Application note:

How to obtain the best from SP5/SP8 confocal microscopes using HyD*

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Differences between PMT and HyD detector

PMT → analog imaging (Anode Current Measurement)

Quantum efficiency (Capacity of a detector to translate photons into electrical signals) **2-3 times lower than HyD**

Sensitivity of the PMT is adjustable by varying the gain (PMT voltage).

High gain→ high amplification of the signal but also high dark current (noise of the detector) →background signal in the image.

In practice, it is better to use the lowest gain allowing a well contrasted image with good signal to noise ratio. We recommend not to increase the PMT gain beyond 800-900V to avoid too much background.

HyD → photon counting

Quantum efficiency **2-3 times higher than PMT.**

HyD voltage is fixed; sensitivity is not adjustable; HyD are photon counting detectors; the photon count directly depends on the fluorescence emission.

HyDs exhibit a very low noise level and superior sensitivity → high contrast imaging.

Due to the very low background of the HyDs, photon counting allows accumulating as much information as needed.

Unlike PMTs, which intrinsically have a longer flight time for photoelectrons, the HyD generates ultra-short pulses → precise photon counting. Single photon counting is the most accurate approach to the signal quantification.

In conclusion, HyD improves signal to noise ratio. Advantages:

→ highly contrasted images showing sharp details of low fluorescence signals.

→ high speed image capture and/or decreased light dosage resulting in decreased specimen damage and improved cell viability

→precise quantification and reproducible conditions due to the photon counting mode of operating *

**HyDs appear to outperform PMTs in most cases.**
A PMT is only preferable in extremely bright emission situations, when the usage of the PMT gain lower than 600V produces low noise bright images without need of averaging (for example reflectance).**

*At higher count rates (high intensity), separating individual photon arrivals is no longer possible, and the imaging is no longer strictly quantitative. In photon counting mode, the HyD response is linear up to 60 MHz (can detect individual photon up to 60M/s).

**Intense signal from dust or local fluorophore aggregates for example may also exclude HyDs usage (sensitive HyDs can get damaged if they received too much light).

More information about PMts and HyD in the Hamamatsu PMT's Handbook (https://www.hamamatsu.com/resources/pdf/etd/PMT_handbook_v3aE.pdf)

Image comparison: PMT versus HyD

PMT   **HYD**
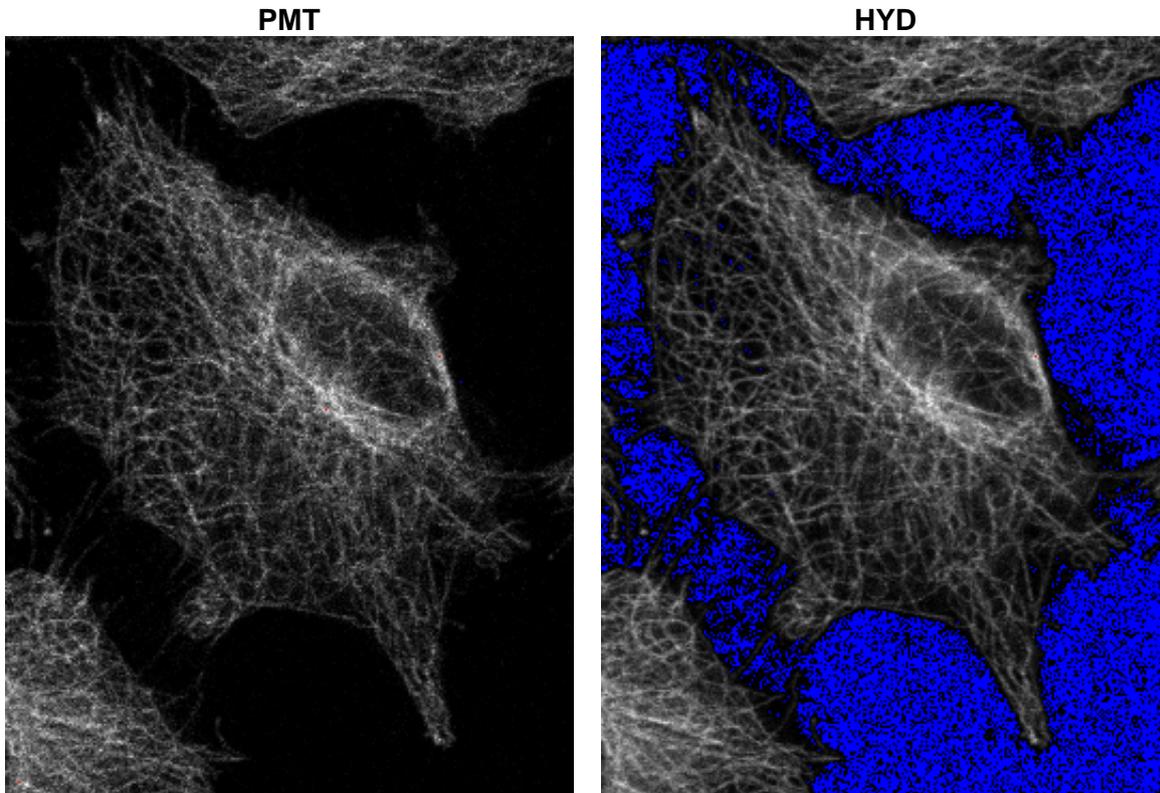
Blue pixels correspond to 0 intensity value
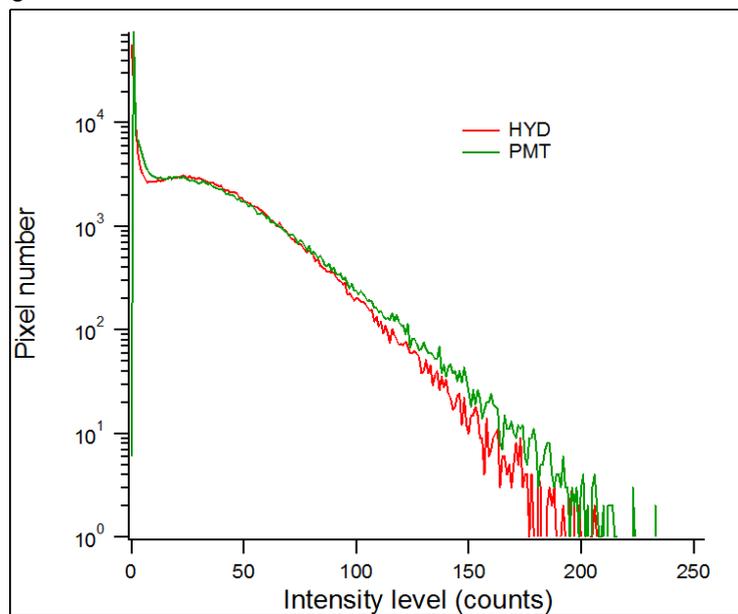
Corresponding histograms

