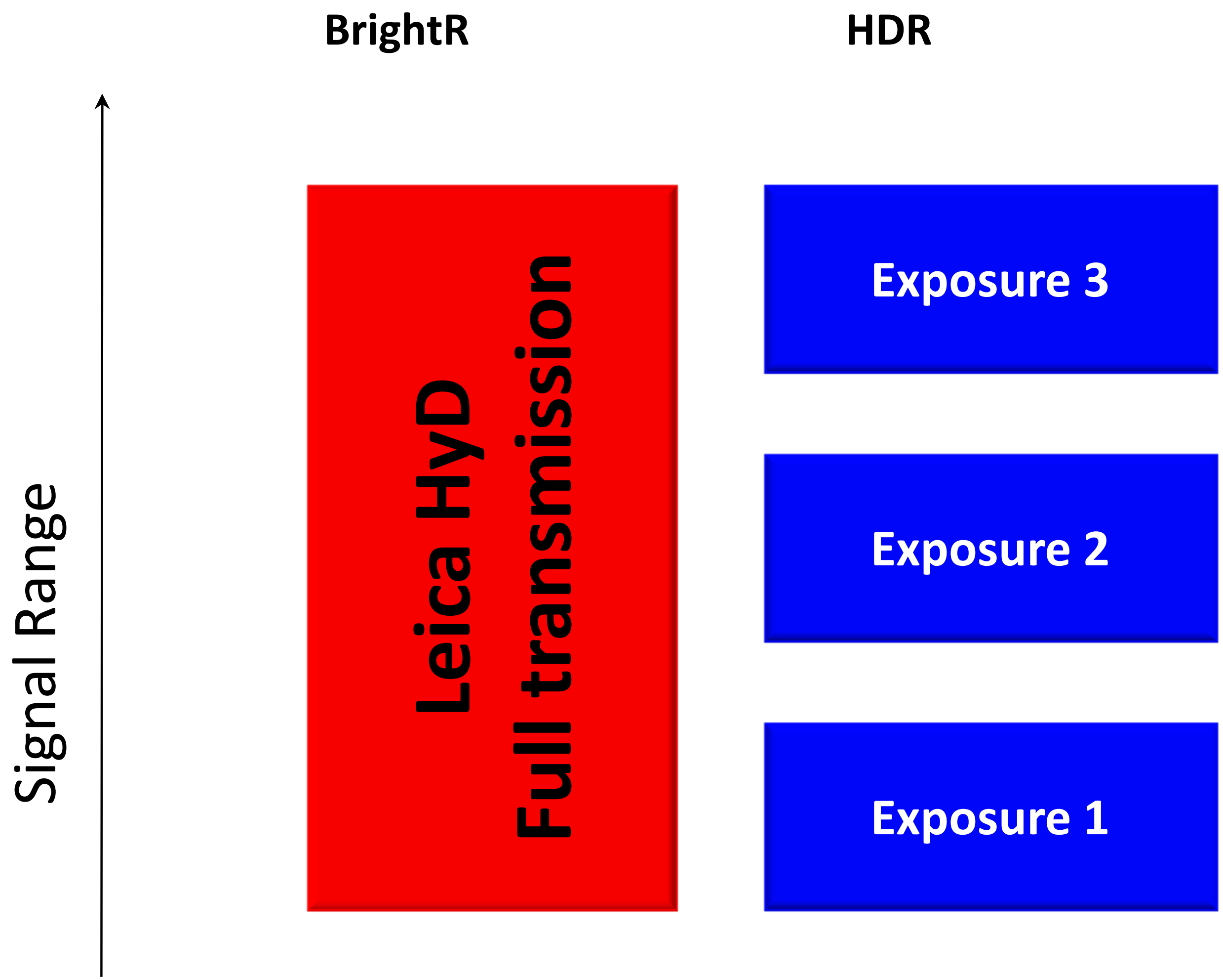
Stained rat primary culture
DAPI
NG2-Cy3
Nestin-Cy2
BetaIII-Tubulin-Cy5

High dynamic range of detector 2/2



- Image bright and dim structures simultaneously
- No need for additional software tools, such as HDR

High dynamic range of detector 4/4



- Transmits a very large dynamic range in one shot
- Obsoletes exposure series and post-processing usually employed by high dynamic range approaches

Imaging with high dynamic sample, comparison of dynamic range:

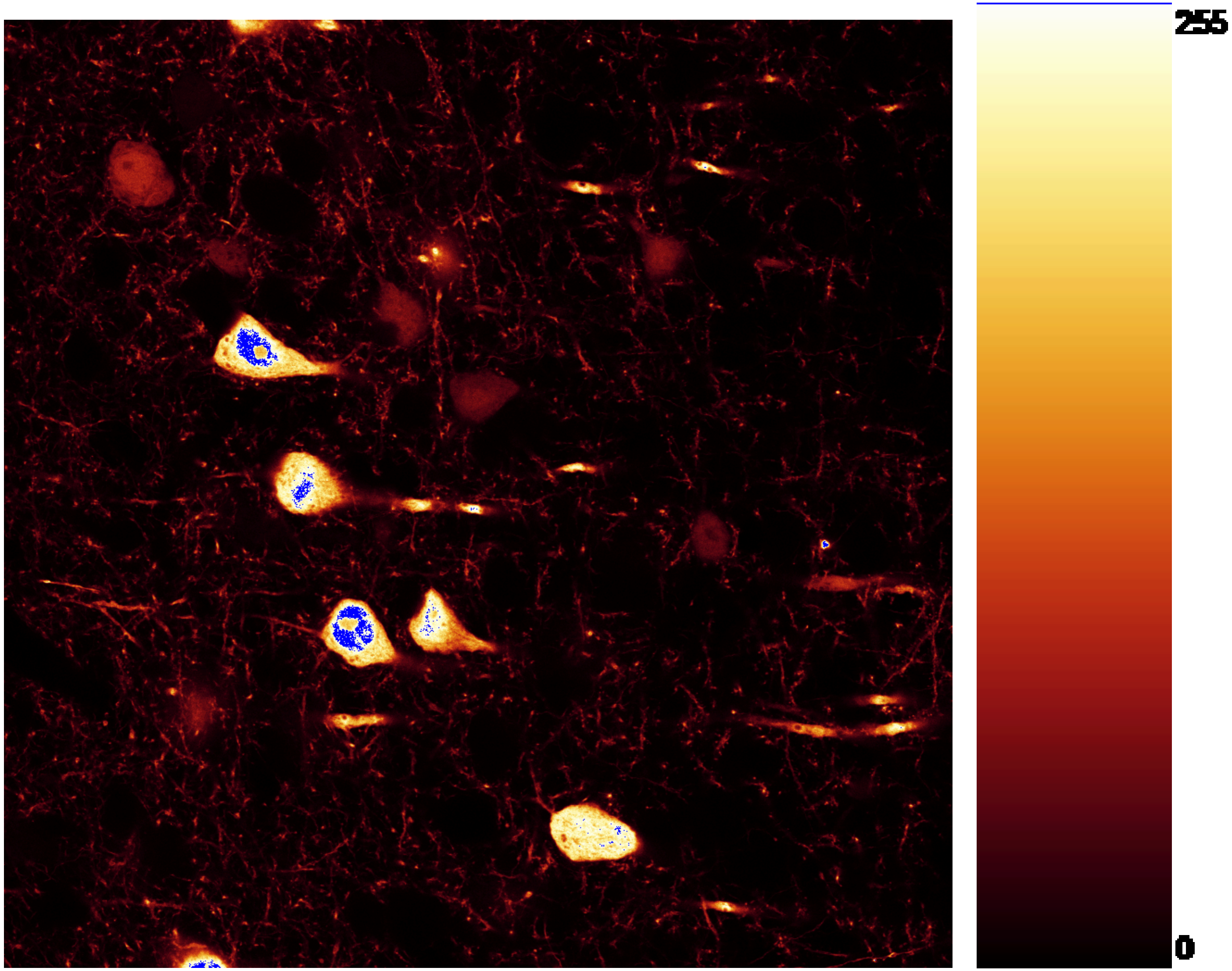
Problem: weak stained structures are not visible if strong stained structures should not be oversaturated

- All images are captured with same imaging settings
  - • 4% laser 514 nm, Argonlaser 11%
  - • 6 line average
  - • 400 Hz
  - • 1024x1024
  - • Detection 520-590 nm
  - • 1 Airy unit
  - • 8 bit resolution
  - • Gamma 1

PMT image

Gain was set just to reach slightly saturated cell bodies.

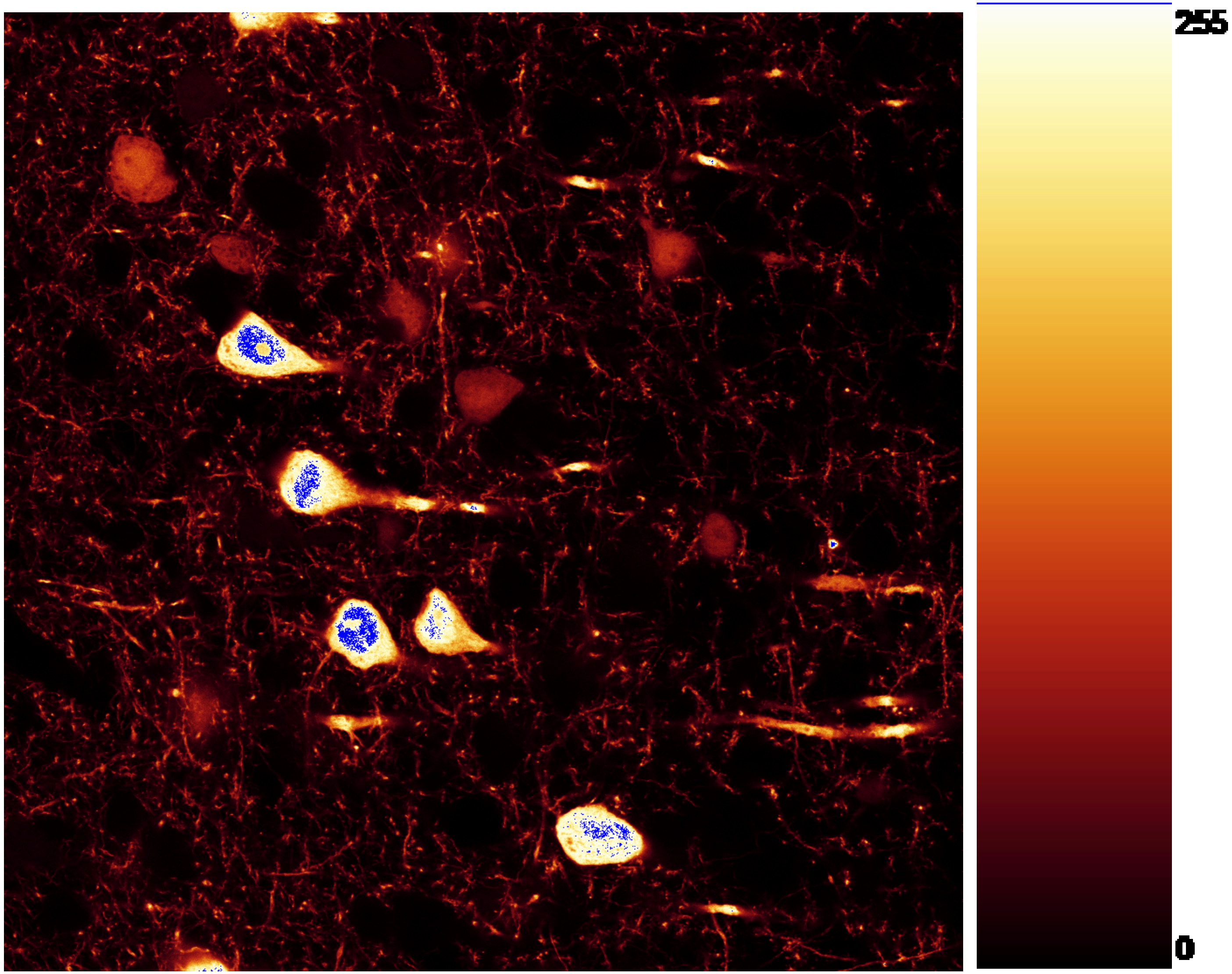
Axonal processes and dendritic spines are hardly visible



HyD image standard imaging mode

Gain was set to have same intensity in the cell bodies.

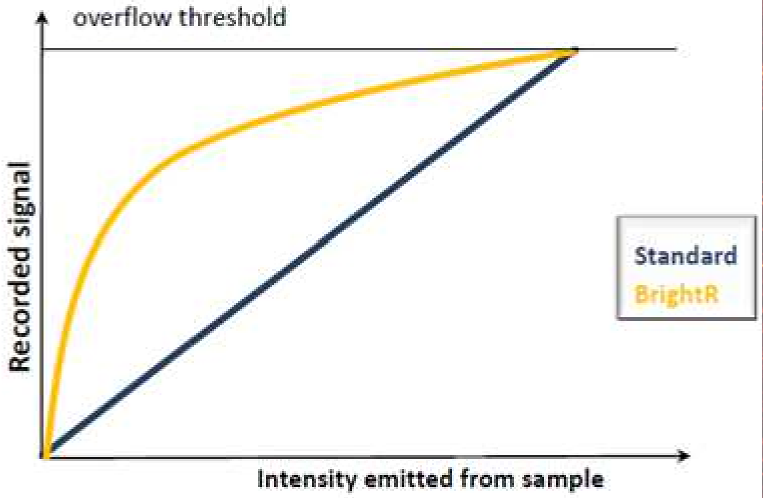
Axonal processes and dendritic spines are better visible

