Table of contents

I. INTRODUCTION

The photometric calculator is a tool that allows to compute extremely important photometric metrics to know for quantitative imaging and reproducibility, for four types of acquisition mode: wide-field, confocal laser scanning, confocal spinning disk and two-photon laser scanning microscopy. The computed photometric metrics are the peak power, the irradiance, the dose and the photon flux.

The photometric calculator also aims at being a pedagogic tool to help understanding how the illumination settings (average power, wavelength, objective, etc.) influence the photometric metrics, and how the different acquisition modes (wide-field, confocal, etc.) can give rise to photometric metrics that can be different from several orders of magnitude for the same input parameters.

II. MICROSCOPY ACQUISITION MODES

A sketch of the four different microscopy acquisition modes in the “Photometric calculator” is shown in Figure 1, and their main intrinsic differences are summarized in Table 1.

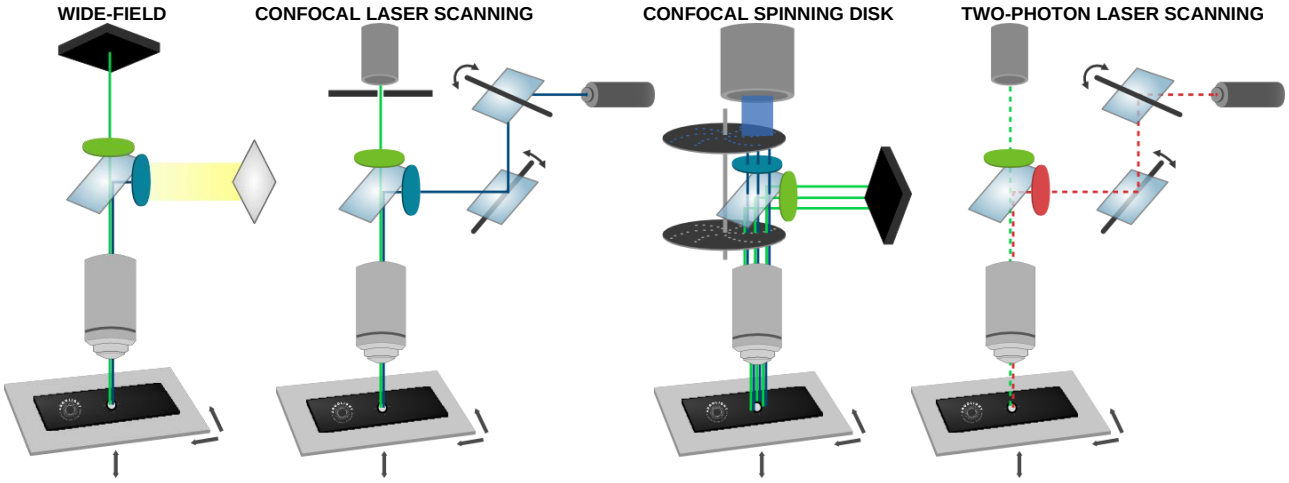


Figure 1: Sketches of the different microscopy acquisition modes. From left to right: wide-field, confocal laser scanning, confocal spinning disk and two-photon laser scanning microscope.

- In a ***wide-field*** microscope, the sample is illuminated by a wide spot, the fluorescence is detected by a camera, so that about 10^6 pixels are illuminated/recorded in parallel.

- In a ***confocal laser scanning*** and a ***two-photon laser scanning*** microscope, the sample is illuminated by a focused spot that is swept with scanning mirrors, the fluorescence is detected by a point detector, so that only 1 pixel is illuminated/recorded at a time.

- In a ***confocal spinning disk*** microscope, the sample is illuminated by about a thousand of focused spots, the fluorescence is detected by a camera, so that about 10^3 pixels are illuminated/recorded in parallel.

Microscopy acquisition mode	Light source type	Illumination shape at sample	Detector type	Number of pixels illuminated
Wide-field	Continuous lamp or LED	1 wide spot	Array detector	~ 10^6 pixels in parallel
Confocal laser scanning	Continuous laser or LED	1 focused spot	Point detector	1 pixel at a time
Confocal spinning disk	Continuous laser	~ 10^3 focused spots	Array detector	~ 10^3 pixels in parallel
Two-photon laser scanning	Pulsed laser	1 focused spot	Point detector	1 pixel at a time

Table 1: Main intrinsic differences between the acquisition modes. A camera is an example of array detector. A photomultiplier tube is an example of point detector.

