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Dynamic Imaging of Chimeric Antigen Receptor T Cells with [18F]Tetrafluoroborate Positron Emission Tomography/Computed Tomography

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TITLE:

Dynamic Imaging of Chimeric Antigen Receptor T Cells with [^{18}F]Tetrafluoroborate Positron Emission Tomography/Computed Tomography

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KEYWORDS:

CAR T cell, NIS reporter, [^{18}F]TFB-PET, non-invasive imaging

SUMMARY:

This protocol describes the methodology for non-invasively tracking T cells genetically engineered to express chimeric antigen receptors *in vivo* with a clinically available platform.

ABSTRACT:

T cells genetically engineered to express chimeric antigen receptors (CAR) have shown unprecedented results in pivotal clinical trials for patients with B-cell malignancies or multiple myeloma (MM). However, numerous obstacles limit the efficacy and prohibit the widespread use of CAR T cell therapies due to poor trafficking and infiltration into tumor sites as well as lack of persistence *in vivo*. Moreover, life-threatening toxicities, such as cytokine release syndrome or neurotoxicity, are major concerns. Efficient and sensitive imaging and tracking of CAR T cells enables the evaluation of T cell trafficking, expansion, and *in vivo* characterization and allows the

development of strategies to overcome the current limitations of CAR T cell therapy. This paper describes the methodology for incorporating the sodium iodide symporter (NIS) in CAR T cells and for CAR T cell imaging using [^{18}F]tetrafluoroborate-positron emission tomography ([^{18}F]TFB-PET) in preclinical models. The methods described in this protocol can be applied to other CAR constructs and target genes in addition to the ones used for this study.

INTRODUCTION:

Chimeric antigen receptor T (CAR T) cell therapy is a rapidly emerging and potentially curative approach in hematological malignancies[1-15]. Extraordinary clinical outcomes were reported after CD19-directed CAR T (CART19) or B cell maturation antigen (BCMA) CAR T cell therapy. This led to the US Food and Drug Administration (FDA) approval of CART19 cells for aggressive B-cell lymphoma (axicabtagene ciloleucel (Axi-Cel), tisagenlecleucel (tisacel), and lisocabtagene maraleucel), acute lymphoblastic leukemia (tisacel)[16], mantle cell lymphoma (brexucabtagene autoleucel)[17], and follicular lymphoma (Axi-Cel)[18]. Most recently, the FDA approved BCMA-directed CAR T cell therapy in patients with multiple myeloma (MM) (idecabtagene vicleucel)[19]. Moreover, CAR T cell therapy for chronic lymphocytic leukemia (CLL) is in late-stage clinical development and is expected to receive FDA approval within the next three years[9-12].

Despite the unprecedented results of CAR T cell therapy, its widespread use is limited by 1) insufficient *in vivo* CAR T cell expansion or poor trafficking to tumor sites, which leads to lower rates of durable response[20], and 2) the development of life-threatening adverse events, including cytokine release syndrome (CRS)[21,22]. The hallmarks of CRS include not only immune activation resulting in elevated levels of inflammatory cytokines/chemokines but also massive T cell proliferation after CAR T cell infusion[23-25]. Thus, the development of a validated, clinical-grade strategy to image CAR T cells *in vivo* would allow 1) CAR T cell tracking in real time *in vivo* to monitor their trafficking to tumor sites and uncover potential mechanisms of resistance, and 2) monitoring of CAR T cell expansion and potentially predicting their toxicities such as the development of CRS.

Clinical features of mild CRS are high fever, fatigue, headache, rash, diarrhea, arthralgia, myalgia, and malaise. In more severe CRS, patients may develop tachycardia/hypotension, capillary leak, cardiac dysfunction, renal/hepatic failure, and disseminated intravascular coagulation[26,27]. In general, the degree of elevation of cytokines, including interferon-gamma, granulocyte-macrophage colony-stimulating factor, interleukin (IL)-10, and IL-6, has been shown to correlate with the severity of clinical symptoms[1,6,21,28,29]. However, the extensive application of "real-time" serum cytokine monitoring to predict CRS is difficult due to the high cost and limited availability. To exploit the beneficial characteristics of CAR T cell therapy, non-invasive imaging of adoptive T cells can be potentially utilized to predict the efficacy, toxicities, and relapse after CAR T cell infusion.

Several researchers have developed strategies to use radionuclide-based imaging with positron emission tomography (PET) or single-photon emission computed tomography (SPECT), which provides high resolution and high sensitivity[30-39] for the *in vivo* visualization and monitoring of CAR T cell trafficking. Among those radionuclide-based imaging strategies, the sodium iodide

symporter (NIS) has been developed as a sensitive modality to image cells and viruses using PET scans[40,41]. NIS$^+$CAR T cell imaging with [^{18}F]TFB-PET is a sensitive, efficient, and convenient technology to assess and diagnose CAR T cell expansion, trafficking, and toxicity. This protocol describes 1) the development of NIS$^+$CAR T cells through dual transduction with high efficacy and 2) a methodology for imaging NIS$^+$CAR T cells with [^{18}F]TFB-PET scan. BCMA-CAR T cells for MM are used as a proof-of-concept model to describe NIS as a reporter for CAR T cell imaging. However, these methodologies can be applied to any other CAR T cell therapy.

PROTOCOL:

The protocol follows the guidelines of Mayo Clinic's Institutional Review Board, Institutional Biosafety Committee, and Mayo Clinic's Institutional Animal Care and Use Committee.

1. NIS$^+$BCMA-CAR T cell production

NOTE: This protocol follows the guidelines of the Mayo Clinic's Institutional Review Board (IRB 17-008762) and Institutional Biosafety Committee (IBC Bios00000006.04).

1.1. Production of BCMA-CAR, NIS, and luciferase-green fluorescent protein (GFP)-encoding lentiviruses.

NOTE: A second-generation BCMA-CAR construct was synthesized *de novo* (see the **Table of Materials**) and cloned into a third-generation lentiviral vector under the control of an elongation factor-1 alpha (EF-1α) promotor. The BCMA-CAR construct (C11D5.3-41BBz) included 4-1BB costimulation and a single-chain variable fragment (scFv) derived from an anti-human BCMA antibody clone C11D5.3[42,43]. The NIS is under the control of the EF-1α promotor and binds to the puromycin resistance gene via self-cleaving peptides (P2A). The lentiviral vector encoding luciferase-GFP (see the **Table of Materials**)[44] is used to transduce tumor cells, which then express GFP and luciferase.

1.1.1. Prepare lentiviral vector plasmids: pLV-EF1α-BCMA-CAR (15 µg), pBMN-CMV-GFP-Luc2-Puro (15 µg), and pLV-EF1α-NIS-P2A-Puro (15 µg).

NOTE: pBMN-CMV-GFP-Luc2-Puro and pLV-EF1α-NIS-P2A-Puro contain the puromycin resistance gene. Therefore, NIS- or luciferase-GFP-transduced cells can be selected with 1 µg/mL or 2 µg/mL of puromycin dihydrochloride, as described previously[21,45,46].

1.1.2. Seed 20 × 10^6 of 293T cells in a T175 flask and incubate for 24 h at 37 °C with 5% CO_2. Confirm that 293T cells are evenly distributed on the flask at 70–90% confluence by direct visualization under the microscope.

1.1.3. Prepare a master mix of 15 µg of the expression vector (e.g., CAR, NIS, or luciferase-GFP linear DNA), 7 µg of the envelope vector (VSV-G), and 18 µg of the packaging vector (gag, pol, rev, and tat). Dilute the DNA master mix in 4.5 mL of the transfection medium, and then add 111 µL of the pre-complexing reagent (Mixture A).

1.1.4. Prepare a new tube, and dilute 129 µL of the liposomal transfection reagent in 4.5 mL of the transfection medium (Mixture B).

1.1.5. Combine Mixtures A and B and flick the tube to mix the contents. Incubate for 30 min at room temperature (RT).

1.1.6. After the incubation, simply aspirate the cell supernatant without detaching the cells and add 16 mL of a growth medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine. Then, add the mixture of Mixtures A and B on 293T cells dropwise. Finally, incubate the transfected cells at 37 °C, 5% CO_2 for 24 h.

1.1.7. On days 1 and 2 post-transfection, harvest the supernatant of 293T, spin down at 900 × *g* for 10 min, and filter through a 0.45 µm nylon filter. Concentrate the filtrate at 24 and 48 h by ultracentrifugation at 112,700 × *g* for 2 h, and freeze at -80 °C.

1.2. *Ex-vivo* T cell isolation (**Figure 1**)

NOTE: Perform all cell culture work in a laminar flow cabinet using aseptic technique and personal protective equipment. Peripheral blood mononuclear cells (PBMCs) are harvested from healthy volunteer donor blood collected during apheresis[47].

1.2.1. Use the standard density gradient technique to isolate PBMCs.

1.2.1.1. Gently add 15 mL of density gradient medium (density of 1.077 g/mL) (containing alpha-D-glucopyranoside, beta-D-fructofuranosyl homopolymer, and, 3-(acetylamino)-5-(acetylmethylamino)-2,4,6-triodobenzoic acid monosodium salt) to a 50 mL density gradient separation tube without creating air bubbles (see the **Table of Materials**).

1.2.1.2. To avoid cell trapping, dilute the blood sample with phosphate-buffered saline (PBS, 0.2 g/L of potassium chloride, 0.2 g/L of potassium phosphate monobasic, 8 g/L of sodium chloride, and 1.15 g/L of sodium phosphate dibasic) containing 2% FBS at a 1:1 volume ratio. Gently transfer the diluted blood on top of the density gradient medium without breaking the interface between the two. Spin down at 1,200 × *g* for 10 min at RT.

NOTE: A 50 mL density gradient separation tube can be used for the isolation of 4–17 mL of a blood sample. The 50 mL density gradient separation tube (see the **Table of Materials**) used in this protocol does not require the "brake off" during centrifugation. However, when standard 50 mL tubes are used, the brake needs to be off and requires 30 min centrifugation.

1.2.1.3. Transfer the supernatant into a new 50 mL conical tube, wash with PBS + 2% FBS by filling up to 50 mL, and then spin down at 300 × *g* for 8 min at RT.

1.2.1.4. Aspirate the supernatant, and resuspend the pelleted cells in 50 mL of PBS + 2% FBS. Count the number of cells, and then spin down at 300 × *g* for 8 min at RT. Repeat the previous step for a total of 2 washes.

1.2.2. Aspirate the supernatant, and resuspend the pelleted cells to a concentration of 50 × 10^6 cells/mL with PBS + 2% FBS.

1.2.3. Perform T cell isolation from PBMCs using a negative selection magnetic bead kit.

NOTE: An ideal negative selection kit includes magnetic beads attached to antibodies against antigens expressed on cells other than T cells. A commonly used kit contains antibodies conjugated to magnetic beads against CD15, CD14, CD34, CD36, CD56, CD123, CD235a, CD19, and CD16 (see **Table of Materials**).

1.2.3.1. Transfer PBMCs to a 14 mL polystyrene round-bottom tube. Then, place the PBMCs and the negative selection antibody cocktail in a fully automated cell separator, and perform T cell isolation according to the manufacturer's protocol.

1.3. T cell stimulation and T cell expansion (**Figure 1**)

1.3.1. To culture the isolated T cells, prepare T cell expansion medium (TCM) made with serum-free hematopoietic cell medium supplemented with 10% human serum albumin and 1% penicillin-streptomycin-glutamine[21]. After T cell isolation, count the cells and culture at a concentration of 2 × 10^6 cells/mL with TCM.

1.3.2. Wash anti-CD3/CD28 beads three times with TCM before culturing with T cells.

1.3.2.1. Mix the vial containing the beads by swirling. Then, pipette the required volume of beads (3:1 beads:cell) (e.g., when stimulating 1.0 × 10^6 cells of T cells, use 3.0 × 10^6 of anti-CD3/CD28 beads) into a sterile microcentrifuge tube (1.5 mL) and resuspend in 1 mL of TCM.

1.3.3. Place the microcentrifuge tube with the beads on a magnet for 1 min, and aspirate the supernatant. Remove the tube from the magnet, and resuspend the washed beads in 1 mL of TCM. Repeat the previous two steps for a total of 3 washes.

1.3.4. Resuspend the beads in 1 mL of TCM and transfer them to the T cells. Then, dilute the T cells to a final concentration of 1.0 × 10^6 cells/mL with TCM. Transfer the T-cell–bead suspension to a tissue-culture-treated 6-well plate and place it in the incubator (37 °C, 5% CO_2).

1.4. Titration of lentiviruses (**Figure 2**)

1.4.1. Prepare T cells for titration assay. Ensure that approximately 1.0 x 10^6 cells are available to titrate one type of virus.

1.4.2. Stimulate T cells as described in section 1.3.2.

1.4.3. Plate 1.0×10^5 cells of stimulated T cells in a 96-well plate (titer plate) and incubate at 37 °C, 5% CO_2 for 24 h (**Figure 2A**). Isolate and stimulate the T cells as described in section 1.2.

1.4.4. Prepare a dilution plate (96-well plate) by adding 100 µL of TCM into the wells of the designated columns and the untransduced control wells (**Figure 2B**).

1.4.5. Thaw one vial of lentiviral particles on ice and gently pipette up and down to mix well. Transfer 50 µL of the virus supernatant into the wells of Column 6 of the 96-well plate (dilution 3) (**Figure 2B**). Pipette up and down to mix well.

1.4.6. Perform serial dilutions (2-fold serial dilution): transfer 50 µL from well A6 to well B6 and then 50 µL from well B6 to well C6; repeat until G6. Then, add 50 µL of the diluted virus to the titer plate (**Figure 2B**).

1.4.7. Incubate the titer plate at 37 °C, 5% CO_2 for 48 h, and determine the percentages of CAR-, NIS-, or GFP-positive cells by flow cytometry (**Figure 2C**).

1.4.7.1. Wash the wells by spinning down the titer plate two times at 650 × *g* at 4 °C for 3 min.

1.4.7.2. Stain the transduced T cells as described in steps 1.5.3 to 1.5.9.

1.4.7.3. Determine the titers based on the percentages of CAR-, NIS-, or GFP-positive cells by using formula (**1**):

Titers = Percentage of BCMA-CAR$^+$ or NIS$^+$ T cells × T cell count at transduction × the specific dilution / volume (**1**)

1.5. Transduction of lentiviruses and NIS$^+$BCMA-CAR T cell expansion

1.5.1. Twenty-four to 48 h after T cell stimulation, perform lentiviral transduction on stimulated T cells (T cells should form clusters).

