A Standardized Light Emitting Diode Device for Photoimmunotherapy

E. de Boer,1,2 J. M. Warram,1 E. Hartmans,3 P. J. Bremer,4 B. Bijl,5 L. M. A. Crane,1 W. B. Nagengast,3, E. L. Rosenthal1 and G. M. van Dam2

1. Department of Surgery, University of Alabama at Birmingham, USA.
2. Department of Surgery, University Medical Center Groningen, University of Groningen, The Netherlands.
3. Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, The Netherlands.

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ABSTRACT

INTRODUCTION
Antibody based photodynamic therapy, i.e. photoimmunotherapy (PIT), is an ideal modality to improve cancer treatment due to its selective and tumor specific mode of therapy. As the use of PIT for cancer treatment is continuing to be described, there is great need to characterize a standardized light source for PIT application. In this work, we designed and manufactured a Light Emitting Diode (LED) / PIT device and validated the technical feasibility, applicability, safety and consistency of the system for cancer treatment.

METHODS
To outline the characteristics and photo biological safety of the LED device multiple optical measurements were performed in accordance with the IEC62471 photo biological safety standard. A luciferase-transfected breast cancer cell line (2LMP-Luc) in combination with panitumumab-IRDye700DX (pan-IR700) was used to validate the in vitro and in vivo performance of our LED device.

RESULTS
Testing revealed the light source to be safe, easy-to-use, and independent of illumination and power output (mW cm$^{-2}$) variations over time. For in vitro studies, a LED-dose (2 J cm$^{-2}$, 4 J cm$^{-2}$, 6 J cm$^{-2}$) dependent cytotoxicity was observed using propidium iodide exclusion and Annexin V staining. Dose-dependent blebbing was also observed during microscopic analysis. Bioluminescence signals of tumors treated with 0.3 mg pan-IR700 and 50 J cm$^{-2}$ decreased significantly (>80%) compared to BLI signals of contralateral non-treated sites at 4 h and 1 day post PIT.

CONCLUSION
To our knowledge, a normalized and standardized LED device has not been explicitly described nor developed. Here, we introduce a standardized light source and validate its usability for PIT applications.
INTRODUCTION

The ultimate goal of cancer treatment is obtaining complete removal of tumor tissue while minimizing damage to surrounding healthy tissue.\textsuperscript{1,2} Antibody-based photodynamic therapy (PDT), i.e., photodynamic immunotherapy (PIT) can become an ideal modality to improve cancer treatment due to its inherent selectivity for targeting tumors. The application can be used for both initial treatment and eliminating residual (microscopic) disease during incomplete resection, which is common in pancreatic cancer (~75% positive margins) or locally advance rectal cancer (~35% positive margins).\textsuperscript{3,4} PIT employs a nontoxic light-sensitive compound (i.e., a photosensitizer) bound to a tumor-targeting antibody, which can serve as both a diagnostic and therapeutic agent.\textsuperscript{5} A near-infrared (NIR) light-emitting diode (LED) is then used to excite the antibody-bound photosensitizer resulting in cell apoptosis and tumor ablation. Numerous preclinical studies have described the therapeutic potential of PIT in multiple cancer types.\textsuperscript{6-8} Its clinical relevance and application is mainly for superficial spreading cancers like skin cancer, melanoma, head and neck cancer, peritoneal metastases (ovarian or colorectal) or (microscopic) residual after an incomplete resection. However, the scientific standardization, performance, tuning, and validation of a light source for PIT has yet to be developed.

Over the past few years, the use of NIR high power LEDs for PIT applications has become more desirable due to the inexpensive and safe nature of the modality.\textsuperscript{6} However, recent findings have shown that the performance can be compromised by illumination variations due to ineffective heat dissipation; especially considering the emitted peak wavelength is highly dependent upon the core temperature of the LED.\textsuperscript{9} With an increase in core temperature, the emitted peak wavelength will be at a higher, less favorable wavelength for photosensitizer excitation of IRDye700DX, a commonly used preclinical PIT agent.\textsuperscript{7,10}

The primary objective of this study was to design and manufacture a standardized, validated and safe Light Emitting Diode (LED) / photodynamic therapy (PDT) device for IRDye700DX based PIT cancer treatment. Our secondary objective was to provide a framework for standardization of in future studies, in which other new developed photosensitizers will be evaluated.

