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SHORT COMMUNICATION

Fluorescence microscope light source stability

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Abstract

The process of fluorescence starts with the efficient generation of light that is required for the excitation of fluorophores. As such, light sources are a crucial component of a fluorescence microscope. Choosing the right illumination tool can not only improve the quality of experimental results, but also the microscope's economic and environmental footprint. While arc lamps have historically proven to be a reliable light source for widefield fluorescence microscopy, solid-state light-emitting diodes (LEDs) have become the light source of choice for new fluorescence microscopy systems. In this paper, we demonstrate that LEDs have superior light stability on all timescales tested and use less electrical power than traditional light sources when used at lower power outputs. They can be readily switched on and off electronically, have a longer lifetime and they do not contain mercury, and thus are better for the environment. We demonstrate that it is important to measure light source power output during warm-up and switching, as a light source's responsiveness (in terms of power) can be quite variable. Several general protocols for testing light source stability are presented. A detailed life cycle analysis shows that an LED light source can have a fourfold lower environmental impact when compared to a metal halide source.

Keywords Stability · Light source · Solid state · Fluorescence · Microscopy · LED

Introduction

Fluorescence microscopy is central to most physical, life science and health science laboratories. Fluorescence microscopy techniques have seen tremendous development and innovation in recent years, including the refinement of solid-state illumination sources. Light-emitting diode (LED) and other solid-state illumination sources are commercially available, and they can be very cost effective to custom-build (Albeanu et al. 2008; Cole and Turner 2008; Sato and Murthy 2012). LED technologies are continually evolving to increase brightness in the green and yellow parts of the visible spectrum and expand capabilities in the ultraviolet (UV) (Tinning et al. 2018) and near-infrared (IR) ranges. Due to these improvements, LED light sources have been expanding to new markets and are now beginning to be used in some confocal microscopy (Vakili et al. 2015), calcium imaging (Tinning et al. 2018) and other applications. Widefield microscopy remains one of the most sensitive and affordable options available to address a plethora of biological questions. Combining the general use of widefield microscopy with the availability of stable LED light sources will improve the quantitative nature of fluorescence microscopy.

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Quantitative microscopy (Jonkman et al. 2014; Waters 2009) and reproducibility in microscopy (Deagle et al. 2017; Lee and Kitaoka 2018) are key topics of interest to researchers across all fields of investigation. A stable light source is a key component for quantitative reproducible fluorescence microscopy. Traditionally, fluorescence illumination has been supplied by mercury arc lamps for widefield applications. Mercury arc lamps (HBO) provide a broad spectrum with a strong UV component and bright intensity peaks from the UV up to about 600 nm red light. For this reason, UV blocking filters should be used for live cell imaging and should be placed in the microscope eyepieces for protection. In turn, metal halide (MH) lamps are an improved version of the original HBO lamps with more uniform power outputs across the spectrum and a longer bulb lifetime (Lichtman and Conchello 2005). HBO and MH lamps pose both an environmental risk and a health risk, in that they introduce mercury into the waste stream. Mercury bulbs have a very limited lifetime of roughly 300 h, while MH bulbs last for roughly 4000 h. HBO and MH blubs both contains mercury although the amount of mercury per bulb per hour of use is reduced in MH light sources. Replacing bulbs is time consuming and expensive, and their laboratory use necessitates the implementation of evacuation and clean-up protocols in case of breakage (Liu and Nolan 2012; Webb and Brown 2013). For more details on how light sources generate light and their spectral intensity outputs, see the Molecular Expressions webpage at https://micro.magnet.fsu.edu/primer/anatomy/sources.html.

Historically, LED-based light sources were too dim to be competitive in the fluorescence microscopy market. Recent versions of LED-based white light solutions have comparable or superior emission spectra when compared to traditional arc lamps (Lichtman and Conchello 2005; Sato and Murthy 2012). Two families of LED-based light sources are available. One has a white light output similar to HBO and MH lamps that uses excitation filters to choose excitation wavelengths. The second family combines multiple individual LEDs of different colours with discreet wavelength peaks that cover the visible spectrum. These latter systems are combined with associated optics, so that they can be directly coupled to the microscope and electronic switching can be used to choose excitation wavelengths sequentially (Liu and Nolan 2012; Webb and Brown 2013). LEDs also have intensity control options to allow for custom excitation power outputs to be set. Thus, a particular fluorescence intensity level can be set for each LED and optimized for each fluorescent dye to ensure optimal image quality and photo-stability (Albeanu et al. 2008; Webb and Brown 2013). This tight intensity control can eliminate the need for neutral density filters although they are often still required for sensitive live cell experiments.

LED systems have a much longer lifetime of up to ~50,000 h so they require limited maintenance and do not generate mercury waste (Liu and Nolan 2012). Studies have shown that LED light sources have an overall reduced operating cost when compared to HBO or MH lamps (Cole and Turner 2008; Kim and Schubert 2008). For upgrades from HBO or MH systems, the LED unit will pay for itself in cost saving mostly from the elimination of the need for replacement bulbs. LEDs also tend to consume less electricity during operation and have reduced maintenance and monitoring costs. It costs approximately $0.45/h to operate an LED system, versus $1/h for HBO lamps (Cole and Turner 2008; Liu and Nolan 2012). LEDs can also eliminate the need for manual or automated shutters to control light exposure because they can be turned on and off electronically (Albeanu et al. 2008; Cole and Turner 2008). Depending on the LED light source and fluorescence microscopy application, excitation filters can often be eliminated. Therefore, it can be an economically sound decision for researchers to invest in updating light sources (Baird et al. 2014). For more information comparing different microscope light sources, see Aswani et al. (2012).

