What is the optimal light source for optical mapping using voltage- and calcium-sensitive dyes?

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Key words: chick, mouse, optical mapping, mercury lamp, LED, xenon lamp, di-4-ANEPPS

Abstract
Optical mapping is a fluorescence-based physiological method to image spreading of action potential in excitable tissues, such as the heart and central nervous system. Because of the requirements for high speed imaging in low light conditions, highly sensitive high-speed cameras together with an optical system with maximum photon efficiency are required. While the optimization of these two components is relatively straightforward, the choice of the perfect light source is less simple; depending on the other (usually fixed) components, various parameters may acquire different weight in decision-making process. Here we describe the rationale for building an optical mapping setup and consider the relative advantages and disadvantages of three different commonly available light sources: mercury vapor lamp (HBO), xenon lamp (XBO), and light emitting diode (LED). Using the same optical system (fluorescence macroscope) and high-speed camera (Ultima L), we have tested each of the sources for its ability to provide bright and even illumination of the field of view and measured its temporal fluctuations in intensity. Then we used each in the actual optical mapping experiment using isolated, perfused adult mouse heart or chick embryonic heart to determine the actual signal to noise ratio at various acquisition rates. While the LED sources have undergone significant improvements in the recent past, the other alternatives may still surpass them in some parameters, so they may not be the automatic number one choice for every application.

Introduction

Optical mapping using voltage-sensitive dyes dates back to 1980s(Kamino et al., 1981), and during its beginnings, various bottlenecks have been overcome. Chemists have focused on improving the dyes in the terms of cell membrane binding by increasing their lipid affinity, photostability, and shift of spectrum towards longer wavelengths that are more penetrant and less phototoxic for the tissues (Berenfeld et al., 2019) Similarly, the optical systems were streamlined by increasing numerical aperture of the objectives (although this is not always desirable as it decreases the depth of focus) and simplifying the light path (100% to the camera, wide diameter filters optimized for the particular dye). The detectors also evolved from a small array (max. 20x20, but typically less) of individually tunable photodiodes (Kamino et al., 1981)to much more user-friendly high speed cameras that excel in sensitivity (large pixels, high quantum efficiency, low noise, electron multiplication) and can run at speeds up to 10,000 frames per second (Vostarek et al., 2014). Also, their resolution is steadily increasing, due to increased demands from other applications requiring rapid readout at low light conditions, such as light sheet imaging.

Optical mapping is a fluorescence-based physiological method to image spreading of action potential in excitable tissues, such as the heart and central nervous system. Because of the requirements for high speed imaging in low light conditions, highly sensitive high-speed cameras together with an optical system with maximum photon efficiency are required (Efimov et al. 2004). While the optimization of these two components is relatively straightforward, the choice of the perfect light source is less simple, as depending on the other (usually fixed) components, various parameters may acquire different weight in decision-making process.

To record the optical signal, the object has to be evenly illuminated by a sufficiently strong source of light at appropriate wavelength. This can be achieved by several means, most of them being used in standard fluorescence microscopes. In addition, for low-magnification applications, incident light sources using either DC-powered halogen lamp with appropriate filters (Hewett et al., 2005) or LED lights (de la Rosa et al., 2013) were used as accessory or sole sources of illumination.

As a result, different labs are using different sources, making often direct comparison of their specific advantages and disadvantages difficult. This is complicated by manufacturers making different claims, emphasizing one or the other parameter (low noise, high luminosity) that are not always easy to verify by the users, especially in their specific settings.

We thus took the opportunity to compare, using the identical optical setup and specimens, relative advantages and disadvantages of three different commonly available light sources: mercury lamp (HBO), xenon lamp (XBO), and green light emitting diode (LED). Using the same optical system (fluorescence macroscope, SciMedia, Japan) and high-speed camera (Ultima L, SciMedia, Japan), we have tested each of the sources for its ability to provide 1) bright and 2) even illumination of the field of view and 3) measured its temporal fluctuations in intensity. Then we used each in the actual optical mapping experiment using isolated, perfused (Hlavacova et al., 2017) adult mouse heart or embryonic chick heart to determine 4) the actual signal to noise ratio at various acquisition rates. While the LED sources have undergone significant improvements in recent past,

the other options may still surpass them in some parameters, so the LEDs may not be the automatic number one choice for each and every application.