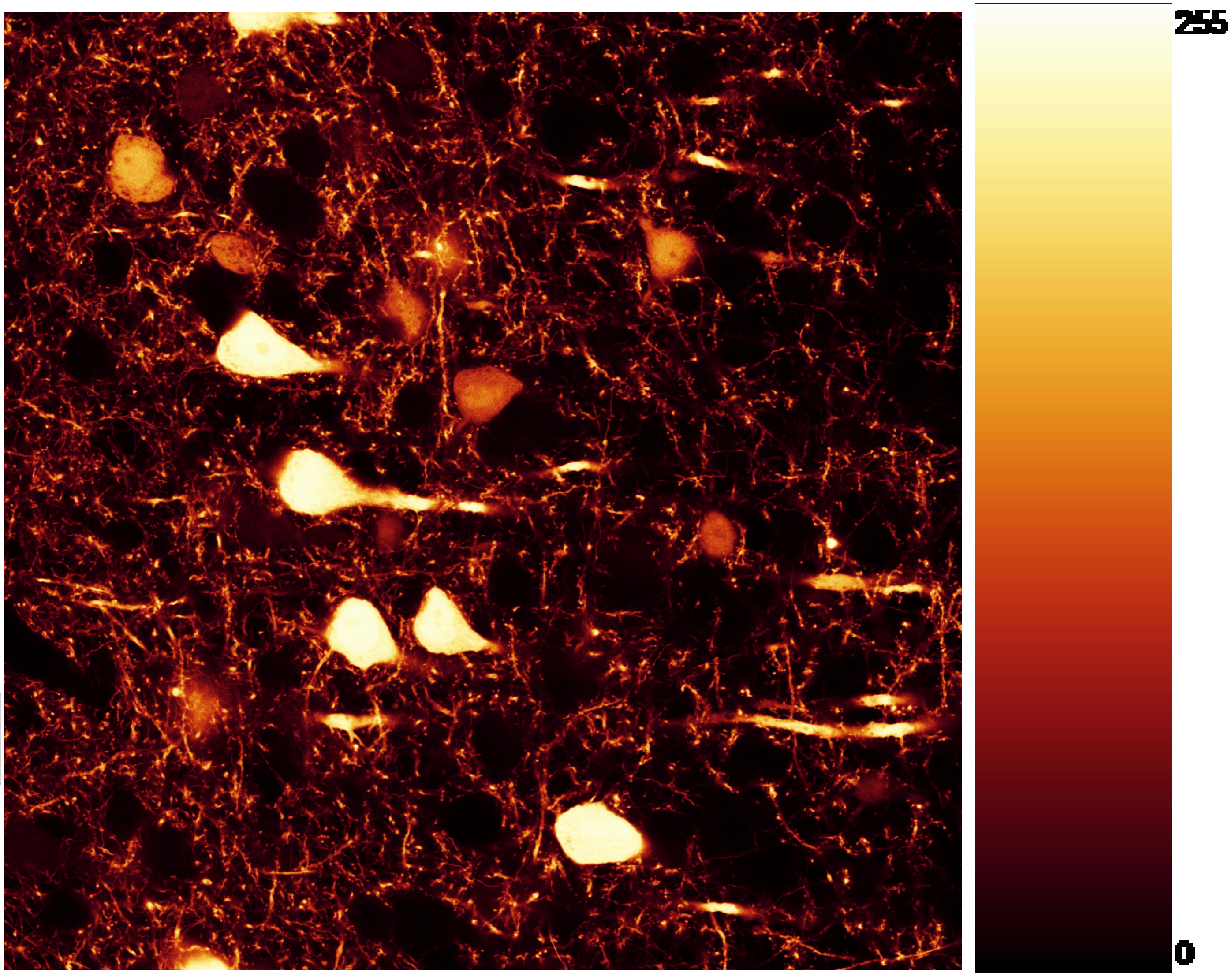


HyD image „Bright R" imaging mode

Exactly the same settings, just switched to Bright R mode.

Axonal processes and dendritic spines are very good visible, cell bodies are not saturated.

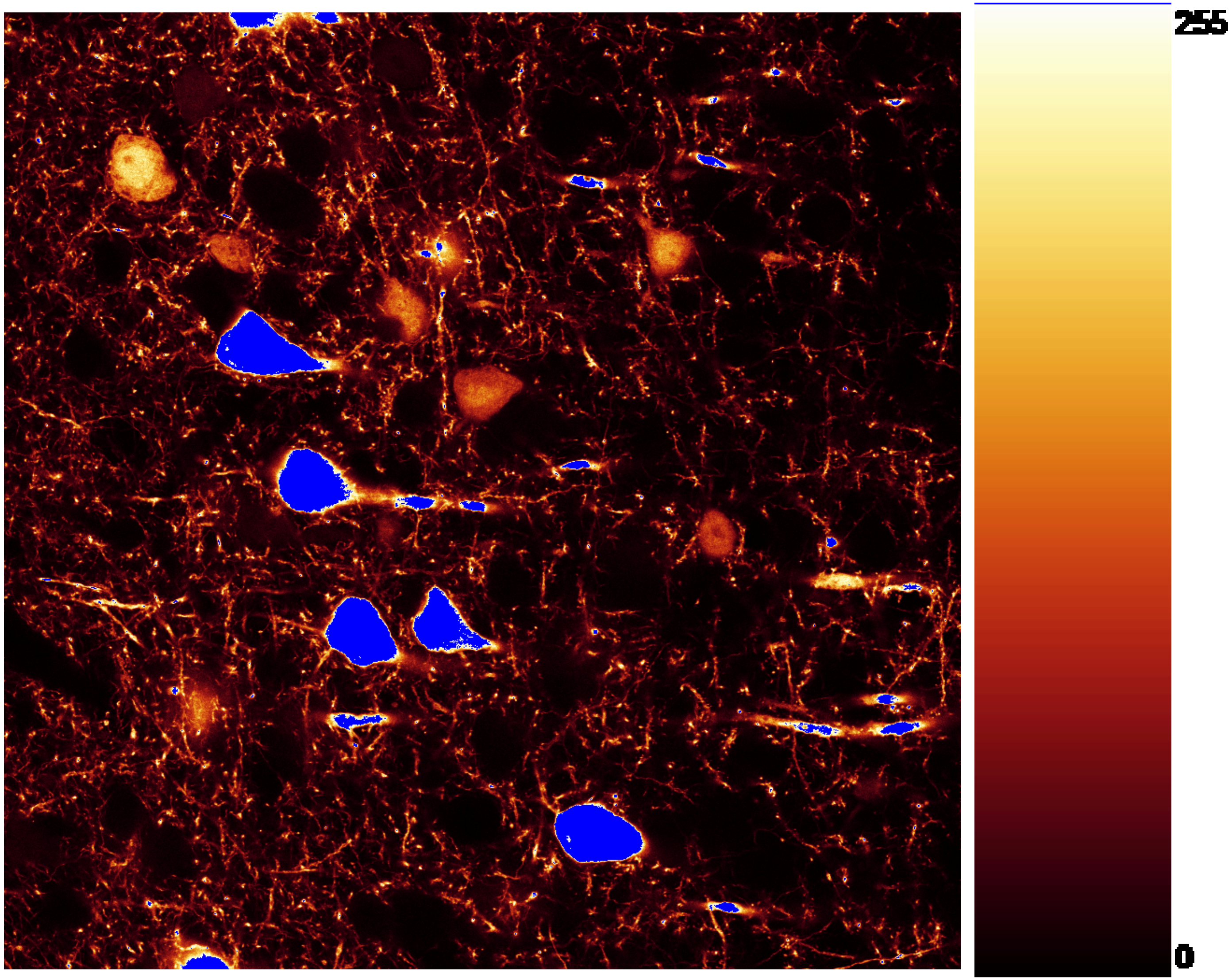




PMT image oversaturated

Gain was set higher to make axonal processes and dendritic spines visible.

Cell bodies are heavily oversaturated.

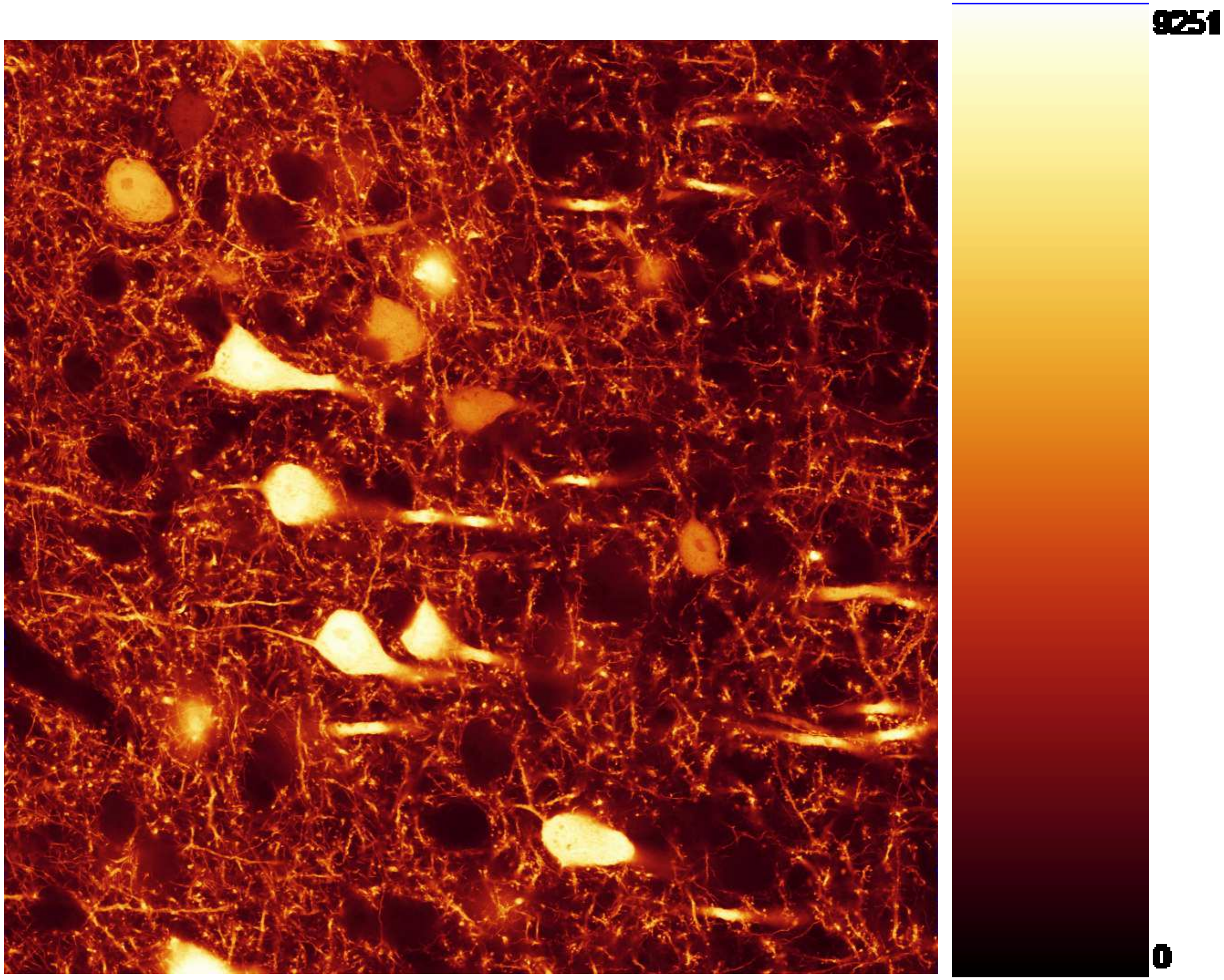


HyD photon counting mode

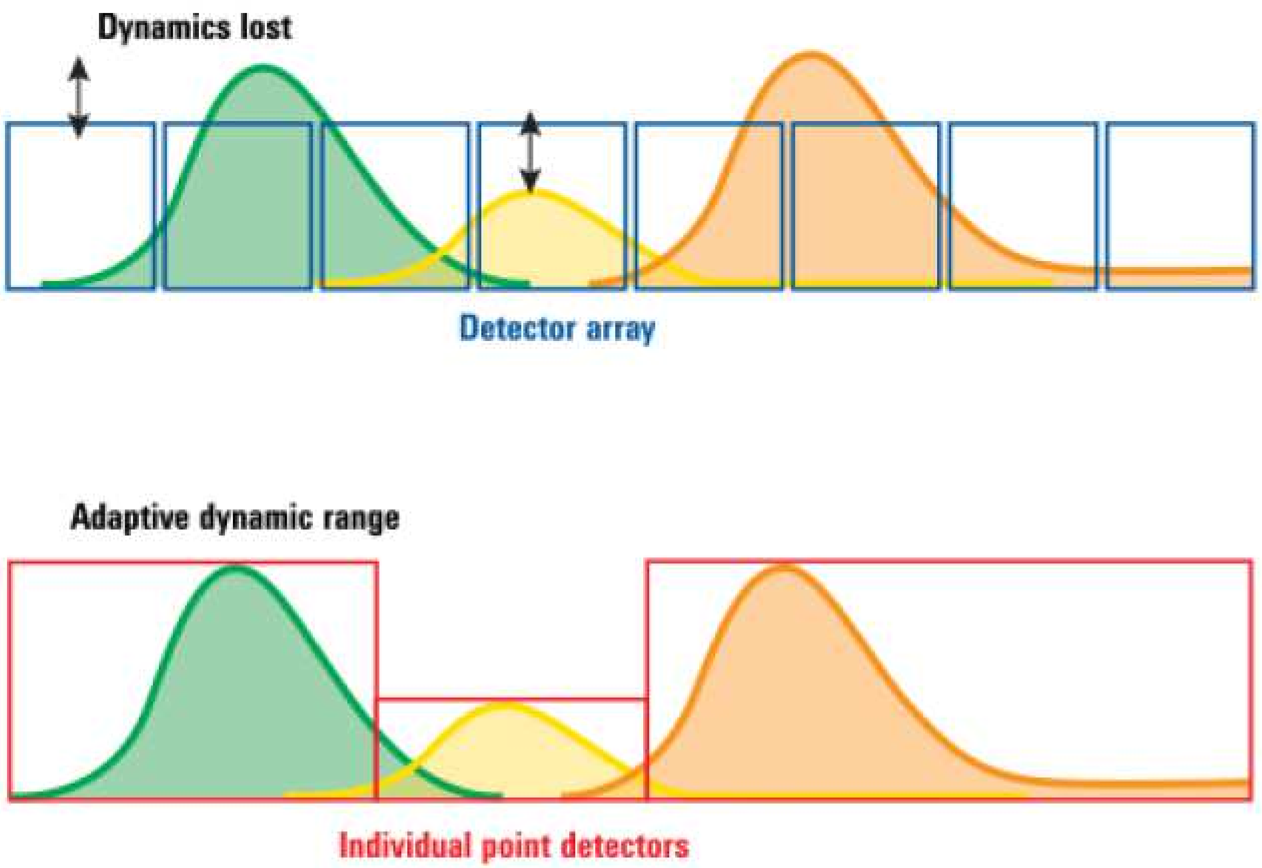
Imaging setting as above, differences are:

- Accumulation 64x
- 16 bit resolution
- Gamma 0,6

LUT displays photon counts



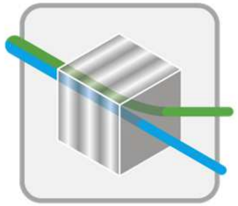
Remember…adaptive dynamic range



THE INNOVATION SYNERGY!

WLL-2

Exclusive!



AOBS

Leica only!

Spectral

Leica only!