1.5.1.1. Thaw the frozen lentiviruses encoding CAR or NIS at 4 °C.

1.5.1.2. Mix the stimulated T cells well to break up the clusters, and simply add freshly thawed virus at a multiplicity of infection (MOI) of 5.0 (when transducing 1.0×10^6 T cells, use 5.0×10^6 of lentivirons). Incubate the transduced cells at 37 °C, 5% CO_2.

1.5.1.3. On days 3, 4, and 5, count the transduced T cells using a hematocytometer[48] or a fully automated cell counter[49] and adjust the cell concentration to 1.0×10^6 cells/mL by adding

fresh, pre-warmed TCM. For NIS-transduced T cells carrying the puromycin resistance gene, treat the cells with 1 µg/mL of puromycin dihydrochloride on days 3, 4, and 5.

1.5.2. On day 6, remove the anti-CD3/CD28 beads from the transduced T cells (from step 1.3.4) by mixing well to break up the T cell clusters and placing them in a magnet for 1 min. Then, simply place the collected transduced T cells back in culture at a concentration of 1.0×10^6 cells/mL. After removing the beads from the T cells, assess the expression of CAR and NIS by flow cytometry.

NOTE: As the single-chain variable fragment of the BCMA-CAR is derived from mouse, it can be stained with goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 647. NIS can be detected using anti-human ETNL [synthetic peptide corresponding to aa625–643 (SWTPCVGHDGGRDQQETNL)]. This antibody recognizes the cytosolic C-terminus of NIS. Therefore, T cells must be permeabilized before incubation with an anti-human NIS antibody.

1.5.3. Perform surface staining of BCMA-CAR using goat anti-mouse IgG (H+L).

1.5.3.1. Take an aliquot of the culture (e.g., 50,000 T cells) and wash with flow buffer (PBS, 1% FBS, and 1% sodium azide). Next, resuspend the cells with 50 µL of flow buffer, and stain the cells with 1 µL of goat anti-mouse antibody for detecting CAR expression and 0.3 µL of live-dead aqua for excluding dead cells.

1.5.3.2. Incubate for 15 min in the dark at RT, wash the cells by adding 150 µL of flow buffer, and centrifuge the cells at 650 × *g* for 3 min at 4 °C.

1.5.4. After surface CAR staining, fix and permeabilize the cells by adding 100 µL of fixation medium (PBS with 4.21% formaldehyde) and incubate for 20 min at 4 °C. Wash the cells twice with 100 µL of a buffer that contains a cell-permeabilizing agent such as saponin (650 × *g* for 3 min at 4 °C).

1.5.5. Resuspend the fixed/permeabilized cells in 50 µL of a permeabilizing buffer. Then, add 0.3 ng of anti-human ETNL NIS antibody in 50 µL of flow buffer and incubate for 1 h at 4 °C.

1.5.6. Add 150 µL of flow buffer, and centrifuge the cells at 650 × *g* for 3 min at 4 °C. Incubate the cells with 2.5 µL of anti-rabbit secondary antibody in 50 µL of flow buffer for 30 min at 4 °C, wash the cells by adding 150 µL of flow buffer, and centrifuge at 650 × *g* for 3 min at 4 °C.

1.5.7. Finally, resuspend in 200 µL of flow buffer and perform flow cytometry to determine the transduction efficiency (**Figure 3A,B**).

1.5.8. On day 8, count and spin down the T cells at 300 × *g* for 8 min at 4 °C. Resuspend the T cells with freezing medium (90% FBS + 10% dimethylsulfoxide [DMSO]) at a concentration of 10×10^6/mL, then transfer 1 mL each to labeled cryovials.

1.5.9. Place the vials in a -80 °C freezer for 48 h. After 48 h (and by day 10), transfer the T cells to liquid nitrogen.

NOTE: See **Figure 1** for the overview of NIS$^+$ CAR T cell production. **Figure 3D** represents examples of *ex vivo* T cell expansion from three different donors.

2. NIS$^+$BCMA-CAR T cell imaging with [^{18}F]TFB-PET scan

NOTE: This protocol follows the guidelines of Mayo Clinic's Institutional Animal Care and Use Committee (IACUC A00001767-16), IRB, and IBC (Bios00000006.04). OPM-2 is a BCMA$^+$ MM cell line, which is often used as a target cell line for BCMA-CAR T cells[50-52].

2.1. Establish luciferase$^+$ BCMA$^+$ OPM-2 cells.

2.1.1. Seed 500,000 OPM-2 cells in a tissue culture-treated 24-well plate. Thaw lentivirus encoding luciferase-GFP at 4 °C.

2.1.2. Add the freshly thawed virus at an MOI of 3.0 to OPM-2 cells and mix well by pipetting. Place the plate in the incubator (37 °C, 5% CO_2).

2.1.3. Forty-eight hours after the transduction, add 2 µg/mL of puromycin to select the transduced cells. Four days after the transduction, assess the GFP-positive cells (luciferase-positive) by flow cytometry (**Figure 3E**).

2.2. Establish BCMA$^+$ OPM-2 xenograft mouse models (**Figure 4**).

2.2.1. Count luciferase$^+$ OPM-2 cells and spin them down twice to remove all cell culture medium. Resuspend the OPM-2 cells at a concentration of 10×10^6 cells/mL with PBS.

2.2.2. On day -21, inject 100 µL (1.0×10^6 cells) of luciferase$^+$ OPM-2 cells into the tails of 8-to-12-week-old immunocompromised NOD-*scid IL2rγ^{null}* (NSG) mice (**Figure 4**).

2.2.3. On day 20 after the OPM-2 cell injection (day -1 of CAR T cell injection), check the tumor burden via bioluminescence imaging (BLI) (**Figure 4**).

NOTE: OPM-2 cells form a slow-growing tumor, which usually takes 2–3 weeks to engraft.

2.2.4. Administer 10 µL/g of D-luciferin to OPM-2 xenograft mice via intraperitoneal (IP) injection. After 10 min, perform BLI on the mice under 2% isoflurane gas (**Figure 5A**). After confirming tumor engraftment, randomize the mice according to the tumor burden.

2.2.5. On day -1 of the CAR T cell injection, thaw NIS$^+$BCMA-CAR T cells, and remove the freezing medium by centrifugation ($300 \times g$, 8 min, 4 °C). Then, the resuspend cells with TCM at 2.0×10^6 cells/mL and incubate overnight (37 °C, 5% CO_2).

2.2.6. On day 0, count and centrifuge the NIS$^+$BCMA-CAR T cells (300 × *g*, 8 min, 4 °C). Resuspend the NIS$^+$BCMA-CAR T cells at 50 × 10^6 cells/mL with PBS.

2.2.7. Administer 100 µL (5.0 × 10^6 cells) of NIS$^+$BCMA-CAR T cells via tail vein injection to the OPM-2 xenograft mice. On days 7 and 15, image the mice using a PET scan.

2.3. NIS$^+$BCMA-CAR T cell *in vivo* imaging using BCMA$^+$OPM-2 xenograft mouse model.

2.3.1. Weigh the mice before the imaging, and remove any metal ear tags to eliminate metal-related artifacts.

2.3.2. Prepare [^{18}F]TFB as previously described[53].

NOTE: [^{18}F]TFB must be produced the day of its use. Radiochemical purity should be >99% and molar activity >5 GBq/mmol.

2.3.3. Inject 9.25 MBq [^{18}F]TFB intravenously via tail vein injection. Allow an uptake period of ~40 min for the radiotracer to be distributed in the body and clear the blood.

2.3.4. Anesthetize the mouse using isoflurane inhalation (2%).

NOTE: Isoflurane is the preferred inhaled anesthetic as it has rapid and reliable onset and recovery.

2.3.5. Prior to anesthetizing the mouse, clean all surfaces of the anesthesia machine with disinfectant cleaners.

2.3.6. Place the mouse inside the induction chamber. Turn the vaporizer dial to 2% and wait for the mouse to become recumbent and non-responsive within 1–2 min.

2.3.7. Monitor the mouse to avoid insufficient anesthesia or excessive depression of respiratory functions. In brief, pinch toe to confirm the insufficient anesthesia.

NOTE: The normal respiratory rate is up to 180/min, and the acceptable drop rate is 50%.

2.3.8. Apply ophthalmic ointment to avoid corneal drying and trauma.

2.3.9. Acquire PET/CT images 45 min post-injection with the anesthetized mouse in a micro PET/CT imaging workstation (see the **Table of Materials**). Next, acquire static PET images for 15 min followed by CT image acquisition for 5 min with 360° rotation and 180 projections at 500 µA, 80 keV, and 200 ms exposure.

2.4. Analyzing acquired imaging data

2.4.1. Analyze the images using PET image processing software (**Figure 5B** and **Supplemental Video S1**).

2.4.2. Define the volume of interest (VOI).

2.4.3. Calculate the standardized uptake value (SUV) using formula (**2**).

SUV in VOI = Concentration of activity in VOI (MBq/mL) × body weight (g) / administered dose (MBq) (**2**)

2.5. Confirmation of NIS^+BCMA-CAR T cell trafficking to the tumor sites with the flow cytometry

2.5.1. After [^{18}F]TFB-PET imaging, place the mouse back into the cage. Following cessation of anesthesia, monitor the animals until they are capable of purposeful movement and ensure that they have access to food and water.

2.5.2. Monitor the mice until the decay of the injected [^{18}F]TFB. Once the radioisotope is not detectable, euthanize the mice with CO_2.

2.5.3. To euthanize, place the mice into the cage (no more than 5 mice per cage).

2.5.4. Expose the mice to CO_2 until complete cessation of breathing in approximately 5–10 min.

2.5.5. To ensure the death of the mice, perform cervical dislocation by grasping the tail with one hand and hold the neck with the other hand. Then, quickly pull the neck.

2.5.6. Harvest the bone marrow to confirm that the NIS^+BCMA-CAR T cells efficiently traffic to the tumor site.

2.5.7. Transfer the harvested femurs and tibia to a 6-well plate containing 5 mL of cell culture medium. Remove the muscles and tendons from the femurs and tibia, and simply cut both ends (above the joints) of the femurs and tibia.

2.5.8. Fill an insulin syringe with the cell culture medium, and flush bone marrow onto the 6-well plate. For femurs, use 22 G needles and 5 mL syringes because the femur diameter is larger than that of the tibia.

2.5.9. Use the flat end of the plunger to grind the bone marrow. Place a 70 µm cell strainer on a sterile 50 mL conical tube, and filter the ground bone marrow. Then, fill up the tube with the flow buffer, and centrifuge the tube at 300 × *g* for 8 min at 4 °C.

2.5.10. Aspirate the supernatant, and resuspend the bone marrow with 5 mL of flow buffer. Transfer 200 µL of bone marrow to a 96-well plate. Centrifuge the plate at 300 × *g* for 3 min at 4 °C. Decant the supernatant, and resuspend the cells with 50 µL of flow buffer.

2.5.11. Stain the bone marrow with flow antibodies against 0.25 µg of mouse CD45, 0.03 µg of human CD45, 0.06 µg of human CD3, 0.24 µg of human BCMA, and 0.3 µL of live/dead aqua. Incubate the plate for 15 min at RT in the dark.

2.5.12. Centrifuge the plate at 300 × *g* for 3 min at 4 °C. Then, add 200 µL of flow buffer, and run on the flow cytometer (**Figure 5C**).

REPRESENTATIVE RESULTS:

Figure 1 represents the steps of generating NIS^+BCMA-CAR T cells. On day 0, isolate PBMCs and then isolate T cells by negative selection. Then, stimulate T cells with anti-CD3/CD28 beads. On day 1, transduce T cells with both NIS and BCMA-CAR lentiviruses. On days 3, 4, and 5, count T cells and feed with media to adjust the concentration to be 1.0×10^6/mL. For NIS-transduced T cells, add 1 µg/mL of puromycin to select NIS^+ cells. On day 6, remove the beads by placing the cells in the magnet for a minute. Then, put the de-beaded cells from the tube into a new flask. Take an aliquot of cells (e.g., 50,000 cells) and stain with antibodies to check the expression of NIS and CAR on the surface of T cells using flow cytometry. On day 8, count the T cells and cryopreserve with freezing media at the concentration of 10×10^6/mL.

Figure 2 represents the outline of titrating the lentiviruses. On day 0, resuspend T cells at the concentration of 1.0×10^6/mL in TCM. Then, stimulate T cells with anti-CD3/CD28 beads at a 1:3 cell:beads ratio. Add 100 µL (100,000 cells) of T cells to the colored wells as indicated in **Figure 2A**. This plate is called a "titer plate." Incubate the titer plate at 37 °C, 5% CO_2 for 24 h. On day 1, prepare the dilution plate. Add 100 µL of TCM to the colored wells as indicated in **Figure 2B**. Then, add 50 µL of freshly thawed lentiviruses into the first row (e.g., A6, A7, or A8 as depicted in **Figure 2B**). Perform serial dilution by transferring 50 µL from A6 to B6, and then 50 µL from B6 to C6, repeating until G6. Perform serial dilution for A7 and A8 as well. Then transfer 50 µL of the diluted virus to the titer plate. Twenty-four hours after transferring the virus from the dilution plate to the titer plate, feed the cells with 100 µL of TCM. On day 3, stain cells with antibodies and analyze the expression of NIS and BCMA on the T cells via flow cytometry.

Figure 3A,B show the representative flow plots of BCMA-CAR T or NIS^+BCMA-CAR T cells. T cells are gated on FSC/SSC, followed by singlet and live-cell discrimination. Over 90% of cells are NIS^+ cells (**Figure 3B**). **Figure 3C** shows the representative flow plot for the composition of NIS^+BCMA-CAR T cells. Similar to **Figure 3A,B**, T cells are gated on FSC/SSC, followed by singlet and live-cell discrimination. **Figure 3D** shows the T cell expansion curve from days 0 to 8. There are no fold expansion differences between UTD, BCMA-CAR T, or NIS^+BCMA-CAR T cells. **Figure 3E** depicts the GFP expression on OPM-2 cells after the transduction of lentivirus that encodes GFP and luciferase followed by puromycin selection.

