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Illumination Power, Stability, and Linearity Measurements for Confocal, Widefield and Multiphoton Microscopes

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Disclaimer

This protocol was developed by the members of **Working Group 1 "Illumination Power"** of the international consortium QUAREP-LiMi.

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This protocol collection has undergone the internal approval process of QUAREP-LiMi.

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Abstract

To obtain accurate, reproducible, and interpretable data when conducting imaging experiments, it is critical to consider external factors affecting data acquisition at various steps of the experimental workflow. Illumination power and stability represent two critical factors, especially when comparing fluorescence intensities between images during a time-lapse experiment or experiments performed at different times or on different microscopes.

The fluorescence signal can be generated by different types of light sources. These light sources and their coupling elements (e.g., fibers) can display varying performances over time as they age, move, or as environmental conditions change.

Unfortunately, microscope users can often only set illumination power as a percentage of its maximal output and may, therefore, not be aware of potential performance changes. It is important to recognize that a set percentage will not always yield the same illumination power in Watts at the objective over the course of an experiment, not to mention between days or systems. This means that selecting for example 10% output may lead to different experimental results over time or even between two microscopes of the same model. In addition to illumination stability, working within the linear range of the illumination power allows to adjust accurately the illumination power absolute value (in mW) using a fraction (or %) of its maximal value through the imaging software.

If you are responsible for system maintenance, routinely measuring the illumination power, stability, and linearity over time can help you detect issues that affect the integrity of the system and thus the reproducibility of an experiment.

This protocol describes how to measure the stability and linearity of the illumination power using calibrated external power sensors. This protocol is intended for confocal systems (raster scanning and spinning disks), Multi-photon systems (used for 2P- or 3P-imaging, SHG-imaging, etc.), and widefield systems. It represents the collective experience of over 50 imaging scientists.

Image Attribution

Quality Assessment and Reproducibility for Instruments & Images in Light Microscopy logo designed by Thao Do.

Guidelines

Laser safety and regulations

- Please refer to the documentation provided by the manufacturer for additional warnings and preventive protective equipment (PPE) requirements (e.g. laser safety goggles). Always consult with your local Laser Safety Officer or Radiation Safety Officer and refer to your laboratory safety documentation for more information.
- You can also consult your Laser Safety Standards ANSI Z136 in North America, SUVA 66049.D in Europe, and BS EN 60825-1 in the UK. Additionally, laser safety standards and regulations are covered by IEC norm 60825-1 and LED eye safety standards and regulations are covered by IEC norm 62471 in Europe.

Materials

1. Raster scanning, spinning disk confocal, wide field or multi-photons microscopes.
2. Objective (e.g. 10x)
3. Power meter and sensor (e.g., Thorlabs power controller and slide sensor or Argolight Argo-Power Slide)

Examples of equipment used to test this protocol:

Equipment

Microscope Slide Thermal Power Sensor	NAME
High-Power Microscope Slide Power Meter Sensor Head	TYPE
Thorlabs	BRAND
S175C	SKU
https://www.thorlabs.com/thorproduct.cfm?partnumber=S175C	LINK

PNG

Equipment

Handheld Optical Power and Energy Meter Console with Multi-Touch Technology	NAME
Power Meter	TYPE
Thorlabs	BRAND
PM400	SKU
https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=10562	LINK

PNG

Equipment

Thermal Power Sensor NAME

Thermal Sensor for high power and pulsed lasers (multiphotons) TYPE

Thorlabs BRAND

S370C SKU

https://www.thorlabs.com/thorproduct.cfm?partnumber=S370C LINK

Equipment

Laser Power Meter PowerMax & Field Mate NAME

Thermal Power Sensor TYPE

Coherent PowerMax BRAND

1097901 SKU

https://www.coherent.com/laser-power-energy-measurement/meters LINK

Model PM10 SPECIFICATIONS

Equipment	
ArgoPower	NAME
Power Meter	TYPE
Argolight	BRAND
argo-power	SKU
https://argolight.com/products/argo-power/	LINK

PNG

Safety warnings

- Hazardous, visible or invisible radiation from lasers, lamps, and other light sources used for microscopy **can cause permanent damage to the retina, skin burns and fire**. Always follow proper laser safety protocols for your equipment and situation.

Before start

Safety information

Before you take any measurements, it is important to familiarize yourself with all laser safety rules and requirements to avoid eye and skin exposure to scattered or direct radiation.

Illumination power measurement

1 Select a low NA, air objective, such as a 10x objective, for this measurement. Ensure it is clean and presents no signs of damage.

Note

This protocol can be used with other objective magnifications if necessary. Be aware that different objective types may have different transmission efficiencies; thus, always compare results from the same objective. This also means that the power detected with the 10x objective may be very different from the power received by the sample when imaged with a higher NA immersion lens.
Also, many power meter sensors (with diffuser) measure different power levels depending on the beam collimation, i.e., the numerical aperture of the objective (see **Power meter Q&A Appendix 1 Q1&2**). Ensure that the objective used can also withstand high illumination powers at the required wavelength if the measurement needs to be made in such a regime.

Widefield systems
In widefield microscopes, the light entering at the back of the objective is not collimated. Therefore, we highly recommend to always perform this protocol with an objective.

Confocal systems
In confocal microscopes, the light entering at the back of the objective is collimated. Therefore, this protocol can also be performed without any objective, provided that the user operates the system in full awareness of laser safety and security standards.

Multiphoton systems
Each objective may induce a different dispersion of the excitation peak. Power measurement, therefore, does not fully reflect the efficiency of the excitation in the sample.

2 Warm up the entire system, including the illumination source.

Note

Warm-up time
The time required for a given system can be determined empirically by measuring how much time is required for illumination power to reach stability (see **Figure 1** for an example with a laser-based system). Monitoring warming-up time should be performed right after the installation of a new system and regularly afterwards, as changes to the warm-up time are also an indicator of laser ageing. Also, make sure the environment meets the operating specifications of the equipment (especially air renewal or cooling around the lasers and the scope, consult **Appendix 3**).

Laser (continuous wavelength) illumination
The recommended standard warming-up time for typical lasers is one hour. However, gas lasers may need more time to warm up than solid-state or diode lasers. For laser-specific recommended warm-up time, refer to the product documentation.

LED illumination
In most cases, stabilization should be reached within a couple of minutes.

Arc lamps illumination (Xenon, Metal Halide, etc.)
It's hard to predict the behavior of the wide variety of systems and technologies available here. We highly recommend measuring the behavior, as shown in **Figure 1**.

Multiphoton systems with a Ti-Sapphire laser
If the laser is used regularly, it is best to always leave it on stand-by; warm-up will be faster. If the laser is off (cold start), multiple hours may be required to reach stability, and Stabilization time should be determined empirically. Also, some power modulators (e.g., Pockels cells) are temperature sensitive, so one should let the laser light pass through for some time to bring these devices to equilibrium.

