Jun 29, 2021

Confocal imaging power settings – protocol and discussion

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dx.doi.org/10.17504/protocols.io.bu73nzqn

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ABSTRACT

Confocal microscopy is a commonly used microscopy technique involving a point laser scanned across a sample for generating a pixel-by-pixel image of (auto)fluorescent samples (see here for a useful explanatory video and slide set on confocal microscopy). Two key parameters in confocal microscopy are the power of the laser used, and the 'scan time'. The latter determines how long the laser 'dwells' at each point on the sample. Together, these two parameters determine how much photons the sample is exposed to. In turn, the amount of photon the sample receives contributes to phototoxicity and photobleaching effects. Phototoxicity refers to inhibitory/damaging effects of light on cell physiology, while photobleaching relates to (auto)fluorescent molecules losing their ability to emit photons. Both phototoxicity and photobleaching are crucial factors in image-based, live-cell analyses, as we do not want to perturb our samples unnecessarily and we want the highest possible signal. While zero phototoxicity and photobleaching are physically impossible, we aim to reduce these effects as much as possible. It is therefore crucial that we understand the laser settings when conducting confocal microscopy. This protocol will;

• motivate you to care about laser settings,
• introduce how to set them, and
• describe how to report them

ATTACHMENTS

dh67ba2ap.pdf OSP28_00_liveImaging_lightConditions.xlsx

DOI

dx.doi.org/10.17504/protocols.io.bu73nzqn

PROTOCOL CITATION

Marco Polin, Antoine Allard, Kelsey Cremin, Emily Skates, Orkun S Soyer 2021. Confocal imaging power settings – protocol and discussion. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bu73nzqn

KEYWORDS

Laser settings, Confocal, microscopy, lasers

CREATED

May 21, 2021

LAST MODIFIED

Jun 29, 2021

OWNERSHIP HISTORY

May 21, 2021 Urmilas

May 25, 2021 Mary Coates University of Warwick

PROTOCOL INTEGER ID

50139

GUIDELINES

Reasons to care:

Given what we said above, one would expect that laser settings would be a readily discussed point in papers using confocal microscope for live cell imaging. This is not necessarily so. In the literature, it is very common that laser settings for live cell imaging are not mentioned at all, or are provided in percent of laser power (e.g. “laser was set to 20%”). The former approach is completely inappropriate, while the latter is sort of meaningless. This is because different microscopes might be setup with different types or brands of laser that have different total power. Thus, 10% of one laser could mean 20% of another, or any other combination. Without the parameters clearly defined, there is no possibility for other researchers to repeat the experiments in any given paper. So, **repeatability of results** is one crucial reason for you to report laser settings properly.

A second key issue is about the results of an experiment. If phototoxicity and/or photobleaching are contributing to the results we are obtaining from a confocal microscopy study, we need to know this and factor it into our analyses. The only way we can know that phototoxicity and/or photobleaching are playing a role or are negligible is by quantifying our laser settings properly. So, **correct interpretation of results** is the other crucial reason for you to report laser settings properly. In the case of non-living (fixed) samples, where phototoxicity won’t be an issue, laser settings are still important to quantify, or at least qualify, photobleaching effects.

Quantifying laser power settings:

It is unlikely that your confocal microscope displays the actual laser power (in Watt). Instead, settings allow you to change the proportion of maximal laser power available (in %, e.g. 10% of maximum Wattage). While maximum Wattage is sometimes known to microscope operators, laser power can change over time at long timescales (e.g. decrease with use) and can also vary on short time scales depending on the laser (e.g. temperature effects). Thus, the use of tabulated maximum Wattage values by microscope provider, and adjusted by % setting, might not be the best approach to report experiment conditions.

A better, and simpler approach would be to directly measure light conditions on (or near) the specimen at the beginning of an experiment or periodically for different laser settings. More specifically, laser settings can be first set for a given experiment as envisioned optimal by the experimenter. At these specific settings, the light power at the specimen plane can be measured just before the experiment. The measurement can be repeated also at the end of experiment to record any variations in light power over experiment conditions. Light measurement can be readily made using a power-meter that is handheld or embedded into a microscopy slide. The latter is obviously better, as the former suffers from inaccuracies in placing the sensor in the light path (steady those hands!). Still, we currently use a ThorLabs PM160 for some of the measurements given in the Appendix.