LED technologies are now widely available commercially. However, to our knowledge, there has not been an extensive study of the power output of available LED light sources on different timescales and of their electrical power consumption. This study aims to provide that information while also comparing LED systems with HBO and MH products. The present study also includes a detailed life cycle analysis (LCA) comparing an LED-based and MH light source. The LCA analysis includes the environmental costs for each system including manufacturing, operation, maintenance and end of life disposal. This detailed analysis can be found in the supplemental materials (Supplemental Document 1). Overall, this work demonstrates that LED-based light sources are better for the environment, save time, money and energy and lead to better quantitative fluorescence microscopy data.

Materials and methods

The fluctuation in power output was the metric used to compare the emission stability of each light source. Each light source was coupled to a Zeiss inverted Axio Observer 200M microscope (Carl Zeiss, Jena, Germany). The HBO, LED 1 and LED 3 were directly coupled to the microscope. The MH, LED 2 and LED 4 were coupled to the microscope via a liquid light guide. A Fieldmax-II-TO power meter (Coherent Inc., Santa Clara, CA, USA) was used to measure power output by directly mounting the sensor on a 10×/0.3 NA EC Plan-NEOFLUAR objective lens. The sensor was firmly secured in place using adhesive putty. The field aperture

was opened to ensure that there was no restriction of incident light. Next, neutral density filters were removed from the light path and the light source was set to 100% output. One set of experiments also looked at lower power outputs of the LED systems between 1 and 50%. One LED system was not tested in this way as there was no option to reduce the power output below 100%. A soft-coated 470/40 excitation filter and FT495 or FT510 dichroic were used to study the LED 1, HBO lamp, and MH. The filter set was later upgraded to a Chroma FITC (ET480/40 excitation filter, T510lp dichroic) for tests on LEDs 2, 3, and 4, with a unique spectrum of white light being delivered by each light source. All data were compared as relative intensity changes; therefore, small differences in filter sets and light source spectral output should not have had a major impact on the results. The Fieldmax-II-TO product software was employed to automate data acquisition at pre-specified intervals. The power emission stability of each light source was measured at four different timescales: (1) 1-s measurement intervals for 20–60 min throughout the light source warm-up following a cold start; (2) 0.1-s measurement intervals for 60 s; (3) 1-s measurement intervals for 3 h; and (4) 15 measurements at 1-min every 40 h for 200–300 h. Additionally, measurements were made every second for 15 min at different output powers and on and off switching experiments were performed by turning the light source off for 2 min, then turning it back on for 5 min. The on and off procedure was repeated three times while continuously recording power output at intervals of 0.1 s. For all tests, each light source was warmed up for 30 min. All experiments were repeated in triplicate.

Electrical power consumption-use experiments were measured using a Kill A Watt EZ power monitor (P3 international, New York, USA). Experiments were conducted three times on different days. The electrical power consumption monitor was plugged into a standard wall outlet, and the light source of interest was plugged directly into the power monitor. Measurements were made at 5–10-min intervals, and the electrical power consumption was recorded at different light source power output settings.

Statistics

Data points on each graph represent the mean of three experiments, with error bars representing the standard deviation (SD) between the means of the three experiments. The percentage SD measurements presented in Table 1 and Fig. 4d are average SD for all data collected within a given time interval for all three experiments. Data were compiled and analysed in Microsoft Excel (2007). For the warm-up test, each subsequent ten points were averaged to produce one measurement per minute. The standard deviation was calculated for these ten-point averages between experimental means. Similarly, 10 measurements were averaged for the 60-s test, every 60 measurements for the 2.5-h test, and 10 measurements for the on and off switching test to produce 1 point per second or minute. For the 300-h stability test, the 15 points captured during each measurement cycle were averaged to produce 1 power output per day, and the SD was calculated between points on different days. Power fluctuation was defined in this study as the variation from the mean or the standard deviation.

Results and discussion

The stability of MH, HBO and LED devices was investigated and compared along several experimental timescales. The first test investigated how long it took for each light source to stabilize, following a cold start. It was determined that the HBO lamp required 30 min to reach its optimal power stability (Fig. 1a) with an average standard deviation (SD) in power output of 0.4% (Table 1). The MH light source required a longer amount of time than HBO to

Table 1 Light source characterization

	Warm-up time (min)	Warm-up power SD (%)	60-s test SD (%)	2.5-h SD (%)	300-h SD (%)	On/off switching SD (%)	Electrical power consumption (W) at 100% power output
HBO	30	0.4	0.03	0.20	5.9	NA	135
MH	30–40	6.9	1.30	7.70	13.6	NA	144
LED 1	1–2	0.2	0.01[a]	0.02	1.0	0.3	230
LED 2	5	1.1	0.07	0.10	3.5	0.7	100
LED 3	1–3	0.6	0.02	0.06	1.6	0.6	83
LED 4	15	1.2	0.02	0.20	1.8	0.6	158

Warm-up time and percentage standard deviation (SD) of power output for the different tests performed on the six corresponding light sources. Experiments were done in triplicate, and the average % SD from the values obtained during each individual test are presented. The warm-up time was determined based on how long it took for the average power output to vary by no more than ~1%

