APPLICATION NOTE

Pre-clinical Imaging

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Comparison of PerkinElmer Vascular Pre-clinical Fluorescent Imaging Agents in Oncology and Inflammation Research

Abstract

Optical-based in vivo imaging of blood vessels and vascular leak is an emerging modality for studying changes that occur in a variety of different cancers and inflammatory states. A number of fluorescent imaging agents that circulate with the blood, but have no target selectivity, have been used to detect tumor leakiness as an indication of abnormal tumor vasculature. Inflammation is also characterized by distinct vascular changes, including vasodilation and increased vascular permeability, which are induced by the actions of various inflammatory mediators. This process is essential for facilitating access for appropriate cells, cytokines, and other factors to tissue sites in need of healing or protection from infection.

Imaging agents that fluoresce in the near infrared (NIR) are particularly useful for detection of changes in vascular permeability because of relatively low tissue absorbance, permitting effective penetration into deep tissue sites. NIR vascular agents can vary in size and physicochemical properties that affect their overall utility for particular imaging applications or affect the time window for optimal imaging. In these studies, we compared three NIR fluorescent agents, Superhance™ (a low molecular weight agent), AngioSense® (a high molecular weight agent), and AngioSPARK® (30-50 nm nanoparticles). Each agent differs significantly in pharmacokinetics, biodistribution, and tissue clearance rates, offering three slightly different imaging tools for cancer and inflammation research. All three agents can detect tumor vascular leak, with AngioSense showing superior signal to background in orthotopic mouse breast cancer. In contrast, Superhance shows superior imaging capability when applied to a mouse model of acute, inflammatory paw edema. AngioSPARK, designed as a highly robust agent for intravital microscopy assessment of vessel morphometry, shows the ability to image both tumors and inflammation with long term accumulation at the imaging site (sometimes a benefit for time course studies). Images and quantitation of timecourses in animal models of cancer and inflammation provide guidance regarding optimal usage of these three vascular imaging agents.

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Materials and methods

Fluorescent Agents for the Detection of Cancer and Inflammation

A variety of non-targeted fluorescent agents (Superhance, AngioSense, and AngioSPARK) were used as vascular agents to image fluorescent signal circulating through tissues, blood leakage and accumulation into tumors, and extravasation of agents during edema. The imaging doses were as recommended in the product inserts, but all data was normalized to reflect a 2 nmol per mouse intravenous dose for easier comparison.

4T1 Syngeneic Tumor Model

Eight week-old female *nu/nu* mice were injected in both upper mammary fat pads with $0.5x10^6$ 4T1 mouse breast adenocarcinoma cells per site. 5-7 days later, mice were injected with vascular agents and imaged (at indicated times) using FMT to detect and quantitate levels of fluorescence throughout the body, including tumor sites, control sites, and different organ systems.

Carrageenan Paw Edema Model

BALB/c mice were injected in the right hind footpad with 30 µL of a carrageenan (CG) solution in PBS (0.125-1%). The left hind footpad was injected with 30 µL PBS and served as an internal control. Paw edema was determined using a spring-loaded caliper to measure paw thickness as compared to controls. Injection of 2 nmoles/mouse of various NIR vascular agents to enable quantitative biological imaging readouts.

In vivo FMT 2500 tomographic imaging

Mice were anesthetized by isoflurane inhalation. For mice with hair, depilatory lotion (Nair® Church & Dwight Co., Inc., Princeton, NJ) is applied thickly on skin over the upper torso (front, back, and sides) of each mouse, rinsed off with warm water, and re-applied until all hair has been removed. As nu/nu mice have no hair, depilation was not required for tumor studies. Tumor-bearing mice were imaged using the FMT® 2500 Quantitative Pre-clinical Imaging System which collected both 2D surface images as well as 3D tomographic imaging data. Paws from carrageenan edema mice were imaged without the requirement for hair removal. Paws were imaged after careful position of mice on an index-matching imaging block.

FMT reconstruction and analysis

The collected fluorescence data was reconstructed by FMT 2500 system software (TrueQuant™) for the quantitation of three-dimensional fluorescence signal within tumors or inflamed paws. Three-dimensional regions of interest (ROI) boxes were drawn to encompass the relevant biology (*i.e.* tumors or hind paws). Control ROIs were placed to capture a control site, either a flank region for tumor mice or contralateral paws for CG mice. The software determined the total pmoles of fluorescence within each 3D ROI. Additional ROIs placed to encompass the heart region provided useful quantitation regarding blood pharmacokinetics.

Introduction

Vascular Agents

Imaging has become a crucial tool in a variety of therapeutic areas in disease research, from preclinical to clinical studies. Historically, anatomical modalities of imaging (e.g. MRI, CT) have paved the way, providing valuable information used in the diagnosis of many diseases, offering quantitative information regarding dimensional changes in important biology such as tumor size and location, joint space narrowing, etc.