Because of these intrinsic differences, the photometric metrics calculated from the same input parameters (illumination power, objective, wavelength) will be highly distinct.

III. INPUT PARAMETERS

1. LIGHT SOURCE FEATURES

1- Average power at sample location

The average power at sample location is the amount of average power coming out the exit pupil of the objective, illuminating the sample. It is expressed in Watts (W) and shall be measured with a power meter.

2- Illumination wavelength

The illumination wavelength is the wavelength of the light source. It is expressed in nanometers (nm) and can be measured with a spectrometer. If you do not have access to a spectrometer, then use the value provided by the manufacturer.

3- Repetition rate

The repetition rate is the number of pulses delivered by the light source per unit of time. It is expressed in Megahertz (MHz) and can be measured with a fast photodiode connected to a high bandwidth oscilloscope. If you do not have access to a fast photodiode and a high bandwidth oscilloscope, then use the value provided by the manufacturer. It applies only to two-photon laser scanning microscopes, equipped with pulsed lasers.

4- Pulse duration at sample location

The pulse duration at sample location is the time width of the pulses delivered by the light source. It is expressed in femtoseconds (fs) and shall be measured with an auto-correlator, as its full width at half-maximum (FWHM). It applies only to two-photon laser scanning microscopes, equipped with pulsed lasers.

5- Number of illumination spots at sample location

The number of illumination spots at sample location is the number of light spots hitting the sample simultaneously. It is different from one only for confocal spinning disk microscopes. In this case, ask you microscope manufacturer to get this information; it is typically around 1000.

2. OBJECTIVE FEATURES

1- Field number

The field number of the objective is the diameter of the field of view at the intermediate image plane. It is expressed in millimeters (mm) and is provided by the manufacturer.

Note: Sometimes, the field number *FN* and the field of view *FOV* can be mixed-up. They are related through the following equation: $FOV = FN/Mag$.

2- Magnification

The magnification is the enlargement factor of the objective. It is unitless and is provided by the manufacturer.

3- Numerical aperture

The numerical aperture is the range of angles over which the objective can accept or emit light. It is unitless and is provided by the manufacturer.

3. TIME FEATURES

1- Exposure time

The exposure time is the time over which the sample is exposed to acquire an image with a camera. It is expressed in milliseconds (ms) and can be found in the image acquisition software and/or the image metadata. It applies to wide-field and confocal spinning disk microscopes, equipped with cameras.

2- Pixel dwell time

The pixel dwell time is the time over which the focused spot dwells a surface equivalent to a pixel. It is expressed in microseconds (µs).
For confocal laser scanning and two-photon laser scanning microscopes, equipped with scanning mirrors and point detectors, the pixel dwell time can be found in the image acquisition software and/or the image metadata.
For confocal spinning disk microscopes, equipped with spinning disks and cameras, the notion of pixel dwell time, strictly speaking, is not relevant. However, we can define a “pseudo” pixel dwell time, that could be compared to the pixel dwell time of laser scanning microscopes, given by the following equation:

$$Pseudo\ pixel\ dwell\ time\ (\mu s) = 10^3 \times \left[\frac{Pinhole\ diameter\ (\mu m)}{Pinhole\ spacing\ (\mu m)}\right]^2 \frac{Exposure\ time\ (ms) \times 1\ fps}{Disk\ frame\ rate\ (fpr) \times Disk\ rotation\ speed\ (rpm)/60}$$

3- Disk rotation speed

The disk rotation speed is the number of disks revolution per unit of time. It is expressed in revolutions per minute (rpm). It applies only to confocal spinning disk microscopes, equipped with spinning disks.

4- Disk frame rate

The disk frame rate is the number of images per disk revolution. It is expressed in frames per revolution (fpr). It applies only to confocal spinning disk microscopes, equipped with spinning disks.

