

## Video Article

# *In Vivo* Optical Imaging of Brain Tumors and Arthritis Using Fluorescent SapC-DOPS Nanovesicles

Zhengtao Chu<sup>1,2</sup>, Kathleen LaSance<sup>3</sup>, Victor Blanco<sup>1</sup>, Chang-Hyuk Kwon<sup>5,6</sup>, Balveen Kaur<sup>5,6</sup>, Malinda Frederick<sup>4</sup>, Sherry Thornton<sup>4</sup>, Lisa Lemen<sup>3</sup>, Xiaoyang Qi<sup>1,2</sup>

<sup>1</sup>Division of Hematology-Oncology, Department of Internal Medicine, University of Cincinnati College of Medicine

<sup>2</sup>Division of Human Genetics, University of Cincinnati College of Medicine

<sup>3</sup>Department of Radiology, University of Cincinnati College of Medicine

<sup>4</sup>Division of Rheumatology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine

<sup>5</sup>Solid Tumor Biology Program, James Comprehensive Cancer Center, The Ohio State University Medical Center

<sup>6</sup>Department of Neurological Surgery, James Comprehensive Cancer Center, The Ohio State University Medical Center

Correspondence to: Xiaoyang Qi at [xiaoyang.qi@uc.edu](mailto:xiaoyang.qi@uc.edu)

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## Abstract

We describe a multi-angle rotational optical imaging (MAROI) system for *in vivo* monitoring of physiopathological processes labeled with a fluorescent marker. Mouse models (brain tumor and arthritis) were used to evaluate the usefulness of this method. Saposin C (SapC)-dioleoylphosphatidylserine (DOPS) nanovesicles tagged with CellVue Maroon (CVM) fluorophore were administered intravenously. Animals were then placed in the rotational holder (MARS) of the *in vivo* imaging system. Images were acquired in 10° steps over 380°. A rectangular region of interest (ROI) was placed across the full image width at the model disease site. Within the ROI, and for every image, mean fluorescence intensity was computed after background subtraction. In the mouse models studied, the labeled nanovesicles were taken up in both the orthotopic and transgenic brain tumors, and in the arthritic sites (toes and ankles). Curve analysis of the multi angle image ROIs determined the angle with the highest signal. Thus, the optimal angle for imaging each disease site was characterized. The MAROI method applied to imaging of fluorescent compounds is a noninvasive, economical, and precise tool for *in vivo* quantitative analysis of the disease states in the described mouse models.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/51187/>

## Introduction

Whole animal imaging has become a powerful tool in the study of animal physiopathology. Among current imaging systems, the MS FX PRO allows researchers to accurately visualize fluorescently labeled (or luminescent) compounds and/or tissues in living mice, and simultaneously obtain X-ray images. With the recently introduced multi modal animal rotation system (MARS) a complete, automated rotation of the mouse is achieved in order to capture both fluorescent/luminescent and X-ray images at specific angles<sup>1</sup>. Image acquisition can be programmed such that sequential image series can be captured at specific, incremental angles as small as 1°. This permits one to identify the optimal orientation of the animal, *i.e.* that in which the distance between the internally generated fluorescent/luminescent signal and the system's detection device is the shortest. This, in turn, facilitates precise repositioning of the animal for subsequent imaging sessions during longitudinal studies.

In this report, we describe the implementation of a multi-angle rotational optical imaging (MAROI) system for *in vivo* quantitation of fluorescent marker intensity. MAROI signal curve analysis can be used in longitudinal studies for direct correlation of fluorescent signal distribution to precisely map diseased sites or biological processes of interest.

This system was used to monitor the absorption of fluorescently labeled SapC-DOPS nanovesicles by orthotopic and spontaneous tumors, as well as by arthritic foci, in living mice; it provided multispectral and multimodal data sets derived from complete rotational coverage of the animals. Among the numerous fluorescent probes currently available for *in vivo* imaging, those emitting in the near-infrared and far-red spectral regions confer the lowest interference with skin and tissues, and provide the highest penetration and image resolution. We used CellVue Maroon (CVM)<sup>2,3</sup>, a far-red fluorescent cell linker (Ex 647/Em 667), to label SapC-DOPS (SapC-DOPS-CVM)<sup>4-12</sup>.