As alterations in blood flow or oxygenation underlie disease at the tissue or cellular level, physiological processes such as blood flow and perfusion have also become very important. Increased tumor vascular permeability can be an early indicator of tumor angiogenesis, tumor progression, and poor

Table 1. **Basic properties of four different PerkinElmer vascular pre-clinical fluorescent imaging agents.**

	Superhance	AngioSense	AngioSPARK
Agent type	Albumin-binding small molecule	PEGylated large scaffold	Nanoparticle
Molecular weight or size	1540 g/mol	250,000 g/mol	30-50 nm particles
Ex/Em	675/692 nm	680/700 nm	673/690 nm
Blood half-life	1.5 h	7 h	20 h
Tissue half-life	5 h	72 h	> 100 h

Agent Summary. Characteristics of the agents (MW/size, excitation/emission [Ex/Em], and blood/tissue pharmacokinetics) were determined in multiple independent studies. Blood half-lives were measured by blood collection from mice at different times post-intravenous injection. Blood samples were measured for fluorescence levels in a fluorescence microplate reader. Tissue half-lives were determined by time course FMT 2500 imaging of tumors.

therapeutic outcome. In inflammatory diseases and conditions, increased vascular permeability is an indication of alterations in fluid homeostasis, either as an acute edema response or in serious, chronic organ conditions. Radiotracers, chromium- or gadolinium-based paramagnetic agents, and radio-opaque agents have often been used as vascular contrast agents, however fluorescent-labeled agents offer a useful and safe alternative, particularly for preclinical imaging. Agents as simple as fluorescent dyes have been used to visualize tumors, achieving contrast enhancement through enhanced permeability of tumor vessels and enlarged interstitial space. Typically, however, larger fluorophore-labeled molecules are often chosen to better extend plasma half-life and to mimic leak of plasma proteins (i.e. provide a relatively slow leakage rate).

In the current studies, three different near infrared fluorescent contrast agents were assessed; Superhance 680, AngioSense 680, and AngioSPARK 680 have different physicochemical compositions, different blood pharmacokinetics (Table 1), and show slightly different imaging uses.

These agents were assessed for their ability to image tumor vascular leak and acute carrageenan paw edema. Agents were assessed by 3D fluorescence molecular tomography and 2D planar imaging at a variety of time points after injection into mice. The 3D imaging offered quantitative detection throughout the tissue, whereas 2D imaging provides a simple and rapid means of detecting surface-weighted fluorescence (*i.e.* mostly the fluorescence in the outer mm of the tissue).

Results

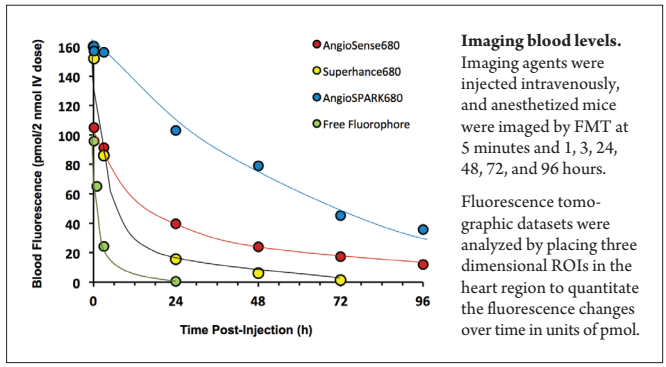
Vascular Agent Properties

Fluorescent vascular imaging agents can vary widely in structure, size, and physiochemical properties (from small molecule dyes to large nanoparticles), and these differences can significantly affect their pharmacokinetics, biodistribution, and metabolic clearance characteristics. These properties will further impact the types of biology that can be imaged as well as the optimal imaging timepoints. In order to achieve an effective level of agent in the target tissues (*i.e.* sites of tumor vascularity, inflammation-based edema, or tumor-associated vascular leak) the agent will be imaged in circulation immediately upon injection, and the circulating levels will drive the extravasation from circulating blood to the tissue of interest, either accumulating in areas of enhances vascular permeability or passively extravasating into normal tissues. Table 1 shows the 3 different agents examined in the present studies.

Circulation levels of each agent were assessed non-invasively by longitudinal imaging of the heart region at select time points. Figure 1 shows the different circulating levels detected and quantitated in living mice by FMT 2500, measurements in close agreement with half-life values in Table 1 determined by ex vivo blood assays.

Imaging Tumors with Vascular Agents

To determine the optimal imaging time points for the different vascular imaging agents we used a syngeneic mouse tumor model, orthotopic implantation of 4T1 mouse breast adenocarcima at two sites (in each upper mammary fat pad). Although this model does not require deep tissue imaging for detection, it provided an experimental approach that permitted the comparison of FMT imaging and planar imaging in the analysis of vascular agent kinetics. As shown in the diagram in Figure 2, tumors were allowed sufficient time to grow prior to injection of imaging agents, after which imaging datasets were acquired at different time points to characterize the kinetics of tumor fluorescence accumulation and tissue washout.



Imaging blood levels. Imaging agents were injected intravenously, and anesthetized mice were imaged by FMT at 5 minutes and 1, 3, 24, 48, 72, and 96 hours.

Fluorescence tomographic datasets were analyzed by placing three dimensional ROIs in the heart region to quantitate the fluorescence changes over time in units of pmol.

Figure 1. Blood levels of vascular agents.

