Improvements in fluorescence microscopy allowed by high power light emitting diodes

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Fluorescence excitation, even in modern microscopes, is generally achieved by means of arc lamps. The peculiar characteristics of high energy and whole spectrum of emission of these lamps are associated with some disadvantages in terms of safety and global efficiency. High pressure Hg and Xe lamps in some circumstances may in fact explode. Furthermore among the total electrical power, less than 1% goes to the sample while the rest 99% is wasted or dispersed through heat. A new high efficient illumination system is proposed based on the use of high power light emitting diodes (LEDs). Both 1 and 5W "Luxeon Star" LEDs have been employed. A specific plastic light condenser lens have been designed and made by Fraen. Blue and green LEDs have been tested to generate the corresponding two excitation bands. These light beams have been compared with similar bands delivered by the HB0 100 W lamp. The measurements of light intensities at the objective lens output allow to state that the Luxeon STAR LEDs can deliver a light beam of radiant intensity similar to the blue and green bands delivered by the standard HBO 100W lamp. Based on these experiments a prototype of an advanced, all solid state, illuminator had been realized. It can replace conventional excitation arc lamp system as well as up-grade conventional bright field instruments to fluorescence microscopes.

Keywords Fluorescence microscopy, instrumental developments, light emitting diodes

1. Introduction

Conventional fluorescence microscopy is up to now largely based on the use of mercury or xenon arc lamps as excitation source. Other than filament lamps delivering only low energy radiations sufficient for transmitted light illumination (bright/dark, phase contrast, etc) fluorescence observation require more energy to stimulate light emission from the sample. Since many decades arc lamps are the more brilliant power light generators widely used in many applications [1,2,3]. For few specific micro-techniques like scanning and confocal microscopy as well as flow cytometry the peculiar characteristics of laser light have been successfully applied [4]. Furthermore the great progress of micro-electronics have recently made available a new generation of solid state lasers having similar optical performance of the previous water or air cooled ion gas lasers. Other than laser improvements also the light emitting diodes (LEDs) have a continuous power growing in these last few years. From general application as light indicators they are now going to replace conventional illumination sources at least in some specific applications. There are now available high power LEDs able to provide optical radiant flux of more than 0.5 Watts, inside a narrow spectral band, over almost the whole visible spectrum. Being the emitted light spatially incoherent but restricted to a narrow color band, high power LEDs can be considered a bridge between arc lamps and lasers. The aim of the present paper is the evaluation of the potential capability of LED to be applied as excitation source in fluorescence microscopy. It is in fact well known that in any fluorescence microscopes equipped with mercury or xenon arc lamps a very limited portion of the total emitted light (all over the optical spectrum) is practically available to the sample under the objective lens. From a 100 W HBO lamp, after a proper filter selection, required to match the absorption band of the fluorochrome(s) involved, few hundreds mW of light is the true available power. Furthermore taking into account the great light losses induced by the optical components the beam light delivered by the objective lens is in the order of few decades of mW (that means less than 1% of the electrical power of the lamp).

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Taking into account that the new generation of high power LEDs can provide a light flux higher than 0.5W some preliminary applications in the field of microscopy are already described in the recent literature [5,6,7,8,9,10,11]. The established standard production of "white LEDs" has recently pushed the competition between solid state and conventional lighting devices. The intrinsic advantages of LED lighting and among others the limited energy consumption as well as the very long life have been already exploited. Furthermore the recent production of established microscope companies include a white LED in the bright field illumination systems. Based on the ultra-bright Luxeon LEDs from Lumiled an advanced illumination solid state device is described aimed to replace Hg and Xe lamps in existing fluorescence microscopes and/or upgrade to fluorescence the normal bright field microscopes. Finally the same illuminator has allowed the design and construction of a prototype of a portable fluorescence microscope fully operated by rechargeable battery.

2. Material and methods

The "Luxeon" series "Stars" of 1 and 5 Watts of high power diodes from Lumiled company (Lumileds Lighting, LLC, USA) have been tested. A power supply with voltage and current regulation has been employed for the preliminary study aimed to the spectral characterization of the diodes. A commercially available 6.5 V, 3A stabilized power supply in combination with rechargeable Ni-Cd battery (3Ah) was employed for the portable microscope. All the electronics as well as all the mechanical parts were made on purpose by Fraen. A special series of plastic lenses were also designed and made on purpose aimed to generate a parallel beam of light to be directed into the epilluminator. A series of interference filters and dichromatic mirrors have been supplied by Chroma (Chroma Technology Corp, USA) matched on the emission peaks of the selected diodes.
For spectrographic analyses of the diode emission a multichannel spectrum analyzer Hamamatsu (Hamamatsu Photonics KK, Japan) model PMA-11 was used. A portable power meter Delta Ohm LP9221 were employed for light intensity determination an comparison tests between lamp and LEDs. For the preliminary study of project feasibility an Orthoplan Leitz (Leitz Germany) equipped with epifluorescence "Ploem Opak" and conventional HBO 100 W mercury arc lamp excitation was employed. A series of biological samples including cell cultures grown on coverglass, were employed as sample test for "visual comparison" trials. Samples were processed and stained by different protocols based on the use of Propidium Iodide and Fluoresceine Isotiocianate.