Figure 4 is the outline of imaging NIS$^+$BCMA-CAR T cells *in vivo* with [^{18}F]TFB-PET. Inoculate six to eight-week-old mice with 1.0×10^6 cells of luciferase$^+$ OPM-2 cells via tail vein injection on day -21. Assess the tumor burden by BLI on day -1. On day 0, randomize the mice according to the tumor burden to be treated with NIS$^+$BCMA-CAR T cells through tail vein injection or monitored without any treatment (untreated xenograft). Image the mice with BLI on day 6. Perform [^{18}F]TFB-PET on day 7 to image NIS$^+$BCMA-CAR T cells.

Figure 5A shows the representative BLI 20 days after the inoculation of luciferase$^+$OPM-2 cells into the NSG mice. **Figure 5B** shows the representative PET imaging a week after administration of NIS$^+$BCMA-CAR T cells. [^{18}F]TFB uptake is observed in the sternum, spines, pelvis, and femurs. In addition, physiological uptake of [^{18}F]TFB is seen in the thyroid and stomach. **Figure 5C** shows the representative flow plots of femur-derived bone marrow harvested from the untreated xenograft or NIS$^+$BCMA-CAR T cell-treated mice. Bone marrow samples are stained with mouse CD45, human CD45, human CD3, and human BCMA. Cells are gated on FSC/SSC, followed by singlet, live, and human-cell discrimination. Bone marrow samples derived from untreated xenograft show BCMA$^+$ cells whereas NIS$^+$BCMA-CAR T cell treated mouse shows CD3$^+$ cells, which support the [^{18}F]TFB-PET finding.

FIGURE AND TABLE LEGENDS:

Figure 1: NIS$^+$BCMA-CAR T cell production schema. Normal donor CD3 T cells (isolated from peripheral blood mononuclear cells) using negative bead selection. T cells are plated at 1.0×10^6/mL and expanded in TCM using anti-CD3/CD28 beads added on day 0 of culture and removed on day 6. T cells are dually transduced with lentiviruses encoding NIS or BCMA-CAR on day 1 (MOI=5). NIS$^+$BCMA-CAR T cells are treated with 1 µg/mL of puromycin on days 3, 4, and 5. T cells are expanded in culture for 8 days. T cells are cryopreserved in FBS with 10% DMSO for future experiments. T cells are thawed and rested overnight at 37 °C before all experiments. Abbreviations: CD = cluster of differentiation; TCM = T-cell expansion medium; BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; GFP = green fluorescent protein; PBMCs = peripheral blood mononuclear cells; MOI = multiplicity of infection; FBS = fetal bovine serum; DMSO = dimethylsulfoxide.

Figure 2: Lentivirus titration. (**A**) On day 0, stimulate T cells with anti-CD3/CD28 beads at a 3:1 beads:cell ratio. Add 100 µL of 1.0×10^6/mL of stimulated T cells to the colored wells as indicated in the cartoon. Then, incubate the titer plate at 37 °C, 5% CO_2 for 24 h. (**B**) Prepare a dilution plate by adding 100 µL of TCM to the colored wells as indicated in the cartoon. Add 50 µL of freshly thawed virus to A6, A7, or A8 (e.g., BCMA-CAR to A6, NIS to A7, and luciferase-GFP to A8). Then, serially dilute the virus by transferring 50 µL from A6 to B6, and then 50 µL from B6 to C6, repeating until G6. Perform serial dilution for A7 and A8 as well. Transfer 50 µL of the diluted virus to the titer plate. (**C**) On day 3, stain the cells with corresponding antibodies and analyze the titer plate by flow cytometry. Abbreviations: CD = cluster of differentiation; TCM = T-cell expansion medium; BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; GFP = green fluorescent protein.

Figure 3: The generation of NIS$^+$BCMA-CAR T cells and luciferase-GFP positive OPM-2 cells. (**A** and **B**) Cells are gated on FSC/SSC, followed by singlet and live-cell discrimination. Representative flow plots of (**A**) untransduced T and BCMA-CAR T cells and (**B**) UTD and NIS$^+$BCMA-CAR T are shown. NIS$^+$BCMA-CAR T cells are generated by co-transduction of two viruses on day 1 of T cell expansion, as described in **Figure 2**. On day 6, cells are stained for CARs and NIS. (**C**) The phenotypic analysis of NIS$^+$BCMA-CAR T. The representative flow plot of NIS$^+$BCMA-CAR T cells is shown. (**D**) Summary of the UTD, BCMA-CAR T, or NIS$^+$BCMA-CAR T cell growth kinetics. Incorporation of BCMA-CAR and/or NIS does not impact T cell expansion (two-way ANOVA, n=3 biological replicates, mean ± SD). (**E**) Flow cytometric analysis of luciferase-GFP-transduced OPM-2. OPM-2 cells are transduced with lentivirus encoding luciferase-GFP with puromycin resistance. Forty-eight hours after transduction, OPM-2 cells are treated with 2 μg/mL of puromycin. Cells are expanded for two more days, and the expression of GFP is analyzed via flow cytometry. Abbreviations: CD = cluster of differentiation; BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; GFP = green fluorescent protein; UTD = untransduced; FSC/SSC = forward scattering/side scattering; ANOVA = analysis of variance; n.s.= not significant; SD = standard deviation; FL-1-A = area of fluorophore 1; FITC-A = area of fluorescein isothiocyanate.

Figure 4: Scheme for i*n vivo* trafficking assay in a systemic OPM2 xenograft model. Inject six to eight-week-old NSG mice with 1.0×10^6 of luciferase-positive OPM-2 cells via the tail vein on day -21. On day -1, perform bioluminescent imaging on the mice to confirm the engraftment of OPM-2 cells. On day 0, inject mice with 5.0×10^6 of NIS$^+$BCMA-CAR T cells. Image mice with [^{18}F]TFB-PET/CT on day 7 to assess the trafficking of NIS$^+$BCMA-CAR T cells. Abbreviations: BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; BLI = bioluminescent imaging; NSG = immunocompromised NOD-*scid IL2rγ^{null}*; luc = luciferase; [^{18}F]TFB-PET/CT = [^{18}F]tetrafluoroborate positron emission tomography/computed tomography.

Figure 5: *In vivo* trafficking assay in a systemic OPM2 xenograft model. (**A** and **B**) BCMA$^+$luciferase$^+$OPM2 cells are intravenously injected into NSG mice. Mice receive NIS$^+$BCMA CAR T cells three weeks after the inoculation of OPM-2 cells. (**A**) BLI confirms the engraftment of OPM-2 cells. (**B**) [^{18}F]TFB-PET reveals NIS$^+$BCMA-CAR T cell trafficking to the bone marrow. (**C**) To confirm that OPM-2 cells engraft in the bone marrow and NIS$^+$BCMA-CAR T cells traffic to the tumor site, mice are euthanized after the imaging, and the bone marrow is harvested. Flow cytometric analysis revealed that OPM-2 cells are engrafted in the bone marrow (*left*), and NIS$^+$BCMA-CAR T cells are present in the bone marrow (*right*). Abbreviations: BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; BLI = bioluminescent imaging; NSG = immunocompromised NOD-*scid IL2rγ^{null}*; [^{18}F]TFB-PET/CT = [^{18}F]tetrafluoroborate positron emission tomography/computed tomography; IVIS = *in vivo* imaging system; CD = cluster of differentiation; SUV = standardized uptake value.

Supplemental Video S1: Three-Dimensional (3D) rendering of PET/CT data showing the *in vivo* distribution of [^{18}F]TFB in the thyroid, stomach, and bone marrow. Abbreviations: BCMA = B cell maturation antigen; CAR = chimeric antigen receptor; NIS = sodium iodide symporter; PET/CT = positron emission tomography/computed tomography.

DISCUSSION:

This paper describes a methodology for incorporating NIS into CAR T cells and imaging infused CAR T cells *in vivo* through [^{18}F]TFB-PET. As proof of concept, NIS^{+}BCMA-CAR T cells were generated via dual transduction. We have recently reported that incorporating NIS into CAR T cells does not impair CAR T cell functions and efficacy *in vivo* and allows CAR T cell trafficking and expansion[54]. As CAR T cell therapies continue to expand beyond the current B-cell malignancies to applications in CLL, there will be a greater need for tools that allow non-invasive *in vivo* imaging and monitoring of infused adoptive T cells. Dynamic imaging of T cells will enable the validation of adoptive T cell trafficking and potentially allow earlier detection of efficacy and toxicity.

NIS has been investigated and validated as a sensitive modality to image cells and viruses in clinical trials[41,55]. Physiological accumulation of tracers for NIS is mainly seen in the thyroid/salivary glands, stomach, and bladder, which are not common organs affected by liquid tumors[56]. Especially in MM, malignant plasma cells are often distributed in the bone marrow or bones, and an extramedullary plasmacytoma in lesions, where the physiological accumulation of tracers for NIS occurs, is a rare phenomenon[57,58]. Furthermore, NIS is non-immunogenic and therefore suitable for longitudinal imaging studies[59]. NIS is an intrinsic membrane protein that transports iodide into the cytosol and contains 13 putative transmembrane segments with an extracellular amino terminus site and cytosolic carboxy terminus[60].

NIS can be visualized with gamma- or positron-emitting radioisotopes such as technetium-99m (^{99m}Tc) pertechnetate, iodide-123 (^{123}I), ^{131}I, ^{124}I, and [^{18}F]TFB[56]. Recently, [^{18}F]TFB has emerged as a promising iodide analog for NIS-based PET imaging, as it has similar biochemical properties and is radiosynthesized[61]. One advantage of TFB is that it does not undergo organification in thyroid cells and therefore has a comparatively mild uptake in normal thyroid tissue[61]. Another advantage of TFB is its short half-life of 109.8 min, while the half-lives of other tracers range from 12 h to 8 days, which could present safety issues for clinical applications[62]. The main limitation of NIS-based CAR T cell imaging is that tracers, including TFB, do not penetrate the blood-brain barrier (BBB), making it difficult to assess neurotoxicity after CAR T cell treatment[63-65].

Neurotoxicity is associated with the infiltration of T cells and the activation of myeloid cells in the central nervous system. However, in most cases of neurotoxicity after CAR T cell therapy, the integrity of the BBB is disrupted[63,66]. Therefore, it is unclear whether the tracer is unable to cross the BBB in this compromised setting. Further studies need to be carried out to validate whether neurotoxicity after CAR T cell therapy can be imaged with [^{18}F]TFB-PET. Although the short half-life of ^{18}F-TFB is safe for patients and staff, it makes its procurement difficult for the hospital. Therefore, institutes must be equipped with cyclotrons or have access to a regional facility. The methodology described in the protocol here can potentially be applied to a variety of other CAR T cells via dual transduction to visualize and assess CAR T cells *in vivo* using [^{18}F]TFB-PET scan.

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SSK is an inventor on patents in CAR immunotherapy licensed to Novartis (through an agreement between Mayo Clinic, University of Pennsylvania, and Novartis) and Mettaforge (through Mayo Clinic). RS and SSK are inventors on patents in the field of CAR immunotherapy that are licensed to Humanigen. SSK receives research funding from Kite, Gilead, Juno, Celgene, Novartis, Humanigen, MorphoSys, Tolero, Sunesis, Leahlabs, and Lentigen.

REFERENCES:

1 Maude, S. L. et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *New England Journal of Medicine.* **371** (16), 1507–1517 (2014).

2 Kochenderfer, J. N. et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *Journal of Clinical Oncology.* **33** (6), 540–549 (2015).

3 Turtle, C. J. et al. Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. *Science Translational Medicine.* **8** (355), 355ra116 (2016).

4 Kochenderfer, J. N. et al. Lymphoma remissions caused by anti-CD19 chimeric antigen receptor T cells are associated with high serum interleukin-15 levels. *Journal of Clinical Oncology.* **35** (16), 1803–1813 (2017).

5 Kochenderfer, J. N. et al. Long-duration complete remissions of diffuse large B cell lymphoma after anti-CD19 chimeric antigen receptor T cell therapy. *Molecular Therapy.* **25** (10), 2245–2253 (2017).

6 Lee, D. W. et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet.* **385** (9967), 517–528 (2015).

7 Turtle, C. J. et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *Journal of Clinical Investigation.* **126** (6), 2123–2138 (2016).

8 Brentjens, R. J. et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Science Translational Medicine.* **5** (177), 177ra138 (2013).

9 Porter, D. L. et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Science Translational Medicine.* **7** (303), 303ra139 (2015).

10 Turtle, C. J. et al. Durable molecular remissions in chronic lymphocytic leukemia treated with CD19-specific chimeric antigen receptor–modified T cells after failure of ibrutinib. *Journal of Clinical Oncology.* **35** (26), 3010–3020 (2017).

11 Porter, D. L., Levine, B. L., Kalos, M., Bagg, A., June, C. H. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *New England Journal of Medicine.* **365** (8), 725–733 (2011).

12 Fraietta, J. A. et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nature Medicine.* **24** (5), 563–571 (2018).

13 Raje, N. et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *New England Journal of Medicine.* **380** (18), 1726–1737 (2019).
14 Brudno, J. N. et al. T Cells genetically modified to express an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of poor-prognosis relapsed multiple myeloma. *Journal of Clinical Oncology.* **36** (22), 2267–2280 (2018).
15 Cohen, A. D. et al. B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. *Journal of Clinical Investigation.* **129** (6), 2210–2221 (2019).
16 Schuster, S. J. et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *New England Journal of Medicine.* **380** (1), 45–56 (2019).
17 Wang, M. et al. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. *New England Journal of Medicine.* **382** (14), 1331–1342 (2020).
18 Jacobson, C. et al. Primary analysis of Zuma-5: a phase 2 study of axicabtagene ciloleucel (Axi-Cel) in patients with relapsed/refractory (R/R) indolent non-Hodgkin lymphoma (iNHL). *Blood.* **136** (Supplement 1), 40–41 (2020).
19 Munshi, N. C. et al. Idecabtagene vicleucel (ide-cel; bb2121), a BCMA-targeted CAR T-cell therapy, in patients with relapsed and refractory multiple myeloma (RRMM): initial KarMMa results. *Journal of Clinical Oncology.* **38** (15 Suppl), 8503–8503 (2020).
20 Sakemura, R., Cox, M. J., Hefazi, M., Siegler, E. L., Kenderian, S. S. Resistance to CART cell therapy: lessons learned from the treatment of hematological malignancies. *Leukemia & Lymphoma.* 1–18, doi:10.1080/10428194.2021.1894648 (2021).
21 Sterner, R. M. et al. GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood.* **133** (7), 697–709 (2019).
22 Grupp, S. A. et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *New England Journal of Medicine.* **368** (16), 1509–1518 (2013).
23 Neelapu, S. S. et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *New England Journal of Medicine.* **377** (26), 2531–2544 (2017).
24 Maude, S. L. et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine.* **378** (5), 439–448 (2018).
25 Teachey, D. T. et al. Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discovery.* **6** (6), 664–679 (2016).
26 Hay, K. A. et al. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T-cell therapy. *Blood.* **130** (21), 2295–2306 (2017).
27 Lee, D. W. et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biology of Blood and Marrow Transplantation.* **25** (4), 625–638 (2019).
28 Davila, M. L. et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Science Translational Medicine.* **6** (224), 224ra225–224ra225 (2014).
29 Maude, S. L., Barrett, D., Teachey, D. T., Grupp, S. A. Managing cytokine release syndrome associated with novel T cell-engaging therapies. *Cancer Journal.* **20** (2), 119–122 (2014).
30 Krekorian, M. et al. Imaging of T-cells and their responses during anti-cancer immunotherapy. *Theranostics.* **9** (25), 7924–7947 (2019).