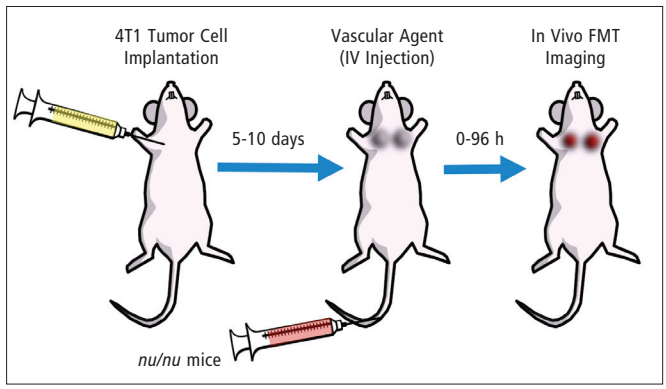
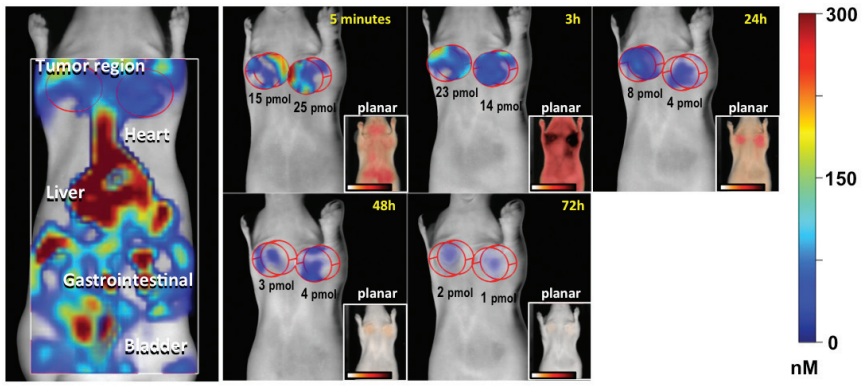


Figure 2. 4T1 orthotopic breast cancer model.



Imaging tumors. FMT and planar images show the patterns of fluorescence that occur in animals bearing tumor masses on the upper mammary fat pads.

A. Whole body signal by FMT imaging.

B. FMT imaging showing only tumor region fluorescence over time. Inset panels represent surface fluorescence detected by planar imaging.

Figure 3. Superhance tumor imaging.

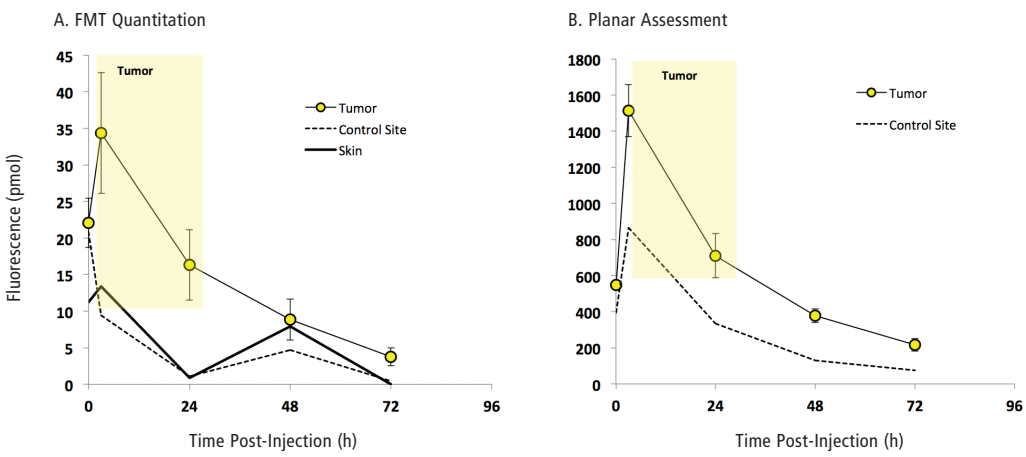
Determining optimal tumor imaging timepoints for Superhance

To determine the optimal imaging timepoint relative to agent injection, we imaged 4T1 tumor-bearing mice at different times post-Superhance 680 injection (Figure 3). Whole body images at 3 h post-injection (Figure 3A) reveal signal throughout the body (including bladder, intestines, liver, and heart) as well as in the tumor periphery.

Careful FMT assessment of the tumor regions at each of the time points reveals high heart and carotid/jugular signal at 5 min post injection, interfering with proper FMT analysis of tumors. The maximal signal over background (Figure 4) was seen with FMT at 3 h, with signal distributed throughout the tumors at all time points rather than localizing only in the tumor margins or periphery. Both FMT and planar imaging can detect differences as compared to control sites, but planar imaging faces difficult background signal due to high skin levels. FMT would offer the additional advantage of the detection of deep tissue tumors undetectable at the surface by 2D planar imaging.