Expected result

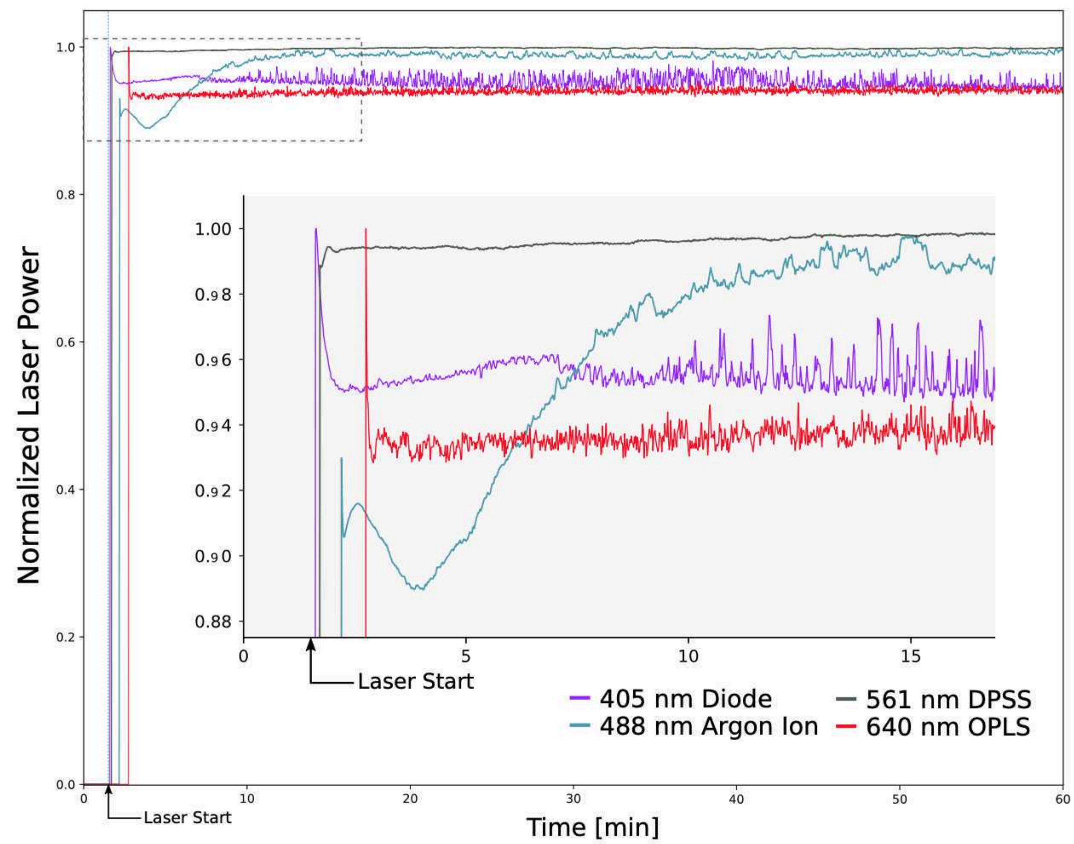


Figure 1. Examples of data collected for laser warm-up and short-term illumination power stability. The confocal microscope was not pre-warmed to illustrate the fluctuation of illumination power during this crucial preparation step. All laser intensities were controlled with an AOTF (acousto-optic tunable filter) and the laser power was measured at the objective with a PM100A power meter and a S170C sensor (Thorlabs). The inset (greyed out) shows the first 25 min with greater details.

3 Place the power meter sensor (choose the right sensor for your light source, see Note) in front of the objective. Make sure the sensor is fixed (in a stage holder) and stable during the measurement. Since the sensor can reflect the incident light, avoid orientating the sensor in such a way that it could reflect the light directly to your eyes, skin or any other reflecting surfaces.

Note

Be very careful which power sensor you use! (consult our **Power meter Q&A Appendix 1 Q11**).

Choose an appropriate power meter sensor:

1. for your light source power: too high powers may destroy the sensor, **check damage threshold in the sensor specifications**.
2. for your laser type: Typically, continuous wavelength lasers are measured with Photodiodes, whereas for pulsed lasers multiphoton applications, if the power at the objective is unknown, we recommend using a higher-power thermal sensors .

We recommend the use of a sensor with the shape and size of a slide, these are easier to fix on classical sample holders. For other sensor shapes, it might be possible to 3D print or have a workshop make a special holder. Examples of stage holders for different power sensors are shown in **Figure 2 and Figure 3**. We recommend higher-power thermal sensors be used for multiphoton light sources (see examples under Materials). Using the wrong type of sensor for multiphoton light sources may destroy the sensor due to the very large power input.

When the sensor is designed for it, an immersion medium may be used.

The operator should not hold or touch the sensor during the measurement, especially thermo-sensors, which should be shielded from any airflow, as they are susceptible to environmental changes (see **Appendix 3: Impact of environmental factors**).

Expected result

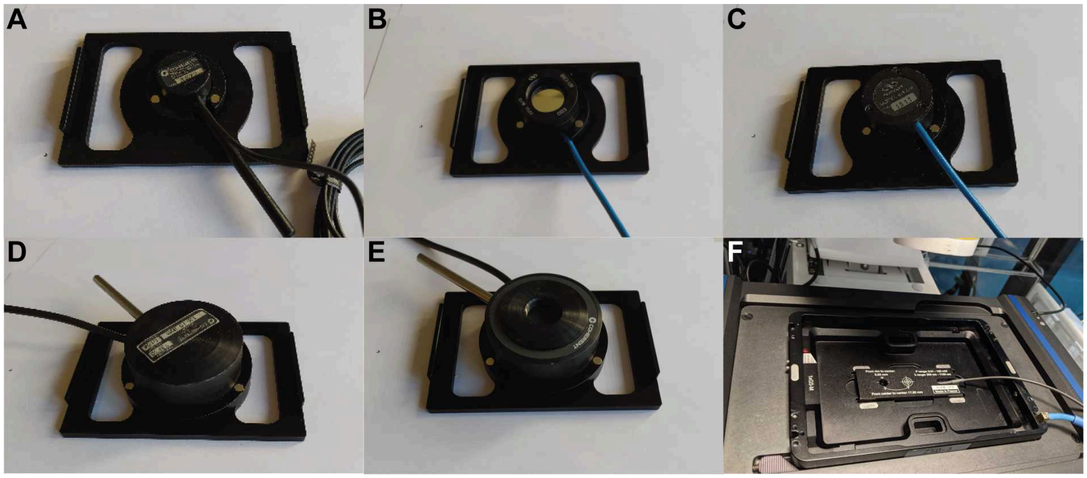


Figure 2. Examples of power sensor holders. Computer Numerical Control (CNC) machined holders in multi-well size for use in upright or inverted microscope configuration for A) inverted and B) upright Coherent LM-2 VIS and C) inverted Newport 840 sensor model 818C, D) inverted, and E) upright Coherent LM-10 HT power sensors and F) commercial slide holder (Oko Lab) for the Argo-Power (Argolight) power meter sensor. Respective CAD files for these holders are available on the QUAREP website (https://github.com/QUAREP-LiMi/WG1).

4 Center the illumination on the sensor. Avoid using the corners or the very periphery of the sensor for the measurement.

Note

The illuminated area on the sensor should be at least $1mm^2$ to avoid local variations within the sensor. If a microscope objective is used, do not focus on the sensor as the focal spot might be too concentrated and damage the sensor and/or yield inaccurate readings (see **Power meter Q&A Appendix 1 Q14**).

No variation in measurements is expected on larger sensors over most of their surface, except at the very periphery and in corners. The illumination is sufficiently centered on the sensor when moving the stage in XY or Z (focus) slightly does not change the measured value. If no objective lens is used, the position of the power meter in the axial direction does not impact the measurement on raster scanning and spinning-disk confocals, as long as the illumination does not spill outside of the sensor area.

On inverted microscopes, when the sensor has a cross mark on its back indicating the center of the sensing area, transmitted light can be used to center the sensor on the illumination axis (see **Figure 3**).