Fig.1: *Image comparison: PMT versus HyD and their corresponding histograms.*
Same acquisition conditions for the 2 images: same % laser and detection.
HyD in the counting mode – max = 120 → 2 accumulations to fill the 8bit range
PMT gain set to fill the 8bit range (max ~255) – average=2

Conclusion: HyD → low noise and sharp image!

Using HyD in the counting mode

Counting mode is the basal mode of operating of the HyD. No amplification of the signal: the photon count depends directly on the fluorescence emission.

How to optimize your confocal settings using HyD:

2 cases:

1- **High quality imaging for localization, structure analysis, highlighting fine details in low fluorescence conditions (no need to quantify the intensity levels).**

2- **Imaging for quantitative intensity measurements.**

Case 1: High quality imaging for localization, structure analysis, highlighting fine details and low fluorescence … (no need to quantify the intensity levels).

Steps to optimize your acquisition:

1- Set the HyD in counting mode. Set your detection bandwidth and set a low laser % (1% for Argon, <10% for white laser).
(HyDs can be damaged if they receive too much light! Always begin with a low laser power and increase the laser depending on the max count value).

2- In the live mode, display the histogram and check the max value.
SP8: max appears in a box case at the top of the intensity/color bar (Fig 2);
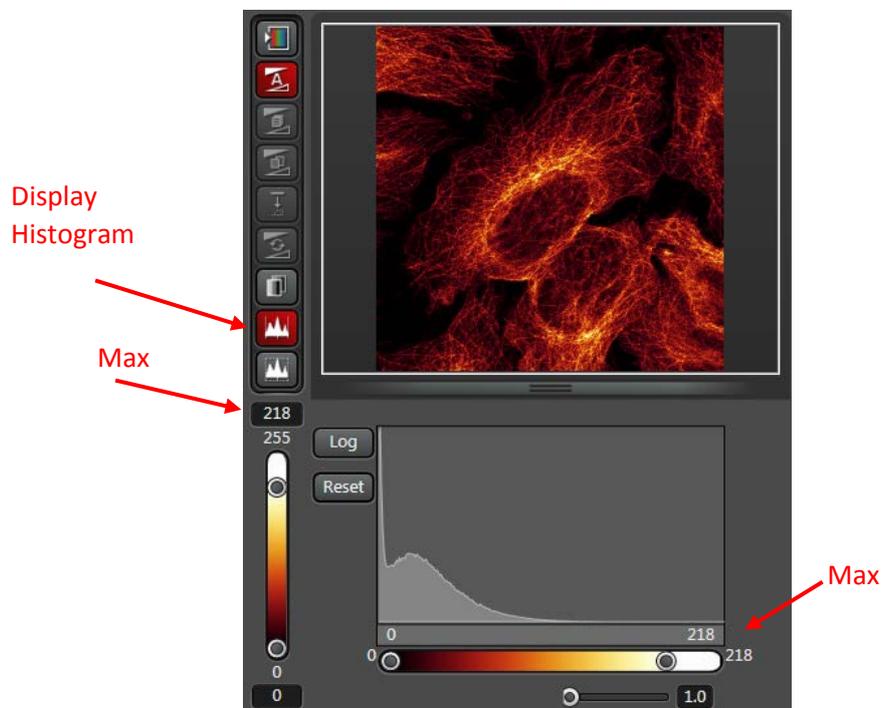SP5: max is written at the top of the histogram window.

Fig. 2: Image acquisition: displaying the histogram and checking for the max intensity value.

3- Increase the laser % (keep the laser % low enough to avoid the photobleaching) to fill as much as possible the 8 bit range (bring the max value close to the 255 value on the brightest part of your sample, for example 200-220 is good enough, 240 would be almost perfect) (Fig3) If the fluorescence is weak, accumulate to fill the 8bit range and to obtain a sufficient signal to noise ratio

(ex: max value = 60 you can accumulate 4 times to reach 60x4= 240 resulting counts, value close to the max level in the 8bit range).