3. Results

From the large family of Lumiled diodes the V stars serie was selected on the basis of the quite complete data sheet available from the producer. Comparing the spectral range and light flux reported, and taking into account our target, i.e. the fluorescence stimulation of biological samples, the following diodes were selected. The 5W blue around 485 nm, the 5W green close to 520nm and the 1W red around 620nm. Finally the normal 1W "white" has been used as bright field illumination of the "portable fluorescence microscope" prototype.

3.1 Lens condenser

The diodes we used are commercially characterized as "dom". In this version the emitting part of the chip is already equipped with a small spherical lens and the radiation pattern shows a lambertian emission. The Fraen Company designs, makes and distributes a quite complete series of additional lenses, made on purpose for many lighting projects, based on the use of this model of Luxeon diodes. In Fig. 1 the plastic lens condenser we used is represented. It is designed on purpose to fit exactly over the surface of the existing lens of the Luxeon Lambertian. The lens is then definitely assembled to the LED board in order to made a compact unique lighting device.

Fig. 1 Pictures representing the LED illuminator. On the left the fully assembled device. On the right the various components: a) the plastic lens condenser, b) the single LED chip and c) the chip mounted on the metal plate.

3.2 Electrical characteristics and power supply

As detailed in the data sheet available from the producer (www.Luxeon.com). The maximum operating current is 350 mA for the 1W and 750 mA for the 5W, the operating voltage for the 1W and 5W Luxeon diodes being variable around 3.5 [1W] and 6.5 (5W) V D C. At maxim current rate the diode generates a consistent quantity of heat that must be dispersed by means of a proper metal radiator. The 1W model only require a simple aluminium plate of 3x3 cm while the 5W model when operated for long time (hrs) at maximum output requires a more efficient thermal radiator able to prevent overheating of the emitters. In our case the metal block visible in Fig. 3 having the main task to fit the illumination device on the microscope body is also an over-sized thermal conductor. Even on stressing condition of ten hours continuous working the temperature of the chip surface was about 50°C, which was always down the upper limit suggested by the producer (from 70°C to 90 °C depending on the thermal parameters).

3.3 Spectral characterization of diodes emission

By means of the spectrum analyser we have tested the spectral distribution of light emitted by several types of diodes we were interested in. In Fig. 2 the spectral curves of four three of 1W diodes as well as two types of 5W are reported. There are important emission differences between 1 and 5W series. All the tested LEDs are anyway characterized by a narrow emission band of around 50 nm centered on the typical frequency of the emission. As far as our specific needs is concerned the three diodes we used have the following spectral range: 5W blue (485 ± 10nm), 5W green (520 ± 12nm), 1W red (640 ± 8nm).

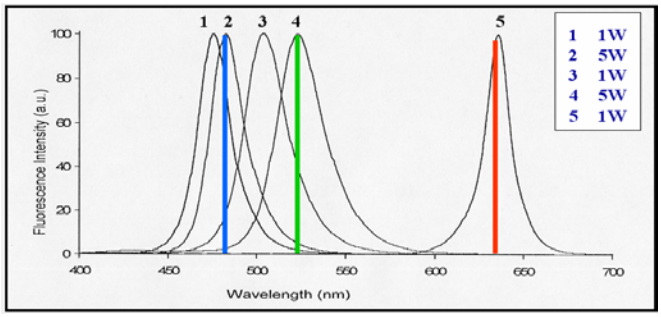

Fig. 2 Spectral distribution of light emitted by few tested 1W and 5W high power Luxeon Star LEDs. The curves n° 2 and 4 refer to the LEDs utilized in the comparison test with the HBO 100W lamp. Together with n° 5 are also mounted in the multi-color illuminator described.