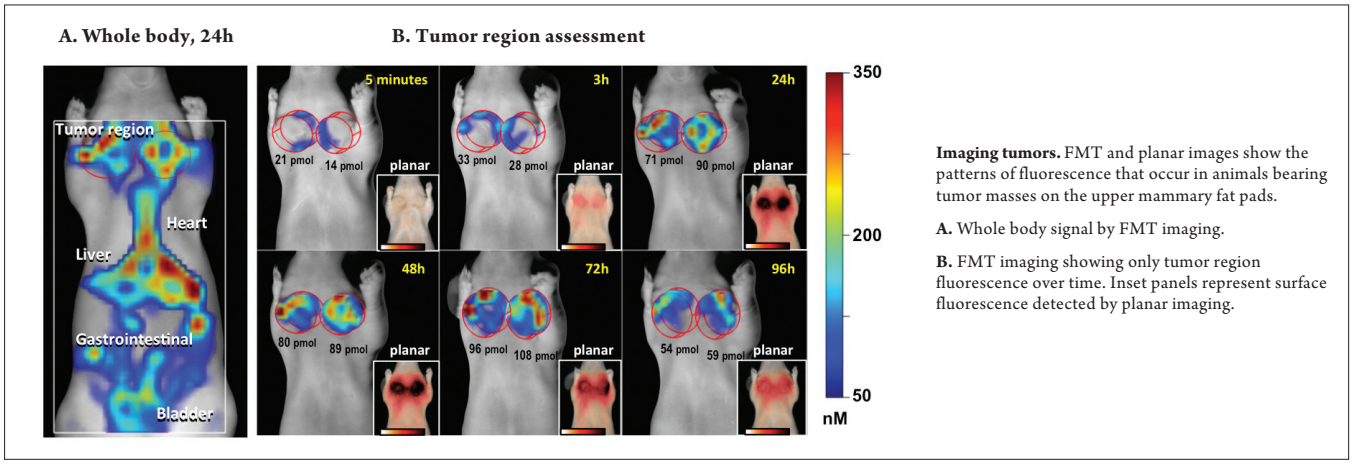
Superhance Comments:

1. Agent provides good tumor definition by planar imaging at 3-24 h. FMT shows heterogeneous signal throughout the tumor region.
2. Earliest time (5 min) in the tumor region is mostly attributed to skin/heart/carotid signal.
3. Fluorescence distribution pattern indicates accumulation throughout the tumor rather than just at tumor margins, suggesting efficient tumor penetration.



Superhance quantitation. Fluorescence within tumor regions as well as control and skin sites were determined by **A.** FMT imaging, and **B.** planar imaging analysis. All fluorescence quantitation in control sites is adjusted to match the volume (FMT) or area (planar) captured by the tumor site analysis.

Figure 4. Superhance tumor fluorescence quantitation.



Imaging tumors. FMT and planar images show the patterns of fluorescence that occur in animals bearing tumor masses on the upper mammary fat pads.

A. Whole body signal by FMT imaging.

B. FMT imaging showing only tumor region fluorescence over time. Inset panels represent surface fluorescence detected by planar imaging.

Figure 5. AngioSense tumor imaging.

Determining optimal tumor imaging timepoints for AngioSense

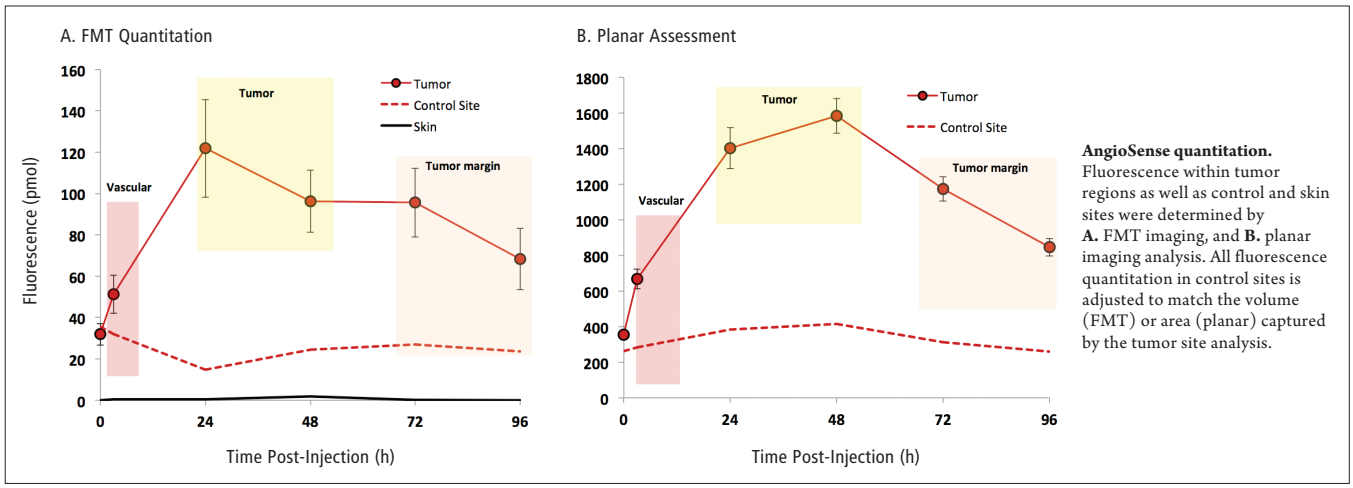
To determine the optimal imaging time point relative to agent injection, we imaged 4T1 tumor-bearing mice at different times post-AngioSense 680 injection (Figure 5). Whole body images at 24 h post-injection (Figure 5A) reveal signal throughout the body (including bladder, intestines, liver, and heart) as well as in the tumor periphery.

Careful FMT assessment of the tumor regions (Figure 6) showed a mostly vascular signal from 5 minutes to 3 h. The maximal tumor signal over background was seen at 24-48 h, with signal distributed throughout the tumors at 24-48 h. At later time points (72-96 h) signal appears to clear from the center of the tumors and localizes in the tumor margins.

