Journal of Visualized Experiments

Near Infrared photoimmunotherapy for mouse models of pleural dissemination

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61593R4
Full Title:	Near Infrared photoimmunotherapy for mouse models of pleural dissemination
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TITLE:

Near Infrared photoimmunotherapy for mouse models of pleural dissemination

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Keyword:

pleural dissemination model, IRDye 700DX, antibody-dye conjugates, near infrared photoimmunotherapy, near infrared light

Summary

Near-infrared photoimmunotherapy (NIR-PIT) is an emerging cancer therapeutic strategy that utilizes an antibody-photoabsorber (IR700Dye) conjugate and NIR light to destroy cancer cells. Here, we present a method to evaluate the antitumor effect of NIR-PIT in a mouse model of pleural disseminated lung cancer and malignant pleural mesothelioma using bioluminescence imaging.

Abstract

The efficacy of photoimmunotherapy can be evaluated more accurately with an orthotopic mouse model than with a subcutaneous one. A pleural dissemination model can be used for

the evaluation of treatment methods for intrathoracic diseases such as lung cancer or malignant pleural mesothelioma.

Near-infrared photoimmunotherapy (NIR-PIT) is a recently developed cancer treatment strategy that combines the specificity of tumor-targeting antibodies with toxicity caused by a photoabsorber (IR700Dye) after exposure to NIR light. The efficacy of NIR-PIT has been reported using various antibodies; however, only a few reports have shown the therapeutic effect of this strategy in an orthotopic model. In the present study, we demonstrate an example of efficacy evaluation of the pleural disseminated lung cancer model, which was treated using NIR-PIT.

Introduction

Cancer remains one of the leading causes of mortality despite decades of research. One reason is that radiation therapy and chemotherapy are highly invasive techniques, which may limit their therapeutic benefits. Cellular- or molecular-targeted therapies, which are less invasive techniques, are receiving increased attention. Photoimmunotherapy is a treatment method that synergistically enhances the therapeutic effect by combining immunotherapy and phototherapy. Immunotherapy enhances tumor immunity by increasing the immunogenicity of the tumor microenvironment and reducing immunoregulatory suppression, resulting in the destruction of tumors in the body. Phototherapy destroys primary tumors with a combination of photosensitizers and light rays, and tumor-specific antigens released from the tumor cells enhance tumor immunity. Tumors can be selectively treated using photosensitizers as they are specific and selective for the target cells. The modality of phototherapy includes photodynamic therapy (PDT), photothermal therapy (PTT), and photochemistry-based therapies[1].

Near-infrared photoimmunotherapy (NIR-PIT) is a recently developed method of antitumor phototherapy that combines photochemical-based therapy and immunotherapy[1,2]. NIR-PIT is a molecularly targeted therapy that targets specific cell surface molecules through the conjugation of a near-infrared silicon phthalocyanine dye, IRdye 700DX (IR700), to a monoclonal antibody (mAb). The cell membrane of the target cell is destroyed upon irradiation with NIR light (690 nm)[3].

The concept of using targeted light therapy by combining conventional photosensitizers and antibodies or targeted PDT is over three decades old[4,5]. Previous studies have attempted to target conventional PDT agents by conjugating them to antibodies. However, there was limited success because these conjugates were trapped in the liver, owing to the hydrophobicity of the photosensitizers[6,7]. Moreover, the mechanism of NIR-PIT is

completely different from that of conventional PDT. Conventional photosensitizers generate oxidative stress that results from an energy conversion that absorbs light energy, dislocates to an excited state, transitions to the ground state, and causes apoptosis. However, NIR-PIT causes rapid necrosis by directly destroying the cell membrane by aggregating photosensitizers on the membrane through a photochemical reaction[8]. NIR-PIT is superior to conventional targeted PDT in many ways. Conventional photosensitizers have low extinction coefficients, requiring the attachment of large numbers of photosensitizers to a single antibody molecule, potentially reducing binding affinity. Most conventional photosensitizers are hydrophobic, making it difficult to bind the photosensitizers to antibodies without compromising their immunoreactivity or *in vivo* target accumulation. Conventional photosensitizers typically absorb light in the visible range, reducing tissue penetration.

Several studies on NIR-PIT targeting intrathoracic tumors such as lung cancer and malignant pleural mesothelioma (MPM) cells have been reported[9–17]. However, only a few reports have described the efficacy of NIR-PIT in pleural disseminated MPM or lung cancer models[9–12]. Subcutaneous tumor xenograft models are thought to be standard tumor models and are currently widely used to evaluate the antitumor effects of new therapies[18]. However, the subcutaneous tumor microenvironment is not permissive for the development of an appropriate tissue structure or a condition that properly recapitulates a true malignant phenotype[19–22]. Ideally, orthotopic disease models should be established for a more precise evaluation of the antitumor effects.

Here, we demonstrate a method of efficacy evaluation in a mouse model of pleural disseminated lung cancer, which was treated using NIR-PIT. A pleural dissemination mouse model is generated by injecting tumor cells into the thoracic cavity and confirmed using luciferase luminescence. The mouse was treated with an intravenous injection of mAb conjugated with IR700 and NIR irradiation to the chest. The therapeutic effect was evaluated using luciferase luminescence.

Protocol

All *in vivo* experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animal resources of Nagoya University Animal Care and Use Committee (approval #2017-29438, #2018-30096, #2019-31234, #2020-20104). Six-week-old homozygote athymic nude mice were purchased and maintained at the Animal Center of Nagoya University. When performing the procedure in mice, they were anesthetized with isoflurane (introduction: 4–5%, maintenance 2–3%); the paw was pressed with tweezers to

confirm the depth of anesthesia.