Scaling adjusted for display
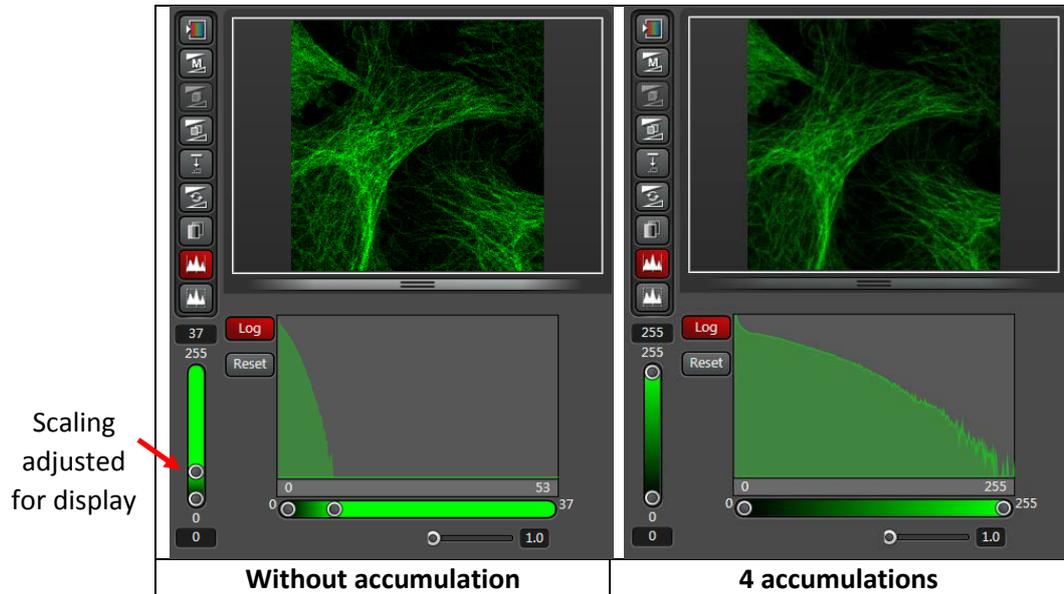
Without accumulation | 4 accumulations

Fig. 3*: optimization of the image acquisition by filling the dynamic range*

Note: check your accumulation factor for each acquisition: too many accumulations can result in saturation (max value exceeding the 255 max level in the 8bit range). To avoid saturation keep the max resulting value below 255 or change for the 12 bit range to extend saturation level at 4095.

For situations where low and high levels of fluorescence coexist, 12bit range will also be chosen to allow accumulation and highlighting of the low signal without saturation in the intense areas.

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Case 2: Imaging for quantitative measurements

Take care! At high count rates, the HyD response curve saturates (becomes non-linear) and the imaging is no longer quantitative.
In photon counting mode, the HyD is linear up to 60 MHz (Fig 4).
That means that you have to maintain the photon count **below 60 counts /µs**!
If not respected, you underestimate the high intensity levels and introduce errors in your quantification.

Reference values:
1µs pixel dwell time is obtained for a **512x512pixels** image at a **485Hz** scan speed.
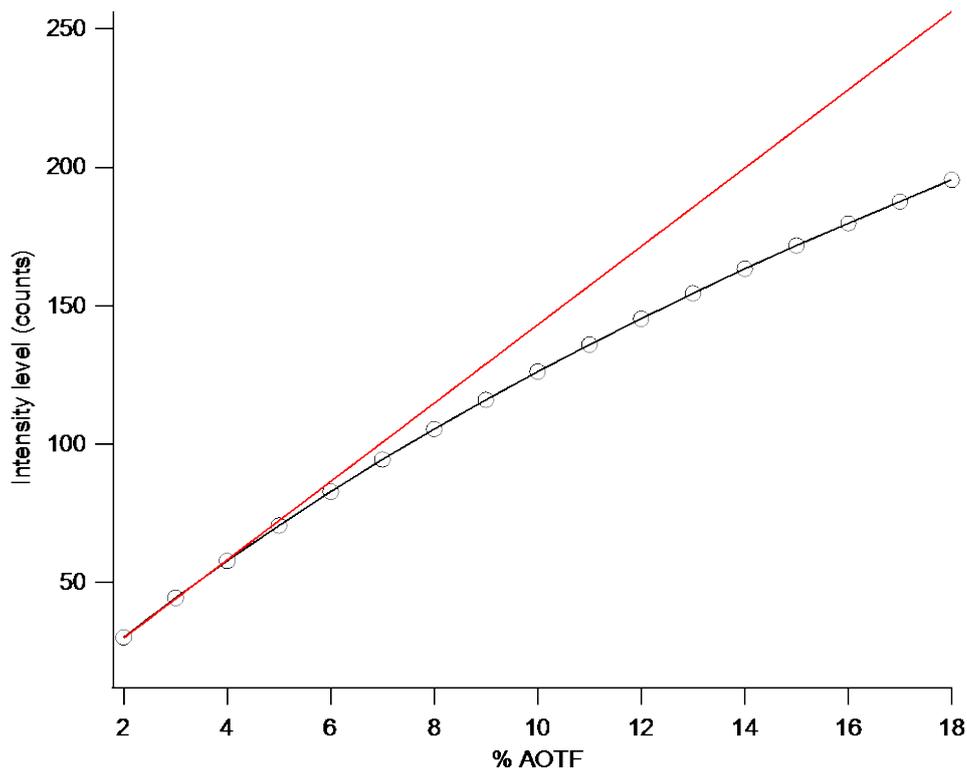For these conditions the **max count value is 60**!