31 Wei, W., Jiang, D., Ehlerding, E. B., Luo, Q., Cai, W. Noninvasive PET imaging of T cells. *Trends in Cancer.* **4** (5), 359–373 (2018).
32 Volpe, A. et al. Spatiotemporal PET imaging reveals differences in CAR-T tumor retention in triple-negative breast cancer models. *Molecular Therapy.* **28** (10), 2271–2285 (2020).
33 Minn, I. et al. Imaging CAR T cell therapy with PSMA-targeted positron emission tomography. *Science Advances.* **5** (7), eaaw5096 (2019).
34 Keu, K. V. et al. Reporter gene imaging of targeted T cell immunotherapy in recurrent glioma. *Science Translational Medicine.* **9** (373), eaag2196 (2017).
35 Moroz, M. A. et al. Comparative analysis of T cell imaging with human nuclear reporter genes. *Journal of Nuclear Medicine.* **56** (7), 1055–1060 (2015).
36 Sellmyer, M. A. et al. Imaging CAR T cell trafficking with eDHFR as a PET reporter gene. *Molecular Therapy.* **28** (1), 42–51(2019).
37 Weist, M. R. et al. PET of adoptively transferred chimeric antigen receptor T cells with (89)Zr-oxine. *Journal of Nuclear Medicine.* **59** (10), 1531–1537 (2018).
38 Vedvyas, Y. et al. Longitudinal PET imaging demonstrates biphasic CAR T cell responses in survivors. *JCI Insight.* **1** (19), e90064, (2016).
39 Sakemura, R., Can, I., Siegler, E. L., Kenderian, S. S. In vivo CART cell imaging: Paving the way for success in CART cell therapy. *Molecular Therapy Oncolytics.* **20**, 625–633 (2021).
40 Penheiter, A. R., Russell, S. J., Carlson, S. K. The sodium iodide symporter (NIS) as an imaging reporter for gene, viral, and cell-based therapies. *Current Gene Therapy.* **12** (1), 33–47 (2012).
41 Msaouel, P. et al. Clinical trials with oncolytic measles virus: current status and future prospects. *Current Cancer Drug Targets.* **18** (2), 177–187 (2018).
42 Kalled, S., L. Hsu, Yen-Ming. Anti-BCMA antibodies. WO/2010/10949 (2010).
43 Carpenter, R. O. et al. B-cell maturation antigen is a promising target for adoptive T-cell therapy of multiple myeloma. *Clinical Cancer Research.* **19** (8), 2048–2060 (2013).
44 Jin, C. et al. Safe engineering of CAR T cells for adoptive cell therapy of cancer using long-term episomal gene transfer. *EMBO Molecular Medicine.* **8** (7), 702–711 (2016).
45 Sterner, R. M., Cox, M. J., Sakemura, R., Kenderian, S. S. Using CRISPR/Cas9 to knock out GM-CSF in CAR-T cells. *Journal of Visualized Experiments.* (149), 59629 (2019).
46 Yang, L. et al. Pirh2 mediates the sensitivity of myeloma cells to bortezomib via canonical NF-κB signaling pathway. *Protein & Cell.* **9** (9), 770–784 (2018).
47 Dietz, A. B. et al. A novel source of viable peripheral blood mononuclear cells from leukoreduction system chambers. *Transfusion.* **46** (12), 2083–2089 (2006).
48 Absher, M. in *Tissue Culture*: Methods and Applications. Kruse, P. F., Patterson, M. K. (Eds), Academic Press Inc., 395–397 (1973).
49 Janakiraman, V., Forrest, W. F., Chow, B., Seshagiri, S. A rapid method for estimation of baculovirus titer based on viable cell size. *Journal of Virological Methods.* **132** (1–2) (2006)
50 Works, M. et al. Anti–B-cell maturation antigen chimeric antigen receptor T cell function against multiple myeloma is enhanced in the presence of lenalidomide. *Molecular Cancer Therapeutics.* **18** (12), 2246–2257 (2019).
51 Smith, E. L. et al. GPRC5D is a target for the immunotherapy of multiple myeloma with rationally designed CAR T cells. *Science Translational Medicine.* **11** (485), eaau7746 (2019).

52 Oden, F. et al. Potent anti-tumor response by targeting B cell maturation antigen (BCMA) in a mouse model of multiple myeloma. *Molecular Oncology.* **9** (7), 1348–1358 (2015).
53 Jiang, H. et al. Synthesis of 18F-tetrafluoroborate via radiofluorination of boron trifluoride and evaluation in a murine C6-glioma tumor model. *Journal of Nuclear Medicine.* **57** (9), 1454–1459 (2016).
54 Sakemura, R. et al. Development of a sensitive and efficient reporter platform for the detection of chimeric antigen receptor T cell expansion, trafficking, and toxicity. *Blood.* **134** (Suppl 1), 53 (2019).
55 Dispenzieri, A. et al. Phase I trial of systemic administration of Edmonston strain of measles virus genetically engineered to express the sodium iodide symporter in patients with recurrent or refractory multiple myeloma. *Leukemia.* **31** (12), 2791–2798 (2017).
56 Ravera, S., Reyna-Neyra, A., Ferrandino, G., Amzel, L. M., Carrasco, N. The sodium/iodide symporter (NIS): molecular physiology and preclinical and clinical applications. *Annual Review of Physiology.* **79**, 261–289 (2017).
57 Varettoni, M. et al. Incidence, presenting features and outcome of extramedullary disease in multiple myeloma: a longitudinal study on 1003 consecutive patients. *Annals of Oncology.* **21** (2), 325–330 (2010).
58 Bladé, J. et al. Soft-tissue plasmacytomas in multiple myeloma: incidence, mechanisms of extramedullary spread, and treatment approach. *Journal of Clinical Oncology.* **29** (28), 3805–3812 (2011).
59 Brunton, B. et al. New transgenic NIS reporter rats for longitudinal tracking of fibrogenesis by high-resolution imaging. *Scientific Reports.* **8** (1), 14209 (2018).
60 Dohán, O. et al. The sodium/iodide symporter (NIS): characterization, regulation, and medical significance. *Endocrine Reviews.* **24** (1), 48–77 (2003).
61 Jiang, H., DeGrado, T. R. [^{18}F]Tetrafluoroborate ([^{18}F]TFB) and its analogs for PET imaging of the sodium/iodide symporter. *Theranostics.* **8** (14), 3918–3931 (2018).
62 Ahn, B.-C. Sodium iodide symporter for nuclear molecular imaging and gene therapy: from bedside to bench and back. *Theranostics.* **2** (4), 392–402 (2012).
63 Gust, J. et al. Endothelial activation and blood-brain barrier disruption in neurotoxicity after adoptive immunotherapy with CD19 CAR-T cells. *Cancer Discovery.* **7** (12), 1404–1419 (2017).
64 Gofshteyn, J. S. et al. Neurotoxicity after CTL019 in a pediatric and young adult cohort. *Annals of Neurology.* **84** (4), 537–546 (2018).
65 Shalabi, H. et al. Systematic evaluation of neurotoxicity in children and young adults undergoing CD22 chimeric antigen receptor T-cell therapy. *Journal of Immunotherapy.* **41** (7), 350–358 (2018).
66 Santomasso, B. D. et al. Clinical and biological correlates of neurotoxicity associated with CAR T-cell therapy in patients with B-cell acute lymphoblastic leukemia. *Cancer Discovery.* **8** (8), 958–971 (2018).

Figure 1

Figure 1

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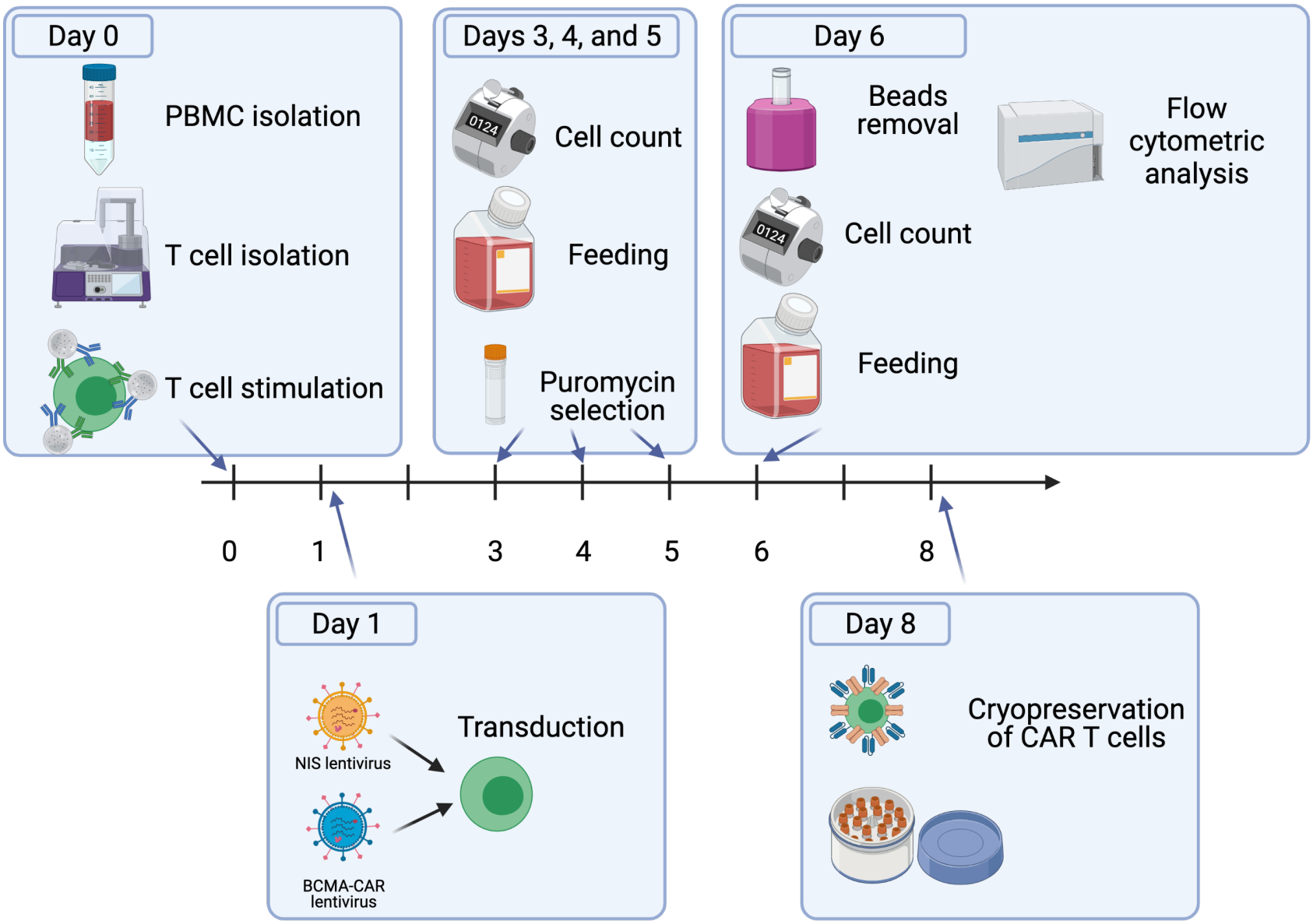

