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TITLE:

Analyzing Tumor and Tissue Distribution of Target Antigen Specific Therapeutic Antibody

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KEYWORDS:

antibody, farletuzumab, clinical antibodies, mouse xenograft, infra-red dye, fluorescence imaging, tumor enrichment

SUMMARY:

Here we present a protocol to study the in vivo localization of antibodies in mice tumor xenograft models.

ABSTRACT:

Monoclonal antibodies are high affinity multifunctional drugs that work by variable independent mechanisms to eliminate cancer cells. Over the last few decades, the field of antibody-drug conjugates, bispecific antibodies, chimeric antigen receptors (CAR) and cancer immunotherapy has emerged as the most promising area of basic and therapeutic investigations. With numerous successful human trials targeting immune checkpoint receptors and CAR-T cells in leukemia and melanoma at a breakthrough pace, it is highly exciting times for oncologic therapeutics derived from variations of antibody engineering. Regrettably, a significantly large numbers of antibody and CAR based therapeutics have also proven disappointing in human trials of solid cancers because of the limited availability of immune effector cells in the tumor bed. Importantly, nonspecific distribution of therapeutic antibodies in tissues other than tumors also contribute to the lack of clinical efficacy, associated toxicity and clinical failure. As faithful translation of preclinical studies into human clinical trails are highly relied on mice tumor xenograft efficacy and safety studies, here we highlight a method to test the tumor and general

tissue distribution of therapeutic antibodies. This is achieved by labeling the protein-A purified antibody with near Infrared fluorescent dye followed by live imaging of tumor bearing mice.

INTRODUCTION:

FDA approved the first monoclonal antibody targeting CD3 (OKT3, Muromonab) in 1986[1,2]. Since then for the next twenty years, there has been a rapid explosion in the field of antibody engineering due to the overwhelming success of antibodies against immune checkpoint inhibitors[3]. Beside indirect activation of immune system, antibodies are being aimed to directly flag cancer cells to precisely engage immune effector cells, trigger cytotoxicity via death receptor agonist, block tumor cell survival signaling, obstruct angiogenesis (growth of blood vessels), constrain immune checkpoint regulators, deliver radioisotopes, chemotherapy drugs and siRNA as a conjugated agents[2]. In addition, studying the single chain variable fragments (scFv) of various antibodies on the surface of patient derived T-cells and NK cells (CAR-T and CAR-NK) is a fast growing area of clinical investigations for cell-based therapies[4].

The ultra-high affinity of antibody-based drugs that provides selectivity to antigen expressing tumor cells makes it an attractive agent. Likewise, the targeted delivery and tumor retention of a therapeutic antibody (or a chemical drug) is the key to balance efficacy over toxicity. Therefore, a large number of protein engineering based strategies that include but are not limited to bispecific[5] and tri-specific antibodies[6] are being exploited to significantly enhance avidity optimized tumor retention of intravenously (IV) injected therapeutics[5,7]. Here, we describe a simple fluorescence-based method to address the tumor and tissue distribution of potentially effective anti-cancer antibodies.

Because animal tissues possess auto-fluorescence when excited in visible spectrum, the antibodies were initially labeled with near Infrared dye (e.g., IRDye 800CW). For proofs of concept investigations, we have made use of folate receptor alpha-1 (FOLR1) targeting antibody called farletuzumab and its derivative called Bispecific anchor Cytotoxicity activator (BaCa)[7] antibody that co-targets FOLR1 and death receptor-5 (DR5)[8] in one recombinant antibody. FOLR1 is a well-defined overexpressed target receptor in ovarian and TNBC cancer cells, tumor xenografts and patient tumors[9]. Notably, there are multiple efforts to clinically exploit FOLR1 using antibody-based approaches to engage immune effector cells and antibody drug conjugates (ADC) for ovarian and breast cancers[10,11].

In this methods paper, we cloned, expressed and purified clinical anti-FOLR1 (farletuzumab) along with other control antibodies using CHO expression system. IgG1 isotype and a clinical anti-idiotype mucin-16 antibody called abagovomab[12] were used as negative controls. Following protein-A purification, indicated antibodies were labeled with IRDye 800CW and were administered into the tail vein of nude mice either bearing ovarian tumor xenografts or stably transfected human FOLR1 expressing murine colon cancer xenografts. The antibody localization was tracked by live imaging using in vivo imaging spectrum at multiple different time points[7]. This method does not require any

genetic modification or injection of the substrate to enable light emission and is significantly quicker, cost effective and efficient. The general cloning, expression, purification and labeling protocol described below can be applied to any clinical and nonclinical antibody if heavy and light chain sequences are available.

PROTOCOL:

All the procedures involving animals handling and tumor xenografts studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) here at the University of Virginia and conform to the relevant regulatory standards

1. Expression and purification of antibodies

1.1. Maintenance of CHO cells

1.1.1. Grow CHO cells in FreeStyle CHO Media supplemented with commercially available 1x glutamine supplement at 37 ˚C shaking at 130 rpm with 5% CO_2 using delong Erlenmeyer flasks either glass or disposable.

NOTE: It is highly recommended to use a baffled flask with vented cap for increased agitation and to improve gas transfer during shaking condition. Significantly reduced antibody yield has been obtained with regular flasks (non-baffled) due to limited agitation of the suspension culture.

1.1.2. Maintain the cell number between 1-5 x 10^6 cells/mL with >95% cell viability. If the cell number increases over 5 x 10^6 cells/mL, split the cells. Never allows CHO cells to reach below 0.2 x 10^6 cells/mL.

1.2. Transfection of CHO cells

1.2.1. Grow CHO cells in 200 mL of media (2 x 10^6 cells/mL) in delong Erlenmeyer baffled flasks.

NOTE: Suspension cultures grown in baffled flasks have always produced higher protein yields vs when grown in non-baffled flasks.

1.2.2. In a 15 mL tube, take 5 mL of CHO FreeStyle media and add 50 µg of VH clone DNA and 75 µg of VL clone DNA. Vortex to mix well.

1.2.3. Incubate the DNA mixture at room temperature for 5 min.

NOTE: Longer incubation reduces the protein yields.

1.2.4. Add 750 µL of 1 mg/mL polyethyleneimine (PEI) stock to the DNA solution and aggressively vortex the mixture for 30 s. Incubate at room temperature for additional 5

min.

NOTE: PEI must be made fresh. Multiple freeze thaw cycle of PEI reduces overall yield significantly.

1.2.5. Add the entire mixture of DNA and PEI on the cells while manually shaking the flask. Immediately incubate the delong Erlenmeyer baffled flasks with cells at 37 ˚C shaking at 130 rpm.

1.3. Expression

NOTE: The antibody has secretory signal peptide engineered to its N-terminal end, which helps antibodies to be secreted out into the media.

1.3.1. Grow the transfected cells at 37 ˚C, with shaking at 130 rpm on the Day 1.

1.3.2. On the Day 2, add 2 mL of 100x anti-clumping agent and 2 mL of 100x anti-bacterial-anti-mycotic solution. Shift the flask to lower temperature (32-34 ˚C), with shaking at 130 rpm.

1.3.3. On every fifth day, add 10 mL of Tryptone N1 feed, and 2 mL of 100x glutamine supplement.

1.3.4. Keep counting the cells every third day using hemocytometer after staining an aliquot of cells with trypan blue stain. Ensure that the cell viability stay above 80%.

1.3.5. On the Day 10 or 11, harvest the medium for antibody purification. Spin the culture at 3000 *x g*, 4 ˚C for 40-60 min and then filter the clear media using 0.22 µM bottle filters.

1.4. Purification

NOTE: Antibody purification is performed using commercially available Protein-A column (see **Table of Materials**), using a peristaltic pump.

1.4.1. Equilibrate the column with two column volume of binding buffer (20 mM sodium phosphate at pH 7.4).

1.4.2. Pass the filtered media containing antibody (obtained in step 1.3.4) through the column at the flow rate of 1 mL/min.