Expected result

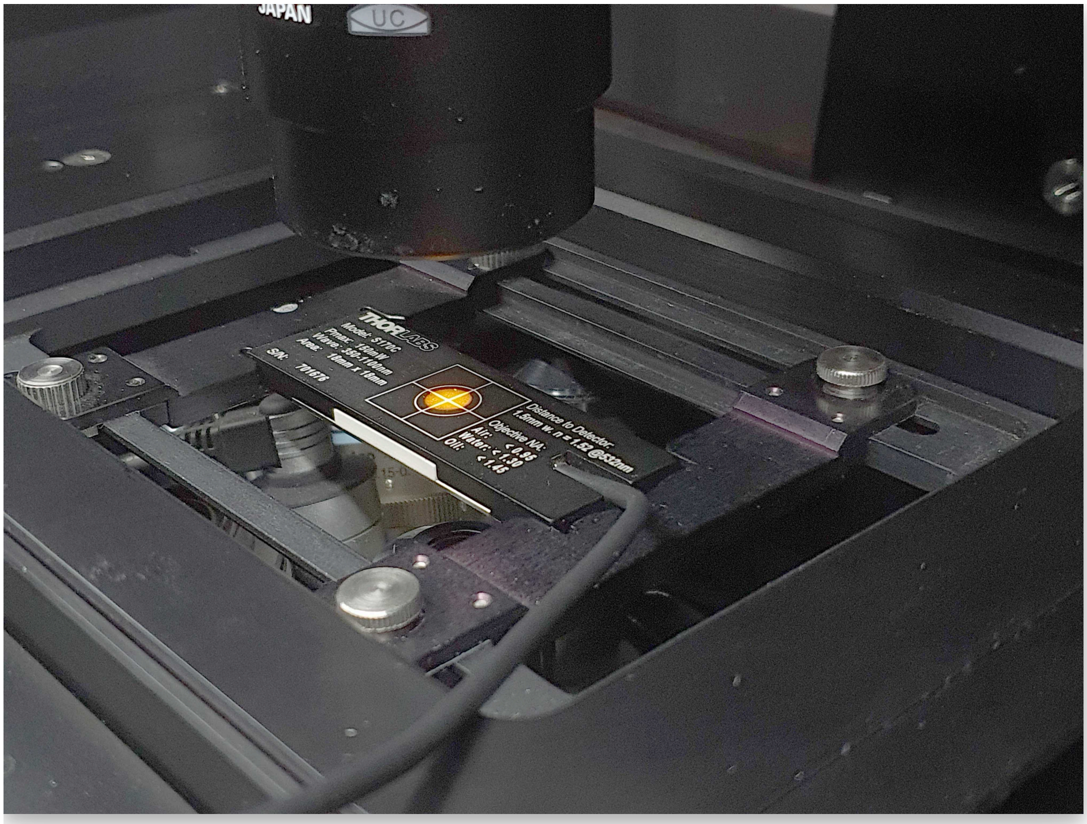


Figure 3. Slide power meter sensor mounted on the stage of an inverted microscope and aligned to the center of the sensing area using transmitted light and the cross mark located at the back of the sensor.

5 Select the wavelength of interest on the power meter control panel (see examples in **Figure 4**).

Note

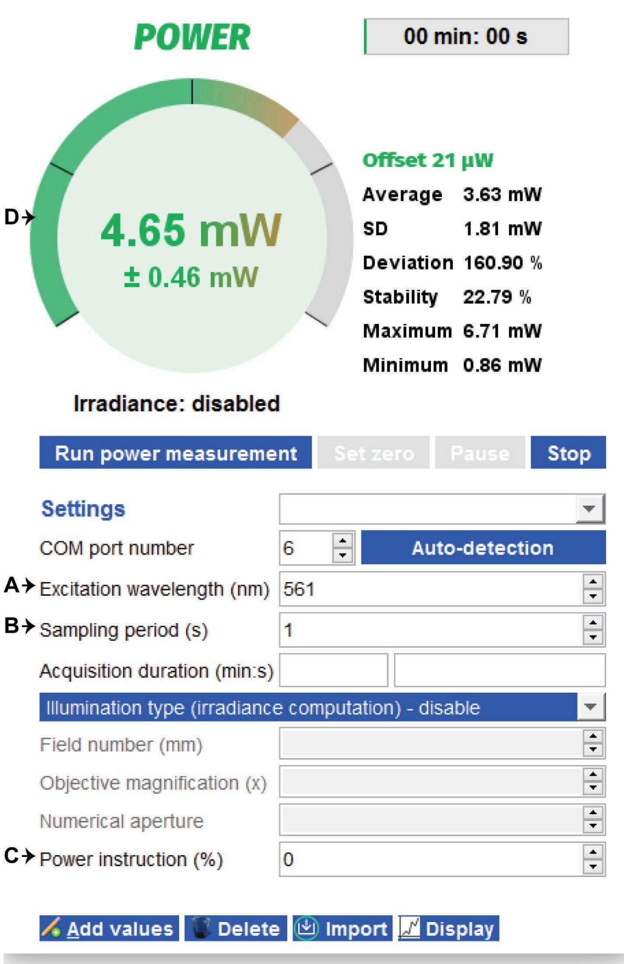
The measurement has to be done for each selected wavelength along with the chosen filter sets used for the imaging experiments.

Broad-band light sources
For broadband illuminations, like LEDs or other lamps in combination with excitation filters, select the wavelength in the middle of the bandpass of the excitation filter (see **Power meter Q&A Appendix 1 Q19** for explanations).

Non-tunable lasers
Select the same wavelength on the power meter as the laser.

Tunable lasers
Choose the wavelength used for your experiment, or select a series of wavelengths (i.e.,3 to 4) over the tunable range of your laser, as power may decrease in one part of the spectrum while another wavelength may still give consistent results over time.
Many tunable lasers have a display with the nominal power for a given wavelength. We recommend saving this value along the one measured at the objective, as a divergence between the two over time may reveal alignment or beam shape issues.