[a]It is likely that the power variability of this light source is below the detection limit of the power meter

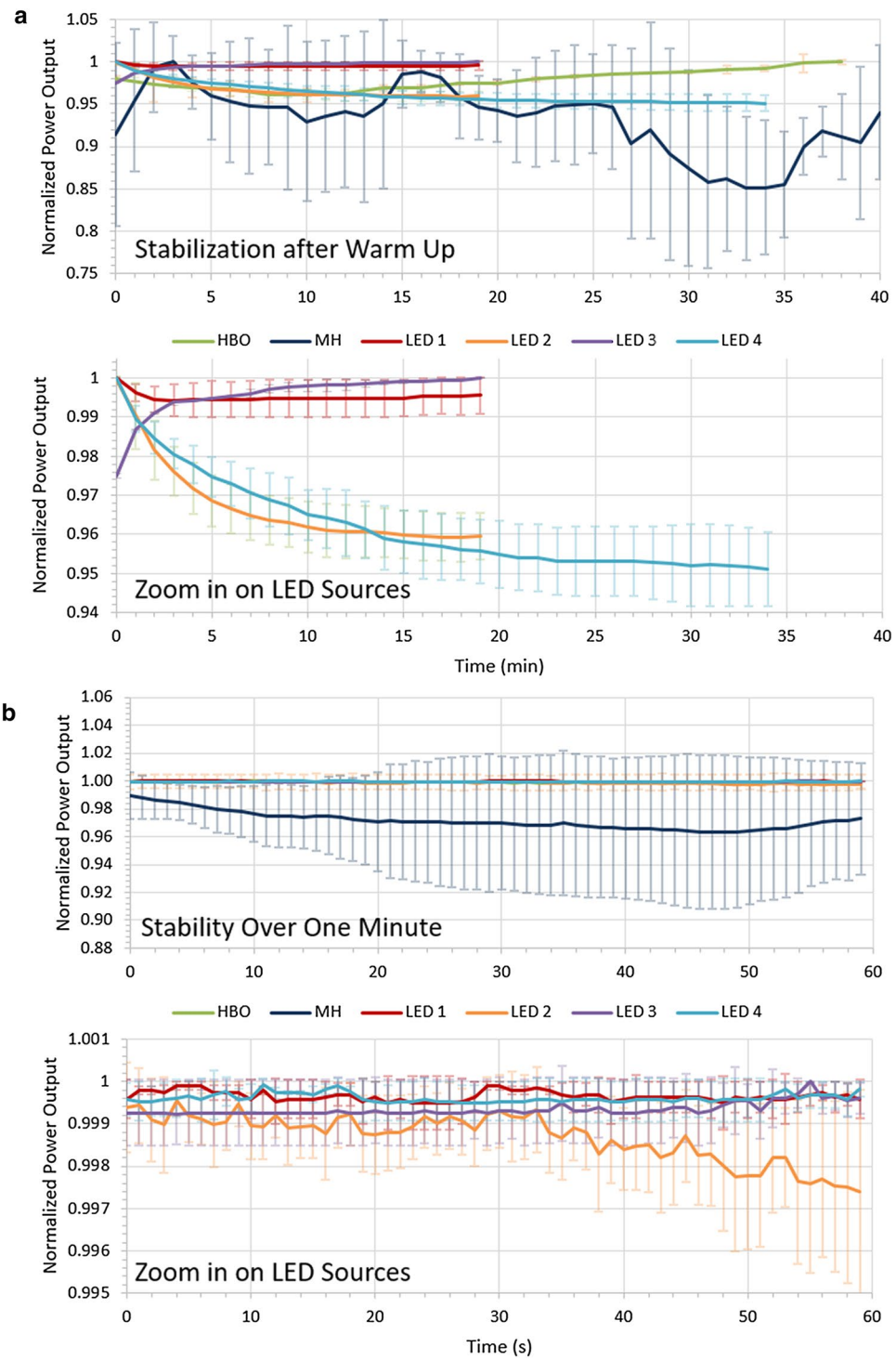

Fig. 1 Light source warm-up and short-term stability. **a** Normalized power output of each light source tested following a cold start at 100% power. Top panel shows all light sources from 75 to 100% intensity. The bottom panel is a rescaled graph with only the LED light sources showing 94–100% intensity. Power output was measured using a Coherent Fieldmax-II TO power meter mounted directly on a 10×/0.3 NA objective of an inverted microscope. Data points were acquired at 1-s intervals and then every 60 points were averaged to plot 1-point/minute. **b** Power output stability of each light source over 1 min. Data were taken every 0.1 s at 100% power. Every ten points were averaged to plot one-point per second. The top panel is all light sources showing 88–100% intensity. The bottom panel is only LED light sources scaled to show 99.5–100% intensity. Plots are averaged from three trials and error bars are the SD between the trials

warm up and its power output did not completely stabilize even after 40 min (Fig. 1a) and it exhibited high power output variations with a SD of 6.9% (Table 1). In contrast, all four LED light sources reached a stable power output within 5–10 min (Fig. 1a) with a much lower SD ranging from 0.2 to 1.2% (Fig. 1a; Table 1). LED light sources 1 and 3 stabilized in as little as 2–3 min, while LEDs 2 and 4 stabilized after ~ 10 min (Fig. 1a, lower panel).