1.4.3. Wash the column with two-column volume of binding buffer.

1.4.4. Elute the antibody into 500 µL fractions using 5 mL elution buffer (30 mM sodium acetate at pH 3.4).

1.4.5. Neutralize the pH of the eluted antibody by adding 10 µL of neutralization buffer (3 M sodium acetate at pH 9) per fraction.

NOTE: Fraction number 3-6 contains most of the antibody. It is advisable to keep all the fractions in case the antibody is eluted in a later fraction than expected.

1.4.6. Measure the concentration of purified antibody using a spectrophotometer by selecting the default protocol for IgG. The final concentration of the antibody is obtained in mg/mL by considering the molecular weight and absorption coefficient.

2. Fluorescent labeling

NOTE: Antibodies are labeled with the infrared dye that contains an NHS ester reactive group, which couples to proteins and form a stable conjugate. This reaction is pH sensitive and works best at pH 8.5. Fluorescent conjugates labeled with the dye display an absorption maximum of 774 nm, and an emission maximum of 789 nm. pH 8.5 is key for effective conjugation.

2.1. Dialyze 0.5 mL of the antibody using a dialysis cassette (0.1-0.5 mL) in 1 L of conjugation buffer (50 mM phosphate buffer at pH 8.5). After 4 h transfer the dialysis cassette to fresh buffer and dialyze overnight.

2.2. Setup a conjugation reaction by adding 0.03 mg of IRDye 800CW per 1 mg of antibody in a reaction volume of 500 µL.

NOTE: IRDye 800CW is dissolved in DMSO at a concentration of 10 mg/mL.

2.3. Carry out labeling reactions for 2 h at 20 °C.

NOTE: Increasing time beyond 2 h does not improve the labeling.

2.4. Purify labeled conjugates by extensive dialysis against 1x PBS.

2.5. Estimate the degree of labeling by measuring the absorbance of the dye at 780 nm and absorbance of the protein at 280 nm. The dye contribution to the 280 nm signal is 3%.

2.6. Calculate the dye/protein ratio using this formula:

$$D/P = \{A780/\varepsilon Dye\} / \{A280 - (0.03XA780)/\varepsilon Protein\}$$

where 0.03 is a correction factor for the absorbance of the dye used at 280 nm (equal to 3.0 % of its absorbance at 780 nm), ε_{Dye} and $\varepsilon_{Protein}$ are molar extinction coefficients for

the dye and the protein (antibody) respectively.

NOTE: ε_{Dye} is 270,000 M^{-1} cm^{-1} and $\varepsilon_{Protein}$ is 203,000 M^{-1} cm^{-1} (for a typical IgG) in 1:1 mixture of PBS: methanol. Proteins other than IgG may have very different molar extinction coefficients. Use of correct extinction coefficient for the protein of interest is essential for accurate determination of D/P ratio.

2.7. Calculate the final protein concentration using this formula:

$$Protein\ Conc.\ (mg/mL) = \{A280 - (0.03XA780)/\varepsilon Protein\}\ x\ MWprotein\ x\ Dilution\ factor$$

NOTE: Always confirm the antigen-binding efficacy of labeled and unlabeled antibody using ELISA (Enzyme Linked Immunosorbent Assay) or FACs before proceeding to in vivo studies.

3. Mouse xenograft studies

3.1. Preparation of tumor cells for injection

3.1.1. Grow Ovcar-3 cells in RPMI-1640 Medium, supplemented with 10% of FBS and 1x penicillin-streptomycin.

3.1.2. The day before the injection, subculture cells into new 100 mm culture dishes with 10 mL of complete medium/dish. Use cell number of 0.5-1 x 10^6 cells/dish.

3.1.3. Incubate cultures at 37 °C temperature, 95% humidity and 5% CO_2 for 20-24 h.

3.1.4. On the day of injection, remove the growth medium from culture dishes. Thoroughly rinse the cell layer with Ca^{2+}/Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS) to remove dead cells, cellular debris and all traces of serum, which may interfere in trypsin action.

3.1.5. Trypsinize the cells by adding 1.0-1.5 mL of Trypsin-EDTA solution to each dish and try to spread the solution by tilting the dish all around followed by incubation at 37 °C for 5-10 min.

NOTE: Observe cells under an inverted microscope to check the actual trypsinization status. Under trypsinization results in lower number of cell detachment from the surface of culture dish, over trypsinization induces cellular stress. So, proper trypsinization is important.

3.1.6. Add 1.0-1.5 mL of complete growth medium to each dish to stop the trypsin action, after that re-suspend cells by pipetting gently.

NOTE: Gentle pipetting is important to maintain cell health.

3.1.7. Collect the cell suspension into a 15 mL conical tube and spin at 250 *x g* for 5 min at room temperature.

3.1.8. Collect the cell pellet after removing the supernatant and wash the cells by re-suspending the pellet in 1x DPBS.

3.1.9. Spin the cell suspension at low speed 250 *x g* for 5 min.

3.1.10. Add 500 µL of DPBS and re-suspend cells by gentle pipetting to get single cell suspension.

3.1.11. Count cells using hemocytometer or automated cell counter.

NOTE: For better accuracy, repeat the counting for three times and take an average.

3.1.12. Adjust the volume in such a way so that the final cell density will be 1 x 10^8 cells/mL.

3.2. Subcutaneous injection of cells to develop mouse xenografts

NOTE: All procedures should be done in a BSL2 safety cabinet. Athymic Nude *Foxn1*^{nu}*/Foxn1*$^{+}$ mice have been used in the current study.

3.2.1. Take 50 µL of the cell suspension into a 1.5 mL tube and mix with 50 µL of basement membrane matrix medium.

NOTE: Basement membrane matrix medium tends to form a gel like state at room temperature, so carefully maintain the cells and the matrix medium mixture on ice. It is advisable to keep tubes, tips and syringes in the fridge and then transfer on ice prior to the animal injection.

3.2.2. Agitate the mixture to avoid any cell clumping. Then, take this 100 µL of the cell suspension-matrix medium mixture that contains 5 x 10^6 cells, into a 1 cc syringe.

3.2.3. Gently lift the skin of the animal to separate the skin from the underlying muscle layer and slowly inject the cell suspension (100 µL) under the skin (5 x 10^6 cells), with a 26 G needle. Wait for a few seconds before taking the needle out, so that basement matrix medium can form the semi-solid gel like structure along with cells under the skin, preventing the mixture coming out from the site of injection.

NOTE: Cells needs to be tested prior to injection for any contamination which may harm

to immunodeficient mice. While injecting do not put the needle too deep into the skin as this may form the tumor deeper than expected.

3.2.4. Keep the animal in a sterile cage and observe for around 20 min.

3.2.5. Observe the mice for 2-3 weeks and allow the tumor to grow up to 500 mm^3 size.

4. Antibody localization using an in vivo imaging system

NOTE: In vivo imaging equipment (see **Table of Materials**) used in this experiment uses a set of high efficiency filters and spectral un-mixing algorithms for noninvasive visualization and tracking of cellular and genetic activity within a living organism in real time. System provides both fluorescence and bioluminescence monitoring capability.

4.1. Inject 25 µg of dye labeled antibody via tail vein.

4.1.1. Anesthetize tumor bearing mice using 2% isoflurane. Check for the lack of response to pedal reflexes.

4.1.2. Once mice stop moving, dilate the lateral tail vein by applying worm water.

4.1.3. Inject 25 µg (in 100 µL) of labeled antibody using 1 cc insulin syringe with a 26 G needle.

4.1.4. Similarly, as a negative control, label and inject the non-specific IgG1 Isotype antibody which does not target cancer cells.

4.2. Perform in vivo live imaging after 8, 24, 48 h, etc. of antibody injections.

4.2.1. In the associated software, click **Initialize** located in the control panel and confirm that the stage temperature is 37 °C.

