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Effective Fluorescence-Guided Surgery of Liver Metastasis Using a Fluorescent Anti-CEA Antibody

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Abstract

Background and Objectives—Delineation of adequate tumor margins is critical in oncologic surgery, particularly in resection of metastatic lesions. Surgeons are limited in visualization with bright-light surgery, but fluorescence-guided surgery (FGS) has been efficacious in helping the surgeon achieve negative margins.

Methods—The present study uses FGS in a mouse model that has undergone surgical orthotopic implantation (SOI) of colorectal liver metastasis tagged with green fluorescent protein (GFP). An anti-CEA antibody conjugated to DyLight 650 was used to highlight the tumor.

Results—The fluorescent antibody clearly demarcated the lesion at deeper tissue depth compared to GFP. Fluorescence of the anti-CEA-DyLight650 showed maximal tumor-to-liver contrast at 72 hr. Fifteen mice underwent bright-light surgery (BLS) versus FGS with GFP versus FGS with anti-CEA-DyLight650. Mice that underwent FGS had a significantly smaller area of residual tumor ($P < 0.001$) and significantly longer overall survival ($P < 0.001$) and disease-free survival ($P < 0.001$). Within the two FGS groups, mice undergoing surgery with anti-CEA-DyLight650 improved survival compared to only GFP labeling.

Conclusions—In the present report, we demonstrate that an anti-CEA antibody conjugated to a DyLight 650 nm dye clearly labeled colon cancer liver metastases, thereby enabling successful FGS.

Keywords

fluorescence-guided surgery; colon-cancer liver metastasis; orthotopic-liver metastasis model; fluorescent anti-CEA antibody; survival; disease-free survival

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INTRODUCTION

Adequate tumor margins are essential for curative resection oncologic surgery. It is particularly important in more complex oncologic resections such as metastasectomy of liver lesions in colon cancer. To meet this challenge, our laboratory has pioneered fluorescence-guided surgery (FGS) using patient-derived orthotopic xenograft mouse models of human cancers that better recapitulate tumor growth and metastasis [1–3].

FGS has shown significant benefit to extend disease-free survival (DFS) and overall survival (OS) and to greatly reduce residual cancer in a variety of orthotopic tumor models including colon cancer [4–7], pancreatic cancer [8–14], breast cancer [15], lung cancer [16], glioblastomas [17,18], melanomas [19], gastrointestinal stromal tumors [20], soft tissue sarcomas [21,22], and osteosarcomas [23,24].

In colon cancer specifically, FGS technology has been shown to be efficacious in a PDOX model [7]. Anti-CEA conjugated with AlexaFluor 488 dye was delivered intravenously prior to surgery. The fluorescent antibody selectively labeled the tumor that was accurately visualized during laparotomy and enabled complete resection. No cancer cells were seen at the histologic margins and the FGS group remained tumor-free after 6 months.

FGS was used for colon cancer metastases in an orthotopic nude mouse model with a green fluorescent protein (GFP)-expressing colon cancer cell line- HT29 (HT-29 GFP) [25]. Surgical resection of the liver metastasis using bright-light surgery (BLS) and FGS was performed. After BLS, residual GFP-labeled cancer cells were present at the resection bed. Residual tumor was not seen in the FGS group, even at high magnification. Repeat laparotomy 28 days after initial surgery showed large recurrent tumor in the BLS group, but not in the FGS group. FGS significantly reduced residual tumor and decreased the rate of recurrence.

We also developed an in situ method of labeling orthotopic colon-cancer liver metastases with fluorescence using OBP401, an adenovirus vector [26]. OBP-401 is a telomerase-dependent adenovirus previously shown to be efficacious in labeling solid tumors such as colon cancers [26], pancreatic cancers [14], lung cancers [16], glioblastomas [17], melanomas [19], soft-tissue sarcomas [22], and osteosarcomas [24]. In an orthotopic colon-cancer liver metastasis model, OBP-401 clearly labeled the hepatic lesion in situ as well as satellite lesions and allowed successful FGS. Compared to BLS, recurrence and survival were improved after FGS of the metastasis.

In the present report, we demonstrate that an anti-CEA antibody conjugated to a DyLight 650 nm dye clearly labeled colon-cancer liver metastases, thereby enabling successful FGS.

MATERIALS AND METHODS

Animal Care

Athymic nude mice (AntiCancer, Inc., San Diego, CA) at 4–6 weeks of age were used for this study. These mice were kept in a positive-pressure facility with high-efficiency particulate arrestance (HEPA) filtration and cared for in accordance with principles and

procedures outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals under Public Health Service Assurance Number A 3873-1. All procedures were performed under anesthesia with subcutaneous injection of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acetapromazine maleate. Postoperative analgesia was provided via ibuprofen 7.5 mg/kg orally in drinking water for 7 days postoperatively. Mice were sacrificed by CO₂ inhalation when meeting humane end point criteria of skin ulceration, cachexia, difficulty breathing, and prostration.

Establishment of Liver Metastasis Model

HT-29 GFP-expressing cells were maintained and cultured in DMEM as described previously [25]. Cells (5×10^5) were trypsinized and harvested. The cells were washed twice and suspended in 50 μ l serum-free media with 50% Matrigel and injected into the spleen of athymic nude mice (4–5 weeks old) to produce splenoportal seeding of the liver. Four weeks after splenic injection, multiple-lobe liver metastases developed in the mice. The animals were sacrificed and the liver metastases were harvested.

To establish an orthotopic liver metastasis model for subsequent transplantation, a small 6–8 mm laparotomy incision was made in recipient nude mice. The left lobe of the liver was then exteriorized through this incision and a single 3 mm³ tumor fragment was surgically orthotopically implantated (SOI) in the subcapsular space of the liver. The liver was then returned to the abdomen and the laparotomy closed in one layer with 6-0 nylon sutures (Ethilon, Ethicon, Inc., Somerville, NJ). The solitary tumor metastasis was allowed to grow for 3 weeks.

Antibody Conjugation With Fluorescence Dye

Monoclonal antibodies specific for CEA were labeled with the DyLight650 protein labeling kit according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA).

BLS and FGS of Liver Metastases

Intravenous injection of 50 μ g of the anti-CEA antibody conjugated with DyLight650 was delivered via the tail vein into each of the mice in the anti-CEA-DyLight650 FGS group with orthotopic liver metastasis 24 hr before surgery. Tumor imaging was performed at various time points after administration to determine peak intensity of dye fluorescence.