## Protocol

Ethics statement of animal use. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (IACUC Protocol Number: 11-05-05-02) and the Cincinnati Children's Hospital Research Foundation (Animal Welfare Assurance Number A3108-01). All experiments involving mice followed the animal care guidelines of the University of Cincinnati and the Cincinnati Children's Hospital Research Foundation.

### 1. Prepare Animal Models

**Note:** Three different animal models outlined below have been used in our prior studies:

1. Orthotopic brain tumor mouse: Use Nu/Nu athymic female mice that have been intracranially injected with human U87-ΔEGFR-Luc cells. These mice develop an aggressive tumor showing typical features of human glioblastoma.
2. Genetically engineered brain tumor mouse models<sup>13</sup>: Breed Mut3 (GFAP-cre; Nf1loxP/+; Trp53-/-) male mice with Trp53loxP/loxP; PtenloxP/loxP females to generate Mut6 mice (GFAP-cre; Nf1loxP/+; Trp53-/-loxP; PtenloxP/+). Maintain Mut3 mice in B6CBAF1/J strain by breeding male Mut3 mice with female B6CBAF1/J mice. Genotype the mice between P9 and P12 and confirm the genotypes after harvesting their tissues.
3. K/BxN arthritis: Use C57Bl/6J mice that have been intraperitoneally administered with 150 µl sera from KRN x NOD F1 mice. These mice develop arthritis 24 to 48 hr following sera injection. Imaging of the arthritic mice is performed on day 7 following sera administration, a time point at which mice exhibit overt macroscopic arthritis. Mice should be evaluated using the criteria outlined in the next step.
  1. Evaluation of mice for macroscopic arthritis using an arthritic index macroscopic scoring system as follows: 0 = no detectable arthritis, 1 = swelling and/or redness of paw or one digit, 2 = two joints involved, 3 = three joints involved, and 4 = severe arthritis of the entire paw and digit. The arthritic scoring system is used to determine the number of joints affected and the severity of arthritis in the mouse paws. Even mice with the highest possible arthritic score rarely show signs of immobility. However, arthritis is monitored 3x/week and mice in excessive pain (such as severe immobility from swollen paws that inhibits food and water consumption) are sacrificed.
  2. Note: Fluids injected IV into the mouse tail vein have sterility maintained throughout the experiment. Clean, sterile, disposable syringes and vials are used for study solution preparation and administration.

### 2. Preparation of Fluorescently-labeled SapC-DOPS Nanovesicles

1. SapC protein production: Recombinant SapC protein with exact human SapC sequence was produced in *E. coli* cells as previously described with modifications<sup>4</sup>. SapC was precipitated by ethanol followed by high performance liquid chromatography purifications. After lyophilization, dry SapC was used and its concentration was determined by its weight.
2. Mix SapC protein as previously described<sup>7,10,11</sup>. Mix DOPS (0.18 mg) and CVM (0.03 mg) in a glass tube and use nitrogen gas to evaporate lipid solvents.
3. Add SapC protein powder (0.32 mg) to the mixture, suspend the dry mixture in 1 ml of PBS buffer and bath sonicate for about 15 min as previously described<sup>7,10,11</sup>. Then pass the suspension through a Sephadex G25 column (PD-10) to remove free CVM dye. Excitation and emission maxima of the final product, *i.e.* SapC-DOPS-CVM nanovesicles, are 653 nm and 677 nm, respectively.