Although small, the variability between LED light sources can be explained by differences in manufacturing, electronics, cooling, and overall implementation of the various technologies. For instance, high-power LEDs are quite

sensitive to heat, and they can consume high amounts of electrical power. Often only a small portion of that energy is converted to optical light, with much of the energy being emitted as heat. This heat generation can take up to 50 W of power (Sato and Murthy 2012). As such, it would be expected that LEDs with inferior heat management capabilities would take longer to reach thermal stability, and stable light output. Nevertheless, these results reveal that LED technologies stabilize quickly with minimal variation in power output from 0.2 to 1.2%, as opposed to high power variability for the MH light source.

For reliable quantitative imaging, it is imperative that fluctuations in incident light power are kept to a minimum so that any changes in light intensity in the images are due to biological processes and not instrument instability. As such, tests were performed to evaluate power output stability during short-term, intermediate-term and long-term usage. The 60-s test is relevant for fast time-lapse image acquisition for applications such as collection of 3D image stacks or measurement of photo-bleaching recovery curves. On this timescale, all light sources displayed average power output SD under 0.10%, except for the MH system (Fig. 1b; Table 1). The MH bulb had a power output SD of ~ 1.3%. Three of the four LED light sources showed high stability with a maximum power output SD of ~ 0.02% while LED 2 deviated up to 0.07%. The HBO light source also performed well with a power output SD of ~ 0.03%.

The 2.5-h (intermediate-term) stability test is relevant for experiments such as live cell tracking, protein trafficking or imaging of multiple live or fixed samples within a single microscope imaging session. For example, if the power drifts or fluctuates significantly on this timescale, sample slides of controls may look brighter than sample slides with treatments but only because the light source power changed over the course of the experiment. All the light sources exhibited minimal power variability aside from the MH with a power output SD of ~ 7.7% (Fig. 2a). LED 1 displayed the minimal power output variation with a SD of 0.02%, while LEDs 2, 3 and 4 had SDs of 0.10%, 0.06%, and 0.20%, respectively, and the HBO light source had a SD of 0.20% (Fig. 2b). Note that the HBO light source used in these studies had a bulb with ~ 100 h of usage and an electronic control unit with a feedback loop to maintain stable power output over time. This could explain its high stability.

In general, power output fluctuations are undesirable in quantitative fluorescence microscopy because they can have a major impact on experimental data and be a source of error that affects relative intensity values and reproducibility. Power increases over time, for instance, mean the sample will be exposed to more light than initially anticipated, introducing spikes in intensity data that make quantification difficult and inaccurate. Intensity spikes can also increase the photo-toxic effects live samples are subjected to, in turn affecting cellular processes (Boudreau et al. 2016; Mubaid and Brown 2017). We have recently shown that linearly increasing incident light power exponentially elevates the bleaching rate of fluorophores, which results in an accelerated loss of fluorescence signal and a premature end to live cell imaging experiments and/or reduced image quality (Mubaid and Brown 2017).

The light source long-term stability was tested over 300 h, corresponding to the lifetime of a standard HBO bulb. This timescale parallels that for comparison of data sets collected over several weeks or even months as part of a continuing research project. Over that timescale, it is important that the intensities of images are comparable in terms of the excitation light source power output, so the intensity of samples from multiple replicates of the same experiment can be compared. The test showed that over 300 h, the HBO bulb's power output decreased by ~15%, while the MH gradually lost ~ 38% of output power (Fig. 2c). In contrast, the power output of all four LED light sources tested remained within 1–3.5% of average levels after 300 h with no evidence of a systematic decrease (Fig. 2c). LED 1 and LED 3 were directly coupled to the microscope and were very stable over time. It is possible that liquid light guide alignment and decay could play some role in the reduction of power output for the MH, LED 2 and LED 4. Liquid light guides can get damaged, develop air pockets and the fluid in the guide degrades over time and becomes opaque reducing light throughput. Ideally, light sources should be directly coupled to the microscope whenever possible to avoid issues with reduced light output from the liquid light guide over time and the cost of light guide replacement every couple of years.

LED light sources are known to be less stable when operated at lower powers. Previous experiments and the data in Table 1 were all collected with 100% lamp power. Experiments on LED 1, LED 2 and LED 3 show that although power variation is higher when operating the light sources at lower power, the light sources are still very stable with variability in power of less than 0.40% (Fig. 3a–d).

Overall, these results demonstrate valid concerns with MH and HBO bulbs, owing to their marked decrease in intensity over time. In addition, when the bulbs are exchanged, the output power increases significantly and users may not be aware that a new bulb has been put in place. One way to get around these issues is for users to have a control slide that is imaged at each experimental session and used to normalize the intensity for each data set. An even more superior solution is to measure the light source power with a power meter and set the light power to the same value for each session. Since there are no burning components in LED light sources, they do not suffer from the same power output decay over time and there are no routine bulb replacements. This saves on staff time and provides a