The key design parameters of the system developed were: To select a light source suitable for excitation of the IRDye700DX, illuminate a large field of view, obtain sufficient cooling to sustain light source within operating temperature, and achieve manageable power output levels (20 mW cm\textsuperscript{-2} – 200 mW cm\textsuperscript{-2}) for application in various in vitro and in vivo conditions. To
reach these functional specifications, a system, hereafter referred to as 'LED device', was developed. The device was characterized using the standardized testing environment of domestic appliances, in vitro models, and in vivo xenograft mouse models of breast cancer.

MATERIALS & METHODS

SIMULATION AND NORMALIZATION
IRDye700DX (LiCor Biosciences) was utilized as the fluorescent photosensitizer. Illumination was provided by a 690nm (SMBB690D-1100-02) high output light-emitting diode (LED) for fluorochrome excitation (Marubeni). The LED specifications are shown in Table 1.

<table>
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<tr>
<th>Item</th>
<th>Symbol</th>
<th>Condition</th>
<th>Minimum</th>
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<th>Maximum</th>
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<td>(3.0)</td>
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<td>V&lt;sub&gt;F-PEAK&lt;/sub&gt;</td>
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<td>mW</td>
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<td>690</td>
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Table 1 – System specifications

To ensure homogenous illumination of the area of interest to be photosensitized in the surgical field, predefined as 5 x 3 cm, with a power output ranging 20–200 mW cm<sup>2</sup>, a total of 126 individual LED bulbs were needed. To verify the design of the LED device, shown in Figure 1A, the optical design simulation tool ‘LightTools’ of Synopsys was used (Synopsys).

As demonstrated in Figure 1B, unlike power output, peak wavelength is highly dependent upon the LED temperature, therefore to assure optimal heat dissipation, 690nm high output LEDs (126 total) were mounted on Metal Core Printed Circuit Board (MCPCB) attached to the semiconductor-mounting surface of the heatsink (Fischer elektronik), after exact optical alignment. The special heat sink geometry, consisting of a hollow fin, optimizes the airflow for guaranteed effective heat dissipation, and as such will keep the temperature of the light sources within the operating temperatures to stabilize the peak wavelength. The LED system is provided with a tunable LED power supply module (HLG-240H-54B), which enables the user to adjust the emitted power output (mW cm<sup>2</sup>) (Meanwell).

To outline the capabilities and photo biological safety of the LED device multiple optical measurements were performed (Optonic Laboratories) by Philips Lighting in accordance with the IEC62471 photo biological safety standard (Philips Lighting B.V). The LED device was tested
for 3 potential hazards by calculating the Emission Hazard Value (EHV), which represents the ratio between the ‘emission level’ and the ‘emission limit’. The EHV classifies related risk into four groups, ranging from no photo biological hazard (exempt) to hazardous for momentary exposure (risk groups 3).

CELL LINE AND CULTURE
To validate the in vitro and in vivo performance of our LED system, 2LMP-Luc (a 2x lung metastatic pooled subclone of MDA-MB-231, a gift from Dr. Donald Buchsbaum, UAB), human breast carcinoma cell line was used. The 2LMP-Luc cells were previously transformed using the ViraPort retroviral vector (Stratagene). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in tissue culture flasks in a humidified incubated in an atmosphere of 37°C, 95% air and 5% carbon dioxide.