Methods

Light sources

The following light sources with the following parameters were used:

1) Two different HBO lamps (Ushio, Japan, rated at 300 h) connected alternatively to the same power source were used: one with 25 hours of life ("new"), and one with 250 hours of life ("old").
2) A 150 W XBO lamp (Ushio, Japan, rated at 1200 h) connected to a Cairn power supply had 60 h of life elapsed. Both lamps were directly coupled to the photomacroscope via a Uniblitz shutter.
3) A LED light source LEX2-LZ4-G (SciMedia, Japan) with internal electronic shutter and power control was coupled via a fiberoptic lightguide. The LED emission spectrum had a single peak with center wavelength of 530 nm, and >95% of the emission was between 500 and 550 nm (Figure 1).

Optical setup

A photomacroscope with 50 mm light path with tandem Leica lenses was used. The system uses epifluorescence illumination cube with 531/40 nm band pass excitation filter, 580 nm dichroic mirror, and 600 nm LP emission filter (Figure 1; optimized for voltage imaging using di-4-ANEPPS). For measurements on sham sample (fixed, di-4 ANEPPS stained rat heart) and adult beating mouse heart, the objective lens was 0,63x, and the projective 1.0x, resulting in field of view 16x16 mm. For mapping of the chick embryonic heart, the combination of 1.6x objective lens with 1.0x projective was used (field of view 6.3x6.3 mm).

Camera recordings

MicamULTIMA L CMOS camera (SciMedia, Japan) was used in Dif mode at rates of 500, 1000, 2000 and 10000 frames / second. The chip was 1x1 cm in size and of 100x100 pixels resolution.

The HBO and XBO lamps were turned on at least 30 min before recording. Since the LED light has a declared time to peak/stability of 3 ms, our standard shutter delay (500 ms) was more than sufficient to prevent any variation in intensity due to turning it on. Using the camera live mode, fluorescence sham plastic plate, and centering screws on the lamp housing and dichroic mirror, the light was centered carefully, and focusing lens was used to achieve as homogeneous field illumination as possible. Short recordings of 1024 frames were then made at various speeds, adjusting the light source (if possible) to obtain 80% of maximum sensor saturation (per camera manufacturer's recommendations) on the sham specimen. For determining the camera-related (dark) noise, recordings at 1 kHz and 10 kHz were made with the shutter closed. Same approach, but with higher recording time (1 - 4 seconds, depending on frame rate) was then used for recordings of a perfused adult mouse heart, prepared as described (de la Rosa et al., 2013) or ED4 chick embryonic heart (Sankova et al., 2010).

Data processing and analysis

Data were sampled at 1-10 kHz frequency and analyzed using BV_Ana software tools (SciMedia Brain Vision, Tokyo, Japan). Images from different light sources

were merged to ensure spatial correspondence of analyzed pixels and area from image center was selected for subsequent analysis (Fig. 3). Temporal intensity fluctuation in signal was measured without any spatial or temporal filtration. Signal wave was exported as a .csv file, time course of intensity was reconstructed, and intensity fluctuation was then measured using Microsoft Excel. Spatial homogeneity in signal amplitude over the mapped view was analyzed using strip map tool. Signal to noise ratio (SNR) from beating heart was measured by comparing amplitude change of base line (between two action potentials) with the maximum amplitude change during action potential (Laughner et al. 2012). Homogeneity of field illumination was evaluated on 16 bit grayscale images exported from the recordings in FIJI, where they were (at the same scale) reduced to 8-bit pseudocolor display (Black Body) for visualization (Figure 2). Line profiles of pixel intensity across the field were also inspected in the original 16-bit images in FIJI.

Statistical analysis

Results are expressed as mean ± S.E.M. Data were assembled and statistically analyzed in GraphPad Prism® 5 (version 5.01, GraphPad Software, Inc., San Diego, CA, USA) using ANOVA with Tukey *post-hoc* test. Differences were considered statistically significant when $P<0.05$, 0.01 and 0.001, respectively.

Results

Homogeneity of the field illumination achieved with different light sources is graphically represented in Figure 2. All images show a significant vignette effect due to high fill factor of the 1 cm^2 chip within the field of view. The most uniform appeared the LED source, while the highest variation between the center (peak) and the periphery of the image was with the HBO source. The XBO lamp appeared the least bright of the three sources.