Reporting and interpreting laser light measurements:

A power meter usually measures light power (that is energy per unit of time) at a given light wavelength, λ. The PM160 mentioned above, for example, is capable of and calibrated for measuring power within a wavelength range of 400-1100nm and a range of powers from 10nW to 2mW (a sliding neutral density filter can extend the max power measurable to 200mW). This covers visible light (~400-700nm) and near infrared. PM160 reports power in Watts at a chosen wavelength (Note that PM160 software uses the user-defined wavelength info to compensate for the different sensitivities of the sensor to different wavelengths. This means that this power meter is not well suited to measure power of a multispectral source. Here, we are using it for lasers, so it should not be an issue, but this point should be kept in mind. PM160 should be calibrated every year, as is the case with all available power meter units. So, do make sure **your power meter is within calibration** before use.

Light power in Watts can be converted to energy of photon and subsequently to moles of photon, using the

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following approach (see also attached Excel sheet with same file name).

1. **Energy of a photon**. This needs to be determined at the wavelength you are working in.

Energy of photon (in Joules, J) = $h \cdot c / \lambda$ (Eq.1)

where, λ is the light's wavelength, h is Planck's constant ($6.62607015 \cdot 10^{-34}$ J·s) and c is speed of light (299792458 m·s^{-1}). Expressing light speed in µm/s, we can re-write Eq. 1 as ~ $2 \cdot 10^{-16}$ J·nm / λ, where, the light wavelength is expressed in nm. As an example, for blue light with λ = 405nm, we get:

Energy of photon = $2 \cdot 10^{-16}$ / 405 = **5·10-19 J**.

2. Light at sample. This needs to be measured at the specimen. Most power-meters report light power in Watts (= J per second) or in Watts per unit of area. If, like the hand-held meter PM160, your light meter reports values only in Watts, you will need to convert this into Watts per unit area, i.e. light density. To do so, you will need to measure the area of your light beam. This can either be approximated or directly measured, e.g. by photobleaching a sample and then observing and measuring the dark spot generated at a lower magnification.

Note: There are possible complications with this manual measurement approach: 1) the "observation" that one makes after the photobleaching is limited by the point-spread-function of the microscope. If the bleached spot is below a certain size, the measurement won't be accurate; 2) depending on the setup, the photobleached fluorophore can diffuse around, causing distortion of the measurement; 3) the longer the exposure time, the larger is the part of the beam that will cause photobleaching. Some of these issues can be reduced by embedding an fluorophore in low percentage agar for this measurement.

For a confocal microscope, the excitation beam behaves like a point source, the radius of which can be approximated by 0.6 • λ/NA [1] (see also microscopyU), where NA is the numerical aperture of the objective.

Note: This is an important point, and there are at least two things that should be kept in mind. The first one is that the size of the diffraction limited spot is the theoretical best. In reality, the actual area of light will be larger than that. This topic is actually worth exploring on its own, and there exist already tools that can be implemented in ImageJ that try to 'simulate' the diffraction shape depending on microscope settings. See for example the PSF generator plugin. Secondly, this is the size of the "tip" of the cone of light that comes out of the objective. As one moves out of this tip, either above or below, the "spot" expands. The divergence of the cone can be quite large. Think about the fact that an oil objective with a NA of 1.4 will converge the laser light on a cone with an angle of (roughly) 2 x arcsin(1.4/1.5) ~ 70deg. Here 1.5 is the refractive index of the oil. So the light that a cell is exposed to is not a simple circular beam of diffraction-limited size, but a cone of light. What is the role of the different parts of the beam on phototoxicity? Is it correct to focus only on the vertex of the cone? These are points to consider, if one wants to define phototoxicity in detail.