1. Conjugation of IR700 with mAb

1.1. Incubate MAb (1 mg, 6.8 nmol) with IR700 NHS ester (66.8 mg, 34.2 nmol, 5 mmol/L in DMSO) in 0.1 mol/L Na_2HPO_4 (pH 8.6) at 15-25 °C for 1 h.

1.2. Purify the mixture using a column (e.g., Sephadex). Prepare and wash the column with PBS. Then, apply the mixture onto the column and collect the drop, which contains the purified IR700-conjugated antibody. This IR700-conjugated antibody is referred to as the antibody photosensitizer conjugate (APC).

1.3. Measure the protein and IR700 concentration in the APC.

1.3.1. Prepare calibration curves for protein and IR700 using a spectrophotometer.

1.3.2. Mix standard concentrations of albumin with a protein assay kit following the kit protocol (see **Table of Materials**, CBB protein staining). Measure the absorbance of albumin at 595 nm wavelength, and plot the calibration curve (a linear approximation formula) for the protein using the following equation: y = ax + b (x: concentration, y: absorbance).

1.3.3. Obtain calibration curves for IR700 with absorption at 690 nm using the same procedure. The standard concentration of IR700 is recommended at 0.1–5 µM (0.1954–9.77 µg/mL).

1.3.4. Measure the protein concentration and IR700 concentration in the APC using a calibration curve [x = (y-b)/a (x: concentration, y: absorbance)].

1.3.5. Determine the number of IR700 dyes bound per mAb with the results of the molar concentration.

NOTE: It is important to determine the optimal conjugation number of IR700 molecules per mAb molecule. Generally, approximately three IR700 molecules bound on a single mAb molecule would be effective both *in vitro* and *in vivo*. Many IR700 bound per antibody (e.g., six) makes it easier to be trapped in the liver during *in vivo* experiments. The ratio of antibody bound to IR700 was in the range of 1:2–1:4. The proportion of IR700 was reduced, if necessary.

1.4. Perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a confirmation for the formation of an APC. Image the gel at 700 nm using a fluorescent imager, and stain the protein in the gel using a protein staining kit following the kit protocol (see **Table of Materials**, CBB protein staining).

2. Generation of a pleural dissemination model

2.1. Prepare luciferase-expressing target cells and suspend 1.0×10^6 target cells in 100 µL of phosphate-buffered saline (PBS)

NOTE: Intrathoracic cancer cells such as lung cancer and MPM are suitable as target cells. Luciferase-expressing cells were prepared via luciferase gene transfection, and high expression of luciferase was confirmed after > 10 cell passages. Cells were cultured in a medium supplemented with 10% fetal bovine serum and penicillin (100 IU/mL) and streptomycin (100 mg/mL). The number of cells was adjusted according to the tumor growth rate and the time course of treatment ($1.0. \times 10^5$–6.0×10^6 cells/body weight).

2.2. Prepare 8–12-week-old female homozygote athymic nude mice, with a preferable body weight of 19–21 g.

2.3. Anesthetize mice during the procedure with isoflurane (introduction: 4–5%, maintenance 2–3%); press the tail with tweezers to confirm that there is no reaction.

2.4. Make a stopper with polystyrene foam and attach the stopper to the 30 G needle so that the tip remains at 5 mm to prevent lung injury. Bend the needle tip by pressing it against a hard object to avoid pneumothorax (**Figure 1**).

CAUTION: Be careful not to pierce oneself. Use forceps to bend the needle. Do not hold the stopper when attaching it to the needle. It is safer to stick the stopper before filling the cells into the syringe.

2.5. Fill a syringe (1 mL) with target cells, and attach a 30G needle with a stopper.

2.6. Pierce a needle into the chest of the mouse through the intercostal space. Owing to the resistance while hitting against the ribs at that time, the needle tip moved up and down. After passing through the intercostal space, press the syringe against the mouse and inject 100 µL of target cells (**Figure 2**).

NOTE: The mouse breaths deeply when the needle properly enters the chest cavity. With the bending of the needle tip, pneumothorax and inappropriate injection of cells into the lung could be avoided.

2.7. Roll the mouse 2–3 times to spread the cells throughout the thoracic cavity.

2.8. Return the mouse to the cage. After the procedure, the mouse will wake up from anesthesia and behave normally.

3. Measurement of bioluminescence

NOTE: The software used for data acquisition is listed in the **Table of Materials**.

3.1. To confirm the generation of the pleural dissemination model, evaluate the bioluminescence images every day after injecting the cells into the thoracic cavity.

3.2. Anesthetize mice (step 2.3) and inject intraperitoneally with D-luciferin (15 mg/mL, 200 µL).

3.3. Ten minutes after the injection, set the mouse in the bioluminescence imaging (BLI) measuring equipment. For image acquisition, open the **Acquisition Control Panel** of the software. Select **Luminescent**, **Photograph**, and **Overlay** (**Figure 3**).

3.4. Set exposure time as **Auto**. Set Binning as small.

3.5. Set f/stop as **1** for luminescent and **8** for photograph; f/stop controls the amount of light received by the charged-coupled device detector.

3.6. Set the Field of View as **C**.

3.7. Once the mouse sample is ready for imaging, click **Acquire** for imaging acquisition. Mice with sufficient luciferase activity were selected for further studies.