4.2.2. Turn on the oxygen supply, all the pumps on the anesthesia system, isoflurane gas supply to anesthetic chamber and set the isoflurane vaporizer valve to 2%.

4.2.3. Transfer the mice to anesthetic chamber and wait till mice are completely anesthetized. Apply the eye lubricating ointment to avoid drying of their eyes.

4.2.4. Go to the **Control panel**, set up the fluorescence imaging through the **Imaging Wizard** option and select the excitation at 773 nm and emission at 792 nm.

NOTE: The default auto exposure settings provide a good fluorescent image. However, the auto exposure preferences can be modified as per the needs.

4.2.5. Transfer the anesthetized mice into the imaging chamber and assemble it on the imaging field using nose cone. The imaging stage provides the option to accommodate 5 mice at a time.

NOTE: Have control mice imaged along with the test mice to have a similar amount of exposure and other settings while analysis.

4.2.6. Once everything is ready, select **Acquire** option on the control panel for the image acquisition.

4.2.7. Click **Auto-expose** and the system generate the image within a minute. The generated image is the overlay of fluorescence on photographic image with optical fluorescence intensity displayed in units of counts or photons, or in terms of efficiency.

4.2.8. After acquiring the image, transfer the mice from the imaging chamber back to their cage and observe for their recovery for 1 to 2 min.

4.3. Fine tune the image further with the tools and functions provided within the software for image analysis. Image analysis tools are in the menu bar and tool palette.

4.3.1. Under **Image Adjust**, adjust the brightness, contrast, or opacity and select the color scale.

4.3.2. Use the **ROI Tools** to specify a region of interest (ROI) in an optical image and measure the signal intensity within the ROI. If needed, export the quantified signal data to a spreadsheet software and plot the data.

4.3.3. For follow-up studies, analyze mice necropsies of various tissues (e.g., liver, lung, heart, kidney, spleen, brain etc.) side-by-side for a detailed non-specific distribution of fluorescently labeled antibody.

NOTE: Data can be further supported with tissue specific ELISA by making use of antigen (FOLR1 in this case) in 96 well assays.

REPRESENTATIVE RESULTS:

In the described methodology, first we cloned antibodies targeting folate receptor alpha-1 (FOLR1) named farletuzumab, and a bispecific antibody called BaCa consisting of farletuzumab and lexatumumab along with control antibodies such as abagovomab (sequences provided in **Supplementary File 1**). Details of representative variable heavy (VH) and variable light (VL) domains in DNA clones (pVH, pVL) are shown in **Figure 1A**. To confirm the positive clones, we carried out colony PCR using signal peptide forward (SP For) and CK Rev/CH3 Rev primers (sequences provided in **Supplementary File 1**). Representative results of colony PCR confirm the expected sizes of light and heavy chains (**Figure 1C**). Positive antibody clones were, also, confirmed using Sanger sequencing.

Following confirmed pVH and pVL DNA cloning, transfections were carried out using CHO suspension cultures, followed by protein-A column affinity purifications of antibodies at 4 °C (see **Figure 1B** and protocol for detail steps).

Representative results of purified farletuzumab along with other control IgG1 (except BaCa antibody run on non-reducing and reducing SDS PAGE are shown in **Figure 2A**. As evident, heavy and light chain produced 50 and 25 KDa bands after reduction. This was followed by binding confirmation of antibodies to native proteins on the cell surface. Representative farletuzumab binding to human FOLR1 on OVCAR3 cells surface is shown using flow cytometry (**Figure 2B**).

Next fluorescently labeled antibodies were tail vein injected into animals grafted with FOLR1 expressing tumors (**Figure 3**). Animals were live imaged at multiple time points using IVIS. The data confirms selective enrichment of FOLR1 and BaCa antibodies into the $FOLR1^{+}$ tumors (**Figure 4** and **Figure 5**). Importantly control antibodies (negative for tumor antigen) did not localize into tumors.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of antibody cloning, expression and purification. (**A**) Shows the detail schematic of heavy (pVH) and light (pVL) chain vectors. (**B**) pVH vector carrying variable domain sequence of an IgG1 heavy chain and pVL vector carrying variable domain sequence of a light chain were mixed together (1:2 ratio) along with transfection reagents (such as mirus or PEI) before adding to the suspension culture of CHO or HEK cells. Cells were fed with supplemental feed next day and cultures were monitored for 10 additional days with intermittent feeding. At day 11, cells were harvested through 0.2 µm PES filters followed by affinity chromatography using protein-A. Purified antibodies were next analyzed for % monomer (FPLC), binding activity (ELISA or SPR), and binding to the target antigen (FACS) on live cells. Antibodies can also be checked for in vivo assays (e.g., cell growth inhibition, cell viability assays, or inhibition of signaling intermediate phosphorylation etc). Antibodies can also be conjugated with far-red dyes e.g., IRDye 800CW for in vivo imaging. ± IRDye 800CW must be tested for activities prior to tumor and tissue distribution studies. (**C**) Agarose gels of colony PCR confirming the sizes of positive heavy (1.4 Kb) and light chain (0.8 Kb) clones.

Figure 2: A gel-based reduction assay to confirm antibody integrity. (**A**) Four different IgG1 antibodies (schematic shown on top) were added ± reducing agent (such as BME or DTT) at 95°C for 10 min. Antibodies were next loaded on a 10% SDS-PAGE gel followed by protein staining and imaging. Gel image in left and right clearly show the intact antibody and two separate polypeptides (~50 KDa and ~25 KDa) respectively. Please also see pVH and pVL vector maps (Figure 1) carrying cDNA corresponding to VH/VL, CH1/CK, CH2 and CH3 domains. (**B**) Flow cytometry confirmation of unlabeled and IRDye 800CW labeled farletuzumab binding to native FOLR1 on ovarian cancer cells.
Non-reducing = Antibody run on gel with non-reducing dye, Reducing = Antibody run on

gel with reducing dye, HC = Heavy chain, LC = Light chain, VL = Variable domain of light chain, VH = Variable domain of heavy chain, CK = Kappa chain

Figure 3: Experimental schematic of tumor generation and antibody treatments. 6-8 weeks old mice strains such as: Immunodeficient athymic nude/NSG/ Immunocompetent C57BL/6 or Balb/C mice could be easily grafted with tumor cells via subcutaneous (SQ) tumors. Similar studies could be carried out using breast fat-pad and intraperitoneal (IP) tumors. 3-4 weeks later (tumor ~200 mm^3), mice were IV injected with an IRDye labeled indicated antibodies that were ± selective against tumor-overexpressed receptor. This was followed by live in vivo imaging.

Figure 4: Live in-vivo imaging of human OVCAR3 tumor bearing mice. Randomly selected 6 to 8 weeks old (Age) and 20-25 gram (Weight) hairless athymic Nude $Foxn1^{nu}/Foxn1^{+}$ (*Envigo*) were grafted with $FOLR1^{+}$ ovarian tumors (OVCAR-3 cells). After 3 weeks with evident tumors, mice were tail vein injected with IRDye 800CW labeled IgG1 control, abagovomab (CA-125 anti-idiotypic antibody), farletuzumab (anti-FOLR1 antibody) and BaCa (anti-FOLR1-DR5 antibody) followed by live imaging at indicated times.

Figure 5: Live in-vivo imaging of human FOLR1 expressing murine MC38 cell derived tumor bearing mice. (**A**) Randomly selected 6 to 8 weeks old (Age) and 20-25 g NOD.Cg $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ or nude mice were SQ injected with murine MC38 cells stably expressing human FOLR1. Upon tumor appearance, mice were tail vein injected with IRDye 800CW labeled IgG1 control, abagovomab (CA-125 anti-idiotypic antibody), farletuzumab (anti-FOLR1 antibody) and BaCa (anti-FOLR1-DR5 antibody) followed by live imaging at indicated times. (**B**) After 7 days animals were euthanized and isolated key organs (as indicated) were imaged together along with grafted tumors for relative antibody signal (IRDye 800CW) distribution. As expected, tumors remained negative with IRDye 800CW signal in IgG1 control and CA-125 anti-idiotypic antibody, abagovomab injected animals.