Fig. 4*: variation of the photon count depending on the laser power (% AOTF*) measured on the SP8MP confocal microscope. Image format: 512x512 pixels, scan speed 400Hz. HyD response is linear until ~70 counts.*
* On SP8 systems, the laser power varies linearly with the %AOTF.

From the reference values you can easily calculate the max value for other format or speed.
To help, few standard values are summarized in the table below:

Format (pixels)	Speed (Hz)	Pixel dwell time (µs)	Max counts
512 x 512	485	1	60
512 x 512	**400**	**1.20**	**72**
512 x 512	700	0.675	40
256 x 256	400	2.43	146
1024 x 1024	400	0.6	36
2048 x 2048	400	0.3	18

In practice, you can easily check the max count value by looking at the histogram.

SP8 system running with the LAS X software have a LUT table showing the intensity level that exceed the linear range (Fig 5)
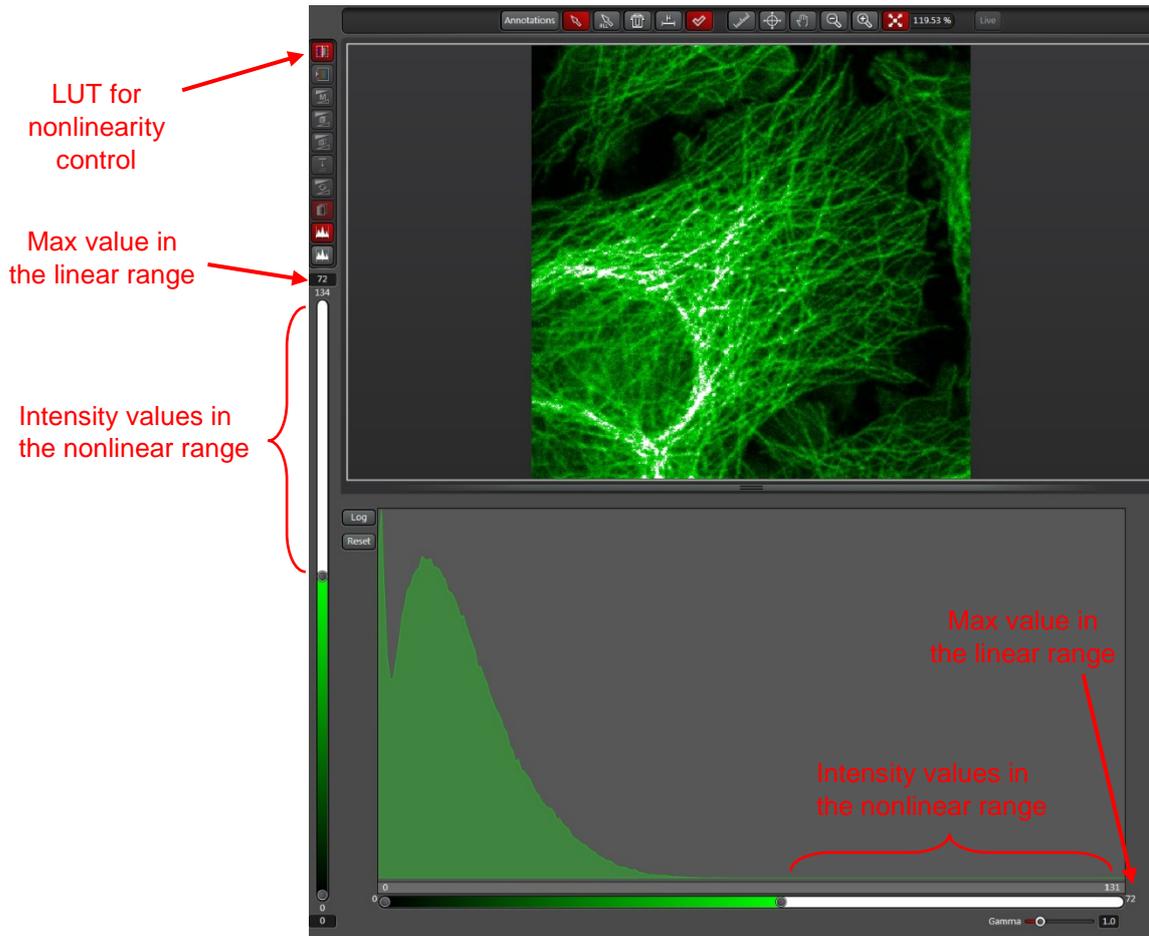

Fig.5: *LUT table for nonlinearity control. Intensity values exceeding the linear range are coding in a specific color.*

To optimize your acquisition for quantification using the HyD, one can follow the procedure that is described in case 1 (high quality imaging for localization) except that you have to maintain the count values in the HyD linear range:

1- Set the HyD in counting mode. Set your detection bandwidth and set a low laser % (1% for Argon, <10% for white laser). HyDs can be damaged if they receive too much light! Always begin with a low laser power and increase the laser depending on the max count value.

2- In the live mode, display the histogram and check if the max value (Fig 2) (in the areas where you need to quantify) is below the nonlinear range values (look at the table)

SP5: max appears at the top of the histogram window.

SP8: max appears in a box case at the top of the intensity/color bar / Intensity values in the "nonlinear" range are coded with the specific LUT for SP8 running with LAS (Fig5).

3- Adjust the laser % to stay in the linear range (check table).

Perform accumulation to obtain a sufficient signal to noise ratio or to fill the 8bit range (Fig. 3).