### 3. Imaging

1. Use brain tumor and arthritis mouse models (described above in Step 1) to test the MAROI system. Anesthetize mice to effect with 2% isoflurane. 1-2% isoflurane is maintained for the duration of the imaging procedure. Warm air is continuously and gently delivered into the imaging chamber for the duration of imaging. A small bead of sterile artificial tears ointment is applied to each eye of the mouse so as to cover and lubricate the eye. Place mice into the MARS system by positioning the mice in a supine position with their spine initially directed towards the camera (**Figure 1**). Calibrate the MARS 380° support film and position the mouse using the rotation software in the Bruker MI protocol tab. Obtain baseline images of mice prior to SapC-DOPS-CVM administration in the manner described below.
2. Inject 200 µl of SapC-DOPS-CVM intravenously into the tail vein of the mouse. Administer to control mice and arthritic or brain tumor-bearing mice.
3. Image mice 24 hr post injection and again at 7-9 days post injection by taking fluorescence (25 sec exposure time) and X-ray (10 sec exposure time) images at 10° increments over a course of 380°, creating a slight overlap to ensure there are no gaps in the rotational dataset. Using Bruker MI software, superimpose fluorescent onto X-ray images for anatomical localization.

### 4. Image Analysis

1. Draw a rectangular ROI encompassing the width of the field of view (FOV) of the disease site (tumor and arthritis). The ROI must be large enough to keep the disease feature within the FOV as the animal moves in the course of the 380° rotation. For brain tumor mice (orthotopic and transgenic models), use the same rectangular ROI on each tumor model and its three (3) respective control mice for all time points (baseline, 24 hr, and 9 days). The positioning of the rectangular ROI for each mouse is preserved over all time points by utilizing anatomical landmarks on each animal's corresponding X-ray images. The anatomical landmark(s) identified on the tumor model must also be used to place identical rectangular ROIs on each model's respective controls. Anatomic landmarks identified on the X-ray images allowing for consistent ROI placement include the base of the skull and the posterior aspect of the zygomatic arch. They are visualized on the right and left lateral skull in the posterior-anterior (PA) image.

- After automatic background subtraction, determine mean fluorescence intensity for every image. Convert the fluorescence images to photons/s/mm<sup>2</sup> using Bruker MI imaging software. Plot the fluorescence values as a function of the imaging angles, and apply as error bars the standard deviation of the averaged fluorescence values obtained from control mice using Excel or other graphing software.

## Representative Results

We demonstrate here that SapC-DOPS nanovesicles labeled with a far-red dye (CVM) specifically accumulate in orthotopic and spontaneous mouse brain tumors, as well as in arthritic joints of K/BxN mice. Serial fluorescence/X-ray images acquired from an ROI placed over each disease site during complete rotations of the mice were subjected to MAROI curve analysis, which revealed the optimal imaging angle with the highest fluorescence intensity.

The primary purpose for using the MARS system is to determine the optimum angle of fluorescence so that the most accurate measurements can be taken. Representative results from three experiments using mice with brain tumors or arthritis are shown. Using SapC-DOPS-CVM and the MARS system (**Figure 1**), the best possible image angle for observing the tumor or inflammation due to arthritis was determined. Fluorescence images, followed by an X-ray acquisition, were acquired every 10° during a 380° rotation of the mouse. Fluorescence images were overlaid onto the corresponding X-ray images for image display and rotational movie generation.

Results from the orthotopic brain tumor model are demonstrated in **Figure 2**. The fluorescence image of a representative orthotopic tumor-bearing mouse (Ortho1) is shown in **Figure 2A**. The optimal image angle for this animal is 10°, the position at which the fluorescence photon intensity is the greatest (**Figure 2B**). Measurements were taken before injection with SapC-DOPS-CVM (baseline) and 24 hr after injection. Control mice (tumor free) received a similar treatment.

**Figure 3** shows comparable data from the genetically engineered brain tumor mouse model. The fluorescence images and photon measurements were taken before injection with SapC-DOPS-CVM (baseline) and 24 hr (**Figures 3A and 3B**) and 9 days (**Figure 3C**) after injection. These graphs show that the optimal imaging angle in the tumor-bearing animal (Tumor Mut49) is 20° 24 hr post injection but changes to 10° 9 days post injection. This suggests that fluorescence signal alteration correlated with morphological changes, likely reflecting tumor growth.

As shown in **Table 1**, the MAROI method clearly demonstrates that the fluorescent signal decreases for projections at increasing rotation away from the optimal imaging angle. In brain tumors, a 7% average decrease in fluorescent signal was obtained if the animal's physical orientation was ±10° offset from the optimal imaging angle. An average 21% decrease in fluorescent signal was measured at ±20°. Thus relatively small offsets from the optimal angle can result in significant signal attenuation. Utilizing the MAROI technique for image positioning will allow investigators to produce more consistent and reliable data.