Both FMT and planar imaging can detect differences as compared to control sites, although multiple studies have shown detection of deep tissue tumors with AngioSense that are not detectable by 2D imaging.

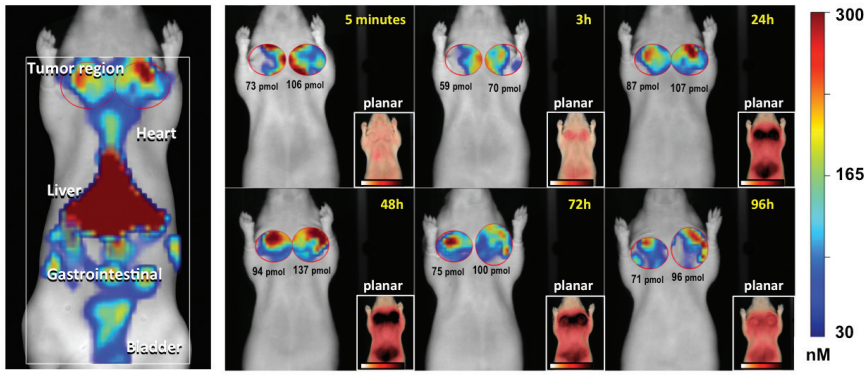
AngioSense Comments:

1. For superficial tumors, 3D and 2D tumor datasets generally align well.
2. Regions of low-grade background signal can be visualized.
3. Maximal signal is at 24-48 h with clearance to the margins occurring at a steady rate thereafter.



AngioSense quantitation. Fluorescence within tumor regions as well as control and skin sites were determined by **A.** FMT imaging, and **B.** planar imaging analysis. All fluorescence quantitation in control sites is adjusted to match the volume (FMT) or area (planar) captured by the tumor site analysis.

Figure 6. AngioSense tumor fluorescence quantitation.



Imaging tumors. FMT and planar images show the patterns of fluorescence that occur in animals bearing tumor masses on the upper mammary fat pads.

A. Whole body signal by FMT imaging.

B. FMT imaging showing only tumor region fluorescence over time. Inset panels represent surface fluorescence detected by planar imaging.

Figure 7. AngioSPARK tumor imaging.

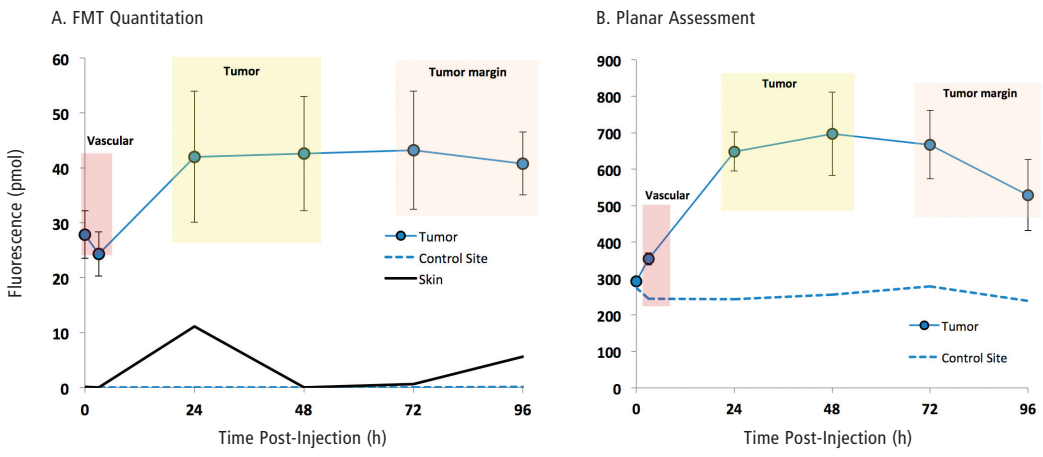
Determining optimal tumor imaging timepoints for AngioSPARK

To determine the optimal imaging time point relative to agent injection, we imaged 4T1 tumor-bearing mice at different times post-AngioSPARK 680 injection (Figure 7). Whole body images at 24h post-injection (Fig 7A) reveal signal throughout the body (including bladder, intestines, liver, and heart) as well as in the tumor.

Careful FMT assessment of the tumor regions (Figure 8) showed a mostly vascular signal from 5 minutes to 3 h. The maximal tumor signal over background was seen at 24 h, with signal distributed throughout the tumors at 24-48 h. At later time points (72-96 h) signal appears to clear from the center of the tumors and localizes predominantly in the tumor margins. Both FMT and planar imaging can detect differences as compared to control sites. FMT would offer the additional advantage of the detection of deep tissue tumors undetectable at the surface by 2D planar imaging.

AngioSPARK Comments:

1. For superficial tumors, 3D and 2D tumor datasets generally align well.
2. Regions of low-grade background signal can be visualized.
3. Maximal signal is at 24-48 h with clearance to the margins occurring but little overall clearance prior to 96 h.



AngioSPARK quantitation. Fluorescence within tumor regions as well as control and skin sites were determined by **A.** FMT imaging, and **B.** planar imaging analysis. All fluorescence quantitation in control sites is adjusted to match the volume (FMT) or area (planar) captured by the tumor site analysis.