(ex: 512 x 512 images 485 Hz, max value = 60 you can accumulate 4 times to reach 60x4= 240 resulting counts, value close to the max level with a 8bit range)

Note: check your accumulation factor for each acquisition: too many accumulations can result in saturation (max value exceeding the 255 max level in the 8bit range). To avoid saturation keep the max resulting value below 255 or change for the 12 bit range to extend saturation level at 4095.

HyD standard vs counting mode

Standard mode - The digital gain

In the "standard" mode, the LEICA confocal software offers the possibility to set a digital gain (tunable between 10 and 500%).

This gain is "artificial" as it has no influence on the detector itself. The HyD still works in its basal photon counting mode. The output signal is numerically manipulated (changing the gain is just multiplying the intensity of each pixel by a numerical factor).

As a consequence, if your original image (counting mode) contains 30 grey levels (counts), applying a digital gain will stretch the histogram without any information gain in the image. The resulting image will still contain 30 intensity levels but now with a discontinuous distribution and a larger difference in the apparent intensity between 2 consecutive pixel (Fig 6)
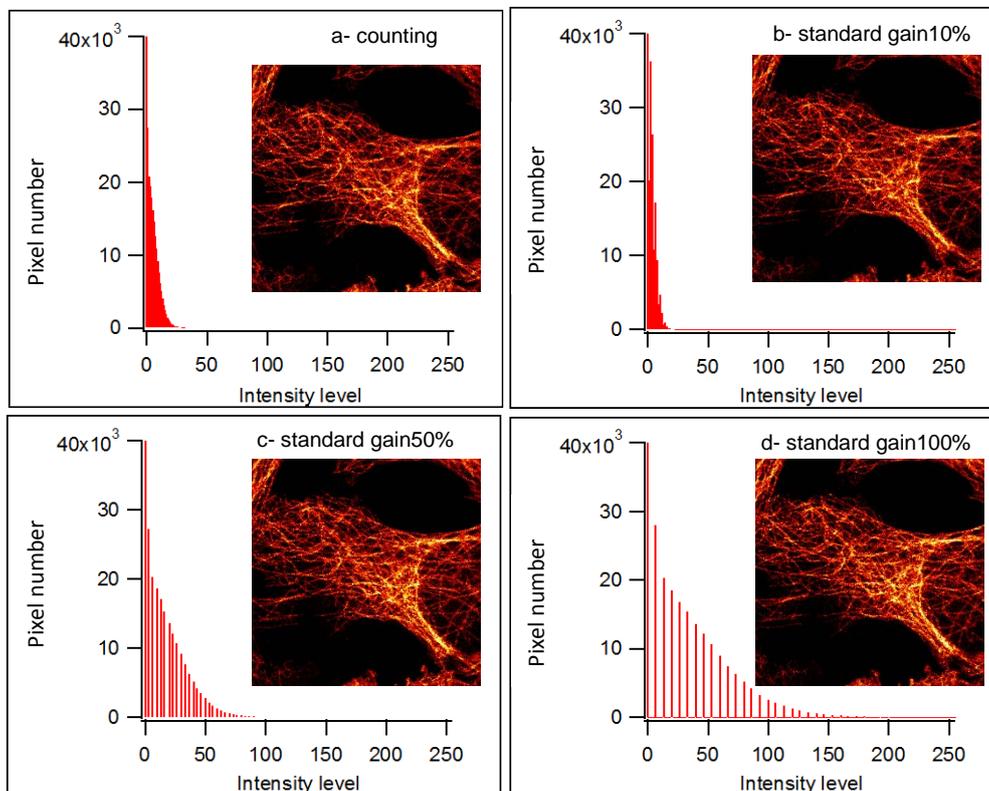

Fig .6: Histograms corresponding to images acquired with HyD in the counting mode (a) or in standard 10% gain (b), 50% gain (c) and 100% gain (d).The acquisition conditions (laser, detection bandwidth, no average, no accumulation) were fixed for the 4 acquisitions. The images obtained for the 4 conditions appears identical (contrast have been adjusted depending on the intensity range).

To improve the image quality after applying a gain, you have to perform averaging (in the same way to optimize a PMT acquisition…)

The best image quality and/or the best situation for quantification are obtained when the resulting histogram becomes continuous (→ statistically, all the intensity levels become well represented) (Fig7)