The MAROI method was finally used to assess the targeting of arthritic joints by SapC-DOPS-CVM 24 hr after SapC-DOPS-CVM injection. This animal scored 3 with three arthritic joints. Fluorescence images of toe and ankle of the arthritic mouse are shown in **Figures 4A and 4B**. Corresponding photon measurements at 10° rotation intervals are graphed in **Figures 4C and 4D**. The optimal imaging angles found for the toe and ankle are 140° and 120°, respectively.

In summary, the combination of the MAROI system with fluorescent SapC-DOPS nanovesicles represents a noninvasive, accurate and highly sensitive strategy for live imaging, which allows for quantitative studies of tumor and arthritis progression in small animals. The possibility of acquiring a 360° multimodal imaging dataset considerably improves data analysis and interpretation, as compared with what is achievable using single angle imaging techniques.

Mouse	Timepoint	FLR Optimum Angle	Standard Anatomic Angle (x-ray)	% signal change FLR angle vs x-ray angle	FLR signal change at +/-10° of Optimum	FLR signal change at +/-20° of Optimum
Ortho1	24hr	10	10	0	9-12%	19-21%
Mut49	24hr	20	20	0	3-5%	15-18%
	9-day	10	20	3%	3%	8-10%

**Table 1. Optimum imaging angles for each mouse model.** The differences between the angle of maximum photon fluorescence (FLR optimum angle) and the standard anatomic angle (X-ray) can be seen. When these two angles become increasingly different, the measured signal changes significantly. [Click here to view larger image.](#)

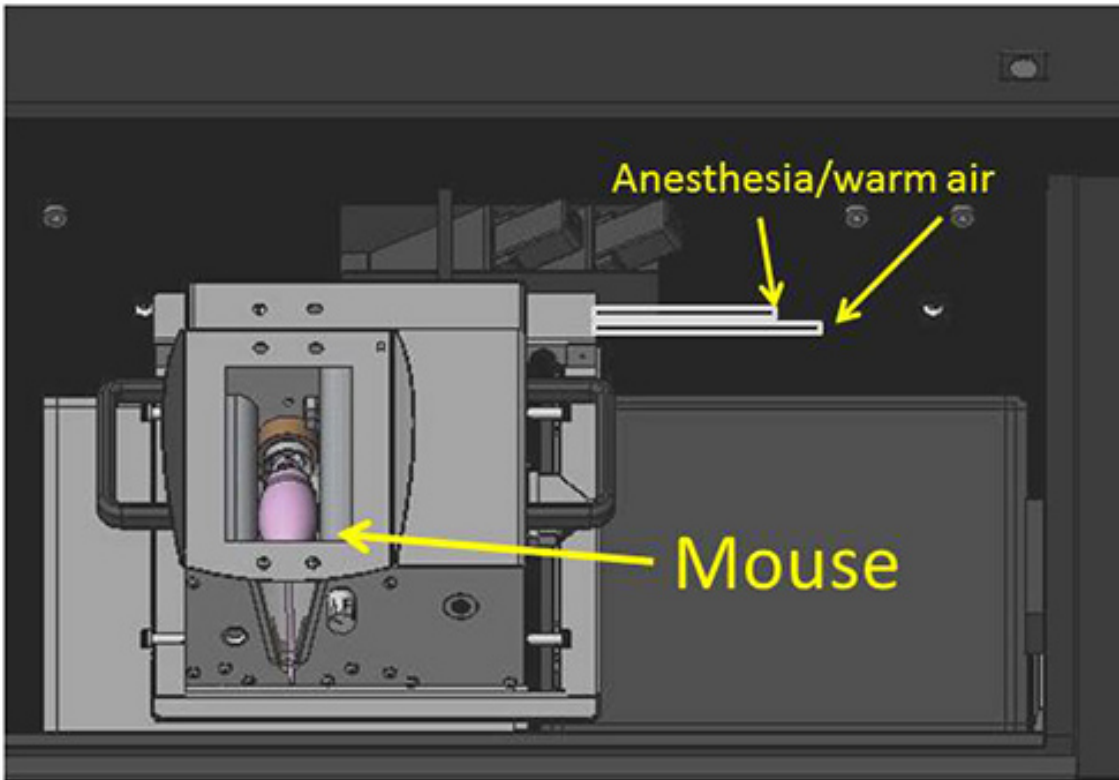


Figure 1. Multi-angle rotational optical imaging (MAROI) device. [Click here to view larger image.](#)