PANITUMUMAB-IRDYE700 CONJUGATION
The antibody used, panitumumab (Vectibix, Amgen; 177 kDa) is a fully humanized monoclonal antibody (mAb) directed specifically to the epidermal growth factor receptor (EGFR). The photosensitizer IRDye700DX NHS ester (IR700; 2,0 kDa) was purchased from LI-COR Bioscience. Panitumumab was diluted to 5 mg mL\(^{-1}\) in PBS and incubated with the IR700 for 2 h at room temperature, according to manufacturer instruction. The mixture was purified with a desalting column (Pierce). After purification, the protein concentration and the number of dye molecules per protein were determined by absorption with UV-Vis spectroscopy (ThermoScientific).
In order to determine in vitro immunoreactivity of the panitumumab-IR700 (pan-IR700) after conjugation, a binding assay was performed. Briefly, 3.0x10^5 cells were resuspended in phosphate buffer solution (PBS) containing 5% FBS. Pan-IR700 was added (10 μg mL\(^{-1}\)) and incubated for 1 h at 37°C. Cells were washed 3x and resuspended in 200 μL of PBS followed by flow cytometry (Accuri C6, BD Biosciences). Nonspecific binding to the cells was examined by adding a 100-fold excess of cold nonlabeled panitumumab. This was repeated three times to obtain a Mean Fluorescence Intensity (MFI) ± Standard Error of the Mean (s.e.m.).

**IN VITRO CELL VIABILITY ASSAY**

To assess PIT effects using the LED device in vitro, cells were harvested and seeded into two 24 well, black welled plates (Wallac) at 2.0x10^5 cells per well for the following treatments: (1) no treatment; (2) PIT at 2 J cm\(^{-2}\), 4 J cm\(^{-2}\) and 6 J cm\(^{-2}\); (3) pan-IR700 only; (4) pan-IR700 and PIT at 2 J cm\(^{-2}\), 4 J cm\(^{-2}\) and 6 J cm\(^{-2}\); (5) pan-IR700 with blocking panitumumab (100-fold excess) and PIT at 2 J cm\(^{-2}\), 4 J cm\(^{-2}\) and 6 J cm\(^{-2}\). Pan-IR700 dose was 10 μg mL\(^{-1}\). Light microscopy (40x: Olympus IX70) was used to visualize morphological changes between groups. To determine the cell viability after PIT cells were harvested and resuspended in 0.1 mL of flow cytometry staining buffer containing propidium iodide (PI) (Southern Biotech) and Annexin V-FITC (Southern Biotech). The samples were then incubated for 15 min on ice, protected from the light and analyzed by flow cytometry (Accuri C6, BD Biosciences).

**IN VIVO STUDY DESIGN**

Athymic NCr-nu/nu female mice, aged 5-6 weeks (Frederick Cancer Research) were obtained and housed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). All animal experiments were conducted according to approved IACUC protocols. Mice received a 0.1 mL subcutaneous bilateral flank injection of 2LMP-Luc cells (2x10^6 cells per flank) suspended in FBS-free base media. Tumor growth was monitored by bioluminescence imaging (BLI) using IVIS 100 Imaging System (Caliper Life Sciences) and visual inspection of the flank 2 times a week. At day 21 baseline BLI measurements were collected and mice were sorted into 2 groups (n=3) based on BLI counts to achieve equal distribution of tumor size. For the treatments, group 1 received 0.1 mg pan-IR700 (intravenous via tail vein) 24h prior to LED treatment while group 2 received 0.3 mg pan-IR700. Twenty-four hours post injection, LED treatment was applied to left and right flank tumors, respectively receiving 0 J cm\(^{-2}\) and 50 J cm\(^{-2}\). Untreated tumor was shielded during contralateral tumor LED treatment. To monitor PIT effects on tumor size, BLI was performed at 4 h and 24 h post PIT. Quantitative analysis of total luciferase counts was calculated using tumor-specific ROI analysis performed using integrated instrument software. Therapeutic effect is expressed as
the percentage BLI reduction compared to baseline measurements.