NOTE: A suitable pleural dissemination model shows strong luminescence on the diffused site in the chest when viewed from the ventral side. If the BLI images are not diffused in the thorax, and only at the injection site, the tumor may be transplanted subcutaneously.

3.8. After displaying the image, set the display format to **Radiance**. Open the **Tool Palette** panel (**Figure 4A**).

3.9. Select **ROI Tools**. We recommend using the **Circle** to range the bioluminescent area on images.

3.10. Click **Measure ROIs** to measure the surface bioluminescent intensity (**Figure 4B**).

3.11. Use **Configure Measurement** on the left corner of the ROI measurement panel to select the values/information needed. Export this data table as a .csv file (**Figure 4C**).

3.12. Use the values of **Total Flux (p/s)** as the bioluminescent intensity quantification in the .csv file.

4. Diffuse luminescence imaging tomography (DLIT)

NOTE: The software used for data acquisition is listed in the **Table of Materials**.

4.1. Turn on the **X-ray Armed** button.

4.2. Anesthetize the mice (step 2.3) and then inject D-luciferin (15 mg/mL, 200 µL) intraperitoneally into the mice. To shoot DLIT continuously from 3.2 to 3.7, skip this step.

4.3. Ten minutes after injection, set the mouse in the BLI equipment.

4.4. Open the **Acquisition Control Panel** of the software. Select **Luminescent**, **Photograph**, **CT**, **Standard-One Mouse**, and **Overlay**. Other settings were the same as in 3.4–3.6 (**Figure 5A**).

4.5. Select the **Imaging Wizard** on the **Acquisition Control Panel**.

4.6. Select **Bioluminescence** and then **DLIT** (**Figure 5B**).

4.7. Select **Firefly** as the wavelength to measure (**Figure 5C**).

4.8. Set the Imaging Subject as **Mouse**, Exposure parameters as **Auto Settings**, Field of View as **C-13.4 cm**, and Subject Height as **1.5 cm** (**Figure 5D**).

4.9. Push the **X-rays will be produced when energized. Acquire**.

4.10. Open the CT sequential image data.

4.11. Open **Surface Topography** on the Tool Palette. Select **Show** (**Figure 6A**).

4.12. Adjust the threshold as the purple display shows only the body surface (**Figure 6B**). Then, select the Subject **Nude Mouse** and click the **Generate Surface**. Make sure that the outline of the mouse is accurately drawn (**Figure 6C**).

4.13. Open the **Tool Palette**, **DLIT 3D Reconstruction Properties** tab. Select Tissue Properties as **Mouse Tissue** and Source Spectrum as **Firefly** (**Figure 6D**). Next, open the **Analyze** tab and confirm the data for each selected wavelength data. Finally, click the **Reconstruct** button (**Figure 6E**).

4.14. Confirm the presence of BLI in the chest cavity in the configured DLIT image.

5. NIR-PIT for *in vivo* pleural dissemination model

5.1. Measure the light dose of 690 nm wavelength (NIR) laser with a power meter, and adjust the output to 100 mW/cm^2.

NOTE: The laser light is coherent with a precise coil size; thus, the light energy hardly changes regardless of the distance within 50 cm. If there are many adverse events, such as burns, reduce the output within the range of 40 mW/cm^2.

5.2. Intravenously inject APC (100 µg) *via* the tail vein 24 h before NIR irradiation.

NOTE: Adjust the volume of APC to 50–200 µL for injection.

5.3. Anesthetize the mice (step 2.3), and lay it on its back. To avoid NIR irradiation to the non-target site, shield other sites with aluminum foil (**Figure 7A**). Irradiate with NIR light with a laser of 100 J/cm^2; if the tumor is disseminated back to the belly, the NIR-light irradiation dose could be divided in multiple directions (**Figure 7B**).

NOTE: Adjust the dose at 30–150 J/cm^2 depending on the *in vitro* results and adverse events such as burns.

5.4. When the NIR irradiation is complete and the mouse is awake, return it to the cage.

5.5. Observe the BLI and measure the ROI over time (every day) (See 3.2–3.12).

5.6. For *ex vivo* imaging, euthanize mice with carbon dioxide 24 h after the APC injection, immediately before the NIR irradiation.

5.6.1. To observe the inside of the chest of the mouse, remove the thorax and cut the ribs and sternum. Capture the fluorescence image (700 nm) alongside the control without APC administration. Then, apply D-luciferin (150 µg/mL) over the exposed thorax, and the BLI was taken (refer 3.2–3.7).

Representative results

Anti-podoplanin antibody NZ-1 was conjugated with IR700 to generate NZ-1-IR700. We confirmed the binding of NZ-1 and IR700 on an SDS-PAGE (**Figure 8**). Luciferase-expressing H2373 (H2373-luc) was prepared by transfecting malignant mesothelioma cells (H2373) with a luciferase gene[10].

We anesthetized 8–12-week-old female homozygote athymic nude mice and injected 1×10^5 H2373-luc cells into the thoracic cavity. The day of injection of tumor cells into the mice was indicated as day 1.

At day 4, BLI and DLIT were performed after D-luciferin (15 mg/mL, 200 µL) was injected intraperitoneally, and mice with sufficient luciferase activity in the chest cavity were selected for further studies (**Figure 9**). Hundred micrograms of NZ-1-IR700 (100 µL) was intravenously injected *via* the tail vein. The control group was injected with PBS (100 µL).

At day 5, two mice were sacrificed using carbon dioxide asphyxiation for *ex vivo*. The NZ-1-IR700 injected mouse showed both high IR700 fluorescence and luciferase activities in thoracic tumors, indicating that intravenously injected NZ-1-IR700 reached the disseminated pleural tumor sites (**Figure 10**).

