Abstract. Background/Aim: Near-infrared photoimmunotherapy (NIR-PIT) is a newly approved cancer phototherapy. We aim to confirm whether a clinically approved camera for indocyanine green (ICG) could monitor IR700 fluorescence in real time during exposure to therapeutic NIR light. Materials and Methods: An NIR camera, LIGHTVISION, designed to image ICG fluorescence, was used. A431-GFP/luc tumor-bearing mice were exposed to therapeutic NIR light and real-time fluorescence imaging (RT-FI) was obtained and measured with LIGHTVISION. Bioluminescence imaging (BLI) was performed to confirm cell death. Results: RT-FI during NIR-PIT revealed an initial rapid loss of fluorescence, followed by a plateau which occurred at a light dose of approximately 30 J/cm². Correlation between BLI and IR700 fluorescence loss showed that loss of fluorescence was associated with increased cell death. Conclusion: The efficacy of NIR-PIT could be monitored non-invasively and in real-time using weak fluorescence at wavelengths much longer than the peak fluorescence of IR700. This technique can achieve precise light dosimetry that allows us to decide on the optimal exposure.

Real-time IR700 Fluorescence Imaging During Near-infrared Photoimmunotherapy Using a Clinically-approved Camera for Indocyanine Green

SHUHEI OKUYAMA1,2, DAIKI FUJIMURA1,2, FUYUKI INAGAKI2, RYUHEI OKADA2, YASUHIRO MARUOKA2, HIROAKI WAKIYAMA2, TAKUYA KATO2, AKI FURUSAWA2, PETER L. CHOYKE2 and HISATAKA KOBAYASHI2

1Shimadzu Corporation, Kyoto, Japan; 2Molecular Imaging Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

This article is freely accessible online.

Correspondence to: Hisataka Kobayashi, Molecular Imaging Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A. Tel: +1 2408583069, Fax: +1 240541452, e-mail: kobayash@mail.nih.gov

Key Words: Near infrared, photoimmunotherapy, real-time fluorescence imaging, IR700, ICG camera.

©2021 International Institute of Anticancer Research
www.iiar-anticancer.org
longer than the peak fluorescence wavelength of 700 nm. Highly sensitive cameras have been developed to measure indocyanine green (ICG) fluorescence at > 820 nm as this agent is increasingly used to assist in surgical procedures. Therefore, measurement of IR700 fluorescence loss in a tumor at > 820 nm could be a useful indicator that a sufficient amount of light has been delivered and further light treatment is unnecessary.

In this study, we performed real-time fluorescence imaging (FI) at 820 nm during NIR-PIT in a A431-GFP/luc tumor-bearing mouse model as the first real-time theranostic imaging for cancer phototherapy using the LIGHTVISION camera, which was designed to measure the fluorescence of ICG. Following NIR-PIT, we performed bioluminescence imaging (BLI) to evaluate the correlation between fluorescence loss and cell death.

Materials and Methods

Reagents. Water soluble, silica-phthalocyanine derivative,IRDye700DX NHS ester (IR700; C_{37}H_{60}N_{12}Na_{5}O_{27}S_{6}Si_{3}, molecular weight of 1954.22) was obtained from LI-COR Bioscience (Lincoln, NE, USA). Panitumumab, a fully humanized IgG2 monoclonal antibody (mAb) directed against EGFR, was purchased from Amgen (Thousand Oaks, CA, USA). All other chemicals were of reagent grade.

Synthesis of IR700-conjugated Panitumumab. Conjugation of dyes with mAb has been previously described (9). Briefly, panitumumab (1 mg, 6.8 nmol) was incubated with IR700 [66.8 μg, 34.2 nmol, 10 mmol/L in dimethyl sulfoxide (DMSO)] in 0.1 mol/L Na_{2}HPO_{4} (pH 8.5) at room temperature for 1 h. Subsequently, the mixture was purified with a Sephadex G25 column (PD-10; GE Healthcare, Piscataway, NJ, USA). The protein concentration was determined with a Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL, USA) by measuring the absorption at 595 nm (8453 Value System; Agilent Technologies, Santa Clara, CA, USA). The concentration of IR700 was measured by its absorption to confirm the number of fluorophore molecules conjugated to each mAb. IR700-conjugated Panitumumab is abbreviated as Pan-IR700.

Cell lines and culture. A431-GFP/luc cells expressing EGFR were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in tissue culture flasks in a humidified incubator at 37°C with 5% CO_{2}.

