

Surface Engineering and Multimodal Imaging of Multistage Delivery Vectors in Metastatic Breast Cancer

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[Abstract] The design of effective nanoformulations that target metastatic breast cancers is challenging due to a lack of competent imaging and image analysis protocols that can capture the interactions between the injected nanoparticles and metastatic lesions. Here, we describe the integration of *in vivo* whole-body PET-CT with high temporal resolution, *ex vivo* whole-organ optical imaging and high spatial resolution confocal microscopy to deconstruct the trafficking of injectable nanoparticle generators encapsulated with polymeric doxorubicin (iNPG-pDox) in pulmonary metastases of triple-negative breast cancer. We describe the details of image acquisition and analysis in a step-wise manner along with the development of a mouse model for metastatic breast cancer. The methods described herein can be easily adapted to any nanoparticle or disease model, allowing a standardized pipeline for *in vivo* preclinical studies that focus on delineating nanoparticle kinetics and interactions within metastases.

Keywords: *In vivo* imaging, Positron emission tomography, Optical imaging, Cancer, Metastasis, Mouse models

[Background] Metastatic cancer accounts for >90% of mortality in cancer patients; yet, effective therapeutic strategies are missing due to the small size, heterogeneity, and dispersed nature of metastatic cancers (Bianchini *et al.*, 2016; Elia *et al.*, 2019). A series of biological barriers hinder the accumulation, and hence, the efficacy of conventional small molecule drugs and nanoparticle-based delivery systems in metastatic cancers (Blanco *et al.*, 2015). Our group has previously demonstrated the sequential and successful negotiation of most, if not all, biological barriers using iNPG-pDox composed of porous silicon microparticle-based injectable nanoparticle generators (iNPG) packaged with poly(lactic-co-glycolic acid) polymer-doxorubicin (pDox) conjugates (Xu *et al.*, 2016). pDox molecules self-assemble into nanoparticles after their release from iNPGs, and iNPG-pDox treatment significantly inhibited tumor metastasis, including a functional cure in 40-50% of mice with pulmonary metastatic breast cancer (Xu *et al.*, 2016). Visualization of the spatiotemporal kinetics of such nanomedicines that effectively target metastases can allow the directed development and rational improvement of other advanced therapies in the future (Goel, 2017).

Despite significant progress in the development of drug delivery systems, limited studies have

evaluated the distribution and interactions of such systems in *in vivo* metastatic settings. Molecular imaging modalities can uncover a wealth of information across multiple length- and time-scales in disease settings; however, the application of rationally combined multiscale imaging methods to assess the biodistribution of complex drug delivery systems in metastatic tumors remains to be performed (Goel, 2017). Such knowledge, in conjunction with mathematical modeling approaches, can predict and evaluate therapeutic responses and efficacies of complex drug delivery systems, eventually being employed to guide the reverse engineering of advanced therapies (Michor, 2011). In this protocol, we provide a detailed guide to designing and implementing a multiscale and multimodal imaging toolkit for the systematic deconstruction of the trafficking and interactions of nanotherapeutics in a metastatic model of breast cancer, as described in our earlier work (Goel *et al.*, 2020).

We first designed a modular orthogonal surface engineering approach for the modification of iNPG-pDox to render them amenable to multimodal imaging (both nuclear and fluorescence imaging) and microscopy without affecting their intrinsic biophysical properties. Such an approach is independent of carrier type, cargo, formulation method, or delivery route, and can be adopted for any nano- or bioengineered platform and implemented in any disease model. Next, we designed and implemented a systematic imaging toolkit composed of: (1) positron emission tomography/computed tomography (PET/CT) for longitudinal *in vivo* whole-body imaging at a high spatiotemporal resolution; (2) multispectral optical imaging (OI) for *ex vivo* whole-organ imaging of metastatic lungs; and (3) multispectral confocal microscopy for intra-tissue imaging of iNPG-pDox distribution in metastatic lungs with high spatial resolution (Goel *et al.*, 2020). This protocol includes methods for each of the approaches described above.