For the evaluation of the effect of NIR-PIT in the pleural disseminated mouse model, the NIR light was applied at 15 J/cm^2 from two directions (total of 30 J/cm^2) at 40 mW/cm^2 transcutaneously on day 5 (the NIR light was irradiated externally), followed by serial BLI. The control group was not irradiated with NIR light.

After treatment of mice with NIR-PIT, the treated group showed decreased luciferase activity. However, the relative light unit in the control group showed a gradual increase (*p < 0.05 versus control, t-test) (**Figure 11**).

Figure 1: Easy hand-made device for cell transplantation. Attach the stopper made with polystyrene foam to the 30G needle so that the tip remains at 5 mm. The tip of the needle should be bent to avoid pneumothorax.

Figure 2: Injection of target cells into the thoracic cavity. Turn the mouse sideways and pierce the needle into the mouse toward the lung. Since the stopper and needle tip are bent, the needle enters the thoracic cavity without sticking to the lungs. Inject target cells while pressing the needle against the mouse.

Figure 3: Acquisition Control Panel. Select **Luminescent**, **Photograph**, and **Overlay**. Set Exposure Time as **Auto**, Binning as **Small**, f/stop as 1 for luminescent and 8 for photograph, and Field of View as **C**. Once the mouse sample is ready for imaging, click **Acquire** for imaging acquisition.

Figure 4: Measurement (BLI). (**A**) Tool Palette panel. Select ROI Tools. We recommend the Circle to range the bioluminescent area on images. (**B**) BLI quantification. After selecting the ROI in each image, click **Measure ROIs** to analyze. (**C**) Quantification information. Use Configure Measurement on the left corner of the ROI measurements panel to select the values/information needed. Export this data table as .csv file.

Figure 5: Acquisition of DLIT. (**A**) Acquisition Control Panel for DLIT. Select **Luminescent**, **Photograph**, **CT**, **Standard-One Mouse**, and **Overlay**. Other settings are the same as 3.4–3.6 (Figure 3). (**B**) Imaging Wizard panel. Select **Bioluminescence**, and **DLIT**. (**C**) Select measurement wavelength. Select the wavelength as **firefly**. (**D**) Set the Imaging Subject as **Mouse**, Exposure Parameters as **Auto Settings**, Field of View as **C-13.4 cm**, and Subject Height as **1.5 cm**. Then Click the **X-rays will be produced when energized. Acquire.**

Figure 6: Reconstruction of DLIT. (**A**) Tool Palette panel. Open **Surface Topography** on the Tool Palette. Select **Show**. (**B**) Adjusting mouse surface recognition. Adjust the **Threshold** as the purple display shows only the body surface. Select the subject **Nude Mouse**, then click the **Generate Surface**. Make sure that the outline of the mouse is accurately drawn. (**C**) Tool Palette. Open the **DLIT 3D reconstruction Properties** tab, select Tissue Properties as **Mouse Tissue** and Source Spectrum as **Firefly**. (**D**) Open the **Analyze** tab and select the data for each wavelength data. (**E**) Click the **Reconstruct** button.

Figure 7: NIR irradiation. (**A**) Shield its belly with aluminum foil to prevent NIR irradiation to belly. (**B**) Irradiate NIR light using laser where BLI is strong; in some cases, NIR laser is divided in multiple directions.

Figure 8: SDS-PAGE. Successful confirmation of conjugated NZ-1-IR700 on an SDS-PAGE gel (left, colloidal blue staining; right, fluorescence at 700 nm channel). Diluted NZ-1 served as the control.

Figure 9: DLIT. Confirmation of the luciferase-expressing tumor cells in the thoracic cavity.

Figure 10: *Ex vivo* imaging. Characterization of the pleural disseminated MPM model 24 h after NZ-1-IR700 injection with BLI.

Figure 11: Antitumor effect of NIR-PIT on pleural disseminated model. (**A**) The podoplanin-targeted NIR-PIT regimen is showen in a line. Podoplanin-targeted NIR-PIT with NZ-1-IR700 on pleural disseminated model with H2373-luc tumors. BLI of the pleural disseminated model is shown. (**B**) While luciferase activities measured with BLI did not increase in the NIR-PIT group, the control group showed a gradual increase along with tumor growth. ($n \geq 3$ in both groups, t-test).

Discussion

In this study, we demonstrated a method for measuring the therapeutic effect of NIR-PIT on the pleural dissemination model of MPM. Highly selective cell killing was performed with NIR-PIT; thus, the normal tissue was hardly damaged[23–25]. With this type of selective cell killing, NIR-PIT was demonstrated to be safe in disseminated models[9,26]. However, alternative methods are possible in some steps. Various methods have been reported for the pleural dissemination model[27–30]. We selected the injection model because it is a simple procedure that is least burdensome to mice. We used BLI to measure the therapeutic effect of NIR-PIT because we can evaluate quantitatively with live mice. For example, positron emission tomography/computed tomography (PET/CT)[27] could be an alternative way to evaluate the tumor volume of the pleural dissemination model. NIR-PIT with BLI in other orthotopic models has been reported; NIR-PIT can be used for the abdominal dissemination model, lung multiple metastatic tumor model, and brain tumor[12,26,31–36]. Even small metastatic tumor foci can be observed with BLI, and NIR-PIT can be performed[11,12].