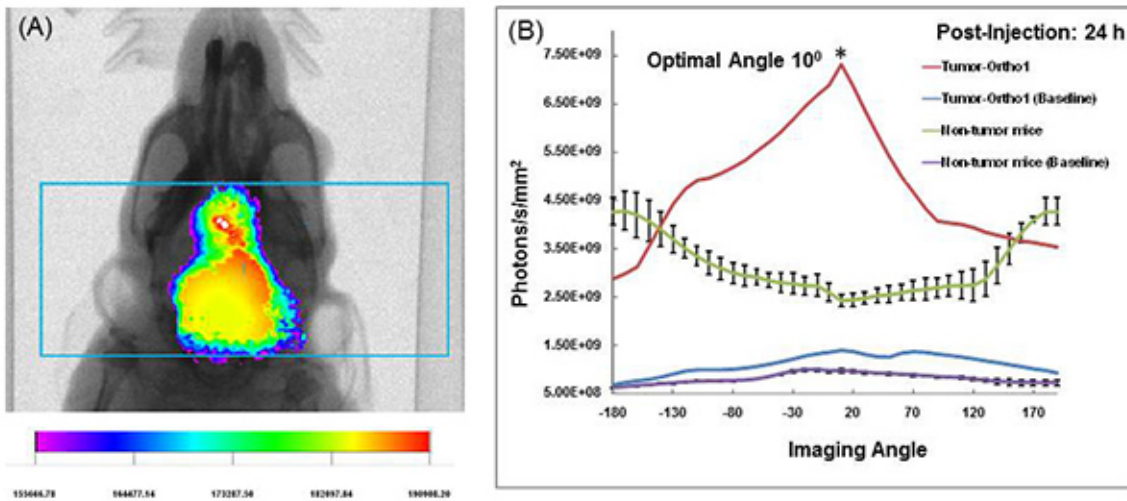
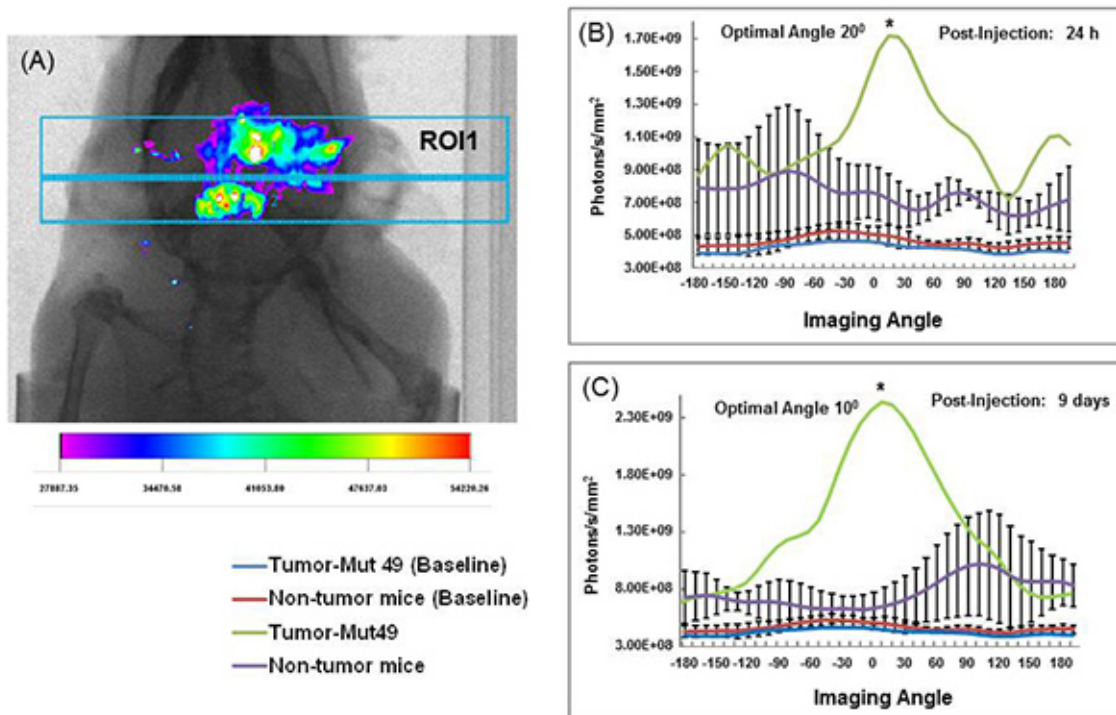