4. SPATIAL FEATURES

1- Pinhole diameter

The pinhole diameter is the diameter of the confocal pinhole. It is expressed in micrometers (µm). If you know the pinhole diameter in Airy unit (AU), you can convert it into micrometers (µm) through the following relation:

$$Pinhole\ diameter\ (\mu m) = Pinhole\ diameter\ (AU) \times Mag \times \frac{1.22 \times \lambda_{em}}{NA}$$

Where λ_{em} is the emission wavelength (µm), *Mag* the objective magnification (unitless) and *NA* the objective numerical aperture (unitless).

2- Pinhole spacing

The pinhole spacing is the distance between two adjacent pinholes of a spinning disk. It is expressed in micrometers (µm). It applies only to confocal spinning disk microscopes, equipped with spinning disks.

3- Theoretical illuminated surface

For wide-field microscopes, the theoretical illuminated surface, expressed in square micrometers (μm^2), of the illumination circular area is given by the following formula:

$$S_{WF} = 10^6 \times \pi \times \left(\frac{FN}{2 \times Mag}\right)^2$$

Where *FN* is the objective field number (mm) and *Mag* the objective magnification (unitless).

For confocal laser scanning, confocal spinning disk and two-photon laser scanning microscopes, the theoretical illuminated surface, expressed in square micrometers (μm^2), of the focused laser beam is given by the following formula:

$$S_{CLS} = S_{CSD} = S_{2PLS} = 10^{-6} \times \pi \times \left(\frac{0.515 \times \lambda_{exc}}{NA}\right)^2 \times \frac{1}{4 \times ln(2)}$$

Where λ_{exc} is the excitation wavelength (nm) and *NA* the objective numerical aperture (unitless). The factor $4 \times ln(2)$ comes from the fact that the spatial distribution of the laser beam is considered as Gaussian.

IV. PHOTOMETRIC METRICS

1. PEAK POWER

The peak power, also called instantaneous power, is the amount of energy concentrated inside a time duration. It is expressed in Watts (W). It concerns only 2-photon laser scanning microscopes, equipped with lasers delivering short pulses in which the energy is concentrated. The peak power and the energy are related through the following general formula:

$$Energy = \int_0^{Pulse\ duration} Power(t)\ dt$$

For two-photon laser scanning microscopes, equipped with pulsed lasers, the irradiance is given by:

$$Peak\ power\ (W) = 10^6 \times 4 \times \sqrt{\frac{ln(2)}{\pi}} \times \frac{Average\ power\ (mW)\ at\ sample\ location}{Pulse\ duration\ (fs) \times Repetition\ rate\ (MHz)}$$

The factor $4 \times \sqrt{ln(2)/\pi}$ comes from the fact that the temporal shape of the laser pulse is considered as Gaussian.

2. IRRADIANCE

The irradiance, also called surface power density, is the amount of power (average or peak) distributed over a surface area. It is expressed in Watts per square centimeter (W.cm^{-2}). The irradiance and the power are related through the following general formula:

$$Power = \int_0^{Surface} Irradiance(x;y)\ dS$$

For wide-field, confocal laser scanning and confocal spinning disk microscopes, equipped with continuous light sources, the irradiance is given by:

$$Irradiance\ (W.cm^{-2}) = 10^5 \times \frac{Average\ power\ (mW)\ at\ sample\ location}{Surface\ (\mu m^2) \times Number\ of\ illumination\ spots\ at\ sample\ location}$$

For two-photon laser scanning microscopes, equipped with pulsed lasers, the irradiance is given by:

$$Irradiance\ (W.cm^{-2}) = 10^8 \times \frac{Peak\ power\ (W)\ at\ sample\ location}{Surface\ (\mu m^2) \times Number\ of\ illumination\ spots\ at\ sample\ location}$$

3. DOSE

The dose, also known as the dosage rate, is the amount of irradiance received over an illumination duration. It is expressed in Joules per square centimeters (J.cm^{-2}). The dose and the irradiance are related through the following general formula:

$$Dose = \int_{0}^{Illumination\ duration} Irradiance(t)\ dt$$

For wide-field microscopes, the dose is given by:

$$Dose\ (J.cm^{-2}) = 10^{-3} \times Irradiance\ (W.cm^{-2}) \times Exposure\ time\ (ms)$$

For confocal laser scanning, confocal spinning disk and two-photon laser scanning microscopes, the dose is given by:

$$Dose\ (J.cm^{-2}) = 10^{-6} \times Irradiance\ (W.cm^{-2}) \times [Pseudo]\ pixel\ dwell\ time\ (\mu s)$$