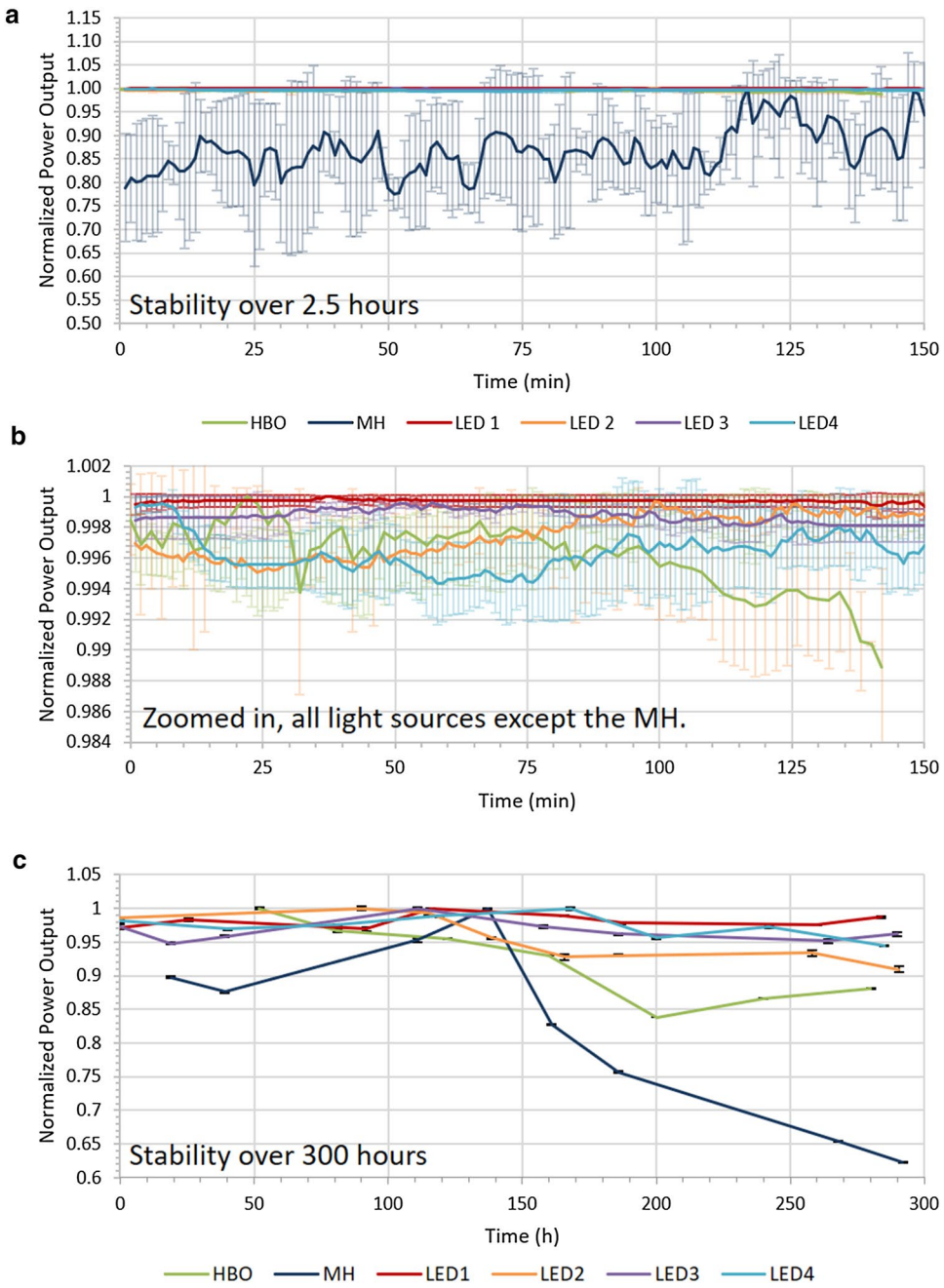

Fig. 2 Intermediate- and long-term light source stability. **a** Power output of each light source over 2.5 h at 100% power. Data points were averaged to plot one-point per minute. Normalized power output is shown from 50 to 100% intensity. **b** Normalized power output from all light sources except the MH showing power output from 98.4 to 100% intensity. Plots in **a** and **b** are averaged from three trials and error bars are the SD between the trials. **c** Power output of each light source over 300 h at 100% intensity. Data were acquired at 1-min intervals for 15 min and averaged at different timepoints. Due to the long duration, this experiment was only done once for each light source. SD is for successive measurements over the 15-min time interval

more consistent power output over the lifetime of the light source. Taken together, these results support that LEDs are ideal light sources for accuracy, consistency, reliability and reproducibility for quantitative fluorescence microscopy.

Manufacturers often promote LED light sources for their "instant" on and off light switching capabilities. Whereas HBO and MH light sources rely on physical shutters for on and off switching, LEDs can be switched on and off electronically. Theoretically, this could happen with little or no impact on the light source's lifetime or power output. The on and off switching test assessed how quickly maximal stable power was reached and if that maximal power was consistent for each shuttering cycle. The test consisted of a 20–30-min warm-up time, monitoring of power output for 5 min followed by a switch off, monitoring of power output for 2 min followed by a switch on and repeating this process several times (Fig. 4a). Interestingly, the four different LEDs exhibited different responses after being switched on. LED 1 and LED 4 displayed an initial spike in intensity, when turned on (following each 'off' period) before the output fluctuation re-stabilized. Power re-stabilized to within ~0.5% of average power output in a few seconds (Fig. 4b). LED 3, once turned on, initially exhibited a lower power output, followed by a steady increase until it stabilized after ~50 s