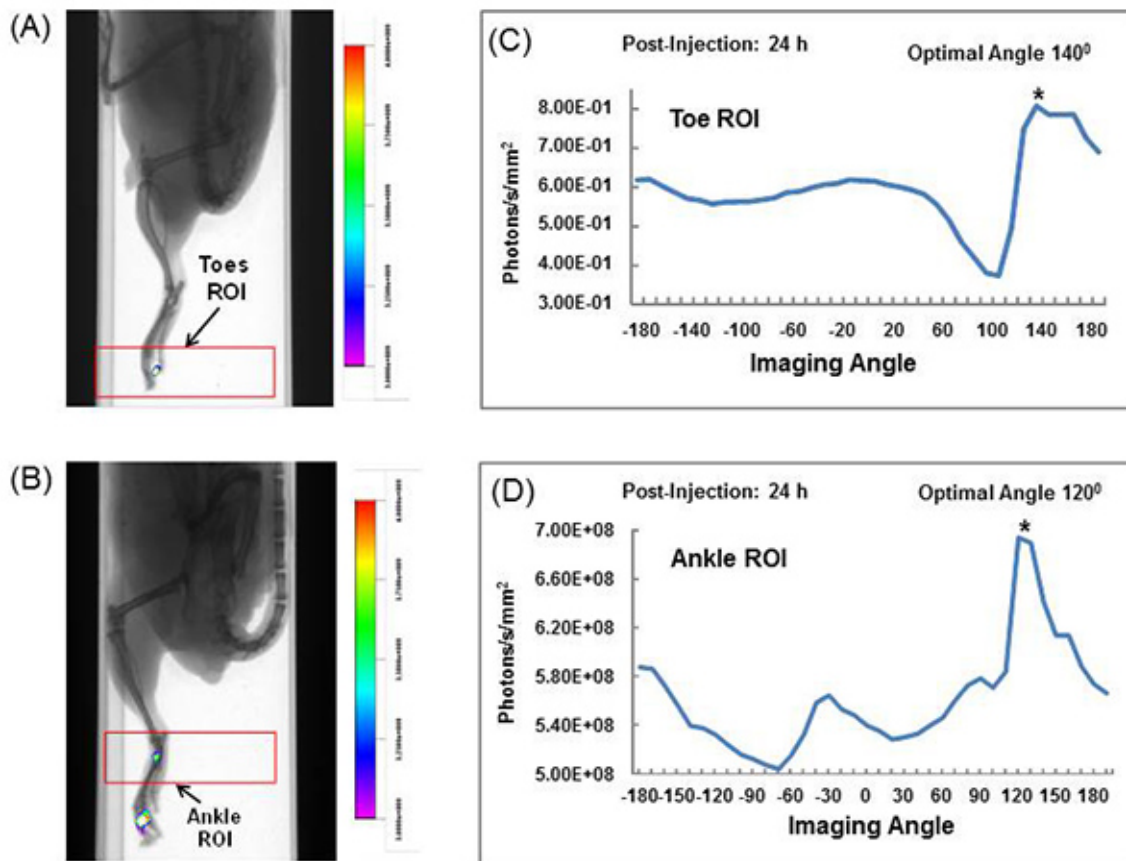