Materials and Reagents

1. T-75 cell culture flasks
2. Serological pipettes (Thermo Fisher Scientific, catalog numbers: 13-676-10F and 13-676-10M)
3. Falcon™ conical centrifuge tubes (Thermo Fisher Scientific, catalog number: 14-959-53A)
4. 1 cc Exel insulin syringes (28 G, 1 ml, Fisher, catalog number: 14-841-31)
5. BD 1 ml insulin syringe with slip tip (Fisher, catalog number: 22-253-260)
6. Sterile filter pipette tips
7. Eppendorf™ tubes (various sizes)
8. Coverslips
9. 31 G disposable insulin syringes (BD, catalog number: 324921)
10. Parafilm™ M PM996 all-purpose laboratory film
11. MilliporeSigma™ silica gel 60G TLC plates, glass-backed (MilliporeSigma, Catalog number: 1.00384.0001)
12. 26 G × ¾" mouse and rat tail vein Monoject IV catheter (Patterson Veterinary, catalog number: 07-836-8403)

13. BalB/c mice: Female, 4-6 weeks old (Invigo or The Jackson Laboratory) or another strain suitable for the selected cancer cell line
14. Murine triple-negative breast cancer cell line expressing luciferase: 4T1-GFP-Luc (ATCC) or another cancer cell line
15. Discoidal porous silicon particles (iNPG) and pDox nanoparticles (prepared in-house) or another amine-modified nanoparticle of choice (Xu *et al.*, 2016)
16. 2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SC-Bn-NOTA) (MacroCyclics, Dallas, ID: B-605)
17. 0.9% sodium chloride injection USP, 100 ml fill in 150 ml PAB[®] (B. Braun, NDC number: 00264-1800-32)
18. Sterile de-ionized water
19. Phosphate-buffered saline (PBS) solution, pH 7.4 (Thermo Fisher Scientific, catalog number: 10010023)
20. 100 ml filter-sterilized 50 mM EDTA solution
21. N,N'-dimethylformamide (DMF; MiliporeSigma, catalog number: 227056-100ML)
22. Dimethyl sulfoxide (DMSO; MiliporeSigma, catalog number: 317275-100ML)
23. Diisopropylethylamine (MiliporeSigma, catalog number: 387649-100ML)
24. Isopropanol
25. 0.1 M sodium acetate solution
26. 0.1 M HCl solution
27. Copper-64 (⁶⁴Cu; Washington University St. Louis)
28. AlexaFluor[™] 647 NHS ester (Thermo Fisher, catalog number: A20006)
29. Mouse serum (MiliporeSigma, catalog number: M5905-5ML)
30. OCT freezing compound (Tissue-Tek[®] O.C.T. Compound, Sakura[®] Finetek; VWR, catalog number: 25608-930)
31. Tissue-Tek[®] Cryomold[®] molds/adapters (Sakura[®] Finetek)
32. 4',6-diamidino-2-phenylindole (DAPI; Thermo Scientific, prepared according to vendor instructions; catalog number: 62247)
33. ProLong[™] Gold Antifade Mountant (ProLong, catalog number: P36930)
34. RPMI-1640 medium (ATCC, catalog number: ATCC[®] 30-2001[™])
35. Fetal bovine serum (FBS) (Gibco[™], catalog number: 10-082-147)
36. Pencillin-streptomycin antibiotics (Gibco[™], catalog number: 15-640-055)
37. Isoflurane (MiliporeSigma, catalog number: Y0000858)
38. Aluminium stubs (PELCO[®] pin mount starter kit; PELCO, catalog number: 76250-10)
39. Recombinant Anti-CD31 antibody [EPR17260-263] (Abcam, catalog number: ab222783)
40. Goat anti-rabbit IgG H&L (FITC) (Abcam, catalog number: ab6717)
41. Complete culture medium (see Recipes)
42. 50 mM EDTA solution (see Recipes)