In practice, the number of averages to obtain the best result (i.e. an image with a corresponding continuous histogram) is generally equal to the number of accumulations to perform in the HyD basal counting mode (Fig7).
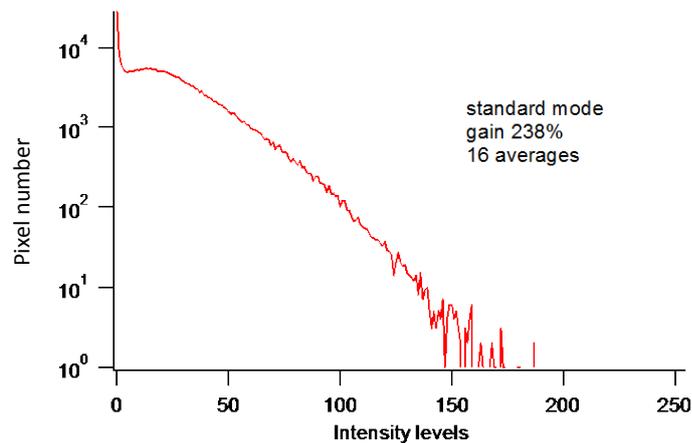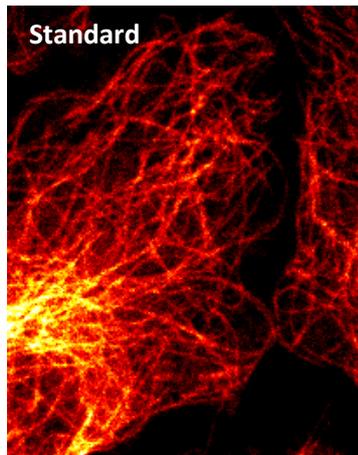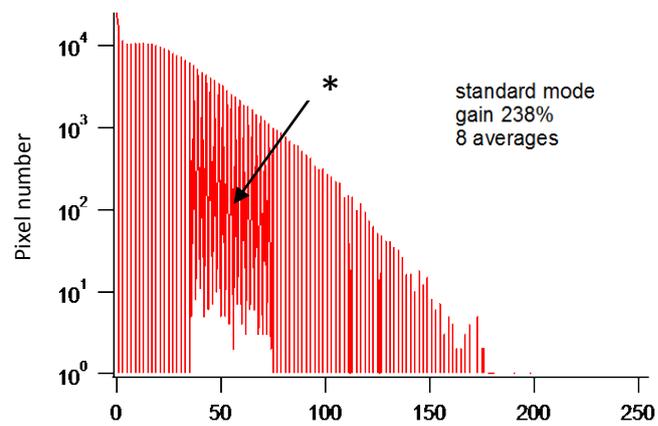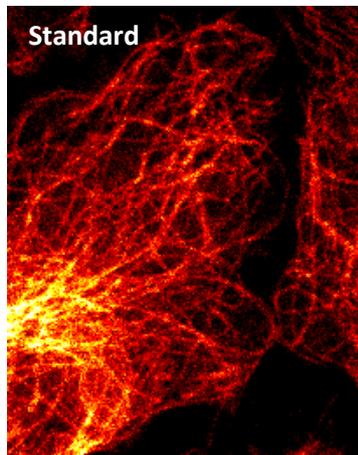

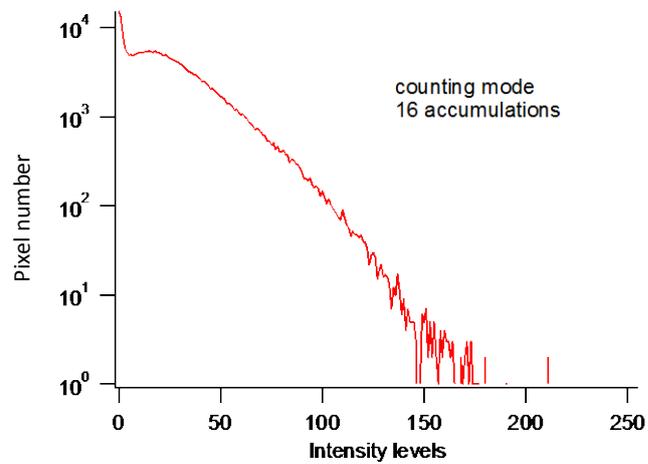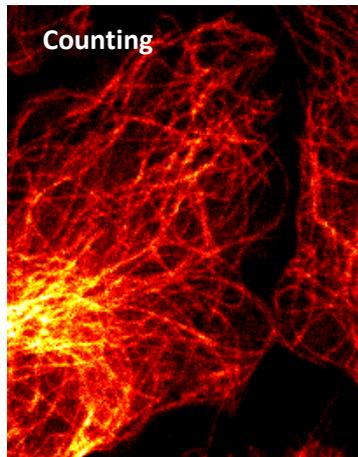*Fig 7* images and corresponding histogram obtained in a low signal situation. Comparison between the HyD counting and standard modes. Confocal SP8X - Excitation at 488nm, 0.2% laser power. Fixed detection bandwidth. In the standard mode, the digital gain was adjusted to obtain the same intensity range as in the counting mode.

* see the note at the end of the standard mode paragraph concerning the "artifacts".

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Using a too high gain can lead to information loss (misuse of the system): high gain value will lead to saturation of the 8bit dynamic range despite a low number of represented intensity levels! Users accustomed to using PMT could react by decreasing the laser power and consequently loose intensity levels!

In comparison with the HyD counting mode:

→You don't gain time by using the digital gain

→You don't obtain more information in the image; you don't extract more details from your specimen

→You don't obtain the best image quality if the averaging number is not well suited

→ The intensity signal is no longer the real photon count. Take care to take into account the gain factor in your quantifications analysis

→You take a risk to misuse the system. A too high gain will not allow a good optimization of the laser power (saturation of the 8bit range with only few intensity levels) resulting in a loss of information.