To compare BLS with FGS, 14 mice underwent surgery: seven mice underwent BLS, seven mice underwent FGS using anti-CEA-DyLight650. The HT-29 GFP-expressing tumor was exposed and macro imaging was performed preoperatively with the Olympus OV100 small animal imaging system [27] (Olympus, Tokyo, Japan). Three images were taken of each mouse: Bright light, 490 nm filter (GFP) and 650 nm filter (DyLight). For FGS of the mice with liver metastases labeled with anti-CEA antibodies conjugated to DyLight650, a Mini Maglite LED pro flashlight (Mag instrument, Ontario, CA) coupled to an excitation filter (ET 640/30x, Chroma Technology Corporation, Bellows Falls, VT) was used as the excitation source. A canon EOS 60D digital camera with an EF-S18-55 IS lens (Canon, Tokyo, Japan) coupled with an emission filter (Hq700/75M-HCAR, Chroma Technology Corporation) was used for FGS. BLS surgery was performed under standard bright-field

using an MVX10 microscope (Olympus) [28]. For hemostasis, a single 6-0 nylon suture (Ethilon) was placed on cranial side of the liver. The resection line was beyond the limit of the tumor margin that was visible during FGS, but not during BLS. Manual pressure was applied to the resection bed for further hemostasis at the conclusion of the procedure and the liver suture was removed. The surgical resection bed was imaged with the OV100 system to determine residual cancer cells immediately after surgery.

To compare BLS with FGS using fluorescence from GFP alone and FGS with anti-CEA-DyLight650, 15 mice underwent surgery: The mice were randomized into three groups. Five mice underwent BLS, five mice underwent FGS with fluorescence from GFP alone, and five mice underwent FGS with fluorescence labeling using anti-CEA-DyLight650. For FGS of the mice with liver metastases labeled with anti-CEA antibodies conjugated to DyLight650, the Mini Maglite LED pro unit was used as described above. FGS for the mice with liver metastases expressing GFP was performed using a hand-held imaging system (AM4113TGFBW Dino- Lite Premier; AnMo Electronics Corporation, Taipei, Taiwan). BLS surgery was performed under standard bright-field using an MVX10 microscope as above. To assess for recurrence after surgery, weekly noninvasive imaging was performed with the LT-9900 Illumatool (Lighttools Research, Encinitas, CA). Mice were followed until humane end point criteria were reached to monitor DFS and OS.

RESULTS AND DISCUSSION

Schema of liver metastasis mouse model is shown in Figure 1A. Images of the multiple-lobe (Fig. 1B) and single-lobe (Fig. 1C) liver metastasis mouse model show the surface-exposed tumors in bright field (BF) light where it is not possible to discern the margins. GFP clearly illuminated exposed tumors and was able to delineate surface tumor margins. However, the anti-CEA antibody conjugated with DyLight 650 (CEA650) was able to display deeper portions of liver tumors, which were not clearly seen with GFP fluorescence.

Tumor imaging was performed at various time points after administration of anti-CEA antibodies conjugated to DyLight650 via tail-vein injection. Figure 2A shows the liver metastasis illuminated most brightly at 48 hr. However at 48 hr, the liver was also brightly illuminated. The best contrast between tumor and background liver peaked at 72 hr when the fluorescent antibody has washed out from normal liver tissue. Time-intensity curves of fluorescence kinetics in the tumor, liver, and contrast (difference between intensity values from the tumor and liver) are summarized in Figure 2B. FGS was performed using the Mini Maglite and digital camera with the filters described above. A Photograph of the setup is shown in Figure 2C. White-light imaging did not clearly image the tumor, but fluorescence imaging was able to (Fig. 2D).

The tumor surface was clearly visible under both GFP and anti-CEA-DyLight650 navigation (Figs. 2D and 3A). Deeper tissue penetrance was limited with GFP, but not with anti-CEA-DyLight650. Tumors covered by normal liver tissue were detectable only under anti-CEA-DyLight650 navigation. No residual tumor was detected after FGS. In the area of the metastasis covered by normal liver tissue, the GFP fluorescence drops off. Using anti-CEA-DyLight650, the fluorescence signal was able to penetrate normal liver tissue and the deep

part of the tumor was demarcated in representative gross images of excised tumors (Fig. 3B).

The study schema to compare BLS and FGS is shown in Figure 4. Four weeks after surgical orthotopic implantation, 14 mice were randomized into the BLS group ($n = 7$) and the FGSS group ($n = 7$). FGS was performed using the imaging system as described above. Recurrence was assessed after surgery with weekly noninvasive imaging using the LT-9900 Illumatool (Lighttools Research) until the end of the experiment. Both imaging systems excited GFP fluorescence at approximately 490 nm.

Whole-body images after BLS (Fig. 5A) or FGS under anti-CEA-DyLight650 navigation (Fig. 5B) do not show residual tumor at low magnification. However, residual tumors can be visualized at high magnification with the OV-100. Multiple small residual tumors were observed in the resection bed after BLS (Fig. 5C) and a smaller residual tumor was detected at high magnification even after FGS (Fig. 5D).

The residual tumor area was detected at high magnification with the OV-100 and measured with ImageJ. Bar graphs of residual tumor area after surgery show that the residual tumor area was significantly larger after BLS compared to FGS (Fig. 6).

Disease recurrence was measured by weekly noninvasive imaging with the LT-9900 Illumatool (Lighttools Research). Mice that underwent FGS with anti-CEA-DyLight650 had significantly longer DFS ($P < 0.001$) (Fig. 7A) and OS ($P < 0.001$) (Fig. 7B) compared to the mice treated with BLS.

Figure 8 outlines the overall study schema to compare BLS with FGS under GFP navigation and FGS under anti-CEA-DyLight650 navigation.

DFS and OS for the mice treated with BLS, FGS under GFP navigation (FGS-GFP), or FGS under anti-CEA-DyLight650 navigation (FGS-650) are shown in Figure 9. Mice undergoing FGS using anti-CEA-DyLight650 navigation had significantly longer DFS ($P < 0.001$) (Fig. 9A) and OS ($P = 0.001$) (Fig. 9B) compared to the other two groups.

We have previously shown that FGS of liver metastases improves resection margins and decreases recurrent tumor burden with cancer cells labeled with GFP. In the present report, we used DyLight650 conjugated to an anti-CEA antibody. We observed that the in situ labeling with CEA650 better labeled deep-seated lesions compared to GFP alone.

Our results showed that GFP visualization was equivalent to anti-CEA-DyLight650 in identifying superficial lesions, but demonstrated the limitations of GFP for visualization of areas of metastases covered by normal tissue. This was reflected in the survival data with the FGS-GFP group having improved DFS and OS compared to BLS. The FGS-anti-CEA-DyLight650 had superior DFS ($P < 0.001$) and OS ($P < 0.001$) compared to both these groups. Results from the FGS-GFP group are consistent with our previously published data [5–11,21,26,29–34]. Further studies are needed to determine the limits of contrast under tissue depth and other improvements with a number of different fluorophores.