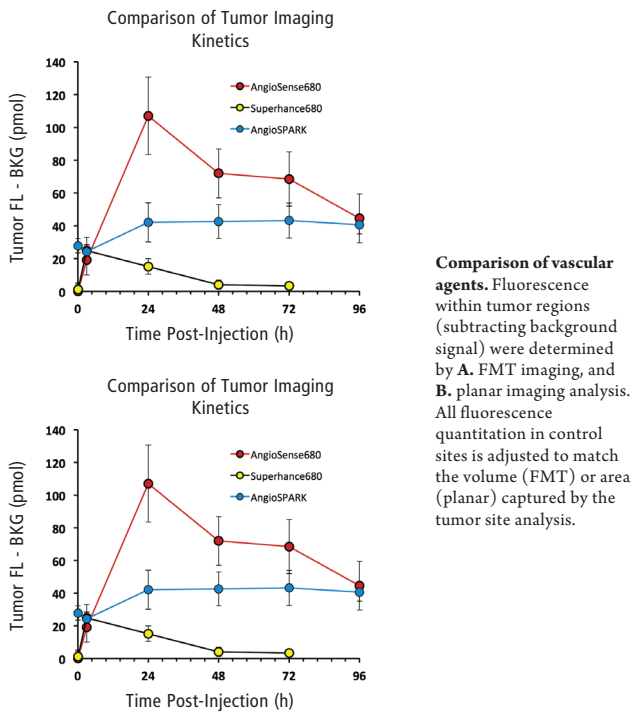
Figure 8. AngioSPARK tumor fluorescence quantitation.

Comparison of three vascular agents in oncology imaging

Superhance, AngioSense and AngioSPARK show clear differences in physicochemical properties, blood pharmacokinetics (Table 1, Figure 1) as well as in optimal imaging time points (indicated by arrows) and tissue clearance. All three agents provide good tumor definition both by FMT and planar imaging of these superficially-implanted tumors, and (when using FMT) deep tissue tumors can be detected as well.

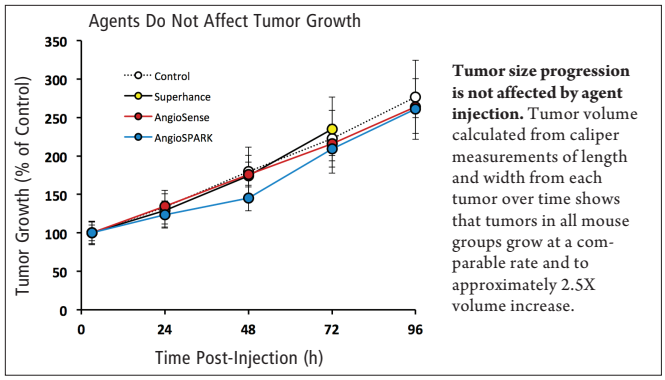
It is interesting to note that AngioSense appears to provide the optimal combination of pharmacokinetics and efficiency in tumor accumulation, as shown by its ability to achieve the highest levels of tumor signal. AngioSPARK shows greatly extended blood pharmacokinetics, and because of its large size it appears to be less efficient at accumulating within tumors. Its slow overall clearance from tissue, however, offers a method of long term tumor imaging. Superhance, because of its very short circulation half-life shows the lowest tumor signal, yet it provides a very early readout (3 h).

It is important to note that these three agents show no effect on 4 day tumor growth (by direct physical measurement of tumor dimensions) as compared to tumors in animals receiving no imaging agent injection (Figure 10). This indicates that these agents are not altering tumor biology by passively accumulating within the tissue.



Comparison of vascular agents. Fluorescence within tumor regions (subtracting background signal) were determined by **A.** FMT imaging, and **B.** planar imaging analysis. All fluorescence quantitation in control sites is adjusted to match the volume (FMT) or area (planar) captured by the tumor site analysis.

Figure 9. Vascular agent tumor imaging summary.



Tumor size progression is not affected by agent injection. Tumor volume calculated from caliper measurements of length and width from each tumor over time shows that tumors in all mouse groups grow at a comparable rate and to approximately 2.5X volume increase.

Figure 10. Effects on tumor progression.

Imaging acute edema with vascular agents

To determine the optimal imaging time points for the different vascular imaging agents we used a mouse model of acute neutrophil-driven paw edema induced by injection of carrageenan (CG) into the right hindpaws (Figure 11). This provided an experimental approach that permitted the comparison of FMT imaging and planar imaging in the analysis of vascular agent kinetics. Superhance was injected at t = 2 h post carrageenan, whereas AngioSense and AngioSPARK were injected at t = 3 h, to accommodate the differences in blood pharmacokinetics and to permit imaging at the peak edema response (3 h). As shown in Figure 12, all three agents showed increased signal in carrageenan paws, although AngioSPARK showed high background signal in control paws. Superhance cleared quickly from tissue, making this agent ideal for imaging acute edema. Both AngioSense and AngioSPARK showed continued carrageenan paw accumulation at 24 h, a time at which there is less edema and more cellular inflammation.