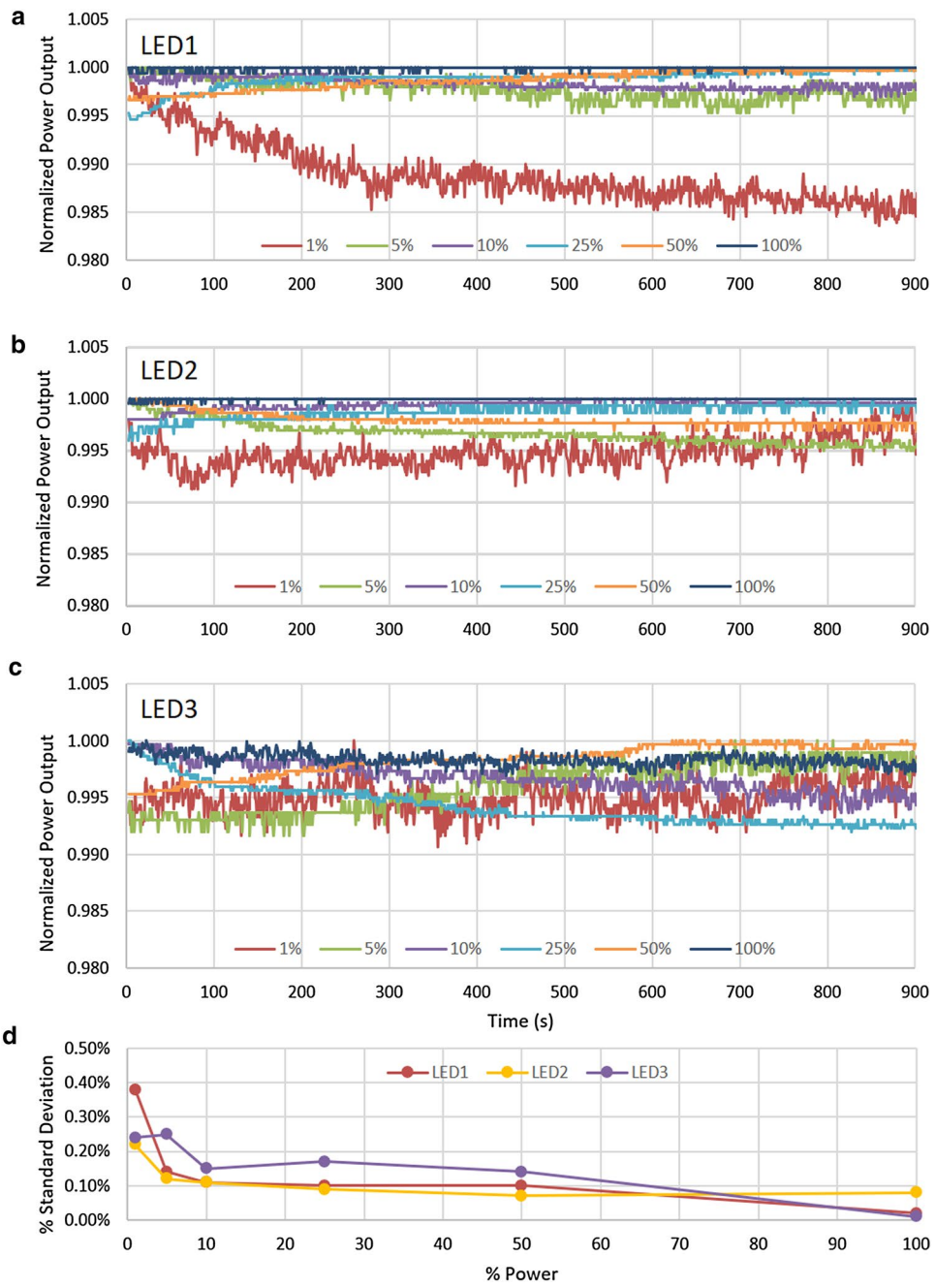

Fig. 3 Stability of LED light sources at different power outputs. Normalized power output of each light source over a 15-min time period at 1–100% power output. Plots were averaged over three trials for **a** LED 1, **b** LED 2 and **c** LED 3. LED 4 did not have the capability to change the power output so it was not tested. **d** Average % SD in power versus power output for the three LEDs

(Fig. 4b). Once turned on, LED 2 exhibited a high peak power, and then slowly decreased in power output by ~5% until a stable output was reached after ~5 min (Fig. 4b, only first 100 s are shown). In all cases, these patterns of power output stabilization were very reproducible with each on and off cycle (Fig. 4c). Thus, if the switching characteristics are well characterized and reproducible then the intensity variations could be corrected for after the fact using imaging software.

These tests show that the shape of the power output recovery of the "instant" switching of the LED light sources is different depending on the supplier. Some of the systems show significant time to stabilize and significant changes in output power. This could be a limitation of LEDs for measuring rapid biological processes such as calcium spikes or membrane potential changes. It should also be noted that for highly sensitive applications such as fluorescence resonant energy transfer (FRET) where dyes may only change in intensity by 10–20%, the time to reach power output stability after being switched on could be limiting. These experiments were conducted with manual on and off switching so further testing should be done for light sources that allow for