With an exemplar NA of 1.4 and 405nm light, we get a radius of about 173nm and an area of $9.46 \cdot 10^{-10}$ cm^2. Thus, a measurement of 1µW converts to **1060 W·cm^{-2}**.

3. Number of photons. Knowing the power per unit area and the energy of a single photon, we can calculate the corresponding flux of photons at the focal point of the objective. For the above example, we get:

Photon flux at focal point = 1060 W·cm^{-2} / $5 \cdot 10^{-19}$ J = **$2.2 \cdot 10^{21}$ s^{-1}·cm^{-2}**.

So, in this example, we are feeding **about 3.5 millimoles of photons per cm2 of sample per second**, or about $2.04 \cdot 10^{12}$ photons (=3.38 picomoles of photons) every second onto the beam area.

At higher wavelengths, the energy of each photon is lower. However, the excitation area also increases. So, if we had a similar energy measurement in Watts, that can still mean we have less power per cm2. For example, the same calculations above, with 561nm light and with a power measurement of 1µW gives us 0.55 kW·cm^{-2}, which is 2.5 millimoles photons per second per cm^2 or 4.69 picomoles of photons per second onto the beam area (as

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calculated
using above formula).

Relation to phototoxicity and photobleaching:

Now that we have a route to convert power measurements on our samples to number of photons that they receive, we can start making connections between light imaging and phototoxicity and photobleaching effects. Luckily for us, (bio)physicist have been studying these relations and we can gain from existing literature a ballpark 'feel' for the right levels of light for imaging.

As an example, for photobleaching, Kiepas et. al. [2] shown direct relation between light power and photoxicity (measured as ROS production from cells using the CellRox dye) and photobleaching. The conditions they tested included 3.814W·cm^{-2} for 24ms per frame with 590-650nm excitation. Under these conditions they found significant photobleaching (of a EGFP construct) within 100 frames of imaging. If we apply the above calculations with a wavelength of 600nm, we find that these settings equate to **about 0.019 millimoles of photons per cm^2 of sample per second** (or 0.05 millimoles of photons per cm^2 over a 100*24ms exposure period, i.e. 100 frames at 24ms).

Similarly, Waeldchen et al [3] has shown that HeLa cells irradiated at 0.49kW·cm^{-2} at 514nm for 240s (20ms, 12,000 frames) display apoptotic behaviour. If we apply the above calculations, we find that these settings equate to about **2.1 millimoles of photons per cm2 per second**, and 505 millimoles of photons per cm^2 over the course of a 240s timelapse (20ms, 12,000 frames). The same study found a sigmoidal relation between light intensity and fraction of dead cells, with the steep part of the curve falling between 0.5 and 1.0kW·cm^{-2} when using 514nm and a 240s timelapse (20ms, 12,000 frames). Converting again using our approach above, the 1.0kW·cm^{-2} mark at 514nm corresponds to **4 millimoles of photons per cm^2 per second**, and 1032 millimoles of photons per cm^2 over experiment time.

In summary and based on the above and similar studies [4,5], a general rule of thumb suggest that live cell imaging should ideally be conducted below 0.5 kW·cm^{-2}. Assuming a confocal microscope, with light source of 405nm (561nm), considered as a point source and an objective with an NA of 1.4, this corresponds to about 0.15µW (0.3µW) measured on PM160 and 1-2 millimoles of photons per cm^2 per second.

Confocal imaging power settings-step-by-step protocol

1 Prepare sample.

2

Use sample to set your microscope up for correct laser and scanning settings.

3 Make sure you use a calibrated power-meter and that it is zeroed before measurement and set to the correct wavelength matching that of your laser.

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4 Remove sample and place power meter on to the sample holder or place power-meter slide, in case you are using a power-meter embedded in a slide.

5

Turn laser on and record power measurement.

6 Repeat measurement few times to make sure you get an average / approx. value at andaround the specimen position.

7 **BE AWARE of lasers**, and do not look into light path, while holding the power-meter in place.

8

Record laser power value and perform experiment.

9 At end of experiment, repeat 3 to 8 again and record laser power for end of experiment.

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