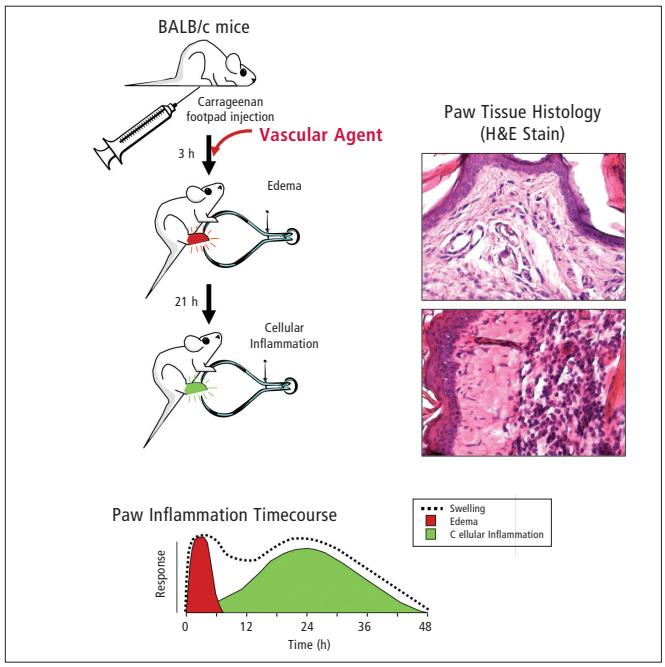
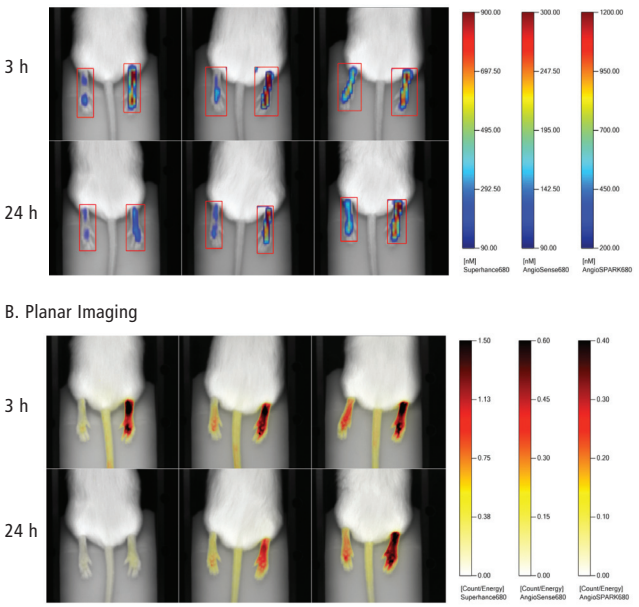


Figure 11. Carrageenan Paw Edema (CPE)



Imaging Acute Paw Edema Induced by Carrageenan Injection. FMT and planar images show the patterns of fluorescence of three different vascular imaging agents that accumulate in paws of animals with inflammatory edema induced in the right footpad.

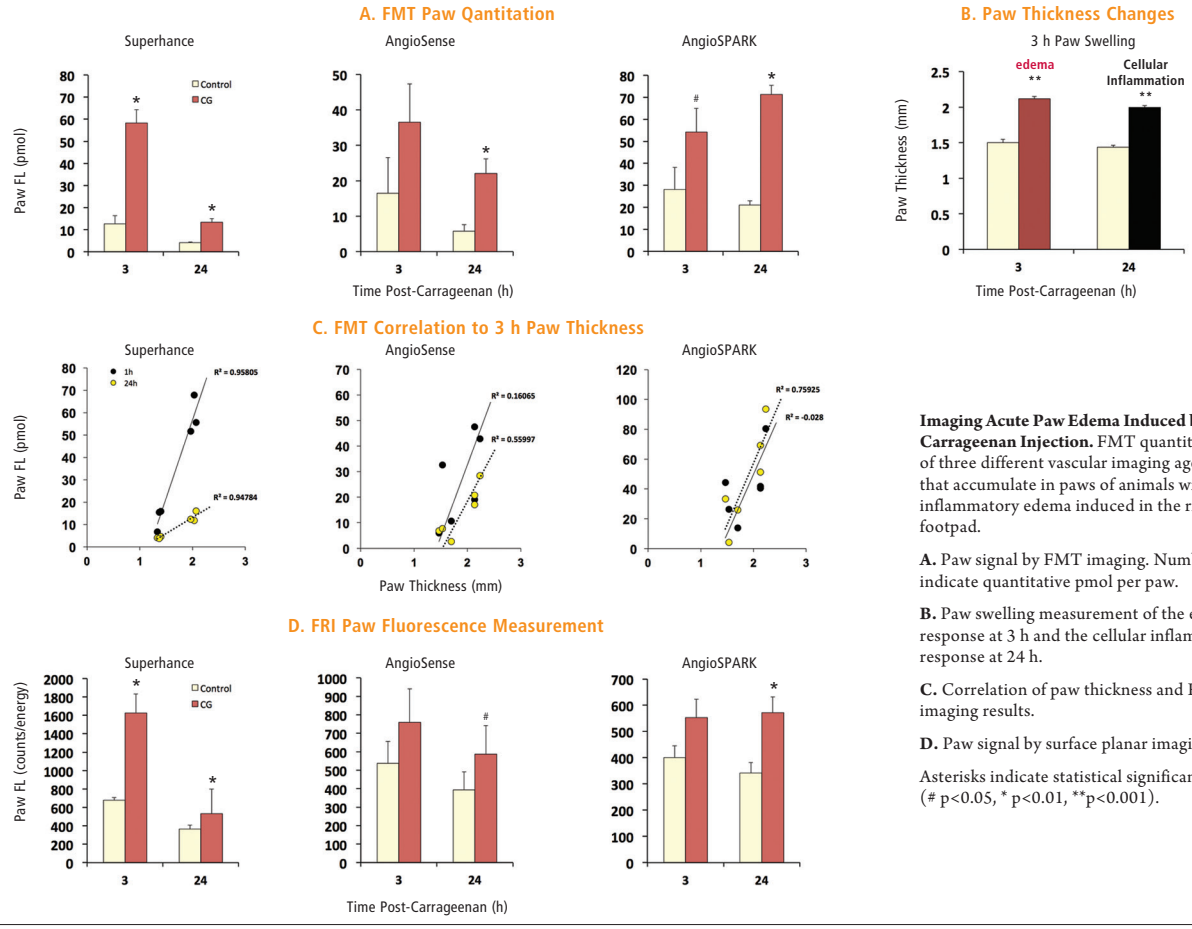
A. Paw signal by FMT imaging. Numbers indicate quantitated pmol per paw.