All light sources had sufficient intensity for imaging even at the maximum frame rates, although only the LED source was able to achieve 80% pixel saturation at 10 000 frames/second. The noise due to intensity fluctuations was the lowest in the LED (Figure 2). However, the difference between LED and XBO was minimal, and SNR was comparable at about 20:1 in the perfused adult mouse heart (Figure 3) and 12:1 in the chick embryonic heart (Figure 4). However, the area from which it was possible to generate the map was smaller in the XBO-illuminated mouse heart due to worse illumination homogeneity and thus insufficient signal from the curving portions of the heart near the edge (Figure 3). Both illumination intensity and SNR were good with the fresh (25h of life) HBO; however, in the aged one (250h), the flicker noise became significant, resulting in SNR deterioration to 4:1in the chick embryonic heart (Figure 4). From recordings with all three light sources, it was possible to generate activation maps thanks to signal filtering, and those were comparable at all sampling frequencies (Figure 3 and 4).

Dark noise of the camera was uniform at different recording speeds and oscillated 6 levels above and below the mean level, thus not contributing significantly to the noise from the illumination source.

Discussion

Spectrum characteristics and illumination method

The spectrum of light sources used in this study and its relationship to the excitation and emission spectrum of the most popular voltage-sensitive dye di-4-ANEPPS could be appreciated in Figure 1. The excitation filter used spans almost the entire green peak of the HBO, making it appear sufficiently bright despite its otherwise discontinuous spectrum compared with the XBO. Interestingly, although often considered as monochromatic, the green LED has a peak at 530 nm with 95% of its emission contained in 50 nm band between 500 and 550 nm. This means that it can be used also directly as an incident illumination source for this purpose without excitation filter, and such solutions are popular by some labs either as hand-made rigs (e.g. Efimov lab) or commercial solutions (Cairn Macro LED green, SciMedia LEX2-LZ4-G). However, the epifluorescence excitation has the advantage on concentrating the light exactly on the imaged area using high-quality optics of the objective lens and avoids additional holders around the specimen. This is advantageous especially if other elements (stimulation or recording electrodes, perfusion system components) are in place. It also allowed us direct comparison with the other sources (HBO, XBO).

Biological factors

Photobleaching occurs in all biological samples with sufficient intensity of illumination and could pose problems in both morphological applications (e.g., confocal microscopy) as well as optical measurements of physiological parameters such as voltage or calcium concentration. In our experience, the gradual drift in baseline fluorescence is noticeable during all recordings, and within the typical 4 s recordings represents typically less than 2%. The BV_Ana software (as well as other applications for analyzing optical action potentials) has a built-in feature for correcting this systematic problem by compensating for this drift. The magnitude of photobleaching depends on intensity of illumination (light energy concentration) and is more pronounced with high magnification, high NA objectives (e.g. water immersion 20x lens). In practice, we use in our experiments at most 8 s long recordings, and in many different species analyzed to date (from fish to mammals), we never saw a significant decrease in S/N ratio during this period. In repeated measurements on chick embryonic hearts (effect of temperature changes, spontaneous vs. paced beat, (Vostarek et al., 2016)), we noticed the first decrease in S/N ratio after more than 30 s of cumulative illumination with 10x, NA 0.4 water immersion lens. Much larger problem was phototoxicity of dye degradation product and free radicals, resulting after this period in bradycardia, cessation of spontaneous heart beat or atrioventricular block. In Langendorff-perfused mouse heart, decrease in S/N was noticed 30 min after staining and was described to dye internalization (Hewett et al., 2005) and could be reverted by re-staining of the samples.

To minimize all these problems, we used the shortest possible illumination times for the samples in this study, and the maximum total illumination time (divided in several doses) was 12 seconds. Alternatively, to distinguish the artifacts from plain specimen fatigue, golden standard electrode recordings (Kittnar et al., 2018) could be used.

Alternative light sources

While we chose the three most popular epifluorescence sources, there are other alternatives for the optical mapping, one being a 150 W halogen lamp. It needs

very tight power supply control to avoid any AC noise (in fact, we used a model operated by a rechargeable car battery in the past), and is still fairly dim at the wavelengths required (according to manufacturer's measurements, the LED light we used is approximately 3 times brighter than the tungsten lamp). Since it has low output at shorter wavelengths, wide bandpass excitation filters are helpful (same for the XBO). Thus, we used the halogen source primarily as an additional illumination to further increase the signal and improve homogeneity of illumination of the curved heart surface (Hewett et al., 2005).