The value of FGS is evident in open surgeries: improving resection margins while limiting the resection of non-neoplastic tissue. Translational use of FGS will also be particularly useful in laparoscopic oncologic surgeries. Many colon surgeries are performed via laparoscopy and some liver resections are as well. We have also shown that the use of fluorescent dyes in laparoscopy aids in rapid and accurate identification and localization of tumors, especially at the sub-millimeter resolution [29,31,35–36]. The goal of oncologic FGS, with any modality, is cure. Laparoscopic resection of liver metastases is an important new area and the use of FGS in this setting with development of tumor-specific fluorophores is especially promising [37,38].

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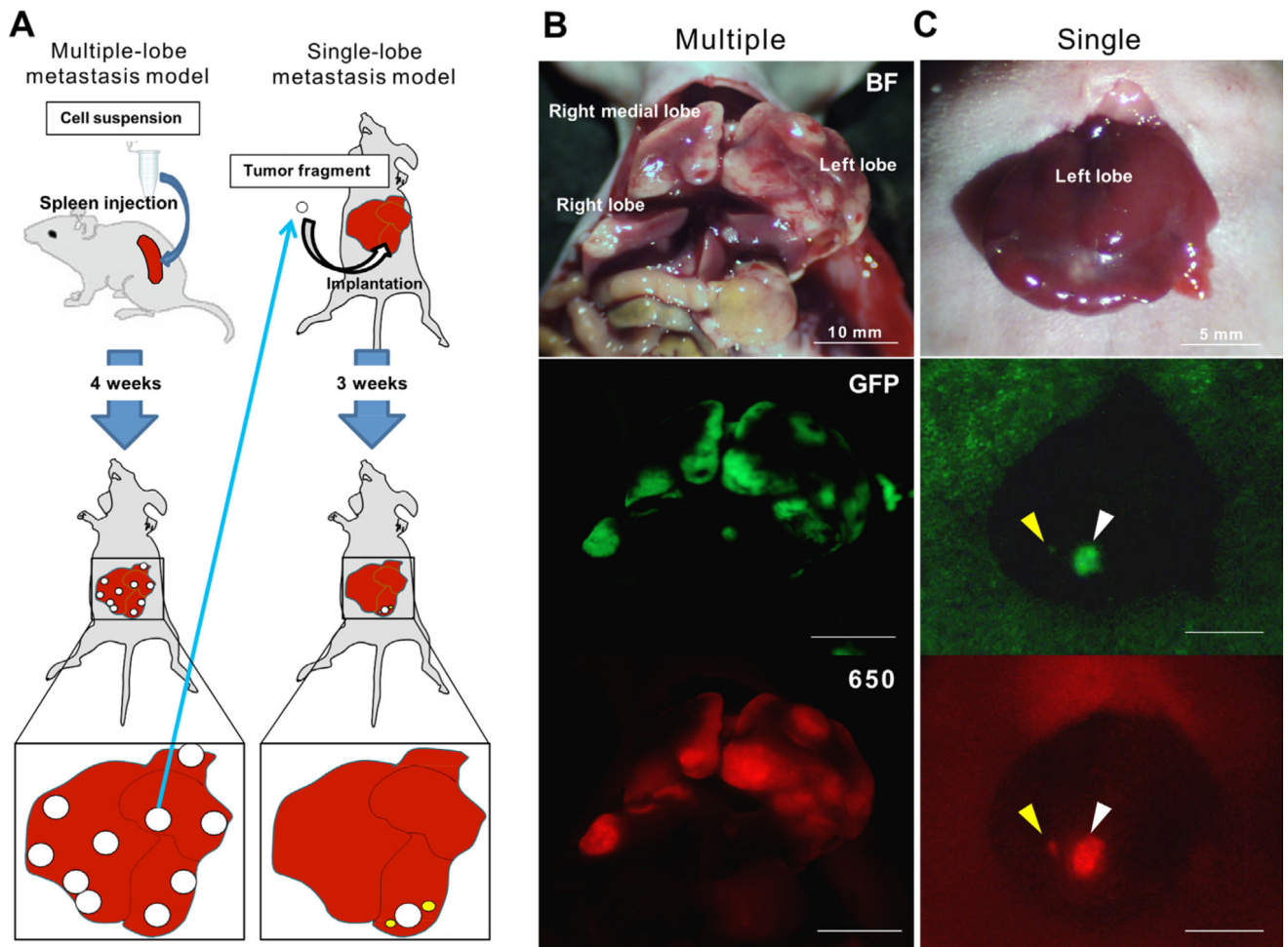


Fig. 1.

(A) Schema of liver-metastasis mouse model. HT-29-GFP cancer cells expressing CEA were harvested by trypsinization and washed twice with serum-free medium. Cells (5×10^5 cells in 50 μ l serum-free medium with 50% Matrigel) were injected into the spleen in mice. Four weeks after spleen injection, multiple-lobe liver metastases developed in the mice. Experimental liver metastases were resected and cut into fragments for subsequent orthotopic implantation. In order to develop a single-lobe liver-metastasis model, a small 6–8 mm midline incision was made in other 14 nude mice to access the liver. The left lobe of the liver was exposed through this incision, and a single tumor fragment was orthotopically implanted to the left lobe of the liver. Images of multiple-lobe (B) and single-lobe (C) liver-metastasis mouse model. Surface-exposed tumors were detected in bright field (BF). GFP clearly illuminated exposed tumors, whereas labeling with anti-CEA antibody conjugated with DyLight 650 (CEA650) was able to visualize deeper parts of liver tumors, which were not detected in GFP fluorescence. Yellow arrowhead indicates intrahepatic metastasis from the implanted tumor (white arrowhead).