HyD

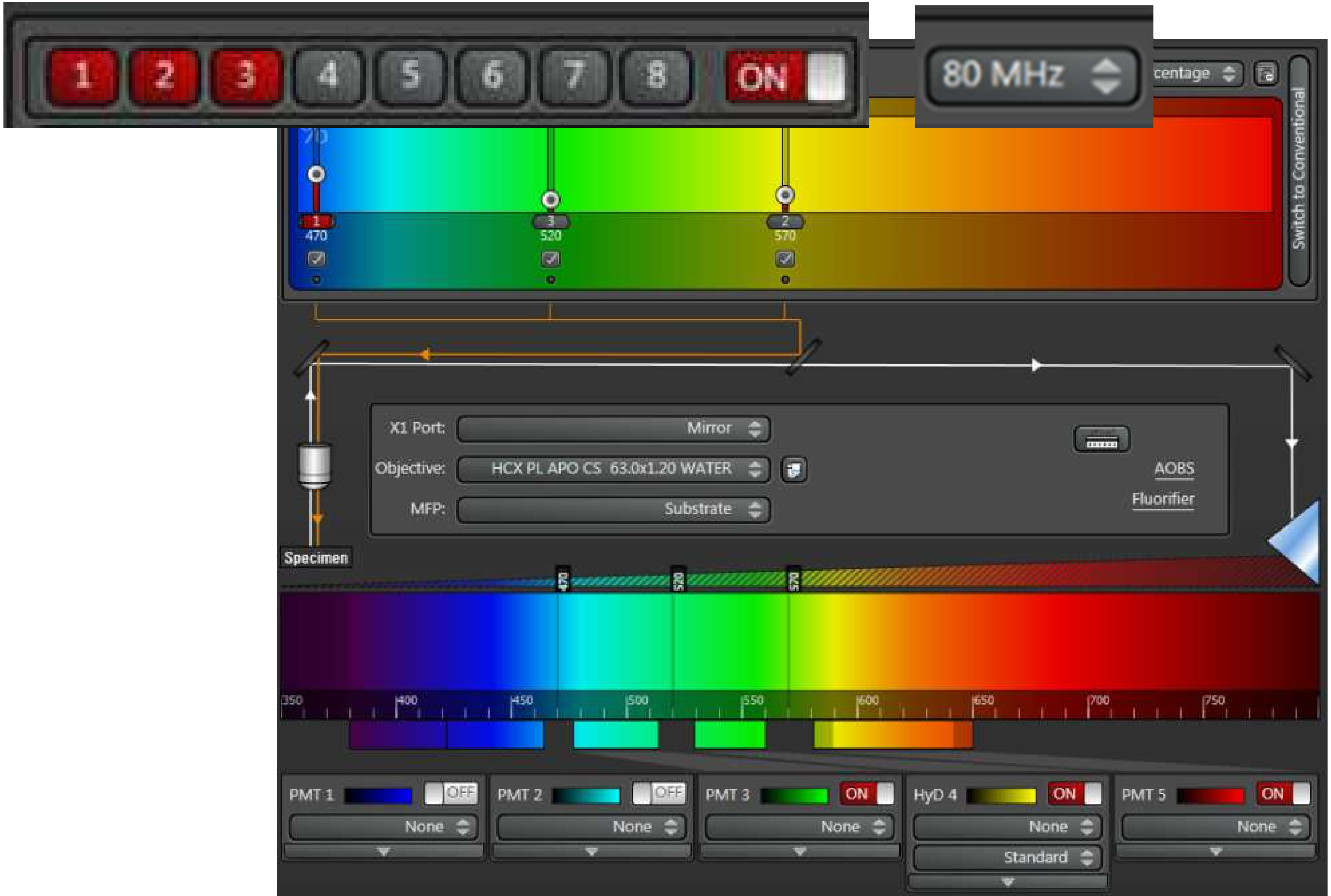
Exclusive!

Combing Leica technologies to reach the next mile stone in confocal Imaging!

LightGate

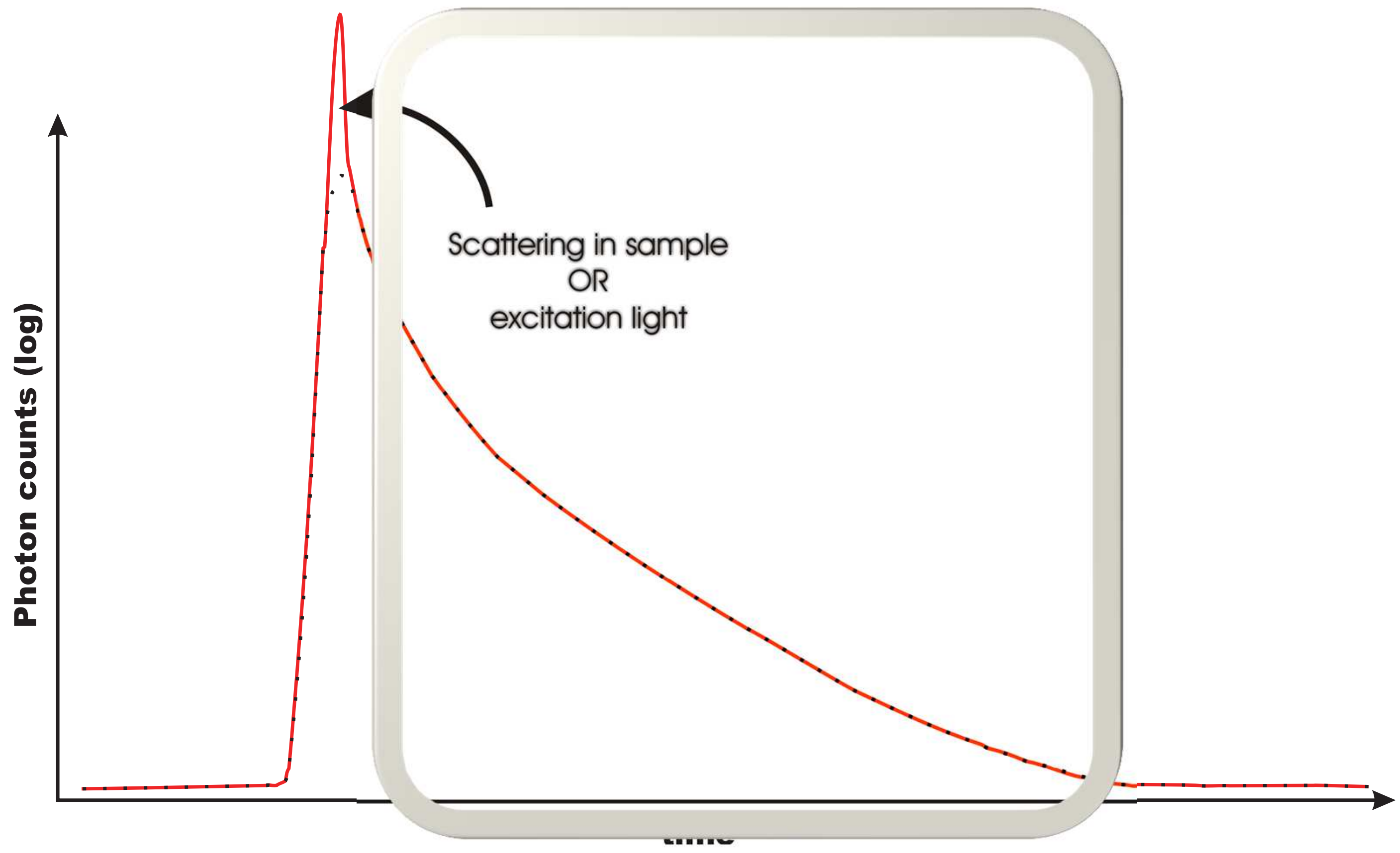
Experiment Optimization for Best Data Quality

WLL-2

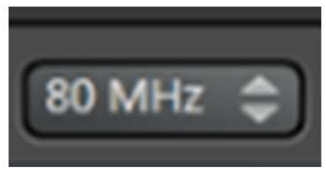


LightGate

- Non-optical removal of reflection light
- Based on Time-gating mechanism
- Available with WLL and HyD only



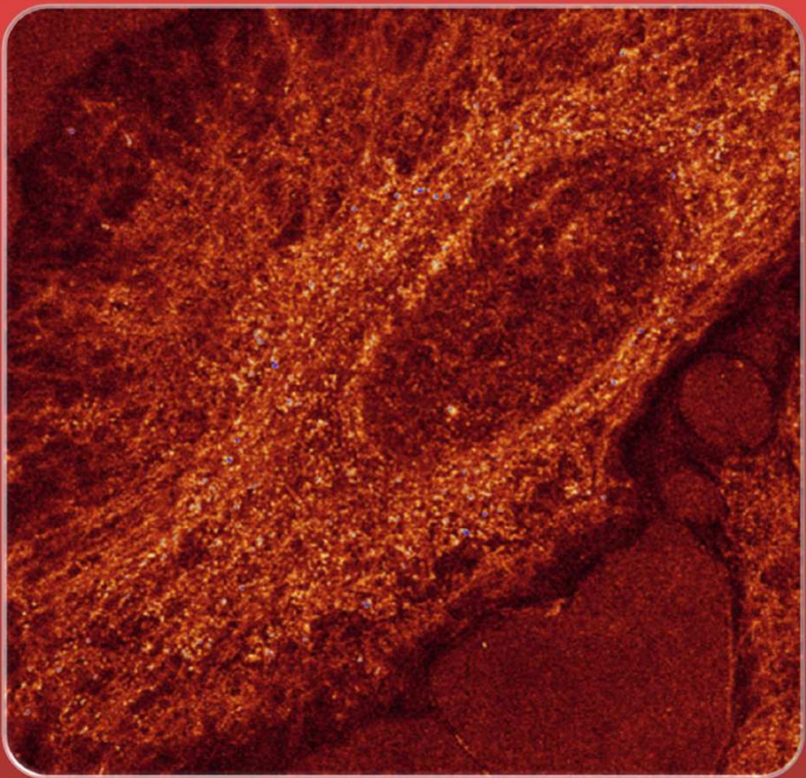
„Detection Window“

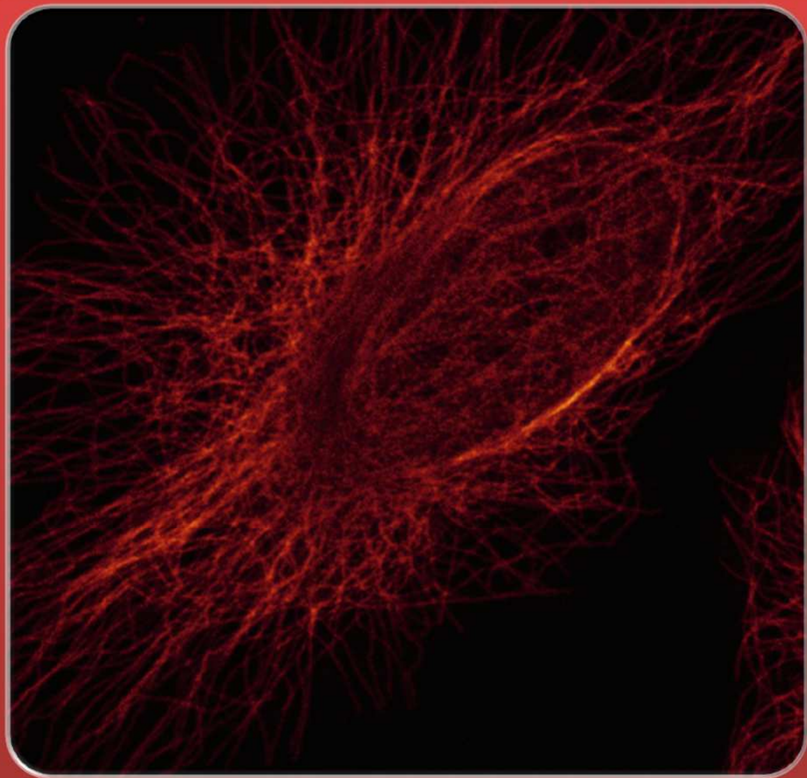


LightGate - Removal Of Back Scattered Light

LightGate off

LightGate on

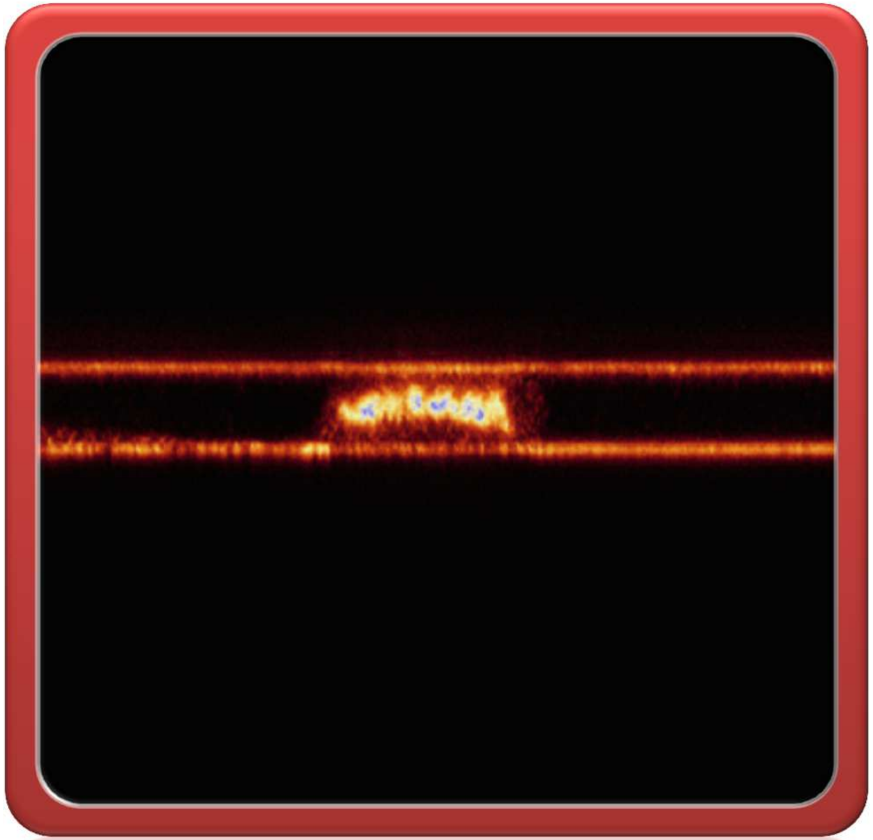




LightGate - Removal Of Cover Glass Reflection

LightGate off

LightGate on



LightGate – What Is It Good For?