Limitations of current light sources

We would caution all potential LED users to insist on demonstration and testing in their actual setup, as not all LEDs are created equal. For example, we found the previous model (according to manufacturer also about 3 times less bright) so dim that it was only useable as a booster light; however, this might be partly due to suboptimal coupling and focusing on the specimen, which is also essential for good results (and main reason why we use and recommend the epifluorescence illumination mode that elegantly takes care of this problem).

Another potential problem with LEDs is their limited versatility – while performing indeed spectacularly at particular wavelength (in this case, for the rhodamine channel), a different, similarly expensive LED needs to be obtained for fluorescein channel applications, since the white LED (popular nowadays for visible light observation) is currently too weak for filter-based applications. LED control with pulse modulation generating less heat is common in room illumination because human eyes can't recognize the flashes. However, high stability LED illumination system used controls LED intensity by the amount of current. The LED intensity feedback system is driven at more than 10kHz frequency so that fast cameras operating typically at 1-2 kHz does not register the minute flickering.

Larger versatility is offered by the HBO, which is particularly strong in the UV region, used by some calcium probes. However, by testing side by side different calcium indicators, we found that rhod-2, which uses the same channel as the di-4-ANEPPS, showed the most reproducible measurements and also specimen stability due in part to longer wavelength used (Vostarek et al., 2016). XBO, on the other hand, is especially useful for higher, less cytotoxic and more penetrant wavelengths used by a new generation of voltage-sensitive dyes (Salama et al., 2005).

Regulation of power output

A common feature of all high-speed cameras is a limited possibility to control exposure time at maximum recording rate. The exception are EM-CCDs, where one can meaningfully fine-tune the gain. It is thus useful to have some control of output power regulation (although in the past, it was seldom a problem as there was never too much light). HBO does not offer this feature, so the only way is to use attenuation filters, which are usually included on a standard fluorescence microscope. The power regulation option of the XBO was particularly useful, as this allowed us to save the lamp life by running it at lower power (80-100 W) and then switching to higher (or maximum) power only during the actual recording. Regulation of sample illumination intensity (in addition to temporal limitation of exposure to light) also improves its viability and decreases

formation of the reactive oxygen species, detrimental for heart function (Sarre et al., 2005). The LED source offers the ultimate control (0-150% of rated power), allowing both continuous observation as well as burst recording (power over 100% leads to quick overheating and shuts the diode off automatically after several seconds).

Additional factors to consider in the light source selection

Another variable to ponder when selecting a light source is the cost of its operation (Table 1). The weight of this factor varies depending on how much the system is actually used, whether it is a single-purpose setup, or some versatility is required (different magnifications, cells or whole organs, different indicators, etc.). Another consideration is the environmental impact, which is significant in the case of HBO where one has to dispose of the used mercury bulbs appropriately.

Other sources might be also considered (and tested), such as mixed mercury lamp offered by some manufacturers (e.g. Olympus) and touted for its stability and longer bulb life for time-lapse recordings, or even lasers (Dillon and Morad, 1981) were reported by some investigators as a viable light source for some applications. The quest for the perfect light source is thus not finished and there is still room for further improvement. The relative advantages and disadvantages of the light sources tested in this study for optical mapping are summarized in Table 2. At the present time, we would recommend the HBO as a versatile, widely available startup option, while LED might be the best choice for a dedicated setup due to its brightness, stability, and long life.

Acknowledgements
We would like to express our thanks to the manufacturers (Olympus, Cairn, SciMedia) for making available the equipment for testing, and for providing various tips and detailed technical information. Technical assistance of Ms. Marie Jindrakova with the animal maintenance (mice) and solution preparation is also appreciated. This research was supported by grant no. 18-03461S from the Grant Agency of the Czech Republic, Ministry of Education, Youth and Sports of the Czech Republic - PROGRES-Q38, INTER-COST LTC17023, and LM2015062 Czech-BioImaging (Modernization and support of research activities of the national infrastructure for biological and medical imaging Czech-BioImaging funded by OP RDE, grant number: CZ.02.1.01/0.0/0.0/16_013/0001775), Charles University UNCE, Grant agency of the Charles University 1456217, and institutional financing from the Czech Academy of Sciences RVO: 67985823.