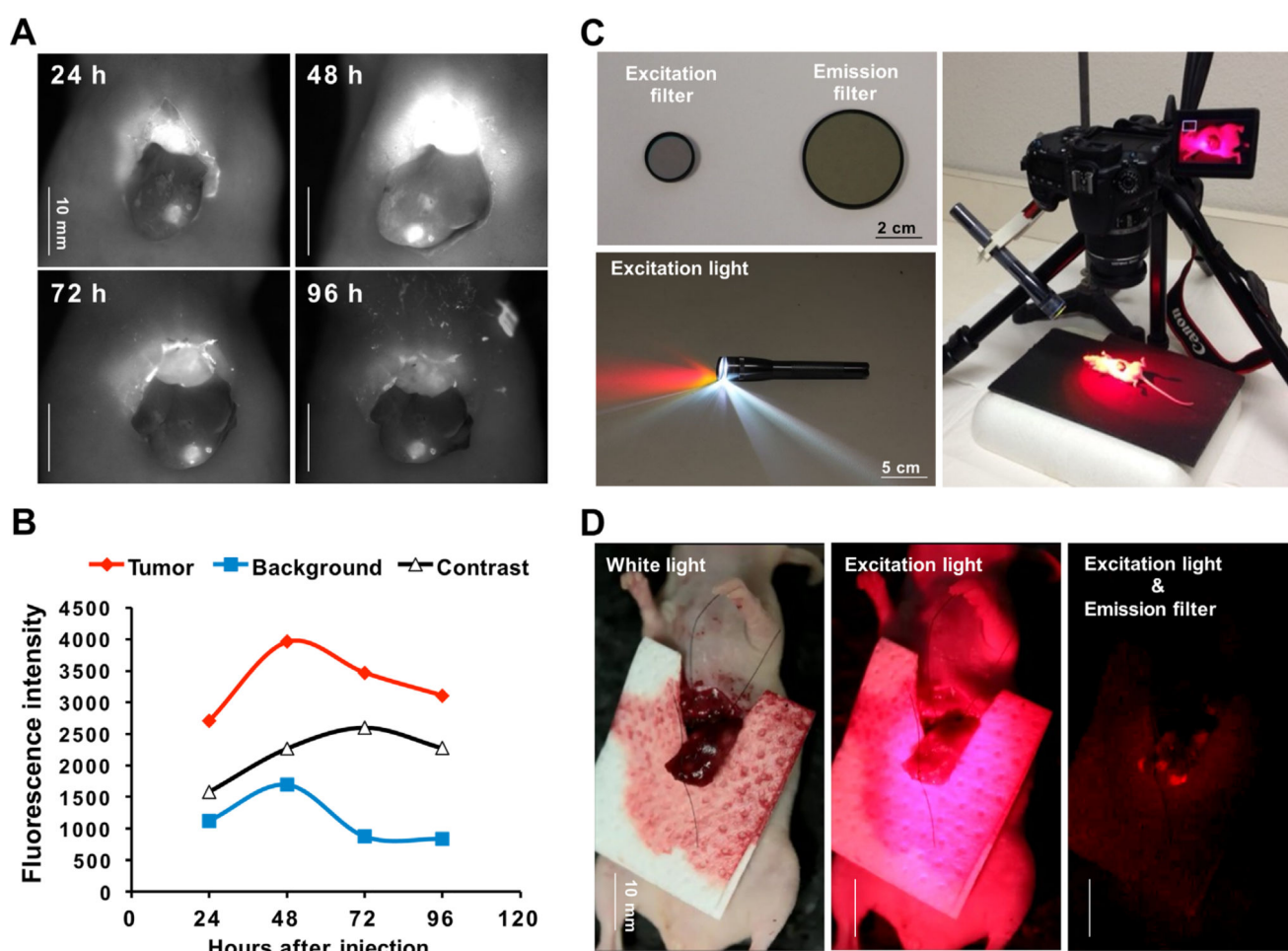


Fig. 2.

Tumor imaging using anti-CEA-DyLight650. Fifty micrograms of anti-CEA-DyLight650 was injected from the tail vein of the mice with liver tumor before imaging. Tumor imaging was performed at various time points after administration (A). The liver tumor was illuminated most brightly at 48 hr, when CEA650 was not fully washed out from normal live tissue. (B) Time–intensity curve of imaging using CEA-650. Tumor and background fluorescence had peak intensity at 48 hr, and background fluorescence decreased rapidly after that. The best contrast for tumor imaging was obtained at 72 hr. (C) In vivo imaging system for CEA-650. Imaging was performed using the Mini Maglite® LED Pro flash light (Mag Instrument) with an excitation filter (ET640/30X, Chroma Technology Corporation) and a Canon EOS 60D digital camera with an EF-S18-55 IS lens (Canon) and an emission filter (HQ700/75M-HCAR, Chroma Technology Corporation). FGS under CEA-650 navigation was performed using this imaging system. (D) In vivo images of liver metastasis. Only exposed tumors were detected under white light (bright field), whereas buried tumors were clearly detected with an excitation light and emission filter for CEA-650 fluorescence. Scale bars: 10 mm (A and D), 2 cm (C; filters), and 5 cm (C; flash light).