Expected result



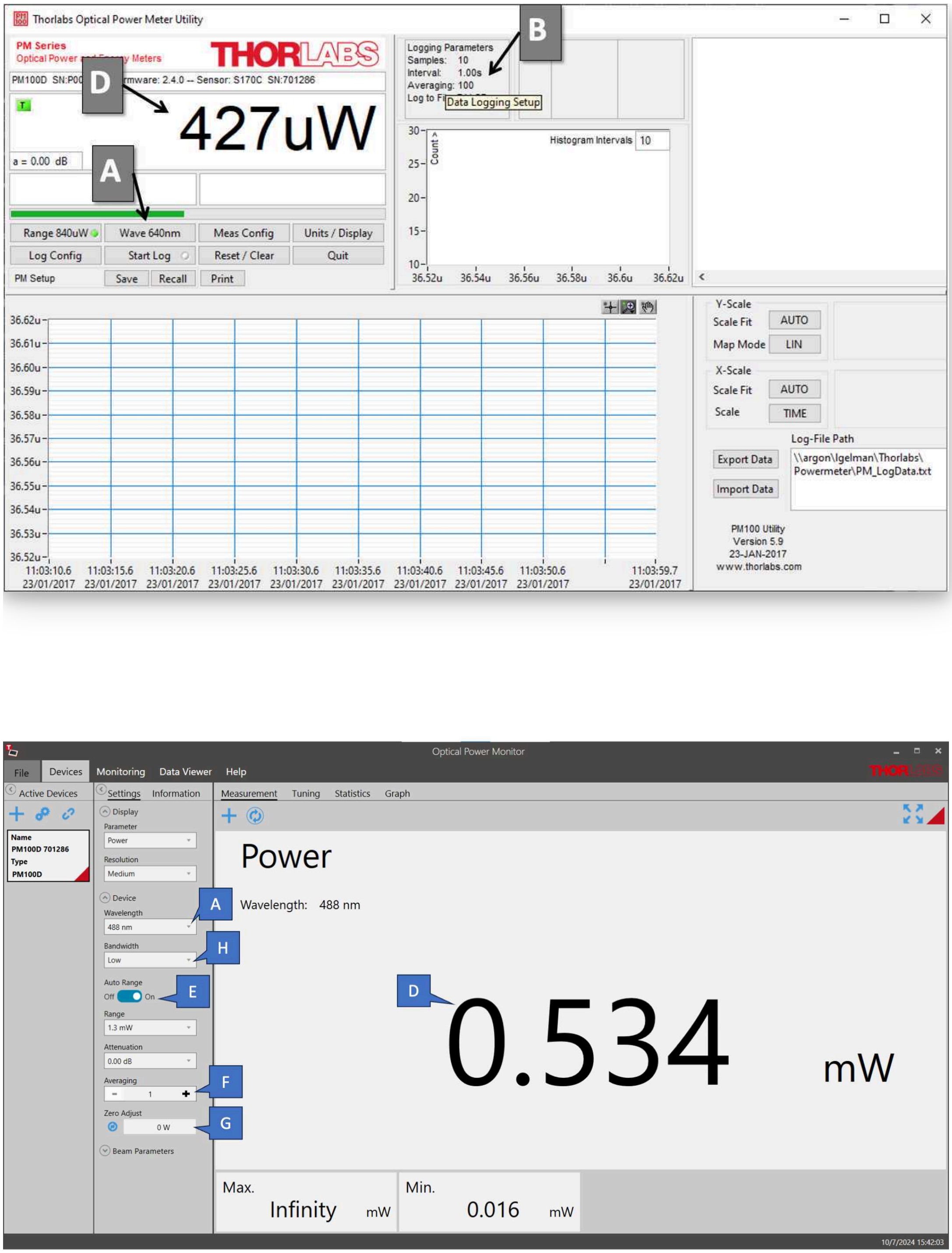


Figure 4. Examples of control panels for power meters. Upper panel: Argo-Power (Argolight). Middle & lower panels: different Thorlabs software versions. A) Excitation wavelength selection, B) sampling period adjustment, C) power instruction in % selected from the imaging system software, D) power measurement in mW, E) auto range , F) averaging G) zeroing, and H) bandwidth. See **Power meter Q&A Appendix 1 Q14**, for explanations.

6 Zero the power meter while the illumination is off (**Figure 4G**, see also **Power meter Q&A Appendix 1 Q15**). Ensure ambient light is kept to a minimum and remains consistent throughout the measurements (i.e. room light off, while a small light source on a desk can stay on to provide some visibility and avoid accidents).

Note

It is better to cover the oculars and block any stray light path that may reach the sensor. Try to perform measurements with stable, consistent, and reproducible temperature, airflow, and humidity, especially for open systems (see more about the impact of environmental parameters in **Appendix 3**).

7 Switch on the illumination and read the power output on the power meter (**Figure 4D**) setting a suitable range on the power meter (in most cases, best set your power meter range to "automatic") (**Figure 4E**).

Note

At this stage and without logging the data yet, observe whether the power is stable, drifting or fluctuating over 10 seconds, as this can be an immediate indication of problems with illumination control or certain optical elements.

Single-Beam Laser Scanning Confocal Microscopes (including Multiphoton systems)
Ideally, the laser beam should be stationary (meaning not scanning but pointing to the same position). Indeed, when scanning, blanking of the laser between lines and frames, along with scanning speed, will affect the measured values. If it is not possible to keep the laser beam stationary, you may want to use the Fluorescence Correlation Spectroscopy (FCS) or single point stimulation option, if available. If no FCS or single point stimulation option are available, ensure you reuse the same settings each time you make the recordings, e.g., record pixel size, pixel dwell-time, and frame size (in pixel).
Some systems (e.g. for STED or SHG microscopy) are equipped with a polarizer, which can be slided in or out the light path, or whose orientation can be turned. This will have an impact on the absolute power measured at the objective. Make sure that the position of the polarizer is always the same when performing the tests.

Spinning-Disk Microscopes
Rotation speed, disk position (confocal or wide-field mode), as well as the size of the field diaphragm may affect the measurement. Ensure the very same microscope settings are used when measurements need to be compared.

Illumination stability measurement

8 Proceed with the power measurement and measure illumination power over time for each excitation wavelength or wavelength range.

For short-term stability: record every second for 5 minutes.

For long-term stability: record every 30 seconds, with 1 second integration time, over 120 minutes. Sampling, integration time, and duration have to be set on the power meter before recording (**Figure 4**). See also **Appendix 1**.

Note

These recommendations are mainly for routine maintenance. If the quality control is made to check the system's behavior for a specific experiment, use a frequency and duration that match your time-lapse conditions (see **Figure 4B&C**).

For routine maintenance, measure the illumination power stability at different intensities. We recommend starting with intensities corresponding roughly to 5%, 20% and 80% of maximum power. Record the powers measured in mW at the objective for these different percentages. When repeating the stability measurements at later times, set up your system to get the same values in mW at the objective (i.e., not necessarily the same percentage). You need to measure the stability of a given illumination power (mW) at the objective, not of a given percentage.

Assessing long-term stability for multiple wavelengths takes time. For some combinations of microscope and power meter brands, scripts allowing the automation of the measurements have been developed and are publicly available on our **QUAREP Github** repository. For more information, consult **Appendix 2: Automation of measurements**.

9 Calculate illumination stability (as a % variation) using the following formula:

$$\Delta Power\ (\%) = 100 \times (1 - ((P_{max} - P_{min}) / (P_{max} + P_{min})))$$

where Pmax and Pmin are the maximal and minimal powers recorded during the time interval measured.

Expected result

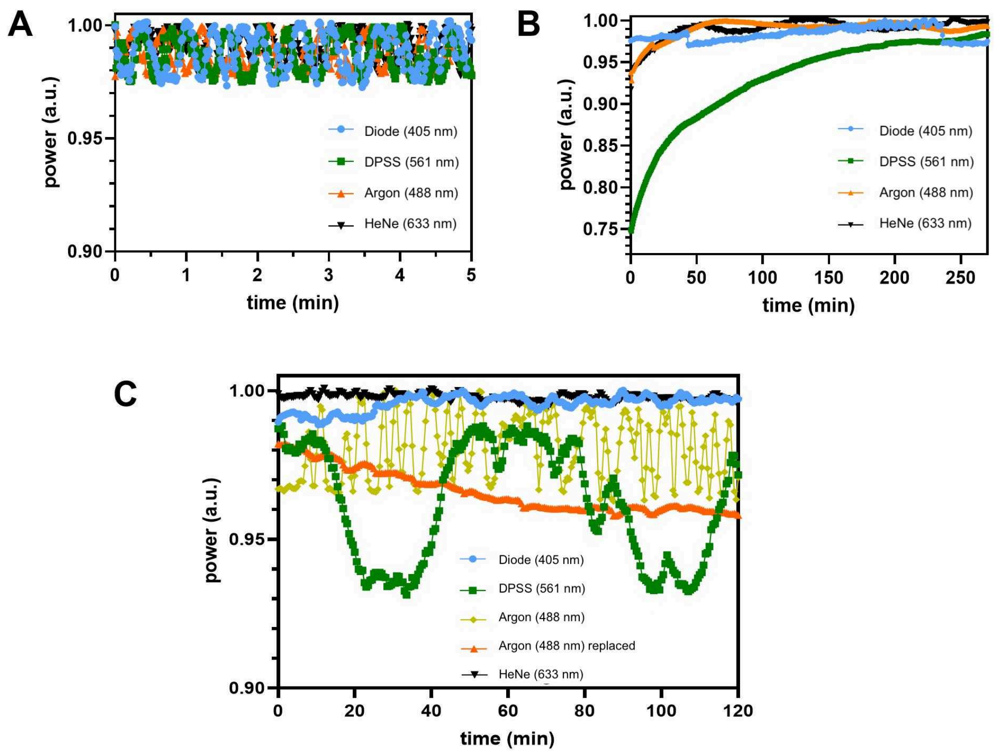


Figure 5. Examples of data collected for short and long-term illumination power stability. A) Short-term stability monitoring for a spinning-disk equipped with 4 diode lasers. B&C) Long-term monitoring for a spinning-disk confocal and a raster scanning confocal microscope, respectively. The spinning-disk in (B) was not pre-warmed to show the warming up time of the lasers. The raster scanning in (C) was equipped with diode (405nm), DPSS (561nm), Argon (488nm), or HeNe (633nm) gas lasers. The Argon laser was particularly unstable due to aging and was later replaced. Measurements of the replacement laser are also shown (yellow triangles), displaying a better, but not perfect stability. Laser intensities for (B&C) were controlled with an AOTF. The power values of each laser line were normalized to the maximal value for each case.