3.4 Comparison between HBO lamp and LEDs

In order to evaluate the excitation capability of the LEDs, i.e. their efficiency in fluorescence stimulation on a conventional fluorescence microscope, the special holder shown in Fig. 3 had been realized. The aluminium block has a diameter fitting the inside of the microscope hole normally used for the lamp excitation inlet. The LED is mounted at one side of the block while the wires come out the opposite side of the holder, to be connected to the external power supply. The blue and green LEDs had been alternatively easily replaced on the holder. This design allows the LED to be located just in front the entrance of the epilluminator with minimal light lost. The metal mass of the holder is itself enough to disperd the heat generated by the LED. The mirrors/filters combination of Leitz epilluminator has resulted compatible with the light bands delivered by the LEDs. The "blue position" is in fact equipped with an interference filter 450-490 and a dichroic mirror DM 510 while the "green position" has an IF 530-560 and DM 580. Table 1 reports the measurements performed on the Leitz Orthoplan microscope equipped alternatively with the HBO 100W arc lamp and with the LEDs (blue and green). The light intensity has been measured by the power-meter probe placed in different positions of the microscope stage: a) at the entrance of the epilluminator device (before the excitation filter); b) at the epilluminator exit (after the interference filter and dichromatic mirror reflection); c) at the exit of the objective lens (at the sample plane). Note that in case of the lamp the first measurement is omitted while without filter selection the whole lamp emission "saturate" the power-meter. Measurements were performed in the same instrumental conditions in position 2 (blue) and 3 (green) of the epilluminator just replacing the lamp with the LEDs. In this instance the LEDs were used without any additional interference filters.

Table 1 Light intensity values (in mW) measured by a power meter in various positions of the optical pathway of the Orthoplan Leitz fluorescence microscope. Alternatively the HBO 100W arc lamp or LEDs (n° 2 blue and n° 4 green as in Fig. 1) had been installed as excitation sources. Comparative mesurements were performed in the same instrumental conditions.

	LED blue	HBO blue		LED green	HBO green
EPILL. in	362	Over Flow		244	Over Flow
EPILL. out	60	56		50	25
OBJECT. out	20	17		18	13

3.5 Comparative observation

Few biological samples, preliminary stained by means of PI and FITC, were used in order to evaluate the excitation performance of the LEDs as compared to the conventional mercury arc lamp. As already demonstrated by the light intensity measurements both the blue and green diodes allow to see a bright green and red emission from the above mentioned samples. Comparative observation of the same biological specimen sequentially excited by LEDs and lamp in the same optical setting didn't allow to appreciate any "visual" difference in the emitted fluorescence intensity.

3.6 The portable microscope prototype

Being verified the capability of LEDs to provide similar excitation power as allowed by conventional arc lamp we further proceed with the project of a fully portable fluorescence microscope. First step was the

design of a multi-source excitation device able to allocate different colour emitting LEDs. Fig. 4 reports the final version of the realized prototype carrying the blue, green and red diodes. It is made in aluminium to provide a metal body working as a thermal radiator. Once installed in the microscope body the illuminator can be rotated and the mobile contact turns on the LED fitting the "work" position, while the other two remain off. In front of the three condenser lenses the interference filters are mounted in order to further restrict the light emission band. Fig. 5 shows the microscope made inside of a commercial rigid bag (50x40x20 cm) for a fully portable out-lab use. The lower half bag houses the battery and the electronics for both fluorescence as well as the "bright field" illumination. This latter is also made by 1W white LED equipped with a specific plastic lens . The 220 V AC external power supply is used for both battery re-charging and for in-door direct supplier of the illumination systems. With fully charged battery the microscope can operate in fluorescence for at least three hours without external electrical connection. In order to have a compact-basic microscope the single ocular version was build up based on the use of a simple metal cube. This latter houses the place of interchangeable dichromatic mirror, the barrier filter holder and fits the objective lens below and the ocular in the upper position. The cube and the illuminator are fixed to a mobile arm able to rotate 90° down to the plane of the microscope stage. This allows the upper part of the microscope to be housed down to the closed bag. Before to make this step the ocular and its holder have to be removed and placed in a dedicated place in the bag. The microscope stage allows the x y movements for specimen handling as well as the z movement for focusing. A control panel on the left of the stage provides all the necessary switches for the LEDs controls as well as for the external 220 AC or internal DC operating mode.

Fig. 3 Application of LED excitation on the Orthoplan Leitz fluorescence microscope. On the left the diode with its condenser lens is shown outside the microscope. On the right the solid state illuminator in "working" mode.

Fig. 4 The portable fluorescence microscope. On the left, details of the rotating multicolor solid state illuminator housing the three LEDs (upper) coupled with the corresponding interference filters (below). On the right the view of the microscope turrette holding the illuminator. The microscope stage with the sample movements as well as the switches controlling bright field and fluorescence excitation can be seen at the bottom.

Fig. 5 The portable fluorescence microscope. On the left, the open bag with the microscope "ready-to-use". In the central picture the microscope is turned in the "close-mode". On the right the top mobile part of the microscope is placed down to the bottom of the bag. The external 220 vAC can also be recovered inside the bag.