Equipment

1. Inveon™ PET/CT (Siemens Medical Inc., INVEON, catalog number: 138757)
2. IVIS® Spectrum *In vivo* Imaging System (PerkinElmer, catalog number: 124262)
3. Bioscan AR-2000 Radio-TLC Imaging Scanner (Eckert & Ziegler, Valencia, CA)
4. Scanning electron microscope (Nova NanoSEM 230, Thermo Fisher Scientific, USA)
5. Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK)
6. Ultraviolet-visible-NIR spectrophotometer (Synergy H4 Hybrid, BioTek, USA)
7. Wizard² 2-Detector Gamma Counter (PerkinElmer, catalog number: 2470-0020)
8. EVOS Auto FL System (Thermo Fisher Scientific, USA)
9. Nikon Eclipse Ti microscope
10. Centrifuge (Beckman Coulter, model: Avanti® J-26 XPI, catalog number: 393127)
11. Tissue culture incubator (Sanyo Scientific, catalog number: 133060)
12. Biological safety cabinet (The Baker company, SterilGARD, catalog number: 101951)
13. Fisherbrand™ Isotemp™ Hot Plate Stirrer, Ambient to 540 °C, Ceramic (Fisherbrand, catalog number: SP88854200)
14. PCR thermal cycler (Bio-Rad Laboratories, model: T100™, catalog number: 1861096)
15. Braintree Scientific Diversity Partner Mouse Tail Illuminator Restrainer (Fisher, catalog number: NC0772753)
16. Simport™ Scientific StainTray™ Slide Staining System (Fisher, catalog number: 22-045-035)

Software

1. Inveon Research Workplace (INVEON, Siemens)
2. Living Image Software (PerkinElmer)
3. Nikon Elements software (Nikon)
4. FIJI (NIH)
5. Microsoft Office Suite
6. GraphPad Prism

Procedure

- A. Animal models for pulmonary metastasis of triple-negative breast cancer
 1. Grow and expand the cells according to the recommended conditions. Typically, 4T1-GFP-Luc cells are cultured in RPMI-1640 medium supplemented with 10% FBS and 1% Pencillin-streptomycin.
 2. Harvest cells while in the exponential growth phase (approximately 80-90% confluence) using trypsin or an appropriate enzyme for the specific cell line. Resuspend cells in medium containing serum.

3. Count cells.
4. Centrifuge cells at $225 \times g$, 4°C for 5 min. Resuspend cells in $1 \times$ PBS to a concentration of 1×10^5 cells/200 μl .
5. Place cells on ice and transport to the vivarium.
6. Restrain the mouse in a restrainer and position the tail such that the vein is facing upwards.
7. Draw 200 μl cells into a 28 G disposable insulin syringe and gently inject into the tail vein. The contents of the needle should inject easily without resistance.
8. Monitor tumor development regularly via bioluminescence imaging. Typically, 4T1-Luc cells take 10 days to develop lung metastases.

B. Surface modification of iNPG-pDox or other amine-modified nano/microparticles

1. For details of the structure of iNPG-pDox vectors and chemistry, readers are referred to the following references (Goel *et al.*, 2020; Xu *et al.*, 2016).
2. Dissolve 3×10^9 amine-modified iNPG (or other nanoparticles of choice) in 0.5 ml DMF in a 1.5-ml Eppendorf™ tube. Shake gently.
3. Dissolve 0.8 mg p-SCN-Bn-NOTA in 0.5 ml DMSO and add to the iNPG solution.
4. Sonicate the mixture until a homogenous solution is formed.
5. Add 20 μl diisopropylethylamine and incubate the reaction mixture at 25°C for 4 h under vigorous shaking at 700 rpm. This step can be performed on a hot plate stirrer or in a thermal cycler.
6. After the 4-h incubation, wash the iNPG-NOTA conjugates by centrifugation at 5,000 rpm for 5 min with DMSO as the solvent. Briefly, spin the conjugates down, carefully discard the supernatant and resuspend the pelleted nanoparticles in 0.5 ml DMSO by sonication. Repeat the wash step with DMSO (for a total of 2 times), followed by 0.5 ml isopropanol (one time) and 0.5 ml DI water (one time). The speed and time of the centrifugation can be adjusted according to the nanoparticles used.
7. After the final wash step, resuspend the iNPG-NOTA pellet in DI water and freeze at -80°C or freeze-dry (depending on the nanoparticle used) for further use (for up to 1 week).
8. Use the same process to obtain iNPG-AF647 by conjugating fluorescent dyes (e.g., AlexaFluor™ 647 NHS ester) for optical imaging.