Enhancement of image contrast

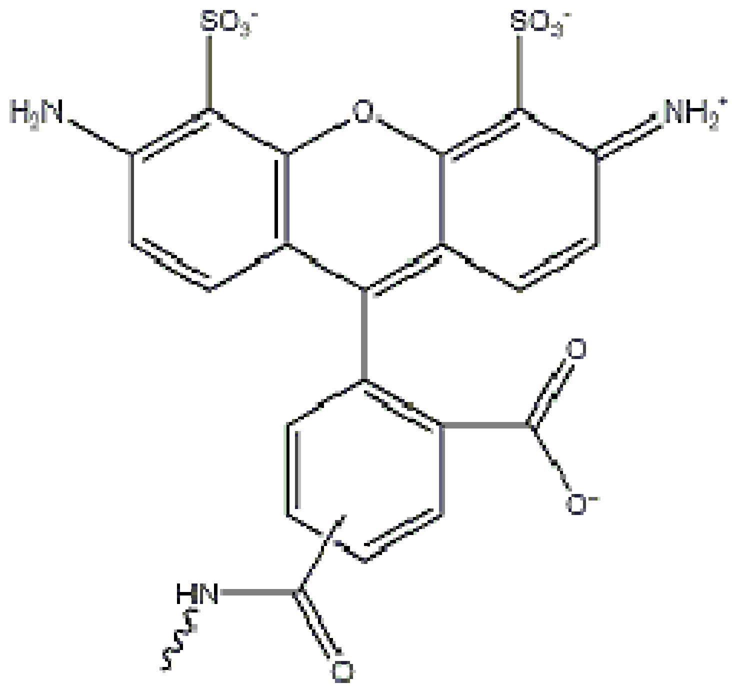
Full spectral characterization

High SNR even at "difficult" samples

Application

Dye properties; endless photons????

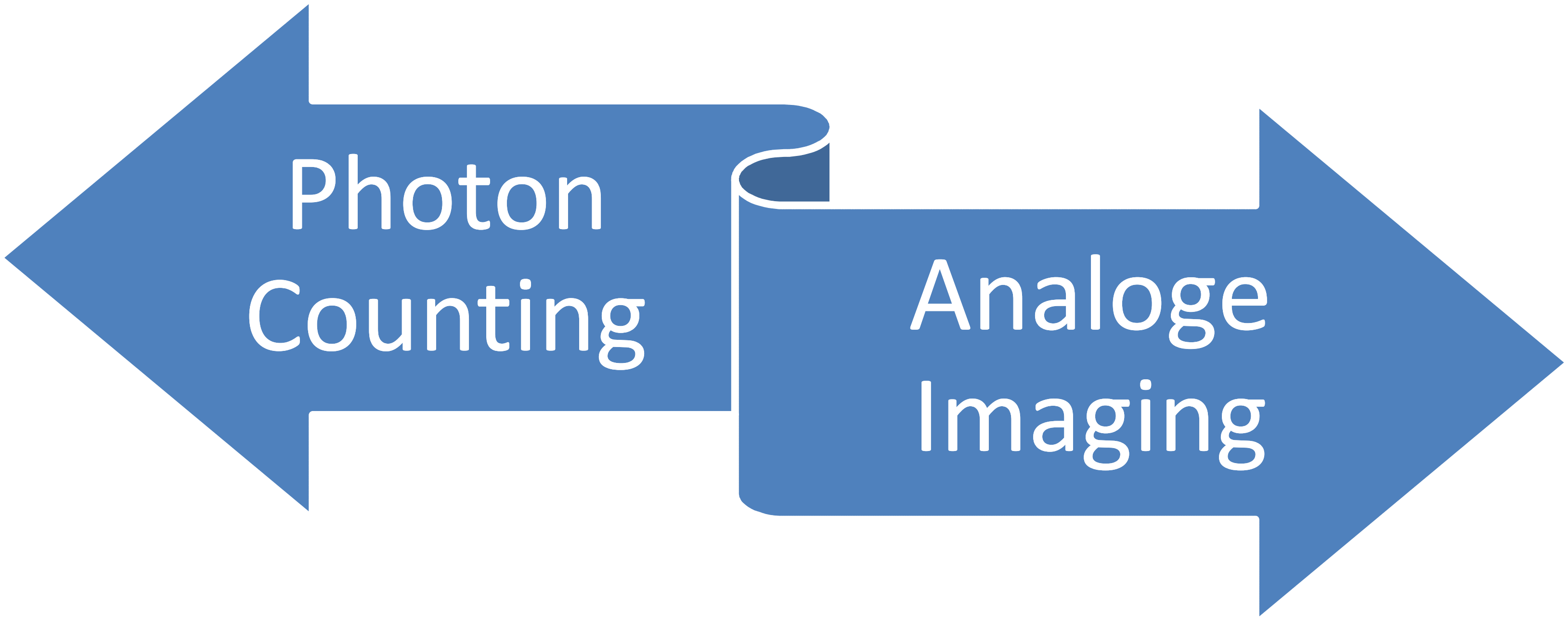
- Solar Energie/day = 165 W/m²
  - 230 Count/area (cpa)
- Laser Energie = 20 mW (488nm)
  - 140 x10^{13} Counts Per Second



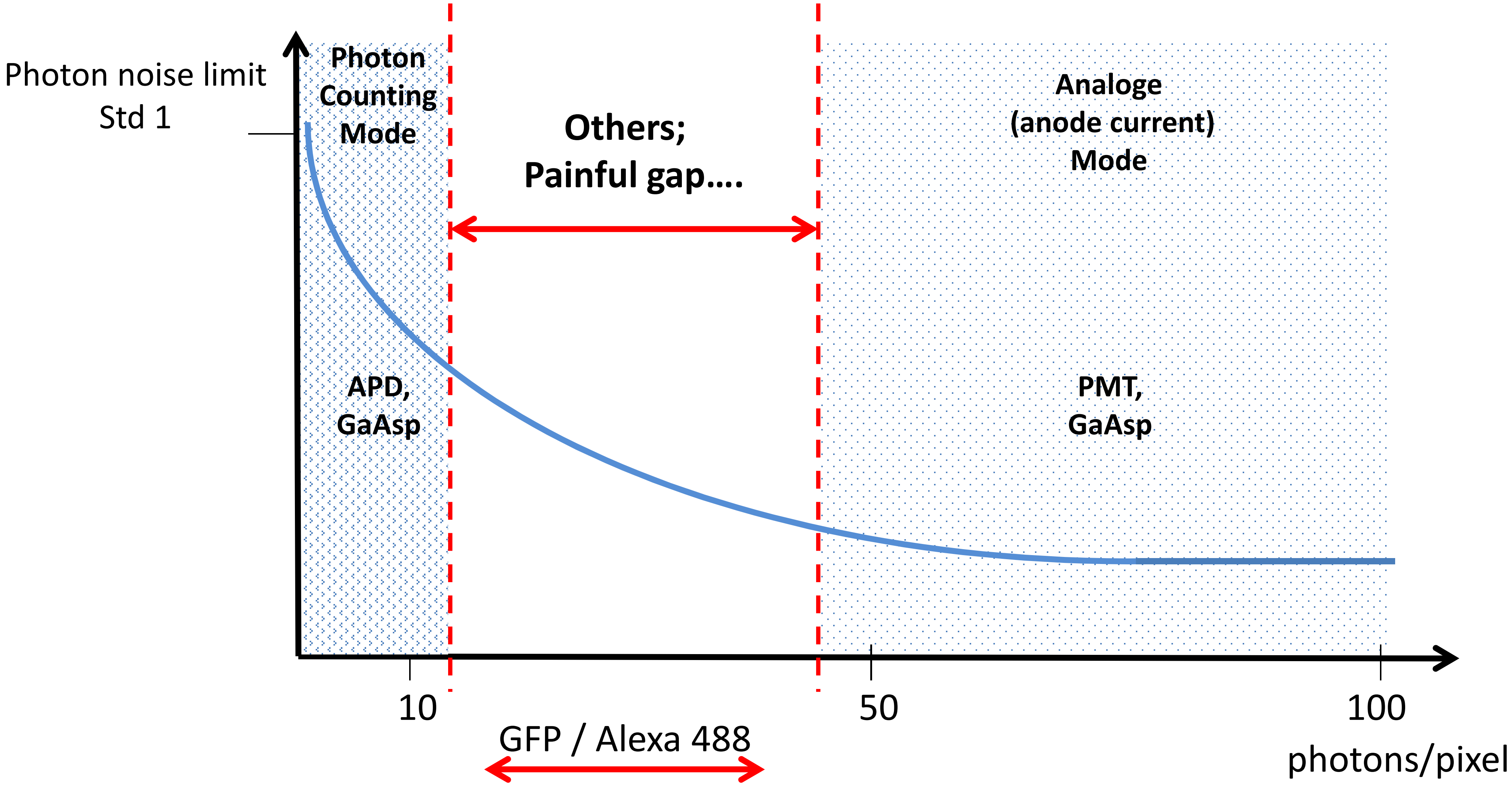
- **Dye properties**
  - **Alexa 488 = 42 Mcps (Mega counts per sec.)**
  - **GFP = 25 Mcps**

Traditional (GaAsP) detector choice

- Detector choice/mode
- Only ONE choice per experiment;
  - Photon Counting (15 MHz) or
  - Analoge Imaging (40 MHz)



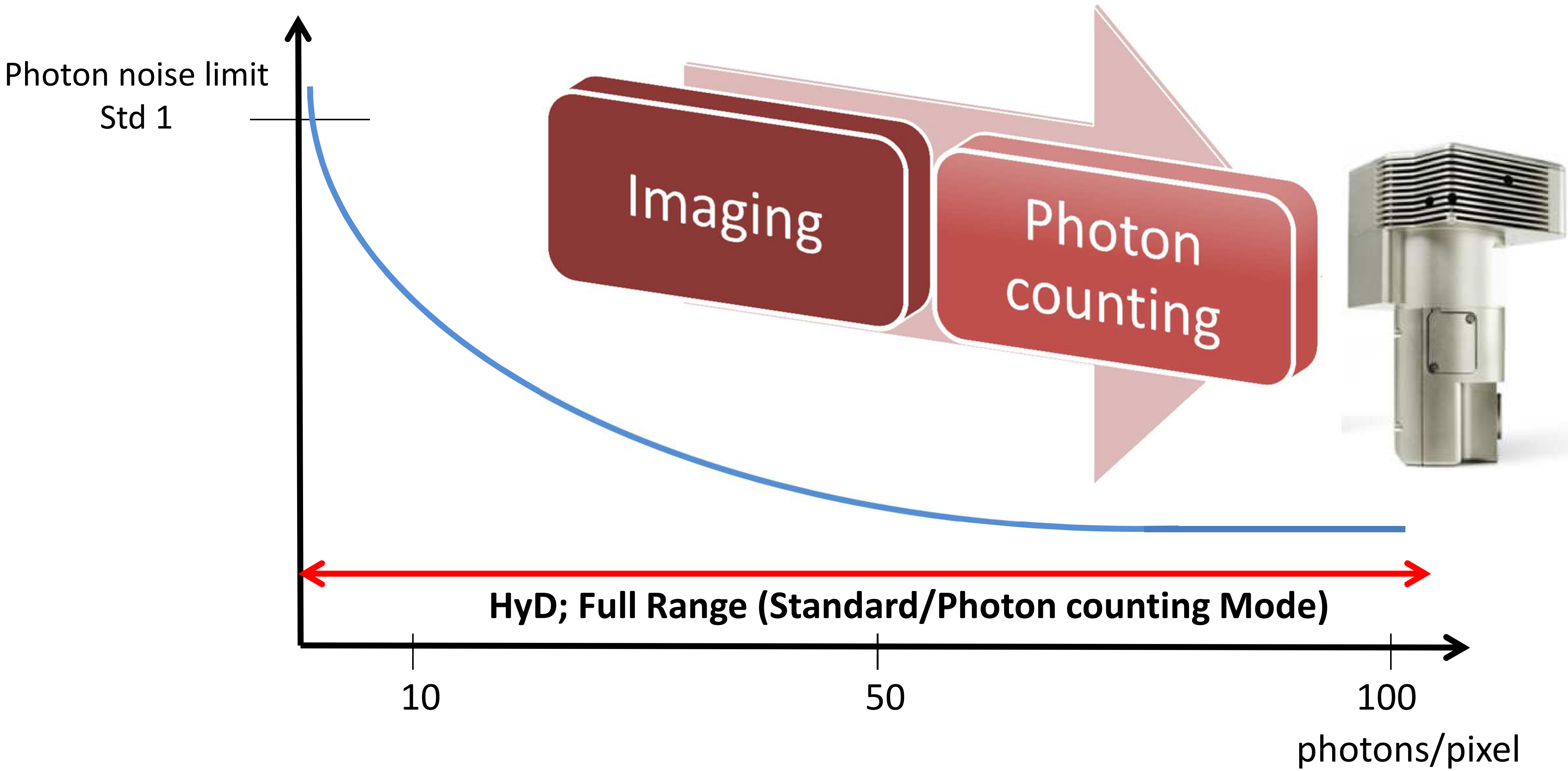
The painful gap....