We described some parts of the protocol based on preliminary experiments. First, the time from APC administration to NIR irradiation. The pharmacokinetics were evaluated in

advance using a subcutaneous tumor model. APC peaks in the tumor 24 h after intervention *via* the tail vein using mAb; NIR irradiation can be performed 24 h after the APC administration[9–12]. Second, the NIR light dose required for killing tumor cells in NIR-PIT differs depending on the antibody and target cell lines, which is predicted using the *in vitro* results.

This study has a few limitations. First, tumors were widely distributed in the thoracic cavity, and NIR-irradiated energy could not be measured precisely. The wavelength of the NIR excitation light (peak at 690 nm) allows penetration of at least 2–3 inches into the tissue[37]. Therefore, in the case of mice, NIR light reaches the thoracic cavity even externally. Currently, NIR laser devices are used for the mouse pleural dissemination model[9–12]. However, in actual clinical use, we intend to use precise fiber optics to irradiate the entire intrathoracic cavity *via* the thoracic drainage tube[34]. Second, the NIR-irradiation dose is limited depending on the specificity of the mAb to antigens expressed on tumor cells. Non-specific binding of APC could cause unexpected organ damage.

In conclusion, we presented a method to evaluate the therapeutic effect of NIR-PIT with BLI in the pleural dissemination model of MPM.

Acknowledgements:
None

Disclosures

The authors declare that they have no competing financial interests.