B. Planar imaging of the same mice shows the superficial fluorescence associated with the edema response.

Figure 12. Carrageenan Paw Edema imaging.

Imaging Acute Edema with vascular agents

Comparison of 3 h and 24 h imaging results (Figure 13A) with paw swelling levels (measured by caliper) shown in Figure 13B suggests that Superhance imaging provides good detection and signal to noise at 3 h with excellent statistical significance despite using only 3 mice per group. The Superhance results agree well with the 3 h paw swelling results, and signal washes out appreciably by 24 h. In contrast, both AngioSense and AngioSPARK detected paw inflammation best 24 h post-injection. Thus, for these two agents, although they accumulate during the edema phase of the inflammation, their signals are assessed during the cellular inflammation phase. It is clear however, that Superhance (1 h post injection) is ideal for assessing acute inflammatory edema, and the results for individual paws correlate very well with paw thickness measurements (Figure 13C). The other agents require imaging long after the peak edema response and do not correlate as well with peak edema paw thickness. Other studies in more chronic forms of cellular inflammation, such as arthritis, show a strong benefit with AngioSense and AngioSPARK imaging (data not shown). Planar imaging was less sensitive than FMT imaging at detecting differences between edema and control paws, with significant interference by high reflectance background signal.



Imaging Acute Paw Edema Induced by Carrageenan Injection. FMT quantitation of three different vascular imaging agents that accumulate in paws of animals with inflammatory edema induced in the right footpad.

A. Paw signal by FMT imaging. Numbers indicate quantitative pmol per paw.

B. Paw swelling measurement of the edema response at 3 h and the cellular inflammation response at 24 h.

C. Correlation of paw thickness and FMT imaging results.

D. Paw signal by surface planar imaging.

Asterisks indicate statistical significance (# $p<0.05$, * $p<0.01$, ** $p<0.001$).

Figure 13. Comparison to paw swelling responses.

Conclusions

Superhance, AngioSense and AngioSPARK show clear differences in physicochemical properties, blood pharmacokinetics, optimal imaging time points, tissue clearance, and overall efficacy in tumor and inflammation imaging.

Table 2 summarizes the findings for these agents in tumor and paw edema imaging as well as including supportive summaries from arthritis and intravital microscopy studies. All three agents provide good tumor definition, with Superhance affording very early imaging opportunities, AngioSense generating the greatest signal to background, and AngioSPARK showing long-lasting signal in the tumor tissue. For inflammatory edema imaging, Superhance shows the greatest potential in acute paw edema (highest signal to background, early time point imaging), whereas AngioSense and AngioSPARK require longer time for optimal accumulation, making them more suitable for chronic inflammation models such as arthritis (data not shown). There are multiple other applications currently being explored for these imaging agents in inflammation models (*e.g.* asthma, COPD, arthritis, peritonitis, wound healing), cancer (*e.g.* tumor xenografts, orthotopic tumor models, metastasis models), and other disease conditions (*e.g.* graft-versus-host disease, graft rejection, blood-brain-barrier breakdown, organ failure).

Table 2. **Summary information for four applications for Superhance, AngioSense, and AngioSPARK.**

	Superhance	AngioSense	AngioSPARK
Tumor imaging	+	+++	++
Tumor imaging time	3 h	24-48 h	24-48 h
Tumor washout	48 h	144 h	>192 h
Response to anti-angiogenic agents	*nd*	+++	*nd*
Acute edema imaging	+++	+	+
Tissue imaging time	3 h	24 h	24 h
Tissue washout	48 h	144 h	>192 h
Arthritis imaging	*nd*	+++	+++
Tissue imaging time	*nd*	24 h	24 h
Tissue washout	*nd*	144 h	>192 H
Intravital microscopy	++	+++	+++
Optimal imaging time	5-15 min	5-60 min	5 min – 3 h

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