Animal and tumor models. All procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the local Animal Care and Use Committee. Six- to eight-week-old female homozygote athymic nude mice were purchased from Charles River (Frederick, MD, USA). During the procedure, mice were anesthetized with isoflurane. Two million A431-GFP/luc cells in 200 μL phosphate-buffered saline (PBS) were injected just under the skin on the right dorsum of the mouse. Mice were used in the experiments when tumors reached 7-10 mm in the longest diameter.

In vivo NIR-PIT. We administered 100 μg Pan-IR700 to A431-GFP/luc tumor-bearing mice intravenously, 24 h before NIR light exposure. Mice were anesthetized with isoflurane during the experiments (n = 6). A frontal diffuser (M113L02; Thorlabs, Newton, NJ, USA) was connected to a NIR laser system (ML7710, Modulight, Inc. Tampere, Finland) emitting 690 ± 5 nm light at a maximum of 2 W; NIR light exposure conditions such as spot diameter, exposure intensity (mW/cm²) and total energy (J/cm²) were adjusted manually. After calibration of the exposure intensity by the system, the diffuser tip was connected to a collimator (COL-SMA-BK7-25.4mm; Us Fiberoptec Technology Inc., San Jose, CA, USA) and placed vertically, approximately 30 cm from the target tumor.

IR700 fluorescence real-time imaging during NIR-PIT. For obtaining fluorescence images, LIGHTVISION, a near-infrared FI system (Shimadzu Corp, Kyoto, Japan) originally designed for measuring ICG fluorescence, was used (collection wavelength of 820 nm and above). After placing anesthetized mice on the lab desk, the camera of the system was placed approximately 50 cm from the target, at an angle of ~20 degrees, to ensure adequate field of view, while focus and zoom was adjusted to encompass the tumor. The detector sensitivity for NIR was adjusted so as not to saturate its signal. We recorded fluorescence images using LIGHTVISION at 1 frame per second while NIR light was administered via the 690-nm laser with an output of 100 J/cm² at 150 mW/cm² to the tumor surface. After NIR light exposure, images were analyzed by Fiji Software (10). Images corresponding to a dose of 0, 20, 40 and 80 J/cm² were selected. To calculate the fluorescence intensity of each image, we set a region of interest (ROI) on the tumors. All pixel values were normalized to the initial intensity (equal to 100%); fluorescence intensity curves were then drawn. In addition, we set another ROI at the center of the body with the same height in the image so as not to be affected by scattering fluorescence signal from the tumor. The fluorescence intensity ratio of tumor and normal tissue (T/N ratio) was also calculated using the fluorescence intensities on each area.

Bioluminescence imaging (BLI). For BLI, D-luciferin (15 mg/mL, 200 μL) was injected intraperitoneally and imaging was performed just before and 6 h after NIR light exposure using Photon Imager (Biospace Lab, Nesles la Vallée, France). Luciferase activity per unit area and time were quantified by placing ROIs on the tumor implanted on the right dorsum with a background ROI over the corresponding left dorsum. To estimate anti-tumor efficacy, tumor to background ratio (TBR) was calculated as following: TBR = (luciferase activity of target)/(luciferase activity of background). Each TBR was normalized to the BLI intensity before NIR-PIT.

Statistical analysis. Data are expressed as mean ± standard error. Approximate straight lines and Pearson’s correlation coefficient were determined for the correlation of IR700 fluorescence and BLI using Microsoft Excel software (Microsoft, Redmond, WA, USA). p-values of less than 0.05 were considered statistically significant.