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Figure 1

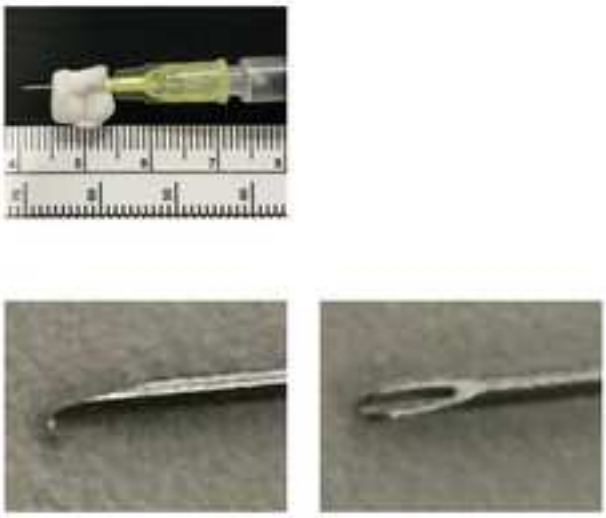


Figure 2

Figure 3

Figure 4

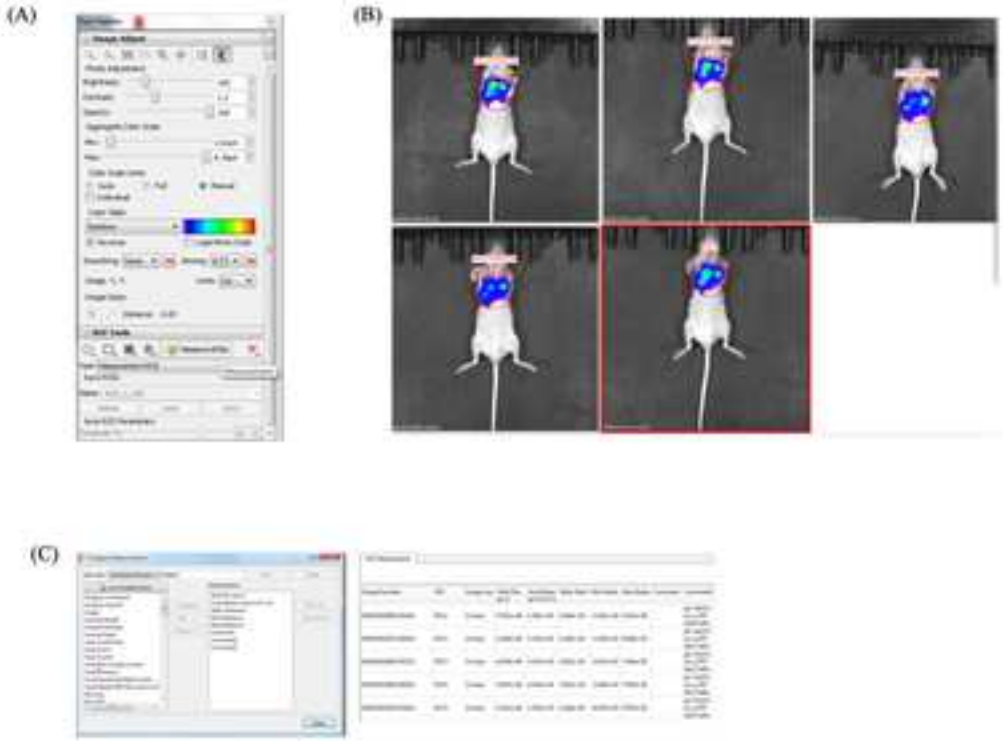


Figure 5

Figure 6

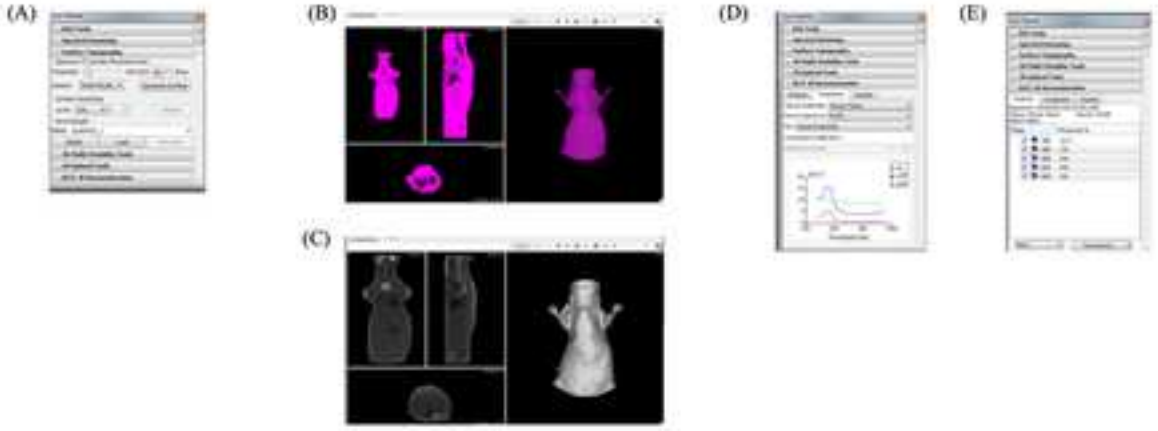


Figure 7

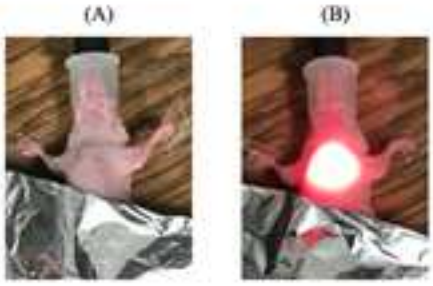


Figure 8

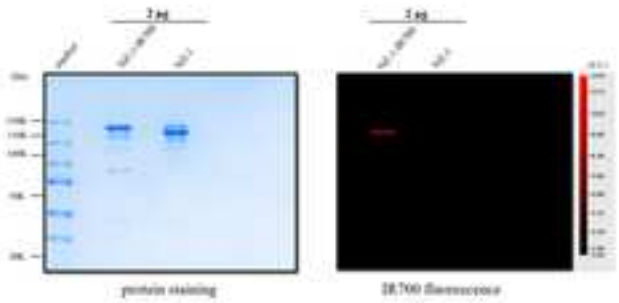


Figure 9

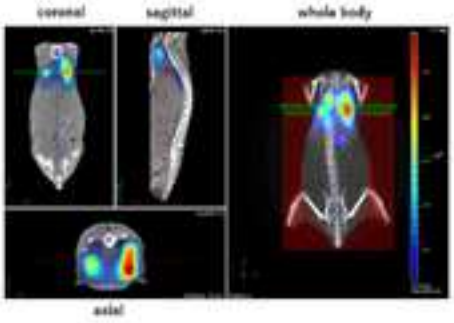


Figure 10

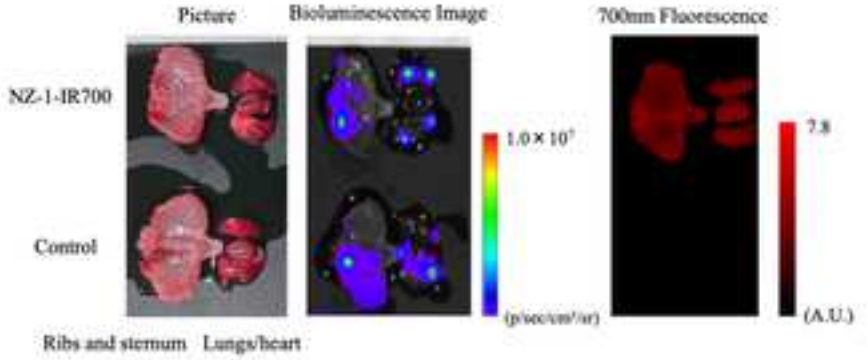
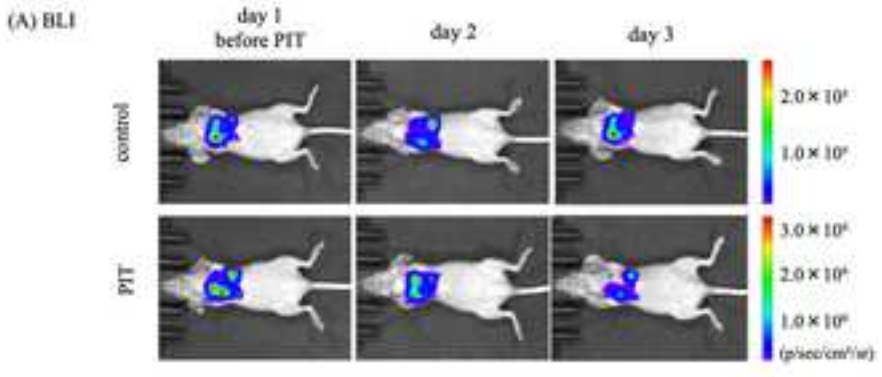