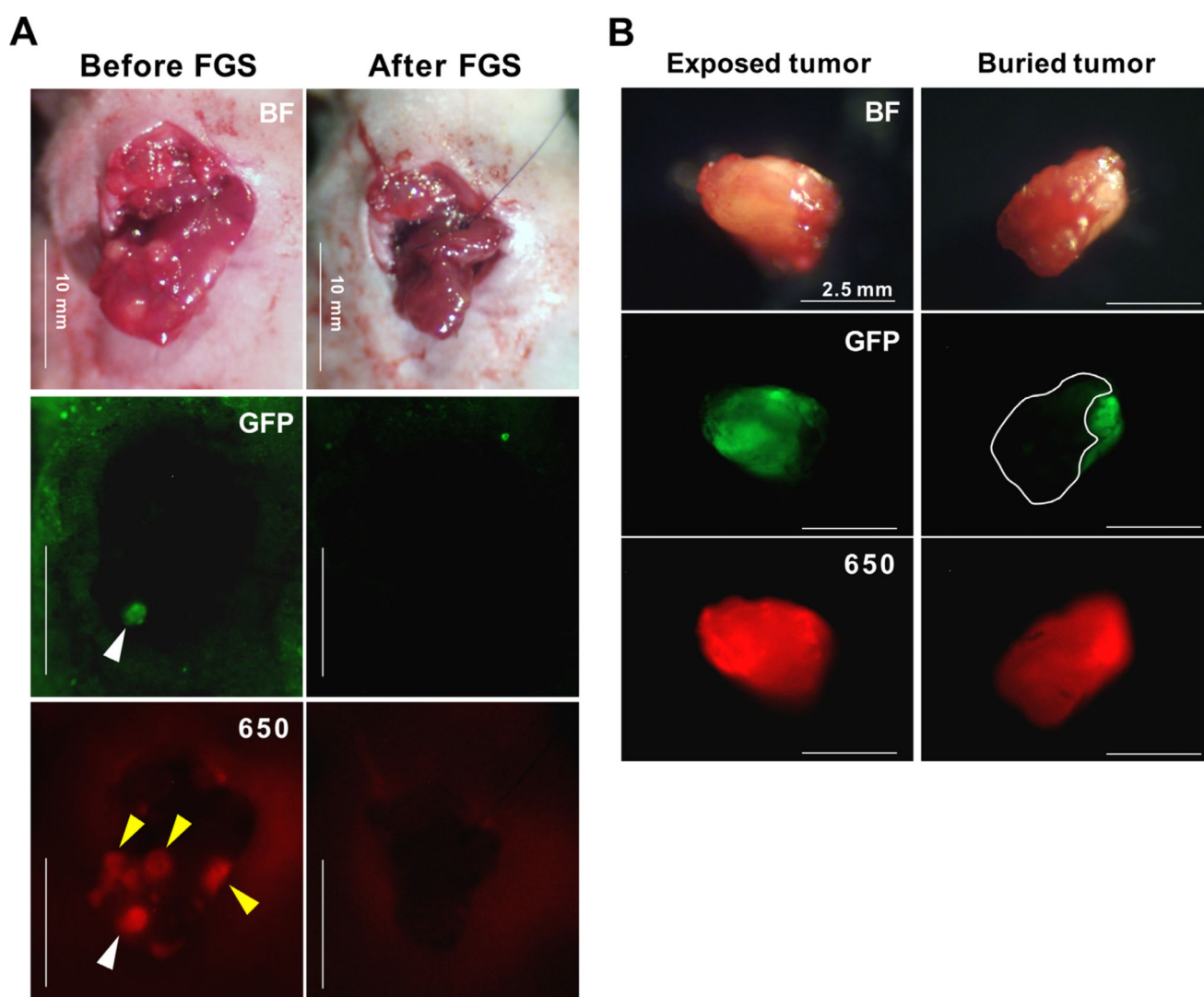
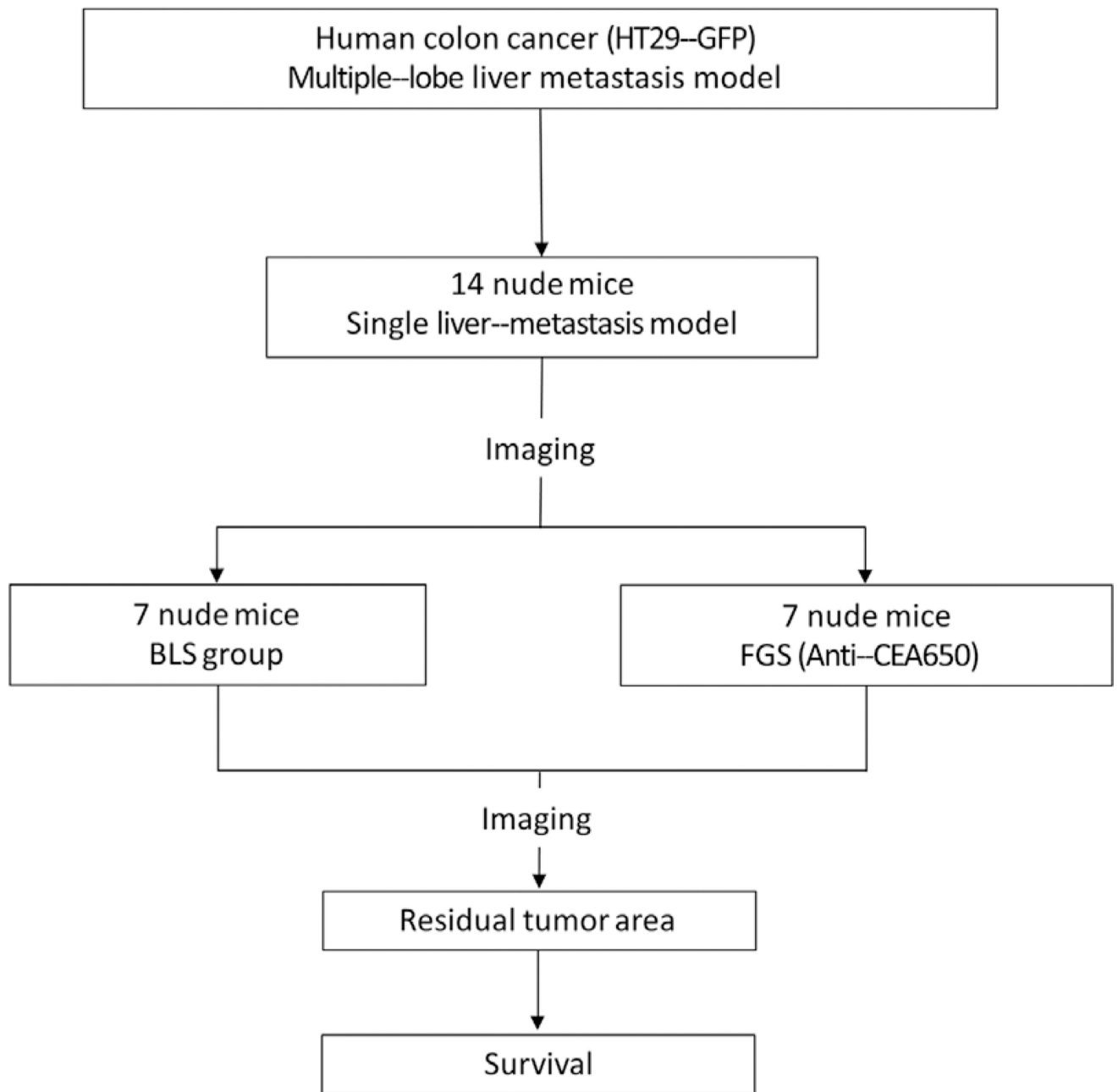


Fig. 3. (A) Whole-body images before and after FGS under anti-CEA-DyLight650 navigation. The surface-exposed tumor (white arrow head) was clearly detected under both GFP and anti-CEA-DyLight650 navigation. In contrast, the tumors covered with normal tissues (yellow arrow heads) were detected only under anti-CEA-DyLight650 navigation. No residual tumor was detected after FGS. (B) Representative gross images of excised tumors (left panel: exposed tumor, right panel: buried tumor). Upper panels indicate bright field (BF) images, middle and lower panels indicate fluorescence images for GFP and DyLight 650 (650), respectively. The area surrounded by a white broken line indicates the buried part of the excised tumor. CEA650 fluorescence was able to penetrate normal liver tissue and visualize the buried part of the tumor, which was not detected in GFP fluorescence. Scale bars: 10 mm (A) and 2.5 mm (B).

**Fig. 4.**

Overall study schema to compare BLS with FGS under anti-CEA-DyLight650 navigation. Four weeks after surgical orthotopic implantation, 14 mice were randomized into BLS group ($n = 7$) and FGSS group ($n = 7$). BLS was performed under bright light. FGS was performed using the imaging system as described above. The resection line was designed beyond the limit of the tumor margin which was visible during FGS, but not during BLS. Residual GFP tumor fluorescence after surgery was detected with the OV100 Small Animal Imaging System with variable magnification. To assess for recurrence after surgery, weekly noninvasive imaging was performed with the LT-9900 Illumatool (Lighttools Research) until

the end of the experiment. Both imaging systems excited GFP fluorescence at approximately 490 nm.