Table 1. Comparison of different light sources for optical mapping.

Source/parameter	HBO 100 W	XBO 150 W	LED 30 W
Spectrum	multiple peaks	continuous	single peak
Center intensity	+++	+	++
Field homogeneity	+	++	+++
Temporal oscillations	moderate, worsen with time	moderate, worsen with time	small
Output control	no (ND filters)	within range (60-100%)	continuous
Lamp life	200 h (approx. 70 in practice)	1200 h (approx. 600 in practice)	10000 h (?)
Cost of purchase/ bulb replacement	$5000 / $400	$8000 / $ 700	$7500/?

Table 2. Relative advantages and disadvantages of different light sources for optical mapping.

Source	HBO 100 W	XBO 150 W	LED 30 W
+	•General availability •Low purchase cost •Good peak at desired wavelength •High brightness at the center	•Continuous spectrum •Regulatable (within limits)	•Very low noise •Excellent field homogeneity •Complete intensity control •High brightness
-	•Short usable life •Environmental concerns •Uneven field homogeneity	•Replacement cost significant	•Low versatility •High purchase cost

Figures with legends

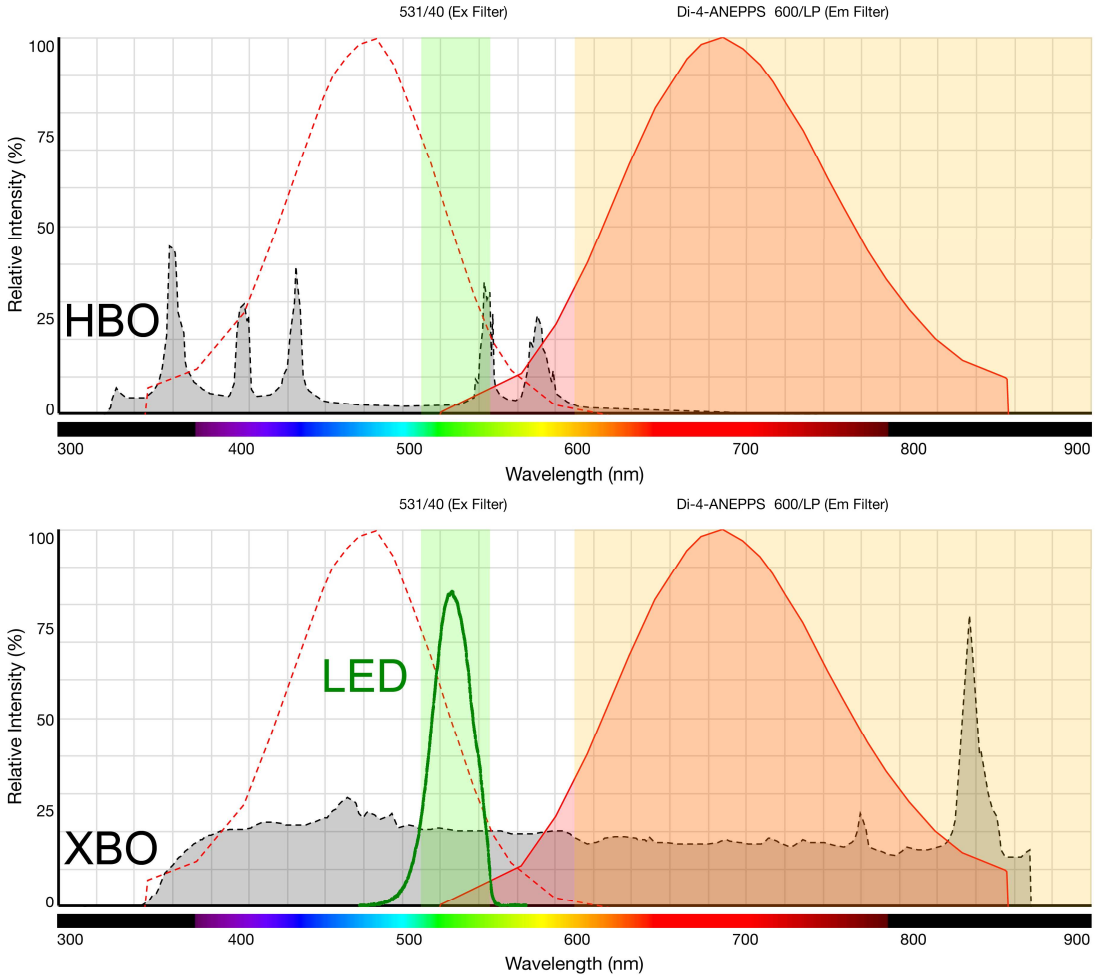

Figure 1. Spectrum of the light sources used related to the excitation (dashed red line) and emission (solid red line) of the voltage-sensitive dye di-4-ANEPPS. The bands of the excitation (green) and emission (salmon) filters used are also indicated. Note that only the green portion of the broad excitation spectrum shows voltage dependence (drop of emission intensity with membrane depolarization, (Witkowski et al., 1997)).