Figure 2

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Figure 2

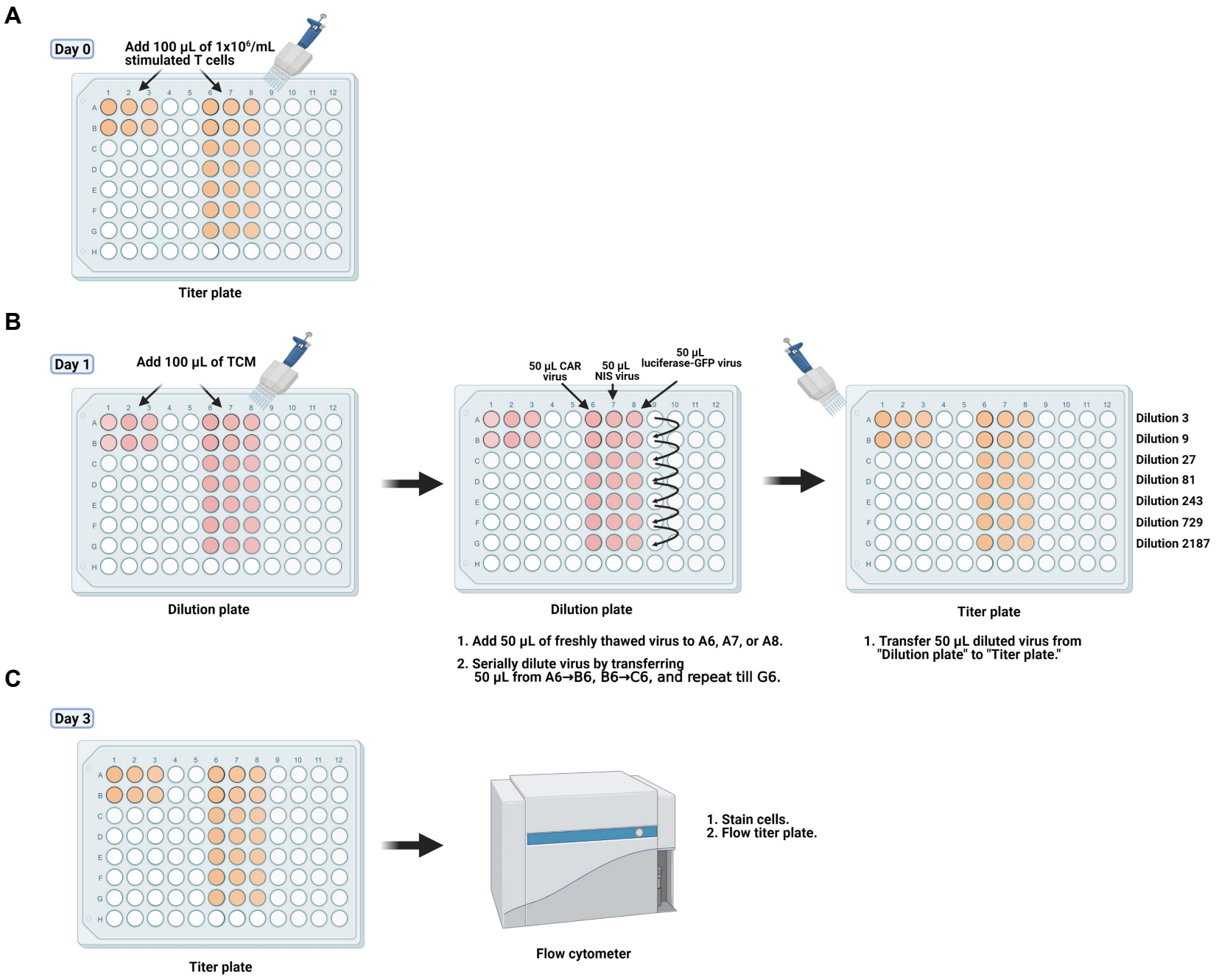

Figure 3

Figure 3

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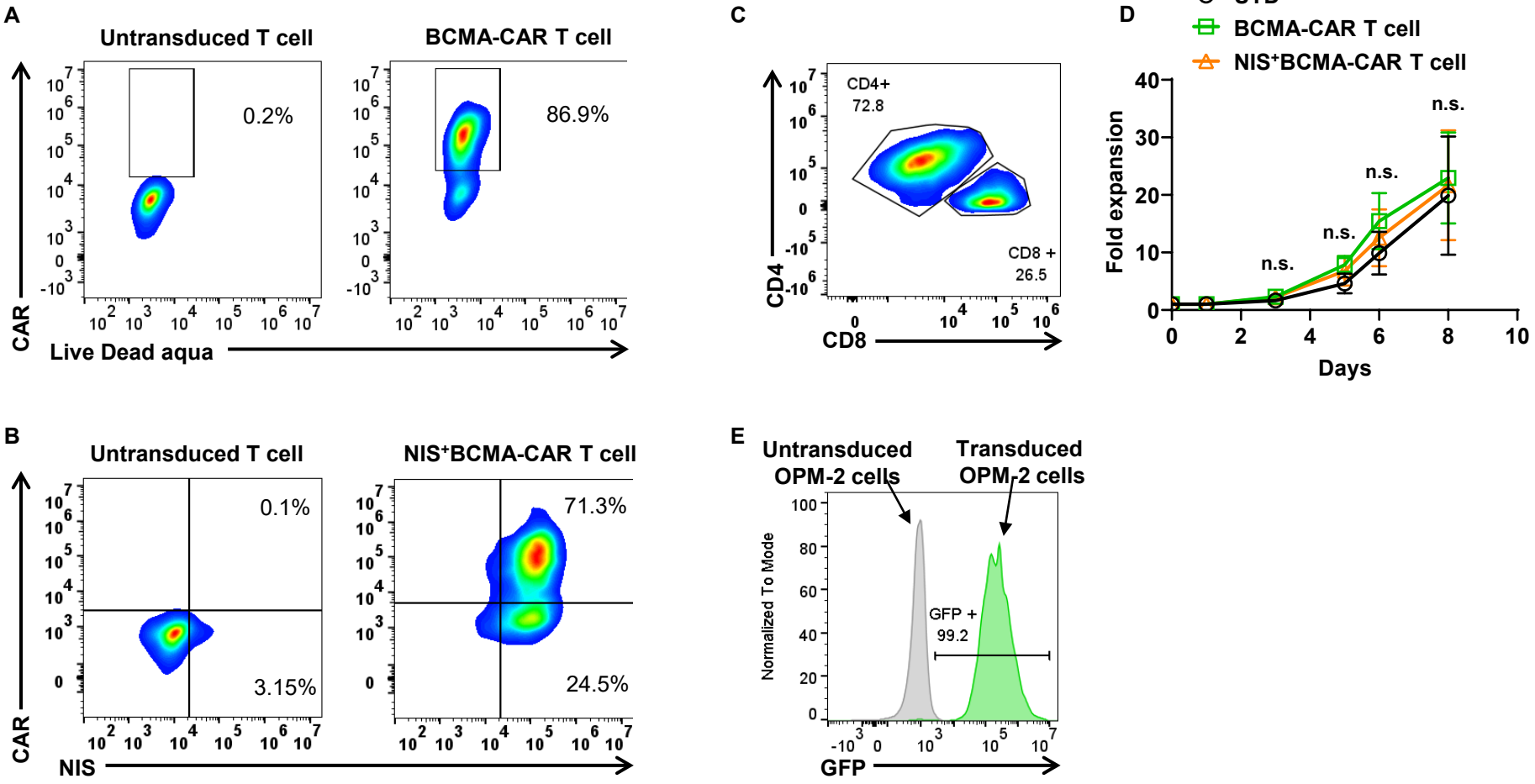

Figure 4

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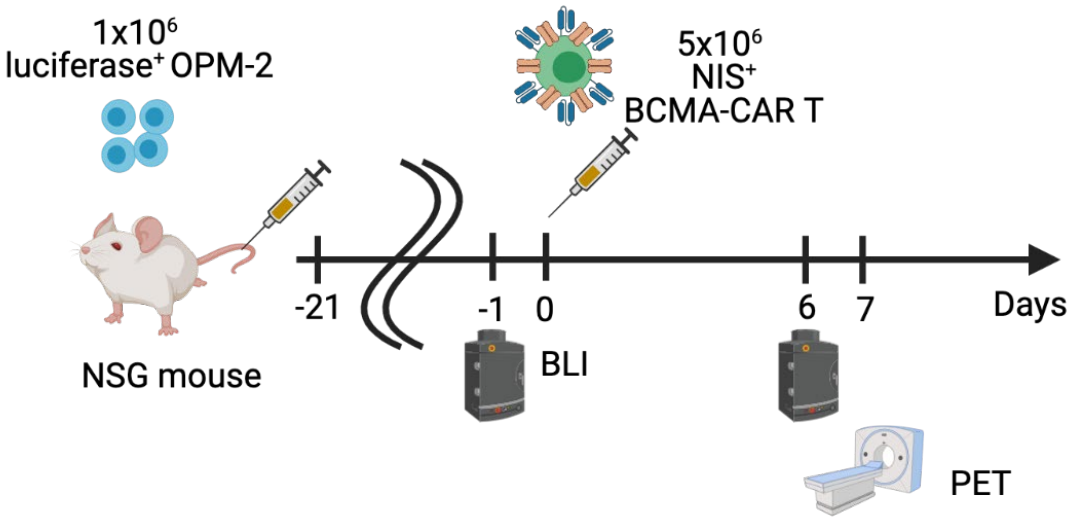

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Figure 5

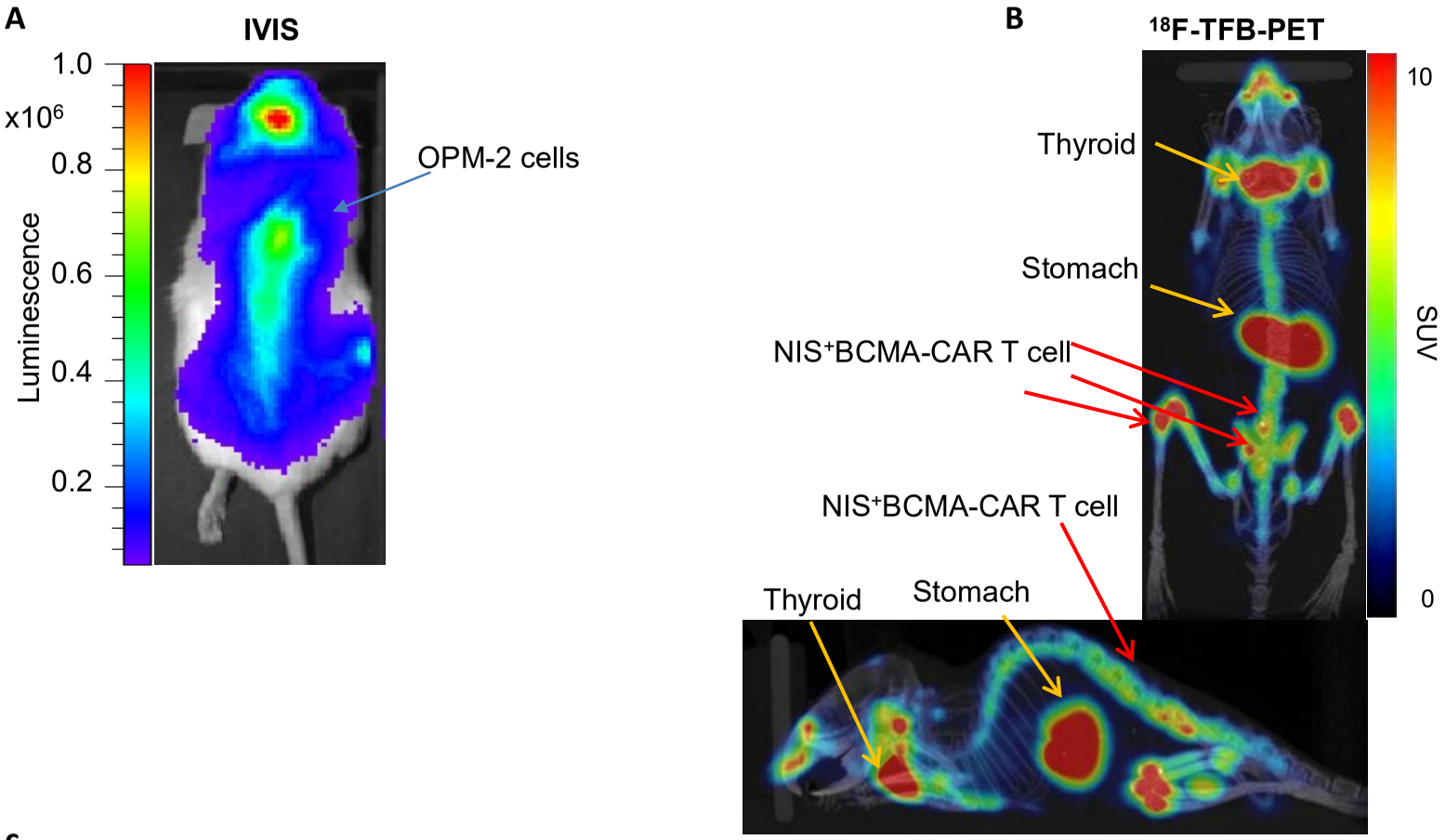


Figure 5

Click here to access/download
Table of Materials
Sakemura et al JoVE_Revised Materials_Final.xls

Rebuttal letter

Click here to access/download;Rebuttal Letter;JoVE Response to reviewers.docx

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

➔ *Thank you for your comment. Revised highlighted manuscript incorporating the changes, highlighted in green. We have gone through the manuscript to confirm that we corrected spelling and grammar mistakes.*

2. Please provide an institutional email address for each author.

➔ *We added authors' emails next to authors' names.*

3. Please revise the following lines to avoid previously published work: 48-50, 94-96, 107-110, 115-117, 122, 137-139, 151-154, 167-168, 212-213.

➔ *Thank you for your comment. We revised the sentences where you indicated.*

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

➔ *We appreciate the editor for the careful review. We excluded all the personal pronouns and changed the sentences accordingly.*

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

➔ *Thank you for your comment. We have added the ethics statement for each protocol step.*

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

➔ *We apologize to the editor for not providing the details of protocol steps. According to your advice, we added extensively to each step in the main text as well as in the figures.*

7. Line107: Please include more details regarding PBMC harvesting. How are the cells harvested? What media is used?

➔ *We added details and steps of PBMC harvesting and isolation. Please see step 1.3.*

8. Line 122: Please elaborate the isolation of T cells.

➔ *We added details regarding T cell isolation. Please see step 1.3.*

9. Line 140/180-185: Please use standard abbreviations for time units preceded by a numeral (e.g., 5 min, 4 h. 10 s).

➔ *We changed the time units to minutes, hours, and seconds.*

10. Line 166: Please define the abbreviations before use (ETNL).

➔ *We apologize to the editor for not defining ETNL, we revised the manuscript and described details in step 1.4.6.*

11. Line 168: Please mention how is the transduction efficiency quantified using flow cytometry.

➔ *We added a section where we described how to titrate viruses in section 1.2 as well as in Figure 1.*

12. Please include a one line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

➔ *We highlighted in yellow where we defined the essential steps of the protocol for the video.*

13. Please move the Figure Legends to the end of the Representative Results.

➔ *Figure legends are now moved to after the discussion section.*

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, +F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not use abbreviations for journal titles and book titles. Article titles should appear exactly as they were published in the original work, without any abbreviations or truncations.

➔ *References are now changed to meet the JoVE requirements.*

15. Please sort the Table of Materials in alphabetical order.

➔ *The Table of Materials is now sorted in alphabetical order.*

Reviewer #1:
TO AUTHORS:
The authors propose a manuscript focused on methodology to non-invasively track chimeric antigen receptor T cells (CAR-T) in an animal model. The authors report methodology based on using the human sodium iodide symporter as a radionuclide reporter gene to enable non-invasive and longitudinal PET imaging. The approach requires a radiotracer to detect reporter gene expressing cells in vivo, and this radiotracer is F-18-labelled tetrafluoroborate (TFB).

➔ *We thank the reviewer for summarizing our work, for the comprehensive and detailed review, and for the constructive feedback which we believe has improved our methods paper.*

In vivo cell tracking with TFB/NIS-PET has previously been reported in cancer models (Diocou et al Sci Rep 2017). Non-invasive CAR-T tracking by PET has also been reported (Keu et al Science Trans Med 2017, Minn et al Sci Adv 2019) including the use of NIS (Emami et al Nat Comm 2018 using NIS-SPECT) and including the use of TFB-afforded NIS-PET (Volpe et al Mol Ther 2020). Notably, all these original reports have been reported by teams different to the authors who propose this methods paper. While novelty is not really a criterion for a methods paper, unfortunately, the suggested method/protocol is nothing more than what has previously been published already as methods/protocols (e.g. several methods/protocols how to make CAR T cells, for example Riccione et al JoVE 2015; Ghassemi and Milone JoVE 2019; Xu et al Experiments (Springer Nature) 2019) and a detailed protocol how to use NIS for cell tracking by PET using TFB (Volpe et al JoVE 2018).