Prior to LED treatment, all animals were imaged using Pearl Impulse Small Animal Imaging System (LI-COR Biosciences) in the 700 nm fluorescence emission channel. Average group tumor fluorescence (mean fluorescence intensity: defined as total counts / ROI pixel area) was calculated for each group using integrated instrument software. To evaluate histological changes after PIT, a standard hematoxylin/eosin stain (H/E) microscopic study (serial 20 μm slice sections) was performed. Tumors were surgically removed and fixed in 10% formalin overnight at day 4 post-PIT treatment of both the internal negative control and the treated tumor within the same animal.

STATISTICAL ANALYSIS
Data are expressed as means ± s.e.m. from triplicate experiments, unless otherwise indicated. Independent- and paired samples T-tests were used to compare treatment effect with that of control. For statistical analysis, SPSS version 21.0 was used. P < 0.05 was considered to indicate a statistically significant difference.

RESULTS

SYSTEM CHARACTERIZATION
After placing a receiver at various distances from the light source the peak and average light intensities were simulated (Synopsys). Figure 2A demonstrates that at a distance of 20 cm on an area of 5 x 3 cm both the peak and average power output were 200 mW cm⁻². Furthermore, the simulation shows an equal energy distribution (Fig. 2B) at the predefined area of interest (5 x 3 cm), making this device suitable for in vitro and in vivo applications of PIT. Moreover the special heat sink geometry was able to keep the temperature within the necessary operating temperature of 50°C (Fig. 2C) to stabilize the peak wavelength over time (Fig. 2D).
To verify the optical design and safety of the LED device, a series of system and safety characterizations was performed. LED system specifications are shown in Table 1. The measured maximum light output was concordant with the simulation and was determined to be 206 mW cm⁻², with the detector at a distance of 20 cm of the light source. As demonstrated in Table 2, there was no potential hazard concerning the eye or skin to disclose. The EHV for retinal thermal- and thermal skin injury were respectively at 34% and 49% of the emission limit for exempt. Moreover, the EHV for potential hazard caused by infrared exposure to the eye was below the measuring capability range of the used system, and therefore far below the emission limit for exempt.
Table 2 – Hazards

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<th>Emission Level Unit</th>
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<th>Emission Hazard Value</th>
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<td>Exempt</td>
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<tr>
<td>Thermal Skin</td>
<td>$2.12 \times 10^3$</td>
<td>$3.5 \times 10^3$</td>
<td>W/m²</td>
<td>Pass</td>
<td>0.49</td>
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</table>

Figure 2 Energy distribution, temperature normalization and wavelength distribution. (A) At 20 cm both the peak and average power output were 200 mW cm⁻². (B) Simulation shows equal energy distribution at the predefined 5 x 3 cm area of interest. (C) After 1 minute the LED device is capable of stabilizing its core temperature and (D) emitted wavelength distribution.
SYSTEM VALIDATION

Pan-IR700 Conjugate. Conjugation of panitumumab to the fluorescent photosensitizer IRDye700DX, resulted in a Dye to Protein ratio (D/P) of 1:3 (data not shown). The immunoreactivity of the EGFR targeting pan-IR700 conjugate was validated in vitro by a binding assay (Fig. 3A). Direct staining of the cell surface epitope of the EGFR 2LMP-Luc cells by the pan-IR700 conjugate caused a significant increase in Mean Fluorescence Intensity (MFI: 4.6x10^3 ± 51.8) compared to the MFI of the control (0.4x10^3 ± 11.6; P < 0.001). Additionally, after saturating the EGFR antigen binding sites by adding an excess native unconjugated panitumumab, non-specific binding was considered neglectable (MFI 0.7x10^3 ± 51.8).

Figure 3 Pan-IR700 mediated in vitro PIT for 2LMP-Luc cells. (A) Target specific binding confirmed by flowcytometer based immunoreactivity assay. (B) Target specific cell death in response to pan-IR700 mediated PIT in 2 LMP-Luc breast cancer cell line. (C) Microscopic observation of before and directly after 6 J cm^-2 PIT. Scale bar, 50 μm. Data are mean ± s.e.m. (n=3, * P < 0.001 vs. non treatment control).
Pan-IR700 Mediated PIT Using Standardized LED System Leads to Rapid Cell Death In Vitro.