Supplementary File 1: Sequences of all antibodies and primers.

DISCUSSION:

Selective and tumor tissue specific delivery of anti-cancer therapeutic agent is the key to measure efficacy and safety of a given targeted therapy[13]. Here we have described a quick and efficient approach to investigate the detailed tissue and tumor distribution of clinical, farletuzumab and a nonclinical BaCa antibody. The described approach is applicable to any newly generated antibody and can be used alongside of a clinically effective antibody (with desired qualities) for its tumor/organ distribution properties. Considering most antibody target receptors (such as HER2 in breast cancer) are highly overexpressed in tumor cells (tissues), in most cases their suboptimal expression and function is also critical in cell types other than tumor cells[14]. For example, a significant proportion of EGFR targeting clinical antibodies in colon cancer patients accumulate and cause toxicity to skin tissue[15], a noncancerous tissue whose growth and differentiation requires EGFR signaling

and function. Therefore, we strongly believe these sorts of preliminary tissue distribution investigations in combinations with hepatotoxicity and tissue histochemistry assays in a larger cohort of animals are key to comprehensively assess safety and therapeutic viability of the newly generated antibody. Moreover, described tissue distribution studies would also be highly applicable in immune competent mice xenograft studies, if the newly generated antibody maintains cross-reactivity to murine counterpart antigen/receptor. In syngeneic animal studies, along with tumor distribution and tissue histochemistry studies, detailed blood cytokine analysis can used to strengthen the efficacy and safety data. An attractive feature of the described approach is that it allows near accurate quantitation of antibody distribution if data is additionally supported with ELISA (against target antigen) from the tumor and other significant tissue lysates (such as liver, heart, lung, spleen, kidney etc)[7]. Another important feature of described method over single photon emission computed tomography (called SPECT) and position emission tomography (PET) is the cost-effectiveness[16]. Both SPECT and PET are very expensive and makes use of radioactive tracers for imaging, making the whole process cumbersome if testing a large cohort of animals[17]. In addition SPECT and PET imaging facilities are not very standard in laboratories and vivariums to study small animal models of diseases such as mice[18].

One limitation with described method to achieve a near accurate quantitation of antibody tumor distribution is the dependence on high affinity target antigen binding. It is because high affinity antigen-antibody interactions may result in "target-mediated drug disposition (TMDD)" by enhanced endocytosis and shuttling to lysosomes[19]. Therefore, the results of the described approach will vary depending on the particular target receptor in a particular tumor type. Thus, we strongly recommend testing labeled antibody/antibodies in a large cohort of animals with tumor xenografts generated with more than one tumor cell line(s) having variable (heterogeneous) expression of target antigen receptor. It is also greatly recommended to make use of more than one fluorescent conjugate dye(s) and mice strain(s) for the proposed studies.

Considering that small size antibodies such as Fabs, scFvs, BiTes, DARTs, etc. (lacking salvage recycling by neonatal Fc receptor (FcRn) clear more rapidly from tumors (with serum half times being minutes to hours), care should be taken to compare data between different tumor types having highly variable FcRn expression. Furthermore, larger molecules (such as dual and trispecificity antibodies) that are engineered with Fc domain for salvage recycling have tissue/tumor penetration issues. In those scenarios, the described approach will not be suitable to compare tissue/tumor distribution of antibodies that differ significantly in sizes[6]. In terms of significance however, the described tumor and detailed tissue distribution studies along with their counterpart monospecific antibodies would serve as a key factor in an effective dual and trispecificity antibody platform design. Finally, since higher affinity and avidity-optimized antibodies generally have a significantly homogenous distribution in tumors, the target tumor epitope selection (lacking TMDD), overall antibody affinity and biological activity in a particular cancer model should always be considered before making conclusion of tumor

penetration, safety and efficacy.

In summary, we have described a quick and simple method for monitoring the tumor and tissue distribution of intravenously injected antibodies. The described approach has added potential to analyze antibody-siRNA conjugates (where siRNA is labeled), antibody-drug conjugates (where drug is labeled) and antibody-nanoparticles (where nanoparticle lipids are labeled with fluorescent dye). Likewise, a uniquely engineered cysteine residue in a tumor targeting scFv (if fluorescently labeled with melamide chemistry) of chimeric antigen receptor T-cells (CAR-T) and CAR-NK will be a cost effective approach to analyze tumor/tissue distribution of these cell based therapies independent of viral transfection based GFP/RFP signals strategies.

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DISCLOSURES:

Authors have no competing financial interests.