Figure 2. Fluorescence signal vs. image angle in an orthotopic brain tumor mouse model. (A). Image of the peak fluorescent signal at the optimal image angle of 10°. The blue box shows the ROI used to quantify the emitted photons. (B). Graph of the image angle versus photon emission. The representative orthotopic tumor-bearing mouse (Ortho1) is graphed against averaged fluorescence values from identical ROIs in three nontumor mice. Measurements were taken at baseline (before injection) and 24 hr post injection. Error bars represent Standard Deviation. [Click here to view larger image.](#)



**Figure 3. Fluorescence signal vs. image angle in a spontaneous brain tumor of a genetically engineered mouse model. (A).** Image of the peak fluorescent signal of ROI 1 (top blue box) at the optimal image angle of 20°. **(B) and (C).** Graphs of the image angle versus photon emission from ROI 1. Values from a representative spontaneous brain tumor-bearing mouse, Tumor-Mut 49, are graphed against averaged values from three non tumor mice. Measurements were taken at baseline (before injection) and 24 hr (B) and 9 days (C) post injection. Error bars represent Standard Deviation. [Click here to view larger image.](#)



**Figure 4. Fluorescence signal vs. image angle in a mouse with arthritis of the toe and ankle joints.** (A) and (B). Images showing the peak fluorescent signal for the toes (A) and ankle joints (B), within the ROIs shown in the red box. (C). Graph of angle versus mean photon emission for the toe. Peak photon emission can be seen at an angle of 140°. (D). Graph of angle versus mean photon emission for the ankle; maximum intensity occurs at an angle of 120°. [Click here to view larger image.](#)

## Discussion

Accurate determination of the location and magnitude of solid tumors and inflammatory foci in rheumatic conditions is critical to implement adequate treatment and follow up disease progression or remission. While valuable, current imaging strategies (X-rays; MRI; ultrasound; X-ray computed tomography) provide incomplete assessments of disease status. For instance, arthritic joint damage is commonly assessed by X-rays, which provides information on bone structure but not on soft tissue inflammation and destruction, characteristic of early stages of the disease. The MAROI method presented here combines the advantages of both X-rays and sophisticated soft tissue imaging modalities (e.g. MRI or ultrasound) in an integrated, noninvasive and simpler platform that also allows for a full 3D mapping and reconstruction of the diseased tissue or organ in small animals such as mice.

This method takes advantage of the selective affinity of SapC-DOPS nanovesicles for exposed phosphatidylserine residues, which are abundant in the membranes of cancer and inflammatory cells. The determinant of this binding is SapC, a fusogenic lysosomal protein with a strong affinity for anionic phospholipids such as phosphatidylserine<sup>7,10,11</sup>. When conjugated to a fluorescent probe (CVM), systemically injected SapC-DOPS can be traced to tumor and arthritic sites by fluorescence imaging.

Limitations of our method are related to its sensitivity, which at present restricts its use to imaging of small animals like mice. As with other imaging methods, optimal fluorescent signal to noise ratio is constrained by the size of the tumor or the extent of arthritis, and may be compromised when imaging tissues or organs with high background (autofluorescence) such as ears (brain imaging), intestines/feces (abdominal imaging) and paws (hind limb imaging). In this respect, we found that a far-red dye such as CVM provides better spectral separation and resolution in the *in vivo* setting than other fluorescent probes in the visible range.

Other pitfalls include potential movement of the animal during imaging, both while anesthetized and *post mortem* (*rigor mortis*). Hind limb positioning, particularly, is often difficult to stabilize to avoid movement during rotation. In its present state the technique is also time consuming, with scan times as long as 60 min needed to complete a full rotation and acquire high quality images.

The MAROI method presents a number of advantages over other imaging modalities. The ability to image diseased tissue from 38 (or more) different angles allows visualization of fluorescence that may be hindered when assessing it from a single plane; this is valuable in animal studies because it may help minimize the number of false negatives that result from imaging at inappropriate angles. By overlying X-ray and

fluorescence images, a precise anatomical localization of the diseased site can be determined. Finally, the possibility of live (*in vivo*) imaging allows for longitudinal studies to be performed.

## Disclosures

There is nothing to disclose.

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