Standard mode – correction of the non-linearity

Reminder: in photon counting mode, the HyD is linear up to 60 MHz.
In standard mode, a calibration is applyed to ensure linearity up to 300 MHz (Fig.8) (i.e. beyond 256 levels at 400 Hz scan speed). That means that your signal is digitally corrected for count numbers (intensity levels) beyond the HyD linear range.
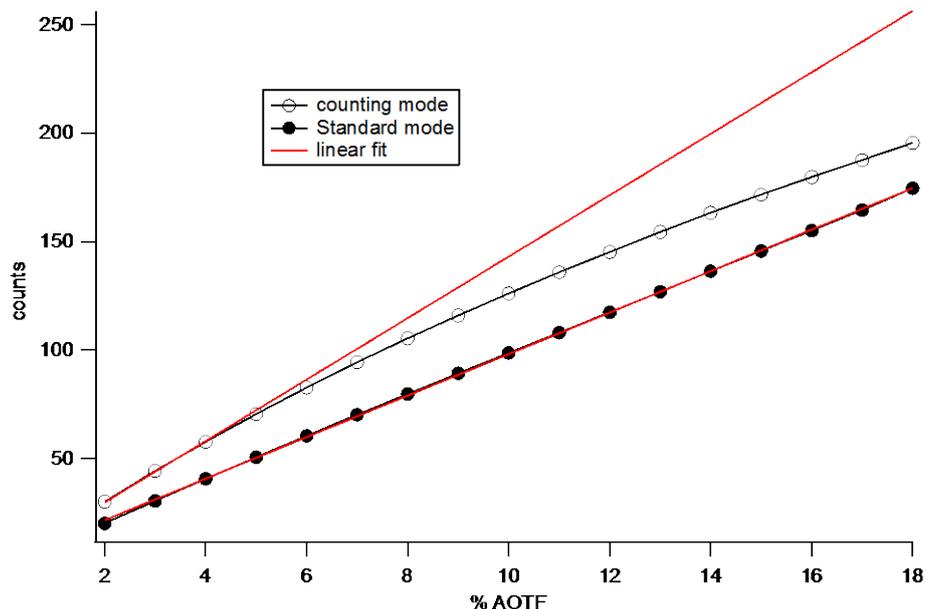

Fig. 8*: Variation of the photocount (grey level) depending on the laser power (% AOTF*) measured with HyD dector in the counting mode and in the standard mode (10% gain). SP8MP confocal microscope. Image format: 512x512 pixels, scan speed 400Hz.*

Note also that an additional correction factor is added to obtain an apparent intensity signal independent of the pixel dwell time. Change of the speed or the format will not have any effect anymore on the resulting apparent intensity (if the speed increases by a factor 2, the signal is multiplied by a factor 2).

In comparison with the HyD counting mode:

→ Corrections are introduced to compensate

- the intensity changes with the pixel dwell time/speed/format,

- the non-linearity of the HyD at high count rates.

→ for quantification applications, users doesn't risk anymore to underestimate the fluorescence intensity due to nonlinearity at high count rates. No need to check carefully that the max count rate is below the nonlinear threshold!

But keep in memory that the photon count doesn't directly depends on the fluorescence emission.

Using the HyD standard mode - Steps to optimize your acquisition

1- Set HyD in standard mode. Set your detection bandwidth and set a low laser %. (HyDs can be damaged if they receive a high intensity level! Always begin with a low laser power and increase the laser depending on the max count value)

2- In the live mode, display the histogram (Fig 2.)

3- Adjust the %laser (take care of avoiding photobleaching) and set the digital gain

2 possibilities for optimizing the image:

Possibility 1: Increase the gain to fill as much as possible the 8 bit range (bring the max value close to the 255 value). Perform averaging until the histogram becomes continuous (smooth, Fig 7 middle and bottom). Difficult to initially judge how many averages will be sufficient to obtain the best…

Possibility 2: put the gain at the minimal 10% value. This situation is closest to the counting mode with additional correction for the nonlinearity and pixel dwell time/format/speed. Perform accumulations to fill as much as possible the 8 bit range (bring the max value close to the 255 value on the brightest part of your sample, for example 200-220 is good enough, 240 would be almost perfect) in the same way than the counting mode (Fig3)
If the fluorescence is weak, perform accumulation to fill the 8bit range and to obtain a sufficient signal to noise ratio
(ex: max value = 60 you can accumulate 4 times to reach 60x4= 240 resulting counts, value close to the max level with a 8bit range).

Note: check your accumulation factor for each acquisition: too many accumulations can result in saturation (max value exceeding the 255 max level in the 8bit range). To avoid saturation keep the max resulting value below 255 or change for the 12 bit range to extend saturation level at 4095.
For situations where low and high levels of fluorescence coexist, 12bit range will also be chosen to allow accumulation and highlighting of the low signal without saturation in the intense areas.

The procedure can also be apply for quantification applications. The non-linearity of the HyD is corrected (no risk to enter the nonlinear range and underestimate the fluorescence signal). Users just have to consider their gain settings.

In conclusion:

To obtain high apparent quality images, standard mode and counting mode are almost equivalent if the imaging conditions are well suited. In practice, optimizing image in the counting mode is more straightforward.

Imaging for quantification can be facilitate with the standard mode as the non-linearity of the HyD is corrected (no risk to enter the nonlinear range and underestimate the fluorescence signal)

In comparison with the basal counting mode, standard mode manipulates digitally the raw signal from the detector (digital gain, numeric correction for the non-linearity, pixel dwell time compensation).

Corrections give rise to small artifacts (see below). However they should have a negligible effect on the quantification.

Standard mode artifacts:

1- Counting and standard mode (10% gain) comparison

Fewer levels are represented in the images in standard mode 10% gain (resulting in a lower mean intensity in the image) in comparison with the counting mode (Fig 9). This result depends on the microscope (some systems exhibit an intensity increase).