C. Radiolabeling of iNPG-NOTA

1. Resuspend iNPG-NOTA in 0.5 ml 0.1 M sodium acetate solution.
2. Adjust the pH of the solution to 5.5 using 0.1 M HCl.
3. Add 37 MBq $^{64}\text{CuCl}_2$ to the nanoparticle solution. All handling of radioisotopes and radiolabeled solutions should be performed behind a lead shield in a fume hood, in accordance with institutional radiosafety guidelines.
4. Carefully wrap parafilm around the cap and incubate the tube at 37°C for 1 h with gentle shaking. This step should be performed in a thermal cycler or on a hot plate stirrer behind a protective

lead shield.

- Remove the excess unchelated ^{64}Cu by centrifugation at $\sim 1,610 \times g$ for 5 min. Wash twice with DI water to obtain the final radiolabeled constructs (^{64}Cu]NOTA-iNPG). All radioactive waste should be appropriately discarded.
- To calculate radiolabeling yield, radio thin-layer chromatography (radio TLC) is performed (Figure 1). Briefly, drop 2 μl ^{64}Cu]NOTA-iNPG on one end (origin) of a silica gel TLC plate. Dip the strip vertically in 2 ml 50 mM EDTA solution in a 50-ml conical tube.

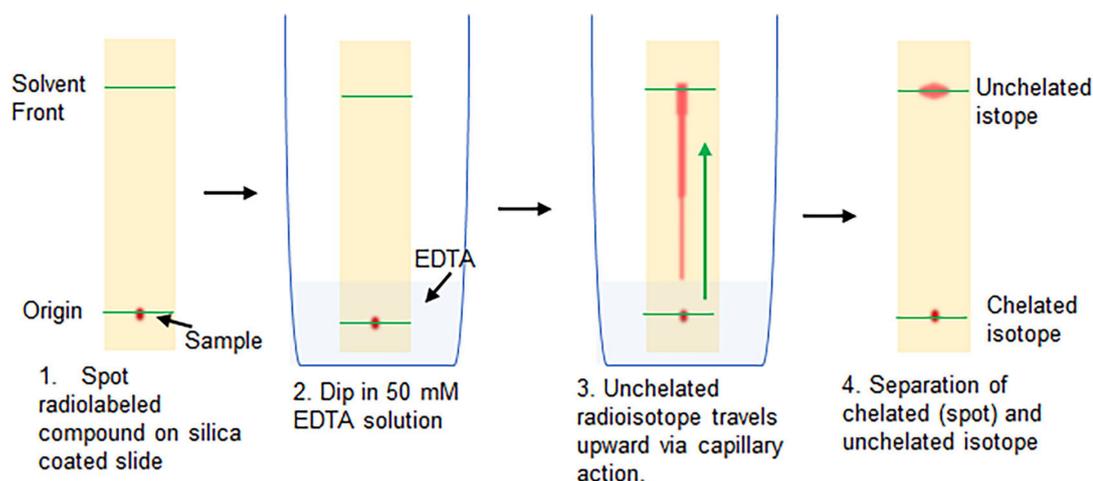


Figure 1. Schematic of the radio TLC procedure to calculate radiolabeling yield

- The solution will slowly travel upward by capillary action. Once the solution has moved 2–4 cm upward (or 1 cm from the top of the strip; solvent front), remove the strip from the EDTA solution and allow to dry.
- Scan the strip on a Bioscan AR-2000 RadiobTLC Imaging Scanner according to the vendor's instructions.
- Typical radiolabeling yields using with this procedure range from 50% to 70%, with >99% radiochemical purity.

D. Loading pDox into ^{64}Cu]NOTA-iNPG or iNPG-AF647

- Suspend 3 mg polymeric pDox monomers in DMSO (70 mg/ml) and add the solution to the ^{64}Cu]NOTA-iNPG or iNPG-AF647 conjugates.
- Incubate the mixture for 3 h at room temperature with vigorous shaking. This step can be performed in a thermal cycler.
- Remove unloaded pDox polymers by washing twice with DI water using centrifugation, as described in B above, to obtain the final constructs: ^{64}Cu]NOTA-iNPG-pDox for PET/CT imaging and iNPG-AF647-pDox for optical imaging and confocal microscopy. Resuspend the final conjugates in 1 ml PBS.
- Draw 200 μl ^{64}Cu]NOTA-iNPG-pDox ($\sim 60\text{--}80 \mu\text{Ci}$ per mouse) solution into a 1-ml syringe for

injections in the next step.