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Figure 1

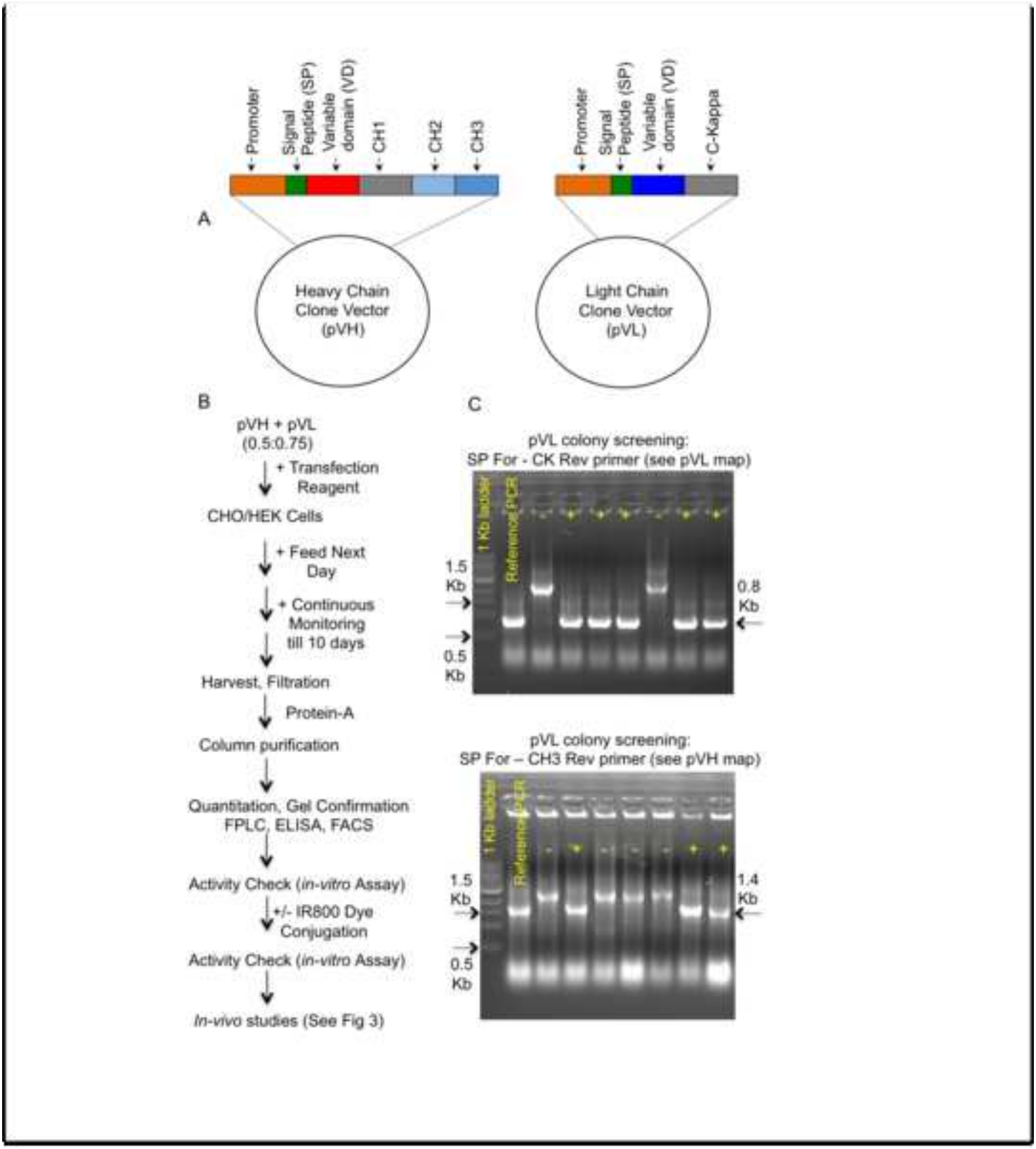

Figure 2

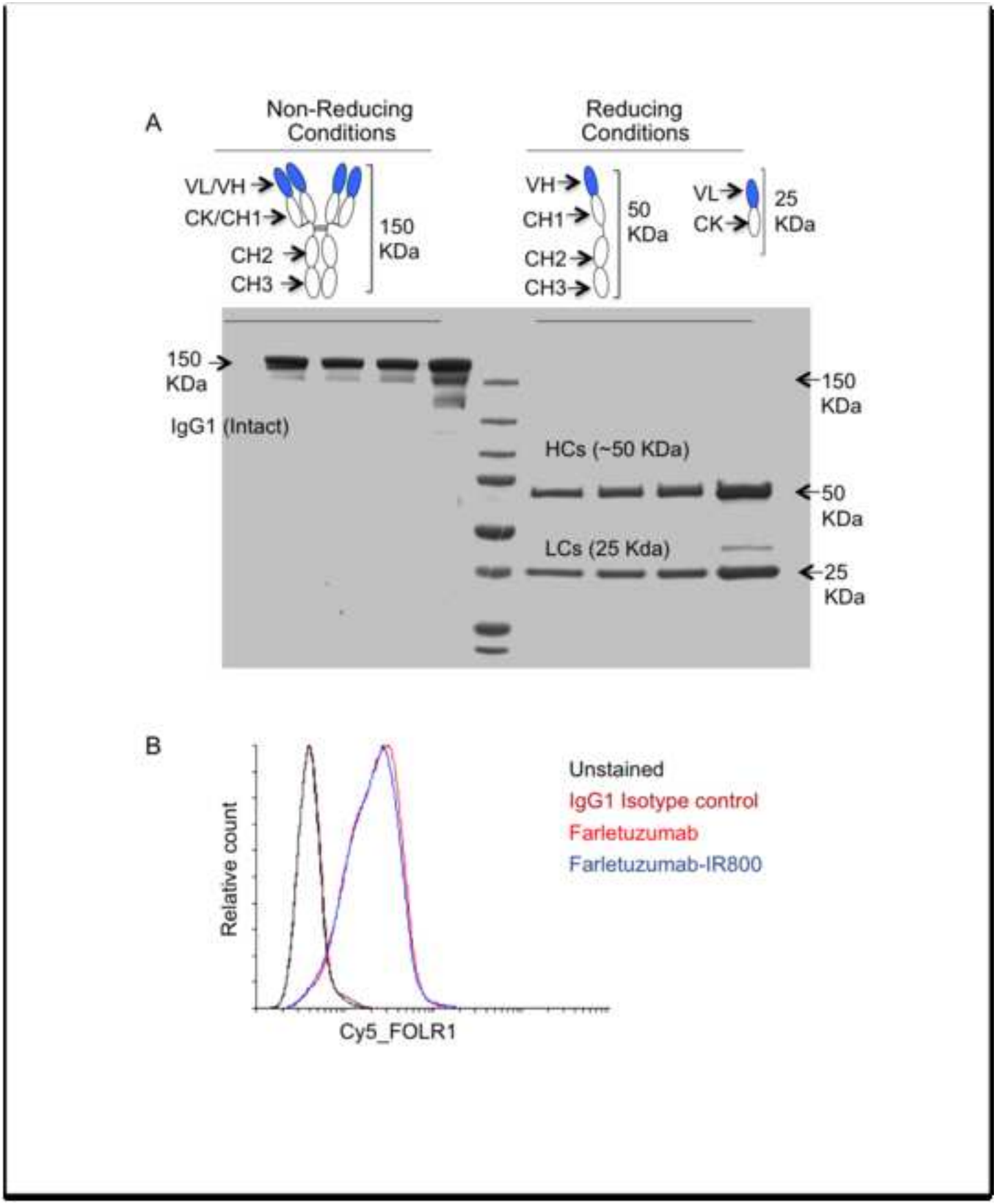

Figure 3

Click here to access/download;Figure;Fig 3.png

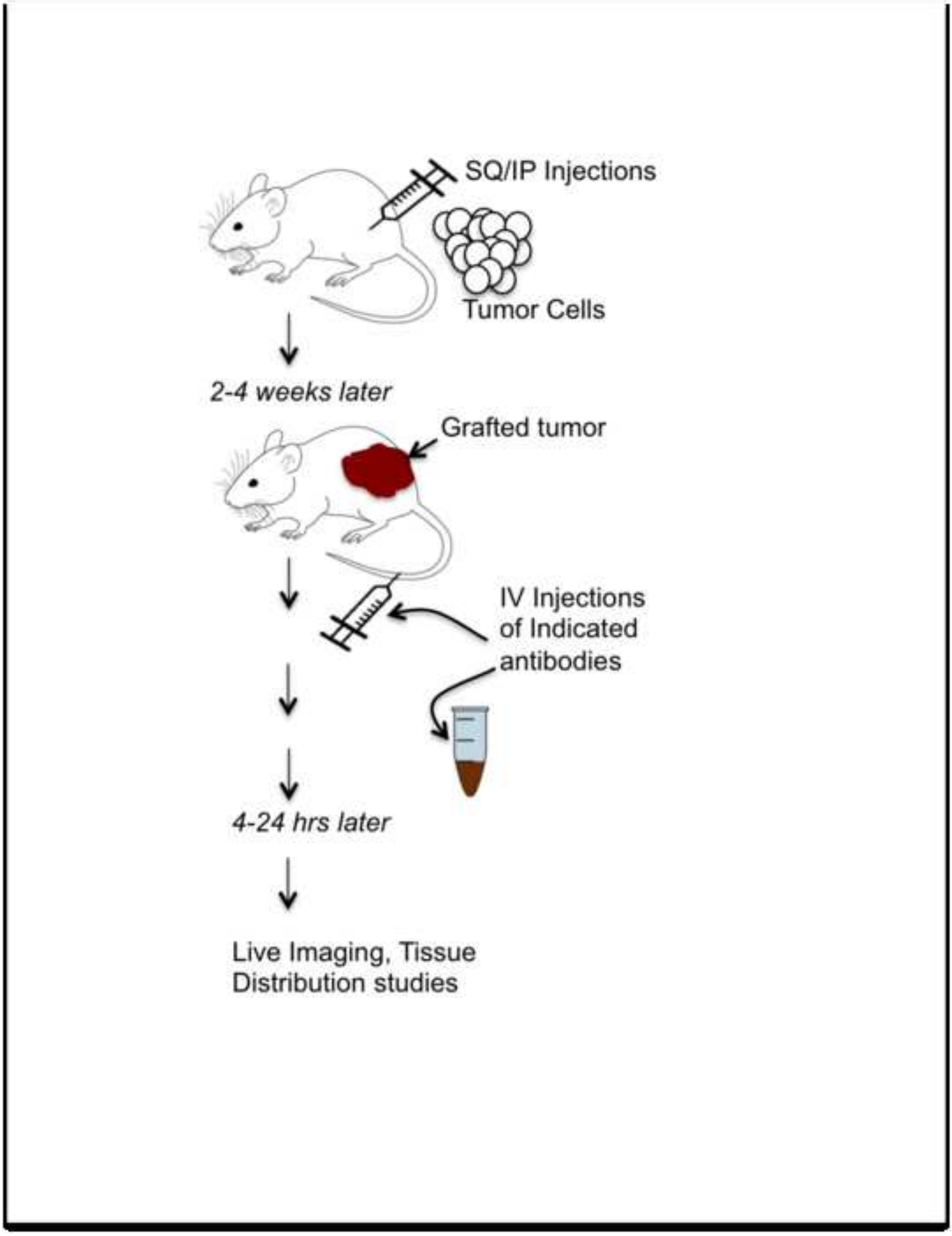

Figure 4

Click here to access/download;Figure;Fig 4.png

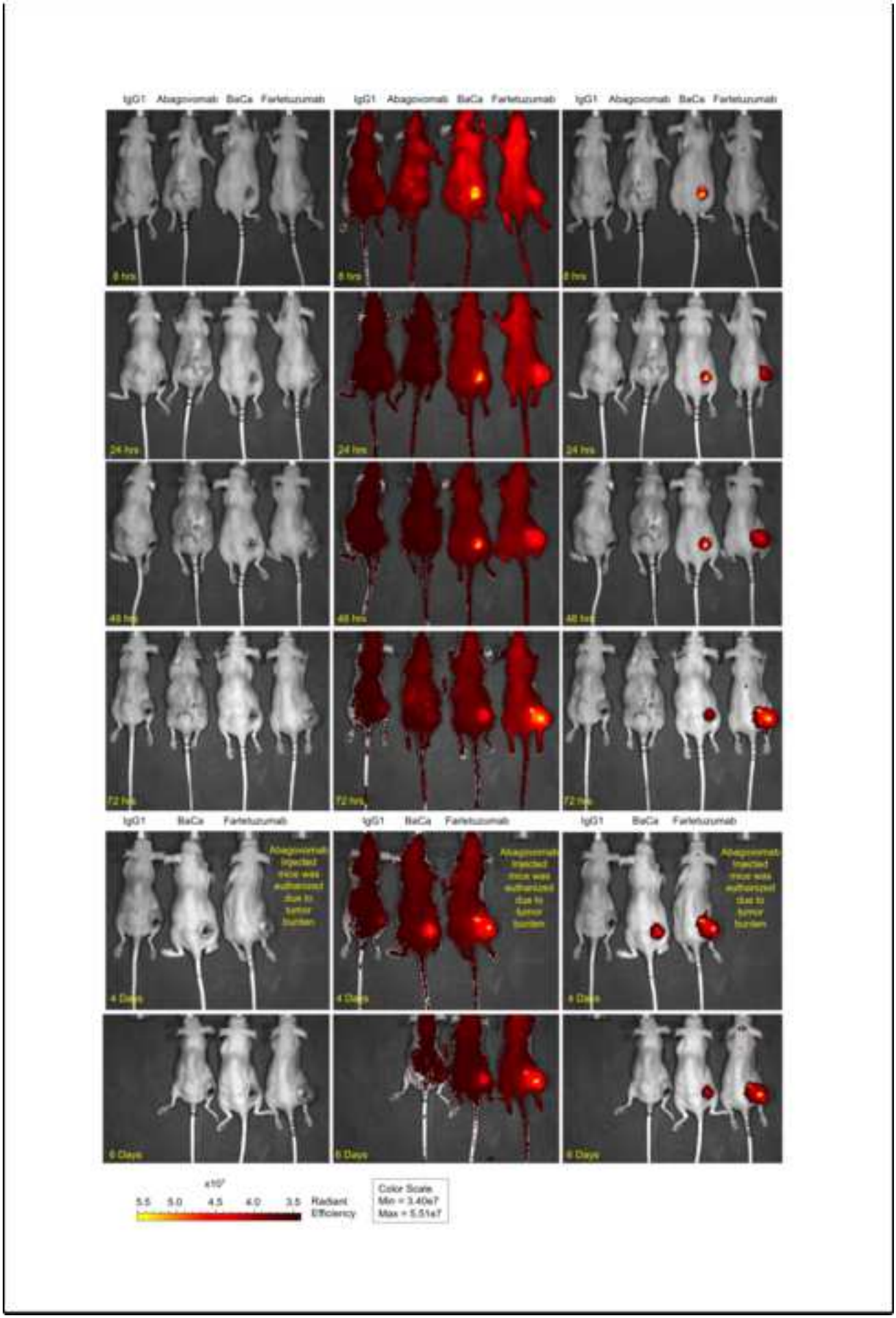

Figure 5

Click here to access/download;Figure;Fig 5 (1).png

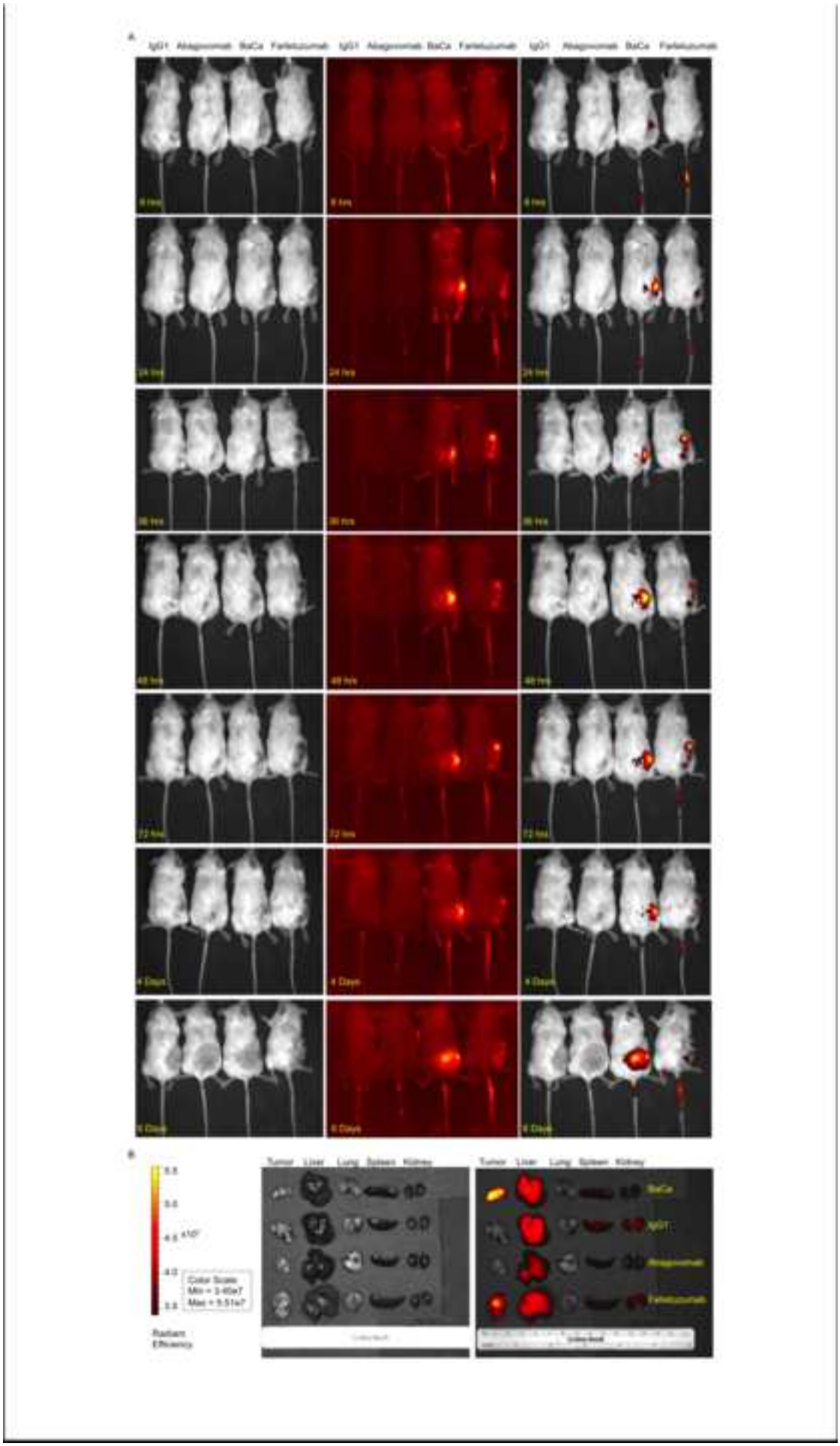

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
FreeStyle CHO media	Gibco Life Technologies	Cat # 12651-014	
Anti-Anti (100X)	Gibco Life Technologies	Cat # 15240-062	
Anti-Clumping Agent	Gibco Life Technologies	Cat # 01-0057DG	
BD Insulin Syringe	BD BioSciences	Cat #329420	
Caliper IVIS Spectrum	PerkinElmer	Cat #124262	
CHO CD EfficientFeed B	Gibco Life Technologies	Cat #A10240-01	
Corning 500 mL DMEM (Dulbecco's Modified Eagle's Medium)	Corning	Cat # 10-13-CV	
Corning 500 mL RPMI 1640	Corning	Cat # 10-040-CV	
Cy5 conjugated Anti-Human IgG (H+L)	Jackson ImmunoResearch	Cat # 709-175-149	
GlutaMax-I (100X)	Gibco Life Technologies	Cat # 35050-061	
HiPure Plasmid Maxiprep kit	Invitrogen	Cat # K21007	
HiTrap MabSelect SuRe Column	GE Healthcare	Cat # 11-0034-93	
Infusion	Takara BioScience	STO344	
IRDye 800CW NHS Ester	LI-COR	Cat # 929-70020	
Isoflurane, USP	Covetrus	Cat # 11695-6777-2	
Lubricant Eye Ointment	Refresh Lacri-Lube	Cat #4089	
Matrigel	Corning	Cat # 354234	
PEI transfection reagent	Thermo Fisher	Cat # BMS1003A	

Slide-A-Lyzer Dialysis Cassettes	Thermo Scientific	Cat # 66333
Steritop Vacuum Filters	Millipore Express	Cat #S2GPT02RE
Trypsin-EDTA	Gibco Life Technologies	Cat # 15400-054

Experimental Models: Cell lines

Human: OVCAR-3	American Type Culture Collection	ATCC HTB-161
Human: CHO-K cells	Stable transformed in our lab	ATCC CCL-61
Mouse: 4T1	Kind gift from Dr. Chip Landen, UVA	
Mouse: MC38	Kind gift from Dr. Suzanne Ostrand-Rosenberg, UMBC	Authenticated by STR profiling
Mouse: MC38 hFOLR1	Generated in our laboratory (This paper)	

Experimental Models: Animal

Mice: athymic Nude $Foxn1^{nu}/Foxn1^{+}$	Envigo	Multiple Orders
Mice: NOD.Cg $Prkdc^{scid}$ $Il2rg^{tm1Wjl}/SzJ$	Jackson Laboratory	Multiple Orders

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April 2, 2020

Dear Phillip Steindel,

Hope all is well and safe at your end.

Thank you so much for your patience during this process.

Please find attached resubmission of the invited method manuscript entitled "A quick method to analyze tumor and tissue distribution of target antigen specific therapeutic antibody" to JOVE. Additional data sets have been included. Discussion and protocol has been updated as requested by reviewers.

Here is our response to specific comments (*Italics and blue font color*) raised for this resubmission:

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