Results

IR700 Fluorescence Real-Time Imaging during NIR-PIT. Real-time IR700 FI using LIGHTVISION was performed during NIR-PIT (Figure 1A and 1B). NIR fluorescence loss was compared to the anti-tumor effect as measured by BLI.
at 6 h post NIR-PIT (Figure 2A). Tumor bearing mice were exposed to NIR laser light at 150 mW/cm² and FI snapshots were obtained at light doses of 0, 20, 40 and 80 J/cm², corresponding to increasing time of laser light excitation. Fluorescence loss was rapid at energies ≤ 20 J/cm² (Figure 2B) but reached a minimum plateau ≥ 30 J/cm². We also calculated average fluorescence intensity by placing ROIs on a series of images during NIR-PIT and compared NIR fluorescence to laser light dose (n = 6) (Figure 2C). Longer exposure times of laser light resulted in higher light doses. This showed that IR700 fluorescence decreased rapidly in the early phases of NIR light exposure, attenuated to under 20% or less compared to the initial intensity at the light dose of approximately 40 J/cm²; it then gradually decreased, reaching a minimum of 15% by the end of NIR light exposure. Furthermore, we calculated the IR700 fluorescence T/N ratio by using an averaged fluorescence signal in ROIs of normal tissue (Figure 2D). These comparisons showed a similar rapid fluorescence decrease followed by a slow decrease. The rapid loss phase occurred when light exposures were ≤ 20 J/cm² and the slow phase began at exposures ≥ 30 J/cm² (n = 6). To determine the light dose at which the light energy rapid decrease of tumor fluorescence ended, we performed a linear curve fit for each phase (Fast: 5-20 J/cm²; Slow: 30-100 J/cm²) and calculated the crossing point of two lines in each tumor, resulting in a crossing point of 22.7 J/cm² (Figure 2E). The crossing points were calculated in individual mice used for Figure 2D, resulting in an average light dose of 26.5 J/cm² to reach the crossing point in fluorescence loss.

**Correlation between IR700 Fluorescence loss and therapeutic effects of NIR-PIT.** Beyond the NIR light dose of 40 J/cm², fluorescence intensity loss became minimal. This corresponded to cell killing as measured by BLI which indicated a relative decrease in signal compared to baseline (Figure 3A). As shown in Figure 3B, a positive correlation was found between IR700 loss of fluorescence and bioluminescence loss (n = 25, r = 0.6838, p < 0.001 using Pearson’s correlation coefficient) indicating successful cancer cell death.

**Discussion**

The major cytotoxic mechanism of NIR-PIT is based on a photo-induced ligand release reaction of IR700 after NIR light exposure that leads to biophysical changes in the APC, resulting in physical damage to the cell membrane (6). At the same time, this photo-induced ligand release results in irreversible loss of IR700 fluorescence, indicating that NIR-PIT has been effective. Excess light exposure beyond the point where fluorescence is lost is unlikely to contribute further to the effectiveness of NIR-PIT and may even cause harm by non-specific production of ROS. Therefore, fluorescence images of IR700 during NIR-PIT could be used to monitor the process and provide useful feedback to the operator regarding the point when NIR-PIT laser light

---

**Figure 1. (A) The NIR FI system, LIGHTVISION. (B) Experimental setup with LIGHTVISION. A laser was placed vertically above the tumor, approximately 30 cm from the observing target. The camera was also placed ~50 cm from the observing target at an angle of ~20 degrees to ensure a complete field of view.**
Figure 2. (A) Schema of the FI and BLI study. (B) IR700 fluorescence of A431-GFP/luc tumor-bearing mice was detected during application of laser light at 689 nm. The fluorescence intensity decreased in a light dose-dependent manner. (C) Time intensity curve was obtained by analysis of sequentially obtained images (average and standard error are shown in each point; n = 6). The relative IR700 fluorescence intensity was calculated with the initial intensity as 100%. IR700 fluorescence rapidly decreased early after NIR light exposure and then plateaued at light doses approximately 40 J/cm² or greater. (D) An average IR700 fluorescence intensity ratio between tumor and normal tissue (T/N ratio) was calculated (n = 6). The ratio rapidly decreased at the early phase of NIR light exposure (≤ 20 J/cm²) and then plateaued approximately at doses ≥40 J/cm². (E) The crossing point was calculated from the T/N ratio curve using approximate straight lines obtained by the typical area of each phase (5-20 J/cm² and 40-100 J/cm², respectively). The crossing points occurred at 22.7 J/cm².
exposure could be stopped. However, measuring IR700 fluorescence directly while high intensity laser light is activating the APC at a peak of 690 nm ± 5 nm is not possible due to the overlap of the excitation and peak emission spectra. However, while IR700 exhibits peak fluorescence emission at 700 nm, its complete spectrum includes wavelengths > 700 nm which extends beyond 800 nm, albeit at vastly reduced intensities.