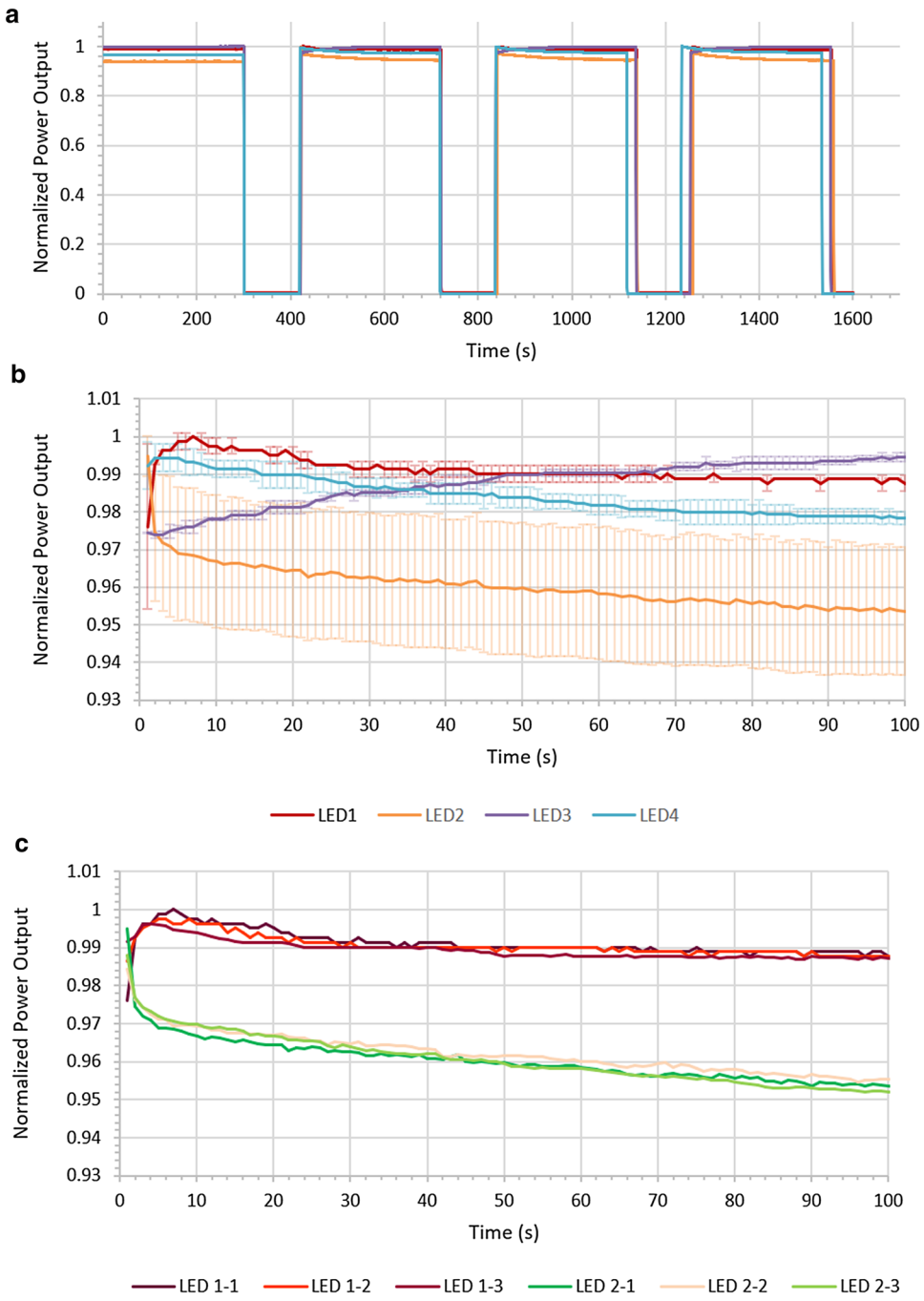

Fig. 4 Electronic shuttering with LED light sources. Power output of each LED was measured every 0.1 s during manual on and off switching cycles at 100% power. Experiments were repeated in triplicate. **a** Normalized power output was measured continuously while each LED light source was manually cycled on for 5 min and off for 2 min. **b** Normalized power output from 93 to 100% intensity. Plots in **a** and **b** were averaged from three trials and error bars are the SD between the trials. **c** Three consecutive intensity peaks for two of the LED light sources after being turned on show reproducibility of the intensity and shape of the power output curves

electronic on and off switching. Despite this downside, the ability to turn LEDs on and off rapidly and only turn them on when they are needed during experiments can save countless working hours for the unit and dramatically increase the light source lifetime and reduce power consumption.

Another major advantage with electronic shuttering is the ability to bypass manual microscope shutters that can have significant delays in opening and closing leading to excess light exposure to samples (Cole and Turner 2008). Rapid electronic shuttering is especially important for high-resolution 3D imaging, long-term live sample time-lapse imaging and low-brightness or low-stability dyes and can be used to automate manual microscopes for multicolour imaging. In principle, electronic shuttering can bring the switching speed down to microsecond or even nanosecond scales (Albeanu et al. 2008; Wessels et al. 2012). Transistor–transistor logic (TTL) shuttering directly linking LED control with camera exposure times improves accuracy of light exposure. This opens the door

to rapid ratiometric imaging of dyes like Fura2 without the need for moving parts and shutters (Tinning et al. 2018).

Another cited benefit of LEDs is their low electrical power consumption. In the final light source test, the electrical power consumption of each light source was measured. Most LED light sources had variable intensity options and the power consumption was linearly related to the light source intensity (Fig. 5). The different light sources did have variable overall power consumption and if run at the 100% light intensity have similar power consumption to MH and HBO light sources (Table 1). However, LED light sources can routinely be used in the range of 10–20% light intensity and then offer significantly lower power consumption relative to MH and HBO (Fig. 5).