Alexa 488= 42 Mcps
GFP = 25 Mcps

HyD offers the best of both worlds

Full range for Imaging/photon counting without switching hardware or mode setting for detector

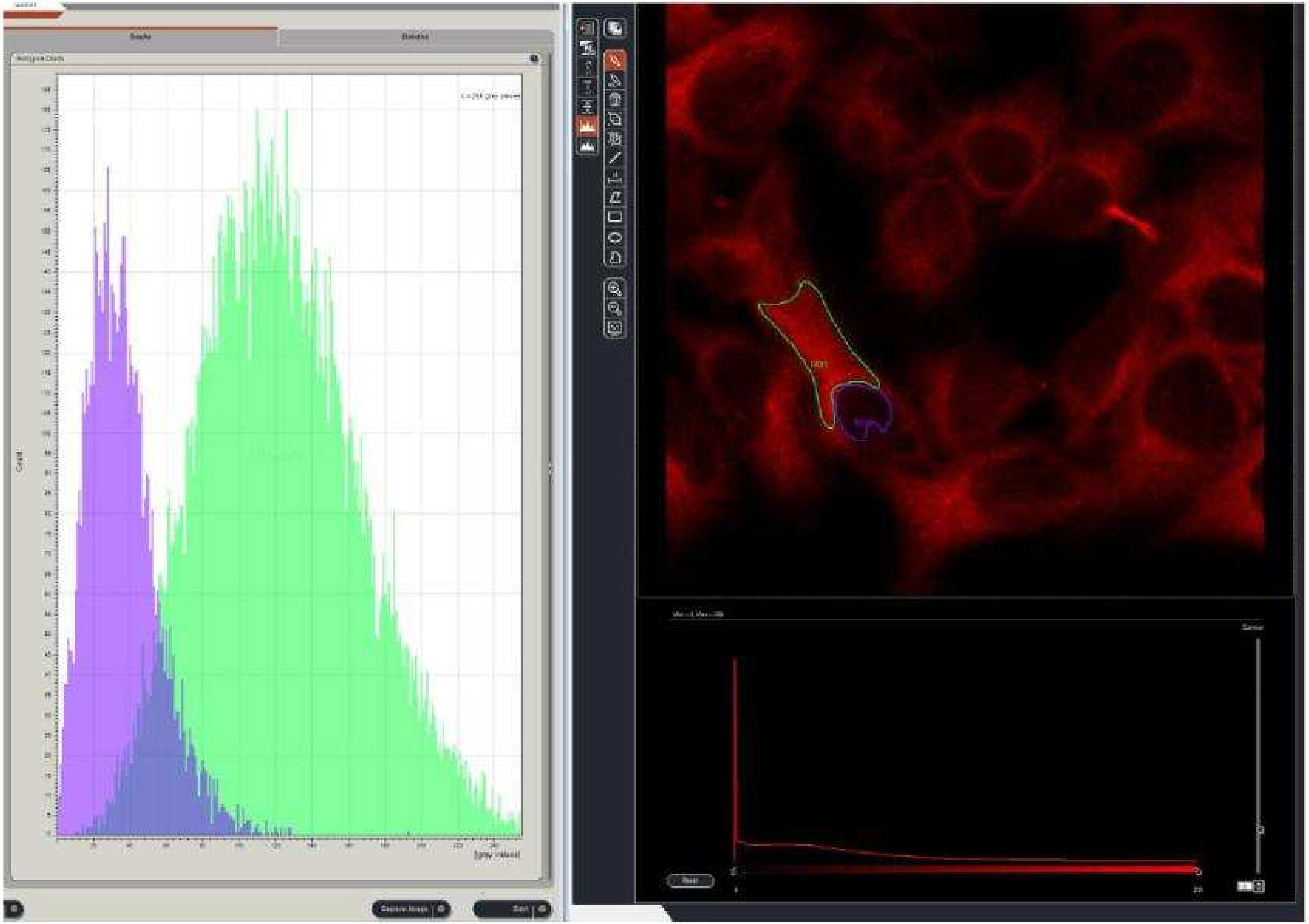


In-depth „Histo-Logy“

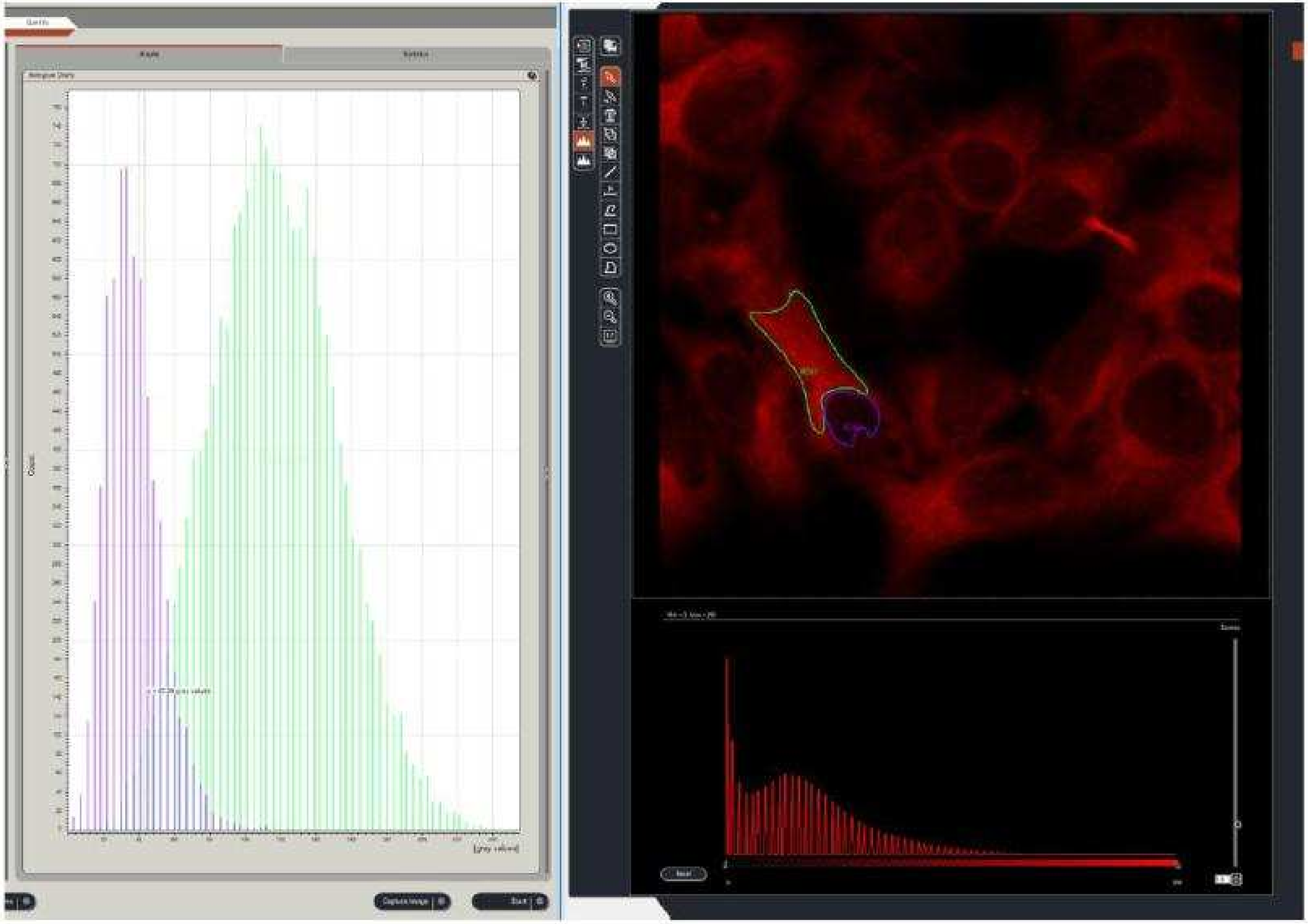
The experiment; GFP cells

- Recorded using Ex 488 nm , Em 495-555 nm
- Single pass, no averaging
- PMT @ 750 V, HyD @ Gain 28 (if applicable)
- Use identical conditions for
  - PMT
  - HyD Standard
  - HyD BrightR
  - HyD Photon Counting
- Calculate ratio between bright and dark area

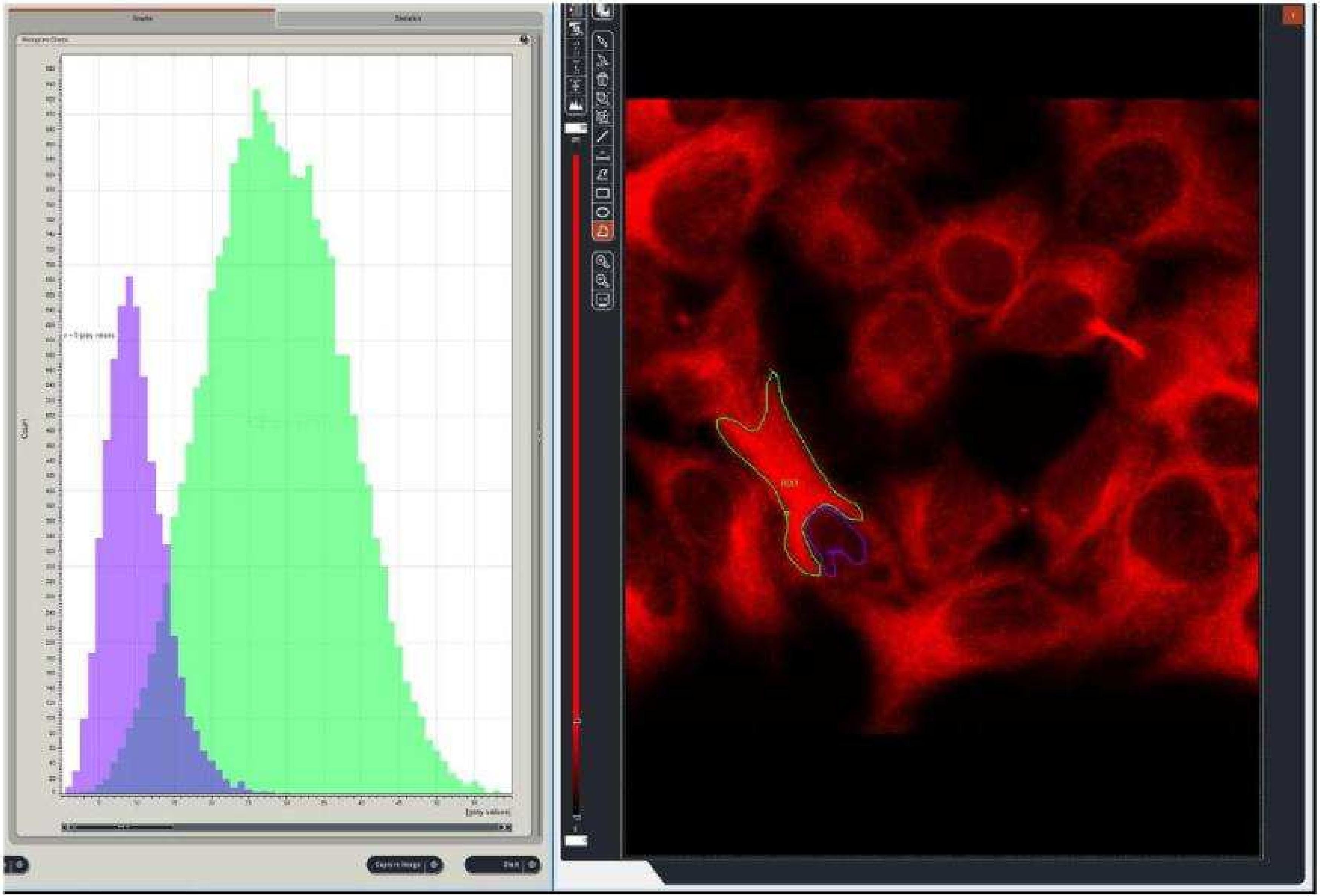
PMT...



HyD Standard…

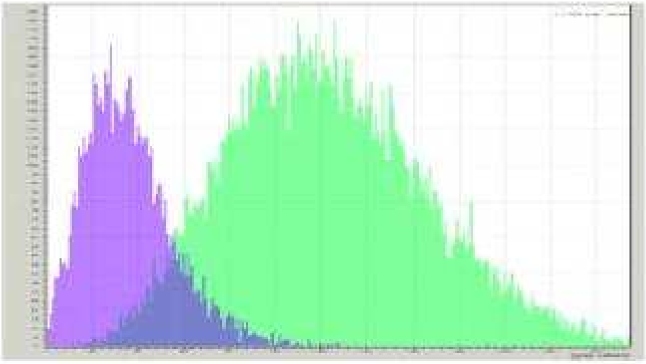


and Photon Counting

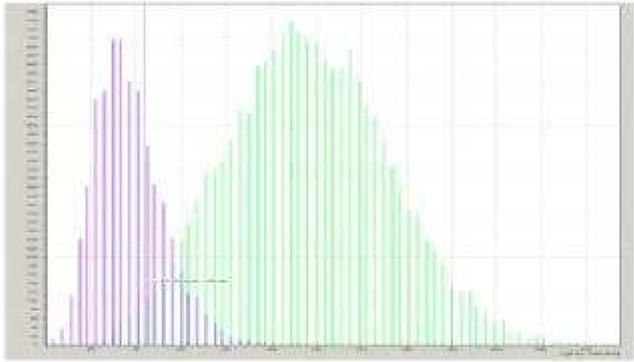


The Result; GFP cells

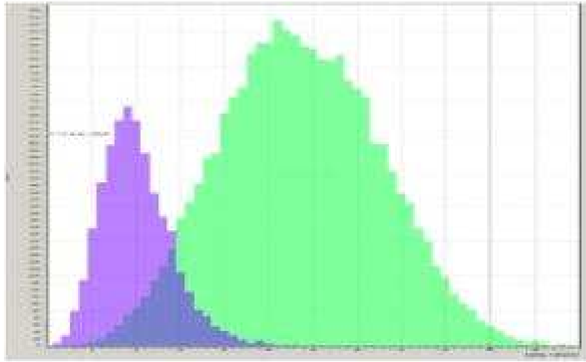
PMT



HyD Standard



HyD Photon Counting



ROI1/ROI2 = 3,2

ROI1/ROI2 = 3,1

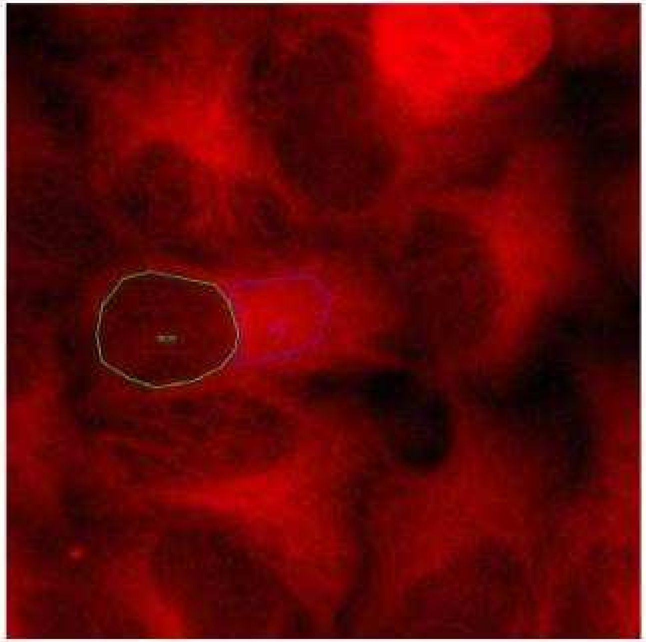
ROI1/ROI2 = 2,9

Another experiment with GFP cells

Do bit depth or mode have an effect on quantification?

Detec	Gain	Mode	Bit Depth	ROI1	ROI2	Ratio
HyD	28	Standard	12	304	1090	0,28
HyD	28	Standard	8	18,5	66,6	0,28

Detec	Gain	Mode	Bit Depth	ROI1	ROI2	Ratio
HyD	-	PC	8	4,8	17,1	0,28
HyD	-	PC	12	4,96	18,1	0,27



No, they don't!