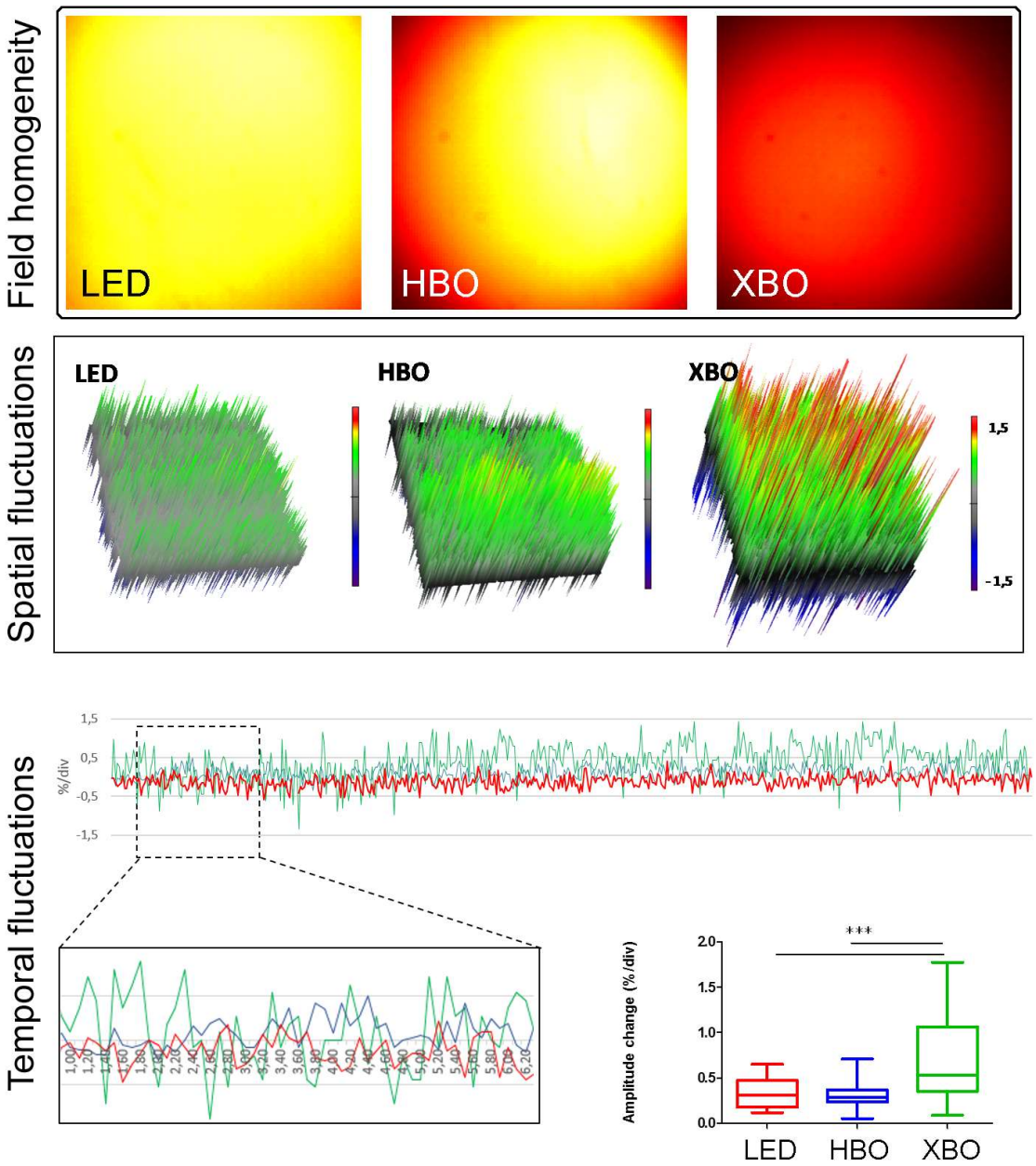

Figure 2. Graphic comparison of pertinent parameters of light sources. Top row shows the homogeneity of the field of illumination (pseudocolor display BlackBody), which is the best for LED. HBO has a bright center and dim periphery. XBO is the dimmest, but with less center-periphery difference than HBO. Comparison of spatial (middle row) and temporal (lower row) fluctuations of light source intensity show that the most stable is LED, followed by HBO (new lamp) and XBO.