Illumination linearity measurement

10 For illumination power linearity measurement (or illumination linearity): Measure for 30 seconds, 10 incremental fractions of the total illumination power in steps of 10%, from 0 to maximum power value. See **Power meter Q&A Appendix 1 Q15, Q18 and, Q19** for explanations.

11 Plot the average power values measured versus the set power fraction (or %) and determine the goodness of fit of a simple linear regression by calculating the coefficient of determination (r^2). A perfectly calibrated system should have an r^2 value very close to 1, as shown below in **Figure 6**. We recommend making multiple measures (replicates) for accurate results.

Expected result

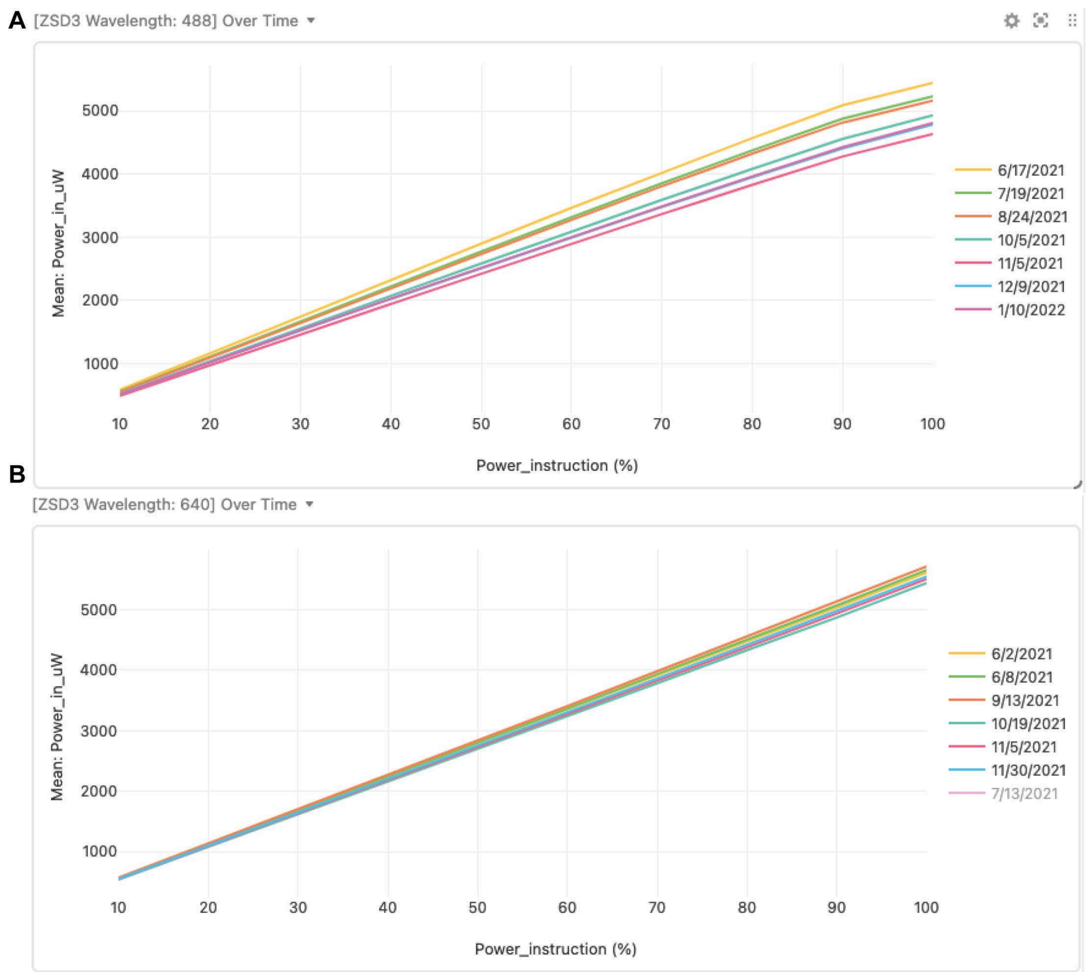


Figure 6. Example of laser linearity assessment of illumination power data acquired on a spinning-disk confocal. Each plot shows the laser power in µW for a given power set in % from the software. This relationship can be tracked over time (dates of measurements shown in the legend on the right of each graph) as shown here for A) the 488 nm laser line and B) the 640 nm laser line.

Note

If extremely low or extremely high illumination powers are measured to match experimental settings, keep in mind that power-modulating elements (e.g., AOTFs) are more likely to behave non-linearly at the extreme ends of their power range. To catch issues on the low and high ends of the percentages settings you can measure with the following increments: 0, 1, 2, 3, 4, 8, 16, 32, 44, 56, 68, 84, 92, 96, 98, 99, 100 (%).

Results display and tracking

12 Enter your measurements in your database, see example on **Figure 7**.

Expected result

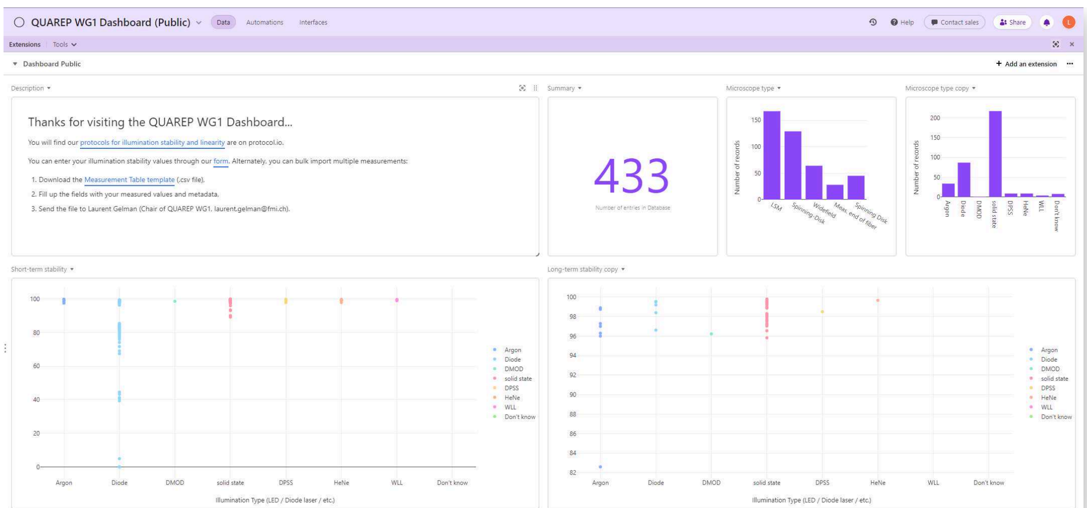


Figure 7. Starting mid-2020, members of the QUAREP-LiMi WG1s tested this protocol, and started collecting these measurements in a QUAREP-LiMi database. Stability measurements communicated by QUAREP members were displayed on the QUAREP WG1 dashboard for different illumination source types. These results do not constitute a norm but may help microscope users to evaluate the performance of their system in comparison to other systems in the field. This database is no longer active.