Figure 11



(B) Luciferase activity ratio

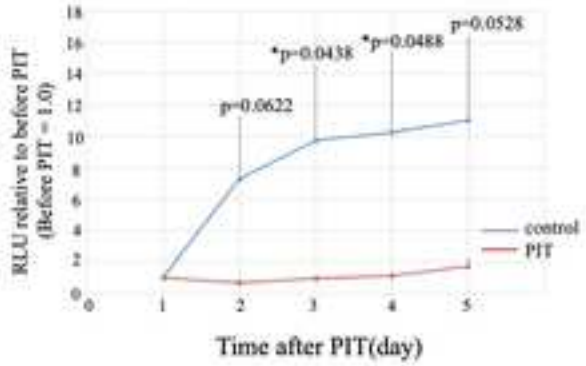


Table of Materials

Click here to access/download;Table of Materials;20200803 Jove materials.xlsx

Name

0.25w/v% Trypsin-1mmol/l EDTA 4Na Solution with Phenol Red
1mL syringe
30G needle
BALB/cSlc-nu/nu
Collidal Blue Staining Kit
Coomassie (bradford) Plus protein assay
Dimethyl sulfoxide (DMSO)
D-Luciferin (potassium salt)
GraphPad Prism7
Image Studio
IRDye 700DX Ester Infrared Dye
isoflurane
IVIS Spectrum CT
Living Image
Na2HPO4
NIR Laser
Novex WedgeWell 4 to 20%, Tris-Glycine, 1.0 mm, Mini Protein Gel, 12 well
NuPAGE LDS Sample Buffer (x4)
Optical power meter
PBS(-)
Pearl Trilogy imaging system
Penicilin-Streptomycin Solution (x100)
Puromycin Dihydrochloride
RediFect Red-Fluc-Puromycin Lentiviral Prticles
RPMI-1640 with L-glutamine and Phenol Red
Sephadex G25 column (PD-10)
UV-1900i

Company	Catalog Number
Wako	209-016941
TERUMO	SS-01T
Nipro	1907613
Japan SLC	
Invitrogen	LC6025
Thermo Fisher Scientific Inc (Waltham, MA, USA)	PI-23200
Wako	043-07216
Cayman Chemical	14681
GraphPad software	
Li-Cor Biosciences	
LI-COR Bioscience (Lincoln, NE, USA)	929-70011
Wako	095-06573
PerkinElmer	
PerkinElmer	
SIGMA-ALDRICH (St. Louis, MO, USA)	S9763
Changchun New Industries Optoelectronics Technology	MRL-III-690R
Invitrogen	XP04202BOX
Invitrogen	NP0007
Thorlabs (Newton, NJ, USA)	PM100
Wako	166-23555
Li-Cor Biosciences	
Wako	168-23191
ThermoFisher	A1113803
PerkinElmer	CLS960002
Wako	189-02025
GE Healthcare (Piscataway, NJ, USA)	17-0851-01
Shimadzu	

Comments

for cell culture
for mice experiment
for mice experiment

use for gel protein staining
for measuring the APC concentration
use for conjugation of IR700
for bioluminescence imaging and DLIT
for statistical analysis
for analyzing 700 nm fluorescent image

for mice anesthesia
for capturing bioluminescent image and DLIT
for analyzing bioluminescent image and DLIT
use for conjugation of IR700
for NIR irradiation
use for SDS-PAGE
use for SDS-PAGE
for measuring the output of the NIR laser

for capturing 700 nm fluorecent image
for cell culture
for luciferase transfection
for luciferase transfection
for cell culture
use for conjugation of IR700
for measuring the APC concentration

Dear Dr. Vineeta Bajaj, Review Editor, JoVE

We were gratified to receive the request for a revision of our review**, Near Infrared photoimmunotherapy for mouse models of pleural dissemination**, by Yasui H, et al. We are glad the reviewers and editors found sufficient merit in our work to justify further revision and we wish to express our appreciation to the reviewers for insightful comments. We believe we can address all the points raised and below, we respond on a point-by-point basis to the reviewers' comments.

Your manuscript, JoVE61593R1 "Near Infrared photoimmunotherapy for mouse models of pleural dissemination," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Oct 19, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Nam Nguyen, Ph.D.
Manager of Review
JoVE
nam.nguyen@jove.com
617.674.1888

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

1. Please revise the following lines to avoid previously published text: 42-44, 51-53, 64-69

We revised.

2. Additional details are needed:
1.2: What type of column is used?

We revised to Sephadex column.

1.4: What standard concentrations are used here?

The standard concentration of IR700 is recommended to be 0.5 – 5 µM.

1.4.4: How is this determined? Please provide an equation here.

We added equations to 1.3.2, 1.3.4.

2.1: How are the cells prepared?

Luciferase-expressing cells were prepared by luciferase gene transfection and confirmed high expression of luciferase after performing more than 10 cell passages.

Cells are cultured in medium supplemented with 10% fetal bovine serum and penicillin (100 IU/mL) and streptomycin (100 mg/mL).

2.3: What concentration of isoflurane is used here? How is sufficient depth of anesthesia determined?

Added to protocol about how to check anesthesia concentration and anesthesia depth. “When performing the procedure to mice, anesthetize them with isoflurane (introduction: 4-5%, maintenance 2-3%) and pressed the tail with tweezers to confirm that there was no reaction.

”

2.8: What happens to the mouse after?

The mouse will wake up from anesthesia and walk normally.

3. Please highlight up to 3 pages of essential protocol steps to be included in the protocol section of the video.

The next section is an important protocol step. (Page 2.5)

2. Creating a pleural dissemination model (1.5 pages)

5. In vivo NIR-PIT for pleural dissemination model. (1 page)

4. Please consolidate or remove some of the figures (especially the menu screenshots). If we are filming those steps, the menu screenshots would not be needed.

We integrated some of the screenshot content.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The MS has been greatly improved since the original version. The protocol is more detailed and the additional reference and context are appreciated.

Minor Concerns:

It is stated in the rebuttal that any mAb can be used; but presumably it should be directed to a cell surface/transmembrane protein?