E. Small animal PET/CT imaging

1. Anesthetize 4T1 tumor-bearing mice under 2% isoflurane (in 100% O₂ gas).
2. Transfer each mouse to a mouse restrainer, carefully placing the nose cone to maintain anesthesia.
3. Insert the micro-catheter into the tail vein and flush with 50 μ l PBS. Leave the syringe with PBS attached to the catheter.
4. Secure the mouse onto the microPET/CT scanner bed under 2% isoflurane anesthesia administered via the nose cone manifold. To minimize movement, the limbs can be secured to the side of the bed with tape.
5. Switch the PBS syringe in the catheter with the syringe containing [⁶⁴Cu]NOTA-iNPG-pDox.
6. Acquire a whole-body CT calibration image of the mouse.
7. The PET/CT acquisition protocol will depend on the particular experiment and the requirements of the researcher.
8. For dynamic PET scans, a 30-min dynamic PET mode, data are acquired using the 3D-OSEM iterative algorithm with a 256 \times 256 \times 256 voxel volume.
9. Immediately after the dynamic PET scan is started, slowly inject the radiolabeled nanoparticle solution as a single bolus dose over 30 s.
10. Continue the dynamic PET acquisition.
11. If PET images are required at more time points, data are acquired using the standard 3D PET acquisition protocol. To account for radioactive decay, longer scan times may be necessary.
12. Datasets are acquired as: standard static 3D PET reconstruction and dynamic 3D PET reconstruction with 6 \times 300 s frames. CT attenuation correction is applied to all PET images.

F. *Ex vivo* gamma counting

1. When the PET/CT scans are at the final time point, euthanize the animal according to institutional protocols.
2. Harvest and weigh the major organs including the tumor-bearing lungs.
3. Radioactivity in the organs is measured using a gamma counter per the manufacturer's instructions.

G. *Ex vivo* whole-organ optical imaging

1. In a separate cohort of 4T1-tumor bearing mice, inject 200 μ l iNPG-AF647-pDox prepared in section D via the tail vein (described in section A).
2. At the desired time point post-injection, euthanize the mouse according to institutional protocols.
3. Harvest the lungs and place on a black sheet in the IVIS Spectrum Scanner.
4. Acquire multiplexed *ex vivo* fluorescence images using the vendor's preset filter sets encompassing the excitation and emission spectra for GFP (tumor), doxorubicin (pDox), and

AF647. For this combination of fluorophores, 18 filter sets are automatically selected when using the IVIS Spectrum system.

5. Set the field of view, acquisition time, and binning number. Data acquisition settings will vary from user to user but should be kept constant throughout the experimental study.
6. After image acquisition, rinse the tissues in PBS, preserve in OCT compound in cryomolds, and store at -80°C for sectioning.

H. Immunofluorescence microscopy

1. Cryo-section the frozen tissues into 6- μ m-thick sections using a cryotome.
2. Incubate the sectioned tissues in cold acetone for 10 min. All steps for staining and incubation in this section should be performed in a staining tray or similarly fashioned light-blocking device.
3. Prepare the blocking solution as follows: Mix horse and goat serum at a 1:1 v/v ratio and add 0.1% (by volume) Triton X-100.

Note: The choice (species) of blocking serum depends on the host of the primary and secondary antibodies used for staining.