Note

Other recommendations and future directions
The recommended frequency of this measurement can vary, e.g., depending on the illumination type. Manufacturers recommend monitoring every 2-4 weeks for a gas laser, every two months for a Diode-Pumped Solid-State (DPSS) laser and every 3-4 months for a diode laser. A higher frequency of this measurement may be needed if other factors influencing the sample illumination are at play, like for example the stability of the coupling system and the age and polarization of the laser fiber, the stability of the AOTF, etc..

The formula chosen for stability calculation is very sensitive to any outlier. Be mindful that a single value can significantly decrease the percent of stability calculated.

Illumination power can be modulated in different manners (e.g., directly at the laser, or indirectly through an acousto-optic modulator (AOM) or acousto-optic tunable filter (AOTF), optical elements like neutral density (ND) filters or polarization filters, prisms or combinations thereof), and hence different components may be the source of instability. Consider potential additional sources of error when interpreting results.

Some illumination sources can be operated in different operation modes (e.g., run/standby, constant power, or constant current mode), which may impact output power. If present, the so-called “constant power mode” is expected to reduce fluctuations.
Power measurements before starting and after completing a sensitive experiment could be helpful to ensure that the illumination power or stability did not change.

Multiphoton system users can find more specific troubleshooting tips and advice in the article by Lees et al. (DOI: **10.1101/2024.01.23.576417**).

Appendix 1: Power meter settings Q&A

13 *Some of the answers below are specific to Thorlabs' power meters.*

1. How much of the sensor area needs to be illuminated for accurate measurement?
It is recommended to not focus on the sensor and to keep the diameter of the illuminated area within 1 to 10 mm (except if the measurement depends on the creation of a second-harmonic generation signal like with the sensor NS170C for multiphoton illumination power measurements).

2. Should I use an immersion medium for my objective when measuring illumination power?
When measuring the power through a high NA objective (NA>1) it is important to check whether the sensor is designed to be used in air or whether an immersion medium should be used between the front objective lens and the sensor. Using an appropriate immersion medium allows the sensor to detect the total laser power over a high NA, without losses

arising from deflection or reflection. Most power meter sensors are not waterproof and can be damaged by exposure to water or oil. However, some sensors were designed specifically for microscopy applications and can accept a drop of immersion media (for example, the S170C sensor from Thorlabs).

There will be a difference in power measurements with and without immersion media when the NA of the objective is above 1.1. This is caused by:
Total internal reflection (which can be mitigated in the S170C via an index-matching gel in between the silicon sensor and the cover window) and/or
Absorption of light from the immersion medium (i.e.10% less for water and 15-20% less for oil when compared to air).

3. Is the detection linear across the sensor?
The measured power response may not be linear near the edges of the sensor. Hence, it is important to center the beam to improve linearity but also to avoid clipping the beam on the edges of the sensor.

4. How to prevent damage and saturation of the sensor?
Keeping the optical power below the maximum rating in the specification sheet prevents saturating the sensor. Higher powers than specified will saturate the detector or create zones of critical saturation on the sensor, leading to a non-linear measurement of the signal. You can use a neutral density filter to lower illumination power.

5. How does the integration and readout time work and what should be the preferred power meter bandwidth settings?
A photodiode has a response time of 1 µs, but the electronics behind it are slower depending on the readout device used. The rule of thumb is to set the bandwidth to "low". For very fast fluctuation measurements, it is best to use a very fast photodiode with an oscilloscope rather than a power meter, power meters being designed for slower changes (see **Figure 4**, Lower panel, H).
Very different speeds also require the usage of different photodiodes. When a photodiode converts photons to electrons, the flow of electrons out of the sensor is governed by its capacitance. Thus, large photodiodes with high capacitance are poor at detecting fast dynamics, but their large area makes it easier to capture the light beam, especially when it comes out of a microscope objective with a low NA or when no objective at all is used. For fast dynamics, you will need a smaller photodiode, which has lower electrical capacitance and responds faster, and you will need electronics designed to sample quickly as well. For this, a high-speed oscilloscope is preferred.

6. Does the power meter need to be calibrated or just the sensor?
The power meter does not change over time; thus, only the sensor needs to be calibrated regularly. Calibration data is typically saved in a chip in the DB9 (red) connector of the sensor for Thorlabs power meters. Other vendors may store this information elsewhere. We

recommend checking the calibration status of your sensor by comparing the results to those obtained with a different sensor.

7. How often does a power meter need to be calibrated?
It is different depending on the power meter you use, but typically, the measurement accuracy may change by 2-3% over 3-4 years but could also deviate by up to 10% over a longer time. In general, it is recommended that a power meter be calibrated yearly. However, if you do not exceed the specified power rating, the calibration should be stable for 2-3 years. This is within the wavelength range of 300 to 1060 nm; shorter wavelengths may incur further deviation.

8. Is using a power meter safe?
For standard confocal microscopes, it is generally safe to take power meter measurements, but before you do so, make sure you check with the vendor or the person who built your system. Also, check your organization's regulations around laser safety, as they vary by country and jurisdiction. Precautions regarding the reflectivity of the sensor coating should be taken. Up to 0.5-1.5%, reflection may occur when delivering high powers to the sensor. You can damage your eyes using a power meter through stray reflections from a significantly powerful lamp or laser, and you must be trained to carry out this procedure using eye protection when necessary. Under no circumstances should you check high-powered lasers such as those used in multiphoton systems, TIRF systems, or point localization systems without training. Typically, less-reflective thermal sensors are used with a higher power as they have higher maximum power ratings and are designed to measure such lasers.

9. Can you capture the data and change settings on a computer?
If your power meter uses a standard Universal Serial Bus (USB) to a Common (COM) terminal, you can send Standard Commands for Programmable Instruments (SCPI) to the power meter, query data, and other parameters. For example, the PM400 (Thorlabs) allows for different capture intervals to the internal memory via SCPI commands. Most common programming languages support serial terminal commands so writing a simple program to query the power meter from a personal computer is often straightforward. Some power meter consoles also support storage media, such as a removable USB drive or Secure Digital (S.D.) card, which can log data for later review.

10. How to calculate the photon flux from power measurements?
The number of photons (n) observed per unit time (t) is the photon flux (Φq).
<u>Formula:</u>
Photon Energy: $E_p = hc/\lambda$
Measured Power: $P_{meas} = n \cdot E_p/\lambda$
Photon Flux: $\Phi q = n/t = P_{meas}/E_p = P_{meas} \cdot \lambda / hc$
($c = 3 \cdot 10^8$ ms and Planck constant $h = 6.62607015 \cdot 10^{-34}$ Js)

11. Are there different types of power meter sensors?

Yes, three different types: photodiode, thermal power, and pyroelectric energy sensors.

Photodiode sensors have a strong spectral sensitivity dependence and must be calibrated over the entire wavelength range. On the other hand, they have a high dynamic range (70dB), very low noise, and high response speed. Photodiodes are made of different semiconductor materials like silicon (190-1100 nm), germanium (400-1700nm), and indium gallium arsenide (800-2600nm). They are common for low-to-medium power measurements typical of visible light confocal microscopes.