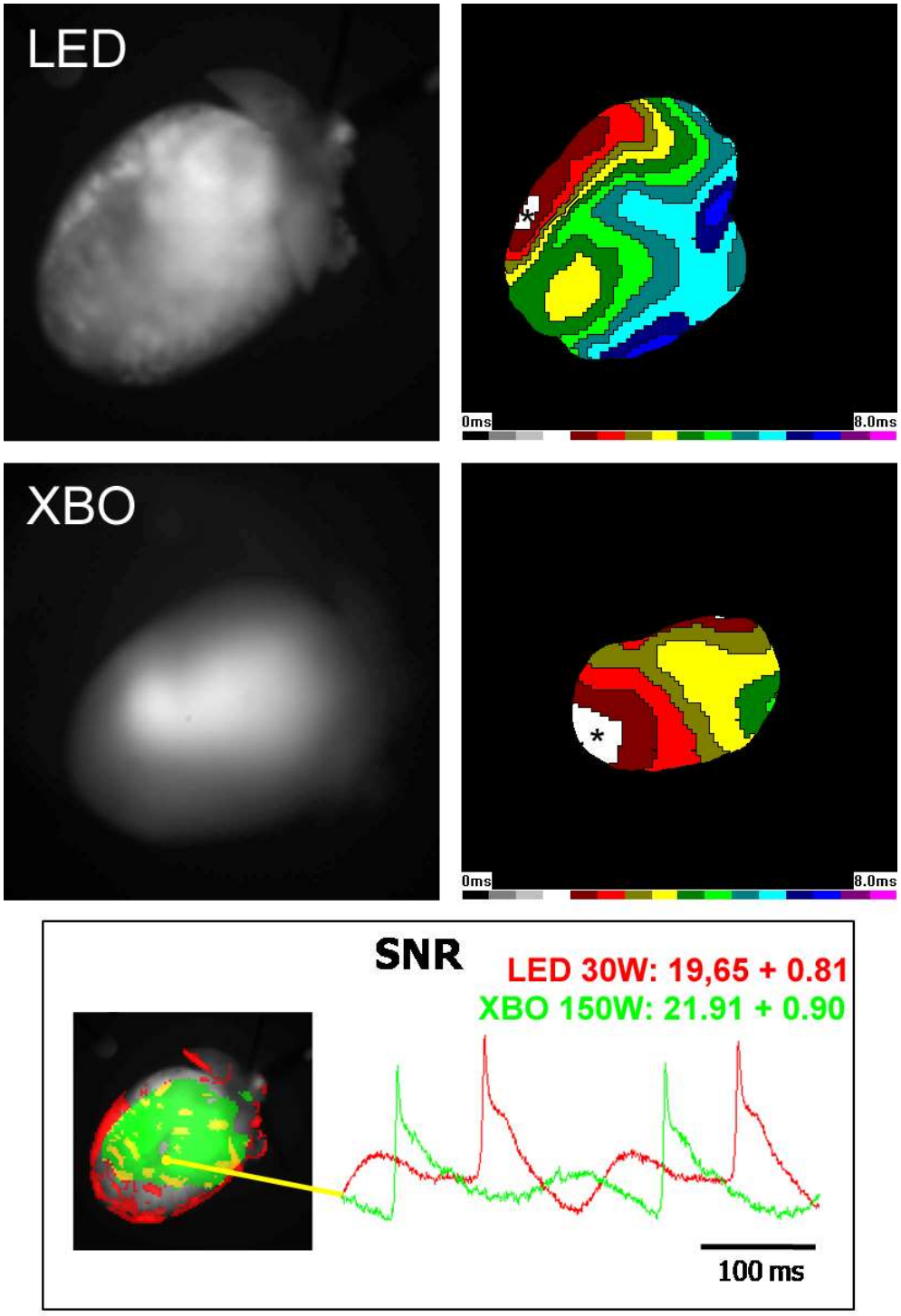

Figure 3. Comparison of epicardial activation maps of the adult mouse heart generated from the recordings at 2000 frames per second and individual optical action potentials (bottom), obtained from the center of the heart. While the SNR and maps are comparable between the sources, the image from XBO results in smaller area with useable SNR for map generation due to less even field illumination than obtained with the LED source. Isochronal interval 0.5 ms, asterisk indicates the first place of activation. Merged raw 2D data (yellow) from LED (red) and XBO (green) light source image (lower row). The action potential tracings are highly similar (successive recording from the same heart).