4. Add 100 μ l blocking solution per tissue section. Incubate at 4°C for 1 h.
5. Prepare the solution of primary antibody for a total volume of 100 μ l per tissue section. The anti-CD31 antibody can be used at a 1:200 dilution. For other primary antibodies, refer to the vendor's instructions for the suggested dilution.
6. After 1 h, gently tap the slide on its side to discard the blocking solution.
7. Add 100 μ l primary antibody cocktail to each tissue section and incubate overnight at 4°C.
8. After overnight incubation, wash the tissue sections gently in PBS. For each wash, incubate the tissue section in PBS for 3 min, tap the slide on its side to drain the solution, and add fresh PBS. Repeat the wash step 3 times.
9. Prepare the secondary antibody in blocking solution at the vendor's recommended dilution. For the goat anti-rabbit-FITC secondary antibody, a 1:50 dilution is recommended.
10. Incubate the sections in secondary antibody solution for 1 h at 4°C.
11. Wash the secondary antibody with PBS as described in Step H8.
12. Add 50 μ l DAPI solution to the tissue sections and incubate for 10 min.
13. Wash twice with PBS as outlined in Step H8.
14. Clean and dry the portions of the slide adjacent to the tissue, taking care not to disturb or dislodge the tissue.
15. Add a drop (or 5 μ l) Gold Anti-fade Mounting Medium and gently place a coverslip on the tissue sections. Care must be taken to prevent the introduction of bubbles.
16. Dry the slide by tapping gently with Kimwipes and keep protected from light in a black box or slide cassette covered with aluminum foil. It is recommended to perform confocal microscopy within a day of staining.
17. Observe and photograph immunofluorescent slides by mounting on the stage of a confocal microscope according to the vendor's instructions, using the appropriate objectives. Typically,

10× and 40× objectives are sufficient for nanoparticle uptake studies. Take images of 5-10 fields of view per tissue section distributed over the entire section.

18. Prevent overexposure or stain bleaching of the samples. All images are acquired under identical conditions.
19. FITC and DAPI can be observed with their dedicated filter sets. To observe iNPG-AF647, use the cy5 common filter set. To observe fluorescence from pDox nanoparticles, the RFP common filter can be used.

Data analysis

A. MicroPET/CT analysis

1. PET/CT analysis varies by the user experimental system and institutional software and hardware set up. In this work, the following steps were followed:
2. Input the injected radioactivity dose, time of injection, name of isotope, and time of scan into the software.
3. Analyze the whole-body PET images of the mouse using the Inveon Workstation Software (Siemens Medical Inc.).
4. Manually draw the region-of-interest (ROI) centered around the organs of interest (*e.g.*, lungs, liver, spleen, kidney, bone, muscle, and heart) on the co-registered PET/CT images (Figure 2). The decay-corrected radioactivity in each ROI is computed automatically by the software as the percentage of the injected dose per gram of organ (%ID/g).

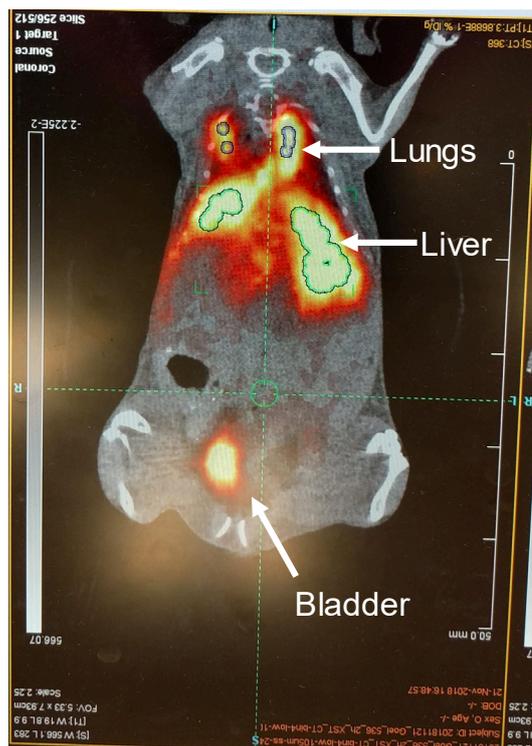


Figure 2. Example of PET image quantitation using the Inveon Workstation Software (Siemens Medical Inc.). Coronal view of the whole-body distribution of $[^{64}\text{Cu}]$ NOTA-iNPG-pDox in a mouse bearing lung 4T1 lung metastases. White arrows depict the different organs, while ROI outlines are shown in green.

5. Dynamic time-activity curves are plotted for each ROI using the dynamic 3D dataset.
6. To save images, select a coronal, axial, or sagittal view and export as .TIFF files. 3D maximum intensity projection (MIP) reconstructions can be also obtained using the software.

B. Optical imaging analysis

1. Analysis of multiplexed optical imaging will vary from user to user depending on the experimental and hardware/software setups. The following steps were used in this protocol.
2. Analyze the data using the LivingImageTM software (PerkinElmer).
3. Perform spectral unmixing using the automatic mode with four-component principal components analysis. Autofluorescence is subtracted from all images during analysis.
4. Draw volume- and area-matched ROIs on the spectrally unmixed images for each component (GFP for the tumor, AF647 for iNPG, and doxorubicin for pDox) (Figure 3).