Thermal power sensors use the Seebeck effect that turns heat flow into a power-proportional voltage. They can be used up to very high power levels. Their useful dynamic range is much lower than the range of the photodiode sensors (30dB). Another drawback of the type is that they are very sensitive to ambient temperature or airflow (drafts). Hence, they should be shielded from moving air during measurements. In general, the speed of response is low. Therefore, a few seconds of settling time is often required.

Pyroelectric energy sensors can only handle pulsed signals (no continuous waves). A pyroelectric crystal converts the heat impact of a laser pulse into its energy-proportional voltage. They are more commonly used for high-energy, lower repetition rate pulse lasers, which are not common in microscopy except for certain photo-activation and microsurgery applications.

12. Using the same type of sensor, will all power meters give me the same values?

Yes, most of the time. However, different values can be observed between different models of power meter sensors due to the sensor's calibration, electronics, and composition (a sensor without gel filling the gap between the filter glass and sensor surface will show lower power levels with high NA objectives).

Different values can also be obtained when measuring broadband light sources. Different detectors or filters have different spectral curves, which can run in opposite directions. Since you can only set one wavelength point from the entire incident light spectrum, the light outside the wavelength set point gets weighted differently and causes a difference in the reading.

13. Do different light sources (e.g., laser, LED, lamp) require a different type of sensors?

Thermal sensors best measure broadband light sources, such as white lamps, as this type of sensor is not wavelength-sensitive.

Narrower-band light sources, such as filtered lamps, lasers, and LEDs, can be measured with photodiode sensors, but the accuracy of the measurement will depend on the bandwidth of the source. Most lasers and LEDs are narrow band enough that the error will be negligible, but some broadband LEDs (phosphor type without a bandpass filter) may be more accurately measured with a thermal sensor if sufficiently powerful. If a photodiode is used for a broadband light source, the error may be compensated for by considering the wavelength response of the photodiode, as discussed above.

14. Is the electric grounding of a power meter essential?

Power meters typically filter interference from nearby electronics or power cords. For very sensitive measurements, or particularly electrically noisy environments (rare), additional measures may be necessary (like grounding your sensor). It is also preferable to use the power meter in battery-operation mode (unplugged from wall power) as this will isolate the meter electronics from any noise coming through the power lines.

15. Is zeroing the power meter at each wavelength important?
The zeroing feature measures dark current (if the sensor is covered and is not detecting ambient light), and subtracts it from future measurements. It is not wavelength-dependent. Therefore, you can simply do it once when you switch it on.
The zeroing feature can also be used to compensate for room lighting by performing the "zero" sampling with the sensor uncovered, but the light source to be measured shuttered or blocked.
After zeroing, keep the room light settings constant during your measurement.

16. How to measure the pulsed input and peak power of your light source?
You need a fast response/reacting power meter (like the new Thorlabs PM103) and sensor, or a pyroelectric energy sensor for these measurements. For microscopy applications, typically only some fast-flashing stimulation or uncaging light sources require these types of measurements. If the power meter response speed cannot follow the pulses, you will get an average measurement as the sensor will average the effect of the pulses over time in its reading.

17. How can the circularity, shape, and diameter of the beam affect the measurements?
The power reading (Watts) should not be affected by the shape and diameter of the illumination spot except when the illumination spot overfills the sensor or is focused so tightly on the sensor that it creates local variation or saturates the sensor area (this can damage the sensor). It is best to have a defocused illumination spot, as mentioned in Question 1.
The power per unit area (usually W or mW per cm2) is impacted by the illumination spot's shape, intensity profile, and diameter. If the geometry and profile parameters of the illumination spot are known, they can be used to calculate irradiance (mW per cm2) once the overall power in Watts is measured.

18. What does "averaging" exactly do?
Depending on your chosen settings, it averages the results of multiple readings, reducing measurement noise. For example, if you set the power meter with an average of 100, it will take 100 readings and report their average. Note that averaging slows down the speed of measurement, as the meter must acquire all the readings to be used in the average before performing the average and displaying the result, although most meters are fast enough that 10s or 100s of readings for an average is not excessive. (**Figure 4F**) The averaging in the new Thorlabs software (OPM - released around 2018) is done externally in the software. It takes the number of set samples and calculates the average value. The

Thorlabs manual may however still refer to an old software application. This application uses the internal averaging capability of the power meter - the meter measures all 300µs so that the result of a set value of 3000 results in a 1-second measurement interval. The command for this internal averaging is still available, but not used in the new software.

19. What does integration time refer to?
The integration time, i.e., the time in which the measurement has been made (measurement time interval) is used in this protocol as the signal integration time referring to the duration over which the sensor collects the incoming laser light.

20. How to measure Broadband Light Sources?
Every sensor has a wavelength-dependent responsivity (see **Figure 8**). To achieve a correct power measurement, the current or voltage measured by the power meter must be multiplied by the responsivity factor of that sensor at the illumination wavelength used (if the power meter model used does not do it automatically).

When using lasers, this operation is directly taken care of by the power meter once one has entered the laser wavelength into the power meter (**Figure 8A**). If not, to do the calculation without a power meter, just take the measured current or voltage measured by the power meter and divide it by the responsivity value at your laser wavelength from the calibration certificate of your sensor. Ex:

$Power = I / R(\lambda)$ for photodiode sensors

$Power = V / R(\lambda)$ for thermal and pyroelectric sensors

with I being the current and V the voltage measured.

For example, using the responsivity curve shown in Figure 8A, if the current measured at 830nm is 3mA, the power is 3[mA]/0.52[mA/W]=5.77[W].

For broadband illuminations, like LEDs or other lamps in combination with excitation filters, one must take into account at least three important parameters that may influence the absolute power measurement: the spectrum of the light source, the specifications of the excitation filter, and the dichroic, and the responsivity of the power meter sensor (see curves in **Figure 8B**). However, to make the measurement easier and more robust, WG1 agreed on a convention: the wavelength to be set on the power meter should be in the middle of the bandpass of the excitation filter. Note that if the measurement is done within the linear range of the responsivity curve of the power meter (**Figure 8**), using the center of the bandpass is a way of averaging responsivity values. The specific spectrum of the light source within the excitation bandpass should not have any significant influence on a biological experiment and is not getting further consideration here. For the measurement and calculation of power from the current or voltage value, proceed as for the lasers above. The measurement has to be done for every channel (i.e., a combination of LED/laser and filter set) used. In the case where a multi-band pass excitation filter is used, the wavelength set on the power meter should be in the middle of the band-pass region, matching the LED used.

There are thermopile sensors designed to measure the light power of light sources with a bandwidth of wavelengths. For the width of bandpass filters and powers typically used in light microscopy, the predicted divergence of thermopile sensors from the above-proposed method is expected to be moderate. (There will only be a negligible deviation (~1%) between thermal or photodiode sensors for narrow bandwidth (<1nm) light sources. For low power levels (<100 mW range), the photodiode will consistently deliver more stable readings and lower noise levels, although thermal sensors with sub-microW resolution exist.) Thermopile sensors are also not sensitive to the entrance angle of light. There is a specialized detector (S175C) from Thorlabs for use with immersion microscope objectives.