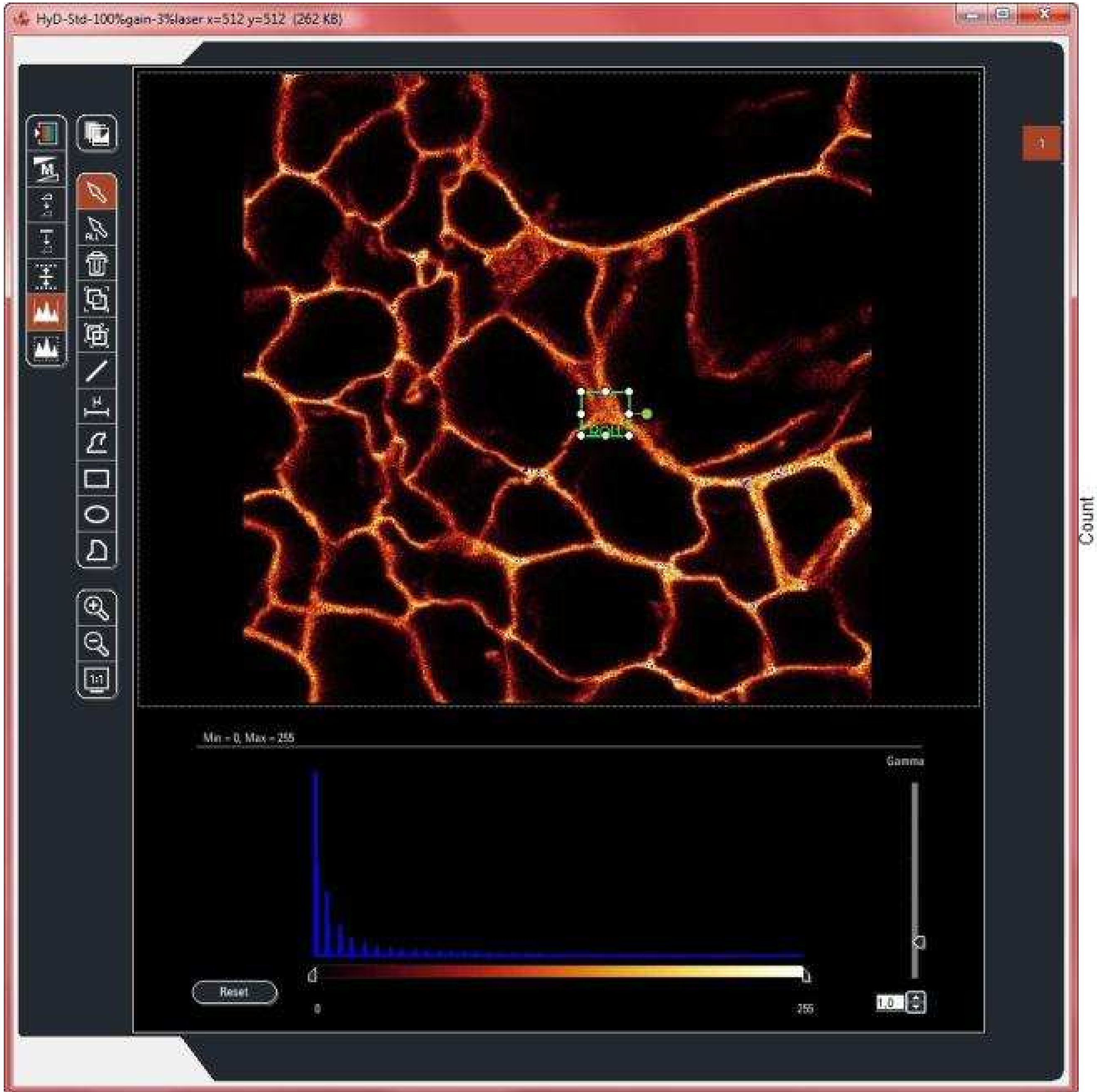
How about BrightR for quantification?

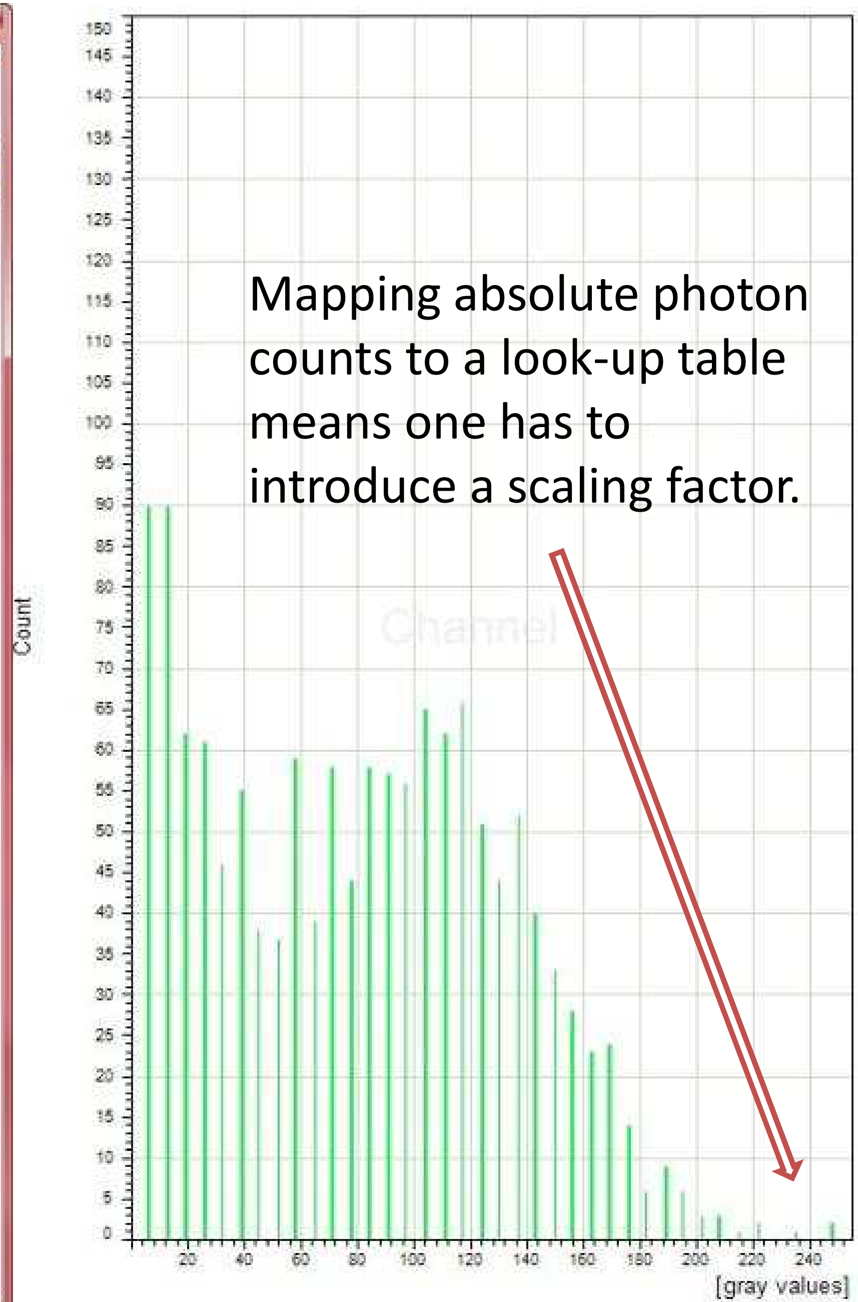
Detec	Gain	Mode	Bit Depth	ROI1	ROI2	Ratio
HyD	28	BrightR	12	537	1970	0,27

No effect within the image,
BUT... do not mix Standard and BrightR in multicolor!

LASAF examples

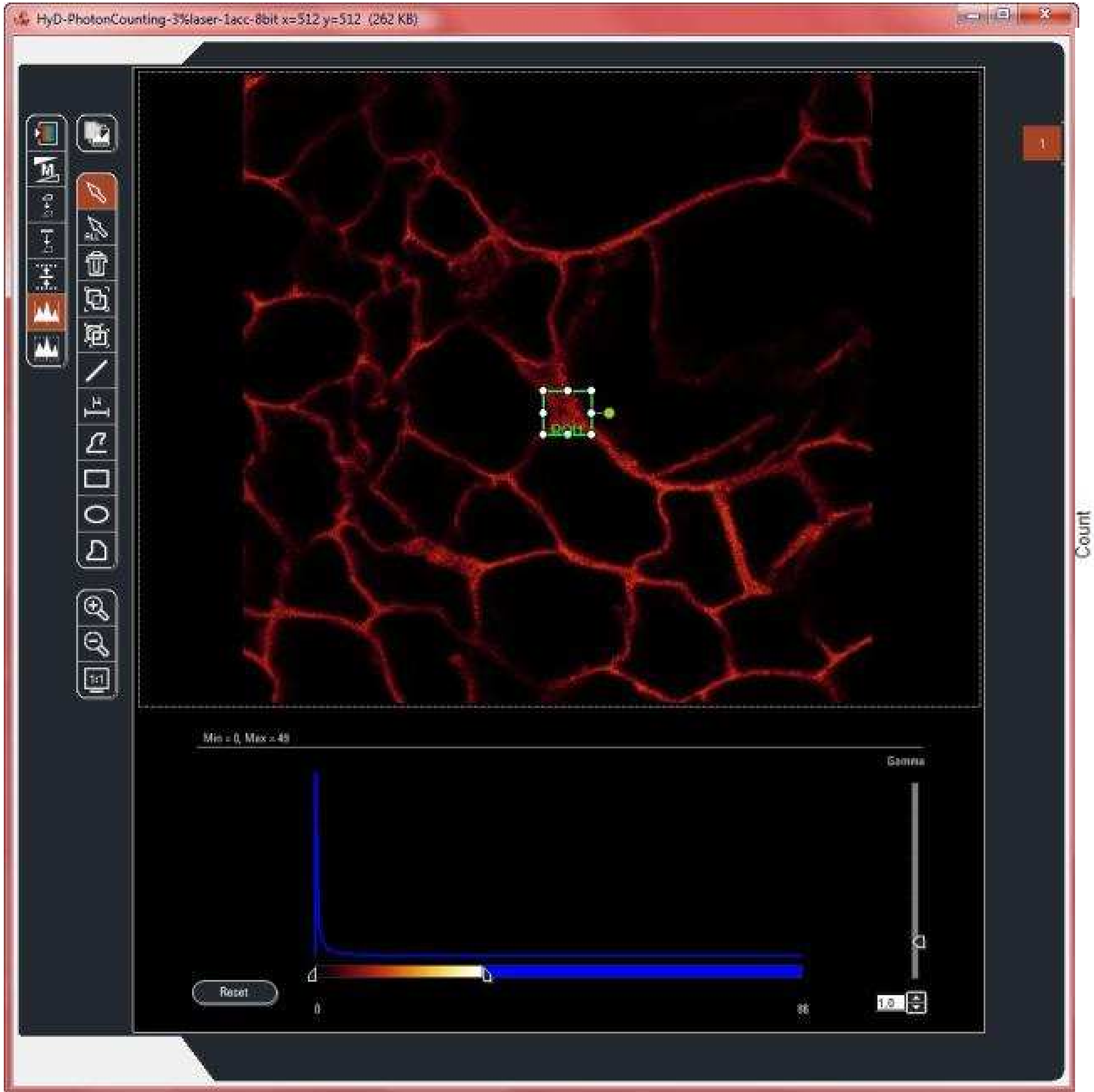
HyD :Standard Mode

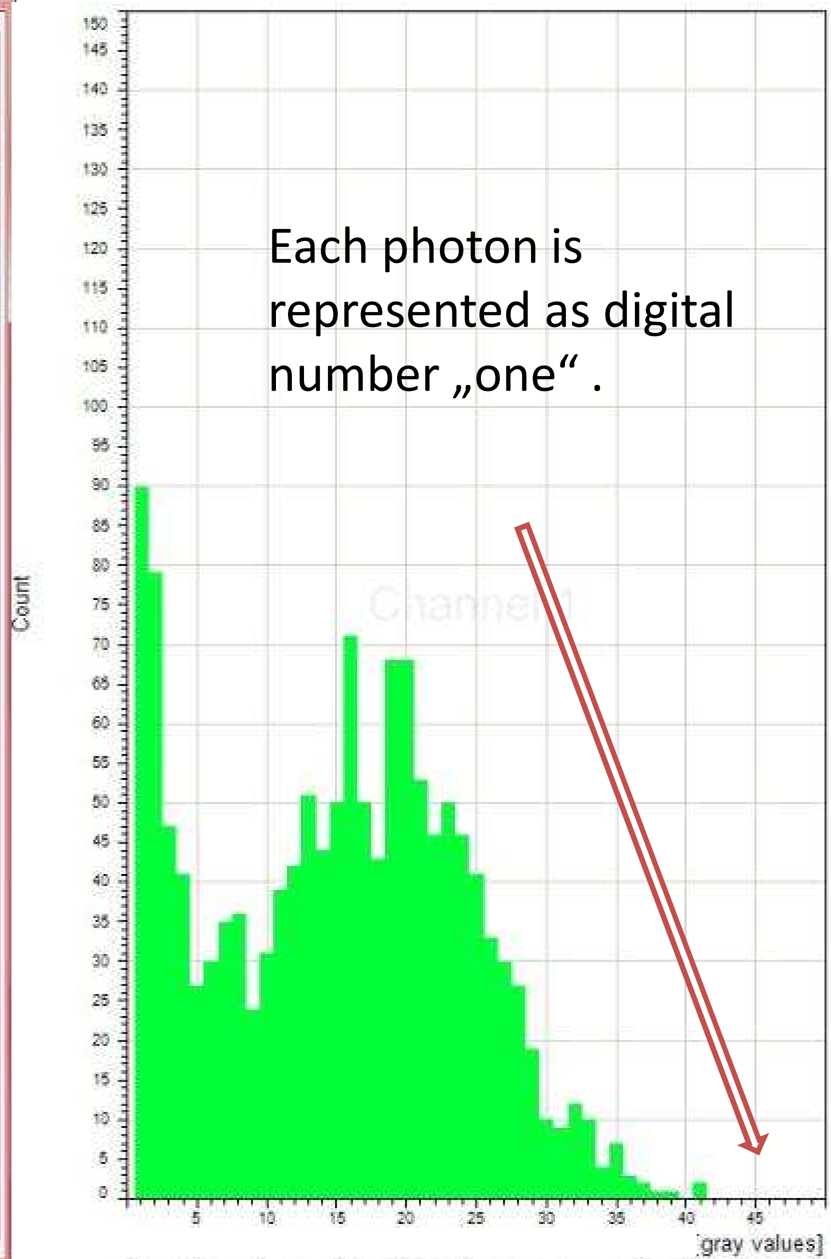




Mapping absolute photon counts to a look-up table means one has to introduce a scaling factor.