As demonstrated in Figure 3B, 2 J cm\(^{-2}\), 4 J cm\(^{-2}\) and 6 J cm\(^{-2}\) LED exposure of 2LMP-Luc cells incubated with pan-IRD700 (10 μg ml\(^{-1}\)) induced a significantly higher percentage of cell death (32.4% ± 1.4%; 68.5% ± 1.5%; 89.2% ± 2.4% respectively) in comparison to untreated control cells (5.7% ± 1.0%; P < 0.001). We did not observe significant cytotoxicity without pan-IR700 due to light exposure of 2 J cm\(^{-2}\) (5.3% ± 1.0%), 4 J cm\(^{-2}\) (3.9% ± 0.5%), and 6 J cm\(^{-2}\) (5.3% ± 0.6%). Treatment with pan-IR700 in absence of light from the LED device induced no significant cytotoxicity (9.9% ± 0.2%) relative to the PIT treated groups.

To confirm binding-specific pan-IR700 mediated phototoxicity, 2LMP-Luc cells were incubated with an excess of unlabeled panitumumab to saturate the EGFR target antigen, prior to incubating the cells with the pan-IR700 conjugate (10 μg mL\(^{-1}\)) and exposure to light of the LED device. Blocking the EGFR antigen-binding site significantly (P < 0.001) reduced the percentage of cytotoxicity at PIT energy of 2 J cm\(^{-2}\) (9.6% ± 0.9%), 4 J cm\(^{-2}\) (11.3% ± 0.8%) and 6 J cm\(^{-2}\) (11.8% ± 0.9%) after LED illumination. Microscopy studies directly following a 6 J cm\(^{-2}\) treatment dose revealed cellular bleb formation and swelling, which are indicators of necrotic cell death induced by PIT (Fig. 3C).

Pan-IR700 Mediated PIT by the LED System In Vivo. To examine the distribution of pan-IR700 in bilateral 2LMP-Luc tumors, fluorescence imaging was performed at day one after intravenous injection of the conjugate. There was a dose dependent distribution of pan-IR700 with 2 fold higher MFI signals of 0.3 mg versus 0.1 mg injection (Fig. 4A). Representative fluorescent imaging at the 700nm channel is shown in Figure 4B. 2LMP-Luc tumors treated with 0.3 mg pan-IR700 showed a significant decrease in percentage change of BLI signals in treated tumors compared with contralateral non-treated control tumors (Fig. 4C). Figure 4D shows representative BLI signals. No significant decrease in BLI signals was observed in mice that received a 0.1 mg pan-IR700 treatment dose with 50 J cm\(^{-2}\) PIT. Histopathological analysis performed 4 days post PIT, revealed that only a small amount of viable 2LMP-Luc cells were present after 0.3 mg pan-IR700 mediated PIT (Supplemental Figure 1).
Figure 4 Pan-IR700 mediated PIT in vivo. (A) MFI of IR700 in 2LMP-Luc tumors 1 day after injection at two different doses of pan-IR700. (B) EGFR-positive 2LMP-Luc (bilateral flank) tumors were clearly visualized at 1 day after intravenous (i.v.) pan-R700 injection (respectively, 0.1 mg; 0.3 mg). (C) BLI signals of tumors treated with 0.3 mg pan-IR700 (50 J cm\(^{-2}\)) decreased significantly compared to BLI signals of contralateral non-treated sites at 4 hour and 1 day post PIT. No significant decrease in in BLI signal was observed in tumors treated with 0.1 mg pan-IR700 (50 J cm\(^{-2}\)) compared to contralateral non-treated sites. (D) One day post injection of 0.3 mg pan-IR700 i.v., the right tumor was exposed to light of the LED device (white arrow), while the left sided tumor was shielded from light (black arrow). Data are mean ± s.e.m. (n=3 mice, * P < 0.001 vs. non illuminated contralateral control tumor, t test).
DISCUSSION