Yes, that's right. Although not published in the paper due to negative data, NIR-PIT has no effect when targeting proteins “distant” from the cell membrane.

I am not clear on the response to question 23 in the rebuttal letter. Why is it not necessary to show the effect of the mAb alone as compared to the control and PIT-treated (Fig. 18B-C)?

We evaluate the group with dorsal tumor xenografted model, in advance. The result is no effect compared to the control. Therefore, the therapeutic effect with the disseminated pleural model was shown by comparing the two groups of control and NIR-PIT.

コメントの追加 [べ1]: 反論の内容は大丈夫でしょうか？

Fig.1: Should show the bent needle so the reader can see the degree to which the needle is bent. Suggestion for safety (line 148): Use forceps to bend the needle.

Thank you very much. We added a figure and added a description.

Line 145: Toe pinch to test depth of anesthesia prior to injection?

Yes, we anesthetize and check the depth of anesthesia before treatment. Added after the anesthesia concentration of the protocol.

Line 194-195: Do you mean that if the BLI image is not diffused in the thorax then the tumor may be mistakenly transplanted subcu?

Yes, that's right. As far as we experimented, all mice that showed luminescence only at the insertion site were subcutaneously transplanted. Even after continuing the observation, no tumor was formed in the thoracic cavity, and a subcutaneous tumor developed at the insertion site.

Line 312: Do you mean "pierce the lung with the needle angled towards the lung"?

No, it sticks the needle towards the lungs, but not pierce the lungs.

I changed it to the following expression to avoid misunderstanding.

Turn the mouse sideways and stick the needle into the mouse toward the lung. Since the stopper and needle tip are bent, the needle enters the thoracic cavity without sticking to the lungs. Inject target cells while pressing the needle against the mouse. Thank you.

Reviewer #2:

This is a study for which the PDT-related details remain sparse, poorly described and will not hold up to scrutiny from experts in the field.

The main issue is that the authors seem averse to constructive critiques and are more interested in insisting that they are right than in improving the manuscript.

The light dosimetry parameters, the photosensitizer dose, and numerous details related to background in the field remain missing or poorly considered. This is not a high quality submission and is not an article that, in current form, will reflect well on JoVE. It is also not evident that the authors will take criticism in the spirit of the peer review process that is designed to help their study hold up to scrutiny in the scientific community.

コメントの追加 [べ2]: なんて返事したらいいかわからないです・・・

We responded your comments with honest. I'm sorry for such a terrible reply. Also, our study is totally different from the PDT tratment. The mechanism of NIR-PIT is completely different from the conventional PDT.

Please see below.

Near-Infrared Photoimmunotherapy of Cancer.
Kobayashi H, Choyke PL. Acc Chem Res. 2019 Aug 20;52(8):2332-2339. doi: 10.1021/acs.accounts.9b00273.

Reviewer #3:

Manuscript Summary:

The paper described a protocol using NIR irradation for photo-immunological treatment to cancer.

Major Concerns:

No

Minor Concerns:

1. The introduction should be more informative on the Photoimmunotherapy towards cancers. A review paper can be referred, Near Infrared Light Triggered Photo/Immuno-Therapy Toward Cancers. The paragraphs in Introduction section should be more coherent. Last paragraph needs provide more details of the work.

Thank you for introducing the references.

We organized paragraphs and added consideration about photoimmunotherapy.

2. The way to determine the ratio of antibody to IR700 dyes in the APC after they bounded using UV-Vis is not very direct. When there are free antibody or IR700 dyes

molecules in the solution, the UV-Vis results would not be accurate. Can author explain how much accuracy the measurement can be achieved.

Previous reports of NIR-PIT have examined the purity of mAb-IR700 by multiple methods such as mass spectrometry (Cancer Cell-Selective In Vivo Near Infrared Photoimmunotherapy Targeting Specific Membrane Molecules; Makoto M, Nat Med. 2011; 17 (12): 1685-91). Thus the accuracy is already confirmed.

3. The irradiation power of the NIR light is not consistent, 40-100 mW/cm2 and 30-150 J/cm2.

Yes, that's right. Irradiation energy and total irradiation amount vary depending on the APC used. Most reports irradiate at 100 mW/cm^2, but in this paper it is treated at 40 mW/cm^2, so the conditions are broader.

4. The protocol can be described in a more quantitative way. It is a bit too general.

Specific values are described in the protocol, and the conditions to be adjusted are described in NOTE. Please refer to the section. Thank you.

差出人: **Christina Zhang** christina.zhang@mdpi.com
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宛先: k-sato@med.nagoya-u.ac.jp
CC: 安井裕智 yh0814@med.nagoya-u.ac.jp

Dear Professor Sato,

Thank you for getting in touch with us.

I hope the information is helpful. Don't hesutate to contact me if any question. Have a good day!

Kind Regards,
Christina Zhang
Section Managing Editor, MDPI AG
Skype: live:1179299252
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> > Title: Targeted Phototherapy for Malignant Pleural Mesothelioma: Near Infrared Photoimmunotherapy Targeting Podoplanin
> > Authors: Yuko Nishinaga, Kazuhide Sato *, Hirotoshi Yasui, Shunichi Taki, Kazuomi Takahashi, Misae Shimizu, Rena Endo, Chiaki Koike, Noriko Kuramoto, Shota Nakamura, Takayuki Fukui, Hiroshi Yukawa, Yoshinobu Baba, Mika K. Kaneko, Toyofumi F. Chen-Yoshikawa, Hisataka Kobayashi, Yukinari Kato, Yoshinori Hasegawa
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