In the specific case below, the digital manipulation and correction factors introduce an alternation of high and low count values!
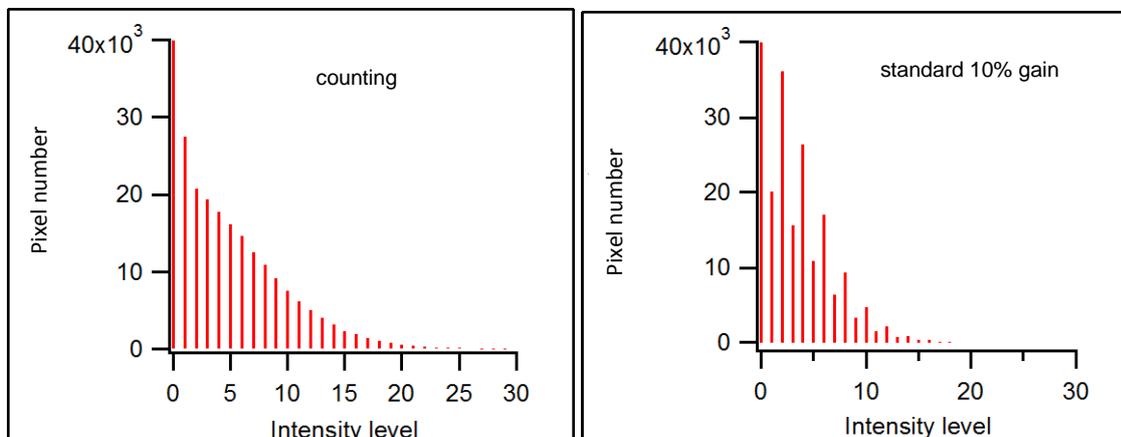

Fig9: Counting and standard mode (10% gain) comparison. Same conditions (laser, detection bandwidth, no average, no accumulation) for both acquisitions.

2 – Standard mode creates a number of artefactual intensity levels.

In comparison with the "normal" continuous histogram obtained in the counting mode, increasing the digital gain to fill the dynamic range in the standard mode gives rise to some artefactual levels (Fig10 and fig7 - middle histogram).
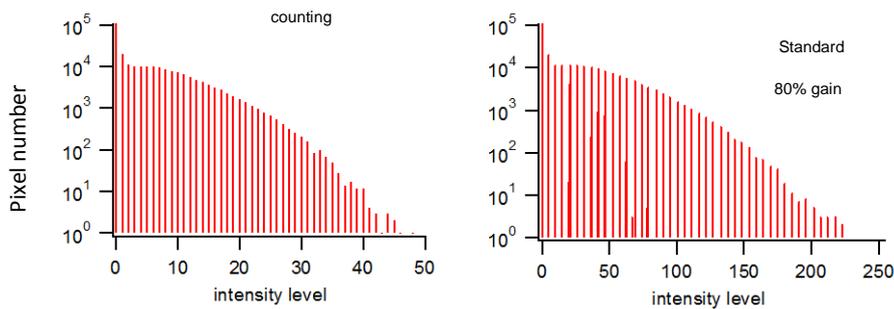
Fig10: comparison of histograms obtained in the counting and in the standard mode (80% gain). Same conditions (laser, detection bandwidth, no average, no accumulation) for the 2 acquisitions. 7 artefactual levels appear in the standard mode.

These levels are negligible as they rarely occur (1/1000 less represented than the neighboring levels). They are caused by the mathematical manipulation of the raw data when applying corrections (decimal intensity values resulting from the corrections are rounded). Thus the acquisition is not very "clean" but these levels will not make a quantification flawed.

In practice: Counting mode or Standard mode?

In practice, it appears more straightforward to optimize the image in the basal counting mode, without any manipulation of the raw data! If you don't need quantification, just use the counting mode!

For users who need quantification and who are not able to insure that the whole experiment will respect the linear threshold (for example if you expect a change of intensity during time..), the preferable protocol would be to use the standard mode with the minimal gain!

HyD BrightR mode

BrightR mode is an option for brightness reinforcement to use in the situation where fluorescent labels are not distributed homogenously. It allows visualizing both weak and strong signals using Specimens that exhibit very bright structures in one part and low signals in others part are difficult to image: the weak part stay almost invisible while the brighter parts give rise to saturation.

BrightR mode give the possibility to amplify (numerically) dim structures more than bright ones.
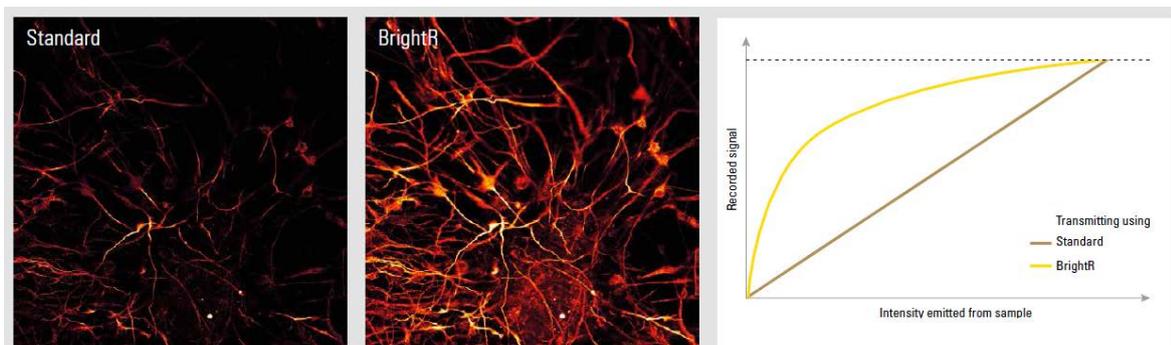
Of course, this mode can be useful to highlight details but make quantification analysis impossible!

Fig from Leica HyD brochure