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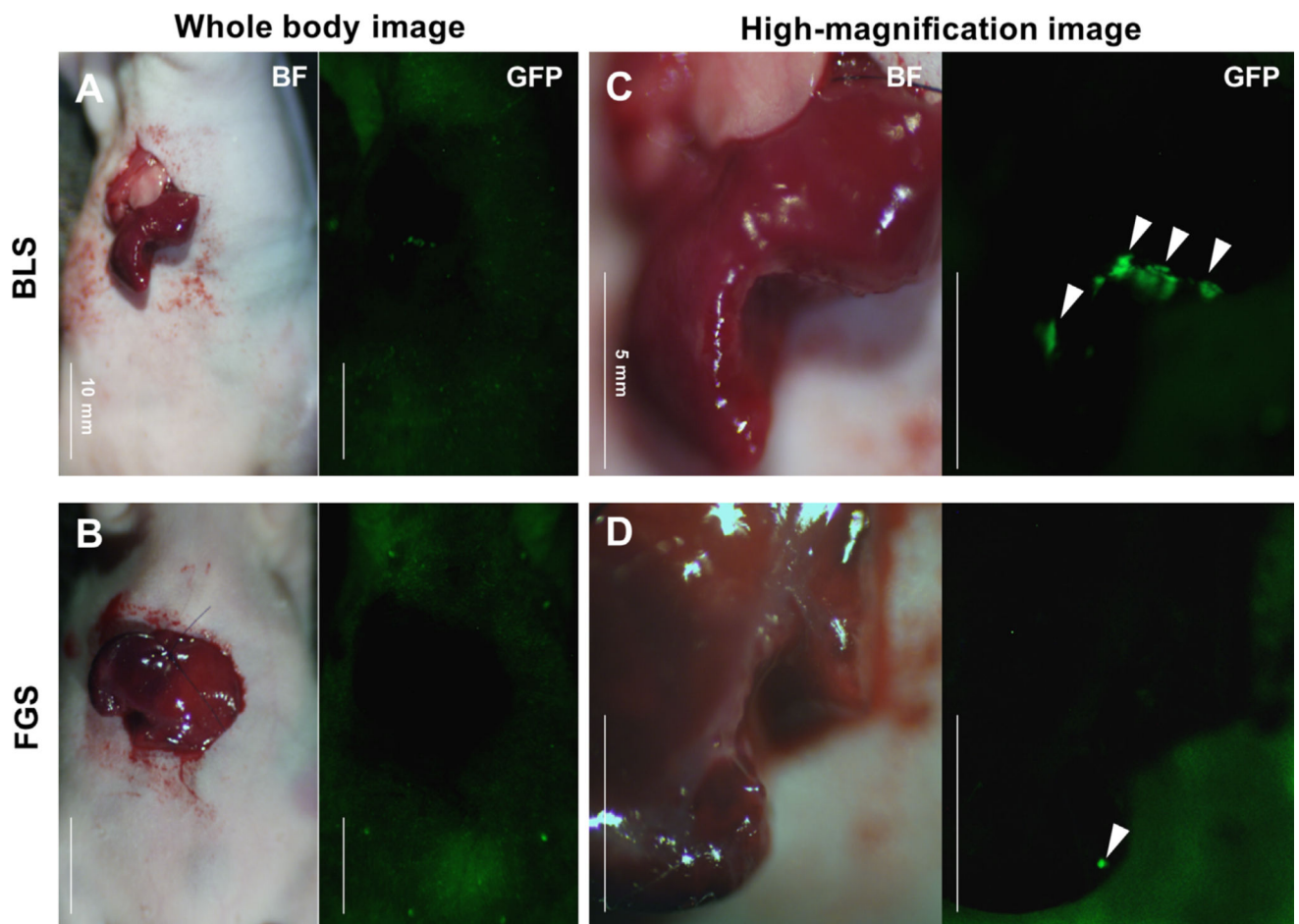


Fig. 5. Whole body images after BLS (A) or FGS under CEA-650 navigation (B). No residual tumor was detected in whole body images (at low magnification). In contrast, some residual tumors (white arrowheads) after BLS were visualized at high magnification with the OV-100 (C). A very small residual tumor nodule was detected at high magnification even after FGS (D). Scale bar: 10 mm (A and B) and 5 mm (C and D). ** $P < 0.01$.