4. Discussion and conclusion

Light emitting diodes were constantly improved in the recent past and their technological development is continuously growing up. The efficiency they have in the electro-optical conversion is definitely higher as compared to filament and other lighting systems. On the other hand the peculiar characteristics of LEDs to be "solid state" and therefore compact, small and practically insensitive to shock made them very attractive for lighting purposes. Furthermore the low DC voltage and current they need combined with a limited amount of heat produced made the new generation of high power LEDs very interesting in many specific applications. Particularly in those cases where coloured light is required (light crosses and road indicators) they are going to replace conventional illumination devices. Among the various LEDs commercially available, the family produced by Lumiled on the name LUXEON seems to be, this time, the more brillant and therefore suitable for innovative applications. In the frame-work of a fruitful cooperation between the expertise of Fraen Company leader in lenses design now dedicated to LED lighting and a group in Pavia dealing with fluorescence microscopy a new project was exploited. It was

focussed on the evaluation of the possibility to replace arc lamps with LEDs illumination for fluorescence microscopy. In some modern commercial microscopes white LEDs have replaced filament lamps for the bright field illumination. On the contrary fluorescence microscopes are basically equipped with arc lamps or upgraded, for sophisticated applications, with very expences lasers. Preliminary experiences are reported in the recent literature exploiting the excitation performances of the high power LEDs especially in the area of time decay studies [8,9,12,13,14,15]. Our results confirm that the actual production of Lumiled LEDs has enough power to stimulate fluorescence of biological samples normally observed by means of conventional fluorescence microscopy. The comparison we made by both light intensity measurements as well as by visual observation did validate our hypothesis that LEDs are bright enough to excite fluorescence in microscopy. Taking into account the very limited power of the ultraviolet LEDs actually available on the market we didn't include these emitters in our trial. We therefore focussed on the blue and green LEDs being the most common fluorochromes employed in cytochemistry as well as in immunostaining excited with these two light bands. In addition we included in the illuminator prototype the red LED 640 nm while there is emerging interest in few preliminary application based on the use of the far red emitting molecules whose absorption is in the near red where some diodes have their emission peak. By the spectral study we have verified that the LEDs emission in the series of few similar pieces is quite constant. May be a more important variation may occur for different production lots. The spectral characterization of the LEDs allowed us to choose a dedicated interference filter to put in front of each diode. We can therefore be sure to cut out any undesidered secondary emission of light who can compromise the quality of fluorescence observation. The preliminary experiments we have carried out on a Leitz microscope validated, on the practical point of view, the possibility to replace arc lamps with high power LEDs. The solid state illuminator we have described (TM by Fraen) is now ready to be mechanically adapted to any existing fluorescence microscopes. The preliminary experience we made on the Leitz Orthoplan microscope allows to start up a project of a fully portable fluorescence microscope [16,17]. His actual design was drown with the goal to have the instrument portable and "working" in a hard bag of a convenient sizes and weight. Therefore the solution we found is based on a folding microscope body using the lower part of the bag as a mechanical base including the electronics and holding the sample stage. The upper part of the microscope is up in the "working mode" or turned and replaced in the lower part of the bag when this latter is closed. The evident advantage of this design is to have a very compact, small (50x37x21 cm) relatively light (less than 10 Kg, battery and AC supply included) instrument in a easily portable bag. Further improvements are planned dealing with pulsed fluorescence excitation. Being the switching time of diodes very short (nanoseconds) they can be operated from DC or high frequency [5,14] till few Hertz. In these last case a low flashing excitation can be applied resulting in a less energy delivered to the sample. Matching a suitable frequency of excitation the fluorescence image will remain stable to the observer eyes, while the sample will have a reduced photo-bleaching effect. Other innovative project based on the portable fluorescence microscope deals with the counting of cell subpopulation present in very low frequency in biological samples (absolute counting of "rare events"). Actually these applications(including the monitoring of the AIDS risk population)fall down the application field of flow cytometry. We are working both on the side of a sensitive compact fluorescence microscope-counter PC assisted as well as on a "cell capture" device aimed to collect a specific cell subpopulation in a small area of a modified cover-glass. The microscope will focus, at low magnification, a defined area and the fluorescing cells will be viewed by a sensitive CCD camera. Whenever the number of cells to be enumerated is in the order of hundreds the PC will counts in seconds. Few preliminary data seem to validate the possibility to upgrade the fully portable fluorescence microscope here described to a simple cell counter, who will have interesting clinical applications (e.g. monitoring of AIDS risk population in the poor emerging countries).

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