Image #: [REDACTED]
Thu, Dec 6, 2018 16:42:51
Level=High, Em=560, Ex=535, Epi-Illumination, Bin:(M)8, FOV:6.5, F4, 1s
Living Image Version: 4.5.5.19626(Aug 3 2017)
Camera: IVIS 23280, [REDACTED]

User: [REDACTED]
Group: [REDACTED]
Experiment: [REDACTED]
Comment 1: inpg pdox 24 h pi trams spectral unmix

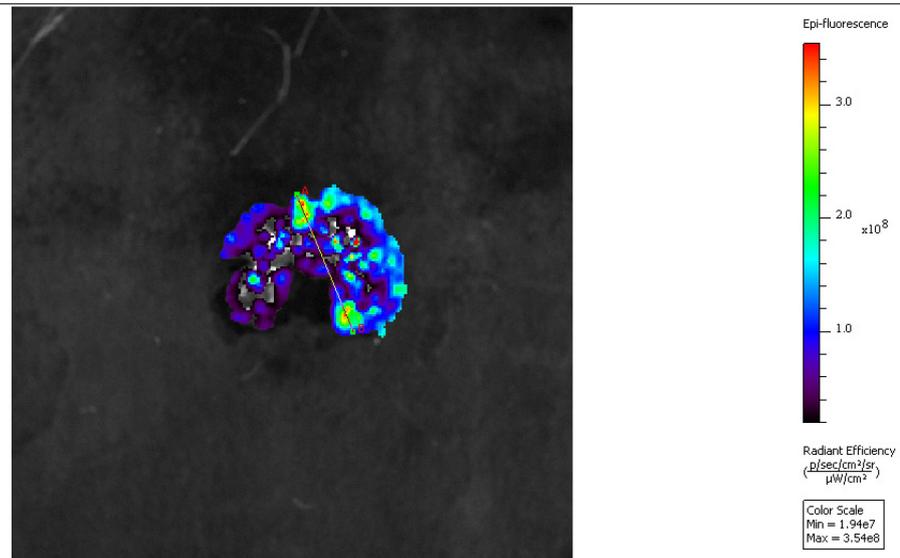


Figure 3. Example of *ex vivo* multiplexed optical imaging (IVIS[®] Imaging System) depicting the distribution of AF647-INPG-pDox in 4T1 lung metastases using the PerkinElmer Living Image[™] software. Fluorescence signal from Dox (excitation: 535 nm, emission 560 nm) is shown.

5. ROIs are computed automatically and are presented as the average radiant efficiency [units: $(p/s/cm^2/sr)/(\mu W/cm^2)$].
6. Pixelwise line analysis can also be performed using the software.
7. Export data as .TIFF files.