➔ *We agree with the reviewer that in vivo tracking of CART cells with NIS and PET imaging has been previously reported, and we agree that novelty is not a criterion for a method paper. However, we respectfully, but strongly, disagree with the reviewer's statement that this protocol is nothing more than what has previously been published in the past. First, it is important to note that our group at the Mayo Clinic has been developing and optimizing the use of NIS as a reporter for cell and gene therapy for the last two decades (PMID 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108, 28439108). Secondly, our group has recently reported the use of PET scan imaging of NIS^+ CART cells as a strategy to track both CART cell trafficking and expansion and to predict the development of CART cell toxicities in preclinical models (Sakemura et al, presented at the American Society of Hematology annual meeting in 2019). This manuscript is under advanced stages of revision and will likely be published before this method paper is published. Our strategy of using PET scan to track CART cell expansion and predict the development of toxicities in preclinical models is novel and has not been reported in the past, and we believe it represents a major advance in the field, compared to other reports that have only focused on CART cell trafficking to tumor sites. The*

platforms and potential applications of in vivo CART cell imaging were also recently reviewed by our group (Sakemura et al, Molecular Therapy Oncolytics 2021).

In addition, what is presented here is by no means compatible with a scientific protocol/methods paper in line with the publication criteria of JoVE. The reviewer does not see the point of combining existing methods/protocols into new combinations thereof, and certainly not if this is very poorly performed with an obvious lack of expertise and diligence towards large aspects of the reported methodologies. The idea of JoVE is to provide a reproducible protocol and thereby foster education and enhance the reliability of science; neither of this is accomplished here.

➔ *We appreciate the reviewer's time to provide constructive comments below, which we believe have significantly improved our work. However, we also respectfully disagree and condemn this reviewer's unprofessional characterization of our team as lacking expertise.*

Introduction
Up to line 86 this is very much focused on CAR-T therapy, but the emphasis is clearly on blood cancers with little (if any) regard of the issues CAR-T (or other anti-cancer T cell therapies) suffer when tried in solid tumors. The argument for imaging in CAR-T development could be presented much stronger. However, it is in solid tumors, where imaging and cell tracking can be of real additional value. Prediction of toxicities is a stark claim as is the detection or prediction of resistance phenomena at this point.
Line 90: "several" is correct, but the authors should not cite reviews but instead the original papers. The minimum to be cited are CAR-T tracking by PET by Keu et al Science Trans Med 2017 (first clinical example), CAR-T tracking using a non-immunogenic reporter by Minn et al Sci Adv 2019 and TFB-afforded NIS-PET by Volpe et al Mol Ther 2020.

➔ *We have clarified in the revised manuscript the potential roles of CART cell imaging for the assessment of both CART cell trafficking to tumor sites, and CART cell expansion/prediction of their toxicities, and the use of blood cancers as a proof-of-concept model to measure these two different CART cell functions. We have made sure to cite all prior publications as requested by the reviewer.*

Line 94: There is nothing to "propose"; everything shown here has in principle already been done and published. This is a methods/protocol journal and what is provided should be based on prior published and peer-reviewed developments, ideally by experts in the field.

➔ *We agree with the reviewer and have therefore avoided the use of propose in the introduction section of the revised manuscript.*

Protocol

Line 109/110: give details and composition of 'density gradient medium'

➔ *Thank you for your comment. The composition of "density gradient medium" is now described on page 6.*

Line 111: give composition of PBS, is it important to keep the sodium : potassium ratio in check?

➔ *Thank you for your comment. The composition of "PBS" is now described on page 6.*

Line 122: 'negative isolation magnetic bead kit' is an insufficient description; what is the principle of the negative isolation based on, how is it done? Is a kit necessary or can it be done without the proprietary materials? which kits from what companies can be used? Which ones are not performing well? Is it wise to stick to the manufacturer's recommendations or are tweaks necessary?

➔ *Thank you for your comment. Details regarding the principle of negative selection and options for negative selection were added to the protocol. According to the author guidelines for publishing a manuscript in JoVE, the commercial/brand names of reagents are not allowed to be part of the manuscript. This, however, is included in the Table of Materials.*

Line 124: tell the reader what TCM is and give all details. There is no point writing a protocol that relies on the user to search for other publications to find the recipe of a component.

➔ *Thank you for your comment. The details of TCM is now described in main text, page 7.*

Line 128: inaccurate description; clearly the CD3/CD28 must be on the beads. Here this is preparation for bead washing - how does the user calculate how much beads they need when there is nowhere a recommendation for the amount of T cells to be used. Make it clear in the protocol what to do and present instructions for your recommended 3:1 ratio and the scale of T cell numbers to operate on. Which other ratios are acceptable in addition to the recommended ratio of 3:1?

➔ *Thank you for the careful review. We have clarified in the revised manuscript the composition of beads and have described the ideal bead:cell ratio.*

Line 140: how long can T cells be cultured from here until transduction? Is transduction envisaged to happen immediately? What about re-stimulation of beads? What about IL-2 or IL-15 addition to the media? As it stands the protocol proceeds to lentivirus production after T cell isolation, which does not seem to be smart as the time needed

for lentivirus production is time the T cells need to be in culture. This is either an error or an unfortunate way of description. A figure with a timeline indicating which processes/steps happen when and how they are recommended to achieve best experimental convergence and success is certainly needed here.

➔ *We have included a figure to clarify the process. For the reviewer's information, the protocol we use to generate CART cells is widely used in multiple laboratories and in the manufacturing of clinical-grade CART cells. Transduction usually happens within 24-48 hours after T cell stimulation. There is no role for re-stimulation during CART cell production. Re-stimulation of T cells increases their susceptibility to exhaustion. Similarly, the use of cytokines does not impact the overall fitness of CART cells and therefore is not routinely included in our and others' protocols for CART cell generation.*

The protocol step "1.2.2 Perform lentiviral production as previously described" is pointless. Either the authors describe a protocol in such a manner that researchers/colleagues with some basic understanding of biological procedures can repeat it, or they should refrain from publishing methods. Protocols should be as stand-alone as possible.
There is no description whatsoever which constructs are used to generate the viruses, not even with NIS ortholog, not even if and which selection marker. What about the CAR? How does it get into the T cells here?

➔ *Thank you for your comment. We have incorporated in the revised manuscript a protocol for lentiviral production and details with regard to the CAR construct and transduction.*

Step 1.3.1 includes virus titer determination - this cannot simply be mentioned only. It is integral part of 1.2.2 and must be described if it is crucial to the method. Particularly, as there are different approaches to determine virus titers and they can lead to vastly differing results. Notably, the authors constantly cite their own paper (#17) which does not well describe the methodologies either (this paper is not original in terms of developing the relevant methods cited here).

➔ *To address the reviewer's concerns, we have incorporated the virus titration protocol in this manuscript and cited our prior methods paper as well (Sterner et al, JoVE 2019, which describes the methodology in details.)*

Line 148: Nobody new to the methodology and reading this with the aim to learn how to perform the procedure will understand the jargon used, e.g. "rosettes". Please explain and clarify the steps. Moreover, the concept of MOI is not explained, and the new user will not know what to do with this information. There is no example to help and there is

no note (as is routinely used in JoVE papers) to explain more in-depth certain aspects and warn of potential pitfalls.

➔ *The description of MOI is now included in the revised manuscript under the virus titration protocol, under T cell transduction and Figure 1. We have clarified T cell clustering.*

Line 151-152: this sound like accounting for T cell growth post transduction and trying to keep the T cell concentration constant. That should be said, and it should be said why this is important. Give example data so the user can judge when performing the steps whether things are working or not. T cell expansion data with and without transduction should be shown as part of Figure 1.1

➔ *The T cell growth is a result of T cell stimulation during their ex vivo expansion. We have included a summary of CART cell kinetics after bead stimulation in revised Figure 3D as suggested by the reviewer.*

Line 153: Where does the puromycin resistance gene come from - this is nowhere mentioned before? Does it need to be the puromycin resistance system? Why 1ug/mL? (other papers report selection with 2ug/mL) - are T cells particularly sensitive to this? What are the consequences of this us on T cell function? Explain, show example data, give hints and notes as otherwise this is useless.

➔ *Thank you for the careful review. In the revised manuscript, we have explained the use of puromycin gene resistance in the NIS vector backbone to select for NIS^+ cells. The use of puromycin for selection does not impair CART cell functions. A footnote was added to clarify in section 1.1.2.*

Step 1.3.3. get rid of the lab jargon ("rosettes", "debeading")…

➔ *Thank you for your comment; this is now changed to T cell clustering as requested by the reviewer.*

Step 1.3.3. where does the CAR suddenly come from - it has not been mentioned anywhere above in the protocol. So far it is T cells and a NIS construct (albeit unknown which one). In the abstract the authors claim transduction with two viruses (which in itself is a debatable approach). Are they here now using a construct that contains both CAR and NIS (as reported by others previously)? If so, give details and the relevant references!

➔ *Dual transduction was used in this study. Details regarding the NIS construct and CAR construct are included in the revised manuscript.*

Step 1.3.4. there is no way this can be done by anybody, even by an expert. There is no description of the antibodies and what they are directed against, where they are available from or how they are used (concentration, buffers, times etc). Why goat anti-Mouse? What is the primary antibody? For flow cytometry, fluorophores are needed - where are they? Which ones are used? Fix cells how? Permeabilize cells how? What is ETNL describing? First indication of the use of human NIS here. Is this important? Would that change if mouse T cells are used? How was flow cytometry done (cell density in samples important? Any anti-sticking admixtures? Media? Only analytical sorting or preparative sorting? If the latter, what speed, pressure, nozzle etc.)?

➔ *Thank you for the careful review. The requested details regarding detection of CAR and NIS were added to the revised manuscript, section 1. NIS^{+}BCMA-CART cell production.*

Step 2: This needs to separated into cancer model generation and imaging administered CAR-T cells.
Luc+OPM-2 cells are not described (source?) explain why Luc+ is important. How to confirm tumor engraftment? How long does it take? How to monitor? What are adverse effects to watch out for? How is the BLI done, in what intervals etc. This is all impossible to reproducibly do as described.

➔ *Thank you for the careful review. This section was separated into two paragraphs in the revised manuscripts and details regarding the experimental schema were added.*
- *Luciferase-GFP production is now clearly described in section 1.1.*
- *Titration of produced lentivirus is now described in section 1.2. and Figure 1.*
- *Transduction of luciferase-GFP is now described in section 2.1.*
- *A protocol for how to establish OPM-2 xenograft mouse is now described in section 2.2 and Figure 4.*

Step 2.1.2 Again, instead of a description, in fact the very description advertised in this protocol, there is only a reference. This is simply unacceptable and frankly pointless.
Step 2.1.3-2.3.4 Why 45min ?(where are the optimization data? Where is the reference to the paper that demonstrated that this works?) Time must be kept constant here. Radioactivity should be given in SI units (i.e. Bq or for convenience MBq). There is no mention where TFB comes from, how it was made (and it has to be made on the day on site!!!), with which method, with what specific activity -and SA is important for preclinical imaging as has been explained in the literature using TFB as an example! (see Khoshnevisan et al EJNMMI Res 2016) - or how long after production TFB can be used and what the consequences for imaging throughput are. No word about PET image acquisition parameters. No word about animal preparation and anesthesia or monitoring etc. (the latter aspects are totally

unacceptable from a veterinary point of view). Why PET imaging for 20min? What are the implications of this? Would dynamic imaging be of use? If not, why not? Step 2.2. This is again not described properly, not even the basics of image reconstruction or image registration let alone any relevant image processing or even image quantification (which is the strength of PET).

➔ *Details regarding imaging duration and tracer generation were added to the revised manuscript. However, we believe the optimization data for NIS reporters is extensive and do not belong in this methodology paper; therefore, we have cited prior work in regard to this. TFB is generated in house, and the methods for its generation are also beyond the scope of this manuscript, which is focused on CART cell imaging in vivo. We also cited prior work regarding this.*

Figure 1: far too rudimentary. It should show the whole characterization so the user can appreciate the quality of the produced CAR-T in comparison to non-transduced and non-traceable CAR-T cells. There is a lack of phenotypic information (CD3, CD4, CD8); there is no information about the activation state of these CAR-T after transduction. There is no information about reporter gene function or stability. There is no information about the dual transduction strategy. Technically, the flow cytometry quantification is wrong as clearly the double-positive cell populations are overestimated by drawing the quadrant too low (clearly there is a second population, negative one, that is half added to the positive cells).

➔ *Please note that this is now moved to Figure 3. Please note that gating is set up based on negative controls. We have modified the gating strategies to clearly reflect this and added CD4:CD8 composition. However, we believe CART cell activation status is irrelevant to this manuscript which is focused on reporting a methodology for CART cell imaging in vivo – and since we report potent CART cell activity in vivo.*

Figure 2: This is simply unacceptable from an imaging perspective. For PET, a maximum intensity projection must be shown as well as coronal/sagittal/axial sections, ideally all PET only plus PET/CT overlays. The various sites of what appears as bone uptake in the pelvic area are suspicious - this clearly points towards free F-18 and this is most likely due to poor radiotracer production (it could also be defluorination in vivo although the latter is less likely for well-produced TFB judging from other published reports and their data). Why are CAR-NIS-T cells in the 'nose' region of the animal? The BLI image does not add anything here; it is confusing as it is unclear what the massive amount of signal in lower body half indicates. Moreover, the 2D planar imaging of BLI is by no means comparable to the 3D tomography PET-CT images.
For the reader a serial image of the same mouse would be useful to see, e.g. shortly after CAR-NIS-T injection (e.g. 1h) and at least one if not two later time points, e.g. 24h and 48h. The reader would then be able to appreciate the dynamic changes of CAR-NIS-T cells populations compared to the endogenous signals observed (stomach, thyroid) and excretion signals (bladder).