The use of PIT for treatment of solid tumors is highly relevant for either superficially located cancer types (primary tumor and locoregional metastases) such as skin cancer, melanoma, head and neck cancer, or colorectal or ovarian peritoneal metastases and also for treatment of (microscopic) residual disease after an incomplete microscopic R1 resection or macroscopic R2 resection. As PIT advances rapidly from pre-clinical validation towards clinical use, there is great need for a standardized light source to be introduced. A viable candidate for FDA-consideration will be safe, cheap, easy-to-build, robust, reliable, and independent of (illumination) and temperature variations. To our knowledge, a normalized and standardized LED device has never been explicitly described, developed, or validated.

We developed a device appropriate for IR700 excitation that is safe, universal and standardized. While high output laser devices are getting cheaper with costs ranging from $10,000 to $25,000, we were able to build a cost friendly and easy to build LED (prototype) device for less than $1,500. We anticipate that in next generation build devices this will be even cheaper due to reduction in costs of manufacturing larger quantities of components such as LEDs and electronic constituents.

Light in the NIR suffers from little attenuation, with penetration depth up to 1 cm. Therefore the skin and eyes of the human body are most at risk when exposed to optical radiation. Although these hazards have been recognized for laser light for many years, its implication in LED light is relatively new, though necessary since the introduction of high-power LEDs. Hazard test confirmed that there were no potential hazards concerning the eye or skin to disclose for the device as tested. Moreover, we found that the multiple LEDs mounted on heat exchanger guarantees optimal thermal management of the LEDs, permitting the device to remain within its operating temperatures to deliver consistent peak wavelength at IR700 optimal peak excitation wavelength (nm).

Consistent with literature, we show that target-specific killing by the LED device was achieved in response to a single dose of pan-IR700 and PIT for both in vitro and in vivo studies. Pan-IR700 localized specifically in EGFR-positive 2LMP-Luc tumors as determined by non-invasive optical imaging. This study demonstrates furthermore, that target-selective accumulation of pan-IR700 in the EGFR-positive 2LMP-Luc tumors was dose dependent. In vivo decrease in tumor growth was confirmed after one dose of 0.3 mg pan-IR700 followed by one single bolus exposure to light from the LED device (50 J cm⁻²).
Numerous reports have been published about the clinical implications of fluorescence imaging, providing the surgeon with real time visualization of tumors deposits in order to improve resection rates and thus positively influence prognosis. Nevertheless, due to anatomical restraints such as vital tissues, the risk of minimal residual disease remains after an incomplete microscopic resection (R1 resection) or even a macroscopic irradical resection (R2 resection). PIT can provide clinical applicability not solely for superficial spreading tumors but also as an adjuvant treatment modality after surgery for irradiating the remaining tumor cavity and its in situ resection margins. We envision the application used for treatment of localized disease, such as in peritoneal metastases, with a hand-held device or in an outpatient clinic setting, such as treatment for superficial cutaneous metastases. A highly advantageous feature of the IR700 photosensitizer is that upon excitation it also generates fluorescence. Extending our LED device with a specialized camera system (sensitive charge-coupled device: CCD) enables simultaneous registration and processing of the signal for intraoperative real-time imaging purposes combined with PIT capabilities. Moreover making several technical adjustments may optimize the efficacy of our LED device even more. For example, the bandwidth of the LED presented here is 20nm. Including additional cut-off filters or selecting LEDs with a narrower bandwidth may be able to increase specificity. Moreover further research is necessary to evaluate whether employing a pulse controller will increase the efficacy of our LED device. We envision that PIT allows for delineation of tumor margins through optical imaging, and subsequent light based phototherapy to eliminate residual microscopic disease thereby aiding the surgeon in obtaining a more radical resection while preserving as much as possible functionality with minimal collateral damage.

CONCLUSION

To our knowledge, a normalized and standardized LED device has not been explicitly described nor developed. Here, we introduced a standardized light source and validated its usability for PIT applications.
ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:
Supplementary Figure 1 Histology
REFERENCES