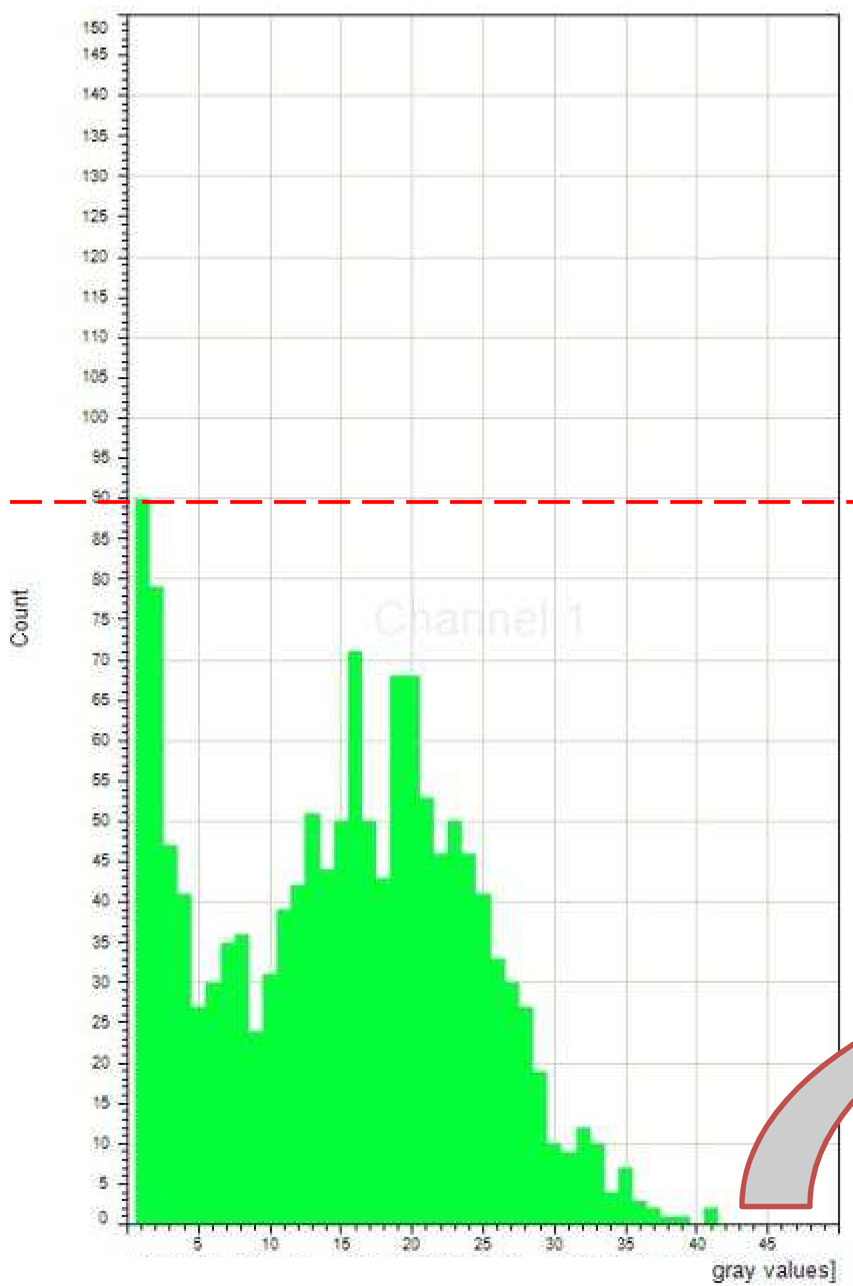
HyD : Photoncounting Mode



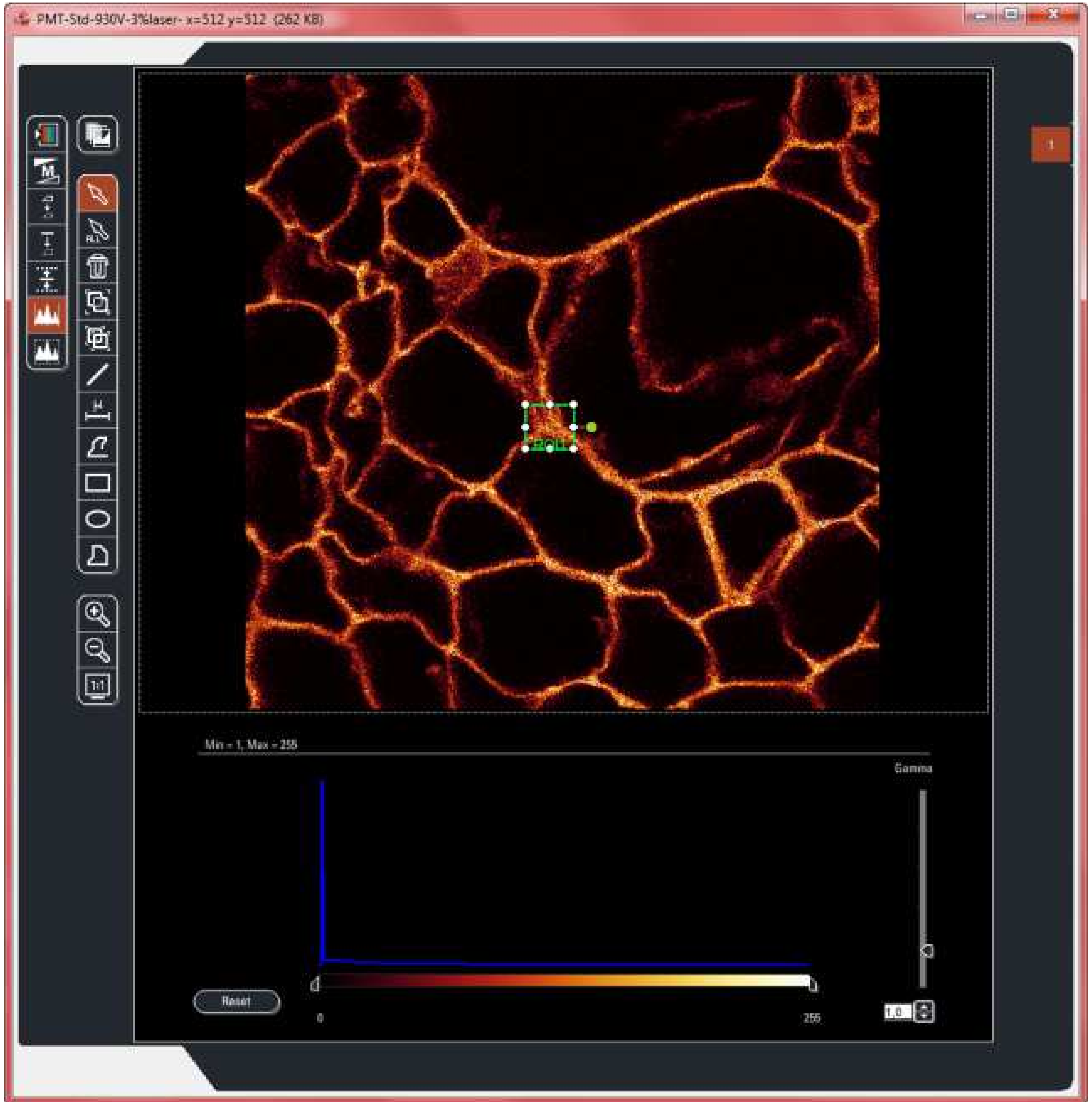


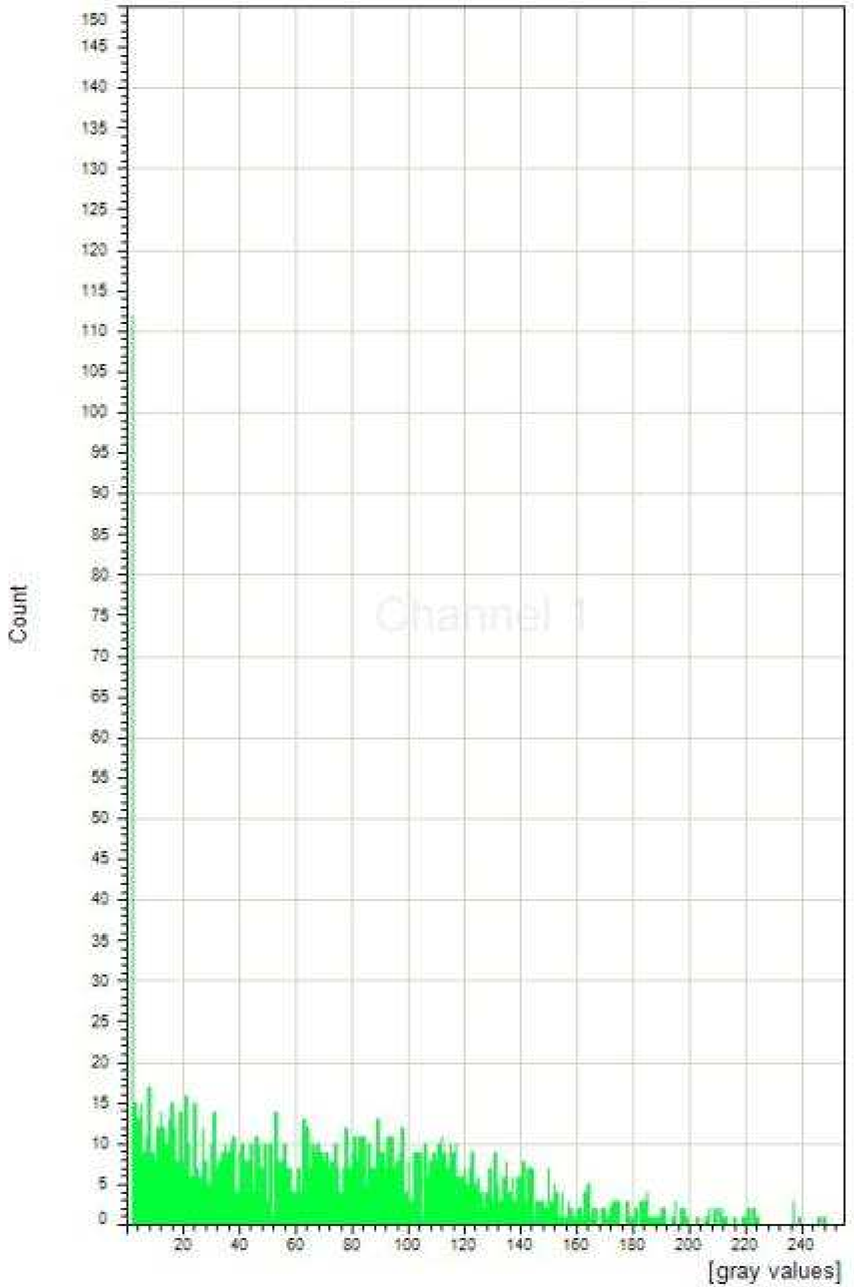
Each photon is represented as digital number „one“ .