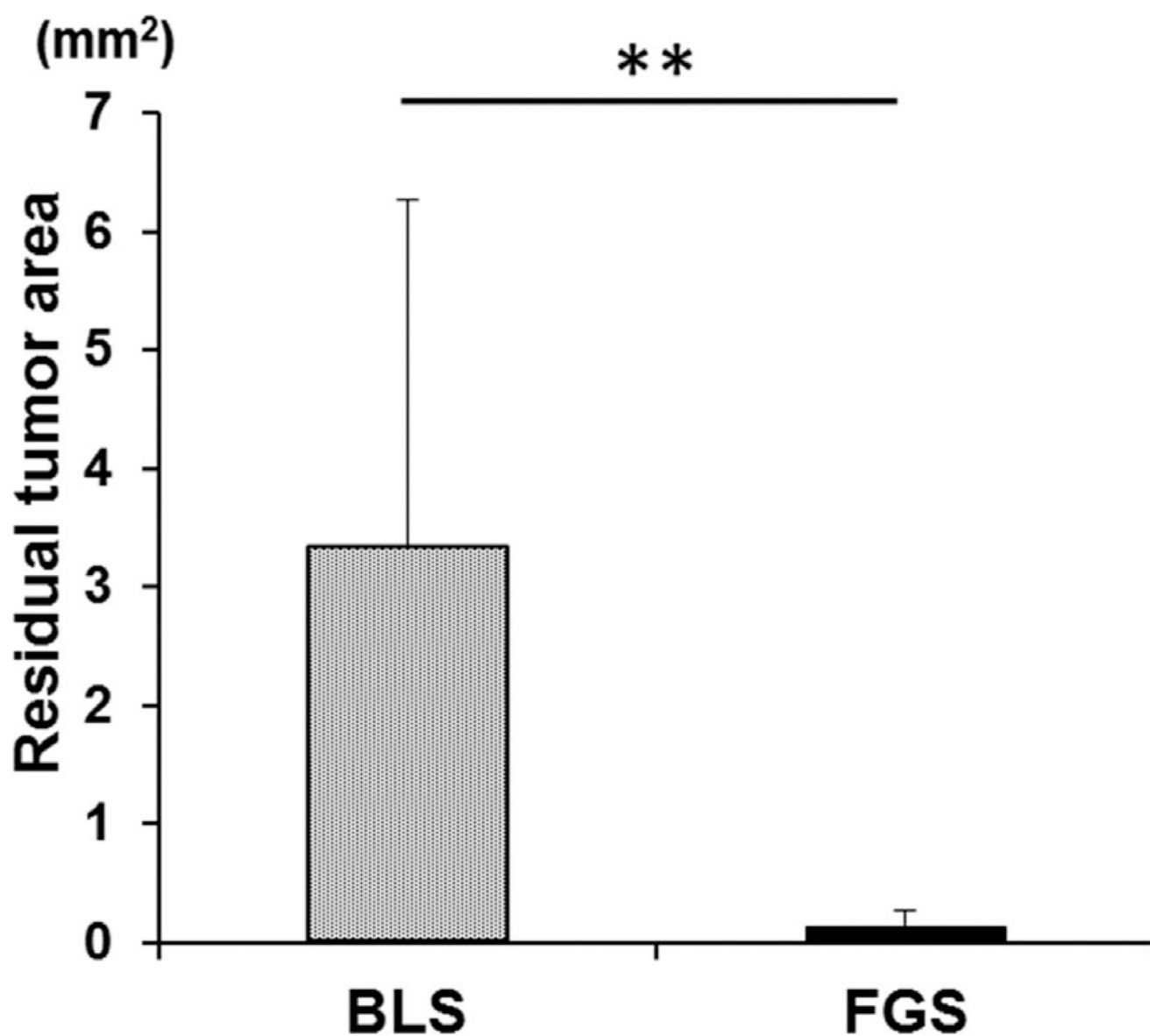
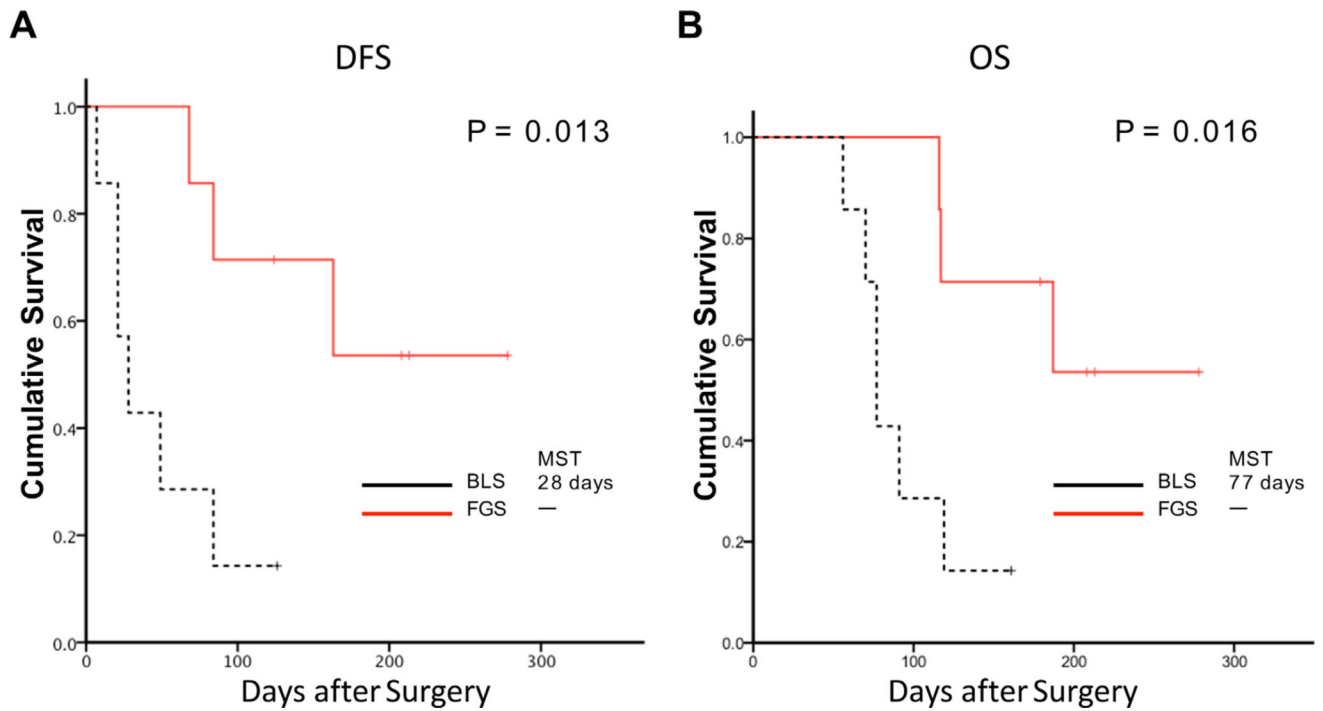
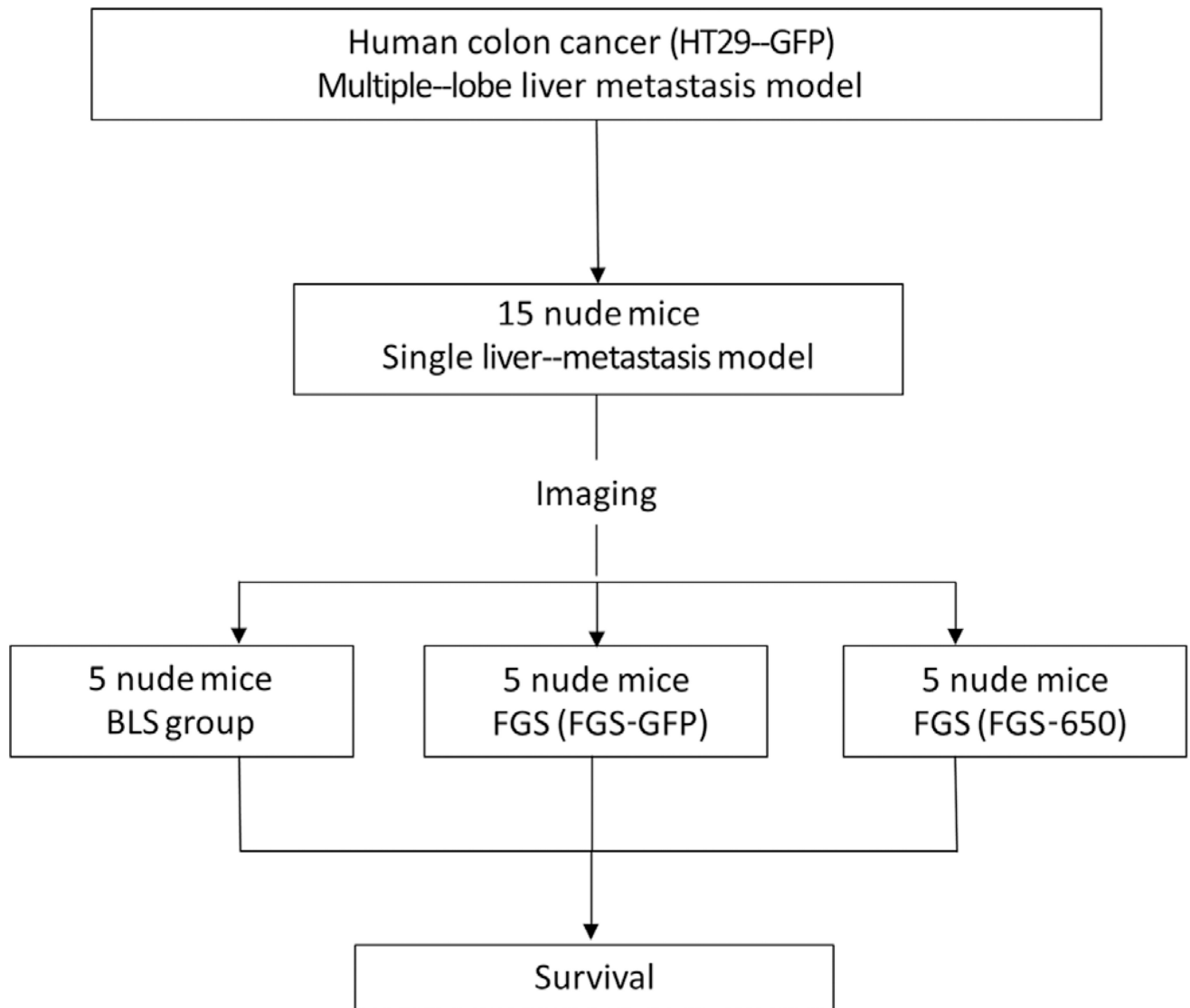