➔ *We agree with the reviewer that images needed more clarity and have therefore substantially revised the figure. The BLI imaging is relevant to show trafficking of NIS^{+} CART cells (PET^{+} cells) to tumor sites (BLI^{+} sites).*

Discussion
The first sentence appears to reveal the authors' real intention, namely to "report" a methodology. But this is not the place to do so and clearly not the scope of this journal - instead, it is to *describe* methodology such that *others can use it*.
The statement "We have recently reported that incorporating NIS into CART cells does not impair CART cell functions and efficacy in vivo and is able to track CART cell trafficking and expansion. (32)" is not supported by any evidence. As far as the reviewer can see from PubMed, the authors did not publish a paper demonstrating this, certainly not the paper they cited as #32 (it is from the authors but does not mention NIS or PET imaging). A quick Google search, however, revealed that Volpe et al Mol Ther 2020 in fact did report on the impact of using NIS expressed in CAR-T for imaging - but this is data from a different laboratory and interestingly not cited here.
The reference to clinical trials using NIS as a reporter gene is simply wrong - no such trials have been reported yet. The only clinical trial with in vivo traceable therapeutic cells is Keu et al Science Trans Med 2017. NIS methodology has great potential for clinical translation though and its tracers for SPECT and PET have been translated to human use already (although for different purposes such as thyroid imaging and imaging thyroid cancer metastasis locations) and NIS is a host protein, hence non-immunogenic as the authors correctly state (without reference).

➔ *We respectfully disagree with the reviewer on multiple comments here. Our group did report that the incorporation of NIS does not impair CART cell efficacy and presented this work at the American Society of Hematology annual meeting in December 2019. Our full manuscript is under advanced stages of revision. In addition, clincial trials using oncolytic measles virus expressing the human NIS in patients with myeloma have been reported (Dispenzieri et al, PMID 28439108, and, Russell et al, 28439108).*

➔ *At the time of submission of this JoVE methods manuscript, the paper by Volpe and colleagues was not published. We have appropriately cited this paper in the revised manuscript.*

The description of NIS in the Discussion is off-topic; if at all it should be in the introduction. Instead, several aspects relevant to a protocol are missing, for example: Sensitivity of cell detection; Availability of radiotracers; Effects of the reporter on T cell function; Long-term expression stability of the reporter; Advantages of this methodology over competing approaches; Common pitfalls of using this methodology etc.
(NB Tc-99m is not a radiotracer for NIS; in fact the radiotracer is Tc-99m-pertechnetate - this a minor aspect, but wrong, and highlights the lack of expertise of the authors).

➔ *We appreciate the reviewer's request for additional details concerning the pros and cons of this platform, which we have now briefly included in the revised manuscript. However, we once more respectfully condemn this reviewer's unprofessional characterization of our team as lacking expertise.*

The apparent limitation regarding the blood brain barrier appears to be the only critical aspect the authors mention. Notably, it would be beneficial to enrich this aspect with a discussion to what extent the blood brain barrier remains intact during tumor progression and what are the consequences for CAR-T penetration/infiltration into the tumors as well as imaging (if the BBB is broken down, would NIS imaging work?). If they want to image in the brain, why would they choose a reporter which radiotracer is not crossing the BBB when there are explicit publications reporting on options compatible with this purpose (Haywood et al PNAS 2019)?

➔ *We agree with the reviewer regarding this limitation. We plan to address this in a follow up study using our novel xenograft model of CART cell neurotoxicity (Sterner et al, Blood 2019). We have added this as a future direction in the discussion section of this manuscript).*

Reviewer #2:
Manuscript Summary:
The submitted article is an interesting, straight-forward approach to image CART cells in vivo by incorporating NIS into the cells to be exploited for PET/CT imaging with 18F-TFB. Although most of the protocol is well written and understandable, I have some comments/questions.

➔ *Thank you very much for your positive feedback. We have addressed all of your comments below.*

Major Concerns:
I have no major concerns.

Minor Concerns:
General remarks:
In general, it would be nice if the authors could briefly summarize each step of the protocol even if it was previously described somewhere else. The focus of JoVe is to publish enhanced versions of these rather short protocols in result-based papers so that researchers can learn new techniques step-by-step. Also, I think it would be of great value to explain each step (or at least the major ones) in a half-sentence: Why is this step necessary, what does it do to the cells/sample? Furthermore, abbreviations should be explained and spelled out when being mentioned first.
Protocol:
I have isolated PBMCs myself many times and my protocol slightly differs from this one, but of course that does not mean that one is superior, so most of the following comments/questions regarding PBMC isolation can be seen as optional or out of pure interest. Nonetheless, I tried to view the protocol with the eyes of someone who has little experience in this field and would like to learn a new technique.
1) Line 109: The density gradient medium used should be specified (regarding the optimal density, I assume something like Histopaque 1.077 g/ml?)

➔ *Thank you for your comment. We have been using Lymphoprep, which is similar to Histopaque. Since JoVE does not allow to add the brand name in the text, it is stated in the Table of Materials.*

2) Line 110: Shouldn't there be a short centrifugation step after adding the density gradient medium to the separation tube so that the medium is below the filter? Or are other tubes without a filter being used? Also, the volume the tubes should have should be specified as well as the amount of blood usually used to get a sufficient amount of PBMCs.
3) Line 114: I remember having to turn off the brakes of the centrifuge to ensure the different cell discs are not disturbed after centrifugation. Is that not the case in this protocol?

➔ *Thank you for your comment. We are using Sepmate tubes, which do not require "brakes off." This is now described in section 1.3.4.*

4) Line 115: Shouldn't the top layer containing serum and media first be aspirated before decanting the light density white cells into a new tube?

➔ *Thank you for your comment. When the Sepmate tubes are used to isolate PBMCs, we just simply pour off the top layer. It could have been much clearer if we could explain the actual kit name.*

5) Line 118/Step 1.1.6: I would recommend a 3rd or even 4th PBS washing step to ensure that thrombocytes are washed out sufficiently.

➔ *Thank you for your comment, and we would like to add a third wash according to the reviewer's suggestion.*

6) Line 122-124/Step 1.1.8/1.1.9.: A brief summary of this step should be provided.

➔ *Thank you for your comment. We have been using RoboSep-S (a fully automated cell separator) to isolate T cells. It is now described more details in the main text, page 7.*

7) Line 127: The kind of dish used to culture the cells should be specified.

➔ *Thank you for your comment. It is now specified in the main text, page 8.*

8) Line 144: so, please provide a brief summary of the lentivirus production.

➔ *Thank you for your comment. It is now described in the main text, page 5.*

9) Line 147: A brief summary of this step should be provided.

➔ *Thank you for your comment. A brief summary of this step is now described in Figure 2.*

10) Line 161/Step 1.3.4: I am a bit confused at what is stained here and what kinds of antibodies are used. Isn't goat-anti-mouse IgG (H+L) just a secondary antibody? Why are anti-human and anti-rabbit antibodies used in the next step? Also, BCMA is introduced here for the first time --> hasn't even been mentioned in the introduction. The term CAR19 and ETNL has also been used for the first time without spelling it out. It should also be specified how you would fix and permeabilize the cells and at what speed you centrifuge during the washing steps.

➔ *To avoid confusion, we excluded CAR19 from this manuscript since we were focusing on imaging BCMA CART cells in a OPM-2 xenograft model in Figure 2 (which is now Figure 4). We used goat-anti-mouse (GAM) IgG (H+L) antibody to detect CAR receptors since the scFv portions are derived from mouse. The GAM antibody is conjugated with Alexa Fluor 647.*

➔ *We apologize for not introducing BCMA CART cells properly. It is now briefly mentioned in the introduction section, page 3.*

11) Line 171: OPM-2 is mentioned the first time --> spell out and briefly mention the type of cell line

➔ *Thank you for your comment. We briefly mentioned in the main text, page 11.*

12) Line 177: It would be nice to have a brief reasoning for why the others picked day 22 and day 29 for PET imaging.

➔ *Thank you for your comment. OPM-2 cells are a slow growing tumor, and it takes 2-3 weeks to engraft. We briefly mentioned in the main text, page 11.*

13) Line 179: A brief summary of this step should be provided.
14) Line 180: I think, 2.1.3 should be mentioned before 2.1.2. Also, mCi is a rather old unit and I would use the SI unit Mbq instead.
15) Line 184/185: This sentence might be misleading, because one could think that image acquisition should last for 45 min. Rephrasing should be considered.
Discussion:
Line 208: References for the different isotopes that NIS can be labelled with should be provided.
Figures:
Fig. 2: If I am not mistaken the IVIS image just shows BCMA+ luciferase+ OPM2 cells to confirm that OPM2 cells were engrafted, whereas the PET image shows the CART cells. I think it would be helpful to make it a bit clearer in the figure itself what kind of cells are imaged in IVIS and in PET. Also, why is there such a strong signal in the head in BLI but not in PET?

➔ *Thank you for your comment. We agree with the reviewer that the quality of the figure is not impressive and could be misleading. Therefore, we performed another round of PET imaging. We stated "OPM-2 cells" on the IVIS image so that the readers would not misunderstand.*

Typos:
1) Line 66: space between "therapy," and "its" is missing.
2) Line 88: "using" is used twice --> rephrase the sentence to avoid misunderstandings

➔ *Thank you for your comment. It is now changed to "with."*

3) Line 107: I think it's supposed to say "Harvest".

➔ *Thank you for your comment. We restructure the sentence.*

4) Line 116: space is missing between "volume" and "up"

➔ *Thank you for your comment. We change the phrase to "filling up."*

5) Line 128: I think this sentence and the following might be a bit misleading because the word "beads" is missing in line 128 behind CD3/CD28?!

➔ *Thank you for your comment. We apologize for not defining beads. It's now changed to anti-CD3/CD28 beads.*

6) Line 222: unclear is spelled wrong

➔ *Thank you for your comment. We fixed the typo.*

7) Line 387: I assume between "three" and "after" the word "days" is missing.

➔ *Thank you for your comment. We added "days" in the figure legend.*

Combined Figures

Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Sakemura et al._Jove_NIS CART_Revised_Figures_6-17-21.pdf