Photoncounting vs Standard

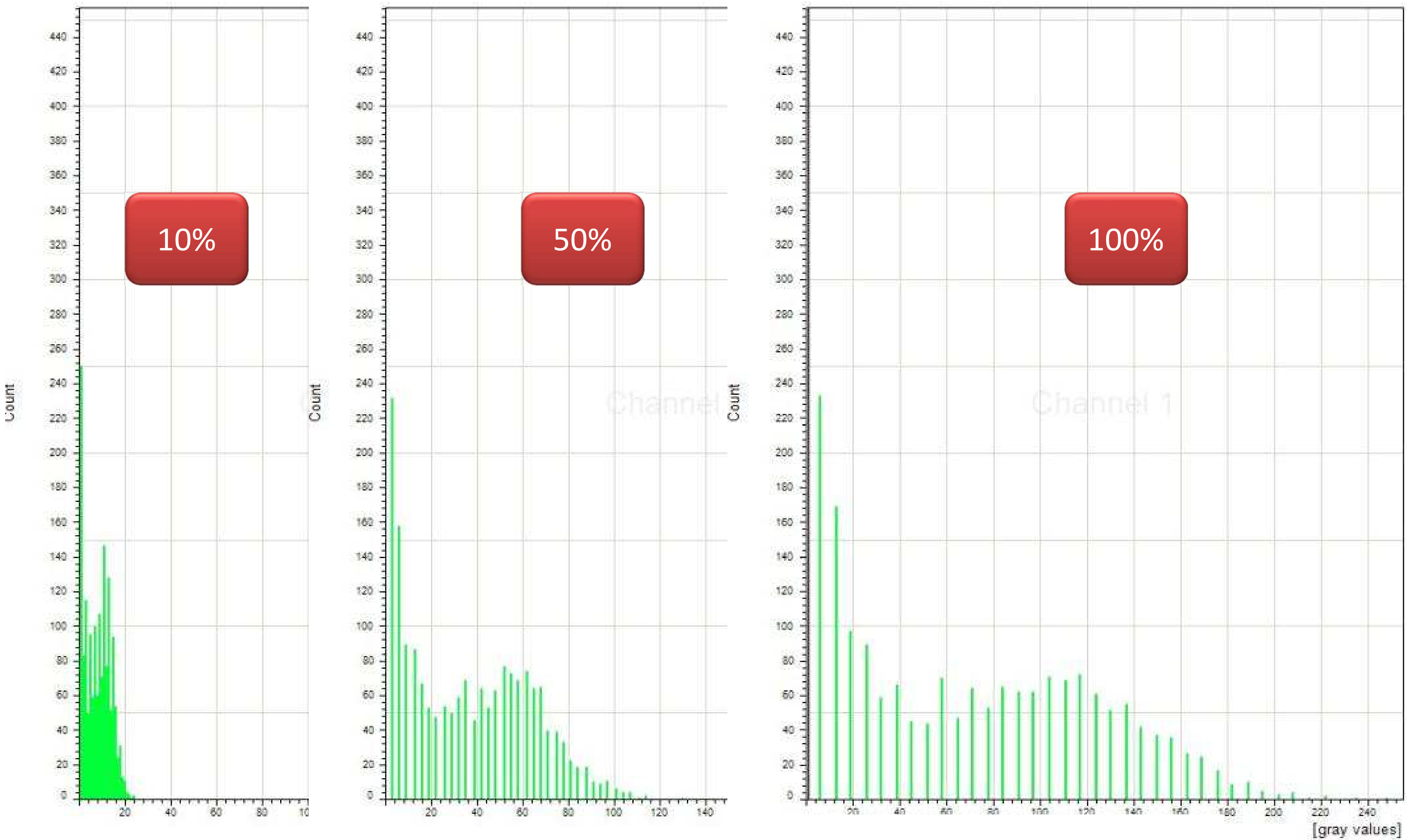


PMT

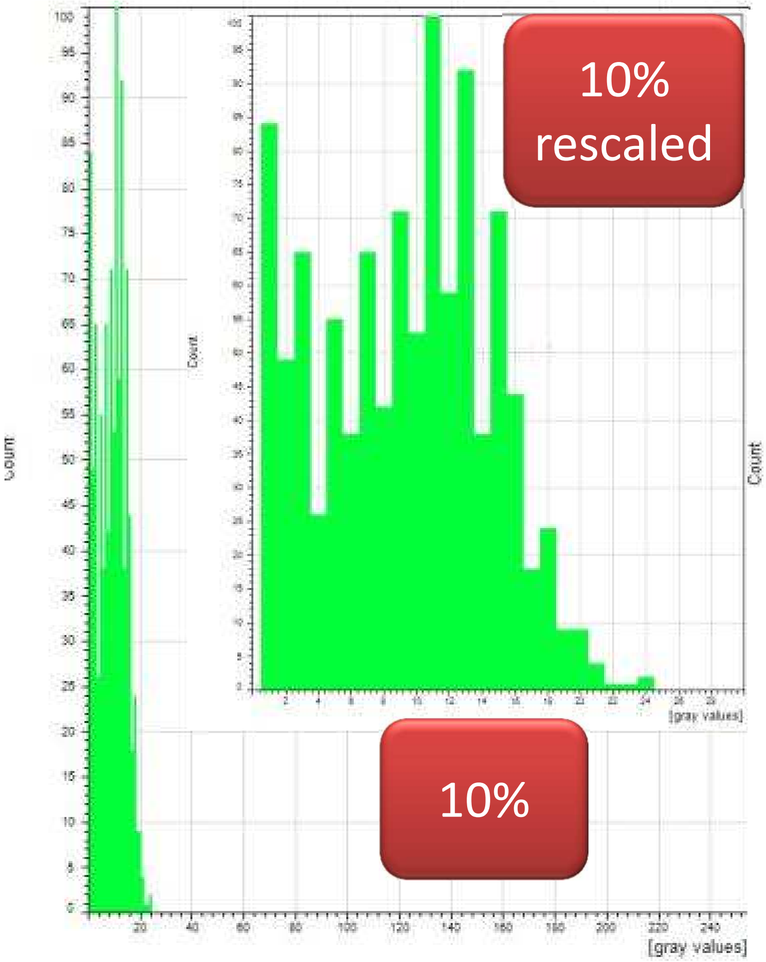

PMT-Std-930V-3%laser- x=512 y=512 (262 KB)
Min = 1, Max = 255
Gamma
Reset
0
255

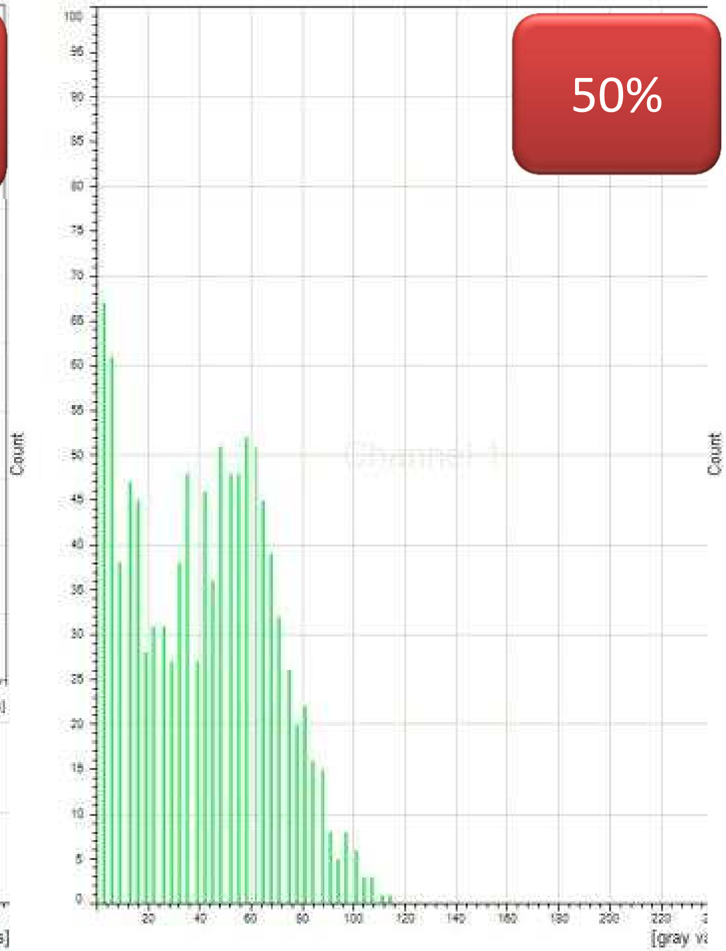

Count
Channel 1
[gray values]
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
20
40
60
80
100
120
140
160
180
200
220
240

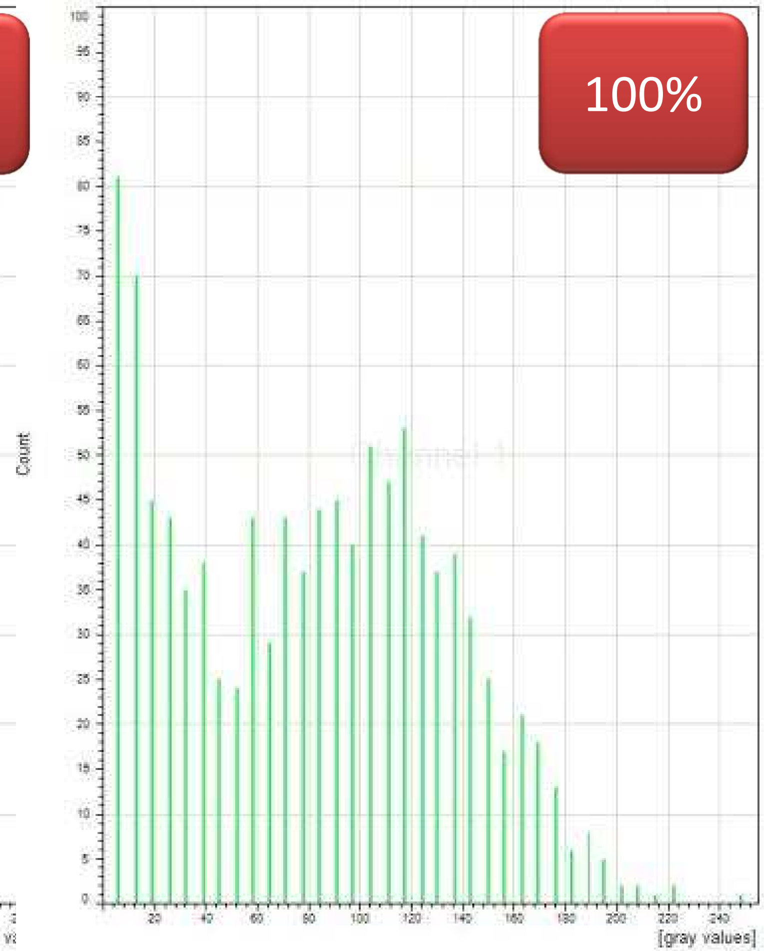
HyD Standard mode; gain 10%-50%-100%



HyD Standard mode with low light ; 3%laser







Practical example

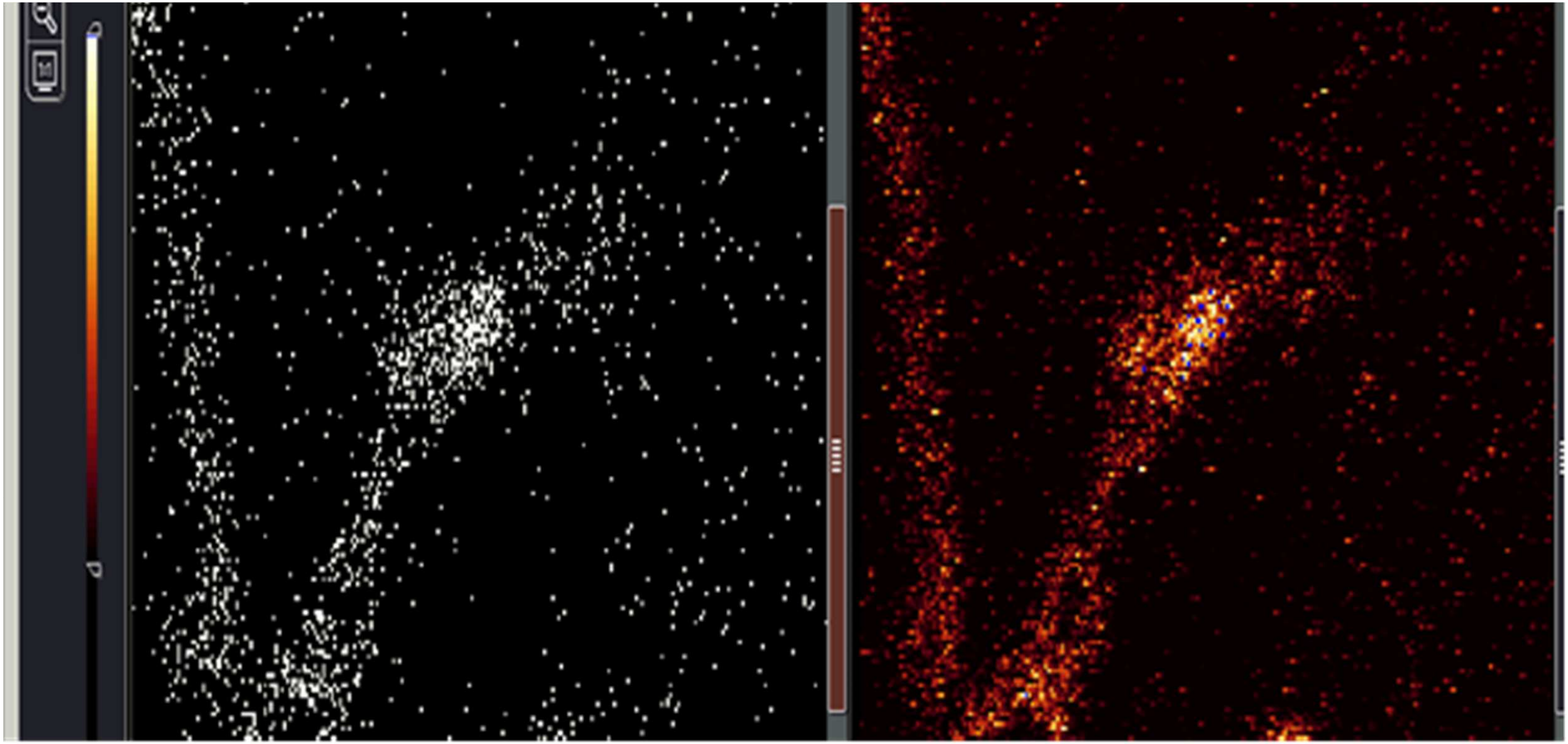
Hyd-PMT practical example;

When scanning very fast, there is very little light per pixel, because the dwell time is short.
In this example, in fact, there is so little light, that the HyD captures only one photon per pixel.
At a resolution of 1024 pixels you have about 40 ns pixel dwell time (estimated).
1 photon count / 40 ns = 25 Mcounts/s.

This is a fairly large photon stream (for example in FLIM the maximum is 1 Mcounts/s),
so the sample is bright enough, but at this short pixel time there is only really little information being picked up.
So the image looks pretty much binary.

Now, the interesting part - why does the PMT apparently capture more dynamic information ??
The answer is revealed only when zooming in very much (see next).
If you do, you can see that HyD produces completely square pixels.
PMT on the other hand produces very rectangular pixels.
Reason: The PMT is too slow for this high pixel time. So, it smears out the pixels, because it can not switch from bright to dark fast enough. The effect of that is very interesting, however.

HyD vs PMT



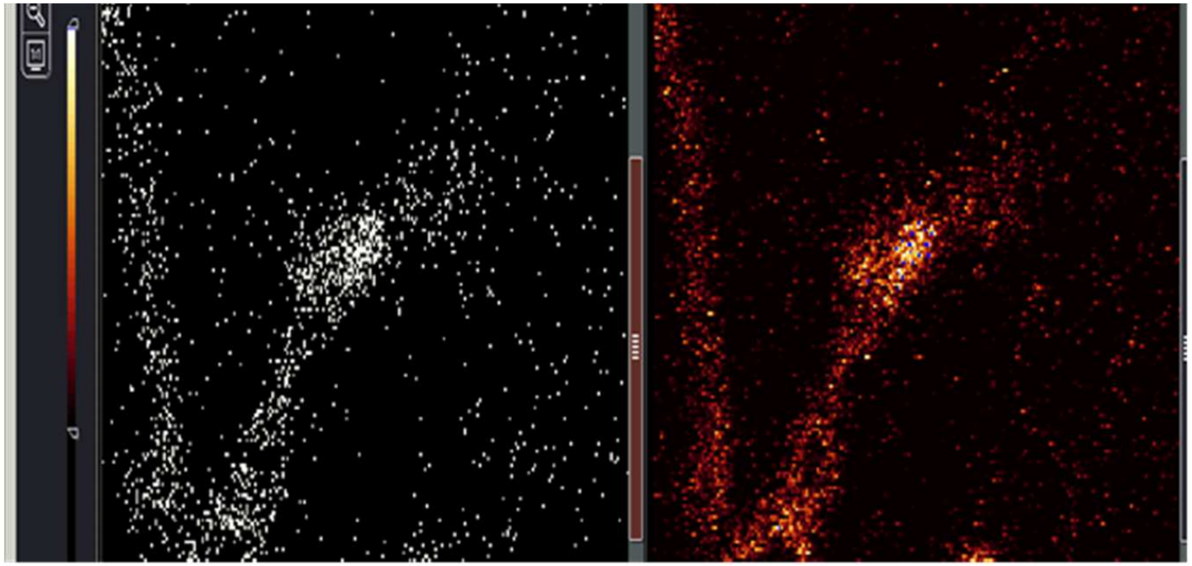
Hyd-PMT practical example;

The effect is, that neighboring pixels influence each other in the PMT image. It works a bit like a Gaussian blur filter, although in one dimension only. The result is, that higher pixel DENSITIES render apparent (but artificial) centers of INTENSITY in the PMT case.

The PMT in this situation makes the insides of a structure look brighter than the outsides.

So, interestingly, the apparent dynamic steps in the PMT image are artificial and caused by a shortcoming (being slow) of the PMT!

In our human perception, bright insides and dark outsides appear very believable, however, because nature is perceived as a continuum, but HyD, being a photon counting detector, reveals the underlying quantized nature of things.



What you can do about it:

Reduce Scan format and Zoom in more

Use Accumulation

Scan more slowly

Low excess noise

- Each photon generates the same charge
- Crisp images, High speed imaging
- More information

High dynamic range

- Versatile detector, Bright and dark regions visible
- One detector for all applications, More info on screen

High sensitivity

- Lower laser power, Extend towards bottom end of intensities
- One detector for all applications, More info on screen

Small TTS

- Suitable for single photon counting
- Bigger dynamic range

Small dead time

- Better photon efficiency, Better duty cycle of detection , More information

  Increased cell viability -> Better turn-over rate for experiments

Less ionization

- More photostable
- Longer detector lifetime

End