C. Confocal microscopy analysis

1. In the present work, images were analyzed using the Nikon Elements (Figure 4) and FIJI software.

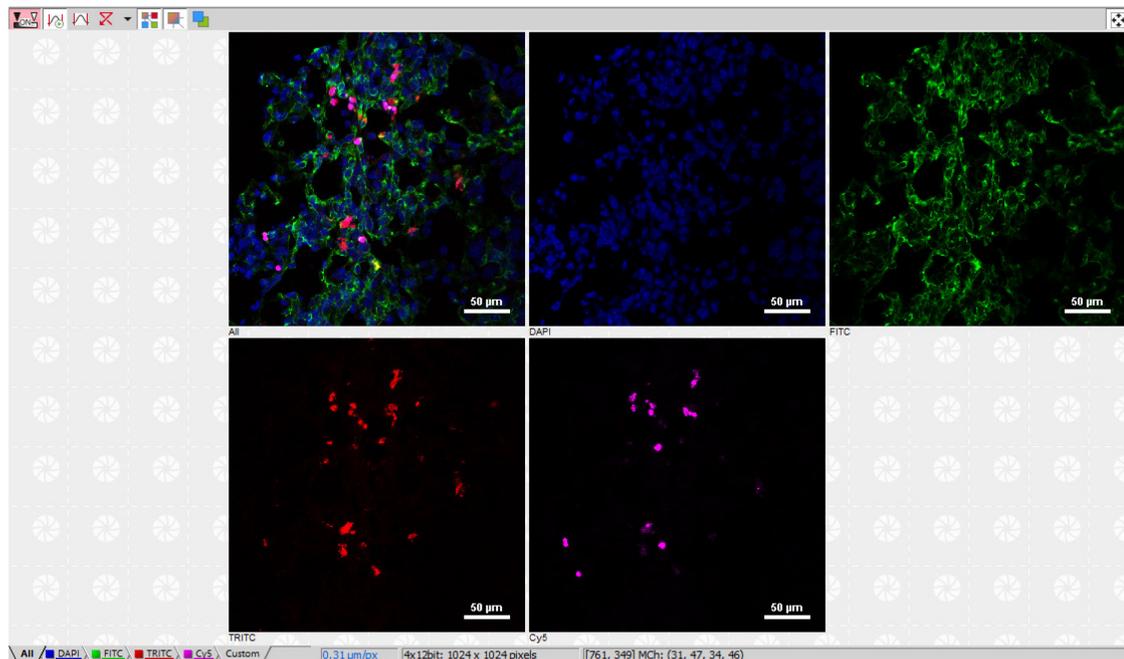


Figure 4. Example of multiplexed confocal microscopy immunofluorescence imaging analysis using the Nikon NIS-Elements™ software. Merged (first panel) and single-channel images of DAPI (blue), blood vessels (CD31, green), AF647-iNPG (magenta), and pDox (red) in 4T1 metastases-bearing lungs. Scale bars as shown.

2. Use constant settings for image analysis throughout the study.
3. Apply Gaussian blur and automatic thresholding to segment the images for quantitation.
4. Perform pixelwise quantitation of fluorescence intensity and area fraction coverage by drawing an ROI over the entire organ or individual tumor nodules.
5. Typically, quantitation is performed over 5 random fields of view per stain.

Recipes

1. Complete culture medium for murine 4T1 triple-negative breast cancer cell line:
500 ml RPMI 1640 Medium
50 ml 1:10 Gibco fetal bovine serum, Premium Plus
5.5 ml 1:100 Penicillin-Streptomycin (Pen/Strep) antibiotics

Notes:

- a. We recommend aliquoting FBS and Pen/Strep into 50 5.5-ml batches (sufficient to make one batch of 500 ml complete cell culture medium) to prevent repeated freezing/thawing of the components.*
- b. The complete medium can be pre-prepared in advance and stored in 4°C for an extended period.*
- c. We recommend warming the complete cell culture medium in a water bath at 37°C prior to use.*

2. 100 ml filter-sterilized 50 mM EDTA solution:
 - 1.87 g EDTA (disodium ethylenediaminetetraacetate·2H₂O)
 - De-ionized water
 - a. Add sterile de-ionized water to EDTA powder to a final volume of 100 ml
 - b. Pass the solution through a 0.2-µm Nalgene bottle top sterile filter unit
 - c. Store in 4°C for an extended period

Acknowledgments

The protocols described in this work are derived from the original research publication, *Sci Adv.* 2020 Jun 24;6(26):eaba4498 (Goel *et al.*, 2020). This work was partially supported by the NIH grants U54CA210181, R01CA193880, and R01CA222959, in addition to the U.S. Department of Defense grant W81XWH-17-1-0389.

Competing interests

Mauro Ferrari is the inventor of a U.S. patent from The University of Texas-Houston (patent no. US 8,920,625 B2, granted 30th December, 2014). Mauro Ferrari and Haifa Shen are inventors of a non-provisional patent application from the Houston Methodist Hospital (PCT/US 2014/0010879 A1). Both inventions are related to the scientific research described herein. More recently, Mauro Ferrari has formed a company (BrYet LLC) and has secured certain commercial rights on said patents. Though not an executive officer, he remains a majority shareholder, director, and scientific advisor of the company. All other authors declare that they have no competing interests.

Ethics

All animal studies were performed in accordance with the guidelines from the Institutional Animal Care and Use Committee (IACUC ID: IS00004452; Validity: 01/02/2018-01/01/2021) at the Houston Methodist Research Institute.

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