In this study, real-time monitoring of IR700 fluorescence during NIR-PIT was accomplished with a highly sensitive NIR FI camera system (LIGHTVISION) which is normally used clinically for indocyanine green ICG imaging (11), a dye that fluoresces at > 820 nm. We showed that NIR FI at > 820 nm successfully detected fluorescence loss of IR700 in a light dose dependent manner (Figure 2B, C). IR700 showed a 2-phase fluorescence loss pattern of a rapid early decrease at low NIR light exposures, followed by a slower decrease resulting in a flattening of the curve beginning at approximately 40 J/cm² (corresponding with a light exposure time of approximately 267 s) (Figure 2C). As the LIGHTVISION camera is tuned to detect fluorescence at much longer wavelengths than the NIR laser, it avoids signal cross contamination during NIR-PIT. Similarly, the T/N ratio also showed the same pattern of fluorescence loss, resulting in a fluorescence plateau ≥40 J/cm² (Figure 2D). Theoretically, the fluorescence intensity only decreases during NIR light exposure due to irreversible photobleaching of IR700. However, we observed that the T/N ratio increased at exposures greater than 30 J/cm² (Figure 2D and 2E). This is likely explained by the super-enhanced permeability and retention (SUPR) effect that has been previously reported with NIR-PIT (12). The SUPR effect is caused by rapid perivascular cancer cell killing that enhances delivery of macromolecules, including the APC itself, into the tumor (13). In the early phases of NIR-PIT, the SUPR effect is minimal and thus the T/N is monotonically decreased. However, as cell killing increases the SUPR effect allows more circulating intact APC to enter the tumor (Figure 2E) leading to slight increases in fluorescence. The two-phase T/N curve shown in Figure 2D and 2E is thus explained by the onset of the SUPR effect within the tumor. Additionally, BLI signal loss was positively correlated with IR700 fluorescence loss (Figure 3B). BLI is a reliable indicator of cell viability and loss of BLI signal indicates tumor killing. Real-time fluorescence monitoring at > 820 nm could indicate when the excitation of the APC is complete and no further NIR light is needed. This could provide useful feedback to an operator in a clinical situation.

There are several limitations to this study. First, the observable depth of FI is limited and the camera only images the most superficial parts of this subcutaneous tumor model. Fluorescence of deeper tissue was not obtained in this study. Although NIR light penetrates deeper than visible range light, even NIR light is highly attenuated in tissue. Therefore, to extend this method to interstitial light delivery to treat deeper lesions may require fluorescence detectors to be built into the interstitial light fibers. Additionally, fluorescence loss plateaued at 15-20% of maximum. At this point it becomes difficult to visualize the tumor compared to background fluorescence. Therefore, it is important not to move the camera during this procedure. Since FI using LIGHTVISION operates at >820 nm, there is a maximal exposure of fluorescence.
depth penetration of light and minimal autofluorescence compared to shorter wavelengths; nonetheless there is a signal floor below which the tumor can no longer be seen.

**Conclusion**

Using a commercially available NIR camera, the effectiveness of NIR-PIT could be monitored non-invasively and in real time, taking advantage of the high intensity of the laser light excitation and the long tail of IR700 fluorescence at this high intensity light. Loss of IR700 fluorescence demonstrates a 2-phase pattern with rapid initial loss of fluorescence and then a delayed slower response, leading to a gradual flattening of the curve beyond 40 J/cm² of delivered light. Fluorescence loss correlated with tumor cell death and thus, FI imaging could be a non-invasive method for monitoring the dose of laser light needed to have effective cell killing with NIR-PIT.

**Conflicts of Interest**

The Authors have no conflicts of interest to disclose.

**Authors’ Contributions**

All Authors read and approved the final version of the manuscript. S.O. and D.F. mainly designed and conducted experiments, performed analysis, verified data and wrote the manuscript; F.I., R.O., Y.M., H.W., T.K., and A.F., performed experiments and analysis; P.L.C. wrote the manuscript and supervised the project; H.K. planned and initiated the project, designed and conducted experiments, verified data, wrote the manuscript, and supervised the entire project.

**Acknowledgements**

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research (ZIA BC011513). F.I. was also supported by a grant from National Center for Global Health and Medicine Research Institute, Tokyo, Japan.

**References**

7. ASP-1929 photoinmunotherapy (PIT) study in recurrent head/neck cancer for patients who have failed at least two lines of therapy. Available at: https://clinicaltrials.gov/ct2/show/NCT03769506 [Last accessed on April 27, 2021]
10. Fiji, Image J. Available at: https://imagej.net/Fiji [Last accessed on April 27, 2021]

Received April 19, 2021
Revised April 25, 2021
Accepted April 27, 2021