Our group performed a detailed life cycle analysis (LCA) comparing the environmental impact of a MH and an LED-based light source from the same supplier (see Supplemental Materials). The overall conclusions from the LCA were that the LED light source was a better choice as it does not contain mercury and it uses significantly less energy over its lifetime. More specifically, over the total lifetime of each unit, the LED system had a fourfold reduction in the energy consumption of use, a 2.8-fold reduction in energy consumption of manufacturing (mostly due to the energy required to manufacture many MH bulbs while only one LED source needs to be made), and an almost threefold reduction in the acidification potential. The LED light source also had an almost threefold lower impact on global climate change, soil ecotoxicity and water ecotoxicity (see Supplemental Materials). In general, the LED light source is a better choice for reducing environmental impact as well as saving time, money and energy.

The results presented here demonstrate that all LED units have high stability of power output but not all LED units perform the same. For sensitive applications, power outputs should be tested to ensure accurate quantitative fluorescence imaging and some systems may be better for certain applications than others. Overall, the results presented here support the fact that LED light sources have a highly stable power output and are a good alternative to traditional HBO and MH bulb-based systems. They have a more rapid warm-up period from a few minutes up to about 10 min. They are highly stable on all the timescales tested from seconds (SD < 0.07%) to minutes (SD < 0.20%) to hours (SD < 3.5%) when compared to bulb-based systems. They can be rapidly switched on and off electronically with some variability in intensity over time depending on the LED light source. When operated at optimal powers (< 20% of maximum power output) they use

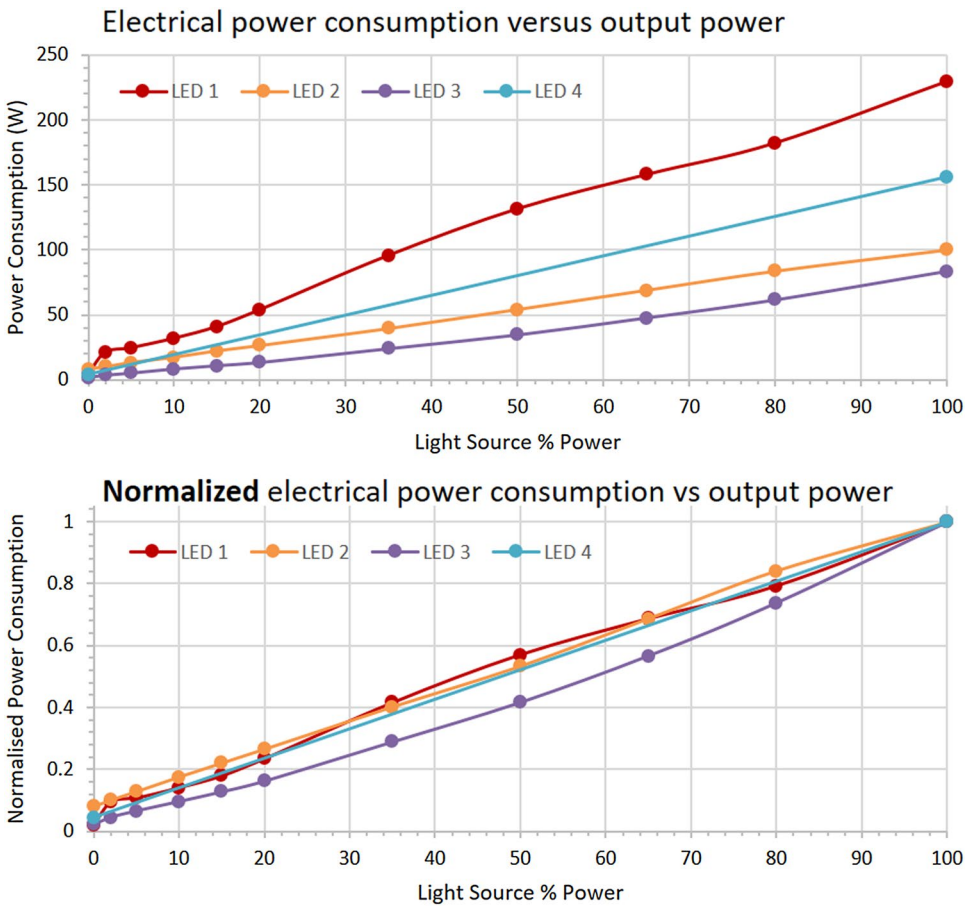

Fig. 5 Electrical power consumption of each light source. Electrical power consumption of each LED light source at various power outputs. Upper panel is the actual power consumption in Watts. Bottom panel is the normalized power consumption. Plots were averaged from three trials and error bars are the SD between trials but are too small to see on the plots

significantly less energy. Taken together LED light sources are recommended for quantitative fluorescence microscopy due to the stability of their light output. They are also recommended for reduced environmental impact, maintenance time, cost and energy savings.

Moving forward, LEDs with increasing power outputs are now starting to champion applications usually limited to laser illumination such as spinning disk confocal microscopy (Sato and Murthy 2012). The ultrafast electronic switching ability of LEDs makes them an ideal candidate for FRET and FLIM applications (Wessels et al. 2012). The small size of LEDs has allowed for compact optical designs ideal for miniaturized fluorescence imaging tools and for deployment in field work such as microscopy on space missions or in remote telemedicine applications (Wessels et al. 2012). The protocols presented here should be helpful to guide researchers to thoroughly compare and choose the best light source option for their applications.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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