4. PHOTON FLUX

The photon flux is the amount of irradiance per photon energy, *i.e.* the number of photons per unit of time per unit of surface, at a given wavelength. It is expressed in photons per second per square centimeter (photons.s^{-1}.cm^{-2}). The photon flux and the irradiance are related through the following general formula:

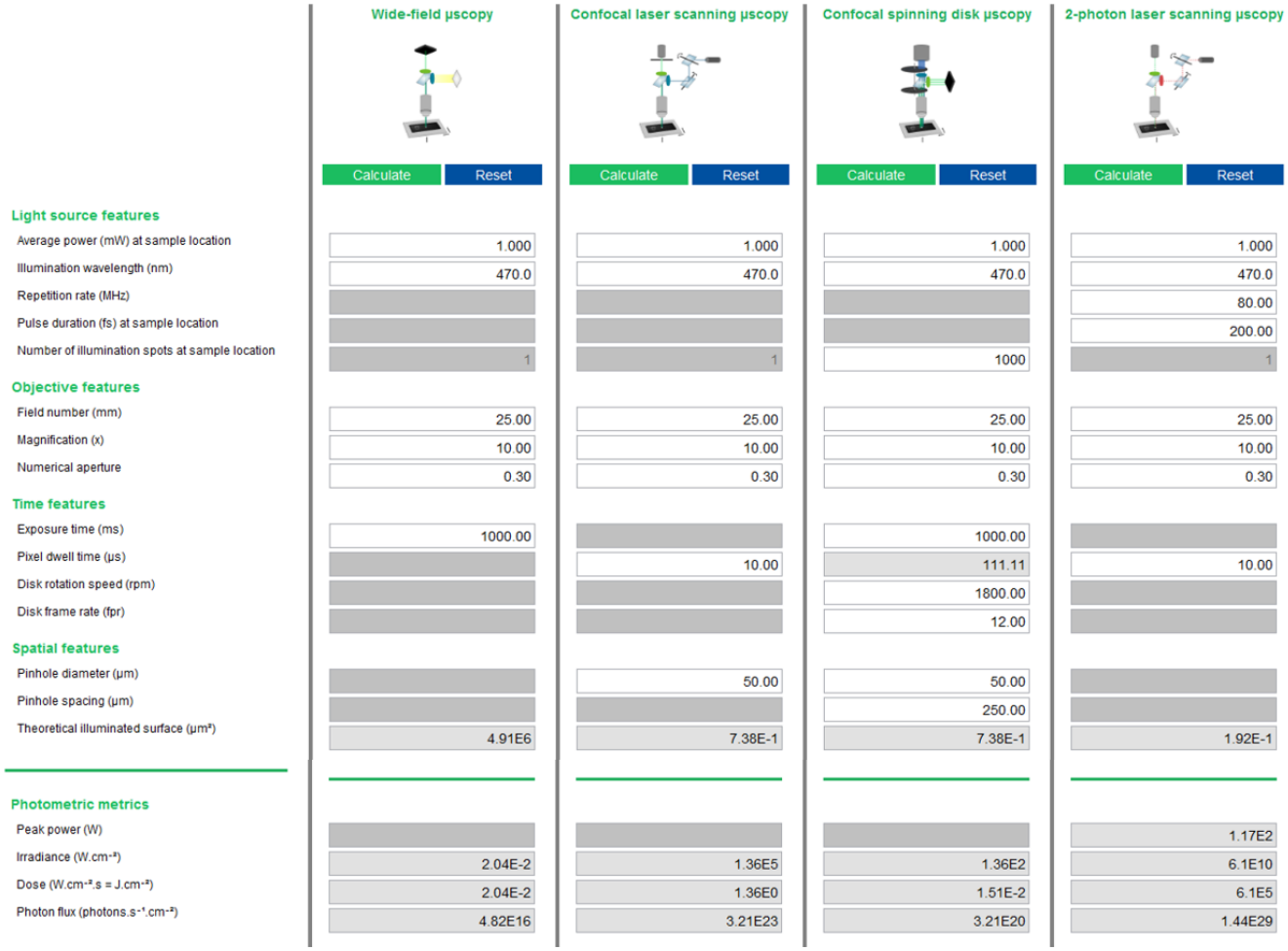
$$Photon\ flux = \frac{Irradiance}{Photon\ energy}$$

$$Photon\ flux\ (photons.s^{-1}.cm^{-2}) = \frac{Irradiance\ (W.cm^{-2}) \times \lambda_{exc}}{h \times c}$$

Where λ_{exc} is the excitation wavelength (nm), h the Planck constant, equal to 6.626e^{-34} J.s and c the light vacuum speed, equal to 29979245800 cm.s^{-1}.