Expected result

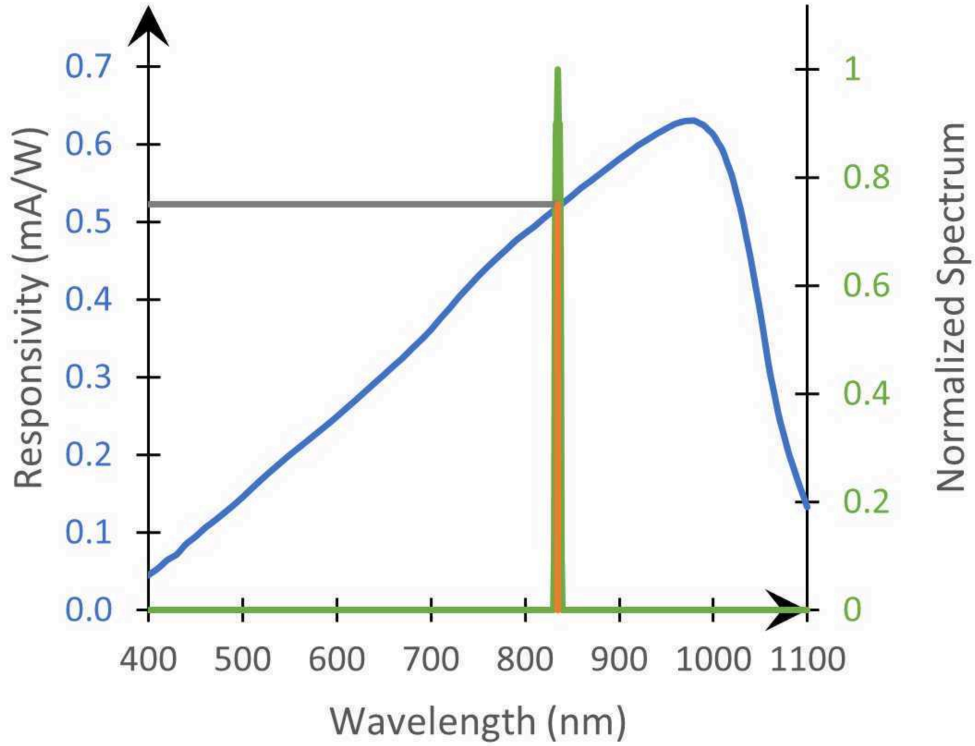


Figure 8A. Plot showing the wavelength-dependent responsivity of a power meter sensor and the normalized spectrum of a single wavelength light source.

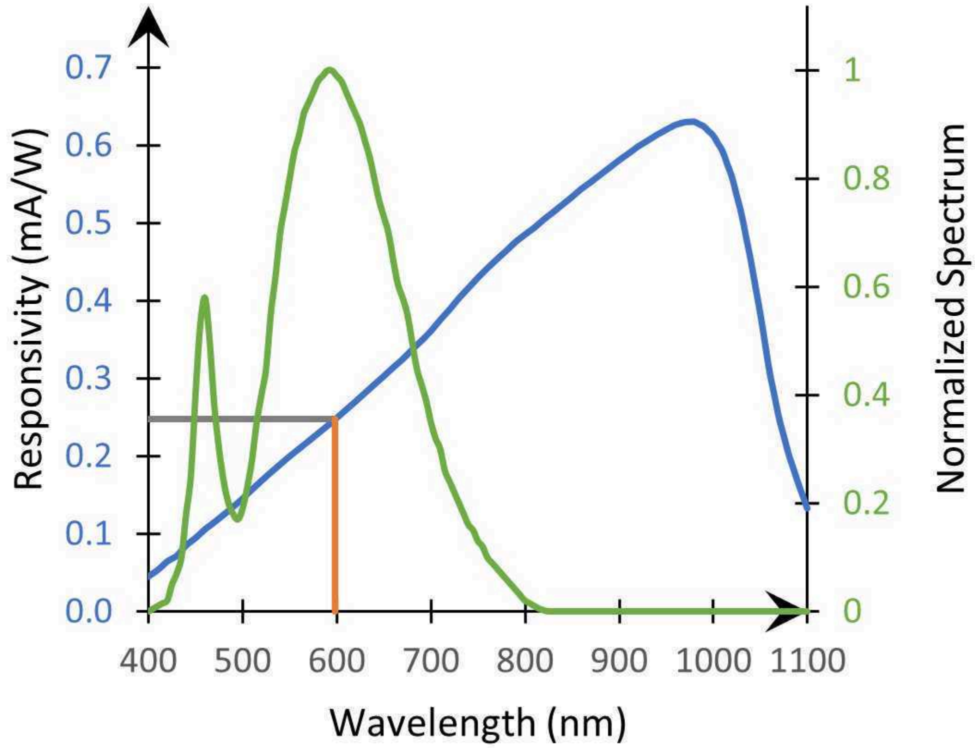


Figure 8B. Plot showing the wavelength-dependent responsivity of a power meter sensor and the normalized spectrum of a broad wavelength light source.

Appendix 2: Automation of Measurements

14 *The outlined illumination power and stability assessment protocol requires measurements repeated over different conditions for each microscope and its respective excitation light sources. Therefore, extensive time will be consumed, if the measurements are conducted manually in a sequential manner. However, most modern microscopes provide automation programming interfaces to communicate with a calibrated external power sensor. The automated measurement has a two-fold aim: to make time-intensive measurements feasible through automatic interleaving of the different measurement conditions and to improve reproducibility by repeating measurements in an identical fashion. The QUAREP-LiMi Tool Kit provides an automatic power assessment interface for Nikon and Zeiss microscopes. For microscopes providing no automation interfaces, the toolkit includes another utility that can either reassign measurements* from *stored data files or directly* recognize *patterns during the acquisition. This* open-source *tool can be downloaded from* ***Github**.*

Appendix 3: Impact of the environmental factors

15 The power of illumination delivered to a sample depends on environmental conditions such as temperature fluctuations of the equipment and the surrounding environment, airflow, humidity changes in the laboratory, and instrument vibrations. Hence, all these parameters should be inspected and measured routinely.

For **temperature** measurements: Ensure that the entire system is turned on and sufficient time has elapsed for the equipment to warm up (approx. 1 hour) to reach a steady-state.

Caution: The temperature measurements should be performed as close to the system as possible to account for local temperature fluctuations in the room. Also, special care has to be brought when using thermo-sensors, which are very sensitive to temperature (value delivered changes with temperature), and should be shielded also from temperature changes.

Additional recommendation: If you routinely carry out live-cell imaging and/or require temperatures different from room temperature, it is advisable to carry out power measurements under these conditions since they may introduce fluctuations in the illumination power, as mentioned above.

Measuring **relative humidity** at the same interval is also recommended.

If power fluctuates, but the temperature is stable within +/- 1 degree Celsius/hour, and the relative humidity is within 40 +/- 20 %, other environmental factors, such as **airflow** (including drafts) or **vibrations**, should be considered and measured.

Avoid direct airflow towards the equipment, as drafts and gusts will cause transient temperature changes, which will impact stability. If the air vents in the room are directly above the equipment or otherwise blowing air towards it, consider adding a deflector or metal or filter cloth diffuser to the outlet to divert the airflow.

Caution: Ensure that these adjustments do not create fire hazards or other safety issues when placed close to light fixtures or other heat sources. Check with your facilities administration before implementing ventilation modifications.

Additional recommendations:
If constructing a new space, a laminar flow ventilation scheme for the room is best, as this delivers regular air movement concentrated around the edges of the room, away from the equipment. Otherwise, it is good practice to avoid placing equipment directly underneath vents when planning your equipment layout.

Doors should be closed during an experiment or protected by an inside curtain to block drafts.

Appendix 4: Tutorials

16 **How to place a power meter sensor on a microscope**

How to set up a Power Meter for Illumination Intensity Measurement on a Light Microscope

Short-term laser stability (using Zen Blue (Zeiss) and Argopower meter (Argolight)

Laser Power readings on a Nikon confocal with a Thorlabs Power Meter

How to set up a power measurement for a two-photon microscopy system