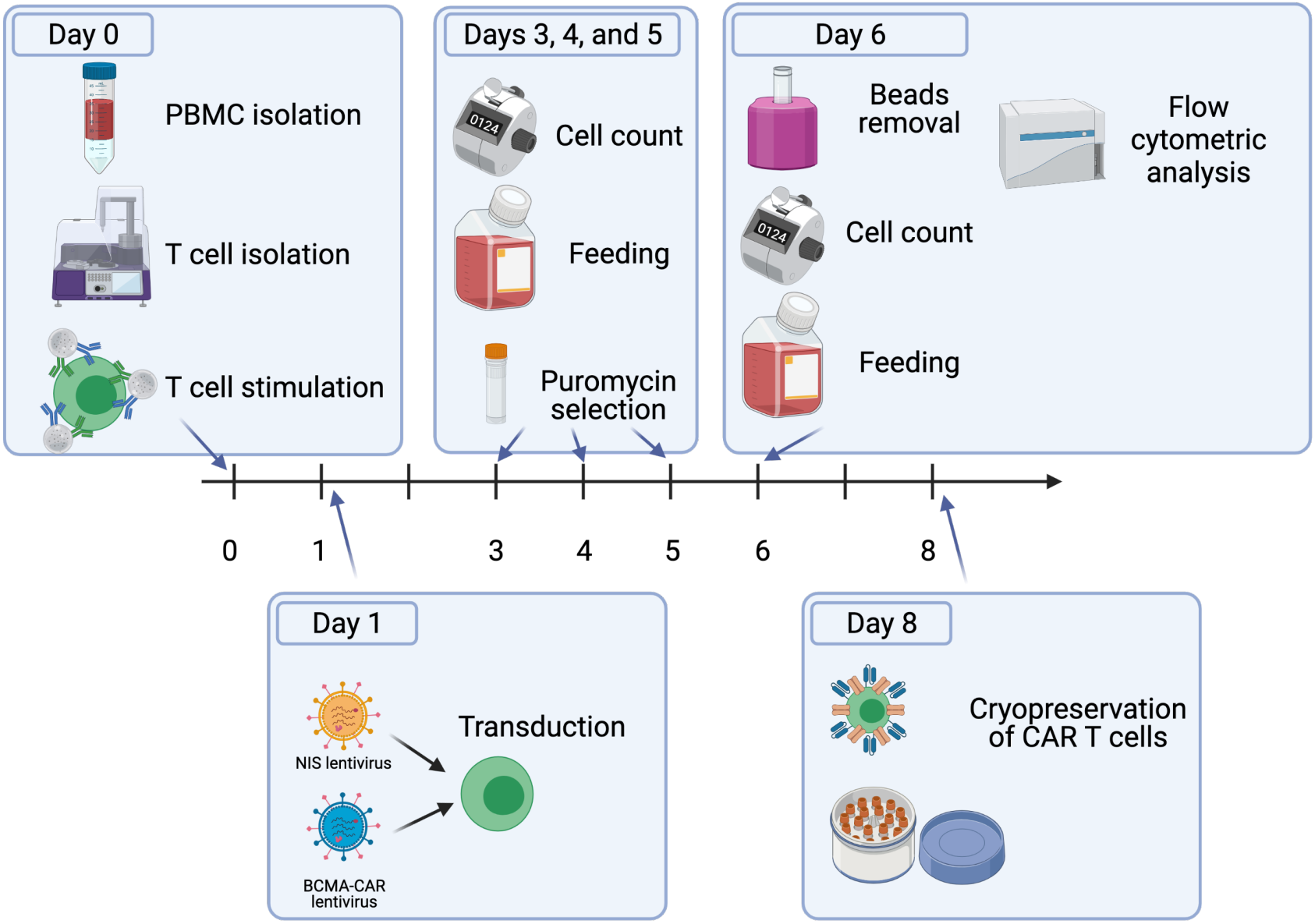


Figure 2

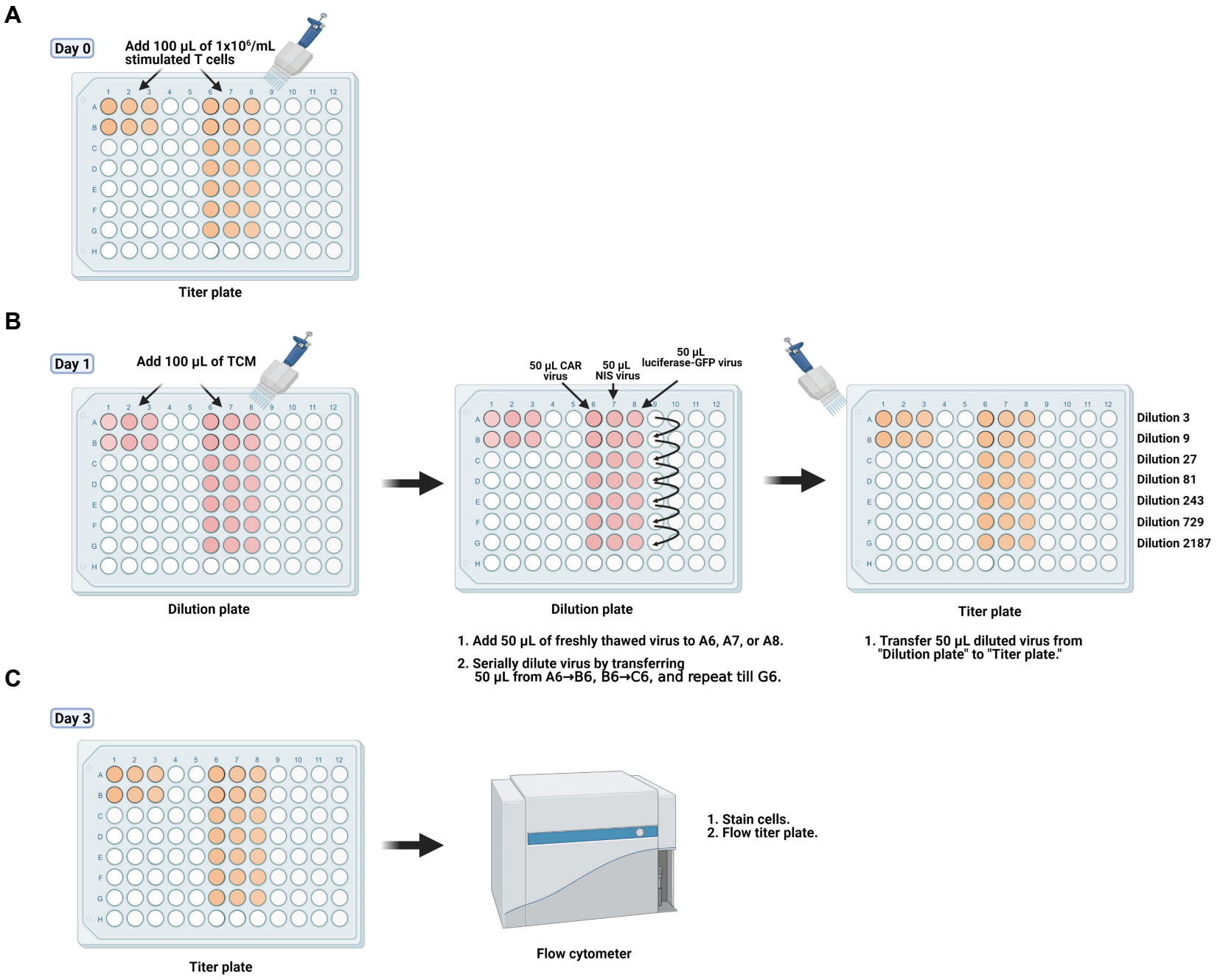

A
Day 0
Add 100 µL of 1x10⁶/mL stimulated T cells
Titer plate
B
Day 1
Add 100 µL of TCM
Dilution plate
50 µL CAR virus
50 µL NIS virus
50 µL luciferase-GFP virus
Dilution plate
1. Add 50 µL of freshly thawed virus to A6, A7, or A8.
2. Serially dilute virus by transferring 50 µL from A6→B6, B6→C6, and repeat till G6.
1. Transfer 50 µL diluted virus from "Dilution plate" to "Titer plate."
Titer plate
Dilution 3
Dilution 9
Dilution 27
Dilution 81
Dilution 243
Dilution 729
Dilution 2187
C
Day 3
Titer plate
Flow cytometer
1. Stain cells.
2. Flow titer plate.

Figure 3

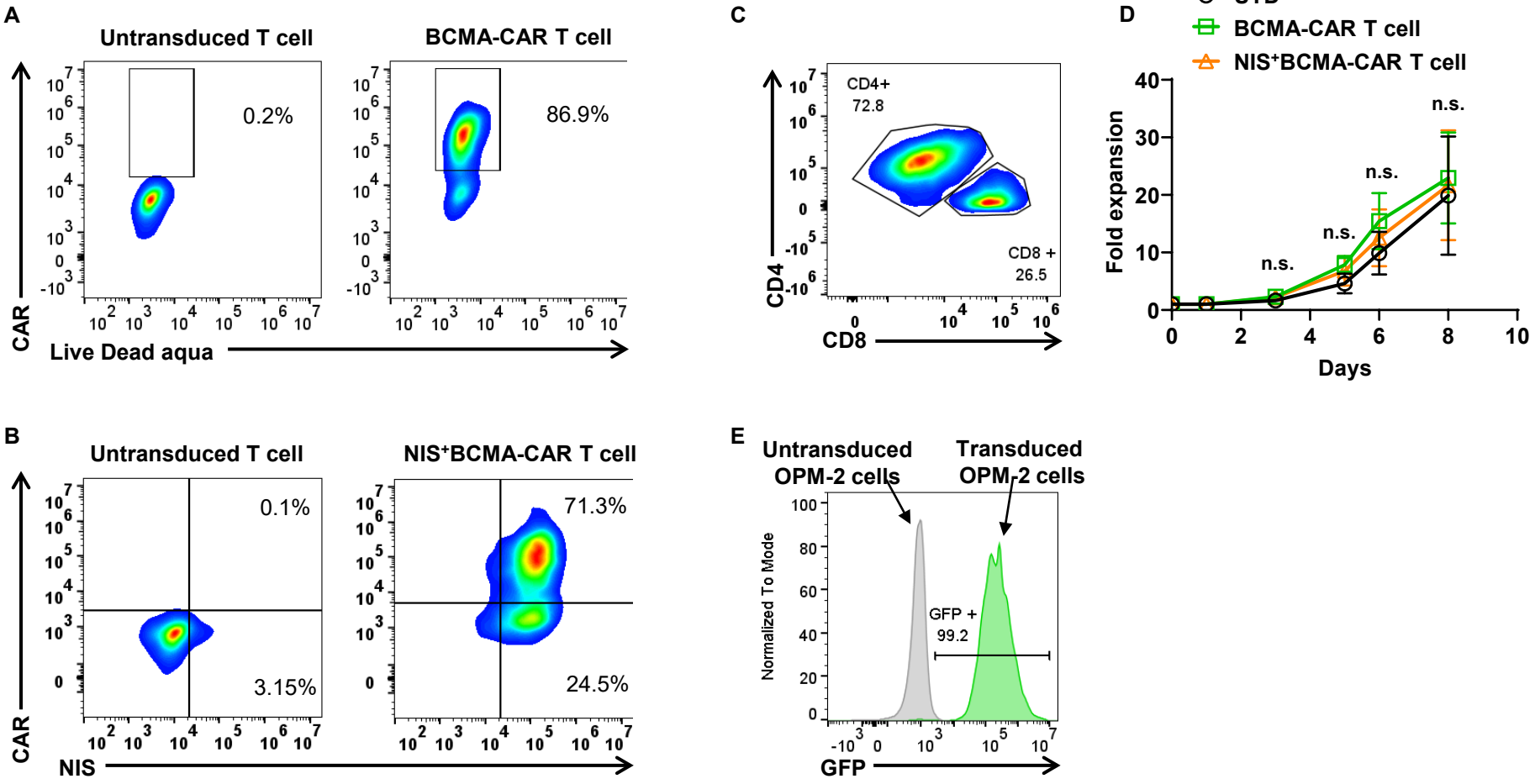

A
Untransduced T cell
BCMA-CAR T cell
0.2%
86.9%
CAR
Live Dead aqua
B
Untransduced T cell
NIS+BCMA-CAR T cell
0.1%
3.15%
71.3%
24.5%
CAR
NIS
C
CD4+
72.8
CD8 +
26.5
CD4
CD8
D
UTD
BCMA-CAR T cell
NIS+BCMA-CAR T cell
Fold expansion
n.s.
Days
E
Untransduced
OPM-2 cells
Transduced
OPM-2 cells
Normalized To Mode
GFP +
99.2
GFP

Figure 4

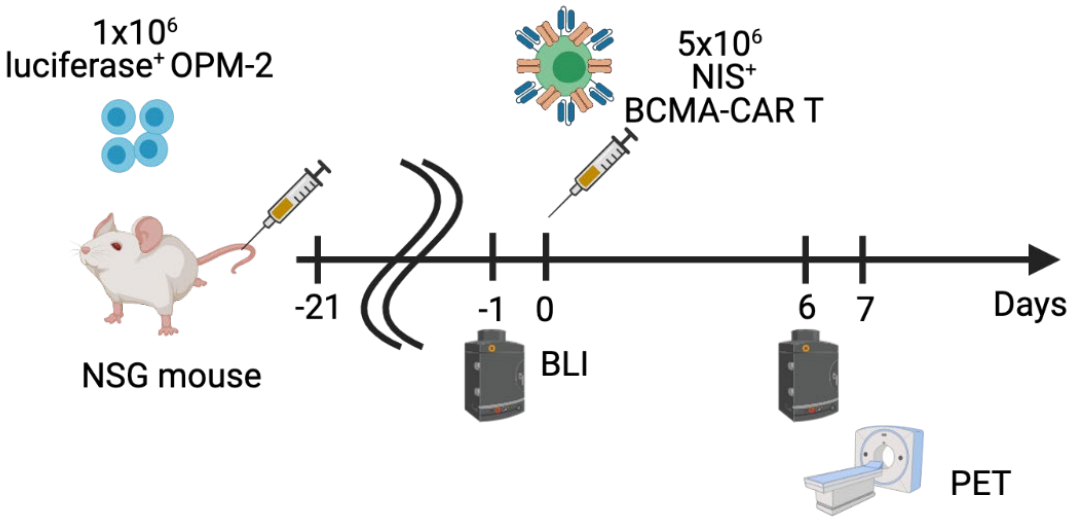
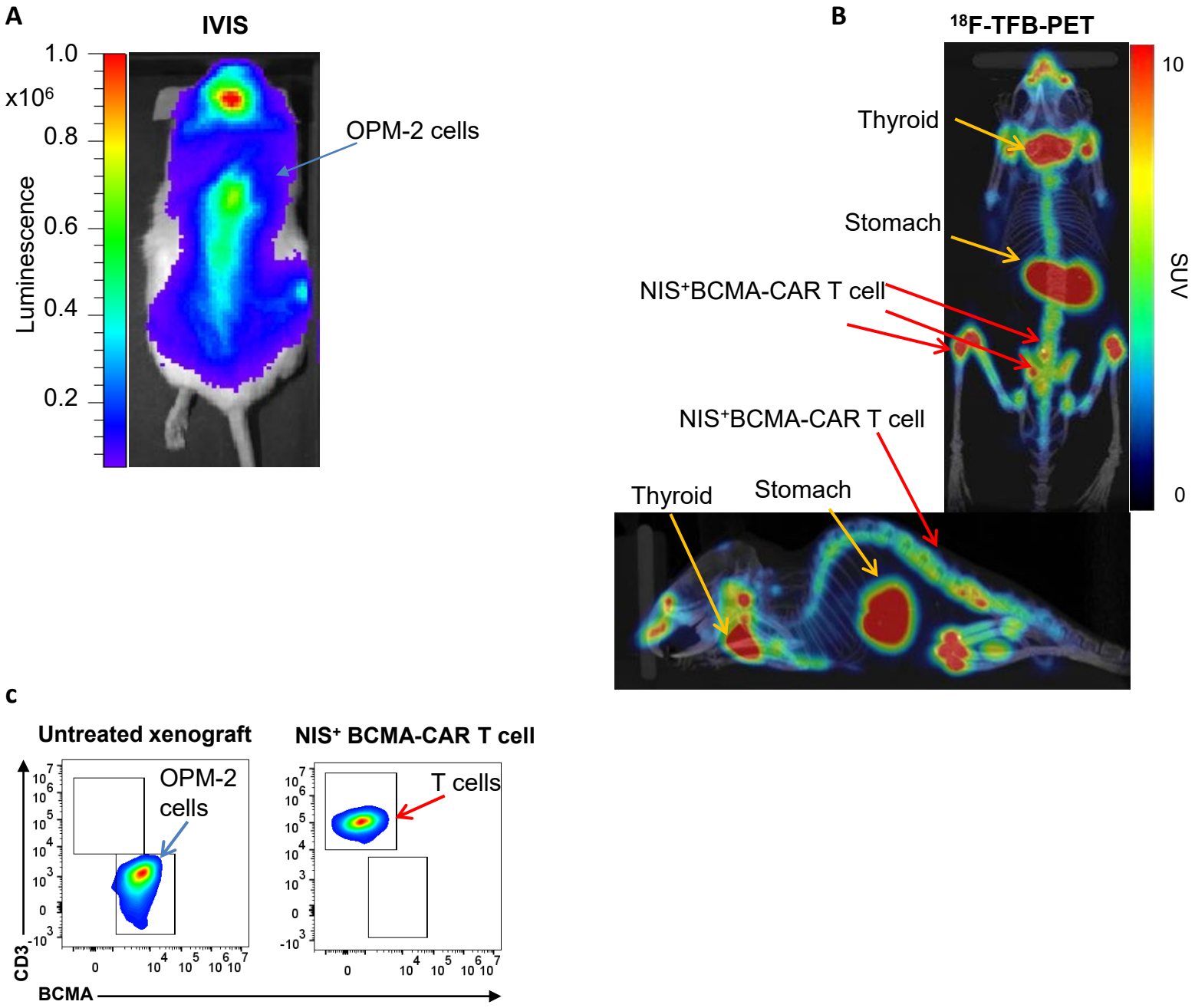

1x10⁶
luciferase⁺ OPM-2
5x10⁶
NIS⁺
BCMA-CAR T
NSG mouse
-21
-1
0
6
7
Days
BLI
PET

Figure 5


A
IVIS
1.0
x10^6
0.8
0.6
0.4
0.2
Luminescence
OPM-2 cells
B
18F-TFB-PET
Thyroid
Stomach
NIS+BCMA-CAR T cell
NIS+BCMA-CAR T cell
Thyroid
Stomach
10
SUV
0
C
Untreated xenograft
NIS+ BCMA-CAR T cell
OPM-2 cells
T cells
CD3
BCMA

Video

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