V. EXAMPLE AND GUIDELINE

An example of photometric metrics calculation is displayed in Figure 2 for the four microscopy acquisition modes with the same following input parameters:
- Average power at sample location: 1 mW
- Illumination wavelength: 470 nm
- Objective: 100×/1.4
- Exposure times and pixel dwell times that provide about the same time to acquire an image: 25 ms and 1 µs, respectively.



	Wide-field µscopy	Confocal laser scanning µscopy	Confocal spinning disk µscopy	2-photon laser scanning µscopy
	Calculate / Reset	Calculate / Reset	Calculate / Reset	Calculate / Reset
Light source features				
Average power (mW) at sample location	1.000	1.000	1.000	1.000
Illumination wavelength (nm)	470.0	470.0	470.0	470.0
Repetition rate (MHz)				80.00
Pulse duration (fs) at sample location				200.00
Number of illumination spots at sample location	1	1	1000	1
Objective features				
Field number (mm)	25.00	25.00	25.00	25.00
Magnification (x)	10.00	10.00	10.00	10.00
Numerical aperture	0.30	0.30	0.30	0.30
Time features				
Exposure time (ms)	1000.00		1000.00	
Pixel dwell time (µs)		10.00	111.11	10.00
Disk rotation speed (rpm)			1800.00	
Disk frame rate (fpr)			12.00	
Spatial features				
Pinhole diameter (µm)		50.00	50.00	
Pinhole spacing (µm)			250.00	
Theoretical illuminated surface (µm²)	4.91E6	7.38E-1	7.38E-1	1.92E-1
Photometric metrics				
Peak power (W)				1.17E2
Irradiance (W.cm⁻²)	2.04E-2	1.36E5	1.36E2	6.1E10
Dose (W.cm⁻².s = J.cm⁻²)	2.04E-2	1.36E0	1.51E-2	6.1E5
Photon flux (photons.s⁻¹.cm⁻²)	4.82E16	3.21E23	3.21E20	1.44E29

Figure 2: Example of photometric metrics calculated with the same input parameters for the four different acquisition modes.

One can see that the irradiance, dose and photon flux for each acquisition mode are different from several orders of magnitude (*cf.* Figure 2 and Table 2).

In terms of irradiance and photon flux, the wide-field microscope has the lowest value, followed by the confocal spinning disk, the confocal laser scanning and the two-photon microscopes.

In terms of dose, yet not intuitively, the confocal spinning disk microscope has the lowest value, followed by the wide-field, the confocal laser scanning and the two-photon microscopes.

Both the wide-field and the confocal spinning disk microscopes have much lower photo-toxicity than the confocal laser scanning microscope, that is itself less photo-toxic than the two-photon laser scanning microscope.

Microscopy acquisition mode	Irradiance ($W.cm^{-2}$)	Dose ($J.cm^{-2}$)	Photon flux ($photons.s^{-1}.cm^{-2}$)
Wide-field	2.04×10^{0}	5.09×10^{-2}	4.82×10^{18}
Confocal laser scanning	2.95×10^{6}	2.95×10^{0}	6.99×10^{24}
Confocal spinning disk	2.64×10^{3}	2.64×10^{-3}	6.24×10^{21}
Two-photon laser scanning	1.33×10^{12}	1.33×10^{6}	3.15×10^{30}

Table 2: Comparison of the photometric metrics calculated for the four different acquisition modes, with the following input parameters: 1 mW of average power at sample location, 470 nm illumination wavelength and 100×/1.4 objective.

From this example, one can notice that it is not sufficient to provide only the average power at sample location, nor only the irradiance, the dose or the photon flux. We recommend providing, in any publication that claims to perform quantitative imaging and aims at being reproduced, the measured average power at sample location, the irradiance and the dose as photometric metrics.

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