We have read the manuscript few times to check spelling/grammar errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: FreeStyle, Glutamax, Falcon, HiTrap, Matrigel

Thanks for pointing out. symbols (™), registered symbols (®) have been removed. For reagents, a key resource table has been added to the manuscript.

Summary:
1. Please include a separate Summary section (before the abstract) that clearly describes the protocol and its applications in complete sentences between 10¬ and 50 words, e.g., "Here, we present a protocol to …

Protocol:

Summary Section has been included.

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1. Please convert all centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Thanks for pointing out. Details are updated.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers 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.

In the manuscript protocol section, the 2.75 page limit filmable area is blue underlined.

3. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thanks for pointing out. Details are updated.

Specific Protocol steps:
1. 2.1, 2.4: How long to dialyze? What volumes and cassettes/tubing are used?

Following has been added to protocol.

Dialyze 0.5ml antibody using dialysis cassette (0.1-0.5ml Thermo Scientific) in 1L of conjugation buffer (50mM Phosphate buffer pH 8.5). After 4 hours transfer to fresh buffer and dialyze overnight.

2. 3.1.5: The Trypsin-EDTA solution is not in the Table of Materials.

Trypsin EDTA solution is added to the Table of Material called Key Resource table

3. 4.1: Tail vein injection, specifically, is mentioned in the Results; please provide more details here.

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Tail vein injections has been elaborated in protocol section 4.1

4. 4: More details about animal handling are needed in this section: What concentration of isoflurane is used and how is anesthetization confirmed? What happens to the mice after imaging? If applicable, please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

More details about animal handing and IVIS imaging has been added in protocol section 4.

Acknowledgment and Disclosures:
1. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.
2. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Acknowledgment and Disclosures have been updated.

References:
1. Please do not abbreviate journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
Key Resource table has been added.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The protocol investigated folate receptor alpha-1 (FOLR1) targeting antibody for tumor xenografts of ovarian imaging.

Major Concerns:
However, the title, Non-invasive In vivo method for monitoring the tumor and tissue distribution of therapeutic antibodies, is not proper to reflect the content of this manuscript.

The manuscript title had been updated as follows:

"A quick method to analyze tumor and tissue distribution of target antigen specific therapeutic antibody"

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In vitro experiment (imaging), such as the antibody bind to cell should be investigated;

We thank the reviewer for pointing out the data lacking in-vitro antibody binding to tumor cells. In response, we have added flow cytometry data (Fig 2) of surface binding of antibodies before and after the IR800 labeling to assure the conjugation of dye does not alter the antigen recognition ability of antibody. Additional data is there in Fig. 4 and Fig. 5.

4.2 imaging after 24 hours, much more time point should be tested, e.g. 12, 36,48h

We thank reviewer for the comment. In response, additional time points (such as 8 hrs, 24 hrs, 42, hrs, 72 hrs, 120 hrs etc.) of animal imaging have been added. In addition, we have added NOD.Cg Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG, Jackson Labs) strain of mice along with athymic Nude Foxn1^{nu}/Foxn1^{+} (envigo) strain to re-confirm our findings (Fig. 4, Fig. 5)

Minor Concerns:
Abstract: Infra-ed is not correct;

Thank you for notifying the typing mistake. The correction has been incorporated into revised manuscript.

1.1 >95% survival density, should be cell viability;

Thank you for suggesting the correct alternative world. The correction has been incorporated into revised manuscript.

3.2.3 (5 X 10^6 cells) were injected, too much cells used for tumor formation, special with matrigel

We agree with the review. It was a type and has been updated with 0.5-1.0 X 10^6 cells the tumor will generate rapidly and aggressively, however in the present study the aim is to just generate the tumor and study for antibody localization.

4.1 antibody intravenously, tail vein?

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The protocol has been updated. Antibodies were injected via tail vein. The details are now elaborated in protocol section 4.1

Reviewer #2:

Manuscript Summary:

This manuscript describes a methodology for generating full-length antibodies with the description of their subsequent labelling with infra-red fluorophores. This allows the Authors to perform a biodistribution analysis of antibody binding in an ovarian cancer mouse xenograft models assessed via IVIS in vivo imaging. The IVIS methodology is applicable to the wider JoVE audience and could provide a valuable resource for researchers entering into the antibody discovery and characterisation field. This current version of the text however would require revision prior to publication to meet the needs of these researchers.

Major Concerns:

*The uniqueness of this manuscript would be enhanced by a focus and description of the IVIS protocols used by the Authors. Special attention should be given to the methods utilised for data visualisation and especially the analysis of antibody distribution in the models explored.