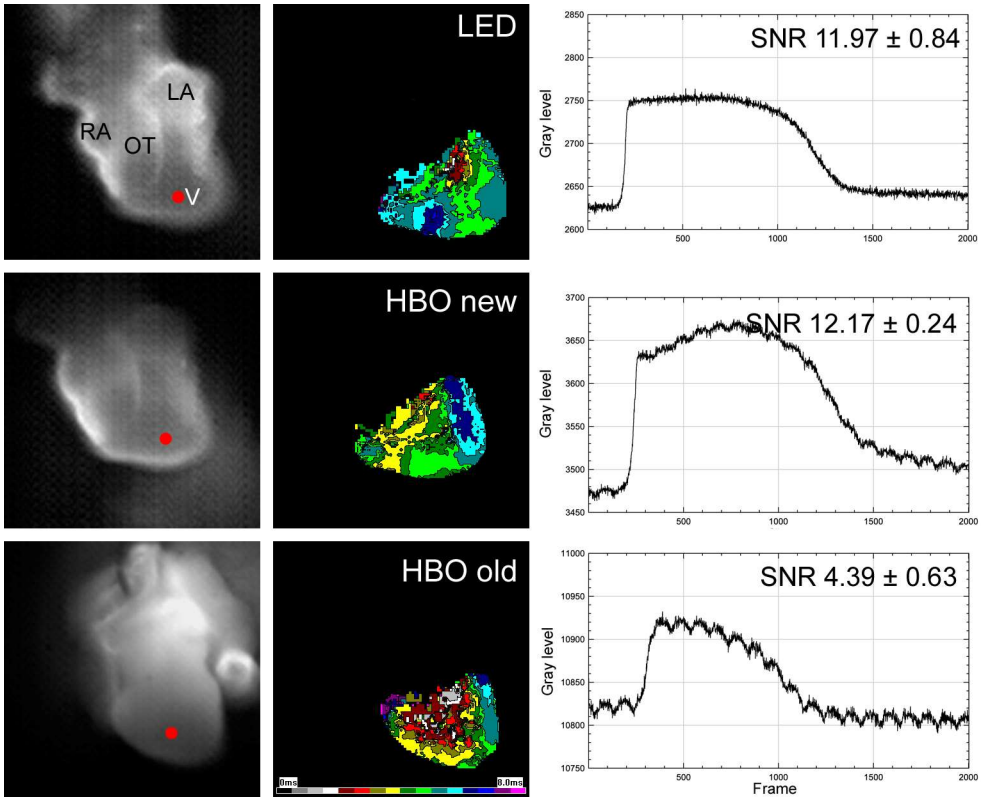

Figure 4. Effect of ageing on HBO performance. Activation maps (left) and optical action potentials (right, unfiltered raw data, gray levels at the same scale) obtained from the region indicated by the red dot show the superior quality of recordings with the LED. While the new HBO had a comparable SNR (recording performed on the same heart), decreased amplitude of the action potential and increased noise is noticeable in the old lamp. All recordings were made at 10 000 frames per second, the maps were generated with 0.5 ms isochronal intervals.

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