Fig. 6. Bar graphs of residual tumor area after surgery. The residual tumor area was detected at high magnification with the OV-100 and measured with ImageJ. The residual tumor area was significantly larger after BLS compared to FGS.

**Fig. 7.**

Disease-free survival (DFS) and overall survival (OS) for the mice treated with BLS or FGS-650. FGS-650-treated mice significantly had longer DFS ($P = 0.013$) (A) and OS ($P = 0.016$) (B) compared to BLS-treated mice. MST, median survival time.

**Fig. 8.**

Overall study schema to compare BLS with FGS under GFP navigation and FGS under anti-CEA-DyLight650 navigation. Four weeks after surgical orthotopic implantation, 15 mice were randomized into three groups (BLS, FGS under GFP navigation, FGS-GFP or FGS under CEA-650 navigation: FGS-650). BLS was performed under bright light. FGS-GFP was performed using a hand-held imaging system (AM4113TGFBW Dino-Lite Premier; AnMo Electronics Corporation). FGS-650 was performed using the imaging system as described above. To assess for recurrence after surgery, weekly noninvasive imaging was performed with the LT-9900 Illumatool (Lighttools Research) until the end of the experiment.

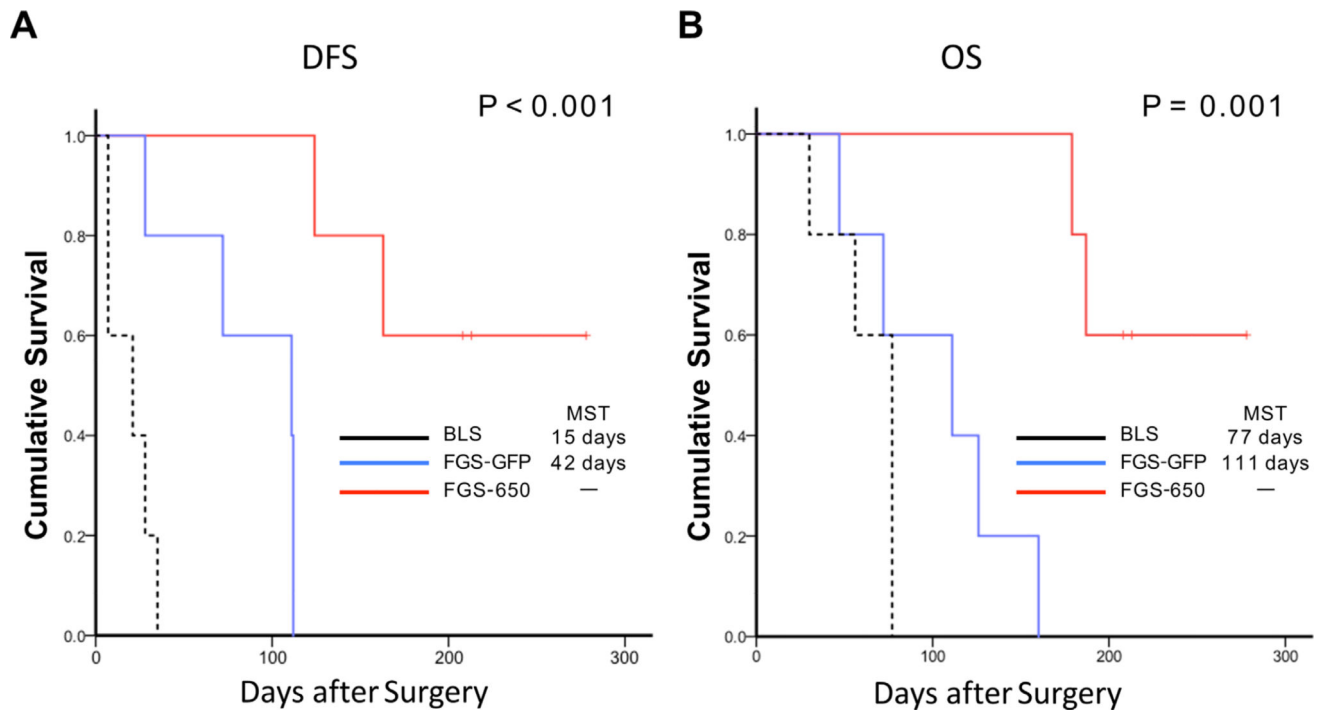


Fig. 9. Disease-free survival (DFS) and overall survival (OS) for the mice treated with BLS, FGS under GFP navigation (FGSGFP), or FGS under anti-CEA-DyLight650 navigation (FGS-650). Mice undergoing FGS-650 had significantly longer DFS ($P < 0.001$) (A) and OS ($P = 0.001$) (B) compared to other two groups. MST, median survival time.