We thank reviewer's comment. As suggested by the review, more detailed description about IVIS imaging protocol, data visualization and analysis has been incorporated in protocol section 4.

*More emphasis is required in the description of the underlying IVIS technique throughout the document. Text to describe the strengths, weaknesses and comparisons with other biodistribution based techniques (SPECT, PET for example) to IVIS would be suggested.

We thank reviewer's comment. Discussion has been updated that include our methods strength and weaknesses over SPECT, PET etc.

*The description of the negative model is not made within the text.

We thank reviewer for noticing the missing description of negative model. We have used IgG1 Isotype control and an anti idiotypic antibody as a negative control, which does not bind to cancer cells. The respective description has been incorporated in the section 4.1.4 and additional data is added in Fig. 4 and Fig. 5.

Minor Concerns:

*There are some minor grammatical corrections required in the text. The capitalisation of some units of measure is incorrect mM = mm. Some typos can be corrected (IVOS = IVIS).

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Thank you for notifying the typing mistake. The correction has been incorporated into revised manuscript.

*There are additional references required in the introduction. Some of the larger reviews of antibody-based therapies or antibody-drug conjugates development would be warranted.

We agree with reviewer. Additional references have been updated

*Introduction: The development of bi-specific and tri-specific antibodies does have merit. The text should also mention other important factors for targeted antibody success and antibody retention in vivo.

We agree with reviewer. The text has been updated.

*As this is a biodistribution methodology, a description of the presence of target antigen in normal tissues would be important to make and why protocols exploring this in the context of animal model prior to clinical trials are critically important.

We agree with reviewer .The chosen antigen, FOLR1, which is highly expressed (>10 fold in on tumor cells). In response, a few lines in discussion section has been updated by including EGFR targeting antibodies and skin toxicities as an example.

*Recommendations or Notes should be separate from the protocol step (1.1).

The notes has been separated from the protocol step 1.1

*Some introduction text should be included to describe the fluorophore options, the issues of penetration and background (and background correction for analysis) as well as differences depending on mouse strain (hairless or not) using this technique. These could be included as paragraphs of text above the specific protocol steps to provide adequate context.

New data with another mouse strain has been added in Fig 5. Additional fluorophore has been recommended. According to manufacture, the body fluids and tissue has absorption minima at 800nm which helps in the better tissue penetration when excited at 800nM making this dye ideal for imaging. In our lab, we have successfully used this imaging technique in hairless nude mice and hairy nude and immunocompetent mice (Fig 4 and Fig 5)

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*Protocol 1.4. A note step should advise to keep all fraction tubes rather than disposing, if antibody is eluted in a later fraction than expected.

We thank the reviewer for suggestion. Note has been incorporated to keep all fraction tubes rather than disposing.

*Protocol 1.4. Steps for antibody concentration calculation is a suggested addition to this section as this is required for step 2.2

We thank the reviewer for this important suggestion. Steps for antibody concentration calculation has been incorporated in section 1.4

*Protocol 3.2 An additional note could include the importance of pre-chilling all consumables for this step. Tubes, tips and syringes can be stored in the fridge and then kept on ice prior to animal injection.

We thank the reviewer for this important suggestion. An advice has been incorporated into the notes suggesting pre-chilling of Tubes, tips and syringes.

*Protocol 4. Has a comparison of the dynamics of distribution of this antibody to target been made in this model? What is the shortest interval used? What is the duration of the fluorescent signal in this model?

In different study, we have monitored the distribution of antibody within 12 hours of post antibody administration and we have noticed the enrichment of antibody in the tumor. In response to reviewer, additional data and additional time points have been added (See Fig. 4 and Fig. 5)

*An organ biodistribution image would be impactful for this manuscript to compare it to the whole-body image.

We agree with reviewer. In response, additional organ distribution data has been added. (See Fig. 4 and Fig. 5)

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*The figure legends contain a lot of conjecture rather than a pure description of the figures themselves. A lot of this text could be included in the discussion instead.
*Figure 4 legend could be expanded with more experimental detail.
*The figures are well constructed. The uploaded versions which included the vector diagrams or flowcharts have blurred. Figure 1 gel labels could be made easier to read.

Figure legends have been updated and new data has been added.

Reviewer #3:
The article by Shivange et al. presents a timely and elegant description of the methodology and utility of antibody-based tumor imaging and therapeutic antibodies. I have the following minor concerns:

1. Please expand the acronym ELISA, IVOS

We thank reviewer for the suggestion. The respective acronyms have been expanded in the revised manuscript.

2. Please include the R01 grant number in acknowledgements.

R01 grant number has been added to acknowledgements.

3. Please describe the equipment used for IVOS imaging.

Details of IVIS imaging has been incorporated in protocol section 4

4. Please be consistent in using VH or pVH and VL or pVL clone acronyms.

We agree with reviewer. Consistency in terms of naming has been updated.

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Once again, we sincerely appreciate your consistent request to submit the method article.

We also sincerely “Thank you” for your patience during this process.

Looking forward to seeing the manuscript proofs.

Respectfully with Kind Regards

Jogender Tushir-Singh, PhD
DoD, Ovarian Cancer Academy Early Career Investigator
Assistant Professor and Member UVA Cancer Center
Laboratory *of* Novel Biologics
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Supplementary File 1

1a. Farletuzumab VH Sequence

EVQLVESGGGVVQPGRSLRLSCSASGFTFSGYGLSWVRQAPGKGLEWVAMISSGGSYTYYADSV
KGRFAISRDNAKNTLFLQMDSLRPEDTGVYFCARHGDDPAWFAYWGQGTPVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS
LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSLG

1b. Farletuzumab VL Sequence

DIQLTQSPSSLSASVGDRVTITCSVSSSISSNNLHWYQQKPGKAPKPWIYGTSNLASGVPSRFSGS
GSGTDYTFTISSLQPEDIATYYCQQWSSYPYMYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA
SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGEC

2a. Abagovomab VH Sequence

QVKLQESGAELARPGASVKLSCKASGYTFTNYWMQWVKQRPGQGLDWIGAIYPGDGNTRYTH
KFKGKATLTADKSSSTAYMQLSSLASEDSGVYYCARGEGNYAWFAYWGQGTTVTVSSASTKGPS
VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
SLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

2a. Abagovomab VL Sequence

DIELTQSPASLSASVGETVTITCQASENIYSYLAWHQQKQGKSPQLLVYNAKTLAGGVSSRFSGSG
SGTHFSLKIKSLQPEDFGIYYCQHHYGILPTFGGGTKLEIKTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

3a. BaCa VH Sequence

EVQLVESGGGVVQPGRSLRLSCSASGFTFSGYGLSWVRQAPGKGLEWVAMISSGGSYTYYADSV
KGRFAISRDNAKNTLFLQMDSLRPEDTGVYFCARHGDDPAWFAYWGQGTPVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS
LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVEHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSLGKGGGS
GGGSGGGSSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSG
IPDRFSGSSSGNTASLTITGAQAEDEADYYCNSRDSSGNHVVFGGGTKLTVLGGGGSGGGDSGG
GGSGGGGSEVQLVQSGGGVERPGGSLRLSCAASGFTFDDYGMSWVRQAPGKGLEWVSGINW
NGGSTGYADSVKGRVTISRDNAKNSLYLQMNSLRAEDTAVYYCAKILGAGRGWYFDLWGKGTT
VTVSS

3b. BaCa VL Sequence

DIQLTQSPSSLSASVGDRVTITCSVSSSISSNNLHWYQQKPGKAPKPWIYGTSNLASGVPSRFSGS
GSGTDYTFTISSLQPEDIATYYCQQWSSYPYMYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA
SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGEC

1c. Primers

SP FOR Primer

ATGGGCTGGTCCTGTATCATCCTGT

CK REV Primer

AGAGCTTCAACAGGGGAGAGTGT

CH3 REV Primer

AAGAGCCTCTCCCTGTCTCTGGGT